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Microbiota in inflammatory bowel disease

Doctoral thesis

NTNU
Norwegian University of Science and Technology
Thesis for the Degree of
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Mikrobiota ved inflammatorisk tarmsykdom

Mikrobiota er fellesbetegnelsen på bakterier, sopp, virus, bakteriofager og arkebakterier som eksisterer i et miljø. Inflammatorisk tarmsykdom (IBD) er kroniske betennelsestilstander i gastrointestinaltraktus og består hovedsakelig av sykdommene Crohns sykdom (CD) og ulcerøs kolitt (UC). Årsaken til IBD ukjent, men den rådende hypotesen er at betennelse utvikles hos genetisk disponerte individ utsatt for miljøfaktorer og mikrobiota som fører til en overaktivering av tarmens immunsystem. Eksisterende behandling er ofte utilstrekkelig. Det nedlegges store ressurser i forskning på sykdomsårsak, sykdomsmekanismer og videreutvikling av behandling.

I artikkel I målte vi konsentrasjonen av medisinen 5-aminosalisylsyre (5-ASA) i tarmslimhinnen og undersøkte slimhinne-assosiert mikrobiota hos 42 pasienter med UC. 5-ASA er grunnsteinen i behandling av UC og medisinen utøver sin virkning i tarmslimhinnen. Vi sammenlignet tre forskjellige 5-ASA preparater med ulik frigjøringsmekanisme; Mezavant, Asacol og Pentasa. Vi fant stor variasjon av 5-ASA konsentrasjon i tarmslimhinnen mellom pasienter. Pasienter som brukte Mezavant hadde høyere 5-ASA konsentrasjoner enn pasienter som brukte Pentasa. Videre fant vi at pasienter med høye 5-ASA konsentrasjoner i slimhinnen hadde økt bakteriemangfold og en antatt gunstig bakteriesammensetning i tarmslimhinnen.

I artikkel II undersøkte vi bakteriell mikrobiota i tynntarmen hos 51 pasienter med CD og 40 friske kontroller (FK). Vi fant at pasienter med CD hadde redusert bakteriemangfold og en annerledes bakteriesammensetning i tynntarm sammenlignet med FK. Pasienter med CD hadde også bemerkelsesverdig høye mengder av bakterien *Tyzzerella 4* i tarmslimhinnen. Når vi sammenlignet bakteriesammensetningen innad i en pasientgruppe som hadde betent slimhinne nederst i tynntarmen og ikke-betent slimhinne lengre oppe fant vi ingen forskjell i bakteriesammensetning eller mangfold mellom betent slimhinne og ikke-betent slimhinne. Bakteriesammensetningen og mangfoldet var også lik uavhengig av betennelsesgrad og tynntarmslokalisasjon for hele gruppen av CD pasienter.

I artikkel III analyserte vi soppmikrobiota i tynntarmen hos 44 pasienter med CD og 40 FK (samme pasientmateriale som artikkel II). Vi fant at pasienter med CD hadde en endret soppflora med endret fordeling av ulike sopparter sammenlignet med FK. Spesifikt fant vi økt mengde *Malassezia* og redusert mengde *Saccharomyces* hos pasienter med CD. Når vi sammenlignet soppsammensetningen innad i en pasientgruppe som hadde betent slimhinne nederst i tynntarmen og ikke-betent slimhinne lengre oppe fant vi likt soppmangfold, men ulik soppsammensetning. I betent slimhinne var *Candida sake* overrepresentert, mens *Exophiala equina* and *Debaryomyces hansenii* var overrepresentert i ikke-betent slimhinne lengre oppe i tynntarmen. For hele gruppen av CD pasienter hadde ikke betennelsesstatus i slimhinnen eller lokalisasjon i tynntarm betydning for soppmangfold eller sammensetning.

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Contents

1. Acknowledgement

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2. Abbreviations

5-ASA	5-aminosalicylic acid
Ac-5-ASA	N-acetyl-5-aminosalisylic acid
ACE	Abundance Coverage Estimator
AMPs	Antimicrobial peptides
ANOVA	Analysis of variance
anti-TNF	Anti-tumour necrosis factor
ASV	Amplicon sequence variant
ATG16L1	Autophagy-related protein 16-1
BSG	British Society of Gastroenterology
CARD9	Caspase recruitment domain-containing protein 9
CBC	Crypt-base-columnar
CCK	Cholecystokinin
CD	Crohn's disease
CFU	Colony forming units
CT	Computer tomography
dATP	Deoxyadenosine triphosphate
DCs	Dendritic cells
dCTP	Deoxycytosine triphosphate
ddNTP	Dideoxynucleotide triphosphate
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
dTTP	Deoxythymidine triphosphate
dNTP	Deoxynucleotide triphosphate
ECL	Enterochromaffin-like
FAE	Follicle-associated epithelium
FMT	Faecal microbiota transplantation
GI	Gastrointestinal
H&E	Hematoxylin and eosin
HC	Healthy control
HCl	Hydrochloric acid
HPLC	High-performance liquid chromatography
HTS	High throughput sequencing
IBD	Inflammatory bowel disease
ICR	Ileocecal resection
IEL	Intraepithelial lymphocytes
IRGM	Immunity-related GTPase M
JAK	Janus kinase
LC-MS/MS	Liquid chromatography- tandem mass spectrometry

LGR5	Leucine-rich repeat-containing G protein-coupled receptor 5
MMX	Multi Matrix System
MRI	Magnetic resonance imaging
MS/MS	Tandem mass spectrometry
NAT	N-acetyltransferase
NF-κB	Nuclear factor κB
NGS	Next-generation sequencing
NMDS	Non-metric multidimensional scaling
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
OTU	Operational taxonomic unit
PAMPs	Pathogen-associated molecular patterns
PCA	Principal Component Analysis
PCoA	Principal coordinates analysis
PD	Phylogenetic diversity
PPAR-γ	Peroxisome proliferator-activated receptor-γ
PPI	Proton pump inhibitor
PPK	Polyphosphate kinase
PRRs	Pattern recognition receptors
QIIME	Quantitative Insights into Microbial Ecology
RCTs	Randomized controlled trials
REG	Regenerating islet- derived protein
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SCFA	Short chain fatty acids
sIgA	Secretory immunoglobulin A
SNP	Single nucleotide polymorphism
$sPLA_2$	Secretory phospholipase A_2
ssDNA	Single stranded DNA
TFF3	Trefoil factor 3
TLR4	Toll-like receptor 4
TLRs	Toll-like receptors
TNFα	Tumour necrosis factor-α
UC	Ulcerative colitis
UCDAI	Ulcerative Colitis Disease Activity Index
UHPLC	Ultra-high performance liquid chromatography
UHPLC-MS/MS	Ultra-high performance liquid chromatography- tandem mass spectrometry

3. List of papers

This thesis is based on the following papers and will be referred to in the text by their Roman numerals (I, II, III).

I. **Olaisen M**, Spigset O, Flatberg A, Granlund AVB, Brede WR, Albrektsen G, Røyset ES, Gilde B, Sandvik AK, Martinsen TC, Fossmark R.
Mucosal 5-aminosalicylic acid concentration, drug formulation and mucosal microbiome in patients with quiescent ulcerative colitis.
Aliment Pharmacol Ther. 2019 May;49(10):1301-1313.

II. **Olaisen M,** Flatberg A, Granlund AVB, Røyset ES, Martinsen TC, Sandvik AK, Fossmark R.
Bacterial Mucosa-associated Microbiome in Inflamed and Proximal Noninflamed Ileum of Patients With Crohn's Disease.
Inflamm Bowel Dis. 2020 May 25:izaa107. doi: 10.1093/ibd/izaa107. Epub ahead of print. PMID: 32448900.

III. **Olaisen M*,** Richard ML*, Beisvåg V, Granlund AVB, Røyset ES, Martinsen TC, Sandvik AK, Sokol H, Fossmark R.
Fungal microbiota in the ileal mucosa of patients with Crohn's disease
Manuscript. *Shared first authorship

4. Summary

Microbiota is the microorganisms residing in a specific environment, including bacteria, fungi, viruses, archaea and phages. Inflammatory bowel disease (IBD) is chronic inflammatory diseases encompassing Crohn's disease (CD) and ulcerative colitis (UC). The pathogenesis of IBD is still not understood, but disease occurs in genetically predisposed individuals exposed to environmental factors including microbiota, causing an aberrant immune reaction towards the gut epithelium. The last decade increasing amounts of IBD microbiome studies have been published. However, most studies have analysed the faecal microbiota which is different from the mucosa-associated microbiota and suggested to be less relevant in terms of understanding IBD pathogenesis as the majority of faecal microbes bypasses the gastrointestinal tract and are not adjacent to the epithelium or the enteral immune system. The mucosa-associated microbiota in patients with IBD is different from healthy controls, however causality between microbiota alterations and IBD development has not been established. Additionally, it is uncertain to what extent medical therapies influence gut microbiota composition.

In paper I, we measured the mucosal concentration of 5-aminosalicylic acid (5-ASA) in 42 patients with quiescent UC using three different 5-ASA preparations (Mezavant, Asacol and Pentasa) and we correlated mucosal 5-ASA concentration to the mucosa-associated bacterial diversity and composition. We found large inter-individual variations in mucosal 5-ASA concentration. Patients using Mezavant had higher mucosal 5-ASA concentrations than patients using Pentasa. Further, the mucosal 5-ASA concentration was positively associated with mucosa-associated bacterial diversity and presumed beneficial alterations in mucosa-associated bacterial composition.

In paper II, we assessed the mucosa-associated bacterial microbiota in the ileum 51 CD patients and 40 healthy controls (HC). Paired samples were taken 5 cm and 15 cm proximal of the ileocecal valve or anastomosis. CD patients displayed lower α-diversity

and altered microbiota composition compared to HC. The species *Tyzzerella 4* was strongly overrepresented in CD patients. In CD patients with terminal ileitis and no history of upper CD involvement, the bacterial diversity and composition in the inflamed and proximal non-inflamed ileum were similar. Endoscopic inflammation and ileal sub-location did not influence bacterial microbiota biodiversity or composition in the whole CD cohort.

In paper III, we examined the mucosa-associated mycobiota in 44 CD patients and 40 HC (same patient cohort as paper II). We found that CD patients had an altered mycobiota composition compared to HC, characterised by reduced fungal evenness, increased Basidiomycota-to-Ascomycota ratio, enrichment of *Malassezia* and *Candida albicans* and depletion of Chytridiomycota and *Saccharomyces*. The fungal composition in the inflamed ileal mucosa was compared to the proximal non-inflamed mucosa within CD patients without a history of upper CD involvement. We found similar fungal α-diversity, but a more dysbiotic fungal composition in inflamed mucosa with an expansion of *Candida sake* and depletion of *Exophiala equina* and *Debaryomyces hansenii* spp. Inflammation and location did not impact overall fungal microbiota biodiversity or composition in the total CD cohort.

In summary, the studies describe the mucosa-associated microbiota in patients with established IBD.

5. Introduction

5.1. The gastrointestinal (GI) tract

5.1.1. Anatomy

The GI tract consists of the oesophagus, stomach, small intestine, colon and rectum. The small intestine is subdivided into the duodenum, jejunum and ileum, whereas the colon is subdivided into the caecum, ascending colon, transverse colon, descending colon and sigmoid colon (in oral to rectal direction). The small intestine is approximately 5 meters in length and the luminal surface heavily folded, while the colon is wider and approximately 1.5 meters in length and luminal surface relatively flat (1, 2). The intestinal wall is divided into different layers, the mucosa being closest to the lumen, muscularis mucosae, submucosa, muscle layer (muscularis propria) and the serosa (Figure 1). The mucosa is further subdivided into the epithelium and lamina propria. The epithelium consists mainly of tightly connected columnar epithelial cells, the different cell types in the epithelium are described in detail in section 5.1.2. The lamina propria consists of connective tissue, blood vessels, lymph vessels, nerves and immune cells. The submucosa contains a plexus of parasympathetic nerves. The muscularis propria of the intestine is covered by an outermost fibrous layer called the serosa.

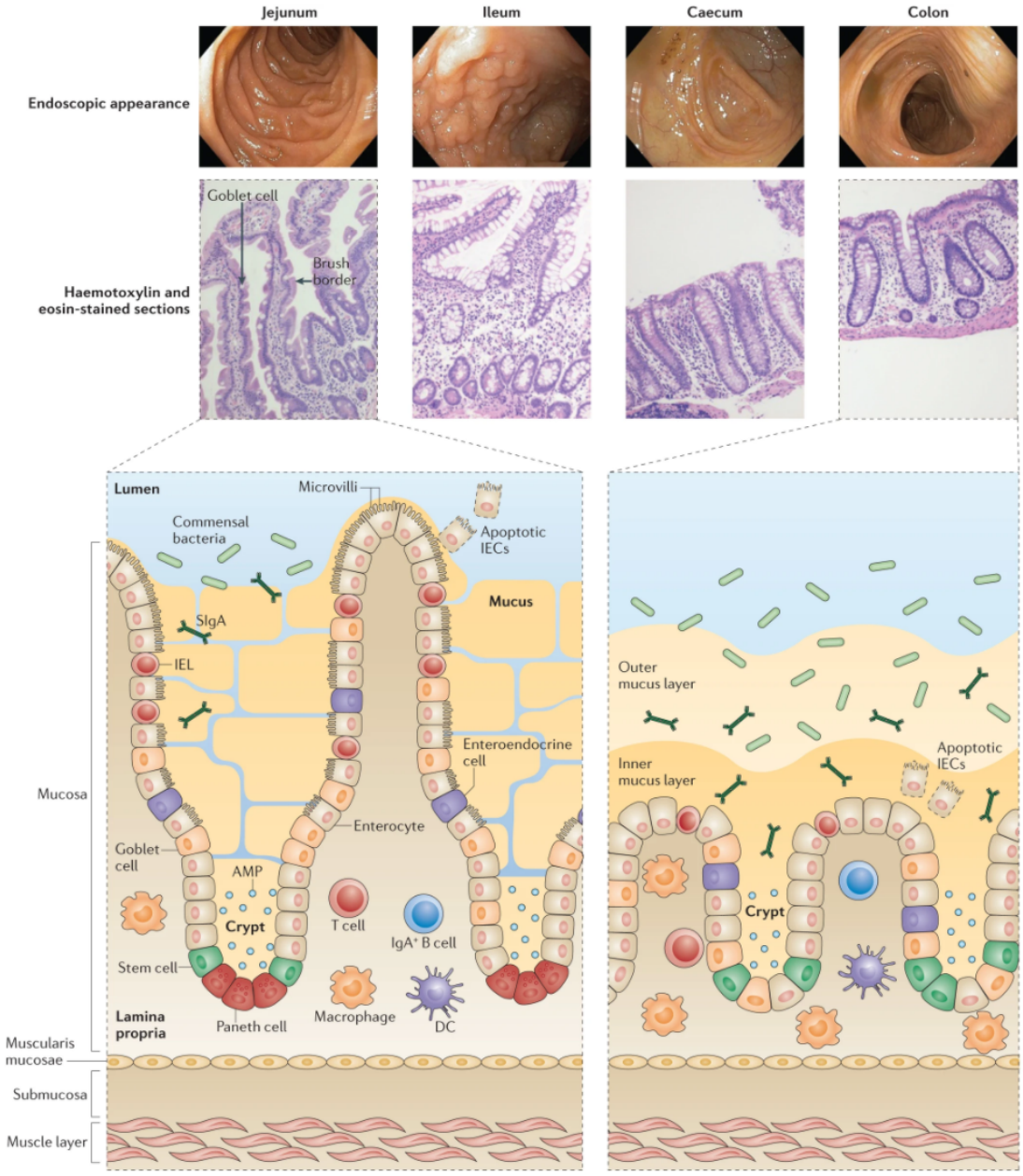

Figure 1. Illustration of endoscopic appearance, histologic appearance and organization of cells in the small intestine and colon. Figure from Mowat et al. (1). Reprinted with permission from Nature Springer. IEL; intraepithelial lymphocytes, SIgA; secretory immunoglobulin A, AMP; antimicrobial peptides, IECs; intestinal epithelial cells.

5.1.2. Cell types in small intestine and colon

As this thesis focuses on the microbiota in the small intestine and colon, the cell types in the small intestine and colon will be described in the following section.

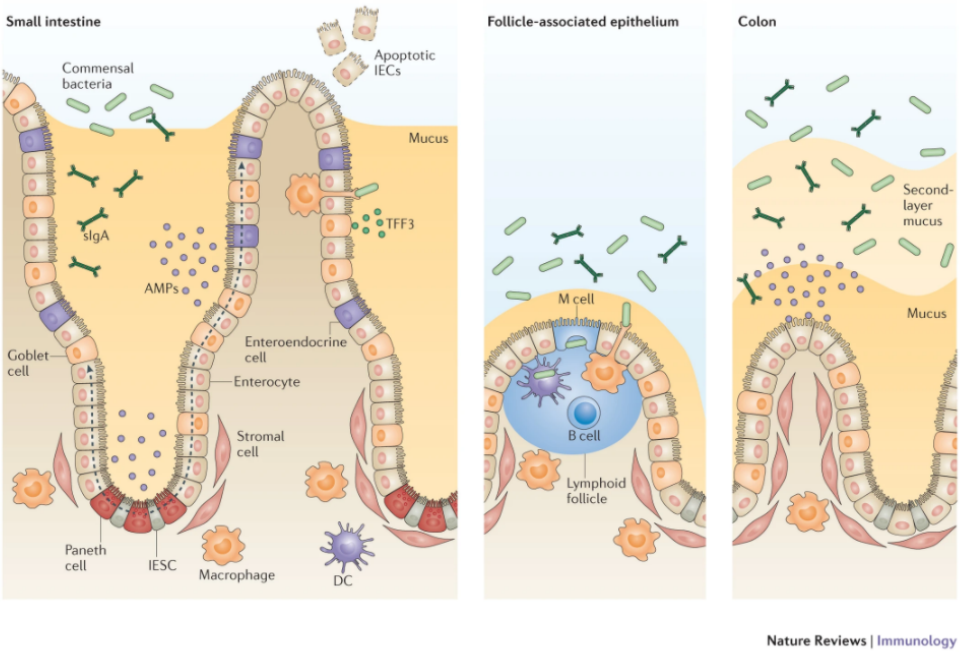

Figure 2. Overview of epithelial cells and immune cells in the small intestine (left) and colon (right). In the middle, a Peyer's patch with follicle-associated epithelium. Figure from Peterson and Artis 2014 (3). Reprinted with permission from Nature Springer. IEC; Intestinal epithelial cell, IESC; intestinal epithelial stem cell niche incl crypt-base-columnar (CBC) stem cells and transit amplifying cells. AMPs; antimicrobial peptides, sIgA; secretory Immunoglobulin A, TFF3; trefoil factor 3, DC; dendritic cell.

5.1.2.1. CBC stem cell

Crypt-base-columnar (CBC) stem cells are pluripotent intestinal epithelial stem cells, which can self-renew and differentiate into any specialised intestinal epithelial cell (4). The marker for CBC cells is Leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5), and sometimes these cells are referred to as LGR5+ stem cells (5). CBC stem cells

are located in the base of the crypts and divide every 24 hours into transit amplifying cells which further divides and differentiate or provide new CBC stem cells (5).

5.1.2.2. Transit amplifying cell

Transit amplifying cells are daughter cells of the CBC stem cells and are localised further up in the crypts towards the villi (3). Transit amplifying cells divide every 12 hours (4). The cells then differentiate into specialised cells such as enterocytes and migrate up along the villi as the surface cell-layer of the intestine shred off (3). The life span of a cell from birth, differentiation, migration and until it is shred of is five days (4).

5.1.2.3. Enterocyte

Absorptive enterocytes are the most abundant cell-type in the intestine (Figure 2). Enterocytes are columnar cells, in the small intestine, they have a characteristic brush border consisting of microvilli protruding into the lumen (1). Enterocytes are specialised cells absorbing and digesting luminal contents, but can also secrete some AMPs such as C-type lectin regenerating islet derived protein (REG) 3γ which stimulate segregation between gut microbiota and epithelium (3, 6).

5.1.2.4. Enteroendocrine cell

Enteroendocrine cells are secretory intestinal epithelial cells producing hormones that regulate digestive functions, and they act as a mediator between the central and enteric neuroendocrine system (Figure 2) (3). Numerous subtypes exist, producing hormones such as gastrin, somatostatin, ghrelin, serotonin, cholecystokinin (CCK), glucose-dependent insulinotropic peptide, glucagon-like peptides and peptide YY (7).

5.1.2.5. Goblet cell

Goblet cells are secretory intestinal epithelial cells which produce and secrete glycoproteins, including mucin2 (MUC2) into the intestinal lumen forming mucus layers which act as the first line defence against luminal contents including microbiota (3, 6).

Goblet cells also produce trefoil factor 3 (TFF3), which contributes to mucin organization by mucin crosslinking and epithelial repair stimulation (Figure 2). Additionally, goblet cells allow passage of antigens from the lumen to specialised dendritic immune cells (8). The number of goblet cells increases throughout the GI-tract and is much more prevalent in the colon than in the small intestine (1).

5.1.2.6. Paneth cells

Paneth cells are localised in the crypt base and are long-lived differentiated intestinal epithelial cells which produce and secrete antimicrobial peptides (AMPs) (8). The Paneth cell is the only cell type that differentiates and migrates down towards the crypt base, where the oldest Paneth cells are localized (5). Paneth cells are renewed every 3-6 weeks (5). Paneth cells are primarily localised in the small intestine and not in the colon, however during inflammatory processes, metaplastic Paneth cells occur in the colon. The AMPs are located in the granules of Paneth cells. Secretion of AMPs might occur continuously, with increased secretion after various stimulation, such as bacteria, bacterial products or cholinergic agonists (9). AMPs produced in Paneth cells include α-defensins, lysozyme, secretory group IIA phospholipase A2 (sPLA2), REG3α (9). AMPs act bactericidal and many kill targeted microorganisms, some also act towards fungi, viruses and protozoa (9).

5.1.2.7. Peyer's patch

Peyer's patches consist of aggregated lymphoid follicles encircled by follicle-associated epithelium (FAE) that contains M cells (Figure 2) (10). Peyer's patches are dominantly located in the distal ileum and increase in size and density from the jejunum to ileum (1). The number of Peyer's patches declines after youth. The Peyer's patches communicate with mesenteric lymph nodes (MLN) through lymphocytes that enter the Peyer's patch as naïve lymphocytes and leave as either naïve or active lymphocytes (10). The FAE also harbour an extensive abundance of immune cells; infiltrated B-cells, T-cells, macrophages and dendritic cells.

5.1.2.8. M cell

Microfold cells, or M cells, are specialized intestinal epithelial cells are primarily located in FAE in close proximity to the Peyer's patches (Figure 2). M cells transport live bacteria, fungi, viruses, parasites as well as non-infectious particles and antigens from the lumen through the apical membrane to the basolateral surface for presentation to the underlying immune system, both through non-specific transcytosis and by specific receptor-mediated microbial uptake (3, 11). Additionally, M cells transport secretory IgA (sIgA) produced in plasma cells from the basolateral membrane to the intestinal lumen. Interestingly, M cells also have IgA receptors enabling them to capture IgA coated bacteria and present them to the immune system (10). On the contrary, M cells can also be a gateway exploited by intestinal pathogens. In fact, many typical enteric pathogen bacteria have been found to adhere to M cells during invasion, among these are *Escherichia coli* and *Yersinia* (10).

5.1.2.9. Tuft cell

Tuft cells, or brush cells, are chemosensory cells. They were initially identified by electron microscopy due to their characteristic morphology consisting of a tubulovesicular system connected to a tuft of long microvilli protruding into the lumen (8). The number of tuft cells increases significantly during parasite infections, where tuft cells produce IL-25 initiating a type 2 immune response (8).

5.1.3. Intestinal epithelium and mucosal barriers

The gut lumen is the largest microbial reservoir within the human body (12). The intestinal epithelium, mucosal immune system and gut microbiota interact closely. The mucosal immune system is continuously presented to residual microorganisms in the gut, however it should only assemble an immune reaction towards invading microorganisms (6). Intestinal homeostasis is organised by limiting the contact between gut microbiota and epithelial cells by three mucosal barriers consisting of the mucus

layer, AMPs and sIgA (Figure 3) (6, 13). Goblet cells produce the mucus layers, which in the small intestine consists of a single layer. In contrast, the mucus layer of the colon consists of a dense inner layer attached to the epithelial surface containing few microorganisms and a loosely organised outer layer which is more densely populated with bacteria (13) (Figure 3).

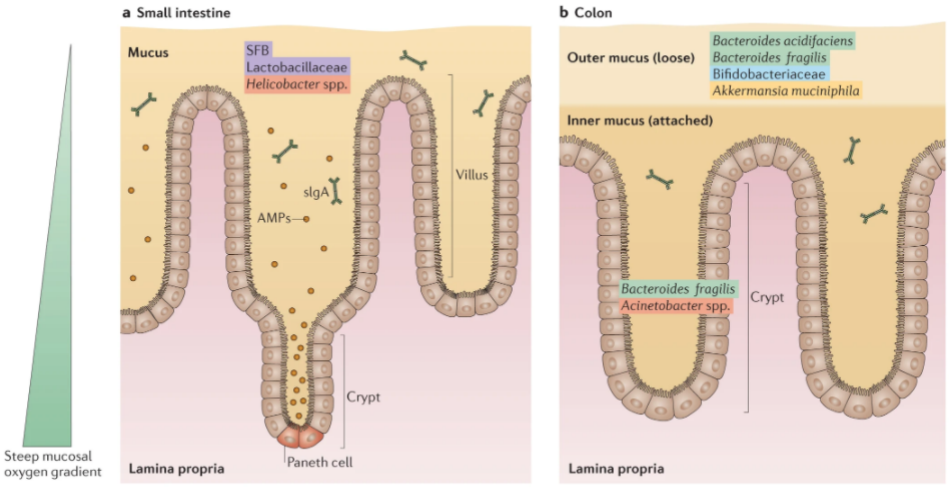

Figure 3. Illustration of the mucosal barriers consisting of the mucus layer, secretory immunoglobulin A (sIgA) and antimicrobial peptides (AMPs). Organization of mucus layers in the small intestine (a) and the colon (b) and the bacteria inhabiting the mucus layers. sIgA produced in plasma cells in lymphoid tissues. AMPs are produced in Paneth cells. Figure from Donaldson et al. (13). Reprinted with permission from Nature Springer.

Plasma cells produce sIgA, which is secreted into the lumen where it coats bacteria and binds to microbial antigens and toxins. AMPs are mainly produced in Paneth cells and act bactericidal (9). Additionally, intestinal epithelial cells are connected through tight junctions forming an evident barrier. Intestinal epithelial cells express different pattern recognition receptors (PRRs) which can identify microorganisms' trough pathogen-associated molecular patterns (PAMPs) found on bacteria, fungi and viruses. Activation of PRRs enables the production of mediators which activate immune cells (1). Toll-like

receptors (TLRs), NOD-like receptors, C-type lectin receptors, scavenger receptors and Galectin-3 are different types of PRRs (14, 15).

5.1.4. Immune cells

The enteric immune system consists of immune cells such as dendritic cells (DCs), macrophages, innate lymphoid cells, eosinophils, neutrophils, mast cells, plasma cells (IgA+ B cells being dominating) and T-cells. The immune cells are localised in the epithelium, in Peyer's patches and the lamina propria (Figure 1 and 2). Primarily T-cells are located in the epithelium (1). The number of intraepithelial lymphocytes (IEL) is highest in the proximal GI tract, declining towards the colon (1).

5.1.5. The intestinal immune system

The intestinal immune system is complex and will only be described in brief. DCs are antigen-presenting cells localised in Peyer's patches and lamina propria where they sense, sample and presents microorganism antigens to B- and T- cells (16). DCs have projections called dendrites which can sample the antigens; transepithelial dendrites can extend between intestinal epithelial cells to sample antigens in the gut lumen (1, 16). DCs also engulf live microorganisms, which reside inside the DC as it migrates towards draining lymph nodes, where the microorganism is presented to B- and T-cells (1, 16). Macrophages degrade microorganisms and dead tissue cells through phagocytosis in addition to stimulating epithelium proliferation (1, 16). There are numerous macrophages in the lamina propria, which kills microorganisms which have penetrated the mucosal barrier (6). T-cells comprises of two major types, CD4+ helper cells and CD8+ cytotoxic cells, both found in the lamina propria (16). CD4+ cells can be further sub-divided into T_H1 cells, T_H2 cells, T_H17 cells, T_R1 cell and T_{Reg} cell depending on cytokine production profile (16). B-cells are most prevalent in the lamina propria in the proximal and distal GI tract. The majority of B-cells produce IgA while the rest mainly produce IgM (1). However, during inflammation large amounts of IgG is produced (6).

5.1.6. Physiology and microbiota along the GI tract

The physiological processes in the intestine include transport and uptake of nutrition, barrier function against microorganisms and toxins as well as immune, paracrine and endocrine functions. The human GI tract is the largest reservoir of microorganisms in the human body, and it is estimated that 3.9×10^{13} bacteria reside in the GI tract, with the majority residing in the colon (12). The digestion starts in the mouth by mechanical and enzymatic degradation by chewing and secretion of salvia containing amylase, which catalyses the degradation of starch into glucose. When food is swallowed, it passes through the oesophagus into the stomach. In the stomach, amino acids and increased pH cause increased gastrin release. Gastrin subsequently stimulates histamine release from enterochromaffin-like (ECL) cells which finally stimulates hydrochloric acid (HCl) secretion from parietal cells. The massive production of HCl causes an acidic environment with a pH below 2 (17) that kills most of the ingested microbiota. The other constituents of gastric juice seem to have only little effect on the destruction of infective agents (18, 19).

The processed food is then passed to the duodenum. In the duodenum fat and proteins stimulate cholecystokinin (CCK) release from enteroendocrine I-cells, which stimulates secretion of pancreatic fluid and bile acids from the hepatopancreatic duct into the duodenum. The pancreatic fluid contains amylase, lipase and trypsin. Amylase catalyses the degradation of starch, lipase breaks down lipids into glycerol and fatty acids, and trypsin acts to degrade proteins. The bile acids act to emulsify fat, enabling digestion and absorption of lipids in the small intestine (20). Bile acids also have a great impact on gut microbiota, by both direct antimicrobial effects and by stimulating the production of antimicrobial peptides (AMPs) (20). The majority (95%) of bile acids are reabsorbed in the ileum (20). When acidic content from the stomach enters the duodenum, secretin is released from duodenal S-cells. Secretin regulates pH by direct inhibition of gastric acid secretion and by stimulating bicarbonate release from the pancreas (21). Mean pH in the proximal small intestine has been reported to be around 6 (22, 23). The surface of the small intestine is constructed of millions of villi, 0.5-1.6 mm finger-like protrusions

of the intestinal epithelium making the surface immense. The nutritional uptake occurs along the villi, in the absorptive enterocyte by diffusion or active transportation. The absorptive enterocytes also possess microvilli expanding the surface additionally. Nutritional uptake and absorption of water continue throughout the small intestine.

The bacterial density increases along the GI tract (24), a recent study found that the jejunum harboured 10^3-10^6 colony forming units (CFU) per mL content (CFU/mL) (25). Both luminal and mucosa-associated jejunal flora, investigated by culture-dependent technique and 454-pyrosequencing respectively, are dominated by *Streptococcus*, *Veillonella, Prevotella*, *Rothia, Escherichia*, *Fusobacterium*, *Haemophilus* and *Fusobacterium* genera (25, 26). Proteobacteria, Actinobacteria and Fusobacteria phyla are more abundant in the jejunum vs in the colon, while *Ruminococcus* and *Faecalibacterium* and other anaerobes frequently found in the colon were nearly absent in the jejunum (25). Kashiwagi et al. (27) investigated the mucosa-associated microbiota in the upper and lower GI tractus of 17 healthy individuals by luminal brush cytology and found lower α-diversity (observed species and Chao1 index) in the upper GI tract compared to the lower GI tract as well as differences in β-diversity (weighted UniFrac) between the upper and lower GI tract. In addition, they found no intra-individual differences in microbiota composition between different locations within the upper (incl. intraoral, mid-oesophagus, gastric corpus, gastric antrum, and duodenum) nor lower GI tract (27). In contrast, differences in the relative abundance of genera at different locations within the ileum and colon have been reported by others (28).

Parallel to the increase in pH from the duodenum to the ileum, the abundance of bacteria increases towards the distal ileum (Figure 4). This can be explained by several factors additional to the pH, including reduced level of AMPs along the small intestine, lower oxygen levels, reduced effect and concentration of bile acids which has antimicrobial effects and due to shorter transit time compared to the colon transit time, despite longer length of the small intestine (13, 20) (Figure 4).

The intestinal content passes the ileocecal valve into the colon. In the colon water, electrolytes and 5-10% of energy requirements are absorbed (29). Intestinal bacteria in the colon participate in digestion by degrading fibre or polysaccharides and protein into the small chain fatty acids (SCFA) acetate, propionate and butyrate which are absorbed in the colon and constitute the colonic energy uptake (20, 29). Colonic bacteria also synthesise vitamin K2 from K1, and Clostridial species convert primary bile acids to secondary bile acids (20). Secondary bile acids are presumed to be protective in inflammatory bowel disease (IBD) (20), but have found to be reduced in IBD dysbiosis (30). Also, the gut microbiome participates in xenobiotic metabolism, and the microbiota composition may affect energy utilisation (31).

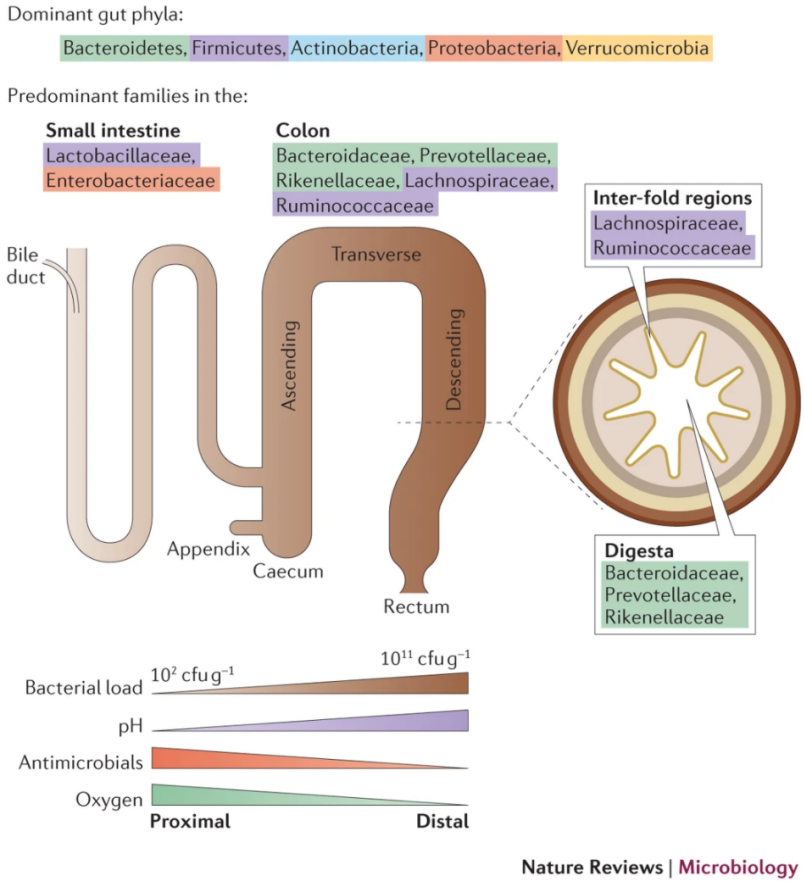

Figure 4. Illustrating bacterial gut microbiota abundance and composition from small intestine to rectum in relation to pH, antimicrobials and oxygen. Figure from Donaldson et al. (13). Reprinted with permission from Springer Nature.

5.2. Inflammatory bowel disease

5.2.1. Aetiology, pathogenesis and epidemiology

IBD are chronic inflammatory diseases of the gastrointestinal (GI) tract and include ulcerative colitis (UC), Crohn's disease (CD) and a smaller proportion of IBD named indeterminate colitis. The exact pathogenesis of IBD is not known, but the leading consensus is that a complex interaction between genetic factors and the environment causes an inappropriate chronic activation of the mucosal immune system. The last decade, it has been suggested that the activation of the immune system is caused by a microbial shift, commonly termed dysbiosis. IBD are chronic diseases evolving in a relapsing and remitting pattern (32, 33). The highest reported incidence and prevalence for CD and UC are 29.3 and 322 per 100.000 for CD in Australia and Europe respectively and 24.3 and 505 per 100.000 for UC in northern Europe and Europe respectively (32, 33). Worldwide there has been an increased incidence of IBD, which substantiates environmental factors' contribution to the pathogenesis (32, 33). IBD affect both genders equally, and peak disease onset is between 20-40 years for CD and 30-40 for UC (32, 33).

5.2.2. Ulcerative colitis

UC is characterized by inflammation limited to the mucosal layer in the colon presenting as continuous inflammation extending from the rectum and proximally, with various extensions (32, 34). From the Norwegian IBSEN-study, it was reported that 33 % had rectosigmoid disease involvement, 35 % left-sided disease involvement and 32 % had pancolitis at diagnosis respectively (35). Typical symptoms of UC include increased frequency of defecations, rectal bleeding, increased mucus discharge, incontinence,

urgency, nocturnal defecations and fatigue. Abdominal pain is not primarily a symptom of UC, however some patients experience abdominal discomfort. Progression of disease is common, 10 years after diagnosis 21.2 % of patients with rectosigmoid or left-sided involvement progressed to pancolitis (35). UC can develop into fulminant colitis with a risk of bowel perforation or toxic megacolon. The cumulative rate of colectomy after 10 years was reported to be 9.8 % in Norway in 2009 (35). Dysplasia development may complicate long-standing UC, and the risk correlates with disease extension with pancolitis having the highest risk of colorectal cancer, left-sided colitis intermediate risk and proctitis having no increased risk (36). Currently, surveillance colonoscopies are recommended to start 8 years after onset of symptoms and surveillance interval determined based on the severity of inflammation, disease extension, presence of dysplasia, polyps or strictures, primary sclerosing cholangitis and family history of colorectal cancer (36, 37).

5.2.3. Crohn's disease

CD is characterized by transmural inflammation with a skip lesion pattern, or discontinuous involvement, that can manifest anywhere in the GI tract (34). Approximately 1/3 of all patients have isolated involvement of the terminal ileum, 1/3 have ileocolonic involvement, and 1/3 have colonic involvement only (38). Additionally, a small subset (<5 %) of CD patients has upper GI tract involvement, and 30 % has perianal involvement, which may include fistulas and abscess formation (33). Typical symptoms of CD depend greatly on affected GI segment, but abdominal pain, diarrhoea, fatigue and weight loss are typical. If the distal colon or rectum is involved, the patient can present with bloody diarrhoea or rectal bleeding. Up to 80 % of CD patients require surgical intervention for their disease (39). The majority of CD patients undergoing ileocecal resection experience recurrence of the disease within a year (75-80 %), and the most frequent location for recurrence is immediately proximal to the surgical anastomosis (33, 39-41). Similarly to UC, CD patients have increased risk for development of colonic dysplasia and surveillance ileocolonoscopy is recommended 8

years after onset of symptoms with surveillance intervals determined by the same criteria as for UC (36, 37).

5.2.4. Diagnosis

Diagnosis of IBD is based on a combination of symptoms, endoscopic, radiologic and histologic findings (32, 33). A blood test can reveal anaemia, iron deficiency, increased CRP and leucocytosis. Also, faecal calprotectin can be used as a diagnostic tool. Faecal calprotectin correlates with the release of neutrophilic granulocytes in faeces and can be used for diagnostic purposes, low calprotectin values make IBD unlikely, whereas high levels indicate an inflammatory condition in the GI tract (32). However, an airway infection may also result in elevated faecal concentrations of calprotectin.

Endoscopic evaluation by ileo-colonoscopy is the most useful examination to diagnose IBD. Ileo-colonoscopy provides information regarding degree of inflammation, localization of inflammation and possibility to take mucosal pinch biopsies which can be used for histologic evaluation. Endoscopic findings in UC include erythema, decreased or loss of vascular patters, varying degrees of friability, erosions, ulcerations and spontaneous bleeding (36). Endoscopic findings in CD include segmental inflammation with normal mucosa in between inflamed segments, aphthous ulcers, serpiginous and longitudinal ulcerations, cobblestone pattern inflammation and strictures (33, 36).

Common histologic findings in IBD are epithelial damage, destruction of crypts, infiltration of immune cells such as neutrophils, eosinophils and lymphocytes in the lamina propria and epithelium, erosions and ulcerations (42-44). Histologic hallmarks can if found, help to differentiate between UC and CD (Figure 5). In UC crypt abscess formation is common (34), whereas in CD granulomas are typical, granulomas are found in 25-37 % of CD patients, in 14-56 % of endoscopic biopsies and 37-87 % of surgical specimens (45).

Cross-sectional imaging such as computer tomography (CT) and magnetic resonance imaging (MRI) can also help diagnose and establish disease extension in CD (36). CT and MRI can reveal thickened and ahaustral colon in UC, but sensitivity and specificity are insufficient to be used alone as diagnostic tools for UC (32).

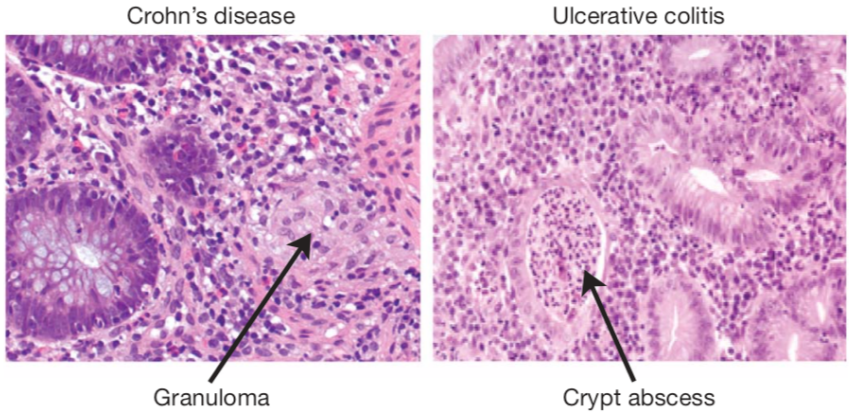

Figure 5. Histologic hallmarks of Crohn's disease and ulcerative colitis. Figure from Xavier et al. (34). Reprinted with permission from Nature Springer. To the left a granuloma (arrow), to the right crypt abscess (arrow).

5.2.5. Genetics

Genome-wide association studies have as of 2020 identified around 240 IBD loci, most of them associated with both CD and UC, whereas 49 were CD specific and 32 UC specific (46-50). Mutations in for instance NOD2, ATG16L1, IRGM, TLR4 and CARD9 support the idea that microbiota is important in IBD pathogenesis, as genetic variants cause impaired sensing and handling of intestinal bacteria (51), these will be discussed in more detail below. An overview of IBD genetic susceptibility genes associated with impaired handling of gut microbiota is provided in Figure 6.

In European IBD twin studies the concordance rate for CD have been reported to be 20-50% and <10% for monozygotic and dizygotic twins respectively, whereas for UC the concordance rates was 16% for monozygotic twins and 4% for dizygotic twins (reviewed by (52)). Higher concordance rates for monozygotic twins versus dizygotic twins

demonstrate the degree of genetic contribution. Higher concordance rates for CD vs UC suggests that genetic predisposition plays a more prominent role in the pathogenesis of CD.

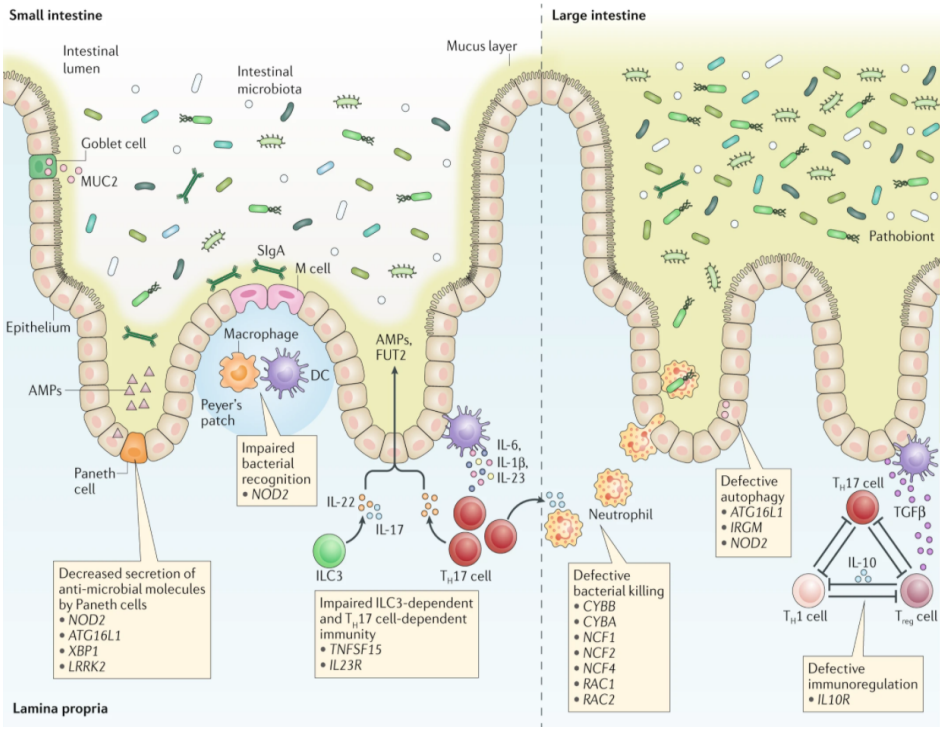

Figure 6. Illustration of IBD genetic susceptibility genes associated with impaired handling of gut microbiota. Figure from Caruso et al. (6). Reprinted with permission from Nature Springer. MUC2; mucin 2, SIgA; secretory immunoglobulin A, AMPs; antimicrobial peptides, FUT2; fructosyltransferase 2.

5.2.5.1. NOD2/CARD15

Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) is an intracellular receptor that recognizes bacterial peptidoglycans which belong to the class of PRRs. Simplified NOD2 acts as a detector for intestinal bacteria. Activation of NOD2 receptor causes a cascade of downstream signalling, including activation of nuclear factor κB (NF-κB) (53) that causes an appropriate immune reaction. Mutations in the

NOD2/CARD15 gene may cause altered microbial recognition, sensing and handling of intestinal bacteria. NOD2 mutations are the genetic variant most strongly associated with CD, and it has been estimated that NOD2 accounts for approximately 20% of the heritability in CD (54). The three most common NOD2 single nucleotide polymorphism (SNP)'s are SNP8 (SNP: rs2066844, protein coding variant: R702W), SNP12 (SNP: rs2066845, protein coding variant: G908R) and SNP13 (SNP: rs2066847, protein coding variant 1007fs), these variants account for 81% of NOD2 mutations, whereas the remaining 19% is due to rare variants (55).

NOD2 is expressed in Paneth cells and crypt epithelial cells (56). It has been suggested that NOD2 mutations are associated with a reduced α-defensin expression in CD patients (57). NOD2 activation is linked to activation of ATG16L1 and IRGM, and thereof involved in the regulation of autophagy of intestinal bacteria (54, 58). Autophagy is a cellular degradation process where cytoplasmatic material is wrapped into autophagosomes and degraded by lysosomal degradation. Mutations in NOD2 inhibits autophagy activation, thereby compromising autophagy of possible pathogen intestinal or intracellular bacteria.

5.2.5.2. ATG16L1

Autophagy-related protein 16-1 (ATG16L1) is a protein that is involved in the initiation of the autophagy process by formation of autophagic vacuoles in dendritic and epithelial cells. ATG16L1 mutations are similar to NOD2 associated with increased risk for CD, but not UC. The most common SNP associated with CD is (SNP: rs2241880, protein coding variant T300A). ATG161L mutation cause impaired bacterial clearance and antigen presentation (59). CD patients homozygous for T300A in ATG16L1 show abnormal Toll-like receptor signalling and Paneth cell dysfunction (59).

5.2.5.3. IRGM

Immunity-related GTPase M (IRGM) is also involved in autophagy of intestinal bacteria and polymorphisms associated with CD (46, 60). It has been shown that IRGM interacts with NOD2 and ATG16L1 to form a complex which regulates the autophagic machinery activation in response to PAMPs (58). IRGM both acts an activator of the autophagy through phosphorylation and activation of autophagy mediators, but also as an inhibitory effect by reducing NOD2 levels, thereby stabilizing the autophagic circuit (58). Knockout of IRGM in human macrophages infected with *Mycobacterium tuberculosis* is associated with increased bacterial survival (60).

5.2.5.4. TLR4

Toll-like receptor 4 (TLR4) belongs to the family of PRRs. TLR-4 recognizes lipopolysaccharides (LPS) in bacteria and several fungal PAMPs (15, 61). TLR-4 is expressed in the intestinal epithelium and mononuclear cells in the lamina propria, and increased expression of TLR-4 are found in IBD (61). TLRs activation is essential for the initiation of immune responses to infections, however prolonged activation can be deleterious and associated with inflammatory disease (61). TLR4 Asp299gly polymorphism is associated with IBD (62). TLR4 polymorphisms are associated with a predisposition for systemic *Candida* infections in humans (63).

5.2.5.5. CARD9

Polymorphisms in Caspase recruitment domain-containing protein 9 (CARD9) is associated with both UC and CD (46, 64). The standard CD disease risk allele is rs4077515 (65). The C-type lectin receptors belonging to the family of PRRs are essential in detecting fungi, for instance, Dectin-1 detects β-glucan in the fungal cell wall, after ligation to Dectin-1 a signalling cascade begins which normally activates CARD9 which enables an appropriate immune response (66, 67). Similarly, the C-type lectin receptor Dectin-2 and macrophage-inducible C-type lectin also detect fungi, and both depend on

the CARD9 pathway (66, 67). Genetic defects in CARD9 in humans is associated with susceptibility to fungal infections and a reduced number of Th17-cells (66).

5.3. Microbiota

5.3.1. Terms and definitions

The terms microbiome and microbiota are often used interchangeably. In an early publication from The Human Microbiome Project, the microbiome was defined as the genomes of microbial symbionts and microbiota is defined as microorganisms living inside or on humans (68). As the field of microbiome research has evolved, inaccurate definitions have been clarified, and definitions drafted more precisely (14). The term microbiome comprehends a wider set of information than microbiota; it includes all microorganisms (bacteria, fungi, viruses, archaea and bacteriophages), their genome sequences and metabolomics of a defined habitat (14). Bacteriophages or phages are viruses which infect bacteria, these are the most abundant viruses in the GI tract (69). Microbiota is confined to the microorganisms in a specific environment, including bacteria, fungi, viruses, archaea and bacteriophages (14). The terms microbiome and microbiota have often been used inaccurately referring only to the bacterial population. However, both terms can be used with sub-specification such as bacterial microbiome or bacterial microbiota. Other designations such as bacteriome, fungal microbiome or mycobiome, viral microbiome or virome can be used to describe the microbiome. Finally, the fungal microbiota is also referred to as mycobiota.

The designation symbionts are used for organisms that live in close interaction with other organisms (13). The relationship between symbionts can be divided into three categories; mutualism, commensalism and competition (14). Mutualism describes a relationship where both organisms benefit from each other. In commensalism (organisms designated commensals) one organism benefit while the other is not affected by the relationship. The designation commensals have also been used as a term for the resident gut microbiota (13). Competition involves negative effects for both

organisms. Further, the term pathobionts are used about microorganisms that generally live as symbionts, but during certain circumstances can induce pathology (6).

5.3.2. Microbial taxonomy and nomenclature

In biology, different organisms are grouped based on their similarities, both morphological, molecular, genetic, metagenomic and metabolomic, this is called taxonomic ranking (70). A taxon (plural; taxa) is a group of organisms that forms a unit or a named group (71). Many different ranking systems have been suggested throughout the centuries. In this thesis, organisms are classified based on the Catalogue of Life ranking system (70) and the International Code of Nomenclature of Prokaryotes (71). Table 1 gives an overview of the different taxonomic levels from phylum to strain.

Taxonomic rank	**Suffix**	**Example**
Phylum		*Proteobacteria*
Class	-ia	*Gammaproteobacteria*
Order	-ales	*Enterobacteriales*
Family	-aceae	*Enterobacteriaceae*
Genus		*Escherichia*
Species		*Escherichia coli (E.coli)*
Strain		NCTC 90001 / ATCC 11775

Table 1. Overview of suffixes and bacterial taxa names within the different taxonomic orders. NCTC; National Collection of Type Cultures, ATCC; American Type Culture Collection.

Above the rank of phylum is kingdom. The different kingdoms include Archaea, Bacteria, Protozoa, Chromista, Fungi, Plantae and Animala. Above the rank kingdom is superkingdom, which includes Prokaryota (including Archaea and Bacteria) and Eukaryota (including Protozoa, Chromista, Fungi, Plantae and Animala). (70). Viruses are currently not a part of the tree of life as they are not able to live or replicate without a host.

The nomenclature of microorganisms follows specific rules. The name of a taxon within the ranks of phylum, class, order, family and genus should be capitalised. Both genus and species name should be used, whereas only the genus name is capitalised and the species name is not (71). According to recommendations from International Code of Nomenclature of Prokaryotes scientific names of taxa (any rank) should preferably be written in a different font, for example, italic (71). However, The Chicago Manual of Style recommends that ranks higher than genus (phylum, class, order, family) should be capitalised, but not italicised (72). This thesis follows the recommendations from The Chicago Manual of Style. After the first use, the genus name can be abbreviated to its initial capital letter, for example, *E.coli* instead of *Escherichia coli* (71).

5.3.3. Microbial diversity

The microbiome diversity is a measure of the number of different taxa and the abundance of them (73). A distinction is made between α-diversity and β-diversity; α-diversity is a measure of diversity within a sample or environment, also called biodiversity. Whereas β-diversity is a measure of diversity between different samples or different environments (73, 74).

Several different α-diversity indices or measures exist, however a consensus regarding which method or measure that should be used in various settings has not been reached (74). Some standard α-diversity measures are presented below.

- Richness is a quantitative measure of the number of different taxa or organisms within a particular sample or environment, for example, the number of species detected within a biopsy sample is called species richness (73). The richness is simply a count and does not take into account the abundances of the different taxa (Figure 7).
- Evenness describes the abundances of taxa in a sample and gives information regarding the distribution of taxa; are the taxa equally distributed or are some

taxa dominating? Low evenness describes an environment where some taxa are dominating, illustrated in the lower left box in Figure 7.

- Chao1 index is a non-parametric method which estimates species richness but also intends to correct for underestimation of species richness due to loss of species during sampling or sequencing (75). Chao1 index uses the number of species with one or two counts to correct the observed number of species in order to estimate a more realistic number of species within a sample (74).
- Shannon diversity is a complex index which takes both the species richness and relative abundance of each species into account, the index is calculated on a logarithmic scale and can therefore not be directly interpreted by its number (74).

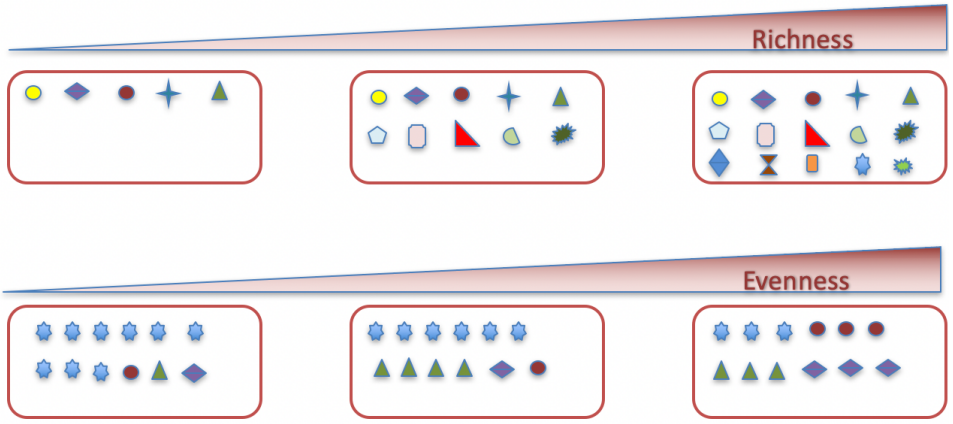

Figure 7. Illustrating the richness and evenness as measures of α-diversity. Increasing richness from left to right (upper three boxes). Increasing evenness (lower three boxes) from left to right. Each symbol illustrates one taxon, similar symbol equals similar taxon.

- Simpson index also takes the species richness and relative abundance of each species into account, but whereas Shannon index emphasises species richness, the Simpson index emphasises species evenness (76).

- Phylogenetic diversity (PD) is a measure that reflects the molecular or evolutionary diversity of taxa within a sample; it estimates diversity by summing the branch lengths along the tree of life covered by one sample (77). PD provides information about the relatedness of the species or taxa within a sample based on evolutionary similarity, as opposed to the other α-diversity measures which only give information regarding the count and distribution of taxa within a sample. Higher PD numbers reflect a more diverse sample covering a larger part of the tree of life (77).

β-diversity measures the diversity between different samples or environments and gives an estimate of how different two communities are (74). A high β-diversity indicates that the two samples or environments have a low number of shared few taxa or species, whereas a low β-diversity indicates that the samples are similar and share most of their taxa (74). The β-diversity is often graphically visualised in Principal Coordinates Analysis (PCoA) plots or Non-metric multidimensional scaling (NMDS) plots. PCoA is based on eigenvalue equations to calculate distance matrix between variables or observations and visualise the distances in a low-dimensional Euclidian space (78). As opposed to Principal Component Analysis (PCA), PCoA can use different measures of association to calculate distance matrix, while PCA is based on covariance/correlation coefficient and requires a linear relationship between the observations/variables (79). The difference between PCoA and NMDS is the distance matrix calculation, PCoA is based on eigenvalue, while in NMDS uses order or rank between observations (78).
The most common β-diversity measures of association are unweighted UniFrac, weighted UniFrac, Jaccard index and Bray-Curtis dissimilarity.

- UniFrac or unique fraction metric measures the phylogenetic distance between taxa on the phylogenetic tree by measuring the percentage of branch lengths of the tree that is unique to one sample or environment (80). If two samples or environments have no unique branches, they are considered phylogenetic similar, contrary if two samples share no

branches and each sample only contains unique branches they are considered phylogenetic maximum different (80).

- Weighted UniFrac emphasises the abundance of taxa in the calculation so that the most abundant taxa are considered more important (81).
- Unweighted UniFrac only accounts for the presence or absence of different taxa and does not use abundance information in the calculation. Therefore abundant and rare taxa are similarly emphasised. Unweighted UniFrac is therefore efficient in terms of accounting for changes in abundance of rare taxa (81).
- Bray-Curtis dissimilarity is a metric which quantifies the compositional dissimilarity between two samples based on the taxa counts in each sample. Bray-Curtis is considered an abundance-based β-diversity index (82). The Bray-Curtis dissimilarity is calculated by the formula:

$$BC = \frac{2C_{ij}}{(S_i + S_j)}$$

where j and i are the two samples, C_{ij} is the sum of the minimum value of each species found in both samples, S_i and S_j are the total number of taxa present in sample i and j respectively (Figure 8) (83). The Bray-Curtis dissimilarity is bound to be between 0 and 1, where 0 implies the two samples have the same composition (share all taxa), and 1 implies that the two samples do not share any taxa.

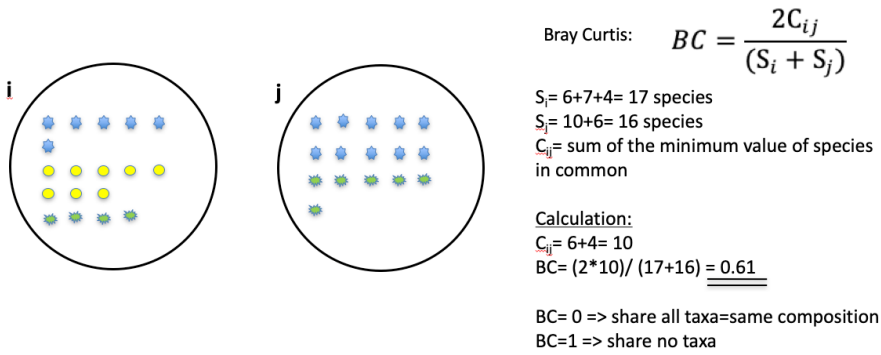

Figure 8. Illustrating calculation of Bray Curtis measure of association. The circles (i and j) represent two different samples (S_i and S_j). Each of the symbols within each circle represent one species, similar symbols equal similar species. Sample i consist of 17 species in total and three different species. Sample j consist of 16 species and two different species. C_{ij} is the sum of the minimum value of each species found in both samples, S_i and S_j are the total number of taxa present in sample i and j respectively.

- Jaccard index is a so-called presence-absence index which focuses on more on rare species in comparison to abundance-based indices such as Bray-Curtis (82). Jaccard index is calculated by the formula:

$$dJ = [1 - \frac{a}{a + b + c}]$$

where *a* is the number of shared species between samples, *b* is the number of species occurring exclusively in sample i, *c* is the number of species occurring exclusively in sample j (84).

5.3.4. Healthy gut microbiota

Gut microbiota is a common designation for all microorganisms harbouring the GI tract including bacteria, fungi, protozoa and viruses including bacteriophages. The core gut microbiota, is the microbiota shared between individuals. The bacterial component is stable and is estimated to consists of approximately 40 bacterial species which constitute 75% of the bacterial abundance (13). The gut bacterial microbiota is dominated by Firmicutes and Bacteroidetes phyla (13). One gram of stool from healthy

individuals contains 10^{11} and 10^{2}-10^{6} cells of bacteria and fungi, respectively (12, 85). However, the fungal cell is approximately 10-fold longer and have a 100-fold larger volume than most bacteria making the fungal biomass substantial (14). The most common fungal phyla are Ascomycota and Basidiomycota, the normal allocation is 70% and 30%, respectively (14). Fungal diversity is lower than bacterial diversity, less than 20 fungal species are generally identified with high inter-individual variability (14, 86).

5.3.5. The role of microbiota in IBD

Several findings support the importance of the microbiota in IBD pathogenesis. First, the increasing incidence of IBD worldwide after World War II and increasing incidences in developing countries substantiates the importance of environmental factors (87). From twin studies, we know that the concordance rate for CD and UC in monozygotic twins are 20-50% and 14-19% respectively (52), advocating the significance of environmental factors. The hygiene hypothesis postulates that the increasing incidence of allergic and autoimmune diseases, including IBD, can be explained by decreased exposure to microorganisms in childhood due to better sanitary conditions (87, 88). The gut microbiota is vital for the development and regulation of the immune system and maintenance of intestinal epithelial barrier homeostasis (6). Several of the genetic polymorphisms associated with IBD are involved in recognition and eradication of bacteria in the intestine, supporting that IBD patients have genetic variants that lead to impaired defence against gut microbiota (6). The genetic defects in IBD in combination with triggering alterations in the gut microbiome composition can cause increased invasion of pathobionts into the epithelium and lamina propria causing a cascade reaction with inflammation and additional gut microbiome perturbations (6).

Even though colitis in mice does not exhibit human IBD characteristics, research from several germfree mouse models has found that mice genetically susceptible for colitis do not develop colitis until they are exposed to an IBD-like dysbiotic microflora (89). After ileocecal resection in patients with CD, diversion of the faecal fluid to bypass the neoterminal ileum prevents recurrence of disease and restoration of the gastrointestinal tract or infusion of intestinal content lead to recurrence of the

inflammation (43, 90). In UC, faecal microbiota transplantation (FMT) has proved to be significantly better than placebo for inducing remission (91). A recent Swedish national case-control study found an association between the cumulative antibiotic exposure and development of IBD, arguing that microbiota disruptions can be detrimental (92). On the contrary, selected antibiotics and probiotics in patients with established IBD can induce remission or reduce disease activity. A correlation between IBD risk and use of antibiotics is not equivalent to causation, but these data supports that the microbiota composition is of relevance in the pathogenesis (93-96). The microbiota in IBD patients differs from healthy controls, however if these differences occur before the inflammation or is a consequence of inflammation is not yet fully understood.

5.3.6. Microbiota alterations in IBD

Microbiota alterations in IBD are commonly called dysbiosis. Dysbiosis is defined as a shift or imbalance in the microbial composition or community (97). IBD dysbiosis is characterised by reduced microbial diversity, an increase of potentially harmful bacteria and a decrease of bacteria characterised as beneficial (94, 98-100). The majority of studies within the field of IBD gut microbiota have analysed faecal samples. However, the faecal and mucosa-associated microbiota are very different (24, 101-105). The accessibility of faeces compared to mucosa samples is indisputably the most important reason why most microbiota studies are based on faecal samples. Nevertheless, as the epithelium is central in IBD, knowledge about the microbes adjacent to the epithelium seems pivotal in terms of understanding the microbial contribution to the disease pathogenesis (106). Therefore, this thesis has focused on the mucosa-associated microbiota in IBD.

Reduced mucosal bacterial diversity has been reported as the main feature of IBD dysbiosis (99, 107, 108). It has been debated whether reduced diversity is due to the disease, inflammation or a combination. Both Liguori et al. and Kansal et al. found mucosal diversity in CD to be the same in inflamed and non-inflamed regions, suggesting that loss of diversity cannot be driven by inflammation alone (107, 109). Mucosal IBD

microbiota (including both UC and CD) has further been described by increases in Proteobacteria phylum and *Clostridium* genera as well as decreases in *Roseburia* genus and the species *Faecalibacterium prausnitzii* (99, 101, 110, 111). The Proteobacteria phylum includes the majority of well-known enteropathogens and increased abundances are associated with increased IBD disease activity (110, 112). The Firmicutes phylum includes the majority of short chain fatty acid (SCFA) producing bacteria. SCFA are beneficial metabolites, especially butyrate, as it supplies colonocytes with nutrition and strengthens epithelial integrity (113). The depletion of *F. prausnitzii* is particularly thought to be of importance due to its anti-inflammatory effects, inverse correlation with IBD disease activity and that increased faecal abundances are associated with long-term remission (111, 114, 115).

5.3.7. Mucosal microbiota in UC

At phylum level, the abundance of Proteobacteria is increased in UC patients compared to HC (110, 116, 117), whereas conflicting results regarding abundances of Bacteroidetes and Firmicutes have been reported (108, 116-119). Further, reductions in *Faecalibacterium prausnitzii* species and the genera *Roseburia*, *Akkermansia* and *Odoribacter* have been described, the latter being depleted in UC pancolitis patients (99, 101, 120). Several papers have found the microbial alterations in UC to be less pronounced than in CD (28, 99, 101). A recent Nature publication reported a greater loss of potentially beneficial taxa in CD in comparison to UC. When comparing the microbial communities of UC and CD they found increased abundances of Ruminococcaceae, Lachnospiraceae and Faecalibacterium families as well as *Coprococcus*, *Lachnospira*, *Roseburia*, *Bifidobacterium* and *Prevotella* genera in UC vs CD in two separate cohorts (99). They also found UC patients to be more similar to HC in terms of β-diversity (Bray-Curtis) vs CD patients (99). Only a minority of papers describing the mucosal microbiota analysed by high-throughput sequencing have exclusively included UC patients (120, 121).

Fungal diversity in the colonic mucosa of UC patients has been reported to be similar to HC (122), while the fungal genus *Aspergillus* was significantly increased in UC patients compared to HC (122). However, the mucosa-associated mycobiota in UC, has only to a small extent, been investigated. The viral mucosa-associated microbiota in UC patients is characterised by a higher abundance of Hepadnaviridae family and a correlated decreased abundance of Polydnaviridae and Tymoviridae families in comparison to HC (123). Additionally, increased abundances of Caudovirales bacteriophages, *Escherichia phage* and *Enterobacteria phage* have been found and decreased viral diversity in the inflamed UC mucosa has been described (124).

5.3.8. Mucosal microbiota in CD

Increased abundances of Proteobacteria and Fusobacteria are found in the mucosa of CD patients (100, 109, 110, 125), whereas reported abundances of Firmicutes and Bacteroidetes have been conflicting (109, 118, 126). In addition to reduced bacterial α-diversity significant differences in β-diversity between CD patients and HC have been found (99, 100). Many specific alterations in the mucosal bacterial microbiota on family and genus level have been reported, but most frequently an expansion of Enterobacteriaceae, Fusobacteriaceae and reductions in Lachnospiraceae, Ruminococcaceae, Bifidobacteriaceae and Erysipelotrichaceae families are found (28, 102, 110, 125, 127). On genus level expansion of *Fusobacterium*, *Escherichia*, *Enterococcus*, *Haemophilus* and reductions in *Roseburia* and *Faecalibacterium* are reported (99, 100, 102, 107, 109, 125, 127). Yilmaz et al. (99) have described a cluster of bacteria occurring together, which they call CD_A cluster, consisting of *Lachnospira*, *Blautia*, *Dorea*, *Coprococcus*, *Ruminococcus*, *Faecalibacterium*, *Roseburia*, *Oscillospira* and *Bilophila*. They report that reductions in this cluster are associated with worse outcome, poor treatment response, less healthy lifestyle and increased risk of relapse after surgery in two separate cohorts. In treatment-naïve paediatric patients increased abundance of Enterobacteriaceae, Pasteurellaceae, Veillonellaceae and Fusobacteriaceae and decreased abundance of Erysipelotrichales, Bacteroidales and

Clostridiales are strongly correlated to disease severity (102). In CD patients undergoing ileocecal resection endoscopic recurrence has been associated with increased abundance of *Proteus* and reduced abundance of *Faecalibacterium* genera (100). Similarly, a recent study found increases in Proteobacteria, especially Alphaproteobacteria, Coribacteriaceae family and *Enterococcus* genus and decreases in Firmicutes phylum, especially Ruminococcaceae and Lachnospiraceae family and *Eubacterium*, *Ruminococcus*, *Butyricoccus*, *Dorea* and *Blautia* genera in samples from patients with postoperative recurrence vs patients without recurrence (128). The same study identified increased abundances of Gammaproteobacteria and *Ruminococcus gnavus* and decreased abundance of *Ruminiclostridium 6* at the time of ileocecal resection to be predictive of postoperative disease recurrence (128). Decreased abundance of *Faecalibacterium prausnitzii* at ileocecal resection has also been found to be predictive of disease recurrence six months postoperatively (114).

The fungal diversity in CD mucosa has been reported to be similar to HCs (109, 129). A characteristic of CD mycobiota is a reduced Ascomycota/Basidiomycota ratio compared to HC (67, 129). CD mycobiota alterations also include increased abundances of Psathyrellaceae, Cortinariaceae and Cystofilobasidiaceae families and *Psathyrella*, *Gymnopilus*, *Malassezia*, *Cladosporium*, *Aureobasidium* and *Dioszegia* genera together with decreased abundances of *Fusarium*, *Leptosphaeria* and *Trichosporon* genera *(67, 109, 129).* On species level, increased abundances of *Malassezia globosa, Malassezia restricta* and *Candida glabrata* have been reported (67, 109). The mucosa-associated viral microbiota in patients with CD is characterised by increased abundance of Hepeviridae family together with a significantly negatively correlated reduced abundance of Virgaviridae family in comparison to HC (123).

5.4. IBD treatment

5.4.1. 5-ASA

5-aminosalicylic acid (5-ASA) or mesalazine acts locally in the intestinal mucosa, where it is metabolised to its inactive compound N-acetyl-5-aminosalicylic acid (Ac-5-ASA) (130, 131) by N-acetyltransferase 1 (NAT1) and to a small degree by N-acetyltransferase 2 (NAT2) (132, 133). 5-ASA has been suggested to act through numerous mechanisms inhibiting pro-inflammatory mediators such as leukotrienes (134, 135), prostaglandin (134), interleukin 1 (136), NF-κB (137, 138) and tumour necrosis factor-α (TNFα) (137). Additionally, 5-ASA acts as a peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist, and expression of PPAR-γ is reduced in UC patients (139, 140). Recent studies also suggest that 5-ASA has effects on various bacteria as a polyphosphate kinase (PPK) inhibitor that decreases some bacteria's ability to colonise and increase their susceptibility to oxidative stress (141, 142).

Oral administration of unbound 5-ASA leads to quick absorption of 5-ASA from the upper GI-tract followed by rapid acetylation and inactivation in the intestinal epithelium and liver by NAT1 (130, 131), thus will 5-ASA not reach the inflamed intestinal segments and exert an effect. Similarly, only small fractions of intravenously administered 5-ASA reach the intestine (131, 143). Therefore, several pharmaceutical delivery systems have been developed to transport orally administered 5-ASA to the colon. Following oral administration of various 5-ASA formulations, the highest mucosal concentrations are found in the proximal colon segments and the lowest in the rectum (144-146). Mucosal 5-ASA concentrations reflect the amount of therapeutically active drug at the site of action, and mucosal 5-ASA concentrations are inversely correlated to UC disease activity (144, 147-150). The principle is illustrated by studies of combination therapy with both oral and rectal 5-ASA, which significantly increase mucosal 5-ASA concentrations in the rectum and sigmoid colon and improve the clinical course of UC compared to oral administration alone (146, 151).

The different oral 5-ASA formulations lead to absorption of 5-ASA in the stomach, small intestine or the colon depending on the pharmaceutical drug release mechanism (152). Absorption of released 5-ASA occurs more rapidly in the small intestine than in the colon (143, 153). Metabolism of 5-ASA to Ac-5-ASA occurs in the intestinal epithelium, however some 5-ASA reach the portal circulation and is later metabolised in the liver (Figure 9). 5-ASA is also to a small extent (<4 % compared to mucosal acetylation) metabolised by bacteria in the faeces (154). Ac-5-ASA is either secreted into the gut lumen and excreted in faeces, or absorbed into the portal circulation and excreted in the urine (130).

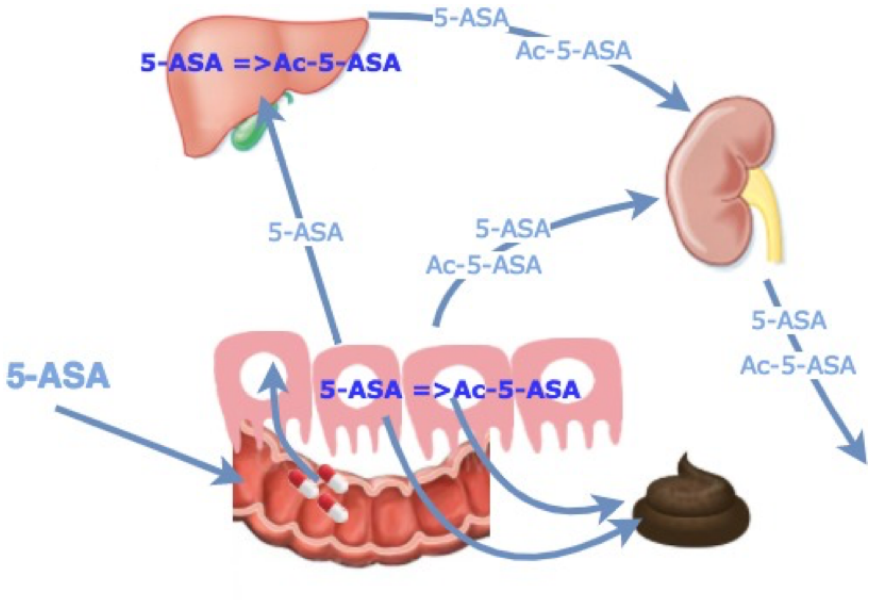

Figure 9. Metabolization of 5-ASA in the intestine and liver and excretion in kidney and faeces. Figure modified from Bondesen et al. (155)

Some 5-ASA will pass through the GI-tract without being absorbed or metabolised and is excreted in the faeces unmetabolised. Pharmaceutical drug formulations that release 5-ASA more distally in the GI tract results in reduced urinary excretion and increased faecal excretion of 5-ASA and Ac-5-ASA (130). Systemic absorption of 5-ASA should be

kept as low as possible, as serum concentrations are not associated with therapeutic effect, but rather a risk of systemic side effects such as nephrotoxicity, hepatitis, pancreatitis, and blood dyscrasias even though rarely occurring (153, 156).

Large inter-individual differences in 5-ASA mucosal concentrations have been reported in patients using the same 5-ASA formulation and dosage (144, 147-149). The underlying mechanism is not understood, but GI transit time, pH alterations and genetic variation in N-acetylation have been suggested. NAT1 is reported to be 19.000 fold more active than NAT2 in N-acetylation/metabolisation of 5-ASA (132). Theoretically, patients with different genotypes leading to different NAT-acetylator velocity could affect the 5-ASA concentration levels, with rapid acetylators being exposed to lower mucosal 5-ASA concentrations. However, NAT1 and NAT2 genotypes have previously not been found to influence 5-ASA treatment efficacy (157).

Based on previous studies of mucosal 5-ASA concentration there seems to be an association between oral 5-ASA dose and mucosal 5-ASA concentration;

- Hussain et al. (158) found an increase in mucosal 5-ASA concentration when the dose was increased from 1-2 g/day to 2.4 g/day of Asacol but found no further increase in mucosal 5-ASA concentration with dose escalation to 4.8 g/day
- D'Haens et al. (148) found that mucosal 5-ASA concentration was greater in patients using 4.8 g/day (48.8 ng/mg) of Mezavant in comparison to 1.2 g/day (11.2 ng/mg) and 2.4 g/day (6.9 ng/mg).
- Frieri et al. (151) found the rectal mucosal 5-ASA concentration to increase after 5-ASA dose escalation from oral 2.4-3.2 g/day (3.065 ng/mg) to oral 3.2-4.8 g/day in addition to topical 5-ASA 4 g/day (259.81 ng/mL).

5-ASA can be administered orally or rectally, and several different 5-ASA formulations exist on the marked (oral mesalazine formulations in Norway as of 2020; Pentasa, Asacol, Mezavant, Salofalk). Pentasa is classified as a time-dependent formulation, consisting of 5-ASA coated with a semipermeable membrane of ethyl cellulose. 5-ASA

discharge distribution has been estimated to be 35% in the small intestine, 25% in the colon and 40% in faeces (159), however a more recent study found that Pentasa also released a significant proportion of 5-ASA in the stomach (152). Asacol and Mezavant both have a pH-dependent capsule dissolving around pH 7, usually occurring in the terminal ileum (130, 153). Furthermore, Mezavant has a Multi Matrix System (MMX), which in addition to the pH-dependent coating includes hydrophilic and lipophilic excipients causing slow discharge throughout the colon (153, 160). The small intestinal 5-ASA absorption of Asacol and Mezavant is estimated to be 20% in pharmacokinetic and scintigraphy studies (153, 161, 162).

5-ASA is the first-line therapy for UC (163), but there are conflicting results regarding effect in CD (164, 165) and the ECCO guidelines do not recommend 5-ASA treatment in CD (165). Current guidelines recommend ≥2.4 g/day to induce remission of UC (163, 166). The ECCO guidelines recommend combination therapy with oral 5-ASA ≥2.4 g/day and rectal 5-ASA ≥1.0 g/day for induction of remission, however, rectal administration may be inconvenient, and adherence to treatment with rectal 5-ASA is low (163). The guidelines also state that doses of 4.8 g/day may be beneficial to induce remission in patients with moderately active UC (163, 167). For maintenance of remission 2 g/day is the recommended dose, but doses ≥2.4 g/day might benefit patients with extensive disease, prior glucocorticoid therapy and age <40 years (163, 168-171). The latest Cochrane report found a trend towards increased effect with higher dosages (169). Also, the British Society of Gastroenterology (BSG) recommend high-dose 5-ASA to UC patients who have required ≥2 courses of corticosteroids the last year, to corticosteroid dependent or refractory UC patients, and UC patients requiring a thiopurine, anti-tumour necrosis factor (anti-TNF) alpha antibodies, vedolizumab or tofacitinib (36). High-dose 5-ASA is not associated with more adverse effects than low dose 5-ASA (168).

A recent ECCO review recommend considering dose reduction down to 2.0 g/ day in patients with endoscopic Mayo score of 0 and avoiding dose reduction in patients with increased faecal calprotectin or patients with either endoscopic or histologic

inflammation, or both (172). Besides, the reviewers discourage 5-ASA withdrawal in patients with a history of repeated relapses or in patients with extensive disease (172). Another recent review found 5-ASA withdrawal to be safer in patients >40 years, no history of frequent relapses, UC patients without extensive disease and patients in remission for 2 years or more (173). Additionally, 5-ASA has a protective effect against colorectal cancer in IBD patients, which is dose-dependent and more pronounced in UC patients than CD patients (36, 163, 174). Consequently 5-ASA is recommended for long-term use in UC patients.

5.4.2. Budesonide

Budesonide is a corticosteroid drug with reduced systemic bioavailability due to extensive first-pass metabolism in the liver (175). Different pharmaceutical delivery systems for budesonide have been developed, time-dependent release and pH-dependent release, causing the release of budesonide to start in the ileum (175) as well as an MMX formula designed to release budesonide throughout the colon (176). Orally administered budesonide 9mg/day is the first-line treatment to induce remission in localised ileocecal CD, which is less effective than conventional steroids, but has less severe side effects (165). Budesonide is not recommended for treatment of colonic CD unless primarily the proximal colon is affected (165). In severe CD, systemic corticosteroid treatment is preferred rather than budesonide (165). Both ECCO guidelines and a Cochrane review concluded that budesonide is not effective for maintenance of remission in CD, despite being associated with longer time to relapse and lower CDAI scores, it should not be used to maintain remission due to its adverse effects (165, 175). In patients with mild to moderate left-sided UC budesonide MMX 9 mg/day can be considered in patients intolerant or refractory to 5-ASA therapy for induction of remission, whereas budesonide is not recommended in UC patients with pancolitis (163, 176).

5.4.3. Systemic corticosteroids

Corticosteroid drugs act through binding to the glucocorticoid receptor and thereby producing widespread anti-inflammatory effects and suppressing the immune system (177). Systemic corticosteroid therapy is also associated with a wide range of adverse effects, including Cushing's syndrome with abdominal obesity, hypertension, acne, moon face, skin striae and muscular atrophy, among others. Corticosteroid therapy is also associated with sleep and mood disturbance, glucose intolerance, osteoporosis and increased susceptibility to infections (165). In CD, systemic corticosteroids are recommended for induction of remission in moderately and severely active ileocecal CD, active colonic CD, CD with extensive small bowel involvement and in CD patients with oesophageal or gastroduodenal involvement and severe disease (165). Corticosteroids are not considered effective for maintenance of remission in CD (165). In UC, systemic corticosteroids are used for induction of remission in moderate to severe UC and intravenous corticosteroids is the mainstay of treatment of severe UC (163).

5.4.4. Azathioprine

Azathioprine is classified as a thiopurine together with mercaptopurine, which is the first metabolite in azathioprine degradation. Azathioprine is an immunosuppressive agent which inhibits ribonucleotide synthesis and induce T-cell apoptosis (165). Azathioprine has a slow onset of action, and it is therefore recommended primarily for maintenance of remission in both CD and UC, however it is also used as a steroid-sparing agent or adjunctive therapy in combination with anti-TNF for induction of remission (163, 165). Azathioprine is superior to placebo for maintenance of remission in both CD and UC (163, 165).

5.4.5. Methotrexate

Methotrexate is a chemotherapeutic agent and an immunomodulator which acts by inhibiting dihydrofolate reductase, essential for the synthesis of purines and pyrimidines, thereby inhibiting both ribonucleic acid (RNA) and deoxyribonucleic acid

(DNA) synthesis. In contrast, the immunomodulatory effect most likely comes from increased adenosine levels and agonistic effect on adenosine A_{2A} and A_3 receptors (178). Methotrexate is proven effective for both induction of remission and maintenance of remission in CD (165) with parenteral administration proven more effective than peroral administration. Methotrexate is not recommended for maintenance of remission in UC but can be considered in patients with steroid-dependent UC for induction of remission (163).

5.4.6. Anti-TNF

Anti-TNFs have potent anti-inflammatory effects through inhibition of TNFα. Anti-TNFs approved for IBD treatment in Norway include infliximab, adalimumab and golimumab. The use of anti-TNF has increased in Norway during the past decade (179). In CD, anti-TNF therapy is considered particularly in patients with moderately active localised CD who are steroid-refractory or steroid–intolerant, in patients with severely active localised or colonic CD who have relapsed after initial corticosteroid treatment or are intolerant to corticosteroids (165). Furthermore, it is recommended that early anti-TNF therapy should be evaluated in patients with extensive small bowel disease and CD patients with high disease activity and poor clinical prognostic factors and in CD patients relapsing while treated with azathioprine (165).

In UC, anti-TNF therapy is recommended in steroid-dependent patients, patients refractory to systemic corticosteroids and in moderate UC refractory to azathioprine (163). For both CD and UC patients, anti-TNF therapy has proven effective for both induction of remission and maintenance of remission (163, 165).

5.4.7. Vedolizumab

Vedolizumab is an antibody specifically targeting $\alpha4\beta7$ integrin, also called anti-integrin therapy (165) and is proven effective both for induction of remission and maintenance of remission in both CD and UC (163, 165). Vedolizumab is generally recommended to

CD and UC patients as an alternative to anti-TNF therapy and to patients refractory to anti-TNF therapy (163, 165).

5.4.8. Antibiotics

Some antibiotics have shown to be effective for induction of remission in CD and UC (94, 163, 165), however the ECCO guidelines do not recommend antibiotic therapy for maintenance of remission in either disease and are also reluctant to recommend it for induction of remission due to side effects and questionable efficiency (163, 165). The ECCO consensus recommends evaluating antibiotics in severe UC only if an infection is likely additionally, two-week antibiotic therapy with amoxicillin, tetracycline and metronidazole can be considered in steroid-refractory UC (163). For steroid refractory CD, data from some clinical trials find metronidazole, ciprofloxacin or the combination of these to have some effect (165). In a randomised controlled trial including patients with moderately active CD, 800 mg of rifaximin twice daily for 12 weeks was significantly better than placebo for inducing remission, however remission rates for doses of 400 mg and 1200 mg were not better than placebo (93). The ECCO consensus recommends antibiotics to be used for septic complications, bacterial overgrowth and treatment of perianal disease in CD (165).

5.4.9. Surgery

In CD patients with extensive disease, surgery is generally avoided due to the risk of short bowel syndrome. ECCO guidelines recommend surgery in CD patients with limited ileocecal disease as well as considering surgery in patients with disease refractory to medical treatment. (165). In cohorts with long-term follow up 75-80% of CD patients have required surgical treatment during the observation period, the most common surgical procedure has been ileocecal resection (ICR) (33, 39, 180). In CD patients with stricturing disease the surgical treatment options are strictureplasty (surgical treatment without losing bowel length) and intestinal resection, however in short strictures (length <4 cm) endoscopic balloon dilatation may be performed, however the number of

strictures, length of involved intestine and total small bowel length also need to be considered before choosing treatment approach (36).

UC can be cured by removing the colon surgically, however due to the implications of total colectomy, medical treatment is preferred when it is effective and tolerated. In patients with disease resistant to medical therapy, colectomy should be considered (163). From the Norwegian IBSEN study, it was reported that 9.8 % of UC patients underwent colectomy during the first 10 years after diagnosis and that the 10-year colectomy rate for UC patients with pancolitis was 19% (35). In UC patients with acute severe colitis, surgery is indicated if the disease is resistant to medical therapy, i.e. insufficient response to rescue therapy, or if the adverse effects of medication are not tolerable or in cases with life-threatening haemorrhage, toxic megacolon or perforation (36). In acute severe UC delayed surgery associated with increased risk of surgical complications (36).

5.4.10. Other medical treatments for IBD

Ustekinumab, an anti IL12/23 p40 antibody, has shown efficacy in inducing remission in active CD (165). Tofacitinib is a Janus kinase (JAK) inhibitor, which is administered orally and has proven effect over placebo in UC patients, but no comparisons have been made with other biologic treatment (36). Indication for treatment with Tofacitinib is moderate to severe UC with intolerance or failure on medical treatment or biological treatment (36). Treatment with anti-Madcam antibodies, anti-IL-6 antibodies and SMAD7 antisense oligonucleotides are currently tested in CD (165).

5.5. Factors impacting the gut microbiota

5.5.1. Age

The scientific studies of microbiota concerning ageing are primarily based on analyses of faecal samples. Studies investigating the mucosa-associated microbiota in children are lacking, due to the ethical aspects preventing invasive sampling. The gut microbiota

undertakes great reorganisation throughout the first year of life. During delivery, the infant is exposed to vaginal and faecal microflora or skin flora depending on delivery method, vaginal or caesarean section, respectively. Delivery method affects the microbiome development at least the first year of life, and vaginal delivery is associated with increased abundances of *Bacteroides* spp and *Candida albicans* (14, 181). Breastmilk contains 8.9 x 10^5 bacterial and 3.5 x 10^5 fungal cells/mL and is rich in *Bifidobacterium* spp. and *Lactobacillus* spp. as well as the fungal genera *Malassezia*, *Candida* and *Saccharomyces* (181, 182). Fungal α-diversity has been reported to be higher in infants and children compared to adults (14) whereas the bacterial α-diversity is lower (183). Especially breastfeeding is associated with lower bacterial diversity due to *Bifidobacterium* dominance (181). During breastfeeding high abundances of the fungi, *Debaryomyces hansenii* has been described (184). Breastfeeding cessation greatly impacts the gut microbiota, interestingly to a more considerable extent than introduction of solid foods and is associated with increased levels of Firmicutes (181, 185). Introduction of solid foods increases the total number of bacteria, bacterial α-diversity and is associated with dominance of Bacteroidetes and Firmicutes phyla and increased abundances of SCFA and the fungi *Saccharomyces cerevisiae* (184, 185). It has been suggested that maturation of gut microbiota occurs in phases, a developmental phase (3-14 months), a transitional phase (15-30 months) and a stable phase (31-46 months) (181). High abundances of Bifidobacterium characterised the developmental phase, whereas Firmicutes and increased bacterial α-diversity dominated the stable phase (181). A recent review described persistently reduced bacterial α-diversity in 5-year-olds and increased abundances of *Bifidobacterium* and *Faecalibacterium* spp. in children 7-12 years of age in comparison to adults (185).

It is generally accepted that the microbiota composition is altered in the elderly. In 2018 An et al. (186) reviewed the findings from faecal microbiota studies in elderly, they concluded that inter-individual variations in microbiota composition were large and harboured many confounding factors such as physical condition, medical treatment, lifestyle including smoking and diet and living situation (health care facility or private

home). Bacterial α-diversity have both been reported to be increased and decreased in elderly, increased abundances of Enterobacteriaceae and decreased abundances of *Bifidobacterium* are generally found, but with a few exceptions. Altogether the authors found it hard to define characteristic microbiota alterations for elderly (186).

5.5.2. Diet and obesity

Diet is one of the factors with the strongest influence on gut microbiota composition (14, 187, 188). Dietary habits are associated with IBD risk in epidemiological studies, for instance, are consumption of soft drinks and red meat associated with an increased risk of UC, sucrose intake associated with both UC and CD while fibre and fruit associated with reduced CD risk and consumption of tea, fruit and vegetables associated with reduced risk of UC (reviewed by (189)). Exclusive enteral nutrition is an effective therapy for induction of remission in paediatric CD patients and is associated with alterations in the mucosa-associated microbiota (190, 191). Liu et al. have recently assessed the bacterial microbiota in colonic biopsies from 34 healthy participants, whom self-reported dietary consumption using a validated food frequency questionnaire (192). Poor dietary quality was associated with lower bacterial α-diversity, reduced abundance of *Roseburia*, *Subdoligranulum* and *Parabacteroides* and increased abundance of *Fusobacterium*, *Escherichia*, *Bilophila* and *Tyzzerella* (192). Summarised, the authors found that dietary quality and intake of fruit, soy and milk products, added sugar, alcohol and saturated fat had the highest impact on the bacterial colonic microbiota (192).

Similar to most other fields, most papers investigating the effect of diet on microbiota have assessed faecal samples (193, 194). The faecal gut microbiome is reported to be rapidly altered depending on diet, an animal-based diet is associated with increased abundance of the bacterial genera *Alistipes*, *Bilophila* and *Bacteroides* and *Bilophila wadsworthia* species as well as the concentration of viable fungi, whereas a plant-based diet associated with increased the concentration of the SCFAs acetate and butyrate and fibre intake positively associated with *Prevotella* (187). Of these taxa, *Bilophila*

wadsworthia is associated with development of colitis in mice models (195). While SCFAs are favourable metabolites in the gut, of which butyrate is the crucial energy source for colonic epithelial cells (20). These findings support that reduced intake of milk and meat and increased plant-based foods can be beneficial for IBD. Interestingly, a low fibre diet in mice is associated with increased abundance of mucus degrading bacterial strains and decreased mucus thickness (196).

Obese individuals have a shifted composition of Firmicutes and Bacteroidetes phyla compared to healthy individuals with decreased abundances of Bacteroidetes (197). Ley et al. (197) showed that two different calorie restriction diets in 12 obese individuals caused increased abundances of Bacteroidetes in faecal samples, which correlated with weight loss and not with changes in calorie content in diet over time. Mice studies have found that colonisation with microbiota from obese individuals to lean individuals causes the lean mice to gain weight due to increased energy harvest, not increased consumption or reduced activity patterns (31, 198). Interestingly, Kootte et al. found that FMT from lean donors to 38 male metabolic syndrome recipients caused improved insulin sensitivity and altered microbiota composition in duodenal and faecal samples six weeks after FMT (199).

5.5.3. Smoking

Tobacco smoking is associated with reduced α-diversity, increased abundances of *Streptococcus*, *Veillonella* and *Rothia* and decreased abundances of *Prevotella* and *Neisseria* in the duodenal microbiota in comparison to never smoking (200). Smoking has also been associated with reductions in the butyrate-producing genus *Anaerostipes* (101). Smokers have increased risk of developing CD and increased risk of complicated disease (33). Smoking is also the most substantial risk factor for disease recurrence in CD, and the microbiota alterations caused by smoking may be instrumental (201, 202).

5.5.4. Antibiotics

A substantial number of studies have found antibiotics to alter the gut microbiota significantly by reducing the bacterial α-diversity (richness and abundance) and altering bacterial composition according to β-diversity (reviewed by (203)). However, the majority have studied the faecal microbiota. In a Cell paper from 2018 (204), 21 healthy participants were given oral antibiotic treatment with ciprofloxacin and metronidazole for seven days. After treatment, biopsy samples from the upper and lower GI tract and faecal samples were collected. Antibiotic therapy caused a disrupted microbial community composition in the faeces and mucosa. Interestingly, the antibiotic treatment caused more profound effects on the microbial composition in the lower GI than in the upper GI (204).

Swidsinski et al. (205) also evaluated changes in the mucosa-associated microbiota after combined treatment with ciprofloxacin and metronidazole by evaluating the number and type of mucosa-adherent bacteria with fluorescent in situ hybridisation and DAPI stain. They found antibiotic therapy to cause a substantial reduction of mucosal-adherent bacteria. Additionally, cessation of antibiotics caused a rebound effect, increasing the number of mucosa-adherent bacteria significantly up to 4.5 months after cessation, especially the abundance of Enterobacteriaceae family (205). Morgan et al. (101) analysed both faecal and biopsy samples from patients with IBD and found that antibiotic treatment was associated with significant reductions in bacterial diversity and abundance of *Dorea*, *Butyricicoccus*, *Collinsella*, *Subdoligranulum* and *Acetivibrio* genera. Antibiotic therapy has also been found to exacerbate mucosal dysbiosis in paediatric CD patients (102). Microbial alterations in the faeces after oral antibiotic treatment have been reported to last up to 4 weeks after ciprofloxacin and amoxicillin treatment, up to 4 years after clindamycin or clarithromycin treatment, whereas microbial alterations after metronidazole monotherapy seem to be minor (reviewed by (206)).

Antibiotic treatment also leads to a shift in the fungal-bacterial ratio in the GI tract with increased fungal load after antibiotic treatment, especially increased *Candida* spp. in faecal samples are associated with various antibiotic treatments (14).

5.5.5. Proton pump inhibitors (PPI)

Most studies reporting microbial alterations after PPI use have studied the faecal microbiota (207, 208), few have investigated mucosa-associated microbiota alterations after PPI therapy. PPI use has in meta-analysis of epidemiological studies been associated with increased risk of GI infections, likely due to increased gastric pH, especially *Clostridium difficile* infections have been associated with PPI use (207). In a study comparing 109 PPI users with 75 controls, using culture techniques on gastric juice and staining techniques on stomach biopsy specimens, PPI use was found to be associated with increased abundances of bacteria (CFU/mL) in gastric juice as well as increased prevalence of non- H. pylori bacteria in the mucosa (209). Another study compared the mucosa-associated microbiota in 12 PPI users and 12 controls with 454 pyrosequencing and found PPI use to be associated with increased abundance of Firmicutes phylum and the *Streptococcus* genus in particular (210).

5.5.6. Other drugs affecting gut microbiota

It seems likely that oral administration of most drugs influences the gut microbiota to various extents. Drugs such as NSAIDs, metformin, statins, antipsychotics and opioids have been associated with alterations in the faecal microbiota (207, 211).

5.5.7. Probiotics

Probiotics are microorganisms prepared for consumption in order to “normalise” the gut microbiota (95). Probiotics have been reported to change the microbial composition in both faecal and mucosal samples (204). Many different probiotic formulas exist, containing single or multiple strains of bacteria or fungi in different concentrations (94, 212). The probiotic fungi *Saccharomyces boulardii* is used to reduce diarrhoea in cholera

patients and has in some pilot studies showed an effect in inducing and maintaining remission in UC patients and maintaining remission in CD patients (14, 94, 213). The probiotic VSL#3 contains a combination of eight bacterial strains; *Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus paracasei, Lactobacillus bulgaricus,* and *Streptococcus thermophilus*, and is considered the probiotic formula with most evidence of effect in UC treatment (94-96). In a randomised, double-blind placebo-controlled trial, 147 UC patients received either VSL#3 or placebo for 12 weeks, 42.9% in the VSL#3 group and 15.7% in the placebo group achieved remission after 12 weeks ($p< 0.001$) (96). Patients were allowed to continue 5-ASA and thiopurine therapy, but administration of rectal corticosteroids was not allowed.

Accordingly, most probiotic studies are challenging to interpret as probiotics have been used alongside 5-ASA or thiopurine therapy, which could potentially influence the efficiency of the probiotics (212). The most recent Cochrane review did not find evidence to support the use of probiotics as UC maintenance therapy, however the study from Sood et al. (96) was not included (212). The BSG consensus guidelines state that probiotics possibly give modest benefits in UC patients, but is not recommended for routinely use (36). The latest Cochrane report on probiotics for induction of remission in CD emphasises lack of scientific reports regarding efficacy and safety, but based on the two studies meeting criteria for inclusion there was no difference between probiotics and placebo (214).

However, use of probiotics does not necessarily improve outcome, as probiotic treatment after a seven days course of antibiotic therapy was found to cause prolonged antibiotic-associated dysbiosis, lower bacterial α-diversity and delayed reconstruction of the pre-antibiotic mucosa-associated microbiota (204). The complexity of microbiota targeted treatment was also illustrated in a study investigating the effect of probiotics on healthy participants in stool, luminal and mucosal samples by both 16sRNA sequencing, quantitative Polymerase Chain Reaction (PCR) and shotgun metagenome

sequencing (24). Main findings were that detection of probiotic strains in faecal samples or faecal microbiota composition did not resemble mucosal colonisation, but rather a washout of non-adhering strains. Furthermore, there were significant intra-individual variations in mucosal colonisation patterns of probiotic strains which correlated with host genetics and baseline gut microbiota composition (24).

5.5.8. Faecal microbiota transplantation (FMT)

FMT involves transplantation of faeces into the GI tract. FMT can either be autologous (own faeces, usually sampled and stored at an earlier time) or allogeneic (from a donor). Administration can be through installation into the duodenum via either upper endoscopy or a nasoduodenal tube, into the ileum or colon during ileo-colonoscopy, by rectal enema or enteric-coated capsules (94). The rationale behind FMT is to reorganise the gut microbiota to a healthier composition.

Preparation of the faeces is another methodological aspect, as some studies have prepared the faeces in an aerobic environment, whereas others claim that anaerobic preparation is beneficial to preserve anaerobic bacteria (91). The faeces can also be derived from one or multiple donors. FMT is an effective treatment of recurrent clostridium difficile infection (reviewed by (94)).

Four randomised controlled trials (RCTs) have assessed FMT for induction of remission in UC patients, of which three found FMT to be superior to placebo (91, 215, 216), whereas one found no significant difference (217). In the most recent RCT, comparing donor and autologous FMT, the FMT was prepared anaerobically and installed in the right colon via colonoscopy followed by two enemas within seven days after colonoscopy (91). The primary endpoint was steroid free remission (total Mayo score $\leq$2 of and endoscopic Mayo score $\leq$1) 8 weeks after transplantation, this was reached in 12 of 38 patients receiving donor FMT and 3 of 35 receiving autologous FMT p=0.03 (91). The first published randomised controlled pilot study assessing the effect of FMT in CD patients included 17 CD patients in remission after corticosteroid therapy (218). Eight

patients received donor FMT and nine physiological serum administered into the caecum by colonoscopy, a higher rate of steroid-free clinical remission as well as a significant reduction in Crohn's Disease Endoscopic Index of Severity was found in the FMT group.

The major concern regarding FMT is the potential of serious adverse effects caused by transplantation of unknown microbes or microbiome associated metabolites which could harm the recipient on both short- and long-term basis and the possible transfer of diseases linked to gut bacteria. The BSG consensus guidelines characterise FMT as an experimental treatment for use in clinical IBD trials and emphasise that the effect of FMT does not last after one year (36). ECCO guidelines describe FMT as promising for use in UC but call for more studies to define the best FMT protocol, including administration site, donor characteristics and number of transplantations (163).

6. Aims of the studies

UC is a disease that affects the colonic mucosa, and 5-ASA is the first-line therapy for patients with UC. 5-ASA acts through mechanisms in the colonic mucosa, and it is the mucosal 5-ASA concentration that reflects the therapeutic potential. Previous studies have found large inter-individual variations in mucosal 5-ASA concentrations and significant differences in rectal 5-ASA concentrations between Asacol and Pentasa, with Asacol providing higher concentrations (145, 147). After Mezavant was marketed, mucosal 5-ASA concentrations studies comparing Mezavant and other 5-ASA formulations have not been conducted. 5-ASA is metabolized in the intestinal mucosa, the majority of 5-ASA is metabolized by NAT1 and a small proportion by NAT2. Different NAT genotypes lay the foundation for different metabolizing rates, which could explain the large inter-individual variations in mucosal 5-ASA.

Several papers suggest that 5-ASA have an effect on bacteria; by inhibiting growth in-vitro (219, 220), affecting gene expression (221), decreasing concentrations of mucosal adherent bacteria (222) and by altering faecal bacterial profiles (223).

In paper I, we aimed to:

1. Measure and compare mucosal 5-ASA concentrations in the left hemicolon and rectum in patients with quiescent UC using monotherapy with different oral 5-ASA formulations (Mezavant, Asacol, Pentasa).
2. Explore if NAT1 and NAT2 genotypes could explain variations in mucosal 5-ASA concentration.
3. Explore the interrelation between mucosal 5-ASA concentration and the bacterial composition in mucosa and faeces.

The terminal ileum is the most common site of inflammation in patients with CD. After ICR in patients with ileocecal CD, the majority of patients experience disease recurrence in the neo-terminal ileum, at and immediately proximal to the surgical anastomosis (39, 40). Diversion of the faecal stream by stoma prevents CD recurrence, while stoma reversal or infusion of faecal contents triggers CD recurrence (43, 90). It is thought that

the microbiota in the faecal contents contributes to recurrence of disease, as disease recurrence has been associated with the mucosa-associated microbiota composition at the time of ICR and postoperatively (99, 114, 128). To investigate the role of microbiota in ileal CD, we aimed to investigate the microbiota in the inflamed terminal ileum and the proximal non-inflamed ileum.

In paper II, we aimed to:

1. Describe the mucosa-associated bacterial microbiota in the ileum of CD patients in comparison to HC.
2. Compare the mucosa-associated bacterial microbiota in the inflamed and proximal non-inflamed ileal mucosa within the same patients.
3. Assess the effect of inflammation on the ileal mucosa-associated bacterial microbiota.
4. Assess the effect of ileal biopsy sampling location on the mucosa-associated bacterial microbiota.

Mycobiota has for long thought to be involved in gut inflammation in IBD. CD patients harbour genetic polymorphisms, causing aberrant recognition and immune responses to fungi and detection of antibodies specific for fungi (ASCA) can help discriminate IBD subtypes and diagnose patients with CD. Few studies have investigated the mucosa-associated mycobiota in CD patients, and none the adult ileal mucosa. To investigate the role of mycobiota in ileal CD, we aimed to investigate the mycobiota in the inflamed terminal ileum and the proximal non-inflamed ileum in the same patient cohort as in paper II.

In paper III, we aimed to:

1. Describe mucosa-associated fungal microbiota in the ileum of CD patients in comparison to HC.
2. Compare the mucosa-associated fungal microbiota in the inflamed and proximal non-inflamed ileal mucosa within the same patients.

3. Assess the effect of inflammation on the ileal mucosa-associated fungal microbiota.
4. Assess the effect of ileal biopsy sampling location on the mucosa-associated fungal microbiota.

7. Summary of papers

7.1. Paper I: Mucosal 5-aminosalicylic acid concentration, drug formulation and mucosal microbiome in patients with quiescent ulcerative colitis

We measured the mucosal 5-ASA concentration at three different locations (10, 25 and 40 cm proximal of the anal verge) in the distal colon of 42 patients with UC in remission using three different oral 5-ASA formulations; Mezavant, Asacol and Pentasa, in dosages 4.0-4.8g once daily. Disease activity was assessed by Mayo score and histologically by Geboes score. All patients were genotyped for NAT1 and NAT2, which encode the enzymes metabolising 5-ASA in the intestinal mucosa and liver. Faecal and mucosa-associated bacterial microbiota were assessed by 16S rRNA sequencing. We found large inter-individual variations in mucosal 5-ASA concentrations. Patients using Mezavant had significantly higher mucosal 5-ASA concentrations in the distal colon and rectum than patients using Pentasa, while there was no difference in mucosal 5-ASA concentration between patients using Mezavant and Asacol. We found no correlation between NAT genotypes and mucosal 5-ASA concentration, and different NAT genotypes could not explain the large inter-individual variations in 5-ASA concentration. Mucosal 5-ASA concentration was associated with alterations in the mucosa-associated bacterial microbiota. High 5-ASA concentration was associated with high bacterial diversity, decreased abundances of Proteobacteria and increased abundances of Firmicutes and Bacteroidetes phyla in addition to increased abundance of 10 bacterial families and 18 bacterial genera and decreased abundance of six bacterial families and one bacterial genus. High mucosal 5-ASA was associated with a presumed favourable bacterial microbiota composition. Mucosal 5-ASA concentration was not associated with alterations in the faecal bacterial microbiota.

7.2. Paper II: Bacterial mucosa-associated microbiome in inflamed and proximal non-inflamed ileum of patients with Crohn's disease

CD patients (n=51) and HC (n=40) scheduled for endoscopic examination were recruited. Paired mucosal pinch biopsies were sampled approximately 5 cm and 15 cm orally of the ileocecal valve or ileocolic anastomosis. CD patients where the 5-cm location was endoscopically inflamed, and the 15-cm location was normal were termed terminal ileitis. CD patients with endoscopic inflammation at both 5-cm and 15-cm location were termed active disease. In CD patients in remission and HC, both 5-cm and 15-cm location were endoscopically normal. In CD patients with ileal stenosis, biopsies were only sampled on 5-cm location as the stenosis prevented further intubation into the ileum. The mucosa-associated microbiota in CD patients was characterised by reduced α-diversity and clear separation from HC on β-diversity plots. CD patients displayed increased abundances of Proteobacteria, *Tyzzerella 4*, *Escherichia shigella* and decreased abundances of *Ruminiclostridium 5*, *Ruminiclostridium 6*, *Eisenbergiella* and *Faecalibacterium*. The abundance of *Tyzzerella 4* was profoundly increased. Comparison of the inflamed and proximal non-inflamed mucosa in 20 CD patients with terminal ileitis and no history of upper CD involvement revealed no difference in α-diversity and no separation according to β-diversity. Also, differential expression analyses did not identify any taxa to be differentially expressed between inflamed and proximal non-inflamed mucosa in these patients. α-diversity and microbiota composition did not differ between 5-cm and 15-cm location within all CD patients. α- and β-diversities were also similar regardless of endoscopic inflammation. Biopsies characterised as histologically inflamed had lower α-diversity, but β-diversity did not differ according to histological inflammation. Patients with ileal stenosis clustered further away from HC than CD patients with terminal ileitis and CD patients in remission on β-diversity plots. In addition, the presumed favourable species *Bacteroides massiliensis B84634* and unidentified species of *Sutterella* and *Akkermansia* were underrepresented in stricturing CD. Patients who had undergone ICR had lower α-diversity, but the bacterial composition in ICR patients did not differ from non-ICR CD patients. Summarised, our

findings suggested that the altered ileal mucosa-associated microbiota in CD patients is present across different locations in the ileum and is independent of inflammation status at biopsy location.

7.3. Paper III: Fungal microbiota in the ileal mucosa of patients with Crohn's disease

CD patients and HC scheduled for endoscopic examination were recruited (same patient cohort as paper II). Paired mucosal pinch biopsies from approximately 5 cm and 15 cm proximal of the ileocecal valve or ileocolic anastomosis were collected. The study comprised of 44 CD patients of which 22 had terminal ileitis with endoscopic inflammation at 5-cm location and normal endoscopic appearance at 15-cm location, 10 with endoscopic inflammation at both 5- and 15-cm location and 12 with normal endoscopic appearance at both 5- and 15-cm location. Forty HC were also included. CD associated mycobiota were characterised by reduced fungal evenness, increased Basidiomycota-to-Ascomycota ratio and altered β-diversity and fungal composition compared to HC. Also, an expansion of *Malassezia* and a depletion of *Saccharomyces*, as well as increased abundances of *Candida albicans* and *Malassezia restricta* were found in CD patients compared to HC. We separately analysed the inflamed and proximal non-inflamed mucosa of 20 CD patients with terminal ileitis without a history of upper CD involvement. There was no difference in α-diversity, but the inflamed and proximal non-inflamed mucosa separated on β-diversity plots, with the inflamed mucosa harbouring a more dysbiotic mycobiota composition compared to HC and non-inflamed mucosa. Several fungal taxa were found to be differentially abundant between inflamed and the proximal non-inflamed mucosa. *Candida sake* was increased in the inflamed mucosa, whereas *Exophiala equina* and *Debaryomyces hansenii* were increased in the proximal non-inflamed mucosa. In the whole CD cohort, neither inflammation (endoscopic and histologic) nor sub-location (5- or 15 cm) influenced fungal α- or β-diversity. Summarised, this study confirmed CD specific alterations in the mucosa-associated mycobiota and described structural alterations in

the mycobiota composition between the inflamed and proximal non-inflamed ileal mucosa within the same CD patients.

8. Methodological considerations

8.1. Patient material

All papers (I-III) are cross-sectional studies based on human samples. For paper I, nine mucosal pinch biopsies from the left colon (formalin-fixated or snap-frozen), faecal samples, serum and plasma samples were collected. For paper II and III (same patient cohort) a total of six mucosal pinch biopsies from the terminal ileum (formalin fixated or snap frozen), serum and plasma samples were collected. For all papers, informed and written consents were given from all patients, and the studies were approved by the Regional Committee for Medical and Health Research Ethics, Central Norway. Patients were recruited at the outpatient clinic, Department of Gastroenterology, St. Olav's Hospital.

In paper I, patients with established UC, confirmed by endoscopic, histologic and radiological findings using monotherapy with oral 5-ASA and in clinical remission were invited to participate. Exclusion criteria were use of rectal 5-ASA formulations, prednisolone, azathioprine, methotrexate, TNF-α medication and antibiotic or antifungal therapy within the last 3 months.

For paper II and III, patients with established CD or patients referred to ileocolonoscopy due to symptoms which could represent CD and controls referred to colonoscopy due to rectal bleeding or screening for disease were invited to participate. Exclusion criteria were use of antibacterial or antifungal treatment for the past 2 months, diabetes mellitus, primary sclerosing cholangitis, primary biliary cholangitis or celiac disease. Additional exclusion criteria for the controls were gastrointestinal polyps, cancer, diverticulitis, irritable bowel disease fulfilling ROME IV criteria (224) or previous gastrointestinal surgery. Patients with CD confirmed by endoscopic, histologic and radiological findings and controls with completely normal ileocolonoscopy (rectal bleeding permitted) were included.

8.2. Effect of bowel cleansing on mucosal 5-ASA concentration

De Vos et al. (145) studied the effect of bowel cleansing on serum 5-ASA concentrations and found that the serum concentrations during bowel cleansing were reduced to approximately 1/10 of the concentration found in steady-state before bowel cleansing was initiated. They concluded that bowel cleansing induced diarrhoea which evoked mechanical removal of intraluminal 5-ASA. Therefore, we chose to use a sorbitol enema 30-45 minutes before sigmoidoscopy in paper I. Sorbitol enema is considered to be a milder bowel emptying method and additionally, less bothersome and time consuming for the patients. However, enema-induced bowel emptying most likely removes some mucosal 5-ASA, but it is unlikely that the enema had a different effect on the different 5-ASA formulations. Additionally, all mucosal biopsies were cleansed briefly in 0.9% NaCl and dried for a few seconds on paper before being snap frozen on liquid N_2, this was done to reduce contamination from luminal 5-ASA in faecal remnants.

8.3. Effect of bowel cleansing on gut microbiota

Bowel cleansing is necessary to empty the bowel from faecal contents before an endoscopy procedure and allows safe scope intubation and visualisation of the mucosa. Bowel cleansing induces watery diarrhoea which has been reported to affect the mucosa-adherent gut microbiota (24, 225, 226). Shobar et al. (225) investigated the effect of bowel cleansing on both faecal and mucosa-associated microbiota in HC and IBD patients and found that α-diversity was reduced in biopsy samples after bowel cleansing. Additionally, bowel cleansing was associated with reductions in Actinobacteria and Tenericutes and an increase of Bacteroidetes in HC, whereas in IBD patients bowel cleansing were associated increases in Bacteroidetes and reductions in Lactobacillales order and unclassified *Streptococcaceae* and unclassified *Clostridiaceae* genera. Harrell et al. (226) investigated the effect of bowel cleansing on microbiota by comparing biopsies from HC sampled during an un-prepped sigmoidoscopy with biopsies sampled post bowel cleansing. They also included two control groups which underwent two un-prepped sigmoidoscopies with biopsy collection (either on clear

liquid diet or regular diet). In accordance with Shobar et al. (225), the authors found bowel cleansing to be associated with reduced α-diversity (richness), decreased diversity was neither found in the control groups nor in controls on liquid diet (226). The majority of papers which have studied the effect of bowel cleansing have done this in faecal samples, however based on the findings from Shobar et al. and Harrell et al. bowel cleansing appear to affect the mucosa-associated microbiota. Patients included in paper I received a 240mL sorbitol enema, which is considered to be a very mild form of bowel cleansing resulting in one or two defecations within less than 30 minutes in comparison to 10-20 after full bowel cleansing that may take hours (225). All study participants in paper II and III, both CD patients and controls underwent full bowel cleansing as it was a necessity in order to reach the terminal ileum and perform the ileocolonoscopy with good clinical quality. We and the majority of microbiota studies cannot exclude confounding effects of bowel cleansing.

8.4. Ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS)

To analyse the 5-ASA concentration in the mucosa and serum in paper I ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) was applied. UHPLC-MS/MS is a method enabling quantification of drugs at high speed with high sensitivity, specificity and reproducibility (227-229). UHPLC-MS/MS combines ultra-high performance liquid chromatography (UHPLC) and two mass spectrometry steps (tandem mass spectrometry (MS/MS)) (230). High-performance liquid chromatography (HPLC) separates different compounds on a stationary phase column filled with absorbent particles, UHPLC offers enhanced separation and column capacity compared to HPLC by using columns with smaller particle diameters and higher pressure, thereby increasing the sensitivity (228, 229). Mass spectrometry separates ions according to their mass to charge ratio (m/z) (230).

8.5. NAT genotyping

NAT is the enzyme which metabolises 5-ASA to its inactive metabolite Ac-5-ASA in the intestinal mucosa. 5-ASA is mainly metabolised by NAT1, and to a small degree by NAT2 (132, 133). Different genotypes for NAT1 and NAT2 give rice to phenotypes with different enzyme activity or acetylator status. A total of 28 sequence variants or NAT1 alleles have been described to date (231). Patients, both heterozygous and homozygous of NAT1 *14A, *14B, *15, *17, *19A, *19B and *22 alleles have a reduced enzyme activity and were considered slow acetylators, while patients with other allele combinations were considered rapid acetylators (232). NAT2 genotypes are classified into rapid, intermediate and slow acetylators (232). Patients homozygous for NAT2*4 are considered rapid acetylators, patients heterozygous for NAT2*4 or NAT2*12 are considered intermediate acetylators, other allele combinations are considered slow acetylators (157, 232, 233). Rapid acetylators could potentially have lower mucosal 5-ASA concentrations than patients with a slow acetylator phenotype. Ricart et al. (157) genotyped patients using 5-ASA or sulfasalazine and found that NAT1 and NAT2 genotypes were not associated with clinical response to 5-ASA or sulfasalazine treatment. In paper I, all patients underwent both NAT1 and NAT2 genotyping. NAT genotypes were determined using Sanger sequencing on DNA isolated from EDTA preserved whole blood.

8.6. Sanger sequencing

DNA sequencing was first made available by the invention of Sanger sequencing in 1977 (234, 235). In Sanger sequencing the single-stranded DNA (ssDNA) strand is amplified by adding a primer, DNA polymerase, the four deoxynucleotide triphosphate(s) (dNTP) including deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxythymidine triphosphate (dTTP) and deoxyguanosine triphosphate (dGTP) as well as one of the four chain-terminating dideoxynucleotide triphosphate (ddNTP); ddATP, ddCTP, ddTTP or ddGTP together with the ssDNA in a mixture (236, 237). When the ddNTP is incorporated into the growing strand, it inhibits the DNA polymerase, causing

DNA elongation to stop. Four mixtures, each containing one ddNTP, are prepared. In each mixture, DNA strands of different lengths are made depending on when the ddNTP is incorporated. All four mixtures are then fractioned by electrophoresis; the shortest DNA fragments will move further down the gel. By comparing the bands on gel electrophoresis from all four mixtures, the DNA sequence can be read (235). Automated Sanger sequencing platforms were later made available, providing up to 96kb of data per run (236). Sanger sequencing was performed to NAT genotype patients in paper I.

8.7. DNA isolation of mucosal biopsies and faecal samples

In order to analyse the microbiota composition in biopsy samples or faeces, DNA needs to be extracted from the sample and purified. Mucosal pinch biopsies collected for paper I were snap frozen on liquid N_2 and stored on -80°C until DNA isolation analysis. Biopsies collected in paper II and III were directly snap-frozen on liquid N_2 and stored on liquid N_2 until DNA isolation analysis. Patients in paper I were also instructed to bring a fresh faecal sample, which were stored directly on -80°C until DNA isolation analysis.

Before DNA isolation of samples for paper I the literature was reviewed before choosing a DNA isolation kit. Previous studies investigating the mucosal microbiota in CD patients using high throughput sequencing techniques had been using a variety of different kits. For example, the paediatric studies by Gevers et al. 2014 (102) and Haberman et al. 2014 (110) used Qiagen Allprep RNA/DNA kit (Qiagen, Hilden, Germany). Morgan et al. 2012 (101) analysed both faecal and biopsy samples using QIAamp DNA stool mini kit (Qiagen), Chiodini et al. 2015, 2016 and 2018 analysed surgical resection samples from CD patients using DNeasy Powersoil Max (Qiagen) (126, 238, 239). El Mouzan et al. analysed fungal microbiota in stool and biopsies from paediatric patients using MO BIO Powersoil (MO BIO, San Diego, CA) now sold as DNeasy Powersoil (Qiagen) (129).

Only a few studies had compared relevant DNA isolation kits, and no study had compared the efficiency of the kits on mucosal biopsy samples from the GI tract. Vesty

et al. 2017 compared four DNA extraction methods including MO BIO Powersoil (now sold as DNeasy Powersoil (Qiagen)) and QIAamp DNA Mini Kit (Qiagen) on dental plaque and saliva samples from the oral cavity (240). They evaluated DNA quality and yield as well as bacterial and fungal microbiota structures after 16S ribosomal RNA (rRNA) sequencing of the V3-V4 region. The main findings were that the QIAamp DNA Mini kit produced greater DNA yield compared to the three other methods, but that the kits were similar in terms of bacterial diversity and abundance yield (240).

Since commercial kits used in previous mucosal microbiota papers were all owned by Qiagen, we requested Qiagen support for advice. According to recommendations by the manufacturer, the QIAamp DNA Mini Kit would isolate genomic DNA from patients and gram-negative bacteria, however the lysis procedure was not compatible with isolating gram-positive bacteria. Whereas, DNeasy Powersoil and QIAamp DNA PowerSoil both have a lysis strategy based on bead beating, which would allow isolation of both gram-negative and gram-positive bacteria, including genomic DNA from humans (personal communication). Inclusion of a Proteinase K digestion step after homogenisation with bead beating was also recommended (personal communication).

We chose to use QIAamp PowerFecal (Qiagen) and DNeasy PowerSoil (Qiagen) to isolate DNA from faecal samples and biopsy samples, respectively, in paper I and II. Manufacturer's protocol was followed except from the following adjustments; step 3 and 4 in the protocol (vortexing) was replaced by bead-beating using Precellys 24 tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) at 5000 rpm x 3 rounds of 40 seconds. After bead beating and before centrifuging (Step 5) 20 µL of Proteinase K 20mg/mL were added and samples incubated at 65°C for 30 minutes (paper I and II).

After DNA isolation was performed for paper I, a study comparing DNA isolation kits in faecal samples was published (241), this study compared QIAamp Stool Mini kit and QIAamp PowerFecal. The main findings were that all methods generated adequate DNA

concentrations and DNA quality for sequencing. However, the inclusion of a bead-beating step generated higher microbial diversity and caused increased abundances of gram-positive bacteria (241), further supporting that bead-beating was of importance to regenerate the true microbial composition in the sample. Bead-beating was performed in all studies (I-III).

DNA isolation performed in previous mucosa-associated mycobiota papers varied, but two papers reported to use MO BIO Powersoil (67, 129). A co-author had performed ITS-1 sequencing of faeces from IBD patients (242) using a DNA isolation protocol provided from David Underhill (243) which was designed to destroy the fungal cell wall which is well-known to be challenging to lyse. Therefore, we performed a pilot-study were paired samples from the ileum of IBD patients and HC were isolated with DNeasy PowerSoil (Qiagen) including bead-beating step and proteinase K digestion step and the Underhill protocol including a lyticase treatment step, a bead-beating step and QIAamp DNA Mini Kit (Qiagen). PCR products from 18S rRNA PCR amplification were run on a Bioanalyzer (DNA 1000) chip (Agilent Technologies, Santa Clara, CA). Based on the result's samples isolated with the Underhill protocol harboured more fungal DNA and less contamination and the Underhill DNA isolation protocol was chosen to isolate samples for paper III.

After DNA isolation, the samples for paper I and II were quality tested using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Nanodrop uses UV-light to give an estimate of the total DNA concentration (ng/μL) (including all DNA, both human, bacterial, fungal) and the purity of the DNA (260/280 and 260/230 nm ratio). Since DNA absorb light at around 260 nm, and contaminants at around 280 and 230, a low 260/280 or 260/230 ratio indicate contamination. A DNA concentration above 30 ng/μL together with 260/280 ratio $\geq$1.8 and 260/230 ratio $\geq$2.0 was considered adequate. For paper I-III, DNA in samples were quantified using Qubit (Thermo Fisher Scientific). Qubit is more specific than UV absorbance measurements as the Qubit assay kits only fluorescence when bound to the selected molecule in this case DNA. Qubit can

detect low DNA concentrations. The isolated samples for paper I-III were stored on -80°C until library preparation and amplicon sequencing.

8.8. High throughput sequencing

High throughput sequencing (HTS) or so-called next-generation sequencing (NGS) technologies have been available from 2005, enabling massive DNA sequencing in a short time, the first NGS platforms provided up to 1 GB of data per run. However, NGS also enabled massive parallel sequencing (sequencing millions of fragments instead of one DNA fragment simultaneously) and simultaneously sequencing of multiple genes in the same run with high data output (236). By 2014 sequencing platforms generated up to 1.8 Tb of data per run (244, 245).

HTS/NGS include different sequencing strategies, for example, whole metagenomic shotgun sequencing targets to sequence all available DNA, while amplicon sequencing only targets to sequence the DNA of interest captured by the sequencing primer. The most widely used sequencing method in microbiota research is amplicon sequencing.

8.9. 16s rRNA and ITS primers

The 16S rRNA gene is highly conserved between Bacteria and Archaea, meaning that the DNA sequence is identical within different taxa of Bacteria. The 16S rRNA gene consists of nine hypervariable regions, within these regions, the DNA sequence (order of nucleotides) varies, enabling characterisation of different taxa (73). 16S rRNA sequencing is based on identification and capturing of the 16S gene in a sample by binding a primer in the conserved region upstream to the hypervariable region of interest, following amplifying the 16S rRNA DNA strands by PCR and subsequent sequencing. Choice of 16S primers significantly affect the sequencing result, for example, the V5 region enables identification of bacteria on phylum level whereas the Shannon diversity index is higher in the V3/V4 region (244). A recent review paper

recommended to sequence the V3/V4 region or the V4 region (244). In paper I and II primers targeting the V3 and V4 region were chosen.

In fungi, the nuclear ribosomal internal transcribed spacer (ITS) is the standard DNA barcode (246). The preferred ITS region for fungi amplicon sequencing is not established (14), both ITS1 and ITS2 can be used for identification of fungi. Figure 10 provide a visualisation of the fungal rRNA locus and localisation of the ITS regions. A challenge with the ITS regions is that they vary in length between fungi from 200 to 800 base pairs (14), in comparison to the 16S rRNA in bacteria. Primers against both ITS regions have been used in previous publications, alone (109, 247, 248) or in combination (249). There is no evidence to support that one ITS region should be preferred over the other (14). In paper III, we performed ITS2 sequencing.

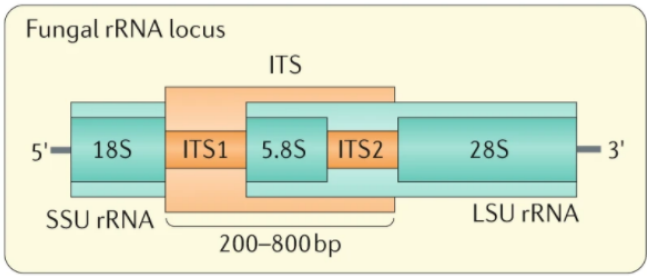

Figure 10. Fungal rRNA locus with location of ITS1 and ITS2 sequences. Figure from Richard and Sokol (14). Reprinted/modified with permission from Springer Nature.

8.10. Amplicon sequencing

Amplicon sequencing is a targeted approach to analyse a specific genomic region by ultra-deep sequencing of PCR amplicons. The first step of amplicon sequencing is library preparation (250). Library preparation includes fragmentation of the DNA, binding of primers, amplification by PCR and ligation of adapters to the flanks of the DNA (Figure 11) (250). Illumina 16s metagenomics sequencing library preparation was used for paper I and paper II, while Illumina fungal metagenomic sequencing protocol (251) was used in paper III. In these protocols, indices and the adapters are ligated to the DNA fragments. The indices are tags which are unique for each sample enabling identification

after pooling. The adapters are complementary to the surface-bound oligonucleotides of the flow cell (245).

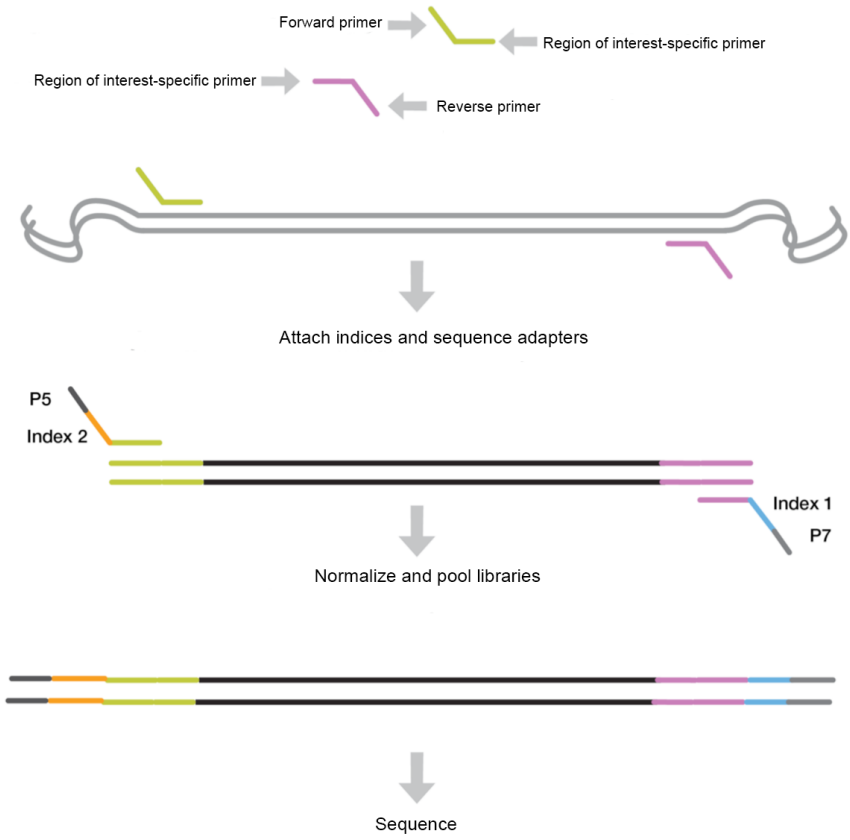

Figure 11. Figure modified from Illumina 2014, 16s Metagenomic Sequencing Library Preparation (252). Used under license from Illumina, Inc. All rights Reserved. Illustrating library preparation with binding of forward and reverse 16S rRNA primer (green and purple respectively) capturing the DNA sequence of interest (grey). Then index tags (orange and blue) and sequencing adapters (P5 and P7) are ligated to the flanking DNA. Libraries are then normalized and pooled.

After library preparation, the samples are ready to be sequenced. There exist several sequencing platforms, for all papers we have used Illumina MiSeq platform (Illumina Inc., San Diego, CA) which has been recommended as a standardised protocol by the Earth Microbiome Project (244). However, several papers investigating the mucosal

microbiota in IBD with HTS have used 454 pyrosequencing or Ion Torrent sequencing (99, 111, 117, 253).

For Illumina sequencing platforms, the first sequencing step is cluster amplification; the sequencing library is loaded into a flow cell where the ligated adapters attach to the complementary oligonucleotides on the flow cell surface (245). Each DNA fragment is then amplified into separate clusters by a process called bridge amplification (Figure 12B). After bridge amplification, fluorescently labelled dNTPs are added together with sequencing reagents. All four dNTPs are present and compete for binding to the elongating DNA strand, every time a dNTP is incorporated to a cluster colour is emitted and recorded by imaging, identifying the newly incorporated base by its colour (Figure 12C) (254). The cycle is repeated multiple times.

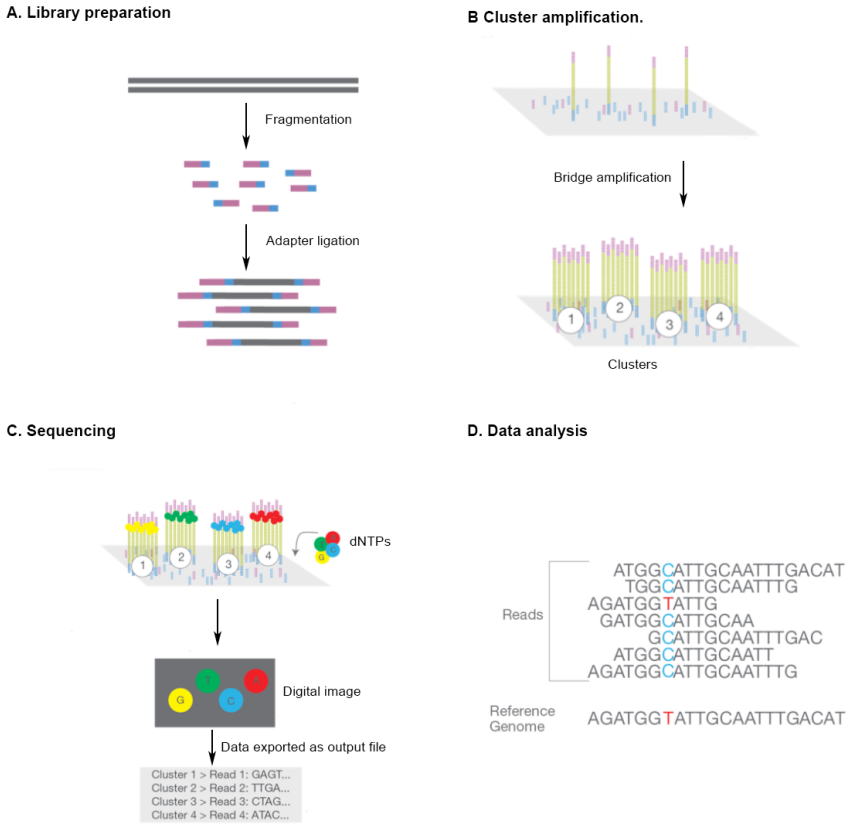

Figure 12. Figure modified from Illumina 2017, An introduction to Next-Generation Sequencing Technology (245). Used under license from Illumina, Inc. All rights Reserved. The figure illustrates Illumina next generation sequencing workflow with the four steps: A: Library preparation, B: Cluster amplification, C: sequencing, D: data analysis.

8.11. Bioinformatic analysis

The bioinformatic pipeline contains detailed information regarding all steps in the bioinformatic analysis of the sequencing data. Many different pipelines and software packages are used (255). The bioinformatic pipeline typically includes quality filtering, taxonomic classification of sequences and statistical analysis of data (73). Quality filtering involves removal of low-quality sequences and chimeric sequences, the latter being a PCR artefact forming hybrid sequences which originates from two unrelated parent sequences. Further, raw sequences are trimmed, which implies removal of the sequenced primer adapters (which enabled binding to the flow cell) (255). The taxonomic classification involves amplicon sequence identification by using a reference database of previously classified sequences, such as Ribosomal Database Project (RDP), Greengenes or SILVA (255, 256). It exists two main approaches for sequence identification, either via direct assignment of amplicon sequences to phylotypes or indirectly by comparing the operational taxonomic unit (OTU) reference sequence (73) to the reference database. An OTU is a cluster of similar sequences grouped together based on sequence similarity, 97% similarity is normally required to form an OTU (73, 255).

In paper I, Quantitative Insights into Microbial Ecology (QIIME) pipeline was applied to cluster sequences into OTUs, and taxonomic classification was performed using RDP trained on Greengenes database. OTU tables were generated and subsequently quality filtered to include only OTUs from Bacteria kingdom. Core microbiome was estimated by requiring 10% prevalence of detected OTUs (>1 sequence). DESeq2 R package was applied for statistical analysis (257). α-diversity was assessed by Shannon entropy.

Count tables of the core microbiota at different taxonomic ranks were imported into DESeq2 R package to estimate the correlation between OTUs and the log-transformed mucosal 5-ASA concentrations. DESeq2 regression model was used for identifying significant OTUs.

In paper II, QIIME II pipeline was applied, and sequence data were filtered using DADA2, which is a software package that acts as a denoiser and corrects Illumina amplicon errors without constructing OTUs (258). Direct taxonomic classification was performed matching the sequences to phylotypes using the SILVA database. DESeq2 was applied for statistical analysis (257) and count tables at a given taxonomic rank were imported into DESeq2 R package to estimate differential expression. α-diversity was assessed by Shannon entropy and β-diversity by Bray- Curtis dissimilarity index.

For all bioinformatic analyses performed in paper I-II, p-values were estimated using a Wald test and adjusted for multiple testing by Benjamini-Hochberg false discovery rate correction, p-values of <0.05 was considered statistically significant.

In paper III, the FROGS pipeline established in Toulouse, France (259) was applied for sequence quality control, filtering and affiliation of taxa. Sequences were taxonomically identified using the UNITE database, which has undergone quality improvements (260). DESeq2 was applied for statistical analysis of OTUs according to phenotype (257). The linear discriminant analysis (LDA) effect size (LEfSe) algorithm (261) was used to identify taxa that were specific to phenotype or inflamed vs proximal non-inflamed mucosa. α-diversity was assessed by observed OTUs and Simpson index and β-diversity by Bray-Curtis dissimilarity index and Jaccard index. P-values of <0.05 was considered statistically significant.

8.12. Challenges with amplicon sequencing

All steps, from sampling (mucosal tissue or faeces, mucosal pinch biopsy location), sampling storage (liquid N_2 or freezing, preservatives), DNA extraction, 16S rRNA or ITS

primer choice, library preparation, sequencing platform and bioinformatic pipeline will influence the result of microbiota composition (73), illustrated in Figure 13. DNA isolation method determines what DNA is isolated as well as DNA quality. The primer will influence what part of the DNA is "captured" and subsequently sequenced. Library preparation and sequencing method will influence sequencing quality and amplification of the sequences. Finally, the quality filtering, reference database for taxonomic classification and statistical analysis will impact the final results. Even small differences in the methodological steps can affect the reported microbial composition (73). Due to large methodological differences, it can be challenging to compare results from different microbiota studies. Another challenge with amplicon sequencing is that sequencing is performed on PCR products (amplicons) and the abundance of different amplicons does not necessarily reflect the natural abundances at the sample origin.

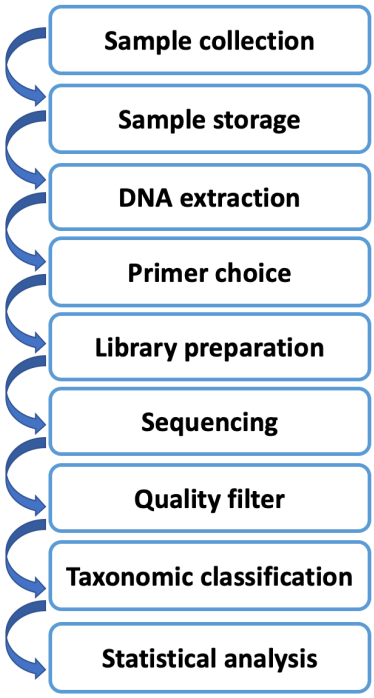

Figure 13. Methodological steps influencing results of high-throughput sequencing. Figure modified from Tyler et al. (73). Modified with permission from Wolters Kluwer.

There is also uncertainty regarding whether one or a few sample(s) contain the complete or a representative microbiota composition compared to the actual biological diversity of the environment they were sampled from.

Furthermore, each sample gains different numbers of sequences (library size) which reflects the sequencing process rather than the true biological variation between samples (262). The taxonomic classification reference databases are accurate down to genus level, however species level classification is associated with less accuracy and specificity (255). Another challenge is that OTU/amplicon sequence variant (ASV) files do not follow normal distribution. Values are often bound by 0 or 1, and many microbial organisms are detected only in a few samples resulting in a sparse OTU file meaning that it contains many zeros, altogether making the bioinformatic statistical analysis complicated (73, 262).

8.13. H&E staining and histological classification

For paper I, three biopsy samples (one from each location; 10 cm, 25 cm and 40 cm proximal of the anal verge) were formalin-fixed and stained with hematoxylin and eosin (H&E). For paper II and III, two biopsy samples (one from each location: 5 cm and 15 cm proximal to the ileocecal valve or anastomosis) were formalin-fixed and stained with H&E. The pathology assessment was for all papers (I-III) blinded and performed by an experienced pathologist. For paper I, biopsies were assessed by Geboes histological score (42), histological remission was defined as Geboes score <2.1 in accordance with deep remission defined by Magro et al. (263). For paper II and III, biopsies were evaluated by Global Histologic Disease Activity Score (GHAS) and Robarts score (43, 44, 264). However, a validated histological scoring index for evaluation of CD disease activity does not exist. Histological evaluation of all mucosal pinch biopsies in paper II and III guaranteed normal histological classification of HCs.

8.14. Statistical analysis

For all statistical analyses, a p-value <0.05 was considered statistically significant.

8.14.1. Descriptive statistics

Demographic and clinical characteristics of patients in papers I-III were presented using mean value (standard deviation (SD)) for normally distributed variables, median (interquartile range (IQR)) for variables with skewed distribution and n (%) for categorical variables. The mean value is the average value of all measurements. SD is a measure for variation between sample measurements and is calculated by the square root of the sample variance. The median value is the middle value of all measurements. IQR is also a measure of variation between sample measurements, it is calculated by the difference between the 75th percentile and 25th percentile. The 25th percentile or first quartile is defined as the value where one forth or 25% of all measurements are below the value similarly, the 75th percentile or third quartile is the value where 25% of the measurements are above the value.

Concentrations are often presented as log concentrations which implies that each concentration value differs by a constant, making the values equally spaced from each other and thus the distribution less skewed. In paper I, mucosal 5-ASA concentrations in the different 5-ASA formulation groups were presented as geometric mean (95% confidence interval (CI)). The geometric mean is applied to find the mean value on a logarithmic scale and calculating the value back to the original scale. The geometric mean is calculated by the antilogarithm of log x. The 95% CI is defined as a calculated interval which will contain the true parameter in 95% of all random samples obtained from a reference population.

8.14.2. Parametric tests

Parametric tests depend on normal distribution of variables.

The Independent Samples *t* Test or two-sample independent *t* Test tests if there is a statistical difference between the means of two groups. The test requires samples and

groups to be independent of each other. Independent Samples *t* Test was applied in paper II and III.

One-way Analysis of variance (ANOVA) is applied to determine if the means of two or more groups are different, the test requires variables to follow normal distribution and have the same variance. The ANOVA *F* test tests if the overall mean between for example three groups are different, it is important to notice that if the test shows a statistical difference, we still do not know which mean is different. ANOVA was applied in paper I.

8.14.3. Non-parametric tests

Non-parametric tests are applied when the sample size is small, or the variable is not normally distributed, or a combination of the two. The advantage of non-parametric tests are that they do not depend on normal distribution, however if the data is truly normally distributed, some statistical power will be lost by using a non-parametric test. The Mann Whitney *U* test is the non-parametric analogue to the two-sample independent *t* test, and it is used to compare the means of two groups. The test is based on ranks of observation, meaning that each observational value from the two groups is replaced by a rank and the test calculated based on the rank sum. The Kruskal-Wallis test is analogous to the Mann Whitney *U* test and allows comparisons between more than two groups. We applied the Mann Whitney *U* test in paper I-III and Kruskal Wallis test in paper I.

The Chi-square test or X^2 test is a non-parametric test which is applied to test for associations between two categorical variables. Chi-square test is based on the arrangement of a contingency table and computation of the observed and expected count in each cell. The null hypothesis is that the expected and observed values are not significantly different. The Chi-square test require that the expected values are not too low; not more than 1/5 of cells can have an expected value <5 and no cell can have an expected value of <1. If the expected values of some cells are low, a Fisher-Exact test should be performed. The Chi-square test was performed in paper I-III, and Fisher-Exact test was performed in paper II and III.

8.14.4. Multilevel linear mixed model

Multilevel linear mixed model or linear mixed-effect models are suitable for datasets with repeated measurements, missing data and datasets with an unbalanced data structure. The multilevel linear mixed model is an extension of standard linear regression where hierarchical data structure (repeated measurements, for instance) are taken into account.

In paper I, we had repeated 5-ASA concentration measurements at different locations in the distal colon within each patient, also the number of measurements at the different locations were not the same (two samples at 10 cm, one at 25 cm and two at 40 cm proximal of the anal verge), which made the multilevel mixed linear model suitable. 5-ASA formulation and biopsy location were included in the model as fixed factors, whereas subject was defined as a random factor to account for the dependency of the repeated measurements (10, 25 and 40 cm) and order of observation (two samples at 10 cm and 40 cm and one sample at 25 cm). An interaction term between 5-ASA formulation and location was included in the model to test whether the difference in mean 5-ASA concentration by location differed between 5-ASA formulations, and conversely, whether the difference in mean values between 5-ASA formulations differed by location.

9. Results and discussion

9.1. Mucosal 5-ASA concentration in patients with UC

5-ASA is the first-line therapy in patients with UC and acts locally in the intestinal mucosa. In paper I, we measured mucosal 5-ASA concentration in the distal colon and rectum (at the following locations: 10, 25 and 40 cm proximal of the anal verge) of patients with UC using high dose (4.0-4.8 g/day) oral 5-ASA. We found large inter-individual variations in mucosal 5-ASA concentration in the distal colon and declining concentrations towards the rectum, in accordance with previous studies (144, 146-149). However, the intra-individual variations in mucosal 5-ASA concentration, i.e. variation in mucosa 5-ASA concentration between the different locations in the colon and rectum within the same patient were small, suggesting that individual factors contribute to the variations and not location-dependent factors. It has been proposed that intraluminal pH, NAT genotype, GI transit time and disease pattern can contribute to the variation (153, 157, 265). However, we ruled out differences in NAT genotype as influential on mucosal 5-ASA concentration (paper I).

9.2. Effect of 5-ASA formulation on mucosal 5-ASA concentration

Previous studies have found Asacol to yield higher mucosal 5-ASA concentrations than Pentasa (145, 147). Very few randomised controlled trials (RCTs) have compared pH-dependent and time-dependent 5-ASA formulations in terms of clinical efficacy (169, 266), and to the best of our knowledge, none have compared these formulations in maximum dosages. A recent meta-analysis concluded that the different 5-ASA formulations are equally efficient, however this the conclusion was based on comparison of 6 RCTs, of which none included Mezavant formulation, altogether the evidence was considered low quality (169). We aimed to compare mucosal 5-ASA concentration in patients using three different 5-ASA formulations, Mezavant, Asacol and Pentasa (paper I). We included 18 patients using Mezavant, 14 using Asacol and 10 using Pentasa. We found no significant difference in overall mean 5-ASA concentration averaged over location and order of sampling between the three 5-ASA formulations

(Mezavant 2.39 ng/mg, Asacol 1.60 ng/mg and Pentasa 0.57 ng/mg, p=0.099). However, pairwise comparison showed a significant difference between Mezavant and Pentasa (p=0.033), but not between Mezavant and Asacol (p=0.50). Mezavant and Asacol both depend on a pH-dependent release of 5-ASA, while Pentasa utilises a time-dependent release. Yu et al. measured 5-ASA concentrations in intestinal fluids collected with a GI tube, the authors found Pentasa to start releasing 5-ASA in the stomach, while 5-ASA release from Mezavant was minimal in the upper GI tract (152). Our results suggest that the availability of 5-ASA in the colonic mucosa is significant for developing high mucosal 5-ASA concentrations in the distal colon and rectum. However, a minimum therapeutic 5-ASA mucosal concentration equivalent to minimum inhibitory concentration in antibiotic therapy or trough level for biologic treatment has not been established (146, 267). Nevertheless, several studies have found mucosal 5-ASA concentration to be inversely correlated to UC disease activity (144, 148) and our findings support that pH-dependent 5-ASA formulations should be preferred when prescribing 5-ASA to UC patients with inflammation in the distal colon.

9.3. Effect of 5-ASA concentration on the mucosa-associated microbiota

In addition to measuring the mucosal 5-ASA concentration in 42 UC patients, we also sequenced the mucosa-associated and faecal bacterial microbiota (paper I). We found an association between mucosal 5-ASA concentration and mucosal bacterial diversity and abundance of bacterial taxa at all taxonomic levels. Mucosal 5-ASA concentration was positively associated with bacterial diversity and presumed beneficial alterations the mucosa-associated microbiota such as decreased abundances of Proteobacteria phylum, increased abundances of Firmicutes phylum and Lachnospiraceae and Ruminococcaceae families. *Faecalibacterium prausnitzii* was also found to be positively associated with mucosal 5-ASA concentration. *F. prausnitzii* is considered an anti-inflammatory bacterium as it produces butyrate which supplies colonocytes with nutrition, has immunomodulatory effects and anti-inflammatory attributes (20, 114, 268). *F. prausnitzii* also produces an anti-inflammatory protein itself (269). An inverse

relationship between *F. prausnitzii* levels and IBD disease activity has been described in several studies (115, 268, 270). Mucosal 5-ASA concentration was not associated with faecal bacterial diversity, and the association between mucosal 5-ASA concentration and faecal bacterial composition was minor compared to the alterations in the mucosa-associated microbiota. The association between 5-ASA concentration and mucosa-associated microbiota alterations could be explained by a direct effect of 5-ASA on bacteria's ability to attach and live in close proximity to the mucosa, this could be supported by the following previous findings I) direct effect of 5-ASA on bacterial in vitro growth and growth in cell cultures (219, 220) II) 5-ASA effect on bacterial gene expression causing reduced bacterial invasiveness (221) III) 5-ASA acting as a polyP-kinase (PPK) inhibitor and thereby decreasing some bacteria's ability to colonise and increase their susceptibility to oxidative stress (141) and IV) decreasing concentrations of mucosal adherent bacteria after 5-ASA use (222). Another explanation could be that 5-ASAs effect on inflammatory mediators possibly causes alterations in the intestinal permeability or defence against commensal bacteria in the gut lumen, for instance through altering the synthesis and secretion of AMPs. However, even though we found an association between mucosal 5-ASA concentration and mucosa-associated bacterial microbiota, we have not proven a causal relationship or a mechanistic explanation. We did not assess the mucosa-associated microbiota before 5-ASA treatment, however two other studies have assessed this and found that 5-ASA treatment indeed alters bacterial microbiota in the faeces and the mucosa (223, 271).

Altogether our results demonstrate that mucosal 5-ASA concentrations are correlated with postulated favourable alterations in the mucosa-associated bacterial microbiota composition. Our findings accentuate 5-ASAs cornerstone role in UC treatment and are in support of high-dose 5-ASA treatment. The positive microbiota alterations can possibly be beneficial even though treatment with immunomodulators or biologic treatment is needed. However, a recent ECCO review stated that it is uncertain if continuation of 5-ASA therapy after initiation of immunomodulators or anti-TNF therapy gives additional benefit beyond the chemoprotective effects of 5-ASA (172).

Interestingly, a recent study reported that 5-ASA also had an impact on the mucosa-associated mycobiota decreasing the fungal genera *Scytalidium*, *Fusarium*, *Sporobolomyces*, *Paecilomyces*, *Morchella* and *Mortierella* and increasing *Wickerhamomyces*, altogether suggesting that 5-ASA decreased pathogenic fungal colonisation in the mucosa of UC patients (272).

9.4. Alterations in mucosa-associated ileal microbiota of patients with CD

Many CD patients have genetic polymorphisms causing impaired recognition and defence against gut microbes. An altered microbiota composition, commonly called dysbiosis, is found in CD patients. In paper II and III, we assessed the bacterial and fungal mucosa-associated microbiota in the ileum of CD patients, respectively. We found that CD patients displayed lower bacterial α-diversity and an altered bacterial microbiota composition compared to HC (paper II). Proteobacteria, Enterobacteriaceae, *Tyzzerella 4* and *Escherichia shigella* were overrepresented in our CD patient cohort, while *Ruminiclostridium 5*, *Ruminiclostridium 6*, *Eisenbergiella* and *Faecalibacterium* were reduced. Increased abundances of Proteobacteria, Enterobacteriaceae and *Escherichia shigella* and reduced abundances of *Ruminiclostridium* and *Faecalibacterium* are in accordance with previous literature (99, 101, 102, 110, 117, 125, 249). The remarkably increased abundance of *Tyzzerella 4* (27-fold (log2), $p=4.1 \times 10^{-68}$) in CD patients was a novel finding. From the limited literature that exist on *Tyzzerella 4*, increased abundances have been found in the colonic mucosa of persons with low dietary quality according to the Healthy Eating Index as well as increased abundances in the stool of patients with high cardiovascular risk (192, 273). Recently, faecal abundances of *Tyzzerella 4* has been associated with antibiotic use and childhood obesity (274). The role of *Tyzzerella 4* in CD remains to be resolved, and further research is warranted. The alterations in the mucosa-associated bacterial microbiota of CD patients found in our study and previous literature points toward a dysbiotic state in CD characterised by increased abundances of potentially

pathogenic facultative anaerobe bacteria and reduced abundances of beneficial obligate anaerobes (30).

The ileal mucosa-associated mycobiota was also altered in CD patients compared to HC (paper III). CD patients displayed reduced fungal evenness, increased abundances of Basidiomycota along with decreased abundances of Ascomycota and Chytridiomycota and an altered fungal composition according to β-diversity. We also identified several differentially abundant fungal taxa between CD patients and HC. In CD patients, we found an enrichment of *Malassezia* and a depletion of *Saccharomyces* at genus level, while at species level *Candida albicans* and *Malassezia restricta* were enriched. Our findings are in consistence with previous findings in faeces, mucosa and water-lavage samples of CD patients (67, 109, 129, 248). Interestingly, our findings unify the different findings from previous fungal studies confirming an altered mycobiota profile in CD patients. There is evidence to suggest that the CD associated mycobiota alterations are, similar to alterations in the bacterial microbiota, unfavourable. In mice models, both *Malassezia restricta* and *Candida albicans* have been shown to deteriorate colitis (67, 275). Whereas, *Saccharomyces* has been shown to have anti-inflammatory attributes (14, 213, 248). Genetic polymorphisms may influence the mycobiota alterations in CD, at least in part as a positive association between the abundance of *Malassezia restricta* and CARD9 polymorphism are found (67). Furthermore, as fungi and bacteria interact closely, the perturbations in mycobiota and bacterial microbiota composition are likely to have co-evolved (14, 276). We did not correlate the bacterial and fungal microbiota composition to each other, however this would be interesting to do in the future. Also, CD patients have an altered composition of viruses, and we have not assessed the viral microbiota in CD patients (123).

9.5. The mucosa-associated microbiota in the inflamed and proximal non-inflamed ileum of patients with CD

To investigate the role of ileal microbiota composition on CD pathogenesis we compared the mucosa-associated microbiota in the inflamed terminal ileum (5-cm location) and the proximal non-inflamed ileum (15-cm location) in CD patients with no history of upper GI CD (paper II and III). In paper II, we assessed the mucosa-associated bacterial microbiota. We found no difference in bacterial α-diversity, β-diversity or differential expression of any bacterial taxa between the inflamed and proximal non-inflamed mucosa. This suggests that the bacterial microbiota is similar within CD patients across ileal sub-locations and regardless of endoscopic inflammation.

In paper III, we assessed the mucosa-associated mycobiota. We found no difference in fungal α-diversity between the inflamed and proximal non-inflamed mucosa. However, on β-diversity plots, there was a clear separation with inflamed samples clustering furthest away from HC and non-inflamed samples clustering in between, suggesting a more profoundly altered fungal composition, possibly dysbiotic, within the inflamed mucosa compared to the proximal non-inflamed mucosa. The fungal composition between inflamed and proximal non-inflamed mucosa differed. We identified six fungal taxa which were increased in inflamed samples and four taxa which were increased in non-inflamed samples. Within the inflamed mucosa Cordycipitaceae and Sporidiobolaceae families, *Lecanicillium* genus and *Candida sake* were increased, while *Exophiala* and *Debaryomyces* genera, identified as *Exophiala equina and Debaryomyces hansenii* at species level were increased in the proximal non-inflamed mucosa. We are, to the best of our knowledge the first to assess differences in the mycobiota between the inflamed terminal or neoterminal ileum and the proximal non-inflamed ileum of the same CD patients.

In comparison, Liguori and colleagues assessed the bacterial and fungal microbiota in inflamed and non-inflamed colon samples from CD patients during relapse (109). They found similar fungal OTU number, but increased abundances of *Saccharomyces cerevisiae* and *Filobasidium uniguttulatum* in non-inflamed mucosa and increased Xylariales order in inflamed mucosa. Interestingly, they also found changes in the mycobiota but not in bacterial microbiota between inflamed vs non-inflamed samples from the same patients.

Summarised, our findings suggest that the fungal microbiota, but not the bacterial microbiota is altered in the inflamed ileum compared to the proximal non-inflamed ileum within the same CD patients. It is difficult to interpret the implications these mycobiota alterations have without mechanistic studies. It can be speculated that fungi overrepresented in the inflamed mucosa trigger inflammation. However, the altered fungal composition could also be a result of altered immunological activity or mucosal barrier between inflamed and non-inflamed mucosa. Future studies are needed to conclude which impact the differentially abundant taxa have on the mucosa and mucosal immune system, this could also possibly elucidate fungi's role in CD pathogenesis. Nevertheless, we have not assessed the viral microbiota and cannot exclude viral factors to contribute to why the terminal ileum and neoterminal ileum is the predilection site for CD inflammation.

9.6. Impact of ileal sub-location and inflammation on the mucosa-associated microbiota

We specifically assessed the impact of ileal sub-location, endoscopic and histologic inflammation in CD patients with respect to bacterial and fungal microbiota in paper II and III, respectively. In paper II, we found that CD patients with histological inflammation had a lower α-diversity than CD patients without histological inflammation. However, β-diversity did not differ according to histological inflammation. Due to the focality of CD, histology can often be normal despite endoscopic inflammation. In our cohort,

histologic inflammation concurred in the majority of cases with endoscopic inflammation, possibly suggesting that patients with histologic inflammation had a higher degree of inflammation. Previously, Sokol and co-authors (128) have found reduced bacterial α-diversity in mucosa with Rutgeerts score i2-i4, but not in mucosa with Rutgeerts score i0-i1. Altogether this may indicate that bacterial α-diversity is only reduced in severely inflamed mucosa. Endoscopic inflammation did not impact bacterial α-diversity or β-diversity in our cohort. Similarly, did not ileal sub-location influence bacterial diversity and no differentially abundant taxa between sample locations in the ileum were identified.

In paper III, the effect of inflammation and ileal sub-location on the mucosa-associated mycobiota was assessed. Mycobiota was similar both according to α-diversity and β-diversity regardless of endoscopic inflammation, histologic inflammation and ileal sub-location. Ileal sub-location influenced neither mycobiota in CD patients nor HC. Altogether, neither inflammation nor ileal sub-location seemed to alter the mycobiota biodiversity or composition alone.

There have been conflicting results regarding the effect of inflammation on mucosa-associated bacterial microbiota composition. Some studies have reported differences in microbiota composition between inflamed and non-inflamed ileal and colon segments (111, 277), while others have found the mucosal microbiota composition to be similar irrespective of inflammatory state (107, 116, 121). There are few studies that have assessed mycobiota in inflamed vs non-inflamed mucosa, Liguori found differences in fungal composition between inflamed and non-inflamed colonic mucosa in the same CD patients (109). Another study assessing the fungal microbiota between inflamed and non-inflamed mucosa by PCR and Denaturing Gel Gradient Electrophoresis found increased fungal richness and diversity in inflamed mucosa (278). However, these methods are less sensitive for detection of fungal taxa in comparison to ITS2 sequencing.

The causal relationship between IBD and dysbiosis is unresolved, however our findings have delineated the role of mucosal inflammation on IBD dysbiosis. Based on our findings in paper II and III, we argue that sub-location in the ileum and inflammation does not influence bacterial or fungal α-diversity or composition in the ileal mucosa of CD patients.

9.7. Bacterial microbiota alterations associated with CD phenotype and ICR

The majority of CD patients in our cohort had undergone ICR. Previous analyses of the bacterial microbiota at ICR and postoperatively have found specific bacterial microbiota profiles associated with disease recurrence (99, 128). Therefore, we assessed if ICR was associated with a distinct bacterial microbiota profile in our cohort (paper II). We found ICR to be associated with decreased bacterial α-diversity, but not with β-diversity. Within our cohort of ICR CD patients, disease recurrence was associated with increased abundances of *Parasutterella*, which belongs to the class of Gammaproteobacteria, which previously has been associated with disease recurrence (100, 128). However, most of the patients in our cohort had undergone ICR a long time before the assessment of the mucosa-associated bacterial microbiota and it is therefore difficult to draw further conclusions of our findings.

We also assessed the mucosa-associated bacterial microbiota between different CD phenotypes in paper II. We found that patients with stricturing CD (n=7) clustered furthest away from HC compared to the other CD phenotypes (remission and terminal ileitis) on β-diversity plots. Additionally, we found a depletion of the presumed beneficial species *Bacteroides massiliensis B84634*, unidentified species of *Sutterella* and unidentified species of *Akkermansia* in CD patients with stricturing disease compared to CD patients with terminal ileitis. These findings were based on a very small subset of patients and must be interpreted with caution. However, several studies point towards specific microbiota alterations in CD phenotypes (279, 280). Increased abundances of *Ruminococcus* and decreased abundances of *Rothia* has been

found in ileal biopsies from patients with stricturing CD in comparison to CD patients without stricturing or penetrating disease behaviour (279). Based on the findings in paper II, we speculate that CD patients with stricturing disease have a more profoundly altered bacterial microbiota with increased depletion of beneficial bacteria.

10. Conclusions

1. Large inter-individual variations in mucosal 5-ASA concentration in the left hemicolon and rectum exist despite intake of equivalent oral 5-ASA dose, Mezavant yields higher 5-ASA concentrations than Pentasa, no significant differences between Mezavant and Asacol was found.

2. NAT1 and NAT2 genotype could not explain inter-individual variations in mucosal 5-ASA concentration.

3. Mucosal 5-ASA concentration was positively associated with bacterial diversity and a presumed beneficial bacterial composition the mucosa.

4. CD patients have an altered ileal mucosa-associated bacterial microbiota in comparison to HC.

5. The mucosa-associated bacterial microbiota in the inflamed and proximal non-inflamed ileal mucosa of the same patients did not differ according to bacterial α- or β-diversity or differential expression of bacterial taxa.

6. Endoscopic inflammation does not influence mucosa-associated bacterial microbiota according to α- or β-diversity or differential expression of bacterial taxa. Histologic inflammation was associated with reduced bacterial α-diversity, but not associated with an altered β-diversity.

7. Ileal sub-location did not influence bacterial α- or β-diversity or differential expression of bacterial taxa in CD patients.

8. CD patients display an altered ileal mucosa-associated mycobiota profile compared to HC.

9. The mucosa-associated mycobiota in the inflamed and proximal non-inflamed ileum of the same CD patients are structurally different.

10. Inflammation, both endoscopic and histologic did not impact α- or β-diversity of the mucosa-associated mycobiota of CD patients overall.

11. Ileal sub-location did not impact α- or β-diversity of the mucosa-associated mycobiota, neither in CD patients nor HC.

11. Future perspectives

The large inter-individual variations in mucosal 5-ASA concentrations are not understood but may be important to optimize treatment with oral 5-ASA preparations. In future work, it would be valuable to measure the luminal pH in the ileum and colon of patients using pH-dependent 5-ASA preparations and correlate pH with measured mucosal 5-ASA concentrations. It would also be interesting to measure the effect of GI transit time on mucosal 5-ASA concentration in order to broaden our understanding of underlying mechanisms associated with variation in mucosal 5-ASA concentration.

In paper II and III, we assessed the bacterial and fungal microbiota in patients with established CD at one-time point. It is challenging to design studies that clearly delineate the cause-and-effect relationships between microbiota and IBD. However, measuring the mucosa-associated microbiota at diagnosis, before treatment initiation and during follow-up as well as assessing microbiota characteristics in different CD phenotypes in multivariate analyses accounting for medical therapy, diet and smoking would be interesting.

Alteration of gut microbiota has an important therapeutic potential in many diseases including IBD, obesity, Clostridium difficile colitis and colorectal cancer (69, 199, 218). Alteration of the microbiota can be achieved through FMT and possibly by bacteriophages and pharmaceutically produced microbiota- cocktails or capsules in the future. There are many drawbacks with FMT, as we are oblivious to which microorganisms and microbiota-linked diseases we may transfer. However, microbiota alteration is a promising therapeutic tool as many diseases seems to be linked to GI microbiota and modifications of the microbiota towards HC seem to improve the disease (215, 216, 218). Nevertheless, more research is warranted to characterize the complex interplay between microbial components such as bacteria, fungi, phages and viruses, including their metabolites in the gut. Increased knowledge about the complex interplay between the microbiota and host immune system is also needed. Large research groups

and networks aim to characterize the microbiota, the interplay between bacteria, fungi, viruses and their metabolites in IBD patients using multi-omics platforms with subsequent hypothesis testing in ex vivo models. Such studies could contribute to tailoring targeted microbiota alterations that could affect the course of IBD.

12. References

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Paper I

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Mucosal 5-aminosalicylic acid concentration, drug formulation and mucosal microbiome in patients with quiescent ulcerative colitis

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Summary

Background: 5-aminosalicylic acid (5-ASA) is the first-line therapy for ulcerative colitis (UC). 5-ASA acts locally in the colonic mucosa by numerous proposed mechanisms, and is metabolised by N-acetyltransferase (NAT). Large variations in mucosal 5-ASA concentrations have been reported, but the underlying mechanisms are not understood.

Aim: To study the relationship between 5-ASA concentration, 5-ASA formulation, NAT genotype and bacterial microbiome in patients with UC.

Methods: Patients with quiescent UC, using monotherapy of Mezavant (n = 18), Asacol (n = 14) or Pentasa (n = 10), 4.0-4.8 g/day were included. 5-ASA was measured in colonic mucosal biopsies and serum by ultra-high performance liquid chromatography. NAT genotypes were determined by Sanger sequencing. Bacterial microbiome was sequenced from faeces and mucosa by 16S rRNA sequencing using Illumina Miseq.

Results: Mezavant provided the highest mucosal 5-ASA levels (geometric mean 2.39 ng/mg), followed by Asacol (1.60 ng/mg, 33% lower, $P = 0.50$) and Pentasa (0.57 ng/mg, 76% lower, $P = 0.033$). Mucosal 5-ASA concentration was not associated with NAT genotype, but serum 5-ASA concentration and NAT1 genotype was associated ($P = 0.044$). Mucosal 5-ASA concentration was positively associated with mucosal bacterial diversity ($P = 0.0005$) and bacterial composition. High mucosal 5-ASA concentration was related to reduced abundance of pathogenic bacteria such as Proteobacteria, and increased abundance of several favourable bacteria such as Faecalibacterium.

Conclusions: Mucosal 5-ASA concentration is positively associated with bacterial diversity and a mucosal bacterial composition that are perceived favourable in UC. Mezavant yielded higher mucosal 5-ASA concentrations than Pentasa. 5-ASA may have beneficial effects on the mucosal microbiome, and high concentrations possibly amend dysbiosis in UC.

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1 | INTRODUCTION

Mesalazine or 5-aminosalicylic acid (5-ASA) is the first line therapy for patients with ulcerative colitis (UC), and is effective both for inducing and maintaining remission.[1,2] 5-ASA is proposed to act through numerous mechanisms, including inhibition of pro-inflammatory mediators such as leukotriens, prostaglandin, interleukin-1, Nuclear factor-κB (NF-κB) and tumour necrosis factor alpha (TNFα), as well as a peroxisome proliferator-activated receptor gamma (PPAR-γ) receptor agonist (reviewed by Lichtenstein and Kamm[3]).

5-ASA exerts its effect in the intestinal mucosa, and mucosal 5-ASA concentrations are inversely correlated to disease activity.[4–7] It is therefore important to identify and understand the determinants of mucosal 5-ASA concentration. In the intestinal mucosa, 5-ASA is acetylated to its inactive metabolite N-acetyl-5-ASA (Ac-5-ASA), mainly by the enzyme N-acetyl-transferase 1 (NAT1), and to a small degree by N-acetyl-transferase 2 (NAT2).[8,9] Orally administered unbound 5-ASA is absorbed and inactivated in the small intestinal mucosa and in the liver, thus only small amounts 5-ASA will reach the colonic mucosa.[3,10] Therefore, several pharmaceutical delivery systems have been developed to transport orally administered 5-ASA to the colon. In a time-dependent formulation (Pentasa, Ferring Pharmaceuticals, Saint-Prex, Switzerland), 5-ASA is coated with a semipermeable membrane of ethyl cellulose, providing a pH-independent release of approximately 35% of its content in the small bowel, 25% in the colon, while the last 40% eliminates in the faeces.[11] In a pH-dependent delivery system (Asacol, Tillots Pharma AG, Rheinfelden, Switzerland), 5-ASA is coated with Eudragit S which dissolves at a pH >7,[12] normally occurring in the terminal ileum and caecum. The Multi Matrix System (Mezavant, Shire Pharmaceutical Contracts Ltd, in partnership with Cosmo SpA, Milan, Italy) consists of hydrophilic and lipophilic excipients, covered by a pH-dependent coating dissolving at pH 7, causing slow diffusion of the drug into the gut lumen.[3] For both Asacol and Mezavant absorption in the ileum is estimated to be around 20%.[13,14] Faecal elimination for Asacol and Pentasa has been found to be similar.[12]

After oral administration of 5-ASA, mucosal concentrations are highest in the proximal colon segments and lowest in the rectum for the previously examined preparations.[7,15,16] Higher concentrations in the rectum and left hemicolon have been achieved by combining oral and rectal 5-ASA formulations.[16,17] However, rectal administration may be inconvenient and adherence to treatment over time is low. As 65%-85% of patients with UC have rectosigmoid and left-sided involvement at the time of diagnosis,[18,19] it seems essential to assure optimal concentrations of 5-ASA in these bowel segments. Although previous studies suggest that Asacol yields higher mucosal 5-ASA concentrations than Pentasa,[4,15] the different 5-ASA formulations are by many considered clinically equally efficient.[2] Studies comparing mucosal 5-ASA concentrations of the more recently marketed Mezavant with other 5-ASA formulations, have to the best of our knowledge, not been published.

Patients with UC have an altered gut microbiome, commonly called dysbiosis. Microbiome alterations in UC are characterised by reduced alpha-diversity[20–23] combined with increases in the Proteobacteria phylum[22,24,25] and decreases in the Firmicutes phylum.[21,25,26] At genus level, Roseburia is reported to be decreased and Haemophilus increased.[27] The effect of selected antibiotics, probiotics and faecal microbiota transplantation[28] supports that dysbiosis is a part of the pathogenesis in UC. 5-ASA has previously also been reported to affect intestinal bacteria, by inhibiting growth of anaerobic strains, reducing bacterial invasiveness and total faecal bacterial abundance,[29–31] as well as reducing bacterial adherent biofilm thickness and Escherichia and Shigella abundances in patients with inflammatory bowel disease (IBD).[21,32] Results from a recent study also suggest that 5-ASA acts as a polyP-kinase inhibitor, thereby decreasing some bacteria's ability to colonise and increase their susceptibility to oxidative stress.[33]

In the current study, we aimed to measure and compare mucosal 5-ASA concentrations in the left hemicolon and rectum in patients with quiescent UC using different oral 5-ASA formulations (Mezavant, Asacol or Pentasa). We also aimed to explore underlying mechanisms which could explain variations in mucosal 5-ASA concentration as well as exploring the interrelation between 5-ASA mucosal concentration and bacterial composition.

2 | MATERIALS AND METHODS

2.1 | Study population

Patients were recruited from the Department of Gastroenterology and Hepatology, St. Olav's Hospital, Trondheim, Norway from 2015 to 2017. Inclusion criteria were confirmed diagnosis of UC in clinical remission prior to invitation, 18-70 years of age and use of oral 5-ASA (Asacol, Pentasa or Mezavant) 4.0-4.8 g once daily. Exclusion criteria were use of rectal 5-ASA formulations, prednisolone, azathioprine, methotrexate and TNF-α medication within the last 3 months. None of the patients had been using antibiotics or probiotics during the last 3 months prior to inclusion. The study was approved by the Regional Committee for Medical and Health Research Ethics, Central Norway (approval reference 2014/2043).

2.2 | Blood samples, endoscopic procedure and collection of mucosal biopsies

Eight hours after daily 5-ASA dose ingestion, patients underwent blood sample collection, enema bowel preparation and sigmoidoscopy. Blood haemoglobin (Hb), leucocyte concentration, plasma erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) were analysed successively. Serum was stored at −80°C until analysis of 5-ASA and Ac-5-ASA concentrations. EDTA blood was used for NAT1 and NAT2 genotyping using Sanger sequencing, as described in Supporting Information. A 240 mL sorbitol enema (Klyx,

Ferring AS, Copenhagen, Denmark) was administered 30-45 minutes before sigmoidoscopy (Olympus Exera II GIF H180, Olympus Europa GmbH, Hamburg, Germany) up to 40 cm from the anal verge. Sets of three mucosal biopsies were collected at 10, 25 and 40 cm from the anal verge (total of nine biopsies) using endoscopy forceps (EndoJaw, Olympus Medical Systems, Tokyo, Japan) after cleaning of the mucosa with water to remove visible faecal remnants. Three biopsies were put on formalin for subsequent histological scoring of inflammation. The remaining six biopsies were briefly rinsed in NaCl 9 mg/mL solution, dried, weighed, snap frozen on liquid N_2, and stored at −80°C for subsequent measurement of 5-ASA and Ac-5-ASA concentration and sequencing of the mucosal microbiome.

2.3 | Analysis of 5-ASA and Ac-5-ASA concentrations

5-ASA and Ac-5-ASA concentrations were analysed in five mucosal colonic biopsies (two biopsies from 10 cm, one biopsy from 25 cm and two biopsies from 40 cm from the anal verge) and in serum from each patient, using an ultra-high performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method, as described in Supporting Information.

2.4 | Evaluation of disease characteristics and inflammation

Demographic and clinical characteristics were recorded. Disease activity was assessed by clinical and endoscopic Mayo score,[34] Geboes histological score[35] and faecal calprotectin. Endoscopic remission was defined as an endoscopic Mayo score ≤1, histological remission as a Geboes score <2.1 and remission according to total Mayo score as a total Mayo score ≤2. Patients that fulfilled both endoscopic Mayo score ≤1 and Geboes score <2.1 were classified as being in deep remission.[36] Patients were instructed to bring a fresh (<24 hours) faecal sample to the appointment. An aliquot was used for calprotectin measurement using a commercial ELISA method (Calpro AS, Lysaker, Norway), normal range <50 mg/kg. Three biopsies from each patient, one from each location (10, 25 and 40 cm from the anal verge) were histologically evaluated by an experienced pathologist. The pathology assessment of inflammation was blinded and scored according to Geboes histological score.[35]

2.5 | Microbiome analysis

The bacterial microbiome was analysed in one mucosal biopsy (sampled 25 cm from the anal verge) and in one faecal sample from each patient (stored at −80°C until analysis). Bacterial DNA was isolated using QIAamp Powerfecal (faecal samples) (Qiagen, Hilden, Germany) and DNeasy Powersoil kit (biopsy samples) (Qiagen), further described in Supporting Information. Libraries were constructed according to Illumina's 16S Metagenomic Sequencing Library Preparation. 16S rRNA amplicon sequencing was performed on an Illumina MiSeq sequencer (Illumina Inc, San Diego, CA). The short read sequencing data from both faecal and mucosal origin were analysed using the operational taxonomic unit (OTU) approach. The QIIME software pipeline (version 1.9.1)[37] was used to cluster the gene sequences into OTUs based on sequence similarity. The SortMeRNA_SUMACLUST method of open-reference OTU picking was applied to a total of 6207583 and 296047 sequencing reads and clustered into 1043 and 770 OTUs for faecal and mucosal samples respectively, at 0.8 SortMeRNA coverage threshold. The OTU taxonomy was identified using the Ribosomal Database Project classifier trained on the Greengenes database (version 13.8).

2.6 | Statistical analysis

IBM SPSS Statistics version 24.0 (IBM Corp., Armonk, NY) was used to conduct the statistical analysis. Demographic and clinical characteristics are presented as median (IQR, interquartile range) for skewly distributed variables, mean value (SD) for normal distributed variables, and as % (n) for categorical variables. Accordingly, Kruskal-Wallis test, *F*-test (ANOVA) and chi-squared test were used for comparing 5-ASA formulations groups with respect to these measures. A multilevel linear mixed model was applied to test for difference in mean mucosal concentrations of 5-ASA and Ac-5-ASA (log-transformed data) by type of 5-ASA formulation, adjusted for (no-interaction model), or specific to (interaction model) sample locations in the left hemicolon and rectum (10, 25 and 40 cm from the anal verge), further described in Supporting Information. Kruskal-Wallis test was used for comparing serum concentrations between the three 5-ASA formulations. Regardless of 5-ASA formulation (n = 42), mean mucosal 5-ASA concentration (individual level defined by mean of five repeated measures, log-transformed) and mean serum 5-ASA concentration was compared according to NAT genotype (two phenotypes for NAT1 and three phenotypes for NAT2) and disease activity (deep remission or remission according to endoscopic or total Mayo score) using one-way ANOVA. *P*-values <0.05 were considered statistically significant. A power analysis was performed in which mean mucosal 5-ASA concentration estimates were based on previous studies comparing Pentasa and Asacol[4,7,15] by one-way ANOVA and two pair-wise comparisons. With mean 5-ASA concentrations of 60 and 20 ng/mg, a SD of 30 ng/mg, $\alpha = 0.05$ and $\beta = 0.8$, 11 patients in each group were needed.

Associations between 5-ASA mucosal concentrations and bacterial composition in the mucosa and faeces were examined using a linear regression model. The OTU tables generated by the QIIME pipeline were imported into the R software environment version 3.4.1 (R Foundation for Statistical Computing, Vienna, Austria) using the phyloseq package and subsequently filtered as to include only OTUs from the Bacteria kingdom and excluding OTUs classified as Mitochondria, Chloroplast or Cyanobacteria/Chlorplast. Alpha diversity values were estimated from the filtered datasets using Shannon entropy. The core microbiome was estimated by requiring 10% prevalence of detected (>1 sequence) OTUs. The core microbiome had a total of 822 and 156 OTUs in faecal and mucosal samples respectively. The count tables at a given taxonomic rank of the core

TABLE 1 Demographic and clinical characteristics of patients with ulcerative colitis using three different oral 5-aminosalicylic acid (5-ASA) formulations

	Mezavant	Asacol	Pentasa	*P*-value
Number of patients, n	18	14	10	—
Dose (g/d), median (IQR)	4.8 (0.0)	4.8 (0.2)	4.0 (0.0)	—
Gender (M-F) (n-n)	11-7	5-9	7-3	0.19
Age at diagnosis (y), mean (SD)	35.8 (9.7)	31.4 (12)	37.2 (13.7)	0.325
Duration of disease (y), median (IQR)	8.5 (5)	11.0 (6)	7.0 (15)	0.076
Disease extension, n (%)				
Rectosigmoid involvement	6 (33)	3 (22)	3 (30)	0.796
Left sided	3 (17)	1 (7)	1 (10)	
Pancolitis	9 (50)	10 (71)	6 (60)	
Previous medical therapy (≥3 mo ago), n (%)				
Prednisolone	17 (94)	14 (100)	8 (80)	—
Methotrexate	0	1 (7)	0	—
Azathioprine	1 (5.5)	2 (14)	0	—
Anti-TNFα	0	2 (14)	0	—
Current co-medication, n (%)[a]				
No co-medication	10 (56)	10 (72)	7 (70)	0.802
Laboratory values				
Hb[b] (g/dL), mean (SD)	14.4 (1.3)	13.9 (1.4)	14.9 (1.1)	0.168
CRP[c] (mg/L), median (IQR)	<5 (0)	<5 (4)	<5 (0)	0.074
ESR[d] (mm/h), median (IQR)	3,0 (4)	7.0 (12)[e]	4.5 (6)	0.210
Leukocytes ($\times 10^9$/L), mean (SD)	6.7 (1.4)	8.1 (2)	6.6 (1.1)	0.036[f]
Faecal calprotectin (mg/kg), median (IQR)	33 (243)	93 (175)	55 (410)	0.253
Disease activity				
Total Mayo score, median (IQR)	1.5 (3.0)	1.0 (2.25)	0.5 (1.25)	0.055
Clinical Mayo score, median (IQR)	1.0 (2.0)	0.5 (2.0)	0.0 (1.0)	0.311
Endoscopic Mayo score, median (IQR)	1.0 (2.0)	0.0 (1.0)	0.0 (1.0)	0.010[g]
Endoscopic remission[h], n (%)	12 (67%)	13 (93%)	10 (100%)	0.021[i]
Histologic Geboes score, median (IQR)	1.1 (1.1)	1.1 (0.1)	0.1 (1.3)	0.328
Histologic remission[j], n (%)	14 (78)	11 (79)	7 (70)	0.873
Deep remission[k], n (%)	12 (67)	11 (79)	7 (70)	0.750

[a]Percentage of patients on monotherapy with oral 5-ASA and no co-medication. For patients using co-medication, the following drugs were used: amiloride/hydrochlorothiazide (n = 1), lisinopril/hydrochlorothiazide (n = 1), losartan/ hydrochlorothiazide(n = 1), calcium and cholecalciferol (n = 3), zoledronate (n = 1), alendronate (n = 2), pantoprazole (n = 1), esomeprazole (n = 1), drospirenone/ethinylestradiol (n = 1), estradiol/norethindrone acetate (n = 1), ethinylestradiol/levonorgestrel (n = 1), venlafaxine (n = 2), levothyroxine (n = 2), tiotropium bromide (n = 1), simvastatin (n = 1), cetirizine (n = 1), paracetamol (n = 1), zolmitriptan (n = 1), sumatriptan (n = 1), desoximetasone crème 2.5 mg/g (n = 1), terbinafine (n = 1).
[b]Blood haemoglobin.
[c]C-reactive protein.
[d]Plasma erythrocyte sedimentation rate.
[e]For one Asacol patient SR-value is missing.
[f]Asacol group had higher leukocyte concentration.
[g]Mezavant group had higher endoscopic Mayo scores.
[h]Defined as a endoscopic Mayo score ≤1.
[i]Mezavant patients had lower rates of endoscopic remission.
[j]Defined as a Geboes score <2.1.
[k]Defined as a endoscopic Mayo score ≤1 and a Geboes score <2.

microbiome were imported into the DESeq2 R package to estimate which OTUs that significantly correlated with log-transformed mucosal 5-ASA concentrations.[38] Significant OTUs were identified using a regression model in DESeq2 using default options and *P*-values were estimated using a Wald test and adjusted for multiple testing by Benjamini Hochberg false discovery rate correction.

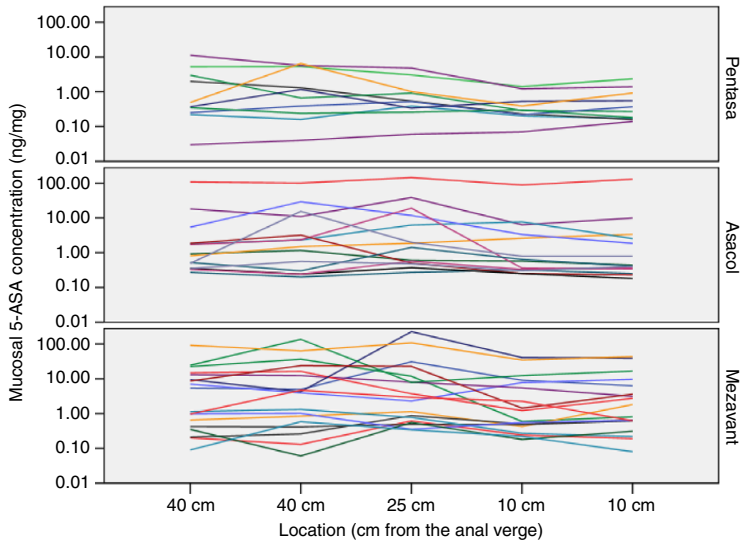

FIGURE 1 Mucosal 5-aminosalicylic acid (5-ASA) concentration in five colonic biopsies sampled 40, 25 and 10 cm from the anal verge in patients with ulcerative colitis using three different oral 5-ASA formulations. Each line represents one patient

3 | RESULTS

3.1 | Patient characteristics

Demographic and clinical characteristics are presented in Table 1. Overall the three 5-ASA formulation groups were similar. There were no significant differences in total Mayo score ($P = 0.055$) or proportion of patients in deep remission ($P = 0.750$). However, the Asacol group had higher leukocyte concentrations ($P = 0.036$), and the Mezavant group had higher endoscopic Mayo scores ($P = 0.01$) and fewer patients in endoscopic remission ($P = 0.021$). All Pentasa patients were classified to be in endoscopic remission, while 12 (67%) Mezavant patients and 13 (93%) Asacol patients were in endoscopic remission ($P = 0.021$). The proportion of patients in histological remission was, however, lowest in the Pentasa group, n = 7 (70%).

3.2 | 5-ASA drug formulation was associated with mucosal 5-ASA concentration

A graphical display of the individual 5-ASA concentrations at different locations (Figure 1) illustrates large inter-individual variations, but small intra-individual variation between segments. Boxplots of mucosal 5-ASA concentration at all locations are shown in Figure S4.

The overall test for difference in adjusted mean 5-ASA concentration (averaged over location) between the three 5-ASA formulation groups (Table 2) did not reach statistical significance ($P = 0.099$), but pairwise comparisons revealed a significant higher mean concentration in patients using Mezavant compared with Pentasa (geometric mean 2.39 ng/mg vs 0.57 ng/mg, $P = 0.033$) and a nonsignificant higher mean value compared to the Asacol group (2.39 ng/mg vs 1.60 ng/mg, $P = 0.50$). Recalculated into % difference, the Pentasa patients had 76% lower 5-ASA concentrations compared with Mezavant patients, and Asacol patients had in average 33% lower concentrations. The mean 5-ASA concentration level differed significantly by location ($P \leq 0.001$), with the lowest concentrations in the rectum regardless of formulation. Despite a more clear decreasing trend according to location in the Pentasa group, the test for interaction between location and formulation did not reach statistical significance ($P = 0.68$). Consequently, the difference between the three 5-ASA formulations was consistently observed at each location, although power of test within subgroups was low. Adjustment for deep remission did not change the results notably, but provided more precise estimates (slightly lower *P*-values and smaller CI, results not shown).

The mucosal concentration of Ac-5-ASA decreased significantly from oral to anal direction, and did not differ between the formulations overall ($P = 0.47$) or in pairwise comparisons (Mezavant vs Pentasa $P = 0.23$ and Mezavant vs Asacol $P = 0.48$). There were no significant differences in serum 5-ASA ($P = 0.20$) or serum Ac-5-ASA ($P = 0.20$) between the different 5-ASA formulations (Table 3).

3.3 | Mucosal 5-ASA concentration was positively associated to abundance of numerous beneficial bacteria

Mucosal 5-ASA concentration was significantly associated to the mucosal bacterial abundance on all taxonomic levels (Table 4), whereas the association to bacterial abundance in faecal samples was weaker (Table 5). Mucosal 5-ASA concentration and alpha-diversity was positively associated in mucosal biopsies ($P = 4.7 \times 10^{-4}$), whereas no association was found in faecal samples (Figure 2). Regression analysis of bacterial abundances on phylum level and mucosal 5-ASA concentration (Figure 3) revealed that mucosal 5-ASA mucosal concentrations were associated with lower abundances

TABLE 2 Mucosal concentrations (ng/mg) of 5-aminosalicylic acid (5-ASA) and acetylated 5-ASA (Ac-5-ASA) in the left hemicolon and rectum in patients with ulcerative colitis using three different oral 5-ASA formulations

	Geometric mean (95% confidence interval)[a]			*P*-value	
	Mezavant (n = 18)	Asacol (n = 14)	Pentasa (n = 10)	By location[b]	By formulation[c]
5-ASA concentrations					
Adjusted mean[d]	2.39 (1.09-5.28)	1.60 (0.65-3.91)	0.57 (0.20-1.64)		0.099
Difference (%)	1.0 (reference)	−33.2 (−79.8, 119.7)	−76.3 (−93.7, −11.2)		
P-value[e]		0.50	0.033		
By location (cm from the anal verge)				<0.001	
40	2.71 (1.20-6.10)	1.71 (0.68-4.31)	0.76 (0.26-2.26)		0.24
25	3.22 (1.36-7.59)	2.31 (0.88-6.10)	0.58 (0.18-1.81)		0.064
10	1.61 (0.71-3.63)	1.09 (0.43-2.74)	0.37 (0.13-1.10)		0.081
P-value, location[f]	0.013	0.011	0.006		
P -value, interaction[g]	0.68				
Ac-5-ASA concentrations					
Adjusted mean[d]	1.56 (0.98-2.49)	1.03 (0.61-1.74)	1.19 (0.64-2.22)		0.47
Difference (%)	1.0 (reference)	−34.3 (−67.4, 32.4)	−23.9(−64.9, 65.2)		
P-value[e]		0.23	0.48		
By location (cm from the anal verge):				<0.001	
40	2.03 (1.24-3.30)	1.68 (0.97-2.93)	2.02 (1.05-3.89)		0.85
25	1.50 (0.88-2.57)	0.88 (0.48-1.61)	1.05 (0.52-2.15)		0.47
10	1.29 (0.79-2.10)	0.71 (0.41-1.24)	0.78 (0.40-1.50)		0.27
P-value, location[f]	0.009	<0.001	<0.001		
P-value, interaction[g]	0.23				

[a]Results based on analyses of log-transformed data in multilevel linear mixed model, with and without interaction between 5-ASA formulation type and location, and with additional adjustments for pinch biopsy site and replicates (replicate samples at 40 and 10 cm).
[b]*F*-tests for difference in mean values between locations; 10, 25 and 40 cm from the anal verge (no-interaction model).
[c]*F*-tests for difference in mean values between 5-ASA formulation groups, overall (no-interaction model) and at each specific location (interaction model).
[d]Overall mean, averaged over location and order of sampling (estimated marginal means from no-interaction model).
[e]Pairwise tests (each group compared with Mezavant).
[f]*F*-tests for difference in mean values between locations within each 5-ASA formulation (interaction model).
[g]*F*-test for interaction between location and 5-ASA formulation type.

TABLE 3 Serum concentrations (ng/mL) of 5-aminosalicyclic acid (5-ASA) and acetylated 5-ASA (Ac-5-ASA) in patients with ulcerative colitis using three different oral 5-ASA formulations

	Mezavant	Asacol	Pentasa	*P*-value[a]
Serum concentrations (ng/mL), median (interquartile range)				
5-ASA	3420 (5085)	1658 (3347)	3028 (6317)	0.202
Ac-5-ASA	2319 (2769)	1770 (2439)	4634 (5555)	0.200

[a]Compared using Kruskal-Wallis test.

of the Proteobacteria phylum ($P = 1.2 \times 10^{-15}$) and higher abundances of Firmicutes ($P = 2.6 \times 10^{-6}$) and Bacteroidetes ($P = 3.1 \times 10^{-4}$) in mucosal biopsies. There were no associations between faecal bacterial abundance on phylum level and 5-ASA mucosal concentration.

In the mucosal biopsies, 10 bacterial families were positively and six families were negatively associated to mucosal 5-ASA concentration (Figures S5 and S6), the most significant association was seen for Lachnospiraceae and Ruminococcaceae families (Figure 4A,B). Totally 19 mucosal bacterial genera were associated with 5-ASA concentration. The bacterial genus in the mucosa that was most significantly associated to 5-ASA concentration was Faecalibacterium (4.8×10^{-16}) (Figure 4C), followed by the genera Blautia (Figure 4D), Bacteroides, Coprococcus and Dorea. Of the genera associated with 5-ASA concentration, 18 of 19 were positively associated (Figure S7). Cloacibacterium was the only genus negatively associated ($P = 0.048$) with mucosal 5-ASA concentration in the mucosal biopsies (Figure 4F). On species level, *Faecalibacterium prausnitzii* was positively correlated with mucosal 5-ASA concentration ($P = 1.3 \times 10^{-6}$) (Figure 4E). In faeces, two bacterial genera were associated with 5-ASA mucosal concentration; Prevotella ($P = 7.2 \times 10^{-5}$) and Sutterella ($P = 0.03$), both genera were negatively associated with 5-ASA concentrations (Table 5).

TABLE 4 Mucosal bacteria abundances significantly associated with 5-aminosalicylic acid (5-ASA) concentration on different taxonomic levels (regression analysis)

Phylum	Class	Order	Family	Genus	*P*	*B*
Proteobacteria	—	—	—	—	1.2×10^{-15}	−1.29
Firmicutes	—	—	—	—	2.6×10^{-6}	0.48
Bacteroidetes	—	—	—	—	3.1×10^{-4}	0.99
Firmicutes	Clostridia	—	—	—	6.1×10^{-21}	2.33
Bacteroidetes	Bacteroidia	—	—	—	6.9×10^{-9}	2.10
Proteobacteria	Alphaproteobacteria	—	—	—	1.2×10^{-6}	−0.87
Firmicutes	Bacilli	—	—	—	1.2×10^{-5}	−0.70
Proteobacteria	Betaproteobacteria	—	—	—	5.5×10^{-5}	−0.65
Bacteroidetes	Flavobacteriia	—	—	—	4.8 0.10^{-3}	−2.34
Firmicutes	Clostridia	Clostridiales	—	—	2.4×10^{-20}	2.62
Bacteroidetes	Bacteroidia	Bacteroidales	—	—	8.2×10^{-10}	2.10
Actinobacteria	Actinobacteria	Bifidobacteriales	—	—	1.3×10^{-4}	1.94
Firmicutes	Bacilli	Lactobacillales	—	—	1.4×10^{-4}	3.37
Proteobacteria	Alphaproteobacteria	Rhizobiales	—	—	9.8×10^{-4}	−0.57
Firmicutes	Bacilli	Bacillales	—	—	0.002	−0.49
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	—	7.8×10^{-13}	1.73
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	—	1.6×10^{-12}	2.01
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	—	9.8×10^{-12}	−1.27
Firmicutes	Bacilli	Bacilliales	Bacillaceae	—	6.2×10^{-12}	−1.32
Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	—	2.8×10^{-8}	−1.51
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	—	3.9×10^{-5}	1.46
Bacteroidetes	Flavobacteriia	Flavobacteriales	Weeksellaceae	—	5.7×10^{-4}	−2.95
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	—	8.2×10^{-4}	2.04
Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	—	0.011	2.38
Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	—	0.015	−1.11
Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	—	0.015	2.10
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	—	0.015	1.76
Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	—	0.029	−0.74
Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	—	0.031	3.60
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	—	0.031	0.99
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	—	0.035	1.87
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Faecalibacterium	4.8×10^{-16}	3.16
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	2.1×10^{-14}	2.60
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	1.8×10^{-10}	2.72
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Coprococcus	3.6×10^{-10}	2.41
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Dorea	1.8×10^{-8}	3.05
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Ruminococcus	6.1×10^{-8}	3.97
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	6.2×10^{-8}	3.94
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Roseburia	9.6×10^{-8}	3.39
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides	1.0×10^{-7}	3.40
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	1.9×10^{-5}	2.10
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	5.5×10^{-5}	3.55
Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Sutterella	1.2×10^{-4}	3.36
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Lachnospira	1.2×10^{-4}	2.02
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	2.1×10^{-4}	2.76

(Continues)

TABLE 4 (Continued)

Phylum	Class	Order	Family	Genus	*P*	*B*
Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	Collinsella	1.2×10^{-3}	2.17
Bacteroidetes	Bacteroidia	Bacteroidales	Odoribacteraceae	Odoribacter	9.3×10^{-3}	2.48
Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	0.021	4.51
Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Dialister	0.028	1.40
Bacteroidetes	Bacteroidia	Bacteroidales	Weeksellaceae	Cloacibacterium	0.048	−1.62

Bacteria within each taxonomic level are listed by increasing *P*-values. *P* = adjusted *P*-value, *B* = regression coefficient. Red text = increased abundance (positive association), blue = decreased abundance (negative association).

TABLE 5 Faecal bacteria abundances significantly associated with 5-aminosalicylic acid (5-ASA) concentration on different taxonomic levels (regression analysis)

Phylum	Class	Order	Family	Genus	*P*	*B*
Proteobacteria	Betaproteobacteria	—	—	—	0.004	−2.02
Bacteroidetes	Betaproteobacteria	Burkholderiales	—	—	0.007	−2.02
Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	—	7.7×10^{-5}	−5.17
Firmicutes	Betaproteobacteria	Burkholderiales	Alcaligenaceae	—	0.02	−1.92
Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	7.2×10^{-5}	−5.27
Firmicutes	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Sutterella	0.025	−1.91

Bacteria within each taxonomic level are listed by increasing *P*-values. *P* = adjusted *P*-value, *B* = regression coefficient, Blue text = decreased abundance (negative association).

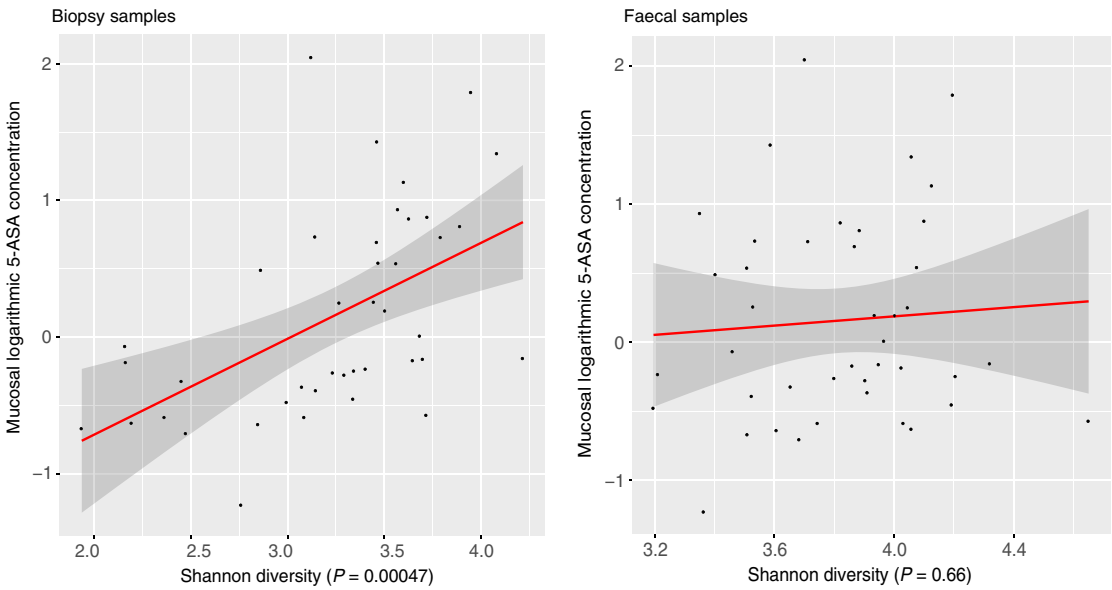

FIGURE 2 Bacterial diversity analysis in biopsies and faecal samples in patients with ulcerative colitis using 5-aminosalicylic acid (5-ASA). Red line = regression line, dark grey area = 95% confidence interval

3.4 | Mucosal 5-ASA concentration was not associated with mucosal inflammation

Mucosal 5-ASA concentrations and disease activity were not significantly associated in our patients. Mucosal 5-ASA concentration was neither associated with deep remission (*P* = 0.106), total Mayo score (*P* = 0.114) nor endoscopic Mayo score (*P* = 0.055). The inflammation variables tended to be higher in patients with high mucosal 5-ASA concentration.

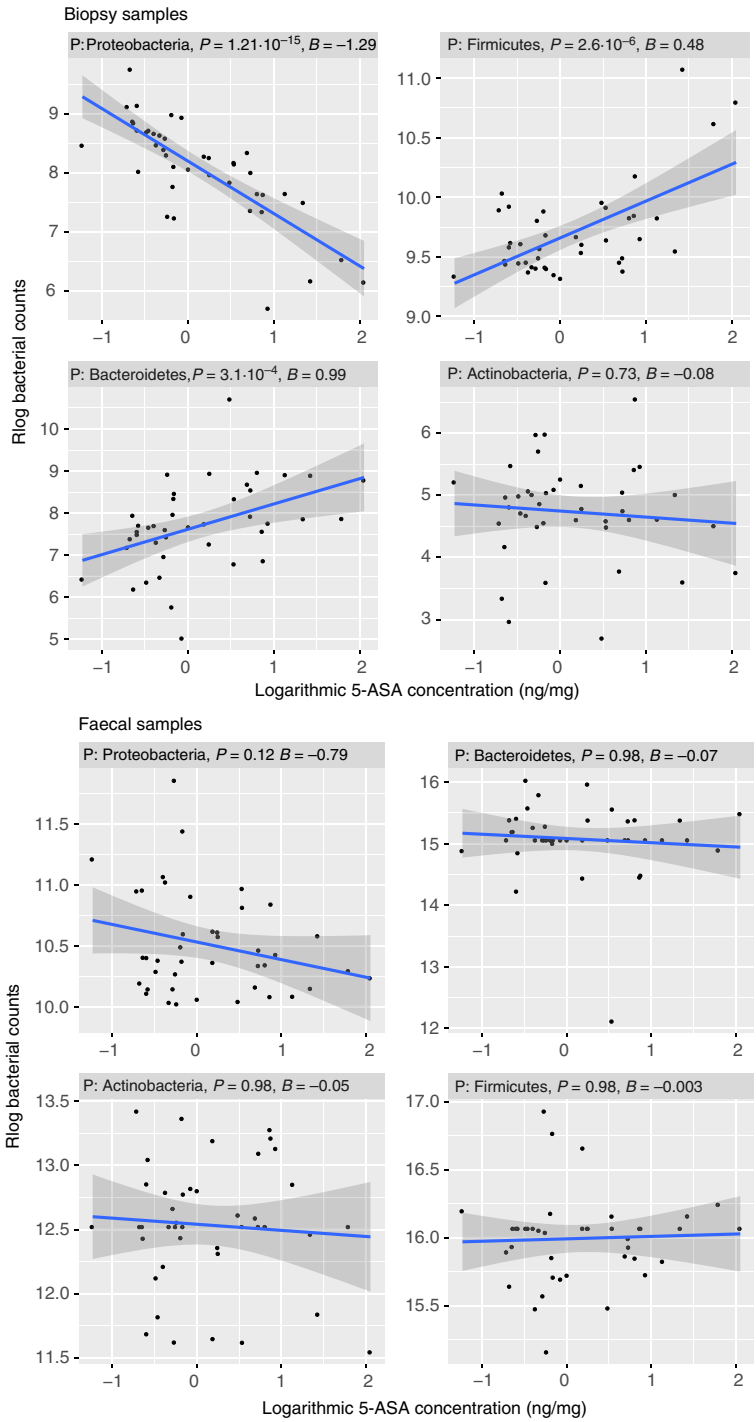

FIGURE 3 Bacterial phyla correlated to 5-aminosalicylic acid (5-ASA) mucosal concentration in biopsies and faecal samples in patients with ulcerative colitis (UC). P: phylum, *P* = adjusted *P*-value, B = regression coefficient, Rlog = regularised logarithm transformation. Blue line = regression line. Dark grey area = 95% confidence interval

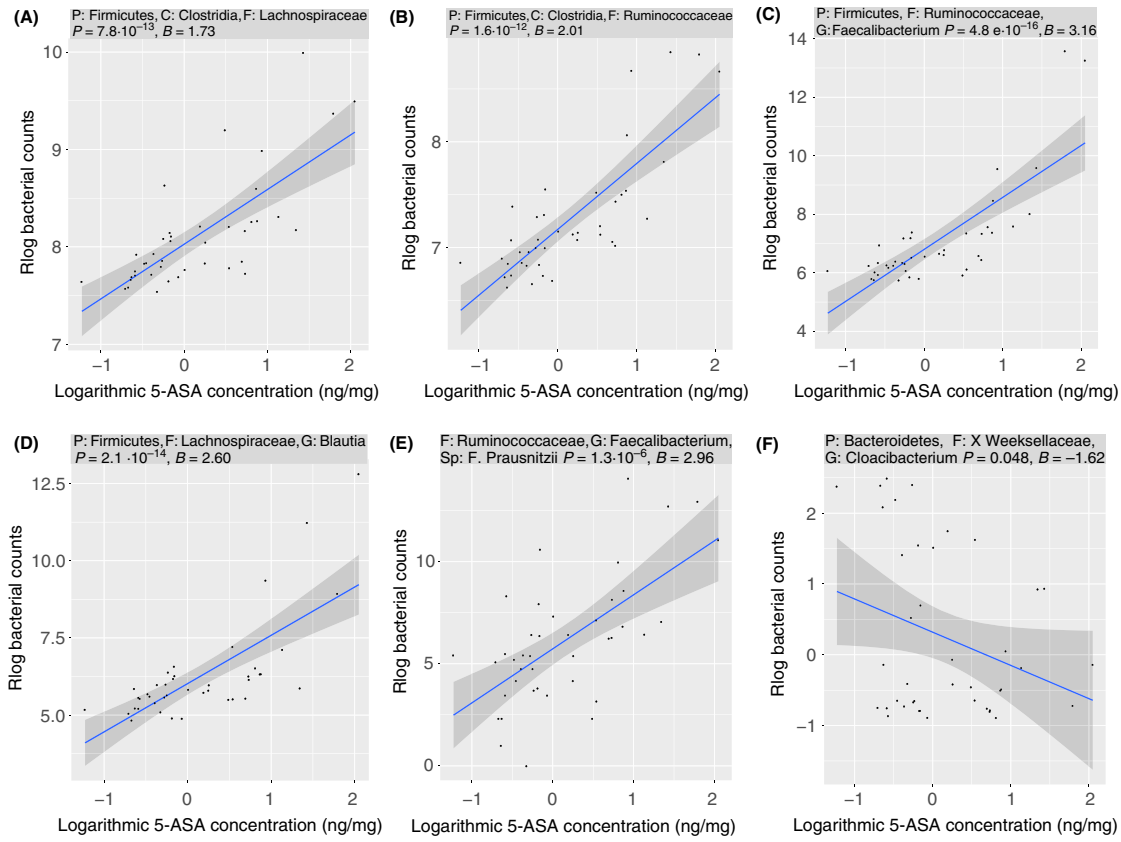

FIGURE 4 Selected bacterial families, genera and species significantly correlated to 5-aminosalicylic acid (5-ASA) mucosal concentration in mucosal biopsies in patients with ulcerative colitis; (A) Lachnospiraceae, (B) Ruminococcaceae, (C) Faecalibacterium, (D) Blautia, (E) Faecalibacterium prausnitzii, (F) Cloacibacterium (for complete list see Figures S5-S7). P, phylum; C, class; F, family; G, genus; Sp, species. *P* = adjusted *P*-value; *B* = regression coefficient, Rlog = regularised logarithm transformation. Blue line = regression line. Dark grey area = 95% confidence interval

3.5 | NAT1 genotype influences serum, but not mucosal 5-ASA concentration

All patients were successfully genotyped for NAT1 and NAT2. Eight patients (19.1%) had a slow acetylator NAT1 phenotype (Table 6). Thirty-one patients (73.8%) had a slow acetylator NAT2 phenotype (Table S3). Patients with NAT1 slow acetylators status had significantly higher 5-ASA serum concentrations ($P = 0.044$) than patients with NAT1 rapid acetylator status. There was no significant association between serum 5-ASA concentration and NAT2 genotype ($P = 0.708$). Mucosal 5-ASA concentration was not significantly associated with neither NAT1 ($P = 0.276$) nor NAT2 ($P = 0.488$) genotype.

4 | DISCUSSION

This is the first study to measure mucosal 5-ASA concentration in UC patients using different oral 5-ASA formulations, and correlating it to NAT genotype and bacterial microbiota. Concurring with previous findings, our patients had declining concentrations of 5-ASA towards the rectum.[16] A novel finding was that patients using Mezavant had higher overall mucosal 5-ASA concentrations, compared to patients using Pentasa, while there was no significant difference between patients using Mezavant and Asacol. All patients used high doses of 5-ASA, however the manufacturers' maximal recommended dose of Pentasa (4.0 g) is 17% lower than of Mezavant (4.8 g) and Asacol (4.8 g), but this could only explain a minor proportion of the observed differences. The large inter-individual variation in concentration was consistent with previous reports,[4–7] whereas the intra-individual variation between bowel segments was small. Factors such as colonic transit time, intraluminal pH and disease pattern may affect dissolution and uptake of 5-ASA in the colon[39] and could explain some of the inter-individual variation in 5-ASA concentration reported in the present and previous studies.[4,15,16] The mucosal Ac-5-ASA concentration reflects the amount of 5-ASA metabolised by NAT1 in the colonic mucosa. There were no significant differences

TABLE 6 Frequencies of N-acetyl-transferase 1 (NAT1) genotypes and phenotype/acetylator status in patients with ulcerative colitis using oral 5-aminosalicylic acid

NAT1 Genotype	Number of patients (n)	Phenotype/ Acetylator status	Observed frequency (%)
NAT1*3/*4	1	Rapid	2.4
NAT1*4/*4	24	Rapid	57.1
NAT1*4/*10	7	Rapid	16.7
NAT1*4/*14B	2	Slow	4.8
NAT1*4/*15	1	Slow	2.4
NAT1*4/*16	1	Rapid	2.4
NAT1*4/*17	4	Slow	9.5
NAT1*10/*10	1	Rapid	2.4
NAT1*10/*14A	1	Slow	2.4

in Ac-5-ASA mucosal concentrations between the three 5-ASA formulations. Similarly, the serum concentrations of 5-ASA or Ac-5-ASA did not differ between the formulations.

The intestinal bacterial composition and metabolic products may affect disease activity in UC. 5-ASA has been found to inhibit in vitro growth of Mycobacterium avium paratuberculosis as well as anaerobic bacteria.[31,40] 5-ASA also influence bacterial gene expression causing reduced bacterial invasiveness.[30] Furthermore, in patients with irritable bowel syndrome, 5-ASA reduces overall faecal bacterial abundance, increases Firmicutes and decreases Bacteroidetes abundances.[29] In the present study, mucosal 5-ASA concentration was remarkably associated with alterations in the mucosal bacterial composition, but to a lesser degree with alterations in faecal microbiota. High mucosal 5-ASA concentration was associated with high bacterial diversity, decreased mucosal abundances of Proteobacteria phylum and increased mucosal abundances of Firmicutes and Bacteroidetes phyla. Reduced bacterial diversity is a hallmark of dysbiosis in UC, whereas increased bacterial diversity is perceived beneficial.[20,21,25,41] Proteobacteria are increased in patients with UC,[25,42,43] and linked to increased disease activity and relapse frequency.[24,42] Overall, the bacterial genera positively associated with high 5-ASA concentrations have previously been associated with beneficial effects in IBD: (a) the abundances of the butyrate producing Faecalibacterium and Roseburia have been inversely correlated to disease activity in UC.[44] (b) Blautia, Bacteroides, Parabacteroides and Sutterella have all been reported to be inversely correlated with inflammation in patients with ileal-pouch anal anastomosis.[45] (c) Ruminococcaceae and Ruminococcus spp in donor faeces transplanted to UC patients is associated with induction of remission[46] and (d) the Lachnospiraceae and Ruminococcaceae family, to which most genera in Table 4 belong, may protect against UC as they are increased in healthy twins discordant for UC.[22] One genus, Cloacibacterium, was found to be negatively associated to mucosal 5-ASA concentration, recently this genus has been reported to be more abundant in inflamed vs. non-inflamed tissue of UC patients.[23]

Although 16S rRNA sequencing may not be the preferred method for analysing bacteria on species level, the mucosal 5-ASA concentration was positively associated with the mucosal abundance of *F. prausnitzii*. *Faecalibacterium prausnitzii* is found to be depleted in UC patients and inversely correlated with disease activity, whereas increased abundance is associated with long-term remission.[44,47]

The mucosal 5-ASA concentration was not associated with alterations in the faecal bacteriome to the same extent as the mucosal bacteriome. The faecal bacterial diversity, and bacterial abundance on phylum level, were not associated with mucosal 5-ASA concentrations. However, abundances of the Betaproteobacteria class and Burkholderiales order in faeces were negatively associated to mucosal 5-ASA concentration, the former association was also found in the mucosa and is presumed favourable in UC.[24,42]

5-ASA concentration have previously been found to be inversely correlated to disease activity index scores,[5,7] endoscopic and histologic remission[4,6] in UC patients with mild to moderate disease activity. In the present study, patients had low disease activity, and in contrast to previous findings, the 5-ASA concentration was not significantly associated with the combined parameters of disease activity. Such associations may not be evident within a relatively homogenous patient group with low disease activity. In fact, there was a trend towards higher mucosal 5-ASA concentrations in patients with higher endoscopic scores and it is unlikely that the microbiome features associated with mucosal 5-ASA concentration were confounded by the degree of inflammation. NAT1 and NAT2 genotypes have previously not been found to influence treatment efficacy,[48] but studies correlating NAT genotypes to 5-ASA mucosal concentrations have previously not been published. We found no correlation between NAT genotypes and mucosal 5-ASA concentration, thus NAT1 genotype cannot explain the large inter-individual variations in mucosal 5-ASA concentrations in our study. Interestingly we found that patients with the NAT1 slow acetylator genotype had significantly higher serum 5-ASA concentrations compared to patients with NAT1 rapid acetylator genotype, which could theoretically influence the risk of concentration dependent systemic adverse effects.

5 | CONCLUSIONS

Patients using Mezavant had significantly higher mucosal 5-ASA concentrations than patients using Pentasa. Our results suggest that 5-ASA increases bacterial diversity, favours numerous beneficial bacteria and inhibits disadvantageous bacteria in UC patients. In conclusion, our novel findings indicate that 5-ASA may have positive effects on the mucosal microbiome and could amend dysbiosis in UC patients.

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AUTHORSHIP

Guarantor of the article: Reidar Fossmark.

Author contributions: TCM and RF designed the study. MO, TCM and RF have collected the data. OS, AF, WRB, GA, ESR, and BG have contributed to analyses of biological material and data. MO, OS, AF, AvBG, GA, AKS, TCM and RF have interpreted the results and drafted the manuscript. All authors have approved the final version of the manuscript including the author list.

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Supporting information

NAT1 and NAT2 genotyping

NAT1 *4 and NAT2 *4 have been defined as reference alleles (1). A total of 28 sequence variants or NAT1 alleles have been described to date (2). Carriers, both heterozygous and homozygous of NAT1 *14A, *14B, *15, *17, *19A, *19B and *22 alleles have a reduced enzyme activity and were considered slow acetylators, while all other allele combinations were considered rapid acetylators (1). NAT2 genotypes are classified into rapid, intermediate and slow acetylators phenotypes according to previous publications (1).

DNA was isolated from EDTA preserved whole blood using QIAsymphony SP/AS and QIAsymphony DSP DNA Midi kit, version 1.0 (Qiagen, Hilden, Germany), quality tested using NanoDrop (Termo Fisher Scientific, Waltham, MA), and stored at -20 °C until analysis of NAT1 and NAT2 genotype. NAT1 and NAT 2 genotypes were determined using Sanger sequencing. All primers were designed using Oligo® Primer Analysis Software version 7.57 (Molecular Biology Insights, Inc., Colorado Springs, CO) and Alamut® Visual version 2.10 (Interactive Biosoftware, Rouen, France). The primers were ordered from Eurogentec (BioNordika AS, Oslo, Norway). Four primers pairs (Supplementary information table S1) were designed to identify all known NAT1 genotypes (2). The primers amplify the region c.-6-405 – c.*297 of the reference sequence NG_012245.2 (NM_001160170.3) with overlapping regions. Two primer pairs (Supplementary information table S2) were designed to identify the most common six NAT2 alleles; NAT2*4, NAT2*5, NAT2*6, NAT2*7, NAT2*10 and NAT2*14 (3). The primers amplify the region c.-5-c.-91 of the reference sequence NG_012246.1 (NM_000015.2).

30 ng of isolated DNA from EDTA preserved whole blood and 7µM of each primer were added to the polymerase chain reaction (PCR) mix of 25 µL. PCR was preformed using AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster city, CA) as per manufacturer's instructions. The PCR products were purified with A'SAP (ArcticZymes®, Tromsø, Norway) before sequencing reaction was performed with BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems) as per manufacturer's instructions. The PCR and sequencing were done on a 2720 Thermal Cycler (Applied Biosystem). The sequencing products were purified with BigDye XTerminator Purification Kit (Applied Biosystems) and subsequent capillary electrophoresis was performed on 3730 DNA Analyzer (Applied Biosystems). Sanger sequencing data was analysed using SeqScape Software version 3.0 (Applied Biosystems) and Alamut® Visual, version 2.10 (Interactive Biosoftware).

Analysis of 5-ASA and Ac-5-ASA concentrations

5-ASA and Ac-5-ASA concentrations were analysed in five mucosal colonic biopsies and in serum from each patient. Two biopsies were sampled from 10 cm, one biopsy from 25 cm and two biopsies from 40 cm from the anal verge. The mean net weight of the biopsies was 7.5 mg (IQR 3.33). 5-ASA and Ac-5-ASA concentrations were analysed using an ultra-high performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method. Reference standards of both compounds were purchased from Sigma-Aldrich (St. Louis, MO), whereas the internal standard 5-ASA-d_3 was from Alsachim (Illkirch Graffenstaden, France). For the preparation of spiked standards and quality controls, blank intestinal tissue was collected from patients undergoing colectomy without using 5-ASA. Blank human serum was collected from healthy medication-free blood donors.

On the day of analysis, preparation of the thawed intestinal biopsy started with the addition of 100 µL water and 10 µL of the internal standard 5-ASA-d_3 at a concentration of 15 µg/mL. Thereafter, the biopsy was finely cut with a stainless steel scissor, mixed and frozen at -80 °C for 20 minutes. After thawing, 0.7 mL of ice-cold acetonitrile with 1% formic acid was added and the sample was shaken, centrifuged, transferred to new tubes and evaporated to dryness. The dry residue was dissolved in 50 µL 0.1% formic acid in water and 0.5 µL was injected onto the UPLC-MS/MS system.

A Waters Acquity UPLC I-class FTN system equipped with an Acquity UPLC HSST3 1.8µm, 2.1 x 100mm column (Waters, Milford, MA) was used for chromatographic separation. Mobile phase A consisted of 0.1% formic acid in water and mobile phase B consisted of methanol. After isocratic conditions with 95% A and 5% B for 2.5 minutes, a step gradient to 5% A and 90% B followed. At 4.5 minutes, the composition was reset to the initial conditions, and a 1.5 min equilibration time was allowed. The compounds were detected on a Xevo TQ-S tandem-quadrupole mass spectrometer (Waters, Manchester, UK) equipped with an electrospray source. Positive electrospray ionization was performed in the multiple reaction monitoring mode. Mass transitions were *m/z* 154.0>136.0 for 5-ASA, *m/z* 196.0>136.0 for Ac-5-ASA and *m/z* 157.0>139.0 for the internal standard 5-ASA-d_3.

The limit of quantification was 25 ng/mL for both 5-ASA and Ac-5-ASA, corresponding to 0.5 ng/mg in a biopsy weighing 5 mg and 0.25 ng/mg in a biopsy weighing 10 mg. The method was linear at least up to 10,000 ng/mL. Between-day coefficients of variation were assessed at three different concentrations (low, medium and high), and were <6% for 5-ASA and <19% for Ac-5-ASA.

DNA isolation, microbiome analysis

Faecal DNA was isolated from 250 mg samples of faeces using QIAamp® Powerfecal DNA (Qiagen) according to manufacturer's protocol with the following adjustment; Step 5 (Vortex) was replaced, instead the provided PowerBead tubes were vortexed using Precellys 24 tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) at 5000 rpm x 3 rounds of 40 seconds. The DNA from the mucosal biopsy, mean net weight was 7.3 mg (IQR 3.98), were isolated using the DNeasy PowerSoil kit® (Qiagen) according to manufacturer's protocol with the following adjustments; Step 3 and 4 (vortexing) were replaced and instead the provided PowerBead tubes were vortexed using Precellys 24 tissue homogenizer (Bertin Technologies) at 5000 rpm x 3 rounds of 40 seconds, after bead beating and before centrifuging (Step 5) 20 µL of Proteinase K 20mg/mL were added and samples incubated at 65°C for 30 minutes, according to Qiagen's recommendations. The isolated DNA were quality tested using NanoDrop (Termo Fisher Scientific) and Qubit (Termo Fisher Scientific).

Statistical analysis

5-ASA formulation and biopsy location (pinch biopsy site and replicates) were included in the multilevel linear mixed model as fixed factors (categorical variables), whereas subject was defined as random factor to take account of the dependency (equal covariance assumed) in the repeated measurements over location (10, 25, 45 cm) and order of observation (2 replicates at each location except at 25 cm). An interaction term between formulation type and location was included in the model to examine whether the difference in mean mucosa 5-ASA concentration by location differed between formulation type, and conversely, whether the difference in mean values between formulation types differed by location. The analyses were

performed on log-transformed data, but estimated parameters were back-transformed (geometric mean and relative difference in mean values, respectively).

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Table S1. N-acetyltransferase 1 (NAT1) primers designed to genotype NAT1 haplotypes on reference sequence NG_012245.2 (NM_001160170.3) with overlapping regions. S = sense strand. AS = antisense strand, PCR = Polymerase chain reaction, bp = basepairs.
- / + = bp before/after coding sequence.

Primer	Primer sequence	Coding sequence	PCR product length (bp)
Primer 1 S	5'-TTGAGTGGGTCAGGTACCACG-3'	-6-405	479
Prime 1 AS	3'-GTTTCCAAGTCCAATTTGTTCC-5'	68	
Primer 2 S	5'-GTCTAGGAACAAATTGGACTTGG-3'	42	511
Primer 2 AS	3'-GCTGTCTTCTAGGAGATCAGAATG-5'	552	
Primer 3 S	5'-GATTCTGGTATCTAGACCAAATC-3'	470	372
Primer 3 AS	3'-GCACAAGCTTTCTCTGCAAG-5'	841	
Primer 4 S	5'-GTTCCTTGCAGACCCCAG-3'	668	503
Primer 4 AS	3'-CAACAATAAACCAACATTAAAAGC-5'	+297	

Table S2. N-acetyltransferase 2 (NAT2) primers designed to genotype NAT2 haplotypes on reference sequence NG_012246.1 (NM_000015.2) with overlapping regions. S = sense strand. AS = antisense strand, PCR = Polymerase chain reaction, bp = basepairs. - / + = bp before/after coding sequence.

Primer	Primer sequence	Coding sequence	PCR product length (bp)
Primer 1 S	GGATCATGGACATTGAAGC	-5	468
Primer 1 AS	CTTCTGTCAAGCAGAAAATG	463	
Primer 2 S	ATTGTCGATGCTGGGTCTG	358	607
Primer 2 AS	ACGTGAGGGTAGAGAGGAT	+91	

Table S3. Frequencies of N-acetyltransferase 2 (NAT2) genotypes and phenotype/acetylator status in patients with ulcerative colitis using using oral 5-aminosalicylic acid.

NAT2 Genotype	Number of patients (n)	Phenotype/Acetylator status	Observed frequency (%)
NAT2*4/*4	3	Rapid	7.1
NAT2*4/*5	3	Intermediate	7.1
NAT2*4/*6	4	Intermediate	9.5
NAT2*4/*7	1	Intermediate	2.4
NAT2*5/*5	7	Slow	16.7
NAT2*5/*6	14	Slow	33.4
NAT2*5/*7	4	Slow	9.5
NAT2*6/*6	4	Slow	9.5
NAT2*6/*7	2	Slow	4.8

Figure S4. Logarithmic mucosal 5-aminosalisylic acid (5-ASA) concentration 40, 25 and 10 cm from the anal verge in patients with ulcerative colitis (UC), stratified by 5-ASA formulation. The vertical line inside the box represents the median value, the length of the box represents the interquartile range (IQR) and the whiskers represent the highest and lowest values, values more than 1.5 x IQR are defined as outliers.

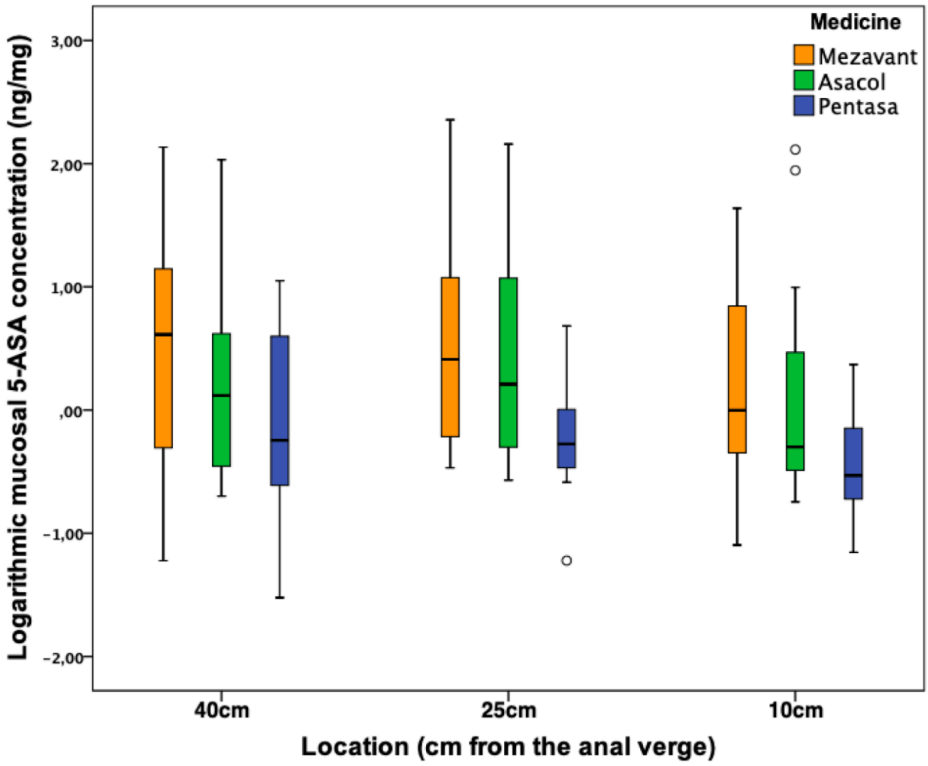


Figure S5. Bacterial families positively correlated to 5-aminosalisylic acid (5-ASA) mucosal concentration in mucosal biopsies. P: phylum, C: class, F: family, p = adjusted p-value, B = regression coefficient, Rlog = regularized logarithm transformation. Blue line = regression line. Dark grey area = 95% confidence interval.

Figure S5. Bacterial families positively correlated to 5-ASA mucosal concentration in mucosal biopsies

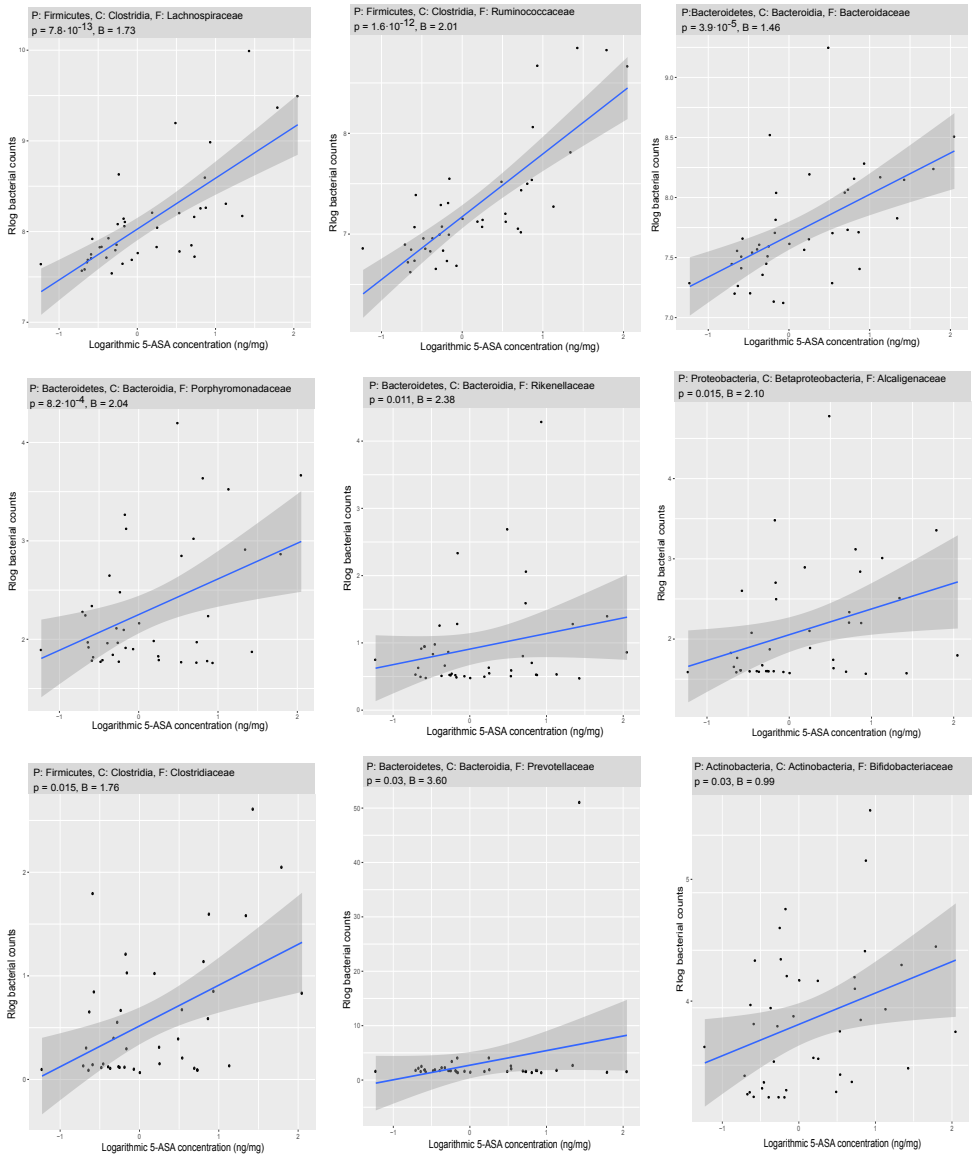


Figure S6. Bacterial families negatively correlated to 5-aminosalisylic acid (5-ASA) mucosal concentration in mucosal biopsies. P: phylum, C: class, F: family, p = adjusted p-value, B = regression coefficient, Rlog = regularized logarithm transformation. Blue line = regression line. Dark grey area = 95% confidence interval.

Figure S6. Bacterial families negatively correlated to 5-ASA mucosal concentration in mucosal biopsies

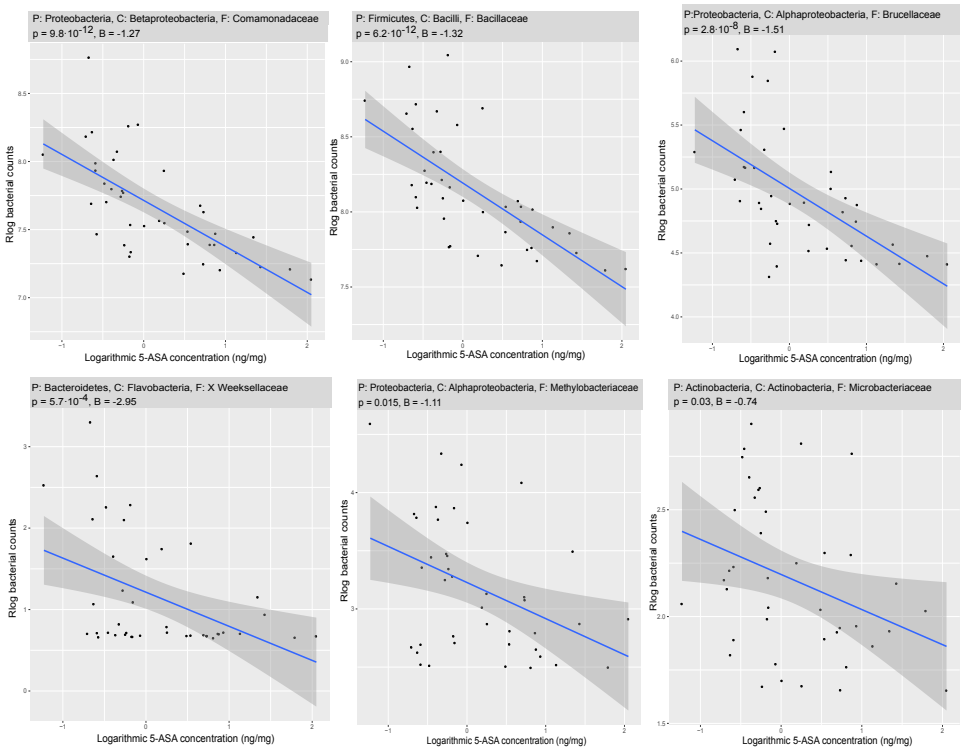
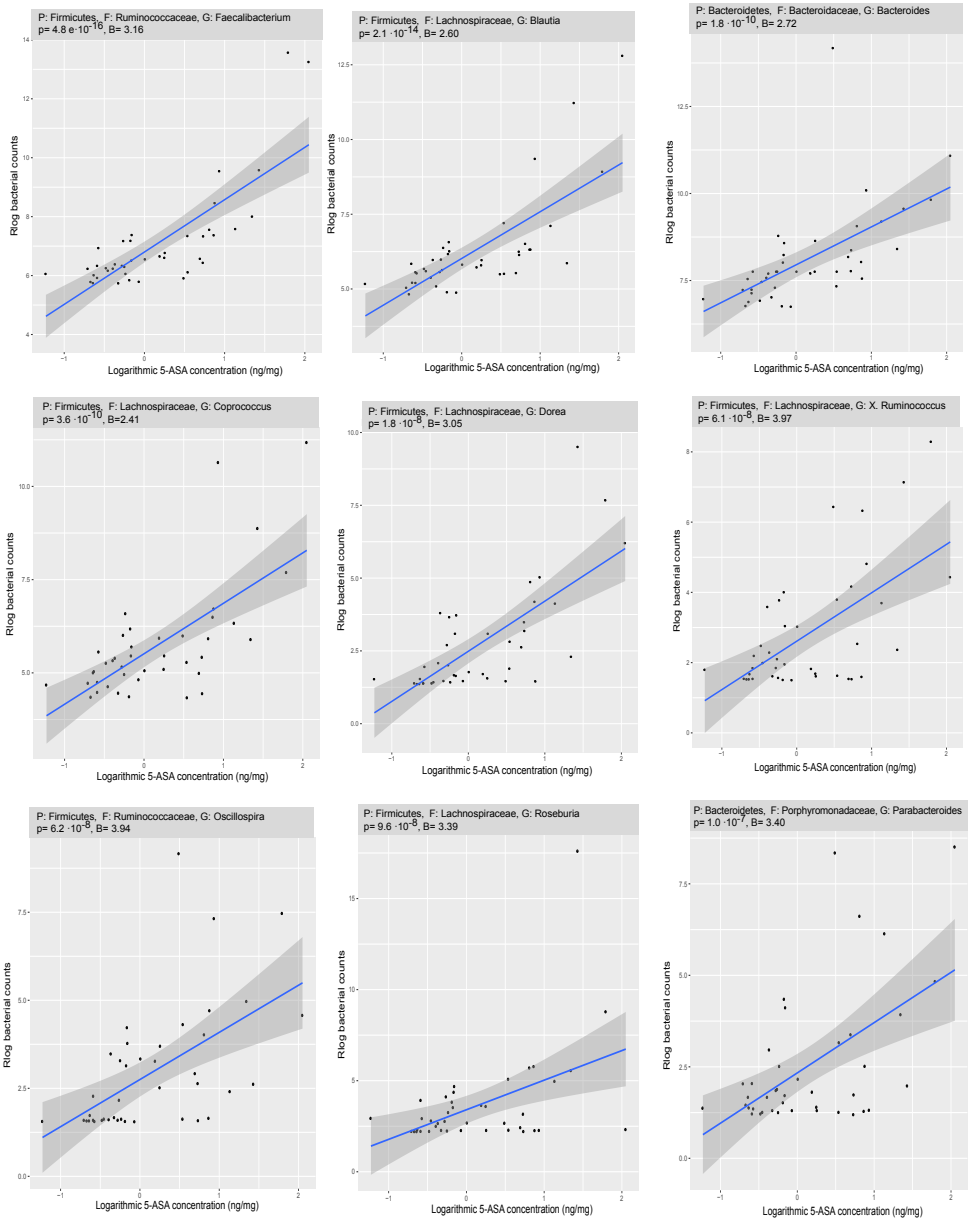
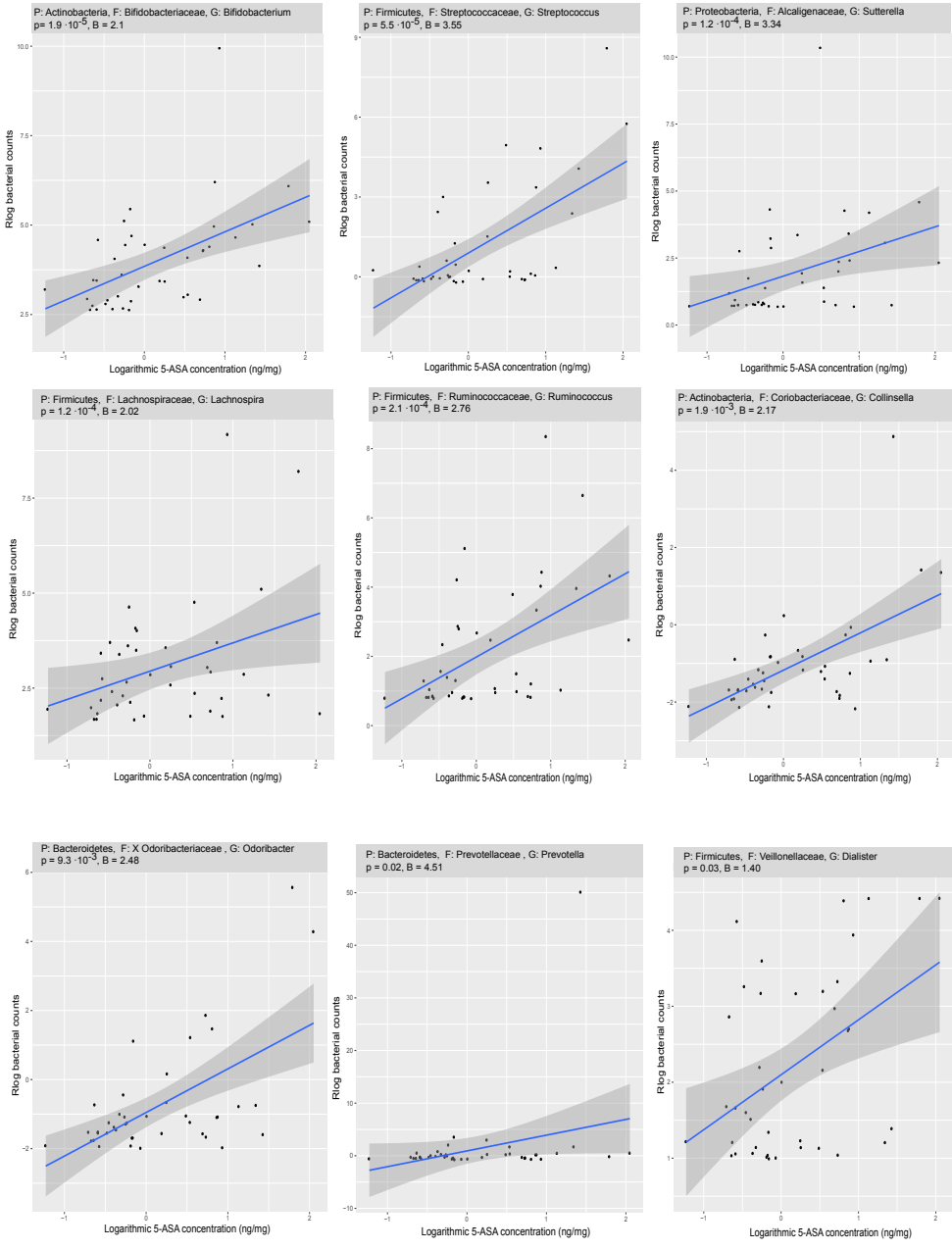


Figure S7. Bacterial genera positively correlated to 5-aminosalisylic acid (5-ASA) mucosal concentration in mucosal biopsies. P: phylum, C: class, F: family, G: genus, p = adjusted p-value, B = regression coefficient, Rlog = regularized logarithm transformation. Blue line = regression line. Dark grey area = 95% confidence interval.

Figure S7. Bacterial genera positively correlated to 5-ASA mucosal concentration in mucosal biopsies




P: Actinobacteria, F: Bifidobacteriaceae, G: Bifidobacterium
p= 1.9 ·10-5, B = 2.1
P: Firmicutes, F: Streptococcaceae, G: Streptococcus
p = 5.5 ·10-5, B = 3.55
P: Proteobacteria, F: Alcaligenaceae, G: Sutterella
p = 1.2 ·10-4, B = 3.34
P: Firmicutes, F: Lachnospiraceae, G: Lachnospira
p = 1.2 ·10-4, B = 2.02
P: Firmicutes, F: Ruminococcaceae, G: Ruminococcus
p = 2.1 ·10-4, B = 2.76
P: Actinobacteria, F: Coriobacteriaceae, G: Collinsella
p = 1.9 ·10-3, B = 2.17
P: Bacteroidetes, F: X Odoribacteriaceae , G: Odoribacter
p = 9.3 ·10-3, B = 2.48
P: Bacteroidetes, F: Prevotellaceae , G: Prevotella
p = 0.02, B = 4.51
P: Firmicutes, F: Veillonellaceae, G: Dialister
p = 0.03, B = 1.40
Rlog bacterial counts
Logarithmic 5-ASA concentration (ng/mg)

Paper II

Bacterial Mucosa-associated Microbiome in Inflamed and Proximal Noninflamed Ileum of Patients With Crohn's Disease

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Background: Microbiota is most likely essential in the pathogenesis of Crohn's disease (CD). Fecal diversion after ileocecal resection (ICR) protects against CD recurrence, whereas infusion of fecal content triggers inflammation. After ICR, the majority of patients experience endoscopic recurrence in the neoterminal ileum, and the ileal microbiome is of particular interest. We have assessed the mucosa-associated microbiome in the inflamed and noninflamed ileum in patients with CD.

Methods: Mucosa-associated microbiome was assessed by 16S rRNA sequencing of biopsies sampled 5 and 15 cm orally of the ileocecal valve or ileocolic anastomosis.

Results: Fifty-one CD patients and forty healthy controls (HCs) were included in the study. Twenty CD patients had terminal ileitis, with endoscopic inflammation at 5 cm, normal mucosa at 15 cm, and no history of upper CD involvement. Crohn's disease patients (n = 51) had lower alpha diversity and separated clearly from HC on beta diversity plots. Twenty-three bacterial taxa were differentially represented in CD patients vs HC; among these, *Tyzzerella 4* was profoundly overrepresented in CD. The microbiome in the inflamed and proximal noninflamed ileal mucosa did not differ according to alpha diversity or beta diversity. Additionally, no bacterial taxa were differentially represented.

Conclusions: The microbiome is similar in the inflamed and proximal noninflamed ileal mucosa within the same patients. Our results support the concept of CD-specific microbiota alterations and demonstrate that neither ileal sublocation nor endoscopic inflammation influence the mucosa-associated microbiome.

Key Words: Crohn's disease, mucosal microbiota, microbiome.

INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory bowel disease (IBD) characterized by transmural inflammation of the gastrointestinal (GI) tract. It may involve the entire GI tract from the oral cavity to the perianal area. Approximately 75% percent of patients have small bowel involvement, usually in the distal ileum and 25%–30% of all patients have ileitis exclusively.[1, 2] The etiology of CD is not known; however, an abnormal immune reaction toward environmental factors, including gut microbiota, in genetically predisposed individuals is the most widely accepted hypothesis. During their lifetime, 75%–80% of CD patients will require surgical intervention,[1, 3] most commonly ileocecal resection (ICR).[2, 4] Furthermore, 75%–80% of patients experience endoscopic recurrence of disease, usually in the neoterminal ileum, proximal to the surgical anastomosis.[1–3, 5]

Patients with CD have an altered gut microbiome composition compared with healthy controls, characterized by decreased bacterial diversity and alterations in bacterial composition, including increased abundances of potentially harmful bacteria and decreased abundances of protective bacteria.[6–9] More specifically, the altered mucosal microbiome in the ileal mucosa of CD patients is characterized by increased abundances of Proteobacteria and Fusobacteria phyla, in addition to Enterobacteriaceae, Veillonellaceae, Gemellaceae, and Fusobacteriaceae families, in combination with decreased abundance of Firmicutes phylum and Lachnospiraceae, Bifidobacteriaceae, and Erysipelotrichaeceae families.[7, 10–14]

Previous studies have found large differences between the bacterial composition in fecal samples and in mucosal biopsies from the colon.[14, 15] Because microbes found in fecal samples may be derived from any part of the GI tract, fecal samples are not ideal for studying microbial changes within the small bowel.

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Conflicts of interest: The authors report no conflicts of interest.

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Investigating the ileal microbiome may be crucial to understand etiologic aspects of CD, for instance, why fecal diversion after ileocecal resection prevents downstream CD recurrence, whereas reestablishment of bowel continuity or infusion of intestinal content is associated with recurrence of disease.[16, 17] Analyses of ileal mucosal microbiome both at the time of ICR and postoperatively have identified characteristics of the microbiome that are associated with postoperative recurrence.[18–20] Sokol et al[20] found that increased abundances of Gammaproteobacteria, *Corynebacterium*, and *Ruminococcus gnavus* and reduced abundance of *Ruminoclostidium 6* at the time of ileocecal resection were predictive of disease recurrence. Additionally, a recent analysis of 2 separate cohorts found that reductions in a specific cluster of bacteria in postoperative CD patients was associated with increased risk of disease recurrence.[6] Postoperative recurrence of disease most often occurs in the surgical anastomosis and immediately proximal to the anastomosis,[1, 3, 5] and analyses of the mucosal microbiome in the inflamed and proximal noninflamed ileum could increase our understanding of the microbial role in disease recurrence.

In the current study, we assessed the ileal bacterial microbiome of adult CD patients and compared the bacterial mucosa-associated microbiome in the inflamed ileum with the proximal noninflamed mucosa within the same patients, which to the best of our knowledge, has not been performed previously.

MATERIALS AND METHODS

Patients and Control Subjects

Patients were recruited from the Department of Gastroenterology, St. Olav's Hospital, Trondheim, Norway, between 2017 and 2019. Patients with Norwegian ethnicity who were 18 to 70 years old and referred to an endoscopic examination involving the small intestine were invited to participate if they were eligible. Inclusion criteria included an established diagnosis of CD based on clinical, endoscopic, and histological criteria or a clinical suspicion of CD that was confirmed after both endoscopic and histologic evaluation. The Montreal classification was used to describe CD characteristics.[21] Age- and sex-matched patients referred to ileocolonoscopy due to rectal bleeding or screening for disease were included as healthy controls (HCs) if the endoscopy and histologic evaluation of biopsies were normal. Exclusion criteria included use of antibacterial or antifungal treatment for the past 2 months or a diagnosis of either diabetes mellitus, liver diseases including primary sclerosing cholangitis and primary biliary cirrhosis, or celiac disease. Additional exclusion criteria for the HC group were history of gastrointestinal surgery, gastrointestinal polyps, cancer, diverticulitis, or irritable bowel disease fulfilling ROME IV criteria.[22]

Endoscopic Procedure

Ileocolonoscopy was performed using either Olympus Exera II GIF HQ190 or PH190L or enteroscope SIF-Q180 (Olympus Europa GmbH, Hamburg, Germany). A total of 4 ileal pinch biopsies were collected from each study participant. An overview of the study design is provided in Figure 1. In CD patients, 2 biopsies were sampled from the inflamed area (approximately 5 cm orally of the ileocecal valve or ileocolic anastomosis) and 2 from normal appearing mucosa (approximately 15 cm from the ileocecal valve or anastomosis). In CD patients with active inflammation where the proximal limit of inflammation could not be reached by the endoscope, in CD patients in remission and in the HC group, biopsies were sampled 5 cm and 15 cm proximal to the ileocecal valve or anastomosis. In the CD patients with an ileal stenosis preventing further intubation of the ileum, 5-cm samples were collected exclusively. Degree of endoscopic ileal inflammation was evaluated according to Rutgeerts score;[23] inflammation was defined as Rutgeerts score ≥1. One pair of mucosal pinch biopsies sampled at 5 and 15 cm from the ileocecal valve were put on formalin for histological grading of inflammation, and the remaining pair were put directly on liquid N_2 and stored on N_2 until subsequent DNA isolation and sequencing of the mucosal bacterial microbiome.

Histological Evaluation of Biopsies

Formalin-fixed biopsies were stained with hematoxylin and eosin (H&E) and evaluated blindly by an experienced pathologist and scored according to Global Histologic Disease Activity Score (GHAS) and Robarts score.[17, 24, 25] No validated histological scoring index for evaluation of disease activity in CD exists. Due to the focality of CD and poor correlation between histological activity and other measurements of disease activity, it is claimed that the significance of histologic disease activity is uncertain.[25, 26] However, blinded histological evaluation of all biopsies ensured classification of a histological, normal appearing ileal mucosa in biopsies from HCs.

Microbial Analyses

DNA was isolated from the mucosal biopsies using DNeasy Powersoil kit (Qiagen, Hilden, Germany) according to manufacturer's protocol with the following adjustments: steps 3 and 4 (vortexing) were replaced, and instead the provided PowerBead tubes were vortexed using Precellys 24 tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) at 5000 rpm × 3 rounds of 40 seconds (step 3). After bead beating, 20 µL of Proteinase K 20 mg/mL was added, and samples were incubated at 65°C for 30 minutes according to Qiagen's recommendations before centrifugation (step 5). The isolated DNA was quality tested using NanoDrop (Thermo Fisher Scientific, MA) and Qubit (Thermo Fisher Scientific). Then 16S metagenomic

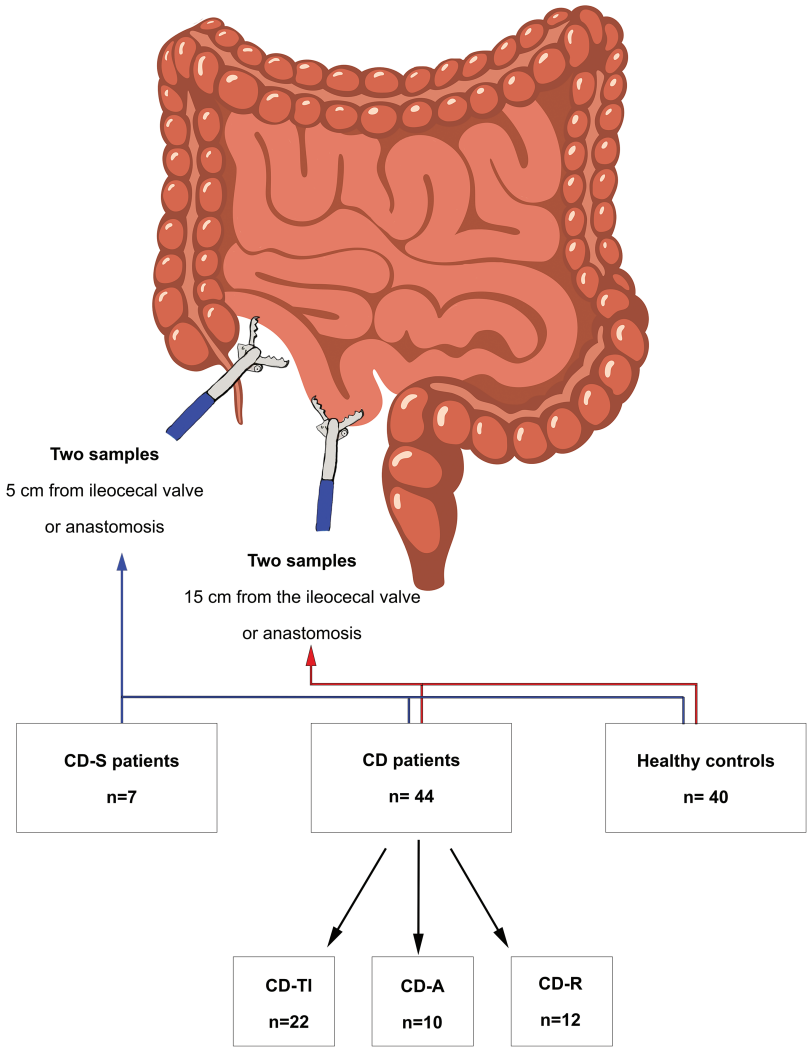

FIGURE 1. Illustration of study design with mucosal pinch biopsy location and number from the study participants. Fifty-one Crohn's disease patients including 7 CD patients with ileal stenosis (CD-S), 22 CD patients with terminal ileitis (CD-TI) with endoscopic inflamed mucosa at 5-cm location (Rutgeerts score ≥1) and normal endoscopic appearing mucosa at 15-cm location, 10 CD patients with active disease (CD-A) with endoscopic inflamed mucosa at both 5-cm and 15-cm location, and 12 CD patients in remission (CD-R) with endoscopic normal appearing mucosa at both 5-cm and 15-cm location and 40 healthy controls (HC) were included in the cohort. Two mucosal pinch biopsies were collected on each location in the 44 CD patients and HCs, in total 4 mucosal pinch biopsies per study participant. Two mucosal biopsies were collected in the 7 CD-S patients at 5-cm location because of ileal stenosis mucosal pinch biopsies could not be collected at 15-cm location.

sequencing libraries were prepared according to the "16S Illumina Demonstrated Library Prep Guide,"[27] with minor adjustments. In brief, 22.5 ng genomic DNA (extracted from biopsies samples) was used as a template for polymerase chain reaction (PCR) amplification (25 cycles) of the 16S V3 and V4 regions. The 16S ribosomal RNA gene PCR primers were based on sequences first published by Klindworth.[28] Illumina adaptor compatible overhang nucleotide sequences were added to the gene/locus specific sequences (16S Amplicon PCR Forward Primer = 5' TCG TCGGCAGCGTCAGATGTGTATAAGAGACAGCCTA CGGGNGGCWGCAG and 16S Amplicon PCR Reverse

Primer = 5' GTCTCGTGGGCTCGGAGATGTGTAT AAGAGACAGGACTACHVGGGTATCTAATCC), resulting in a PCR product of the expected size of approximately 550 bp. The PCR products were then cleaned using AMPure XP beads to purify 16S V3 and V4 amplicons from free primers and primer-dimer products. In a second PCR amplification step (8 cycles), dual indices and Illumina sequencing adaptors were added by using the Nextera XT indexing kit (Illumina Inc., San Diego, CA) according to the manufacturer's instructions. A second PCR clean-up step was performed using AMPure XP beads before validation of the library by a LabChip GX DNA high sensitivity assay (PerkinElmer, Inc., Waltham, MA). Libraries were normalized and pooled to 10 pM and subjected to clustering on 1 MiSeq v3 flowcell. Finally, paired end read sequencing was performed for 2X300 cycles on a MiSeq instrument (Illumina Inc.) according to the manufacturer's instructions. Base calling was done on the MiSeq instrument by RTA v1.18.54. FASTQ files were generated using bcl2fastq2 conversion software v2.17 (Illumina Inc.).

Statistics

IBM SPSS Statistics version 25.0 (IBM Corp., Armonk, NY) was used to conduct the statistical analysis apart from the sequencing data. Demographic and clinical characteristics are presented as percentages (n) for categorical variables, median (interquartile range [IQR]) for skewed distributed variables, and mean value (SD) for normally distributed variables; $\times^2$ test, Mann-Whitney *U* test, or independent *t* test were used for comparing CD patients with HCs. A *P* value <0.05 was considered statistically significant.

Sequencing data were processed using QIIME II pipeline and denoised using DADA2. Data generated by the QIIME II pipeline were imported into the R software environment version 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria) using the phyloseq package and subsequently filtered to include only operational taxonomic units from the bacteria kingdom and excluding operational taxonomic units classified as mitochondria, chloroplast or cyanobacteria/chloroplast. Sequences were classified taxonomically using Silva (release 132) reference database. Alpha diversity was assessed by Shannon entropy and beta diversity by Bray-Curtis dissimilarity index. The count tables merged at a given taxonomic rank were imported into the DESeq2 R package to estimate differential expression. *P* values were estimated using a Wald test and adjusted for multiple testing by Benjamini-Hochberg false discovery rate correction. For all statistical analysis, adjusted *P* values <0.05 were considered statistically significant.

Ethical Considerations

The study was approved by the Regional Committee for Medical and Health Research Ethics, Central Norway (approval reference, 2016/2164). All study participants provided written informed consent.

RESULTS

Patient Characteristics

A total of 51 CD patients and 40 HCs were included in the study. Demographic and clinical characteristics of all study participants are presented in Table 1. Overview of study participants is provided in Figure 1. Seven of the 51 CD patients had a stenosis preventing intubation 15 cm into the ileum (CD-S). For the remaining 44 CD patients, biopsy specimens were sampled from both 5 and 15 cm proximal to the ileocecal valve or anastomosis; 22 of these had terminal ileitis (CD-TI) with endoscopic inflammation at 5 cm and normal appearing mucosa at 15 cm; 10 had endoscopic active inflammation both at 5 and 15 cm (CD-A); and 12 were in endoscopic remission with normal appearing mucosa at 5 and 15 cm (CD-R). Crohn's disease–specific characteristics are presented in Table 2. The majority of CD patients had previously undergone ileocecal resection (n = 32), 63.6% and 57.1% in the CD and CD-S groups, respectively. A total of 190 pinch biopsies were sampled from all CD patients; of these, 95 biopsy specimens underwent histologic evaluation, and the remaining 95 underwent 16S rRNA sequencing for analysis of the bacterial microbiome (Supplementary Table 1).

We found 76% agreement between endoscopic and histologic characterization of inflammation. In the majority of cases where endoscopic and histologic conclusion differed, endoscopy disclosed inflammation, but histology was described as normal, plausibly due to the focality of CD.

Mucosa-associated Bacterial Microbiota in CD Patients vs HC

Crohn's disease patients (n = 51) had lower alpha diversity compared with HC ($P = 2.4 \times 10^{-7}$; Fig. 2A). There was also a clear separation between CD patients and HCs on nonmetric multidimensional scaling (NMDS) plots of Bray-Curtis dissimilarity, reflecting differences in bacterial microbiome composition (Fig. 2B). Differential expression analysis identifying taxa differentially expressed between CD patients and HCs showed that CD significantly increased abundances of Proteobacteria phylum ($P = 1.2 \times 10^{-12}$) and Enterobacteriaceae family ($P = 9.9 \times 10^{-7}$) compared with HCs. At genus level, CD patients had higher abundances of *Tyzzerella 4* (27-fold [log2]; $P = 4.1 \times 10^{-68}$) and *Escherichia shigella* and lower abundances of *Ruminiclostridium 5, Ruminiclostridium 6, Eisenbergiella* and *Fecalibacterium*. In total, 7 bacterial families and 15 bacterial genera were significantly differently expressed between CD and HC (Fig. 2C). The increased abundance of *Tyzzerella 4* in CD patients was remarkable; this genus was further identified as *Tyzzerella sp. Marseille-P3062*. However, 16S rRNA sequencing is not the

TABLE 1. Demographic and Clinical Characteristics of Crohn's Disease Patients and Healthy Controls

	CD	HC	P[a]
Number of patients, n	51	40	
Male gender, n (%)	26 (51%)	19 (47.5%)	0.785
Age, years, mean (SD)	41.5 (14.2)	36.6 (12.9)	0.94
BMI, mean (SD)	25.9 (4.7)	26.6 (4.7)	0.502
Acid reflux medication, n (%)			0.839
PPI	5 (9.8%)	2 (5%)	
H_2 blockers	1 (2.0%)	0	
PPI on demand	1 (2.0%)	0	
H_2 blockers on demand	1 (2.0%)	1 (2.5%)	
Smoking			0.560
Never smoker	25 (49%)	25 (62.5%)	
Active smoker	6 (11.8%)	5 (12.5%)	
Snuff	12 (23.5%)	8 (20%)	
Ex-smoker	8 (15.7%)	2 (5%)	
Laboratory values			
Hb (g/dL), mean (SD)	13.9 (1.4)	14.5 (1.7)	0.09
Leukocytes (x10^9/L), median (IQR)	6.7 (2.0)	6.5 (2.3)	0.261
CRP (mg/L), median (IQR)	<5 (5)	<5 (0)	**0.006**

[a]Comparing CD with HC using Mann-Whitney *U* test for skewly distributed continuous variables, independent *t* test for normal distributed continuous variables, and Fisher exact test for categorical variables.

preferred method for analyzing bacteria on species level. The microbiome community composition shift within individual patients is visualized in Supplementary Figure 1.

Mucosa-associated Microbiota at Different Locations in the Ileum

Alpha diversity did not differ between 5-cm and 15-cm biopsy samples within CD patients (P = 0.83; Fig. 3A). Similarly, bacterial composition did not differ between 5 cm and 15 cm within CD patients (Fig. 3B). Differential expression analysis did not identify any bacterial phyla, families, or genera that differed in expression at 5 cm and 15 cm in the ileum in CD patients. When CD and HC samples were pooled together, there was no separation between 5 cm and 15 cm regarding beta diversity, nor within the HC or CD groups (Fig. 3C). We also performed differential expression analysis comparing abundances of bacterial taxa at 5 cm and 15 cm within the HC samples. We found the Peptostreptococcaceae family to be overrepresented at 15 cm vs 5 cm (P = 0.02); however, this was the only taxa on phylum, family, genus and species level that was differentially expressed between 5 cm and 15 cm in the ileum of HC.

Mucosal Microbiome in the Inflamed and Proximal Noninflamed Ileum in CD Patients

To separate the effects of localized inflammation itself from a potentially disseminated alteration in mucosa-adjacent bacteria, we compared the microbiome in inflamed ileal mucosal microbiome at 5 cm and the orally noninflamed mucosal microbiome 15 cm from the ileocecal valve or anastomosis in CD patients. Twenty-two CD patients had terminal ileitis with inflammation at 5 cm and endoscopic normal appearing mucosa at 15 cm (CD-TI); however, 2 patients had a history of concomitant upper gastrointestinal CD and were excluded from these particular subanalyses. There was no difference in alpha diversity between 5-cm samples and 15-cm samples from the 20 CD-TI patients (P = 0.88; Fig. 4A). Similarly, there was no separation between 5-cm and 15-cm samples according to beta diversity (Fig. 4B), reflecting a similar bacterial microbiome composition in inflamed and proximal noninflamed ileal mucosa. Differential expression analysis provided similar results; no taxa (phylum, family, genus, or species level) were differentially represented between inflamed 5 cm vs noninflamed 15 cm ileal location. However, the 20 CD-TI patients had significantly lower alpha diversity (P = 0.0013) than HCs (Fig. 4C), and beta diversity analysis demonstrated a clear separation between CD-TI patients and HCs (Fig. 4D).

Effects of Inflammation on Mucosa-Associated Microbiome

To further explore if microbiome differences found in CD patients vs HC were associated with inflammation, we compared the microbiome in CD biopsies from locations characterized as inflamed at endoscopy (n = 49) and

TABLE 2. Crohn's Disease Characteristics, Current and Previous Medical Treatment, and Surgical History of CD Patients

	Crohn's disease (5 + 15 cm samples)	**Crohn's disease with stenosis (CD-S, only 5 cm sample)**
Number of patients, n	44	7
Disease duration, years (median (IQR))	10.0 (19.8)	8.0 (13.0)
Subclassification of patients,[a] n (%)		
CD-TI[b] (inflamed 5 cm, normal 15 cm)	22 (50.0%)	0
CD-A[c] (inflamed 5 cm and 15 cm)	10 (22.7%)	0
CD-R[d] (noninflamed 5 cm and 15 cm)	12 (27.3%)	0
CD-S[e] (ileal stenosis 5 cm)	0	7 (100%)
Montreal location, n (%)		
Terminal ileum (L1)	23 (52.3%)	1 (14.3%)
Ileocolonic (L3)	16 (36.4%)	4 (57.1%)
Ileocolonic + Upper GI (L3 + L4)	5 (11.4%)	2 (28.6%)
Montreal behaviour, n (%)		
Nonstricturing, nonpenetrating (B1)	8 (18.2%)	0
Nonstricturing, nonpenetrating + perianal (B1p)	2 (4.5%)	1 (14.3%)
Stricturing (B2)	15 (34.1%)	3 (42.9%)
Stricturing + perianal (B2p)	6 (13.6%)	2 (28.6%)
Penetrating (B3)	11 (25%)	1 (14.3%)
Penetrating + perianal (B3p)	2 (4.5%)	0
Montreal age (age at diagnosis), n (%)		
16 years or younger (A1)	12 (27.3%)	1 (14.3%)
17–40 years (A2)	22 (50%)	5 (71.4%)
Over 40 years (A3)	10 (22.7%)	1 (14.3%)
CD-medication, n (%)	[f]	[g]
No medical therapy for CD	18 (40.9%)	1 (14.3)
Budesonide	7 (15.9%)	4 (57.2%)
Prednisolone	4 (9.1%)	0
5-ASA	3 (6.8%)	1 (14.3%)
Azathioprine	6 (13.6%)	0
Methotrexate	3 (6.8%)	0
Adalimumab	4 (9.1%)	2 (28.6%)
Infliximab	7 (15.9%)	1 (14.3%)
Vedolizumab	1 (2.3%)	1 (14.3%)
Treatment naïve, n (%)	6 (13.6%)	0
TNFα naïve, n (%)	23 (52.3%)	2 (28.6%)
Rutgeerts score, n (%)		
i0	12 (27.3%)	0
i1	12 (27.3%)	0
i2	5 (11.4%)	0
i3	6 (13.6%)	0
i4	9 (20.5%)	7 (100%)
Ileocecal resection	28 (63.6%)	4 (57.1%)

[a]Based on endoscopic evaluation of inflammation
[b]CD-TI; Crohn's disease patients with terminal ileitis
[c]CD-A; Crohn's disease patients with endoscopic active inflammation
[d]CD-R; Crohn's disease patients in endoscopic remission
[e]CD-S; Crohn's disease patients with ileal stenosis
[f]Comedication: n = 8 (18.2%) used 2 CD medications, n = 1 (2.3%) used 3 CD medications
[g]Comedication: n = 3 (42.9%) used 2 CD medications, n = 0 used 3 CD medications

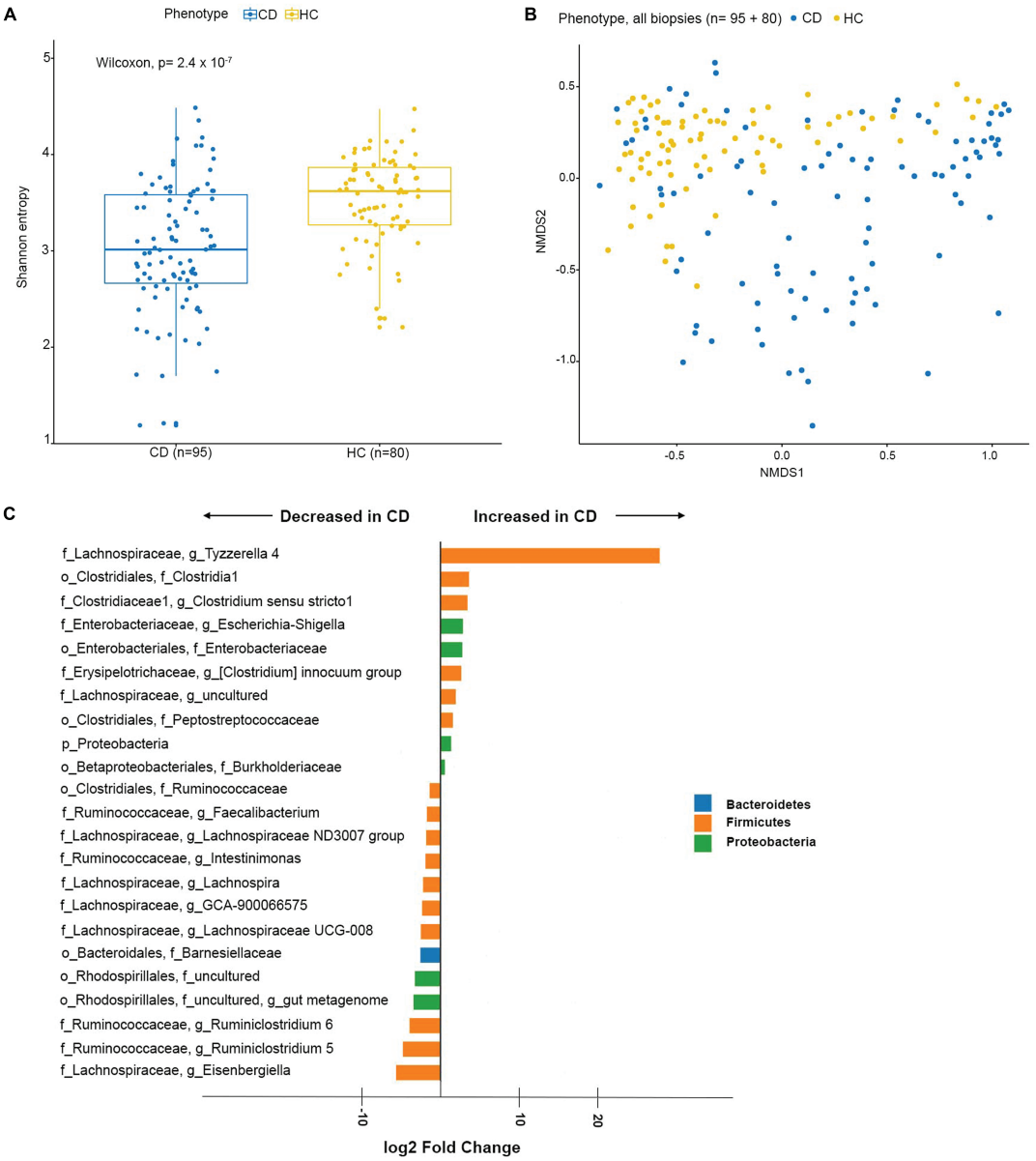

FIGURE 2. Ileal mucosa-associated bacterial microbiome in 51 Crohn's disease patients and 40 healthy controls, mucosal biopsies sampled 5 and 15 cm proximal from the ileocecal valve or anastomosis, respectively (except for 7 CD patients with stenosis where only 5 cm sample was obtained). In total, 95 biopsy specimens from 51 CD patients and 80 biopsy specimens from 40 HCs. A, Alpha diversity illustrated by Shannon entropy index in CD patients vs HC compared with Wilcoxon test. B, Beta diversity illustrated by Bray-Curtis dissimilarity index on nonmetric multidimensional scaling plot, each sample colored according to phenotype (CD and HC). C, Bacterial taxa significantly differentially represented (adjusted $P < 0.05$) in patients with CD vs HC, illustrated by log2 fold change.

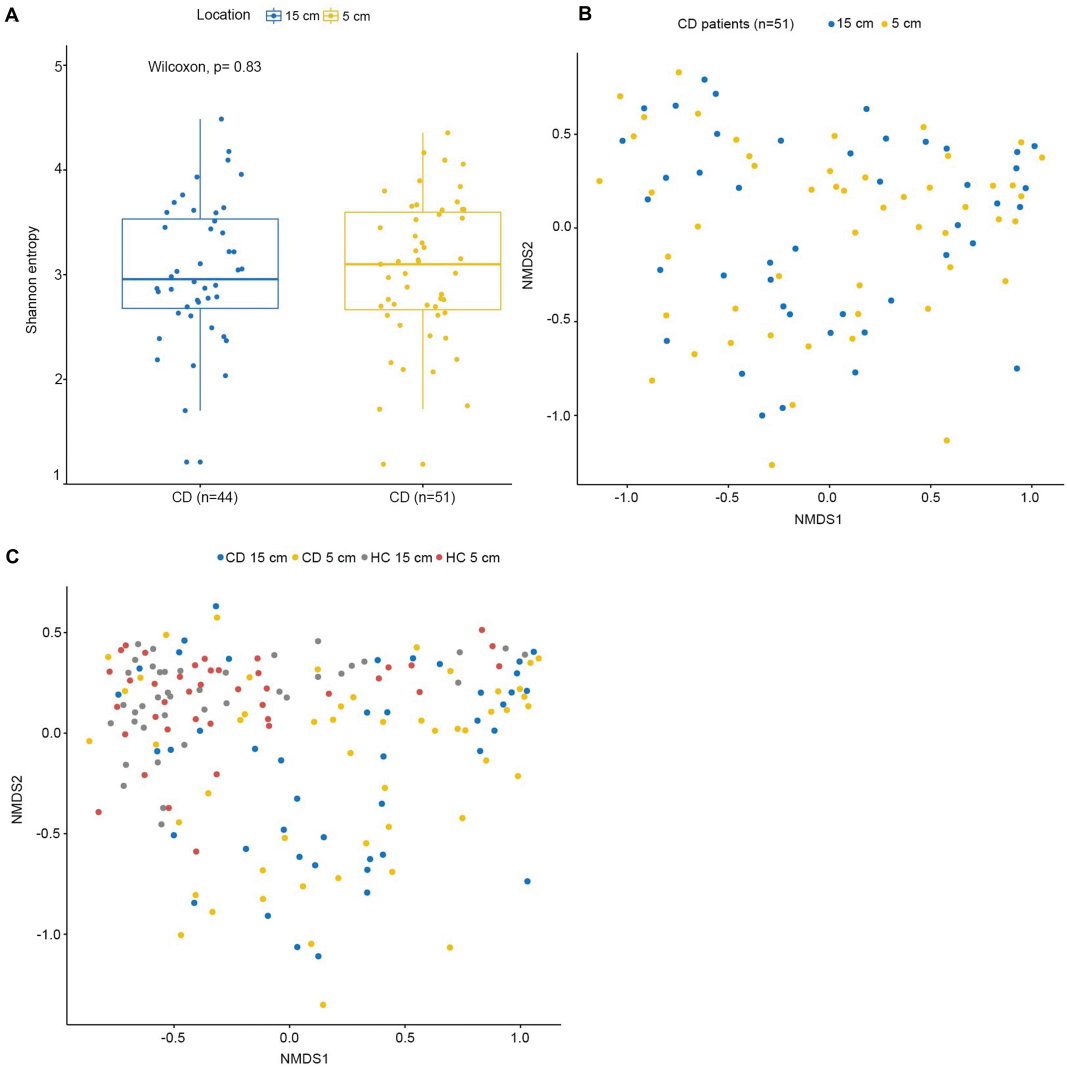

FIGURE 3. Ileal mucosa-associated bacterial microbiome at 5-cm and 15-cm location (proximal from the ileocecal valve or anastomosis respectively) in 51 Crohn's disease patients; biopsies not collected from 7 CD patients at 15-cm location due to ileal stenosis. A, Alpha diversity, illustrated by Shannon entropy index, at 5-cm and 15-cm location compared with Wilcoxon test. B, Beta diversity illustrated by Bray-Curtis dissimilarity index on nonmetric multidimensional scaling plot; each sample colored according to mucosal pinch biopsy location; 5 and 15 cm. C, Beta diversity in 51 CD patients and 40 healthy controls, illustrated by Bray-Curtis dissimilarity index on nonmetric multidimensional scaling plot; each sample colored according to phenotype and location; CD 15 cm, CD 5 cm, HC 15 cm, and HC 5 cm.

histology (n = 32), with biopsies from locations that were noninflamed at endoscopy (n = 46) and with normal histology (n = 63). There was no significant difference in alpha diversity between biopsies from endoscopically inflamed vs noninflamed locations ($P = 0.54$; Fig. 5A). However, biopsies from locations with histological inflammation had significantly lower alpha diversity than locations where biopsies were normal ($P = 0.03$; Fig. 5B). There were no differences in beta diversity between inflamed and noninflamed locations, according to neither endoscopic nor histologic evaluation

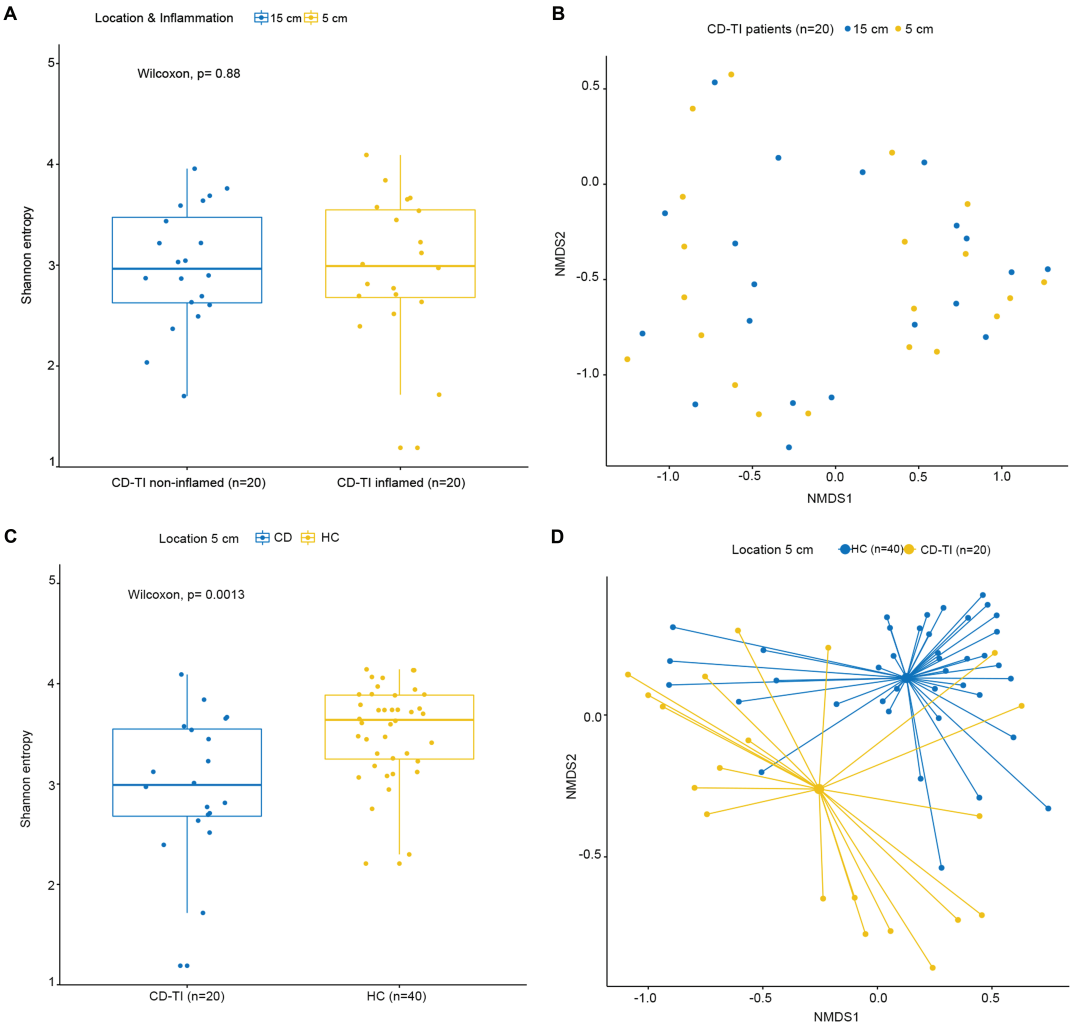

FIGURE 4. Ileal mucosa-associated bacterial microbiome in 20 Crohn's disease patients with terminal ileitis with endoscopic inflamed mucosa at 5 cm proximal from the ileocecal valve or anastomosis and normal endoscopic appearing mucosa at 15 cm proximal from the ileocecal valve or anastomosis. A, Alpha diversity, illustrated by Shannon entropy index according to location, compared with Wilcoxon test. B, Beta diversity illustrated by Bray-Curtis dissimilarity index on nonmetric multidimensional scaling plot, each sample colored according to mucosal pinch biopsy location; 5 and 15 cm. C, Alpha diversity, illustrated by Shannon entropy index, at 5-cm location in 20 CD-TI patients vs 40 healthy controls compared with Wilcoxon test. D, Beta diversity, illustrated by Bray-Curtis dissimilarity index on nonmetric multidimensional scaling (NMDS) plot, in 5-cm samples from CD-TI patients and HCs; each sample colored according to phenotype; HC and CD-TI.

of inflammation (Fig. 5C and D). Similarly, differential expression analysis did not find any bacterial taxa on phylum, family, or genus level to be differentially expressed between CD patients with endoscopic inflammation and endoscopic remission.

The Microbiome in Patients with Ileal Stenosis (CD-S)

Alpha diversity in CD-S patients was similar to that of other CD subgroups (Supplementary Fig. 2); however, on beta diversity NMDS plots, CD-S patients clustered furthest

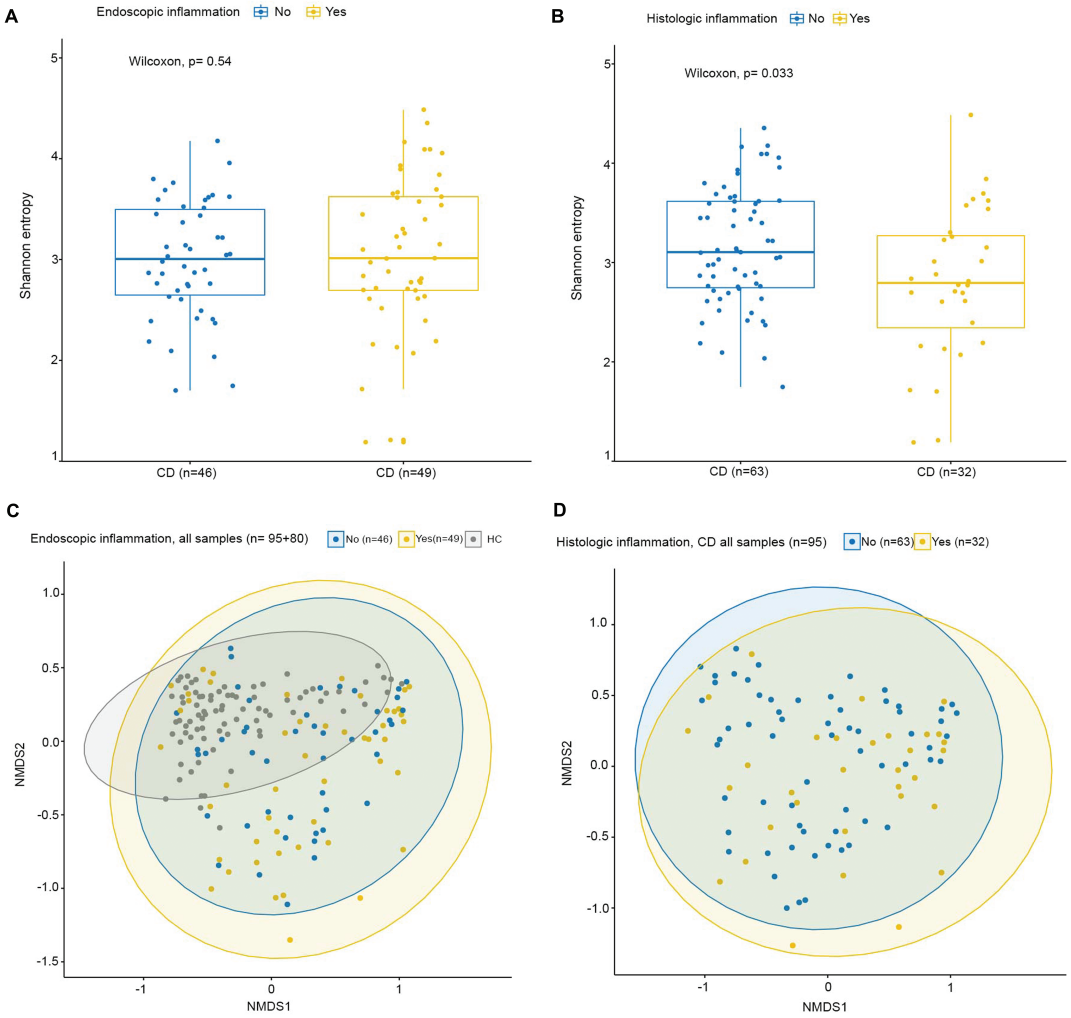

FIGURE 5. Ileal mucosa-associated bacterial microbiome in 95 mucosal pinch biopsy specimens (sampled from 5 and 15 cm proximal from the ileocecal valve or anastomosis) from 51 Crohn's disease patients according to endoscopic inflammation (Rutgeerts score ≥1) and histologic inflammation (GHAS and Robarts score ≥1) at biopsy sample location. A, Alpha diversity, illustrated by Shannon entropy index, according to endoscopic inflammation compared with Wilcoxon test. B, Alpha diversity, illustrated by Shannon entropy index, according to histologic inflammation compared with Wilcoxon test. C, Beta diversity illustrated by Bray-Curtis dissimilarity index on nonmetric multidimensional scaling plot in 95 mucosal pinch biopsies from 51 CD patients and 80 mucosal pinch biopsies from 40 healthy controls; each sample colored according to endoscopic inflammation (yes or no) or HC. D, Beta diversity illustrated by Bray-Curtis dissimilarity index on nonmetric multidimensional scaling plot in 95 mucosal pinch biopsies from 51 CD patients; each sample colored according to histologic inflammation (yes or no).

away from HCs compared with patients with terminal ileitis (CD-TI) or in remission (CD-R; Fig. 6). In a differential expression analysis identifying taxa that were differentially expressed between CD-TI patients and CD-S patients, we found a trend toward higher abundances of Akkermaniaceae family in CD-TI compared with CD-S patients (P = 0.098). The same trend was found for Akkermansia genus, but neither was statistically significant. At species level, three species were significantly overrepresented in CD-TI patients in comparison with CD-S patients: *Bacteroides massiliensis B84634,* unidentified species of *Sutterella*, unidentified species of *Akkermansia*.

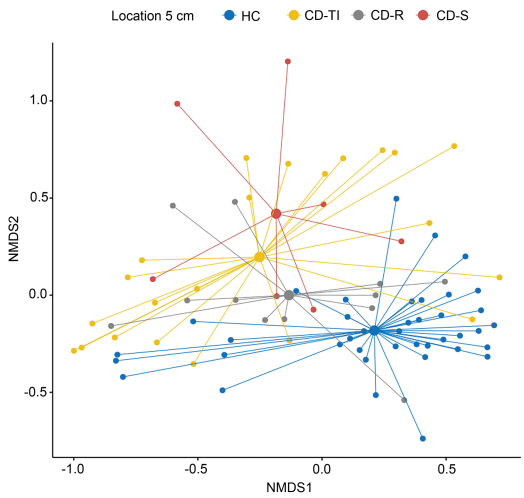

FIGURE 6. Ileal mucosa-associated bacterial microbiome composition in 40 healthy controls, 20 Crohn's disease patients with terminal ileitis and endoscopic inflammation, 12 CD patients in remission and endoscopic normal appearing mucosa, and in 7 CD patients with ileal stenosis. Beta diversity illustrated by Bray-Curtis dissimilarity index on nonmetric multidimensional scaling plot; each biopsy sample colored according to phenotype; HC, CD-TI, CD-R and CD-S. Each study participant represented with 1 mucosal pinch biopsy sampled 5 cm proximal to the ileocecal valve or anastomosis, respectively.

Effects of ICR on Ileal Mucosa-Associated Microbiome

In our cohort, 62.7% of CD patients had previously undergone ICR (n = 32). An overview of CD patients' surgical history is provided in Supplementary Table 2. When we analyzed samples according to ICR, we found lower alpha diversity in the ICR group ($P = 0.021$) compared with CD patients who had not undergone ICR (Supplementary Fig. 3A). However, bacterial composition did not seem to differ according to ICR status (Supplementary Fig. 3B). Furthermore, bacterial composition at 5 cm and 15 cm proximal to the anastomosis did not differ in patients having undergone ICR (Supplementary Fig. 3C). We performed a differential expression analysis comparing the microbiome of ICR patients in remission (n = 6) with ICR patients with disease recurrence (endoscopic inflammation, n = 26) and found increased abundances of *Parasutterella* genus to be associated with disease recurrence ($P = 6.8 \times 10^{-18}$).

DISCUSSION

This is the first study to assess the mucosa-associated microbiota in the inflamed and proximal noninflamed ileum within the same patients. We did not find differences in alpha diversity or beta diversity when comparing inflamed with proximal noninflamed locations within the same patients. Furthermore, no bacterial taxa were differentially expressed in the inflamed vs proximally noninflamed mucosa. Our findings suggest that the altered ileal mucosa-associated microbiota in CD patients is present across locations and independent of inflammation itself at the biopsy location. In consistence, the beta diversity in mucosal biopsies from CD and ulcerative colitis (UC) patients pooled according to inflammation status, and intestinal location did not seem influenced by these factors.[29] Our findings are also supported by analyses of ileal biopsies from pediatric treatment-naïve IBD patients, where dysbiosis seemed to exist in absence of inflammation.[10] The present findings of an altered microbiome in CD patients also proximal to the upper border of inflammation suggests that the ileal mucosa-associated microbiota is altered regardless of inflammation status and location and contributes to delineate the role of bacteria in CD pathogenesis.

Crohn's disease–specific alterations in ileal mucosa–associated microbiota were confirmed in our cohort; CD patients had lower alpha diversity and separated clearly from HCs on beta diversity plots. Furthermore, we identified 23 bacterial taxa that were differentially represented in CD patients vs HCs. In accordance with previous reports, Proteobacteria phylum and Enterobacteriaceae family were increased, and Ruminococcaceae family and several genera from the Lachnospiraceae family were depleted in CD patients.[7, 10, 12, 13] Interestingly, we found *Tyzzerella 4* to be profoundly overrepresented in CD patients. At species level, this genus was identified as *Tyzzerella sp. Marseille-P3062*. Previously, *Tyzzerella 4* has been reported to be increased in a cohort of UC patients from China.[8] The literature describing *Tyzzerella 4* is very limited, but previous reports have found this genus to be increased in patients with a high-risk profile of cardiovascular disease and associated with an increased lifetime risk of cardiovascular disease.[30] *Tyzzerella 4* is also overrepresented in patients with a diet that was characterized as unhealthy by a healthy eating index (HEI).[31] Interestingly, numerous recent studies have identified IBD as a risk factor of cardiovascular disease.[32] Hypothetically, increased abundances of *Tyzzerella 4* could mediate this risk, but further research on CD patients with and without cardiovascular disease in addition to dietary patterns could clarify this. Two of the most decreased taxa in CD patients were the genera *Ruminiclostridium 5* and *Ruminiclostridium 6*. *Ruminiclostridium* has been described to be depleted in the mucosa of newly diagnosed and treatment-naïve CD patients,[33] and increased abundances of *Ruminiclostridium 6* seem protective with respect to endoscopic recurrence of CD after ICR.[20]

In analysis of all CD patients in the cohort, we confirmed that ileal sublocation did not impact microbiome diversity or composition, neither within CD patients nor within HCs. When we assessed the bacterial microbiome according to inflammatory variables, we found that biopsy samples evaluated as histologically inflamed had a lower alpha diversity in comparison with samples from CD patients that were histologically

normal, although alpha diversity in endoscopically inflamed biopsies vs endoscopically normal tissue was similar. Biopsies that were histologically inflamed were, in the majority of cases, also evaluated as endoscopically inflamed. This could suggest that alpha diversity is reduced in patients with severely inflamed ileal mucosa but not in modestly inflamed ileal mucosa, supported by Sokol et al[20] who found alpha diversity only to be reduced in patients with Rutgeerts score i2–i4 and not in patients with Rutgeerts score i0–i1. Ileal mucosa-associated microbiome composition did not differ between inflamed and noninflamed locations, neither according to histological nor endoscopic inflammation status.

Crohn's disease phenotype subgroups separated on beta diversity plots, and there was a trend toward reduced abundance of Akkermaniaceae family in CD-S patients compared with CD-TI patients. Although our cohort only contained seven patients with ileal stenosis (CD-TI), we assessed if there were differences in the mucosa-associated microbiota between CD-S patients and patients with terminal ileitis without stenosis (CD-TI). Our findings indicate that *Bacteroides massiliensis B84634* and unidentified species of *Sutterella* and *Akkermansia* are underrepresented in patients with stricturing CD. Previous research has found the abundance of *Akkermanisa muciniphila* to be correlated with time in remission in UC patients and increased in HC and UC patients in long-term remission.[34] Similarly, *Sutterella* abundance has been found to be inversely correlated to ileal-pouch inflammation.[35] Specific microbiome alterations in stricturing CD have been reported previously. In a prospective pediatric cohort study assessing the ileal mucosa-associated microbiome in patients before treatment, increased abundances of *Ruminococcus* was associated with development of stricturing disease.[36] Unique microbial profile in stricturing CD was also found in 2 data sets from separate cohorts.[37] In conclusion, our results suggest that patients with stenosing CD have a more profound loss of presumed beneficial bacteria compared with CD patients with inflammation but without stenosis. Although the current study has a cross-sectional design that prevents separation of primary and secondary alterations in microbial signatures, it is possible that stenosing disease behavior may be caused by specific bacteria and their products. Previous reports have found specific microbiome characteristics both at the time of ICR and postoperatively to be associated with postoperative recurrence.[6, 18–20] We found increased abundances of *Parasutterella* to be associated with disease recurrence after ICR in our cohort. *Parasutterella* genus belongs to the Gammaproteobacteria class (according to SILVA database but classified as a betaproteobacteria in GenBank, for example). Increased abundances of Gammaproteobacteria have previously been identified as a part of the microbial signature for postoperative recurrence.[20] However, it should be noted that in our patients, ICR was performed months to years ahead of study sampling.

Our study has some limitations. We have assessed the ileal mucosa-associated bacterial microbiome in a heterogeneous population of CD patients with longstanding disease, and the majority of patients had undergone ICR. Although we did not find that ICR impacted the ileal bacterial composition, it would be desirable to repeat the analyses on a cohort of newly diagnosed treatment-naïve patients. Even so, pediatric studies on treatment-naïve CD patients have found similar alterations in the mucosa-associated microbiota,[10, 14] arguing against medical treatment and disease duration as major contributors to the observed microbiome alterations in CD patients.

CONCLUSION

In conclusion, this study demonstrates that the ileal mucosa-associated microbiota alterations in CD patients do not seem affected by inflammatory status or sublocation in the terminal ileum. The abundance of *Tyzzerella 4* is profoundly increased in CD patients, and depleted abundances of presumed favorable bacteria are found in CD patients with ileal stenosis.

SUPPLEMENTARY DATA

Supplementary data is available at *Inflammatory Bowel Diseases* online.

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Supplementary table 1 Number of mucosal pinch biopsies sampled from Crohn's disease (CD) patients and healthy controls (HC)

	CD	HC
Number of patients, n	51	40
Number of biopsies, n	190	160
Histologic evaluation, n	95	80
16S rRNA sequencing, n	95	80
Endoscopic inflammation[1], n (%)	49 (51.6%)	0
Histologic inflammation, n (%)	32 (33.7%)	0
Agreement between endoscopic and histologic grading of inflammation, n (%)	72 (76%)	80 (100%)

1 Endoscopic inflammation at mucosal pinch biopsy sample location (5 or 15 cm from the ileocecal valve)

Supplementary table 2

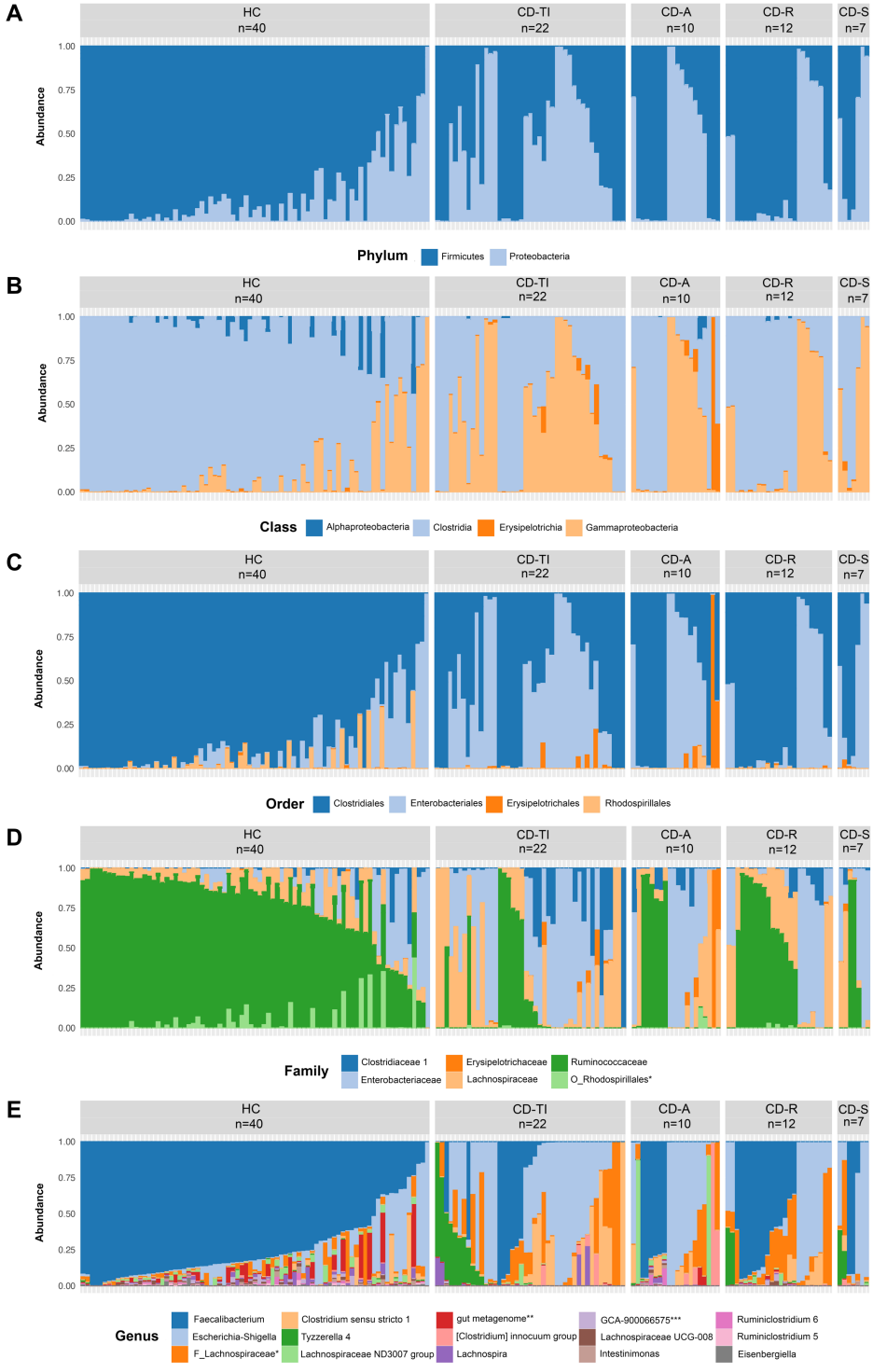
Surgical history	**Crohn's disease** *5 + 15 cm samples* **n=44**	**Crohn's disease patients with stenosis** *Only 5 cm sample* **n=7**
Number of patients, *n*	44	7
Surgical history		
Never undergone CD-surgery, n (%)	10 (22.7%)	1 (14.3%)
Undergone ileocecal resection (ICR), n (%)	28 (63.6%)	4 (57.1%)
Number of surgical interventions (SI)		
Number of SI due to CD, *mean (95%CI)* [1]	1.7 (1.2-2.2)	2.7 (-0.1-5.6)
Bowel resections, *mean (95%CI)*	1.2 (0.9-1.5)	1.7 (-1.3-4.7)
Perianal surgery, *mean (95%CI)*	0.34 (0.05-0.6)	1.0 (-.03-2.3)
Number of patients in remission[2] after ICR	6 (13.6%)[3]	0

1 Include all surgical interventions due to CD (operations due to surgical complications or adenocarcinoma (n=2) not included), these surgical interventions included bowel resection, stoma surgery, surgery due to bowel perforations, intra-abdominal abscesses, perianal fistula/abscess surgery where an incision was made (changing of steton threads was not considered perianal surgery).
2 Crohn's disease patients in endoscopic remission (termed CD-R in the manuscript)
3 Of the 12 CD patients in remission termed CD-R in the manuscript, 6 had undergone ICR previously.

Surgical history by CD phenotype	**CD-TI[1]**	**CD-A[2]**	**CD-R[3]**
Number of patients, *n*	22	10	12
Surgical history			
Never undergone CD-surgery, n (%)	3 (13.6%)	4 (40%)	3 (25%)
Undergone ileocecal resection (ICR), n (%)	17 (77.3%)	5 (50%)	6 (50%)
Number of surgical interventions (SI)			
Number of SI due to CD, *mean (95%CI)* [4]	1.8 (1.1-2.5)	1.5 (-0.2-3.2)	1.75 (0.7-2.8)
Bowel resections, *mean (95%CI)*	1.3 (0.9-1.7))	1.0 (0.3-1.8)	1.2 (0.3-2.1)
Perianal surgery, *mean (95%CI)*	0.2 (-0.2-0.6)	0.4 (-0.5-1.3)	0.5 (-0.7-1.1)
Number of patients in remission[5] after ICR, n (%)	0	0	6 (50%)

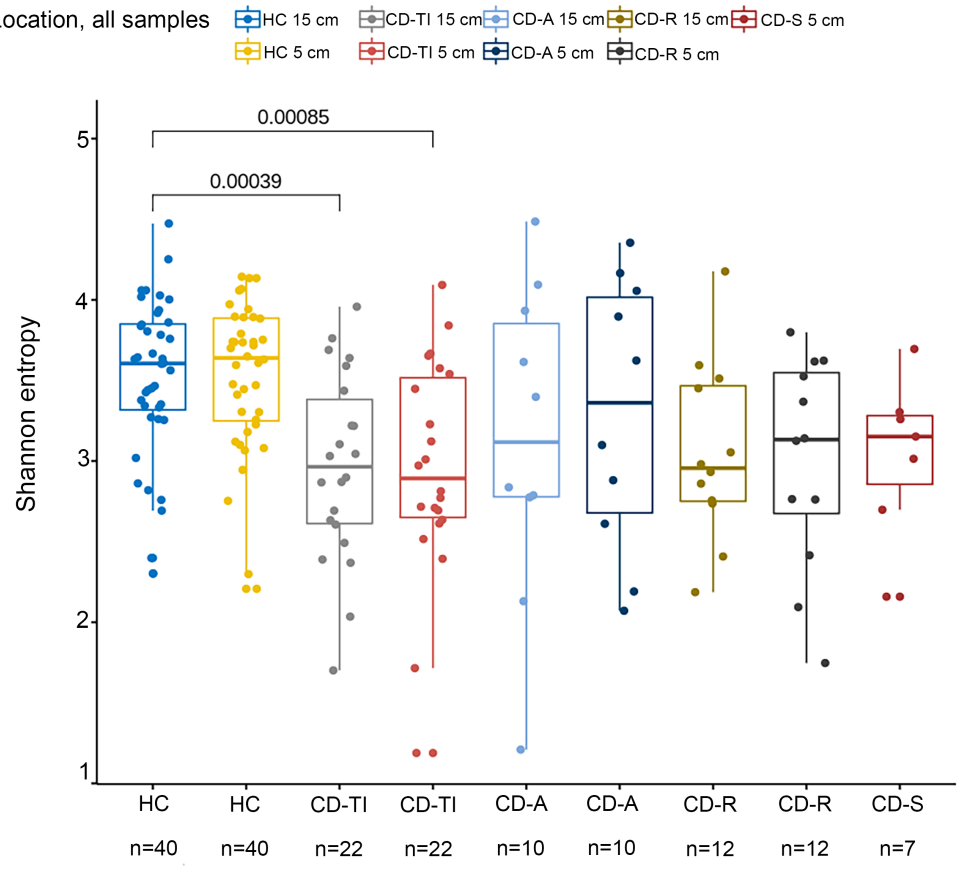
1 CD-TI; Crohn's disease patients with terminal ileitis
2 CD-A; Crohn's disease patients with endoscopic active inflammation
3 CD-R; Crohn's disease patients in endoscopic remission
4 Include all surgical interventions due to CD (operations due to surgical complications or adenocarcinoma (n=2) not included), these surgical interventions included bowel resection, stoma surgery, surgery due to bowel perforations, intra-abdominal abscesses, perianal fistula/abscess surgery where an incision was made (changing of steton threads was not considered perianal surgery).
5 Endoscopic remission (Rutgeerts score <1)

Supplementary Figure 1



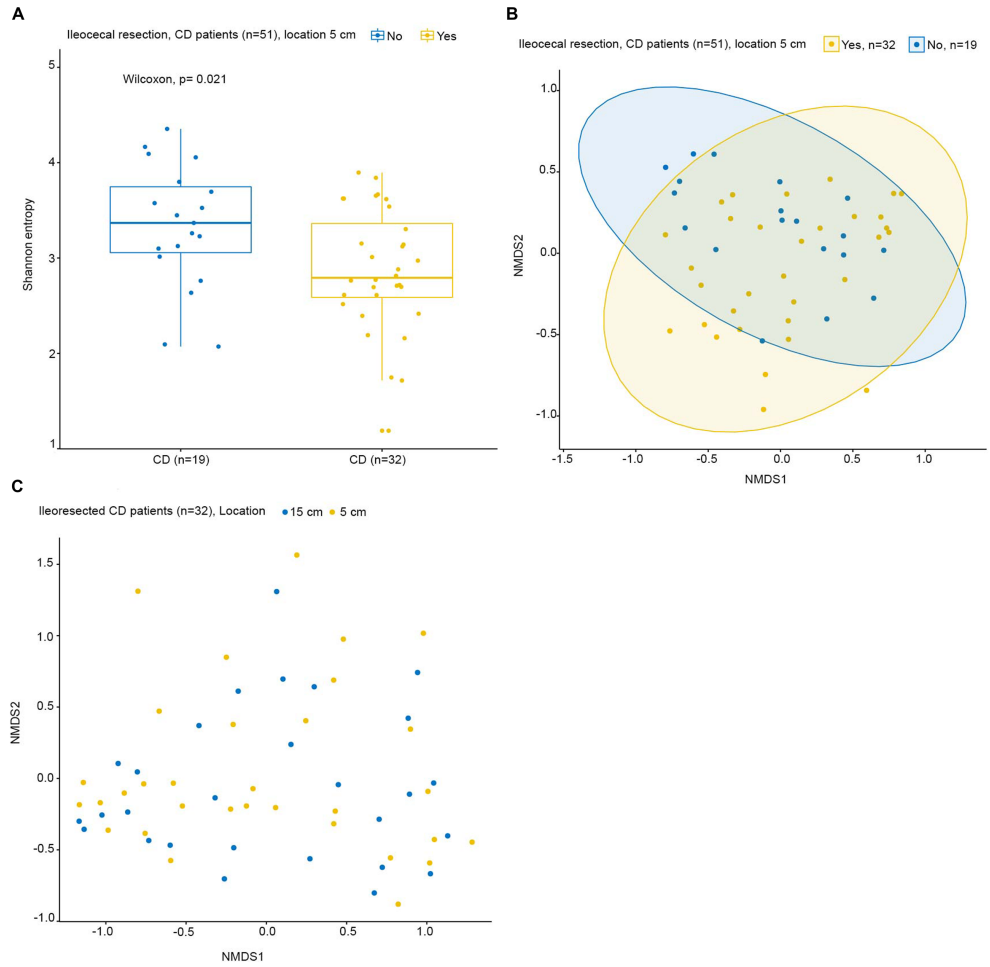
Supplementary Figure 1 Bar chart illustrating the ileal mucosa-associated bacteriome community composition within individual Crohn's disease (CD) patients and healthy controls (HC). Bacterial taxa in this figure were significantly differentially represented (adjusted p-value <0.05) in patients with CD vs. HC. Abundance of bacterial taxa on y-axis, each study participant accounts for one bar on the x-axis. CD patients categorized as CD phenotype subgroups: CD patients with terminal ileitis (CD-TI), CD patients with endoscopic active inflammation (CD-A), CD patients in endoscopic remission (CD-R) and CD patients with ileal stenosis (CD-S). (A) Phylum level. (B) Class level. (C). Order level. (D) Family level *uncultured family of the order Rhodospirillales. (E) Genus level * uncultured genus of the family Lachnospiraceae, ** gut metagenome genus belonging to uncultured family of the order Rhodospirillales *** genus GCA-900066575 belonging to Lachnospiraceae family.

Supplementary Figure 2



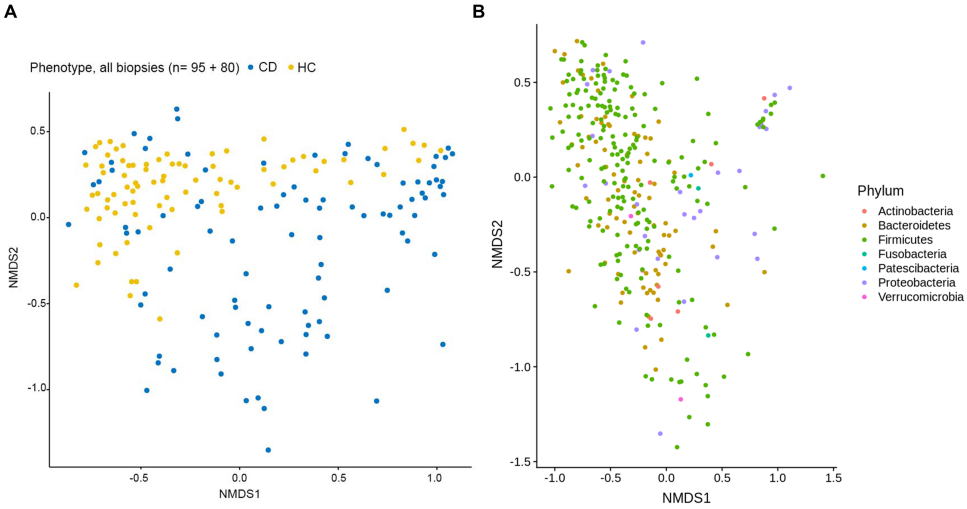
Supplementary Figure 2 Ileal mucosa-associated bacterial alpha diversity, illustrated by Shannon entropy index, in 51 Crohn's disease (CD) patients and 40 healthy controls (HC) according to phenotype and mucosal pinch biopsy location. Two mucosal pinch biopsies were sampled from 5 and 15 cm proximal from the ileocecal valve or anastomosis respectively in each study participant, except for 7 CD patients were only 5 cm sample was obtained. In total 95 biopsy specimens from 51 CD patients and 80 biopsy specimens from 40 HC. CD patients divided into disease phenotype groups: CD-TI, CD patients with terminal ileitis and endoscopic inflamed mucosa at 5 cm and normal appearing mucosa at 15 cm; CD-A, CD patients with active disease and endoscopic inflammation at 5 and 15 cm; CD-R, CD patients in remission with endoscopic normal appearing mucosa at 5 and 15 cm; CD-S, CD patients with ileal stenosis and endoscopic severely inflamed ileal mucosa (Rutgeerts score i4). HC 15 cm vs CD-TI 15 cm and HC 5 cm vs. CD-TI 5 cm, compared by Wilcoxon test, p-values not corrected for multiple comparisons.

Supplementary Figure 3



Supplementary Figure 3 Ileal mucosa-associated bacterial microbiome in 51 Crohn's disease (CD) patients according to ileocecal resection. (A) Alpha diversity, illustrated by Shannon entropy index, according to ileocecal resection status compared by Wilcoxon test, one biopsy sampled 5 cm proximal from the ileocecal valve or anastomosis representing each patient. (B) Beta diversity illustrated by Bray-Curtis dissimilarity index on non-metric multidimensional scaling (NMDS) plot, each sample coloured according to ileocecal resection status, one biopsy sampled 5 cm proximal from the ileocecal valve or anastomosis representing each patient. (C) Beta diversity illustrated by Bray-Curtis dissimilarity index on non-metric multidimensional scaling (NMDS) plot, according to ileal location in 32 ileocecal resected CD patients, two mucosal pinch biopsies sampled 5 and 15 cm proximal from the ileocecal valve or anastomosis respectively were obtained from each patient, except for 4 patients where only 5 cm sample was obtained due to ileal stenosis, each sample coloured according to mucosal pinch biopsy location; 5 and 15 cm.

Supplementary Figure 4



Supplementary Figure 4 Complementary information regarding ileal mucosa-associated bacterial microbiome composition in Crohn's disease (CD) patients and healthy controls (HC) provided in Figure 1B. (A) Similar to Figure 1B (see Figure 1B for details) (B) Non-metric multidimensional scaling (NMDS) plot illustrating phylum position contributing to sample organization on NMDS plot (A)/Figure 1B.

Paper III

Fungal microbiota in the ileal mucosa of patients with Crohn's disease

Short title: Mycobiota in CD

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Disclosures: Authors have no conflict of interest to declare.

Author contributions: MO, TCM and RF were responsible for study design. MO and RF were responsible for data acquisition. MO, MLR, VB, AvBG and ES were responsible for analyses of biological material and data analysis. MO, MLR and RF interpreted the results and drafted the manuscript. MO, MLR, VB, AvBG, ES, TCM, AKS, HS and RF contributed to critical revision of the manuscript.

Word count: 3589

Abstract

Background: The gut mycobiota is thought to be involved in the pathogenesis of Crohn's disease (CD). However, its role is incompletely understood. The terminal ileum is a predilection site for CD, both for primary involvement and recurrences. We have assessed the mucosa-associated mycobiota in the inflamed and non-inflamed ileum in patients with CD.

Methods: In this cross-sectional study, the mucosa-associated mycobiota was assessed by ITS2 sequencing in a total of 168 biopsies sampled 5 and 15 cm proximal of the ileocecal valve or ileocolic anastomosis in 44 CD patients and 40 healthy controls (HC). CD patients with terminal ileitis, with endoscopic inflammation at 5 cm and normal mucosa at 15 cm and no history of upper CD involvement were analysed separately.

Results: CD patients had reduced mycobiota evenness, increased Basidiomycota/Ascomycota ratio and reduced abundance of Chytridiomycota compared to HC. The mucosa-associated mycobiota of CD patients were characterised by an expansion of *Malassezia* and a depletion of *Saccharomyces*, along with increased abundances of *Candida albicans* and *Malassezia restricta*. When the mycobiota in the inflamed and proximal non-inflamed mucosa within the same patients were compared, alpha diversity was similar. However, the inflamed mucosa had a more dysbiotic composition with increased abundances of *Candida sake* and reduced abundances of *Exophiala equina* and *Debaryomyces hansenii*.

Conclusions: The ileal mycobiota in CD patients is altered compared to HC. The mycobiota in the inflamed and proximal non-inflamed ileum within the same patients harbour structural differences which may play a role in the CD pathogenesis.

Introduction

Crohn's disease (CD) is a chronic inflammatory bowel disease (IBD) characterised by transmural and segmental inflammation of the gastrointestinal tract. Currently, CD is thought to develop in genetically susceptible individuals exposed to environmental factors and gut microbiota, causing an aberrant immune response that leads to inflammation and subsequent tissue damage (1). CD may affect any part of the gastrointestinal tract, however most commonly the terminal ileum and colon (1). After ileocecal resection (ICR), approximately 75% of CD patients experience disease recurrence after 10-years of follow-up (2). An ileostomy diverting the intestinal contents has a well-known protective effect, whereas reestablishment of bowel continuity or infusion of faecal content triggers recurrence (3, 4). Disease recurrence typically manifests at and immediately proximal to an anastomosis (2, 5). Studies of the terminal and neo-terminal ileum are therefore of particular interest with regards to understanding CD pathogenesis. Furthermore, alterations in the ileal bacterial mucosa-associated microbiota at the time of ICR and postoperatively have been associated with risk of disease recurrence (6-8).

The mycobiota has for long thought to be implicated in CD pathogenesis. For instance, the presence of Anti-*Saccharomyces cerevisiae* antibodies (ASCA) was an early biomarker for identification of CD (9). Genome-wide association studies (GWAS) have later found polymorphisms in *CARD9* to be associated with CD (10, 11). Identification of intestinal fungi through C-lectin receptors depends on *CARD9* in the signalling pathway to stimulate a pro-inflammatory response to commensal fungi (12, 13) and a defect in *CARD9* is associated with susceptibility to fungal infections and a lower number of Th-17 cells in humans (12). In addition, Toll-like receptor 4 polymorphisms associated with both CD and UC also predispose for systemic Candida infections in humans (14).

After the introduction of high throughput sequencing enabling culture-independent analyses of fungal populations, the majority of studies within the field have analysed the faecal mycobiota. However, the mucosa-associated and faecal bacterial microbiotas are different (15-17), and the mucosa-associated microbiota is by many considered more relevant in CD pathogenesis (18). Only a few studies have described the mucosa-associated mycobiota in CD patients (13, 19, 20). Mucosa-associated mycobiota alterations in CD are characterised by a skewed Ascomycota to Basidiomycota ratio compared to HC, with increased abundances of Basidiomycota and decreased abundances of Ascomycota phyla in CD (13, 20). Liguori et al.

(19) found inflamed tissue in CD patients to have a 40-fold higher load of fungi compared to healthy controls (HC), furthermore CD patients had increased abundances of Cystofilobasidiaceae family and *Candida glabrata* species. Limon et al. (13) have recently investigated water-lavage samples obtained from CD patients during colonoscopy and found increased abundances of *Malassezia*, *Cladosporium* and *Aureobasidium* and decreased abundances of *Fusarium* compared to HC. Notably, *Malassezia* was overrepresented in patients carrying a CARD9 allele which is associated with an increased risk of CD (13). El Mouzan et al. (20) found CD to be associated with increased abundances of Psathyrellaceae and Cortinariaceae families and *Psathyrella* and *Gymnopilus* genera. The identification of these unusual fungal strains was probably due to the specific cohort (teenagers) and also possibly to a specific diet in Saudi Arabia compared to the previous studies.

In the current study, we have assessed the mycobiota of patients with CD and HC, with a particular focus on differences between inflamed and proximal non-inflamed ileal mucosa within CD patients, which to the best of our knowledge has not been performed previously.

Material and methods

Patients and control subjects:

We have previously assessed the bacterial ileal microbiota of the same patient cohort (21). Study participants were recruited from the Department of Gastroenterology, St. Olav's Hospital, Trondheim, Norway between 2017-2019. Patients 18-70 years with Norwegian ethnicity referred to ileocolonoscopy were invited to participate if they were eligible. Inclusion criteria were an established diagnosis of CD based on clinical, endoscopic and histological criteria or patients with CD symptoms where the diagnosis was confirmed after both endoscopic and histologic evaluation. CD characteristics were registered according to the Montreal classification (22). Age- and sex-matched subjects referred to colonoscopy due to rectal bleeding or screening for disease were included as healthy controls (HC) if the ileocolonoscopy and histologic evaluation of biopsies were normal. Exclusion criteria were identical to those described in (21) i.e. use of antibacterial or antifungal treatment for the past 2 months or comorbidity with diabetes mellitus, celiac disease or liver diseases including primary sclerosing cholangitis and primary biliary cholangitis. Additional exclusion criteria

for HCs were previous gastrointestinal surgery, gastrointestinal polyps, cancer, diverticulitis or irritable bowel disease fulfilling the ROME IV criteria (23).

Endoscopic procedure

The ileum was reached during endoscopy using either a colonoscope (Olympus Exera II GIF HQ190 or PH190L, Olympus Europa GmbH, Hamburg, Germany) or a single-balloon enteroscope (Olympus SIF-Q180). A total of six ileal pinch biopsies were collected from each study participant, three biopsies from approximately 5 cm and 15 cm proximal of the ileocecal valve or ileocolic anastomosis, respectively. In CD patients with terminal ileitis, the 5-cm samples were taken from an endoscopically inflamed area and 15-cm samples from normal appearing mucosa. In CD patients categorised as having active disease, both biopsy locations (5- and 15-cm) were endoscopically inflamed. For CD patients in remission and the HC group, both biopsy locations (5- and 15-cm) appeared endoscopically normal. Endoscopic inflammation was evaluated using Rutgeerts score (24) whether the patients were operated by ICR or not, with inflammation defined as Rutgeerts score ≥1. One pair of mucosal pinch biopsies from the 5- and 15-cm locations were put on formalin for histological grading of inflammation. The two remaining biopsy pairs were put directly on liquid N_2 and stored on N_2 until subsequent bacterial or fungal DNA isolation and sequencing of the bacterial (21) and fungal microbiota, respectively.

Histological evaluation of biopsies

Formalin-fixed biopsies were stained with haematoxylin and eosin (H&E). Histological examination was performed blinded for phenotype by an experienced pathologist and scored according to Global Histologic Disease Activity Score (GHAS) and Robarts score (4, 25, 26). A validated histological scoring index for evaluation of disease activity in CD is lacking, and the reciprocity between histological scoring and disease activity measures is deficient (26, 27). However, histological evaluation blinded for phenotype verified all biopsies from HC as histologically normal.

DNA isolation

The fungal cell wall is particularly robust and is known to be hard to lyse (28, 29). A DNA isolation protocol specially designed to lyse the fungal cell wall, with both a chemical and mechanical lysis step, was therefore chosen. DNA from two mucosal biopsies (at 5- and 15-cm location) was isolated according to a previously described protocol (30) with the following adjustments; bead beating was performed with Precellys 24 tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) at 6500 rpm for 60 seconds twice. Centrifugation steps were performed at 21000 g, otherwise the original protocol was followed (30). The DNA samples were quantified using Qubit (Thermo Fisher Scientific, Waltham, MA).

ITS2 sequencing

ITS2 metagenomic sequencing libraries were prepared according to the "Illumina Metagenomics Sequencing Demonstrated Protocol" (31) with minor adjustments. In brief; 200 ng genomic DNA (extracted from biopsy samples) was used as a template for PCR amplification of the ITS2 region (98°C at 30 sec, followed by 34 cycles with: 15 sec at 98°C, 53°C for 30 sec and 72°C for 45 sec, followed by 7 min at 72°C). The ITS2 PCR primers were based on sequences first published by Liguori et al. (19). Illumina adaptor compatible overhang nucleotide sequences were added to the gene/locus specific sequences (ITS2 Amplicon PCR Forward Primer = 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGARTCATCGAATCTTT and ITS2 Amplicon PCR Reverse Primer = 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGATATGCTTAAGTTCAGCGGGT). The PCR products were then cleaned up by using AMPure XP beads (Beckman Coulter, Woerden, Netherlands) to purify ITS2 amplicons away from free primers and primer dimer species. In a second PCR amplification step (9 cycles) dual indices and Illumina sequencing adaptors were added by using the Nextera XT indexing kit (Illumina Inc., San Diego, CA) according to the manufacturer's instructions. A second PCR clean up step was performed using AMPure XP beads (Beckman Coulter), before validation of the library by a LabChip GX DNA high sensitivity assay (PerkinElmer, Inc., Waltham, MA). Libraries were normalised and pooled to 12 pM and subjected to clustering on two MiSeq V3 flowcells. Finally, paired-end read sequencing was performed for 2 x 300 cycles on a MiSeq instrument (Illumina, Inc.), according to the manufacturer's instructions. Base calling was done on the

MiSeq instrument by RTA v1.18.54. FASTQ files were generated using bcl2fastq2 conversion software v2.17 (Illumina, Inc.).

Bioinformatics

Sequencing data were processed using FROGS pipeline (32), established in Toulouse France (33) for sequence quality control, filtering and affiliation of taxa. The sequences were assigned to OTUs with 97% threshold of pairwise identity and classified taxonomically using the UNITE ITS database (version 8_2) (34). Five biopsy samples were removed from the study due to a low number of sequences. This included two 5-cm samples from HC and three 5-cm samples from CD patients. Phyloseq Package for R analysis was used for alpha and beta diversity analyses as well as illustration. Deseq2 package for R analysis was used for differential analysis of OTUs in respect to the different phenotypes (35). The linear discriminant analysis (LDA) effect size (LEfSe) algorithm (36) was used to identify taxa that were specific to phenotype or inflamed vs proximal non-inflamed mucosa.

Statistics

IBM SPSS Statistics version 25.0 (IBM Corp., Armonk, NY) was used for statistical analysis apart from analyses of sequencing data. Demographic and clinical characteristics are presented as % (n) for categorical variables, median (interquartile range (IQR)) for skewly distributed variables and mean value (standard deviation (SD)) for normally distributed variables. Accordingly, the chi-squared test, Mann-Whitney U test or independent t-test were used for comparing CD patients with HC. For all statistical analyses, a p-value <0.05 was considered statistically significant.

Ethical Considerations

The study was approved by the Regional Committee for Medical and Health Research Ethics, Central Norway (approval reference, 2016/2164). All study participants provided written informed consent.

Results

Patients

Forty-four CD patients and 40 HC were included. Demographic and clinical characteristics are presented in Table 1. CD patients had higher CRP levels compared to HC (p= 0.017), apart from that the groups were similar. The bacterial microbiota characteristics in this cohort have been described previously (21). CD characteristics are provided in Table 2. Twenty-two CD patients had terminal ileitis with endoscopic inflammation at 5-cm location and normal mucosa at 15-cm location, of which 20 had no history of upper gastrointestinal CD involvement. Of the remaining CD patients, ten had active disease, and 12 were in remission.

Ileal mycobiota in CD patients vs HC

CD patients had a lower fungal alpha diversity compared to HC based on the Simpson diversity index (p= 0.025), whereas the observed numbers of operational taxonomic units (OTUs) were similar (p= 0.21). This implies that the fungal species richness was similar, but that the evenness of fungi was reduced within the CD group compared to HC (Figure 1A). The most prevalent phyla in the samples overall were Ascomycota, Basidiomycota and Chytridiomycota, some Rozellomycota were also detected (Figure 1B). In CD patients, the Basidiomycota-to-Ascomycota ratio was increased compared to HC (Supplementary figure 1A). CD patients also had lower abundances of Chytridiomycota phyla (Supplementary Figure 1A). Beta diversity analysis assessed by Bray-Curtis dissimilarity showed a clustering of the samples according to the disease status (p< 0.001), confirming structural differences in the mycobiota composition between CD patients and HC (Figure 1C). Using LEfSe (36), fungal composition in CD patients and HC were compared and differentially abundant fungi identified (Figure 2). *Malassezia* and *Vishniacozyma* genera were increased in CD patients, while *Saccharomyces*, *Paludomyces* and *Oculimacula* were depleted in comparison to HC (Figure 2A and Supplementary figure 1B). When the comparison was performed at species level, CD patients had increased abundances of *Malassezia restricta* as well as *Malassezia sympodialis* and two other *Malassezia* species (Figure 2B). *Candida albicans and Vishniacozyma victoriae* were also increased in CD patients (Figure 2B). In HC *Trichosporon asahii*, *Paludomyces mangrovei* and a species from the Chaetomiaceae family were overrepresented compared to CD patients.

Mycobiota in the inflamed and proximal non-inflamed ileum in CD patients

Twenty CD patients had terminal ileitis with inflamed 5-cm location and non-inflamed 15-cm location, and no history of upper CD involvement, these patients were analysed separately. Alpha diversity in inflamed 5-cm samples and non-inflamed 15-cm samples are presented in Figure 3A. There was no statistically significant difference in fungal alpha diversity between 5- and 15-cm locations in CD patients with terminal ileitis based on observed OTUs and Simpson index. Interestingly, on the beta diversity plot assessed by Jaccard index, which focuses more on low abundant OTUs in comparison to Bray-Curtis dissimilarity, inflamed 5-cm samples clustered furthest away from HC with non-inflamed CD 15-cm samples in an intermediate location (Figure 3B). In a beta diversity plot including only CD patients with terminal ileitis, 5- and 15-cm samples separated clearly ($p < 0.05$) according to Jaccard index (Figure 3C). When we compared the fungal composition in inflamed 5-cm samples with non-inflamed 15-cm samples using LEfSe, we identified six taxa which were increased at 5-cm location and four taxa which were increased at 15-cm location (Figure 4). Cordycipitaceae and Sporidiobolaceae families and *Lecanicillium* genus were overrepresented at the inflamed 5-cm location, whereas *Exophiala* and *Debaryomyces* genera were overrepresented at non-inflamed 15-cm location. Differentially abundant species were identified using LEfSe are presented in a heatmap (Figure 4B). *Candida sake* was overrepresented at inflamed 5-cm location. The *Exophiala* and *Debaryomyces* genera, which were increased at non-inflamed 15-cm location, were identified as *Exophiala equina and Debaryomyces hansenii* (Figure 4B).

Mycobiota according to ileal inflammation and sub-location in CD patients overall

The mycobiota was similar according to both alpha diversity (Observed OTUs and Simpson index) and beta diversity (Bray-Curtis dissimilarity), regardless of endoscopic or histologic inflammation in the whole cohort of CD patients including all biopsy samples (Figure 5). This argues that endoscopic and histologic inflammation per se does not alter the fungal mycobiota tremendously. Similarly, we compared the mucosa-associated mycobiota at 5-cm and 15 cm-locations in the CD study cohort in order to assess if differences found in CD patients with terminal ileitis could be explained by effect of location alone (Figure 6). There was no difference in alpha diversity (Observed OTUs and Simpson index) nor beta diversity

(Bray-Curtis dissimilarity) between 5-cm and 15-cm location when all CD patients and biopsy samples were included. Correspondingly, when all samples were pooled (both CD and HC), there were no differences in alpha nor beta diversity between ileal sublocation (5- and 15-cm) (Supplementary Figure 2). This is in accordance with our previous findings suggesting that the mucosa-associated bacterial microbiota alterations in CD patients are present across locations and independent of inflammation and ileal sublocation (21).

Discussion

The mechanisms by which fungi may influence the pathogenesis of CD are uncertain, but both genetic and environmental factors may exist. A proportion of IBD patients have genetic polymorphisms which increase the susceptibility to fungal infections, also the fungal load and richness are elevated in CD patients (19, 37). Factors known to affect the mycobiome include diet, antibacterial and antifungal agents as well as interaction between fungi and bacteria (38-41). The risk of CD was recently reported to be associated with cumulative antibiotic exposure in a Swedish national cohort (42). Antibacterial therapy increases fungal abundances in faecal samples (40, 43) and fungi may mediate the increased risk of CD observed after exposure to antibacterial agents (44). An increase in faecal fungi has also been associated with a diet based on meat, eggs and cheeses compared to a vegetarian diet (45) and correspondingly, epidemiological studies have shown that intake of fibre and fruit reduces CD-risk (46). This is the first study to assess the mucosa-associated fungal microbiota in the ileum of adult CD patients. Previous reports have found CD patients to have an altered fungal composition compared to HC (13, 19, 20, 47).

In this study, we found that that the mucosa-associated mycobiota in the ileum of CD patients had reduced alpha diversity based on the Simpson index, but a similar number of observed OTUs compared to HC, implicating similar species richness, but reduced evenness in CD patients. As reported previously, CD patients had an increased Basidiomycota-to-Ascomycota ratio (13, 47). Furthermore, CD was associated with an altered mycobiota composition characterised by a significant gain of *Malassezia* and loss of *Saccharomyces*. At species level, *Malassezia* was identified as *Malassezia restricta* and *Malassezia sympodialis*. We also found *Candida albicans* to be overrepresented in CD patients vs HC. The expansion of *Malassezia restricta* has previously been reported by others (13, 48). Interestingly increased abundance of *M. restricta* was found in CD patients carrying the *CARD9* risk allele and *M. restricta*

aggravated dextran sodium sulphate-induced colitis in mice (13). The depletion of *Saccharomyces*, both at genus level and higher taxonomic levels have also been described in faecal samples from IBD patients, where *Saccharomyces* was positively correlated with abundances of bacteria depleted in IBD, such as the butyrate-producing *Roseburia*, *Blautia* and *Ruminococcus* genera (47, 49). Several *Saccharomyces* spp. have been suggested to have anti-inflammatory effects (39, 47, 50, 51).

Candida albicans has been proposed to participate in IBD through increasing the inflammation response and by increased abundance in the gut during inflammation, thereby creating a vicious circle (39, 44). We found increased abundances of *C. albicans* in the ileal mucosa of CD patients. Faecal abundance of *Candida* prior to faecal microbiota transplantation in UC patients has been associated with therapeutic response, and the observed effect of faecal microbiota transplantation (FMT) may be mediated by a reduction in *Candida* abundance (52). Increased abundances of *Candida* and *Candida glabrata* have previously been found in faecal and colonic samples, respectively, from CD patients (19, 47, 53). Interestingly, increased abundances of *Candida tropicalis* in faecal samples from CD patients have been found to be positively correlated to ASCA concentrations (41). *Candida* is also extensively involved in bacterial interactions and has a significant influence on microbiome composition (39, 41).

We specifically analysed the mucosa-associated mycobiota in the inflamed and proximal non-inflamed mucosa of CD patients with terminal ileitis as the terminal ileum is the predilection site of primary and recurrent CD. We did not find differences in alpha diversity between the inflamed and proximal non-inflamed mucosa, however there was a separation on beta diversity plots suggesting an altered and more dysbiotic fungal composition in the inflamed ileum compared to the proximal-non inflamed ileum and healthy mucosa of controls. In the inflamed mucosa *Lecanicillium* genera and *Candida sake* sp. were increased, whereas *Exophiala equina and Debaryomyces hansenii* were increased in the proximal non-inflamed mucosa. *C. sake* is frequently found in faeces of healthy humans (28), but can also cause invasive candidemia (54). In the food industry *C. sake* is used as a biocontrol agent to limit decay of apples due to mould (55, 56). *D. hansenii* is a commensal gut fungus which is found in faeces of healthy adults and reported to be increased in faeces of infants (28, 57). *D. hansenii* is also a frequently found in foods such as meat, fruit, cheese, beer and wine (58). The literature on *E. equina* is scarce, but it has been isolated from drinking water (59). However, one report identified *E. equina* in subcutaneous abscesses with histologically

granulomatous inflammation (60). *E. equina* has also been described to cause widespread granulomatous inflammation in a Galapagos tortoise (61). Recently, *Exophiala* has been associated with primary sclerosing cholangitis (62).

Fungal richness and diversity have previously been found to be increased in inflamed vs non-inflamed mucosa assessed by PCR and Denaturing Gel Gradient Electrophoresis (DGGE), however the method is less sensitive in terms of taxa identification and diversity measures compared to ITS-sequencing (63). In the current study, neither endoscopic nor histologic inflammation was associated with an increased number of OTUs or altered mycobiota according to other alpha diversity or beta diversity measures. Similarly, ileal sub-location did not seem to impact mycobiota diversity.

Strengths of the study include that the mucosa-associated mycobiota was assessed in the highest number of CD patients to date (13, 19, 20), the sequencing analysis was of good quality, and the majority of sequences were taxonomically classified. Limitations include heterogeneity of the disease duration, medical and surgical treatment which may affect the mycobiota composition. We have not correlated the bacterial and fungal microbiota, interactions between bacteria and fungi occur and these have not been assessed.

Summarised, CD patients exhibit an altered mucosa-associated mycobiota composition compared to HC. Within CD patients with terminal ileitis, the mycobiota in inflamed and proximal non-inflamed mucosa harboured structural differences in terms of beta diversity and fungal composition. The significance of a different fungal taxa composition in the inflamed and proximal non-inflamed mucosa may be a result of different immunological activity between inflamed and non-inflamed ileal sites or that fungi overrepresented in the inflamed mucosa trigger inflammation onset or maintenance. However, in the whole cohort of CD patients, neither inflammation nor ileal sub-location impacted mycobiota composition, arguing that inflammation or location it-self does not alter immunological activity. Further studies should focus on how the differentially abundant fungi identified may influence the mucosa and immune system, thus providing mechanistic explanations of the role of fungi in CD pathogenesis.

Conclusion

In conclusion, this study confirms CD associated alterations in the mucosa-associated mycobiota. The mycobiota composition in the inflamed ileum and proximal non-inflamed ileum differ according to beta diversity, and differentially abundant fungal taxa were identified. The significance of a different mycobiota composition between the inflamed and proximal non-inflamed mucosa within the same patients may play a role in CD pathogenesis and warrants further research.

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Table 1 Demographic and clinical characteristics of Crohn's disease (CD) patients and healthy controls (HC)

	CD	**HC**	**p-value** [a]
Number of patients, n	44	40	
Male gender, n (%)	24 (54.5%)	19 (47.5%)	0.52
Age, *years*, mean (SD)	42.2 (14.4)	36.6 (12.9)	0.07
BMI, mean (SD)	25.8 (4.8)	26.6 (4.7)	0.40
Acid reflux medication, n (%)			0.72
PPI	5 (11.4%)	2 (5%)	
H_2 blockers	0	0	
PPI on demand	0	0	
H_2 blockers on demand	1 (2.3%)	1 (2.5%)	
Smoking, n (%)			0.57
Never smoker	23 (52.3%)	25 (62.5%)	
Active smoker	5 (11.4%)	5 (12.5%)	
Snuff	10 (22.7%)	8 (20%)	
Ex-smoker	6 (13.6%)	2 (5%)	
Laboratory values			
Hb (g/dL), mean (SD)	14.1 (1.5)	14.5 (1.7)	0.197
Leukocytes (x10^9/L), median	6.4 (2.3)	6.5 (2.3)	0.50
CRP (mg/L), median (IQR)	<5 (4)	<5 (0)	**0.017**

[a] Comparing CD (n=44) with HC (n=40) using Mann-Whitney U test for skewly distributed continuous variables, independent *t* test for normal distributed continuous variables and Chi square/Fisher exact test for categorical variables.

Table 2 Crohn's disease (CD) characteristics, medical treatment, endoscopic evaluation and surgical history

CD characteristics	**CD (n=44)**
Disease duration, *years (median, IQR)*	10.0 (19.8)
Subclassification of patients, *n (%)* [a]	
Terminal ileitis (Inflamed 5-cm + normal 15-cm)	22 (50.0%)
Active disease (Inflamed 5 cm + 15 cm)	10 (22.7%)
Remission (Normal 5 + 15 cm)	12 (27.3%)
Montreal location, *n (%)*	
Terminal ileum (L1)	23 (52.3%)
Ileocolonic (L3)	16 (36.4%)
Ileocolonic + Upper GI (L3 + L4)	5 (11.4%)
Montreal behaviour, *n (%)*	
Non-stricturing, non-penetrating (B1)	8 (18.2%)
Non-stricturing, non-penetrating + perianal (B1p)	2 (4.5%)
Stricturing (B2)	15 (34.1%)
Stricturing + perianal (B2p)	6 (13.6%)
Penetrating (B3)	11 (25%)
Penetrating + perianal (B3p)	2 (4.5%)
Montreal age (age at diagnosis), n (%)	
16 years or younger (A1)	12 (27.3%)
17-40 years (A2)	22 (50%)
Over 40 years (A3)	10 (22.7%)
CD-medication, n (%) [b]	
No medical therapy for CD	18 (40.9%)
Budesonide	7 (15.9%)
Prednisolone	4 (9.1%)
5-ASA	3 (6.8)
Azathioprine	6 (13.6%)
Methotrexate	3 (6.8%)
Adalimumab	4 (9.1%)
Infliximab	7 (15.9%)
Vedolizumab	1 (2.3%)
Treatment naïve, *n (%)*	6 (13.6%)
TNFα naïve, *n (%)*	23 (52.3%)
Rutgeerts score, *n (%)*	
i0	12 (27.3%)
i1	12 (27.3%)
i2	5 (11.4%)
i3	6 (13.6%)
i4	9 (20.5%)
Ileocecal resection	28 (63.6%)

[a] Based on endoscopic evaluation of inflammation
[b] Co-medication: n=8 (18.2) used two CD medications, n=1 (2.3%) used three CD medications

Figure legends

Figure 1 Altered mucosa-associated mycobiota in Crohn's disease (CD) patients in comparison to healthy controls (HC). (A) Fungal alpha-diversity, according to observed operational taxonomic units (OTUs) (left) and Simpson index (right), boxplots coloured according to disease phenotype. (B) Relative abundance of fungal phyla in HC and CD patients. (C) Beta-diversity. Principal coordinates analysis of Bray-Curtis dissimilarity with samples coloured according to disease phenotype. The fraction of diversity captured by the coordinate is given in percentage on axes 1 and 2. Groups compared using Permanova method.

Figure 2 Differentially abundant fungal taxa in Crohn's disease (CD) patients in comparison to healthy controls (HC) identified using linear discriminant analysis effect size (LEfSe). (A) Fungal taxa overrepresented in CD patients (red) and HC (green) illustrated in a histogram which shows Linear Discriminant Analysis (LDA) score computed for the differentially abundant fungal taxa. (B) Heatmap illustrating the distribution of differentially abundant fungal species between CD and HC mucosal pinch biopsies sampled 5 cm proximal of the ileocecal valve or ileocolic anastomosis.

Figure 3 Fungal mucosa-associated mycobiota in a subset of 20 Crohn's disease (CD) patients with terminal ileitis and without upper CD involvement. Biopsies sampled at inflamed 5-cm and non-inflamed 15-cm proximal to the ileocecal valve or ileocolic anastomosis. (A) Alpha diversity, according to observed operational taxonomic units (OTUs) (left) and Simpson index (right), boxplots coloured according to biopsy location. (B) Beta-diversity in CD patients with terminal ileitis and healthy controls (HC). Principal coordinates analysis of Jaccard index with samples coloured according to disease phenotype and ileal location. The fraction of diversity captured by the coordinate is given in percentage on axes 1 and 2. (C) Beta-diversity. Principal coordinates analysis of Jaccard index with samples coloured according to ileal location. The fraction of diversity captured by the coordinate is given in percentage on axes 1 and 2. Groups compared using Permanova method.

Figure 4 Differentially abundant fungal taxa between inflamed 5-cm vs proximal non-inflamed 15-cm ileal biopsies from 20 Crohn's disease (CD) patients with terminal ileitis and no history of upper CD involvement. Biopsies sampled from 5- and 15 cm proximal of the

ileocecal valve or ileocolic anastomosis within the same patients. (A) Fungal taxa overrepresented in 5-cm biopsies (green) and in 15-cm biopsies (red) illustrated in a histogram with Linear Discriminant Analysis (LDA) score computed using linear discriminant analysis effect size (LEfSe). (B) Heatmap showing the distribution of differentially abundant fungal species in 5-cm samples (right) and 15-cm samples (left) identified using LEfSe.

Figure 5 Inflammation does not impact mucosa-associated mycobiota in CD patients overall (n=44). (A and B) Alpha-diversity, according to observed operational taxonomic units (OTUs) (left) and Simpson index (right), boxplots coloured according to endoscopic (A) or histologic (B) inflammation. (C and D) Beta-diversity. Principal coordinates analysis of Bray-Curtis dissimilarity with samples coloured according to endoscopic (C) or histological (D) inflammation. The fraction of diversity captured by the coordinate is given in percentage on axes 1 and 2. Groups compared using Permanova method.

Figure 6 Mucosal pinch biopsy location in the ileum of CD patients does not impact mucosa-associated mycobiota. 44 CD patients, biopsies sampled from 5- and 15-cm from the ileocecal valve or ileocolic anastomosis. (A) Alpha-diversity according to observed operational taxonomic units (OTUs) (left) and Simpson index (right), boxplots coloured according to biopsy location 5-cm or 15-cm. (B) Beta-diversity. Principal coordinates analysis of Bray-Curtis dissimilarity with sample colour according to biopsy location. The fraction of diversity captured by the coordinate is given in percentage on axes 1 and 2. Groups compared using Permanova method.

Supplementary Figure 1 Altered mucosa-associated mycobiota Crohn's disease (CD) patients in comparison to healthy controls (HC). (A) Abundances of fungal phyla and Basidiomycota/Ascomycota ratio, boxes coloured according to disease phenotype. Groups compared using Independent Sample t-test. (B) Abundances of fungal genera, boxes coloured according to disease phenotype. Groups compared using Independent Sample t-test.

Supplementary Figure 2 Mucosa-associated mycobiota is similar across different ileal sub-locations in both Crohn's disease (CD) patients (n=44) and healthy controls (HC) (n=40). (A) Alpha-diversity according to observed operational taxonomic units (OTUs) (left) and Simpson index (right), boxplots coloured according to biopsy location 5-cm or 15-cm. (B)

Beta-diversity. Principal coordinates analysis of Bray-Curtis dissimilarity with sample colour according to biopsy location. The fraction of diversity captured by the coordinate is given in percentage on axes 1 and 2. Groups compared using Permanova method.

Figure 1

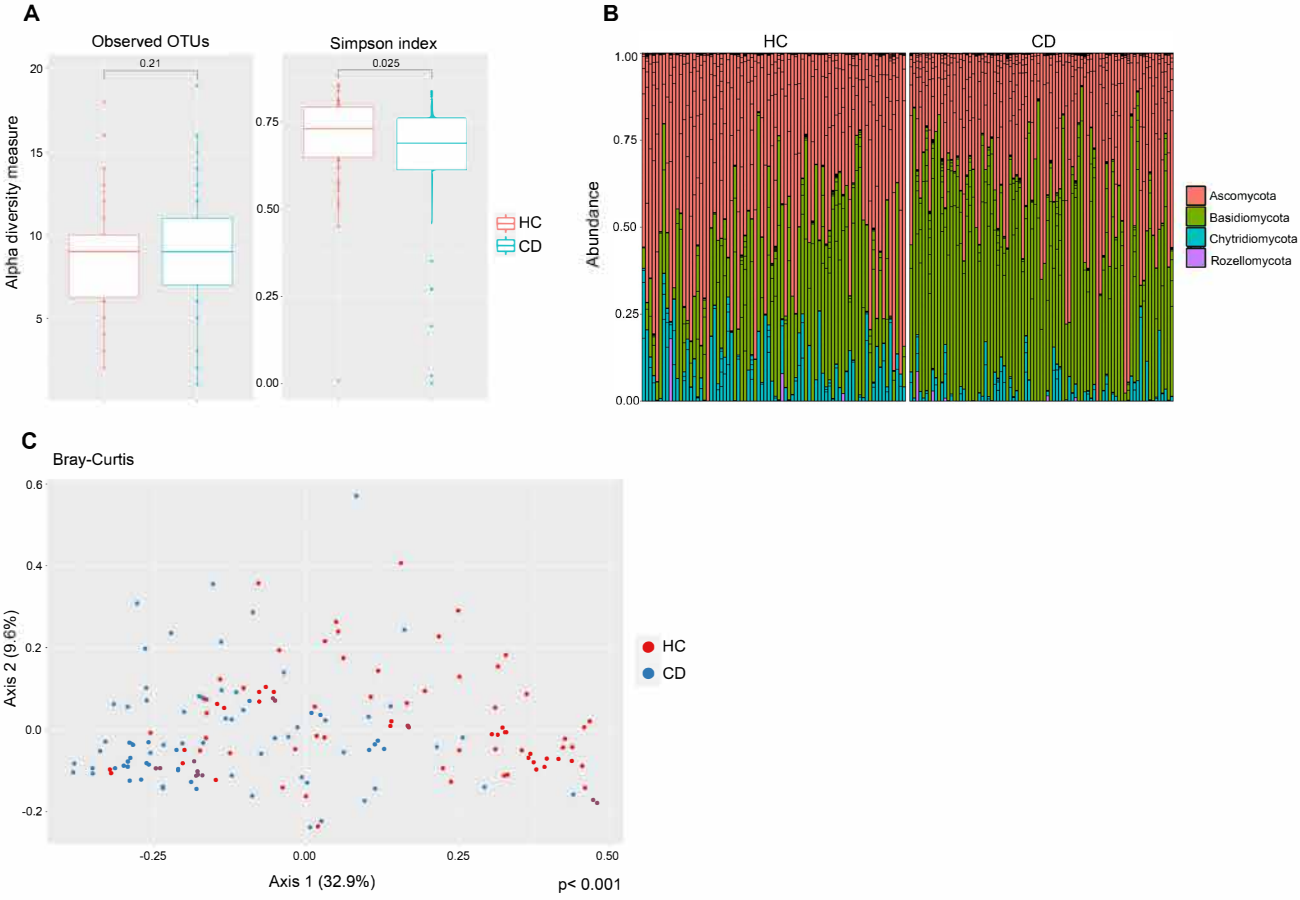

A
Observed OTUs
Simpson index
0.21
0.025
Alpha diversity measure
HC
CD
B
HC
CD
Abundance
Ascomycota
Basidiomycota
Chytridiomycota
Rozellomycota
C
Bray-Curtis
Axis 2 (9.6%)
Axis 1 (32.9%)
p< 0.001
HC
CD

Figure 2

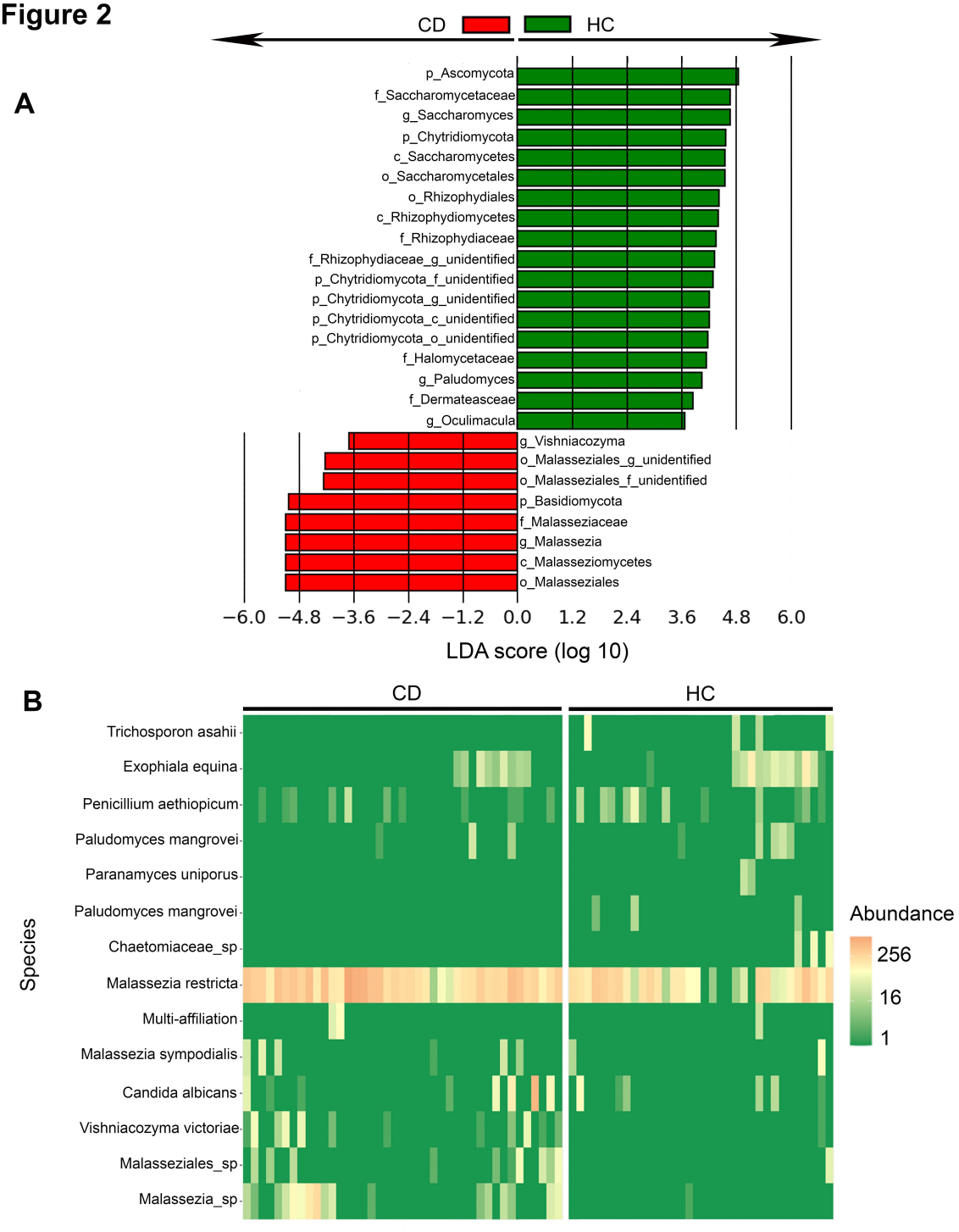

A
CD
HC
p_Ascomycota
f_Saccharomycetaceae
g_Saccharomyces
p_Chytridiomycota
c_Saccharomycetes
o_Saccharomycetales
o_Rhizophydiales
c_Rhizophydiomycetes
f_Rhizophydiaceae
f_Rhizophydiaceae_g_unidentified
p_Chytridiomycota_f_unidentified
p_Chytridiomycota_g_unidentified
p_Chytridiomycota_c_unidentified
p_Chytridiomycota_o_unidentified
f_Halomycetaceae
g_Paludomyces
f_Dermateasceae
g_Oculimacula
g_Vishniacozyma
o_Malasseziales_g_unidentified
o_Malasseziales_f_unidentified
p_Basidiomycota
f_Malasseziaceae
g_Malassezia
c_Malasseziomycetes
o_Malasseziales
−6.0 −4.8 −3.6 −2.4 −1.2 0.0 1.2 2.4 3.6 4.8 6.0
LDA score (log 10)
B
CD
HC
Species
Trichosporon asahii
Exophiala equina
Penicillium aethiopicum
Paludomyces mangrovei
Paranamyces uniporus
Paludomyces mangrovei
Chaetomiaceae_sp
Malassezia restricta
Multi-affiliation
Malassezia sympodialis
Candida albicans
Vishniacozyma victoriae
Malasseziales_sp
Malassezia_sp
Abundance
256
16
1

Figure 3

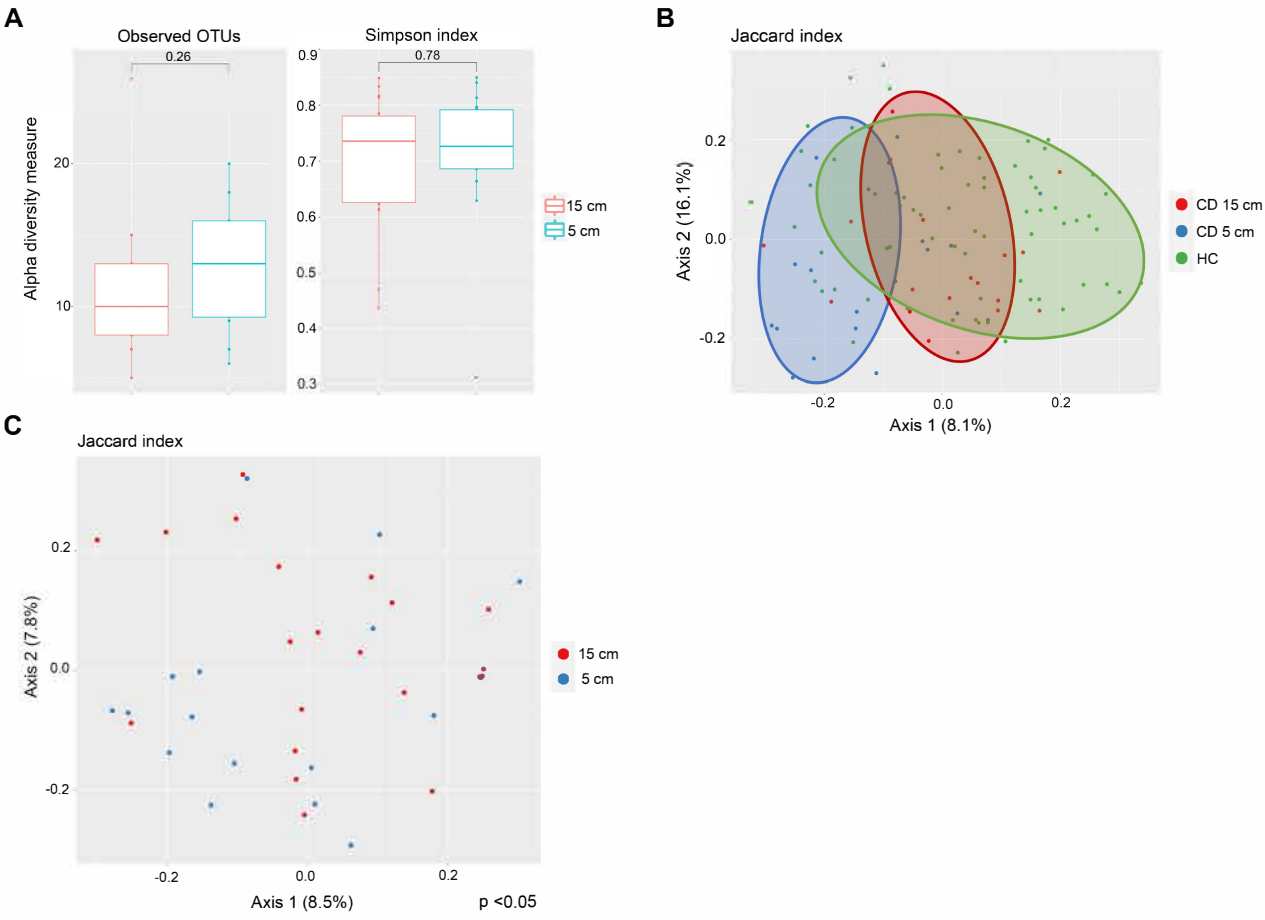
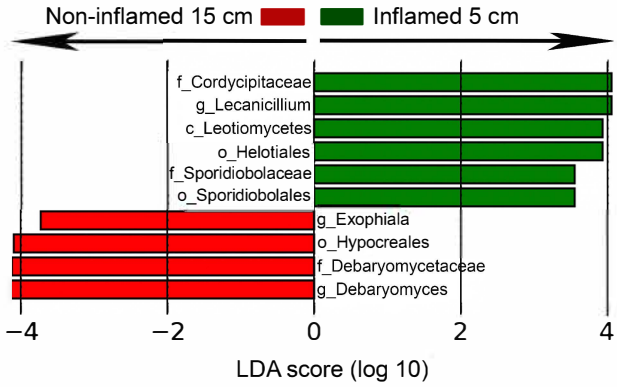

A
Observed OTUs
Simpson index
0.26
0.78
Alpha diversity measure
20
10
0.9
0.8
0.7
0.6
0.5
0.4
0.3
15 cm
5 cm
B
Jaccard index
Axis 2 (16.1%)
0.2
0.0
-0.2
-0.2
0.0
0.2
Axis 1 (8.1%)
CD 15 cm
CD 5 cm
HC
C
Jaccard index
Axis 2 (7.8%)
0.2
0.0
-0.2
-0.2
0.0
0.2
Axis 1 (8.5%)
p <0.05
15 cm
5 cm

Figure 4

A


Non-inflamed 15 cm
Inflamed 5 cm
f_Cordycipitaceae
g_Lecanicillium
c_Leotiomycetes
o_Helotiales
f_Sporidiobolaceae
o_Sporidiobolales
g_Exophiala
o_Hypocreales
f_Debaryomycetaceae
g_Debaryomyces
−4
−2
0
2
4
LDA score (log 10)

B

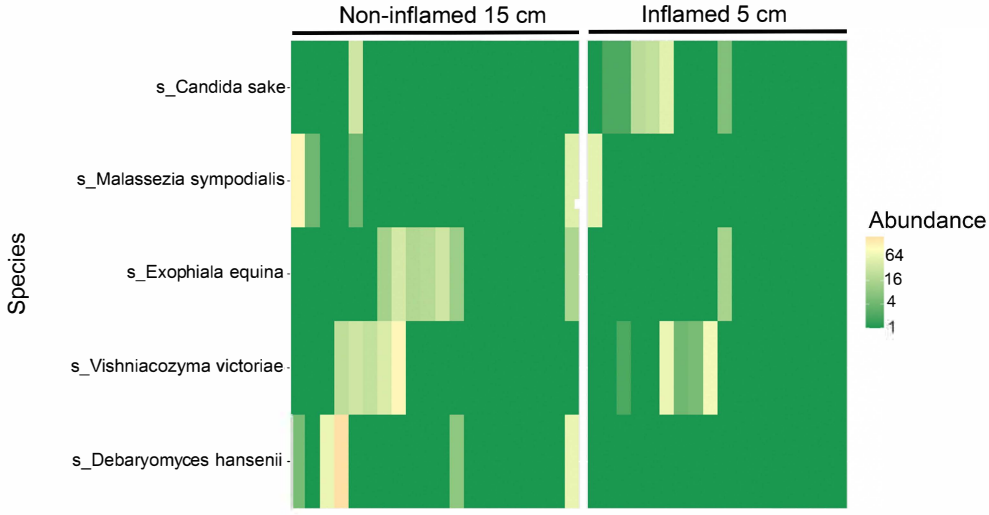

Non-inflamed 15 cm
Inflamed 5 cm
Species
s_Candida sake
s_Malassezia sympodialis
s_Exophiala equina
s_Vishniacozyma victoriae
s_Debaryomyces hansenii
Abundance
64
16
4
1

Figure 5

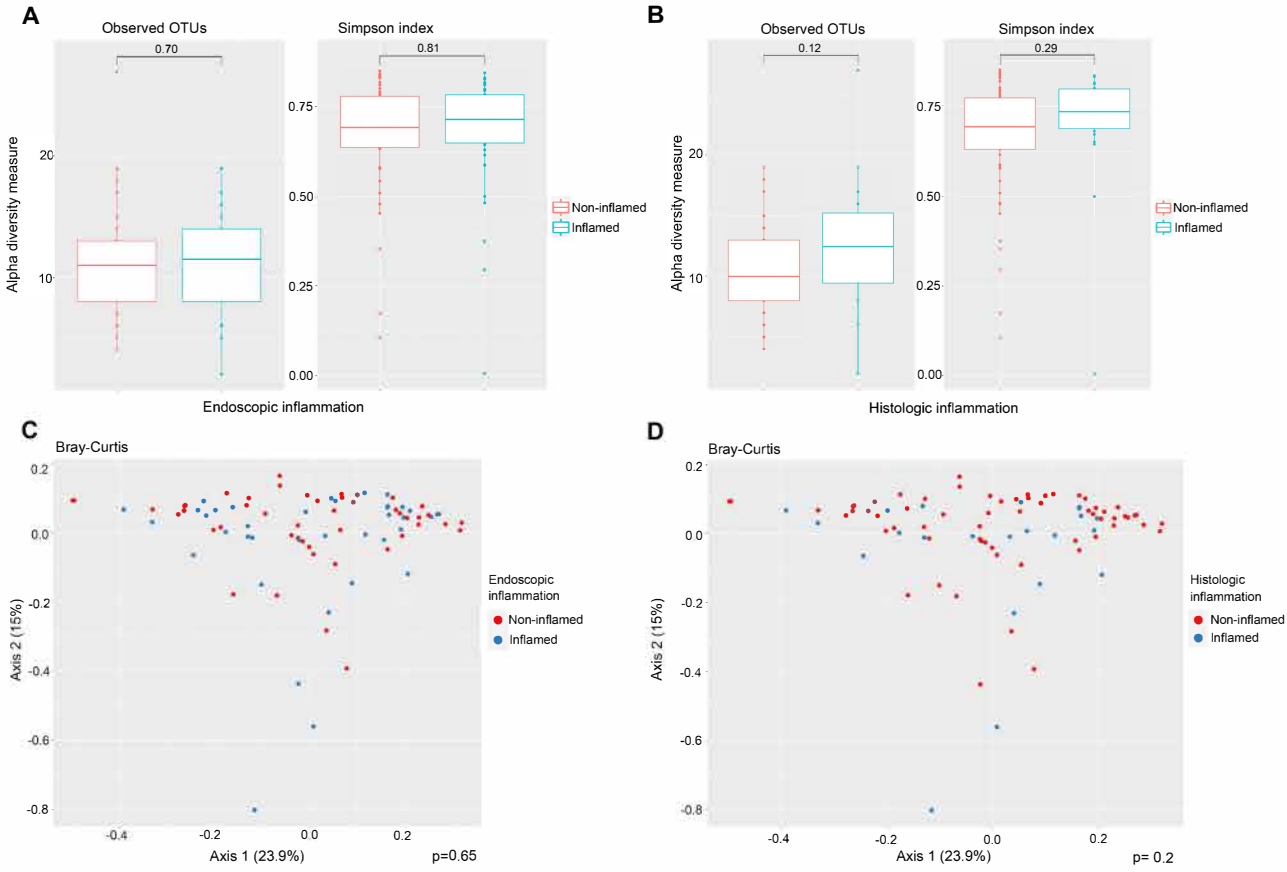
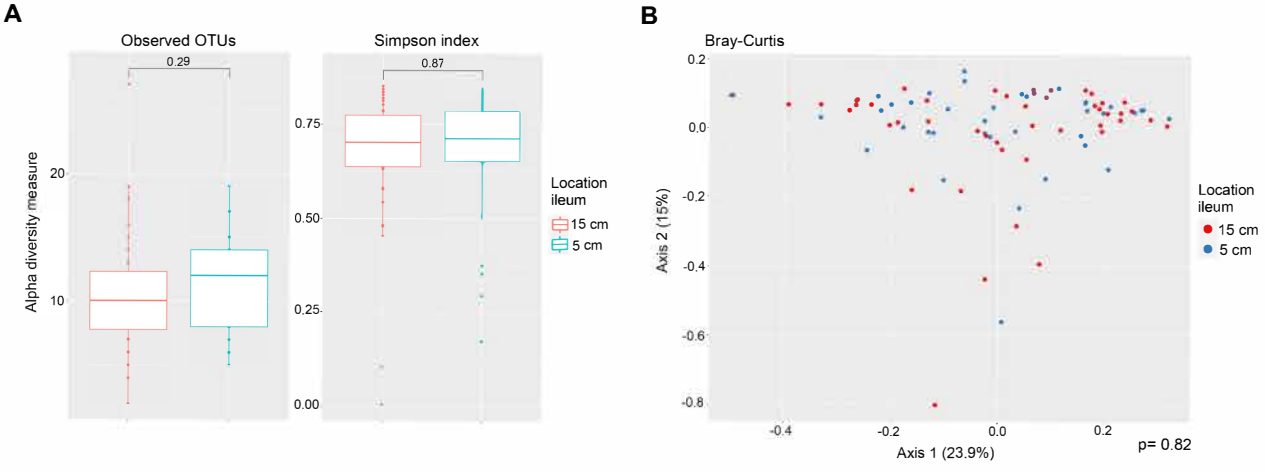
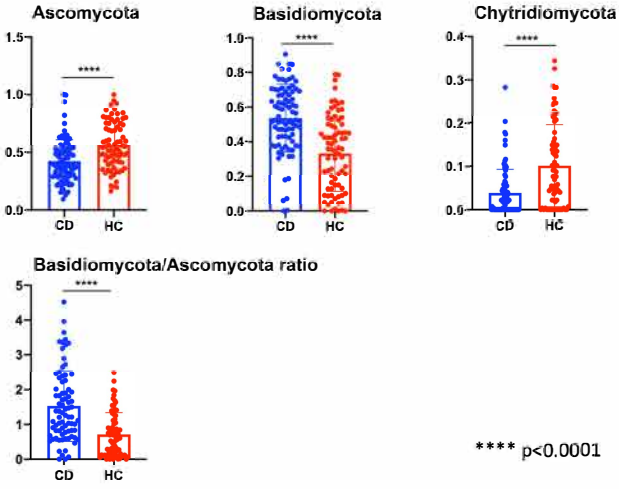

A
Observed OTUs
Simpson index
0.70
0.81
Alpha diversity measure
Endoscopic inflammation
Non-inflamed
Inflamed
B
Observed OTUs
Simpson index
0.12
0.29
Alpha diversity measure
Histologic inflammation
Non-inflamed
Inflamed
C
Bray-Curtis
Axis 2 (15%)
Axis 1 (23.9%)
p=0.65
Endoscopic inflammation
Non-inflamed
Inflamed
D
Bray-Curtis
Axis 2 (15%)
Axis 1 (23.9%)
p= 0.2
Histologic inflammation
Non-inflamed
Inflamed

Figure 6

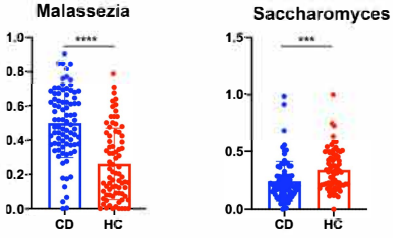

A
Observed OTUs
Simpson index
0.29
0.87
Alpha diversity measure
20
10
0.75
0.50
0.25
0.00
Location
ileum
15 cm
5 cm
B
Bray-Curtis
0.2
0.0
-0.2
-0.4
-0.6
-0.8
Axis 2 (15%)
-0.4
-0.2
0.0
0.2
Axis 1 (23.9%)
p= 0.82
Location
ileum
15 cm
5 cm

Supplementary Figure 1

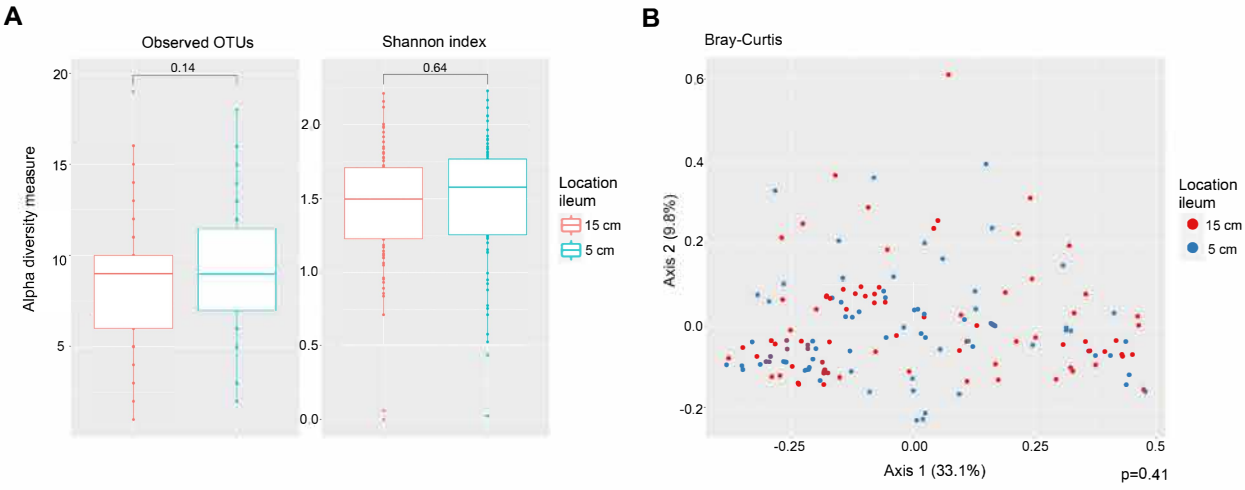
A



B



Supplementary Figure 2



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