Gut Microbiota and Antibiotic Resistance Gene Profiles in Ulcerative Colitis

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Gut Microbiota and Antibiotic Resistance Gene Profiles in Ulcerative Colitis

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Abstract:

The gut microbiota plays a key role in the pathogenesis of ulcerative colitis (UC), a form of inflammatory bowel disease (IBD) characterised by chronic relapsing inflammation of the gastrointestinal tract. The onset and progression of UC have been associated with intestinal dysbiosis and various environmental factors. Despite extensive research, the precise aetiology of IBD remains incompletely understood, presenting challenges in the development of effective treatment strategies.
This study aimed to investigate the bacterial community structure and its correlation with the presence of antibiotic resistance genes (ARGs) in DNA isolated from individuals diagnosed with UC at varying stages of disease severity. To achieve this, 16S rRNA gene amplicon sequencing was used to profile microbial taxonomy, followed by bioinformatic analysis to explore differences associated with disease status and current use of medications. In parallel, a highly sensitive method, qPCR was used to detect the presence of selected ARGs. The results revealed notable differences in the composition of the gut microbiota of UC patients compared to healthy controls. Furthermore, the presence of ARGs appeared to be patient-specific and reflective of microbiota dysbiosis.

Preface and Acknowledgements

This project was written by Julia Zrąbkowska from 1 September 2023 to 9 May 2025 as part of the Master of Science Program in Biotechnology at the Faculty of Engineering and Science at Aalborg University. The main objective of this project was to investigate the structure of the bacterial community and the presence of selected antibiotic resistance genes in ulcerative colitis.

The project was conducted under the guidance of Professor Jeppe Lund Nielsen from the Department of Chemistry and Bioscience at AAU, to whom I extend my sincere thanks for his guidance and input. Special thanks go to the PhD. Stine Karstenskov Østergaard, AAU, for her supervision, guidance, and assistance with both the experimental work and data analysis. I also extend my gratitude to the Ph.D. Patrick Skov Schacksen, AAU, for his valuable introduction to server work and his input on the bioinformatics aspect of the data analysis. I cannot omit thanking my fellow students—Lærke, Silvia, Stefania, and Math—for their support and understanding throughout the project period. I could not have succeeded without you on my side.

Julia Zrąbkowska
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Abbreviations

5-ASA	5-aminosalicylic acid
ABC	ATP-binding cassette
ARG	Antibiotic resistance gene
AZA	Azathioprine
CD	Crohn's disease
Ct	Cycle threshold
FMT	Faecal microbiota transplantation
GI	Gastrointestinal
GWAS	Genome-wide association studies
HC	Healthy control
HDAC	Histone deacetylases
HGT	Horizontal gene transfer
HMOs	Human milk oligosaccharides
IBD	Inflammatory bowel disease
IBS	Inflammatory bowel syndrome
ICD	International classification of diseases
IL	Interleukin
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MATE	Multidrug and toxic compound extrusion
MDR	Multidrug efflux pumps
MFS	Major facilitator superfamily
NTC	No-template control
OTUs	Operational taxonomic units
PCA	Principal component analysis
PCR	Polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RDA	Redundancy analysis
RND	Resistance-nodulation-cell division
SCFAs	Short chain fatty acids
SMR	Small multidrug resistance
TNF	Tumor necrosis factor
UC	Ulcerative colitis

Contents

Introduction 1

1.1 Importance of the Gut Microbiota

The mature gut microbiota comprises more than 1,500 species across 50 different phyla. It is predominantly composed of *Firmicutes* and *Bacteroidetes*, followed by *Proteobacteria*, *Fusobacteria*, *Tenericutes*, *Actinobacteria*, and *Verrucomicrobia* [1, 2, 3]. In addition to bacteria, the healthy gut microbiota also hosts diverse populations of fungi, protists, archaea, and viruses, all of which contribute to the overall microbial ecosystem [1].

The colonisation of the gut begins at birth, driven by the initial exposure of the newborn to the maternal microbial ecosystem. The mode of delivery significantly influences the composition and establishment of the infant's microbiota. During vaginal delivery, the microbes present in the birth canal are transmitted through direct contact [4]. As a result, the neonatal microbiome is typically dominated by genera such as *Bifidobacterium* and *Lactobacillus* [5]. In contrast, babies born by caesarean section acquire a microbiota that more closely resembles the skin, as skin-associated microbes constitute their primary source of colonisation [6]. Breastfeeding also supports the development of neonatal immune cells by introducing beneficial microbes from the mother, for instance *Actinobacteria*, which subsequently use human milk oligosaccharides (HMOs), resulting in increased short-chain fatty acids (SCFAs) [1, 4]. The gut of infants fed formula milk tends to be more abundant in *Clostridia*, *Streptococci*, *Bacteroides* and *Enterobacteria* [1]. Early-life microbial interactions have been shown to play a key role in the formation of a balanced gut microbiota, which in turn enhances immune resilience by promoting tolerance to commensals and effective responses to pathogens. A well-established microbiome contributes to immune defence by responding effectively to antigens and has also been associated with a reduced risk of developing autoimmune diseases later in life [7]. A key factor influencing the composition of the gut microbiota is the transition to a regular diet and continues to be an important factor throughout life (Figure 1.1). A vegetarian diet has been widely recognised for its health benefits and is associated with an increase in relative abundance of certain *Firmicutes* and *Bacteroidetes*. This is attributed to the high daily intake of dietary fibre, which is further metabolised into beneficial SCFAs [1]. In contrast, a western diet, characterised by a high intake of processed foods, fats, sugars, and animal proteins, is linked to a microbial composition, favouring the increased abundance of *Bacteroides*, *Bilophila* and *Alistipes*, while simultaneously suppressing beneficial bacteria from *Firmicutes* phyla [1, 5]. The western diet pattern has been associated with reduced microbial diversity, low-grade chronic inflammation, and metabolic disturbances. In particular, prolonged consumption of this diet has been associated with weakened immune function, increased susceptibility to infections, and increased risk of metabolic disorders such as obesity and type 2 diabetes [8]. Internal factors, such as host genetics, age, and overall health status, also play crucial roles in the composition and stability of the microbiome [9]. Age-related physiological changes, including altered immune function and intestinal motility,

can lead to changes in microbial diversity and composition over time [1]. Moreover, microbes present in the gastrointestinal (GI) tract can affect host gene expression [10]. Colonising bacteria protect against external pathogens by maintaining epithelial integrity and produce antimicrobial compounds that suppress harmful microorganisms. The microbiota regulates immune responses, supports immune cell development, and modulates inflammation by interacting with epithelial and immune cells [11]. For example, key bacteria, such as *Faecalibacterium prausnitzii*, *Roseburia intestinalis*, *Prevotella* spp., break down dietary fibres into SCFAs such as acetate, propionate and butyrate [12, 13]. These acids serve as an energy source for intestinal epithelial cells, strengthen the mucosal barrier, and help maintain intestinal homeostasis [1]. Disruptions in the microbial composition have been associated with various diseases, including inflammatory bowel disease (IBD) and colon cancer, where butyrate-producing bacteria have previously been shown to be significantly reduced [14]. Through the production of neurotransmitters, SCFAs, and other bioactive compounds, the microbiota communicates with the central nervous system via the gut-brain axis, influencing mood and cognition.

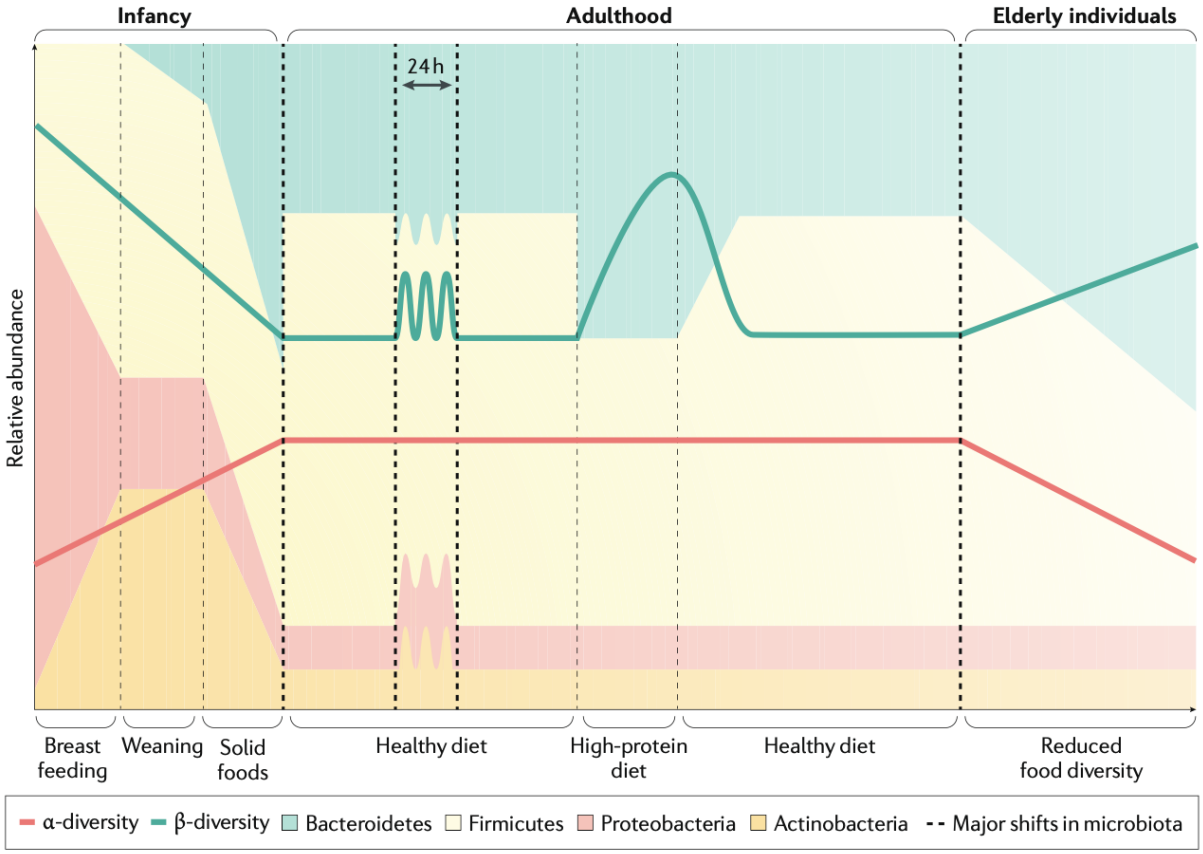

Figure 1.1. Conceptual presentation of changes in bacterial community structure over lifespan with the focus on diet impact on the microbiota composition. Green and red lines present β and α diversity respectively, and the colours of the background present the dominance of specific taxa in each phase. Reproduced from Zmora, *et al.*, 2019 [15].

As the collective genetic and functional content of the microbiota, the microbiome [16] plays a pivotal role in health by modulating digestion, metabolism and immune responses, as well as contributing to neurological function; it also underlies key processes such as vitamin production and insulin-mediated glucose regulation [1]. Given the critical role of the microbiome,

maintaining its health is essential for physical and mental well-being [1, 4]. However, the state of imbalance where the relative abundance of beneficial microbes is reduced and pathogenic or opportunistic microorganisms proliferate is termed dysbiosis. It is closely related to a variety of health conditions, particularly those involving chronic inflammation and immune system dysfunction [5].

1.2 Introduction to Inflammatory Bowel Disease

IBD is a chronic disease characterised by recurrent inflammation of the GI tract, often associated with dysbiosis and multiple environmental triggers. Management typically requires ongoing pharmacological intervention, and in severe cases, surgical treatment may be necessary [10]. The primary symptoms of IBD include abdominal pain, diarrhoea, rectal bleeding, and weight loss. IBD encompasses two main subtypes: ulcerative colitis (UC) and Crohn's disease (CD), which differ in terms of severity and location within the GI tract. CD is marked by transmural inflammation that can occur anywhere in the GI tract, while UC is characterised by restricted mucosal inflammation in the colon and rectum [17]. In IBD, dysbiosis is believed to contribute to the initiation and progression of inflammation in the intestine. In addition, dysbiosis may lead to impaired immune responses, increasing susceptibility to infections [1].

Urbanisation and modernisation of life have introduced factors that were previously absent, such as a diet consisting of highly processed foods, smoking, and exposure to pollutants. These elements have been shown to alter the balance of the microbiota, which seem to be correlated with an increase in the incidence of IBD [7]. The use of antibiotics is particularly impactful as these medications indiscriminately eliminate both beneficial and harmful bacteria. This alteration can have long-term consequences, including increased susceptibility to opportunistic infections and reduced microbial resilience [18]. The establishment of a healthy gut microbiota can be severely impacted by antibiotic exposure. Researchers suggest an association between such disturbance and a higher incidence of IBD in adulthood [19]. Similarly, environmental pollutants, including heavy metals and chemical contaminants, have been shown to negatively impact intestinal microbial balance, further contributing to dysbiosis and its associated health risks [5]. Another hypothesis suggests that reduced exposure to microbes in early life, due to high hygiene standards, may alter immune system development, increasing susceptibility to IBD and other diseases later in life [20]. Despite extensive research, the precise aetiology of IBD remains unclear, which poses challenges in identifying optimal treatment strategies [19].

The global burden continues to rise, particularly in countries undergoing rapid urbanisation and those that are already highly industrialised. Although the absolute number of cases is increasing, recent analyses show a declining trend in the global age-standardised prevalence rate, likely due to demographic changes such as population growth and changes in age distribution [21, 22, 23]. In particular, the most pronounced increase in prevalence has been observed in East Asia, a region undergoing significant socio-economic transformation [24, 23]. This trend supports the hypothesis that industrialisation and westernisation of the lifestyle are key drivers of the increasing incidence of IBD [7]. Improvements in diagnostic infrastructure, such as increased accessibility to endoscopy and the implementation of electronic documentation using International Classification of Diseases (ICD) systems, have contributed to the observed increase in reported cases [23].

Due to the complex interplay of contributing factors, accurately modelling the risk of IBD remains a challenge [25]. However, an association has been established between certain genetic variants and a higher risk of developing the disease [19]. Genome-wide association studies (GWAS) have identified more than 200 genetic loci associated with IBD, with almost half also linked to other immune-related diseases. Many of these genes are involved in key immune defence mechanisms, mucosal barrier integrity, autophagy, epithelial repair, microbial defence, and adaptive immunity regulation [7]. The probability of developing IBD increases significantly in individuals with a first-degree relative affected by the disease [7, 17]. Furthermore, certain ethnic groups, such as Ashkenazi Jews, exhibit a three to five times higher susceptibility [7], which is hypothesised to be related to genetic variants involved in bacterial recognition and immune responses, including *NOD2, IRGM, ATG16L1, CARD9* and *FUT2* [25]. The strongest genetic associations with IBD, such as mutations in *NOD2* and *ATG16L1*, have been observed primarily in individuals of European descent, while Asian populations exhibit distinct *NOD2* mutations and no significant association with *ATG16L1*, suggesting population-specific genetic risk factors for IBD [26]. Therefore, genetic predispositions have been hypothesised to contribute to the development of a more pro-inflammatory gut microbiota, which ultimately leads to altered immune responses and increased susceptibility to IBD.

Although exact aetiology is unknown, advances in high-throughput genomic sequencing illuminate predispositions in host genetics and their influence on the gut microbiome and its metabolic phenotype. Twin studies have shown that only 1.8 and 8.1% of bacterial taxa are heritable, whereas around 20% are related to environmental factors [3]. IBD is understood to be the result of a complex interaction of genetic predisposition, environmental influences, and dysregulation of the immune system (Figure 1.2) [20, 27]. The microbiomes of subjects with IBD fluctuate more than those of healthy individuals, with the greatest deviations observed in individuals with surgical resection [10]. Patients diagnosed with UC often present overgrowth of *Fusobacteria*, particularly species like *Fusobacterium nucleatum* and *Fusobacterium varium* [28, 29]. These species are believed to contribute to the inflammatory processes that drive disease progression. In addition, a reduction in protective genera known as SCFAs producers, such as *Bacterioides* [30], as well as *Faecalibacterium* and *Roseburia* is commonly observed [28, 31]. This aligns with the findings that faecal SCFA concentrations are reduced in patients with UC [28]. They are involved in various processes, including activation of the *NOD*-like receptor family,

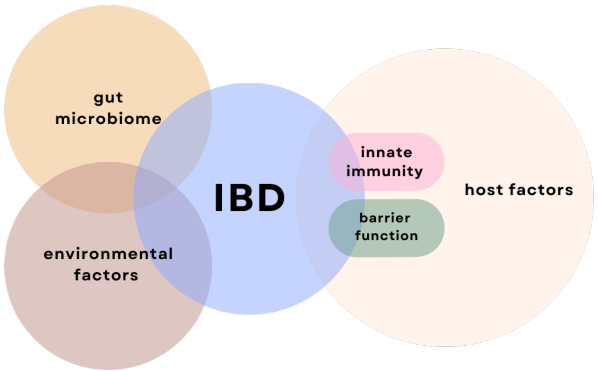

Figure 1.2. Visual presentation of all factors influencing pathogenesis of inflammatory bowel disease. Figure based on Hold *et al.*, 2014 [27].

induction of ion efflux pumps, epithelial repair, and the development of B cells, all of which are important in intestinal immunity [7, 32, 33]. SCFAs influence immune responses by activating G-protein receptors and inhibiting histone deacetylases (HDACs), thus promoting interleukin (IL) 22 production and suppressing inflammation [28]. Higher fibre intake - and the resulting increase in SCFAs levels - has been suggested to reduce the risk of CD; however, no such association has been established for UC[7].

1.2.1 The Assessment of Ulcerative Colitis

The diagnosis of UC typically begins with a sigmoidoscopy to examine the distal colon. If UC is suspected, an ileocolonoscopy is often performed to assess the full extent and severity of the disease. A histological evaluation is also essential for a definitive diagnosis. Clinicians commonly use the Mayo score to classify disease severity, as it integrates both clinical and endoscopic findings. This scoring system is simple and widely used in clinical practice. The specific criteria are presented in Table 1.1 [34].

Table 1.1. Mayo Score Assessment as described in Lamb *et al.*, 2019 [34]

Mayo Index	**0**	**1**	**2**	**3**
Stool Frequency	Normal	1–2/day more than normal	3–4/day more than normal	≥5/day more than normal
Rectal Bleeding	None	Streaks of blood with stool <50% of the time	Obvious blood with stool most of the time	Blood passed without stool
Mucosa (Endoscopic Subscore)	Normal or inactive disease	Mild disease (erythema, decreased vascular pattern, mild friability)	Moderate disease (marked erythema, lack of vascular pattern, friability, erosions)	Severe disease (spontaneous bleeding, ulceration)
Physician's Global Assessment	Normal	Mild disease	Moderate disease	Severe disease

1.2.2 Current Ulcerative Colitis Treatment Strategies

Due to the intricate interplay of genetic, environmental, and immune factors in the pathogenesis of IBD, choosing an appropriate treatment regimen remains a challenge [28]. A standard medical approach for the treatment of moderate to severe UC, as well as for the maintenance of remission, involves the use of compounds that affect the immune response. One of the most commonly used is 5-aminosalicylic acid (5-ASA). The dosage of 5-ASA typically ranges from 2.4 to 4.8 g/day, depending on the severity of the disease [34]. Although the exact mechanism of action of 5-ASA remains unclear, it is known to modulate the immune response by inhibiting pro-inflammatory cytokines and reducing oxidative stress [35, 36]. Other therapeutic

strategies include corticosteroids, such as prednisolone and budesonide, which suppress the inflammatory response and are commonly used to induce remission. To maintain steroid-free remission, thiopurines such as azathioprine (AZA) and mercaptopurine have been widely used [37]. Biological agents, such as anti-tumour necrosis factor (TNF) therapies, including infliximab and adalimumab, target TNF-α, a key cytokine in the inflammatory cascade [38]. Vedolizumab, a monoclonal antibody that targets the $\alpha 4\beta 7$ integrin, works by blocking the migration of leukocytes to the intestinal mucosa, offering a selective intestinal mechanism that can contribute to its favourable long-term safety profile [39]. Another biological agent, ustekinumab, targets the p40 subunit shared by IL-12 and IL-23, thus modulating the immune response in patients with UC [40].

Beyond conventional pharmacological treatments, emerging therapeutic strategies aim to modulate the intestinal microbiota to enhance clinical outcomes. These include the use of probiotics, prebiotics, and synbiotics, all of which are being explored for their potential to restore microbial balance and support disease management. Probiotics consist of beneficial microorganisms that help restore the balance of the gut microbiota by competing with pathogenic microbes and enhancing immune function. They contribute through mechanisms such as SCFAs production, and modulation of inflammatory pathways [41, 42, 43]. Common bacteria used in UC treatment are *Lactobacillus* spp., *Bifidobacterium* spp., *Sacchromyces bouladrii*, *E. coli* Nissle 1917 [44, 43]. Probiotics have shown to be effective in the treatment of postoperative pouchitis in UC patients [45]. Prebiotics, composed mainly of oligosaccharides, serve as fermentable substrates, leading to the production of SCFAs. By selectively stimulating the growth and activity of probiotics, prebiotics further reinforce their beneficial effects [27]. Synbiotics, which combine probiotics and prebiotics, have shown superior efficacy compared to either component alone in supporting intestinal health and modulate immune responses [42]. Although probiotics are more effective in maintaining remission than inducing it, synbiotics and prebiotic-based dietary interventions can offer additional therapeutic benefits [28].

Faecal microbiota transplantation (FMT) is a therapeutic approach in which the stool of a healthy donor is transferred to a recipient with the aim of restoring a balanced intestinal microbiota. Unlike probiotics, which contain a limited number of bacterial strains, FMT introduces a more diverse microbial community, closely resembling that of a healthy gut. In addition, it is an established treatment for *Clostridioides difficile* infection [27]. It has the potential to maintain remission and prevent relapses in UC. However, despite its promise, FMT remains controversial, as concerns remain regarding possible adverse effects and long-term safety. More research is needed to determine its efficacy and safety profile in the management of UC [28].

In contrast to probiotics or FMT, which aim to restore microbial balance by increasing beneficial strains, antibiotics focus on reducing the overall microbial diversity, which can help control inflammation and improve clinical outcomes [28]. However, this approach remains problematic, as antibiotics not only reduce harmful bacteria but also deplete beneficial microbial populations, potentially disrupting intestinal homeostasis [27]. It has been suggested that such as metronidazole, rifaximin, and ciprofloxacin may provide therapeutic benefits to patients with IBD [27]. However, the benefits are not significant compared to conventional therapies [46].

1.3 Antibiotic Resistance

The discovery of antibiotics marked a turning point in medical history, allowing the effective treatment of previously fatal bacterial infections. As a result, global morbidity and mortality have decreased significantly and average life expectancy has increased [47]. Antibiotics may be naturally derived from microorganisms, semi-synthetic, or fully synthetic compounds [48]. With advances in pharmaceutical production, antibiotics have become widely accessible and economically viable. However, their extensive and, at times, indiscriminate use has contributed to the emergence and proliferation of antibiotic resistance genes (ARGs) in bacteria [49, 50]. Unlike many chemical compounds that degrade over time, ARGs can persist in the environment both within bacterial genomes and as extracellular DNA, facilitating their horizontal transfer and long-term survival [49]. Recognising its critical implications, the World Health Organisation has classified antibiotic resistance as one of the top three global public health threats of the 21st century [51]. An overview of commonly used antibiotic classes, their molecular targets, and associated resistance mechanisms is provided in Table 1.2, adapted from Morar and Wright [52].

Table 1.2. Modes of action and resistance mechanisms of commonly used antibiotics adapted from Morar and Wright, 2010 [52]

Antibiotic class	**Example(s)**	**Target**	**Mode(s) of resistance**
β-Lactams	Penicillins (ampicillin), cephalosporins (cephamycin), penems (meropenem), monobactams (aztreonam)	Peptidoglycan biosynthesis	Hydrolysis, efflux, altered target
Aminoglycosides	Gentamicin, streptomycin, spectinomycin	Translation	Phosphorylation, acetylation, nucleotidylation, efflux, altered target
Glycopeptides	Vancomycin, teicoplanin	Peptidoglycan biosynthesis	Reprogramming peptidoglycan biosynthesis
Tetracyclines		Translation	Monooxygenation, efflux, altered target
Macrolides		Translation	Hydrolysis, glycosylation, phosphorylation, efflux, altered target
Lincosamides	Clindamycin	Translation	Nucleotidylation, efflux, altered target
Streptogramins	Synercid	Translation	C-O lyase (type B streptogramins), acetylation (type A streptogramins), efflux, altered target

Antibiotic class	**Example(s)**	**Target**	**Mode(s) of resistance**
Oxazolidinones	Linezolid	Translation	Efflux, altered target
Phenicols	Chloramphenicol	Translation	Acetylation, efflux, altered target
Quinolones	Ciprofloxacin	DNA replication	Acetylation, efflux, altered target
Pyrimidines	Trimethoprim	C metabolism	Efflux, altered target
Sulfonamides	Sulfamethoxazole	C metabolism	Efflux, altered target
Rifamycins	Rifampin	Transcription	ADP-ribosylation, efflux, altered target
Lipopeptides	Daptomycin	Cell membrane	Altered target
Cationic peptides	Colistin	Cell membrane	Altered target, efflux

Antibiotic resistance is a naturally occurring phenomenon rooted in the evolutionary arms race between microorganisms; many bacteria produce antimicrobial compounds, and others have developed intrinsic mechanisms to resist them as a form of self-defence [53]. Intrinsic resistance, which is universally present within certain bacterial species and is not acquired through horizontal gene transfer (HGT), typically involves mechanisms such as (i) limited drug uptake, especially in Gram-negative bacteria, (ii) drug inactivation, and (iii) efflux pump activity [54, 55]. In contrast, acquired resistance arises from genetic mutations or the uptake of external genetic material through HGT mechanisms that include transformation, conjugation, and transduction. Among these, plasmid-mediated gene transfer is the most common, although naturally competent bacteria such as *Acinetobacter* spp. can directly incorporate environmental DNA [53]. Acquired antibiotic resistance in bacteria arises through the activation or acquisition of specific mechanisms that interfere with the antimicrobial's ability to function. The three primary mechanisms involved include: (i) modification of the drug target, (ii) enzymatic inactivation of the drug, and (iii) the activity of efflux pumps. The molecular mechanisms behind antibiotic resistance are presented in Figure 1.3.

Target modification may occur through alterations to cell wall structures, which can hinder antibiotic penetration, or through mutations in intracellular targets such as the ribosome. Resistance to antibiotics targeting ribosomal subunits often involves ribosomal mutations, methylation, or ribosome protection mechanisms [55]. For example, resistance to tetracycline is mediated by genes that encode GTPase proteins capable of displacing the antibiotic from the ribosome using energy derived from GTP hydrolysis [57]. In other cases, resistance may result from the modification of key metabolic enzymes, thereby inhibiting antibiotic efficacy through altered metabolic pathways [55].

Drug inactivation is another widespread mechanism and can be achieved through either degradation or chemical modification of the antibiotic. Hydrolysis, such as the action of β-lactamases on β-lactam antibiotics, leads to irreversible drug inactivation [58, 59]. Alternatively, antibiotics can be rendered ineffective by the transfer of chemical groups (e.g., acetylation,

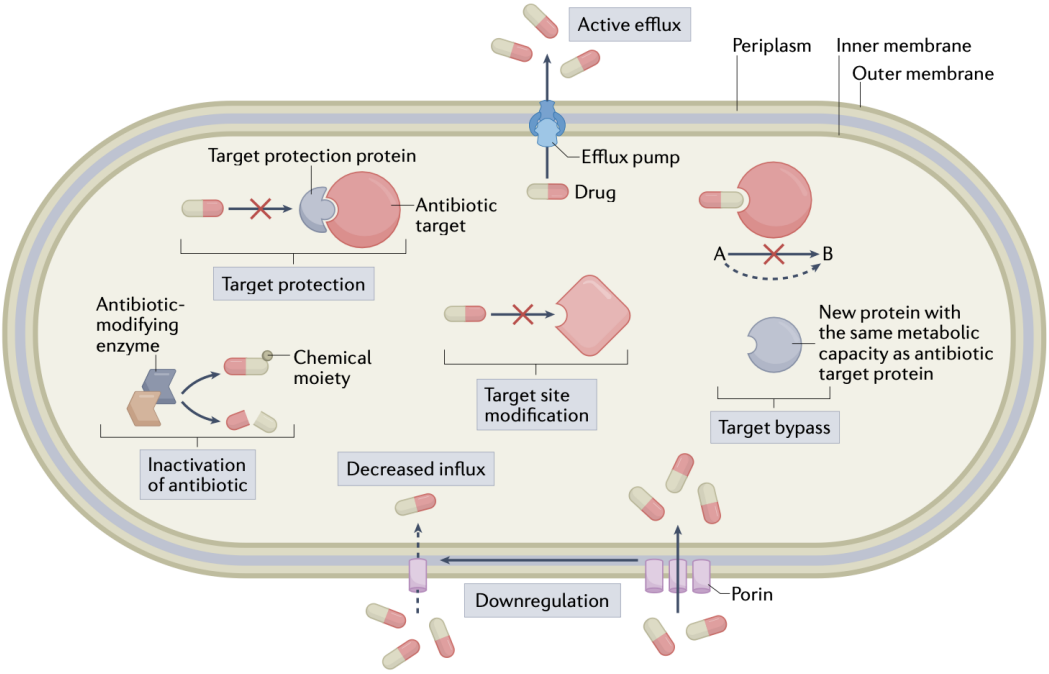

Figure 1.3. Overview of antibiotic resistance mechanisms. Reproduced from Darby *et al.*, 2023 [56].

phosphorylation, or adenylation), which alters their structure and prevents them from interacting with their targets [59].

Efflux pumps, encoded by chromosomal genes, constitute a major mechanism by which bacteria expel toxic compounds, including antibiotics. Although some of these pumps are constitutively expressed, others are inducible or overexpressed in response to environmental stimuli or the presence of specific substrates. The level of resistance they confer can also be influenced by the available carbon sources. Multidrug efflux pumps (MDR) are capable of transporting structurally diverse compounds and are significant contributors to broad-spectrum resistance. There are five main families of efflux pumps in bacteria, classified based on their structure and energy source: the ATP-binding cassette (ABC) family, the multidrug and toxic compound extrusion (MATE) family, the small multidrug resistance (SMR) family, the major facilitator superfamily (MFS), and the resistance-nodulation-cell division (RND) family [60].

It is important to note that the presence of ARGs does not necessarily imply their active expression. Many ARGs may remain transcriptionally silent under normal conditions and are expressed only in response to specific environmental cues, such as exposure to antibiotics. This latent reservoir of resistance potential poses a unique challenge, as bacterial populations can appear susceptible until activated by selective pressure. Moreover, recent research suggests that gene expression in bacteria may also be influenced by epigenetic mechanisms, such as DNA methylation or RNA modifications, which can regulate ARG expression without altering the underlying DNA sequence [49]. The selective pressure exerted by the widespread use of antimicrobials exacerbates this problem. Even sub-inhibitory concentrations of antibiotics can promote the emergence of resistant strains, enhance mutagenesis, and increase the mobilisation of resistance genes [55]. Anthropogenic activities contribute significantly to this burden, as antibiotics and resistant bacteria are introduced into the environment primarily through hospital effluents, agricultural runoff, and animal farming waste [61].

1.3.1 Methods of Detecting Antimicrobial Resistance

The emerging threat of antimicrobial resistance requires the development of effective tools for the detection of ARGs. This challenge can be addressed using both classical and advanced molecular techniques [62]. Traditional culture-based methods remain valuable, particularly due to their ability to determine the minimum inhibitory concentration (MIC) of antibiotics and to facilitate the identification of specific pathogens [63]. However, these methods are labour-intensive, time-consuming, and constrained by the fact that many microorganisms are difficult or impossible to culture under standard laboratory conditions [62]. To address these limitations, molecular approaches such as polymerase chain reaction (PCR) have become increasingly prominent [64]. PCR allows for the rapid and sensitive detection of ARGs directly from extracted DNA, including that of non-culturable organisms. The application of multiplex PCR further enhances efficiency by enabling the simultaneous detection of multiple ARGs within a single assay. Quantitative PCR (qPCR) extends these capabilities by quantifying ARG abundance by normalising to reference genes of known copy number, providing insight not only into the presence but also in the concentration of ARGs in a sample. However, PCR-based methods do not provide MIC values and are generally not suitable for comprehensive pathogen identification [62]. Although metagenomic approaches are more costly, they offer broader and more comprehensive detection [65]. This method is particularly valuable given the continuous emergence and dissemination of novel resistance determinants that may not be captured by conventional assays. An accurate assessment of the ARG burden requires the consideration of both known and potentially uncharacterised genes. As Bengtsson-Palme emphasises, the strategic selection of representative genes can provide a predictive overview of the abundance and diversity of ARGs within the environment tested [66]. In addition, several emerging technologies are being explored for their potential in antimicrobial resistance surveillance, including matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS), lateral flow immunoassays, and CRISPR-based diagnostics. Although these tools are promising, their wider implementation remains hindered by high costs, technical complexity, and the need for specialised personnel and infrastructure [62, 67].

Study Objective 2

The objective of this study is to characterise the composition of the gut microbiota and to analyse the occurrence of antibiotic resistance genes to examine differences in patients diagnosed with ulcerative colitis. The analysis aims to identify variations based on the severity of the disease, providing insight into microbial profiles and antibiotic resistance patterns in relation to disease progression. It is hypothesised that the severity of ulcerative colitis is associated with the structure of the bacterial community in terms of diversity and taxonomy compared to healthy participants. Previous studies have suggested a correlation between the presence of ARGs in the gut microbiota and the reduced microbial diversity, increasing susceptibility to disease. The selective pressure induced by antibiotic use often results in decreased microbial diversity and overgrowth of opportunistic bacteria, potentially disrupting immune responses and exacerbating disease severity. To explore these associations, sequencing of the highly conserved bacterial 16S rRNA gene was used, as it enables taxonomic identification of a wide range of bacterial species. Subsequently, these data will be used for bioinformatic analysis to investigate the structure and diversity of the community, as well as to explore the correlations between bacterial abundance and the presence of ARGs detected by a highly sensitive technique to investigate antimicrobial resistance genes, qPCR.

Materials and Methods 3

3.1 Overview of the Experiment

This study was approved by the North Denmark Region Committee on Health Research Ethics and constitutes a cohort of 34 patients diagnosed with ulcerative colitis (Table A.1) from Aalborg University Hospital recruited between February 22, 2022, and March 31, 2024. Subjects had to meet the following criteria: (1) above 18 years of age; (2) diagnosed with UC; and (3) referred to a status sigmoidoscopy due to symptoms consistent with the UC flare-up. Exclusion criteria included the presence of at least one of the following conditions: (1) history of colectomy; (2) symptoms attributable to causes other than ulcerative colitis; (3) administration of corticosteroids locally to the rectum and/or distal colon; (4) use of antibiotics within six months prior to enrolment; and (5) bacterial infection within three months before enrolment. These criteria were implemented to minimise confounding factors that could alter the composition of the gut microbiota, ensuring that the study population more accurately reflects a representative cohort of UC.

The physician assessed the Mayo score based on symptoms that determined the severity of the disease. Patients received faecal collection tubes by mail and were instructed to immediately freeze the samples at -20°C for microbiome investigation. Subsequently, upon receiving, the samples were placed at -80°C until further processing.

The HC group was based on faecal samples collected from 16 healthy volunteers participating in a study by Nilsson *et al.* [68], and the data generated by Lærke Valsted Bak Nielsen in her Master's thesis project titled "Bacterial Community Composition and Functional Potential in Opioid-induced Gut Microbial Dysbiosis" in June 2024 under the supervision of Jeppe Lund Nielsen and Stine Karstenskov Østergaard.

A total of 50 samples, comprising 34 UC and 16 HC, were used for DNA extraction, followed by sequencing of the 16S rRNA bacterial gene amplicon to facilitate microbial profiling. The resulting data enabled bioinformatic analysis of the structure of the microbial community, focussing on discovering differences between the tested groups.

In parallel, a subset of 24 samples was selected for qPCR analysis to assess the presence and abundance of specific ARGs. To optimise material use and obtain proportional groups, 12 DNA extracts from healthy patients and 12 extracts from patients diagnosed with UC were chosen. The UC group was further divided into three groups, each constituted of four patients representing Mayo scores 1, 2 and 3, respectively (Table A.1). The data were then used to compare the prevalence of resistance genes between healthy individuals and UC patients, offering information on potential correlations between microbial dysbiosis and antimicrobial resistance.

The experimental workflow is presented in Figure 3.1.

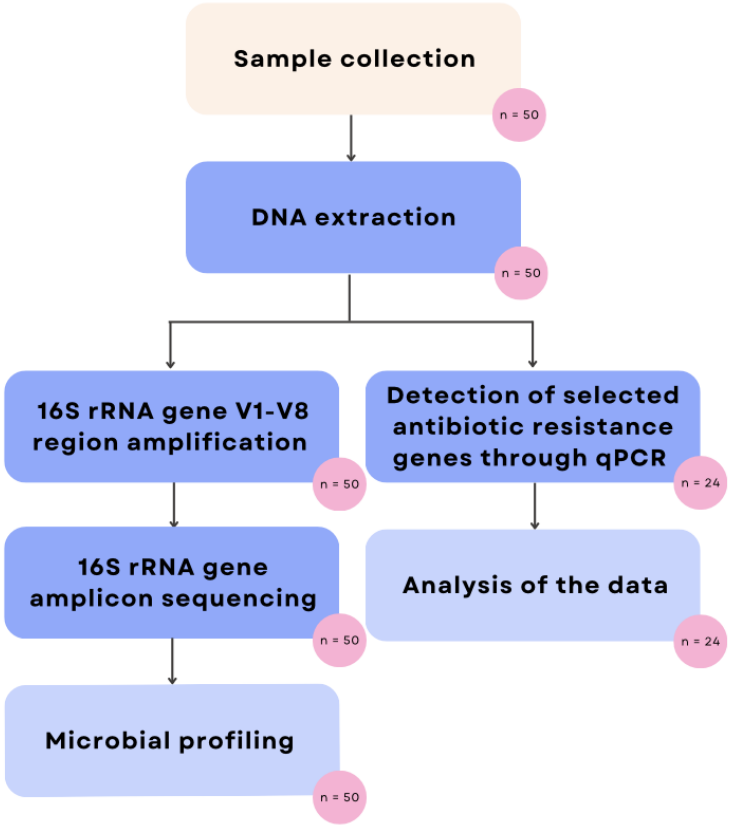

Figure 3.1. Schematic representation of the workflow. 50 faecal samples were processed for DNA extraction, 16S rRNA gene amplification and sequencing for further bacterial community structure analysis. Simultaneously, 12 control samples from healthy patients and 12 samples from patients diagnosed with UC based on severity score were chosen for qPCR to investigate the presence of selected ARGs.

3.2 Total Genomic DNA Extraction

Total genomic DNA was extracted and purified using the DNeasy® PowerLyzer® PowerSoil® Kit (100) (Qiagen) according to the producer's protocol [69] with slight modifications. Initially, approximately 200 mg of faecal sample was transferred to PowerBead tubes with 0.1 mm glass beads and 750 μL of PowerBead Solution was added. The homogenisation step was performed by bead-beating for 15 minutes in Vortex-Genie 2 (Scientific Industries). Further stages were performed exactly as stated in the protocol at room temperature and without the addition of incubation time. The elution was carried out with 100 μL of the C6 solution provided. DNA extracts were stored at -20°C for future use.

3.3 16S rRNA Bacterial Gene V1-V8 Regions Amplification and Sequencing

DNA concentrations were measured using the Qubit™1X dsDNA HS Assay Kit (ThermoFisher Scientific) in the infinite F200 PRO Fluorescence Top Reading mode of TECAN with Tecan i-control software 1.10.4.0 at 485 nm (Tecan Trading AG). The V1-V8 region of the bacterial 16S rRNA gene was amplified using 27F forward 5′ AGRGTTYGATYMTGGCTCAG 3′ [70] and 1392R reverse 5′ GACGGGCGGTGWGTRCA 3′ primers [71]. The PCR reaction (Thermal cycler, UNO96, VWR) was conducted in duplicates of 25 μL total reaction volume using PCRBIO 1x Ultra Mix (PCR BIOSYSTEMS), 400 nM of each primer, 10 ng of DNA template and run under conditions described in Table 3.1. The quality of the amplification process was assessed by the addition of a positive and negative control in the reaction.

Table 3.1. PCR reaction conditions.

Step	Temperature	Time	Number of cycles
Initial denaturation	95 ℃	2 minutes	1
Denaturation	95 ℃	15 seconds	30
Annealing	55 ℃	15 seconds	30
Elongation	72 ℃	90 seconds	30
Final elongation	72 ℃	5 minutes	1

The PCR products were purified using CleanNGS beads (CleanNA) in a 1:0.7 sample:bead ratio, and the DNA concentration was measured as previously described. Libraries were prepared with Ligation Sequencing Kit SQK-LSK114 with the PCR Barcoding Expansion EXP-PBC096 following the manufacturer's protocol (Oxford Nanopore Technologies). Purified PCR products were barcoded and pooled in equimolar concentration. The pooled library was end-prepped, adapter ligated, cleaned, and 8 fmol loaded onto the MinION R10.4.1 flow cell (Oxford Nanopore Technologies). The library was sequenced for 72 hours on a GridION device (Oxford Nanopore Technologies).

3.4 Pre-processing of the Amplicon Sequencing Data

The raw reads were basecalled and demultiplexed using Dorado v.0.5.0 with sup v.4.3.0 [72] with standard settings in MinKNOW software with the addition of required barcodes on both ends to ensure accurate assignment. The bioinformatic processing of the amplicons was based on the ONT-AmpSeq workflow [73]. The reads were visualised using Nanoplot v1.24.0 [74] and low-quality reads (Q-score $>$ 20) were filtered using Chopper [75] keeping more reliable reads $\pm$100 bp of the target amplicon. Polishing was performed using minimap2 [76] and Racon [77]. The reads were then clustered into operational taxonomic units (OTUs) (99% OTUs similarity threshold) and denoised using VSEARCH v.2.13.4 [78] using the UNOISE3 algorithm [79]. The OTUs were taxonomically classified using the Unified Human Gastrointestinal Genome (UHGG) collection [80].

3.5 Quantitative Analysis of Chosen Antibiotic Resistance Genes

ARGs were selected based on the prevalence in environmental samples, as Bengtsson-Palme et al. [66] and the primers used to target them are presented in Table 3.2. The qPCR was performed using the Brilliant III Ultra-Fast SYBR Green qRT-PCR Master Mix (Agilent Technologies) using 20 ng of template DNA per reaction according to the manufacturer's protocol [81]. Amplification was carried out on a Stratagen™ Mx3005P thermocycler (Agilent Technologies) using a two-step programme: initial denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C and elongation at 60°C. The time of denaturation and elongation was adjusted depending on the length of the gene tested. Consequently, the reaction products were purified with CleanNGS (CleanNA) in a 1:1 ratio. The DNA concentration obtained was measured as previously described along with the determination of the amplicon size on Agilent 4150 TapeStation (Agilent Technologies) and High Sensitivity D-1000 ScreenTape (Agilent Technologies). Standard curves were generated for each gene by serially diluting DNA standards from 10^8 to 10^1 gene copy number per μL, and were performed in triplicate. Each qPCR run consisted of standard dilution, no-template control (NTC) to detect potential contamination, and triplicates of selected samples. The abundance of genes was generated by plotting the cycle threshold (Ct) values against the logarithm of the known copy numbers of the standards. The abundance of genes was calculated using gene copy numbers normalised to the 16S rRNA gene copy number: $(\frac{\text{ARG copy number}}{\text{16S rRNA gene copy number}}) \times 4 \times 100$, where the factor 4 corresponds to the average number of 16S rRNA gene copies per prokaryote cell, as estimated in Stalder et al. [82].

Table 3.2. Summary of primer sequences and product lengths used for detecting of chosen genes.

Gene	**Primer**	**Sequence [$5' \rightarrow 3'$]**	**Length [bp]**	**Source**
16S rRNA	341F	CCTACGGGAGGCAGCAG	193	[83]
	518R	ATTACCGCGGCTGCTGG		
tet(W)	forward	GAGAGCCTGCTATATGCCAGC	168	[84]
	reverse	GGGCGTATCCACAATGTTAAC		
erm(B)	forward	GATACCGTTTACGAAATTGG	364	[85]
	reverse	GAATCGAGACTTGAGTGTGC		
blaTEM	forward	CACTATTCTCAGAATGACTTGGT	90	[86]
	reverse	TGCATAATTCTCTTACTGTCATG		
sul1	forward	TGTCGAACCTTCAAAAGCTG	113	[87]
	reverse	TGGACCCAGATCCTTTACAG		
aac(6′)-Ib	forward	TTGCGATGCTCTATGAGTGGCTA	482	[88]
	reverse	CTCGAATGCCTGGCGTGTTT		

3.6 Data Analysis and Visualisation

The analysis was conducted in R v. 4.3.1 through Rstudio v. 2024.09.1-394 [89]. The 16S rRNA gene data was analysed with ampvis2 v. 2.8.7 [90] and ggplot2 v. 3.5.0 [91] packages, which were used to make rarefaction curves, alpha and beta diversity plots, and heatmaps, along with the species richness analysis based on the number of observed OTUs. Beta diversity was examined with principal component analysis (PCA) and constrained analysis was assessed with the use of redundancy analysis (RDA). The Wilcoxon rank sum test was used to assess statistically significant differences between the two groups, as it is appropriate for small sample sizes. Statistical significance was determined by P-values ($P < 0.05$).

Results 4

4.1 The Structure of the Bacterial Community In Ulcerative Colitis

The microbiota of 34 patients diagnosed with UC and 16 HC was analysed by sequencing the near-full-length V1-V8 region of the bacterial 16S rRNA gene. The data analysis in this report is based on OTUs with a reading similarity threshold of 99% + 1. In general, sequencing resulted in a total of 330,658 reads with an average of 7035 reads per sample. Rarefaction curves were generated to assess whether an adequate sequencing depth was reached to reflect the richness (Figure A.1). Figure A.1 implies that there were visible differences in the depth of the sequencing between samples. Thus, the number of observed OTUs fluctuated widely, where the lowest value was 1,117 and the highest was 17,673 OTUs per individual. To standardise sequencing depth the data was rarefied to 10,000 reads per sample.

4.1.1 Alpha Diversity

Microbial diversity was assessed in the tested groups (Table A.1) by calculating alpha diversity. The number of observed OTUs varied notably between samples (Figure 4.1). However, no significant differences were observed when comparing HC with all UC patients and when patients were grouped based on disease state or medication intake ($P > 0.05$, data not shown). Furthermore, the Chao1 and Shannon diversity indices were used to investigate the species richness and evenness of the bacterial community. There were no significant differences between the tested groups (data not shown).

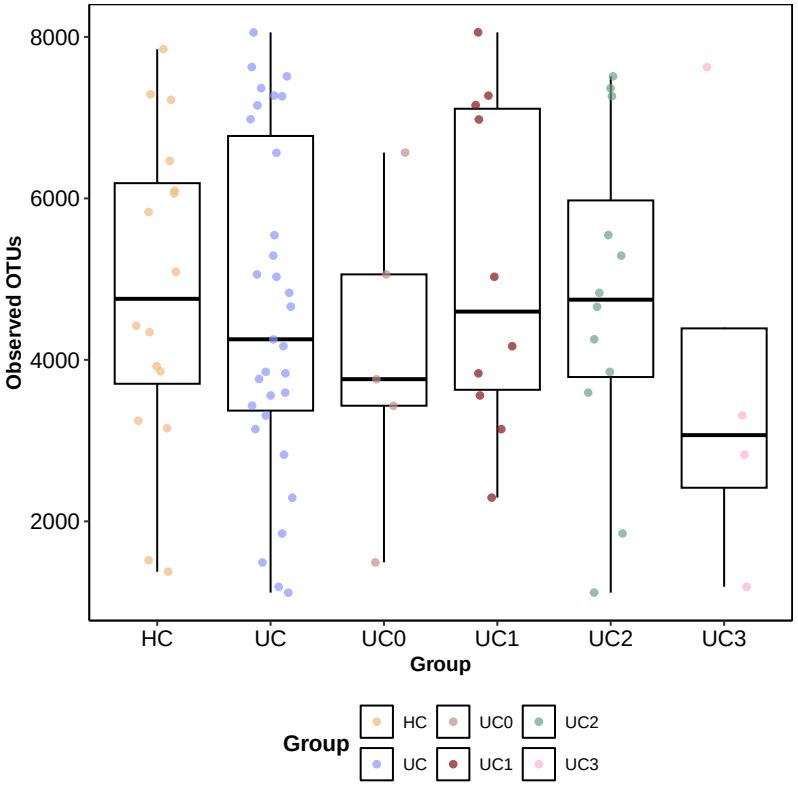

Figure 4.1. Observed richness for healthy control (HC) and all patients diagnosed with UC collectively (UC) as well as grouped by Mayo score (UC0 which represents non-inflamed patients and UC1, UC2, UC3 based on Mayo score 1, 2 and 3, respectively). There are no observable trends in richness among the presented groups.

4.1.2 Bacterial Community Composition

Heatmaps were constructed to investigate the structure of the bacterial community for each patient and the state of the disease. The seven predominant phyla across all patients were *Firmicutes* A (36.4% - 85.7%), *Bacteroidota* (0.9% - 49%), *Firmicutes* (0.9% - 18%), *Actinobacteriota* (0.1% - 11.1%), *Firmicutes* C (0% - 4.3%), *Proteobacteria* (0.1% - 3.2%) and *Fusobacteriota* (0% - 1%) (Figure A.2) proving that the relative abundance among samples varies significant. To examine whether there were differences between the HC and the UC group, the relative abundance of the top phyla was assessed and it was respectively *Firmicutes* A (64.4% vs 63.8%), *Bacteroidota* (15.5% vs 12.3%), *Firmicutes* (3.6% vs 5.6%), *Actinobacteriota* (3.1% vs 3.8%), *Firmicutes* C (1.1% vs 1.3%), *Proteobacteria* (0.6% vs 0.9%).

Heatmap clustered at genus level was made for each sample across HC and UC patients grouped by disease state (Figure A.3). The mean relative abundance of the top genera in samples from the healthy group were *Faecalibacterium* (10% ± 2.6%), *Blautia* A (3.6% ± 1.4%), *Bacteroides* (3.4% ± 2.35%), *Prevotella* (1.3% ± 1.5%), *Alistipes* (1.3% ± 1.2%), *Bifidobacterium* (1.2% ± 1.2%), *Phocaeicola* (1% ± 0.7%), *Anaerobutyricum* (0.9% ± 0.5%) and from all the UC patients combined were *Faecalibacterium* (7.9% ± 4.7%), *Blautia* A (5.1% ± 3.8%), *Bacteroides* (3.1% ± 3.4%), *Ruminococcus* E (1.5% ± 1.6%), *Bifidobacterium* (1.5% ± 1.3%), *Streptococcus* (1% ± 1%), *Prevotella* (1% ± 0.7%), *Alistipes* (0.9% ± 0.7%). The less abundant genera varied more among patients with UC compared to HC, where the less abundant genera appeared to be more consistent.

The structure of the bacterial community at the species level showed more details of the differences between the patients and the tested groups (Figure 4.2). The most abundant species across all samples was *Faecalibacterium prausnitzii* with mean relative abundance 4.8% ± 1.3% for HC and 3.6% ± 2.2% for all UC patients. HC patients 36 and 37 showed higher biodiversity compared to other healthy individuals. UC patients presented a higher abundance of *Ligilactobacillus ruminis* that belongs to *Lactobacillaceae* family within *Firmicutes* A phylum, *Blautia wexlerae* classified into the *Lachnospiraceae* family within *Firmicutes* A phylum, *Peptoniphilus harei* from *Peptoniphilaceae* family within *Firmicutes* A phylum and *Bulleidia moorei* from *Erysipelotrichaceae* family, *Firmicutes* phylum, specifically among patients with Mayo score above 1. Furthermore, patient 4 assigned to the UC2 group and patient 6 from UC3 had an notable abundance of *Parvimonas micra* classified to *Peptoniphilaceae* family enclosed in *Firmicutes* A phylum and *Anaerococcus senegalensis* from *Peptoniphilaceae* family within *Firmicutes* phylum. In addition, there was a correlation in the structure of the bacterial community when patients were grouped by medication intake (Appendix, Figure A.4).

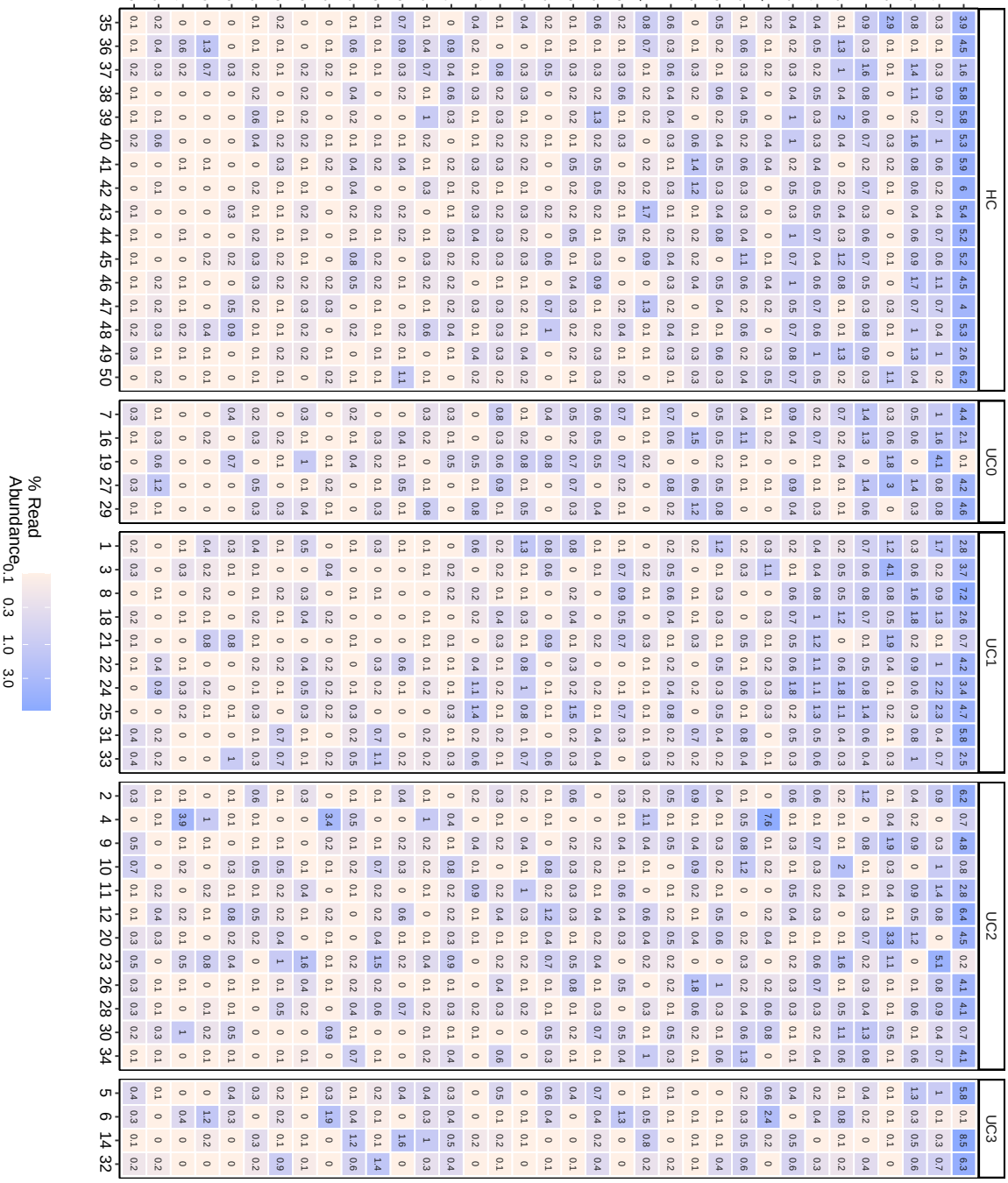

Figure 4.2. Relative abundance of the 35 most abundant species with a minimum read abundance of 0.1% in the faecal samples across all patients. The heatmap is grouped by participants and split to healthy controls (HC) and patients diagnosed with UC with numbers representing Mayo score describing state of the illness.

4.1.3 Differentially Abundant Bacteria

A differential plot was made to assess whether the structure of the bacterial community of patients with UC differed significantly compared to HC. Consequently, 16 healthy individuals and 16 patients with UC with moderate to severe symptoms (Mayo scores 2 and 3) were analysed. The mean relative abundance in the HC group is shown against the mean relative abundance difference between the UC group and the HC (Figure 4.3). In total, six different OTUs had a mean relative abundance difference $\geq 0.1\%$ (Figure A.5), although none varied significantly. However, the most pronounced difference was observed in OTU 79214 assigned to the *Bacterioides* genus.

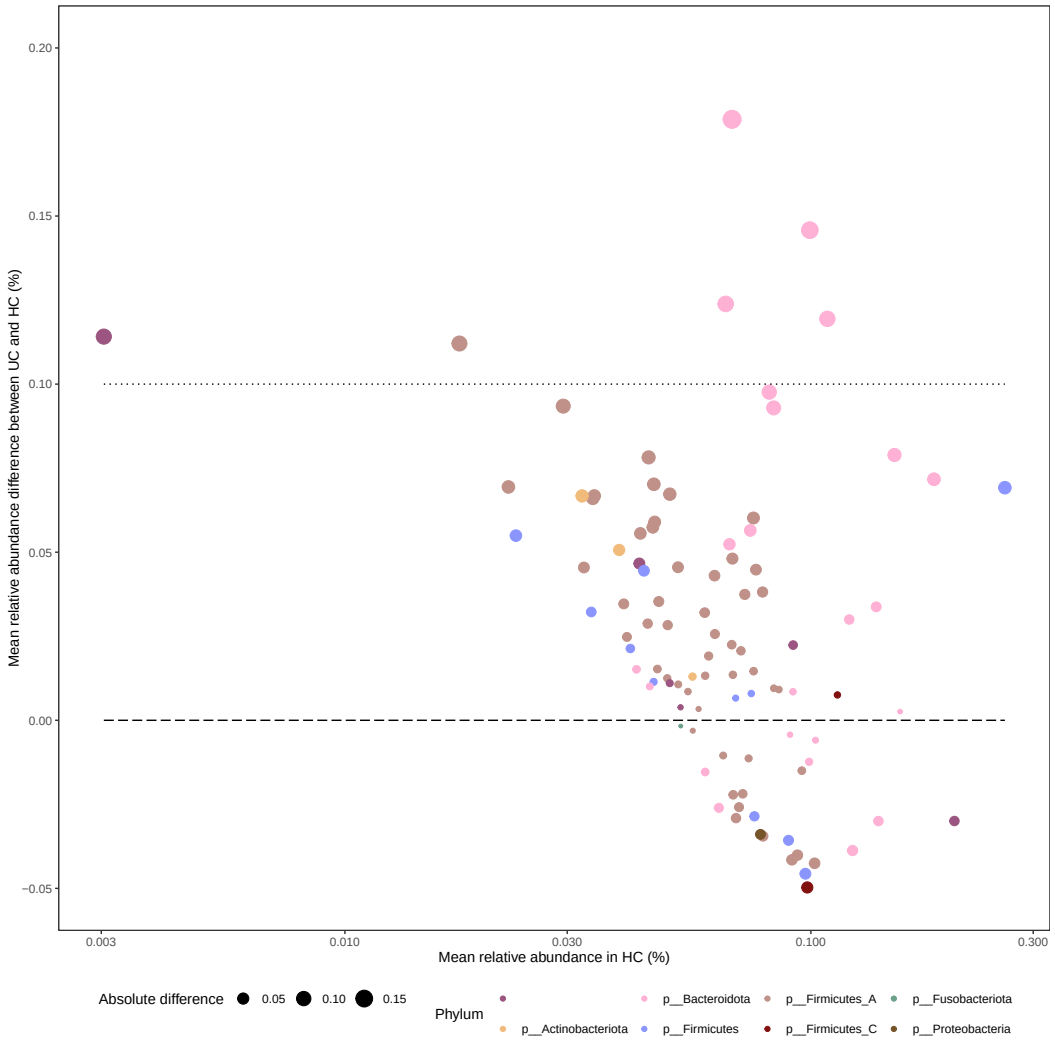

Figure 4.3. Differentially abundant bacteria in healthy control (HC) and moderate to severe ulcerative colitis (UC; Mayo score 2 and 3). The mean relative abundance of HC alongside with the mean relative abundance difference between UC and HC groups. Bacteria with a mean relative abundance difference $\geq 0.1\%$ were considered different between tested groups. The differential abundance plot was made for the 100 most abundant OTUs.

4.1.4 Ordination Analysis

Principal component analysis (PCA) was performed to visualise the variability of the microbial composition between HC and UC patients (Figure 4.4). OTUs with an abundance >0.1% underwent Hellinger transformation to normalise the data distribution. The PCA determined principal components where two main ones explained 8.3% and 6.3% of the variance, respectively. The samples of the HC group cluster closer together compared to the UC samples, which appear more dispersed, indicating greater variability in the community structure (Figure 4.4). In the UC group, the variation in the microbial composition was greater, although the overall microbial diversity was not significantly different. These results align with observations of the composition of the bacterial community presented earlier. In addition, differences were observed when grouping the samples according to the dosage of the medication (Figure A.6). UC patients receiving medication exhibited greater microbial variability than both untreated patients and HC. In addition, these patients were assigned a Mayo score of 2 or 3 more frequently.

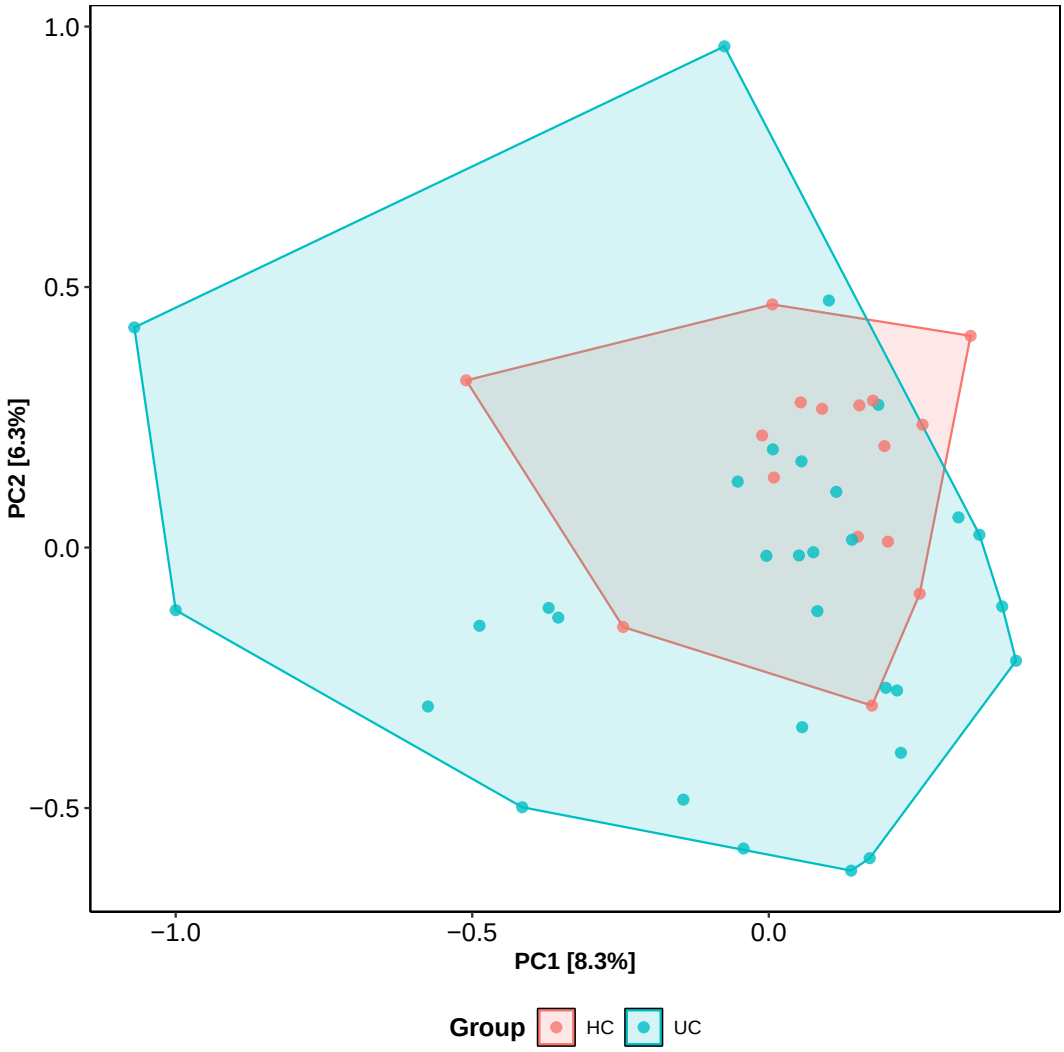

Figure 4.4. Principal Components Analysis (PCA) of 1199 OTUs and 47 samples coloured by healthy control (HC) and patients diagnosed with UC collectively (UC). Prior to the analysis, OTUs that are not present in more than 0.1 % relative abundance in any sample have been removed. The data has been transformed initially by applying the Hellinger transformation [92]. The relative contribution (eigenvalue) of each axis to the total inertia in the data is indicated in percent at the axis titles.

Redundancy analysis (RDA) was applied to OTUs with a relative abundance greater than 0.1% to examine the variation in microbial community composition constrained by disease state. The RDA plot (Figure 4.5) shows the clustering of sample groups, where 3.7% of the variance along the first component could be explained by the state of the disease. UC3 appeared to be the most distant along the x-axis, indicating greater dissimilarity. The clustering of HC, UC0, UC1, and UC2 relative to UC3 along the y-axis highlighted the greatest compositional difference. In addition, species points were added to link the variation between groups with the bacteria taxa at the species level. HC, UC0, UC1, and UC2 groups share a large proportion of species. *Faecalibacterium prausnitzii*; and members of the *Bacteroidaceae* and *Lachnospiraceae* familes were observed in the HC group. Species *Blautia wexlerae*, *Gemmiger formicilis* and members of the genus *Bifidobacterium* genus were shared among patients with UC0 and UC1. Bacteria classified to *Blautia* A, *Faecaliococcus* and *Ruminococcus* genera were found in group UC2.

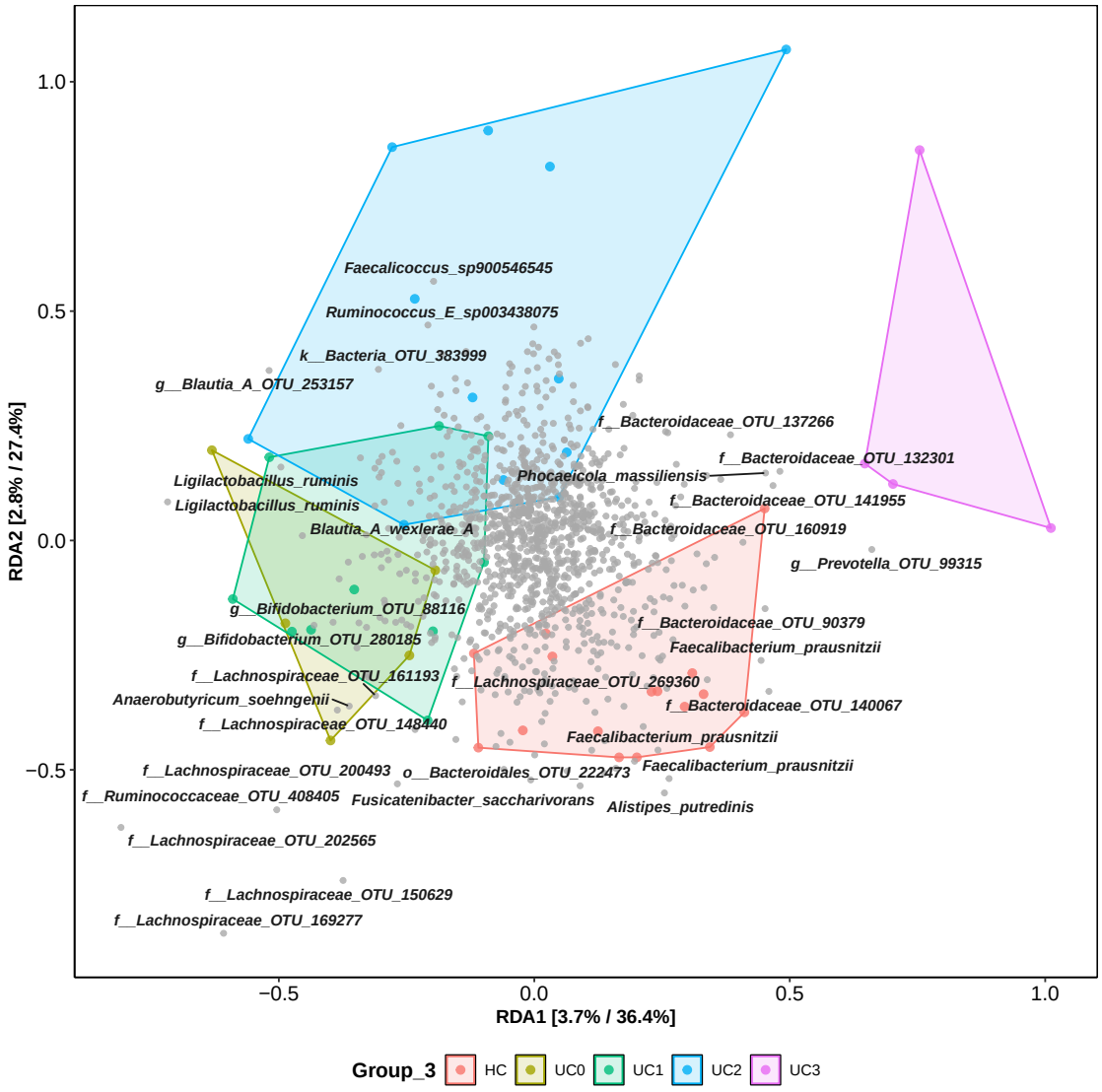

Figure 4.5. Redundancy Analysis (RDA) of 1199 OTUs and 47 samples constrained to the disease state variable. Prior to the analysis, OTUs that are not present in more than 0.1% relative abundance in any sample have been removed. The data has been transformed initially by applying the Hellinger transformation [92]. The relative contribution (eigenvalue) of each axis to the total inertia in the data as well as to the constrained space only, respectively, are indicated in percent at the axis titles.

4.2 Quantitative Analysis of Antibiotic Resistance Genes

The number of ARGs was determined by normalising the number of copies of the ARG to the estimated number of cells in each sample, calculated based on an average of 4 16S rRNA gene copies per cell, with the assumption that each ARG corresponds to one gene per cell (Table A.2). During the experimental process, a sample from HC was lost while testing the presence of tetW due to a mistake in laboratory handling. As a result, it was excluded from further analysis of this ARG. The mean number of ARG copies per 100 cells and the standard deviations of all tested ARGs are presented in Table 4.1.

Table 4.1. The mean gene copy number based on the ARGs copy number to the estimated number of cells in each sample, calculated based on an average of 4 16S rRNA gene copies per cell, with the assumption that each ARG corresponds to one gene per cell and standard deviation of ARGs per 100 cells across groups: healthy control (HC), all UC patients combined (UC) and same individuals grouped by Mayo score describing disease state (UC1, UC2, UC3 - representing respectively Mayo score 1, 2 and 3).

Group	**The mean number of gene copies ± standard deviation**			
	tetW	***ermB***	***blaTEM***	***sul1***
HC	7.2 ± 7.1	5.4 ± 6.0	0.02 ± 0.06	0.001 ± 0.005
UC	6.7 ± 4.8	7.1 ± 9.5	0.09 ± 0.2	0.01 ± 0.05
UC1	9.2 ± 6.5	13.9 ± 13.9	0.0004 ± 0.0008	0.000006 ± 0.000004
UC2	6.4 ± 4.3	4.3 ± 4.4	0.3 ± 0.3	0.04 ± 0.08
UC3	4.5 ± 2.8	3.2 ± 4.9	0.002 ± 0.002	0.0003 ± 0.0003

The mean copy number of ARG between HC was 7.22 ± 7.12 for *tetW*, 5.42 ± 6.06 for *ermB*, 0.02 ± 0.06 for *blaTEM*, and 0.001 ± 0.005 for *sul1*. Among UC samples, the highest mean was observed for *ermB* 7.1 ± 9.14, followed by *tetW* 6.7 ± 4.77, *blaTEM* 0.09 ± 0.2, and *sul1* 0.01 ± 0.05. The mean ARG copy numbers for both *tetW* and *ermB* were higher across all groups, surpassing those for other ARGs. UC1, which includes patients with Mayo score 1, had the highest, while the group of patients with Mayo score 3 had the lowest mean abundance of ARGs of both *tetW* and *ermB*. There were no significant differences between tested groups ($P > 0.05$, data not shown). However, some individuals exhibited a significantly higher abundance of ARGs compared to others (Figure 4.6). In HC, the individuals who collected the highest *tetW* copy number were: patient 42 (26), patient 46 (10.5), patient 37 (10.3) and patient 41 (9.7), while *ermB* was detected the most in patients 42 (22.4), 41 (10.5) and 46 (6.5). The genes *blaTEM* and *sul1* were negative in most of the HC samples, except for individuals 36 (0.2 *blaTEM)* and 46 (0.02 *sul1*). The highest number of copies of the *tetW* gene was detected within UC patients in samples from patients 24 (15.3) and 21 (14.4) assigned to the UC1 group, 10 (10.7) and 9 (7.4) from the UC2 group, as well as patients 6 (7.5) and 5 (6.1) from the UC3 group. Values above 10 for the *ermB* gene were observed in patients 24 (33.8) and 33 (12.3) of the UC1 group, 23 (10.7) of the UC2 and 32 (10.4) of UC3. Consequently, the genes *blaTEM* and *sul1* were detected only in two patients, both assigned to the UC2 group. Patient 4 displayed a value of 0.7 ARG copy number per 100 cells for the *blaTEM* gene and 0.2 for the *sul1* gene, while patient 10 had a copy number of 0.4 *blaTEM* gene per 100 cells. Details about each patient tested are presented in Table A.2.

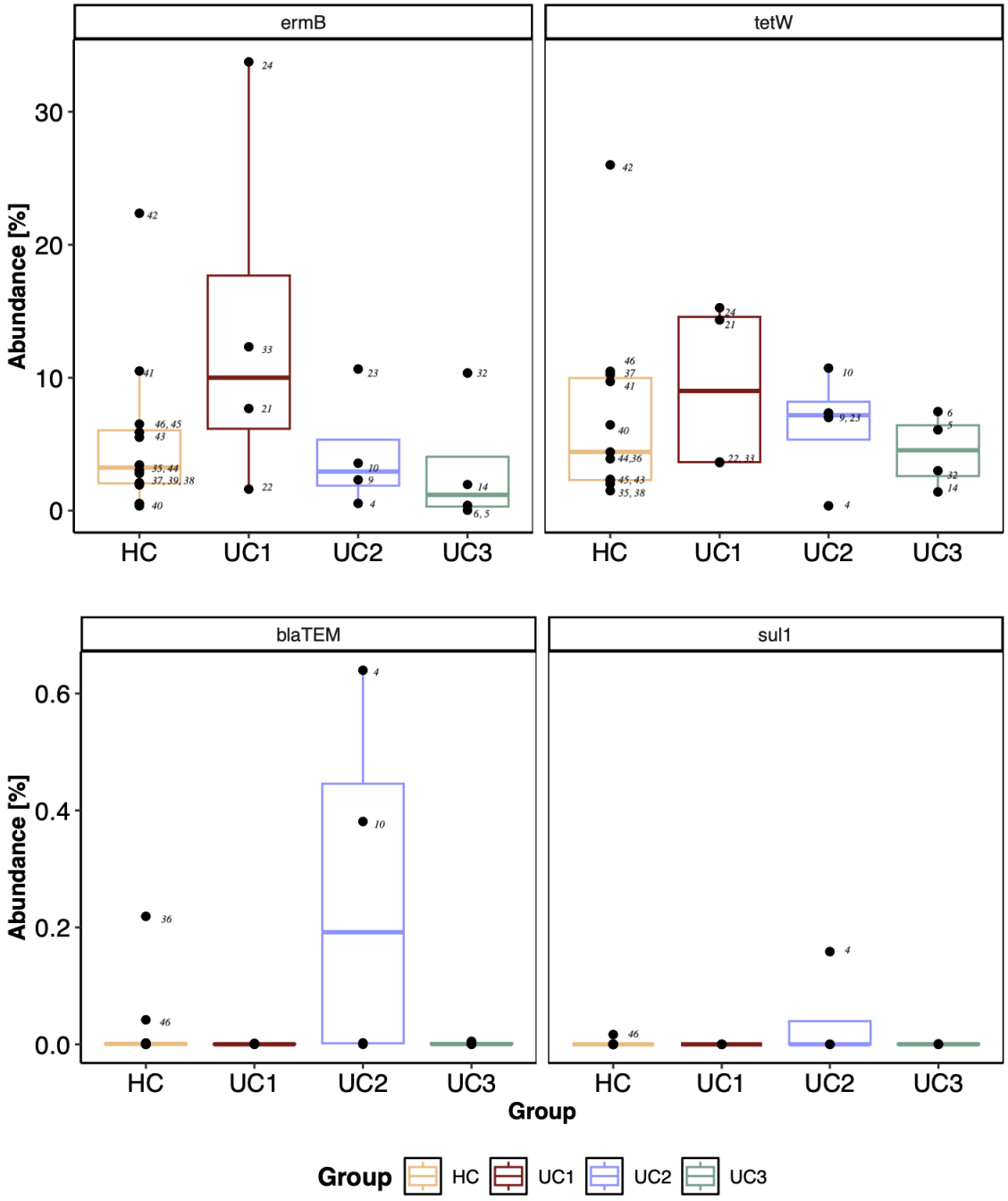

Figure 4.6. The abundance of each tested antibiotic resistance genes calculated based on the gene copy number of the ARG per 100 prokaryote cells based on the assumption of presence of 4 copies of bacterial 16S rRNA gene per one prokaryote cell. Patients are presented in four groups: healthy control (HC) consisting of 12 individuals, followed by 12 patients diagnosed with ulcerative colitis displayed based on disease state described by the Mayo score ranging from 1 to 3 (UC1, UC2, UC3). Each dot represents one patient.

Furthermore, the patients mentioned above showed variances in the analysis of the structure of the bacterial community, where seven of them presented a lower abundance of *Faecalibacterium prausnitzii*. However, only one HC matched this pattern. Within the HC group, there was observed an increase in relative abundance of *Gemmiger formicilis* in three samples carrying *tetW, ermB* and *sul1* ARGs; *Ruminococcus bicirculans* in two samples with both *tetW* and *ermB*; *Faecalibacillus intestinalis* in two samples with *tetW* and *blaTEM*; and *Phocaeicola coprocola* in two samples with higher gene copy number of *tetW* and *blaTEM*. Species observed in higher abundance only once among HC samples with detected ARGs were *Blautia wexlerae*, *Alistipes putredinis*, *Fusicatenibacter saccharivorans*, *Bacteroides uniformis*, *Anaerobutyricum hallii*, *Bulleidia moorei*, *Parabacteroides merdae*, while *tetW* was present in seven, *ermB* in four, *sul1* in three and *blaTEM* in one of eight cases.

Among UC patients, the presence of ARG could be related to the higher relative abundance of *Faecalibacillus intestinalis*, which was demonstrated in five samples with ARG *tetW, ermB and blaTEM*. Species *Blautia wexlerae*, *Gemmiger formicilis*, *Bacteroides uniformis*, *Holdemanella biformis*, *Ligilactobacillus ruminis* and *Parabacteroides merdae* were each observed three times in samples carrying *tetW, ermB, blaTEM*. *Ruminococcus bicirculans*, *Fusicatenibacter saccharivorans*, *Catenibacterium mitsuokai*, *Bulleidia moorei*, *Anaerobutyricum hallii*, *Parvimonas micra*, *Anaerostipes hadrus*, *Phocaeicola coprocola* and two species within the *Lachnospiraceae* family each presented higher abundance twice among samples carrying *tetW*, *blaTEM, ermB,* and *sul1* ARGs.

Discussion 5

5.1 Microbial Diversity in Ulcerative Colitis

Among various environmental and host-related factors, the composition and diversity of the intestinal microbiota have emerged key contributors to the pathogenesis of IBD [93]. A balanced microbial ecosystem is essential for maintaining mucosal immunity, intestinal barrier integrity, and protection against pathogens [19, 27, 33]. Dysbiosis has been consistently associated with the initiation and progression of IBD, including UC [94]. Maintaining microbial homeostasis is therefore crucial to ensure appropriate immune responses and prevent chronic gastrointestinal inflammation.

In this study, the gut microbiota composition of 34 patients diagnosed with UC was analysed using 16S rRNA bacterial gene amplicon sequencing. Notable variability in sequencing depth was observed across samples, with the number of observed OTUs ranging from 1,117 to 17,673 per individual, which was further rarefied to standardise the sequencing depth for all samples. The highest OTU count was detected in a sample from UC patients. However, it is important to note that a high number of OTUs does not necessarily imply higher taxonomic diversity, as multiple OTUs may correspond to the same bacterial species [95]. To more accurately assess microbial diversity, alpha diversity metrics were calculated. The Chao1 and Shannon indices were used to assess species richness and their relative abundance, respectively. No statistically significant differences were found in alpha diversity between UC patients and HC, nor among UC subgroups stratified by disease severity or medication status (data not shown). These findings suggest a similar overall diversity between the groups. However, caution is warranted in interpreting these results due to imbalances in sample sizes. For example, the subgroup with Mayo score 3 consisted of only four individuals, which may limit statistical power and the ability to detect true differences. Therefore, the absence of significant results should not be interpreted as conclusive evidence of biological similarity, but rather as a reflection of limited subgroup representation. Furthermore, the values obtained by calculating alpha diversity were higher than expected (data not shown), suggesting a potential influence of the taxonomic resolution of the method used and possibly reflecting the complex microbial community of the GI tract.

These findings align with those of Berbisá *et al.* [96], who examined the microbiota of 41 UC patients and similarly reported no significant differences in alpha or beta diversity. Their subgroup analyses by sex, smoking status, BMI, and disease extent also yielded non-significant results. However, they reported that patients who underwent standard treatment exhibited a significantly lower Shannon diversity index compared to untreated individuals, suggesting that therapeutic interventions may influence microbial diversity. Additional support for this trend comes from a recent large-scale meta-analysis that aggregated data from 2,518 individuals, including 934 IBD patients. The study found a consistent reduction in alpha diversity among UC

and CD patients compared to HC [97]. The reduction was more pronounced in CD, indicating a possible gradient of microbial disturbance across IBD subtypes. This difference might explain why no significant reduction in alpha diversity was observed in our UC cohort. It could reflect a relatively greater microbial stability in UC or be influenced by external factors such as treatment status, disease location, or host-specific characteristics. As such, more research involving larger and more balanced cohorts is needed to confirm these trends and understand their biological implications. A separate meta-analysis by Abdel-Gadir *et al.* [98] reinforced these findings, highlighting that reduced alpha diversity is a consistent feature in both UC and CD, although it was more pronounced in CD. They also reported that the disease status contributed significantly to the variation in beta diversity, although the magnitude of this effect differed between studies. Interestingly, the type of sample emerged as a greater source of variability than the disease status itself. For example, stool samples showed lower heterogeneity compared to biopsy samples, particularly in CD studies.

To gain deeper insights into the structure of the bacterial community, a PCA was conducted. The analysis was based on Hellinger-transformed abundance data, a transformation widely used in ecological studies to reduce the influence of highly abundant taxa while maintaining the Euclidean characteristics of the data set [92]. To further minimise noise from rare taxa and focus on the dominant members of the microbiota, only those with a relative abundance greater than 0.1% were included. The PC1 represented 8.4% of the total variance, while the PC2 explained 6.4%. The resulting PCA plot (Figure 4.4) showed greater dispersion among samples from UC patients compared to HC, indicating increased interindividual variability within the UC group. This increased variation suggests that UC may be associated with instability or dysbiosis of the microbial community. In particular, the greatest spread was observed in people receiving treatment (Figure A.6). This raises the possibility that pharmacological interventions may alter the intestinal environment, potentially allowing the expansion of taxa that are normally kept under control by a healthy immune response. To support this interpretation, Berbisá *et al.* [96] reported significant differences in microbial diversity according to treatment status. Their findings indicated that patients who received standard treatment exhibited lower alpha diversity compared to those who did not receive any medication, highlighting the potential impact of therapeutic regimens on microbiota diversity. However, it remains unclear whether the observed microbial changes are a cause or consequence of therapeutic intervention, particularly in light of evidence that medications such as corticosteroids and immunomodulators can significantly influence the microbial composition [99].

To investigate the composition of the intestinal microbiota in relation to disease severity, an RDA was performed using Hellinger transformation [92]. Taxa with a relative abundance below 0.1% were excluded to highlight more functionally relevant changes in microbial composition. The RDA was constrained by Mayo score subgroups (UC0 to UC3), and the results are presented in Figure 4.5. The first component explained 3.7% of the variance, while the second component accounted for 2.8%. A discernible gradient along the first component was observed, with UC0, UC1 and UC2 samples forming overlapping clusters, while UC3 formed a distinct group with minimal overlap. The second component partially separated UC2 and UC3, supporting the notion that the microbiota composition is modulated by increasing the severity of the disease. However, the overlap between UC1 and UC2 suggests transitional community structures and due to the small sample size in the UC3 group (n = 4), these findings must be interpreted with caution. Although these results suggest compositional changes related to the disease, they

require validation in larger cohorts. These findings are consistent with the meta-analysis of a larger cohort [97]. Furthermore, studies involving longer durations with multiple sampling points within the same cohort suggest that the overall microbial communities in patients with IBD fluctuate more than in HC. Specifically, the composition of the gut microbiota in the early stages of IBD can exhibit greater variability than in healthy individuals [10].

These observations underscore the dynamic and unstable nature of the microbiome associated with UC. Although the absence of significant differences in alpha diversity between the UC and HC groups may seem counterintuitive, the greater variability within the UC, could reflect underlying ecological instability. From a clinical perspective, this instability has potential implications for using microbial diversity as a biomarker. For example, longitudinal monitoring of diversity metrics or temporal variance could help identify early disease activity or predict flare-ups. However, given that increased variability does not align consistently with clinical outcomes, caution should be exercised when interpreting diversity metrics in isolation. Rather, they should be integrated with functional and taxonomic data in a broader monitoring framework.

5.2 Microbial Taxonomic Shifts Associated With Ulcerative Colitis

To gain a deeper understanding of taxonomic differences between the groups of patients, a heatmap of relative abundance was generated at the phylum level (Figure A.2). The general distribution of the dominant phyla, including *Firmicutes*, *Bacteroidota*, *Actinobacteriota*, and *Proteobacteria*, appeared similar between the HC and UC groups. These findings are consistent with previous studies, which have also identified *Firmicutes* and *Bacteroidota* as the most prevalent phyla in healthy individuals affected by UC [100, 101, 102]. Despite the similarity in composition, the relative abundance of first three dominant phyla was lower in patients with UC compared to HC [103]. Furthermore, some UC samples exhibited an increased presence of *Proteobacteria*, a phylum comprising numerous opportunistic pathogens that have been implicated in the pathogenesis of inflammatory bowel disease [100, 104]. Elevated levels of *Proteobacteria* have also been associated with diets high in simple sugars, suggesting that diet patterns may influence their proliferation [105]. In addition, an increased abundance of *Actinobacteriota* was observed in UC individuals, which is consistent with previous studies [102, 104]. Given the immunomodulatory functions attributed to this phylum, its elevated presence in UC patients could reflect the dysregulated immune responses characteristic of IBD [102].

The heatmap clustered at genus level (Figure A.3) further illustrates microbial community structure, with the mean relative abundance in HC samples showing dominance of genera such as *Faecalibacterium* (10% ± 2.6%), *Blautia* A (3.6% ± 1.4%), *Bacteroides* (3.4% ± 2.4%), *Prevotella* (1.3% ± 1.5%), *Alistipes* (1.3% ± 1.2%), *Bifidobacterium* (1.2% ± 1.2%), *Phocaeicola* (1% ± 0.7%), and *Anaerobutyricum* (0.9% ± 0.5%). In contrast, UC samples exhibited an altered microbial landscape with *Faecalibacterium* (7.9% ± 4.7%), *Blautia* A (5.1% ± 3.8%), *Bacteroides* (3.1% ± 3.4%), *Ruminococcus* E (1.5% ± 1.6%), *Bifidobacterium* (1.5% ± 1.3%), *Streptococcus* (1% ± 1%), *Prevotella* (1% ± 0.7%), and *Alistipes* (0.9% ± 0.7%). This change in microbial composition supports previous literature indicating reduced *Prevotella* and *Alistipes* in UC patients [101], alongside increased *Blautia*, *Ruminococcus*, and *Streptococcus* [100, 102].

Both *Faecalibacterium* and *Bacteroides* are important producers of SCFAs, which are known for their anti-inflammatory properties [12]. In several UC samples, a marked reduction in *Faecalibacterium* was observed, often accompanied by an increased relative abundance of other genera such as *Blautia* A. Although reduced levels of this genus have been reported in the mucosal microbiota of CD patients [106], higher abundances have been observed in fecal samples from people with UC and irritable bowel syndrome (IBS) compared to HC [107]. This suggests that its abundance may vary depending on the disease and the sampling site. As a genus known to produce SCFAs, particularly acetate, and exhibit immunomodulatory properties, the role of *Blautia* in intestinal inflammation is likely complex and context dependent. The genus *Prevotella* has been associated with the onset of inflammatory disorders, however, it is frequently found in healthy individuals. Therefore, its role in inflammation remains uncertain and may depend on complex ecological interactions within the microbiota [108]. The abundance of *Bifidobacterium* varied considerably across individuals in both groups. This variability may be influenced by the use of probiotic supplements [100], but due to the lack of information on patient supplementation, conclusions cannot be drawn regarding its significance in this dataset. Environmental and dietary factors are known to influence the microbial composition. For example, a cross-population study comparing traditional and westernised diets found higher *Prevotella* and lower *Bifidobacterium*, *Faecalibacterium*, and *Bacteroides* in high-fibre diets [54]. Similarly, short-term nutritional interventions demonstrated that a fibre-rich plant-based diet significantly increases saccharolytic genera such as *Prevotella* and *Roseburia* [109].

Analysis at the species level (Figure 4.2) revealed that *Faecalibacterium prausnitzii* was the most abundant species in all individuals, although its abundance varied more markedly between UC patients. This is consistent with previous studies showing depletion of *F. prausnitzii* in UC and CD, with its reduced levels associated with increased postoperative recurrence [110]. *Blautia wexlerae*, a prominent member of the *Blautia* genus known for SCFA production [111], also varied in abundance among UC patients; whereas, reductions in *Blautia* abundance have been linked to IBD and colorectal cancer [112]. Similarly, lower levels of *Gemmiger formicilis* were detected in some individuals, which is consistent with its under-representation in IBD cohorts [113]. Other notable taxa included *Ligilactobacillus ruminis*, recognised for ameliorating symptoms of colitis in a mouse model [114]; its increased abundance in some cases could reflect probiotic intake. Furthermore, some UC patients exhibited lower levels of *Anaerobutyricum hallii*, an SCFA producer capable of metabolising dietary carcinogens into less harmful compounds [115]. Likewise, *Anaerostipes hadrus*, another SCFA-producing bacteria, was observed in reduced abundance among patients with UC and colorectal cancer [116, 117]. In contrast, certain individuals showed elevated abundances of *Parvimonas micra*, a bacterium associated with infections and reported more frequently in CD than UC [118], together with increased levels of *Phocaeicola coprocola*, *Anaerococcus senegalensis*, and *Peptoniphilus harei*, suggesting a state of dysbiosis. Notably, *P. harei* has been implicated in peritoneal infections after intestinal occlusion [119]. Reduced levels of *Ruminococcus bicirculans*, associated with lower starch metabolism [120], were also observed. Furthermore, *Bacteroides uniformis*, involved in carbohydrate metabolism and SCFA production, was decreased in patients with UC, potentially altering nutrient fermentation and immune regulation; administration of this species has been shown to alleviate colitis and restore barrier integrity [121]. *Anaerobutyricum soehngenii*, another butyrate producer, has demonstrated beneficial effects on glucose metabolism in humans with metabolic syndrome, supporting its probiotic potential [122]. *Dialister succinatiphilus*,

typically a commensal species, was found to be in greater abundance in UC patients and has been implicated in the promotion of intestinal inflammation [123]. Collectively, many of the observed species, as key SCFA producers, are critical for maintaining gut homeostasis through modulation of immune responses, intestinal permeability, and motility. The diet also exerts a significant influence; protein rich diets may enhance *Alistipes* abundance, whereas fibre intake supports SCFA-producing *Bacteroides* and *Prevotella* spp. [124, 125, 126]. However, not all *Bacteroides* species are beneficial; for instance, *Bacteroides fragilis* can induce chronic intestinal inflammation under specific conditions [127]. In general, the species involved in SCFA production showed considerable variability across UC patients, suggesting altered intestinal microbiota stability. A discernible pattern emerged where higher Mayo scores, indicative of greater disease severity, correlated with larger fluctuations in bacterial composition. This observation is consistent with the findings of Halfvarson *et al.* [10], demonstrating that the microbiota of UC patients exhibits greater variability compared to HC. Furthermore, patients undergoing medication treatment presented more pronounced microbiota fluctuations than those without pharmacological intervention (Figure A.4 and A.6), consistent with previous reports. Further investigation into the specific types of medications is warranted, since, different therapeutic agents, such as immunosuppressants or biological therapies, could selectively influence the structure of the microbial community by creating environments more favourable for specific bacterial taxa.

A differential abundance analysis was performed between 16 healthy individuals and 16 patients diagnosed with UC who had moderate to severe symptoms (Mayo scores 2 and 3) (Figure 4.3). Although none of the six OTUs with a relative abundance difference $\geq 0.1\%$ reached statistical significance (Figure A.5), a closer examination of abundance trends offers valuable insight into the potential dynamics of the microbiota associated with UC. OTU 79214, assigned to the genus *Bacteroides*, showed a trend towards increased abundance in UC patients ($P = 0.079$). Similarly, an OTU classified as *Parabacteroides merdae* (phylum *Bacteroidota*, genus *Parabacteroides*; $P = 0.12$) was also more abundant among UC patients. *Parabacteroides* species have a complex role in human health: they are part of the healthy gut microbiota and have been linked to immunomodulatory effects, including anti-inflammatory activity and SCFA secretion. However, elevated *Parabacteroides* abundance has also been associated with various pathologies, suggesting that its role is context-dependent and may change under inflammatory conditions such as UC [128]. Other genera identified among differentially abundant OTUs included *Anaerococcus*, *Blautia*, and *Bacteroides*, all of which have been implicated in intestinal health and disease.

The observed changes, although statistically non-significant, suggest a subtle but biologically important restructuring of the gut microbiota in UC. These findings align with broader ecological theories, which propose that in inflammatory states, such as UC, microbial communities undergo selective pressures that favour taxa capable of surviving under hostile conditions. As discussed earlier, overall diversity may not serve as a reliable marker of UC, whereas instability in the microbiota appears to be more informative. Here, the analysis of differentially abundant OTUs revealed taxa such as increased *Parabacteroides merdae* and trends in specific *Bacteroides* OTUs that could hold promise as early indicators of disease activity or therapeutic response. The observed correlation between microbiota fluctuations and medication use further supports the idea that therapeutic interventions influence the dynamics of the microbial community, with potential implications for patient stratification. Although these findings should be interpreted with caution due to the limited sample size and lack of statistical significance, they nonetheless

suggest that fine-scale microbial profiling may uncover biologically relevant signals associated with UC.

5.3 Antibiotic Resistance Genes Quantification and Correlation with Microbial Composition

Antibiotic resistance is a naturally occurring mechanism that reflects the adaptive capacities of bacterial populations when placed under environmental pressure [53]. In patients with UC, chronic inflammation adds another layer of environmental stress, potentially intensifying selective pressure that favours the survival of better-adapted microbial species. These conditions can facilitate the persistence and proliferation of bacteria that harbour ARGs, which contributes to dysbiosis and potentially exacerbating the progression of the disease.

In this study, the abundance of ARGs was quantified using qPCR, a technique well known for its sensitivity and specificity in the detection of low-abundance genetic targets in complex microbial communities [129]. qPCR enables real-time amplification monitoring and offers accurate quantification; however, its performance is highly dependent on the design and specificity of primers, which can limit its scope relative to metagenomic approaches [65]. The selection of primers was a critical aspect of the experimental design, as it determines the accuracy and range of detectable ARGs [66]. Of the 18 primers initially chosen to detect specific ARGs, only eight produced acceptable amplification profiles and five were ultimately included in the final analysis (data not shown). Several primer sets failed to produce reliable results, primarily due to limitations in assay optimisation and resource constraints. In particular, the gene *aac(6')-Ib* initially passed verification and was included in the experimental workflow; however, subsequent analysis revealed nonspecific amplification products, rendering the results invalid. These challenges underscore the influence of methodological decisions on the interpretation of the prevalence of ARG. Although qPCR is effective for targeted quantification, metagenomic approaches can provide a more comprehensive and unbiased overview of ARG diversity. In addition, the selection of specific ARG targets plays an important role in shaping the conclusions drawn about the dissemination of resistance genes within microbial communities [66].

The abundance of ARGs was determined by normalising the number of copies of ARGs to the estimated number of bacterial cells in each sample, assuming an average of four copies of 16S rRNA gene per bacterial cell (Table A.2). Across HC, the mean copy numbers of ARGs were 7.22 $\pm$ 7.12 for *tetW*, 5.42 $\pm$ 6.06 for *ermB*, 0.02 $\pm$ 0.06 for *blaTEM*, and 0.001 $\pm$ 0.005 for *sul1*. Among UC patients, the highest mean abundance was observed for *ermB* (7.1 $\pm$ 9.14), followed by *tetW* (6.7 $\pm$ 4.77), *blaTEM* (0.09 $\pm$ 0.2), and *sul1* (0.01 $\pm$ 0.05). The mean number of ARG copies per 100 cells and the standard deviations for all genes tested are presented in Table 4.1. It is well-established that *tet* and *erm* genes are widely distributed in environmental and commensal bacterial reservoirs due to the historical and ongoing selective pressure from antibiotic use in both clinical and agricultural settings [130]. Although apparent differences between genes were observed, particularly in the low abundance of *blaTEM* and *sul1* in HC versus UC patients. Some individuals exhibited markedly elevated copy numbers in multiple ARGs, suggesting the potential presence of shared genetic platforms, such as plasmids or integrons, that carry multiple resistance determinants. These mobile genetic elements may facilitate the HGT of ARGs, especially under inflammatory and antimicrobial selective pressures characteristic of the intestinal environment

of the UC [131].

In general, the distribution of *tetW* and *ermB* was more consistent between individuals than that of *blaTEM* and *sul1*. Among UC patients, those with a Mayo score of 1 (UC1 subgroup) exhibited the highest mean abundances of *tetW* and *ermB*, whereas individuals with a Mayo score of 3 showed the lowest levels. This inverse relationship suggests that the abundance of ARG does not necessarily correlate with the severity of the disease. Instead, it may reflect more complex interactions between the composition of the microbial community. When stratifying UC samples by disease severity, patients with a Mayo score of 2 showed the highest mean copy numbers of *blaTEM* and *sul1*. However, this increase was mainly attributable to two individuals (patients 4 and 10), indicating that ARG abundance may be strongly influenced by patient-specific factors. In particular, patients 4 and 10 showed pronounced changes in microbial composition, raising the possibility that changes in taxonomic structure may contribute to increased carriage of ARG. As shown in Figure A.3, both patients had elevated relative abundances of the genus *Bacteroides*. In addition, patient 4 - who exhibited particularly high levels of both *sul1* and *blaTEM* - also showed increased abundances of *Anaerococcus* and *Parvimonas*. These genera may represent potential carriers of ARGs. *Sul1* has been associated with class one integron, suggesting that it may be transmitted between bacteria [132].

Further analysis of microbial composition revealed several bacterial species that can be more frequently associated with ARG-positive samples (Figure **??**). In HC samples carrying *tetW*, *ermB*, *sul1*, and *blaTEM*, increased relative abundance was observed for *Gemmiger formicilis*, *Ruminococcus bicirculans*, *Faecalibacillus intestinalis*, and *Phocaeicola coprocola*. Species such as *Blautia wexlerae*, *Alistipes putredinis*, and *Bacteroides uniformis* were also sporadically associated with ARG-positive individuals.

Among UC patients, the presence of ARGs was most frequently associated with a higher relative abundance of *Faecalibacillus intestinalis* across samples carrying *tetW*, *ermB*, and *blaTEM*. Other recurrently observed species included *Blautia wexlerae*, *Gemmiger formicilis*, and *Parabacteroides merdae*. In particular, several species within the *Lachnospiraceae* family were identified as recurring taxa in ARG positive samples.

Importantly, some bacterial species are inherently more prone to ARG carriage. For instance, *Blautia obeum*, *Blautia producta*, and *Blautia wexlerae* have been reported to possess a higher number of mobile genetic elements, such as prophages and transposons, which facilitate the acquisition of ARG and environmental adaptability [112]. Similarly, *Parabacteroides distasonis* and *Parabacteroides merdae* have demonstrated notable resistance to clindamycin, largely attributed to the presence of the *ermF* gene [128].

Furthermore, recent studies suggest that ARG dynamics in UC may be influenced not only by bacteria but also by other microbial kingdoms. A study by Akiyama *et al.* [133], using multi-omics approaches, reported fluctuations not only in the bacterial microbiome but also in the virome and mycobiome of UC patients. These findings indicate that a comprehensive understanding of the dynamics of microbial and resistance genes must consider the larger microbial ecosystem.

In summary, the abundance of ARGs in patients with UC is not uniformly elevated, but appears to reflect individual-specific microbiome structures and environmental exposures. The increased dispersion of ARG in the UC may mirror the broader instability and dysbiosis characteristic

of these microbial communities. A major limitation of this study is the narrow gene panel used: in the end only four ARGs were analysed, which limits the completeness of the findings [66]. Furthermore, although qPCR is a useful and sensitive method, cross-study comparisons remain challenging due to non-standardised protocols and conditions [134] . Therefore, broader gene targets and harmonised methodologies are needed to more accurately characterise ARG distributions across different populations and environments.

Conclusion 6

This study aimed to characterise the intestinal microbiota and assess the prevalence of ARGs in individuals diagnosed with UC, using 16S rRNA gene amplicon sequencing and qPCR. Although no significant differences in alpha diversity were observed, constrained ordination analyses revealed discernible clustering patterns associated with disease severity and medication use. These patterns suggest greater variability in the structure of the microbial community among UC patients, likely reflecting complex interactions between the host's condition and therapeutic interventions.

At the phylum level, the structure of the microbial community remained largely comparable between the UC and HC groups. However, taxonomic differences became apparent at lower levels, particularly at the genus and species levels. Certain taxa appeared over or under-represented in patients with UC, but rather than being associated with specific pathogens, these changes likely reflect broader dysbiosis, a symptom of IBD. Whether such dysbiosis is a cause or a consequence of UC remains unresolved; indeed, the interplay of host genetics, environmental exposures, and immune responses complicates causal interpretation.

Crucially, the instability observed in the microbiota associated with UC can facilitate HGT, thus promoting the spread of ARGs. In this study, a targeted qPCR analysis indicated that the abundance of ARGs is not consistently elevated in UC patients, but varies between individuals, possibly reflecting differences in microbiota composition, environmental exposures or medication history. However, the scope of ARG detection was limited to four genes, which significantly constrained the breadth of the findings. Moreover, the lack of standardisation in qPCR protocols hampers inter-study comparability.

In summary, this study highlights the intricate links between microbial dysbiosis and ARG dissemination in UC, reinforcing the need for high-resolution, functional, and longitudinal analyses.

7 Perspectives

Future studies should consider expanding cohort sizes to improve subgroup analyses and statistical power. Dietary influences also warrant further investigation, as fibre-rich diets are associated with the proliferation of beneficial taxa, such as SCFA producers. Although 16S rRNA gene sequencing provided valuable information on microbial community structure, its resolution is insufficient for strain-level identification. To overcome this, metagenomic and other multi-omics approaches, such as metatranscriptomics, metabolomics, and metaproteomics, should be employed to gain deeper insight into both taxonomic composition and functional potential [135].

Broader gene panels and harmonised detection methods are needed to enable accurate and reproducible assessments of the prevalence of ARGs. Furthermore, expanding the cohort size would improve the reliability of the findings, as testing only 12 UC patients - four per disease severity group - may be insufficient given the high variability in the microbiota between UC patients. Furthermore, many ARGs are co-located on mobile genetic elements such as plasmids or integrons, which facilitates their dissemination under selective pressures and underscores the need for integrative profiling of both the microbiota and ARGs.

As IBD continues to emerge as a global health concern, current therapies—such as anti-inflammatory drugs —remain limited in efficacy for many patients and often come with adverse effects. In severe cases, surgical intervention becomes necessary, further complicating disease management and disrupting microbial homeostasis. Therefore, advancing our understanding of the role of the microbiota in the pathogenesis of IBD and the dynamics of resistance genes is critical to develop effective and sustainable treatment options.

The complex and multifactorial nature of UC requires a move toward personalised medicine. Integrating host genotypes, disease phenotypes, immune profiles, and microbiome data could facilitate tailored interventions. Ultimately, the goal is to move beyond symptomatic treatment to precision therapies that address underlying microbial imbalances. Given the observed instability in microbial communities and the presence of ARGs, therapeutic interventions targeting the microbiome warrant further exploration. Approaches such as FMT have shown promise in animal models, but their efficacy in UC patients remains variable. Furthermore, microbial metabolites, key modulators of host immunity and inflammation, may serve as promising targets for precision therapy. As discussed by Li *et al.* [28], understanding host–microbiota co-metabolism could unlock novel treatment pathways, particularly through the modulation of metabolic outputs relevant to disease progression.

Broader application of multi-omics technologies, standardisation of analytical methods, and integrative research frameworks will be essential to advance our understanding of the microbial underpinnings of IBD and to guide the development of next-generation therapeutic strategies.

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Appendix A

A.1 Enrolled Ulcerative Colitis Patients

The 34 UC patients enrolled in the study with a MAYO score assigned that describes the state of the disease and is grouped based on its value are presented in Table A.1. In addition, this table includes information on medication intake, gender, and whether the sample was used for qPCR analysis.

Table A.1. Characteristics of 34 patients diagnosed with UC that met inclusion criteria with assigned groups used for data analysis. Samples used for qPCR data analysis are marked with "X".

No.	MAYO score	Group	Medication	Gender	qPCR
1	1	UC1	5-ASA[1]	Female	
2	2	UC2	No	Female	
3	1	UC1	No	Female	
4	2	UC2	5-ASA, Ustekinumab[2]	Female	X
5	3	UC3	5-ASA	Male	X
6	3	UC3	5-ASA	Female	X
7	0	UC0	5-ASA	Female	
8	1	UC1	5-ASA	Female	
9	2	UC2	5-ASA, Adalimumab[3]	Male	X
10	2	UC2	No	Female	X
11	2	UC2	Vedolizumab[4]	Female	
12	2	UC2	5-ASA	Male	
13	0	UC0	5-ASA, AZA[5], Citalopram[6]	Female	
14	3	UC3	Vedolizumab	Male	X
15	0	UC0	5-ASA	Female	
16	0	UC0	No	Female	
17	1	UC1	5-ASA	Female	
18	1	UC1	No	Male	

[1] 5-aminosalicylic acid (5-ASA): Anti-inflammatory agent [36].
[2] Ustekinumab: Targets IL-12 and IL-23 [40].
[3] Adalimumab: Targets TNF [38].
[4] Vedolizumab: Targets $\alpha 4\beta 7$ integrin [39].
[5] Azathioprine (AZA): Immunosuppressant drug [136].
[6] Citalopram: SSRI used in depression treatment [137].

No.	MAYO score	Group	Medication	Gender	qPCR
19	0	UC0	Infliximab[7]	Male	
20	2	UC2	No	Female	
21	1	UC1	No	Female	X
22	1	UC1	No	Male	X
23	2	UC2	Vedolizumab	Male	X
24	1	UC1	5-ASA, Vedolizumab	Female	X
25	1	UC1	Infliximab	Female	
26	2	UC2	5-ASA	Female	
27	0	UC0	5-ASA	Female	
28	2	UC2	No	Female	
29	0	UC0	No	Female	
30	2	UC2	5-ASA	Male	
31	1	UC1	5-ASA, AZA, Vedolizumab	Female	
32	3	UC3	5-ASA	Male	X
33	1	UC1	5-ASA, Citalopram	Female	X
34	2	UC2	5-ASA	Female	

[7]Infliximab: Targets TNF, a pro-inflammatory cytokine [138].

A.2 Bacterial Community Composition at Different Taxonomic Levels

Rarefaction curve of the 99% + 1 read OTU data was made to investigate whether adequate sequencing depths were reached (Figure A.1).

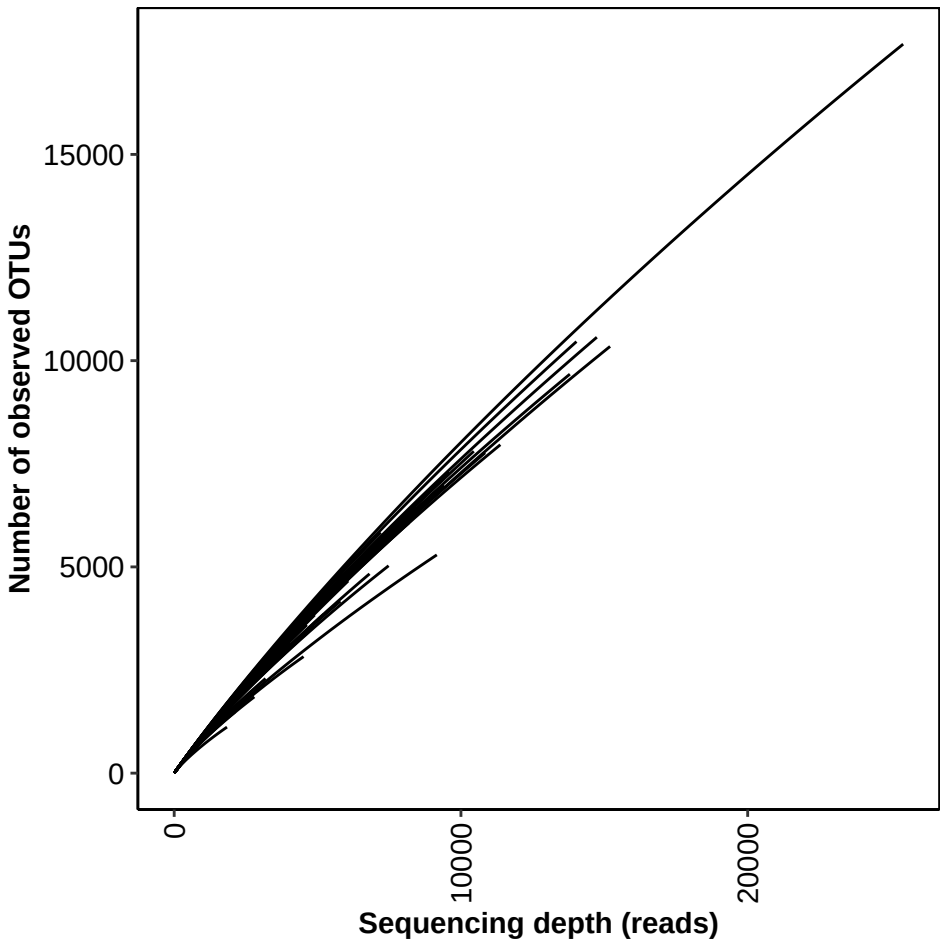

Figure A.1. Rarefaction curves of the 50 faecal samples estimating the number of observed OTUs as a function of sequencing depth.

To identify the most abundant phyla in the gut microbiota of all patients, a heatmap was made (Figure A.2).

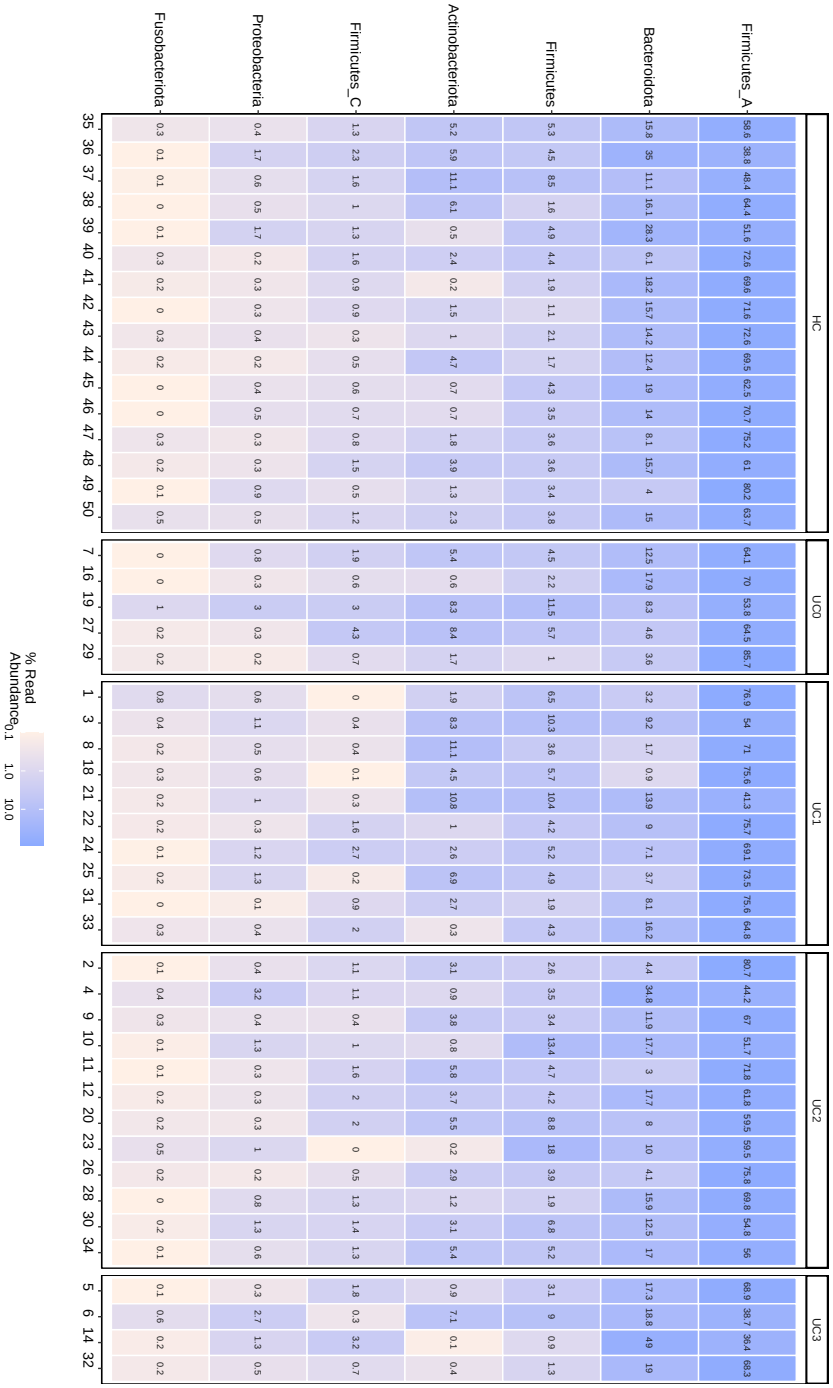

Figure A.2. Relative abundance of the top seven phyla with a minimum read abundance of 0.1% in the faecal samples across patients. The heatmap is grouped by participants and divided to healthy control (HC) and patients diagnosed with UC with numbers representing MAYO score describing the state of the illness (UC0, UC1, UC2, UC3, where score 0 represents the least and 3 the most inflamed group).

The most abundant genera among all patients were visualized with the use of a heatmap (Figure A.3).

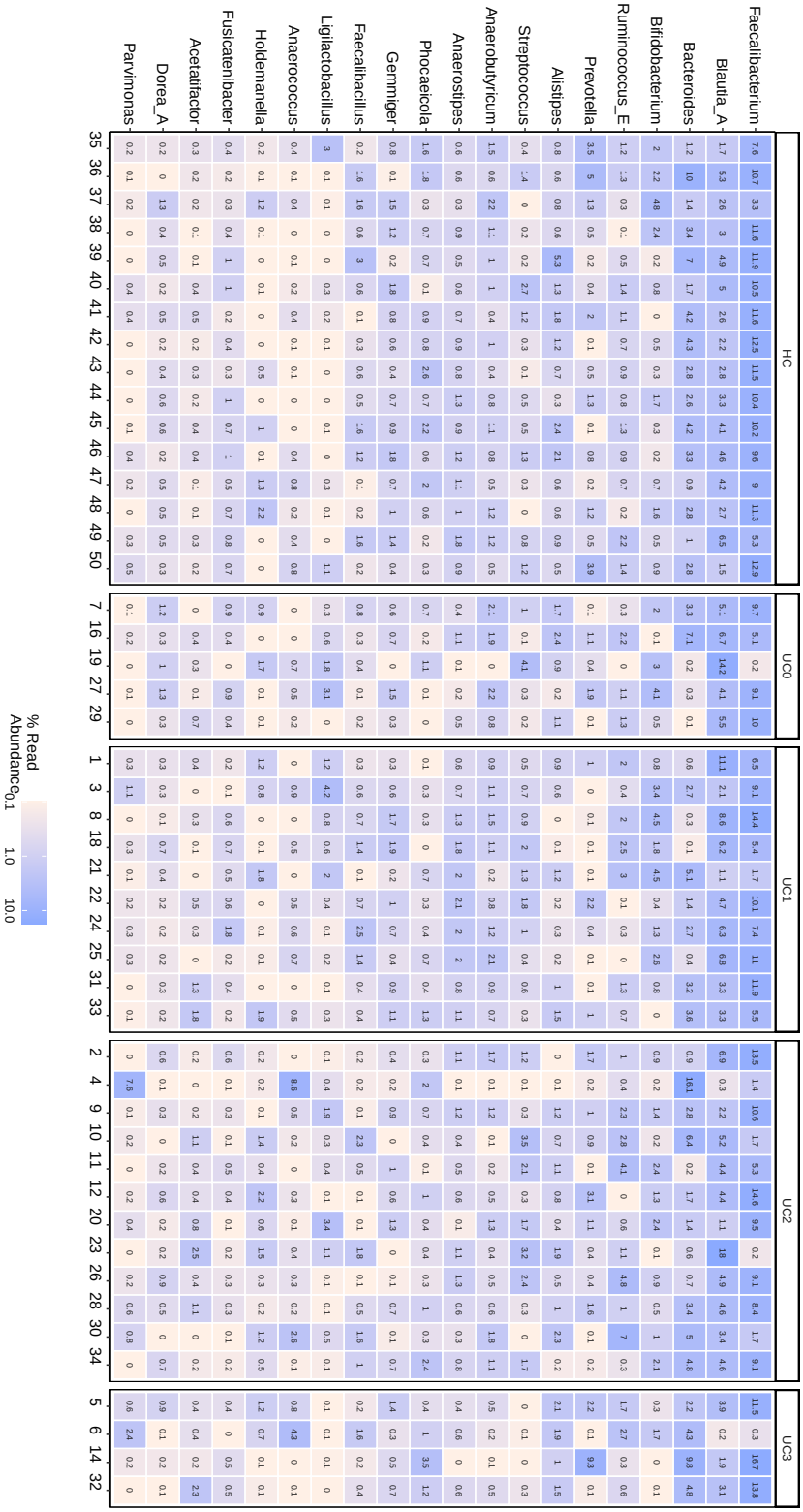

Figure A.3. Relative abundance of the top 20 genera with a minimum read abundance of 0.1% in the faecal samples across patients. The heatmap is grouped by participants and divided to healthy control (HC) and patients diagnosed with UC with numbers representing MAYO score describing the state of the illness (UC0, UC1, UC2, UC3, where score 0 represents the least and 3 the most inflamed group).

The 35 most abundant species in the gut microbiota of all patients grouped by medication intake were visualized by a heatmap (Figure A.4).

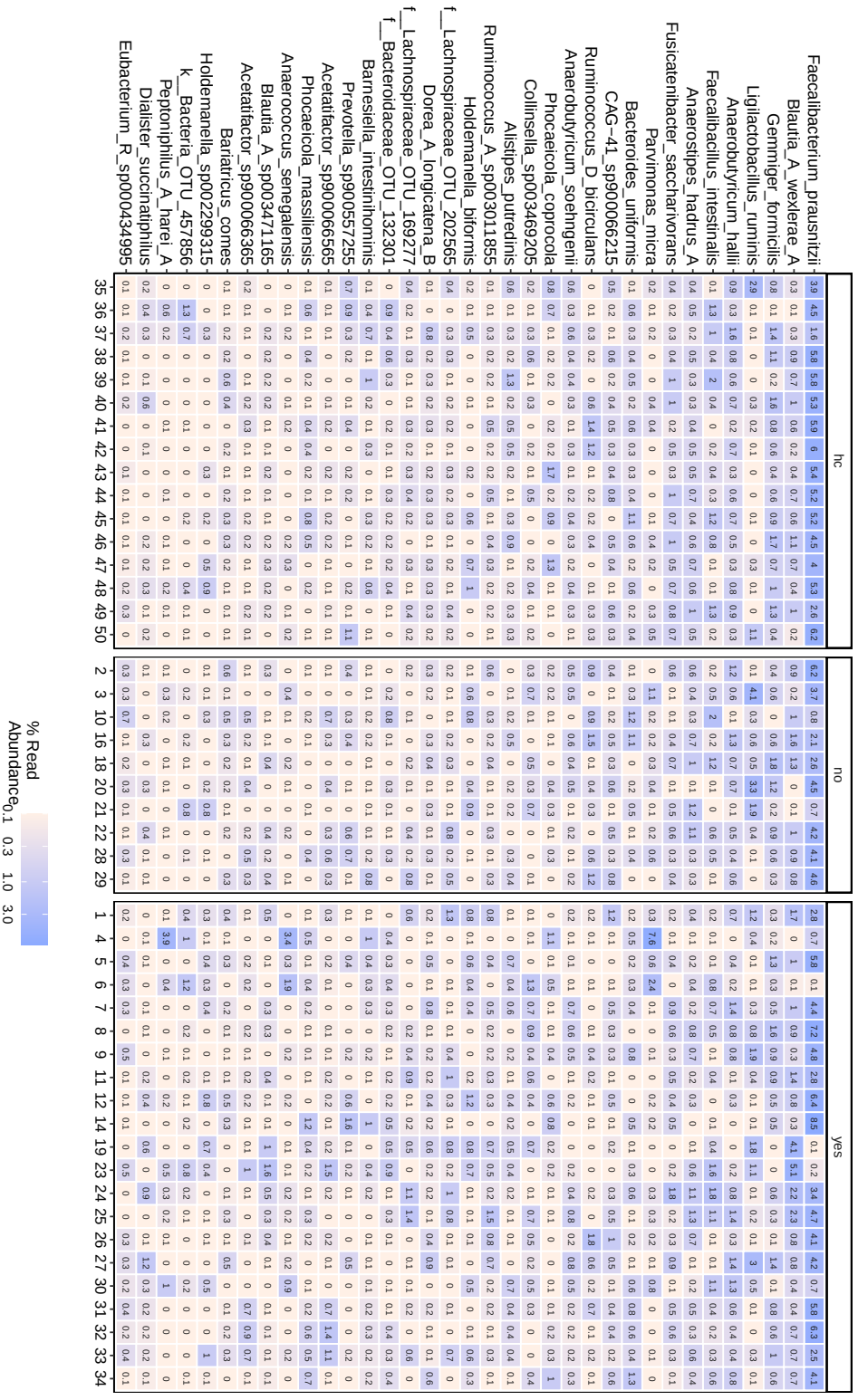

Figure A.4. Relative abundance of the 35 most abundant species with a minimum read abundance of 0.1% in the faecal samples across all patients. The heatmap is grouped by participants and split to healthy control (hc) and patients diagnosed with UC grouped by medication intake (yes/no).

In total, 6 different OTUs had a mean relative abundance difference $\geq 0.1\%$ in the UC compared to the HC group (Figure A.5).

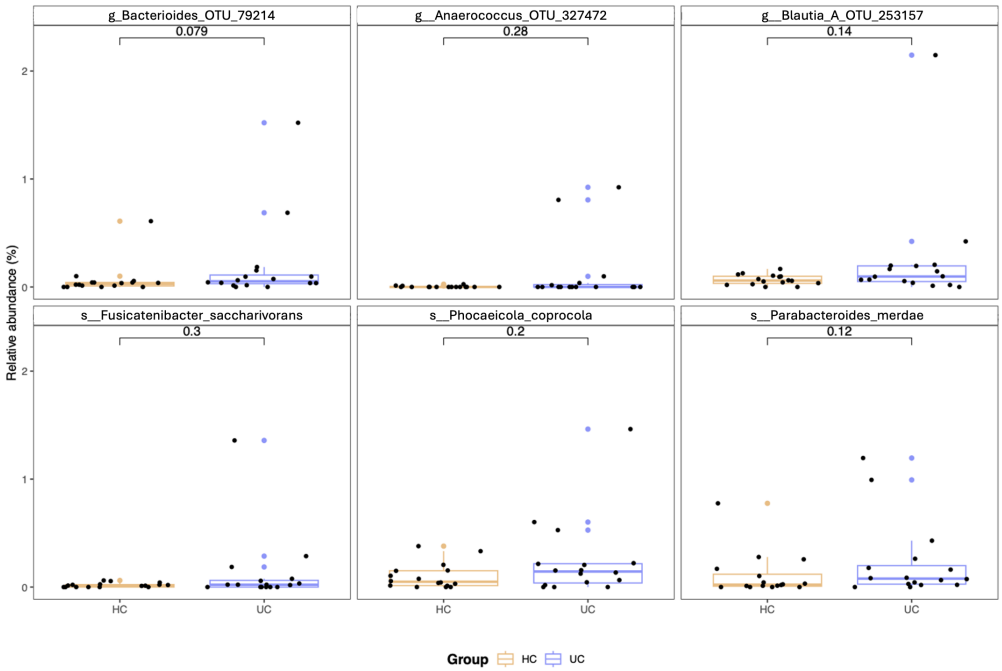

Figure A.5. The OTUs with a mean relative abundance difference $\geq 0.1\%$ between HC and moderate to severe UC group. The bacteria were assigned at different taxonomic levels. The Wilcoxon test was used to calculate whether the mean relative abundance of each taxon was significantly different.

PCA performed on OTUs with a relative abundance >0.1% showed clustering between groups (Figure A.6).

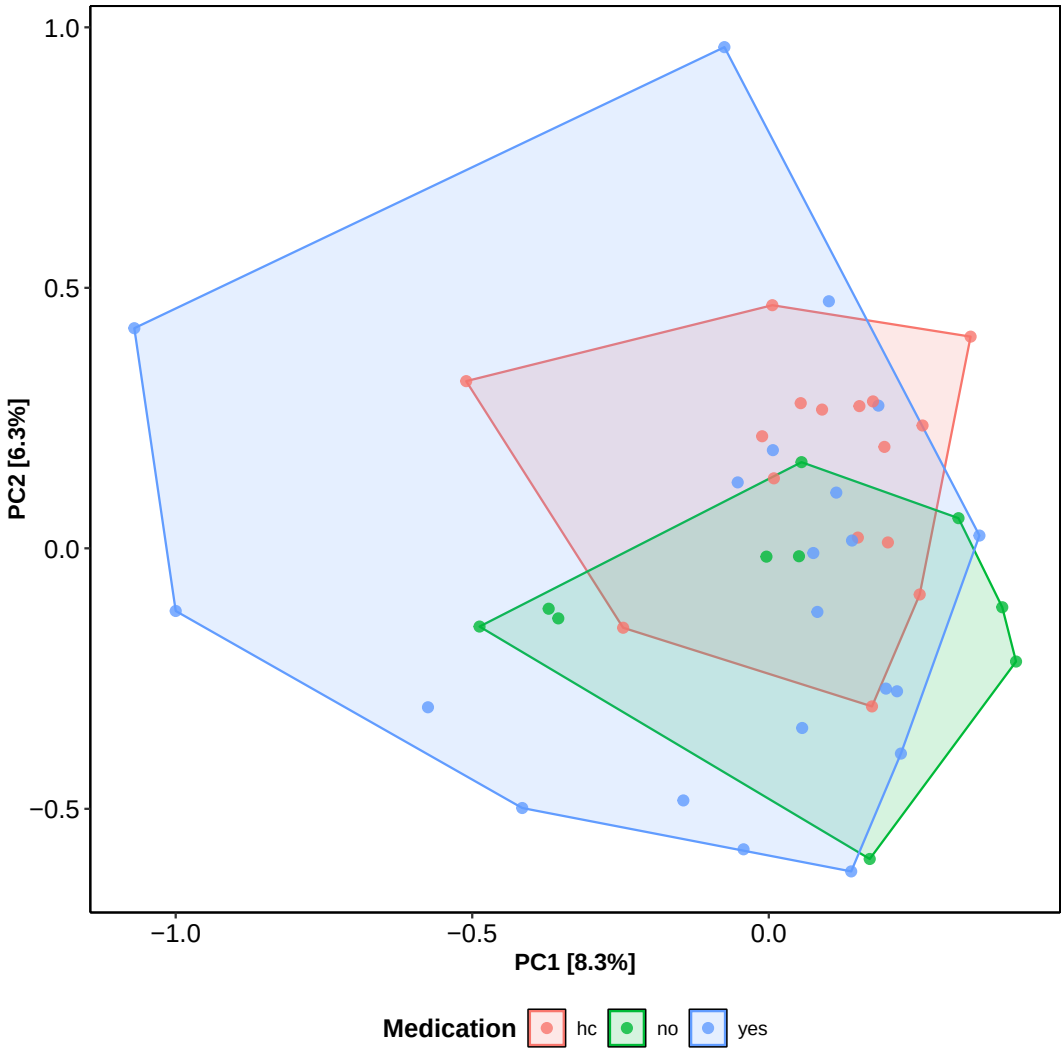

Figure A.6. Principal Components Analysis (PCA) of 1190 OTUs and 47 samples coloured by healthy control (HC) and UC patients based on medication intake. Prior to the analysis, OTUs that are not present in more than 0.1 % relative abundance in any sample have been removed. The data has been transformed initially by applying the Hellinger transformation [92]. The relative contribution (eigenvalue) of each axis to the total inertia in the data is indicated in percent at the axis titles.

A.3 The Abundance of Selected Antibiotic Resistance Genes

The abundance of ARGs present in each sample tested (Table A.2).

Table A.2. The gene copy number of tetW, blaTEM, ermB and sul1 genes per 100 cells, present in 12 HC and 12 UC patients. It was calculated using ARGs copy number normalized to 16S rRNA gene copy number with the assumption of 4 copies of 16S rRNA gene per prokaryote cell: $(\frac{\text{ARG copy number}}{\text{16S rRNA gene copy number}}) \times 4 \times 100$ [82].

No.	MAYO score	Group	Gene copy number per 100 cells			
			tetW	blaTEM	ermB	sul1
35		HC	2.01	0.0018	3.45	0
36		HC	3.91	0.2189	0.53	0
37		HC	10.25	0	2.82	0
38		HC	1.50	0	1.93	0
39		HC	-	0	2.11	0
40		HC	6.47	0.0023	0.35	0
41		HC	9.73	0.0021	10.51	0.0003
42		HC	26.01	0.0008	22.37	0
43		HC	2.26	0.0006	5.53	0
44		HC	4.43	0	3.05	0
45		HC	2.37	0	5.90	0
46		HC	10.49	0.0419	6.52	0.0167
21	1	UC1	14.35	0	7.69	0
22	1	UC1	3.63	0.0016	1.61	0
24	1	UC1	15.27	0.0002	33.75	0
33	1	UC1	3.67	0	12.33	0
9	2	UC2	7.36	0	2.33	0
10	2	UC2	10.73	0.3811	3.58	0
23	2	UC2	7.02	0.0024	10.66	0
4	2	UC2	0.36	0.6398	0.54	0.1586
5	3	UC3	6.09	0.0001	0.03	0.0007
32	3	UC3	3.02	0	10.36	0
6	3	UC3	7.46	0.0009	0.39	0
14	3	UC3	1.41	0.0051	1.97	0.0003