Harnessing Gut Microbial Modulation in Chronic Inflammatory Gastrointestinal Disease

by

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Abstract

Background

A growing body of work has emerged which suggests that not only is the gut microbiome integral to maintaining human health, but also promising in the treatment of chronic inflammatory gastrointestinal diseases. Obesity and inflammatory bowel disease (IBD) are two such conditions with perhaps the greatest evidence for microbial intervention. However, our understanding of the role gut microbial modulation has in the management of these diseases remains in its infancy thereby limiting our ability to harness its therapeutic potential.

Aims

The aim of this thesis is to explore the role of gut microbial modulation for two chronic inflammatory gastrointestinal diseases in which we face the biggest therapeutic challenges to date - obesity and inflammatory bowel disease.

Hypothesis

The overarching hypothesis of the enclosed thesis is that gut microbial modulation can be utilized to improve metabolic and gastrointestinal health in obesity and inflammatory bowel disease.

Methods

In our first study, a randomized double-blinded placebo-controlled trial was performed to evaluate whether gut microbial modulation through fecal microbial transplantation (FMT)

combined with fiber supplementation could improve insulin resistance in patients with severe obesity and metabolic syndrome. Secondly, we conducted a systematic review and pooled proportion meta-analysis to evaluate whether two gut microbial modulation strategies, repeated FMT delivery and antibiotic pre-treatment, could improve IBD response and remission. Lastly, we utilized an established murine model of ileocecal resection to evaluate if peri-operative tributyrin supplementation, an adjunct chosen to restore luminal hypoxia and gut barrier integrity, could improve post-operative inflammation by fostering a recolonization of anti-inflammatory anaerobic microbes.

Results

Results of our randomized trial demonstrated that microbial modulation imparted improvements on insulin resistance using a safe and tolerable oral FMT delivery method in a North American bariatric population undergoing concurrent medical therapy. Interestingly, fiber fermentability was found to differentially modulate metabolic response, with patients receiving low-fermentability fiber following FMT demonstrating significant improvements for insulin resistance, insulinemia, and enteroendocrine physiology. These benefits were associated with increased microbial richness and a bloom in select microbial taxa such as *Phascolarctobacterium, Christensellaceae, Bacteroides,* and *Akkermansia*.

Results of our systematic review on the efficacy of repeated FMT and antibiotic pre-treatment with respect to improvement of IBD outcomes revealed a potential for both strategies in modulating IBD response and remission rates. That these benefits were also associated with an enrichment in select bacterial taxa like *Bifidobacterium, Roseburia, Lachnospiraceae,*

Prevotella, Ruminococcus, and *Clostridium* related species which are associated with anti-inflammatory metabolite production further supports an adoption of these strategies in future clinical trials.

Lastly, our ileocecal mouse model study provided evidence that timing of tributyrin delivery in the peri-operative period was associated with differences in gastrointestinal inflammation and gut microbial recolonization. Notably, mice receiving tributyrin postoperatively demonstrated improvements in colonic inflammation and a bloom of anti-inflammatory anaerobic taxa including *Bacteroides thetaiotomicorn, Bacteroides caecimuris, Parabacteroides distasonis, Clostridia,* and *Turicibacter*.

Conclusion

These studies add further backing to the growing body of evidence supporting the ongoing pursuit of gut microbial modulation strategies as a novel therapeutic modality for management of chronic inflammatory gastrointestinal diseases. In so doing, they also serve as a framework for the ongoing development of novel microbial biotherapeutic strategies aimed at combatting the growing obesity and IBD epidemics through the future delivery of safe, effective, and affordable designer bacterial consortia.

Preface

This thesis is an original work by Valentin Mocanu. The proof-of-concept pilot trial presented in Chapter 2 was approved by the University of Alberta Health Research Ethics Board policies (Pro00076642), Health Canada (control #212903) and registered with clinicaltrials.gov (NCT03477916). Informed consent was obtained from all participants and safety monitoring was conducted independently by the University of Alberta Quality Management in Clinical Research Department (QMCR). The study complied with all relevant institutional and national regulatory body requirements. For mouse experiments presented in Chapter 4, experimental protocols were approved by the University of Alberta animal ethics committee (AUP00000293) with peri-operative mouse husbandry protocols approved by the university's Health Sciences Laboratory Animal Services.

Chapter 2 of this thesis is accepted as: **Mocanu V**, Zhang Z, Deehan EC, Kao DH, Hotte N, Karmali S, Birch DW, Samarasinghe KK, Walter J, Madsen KL. **Nature Medicine.**
K.L.M conceived the project. K.L.M, J.W., E.C.D., D.H.K, N.H., and V.M. designed the proof-of-concept study. K.L.M, J.W. and E.C.D designed the fiber mixture. K.L.M. and D.H.K. supervised the clinical aspect of the study. D.H.K. supervised the FMT donor program. D.W.B and S.K. helped identify patients enrolled in the bariatric clinic for study inclusion. V.M. and K.K.S. recruited, coordinated, and conducted clinical visits. N.H. performed study randomization and created the fiber sachets. V.M. performed the analysis and interpretation of anthropometric, biochemical, immunologic, and enteroendocrine results. E.D.C. conducted the analysis and interpretation of dietary data. Z.Z. performed the fecal microbiome sequencing and analysis. All authors discussed the study findings, interpretations and approved the final manuscript.

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Chapter 1: Introduction

1.0 Background

Our understanding of microbes and their implications on human gastrointestinal health and disease has experienced a dramatic evolution over the course of the last few decades[1,2]. Recent culture-independent technologies have broadened our understanding of microbes from that of single organisms responsible for acute pathology to one of complex dynamic communities essential in the maintenance of host immunologic and metabolic homeostasis[3].

The collection of bacteria in our gastrointestinal tract- our gut microbiome- has indeed been associated with a number of critical functions ranging from maturation and education of the host immune response to intestinal enteroendocrine regulation and providing colonization resistance against bacterial pathogens [4]. The relevance of these functions on human health and disease have led many to reconsider the human gut microbiome as a novel 'microbial organ'[5], one with which we have co-evolved over the span of millions of years and are just now beginning to harness to our benefit.

A growing body of evidence has emerged which suggests that not only is the gut microbiome integral to maintaining human health, but also promising in the treatment of chronic inflammatory gastrointestinal diseases[6]. Obesity and inflammatory bowel disease are two conditions with perhaps the greatest evidence for microbial intervention and are the topic of the enclosed thesis. Together, obesity and IBD are of particular importance because while their prevalence is rapidly growing worldwide[7,8], they both have limited response to modern medical management [9,10]. In addition, current therapies for obesity and IBD are costly, often poorly

tolerated, and are associated with life-threatening complications when used long-term[10–12]. Challenges in managing these diseases thus necessitate the development of new therapeutic paradigms.

Modulation of the gut microbiome presents a promising novel target with which to potentially alter the disease course for both obesity and IBD[13–15]. This concept will be explored in greater detail in the following sections while discussing the history of the microbiome, its relationship with obesity and IBD, and its role in ultimately developing novel personalized biotherapeutics.

1.1 The Human Gut Microbiome and Chronic Inflammatory Gastrointestinal Disease

1.1.1 History of the Microbiome

The human microbiome was first studied by Antonie van Leewenhoek in the 1680s. He noted what now seems obvious, that remarkable differences exist between oral and fecal microbiota and that differences in the microbiota also exist between individuals, and for different pathologic states[16]. Perhaps the first theory that postulated gut microbial modulation could impart benefits to the human host was proposed by Elie Metchnikoff, at the turn of the last century. Metchnikoff, a Nobel Prize winner for his studies on phagocytosis, hypothesized that gut microbial metabolites were responsible for the toxic substances involved in aging and that supplementation with fermented milk products would reverse the deleterious effects of these microbial by-products[17]. His theories were based on the observation that Bulgarian peasants who lived in harsh unsanitary conditions and consumed fermented milk had unexpectedly long-life expectancies when compared to more affluent Bulgarian citizens.

Following these initial theories, the first causal link between a specific microbe and human disease was eventually established in 1983 by Marshal and Warren with the discovery of *Helicobacter pylori* as a cause of gastric ulcers[18]. The majority of scientific efforts remained focused on the pathologic implications of bacteria until the 21st century despite the fact the vast proportion of gut microbiomes are not associated with disease and may, in fact, prove beneficial[17,19]. The prevailing theories regarding the gut microbiome solely acting as a contributor to human disease remained predominantly shaped by the culture of single microbes[16] - a major limitation since most microbes residing in our gastrointestinal tract cannot be cultured through typical laboratory conditions[20].

This philosophy changed in 2007 after the Human Microbiome Project (HMP) set out to create a healthy cohort microbiome reference database using culture-independent microbial analytical techniques[21].The HMP recruited 300 healthy adults and sampled their microbiome up to three times over two years from 15 different body sites. Microbiomes were then characterized using 16S ribosomal ribonucleic acid (rRNA) and meta-genomic sequencing, providing the foundational catalogue for the adult human gut microbial genome. This work has not only been pivotal in advancing our knowledge of gut microbial composition in a human host, but also in our understanding of a 'healthy' gut ecology that is perturbed in chronic inflammatory gastrointestinal diseases such as obesity and IBD[22–24].

1.1.2 Composition of Human Gastrointestinal Microbiome

The gut microbiome consists of over 100 trillion organisms that have co-evolved in our gastrointestinal tract over millions of years[3,25,26]. This amounts to nearly as many microbial cells as there are somatic cells present in the human body[27]. The highest concentration of microbes resides in the colon and contribute up to 1.5kg of our total body weight[28]. Studies have demonstrated that over 3 million genes compose the microbiome while approximately only 23, 000 genes make up the human genome[16,29]. In other words, the gut microbiome has nearly 200 times the genetic potential of our own genome[16]. Up to 10% of all circulating metabolites are bacterial derived[30], a feature that has helped establish the symbiotic relationship responsible for modulating a number of essential host functions including energy metabolism and immune system regulation – key functions underlying the aetiologies of both obesity and IBD[31].

The human gut microbiome is primarily composed of four phyla[21,32,33] existing not in isolation, but within a complex dynamic ecosystem. Of the four phyla, *Bacteroidetes* and *Proteobacteria* are gram negative, whereas *Actinobacteria* and *Firmicutes* are gram positive bacteria. The majority of the human microbiome is composed of *Bacteroidetes* and *Firmicutes* which account for over 90% of bacterial taxa[6]. Interestingly, while the gastrointestinal tract contains anywhere from 500 to 1000 distinct species, only 40 stable species make up 75% of the total bacterial population and 60 to 80 species compose 99% of our overall microbiome[34–37]. This suggests that although each person has a unique 'core' microbiome, the functional capacity of the gut microbiome remains relatively consistent across healthy individuals and remarkably conserved at high taxonomic levels[16,34].

1.1.3 Acquisition and Development of the Gut Microbiome

A number of environmental and host factors are responsible for the inheritance and assembly of our gut microbial communities in the perinatal period[38]. These include mode of delivery, nutritional intake, and geographic location[3].

While debate exists regarding the exact timing of microbial colonization in the neonate, the prevailing consensus, including that of this author, is that of microbial acquisition at the time of delivery. Infants delivered vaginally are exposed to and are subsequently initially colonized by the maternal fecal and vaginal microbiota associated with the birth canal[36]. Evidence to support this acquisition is that the meconium composition of vaginally delivered babies consists of *Lactobacillus* and *Prevotella*- microbes common within the vaginal flora. In contrast, infants delivered surgically by caesarean section exhibit a meconium composition more closely resembling that of skin flora[39,40]. Although differences in the gut microbiota between vaginally and surgically delivered children decrease over time, some have reported detectable differences in children up to 7 years of age[41].

Following the first critical colonization event of delivery route, the next major factor influencing the development of neonatal microbial communities is nutritional intake[42]. Breastfed infants have a reduced microbial diversity versus formula-fed infants and a higher proportion of *Lactobacillus*, *Staphyloccoccus*, and *Bifidobacterium*[38,43]. In addition, breastfeed infants also demonstrate an increased expression of genes associated with milk oligosaccharide metabolism. Formula fed infants on the other hand have more diverse microbiomes with higher proportions of *Bacteroides* and lower proportions of *Bifidobacteria*[42,44]. The introduction of solid food next

shifts the infant microbial composition from one that is *Bifidobacterium* dominated to one that is characterized by increased diversity and increased abundance of *Bacteroides*, and *Firmicutes*. The initially heterogenous infant microbiome eventually reaches stability around four years of age and continues to increase in diversity and decrease in interindividual variability until adulthood[45].

In contrast to the vertical microbial transmission discussed above, horizontal transmission is dictated by a variety of environmental and geographic factors. In a recent Danish cohort study, infants with older siblings were found to have increased microbial diversity[46]. Other trials have also demonstrated differences due to family structure with the KOALA Birth Cohort trial showing an increased *Bifidobacteria* in infants with older siblings[47].

Lastly, geographic factors such as industrialization have led to an adoption of a calorie dense, nutrient poor diet deficient in microbiota accessible carbohydrates[48]. Together with differences in sanitation and antibiotic use, these factors are thought to be major contributors to the microbial changes present between rural and industrial populations. For instance, in comparison to rural populations, industrialized populations have a decrease in phylogenetic and carbohydrate-active enzyme diversity[49]. The industrialized microbiome is also associated with an increased abundance of *Bacteroides* and *Akkermansia muciniphilia*. Such shifts have been implicated in the deterioration of human health leading to the concept of microbial 'dysbiosis'- a pathologic imbalance in native microbial communities thought to be key predisposing factor to chronic inflammatory gastrointestinal disease.

1.1.4 Microbiome in Health and Inflammatory Gastrointestinal Disease

The combination of our innate and adaptive gastrointestinal host response together with our commensal gut microbiome have been termed the "mucosal firewall"[50] (Figure 1.1). In a state of health, the epithelial mucous layer presents the initial barrier between our microbes and host immunity. A healthy mucous layer allows resident macrophages and dendritic cells to constantly sample commensal microbiota. Meanwhile, intestinal epithelial cells secrete antimicrobial peptides, ensuring only adequate exposure of potential microbial pathogens to the underlying immune cells of the lamina propria[51]. This controlled sampling of commensals allows for a dynamic regulation of both pro- and anti-inflammatory immune regulation.

Even in healthy gastrointestinal tissues, translocation of luminal gut bacteria occurs, albeit in a controlled fashion. These microbes are quickly engulfed by resident macrophages with subsequent antigen presentation to either resident dendritic cells of local Peyer's patches, or to more distant mesenteric lymph nodes. Depending on the presented commensal antigen, activated dendritic cells are able to induce an array of pro-inflammatory or anti-inflammatory pathways through modulation of T-cell differentiation into Th1, Th2, Th17, and regulatory T-cells[52,53]. These responses are further coordinated by anti-inflammatory microbial metabolite by-products like short chain fatty acids. Differentiated T-cells also coordinate B-cell immunoglobulin class switching towards IgA-production further modulating commensal microbial composition. The sum of this "mucosal firewall" results in a gastrointestinal immune homeostasis responsible for the development, regulation, and propagation of our gut health[54].

In states of gastrointestinal chronic inflammation, as exist in obesity and IBD, the harmony between pro-inflammatory and anti-inflammatory responses is lost. The "mucosal firewall" responsible for homeostasis is perturbed. While the combination of genetic and environmental factors between these two diseases differ, the resulting picture is remarkably similar.

The once effective primary mucous barrier becomes defective leading to an increased and uncontrolled translocation of commensal bacteria. Innate resident macrophages and dendritic cells in the lamina propria continue the process of phagocytosis, antigen presentation, and adaptive immune response activation[55]. Initially, intestinal epithelial cells also continue production of regulatory antimicrobial peptides and provide an effective microbial barrier through expression of tight junction proteins[54]. Yet, with continued loss of gut barrier integrity and immune system activation, the once harmonious environment eventually turns into a battle ground.

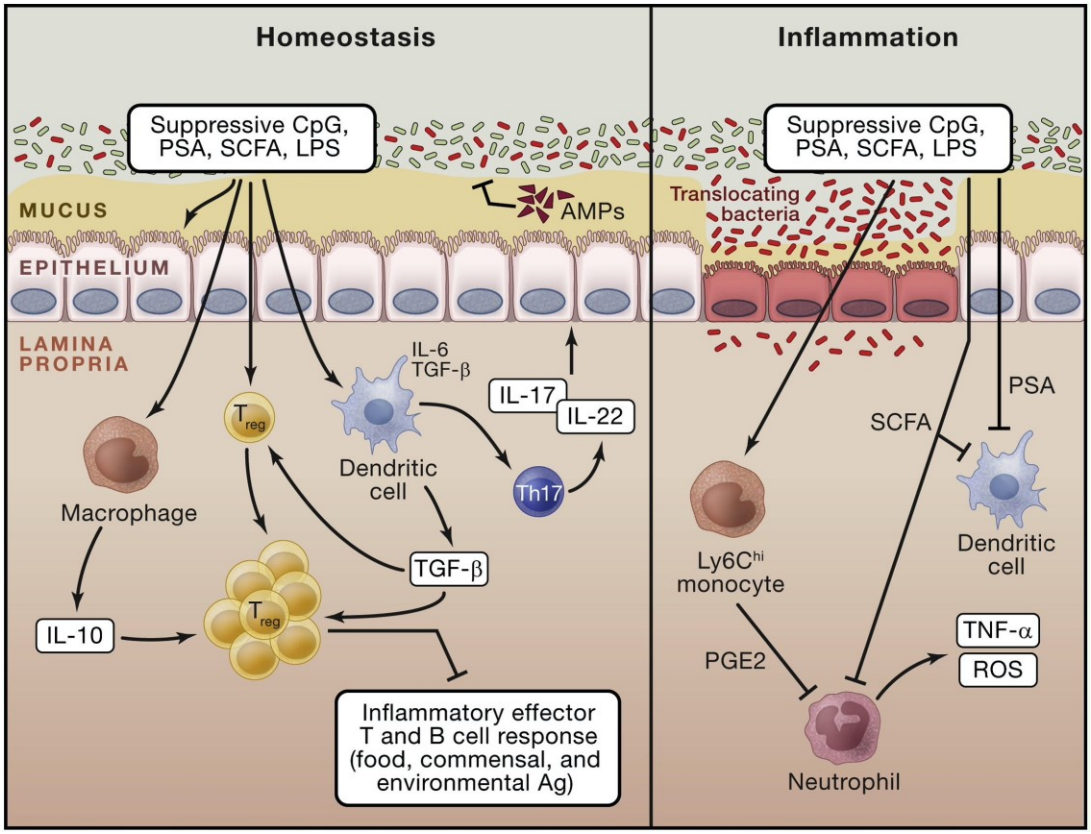

Figure 1.1 Overview of gut microbiome in a state of homeostasis in comparison to a state of inflammation.

(Reprinted from Cell, Vol 157, Issue 1, Belkaid Y. and Hand T. Role of the Microbiota in Immunity and Inflammation, Pages 121-141, Copyright 2014, with permission from Elsevier to publish in enclosed PhD thesis both electronically and in-print, License Number 5044361094592)

Continued translocation leads to a decreased synthesis of anti-inflammatory microbial compounds and an increased presentation of microbial antigens. This results in a vicious cycle leading to persistent pro-inflammatory immune activation, loss of intestinal epithelial barrier integrity, ongoing loss of mucous thickness, and adverse changes to the once stable commensal microbial communities[56] (Figure 1.1). The fact that the perturbation of the "mucosal firewall"

coincides with a western diet, microbial dysbiosis, and increased prevalence of chronic gastrointestinal inflammatory diseases like obesity and IBD presents a unique therapeutic opportunity we are just beginning to harness[57].

1.1.5 Strategies for modulating the gut microbiome

A number of current strategies exist with which to modulate the gut microbiome and improve human health with regards to chronic gastrointestinal inflammatory disease. These include dietary interventions, fecal microbial transplantation (FMT), prebiotics, probiotics, and other supplements. General mechanisms of each will be explored in the following paragraphs with more detailed disease-specific evidence provided in the subsequent obesity and IBD sections.

1.1.5.1 Fecal Microbial Transplantation

Fecal microbiota transplantation seeks to achieve a reestablishment of balanced gut microbial composition and can be performed through a number of different techniques[58]. The use of FMT in modulating the host microbiota has become a field of significant research and has shown extraordinary promise in the treatment of a variety of medical diseases [59–63]. In addition to obesity and IBD, case reports have indeed reported benefit for numerous conditions from multiple sclerosis to Parkinson's disease, chronic fatigue, and idiopathic thrombocytopenic purpura[64].

While a novel therapy in contemporary Western medicine, the concept of FMT can be traced back as early as the 4th century China[60,61]. Originally known in China as the 'yellow soup' or 'golden syrup', FMT was used to treat cases of severe diarrhea. Evidence of fecal transplantation

in European culture was first demonstrated by Italian surgeon Acquapendente (1537-1619) after he noticed benefit when transferring stool from healthy animals to sick ones[65]. The importance of gut bacteria modulation would later be revealed during the Second World War, when German soldiers began dying of dysentery. After noticing improvement of symptoms by locals who ingested fresh camel stools[66], German scientists isolated *Bacillus subtilis* and observed resolution of dysentery with its administration.

Currently, the only indication for fecal microbiota transplantation is refractory *Clostridioides difficile* infection (CDI). CDI is a gastrointestinal disease provoked by antibiotic use leading to a state of microbial dysbiosis that is characterized by a pathologic bloom of *C. difficile* [59–61]. FMT was first performed by Dr. Eiseman in a group of 4 critically-ill CDI patients with pseudomembranous colitis induced toxic mega-colon [67,68]. These patients were treated with fecal enemas with stool obtained from healthy donors. Conventional surgical treatments at the time were associated with a mortality of 50% or greater, yet Eiseman et al. were able to demonstrate a remarkable cure rate in excess of 90%. Randomized controlled trials have since validated these initial case studies, confirming the safety and long-term efficacy of FMT treatment[69].

The resounding success of FMT therapy in improving outcomes for patients with CDI has recently drawn attention to its potential role in modulating outcomes for additional gastrointestinal diseases associated with perturbed microbial ecology [61,62]. Indeed, it stands to reason that reversing the gut dysbiosis implicated in propagating other inflammatory gastrointestinal diseases like obesity and IBD with a healthy microbiome may impart similar benefits. However, unlike *C. difficile*, the evidence for FMT in these diseases is still very much

in its infancy. Major limitations include only short-term donor microbial engraftment, inconsistent clinical efficacy, and lack of information regarding optimal donor-recipient combinations[70].

To date, expert consensus does not recommend the use of FMT for obesity or IBD treatment given lack of high-quality evidence. Yet, current FMT studies are underway to determine presence of clinical efficacy as well as optimal delivery, dosing, and treatment duration.

1.1.5.2 Prebiotics

The advent of high-throughput sequencing technologies together with our expanding knowledge of the gut microbiome have helped shaped the definition of the term prebiotic. Originally defined in 1995 as a "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria already resident in the colon"[71], it soon became evident that the impact of prebiotics on gut microbial ecology is more complex and not just limited to only select bacterial taxa. This knowledge led to a number of subsequent revisions culminating in an International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus whereby prebiotics are defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit"[72].

Dietary fibers, or prebiotics, are carbohydrate compounds which have a degree of polymerization of ten or more monomers and are not absorbed or digested in the human gastrointestinal tract[73]. Since prebiotics are not digestible, they resist stomach acid breakdown as well as pancreatic and brush border enzyme digestion. Fibers thus reach the colon where they are selectively utilized by

commensal gut bacteria. This selective utilization is thought to differentially modulate microbial ecology as well as the production of health-promoting microbial end-products which have been shown to confer a health benefit[74].

Ample epidemiologic studies have provided support for use of prebiotics in prevention of inflammatory gastrointestinal diseases on a population level. For example, a recent 2019 meta-analysis of 185 cohort studies demonstrated that patients with increased fiber intake were associated with improved overall mortality, diabetes, and cardiovascular disease in comparison to those with decreased fiber intake[75]. A large prospective cohort study of 170,776 women with a 26-year follow-up further found that women with higher intake of dietary fiber were associated with reduced risk of Crohn's disease[76]. However, while promising, interventional human studies evaluating the clinical impact of fiber as a therapy for obesity and IBD remain conflicting.

A number of psychochemical properties dictate the classification and mechanisms of prebiotic function in the human gastrointestinal tract: solubility, viscosity and fermentability[73]. The degree to which different prebiotics express these characteristics affects a range of host factors from gut transit to metabolite bioavailability, gut barrier integrity, and intestinal inflammation. Of these properties, fiber fermentability has been a topic of great interest due to the ability to selectively modulate specific microbial communities and, in turn, their potentially beneficial metabolites[77].

High-fermentability fibers are differentially degraded in the colon by microbial taxa. These include resistant starches, arabinoxylans, pectins, inulin, galacto-oligosaccharides amongst others[78]. Greater intake of high-fermentability starches have been associated with increased anti-

inflammatory SCFA concentrations in numerous human studies and have been purported to improve human health[79]. In contrast, low-fermentability fibers such as cellulose and maltodextrin are resistant to mirobial degradation and modulate gut health through changes in fecal pH, stool transit time, and branched-chain amino acids metabolites (BCAAs)[80].

Despite their promise, a variety of questions remain to be answered regarding the role of prebiotics in chronic inflammatory gastrointestinal diseases. Namely, we do not yet understand from which bacterial communities we stand to gain most benefit nor the optimal dose or duration of prebiotic intervention. Additionally, and of particular importance to this thesis, the concept of using of prebiotics to selectively tailor microbial communities as an adjunct to FMT has been much discussed in literature[81–83]. However, no real-world clinical trials which evaluate this concept currently exist.

1.1.5.3 Probiotics

According to the World Health Organization, probiotics are defined as "live microorganisms, that when administered in adequate amounts, confer a health benefit to the host"[84]. Commercially, most probiotic strains currently in use include *Lactobacillus* and *Bifidobacterium*[85]. While they do occur naturally, probiotic strains are often produced separately and either then added to foods or sold separately in many health food stores and pharmacies. Unfortunately, these strains are often selected for factors like safety and shelf-life instead of desired metabolic function which may explain the conflicting efficacy surrounding their administration. It is therefore unsurprising that many probiotics have been found to have limited effect on the gut microbiome or health benefits.

Recent landmark work, however, has demonstrated that novel designer probiotics can indeed impart specific microbiome-mediated benefits to outcomes like metabolic parameters. For example, in a study by Depommier et al., supplementation of *Akkermansia muciniphilia* in patients with metabolic syndrome was associated with improvements in insulin sensitivity, body weight, and other inflammatory markers[86]. Interestingly, these changes were independent of shifts in gut microbial structure suggesting a failure for probiotics to engraft new microbes in niches already colonized by host commensal bacteria. Failure of sustained colonization is therefore one reason for the transient observed effects of probiotics seen after cessation of supplementation[87]. Elaborate work to culture and supplement 'autochthonous' taxa, or bacteria normally occupying human microbial communities, together with strategies which sustain their engraftment are currently underway.

1.1.5.4 Antibiotics

The gut microbial revolution is in large part due to both the successes and failures of antibiotic utilization. On one hand, the ability to selectively culture bacteria and study their functions could not be possible without antibiotics. On the other, the uncontrolled prescribing of antibiotics worldwide has led to widespread antimicrobial resistance and an unprecedented increase in multi-drug resistant organisms. As mentioned earlier, one of these organisms, *C. difficile*, has indeed been responsible for the current transition of FMT to both obesity and IBD[88].

Paradoxically, while antibiotics have been clearly shown to adversely alter gut microbial ecology, they may also prove essential in optimizing restoration of normal ecology following FMT[78]. FMT literature in both obesity and IBD has been conflicting and potentially limited by

lack of host engraftment of donor microbes[90]. If colonization resistance by native gut microbial communities is a barrier to FMT efficacy, it stands to reason that pre-clearing these communities with antibiotics prior to FMT may serve to open new ecological niches for donor microbes to successfully engraft. Emerging evidence indeed suggests potential for this approach, but caution and further evidence are needed as to not repeat our prior failures of over-utilization.

1.1.6 Concluding Summary

This section has been a broad overview of the microbiome and its implications on health and chronic inflammatory gastrointestinal disease. Topics and rationale for the enclosed experiments will be discussed in greater detail in the following specific sections on obesity and IBD.

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1.2 Obesity and the Gut Microbiome

1.2.1 Epidemiology and Economic Costs of Obesity

Obesity is one of the greatest health epidemics of the 21st century. According to recent World Health Organization (WHO) estimates, the worldwide prevalence of obesity has nearly tripled from 1975 to 2016[1]. Currently, over 13% of the world's adult population suffers from obesity, with 39% of adults aged 18 years and older being overweight[2–4]. In 2016 alone, nearly 2 billion adults were overweight, while over 650 million adults were diagnosed with obesity. The economic impact of this epidemic is overwhelming, with the total global cost of obesity estimated at $2.0 trillion, nearly surpassing the economic costs of smoking, war, and terrorism[5]. With rates of obesity rapidly continuing to climb, the management of obesity is at the forefront of modern research. Outside of bariatric surgery, current medical therapies have limited efficacy, are costly, and are poorly tolerated[6,7]. Thus, novel therapeutic approaches to combat this evolving epidemic are urgently needed.

Subsequent sections will discuss, from a gut microbiome perspective, the complex nature of obesity. This includes its definition, its etiology and link to chronic systemic inflammation, and the ongoing limitations of current management approaches. Using this framework, these sections will help provide justification for the active pursuit of the novel microbial based biotherapeutic strategies presented in Chapter 2.

1.2.2 Defining Obesity and Metabolic Syndrome

Obesity is a complex chronic progressive inflammatory gastrointestinal disease defined by the WHO simply as excessive fat accumulation that poses a risk to one's health[8]. It is measured

using the body mass index (BMI) which is calculated by dividing an individual's body weight in kilograms by the square of their height in meters. Adults are considered obese if their BMI is 30 kg/m^2 or greater, and overweight if their BMI is $25kg/m^2$ or greater. Using BMI, obesity can be further categorized into three distinct classes using the National Institutes of Health Classification (NIH) (Table 1)[9]. Obesity classes are then stratified based on the health risks associated with increasing BMI and are further used to help guide therapeutic interventions[10].

BMI (kg/m^2)	Weight Classification
<18.5	Underweight
18.5-24.9	Normal weight
25-29.9	Overweight
30.0 – 34.9	Class I Obesity
35 – 39.9	Class II Obesity
>40	Class III Obesity

Table 1.1 National Institutes of Health Classification of Overweight and Obesity by BMI

1.2.3 Metabolic Syndrome: An Obesity Phenotype

Metabolic syndrome was first described in 1956 when Vague identified an association between a pattern of adiposity distribution and development of future metabolic complications such as diabetes, dyslipidemia, and cardiovascular disease[11–14]. Vague described two phenotypes, gynoid and android, each with different patterns of subcutaneous fat distributions. Gynoid, or female, obesity phenotypes were observed to have an increase in lower body adipose storage with a decreased truncal fat distribution. Android, or male, obesity patterns displayed the opposite fat

localizing pattern- an increased truncal fat deposition with a proportional reduction in lower body adipose storage.

Both obesity phenotypes have since been shown to be strong predictors of weight gain and subsequent development of diabetes, vascular disease, calculous disease, and gout. Additional studies have further expanded on Vague's initial metabolic syndrome list of key traits to currently include hypertriglyceridemia and hyperinsulinemia. Metabolic syndrome is now understood to be an obesity phenotype, with the presence of each additional trait incrementally increasing the risk of developing subsequent health complications[2,12,14,15].

The National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III), ultimately unified the diagnosis of the metabolic syndrome in 2001[16]. To meet NCEP criteria for metabolic syndrome, the presence of three of the following five factors is required: abdominal obesity (waist circumference >102 cm for men and >88 cm for women), triglycerides > 1.7 mmol/L, high density lipoprotein (HDL) cholesterol < 1.03 mmol/L (male) and < 1.29mmol/L (female), blood pressure >130/85 mmHg or treatment for hypertension, or fasting plasma glucose >6.1 mmol/L. In 2003, the American Association of Clinical Endocrinologists (AACE) later modified the metabolic syndrome definition such that a minimum number of traits are no longer required to establish a diagnosis[17]. A variety of metabolic syndrome definitions now exist with each still dependent on the presence of the original key traits: obesity, dyslipidemia, hypertension, and insulin resistance.

Today, metabolic syndrome is also known by a variety of terms including Syndrome X, Reaven's syndrome, and insulin resistance syndrome[12,13,18,19]. These terms are interchangeable in literature and underscore the importance of promptly identifying at-risk individuals in order to prevent the future development of diabetes and cardiovascular disease. While a consensus has yet to be reached regarding a unified definition of metabolic syndrome, it is evident that understanding its pathophysiology will help foster the ongoing development of effective therapies.

1.2.4 History of Obesity

Initial theories of obesity were first proposed by Hippocrates as early as the 5th century BC[20–23]. Hippocrates originally prescribed exercise and reducing dietary intake to one fatty meal per day as a treatment for obesity. Implicit in this treatment approach was a theory that decreasing dietary consumption and increasing energy expenditure would promote weight loss. This energy imbalance theory prevailed until the late 18th century when it was observed that some individuals with obesity only ate moderately, while other lean individuals were able to consume large amounts of food without gaining weight[2,24].

Such observations lead to the "Luxus Konsumption" theory of obesity[11,20,23]. A healthy individual ingesting excess food, this theory argued, was able to appropriately increase their metabolic consumption to maintain a lean body weight. Individuals with obesity, however, were believed to lack this homeostatic mechanism. This theory was later refuted with the advent of metabolomics and human calorimetry[25]. Benedict et al., at the turn of the first World War, restricted the intake of 22 healthy subjects for 3 months[26]. Subjects lost only 7-11% of their body

weight despite much more dramatic reductions seen in their basal metabolic rates. This observation made it clear that food intake and metabolic rate were not the sole players in development of obesity and metabolic syndrome.

The early 20th century brought a more comprehensive, albeit still naïve understanding of the pathophysiology of obesity. Medical literature, including Osler's textbook of medicine identified hereditary, endocrine, and patient sex as additional factors associated with obesity[20]. Osler concluded that diet and exercise were responsible for less than half of all obesity cases with the other half presumed to be multifactorial in nature. Despite this conclusion, ongoing theories remained limited by a lack of available technology and population-based research studies at the time. Indeed, it was not until the 21st century with the advent of genomic sequencing and the Human Microbiome Project that a more complete understanding of the etiology of obesity began to unfold[14,18,21,27].

1.2.5 Obesity and Chronic Systemic Inflammation

Demonstrated associations between systemic inflammation, the human gut microbiome, and obesity have helped to further our understanding of how the gut microbiome may contribute to the pathogenesis of obesity and metabolic syndrome [28–30]. A key step in identifying the now well acknowledged link between obesity and the gut microbiome was first establishing obesity as a chronic inflammatory gastrointestinal disease.

Cross-sectional population studies have long provided support for systemic inflammation as an important underlying factor associated with the etiology and progression of obesity and

metabolic disease[31]. A recent review of 51 studies, for example, demonstrated that C-reactive protein (CRP), a well-studied clinical marker of inflammation, was positively correlated with increasing body weight[32]. Similar correlations have also been found between obesity and other pro-inflammatory markers including erythrocyte sedimentation rate, interleukin 6, and tumor necrosis factor-α[33]. In contrast, health-promoting anti-inflammatory markers such as adiponectin have been found to be reduced in patients with metabolic syndrome, with these lower levels also linked to increased risk of atherosclerosis[33].

Strong clinical evidence further exists that obese patients with elevated inflammatory markers are at an increased risk of developing cardiovascular complications. A 2010 meta-analysis demonstrated that for every standard deviation increase in CRP, patients were associated with an 60% higher risk of developing metabolic complications[34]. That these markers increase with progressive severity of obesity and are ameliorated by interventions like bariatric surgery, further highlight the importance of incorporating strategies which mitigate systemic inflammation as novel targets for obesity intervention. The gut microbiome presents a remarkable opportunity with which to modulate this pathologic state of chronic systemic inflammation. In so doing, it can be harnessed to potentially reverse the associated risk of adverse metabolic complications. Rationale for this concept and mechanistic insight will be provided in greater detail below.

1.2.6 Obesity and the Gut Microbiome

Landmark work by Turnbaugh et al. in 2006 first implicated the gut microbiome in the development of obesity by demonstrating the existence of an obesity-associated microbiome[35] . Comparing the microbial metabolic potential of genetically obese mice to those of lean mice

revealed that an "obese microbiota" that harbored an increased ability to harvest energy from the diet. Furthermore, this observed obesogenic microbial phenotype was found to be surprisingly transmissible. Germ-free mice provided with "obese microbiota" from mice fed a Western diet were also found to demonstrate greater weight gain than those provided with "lean microbiota" despite a decrease in food consumption[25,35].

Convincing human studies exist which also support the link between obesity and the gut microbiome[25,30]. A recent metagenomic study of 154 monozygotic and dizygotic twins revealed that obesity was associated with reduced bacterial diversity, a reduction of *Bacteroidetes*, and an increase in *Actinobacteria* versus lean individuals[36]. Transfer of fecal content from human twins of different obesity phenotypes to germ-free mice demonstrated that the mice adopted their human donor phenotypes[37]. Additional studies also revealed that the imbalanced microbial ecology, or dysbiosis, seen with obesity and metabolic syndrome was restored with improvements in weight-loss achieved through lifestyle, pharmacologic, or surgical therapies[38]. Together, these studies implicate the human microbiome as an important contributor in the pathophysiology of obesity and metabolic syndrome[39,40].

Although still debated, specific bacterial phyla have been linked to obesity and metabolic syndrome[28,41,42]. In particular, phylum *Firmicutes* has been associated with obesity while *Bacteroidetes* has been linked to weight loss[43–47]. *Bacteroidetes sp.* have been demonstrated to enhance host nutrient absorption and be reduced in mice fed a Western diet[48]. This shift is thought to be a result of the inability of *Bacteroidetes sp.* to efficiently metabolize and promote absorption of lipids and carbohydrates. On the other hand, *Firmicutes* species are found to be

more abundant in those with a western diet possibly due to their increased capacity to degrade complex carbohydrates[49]. *Bifidobacterium* has also been shown to have a role in obesity, as several human studies have revealed an association between excess body weight and lower levels of *Bifidobacterium*[47,50]. Lastly, *Akkermansia muciniphilia*, a mucin-degrading bacterium, has also been associated with improved metabolic outcomes in both human and murine studies[51].

Exact mechanisms of how the gut microbiome may be responsible for developing or propagating obesity and metabolic syndrome have yet to be fully elucidated. A prevailing theory is that of an obesity-mediated gut dysbiosis which has been linked to an increase in nutrient absorption and storage. In addition, the microbial dysbiosis concept is thought to foster a state of chronic gastrointestinal inflammation which is associated with bacterial translocation and gut barrier dysfunction[44,46,52]. If this is true, reversal of dysbiosis may therefore have significant therapeutic implications by ameliorating the pathologic shifts in microbial ecology. These concepts and supporting evidence for microbial modulation will be discussed below.

1.2.6.1 The Storage Hypothesis

The storage hypothesis postulates that an obesity microbiome exists that is more capable absorbing dietary calories for the human host [25,38,53,54]. Germ-free mice lacking a gut microbiome have demonstrated a resistance to weight gain when supplied with a high-calorie obesogenic diet. As discussed previously, re-colonization of lean germ-free mice with microbiota from obese mice resulted in greater weight gain when compared to germ-free mice receiving gut microbiota from lean mice. These results in weight gain were independent of mice caloric intake suggesting a mechanism of increased total caloric absorption[55–57]. In murine studies, an "obese microbiota"

thus appears to facilitate excess caloric extraction and fat deposition independent of caloric intake.

1.2.6.2 The Metabolic Endotoxemia Hypothesis

Obesity is a chronic disease characterized by progressive low-grade gastrointestinal and systemic inflammation in addition to gut barrier dysfunction [58,59]. Individuals with obesity, for example, have been shown to have higher proportions of cytotoxic T cells, pro-inflammatory monocyte populations, and neutrophils primed to produce increased concentrations of free radicals[60]. Studies have recently provided evidence for the correlation between gut dysbiosis and an elevation in proinflammatory cytokines such as IL-6, and TNF-α in patients with obesity and metabolic syndrome[25,38,61]. One explanation for this detrimental pro-inflammatory cascade is believed to be associated with the translocation of gram-negative bacterial endotoxins.

Microbiota in obese individuals produce an increase in lipopolysaccharide (LPS), a pro-inflammatory endotoxin released upon degradation of gram-negative bacteria cell walls. LPS interacts with the human host immune system by binding to the toll-like receptor-4 (TLR-4) complex[28,38,62–64](Figure 1.2). Cani et al. were able to correlate LPS-mediated metabolic endotoxemia in mouse models by inducing obesity with a high fat diet[58]. Subcutaneous infusion of LPS in mice has also been shown to induce the metabolic abnormalities found with obesity. Moreover, LPS knockout mice displayed resistance to development of metabolic complications[65,66].

Anderson et al. were also able to demonstrate similar physiologic effects of LPS endotoxemia in human subjects[66]. LPS in obese patients was associated with elevated TNF and IL-6 concentrations and subsequent development of metabolic syndrome. Of note, metabolic endotoxemia was not present in patients receiving a diet rich in fruits and vegetables. The finding that chylomicrons, which are increased with a high fat diet, increase gastrointestinal LPS absorption provide further support for the endotoxemia hypothesis[67].

1.2.6.3 The Microbial Metabolite Hypothesis

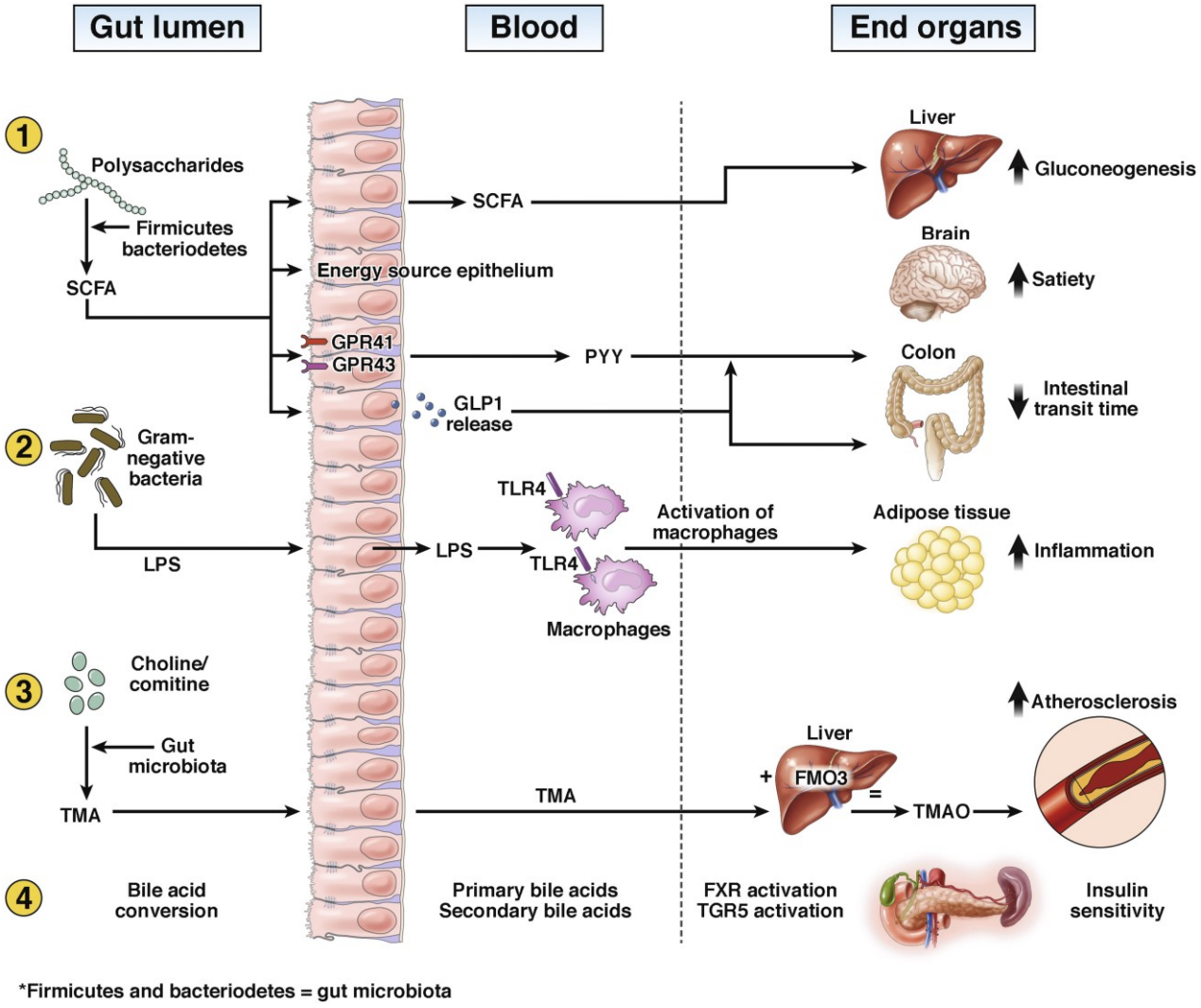

Figure 1.2 Implications of gut microbial metabolites on pathogenesis of obesity.

(Reprinted from Gastroenterology, Vol 152, Issue 7, Kristien E. Bouter, Daniël H. van Raalte, Albert K. Groen, Max Nieuwdorp. Role of the Gut Microbiome in the Pathogenesis of Obesity and Obesity-Related Metabolic Dysfunction, Pages 1671-1678, Copyright 2017, with permission from Elsevier to publish in enclosed PhD thesis both electronically and in-print, License Number 5043170209564)

A theory proposed by the author of this thesis which combines the totality of potential metabolic and immune effects of the gut microbiome on the human metabolome is the microbial metabolite hypothesis. This theory builds upon the prior two yet incorporates the more dynamic and complex function of the microbiome in postulating that a shift in microbial-derived metabolites is associated with the development and propagation of obesity.

The most well understood microbial metabolites are short-chain fatty acids[68]. SCFAs are primary end products of fermented carbohydrates which are not digested by our gut. They exist in a 3:1:1 proportion of acetate, propionate and butyrate and have a wide range of functions including antimicrobial activity, energy source for colonic epithelial cells, regulation of gut barrier integrity, and anti-inflammatory properties[69,70]. In addition, SCFAs also account for nearly 70% of the colonic epithelial cell energy requirements[71,72]. The mechanisms below, together with the fact that patients with obesity have decreased levels of SCFA-producing bacteria provide strong support for their role the pathogenesis of obesity.

An example of the complex immunologic role for SCFAs is their ability to regulate the differentiation, recruitment and activation of neutrophils, dendritic cells, and macrophages[73,74]. Through free fatty acid receptor (FFAR2) mediated mechanisms, SCFAs have also been shown to induce anti-inflammatory regulatory T-cells which reduce macrophage infiltration in white adipose tissue, preventing the development of insulin resistance[70]. Additional functions include regulation of tight junction proteins by increasing expression of claudin, zonula and occludin.

SCFAs are also potent regulators of the enteroendocrine system through activation of G-protein coupled receptors and inhibition of histone deacetylases[75]. FFAR2 receptors are highly expressed in enteroendocrine colonic and pancreatic beta-cells. Activation of these receptors has been linked with increased glucagon like peptide 1 (GLP-1), peptide YY (PYY), two anorexic neuropeptides[75,76].

In addition to LPS which was discussed in the inflammatory theory above, other important metabolites implicated in obesity and its metabolic complications are bile acids, trimethylamine N-oxide (TMAO), and BCAAs[69]. Secondary bile acids are deconjugated by our gut microbes and regulate lipid, glucose and bile acid metabolism through stimulation of farnesoid X receptor (FXR) as well as TGR5[77]. These receptors are highly expressed in enteroendocrine cells, hepatic tissue and adipose tissue where they have been shown to promote GLP-1 secretion, decrease gluconeogenesis, and improve insulin sensitivity. TMAO is an important microbe-dependent metabolite associated with atherosclerotic disease through activation of NF-kappa B pathways and implicated in the pathogenesis of systemic inflammation[78]. Lastly, BCAAs, which have been shown to be associated with an increased risk of developing diabetes, are also microbial-derived

metabolites which interfere with insulin signaling by stimulation of the mammalian target of rapamycin (mTOR) pathway[79]. Multi-omics studies have shown that patients with metabolic syndrome have microbial species with enriched BCAA synthesizing potential including *Prevotella copri* and *bacteroides vulgatus*[80].

Since nearly 10% of circulating metabolites are derived from our microbiome[81], it stands to reason that abnormal shifts in microbial populations responsible for the synthesis of these products would be implicated in the pathogenesis of obesity. Unfortunately, no modern medical therapies currently target the microbiome and its functional potential, a factor which may be responsible to their current limited efficacy.

1.2.7 Obesity Current Therapeutic Options

1.2.7.1 Lifestyle Intervention: Diet and Exercise

Lifestyle intervention is first offered to patients with the aim of modifying dietary intake and increasing physical activity. These interventions are not simply just unilateral recommendations provided by primary care physicians but involve high-intensity behavioral counselling requiring ongoing monthly assessments. Lifestyle intervention trials such as the Diabetes Prevention Program (DPP) have demonstrated successful reduction in incidence of diabetes for high risk individuals[82]. The DPP trial randomized 3234 patients with metabolic syndrome to placebo, metformin or a lifestyle modification therapy over a mean follow-up of 2.8 years. Lifestyle intervention was found to reduce incidence of diabetes by 58% versus 31% for metformin groups. These interventions, however, required significant resource utilization including

behavioral self-management training, individual case managers, and development of individual adherence strategies.

Recent research has examined a variety of dietary trends, hoping to find the optimal diet required to induce sustained weight loss[83]. These range from severe low-calorie diets to specific macronutrient restriction. Severe caloric restriction has been associated with more rapid weight loss, but also with a reduction in resting metabolic rate affecting minimizing long-term weight loss. Consensus recommendations now advise diets with a minimum of 800 kcal/day[84,85]. Dansinger et al. performed a randomized trial to compare a variety of diets including Atkins, Weight Watchers, and Zone diets and their impact on weight loss and heart disease reduction[86]. Each diet produced a modest reduction in body weight, and cardiac risk factors at one year. There was no statistical significance in weight loss between diet types. The greatest predictor of weight loss was importantly found to be dietary adherence and not the type of diet chosen. This suggests that the type of diet is not as important an individual's ability to consistently adhere to a particular diet plan.

Exercise therapy alone is not as successful at inducing weight loss in comparison to caloric restriction. Combination therapy appears to produce the most consistent results. Villareal et al. conducted a clinical trial demonstrating that aerobic and resistance exercise was superior to either treatment alone in improving functional status and weight loss for obese adults[87]. Given such promise, the AHA has recently increased their exercise recommendations for adults to include at least 150 minutes per week of moderate-intensity aerobic exercise while also encouraging at least moderate-intensity strength training at least two days per week. Modern

interdisciplinary programs also now incorporate fitness apps, and wearable activity tracking devices[88]. Studies assessing the benefit of these devices are promising, however have failed to demonstrate a sustained clinical benefit[89].

While effective, lifestyle modifications are resource intensive and are prone to weight recidivism for the majority of patients[90]. For this reason, pharmacotherapy adjuncts are typically started concurrently in obese patients at time of initial assessment.

1.2.7.2 Obesity and current Pharmacologic Therapy

In the United States, only five medications are approved for long-term weight management. Of those, only the major three medications used at Edmonton's Bariatric Weight Wise Clinic will be discussed in detail below[91]. Each medication should be trialed for 3 to 4 months and continuously reassessed for efficacy. Patients who do not demonstrate at least a 5% weight loss during this time are unlikely to receive long-term benefit.

1.2.7.2.1 Orlistat

Orlistat is a pancreatic and gastric lipase inhibitor that works to improve weight loss by inhibiting fat digestion. A systematic review and meta-analysis by Leblanc et al. performed from 2005 to 2010 identified that combination therapy of orlistat with lifestyle modification resulted in 3kg more weight loss at one year than placebo[13,14,21]. A recent randomized trial by Yancy et al. examined the effect low-carbohydrate diet versus orlistat plus a low-fat diet[92]. Findings revealed similar changes in weight, lipid and glycemic parameters suggesting that even patients on low fat diets receive benefit. Long-term therapy with orlistat also shows promise. Trials have

demonstrated decreased incidence of diabetes versus placebo with improved glycated hemoglobin A1C after 3 years of follow up[93]. Orlistat is relatively well tolerated, with common side effects including flatulence and oily stools.

1.2.7.2.2 Incretin Peptide Therapy: Liraglutide and Sitagliptin

Liraglutide and sitagliptin both act as GLP-1 agonist[14,92]. Liraglutide is a direct GLP-1 agonist, while sitagliptin is a DPP4 inhibitor that works by preventing GLP-1 degradation by the DPP4 enzyme. Indications for liraglutide use include all patients with metabolic syndrome that are not pregnant or have multiple endocrine neoplasia. In a randomized, double-blinded trial, liraglutide demonstrated significantly greater weight loss than placebo at 3 years in patients with type 2 diabetes [13,14,94]. Le Roux et al. not only demonstrated 4 kg weight reduction versus placebo, but also statistically significant reduction in Hgb A1c and improvement in quality of life. Another recent landmark randomized trial further evaluated the role of once-weekly liraglutide versus placebo on sustaining longterm weight loss[95]. In this trial, Wilding et al. demonstrated that liraglutide was associated with sustained weight loss at 68 weeks along with improvements in cardiometabolic comorbidities and patient-reported outcomes.

Although these medications are generally well tolerated, they are costly, associated with adverse gastrointestinal symptoms, and are not universally covered by healthcare plans[96].

1.2.7.2.3. Buproprion-Naltrexone

Combination therapy of buproprion and naltrexone is not first-line therapy for obesity or metabolic syndrome but has a role in patients with obesity who also desire to quit smoking.

Naltrexone is a dopamine and norepinephrine reuptake inhibitor while bupropion acts to reduce hunger and improve satiety. One-year outcomes have demonstrated up to a 5% weight reduction, but also a significant elevation in blood pressure when compared to placebo[97].

1.2.7.3 Obesity and Bariatric Surgery

Bariatric surgery is currently the most effective sustained long-term therapy for obesity and metabolic syndrome [98]. The number of bariatric procedures performed have increased dramatically over the past decade with laparoscopic sleeve gastrectomy (LSG), and Roux-en-Y gastric bypass (RYGB) being the two most commonly performed procedures worldwide. Indications for bariatric surgery include BMI >= 40 or more than 100 pounds overweight, BMI >=35 with at least one feature of metabolic syndrome, and inability to achieve a healthy weight despite appropriate efforts[99–101]. While significantly more effective than lifestyle or pharmacologic therapy, bariatric surgery comes with operative risks and long-term nutritional complications[102,103].

To date, RYGB is the bariatric procedure with the most proven long-term results. In an observational US study, mean weight loss at 12 years was 45 kg versus 2.9kg in patients who elected not to undergo surgery. Other studies have reported similar success, with five year sustained weight loss up to 70% of the excess body weight [104–106]. Rates of diabetes resolution are also unrivaled in contrast to other therapeutic interventions. The landmark Surgical Therapy and Medications Potentially Eradicate Diabetes Efficiently (STAMPEDE) trial demonstrated that 80% of diabetics were medication free at three years versus 0% of patients treated nonoperatively[107–109]. Furthermore, the incidence of new diabetes was approximately 90% lower

than in patients without surgery. In addition to improvements in weight and diabetes, RYGB patients also experience drastic improvement in dyslipidemia, OSA, and reflux.

1.2.8 Evidence and Future Directions for Obesity and Microbial Modulation

1.2.8.1 FMT and Obesity and Metabolic Syndrome

Since obesity and metabolic syndrome are associated with gut dysbiosis, resulting in a complex metabolic and immune dysregulation, it is therefore intuitive that the first step in microbiota therapy would be the direct modulation of dysbiosis via FMT.

The first landmark trial by Vrieze et al. provided great initial excitement and promise for this strategy[110]. Vrieze performed a randomized double-blind controlled trial of 18 patients with metabolic syndrome allocated to either autologous or allogenic transplant from lean donors. Recipients of lean donor transplants noticed marked improvement of insulin sensitivity, gut microbiota diversity, and a 2.5-fold increase in *Roseburia intestinalis*, a butyrate producing bacteria. In summary, Vrieze et al. provided the first high-quality evidence that direct intestinal microbiota modulation may be successfully utilized as a novel obesity and metabolic syndrome therapy.

The initial excitement for the therapeutic potential of FMT has since been quelled after subsequent trials have revealed conflicting results. A recent systematic review of current literature performed by our group identified a total of three randomized placebo-controlled trials where patients with obesity and metabolic syndrome received FMT[111]. Only two studies reported improvements in peripheral insulin sensitivity for patients receiving FMT at 6-weeks. No

differences in other metabolic parameters such as serum lipids, BMI, glycemia, or blood pressure were observed.

Significant limitations exist with current FMT study designs[112]. So far, only one group has been able to demonstrate metabolic benefit of FMT using solely European male patients with limited metabolic dysfunction and a nasojejunal route of FMT delivery[113]. Included patients also did not undergo concurrent medical therapy, limiting our ability to evaluate the real-world impact of FMT therapy. Further, FMT benefits appear to be short-lived due to loss of donor microbial engraftment after six weeks, adding further impracticality to adoption of FMT as a therapeutic intervention[112,114]. This suggests that utilization of complementary microbial modulation strategies which help to sustain donor microbes are warranted.

1.2.8.2 Prebiotics as an adjunct to FMT

The use of dietary fibers, or prebiotics, to selectively modulate the gut microbiota have been recently described in healthy individual, but evidence for their use in patients with obesity and metabolic syndrome is lacking[115]. In a trial of healthy subjects receiving either fermentable or non-fermentable fiber supplementation, Deehan et al. demonstrated that physicochemical properties of supplemented fibers were associated with precise dose-dependent microbial responses[116,117]. Crystalline maize resistant starch supplementation was associated with a bloom of *Eubacterium rectale*, a well-known butyrate producer. Meanwhile, patients receiving cross-linked tapioca resistant starch supplementation demonstrated an increase in *Parabacteroides distasonis*. Importantly, these microbial shifts were highly specific and associated with changes in both propionate and butyrate SCFA metabolites. In contrast, non-fermentable starches had no

effects on the microbiome or fecal SCFA concentrations. This work is just one trial which provides compelling evidence for the potential use of prebiotics for microbial modulation either solely or as a supplement to concurrent microbial strategies.

1.2.8.3 Rationale for FMT and fiber trial

If we hope to reach a point where fecal microbial transplantation and other novel personalized microbial biotherapeutics are incorporated into current clinical obesity management, a number of current limitations need to be addressed. Firstly, novel pragmatic and generalizable studies which incorporate North American patients, with an emphasis on gender equity, are required. These studies must also emphasize the concept of clinical equipoise, where concurrent medical therapy should not be withheld in order to allow us to evaluate the potential efficacy of FMT as an adjunct to modern intervention. Secondly, new safe and practical delivery methods of FMT need to be studied given the lack of practicality and potential life-threatening risks of nasojejunal delivery. Lastly, microbe-centered strategies like prebiotic supplementation which are aimed to sustain and enhance the efficacy of FMT by enhancing microbial engraftment are needed.

These concepts are the topic of Chapter 2, wherein the findings of this author's study, and the first landmark North American randomized placebo controlled clinical trial using oral-encapsulated FMT and fiber supplementation to modulate metabolic outcomes in patients with metabolic syndrome and severe obesity are presented.

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1.3 Inflammatory Bowel Disease and the Gut Microbiome

1.3.1 Epidemiology and Economic Costs of IBD

Inflammatory bowel disease (IBD) is a chronic idiopathic progressive inflammatory condition of the gastrointestinal tract and most often used to describe Crohn's disease (CD) and ulcerative colitis (UC)[1]. The prevalence and global burden of IBD have risen dramatically over the last several decades with nearly 7 million confirmed cases of IBD present worldwide as of 2017. A recent analysis on the impact of IBD in 195 countries and territories demonstrated a near doubling of years lived with IBD-related disability from 1990 to 2017[2]. In Canada, the rates of IBD are nearly 1% of the overall population, amongst the highest rates in the world[3]. Further, the economic impact of IBD in Canada alone approached nearly $3.0 billion in 2018 after accounting for both direct and indirect costs[4,5].

Despite the rising prevalence, morbidity, and economic costs of IBD, current modern therapeutic approaches – much like obesity- have shown a lack of long-term efficacy in addition to a number of other significant limitations[4,6]. For example, current strategies depend on non-selective systemic immunosuppression therapy which is associated with life-threatening complications like malignancy, hepatotoxicity and malignancy[7]. Surgical intervention, while curative for UC, only serves to stabilize CD with over 50% of CD patients requiring repeated surgical resection within 10 years[8]. New tailored therapeutic approaches for the management of IBD which are safe, cost effective, and have more favorable side effect profiles are thus required.

Following a similar approach to the obesity sections above, the subsequent IBD sections will discuss the following through the lens of the gut microbiome with an emphasis on gut microbial

modulation: history and definition of IBD, etiology of IBD, current IBD therapies and their limitations. Together, these will serve as a framework for the work discussed in Chapter 3 and Chapter 4.

1.3.2 IBD History and Definition

The term "ulcerative colitis" was first noted in a case report by Sir Samuel Wilks in 1859 where Sir Wilks described a clinical presentation very much in keeping with what we expect of modern UC today- a presentation of non-infectious bloody diarrhea, fevers, and pan-colonic inflammation leading to the death of a 42 year old female[9]. Crohn's disease was identified many years later in a landmark article by Burrill Crohn in 1932 which described a case series of 14 patients with what he termed was "regional ileitis"[10]. Although these diseases share similar medical therapeutic approaches and are both grouped under the umbrella term of inflammatory bowel disease, they are characterized by different endoscopic and histologic findings.

Significant overlap exists in the clinical presentation of CD and UC limiting the ability to diagnose each disease solely on clinical history or physical examination alone. Endoscopic findings demonstrating skip lesions and cobblestone mucosa are more in keeping with CD while continuous disease extending from the rectum proximally is a feature suggestive of UC. Histologic findings of CD reveal focal transmural granulomatous inflammation alongside crypt distortion. On gross examination, CD may further demonstrate "creeping fat" of the bowel mesentery. UC on the other hand, traditionally was thought to be associated with a distribution of inflammation primarily involving the submucosa or mucosa[11]. Recent guidelines now suggest

that no histologic feature is diagnostic of UC and that both clinical and histologic features need to be contextualized for accurate diagnosis[7].

1.3.3 The Gut Microbiome and IBD

The etiology of IBD remains elusive and is thought to be multifactorial, involving a complex interplay of host genetics, environmental factors, and nutrition [12,13]. The advent of affordable high-throughput microbial sequencing facilitating the microbial revolution has produced convincing evidence in both animal and human studies for the role of the gut microbiome in IBD[14–17].

Germ-free murine studies have shown that knockout mice which are genetically engineered to develop colitis are protected in the absence of gut microbes[18]. Further evidence supporting Koch's postulates for causality is that pro-inflammatory microbes obtained from colitis-induced mice and transferred into healthy animals subsequently initiate colitis[19]. Together, this evidence suggests that alterations in the gut microbiome are implicated in the aberrant mucosal response characteristic of IBD[20].

A number of elegant human studies have also provided convincing evidence which highlight the gut microbiome as a causal player in the etiology of IBD. For ethical reasons, fecal transplantation of dysbiotic communities to induce colitis in healthy subjects has not been conducted in human studies. However, antibiotic studies have demonstrated success in obtaining disease remission and preventing postoperative recurrence by eliminating purported pathobionts[21]. Further, the dysbiotic microbial ecology observed in IBD is improved with fecal

diversion (via ostomy) from the diseased bowel with the disease re-activating after intestinal continuity is restored[20,22,23]. Lastly, a large proportion of genetic polymorphisms associated with IBD have been found to involve mucosal barrier dysfunction[20,24] – the key interface regulating our host-microbiome relationship.

Together, this culmination of work has led to four prevailing theories linking the gut microbiome to the pathogenesis of IBD[25]: (a) an increase in mucosally adherent and invasive pathogenic bacteria, (b) microbial dysbiosis leading to a pro-inflammatory environment, (c) defective gut barrier integrity causing increased bacterial translocation, and (d) an exaggerated immunologic response to normal commensal bacteria (Figure 1.3). Research is ongoing to determine the implications of these theories and our ability to utilize them to design novel microbial therapies and improve IBD outcomes. Various iterations of these theories are thought to be dictated by a patient's predisposing dietary, environmental and genetic factors.

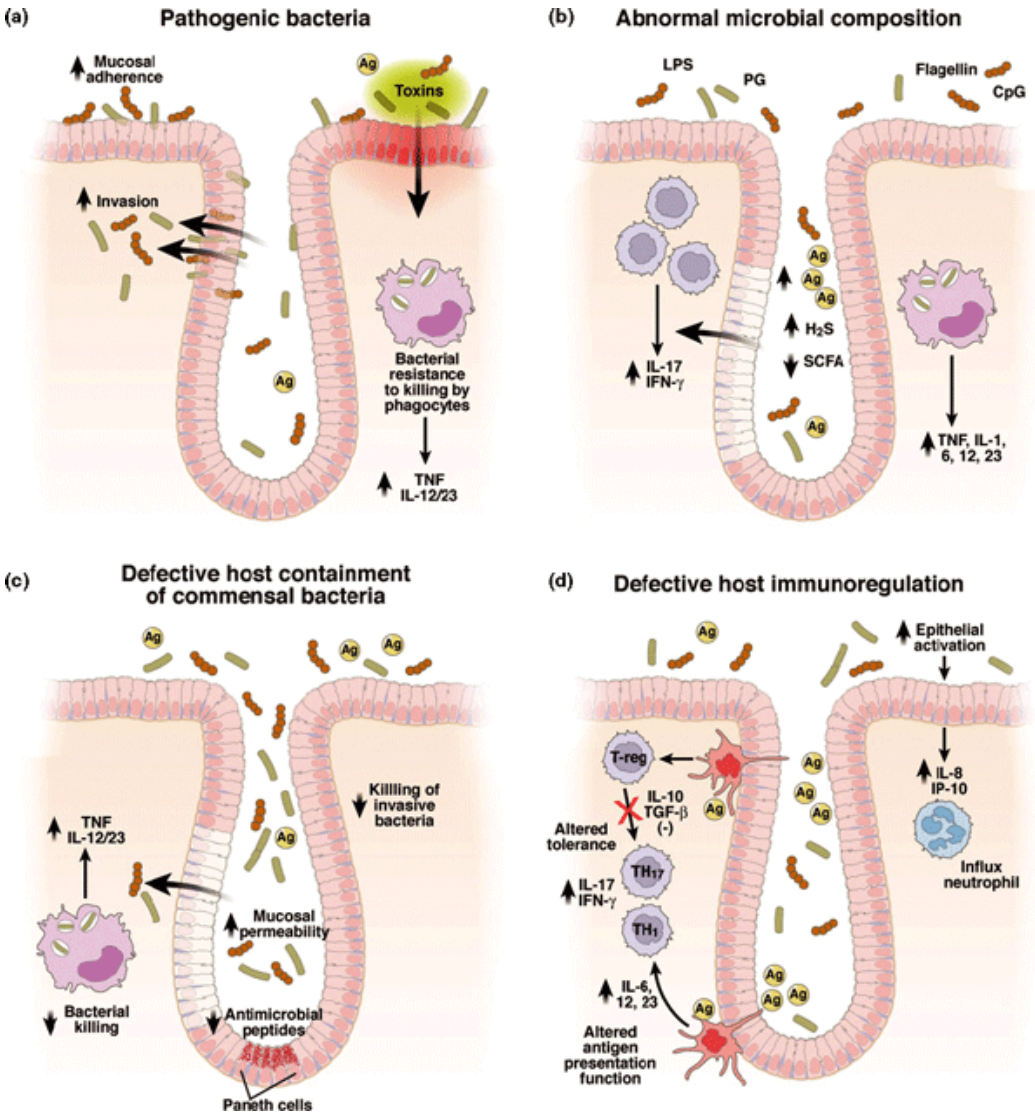

Figure 1.3 Summary of prevailing theories implicating the gut microbiome in the pathogenesis of IBD.

(Reprinted from Journal of Internal Medicine, Vol 263, Issue 6, Sartor R.B., and Packey C.D. Interplay of commensal and pathogenic bacteria, genetic mutations, and immunoregulatory defects in the pathogenesis of inflammatory bowel diseases, Pages 10,

Remarkably consistent differences in microbial ecology have been demonstrated when comparing the gut microbiome of IBD patients versus healthy controls[16,26–28], especially for Crohn's disease[29]. Much like the "obesity microbiota", the microbial signature of IBD is one of reduced bacterial alpha diversity, a reduction of anti-inflammatory anerobic bacteria, and a bloom of pro-inflammatory aerobic species[29–31]. Specific bacteria routinely found to be decreased in abundance in IBD patients include *Bacteroides, Bifidobacterium, Clostridium XIVa* and *IV,* and *Faecalibacterium prausnitzii*[32–34]. Notably, these bacteria are all well-known SCFA producers which provides support for current modulation strategies aimed at replenishing microbes capable of SCFA synthesis[27,35]. Taxa associated with pro-inflammatory host immune shifts are found to bloom in patients with IBD and include *Fusobacterium, Ruminococcus* along with the major phylum Proteobacteria[30,36].

Current work is underway to evaluate whether the reversal of these purportedly pathologic microbial shifts through tailored microbial modulation approaches can safely and effectively alter the course of IBD[37–39]. Until such real-world evidence exists, the management of IBD will continue to rely on use of costly therapies resulting in long-term immunosuppression and potential risk of fatal side effects.

1.3.4 IBD and current Pharmacologic Therapy

The management of IBD is complex and dictated by disease type, disease severity, medication safety profile and patient preferences. A brief general overview of the classes of medications and their limitations are provided below.

1.3.4.1 5-Aminosalicylic acid

Mesalazine or 5-Aminosalicyclic Acid (5-ASA) has been in use for management of IBD for over 50 years[40]. It's mechanism of action involves reducing the production of inflammatory prostaglandin and leukotriene metabolites, reducing leukocyte activation, and improving reactive oxygen species elimination[41]. Currently oral 5-ASA is the first line therapy for induction and maintenance of mild to moderate UC with its use supported by high quality Cochrane analyses[42]. In contrast, a Cochrane review of CD literature have shown that 5-ASA has no efficacy in either disease induction or maintenance in patients managed with medical therapy[43]. 5-ASA therapy is generally well tolerated but is associated with nephrotoxicity requiring periodic monitoring of renal function[44].

1.3.4.2 Corticosteroids

Corticosteroids come in topical or systemic oral formulation and are the next step in management of IBD[7]. A meta-analysis of randomized trials demonstrated that corticosteroids are indeed superior to placebo for inducing remission in patients with UC[45]. They are also the treatment of choice for the initial treatment of moderate to severe CD flares[46]. Corticosteroids have both anti-inflammatory and immunomodulating properties through inhibition of the pro-inflammatory arachidonic acid pathway, suppression of leukocyte migration, and stimulation of lymphocyte

apoptosis. Side effects of corticosteroids are seen in every organ system with nearly 50% of IBD patients experiencing at least short-term adverse events[47].

1.3.4.3 Thiopurines

Failure of 5-ASA and requirement of chronic corticosteroids necessitates evaluation for thiopurine therapy. Azathioprine, a thiopurine drug, has shown benefit when compared to placebo in patients with UC. A Cochrane review demonstrated a near 30% relative risk reduction for UC patients receiving Azathioprine versus placebo[48]. For CD, thiopurines have shown limited benefit for inducing remission[49]. However, they have shown a near two-fold improved odds of improved maintenance of remission, once successfully induced, when compared to placebo[50]. Like corticosteroids, adverse effects of thiopurines are many and include pancreatitis, hepatotoxicity, and myelosuppression[7].

1.3.4.4. Anti-TNF monoclonal antibodies

The advent of anti-TNF monoclonal antibodies has revolutionized the disease course of IBD in addition to many other chronic inflammatory conditions[51]. Through different mechanisms, these drugs work by blocking the host immune response to high levels of pro-inflammatory systemic TNF present in these conditions[52]. While numerous trials have demonstrated their efficacy in both UC[53] and CD[54], they are costly [55]and prolonged use of such systemic immunotherapy comes with severe side effects including infection and malignancy[7].

1.3.5 Evidence for Gut Microbial Modulation in IBD

1.3.5.1 FMT and IBD

In comparison to the fields of obesity and metabolic syndrome, evidence for the use of FMT in modulating IBD outcomes is much more established. A recent review by Paramsothy et al. which investigated the efficacy of FMT in management of IBD identified a total of 53 studies[56]. Using a limited pooled proportion analysis, the authors concluded that FMT appears most effective for UC remission with limited efficacy observed for CD. In contrast, another systematic review conducted by Caldeira et al. identified 36 studies of which only six could be used for meta-analysis. CD patients using this approach appeared to benefit more from FMT than those with UC[57].

It is evident that a signal towards clinical benefit for FMT has been demonstrated in current IBD literature[58]. Yet, substantial barriers preventing the adoption of FMT in routine IBD cases remain[59]. One example is the problem of extensive inter-study heterogeneity potentially stemming from variable engraftment of donor microbes or persistence of host pathobionts. Two approaches to address such barriers relating FMT engraftment have been increasingly utilized in clinical trials- antibiotic pretreatment[60] and increasing frequency of FMT delivery[61]. However, no systematic reviews have been conducted to evaluate their efficacy which limits the ability to optimize IBD outcomes in patients receiving FMT. Addressing this gap in knowledge is another focus of this author's work and is presented in Chapter 3 of the enclosed thesis.

1.3.5.2 Efficacy of Prebiotics and Probiotics in IBD

The promise of using prebiotics and probiotics to modulate host microbial communities and improve IBD outcomes has unfortunately been met with limited success. A systematic review and meta-analysis by Derwa et al. provides perhaps the best evidence regarding the limited clinical efficacy of probiotics[62]. Evaluation of 22 eligible randomized trials revealed no benefit in inducing remission or presenting relapse in CD. Only trials utilizing VSL#3 in patients with UC suggested a potential benefit for induction of remission. A similar appraisal of clinical trials investigating the clinical efficacy of prebiotics by Wedlake et al. demonstrated further demonstrated weak evidence for use in IBD[63]. There is therefore insufficient evidence at present for adoption of either prebiotic or probiotic therapies into routine clinical care.

1.3.6 The Microbiome and postoperative Crohn's disease recurrence

Particular interest has been drawn to the peri-operative microbiome of patients with Crohn's disease[64,65]. Unlike UC, which is cured by surgical resection, CD is a chronic relapsing disease in which affected patients often requiring multiple gastrointestinal surgeries during their lifetime. Studies by our group[66] and others[67,68] have demonstrated that the pre-operative microbial ecology is predictive of post-operative disease recurrence. This feature of the gut microbiome is of great interest as it may present both a predictive tool to identify patients at high-risk for disease recurrence and as a potential strategy for intervention.

In a recent prospective study by Hamilton et al., the fecal samples of 130 CD patients were obtained before surgery and compared at routine intervals until 18-months following surgical resection[69]. Microbial composition was then compared with endoscopic findings to evaluate for

specific associations between bacterial taxa and disease activity. The *Lachnospiracae* family was found to be enriched in patients with disease remission while *Enterobacteriaceae* was associated with a six-fold increased odds of recurrence. Additional work by Machiels et al. also demonstrated dramatic differences in the postoperative microbial recolonization between disease remission and recurrence using fecal samples from 121 CD patients who underwent ileocecal resection and were followed for six-months[70]. Patients with disease recurrence were found to have a higher abundance of *Fusobacteria* and *Negativicutes* while those in remission had a decrease in *Streptococcaceae* and *Actinomycineae*.

Challenges remain when attempting to draw conclusions from such studies given the high heterogeneity of CD patients. Additional factors like geographic differences may further influence the observed microbial changes through confounders like dietary intake or variations in clinical management. Regardless, it is clear that the peri-operative microbial shifts appear to significantly influence the disease course of CD. Given the wide variety of microbial shifts noted between current studies, a consensus target for specific bacterial strain level modulation remains elusive. Instead, current approaches aim to more broadly influence microbial shifts towards a bloom of anaerobic bacteria with increased capacity for anti-inflammatory SCFA synthesis.

An example of such an approach is provided by work from our group where the prebiotic Fructooligosaccharide (FOS) supplementation following murine ileocecal resection aimed to facilitate a bloom of postoperative *Bifidobacterium*[71]. Since *Bifidobacterium* are decreased in abundance in patients with IBD and are associated with the production of anti-inflammatory metabolites, it presented an optimal target for microbial modulation. Interestingly, although FOS

supplementation produced the desired increase in *Bifidobacterium*, FOS-supplemented post-operative mice instead experienced a worsening in inflammatory makers and reduced bacterial diversity.

Findings from this work highlighted the complex ecological challenges present when aiming to restore a number of bacterial communities using a supplement which may preferentially facilitate the bloom of only select bacterial taxa[72]. In clinical terms, the narrow-spectrum of activity from a single fiber supplement may thus not be sufficient to reverse the global ecologic microbial imbalances induced by IBD or the aerobic insult of gastrointestinal surgery. If this is true, a different strategy which instead aims to facilitate a more anaerobic environment fostering the broader recolonization of desirable SCFA anaerobic communities may prove promising. This concept is the focus of Chapter 4.

1.3.7 Linking IBD, Obesity and Microbial Modulation

Although IBD and obesity are traditionally thought of as two distinct diseases, it is clear that they both share many important similarities when considered through the lenses of chronic inflammation and microbial modulation presented above.

However, further promising links between these two diseases exist. Retrospective studies have shown that contrary to classical teaching, nearly 40% of patients with IBD also suffer from obesity[73]. This coexistence between both diseases is important since observational studies have suggested that obesity may adversely influence the natural course of IBD through presence of excess pro-inflammatory adipose tissue[74]. Obesity has also been shown to be a risk for poor

medical response to IBD therapy by adversely affecting biologic agent pharmacokinetics in addition to increasing the technical difficulty of IBD surgical intervention and increasing risks of post-operative complications[73,75]. Conversely, supplementation of diet-induced metabolic syndrome mice with anti-inflammatory 5-ASA IBD therapy has demonstrated improvements in gut inflammation and insulin resistance[76]. Taken together, these findings suggest a complex link between obesity and IBD which, while not yet entirely understood, presents a promising target for microbial modulation therapy and justification for the enclosed studies below.

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Chapter 2: Fiber fermentability differentially modulates responses to oral encapsulated fecal microbial transplantation in patients with metabolic syndrome and severe obesity: a randomized double-blinded placebo-controlled pilot trial

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Contributions

K.L.M conceived the project. K.L.M, J.W., E.C.D., D.H.K, N.H., and V.M. designed the proof-of-concept study. K.L.M, J.W. and E.C.D designed the fiber mixture. K.L.M. and D.H.K. supervised the clinical aspect of the study. D.H.K. supervised the FMT donor program. D.W.B and S.K. helped identify patients enrolled in the bariatric clinic for study inclusion. V.M. and K.K.S. recruited, coordinated, and conducted clinical visits. N.H. performed study randomization and created the fiber sachets. V.M. performed the analysis and interpretation of anthropometric, biochemical, immunologic, and enteroendocrine results. E.D.C. conducted the analysis and

interpretation of dietary data. Z.Z. performed the fecal microbiome sequencing and analysis. All authors discussed the study findings, interpretations and approved the final manuscript.

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2.0 Abstract

Fecal microbial transplantation (FMT) from lean donors to obese patients has been associated with promising yet inconsistent metabolic benefits. In this study we tested the application of daily fiber supplementation as an adjunct to FMT therapy aimed to modulate cardiometabolic markers. We performed a double-blinded randomized trial in patients with metabolic syndrome (MS) and severe obesity receiving oral FMT followed by 6-wk supplementation with either high-fermentability or low-fermentability fibers (NCT03477916). Primary outcomes were changes in insulin resistance (IR) from baseline to 6-wks evaluated using the homeostatic model assessment (HOMA2-IR). At six weeks, subjects receiving FMT and low-fermentability fiber demonstrated significant improvements in IR and insulinemia. Metabolic benefits were associated with increases in microbial richness and select bacterial taxa, including *Phascolarctobacterium, Christensellaceae, Bacteroides, and Akkermansia*. Interventions were safe and well tolerated with no attributed serious adverse events. In conclusion, we provide proof-of-concept for use of a single-dose oral FMT combined with daily low-fermentability fiber supplementation to attenuate IR in patients with MS and severe obesity.

2.1 Introduction

Obesity and metabolic syndrome (MS) comprise one of the greatest health epidemics of the twenty-first century[1–3] with current medical strategies having limited efficacy, poor tolerance, and high cost[4–8]. Continued lack of progress in curbing this epidemic has drawn growing interest into new approaches such as fecal microbial transplantation (FMT) which target the gut microbiome[9–14]. Recent landmark studies where MS patients received FMT from lean healthy donors have indeed demonstrated metabolic improvements, but effects have been inconsistent

and short-term, likely due to progressive loss of donor microbe engraftment[15–17]. While promising, only one group has demonstrated these benefits to date and solely in male subjects with mild metabolic dysfunction through invasive FMT delivery techniques[18–21].

In order to establish FMT as a pragmatic therapy for obesity and MS, novel strategies that enhance efficacy using non-invasive delivery methods in both male and female patients with a diverse spectrum of metabolic dysfunction are needed[22–26]. The concept of using dietary fiber supplementation to maintain healthy microbial communities introduced by FMT is one strategy that has been proposed, but no human trials have examined this concept[27–30]. The aim of our work was thus to evaluate orally encapsulated FMT with adjunctive fiber supplementation in a representative North American population of MS and severe obesity – the most rapidly escalating form of obesity worldwide[31].

Dietary fibers are non-digestible carbohydrates that exert a variety of health benefits dependent on their physicochemical properties[32]. High-fermentability fibers, for example, are readily fermented by select microbial strains and produce beneficial metabolites such as short chain fatty acids while low-fermentability fibers are largely resistant to gut microbial fermentation and can act through fecal bulking and modulating gastrointestinal transit[33]. Together, these diverse properties provide opportunities with which to influence engraftment of an FMT and microbiome assembly in the recipient to enhance metabolic benefits[34].

In this study we present the primary outcomes of a four-arm randomized, double-blind, placebo-controlled proof-of-concept safety and feasibility study where patients with severe obesity and

MS received either FMT or placebo followed by 6 weeks of daily fermentable or non-fermentable fiber supplementation (*Figure 2.1).* Participants were consecutively enrolled, block randomized and stratified by sex into one of four groups: (1) FMT and high-fermentability fiber (FMT-HF), (2) FMT and low-fermentability fiber (FMT-LF), (3) placebo FMT and high-fermentability fiber (HF), and (4) Placebo FMT and low-fermentability fiber (LF) (*Figure 2.2*). All patients underwent concurrent medical therapy but were asked to discontinue alternative prebiotic or probiotic therapies. Fermentable fiber supplementation consisted of an equal mixture of soluble corn fiber, type-IV resistant starch, and acacia gum while non-fermentable fiber supplementation consisted of microcrystalline cellulose at doses of 33g/day for males and 27g/day for females.

We hypothesized that orally administered FMT would alter recipient microbial ecology thereby improving insulin resistance and that dietary fiber supplementation would enhance or maintain these effects. Our primary outcome was to evaluate changes in insulin resistance and sensitivity between baseline (BL) and 6 weeks (T6) after treatment using the homeostatic model assessment (HOMA2-IR/IS). Secondary outcomes included evaluating safety and tolerability in addition to changes in anthropometrics, glycemic markers, lipid profile, quality of life measures, dietary intake, serum inflammatory markers, fecal short-chain fatty acids, and fecal microbiota composition.

2.2 Methods

2.2.1. Trial Design Overview

This pragmatic proof-of-concept feasibility pilot trial was approved by the University of

Alberta Health Research Ethics Board policies (Pro00076642), Health Canada (control #212903) and registered with clinicaltrials.gov (NCT03477916). Informed consent was obtained from all participants and safety monitoring was conducted independently by the University of Alberta Quality Management in Clinical Research Department (QMCR). The study complied with all relevant institutional and national regulatory body requirements.

The trial utilized an exploratory four-arm parallel double-blinded randomized placebo-controlled study design *(Figure 2.2).* All patients with obesity and metabolic syndrome who provided informed consent were consecutively enrolled through the Weight Wise Bariatric Clinic (Edmonton, Alberta, Canada) from July 2018 to October 2019 inclusive. Participants underwent a two-week screening and clinical baseline assessment prior to being randomized to one of four groups: (1) FMT and HF, (2) FMT and LF, (3) placebo FMT and HF, and (4) placebo FMT and LF.

Randomization was performed at a 1:1:1:1 ratio in blocks of 4 and stratified by sex via computer-generated codes using REDCap cloud (Version 1.6). The individual generating the random allocation sequence (NH) was different from staff who enrolled and assessed patients. Participants and all study personnel were blinded to study allocation for the duration of the trial. Interventions included a single FMT dose (or placebo) followed by a 6-week study period during which high-fermentability or low-fermentability fibers were added daily to the participant's normal diet. All patients were provided concurrent medical therapy by the bariatric team through the duration of the trial.

In total, participants attended 5 clinic appointments (baseline, FMT, 2, 6, and 12 weeks) for anthropometric measurements, vitals assessment, assessment questionnaires (dietary intake, hunger and satiety, quality of life), as well as blood and stool sample collection (*Figure 2.3*). Telephone interviews were conducted at 1 and 4 weeks to assess for adverse events and compliance monitoring. A two-hour 75g oral glucose tolerance test was performed at baseline and 6-weeks. Compensation was provided in the form of cash stipends ($10 CDN) at each in-person clinic appointment.

2.2.2. Bariatric Management

Edmonton's Weight Wise Bariatric Clinic is a diverse multidisciplinary clinic staffed with bariatricians, endocrinologists, psychologists, physiotherapists, and dieticians. Clinical teams were blinded to study allocation and all patients underwent concurrent bariatric therapy including medical pharmacotherapy, nutritional modification, and mental health counselling.

2.2.3. Study Population

Our inclusion criteria were: (1) age between 18 and 65 years; (2) BMI greater than 30 kg/m^2; (3) total body weight fluctuation over the last 6 months less than 10%; (4) a fasting plasma glucose > 5.6 mmol/L or a HgbA1c ≥5.5% or antidiabetic medication use; and (5) at least one of (a) fasting triglycerides ≥ 1.7 mmol/L, (b) HDL cholesterol <1.03 mmol/L in males or < 1.29mmol/L in females, (c) known diagnosis of hypertension or systolic blood pressure ≥ 130mmHg or diastolic blood pressure ≥85mmHg, (d) dyslipidemia medication use, or (e) antihypertension medication use.

Exclusion criteria were: systolic blood pressure ≥ 180mmHg or diastolic blood pressure ≥ 110 mmHg at screening, fasting triglycerides ≥6mmol/L, acute infection or inflammatory condition over the presiding 4 weeks, antibiotic use within the past 4 weeks, current or use of insulin within the last 6 months, prior weight loss surgery, dysphagia, inflammatory bowel disease, colon cancer, colonic polyps with high grade dysplasia, history of autoimmune or chronic inflammatory conditions (rheumatoid arthritis, chronic/active hepatitis B or C, HIV, pancreatitis, advanced non-alcoholic steatohepatitis, or liver cirrhosis), active malignancy, pregnancy, active substance or alcohol abuse (>2 8oz drinks/day).

2.2.4. Sample Size and Recruitment

Assuming a treatment allocation factor of 1:1 and accepting a type 1 error of 0.05 and a correlation between repeated measurements of 50%, a sample size of 15 subjects per arm gave the study a power of 80% to detect a change of 0.8 log [SD=1] in the logHOMA2-IR value, or 80% power to detect a minimal difference of 1.1 [SD 1.1] in HbA1c levels between groups. Accounting for an expected 10% drop-out rate, we enrolled a total of 68 subjects for this feasibility study. All randomized subjects who complete 6 weeks of follow-up were included for analysis.

2.2.5 Study Endpoints

The primary outcome was a change in insulin sensitivity between baseline and 6 weeks as estimated by the homeostatic model assessment of insulin resistance (HOMA2-IR) (*HOMA2 Calculator, University of Oxford*). Secondary outcomes included changes in vitals (blood pressure, heart rate), anthropometric parameters (BMI, hip and waist circumference), glycemic

parameters (fasting plasma glucose, glycated hemoglobin, serum insulin, GLP-1), fasting lipid profile (total cholesterol, HDL, LDL, triglycerides), quality of life assessed by EQ-5D-5L questionnaires, perceived satiety assessed by the Satiety Labeled Intensity Magnitude (SLIM) questionnaire, gastrointestinal tolerance assessed by a gastrointestinal tolerance questionnaire, dietary habits assessed by dietary history questionnaire III (DHQ3), gut hormones (leptin, ghrelin), serum inflammatory markers (CRP, TNF-∝, IL-6, IL-8, IL-10, LPS), fecal short chain fatty acids, and fecal microbiota composition between baseline, 6, and 12 weeks.

2.2.6 Study Interventions.

2.2.6.1 FMT manufacturing

Four lean healthy volunteer stool donors with no metabolic comorbidities provided stool for study participants. The limited number of donors did not allow for equal stratification of donors across all four groups (*Table 2.1*). Donor selection was done by availability and screening was in compliance with Health Canada regulations. Oral FMT capsules were manufactured as previously described[35]. Placebo FMT pills consisted of cellulose and were identical in appearance to ensure blinding of patients and study staff.

2.2.6.2 FMT administration

The day prior to FMT delivery, patients were instructed to undergo a 24-hr clear fluid fast and a bowel preparation involving two doses of Pico-Salax®, a routine colonoscopy preparation. On the day of transplantation, 20 FMT capsules (50g) from a one of four universal donor or 20 placebo capsules were administered orally. Patients were instructed to restart a solid diet one hour following the procedure as tolerated and to stop any other prebiotic or probiotic

supplementation until trial completion.

2.2.6.3 Fiber selection, manufacturing, and administration

Fibers were chosen by expert consensus based on criteria of metabolic efficacy, tolerability, and safety[36–40]. High-fermentability fiber supplementation consisted of an equal mixture by-weight of soluble corn fiber *(*PROMITOR®, Tate&Lyle, 114 Kcal / 100 g**)**, resistant wheat starch 4 (Fibersym®, MGP Ingredients, 35 Kcal / 100 g), and Acacia gum (Pre-Hydrated Gum Arabic**,** TIC GUMS, 17 Kcal / 100 g). In addition to the previous factors, these components were selected due to their ability to promote beneficial gut microbial and immune modulation (REF). Low-fermentability fiber supplementation consisted of microcrystalline cellulose (Microcel MC-12, Blanver Farmoquimica Ltd, 0 Kcal/100g).

Fibers were weighed and proportioned into 27g (females) or 33g (males) foil packets. The first three sachets (days 1-3) contained half the dose by weight to minimize gastrointestinal side effects. Three-week batches were provided on the day of FMT and at the time of the 2-week visit. Participants were instructed to start fiber supplementation the day following FMT and were given dietary instructions from registered dietitians to optimize adherence. All sachets and fibers were identical in appearance to ensure blinding.

2.2.7 Anthropometric and Serology collection

Anthropometric assessment was performed by trained personnel following the recommendations made by the CDC and published in the Anthropometry procedures Manual of the National Health and Nutrition Examination Survey (available at www.cdc.gov). Body weight was

measured using a validated, calibrated bariatric scale (Scale Tronix®) and recorded to the nearest 0.1 kg. Height was measured to the nearest 0.1 cm using a wall-mounted stadiometer. Waist and hip circumferences were measured following the recommendations of the Center for Disease Control (CDC) and Prevention Anthropometry Procedures Manual [41] and the National Institutes of Health (NIH) guidelines[42].

Blood samples were collected after an overnight fast (>8hr) with aliquots of plasma and serum snap frozen in liquid nitrogen and stored at -80C. Routine laboratory investigations were completed using standardized laboratory techniques and included: Cell blood count and differential, electrolyte panel (sodium, potassium, chloride, and carbon dioxide), renal panel (creatinine, blood urea nitrogen), liver panel (aspartate transaminase, alanine transaminase, alkaline phosphatase, albumin, international normalized ratio for pro-thrombin time), glycemic panel (glycated Hemoglobin HbA_1C, blood glucose, insulin), lipid panel (total cholesterol, HDL, LDL, and triglycerides), and C-Reactive Protein.

2.2.8 Enzyme-linked immunosorbent Assay (ELISA) analysis

All assays were conducted in singlet following manufacturer protocol and storage recommendations. Immunologic evaluation included IL-1β (R&D Systems DuoSet ELISA, catalog DY201-05), IL-6 (R&D Systems DuoSet ELISA, catalog DY206-05), IL-10 (R&D Systems DuoSet ELISA, catalog DY217B-05), TNFα (R&D Systems DuoSet ELISA, catalog DY210-05), and LPS (Abbexa Endotoxin (ET) ELISA Kit, catalog ABX514093). Glycemic markers were assessed using insulin (R&D Systems DuoSet ELISA, catalog DY8056-05) assays using a two-fold serum dilution. Enteroendocrine markers were evaluated using total GLP-1

(EMD Millipore Corporation, catalog EZGGLP1T-36K). Hormones influencing hunger and satiety were conducted using ghrelin (R&D Systems DuoSet ELISA, DY8149-05) and leptin (R&D Systems DuoSet ELISA, DY398-05) assays using a 20-fold serum dilution.

2.2.9 Stool collection, Short-Chain Fatty Acid, and Microbiome analysis

Participants were provided stool collection kits and instructed to collect stool samples within two days of their scheduled visits. Written instructions were provided to preserve at least 30 grams of stool, store at 4C prior to transport, and to deliver the sample on ice. On arrival, samples were subsequently aliquoted, and flash frozen at -80C for storage.

Stool short-chain fatty acid concentrations were analyzed using gas chromatography at the Agricultural, Food and Nutritional Science chromatography core facility as previously described[43]. Briefly, 800µL of 0.1N hydrochloric acid and 200µL of 25% phosphoric acid were added to approximately 0.2g of stool. The contents were vortexed until fully homogenized and centrifuged at 5000g for 15 minutes or until the obtaining a clear supernatant. An internal standard solution (150mg of 4-methyl-valeric acid, S381810, Sigma-Aldrich), 5% phosphoric acid, and supernatant were then added to glass chromatography tubes and stored at -80C prior to analysis. Samples were analyzed with a gas chromatograph (Bruker SCION 456-GC, Bruker Corporation, Billerica, MA, USA) using a 30m x 0.53 mm inner diameter x 0.5 µm film thickness capillary column (Stabilwax-DA, Restek Corporation, Belefonte, PA, USA).

Fecal DNA extraction for microbiome analysis was conducted using a modified MultiTarget

Pharmaceuticals protocol. Bleached beads were added to tubes in combination with 200µL of AquaStool (MultiTarget Pharmaceuticals, USA), approximately 100mg of thawed stool, and homogenized. The resultant homogenate was centrifuged (14000g for 5 mins) followed by addition of 100µL of AquaRemove (MultiTarget Pharmaceuticals, USA). After re-centrifugation, the supernatant was collected, and isopropanol was added prior to precipitation on ice for 10 min. The DNA pellet was collected and washed three times with 70% ethanol. A total of 100µL of EB Buffer (Qiagen, USA) was then added to solubilize the DNA followed by 1µL of RNASE A (Qiagen, catalog 1007885). The mixture was incubated at 37C for 1 hour and then recentrifuged. DNA precipitation was obtained with 10µL of 5M NaCl, 100µL of ice cold 100% ethanol, and a 30-minute incubation at -20C. The mixture was recentrifuged and the pellet was rinsed three times with 70% ethanol. An additional 50µL of EB buffer were added after removal of excess ethanol, and the solution was left overnight at 4C for solubilization.

After ensuring appropriate extraction quality using a Nanodrop 1000 Series device (Thermo Fisher Scientific, USA), samples were sent for 16s rRNA gene amplicon sequencing (Microbiome Insights, Canada). Microbial composition was characterized by 16S rRNA gene amplicon sequencing of the v4 region using MiSeq Illumina technology (2x300bp) and the following primers: 515F 'GTGCCAGCMGCCGCGGTA' and 806R 'GGACTACHVGGGTWTCTAAT'. QIIME2 and DADA2 were used to perform quality control and feature table construction [44]. Taxonomic assignment (from kingdom to genus level) of the representative sequences of each sample were performed using the Silva 132 pre-trained Naive Bayes classifier and the q2-feature-classifier plugin in the QIIME2 pipeline.

2.2.10 Statistical Analysis

2.2.10.1 General Analyses

A modified-intent-to-treat analysis was performed to assess the effect of intervention from baseline (T0) to 6-weeks (T6). Continuous variables were reported as means ± standard deviations if normally distributed or medians and interquartile ranges if non-normally distributed. Normality was evaluated using Shapiro-Wilk tests. Categorical data were reported as proportions and analyzed using the Cochran-Mantel-Haenszel[2] test. Figures reported data as mean ± SEM. unless otherwise stated. Percentage change in variables was calculated by dividing the difference between T6 and baseline by the baseline value. The reported mean difference of primary outcomes was calculated using the difference in percentage change between interventions. Within-group paired changes between baseline and T6 were conducted using two-tailed Wilcoxon Signed-rank test while between-group comparisons were conducted using the Mann-Whitney U test. Where more than two measures were compared over time, repeated measure linear regression were applied to compare within-group differences relative to baseline. Data not normally distributed were transformed using cube root method before analysis by the linear regression model. Data greater than five standard deviations from the mean were removed as outliers.

A non-parsimonious logistic regression model was developed using a prospective hypothesis driven methodologic approach to identify factors which independently predict changes in our primary outcomes. HOMA2-IR and HOMA2-IS outcomes were dichotomized into responder and non-responder groups. Responders were defined by improved HOMA2 percent change

parameters while non-responders were defined by worsened percent change in HOMA2 markers. Variables which demonstrate a significance of $p<0.1$ were included in the main effects models unless otherwise deemed clinically relevant. Analysis for clinical, biochemical, and immunologic data was be conducted using STATA 15 (StataCorp 2017; College Station, TX) and figures were designed using Prism 9.0.0 (GraphPad Software, San Diego, CA, USA). Statistical significance was defined using two-tailed tests with a p value < 0.05.

2.2.10.2 Microbiome Bioinformatics and Statistical Analyses

Alpha diversity was calculated by Chao1 and Shannon indices using QIIME2. To compare microbial beta-diversity between samples, Bray-Curtis distance matrices were measured using the 'vegan' package in R[45], and Unifrac distance matrices were measured using QIIME2. Principal coordinate analysis (PCoA) was applied on the resulting distance matrices to generate two-dimensional plots using the 'ape' R package [46]. Significant differences of beta-diversity via permutational multivariate analysis of variance (PERMANOVA, n = 999) using the 'Adonis' function in 'vegan'. For the microbiota compositional features (alpha diversity index and centered log-ratio-transformed bacterial taxa counts, repeated measures linear regression and paired t-tests were applied to compare within-group differences relative to baseline. Between-group differences were assessed by linear regression, and pairwise comparisons (FMT-HF vs. HF; FMT-LF vs. LF) were used unpaired t-tests. P-values were adjusted by Benjamin-Hochberg false discovery rate (FDR) method. The microbiome analysis was conducted using R (Version 3.5.1).

2.3 Results

2.3.1 Overview of Trial Enrollment

From May 7, 2018 to Oct 9, 2019, 5200 patients were electronically screened, 780 were assessed for eligibility, 70 were randomized, and 68 received study intervention (*Figure 2.1*). Randomization and study enrollment continued until 68 patients received study intervention. A total of 61 patients completed their T6 visit and were analyzed using a modified intent-to-treat protocol. Seven patients withdrew before the primary outcome (FMT-HF = 2; FMT-LF = 3; HF = 2) with three patients citing time commitment concerns, two requiring prolonged antibiotic courses (leg cellulitis = 1, prostatitis =1), and two offered early surgery.

2.3.2 Characteristics of Study Population

Basic characteristics of study participants revealed a mean age of 47.8 ± 10.0 years, a mean body mass index of 45.3 ± 7.0 kg/m^2, with a female sex predominance (83.6%) (*Table 2.2)*. The majority of patients received novel GLP-1 (n = 39, 63.9%) and SGLT2 (n = 3, 4.9%) antihyperglycemic medications while a minority received conventional metformin (n = 15, 24.6%) therapy. No differences in basic characteristics, degree of metabolic dysfunction, medications, or dietary intake were observed between groups at baseline.

2.3.3 FMT and low-fermentability fiber associated with improved HOMA2 and insulinemia

After six weeks, participants receiving FMT with low-fermentability fiber supplementation demonstrated improvements in the mean difference (MD) for HOMA2-IR (MD -24.0% ± 12.0%; *p*=0.02), HOMA2-IS (MD 27. 6% ± 12.3%; *p*=0.02), and insulinemia (MD -25.4% ± 12.3%; *p*=0.02) (Figure 2.4 a-c). There was no difference in FMT donor allocation between FMT-HF

and FMT-LF groups *(Table 2.1)*. No differences in fasting glycemia or glycated hemoglobin were seen either within or between interventions. These findings occurred in the absence of any significant changes in anthropometric values (*Figure 2.*), lipid parameters (*Figure 2.6*), or dietary intake (*Table 2.3)*. Follow-up at 12 weeks revealed that these beneficial effects were not maintained in the absence of daily fiber intake.

2.3.4 HOMA2 benefits observed after adjusting for clinical covariates

We then examined whether our findings could have been confounded by factors unrelated to our study intervention including differences in basic characteristics, medication regimen, or degree of metabolic dysfunction. Two multivariable logistic regression models were developed to evaluate for changes between baseline and T6 for insulin resistance (IR) and insulin sensitivity (IS) (Table 2.4). After adjusting for these clinical covariates, the greatest independent predictor of improvements in both IR and IS was allocation to FMT-LF intervention (p=0.003). Interestingly, baseline hyperinsulinemia was also an independent predictor of improved IR and IS.

2.3.5 Patient Reported Outcomes, Intervention Safety and Tolerability

Patient reported outcomes including quality of life, hunger and satiety, and gastrointestinal symptoms (flatulence, bloating, stool consistency, stool frequency) did not differ between groups (*Figures 2.7-2.9*). FMT and fiber tolerability were excellent with only one patient per group unable to tolerate fiber therapy and two patients reporting emesis following placebo FMT *(Table 2.5)*. There were no serious adverse events attributed to any study intervention.

2.3.6 Improvements in glycemic parameters were independent of changes in inflammatory markers

As systemic inflammation has been linked with altered gut barrier function and the development of insulin resistance[47,48], we measured serum markers of inflammation and endotoxemia (*Table 2.6*). While subjects receiving high-fermentability fiber showed reduced levels of TNFα and IL-8 at T6, these changes did not translate to observed benefits in glycemic outcomes. Circulating plasma LPS which is an established marker of gut barrier integrity and associated with insulin resistance was also not altered by treatment in any of the groups. Notably short-chain fatty acids (SCFAs) hypothesized to improve gut barrier function and insulin resistance were not influenced by either FMT or fiber intervention (*Table 2.7)*.

2.3.7 FMT-LF was associated with improved oral glucose tolerance test

Blunted enteroendocrine responses to glycemia have long been implicated in the pathophysiology of insulin resistance in patients with obesity and metabolic syndrome[49]. To elucidate potential mechanisms through which FMT-LF attenuated insulin resistance, we compared serum GLP-1 and insulin responses to oral glucose tolerance tests at baseline and T6 (*Figure 2.10*). After 6 weeks, FMT-LF was associated with restoration of physiologic patterns of GLP-1 secretion as evidence by significant increases between 0-, 1- hours and decreases between 1- and 2- hours ($p < 0.05$). Peak 1-hour serum GLP-1 levels at T6 also significantly increased compared to FMT-HF ($p=0.04$) and approached significance in the LF group ($p=0.08$). Marked reductions in peak insulinemia were also demonstrated at 1- hour (BL to T6, $p < 0.05$) in all patients receiving non-fermentable fiber therapy with these effects notably more pronounced in the FMT-LF group ($p=0.052$).

2.3.8 FMT-LF was associated with altered microbial ecology

We then evaluated the extent to which gut microbiota correlated with our observed metabolic improvements. α-diversity analysis revealed that FMT-LF was associated with increases in bacterial richness (Chao1 index) from BL to T6 (*Figure 2.11a*, $q<0.01$). There were no differences in evenness (Shannon index) from BL to T6. At T6, β-diversity for both FMT groups demonstrated significant shifts in microbiota composition (*Figure 2.11b*, $p<0.02$) with the FMT-LF group showing a greater degree of compositional change (*Figure 2.11b*). When focusing on specific taxa, FMT-LF intervention led to changes in seven genera and twelve amplicon sequence variants (ASVs), several of which were detectable at T6 including increases in relative amounts of *Phascolarcobacterium, Christensenellaceae, Bacteroides,* and *Akkermansia muciniphilia* and decreases in *Dialister* and *Ruminococcus torques* (*Figure 2.11c)*.

2.3.9 Select baseline taxa independently predicted improvements in HOMA2

To assess whether the abundance of particular bacterial taxa at baseline was able to independently predict improvements in HOMA2-IR and insulin sensitivity in patients receiving FMT-LF after adjusting for baseline characteristics, we conducted a repeated linear mixed model. This revealed that *Phascolarctobacterium, Bacteroides stercoris, and Bacteroides caccae (Figure 2.11d)* demonstrated significant associations. Since these taxa have been associated with insulinemic and enteroendocrine benefits in MS and obesity [50–52], they may represent an adaptive host microbial response particularly responsive to microbial biotherapeutic intervention and be used as treatment.

2.3.10 Fiber fermentability differentially modulates FMT engraftment

Lastly, we conducted a *post hoc* analysis to evaluate if donors or fibers differentially modulated FMT engraftment. Donor oral capsules retained the microbial composition of the corresponding stool samples (*Figures 2.12 -2.13)* and differed from recipients at BL and T6 (*Figure 2.13)*. FMT-LF was the only group in which we observed significant increases in bacterial richness as well as shifts in microbial composition more closely resembling donor ecology over time (*Figures 2.15-2.16, $q < 0.05$*). Engraftment of specific taxa (*Figure 2.15f*) in the FMT-LF group was also donor-meditated with donor 28 engrafting the highest number of unique ASVs including the primary fiber degrader (ASV50 *Bifidobacterium breve/longum*), SCFA producing members (ASV79 *Phascolarctobacterium succinatutens*, ASV144 *Odoribacter splanchnicus*[53,54], and the taxa with modulatory characteristic in the suppression of tumor growth (ASV93 *Alistipes shahii*[55]. However, no significant associations between HOMA2 response and specific donor-recipient combinations were found *(Figure 2.17)*.

2.4 Discussion

Taken together, we show that patients receiving low-fermentability fiber supplementation following FMT had improved glycemic markers from baseline to 6-weeks. The fact that these changes were independent of factors like diet or medications but were associated with improved enteroendocrine responses, altered microbial ecology, and increased engraftment of donor microbes provides further support for gut microbial modulation as a promising strategy to ameliorate metabolic dysfunction.

Our understanding of the efficacy of FMTs in pathologies other than *Clostridioides difficile* infections is still very much in its infancy. However, there is increasing evidence that targeting

the microbiome can impact metabolic health. Regarding our study, key questions remain as to the underlying mechanisms whereby patients receiving low-fermentability microcrystalline cellulose fiber supplementation, after FMT therapy demonstrated benefits to insulin resistance.

Possible explanations include the ability of cellulose to act as a bulking and binding agent which could alter metabolite luminal concentrations, influence gastrointestinal transit and modulate the donor microbial-host mucous layer interface. Cellulose supplementation may also directly alter the function of specific taxa including cellulose-degrading H2-producing methanogens leading to changes in gut microbial fermentation efficiency and by-products [56,57]. Together these factors may constitute mechanisms through which FMT-LF increased microbial diversity and richness while also potentially inducing functional changes in taxa associated with host HOMA2-IR/IS improvements.

Limitations of our study are in keeping with the nature of our proof-of-concept study design. Given our relatively small sample size, we were not sufficiently powered to evaluate for small differences across the full spectrum of metabolic outcomes or for other plausible mechanisms associated with the two fiber therapies. The study was also only powered to evaluate for changes occurring from BL to T6, limiting our ability to identify why the effects were not maintained following fiber cessation. As we did not conduct any 'omics' analysis, the complex functional implications of our microbial modulation could not be fully evaluated. Hence, caution should be taken while interpreting the results from the inferential analysis. It is also possible that factors such as medications, diet and exercise could have confounded our findings. All patients, however, were managed by the same clinical care team which provided standardized nutritional,

exercise, and psychosocial guidance thereby minimizing the potential confounding effects of these variables. Lastly, due to scarcity, donors could not be stratified across groups. Nevertheless, the proportion of donors did not differ significantly between groups arguing against a super-donor phenomenon which may have confounded our results.

2.5 Conclusion

In conclusion, this proof-of-concept pilot trial provides novel evidence that (1) a single-dosed oral FMT can be safely and feasibly engrafted in patients with metabolic syndrome and severe obesity and that (2) daily low-fermentability fiber supplementation can attenuate insulin resistance following FMT by differentially modulating engraftment of select bacterial taxa and the enteroendocrine axis. Results from our trial will serve as a basis for the ongoing development of novel microbial biotherapeutic strategies aimed at combatting the growing MS and obesity epidemics through the future delivery of safe, effective, and affordable designer bacterial consortia.

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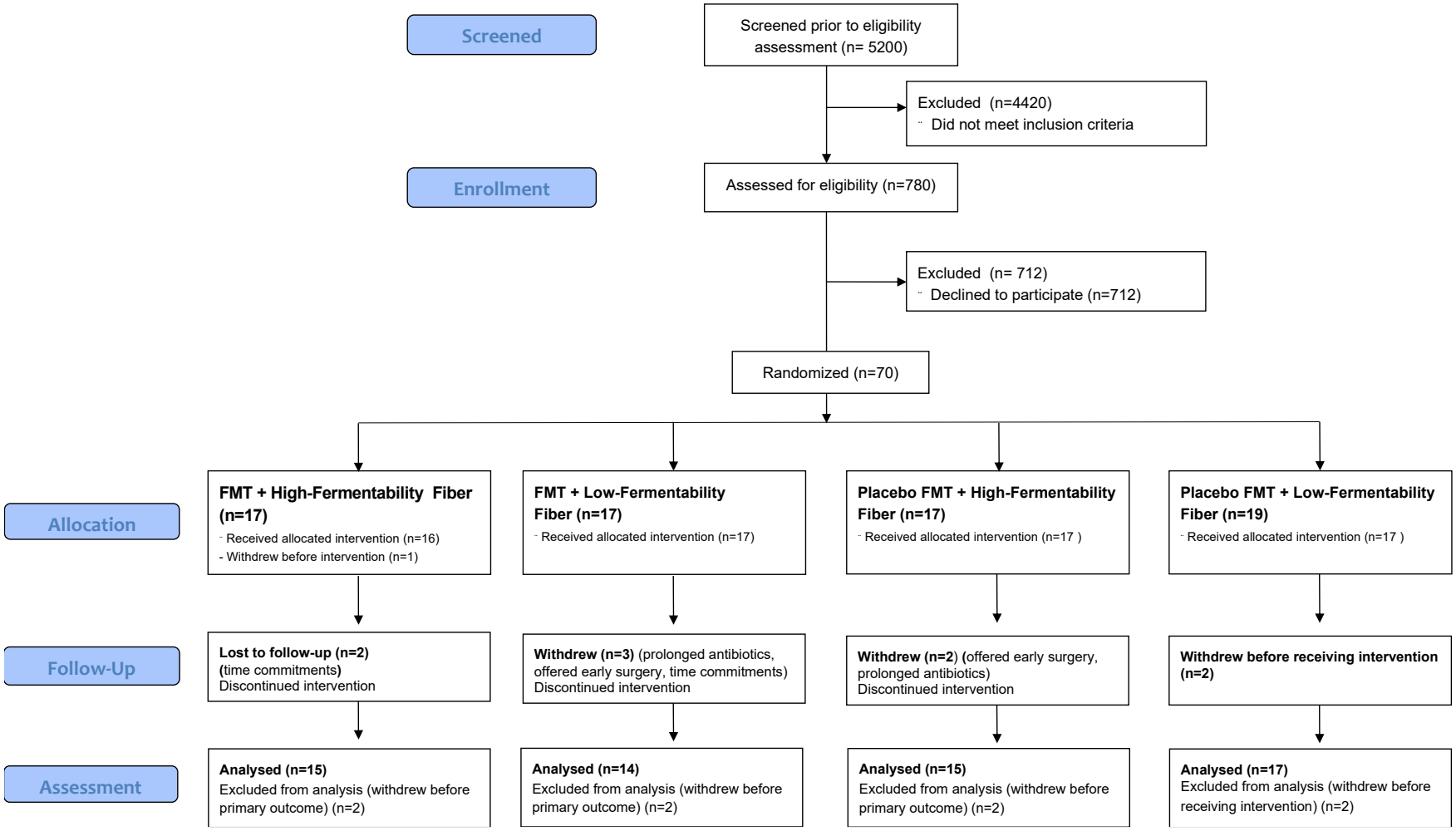
Figure 2.1. Study Consort diagram.



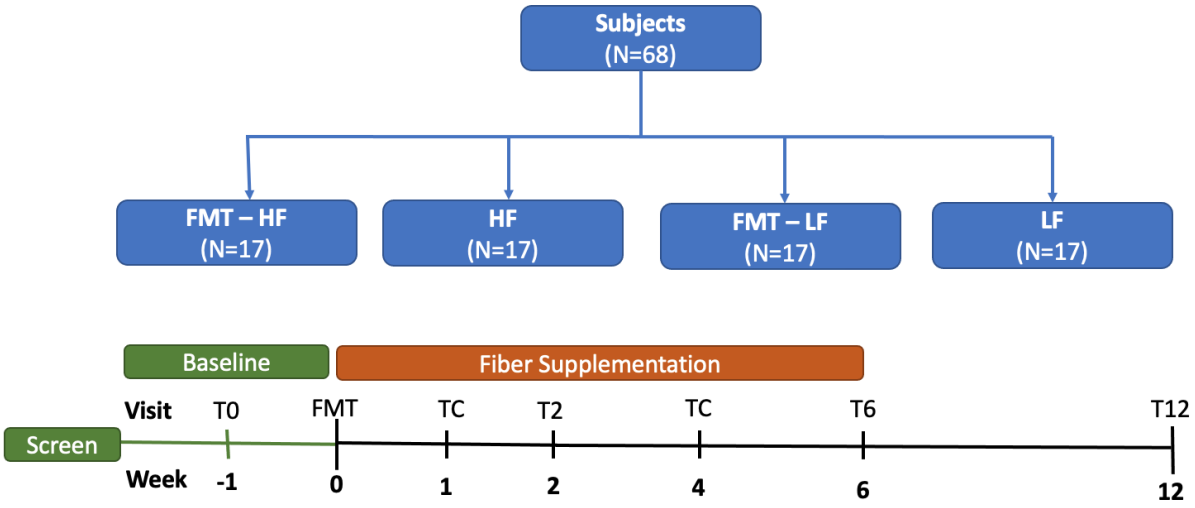


Figure 2.2. Overview of Study Design by week and study visit. FMT-HF, FMT and high-fermentability fiber group. **HF**, high-fermentability fiber group. **FMT-LF**, FMT and low-fermentability fiber group. **LF**, low-fermentability fiber group.

	Visit	S1	S2	BL	1 (TC)	T2	4 (TC)	T6	T12
Interview and assessment									
	Interview	X	X	X	X	X	X	X	X
	Anthropometric assessment	X	X	X				X	X
	Vital signs (HR, BP)	X	X	X		X		X	X
	Consent	X	X						
Specimen sampling and Laboratory work	TSH		X*						
	CBCd	X*	X						
	Renal Function (Cr, BUN)	X*	X						
	Electrolytes	X*	X						
	LFTs: (AST, ALT, ALP, Albumin, Bilirubin, INR)	X*	X						
	Hs-CRP		X			X		X	
	Glucose homeostasis (FPG, HbA1c, Serum insulin).	X*	X			X		X	X
	Fasting lipids profile.	X*	X			X		X	X
	Infectious markers: HIV, HVB, HVA, HVC.	X*	X						
	Adipocytokines: Leptin, Ghrelin, GLP-1, TNFα, IL-6, IL-1β, IL-10, IL-8		X			X		X	X
	Intestinal Barrier: Plasma level of LPS,		X			X		X	X
	Stool Sampling: SCFA, 16S rRNA.		X			X		X	X
Investigations	75g OGTT		X					X	
	Hunger and satiety questionnaire		X		X	X	X	X	X
	Gastrointestinal Tolerance		X		X	X	X	X	X
	EQ-ED5		X		X	X	X	X	X
	Dietary Intake (24-h recalls)		X		X	X	X	X	
	Diet History Questionnaire (DHQ)		X						
	FMT			X					

Figure 2.3. General schedule for assessment and intervention.
X: Sample collection, S1- Screening visit 1, S2- Screening visit 2, BL- baseline visit, TC – telephone call, T2/6/12- Weeks 2/6/12.
***** Not re-drawn if previous result available within 3 months

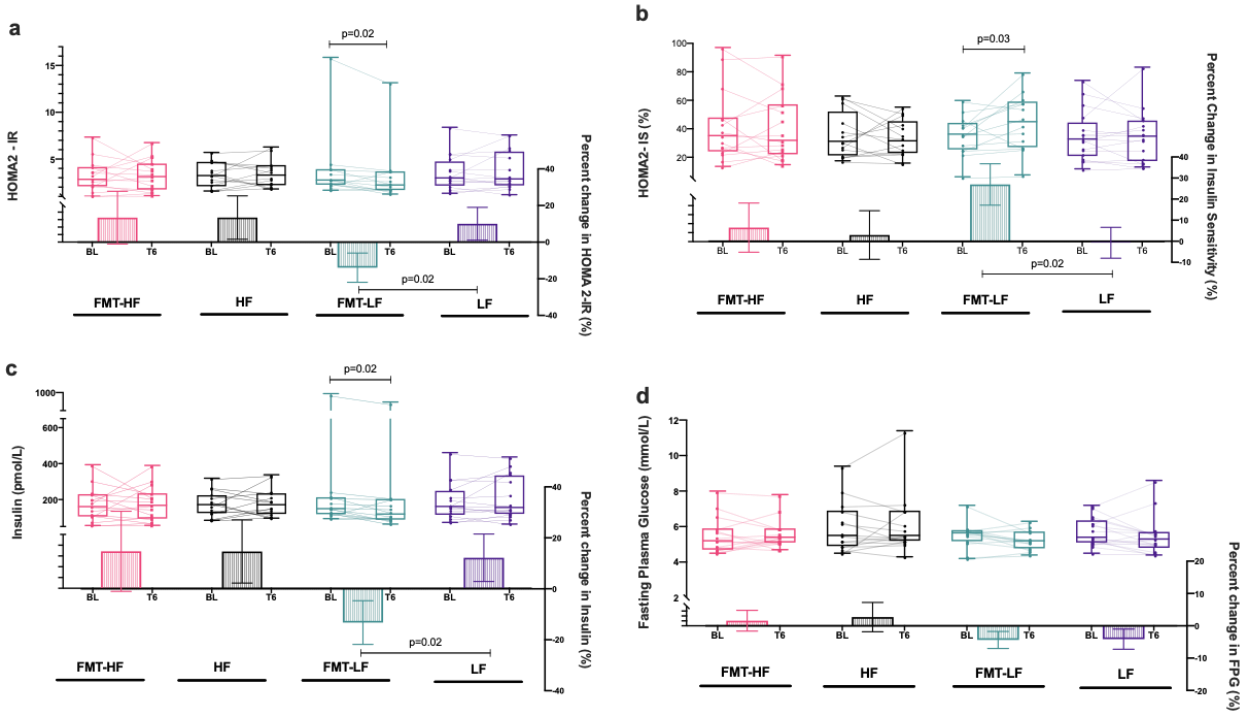


Figure 2.4. Changes in glycemic parameters across intervention groups. a, HOMA2-IR. **b**, HOMA2-IS. **c**, Serum insulin. **d**, Fasting Plasma Glucose (FPG). Values are reported as mean ± SEM or as percent change from BL to T6. Lines represent raw unadjusted values at baseline and week 6 following FMT and/or fiber supplementation. Box-and-whisker plots represent the distribution of each group from baseline to week 6 by intervention group. The median is represented by the middle line while the upper and lower borders of the box plot identify the 75th and 25th percentile, respectively. The whiskers correspond to the maximal and minimal values. **FMT-HF (*n*=15)**, FMT and high-fermentability fiber group. **HF (*n*=15)**, high-fermentability fiber group. **FMT-LF (*n*=14)**, FMT and low-fermentability fiber group. **LF (*n*=17)**, low-fermentability fiber group. All tests were two-tailed with a *p*-value <0.05 identifying statistical significance.

Figure 2.5. Changes in anthropometric parameters across intervention groups. a, Weight. **b**, Waist circumference. **c**, Systolic Blood Pressure (SBP). **d**, Diastolic Blood Pressure (DBP). Values are reported as mean ± SEM or as percent change from BL to T6. Lines represent raw unadjusted values at baseline and week 6 following FMT and/or fiber supplementation. Box-and-whisker plots represent the distribution of each group from baseline to week 6 by intervention group. The median is represented by the middle line while the upper and lower borders of the box plot identify the 75th and 25th percentile, respectively. The whiskers correspond to the maximal and minimal values. **FMT-HF (n=15)**, FMT and high-fermentability fiber group. **HF (n=15)**, high-fermentability fiber group. **FMT-LF (n=14)**, FMT and low-fermentability fiber group. **LF (n=17)**, low-fermentability fiber group. All tests were two-tailed with a p-value <0.05 identifying statistical significance.

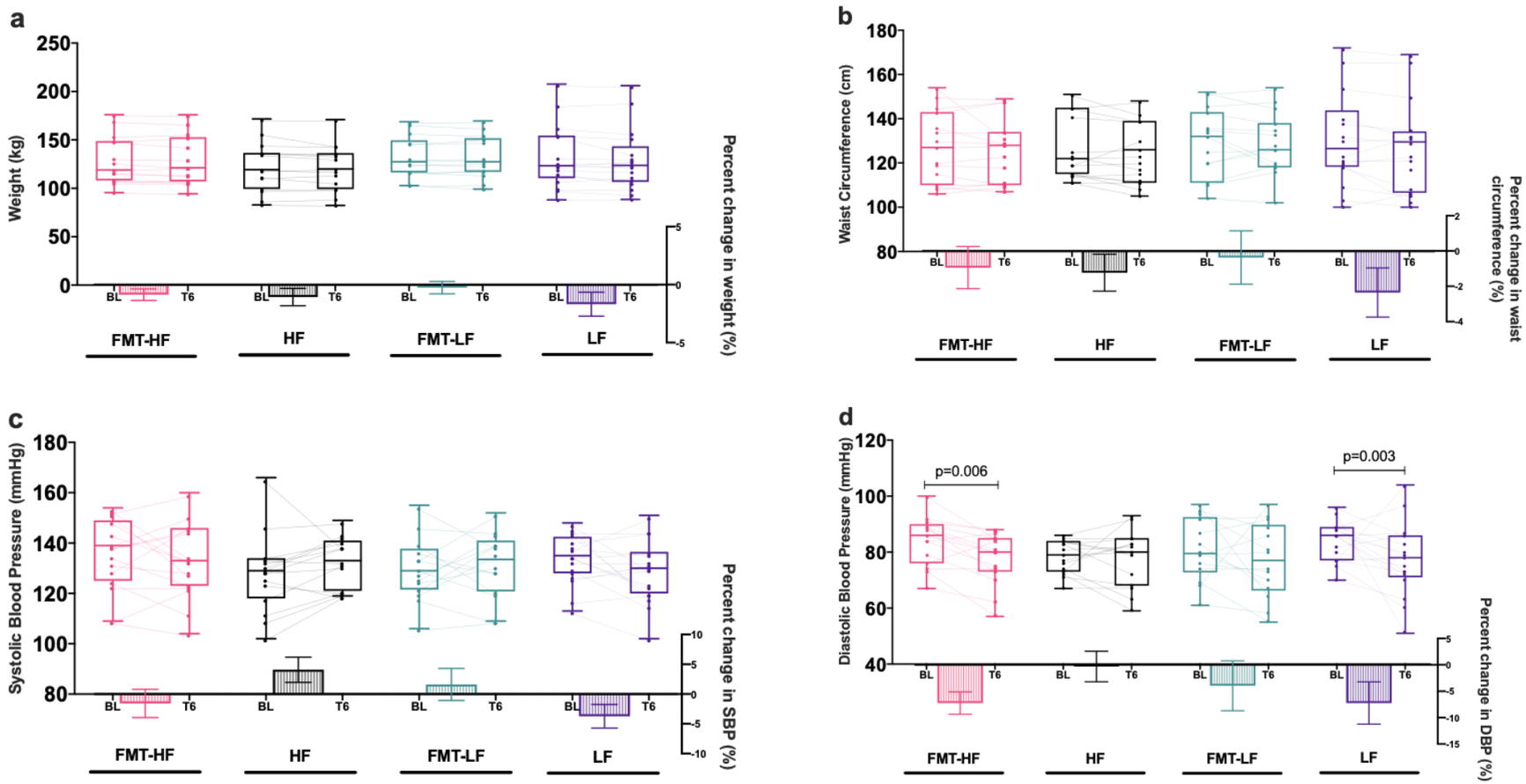
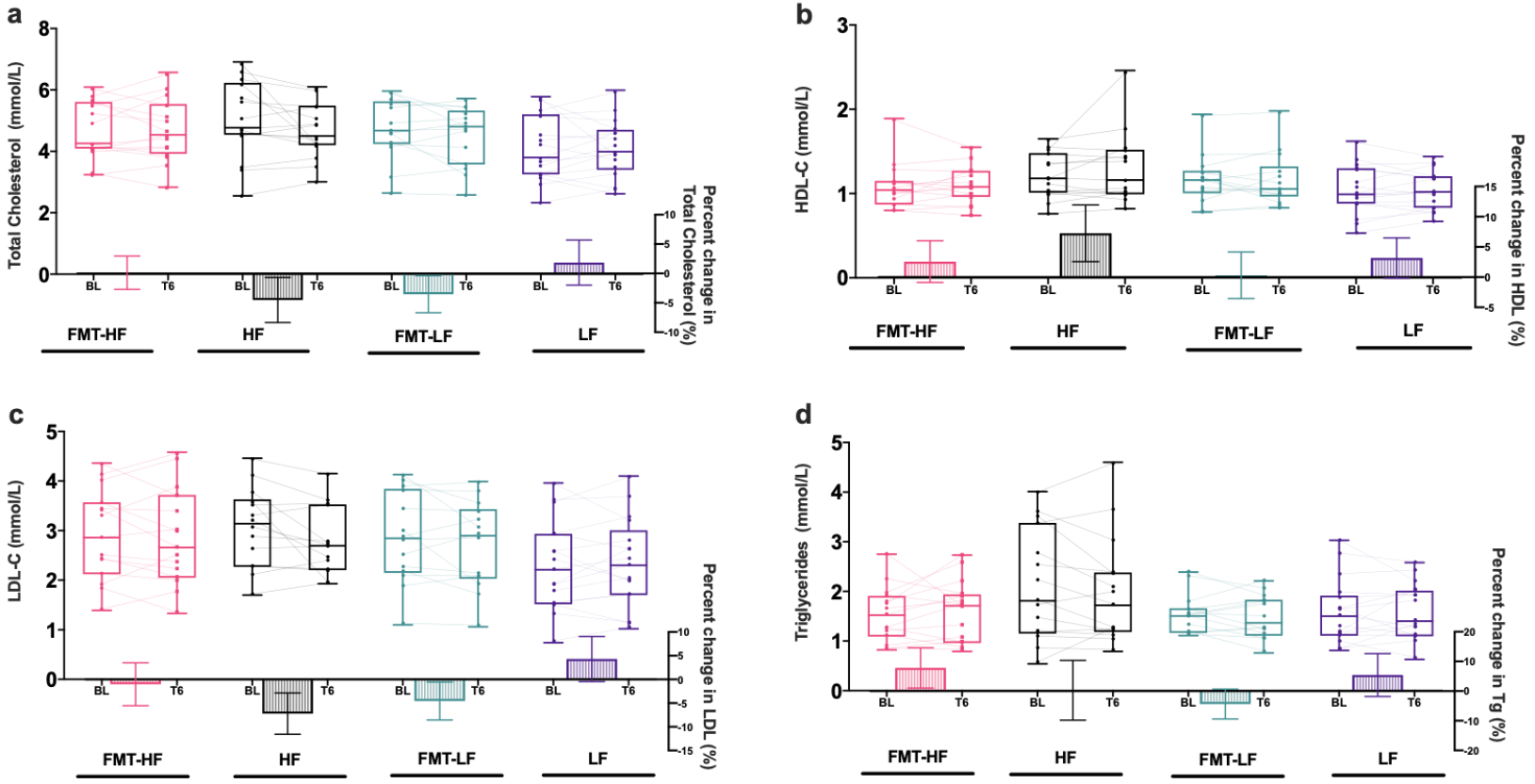


Figure 2.6. Changes in lipid parameters across intervention groups. a, Total Cholesterol. **b**, High-density lipoprotein (HDL-C). **c**, Low-density lipoprotein (LDL-C). **d**, Triglycerides (Tg). Values are reported as mean ± SEM or as percent change from BL to T6. Lines represent raw unadjusted values at baseline and week 6 following FMT and/or fiber supplementation. Box-and-whisker plots represent the distribution of each group from baseline to week 6 by intervention group. The median is represented by the middle line while the upper and lower borders of the box plot identify the 75th and 25th percentile, respectively. The whiskers correspond to the maximal and minimal values. **FMT-HF (n=15)**, FMT and high-fermentability fiber group. **HF (n=15)**, high-fermentability fiber group. **FMT-LF (n=14)**, FMT and low-fermentability fiber group. **LF (n=17)**, low-fermentability fiber group. All tests were two-tailed with a p-value <0.05 identifying statistical significance.



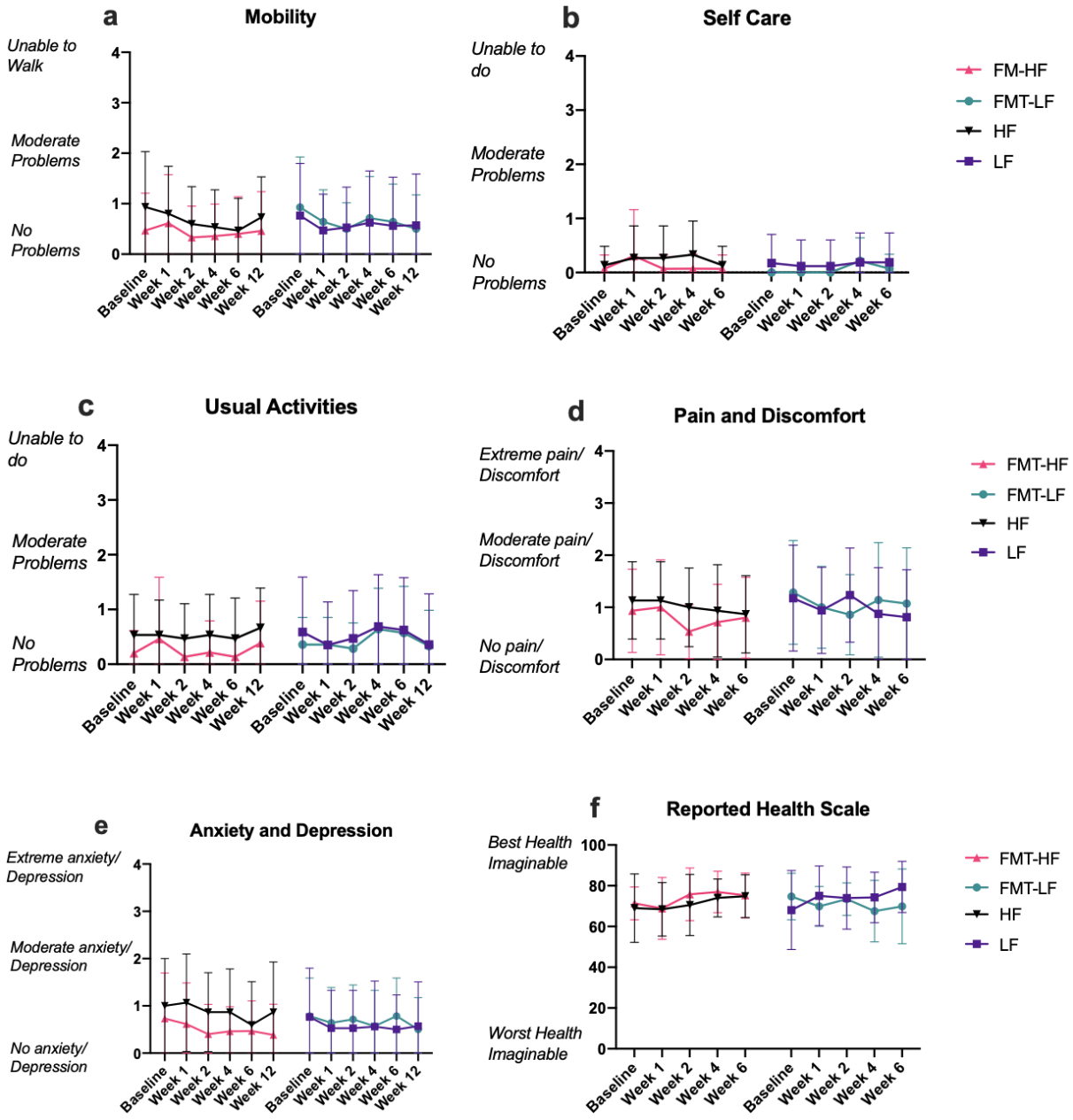


Figure 2.7. Composite scores of 5-level EQ-5D (EQ-5D-5L) survey domains from BL to T6. **a**, Mobility domain. **b**, Self-Care domain. **c**, Usual Activity domain. **d**, Pain and Discomfort domain. **e**, Anxiety and Depression domain., **f**, Visual Analogue Health Scale. Data reported as mean ± SEM. Pairwise comparisons between two groups were conducted using Mann-Whitney U-tests and Kruskal-Wallis tests for four group comparisons. **FMT-HF (n=15)**, FMT and high-fermentability fiber group. **HF (n=15)**, high-fermentability fiber group. **FMT-LF (n=14)**, FMT and low-fermentability fiber group. **LF (n=17)**, low-fermentability fiber group. All tests were two-tailed with a p-value <0.05 identifying statistical significance.

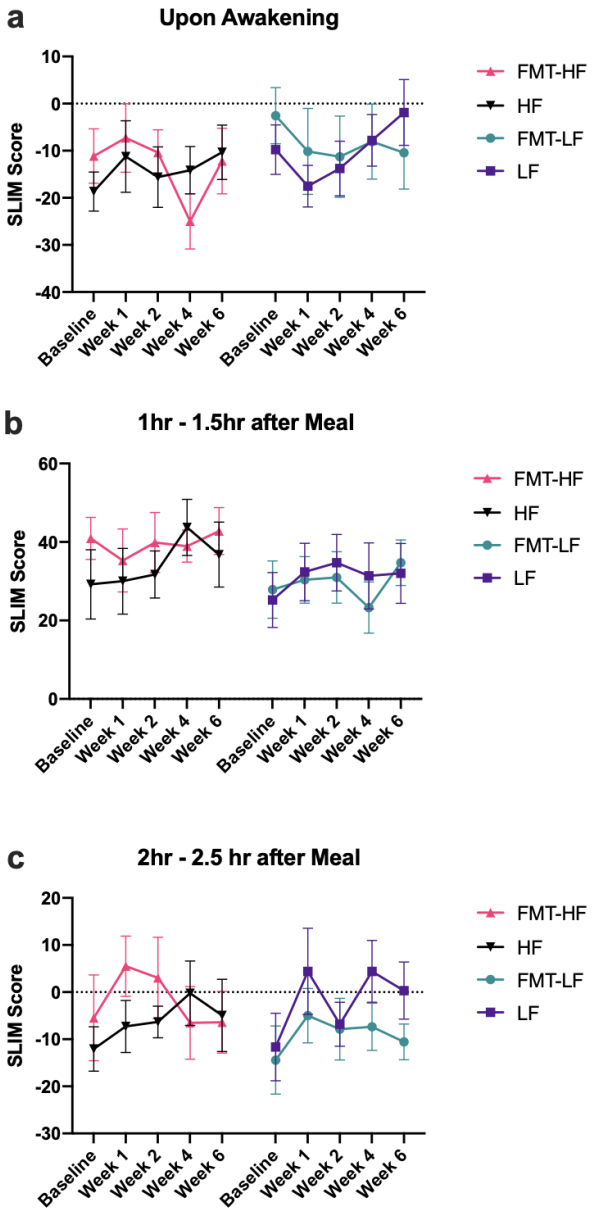


Figure 2.8. Responses to Hunger and Satiety SLIM from BL to T6. a, Hunger and satiety SLIM scoring upon waking. **b**, Hunger and satiety SLIM scoring 1-1.5 hours after a meal. **c**, Hunger and satiety SLIM scoring 2-2.5 hours after a meal. Data reported as mean ± SEM. Pairwise comparisons between two groups were conducted using Mann-Whitney U-tests and Kruskal-Wallis tests for four group comparisons. Paired data was analyzed by fitting a mixed model. **FMT-HF (n=15)**, FMT and high-fermentability fiber group. **HF (n=15)**, high-fermentability fiber group. **FMT-LF (n=14)**, FMT and low-fermentability fiber group. **LF (n=17)**, low-fermentability fiber group. All tests were two-tailed with a p-value <0.05 identifying statistical significance.

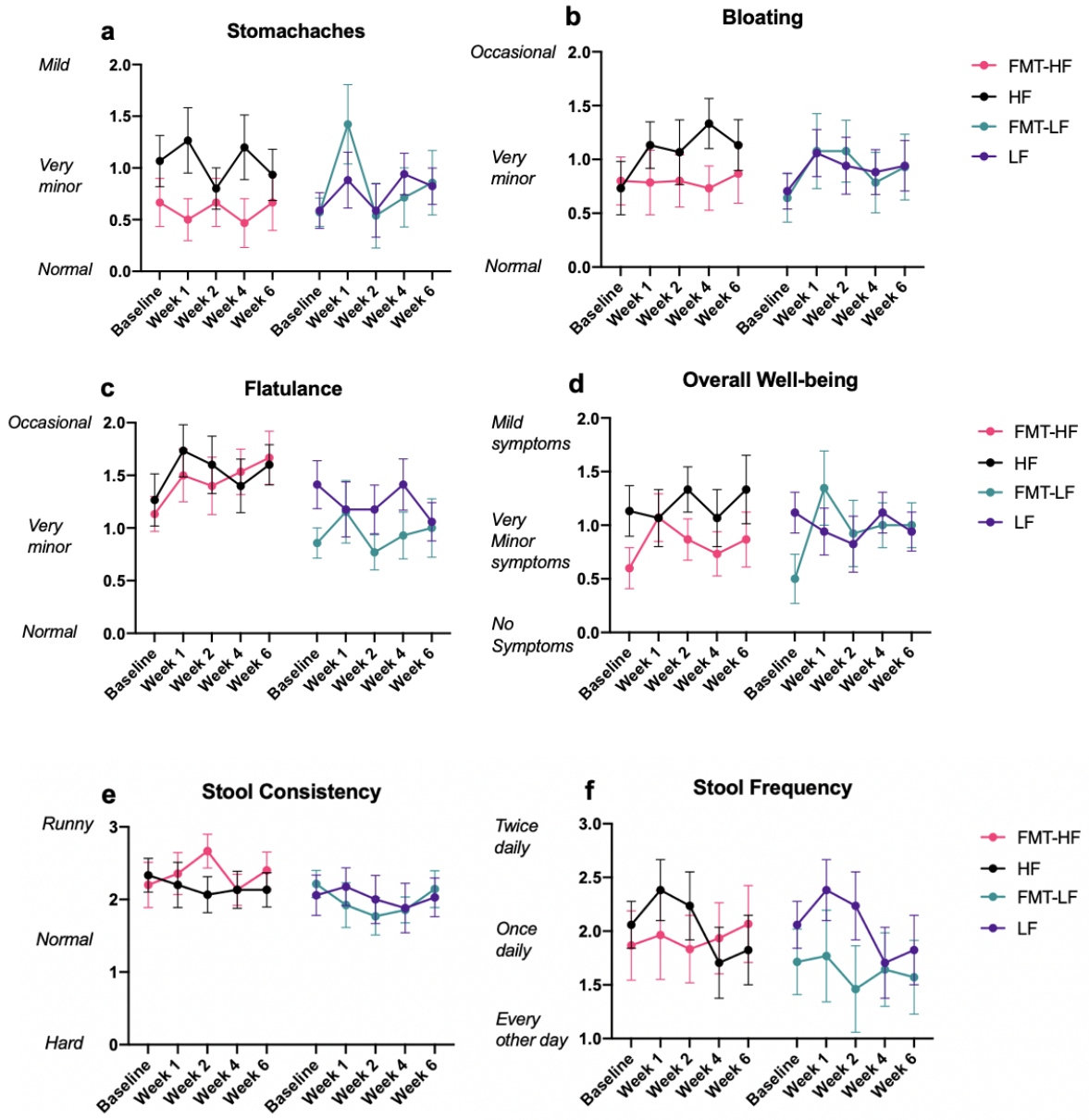


Figure 2.9. Responses to Gastrointestinal Tolerance questionnaire from BL to T6. a, Stomach aches. **b**, Bloating. **c**, Flatulence. **d**, Overall Well-being. **e**, Consistency. **f**, Frequency. Data reported as mean ± SEM. Pairwise comparisons between two groups were conducted using Mann-Whitney U-tests and Kruskal-Wallis tests for four group comparisons. Paired data was analyzed by fitting a mixed model. **FMT-HF (n=15)**, FMT and high-fermentability fiber group. **HF (n=15)**, high-fermentability fiber group. **FMT-LF (n=14)**, FMT and low-fermentability fiber group. **LF (n=17)**, low-fermentability fiber group. All tests were two-tailed with a p-value <0.05 identifying statistical significance.

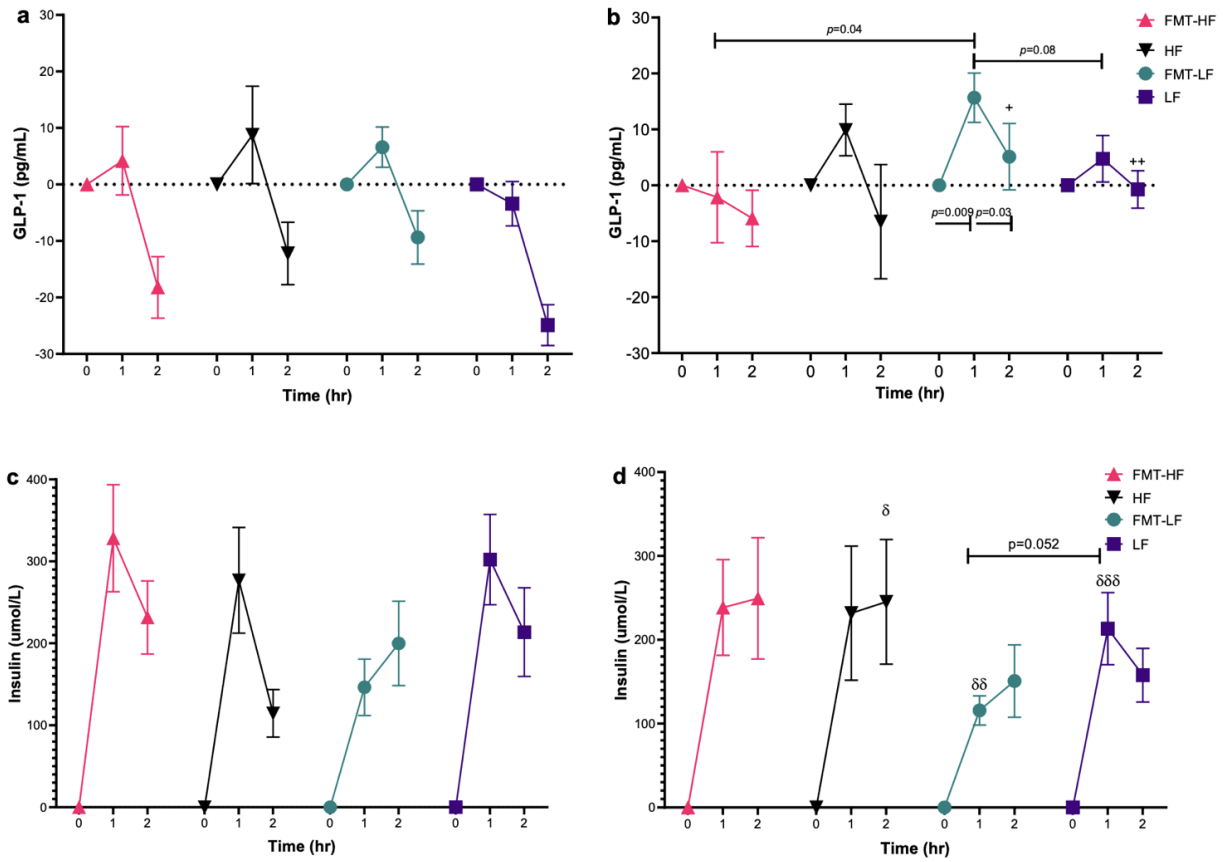


Figure 2.10. Changes in Glucagon-like peptide-1 (GLP-1) and Insulin during 75g Oral Glucose Tolerance Test (OGTT) at 0, 1, and 2 hours across intervention groups performed at BL and T6. a, baseline GLP-1 response to OGTT. **b**, week 6 GLP-1 response to OGTT. **c**, baseline insulin response to OGTT. **d**, week 6 insulin response to OGTT. GLP-1 and insulin levels were normalized to each group's respective baseline values and reported as mean ± SEM. Pairwise comparisons between groups were conducted using Mann-Whitney U-tests. Paired time points were compared to baseline values using the Wilcoxon signed-rank test without adjustment for multiple comparisons. **FMT-HF (*n*=15** patients**)**, FMT and high-fermentability fiber group. **HF (*n*=15** patients**)**, high-fermentability fiber group. **FMT-LF (*n*=14** patients**)**, FMT and low-fermentability fiber group. **LF (*n*=17** patients**)**, low-fermentability fiber group. All tests were two-tailed with a *p*-value <0.05 identifying statistical significance.

+ represents significant differences between BL and T6 normalized GLP-1 values (+ $p = 0.004$; ++ $p = 0.0007$).

δ represents statistically significant differences between BL and T6 normalized insulin values (δ $p = 0.06$; $\delta\delta$ $p = 0.04$; $\delta\delta\delta$ $p = 0.01$).

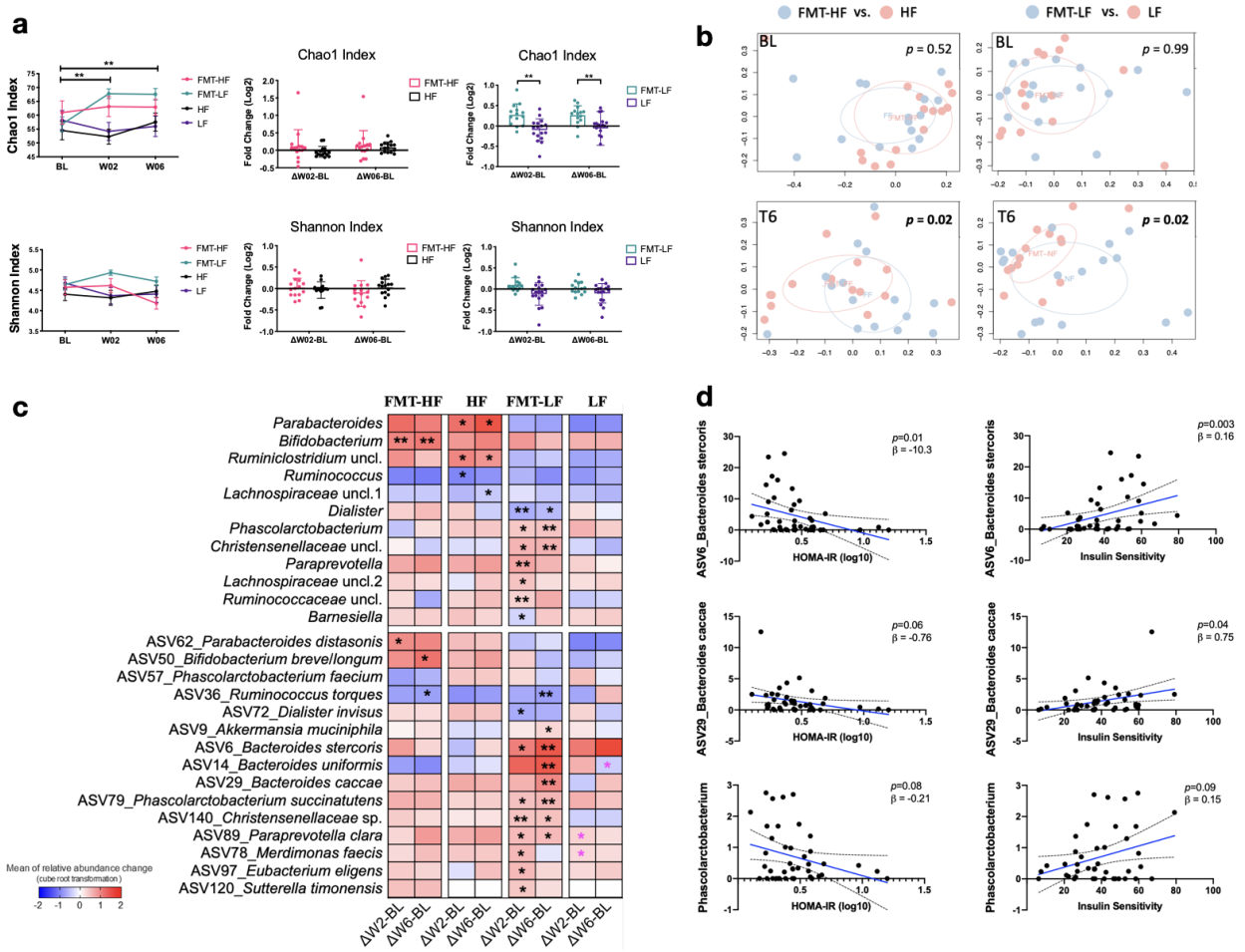


Figure 2.11. Changes in gut microbiome and associated predictors of improved HOMA2-IR and insulin sensitivity from BL to T6. a, Differences in alpha diversity using microbial richness (Chao1) and microbial diversity (Shannon) indices. $^{**}q$ <0.01, $^{*}q < 0.05$ for within and between group comparisons. **b**, Differences in Beta diversity between baseline (T0) and 6 weeks (T6). **c**, Changes in bacterial species between groups across all time points. **d**, Bacterial taxa independently predict improvements in HOMA2-IR and insulin sensitivity in patients receiving FMT-LF. Analysis conducted using linear mixed model regression after adjusting for patient sex and age. Changes within-group relative to baseline (e.g. ΔW6-BL) were assessed by repeated linear regression with FDR correction. Between-group comparison (FMT-HF vs. HF; FMT-LF vs. LF) of bacterial shifts were conducted by linear regression with FDR correction. ** $q < 0.05$ * $q < 0.10$ for within-group comparisons relative to baseline. * $q < 0.15$ for between-group comparisons. **FMT-HF (*n*=15)**, FMT and high-fermentability fiber group. **HF (*n*=15)**, high fermentability fiber group. **FMT-LF (*n*=14)**, FMT and low-fermentability fiber group. **LF (*n*=17)**, low-fermentability fiber group. All tests were two-tailed with a *p*-value <0.05 identifying statistical significance.

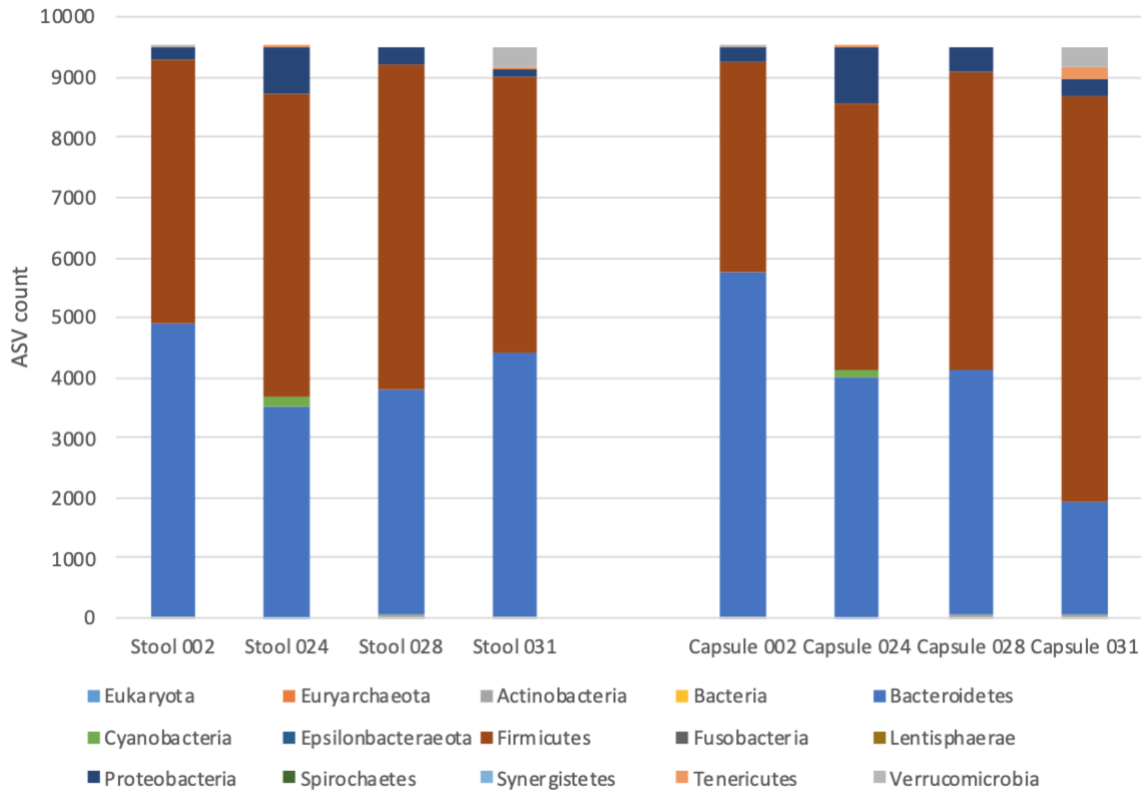


Figure 2.12. Donor composition at Phylum level of stool and oral capsules. Bar chart represents proportion of ASV counts at the phylum level.

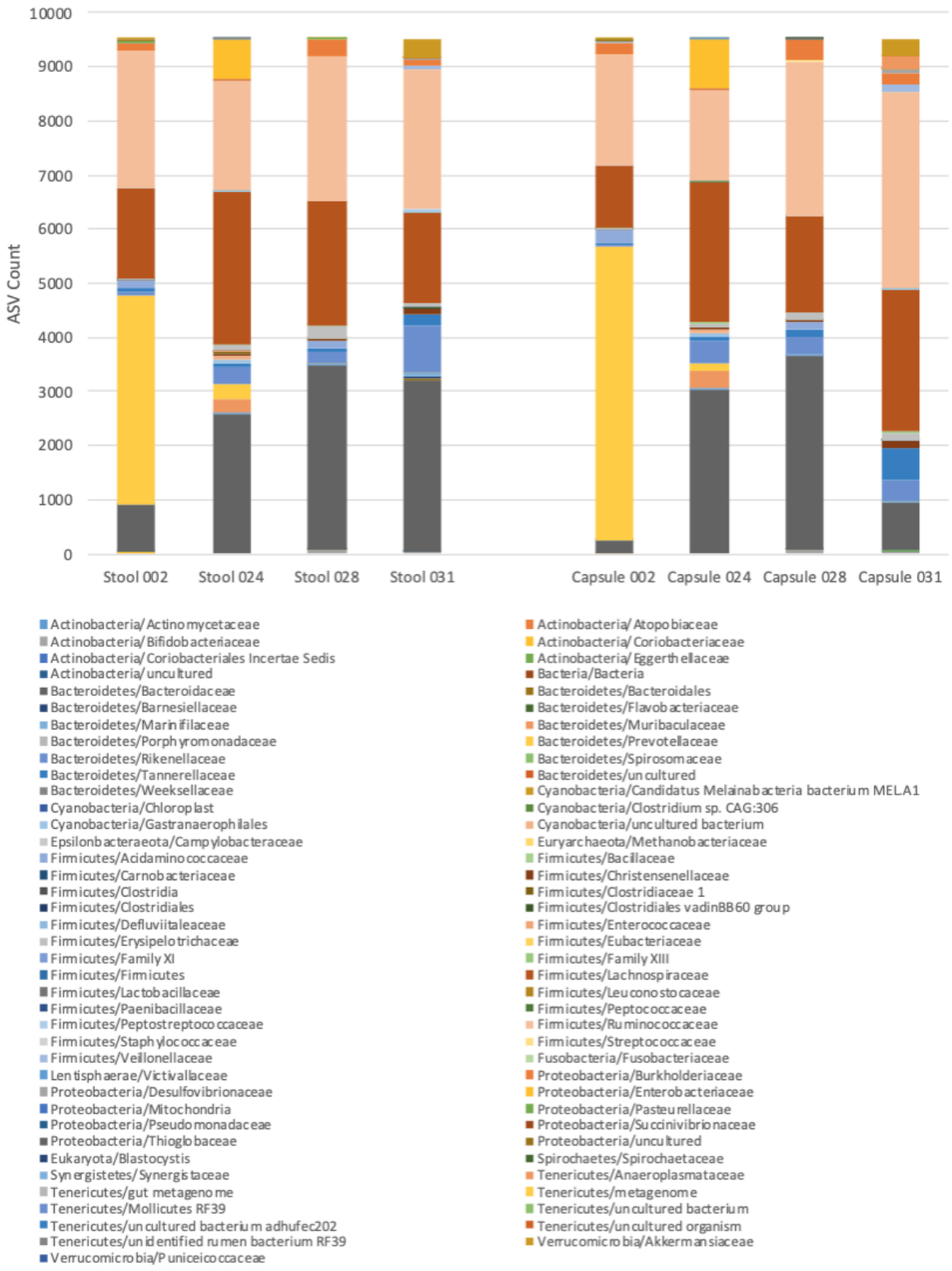


Figure 2.13. Donor composition at Family level of stool and oral capsules. Bar chart represents proportion of ASV counts at the phylum level.

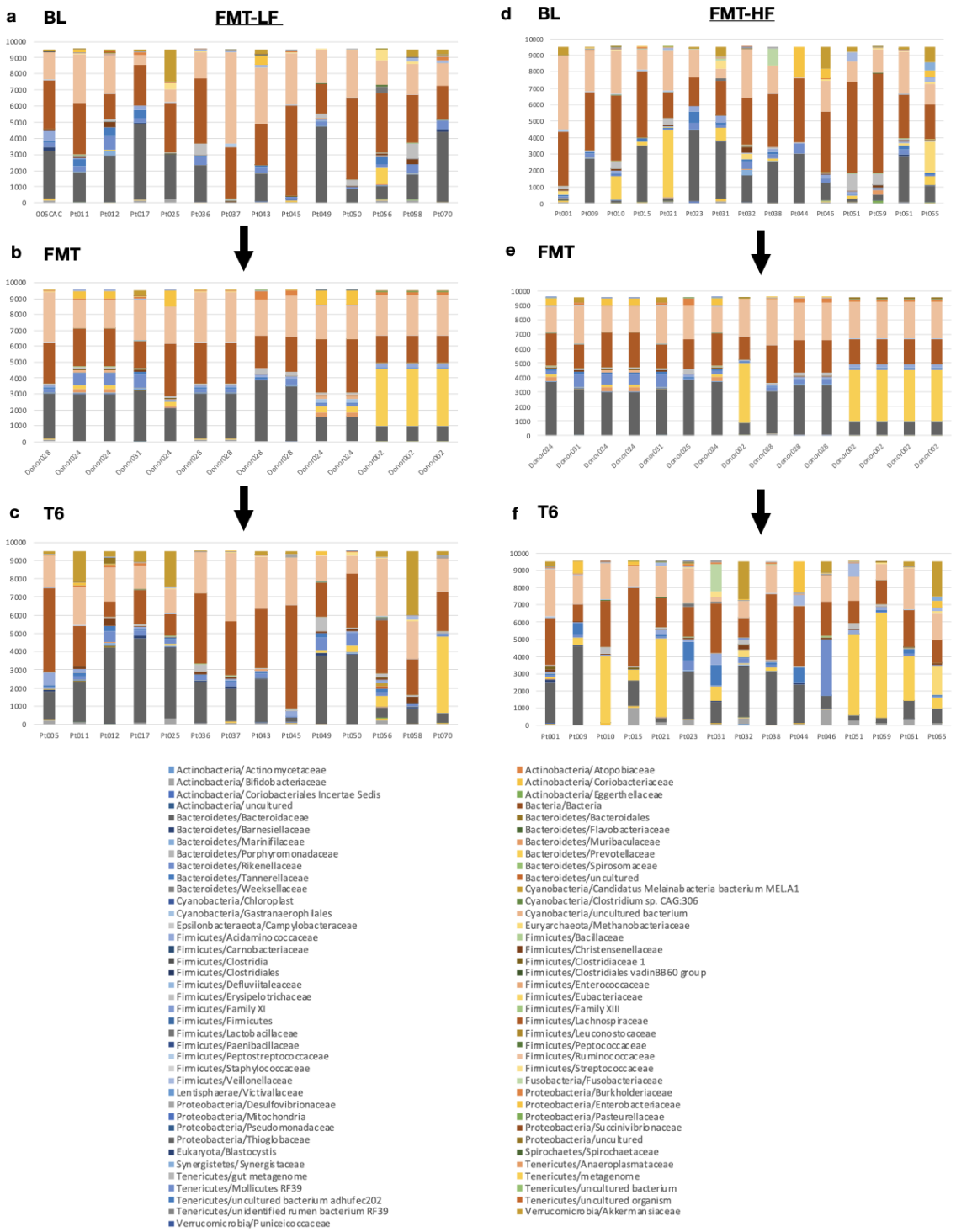


Figure 2.14. Family level microbial composition of FMT recipients and paired donors from BL to T6. a, BL FMT-LF composition. **b,** Composition of matched FMT-donor in patients receiving FMT and low-fermentability fiber. **c,** T6 FMT-LF composition. **d,** BL FMT-HF composition. **e,** Composition of matched FMT-donor in patients receiving FMT and high-fermentability fiber. **f,** T6 FMT-HF composition.

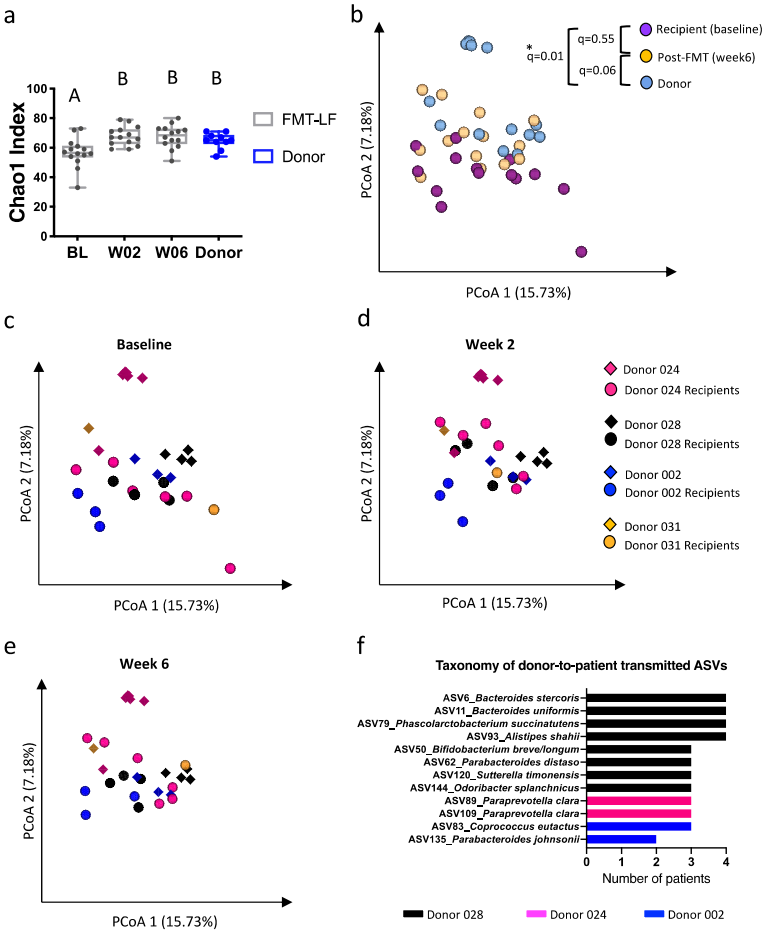
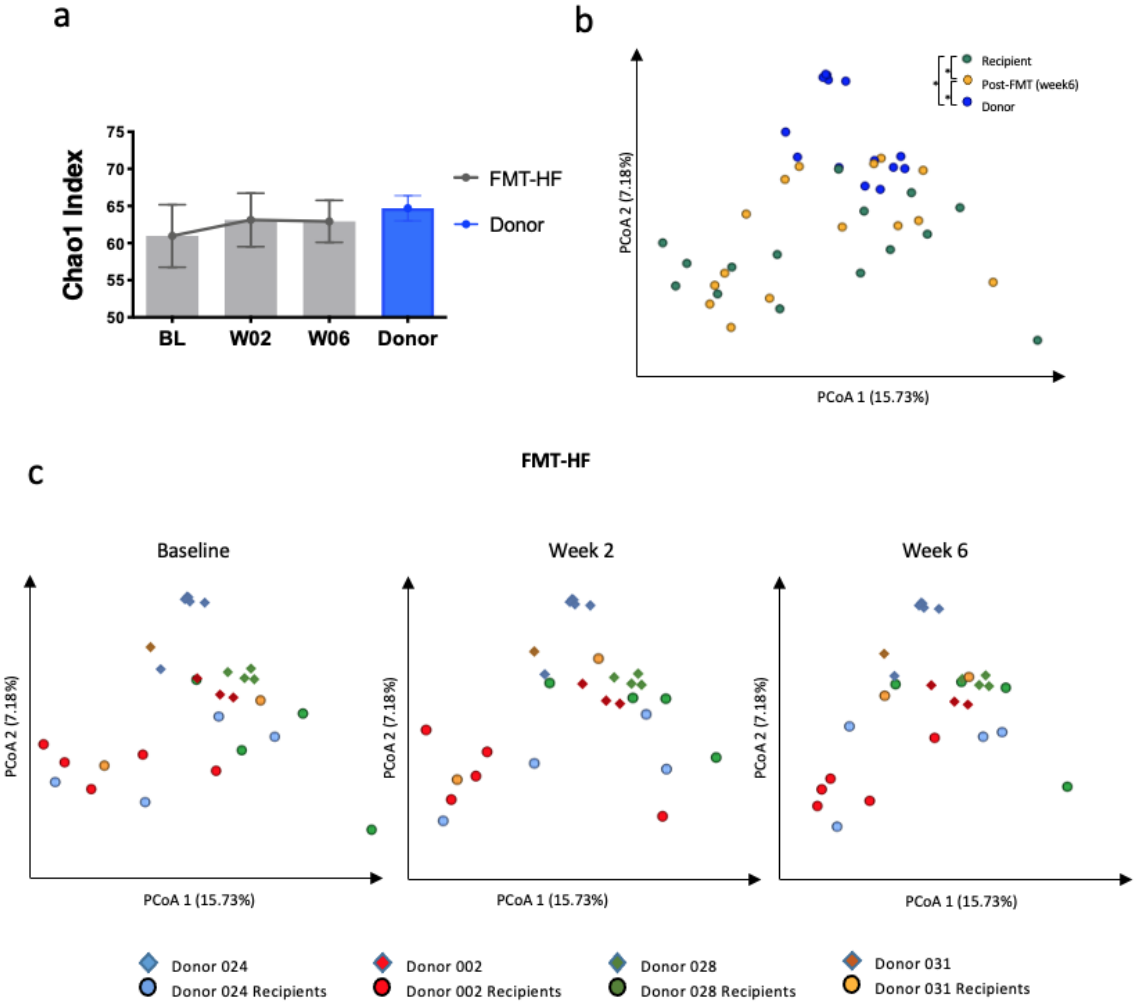


Figure 2.15. Evidence of fecal microbial engraftment in FMT-LF patients from baseline to week 6. a, Recipient Chao1 index from baseline to week 6 versus donor FMT. Box-and-whisker plots represent the distribution of each group's Chao1 index from baseline to week 6. The median is represented by the middle line while the upper and lower borders of the box plot identify the 75th and 25th percentile, respectively. The whiskers correspond to the maximal and minimal values. Between-group differences were assessed by linear regression, and pairwise comparisons were conducted using unpaired *t*-tests. P-values were adjusted by Benjamin-Hochberg false discovery rate (FDR) method. **b,** Principal-coordinate analysis (PCoA) of ASVs demonstrating that at baseline, recipients have significantly different microbial clustering patterns than donors. At week 6, no significant differences in microbial structure were observed between recipients and donors. Significant differences were assessed by PERMANOVA based on unweighted UniFrac distances with FDR correction (* $q < 0.05$). **c-e,** PCoA grouping of patients and donors (baseline, week 2, and week 6) demonstrating substantial increases in similarity between recipient and donor microbial structure over time. **f,** Evidence of donor specific ASV engraftment obtained if taxa were shared between donor and recipients at week 6 but not present in recipients at baseline ASVs with the highest number of post-FMT patients received showing evidence of acquisition of the donor's taxa. **FMT-LF (n=14** patients**)**, FMT and low-fermentability fiber group. All tests were two-tailed with a *q*-value <0.05 identifying statistical significance.

Figure 2.16. Evidence of fecal microbial engraftment in FMT-HF patients from BL to T6. **a,** Recipient Chao1 index from BL to T6 versus donor FMT. Error bars reflect standard deviation with time points sharing the same letter indicating no significant differences in α-diversity. **b,** Principal-coordinate analysis (PCoA) of ASVs demonstrating that at BL, recipients have significantly different microbial clustering patterns than donors. At T6, no significant differences in microbial structure were observed between recipients and donors. **c,** PCoA grouping of patients and donors (baseline, week 2, and week 6) demonstrating increasing similarity between recipient and donor microbial structure over time. **FMT-HF (n=15)**, FMT and high-fermentability fiber group. Significant differences were assessed by PERMANOVA based on unweighted UniFrac distances (* $q < 0.05$).



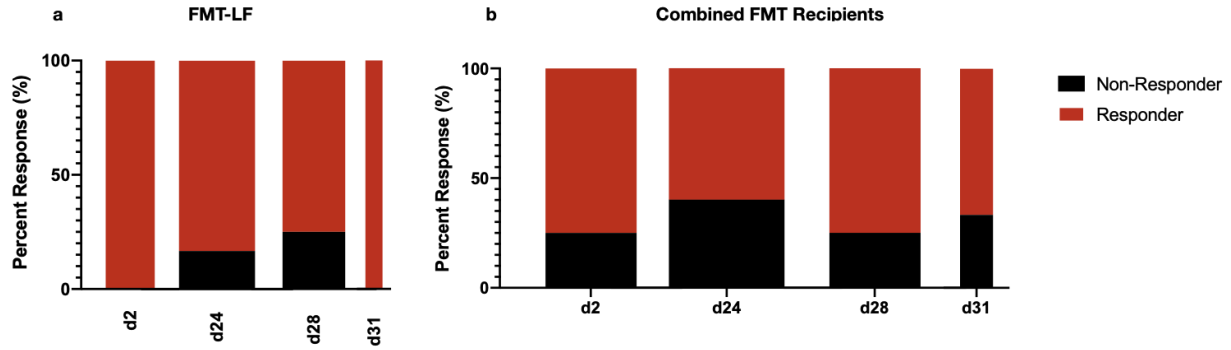


Figure 2.17. Mosaic bar graph of responders and non-responders for FMT-LF and combined FMT recipient groups by FMT donor. a, FMT-LF HOMA2 response to the four donors. **b,** HOMA2 response of all FMT recipients to the four donors. The width of bars for each donor is weighed by the number of corresponding donations for each group. Responders were defined by patients having a negative percent change in HOMA2 from BL to T6 while non-responders were defined as those having a positive percent change.

	FMT-LF		FMT-HF		Combined FMT groups	
Donor	**Responders (n, %)**	**Non-Responders (n, %)**	**Responders (n, %)**	**Non-Responders (n, %)**	**Responders (n, %)**	**Non-Responders (n, %)**
2	3 (100)	0 (0)	3 (60.0)	2 (40.0)	6 (75.0)	2 (25.0)
24	5 (83.3)	1 (16.7)	1 (25.0)	3 (75.0)	6 (60.0)	4 (40.0)
28	3 (75.0)	1 (25.0)	3 (75.0)	1 (25.0)	6 (75.0)	2 (25.0)
31	1 (100)	0 (0)	1 (50.0)	1 (50.0)	2 (66.7)	1 (33.3)

Table 2.1. Overview of donor allocation between FMT-LF and FMT-HF groups. FMT-HF, FMT and high-fermentability fiber group. **FMT-LF**, FMT and low-fermentability fiber group. Responders were defined by patients having a negative percent change in HOMA2 from BL to T6 while non-responders were defined as those having a positive percent change. No significant difference between groups was observed with regards to donor allocation using the Chi-square test ($p=0.75$)

	FMT-HF (n = 15)	HF (n=15)	FMT-LF (n = 14)	LF (n=17)
Sex (females, %)	14 (93.3)	13 (86.7)	12 (85.7)	12 (70.6)
Age (years)	47.6 (10.2)	48.3 (10.4)	46.9 (11.8)	48.4 (8.8)
Systolic pressure (mm Hg)	135 (15)	128 (15)	130 (12)	134 (10)
Diastolic pressure (mmHg)	84 (9)	78 (6)	81 (11)	84 (7.0)
Heart Rate (bpm)	75 (8)	76 (11)	77 (13)	79 (10)
Weight (kg)	128.6 (25.0)	122.8 (25.5)	132.2 (21.7)	131.3 (32.0)
Height (cm)	170.1 (6.8)	166.2 (9.8)	165.4 (6.1)	170.3 (8.2)
Medications				
Metformin (n, %)	3 (20.0)	4 (26.7)	3 (21.4)	5 (29.4)
GLP-1 (n, %)	10 (66.7)	9 (60.0)	10 (71.4)	10 (58.8)
SGLT2 (n, %)	1 (6.7)	0 (0)	1 (7.1)	1 (5.9)
Body mass index (kg/m^2)	44.2 (6.1)	44.2 (6.8)	48.3 (7.0)	44.8 (7.6)
Waist circumference* (cm)	127 (33)	119 (27)	133 (23)	123 (22)
Hip circumference (cm)	136.9 (10.0)	134.4 (21.4)	144.8 (13.8)	136.2 (18.7)
Waist to Hip ratio*	0.89 (0.17)	0.92 (0.22)	0.90 (0.10)	0.94 (0.22)
Hemoglobin A1c* (%)	5.9 (0.8)	5.8 (0.6)	5.6 (0.4)	5.8 (0.7)
Fasting glucose* (mmol/L)	5.2 (1.2)	5.5 (2.0)	5.7 (0.6)	5.4 (1.1)
Insulin* (pmol/L)	161 (124)	162 (121)	150 (90)	166 (113)
HOMA2 IR*	2.8 (2.1)	3.2 (2.8)	2.8 (1.6)	3.1 (1.9)
HOMA2 %S* (%)	35.2 (23.8)	31.2 (30.8)	36.3 (17.9)	32.8 (17.5)
C-reactive protein* (mg/L)	5.5 (9.7)	9.4 (11.8)	5.8 (7.4)	11 (10.8)
Total Cholesterol (mmol/L)	4.7 (0.9)	5.0 (1.3)	4.7 (1.0)	4.1 (1.1)
HDL-C (mmol/L)	1.1 (0.3)	1.2 (0.3)	1.2 (0.3)	1.0 (0.3)
LDL-C (mmol/L)	2.9 (0.9)	3.1 (0.8)	2.9 (0.9)	2.3 (0.9)
Triglycerides* (mmol/L)	1.5 (0.8)	1.8 (2.2)	1.5 (0.5)	1.5 (0.7)
HGB (g/L)	136 (10)	141 (11)	138 (10)	143 (6)
WBC (10^9/L)	7.3 (1.5)	7.1 (1.3)	7.4 (1.5)	7.69 (1.9)

PLT (10^9/L)	305 (67)	282 (60)	297 (46)	261 (59)
ALT* (U/L)	25 (19)	23 (18)	21 (9)	24 (6)
AST* (U/L)	23 (11)	22 (14)	21 (4)	22 (7)
ALK* (U/L)	65 (20)	64 (16)	64 (21)	62 (21)
Total Bilirubin* (µmol/L)	8.5 (5)	11 (5)	13 (6)	12 (6)
INR (Units)	1.0 (0.1)	1 (0.1)	1.0 (0.1)	1.0 (0.1)
Creatinine (µmol /L)	69 (14)	73 (12)	70 (9)	73 (15)
eGFR (mL/min/1.72m^2)	92.5 (18.8)	88.2 (14.6)	92.4 (12.9)	92.3 (19.2)
Urea* (µmol /L)	4.6 (1.4)	5.1 (1.6)	4.1 (1.8)	4.7 (4.6)
Albumin (g/L)	42 (3)	42 (4)	42 (3)	41 (3)
MCV (fL)	86 (4)	88 (5)	87 (4)	89 (3)
RDW* (%)	13.3 (1.7)	13.2 (1.2)	13.1 (2.5)	13.4 (0.5)
TSH* (µIU/mL)	1.7 (0.9)	2.4 (2.4)	1.7 (1.2)	2.3 (1.3)

GLP-1 *glucagon-like peptide-1;* ***SGLT2****- sodium-glucose transport protein 2 inhibitors;* ***HOMA2-IR*** *homeostatic model of insulin resistance;* ***HOMA2-IR*** *homeostatic model of insulin sensitivity;* ***HDL-C*** *high-density lipoprotein cholesterol;* ***LDL-C*** *low-density lipoprotein cholesterol;* ***HGB*** *serum hemoglobin count;* ***WBC*** *serum white blood cell count;* ***PLT*** *serum platelet count;* ***ALT*** *alanine aminotransferase;* ***AST****- aspartate aminotransferase;* ***ALK*** *alkaline phosphatase; INR international normalized ratio;* ***eGFR*** *estimated glomerular filtration rate;* ***MCV*** *mean corpuscular volume;* ***RDW*** *red cell distribution width;* ***TSH*** *thyroid stimulating hormone*

Table 2.2. Baseline characteristics of study cohort. Values represent mean ± standard deviations or absolute values and percentages unless otherwise stated. Differences between cohorts were analyzed using Kruskal-Wallis or Chi-square test as appropriate. **FMT-HF**– FMT and high-fermentability fiber group; **HF** – high-fermentability fiber group**; FMT-LF** – FMT and low-fermentability fiber group; **LF**- low-fermentability fiber group. All tests were two-tailed with p values less than 0.05 deemed significant. *Values presented as median and IQR after normality evaluated using Shapiro-Wilk test.

Supplemental Table 2.3. Macronutrient Intake at Baseline and Week 6 of Intervention as Estimated by the Diet History Questionnaire III.

	FMT - HF (*n*=12)				HF (*n*=13)				Between Group Change *p* value	FMT - LF Fiber (*n*=10)				LF (*n*=12)				Between Group Change *p* value
	Baseline	Week 6	Within Group *p* value	Change (%)	Baseline	Week 6	Within Group *p* value	Change (%)		Baseline	Week 6	Within Group *p* value	Change (%)	Baseline	Week 6	Within Group *p* value	Change (%)	
Energy (kcal)	1660 ± 577	1539 ± 419	0.7	2±32	1755 ± 870	1679 ± 542	0.5	11±73	0.8	1602 ± 496	1603 ± 463	0.7	8±37	1969 ± 1025	1673 ± 803	0.08	-9±32	0.3
Carbohydrates (g)	226±97	205±53	0.6	8±52	206±112	223±116	0.8	29±92	0.9	195±82	214±66	0.4	34±79	231±116	220±117	0.8	0±35	0.4
Total sugar (g)	131±74	118±44	0.5	29±117	111±70	129±86	0.7	50±138	0.7	105±58	130±56	0.3	45±97	118±65	116±74	0.9	2±37	0.4
Added sugar (g)	97±57	90±46	0.9	74±240	77±57	91±69	0.5	131±380	0.4	75±52	98±51	0.4	60±138	87±58	87±69	0.9	2±45	0.3
Dietary fiber (g) [a]	19±8	17±6	0.5	-5±25	19±9	20±10	0.7	11±41	0.2	19±8	17±6	0.9	17±53	18±8	17±7	0.8	9±49	0.7
Insoluble fiber (g)	13±6	11±4	0.6	-4±27	12±5	12±5	0.8	4±36	0.6	12±5	11±4	0.4	18±47	12±6	11±4	0.6	4±50	0.4
Soluble fiber (g)	7±3	6±3	0.3	-5±27	7±5	8±5	0.3	29±56	0.1	7±3	6±2	0.8	20±75	6±3	6±3	0.3	21±50	0.8
Proteins (g)	70±27	66±24	0.5	-1±32	77±40	63±14	0.1	14±126	0.4	64±22	62±16	1	5±28	90±45	72±31	0.08	-13±40	0.2
Animal proteins (g)	50±23	47±18	0.5	1±40	56±31	44±12	0.06	121±525	0.3	46±18	44±11	1	2±27	68±36	52±23	0.07	-16±42	0.2
Plant proteins (g)	20±6	19±6	0.6	-1±29	21±11	19±8	0.4	2±59	0.7	18±8	17±6	0.5	25±62	22±11	21±10	0.60	-2±40	0.4
Total fats (g)	55±19	51±22	0.5	-3±33	70±40	61±18	0.3	9±80	0.8	64±28	56±21	0.6	1±43	77±46	57±27	0.08	-16±29	0.4
Saturated fat (g)	19±8	17±8	0.5	-1±38	23±14	21±8	0.4	23±125	0.8	21±10	20±9	0.9	4±40	26±17	19±10	0.07	-16±27	0.2
Unsaturated fat (g)	31±10	29±13	0.3	-3±34	41±23	35±11	0.3	6±69	0.6	38±16	32±10	0.4	1±48	43±25	33±16	0.1	-16±30	0.4
Cholesterol (mg)	262±130	242±111	0.6	9±64	257±125	223±123	0.1	187±756	0.2	251±128	235±89	0.6	3±45	337±191	259±126	0.1	-15±41	0.4

Statistical significance of changes within-group were determined by the Wilcoxon signrank test while between-group differences (FMT-HF vs HF, FMT-LF vs. LF; [week 6 – baseline]/baseline*100) were determined by the Mann-Whitney test. Data are presented as means ± SD. Statistical significance was set at $p<0.05$.

[a] Total dietary fiber provided by the background diet without consideration of the supplemented fiber.

	HOMA2-IR Response			HOMA2-IS Response		
	Odds Ratio	95% CI	P-Value	Odds Ratio	95% CI	P-Value
Study Allocation						
FMT-HF vs. HF	2.86	0.54-15.2	*0.22*	2.06	0.41-10.3	*0.38*
FMT-LF vs. LF	23.4	2.8-193.3	***0.003***	16.6	2.19-125.8	***0.007***
FMT-LF vs. FMT-HF	5.89	0.75-42.9	*0.09*	3.32	0.63-17.3	*0.15*
HF vs. LF	1.46	0.28-7.68	*0.65*	1.61	0.33-7.98	*0.58*
Male Sex	0.69	0.10-4.77	*0.71*	1.35	0.20-8.98	*0.75*
Increasing Age (per year)	1.05	0.98-1.12	*0.15*	1.03	0.96-1.10	*0.44*
Increasing BMI (per kg/m^2)	0.99	0.89-1.10	*0.85*	0.97	0.88-1.07	*0.58*
Increasing baseline serum insulin (per 50pmol/L)	2.01	1.19-3.41	***0.009***	1.87	1.13-3.11	***0.015***
Use of novel anti-hyperglycemic medications	1.24	0.31-5.00	*0.8*	1.06	0.28-4.02	*0.94*

Table 2.4. Predictors of beneficial response in percent change of insulin resistance (HOMA2-IR) and insulin sensitivity (HOMA2-IS) from baseline to week 6 using multivariate logistic regression. FMT-HF (*n*=15), FMT and high-fermentability fiber group. **HF (*n*=15)**, high-fermentability fiber group. **FMT-LF (*n*=14)**, FMT and low-fermentability fiber group. **LF (*n*=17)**, low-fermentability fiber group.

	FMT-HF (n = 15)	HF (n=16)	FMT-LF (n = 15)	LF (n=17)
No. of AEs related to study intervention	1	3	2	1
Bloating requiring fiber cessation	1	1	1	1
Constipation	0	0	1	0
Emesis following FMT	0	2	0	0
No. AEs unrelated to study intervention	1	2	0	2
Ear, Throat and Respiratory Infections [a]	1	1	1	1
Prostatitis [b]	0	1	0	0
Lower Extremity Cellulitis	0	0	1	1
No. Serious AEs	0	0	0	0

Table 2.5. Overview of recorded Adverse Events (AE) and Serious Adverse events of all patients receiving either FMT or fiber Intervention. FMT-HF, FMT and high-fermentability fiber group. **HF**, high-fermentability fiber group. **FMT-LF**, FMT and low-fermentability fiber group. **LF**, low-fermentability fiber group. Two patients required prolonged antibiotics prior to the T6 visit and were excluded from the modified intent-to-treat analysis as they were withdrawn from the trial and did not reach their T6 visit.

[a] Ear, throat, and respiratory infections included upper respiratory tract infections, nasopharyngitis, laryngitis, sinusitis, bronchitis, and otitis.

[b] Diagnosed by cystoscopy following urologic consultation

	FMT- HF (*n*=12)				HF (*n*=13)				Between group change p value	FMT – LF (*n*=10)				LF (*n*=12)				Between group change p value
	Baseline	Week 6	Within Group p value	Change (%)	Baseline	Week 6	Within Group p value	Change (%)		Baseline	Week 6	Within Group p value	Change (%)	Baseline	Week 6	Within Group p value	Change (%)	
Cytokines																		
LPS (EU/mL)	2.0 (1.7)	1.1 (0.7)	*0.054*	- 34.8 (38.5)	1.5 (2.0)	1.6 (1.3)	*0.4*	45.7 (131.2)	*0.1*	1.6 (1.7)	1.3 (1.1)	*0.7*	5.3 (163.0)	1.0 (0.8)	1.0 (1.0)	*0.6*	4.3 (129.3)	*0.7*
IL1B (pg/mL)	14.5 (193.2)	15.5 (177.7)	*0.5*	-1.9 (20.7)	5.2 (53.2)	5.3 (49.7)	*0.2*	-1.9 (18.7)	*0.6*	2.7 (28.6)	4.1 (22.0)	*0.8*	1.7 (15.4)	4.8 (18.5)	5.1 (16.0)	*0.4*	-1.3 (25.3)	*0.6*
IL-6 (pg/mL)	9.6 (268.3)	11.4 (205.4)	*0.5*	-9.6 (49.1)	7.8 (18.8)	7.5 (19.8)	*0.5*	-8.3 (31.7)	*0.6*	4.4 (4.6)	5.7 (7.2)	*0.2*	6.0 (38.2)	6.6 (6.7)	5.4 (5.6)	*0.1*	-10.3 (20.3)	***0.03***
IL-8 (pg/mL)	5.0 (36.3)	4.0 (30.9)	***0.01***	-14.1 (35.8)	4.2 (9.7)	3.8 (4.4)	*0.06*	-19.3 (35.2)	*0.9*	4.1 (6.9)	5.8 (7.5)	***0.056***	14.5 (74.0)	2.4 (0.9)	3.0 (2.1)	*0.4*	24.2 (83.1)	*0.7*
IL-10 (pg/mL)	38.2 (1915.0)	32.1 (1772.0)	*0.3*	-7.4 (20.1)	31.2 (96.3)	29.5 (85.6)	*1*	-3.1 (17.1)	*0.4*	21.6 (69.4)	20.8 (69.8)	*0.6*	0.8 (18.6)	26.2 (20.9)	28.9 (34.7)	*0.8*	-2.8 (15.7)	*0.6*
TNF-a (pg/mL)	60.4 (433.3)	24.2 (219.3)	***0.005***	-25.6 (69.0)	29.2 (144.4)	13.1 (127.7)	***0.054***	-12.3 (45.8)	*0.4*	13.0 (16.2)	14.8 (16.5)	*0.6*	-1.2 (14.2)	18.9 (29.6)	21.2 (29.8)	*0.7*	1.6 (29.4)	*0.7*
SCFA																		
Total (umol/mL)	9.7 (4.5)	11.5 (6.6)	*0.1*	15.5 (77.1)	9.2 (4.6)	12.6 (3.6)	*0.4*	16.0 (60.6)	*0.9*	9.4 (3.1)	11.5 (6.6)	*0.5*	15.5 (77.1)	11.4 (3.0)	12.3 (5.3)	*0.7*	8.2 (57.7)	*0.7*
Acetate (umol/mL)	6.2 (3.2)	6.8 (3.6)	*0.1*	22.5 (56.1)	6.2 (3.1)	7.7 (2.0)	*0.4*	10.4 (57.7)	*0.7*	6.1 (1.3)	5.4 (2.2)	*0.2*	-15.5 (56.9)	6.9 (1.9)	7.7 (2.1)	*0.9*	8.8 (48.1)	*0.3*
Propionate (umol/mL)	1.9 (1.7)	2.4 (0.8)	*0.2*	12.6 (66.5)	1.7 (1.1)	2.3 (1.3)	*0.2*	32.9 (72.6)	*0.8*	1.4 (1.4)	1.6 (0.7)	*0.9*	-4.8 (77.2)	2.2 (1.0)	2.5 (1.3)	*0.9*	5.4 (61.4)	*1*
Butyrate (umol/mL)	1.2 (0.9)	1.8 (2.0)	*0.1*	42.1 (131.1)	1.0 (0.8)	1.5 (1.2)	*0.6*	16.2 (145.9)	*0.7*	0.9 (0.5)	1.1 (1.1)	*0.3*	32.7 (165.4)	1.5 (1.2)	1.5 (2.1)	*0.5*	17.8 (146.2)	*0.8*
Hormones																		
Ghrelin (pg/mL)	81.3 (5942.7)	90.4 (8933.9)	*0.7*	0.3 (18.8)	73.2 (587.6)	80.0 (362.0)	*0.3*	-3.8 (14.6)	*0.6*	61.1 (27.7)	61.9 (24.2)	*0.6*	2.0 (9.1)	65.8 (32.3)	65.1 (49.1)	*0.6*	2.5 (17.4)	*0.9*
Leptin (pg/mL)	3075.7 (838.5)	2959.3 (1117.6)	*0.6*	-0.5 (18.7)	3069.6 (966.1)	3193.0 (867.4)	*0.5*	0.6 (24.9)	*0.4*	3694.6 (1414.5)	3595.6 (1474.5)	*0.9*	0.6 (11.4)	3240.6 (983.1)	3074.9 (975.5)	*0.6*	0.1 (8.0)	*0.4*

Table 2.6. Summary of changes to cytokines, SCFAs, and satiety hormones from baseline to week 6. Statistical significance of changes within-group were determined by Wilcoxon sign-rank tests while between-group differences (FMT-HF vs HF, FMT-LF VS LF; [week 6 – baseline]/baseline*100) were determined by Mann-U Whitney tests. Data are represented as medians and IQR. **FMT-HF(n=15)**, FMT and high-fermentability fiber group. **HF (n=15)**, high-fermentability fiber group. **FMT-LF (n=14)**, FMT and low-fermentability fiber group. **LF (n=17)**, low-fermentability fiber group. All tests were two-tailed with a p-value <0.05 identifying statistical significance.

Chapter 3: Repeated Fecal Microbial Transplantations and Antibiotic Pre-Treatment are Linked to Improved Clinical Response and Remission in Inflammatory Bowel Disease: A Systematic Review and Pooled Proportion Meta-Analysis

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Institutional Review Board Statement: Ethical review and approval were waived for this study due to the systematic review study design

Informed Consent Statement: Informed consent was waived for this study as this was a systematic review.

3.0 Abstract

The response of patients with inflammatory bowel disease (IBD) to fecal microbial transplantation (FMT) has been inconsistent possibly due to variable engraftment of donor microbiota. This failure to engraft has resulted in the use of several different strategies to attempt optimization of the recipient microbiota following FMT. The purpose of our study was to evaluate the effects of two distinct microbial strategies—antibiotic pre-treatment and repeated FMT dosing—on IBD outcomes. A systematic literature review was designed and implemented in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. A medical librarian conducted comprehensive searches in MEDLINE, Embase, Scopus, Web of Science Core Collection, and Cochrane Library on 25 November 2019 and updated on 29 January 2021. Primary outcomes of interest included comparing relapse and remission rates in patients with IBD for a single FMT dose, repeated FMT dosages, and antibiotic pre-treatment groups. Twenty-eight articles (six randomized trials, 20 cohort trials, two case series) containing 976 patients were identified. Meta-analysis revealed that both repeated FMT and antibiotic pre-treatment strategies demonstrated improvements in pooled response and remission rates. These clinical improvements were associated with increases in fecal microbiota richness and α-diversity, as well as the enrichment of several short-chain fatty acid (SCFA)-producing anaerobes including *Bifidobacterium, Roseburia, Lachnospiraceae, Prevotella, Ruminococcus,* and *Clostridium* related species.

3.1 Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the gastrointestinal tract categorized by Crohn's disease (CD), ulcerative colitis (UC), and indeterminate colitis [1,2].

The incidence of IBD is steadily increasing worldwide [3], as are its extensive healthcare and economic burdens. While IBD is believed to involve a host's genetic predisposition, environmental factors, and an imbalanced gut microbial community, the etiology of IBD has yet to be fully elucidated [4–8]. The complex pathophysiology underlying IBD has led to the current implementation of non-specific therapeutic strategies centered on systemic immunosuppression [9,10]. Despite the significant complications associated with these strategies, ongoing high rates of refractory disease remain [11–13] suggesting that alternative targeted approaches are needed to enhance the clinical efficacy and safety of modern IBD therapies[14].

Accumulating evidence suggests that imbalances in the gut microbiome, a highly diverse community of microorganisms that inhabits the gastrointestinal tract of humans, plays a causative role in the pathogenesis of IBD[15–17]. In general, gut microbial communities of patients with IBD are characterized by reduced microbial diversity, an increased abundance of aerobic pro-inflammatory bacteria, and a reduction in anaerobic bacteria that generate beneficial anti-inflammatory metabolites, such as short-chain fatty acids (SCFA). These findings have fostered growing interest in adopting microbiota-targeted strategies into the forefront of modern IBD therapeutics[18–20] in order to reduce the need for long-term immunosuppressants and their associated adverse complications.

Fecal microbial transplantation (FMT) is one such microbiota-targeted strategy that has shown initial promise for the management of IBD by implanting members of microbiota from healthy donors in an attempt to restore imbalances in host-microbial ecology[21]. However, clinical response of IBD to FMT has shown extensive inter-study heterogeneity[22], which might stem

from the variable engraftment of donor derived microbes and the high or persistent populations of unfavorable pathobionts in the host[23–26]. In this regard, both antibiotic pre-treatments (to lessen competitive interactions) and increased frequency of FMT delivery may both enhance the engraftment of putatively beneficial microbes, correcting dysbiotic populations, and promoting clinical response and disease remission[27–30]. While several trials utilizing either antibiotic pre-treatments[31–34] or repeated FMT regimens[35,36] have been conducted in patients with IBD, no pooled analyses of these findings exist, therefore hindering the optimization of FMT-based IBD therapies.

The purpose of our study was to address this important gap in knowledge by conducting a systematic review and meta-analysis to characterize the effects of antibiotic pre-treatment and repeated FMT dosing on IBD response and remission. Our primary outcome was to compare differences in pooled relapse and remission rates between antibiotic pre-treatment and repeated FMT dosing strategies. Secondary outcomes included comparing differences in fecal microbiota composition associated with disease response and remission for these two approaches.

3.2 Methods

3.2.1 Eligibility Criteria

A systematic literature search strategy was designed using the Population, Intervention, Comparison, Outcome, and Study Design (PICOS) framework and implemented in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. FMT was defined as the administration of a fecal matter solution from a healthy donor to the gastrointestinal tract of a recipient to confer a health benefit. Our inclusion criteria

included studies with adults (age ≥ 18 years) that had a diagnosis for IBD and received FMT. All modalities of FMT delivery, such as colonoscopy, nasogastric tube, oral capsules, or enemas, and any regimen of antibiotic pre-treatment were included. Studies were excluded if disease was localized to the surgical pouch (i.e., pouchitis), patients had concurrent *Clostridioides difficile* infection, less than six patients were enrolled, or in a pediatric population. Duplicate studies, kin studies, studies using animal models, and non-English studies were also excluded.

3.2.2 Search Strategy

A medical librarian (JK) systematically searched the MEDLINE (via Ovid), Embase (Ovid), Scopus, Web of Science Core Collection, and Cochrane Library (via Wiley) databases on 25 November 2019 (see *Table S3.1* for full-text search strategy) and updated on 29 January 2021. No language or date limits were applied. To complement this approach, the research team also screened the first 200 results from Google Scholar for inclusion. Manual searches of references from included studies were further performed to identify potentially missed articles.

3.2.3 Study Selection

Titles and abstracts of relevant articles were first manually screened for inclusion by two independent reviewers (VM, SR). Studies meeting initial screening criteria by at least one reviewer were selected for a full text review by two independent reviewers (VM, SR) using pre-specified inclusion and exclusion criteria. Disputes were resolved by a third reviewer (JD). Data were extracted independently by two reviewers (VM, SR) into separate Excel spreadsheets and cross-examined for accuracy. Studies were then assessed for methodological quality and bias

using the Newcastle-Ottawa[37] tool for cohort studies and the Cochrane Risk of Bias[38] evaluation for randomized controlled trials (RCTs).

3.2.4 Data Extraction

Study characteristics were evaluated for study design, year, and country of origin. Primary outcomes of interest included relapse and remission rates following FMT. Secondary outcomes included differences in fecal microbiota composition, and adverse events. Patient characteristics included age, sex, mean disease duration, type of IBD, histology disease scoring, and current medications. FMT strategy-specific variables included donor stool processing, mode of delivery, type of FMT regimen, and type and duration of antibiotic pre-treatments.

3.2.5 Data Synthesis

Continuous data were expressed as mean ± standard deviation (SD). For the purpose of meta-analysis, data extracted as medians and interquartile ranges were converted to mean ± SD using methods outlined by Hozo et al.[39]. Meta-analyses of pooled proportions were conducted using a random effects models by the DerSimonian-Laird method[40]. Estimates of heterogeneity were obtained from inverse-variance fixed-effect models. Pooled estimate variances were stabilized using the Freeman-Tukey Double Arcsine Transformation. Heterogeneity was assessed using the Chi-squared test with significance set at $p < 0.10$ and the amount of heterogeneity quantified by the I^2 statistic as low <50%, moderate 50–75%, or high >75%[41]. Categorical data were assessed using either Chi-squared or Fischer's exact tests. A two-sided α of less than 0.05 was considered statistically significant. Meta-analysis was conducted using the metaprop function in STATA (v15.1; StataCorp, College Station, TX, USA).

3.3. Results

3.3.1 Search Results and Study Characteristics

Comprehensive search of the five databases yielded a total of 4220 results, and after duplicate records were removed, 3624 articles remained (*Figure 3.1*). After initial screening of the titles and abstracts, the text of 45 articles were fully reviewed. Following full text review, 28 articles were eligible for inclusion in the final systematic review. No prior systematic reviews examining FMT outcomes with respect to antibiotic pre-treatment or repeated FMT regimens were identified. Of the included articles, six were randomized controlled trials, 20 were prospective cohort trials, and two studies were case series.

Of the 28 studies reviewed, 22 included patients with UC, four included patients with CD, and two studies assessing both UC and CD. Most studies examined disease response in patients with mild to moderate disease (n = 9 studies), with twelve studies assessing patients with severe disease (*Table 3.1*). Study duration and follow-up ranged from 4 weeks to 13 years with most studies having a follow up $\leq$ 12 weeks (n = 17). Five studies utilized pre-operative antibiotics prior to FMT, with only two studies utilizing the same antibiotic regiments. Nearly half of the studies included a single FMT delivery (n = 12), while the remaining trials use varied regimens.

3.3.2 Risk of Bias Assessment

Risk of bias for cohort studies was characterized using an adjusted 7-point Newcastle-Ottawa scale of selection, comparability, and study outcome categories (*Table S3.2*). The 19 included cohort studies demonstrated low to moderate risk of bias due to a lack of long-term follow-up

greater than three months (*n* = 7 studies), and inadequate description or evaluation fecal microbiota changes (*n* = 8 studies). The six randomized trials were assessed for bias using the Cochrane Risk of Bias tool and together demonstrated low risk of bias.

3.3.3 Baseline Demographics

A total of 976 patients were identified from the 28 studies included (*Table 3.2*). Twenty-two studies included only patients with UC (*n* = 767), while three studies included patients with CD (*n* = 87) alone. The mean weighted age of all patients was 40.0 years, of which 59% were on average male with a mean weighted disease duration of 6.2 years. The proportion of patients receiving concurrent corticosteroids varied extensively from 7% to 100%. Patients with a diverse spectrum of IBD severity were included although the majority of included patients had mild-moderate disease (*n* = 439; 9 studies). Prior to FMT, total Mayo scores for UC activity ranged from 6.1 to 11.1 and CD activity index ranged from 275 to 345. No significant differences in clinical characteristics were observed between CD and UC patients prior to FMT.

3.3.4 FMT Administration, Dosing, and Donor Characterization

FMT methodologies varied substantially across all studies. The most frequent mode of FMT was via colonoscopy (*n* = 19 studies), followed by nasoduodenal/naso-jejunal tube (*n* = 4 studies), enemas (*n* = 4 studies), gastroscopy (*n* = 3 studies), and oral capsules (*n* = 1 study). The dosage of FMT ranged from 12 g to 300 g of stool per administration with 50% (n = 10 studies) of all studies delivering multiple doses. Antibiotic pre-treatment regimens ranged from three to 14 days prior to FMT (*n* = 5 studies), with most studies using a combination of antibiotics (*n* = 4 studies) and specifically vancomycin (*n* = 3 studies). FMT donors of included studies were

typically healthy donors unrelated to the recipients. Nine studies utilized donors that were either relatives or specifically chosen by the patients.

3.3.5 Response and Remission Rates for Repeated FMT Regimens

Of the 976 patients included, 41.9% (*n*=409) were treated with a single FMT and 30.0% (*n*=229) with repeated FMT (*Tables 3.1 and 3.3*). Meta-analysis of all included studies revealed that repeated FMT studies had higher pooled response rates (15 studies; 70%; 95% CI 59–80%; I^2 = 72%; *Figure 3.2a*) than those with single FMT (13 studies; 53%; 95% CI 39–67%; I^2 = 80%; *Figure 3.2b*). Pooled remission rates for studies with multiple FMTs (15 studies; 43%; 95% CI 31–56%; I^2 = 82%; *Figure 3.2c*) were also higher than for studies with a single FMT (13 studies; 30%; 95% CI 15–47%; I^2 = 88%; *Figure 3.2d*).

Subgroup analysis of UC studies revealed more pronounced differences in pooled response (12 studies; 72%; 95% CI 61–83%; I^2 = 71% vs. 10 studies; 47%; 95% CI 34–61%; I^2 = 75%) and remission rates (12 studies; 43%; 95%CI 30–57%; I^2 = 82% vs. 10 studies; 19%; 95% CI 8–34%; I^2 = 83%) when comparing repeated and single FMT regimens, respectively. Taken together, pooled response and remission rates were more favorable for patients receiving repeated FMT regimens than single FMT alone. Heterogeneity for all pooled analyses was high with all I^2 values greater than 70%.

3.3.6 Response and Remission Rates for Antibiotic Pre-Treatments

Antibiotics were not frequently administered as pre-treatments, with only 11.2% (*n*=109) of patients receiving an antibiotic regimen prior to FMT. Meta-analysis of included studies revealed

that pooled response rates for antibiotic pre-treatment (five studies; 82%; 95% CI 58–98%; I^2 = 82%; *Figure 3.3a*) were higher than for no pre-treatment (23 studies; 58%; 95% CI 48–68%; I^2 = 77%; *Figure 3.3b*). Likewise, antibiotic pre-treatment was also associated with improved remission rates (five studies; 66%; 95%CI 31–94%; I^2 = 91%; *Figure 3.3c*) when compared to no pre-treatment (23 studies; 31%; 95%CI 21–43%; I^2 = 86%; *Figure 3.3d*).

The favorable effect of antibiotic pre-treatment on pooled response (four studies; 73%; 95% CI 52–90%; I^2 = 68% vs. 17 studies; 58%; 95% CI 48–70%; I^2 = 80%) and remission rates (four studies; 51%; 95% CI 24–77%; I^2 = 81% vs. 18 studies; 29%; 95% CI 17–42%; I^2 = 88%) was also observed on subgroup analysis of UC studies. Similar to the repeated FMT analysis, heterogeneity for the pooled proportion analyses of antibiotic pre-treatment was high.

3.3.7 Fecal Microbiota Compositional Changes Following FMT

3.3.7.1 Overview of Microbiota Reporting of Included Studies

Although FMT aims to shift the gut microbial communities of patients with IBD, only 64% of studies (*n* = 18 studies) characterized the recipient's fecal microbiota following FMT and only two studies directly assessed associations between IBD remission and fecal microbiota compositional changes (*Table 3.4*). Further, no study directly compared microbial changes of antibiotic pre-treatment vs. no pre-treatment or repeated FMT vs. single-dose FMT. Only five studies provided donor microbial characterization. The majority of studies (*n*= 14 studies) used 16 s rRNA gene amplicon sequencing methods, with three studies using whole-genome sequencing and one using *Bacteroides* HSP60 sequencing.

3.3.7.2 Changes in Alpha and Beta Diversity Following FMT

Of these 18 studies, nine (50%) reported an increase in microbial richness and α-diversity following FMT, as estimated by the abundance of operational taxonomic units (OTUs), Chao1, Simpson and Shannon indices. Six studies reported no change in α-diversity after FMT. Changes in β-diversity evaluated using Bray-Curtis dissimilarity were reported in five studies, with the majority (n = 4 studies) showing that the microbial ecology of FMT recipients underwent shifts towards those of their respective donors. Within these four studies, increased engraftment was associated with improved clinical outcomes.

In terms of specific bacterial shifts, the effects of FMT were shown to be highly variable (Table 3.4). Nonetheless, 15 of the 18 studies (83%) that evaluated for shifts in specific gut microbial taxa reported increases in the abundance of anaerobes purported to produce health promoting anti-inflammatory SCFAs, such as *Bifidobacterium*, *Roseburia*, *Lachnospiraceae*, *Prevotella*, *Ruminococcus*, and *Clostridium* related species.

3.3.7.3 Recipient and Donor Microbial Ecology Associated with IBD Outcomes

Findings from the two studies that assessed associations between IBD remission and fecal microbiota compositional and functional changes were also variable. Parmsothy et al. provided the best assessment of bacterial taxa and corresponding metabolic pathways related to specific IBD outcomes. Following intensive multi-donor FMT, patients with sustained remission had increased relative abundance of *Eubacterium halii*, *Roseburia inulivorans*, and *Ruminococcus* while those who relapsed had higher proportions of *Fusobacterium*, *Escherichia*, and *Prevotella.* Metabolomics of remission patients further revealed increased activation of metabolic pathways

associated with the biosynthesis of SCFAs and secondary bile acids. In addition, only one study by Kump and colleagues explored the role of donor microbiota with respect to IBD outcomes following FMT. Patients that received donor fecal microbiota of greater bacterial richness and α-diversity (assessed by OTU abundance and Shannon diversity) and with increased *Ruminococcus* and *Akkermansia* abundances were shown to have higher rates of IBD remission.

3.3.8 Reported Adverse Events

Overall, FMT in patients with IBD was shown to be safe and well tolerated. Frequently reported symptoms related to FMT included a transient self-limiting fever alleviated with paracetamol, and non-specific transient gastrointestinal symptoms such as abdominal discomfort, bloating, nausea, vomiting, and diarrhea (*Table 3.5*). Of 26 studies that reported serious adverse events, 13 patients with UC required colectomies and one required hospitalization due to disease progression. One patient also contracted *Clostridioides difficile* requiring a colectomy and one patient contracted cytomegalovirus infection seven weeks after FMT. Overall, the reported serious adverse events were suggested by the authors to be unrelated to the FMT therapy. No patient receiving FMT intervention in the included studies suffered mortality.

3.4 Discussion

To our knowledge, we present the first systematic review and meta-analysis evaluating the effects of antibiotic pre-treatment and repeated FMT approaches on improving response in patients with IBD response. Notably, our meta-analysis revealed that repeated FMT and antibiotic pre-treatment were associated with improvements in both pooled IBD response and pooled remission rates. These improvements were associated with key changes in fecal microbial

composition such as increased bacterial richness, α-diversity and relative abundance of anaerobes purported to produce SCFAs. Taken together, our findings are novel in that they highlight the potential of these microbiota-targeted strategies to optimize the efficacy of FMT for the management of IBD.

Our findings are in agreement with previous systematic reviews and meta-analyses examining the impact of FMT as a therapy for IBD. In 2014, Colman et al. first identified a lack of literature characterizing FMT treatment efficacy despite publications investigating FMT therapy for IBD more than doubling since 2012 [64]. The systematic review and meta-analysis of 18 studies consisting of 122 IBD patients by Colman and colleagues further revealed that the pooled proportion of patients achieving clinical remission was 36.2% (95% CI 17.4–60.4%). The authors concluded that, while FMT demonstrated variable efficacy, further rigorously designed RCTs were needed to determine efficacy, with a particular need for studies that investigate the effects of FMT frequency and route of administration. More recently, Imdad et al. conducted a 2018 Cochrane review examining FMT therapy on IBD response and remission [65]. Four studies with a total of 277 UC patients were identified and revealed an improved clinical response (RR 1.70; 95% CI 0.98–2.95) and endoscopic remission (RR 2.96; 95% CI 1.60–5.48) for patients receiving FMT vs. placebo. These systematic reviews were, however, limited by a lack of high-quality RCTs and standardized fecal microbiota analysis. Our study addresses a number of these gaps by evaluating both high-quality RCTs and cohort studies, which allowed us to specifically characterize the impact of FMT frequency and antibiotic pre-treatment on IBD outcomes.

Repeated FMT strategies have been employed with variable success in a number of different clinical entities thought to be associated with imbalances in host-microbial ecology[42–44]. Perhaps the most compelling evidence for repeated FMT is observed in the *Clostridiodes difficile* infection (CDI) literature. In a recent systematic review and meta-analysis by Baunwall et al., repeated FMT was found to be superior to single-dose FMT in management of recurrent CDI (91% vs. 84%)[44]. Similarly, El-Salhy et al. demonstrated an increased clinical efficacy for repeated FMT dosing in patients with irritable bowel syndrome, albeit in a small case series of 10 patients[43]. Lastly, in a double-blinded placebo-controlled pilot trial, repeated FMT in patients with obesity and metabolic syndrome demonstrated successful engraftment of donor derived microbes, but without any clinical improvements in host metabolic parameters[42]. These inconsistencies are in large part due to the dramatic study heterogeneity with respect to donor selection, FMT preparation and route of delivery, as well as underlying differences in host-gut microbiome interactions implicated in disease pathophysiology[45]. Notwithstanding, our study findings indeed suggest that repeated FMT dosing provides a promising approach to improve IBD outcomes by facilitating donor microbe engraftment, increase α-diversity, and promote SCFA producing taxa.

Ongoing debate exists regarding the pre-treatment of recipients with antibiotics prior to FMT to increase efficacy[46,47]. Conceptually, antibiotic pre-treatment helps provides a proverbial ecological clean slate for the engraftment of donor microbes by freeing up otherwise occupied niches. Elegant work by Ji et al. compared antibiotic pre-treatment versus bowel cleansing or no pre-treatment in mice prior to FMT. The authors demonstrated that FMT efficacy was dependent on the number of niches available for donor microbe engraftment[48]. Further, they found that

antibiotic pre-treatment proved to be the most effective strategy for enhancing host gut microbiota reprogramming by increasing donor microbe colonization. Work by Freitag et al., on the other hand, demonstrated that antibiotic pretreatment prior to FMT in mice had only minor effects on overall donor microbial engraftment[46]. Antibiotics disrupted pre-FMT host microbial communities, yet only select donor-derived bacterial taxa such as *Bifidobacterium* were increased and no improvements in overall similarity to the donor microbiota were noted. Indeed, questions remain regarding the optimal antibiotic regimens required to make niches accessible, which niches should be targeted for FMT re-colonization, and whether the potential benefit surpasses the potential harm associated with antibiotic resistance and CDI. While our findings are promising as they show improvements in IBD remission and relapse for groups receiving antibiotic pre-treatment prior to FMT, further studies are needed that evaluate the mechanisms and implications of similar approach on IBD.

We acknowledge that our systematic review and meta-analysis has a number of important limitations. Pooled analysis of our primary outcomes demonstrated a high degree of heterogeneity and does not allow for direct comparison of effect size associated with either repeated FMT or antibiotic pre-treatment regiments. The heterogeneity of our results was extensive and, in a large part, due to differences in study design, FMT regimens and individualized responses to FMT. In general, the administration and preparation of FMT is not standardized with practice patterns varying dramatically. Major differences in route of delivery, donor selection, dosing rationale, and antibiotic pre-treatment regimen are all likely to promote inter-study heterogeneity in our review. Follow-up timeframes also ranged from two weeks to 13 years, with nearly half of the studies having a follow up <3 months. This may have introduced a

bias towards more favorable clinical response and remission rates following FMT therapy. Therefore, arguments can be made that, given the immense variability of such disparate study interventions, more focused inclusion criteria are warranted in future studies. As this is the first IBD review to evaluate repeated FMT and antibiotic pretreatment concepts, we elected a priori to broadly include all potentially relevant literature in order to highlight current limitations and to allow for explorative hypothesis generation.

Correlations regarding outcomes and antibiotic pre-treatment should also be interpreted with caution given the small proportion of patients within included studies and the lack of direct comparison with patients receiving FMT alone. Histologic assessments pre- and post- FMT were also not consistently reported across studies hindering our ability to evaluate the histologic effects of FMT on disease activity, or the effects of FMT on mucosal adherent bacterial communities. The findings of our review also heavily favored patients with UC and are therefore less generalizable to CD. Additionally, consistent reporting and analysis of fecal microbiota compositional data for both donors and patients were not reported across all studies, which limits the ability to elucidate potential underlying features of the gut microbiome important for optimizing clinical efficacy. Finally, our literature search revealed a number of abstracts and protocols not ultimately published as final manuscripts, which is indicative of publication bias in the FMT literature.

Despite these limitations, our study provides the first systematic review and meta-analysis that evaluates the impact of two key microbial-based strategies which optimize the efficacy of FMT on IBD outcomes. Results of this study may have a number of important implications. Firstly, we

demonstrate that repeated FMT dosing and antibiotic pre-treatment approaches have a promising role in optimizing IBD remission and response rates following FMT. Second, results of this study also highlight a need for standardization of FMT therapy protocols (donor, dose, delivery, and pre-treatment) and reporting of microbial data as the lack of this data seen in current practices preclude meaningful meta-analysis of microbial ecology. Lastly, additional high quality randomized trials are needed which directly compare these two strategies in order to help overcome the high degree of heterogeneity in present studies and to elucidate the mechanisms through which these improved outcomes occur. Only through such standardization practices can we eventually bring tailored microbial transplant therapies from the forefront of current IBD research to standard clinical practice.

3.5 Conclusions

Repeated fecal microbial transplantation and antibiotic pre-treatment engraftment strategies in patients with IBD were associated with improvements in pooled response and remission rates following FMT. These improvements were associated with an increase in fecal microbiota richness, α-diversity, and several SCFA-producing anaerobic taxa. Further standardization of FMT therapies is required to bring microbial-targeted therapies based on FMT from the forefront of current IBD research to modern clinical practice.

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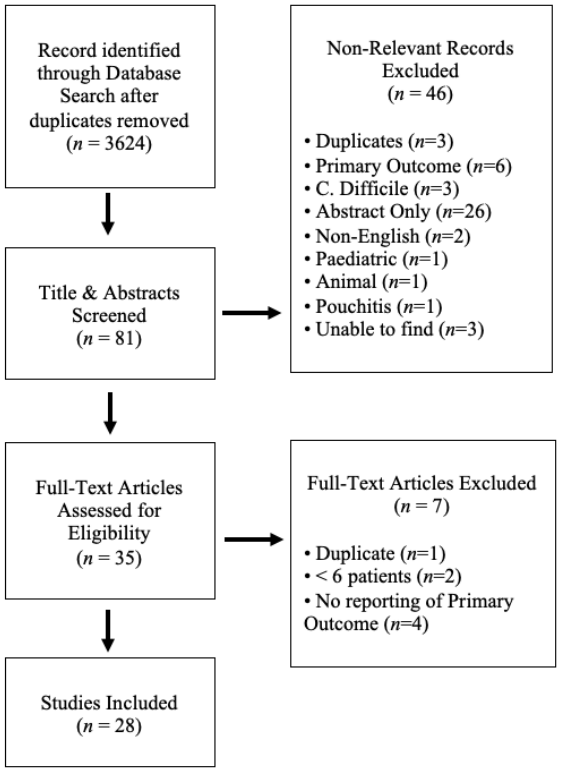

Figure 3.1. PRISMA flow chart of assessed studies.

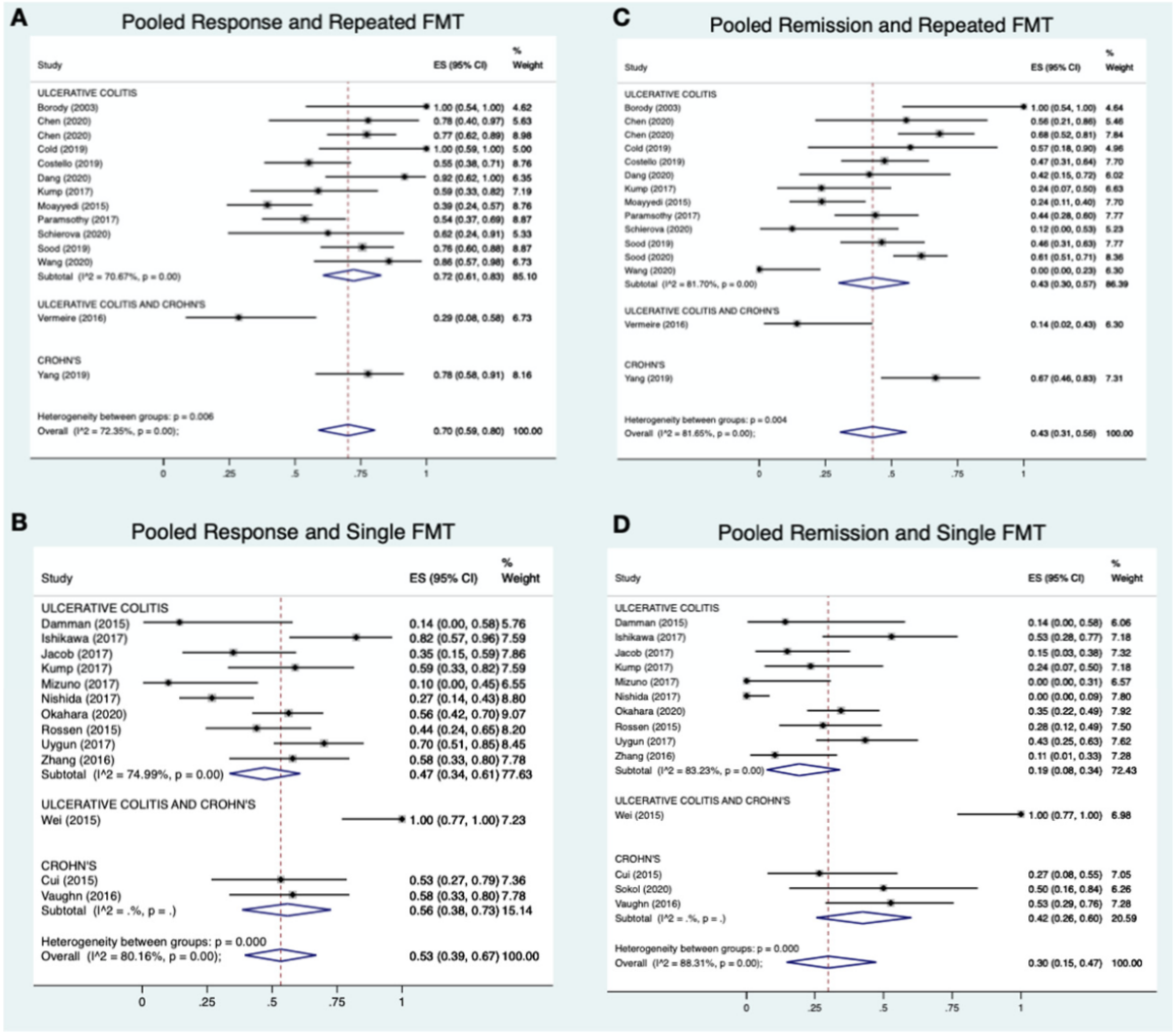

Figure 3.2. A-2D: Meta-analysis of pooled response and remission rate for repeated vs. single FMT.

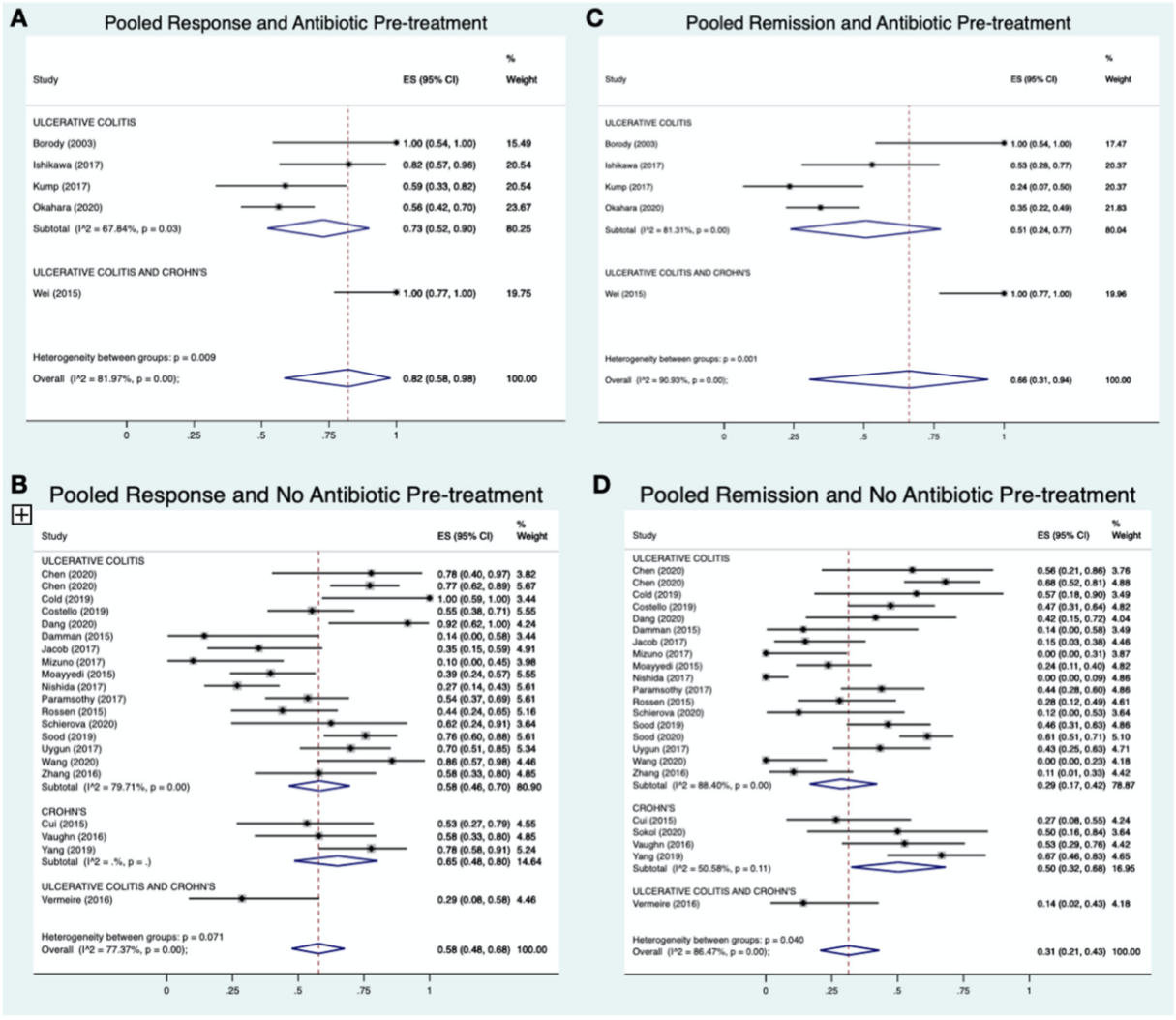

Figure 3.3. A-3D**:** Meta-analysis of pooled response and remission rate for antibiotic pre-treatment vs. no pre-treatment.

Table 3.1. Study design and FMT regimen characteristics.

Study	Study Design	Patients (*n*)	Country	Disease	Severity	FMT Delivery	FMT Donor	FMT Dosage	FMT Frequency	Pre-Treatment Antibiotics	Antibiotic Frequency	Total Follow-up (Weeks)
Borody 2003[31]	Case series	6	Australia	UC	Severe	Enema	Healthy donors chosen by patient	200–300 g/200–300 mL saline	Daily for 5 days	Vancomycin (500 mg bid), metronidazole (400 mg bid), rifampicin (150 mg bid)	7–10 days	676
Chen 2020[49]	Prospective cohort	9	China	UC	Moderate-severe	Naso-jejunal	Healthy donor	200–250 mL of fecal suspension	3 doses at 1, 3 and 5 days	-	-	12
Chen 2020[50]	Prospective cohort	44	China	UC	Mild-moderate	Colonoscopy	Healthy donor	150–200 g stool/1000 mL saline	x3 in 1 week	-	-	12
Cold 2019[51]	Prospective cohort	7	Denmark	UC	Active	Oral capsules	Healthy volunteers	12 g daily dose of 25 capsules	25 capsules/day for 50 days	-	-	24
Costello 2019[35]	RCT	73	Australia	UC	Mild-moderate	Colonoscopy and enema	Healthy volunteers recruited by advertisement	50 g/200 mL saline colonoscopy, 25 g/100 mL saline enema	1x colonoscopy then 2x enemas over 1 week	-	-	8
Cui 2015[52]	Prospective cohort	30	China	CD	Moderate-severe	Gastroscopy	Related or unrelated volunteer	60 mL/100 mL saline	x1	-	-	65
Dang 2020[53]	Case series	12	China	UC	Moderate-severe	Colonoscopy	Healthy volunteers	15 mL bacterial pellet in 75 mL saline	multiple, exact frequency NR	-	-	52
Damman 2015[54]	Prospective cohort	7	USA	UC	Mild-moderate	Colonoscopy	Chosen by patient	Diluted with 2–3 mL saline/g of stool	x1	-	-	12
Ishikawa 2017[55]	Prospective cohort	36	Japan	UC	Mild-severe	Colonoscopy	Spouse or relative	150–250 g/350–500 mL saline	x1	Amoxicillin (1500 mg/day), Fosfomycin (3000 mg/day), metronidazole (750 mg/day)	2 weeks until 2 days before FMT	4
Jacob 2017[56]	Prospective cohort	20	USA	UC	Active	Colonoscopy	Healthy donor	60 mL	x1	-	-	4
Kump 2017[57]	Prospective cohort	27	Austria	UC	Mild-severe	Colonoscopy	Related or unrelated volunteer	50 g/200–500 mL saline	5x, 14 days apart	Vancomycin (250 mg qid), paromomycin (250 mg tid), nystatin (10 mL, 1 million IE qid)	10 days	13
Mizuno 2017[58]	Prospective cohort	10	Japan	UC	Moderate-severe	Colonoscopy	Healthy relatives	50–300 g/50–100 mL saline	x1	-	-	12
Moayyedi 2015[59]	RCT	70	Canada	UC	Mild-moderate	Enema	Healthy donors	50 g/300 mL water	x6; 0,1,2,3,4,5,6 weeks	-	-	7
Nishida 2017[60]	Prospective cohort	41	Japan	UC	Mild-moderate	Colonoscopy	Healthy relatives	150–200 g/500 mL saline	x1	-	-	12
Okahara 2020[61]	Prospective cohort	92	Japan	UC	Mild-severe	Colonoscopy	Spouses and relatives	350–500 mL filtered bacterial suspension infusion	X1	Amoxicillin (1500 mg/day), Fosfomycin (3000 mg/day), metronidazole (750 mg/day)	2 weeks prior to FMT	104

Paramsothy 2017[62]	RCT	85	Australia	UC	Mild-moderate	Colonoscopy and enema	Healthy volunteers recruited by advertisement	37.5 g	x5/week for 8 weeks	-	-	8
Rossen 2015[63]	RCT	50	Finland	UC	Mild-moderate	Nasoduodenal tube	Relatives, partner, or volunteer	120 g	x2; 3 weeks apart	-	-	12
Schierova 2020[64]	Prospective cohort	16	Czech Republic	UC	NR	Enema	Healthy donors	50 g stool/150 mL saline	5x/week for 1 week then weekly x 6 weeks	-	-	12
Sokol 2020[65]	RCT	17	France	CD	NR	Colonoscopy	Healthy donors	50–100 g/250–350 mL saline	X1	-	-	24
Sood 2019[66]	Prospective cohort	41	India	UC	Mild-moderate	Colonoscopy	Two healthy unrelated volunteers	NR	x7; 0,2,6,10,14,18,22 weeks	-	-	22
Sood 2020[67]	Prospective cohort	140	India	UC	Moderate-severe	Colonoscopy	Healthy donors	80 g stool/ 200 mL saline	X7; 0, 2, 6, 10, 14, 18, 22 weeks	-	-	30
Uygun 2017[68]	Prospective cohort	30	Turkey	UC	Moderate-severe	Colonoscopy	Relatives, partner, or volunteer	120–150 g	x1	-	-	12
Vaughn 2016[69]	Prospective cohort	19	USA	CD	Active	Colonoscopy	Healthy unrelated volunteers	50 g/250 mL saline	x1	-	-	4
Vermeire 2016[70]	Prospective cohort	14	Belgium	UC+ CD	Refractory	Naso-jejunal or rectal tube	Family, friend, or partner	200 g/400 mL saline	x2; 2 consecutive days	-	-	8
Wang 2020[71]	Prospective cohort	16	China	UC	Moderate- severe	Colonoscopy	Healthy donor	100 g stool/ 500 mL saline	x3; 2–3 month intervals	-	-	>24
Wei 2015[34]	Prospective cohort	14	China	UC+ CD	NR	Colonoscopy or naso-jejunal tube	Healthy unrelated donor	60 g/350 mL saline	x1	Vancomycin (500 mg)	Twice a day for 3 days before FMT	4
Yang 2019[72]	RCT	27	China	CD	Mild-moderate	Gastroscopy or colonoscopy	Healthy donors	200 g in saline	x2; 1 week apart	-	-	2
Zhang 2016[73]	Prospective cohort	19	China	UC	Moderate-severe	Gastroscopy	NR	NR	x1	-	-	13

IBD—Inflammatory Bowel Disease; FMT—Fecal Microbiota Transplantation; UC—Ulcerative Colitis; CD—Crohn's Disease; NR—Not recorded; RCT—Double-blinded, randomized controlled trial.

Table 3.2. Baseline characteristics of patients for included studies.

Study	Disease	Intervention Arm	Patients (*n*)	Age	Sex (% male)	Disease Duration (years)	Ongoing Systemic Corticosteroids (%)	Total Mayo Score	CDAI
Borody 2003	UC	**Antibiotic pre-treatment and repeated FMT**	6	35.8 (11.0)	50.0	11.7 (5.8)	NR	NR	-
Chen 2020	UC	**Repeated FMT**	9	47.9 (10.6)	77.8	5.3 (5.1)	33.3	5.9 (2.0)	-
Chen 2020	UC	**Repeated FMT**	44	44.4 (15.5)	57	4.6 (2.1)	25.0	5.9 (2.0)	-
Cold 2019	UC	**Repeated FMT**	7	38.3 (5.8)	71.4	10.8 (3.8)	NR	NR	-
Costello 2019	UC	**Repeated donor FMT**	38	38.5 (6)	53.0	4.9 (4.8)	21.0	7.2 (1.7)	-
		Repeated autologous FMT	35	35.0 (5.25)	57.0	5.8 (2.2)	31.0	7.4 (1.9)	-
Cui 2015	CD	**Single FMT**	30	38.0 (13.8)	64.5	7.4 (5.3)	56.7	NR	NR
Damman 2015	UC	**Single FMT**	8	41.1 (15.5)	25.0	16.6 (13.1)	NR	NR	-
Dang 2020	UC	**Repeated FMT**	12	51 (14.0)	66.0	NR	41.7	NR	-
Ishikawa 2017	UC	**Antibiotic pre-treatment and single FMT**	17	40.4 (14.2)	76.5	7.8 (8.4)	29.4	7.5 (1.9)	-
		Antibiotic pre-treatment only	19	44.8 (14.9)	63.2	7.0 (8.0)	47.4	8.2 (2.2)	-
Jacob 2017	UC	**Single FMT**	20	38.4 (12.6)	60.0	NR	30.0	8.1 (2.4)	-
Kump 2017	UC	**Antibiotic pre-treatment and repeated FMT**	17	44.0 (18.0)	82.0	8.0 (8.0)	59.0	8.9 (1.6)	-
		Antibiotic pre-treatment only	10	36.0 (13.0)	30.0	7.0 (6.0)	30.0	8.1 (3.1)	-
Mizuno 2017	UC	**Single FMT**	10	31.8 (7.8)	70.0	6.25 (3.5)	NR	6.1 (1.0)	-
Moayyedi 2015	UC	**Repeated FMT**	38	42.2 (15.0)	47.0	7.9 (5.6)	39	8.2 (2.6)	-
		Placebo	37	35.8 (12.1)	70.0	7.0 (6.8)	35	7.9 (2.3)	-
Nishida 2017	UC	**Single FMT**	41	39.6 (16.9)	68.3	7.6 (8.6)	26.8	5.6 (2.4)	-

Okahara 2020	UC	**Antibiotic pre-treatment Single FMT**	55	40.1 (13.3)	69.1	8.6 (7.4)	43.2	6.3 (4.1)	-
Paramsothy 2017	UC	**Repeated FMT**	41	35.6 (5.3)	54.0	5.8 (1.4)	22.0	8 (0.8)	-
		Placebo	40	35.4 (4.5)	63.0	5.8 (1.4)	28.0	8 (0.8)	-
Rossen 2015	UC	**Single donor FMT**	23	42.3 (5.8)	47.8	7 (NR)	21.7	NR	-
		Single autologous FMT	25	41 (4.5)	44.0	9 (NR)	20.0	NR	-
Schierova 2020	UC	**Repeated FMT**	8	41.3 (10.1)	50.0	NR	0	5.8 (1.7)	-
		Medical therapy	8	44.3 (10.4)	50.0	NR	25.0	6.0 (1.5)	-
Sokol 2020	CD	**Single FMT**	8	31.8 (6.8)	62.5	8.5 (8.1)	100	NR	89 (30.5)
		Placebo	9	38.3 (6.0)	44.4	11.3 (2.0)	100	NR	61.5 (20.1)
Sood 2019	UC	**Repeated FMT**	41	36.5 (10.7)	58.5	4.6 (4.2)	100	8.8 (2.6)	-
Sood 2020	UC	**Repeated FMT**	93	35 (11)	62.4	5.2 (4.6)	78.5	8.1 (2.0)	-
Uygun 2017	UC	**Single FMT**	30	34.6 (10.3)	46.7	5.3 (3.3)	NR	11.1 (1.1)	-
Vaughn 2016	CD	**Single FMT**	19	36 (12.3)	63.0	12.5 (10.6)	42.0	NR	NR
Vermeire 2016	UC and CD	**Repeated FMT**	14	38.6 (8.2)	50.0	10.2 (7.5)	21.4	8.4 (0.6)	290 (29)
Wang 2020	UC	**Repeated FMT**	16	39.5 (4)	62.5	7.5 (5.8)	NR	9.9 (2.2)	-
Wei 2015	UC and CD	**Antibiotic pre-treatment and single FMT**	14	43.5 (16.4)	42.9	4.1 (3.2)	7.1	5.8 (1.9)	345 (77.8)
Yang 2019	CD	**Repeated FMT**	30	72.2 (10.8))	57.9	1.3 (0.4)	NR	NR	283 (131)
Zhang 2016	UC	**Single FMT**	19	39.2 (14.1)	36.8	8.0 (5.8)	NR	10.5 (1.7)	-

Values are presented as mean +/−SD; UC- ulcerative colitis; CD- Crohn's disease; NR—Not Recorded; CDAI- Crohn's Disease Activity Index.

Table 3.3. Response and remission rates for included studies.

Study	Intervention Arm	Follow-Up at Response/Remission (weeks)	Patients (*n*)	Response (%)	Remission (%)
Borody 2003	Antibiotic pre-treatment and repeated FMT	676	6	6 (100%)	6 (100%)
Chen 2020	Repeated FMT	2 weeks for response 12 weeks for remission	9	7 (77.8%)	5 (55.6%)
Chen 2020	Repeated FMT	12	44	34 (77.3%)	30 (68.2%)
Cold 2019	Repeated FMT	24	7	7 (100%)	4 (57.1%)
Costello 2019	Repeated donor FMT	8	38	21 (55%)	18 (47%)
	Repeated autologous FMT	8	35	8 (23%)	6 (17%)
Cui 2015	Single FMT	12–72	15	8 (53.3%)	4 (26.7%)
Dang 2020	Repeated FMT	52	12	11 (91.7%)	5 (41.7%)
Damman 2015	Single FMT	4	7	1 (14.3%)	1 (14.3%)
Ishikawa 2017	Antibiotic pre-treatment and single FMT	4	17	14 (82.3%)	9 (52.9%)
	Antibiotic pre-treatment only	4	19	13 (68.4%)	3 (15.8%)
Jacob 2017	Single FMT	4	20	7 (35%)	3 (15%)
Kump 2017	Antibiotic pre-treatment and repeated FMT	13	17	10 (59%)	4 (24%)
	Antibiotic pre-treatment only	13	10	1 (10%)	0 (0%)
Mizuno 2017	Single FMT	12	10	1 (10%)	0 (0%)
Moayyedi 2015	Repeated FMT	7	38	15 (39%)	9 (24%)
	Placebo	7	37	9 (24%)	2 (5%)
Nishida 2017	Single FMT	8	41	11 (26.8%)	0 (0%)
Okahara 2020	Single FMT	4	55	31 (56.3%)	19 (34.5%)
Paramsothy 2017	Repeated FMT	8	41	22 (54%)	18 (44%)
	Placebo	8	40	9 (23%)	8 (20%)
Rossen 2015	Repeated donor FMT	12	23	11 (47.8%)	7 (30.4%)
	Repeated autologous FMT	12	25	13 (52.0%)	8 (32.0%)
Schierova 2020	Repeated FMT	12	8	5 (62.5%)	1 (12.5%)
Sokol 2020	Single FMT	24	8	NR	4 (50%)
Sood 2019	Repeated FMT	22	41	31 (75.6%)	19 (46.3%)
Sood 2020	Repeated FMT	30	93	NR	57 (61.3%)
Uygun 2017	Single FMT	12	30	21 (70%)	13 (43.3%)
Vaughn 2016	Single FMT	4	19	11 (58%)	10 (53%)
Vermeire 2016	Repeated FMT	6 weeks for response 8 weeks for remission	14	4 (50%)	2 (14.3%)
Wang 2020	Repeated FMT	>6 mo	16	14 (87.5%)	0 (0%)

Wei 2015	Antibiotic pre-treatment and single FMT	4	14	14 (100%)	14 (100%)
Yang 2019	Repeated FMT	*2*	27	21 (77.8%)	18 (66.7%)
Zhang 2016	Single FMT	13	19	11 (57.9%)	2 (10.5%)

Table 3.4. Effect of fecal microbial transplant therapy on microbiota composition.

Study	Methods	Donor Microbiota Differences vs. Recipient	Recipient Microbiota Changes Following FMT	Recipient Microbiota Changes Associated with Response/Remission
Borody 2003	NR	NR	NR	NR
Chen 2020	NR	NR	NR	NR
Chen 2020	16 s rRNA	↑ α—diversity (Shannon, Chao1)	↑ α—diversity (Shannon, Chao1) ↑ *F. Prausnitzii*	NR
Cold 2019	16 s rRNA	NR	No change in α—diversity (Shannon, Simpson)	NR
Costello 2019	16 s rRNA	NR	↑ α—diversity (operational taxonomic units - OTUs) ↑ *Peptococcus niger*, ↑ *Faecalicoccus pleomorphus*, ↑ *Olsenella* sp., ↑ *Acidaminococcus intestini*, ↑ *Prevotella copri*, ↑ *Clostridium methylpentosum*, ↑ *Allistipes indistinctus*, ↑ *Odoribacter splanchnicus* ↓ *Anaerostipescaccae*, ↓ *Clostridium aldenense*	NR
Cui 2015	NR	NR	NR	NR
Damman 2015	Metagenomic Shotgun Sequencing	NR	No significant difference in α diversity (Shannon) ↑ *Actinobacteria*, ↑ *Bacteroidetes (Prevotella copri)*	NR
Dang 2020	NR	NR	NR	NR
Ishikawa 2017	16 s rDNA	NR	↑ *Bacteroidetes*	NR
Jacob 2017	16 s rRNA	NR	↑ α—diversity (OTUs, Shannon) Change in β—diversity (Bray-Curtis) towards donor	NR
Kump 2017	16 s rRNA	↑ unclassified *Ruminococcus* sp., ↑ *Akkermansia muciniphila*	No change in α—diversity (Chao1) Change in β—diversity (Bray-Curtis) towards donor	↑ *Akkermansia*, ↓ *Dialister* sp. Change in β—diversity (Bray-Curtis) towards donor in responders
Mizuno 2017	16 s rRNA	NR	No significant difference in diversity or composition	NR
Moayyedi 2015	16 s rRNA	↑ *Lachnospiraceae*, ↑ *Ruminococcus*	Change in β—diversity (Bray-Curtis) towards donor	Change in β—diversity (Bray-Curtis) towards donor
Nishida 2017	16 s rRNA	↑ *Bifidobacterium*	No significant difference in α—diversity (Shannon) or β—	NR

			diversity (Bray-Curtis) between responders and non-responders	
Okahara 2020	HSP60 Bacteroidetes Sequencing	NR	Increase in similarity of *Bacteroidetes* species to donor	↑ *Bacteroides uniformis*, ↑ *Parabacteroides distasonis*, ↑ *Bacteroides dorei*
Paramsothy 2017	16 s rRNA shotgun sequencing	NR	↑ α—diversity (OTUs, Shannon) Shift towards donor at OTU level ↑ *Prevotella* spp., ↓ *Bacteroides* spp.	↑ *Barnesiella* spp., ↑ *Parabacteroides* spp., ↑ *Clostridium cluster IV*, ↑ *Ruminococcus* spp.
Rossen 2015	16 s rRNA	NR	↑ α—diversity (OTUs, Shannon) ↑ *Clostridium clusters IV*, *XIVa, XVIII* ↓ *Bacteroidetes*	NR
Schierova 2020	16 sRNA	NR	No difference in α—diversity (Shannon, Chao1, Faith's phylogenetic diversity) or β—diversity	↑ *Lachnospiraceae*, ↑ *Ruminococcaeae*, ↑ *Clostridaceae*, ↑ *Bifidobacteriaceae*, ↑ *Coriobacteriaceace* ↑*Faecalibacterium* ↑ *Blautia*, ↑ *Coriobacteria*, ↑ *Collinsella*, ↑ *Slackia*, ↑ *Bifidobacterium*
Sokol 2020	16 s rRNA	NR	Transient ↑ α—diversity (Shannon, Chao1) Trend towards change in β—diversity (Bray-Curtis, Sorensen similarity index) between donor/recipient correlated	Sorensen index similarity showing improved engraftment; ↑ *Ruminococcaecea*, ↑ *Coprococcus*, ↑ *Desulfovibrio*
Sood 2019	NR	NR	NR	NR
Sood 2020	NR	NR	NR	NR
Uygun 2017	NR	NR	NR	NR
Vaughn 2016	Whole-genome shotgun sequencing	NR	↑ α—diversity (Shannon) ↑ *Bacteroides cellulosilyticus*, ↑*Bilophila* unclassified, ↑ *Desulfovibrio piger*, ↑ *Bilophila wadsorthia*, ↑ *Clostridium leptum*, ↑ *Odoribacter splanchnicus*, ↑ *Bacteroides dorei*, ↑ *Parasutterella excrementihominis*,	Change in β—diversity (Bray-Curtis) towards donor in responders

			↑ *Lachnospiraceae bacterium 7 1 58FAA*, ↑ *Eubacterium ventriosum*, ↑*Burkholderiales bacterium 1 1 47*, ↑ *Dorea longicatena*, ↑ *Alistipes finegoldii* ↓ *Coprobacillus* unclassified, ↓ *Bacteroides massiliensis*, ↓ *Ruminococcus lactaris*, ↓ *Veillonella dispar*, ↓ *Lachnospiraceae bacterium 5 1 57FAA*, ↓ *Bifidobacterium adolescentis*, ↓ *Bacteroides vulgatus*, ↓ *Bacteroides ovatus*, ↓ *Streptococcus parasanguinis*, ↓ *Streptococcus salivarius*, ↓ *Clostridium scindens*	
Vermeire 2016	16 s DNA	↑ α—diversity (OTUs)	↑ α—diversity (OTUs), ↑ *Roseburia*, *Oscillibacter*, ↑ unclassified *Lachnospiraceae*, ↑ unclassified *Ruminococcaceae*	NR
Wang 2020	NR	NR	NR	NR
Wei 2015	NR	NR	NR	NR
Yang 2019	16 s RNA	NR	↑ α—diversity (OTUs, Shannon)	NR
Zhang 2016	NR	NR	NR	NR

NR—Not recorded.

Table 3.5. Adverse events and interventions reported for included studies.

Study	FMT or Antibiotic Treatment Delivery and Frequency	Patients (*n*)	Adverse Events Per Patient	Action
Borody 2003	Daily enema for 5 days	6	NR	NR
Chen 2020	Naso-jejunal 3 doses at 1, 3 and 5 days	9	Mild bloating (*n* = 3) Treatment failure (*n* = 1)	Colectomy (*n* = 1)
Chen 2020	Colonoscopy x3 in 1 week	44	NR	NR
Cold 2019	25 oral capsules per day for 50 days	7	No adverse events	No adverse events
Costello 2019	Single donor FMT (colonoscopy and 2 enemas over a week)	38	After 8 weeks: Worsening colitis (*n* = 1) *C. difficile* infection (*n* = 1) Pneumonia (*n* = 1) New anemia (*n* = 1) Mild elevation of alkaline phosphatase (*n* = 2) and alanine aminotransferase (*n* = 1)	Colectomy (*n* = 1)
	Single autologous FMT (colonoscopy and 2 enemas over a week)	35	After 8 weeks: Worsening colitis (*n* = 2) New anemia (*n* = 2) Mild elevation of alanine aminotransferase (*n* = 3)	NR
		61	After 12 months: Worsening colitis (*n* = 13) Infections (*n* = 8) New psoriatic arthritis (*n* = 2) Entero-pathic arthritis (*n* = 1) Crohn's disease (*n* = 1) Allergy to infliximab (*n* = 1) Weight gain (*n* = 13) Weight loss (*n* = 8)	Colectomy (*n* = 9)
Cui 2015	Single gastroscopy	30	Fever (*n* = 2)—1–6 h after FMT Increased diarrhea (*n* = 7)—1–6 h after FMT	NR
Damman 2015	Single colonoscopy	7	Abdominal cramping, increase in stool output (NR)—immediately after FMT Abdominal pain (*n* = 1)—after 5 days	None
Ishikawa 2017	Single colonoscopy	21	Transient borborygmus (*n* = 10)—during or soon after FMT	Resolved after end of

				treatment (*n* = 10)
	Antibiotic pre-treatment only	20	Nausea and watery diarrhea—after antibiotic treatment (*n* = 8)	Discontinued antibiotic treatment (*n* = 3)
Jacob 2017	Single colonoscopy	20	Fever (*n* = 1) Chills (*n* = 1) Fatigue/malaise (*n* = 4) Abdominal pain (*n* = 3) Anorexia (*n* = 1) Diarrhea (*n* = 2) Constipation (*n* = 1) Transient febrile response (*n* = 1) Increase in Mayo score (*n* = 2)—at week 4	Conservative care Anti-TNF alpha blockade therapy or colectomy
Kump 2017	Colonoscopy (5 times, 14 days apart)	17	Worsening colitis (*n* = 1)—after day 3	Required additional therapy (*n* = 1)
	Antibiotic pre-treatment only	10	*C. difficile* infection (*n* = 3)—after 14 days Antibiotic-associated diarrhea (*n* = 1) Worsening colitis (*n* = 1)	Required additional therapy (*n* = 5)
Mizuno 2017	Single colonoscopy	10	Worsening colitis (*n* = 6)	
Moayyedi 2015	Enema (once per week for 6 weeks)	38	Patchy inflammation and rectal abscess (*n* = 2) Abdominal discomfort (*n* = 1) *C. difficile* infection (*n* = 1)—after end of study	Antibiotic therapy (*n* = 2)
	Placebo	37	Worsening colitis (*n* = 1) Patchy inflammation and rectal abscess (*n* = 1)	Colectomy (*n* = 1) Antibiotic therapy (*n* = 1)
Nishida 2017	Single colonoscopy	41	No adverse events	
Okahara 2020	Single colonoscopy	55	Nausea (*n* = 20)	None
Paramsothy 2017	Colonoscopy and enema (x5 per week for 8 weeks)	41	Infection-related adverse event (*n* = 10) Serious adverse event (*n* = 2) Abdominal pain (*n* = 12) Colitis (*n* = 10) Flatulence (*n* = 10) Bloating (*n* = 8) Upper respiratory tract infection (*n* = 7)	Colectomy (*n* = 1), intravenous corticosteroid therapy (*n* = 1)

			Headache (*n* = 4) Dizziness (*n* = 3) Fever (*n* = 3) Rash (*n* = 3)	
	Placebo	40	Infection-related adverse event (*n* = 14) Serious adverse event (*n* = 1) Abdominal pain (*n* = 11) Colitis (*n* = 9) Flatulence (*n* = 8) Bloating (*n* = 11) Upper respiratory tract infection (*n* = 6) Headache (*n* = 2) Dizziness (*n* = 3) Fever (*n* = 2)	Hospitalization (*n* = 1)
Rossen 2015	Donor FMT by nasoduodenal tube (twice, 3 weeks apart)	23	Discomfort with tube placement (*n* = 1) Fever (*n* = 2) Nausea (*n* = 2) Diarrhea (*n* = 5) Headache (*n* = 1) Vomited fecal infusion (*n* = 2) Vomiting (*n* = 1) Abdominal pain (*n* = 1) Transient borborygmus (*n* = 4) Mild constipation (*n* = 1)	
	Autologous FMT by nasoduodenal tube (twice, 3 weeks apart)	25	Discomfort with tube placement (*n* = 1) Nausea (*n* = 1) Malaise (*n* = 1) Diarrhea (*n* = 1) Headache (*n* = 1) Abdominal cramps (*n* = 6) Abdominal pain (*n* = 4) Transient borborygmus (*n* = 8) Dizziness (*n* = 1) Cytomegalovirus infection (*n* = 1)—7 weeks after the first FMT; unrelated to treatment	Ganciclovir (*n* = 1)
		50	Severe small bowel Crohn's disease (*n* = 1) Abdominal pain (*n* = 1)—after 11 weeks Cervix carcinoma (*n* = 1)—after 6 weeks; unrelated to treatment	Antibiotics (*n* = 1)
Schierova 2020	Enema 5x for first week then weekly x 6 weeks	8	No adverse events	None
Sokol 2020	Single colonoscopy	8	Gastroenteritis (*n* = 2) Transient asthenia (*n* = 1) Cutaneous abscess (*n* = 1)	Self-limiting

Sood 2019	Colonoscopy at 0, 2, 6, 10, 14, 18, 22 weeks	41	After FMT, at 0 weeks: Abdominal discomfort (*n* = 26) Abdominal distension (*n* = 14) Fever (*n* = 4) Worsening diarrhea (*n* = 4) Flatulence (*n* = 2) Fatigue (*n* = 2)	Symptoms were self-limiting Oral rehydration solution (*n* = 4)
Sood 2020	Colonoscopy at 0, 2, 6, 10, 14, 18, 22 weeks	93	Abdominal discomfort (*n* = 28) Flatulence (*n* = 12) Borborygmi (*n* = 10) Low grade fever (*n* = 8) Diarrhea (*n* = 7)	Self-limiting
Uygun 2017	Single colonoscopy	30	Nausea, vomiting, abdominal pain, diarrhea (*n* = 7)	NR
Vaughn 2016	Single colonoscopy	19	Hives (*n* = 1)	Oral steroids (*n* = 1)
Vermeire 2016	Naso-jejunal or rectal tube (twice one day, then the following day)	14	High fever (*n* = 4)—few hours after FMT Vomited and pneumonia (*n* = 1)—after FMT	Paracetamol (*n* = 4) Broad-spectrum antibiotics (*n* = 1)
Wang 2020	Colonoscopy x3; 2–3 month intervals	16	None	None
Wei 2015	Single colonoscopy or naso-jejunal tube	14	Intolerance with FMT (*n* = 1) Moderate fever (*n* = 2)—after FMT	Self-limiting
Yang 2019	Gastroscopy or colonoscopy (twice, one week apart)	31	Nausea (*n* = 1) Reflux (*n* = 4) Belching (*n* = 2) Diarrhea (*n* = 10) Constipation (*n* = 1) Fever (*n* = 2) Aggravation of abdominal pain (*n* = 5) Abdominal distension (*n* = 3)	NR
Zhang 2016	Single endoscopy	19	Transient increased diarrhea (*n* = 7) Mild skin pruritus (*n* = 1) Borborygmus (*n* = 2)	-

NR—Not recorded.

Table S3.1: Full-text search strategy of included databases.

Database	Search Strategy
MEDLINE **Ovid MEDLINE(R) ALL** 1946 to January 28, 2021	1. Fecal Microbiota Transplantation/ 2. FMT.ti,ab. 3. feces infusion*.mp. 4. donor feces.mp. 5. (stool adj2 transplant*).mp. 6. f?ecal transfusion*.mp. 7. f?ecal bacteriotherap*.mp. 8. (f?ecal adj3 transplant*).mp. 9. or/1-8 10. exp Inflammatory Bowel Diseases/ or inflammatory bowel disease*.mp. 11. Crohn*.mp. 12. ulcerative colitis.mp. 13. IBD.ti,ab. 14. indeterminate colitis.mp. 15. or/10-14 16. 9 and 15 17. Animals/ or (veterinary or rabbit or rabbits or animal or animals or mouse or mice or rodent or rodents or rat or rats or pig or pigs or porcine or horse* or equine or cow or cows or bovine or goat or goats or sheep or ovine or canine or dog or dogs or feline or cat or cats or zebrafish).ti. 18. Humans/ 19. 17 not (17 and 18) 20. 16 not 19
Embase **Ovid Embase** 1974 to 2021 January 28	1. fecal microbiota transplantation/ 2. FMT.ti,ab. 3. feces infusion*.mp. 4. donor feces.mp. 5. (stool adj2 transplant*).mp. 6. f?ecal transfusion*.mp. 7. f?ecal bacteriotherap*.mp. 8. (f?ecal adj3 transplant*).mp. 9. or/1-8 10. exp inflammatory bowel disease/ or inflammatory bowel disease*.mp. 11. Crohn*.mp. 12. ulcerative colitis.mp. 13. IBD.ti,ab. 14. indeterminate colitis.mp. 15. or/10-14 16. 9 and 15 17. animal/ or (veterinary or rabbit or rabbits or animal or animals or mouse or mice or rodent or rodents or rat or rats or pig or pigs or porcine or horse* or equine or cow or

	cows or bovine or goat or goats or sheep or ovine or canine or dog or dogs or feline or cat or cats or zebrafish).ti. 18. human/ 19. 17 not (17 and 18) 20. 16 not 19
Scopus	TITLE-ABS-KEY (fmt OR "feces infusion*" OR "donor feces" OR (stool W/2 transplant*) OR "fecal bacteriotherap*" OR "faecal bacteriotherap*" OR (fecal W/3 transplant*) OR (faecal W/3 transplant*)) AND TITLE-ABS-KEY ("inflammatory bowel disease*" OR crohn* OR "ulcerative colitis" OR ibd OR "indeterminate colitis") AND NOT TITLE (veterinary OR rabbit OR rabbits OR animal OR animals OR mouse OR mice OR rodent OR rodents OR rat OR rats OR pig OR pigs OR porcine OR horse* OR equine OR cow OR cows OR bovine OR goat OR goats OR sheep OR ovine OR canine OR dog OR dogs OR feline OR cat OR cats OR zebrafish)
Web of Science Core Collection	#1 TS= (fmt OR "feces infusion*" OR "donor feces" OR (stool NEAR/2 transplant*) OR "fecal bacteriotherap*" OR "faecal bacteriotherap*" OR (fecal NEAR/3 transplant*) OR (faecal W/3 transplant*)) AND TS= ("inflammatory bowel disease*" OR crohn* OR "ulcerative colitis" OR ibd OR "indeterminate colitis") #2 TI=(veterinary OR rabbit OR rabbits OR animal OR animals OR mouse OR mice OR rodent OR rodents OR rat OR rats OR pig OR pigs OR porcine OR horse* OR equine OR cow OR cows OR bovine OR goat OR goats OR sheep OR ovine OR canine OR dog OR dogs OR feline OR cat OR cats OR zebrafish) #3 #1 NOT #2
Cochrane Library via Wiley	#1 MeSH descriptor: [Fecal Microbiota Transplantation] this term only #2 FMT:ti,ab #3 feces infusion* #4 donor feces #5 stool NEAR/2 transplant* #6 f?ecal next transfusion* #7 f?ecal next bacteriotherap* #8 f?ecal NEAR/3 transplant* #9 #1 OR #2 OR #3 OR #4 OR #5 OR #6 OR #7 OR #8 #10 MeSH descriptor: [Inflammatory Bowel Diseases] explode all trees #11 inflammatory next bowel next disease* #12 Crohn* #13 ulcerative colitis #14 IBD:ti,ab #15 indeterminate colitis #16 #10 OR #11 OR #12 OR #13 OR #14 OR #15 #17 #9 AND #16

	#18 (veterinary OR rabbit OR rabbits OR animal OR animals OR mouse OR mice OR rodent OR rodents OR rat OR rats OR pig OR pigs OR porcine OR horse* OR equine OR cow OR cows OR bovine OR goat OR goats OR sheep OR ovine OR canine OR dog OR dogs OR feline OR cat OR cats OR zebrafish):ti #19 #17 NOT #18
Google Scholar	("fecal transplant" OR "fecal transfusion" OR FMT OR "feces infusion" OR "donor feces" OR "stool transplant") AND ("inflammatory bowel disease" OR Crohn's OR "ulcerative colitis" OR "indeterminate colitis")

Table S3.2. Newcastle-Ottawa scale for assessing risk of bias for included cohort studies.

a-Inadequate microbiome description.

Quality assessment scale	**Accepted criteria**	**Chen 2020**	**Chen 2020**	**Cold 2019**	**Cui 2015**	**Damman 2015**	**Ishikawa 2017**	**Jacob 2017**	**Kump 2017**	**Mizuno 2017**	**Nishida 2017**	**Okahara 2020**	Schierova 2020	**Sood 2019**	**Sood 2020**	**Uygun 2017**	**Vaughn 2016**	**Vermeire 2016**	**Wei 2015**	**Zhang 2016**	**Wang 2020**
Selection																					
Representativeness of the exposed cohort	Representative of average IBD adults	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Ascertainment of FMT	Secure records	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Demonstration that outcome of interest was not present at start of study	Evidence of no prior FMT exposure	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Comparability																					
Comparability of cohorts on the basis of the design or analysis	Described FMT regiment and delivery, current medications, disease severity, microbiome analysis	a	*	*	a	*	*	*	*	*	*	*	*	a	a	a	*	*	a	a	a
Outcome																					
Assessment of outcome	Pre-defined cut-off points for response and remission	*	*	*	*	*	*	*	*	*	*	*	*	*	-	*	*	*	*	*	*
Was follow-up long enough for outcomes to occur?	3 months	*	*	*	*	-	-	-	*	*	-	-	*	*	*	*	-	-	-	*	-
Adequacy of follow-up of cohorts	Follow up of complete cohort or appropriate characterization of dropouts	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Total (Max = 7)		6	7	7	6	6	6	6	7	6	6	6	7	6	5	6	6	6	5	6	5

a-Inadequate microbiome description

Chapter 4: Timing of Tributyrin supplementation differentially modulates gastrointestinal inflammation and gut microbial recolonization following murine ileocecal resection

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4.0 Abstract

Background: Gastrointestinal surgery imparts dramatic and lasting imbalances, or a dysbiosis, to the composition of finely tuned microbial ecosystems. Current strategies aimed to restore these imbalances have been met with mixed success due to the inherent challenges of re-establishing anaerobic bacterial communities in an aerobic environment that is unable to successfully foster recolonization of native microbes.

Aims: The aim of the present study was to use an established mouse ileocecal resection (ICR) model to determine if peri-operative tributyrin (TBT) supplementation, a butyrate analogue shown to improve tissue hypoxia and gut barrier integrity, could prevent the onset of postoperative microbial dysbiosis or alternatively enhance recovery of the gut microbiota and reduce gastrointestinal inflammation.

Methods: Male wild type (129s1/SvlmJ) mice aged 8-15 weeks were separated into single cages and randomized 1:1:1:1 to each of the four experimental groups: control (CTR), 1-week of pre-operative TBT supplementation (PRE), 1-week of post-operative TBT supplementation (POS), and combined two-weeks of pre- and post-operative supplementation (TOT) (Figure 4.1a). ICR was performed one week from baseline assessment with mice assessed at 1-, 2-, 3-, and 4- weeks postoperatively. Primary outcomes included evaluating changes to gut microbial communities due to study intervention occurring from ICR to 4-weeks. Secondary outcomes included evaluating for differences inflammatory cytokines, fecal short-chain fatty acid concentrations, and histologic injury scoring.

Results: A total of 34 mice that underwent ICR (CTR *n* = 9; PRE *n* = 10; POS *n* = 9; TOT *n* = 6) and reached the primary endpoint were included in the analysis. PRE mice had a trend towards decreased ileal inflammation as evidenced by decreased levels of IL-1β ($p = 0.09$), IL-6 ($p=0.03$), and TNF-α ($p < 0.05$) versus CTR. In comparison to PRE mice, POS mice had trends towards reduced colonic inflammation demonstrated by decreased levels of IL-6 ($p =0.07$) and TNF-α ($p=0.07$). Notably, postoperative TBT supplementation was associated with an increased bloom of anaerobic taxa recolonization. The microbial recolonization of PRE mice was characterized by a bloom of *Staphylococcus, Lactobacillus, Enteroccaceae* and *Peptostreptococcacea.* In contrast, mice treated with post-operative TBT had a bloom of anaerobes including *Bacteroides thetaiotomicorn, Bacteroides caecimuris, Parabacteroides distasonis, Clostridia,* and *Turicibacter*.

Conclusion: Taken together, the results of our work demonstrate that timing of TBT supplementation differentially modulates gastrointestinal inflammation and gut microbial recolonization following murine ICR. Results of this trial build upon our understanding of peri-operative gut microbial shifts and provide evidence for the ongoing pursuit of gut microbial modulation strategies as a novel therapeutic modality following gastrointestinal surgery.

4.1 Introduction

The human gut microbiome contains over 100 trillion microorganisms, species which are not simply innocent bystanders, but have co-evolved with their human hosts to achieve a complex symbiotic relationship integral to human health[1–6]. In a healthy state, commensal bacteria provide a number of essential functions from producing anti-inflammatory short-chain fatty acid (SCFA) metabolites to regulating both adaptive and innate immunity, conjugating bile acids, maintaining gut-barrier integrity, synthesizing antimicrobial peptides, and providing colonization resistance to gut pathogens[2,7,8].

Gastrointestinal surgery imparts dramatic and lasting imbalances, or a dysbiosis, to the composition of these finely tuned microbial ecosystems[9–11]. Luminal exposure to oxygen facilitates a depletion of anti-inflammatory obligate anaerobes and a bloom of pro-inflammatory aerotolerant organisms[12]. Animal models have demonstrated that perturbation of these commensal microbial communities triggers loss of anti-inflammatory SCFAs, increased expression of bacterial virulence genes, and heightened protease activity. [9,12,13]. The cumulative effect of these changes are important since they have been implicated in a variety of adverse clinical outcomes including surgical site infections, anastomotic leak, and recurrence of Crohn's disease.

Although our group and others have shown the importance of these factors in mitigating adverse surgical outcomes and maintenance of host immune homeostasis[10,11], it is not known whether this loss of microbial ecology can be avoided nor the degree to which these shifts can be manipulated following surgery. Approaches that harness the gut microbiome in the immediate

post-operative period thus present a novel opportunity to optimize the physiological stress imparted by surgery[14]. Current strategies including prebiotic, probiotic and synbiotic supplementation, however, have been met with mixed success[10,15]. This is thought to be due to the inherent challenges of re-establishing anaerobic bacterial communities in an aerobic environment that is unable to successfully foster recolonization of native microbes[10,11,16].

An approach which instead emphasizes the re-establishment of an anaerobic environment may thus prove more effective in restoring pre-surgical microbial ecology. A promising supplement to facilitate this recovery is tributyrin (TBT), a butyrate analogue shown to increase butyrate delivery at the level of the colon and terminal ileum. Butyrate has been recently demonstrated to augment luminal hypoxia through modulation of hypoxia-inducible factor-1 (HIF-1)[17,18]. In dextran sulphate sodium-induced colitis mice, HIF-1 has been found to play a protective role in maintenance of gut barrier integrity through butyrate-mediated tight junction protein upregulation[19]. Yet, while TBT has demonstrated benefit in a number of acute and chronic inflammatory gastrointestinal diseases, its effects on gut barrier health or microbial recolonization following surgery are currently not known.

To evaluate the above concept, the aim of the present study was to use an established mouse ileocecal resection (ICR) model to determine if peri-operative TBT supplementation could prevent the onset of postoperative microbial dysbiosis or alternatively enhance recovery of the gut microbiota and reduce gastrointestinal inflammation.

4.2 Methods

4.2.1 Experimental Design

Experimental protocols were approved by the University of Alberta animal ethics committee (AUP00000293) with peri-operative mouse husbandry protocols approved by the university's Health Sciences Laboratory Animal Services.

To evaluate for the inflammatory and microbial effects of peri-operative tributyrin supplementation on a mouse model of ileocecal resection (ICR), a parallel four arm study design was utilized. The four intervention groups were control (CTR), pre-operative TBT supplementation (PRE), post-operative TBT supplementation (POS), and combined pre- and post-operative supplementation (TOT) (Figure 4.1a). PRE mice received one week of pre-operative TBT followed by ICR, POS mice received ICR followed by one week of post-operative TBT, and TOT mice received a one week of pre-operative TBT followed by ICR and an additional one week of post-operative TBT. CTR mice received surgery but no TBT supplementation.

At baseline (BL), male wild type (129s1/SvlmJ) mice aged 8-15 weeks were separated into single cages to avoid cage effects biasing microbial analysis and were randomized 1:1:1:1 to each of the four experimental groups. ICR was performed one week from baseline assessment with mice assessed at 1-, 2-, 3-, and 4- weeks postoperatively. Mouse weights along with water and chow consumption were measured at BL and then weekly until sacrifice. Stool samples were immediately collected and frozen at -80C at baseline, prior to start of the surgical liquid diet, and then weekly until our end point. At the start of four weeks, mice were sacrificed with the

following collected and frozen at -80C for immunologic analysis: ocular blood, ileal tissue, colonic tissue, and anastomotic tissue.

4.2.2 Study Interventions

4.2.2.1 Tributyrin Supplementation

All TBT intervention groups were provided tributyrin (Tributyrin 97% FG, Sigma Aldrich, Product# W222305) at a concentration of 10mM. Rationale for TBT dosing was based on prior ethanol-induced gut injury mouse models which demonstrated evidence for improved gut epithelial integrity at the level of the ileum and proximal colon. To minimize animal stress during the peri-operative period, TBT supplementation was added to existing water-bottles and provided *ad libitum* while CTR mice received water alone in identical delivery systems. Standard mouse chow Labdiet 5001 (LabDiet, USA) was further provided *ad libitum* throughout the course of the experiment with the exception of the four peri-operative days where mice were kept on liquid diet. Throughout, animals were housed in filter-top cages in humidity and temperature-controlled facilities with regulated day/night cycles at the University of Alberta (Edmonton, AB, Canada).

4.2.2.2 Ileocecal Resection

The ileocecal resection procedure was conducted using a modified version of a protocol previously described by our group[20]. Two days prior to ICR, mice were transitioned to a Lieber-DeCarli '82 liquid diet (Bio-Serv, Product#F1259SP) to minimize risk of post-operative obstruction. Following induction of anesthesia, Meloxicam (Metacam ®, 3mg/kg) was administered subcutaneously. Induction and maintenance of anesthesia was achieved using

isoflurane and titrated to a respiratory rate of approximately 50 breaths/min and absent pedal pain responses. A bolus of 30ml/kg of Normal Saline was given subcutaneously with mice then positioned in a supine position and immobilized on a warming pad. Hair was depilated over the site of the incision and the abdomen was then prepped with betadine and draped with sterile gauze.

A 1 cm midline incision was then made sharply and extend through the skin and peritoneum. The cecum was identified and delivered through the incision with the proximal colon and distal ileum. The ileocolic vascular pedicle was first identified and ligated. Next, the segmental blood supply to the terminal ileum located approximately 2cm proximal to the ileocecal valve was ligated. The ischemic tissue was allowed to demarcate, and the colon and ileum were then transected at the borders of ischemia. The resulting ends of the colon and terminal ileum were inspected for perfusion, gently dilated, and then generally re-approximated on moistened sterile gauze using stay-sutures.

Using a dissecting microscope an end-to-end anastomosis using simple interrupted 8-0 monofilament Prolene sutures was then created. Upon completion, the anastomosis was checked for leak and re-inserted into the abdomen. The abdomen was then irrigated with sterile saline (~2mL) to ensure clear effluent. The abdominal wall and skin were then closed in separate layers using 5-0 Vicryl in a running continuous fashion for the abdominal wall and in a simple interrupted fashion for the skin. Vet-bond (3M, USA) was then applied to all external visible suture knots to prevent wound dehiscence.

Postoperatively, each mouse was recovered under a heat lamp until alert, placed in a clean cage, and maintained on the liquid diet for two days. Analgesia was continued for three days via daily Meloxicam subcutaneous injections at 3mg/kg. Water (CTR) and tributyrin supplementation were continued in the perioperative phase *ad-libitum.* Solid chow diet was reintroduced two days postoperatively during which animals were monitored twice daily for lack of stooling or other signs of obstruction. When followed closely, survival rates of this protocol approached 90%.

4.2.3 Primary and Secondary Outcomes

Primary outcomes included evaluating changes to gut microbial communities due to study intervention occurring from ICR to 4-weeks evaluated by 16s rRNA sequencing. Secondary outcomes included evaluating for differences in the following from ICR to 4-weeks: weight, water and food intake, inflammatory cytokines (LPS, IL-1β, IL-6, IL-10, TNF-α), fecal short-chain fatty acid concentrations, and histologic injury scoring.

4.2.4 Analytical Techniques

4.2.4.1 Microbiome Extraction

Fecal DNA extraction for microbiome analysis was conducted using a modified MultiTarget Pharmaceuticals protocol. Bleached beads were added to tubes in combination with 200uL of AquaStool (MultiTarget Pharmaceuticals, USA), approximately 100mg of thawed stool, and homogenized. The resultant homogenate was centrifuged (14000g for 5 mins) followed by addition of 100uL of AquaRemove (MultiTarget Pharmaceuticals, USA). After re-centrifugation, the supernatant was collected, and isopropanol was added prior to precipitation on ice for 10 min. The DNA pellet was collected and washed three times with 70% ethanol. A

total of 100uL of EB Buffer (Qiagen, USA) was then added to solubilize the DNA followed by 1uL of RNASE A (Qiagen, catalog 1007885). The mixture was incubated at 37C for 1 hour and then recentrifuged. DNA precipitation was obtained with 10uL of 5M NaCl, 100uL of ice cold 100% ethanol, and a 30-minute incubation at -20C. The mixture was recentrifuged and the pellet was rinsed three times with 70% ethanol. An additional 50uL of EB buffer were added after removal of excess ethanol, and the solution was left overnight at 4C for solubilization.

After ensuring appropriate extraction quality using a Nanodrop 1000 Series device (Thermo Fisher Scientific, USA), samples were sent for 16s rRNA gene amplicon sequencing (Genome Canada, QC, Canada). Microbial composition was characterized by 16S rRNA gene amplicon sequencing of the v4 region using MiSeq Illumina technology (2x300bp) and the following forward and reverse primers: 341F
'CCTACGGGNGGCWGCAGTCCTACGGGNGGCWGCAGACCCTACGGGNGGCWGCAGCTACCTACGGGNGGCWGCAG'
and 805R
'GACTACHVGGGTATCTAATCCTGACTACHVGGGTATCTAATCCACGACTACHVGGGTATCTAATCCCTAGACTACHVGGGTATCTAATCC'.

4.2.4.2 Enzyme-Linked Immunosorbent Assay (ELISA)

Assays were conducted in singlet using commercially available kits while following manufacturer protocol and storage recommendations. Preparation of frozen tissue homogenate for ELISA analysis was performed by combining the frozen tissue together with 0.5mm silica

beads (BioSpec Products, catalog 11079105) and 400uL an extraction buffer mixture of Tween-20 (0.05%), Bovine Serum Albumin (BSA) 0.1%, and 1uL/mL protease inhibitor (Sigma P-8340) solubilised in 1x phosphate buffered saline (PBS). Samples were then homogenised with a beadmill (MPBiologicals, Fast-Prep 24) for 40s at 6m/s and centrifuged at 10000g to pellet excess debris. Resulting supernatant was then used for ELISA analysis with concentrations corrected for dry weight of tissue. Immunologic evaluation included IL-1β (R&D Systems DuoSet ELISA, catalog DY201-05), IL-6 (R&D Systems DuoSet ELISA, catalog DY206-05), TNF-α (R&D Systems DuoSet ELISA, catalog DY210-05), and LPS (Abbexa Endotoxin (ET) ELISA Kit, catalog ABX514093).

4.2.4.3 SCFA extraction

Fecal short-chain fatty acid concentrations were analyzed using gas chromatography at the Agricultural, Food and Nutritional Science chromatography core facility as previously described[10]. Briefly, 800uL of 0.1N hydrochloric acid and 200uL of 25% phosphoric acid were added to approximately 0.2g of stool. The contents were vortexed until fully homogenized and centrifuged at 5000g for 15 minutes or until the obtaining a clear supernatant. An internal standard solution (150mg of 4-methyl-valeric acid, S381810, Sigma-Aldrich), 5% phosphoric acid, and supernatant were then added to glass chromatography tubes and stored at -80 prior to analysis. Samples were analyzed with a gas chromatograph (Bruker SCION 456-GC, Bruker Corporation, Billerica, MA, USA) using a 30m x 0.53 mm inner diameter x 0.5 um film thickness capillary column (Stabilwax-DA, Restek Corporation, Belefonte, PA, USA).

4.2.4.4 Histology preparation and Analysis

At the time of sacrifice, sections of perianastomotic ileum, colon, and anastomotic tissue were opened and flushed with 1xPBS+Gentamycin 50ug/ml (Gibco 15750-60), then fixed in 10% buffered formalin (Fisher Scientific #245-685) for a minimum of 24hrs. Tissues were processed for paraffin embedding using a Leica Automated Tissue Processor with the following program: 1hr 70% ethanol, 1hr 90% ethanol, 3x 30min 100% ethanol, 4x 40min Xylene then 2x 40hr paraffin under vacuum. Embedded tissues were further cut and processed for Hematoxylin & Eosin staining at the Alberta Diabetes Institute histology with their standard procedures. Slide were blinded and scored by a single pathologist (AT) using a validated scale which evaluates enterocyte injury, epithelial hyperplasia, lamina propria, lymphocytes, and lamina propria neutrophils[21].

4.2.5 Statistical Analysis

Continuous variables are reported as means ± standard deviations if normally distributed or medians and interquartile ranges if non-normally distributed. Categorical data are reported as proportions and analyzed using the Cochran-Mantel-Haenszel[2] test. Within-group paired changes were conducted using two-tailed Wilcoxon Signed-rank test while between-group comparisons were conducted using the Mann-Whitney U test. Outliers were defined as greater than 3 standard deviations and were removed prior to analysis. Analysis for changes in weight, dietary intake, and immunologic data were be conducted using STATA 15 (StataCorp 2017; College Station, TX). Figures were designed using Prism 9.0.0 (GraphPad Software, San Diego, CA, USA). Statistical significance was defined using two-tailed tests with a p value < 0.05.

For microbial analysis, 16s rRNA sequences were first processed using the divisive amplicon de-noising algorithm version 2 (DADA2) pipeline. This pipeline allowed for sequencing quality control and was used for trimming, error correction, exact sequence inference, chimera removal and for generation of an amplicon sequence variant (ASV) table. Taxonomic classification was performed using a native RDP Bayesian classifier alongside the Silva database (version 138). Calculation of α-diversity (Shannon, Chao) and β-diversity (weighted UniFrac) were performed using the 'phyloseq' (v1.28.0) package in R. Samples with a minimum cut-off of 10,000 counts based on α-diversity rarefaction or where rarefaction curves plateaued were included for analysis. Changes in β-diversity were evaluated using the permutational multivariate analysis of variance (PERMANOVA), a non-parametric test which determines if the centroids of sample clusters differ. Differences in bacterial ASV abundance were analyzed using DESeq2, an estimate of variance-mean dependence based on a negative binomial distribution model. Microbial analysis was conducted using R (Version 3.5.1).

4.3 Results

4.3.1 Tributyrin is associated with a quicker restoration of postoperative weight loss

A total of 34 mice that underwent ICR (CTR $n = 9$; PRE $n = 10$; POS $n = 9$; TOT $n = 6$) and reached the primary endpoint were included in the analysis. There were no differences for mouse weight or food intake between groups either at baseline or across any other time points (Figure 4.1b, Figure 4.1d). PRE and TOT mice receiving preoperative TBT had increased water intake from BL to ICR ($p<0.05$) when compared to CTR (Figure 4.1e). Relative to baseline weights, significant differences in the percent weight change were observed between groups (Figure 4.1c). All groups demonstrated significant differences in precent weight change between ICR and W1

($p<0.05$), and between ICR and W2 ($p<0.05$). No significant differences were observed at W3 for any groups receiving TBT supplementation or for all groups at W4 suggesting that TBT may facilitate a quicker weight-regain following ICR.

4.3.2 Peri-operative timing of TBT differentially modulates ileal and colonic inflammation

Analysis of cytokine concentrations for peri-anastomotic ileal and colonic tissue at week 4 revealed at different inflammatory profiles associated with TBT supplementation (Figure 4.2 a-j). PRE mice had a trend towards decreased ileal inflammation as evidenced by decreased levels of IL-1β (Figure 4.2b, PRE vs. CTR; $p = 0.09$), IL-6 (Figure 4.2c, PRE vs. CTR $p=0.03$), and TNF- α (Figure 4.2e PRE vs. CTR/TOT; $p < 0.05$). In contrast, POS mice demonstrated trends towards reduced colonic inflammation, particularly when comparing to PRE mice. This was demonstrated by decreased levels of IL-6 (Figure 4.2h, POS vs. PRE; $p =0.07$) and TNF- α (Figure 4.2j POS vs. PRE; $p=0.07$). These findings suggest that timing of TBT supplementation differentially modulates ileal and colonic inflammation following ICR.

4.3.3 TBT supplementation altered colonic tissue weight to length ratio but not histologic injury scoring or serum pro-inflammatory cytokines

At week four, all groups demonstrated low levels of pro-inflammatory LPS (Figure 4.3a) and IL-6 (data not shown) in the serum with no differences observed between groups. Similarly, histologic injury scoring of ileal and colonic tissue revealed minimal levels of active tissue inflammation (Figure 4.3b-c). Weight to length values of ileal tissue did not differ between groups (Figure 4.3d). However, when comparing PRE mice with those receiving post-operative TBT (PRE vs. POS; $p = 0.09$; PRE vs. TOT $p = 0.04$), a trend towards reduced colonic weight to

length ratio, in keeping with reduced tissue inflammation, was observed for post-operative TBT supplemented mice (Figure 4.3d). Correlation of colonic weight to length ratio with cytokine trends observed in colonic tissue homogenate adds further support to the observation that TBT differentially modulates perianastomotic inflammation.

4.3.4 Effects of ICR and TBT on fecal SCFA concentrations

Neither total nor individual fecal SCFA concentrations differed significantly between groups from BL to W4 (Figure 4.4 a-e). While acetate and total SCFA levels remained relatively unchanged during the course of the experiment, ICR resulted in a significant reduction in propionate and an even more pronounced loss of butyrate. Concentrations of both of these anti-inflammatory metabolites remained decreased from ICR to W4, with no return to preoperative levels observed in any group (Figure 4.4 c-e). Notably, TBT supplementation was not found to alter fecal butyrate concentrations.

4.3.5 ICR imparts a dramatic shift in microbial ecology

A dramatic shift in gut microbial ecology was observed in all groups following ICR (Supplemental Tables 4.1-4.4). In decreasing order of relative abundance, the preoperative microbiome of all mice was composed of the following bacterial phyla: *Bacteroidetes*, *Firmicutes*, *Verrucomicrobia*, and *Proteobacteria (Figure 4.5a)*. Immediately following ICR, at week 1, a complete loss of *Bacteroides* and *Verrucomicrobia* phyla was observed. These changes resulted in a corresponding bloom of *Firmicutes and Proteobacteria* that were sustained throughout the postoperative period. At four weeks, only four mice demonstrated a recovery of *Bacteroidetes*, three of which received postoperative TBT supplementation.

Evaluation of changes in α-diversity and in β-diversity revealed similar dramatic microbial changes imparted by ICR. α-diversity as assessed by Chao1 and Shannon indices significantly decreased from ICR to W1 and did not recover to pre-operative levels at any time point. After one week of TBT supplementation, PRE mice demonstrated a decrease in alpha diversity (q=0.045) in comparison to CTR. No other differences in α-diversity were noted between groups at other time points. β-diversity analysis revealed a significant difference in pre- and post-operative microbial composition (Figure 4.5c, $q<0.05$). Although at four weeks no intervention demonstrated a complete return to pre-operative β-diversity, POS and TOT groups receiving post-operative TBT supplementation were associated with the most substantial shifts towards that of preoperative composition.

4.3.6 Timing of TBT differential modulates recolonization following ICR

Lastly, differences in relative abundance of bacterial taxa between groups from W1 to W4 were assessed using DESeq2 to observe if TBT supplementation was able to facilitate a recolonization of anaerobic bacteria following ICR (Figure 4.6a-d). When comparing group receiving preoperative supplementation to those receiving post-operative TBT, POS and TOT mice were associated with significant increased bloom of specific anaerobic taxa. These included *Bacteroides thetaiotomicorn, Bacteroides caecimuris, Parabacteroides distasonis, Clostridia,* and *Turicibacter.* Since the POS group was associated with trends towards improved inflammatory markers when compared to PRE mice, we next compared their differences in relative abundance at week 4. This analysis revealed that POS groups had a significant increase

in *Clostridia ($p<0.05$)*, a prominent anerobic class of commensal bacteria, while PRE groups had a relative bloom of *Staphylococcus, Lactobacillus, Enteroccaceae* and *Peptostreptococcacea.*

4.4 Discussion

This study evaluated the effects of various peri-operative tributyrin (TBT) regimens on gastrointestinal inflammation and gut microbial ecology using a mouse model of ileocecal resection. Pre-operative TBT supplementation was associated with a reduction in ileal tissue inflammation and an increase in colonic tissue inflammation when compared to mice receiving postoperative TBT. The microbial recolonization of PRE mice was characterized by a bloom of *Staphylococcus, Lactobacillus, Enteroccaceae* and *Peptostreptococcacea.* In contrast, mice treated with post-operative TBT had a bloom of anaerobes including *Bacteroides thetaiotomicorn, Bacteroides caecimuris, Parabacteroides distasonis, Clostridia,* and *Turicibacter*. Taken together, the results of our work demonstrate that timing of TBT supplementation differentially modulates gastrointestinal inflammation and gut microbial recolonization following murine ICR.

Tributyrin is an oral butyrate analogue which has shown substantial promise in ameliorating inflammatory gastrointestinal pathology in murine studies. Perhaps the first high-quality evidence was provided by Vinolo et al. nearly a decade ago using murine models of obesity and metabolic disease[22]. Supplementation of TBT in mice receiving a high-fat diet was found to attenuate systemic inflammation, insulin resistance, and hepatic steatosis. Subsequent studies by Cresci et al. on ethanol-induced murine gut injury further demonstrated a protective role for TBT in modulating gastrointestinal barrier integrity by increasing the expression of tight junction

proteins in the ileum and proximal colon[23]. Lastly, elegant work by Rivera-Chavez et al. showed that TBT was able to restore the epithelial anaerobic environment perturbed by streptomycin treatment and prevent an aerobic expansion of pathogenic *Salmonella enterica*[24].

In healthy colonic mucosa, butyrate inhibits histone deacetylases leading to reduced activation of the pro-inflammatory NF-kB pathway, increased expression of tight junction proteins which promote gut barrier integrity, regulation of antimicrobial peptides, and attenuation of aberrant innate and adaptive host immune responses. Additional *in vivo* evidence in support of butyrate and SCFA supplementation as a promising target for restoring microbial-mediated intestinal epithelial dysfunction exists. For example, administration of oral short-chain-fatty acids have been found to restore the abnormal intestinal epithelial cell turnover present in specific pathogen-free mice after antibiotic depletion of SCFA producing bacteria taxa. Given the well accepted anti-inflammatory properties of butyrate on the intestinal epithelium, our findings of decreased ileal inflammation at the expense of increased colonic inflammation when comparing mice receiving pre-operative and post-operative supplementation are unexpected.

While our study was not specifically designed to evaluate the underlying mechanisms responsible for these findings, a number of possible explanations exist. The degradation of TBT, release of butyrate, and subsequent absorption of butyrate along the length of the gastrointestinal tract are currently not well understood. It is thus possible that in the PRE group, TBT may have been preferentially absorbed in the proximal small intestine prior to reaching the colon and providing any potential benefits. Removal of the terminal ileum resulting in bile acid changes

and altered gastrointestinal motility may have facilitated an increased delivery of butyrate to mice supplemented TBT postoperatively leading to improved colonic inflammatory profiles. Alternatively, it is possible that the timing of TBT in relation to ICR may have altered the concentrations of butyrate at the level of the intestinal crypts to modulate tissue inflammatory responses. Butyrate exerts differential effects on intestinal crypt stem cells by impairing cell proliferation in response to mucosal injury through Foxp3 transcription factor dependent mechanisms[25]. Supra-physiologic butyrate concentrations in the presence of healthy colonic epithelial cells may have overcome endogenous colonocyte butyrate utilization capacity. This would in turn increase crypt concentrations in the PRE and TOT groups leading to impaired response to surgical insult. In POS mice which received only post-operative supplementation, however, these supra-physiologic butyrate concentrations may have instead been fully utilized by injured colonocytes for restoration of barrier integrity and immune regulation.

The advent of high throughput cost effective sequencing technologies has brought a remarkable understanding of the complex gut microbial ecologic shifts imparted by surgery. Yet, our ability to modulate these shifts, including restoring the loss of anti-inflammatory SCFA-producing anaerobes, in the peri-operative period has been met with initial challenges. Through stimulation of hypoxia inducible factor-1 (HIF-1), butyrate presents a particularly promising strategy as it has been demonstrated to restore luminal hypoxia via regulation of enterocyte transcriptional factors and improve gut barrier integrity in colitis models[19,26]. Findings from our study indeed support the promise of this concept. All mice receiving post-operative TBT demonstrated an increased bloom of anaerobic bacteria, with the TOT group showing the greatest bloom and also a trend towards restoration of beta-diversity at four weeks.

Our study was not without its limitations. Mice used in our experiment did not have active gastrointestinal inflammation at the time of surgery which may have limited our ability to observe significant differences in inflammatory markers between intervention groups. In future studies, this limitation could be overcome either by using mouse colitis models, while accepting an increased operative mortality, or by separating arms into early and late cohorts. Early cohorts, between 1-3 weeks postoperatively may allow for a clearer evaluation of inflammatory markers. Late cohorts, on the other hand, may provide more complete characterization regarding the timing and effect of TBT on post-operative microbial re-colonization. Additional limitations were that TBT dosing was provided through pre-existing water supplies, making dosing less accurate than oral gavage. However, the stress of surgery combined with that of daily gavage makes the use of gavage vehicle difficult to justify. Lastly, our study did not elucidate exact mechanisms responsible for our immunologic or microbial findings and should thus serve as hypothesis generating.

Despite these limitations, this study is the first to demonstrate that the peri-operative timing of a a supplement aimed at optimizing the gut microbiome differentially alters gastrointestinal inflammation and gut microbial recolonization following ileocecal resection. Results of this trial build upon our understanding of peri-operative gut microbial shifts and provide evidence for the ongoing pursuit of gut microbial modulation strategies as a novel therapeutic modality following gastrointestinal surgery.

4.5 Conclusion

Timing of tributyrin supplementation differentially modulates gastrointestinal inflammation and gut microbial recolonization following murine ileocecal resection. Results of this trial build upon our understanding of peri-operative gut microbial shifts and provide evidence for the ongoing pursuit of gut microbial modulation strategies as a novel therapeutic modality following gastrointestinal surgery.

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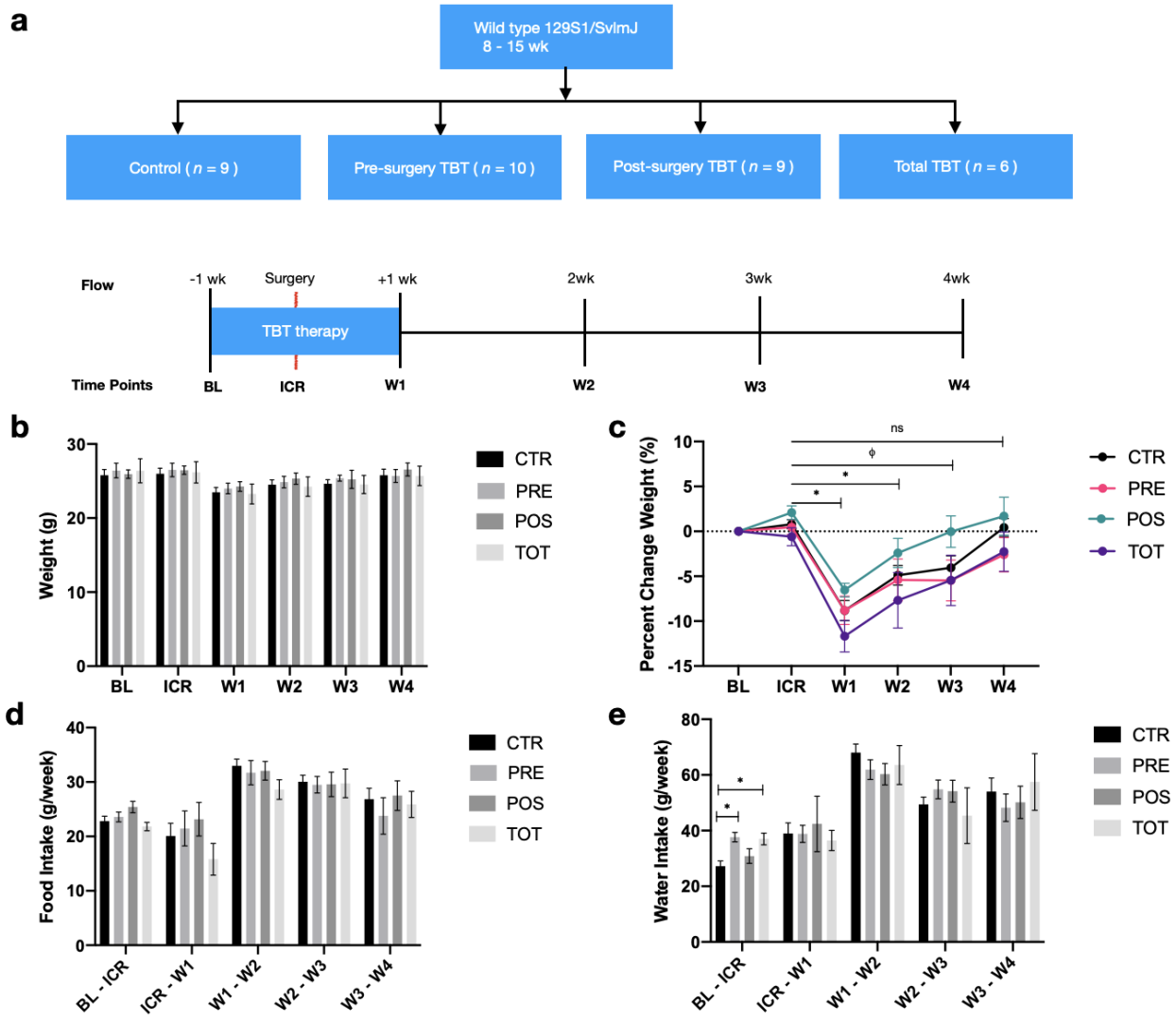

Figure 4.1. Overview of study design and changes in weight, food, and water intake. a. Study design overview. Column graphs represent median +/- SEM. **b.** Mouse weights from BL to W4 across intervention groups **c.** Percent change in weight relative to baseline across intervention groups. **d.** differences in weekly food intake across intervention groups. **e.** Differences in weekly water intake across intervention groups. All p-values were two-sided with statistical significance defined as $p < 0.05$. * represents significance in paired analysis for percent change relative to baseline for all groups; Ø represents significance in paired analysis for percent change relative to baseline for CTR group alone.

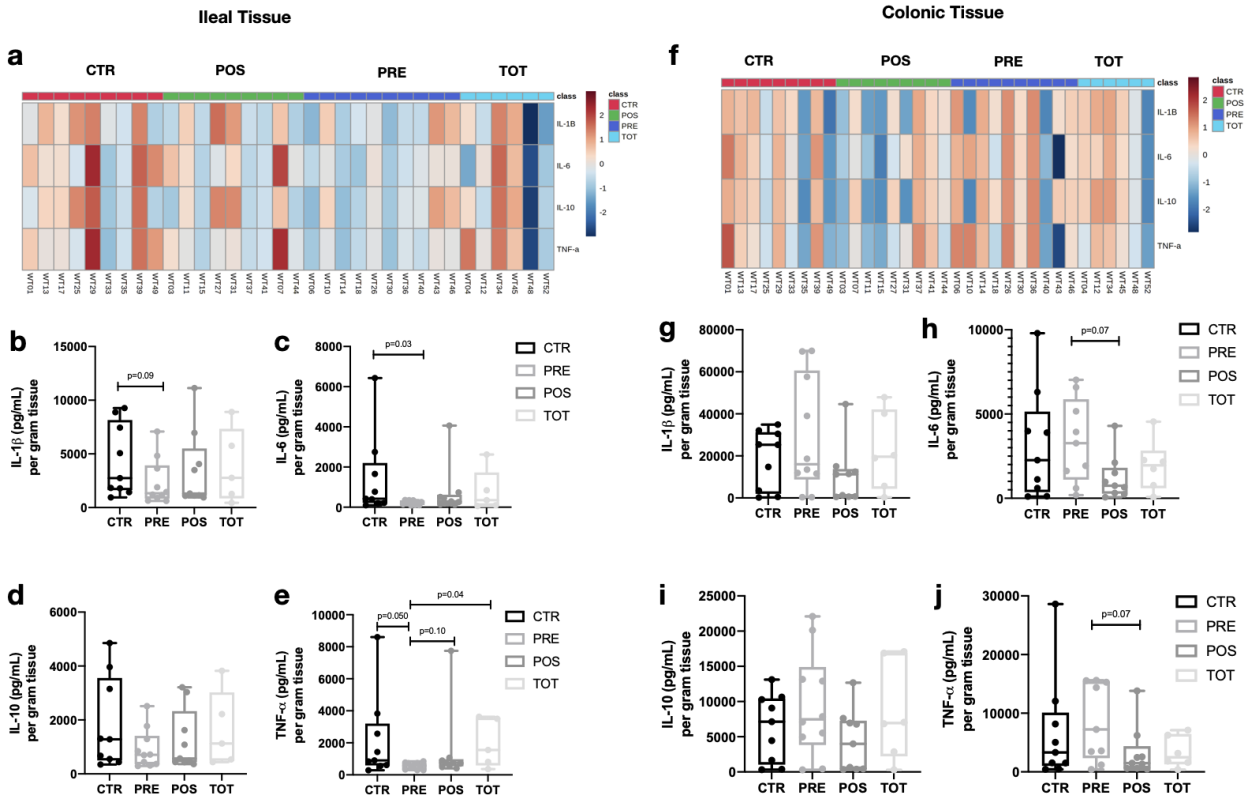

Figure 4.2. Tissue-weight adjusted cytokine concentrations in peri-anastomotic ileal and colonic tissue homogenate. Box-and-whisker plots represent the distribution of each group at W4. The median is represented by the middle line while the upper and lower borders of the box plot identify the 75th and 25th percentile, respectively. The whiskers correspond to the maximal and minimal values. **a**. Heatmap of ileal tissue cytokine concentrations after logarithmic transformation of data. **b-e**. Concentration of IL-1 β, IL-6, IL-10, and TNF- α , respectively, per gram of dry ileal tissue. **f.** Heatmap of colonic tissue cytokine concentrations after logarithmic transformation of data. **g-j**. Concentration of IL-1 β, IL-6, IL-10, and TNF- α , respectively, per gram of dry colonic tissue.

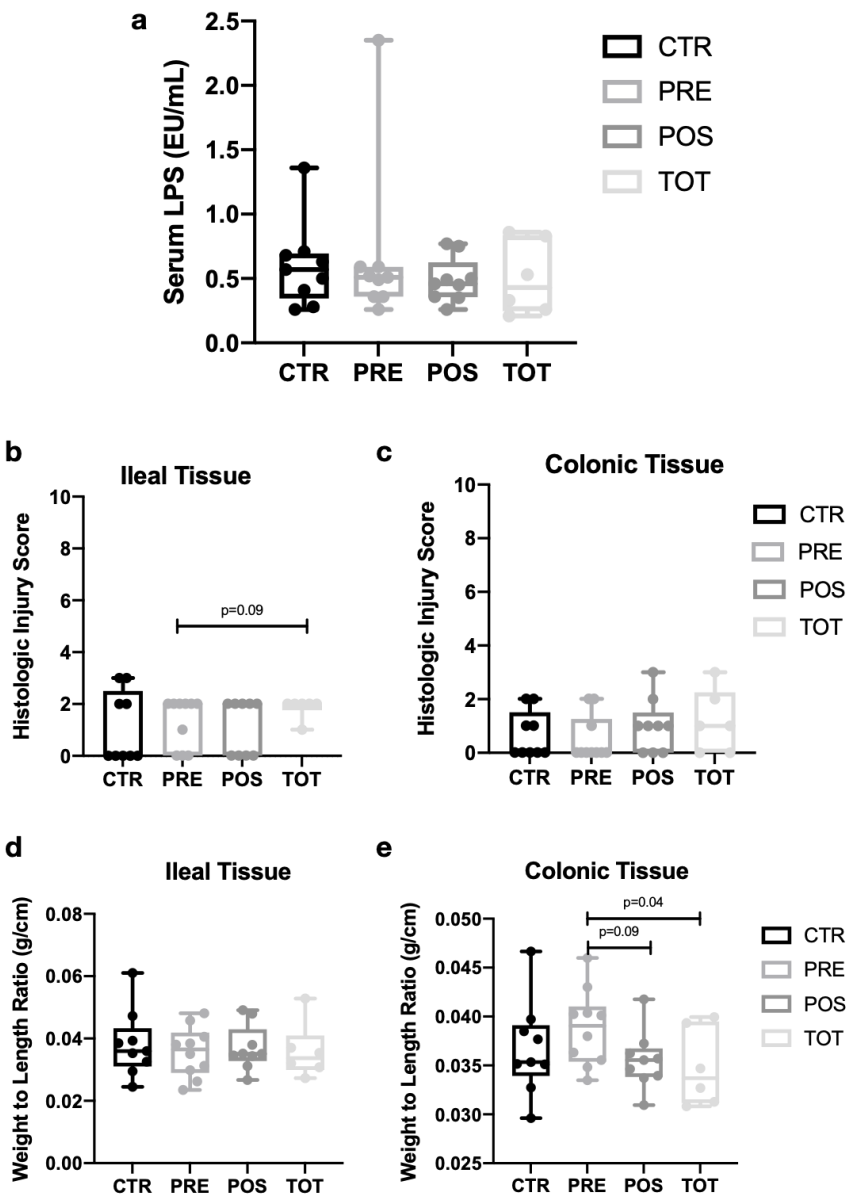

Figure 4.3. Serum pro-inflammatory cytokines, tissue histologic injury scoring, and tissue weight to length ratios. Box-and-whisker plots represent the distribution of each group at W4. The median is represented by the middle line while the upper and lower borders of the box plot identify the 75th and 25th percentile, respectively. The whiskers correspond to the maximal and minimal values. **a.** Serum LPS concentrations obtained from ocular blood at W4. **b-c.** Histologic injury scoring for ileal and colonic tissues, respectively. **d-e.** Weight to length ratio for ileal and colonic tissue, respectively.

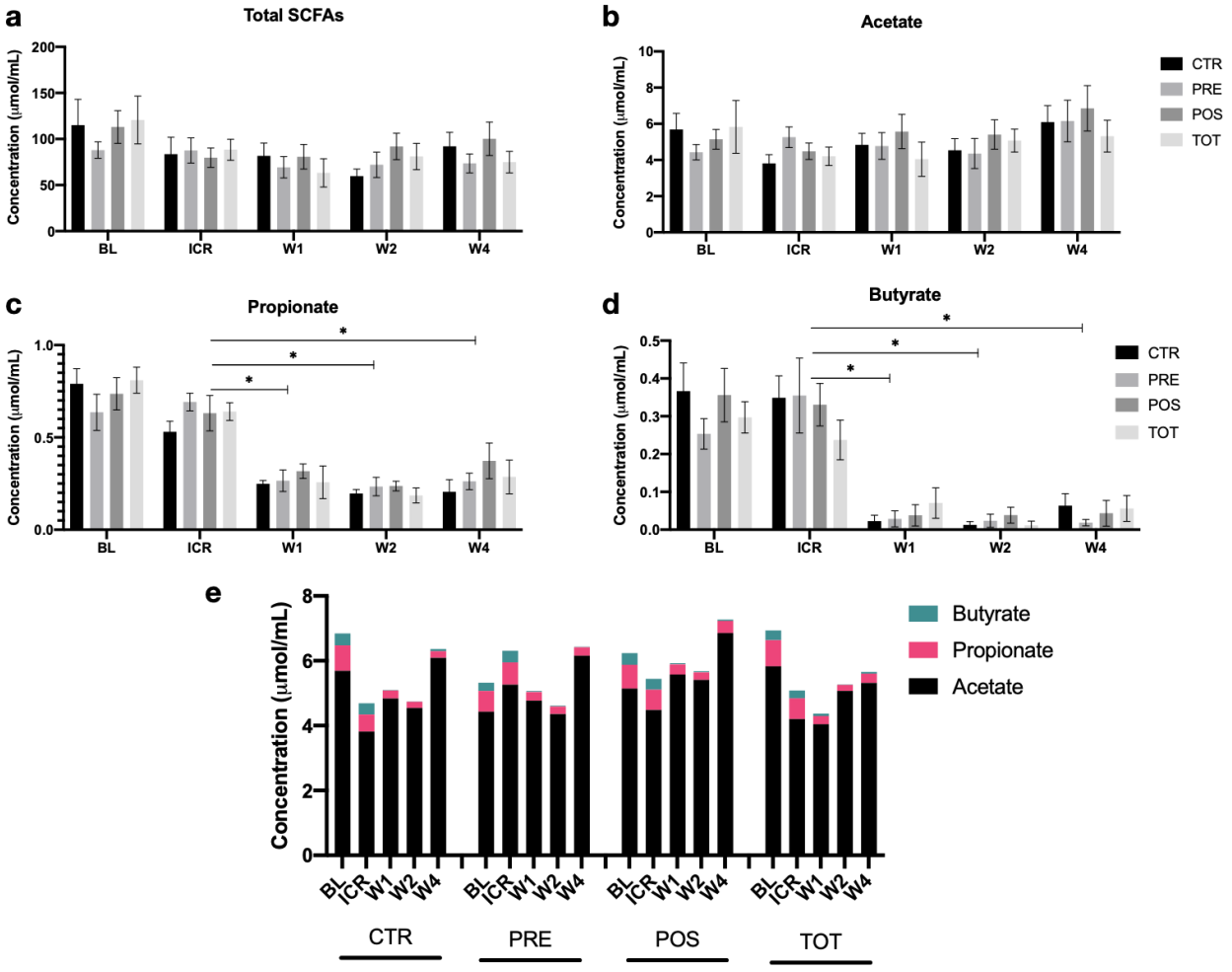

Figure 4.4. Concentrations of fecal short-chain fatty acids (SCFAs) from BL to W4 by intervention group. Column graphs represent median +/- SEM. **a.** Total SCFAs. **b.** Acetate. **c.** Propionate. **d.** Butyrate. **e.** Proportion of SCFAs. *represents $p < 0.05$

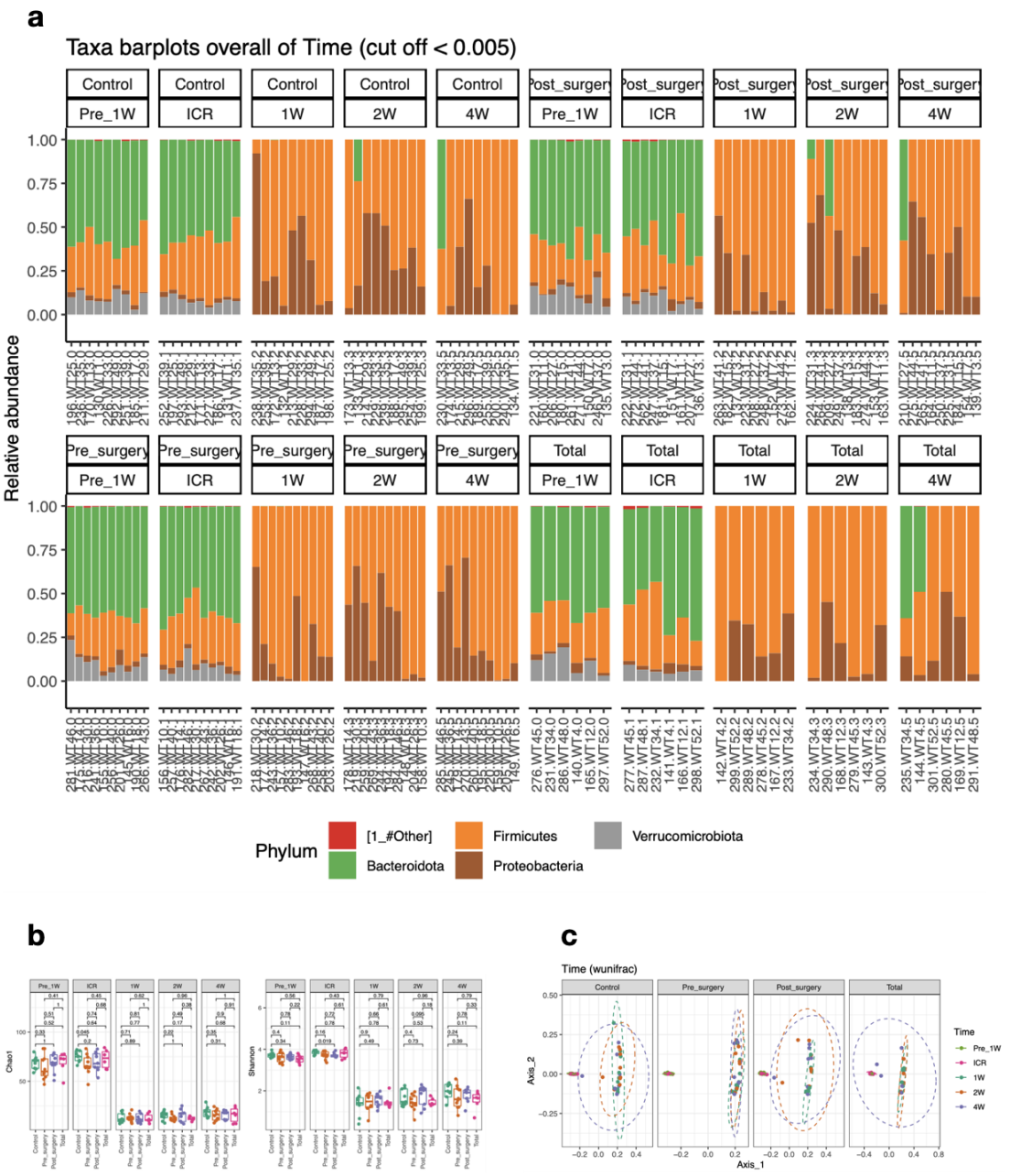

Figure 4.5. Difference in microbial abundance at the phylum level along changes in alpha and beta diversity from BL to W4. a. Phylum level differences in relative microbial abundance between groups over time. **b.** Within- and between- group changes in α-diversity using Chao1 and Shannon indices. **c.** Between-group differences from BL to W4 in β-diversity using weighted UniFrac analysis.

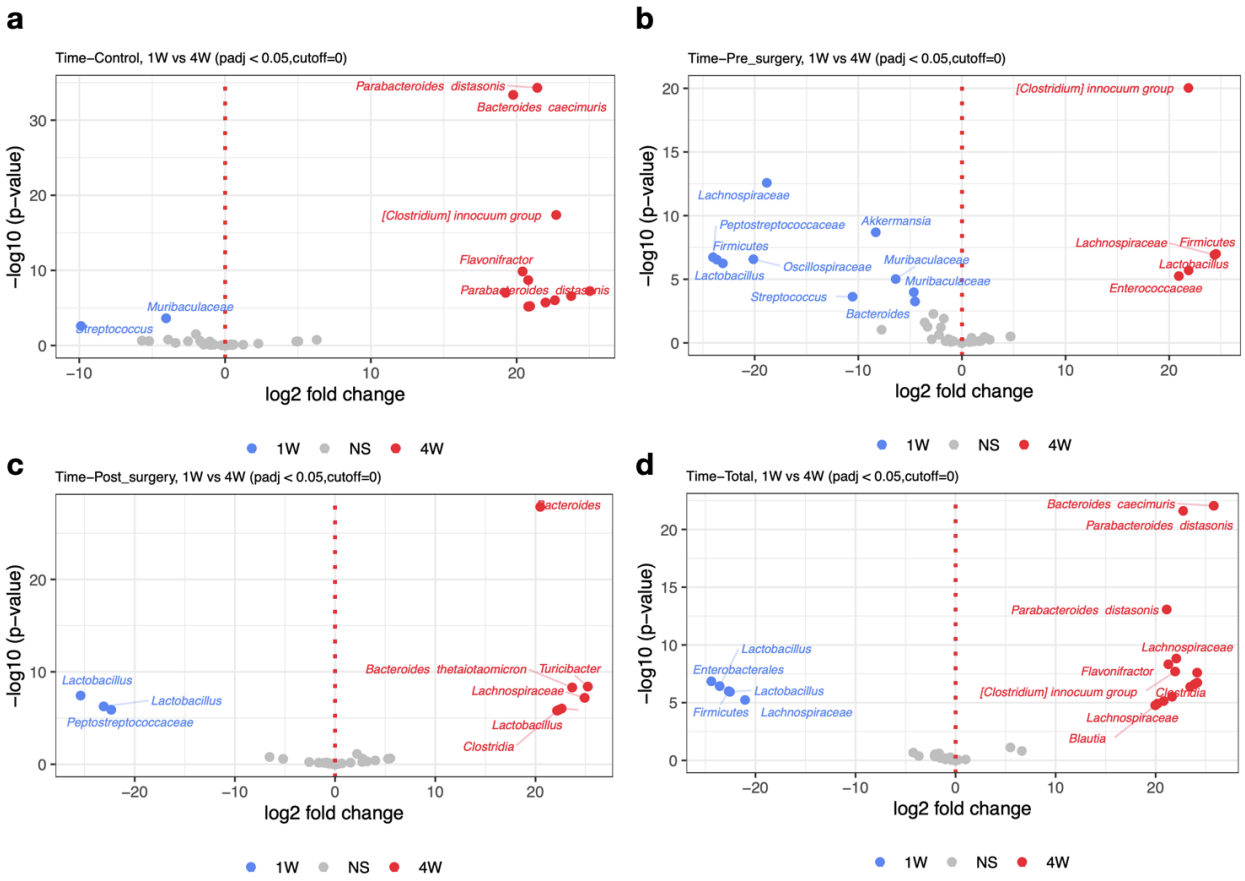

Figure 4.6. Volcano plots demonstrating significant differences (p adj. <0.05) in relative abundance of microbial taxa from W1 to W4. **a.** CTR group volcano plot **b.** PRE group **c.** POS group volcano plot. **d.** TOT group volcano plot.

CTR Group		Time Point				
		BL	ICR	W1	W2	W4
Phylum						
	Bacteroidetes	58.0 (2.2)	56.0 (2.0)	0.03 (0.03)	2.7 (2.5)	6.9 (6.9)
	Firmicutes	29.9 (2.7)	33.5 (2.6)	68.1 (9.7)	64.8 (6.3)	75.3 (8.7)
	Proteobacteria	2.1 (0.3)	2.2 (0.2)	31.9 (9.7)	32.5 (6.6)	17.7 (7.6)
	Verrucomicrobiota	9.7 (1.3)	8.0 (0.8)	0 (0)	0.02 (0.02)	0 (0)
Class						
	Alphaproteobacteria	1.0 (0.2)	1.1 (0.2)	0 (0)	0.01 (0.01)	0 (0)
	Bacilli	3.5 (0.7)	3.2 (0.8)	47.7 (8.9)	48.3 (6.1)	44.8 (7.1)
	Bacteroidia	58.0 (2.2)	56.0 (2.0)	0.03 (0.03)	2.7 (2.6)	7.1 (7.1)
	Clostridia	26.3 (2.7)	30.2 (2.2)	20.1 (7.1)	16.1 (7.7)	29.7 (7.0)
	Gammaproteobacteria	1.1 (0.1)	1.1 (0.1)	32.2 (9.7)	33.0 (6.6)	18.4 (7.8)
	Verrucomicrobiae	9.7 (1.3)	8.0 (0.8)	0 (0)	0.02 (0.02)	0 (0)
Family						
	Akkermansiaceae	9.9 (1.3)	8.1 (0.8)	0 (0)	0.02 (0.02)	0 (0)
	Bacteroidaceae	7.5 (0.7)	7.1 (0.8)	0 (0)	0 (0)	6.3 (6.3)
	Clostridiaceae	0 (0)	0 (0)	3.6 (3.6)	8.3 (8.3)	8.4 (8.4)
	Enterobacteriaceae	0.01 (0.01)	0 (0)	30.6 (9.5)	33.0 (6.7)	18.1 (7.6)
	Enterococcaceae	0 (0)	0 (0)	4.1 (1.4)	8.0 (1.8)	9.6 (2.1)
	Erysipelotrichaceae	0.2 (0.1)	0.1 (0.04)	0.8 (0.6)	2.5 (1.8)	5.2 (3.3)
	Lachnospiraceae	21.8 (2.2)	25.6 (2.3)	0.01 (0.01)	0.3 (0.3)	3.6 (2.3)
	Lactobacillaceae	2.5 (0.7)	2.3 (0.9)	44.0 (8.9)	37.7 (5.0)	30.7 (5.9)
	Muribaculaceae	47.4 (2.1)	46.4 (1.8)	0.03 (0.03)	0 (0)	0 (0)
	Oscillospiraceae	2.9 (0.6)	3.2 (0.3)	0 (0)	0.3 (0.3)	0.2 (0.2)
	Peptostreptococcaceae	0.01 (0.01)	0.01 (0.01)	16.5 (7.3)	7.1 (2.7)	17.1 (3.7)
	Sutterellaceae	1.1 (0.1)	1.1 (0.1)	0 (0)	0 (0)	0 (0)
	Tannerellaceae	3.9 (0.6)	3.1 (0.4)	0 (0)	2.7 (2.7)	0.9 (0.9)
Genus						
	[Clostridium] inoculum group	0.4 (0.2)	0.3 (0.1)	0 (0)	0.6 (0.4)	1.7 (0.7)
	Akkermansia	32.3 (4.0)	28.2 (2.4)	0 (0)	0.02 (0.02)	0 (0)
	Anaeroplasma	2.7 (1.0)	2.6 (1.1)	0 (0)	0 (0)	0 (0)
	ASF356	2.0 (0.4)	3.0 (0.9)	0 (0)	0 (0)	0 (0)
	Bacteroides	0 (0)	24.9 (2.6)	0 (0)	0 (0)	7.3 (7.3)
	Clostridium sensu stricto 1	0 (0)	0 (0)	2.9 (2.9)	8.2 (8.2)	8.3 (8.3)
	Enterococcus	0 (0)	0 (0)	5.1 (1.8)	9.7 (2.0)	13.1 (2.9)
	Escherichia-Shigella	0.02 (0.02)	0 (0)	17.9 (6.3)	16.6 (5.9)	8.6 (4.7)
	Lachnospiraceae NK4A136 group	2.4 (1.0)	4.2 (1.5)	0 (0)	0 (0)	0 (0)

Lactobacillus	7.9 (2.4)	7.8 (2.7)	72.3 (7.8)	59.3 (8.5)	53.7 (9.7)
Muribaculum	2.8 (1.0)	4.1 (1.3)	0 (0)	0 (0)	0 (0)
Parabacteroides	12.3 (1.9)	10.6 (1.4)	0 (0)	2.9 (2.9)	1.0 (1.0)
Parasutterella	3.5 (0.4)	3.9 (0.2)	0 (0)	0 (0)	0 (0)
Turicibacter	0 (0)	0 (0)	1.0 (0.7)	2.2 (1.7)	6.2 (3.9)

Table S4.1. Shifts in microbial taxa from BL to W4 in CTR mice.

PRE Group		Time Point				
		BL	ICR	W1	W2	W4
Phylum						
	Bacteroidetes	61.2 (0.9)	60.8 (2.2)	0 (0)	0 (0)	0 (0)
	Firmicutes	24.6 (2.0)	28.1 (2.0)	79.0 (6.9)	68.3 (7.8)	73.7 (8.3)
	Proteobacteria	3.4 (0.7)	3.0 (0.3)	20.9 (6.9)	31.7 (7.8)	26.3 (8.3)
	Verrucomicrobiota	10.4 (1.9)	7.8 (1.4)	0 (0)	0 (0)	0 (0)
Class						
	Alphaproteobacteria	1.9 (0.6)	1.5 (0.3)	0 (0)	0 (0)	0 (0)
	Bacilli	3.3 (1.0)	2.4 (0.4)	55.8 (6.2)	58.3 (6.9)	56.9 (7.6)
	Bacteroidia	61.2 (0.9)	60.8 (2.2)	0 (0)	0 (0)	0 (0)
	Clostridia	21.3 (2.0)	25.7 (2.1)	23.0 (8.4)	9.5 (3.1)	16.3 (5.4)
	Gammaproteobacteria	1.5 (0.1)	1.5 (0.2)	21.1 (6.9)	32.3 (7.9)	26.8 (8.3)
	Verrucomicrobiae	10.4 (1.9)	7.8 (1.4)	0 (0)	0 (0)	0 (0)
Family						
	Akkermansiaceae	10.8 (1.9)	8.0 (1.4)	0 (0)	0 (0)	0 (0)
	Bacteroidaceae	12.6 (1.8)	11.5 (1.3)	0 (0)	0 (0)	0 (0)
	Clostridiaceae	0 (0)	0 (0)	0 (0)	0.1 (0.1)	0 (0)
	Enterobacteriaceae	0.1 (0.1)	0.02 (0.02)	21.1 96.9)	31.4 (7.7)	26.4 (8.4)
	Enterococcaceae	0 (0)	0 (0)	1.9 (0.9)	4.6 (1.3)	7.2 (1.3)
	Erysipelotrichaceae	0.2 (0.1)	0.08 (0.03)	4.1 (3.1)	5.7 (3.6)	10.4 (5.8)
	Lachnospiraceae	17.9 (2.1)	21.1 (2.2)	0 (0)	1.0 (1.0)	3.0 (2.0)
	Lactobacillaceae	2.6 (1.0)	1.8 (0.4)	49.6 (5.8)	48.8 (8.0)	40.0 (8.6)
	Muribaculaceae	45.2 (1.5)	45.4 (2.1)	0 (0)	0 (0)	0 (0)
	Oscillospiraceae	2.0 (0.4)	2.9 (0.6)	0 (0)	0 (0)	0.2 (0.2)
	Peptostreptococcaceae	0.04 (0.03)	0.1 (0.03)	22.9 (8.4)	8.4 (2.8)	12.8 (4.4)
	Sutterellaceae	1.5 (0.1)	1.4 (0.2)	0 (0)	0 (0)	0 (0)
	Tannerellaceae	5.8 (0.9)	5.4 (0.2)	0 (0)	0 (0)	0 (0)
Genus						
	[Clostridium] inoculum group	0.4 (0.1)	0.2 (0.1)	0 (0)	0.8 (0.5)	1.7 (0.9)
	Akkermansia	28.2 (4.4)	24.3 (3.6)	0 (0)	0 (0)	0 (0)
	Anaeroplasma	1.8 (0.8)	2.0 (1.1)	0 (0)	0 (0)	0 (0)
	ASF356	1.4 (0.3)	3.5 (0.9)	0 (0)	0 (0)	0 (0)
	Bacteroides	33.4 (3.2)	33.8 (2.4)	0 (0)	0 (0)	0 (0)
	Clostridium sensu stricto 1	0 (0)	0 (0)	0.1 (0.1)	0.1 (0.1)	0 (0)
	Enterococcus	0 (0)	0 (0)	2.7 (1.1)	5.3 (1.1)	10.0 (2.9)
	Escherichia-Shigella	0.1 (0.1)	0 (0)	16.6 (5.2)	12.2 (5.6)	8.6 (3.6)
	Lachnospiraceae NK4A136 group	1.3 (0.7)	1.3 (0.7)	0 (0)	0 (0)	0 (0)
	Lactobacillus	6.9 (2.3)	5.1 (0.8)	75.8 (6.3)	75.4 (8.7)	65.2 (8.8)

Muribaculum	1.8 (1.0)	2.2 (1.1)	0 (0)	0 (0)	0 (0)
Parabacteroides	15.2 (1.8)	15.9 (1.8)	0 (0)	0 (0)	0 (0)
Parasutterella	4.1 (0.3)	4.4 (0.5)	0 (0)	0 (0)	0 (0)
Turicibacter	0 (0)	0 (0)	4.3 (3.1)	6.1 (3.7)	14.0 (7.1)

Table S4.2. Shifts in microbial taxa from BL to W4 in PRE mice.

POS Group		Time Point				
		BL	ICR	W1	W2	W4
Phylum						
	Bacteroidetes	59.6 (2.2)	58.5 (3.7)	0 (0)	6.1 (4.9)	6.4 (6.4)
	Firmicutes	24.7 (2.5)	30.2 (3.9)	82.9 (6.7)	64.7 (8.2)	64.2 (7.8)
	Proteobacteria	3.0 (0.5)	2.7 (0.6)	17.1 (6.7)	29.2 (8.2)	29.4 (8.1)
	Verrucomicrobiota	12.5 (1.8)	8.1 (1.4)	0 (0)	0 (0)	0 (0)
Class						
	Alphaproteobacteria	1.6 (0.4)	1.6 (0.5)	0 (0)	0 (0)	0 (0)
	Bacilli	2.6 (0.5)	1.7 (0.4)	62.7 (6.4)	47.4 (6.0)	46.7 (7.4)
	Bacteroidia	59.4 (2.2)	58.5 (3.7)	0 (0)	6.3 (5.1)	6.5 (6.5)
	Clostridia	22.1 (2.6)	28.4 (4.1)	20.1 (8.5)	16.9 (6.2)	16.9 (6.5)
	Gammaproteobacteria	1.4 (0.3)	1.1 (0.1)	17.2 (6.6)	29.4 (8.2)	29.9 (8.1)
	Verrucomicrobiae	12.5 (1.8)	8.1 (1.4)	0 (0)	0 (0)	0 (0)
Family						
	Akkermansiaceae	12.9 (1.9)	8.3 (1.4)	0 (0)	0 (0)	0 (0)
	Bacteroidaceae	9.7 (1.9)	9.4 (1.6)	0 (0)	6.4 (5.1)	6.7 (6.7)
	Clostridiaceae	0 (0)	0 (0)	0 (0)	0.7 (0.7)	0 (0)
	Enterobacteriaceae	0.1 (0)	0 (0)	14.0 (6.4)	16.5 (6.7)	29.7 (8.1)
	Enterococcaceae	0 (0)	0 (0)	3.9 (1.5)	8.2 (0.9)	8.9 (3.5)
	Erysipelotrichaceae	0.2 (0.1)	0.1 (0)	2.8 (2.7)	3.2 (1.9)	3.8 (1.9)
	Lachnospiraceae	18.5 (2.7)	24.3 (4.0)	0 (0)	0.5 (0.5)	2.3 (1.3)
	Lactobacillaceae	2.1 (0.6)	1.4 (0.4)	58.5 (7.6)	49.4 (8.5)	34.4 (4.0)
	Muribaculaceae	46.8 (1.7)	46.5 (3.1)	0 (0)	0 (0)	0 (0)
	Oscillospiraceae	2.0 (0.4)	2.7 (0.6)	0 (0)	0.1 (0)	0 (0)
	Peptostreptococcaceae	0.1 (0)	0.1 (0)	20.0 (8.5)	15.1 (5.6)	14.1 (6.4)
	Sutterellaceae	1.3 (0.2)	1.2 (0.1)	0 (0)	0 (0)	0 (0)
	Tannerellaceae	5.2 (0.6)	4.4 (0.5)	0 (0)	0 (0)	0 (0)
Genus						
	[Clostridium] inoculum group	0.3 (0.1)	0.2 (0.1)	0.2 (0.2)	1.8 (1.3)	1.6 (0.6)
	Akkermansia	37.7 (5.0)	29.4 (4.7)	0 (0)	0 (0)	0 (0)
	Anaeroplasma	1.4 (0.7)	0.9 (0.6)	0 (0)	0 (0)	0 (0)
	ASF356	2.3 (0.7)	4.4 (1.3)	0 (0)	0 (0)	0 (0)
	Bacteroides	27.3 (4.3)	31.5 (4.1)	0 (0)	11.4 (7.6)	8.5 (8.5)
	Clostridium sensu stricto 1	0 (0)	0 (0)	0 (0)	1.1 (1.0)	0 (0)
	Enterococcus	0 (0)	0.1 (0)	4.9 (2.1)	9.0 (1.1)	11.1 (3.3)
	Escherichia-Shigella	0 (0)	0 (0)	14.4 (6.3)	11.6 (5.1)	14.6 (6.3)
	Lachnospiraceae NK4A136 group	1.2 (0.6)	0.7 (0.5)	0 (0)	0 (0)	0 (0)
	Lactobacillus	5.6 (1.3)	4.8 (1.4)	77.0 (7.0)	62.4 (8.2)	60.8 (8.7)

Muribaculum	0.7 (0.7)	0.7 (0.7)	0 (0)	0 (0)	0 (0)
Parabacteroides	14.7 (1.3)	15.3 (1.5)	0 (0)	0 (0)	0 (0)
Parasutterella	3.5 (0.6)	4.1 (0.2)	0 (0)	0 (0)	0 (0)
Turicibacter	0 (0)	0 (0)	2.7 (2.7)	2.4 (1.9)	3.3 (1.6)

Table S4.3. Shifts in microbial taxa from BL to W4 in POS mice.

TOT Group		Time Point				
		BL	ICR	W1	W2	W4
Phylum						
	Bacteroidetes	58.8 (2.0)	59.4 (5.5)	0 (0)	0 (0)	18.8 (12.1)
	Firmicutes	27.1 (2.2)	30.4 (5.9)	77.4 (6.1)	82.1 (7.4)	61.0 (11.3)
	Proteobacteria	2.7 (0.7)	3.1 (0.7)	22.6 (6.1)	17.9 (7.4)	20.2 (7.9)
	Verrucomicrobiota	11.0 (2.5)	6.1 (0.7)	0 (0)	0 (0)	0 (0)
Class						
	Alphaproteobacteria	1.4 (0.6)	2.0 (0.6)	0 (0)	0 (0)	0 (0)
	Bacilli	3.6 (1.3)	2.8 (0.8)	54.4 (6.9)	65.1 (5.9)	48.0 (13.4)
	Bacteroidia	58.9 (2.0)	59.5 (5.5)	0 (0)	0 (0)	18.9 (12.1)
	Clostridia	23.4 (3.0)	27.6 (5.6)	22.6 (10.6)	16.9 (8.4)	12.6 (4.1)
	Gammaproteobacteria	1.3 (0.2)	1.2 (0.1)	23.0 (6.2)	18.0 (7.4)	20.4 (8.1)
	Verrucomicrobiae	11.0 (2.6)	6.1 (0.7)	0 (0)	0 (0)	0 (0)
Family						
	Akkermansiaceae	11.4 (2.6)	6.4 (0.8)	0 (0)	0 (0)	0 (0)
	Bacteroidaceae	15.0 (3.1)	13.1 (4.1)	0 (0)	0 (0)	9.3 (9.3)
	Clostridiaceae	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Enterobacteriaceae	0 (0)	0 (0)	22.4 (5.9)	18.0 (7.5)	20.1 (8.0)
	Enterococcaceae	0 (0)	0 (0)	0 (0.0)	9.4 (6.2)	3.1 (1.0)
	Erysipelotrichaceae	0.1 (0.1)	0 (0)	0 (0)	0.2 (0.2)	0.4 (0.3)
	Lachnospiraceae	19.5 (3.5)	22.9 (4.7)	0 (0)	0 (0)	5.1 (2.7)
	Lactobacillaceae	2.5 (1.0)	1.3 (0.4)	53.4 (6.9)	56.0 (10.0)	44.6 (13.1)
	Muribaculaceae	40.0 (3.3)	42.4 (2.1)	0 (0)	0 (0)	0 (0)
	Oscillospiraceae	1.7 (0.5)	2.6 (1.0)	0 (0)	0 (0)	0.2 (0.2)
	Peptostreptococcaceae	0.1 (0.1)	0 (0)	22.6 (10.6)	16.5 (8.1)	7.1 (2.8)
	Sutterellaceae	1.4 (0.2)	1.2 (0.1)	0 (0)	0 (0)	0 (0)
	Tannerellaceae	5.4 (1.4)	5.4 (1.5)	0 (0)	0 (0)	10.0 (8.2)
Genus						
	[Clostridium] inoculum group	0.2 (0.1)	0.3 (0.1)	0 (0)	0.3 (0.3)	0.5 (0.3)
	Akkermansia	28.0 (7.0)	19.0 (3.1)	0 (0)	0 (0)	0 (0)
	Anaeroplasma	2.4 (0.8)	3.9 (1.2)	0 (0)	0 (0)	0 (0)
	ASF356	2.4 (0.9)	2.6 (1.1)	0 (0)	0 (0)	0 (0)
	Bacteroides	35.8 (7.1)	34.2 (7.7)	0 (0)	0 (0)	12.8 (12.8)
	Clostridium sensu stricto 1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Enterococcus	0 (0)	0 (0)	1.6 (1.2)	14.5 (11.0)	3.1 (0.8)
	Escherichia-Shigella	0 (0)	0 (0)	16.7 (8.4)	11.2 (5.7)	8.4 (5.8)
	Lachnospiraceae NK4A136 group	1.9 (1.1)	3.8 (1.8)	0 (0)	0 (0)	0 (0)
	Lactobacillus	5.7 (2.2)	4.0 (1.4)	81.1 (9.0)	74.0 (10.9)	61.2 (16.0)

Muribaculum	1.6 (1.1)	2.8 (1.8)	0 (0)	0 (0)	0 (0)
Parabacteroides	12.5 (3.3)	14.1 (2.7)	0 (0)	0 (0)	11.8 (9.3)
Parasutterella	3.2 (0.3)	3.6 (0.3)	0 (0)	0 (0)	0 (0)
Turicibacter	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Table S4.4. Shifts in microbial taxa from BL to W4 in TOT mice.

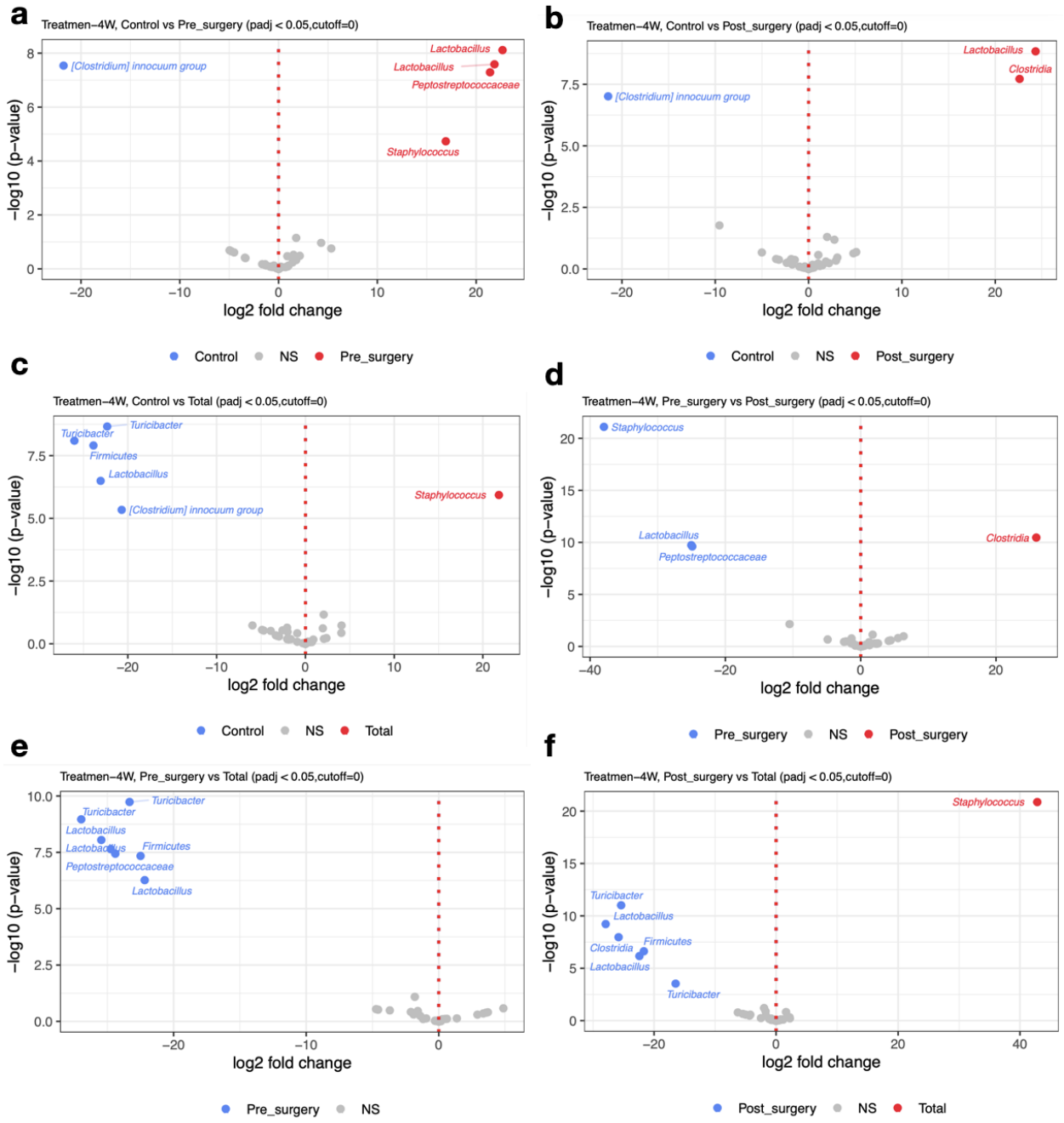

Figure S4.1. Volcano plots demonstrating differences in relative abundance between groups at W4 using DESeq2 analysis. a. CTR vs. PRE. **b.** CTR vs. POS. **c.** CTR vs. TOT. **d.** PRE vs. TOT **e.** PRE vs TOT **f.** POS vs. TOT.

Chapter 5: Conclusion

Gut microbial modulation has profound implications on the management of chronic human disease. In the enclosed thesis, the principles of this novel biotherapeutic strategy are explored with respect to two notable diseases of particular consequence to the health of North American populations- obesity and inflammatory bowel disease.

As mentioned in the introduction of this thesis, obesity remains one of the greatest epidemics of our time with its economic costs nearly surpassing the combined costs of smoking, war, and terrorism. Similarly, the prevalence and burden of IBD has increased markedly worldwide. Canadian rates of IBD are amongst the highest in the world with associated economic costs approaching nearly $3.0 billion in 2018. In addition to the similarities in their rising prevalence and increasing socioeconomic burdens, obesity and IBD also share important communal links essential to the justification of the work presented in this thesis.

Overwhelming evidence in animal and human studies supports a role for the gut microbiome in the development, propagation, and treatment of disease. Further, modern therapeutic approaches for both diseases are currently hampered by related overarching limitations: a lack of long-term efficacy, rising medication costs, and life-threatening complications. Together, these factors highlight that **a pursuit of novel therapeutic strategies which harnesses the principles of gut microbial modulation is therefore warranted.**

The first such strategy is presented in Chapter 2, wherein a landmark trial conducted by this author tested whether daily fiber supplementation used as an adjunct to FMT could provide

metabolic benefit in patients with severe obesity. This randomized double-blinded proof-of-concept trial is the first of its kind for a number of reasons. It demonstrates that microbial modulation could impart improvements in metabolic benefits using a safe and tolerable oral capsule FMT delivery method in a North American bariatric population undergoing concurrent medical therapy. Interestingly, fiber fermentability was found to differentially modulate metabolic response, with patients receiving low-fermentability fibers following FMT demonstrating significant improvements in insulin resistance, insulinemia, and enteroendocrine physiology. These benefits were associated with increased microbial richness and a bloom in select microbial taxa such as *Phascolarctobacterium, Christensellaceae, Bacteroides,* and *Akkermansia* – all of which have been shown by others to be associated with improved metabolic outcomes. Further, fiber fermentability was found to also optimize donor microbial engraftment, a principle that is thought to be a key roadblock to the efficacy of FMT in other inflammatory gastrointestinal diseases like IBD.

In chapter 3, alternative gut microbial modulation approaches which also aim to improve engraftment and optimize clinical IBD responses following FMT are discussed. Using a systematic review and pooled proportion meta-analysis, we evaluated the differences between two increasingly utilized strategies currently employed to overcome challenges with variable engraftment of donor FMT microbes- repeated FMT and antibiotic pretreatment. A total of 28 articles containing 976 patients were identified following a systematic search conducted by a medical librarian. Importantly, pooled analysis indeed revealed potential for both repeated FMT and antibiotic pre-treatment strategies in modulating IBD response and remission rates. That these benefits were also associated with an enrichment in bacterial taxa like *Bifidobacterium,*

Roseburia, Lachnospiraceae, Prevotella, Ruminococcus, and *Clostridium* related species which are associated with anti-inflammatory metabolite production further supports an adoption of these strategies in future clinical trials.

Lastly, in Chapter 4 the principles of gut microbial modulation are applied to an established murine model of ileocecal resection to assess whether an oral supplement designed to restore hypoxia and improve gut barrier integrity could enhance the immunologic dysfunction and microbial dysbiosis imparted by surgery. In this experiment, we provide evidence that tributyrin supplementation in the peri-operative period was associated with a differential modulation in gastrointestinal inflammation and gut microbial recolonization. Notably, mice receiving tributyrin postoperatively demonstrated a bloom of anaerobic taxa including *Bacteroides thetaiotomicorn, Bacteroides caecimuris, Parabacteroides distasonis, Clostridia,* and *Turicibacter*. These changes were also associated with improved trends in colonic inflammatory markers. These findings are the first to show that the peri-operative timing of a supplement differentially modulates gastrointestinal inflammation and gut microbial recolonization following gastrointestinal surgery.

Taken together, these studies add further backing to the growing body of evidence supporting the ongoing pursuit of gut microbial modulation strategies as a novel therapeutic modality for management of chronic inflammatory gastrointestinal diseases. In so doing, they also serve as a framework for the ongoing development of novel microbial biotherapeutic strategies aimed at

combatting the growing obesity and IBD epidemics through the future delivery of safe, effective, and affordable designer bacterial consortia.

Chapter 6: Future Directions

The gut microbial revolution has brought equal parts promise and excitement to the management of various diseases. Indeed, we are currently in what this author regards as the golden era of translational research within the field of gut microbial modulation- one where we hope to establish the gut microbiome as a viable target for population-based medical intervention. In this pivotal era, our goals must be to take the promising concepts learned from current murine studies and proof-of-concept human trials so as to develop novel safe, effective and tolerable biotherapeutic therapies.

In order to achieve these goals, a number of key questions remain to be answered. For example, ongoing debate exists regarding the concept of microbial dysbiosis. Are the imbalances in microbial communities truly causal to the pathogenesis of these diseases or do they result as a consequence of the underlying host pathology?

Currently, the gold-standard methodologic approach in answering these questions is the use of human microbiota-associated (HMA) rodent studies. Using these elegant designs, researchers are able to evaluate if human microbiota transferred to germ-free mice animals can successfully transmit human disease phenotypes. However, a recent review of these studies has raised important concerns regarding their methodological rigor advocating for caution when interpreting causality. Walter et al. found that out of 38 studies an alarming 36 demonstrated a successful transfer of human disease phenotype. This finding, the authors believed, was particularly implausible given the shortcoming in experimental design, inappropriate statistical analysis, lack of negative studies, and failure to provide mechanistic insight. Studies in the

translational era must thus adopt newfound methodological rigor if we aim to successfully establish causality and eventually translate experimental findings to routine clinical practice.

Another important next step in transitioning gut microbial modulation therapy from theory to routine clinical practice is the adoption of standardized practices. Fecal microbial transplantation is an excellent example of why this is needed. In our review of IBD and FMT literature, many of the donors were chosen based on convenience, or donor availability, with some receiving donor stool from healthy family members while other receiving FMT from established donors. Routes of delivery, dosing, and timing of delivery also differed dramatically between studies. Further variations in sequencing methodologies which utilized different primers resulted in high inter-study heterogeneity and precluded effective meta-analysis. If we truly hope to incorporate FMT as a novel medical therapeutic strategy, future standardization of these practices is needed.

In addition to incorporating enhanced experimental rigor and standardization of microbial modulation practices, perhaps the last critical hurdle is the adoption of 'omics' analyses. While currently cost-prohibitive, these technologies remain pivotal in helping us understand the complex ways in which gut microbial modulation can benefit gastrointestinal health. Commonplace 16s sequencing only tell us what bacteria are there and provide little mechanistic information regarding how various therapies are able to change bacterial function within our complex gut microbial ecosystem. It is only with this enhanced mechanistic understanding of our newfound microbial organ that the golden era of translational research can help to usher away the growing epidemics of chronic inflammatory gastrointestinal disease.

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