

THE INTESTINAL MICROBIOME IN INFLAMMATORY BOWEL DISEASE

**THE INTESTINAL MICROBIOME IN INFLAMMATORY BOWEL DISEASE
AND ITS RESPONSE TO THERAPY**

By JOSIE LIBERTUCCI, M.Sc., B.Sc.

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AUTHOR

Josie Libertucci, MSc

SUPERVISOR

Dr. Michael G. Surette

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ABSTRACT

Inflammatory bowel diseases, consisting of both Crohn's disease (CD) and ulcerative colitis (UC), occur when a susceptible host mounts a chronic aberrant immune response to its commensal microbial community resulting in chronic inflammation. CD and UC may exhibit similar clinical manifestations that oscillate between active disease and remission, but these diseases are distinct, and are characterized by specific pathophysiology. The incidence and prevalence of IBD has been increasing over time, with the highest rates occurring in Europe and North America. Although many genome-wide association studies (GWAS) on CD and UC have found loci associated with these diseases, studies conducted on monozygotic twins show low concordance rates, suggesting environmental causes play an important role in the onset of IBD. Current therapies for treatment of IBD focus on suppressing the immune response, but remission rates remain low and relapses occur often. There is a dire need for new treatment options that involve suppressing the immune system stimuli – the commensal gut microbiota. The commensal microbiota triggers the mucosal immune system to drive chronic inflammation in IBD, and studies have shown that patients display a gut dysbiosis compared to healthy individuals. Modulation of the disease through modulation of the gut microbiome, has recently gained interest as a potential treatment option. Little is known about how the microbial community in IBD patients will respond to therapies that attempt to modulate the community. Thus, the overall goal of this thesis was to characterize the IBD microbiome in relation to disease status, severity, and response to therapy. Together these results, (a) provide an in-depth characterization of the intestinal microbiome in IBD,

(b) demonstrate the response of the gut microbiome in UC patients to FMT, providing insight into the successful mechanism, and (c) provide evidence for inflammation related differences in CD giving insight into the mechanism of pathogenesis. These results not only further our understanding regarding dysbiosis in IBD, but also can be used to modify our current treatment protocols to increase efficacy.

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LIST OF ABBREVIATIONS AND SYMBOLS

5-ASA	5-aminosalicylic acid	DMC	Data monitoring committee
16S	Small subunit of 30S ribosome	DMSO	Dimethyl sulfoxide
⁵¹ Cr	Chromium	DNA	Deoxyribonucleic acid
Ang4	Angiogenin, ribonuclease A family, member 4	Eg	Example
ANOVA	Analysis of Variance	ELISA	Enzyme linked immunosorbent assay
BSA	Bovine serum albumin	ESR	Erythrocyte sedimentation rate
bp	Base pair	EQ-5D	EuroQol
C	Controls	Ex	Example
CD	Crohn's Disease	FBT	Fecal biotherapy
CD-I	Crohn's disease inflamed	fCal	Fecal calprotectin
CDI	<i>Clostridium difficile</i> infection	FDR	False discovery rate
CD-NI	Crohn's disease non-inflamed	FISH	Fluorescent <i>in situ</i> hybridization
CDAI	Crohn's disease activity index	FMT	Fecal microbiota transplantation
CFU	Colony Forming Unit	g	Gram
CH ₄	Methane	GALT	Gut-associated lymphoid tissue
CO ₂	Carbon dioxide	GWAS	Genome wide association studies
CRP	C-reactive protein	GI	Gastrointestinal
cTregs	Colonic regulatory T cells	h	hour
DAPI	4',6-diamidino-2-phenylindole	H ₂	Hydrogen gas
df	Degrees of freedom	H&E	Hematoxylin and eosin
		HBI	Harvey-Bradshaw index

IBD	Inflammatory bowel disease	PBS	Phosphate buffered saline
IBDQ	Inflammatory bowel disease questionnaire	PICRUST	Phylogenetic investigation of communities by reconstruction of unobserved states
IBS	Irritable bowel syndrome	PCR	Polymerase chain reaction
IEL	Intraepithelial lymphocytes	PRRs	Pattern recognition receptors
IgA	Immunoglobulin A	QIIME	Quantitative insights into microbial ecology
IL	Interleukin	qPCR	Quantitative PCR
ITS	Nuclear ribosomal internal transcribed spacer	RCDI	Recurrent or refractory <i>Clostridium difficile</i> infection
LPS	Lipopolysaccharide	RDP	Ribosomal Database Project
MAMPs	Microbe-associated molecular pattern	RegIII	Regenerating islet derived protein
MALT	Mucosa-associated lymphoid tissue	rRNA	ribosomal ribonucleic acid
mg	milligram	sIgA	Secretory immunoglobulin A
mL	Milliliter	SCFA	Short chain fatty acid
MUC2	Mucin 2	STAMP	Statistical analysis of metagenomic profiles
Myd88	Myeloid differentiation primary response 88	SNVs	Structural nucleotide variants
NOD2	Nucleotide-binding oligomerization domain-containing protein 2	TLR	Toll like receptor
NGS	Next generation sequencing	TJ	Tight junction
OTU	Operational taxonomic unit	UC	Ulcerative colitis
		V1-V9	Variable regions 1-9

vs	versus
%	Percentage
°C	Degrees Celsius
α	Alpha
β	Beta
γ	Gamma
μL	Microliter
>	Greater than
<	Less than
\geq	Greater than or equal to
\leq	Less than or equal to

CHAPTER 1

INTRODUCTION

1.1 The human intestinal microbiome

Trillions of microbial cells inhabit the gastrointestinal tract (GI) in humans, and yet, the number of bacterial cells in the human body does not exceed the amount of host cells, as the ratio is relatively close to 1:1 (Sender et al., 2016). The microorganisms that reside within humans exist in a symbiotic relationship with the host (Bäckhed et al., 2005) and are essential for human life. These complex and dynamic microbial communities are shaped by external and internal factors including diet (David et al 2013), geography (Yatsunenکو et al., 2012), host genetics (Benson et al., 2010), and disease state (Greenblum et al., 2012). Not only essential to human life, microorganisms may have played a key role in the evolution of higher organisms (Zilber-Rosenberg and Rosenberg, 2008).

1.1.1 Defining a healthy microbiome

The microorganisms that inhabit the human gut differ remarkably between healthy individuals, and because of this there is some difficulty associated with defining a healthy microbiome based on composition alone. Characterizing a healthy microbiome based on a specific set of microorganisms is too simplistic and inappropriate (Bäckhed et al., 2012). If one cannot define a microorganism as a pathogen without knowledge of its host (Casadevall and Pirofski, 2012), it is equally problematic to define a healthy microbiome without considering the host. For this reason, dysbiosis is defined as perturbations of the microbiota and their genes that can lead to a disruption of intestinal homeostasis (Peterson and Round, 2014). Studies have suggested that the function of the microbiome can play a much larger role in defining a healthy individual (The Human Microbiome

Project Consortium, 2012). Many bacterial species can perform the same function, meaning the absence of one group may not correlate with the absence of that group's function.

Studies have shown that the healthy gut microbiome is stable meaning; an individual's microbial composition over time would be more similar to ones self than to another person (Caporaso et al., 2011). This temporal equilibrium can be perturbed, but the healthy microbiota returns to its equilibrium (Lozupone et al., 2012). Resilience, the amount of stress that can be tolerated prior to a change in equilibrium, is characteristic of a stable community (Folke et al., 2004). There are many factors that can perturb the microbiota and challenge the community's resilience. The microbiota is mostly resilient to dietary changes, although long-term changes in diet can lead to an altered equilibrium (Wu et al., 2011). Antibiotics are another factor that are able to alter the composition, and in some cases push the community to dysbiosis, as is seen in *Clostridium difficile* infections (Theriot, 2014). Antibiotics can compromise the abundance of certain microbial communities, and allow the host to be vulnerable to pathogens by creating a niche for foreign species. In the absence of antibiotics, the microbiota is resilient to the invasion of foreign species, whether they are potentially pathogenic (ex. *Campylobacter jejuni*) or potentially beneficial (ex. probiotic) – this mechanism is often referred to as colonization resistance (van der Waaij et al., 1971).

1.1.1.1 Colonization resistance

There are mechanisms that ecosystems utilize that allow for resilience, two being high species diversity and competition. Communities with high species richness are less

susceptible to invasion from exogenous species because there are fewer nutrients available to those invading (Levine and D'Antonio, 1999). Although, true for most ecosystems, it is unknown how diversity affects colonization resistance and resilience in the human gut. Competition of resources may also play a role in stability and resilience of the gut microbiome, as microbes that use the same resources will inhibit one another. This competition can initiate microbe inhibitory interactions, and usually works in favour of the resident microbiota (Hibbing et al., 2010). For example, certain bacterial species, like *Bacteroides thetaiotaomicron*, provide a direct defense against pathogens. *B. thetaiotaomicron* can induce the expression of antimicrobial peptides including regenerating islet-derived III (RegIII) proteins (REGIII γ and REGIII β) within the colon following colonization of foreign species (Sonnenburg et al., 2006).

1.1.1.2 Short chain fatty acid production

Microbial metabolites have a major influence on host physiology and influence human health. Bacterial fermentation of sugars occurs in the absence of oxygen resulting in energy for bacterial growth and metabolism, and by-products that are essential to the host. Products of fermentation include gasses such as methane (CH₄), carbon dioxide (CO₂), and hydrogen gas (H₂), short chain fatty acids (SCFA) and heat (Topping and Clifton, 2001). SCFA are produced in the large intestine by the resident microbiota from food undigested in the small intestine (Ríos-Covián et al., 2016). These fatty acids are volatile, can be straight chained or branched, and contain one to six carbon atoms (Miller and Wolin, 1979). The most abundant SCFA in the colon is acetate (~60%), followed by relatively equal concentrations of propionate (~25%) and butyrate (~15%) (Cummings et

al., 1979). SCFA production can be influenced by the amount (colony forming units per g, CFU/g) of bacteria, the species that are present, substrate source, and intestinal motility. The majority of SCFA production occurs in the proximal colon, as the distal colon is carbohydrate and water depleted. Butyrate is the preferred energy source for colonocytes, with approximately 95% of SCFA being absorbed in the colon (Duncan et al., 2004).

SCFA also harbor an immunogenic effect within the host. Smith and colleagues determined that they also play a role in regulating the size and function of colonic regulatory T cells (cTregs) (Smith et al., 2013). *Bacteroides fragilis* and species within the genus *Clostridium* induce cTregs, and this interaction is essential for proper intestinal inflammation control. However, in the absence of the microbiota, germ-free mice have a reduction in luminal SCFA and show a significant decrease in cTregs, highlighting a communication network between SCFA and the immune system (Smith et al., 2013).

1.2 Maintaining intestinal homeostasis

From an evolutionary point of view, there is a survival advantage to organisms that harbor an indigenous microbiota, as it aids in fending off unwanted infections, and allows the host to harness their indigenous microbiota's metabolic capabilities (Maynard et al., 2012). Since the microbiome provides many advantages to the host, it is hypothesized that the adaptive immune system evolved in mammals to maintain and promote microbial colonization and proliferation, rather than inhibiting this community (McFall-Ngai, 2007). The adaptive immune system is capable of educating immune cells

to allow for recognition of the commensal microbiota, in addition to identifying foreign species. The host maintains and regulates the composition of the microbial community by either initiating or suppressing inflammation in the presence of unwanted or beneficial microbes, respectively. Within the gut the intestinal epithelium, a single-cell layer, provides an important barrier function between the external environment and the host.

1.2.1 Intestinal barrier function

The gut acts as a physical barrier to protect the host from unwanted immune stimulation and subsequent inflammation resulting from the commensal microbiota. The host provides a barrier through the lining of the GI epithelial cells. These cells produce a thick mucus layer, secreted by goblet cells, which physically separates the microbiota from directly interacting with epithelial cells, consequently, inhibiting an immune response (Pelaseyed et al., 2014). The mucus layer is composed of highly glycosylated gel forming mucins, the most prevalent being MUC2 (Pelaseyed et al., 2014). If microorganisms are able to reach the epithelial cell lining despite the presence of the mucus layer, or if the mucus layer is damaged, microbial-associated molecular patterns (MAMPs) can be recognized by pattern recognition receptors (PRRs), which includes toll like receptors (TLRs), formylated peptide receptors (FPRs), or nucleotide-binding oligomerization domain like receptors (NODs) (Hooper et al., 2001). MAMPs can include lipopolysaccharide (LPS), flagellin, peptidoglycans, and formylated peptides. Following binding to their respective ligand, TLRs trigger recruitment of *Myd88* adapter proteins to induce intracellular signaling, which leads to the activation of NF κ B and regulate the

expression of genes to induce a pathogen-driven inflammation response (Lawrence, 1999).

Between two neighbouring epithelial cells, tight junctions (TJ) provide a barrier between the lumen and lamina propria (Liu et al., 2005). TJs consist of a complex of proteins, which are dynamic, and consist of intracellular proteins [(ZO)-1, ZO-2, and ZO-3, cingulin, 7H6, symplekin, and ZA-1) (Wittchen et al., 1999) and surface membrane proteins [occludin, claudin, junctional adhesion molecules (JAMs), coxsackie virus and adenovirus receptor proteins (CAR)] (Hossain and Hirata, 2008). Many enteric pathogens try to reduce the integrity of TJs including, *Escherichia coli* and *Clostridium difficile* by altering the distribution and disorganizing the TJ protein complex (Prasad et al., 2005; Cani et al., 2009). When TJs are compromised paracellular permeability (the transfer of substances between epithelial cells) control is disrupted and barrier function is altered, which may lead to inflammation resulting from an aberrant immune response (Arrieta et al., 2006). Adherens junctions are required for the assembly and stabilization of the tight junctions, and are composed of E-cadherin and catenins (Hartsock and Nelson, 2008).

1.2.2 The early life intestinal microbiome in humans and factors that shape the immune response

During the course of one's life, the microbiome changes and evolves – age influences the composition and diversity of the human microbiome (Saraswati and Sitaraman, 2015; Whelan et al., 2014). It was once thought that prior to birth, humans were effectively sterile; however this notion has been challenged, as studies have reported the presence of a microbial community in the human placenta (Aagaard et al., 2014),

amniotic fluid (Oh et al., 2010), fetal membrane (Steel et al., 2005), and umbilical cord (Jiménez et al., 2005). During gestation, maternal factors such as diet, antibiotic treatment, and the maternal microbiome can play a role in shaping the infant gut microbiota following birth. Mouse models have suggested the possibility of bacteria from the genus *Enterococcus* could be maternally transferred to the fetus (Jiménez et al., 2005). Preliminary studies examining maternal intake of antibiotics during pregnancy for the treatment of Group B streptococcal infections, and its impact on the development of the infant microbiome, have shown a delayed enrichment for the genus *Bifidobacterium* (Stearns et al., 2016; unpublished data). How the delay in abundance of this group will affect the immune development of the child is currently under investigation.

Following birth, the composition of the microbiota during the first weeks of life is driven by oxygen concentrations within the infant intestine, and influenced by the maternal microbiome, specifically the vagina and skin (Mueller et al., 2015), mode of delivery (Dominguez-Bello et al., 2010), antibiotics, and diet (Bokulich et al., 2016). Generally, facultative anaerobes such as bacteria within the family Enterobacteriaceae colonize first, followed by a switch to strict anaerobes including *Clostridium*, *Bacteroides*, and *Bifidobacterium* (Matamoros et al., 2013). Not until infants are exposed to foods that require the digestion of polysaccharides, which are unable to be digested by the host, does the abundance of *Bacteroides*, *Clostridium*, and *Ruminococcus* increase, and a decrease in *Bifidobacterium* and Enterobacteriaceae occurs (Fallani et al., 2011). Up to three years of age, the infant microbiota is highly unstable and dynamic (Koenig et al., 2011).

The neonatal immune system matures under the influence of the microbiota and is regulated by maternal and environmental factors. In addition to influencing the colonization of the infant gut microbiome, additional maternal factors are transferred to the infant such as immunomodulators like secretory immunoglobulin A (sIgA), and play a role in shaping the microbiome. Secretory IgA is the most abundant antibody found in the intestinal lumen, and acts as a first line of defense in protecting the host epithelium from unwanted pathogens and toxins (Mantis et al., 2011). Natural and specific sIgA antibodies can be transferred to the infant through breast milk (Sekirov et al., 2010). The infant commensal microbiota will stimulate the maturation of GALT (gut associated lymphoid tissue), a subcategory of MALT (mucosa-associated lymphoid tissue), which includes Peyer's patches, lamina propria, and intraepithelial leukocyte (IEL) spaces (Cebra et al., 1998; Owen and Jones, 1969; Cerf-Bensussan and Guy-Grand, 1991), and allow for the production of sIgA (Jiang et al., 2004). Infant sIgA will have limited affinity and range to redundant epitopes (Stoel et al., 2005), highlighting the benefit of acquiring maternal sIgA.

1.2.3 The microbiota and its influences on the immune system

The commensal microbiome is essential for the normal development of the gut to allow for proper gut function and for the development of the immune system. One way to examine and characterize its function in development is to understand how the body develops in the absence of the commensal microbial community – this highlights the importance of gnotobiology. Germ free mice, mice born and raised without the commensal microbiota, show defects in the development of GALT (Falk et al., 1998).

Within the small intestine, germ-free mice have fewer Peyer's patches, a thinner lamina propria, fewer B cells within germinal centers, and smaller isolated lymphoid follicles (ILFs) (Round and Mazmanian, 2009). Secretory IgA is also reduced in germ-free mice (Umesaki et al., 2014).

The gut epithelium, in addition to acting as a physical barrier, secretes many antimicrobial peptides; this is the role of Paneth cells within the crypts of the small intestine (Bevins and Salzman, 2011). When in the presence of bacteria, Paneth cells will secrete their granule contents to reduce interactions between microbes and host in an attempt to maintain gut homeostasis. The expression of angiogenin-4 (Ang4), a Paneth cell granule protein, is regulated following birth, and increases to adult levels in mice following weaning (Hooper et al., 2004). Expression of Ang4 is significantly reduced in germ-free mice (Hooper et al., 2004) emphasizing the role the commensal microbial community plays in generating levels of Ang4. RegIII proteins belonging to the family of C-type lectins, are expressed in the intestine (Natividad et al., 2013). RegIII binds to peptidoglycan moieties to induce damage (Cash et al., 2006). RegIII expression is heavily regulated by the intestinal microbiota composition, as it is significantly reduced in germ-free mice (Natividad et al., 2012).

1.3 Investigating the gut microbiome

With the advent of next-generation sequencing (NGS) technologies, investigating the highly diverse gut microbiome has focused on investigating what groups are present, and their respective function. To do so, marker gene sequencing and shotgun

metagenomics are frequently utilized. Marker gene sequencing provides taxonomic information, whereas shotgun metagenomics will provide taxonomic information as well as functional information. This section will provide a brief overview of these common techniques.

1.3.1 16S rRNA marker gene sequencing

Classical studies of the gut microbiota have focused on cultivating techniques for identifying the diverse intestinal community. Traditional culture methods revealed that as little as 10% to 30% (Suau et al., 1999) of the gut microbiome could be cultivated. More recently, groups have tried to capture the gut microbiome by combining culturing techniques with NGS, but only captured a maximum of 50% of the community (Goodman et al., 2011; Rettedal et al., 2014). However, a recent study that used culture-enriched molecular profiling, revealed that the majority of the gut microbiome can be cultured, and at greater diversity than by using marker gene sequencing (Lau et al., 2016). However, culturing the gut microbiome is a highly labour intensive process, and does not allow for high-throughput analysis of samples as compared to NGS. Microbial community analysis using NGS has focused on using marker gene sequencing to identify the community in a sample, using a higher throughput fashion enabling a large number of samples to be analyzed at once. Amplifying one or multiple variable regions within the conserved 16S rRNA gene (Muyzer et al., 1993) is commonly used to identify the gut microbial community. Targeting the 16S rRNA gene has the advantage of containing enough conserved regions to allow for identification of bacteria, and nine hypervariable regions that allow for the specific identification of bacterial species (Bodilis et al., 2012).

Generally, 16S marker gene analysis involves genomic DNA isolation, amplification of one or multiple variable regions, sequencing, and bioinformatic analysis. However, current methods for defining the gut microbiota via amplification of a variable region from the 16S rRNA gene are not standardized in the field, which raises issues regarding data comparison between groups. Recent papers support the notion that extraction efficiencies can vary greatly depending on the protocol used (Rubin et al., 2014). More bias arises depending on the variable region that is amplified within the 16S rRNA gene. Some primers may not be able to target all groups. For example, primers that target the V1-V2 regions do not amplify species from the genus *Bifidobacterium*, which is problematic as the community is then not fully represented, and can lead to biased conclusions that this group of bacteria may not be important in terms of gut function (Palmer et al., 2007), which is now known to be false (Arrieta et al., 2014). Other limitations that can arise from targeting specific variable regions include a lack of taxonomic resolution. Targeting the V3 region of the 16S rRNA gene may not be able to fully identify species that are closely related such as *Roseburia* and *Lachnospira* in some cases, and may over represent some groups like Lachnospiraceae.

Historically, sequencing was completed using the Roche platform, 454 pyrosequencing. This allowed for long sequence reads (up to 500bp) containing information that covered many variable regions and resulted in better taxonomic resolution, however, sequencing depth remained low. Using the Illumina MiSeq technology, sequencing depth has vastly improved, but sequence reads remain smaller than the 454 technology, ~250bp, although error rates are significantly better. Other

marker genes like the internally transcribed spacer (ITS) gene region (Schoch et al., 2012) can be used to identify the fungal community.

1.3.2 Shotgun metagenomics

16S rRNA gene analysis reveals the community that is present in a particular sample, but it fails to provide information regarding the function of the community. This is problematic because the absence of a particular group does not always infer the absence of that group's function, making elucidating the mechanism based on associations difficult. Programs have been made that allow for functional predictions based on 16S rRNA information (Langille et al., 2013), but predictions based on 16S analysis have their limitations. Shotgun metagenomics allow for full functional analysis of microbial communities and allows for the identification of microbes other than bacteria including fungi, archaea, and viruses. Shotgun metagenomics involves the shearing of DNA from the entire community followed by amplification. This sequencing is completed at a very deep level in order to identify genes that are present at low abundance. Following sequencing, read sequences can be assembled into longer consensus sequences called contigs, although assembling does have its limitations and may not always be necessary.

1.4 Inflammatory bowel disease

It is accepted that IBD, consisting of both Crohn's disease (CD) and ulcerative colitis (UC), occurs when a susceptible host mounts a chronic aberrant immune response to its commensal microbial community resulting in chronic inflammation (Sartor, 2008; Abraham and Cho, 2009). CD and UC may exhibit similar clinical manifestations that

oscillate between active disease and remission, but these diseases are distinct, and are characterized by specific pathophysiology (Zhang and Li, 2014). Inflammation characteristics vary between the two; in CD inflammation is transmural (affecting the full thickness of the intestinal wall) and is commonly seen in both the small and large intestine, whereas in UC inflammation is superficial and restricted to the colon (Greenstein et al., 1976; Price, 1978). The incidence and prevalence of IBD has been increasing over time, with the highest rates occurring in Europe and North America (Molodecky et al., 2012). Although many genome-wide association studies (GWAS) on CD and UC have found loci associated with these diseases, studies conducted on identical twins show low concordance rates, suggesting environmental causes play an important role in the onset of IBD (Jostins et al., 2012; Thompson et al., 1996; Orholm et al., 2009).

1.4.1 Ulcerative Colitis

Ulcerative colitis (UC) affects approximately 104,000 Canadians (CCC, 2012), and the disease is characterized by inflammation and ulceration of the colonic mucosa. The incidence of UC has increased steadily in Canada since 1950, resulting in one of the highest incidences of UC worldwide (Molodecky et al., 2012). Symptoms of UC include frequent diarrhea – often bloody – severe to moderate abdominal cramps, fatigue, mild fever, increased risk of colon cancer and as a result, an increase in depression. The cause of UC is unknown, but there are data that strongly suggest the disease arises from a robust immune response to an altered gut microbiome in predisposed individuals (Frank et al., 2011). Current therapy for UC focuses on suppressing the immune response with 5-amino salicylic acid (5-ASA), corticosteroid therapy, purine antimetabolites and anti-tumor

necrosis alpha monoclonal antibodies (anti-TNF α) (Talley et al., 2011), instead of blocking the agent of inflammation – a dysbiosis of the GI microbiota.

Intestinal innate immunity is altered in UC patients because the three innate barriers, mucus layer, epithelium and lamina propria are damaged. MUC2 expression and secretion is lowered in UC patients (Dorofeyev et al., 2013). MUC2^{-/-} deficient mouse models show spontaneous development of colitis causing UC human like symptoms, diarrhea, inflammation, and in some cases colon cancer (Van der Sluis et al., 2006). In UC patients, antimicrobial peptide secretion and antigen presentation are reduced, and intestinal barrier is compromised causing a reduction in immunity and an increase in inflammation (Ho, 2013). Directly below the epithelial layer is the lamina propria, which contains innate and immune cells that are significantly altered in UC patients (La Scaleia et al., 2012). Dendritic cells, which are atypical in UC patients and accumulate in the inflamed intestine, highly promote antigen recognition and cause activation of the immune system to commensal bacteria. Atypical immune cells cause a robust and chronic immune response in UC patients (La Scaleia et al., 2012).

1.4.2 Crohn's Disease

The exact etiology of Crohn's disease (CD), a subclass under inflammatory bowel disease (IBD), remains unknown, but current consensus supports the notion that this disease is due to a complex interplay between the host's genetics, immune system, intestinal microbiome, and other environmental factors (Xavier et al., 2007). Chronic inflammation in CD is not restricted to the colon and does not always involve the rectum, unlike ulcerative colitis (UC), is patchy instead of continuous, transmural as opposed to

superficial, and includes histopathological features such as granulomas, deep fissuring ulcers, and transmural lymphoid aggregates (Bouma and Strober, 2003). The absence of mucosal inflammation and complete mucosal healing often defines remission in CD, and is a strong predictor for improved outcomes (Schnitzler et al., 2009).

CD is associated with impaired barrier function, which has been identified as a key pathogenic mechanism (Baumgart et al., 2012). When the intestinal barrier is compromised, as reported in CD, tight junctions become leaky and transepithelial transport is loosely regulated, bacteria and other antigens can mount an immune response that can lead to intestinal inflammation (Söderholm et al., 1999). Clinical remission in CD is associated with a decrease in intestinal permeability compared to active disease and when in remission, abnormal intestinal permeability can be a predictor of relapse (Tibble et al., 2000). Quiescent CD can display sub-clinical inflammation due to barrier disruption, which presents as inflammatory bowel syndrome (IBS) like symptoms (Vivinus-Nébot et al., 2014). Although impaired permeability is highly associated with CD, it is unknown if it is a result of the disease, or if impaired barrier function leading to low-grade inflammation precedes the clinical diagnosis. Abnormal barrier function due to increased intestinal permeability is reported in many other chronic inflammatory diseases (Sapone et al., 2011) and has emerged as a target for new therapeutics and disease prevention in CD (Bischoff et al., 2014).

Murine models have confirmed that intestinal permeability in genetically susceptible mice is an important pathogenic mechanism that drives inflammation, but inflammation only presents in the presence of the intestinal microbiota (Madsen et al.,

2007). In CD patients, studies have reported that the commensal microbiota triggers the mucosal immune system to cause chronic inflammation (Ohkusa et al., 2009). CD patients display a gut dysbiosis compared to healthy individuals (Joossens et al., 2011; Lewis et al., 2015; Norman et al., 2015). Many studies have reported a decrease in the phylum Firmicutes - subsequently decreasing the abundance of short-chain fatty acid (SCFA) producers within the luminal contents, an increase in pro-inflammatory bacteria such as Fusobacteria, and a notable decrease in *Faecalibacterium prausnitzii*, a bacterial species associated with an anti-inflammatory properties (Sokol et al., 2008). Moreover, commensals play an important role in disease progression as mucins are depleted, rendering the host more susceptible to an immune response (Shaoul et al., 2004; Fyderik et al., 2009), and have been shown to drive intestinal inflammation due to their inflammatory properties identified via IgA coating (Palm et al., 2014).

1.5 Modulating the microbiome via fecal microbiota transplantation to modulate disease activity in inflammatory bowel disease

Fecal microbiota transplantation (FMT), the insertion of a fecal suspension from a healthy donor to a recipient, is a highly effective treatment for recurrent or refractory *Clostridium difficile* infection (CDI) (van Nood et al., 2013; Kassam et al., 2012). Ge Hong first described FMT during the 4th century for the treatment of diarrhea in humans, and in 1958, Eiseman et al., was the first to publish the use of a fecal enema to treat pseudomembranous colitis, most likely due to CDI (Ge, 2000; Eiseman et al., 1958). Since 1958, FMT usage has been reported in other dysbiotic diseases including ulcerative colitis (UC), Crohn's disease (CD), pouchitis, metabolic disorders, autism, and mood

disorders including anxiety and depression (Scaldaferri et al., 2016; Colman and Rubin, 2014; Stallmach et al., 2016; Vrieze et al., 2012; Mangiola et al., 2016).

Current therapy for IBD focuses on suppressing the immune response with remission rates remaining low. It is possible, however, to block the stimuli of chronic inflammation, a dysbiotic microbiota, instead of merely treating the symptoms (Ford et al., 2011a; Khan et al., 2011; Ford et al., 2011b; Ford et al., 2011c). In both diseases, the microbial biogeography of the lumen and mucosa is altered in patients, and has been suggested to contribute to chronic inflammation and a diseased state (Ott et al., 2004; Frank et al., 2007; Sokol et al., 2009; Morgan et al., 2012). Since FMT has been shown to be a successful treatment for treating RCDI by correcting dysbiosis in the host, FMT has gained interest as a potential treatment option for IBD. FMT may restore gut homeostasis and eliminate intestinal inflammation in IBD patients. Many case studies have tested FMT for treatment of both UC and CD, but as these studies used small sample sizes and have differing protocols, drawing conclusions regarding the efficacy of FMT remains challenging. Additionally, very few studies have provided insight into the microbial changes following FMT in IBD patients, or a comparison of patients that entered into remission via FMT and treatment failures.

In this past decade, FMT has gained much interest as a potential therapy for inflammatory bowel disease (IBD), although mixed efficacy results have been reported. It has been suggested that mode of administration, duration of therapy, and donor are important factors that can affect the outcome of successful FMT for treatment of IBD (Kelly et al., 2015). The mechanism of successful FMT remains to be elucidated

however, for the treatment of CDI, it has been suggested that engraftment, microbial competition, increase in secondary bile acid metabolism, and restoration of the gut barrier via cessation of the aberrant mucosal immune response, are possible main mechanisms (Khoruts et al., 2010; Weingarden et al., 2016; Khoruts and Sadowsky, 2016). The mechanism of successful FMT for treatment of IBD is even less well established compared to what is known about FMT for treatment of CDI. Considering these diseases have distinct pathogenesis, one being a chronic inflammatory disease, the other an infectious disease, the mechanism may differ between CDI and IBD.

1.5.1 History of FMT

From 1983–2000, many case series reported the use of FMT (then referred to as fecal biotherapy) for the treatment of CDI via rectal enema, colonoscopy, or nasoduodenal tube, with good success rates. These treatments were usually completed with feces from a relative or cohabitant with no adverse effects reported (Tvede and Rask-Madsen, 1989; Fløtterød and Hopen, 1991; Lund-Tønnesen et al., 1998; Schwan et al., 1983; Persky et al., 2000; Bowden et al., 1981). At the time, stool used from cohabitants of the patient (partner or relative) was preferred over anonymous stool, as it was thought the risk of obtaining an infectious agent from stool obtained from a cohabiting relative or partner would be lower, although, evidence supporting this notion is deficient (Waldum, 1998). In addition to fecal biotherapy, other forms of biotherapy instead of or in conjunction with antibiotic treatment were tested for treating recurrent or refractory CDI. This included using agents such as yeast, *Saccharomyces boulardii*, and bacteria, mainly *Lactobacillus* species, with mixed efficacy (Roffe, 1996).

In the early 2000s, FMT was still an unpopular treatment for CDI, despite reports of good success rates, most likely because of a lack of randomized controlled trials (Aas et al., 2003). With the advent of culture independent identification methods used to identify the gut microbial community, antibiotic associated diarrhea was found to be associated with a severely altered (dysbiotic) microbiome (Young et al., 2004). It was later confirmed that CDI patients have an altered gut microbiome, which assists in the loss of colonization resistance, rendering the host susceptible to infectious agents (Chang et al., 2008). This evidence supported the notion of manipulating the microbiome to prevent further CDI infections through FMT.

Dysbiosis of the microbiota has been extensively characterized in IBD and is hypothesized to drive inflammation and the disease phenotype in IBD patients (Ott et al., 2004; Frank et al., 2007; Sokol et al., 2009; Morgan et al., 2012; Norman et al., 2015). The idea of using FMT for the treatment of IBD originated from the role the microbiota plays in driving the disease and the success of using FMT to treat recurrent or refractory CDI (Borody et al., 2003). The first reported case of FMT to treat IBD was in 2003 by Borody and colleagues. Six patients were treated with a rectal retention enema of 200 – 300 mL of donor suspension once a day for six days. All patients entered remission post therapy (Borody et al., 2003).

1.5.2 FMT efficacy in *Clostridium difficile* infection

CDI is the most common cause of health care associated infections (HAIs) in North America, more common than methicillin-resistant *Staphylococcus aureus* (MRSA)

(Khanna et al., 2012). Magill and colleagues, surveyed 183 hospitals, which included 11,282 patients. Within this group of patients, 452 had 1 or more HAI with *C. difficile* as the most common pathogen causing 12.1% of the HAIs in this population (Magill et al., 2014). Prior to 2000, mortality rates were low, 1.5% reported in some hospitals, since then mortality rates due to CDI have significantly increased (Miller, 2007; Evans and Safdar, 2015). During epidemic periods, mortality rates increased to as high as 16.7%, which is correlated to the geographical distribution of epidemic strains BI/NAP1/027 (Pépin et al., 2005; Abou et al., 2014). Epidemic *Clostridium difficile* strains BI/NAP1/027 are characterized by higher production of toxin A and B, the presence of a third toxin, and high levels of resistance to fluoroquinolone antibiotics (O'Connor et al., 2009).

Since the identification of *C. difficile* in 1935, the standard therapy for treatment of this infection has been the use of antimicrobials, currently standard therapy for CDI is vancomycin and metronidazole (Bagasarian et al., 2015; Hall and O'Toole, 1935). Cure rates of CDI differ depending on the severity of the disease. During mild disease, either treatment is equally effective but during severe disease vancomycin is reported to increase cure rates compared to metronidazole, with equal recurrence rates independent of treatment (Zar et al., 2007; Surawicz et al., 2013). However, antibiotic treatment in addition to hospitalization and age (over 65) are among the top risk factors for acquiring CDI (Freeman and Wilcox, 1999; Owens et al., 2008). It has been shown that antimicrobial therapy alters the gut microbiota, causing a dysbiosis, and results in loss of colonization resistance, which allows for *C. difficile* colonization within the host (Reeves

et al., 2011; Buffie et al., 2012). Antimicrobials cause a global shift in the host's gut metabolic profile to one that favours *C. difficile* germination and growth (Theriot et al., 2014). Specifically, colonization resistance of the host is lost when antimicrobials used result in a depletion of secondary bile acid production, due to a devastating community shift in the large intestine that then promotes *C. difficile* colonization (Theriot et al., 2016). The strategy to cure CDI should focus more on restoring the indigenous microbiota rather than further perturbing and disrupting the gut microbiota with antibiotics.

FMT has proven to be an effective therapy for treating recurrent or refractory CDI (RCDI) (van Nood et al., 2013; Kassam et al., 2012; Eiseman et al., 1958; Tvede and Rask-Madsen, 1989; Fløtterød and Hopen, 1991; Lund-Tønnesen et al., 1998; Schwan et al., 1983; Persky et al., 2000; Bowden et al., 1981; Grzesiowski et al., 2015; Mattila et al., 2012). In 2013, a double blind placebo controlled trial to test FMT for use against RCDI showed FMT was more efficacious than vancomycin, the current standard treatment (van Nood et al., 2013). This trial was stopped early because it became unethical to allow patients to continue to receive vancomycin. These results have been repeated several times and RCDI recurrence rates following FMT remain low (Mattila et al., 2012). In addition to treating RCDI, FMT has been shown to be successful in intestinal decolonization for multiple multidrug-resistant bacterial infections including vancomycin resistant Enterococci (VRE), MRSA, and extended spectrum β -lactamase (ESBL) producing and carbapenemase producing Enterobacteriaceae (Manges et al., 2016). FMT can be administered through rectal retention enema, delivered to the colon by

colonoscopy, delivered to the small intestine through a nasogastric tube (NGT) by esophagogastroduodenoscopy, or delivered orally via frozen encapsulated stool pills (Youngster et al., 2014). Early studies have reported that FMT via colonoscopy or NGT have equivalent efficacy rates although recent studies are suggesting the rectal route may be more efficacious and safer (Postigo et al., 2012; Furuya-Kanamori et al., 2016).

1.5.3 FMT in CD and associated microbial changes

The first published report of using FMT for the treatment of newly diagnosed CD was in 1989 (Borody et al., 1989). The authors make note that patient symptoms commonly begin following a GI infection, are similar to CDI symptoms, and frequently resolve following the use of antimicrobials and bowel lavage. Due to these anecdotal clinical observations the notion of modulating the microbiota to modulate disease via FMT in IBD was introduced. Three separate case reports for the use of FMT in severe CD patients were reported in 2013 and 2014 and all case reports stated successful treatment and cessation of symptoms (Zhang et al., 2013; Gordon and Harbord, 2014; Kao et al., 2014). Only one case study described microbial changes within the lumen following FMT and showed, at both the phylum and genus level, that the community shifts towards the microbial profile of the donor (Kao et al., 2014). A case series consisting of 30 patients with refractory CD who underwent a single FMT via nasoduodenal tube indicated 76.7% (23/30) of patients entered into clinical remission post FMT at a 15-month follow up. However, this was not a controlled trial (Cui et al., 2015). The authors used complete shotgun metagenomics to conclude that both stool and the FMT preparation have a similar microbial composition (Cui et al., 2015). Not all reports on FMT in CD have been

positive however. Interestingly, in CD patients, FMT is less effective at clearing CDI than in non-IBD patients (Khoruts et al., 2016). Also, recently, the first case of extraintestinal manifestations following FMT in a patient with active CD was observed (Teich et al., 2016). As this report was a case study, it is hard to draw conclusions about this observation but caution for the use of FMT on active IBD patients is advised. This result could be due to a stimulatory effect on commensal microbiota, which could lead to other complications including altered intestinal permeability that may cause bacteraemia (Quera et al., 2014).

1.5.4 FMT in UC Cohort Studies and associated microbial changes

In 1989, Bennet and Brinkman reported the first case of the use of FMT for treatment of UC with successful results (Bennet et al., 1989). Following this case study, six patients with established UC (> 5 years of active disease) received 6 rectal enemas of 300 mL donor suspension and achieved clinical remission (defined as lack of clinical symptoms, absent inflammation during endoscopy, and normal histology scores) (Borody et al., 2003). In this study the microbiota was not characterized pre or post FMT. In 2013, another case series study was conducted to test the efficacy of FMT on UC patients but this time opposite results were observed. Angelberger and colleagues administered FMT through both nasojejunal tube and enema for three consecutive days, collectively infusing 300 mL of donor suspension per day per patient – this followed a 5-10 day course of broad-spectrum antibiotics (Angelberger et al., 2013). None of the five patients entered into remission, and only 1 displayed a positive clinical response. In terms of the patient's microbiota, all patients experienced a transient change towards the donor profile, with an

increase in phylotype richness. Interestingly, the patient that responded clinically experienced the most consistent change towards their donor profile. It is hard to draw conclusions based on a mix of case studies and case series reporting opposite results using differing FMT protocols. Randomized controlled trials can provide increased stringency in these variables and thereby offer greater insight into the use of FMT for treatment of UC (Laszlo et al., 2014; Kump et al., 2013; Suskind et al., 2015).

1.5.5 Donor specificity for treatment of CDI

Donor specificity for treatment of recurrent or refractory CDI has not been explicitly studied, and studies have not reported greater success rates due to particular donors. The efficacy of FMT for treatment of RCDI is also not affected by frozen storage of donor fecal matter, as tested in a double blind RCT by Lee and colleagues (Lee et al., 2016). Frozen, encapsulated fecal matter pills have also shown to be effective at curing RCDI (Youngster et al., 2014). Since most fecal suspensions are prepared in aerobic conditions and stored without consideration for maintaining the anaerobic community, the microbial community of the fecal suspension given to patients could be vastly different than the original microbial profile of the donor. This suggests that donor type does not play a role in successful FMT for treatment of CDI. In addition to this, 13 donors were used in the van Nood study in 2013, and there was not one donor whose use led to greater success rates over another, suggesting successful treatment of CDI through usage of FMT is independent of donor composition.

1.5.6 Anaerobic considerations for FMT

The majority of microbes in the GI tract are obligate anaerobes that provide an important function for the host – the digestion of glycans to short chain fatty acids via the fermentation process (Qin et al., 2010; Fischbach et al., 2011). There are two main oxygen gradients that directly influence the composition and subsequently the function of the gut microbiome. Oxygen levels reduce towards the distal gut, and within the intestines, oxygen increases towards the submucosa (Espey, 2013). During states of inflammation, gut epithelial cells enter a state of hypoxic stress, most likely due to recruited immune cells requiring the uptake of oxygen (Campbell et al., 2014). This reduces environmental oxygen levels at the epithelial layer, which disrupts epithelial cell absorption and barrier functions, known to be regulated by oxygen (Peterson and Artis, 2014). Inflammation results in a reversed oxygen gradient, oxygen is present at higher levels within the distal gut, and reduced towards the submucosa. This deregulated oxygen gradient, due to inflammation, has been observed in both non-IBD mouse models and mouse IBD models (Karhausen et al., Xue et al., 2013). As IBD is associated with reduced barrier function, disrupted epithelial cells, and a shift in the microbiome from obligate to facultative anaerobes and the appearance of aerobes within the colon, dysanaerobiosis, plays a large role in IBD pathogenesis (Rigottier-Gois, 2013).

Despite the large role that anaerobic bacteria and a homeostatic oxygen gradient contribute to the function of a healthy gut, most FMT protocols for treatment of IBD or CDI do not consider preserving donor anaerobic bacteria. However, one could argue, with increased oxygen levels within the lumen, the reestablishment of anaerobic bacteria may

not be feasible with an initial FMT treatment. Nonetheless, considering the important role anaerobes play in gut homeostasis, it is important to consider preserving obligate anaerobes for FMT treatment of IBD. Given the success rates of FMT for treatment of CDI, viable obligate anaerobes do not seem to play a role in the successful mechanism of FMT for CDI.

1.5.7 Mechanism of Action in FMT – Engraftment

CDI and IBD are two distinct diseases, one is an acute infection of the GI tract, the other a chronic inflammatory disease that is multifactorial – considering these stark differences, one can postulate that the mechanism of successful FMT will vary depending on the disease. For CDI, it is suggested that engraftment is a key mechanism. Khoruts and colleagues were one of the first to report microbial community changes following successful FMT in patients with CDI (Khoruts et al., 2010). Using terminal restriction fragment length polymorphism (TRFLP) the authors describe a shift in the patient's microbiota towards the donor. Prior to FMT treatment, there was a reduction in Firmicutes and Bacteroidetes, which dramatically changed following FMT to a community reflecting the donor's – enrichment of Bacteroides and uncharacterized butyrate producing bacterium. This characteristic shift towards the donor composition following successful FMT in CDI patients is commonly reported and has been defined as engraftment (van Nood et al., 2013; Shankar et al., 2014; Seekatz et al., 2014).

Although community composition changes have been reported, a shift towards the donor's microbiota is not enough evidence to suggest donor species are being transferred, and engrafting in the patient following successful FMT. Certainly, if displacement of an

abundant OTU occurs post FMT (*C. difficile*), a shift towards the donor profile would occur, but that does not provide evidence for engraftment. It is impossible to decipher if expansion of and OTU following FMT was transferred from the donor. Expansion of species following FMT could be from the indigenous microbiome prior to FMT. There are also limitations to examining engraftment at the OTU level, mostly the lack of resolution. Li and colleagues reported the most intriguing evidence for engraftment following FMT to date (Li et al., 2016). Single nucleotide variants (SNVs) from their respective donor, were detected following FMT in patients with metabolic syndrome. However, it is still unclear if engraftment occurs following FMT in CDI patients. The evidence to support engraftment post FMT in IBD patients is even less abundant than for CDI.

1.5.8 Mechanism of Action in FMT – Other possibilities

For treatment of CDI with FMT, bile acid levels within the gut have shown to play a possible mechanistic role. Weingarden and colleagues detected that levels of primary bile acids are present prior to FMT, which induced germination of *C. difficile* spores whereas, following FMT, secondary bile acid levels were detected and not able to induce germination and inhibited vegetative growth (Weingarden et al., 2016). Now, due to targeted metabolomics studies, there is strong evidence that restoration of bile acid metabolism occurs in CDI patients following FMT (Theroit et al., 2016). Competition, the displacement of *C. difficile*, could also be a key mechanism in successful FMT. This is evident by the success of a RCT to test the effect of non-toxic spores at reducing the risk

of recurrent CDI (Gerding et al., 2015). More studies are required to fully understand the mechanism of successful FMT for both CDI and IBD.

1.6 Thesis Scope

Inflammatory bowel diseases, consisting of both Crohn's disease (CD) and ulcerative colitis (UC), occur when a susceptible host mounts a chronic aberrant immune response to its commensal microbial community resulting in chronic inflammation. The incidence and prevalence of IBD has been increasing over time, with the highest rates occurring in Europe and North America. Although many genome-wide association studies (GWAS) on CD and UC have found loci associated with these diseases, studies conducted on monozygotic twins show low concordance rates, suggesting environmental causes play an important role in the onset of IBD. There is growing evidence that shows the intestinal microbiome in UC patients is different than in healthy individuals and may play a central role in IBD pathogenesis by inducing a chronic inflammatory response.

The overall goal of this thesis was to characterize the IBD microbiome in relation to disease status, severity, and response to therapy. I hypothesize that the IBD microbiome can be defined by a distinct microbial signature based on disease status, severity, and treatment successes. Defining dysbiosis in relation to disease status and response to therapy will help aid in the refinement of treatment protocols that aim to modulate the disease through manipulation of the microbiota.

1.6.1 Thesis aims

Aim 1: To describe the microbiome in inflammatory bowel disease.

Chapter 2: Fecal microbiota transplantation induces remission in patients with active ulcerative colitis in a randomized controlled trial.

Chapter 3: Case Report: Microbial changes and clinical findings following long-term fecal microbiota transplantation in a severe ulcerative colitis patient.

Chapter 4: Characterizing alterations of the intestinal microbiome following fecal microbiota transplantation in ulcerative colitis patients.

Chapter 5: Inflammation related differences in mucosa-associated microbiota and intestinal barrier function in colonic Crohn's disease.

Aim 2: To investigate the stool and mucosa-associated microbiome in patients with ulcerative colitis pre and post fecal microbiota transplantation.

Chapter 2: Fecal microbiota transplantation induces remission in patients with active ulcerative colitis in a randomized controlled trial.

Chapter 3: Case Report: Microbial changes and clinical findings following long-term fecal microbiota transplantation in a severe ulcerative colitis patient.

Chapter 4: Characterizing alterations of the intestinal microbiome following fecal microbiota transplantation in ulcerative colitis patients.

Aim 3: To characterize dysbiosis in inflammatory bowel diseases based on disease severity and status, and identify an associated microbial signature.

Chapter 3: Case Report: Microbial changes and clinical findings following long-term fecal microbiota transplantation in a severe ulcerative colitis patient.

Chapter 4: Characterizing alterations of the intestinal microbiome following fecal microbiota transplantation in ulcerative colitis patients.

Chapter 5: Inflammation related differences in mucosa-associated microbiota and intestinal barrier function in colonic Crohn's disease.

Aim 4: To investigate the spatial relationship between clinical inflammation, dysbiosis, and impaired barrier function in CD.

Chapter 5: Inflammation related differences in mucosa-associated microbiota and intestinal barrier function in colonic Crohn's disease.

CHAPTER 2

FECAL MICROBIOTA TRANSPLANTATION INDUCES REMISSION IN PATIENTS WITH ACTIVE ULCERATIVE COLITIS

Fecal microbiota transplantation induces remission in patients with active ulcerative colitis

Moayyedi P¹, Surette MG¹, Kim PT^{2,3}, Libertucci J¹, Wolfe M¹, Onischi C³, Armstrong D¹, Marshall JK¹, Kassam Z⁴, Reinisch W¹, Lee CH³

1. Farncombe Family Digestive Health Research Institute, Department of Medicine, McMaster University, Hamilton, Ontario, Canada
2. Department of Mathematics and Statistics, University of Guelph, Guelph, Ontario, Canada
3. Department of Pathology and Molecular Medicine, St Joseph's Healthcare, McMaster University, Hamilton, Ontario, Canada
4. Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA, United States

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Preface: All authors contributed to the design of the study. PM and MW were responsible for the recruitment and assessment of most of the participants with support from DA, JKM, WR, and ZK. FMT was administered by CHL and CO and the fecal microbiome extraction and analyses were conducted by MGS and JL (I produced figures 3-5). PTK conducted all other analyses. PM wrote the initial draft of the manuscript with all authors providing interpretation of the analyses and contributions to the final draft. All authors were involved with addressing concerns from reviewers.

Abstract

BACKGROUND & AIMS: Ulcerative colitis (UC) is difficult to treat, and standard therapy does not always induce remission. Fecal microbiota transplantation (FMT) is an alternative approach that induced remission in small series of patients with active UC. We investigated its safety and efficacy in a placebo-controlled randomized trial.

METHODS: We performed a parallel study of patients with active UC without infectious diarrhea. Participants were examined by flexible sigmoidoscopy when the study began and then were randomly assigned to groups that received FMT (50 mL, via enema, from healthy anonymous donors; n 38) or placebo (50 mL water enema; n 37) once weekly for 6 weeks. Patients, clinicians, and investigators were blinded to the groups. The primary outcome was remission of UC, defined as a Mayo score 2 with an endoscopic Mayo score of 0, at week 7. Patients provided stool samples when the study began and during each week of FMT for microbiome analysis. The trial was stopped early for futility by the Data Monitoring and Safety Committee, but all patients already enrolled in the trial were allowed to complete the study.

RESULTS: Seventy patients completed the trial (3 dropped out from the placebo group and 2 from the FMT group). Nine patients who received FMT (24%) and 2 who received placebo (5%) were in remission at 7 weeks (a statistically significant difference in risk of 17%; 95% confidence interval, 2%33%). There was no significant difference in adverse events between groups. Seven of the 9 patients in remission after FMT received fecal material from a single donor. Three of the 4 patients with UC 1 year entered remission,

compared with 6 of 34 of those with UC >1 year ($P = .04$, Fisher's exact test). Stool from patients receiving FMT had greater microbial diversity, compared with baseline, than that of patients given the placebo ($P = .02$, Mann-Whitney U test).

CONCLUSIONS: FMT induces remission in a significantly greater percentage of patients with active UC than placebo, with no difference in adverse events. Fecal donor and time of UC appear to affect outcomes.

ClinicalTrials.gov Number: NCT01545908.

Keywords: IBD; Treatment; Microbe; Colon.

Introduction

Ulcerative colitis (UC) is a chronic inflammatory disorder of the colorectum that has a peak incidence in early adulthood (Kappelman et al., 2007). The cardinal symptom of UC is bloody diarrhea (Danese and Fiocchi, 2013), which is associated with a significant reduction in quality of life (Graff et al., 2009). The etiology of the disease is unknown, but it is thought to arise from an aberrant immune response to a change in colonic environment in a genetically susceptible individual (Danese and Fiocchi, 2013; Ford et al., 2013). Current medical treatment remains imperfect (Talley et al., 2011) and a significant minority of patients need colectomy to manage their disease (Sandborn et al., 2009). There have been advances in therapy (Fegan et al., 2013) but gains have been modest. The focus of drug development has been on altering the immune response (Danese, 2012) rather than reducing factors that stimulate the aberrant immune response (Torres et al., 2013). A likely candidate that could drive the immune response in UC is the colonic microbiome, as this is altered in patients with the disease compared with healthy controls (Michail et al., 2012) and animal models of colitis require gut bacteria to induce inflammation (Nell et al., 2010). Fecal microbiota transplantation (FMT) has emerged as a novel approach to altering the colonic microbiome and can successfully treat antibiotic-resistant *Clostridium difficile* colitis (van Nood et al., 2013; Moayyedi et al., 2014). The concept of FMT has captured the imagination of the public, and this approach is being advocated for a number of diseases, including UC. The efficacy of FMT is unclear in other situations and there have only been a few case reports of FMT in

UC, with conflicting results (Anderson et al., 2012; Angelberger et al., 2013). We report the first randomized trial of FMT to treat active UC.

Materials and Methods

Study Design

This is a double-blind randomized controlled trial of FMT vs placebo in active UC conducted in Hamilton Health Sciences, St Joseph's Healthcare Hamilton, and McMaster University, Hamilton, Canada. The local research ethics committee at McMaster University approved the trial and Health Canada had no objection to the use of FMT for this study. All participants provided written informed consent and an independent Data Monitoring and Safety Committee evaluated the trial annually.

Study Population

Eligible patients were 18 years or older with active UC defined as a Mayo Clinic score¹⁶ ≥ 4 with an endoscopic Mayo Clinic score ≥ 1 . Concomitant treatments for UC, such as mesalamine, glucocorticoids, immunosuppressive therapy (eg, azathioprine), or tumor necrosis factor antagonists were permitted, provided these had been used at a stable dose for at least 12 weeks (4 weeks for glucocorticoids) and disease remained active. Five patients who were previously exposed to topical mesalamine or steroids had a 30-day washout period before being enrolled. Patients were excluded if they had taken antibiotics or probiotics in the last 30 days, had concomitant *C difficile* infection or another enteric pathogen, had a disease severity that required hospitalization, were pregnant, or were unable to give informed consent.

Baseline Assessments

Potentially eligible patients were scheduled for a flexible sigmoidoscopy and also completed baseline questionnaires to obtain demographic information, Mayo score, (D'Haens et al., 2007) Inflammatory Bowel Disease Questionnaire score (a validated disease specific quality of life measure; range of scores from 0 to 224 with higher score indicating better quality of life), (Irvine et al., 1994) and EuroQol (EQ-5D) score (a validated general quality of life measure; range score 0 to 100 with higher score indicating better quality of life) (Konig et al., 2002). Blood samples were drawn for inflammatory markers (complete blood count, erythrocyte sedimentation rate, and C-reactive protein) and serology for human immunodeficiency virus; hepatitis A, B, and C; and syphilis. Stool samples were provided by the participant and were screened for ova, cysts, and parasites, as well *C difficile* toxin gene tested by polymerase chain reaction. In addition, stool samples were collected in a sterile, airtight container for microbiota assessment.

Randomization

Eligible patients were randomized 1:1 according to a computer-generated randomization list that was stratified for patients with UC diagnosed within 1 year. The randomization was held centrally at the McMaster Gastroenterology Clinical Trials Unit to ensure concealment of allocation. The treatment location was masked to the patient, health care workers caring for the patient, and investigators. The technician administering FMT or placebo was aware of the treatment being administered, as the nature of the intervention meant that it was not possible to make it identical to the placebo.

Interventions and Follow-Up

FMT was prepared from stool donated by volunteers who were between 18 and 60 years of age and were otherwise healthy, as assessed by a screening questionnaire (see [Supplementary Material](#)). Donor stool was screened for enteric pathogens, including *Salmonella*, *Shigella*, *Campylobacter*, *Escherichia coli* O157 H7, *Yersinia*, as well as ova, cysts, and parasites and *C difficile* toxin. The donor had to have negative serology for human immunodeficiency, hepatitis A IgM, hepatitis B surface antigen, hepatitis C antibody, syphilis, and human T- lymphotropic virus and be screened negative for vancomycin-resistant *Enterococcus* or methicillin-resistant *Staphylococcus aureus*. All donors were prospectively screened and rescreened every 6 months. Stool was retested whenever a donor returned from travel outside of North America. The donor could not provide stool samples if they had taken antibiotics in the previous 3 months. Donor stool was delivered for processing within 5 h of collection. Fifty grams of donor stool was collected in the preweighed container and mixed with 300 mL commercial bottled drinking water. The mixture was emulsified for 3 to 5 minutes using a clean wooden/plastic spatula and then allowed to settle for 5 minutes. Filter paper was placed over the mixture until the supernatant filtered to the top and this was then decanted using a 60-mL syringe. The supernatant was either administered immediately to the patient or stored at -20°C .

Participants were randomized to receive 50 mL FMT or placebo consisting of 50 mL water given as a retention enema once per week for 6 weeks. The enema was administered with the patient in the left lateral position with instructions to retain this for at least 20 minutes. Patients provided stool samples each week before receiving their

retention enema and samples were stored at -20°C for fecal microbiota analysis. The donor samples that were analyzed were those that were given to the patient. If the samples were frozen-thawed, it was the thawed sample that was sent for microbiome analysis. Adverse events were assessed at every weekly visit and the intervention was administered in a different hospital from where the patient was assessed for response to therapy. Participants returned to complete a further Mayo Clinic score, Inflammatory Bowel Disease Questionnaire, EQ-5D, and have an exit flexible sigmoidoscopy at week 7 (± 3 days). One investigator (PM) performed all flexible sigmoidoscopies at baseline and week 7, with the exception of 2 baseline sigmoidoscopies and 1 exit sigmoidoscopy (performed by JKM). Two rectal, sigmoid, and descending colon biopsies were taken for histology at baseline and at week 7.

Clinical Outcomes

The primary outcome was UC remission at week 7, defined as a full Mayo score < 3 and complete healing of the mucosa at flexible sigmoidoscopy (endoscopic Mayo score = 0). Secondary outcomes included improvement in UC symptoms (defined as ≥ 3 improvement in full Mayo score), as well as change in Mayo, Inflammatory Bowel Disease Questionnaire, and EQ-5D scores. There was a 12-month follow-up phase of the trial planned with re-randomization to either open label no therapy or FMT once per month for 12 months. This part of the trial was discontinued, as there were insufficient patients entering remission at week 7. We did, however, record changes in UC medication, serious adverse events, hospitalization for UC and colectomies, as well as partial Mayo score at month 12.

Assessment and Analysis of the Microbiome

Microbial community profiling was conducted by extracting genomic DNA from patient and donor stool samples using a protocol described previously (Whelan et al., 2014) which enhances total DNA recovery. After genomic DNA extraction, the V3 region of the 16s ribosomal RNA gene was amplified (total polymerase chain reaction volume of 50 μ L [25 pmol of each primer, 200 μ L each deoxynucleoside triphosphate, 1.5 mM MgCl₂, and Taq polymerase] divided into triplicate for greater efficiency). Samples were sequenced using the Illumina MiSeq platform, as per manufacturer's instructions and raw sequence reads were processed as described previously (Bartram et al., 2011). The stool microbiota was sampled to a mean depth of 103,341 reads per sample (median = 87,509). Beta diversity (Bray-Curtis dissimilarity) was calculated using the Phyloseq R package (McMurdie et al., 2013) and statistical differences between pairwise comparisons were calculated using a Mann-Whitney U test, with significance defined as $P < .05$. Relative abundance and taxonomic profiles were computed using Quantitative Insights Into Microbial Ecology (Caporaso et al., 2010).

Statistical Analysis

We aimed to recruit 130 active UC patients (65 in each arm) using sample size calculations that assumed a 25% remission rate in the placebo arm (Ford et al., 2011) and a 50% remission rate in the FMT arm, using 80% power and 5% significance, and assuming a 10% attrition rate (Ford et al., 2011). Pearson's χ^2 or Fisher's exact test was used to analyze the primary outcome of the proportion of patients in remission at study completion. The primary analysis was intention to treat with an evaluable patient analysis

also planned. All other categorical data were analyzed using Pearson's χ^2 or Fisher's exact test. Continuous data were analyzed using t tests and linear regression, with the baseline value being one of the covariates. Logistic regression and least absolute shrinkage and selection operator were used to evaluate independent predictors of response to therapy. All analyses were conducted using R version 3.1.0. PM, MW, and PTK had full access to the data and PM was responsible for submitting the manuscript.

Results

Patients and Termination of the Trial

Patients were recruited during an 18-month period commencing in July 2012. The Data Monitoring and Safety Committee reviewed the data at the approximate 50% recruitment point of the trial and, at that time, there were 4 of 27 patients in remission in the FMT arm and 2 of 26 in the placebo arm. The Data Monitoring and Safety Committee advised that the trial should be discontinued for futility because the primary end point was unlikely to be achieved as specified in the protocol. They also recommended that patients already enrolled in the trial should complete their allocated therapy. At the point that the Data Monitoring and Safety Committee made this decision, there were 22 participants that had been screened and were eligible and awaiting entry to the trial or were already receiving intervention, but had not yet completed the trial. Overall, 85 patients were considered for the trial, 75 were randomized and 70 completed therapy ([Figure 1](#)). Baseline characteristics of the participants were similar, although those allocated to placebo were slightly older, with a female preponderance ([Table 1](#)).

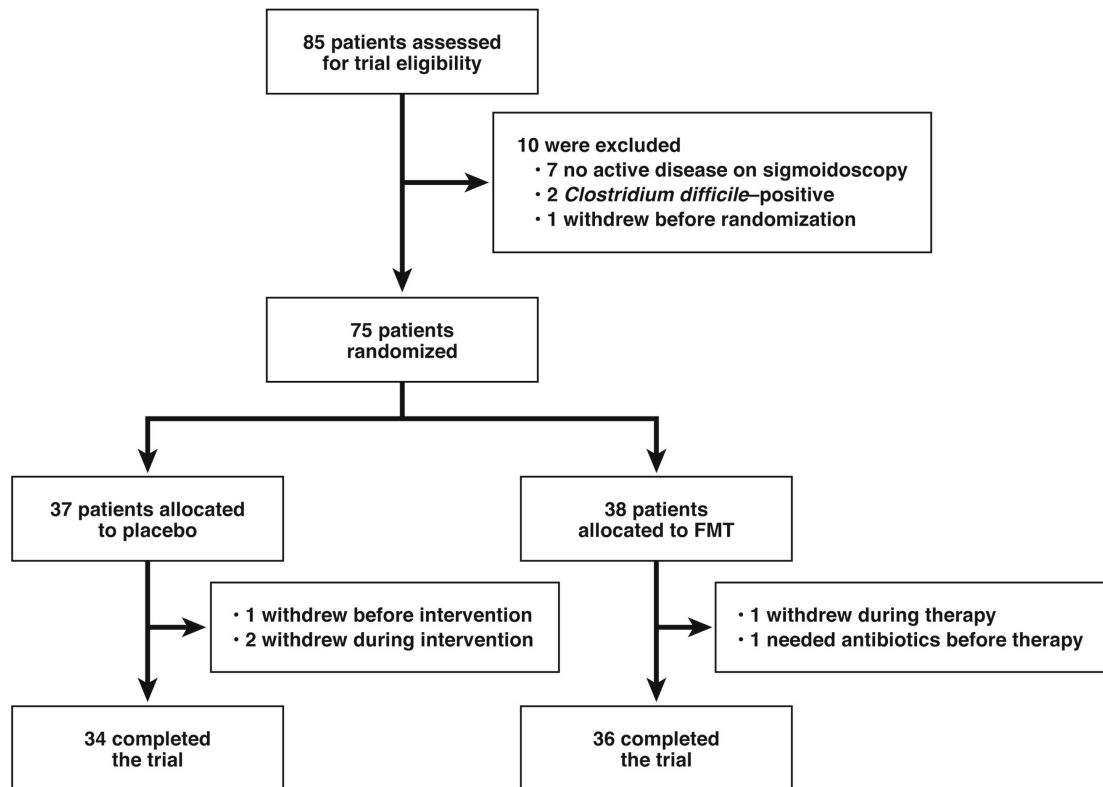


Figure 1: Flow of patients in the trial 1

Table 1: Baseline Characteristics of Patients in the Trial

Variable (denominator: placebo or FMT)	Placebo (n = 37)	FMT (n = 38)
Age, y (37, 38)	35.8 ± 12.1	42.2 ± 15.0 ^a
Male sex, n (%) (37, 38)	26 (70)	18 (47) ^b
White race, n (%) (37, 38)	29 (78)	36 (95)
Nonsmoker, n (%) (37, 38)	21 (57)	19 (50)
UC <1 year, n (%) (37, 38)	4 (11)	4 (11)
Pancolitis, n (%) (30, 36)	12 (37.5)	20 (62.5)
Concomitant medications, n (%)		
Mesalamine therapy (37, 38)	20 (54)	21 (55)
Glucocorticoids (37, 38)	13 (35)	15 (39)
Immunosuppressants (37, 38)	6 (16)	11 (29)
Anti-TNF therapy (37, 38)	2 (5)	5 (13)
Years had UC (37, 38)	7.0 ± 6.8	7.9 ± 5.6
Hemoglobin concentration, g/L (37, 37)	128.6 ± 22.4	129.3 ± 17.3
White cell count, ×10 ⁹ /L (37, 37)	8.8 ± 2.6	8.0 ± 2.5
ESR, mm/h (27, 26)	21.1 ± 16.3	18.9 ± 15.6
CRP, mg/L (27, 26)	7.2 ± 7.7	10.6 ± 16.6
High ESR, n (%) (27, 26)	14 (52)	8 (31)
High CRP, n (%) (27, 26)	13 (48)	11 (42)
Full Mayo Clinic score (37, 38)	7.86 ± 2.28	8.24 ± 2.61
IBDQ score (37, 37)	134.4 ± 32.3	130.3 ± 36.3
EQ-5D score (37, 36)	78.2 ± 15.4	75.7 ± 20.4

NOTE. All values are mean ± SD unless otherwise stated, with missing values not counted. Race, pancolitis, years, hemoglobin, white cell count, ESR, CRP, IBDQ, had missing values in the range of 1 to 23. All $P > .05$ except where indicated.

CRP, C-reactive protein (high CRP >5 mg/L); ESR, erythrocyte sedimentation rate (high ESR >20 mm/h); FMT, fecal microbial transplantation; IBDQ, Inflammatory Bowel Disease Questionnaire; TNF, tumor necrosis factor; UC, ulcerative colitis.

^a $P = .045$.

^b $P = .044$.

Donors

We initially recruited two donors (donor A and B) to provide FMT for the study to minimize the variability of the intervention and aid interpretation of the microbiome analysis. However, after the first 17 participants were enrolled, donor B was prescribed antibiotics and did not donate for 4 months. Four other donors' (donor C to F) stools were used during this period, which ended shortly before the Data Monitoring and Safety Committee's assessment. The remaining participants allocated to active therapy all received FMT from donor B exclusively, as we had not experienced any success with donor A ([Figure 2](#)). In all but one case, donors were anonymous, but 1 patient requested their spouse be used as a donor. This person was randomized to active therapy and was a treatment failure. Twenty-one patients received frozen-thawed stool, 15 patients received fresh stool, and 1 patient was given both fresh and frozen stool on different weeks.

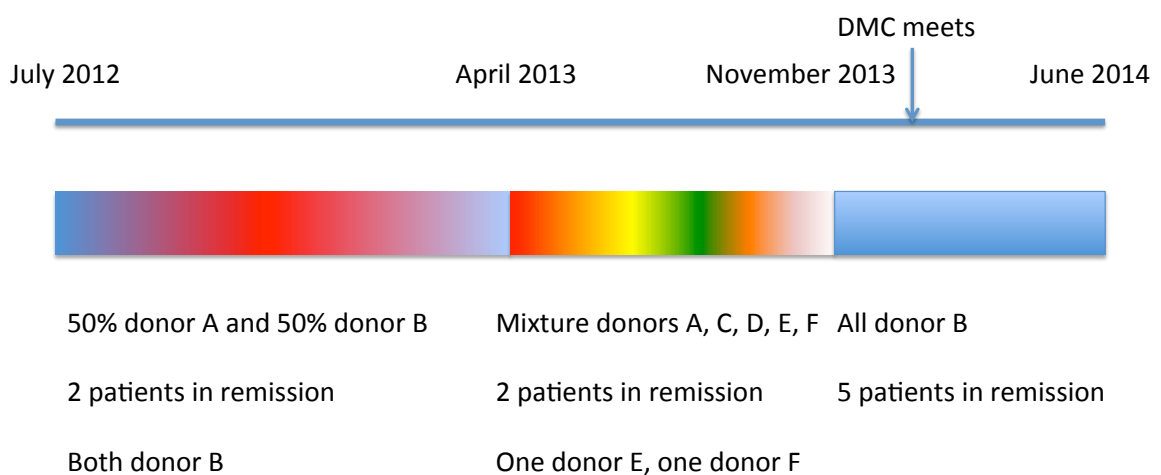


Figure 2: Description of the timeline of the use of donors in the trial.

DMC, Data monitoring committee.

Clinical Outcomes

Overall, there was a statistically significant effect of FMT on inducing remission in UC, with 9 of 38 (24%) patients in the FMT arm vs 2 of 37 (5%) in the placebo arm in remission at the end of treatment ($P = .03$, Pearson's χ^2 ; [Table 2](#)). There were no patients with an endoscopic Mayo score = 0 and a total Mayo score ≥ 3 , so an end point of an endoscopic Mayo score = 0 gave the same result.

Table 2: Outcome Measures Comparing Fecal Microbial Transplantation With Placebo

Outcome	Placebo (n = 37)	FMT (n = 38)	P value
Clinical remission, ^a n (%)	2 (5)	9 (24)	.03
Clinical response, ^b n (%)	9 (24)	15 (39)	.16
Full Mayo score	6.34	6.09	.42
IBDQ score	149.38	152.13	.44
EQ-5D score	70.07	68.52	.99
CRP, mg/L (n = 17 placebo, n = 15 FMT)	3.3 \pm 3.4	4.9 \pm 5.9	.38
ESR, mm/h (n = 17 placebo, n = 15 FMT)	13.1 \pm 11.2	15.9 \pm 17.0	.59
Proportion with high ESR, n (%)	4 (24)	3 (20)	1.0
Proportion with high CRP, n (%)	5 (29)	2 (13)	.40
Patients with serious adverse events n (%)	2 ^c (5)	3 ^d (8)	1.0

NOTE. All continuous data evaluated using analysis of covariance adjusting for baseline value are mean \pm SE. Full Mayo and IBDQ had missing values, which were replaced by their means. The Full Mayo, IBDQ, and EQ-5D are adjusted means for baseline, and P value is the significance of the randomization to placebo or FMT. All analyses are intention to treat.

CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; IBDQ, Inflammatory Bowel Disease Questionnaire.

^aDefined as full Mayo Clinic score <3 and an endoscopic Mayo Clinic score = 0.

^bDefined as a reduction in full Mayo clinic score of at least 3 points.

^cOne patient had the diagnosis changed to Crohn's colitis and one was admitted to hospital with active ulcerative colitis.

^dTwo patients had their diagnoses changed to Crohn's colitis and one was *Clostridium difficile* toxin positive at the end of therapy.

The proportion of patients with improvement in symptoms and the various quality of life scores were not statistically significant ([Table 2](#)). There was no difference in serious adverse events between the FMT and placebo groups ([Table 2](#)). We evaluated factors that might predict treatment success using logistic regression and least absolute shrinkage and selection operator. Of the 19 covariates, 10 were chosen by least absolute shrinkage and selection operator with randomization being one of them. When we ran logistic regression on these covariates, randomization was the only statistically significant positive covariate ($P = .05$, [Supplementary Table 1](#), [Supplementary Table 2](#), [Supplementary Table 3](#), [Supplementary Table 4](#), [Supplementary Table 5](#), [Supplementary Table 6](#), [Supplementary Table 7](#), [Supplementary Table 8](#), [Supplementary Table 9](#)). There were also no statistically significant differences in change scores between groups ([Supplementary Table 10](#)).

We also assessed the histology of patients who were in remission at week 7 in a post-hoc analysis. Seven of the patients in remission in the FMT arm had no active inflammation in any biopsy, and the other 2 patients had mild patchy inflammation in the rectum with no active inflammation in sigmoid and descending colon biopsies (both had inflammation on histologic assessment at these sites at baseline) at week 7. One of the 2 patients that were in remission with placebo had no active inflammation in any biopsy, and the other had mild active inflammation in all biopsies.

There were 38 patients randomized to FMT. Treatment successes attributable to donor B were 7 of 18 (39%) vs 2 of 20 (10%) with other donors ($P = .06$, Fisher's exact test), suggesting statistical evidence for donor dependence. Other notable features

included a trend toward those taking immunosuppressant therapy to have a greater benefit from FMT (5 of 11 [46%] in those taking immunosuppression vs 4 of 27 [15%] in those not on immunosuppressive therapy; $P = .09$, Fisher's exact test). Patients with a recent diagnosis of UC (defined as ≤ 1 year) were statistically significantly more likely to respond to FMT (3 of 4 [75%] compared with 6 of 34 [18%] in those with chronic disease; $P = .04$, Fisher's exact test). There was also a trend for frozen stool to have greater efficacy than fresh stool, although most of the treatment successes were also using donor B ([Supplementary Table 9](#)). We evaluated factors that might predict treatment success in the FMT arm, and there was no impact of sex, age, Mayo score at baseline, disease extent, smoking history, current steroid therapy, or tumor necrosis factor antagonist therapy on treatment success ([Supplementary Table 2](#), [Supplementary Table 3](#), [Supplementary Table 4](#), [Supplementary Table 5](#), [Supplementary Table 6](#), [Supplementary Table 7](#), [Supplementary Table 8](#)).

Five patients had significant adverse events. One person in the placebo group developed worsening colitis and was admitted to hospital 3 weeks into the trial and had an urgent colectomy. Three patients in the trial (2 in the FMT group and 1 in the placebo group) developed patchy inflammation of the colon and also rectal abscess formation, which resolved with antibiotic therapy. One patient in the FMT group with worsening abdominal discomfort tested positive for *C difficile* toxin after study exit.

We have followed up the 37 patients that received FMT in this randomized trial for 12 months, although in 5 patients, follow-up has only been for 9–11 months and 6 patients failed to respond. It is important to note that patients were informed of their

randomization status once all trial information on that patient was obtained, so these data are open label. Eight of the 9 patients that were in remission at week 7 on FMT have remained in remission at week 52 without any relapse in their symptoms. Two of these patients have only been followed up for 9–11 months. Four patients have elected to stop all their UC medications (1 patient on mesalamine, 1 on long-term corticosteroids, 1 on both mesalamine and azathioprine, and 1 on infliximab) and have remained remission free to date. Three of these patients have been receiving FMT once per month (2 electively and 1 as part of the trial that was discontinued). One patient experienced a relapse of their symptoms after taking a course of antibiotic therapy and elected not to have any further FMT therapy. This patient started infliximab and still has some UC symptoms, with a partial Mayo score of 5. Eleven patients believed their symptoms were improving in the trial and when informed that they had been randomized to FMT elected to have further open-label FMT therapy for 6–12 weeks. Four of these patients went into remission with no inflammation at additional 6- to 12-week flexible sigmoidoscopy, and all have remained in remission at week 52. The remaining 20 patients had no further FMT therapy and 14 of these have completed follow-up questionnaires. One patient had a colectomy for failure of UC medical therapy, but there was no significant change in the disease status of the other participants.

Microbiome Outcomes

Patients were separated into their respective cohort (FMT treatment [active] and placebo) and community similarity (or stability) over time was computed using pairwise comparisons of week 0 to week 6. There was a statistically significant change in

microbiota composition with more diversity in the treatment group compared with the placebo group at week 6 vs baseline ($P = .02$, Mann-Whitney U test) (Figure 3). When similarity was compared between the active cohort after FMT (week 6) and their respective donors, there was a statistically significant effect of the active therapy group being more similar to their donor than a control fecal sample ($P = .04$, Mann Whitney U test; Figure 4A). Taxonomic profiles of the donors highlighted distinct microbial differences between the 2 most common donors (A and B) used in this study (Figure 5). Notable differences include a significant enrichment for the family Lachnospiraceae and the genera *Ruminococcus* in donor B, and donor A displayed enrichment for the genera *Escherichia* and *Streptococcus*. Notably donors B and F had similar profiles and both were associated with successful FMT. As most of the responders received donor B, we evaluated whether responders in this group had more similarity at the end of therapy with donor B than nonresponders. There was a trend for responders having microbiota that was more similar to donor B than nonresponders, but this did not achieve statistical significance ($P = .07$, Mann Whitney U test) (Figure 4B).

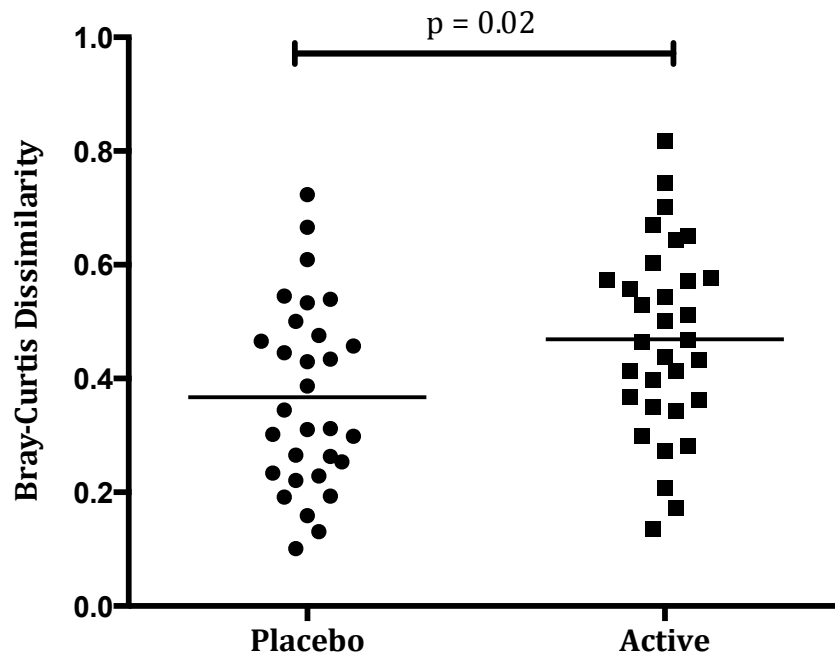


Figure 3: Similarity of microbial composition in those receiving FMT versus placebo at the start and end of the trial.

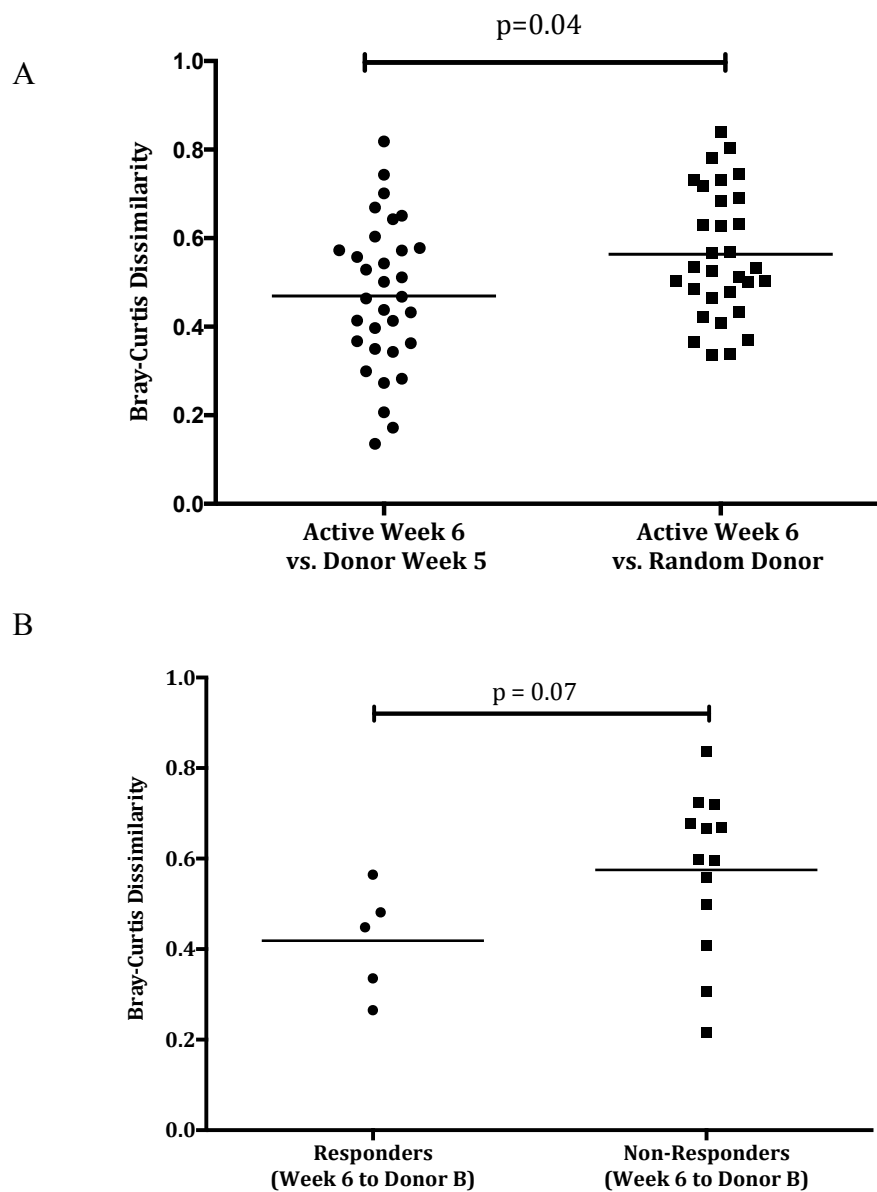


Figure 4: Dissimilarity of the microbial composition compared to donor.

Panel A = all patients receiving FMT compared to the donor they received versus different donor.

Panel B = Similarity of patients to donor B in patients receiving that donor in those that did and did not experience remission at week 7.

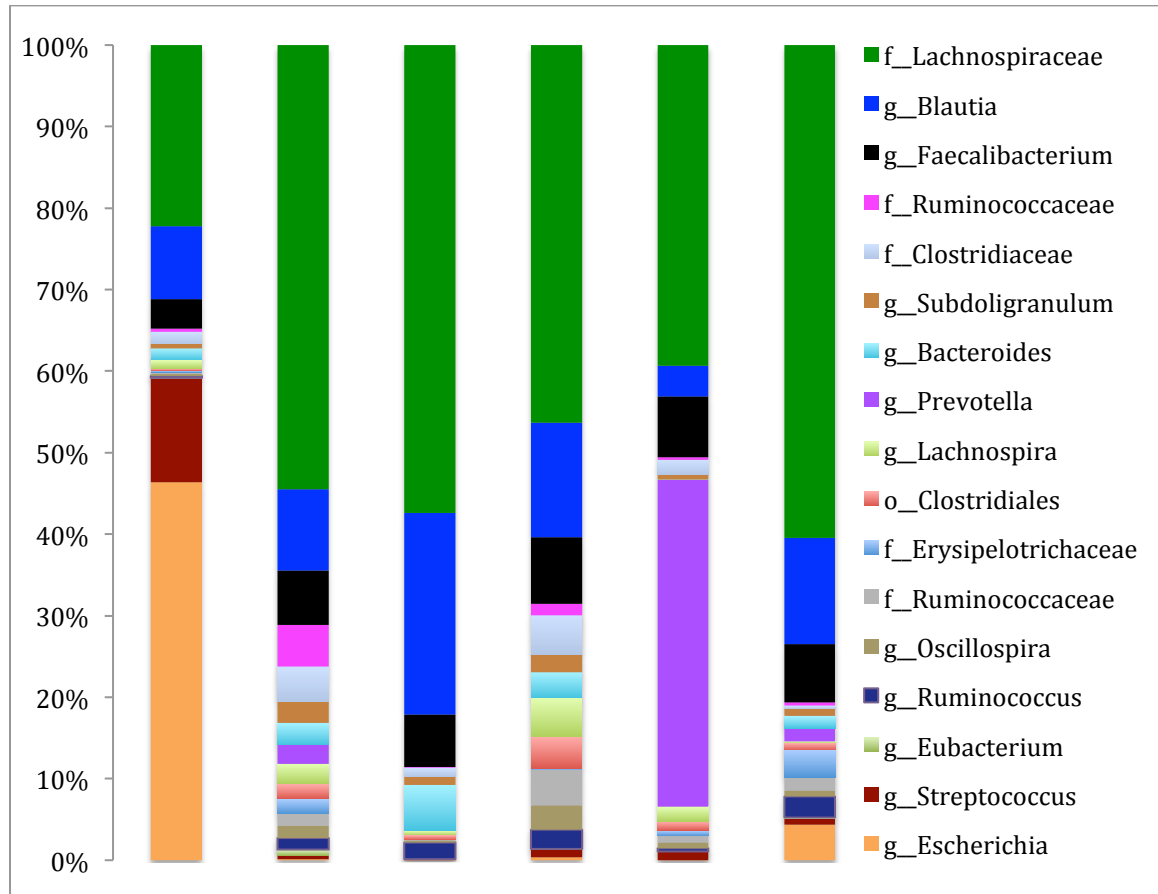


Figure 5: Composite donor profiles for donors used in the study

Discussion

This is the first randomized, placebo-controlled trial, to evaluate the efficacy of FMT in active UC and suggests that this approach induces remission in a statistically significant proportion of cases. FMT may be more efficacious in patients with a recent diagnosis of UC, and this is biologically plausible, as a perturbation in the microbiome might be more easily restored early in the course of the disease. The efficacy of this approach may also be donor dependent and this may explain why some case series have shown promise (Anderson et al., 2012), and others have had disappointing results (Angelberger et al., 2013). The benefit was relatively modest, but our end point for treatment success was more stringent than most trials in UC (Ford et al., 2011) and remission rates seen with FMT were consistent with a similar end point for a novel biologic therapy (Fegan et al., 2013). The secondary end points were not met in this trial. Softer end points often have higher placebo response rates (Talley et al., 2011), and this can limit the power to show a statistically significant difference between active therapy and placebo (Reinisch et al., 2011). There was also no statistically significant effect of FMT over placebo on disease-specific or general quality of life. Quality of life is multidimensional and influenced by other factors, as well as UC activity, and this might explain why there was no significant impact of FMT on these outcome measures (Reinisch et al., 2011).

There are a number of questions on the administration of FMT that remain unanswered. Many protocols recommend giving bowel lavage before donor feces administration and also giving antibiotics before the transplantation (Kassam et al., 2013)

to facilitate the colonization of microbiota from the donor. We did not adopt this in the trial FMT protocol, as currently there is a paucity of data to support this practice (Brandt, 2013), and if colonic lavage and/or antibiotic therapy had any impact on UC activity (Khan et al., 2011), this would complicate the interpretation of the study. There is also uncertainty as to whether fresh or frozen–thawed fecal samples should be used for FMT (Borody et al., 2014). Data from patients with antibiotic-resistant *C difficile* suggest that although the frozen stool may have a different microbiota distribution, both are equally efficacious (Hamilton et al., 2012; Youngster et al., 2014), and there was no difference in outcome seen in this study. There was a trend for frozen–thawed stool to perform better, although evaluation of this is confounded by the fact that most of the treatment successes were also related to donor B. Studies that have evaluated FMT in *C difficile* and in inflammatory bowel disease have used a variety of routes of administration, including retention enemas, colonoscopy, and the nasoduodenal route (Anderson et al., 2012; Angelberger et al., 2013). We chose the retention enema approach, as one systematic review (Kassam et al., 2013) of the *C difficile* literature suggested this was more effective than the nasoduodenal route, and also UC is characterized by a disorder that commences in the rectum but can extend the length of the large bowel (Ford et al., 2013). We speculate that the dysbiosis of UC (Rajilic-Stojanovic et al., 2013) is therefore likely to start in the rectum and it is plausible that this is best targeted by retention enema. It is interesting to note that patients with pancolitis due to *C difficile* colitis have been effectively treated by FMT using a retention enema (Kassam et al., 2012). We also chose the rectal route for safety reasons. A systematic review of the literature suggests that the

nasojejunal approach might have more adverse events (Kassam et al., 2013) and, in a small case series (Talley et al., 2011) all patients were given FMT by the nasojejunal route and developed a fever and rise in C-reactive protein.

A limitation of this trial is that we did not perform colonoscopy at study exit and it is possible that patients with extensive colitis have active disease beyond the limit of the flexible sigmoidoscope. It is, however, unlikely that they would be symptom free and 1 patient with extensive colitis that had successful FMT did require colonoscopy for screening purposes after exiting the trial and had no active inflammation throughout the colon to the cecum either endoscopically or histologically ([Supplementary Figure 1](#)). This is the largest randomized trial of FMT in any disease and, in addition, the number of patients enrolled in the trial is double that seen in a systematic review of all case series of FMT in UC (Anderson et al., 2012). Nevertheless, the sample size of the trial is modest and the magnitude of the effect of FMT in UC remains uncertain. The trial was also stopped early, as we did not anticipate the extent of positive effect seen at completion of the study. This may relate to the effect being donor dependent and it is notable that donor B was not available in the time leading up to the Data Monitoring and Safety Committee meeting and all patients subsequent to this were treated with donor B.

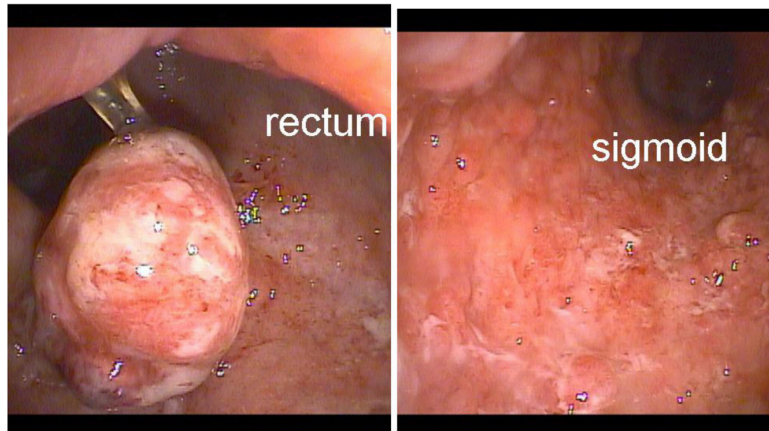
In conclusion, this is the first randomized trial to demonstrate efficacy of FMT in UC. Many questions remain, but this provides interesting data suggesting that altering the gut microbial flora may be promising for treating UC.

Acknowledgments

This trial was supported by grants from Hamilton Academic Health Sciences Organization (HAHSO) and Crohn's and Colitis Canada (CCC). The authors are grateful to Lisa Dunlop and Rita Taraschi for clerical support of the trial and to Dr Ken Croitoru (chair), Josh Neufeld, and Noori Akhtar-Danesh for the services as the Data Monitoring and Safety Committee.

Supplementary Materials

Before therapy



After therapy (week 20)

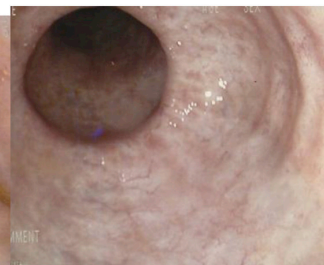
Rectum



Sigmoid



Tranverse colon



Cecum

Supplementary Figure 1: Endoscopy findings pre and post FMT

Supplementary Table 1: Summary of the Logistic Regression Model to Assess Independent Factors Predicting Remission

Logistic regression model:			
Explanatory variables: The following are the variables used in this study and described in Table 1. The units, missing values, and imputation of missing values are described below.			
Age: years; complete			
Sex: 1 = male, 2 = female; complete			
Race: 1 = Caucasian, 2 = Southeast Asian, 3 = other Asians, 4 = Afro-Caribbean, 5 = Middle Eastern, 6 = Other; complete			
Smoke: 1 = nonsmoker, 2 = former smoker; 3 = current smoker, 4 NAs changed to 1			
UC < 1 year: 0 = No, 1 = Yes; complete			
Pancolitis: 0 = No, 1 = Yes; 11 NAs changed to 0			
Mesalamine: 1 = Yes, 2 = No; complete			
Glucocorticoids: 1 = Yes, 2 = No; complete			
Immunosuppressants: 1 = Yes, 2 = No; complete			
Anti-TNF: 1 = Yes, 2 = No; complete			
UC: years, complete			
Hemoglobin: g/L, 1 NA replaced with mean			
White cell count: 10 ⁹ /L, 1 NA replaced with mean			
ESR: mm/h, 22 NAs replaced with mean			
CRP: mg/L, 22 NAs replaced with mean			
Full Mayo: score, complete			
IBDQ: score, 1 NA replaced with mean			
EQ5D: score, complete			
Randomization: 1 = placebo, 2 = FMT, complete			
LASSO: The R package “glmnet” was used on the above explanatory variables for logistic regression. Of the 19 explanatory variables, LASSO using cross-validation selected: age, race, UC <1, pancolitis, immunosuppressants, hemoglobin, C-reactive protein, Full Mayo, and randomization. The intercept was also chosen and the dependent variable was remission.			
Logistic regression: From these selected explanatory variables, we ran logistic regression via “glm” in R and Randomization was the only statistically significant variable.			
Final model: The model is summarized below.			
Remission	Coefficient	SE	<i>P</i> value
Intercept	−4.554	1.503	.002
Randomization	1.692	0.821	.039

Final logistic regression model for UC remission. Null deviance 62.5, degrees of freedom 74; residual deviance 57.2, degrees of freedom 73; Akaike information criterion 61.2.

CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; IBDQ, Inflammatory Bowel Disease Questionnaire; LASSO, least absolute shrinkage and selection operator; NA, not answered; TNF, tumor necrosis factor.

Supplementary Table 2a: Factors That Predict Remission in Evaluable Patients Allocated to Fecal Microbiota Transplantation: Impact of Sex on Remission Rates With Fecal Microbiota Transplantation

	Sex		Total
	Male	Female	
Week 6 remission			
No	14	13	27
Yes	4	5	9
% Within sex	22.2	27.8	
Total	18	18	36

Supplementary Table 2b: Factors That Predict Remission in Evaluable Patients Allocated to Fecal Microbiota Transplantation: Impact of Sex on Remission Rates With Fecal Microbiota Transplantation

χ^2 Tests	Value	df	Asymptotic significance (2-sided)	Exact significance (2-sided)	Exact significance (1-sided)
Pearson χ^2	.148 ^a	1	.700		
Continuity correction ^b	.000	1	1.000		
Likelihood ratio	.148	1	.700		
Fisher's exact test				1.000	.500
Linear-by-linear association	.144	1	.704		
No. of valid cases	36				

df, degrees of freedom.

^aTwo cells (50.0%) have expected count <5. The minimum expected count is 4.50.

^bComputed only for a 2 × 2 table.

Supplementary Table 3a: Factors That Predict Remission in Evaluable Patients Allocated to Fecal Microbiota Transplantation: Impact of Age on Remission Rates With Fecal Microbiota Transplantation

	Age as a category			Total
	<35 years old	35–50 years old	>50 years old	
Week 6 remission				
No	7	9	11	27
Yes	4	4	1	9
% Within age as a category	36.4	30.8	8.3	
Total	11	13	12	36

Supplementary Table 3b: Factors That Predict Remission in Evaluable Patients Allocated to Fecal Microbiota Transplantation: Impact of Age on Remission Rates With Fecal Microbiota Transplantation

χ^2 Tests	Value	df	Asymptomatic significance (2-sided)
Pearson χ^2	2.766 ^a	2	.251
Likelihood ratio	3.135	2	.209
Linear-by-linear association	2.384	1	.123
No. of valid cases	36		

df, degrees of freedom.

^aThree cells (50.0%) have expected count <5. The minimum expected count is 2.75.

Supplementary Table 4a: Factors That Predict Remission in Evaluable Patients
Allocated to Fecal Microbiota Transplantation: Impact of Severity of Disease at Baseline
on Remission Rates With Fecal Microbiota Transplantation

	Baseline Mayo score as a category			Total
	Score = 4 to 5	Score = 6 to 9	Score >9	
Week 6 remission				
No	5	12	10	27
Yes	4	3	2	9
% Within baseline Mayo score as a category	44.4	20.0	16.7	
Total	9	15	12	36

Supplementary Table 4b: Factors That Predict Remission in Evaluable Patients
Allocated to Fecal Microbiota Transplantation: Impact of Severity of Disease at Baseline
on Remission Rates With Fecal Microbiota Transplantation

χ^2 tests	Value	df	Asymptomatic significance (2-sided)
Pearson χ^2	2.459 ^a	2	.292
Likelihood ratio	2.297	2	.317
Linear-by-linear association	1.890	1	.169
No. of valid cases	36		

^aThree cells (50.0%) have expected count <5. The minimum expected count is 2.25.

Supplementary Table 5a: Factors That Predict Remission in Evaluable Patients Allocated to Fecal Microbiota Transplantation: Impact of Disease Extent on Remission Rates With Fecal Microbiota Transplantation

	Extent of UC		Total
	Left-sided colitis	Extensive colitis	
Week 6 remission			
No	12	13	25
Yes	3	6	9
% Within distribution of UC	20.0	31.6	
Total	15	19	34

Supplementary Table 5b: Factors That Predict Remission in Evaluable Patients Allocated to Fecal Microbiota Transplantation: Impact of Disease Extent on Remission Rates With Fecal Microbiota Transplantation

χ^2 Tests	Value	df	Asymptomatic significance (2-sided)	Exact significance (2-sided)	Exact significance (1-sided)
Pearson χ^2	.577 ^a	1	.447		
Continuity correction ^b	.136	1	.713		
Likelihood ratio	.588	1	.443		
Fisher's exact test				.697	.360
Linear-by-linear association	.560	1	.454		
No. of valid cases	34				

^aOne cells (25.0%) have expected count <5. The minimum expected count is 3.97.

^bComputed only for a 2 × 2 table.

Supplementary Table 6a: Factors That Predict Remission in Evaluable Patients Allocated to Fecal Microbiota Transplantation: Impact of Smoking on Remission Rates With Fecal Microbiota Transplantation

	Smoke			NR	Total
	Nonsmoker	Former smoker	Smoker		
Week 6 remission					
No	14	10	2	1	27
Yes	5	3	1	0	9
% Within smoke	26.3	23.1	33.3		
Total	19	13	3	1	36

Supplementary Table 6b: Factors That Predict Remission in Evaluable Patients Allocated to Fecal Microbiota Transplantation: Impact of Smoking on Remission Rates With Fecal Microbiota Transplantation

χ^2 Tests	Value	df	Asymptomatic significance (2-sided)
Pearson χ^2	.488a	3	.922
Likelihood ratio	.723	3	.868
No. of valid cases	36		

aSix cells (75.0%) have expected count <5. The minimum expected count is .25.

Supplementary Table 7a: Factors That Predict Remission in Evaluable Patients Allocated to Fecal Microbiota Transplantation: Impact of Current Steroid Therapy on Remission Rates With Fecal Microbiota Transplantation

	Current therapy steroid		Total
	Yes	No	
Week 6 remission			
No	11	16	27
Yes	3	6	9
% Within current therapy steroid	21.4	27.3	
Total	14	22	36

Supplementary Table 7b: Factors That Predict Remission in Evaluable Patients Allocated to Fecal Microbiota Transplantation: Impact of Current Steroid Therapy on Remission Rates With Fecal Microbiota Transplantation

χ^2 Tests	Value	df	Asymptomatic significance (2-sided)	Exact significance (2-sided)	Exact significance (1-sided)
Pearson χ^2	.156 ^a	1	.693		
Continuity correction ^b	.000	1	1.000		
Likelihood ratio	.158	1	.691		
Fisher's exact test				1.000	.506
Linear-by-linear association	.152	1	.697		
No. of valid cases	36				

^aOne cells (25.0%) have expected count <5. The minimum expected count is 3.50.

^bComputed only for a 2×2 table.

Supplementary Table 8a: Factors That Predict Remission in Evaluable Patients Allocated to Fecal Microbiota Transplantation: Impact of current anti-TNF therapy on remission rates with Fecal Microbiota Transplantation

	Current therapy biologic		Total
	Yes	No	
Week 6 remission			
No	3	24	27
Yes	1	8	9
% Within current therapy biologic	25.0	25.0	
Total	4	32	36

Supplementary Table 8b: Factors That Predict Remission in Evaluable Patients Allocated to Fecal Microbiota Transplantation: Impact of current anti-TNF therapy on remission rates with Fecal Microbiota Transplantation

χ^2 Tests	Value	df	Asymptomatic significance (2-sided)	Exact significance (2-sided)	Exact significance (1-sided)
Pearson χ^2	.000 ^a	1	1.000		
Continuity correction ^b	.000	1	1.000		
Likelihood ratio	.000	1	1.000		
Fisher's exact test				1.000	.702
Linear-by-linear association	.000	1	1.000		
No. of valid cases	36				

^aTwo cells (50.0%) have expected count <5. The minimum expected count is 1.00.

^bComputed only for a 2 × 2 table.

Supplementary Table 9a: Factors That Predict Remission in Evaluable Patients Allocated to Fecal Microbiota Transplantation: Impact of Fresh vs Frozen Stool on Remission Rates of Fecal Microbiota Transplantation

Week 6 remission * stool type cross tabulation				
	Stool type			Total
	Fresh	Frozen	Mixed	
Week 6 remission				
No	14	12	1	27
Yes	1	8	0	9
% Within stool type	6.7	40.0	0.0	
Total count	15	20	1	36

Supplementary Table 9b: Factors That Predict Remission in Evaluable Patients Allocated to Fecal Microbiota Transplantation: Impact of Fresh vs Frozen Stool on Remission Rates of Fecal Microbiota Transplantation

χ^2 Tests	Value	df	Asymptomatic significance (2-sided)
Pearson χ^2	5.422 ^a	2	.066
Likelihood ratio	6.220	2	.045
Linear-by-linear association	3.070	1	.080
No. of valid cases	36		

^aThree cells (50.0%) have expected count <5. The minimum expected count is .25.

Supplementary Table 10: Summary of the Mean Change From Baseline Between Placebo and Fetal Microbiota Transplantation Groups at Week 7 for Continuous Variables (Equal Variances Not Assumed)

Variable	Placebo	FMT	<i>P</i> value
Mayo score	-1.29 ± 2.73	-2.06 ± 3.33	.30
IBDQ score	10.93 ± 25.86	18.69 ± 37.54	.33
EQ-5D score	2.00 ± 15.07	-3.71 ± 18.20	.18
CRP, <i>mg/L</i>	-0.275 ± 6.73	-0.59 ± 25.86	.86
ESR, <i>mm/h</i>	-3.06 ± 11.95	-3.07 ± 7.53	1.00

CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; IBDQ, Inflammatory Bowel Disease Questionnaire.

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Conflicts of interest

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CHAPTER 3

CASE REPORT: MICROBIAL CHANGES AND CLINICAL FINDINGS FOLLOWING LONG-TERM FECAL MICROBIOTA TRANSPLANTATION IN A SEVERE ULCERATIVE COLITIS PATIENT

Case Report:

Microbial changes and clinical findings following long-term fecal microbiota transplantation in a severe ulcerative colitis patient

Josie Libertucci^{1,2}, Paul Moayyedi², Melanie Wolfe², Christine H. Lee³, Md. Sharif Shajib², Catherine Onishi³, and Michael G. Surette^{1,2*}

Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Ontario, Canada¹

Farncombe Family Digestive Health Research Institute, Department of Medicine, McMaster University, Hamilton, Ontario, Canada²

Department of Pathology and Molecular Medicine, St Joseph's Healthcare, McMaster University, Hamilton, Ontario, Canada³

The material in this chapter is prepared for submission to *Anaerobe*

Preface: I am the primary author of this work. Experiments were designed by my supervisor, Michael Surette, and myself. I conducted all experiments with the exception of the ELISA for the fecal calprotectin assay – Md. Sharif Shajib completed that experiment. I wrote this manuscript. Paul Moayyedi and Melanie Wolfe recruited all patients, PM conducted all evaluations for this patient, MW collected all patient data. Christine Lee and Catherine Onishi conducted the FMT administrations. CO shipped all fecal samples to McMaster HSC.

Highlights

- This is the first long-term surveillance of the luminal and mucosa associated microbiota following FMT to treat severe UC refractory to conventional therapy in an adult
- Endoscopic and microscopic remission was achieved following multiple FMT administrations
- After a period of several months in remission on no therapy this patient relapsed, but entered remission again following multiple FMT
- Clinical and endoscopic remission was achieved again, although, histology reports persistent minimal activity
- Microbial community changes occurred following treatment in both the lumen and mucosa-associated microbiota
- Fungal organisms are present, but do not correlate with disease status
- No evidence for major engraftment of donor microbiota following FMT at the level of OTUs
- FMT is a promising treatment for UC but, multiple and repeated FMT may be required to enter and maintain remission

Abstract

Fecal microbial transplantation (FMT) is a highly effective treatment for reoccurring or refractory *Clostridium difficile* infection (CDI); however, it remains unclear if FMT will be as effective for the treatment of ulcerative colitis (UC) as mode of administration, donor, and duration may influence treatment success. In this study, we report a case of a 40-year-old male with UC, refractory to conventional therapy, who entered remission following 20 treatments of FMT. Following an extended treatment free period in remission, this patient relapsed to severe disease. Remission was achieved upon readministration of FMT. Stool and mucosa-associated microbiota, as well as the mycobiome, were monitored over this period. We found alterations of the microbiota following FMT, but no evidence for large-scale transfer of donor microbiota at the operational taxonomic unit (OTU) level. Further investigation is required to better understand the mechanism behind successful FMT for treatment of IBD.

Keywords Ulcerative colitis (UC), fecal microbiota transplantation (FMT), inflammatory bowel disease (IBD), microbiome, microbiota, mycobiome

Introduction

Ulcerative colitis (UC), a chronic inflammatory disease marked by stages of remission and relapse, is characterized by inflammation that is restricted to the colon (De Souza et al., 2015). The exact etiology of the disease remains unknown, but it is generally accepted that in susceptible individuals, an aberrant immune response occurs against the commensal microbiota, with environmental factors also contributing to disease risk (Sartor et al., 2008). Dysbiosis of the microbiota has been extensively characterized in UC (Ott et al., 2004; Mylonaki et al., 2005; Sokol et al., 2009; Morgan et al., 2012; Davenport et al., 2014; Norman et al., 2015). However, when taking into account data collected across studies, few taxa are consistently enriched or depleted relative to healthy controls (Walters et al., 2014). Alteration of the microbiota has yet to be identified as the cause or consequence of chronic inflammation although, at least some of the microbial dysbiosis is a response to inflammation (Lewis et al., 2015). It has been hypothesized that inflammation, and subsequently a diseased state in UC patients, can be resolved through modulation of the immune system stimuli, the gut microbiota, rather than repressing the immune response (Borody et al., 2003).

Fecal microbiota transplantation (FMT) is the administration of a fecal suspension from a healthy donor to a recipient (Eiseman et al., 2003). This treatment has proven to be highly effective in resolving reoccurring and refractory *Clostridium difficile* infections (CDI) (Van Nood et al., 2013; Kassam et al., 2013). Following FMT in CDI patients, studies have shown that the pathogen is displaced, and restructuring of the microbiota occurs (Seekatz et al., 2014; Weingarden et al., 2015). Recently, it has been reported that

FMT can induce remission in severe to moderate UC patients; however, treatment success may be influenced by donor, mode of administration, and duration (Moayyedi et al., 2015; Rossen et al., 2015; Paramsothy et al., 2016). The microbiota in patients that respond to FMT show a shift towards their donor, suggesting a restructuring of the community is associated with positive clinical outcomes. However, it is not known if these changes to the microbiota following FMT in UC patients are transient, and if positive clinical outcomes are associated with a permanent restructuring of the community. There is also a lack of evidence supporting the notion that bacterial taxa are transferred from donor to recipient. For these reasons, long-term studies are needed to address questions surrounding optimal treatment duration, and regarding long-term community changes following FMT, as well as elucidating the key mechanisms that allow for successful FMT treatment in IBD.

In this study, we present a 40-year old male with long term severe ulcerative colitis, refractory to conventional therapy, treated with repeated FMT. This is the first long-term report of microbial changes, from both the stool and mucosa, following FMT in an adult with severe UC over an 86-week period.

Case Report

A 40-year-old white male with a 16-year diagnosis of UC, had been previously treated with 5-ASA and steroids, and reached a point of severe refractory UC. This patient was previously a smoker, with no family history of UC, and had inflammation extending the entire length of the colon (pancolitis). Colectomy was recommended to

improve his condition, but the patient refused, and chose to enroll in a randomized control trial (RCT) testing FMT for treatment of UC, as described in Moayyedi et al., 2015 (Moayyedi et al., 2015). This individual was randomized to placebo, and showed no clinical improvement following the RCT. Succeeding the trial, the patient opted to receive active FMT. This report summarizes the clinical findings as well as the microbiota changes during an 86-week period of repeated FMT.

Materials and Methods

Ethical considerations, donor selection, and FMT protocol

The local research ethics committee at McMaster University and Health Canada had no objection to the use of FMT for use in this patient beyond the clinical trial. All participants (patient and donors) provided written informed consent. FMT protocol, baseline assessment, donor selection, and patient evaluation were completed as in Moayyedi et al., 2015. Donor B from Moayyedi et al., was almost exclusively used to treat this patient. All FMT treatments were completed at St. Joseph's Hospital (Hamilton, Ontario, Canada), and administered by one investigator (CHL). Remission in this patient was defined as complete endoscopic resolution with an endoscopy score of ≤ 1 and a total Mayo score of ≤ 1 . One investigator (PM) performed all flexible sigmoidoscopies and evaluations for this patient.

Sample collection and samples used in this study

Stool samples in this study were collected from this patient at St. Joseph's Hospital, shipped to the McMaster University Health Science Centre (HSC), and processed from this patient starting in July 2012 and ending in July 2014. Upon receiving, stool samples were frozen (-80 °C) until time of processing. Stool samples were collected prior to the first FMT intervention and each week this patient received treatment. Also, at the time of FMT, a donor sample was collected for analysis. Biopsy samples were collected two weeks prior to initial treatment, and at time of evaluation at McMaster University HSC. This study used a total of 46 stool samples, and 7 rectal biopsy samples.

Genomic DNA extraction and PCR amplification of the V3 region of 16S rRNA

For stool samples, genomic DNA extraction and PCR amplification of the V3 region of 16S rRNA gene, was conducted using previously described protocols (Whelan et al., 2014; Bartram et al., 2011; Stearns et al., 2015). For processing of the biopsy samples, the following amendments were made. During genomic DNA extraction, ultrapure reagents made with RNase and DNase free, sterile water, (Qiagen, Hilden, Germany) were used to reduce contaminants. Amplification of the V3 region was conducted by using a nested PCR reaction, to reduce amplification of host DNA. The first reaction, amplified a 918 bp region of the 16S rRNA gene (forward primer, 5'-AGA GTT TGA TCC TGG CTC AG-3'; reverse primer, 5'-CCG TCA ATT CCT TTR AGT TT-3') for 15 cycles. One µL of amplicon was taken from the first reaction, and used as template for the second reaction, carried out as previously described (Bartram et al., 2011). All

samples were sequenced using an Illumina MiSeq platform at the McMaster Genome Facility (Hamilton, Ontario, Canada).

V3 sequence processing and analysis

Raw fastq files were processed using a protocol previously described with the following amendments (Whelan et al., 2014; Lau et al., 2016). Taxonomy was assigned using the Ribosomal Database Project (RDP) classifier against Greengenes reference database, May 2013 release (Wang et al., 2007; DeSantis et al., 2006). In this dataset, 7,756,904 reads were generated with an average depth of 94,596 per sample, a minimum read depth of 13,770, and a maximum read depth of 316,699. Singletons, and operational taxonomic units (OTUs) that were not identified as bacteria were discarded. Taxonomic summaries, alpha and beta diversity (Bray-Curtis dissimilarity), non-parametric t-tests, and Permanova tests, were calculated using quantitative insights into microbial ecology (QIIME) (Caporaso et al., 2010). Statistical tests were always completed assuming a non-normal distribution (non-parametric), and significance defined as a p value ≤ 0.05 , and in cases where multiple tests were completed, False Discovery Rate (FDR) was used to correct for false positives. Some graphs were visualized using Prism v6.0.

Detection and sequencing of the fungal community, mycobiome

Stool and biopsy samples were screened for the presence of fungi by amplification of the internal transcribed spacer 2 (ITS2) region on nuclear ribosome DNA. The region amplified was 300 bp, and primers bound to the 5.8S region (forward 5'-GTG AAT CAT CGA RTC TTT GAA C-3') and the 28S region (reverse 5'-TAT GCT TAA GTT CAG CGG GTA-3'). Total reaction volume was 25 μ L and consisted of 2.5 μ L of 10X buffer,

0.75 μL of 50 mM MgCl_2 , 0.5 μL of 10 mM dNTPs, 0.5 μL of 1 μM of each primer, 0.5 μL of template of genomic DNA (30-50 ng), 0.25 μL of Taq polymerase (1.25U/ μL), and 19.5 μL of ddH₂O. PCR conditions consisted of an initial denaturation at 94 °C for 2 min, 30 cycles of 94 °C for 30s, 50 °C for 30s, 72 °C for 45 s, followed by a final elongation at 72 °C for 10 min.

For identification and sequencing of the fungal community, two PCR reactions were performed prior to sequencing. The first reaction consisted of amplification of the ITS2 region, with primers that included partial Illumina adapters added to 5.8SF and 28SR primers (5'-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT GTG AAT CAT CGA RTC TTT GAA C-3' and 5'-GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC TTA TGC TTA AGT TCA GCG GGT A-3'). The same PCR reaction was conducted as the original ITS2 reaction, with the following amendments. One μL of bovine serum albumin (BSA) and dimethyl sulfoxide (DMSO) were added to the reaction, and 55 °C was used as an annealing temperature. The second reaction, 50 μL total volume, took 23 μL of the purified amplicon from the first reaction, and added the full Illumina adapters, with the specific barcode added on the reverse primer (P5-503 5'-AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT ATC CTC TAC ACT CTT TCC CTA CAC GAC GCT CTT-3' and P7-712 5'-CAA GCA GAA GAC GGC ATA CGA GAT TCC TCT ACG TGA CTG GAG TTC AGA CGT GTG CTC TTC CGA-3'), 0.25 μL of Taq polymerase (1.25U/ μL), 1 μL of each primer, 0.5 μL of 10 mM dNTPs, 0.75 μL 50 mM MgCl_2 , and 2.5 μL of buffer, with ddH₂O added to the final volume.

ITS2 sequence processing

Forward reads of raw sequences, were trimmed using Cutadapt, OTUs were clustered at 97% similarity using uclust, and taxonomy was assigned against the UNITE ITS database (version its_12_11_otus) using QIIME (Martin et al., 2011; Abarenkov et al., 2010). Assignments were confirmed using NCBI blast. A total of 105,711 reads were generated. Taxonomy plots were generated using QIIME.

Quantification of fecal calprotectin

Between 0.1 and 0.3 grams of stool were scooped and processed for detection of fecal calprotectin. Extraction and ELISA assay were conducted as per manufacturers' instructions (Buhlmann, Switzerland).

Quantitative PCR for total fungal load

Total fungal load was detected by 18S quantification using quantitative PCR (qPCR). This was performed using the Bio-Rad Eva Sso Fast supermix chemistry (Bio-Rad Laboratories, Mississauga, Ontario). Ten μL of Bio-Rad Eva Sso Fast supermixed, 1 μL of each primer diluted to 20 pmol, 2 μL of template, and dH_2O up to 20 μL was added to each reaction. Primer sequences for this analysis were used from Dollive et al., 2013 (Dollive et al., 2013). A standard curve was always included in each reaction; seven 10-fold dilutions of *Candida albicans* was used as the standard. A melt curve was completed consecutively to ensure only one product was produced.

Results

Clinical findings

Ten weeks prior to the first FMT treatment, this individual had chronic active colitis that was classified as severe, with a Mayo Score of 12 (Figure 1, Figure 2A and C). Pathology report confirmed features were consistent with chronic active UC, noting swelling in the lamina propria and inverted polyps (Table 1). This patient was initially enrolled in a randomized controlled trial, and received placebo treatment (50 mL of water via rectal enema). Following 6 weeks of placebo treatment, the patient was evaluated again, and showed no clinical improvements (Figure 1 and Table 1). At the end of the trial, the patient opted for FMT, and received treatment as per the RCT protocol. FMT treatment was initiated post second evaluation, with no other medication being administered for treatment of UC. Following 6 treatments of FMT (administered one per week) disease was evaluated (Figure 1). Significant clinical improvement was observed following 6 treatments of FMT, Mayo score decreased from 11 to 5 (Figure 1) and histology reports confirmed the presence of mild disease, instead of severe (Table 1).

Endoscopic and microscopic remission was achieved following an additional 13 FMT treatments from that point (Figure 1, Figure 2B and D). Histology report confirmed quiescent UC in the descending and sigmoid colon however, minimally active UC was found in the cecum, ascending and transverse colon (Table 1). Within the rectum, inflammation was still present at this point, even though endoscope displayed this patient showed mucosal healing (Table 1). Following remission, this patient decided to stop FMT treatment, as he was symptom free for many weeks. The patient, while not receiving FMT eventually

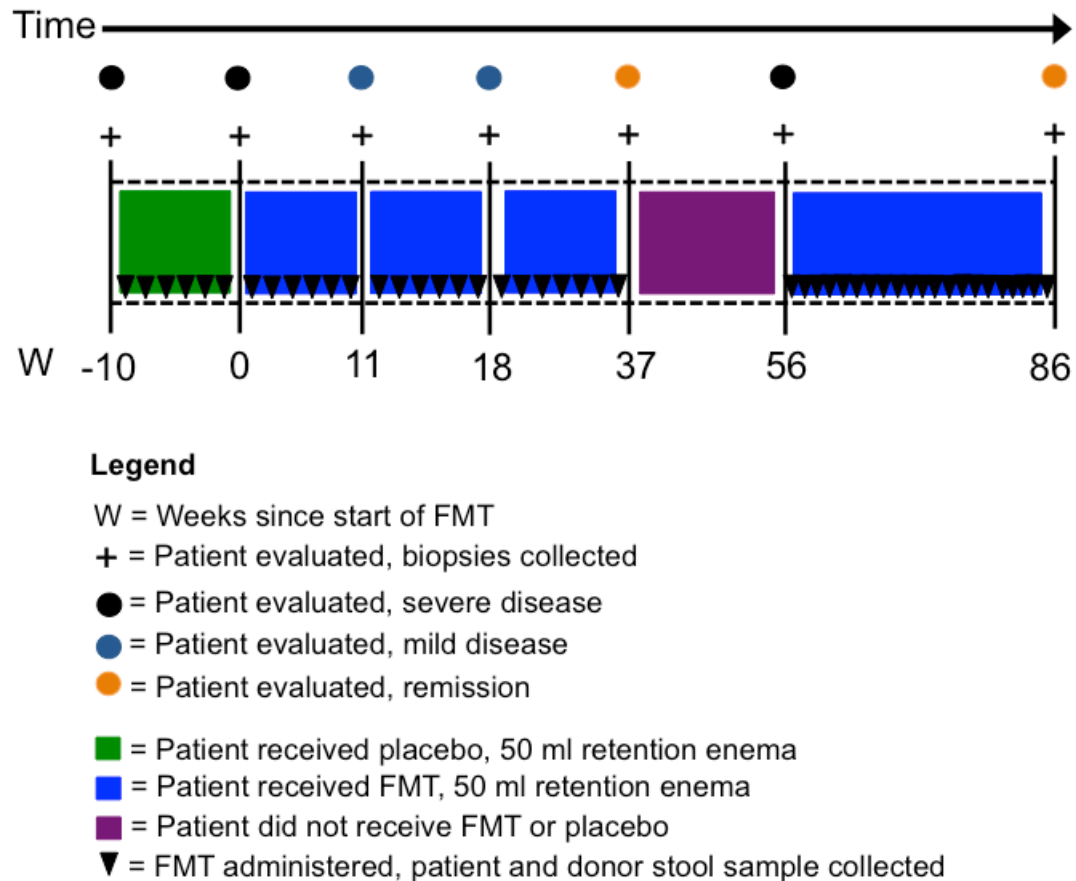


Figure 1: Timeline of sample collection and disease evaluation following FMT. Schematic representing sample collection and administration of FMT over an 86 week period. Placebo (50 ml retention enema containing water) or FMT (50 ml retention enema of donor suspension) was administered when indicated (black triangle), and stool samples collected. Disease was evaluated (+), and classification based on the Mayo score.

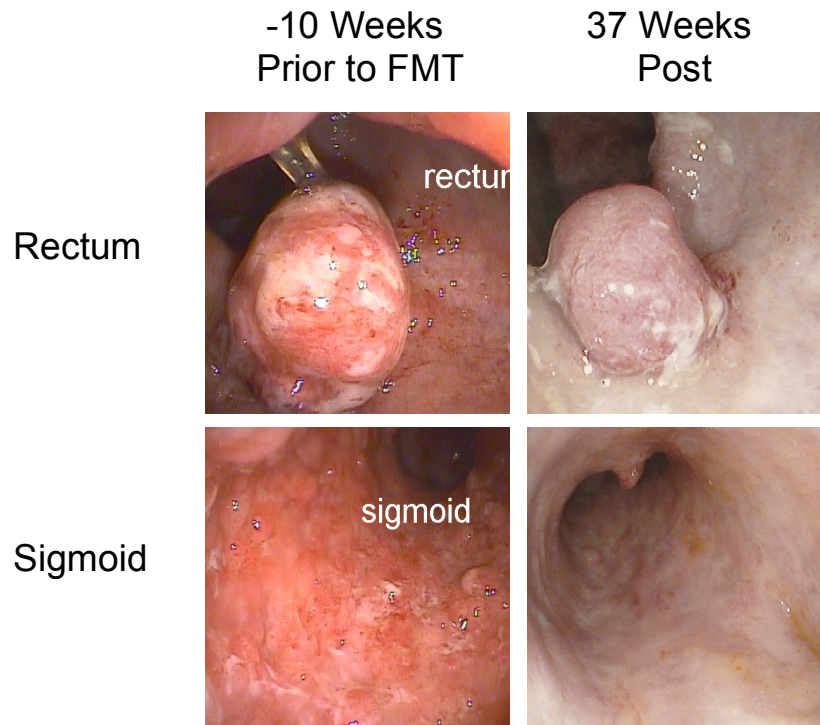


Figure 2: Endoscopy findings prior to and post FMT. Flexibe sigmoidoscopy images of the rectum and sigmoid colon collected -10 weeks prior to FMT treatment, and at a 37 weeks post the initiation of FMT (Points I and IV, respectively Fig 1).

Table 1: Endoscopy score and histology report prior to and following FMT

Histology Score¹	I Week - 10	II Week 0	III Week 11	IV Week 18	V Week 37	VI Week 56	VII Week 86
Cecum	N/A	N/A	N/A	N/A	A	N/A	A
Ascending colon	N/A	N/A	N/A	N/A	A	N/A	A
Transverse Colon	N/A	N/A	N/A	N/A	A	N/A	A
Descending Colon	I	N/A	G	D	B	G	A
Sigmoid Colon	I	E	G	D	B	G	B
Rectum	I	N/A	G	D	C	H	B
Dysplasia	N	N	N	N	N	N	N
Endoscopy Score	3	3	2	1	0	3	0

¹Histology score legend:

N/A: Not applicable, no biopsy provided

A: Chronic active colitis, minimally active

B: Quiescent colitis

C: Superficial colonic type mucosa with scant submucosa showing increased lymphoplasmacytic infiltrate

D: Features consistent with active UC, minimally active

E: Features show chronic active colitis. Reactive changes of the crypt epithelium

F: Features consistent with active IBD. Mild activity noted in the lamina propria. No cryptitis or crypt abscess identified

G: Fragments of colonic mucosa showing features consistent with chronic colitis

H: Colonic mucosa with granulation tissue consistent with ulcer and chronic active colitis.

I: Moderate to severe chronic active colitis with prominent hyperplastic changes and focal edema in the lamina propria

N: Negative for Dysplasia

Endoscopy Score: 0 indicates no disease while 3 indicates severe

relapsed, with endoscopy defined as severe (Table 1) and a Mayo score of 11 (Figure 1). After 18 FMT treatments, clinical remission was achieved again, although histology reported quiescent colitis in the rectum and sigmoid colon, and minimally active UC was observed in the descending, transverse, and ascending colon as well as the cecum (Table 1).

Changes in the mucosa-associated microbiota following FMT

The microbiota associated with the mucosa, at the site of the rectum, was evaluated to identify changes associated with FMT. Prior to FMT and after placebo treatment, the genus *Streptococcus* was found in large proportions, a relative abundance of 92.44% and 68.20%, respectively (Figure 3C). Following 6 FMT treatments, the genus *Streptococcus* reduced to a relative abundance of 0.034%. This decrease in species from the genus *Streptococcus* was mirrored with an enrichment of species from the genera *Ruminococcus* (7.71% to 17.7%), *Bacteriodes* (6.3% to 13.7%), and *Faecalibacterium* (2.49% to 17.9%). The relapse sample biopsy (V) did not revert to the earlier profile (I) with high *Streptococcus* abundance, or shift as dramatically from the previous samples following FMT (Figure 3C). This may indicate that the mucosal adhered community is slower to respond to inflammation and altered intestinal milieu than the stool microbiota. Shannon diversity, richness and evenness, increased over the course of FMT and dissimilarity values indicated a restructuring of the microbiota (Figure 3A and B).

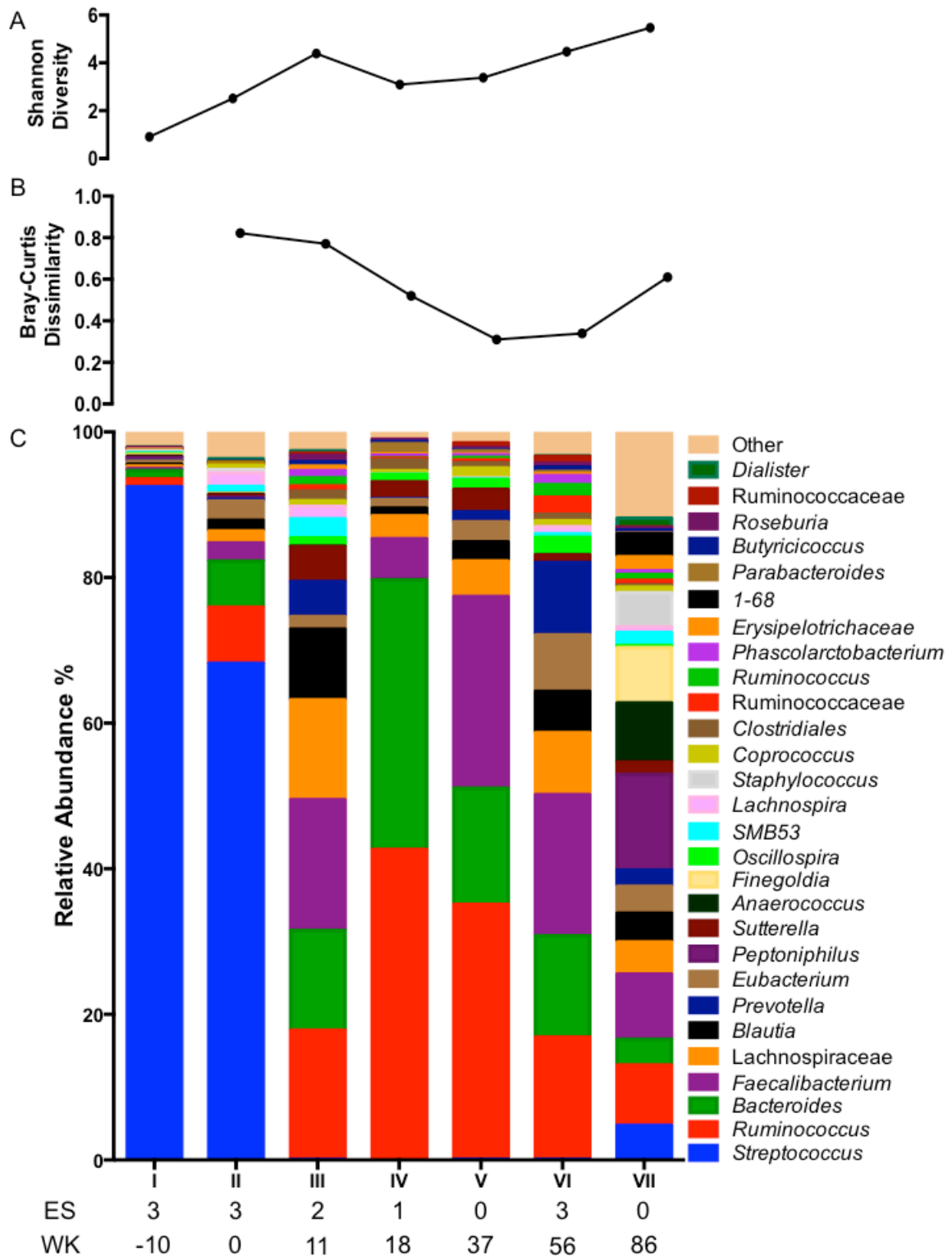


Figure 3: The mucosa-associated microbiota of the rectum. Shannon diversity (A) , and Bray-Curtis dissimilarity, compared to the previous week starting from (B), and taxonomic summary (C) of biopsy samples taken during during evaluation of patient. ES indicates endoscopy score and WK indicates weeks past since the initiation of FMT.

Changes in the stool microbiota following FMT

The stool microbiota was examined at 46 time points (Figure 1). Overall changes in alpha diversity as measured by Shannon Diversity showed a trend to increasing diversity through treatment, and was significantly different between the placebo period and remission state (Fig 4A). Colonic inflammation measured by fecal calprotectin decreased significantly, following the initiation of FMT (Figure 4B). Richness and evenness of the stool microbiota increased significantly, but increased diversity does not correlate with low stool fecal calprotectin levels (Figure S1). The bacterial profiles of these 46 samples is presented in Figure 4C. Within the stool, major microbial changes occurred. A decrease in *Ruminococcus*, *Streptococcus*, and an expansion of Lachnospiraceae and *Blautia* occurred following FMT. (Figure 4C). The expansion of Lachnospiraceae and *Faecalibacterium* occurred following improved disease score. Relapse in this patient was marked by the expansion of genus *Streptococcus*, which was not observed during relapse within the rectum (Figure 3).

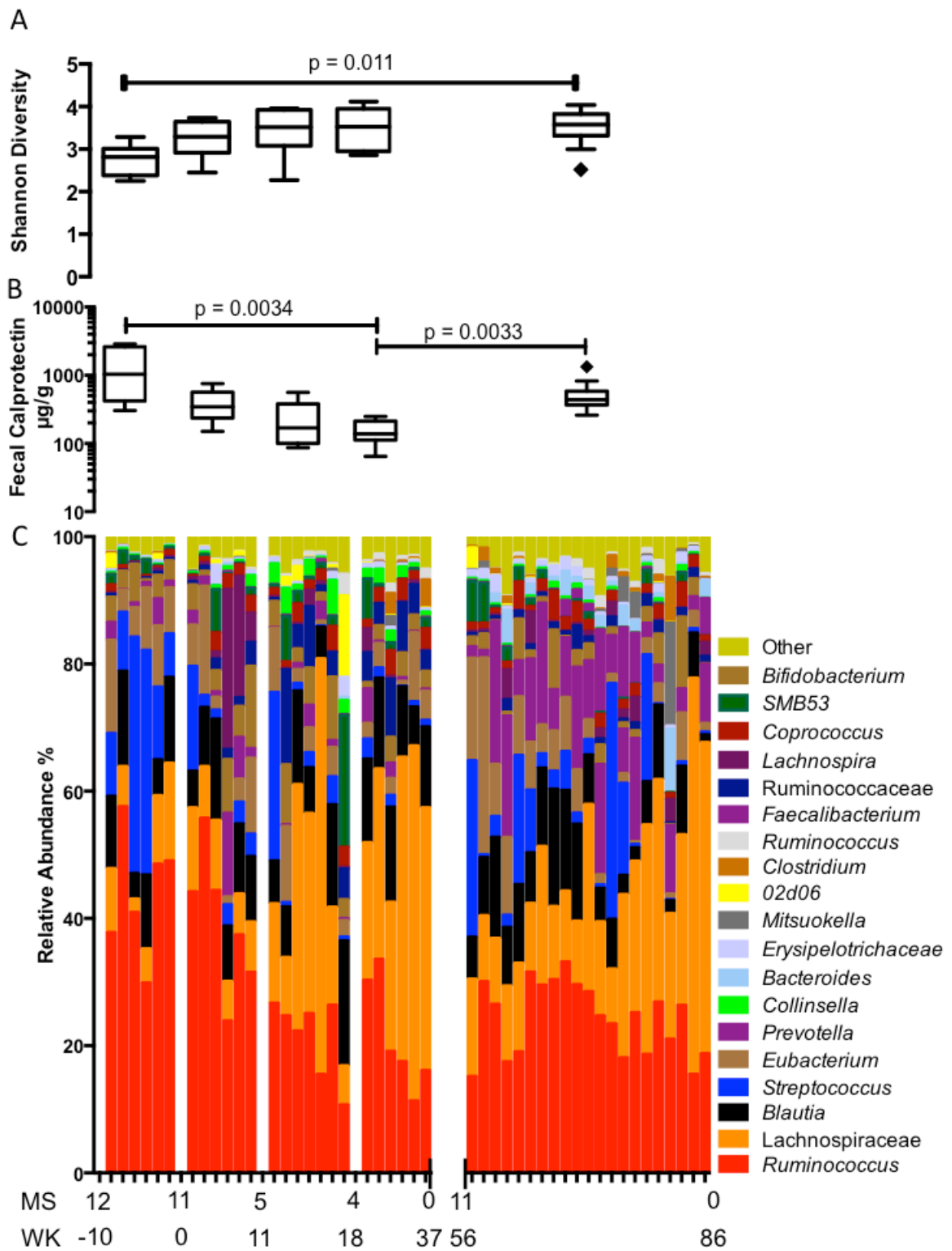


Figure 4: Changes in the gut microbiome following FMT. Shannon diversity (A), fecal calprotectin levels (B), and taxonomic summary chart (collapsed at the genus level) of stool samples based on 16S rRNA marker gene sequencing, prior to and following FMT. MS indicates Mayo score, and WK indicates weeks since the initiation of fecal transplants. A non parametric anova (Kruskal-Wallis) test, followed by Dunn's multiple comparisons was completed to test for significance.

The mycobiome before and after FMT

Presence of fungal species was determined by amplification of the ITS2 region. Prior to treatment and during severe disease, fungal species were present in the stool microbiota. Following FMT however, fungal species remained in the stool. Presence or absence of fungal species was not associated with active disease or remission in this patient (Figure 5A). Fungal species were identified via Illumina MiSeq sequencing, and revealed the majority of the community was *Saccharomyces cerevisiae* (Figure 5B). *Candida albicans*, *Aspergillus vitricola*, and *Claviceps zizaniae* were present in the community as well, but at very low abundance. The association between fungal load found in the stool and fecal calprotectin levels were tested and there was no statistically significant association between colonic inflammation and the presence of fungal species in this patient (Figure 5C).

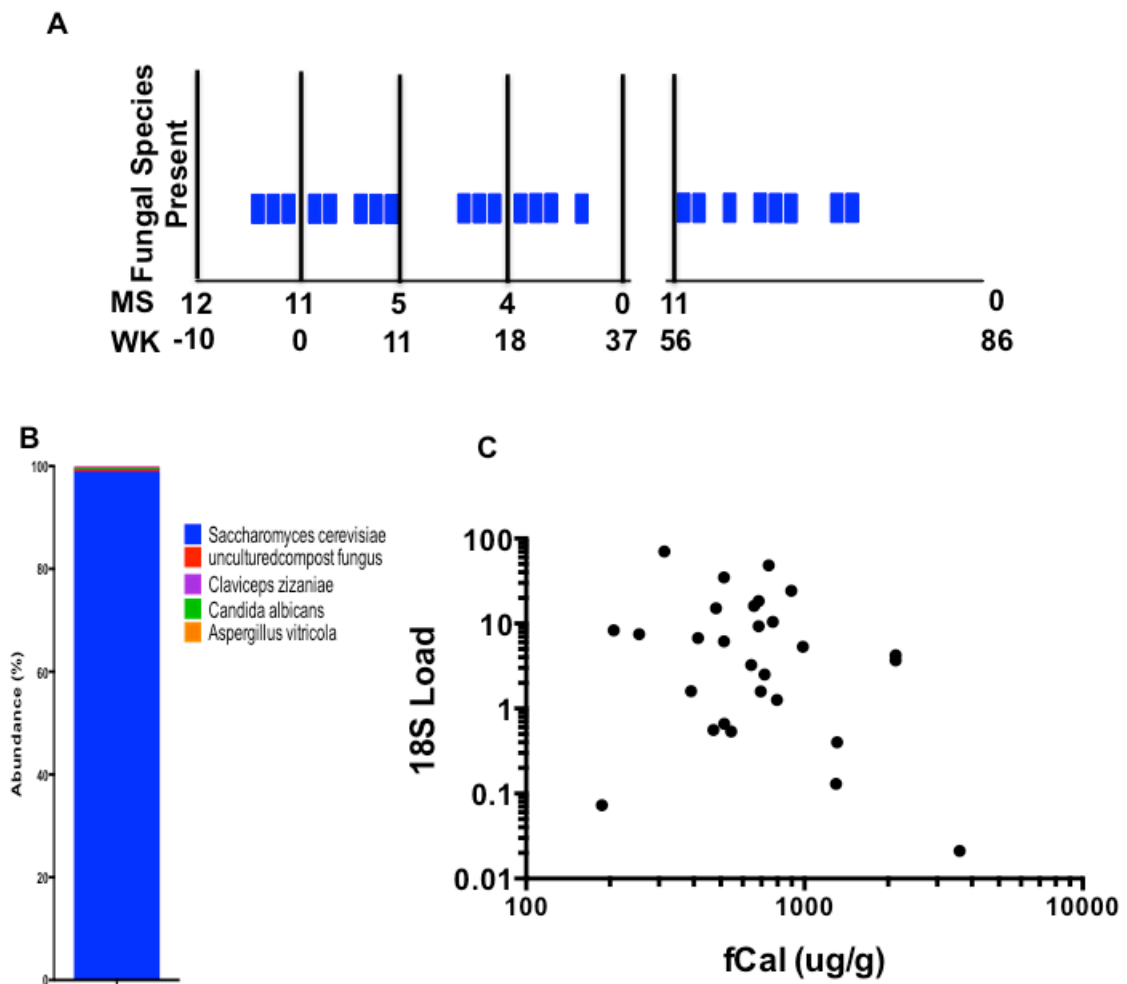


Figure 5: Detection of the mycobiome following FMT. A) Presence of fungal species in stool samples taken before and after FMT. B) Identification of the fungal community. C) Correlation between fungal load and fecal calprotectin levels.

No evidence for large-scale transfer or engraftment at the OTU level

The key mechanism for successful FMT in UC patients remains to be elucidated. To test if large-scale engraftment of donor species into the recipient occurs following FMT, OTUs absent prior to FMT (samples taken during 6 weeks of placebo treatment), but present following FMT, and shared with the donor were identified. This was completed with OTUs at a relative abundance greater than 0.001% across the entire dataset. Only one OTU was identified as being absent prior to FMT and present following at abundance greater than 0.001%. OTU 30 identified as a species from the genus *Phascolarctobacterium* (Figure 6). OTU 30 is the only OTU that met the criteria for engraftment; the most abundant OTUs found in the donor are also shared with the patient prior to FMT.

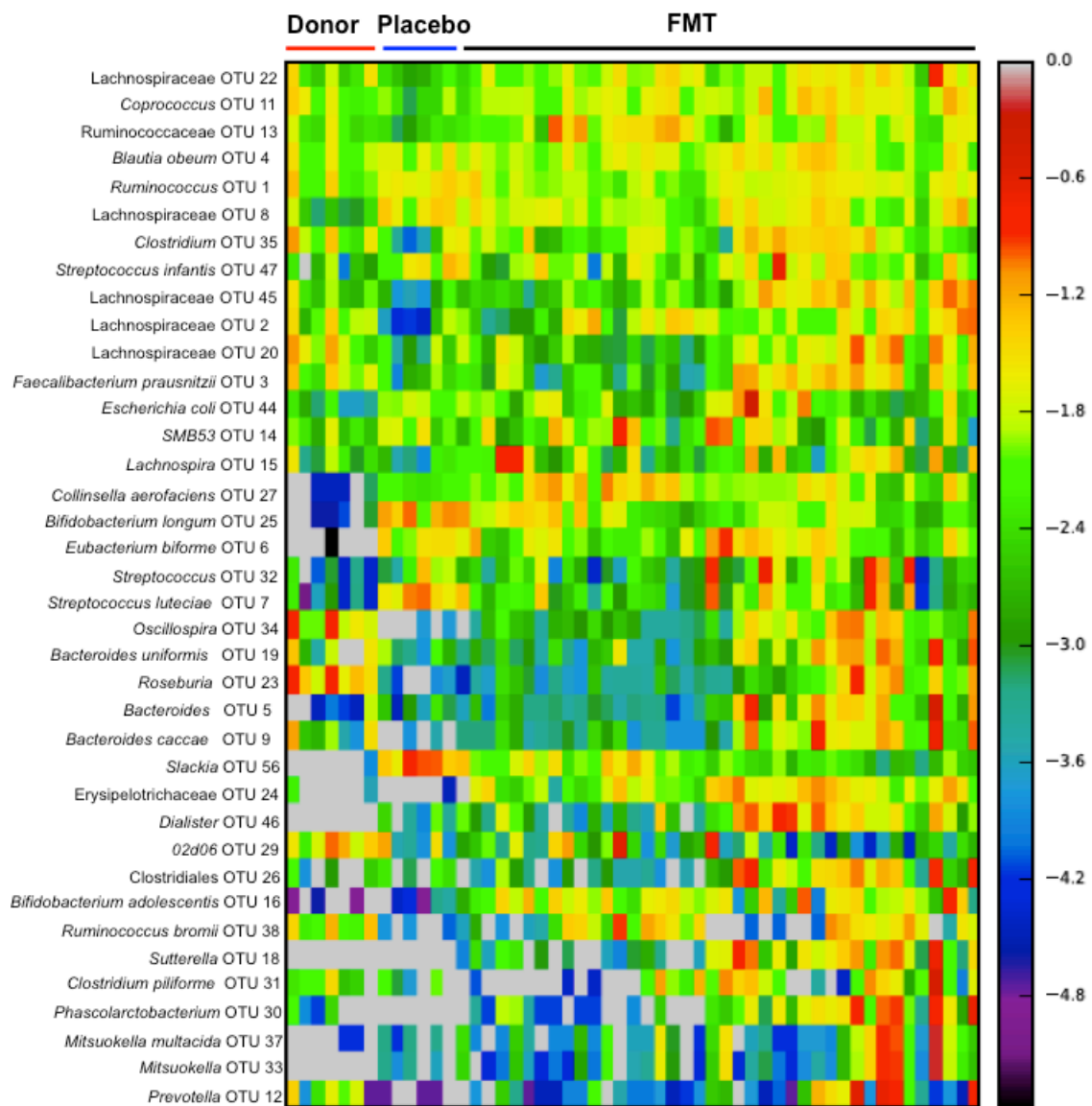


Figure 6: No evidence for engraftment at the OTU level in this individual following FMT. Heatmap of samples from donor, and patient before and after FMT. OTUs were filtered to 0.001% relative abundance, and rows were cluster based on UPGMA.

Discussion

Repeated FMT in a severe UC patient, refractory to conventional medicine, induced remission in this patient, but relapse occurred following cessation of FMT. Remission was induced again after repeated treatment of FMT. Although, this is a single case report, this study provides evidence for the use of repeated FMT in UC patients as opposed to one single FMT, which is commonly used for treating refractory or recurring CDI (Kump et al., 2013). Duration of FMT, the number of FMT over a length of time, is an important consideration for treating UC. Case reports, cohort studies, and RCTs that have used one single FMT, or few FMT treatments, did not achieve high success rates defined by clinical remission (Rossen et al., 2015; Angelberger et al., 2013). Studies that have had the most success, and have induced remission in patients, have used multiple FMT treatments in addition to using the lower route for administration of FMT (Moayyedi et al., 2015; Paramsothy et al., 2016; Borody et al., 2003). Repeated use of FMT has also been shown to be effective for treating refractory UC in a pediatric patient (Shimizu et al., 2016). RCTs should be conducted to test the optimal duration required to induce remission in UC patients via FMT, however this may vary from patient to patient.

As inflammation is superficial in UC, limited to the mucosal layer, mucosal healing defined by endoscopy has remained a clinical therapeutic goal (Peyrin-Biroulet et al., 2011). However, mucosal healing defined by endoscopy does not always correlate with histologic healing – complete mucosal healing defined by the absence of inflammation or structural changes (Peyrin-Biroulet et al., 2014). Histological healing has

been linked with a reduction of symptoms, reduced hospitalization, reduced risk of relapse, and reduced risk of colorectal cancer (Isaacs et al., 2010; Riley et al., 1991; Travis et al., 2011; Gupta et al., 2007). Although there are many benefits to achieving histological healing, a method for evaluating histology reports have not been validated for assessing UC, thus validated scales such as the Mayo score, which includes mucosal healing defined by endoscopy, remains the gold standard (Cooney et al., 2007; D'Haens et al., 2007). Repeated FMT in this individual induced mucosal healing and quiescent colitis, at both the endoscopy and histology level. Interestingly, mucosal healing was seen throughout the colon, rectum to cecum, during both periods this patient achieved remission, but histology reported quiescent colitis in only the descending and sigmoid colon, and sigmoid colon and rectum, respectively. Considering this patient did not achieve complete histologic remission, complete structural colonic healing, before stopping FMT, this may explain why this individual relapsed. It may be beneficial, in future studies, to evaluate FMT based on histology rather than endoscopy, as achieving mucosal healing at the histology level may reduce the risk of relapse.

FMT has been shown to create a transient change within the gut microbiota in UC patients, and remission has been linked to compositional changes towards their respective donor microbiota (Kump et al., 2013; Moayyedi et al., 2015; Rossen et al., 2015). It is unknown if a permanent restructuring of the microbial community would be associated with repeated FMT. In the microbiota associated with the mucosa at the site of the rectum, *Ruminococcus* and *Bacteroides* expanded following a displacement of the genus *Streptococcus*. *Streptococcus* did not expand following relapse to severe disease in this

individual. This is most likely because inflammation in UC is driven by the commensal microbiota, and not driven by a single pathogen (Singh et al., 2016; Hold et al., 2014). The expansion of *Streptococcus* in the rectum may have been a consequence of inflammation (Lewis et al., 2015).

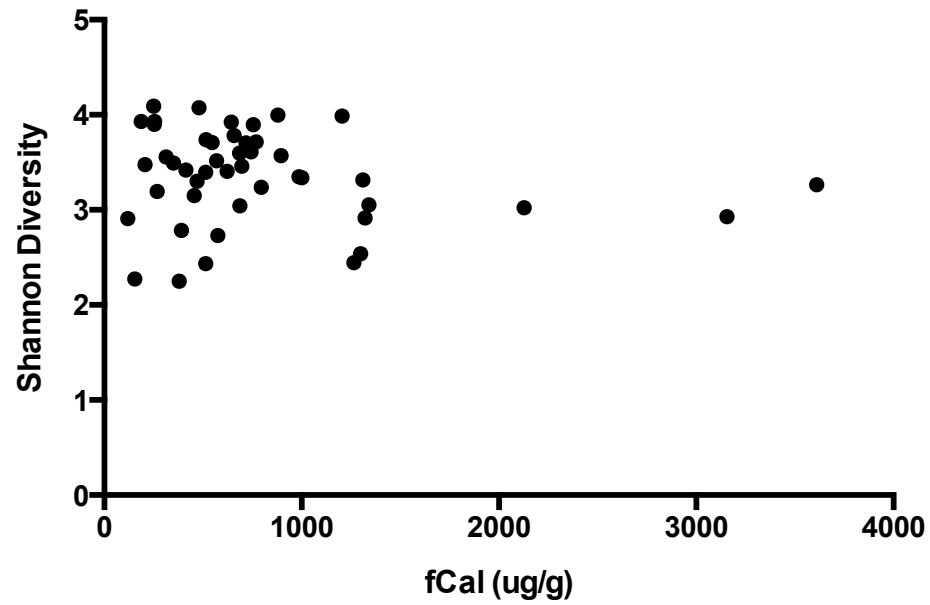
Following successful treatment of CDI using FMT, the pathogen is displaced and the patient's microbiota is similar to the donor's composition (Seekatz et al., 2014). Engraftment has been suggested to be one of the key mechanisms of FMT (Hamilton et al., 2013; Khoruts and Sadowsky, 2016). In this patient, the stool microbiota does show a restructuring, following repeated FMT, but species that are expanded post FMT were present prior to FMT. Currently, these data suggest no evidence for large-scale transfer from the donor microbiota. The most abundant OTUs found in the donor, were shared with the recipient. Only one OTU was found to meet the criteria of engraftment, but it is difficult to discern if this OTU was present prior to FMT at undetectable levels. Also, considering species belonging to the genus *Phascolarctobacterium* are strict anaerobes, it is highly unlikely that it was transferred via the donor suspension, as it was prepared in aerobic conditions. However, it may be that engraftment is occurring following FMT, but at a deeper resolution, undetected by 16S rRNA gene sequencing, although this is questionable (Li et al., 2016). Due to the nature of how the donor suspension is prepared, viable anaerobic bacteria in vegetative form, are most likely absent post processing of donor stool. It may be that only strict anaerobic spores are present in the donor suspension post processing (Young et al., 2016). It is unknown though what role anaerobic spores play in dysbiosis and pathogenesis of UC.

Fungi, once thought to be passive colonizers of the gastrointestinal tract, contribute directly to inflammation and disease severity in patients with IBD (Mukherjee et al., 2014). We were unable to characterize the fungal community at the site of the mucosa and found that within the stool, the community was composed of mostly *Saccharomyces cerevisiae*. It is hard to draw conclusions about fungal dysbiosis in this patient, as colonic inflammation was not associated with increased fungal load. It is also difficult to discern which fungal species are part of the microbiota and which fungal species are from dietary factors.

Conclusion

This is the first long-term case report of using FMT to treat severe UC, refractory to conventional therapy in an adult. Clinical and endoscopic remission was achieved following multiple FMT. Consequently, during cessation of FMT this patient relapsed, but entered remission again following multiple FMT. Clinical and endoscopic remission was achieved again, although, histology reports persistent minimal activity. Microbial community changes occurred following treatment in both the lumen and mucosa-associated microbiota. Fungal community is present, but unclear at this point what role it plays in pathogenesis. No evidence for major engraftment of donor microbiota following FMT at the OTU level. FMT is a promising treatment for UC, but multiple and repeated FMT may be required to enter and maintain remission.

Supplementary Material



Supplementary Figure 1: Alpha diversity is not correlated with fecal calprotectin levels.

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CHAPTER 4

CHARACTERIZING ALTERATIONS OF THE INTESTINAL MICROBIOME FOLLOWING FECAL MICROBIOTA TRANSPLANTATION IN ULCERATIVE COLITIS PATIENTS

**Characterizing alterations of the intestinal microbiome following fecal microbiota
transplantation in ulcerative colitis patients**

Josie Libertucci^{1,2}, Paul Moayyedi², Thomas Gurry³, Jake C. Szamosi², Melanie Wolfe²,
Catherine Onishi⁴, Eric T. Alm³, Christine H. Lee,⁴ and Michael G. Surette^{1,2*}

Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton,
Ontario, Canada¹

Farncombe Family Digestive Health Research Institute, Department of Medicine,
McMaster University, Hamilton, Ontario, Canada²

Department of Pathology and Molecular Medicine, St Joseph's Healthcare, McMaster
University, Hamilton, Ontario, Canada³

The material in this chapter is in preparation for submission

Preface: I am the primary author for this chapter. My supervisor, Mike Surette and I designed all experiments. I conducted all the experiments, analyzed these data, and wrote the manuscript. Paul Moayyedi and Melanie Wolfe recruited patients, PM conducted all patient evaluations, and MW collected all patient data. Christine Lee and Catherine Onishi administered FMT to all patients, CO shipped all fecal samples to McMaster HSC. Thomas Gurry and Eric Alm completed machine learning studies on these data, although since no associations were found, the analysis was not included in this chapter. TG confirmed the analysis for the stool microbiota. Jake Szamosi provided support for the bioinformatics involved in this chapter.

Abstract

Background and Aims: Ulcerative colitis (UC) is a chronic inflammatory disease that is restricted to the colon. Though pathogenesis of this disease is multifactorial, the intestinal microbiota plays a large role in driving disease pathogenesis. Modulating this disease through manipulation of the microbiota by fecal microbiota transplantation (FMT) has recently gained interest as a potential treatment option. A phase II clinical trial, completed in 2015 showed 24% of patients randomized into the FMT arm entered into remission, compared to 5% within the placebo arm. The aim of this study was to characterize microbial changes in UC patients following FMT from the 2015 trial, in order to determine a signature associated with disease or remission. Engraftment of donor microbiota to recipients was also investigated.

Methods: Stool samples were collected from patients prior to and during FMT administrations, as well as donor suspensions. Biopsy samples were taken from patients during evaluation prior to the start of the trial and following. Microbial community was analyzed via 16S rRNA gene sequencing of the V3 and V3V4 regions for stool and biopsy samples, respectively. Fungal analysis was completed through amplification of the ITS2 region. The microbial community from a subset of patients, 4 responders and 4 non-responders pre and post FMT, were characterized using shotgun metagenomics.

Results: Prior to FMT, the stool and rectal microbiota did not show trends stratified based on disease severity or inflammation. Following FMT, a microbial signature was not

observed based on 16S rRNA gene sequencing. There was no relationship between disease status and the presence of fungal species. Within a subset of therapy successes and failures, patients that entered into remission following FMT showed increased abundance of *Roseburia intestinalis*, *Eggerthella lenta*, and the order Selenomonadales, although these associations did not pass FDR corrections. No evidence for engraftment from donor to recipient at the OTU or species level.

Conclusions: Dysbiosis in UC patients is diverse and heterogeneous amongst individuals. Accordingly, large global shifts within responder patients did not occur post FMT, only subtle differences mostly at the species level were observed. No evidence for engraftment of donor microbiota to recipients following FMT at the OTU or species level.

Keywords: Microbiome, microbiota, ulcerative colitis, dysbiosis, engraftment

INTRODUCTION

Ulcerative colitis (UC), a disease that is marked by relapsing inflammation that is restricted to the colon, is heavily influenced by environmental factors including the intestinal microbiome (Molodecky and Kaplan, 2010; Kostic et al., 2014). The commensal intestinal microbiome plays an important role in disease pathophysiology (Round and Mazmanian, 2009) and murine models have provided evidence of this, as the absence of an intestinal microbiota does not induce inflammation (Nell et al., 2010; Sellon et al., 1998). In an active state of UC, the host reacts to the commensal microbial community (Tamboli et al., 2004). For this reason, alterations in UC patients compared to healthy controls have been extensively studied and reported (Norman et al., 2015; Ott et al., 2004; Morgan et al., 2012; Alipour et al., 2015; Sokol et al., 2009) although inconsistencies exist between studies (Butto et al., 2016). After considering data from across studies, UC patients display a general decreased diversity, specifically a decreased diversity within the phylum Firmicutes, reduced abundance of species within the genus *Clostridium* and a reduction of *Faecalibacterium prausnitzii* (Walters et al., 2014).

Modulation of the microbiota through fecal microbiota transplantation (FMT) has gained interest as a treatment option for UC (Borody et al., 2003; Colman et al., 2014). FMT is a revolutionary treatment for recurrent or refractory *Clostridium difficile* infections (R-CDI) (van Nood et al., 2013), however, FMT treatment for chronic diseases like ulcerative colitis (UC) have not shown the same success rates in addition to being inconsistent (Moayyedi et al., 2015; Rossen et al., 2015). Poor success rates may be due to FMT methodologies for the treatment of UC (Cui et al., 2015). Donor, mode of

administration, and duration of treatment may affect success rates and may account for inconsistencies amongst studies, although, FMT methodologies seem to be less important for R-CDI treatment success but still play a role (Furuya-Kanamori et al., 2016).

The mechanism by which FMT works remains poorly understood for successful cases of UC. For treatment of RCDI, the mechanism for successful FMT is hypothesized as a restoration of bile acid metabolism and colonization resistance against *Clostridium difficile* (Theriot et al., 2016). Diversity of the gut microbiome is not always restored in RCDI patients following FMT (Seekatz et al., 2014). Less evidence is provided for characterizing the successful mechanism of FMT for treatment of UC. For UC patients that entered into remission following FMT, their microbial community has been shown to shift towards their donor microbiota (Moayyedi et al., 2015), although it does not provide insight into a mechanism of action, it suggests the community structure does change following FMT. Other reports have shown that the abundance of Clostridium cluster IV is shifted post FMT (Rossen et al., 2015).

In this report, community changes following FMT in both patients that received FMT and placebo are reported. The bacterial community of the lumen and mucosa-associated microbiota was examined in addition to characterizing the microbiome before and after FMT. Evidence for large global transfer of OTUs from donor to recipient was examined in detail as well.

METHODS

Samples collected and genomic DNA extraction

Stool samples in this study were collected from patients at St. Joseph's Hospital (Hamilton, Ontario) and shipped to McMaster University Health Science Centre (HSC), where they were frozen until processing. Samples used in this study were from patients from Moayyedi et al., 2015. Stool samples were collected prior to FMT and each week the patients received FMT, in addition to collecting the donor suspension sample. Biopsy samples were collected at the time of evaluation, 2 weeks prior to and following FMT, at McMaster University HSC. This study used a total of 192 stool samples and 118 biopsy samples. For both stool and biopsy samples, genomic DNA extraction was completed as previously described.²³

PCR amplification of the V3 and V3V4 region of the rRNA gene

For stool samples, amplification of the V3 region of the rRNA gene was completed using previously described protocols (Libertucci et al., 2016; Bartram et al., 2011; Whelan et al., 2014; Lau et al., 2016). To process the biopsy samples, the following amendments were made to the protocol. To reduce host contamination, and increase taxonomic resolution, a nested PCR reaction targeting the V3V4 regions of the 16S rRNA gene was used. The first reaction, amplified a 918bp region of the 16S rRNA gene (forward primer, 5'-AGA GTT TGA TCC TGG CTC AG-3'; reverse primer, 5'-CCG TCA ATT CCT TTR AGT TT-3') for 15 cycles. One µL of amplicon was taken from the first reaction, and used as template for the second reaction, carried out as previously described, but with primers targeting the V3V4 region (forward 5'-CCT ACG GGA GGC

AGC AG-3', reverse 5'-GGA CTA CHV GGG TWT CTA AT-3'). All samples were sequenced using an Illumina MiSeq platform at the McMaster Genome Facility (Hamilton, Ontario, Canada).

Data processing for the V3 and V3V4 region and analysis

V3 and V3V4 raw sequence reads were processed as in Libertucci et al., 2016. Within the stool sample dataset, 15,931,448 reads were generated with an average depth of 82,976 reads per sample, a minimum of 2,497 reads per sample, and a maximum read depth of 322,150. Within the biopsy sample dataset, 11,063,325 reads were generated with an average depth of 33,963 reads per sample, a minimum of 1056, and a maximum of 131,602 reads per sample. Singletons, reads that only appeared once, and operational taxonomic units (OTUs) that were not identified as bacteria were discarded. Taxonomic summaries, alpha and beta diversity (Bray-Curtis dissimilarity), non-parametric t-tests, and Permanova tests, were calculated using quantitative insights into microbial ecology (QIIME) (Caporaso et al., 2010). Statistical tests were always completed assuming a non-normal distribution (non-parametric), and significance defined as a p value ≤ 0.05 , and in cases where multiple tests were completed, False Discovery Rate (FDR) was used to correct for false positives. Some graphs were visualized using Prism v6.0.

Functional predictions based on 16S rRNA marker gene sequencing

Function of the stool and mucosa-associated were predicted from 16S rRNA gene sequence data using PICRUSt (Langille et al., 2013). Operational taxonomic units (OTUs) were picked using closed reference picking with QIIME following trimming and quality control of raw sequence reads. Following OTU picking, OTUs were normalized

by dividing each OTU by the predicted 16S copy number. Following normalization, metagenomic predictions were made by multiplying the normalized OTU abundance by each predicted functional trait. Then predictions were collapsed to the L3 level. Metagenomic predictions were analyzed using STAMP (Bolger et al., 2014).

Identification of the mycobiome

Stool and biopsy samples were screened for the presence of fungi by amplification of the internal transcribed spacer 2 (ITS2) region on nuclear ribosome DNA. The region amplified was 300bp, and primers bound to the 5.8S region (forward 5'-GTG AAT CAT CGA RTC TTT GAA C-3') and the 28S region (reverse 5'-TAT GCT TAA GTT CAG CGG GTA-3'). Total reaction volume was 25 μ L and consisted of 2.5 μ L of 10X buffer, 0.75 μ L of 50mM MgCl₂, 0.5 μ L of 10 mM dNTPs, 0.5 μ L of 1 μ M of each primer, 0.5 μ L of template of genomic DNA (30-50ng), 0.25 μ L of Taq polymerase (1.25U/ μ L), and 19.5 μ L of ddH₂O. PCR conditions consisted of an initial denaturation at 94 C for 2 min, 30 cycles of 94 C for 30s, 50 C for 30s, 72°C for 45s, followed by a final elongation at 72 C for 10 min.

For identification and sequencing of the fungal community, two PCR reactions were performed prior to sequencing. The first reaction consisted of amplification of the ITS2 region, with primers that included partial Illumina adapters added to 5.8SF and 28SR primers (5'-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT GTG AAT CAT CGA RTC TTT GAA C-3' and 5'-GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC TTA TGC TTA AGT TCA GCG GGT A-3'). The same PCR

reaction was conducted as the original ITS2 reaction, with the following amendments.

One μL of bovine serum albumin (BSA) and dimethyl sulfoxide (DMSO) were added to the reaction, and 55 C was used as an annealing temperature. The second reaction, 50 μL total volume, took 23 μL of the purified amplicon from the first reaction, and added the full Illumina adapters, with the specific barcode added on the reverse primer (P5-503 5'-AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT ATC CTC TAC ACT CTT TCC CTA CAC GAC GCT CTT-3' and P7-712 5'-CAA GCA GAA GAC GGC ATA CGA GAT TCC TCT ACG TGA CTG GAG TTC AGA CGT GTG CTC TTC CGA-3'), 0.25 Taq polymerase (1.25U/ μL), 1 μL of each primer, 0.5 μL dNTPs, 0.75 MgCl_2 , and 2.5 μL of buffer, with ddH₂O added to the final volume.

Library preparation for shotgun metagenomics

Genomic DNA was standardized to 5 ng/ μL and sonicated to 500 bp. Using the NEBNext Multiplex Oligos for Illumina kit (New England Biolabs), DNA ends were blunted, adapter ligated, PCR amplified, and cleaned as per manufacturers instructions. Library preparations were sent to the McMaster Genome Facility, and sequenced using the Illumina HiSeq platform.

Taxonomic analysis of shotgun metagenomic data

Raw sequence reads were concatenated, then trimmed and filtered for quality using Trimmomatic (Bolger et al., 2014). Forward and reverse fastq reads were then interleaved and converted to a fasta format. Taxonomy was assigned using MetaPhlAn2

(Truing et al., 2015) and hierarchical cluster analysis based on dissimilarity values was completed using hclust2 (<https://bitbucket.org/nsegata/hclust2>).

RESULTS

Baseline characteristics of the luminal microbiota from UC patients prior to FMT

It is commonly reported that UC patients show a distinct dysbiosis compared to healthy controls. However, it is unknown whether the UC microbiome shows distinct microbial community characteristics based on disease severity. For this reason, the first goal in this work was to examine if the microbial community shows characteristic features such as reduced diversity, reduction of species in the phylum Firmicutes, and increased pro-inflammatory species, depending on disease severity. Disease severity of UC was defined based on the full clinical Mayo score, with mild defined as a full clinical Mayo score from 3-5, moderate 6-9, and severe 10-12. Richness and evenness was examined in all three groups to assess if patients with severe disease had a lower diversity, than those with mild disease. Alpha diversity in UC patients is not correlated to disease severity (Figure 1A). Mean Shannon diversity was found to be relatively equal in all three groups (mean of mild = 3.56, mean of moderate = 3.51, mean of severe = 3.41, $p = 0.99$). Beta diversity was completed to assess the similarity of samples within each group, and if the groups cluster together based on the composition of the community. The microbial community in UC patients was found to be heterogeneous, and does not cluster based on disease severity (Figure 1B). Additionally, the microbiota did not cluster based on disease duration, sex, age, location of inflammation, current drug therapies, and past drug therapies (data not shown). A functional dysbiosis within the stool microbiota has

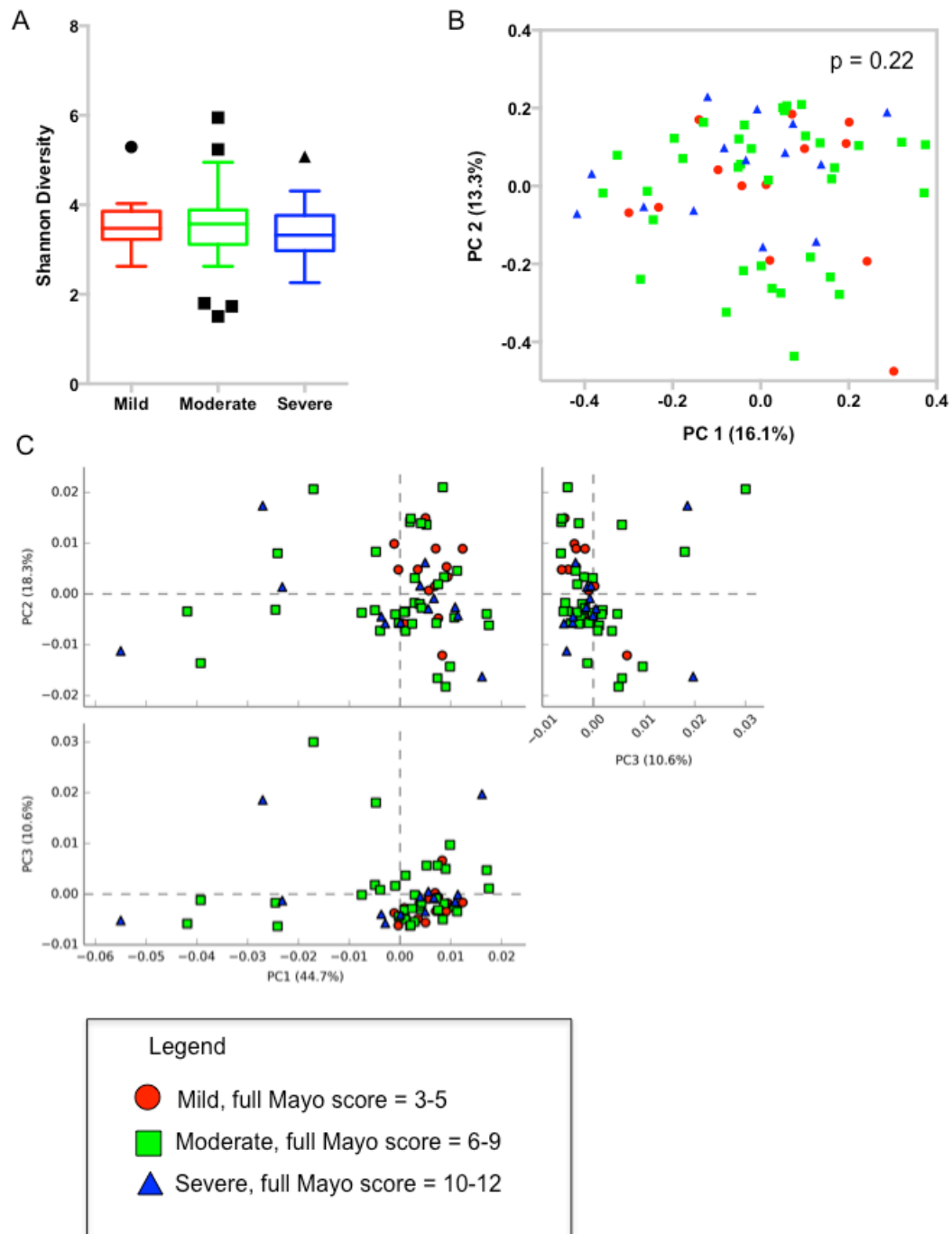


Figure 1: Characterizing dysbiosis in the intestinal microbiota of UC patients. A) Alpha diversity using Shannon Diversity index. B) Principle coordinate analysis at baseline (week 0). C) Principle component analysis of predicted functions.

also been reported in UC patients. For this reason, function of the community was predicted based on 16S data using PICRUSt. A clear separation of the community is not observed (Figure 1C).

Baseline characteristics of the mucosa-associated microbiota from UC patients prior to FMT

Rectal biopsy samples were sequenced using 16S rRNA gene sequencing, though the V3V4 region was targeted to reduce the amount of host DNA. Rectal biopsy samples were separated based on endoscopy score, which provides insight into the amount of colonic inflammation present. Inflammation at the time of evaluation, prior to FMT, was scored as a 1, 2, or 3 (mild, moderate, or severe inflammation) based on the clinical Mayo score characteristics. Within this dataset, the rectum was involved in all patient's disease. Richness and evenness of the microbial community of the rectum was not significantly different compared to the level of inflammation present (Figure 2A). Clustering based on inflammation severity was not observed (Figure 2B) and functional analysis predicted based on 16S gene sequences revealed no clustering based on inflammation severity (Figure 2C).

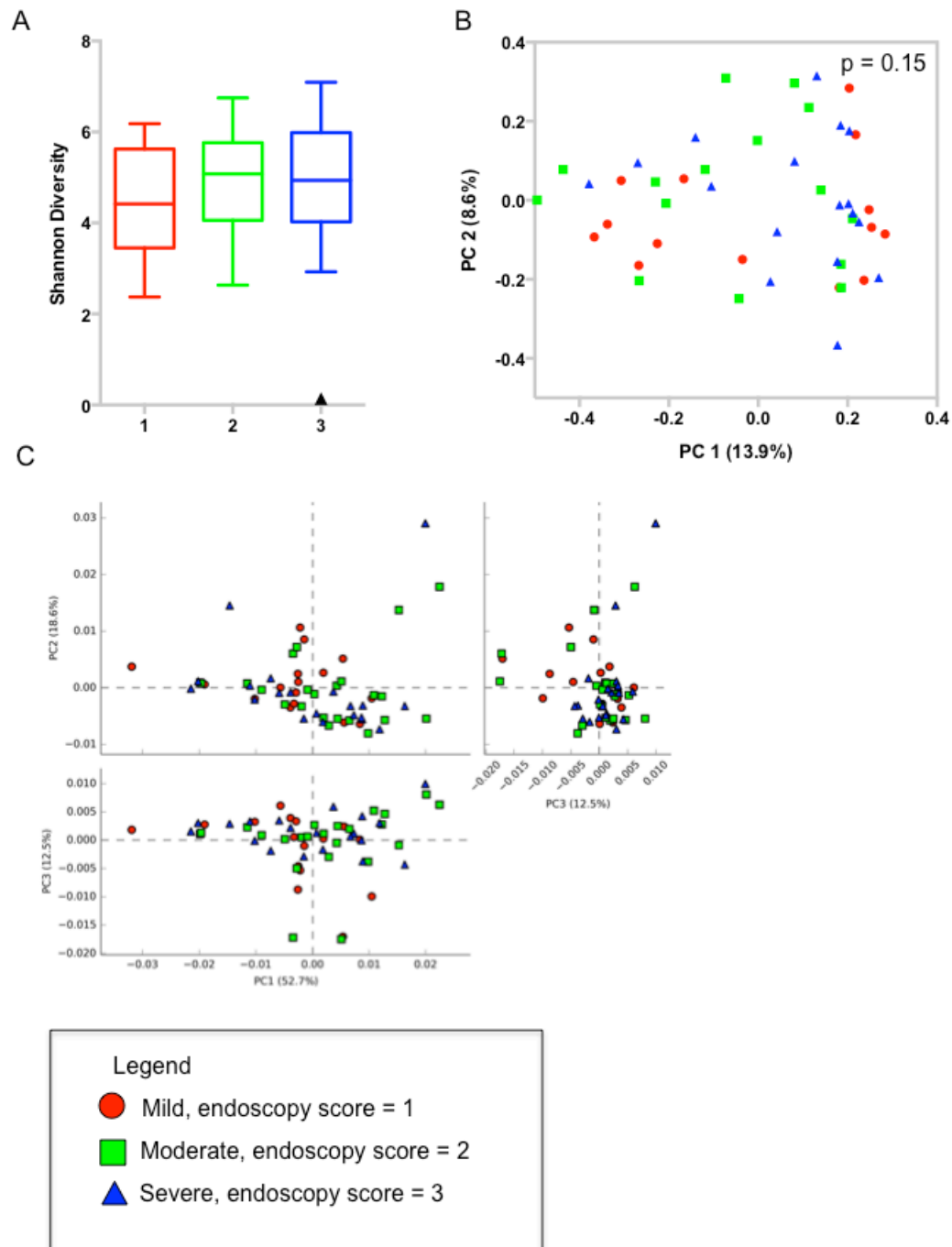


Figure 2: Baseline characteristic of the rectal mucosa-associated microbiota. A) Alpha diversity using Shannon Diversity index. B) Principle coordinate analysis at baseline (week 0). C) Principle component analysis of predicted functions.

Microbial changes of the stool microbiota following FMT

Data represented in this study is from one of only two published phase II clinical trials for treatment of UC. This trial also met their primary end goal, 24% of patients randomized to FMT entered complete endoscopic remission following 6 weeks of FMT administration compared to 5% of patients that were randomized to placebo. A major goal of this research was to investigate microbial changes of the stool microbiota that are associated with remission or conversely, disease. Richness and evenness following FMT was evaluated in patients that received placebo (Figure 3A), those that received FMT (Figure 3B) and those patients that responded (Figure 3C). It was expected that patients that responded to treatment would show an increase in diversity, although this was not observed. Richness and evenness was relatively equal following FMT, in patients that responded, and in those patients that received placebo treatment.

To test for shifts characteristic of responder patients following FMT and placebo, beta diversity was completed. The overall microbial community of placebo patients pre and post FMT revealed no significant differences, confirmed with a Permanova test ($p = 0.9$, Figure 3D). Next, the microbial community of the patients that received FMT was compared from baseline to week 6. Patients that received FMT did not cluster together at week 6 compared to week 0 suggesting their community remains heterogeneous following FMT (Figure 3E). Next, patients that responded to FMT at week 6 were

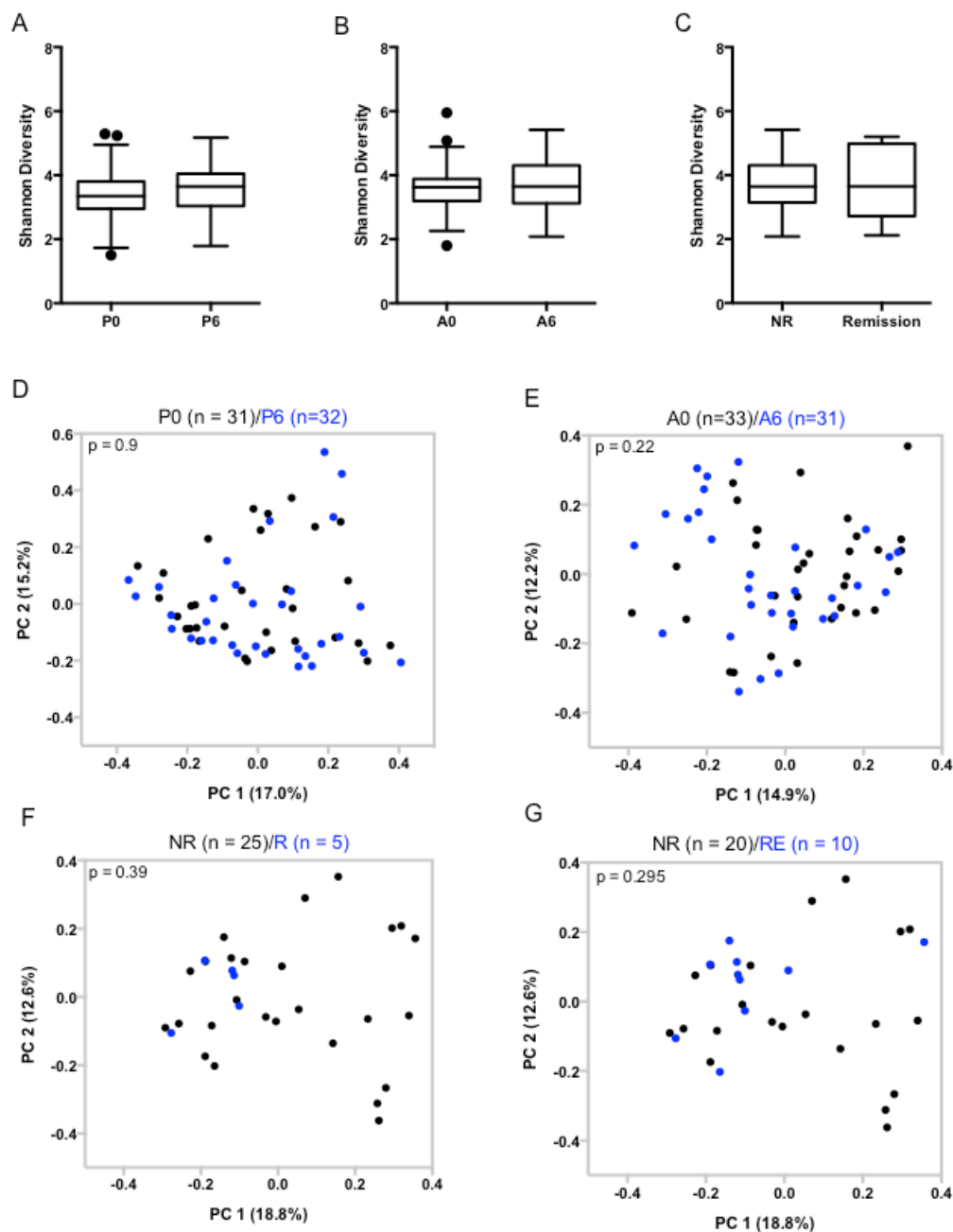


Figure 3: Stool microbiota characteristics in UC patients following FMT. Shannon diversity of UC patients before and after placebo (A), FMT (B), and responders compared to non-responders at week 6 (C). PCoA using Bray-Curtis dissimilarity followed by Permanova analysis at week 0 and week 6 of placebo patients (D), patients that received FMT (E), non responders and responder (F), and non-responders and patients that improved post FMT (G).

compared to those that did not (Figure 3F), no major clustering was observed ($p = 0.39$).

And finally, patients that responded to treatment were compared to patients that did not respond (change of 4 points on the Mayo score), however, response did not cluster as well (Figure 3G). There were no statistical differences comparing responders to non-responders following FMT.

Taxonomic changes compared to their respective donor was examined in patients that received FMT, in addition to the presence of fungal species in both patients and donors (Figure 4 and Figure 5). Figure 4 represents patients that did not exhibit a change in their Mayoscore post FMT, as indicated. Most notable, one of the six donors used contained a large proportion of *Escherichia*. Large global shifts were not observed in patients these patients, and the presence of fungal species does not correlate with disease. Responder patients, and patients in the active arm, did not show large global shifts towards the donor (Figure S1). Figure 5 represents changes at the phylum level in patients that either responded positively to treatment, or patients that responded negatively to treatment. Again, no trend exists compared to fungal species and clinical response. Functional analysis of the microbiota following FMT based on 16S predictions was also examined (Figure 6). Clustering based on the community was not observed (Figure 6A).

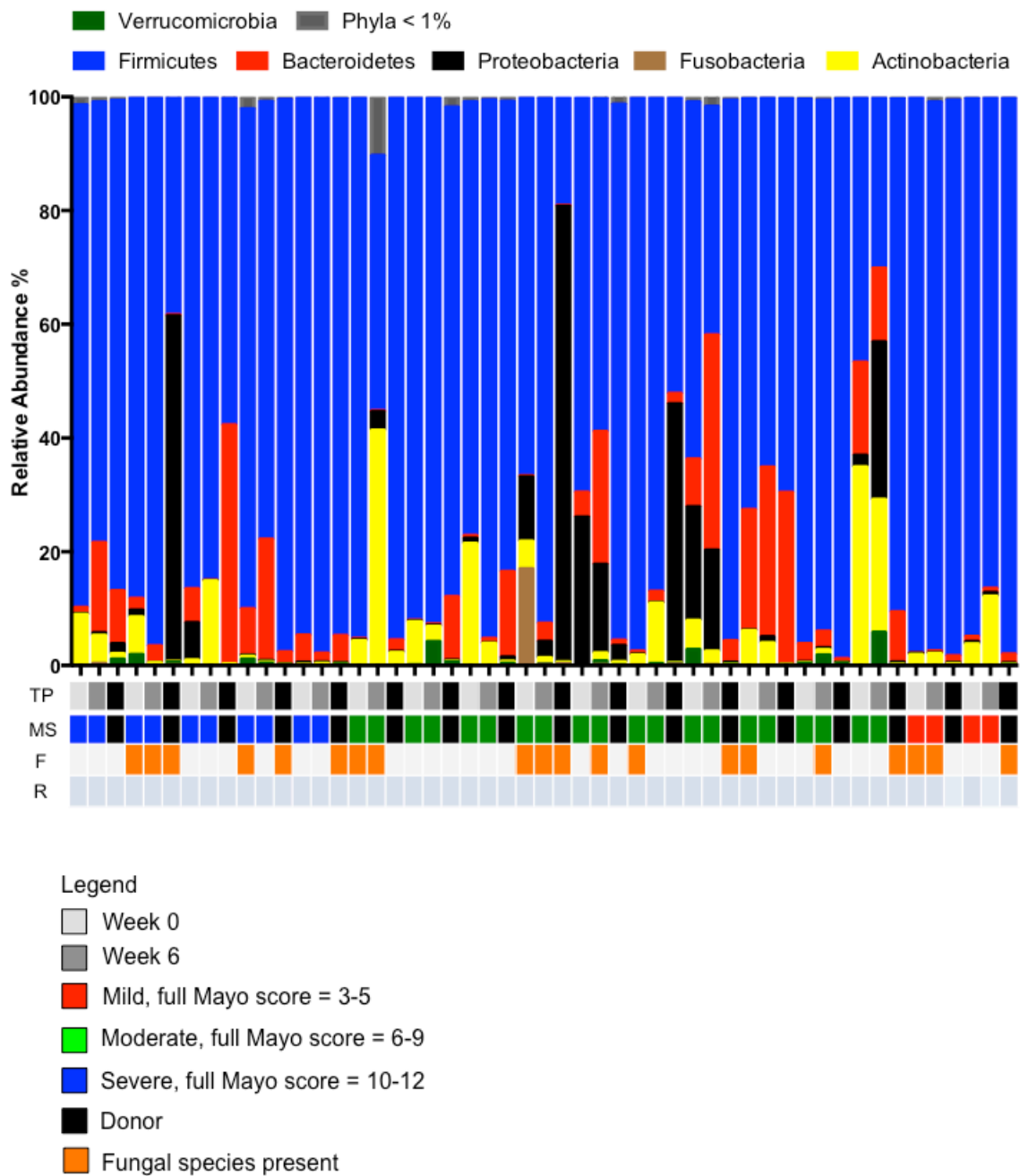


Figure 4: Taxonomic comparisons of the stool microbiota pre and post FMT in patients whose clinical Mayo score did not change.

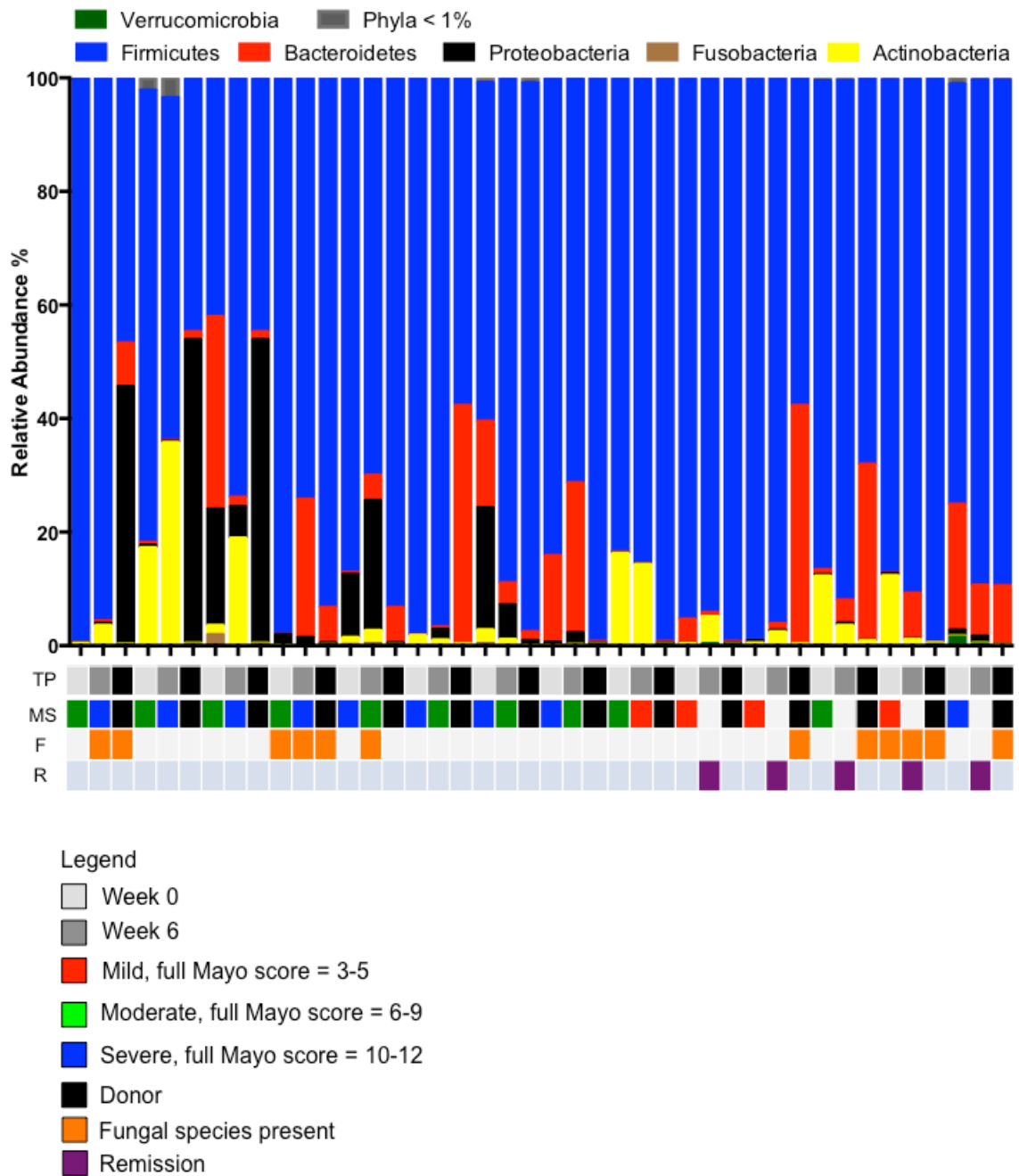


Figure 5: Taxonomic comparisons of the stool microbiota pre and post FMT in patients whose clinical Mayo score changed.

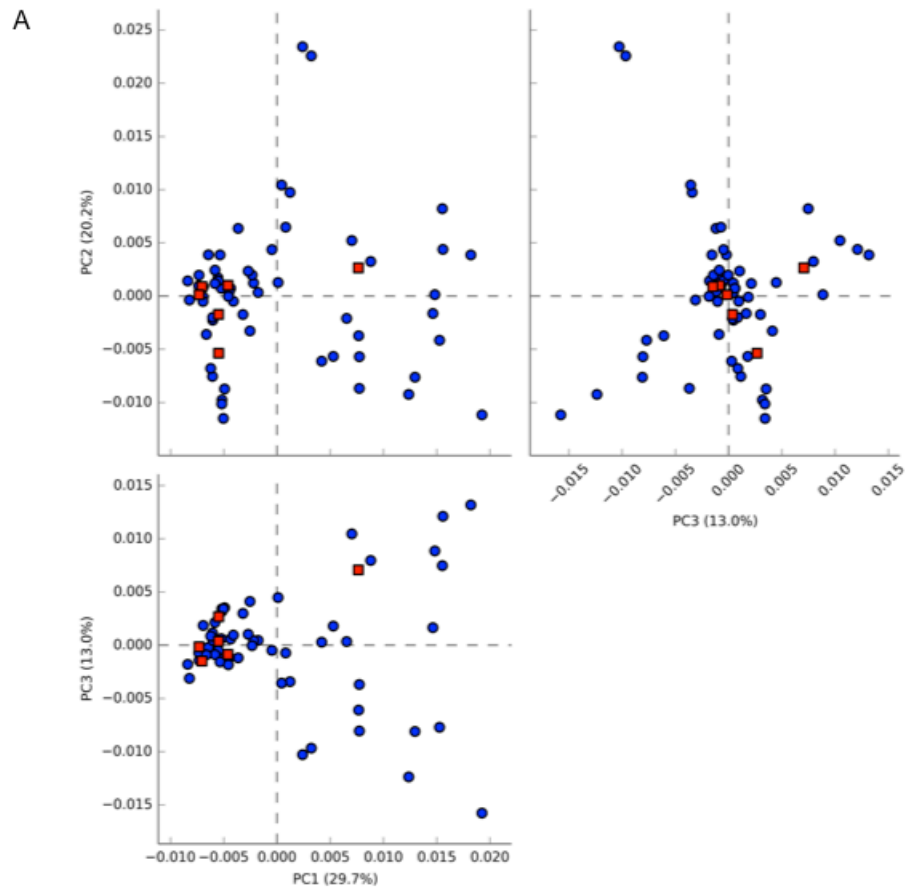


Figure 6: PCA of predicted function base on 16S following FMT

Microbial changes of the mucosal associated microbiota following FMT

Biopsy samples were obtained at time of patient evaluation before and after FMT administration. As within the stool microbiota, alpha diversity did not change in patients within the placebo group, FMT group, or in relation to responder patients (Figure 7A-C). Principle coordinate analysis was also completed to test for large microbial shifts before and after FMT and placebo. No significant differences were observed (Figure 7D-F). Taxonomic changes at the phylum level were compared between pre and post samples, to, in addition to the presence of fungal species (Figure 8). No relationship between disease status and presence of fungal species.

Investigating engraftment of bacterial species from donor microbiota to recipients using shotgun metagenomic

The stool microbial community was examined in a subset of patients, 4 treatment successes and 4 treatment failures pre and post FMT using shotgun metagenomics. Hierarchical cluster analysis based on dissimilarity was completed on this subset (Figure 9). Responder patients did not cluster together, nor did patients with severe disease. Engraftment was tested at the species level (species absent week 0, present week 6, and present in the donor) in the 4 responders and 4 non-responders. Table 1 displays species that meet this description in responder patients, and Table 2 describes the species that meet this criteria in non-responder patients. Engraftment was greater in non-responder patients. Differential abundance testing revealed 3 species were enriched in responder patients *Roseburia intestinalis*, *Eggerthella lenta*, and the order Selenomonadales, although these associations did not pass FDR corrections (Table 3).

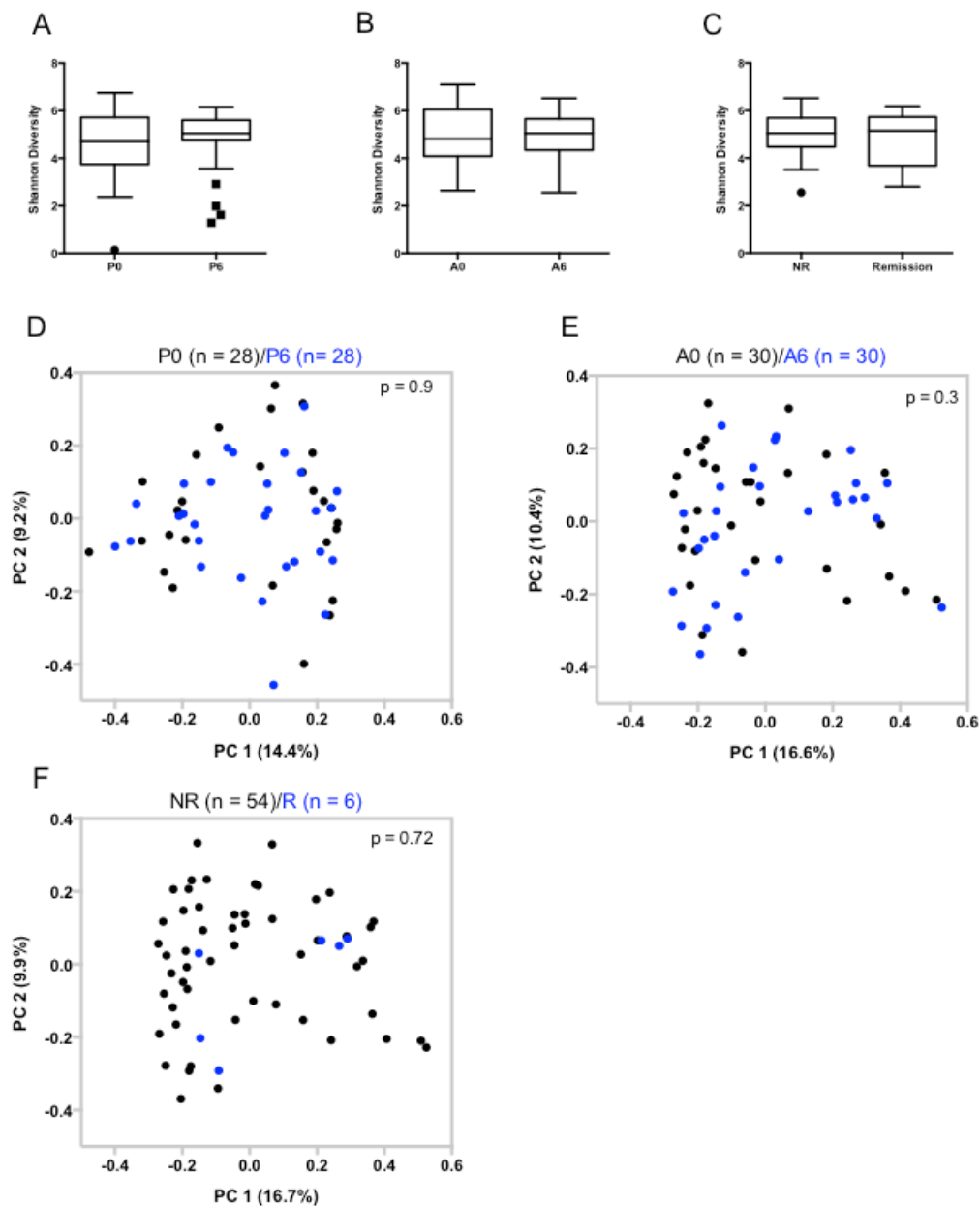


Figure 7: Rectal mucosa-associated microbiota characteristics in UC patients following FMT. Shannon diversity of UC patients before and after placebo (A), FMT (B), and responders compared to non-responders at week 6. (C) PCoA using Bray-Curtis dissimilarity followed by Permanova analysis at week 0 and week 6 of placebo patients (D), patients that received FMT (E), non responders and responder (F)

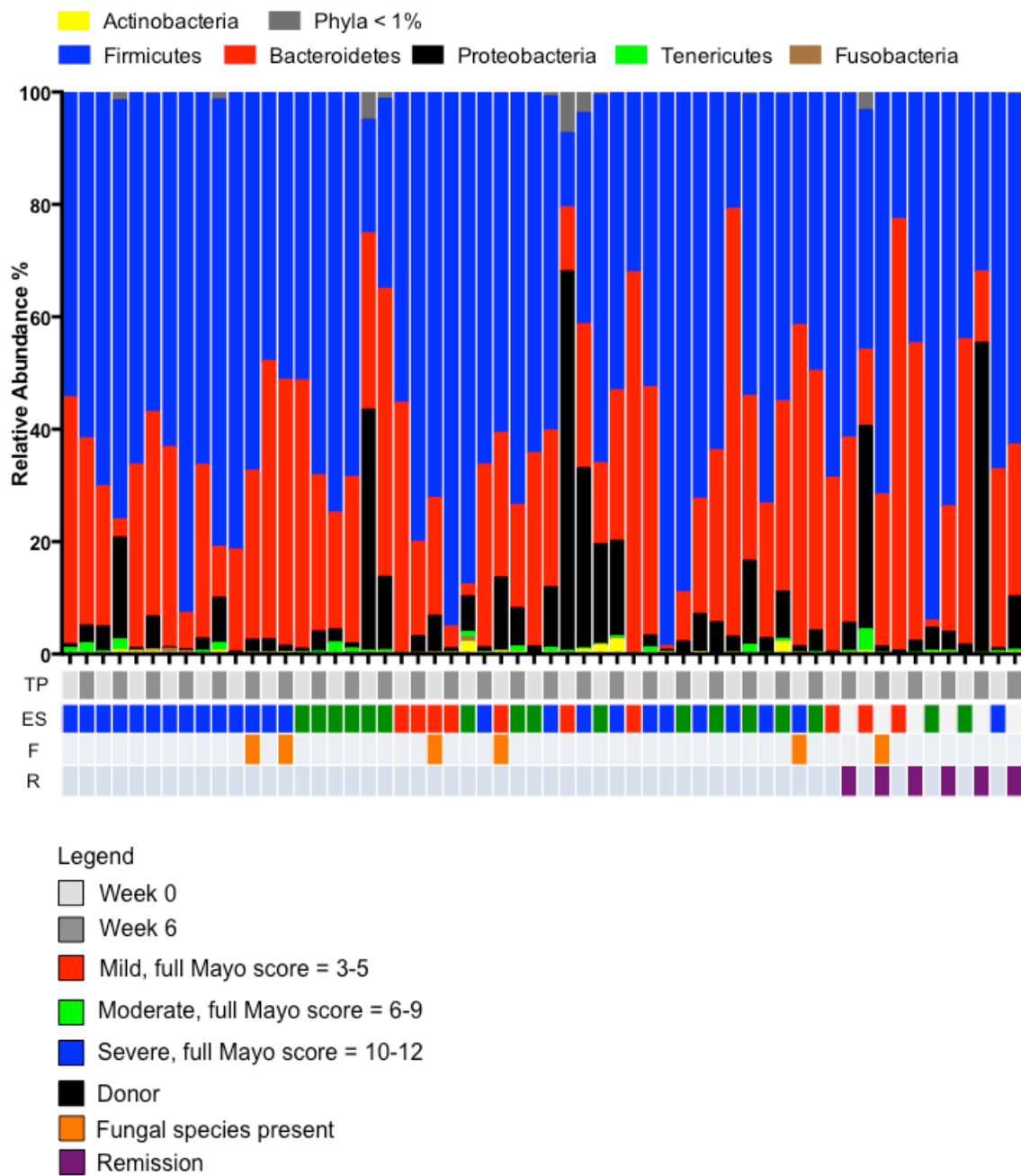


Figure 8: Taxonomic comparisons of the rectal microbiota pre and post FMT in UC patients.

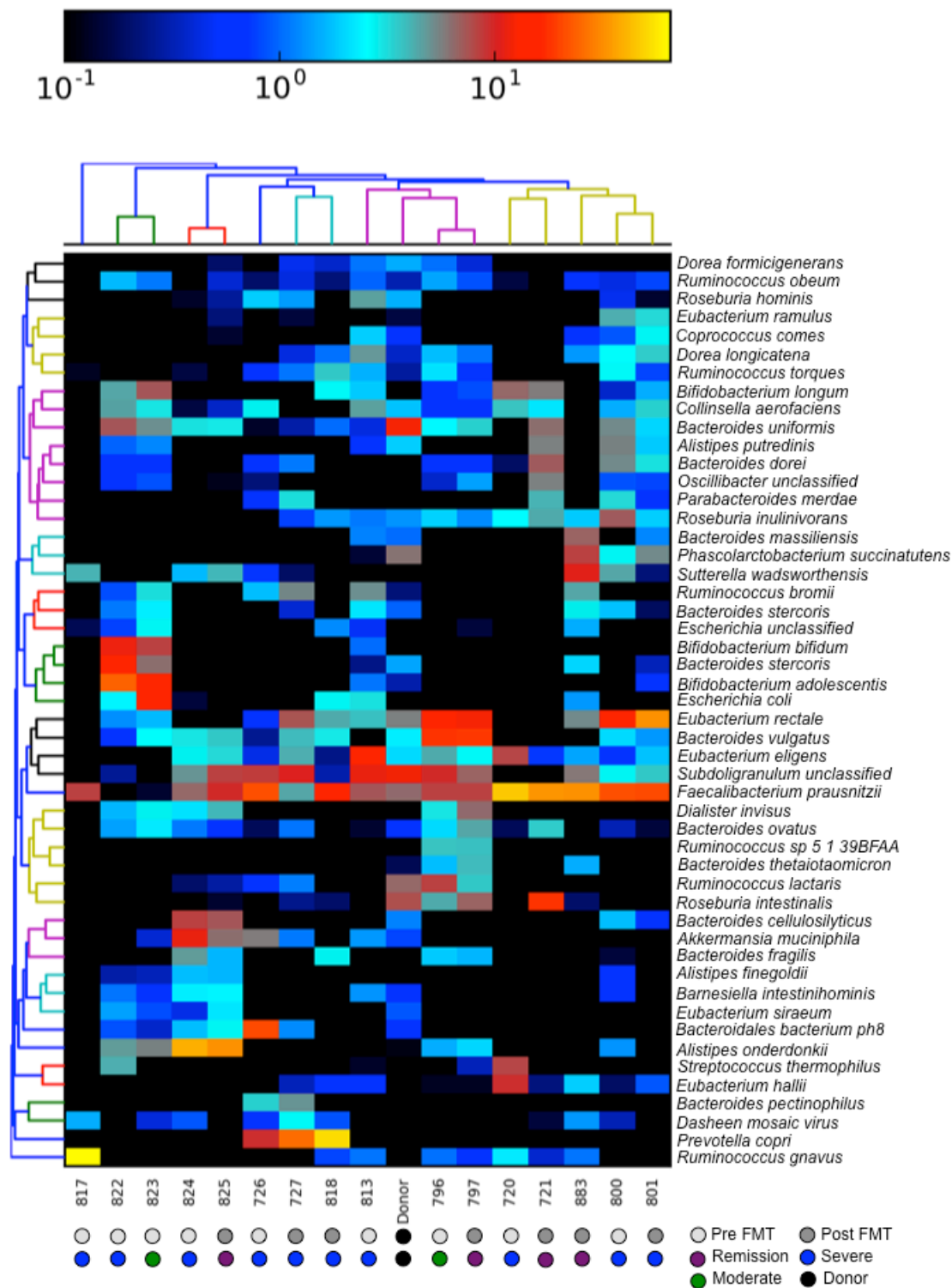


Figure 9: Heat map of stool microbiota in a subset of treatment success and treatment failures using shotgun metagenomics.

Table 1: Relative abundance of species absent at week 0, present at week 6, and present in the donor in remission patients.

Taxonomy	Relative Abundance Week0	Relative Abundance Week0	Donor
Patient #1			
<i>Parabacteroides distasonis</i>	0	0.01022	0.01523
<i>Alistipes putredinis</i>	0	5.67319	1.91241
<i>Roseburia intestinalis</i>	0	17.7185	7.74352
<i>Veillonella unclassified</i>	0	0.03301	0.51417
Patient #2			
<i>None</i>			
Patient #3			
<i>Rothia mucilaginosa</i>	0	0.10222	0.2648
<i>Eubacterium hallii</i>	0	0.00431	0.05873
<i>Anaerostipes hadrus</i>	0	0.01775	0.01546
<i>Dorea formicigenerans</i>	0	0.19653	1.51858
<i>Dorea longicatena</i>	0	0.08375	0.35834
<i>Roseburia intestinalis</i>	0	0.1378	7.74352
<i>Roseburia inulinivorans</i>	0	0.10005	1.30765
Patient #4			
<i>Bacteroides thetaiotaomicron</i>	0	1.5579	0.17068
<i>Eubacterium siraeum</i>	0	0.1016	0.89287
<i>Sutterella wadsworthensis</i>	0	10.82523	0.05794

Table 2: Relative abundance of species absent at week 0, present at week 6, and present in the donor in non-responder patients.

Taxonomy	Relative Abundance Week0	Relative Abundance Week0	Donor
Patient #1			
<i>Methanobrevibacter smithii</i>	0	0.80519	0.06033
<i>Bacteroides caccae</i>	0	0.38434	0.97219
<i>Eubacterium hallii</i>	0	0.33552	0.05873
<i>Eubacterium ramulus</i>	0	0.15076	0.15733
<i>Eubacterium ventriosum</i>	0	0.8865	0.05619
<i>Coprococcus catus</i>	0	0.13145	0.05217
<i>Coprococcus comes</i>	0	0.07509	0.69962
<i>Dorea longicatena</i>	0	0.41594	0.35834
<i>Lachnospiraceae bacterium 3146FAA</i>	0	0.01456	0.01795
<i>Roseburia intestinalis</i>	0	0.24416	7.74352
<i>Roseburia inulinivorans</i>	0	0.77341	1.30765
<i>Eubacterium bifforme</i>	0	0.73846	9.73384
Patient #2			
<i>Rothia mucilaginosa</i>	0	0.04362	0.2648
<i>Bifidobacterium adolescentis</i>	0	0.58902	0.3025
<i>Bacteroides massiliensis</i>	0	1.20651	1.02456
<i>Bacteroides stercoris</i>	0	0.33655	1.49928
<i>Lachnospiraceae bacterium 3146FAA</i>	0	0.02753	0.01795
Patient 3			
<i>Bacteroides fragilis</i>	0	2.80757	0.05786
<i>Bacteroides ovatus</i>	0	0.01529	0.57703
<i>Bacteroides uniformis</i>	0	1.01031	12.12474
<i>Bacteroides vulgatus</i>	0	2.9541	2.35746
<i>Eubacterium hallii</i>	0	0.58067	0.05873
<i>Eubacterium rectale</i>	0	4.33293	5.62744
<i>Ruminococcus obeum</i>	0	0.22342	0.31463
<i>Dorea formicigenerans</i>	0	0.37256	1.51858
<i>Dorea longicatena</i>	0	1.04436	0.35834
<i>Lachnospiraceae bacterium 3146FAA</i>	0	0.35329	0.01795
<i>Roseburia intestinalis</i>	0	0.19616	7.74352
<i>Roseburia inulinivorans</i>	0	1.34251	1.30765
Patient 4			
<i>Rothia mucilaginosa</i>	0	0.00985	0.2648
<i>Parabacteroides unclassified</i>	0	0.08325	0.35825
<i>Bilophila wadsworthia</i>	0	0.00573	0.00198
<i>Akkermansia muciniphila</i>	0	0.38751	0.78087

Table 3: Taxa that are enriched in responder patients following FMT.

Taxonomy	Mean Relative Abundance Remission	Mean Relative Abundance Non- responder	P value
<i>Eggerthella lenta</i>	0.0298075	0.005004167	0.003996004
<i>Roseburia intestinalis</i>	6.2079975	0.40675083	0.007992008
<i>Selenomonadales</i>	4.8010775	1.690806667	0.026973027

DISCUSSION

In this study the microbial community of the lumen and stool was analyzed before and after FMT to characterize alterations that are associated with disease or conversely remission using 16S rRNA gene sequencing and shotgun metagenomics. This study shows, large global shifts do not occur following FMT in either patients that received FMT or patients that responded to treatment. This was observed in both the stool community and the mucosa-associated community. Shotgun metagenomics was completed on a subset of patients, 4 patients that responded and 4 that did not. All patients received FMT from the same donor. Using shotgun metagenomics, subtle differences were found between patients that responded and patients that did not. Remission patients showed an increase in abundance in *Roseburia intestinalis*, *Eggerthella lenta*, and the order Selenomonadales, although these associations did not pass FDR corrections. Given the complex nature of dysbiosis within UC patients, in comparison to patients with CDI, 16S may not be the appropriate tool to tease apart the intricate complexities that occur following FMT. A follow up analysis, detailing the changes in relation to genes and functional pathways should be completed.

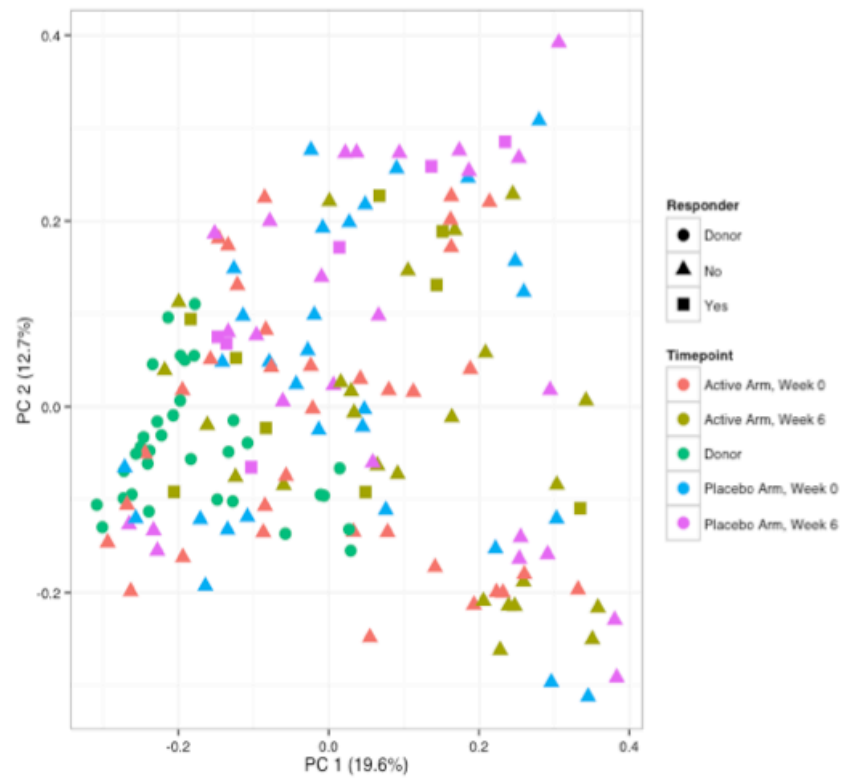
It is not surprising that large global shifts were not observed following FMT in UC patients, as it is seen in patients that receive FMT for treatment in RCDI (Seekatz et al., 2014). These data shown that UC patients, although in dysbiosis, have a diverse and heterogeneous microbiota. This heterogeneity was observed at both the site of the rectum and stool. Considering, not every patient responded, and the fact that within the intestinal

microbiota of the responders large global shifts were not observed, UC patients display a more resilient dysbiosis, unlike patients with RCDI (van Nood et al., 2013; Antharam et al., 2013; Lawley et al., 2012). In a recent case report of a patient with long-term severe UC, it was reported that there was an oversaturation of species from the genus *Streptococcus* in the rectum and lumen (Libertucci et al., 2016). This enrichment of *Streptococcus* was not seen in patients within this study. This patient does not represent the patients that were in this study as this individual had a high stool frequency (>40 per day) and was recommended for colectomy. No other patient in this study was recommended for colectomy, and no other individual was as severe as the individual represented in the case study.

Successful FMT for treatment of RCDI has been shown to be a result of restored bile acid metabolism (Theriot et al., 2016) or competition of non-toxicogenic *C. difficile* spores (Gerding et al., 2015) depending on the treatment. Recently, it has been shown that engraftment of strains, detected by SNVs, following FMT occurs in individuals with metabolic syndrome (Li et al., 2016). Engraftment of donor species to recipients was examined in this study. At the 16S level, there was no evidence for large-scale engraftment. For this reason, engraftment at the species level was tested using shotgun metagenomics. There was evidence of engraftment (species absent at week 0, present at week 6 and present in the donor), but this was observed in both responders and non-responders. Since this phenomenon was not specific to responder patients, and the frequency of acquired species was greater in non-responder patients, this suggests this

mechanism is not associated with response or what was observed was noise. Although, strain replacement may have occurred post FMT, this is a major limitation to this study.

Overall, the findings from this study suggest that the UC microbiota is diverse, heterogeneous amongst individuals, and stable. Given that no trends were observed between individuals, dysbiosis is specific to the individual. Moreover, since it was observed that the UC microbiota is diverse, and there was no evidence for engraftment, this suggests it may be difficult to replace members of the indigenous microbiota through FMT. These data are corroborated by a recent study that showed, engraftment of the probiotic *Bifidobacterium longum* AH1206 occurs depending on the presence of specific features of the resident microbiota (Maldonado-Gómez et al., 2016). Microbial species that are introduced to the human gut microbiota, persist within the gut only in the absence of specific taxa and their functional genes. The use of a universal donor may not be efficacious in treating IBD with FMT, as dysbiosis should be assessed individually, and FMT suspensions prepared based on the characterized dysbiosis.



Supplementary Material

Supplementary Figure 1: PCoA of patients pre and post FMT in relation to donor slurry.

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CHAPTER 5

INFLAMMATION RELATED DIFFERENCES IN MUCOSA-ASSOCIATED MICROBIOTA AND INTESTINAL BARRIER FUNCTION IN COLONIC CROHN'S DISEASE

Inflammation related differences in mucosa-associated microbiota and intestinal barrier function in colonic Crohn's disease

Josie Libertucci^{1,2}, Usha Dutta³, Sandeep Kaur², Jennifer Jury², Laura Rossi², Michelle E Fontes², Michael G. Surette^{1,2}, Elena F. Verdu², and David Armstrong^{2*}

Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Ontario, Canada

Farncombe Family Digestive Health Research Institute, McMaster University, Hamilton, Ontario, Canada.

Department of Gastroenterology, PIGMER, Chandigarh, India

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Preface: I am the primary author for this chapter. Mike Surette, Elena Verdu, David Armstrong, and myself designed all experiments. I conducted all experiments with some technical assistance: Jennifer Jury conducted the Ussing Chamber assays, Michelle Shah conducted the genomic DNA extractions, and Laura Rossi completed the PCR for 16S analysis and ITS2 screening. I analyzed these data and wrote the manuscript.

ABSTRACT

Background and Aims: Crohn's disease (CD) is characterized by patchy lesions, and is associated with an altered microbial composition and increased intestinal permeability. Dysbiosis and its spatial relationship with inflamed areas, as well as altered permeability has not been explored previously. The aim of this study was to analyze the microbial composition of the mucosa-associated microbiota and the permeability characteristics of colonic mucosal biopsies in CD.

Methods: To characterize inflammation, CD patients and controls (C) were evaluated by endoscopy. Patients were characterized as displaying colonic inflammation (CD-I) or not displaying (CD-NI). Microbial community analysis was performed on biopsy samples by 16S rRNA gene sequencing of the V3 region. Encroachment of bacterial cells to host tissue was completed by fluorescent in situ hybridization (FISH). Paracellular permeability was completed using Ussing chambers, and vascular permeability by confocal laser-scanning endomicroscopy (CLE).

Results: The mucosal microbiota in CD-I patients is distinct from controls and CD-NI patients. Compared to controls, CD-I patients showed a decrease in *Lachnospira*, *Faecalibacterium*, and *Parabacteroides*. Compared to CD-NI, a decrease in *Blautia* and an increase in *Escherichia* were observed. Encroachment of bacteria to host epithelial cells was greatest in CD-I patients, and increased in inflamed areas. Altered vascular and

paracellular permeability was present within CD-I patients, and specific to inflamed areas.

Conclusions: Overall CD-I patients showed an altered mucosal microbial community compared to CD-NI patients and controls. Matched biopsy samples from group CD-I showed that inflamed tissue is characterized by increased paracellular and vascular permeability, and increased encroachment of bacteria to host epithelial cells, which may be driving inflammation in CD.

Keywords: Crohn's disease, microbiota, permeability, dysbiosis

INTRODUCTION

The exact cause of Crohn's disease (CD), one of two conditions that define inflammatory bowel diseases (IBD), remains unknown, but it is currently accepted that this disease is due to a complex interplay between the host's genetics, immune system, intestinal microbiome, and other environmental factors (Xavier et al., 2007). Chronic inflammation in CD commonly affects the ileum and colon, and is characterized by patchy and transmural lesions that include granulomas, deep fissuring ulcers, and transmural lymphoid aggregates (Bouma and Strober, 2003). It is unknown what causes certain areas of the intestine to be affected and present as inflamed, while adjacent areas remain unaffected and clinically non-inflamed.

Clinical and animal studies have shown that increased intestinal permeability can precede the onset of intestinal inflammation and may predict relapses (D'Inca et al., 1999; Arnott et al., 2000; Tibble et al., 2000; Kiesslich et al., 2012). Thus, intestinal barrier dysfunction, a broad term that includes alterations in mucosal permeability, has been proposed as a key pathogenic factor in CD (Söderholm et al., 1999; Salim et al., 2011). When intestinal barrier is compromised, containment of bacteria and other antigens is impaired, allowing uncontrolled access to host tissues, which drives inflammation (Shen et al., 2009). Normalization of intestinal permeability has been associated with clinical remission in CD. Although, increased permeability is associated with active CD, a causative role remains unclear. Moreover, clinical determination of intestinal permeability is subject to many confounders, and the patchy nature of CD lesions makes interpretations difficult (Galipeau and Verdu, 2016).

Another pathogenic factor in CD that has recently emerged is the role of intestinal dysbiosis as a driver of chronic inflammation (Zeissig and Blumberg, 2013). Many studies have reported within the stool microbiota, a decrease in the phylum Firmicutes, subsequently decreasing the abundance of short-chain fatty acid (SCFA) producers within the luminal contents (Morgan et al., 2012), an increase in pro-inflammatory bacteria such as Fusobacteria, and a notable decrease in *Faecalibacterium prausnitzii* (Sokol et al., 2008). However as with permeability, a causative effect has been difficult to assess. Little is known about the relationship between the mucosa associated microbiota, and patchy inflammation.

For this reason we analyzed the microbial composition of the mucosa-associated microbiota and intestinal and vascular permeability of mucosal biopsies in patients with colonic CD. We found that although patients with active CD display altered mucosa associated microbial community compared to inactive patients, only inflamed biopsies display increase permeability and disruptive barrier function characterized by encroachment of bacterial cells. We hypothesize that patches of dysbiotic microbial communities disrupt the mucosal and endothelial barrier driving inflammation in CD.

MATERIALS AND METHODS

Inclusion and exclusion criteria, and clinical investigations

Informed consent was taken from all individuals entering the study. Inclusion criteria included patients aged 18 to 70 years and requiring a clinical indication for white

light colonoscopy. Exclusion criteria included, inability to provide informed consent, allergy to fluorescein, pregnancy or breast feeding, presence of serious life threatening co-morbidities, colectomy, toxic megacolon, jaundice, cirrhosis, renal dysfunction, active GI bleeding, and a history of difficult colonoscopy, strictures or extensive diverticulosis. A complete blood count including hemoglobin, leukocyte counts, differential count, platelets, ESR, peripheral blood film will be done. CRP levels will be done by standard methods. CDAI and HBI were calculated.

Identification of Fecal calprotectin levels

Stool samples were collected in a clean container, one day prior to colonoscopy and colonic preparation for colonoscopy. Stool samples were stored by the patient and collected on the day of colonoscopy. One gram of stool was mixed with 4 mL of sterile saline and the supernatant was taken for extraction and subsequent analysis. Supernatants were stored at -80 °C until time of estimation of fecal calprotectin (fCal) using a standard commercially available ELISA protocol, as per manufactures instructions.

16S rRNA gene sequencing of the V3 region

For biopsy samples, genomic DNA extraction and PCR amplification of the V3 region of 16S rRNA gene, was conducted using a previously described protocols.¹⁶⁻¹⁸ For processing of the biopsy samples, the following amendments were made. During genomic DNA extraction, ultrapure reagents made with RNase and DNase free, sterile water, (Qiagen, Hilden, Germany) were used to reduce contaminants. Raw fastq files were processed using a protocol previously described (Whelan et al., 2014; Bartram et al.,

2011; Stearns et al., 2015; Lau et al., 2016). Briefly, raw sequence reads were trimmed using Cutadapt (Martin, 2011), and subsequently aligned using PANDAseq Masella et al., 2012). Sequences were clustered into operational taxonomic units (OTUs) using AbundantOTU (Ye et al., 2012), and taxonomy assigned using the RDP classifier (Cole et al., 2014) against the Greengenes database (DeSantis et al., 2006).

In this dataset, 8,735,095 reads were generated with an average depth of 83,191 per sample, a minimum read depth of 1,004, and a maximum read depth of 211,485. Singletons and unassigned operational taxonomic units (OTUs) were discarded. Taxonomic summaries, alpha and beta diversity (bray-curtis dissimilarity), non-parametric t-tests, and Permanova tests, were calculated using quantitative insights into microbial ecology (QIIME) (Caporaso et al., 2010). Statistical tests were always completed assuming a non-normal distribution (non-parametric), and significance defined as a p value < 0.05, and in cases of multiple tests, False Discovery Rate was conducted to account for false positives. Some graphs were visualized using Prism v6.0.

Identification of the fungal community

Biopsy samples were screened for the presence of fungi via amplification of the internal transcribed spacer 2 (ITS2) region on nuclear ribosome DNA. The region amplified was 300bp, and primers bound to the 5.8S region (forward 5'-GTG AAT CAT CGA RTC TTT GAA C-3') and the 28S region (reverse 5'-TAT GCT TAA GTT CAG CGG GTA-3'). Total reaction volume was 25 µL and consisted of 2.5 µL of 10X buffer, 0.75 µL of 50mM MgCl₂, 0.5 µL of 10 mM dNTPs, 0.5 µL of 1 µM of each primer, 0.5

μL of template of genomic DNA (30-50ng), 0.25 μL (1.25/U) of Taq polymerase, and 19.5 μL of ddH₂O. PCR conditions consisted of an initial denaturation at 94 °C for 2 min, 30 cycles of 94 °C for 30s, 50 °C for 30s, 72 °C for 45s, followed by a final elongation at 72 °C for 10 min.

Calculating paracellular permeability by Ussing Chamber analysis

Paracellular permeability of biopsy specimens was analyzed in Ussing chambers to calculate the total mucosa-to-serosal flux of the probe ⁵¹Cr-EDTA (Silva et al., 2012). Biopsy samples were collected and placed in a cold oxygenated buffer, and equilibrated for 15 min prior to the addition of 6 $\mu\text{Ci/ml}$ of ⁵¹Cr-EDTA (Perkin-Elmer, Boston, MA, USA) to the mucosal buffer in the Ussing chamber. Influx of ⁵¹Cr-EDTA was calculated by sampling 100 μl from the serosal side at time points 0, 30, 60, 90, and 120 min. Values were compared to 100 μl of ⁵¹Cr-EDTA taken from the mucosal buffer at time 0. Influx was calculated as an average over the 2 hr period and expressed as expressed as % Hot/h/cm².

Confocal laser-scanning endomicroscopy

Vascular permeability within biopsy samples was assessed using confocal laser-scanning endomicroscopy (CLE). Five 5 ml of 10% fluorescein was administered intravenously to all participants and images acquired from areas with endoscopically inflamed mucosa (hyperemia, friability, erosions, ulcerations), and from adjacent areas of endoscopically normal mucosa in selected areas (ileum, right colon, transverse colon,

descending colon or rectum). Fluorescein permeability measured by fluorescein leakage was evaluated using IMAGE J. One blinded investigator (JL) evaluated each image twice. A third party not involved in this study randomized the images. The randomization code was broken post evaluation, and values were plotted and analyzed for significance.

Histopathological scoring

Haematoxylin and eosin staining was performed on formalin fixed slides of inflamed and matched non-inflamed regions in active CD patients. Slides were given a code, randomized, and the examiner was blinded during histology scoring. Total score was out of 13, with 0 being completely normal pathology and 13 absolute inflammation. In 10 fields of view, using 400x magnification, inflammation was scored based on the presence or absence of granulomas (0=absent, 1=present), presence of erosion or ulcers (0=no, 1=yes), polymorphonuclear cells in the epithelium (0=none, in surface epithelium only = 1, cryptitis=2, crypt abscess present=3), polymorphonuclear cells in the lamina propria (0=normal, 1=moderate increase, 2=severe increase), infiltration of mononuclear cells in the lamina propria (0=normal, moderate increase=1, severe increase=2), crypt architectural changes (0=normal, 1=focal pathology, 2=extensive pathology), and epithelial damage (0=normal, focal pathology=1, extensive pathology=2).

Fluorescence in situ hybridization (FISH)

Colonic biopsy samples were fixed in Carnoy's solution (60% absolute methanol, 30% chloroform and 10% glacial Acetic Acid) for 2-3 years. Biopsy samples were transferred to 70% ethanol prior to embedding, with the intention of embedding luminal side up. Fixed paraffin embedded biopsy samples were washed and rehydrated by submerging slides into xylene for 5 minutes, followed by multiple ethanol washes, at decreasing concentrations (100%, 90%, 80%, 70%) for 3 minutes each wash. The slides were allowed to air dry for 10 minutes, and then using a wax pencil, circles were drawn around the tissue. Probes were added to a hybridization buffer (NaCl, Trizma base, formamide, SDS, and H₂O), 40 µl of the probe buffer:solution was added to the tissue, and placed at 37°C overnight. Probes used included Cy3 labeled Eub338 (5'-ACUCCUACGGGAGGCAGC-3'), specific for over 90% of known bacteria and targets the 16S rRNA gene sequence, and a scrambled negative probe NON338 (5'-ACTCCTACGGGAGGCAGC-3') used as a measure of non-specific binding. Following the overnight incubation all further steps were carried out in the dark. Slides were washed in hybridization buffer for 15 minutes at 37°C, followed by a wash in wash buffer (NaCl, Trizma base, formamide, and H₂O). Slides were mounted with DAPI solution and observed using fluorescence microscopy.

Measuring encroachment of bacterial cells to the epithelial layer

Slides were observed using oil-emersion at 1000x magnification (100x objective lens, 10x ocular lens) and distance of bacteria to epithelial cells was calculated. In 20

fields of view, closest distance to cells was indicated at three different points. Distance was calculated in microns (μm).

RESULTS

Control and patient characteristics

In total, 10 individuals were classified as controls, 22 as CD patients with no colonic involvement (CD-NI) and 21 with CD and colonic involvement (Table 1). Mean age within those groups varied from 55.1, 48.4, and 36.8, respectively. In both CD-NI and CD-I groups, the proportion of females was higher. Unexpectedly, CDAI score was highest in CD-NI group (184.14 versus 132.47) compared to CD-I group. Within the CD-NI group the majority of patients were on immunosuppressants, compared to the CD-I group that indicated the majority of patients were on 5-ASA. The association between active colonic inflammation defined by endoscopy and CRP, ESR, CDAI, and HBI was tested (Supplementary Figure 3). No significant association was found between active colonic inflammation (group CD-I) and ESR, CRP, CDAI, and HBI (Supplementary Figure 3A-D). The only inflammatory marker associated with active colonic inflammation was found to be fecal calprotectin (Supplementary Figure 3E).

Presence of endoscopic colonic inflammation is associated with dysbiosis of the mucosal microbiota

To investigate the microbial community associated with the mucosa, 16S rRNA gene sequencing of the V3 region was completed for controls (C) and CD patients. Many studies have shown that the stool microbiota in CD patients is altered from healthy

Table 1: Mean characteristics of controls and patients in this study.

Group	Age	Sex	CDAI	Colonic SES-CD sub- score	Previous Treatment	Ongoing Treatment
Control (C) n = 10	55.1	40% F	102.5	N/A	N/A	N/A
CD-NI n = 22	48.4	86.4% F	184.14	0	5-ASA: 32% (7/22) Immunosuppressant: 13.6% (3/22) Steroid: 59% (13/22) Biologics: 0% (0/22) Combination: 45.5% (10/22) None: 0.05% (1/22)	5-ASA: 22.3% (5/22) Immunosuppressant: 50% (11/22) Steroid: 13.6% (3/22) Biologics: 22.3% (5/22) Combination: 27.2% (6/22) None: 13.6% (3/22)
CD-I n = 21	36.8	66.66% F	132.47	7.09	5-ASA: 19.04% (4/21) Immunosuppressant: 38.09% (8/21) Steroid: 71% (15/21) Biologics: 28.6% (6/21) Combination: 47.6% (10/21) None: 23.8% (5/21)	5-ASA: 42.9% (9/21) Immunosuppressant: 38.09% (8/21) Steroid: 0.09% (2/21) Biologics: 28.6% (6/21) Combination: 33% (7/21) None: 19.0% (4/21)

controls, but few have focused on the microbial community associated with the mucosa. Thus, our first aim was to define dysbiosis of the mucosa in CD patients compared to non-IBD controls. Beta diversity was calculated using the Bray-Curtis dissimilarity metric, and a Permanova was subsequently completed to test for statistical significance between groups C and CD. No variation was found within the mucosal microbiota between C and CD patients, $p = 0.051$ (Figure 1A). A non-parametric t-test was also completed to test for variation between groups C and CD, to confirm the Permanova results, and found no statistical differences. Next, diversity of the microbial community was tested as it is commonly reported that CD patients have a reduced diversity within the stool microbiota compared to non-IBD controls, but little is known about diversity of the mucosal community. CD patients did not have reduced diversity, calculated using Shannon diversity index and Chao1, compared to controls (Supplementary Figure 1A and B).

Within this study, CD patients displayed with either endoscopic colonic inflammation (CD-I) or did not display endoscopic colonic inflammation (CD-NI). We wanted to test if the mucosa-associated community varied from controls depending on the presence of colonic inflammation. Again, through utilization of the Bray-Curtis dissimilarity metric, subsequently followed by a Permanova test, it was found that CD-NI patients do not display a dysbiosis compared to controls ($p = 0.054$, Figure 1B). This is in contrast to CD-I patients, as a Permanova test revealed a statistically significant difference compared to controls ($p = 0.038$, Figure 1C). However, diversity of the mucosa microbiota was not reduced in CD-NI or CD-I patients compared to controls

(Supplementary Figure 1C -F). A non-parametric t-test was then completed to identify the taxonomic groups that are enriched or depleted in CD-I patients compared to controls. The genera *Lachnospira*, *Faecalibacterium*, and *Parabacteriodes* were depleted ($p = 0.005$, $p = 0.008$, $p = 0.02$) in CD-I patients compared to controls (Figure 1E). It was also found that variation of the microbiota exists between CD-NI and CD-I patients, as a Permanova revealed the two groups are distinct ($p = 0.032$, Figure 1D). Between these two groups, *Blautia* was significantly decreased ($p = 0.0004$) and *Escherichia* was enriched ($p = 0.03$, Figure 1F). Diversity between these two groups was found to be reduced significantly in CD-I patients based on Chao1 ($p = 0.0003$), but not with Shannon diversity ($p = 0.2$, Supplementary Figure 1G and H).

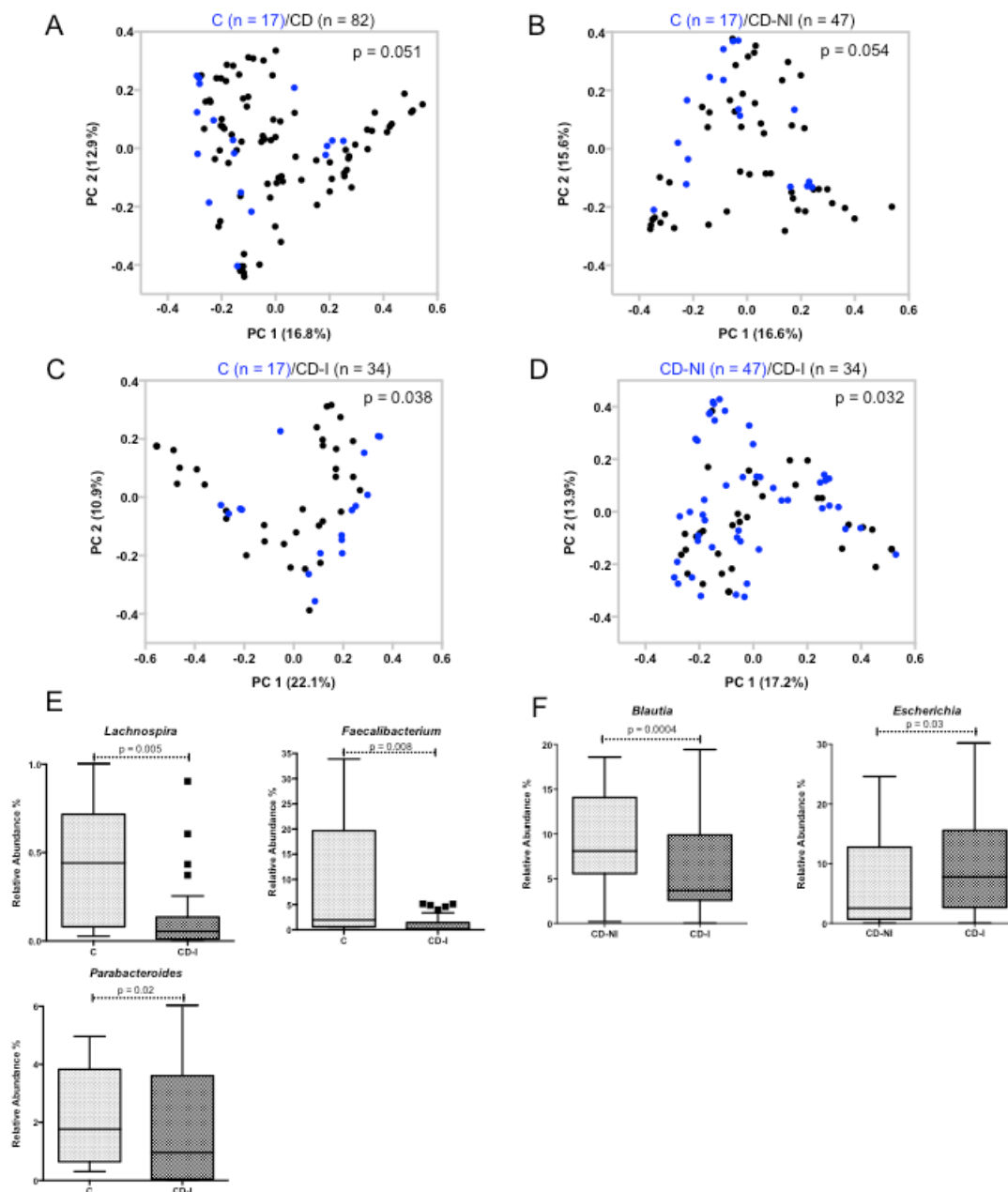


Figure 1: Dysbiosis in CD patients is associated with the presence of endoscopic inflammation. Principle coordinate analysis (PCoA) of Crohn's disease patients (CD) versus controls (A), C versus CD-NI (B), C versus CD-I (C), and CD-NI versus CD-I (D). Differential abundance testing of the mucosa associated microbiota of C versus CD-I (E) and CD-NI versus CD-I (F).

Variation of the mucosal microbiota exists between inflamed and non-inflamed areas in CD patients

Matched biopsy samples were taken from groups C, CD-NI, and CD-I. In controls and CD-NI, specimens were collected 1 cm apart from adjacent non-inflamed areas, but in CD-I patients, samples were collected from a non-inflamed area and a matched inflamed area, also 1 cm apart (Figure 2A). The dissimilarity between matched samples was calculated for C, CD-NI and CD-I using the Bray-Curtis dissimilarity metric. Controls showed the least amount of community variation between matched samples (Figure 2B) with a median distance of 0.24 (Figure 2E). CD-NI patients displayed greater dissimilarity between matched samples (Figure 2C) with a median dissimilarity value of 0.28, although this was not significant to controls ($p = 0.36$, Figure 2E). This is in contrast to CD-I patients, that showed the greatest dissimilarity between inflamed and non-inflamed areas (Figure 2D), median 0.38, trending towards significance, $p = 0.06$ (Figure 2E). Tests were completed to find commonalities between inflamed areas in this cohort of patients, although due to patient heterogeneity, no taxa were found to be consistently enriched or depleted. The presence of the fungal species was tested as well in C, CD-NI, and CD-I groups (Supplementary Figure 2A). Fungal species were found in only one biopsy sample from group C. Within group CD-NI, and CD-I, fungal species were present but, there was no trend associated with the presence of fungal species within the mucosa and inflammation.

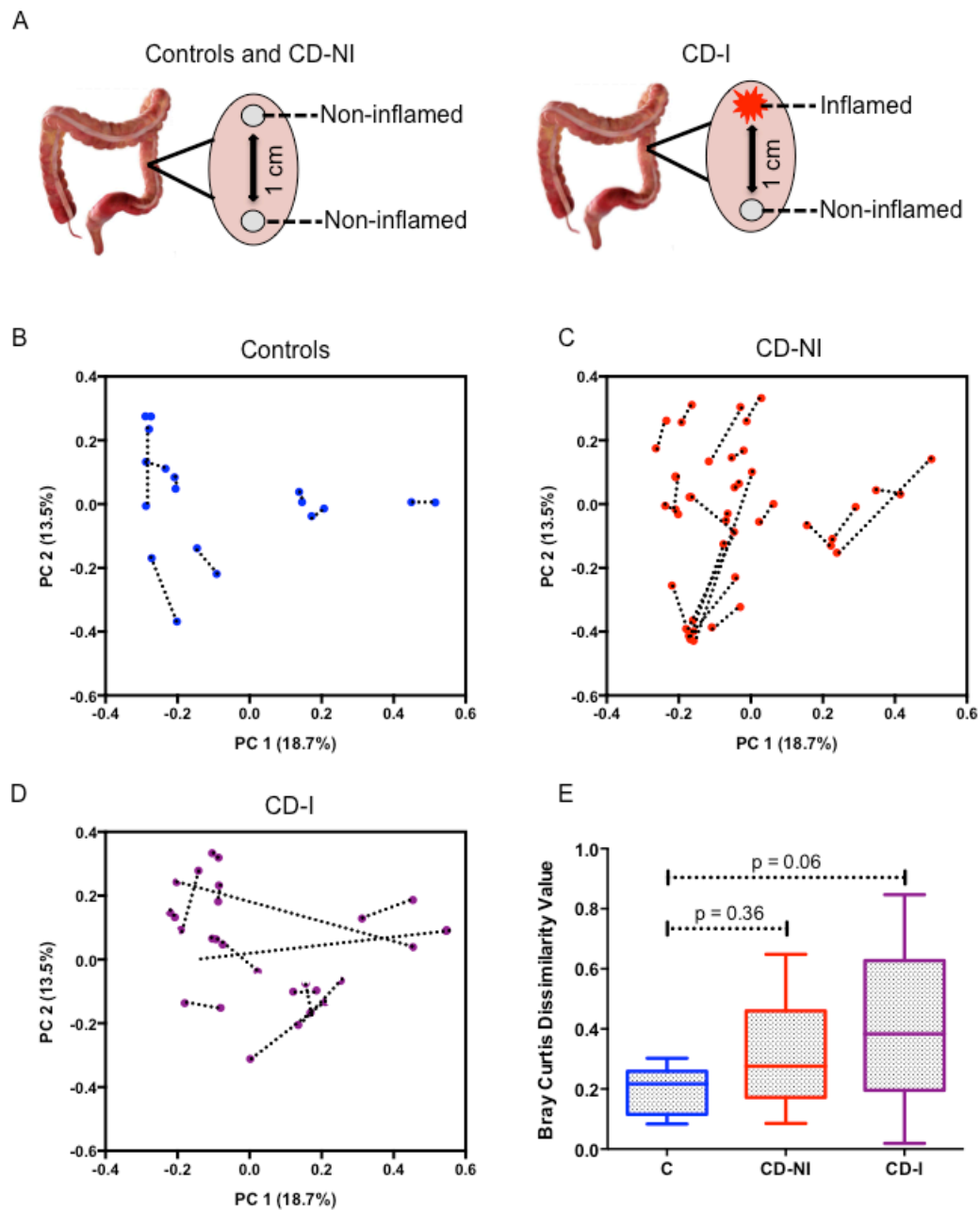


Figure 2: Dissimilarity of the mucosal microbiota exists between matched inflamed and non-inflamed areas in CD patients. (A) Schematic of experimental design. Dissimilarity of matched sites were analyzed and visualized via PCoA in controls (B), CD-NI patients (C), and CD-I (D) patients. Dissimilarity values were plotted for each group and a Kruskal Wallis test followed by a Dunns multiple comparisons was completed to test for significance (E).

Inflamed areas are associated with greater encroachment of bacteria to host epithelial cells

A subset of 5 individuals from each cohort (C, CD-NI, and CD-I) was taken to identify inflammation specific differences between matched samples. Since inflammation is associated with a decreased mucus layer, resulting in bacterial cells lying closer to host epithelial cells, we tested encroachment of bacterial cells in each matched cohort.

Encroachment of bacterial cells to host epithelial cells was calculated using FISH. A probe, labeled with CY3 and able to bind to greater than 90% of bacterial species, showed that in CD-NI patients bacteria do not reside directly on the host epithelia (Figure 3C, left panel), nor are there differences between matched non-inflamed areas ($p = 0.5$, Figure 3A). This is opposed to inflamed areas within CD-I patients (Figure 3C, right panel). Matched inflamed areas show greater encroachment of bacteria to host cells ($p = 0.0001$, Figure B).

Matched inflamed areas are associated with increased histopathological features of inflammation and stool fecal calprotectin

Histopathology was examined for all matched samples within groups C, CD-NI, and CD-I. Pathology was scored based on presence of granulomas, erosion, ulcers, cryptitis, crypt abscesses, polymorphonuclear cells in the lamina propria, infiltration of mononuclear cells in the lamina propria, and crypt architectural changes. No histopathological features of inflammation were found in the control group (Figure 4A, left panel and B). Controls were also found to have a minimal level of fecal calprotectin within their stool (Figure 4C). Compared to controls, low-grade inflammation was present within individuals belonging to group CD-NI (Figure 4A and B). This was reflected with

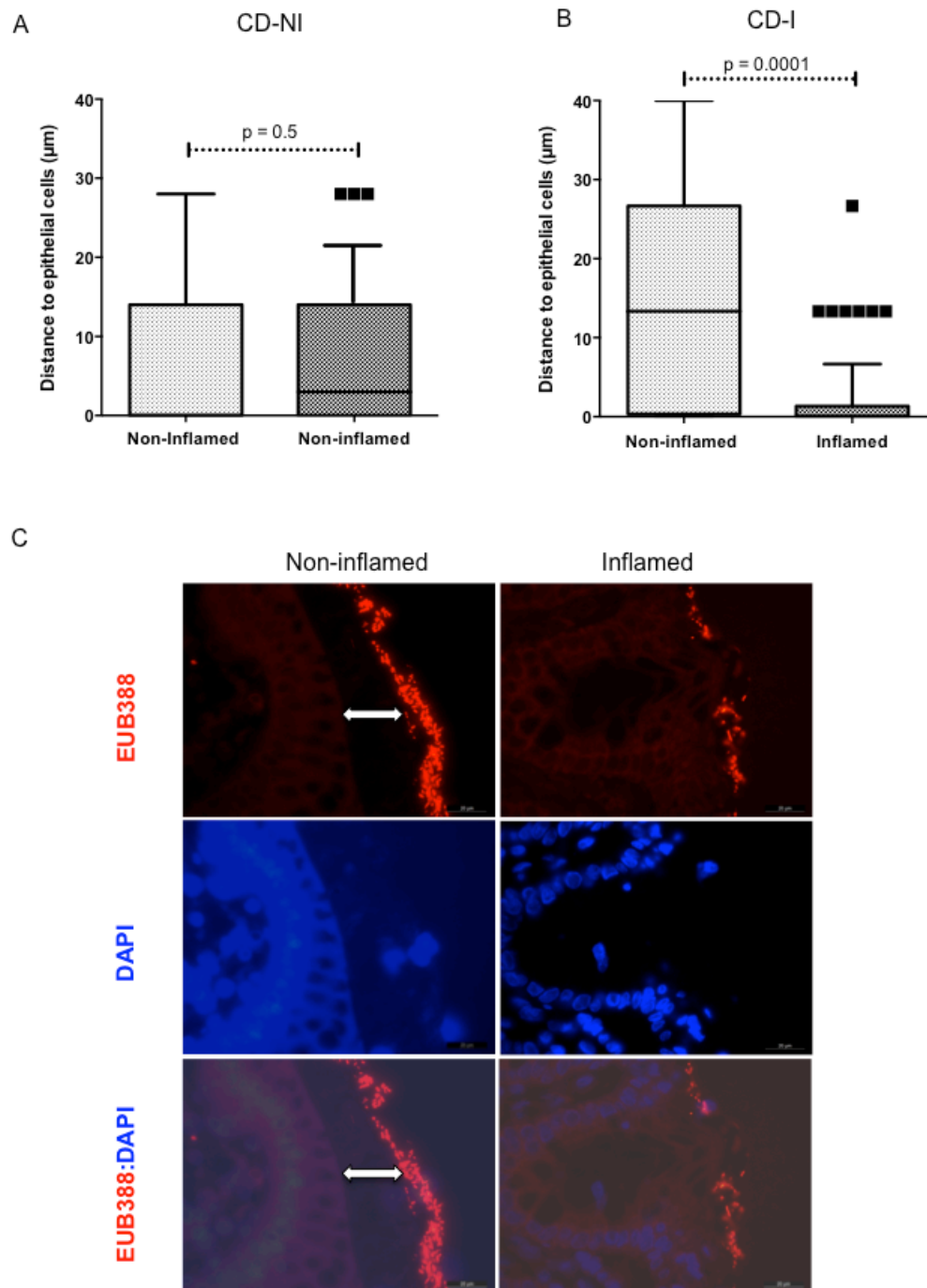


Figure 3: Inflamed areas are associated with greater encroachment of bacteria to host epithelial cells. Matched samples from groups CD-NI and CD-I were tested for encroachment distance by FISH (A and B). Examples from an inflamed and non-inflamed are presented (C).

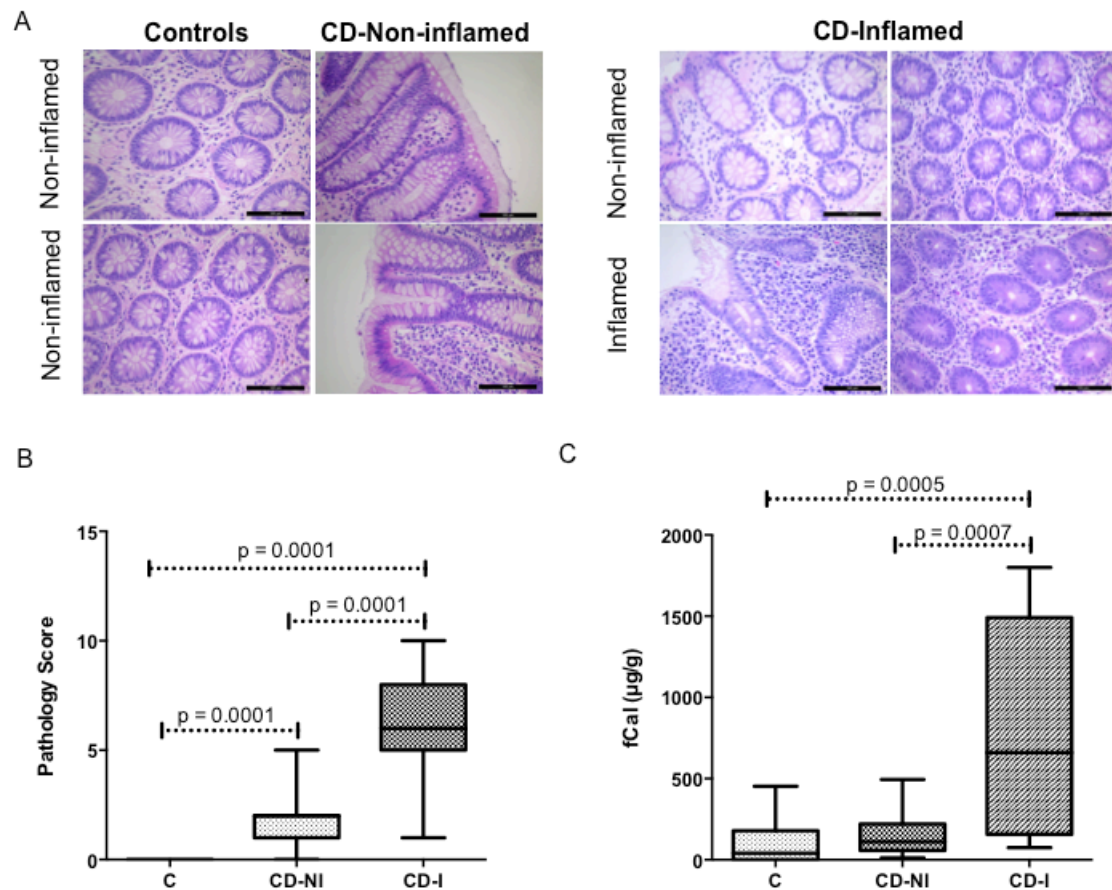


Figure 4: Matched inflamed areas are associated with increased histopathological features of inflammation and stool fecal calprotectin. Crypt architecture, and immune cell infiltration was observed by hematoxylin and eosin staining of C, CD-NI and C-I (A). Pathology was scored for each group (B). Stool fecal calprotectin was calculated (C).

a slightly elevated stool fecal calprotectin (Figure 4C). Evidence of histopathological inflammation and active disease was found in group CD-I (Figure 4A and B), and this was associated with increased stool fecal calprotectin (Figure 4C). In inflamed biopsy specimens, granulomas were commonly present in addition to cryptitis and crypt abscesses. Crypt branching, abnormal crypt architecture such as loss of goblet cells, and an increase in immune cell infiltration was commonly seen in inflamed sites (Figure 4A).

Additional inflammatory markers were measured in all matched groups including Crohn's disease activity index (CDAI), Harvey-Bradshaw index (HBI), erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP). The association between active colonic inflammation define by endoscopy and CRP, ESR, CDAI, and HBI was tested (Supplementary Figure 3). No significant association was found between active colonic inflammation (group CD-I) and ESR, CRP, CDAI, and HBI (Supplementary Figure 3A-D). The only inflammatory marker associated with active colonic inflammation was found to be fecal calprotectin (Figure 4C).

Altered barrier function is associated with colonic inflammation and specific to inflamed areas

Paracellular permeability was calculated through influx of ^{51}Cr EDTA using Ussing chambers. Inflamed biopsy samples from group CD-I showed increased paracellular permeability ($p = 0.01$, Figure 5B). Matched non-inflamed samples did not show an altered intestinal permeability (Figure 5A). Increased paracellular permeability was mirrored by an increase in vascular permeability (Figure 5C and D). The detection of fluorescein was examined via confocal laser endoscanning microscopy (CLE) in patients

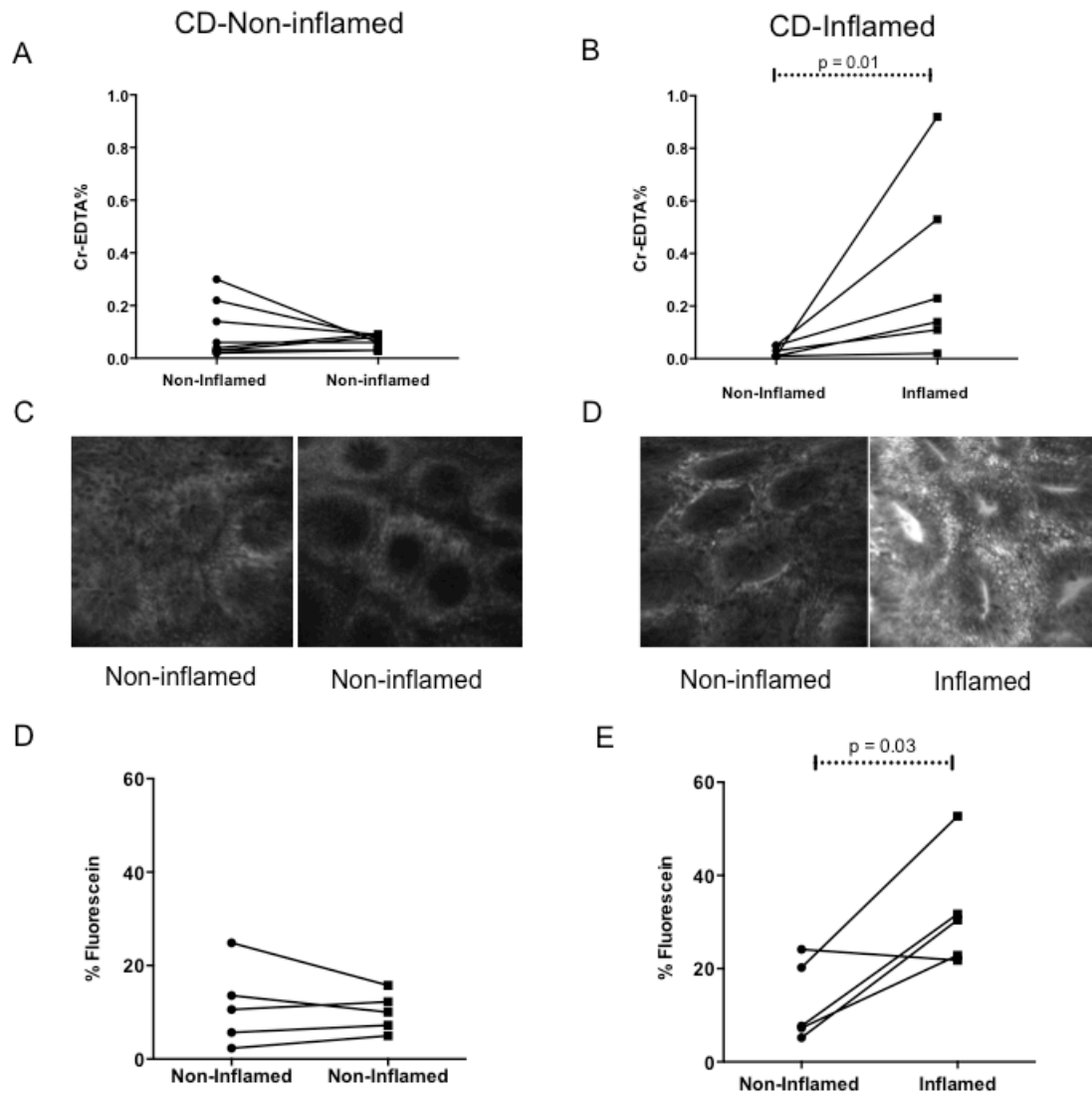


Figure 5: Altered barrier function is associated with colonic inflammation and specific to inflamed areas. Influx of Cr-EDTA was calculated using Ussing chambers in CD-NI (A) and CD-I (B) patients. Fluorescein leakage was observed using confocal laser scanning endomicroscopy (CLE) in CD-NI (C) and CD-I (D) patients. Fluorescein leakage was semi quantified for both cohorts (D and E).

with active colonic inflammation and matched non-inflamed regions. The same cohort of patients that were examined for influx of Cr^{51} -EDTA (Figure 5A and B) was examined for increase of fluorescein by CLE. The matched non-inflamed regions exhibited less fluorescein than the inflamed regions in all patients tested ($p = 0.03$, Figure 5E). Inflamed images (Figure 5D) reveal abnormal crypt architecture that suggests increased vascular permeability within the lumen, which is not observed in patients without colonic inflammation (CD-NI) (Figure 5C and D). Crypts also tend to be elongated in inflamed regions as opposed to circular within non-inflamed regions (Figure 5C and D).

DISCUSSION

Previous studies have reported that CD patients display a dysbiosis compared to healthy controls, although the specific bacterial groups that are altered in these populations vary from study to study (Walters et al., 2014). Most studies completed on defining dysbiosis in IBD patients have focused on examining the stool microbiota (Joossens et al., 2011; Lewis et al., 2015; Norman et al., 2015). Since inflammation presents as patchy in CD, defining dysbiosis in terms of the luminal contents may not capture the groups that are responsible for pathogenesis, and subsequently driving the disease (Willing et al., 2009). Thus, we examined the microbiota associated with the mucosa in controls, and CD patients with and without colonic inflammation. In addition to this, matched biopsy samples were taken from each group, allowing us to examine inflammation specific differences. Here we demonstrate that dysbiosis of the mucosal

microbiota is associated with the presence of colonic inflammation. Compared to controls, significant microbial alterations were observed when CD patients were stratified based on the presence of colonic inflammation. A decrease in *Lachnospira*, *Faecalibacterium*, and *Parabacteroides* was observed in CD patients with active colonic inflammation. Moreover, CD-I patients showed a specific dysbiosis compared to CD-NI meaning, certain bacterial groups may be responsible for driving inflammation.

Matched biopsy samples from inflamed and non-inflamed regions were tested in CD patients. We found no statistically significant variation when comparing different gut compartments of the inflamed and non-inflamed gut. This is in agreement with a recent study that found no specific inflammation related differences of the mucosal microbiota in patchy areas of CD (Forbes et al., 2016), but there were specific differences between CD and ulcerative colitis (UC) patients. However, this does not conclude that specific differences within the inflamed gut do not exist. We did find that matched biopsy samples show greatest dissimilarity between inflamed and non-inflamed sites. This suggests, inflammation related differences are subtle and specific to individuals. It is also possible, that the majority of the differences are related to strains, which cannot be detected by 16S rRNA gene sequencing. Moreover, alpha diversity does not correlate with inflammation, meaning dysbiosis cannot be defined as a reduction of diversity. Using diversity as a metric of dysbiosis is problematic, as it is an oversimplification (Gevers et al., 2014; Ott et al., 2004) of a very complex community, with very complex interactions with the host.

IBD is associated with a thinner mucosa layer however; (Fyderek et al., 2009; Johansson et al., 2014) encroachment of bacteria to host epithelial cells comparing

inflamed and non-inflamed areas has not been previously explored. Encroachment of bacteria to host cells was greater in inflamed areas compared to non-inflamed areas. This is the first report showing an association between encroachment and specific inflamed regions of the intestine in CD patients. Inflamed areas were also found to show an altered intestinal barrier function. An altered barrier function is a key pathogenic mechanism in CD patients however; the spatial relationship between inflammation defined by endoscopy and permeability is poorly defined in humans. We report an association between colonic inflammation in CD and altered barrier function shown through an increase in vascular and paracellular permeability, and increased encroachment.

In summary, we demonstrate specific inflammation related differences in CD-I patients. We hypothesize that dysbiotic communities within inflamed areas disrupt the mucosal and endothelial layer in CD, which as a result drives inflammation. The mechanism that leads to successful degradation of the mucus layer, increased bacterial encroachment, and an increase in intestinal permeability remains to be elucidated. A follow up study is required. An enrichment of *Escherichia* was found in CD-I patients and not in CD-NI patients, although this enrichment is not specific to inflamed areas of the intestine. As many pathogenic *Escherichia* species contain highly conserved metalloproteases that are able to degrade MUC2 (Luo et al., 2014), favourable environmental conditions within the intestine could allow for this degradation, reduction of barrier function, and subsequently an aberrant immune response resulting in patches of inflammation within the intestine.

Supplementary material

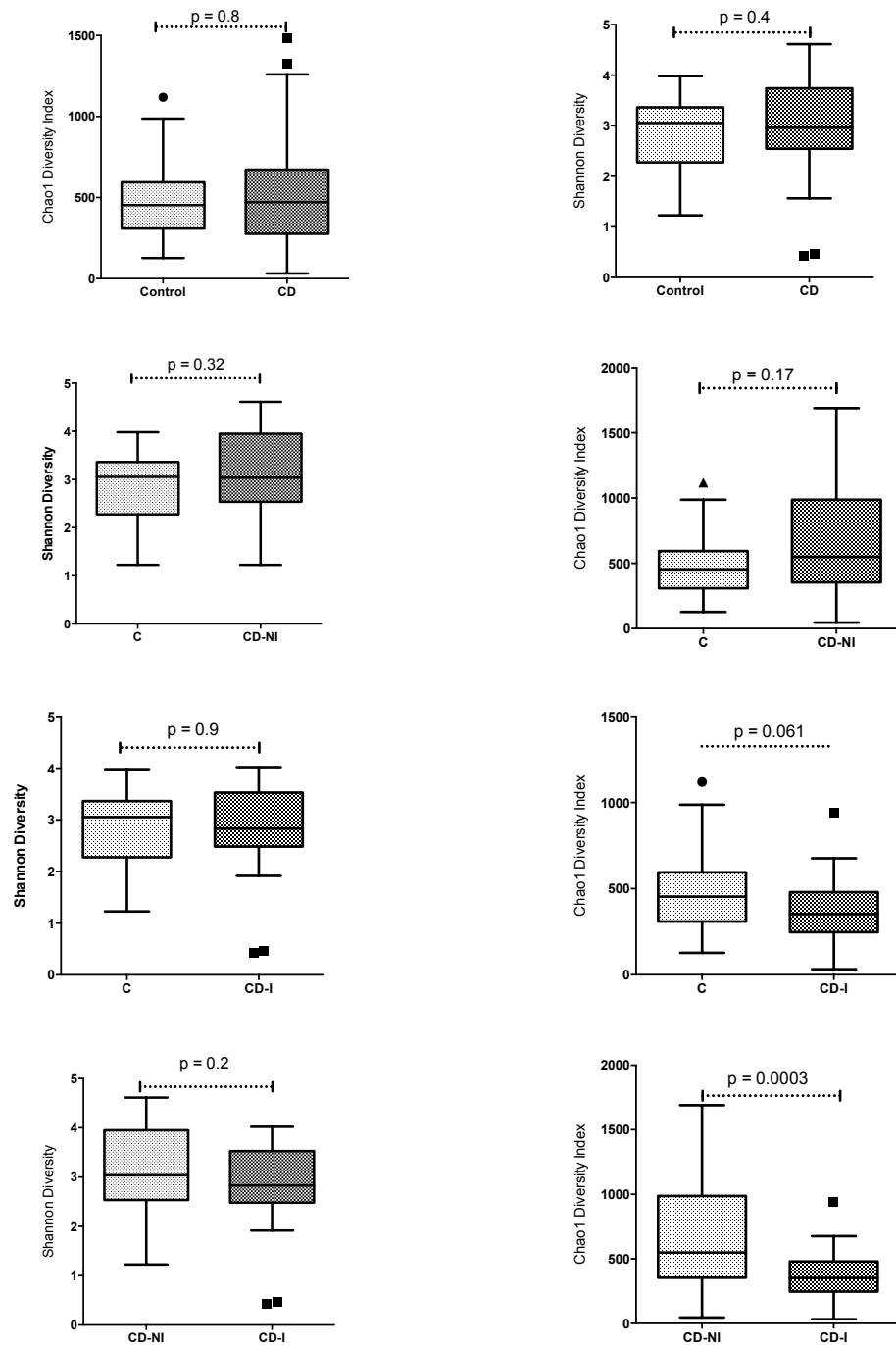
Inflammation related differences in mucosa-associated microbiota and intestinal barrier function in colonic Crohn's disease

Josie Libertucci^{1,2}, Usha Dutta³, Sandeep Kaur², Jennifer Jury², Laura Rossi², Michelle E. Fontes², Michael G. Surette^{1,2}, Elena F. Verdu², and David Armstrong^{2*}

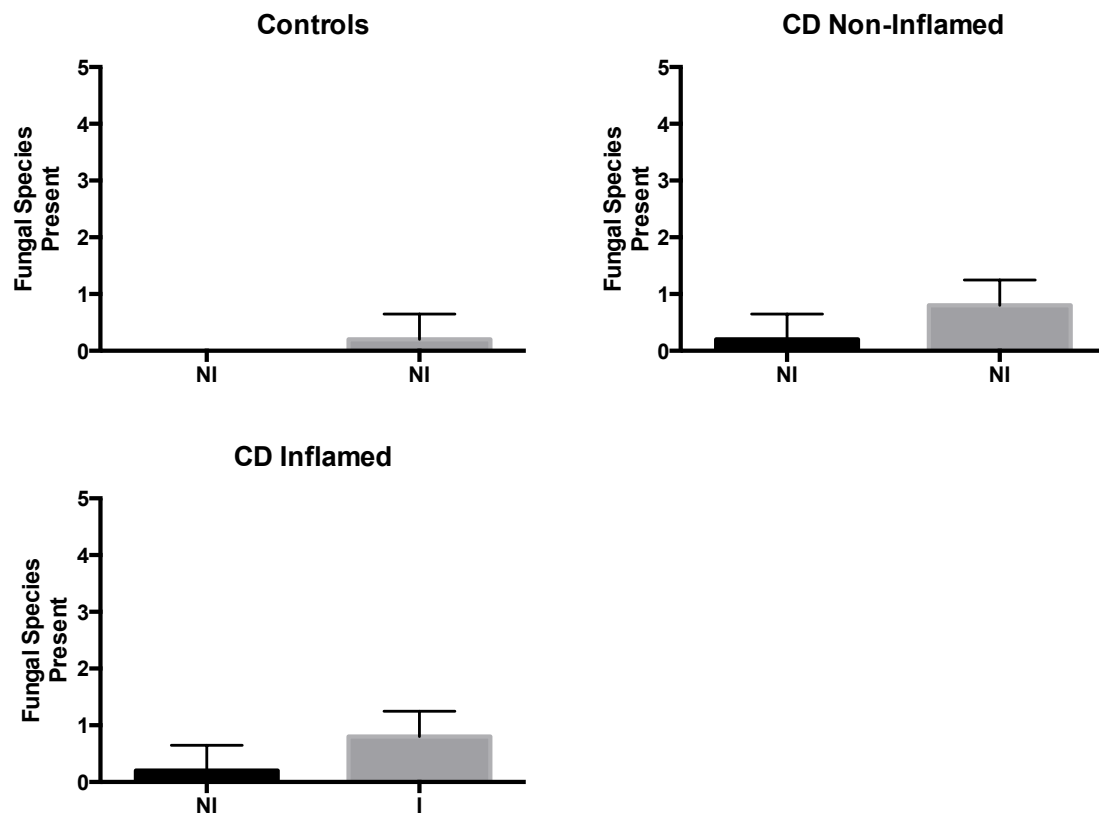
Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Ontario, Canada

Farncombe Family Digestive Health Research Institute, McMaster University, Hamilton, Ontario, Canada.

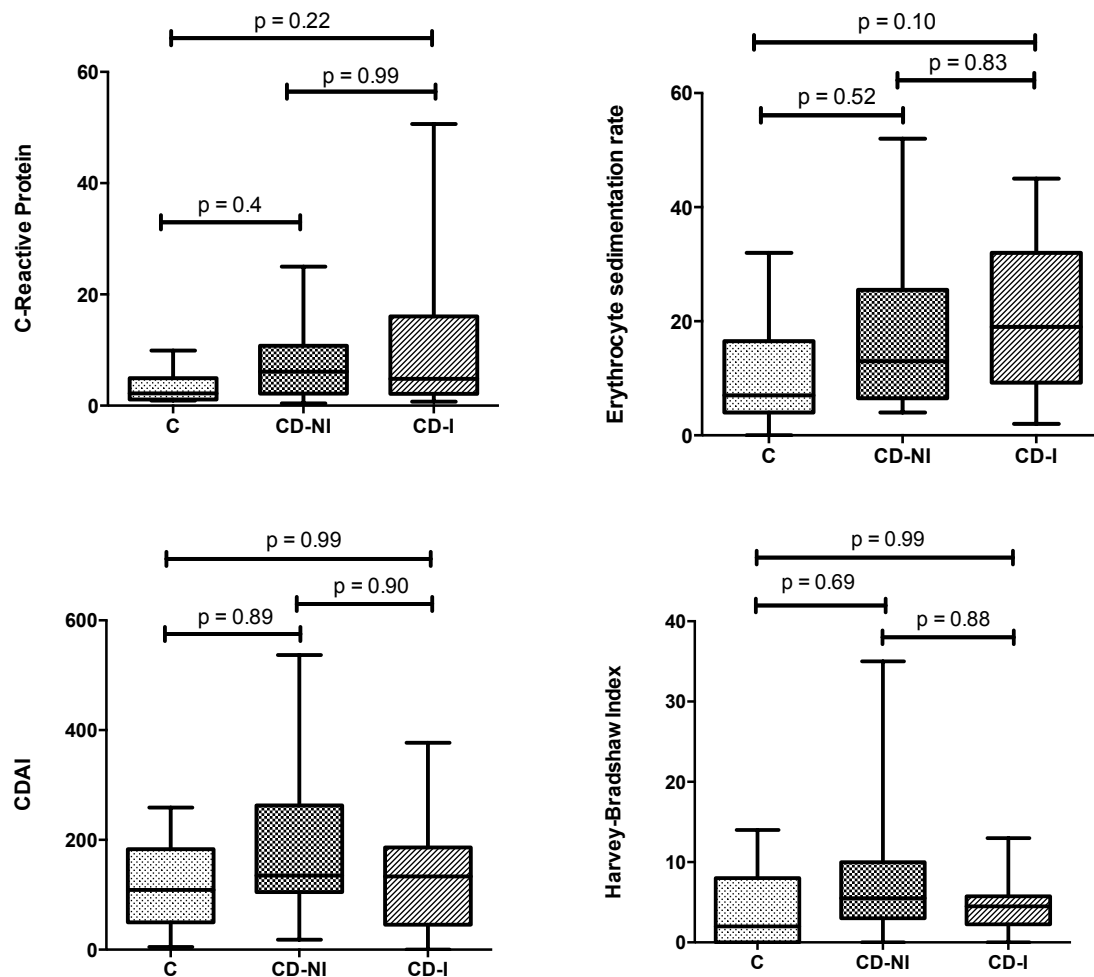
Department of Gastroenterology, PIGMER, Chandigarh, India



Supplementary Figure 1: Alpha diversity of controls, CD-I, and CD-NI



Supplementary Figure 2: Presence of fungal species in biopsy samples



Supplementary Figure 3: Colonic inflammation is not associated with increased CRP, ESR, CDAI, and HBI. (A) CRP measurement and analysis. (B) ESR measurement and analysis. (C) CDAI measurement and analysis. (D) HBI measurement and analysis.

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CHAPTER 6

DISCUSSION

6.1 Summary

Low concordance rates of IBD in monozygotic twins suggests genetics influence the occurrence of this disease, although are not sufficient to cause IBD (Orholm et al., 2009; Halfvarson et al., 2003). Environmental factors, such as the microbiota, play a role in the pathogenesis of this disease. This is highlighted by studies that have taken advantage of germ-free murine models; in those mice inflammation does not occur without the microbiota (Sellon et al., 1998). For these reasons, many studies have focused on characterizing the IBD microbiome in relation to healthy controls. Numerous studies have summarized an overall decrease in species, and a decrease in some species within the phyla Bacteroides and Firmicutes and an increase in some species from the phyla Proteobacteria and Actinobacteria. A meta-analysis completed on IBD patient samples has revealed that when many studies are considered together, few taxa are consistently enriched or depleted in IBD patients, highlighting the variability between studies. Since microbiome methodologies can influence the outcome of the analysis and standardized methods do not exist in the field, it is difficult to assess whether this variation of IBD dysbiosis classification is due to the disease, the sampled population, or methods that are utilized. Modulating the microbiota to influence disease status in IBD has recently gained interest as a potential therapy. Little is known about how the microbiota reacts to modulation therapy, and distinctions in the community based on disease status and severity. The overall goal of this thesis was to characterize the IBD microbiome in relation to disease status, severity, and response to therapy.

In chapter 2 of this thesis, it was shown that FMT induced remission in UC patients following 6 FMT administrations from a non-related healthy donor. Mucosal healing defined by endoscopy occurred in all patients that met the primary end point. Interestingly, secondary outcomes [which included improvement in UC symptoms (defined as ≥ 3 improvement in full Mayo score), as well as change in Mayo, Inflammatory Bowel Disease Questionnaire, and EQ-5D scores] were not met. This means the number of patients that improved in the FMT arm, were equal to the number of patients that improved but did not reach remission in the placebo arm. In terms of the microbiota, dissimilarity increased significantly in patients that had FMT, compared to placebo, suggesting the microbiota did shift following FMT. Dissimilarity compared to their donor decreased following FMT, suggesting a shift towards the donor occurred following FMT. In responder patients this trend was not significant. One of the most important findings from this study was that notion that not all donors are appropriate for use in UC patients, as one donor (Donor A) displayed enrichment for species within the genus *Escherichia*.

In chapter 3, the first long-term surveillance of the luminal and mucosa associated microbiota following FMT to treat severe UC refractory to conventional therapy in an adult was reported. Endoscopic and microscopic remission was achieved following multiple FMT administrations, but following a period of several months in remission on no therapy this patient relapsed. Remission was achieved again following multiple FMT. Clinical and endoscopic remission was achieved although, histology reports persistent minimal activity. Microbial community changes occurred following treatment in both the

lumen and mucosa-associated microbiota. Fungal organisms were present, but did not correlate with disease status. No evidence for major engraftment of donor microbiota following FMT at the level of OTUs. These results were mirrored in chapter 4, which included the analysis for all patients within the trial. Large global shifts within responder patients did not occur post FMT, and only subtle differences mostly at the species level were observed. Large global shifts to the donor microbiota were not observed either. No evidence for engraftment of donor microbiota to recipients following FMT at the OTU or species level.

Lastly, in Chapter 5, inflammation specific differences in the mucosa-associated microbiota within CD patients were analyzed, in relation to areas of impaired barrier function. Overall CD patients with active inflammation showed an altered mucosal microbial community compared to CD patients without inflammation and controls. Matched biopsy samples showed that inflamed tissue is characterized by increased paracellular and vascular permeability, and increased encroachment of bacteria to host epithelial cells, which may be driving inflammation in CD.

6.2 FMT considerations for treatment of UC

In Chapter 2 presented one of first phase II randomized controlled trials testing the efficacy of FMT for the treatment of UC. In that same year, another RCT was published, but the two studies showed opposite results with varying protocols used (Rossen et al., 2015, Moayyedi et al., 2015). In 2016, a third RCT conducted on resistant UC testing FMT was presented at an international conference with positive results (Paramsothy,

2016). This section will compare and contrast the three studies with emphasis on remission rates, FMT protocol, and microbiota characteristics pre and post FMT.

6.2.1 Primary and Secondary Outcomes

RCTs for the induction of remission in UC via FMT have been conducted in Canada, the Netherlands, and Australia, with mixed results (Table 1). The Canadian study, lead by Moayyedi *et al.*, reported a statistically significant ($p = 0.02$) effect of 24% remission rate via FMT, compared to 5% in the placebo arm. Rossen and colleagues from the Netherlands, reported autologous feces induced remission in 20% of the study population compared to the active arm at 30.4%, the difference was not significant ($p = 0.51$). And, in Australia, Paramsothy *et al.*, reported a 27% remission rate in the active arm, compared to 8% in the placebo arm, this was significant ($p=0.02$). All three studies restricted enrollment to patients with active UC however, only Moayyedi *et al.*, included patients with severe UC (Mayo score >10). Of the 9 patients that entered into remission by week 7, only one patient had an initial Mayo Score >10 , all other responders had mild-moderate disease at baseline. This may support the notion that FMT for UC patients should be limited to patients with mild to moderate disease. However, data presented in chapter 3 show severe patients may require longer treatment than 6 FMT administrations. Alternatively, the mixed results, and specifically the unreached clinical endpoint in Rossen *et al.*, could reflect challenges associated with including mild UC patients (that frequently fluctuate between active and remission as compared to moderate to severe patients) in an RCT. It also raises questions regarding the appropriate placebo treatment.

Table 1: Primary and secondary outcomes and remission rates for double blind randomized placebo controlled trials testing the efficacy of FMT to treat UC.

Reference	UC activity for enrollment	No. Patients in study	Primary clinical endpoints	Secondary clinical endpoints	Remission
Moayyedi <i>et al.</i>, 2015	Mayo score ≥ 4 Endoscopic Mayo Clinic score ≥ 1	75 38 FMT 37 Placebo	Remission at week 7 Full Mayo score < 3 and Endoscopic Mayo score = 0		FMT, 24% Placebo, 5% P = 0.02 FMT induces remission in a statistically significant percentage greater than placebo.
Rossen <i>et al.</i>, 2015	Established UC according to Lennard-Jones criteria, Simple Clinical Colitis Activity Index (SCCAI) ≥ 4 , and ≤ 11	48 23 FMT-D 25 FMT-A	Clinical remission at week 12 SCCAI score ≤ 2 ≥ 1 point improvement on endoscopic score of sigmoid and rectum	Clinical response at week 6 or week 12 defined as reduction of ≥ 1.5 points on the SCCAI, clinical remission, endoscopic response, change in median IBDQ** score	FMT-D 30.4% FMT-A 20.0% P = 0.51 No statistically significant difference in clinical and endoscopic remission between FMT-D and FMT-A
Paramsothy <i>et al.</i>, 2016*	Mayo score 4-10 Resistant to standard treatment	81 41 FMT 40 Placebo	Steroid-free clinical remission with endoscopic remission, or endoscopic response at week 8	Clinical remission, endoscopic remission, endoscopic response, increase in quality of life	FMT, 27% Placebo, 8% P = 0.02 FMT induces remission in a statistically greater proportion than placebo

*Abstract presented at DDW and published in Gastroenterology. Full manuscript not released.

** Inflammatory Bowel Disease Questionnaire

6.2.2 Protocol considerations

The mixed results, from the three RCTs discussed are accompanied with varying FMT protocols. This mode of delivery, duration of treatment, preparation of patients, and donors used varied from study to study (Table 2). The mode of delivery of FMT, defined as rectal enema, colonoscopy, or nasoduodenal tube, may impact clinical outcomes in UC patients. It was observed that clinical outcomes were met when FMT was administered through the lower GI, either by rectal enema or colonoscopy, and not met via nasoduodenal tube (Rossen et al., 2015; Angleburger et al., 2013). Moreover, the majority of successful cases in UC have been achieved through use of the lower GI tract – future studies should consider carefully which route to use in their FMT protocol based on the current evidence. Since UC almost always involves the rectum, the lower route may allow for direct interactions between FMT and the host. In addition to mode of delivery, duration of treatment, defined as number of FMTs administered over time, may play a factor in positive outcomes. Two infusions over 3 weeks were unsuccessful in inducing remission compared to placebo, yet 1 rectal enema over 6 weeks and 5 enemas/week for 8 weeks achieved clinical endpoints. It is also unclear from these studies what role donor specificity plays in successful FMT as donor stools were either mixed into one suspension, or a number of different donors were used in a single patient. Surprisingly, bacterial load was not taken into account by any study, meaning the actual quantity of bacteria entering into the patient may vary in each FMT administration. Bacterial load of the donor suspension could play a critical role in successful FMT but at this point it is unclear if load is a factor in successful outcomes.

Table 2: Protocol and placebo choices for RCTs testing efficacy FMT for treatment of

Reference	Patient preparation pre FMT	Donor relationship to patient	No. donors used per patient	FMT preparation	Placebo	Mode of FMT	Duration of FMT
Moayyedi <i>et al.</i> , 2015	None	Unrelated healthy volunteers	6 donors in total, 1 donor per rectal enema. Some patients	500 mL donor suspension or water	Water	Retention enema	1 rectal enema per week for 6 weeks
Rossen <i>et al.</i> , 2016	Bowel lavage the evening before and morning of FMT	Healthy partners, relatives, or unrelated volunteers	1 donor per infusion	500 mL donor suspension	Autologous feces	Naso-duodenal tube	2 duodenal infusions during a 3 week period
Paramsothy <i>et al.</i> , 2016	Unknown	Unrelated volunteers	Mixture of 3 – 7 donors per enema	Unknown	Unknown	Colonoscopic infusion (CI) and rectal enema	CI on day 1 followed by rectal enemas 5/week for 8 weeks

UC

6.2.3 Alterations in the microbiota following FMT in remission patients

In Rossen *et al.*, patients that responded to FMT (in both the FMT-D and FMT-A group) showed an increase in alpha diversity. Richness and evenness within samples increased, but this increase was only significant in the autologous feces arm of patients. This suggests that an increase in alpha diversity does not correlate with successful active treatment but may be a consequence of clinical improvement. However, the mean increase was slight (~ 0.2 in both FMT-A and FMT-D groups), and the mean diversity of non-responders in the FMT-D arm increased as well. In Chapter 4, alpha diversity was not increased in either the FMT arm or in patients that responded positively. It is hard to draw conclusions regarding alpha diversity and UC patients. In both positive studies, responders tended to shift towards their donor profile. Again, in Rossen *et al.*, responders showed a reestablishment of *Clostridium* cluster IV, XIVa, and XVIII, with a reduction in Bacteroidetes. No significant change in *Faecalibacterium prausnitzii* was observed in any of the studies. Responders to FMT-A shifted away from non-responders with an increase in Bacilli, Proteobacteria, and Bacteroidetes suggesting responders to FMT-D displayed a specific compositional shift. This was not observed in chapter 4, as responders displayed very subtle associations. However, this could be due to the limited number of responders.

6.2.4 Donor specificity for treatment of UC

It is unclear at this point how the donor composition affects FMT outcomes in UC. Initial findings from Moayyedi and colleagues, presented at an international conference in 2014, reported interim unmet clinical outcomes, indicating FMT was not significantly

more successful at inducing remission as compared to placebo (Moayyedi et al., 2014). At this time in the trial, 22 additional patients were enrolled but had not completed the study. During the first portion of the trial most patients received Donor A, Donor B, or alternating treatments from either donor. The tail end of the trial, that included the last 22 patients, exclusively used donor B. Interestingly, this phase of the trial had the most success at inducing remission in these patients, and altered this study from having negative findings to a meeting its primary outcome. Does this suggest that donor B is a “super donor” that should be used exclusively? No, it highlights the notion that some donors may not be good candidates for use of FMT to treat IBD. In chapter 2 the microbial composition was reported for all donors. Donor A had a greater proportion of *Escherichia* compared to all other donors. The alpha diversity was not reported, however, the taxonomic summaries suggest donor A has a reduced diversity compared to other donors due to the expansion of *Escherichia*. It could also be likely that having a microbial community enriched with species within the family *Lachnospiraceae*, and the genus *Ruminococcus*, rather than from the genus *Escherichia*, is a better fit for treating patients with UC through modulation of the microbiota via FMT. All donors may not be effective in inducing remission in UC patients however, there is no evidence at this point to suggest one donor is superior compared to others.

In another phase II double blind, placebo controlled trial, to test the efficacy of FMT for the treatment of UC, donor specificity was not taken into account in the protocol but the authors reported that 27% of patients in the active arm achieved steroid free clinical remission (Paramsothy et al., 2016). The authors used a combination of 3 to 7

donors for each patient during the 9 weeks of FMT treatment. These donors were unrelated and their microbial composition was not reported. This suggests duration and frequency of FMT may be more critical for inducing remission.

Recently, it has been suggested that donor species richness determines FMT success in IBD patients (Vermeire et al., 2016). Patients that responded positively to FMT ($n = 3$) were reported to have received fecal suspensions from donors with a significantly higher species richness compared to donors that non-responding patients received (650 vs. 510, $p = 0.012$). Although tempting to make conclusions supporting the notion of a donor microbial community with higher species richness predicts FMT success for UC patients, the study does have limitations. This study was not randomized and has a very low sample size (8 patients with UC, and 5 patients with CD) therefore, the power of these results are questionable, which the authors do acknowledge. A stronger argument for an association between alpha diversity and FMT success would be made if multiple alpha diversity metrics were evaluated and a higher n value used in the study.

It is also interesting to note that the authors did not control for relatedness of the donors to the recipients meaning patients were allowed to choose their donors. This is an interesting point because it has been shown that persons who co-habit have a similar microbial composition (Song et al., 2013). If the recipient was receiving a donor sample that was similar in composition to the recipient's own microbiota, it may be similar to an autologous placebo treatment and may account for poor response rates. It is still uncertain at this point if donors should be related but it is important to address the issue of co-

habiting donors and recipients as this may bring response rates down and negatively bias the effectiveness of FMT for the treatment of UC.

None of the clinical trials took into account anaerobic bacteria, thus it remains unclear if preserving anaerobic bacteria from the donor could increase efficacy rates. Moayyedi and Rossen reported compositional shifts towards donors, and Rossen reported an increase in Clostridial cluster IV. Since species within Clostridial cluster IV and obligate anaerobes and FMT protocols rarely take preservation of viable anaerobes into account during donor fecal suspension preparation and storage, it is questionable if this expansion of *Clostridium* species came from the donor. This could be a result of spores – spores and the relationship to FMT will be discussed in the section. 6.4.

6.3 Dysbiosis in IBD – What was learned?

Dysbiosis in IBD is heterogeneous and diverse. The microbiome in IBD patients is commonly associated with a reduction in alpha diversity. The most commonly reported, is a reduction of observed species in IBD patients. Interesting that when richness and evenness are taken into consideration, a low diversity is not commonly reported in IBD patients. However, low diversity is not always associated with dysbiosis and high diversity is not always associated with health. There were many examples of this throughout this thesis. In Chapter 2, six healthy donors were used to treat patients with moderate to severe UC. Donor A, a healthy donor that was heavily screened prior to the beginning of the trial and was the second most common donor used other than donor B, had low diversity and an enrichment of species within the genus *Escherichia*. This is an

important example, that not only represents the importance of considering the host when defining dysbiosis, but also was one of the first examples that not every healthy person is a good candidate as an FMT donor for treatment of IBD.

This thesis also provides evidence that colonic inflammation is not associated with a reduction of diversity within the lumen – this was seen in chapter 3. Fecal calprotectin levels were calculated at each stool time point and tested for an association with diversity. It was found that low diversity does not correlate with high fecal calprotectin levels. Even though richness and evenness increased following FMT in this individual, taking everything into consideration, an increase in diversity may have been a secondary effect not associated with health outcomes. This result is echoed by data in chapter 4. Patients that entered into remission following FMT did not display an increase in diversity either in the lumen or mucosa. This study was underpowered however, it does question if there is an association between gut homeostasis and microbiome diversity. And in chapter 5, no differences in diversity were observed in the mucosa-associated microbiome between controls and CD patients, with or without colonic inflammation.

As there were no strong associations between low diversity and worsening clinical outcomes, or IBD patients in general, this raises the question of the role of diversity in dysbiosis and IBD. Indeed, a dramatic reduction of diversity in the gut microbiome could signal the enrichment of an infectious agent, as it does in *C. difficile* infections. But, for investigating microbiomes that exhibit diversity while not in homeostasis with the host, what information does a diversity metric provide. Less attention should be given towards measuring alpha diversity in IBD patients, as this measure may be an oversimplification

and fail to capture the complexities that exist in the microbiome of IBD patients. Nor is this measure helping to drive investigation towards developing novel therapies. More attention should be paid toward examining interactions between the host and microbiome, as well as co-occurrences of microorganisms within a diseased state. Oversimplifying dysbiosis in terms of a reduction of diversity in IBD patients is problematic, because it takes focus away from the complex community and the complex interactions that are occurring with the host. Diversity does not predict gut homeostasis with the host in IBD.

6.4 Limitations and future directions

To date, the clinical evidence for FMT as an effective treatment for IBD is conflicting. The findings in Chapter 2 show that FMT was able to induce remission and complete mucosal healing in patients with active UC. Of those patients that were randomized to the active arm, 24% entered into remission following 6 FMT administrations, compared to 5% in the placebo arm that entered into remission. This is in contrast to a study published by Rossen et al., 2015, that showed no statistical improvement for patients that received FMT compared to patients that received autologous feces. Patients that received FMT from a healthy donor reached their primary endpoint 30.4% of the time compared to patients that received their own feces (reached clinical endpoint 20% of the time). Chapter 3 displayed a lack of evidence of engraftment, at the OTU level, as the successful mechanism of FMT. It is important to note that the longitudinal study in chapter 3 followed an individual with severe UC that entered into remission twice following FMT, while not on any other form of medication. Chapter 4

highlighted that there were no taxa commonalities following FMT in patients that entered into remission. What remains unknown is how FMT works. The next few subsections will outline possible mechanisms for FMT success in IBD patients, and experiments for how these hypotheses could be tested.

6.4.1 Strain replacement of related species

A major limitation to this thesis was that engraftment was not examined at the strain level, rather examined at the OTU and species level. In order to analyze strain replacement data generated in chapter 4 could be analyzed to look for structural nucleotide variants (SNVs). This was completed in Li et al., 2016. Generally, reads from patients will be aligned to a reference database (or to donor contigs) and using programs such as mpilup, SNVs can be called to look for what was present pre and post FMT and which strains are shared with the donor. Interestingly, Li et al., claimed strain replacement occurred with closely related species. This is in contrast to Maldonado-Gómez et al., 2016 that showed that species that are missing from the ecosystem are more likely to engraft in the human gut. Li et al., however, did not include a pre FMT sample in their analysis, making their analysis questionable. It would be informative to complete this same analysis in the metagenomic dataset generated in chapter 4.

6.4.2 Competition of non-immunogenic spores

The microbiome that harbors our intestine is diverse, and unique amongst individuals although, mostly anaerobic. It is estimated that within our gut, greater than 99% of the community is composed of anaerobes. Considering all FMT protocols are prepared aerobically, and oxygen is toxic to most microbes that would be found within

the feces that are used to prepared FMT suspensions, anaerobic spore-forming bacteria may play a role in successful FMT for IBD. In chapter 3, it was demonstrated that what was present prior to FMT, was present post FMT. There was no evidence for large-scale engraftment post FMT at the OTU level. The limitation to this finding is that species level replacement could have occurred post FMT but, this may also suggest that competition, and niche exclusion of anaerobic spore forming bacteria could play a role in the mechanism of successful FMT.

Clinical data from studies that used autologous feces as a control support the notion of niche exclusion of anaerobic spore forming bacteria could play a role the mechanism of successful FMT. Two clinical trials, used autologous feces as a control group, and saw success. As mentioned above 20% of IBD patients that received autologous feces entered into remission (Rossen et al., 2015). In a more recent phase two clinical trial, that tested the safety of FMT for RCDI, 90% of patients in the intention to treat arm improved but, 62.5% of patients that received autologous feces improved. Pockets of non-toxigenic *C. difficile* spores could have been produced, possibly through adaptive radiation, and then when re-administered, competed with toxic spores within the host. Non-toxigenic spores have been shown to successfully treat RCDI. It is unknown however, if spores play a role in the pathogenesis of IBD and if they play a role in successful FMT treatment for IBD. I propose to *characterizing spore-forming bacteria in donor suspension and IBD patients before, during, and after FMT through targeted cultured enriched metagenomics of spore-forming bacteria.*

A second phase II clinical trial has started at the McMaster Health Science Centre. The Surette Lab will be receiving fecal samples from patients prior to, during, and following FMT. These samples will be collected and preserved for culture using the methods described in Lau et al., 2016. I propose, that ethanol and heat resistant spores should be characterized in IBD patients, prior to, during, and following FMT. This should also be completed for the donor suspensions that are given to the patients. It is imperative to use the donor suspensions and not the donor stool – one must capture what is going into the patient. This work should be completed post clinical trial, when there is an understanding of what patients responded, and which patients did not respond.

A subset of treatment successes and treatment failures should be taken. Approximately, samples from 10 patients that entered into remission following FMT and 10 patients that did not enter into remission (and whose clinical score did not change following the trial) should be taken for ethanol resistant spore testing. One gram of feces will be treated with 1 mL of ethanol (70%) for 60 minutes to kill all vegetative cells. Then this suspension will be diluted and plated in anaerobic conditions using the methods outlined in Lau et al., 2016, however plates should be incubated for at least 72 hrs. Plates will be pooled and shot gun metagenomics will be completed. Data can be analyzed to not only look for species that form spores, but also genes present that may be required for germination signaling.

6.5 Conclusions

The work in this thesis provides insight into the intestinal microbiome in IBD patients based on disease status, severity and response to therapy. This thesis provided an in-depth characterization of the intestinal microbiome in IBD, demonstrated the response of the gut microbiome in UC patients to FMT, providing insight into the successful mechanism, and provided evidence for inflammation related differences in CD giving insight into the mechanism of pathogenesis. The studies presented in this thesis have significant clinical implications given the increasing prevalence of IBD in Canada. The work in this thesis can be used to not only further our knowledge regarding IBD pathogenesis, but also improve treatment protocols, such as FMT.

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Title: Fecal Microbiota Transplantation Induces Remission in Patients With Active Ulcerative Colitis in a Randomized Controlled Trial

Author: Paul Moayyedi, Michael G. Surette, Peter T. Kim, Josie Libertucci, Melanie Wolfe, Catherine Onischi, David Armstrong, John K. Marshall, Zain Kassam, Walter Reinisch, Christine H. Lee

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