Fecal Microbial Transplantation and its Expansion into the Treatment of Other Diseases

by

Braden Thomas Millan

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

Department of Medicine

University of Alberta

Abstract

Background: Fecal Microbial Transplantation (FMT) has gained popularity due to its efficacy in the treatment of recurrent *Clostridium difficile* infection (RCDI). RCDI is associated with prolonged courses of antibiotics with high recurrence rates following the initial resolution of symptoms. The increased use of antibiotics in both healthcare and agriculture has led to increased prevalence of antibiotic-resistant microbes. Therefore, we first tested the hypothesis that patients with RCDI would harbor high numbers of antibiotic-resistant bacteria and secondly, that FMT would reduce the number of antibiotic-resistant gene containing bacteria. Furthermore, the association between microbial dysbiosis and inflammatory bowel disease (IBD) has enhanced interest in therapies targeting the microbiota. For RCDI, resolution of the infection using FMT correlates with efficient engraftment of the donor microbiota. The purpose of our mouse studies was to determine the efficiency of donor microbiota engraftment into the recipient following a short pre-FMT treatment regimen and investigate if this changed the FMTs ability to reduce the development of colitis in Interleukin-10 knockout (IL-10 -/-) mice.

Methods: Using shotgun metagenomics, we sequenced the microbiota of 20 RCDI patients. The libraries from the RCDI patients and a healthy cohort (n=87) obtained from the Human Microbiome Project (HMP) were aligned against the NCBI bacterial taxonomy database and the Comprehensive Antibiotic Resistance Database (CARD). Results were corroborated through a DNA microarray containing 354 antibiotic resistance (ABR) genes. In mice, the microbiota was analyzed at baseline (day 0), following treatment (day 3), three days after FMT (day 7) and 31 days following FMT (day 35) using real-time PCR and 16S rRNA sequencing. Sequencing data was processed using the QIIME pipeline. Gastrointestinal inflammation was quantified by measuring cytokines from the stool, cecum tissue, small intestine tissue, and serum, as well as histology scoring of the small intestine and colon.

Conclusion: RCDI patients have increased numbers of antibiotic-resistant organisms. FMT is effective in the eradication of pathogenic antibiotic-resistant organisms and elimination of antibiotic-resistance genes. Regardless of the pre-FMT treatment regimen, strong engraftment of the donor microbiota into a healthy recipient was observed and remarkably was maintained after four weeks. In an IBD model, our results indicate that preparation of the host has substantial effects on the efficacy of FMT and should be considered moving forward in FMT research for other diseases.

Preface

This thesis is an original work by Braden T. Millan. All research projects, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board:

Fecal microbiota transplantation (FMT) in the management of recurrent Clostridium difficile infection (CDI), Pro00049006, October 2014.

Probiotic bacteria and epithelial cells and Breeding Colony, AUP00000293, August 2014.

Chapter 2 of this thesis has been published as Millan B, Park H, Hotte N, Mathieu O, Burguiere P, Tompkins TA, Kao D, & Madsen KL. Fecal microbial transplants reduce antibiotic-resistant genes in patients with recurrent Clostridium difficile infection. Clin Infect Dis. 2016 Mar 29.

KM, TT, and DK designed the study; BM and HP conducted the research; BM, HP, NH, OM, PB, JD carried out the data analysis; DK performed patient assessments and fecal microbial transplants; BM, HP, and KM wrote the manuscript; All authors read and approved the final manuscript.

Acknowledgements

First, I would like to thank my supervisor *Dr. Karen Madsen* for all the guidance she has provided me with throughout my graduate studies. Without her, the contents of this thesis would not be possible.

I would also like to thank my committee members, *Dr. Dina Kao* and *Dr. Richard Fedorak* who helped guide me through my graduate studies and provided me with much-needed career guidance.

All the members of the Madsen laboratory helped support me through my graduate studies, in particular, Naomi Hotte whose invaluable knowledge helped guide me through each day; as well as Dr. Heekuk Park who wrote all the bioinformatics pipelines and taught me how to use them.

Finally, I would like to thank my parents for all the opportunities they have provided me with in my life and hope that they understand that my success is a direct indication of how amazing they truly are.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AAD	Antibiotic Associated Diarrhea
ABR	Antibiotic Resistance
Abx	Antibiotics
ANOVA	Analysis Of Variance
AIEC	Adherent Invasive *Escherichia coli*
ATF16L1	Autophagy-related 16-like 1
BFT	Before Fecal Microbial Transplantation
b.i.d.	Twice daily
Bp	Bowel Preparation
BSA	Bovine Serum Albumin
CARD	Comprehensive Antibiotic Resistance Database
CD	Crohn's Disease
C. difficile	*Clostridium difficile*
CDAD	*C. difficile* Associated Diarrhea
CDAI	Crohn's Disease Activity Index
CDI	*Clostridium difficile* Infection
CI	Confidence Interval
CR	Clinical Remission
CRKP	Carbapenem-resistant *Klebsiella pneumoniae*
CRP	C-reactive Protein
DNA	Deoxyribonucleic Acid
DSI	Donor Similarity Index

DSS	Dextran Sodium Sulfate
ELISA	Enzyme-linked Immunosorbent Assay
ESBL	Extended Spectrum Beta Lactamase
FMT	Fecal Microbial Transplantation
g	gram
GWAS	Genome-wide Association Study
HIV	Human Immunodeficiency Virus
HMP	Human Microbiome Project
IBD	Inflammatory Bowel Disease
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
IL10 -/-	IL10 Knock-out 129S1/SvlmJ Mice
IRGM	immunity-related GTPase family M
ISDA	Infectious Diseases Society of America
LCN-2	Lipocalin 2
LDA	Linear Discriminant Analysis
LEfSe	Linear Discriminant Analysis Effect Size
MEGAN	MetaGenome Analyzer
mg	milligram
mL	milliliter
MRSA	Methicillin Resistant *Staphylococcus areus*
NF-κB	Nuclear Factor-kappa B

ng ..... nanogram

NOD2 ..... Nucleotide-binding Oligomerization Domain-containing protein 2

OTU ..... Operational Taxonomic Unit

PBS ..... Phosphate buffered saline

PCA ..... Principal Component Analysis

PCDAI ..... Pediatric Crohn's Disease Activity Index

PCoA ..... Principal Coordinate Analysis

PEG ..... Polyethylene Glycol

PFT ..... Post Fecal Microbial Transplantation

PICRUSt ..... Predictive Functional Profiling of Communities by Reconstruction of Unobserved States

PLS-DA ..... Partial Least Squares-discriminant Analysis

PPI ..... Proton Pump Inhibitor

PUCAI ..... Pediatric Ulcerative Colitis Activity Index

Q-PCR ..... Quantitative-Polymerase Chain Reaction

QIIME ..... Quantitative Insights into Microbial Ecology

RCDI ..... Recurrent *Clostridium difficile* infection

RCT ..... Randomized Controlled Trial

rDNA ..... ribosomal Deoxynucleic Acid

RPM ..... Rotations per minute

rRNA ..... ribosomal Ribonucleic Acid

SES-CD	Simple Endoscopic Activity Score for Crohn's Disease
SDS	Sodium Dodecyl Sulfate
SNP	Single Nucleotide Polymorphism
TcdA	Enterotoxin A
TcdB	Cytotoxin B
t.i.d.	Three times daily
TNF	Tumor Necrosis Factor
UC	Ulcerative Colitis
VRE	Vancomycin Resistant *Enterococci*
µg	microgram
µL	microliter

Chapter 1: The Microbiota in *Clostridium difficile* Infection and Inflammatory Bowel Disease and Implications for Fecal Microbiota Transplantation

1.1 Introduction

All of the bacteria colonizing the human gastrointestinal tract are referred to as our microbiota. It is known that the human gut contains over 1000 different species and 10^{14} total bacteria which are implicated to play a significant role in human health and diseases.(1) Ecology (from the Greek *Oikos,* home, and *logos,* to study) is the scientific study of the interactions between living organisms and the environment they occupy. The interactions exist on a hierarchical scale from the smallest single cell organisms to the biggest multicellular organism and their environment, as well as between organisms that occupy the same environment. The macroscopic interactions between organisms can be described by how the interaction affects each party. They are defined as interactions where both organisms benefit (mutualism), one organism benefits, the other is unaffected (commensalism), one organism benefits and the other is negatively affected (parasitism), one organism is unaffected, and the other is negatively affected (amensalism), and finally where both organisms are adversely affected (competition). There are both biotic and abiotic factors that play a significant role in determining the distribution of organisms within their environment. How a species uses the biotic and abiotic resources in its environment is referred to as the species' ecological niche, whereby competitive exclusion means that no two species can co-exist in the same niche. When two or more

species co-exist within the same geographical area at the same time, this is referred to as a biological community. Species can only co-exist in a community with one another as long as there are one or more significant differences in their niches. The species diversity of a community, or the variety of organisms that make up the community, has two different components. The species richness, or the number of different species in the community, and the relative abundance, or the proportion each species represents of the total population. More specifically, alpha-diversity describes community composition in a single or local environment (one individual), and beta-diversity is used when describing the differences in community composition between environments.(2) When the ecosystem itself resides within an organism, such as the microbiota of the gastrointestinal tract of humans, both ecology and physiology play a fundamental role in determining the community composition. It is well understood that increased fitness results from the mutualistic relationship between the human host and the microbiota residing in the gut, which perform a variety of functions.(3) Many of the interactions between the bacteria within the gut community, and between prokaryotic and eukaryotic cells are hypothesized to have substantial effects on the health of the host, but very few specific relationships have been elucidated to date.

The human gut microbiota is similar to other complex ecosystems in that it is dynamic and has a stable average state. Stability, in the context of a community, is its ability to reach and maintain a relatively constant composition of species in their richness and relative abundance. The human gastrointestinal tract is a complex organism containing up to 100 trillion microbes. Despite the vast number of microbes, the human

gut is extremely selective and is dominated by just two bacterial phyla, the Bacteoidetes, and the Firmicutes.(4) The selective pressures that determine the microbial composition at each location along the intestinal tract remain largely unknown, and there are many environmental and genetic factors that help shape the microbiome of each individual.(5-8) The protective, structural, and metabolic activities of resident commensal gut microbes have some diverse implications on the health of the host, and in recent years, a disturbed microbiome has been associated with many disease states, including inflammatory bowel disease (IBD).(9-14)

The development of a somewhat familiar gut microbiota is the result of basic Darwinism, whereby a precise and stable combination of microbes provide increased fitness for the host, which in turn provides a greater number of environments in which the organism can live.(3) The phylogenetic composition of the human gut microbiota is dominated by Firmicutes and Bacteroidetes, which has been verified using next generation sequencing. Industrialization, western society, including diet, hygiene, and medicine, has had a significant impact on this ecological equilibrium and in the past decade, has been associated with increased incidence of gastrointestinal diseases.(15) Although there are over a thousand different bacterial species that have been found in the gut of humans, each person typically only has approximately 160 different species, suggesting that an individual's microbiota is distinct, and is altered to varying extents by various environmental conditions and their immune system.(16)

Those microbes that appear to be more stable within an individual over time are those that make up a large proportion of the total bacteria.(17-20) These are described as foundation taxon due to their extensive interactions with other taxa and ability to create a locally stable environment for other species. In addition, foundation taxa are numerically dominant while keystone species are present at low abundances and have a high degree of ecological connectedness. Trosvik et al. identified *Bacteroidetes* as an important foundation taxa and *Actinobacteria* as a keystone species.(21) Keystone species, therefore, represent a vulnerable point for the gut microbiota where their loss could have vast implication on ecological homeostasis. Imbalance of the gut microbiota, known as 'dysbiosis,' is a concept which describes the loss of the balance between "protective" versus "harmful" bacterial species, where the host may become more susceptible to infection by opportunistic pathogens due to the loss of colonization resistance.(22) Also, it is now apparent that the microbiota can drive the development of diseases, and in some circumstances, when proper functionality can be re-established, to treat disease. It was recently suggested that this term is being misused in that it is believed it falsely represents a better understanding of gastrointestinal ecology than what we currently have.(23)

New methods of DNA sequencing are unable to differentiate between active and inactive organisms, making it difficult within the context of microbiota dysbiosis to determine the ecological importance of changes in the proportion of particular species. The ability of individual members of the microbiota to induce host innate immune responses is not proportional to their abundance in the community, and, therefore, makes

their functional capacity unknown without further investigation beyond microbiome sequencing.(24) The importance of specific gut microbes is evident in that a single bacterial molecule (polysaccharide A) from *Bacteroides fragilis* has been shown to work actively with the host's immune system to prevent intestinal inflammation.(25) In recent decades, next generation sequencing has created almost as many new questions about the gut microbiota as it has answered, providing scientists with endless opportunities to study the role of the microbiota in health and disease.

1.2 *Clostridium difficile* infection

1.2.1 Introduction

Clostridium difficile infection (CDI) is a leading cause of antibiotic-associated diarrhea (AAD), which has increased in both incidence and severity over the last decade.(17) It is typically transferred via the fecal-oral route and infects the large bowel of the host. Infection ranges from mild symptoms of stomach pain, diarrhea, and dehydration, to fulminant colitis and toxic megacolon, which when untreated may result in death. *C. difficile* is a gram-positive, anaerobic, spore-forming bacterium with the ability to produce two toxins, enterotoxin A (TcdA) and cytotoxin B (TcdB) which confer virulence. *C. difficile* is has not been shown to be invasive, and extra-intestinal infections are extremely rare. Diagnosis is currently based on protein or gene detection of either toxin A or B using enzyme immunoassay or by DNA-based tests on stool samples obtained from the patient. More hospital laboratories have adopted DNA-based tests as they provide higher sensitivity and specificity than the enzyme immune assays. Infection

control and prevention measures for *C. difficile* consist predominately of traditional approaches targeting the bacteria prior to colonization of a host. These include washing surfaces with 10% sodium hypochlorite, use of barriers, and thorough hand washing with chlorhexidine.(26)

1.2.2 Epidemiology and Pathology

There is increasing epidemiology evidence in the literature that the incidence of *C. difficile* infection is worsening at a dramatic rate globally, resulting in higher mortality.(27-30) A systematic review of studies determining risk factors for CDI showed the strongest risk associated with duration of antibiotics, multiple antibiotics, anti-ulcer medications (including antacids, H_2 blockers and proton pump inhibitors (PPIs)), the length of hospital stay and increasing age, providing evidence for its multifactorial etiology.(31) *C. difficile* is present in 3% of the healthy adult population, but up to 20-50% of adults in hospitals and long-term care facilities become colonized, characterizing it as a prevalent nosocomial infection.(32) Levy et al. projected the number of *C. difficile* infections in Canada as 37,900 episodes in Canada in 2012; 7980 (21%) of which were relapses. This infection rate has both a financial and societal burden with an estimated expense to society of $281 million from one study; 92% of which was the result of in-hospital costs, 4% the result of direct medical costs to the community, as well as 4% due to lost productivity.(33)

The ability of *Clostridium difficile* to form spores resistant to a variety of harsh environments is believed to be an important factor in enabling it to persist in patients and

the environment increasing likelihood of transmission. Transmission of *C. difficile* occurs through ingestion of the bacteria and its spores, which can pass through the acidic environment of the stomach and germinate in the small bowel of the gastrointestinal tract when exposed to bile acids. Some patients become asymptomatic carriers of the bacteria while in others it results in *C. difficile*-associated diarrhea (CDAD). Kyne et al. showed that asymptomatic carriers had increased serum levels of IgG antibodies against toxin A.(34) This suggests that there is still an important role of host humoral immunity in the prevention of CDI symptoms in addition to the colonization resistance provided by a healthy microbiota.

The main virulence factors of toxigenic *C. difficile*, are toxins A and B and hydrolytic enzymes. These cause an inflammatory response that increases vascular permeability and immune cell recruitment, followed by epithelial tight junction opening and cellular apoptosis and connective tissue degradation.(35) Also, *in-vitro* experiments showed that both highly and moderately virulent strains of *C. difficile* were able to localize to two cultured human cell lines: Caco-2 cell and the human mucus-secreting HT29-MTX cells.(36) This ability to adhere to the gut epithelium would allow for the efficient delivery of toxin to epithelial cells. In another *in-vitro* study, a novel colonocyte plasma membrane protein, glycoprotein 96, was found to be the integral cell surface receptor responsible for internalization of toxin A.(37) Other virulence factors, such as hydrolase activity, which initially was hypothesized to be important in *C. difficile* virulence, showed no direct correlation between toxigenic status, or virulence and hydrolytic enzyme production.(38) Therefore, in recent years, the pathophysiology of *C.*

difficile has become more understood, with a focus on toxin A and B, epithelial adhesion and host-dependent factors that allow this organism to be virulent.

1.2.3 Treatment and Recurrence

Ironically, first line treatment for CDI has been since the 1970s, a course of antibiotics, starting with metronidazole. If metronidazole does not clear the infection, a course of oral vancomycin or in severe and complicated cases, a combination of the two is used. However, despite their use for such an extended period, clinically relevant resistance to either metronidazole or vancomycin in *C. difficile* has never been reported. The infectious diseases society of America (IDSA) guidelines updated in 2010 suggests that patients presenting for the first time, having recurrent mild to moderate disease should be treated with Metronidazole 500 mg three times per day for ten days while severe infections should be treated with vancomycin 125mg orally four times a day for ten days.(39) Depending on the severity of the symptoms, other co-morbidities, and the number of CDI infections, the ISDA suggest several different approaches for treatment, such as tapering or pulse dosing, and long-term dosing. A study using an *in vitro* human gut model showed that extended dosing regimens of vancomycin may not be more helpful in achieving sustained clinical cure in that it would further exacerbate intestinal microbiota dysbiosis.(40) Vancomycin is recommended for continued treatment of CDI due to the risk of neuropathy from continued metronidazole use. Treatment modalities, therefore, vary based on disease severity, host factors, and the number of recurrences.

Debast et al. summarized the current literature looking at antibiotic treatment for initial CDI and found that a sustained response was maintained in a variable number of patients, as low as only 52% to as high as 93%.(41) It is commonly quoted that the risk of the recurrence for CDI is 20-30% within 30 days of initial treatment, and increases further up to 50% after a second episode.(32, 41, 42) A study looking at the strains isolated from 93 patients showed that less than half (48.4%) of all clinical recurrences were re-infections with a different strain.(43) Therefore, a large proportion of CDI are not simply the result of re-exposure to *C. difficile* but are recurrences that result from the organism persisting in the gastrointestinal lumen following antibiotic treatment. Fortunately, over 90% of first recurrences can be treated in the same way as initial infections, however, the remaining 10% continue to be a challenge for physicians to treat.(44) Experimental treatment options for patients with recurrent CDI (RCDI) and/or refractory disease focus mainly on the development of new antibiotics, such as fidaxomicin, tigecycline, teicoplanin, nitazoxanide, ramoplanin, and rifaximin, although these are not used much clinically and have the potential to develop the same issues as current treatments.

1.2.4 Fecal Microbial Transplantation in CDI and RCDI

Fecal microbial transplantation (FMT) has only recently become a popular and widely accepted approach to treating RCDI, and our understanding of the gut microbiota continues to expand paving the way for its expansion into other diseases and infections. Colonization resistance to *C. difficile* infection is the result of ecological forces alluded to previously which are at play in the gut microbiota of a mature adult. Since their creation,

antibiotics have had massive implications in modern medicine, reducing the number of deaths caused by bacterial infections while causing some adverse side effects, in particular, antibiotic-associated diarrhea (AAD), such as CDI. The microbial dysbiosis and reduced number of bacteria in these individuals caused by repeated courses of antibiotics allow the microbiota from a healthy donor to colonize the intestinal tract following FMT much more efficiently because the bacteria can occupy specific niches, re-establishing colonization resistance in the host, and fighting off the infection.(45)

FMT has been offered via some different delivery methods including colonoscopy, a nasogastric tube, percutaneous endoscopic gastrostomy, retention enema, and orally in the form of frozen pills. Current literature suggests that colonoscopy is the most efficient route of FMT delivery for the treatment of RCDI. (46, 47) A review and meta-analysis which included 18 observational studies found that 531 of 611 patients experienced primary cure (pooled rate, 91.2%; 95% CI, 86.7-94.8%), with overall, early and late recurrence rates of 5.5% (95% CI, 2.2-10.3%), 2.7% (95% CI, 0.7-6.0%) and 1.7% (95% CI, 0.4-4.2%).(48) In these studies, most of the recurrences were the result of the patient being exposed again to antibiotics. In total 38 cases of death were reported in 7 of the 18 studies, where the relation of FMT to death ranged from unrelated to possibly related. In a review of 1190 patients receiving FMT for RCDI, the most common adverse events associated with the procedure were abdominal distension/bloating/cramping (2.35%), flatulence (2.1%), and diarrhea (1.93%).(49) This demonstrates the safety of FMT, although this data comes predominately from case studies and series, and not randomized controlled trials. Also, other factors can complicate this procedure or reduce the efficacy

of CDI eradication. Khoruts et al. showed that having an underlying IBD reduced the efficacy of FMT for RCDI (74.4% vs 92.1%). However, it was still very efficacious where following a second FMT, the success rate increased to 82.9% in IBD patients and 98.7% in non-IBD patients.(50) FMT also posed a risk to the patients with underlying IBD, where 11 of 43 (25.6%) of the patients had an FMT-related flare of their disease. This further emphasizes the complex interactions between the microbiota, the host immune system, and genetics, and whether or not it should be used to treat patients suffering from RCDI who have underlying IBD, as well as whether or not it presents as a possible treatment modality for IBD. The female sex, previous hospitalization, and previous surgeries were other risk factors found to associated with FMT failure in a retrospective study of 201 patients, however, no variables were identified that may help us predict FMT failure.(47)

A retrospective study looking at all published cases showed that FMT via colonoscopy was the most beneficial strategy in 87% of the trials looking at the treatment of RCDI at a willingness-to-pay of $50,000/QALY (quality adjusted life year).(51) Timely delivery of FMT has been shown to be cost-effective (mean cost saving $29,842 CAD per patient) when FMT is given before the onset of a third RCDI recurrence, with similar infection eradication rates.(52) Based on current literature for FMT as a treatment for RCDI, it appears to be the most effective therapy available, with the lowest cited rates of recurrence and few adverse events related to the procedure, however, long-term effects of FMT should be the focus of future investigations. Although useful for CDI and RCDI,

the expansion of FMT as a therapeutic for other diseases still requires a lot of research to determine both its efficacy and safety.

1.3 Inflammatory Bowel Disease

1.3.1 Introduction

The two most studied and characterized forms of IBD are Crohn's disease (CD) and ulcerative colitis (UC); both of which are characterized by relapsing and remitting chronic inflammation of the gastrointestinal tract. Although our understanding of these two predominate forms of IBD has continually been expanding since they were first described, UC in the mid-1800s, and CD later in 1932,(53, 54) we still lack a definitive understanding of the disease etiology. These two forms share many clinical and epidemiological characteristics, suggesting similar underlying causes, and approximately 10% of patients are diagnosed with a colitis that has features of both UC and CD, referred to as indeterminate colitis.(55) Current knowledge suggests a multifactorial etiology, with genetic, environmental, and gut microbial components, resulting in an aberrant immunological reaction and resulting symptoms.(56, 57)

CD can affect any portion of the gastrointestinal tract from the mouth to the anus, however, three phenotypes are predominately observed, ileal (distal small intestine), colonic or ileocolonic CD.(58) CD frequently presents with abdominal pain, fever and clinical signs of bowel obstruction or diarrhea with passage of blood or mucus, or both.(59) In contrast, UC is a disease characterized by inflammation of the mucosal layer

commencing in the rectum (proctitis) and some cases spreading to the rest of the colon. The primary presenting symptom of UC is visible blood in the stool, with or without mucus which is reported in more than 90% of cases.(60) The onset is typically gradual, and the disease course is often characterized by periods of spontaneous remission and subsequent relapse, and that is less prone to complications than CD.

1.3.2 Epidemiology

The incidence and prevalence of IBD vary geographically, with associations to industrialization as seen by high rates observed in the United Kingdom, Northern Europe, Canada, and the United States and lowest in continental Asia, although they are increasing rapidly globally.(56, 61) Previously published studies identified a north-south gradient, however, more recent reports indicate that incidence and prevalence are much the same in the southern hemisphere as in the northern hemisphere.(62) The annual incidence rates vary by geographic region, with UC estimates ranging from zero to 24.3 and CD from zero to 20.2 per 100,000 and prevalence of UC ranging from 4.9 to 505 and CD from 0.88 to 318.5 per 100,000.(63) The observed incidence of UC is more often greater than that of CD, except in Canada and several areas of Europe,(64) although the values are continually changing.(65-67) Several studies have suggested that the levels of IBD in Western countries have plateaued or are even on the decline.(68, 69) Despite this, in the early 2000's it was shown that IBD had emerged in countries in which they had not previously been observed such as South Korea, China, India, Iran, Lebanon, Thailand, the French West Indies, and North Africa.(70-72) The association between epidemiological

data and industrialized and developing countries suggest a substantial role of environmental factors in the etiology of these diseases.

The age of onset reported in the literature is most often between the ages of 20-30 for CD and 30-40 years for UC, with some studies suggesting that a second peak occurrence occurs at 60-70 years old, but this observation is not definitively established.(73) CD is observed in women 20-30% more often than men, especially in high-incidence areas, whereas UC, is observed 60% of the time in men. The distribution of CD and UC among pediatric patients is opposite to that of adults where we saw a predominance of CD among boys and of UC among girls.(64, 66, 74, 75) An analysis of patients listed in a pediatric IBD consortium showed no correlation between sex and age of onset, however CD comprised 60% of the IBD in older children (6-12 years) and 66% of adolescents (13-17 years), with a large increase in frequency beginning at age 10.(76) In addition, a Europe-wide study showed that a younger age at the onset of IBD is associated with a lower quality of life.(77) Needless to say, IBD is a more modern disease with a dire need for new therapies to reduce their incidence and prevalence in the future.

1.3.3 Treatment

Due to incompletely understood pathophysiology, and inter-individual differences, treatment of these disorders remains a clinical challenge for physicians. Current treatment options include anti-inflammatory, immune modulating, and microbiome-modulating therapies including antibiotics, 5-aminosalicylates (5-ASA),

corticosteroids, thiopurines, methotrexate, calcineurin inhibitors, and anti- tumor necrosis factor (TNF) therapy.(64) Although there have been many advances in modern medicine for the management of IBD, surgical options are still required for many patients, which are associated with significant morbidity, cost, and reduced quality of life. Approximately 25-30% of UC patients undergo elective surgery for operative management of their disease, resulting in the cure of the disease.(78) Surgery for CD, in contrast, is not curative and is performed in up to 80% of patients, predominately for complications such as strictures and fistulas. Conservative resection is recommended in the majority of cases to preserve bowel length in case another surgery is required in the future.(79) Therefore, investigation of new treatment modalities such as FMT should be continued to prevent the use of more extreme treatment measures to increase the quality of life and survival of patients diagnosed with these life-long inflammatory diseases.

1.3.4 Economic Costs

IBD are lifelong diagnoses with increasing global incidence and prevalence that will only continue to be a greater burden on the individuals affected and society without the discovery of new therapeutic approaches and disease management. The economic costs of IBD in Canada were estimated to be $2.8 billion in 2012 (nearly $12,000 per IBD patient), with direct costs exceeding $1.2 billion per annum which are driven by the cost of medication ($521 million), hospitalizations ($395 million) and physician visits ($132 million). Indirect costs (society and patient costs) are dominated by long-term work losses and are estimated at a total of $1.6 billion per annum.(80)

1.3.5 Genetics

The concordance rate between monozygotic twins with IBD has been shown to be approximately 30% of CD patients and 10% of UC patients, signifying some genetic effect, but also a strong environmental effect in the etiology of IBD.(81) Twin studies have shown that monozygotic twins have higher concordance rates than dizygotic twins for CD (20.0%-58.3% vs. 0%-6.5%) and UC (6.3%-18.8% vs. 0.0%-4.5%). However, the lack of concordance amongst dizygotic twins confirms that other important etiological factors are important in these chronic diseases.(82-85) Epidemiological data previously published showed that first-degree relatives are 14-15 times more likely of developing IBD than the general population.(86) Recent studies have shown that a child has a 26-fold increased risk of developing CD when another sibling already has CD and the risk in increased 9-fold in the case of UC.(87) Therefore, it is apparent that genetics does play a significant role in the etiology of the IBD, and it appears that CD is more genetically determined than UC.

To date, genome-wide association studies (GWAS) have identified a total of 163 IBD-associated loci in the human genome, 23 associated with UC, 30 with CD, and 110 shared by both diseases.(88-90) Although some loci are unique to each disease, the fact that there is a significant percentage of overlap supports earlier beliefs that these diseases share common genetic risk factors. However, the GWAS evidence shows that the relationship between these genetic loci and the IBD can only explain approximately 14% and 7.5% disease variance in CD and UC, respectively.(91) IBD is associated with other

autoimmune disorders such as ankylosing spondylitis, psoriasis, and primary sclerosing cholangitis, which share more than 50% of the identified IBD risk loci.

One of the first genes associated with IBD, namely CD was the Nucleotide-binding oligomerization domain-containing protein 2 (*NOD2/Card15*). NOD2 is an intracellular receptor which sense microbial patterns such as muramyl peptides that are components of bacterial peptidoglycan. Stimulation of NOD2 results in the activation of nuclear factor NF-κβ and a resultant inflammatory response.(92) Paneth cells responding to microbial signals through NOD2 (93) receptors release α-defensins in order to control the composition of the microbiota in the intestine.(94) Later, gene variants of NOD2/Card15 were shown to be associated with familial risk as well as complicated cases of CD.(95) These results suggest a strong correlation between the genetic risk factors and their association to the intestinal microbiome and the ability of the host's immune system to sense and regulate the commensal prokaryotes that colonize the gut.

In a study comparing 1,855 UC individuals to 3,091 controls, the most significant genetic predictor for UC was variants of the interleukin-10 (IL-10) gene, which regulates the inflammatory process.(96) IL-10 is a potent anti-inflammatory cytokine, and it was shown more than two decades ago that IL-10 deficient mice develop chronic enterocolitis, and are now commonly used as an animal model for studying IBD.(97) IL-10 is an important cytokine for host immune tolerance to innate commensal bacteria of the gastrointestinal tract.(98) For most GWAS identified loci, it cannot be concluded if a single nucleotide polymorphism (SNP) is related to the disease on a biological level, or

whether it is just a marker for the disease.(99-103) Using targeted sequencing methods, we can elucidate the association between illness and specific gene variants. There is currently strong evidence of association to causal variation in IBD (i.e. missense, nonsense, or splice junction variants) in the *NOD2, ATG16L1, IL23R, MST1, CARD9, IL18RAP* and *RNF186* and at least suggestive evidence for causal variation in the *CUL2, C1orf106, PTPN22, MUC19, CEP72, LAMB1, CCR6, JAK2,* and *STAC2* genes. (99-101, 103, 104) In CD, some of the enriched pathways included genes associated with cytokines, host resistance to microbial infection, cell migration, and regulation of immune system, while in UC, the enriched pathways involved leukocyte migration, epithelial-to-mesenchymal transition (EMT) and metabolic and catabolic processes.(105) The identification of these pathways contribute to the working knowledge of the interplay between genetics and the microbiota in the etiology of IBD and offer potential new targets for therapeutics.

1.3.6 Environmental Factors

The high prevalence of IBD in western countries and its diagnosis in developing countries with no previous incidence of these diseases provides support for the hygiene hypothesis.(14) The hygiene hypothesis states that the absence of exposure to infectious and symbiotic prokaryotes and other microorganisms in early childhood prevents the proper development and education of the immune system making individuals more susceptible to disease. Other environmental factors that have been associated with IBD risks are smoking (106), formula feeding, (107) diet, in particular, animal protein and fast food, (5, 6) and many others. Diet studies showed a correlation between the high intake

of total fats as well as red meat with increased incidence of IBD,(7) whereas a semi-vegetarian diet in was associated with a high rate of maintenance of disease remission.(8)

A population-based cohort study looked at the occurrence of IBD in immigrants to Canada, who had an incidence rate of 7.3/100,000 person-years, compared to 23.9/100,000 in non-immigrants. Increased age at immigration was associated with decreased risk of IBD, and a 14% increased risk per younger decade of life at immigration; additionally, children of immigrants born in Ontario assumed the same incidence risk as the children of nonimmigrants.(108) A prospective case-control study that evaluated IBD environmental risk factors of Middle Eastern migrants in Australia compared to matched Caucasians IBD subjects and controls showed that different ethnic groups had a different incidence of IBD when exposed to similar environmental factors, (109) indicating that an environmental component is not sufficient to cause IBD.

1.3.7 Microbiome and the Immune System

In contrast to humans, the role of intestinal microbiota in the development of experimental colitis in animal models has been known for decades, contributing to the interest in the role it plays in human IBD.(110) Patients with IBD have been shown to have reduced diversity of fecal microbiota (12), with much more evidence in CD than in UC.(9-11) Inflammation alters the nutritional environment of the gastrointestinal tract that results in changes in the structure of the microbial community. (Reviewed in (111)) Patterns of dysbiosis in IBD reported in the literature in the past were much more inconsistent. However, this is beginning to change with the increasing number of

metagenomic studies and the enhanced capabilities of next generation sequencing technology. Using Q-PCR, Frank et al. showed a >300-fold and 50-fold reduction in *Lachnospiraceae* and *Bacteroidetes* respectively in an IBD cohort in comparison to a healthy control group.(112) Also, enrichment of *Actinobacteria* and *Proteobacteria* in the dysbiotic IBD cohort contributed to them clustering separately from healthy controls using principal coordinate analysis (PCA). A study comparing the microbiome of twin pairs, some members of the Firmicutes phylum, such as *Faecalibacterium*, were depleted in those with ICD in comparison to healthy controls.(113) They also showed that these changes in the microbiome allowed for higher levels of some bacterial cell surface proteins, which may contribute to the exaggerated immune response observed in CD patients. *Faecalibacterium, Roseburia, Blautia, Bacteroides, Ruminococcus, Lachnospiraceae,* and *Coprococcus* are the genera that have been shown in multiple studies to be a negatively associated to CD.(113-115) The low abundance of some of these bacteria, in particular from the class Clostridia, namely *Faecalibacterium prausnitzii* and *Roseburia hominis* have also been associated with the dysbiosis characteristic of UC, and a corresponding decrease in short-chain fatty acids, potentially including butyrate which is produced by these bacteria.(116) Correlation between the Mayo scores and an overrepresentation of *Enterobacteriaceae* and an underrepresentation of *Lachnospiraceae* was determined in a study of UC patients before their FMT.(117) *Lachnospiraceae* are a member of the phylum Firmicutes and a well-known important commensal bacterium of the human microbiota. An in vitro study using human dendritic cells (hDC) showed that a particular strain of *F. prausnitzii* was able to induce high levels of the anti-inflammatory cytokine IL-10, resulting in a corresponding reduction of pro-

inflammatory cytokines IL-12p70, IL-1β.(118) It was found that the anti-inflammatory properties of *F. prausnitzii* are carried out by the MAM protein, decreasing activation of the NF-κB pathway, providing a pathological explanation for the bacteria's importance in IBD.(119, 120) Therefore, it is established that there are a number of bacterial genera positively and negatively associated with IBD, and evidence for their role in suppressing the immune system and altering metabolite production which may contribute to disease development. However, there is also evidence suggesting that the dysbiosis observed is a consequence of IBD and not a causal factor. In studies using the IL-10 -/- mouse that spontaneously develops colitis, *Salmonella enterica* lacking pathogenic factors is still able to colonize the gut, and deplete the microbiota of Clostridia and Bacteroidia.(121) Therefore, virulence factors are required for pathogenicity, but they are not required for colonization of an inflamed gut, and bacteria associated with dysbiosis may have acquired fitness factors to occupy the new niche created by inflammation. Proponents of the Oxygen Hypothesis suggest that an influx of oxygen from blood entering the gastrointestinal tract creates a more aerobic environment promoting microbial dysbiosis.(122) This could explain the increase in facultative anaerobes such as *Enterobacteriaceae* and the loss of extremely oxygen sensitive bacteria such as *F. prausnitzii*. Nevertheless, the dysbiosis further exacerbates the abnormal immune response results in a constant positive feedback loop, which in turn creates a more abnormal microbiome, perpetuating inflammation.(123)

The immune system is characterized by two different types of responses, innate and adaptive. Innate immunity is an immediate response by immune cells to various classes

of pathogens recognized by their surface molecules. Adaptive immunity, mediated predominately by lymphocytes, is an immune response found only in vertebrates that involves specific recognition of molecules on individual invaders. Upon initial infection by an intruder, the adaptive response is much slower, but it can remember, priming the organism for secondary infections.(124) Inflammation, although helping to protect the host from exogenous threats, can also cause bystander tissue damage and dysfunction, a significant negative consequence of chronic inflammatory conditions like IBD. It has been previously reported that there is increased leakage observed in inflamed intestines, reducing the efficacy of the intestinal barrier keeping microbes within the lumen.(125) Within the physical barrier, the innate immune system is required to distinguish between pathogenic and commensal bacteria to maintain the ecological state of the human intestinal tract.(126) In the healthy individual, this system is extremely effective at detecting, preventing translocation across the epithelial barrier, and eradicating pathogenic or even commensal bacteria before they can cause any harm to the system, without unnecessarily attacking the commensal bacteria on which we rely for some essential functions.

The pathogenesis of IBD appears to be the result of a primary defect in innate immune response resulting from impaired epithelial barrier function and/or bacterial clearance at the epithelium. The first line of defense of the mucosal immune system is a polarized single layer of epithelial cells covered by a mucus layer secreted by goblet cells.(127) Epithelial tight junctions maintain the epithelial barrier while regulating the permeability of ions, nutrients, and water.(125) A failure to maintain this intestinal

barrier can result in the increased translocation of bacteria from the lumen into the lamina propria, which can cause stimulation of the resident immune cells.(128) Several pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-1β, IL-6, and IL-17 have been shown to disrupt tight junctions and increase intestinal permeability.(129-132) Levels of TNF-α, IFN-γ, IL-1β, IL-6, and IL-17 have been shown to be increased in the mucosa of IBD patients, suggesting that these cytokines are involved in the increased gut permeability observed in IBD patients.(133-137)

The possibility of an infectious origin in IBD has been postulated since IBD was first described.(138) Specific strains of *Escherichia Coli* (*E. coli*) have been associated with the pathogenesis of Crohns disease, in particular, and may be able to sustain the inflammation characteristic of these diseases. To classify more precisely *E. coli* associated with IBD, a new pathogenic group was formed, the adherent-invasive *E. coli* (AIEC), (139) stemming from an earlier study in 1998 which was the first to suggest the adherent properties of these *E. coli* from ileal lesions of CD patients.(140) *E. coli* is correlated with both UC and CD, suggested by the findings of some different studies.(140-148) A subset of these studies shows a similar detection rate in both major forms of IBD, with more *E. coli,* detected in patients with active IBD versus inactive.(143, 144, 146, 147) Lopez-Siles et al. performed a retrospective analysis showing a negative correlation between the number of months to the CD patient's next flare-up and the numbers of *E. coli* detected using 16rRNA sequencing.(143) AIEC have also been shown to colonize and exacerbate gut inflammation in mice with DSS-induced gut inflammation.(149) Despite this, there is also evidence that *Enterobacteriaceae*

benefit from the environment of an inflamed gut, and therefore, the increased prevalence of *E. coli* is more likely to be the result inflammatory state rather than being a causal factor of it.(111)

Of the total 163 IBD-associated loci identified in GWAS studies,(99-101, 103, 104) a number of them are polymorphisms in autophagy genes such as ATF16L1 (autophagy-related 16-like 1) (102, 150) and IRGM (immunity-related GTPase family M).(151-153) Impairment of the autophagy process in THP-1 macrophages associated with these CD polymorphisms (NOD2 and ATG16L1) significantly increased the percentage of intra-macrophage AIEC strain LF82, resulting in a more prominent pro-inflammatory response mediated by TNF-α.(154) These IBD loci can lead to an altered interaction between the host immune system and innate commensal bacteria, with a resultant enhancement of a chronic inflammatory response. The associated of these genes to IBD strengthen the association between enteric bacteria and gastrointestinal inflammation.

1.3.8 Fecal Microbial Transplants in IBD

Current success in treating RCDI with FMTs has garnered a lot of attention for its possible use in the treatment of other diseases associated with microbiota dysbiosis, such as IBD. There are currently 19 registered clinical trials on clinicaltrials.gov designed to study the safety and efficacy of FMT for the treatment of IBD. FMT has been shown to significantly improve patients Quality of Life based on changes in their Inflammatory Bowel Disease Questionnaire (IBDQ score) responses before and after FMT, with

stronger evidence for UC than CD.(155, 156) However, the mechanism by which an FMT acts therapeutically in patients with IBD, and also why the observed beneficial effects are not maintained is not yet understood.

In the published randomized controlled trials (RCTs), case and cohort studies on IBD, both fresh and frozen-thawed FMT slurries are used and the delivery method amongst the studies varied from enema administration to nasogastric or nasojejunal, and gastroscopic instillation. (117, 157-159) The case series published by Borody and colleagues demonstrates the long-term efficacy and safety of FMT for UC, where at follow-up appointments 1-13 years following FMT patients had no evidence of active colitis.(159) A meta-analysis of 9 cohort studies, 8 case studies and 1 randomized controlled trial showed that the pooled proportion of patients who achieved clinical remission of their IBD was 45.4%.(160) However, there was a lot of variability in how donors were selected, disease severity at the start of the study, pre and post-FMT treatment regimens and primary end-points, making the comparison of these studies somewhat difficult, demonstrating the need for more RCTs. To date, only three RCTs have been completed, two in UC patients showing 24% and 30% induced remission rates and one in CD patients with a 77% remission rate.(156, 161, 162) Given that remission rates are quite variable in the published literature, more consistency is needed with respect to methodology such that the efficacy of the therapy can be properly determined. The clinical remission rates observed through meta-analysis (160) suggest that like other therapies for IBD, FMT will only be efficacious in a subset of IBD individuals. The complex interplay of many factors including the interaction between the donor

microbiome, the gastrointestinal environment, the immune system, the other microbes, and host genetics, including genes for miRNA, tight junctions, NOD2 receptors and a vast number of other molecules, making it extremely difficult to determine if FMT will be a valuable IBD therapeutic. The methods of FMT processing and delivery have been well researched for *C. difficile* infection, comparing frozen versus fresh transplantations, and delivery via colonoscopy, nasogastric tube, enema, and more recently frozen encapsulation, but whether preparation or type of delivery of the FMT is important in the treatment of IBD remains to be determined.(163)

One of the complexities is the variability of the biotherapy, in that different selection criterion, and inter-individual microbiota differences can result in drastically different FMT slurry, which can alter the efficacy of the therapy. In one study, the FMT donors underwent a mild colonic lavage using polyethylene glycol 3350 (PEG 3350) before donation, and none of the recipients (n=6) responded to the therapy.(157) This may be explained by the fact that short-term changes resulting from colonoscopy bowel preparation have been previously observed (164), or the bowel preparation may have a yet to be determined effect on the FMT altering its therapeutic ability. Vermeire et al. were able to identify that donor microbiota richness was a predictor of successful FMT in their small pilot study, while another study showed that a successful donor had enrichment for the family Lachnospiraceae, and specifically the genera *Ruminococcus,* which have previously been negatively associated with IBD.(161, 165)

The identification of dysbiosis in IBD patients (9-14) has led to many different

hypotheses as to the pathophysiology of IBD and with this a possible mechanism by which FMT might be successful in that it can re-establish a healthy microbiome in the recipient. Interestingly, in one study, both CD and UC responders to FMT had higher baseline richness than those patients that did not respond to FMT, although the richness of the donors was also greater in the slurries given to the responders.(165) In a pediatric population, using the pediatric Crohn's disease activity index (PCDAI) scoring for pediatric CD patients, 7/9 (77.8%) of patients were in clinical remission at two weeks,(166) however, this same research group was unable to achieve remission (0/4) in any of their pediatric UC patients in a separate study.(167) Moayyedi et al. had 3/4 (75%) patients with UC for less than 1-year enter remission, while only 6/34 (18%) with UC for greater than one year entered remission ($p = 0.04$, Fischer's exact test).(161) Gevers et al. showed that increased microbial dysbiosis was an indicator of CD disease severity,(114) and, therefore, it may be possible that patients with early stage disease are more likely to achieve clinical remission following FMT due to the fact that patients with higher richness pre-FMT were more likely to achieve clinical remission. The extent of dysbiosis may also alter the efficacy of engraftment of the donor microbiota into the recipient based on concepts of ecology, where if the perturbed microbiota is well established, healthy commensals are unable to colonize niches that are currently occupied. Angelberger et al. demonstrated in their UC cohort, the sole FMT responder, acquired, and subsequently maintained the similarity to the donor for at least 12 weeks whereas all other patients only transiently increase in similarity to the donor, (117) however, similarity to the donors does not appear to be a consistent trend amongst studies.

Specific changes in taxonomic composition have also been noted following FMT in an attempt to explain differences between responders and non-responders. One study found that a significant reduction in *Proteobacteria* specifically *Enterobacteriaceae* and *Enterocaccaceae* and an increase in *Bacteroidetes* following FMT.(157) In a patient that failed FMT, Suskind et al. observed that the relative abundance of *Escherichia Coli* increased following FMT and experienced clinical deterioration over the course of the follow-up.(166) Vermeire et al. found that patients who responded to the FMT had significantly more bacterial genera (97% similarity OTUs) transferred to their gut.(165) In the two UC patients who had a favorable, long-term response to the FMT, they had 4/5 shared genera, *Roseburia*, *Oscillobacter*, unclassified *Lachnospiraceae* and unclassified *Ruminococcacae* following FMT. However, the difference in engraftment efficiency between responders (median 74%, range 50-76%) and non-responders (median 63%, range 14-83%) was not statistically significant. The study by Angelberger and colleagues also identified patient colonization by *Fecalibacterium prausnitzii*, *Roseburia faecis* and *Bacteroides ovatus* from the donors in UC patients who responded to FMT, reaffirming species-specific trends observed in other studies.(117) The single patient who responded to treatment also maintained four stable, abundant donor phylotypes over the 12-week follow-up. The donor's microbiome in the RCT for UC performed by Moayyedi and colleagues that were significantly enriched with the family *Lachnospiraceae* resulted in the highest rates of remission in the recipients, with responders becoming more similar to the donor at week six than the non-responders ($p = 0.07$).(156, 161) In nearly all the studies and case series published, clinical response and/or remission correlated well with

increased diversity following FMT.(117, 156, 161, 165-168) Cui and colleagues used a step-up strategy to reverse some of the dysbiosis in UC patients, noting an increase in diversity, and a decrease in *Proteobacteria*, although they did not sequence patients who failed to respond to the FMT(s).(168) However, the significance of these changes is brought to question due to the results of the Rossen at al. study, where no significant difference in remission rates was found between cohorts of UC patients who received an FMT from a healthy donor versus and FMT made from their stool.(156) These results suggest that the mechanism of FMT depends on its ability to alter the balance of anti-inflammatory commensals versus potentially pro-inflammatory pathobionts in the gut of IBD patients.

Pre-FMT treatment regimens, where reported, included different antibiotics and variable course lengths, proton pump inhibitors (PPIs), bowel preparation, or none of the above (**Table 1-2**). A literature review of all published reports of FMT for IBD on PubMed (PubMed.gov; **Table 1-2**) showed a difference in efficacy for FMT in UC with a clinical remission rate of 40% with an antibiotic pre-treatment versus 24% clinical remission rate without an antibiotic pre-treatment, and this difference is approaching statistical significance using Fischer's exact test (**Table 1-1**; p-value 0.104). For patients with CD who received an FMT, 64% of those who did not receive pre-FMT antibiotics versus 58% of patients who receive pre-FMT antibiotics achieved clinical remission (**Table 1-1**; p-value 0.748). When the two forms of IBD were combined, 45% of patients receiving a pre-FMT antibiotic regimen achieved remission, while only 35% of those who did not achieve remission (**Table 1-1**; p-value 0.280). By altering the pre-FMT

treatment regimen through the inclusion of antibiotics, it may be able possible to increase the efficacy for IBD, where currently published studies show a trend for increasing effectiveness with antibiotics pre-FMT (**Table 1-1**). Support for this hypothesis comes from ecological theory, as described earlier in the chapter, where antibiotics perturb the gut microbiota, making available previously occupied niches, and allowing for the colonization of commensal bacteria that provide a physiological benefit to the host.

Although the focus is predominately on ecological explanations for the mechanism of FMT and IBD, the immune system must play an integral role in this process. An increase in C-reactive protein (CRP), (a plasma protein which rises in response to inflammation), within the first two weeks of FMT was observed in both non-responding CD and UC patients. They suggest that this rise could be used as an indication for starting patients on rescue therapy due to FMT failure, however, this has yet to be identified in any other study.(165) In an RCT for FMT in UC, 5/11 (46%) of those taking, versus 4/27 (15%) of those not receiving immunosuppressive therapy achieved clinical remission ($p = 0.09$, Fischer's exact test).(161) In addition, anti-TNF therapy has been shown to restore the epithelial barrier in CD patients(169) and alter antimicrobial composition in UC patients.(170) This suggests that concurrent treatment with immunosuppression may be able to enhance the efficacy of FMT. It is interesting that patients can experience a fever following FMT (**Table 1-2**), and although bacterial lipopolysaccharide (LPS) is a pyrogen, FMT should not cause a systemic immune response when introduced into an environment designed to handle this. Therefore, the focus of future studies should be on how FMT activated and alters the recipient's immune system.

Table 1-1: Data summary and statistics for FMT in IBD with and without antibiotic pre-treatment

	N	Clinical Remission	CR Rate	Fischer's Exact Test (p-value)	Odds Ratio	95% CI
UC – no abx	110	26	0.24			
UC - abx	30	12	0.40	0.104	0.4643	0.198-1.089
CD – no abx	44	28	0.64			
CD - abx	12	7	0.58	0.748	1.250	0.340-4.594
IBD – no abx	154	54	0.35			
IBD abx	42	19	0.45	0.280	0.654	0.327-1.306

Abx: antibiotic pre-FMT treatment; CR: clinical remission; CI: confidence interval; UC: ulcerative colitis; CD: Crohn's disease; IBD: inflammatory bowel disease.

1.4 References

1. Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, et al. Metagenomic analysis of the human distal gut microbiome. Science. 2006 Jun 2;312(5778):1355-9.

2. Whittaker RH. Evolution and Measurement of Species Diversity. Taxon. 1972;21(2):213-51.

3. Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-bacterial mutualism in the human intestine. Science. 2005 Mar 25;307(5717):1915-20.

4. Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-bacterial mutualism in the human intestine. Science. 2005 Mar 25;307(5717):1915-20.

5. Jantchou P, Morois S, Clavel-Chapelon F, Boutron-Ruault MC, Carbonnel F. Animal protein intake and risk of inflammatory bowel disease: The E3N prospective study. Am J Gastroenterol. 2010 Oct;105(10):2195-201.

6. Persson PG, Ahlbom A, Hellers G. Diet and inflammatory bowel disease: a case-control study. Epidemiology. 1992 Jan;3(1):47-52.

7. Hou JK, Abraham B, El-Serag H. Dietary intake and risk of developing inflammatory bowel disease: a systematic review of the literature. Am J Gastroenterol. 2011 Apr;106(4):563-73.

8. Chiba M, Abe T, Tsuda H, Sugawara T, Tsuda S, Tozawa H, et al. Lifestyle-related disease in Crohn's disease: relapse prevention by a semi-vegetarian diet. World J Gastroenterol. 2010 May 28;16(20):2484-95.

9. Manichanh C, Rigottier-Gois L, Bonnaud E, Gloux K, Pelletier E, Frangeul L, et al. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. Gut. 2006 Feb;55(2):205-11.

10. Fujimoto T, Imaeda H, Takahashi K, Kasumi E, Bamba S, Fujiyama Y, et al. Decreased abundance of Faecalibacterium prausnitzii in the gut microbiota of Crohn's disease. J Gastroenterol Hepatol. 2013 Apr;28(4):613-9.

11. Hansen R, Russell RK, Reiff C, Louis P, McIntosh F, Berry SH, et al. Microbiota of de-novo pediatric IBD: increased Faecalibacterium prausnitzii and reduced bacterial diversity in Crohn's but not in ulcerative colitis. Am J Gastroenterol. 2012 Dec;107(12):1913-22.

12. Berry D, Reinisch W. Intestinal microbiota: a source of novel biomarkers in inflammatory bowel diseases? Best Pract Res Clin Gastroenterol. 2013 Feb;27(1):47-58.

13. Conlon MA, Bird AR. The impact of diet and lifestyle on gut microbiota and human health. Nutrients. 2014 Dec 24;7(1):17-44.

14. Guarner F, Bourdet-Sicard R, Brandtzaeg P, Gill HS, McGuirk P, van Eden W, et al. Mechanisms of disease: the hygiene hypothesis revisited. Nat Clin Pract Gastroenterol Hepatol. 2006 May;3(5):275-84.

15. Kaplan GG. The global burden of IBD: from 2015 to 2025. Nat Rev Gastroenterol Hepatol. 2015 Dec;12(12):720-7.

16. Lee YK, Mazmanian SK. Has the microbiota played a critical role in the evolution of the adaptive immune system? Science. 2010 Dec 24;330(6012):1768-73.

17. Faith JJ, Guruge JL, Charbonneau M, Subramanian S, Seedorf H, Goodman AL, et al. The long-term stability of the human gut microbiota. Science. 2013 Jul 5;341(6141):1237439.

18. Dethlefsen L, Relman DA. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. Proc Natl Acad Sci U S A. 2011 Mar 15;108 Suppl 1:4554-61.

19. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. Nature. 2012 Sep 13;489(7415):220-30.

20. Vanhoutte T, Huys G, Brandt E, Swings J. Temporal stability analysis of the microbiota in human feces by denaturing gradient gel electrophoresis using universal and group-specific 16S rRNA gene primers. FEMS Microbiol Ecol. 2004 Jun 1;48(3):437-46.

21. Trosvik P, de Muinck EJ. Ecology of bacteria in the human gastrointestinal tract--identification of keystone and foundation taxa. Microbiome. 2015 Oct 12;3:44,015-0107-4.

22. Tamboli CP, Neut C, Desreumaux P, Colombel JF. Dysbiosis as a prerequisite for IBD. Gut. 2004 Jul;53(7):1057.

23. Vangay P, Ward T, Gerber JS, Knights D. Antibiotics, pediatric dysbiosis, and disease. Cell host & microbe. 2015;17(5):553-64.

24. Rolig AS, Parthasarathy R, Burns AR, Bohannan BJ, Guillemin K. Individual Members of the Microbiota Disproportionately Modulate Host Innate Immune Responses. Cell Host Microbe. 2015 Nov 11;18(5):613-20.

25. Mazmanian SK, Round JL, Kasper DL. A microbial symbiosis factor prevents intestinal inflammatory disease. Nature. 2008 May 29;453(7195):620-5.

26. Gerding DN, Muto CA, Owens RC,Jr. Measures to control and prevent Clostridium difficile infection. Clin Infect Dis. 2008 Jan 15;46 Suppl 1:S43-9.

27. McDonald LC, Owings M, Jernigan DB. Clostridium difficile infection in patients discharged from US short-stay hospitals, 1996-2003. Emerg Infect Dis. 2006 Mar;12(3):409-15.

28. Redelings MD, Sorvillo F, Mascola L. Increase in Clostridium difficile-related mortality rates, United States, 1999-2004. Emerg Infect Dis. 2007 Sep;13(9):1417-9.

29. Collins DA, Hawkey PM, Riley TV. Epidemiology of Clostridium difficile infection in Asia. Antimicrob Resist Infect Control. 2013 Jul 1;2(1):21.

30. Cartman ST, Heap JT, Kuehne SA, Cockayne A, Minton NP. The emergence of 'hypervirulence' in Clostridium difficile. Int J Med Microbiol. 2010 Aug;300(6):387-95.

31. Bignardi GE. Risk factors for Clostridium difficile infection. J Hosp Infect. 1998 Sep;40(1):1-15.

32. Fujimura KE, Slusher NA, Cabana MD, Lynch SV. Role of the gut microbiota in defining human health. Expert Rev Anti Infect Ther. 2010 Apr;8(4):435-54.

33. Levy AR, Szabo SM, Lozano-Ortega G, Lloyd-Smith E, Leung V, Lawrence R, et al. Incidence and Costs of Clostridium difficile Infections in Canada. Open Forum Infect Dis. 2015 Jun 3;2(3):ofv076.

34. Kyne L, Warny M, Qamar A, Kelly CP. Asymptomatic carriage of Clostridium difficile and serum levels of IgG antibody against toxin A. N Engl J Med. 2000 Feb 10;342(6):390-7.

35. Poutanen SM, Simor AE. Clostridium difficile-associated diarrhea in adults. CMAJ. 2004 Jul 6;171(1):51-8.

36. Eveillard M, Fourel V, Barc MC, Kerneis S, Coconnier MH, Karjalainen T, et al. Identification and characterization of adhesive factors of Clostridium difficile involved in adhesion to human colonic enterocyte-like Caco-2 and mucus-secreting HT29 cells in culture. Mol Microbiol. 1993 Feb;7(3):371-81.

37. Na X, Kim H, Moyer MP, Pothoulakis C, LaMont JT. gp96 is a human colonocyte plasma membrane binding protein for Clostridium difficile toxin A. Infect Immun. 2008 Jul;76(7):2862-71.

38. Seddon SV, Hemingway I, Borriello SP. Hydrolytic enzyme production by Clostridium difficile and its relationship to toxin production and virulence in the hamster model. J Med Microbiol. 1990 Mar;31(3):169-74.

39. Cohen SH, Gerding DN, Johnson S, Kelly CP, Loo VG, McDonald LC, et al. Clinical practice guidelines for Clostridium difficile infection in adults: 2010 update by the society for healthcare epidemiology of America (SHEA) and the infectious diseases society of America (IDSA). Infect Control Hosp Epidemiol. 2010 May;31(5):431-55.

40. Crowther GS, Chilton CH, Longshaw C, Todhunter SL, Ewin D, Vernon J, et al. Efficacy of vancomycin extended-dosing regimens for treatment of simulated Clostridium difficile infection within an in vitro human gut model. J Antimicrob Chemother. 2016 Jan 10.

41. Debast SB, Bauer MP, Kuijper EJ, European Society of Clinical Microbiology and Infectious Diseases. European Society of Clinical Microbiology and Infectious Diseases: update of the treatment guidance document for Clostridium difficile infection. Clin Microbiol Infect. 2014 Mar;20 Suppl 2:1-26.

42. Goldberg EJ, Bhalodia S, Jacob S, Patel H, Trinh KV, Varghese B, et al. Clostridium difficile infection: A brief update on emerging therapies. Am J Health Syst Pharm. 2015 Jun 15;72(12):1007-12.

43. Barbut F, Richard A, Hamadi K, Chomette V, Burghoffer B, Petit JC. Epidemiology of recurrences or reinfections of Clostridium difficile-associated diarrhea. J Clin Microbiol. 2000 Jun;38(6):2386-8.

44. Gerding DN, Johnson S, Peterson LR, Mulligan ME, Silva J,Jr. Clostridium difficile-associated diarrhea and colitis. Infect Control Hosp Epidemiol. 1995 Aug;16(8):459-77.

45. Keeney KM, Yurist-Doutsch S, Arrieta MC, Finlay BB. Effects of antibiotics on human microbiota and subsequent disease. Annu Rev Microbiol. 2014;68:217-35.

46. Brandt LJ, Aroniadis OC, Mellow M, Kanatzar A, Kelly C, Park T, et al. Long-term follow-up of colonoscopic fecal microbiota transplant for recurrent Clostridium difficile infection. Am J Gastroenterol. 2012 Jul;107(7):1079-87.

47. Meighani A, Hart BR, Mittal C, Miller N, John A, Ramesh M. Predictors of fecal transplant failure. Eur J Gastroenterol Hepatol. 2016 Mar 1.

48. Li YT, Cai HF, Wang ZH, Xu J, Fang JY. Systematic review with meta-analysis: long-term outcomes of faecal microbiota transplantation for Clostridium difficile infection. Aliment Pharmacol Ther. 2016 Feb;43(4):445-57.

49. Baxter M, Colville A. Adverse events in faecal microbiota transplant: a review of the literature. J Hosp Infect. 2016 Feb;92(2):117-27.

50. Khoruts A, Rank KM, Newman KM, Viskocil K, Vaughn BP, Hamilton MJ, et al. Inflammatory Bowel Disease Affects the Outcome of Fecal Microbiota Transplantation for Recurrent Clostridium difficile Infection. Clin Gastroenterol Hepatol. 2016 Feb 22.

51. Lapointe-Shaw L, Tran KL, Coyte PC, Hancock-Howard RL, Powis J, Poutanen SM, et al. Cost-Effectiveness Analysis of Six Strategies to Treat Recurrent Clostridium difficile Infection. PLoS One. 2016 Feb 22;11(2):e0149521.

52. Waye A, Atkins K, Kao D. Cost Averted With Timely Fecal Microbiota Transplantation in the Management of Recurrent Clostridium difficile Infection in Alberta, Canada. J Clin Gastroenterol. 2016 Feb 17.

53. Crohn BB, Ginzburg L, Oppenheimer GD. Regional ileitis: a pathologic and clinical entity. 1932. Mt Sinai J Med. 2000 May;67(3):263-8.

54. Kirsner JB. Historical aspects of inflammatory bowel disease. J Clin Gastroenterol. 1988 Jun;10(3):286-97.

55. Danese S, Fiocchi C. Ulcerative colitis. N Engl J Med. 2011 Nov 3;365(18):1713-25.

56. Lewis JD. A review of the epidemiology of inflammatory bowel disease with a focus on diet, infections and antibiotic exposure. Nestle Nutr Inst Workshop Ser. 2014;79:1-18.

57. Ananthakrishnan AN. Epidemiology and risk factors for IBD. Nat Rev Gastroenterol Hepatol. 2015 Apr;12(4):205-17.

58. Silverberg MS, Satsangi J, Ahmad T, Arnott ID, Bernstein CN, Brant SR, et al. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. Can J Gastroenterol. 2005 Sep;19 Suppl A:5A-36A.

59. Baumgart DC, Sandborn WJ. Crohn's disease. Lancet. 2012 Nov 3;380(9853):1590-605.

60. Stange EF, Travis SP, Vermeire S, Reinisch W, Geboes K, Barakauskiene A, et al. European evidence-based Consensus on the diagnosis and management of ulcerative colitis: Definitions and diagnosis. J Crohns Colitis. 2008 Mar;2(1):1-23.

61. Ahuja V, Tandon RK. Inflammatory bowel disease in the Asia-Pacific area: a comparison with developed countries and regional differences. J Dig Dis. 2010 Jun;11(3):134-47.

62. Abakar-Mahamat A, Filippi J, Pradier C, Dozol A, Hebuterne X. Incidence of inflammatory bowel disease in Corsica from 2002 to 2003. Gastroenterol Clin Biol. 2007 Dec;31(12):1098-103.

63. Molodecky NA, Soon IS, Rabi DM, Ghali WA, Ferris M, Chernoff G, et al. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. Gastroenterology. 2012 Jan;142(1):46,54.e42; quiz e30.

64. Bernstein CN, Wajda A, Svenson LW, MacKenzie A, Koehoorn M, Jackson M, et al. The epidemiology of inflammatory bowel disease in Canada: a population-based study. Am J Gastroenterol. 2006 Jul;101(7):1559-68.

65. Gunesh S, Thomas GA, Williams GT, Roberts A, Hawthorne AB. The incidence of Crohn's disease in Cardiff over the last 75 years: an update for 1996-2005. Aliment Pharmacol Ther. 2008 Feb 1;27(3):211-9.

66. Molinie F, Gower-Rousseau C, Yzet T, Merle V, Grandbastien B, Marti R, et al. Opposite evolution in incidence of Crohn's disease and ulcerative colitis in Northern France (1988-1999). Gut. 2004 Jun;53(6):843-8.

67. Latour P, Louis E, Belaiche J. Incidence of inflammatory bowel disease in the area of Liege: a 3 years prospective study (1993-1996). Acta Gastroenterol Belg. 1998 Oct-Dec;61(4):410-3.

68. Loftus EV,Jr. Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences. Gastroenterology. 2004 May;126(6):1504-17.

69. Logan RF. Inflammatory bowel disease incidence: up, down or unchanged? Gut. 1998 Mar;42(3):309-11.

70. Yang SK, Hong WS, Min YI, Kim HY, Yoo JY, Rhee PL, et al. Incidence and prevalence of ulcerative colitis in the Songpa-Kangdong District, Seoul, Korea, 1986-1997. J Gastroenterol Hepatol. 2000 Sep;15(9):1037-42.

71. Sood A, Midha V, Sood N, Bhatia AS, Avasthi G. Incidence and prevalence of ulcerative colitis in Punjab, North India. Gut. 2003 Nov;52(11):1587-90.

72. Edouard A, Paillaud M, Merle S, Orhan C, Chenayer-Panelatti Dagger M, COGEAG. Incidence of inflammatory bowel disease in the French West Indies (1997-1999). Gastroenterol Clin Biol. 2005 Aug-Sep;29(8-9):779-83.

73. Cosnes J, Gower-Rousseau C, Seksik P, Cortot A. Epidemiology and natural history of inflammatory bowel diseases. Gastroenterology. 2011 May;140(6):1785-94.

74. Devlin HB, Datta D, Dellipiani AW. The incidence and prevalence of inflammatory bowel disease in North Tees Health District. World J Surg. 1980;4(2):183-93.

75. Gearry RB, Richardson A, Frampton CM, Collett JA, Burt MJ, Chapman BA, et al. High incidence of Crohn's disease in Canterbury, New Zealand: results of an epidemiologic study. Inflamm Bowel Dis. 2006 Oct;12(10):936-43.

76. Heyman MB, Kirschner BS, Gold BD, Ferry G, Baldassano R, Cohen SA, et al. Children with early-onset inflammatory bowel disease (IBD): analysis of a pediatric IBD consortium registry. J Pediatr. 2005 Jan;146(1):35-40.

77. Huppertz-Hauss G, Hoivik ML, Langholz E, Odes S, Smastuen M, Stockbrugger R, et al. Health-related quality of life in inflammatory bowel disease in a European-wide population-based cohort 10 years after diagnosis. Inflamm Bowel Dis. 2015 Feb;21(2):337-44.

78. Svaninger G, Nordgren S, Oresland T, Hulten L. Incidence and characteristics of pouchitis in the Kock continent ileostomy and the pelvic pouch. Scand J Gastroenterol. 1993 Aug;28(8):695-700.

79. Lazarev M, Ullman T, Schraut WH, Kip KE, Saul M, Regueiro M. Small bowel resection rates in Crohn's disease and the indication for surgery over time: experience from a large tertiary care center. Inflamm Bowel Dis. 2010 May;16(5):830-5.

80. Rocchi A, Benchimol EI, Bernstein CN, Bitton A, Feagan B, Panaccione R, et al. Inflammatory bowel disease: a Canadian burden of illness review. Can J Gastroenterol. 2012 Nov;26(11):811-7.

81. Halfvarson J, Bodin L, Tysk C, Lindberg E, Jarnerot G. Inflammatory bowel disease in a Swedish twin cohort: a long-term follow-up of concordance and clinical characteristics. Gastroenterology. 2003 Jun;124(7):1767-73.

82. Halfvarson J, Bodin L, Tysk C, Lindberg E, Jarnerot G. Inflammatory bowel disease in a Swedish twin cohort: a long-term follow-up of concordance and clinical characteristics. Gastroenterology. 2003 Jun;124(7):1767-73.

83. Orholm M, Binder V, Sorensen TI, Rasmussen LP, Kyvik KO. Concordance of inflammatory bowel disease among Danish twins. Results of a nationwide study. Scand J Gastroenterol. 2000 Oct;35(10):1075-81.

84. Thompson NP, Driscoll R, Pounder RE, Wakefield AJ. Genetics versus environment in inflammatory bowel disease: results of a British twin study. BMJ. 1996 Jan 13;312(7023):95-6.

85. Tysk C, Lindberg E, Jarnerot G, Floderus-Myrhed B. Ulcerative colitis and Crohn's disease in an unselected population of monozygotic and dizygotic twins. A study of heritability and the influence of smoking. Gut. 1988 Jul;29(7):990-6.

86. Binder V. Genetic epidemiology in inflammatory bowel disease. Dig Dis. 1998 Nov-Dec;16(6):351-5.

87. Bengtson MB, Solberg C, Aamodt G, Jahnsen J, Moum B, Sauar J, et al. Clustering in time of familial IBD separates ulcerative colitis from Crohn's disease. Inflamm Bowel Dis. 2009 Dec;15(12):1867-74.

88. Barrett JC, Hansoul S, Nicolae DL, Cho JH, Duerr RH, Rioux JD, et al. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. Nat Genet. 2008 Aug;40(8):955-62.

89. Franke A, McGovern DP, Barrett JC, Wang K, Radford-Smith GL, Ahmad T, et al. Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. Nat Genet. 2010 Dec;42(12):1118-25.

90. Anderson CA, Boucher G, Lees CW, Franke A, D'Amato M, Taylor KD, et al. Meta-analysis identifies 29 additional ulcerative colitis risk loci, increasing the number of confirmed associations to 47. Nat Genet. 2011 Mar;43(3):246-52.

91. Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature. 2012 Nov 1;491(7422):119-24.

92. Hugot JP, Laurent-Puig P, Gower-Rousseau C, Olson JM, Lee JC, Beaugerie L, et al. Mapping of a susceptibility locus for Crohn's disease on chromosome 16. Nature. 1996 Feb 29;379(6568):821-3.

93. Maynard CL, Elson CO, Hatton RD, Weaver CT. Reciprocal interactions of the intestinal microbiota and immune system. Nature. 2012 Sep 13;489(7415):231-41.

94. Salzman NH, Hung K, Haribhai D, Chu H, Karlsson-Sjoberg J, Amir E, et al. Enteric defensins are essential regulators of intestinal microbial ecology. Nat Immunol. 2010 Jan;11(1):76-83.

95. Helio T, Halme L, Lappalainen M, Fodstad H, Paavola-Sakki P, Turunen U, et al. CARD15/NOD2 gene variants are associated with familially occurring and complicated forms of Crohn's disease. Gut. 2003 Apr;52(4):558-62.

96. Franke A, Balschun T, Karlsen TH, Sventoraityte J, Nikolaus S, Mayr G, et al. Sequence variants in IL10, ARPC2 and multiple other loci contribute to ulcerative colitis susceptibility. Nat Genet. 2008 Nov;40(11):1319-23.

97. Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. Cell. 1993 Oct 22;75(2):263-74.

98. Ueda Y, Kayama H, Jeon SG, Kusu T, Isaka Y, Rakugi H, et al. Commensal microbiota induce LPS hyporesponsiveness in colonic macrophages via the production of IL-10. Int Immunol. 2010 Dec;22(12):953-62.

99. Beaudoin M, Goyette P, Boucher G, Lo KS, Rivas MA, Stevens C, et al. Deep resequencing of GWAS loci identifies rare variants in CARD9, IL23R and RNF186 that are associated with ulcerative colitis. PLoS Genet. 2013;9(9):e1003723.

100. Goyette P, Lefebvre C, Ng A, Brant SR, Cho JH, Duerr RH, et al. Gene-centric association mapping of chromosome 3p implicates MST1 in IBD pathogenesis. Mucosal Immunol. 2008 Mar;1(2):131-8.

101. Momozawa Y, Mni M, Nakamura K, Coppieters W, Almer S, Amininejad L, et al. Resequencing of positional candidates identifies low frequency IL23R coding variants protecting against inflammatory bowel disease. Nat Genet. 2011 Jan;43(1):43-7.

102. Rioux JD, Xavier RJ, Taylor KD, Silverberg MS, Goyette P, Huett A, et al. Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. Nat Genet. 2007 May;39(5):596-604.

103. Rivas MA, Beaudoin M, Gardet A, Stevens C, Sharma Y, Zhang CK, et al. Deep resequencing of GWAS loci identifies independent rare variants associated with inflammatory bowel disease. Nat Genet. 2011 Oct 9;43(11):1066-73.

104. Rioux JD, Xavier RJ, Taylor KD, Silverberg MS, Goyette P, Huett A, et al. Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. Nat Genet. 2007 May;39(5):596-604.

105. Palmieri O, Creanza TM, Bossa F, Palumbo O, Maglietta R, Ancona N, et al. Genome-wide Pathway Analysis Using Gene Expression Data of Colonic Mucosa in Patients with Inflammatory Bowel Disease. Inflamm Bowel Dis. 2015 Apr 21.

106. Mahid SS, Minor KS, Soto RE, Hornung CA, Galandiuk S. Smoking and inflammatory bowel disease: a meta-analysis. Mayo Clin Proc. 2006 Nov;81(11):1462-71.

107. Mikhailov TA, Furner SE. Breastfeeding and genetic factors in the etiology of inflammatory bowel disease in children. World J Gastroenterol. 2009 Jan 21;15(3):270-9.

108. Benchimol EI, Mack DR, Guttmann A, Nguyen GC, To T, Mojaverian N, et al. Inflammatory Bowel Disease in Immigrants to Canada And Their Children: A Population-Based Cohort Study. Am J Gastroenterol. 2015 Mar 10.

109. Ko Y, Kariyawasam V, Karnib M, Butcher R, Samuels D, Alrubaie A, et al. Inflammatory bowel disease environmental risk factors: a population-based case-control study of Middle Eastern migration to Australia. Clin Gastroenterol Hepatol. 2015 Mar 11.

110. Onderdonk AB, Hermos JA, Bartlett JG. The role of the intestinal microflora in experimental colitis. Am J Clin Nutr. 1977 Nov;30(11):1819-25.

111. Winter SE, Lopez CA, Baumler AJ. The dynamics of gut-associated microbial communities during inflammation. EMBO Rep. 2013 Apr;14(4):319-27.

112. Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc Natl Acad Sci U S A. 2007 Aug 21;104(34):13780-5.

113. Erickson AR, Cantarel BL, Lamendella R, Darzi Y, Mongodin EF, Pan C, et al. Integrated metagenomics/metaproteomics reveals human host-microbiota signatures of Crohn's disease. PLoS One. 2012;7(11):e49138.

114. Gevers D, Kugathasan S, Denson LA, Vazquez-Baeza Y, Van Treuren W, Ren B, et al. The treatment-naive microbiome in new-onset Crohn's disease. Cell Host Microbe. 2014 Mar 12;15(3):382-92.

115. Joossens M, Huys G, Cnockaert M, De Preter V, Verbeke K, Rutgeerts P, et al. Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives. Gut. 2011 May;60(5):631-7.

116. Machiels K, Joossens M, Sabino J, De Preter V, Arijs I, Eeckhaut V, et al. A decrease of the butyrate-producing species Roseburia hominis and Faecalibacterium prausnitzii defines dysbiosis in patients with ulcerative colitis. Gut. 2014 Aug;63(8):1275-83.

117. Angelberger S, Reinisch W, Makristathis A, Lichtenberger C, Dejaco C, Papay P, et al. Temporal bacterial community dynamics vary among ulcerative colitis patients after fecal microbiota transplantation. Am J Gastroenterol. 2013 Oct;108(10):1620-30.

118. Rossi O, van Berkel LA, Chain F, Tanweer Khan M, Taverne N, Sokol H, et al. Faecalibacterium prausnitzii A2-165 has a high capacity to induce IL-10 in human and murine dendritic cells and modulates T cell responses. Sci Rep. 2016 Jan 4;6:18507.

119. Quevrain E, Maubert MA, Sokol H, Devreese B, Seksik P. The presence of the anti-inflammatory protein MAM, from Faecalibacterium prausnitzii, in the intestinal ecosystem. Gut. 2015 Dec 15.

120. Quevrain E, Maubert MA, Michon C, Chain F, Marquant R, Tailhades J, et al. Identification of an anti-inflammatory protein from Faecalibacterium prausnitzii, a commensal bacterium deficient in Crohn's disease. Gut. 2016 Mar;65(3):415-25.

121. Stecher B, Robbiani R, Walker AW, Westendorf AM, Barthel M, Kremer M, et al. Salmonella enterica serovar typhimurium exploits inflammation to compete with the intestinal microbiota. PLoS Biol. 2007;5(10):e244.

122. Rigottier-Gois L. Dysbiosis in inflammatory bowel diseases: the oxygen hypothesis. The ISME journal. 2013;7(7):1256-61.

123. Geremia A, Biancheri P, Allan P, Corazza GR, Di Sabatino A. Innate and adaptive immunity in inflammatory bowel disease. Autoimmun Rev. 2014 Jan;13(1):3-10.

124. Marietta E, Rishi A, Taneja V. Immunogenetic control of the Intestinal Microbiota. Immunology. 2015 Apr 24.

125. Lee SH. Intestinal permeability regulation by tight junction: implication on inflammatory bowel diseases. Intest Res. 2015 Jan;13(1):11-8.

126. Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. Nature. 2000 Aug 17;406(6797):782-7.

127. Moussata D, Goetz M, Gloeckner A, Kerner M, Campbell B, Hoffman A, et al. Confocal laser endomicroscopy is a new imaging modality for recognition of intramucosal bacteria in inflammatory bowel disease in vivo. Gut. 2011 Jan;60(1):26-33.

128. Shanahan F. Inflammatory bowel disease: immunodiagnostics, immunotherapeutics, and ecotherapeutics. Gastroenterology. 2001 Feb;120(3):622-35.

129. Ma TY, Boivin MA, Ye D, Pedram A, Said HM. Mechanism of TNF-{alpha} modulation of Caco-2 intestinal epithelial tight junction barrier: role of myosin light-chain kinase protein expression. Am J Physiol Gastrointest Liver Physiol. 2005 Mar;288(3):G422-30.

130. Bruewer M, Utech M, Ivanov AI, Hopkins AM, Parkos CA, Nusrat A. Interferon-gamma induces internalization of epithelial tight junction proteins via a macropinocytosis-like process. FASEB J. 2005 Jun;19(8):923-33.

131. Suzuki T, Yoshinaga N, Tanabe S. Interleukin-6 (IL-6) regulates claudin-2 expression and tight junction permeability in intestinal epithelium. J Biol Chem. 2011 Sep 9;286(36):31263-71.

132. Kinugasa T, Sakaguchi T, Gu X, Reinecker H. Claudins regulate the intestinal barrier in response to immune mediators. Gastroenterology. 2000;118(6):1001-11.

133. Schulzke JD, Bojarski C, Zeissig S, Heller F, Gitter AH, Fromm M. Disrupted barrier function through epithelial cell apoptosis. Ann N Y Acad Sci. 2006 Aug;1072:288-99.

134. Stallmach A, Giese T, Schmidt C, Ludwig B, Mueller-Molaian I, Meuer SC. Cytokine/chemokine transcript profiles reflect mucosal inflammation in Crohn's disease. Int J Colorectal Dis. 2004 Jul;19(4):308-15.

135. Dinarello CA. The interleukin-1 family: 10 years of discovery. FASEB J. 1994 Dec;8(15):1314-25.

136. Louis E, Belaiche J, van Kemseke C, Franchimont D, de Groote D, Gueenen V, et al. A high serum concentration of interleukin-6 is predictive of relapse in quiescent Crohn's disease. Eur J Gastroenterol Hepatol. 1997 Oct;9(10):939-44.

137. Pappu R, Ramirez-Carrozzi V, Sambandam A. The interleukin-17 cytokine family: critical players in host defence and inflammatory diseases. Immunology. 2011 Sep;134(1):8-16.

138. Bosca-Watts MM, Tosca J, Anton R, Mora M, Minguez M, Mora F. Pathogenesis of Crohn's disease: Bug or no bug. World J Gastrointest Pathophysiol. 2015 Feb 15;6(1):1-12.

139. Boudeau J, Glasser AL, Masseret E, Joly B, Darfeuille-Michaud A. Invasive ability of an Escherichia coli strain isolated from the ileal mucosa of a patient with Crohn's disease. Infect Immun. 1999 Sep;67(9):4499-509.

140. Darfeuille-Michaud A, Neut C, Barnich N, Lederman E, Di Martino P, Desreumaux P, et al. Presence of adherent Escherichia coli strains in ileal mucosa of patients with Crohn's disease. Gastroenterology. 1998 Dec;115(6):1405-13.

141. Baumgart M, Dogan B, Rishniw M, Weitzman G, Bosworth B, Yantiss R, et al. Culture independent analysis of ileal mucosa reveals a selective increase in invasive Escherichia coli of novel phylogeny relative to depletion of Clostridiales in Crohn's disease involving the ileum. ISME J. 2007 Sep;1(5):403-18.

142. Kotlowski R, Bernstein CN, Sepehri S, Krause DO. High prevalence of Escherichia coli belonging to the B2+D phylogenetic group in inflammatory bowel disease. Gut. 2007 May;56(5):669-75.

143. Lopez-Siles M, Martinez-Medina M, Busquets D, Sabat-Mir M, Duncan SH, Flint HJ, et al. Mucosa-associated Faecalibacterium prausnitzii and Escherichia coli co-abundance can distinguish Irritable Bowel Syndrome and Inflammatory Bowel Disease phenotypes. Int J Med Microbiol. 2014 May;304(3-4):464-75.

144. Martin HM, Campbell BJ, Hart CA, Mpofu C, Nayar M, Singh R, et al. Enhanced Escherichia coli adherence and invasion in Crohn's disease and colon cancer. Gastroenterology. 2004 Jul;127(1):80-93.

145. Martinez-Medina M, Aldeguer X, Lopez-Siles M, Gonzalez-Huix F, Lopez-Oliu C, Dahbi G, et al. Molecular diversity of Escherichia coli in the human gut: new ecological evidence supporting the role of adherent-invasive E. coli (AIEC) in Crohn's disease. Inflamm Bowel Dis. 2009 Jun;15(6):872-82.

146. Mylonaki M, Rayment NB, Rampton DS, Hudspith BN, Brostoff J. Molecular characterization of rectal mucosa-associated bacterial flora in inflammatory bowel disease. Inflamm Bowel Dis. 2005 May;11(5):481-7.

147. Sha S, Xu B, Wang X, Zhang Y, Wang H, Kong X, et al. The biodiversity and composition of the dominant fecal microbiota in patients with inflammatory bowel disease. Diagn Microbiol Infect Dis. 2013 Mar;75(3):245-51.

148. Willing B, Halfvarson J, Dicksved J, Rosenquist M, Jarnerot G, Engstrand L, et al. Twin studies reveal specific imbalances in the mucosa-associated microbiota of patients with ileal Crohn's disease. Inflamm Bowel Dis. 2009 May;15(5):653-60.

149. Carvalho FA, Barnich N, Sauvanet P, Darcha C, Gelot A, Darfeuille-Michaud A. Crohn's disease-associated Escherichia coli LF82 aggravates colitis in injured mouse colon via signaling by flagellin. Inflamm Bowel Dis. 2008;14(8):1051-60.

150. Hampe J, Franke A, Rosenstiel P, Till A, Teuber M, Huse K, et al. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. Nat Genet. 2007 Feb;39(2):207-11.

151. McCarroll SA, Huett A, Kuballa P, Chilewski SD, Landry A, Goyette P, et al. Deletion polymorphism upstream of IRGM associated with altered IRGM expression and Crohn's disease. Nat Genet. 2008 Sep;40(9):1107-12.

152. Parkes M, Barrett JC, Prescott NJ, Tremelling M, Anderson CA, Fisher SA, et al. Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility. Nat Genet. 2007 Jul;39(7):830-2.

153. Prescott NJ, Dominy KM, Kubo M, Lewis CM, Fisher SA, Redon R, et al. Independent and population-specific association of risk variants at the IRGM locus with Crohn's disease. Hum Mol Genet. 2010 May 1;19(9):1828-39.

154. Lapaquette P, Bringer MA, Darfeuille-Michaud A. Defects in autophagy favour adherent-invasive Escherichia coli persistence within macrophages leading to increased pro-inflammatory response. Cell Microbiol. 2012 Jun;14(6):791-807.

155. Wei Y, Zhu W, Gong J, Guo D, Gu L, Li N, et al. Fecal Microbiota Transplantation Improves the Quality of Life in Patients with Inflammatory Bowel Disease. Gastroenterol Res Pract. 2015;2015:517597.

156. Rossen NG, MacDonald JK, de Vries EM, D'Haens GR, de Vos WM, Zoetendal EG, et al. Fecal microbiota transplantation as novel therapy in gastroenterology: A systematic review. World J Gastroenterol. 2015 May 7;21(17):5359-71.

157. Kump PK, Grochenig HP, Lackner S, Trajanoski S, Reicht G, Hoffmann KM, et al. Alteration of intestinal dysbiosis by fecal microbiota transplantation does not induce remission in patients with chronic active ulcerative colitis. Inflamm Bowel Dis. 2013 Sep;19(10):2155-65.

158. Bennet JD, Brinkman M. Treatment of ulcerative colitis by implantation of normal colonic flora. Lancet. 1989 Jan 21;1(8630):164.

159. Borody TJ, Warren EF, Leis S, Surace R, Ashman O. Treatment of ulcerative colitis using fecal bacteriotherapy. J Clin Gastroenterol. 2003 Jul;37(1):42-7.

160. Colman RJ, Rubin DT. Fecal microbiota transplantation as therapy for inflammatory bowel disease: a systematic review and meta-analysis. J Crohns Colitis. 2014 Dec 1;8(12):1569-81.

161. Moayyedi P, Surette MG, Kim PT, Libertucci J, Wolfe M, Onischi C, et al. Fecal Microbiota Transplantation Induces Remission in Patients with Active Ulcerative Colitis in a Randomized, Controlled Trial. Gastroenterology. 2015 Apr 6.

162. Cui B, Feng Q, Wang H, Wang M, Peng Z, Li P, et al. Fecal microbiota transplantation through mid-gut for refractory Crohn's disease: safety, feasibility, and efficacy trial results. J Gastroenterol Hepatol. 2015 Jan;30(1):51-8.

163. Kassam Z, Lee CH, Hunt RH. Review of the emerging treatment of Clostridium difficile infection with fecal microbiota transplantation and insights into future challenges. Clin Lab Med. 2014 Dec;34(4):787-98.

164. O'Brien CL, Allison GE, Grimpen F, Pavli P. Impact of colonoscopy bowel preparation on intestinal microbiota. PLoS One. 2013 May 1;8(5):e62815.

165. Vermeire S, Joossens M, Verbeke K, Wang J, Machiels K, Sabino J, et al. Donor Species Richness Determines Faecal Microbiota Transplantation Success in Inflammatory Bowel Disease. J Crohns Colitis. 2015 Oct 29.

166. Suskind DL, Brittnacher MJ, Wahbeh G, Shaffer ML, Hayden HS, Qin X, et al. Fecal microbial transplant effect on clinical outcomes and fecal microbiome in active Crohn's disease. Inflamm Bowel Dis. 2015 Mar;21(3):556-63.

167. Suskind DL, Singh N, Nielson H, Wahbeh G. Fecal microbial transplant via nasogastric tube for active pediatric ulcerative colitis. J Pediatr Gastroenterol Nutr. 2015 Jan;60(1):27-9.

168. Cui B, Li P, Xu L, Zhao Y, Wang H, Peng Z, et al. Step-up fecal microbiota transplantation strategy: a pilot study for steroid-dependent ulcerative colitis. J Transl Med. 2015 Sep 12;13:298,015-0646-2.

169. Suenaert P, Bulteel V, Lemmens L, Noman M, Geypens B, Van Assche G, et al. Anti-tumor necrosis factor treatment restores the gut barrier in Crohn's disease. Am J Gastroenterol. 2002 Aug;97(8):2000-4.

170. Magnusson MK, Strid H, Sapnara M, Lasson A, Bajor A, Ung KA, et al. Anti-TNF Therapy Response in Patients with Ulcerative Colitis Is Associated with Colonic Antimicrobial Peptide Expression and Microbiota Composition. J Crohns Colitis. 2016 Feb 19.

171. Agilli M, Ilga U. A methodological approach to fecal microbial transplant via nasogastric tube for active pediatric ulcerative colitis. J Pediatr Gastroenterol Nutr. 2015 Apr;60(4):e36.

172. Damman CJ, Brittnacher MJ, Westerhoff M, Hayden HS, Radey M, Hager KR, et al. Low Level Engraftment and Improvement following a Single Colonoscopic Administration of Fecal Microbiota to Patients with Ulcerative Colitis. PLoS One. 2015 Aug 19;10(8):e0133925.

173. Kunde S, Pham A, Bonczyk S, Crumb T, Duba M, Conrad H,Jr, et al. Safety, tolerability, and clinical response after fecal transplantation in children and young adults with ulcerative colitis. J Pediatr Gastroenterol Nutr. 2013 Jun;56(6):597-601.

174. Vaughn BP, Gevers D, Ting A, Korzenik JR, Robson SC, Moss AC. Mo1228 Fecal Microbiota Transplantation Induces Early Improvement in Symptoms in Patients With Active Crohn's Disease. Gastroenterology 2016/04;146(5):S,591; S-592.

175. Landy J, Al-Hassi H, Mann ER, Peake ST, McLaughlin SD, Ciclitira PJ, et al. Tu1985 A Prospective Controlled Pilot Study of Fecal Microbiota Transplantation for Chronic Refractory Pouchitis. Gastroenterology 2016/04;144(5):S-897.

Table 1-2: Summarized studies for FMT in the treatment of IBD.

Author (reference)	# of UC or CD Patients	Study Type	Donor	Pre-FMT treatment	Delivery Mode (number of infusions)	Definition of remission (primary outcome)	Adverse Events	Efficacy (Clinical Remission)
Angelberger et al. (2013)(117)	5 patients with UC	Prospective, open-label study	Patient suggested; excluded first-degree relatives and health-care workers	Metronidazole 500mg twice a day for 5-10 until the evening before FMT. Bowel lavage using a standard PEG given via NJ tube. Pantoprazole 20 mg before FMT and t.i.d. during FMT procedure.	Nasojejunal tube 60g fresh stool in 250 mL of sterile saline. FMT on 3 consecutive days via NJ tube and enema. Loperamide given PFT and 6 hrs PFT.	Remission defined as a tot score ≤ 2 Response as a decrease from baseline in the total Mayo ≤ 3 and at least 30% with an accompanying decrease in the subscore for rectal bleeding ≥ 1 or subscore of 0-1.	All patients experienced fever, temporary increase in CRP and worsening diarrhea the night after the first day of FMT. Also sore throat due to NJ (N=5), flatulence (N=2), pancreatitis of unknown origin (n=1), itchiness (n=1), erythema (n=1), paresthesia of the hip (n=1), collapse due to orthostatic disorder (n=1), and blisters on the tongue (n=1).	0/5 (0%)
Wei et al. (2015)(155)	11 patients with UC 3 patients with CD	Uncontrolled, single-center pilot study	Healthy unrelated donor.	Vancomycin (500 mg) orally b.i.d. for 3 days until 12 hours BFT. Bowel lavage using a standard PEG solution.	60g stool in 350 mL sterile saline. Colonoscopy or naso-jejunal tube (300 mL).	Clinical remission CDAI score <150 and CRP <10 mg/L in CD. For UC, clinical response defined as Mayo decrease >1 and CR as Mayo <2.	One patient showed intolerance with FMT and immediately leaked donor material. Two patients reported a moderate degree of fever. No serious adverse events were noted.	6/11 (54.5%) UC 0/3 (0%) CD
Rossen et al. (2015)(156)	50 patients with UC randomized to healthy or self FMT	Single-center, double-blind, placebo-controlled, randomized, proof-of-concept phase 2 trial	Healthy partners, relatives or volunteers.	Bowel lavage (2L macrogol solution [Moviprep] and 2 L clear fluids the evening before.	Minimum 60g stool in 500 mL normal saline. 500 mL via nasoduodenal tube twice three weeks apart.	Clinical remission (simple clinical colitic activity index scores ≤ 2 combined with ≥ 1-point decrease in the Mayo score at 12 weeks.	The majority of patients experienced mild adverse events during or shortly after treatment. Serious adverse events occurred in 2 patients in the FMT arm. 1 patient had severe small bowel Crohn's disease, and 1 patient became severely ill from a primo cytomegalovirus infection.	6/23 (26.1%)
Kump et al. (2013)(157)	6 patients with chronic active UC	Prospective, open-label, uncontrolled, single-center pilot study	4 non-related healthy donors Donors underwent a mild colonic lavage using (PEG 3350)	Bowel lavage using a standard PEG solution (Moviprep).	100 to 150 g of stool diluted in normal saline (200-350 mL) 300-500 mL delivered via colonoscopy.	Reduction of the total Mayo score by ≥ 3 points was considered a clinical response, Mayo score ≤ 2 was considered remission.	1 patient on anti-TNF treatment had increased stool frequency and developed a fever up to 39°C 1 day PFT. No other adverse events occurred PFT or during follow-up.	0/6 (0%)
Borody et al. (2003)(159)	6 patients with UC (for >5 years)	Case-series	Patient selected donors	Vancomycin 500 mg b.i.d., metronidazole 400 mg b.i.d. and rifampicin 150 mg b.i.d. for 7-10 d. Bowel lavage using a standard PEG-based oral solution. High fiber diet recommended.	200-300g donor stool diluted in 200-300 mL normal saline. Daily enema for 5 days.	Clinical, colonoscopic and histological assessment.	No adverse events reported.	6/6 (100%)

Moyyedi et al. (2015)(161)	38 patients with UC in FMT arm; 38 patients with UC in placebo arm	Double-blind randomized controlled trial	Anonymous donors	No pre-FMT treatment regimen reported.	50g stool in 300 mL of commercial drinking water. Retention enema 50 mL once per week for 6 weeks.	Mayo scores <3 and complete healing of the mucosa at flexible sigmoidoscopy (endoscopic Mayo score = 0).	3 patients in the treatment group with serious adverse events. Two patients had their diagnosis changed to Crohn's colitis and one was C. diff toxin positive PFT.	9/38 (24%)
Cui et al. (2015)(162)	30 patients with CD	Clinical Trial	Family, friend or physician recommended donor Donors were given Forlax (macrogol 4000)	Mesalazine 3.0 g daily started 1 week before FMT and continued for 3 months PFT. Reduced to 1.5-2.5 g daily.	1 bowel movement in 500-1000mL 0.9% saline, diluted 1.5 fold with 0.9% saline on the day of the procedure 150-200 mL given via gastroscopy to the patients mid-gut.	Clinical improvement defined as a decrease of HBI > 3 Clinical remission was defined as HBI $\leq$ 4.	No serious adverse events 2 patient were observed with fever within 1-6 hours PFT. 7 patients had increased diarrhea within 1-6 hours PFT. 26 patients had abdominal or related back pain.	23/30 (76.7%)
Vermeire et al. (2015)(165)	8 patients with UC 6 patients with CD	Pilot study	Family member or friend	Bowel lavage using a standard PEG solution.	Ileo-colonoscopy through NJ (N=9) or rectal (N=5) tube 200g stool in 400mL sterile saline in two infusions on consecutive days.	The primary endpoint was endoscopic healing defined as a Mayo endoscopic subscore of 0 or 1 for UC and SES-CD <3 for CD.	5 SAEs in a total of 4 patients. 4 patients developed a high fever. 2 patients had a sharp increase in CRP.	0/6 (0%) CD 3/8 (37.5%) UC
Suskind et al. (2015)(166)	9 patients with CD	Prospective, open-label study	Mother or father	Rifaximin 200mg t.i.d. for 3 days. Omeprazole (1mg/kg orally) on the day before and morning of the procedure.	30g of donor stool mixed into 100 to 200 mL of normal saline and filtered, delivered by NG tube.	PCDAI score <10 denotes remission 10-29 mild disease, > 30 moderate to severe disease.	All adverse events were graded as mild except for 1 patient with moderate abdominal pain PFT. 2 patients received standard therapy after 12 weeks due to a flare.	7/9 (78%)
Suskind et al. (2015) (167)	4 pediatric UC patients	Single-center, exploratory open-label study	Not reported	Rifaximin 200mg t.i.d. for 3 days. Omeprazole (1mg/kg orally) on the day before and morning of the procedure. 17g MiraLAX in 8oz water t.i.d. for 2 days BFT.	30g of donor stool mixed into 100 to 200 mL of normal saline and filtered, delivered by NG tube.	PUCAI score <10 denotes remission, 10-35 mild disease, 35-64 moderate activity, >65 severe disease.	All adverse events were graded as mild and self-resolving, such as nasal stiffness, bloating, increased flatulence and vomiting in 2 patients PFT. 2 patients were diagnosed with *C. difficile* infection 3 and 4 months PFT.	0/4 (0%)
Cui et al. (2015)(168)	14 patients with UC	Prospective observational study as a part of a clinical trial	10-17 years old friends or relatives	Specific diet Mesalamine 3.0 g oral daily BFT and PFT. Metoclopramide 10 mg and esomeprazole 40 mg IV one-hour BFT.	1 BM in 500-1000mL 0.9% saline, diluted 1.5 fold with 0.9% saline. 150-200 mL given via gastroscopy.	CR defined as the absence of diarrhea and blood (Montreal Classification scale S0).	No serious adverse events. 2 patients were observed with fever (<39°C) and two had transient diarrhea. 1 patient had mild testicular pain after each of two FMTs, which resolved within 12 hours.	4/14 (28.6%)

Agilli et al. (2015)(171)	4 pediatric patients with UC	Single-center, exploratory open-label study	Not reported; underwent appropriate laboratory tests	rifaximin 200 mg t.i.d. for 3 days. Omeprazole (1mg/kg orally) day before and the morning of MiraLAX (17g in 8 oz water) t.i.d. 2 days BFT.	30g of donor stool with 100 mL of normal saline and then 30 mL via NG tube.	PUCAI score <10 denotes remission, 10-35 mild disease, 35-64 moderate activity, >65 severe disease.	No adverse events.	0/4 (0%)
Damman et al. (2015)(172)	7 patients with UC	Prospective, open-label, uncontrolled, single-center pilot study	Patient selected donors	Bowel lavage using a standard PEG solution.	Single colonoscopic FMT. 2-3 mL of 0.9% normal saline per gram of stool. 175-290 cc of FMT.	Primary endpoint was DSI of greater than 50% UCDAI score decrease of 3 or more and remissions as a total UCDAI score of 2 or less with no individual sub-score greater than 1 at 4 weeks PFT.	Mild increase in abdominal cramping and stool output immediately PFT. 1 patient with persistent abdominal pain.	1/7 (14.3%)
Kunde et al. (2013)(173)	10 pediatric patients with UC	Prospective, open-label, uncontrolled, single-center pilot study	Family members or close friends	No pre-FMT treatment.	~90g stool in 250 mL normal saline. Fecal enemas daily for 5 days (average 165 mL).	Clinical response defined as a decrease in PUCAI >15, and decrease to <10 was considered clinical remission.	No serious adverse events. Mild (cramping, fullness, flatulence, bloating, diarrhea, and blood in stool).	3/9 (33%)
Vaughn et al. (2014)(174)	10 patients with CD	Prospective, open-label trial	Anonymous donors	No pre-FMT antibiotics. Bowel prep or lavage not specified.	Single colonoscopic FMT. 50g of stool in 250mL water.	Clinical response defined as having a Harvey Bradshaw Index (HBI) <5	No serious adverse events.	5/8 (62.5%)
Landy et al. (2013)(175)	8 patients with chronic refractory pouchitis (UC)	Prospective, open-label study	Patient nominated donors	None reported.	30g stool in 50mL of sterile saline. Administered via nasogastric tube.	Not reported.	Not reported.	0/5 (0%)

BFT: before FMT; PFT: post-FMT; CR: clinical remission; DSI: donor similarity index; t.i.d.: three times daily; b.i.d.: two times daily; BM: bowel movement; PEG: polyethylene glycol.

Chapter 2: Fecal microbial transplants reduce antibiotic-resistant genes in patients with recurrent *Clostridium difficile* infection

Braden Millan[1&], Heekuk Park[1&], Naomi Hotte[1], Olivier Mathieu[2], Pierre Burguiere[2], Thomas A. Tompkins[2], Dina Kao[1†], Karen L Madsen[1†*]

Affiliations:

[1] Department of Medicine, University of Alberta, Edmonton, Alberta.

[2]Lallemand Health Solutions, Montreal, Quebec.

†These authors contributed equally to this study.

[&]These authors contributed equally to this study.

A version of this chapter was published in Clinical Infectious Diseases, March 2016.

Running Head: FMT reduces antibiotic-resistance genes

Key words: intestinal microbiome; antibiotics; colitis, antibiotic resistance, C. difficile

MAIN POINTS OF ARTICLES

Patients with recurrent *C. difficile* infection harbor large numbers of microbes with antibiotic resistance genes. Fecal microbial transplantation eradicates pathogenic organisms and eliminates antibiotic-resistance genes suggesting this may be a viable treatment option to eradicate multi-drug resistant bacteria from patients.

Abstract

Background: Recurrent *Clostridium difficile* infection (RCDI) is associated with repeated antibiotic treatment and the enhanced growth of antibiotic-resistant microbes. This study tested the hypothesis that patients with RCDI would harbor large numbers of antibiotic-resistant microbes and that fecal microbial transplantation (FMT) would reduce the number of antibiotic-resistant genes.

Methods: In a single center study, patients with RCDI (n=20) received FMT from universal donors via colonoscopy. Stool samples were collected from donors (n=3) and patients prior to and following FMT. DNA was extracted and shotgun metagenomics performed. Results as well as assembled libraries from a healthy cohort (n=87) obtained from the Human Microbiome Project (HMP) were aligned against the NCBI bacterial taxonomy database and the Comprehensive Antibiotic Resistance Database (CARD). Results were corroborated through a DNA microarray containing 354 antibiotic resistance (ABR) genes.

Results: RCDI patients had a greater number and diversity of ABR genes compared with donors and healthy controls. Beta-lactam, multidrug efflux pumps, fluoroquinolone, and antibiotic inactivation ABR genes were increased in RCDI patients while donors primarily had tetracycline resistance. RCDI patients were dominated by Proteobacteria with *Escherichia coli* and *Klebsiella* most prevalent. FMT resulted in a resolution of symptoms which correlated directly with a decreased number and diversity of ABR genes and increased Bacteroidetes and Firmicutes with reduced Proteobacteria. ABR gene profiles were maintained in recipients for up to a year following FMT.

Conclusion: RCDI patients have increased numbers of antibiotic-resistant organisms. FMT is effective in the eradication of pathogenic antibiotic-resistant organisms and elimination of antibiotic-resistance genes.

2.1 Introduction

The microbiota of the human gut is a complex ecosystem with the potential to be an enormous reservoir of antibiotic resistance (ABR) genes, known as the 'gut resistome' (1). Antibiotic resistance has become a major global clinical problem with the emergence of multi-drug resistant organisms such as vancomycin-resistant Enterococci (VRE), methicillin resistant *Staphylococcus aureus* (MRSA) and extended spectrum beta lactamase (ESBL) (2). Antibiotic resistance may arise in a number of different ways, including the accumulation of point mutations and horizontal gene transfer from other bacterial populations through transformation, transduction and/or conjugation (1, 3 , 4). The increased use of antibiotics in agriculture and healthcare has led to a dramatic increase in the prevalence and incidence of antibiotic resistant bacteria, presenting a severe challenge in the treatment of patients infected with these multidrug resistant organisms.

A major complication associated with antibiotic use is *Clostridium difficile* (*C. difficile*) infection (5). *C. difficile* is an anaerobic, spore-forming, toxin producing bacteria that is present in 3% of the healthy adult population; however, up to 20-50% of adults in hospitals and long term care facilities become colonized (6). *C. difficile* infection is treated with metronidazole or vancomycin but the risk of the recurrence is 20-30% within 30 days of initial treatment, and increases further up to 50% after a second episode (7, 8). Fecal microbiota transplantation (FMT) has emerged as an effective and safe therapy for recurrent *C. difficile* infection (RCDI), with over an 80% success rate (9). This study was designed to analyze the gut resistome in patients with RCDI undergoing FMT and to examine how FMT influences the antibiotic

resistance profile of the recipients. We hypothesized that patients with RCDI would harbor large numbers of antibiotic-resistant microbes and that fecal microbial transplantation (FMT) would reduce the number of antibiotic-resistant genes.

2.2 Materials and Methods

2.2.1 Patient Cohorts

This study was conducted at University of Alberta Hospital in Edmonton, Alberta between October of 2012 and November of 2014. Patients aged 35 to 85 with RCDI, defined as at least 3 episodes of CDI within 6 months were included. Active CDI was defined as diarrhea (>3 loose stools per day) with positive stool *C difficile* toxin test. All participants provided written informed consent for FMT and to provide samples for analysis. This study was approved by the University of Alberta Health Research Ethics Board. Data from 87 healthy individuals between the ages of 18 and 40 was obtained from the Human Microbiome Project Consortium (2012). Extensive medical history was not available for these healthy subjects; however individuals were excluded from participating if they had been exposed to any form of antibiotics, antifungals, antivirals, or antiparasitics within the previous 6 months.

2.2.2 Donor Selection

Stool for FMT was obtained from one of three universal stool donors registered with the Edmonton FMT program. Each donation was matched to a single recipient and donations were stored at -80ºC in a concentrated glycerol stock. All donors were screened by undergoing a full

history and physical exam, specifically screening for GI symptoms and risk factors for viral hepatitis or HIV. Donors were excluded if they had taken any antibiotics in the past 6 months. Donors were tested for HIV, hepatitis A, B and C, syphilis, stool bacterial and feces culture, ova and parasite exam (C & S, O & P), *C difficile* toxin, and VRE and rescreened every 4 months.

2.2.3 Fecal Microbial Transplantation (FMT)

Patients discontinued antibiotics for CDI 24 hours prior to FMT. FMT was performed using a preparation of fresh or frozen fecal slurry via colonoscopy. One day prior, patients took 4L of polyethylene glycol-based bowel preparation (GoLYTELY®). Fecal samples were collected by the patients at home prior to and following FMT. After collection, sample aliquots were placed into the -80°C freezer until DNA extraction.

2.2.4 DNA Extraction and Metagenomic Analysis

Stool samples were physical disrupted using a bead-beating kit and microbial DNA extracted using the Qiagen QIAamp DNA stool kit. Indexed paired-end DNA libraries were constructed using an Illumina Nextera® XT DNA Sample Preparation Kit and sequenced on a MiSeq. Sequencing parameters consisted of paired-end 300bp dual index sequencing chemistry using a MiSeq Reagent Kit-V3 (500 cycles) and the FASTQ Only workflow. There were 17,326,984 total reads from 29 donor and 73 patient samples. Any reads with a length less than 150 base pairs were removed so that the average Phred quality score was greater than thirty (>30; 0.1% error rate). Duplicate reads were collapsed using FASTX-Toolkit (version 0.0.13; http://hannonlab.cshl.edu/fastx_toolkit/index.html). Reads from individual samples were mapped to >5 kb assembled contigs using Bowtie2 against a custom database of bacterial genomes

retrieved from NCBI RefSeq database(10). Outputs were visualized in MEGAN (version 5) for taxonomic assignment and reads aligned using Bowtie2 against the Comprehensive Antibiotic Resistance Database (CARD; http://arpcard.mcmaster.ca). CARD contains 6020 different sequences from 4120 genes related to antibiotic resistance, 3008 of which are tagged specifically for antibiotic resistance, consisting of 31 different antibiotic classes (11). Following alignment to CARD, the total read count was 5,414 reads, with 228 different ABR genes detected as having at least a single read in one sample. In the HMP cohort there were 671 total reads, with 143 different ABR genes detected in at least one sample. Genes with different accession numbers but with ≥98% sequence similarity were grouped together and not considered distinct genes for the analysis. Positive detection of an antibiotic resistance gene was considered if there was >0 read counts in just one of the samples for initial analysis.

2.2.5 DNA Microarray Analysis

DNA was extracted and analyzed on a custom oligonucleotide-based DNA microarray containing 370 probes targeting 354 ABR genes (Lallemand Health Solutions, Montreal, Canada). These were classified into antibiotic resistant gene types, including aminoglycosides (53), beta-lactams (49), tetracycline (44), amphenicols (24), erythromycin (21), vancomycin (20), multidrug resistance (19), trimethoprim (13), macrolides (13), lincosamides (10), integrons (7), and sulfonamides (5). 200ng of DNA was labelled using Cy-5 with the Bioprime® DNA Labeling System (Life Technologies) and purified using the QIAquick PCR purification kit. Slides were washed in 0.2% SDS then incubated in pre-hybridization solution (5X SSC, 0.1% SDS and 0.1 mg/mL BSA). Labeled DNA was added to hybridization buffer (20 µL DIG Easy Hybridization Buffer, 1 µL 10 mg/mL Yeast tRNA and 1 µL 10 mg/mL Salmon Sperm DNA)

and samples hybridized for 18 hours followed by 3X washing in 1X SSC/0.1% SDS at 42^{o}C and drying. Slides were scanned and quantified using Quantarray®.

2.2.6 Statistical analysis

The Shapiro-Wilk test was used to determine if data was normally distributed. If normally distributed, a Student's t-test was used, and if not the non-parametric Mann-Whitney U test was performed. A p-value <0.05 was considered statistically significant. Principal coordinates analysis (PCoA) on the taxonomic data was performed using MEtaGenome ANalyzer (Megan, version 5) using Bray-Curtis dissimilarity which quantifies the compositional dissimilarity between different samples. Cluster analysis was performed on the microarray data using Bionumerics®, where the similarity coefficient was determined by the ratio of the absolute value of the Pearson correlation coefficient. These values were then converted into a percentage value to be graphed.

2.3 Results

2.3.1 Patient Cohorts

Twenty patients underwent FMT for RCDI between September 2012 and December 2014. All 20 patients were cured of RCDI following FMT, but while 11 patients were successfully treated with a single FMT, 9 patients failed the initial FMT and required a second FMT. Patient characteristics are shown in **Table 1**. There was no difference between the two groups in age, gender, or duration of RCDI. Data obtained from the Human Microbiome Project

Consortium for 87 different healthy individuals aged 18-40 was analyzed to determine if the antibiotic profile of donors used in this study was representative of a healthy cohort.

2.3.2 Effects of FMT on Microbial Composition

Prior to FMT, RCDI patients had a larger proportion of Proteobacteria **(Figure 2-1A, B)***;* with *Klebsiella* and *Escherichia* being the most prevalent genera (**Figure 2-1C, D**). Donor samples exhibited higher levels of diversity compared with the RCDI patients (**Figure S2-1**). In the RCDI cohort which responded following a single FMT, microbial profiles at the phyla (**Figure 2-1A**) and genus (**Figure 2-1C**) level increased in similarity to that of the donor (**Figure 2-2A**). In particular, the relative amount of Proteobacteria (*Klebsiella*, *Escherichia*) was reduced and the relative amounts of Bacteroidetes (*Bacteroides*) and Firmicutes (*Ruminococcus*, *Faecalibacterium, Eubacterium*) were increased. In contrast, in the cohort which required a second FMT, the patients generally failed to resemble the donor at both the phyla (**Figure 2-1B**) and genus (**Figure 2-1D**) level following the initial FMT (**Figure 2-1**). However, following the second FMT, their intestinal microbial profile did become more similar to the donor and this correlated with a clinical response (**Figure 2-2B**). The number of raw reads aligned to Proteobacteria was much higher in the patients BFT samples in both cohorts compared with the healthy donors, and decreased significantly following the FMT in the single FMT cohort, and following the second FMT in the cohort which required a second FMT (**Figure S2-4**).

Table 2-1. Clinical Characteristics of Recurrent *Clostridium difficile* Infection Patients Receiving Fecal Microbiota Transplants

	Single FMT	Repeated FMT	P Value
Number of patients	11	9	
Age	67.7 (35.3-84.9)	71.4 (49.5-83.8)	.7039
Gender	7 male; 4 female	5 male; 4 female	1.000
Average duration of RCDI prior to FMT	150.4 d (71-275)	126.2 d (53-246)	.4237
CDI classification	7 community-acquired; 4 hospital-acquired	3 community-acquired; 6 hospital-acquired	.3698

Numbers are given as means with range in brackets.
Abbreviations: CDI, *C. difficile* infection; FMT; fecal microbiota transplantation; RCDI, recurrent *Clostridium difficile* infection.

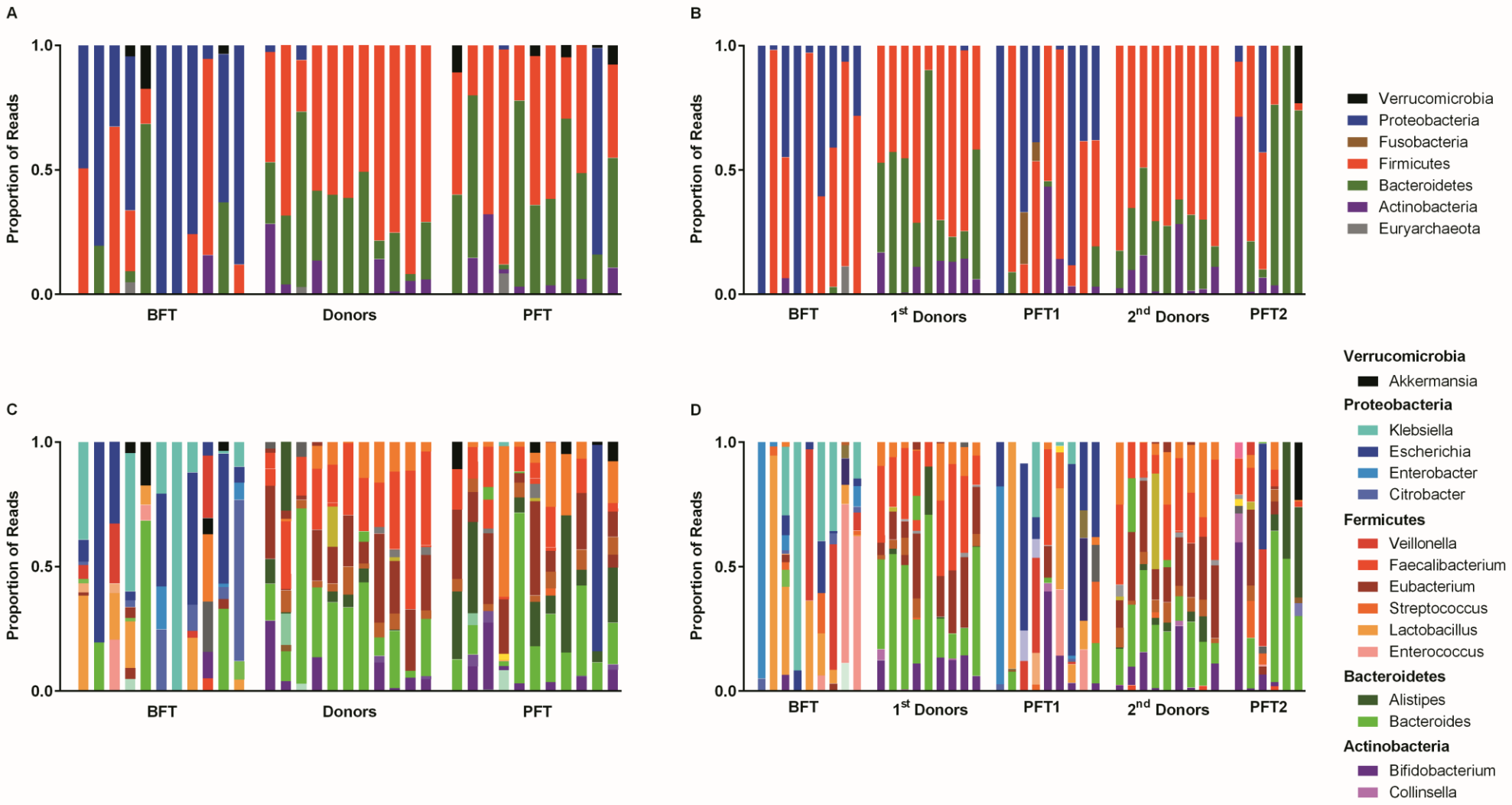


Figure 2-1. Microbial profile of stool samples from RCDI patients and donors. Microbial composition in stool samples from single successful FMT recipients shown at the phyla (A) and genus (C) levels before FMT (BFT; n=11), donor samples (Donors; n=11), and post FMT (PFT; n=11). Microbial composition in stool samples from repeated FMT cohort shown at the phyla (B) and genus (D) level before FMT (BFT; n=9), first donor samples (1st Donors; n=9), patients following initial failed FMT (PFT 1; n=9), second donor samples (2nd Donors; n=9), and patients following successful second FMT (PFT2; n=6). Data is presented as the fraction of total reads in each sample at the phyla level and 14 most prevalent genera.

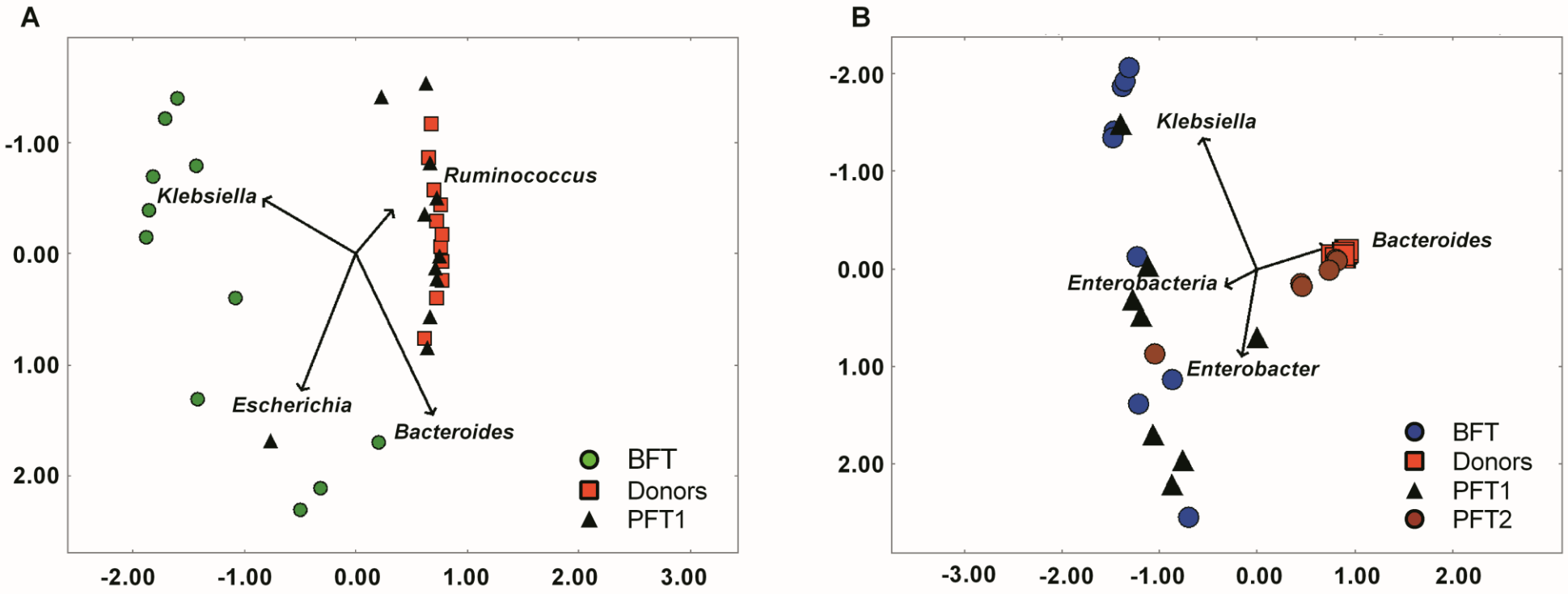


Figure 2-2. Principal coordinates analysis (PCoA) of taxonomic data from RCDI patients and donors. Biplot vectors show the 4 genera with the strongest magnitude that contributed to sample dissimilarity. (A) Prior to FMT, RCDI patients were defined by higher proportions of *Klebsiella* and *Escherichia*, while donors were predominated by *Ruminococcus* and *Bacteroides*. Following FMT, all but one RCDI patient clustered together with donor samples. (B) RCDI patients which failed the initial FMT were also defined by *Klebsiella, Enterobacteria*, and *Escherichia* prior to FMT, and did not resemble the donors until after the second FMT. PCoA was performed using Bray-Curtis dissimilarity to quantify the compositional dissimilarity between samples. (A) Green circles: before FMT samples; Purple triangles: post FMT samples; Red squares: donors samples. (B) Multiple FMT cohort: Blue circles: before FMT samples; Red squares: 1st donors samples; Purple triangles: post 1st FMT recipient samples; Red diamonds: 2nd Donors samples; Brown circle: post 2nd FMT recipient samples. Donor cohort: n = 31; Single FMT Cohort: n = 11; Two FMT Cohort n = 9.

2.3.3 RCDI patients had increased numbers of antibiotic-resistant genes

Stool donors had a mean of 3.4 ± 0.4 and a range of 1-6 ABR genes in their samples. This was similar to healthy controls from the Human Microbiome Project, who had a mean of 6.0 ± 0.9 ABR genes with a range of 0-39 ABR genes. RCDI patients had increased numbers of ABR genes compared with donors (**Figure 2-3A**). There was no correlation between duration of disease or age of RCDI patients and number of ABR genes (**Figure S2-2A**). Prior to FMT, the patients who responded to a single FMT had a mean of 34.5 ± 6.7 different ABR genes. At the first follow-up period (1-3 wks post FMT), the number of ABR genes in the RCDI patients who had a clinical response had significantly decreased to a mean of 12.2 ± 7.0. In subsequent follow-ups, the average number of ABR genes continued to drop to a mean of 5.1 ± 0.74 genes (**Figure 2-3B**). The RCDI cohort which failed to respond to the initial FMT and required a second FMT had a mean of 20.9 ± 4.4 ABR genes prior to FMT and this did not decrease following the initial FMT. However, following the second successful FMT, the number of ABR genes per patient dropped to 10.0 ± 4.9 genes, suggesting that a clinical response was associated with a decrease in ABR genes (**Figure 2-3C**).

Figure 2-3A

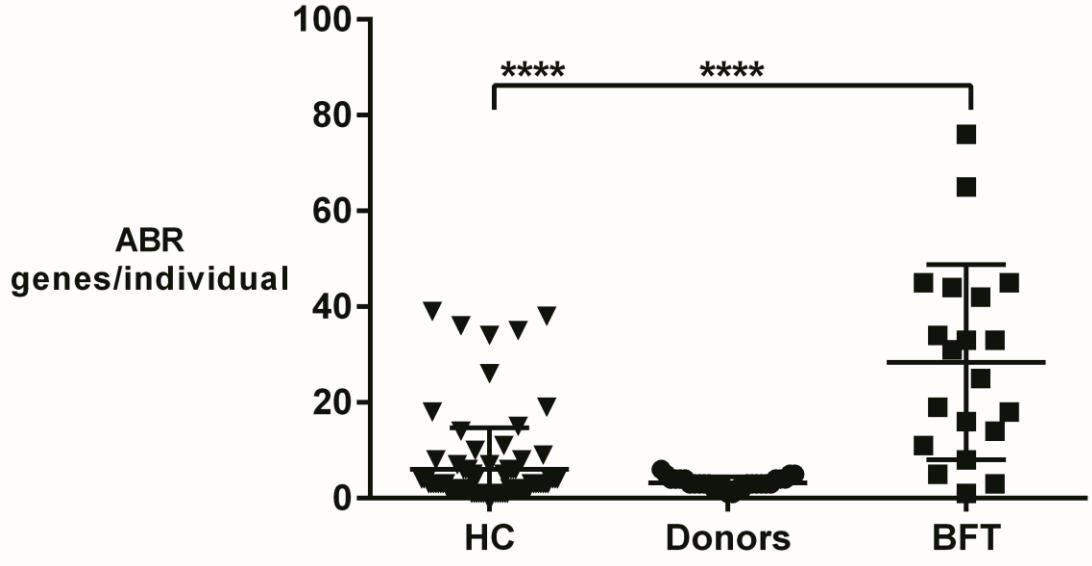
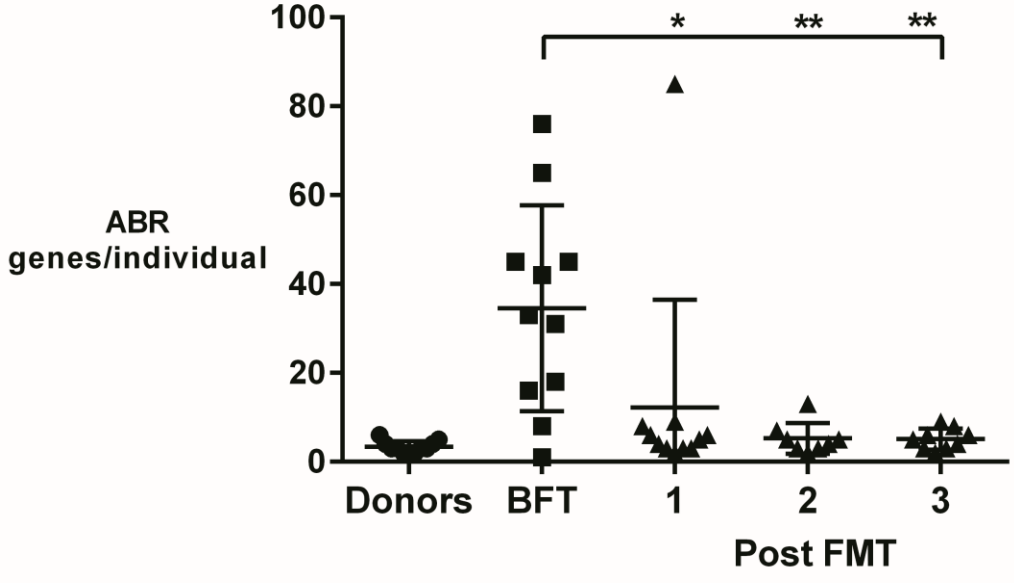


Figure 2-3B



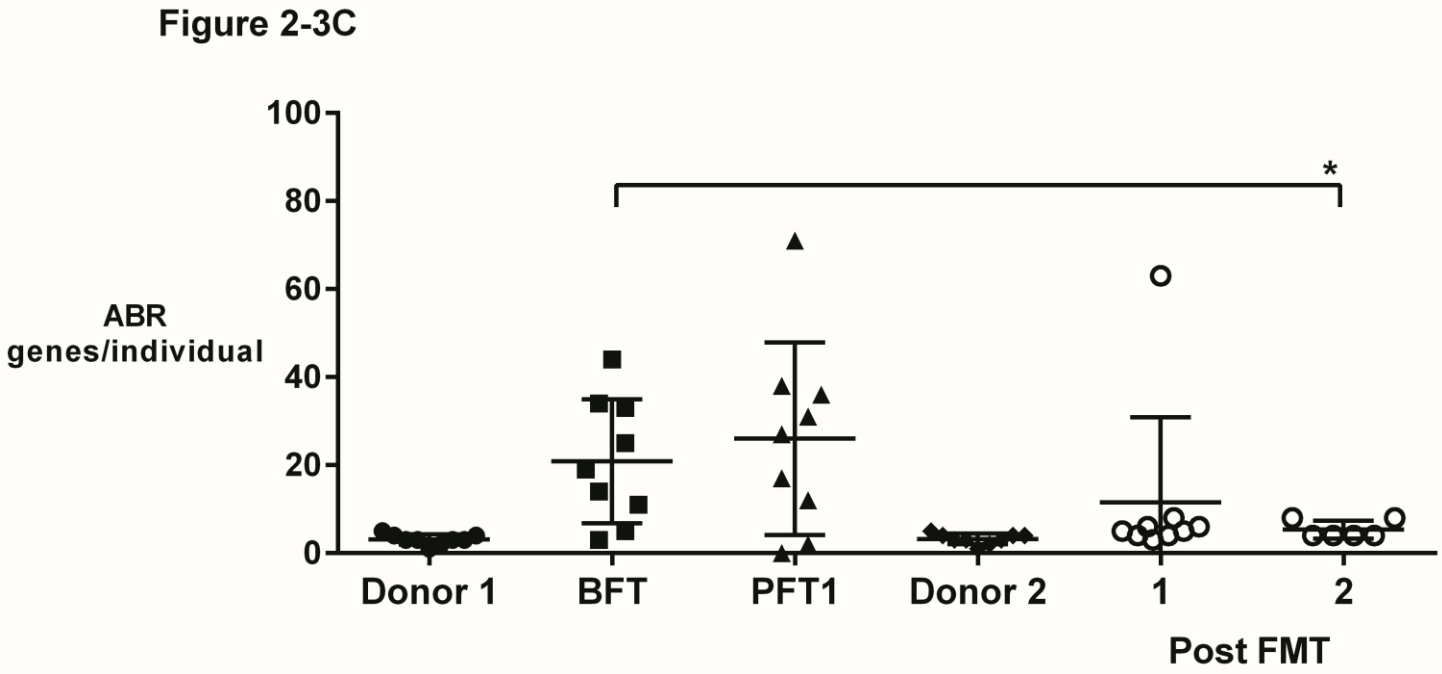


Figure 2-3. Number of antibiotic-resistance genes in RCDI and donor stool samples pre and post FMT. Metagenomic data was aligned using Bowtie2 to the Comprehensive Antibiotic Resistance Database (CARD; http://arpcard.mcmaster.ca) to detect ABR genes. (A) RCDI patients at baseline (BFT) had increased numbers of ABR genes compared with the donors and healthy controls (HC). (B) Following successful FMT, the number of detected ABR genes decreased in the RCDI patients and this was maintained over time. All patients had a post-FMT (PFT) sample taken between 1-4 wks following FMT (Group 1: n=11); 9 had a sample taken between 4 and 8 wks following FMT (Group 2: n=9); 7 had a sample taken between 8 and 22 weeks following FMT and 2 patients had a 1 year PFT sample (Combined in Group 3). (C). In the patients which did not respond to the initial FMT, there was no decrease seen in the number of ABR genes following the first FMT (PFT1). In this cohort, a decrease in the number of ABR genes was seen following the second FMT and this was maintained over time. All patients had a post-FMT (PFMT1) taken between 1-3 wks following FMT2 (Group 1: n=9); 4 patients had a sample taken between 3 and 12 wks following FMT2 and 2 patients had a 28 wk follow-up (Combined in Group 2: n=6). Healthy cohort: n=87; Donor cohort: n = 29; Single FMT Cohort: n = 11; Two FMT Cohort n = 9. Whiskers denote mean with the standard deviation. * *P*-value <0.05; ** *P*-value <0.005; ** P-value <0.0001

2.3.4 RCDI patients had increased diversity of antibiotic-resistant genes

Figure 2-4A shows the main classes of ABR genes in the three cohorts. Beta-lactam, multidrug efflux pumps, fluoroquinolone, and antibiotic inactivation ABR gene classes were increased in the RCDI cohorts compared with donors. In **Figure 2-4B**, it can be seen that the RCDI patient cohort which responded to a single FMT had the highest number of ABR genes with 73 unique and a total of 151 while patients which required a second FMT had 25 unique and a total of 101 ABR genes (**Figure S2-3**). The donor cohort had 14 unique ABR genes and a total of 26, the majority of which were tetracycline antibiotic resistant genes. Although ABR genes were still detected in our donor cohort, 76% of those ABR genes detected were observed in fewer than 10% of the 29 donor samples, and did not appear in the top 100 most detected ABR genes (**Figure S2-3**). Three of the tetracycline genes were detected in 90% of the healthy donors; these included tet (W), tet (O), and tet (Q). In the healthy cohort from the HMP, tet (W) was also highly detected, and was found in 45% of the individuals. The genes detected in the RCDI patients prior to FMT belonged to a total of 31 different ABR classes, where only one was unique to the each of the RCDI cohorts and none to the donor cohorts (**Figure 2-4C**). Seventeen classes were shared amongst all three cohorts, while 11 were unique to the RCDI patient cohorts. These were most evident in the classes of glycopeptide resistance, polymyxin resistance, sulfonamide resistance, lipopeptide resistance, streptothricin resistance and peptide antibiotic resistance (**Figure S2-3**). The sole class that was unique to the single FMT cohort was genes conferring fosfomycin resistance while the class unique to the two FMT cohort was genes conferring trimethoprim resistance.

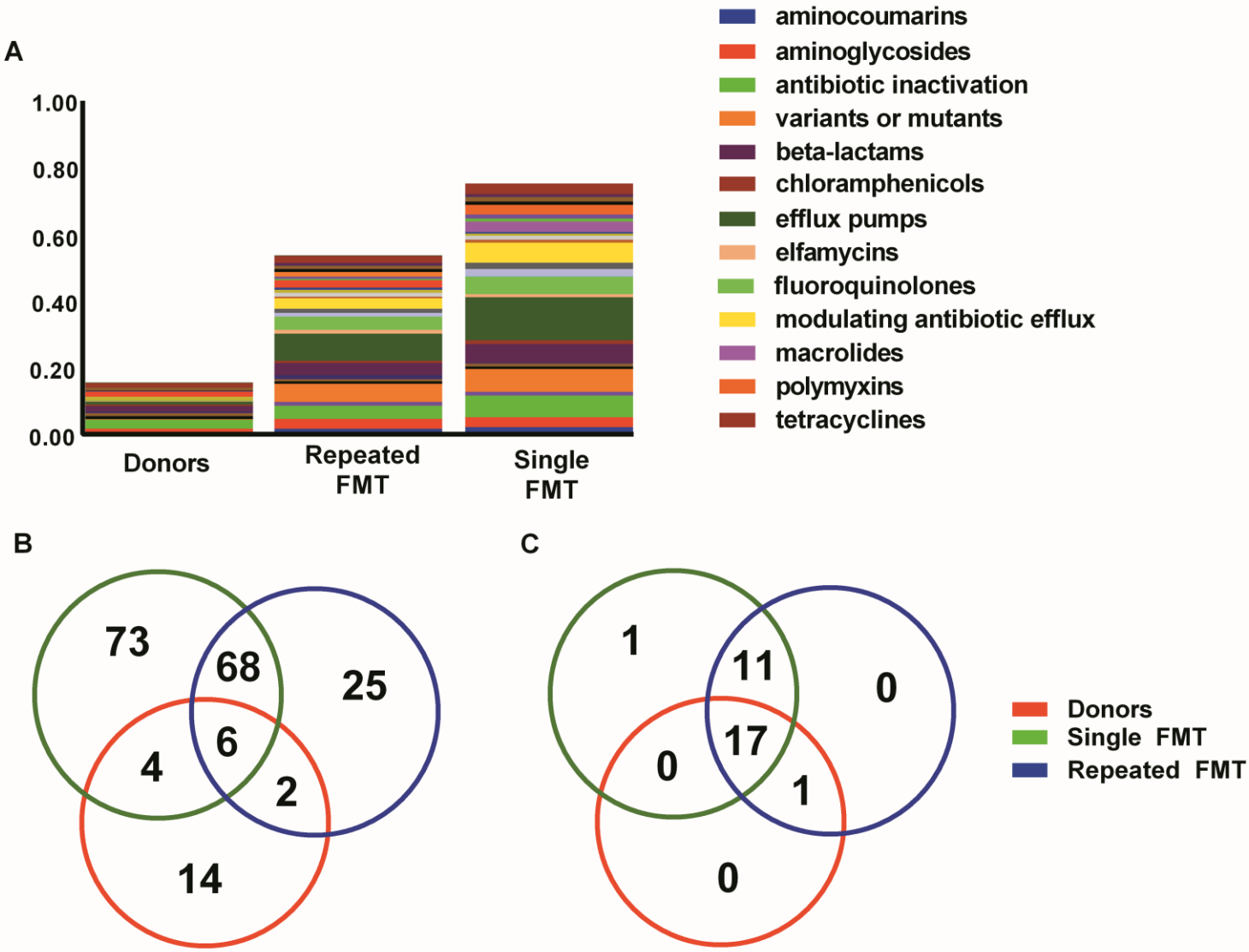


Figure 2-4. Relative abundance of antibiotic resistance gene types assigned to antibiotic class resistance in RCDI patients and donors prior to FMT. Identified antibiotic resistance genes were classified according to CARD (http://arpcard.mcmaster.ca). Genes that confer resistance to multiple antibiotics were included in the analysis. (A) The RCDI patients had a greater diversity of ABR gene classes compared with donors. (B) Venn diagram showing the shared and unique ABR genes in the RCDI patients and donors. (C) Venn diagram showing the shared and unique ABR resistance gene classes in the RCDI patients and donors. Green: single FMT cohort; blue: repeated FMT cohort; red: donors. Donor cohort: n = 29; Single FMT Cohort: n = 11; Two FMT Cohort n = 9.

2.3.5 Antibiotic resistant profiles remained constant over time in donors and in recipients

Microarray analysis was performed to corroborate the metagenomic results and to examine stability over time in the donors and recipients. In total, 229 different genes were detected in at least one of the samples, with the RCDI patients showing positive detection of 178 genes and the donors having positive detection of 26. 90% of the donor samples had all three of the ABR genes, tet (O), tet (Q) and tet (W). Genes within the antibiotic classes of tetracycline, aminoglycosides, beta-lactams, macrolides, erythromycin, and multidrug efflux were the most prevalent antibiotic classes detected on the microarray; similar to what was seen in the metagenomic data. Samples taken from the donors were analyzed for stability over time on the microarray and RDCI recipients for similarity to the donor. Antibiotic resistant profiles in the donors remained relatively constant over the period of donation (**Figure 2-5A**). **Figure 2-5B** shows percent similarity of the single RCDI patients to their respective donor before FMT and post FMT. Following a successful FMT, the resistome of the recipient became more similar to the resistome of the donor and this was maintained up to a year following the FMT. In contrast, in the RCDI patients which failed the first FMT, the resistome did not become more similar to the donor (**Figure 2-5C**). However, following the second successful FMT, the resistome did become more similar to the donor, although there was more variability in this group compared with the single FMT group.

Figure 2-5A

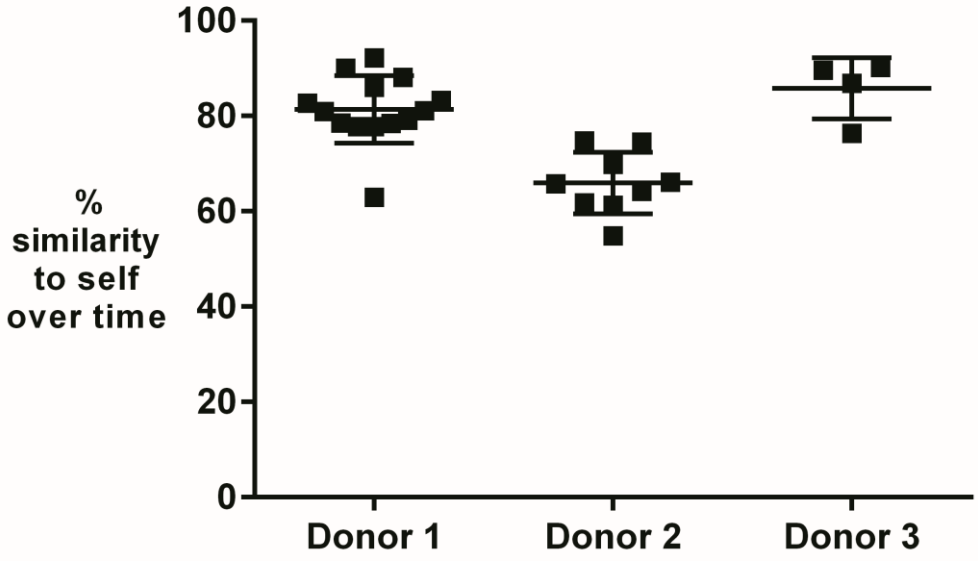
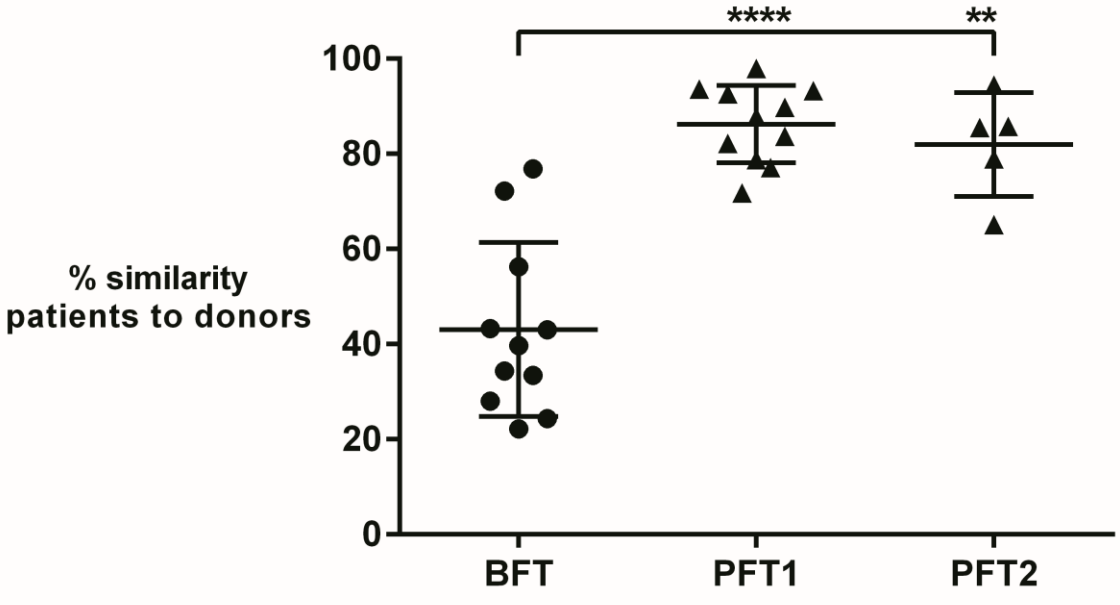


Figure 2-5B



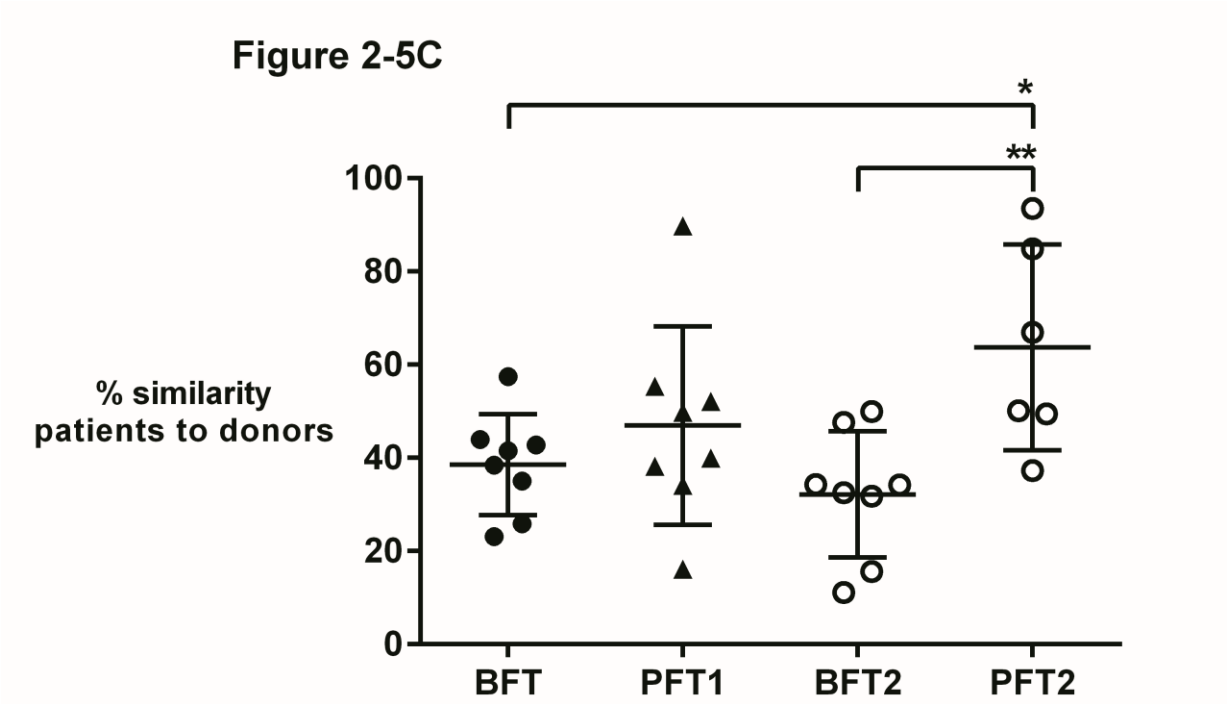


Figure 2-5. Pearson similarity index of donors over time and RCDI patients to donors. Samples were analyzed using a DNA microarray. (A) Donors retained a high degree of similarity to their own samples over their donation time period. Donor 1= 18 months; Donor 2= 12 months; Donor 3= 7 months (B) Percent similarity of the single FMT recipient samples at those time points (1=1-4 wks; 2=8-52 wks post FMT) to their respective donor. Following FMT, the similarity of the RCDI patients increased to more closely resemble the donors; **** p-value < 0.0001 calculated using paired t-test; *** p-value = 0.0005 calculated using Mann-Whitney test. (C) Percent similarity of the repeated FMT recipient samples at those time points to the respective donor of the most recent FMT (PFT 1: 1-2 wks; PFT2: 3-38 wks post FMT2); * p-value < 0.05; ** p-value < 0.005 both calculated using Mann-Whitney test. Whiskers denote mean with the standard deviation.

2.4 Discussion

In this study we demonstrate that patients with RCDI harbor increased numbers and diversity of ABR genes compared with healthy stool donors. FMT was effective in reducing the load of ABR genes in conjunction with resolution of disease and this was maintained up to a year following the transplant. These findings suggest that FMT may have a significant role beyond that of treating RCDI and may be able to eradicate multi-drug resistant bacterial infections or alternatively restore antibiotic susceptibility to individual patients.

RCDI patients had ~30 more ABR genes compared with donors and the healthy cohort. Diversity of the antibiotic genes was also much higher in the RCDI patients, which had all 31 of the identified ABR gene classes, while only 19 were identified in healthy stool donors. In the donors, tetracycline and beta-lactam genes were the most prevalent. In congruence with our data, it has been previously shown that genes conferring resistance to the antibiotic tetracycline are present in the microbiota of the majority of individuals and are also the most abundant family of resistance genes (12, 13). The majority of the ABR genes detected in the healthy stool donors are found predominately within Bacteroidetes and Actinobacteria while ABR genes in the RCDI patients are found almost exclusively in Proteobacteria with a small number in Firmicutes (14). This increased load and diversity of ABR genes likely reflects the significant dysbiosis that was seen in the RCDI patients, with predominance of *Escherichia coli* and *Klebsiella.*

The number of ABR genes was dramatically reduced after the first FMT in the patients who showed a clinical response, but not in the patients who failed the first FMT. This would suggest that the change in the ABR gene profile was likely due to the change in microbial

composition, in particular the reduction in Proteobacteria induced by the FMT. However, although we did show a significant reduction in total Proteobacteria in patients following FMT, it is also possible that FMT reduced their abundance to below our limit of detection (15, 16). However, case reports showing eradication of multidrug-resistant organisms in patients following FMT together with evidence from a murine study suggesting that multi-drug resistant bacteria such as vancomycin-resistant *Enterococcus faecium* (VRE), and Carbapenem-resistant *Klebsiella penumoniae* (CRKP) can be eliminated from the gut following FMT supports the potential for this procedure (17, 18, 19). The elimination of bacteria harboring ABR genes is thought to be a direct result of the ability of FMT to re-establish a healthy gut ecosystem which provides colonization resistance against pathogens by removing favorable growth conditions. (20)

Limited options are available for patients infected with multi-drug resistant organisms. Although selective digestive decontamination together with the intravenous administration of antibiotics has shown benefit as an infection prevention measure in critically ill patients (21-23), the problem arises in that this regimen is associated with dramatic increases in ABR genes (24). In addition, once the treatment is discontinued the patients remained colonized with these multi-drug resistant pathogens and as the gut becomes recolonized following removal of antibiotics an increased horizontal transfer of resistance genes from the surviving organisms to opportunistic aerobic pathogens may occur (25). Eradication of virulent organisms containing ABR genes from the gut may also help in alleviating systemic infections, as studies have shown pathogenic organisms to predominate in the gut prior to translocating to and infecting other body sites (26).

Thus, these findings have significant clinical implications and suggests that FMT may have a role beyond that in treating patients with RCDI.

Although we speculate that the increase in antibiotic resistance genes in the RCDI patients was due to the extensive antibiotic treatment in this cohort, some studies have shown a greater number of ABR genes in the older population (27) which may be related to accumulated exposure over time to factors such as heavy metals (28-30) or to the spread of ABR organisms from animals to humans through the food chain (31). In that the average age of our donors (36 years) was less than that of the patients (67 years), the increased ABR genes in the RCDI cohort could theoretically have arisen even without prolonged exposure to antibiotics. However, in that the linear regression of our data comparing age and number of ABR genes showed no significant correlation, it is more likely that the increase in ABR genes in this cohort was related to their extensive antibiotic usage.

In conclusion, these results demonstrate that patients with RCDI harbor large numbers of microbes that carry a great diversity of antibiotic resistance genes. FMT is effective at both resolving RCDI and in reducing the carriage of multiple ABR genes in these patients.

2.5 Endnote

This work was published in Clinical Infectious Diseases.

Millan B, Park H, Hotte N, Mathieu O, Burguiere P, Tompkins TA, et al. Fecal microbial transplants reduce antibiotic-resistant genes in patients with recurrent Clostridium difficile infection. Clin Infect Dis. 2016 Mar 29.

The authors would like to thank Matt Emberg and Brandi Roach for their contributions towards the fecal microbial transplant program; and Chad MacPherson and Joseph Dimetry for data analysis. This work was supported by Alberta Health Services and Canadian Institutes for Health Research (Grant number: 93675). Olivier Mathieu, Pierre Burguiere, and Thomas Tompkins are employees of Lallemand Health Solutions, Inc.

2.6 References

1. van Schaik W. The human gut resistome. Philos Trans R Soc Lond B Biol Sci **2015**; 370(1670): 20140087.

2. Zhang R, Eggleston K, Rotimi V, Zeckhauser RJ. Antibiotic resistance as a global threat: evidence from China, Kuwait and the United States. Global Health **2006**; 2: 6.

3. Ochman H, Lawrence JG, Groisman EA. Lateral gene transfer and the nature of bacterial innovation. Nature **2000**; 405(6784): 299-304.

4. Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J. Call of the wild: antibiotic resistance genes in natural environments. Nat Rev Microbiol **2010**; 8(4): 251-9.

5. McFarland LV. Antibiotic-associated diarrhea: epidemiology, trends and treatment. Future Microbiol **2008**; 3(5): 563-78.

6. Clabots CR, Johnson S, Olson MM, Peterson LR, Gerding DN. Acquisition of Clostridium difficile by hospitalized patients: evidence for colonized new admissions as a source of infection. J Infect Dis **1992**; 166(3): 561-7.

7. Goldberg EJ, Bhalodia S, Jacob S, et al. Clostridium difficile infection: A brief update on emerging therapies. Am J Health Syst Pharm **2015**; 72(12): 1007-12.

8. Kelly CP. Can we identify patients at high risk of recurrent Clostridium difficile infection? Clin Microbiol Infect **2012**; 18 Suppl 6: 21-7.

9. Rossen NG, MacDonald JK, de Vries EM, et al. Fecal microbiota transplantation as novel therapy in gastroenterology: A systematic review. World J Gastroenterol **2015**; 21(17): 5359-71.

10. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods **2012**; 9(4): 357-9.

11. McArthur AG, Waglechner N, Nizam F, et al. The comprehensive antibiotic resistance database. Antimicrob Agents Chemother **2013**; 57(7): 3348-57.

12. Forslund K, Sunagawa S, Kultima JR, et al. Country-specific antibiotic use practices impact the human gut resistome. Genome Res **2013**; 23(7): 1163-9.

13. Hu Y, Yang X, Qin J, et al. Metagenome-wide analysis of antibiotic resistance genes in a large cohort of human gut microbiota. Nat Commun **2013**; 4: 2151.

14. Liu B, Pop M. ARDB--Antibiotic Resistance Genes Database. Nucleic Acids Res **2009**; 37(Database issue): D443-7.

15. Ni J, Yan Q, Yu Y. How much metagenomic sequencing is enough to achieve a given goal? Sci Rep **2013**; 3: 1968.

16. Rodriguez RL, Konstantinidis KT. Estimating coverage in metagenomic data sets and why it matters. ISME J **2014**; 8(11): 2349-51.

17. Crum-Cianflone NF, Sullivan E, Ballon-Landa G. Fecal microbiota transplantation and successful resolution of multidrug-resistant-organism colonization. J Clin Microbiol **2015**; 53(6): 1986-9.

18. Singh R, van Nood E, Nieuwdorp M, et al. Donor feces infusion for eradication of Extended Spectrum beta-Lactamase producing Escherichia coli in a patient with end stage renal disease. Clin Microbiol Infect **2014**; 20(11): O977-8.

19. Caballero S, Carter R, Ke X, et al. Distinct but Spatially Overlapping Intestinal Niches for Vancomycin-Resistant Enterococcus faecium and Carbapenem-Resistant Klebsiella pneumoniae. PLoS Pathog **2015**; 11(9): e1005132.

20. Buffie CG, Pamer EG. Microbiota-mediated colonization resistance against intestinal pathogens. Nat Rev Immunol **2013**; 13(11): 790-801.

21. de Jonge E, Schultz MJ, Spanjaard L, et al. Effects of selective decontamination of digestive tract on mortality and acquisition of resistant bacteria in intensive care: a randomised controlled trial. Lancet **2003**; 362(9389): 1011-6.

22. Schultz MJ, de Jonge E, Kesecioglu J. Selective decontamination of the digestive tract reduces mortality in critically ill patients. Crit Care **2003**; 7(2): 107-10.

23. de Smet AM, Kluytmans JA, Cooper BS, et al. Decontamination of the digestive tract and oropharynx in ICU patients. N Engl J Med **2009**; 360(1): 20-31.

24. Buelow E, Gonzalez TB, Versluis D, et al. Effects of selective digestive decontamination (SDD) on the gut resistome. J Antimicrob Chemother **2014**; 69(8): 2215-23.

25. Oostdijk EA, de Smet AM, Blok HE, et al. Ecological effects of selective decontamination on resistant gram-negative bacterial colonization. Am J Respir Crit Care Med **2010**; 181(5): 452-7.

26. Ubeda C, Taur Y, Jenq RR, et al. Vancomycin-resistant Enterococcus domination of intestinal microbiota is enabled by antibiotic treatment in mice and precedes bloodstream invasion in humans. J Clin Invest **2010**; 120(12): 4332-41.

27. Lu N, Hu Y, Zhu L, et al. DNA microarray analysis reveals that antibiotic resistance-gene diversity in human gut microbiota is age related. Sci Rep **2014**; 4: 4302.

28. Lubelski J, Konings WN, Driessen AJ. Distribution and physiology of ABC-type transporters contributing to multidrug resistance in bacteria. Microbiol Mol Biol Rev **2007**; 71(3): 463-76.

29. Davis IJ, Roberts AP, Ready D, Richards H, Wilson M, Mullany P. Linkage of a novel mercury resistance operon with streptomycin resistance on a conjugative plasmid in Enterococcus faecium. Plasmid **2005**; 54(1): 26-38.

30. Baker-Austin C, Wright MS, Stepanauskas R, McArthur JV. Co-selection of antibiotic and metal resistance. Trends Microbiol **2006**; 14(4): 176-82.

31. Teale CJ. Antimicrobial resistance and the food chain. J Appl Microbiol **2002**; 92 Suppl: 85S-9S.

2.7 Supplementary Material

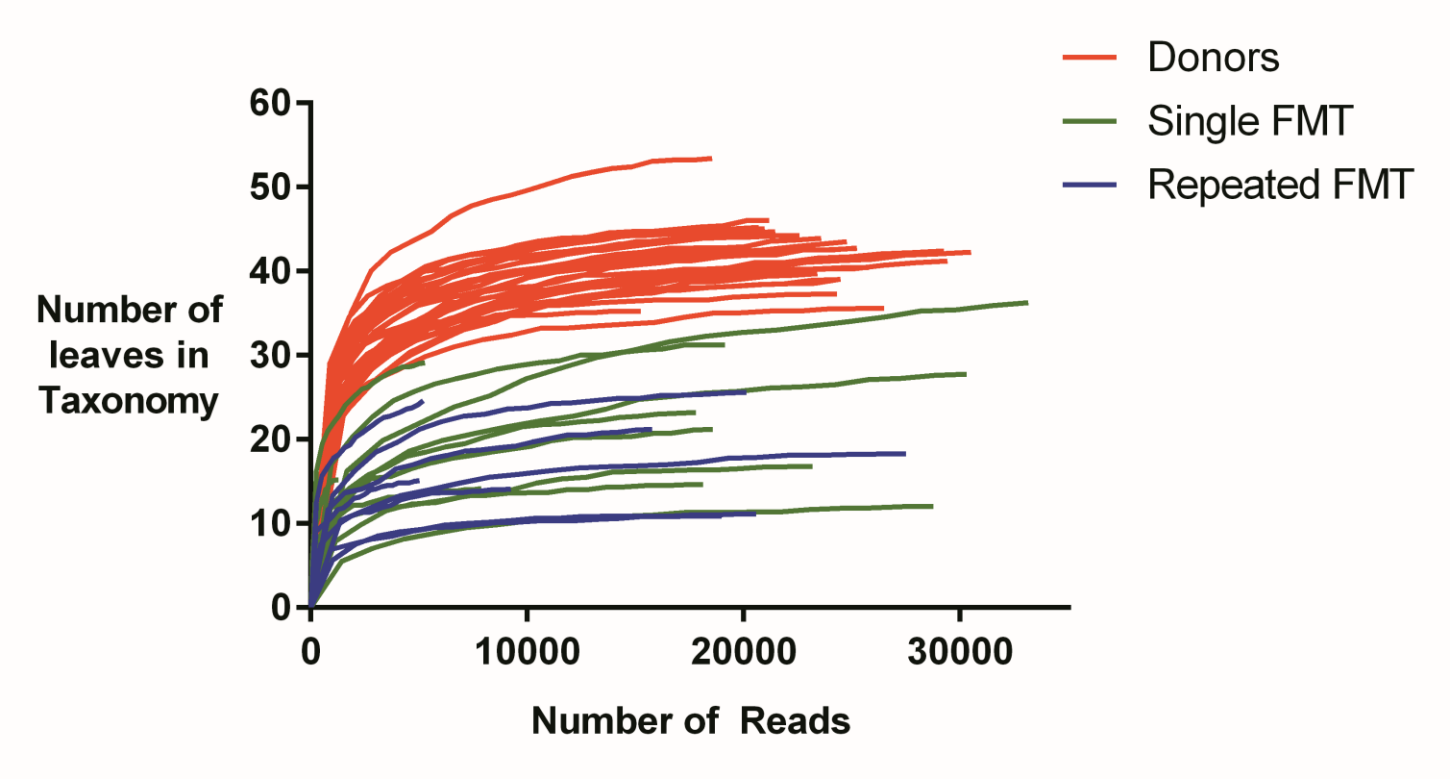


Figure S2-1. **Alpha-diversity as measured by the Shannon-Wiener diversity index using MEtaGenome Analyzer (Megan, version 5).** Donor samples had increased diversity compared with samples from RCDI patients. Donor cohort (red), single FMT cohort (green) and repeated FMT cohort (blue).

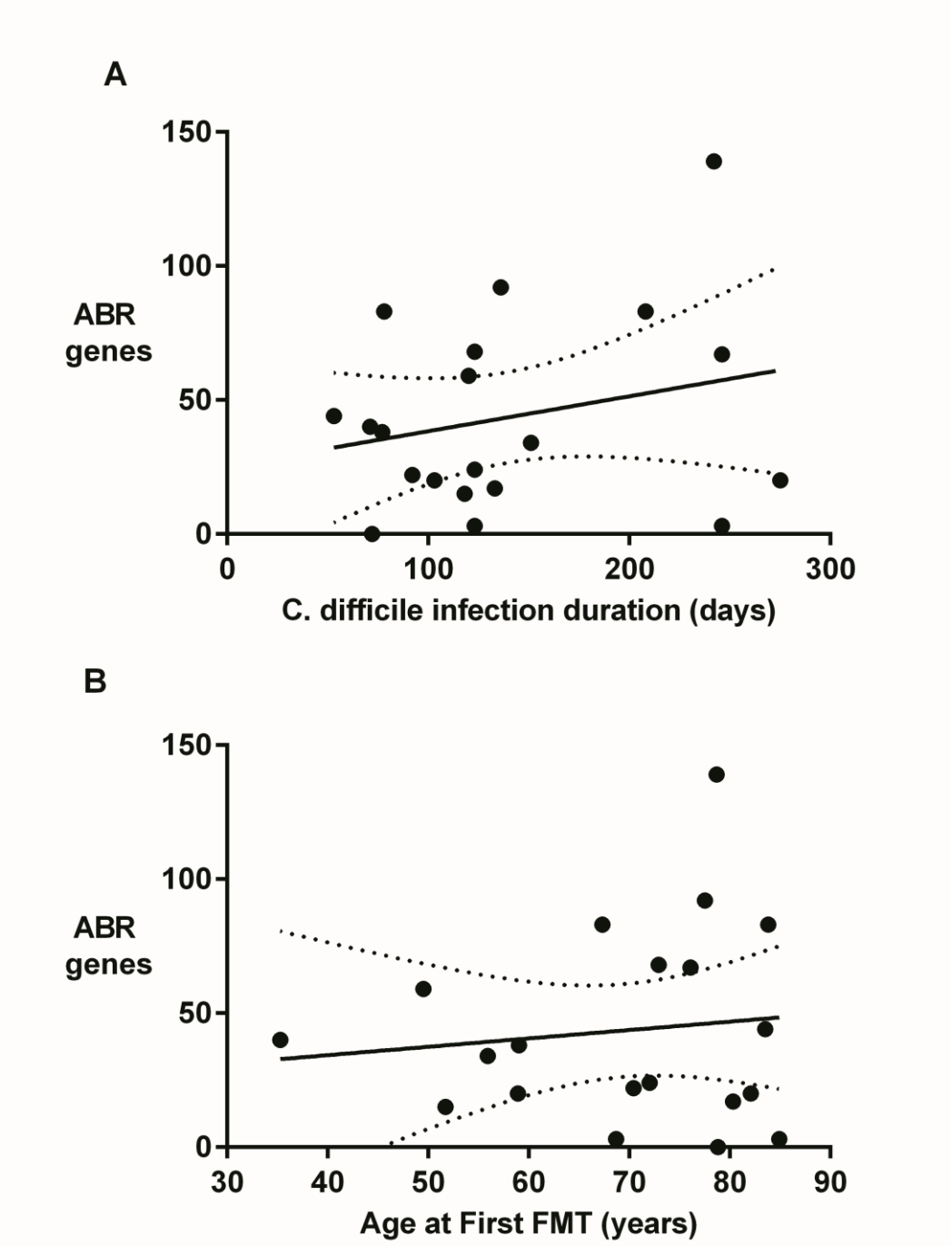


Figure S2-2. **Correlations between the number of ABR genes detected in each individual BFT to *C. difficile* duration and age. (A)** the duration of their *C. difficile* infection ($R_2 = 0.059$; p=0.3) and **(B)** age ($R_2 = 0.014$; p=0.6). Dotted lines show the 95% confidence interval; linear regression was preformed calculating to determine the goodness of fit, and R squared of 1 indicates the regression line perfectly fits the data. There were no significant correlations.

Figure S2-3A

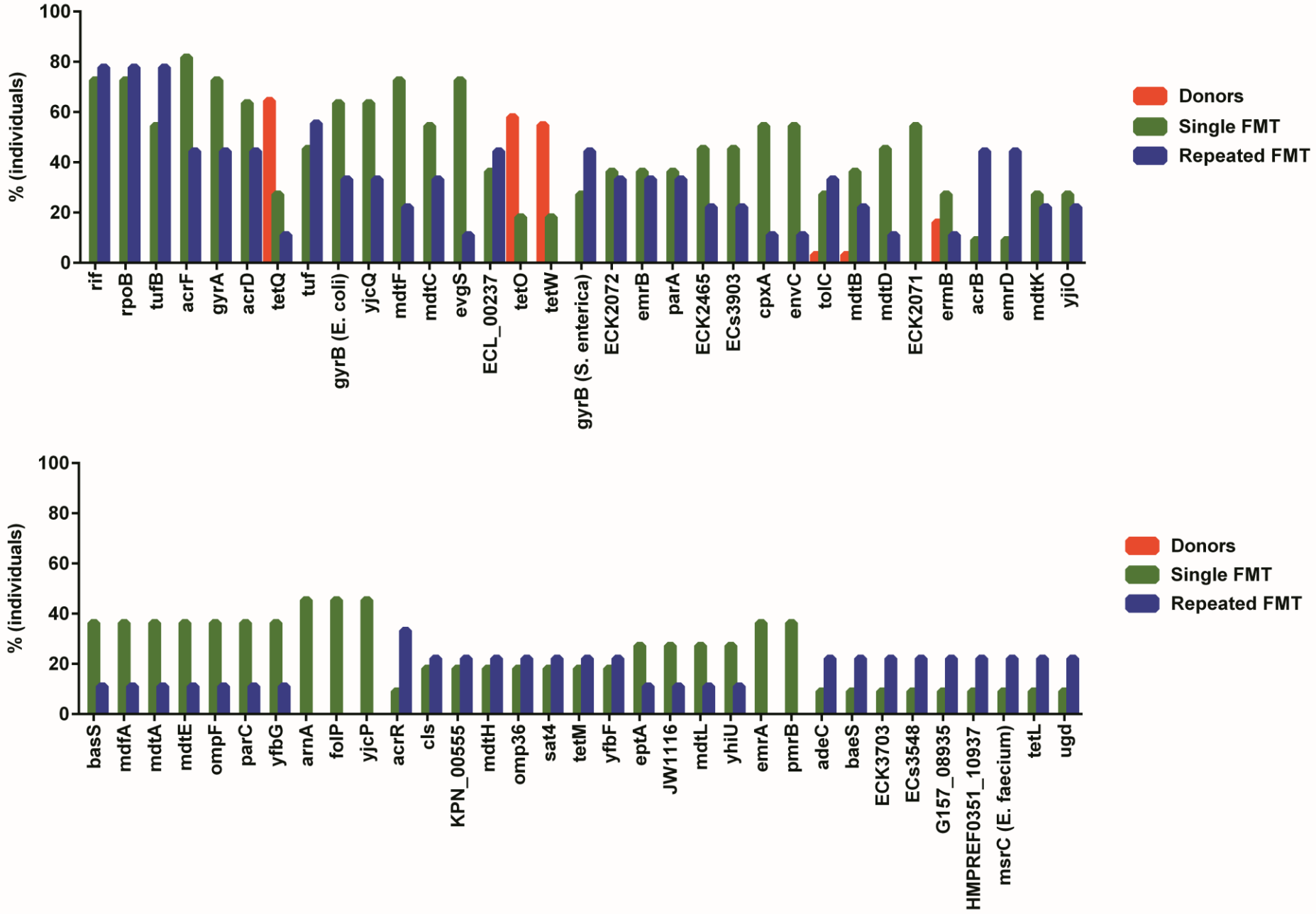

% (individuals)
100
80
60
40
20
0
rif
rpoB
tufB
acrF
gyrA
acrD
tetQ
tuf
gyrB (E. coli)
yjcQ
mdtF
mdtC
evgS
ECL_00237
tetO
tetW
gyrB (S. enterica)
ECK2072
emrB
parA
ECK2465
ECs3903
cpxA
envC
tolC
mdtB
mdtD
ECK2071
ermB
acrB
emrD
mdtK
yjiO
Donors
Single FMT
Repeated FMT
% (individuals)
100
80
60
40
20
0
basS
mdfA
mdtA
mdtE
ompF
parC
yfbG
arnA
folP
yjcP
acrR
cls
KPN_00555
mdtH
omp36
sat4
tetM
yfbF
eptA
JW1116
mdtL
yhiU
emrA
pmrB
adeC
baeS
ECK3703
ECs3548
G157_08935
HMPREF0351_10937
msrC (E. faecium)
tetL
ugd
Donors
Single FMT
Repeated FMT

Figure S2-3C

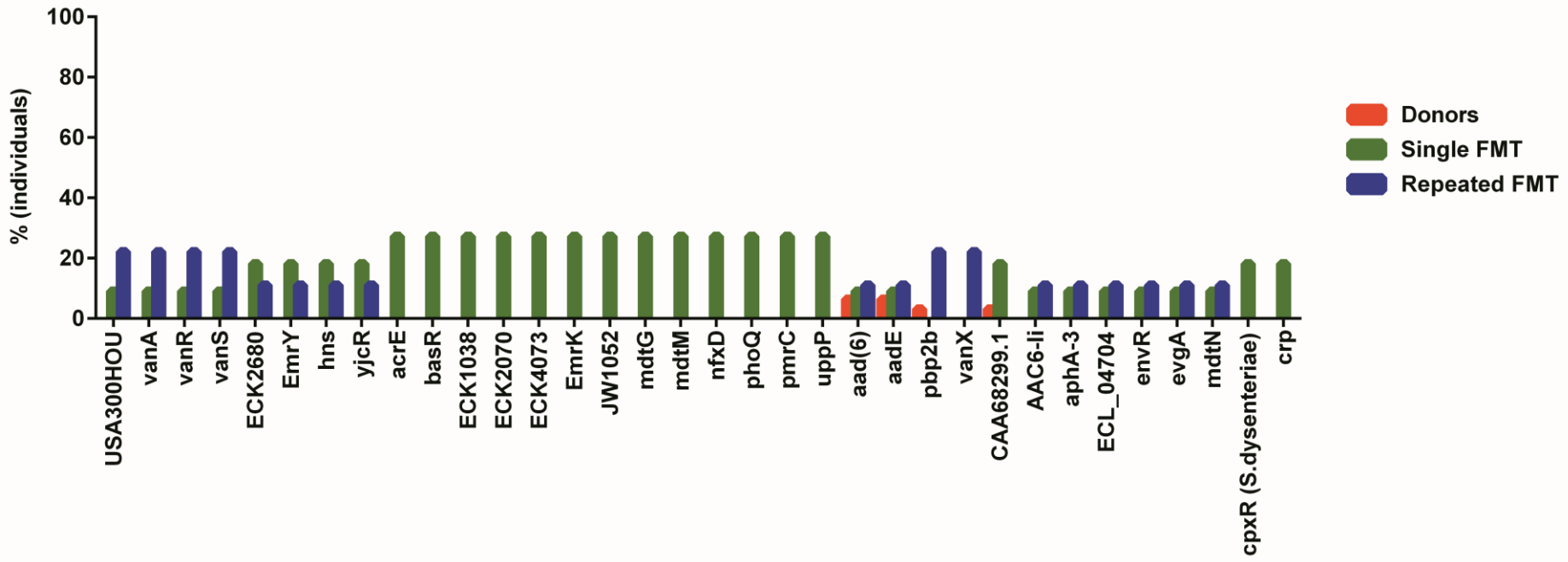


Figure S2-3. **The percentage of individuals containing the top 150 antibiotic resistance genes within each cohort.** The two experimental cohorts, repeated FMT (blue), and single FMT (green), and in donor cohort (red) where samples were taken over time.

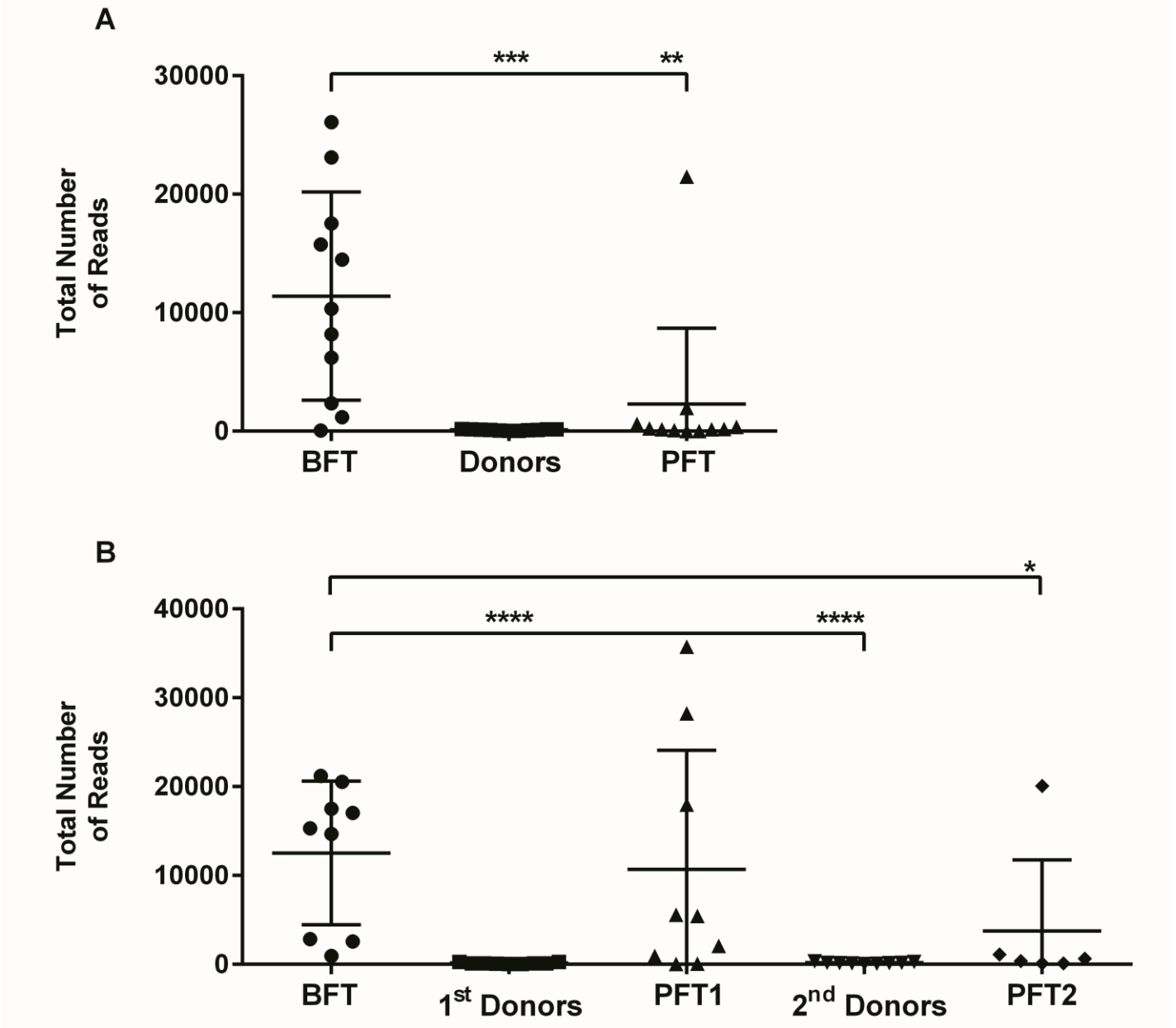


Figure S2-4. The number of raw reads detected in RCDI and Donor samples belonging to the phylum Proteobacteria. (A) Following successful FMT, (PFT: 1-4 wks) the number of reads was reduced significantly. (B). In the patients who did not respond to the initial FMT, there was no significant decrease in the number of reads (PFT1). However, following the second successful FMT, a significant decrease was seen in the number of raw reads belonging to the phylum Proteobacteria (PFT2: 1-3 wks). Donor cohort: n = 29; Single FMT Cohort: n = 11; Repeated FMT Cohort n = 9. Whiskers denote mean with the standard deviation. * *P*-value <0.05; ** *P*-value <0.005; *** *P*-value <0.001; **** *P*-value <0.0001

Chapter 3: Pre-treatment of adult wild-type mice prior to fecal microbial transplantation enhances shifts in microbial abundance but not engraftment

3.1 Introduction

Our understanding of microbial ecology, the interactions between the microscopic living organisms and the environment they occupy, is continually expanding, however, there are still many things left unknown. The number, diversity, and metabolic capacity of the gut microorganisms vary depending on the host animal and anatomical location. Recently characterized correlations between changes in the microbiota and different diseases have ignited interest in the human gut microbiota and with this, the study of the microbiota of model organisms. Experimental manipulation of the microbiota has the potential to help us understand and describe the relationship between host physiology and microbial community ecology. Current methods of altering gut microbial composition include prebiotics, probiotics, synbiotics, antibiotics, and fecal microbial transplantation (FMT). To date, evidence for a health benefit from prebiotics is weak, while probiotics only confer a short-term health benefit, which dissipates when treatment is stopped.(1) FMT has been shown to be effective for recurrent *Clostridium difficile* infection, but it remains an experiment therapeutic for other diseases.(2,3) FMTs expansion as a therapeutic for other health problems is occurring faster than we could have imagined, even going so far as to helping re-establish intestinal barrier function following burn injury.(4) This touches on the endless possibilities for FMTs in treating different indications and the requirement for further research.

The succession of the gut microbiota occurs immediately after birth when the newborns are first exposed to the microbial filled world, and empty niches are occupied by very specialized microbes, many of which are maternally acquired during the process of childbirth.(5) The mutualistic relationship observed between these bacteria and their host can only be explained by co-evolution, the long-term evolutionary adjustments of one species to another, and evidence from the study of *Lactobacillus reuteri*, indicates that species can specialize and become host adapted.(6) Bacterial members of the gastrointestinal tract are classified into two groups, autochthonous, which are long-term residents of the GI tract found in the majority of hosts and allochthonous, which are organisms which transiently occupy the gut but do not colonize it.(7) Autochthonous bacteria play an important role by filling specific niches and adhesion sites thereby preventing colonization of the gut by transient and potentially pathogenic organisms, termed colonization resistance. This occurs through competitive exclusion, where no two species can occupy the same niche indefinitely if the resource they are competing for is limited. When two species do coexist together in a community, either the resources they both depend on must not be limited, or there are one or more major differences in the niche they occupy. These ecological forces, therefore, may make it difficult to manipulate the gut microbiota without first removing the bacteria currently occupying it.

The effect of antibiotics on the gut microbiota is also well established, causing a decrease in diversity and number of bacteria, with spontaneous recovery taking up to three weeks in mice.(8-12) One adverse effect of the overuse of antibiotics has resulted in increasing incidence and mortality caused by antibiotic-associated diarrhea resulting from reduced colonization resistance.(13) However, this could also be harnessed to allow for colonization by beneficial

bacteria, enhancing the efficacy of therapeutic strategies attempting to alter the gut microbiota, such as fecal microbial transplantation (FMT). Li and colleagues were able to show that both fecal microbiota and bacterial consortia transplantation improve recovery of the microbiota following perturbation by antibiotics.(14) Bowel preparations, commonly used by clinicians before colonoscopy or gastroscopy have been shown to increase procedure efficiency, and thereby reduce the cost of colonoscopy.(15) Conversely, it has also been shown to substantially change the microbiota, with the total microbial load being reduced 31-fold in healthy subjects, (16) while in another study, only small short term changes were observed.(17) In our study we hoped to answer if reducing diversity and bacterial load using two different treatment methods could enhance the engraftment of the gut microbiota from the same species of mouse, bred and housed in a different environment.

3.2 Materials and Methods

3.2.1 Experimental design

A total of 15 male 129S1/SvlmJ Jackson Laboratory© mice, were split into three different cohorts, with an average age of 12 weeks, all of which received a fecal microbial transplant (FMT) on the fourth day of the study after stool collection (**Figure 3-1**). All experiments performed with mice follow protocols approved by the University of Alberta Research Ethics Office (Ethics approval: AUC00000293). Mice drank sterilized water and were fed autoclaved, polysaccharide-rich, low fat, chow diet *ad libitum*, with no prevention of coprophagia. Mice were only housed together if they were within the same treatment group. Mice were housed under conventional conditions with light/dark cycles of 12 hours. Mice were

sacrificed by cervical dislocation after inhalation of anesthesia (isopropanol). The three different pre-FMT treatments provided to the mice were as follows: 1) Mice were gavage with 200µL autoclaved helix water for 3 days followed by 200µL FMT (FMT-only); 2) Mice were gavage with 200µL autoclaved helix water for 2 days, followed by a gavage with PegLyte® (Rexall pharmacy Cat. No. 00512496) at a concentration of 70 g/L with 17.8 mmol/L of polyethylene glycol and given it over-night *ad libitum* and fasted followed by 200µL FMT (bp + FMT); 3) Mice were gavage with 200µL of 500 mg/L of Vancomycin Hydrochloride (Calbiochem: Cat. No. 627850) with 1.00 g/L of Metronidazole (Sigma-Aldrich: Cat. No. M1547-5G) for 3 days followed by FMT (abx + FMT). Bowel preparation and antibiotic solutions were made using filtered and autoclaved helix water. Treatment regimens were delivered via gavage using a blunt end needle into the lower esophagus of the mice. Stool collection occurred at baseline (day 0), prior to the FMT procedure (day 4; after treatment), as well as two follow-up time points, three days after the FMT (day 7), and 31 days after the FMT (day 35) (**Figure 3-1**). Upon collection, the stool was snap frozen in liquid nitrogen and placed in the -80°C freezer until analysis was performed.

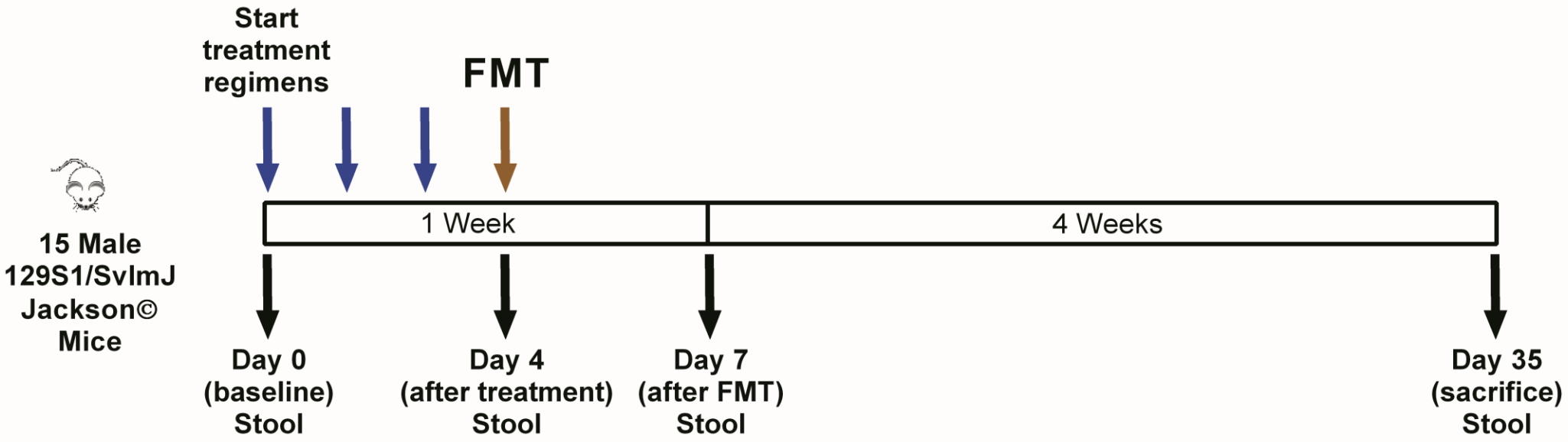


Figure 3-1. Project design and stool sample collection. Blue arrows represent treatment time points with either: **1)** 200µL autoclaved helix water for 3 days (FMT-only); **2)** autoclaved helix water for 2 days, followed by 1 day with PegLyte® (bp + FMT); or **3)** Vancomycin Hydrochloride with Metronidazole for 3 days (abx + FMT). The brown arrow represents the fecal microbial transplant (FMT) all the mice received on day four. Black arrows represent stool collection time points. The total length of the study was 5 weeks.

3.2.2 Fecal Microbial Transplantation (FMT)

Fecal microbial transplantation was performed on day four after the three-day treatment period. 15 healthy 129S1/SvlmJ wild-type mice from our colony at the University of Alberta, with an average age of 13.0 ± 0.7 weeks were used as stool pellet donors to make a stock solution of FMT slurry. FMT slurry was prepared by adding 200 mL of helix water per stool pellet, disruption using an electric mortar and pestle, filtering through a 40 μm nylon cell strainer and then adding 20% volume/volume of glycerol for storage. Before FMT, the stock solution was diluted 1:2 (400 mL of water per stool pellet and 10% glycerol). All mice received an oral gavage of 200μL of pooled stool from healthy 129S1/SvlmJ mice containing 10% glycerol.

3.2.3 DNA Extraction and Metagenomic Analysis

For genomic DNA extraction, one pellet of mouse stool (average 41.71 mg) was suspended in 300μL of AquaStool™. The suspensions were transferred to MP bio Lysing Matrix E (REF: 6914-100) tubes containing 1.4 mm ceramic spheres, 0.1 mm silica sphere, and one 4 mm glass sphere for efficient lysis of bacteria within the stool solution. Tubes were shaken in an MP Bio Fast Prep-24 Bead Beater at 6.5 m/sec for 40 seconds. DNA was extracted following the AquaStool™ designed protocol for mouse fecal DNA extraction and genotyping (MultiTarget Pharmaceuticals, LLC). The FMT stock solution was spun at 14,000 g for 15 minutes to concentrate the bacteria into a pellet and then the same extraction protocol was followed. Immediately following extraction, a simple ethanol precipitation protocol was performed; samples were quantified by Nanodrop as well as Quant-iT™ PicoGreen® double-stranded DNA assay

kit (ThermoFischer; CAT: P11496) to determine the concentration of DNA. For 16S, rRNA sequencing samples were diluted to 10ng/µL before the first PCR reaction.

A total of 2,547,393 total bi-directional reads, a minimum of 25,496, maximum of 48,985 and a median of 42,865 reads were obtained following sequencing of the PCR-amplified V4 region on an Illumina MiSeq sequencer. Processing of the raw sequencing reads in the QIIME pipeline included the normalization to 100,000 reads per sample and the removal of any reads with a Phred quality score (q-score) lower than 20 (1% error rate). Visualization of the core output results was performed also using the QIIME pipeline.(18) See Appendix 1 for the command line used. Operational taxonomic units (OTUs) were defined as having a 97% sequence similarity threshold.

3.2.4 Copy Number

Extracted DNA samples were diluted and quantified by Nanodrop as well as Quant-iT™ PicoGreen® double-stranded DNA assay kit (ThermoFischer; CAT: P11496) for a starting concentration of 10 ng/µL. A standard was made by PCR amplification of the targeted 464 base pair region of the 16S gene using a designed primer set (341mF & 805mmR) based off of the standard Illumina® primers. Approximately 500 ng of DNA was used with the following PCR parameters: 94ºC for 3 minutes followed by 35 cycles of 94ºC for 30 seconds, 57ºC for 30 seconds, 72ºC for 30 seconds, followed by 10 minutes at 72ºC, and 4ºC for ∞. Following PCR amplification, clean up was performed using QIAquick PCR Purification Kit (QIAGEN; Cat. Num. 28104), and the sample was diluted to 0.2 ng/µL, followed by ten-fold dilution to make a standard curve for the RT-

PCR to determine the copy number of each sample. 8µL of each sample at a known concentration was added to the PCR mix and with the same cycle parameters as above. The number of gene copies in the top standard was determined using a simple calculation (www.unitconversion.org), which was used to determine the gene copy number in each of the samples.

3.2.5 Statistical Analysis

Shapiro-Wilk test was used to determine if data were normally distributed, and, therefore, only non-parametric tests were used to determine statistical differences. Multiple comparisons using repeated measures analysis of variance (ANOVA) were used for all data-over-time, with the comparison being to the respective cohorts' baseline values. Using the Qiime pipeline (18), paired-end reads were merged, and sequence reads were normalized to 100,000 per sample, as well as all low quality reads (q-score < 20; 1% error rate) were removed. Partial least squares-discriminant analyses (PLS-DA) were performed on log-transformed abundance values using web-based data processing tool MetaboAnalyst 3.0 (with 95% CI).(19) In MetaboAnalyst, statistical computing and visualization operations are performed using a function from the R and Bioconductor packages.(20) PLS-DA is used to maximize class differentiation using a supervised statistical method. A 1000 permutation test was performed to validate to determine the significance of the models. A permutation test evaluates whether the specific classification of the samples in the designed groups is significantly better than any other random classification.(21) This occurs by first performing the permutation on the classes, then randomly assigning samples with the 'wrong' labels, and performing the

permutation again. The idea is that with the wrong class labels, the newly calculated classification model should not be able to predict the classes very well. The fewer samples it can properly classify, the greater the differences are between the classes being compared.(22) The similarity of microbial samples to the donor FMT slurry microbial composition was also compared by calculating the Jaccard Index and the Pearson Product-Moment Correlation. For all tests a p-value <0.05 was considered statistically significant.

3.3 Results

3.3.1 Microbial Composition

Microbial composition was analyzed using quantitative real-time PCR, and 16S rRNA sequencing was performed on the pooled donors sample, as well as at baseline (day 0), after treatment (day 4), 3 days after FMT (day 7) and 31 days after FMT (day 35) for each mouse in the three different cohorts. By pooling together the stool from 15 different 129S1/SvlmJ mice from our colony, we expected the slurry to have a more diverse composition of microbes. The alpha-diversity as measured by the Shannon-Wiener diversity index was greater in the pooled FMT (2.32) than the average baseline value from any of the three cohorts of Jackson® mice (FMT only: 2.03 ± 0.03; Bp + FMT: 1.86 ± 0.04; Abx + FMT: 1.90 ± 0.04; **Figure 3-2A**). Bowel preparation and the antibiotic treatment regimen did not have a significant effect on alpha-diversity. Following FMT, the alpha diversity of all 3 cohorts increased 3 days following the procedure, however, 31 days after the procedure, the bowel prep + FMT cohort was the

only group to have an increase from day 7 to day 35, although the antibiotics + FMT cohort maintained a significantly higher alpha-diversity than its baseline value. Alpha diversity was only significantly increased in the cohort that received a pre-FMT treatment regimen, with the antibiotics + FMT cohort having the largest increase on day 7 ($p<0.001$), and the bowel prep + FMT cohort having the most significant increase on day 35 ($p<0.0001$).

In contrast, the richness, as defined by the number of different operational taxonomic units (97% similarity) was similar in the pooled FMT sample to the bowel prep + FMT cohort (391 and 394 respectively; **Figure 3-2B**), while it was much lower at baseline in the other two cohorts. Microbial richness was significantly reduced following antibiotic treatment (p-value < 0.05), however, it was only slightly affected by the bowel preparation treatment. All three cohorts experienced an increase in richness following the FMT while a statistically significant increase was only observed on day 35 in the bowel prep + FMT cohort (p-value < 0.01).

The bacterial load, as measured by the copy number of bacterial 16S rRNA genes per gram of stool was dramatically reduced by antibiotic treatment while bowel prep appeared to have little effect (**Figure 3-2C**). Three days following FMT, the antibiotics + FMT had an increased bacterial load ($p < 0.05$) in comparison to the baseline value, whereas 31 days following FMT there was no statistically significant difference.

Figure 3-2A

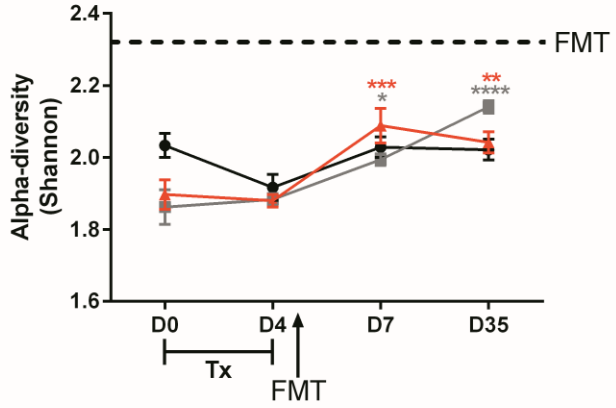


Figure 3-2B

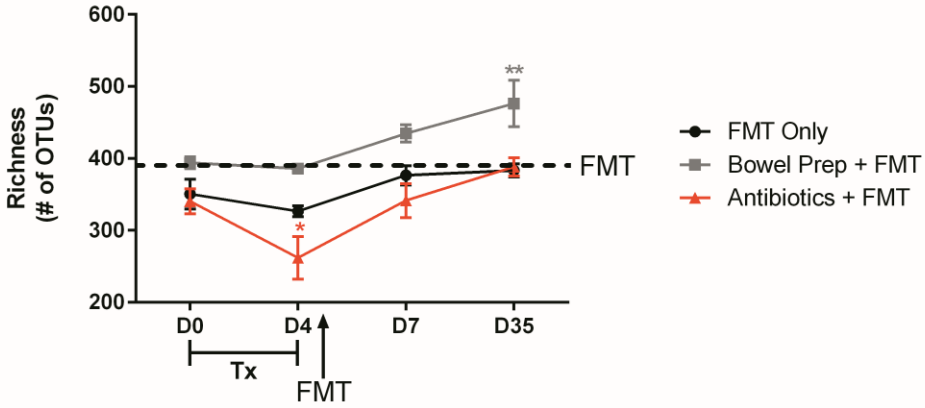


Figure 3-2C

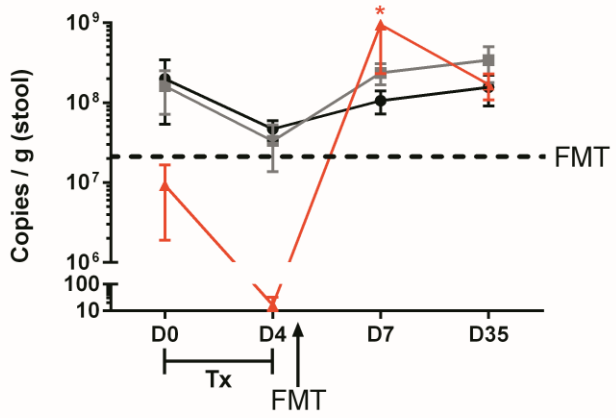


Figure 3-2. Characteristics of the gut microbiome determined through 16S sequencing and quantitative real-time polymerase chain reaction (PCR) of the 16S rRNA gene. **(A)** Alpha-diversity calculated using the Shannon-Wiener index, **(B)** bacterial richness determined by the number of different operational taxonomic units (97% similarity) and **(C)** the 16S rRNA gene copy number or bacterial count per gram of stool. Black= FMT only, Grey = Bowel Prep + FMT, and Red = Antibiotics + FMT. Dotted line represents the donor FMT slurry. (* p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001; **** p-value < 0.0001; p-values determine using two-way ANOVA/Dunnetts' multiple comparisons test)

To determine the amount of microbial engraftment, a Jaccard similarity index and a Person product-moment correlation were calculated, comparing each sample from the different time-points to the donor microbial profile at the genus level (**Figure 3-3**). Both of these similarity measures were used because the Jaccard index only focuses on the presence or absence of the different bacteria, while the Pearson product-moment correlation takes into account both the presence and the relative abundance of the bacteria. The average similarity between the donor FMT slurry and the three cohorts baseline samples using Jaccard was 44.00 ± 1.30% and Pearson was 43.81 ± 1.84%, indicating that at the start of the study the microbial profile of the mice from Jackson© compared with mice raised in our facility were substantially different, and using either indices gave us a similar same result. Jaccard index showed only a slight reduction in the similarity to the donors (43.5 ± 0.02% to 39.3 ± 0.05%), while Pearson saw a decrease of 19% (45.7 ± 2.17% to 24.68 ± 9.43%; p-value < 0.01) following the antibiotic treatment. Different results from the two calculations suggest that our antibiotic treatment was very capable of changing the relative proportion of bacteria, but was not as sufficient in eradicating bacteria completely from the gut. Bowel preparation had very little effect on the Jaccard index, while a slight increase in the Pearson product-moment correlation was observed following treatment. Following FMT, all three cohorts' microbial profile was significantly more similar to the donor FMT slurry using Jaccard, increasing significantly to an average of 69% (p-value < 0.0001). Pearson correlation also saw a significant increase in similarity to the donor in all three cohorts, but to a greater extent in the antibiotics + FMT cohort. This high level of engraftment and similarity to the donor microbial profile was maintained in the recipient, regardless of their pre-FMT treatment

regimen after four weeks using the Jaccard index, however, the significant increase in similarity was only seen in the antibiotics + FMT cohort using Pearson correlation (p-value < 0.05). These results support our original hypothesis, but we expected a more significant difference between the engraftment efficacy between the different cohorts.

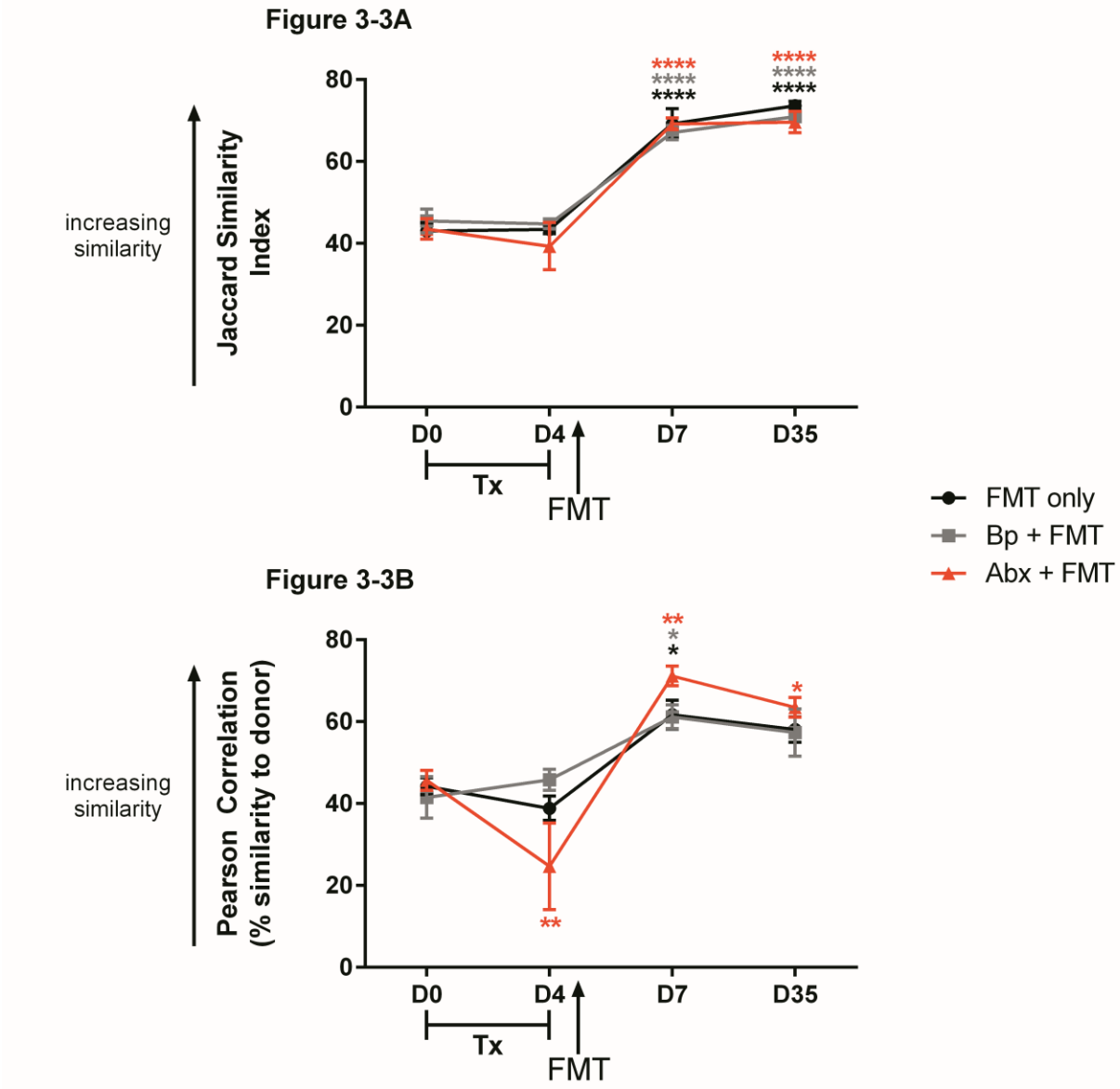


Figure 3-3. **Similarity of genera in recipient microbiota to the donor FMT slurry.** Measured using **(A)** Jaccard similarity index measured and **(B)** Pearson Product Moment Correlation. Black= FMT only, Grey = Bowel Prep + FMT, and Red = Antibiotics + FMT. (* p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001; **** p-value < 0.0001; p-values determine using two-way ANOVA/Dunnetts' multiple comparisons test)

Partial least squares Discriminant Analysis (PLS-DA) was performed on the microbial profiles to see if specific time-points and/or cohorts clustered together using MetaboAnalyst 3.0.(19) At baseline there were no significant differences in the microbial profiles of the different cohorts as expected, although inter-individual differences were observable (**Figure 3-4A**; p-value = 0.905). A significant difference was only detected following the pre-FMT treatment regimen on day 4, where the mice receiving antibiotics clustered separately from the other two cohorts (**Figure 3-4B**). On days 7 and 35, samples grouped more within their cohort, although the differences were not significant (**Figure 3-4C & 4D**). When looking at each cohort, regardless of the pre-FMT treatment regimen, the day 7 and day 35 cluster very separately from the baseline and post-treatment samples (**Figure 3-5**). The microbial profile of mice that received the antibiotic pre-FMT treatment group much closer together on days 7 and 35 than those same time-point samples in the other two cohorts. All samples clustered separately of the donor FMT slurry, with the day 7 and day 35 samples being most similar (**Figures S3-1 and S3-2**).

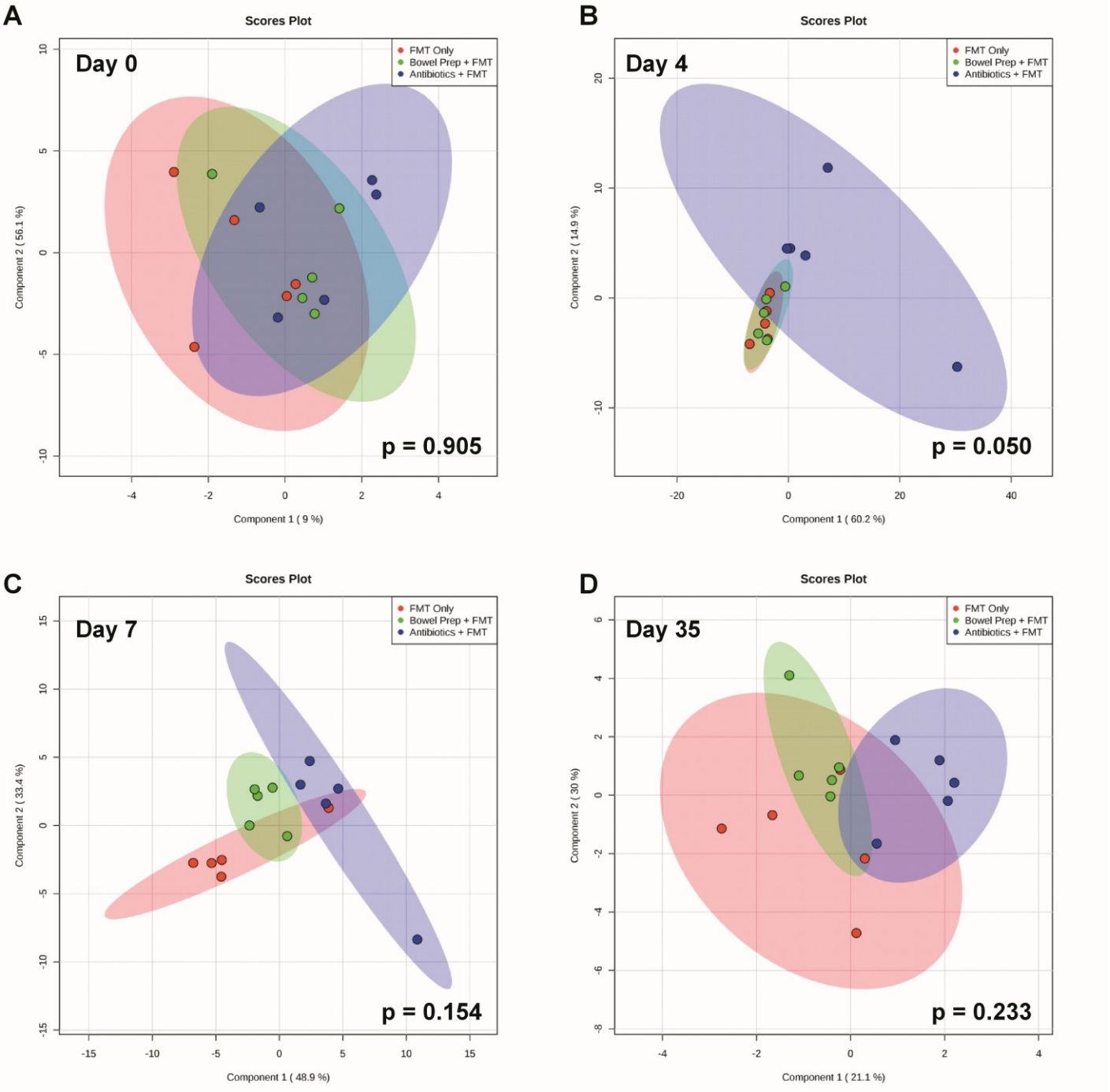


Figure 3-4. **Partial least squares Discriminant Analysis (PLS-DA) of the microbial profiles of the mice at all four time-points made using MetaboAnalyst 3.0.** Red = FMT only, Green = Bowel Prep + FMT, and Blue = Antibiotics + FMT. P-values were calculated using the provided permutation test consisting of 1000 permutations.

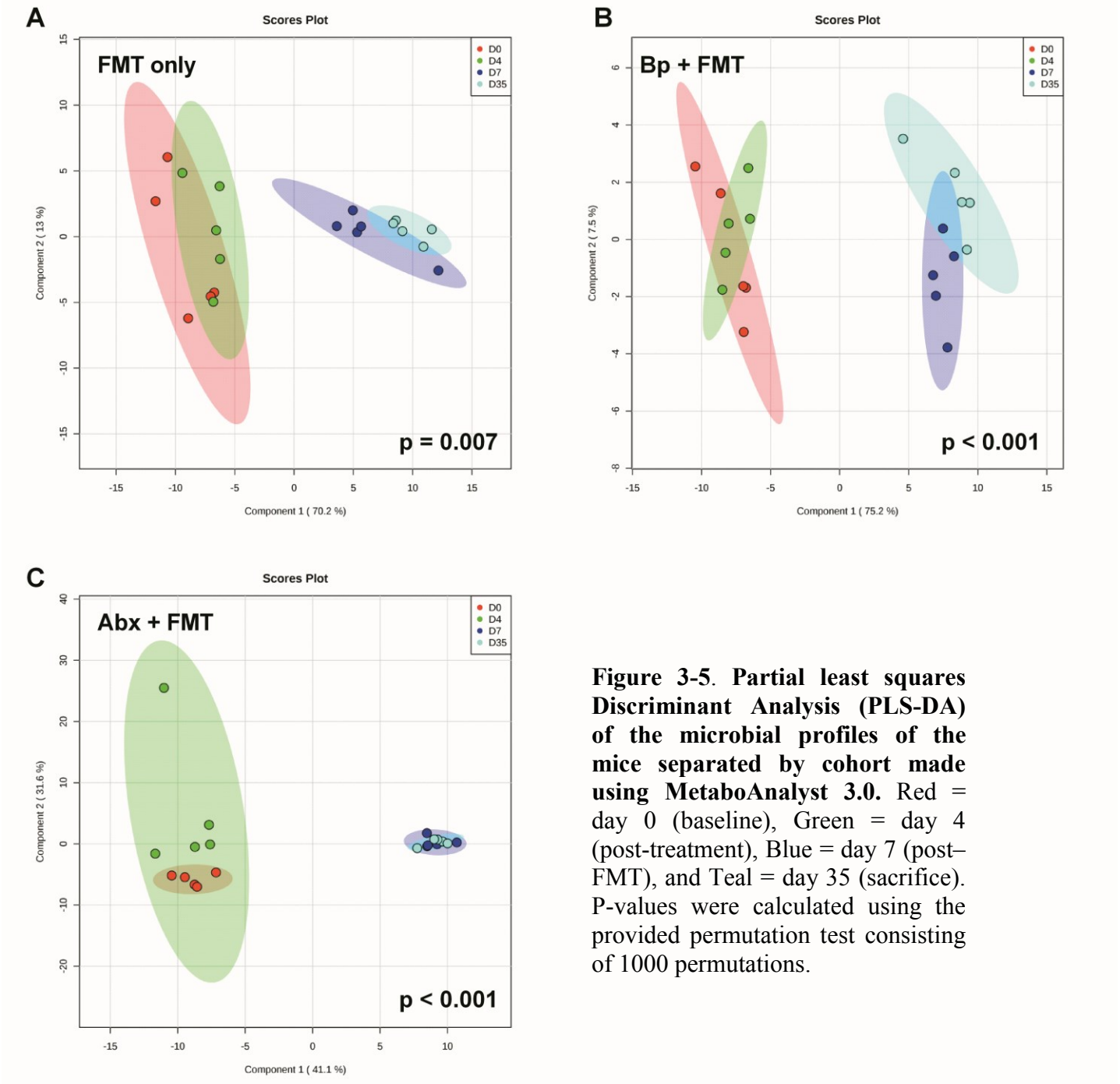


Figure 3-5. Partial least squares Discriminant Analysis (PLS-DA) of the microbial profiles of the mice separated by cohort made using MetaboAnalyst 3.0. Red = day 0 (baseline), Green = day 4 (post-treatment), Blue = day 7 (post–FMT), and Teal = day 35 (sacrifice). P-values were calculated using the provided permutation test consisting of 1000 permutations.

The detected operational taxonomic units showed that at baseline, the microbial community of the recipient mice contained 6 different phyla, dominated by Firmicutes (56.7 ± 1.61%) and Bacteroidetes (32.23 ± 1.55%) (**Appendix 2**). Significant changes in relative abundance as measured by the proportion of total reads were observed in 5 families of bacteria: *Anaeroplamataceae, Clostridiaceae, Bacteroidaceae, S24-7,* and *Lactobacillaceae* (**Figure 3-6**; **Table 3-1** for donor FMT slurry). Very minute changes were seen as a result of the bowel preparation treatment, although there was a slight increase in *S24-7*, and a corresponding decrease in *Clostridiaceae* (**Figure 3-6B**). As expected, the antibiotic treatment resulted in a substantial reduction in *S24-7* and *Clostridiaceae*, and an increase in *Anaeroplasmataceae* at day 4 (**Figure 3-6C**). Following FMT in all three cohorts, the relative abundance of each family moved in the direction of the donor FMT slurry on day 7. However, most trends appeared to return to the direction of their baseline value on day 35. Furthermore, the changes observed were very similar regardless of the pre-FMT treatment regimen. *Bacteroidaceae* were significantly higher in the antibiotics + FMT cohort ($p<0.01$) 3 days following FMT, while *Clostridiaceae* were significantly reduced in comparison to baseline ($p<0.001$; **Figure 3-6**). Significant decreases in *Clostridaceae* were also seen following FMT in the FMT only ($p<0.5$) and the bowel prep + FMT cohorts ($p<0.001$). A significant increase in *Lactobacillaceae* was only observed in the bowel prep + FMT cohort ($p<0.5$) three day following FMT.

Figure 3-6A

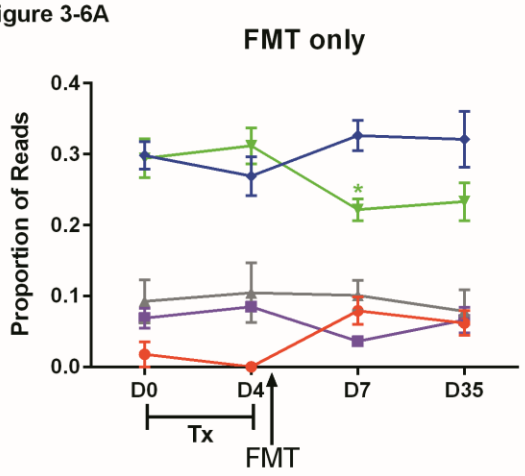


Figure 3-6B

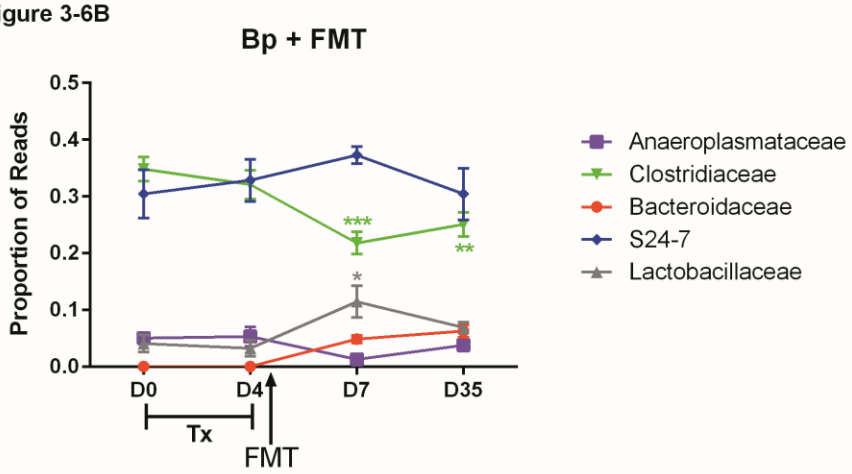


Figure 3-6C

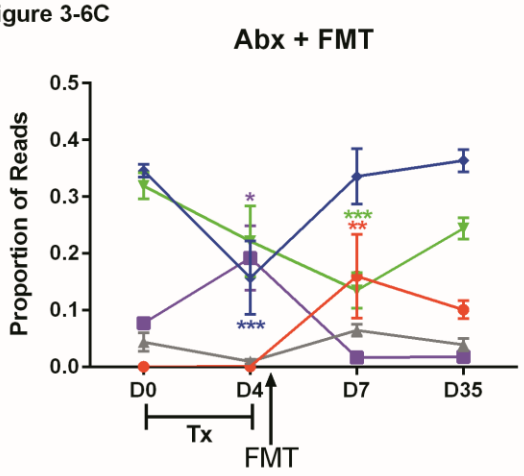


Figure 3-6. **Relative abundance (proportion of total reads) of five bacterial families with the most significant changes following fecal microbial transplantation (FMT).** The relative proportion of each family in the donor FMT slurry is listed in the table below the graphs. Purple = *Anaeroplasmataceae*, Green = *Clostridiaceae*, Red = *Bacteroidaceae*, Blue = S24-7, and Grey = *Lactobacillaceae*. (* p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001; p-values determine using two-way ANOVA/Dunnetts' multiple comparisons test)

Table 3-1. Relative abundance (proportion of total reads) in the donor fecal microbial transplantation (FMT) slurry of five bacterial families with the most significant changes in the recipient mice.

	FMT
Anaeroplasmataceae	0.0000
Clostridiaceae	0.0351
Bacteroidaceae	0.1628
S24-7	0.2221
Lactobacillaceae	0.0424

3.4 Discussion

As expected, mice housed separately (Jackson© versus University of Alberta) regardless of being the same species (129S1/SvlmJ) have different gut microbial composition and cluster very separately via PLS-DA ($p < 0.0001$). This difference in microbiota between mice from the same strain is critical information when designing and performing gut microbial and ecological studies. The only bacterial family that was not present in the donor FMT slurry but was in the Jackson© mice was a family from the Mollicute lineage, the *Anaeroplamataceae* (**Figure 3-6**). These bacteria have been found to have increased abundance following a high-fat diet in animal models, and may suggest that Jackson© mice have a slight gut microbiota dysbiosis.(23, 24)

Pre-FMT treatment with bowel preparation (PEG 3350) only had a slight effect on the gut microbiota, where there was a small reduction in 16s rRNA gene copy number (bacterial load) following treatment, no change in the richness, but a slight increase in alpha-diversity. These results, and the lack of change in the Jaccard Index, and an increase is the Pearson correlation after treatment, suggest that bowel preparation has a larger effect on the more abundant microbes, but does not have the ability in this setting to completely irradicate certain species from the microbial community. However, it is also possible that this change could have been caused by the 24 hours fasting that these mice underwent in addition to the treatment. Currently available studies investigating the effect of bowel preparation on the microbiota suggest that there can be significant or transient effect following bowel preparation treatment.(16, 17) Nevertheless, the effect

of bowel preparation on microbial composition was significant enough to result in the largest increase in alpha-diversity four weeks following FMT.

Vancomycin is an antibiotic that targets Gram-positive bacteria and is poorly absorbed in the gut. Metronidazole is an antibiotic effective against anaerobic bacteria and certain parasites. In **Figure 3-6**, we can see that the relative abundance of the gram-positive *Lactobacillaceae* and *Clostridiaceae* is reduced following antibiotic therapy, which allows for the expansion of the *Anaeroplasmataceae* into a previously occupied niche. This is congruent with the ecological concepts of the completive exclusion whereby in our mice, the niche is likely occupied by a different bacterium which can successful take it over from *Anaeroplasmataceae* following FMT and exclude it from re-establishing itself in the community. The fact that *Anaeroplasmataceae* are not entirely removed for the long term, but persist at lower levels suggests that there may be a sharing of the niche between it and the new occupying organism, reducing its fundamental niche, and accepting a smaller, realized niche.

Regarding engraftment efficacy, all three cohorts experienced statically significant engraftment ($p < 0.0001$) as measured by the Jaccard index and Pearson correlation (**Figure 3-3**), although, only a few changes in the microbiota were noticeable at the family level (**Figure 3-6**). Engraftment efficacy was more significant using Pearson correlation in the antibiotics + FMT cohort, causing it to cluster most closely together (**Figure 3-5**), suggesting that the antibiotic treatment may result in a more stable and specific microbiota following FMT. This may be necessary for certain disease states

to enhance the longevity of fecal microbial transplantation therapy. Reduced diversity has now been associated with a multitude of diseases such as necrotizing enterocolitis (25), increased risk of allergies (26), Crohn's disease (27) while increased diversity and richness are correlated with overall health in an elderly population.(28) If reduced diversity is part of the pathophysiology of these diseases, and not a result of illness, pre-FMT treatment with bowel preparation may be the best approach to enhance the microbial diversity for a longer period with an FMT. However, this requires much more research, as these were healthy wild-type mice with no underlying genetic predisposition to disease. We observed that the relative proportion of each family following FMT (day7) followed a trend towards the relative proportion of that family in the donor FMT. Over time, however, the relative proportion at day 35 suggests that the change was transient and that the microbial profiles are gradually returning to their baseline composition. These results suggest that bacteria from a different host of the same species are still similar enough (or even the same) that they can colonize the gut of the recipient and alter the relative proportion of those microbes. This effect appears to be transient, and it seems that host-determined factors start to return the microbiota back to the baseline. The work by Manichanh and colleagues suggested that antibiotic treatment pre-FMT did not result in enhanced engraftment in their rats.(29) In contrast, our results correlate with ecological principles showing that antibiotic treatment pre-FMT can perturb the gut microbiota enough to allow for enhanced engraftment of the donor microbiota. The differing results may be due to our method of determining donor engraftment, where Pearson correlation not only considers the introduction or removal of bacteria but also their relative proportion within the community. Therefore, with FMT,

we can efficiently alter the balance of the microbial community. However, the introduction or removal of new genera using FMT occurs at the same rate regardless of treatment before FMT with antibiotics or bowel preparation.

There are some limitations with this study that should be addressed. First, cohorts of mice receiving just bowel preparation, or just antibiotics without FMT should have been included to determine if the changes in the microbial profile were the result of the FMT or long-term effects of the pre-FMT treatment regimen. Long-term effects of antibiotics on the gut microbiota in humans have been previously shown (30), and perhaps without an FMT, a microbial profile more similar to the donors could have occurred. FMT is most often used therapeutically for RCDI, with more interest being shown for other diseases, and these patients often have other comorbidities or altered immune responses that would change the hosts' reaction to an influx of microbes, which may alter engraftment efficacy. Culture-independent techniques such as 16S rRNA sequencing provide us with a plethora of information about the microbial diversity within the gut of any organism. The genes for 16S rRNA (16S rDNA) have both conserved regions and variable regions, which allow for the identification of individual species through amplification of the gene using universal primers. The limitations of next-generation sequencing include the detection of both resident and transient organisms, as well as both dead and alive organisms, which may be metabolically active or inactive, giving us just a snapshot of the dynamic gut microbiota. Conclusions made from 16S rRNA sequencing are on the basis of changes in the relative proportion of the reads, but we are not able to determine if these changes result in any functional changes of the

microbiota, in that some bacteria are more metabolically active than others. Furthermore, uncharacterized bacteria are not in the database we used, and therefore would not be detected in our samples.

3.5 References

1. Guarner F, Malagelada J. Gut flora in health and disease. The Lancet. 2003;361(9356):512-9.

2. Colman RJ, Rubin DT. Fecal microbiota transplantation as therapy for inflammatory bowel disease: a systematic review and meta-analysis. J Crohns Colitis. 2014 Dec 1;8(12):1569-81.

3. Li YT, Cai HF, Wang ZH, Xu J, Fang JY. Systematic review with meta-analysis: long-term outcomes of faecal microbiota transplantation for Clostridium difficile infection. Aliment Pharmacol Ther. 2016 Feb;43(4):445-57.

4. Kuethe JW, Armocida SM, Midura EF, Rice TC, Hildeman DA, Healy DP, et al. Fecal Microbiota Transplant Restores Mucosal Integrity in a Murine Model of Burn Injury. Shock. 2015 Dec 14.

5. Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell. 2006 Feb 24;124(4):837-48.

6. Oh PL, Benson AK, Peterson DA, Patil PB, Moriyama EN, Roos S, et al. Diversification of the gut symbiont Lactobacillus reuteri as a result of host-driven evolution. ISME J. 2010 Mar;4(3):377-87.

7. Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell. 2006 Feb 24;124(4):837-48.

8. Dethlefsen L, Huse S, Sogin ML, Relman DA. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS biol. 2008;6(11):e280.

9. Dethlefsen L, Relman DA. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. Proc Natl Acad Sci U S A. 2011 Mar 15;108 Suppl 1:4554-61.

10. Wlodarska M, Willing B, Keeney KM, Menendez A, Bergstrom KS, Gill N, et al. Antibiotic treatment alters the colonic mucus layer and predisposes the host to exacerbated Citrobacter rodentium-induced colitis. Infect Immun. 2011 Apr;79(4):1536-45.

11. Grasa L, Abecia L, Forcén R, Castro M, de Jalón, José Antonio García, Latorre E, et al. Antibiotic-induced depletion of murine microbiota induces mild inflammation and changes in toll-like receptor patterns and intestinal motility. Microb Ecol. 2015;70(3):835-48.

12. Lawley TD, Clare S, Walker AW, Goulding D, Stabler RA, Croucher N, et al. Antibiotic treatment of clostridium difficile carrier mice triggers a supershedder state, spore-mediated transmission, and severe disease in immunocompromised hosts. Infect Immun. 2009 Sep;77(9):3661-9.

13. Redelings MD, Sorvillo F, Mascola L. Increase in Clostridium difficile-related mortality rates, United States, 1999-2004. Emerg Infect Dis. 2007 Sep;13(9):1417-9.

14. Li M, Liang P, Li Z, Wang Y, Zhang G, Gao H, et al. Fecal microbiota transplantation and bacterial consortium transplantation have comparable effects on the re-establishment of mucosal barrier function in mice with intestinal dysbiosis. Frontiers in microbiology. 2015;6.

15. Rex DK, Imperiale TF, Latinovich DR, Bratcher LL. Impact of bowel preparation on efficiency and cost of colonoscopy. Am J Gastroenterol. 2002;97(7):1696-700.

16. Jalanka J, Salonen A, Salojarvi J, Ritari J, Immonen O, Marciani L, et al. Effects of bowel cleansing on the intestinal microbiota. Gut. 2014 Dec 19.

17. O'Brien CL, Allison GE, Grimpen F, Pavli P. Impact of colonoscopy bowel preparation on intestinal microbiota. PLoS One. 2013 May 1;8(5):e62815.

18. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010 May;7(5):335-6.

19. Xia J, Sinelnikov IV, Han B, Wishart DS. MetaboAnalyst 3.0--making metabolomics more meaningful. Nucleic Acids Res. 2015 Jul 1;43(W1):W251-7.

20. Xia J, Psychogios N, Young N, Wishart DS. MetaboAnalyst: a web server for metabolomic data analysis and interpretation. Nucleic Acids Res. 2009 Jul;37(Web Server issue):W652-60.

21. Golland P, Liang F, Mukherjee S, Panchenko D. Permutation tests for classification. In: Learning Theory. Springer; 2005. p. 501-15.

22. Westerhuis JA, Hoefsloot HC, Smit S, Vis DJ, Smilde AK, van Velzen EJ, et al. Assessment of PLSDA cross validation. Metabolomics. 2008;4(1):81-9.

23. Hildebrandt MA, Hoffmann C, Sherrill–Mix SA, Keilbaugh SA, Hamady M, Chen Y, et al. High-fat diet determines the composition of the murine gut microbiome independently of obesity. Gastroenterology. 2009;137(5):1716,1724. e2.

24. Turnbaugh PJ, Bäckhed F, Fulton L, Gordon JI. Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. Cell host & microbe. 2008;3(4):213-23.

25. Wang Y, Hoenig JD, Malin KJ, Qamar S, Petrof EO, Sun J, et al. 16S rRNA gene-based analysis of fecal microbiota from preterm infants with and without necrotizing enterocolitis. The ISME journal. 2009;3(8):944-54.

26. Bisgaard H, Li N, Bonnelykke K, Chawes BLK, Skov T, Paludan-Müller G, et al. Reduced diversity of the intestinal microbiota during infancy is associated with increased risk of allergic disease at school age. J Allergy Clin Immunol. 2011;128(3):646,652. e5.

27. Manichanh C, Rigottier-Gois L, Bonnaud E, Gloux K, Pelletier E, Frangeul L, et al. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. Gut. 2006 Feb;55(2):205-11.

28. Claesson MJ, Jeffery IB, Conde S, Power SE, O'Connor EM, Cusack S, et al. Gut microbiota composition correlates with diet and health in the elderly. Nature. 2012;488(7410):178-84.

29. Manichanh C, Reeder J, Gibert P, Varela E, Llopis M, Antolin M, et al. Reshaping the gut microbiome with bacterial transplantation and antibiotic intake. Genome Res. 2010 Oct;20(10):1411-9.

30. Jernberg C, Lofmark S, Edlund C, Jansson JK. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. ISME J. 2007 May;1(1):56-66.

3.6 Supplementary Material

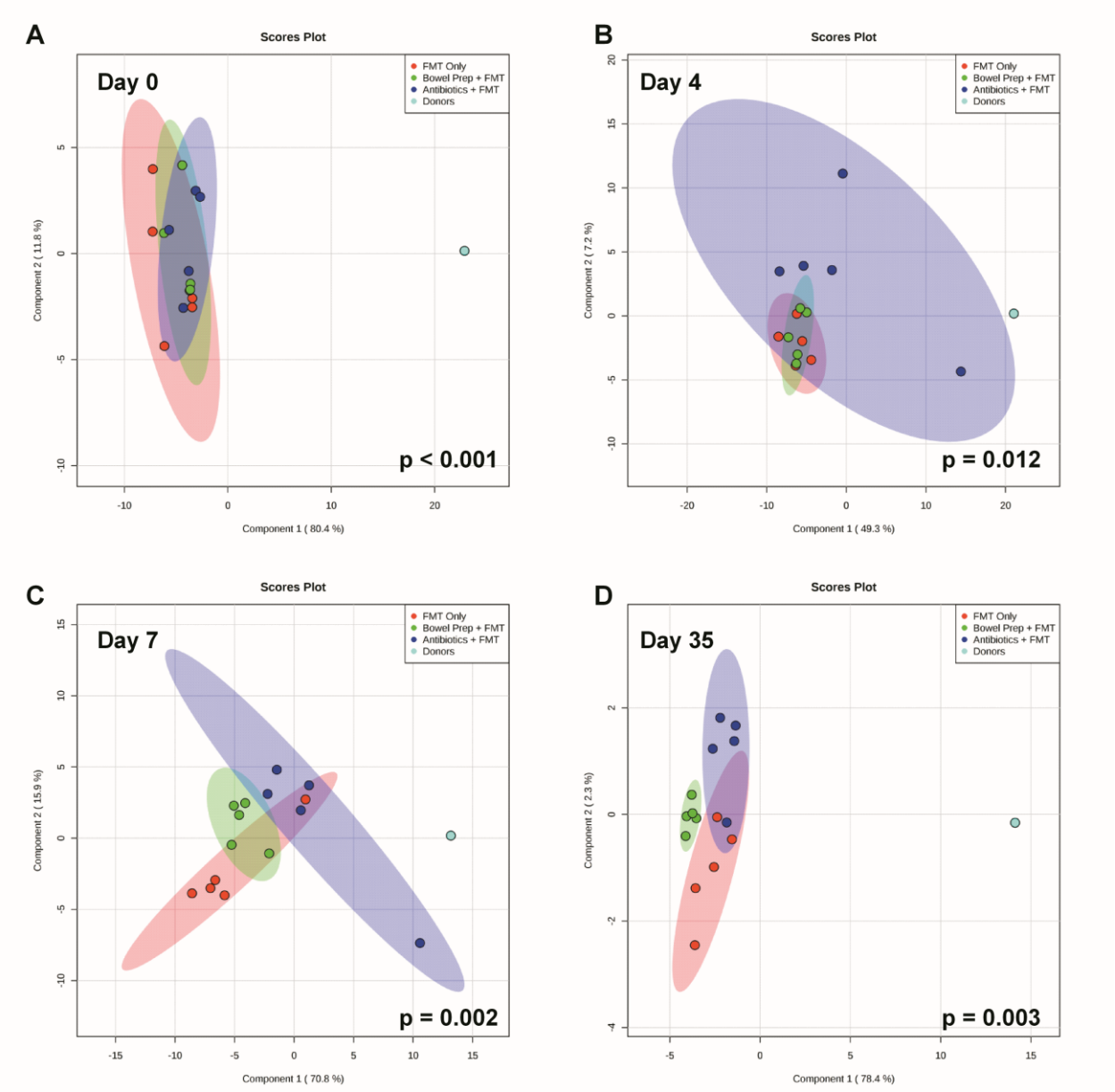


Figure S3-1. **Partial least squares Discriminant Analysis (PLS-DA) of the microbial profiles of the mice at all four time-points with the donors made using MetaboAnalyst 3.0.** Red = FMT only, Green = Bowel Prep + FMT, Blue = Antibiotics + FMT and Teal = Donors. P-values were calculated using the provided permutation test consisting of 1000 permutations.

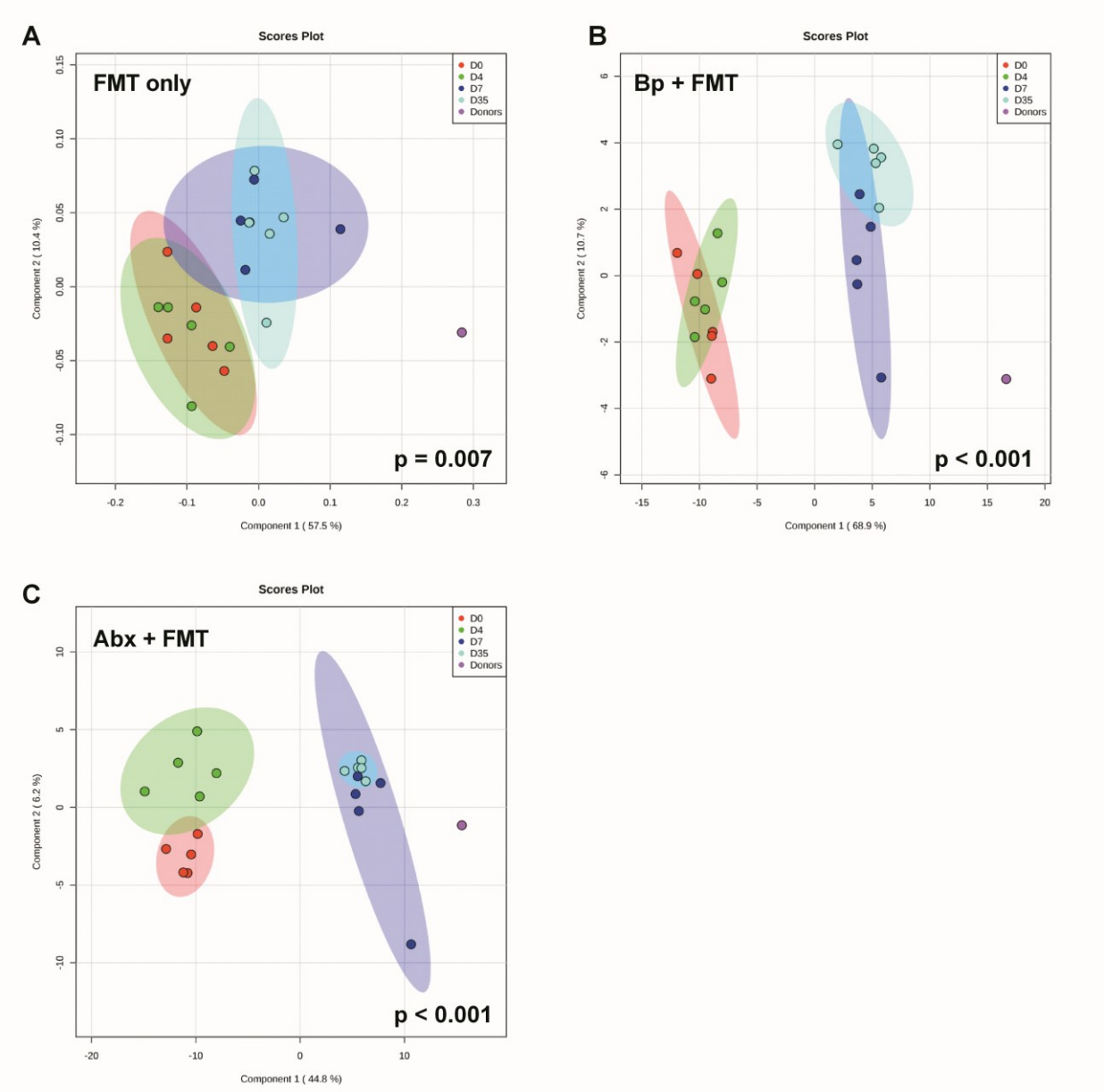


Figure S3-2. **Partial least squares Discriminant Analysis (PLS-DA) of the microbial profiles of the mice separated by cohort with the donors using MetaboAnalyst 3.0.** Red = day 0 (baseline), Green = day 4 (post-treatment), Blue = day 7 (post–FMT), Teal = day 35 (sacrifice), and Purple = Donors. P-values were calculated using the provided permutation test consisting of 1000 permutations.

Chapter 4: Fecal microbial transplantation without pre-treatment regimen is the most effective in reshaping the gut microbiota and reducing colitis in male IL-10 -/- mice

4.1 Introduction

Inflammatory bowel disease (IBD) has two major phenotypes, ulcerative colitis (UC) causing continuous surface inflammation in the colon and rectum, and Crohn's disease (CD), causing transmural inflammation from the mouth to the anus that can result in scarring and stricturing of the gastrointestinal tract.(1, 2) The precise pathogenesis of IBD has yet to be determined, but there is a known association between genetics, the environment, the host immune system and the microbiota. Based on recent interest in fecal microbial transplantation (FMT) as a treatment for IBD, many questions remain unanswered surrounding the approach that would be most efficacious for treating IBD patients. There is little information in the literature specific to the use of FMT as a treatment for IBD. Therefore, there is no standardized approach, and many different pre-procedure treatments have been described including standard colonoscopy preparation with or without antibiotic treatment.(3) Despite this, Coleman and colleagues report a combined clinical remission rate of 45.4% for patients receiving an FMT for IBD, similar to the efficacy of other IBD therapy such as methotrexate (4), anti-TNF antibodies (infliximab) (5), and mesalazine (5-aminosalicyclic acid).(6) If a more standardized approach that took into account ecological and physiological principles could be established, this may result in a more efficacious therapy for patients with IBD.

Antibiotics have a dramatic effect on the intestinal microbiota, with different classes of antibiotics having different specificities and preferred location of action.(7) Antibiotics can result in a substantial decrease in commensal gut microbes; this, in turn, changes community ecology and can cause antibiotic-associated diarrhea.(8) Vancomycin is a glycopeptide antibiotic which ultimately lowers the mechanical strength of the extracellular peptidoglycan layer in gram-positive bacteria rendering it susceptible to lysis as osmotic pressure changes occur.(9) Vancomycin is typically given intravenously, because when taken orally, it is not absorbed in the gastrointestinal tract, although this feature makes it efficacious against gastrointestinal infections. In contrast, metronidazole targets anaerobic bacteria that are both gram-positive and gram-negative. It is also available as intravenous and oral dosage forms, which rapidly kills bacteria and has good tissue penetration.(10) Vancomycin and metronidazole are the primary choice of antibiotics for initial and recurrent episodes of *Clostridium difficile* infection (CDI).(11) Bowel preparations are commonly used before colonoscopy or gastroscopy as they have been shown to increase procedure efficiency and thereby reduce the cost of colonoscopy.(12) However, bowel preps have also been shown to substantially change the microbiota, with the total microbial load being reduced in healthy subjects.(13, 14) Research in both mice and humans has shown that oral vancomycin reduces both total numbers and diversity of the gut microbiota.(15-17) Thus, pre-treatment with antibiotic and bowel prep prior to FMT may help to reduce colonization resistance thus increasing the ability of the donor microbes to survive and engraft.

Interleukin-10 (IL-10) was first shown to inhibit cytokine production by Th1 cells, resulting in its description by Fiorentino and colleagues as a cytokine synthesis inhibitory factor

(CSIF).(18) IL-10 knockout (IL-10 -/-) mice, a long-standing model of colitis, were initially generated by gene targeting, and have normal lymphocyte development and antibody responses, although most animals are growth retarded, anemic, and suffer from chronic colitis.(19) Alterations in the intestine include extensive mucosal hyperplasia, inflammatory reactions, and aberrant expression of major histocompatibility complex class II (MHC-II) molecule on the epithelia (19), as well as defects in primary intestinal permeability.(20) In a healthy animal, immune cells are tolerant to the commensal microbes. This tolerance is maintained in part through the action of IL-10, which suppresses the secretion of pro-inflammatory cytokines such as IFN-γ, IL-1β, and IL-12.(21) In the IL-10 -/- mouse, enteric gut bacteria are required for the development of the colitis.(22) IL-10 -/- mice maintained in germ-free conditions do not develop gastrointestinal inflammation, while in specific pathogen-free conditions, they develop only a mild colitis.(23) Furthermore, antibiotic therapy has been previously shown to reduce the severity of colitis in IL-10 -/- mice.(24) Studies using the probiotic bacterial strain *Lactobacillus* have shown prevention of colitis in the IL-10 -/- model.(25) These data support the concept of altering the gut microbiota as a treatment for either preventing or treating colitis. Although there are obvious limitations with mouse models and translating findings to humans, this study was designed to enhance our knowledge surrounding the potential use of FMT for the treatment of IBD in humans.

4.2 Materials and Methods

4.2.1 Experimental Design

Experiments that were performed followed protocols approved by the University of Alberta Research Ethics Office (Ethics approval: AUC00000293). Twenty male 129S1/SvlmJ IL-10 -/- mice (70 ± 0.9 days old) from our conventional housing colony with 12 hour light/dark cycles at the University of Alberta were separated into the four different cohorts (**Figure 4-1**). The mice drank sterilized water and fed autoclaved, polysaccharide-rich, chow diet *ad libitum*, with no prevention of coprophagia. Mice were housed together if they were within the same treatment group. Due to their territorial behavior, male mice can only be housed together if they are introduced very early in life. The mice were nine weeks of age at the time of study design, therefore, when non-littermates were housed together, fighting ensued. Small sample size, the stress caused by fighting, and the cost of individually housing the mice prevented me from properly separating littermates into the different treatment cohorts. Mice were weighed on days 0, 1, 2, 3, 4, 10, 17, and 35 and then sacrificed by cervical dislocation after inhalation of anesthesia (isoflurane). The four different treatments provided to the mice are as outlined:

1) Control (FMT only): autoclaved helix water followed by FMT (FMT-only);
2) Bowel prep followed by FMT: PegLyte® (Rexall pharmacy Cat. No. 00512496) at a concentration of 70 g/L with 17.8 mmol/L of polyethylene glycol (Bp + FMT);
3) Antibiotics followed by FMT: 0.50g/L of vancomycin hydrochloride (Calbiochem: Cat. No. 627850) with 1.00 g/L of metronidazole (Sigma-Aldrich: Cat. No. M1547-5G; Abx + FMT);

4) Antibiotics plus bowel prep followed by FMT: pre-FMT treatment consisting of both the antibiotic regimen as well as the bowel preparation (bp, abx + FMT).

Solutions were made using filtered and autoclaved helix water. Antibiotic treatment regimen consisted of gavaging 200µL of antibiotic solution using a blunt end needle into the stomach of mice for three consecutive days (~7.3 mg/kg/day of metronidazole & ~3.6 mg/kg/day of vancomycin hydrochloride). Mice in the PegLyte® cohort were gavaged 200µL of autoclaved helix water for the first two treatment days, and 200µL PegLyte® on the third treatment day. They also had their drinking water replaced with the PegLyte® solution and fasted overnight until they received their FMT. The FMT-only group received oral gavages of 200µL autoclaved helix water daily to reduce the stress effect of the gavage procedure on the outcomes. All four cohorts received a 200µL FMT on the fourth day of the study after stool collection. Stool pellets were collected from IL-10 -/- mice for analysis at baseline (day 0), after the treatment period (day 4), as well as two follow-up time points, three days after the FMT (day 7), and four weeks after the FMT (day 35) (**Figure 4-1**). Upon collection, the stool was snap frozen in liquid nitrogen and stored in the -80°C freezer until analysis was performed.

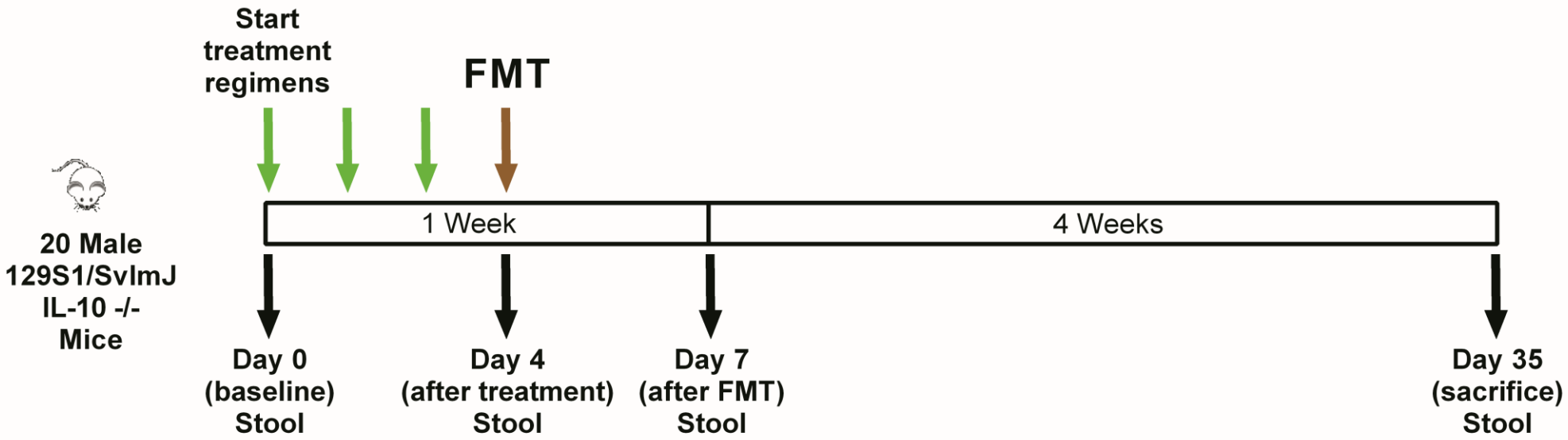


Figure 4-1. Project design and stool sample collection. Green arrows represent treatment time points with either: **1)** Control: 200µL autoclaved helix water for 3 days (FMT-only); **2)** Bowel prep followed by FMT: 200µL autoclaved helix water for 2 days, followed by 200µL PegLyte® for 1 day and PegLyte® over-night (bp + FMT); **3)** Antibiotics followed by FMT: 200µL vancomycin and metronidazole for 3 days (abx + FMT); or **4)** Antibiotics plus bowel prep followed by FMT: 200µL vancomycin and metronidazole for 3 days with PegLyte® over-night (bp, abx + FMT) . The brown arrow represents the fecal microbial transplant (FMT) all the mice received on day four. Black arrows represent stool collection time points. The total length of the study was five weeks.

4.2.2 Fecal Microbial Transplantation (FMT)

Fecal microbial transplantation was performed on day four, after the three-day pre-treatment period. Fifteen healthy 129S1/SvlmJ wild-type mice with an average age of 13.0 ± 0.7 weeks were used as stool pellet donors to make a stock solution of FMT slurry. Stool collected from the mice was prepared into a stock solution by adding 200 mL of helix water per stool pellet and disrupted using an electric mortar and pestle followed by filtering through a 40 μm nylon cell strainer and then adding 20% volume/volume of glycerol for storage. By pooling the donor stool, we aimed to create a consistent transplant with a different diversity and greater richness than the endogenous microbiota of the recipient mice. Before FMT, the stock solution was diluted 1:2 (400 mL of water per stool pellet) for gavage. All mice received an oral gavage of 200μL of pooled stool from healthy 129S1/SvlmJ mice containing 10% glycerol.

4.2.3 DNA Extraction and Metagenomic Analysis

For genomic DNA extraction, one pellet of mouse stool (average 41 mg) was suspended in 300μL of AquaStool™ in an MP bio Lysing Matrix E (REF: 6914-100) tube containing 1.4 mm ceramic spheres, 0.1 mm silica sphere, and one 4 mm glass sphere for efficient lysis of bacteria within the stool solution. The FMT stock solution was spun at 14,000 g for 15 minutes to pellet the bacteria before extraction, and done in duplicate. Tubes were shaken in an MP Bio Fast Prep-24 Bead Beater at 6.5 m/sec for 40 seconds. DNA was the extracted following the AquaStool™ designed protocol for mouse fecal DNA extraction and genotyping (MultiTarget Pharmaceuticals, LLC), followed by an ethanol precipitation. Sample concentration was first quantified by

Nanodrop followed by Quant-iT™ PicoGreen® double-stranded DNA assay kit (ThermoFischer; CAT: P11496).

A total of 3,093,075 total bi-directional reads, a minimum of 22,940, maximum of 58,747 and a median of 38,186 reads were obtained following sequencing of the PCR-amplified V4 region on an Illumina MiSeq sequencer. Processing of the raw sequencing reads in the QIIME pipeline included the normalization to 100,000 reads per sample and the removal of any reads with a q-score lower than 20 (1% error rate). Visualization of the core output results was performed also using the QIIME pipeline.(26) See **Appendix 1** for the command lines used. Operational taxonomic units (OTUs) were defined as having a 97% sequence similarity threshold. With the taxonomic information, predictive functional profiling of communities by reconstruction of unobserved states (PICRUSt) analysis was performed to predict the functional capacity of the microbiota and visualized using linear discriminant analysis (LDA) effect size (LEfSe). (27, 28)

4.2.4 Copy Number

Extracted DNA samples were diluted and quantified by Nanodrop as well as Quant-iT™ PicoGreen® double-stranded DNA assay kit (ThermoFischer; CAT: P11496) for a starting concentration of 10 ng/µL. A standard was made by PCR amplification of the targeted 464 base pair region of the 16S gene using a designed primer set (341mF & 805mmR). Approximately 500 ng of DNA was used with the following PCR parameters: 94ºC for 3 minutes followed by 35 cycles of 94ºC for 30 seconds, 57ºC for 30 seconds, 72ºC for 30 seconds, followed by 10 minutes at 72ºC, and 4ºC for ∞. Following PCR

amplification, clean up was performed using QIAquick PCR Purification Kit (QIAGEN; Cat. Num. 28104), and the sample was diluted to 0.2 ng/µL, followed by ten-fold dilution to make a standard curve for the RT-PCR to determine the copy number of each sample. 8µL of each sample at a known concentration was added to the PCR mix following the same cycle parameters as above. The number of gene copies in the top standard was determined using a simple calculation (www.unitconversion.org), which was then used to determine the copy number of each sample.

4.2.5 Stool Lipocalin-2

Stool samples were reconstituted in 100 mg/mL of phosphate buffered saline (PBS) containing 0.05% Tween 20, and vortexed for 20 minutes to get a homogenous fecal suspension. The fecal suspension was then centrifuged for 10 minutes at 12,000 rotations per minute (rpm) and 4ºC, collecting and diluting the clear supernatant 1:10 (v/v) before immunoassay. Lipocalin (LCN-2) was measured using DuoSet® mouse LCN-2/NGAL ELISA Development kit (R&D Systems, Minneapolis, MN: Catalogue Number DY1857). Manufacturers' protocol was followed using half the suggested volume for each reagent and sample.(29)

4.2.6 Tissue Cytokines

Ileum, cecum, and colon were excised and flushed well with ice-cold 1X PBS and gentamicin (50µ/mL). A piece 1.5-2.0 cm long for the ileum, cecum, and colon were cut into small pieces and placed in a two mL centrifuge tube for protein extraction and analysis. Tubes were pre-weighed such that wet tissue and dry tissue weight were

available for normalization. To prepare samples for the Meso Scale Discovery ©, tissue was suspended in five times the tissue weight of 1X PBS, 0.05% Tween-20, 10% Bovine Serum Albumin (BSA) and 1µL/mL of protease inhibitor (Sigma P-P8340). Samples were sonicated for 20 seconds in two 10 second intervals at intensity level 3 (Fisher Scientific© Sonic Dismembrator Model 100). All samples were then centrifuged at 10,000g for 10 minutes and 4ºC to pellet the cell debris, collecting the supernatant. Serum, cecum, and small intestine samples were loaded onto a pro-inflammatory Panel 1 (mouse) Kit (MSD®: Catalogue Number V-PLEX™ K15048D) to assess the concentration of IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL12p70, and TNF-α. Values are represented in units of a microgram of cytokine protein to a milligram of dry tissue weight (µg/mg).

4.2.7 Histology

Colon, small intestine, and cecum were taken from the mice at sacrifice and washed with ice-cold 1X PBS and gentamycin (50µL/mL). Once void of fecal matter, the length of the colon and small intestine were measured, and tissue samples were dabbed dry before weighing. At sacrifice, 1.5-2.0 centimeter sections of the colon, ileum, and cecum from each mouse were fixed in 10% formalin. The samples were processed in a ©Leica TP1020 (Leica Microsystems, Ballerup, Denmark) histo-processor overnight, embedded in paraffin blocks using a Histostar™ Histocenter (Thermo Fischer Scientific, Waltham, Massachusetts). They were then sectioned at a thickness of 3 µM in a microtome followed by tissue staining with Hematoxylin and Eosin (H&E stain). Histological assessment was performed by a blinded pathologist (Dr. Aducio Thiessen,

University of Alberta) using a histological score ranging from 0 to 10 (**Table 4-1**). The severity of intestinal inflammation was graded taking into account enterocyte injury (0-3), epithelial hyperplasia (0-3), lamina propria – lymphocytes (0-2), and lamina propria – neutrophils (0-2). The grading scheme was based on criteria developed by Berg and colleagues and similar to that used by Madsen et al.(25, 30)

Table 4-1. Grading Scheme for Histology Scores.

Total Histology Score	Score
Enterocyte Injury	
Normal	0
Mild	1
Moderate	2
Severe	3
Epithelial Hyperplasia	
Normal	0
Mild	1
Moderate	2
Pseudopolyps	3
Lamina propria lymphocyte infiltrate	
Normal	0
Slightly increased	1
Markedly increased	2
Lamina Propria neutrophil infiltrate	
Normal	0
Slightly increased	1
Markedly increased	2

Minimal injury, 0; maximal injury 10.

4.2.8 Statistical Analysis

All data provided are given as mean ± standard error. Shapiro-Wilk test was used to define if data were normally distributed. Non-parametric tests were used to determine statistical differences when data was not normally distributed. Multiple comparisons using repeated measures analysis of variance (ANOVA) using Dunnett's multiple comparison tests for all data-over-time, with the comparison being to the respective cohorts' baseline values. Partial least squares-discriminant analyses (PLS-DA) were performed on log-transformed abundance values using web-based data processing tool MetaboAnalyst 3.0 (with 95% CI).(31) In MetaboAnalyst, statistical computing and visualization operations are performed using functions from the R and Bioconductor packages.(32) PLS-DA is used to maximize class differentiation using a supervised statistical method. A 1000 permutation test was performed to determine the significance of the models. A permutation test evaluates whether the specific classification of the samples in the designed groups is significantly better than any other random classification.(33) This occurs by first performing the permutation on the classes, then randomly assigning samples with the 'wrong' labels, and performing the permutation test again. The idea is that with the wrong class labels, the newly calculated classification model should not be able to predict the classes very well. The fewer samples it can correctly classify, the greater the differences are between the classes being compared, and giving us a p-value.(34) The similarity of microbial samples to the donor FMT slurry microbial composition was also compared by calculating the Jaccard Index. For all tests a p-value <0.05 was considered statistically significant.

4.3 Results

4.3.1 Physical Features

The average starting weight of the IL-10 -/- mice before the study began was 25.2 ± 0.4 grams. Over the course of the study, all mice gained weight from their baseline, with a significantly higher weight at sacrifice (p-value <0.0001; **Figure 4-2A**). Mice in the combined bowel prep/antibiotic + FMT cohort had a significant increase in body weight just three days following FMT (D7, $p < 0.05$), and had the most significant weight increase on days 10 and 17. The colon weight to length ratio (g/cm) was elevated in both cohorts that received antibiotics, the antibiotics + FMT, and the bowel prep/antibiotics + FMT cohorts, whereas the other two cohorts had a lower ratio (**Figure 4-2B**). There were no observable trends in the terminal ileum or jejunum weight to length ratio's (**Figure S4-5**).

4.3.2 Histology

The average total histology score for the colon was highest in the antibiotics + FMT cohort, followed by the bowel prep/antibiotics + FMT cohort, with it being the lowest in the FMT-only cohort (**Figure 4-3**). A significant difference in the histology scores was detected between the antibiotics + FMT and the FMT-only cohort (p-value < 0.05). No epithelial hyperplasia was detected in the colonic histology samples from any mice. Histologic assessment of the terminal ileum of the small intestine showed no detection of inflammation where all mice received a histology score of zero.

Figure 4-2A

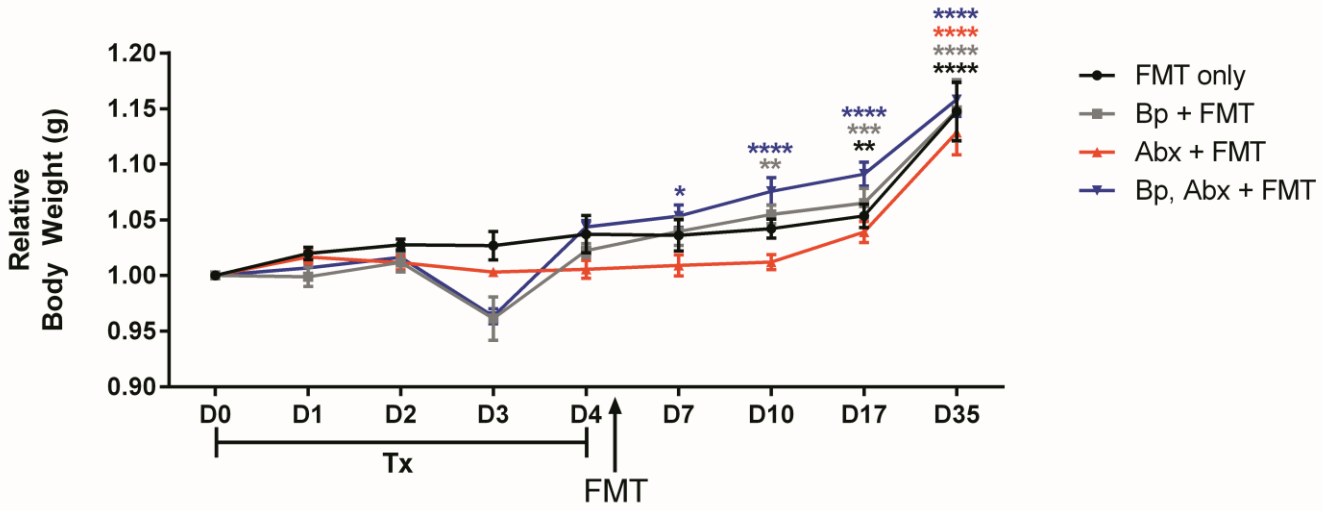


Figure 4-2B

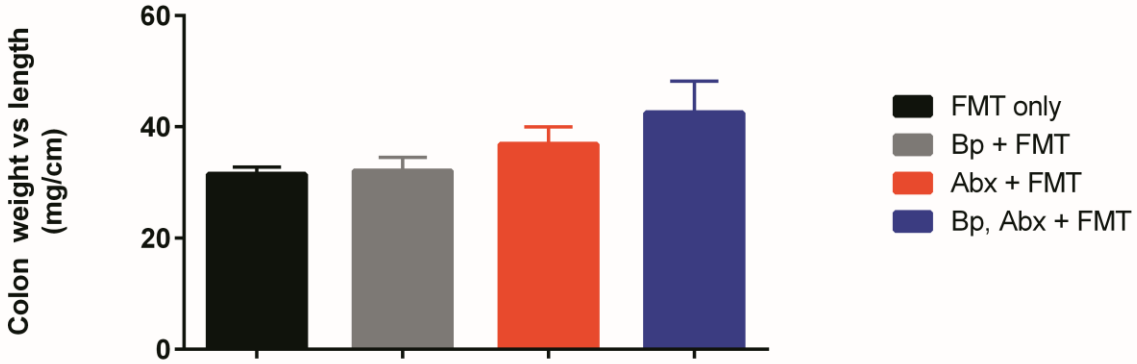


Figure 4-2. Physical features of IL-10 -/- mice. **(A)** Body weight of all mice relative to their starting weight. **(B)** Colon weight to length ratio taken at sacrifice (mg/cm). Black = FMT only (n=5); Grey = bowel preparation + FMT (n=5); Red = antibiotics + FMT (n=5); and Blue = bowel prep/antibiotics + FMT (n=5). (p-values calculated using repeated measures ANOVA/Dunnett's multiple comparison test comparing each time-point to the respective cohorts baseline value; **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$)

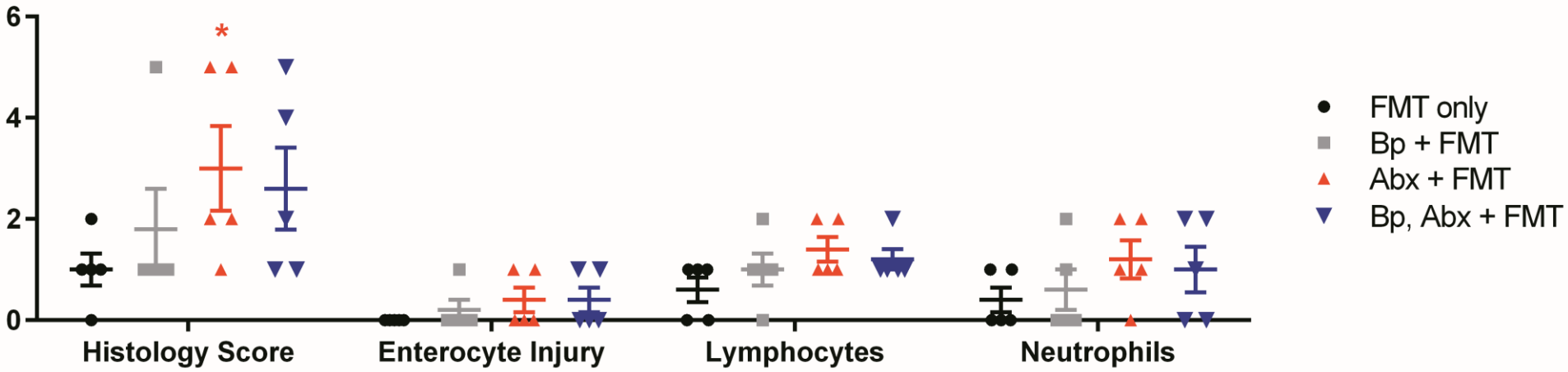


Figure 4-3. Histology scores from IL-10 -/- mice at the end of the study. Ranging from 0 to 10, grading the severity of intestinal inflammation, taking into account Enterocyte injury (0-3), Epithelial hyperplasia (0-3), Lamina propria – lymphocytes (0-2), and Lamina propria – Neutrophils (0-2), which was based on criteria developed by Berg and colleagues.(30) No epithelial hyperplasia was detected in histology samples from any of the mice. Histology scores were worst in cohorts that received antibiotics pre-FMT. Black = FMT-only (n=5); Grey = bowel preparation + FMT (n=5); Red = antibiotics + FMT (n=5); and Blue = bowel prep/antibiotics + FMT (n=5). (* $p < 0.05$ compared to the FMT-only cohort measured using Kruskal-Wallis test)

4.3.3 Cytokine Analysis

Activation of neutrophils locally in the gut when exposed to bacterial antigens results in the release of lipocalin from granules, where it can enter the gastrointestinal lumen. Therefore, it can be used as a surrogate marker of gastrointestinal inflammation associated with bacterial defense. Stool lipocalin (LCN-2) was measured at baseline (day 0), after pre-FMT treatment (day 4), three days after FMT (day 7) and 31 days after FMT (day 35) in all four cohorts (**Figure 4-4**). LCN-2 levels were quite variable at baseline, ranging from 52.5 ± 8.3 ng/g stool in the FMT-only cohort to 256.2 ± 62.3 ng/g stool in the Bowel prep/antibiotics + FMT cohort (p-value < 0.05 using student t-test; not shown). Mean LCN-2 levels were higher at the end of the study than the mean baseline values in all four cohorts, suggesting a progression of colitis regardless of the treatment regimen. However, the increase in stool lipocalin for the bowel prep/antibiotics + FMT cohort had the smallest increase in concentration from baseline to the end of the study (256.2 ± 62.3 ng/g stool to 275.3 ± 37.6 ng/ g stool). The relative increase in LCN-2 was lowest in the bowel prep/antibiotics + FMT cohort while the antibiotics + FMT cohort at the end of the study was the only time point with a significant increase ($p < 0.05$; repeated measures ANOVA/Dunnett's multiple comparison tests) as compared to baseline values. Also, three of the four cohorts had an increase in mean LCN-2 from day 7 to day 35, while the bowel prep/antibiotics + FMT cohort was the only cohort that had a reduction. There is larger variability in the LCN-2 levels following the antibiotic only treatment at day four while bowel preparation and the bowel prep/antibiotics treatment appeared to have no short term effect on LCN-2 levels. Variability in the baseline LCN-2 levels between the

different cohorts may have been due to poor separation of mice from the same litter into separate treatment cohorts (**Figure S4-3**).

The cytokines IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL12p70, and TNF-α, were measured in cecum, terminal ileum and serum samples collected at sacrifice (IFN: interferon; IL: interleukin; KC/GRO: a.k.a. CXCL1 TNF: tumour necrosis factor; **Appendix 4**). IL-1β was significantly increased in the cecal tissue of the antibiotics + FMT cohort (596 ± 97 μg/mg) in comparison to the FMT-only cohort (175 ± 14 μg/mg; **4-5A**; $p < 0.05$). The bowel prep + FMT cohort had significantly increased levels of IFN-γ (120 ± 31 μg/mg versus 55 ± 33 μg/mg;**4-5B**), KC/GRO (226 ± 20 μg/mg versus 180 ± 58 μg/mg;**4-5C**), and TNF-α (127 ± 11 μg/mg versus 100 ± 14 μg/mg**;4-5D**) in the cecum ($p < 0.05$) only when compared to the FMT-only cohort which consistently had the lowest concentration of cytokines in the cecal tissue (**Figure 4-5**). In tissue samples from the terminal ileum and serum samples, there were no significant differences in cytokine concentrations between the four cohorts and levels were consistently lower in both sample types than in the cecal tissues (**Appendix 4**).

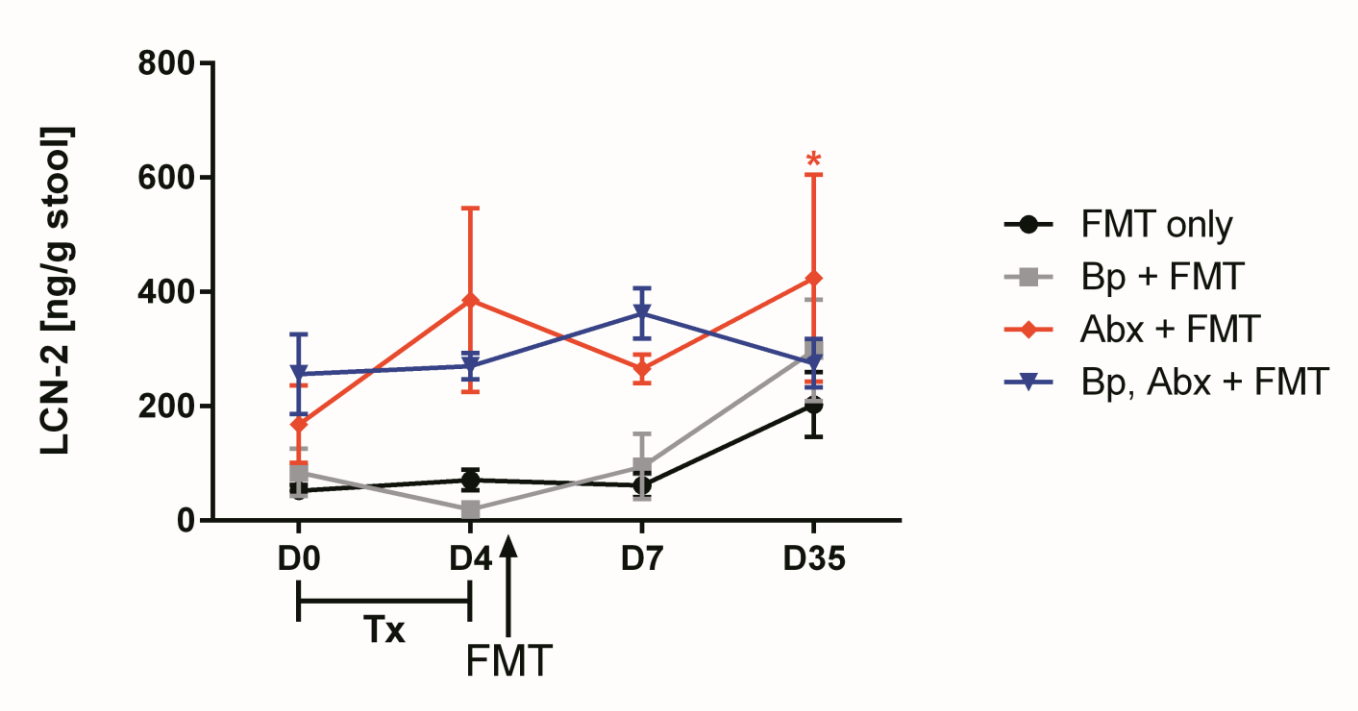


Figure 4-4. Stool lipocalin-2 (LCN-2) concentrations. Measured using enzyme-linked immunosorbent assay (ELISA) at baseline (day 0), after pre-FMT treatment (day 4), 3 days after FMT (day 7) and 31 days after FMT (day 35) in all four cohorts. Black = FMT only (n=5); Grey = bowel preparation + FMT (n=5); Red = antibiotics + FMT (n=5); and Blue = bowel prep/antibiotics + FMT (n=5). (* p-value < 0.05 measured using repeated measures ANOVA/Dunnett's multiple comparison test)

Figure 4-5A

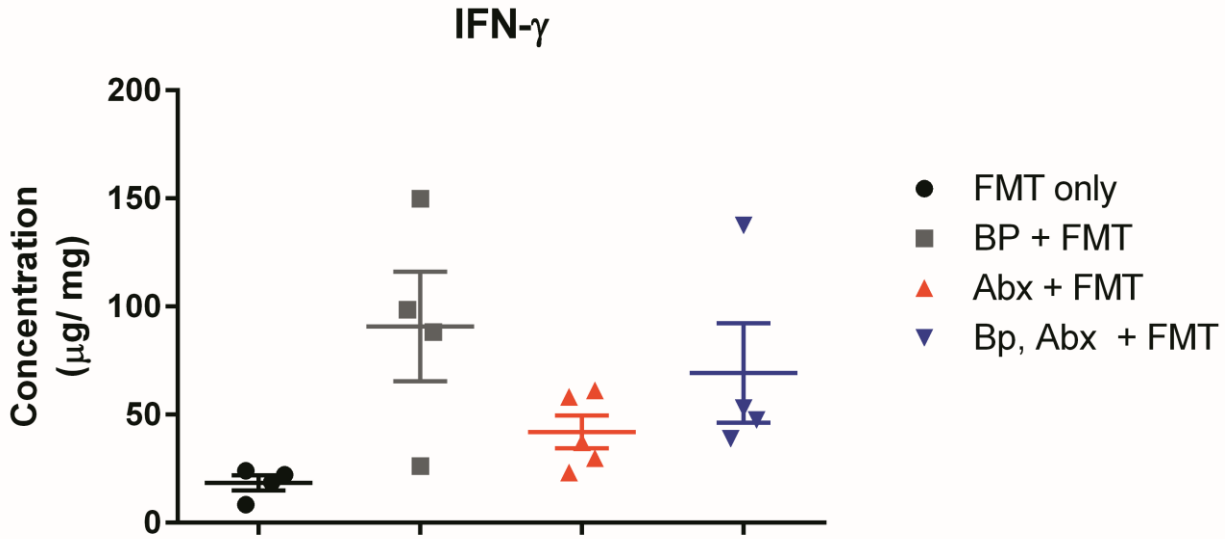


Figure 4-5B

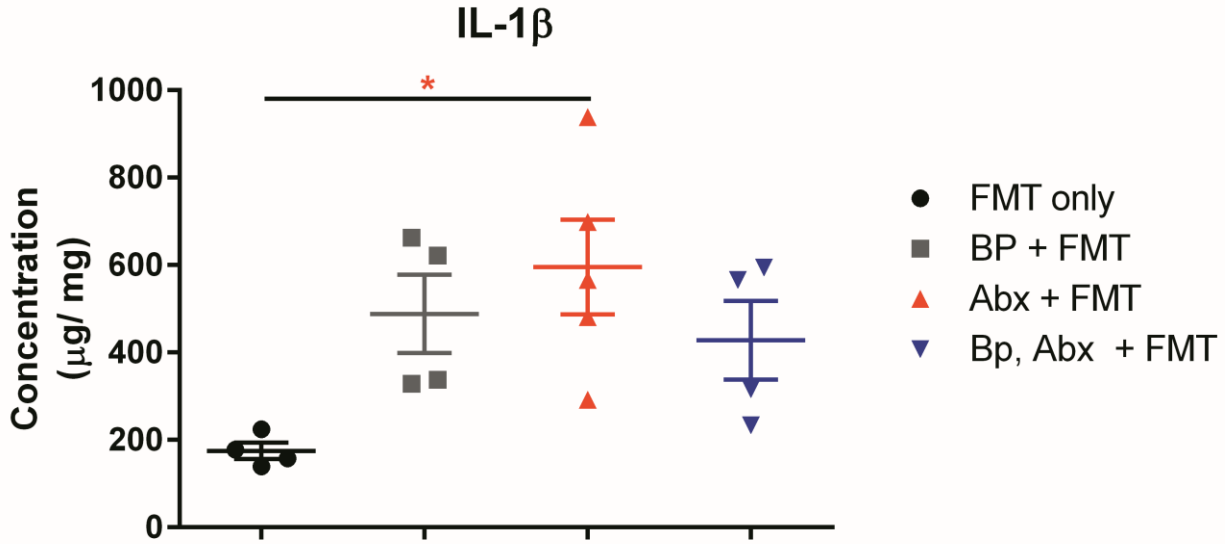


Figure 4-5C

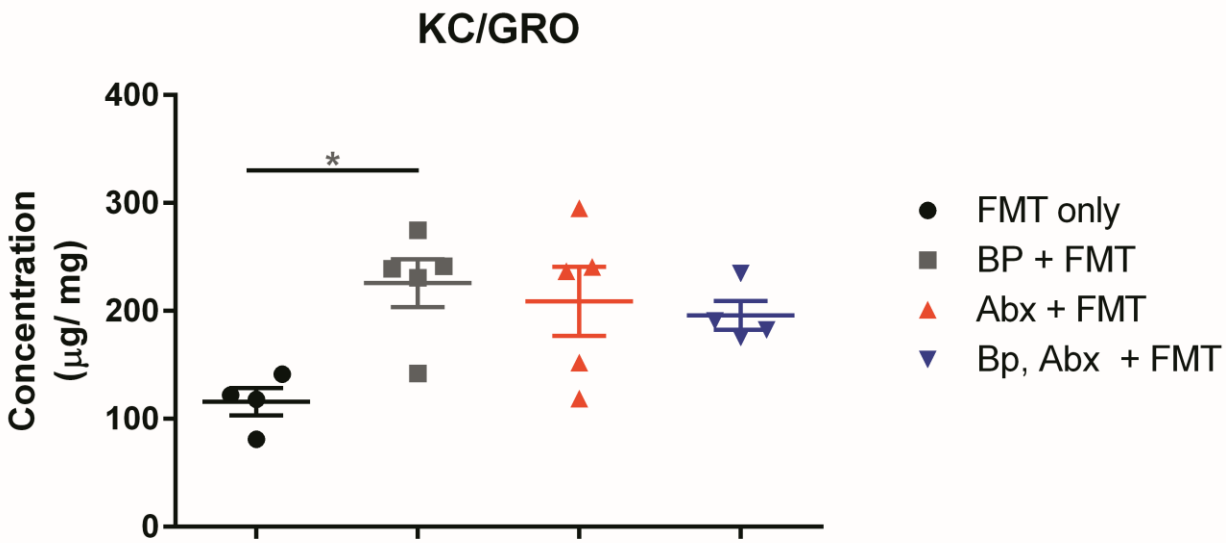


Figure 4-5D

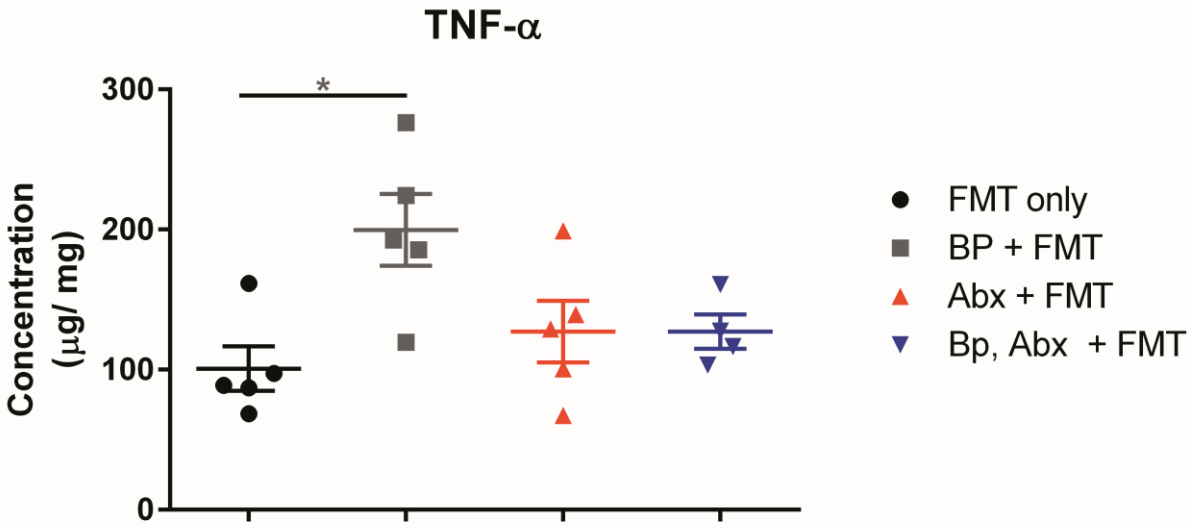


Figure 4-5. Cytokine concentration in cecal tissue from IL-10 -/- mice in micrograms per milligram of dry tissue weight. (A) IL-1β; **(B)** IFN-γ; **(C)** KC/GRO; and **(D)**TNF-α. Black = FMT only (n=5); Grey = bowel preparation + FMT (n=5); Red = antibiotics + FMT (n=5); and Blue = bowel prep/antibiotics + FMT (n=4). (* p-value < 0.05; measured using Kruskal-Wallis test)

4.3.4 Microbial Composition

The microbial composition was analyzed using quantitative real-time PCR and 16S rRNA sequencing of the pooled donors sample and mice in the four cohorts at baseline (day 0), after treatment (day 4), three days after FMT (day 7) and 31 days after FMT (day 35). For complete 16S rRNA sequencing results at all taxonomic levels, for each mouse at each time point, please refer to **Appendix 3**. By pooling together the stool from 15 different 129S1/SvlmJ mice from our colony, we expected the slurry to have a more diverse composition of microbes compared to the IL-10 -/- mice. Alpha-diversity (Shannon-Wiener diversity index) showed that at baseline, the pooled FMT sample from the healthy wild-type mice had a greater alpha-diversity (2.31) than the baseline average values of the mice in all four cohorts, which ranged from (1.96 ± 0.05 to 2.25 ± 0.05) (**Figure 4-6A**). None of the treatments had a negative impact on the alpha-diversity, where the bowel prep/antibiotics cohort had a statistically significant increase from baseline in alpha-diversity on day 4 ($p < 0.0001$). As expected, the cohort that did not receive any pre-FMT treatment regimen had no change in alpha diversity from day 0 to day 4. At the end of the study, the bowel prep/antibiotics + FMT, and the bowel prep + FMT cohorts had a significant increase in alpha diversity in comparison to their baseline values ($p < 0.001$ & $p < 0.01$ respectively). The two cohorts that received antibiotics in their pre-FMT treatment regimen showed a significant reduction in microbial richness (number of OTUs) at day 4 (Abx + FMT: $p < 0.01$; Combo + FMT: $p < 0.001$) while bowel preparation alone did not have an effect on richness (**Figure 4-6B**). Although our measure of alpha-diversity in the donor FMT slurry was higher than baseline values for all the IL-10 -/- mice, the number of operational taxonomic units

(OTUs) detected in the FMT slurry was much lower than the mean values of all cohorts at each time-point, even following antibiotic treatment. Following FMT, the two antibiotic pre-FMT cohorts had an increase in richness, but not to levels surpassing their baseline values while the FMT-only cohort had the largest increase in the number of OTU's (468 ± 25 OTUs versus 546 ± 14 OTUs), although the increase was not statistically significant. Bacterial load as determined by the copy number of 16S rRNA genes per gram of stool was only slightly reduced by the antibiotic pre-FMT treatment regimens, whereas bowel preparation alone had little effect (**Figure 6C**). Copy number had a lot of variability at baseline and the only statistically significant increase in copy number was in the antibiotics + FMT cohort on day 7 ($p < 0.01$), which then saw a drastic decrease in bacterial load from day 7 to day 35. The bowel prep + FMT cohort also saw a decrease in bacterial load, but this occurred in the three days following FMT. In contrast, the bacterial load of the bowel prep/antibiotics + FMT decreased following antibiotic treatment and increased slightly above baseline following FMT. The single cohort that appeared to have a substantial net positive increase in 16S rRNA copy number per gram of stool was the FMT-only cohort, although this change was not statistically significant.

Figure 4-6A

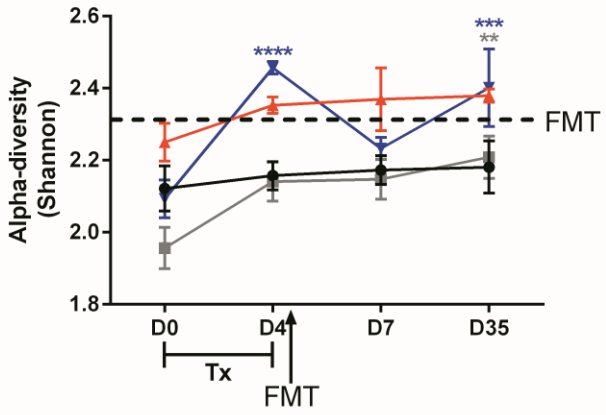


Figure 4-6B

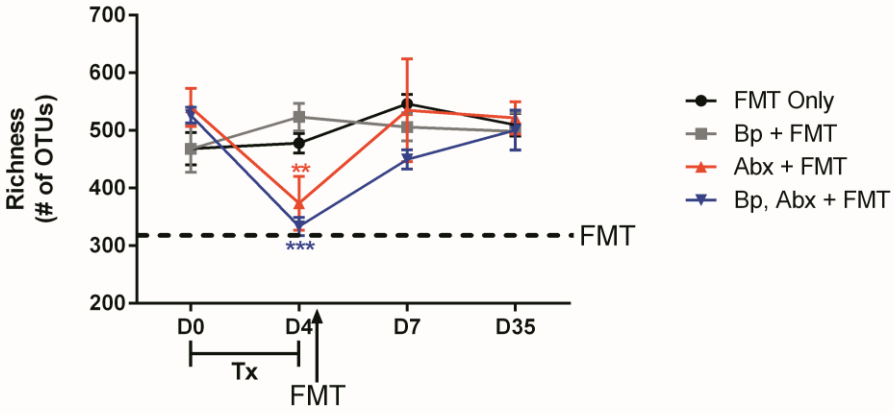


Figure 4-6C

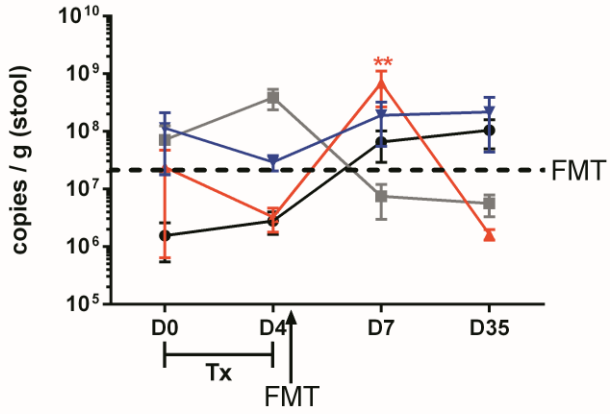


Figure 4-6. Characteristics of the gut microbiome determined by 16S sequencing and quantitative real-time polymerase chain reaction (PCR) of the 16S rRNA gene. **(A)** Alpha-diversity (Shannon-Wiener index), **(B)** bacterial richness determined by the number of different operational taxonomic units (97% similarity) and **(C)** the 16S rRNA gene copy number per gram of stool. Black = FMT only (n=5); Grey = bowel preparation + FMT (n=5); Red = antibiotics + FMT (n=5); and Blue = bowel prep/antibiotics + FMT (n=5). Dotted line represents the donor FMT slurry. (p-values calculated using repeated measures ANOVA/Dunnett's multiple comparison test comparing each time-point to the respective cohorts's baseline value; **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$)

16S rRNA sequencing data showed that the gut microbiota of our IL-10 -/- mice contained a bowel prep/antibiotics of six different phyla, although dominated by Bacteroidetes (mean 56.6%) and Firmicutes (mean 39.4%), and a minute amount of Proteobacteria (mean 1.09%) (**Appendix 3**). The six bacterial families who experienced the most significant changes in abundance (% total reads) in the microbiome as a result pre-FMT treatment regimens and FMT were *Porphyromonadaceae, Verrucomicrobiaceae,* an undefined member of the RF32 order, *S24-7, Clostridiaceae,* and *Ruminococcaceae* (**Figure 4-7**). *Porphyromonadaceae*, which consisted of 14.6% of the total reads of in the donor FMT slurry, increased significantly ($p < 0.0001$) following both pre-FMT treatment regimens that included antibiotics to levels close to those detected in the donors. However, this was not maintained following FMT, with only a small amount remaining on days 7 and 35, although at levels higher than the other two cohorts, and their baseline (**Figure 4-7A**). *Verrucomicrobiaceae* abundance only increased in the bowel prep/antibiotics cohort following treatment, but increased in both groups that received pre-FMT antibiotics following the FMT to levels observed in the donor FMT slurry **(Figure 4-7B**; 0.06). Engraftment of *Verrucomicrobiaceae* was significant in the bowel prep/antibiotics + FMT ($p < 0.0001$) and the antibiotics + FMT ($p < 0.01$) cohorts three days following FMT, however, on day 35 the levels were following a negative trend back towards baseline values. The abundance of the undefined member of the order RF32 was increased following bowel prep/antibiotics pre-FMT treatment ($p < 0.0001$), and following antibiotic treatment alone (**Figure 4-7C**). The proportion of total reads from this bacteria family increased further following the FMT ($p < 0.01$), even though it consisted of a minor proportion of reads (0.01) in the

donor FMT. *S24-7*, a member of the phylum Bacteroidetes, saw a reduction in abundance from baseline to day 4, but the greatest reduction was the result of bowel prep/antibiotics pre-FMT treatment ($p < 0.01$). A decrease at day 35 in the FMT-only ($p < 0.01$) and bowel prep + FMT ($p < 0.05$), and the bowel prep/antibiotics + FMT cohorts, towards the proportion detected in the donor FMT sample was also observed (**Figure 4-7D**). The relative abundance of *Clostridiaceae* is reduced following both antibiotic containing pre-FMT treatments while the abundance following FMT does not change significantly for three of the four cohorts (**Figure 4-7E**). On both days 7 and day 35, the FMT-only cohort has a significant increase from baseline of this bacterial family, even though abundance in the donor FMT slurry is quite low ($p < 0.05$). The relative abundance of *Ruminococcaceae* is also very low in the donor FMT slurry, however, despite this, an increase in relative abundance in this family occurs at day 7 in the FMT only cohort ($p < 0.01$) and the antibiotics + FMT cohort (**Figure 4-7F**). Both cohorts experience a decrease in relative abundance from day 7 to day 35, although the FMT only cohort still has a net increase in this bacterial family over the course of the study. With these changes in the bacterial families, the ratio of two paramount phyla of bacteria changed to different extents in each of the cohorts.

Figure 4-7A

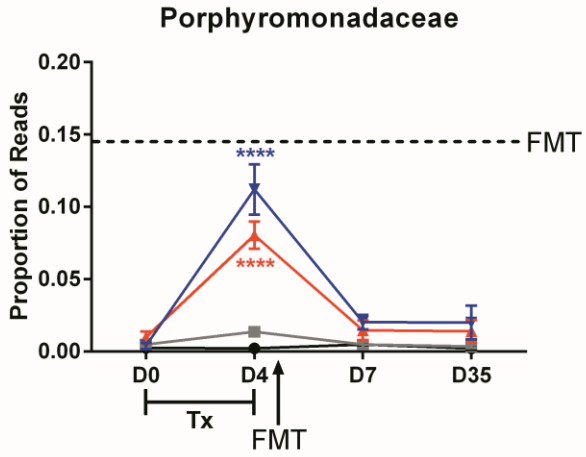


Figure 4-7B

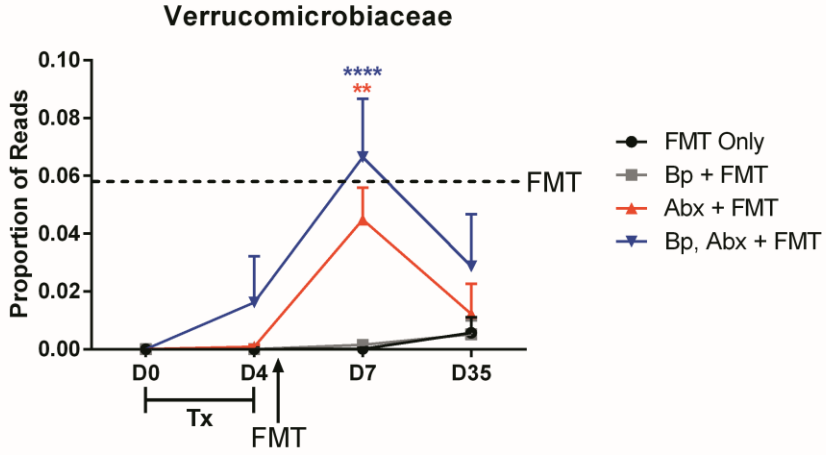


Figure 4-7C

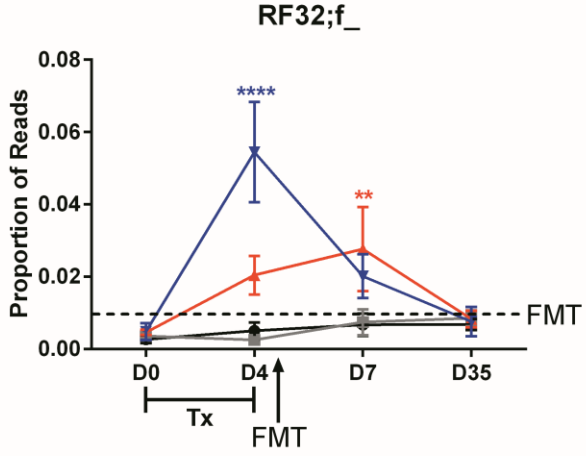


Figure 4-7D

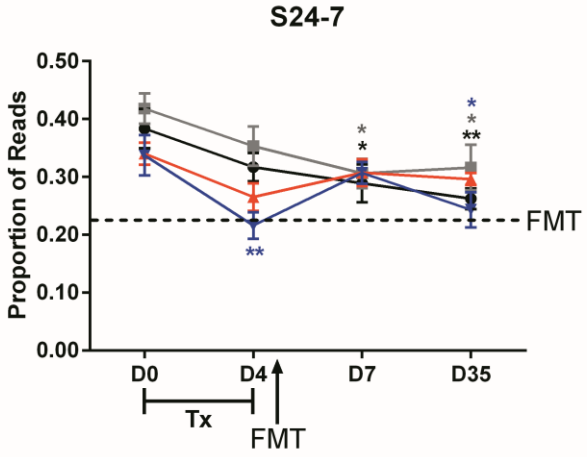


Figure 4-7E

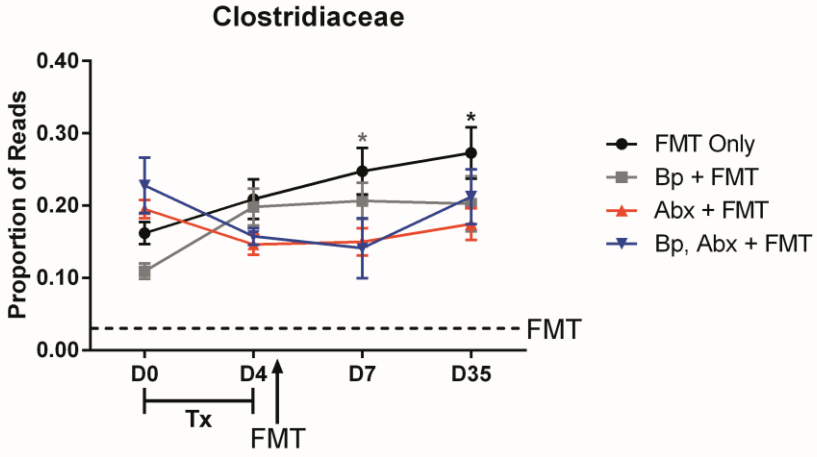


Figure 4-7F

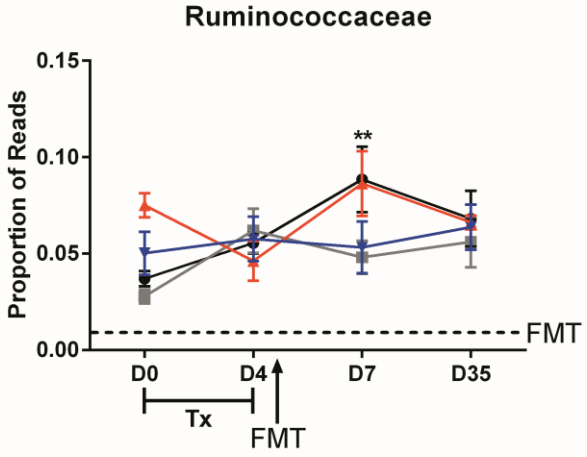


Figure 4-7. The proportion of total reads of the six bacterial families with the most significant changes determined using 16S rRNA sequencing. Black = FMT only (n=5); Grey = bowel preparation + FMT (n=5); Red = antibiotics + FMT (n=5); and Blue = bowel prep/antibiotics + FMT (n=5). Dotted line represents the proportion of reads in the donor FMT slurry. (**** $p < 0.0001$; ** $p < 0.01$; * $p < 0.05$; measured using two-way ANOVA/Dunnett's multiple comparison test comparing each time-point to the respective cohort's baseline value)

The relative ratio of Firmicutes: Bacteroidetes had a net increase in all four cohorts even though the ratio in the donor FMT slurry was relatively low (**Figure 4-8**; 0.66). The FMT-only had the largest increase in this ratio, with the mean peaking at 1.45 on day 7, and staying very high (1.40) at day 35 ($p < 0.001$ for both time points in comparison to baseline). A significant increase ($p < 0.05$) was also observed in this ratio on day 7 for the bowel prep + FMT cohort on day 7. However, this was no longer observed on day 35. The bowel prep/antibiotics + FMT cohort, although the increases were not significant, had an increase in the Firmicutes: Bacteroidetes ratio on day 7 and a further increase detected on day 35 (1.30).

At baseline, the IL-10 -/- mice had on average 57% similarity in microbial composition using the Jaccard similarity index (genus level), although there was variability between the different cohorts (range: 53.5 ± 0.01% in the FMT only cohort to 59.1 ± 0.01% in the bowel prep/antibiotics + FMT cohort; **Figure 4-9**). Antibiotics lowered the Jaccard index in the antibiotics + FMT, and bowel prep/antibiotics + FMT cohorts, although these changes were not significant. Three days following FMT, the Jaccard similarity in the antibiotics + FMT cohort increased to 0.67 ± 0.01 ($p < 0.01$), although this effect was transient and the value returned towards its baseline value. Although not significant, the bowel prep/antibiotics + FMT cohort also saw an increase on day seven to 0.64 ± 0.01, which was also maintained four weeks following the FMT procedure, with a value of 0.63 ± 0.06, although the considerable variability in the values of this cohort led to it not being significant. The only cohort with a significant increase in

similarity to the donor FMT slurry at sacrifice was the FMT only cohort which had a net increase in the Jaccard index of only 6.47% ($p < 0.05$).

Partial least squares - discriminant analysis (PLS-DA) showed that the baseline microbial composition of all cohorts was significantly different at the taxonomic level of genera ($p = 0.012$) before any pre-FMT treatment or the FMT procedure (**Figure 4-10**). The microbial composition clustered separately following the various treatments at day 4 ($p < 0.001$), where the two cohorts receiving antibiotics grouped separately from the other two cohorts, and the bowel preparation pre-FMT treatment regimen caused them to cluster separately from those that received no pre-FMT treatment. Three days following the FMT procedure on day 7, there were significant differences in the microbial composition of the four cohorts ($p = 0.007$). Four weeks following FMT (day 35), the microbial composition was most similar between all groups than any other time point and showed a non-significant p- value (**Figure 4-10**; $p = 0.946$). Although changes in the microbial composition were observed, and the Jaccard similarity index increased significantly in some cohorts, the IL-10 -/- mice did not cluster with the donor FMT slurry at any time point (**Figure S4-1**).

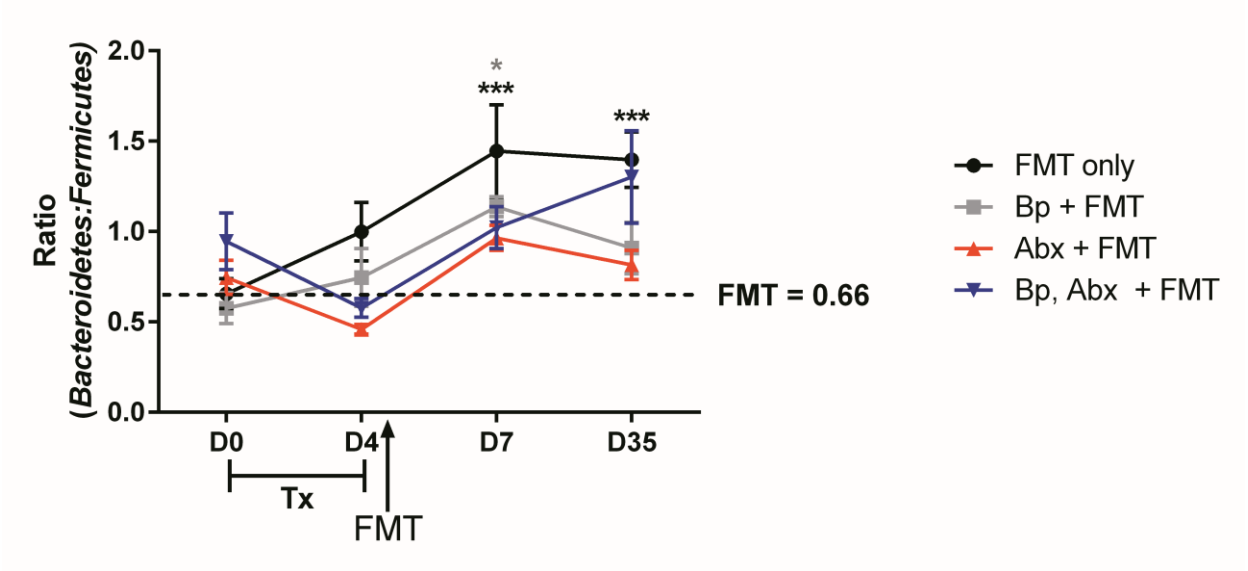


Figure 4-8. The relative ratio of the phyla Fermicutes to Bacteroidetes determined from 16S rRNA sequencing. Black = FMT only (n=5); Grey = bowel preparation + FMT (n=5); Red = antibiotics + FMT (n=5); and Blue = bowel prep/antibiotics + FMT (n=5). The table below shows the absolute values at each time point for each cohort. (*** $p < 0.001$; * $p < 0.05$; measured using two-way ANOVA/Dunnett's multiple comparison test comparing each time-point to the respective mouse's baseline value)

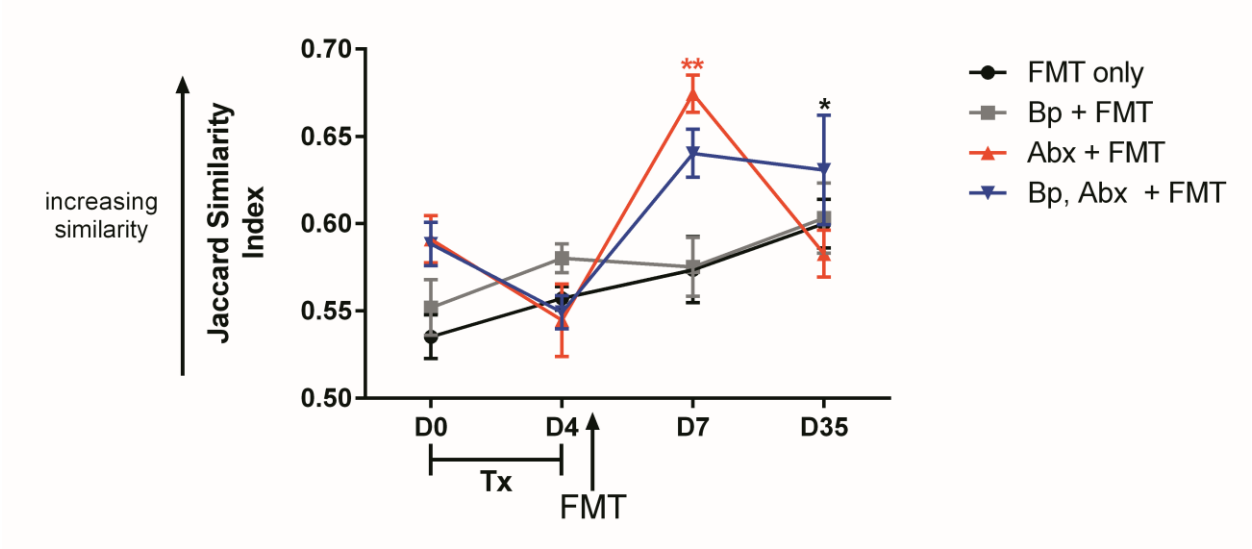


Figure 4-9. Jaccard similarity index measured by comparing the microbial profile of individual samples to the donor FMT slurry at the genus level. Black = FMT only (n=5); Grey = bowel preparation + FMT (n=5); Red = antibiotics + FMT (n=5); and Blue = bowel prep/antibiotics + FMT (n=5). (** p-value < 0.01; * p-value < 0.05; measured using two-way ANOVA/Dunnett's multiple comparison test comparing each time-point to the respective mouse's baseline value)

Significant changes in the microbial composition from treatment and the FMT procedure are observed in the antibiotics + FMT ($p < 0.029$) and the bowel prep/antibiotics + FMT cohort ($p = 0.002$), but not in the FMT-only ($p = 0.207$) and bowel prep + FMT ($p = 0.109$) cohorts (**Figure 4-11**). Directional changes in the microbial composition towards the donor FMT slurry sample in the antibiotics + FMT, and the bowel prep/antibiotics + FMT cohorts can be observed in Figure S4-2, although all samples still cluster very distinctly from the donors. The antibiotic pre-FMT treatment regimens appear to move the cluster in the opposite direction than the FMT moves the cluster along component 2 (**Figures 11 & S2**). Although changes in microbial composition are observed following antibiotics, bowel preparation, a bowel prep/antibiotics and FMT, these changes are minimal, and the donor FMT slurry still has a very different microbial composition than any of the IL-10 -/- mice following treatment.

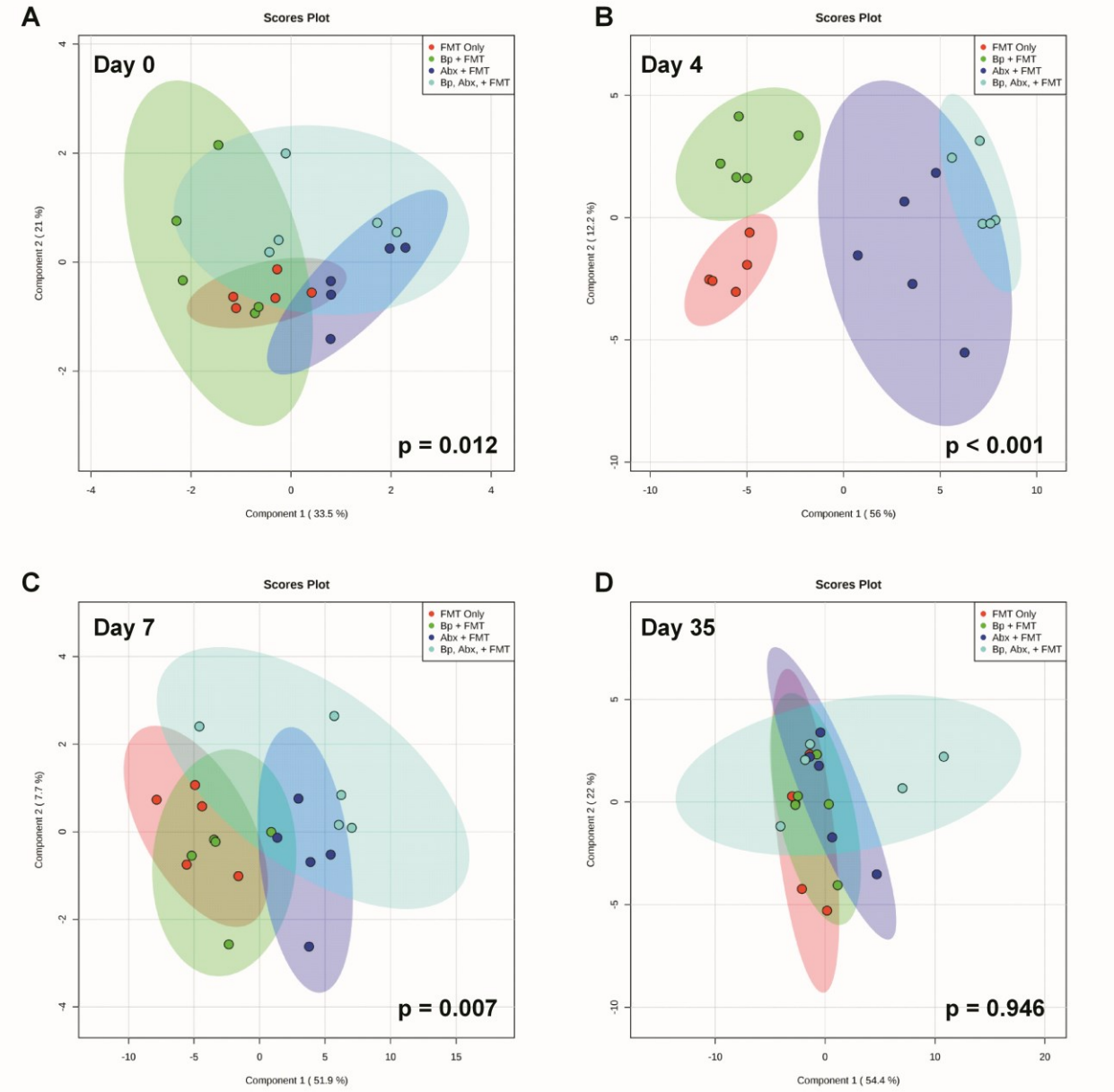


Figure 4-10. Partial least squares Discriminant Analysis (PLS-DA) of the microbial profiles of mice at all four time-points made using MetaboAnalyst 3.0 (with 95% CI). Red = FMT only (n=5); Green = bowel preparation + FMT (n=5); Blue = antibiotics + FMT (n=5); and Teal = bowel prep/antibiotics + FMT (n=5). P-values were calculated using the provided permutation test consisting of 1000 permutations.

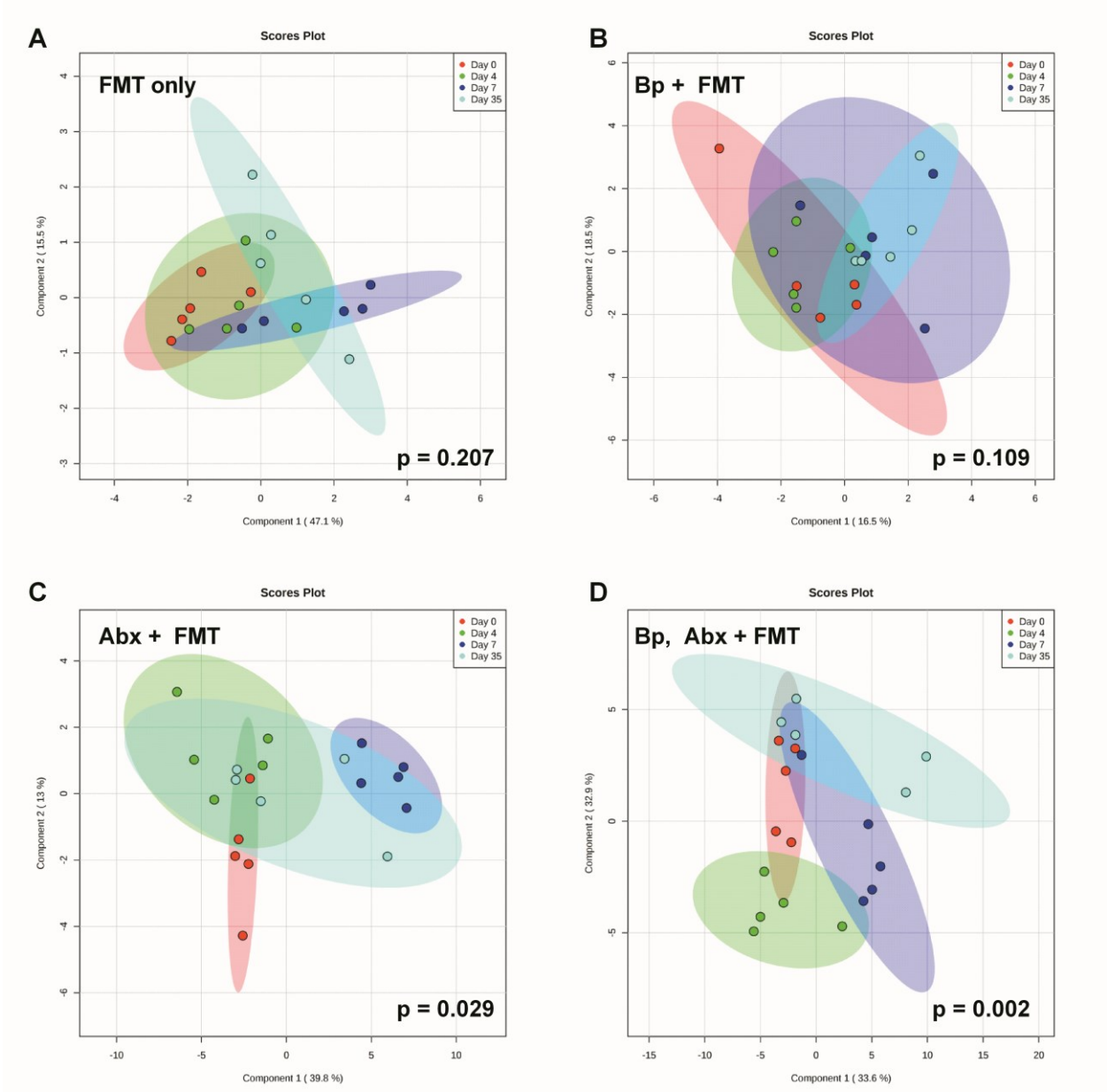
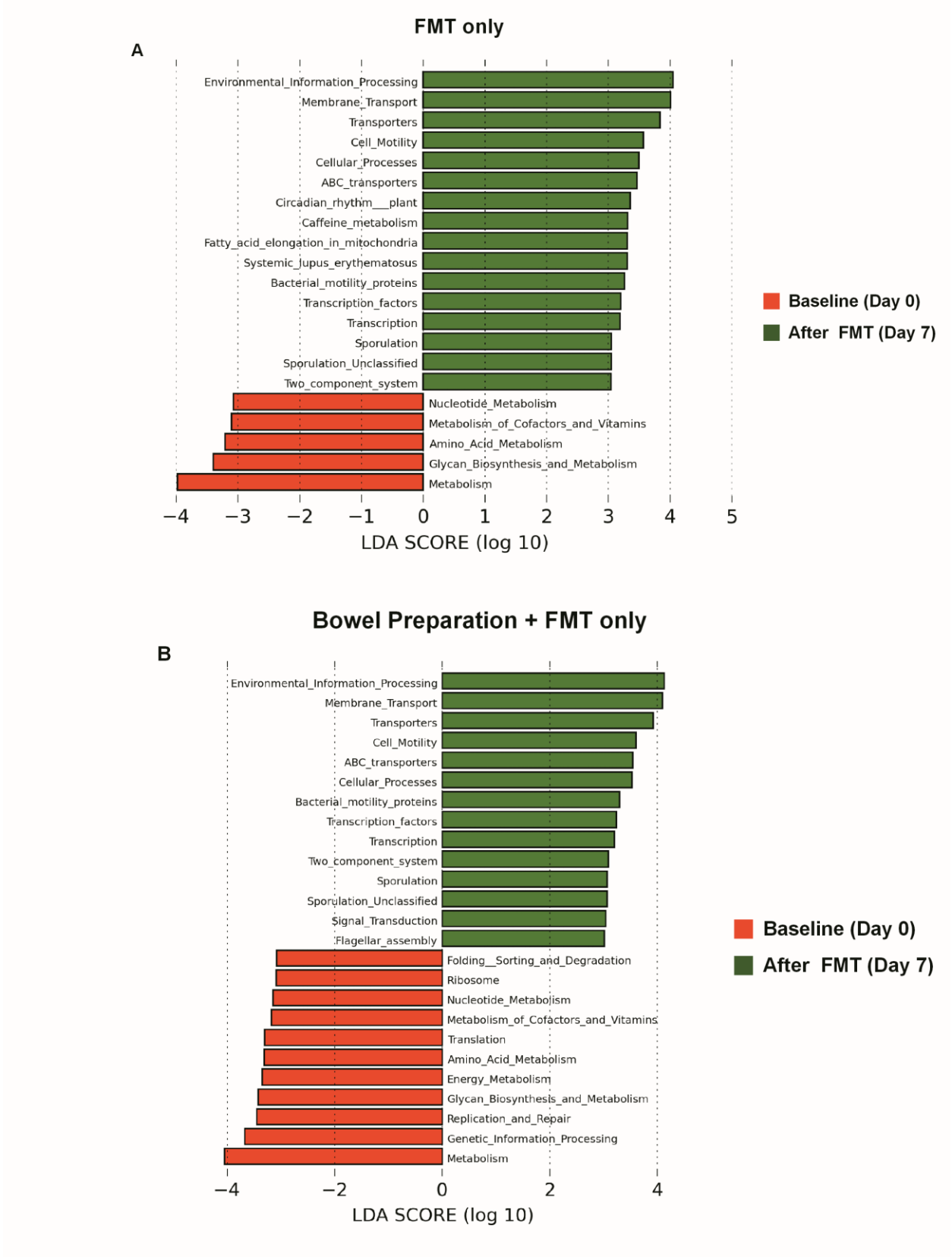
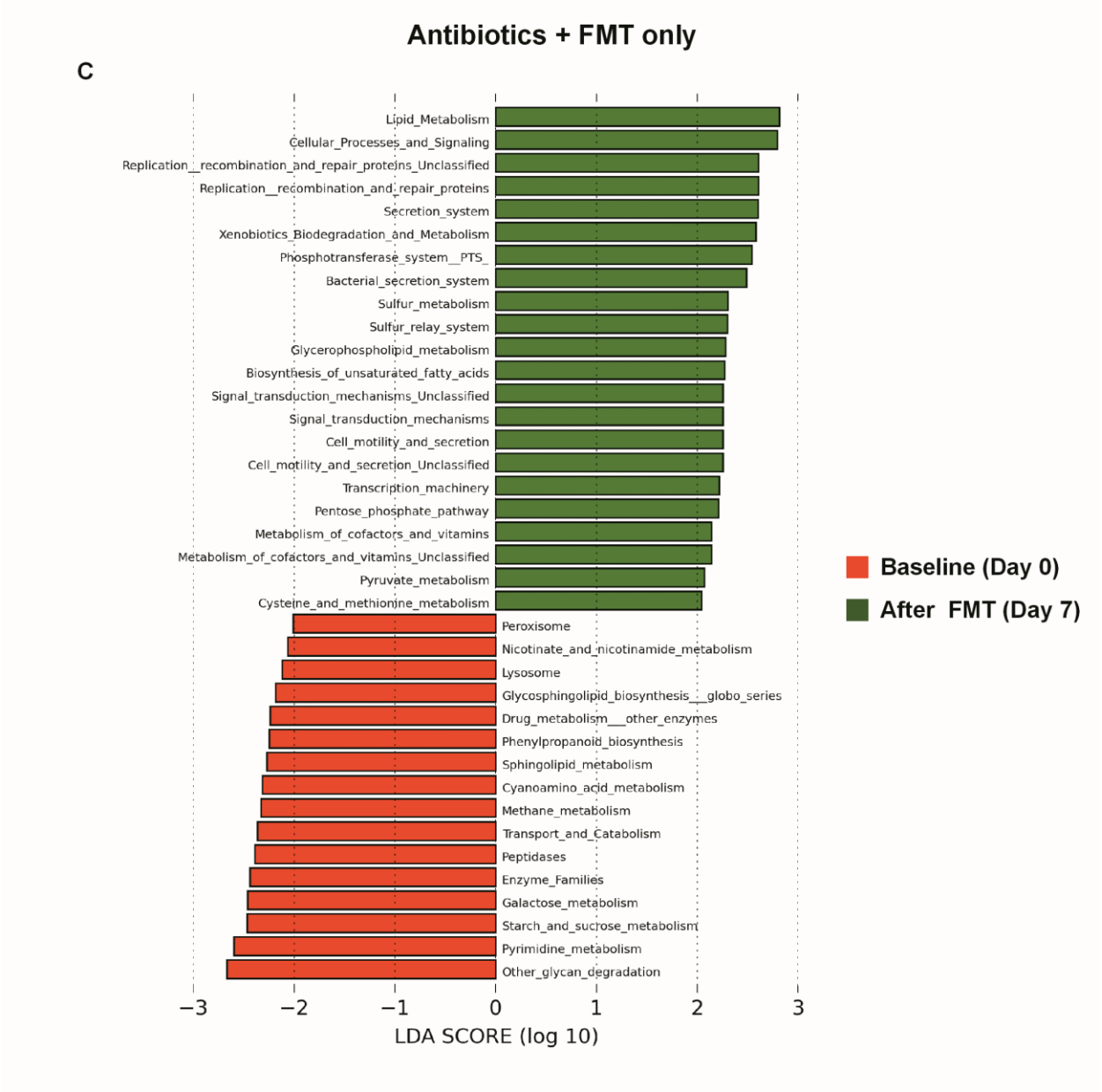


Figure 4-11. Partial least squares Discriminant Analysis (PLS-DA) of the microbial profiles of mice separated by cohort made using MetaboAnalyst 3.0 (with 95% CI). Red = baseline (Day 0; n=5); Green = after treatment (Day 4; n=5); Blue = 3 days following FMT (Day 7; n=5); and Teal = 4 weeks following FMT (Day 35; n=5). P-values were calculated using the provided permutation test consisting of 1000 permutations.

Using phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) we were able to determine changes in the predicted functional content of the microbiota from the 16S rRNA marker gene (LDA log 10; p-value < 0.05). Following the three-day treatment period, there were very minute changes in the functional capacity in the FMT-only cohort, few variations in the antibiotics and bowel prep/antibiotics cohorts, and to our surprise the largest number of changes in the bowel preparation cohort (**Figure S4-4**). The changes observed three days following FMT were nearly identical to the changes following treatment (**Figure 4-12B**), which appeared to predominately increase bacterial movement pathway processes, and a reduction in energy metabolism. Similar functional pathways were altered three days following FMT in the FMT-only cohort, including reduced co-factor and vitamin metabolism, whereas the two cohorts with antibiotic pre-FMT treatment saw a marked increase in this functional capacity of the microbiota (**Figure 4-12**). The bowel prep/antibiotics + FMT cohort also showed an increase in essential amino acid (valine, leucine, and isoleucine), unsaturated fatty acid, and fatty acid biosynthesis, as well as lipid, fructose, and mannose metabolism (**Figure 4-12D**). Four weeks following FMT, the FMT-only, and the bowel preparation + FMT cohorts maintained few functional changes, and modifications predominately belonged to intracellular bacterial processes (**Figure 4-13 A & B**). For the antibiotics + FMT cohort, very few functional changes remained, and the only potentially positive change for the host was increased lipid metabolism (**Figure 4-13C**). In contrast to the other cohorts, the bowel prep/antibiotics + FMT still had several positive changes remaining four weeks following FMT, including unsaturated fatty acid synthesis, alpha-linolenic acid metabolism, and N-glycan biosynthesis (**Figure 4-13D**).


FMT only
A
Environmental_Information_Processing
Membrane_Transport
Transporters
Cell_Motility
Cellular_Processes
ABC_transporters
Circadian_rhythm___plant
Caffeine_metabolism
Fatty_acid_elongation_in_mitochondria
Systemic_lupus_erythematosus
Bacterial_motility_proteins
Transcription_factors
Transcription
Sporulation
Sporulation_Unclassified
Two_component_system
Nucleotide_Metabolism
Metabolism_of_Cofactors_and_Vitamins
Amino_Acid_Metabolism
Glycan_Biosynthesis_and_Metabolism
Metabolism
−4 −3 −2 −1 0 1 2 3 4 5
LDA SCORE (log 10)
Baseline (Day 0)
After FMT (Day 7)
Bowel Preparation + FMT only
B
Environmental_Information_Processing
Membrane_Transport
Transporters
Cell_Motility
ABC_transporters
Cellular_Processes
Bacterial_motility_proteins
Transcription_factors
Transcription
Two_component_system
Sporulation
Sporulation_Unclassified
Signal_Transduction
Flagellar_assembly
Folding__Sorting_and_Degradation
Ribosome
Nucleotide_Metabolism
Metabolism_of_Cofactors_and_Vitamins
Translation
Amino_Acid_Metabolism
Energy_Metabolism
Glycan_Biosynthesis_and_Metabolism
Replication_and_Repair
Genetic_Information_Processing
Metabolism
−4 −2 0 2 4
LDA SCORE (log 10)
Baseline (Day 0)
After FMT (Day 7)


Antibiotics + FMT only
C
Lipid_Metabolism
Cellular_Processes_and_Signaling
Replication__recombination_and_repair_proteins_Unclassified
Replication__recombination_and_repair_proteins
Secretion_system
Xenobiotics_Biodegradation_and_Metabolism
Phosphotransferase_system__PTS_
Bacterial_secretion_system
Sulfur_metabolism
Sulfur_relay_system
Glycerophospholipid_metabolism
Biosynthesis_of_unsaturated_fatty_acids
Signal_transduction_mechanisms_Unclassified
Signal_transduction_mechanisms
Cell_motility_and_secretion
Cell_motility_and_secretion_Unclassified
Transcription_machinery
Pentose_phosphate_pathway
Metabolism_of_cofactors_and_vitamins
Metabolism_of_cofactors_and_vitamins_Unclassified
Pyruvate_metabolism
Cysteine_and_methionine_metabolism
Peroxisome
Nicotinate_and_nicotinamide_metabolism
Lysosome
Glycosphingolipid_biosynthesis__globo_series
Drug_metabolism__other_enzymes
Phenylpropanoid_biosynthesis
Sphingolipid_metabolism
Cyanoamino_acid_metabolism
Methane_metabolism
Transport_and_Catabolism
Peptidases
Enzyme_Families
Galactose_metabolism
Starch_and_sucrose_metabolism
Pyrimidine_metabolism
Other_glycan_degradation
−3
−2
−1
0
1
2
3
LDA SCORE (log 10)
Baseline (Day 0)
After FMT (Day 7)

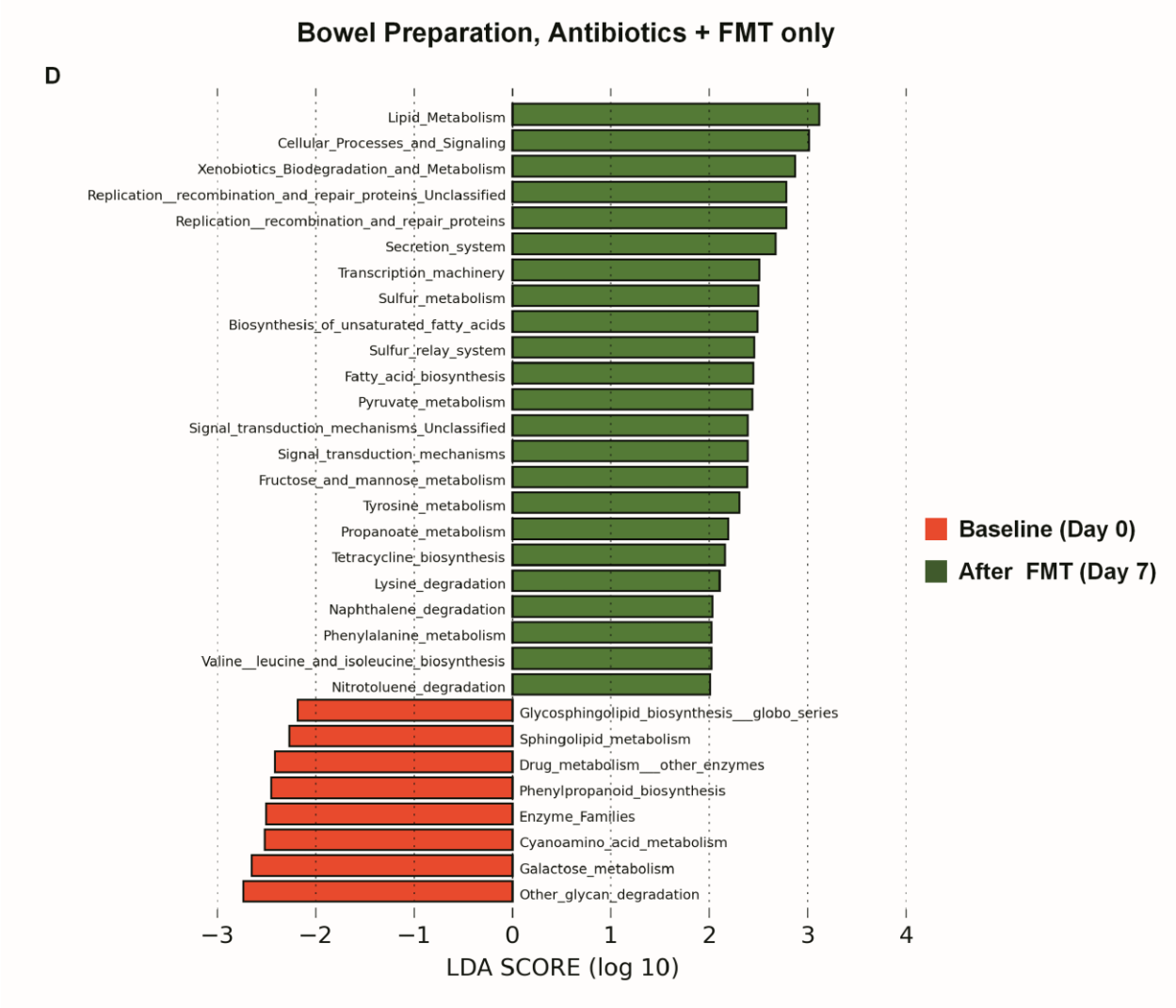
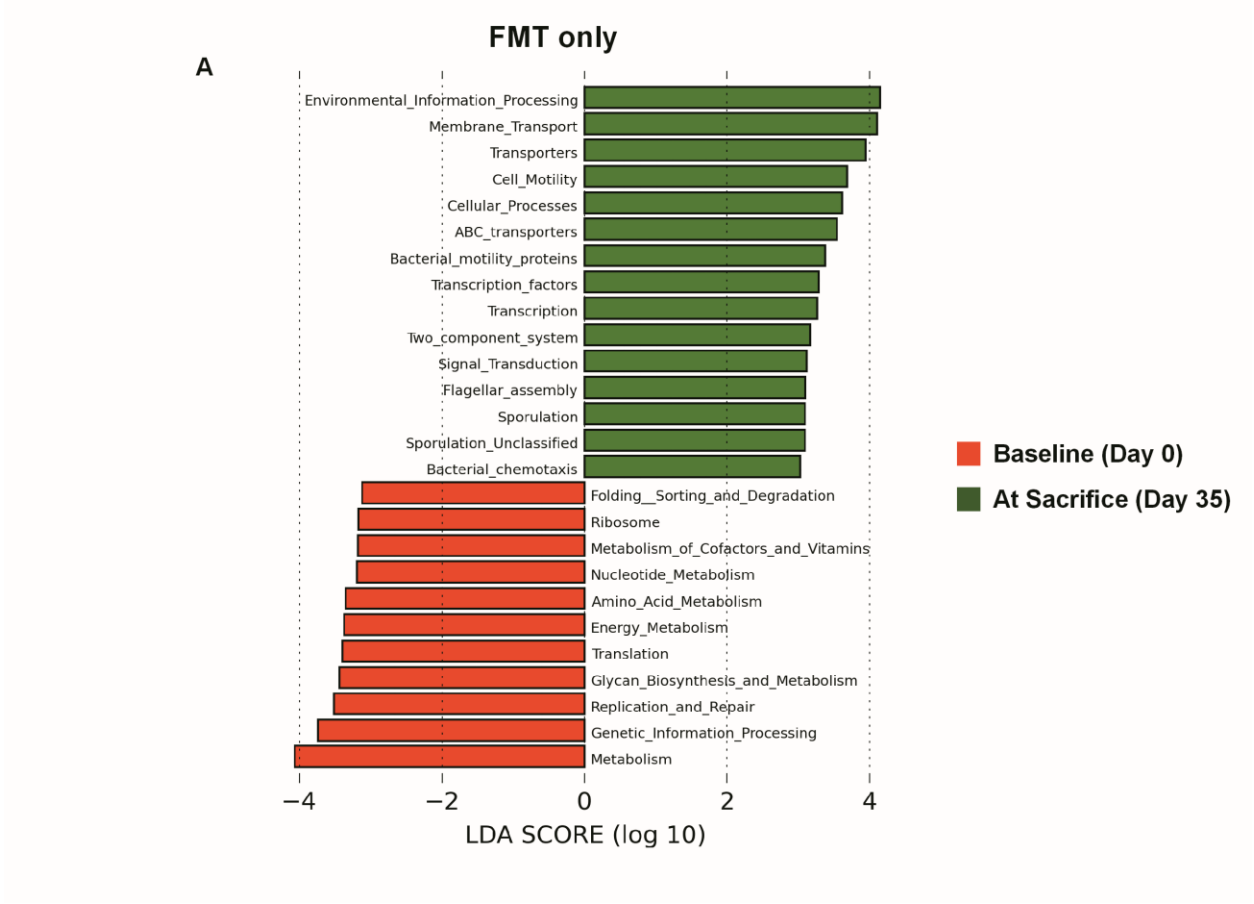
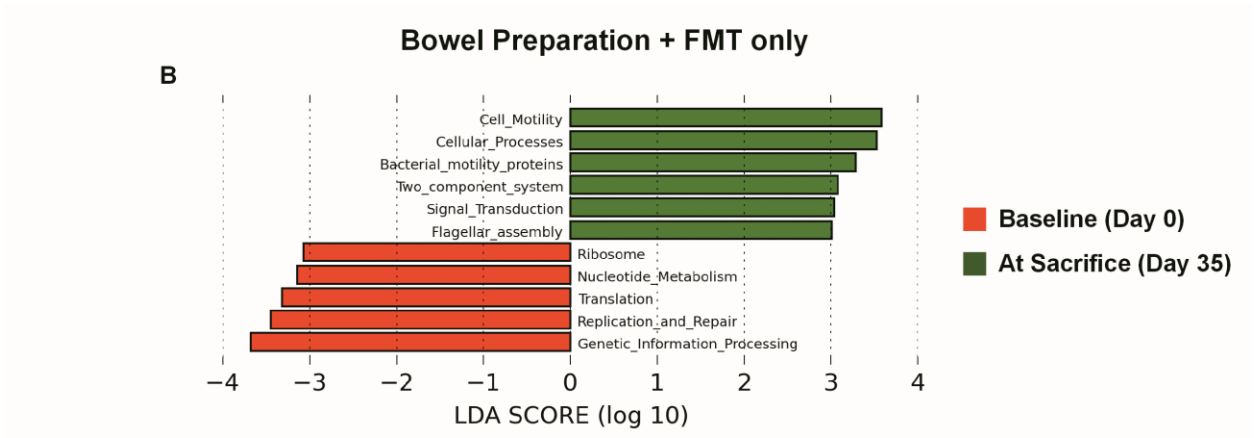
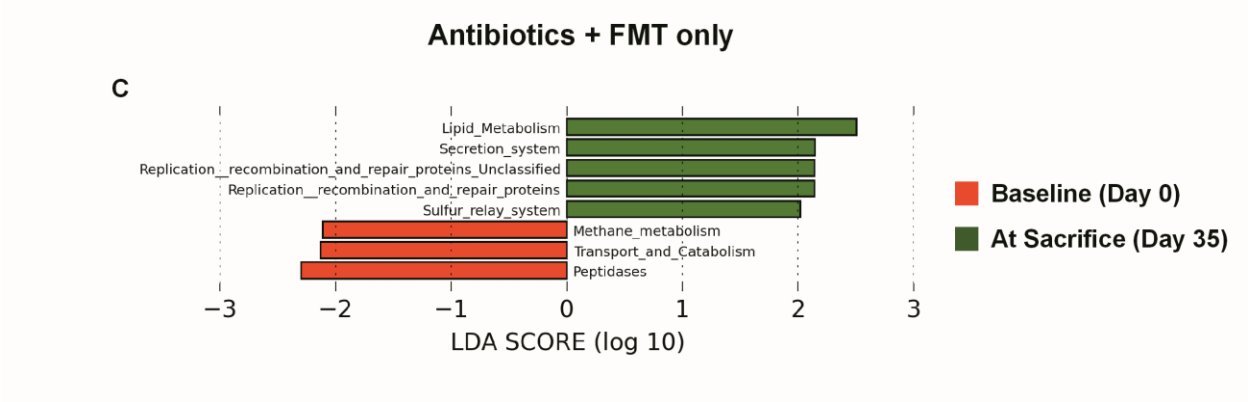


Figure 4-12. **Linear Discriminant Analysis of PICRUSt results comparing the baseline composition to three days following FMT in each of the four cohorts**. Using phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) we were able to determine changes in the predicted functional content of the microbiota from the 16S rRNA marker gene comparing baseline (Red; Day 0) taxonomic composition to taxonomic composition three days after FMT (Green; Day 7). (P-value < 0.05 using Kruskal-Wallis test)


FMT only
A
Environmental_Information_Processing
Membrane_Transport
Transporters
Cell_Motility
Cellular_Processes
ABC_transporters
Bacterial_motility_proteins
Transcription_factors
Transcription
Two_component_system
Signal_Transduction
Flagellar_assembly
Sporulation
Sporulation_Unclassified
Bacterial_chemotaxis
Folding__Sorting_and_Degradation
Ribosome
Metabolism_of_Cofactors_and_Vitamins
Nucleotide_Metabolism
Amino_Acid_Metabolism
Energy_Metabolism
Translation
Glycan_Biosynthesis_and_Metabolism
Replication_and_Repair
Genetic_Information_Processing
Metabolism
−4
−2
0
2
4
LDA SCORE (log 10)
Baseline (Day 0)
At Sacrifice (Day 35)


Bowel Preparation + FMT only
B
Cell_Motility
Cellular_Processes
Bacterial_motility_proteins
Two_component_system
Signal_Transduction
Flagellar_assembly
Ribosome
Nucleotide_Metabolism
Translation
Replication_and_Repair
Genetic_Information_Processing
−4
−3
−2
−1
0
1
2
3
4
LDA SCORE (log 10)
Baseline (Day 0)
At Sacrifice (Day 35)



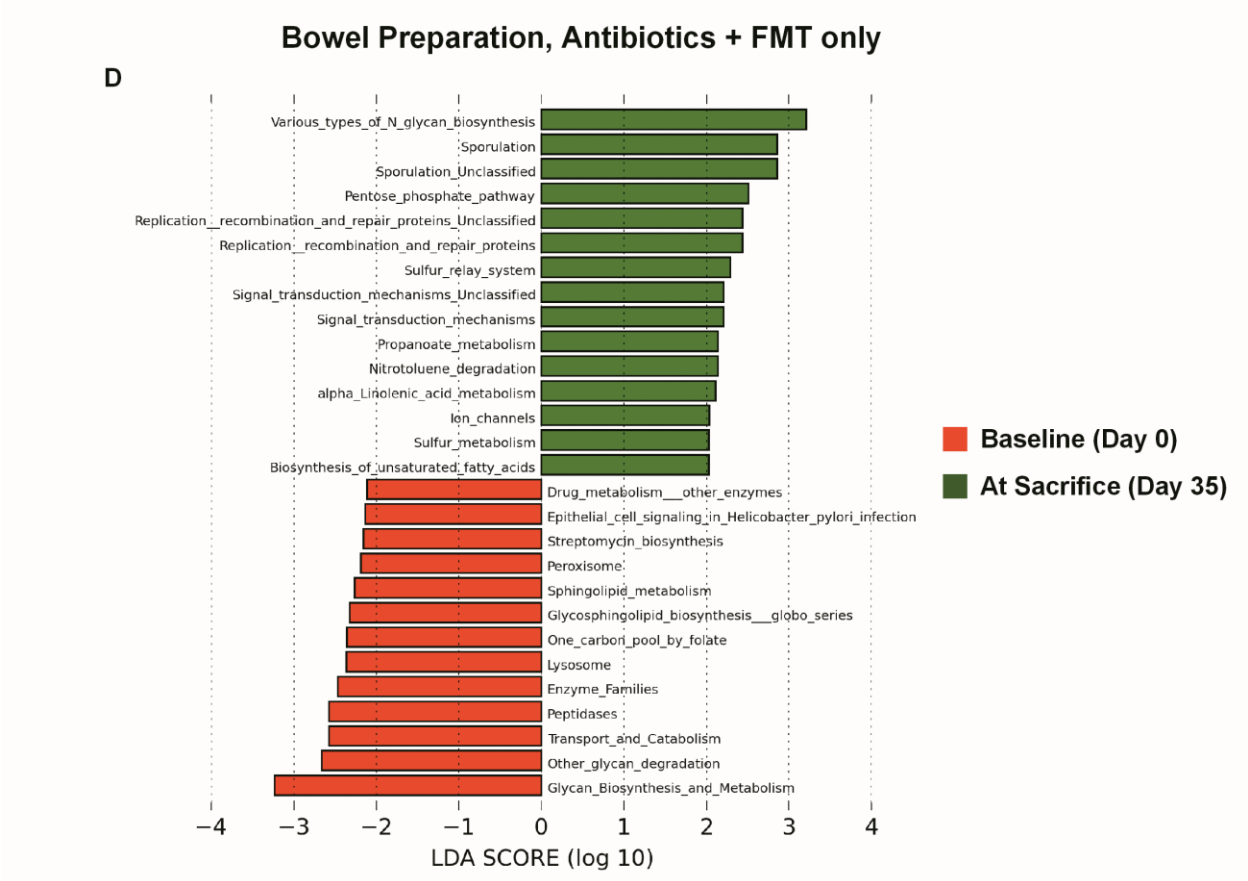


Figure 4-13. **Linear Discriminant Analysis of PICRUSt results comparing the baseline composition to four weeks following FMT in each of the four cohorts**. Using phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) we were able to determine changes in the predicted functional content of the microbiota from the 16S rRNA marker gene comparing baseline (Red; Day 0) taxonomic composition to taxonomic composition 31 days after FMT (Green; Day 35). (P-value < 0.05 using Kruskal-Wallis test)

4.4 Discussion

The primary goal of this study was to determine if we could prevent the development of colitis in IL-10 -/- mice using fecal microbial transplantation, and to see if through altering the pre-FMT treatment regimen, we could enhance its therapeutic potential. It has been established that mice lacking the IL-10 gene have inhibited growth (19), so our first measure of the differing development of disease was body weight. The bowel prep/antibiotics + FMT cohort showed the first signs of a significant increase in body weight, while all four cohorts at the end of the study, four weeks later, had significantly higher body mass than at the start of the study. Madsen et al. showed that at 16 weeks of age IL-10 -/- mouse weight plateaus near 20 grams, whereas the average weight of our four cohorts at 14 weeks of age was 30.4 ± 0.4 grams.(20) This finding suggests that none of our mice were growth retarded; however, sacrifice at 14 weeks may have been before any marked differences could have been observed.

LCN-2 production is part of the innate immune response induced through the activation of Toll-like receptors (TLRs) by bacterial antigens on neutrophils. As neutrophils infiltrate areas of epithelial damage, secreted proteins, such as LCN-2 can enter the gastrointestinal lumen, and therefore can be used as a biochemical marker of inflammatory disease.(35) However, there was a significant difference in the stool LCN-2 levels at baseline between the bowel prep/antibiotics + FMT cohort and the FMT-only cohort. This variability may have been the result of poor separation mice from the same litter into the various treatment cohorts, where it appears the onset/degree of colitis was more similar between littermates than non-littermates at the start of the study (**Figure S4-**

3). Therefore, a possible approach for future studies may be to separate mice into different cohorts based on stool LCN-2 concentrations to reduce the effect of the variable onset of colitis in IL-10 -/- mice. Lastly, from day 7 to day 35, the bowel prep/antibiotics + FMT cohort was the lone cohort to have a reduction in stool LCN-2, nearing the levels detected at baseline, suggesting a long-term positive effect of the bowel prep/antibiotics treatment + FMT on colitis. The colon weight to length ratio (g/cm) was elevated in both cohorts that received antibiotics in their pre-FMT treatment regimen. This correlated with the highest total colonic histology score in the antibiotics + FMT cohort, followed by the bowel prep/antibiotics + FMT cohort, the bowel preparation + FMT cohort, and lastly the FMT-only cohort that showed very minimal signs of inflammation. These results suggest that pre-FMT treatment of IL-10 -/- mice with antibiotics results in a worse disease outcome, while FMT alone, or preceded with a standard polyethylene glycol bowel preparation was sufficient to attenuate the development of colitis in this model.

The cytokines measured in cecum, terminal ileum and serum from samples collected at sacrifice give us a snapshot of the gastrointestinal and systemic immune system at the time of sacrifice. The bowel prep + FMT cohort had the highest concentrations of IFN-γ and TNF-α in the cecum, cytokines that are known to modify epithelial barrier function and are implicated in IBD.(36) These findings could suggest a possible explanation for the increasing levels of LCN-2 detected in this cohort from day 7 to day 35, whereby the development of a leaky gut results in increased localization of neutrophils and passage of neutrophils through the epithelial barrier, resulting in

enhanced immune cell activation and increased levels of luminal LCN-2. Also, higher concentrations of IFN-γ and TNF-α in the antibiotics + FMT and the bowel prep/antibiotics + FMT cohorts could also contribute to the higher levels of LCN-2 and worse histology scores. The FMT-only cohort had minute amounts of IFN-γ, and the lowest levels of TNF-α, which might explain the lower LCN-2 concentrations in this group. These two cytokines have a stronger synergistic effect on the epithelial barrier (37), therefore, having reduced levels of one of the two cytokines may slow the development of colitis. Although concentrations were not extremely high, the antibiotics + FMT cohort also had the highest levels of Interleukin-6, which has also been previously shown to be associated with intestinal barrier dysfunction due to the reduction of the zona occluding protein (ZO-1), allowing increased bacterial translocation across the epithelial barrier.(38) In conjunction with increased levels of IFN-γ and TNF-α, this could explain the increased inflammation detected in this cohort. Increased intestinal permeability has been indicated in early disease development of inflammatory bowel disease, in particular, Crohn's diseases. These findings suggest that FMT can enhance or prevent a reduction in intestinal permeability, and therefore, be a valuable therapeutic for the disease. IL-1β, another colitis associated cytokine was significantly increased in the cecal tissue of the antibiotics + FMT. Research on dextran sodium sulfate (DSS) induced colitis in mice revealed that IL-6, IL-1, and IFN-γ transcripts were significantly reduced following serial FMT, providing further support for enhanced epithelial barrier function following FMT.(39) IFN-γ and TNF-α were also previously shown to negatively correlate with inflammation when IL-10 -/- were given an inhibitor of tight junction opening.(40) Further evidence of the role that bacteria play in this process came from the study on the

probiotic VSL #3, which also resulted in reduced colonic IFN-γ and TNF-α concentrations and histology scores.(41) In tissue samples from the terminal ileum, no significant histological abnormality was detected, and cytokine levels were much lower than in the cecal tissues (**Appendix 4**), demonstrating that FMT does not result in any inflammation of the small intestine. Low serum cytokine levels (**Appendix 4**) suggest that there is no prolonged systemic response to FMT. Differences in disease activity, body weight, and cytokine concentrations suggest that by altering the pre-FMT treatment regimen, we can change the therapeutic effect of FMT on the spontaneous colitis developed by IL-10 -/- mice. To our surprise, the lack of a pre-FMT treatment regimen, avoiding the use of bowel preparation or antibiotics, results in the best outcome on disease activity.

By pooling together the stool from 15 different 129S1/SvlmJ mice from our colony, we expected the FMT slurry to have a more diverse composition of microbes, greater richness, and bacterial load. Alpha-diversity (Shannon-Wiener diversity index) showed the pooled FMT sample from the healthy wild-type mice had a greater diversity, a bacterial load around the average of the IL-10 -/- cohorts, but a much lower richness (number of OTUs). This could have been the result of the FMT processing procedure and the loss of certain bacteria as the stool was processed into its slurry form. The antibiotic solution had the most significant impact on alpha-diversity, richness, and copy number, consistent with the findings of previous studies.(15-17) In contrast, the bowel preparation did not decrease bacterial load or diversity as previously shown (13, 14), although, it is possible that the consumption was not equivalent to the amount

recommended for humans, as the mice were still passing colored diarrhea following treatment. Enhanced diversity at the end of the study was only observed in the bowel prep/antibiotics + FMT cohort, suggesting that emptying the gut of both mucosal associated and luminal bacteria may be necessary for enhanced engraftment of new bacteria. Attenuated development of colitis observed in IL-10 -/- mice in specific pathogen free, germ-free, and following antibiotics may be an explanation for the low disease activity at the end of the study in the FMT-only cohort due to the much lower bacterial load at the start of the study. Furthermore, this could offer an explanation for the high disease activity in the antibiotics + FMT cohort which experienced a significant increase in bacterial load three days following FMT.

The notion behind the use of FMTs as a treatment for IBD comes from the known association between the disease and gut microbial dysbiosis (42-44). The six bacterial families which experienced the most substantial changes in abundance (% total reads) in the microbiome as a result of pre-FMT treatment regimens and FMT were *Porphyromonadaceae, Verrucomicrobiaceae,* an undefined member of the RF32 order, *S24-7, Clostridiaceae,* and *Ruminococcaceae*. A reduction in the family *Porphyromonadaceae* was shown to increase susceptibility to *Clostridium difficile* infection in mice, and to increase the number of tumours in a colorectal cancer model, while enrichment leads to resistance against Salmonella-induced colitis, signifying its role in gastrointestinal health.(45-47) An increased proportion of reads belonging to this family in the antibiotic and bowel prep/antibiotics cohorts following FMT may suggest a positive effect resulting from the pre-treatment regimen although this did not correlate

with reduced inflammation. Counter-intuitively, *S24-7*, a member of the phylum Bacteroidetes, was reduced at day 35 in the FMT-only, bowel prep + FMT, and the bowel prep/antibiotics + FMT cohorts, towards the proportion detected in the donor FMT sample. Although Bateroidetes are critical for gastrointestinal health, their reduced abundance following FMT may have allowed for the expansion of other bacteria essential to host health, whereby the Bacteroides accepted a smaller (realized) niche to allow for the colonization of an important commensal bacteria. Alternatively, *Clostridiaceae* has been both positively and negatively associated with Crohn's disease, (48, 49) while in our study, a significant increase in *Clostridiaceae* was only observed in the FMT-only cohort, which had little to no observed inflammation.

The ratio of two important phyla, the Firmicutes, and Bacteroidetes change over the course of life, being the highest in adulthood.(50) This ratio has also been shown to be significantly higher in healthy subjects versus patients with IBD in remission, and patients with active IBD having the smallest ratio.(51) The ratio of Firmicutes: Bacteroidetes had a net increase in all four cohorts with the largest increase again occurring in the FMT-only cohort while the bowel prep/antibiotics + FMT cohort showed a continually upward trend after being reduced by the pre-FMT treatment regimen. It must be taken into account that these changes in the microbial composition, in our study and the current literature, are correlations and do not necessitate causality. It is therefore important for more precise relationships between the microbiota and health or disease be investigated.

At baseline, the IL-10 -/- mice had on average 57% similarity in microbial composition to the healthy wild-type donors at the level of genera (Jaccard similarity index). Antibiotics before FMT was essential for enhanced similarity to the donor three days after the procedure, however in mice that did not also receive a bowel lavage (antibiotics + FMT), the similarity was only transient. The bowel prep/antibiotics + FMT had the highest mean similarity to the donor. However, the only cohort with increased long-term similarity was the FMT-only cohort, which only had a net increase of 6.47% ($p < 0.05$). Damman and colleagues showed that the patient with the highest percent engraftment of the donor microbiota had the best outcome, although engraftment was transient and did not last longer than one month.(52) These findings, in congruence with our own, suggest that engraftment of the donor microbiota may correlate with efficacy of FMT.

PICRUSt is a commonly used approach to predict the functional content of the microbiota from the 16S rRNA marker gene. Only small changes were observed when comparing the microbial functional capacity before and after treatment in all four cohorts, which were dominated by intracellular bacterial pathways specific to survival and motility. Similar functional pathways were altered three days following FMT in the FMT-only and bowel prep + FMT cohorts, although, reduced glycan, amino acid, cofactor and vitamin metabolism were observed. In the two cohorts with antibiotic pre-FMT treatment, we observed a marked increase in this functional capacity of the microbiota. The bowel prep/antibiotics + FMT cohort showed an increase in essential amino acid (valine, leucine, and isoleucine), unsaturated fatty acid, and fatty acid

biosynthesis, as well as lipid, fructose, and mannose metabolism. In contrast to the other cohorts, the bowel prep/antibiotics + FMT still had several positive changes remaining four weeks following FMT, including unsaturated fatty acid synthesis, alpha-linolenic acid metabolism, and N-glycan biosynthesis. Predicted functional capacity appears to change most significantly in the cohorts that received antibiotic pre-FMT, with the longer-lasting changes in the bowel prep/antibiotics + FMT cohort. Increased energy absorption and greater production of essential metabolites following FMT could explain the differences between the disease outcomes between the four treatment cohorts. In contrast to our original hypothesis, the highest level of engraftment and the correspondingly low disease activity determined through histology, LCN-2, and some pro-inflammatory cytokines occurred in the FMT-only cohort that did not receive any pre-treatment regimen.

A limitation that arose due to the mice available at the time of study design was that we were not able to separate littermates because non-littermates would fight, and housing mice individually was not economically feasible. Therefore, mice from the same litter make up a majority of the mice in each treatment cohort, enhancing the effect of genetic differences. LCN-2 concentrations were significantly different between the FMT-only and bowel prep/antibiotics + FMT cohorts at baseline. This suggests that baseline inflammation was different between the cohorts, and may have contributed to the unexpected results, although there are of course limitations with the use of any biological marker. PLS-DA of the baseline samples showed that the microbial composition of all cohorts was significantly different before any pre-FMT treatment or

the FMT procedure, which could have had an effect on the interpretation of the results. We also did not include cohorts that received only the pre-FMT treatment regimens without an FMT. This makes it difficult to determine if the differences between the cohorts were the result of the pre-FMT treatment regimen or its bowel prep/antibiotics with an FMT. Dethlefsen and Relman have shown that perturbation of the gut microbiota is unique to the individual and that time for recovery back to the pre-antibiotic state is variable.(53) Therefore, although the antibiotic regimens are short, previous evidence of reduced inflammation in IL-10 -/- mice following antibiotic treatment (24) suggests that we are missing relevant control groups within our study, and that the differences observed between our different cohorts could simply be the result of the differing pre-FMT treatment regimens, and not how they alter the therapeutic ability of the FMT. 16S rRNA sequencing enraptures all bacteria, allochthonous or autochthonous, alive or dead, and therefore does not provide an entirely accurate picture of the gut microbiota, and which organisms are essential for host health. PICRUSt, therefore, makes a significant number of assumptions from 16S rRNA data, which must be taken into account when analyzing the findings of these investigations. It has been shown that bacteria have a disproportionate effect on gastrointestinal health and therefore make it tough to predict functional capacities of bacterial communities.(54)

4.5 References

1. Danese S, Fiocchi C. Ulcerative colitis. N Engl J Med. 2011 Nov 3;365(18):1713-25.

2. Silverberg MS, Satsangi J, Ahmad T, Arnott ID, Bernstein CN, Brant SR, et al. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. Can J Gastroenterol. 2005 Sep;19 Suppl A:5A-36A.

3. Colman RJ, Rubin DT. Fecal microbiota transplantation as therapy for inflammatory bowel disease: a systematic review and meta-analysis. J Crohns Colitis. 2014 Dec 1;8(12):1569-81.

4. Lémann M, Zenjari T, Bouhnik Y, Cosnes J, Mesnard B, Rambaud J, et al. Methotrexate in Crohn's disease: long-term efficacy and toxicity. Am J Gastroenterol. 2000;95(7):1730-4.

5. Rutgeerts P, D'Haens G, Targan S, Vasiliauskas E, Hanauer SB, Present DH, et al. Efficacy and safety of retreatment with anti–tumor necrosis factor antibody (infliximab) to maintain remission in Crohn's disease. Gastroenterology. 1999;117(4):761-9.

6. Rachmilewitz D. Coated mesalazine (5-aminosalicylic acid) versus sulphasalazine in the treatment of active ulcerative colitis: a randomised trial. BMJ. 1989 Jan 14;298(6666):82-6.

7. Kohanski MA, Dwyer DJ, Collins JJ. How antibiotics kill bacteria: from targets to networks. Nature Reviews Microbiology. 2010;8(6):423-35.

8. Bartlett JG. Antibiotic-associated diarrhea. Clin Infect Dis. 1992 Oct;15(4):573-81.

9. Walsh CT, Fisher SL, Park I, Prahalad M, Wu Z. Bacterial resistance to vancomycin: five genes and one missing hydrogen bond tell the story. Chem Biol. 1996;3(1):21-8.

10. Freeman CD, Klutman NE, Lamp KC. Metronidazole. Drugs. 1997;54(5):679-708.

11. Cohen SH, Gerding DN, Johnson S, Kelly CP, Loo VG, McDonald LC, et al. Clinical practice guidelines for Clostridium difficile infection in adults: 2010 update by the society for healthcare epidemiology of America (SHEA) and the infectious diseases society of America (IDSA). Infect Control Hosp Epidemiol. 2010 May;31(5):431-55.

12. Rex DK, Imperiale TF, Latinovich DR, Bratcher LL. Impact of bowel preparation on efficiency and cost of colonoscopy. Am J Gastroenterol. 2002;97(7):1696-700.

13. O'Brien CL, Allison GE, Grimpen F, Pavli P. Impact of colonoscopy bowel preparation on intestinal microbiota. PLoS One. 2013 May 1;8(5):e62815.

14. Jalanka J, Salonen A, Salojarvi J, Ritari J, Immonen O, Marciani L, et al. Effects of bowel cleansing on the intestinal microbiota. Gut. 2014 Dec 19.

15. Manichanh C, Rigottier-Gois L, Bonnaud E, Gloux K, Pelletier E, Frangeul L, et al. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. Gut. 2006 Feb;55(2):205-11.

16. Vrieze A, Out C, Fuentes S, Jonker L, Reuling I, Kootte RS, et al. Impact of oral vancomycin on gut microbiota, bile acid metabolism, and insulin sensitivity. J Hepatol. 2014 Apr;60(4):824-31.

17. Yap IK, Li JV, Saric J, Martin FP, Davies H, Wang Y, et al. Metabonomic and microbiological analysis of the dynamic effect of vancomycin-induced gut microbiota modification in the mouse. J Proteome Res. 2008 Sep;7(9):3718-28.

18. Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J Exp Med. 1989 Dec 1;170(6):2081-95.

19. Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. Cell. 1993 Oct 22;75(2):263-74.

20. Madsen KL, Malfair D, Gray D, Doyle JS, Jewell LD, Fedorak RN. Interleukin-10 gene-deficient mice develop a primary intestinal permeability defect in response to enteric microflora. Inflamm Bowel Dis. 1999;5(4):262-70.

21. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol. 2001;19(1):683-765.

22. Sellon RK, Tonkonogy S, Schultz M, Dieleman LA, Grenther W, Balish E, et al. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. Infect Immun. 1998 Nov;66(11):5224-31.

23. Pedrosa E, Lorén V, Cabré E, Doménech E, Ojanguren I, Gassull MA, et al. Bacteria and spontaneous experimental colitis: immunological changes. Eur J Clin Invest. 2011;41(10):1047-53.

24. Madsen KL, Doyle JS, Tavernini MM, Jewell LD, Rennie RP, Fedorak RN. Antibiotic therapy attenuates colitis in interleukin 10 gene–deficient mice. Gastroenterology. 2000;118(6):1094-105.

25. Madsen KL, Doyle JS, Jewell LD, Tavernini MM, Fedorak RN. Lactobacillus species prevents colitis in interleukin 10 gene–deficient mice. Gastroenterology. 1999;116(5):1107-14.

26. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010 May;7(5):335-6.

27. Langille MG, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat Biotechnol. 2013;31(9):814-21.

28. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. Genome Biol. 2011;12(6):R60.

29. Chassaing B, Srinivasan G, Delgado MA, Young AN, Gewirtz AT, Vijay-Kumar M. Fecal lipocalin 2, a sensitive and broadly dynamic non-invasive biomarker for intestinal inflammation. PLoS One. 2012;7(9):e44328.

30. Berg DJ, Davidson N, Kuhn R, Muller W, Menon S, Holland G, et al. Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. J Clin Invest. 1996 Aug 15;98(4):1010-20.

31. Xia J, Sinelnikov IV, Han B, Wishart DS. MetaboAnalyst 3.0--making metabolomics more meaningful. Nucleic Acids Res. 2015 Jul 1;43(W1):W251-7.

32. Xia J, Psychogios N, Young N, Wishart DS. MetaboAnalyst: a web server for metabolomic data analysis and interpretation. Nucleic Acids Res. 2009 Jul;37(Web Server issue):W652-60.

33. Golland P, Liang F, Mukherjee S, Panchenko D. Permutation tests for classification. In: Learning Theory. Springer; 2005. p. 501-15.

34. Westerhuis JA, Hoefsloot HC, Smit S, Vis DJ, Smilde AK, van Velzen EJ, et al. Assessment of PLSDA cross validation. Metabolomics. 2008;4(1):81-9.

35. Xu S, Venge P. Lipocalins as biochemical markers of disease. Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology. 2000;1482(1):298-307.

36. Capaldo CT, Nusrat A. Cytokine regulation of tight junctions. Biochimica et Biophysica Acta (BBA)-Biomembranes. 2009;1788(4):864-71.

37. Fish SM, Proujansky R, Reenstra WW. Synergistic effects of interferon gamma and tumour necrosis factor alpha on T84 cell function. Gut. 1999 Aug;45(2):191-8.

38. Yang R, Han X, Uchiyama T, Watkins SK, Yaguchi A, Delude RL, et al. IL-6 is essential for development of gut barrier dysfunction after hemorrhagic shock and resuscitation in mice. Am J Physiol Gastrointest Liver Physiol. 2003 Sep;285(3):G621-9.

39. Tian Z, Liu J, Liao M, Li W, Zou J, Han X, et al. Beneficial effects of fecal microbiota transplantation on ulcerative colitis in mice. Dig Dis Sci. 2016:1-10.

40. Arrieta MC, Madsen K, Doyle J, Meddings J. Reducing small intestinal permeability attenuates colitis in the IL10 gene-deficient mouse. Gut. 2009 Jan;58(1):41-8.

41. Madsen K, Cornish A, Soper P, McKaigney C, Jijon H, Yachimec C, et al. Probiotic bacteria enhance murine and human intestinal epithelial barrier function. Gastroenterology. 2001;121(3):580-91.

42. Berry D, Reinisch W. Intestinal microbiota: a source of novel biomarkers in inflammatory bowel diseases? Best Pract Res Clin Gastroenterol. 2013 Feb;27(1):47-58.

43. Hansen R, Russell RK, Reiff C, Louis P, McIntosh F, Berry SH, et al. Microbiota of de-novo pediatric IBD: increased Faecalibacterium prausnitzii and reduced bacterial diversity in Crohn's but not in ulcerative colitis. Am J Gastroenterol. 2012 Dec;107(12):1913-22.

44. Fujimoto T, Imaeda H, Takahashi K, Kasumi E, Bamba S, Fujiyama Y, et al. Decreased abundance of Faecalibacterium prausnitzii in the gut microbiota of Crohn's disease. J Gastroenterol Hepatol. 2013 Apr;28(4):613-9.

45. Bassis CM, Theriot CM, Young VB. Alteration of the murine gastrointestinal microbiota by tigecycline leads to increased susceptibility to Clostridium difficile infection. Antimicrob Agents Chemother. 2014 May;58(5):2767-74.

46. Zackular JP, Baxter NT, Iverson KD, Sadler WD, Petrosino JF, Chen GY, et al. The gut microbiome modulates colon tumorigenesis. MBio. 2013 Nov 5;4(6):e00692-13.

47. Ferreira RB, Gill N, Willing BP, Antunes LCM, Russell SL, Croxen MA, et al. The intestinal microbiota plays a role in Salmonella-induced colitis independent of pathogen colonization. PLoS One. 2011;6(5):e20338.

48. Martinez-Medina M, Aldeguer X, Gonzalez-Huix F, Acero D, Garcia-Gil LJ. Abnormal microbiota composition in the ileocolonic mucosa of Crohn's disease patients as revealed by polymerase chain reaction-denaturing gradient gel electrophoresis. Inflamm Bowel Dis. 2006;12(12):1136-45.

49. Kaakoush NO, Day AS, Huinao KD, Leach ST, Lemberg DA, Dowd SE, et al. Microbial dysbiosis in pediatric patients with Crohn's disease. J Clin Microbiol. 2012 Oct;50(10):3258-66.

50. Mariat D, Firmesse O, Levenez F, Guimaraes V, Sokol H, Dore J, et al. The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. BMC Microbiol. 2009 Jun 9;9:123,2180-9-123.

51. Sokol H, Seksik P, Furet J, Firmesse O, Nion-Larmurier I, Beaugerie L, et al. Low counts of Faecalibacterium prausnitzii in colitis microbiota. Inflamm Bowel Dis. 2009;15(8):1183-9.

52. Damman CJ, Brittnacher MJ, Westerhoff M, Hayden HS, Radey M, Hager KR, et al. Low Level Engraftment and Improvement following a Single Colonoscopic Administration of Fecal Microbiota to Patients with Ulcerative Colitis. PLoS One. 2015 Aug 19;10(8):e0133925.

53. Dethlefsen L, Relman DA. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. Proc Natl Acad Sci U S A. 2011 Mar 15;108 Suppl 1:4554-61.

54. Rolig AS, Parthasarathy R, Burns AR, Bohannan BJ, Guillemin K. Individual Members of the Microbiota Disproportionately Modulate Host Innate Immune Responses. Cell Host Microbe. 2015 Nov 11;18(5):613-20.

4.6 Supplementary Material

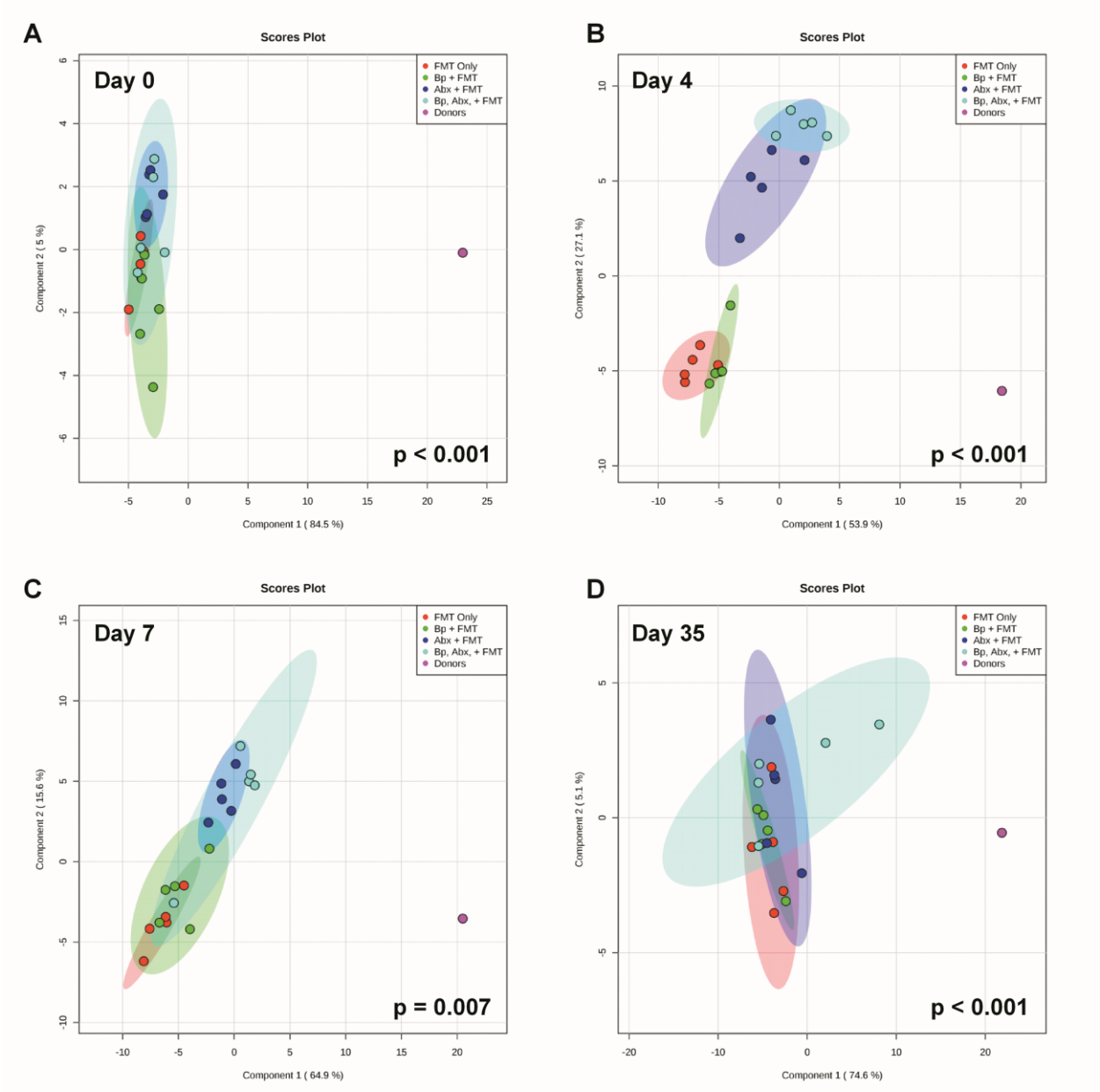


Figure S4-1. Partial least squares Discriminant Analysis (PLS-DA) of the microbial profiles of mice at all 4 time-points eith the donor FMT slurry made using MetaboAnalyst 3.0 (with 95% CI). Red = FMT only (n=5); Green = bowel preparation + FMT (n=5); Blue = antibiotics + FMT (n=5); Teal = bowel prep/antibiotics + FMT (n=5); and Purple = donor FMT slurry (n=1). P-values were calculated using the provided permutation test consisting of 1000 permutations.

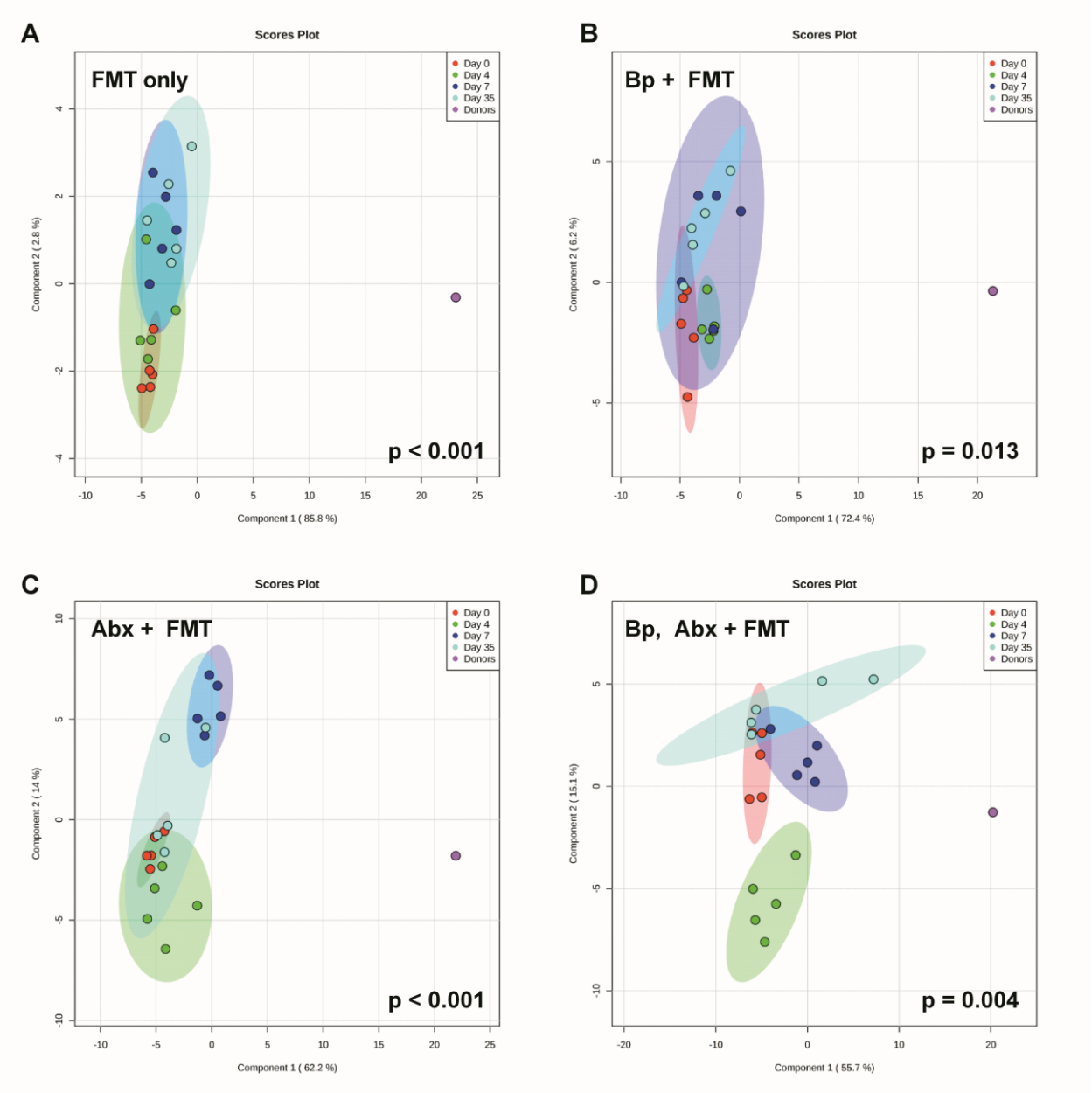


Figure S4-2. Partial least squares Discriminant Analysis (PLS-DA) of the microbial profiles of mice from all 4 cohorts with the donor FMT slurry made using MetaboAnalyst 3.0 (with 95% CI). Red = baseline (Day 0; n=5); Green = after treatment (Day 4; n=5); Blue = 3 days following FMT (Day 7; n=5); Teal = 4 weeks following FMT (Day 35; n=5); and Purple = donor FMT slurry (n=1). P-values were calculated using the provided permutation test consisting of 1000 permutations.

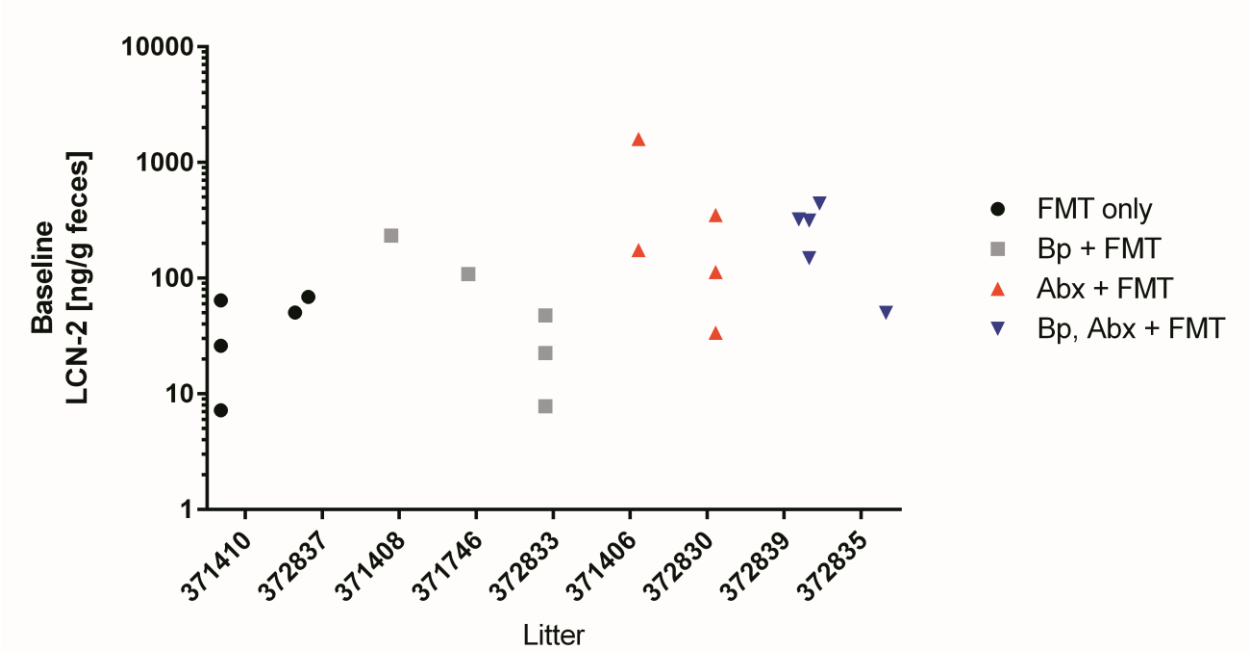
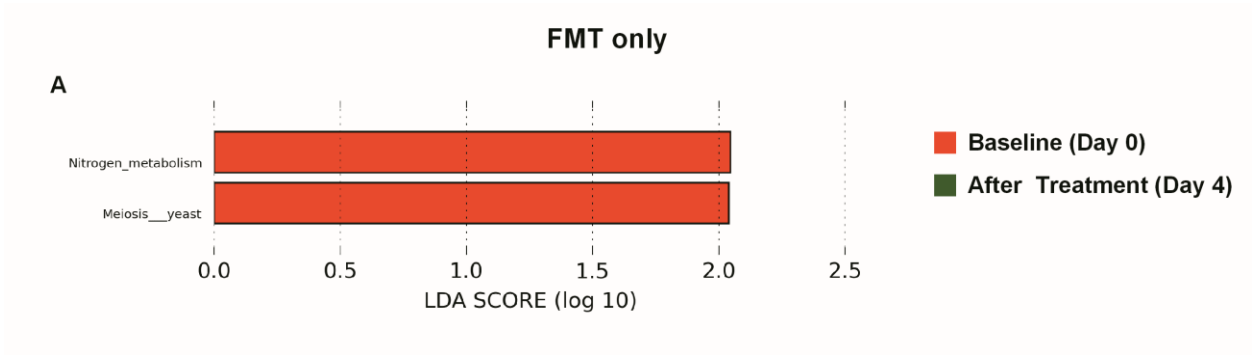
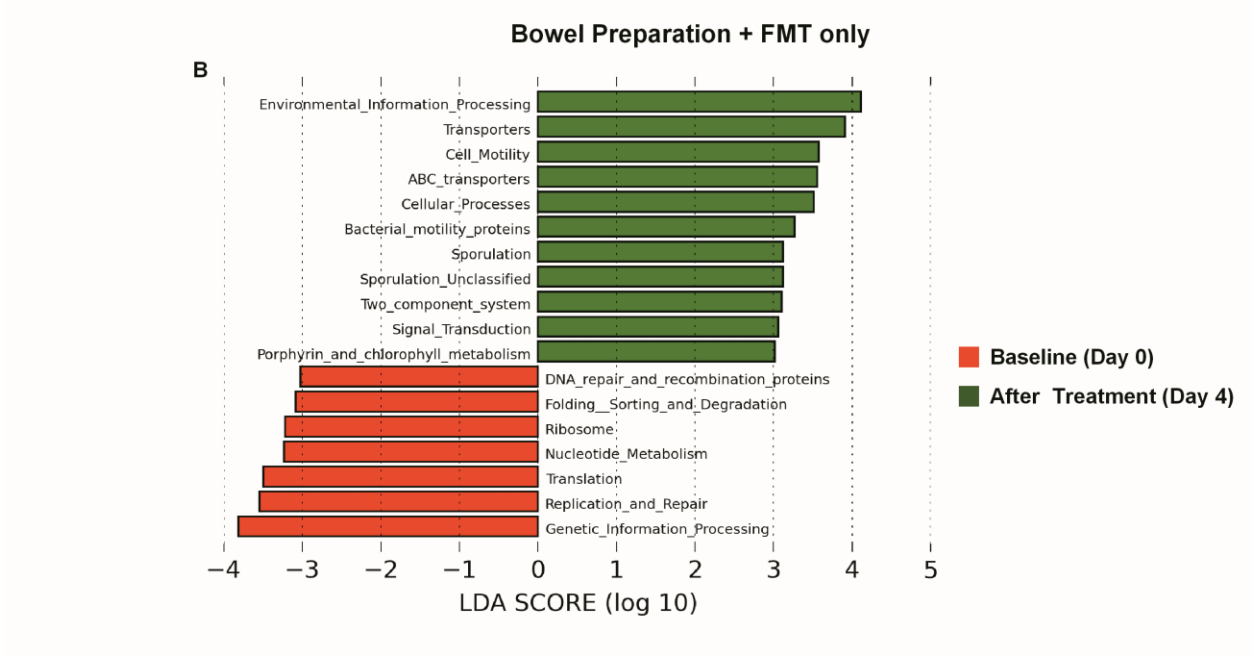
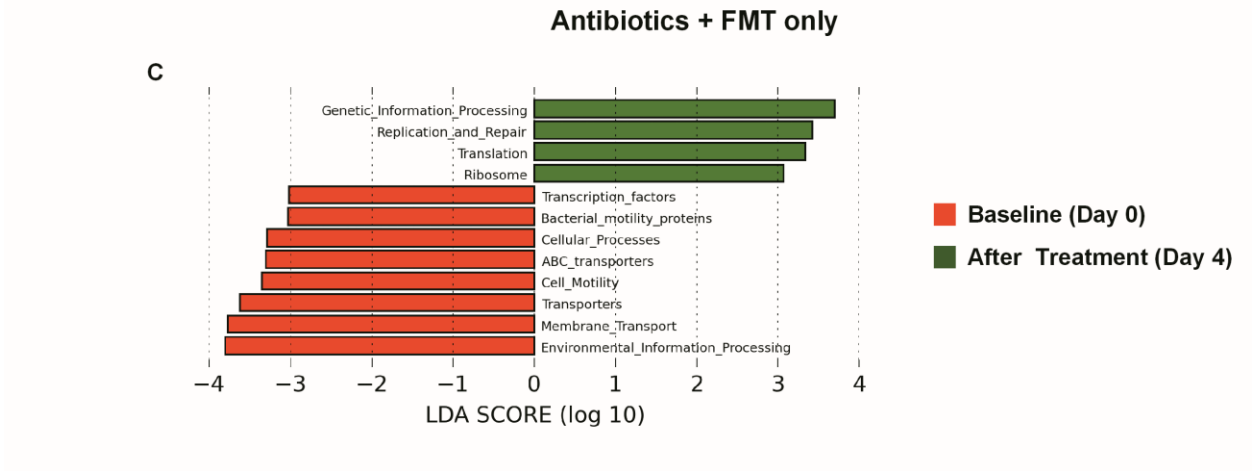


Figure S4-3. Stool lipocalin-2 (LCN-2) levels in mice separated by litter. Measured using enzyme-linked immunosorbent assay (ELISA) at baseline (day 0) in all four cohorts (Log10). Black = FMT only (n=5); Grey = bowel preparation + FMT (n=5); Red = antibiotics + FMT (n=5); and Blue = bowel prep/antibiotics + FMT (n=5).


FMT only
A
Nitrogen_metabolism
Meiosis__yeast
0.0
0.5
1.0
1.5
2.0
2.5
LDA SCORE (log 10)
Baseline (Day 0)
After Treatment (Day 4)


Bowel Preparation + FMT only
B
Environmental_Information_Processing
Transporters
Cell_Motility
ABC_transporters
Cellular_Processes
Bacterial_motility_proteins
Sporulation
Sporulation_Unclassified
Two_component_system
Signal_Transduction
Porphyrin_and_chlorophyll_metabolism
DNA_repair_and_recombination_proteins
Folding__Sorting_and_Degradation
Ribosome
Nucleotide_Metabolism
Translation
Replication_and_Repair
Genetic_Information_Processing
−4
−3
−2
−1
0
1
2
3
4
5
LDA SCORE (log 10)
Baseline (Day 0)
After Treatment (Day 4)


Antibiotics + FMT only
C
Genetic_Information_Processing
Replication_and_Repair
Translation
Ribosome
Transcription_factors
Bacterial_motility_proteins
Cellular_Processes
ABC_transporters
Cell_Motility
Transporters
Membrane_Transport
Environmental_Information_Processing
−4
−3
−2
−1
0
1
2
3
4
LDA SCORE (log 10)
Baseline (Day 0)
After Treatment (Day 4)

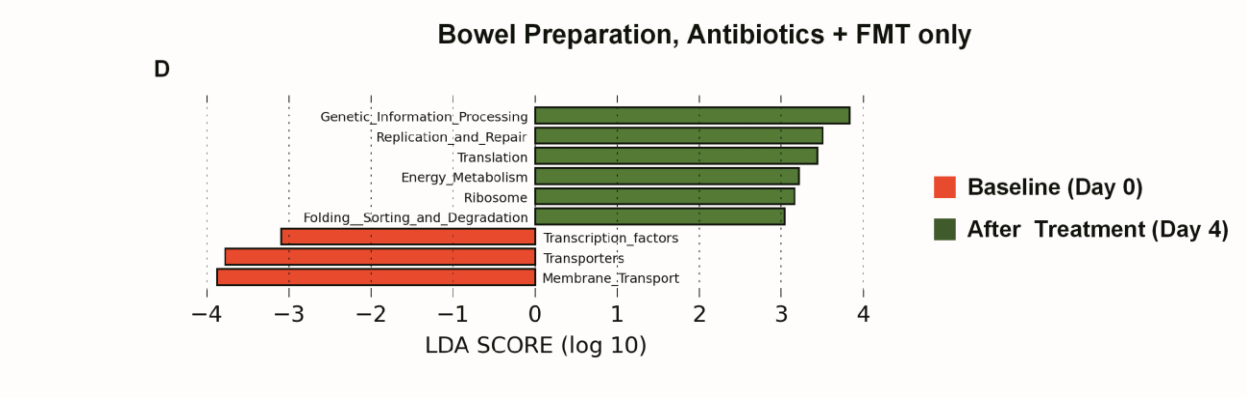


Figure S4-4. **Linear Discriminant Analysis of PICRUSt results comparing the baseline composition to after pre-treatment in each of the four cohorts**. Using phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) we were able to determine changes in the predicted functional content of the microbiota from the 16S rRNA marker gene comparing baseline (Red; Day 0) taxonomic composition to taxonomic composition after treatment (Green; Day 4). (P-value < 0.05 using Kruskal-Wallis test)

Figure S4-5A

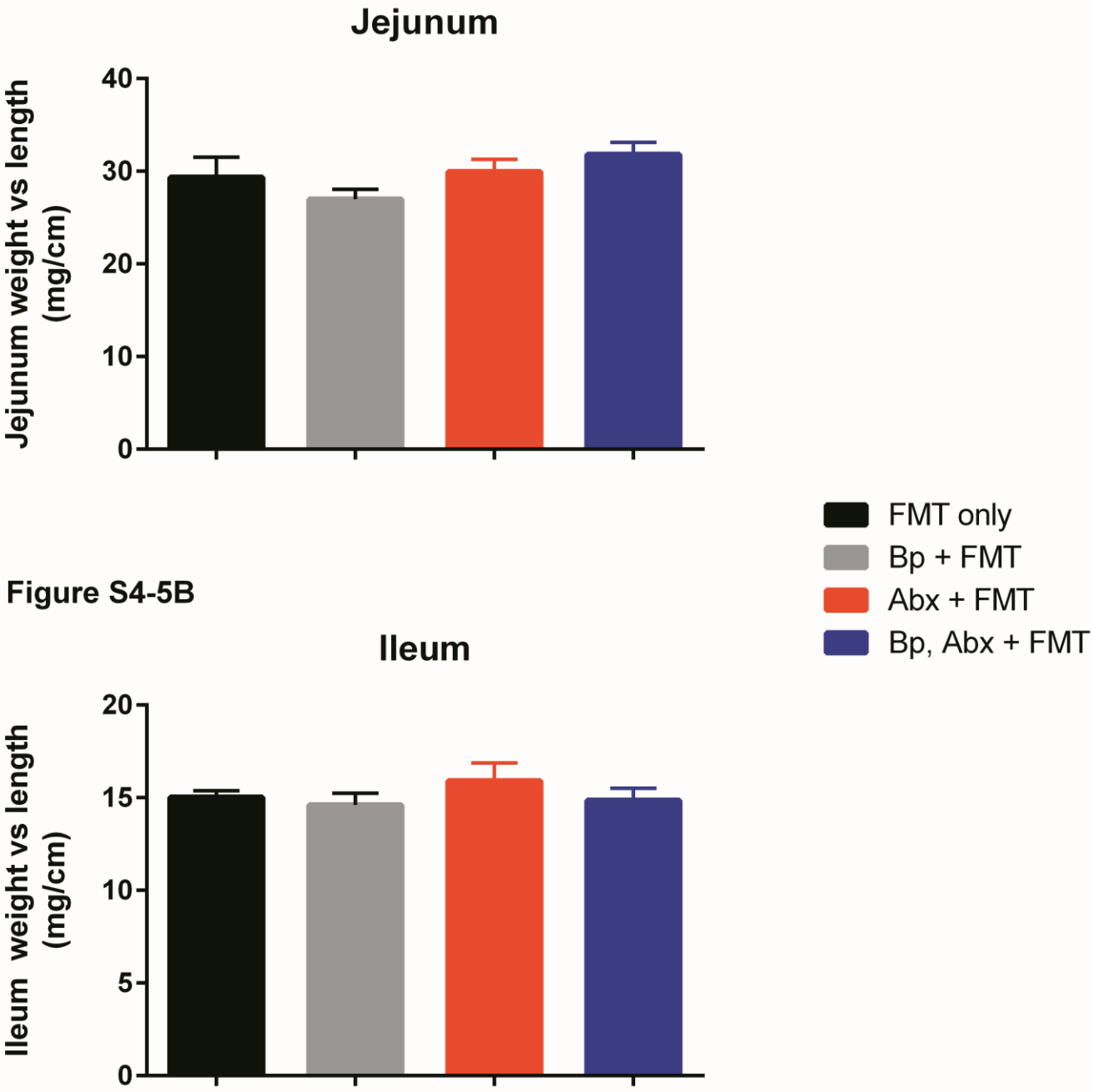


Figure S4-5B

Figure S4-5. Weight to length ratio of two portions of the small intestine. (A) Jejunum weight to length ratio taken at sacrifice (mg/cm). **(B)** Ileum weight to length ratio taken at sacrifice (mg/cm). Black = FMT only (n=5); Grey = bowel preparation + FMT (n=5); Red = antibiotics + FMT (n=5); and Blue = bowel prep/antibiotics + FMT (n=5).

Chapter 5: Discussion and Future Directions

5.1 Discussion

There are a number of diseases with characterized associations to the microbiota and its dysbiosis, which has increased interest in altering the microbiota to treat the underlying conditions. For *Clostridium difficile* infection (CDI) and recurrent CDI (RCDI), the microbiota or lack thereof plays a central role in the pathogenesis of the infection. By re-establishing the microbiota with fecal microbial transplantation (FMT), we can effectively treat the infection, and prevent recurrence.(1) Unlike other diseases in which the dysbiosis of the microbiota has been implicated, altering the microbiota in RCDI is congruent with basic concepts of ecology. FMT restores colonization resistance of the gut microbial community against the pathogen, eradicating the infection, and treating the symptoms. The idea surrounding the treatment of RCDI is quite simple, with many different approaches being effective, and even in patients with underlying gastrointestinal disease such as inflammatory bowel disease (IBD).(2) However, the safety and efficacy of FMTs for IBD is still a subject that requires further investigation.

Increased use of antibiotics in agriculture and healthcare has resulted in an amplified threat of bacterial resistance, in particular in pathogenic bacteria that pose a threat to human health.(3) Selective digestive decontamination together with the intravenous administration of antibiotics in critically ill patients has been shown to be an effective approach for infection prevention (4-6); however, it is also associated with dramatic increases in antibiotic resistance (ABR) genes.(7) Also, once the treatment is

discontinued, the patients remained colonized with these multi-drug resistant pathogens and as the gut becomes recolonized following the removal of antibiotics an increased horizontal transfer of resistance genes from the surviving organisms to opportunistic aerobic pathogens may occur.(8) Eradication of virulent organisms containing ABR genes from the gut may also help in alleviating systemic infections, as studies have shown pathogenic organisms to colonize the gut prior to translocating to and infecting other body sites.(9) Case reports showing eradication of multidrug-resistant organisms in patients following FMT together with evidence from a murine study suggesting that multi-drug resistant bacteria such as vancomycin-resistant *Enterococcus faecium* (VRE), and Carbapenem-resistant *Klebsiella penumoniae* (CRKP) can be eliminated from the gut following FMT.(10-12) Our results demonstrate that patients with RCDI harbor large numbers of microbes that carry a great diversity of ABR genes, which poses the same threat of horizontal transfer of ABR genes to other pathogenic organisms following recolonization of the gut. However, FMT was effective in reducing the load of ABR genes, with no apparent horizontal transfer of ABR genes to microbes introduced via FMT. These findings suggest that FMT may be able to eradicate multi-drug resistant bacterial infections or alternatively restore antibiotic susceptibility to individual patients. FMT may also be used in individuals treated with antibiotics prior to infection to reduce the number of ABR genes in the gut to proactively prevent the transfer of ABR genes to infectious bacteria in the future. Taking into account the threat of horizontal gene transfer of ABR genes, FMT should be considered a rational option for first-line treatment of CDI given the difficulty in treating ABR bacteria and the threat an infection

poses on the elderly population most affect by CDI. Furthermore, FMT has been shown to be economical and effective when provided to patients after fewer recurrences.(13)

Enhanced engraftment of the donor microbiota into the recipient is a defining feature of a successful FMT for the treatment of RCDI.(14) In the treatment of IBD, Damman and colleagues showed that the patient with the highest percent engraftment of the donor microbiota had the best outcome.(15) With these findings in mind, we hypothesized that enhancing the engraftment of donor microbiota using a pre-FMT treatment would result in a more efficient therapy.

Currently available studies suggest that bowel preparation can reduce the diversity of the gut microbiota transiently or long-term.(16, 17) Antibiotics are known to impact diversity, richness, and bacterial load in the gut, reducing colonization resistance and creating an environment more susceptible to colonization by allochthonous bacteria including pathogens.(18-20) Counterintuitively, the work by Manichanh and colleagues suggested that antibiotic treatment pre-FMT did not result in enhanced engraftment in their rats.(21) Our results in healthy wild-type mice showed that engraftment of the donor microbiota, defined by the introduction and/or removal of new genera following FMT occurred to the same amount regardless of treatment before FMT with antibiotics or bowel preparation. However, we also showed that antibiotic treatment followed by FMT results in more changes in diversity (evenness and richness) of the gut microbial composition. Therefore, it is possible that we are focusing on too high of a taxonomic level, and that changes at the species level are occurring, where there are varying degrees

of coexistence between donor and recipient species following different pre-treatment and FMT regimens. Bowel lavage prior to FMT resulted in the largest increase in microbial diversity following FMT, peaking at the end of the study. Reduced diversity has now been associated with a multitude of health issues such as necrotizing enterocolitis (22), increased risk of allergies (23), Crohn's disease (18) while increased diversity and richness are correlated with overall health in an elderly population.(24)

Interest in FMT as a treatment for IBD comes from the documented association between the disease and gut microbial dysbiosis (25-27), although evidence suggests that this may be the result of continuous gastrointestinal inflammation and not the cause of it.(28) Increased intestinal permeability has been indicated in early disease development of IBD, in particular, Crohn's diseases (CD). This association with disease and epithelial barrier function has also been made in IL-10 -/- mice, with evidence that probiotic bacteria can enhance barrier function and reduce disease scores.(29, 30) Our results and the findings of other studies suggest that improving barrier function may contribute to the efficacy of FMT in IBD.(31) Low serum cytokine levels suggest that there is no prolonged systemic response to FMT, however, high incidence of fever following FMT may indicate a short systemic response immediately following FMT (**Table 1-2**).

To our surprise, the lack of a pre-FMT treatment regimen, avoiding the use of bowel preparation or antibiotics, results in the best outcome on disease activity. It is possible that the influx of bacteria from an FMT is distracting the hyperactive immune system from targeting the host and temporarily provides a relief of symptoms. However,

there is evidence that FMT can result in long-term remission in patients with ulcerative colitis (UC).(32) Therefore, the mechanisms of effective FMTs for the treatment of IBD still have to be determined. In human studies, there appears to be a difference in the effectiveness of the FMT depending on the donor, although there are also patients who respond and those who do not respond to an FMT from the same donor (**Table 1-2**). The evidence suggests that induction of clinical remission is both host and donor dependent. Individual members of the gut microbiota have a disproportionate effect on gastrointestinal health based on their abundance and therefore make it challenging to predict functional capacities of bacterial communities.(33) The ratio of the Firmicutes to Bacteroidetes has also been shown to be significantly higher in healthy subjects versus patients with IBD in remission, and patients with active IBD having the smallest ratio.(34) Changes in this ratio differed between our four treatment cohorts in IL-10 -/- mice, which may have implications the differing efficacy in treating the spontaneous colitis between cohorts. It is imperative that more precise relationships between the microbiota and health or disease are investigated.

5.2 Significance of my work

We believe that findings of our research are extremely important as it provides clear scientific evidence that FMT may be a viable treatment option to eradicate multi-drug resistance bacteria from patients, thus expanding the use of FMT into other therapeutic realms. As an experimental therapeutic, a standardized procedure for FMTs has yet to be agreed upon. Our results indicate that a short pre-treatment therapy of antibiotics, bowel preparation, or a combination prior to FMT has a significant effect on

donor microbiota engraftment into the recipient, and consequently a differential effect on therapeutic efficacy. Although we do not provide enough evidence to recommend one approach over another, we did elucidate that pre-treatment regimens are extremely important for FMTs in an IBD model, and should be a major consideration in future research.

5.3 Future Directions

Future research should focus on the following:

1. In human investigations, it is common for patients to experience a fever within 24 hours following an FMT that resolves itself (**Table 1-2**). Valuable information could be collected looking at shorter time intervals following the FMT and how the immune system reacts to the influx of bacteria, and if this immune response changes depending on the type of pre-FMT treatment regimen.

3. For clinical trials using FMT for IBD, information gained in the literature review (**Table 1-2**) would lead me to suggest including both an FMT with antibiotics and FMT without antibiotics arms to the study.

4. Stool substitute preparations containing purified intestinal bacterial cultures have been shown to be as effective as human stool FMTs.(35) Designing and investigating the use

of a particular bacterial consortium for the treatment of colitis in an animal model could elucidate more information regarding the mechanism of FMTs for the treatment of IBD.

5. Given the findings of the mouse projects, a possible approach for future studies may be to separate mice into different cohorts based on stool lipocalin-2 (LCN-2) concentrations to reduce the effect of the variable onset of colitis in IL-10 -/- mice. Repeating similar experiments using this approach may better differentiate the effect of the therapeutic approaches.

5.4 References

1. Colman RJ, Rubin DT. Fecal microbiota transplantation as therapy for inflammatory bowel disease: a systematic review and meta-analysis. J Crohns Colitis. 2014 Dec 1;8(12):1569-81.

2. Khoruts A, Rank KM, Newman KM, Viskocil K, Vaughn BP, Hamilton MJ, et al. Inflammatory Bowel Disease Affects the Outcome of Fecal Microbiota Transplantation for Recurrent Clostridium difficile Infection. Clin Gastroenterol Hepatol. 2016 Feb 22.

3. Neu HC. The crisis in antibiotic resistance. Science. 1992 Aug 21;257(5073):1064-73.

4. de Jonge E, Schultz MJ, Spanjaard L, Bossuyt PM, Vroom MB, Dankert J, et al. Effects of selective decontamination of digestive tract on mortality and acquisition of resistant bacteria in intensive care: a randomised controlled trial. The Lancet. 2003;362(9389):1011-6.

5. Schultz MJ, de Jonge E, Kesecioglu J. Selective decontamination of the digestive tract reduces mortality in critically ill patients. CRITICAL CARE-LONDON-. 2003;7(2):107-10.

6. De Smet A, Kluytmans J, Cooper B, Mascini E, Benus R, Van der Werf T, et al. Decontamination of the digestive tract and oropharynx in ICU patients. N Engl J Med. 2009;360(1):20.

7. Buelow E, Gonzalez TB, Versluis D, Oostdijk EA, Ogilvie LA, van Mourik MS, et al. Effects of selective digestive decontamination (SDD) on the gut resistome. J Antimicrob Chemother. 2014 Aug;69(8):2215-23.

8. Oostdijk EA, de Smet, Anne Marie GA, Blok HE, Thieme Groen ES, van Asselt GJ, Benus RF, et al. Ecological effects of selective decontamination on resistant gram-negative bacterial colonization. American journal of respiratory and critical care medicine. 2010;181(5):452-7.

9. Ubeda C, Taur Y, Jenq RR, Equinda MJ, Son T, Samstein M, et al. Vancomycin-resistant Enterococcus domination of intestinal microbiota is enabled by antibiotic treatment in mice and precedes bloodstream invasion in humans. J Clin Invest. 2010 Dec;120(12):4332-41.

10. Crum-Cianflone NF, Sullivan E, Ballon-Landa G. Fecal microbiota transplantation and successful resolution of multidrug-resistant-organism colonization. J Clin Microbiol. 2015 Jun;53(6):1986-9.

11. Singh R, Nieuwdorp M, ten Berge IJ, Bemelman FJ, Geerlings SE. The potential beneficial role of faecal microbiota transplantation in diseases other than Clostridium difficile infection. Clin Microbiol Infect. 2014 Nov;20(11):1119-25.

12. Caballero S, Carter R, Ke X, Susac B, Leiner IM, Kim GJ, et al. Distinct but Spatially Overlapping Intestinal Niches for Vancomycin-Resistant Enterococcus faecium and Carbapenem-Resistant Klebsiella pneumoniae. PLoS Pathog. 2015 Sep 3;11(9):e1005132.

13. Waye A, Atkins K, Kao D. Cost Averted With Timely Fecal Microbiota Transplantation in the Management of Recurrent Clostridium difficile Infection in Alberta, Canada. J Clin Gastroenterol. 2016 Feb 17.

14. Hamilton MJ, Weingarden AR, Unno T, Khoruts A, Sadowsky MJ. High-throughput DNA sequence analysis reveals stable engraftment of gut microbiota following transplantation of previously frozen fecal bacteria. Gut microbes. 2013;4(2):125-35.

15. Damman CJ, Brittnacher MJ, Westerhoff M, Hayden HS, Radey M, Hager KR, et al. Low Level Engraftment and Improvement following a Single Colonoscopic Administration of Fecal Microbiota to Patients with Ulcerative Colitis. PLoS One. 2015 Aug 19;10(8):e0133925.

16. O'Brien CL, Allison GE, Grimpen F, Pavli P. Impact of colonoscopy bowel preparation on intestinal microbiota. PLoS One. 2013 May 1;8(5):e62815.

17. Jalanka J, Salonen A, Salojarvi J, Ritari J, Immonen O, Marciani L, et al. Effects of bowel cleansing on the intestinal microbiota. Gut. 2014 Dec 19.

18. Manichanh C, Rigottier-Gois L, Bonnaud E, Gloux K, Pelletier E, Frangeul L, et al. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. Gut. 2006 Feb;55(2):205-11.

19. Vrieze A, Out C, Fuentes S, Jonker L, Reuling I, Kootte RS, et al. Impact of oral vancomycin on gut microbiota, bile acid metabolism, and insulin sensitivity. J Hepatol. 2014 Apr;60(4):824-31.

20. Yap IK, Li JV, Saric J, Martin FP, Davies H, Wang Y, et al. Metabonomic and microbiological analysis of the dynamic effect of vancomycin-induced gut microbiota modification in the mouse. J Proteome Res. 2008 Sep;7(9):3718-28.

21. Manichanh C, Reeder J, Gibert P, Varela E, Llopis M, Antolin M, et al. Reshaping the gut microbiome with bacterial transplantation and antibiotic intake. Genome Res. 2010 Oct;20(10):1411-9.

22. Wang Y, Hoenig JD, Malin KJ, Qamar S, Petrof EO, Sun J, et al. 16S rRNA gene-based analysis of fecal microbiota from preterm infants with and without necrotizing enterocolitis. The ISME journal. 2009;3(8):944-54.

23. Bisgaard H, Li N, Bonnelykke K, Chawes BLK, Skov T, Paludan-Müller G, et al. Reduced diversity of the intestinal microbiota during infancy is associated with increased risk of allergic disease at school age. J Allergy Clin Immunol. 2011;128(3):646,652. e5.

24. Claesson MJ, Jeffery IB, Conde S, Power SE, O'Connor EM, Cusack S, et al. Gut microbiota composition correlates with diet and health in the elderly. Nature. 2012;488(7410):178-84.

25. Berry D, Reinisch W. Intestinal microbiota: a source of novel biomarkers in inflammatory bowel diseases? Best Pract Res Clin Gastroenterol. 2013 Feb;27(1):47-58.

26. Hansen R, Russell RK, Reiff C, Louis P, McIntosh F, Berry SH, et al. Microbiota of de-novo pediatric IBD: increased Faecalibacterium prausnitzii and reduced bacterial diversity in Crohn's but not in ulcerative colitis. Am J Gastroenterol. 2012 Dec;107(12):1913-22.

27. Fujimoto T, Imaeda H, Takahashi K, Kasumi E, Bamba S, Fujiyama Y, et al. Decreased abundance of Faecalibacterium prausnitzii in the gut microbiota of Crohn's disease. J Gastroenterol Hepatol. 2013 Apr;28(4):613-9.

28. Winter SE, Lopez CA, Baumler AJ. The dynamics of gut-associated microbial communities during inflammation. EMBO Rep. 2013 Apr;14(4):319-27.

29. Madsen K, Cornish A, Soper P, McKaigney C, Jijon H, Yachimec C, et al. Probiotic bacteria enhance murine and human intestinal epithelial barrier function. Gastroenterology. 2001;121(3):580-91.

30. Madsen KL, Doyle JS, Jewell LD, Tavernini MM, Fedorak RN. Lactobacillus species prevents colitis in interleukin 10 gene–deficient mice. Gastroenterology. 1999;116(5):1107-14.

31. Tian Z, Liu J, Liao M, Li W, Zou J, Han X, et al. Beneficial effects of fecal microbiota transplantation on ulcerative colitis in mice. Dig Dis Sci. 2016:1-10.

32. Borody TJ, Warren EF, Leis S, Surace R, Ashman O. Treatment of ulcerative colitis using fecal bacteriotherapy. J Clin Gastroenterol. 2003 Jul;37(1):42-7.

33. Rolig AS, Parthasarathy R, Burns AR, Bohannan BJ, Guillemin K. Individual Members of the Microbiota Disproportionately Modulate Host Innate Immune Responses. Cell Host Microbe. 2015 Nov 11;18(5):613-20.

34. Sokol H, Seksik P, Furet J, Firmesse O, Nion-Larmurier I, Beaugerie L, et al. Low counts of Faecalibacterium prausnitzii in colitis microbiota. Inflamm Bowel Dis. 2009;15(8):1183-9.

35. Petrof EO, Gloor GB, Vanner SJ, Weese SJ, Carter D, Daigneault MC, et al. Stool substitute transplant therapy for the eradication of Clostridium difficile infection:'RePOOPulating'the gut. Microbiome. 2013;1(1):1.

Appendix 1: Qiime command lines

****QIIME******

1. Preparation of analysis
start fastq.gz file

1-1 Preparation of Map.txt file

#SampleID BarcodeSequence LinkerPrimerSequence SampleType Description
ex1 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG mock unknown_sample

1-2 Paired end read merge
>run_pear.pl -p 4 -o stitched_reads raw_miseq_data/*

2. Normalization of sequence reads
>for m in *.fastq; do subsample_se_fastq.sh $m 100000/${m%*}".fastq" 100000; done

For fastq files:

>grep -c "@" *.fastq

For fasta files:

>grep -c ">" *.fasta

3. Removing low quality reads (<q20)
>for i in *fastq; do split_libraries_fastq.py -i $i --sample_id ${i/_L001_R1_001.fastq/} -o ${i/_L001_R1_001.fastq/}_R1_q20 -m map.txt -q20 --barcode_type 'not-barcoded' ; done

4. Gather seqs.fna file from folder
>for DIR in *_q20; do cat ${DIR}/seqs.fna >>seqs.fna; done
>for DIR in *_q20; do wc -l ${DIR}/seqs.fna; done
>for DIR in *_q20; do grep -c ">" ${DIR}/seqs.fna; done

>wc -l filename.fa

>grep -c ">" *.fasta
4-1 check read count and control read count
Summary
Original: 13.09 GB (usearch61 X) (read count X) 31,286,161
100000: 6 GB (usearch61 X) (read count X)
90000: 4.45 GB (usearch61 X) (read count o) 10,486,814
80000: 3.96 GB (usearch61 X) (read count o) 9,330,776
70000: 3.48 GB (usearch61 X) (read count o) 8,205,092
60000: 2.98 GB (usearch61 o) (read count o) 7,019,645
50000: 2.48 GB (usearch61 o) (read count o) 5,850,118

If below 3.00 GB then Usearch61 is the proper program.

>/home/madsen/Desktop/subsample_se_fasta.sh ALL.fna ALL_1000.fna 10000000

5. Usearch61

>pick_otus.py -i il10.fna -m usearch61 --db_filepath=gold.fa -o usearch_qf_results/ --word_length 64

(gold.fa file from website)-> make sure the gold.fa file is in your working directory.

many group analysis*

>for i in `ls *.fna`
>do
>pick_otus.py -i $i -m usearch61 --db_filepath=/home/madsen/Desktop/gold.fa -o ${i%*}_usearch_qf_results/ --word_length 64
>done

or

>for i in `ls *.fna`; do pick_otus.py -i $i -m usearch61 --db_filepath=gold.fa -o ${i%*}_usearch_qf_results/ --word_length 64; done &

6. Creating the major files
6-1 R1_rep_set.fna
>pick_rep_set.py -i usearch_qf_results/il10_otus.txt -f il10.fna -o il10_rep_set.fna

6-2 Biom file

> assign_taxonomy.py -i il10_rep_set.fna -o il10_taxonomy_results
> make_otu_table.py -i usearch_qf_results/il10_otus.txt -t il10_taxonomy_results/il10_rep_set_tax_assignments.txt -o il10_otu_table.biom

6-3 Tree file

> align_seqs.py -i il10_rep_set.fna -o il10_alignment

(Creates 3 output files)

> filter_alignment.py -i il10_alignment/il10_rep_set_aligned.fasta -o alignment/
(Creates a single output file)

> make_phylogeny.py -i alignment/il10_rep_set_aligned_pfiltered.fasta -o il10_rep_set_tree.tre

(This step takes over 3 hours)

6-4 Create map.file

create_qiime_map.pl *.fastq > map.txt

6-5 Determination of eigenvalue for Core diversity

biom summarize-table -i il10_otu_table.biom -o otu_talbe_summary.txt

7. Core diversity

> core_diversity_analyses.py -i il10_otu_table.biom -o core_output -m il10_map.txt -c SampleType -t il10_rep_set_tree.tre -e 146

(This step takes over 3 hours)

8. Taxonomy result

> summarize_taxa.py -i il10_otu_table.biom -o il10_taxonomy_summaries
> plot_taxa_summary.py -i il10_taxonomy_summaries/il10_otu_table_L3.txt -o il10_taxonomy_plot_L3

9. Extra analysis (optional)

[Make OTU Heatmap]

>make_otu_heatmap.py -i il10_otu_table.biom -o il10_otus/OTU_Heatmap/

[Make OTU Network]

> make_otu_network.py -m il10_map.txt -i il10_otu_table.biom -o il10_otus/OTU_Network

>jackknifed_beta_diversity.py -i il10_otu_table.biom -t il10_rep_set_tree.tre -m map.txt -o wf_jack -e 110

beta_diversity_through_plots.py -i il10_otu_table.biom -m map.txt -o wf_bdiv_even146/ -t il10_rep_set_tree.tre -e 146

echo "alpha_diversity:metrics shannon,PD_whole_tree,chao1,observed_species" > alpha_params.txt

alpha_rarefaction.py -i il10_otu_table.biom -m map.txt -o wf_arare/ -p alpha_params.txt -t il10_rep_set_tree.tre

Appendix 2: Microbial composition of wild-type 129S1/SvlmJ mice at all taxonomic levels

Figure A2-1: Phylum level taxonomy of donor FMT slurry and wild-type mice

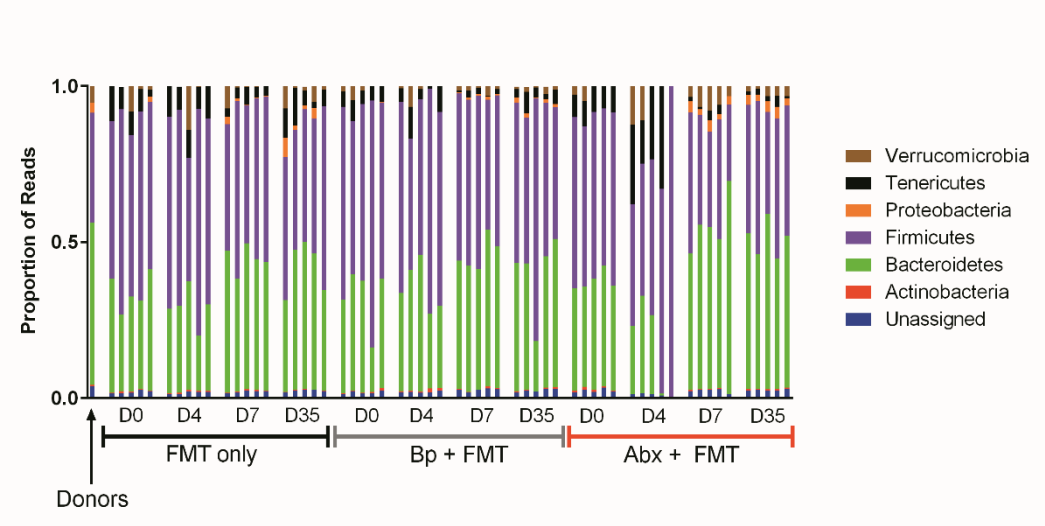


Figure A2-2: Class level taxonomy of donor FMT slurry and wild-type mice

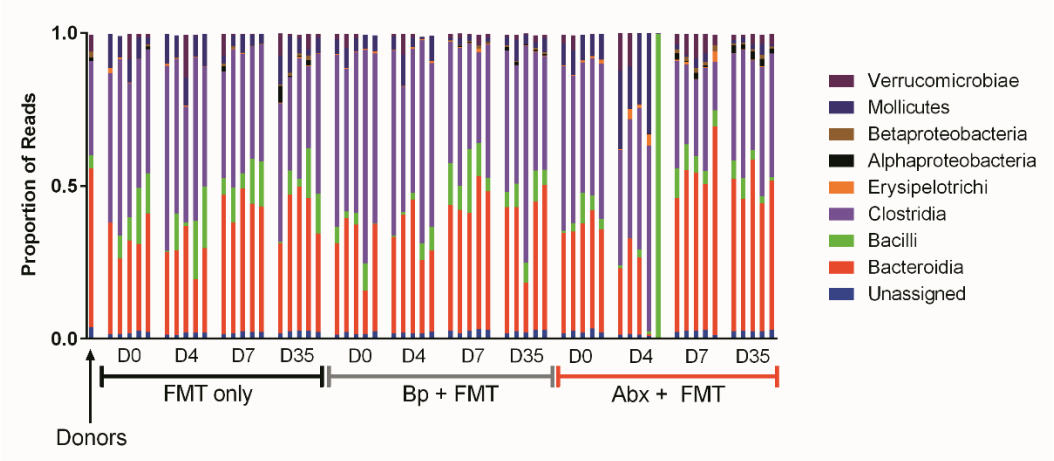


Figure A2-3: Order level taxonomy of donor FMT slurry and wild-type mice

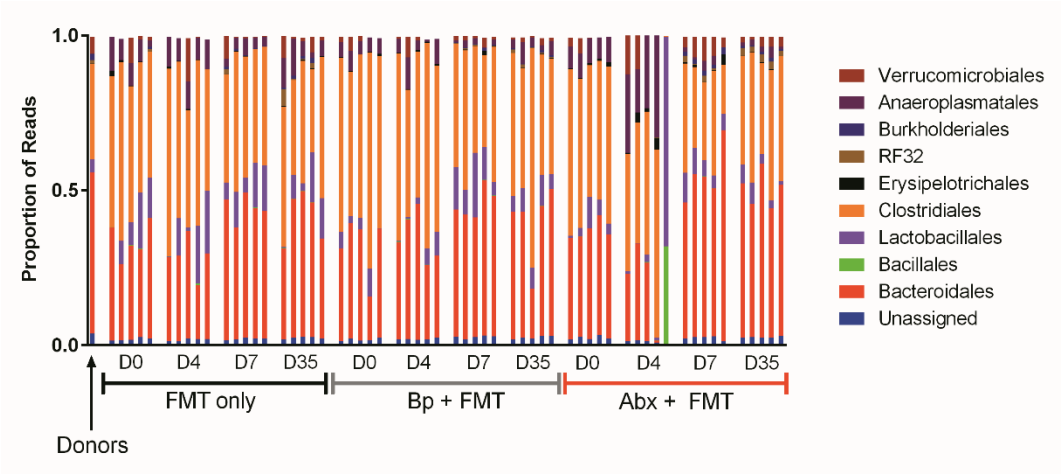


Figure A2-4: Family level taxonomy of donor FMT slurry and wild-type mice

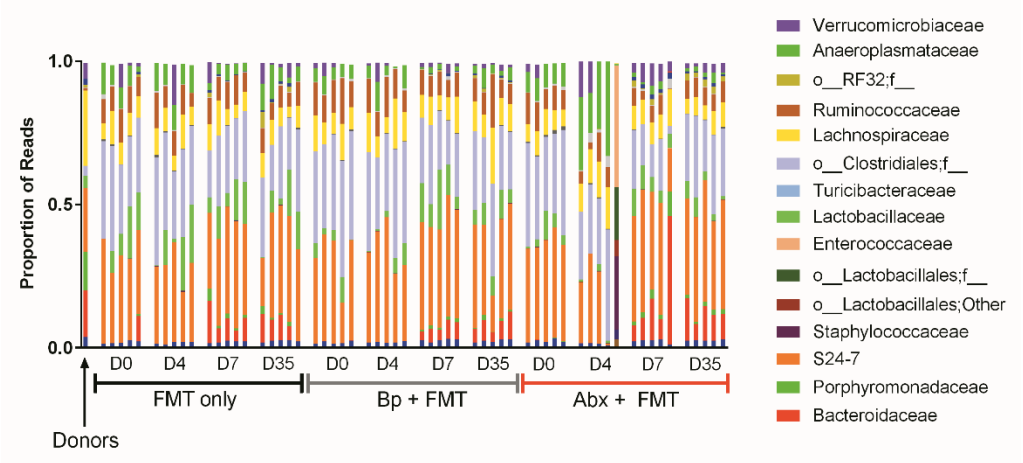
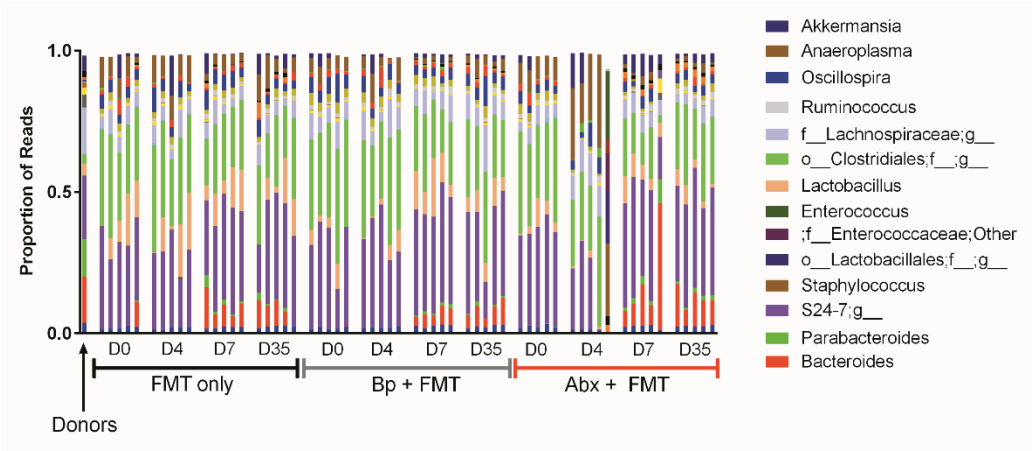


Figure A2-5: Genus level taxonomy of donor FMT slurry and wild-type mice



Appendix 3: Microbial composition of IL-10 Knock-out 129S1/SvlmJ mice at all taxonomic levels

Figure A3-1: Phylum level taxonomy of donor FMT slurry and IL-10 -/- mice

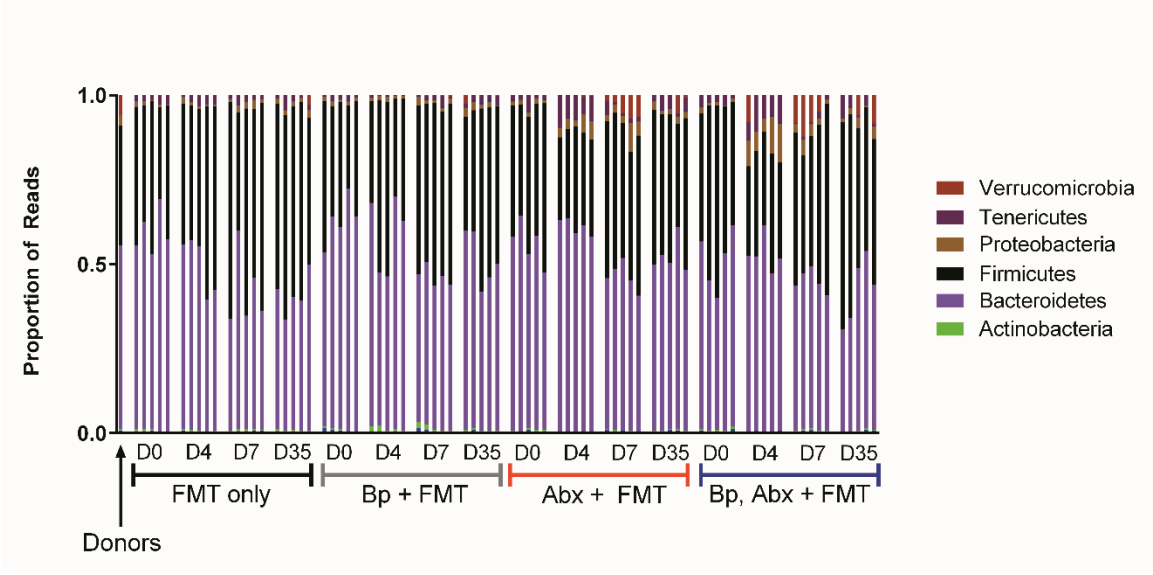


Figure A3-2: Class level taxonomy of donor FMT slurry and IL-10 -/- mice

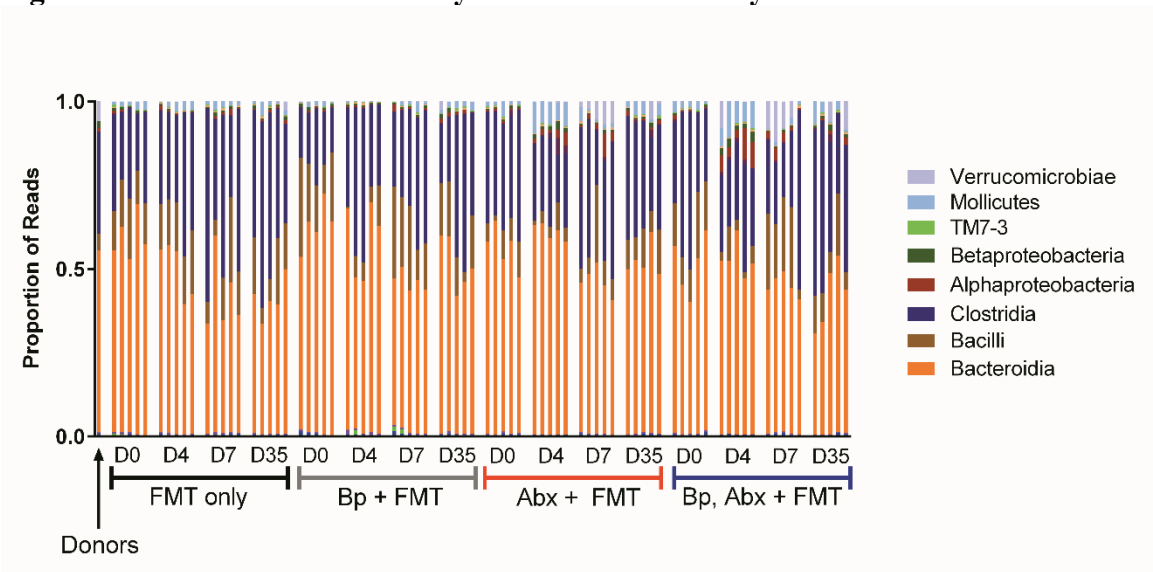


Figure A3-3: Order level taxonomy of donor FMT slurry and IL-10 -/- mice

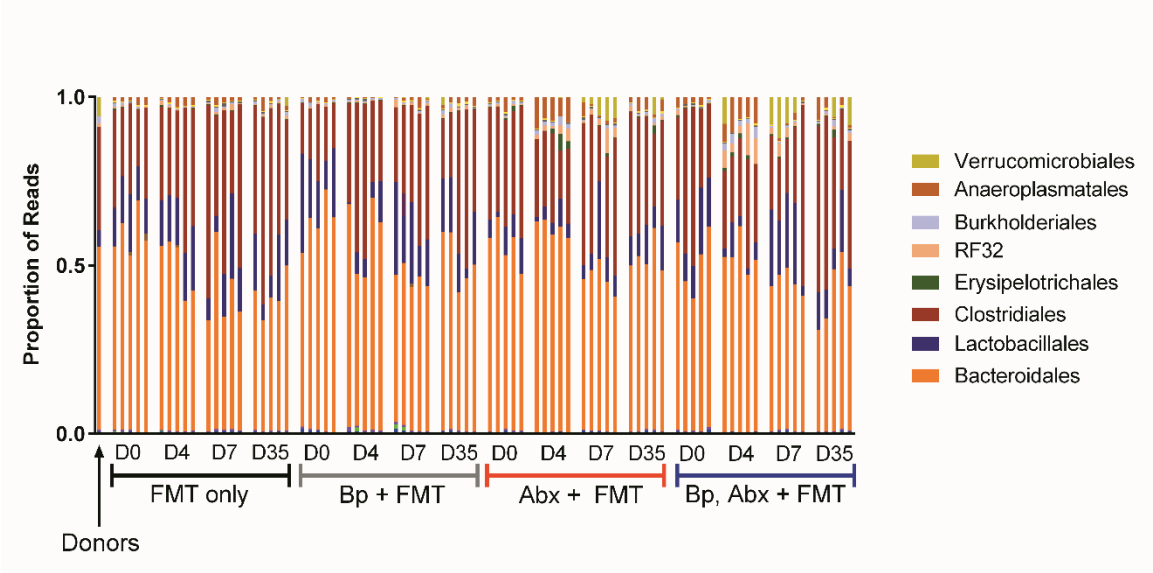


Figure A3-4: Family level taxonomy of donor FMT slurry and IL-10 -/- mice

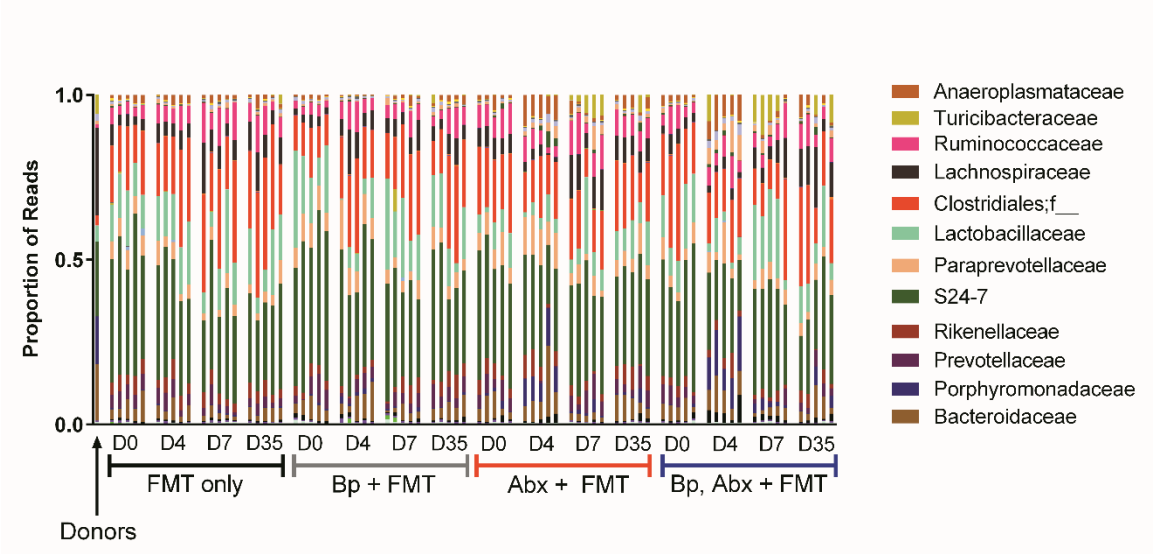
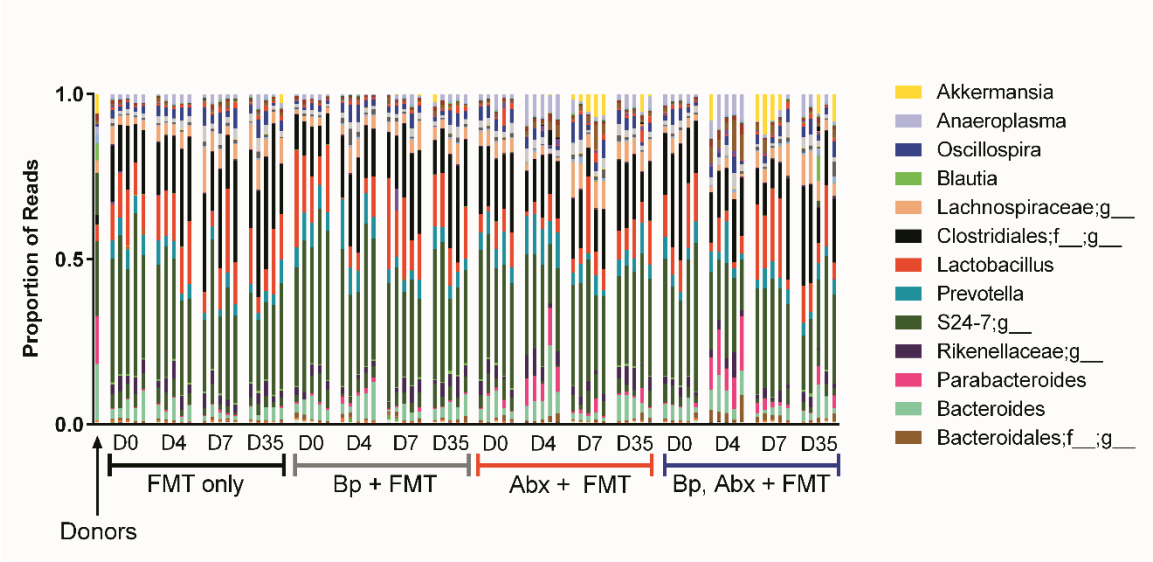


Figure A3-5: Genus level taxonomy of donor FMT slurry and IL-10 -/- mice



Appendix 4: MESO scale Discovery-V-PLEX Validated Assay Results

Figure A4-1: Cecal tissue cytokines measured using Meso Scale Discovery

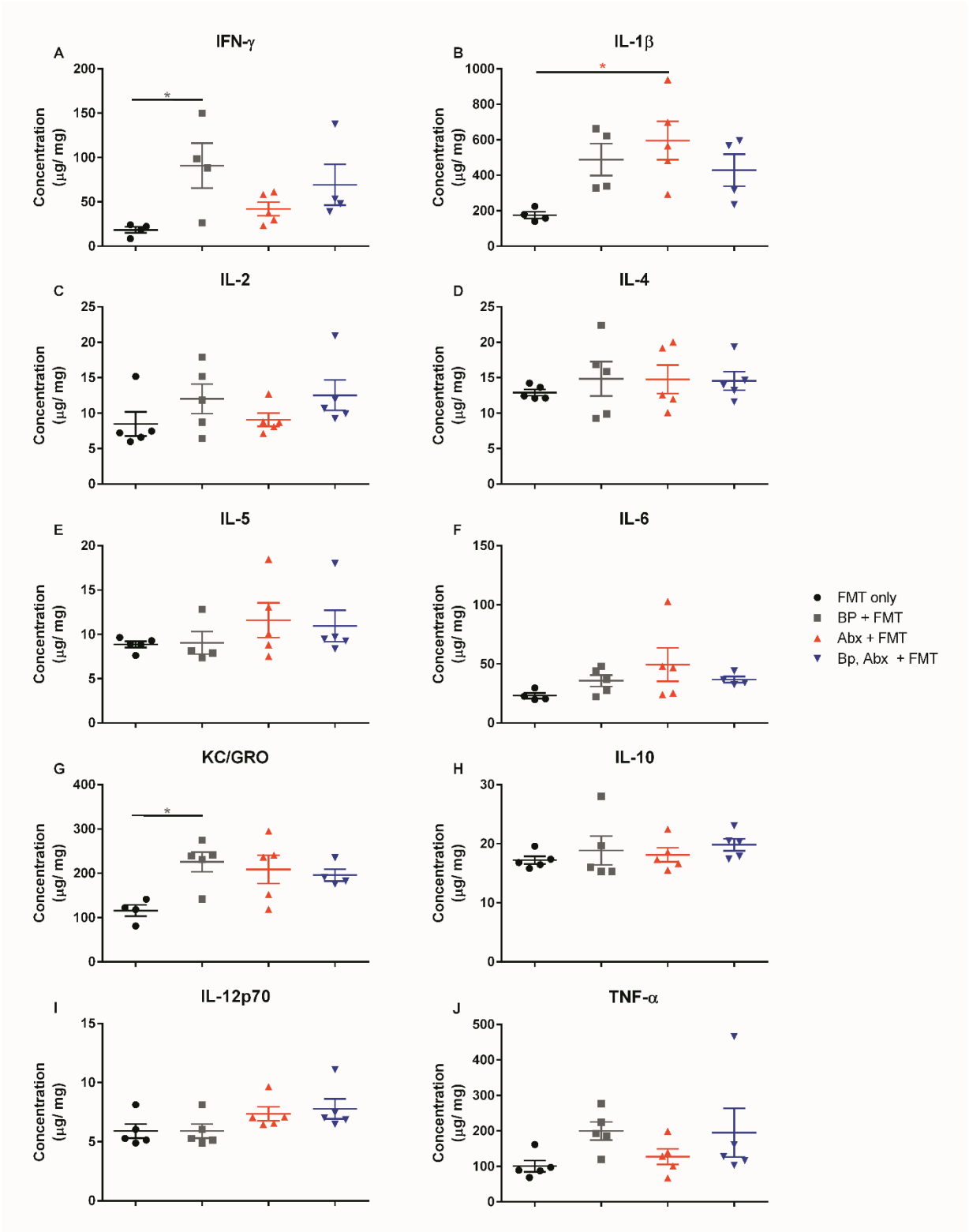


Figure A4-2: Ileal tissue cytokines measured using Meso Scale Discovery

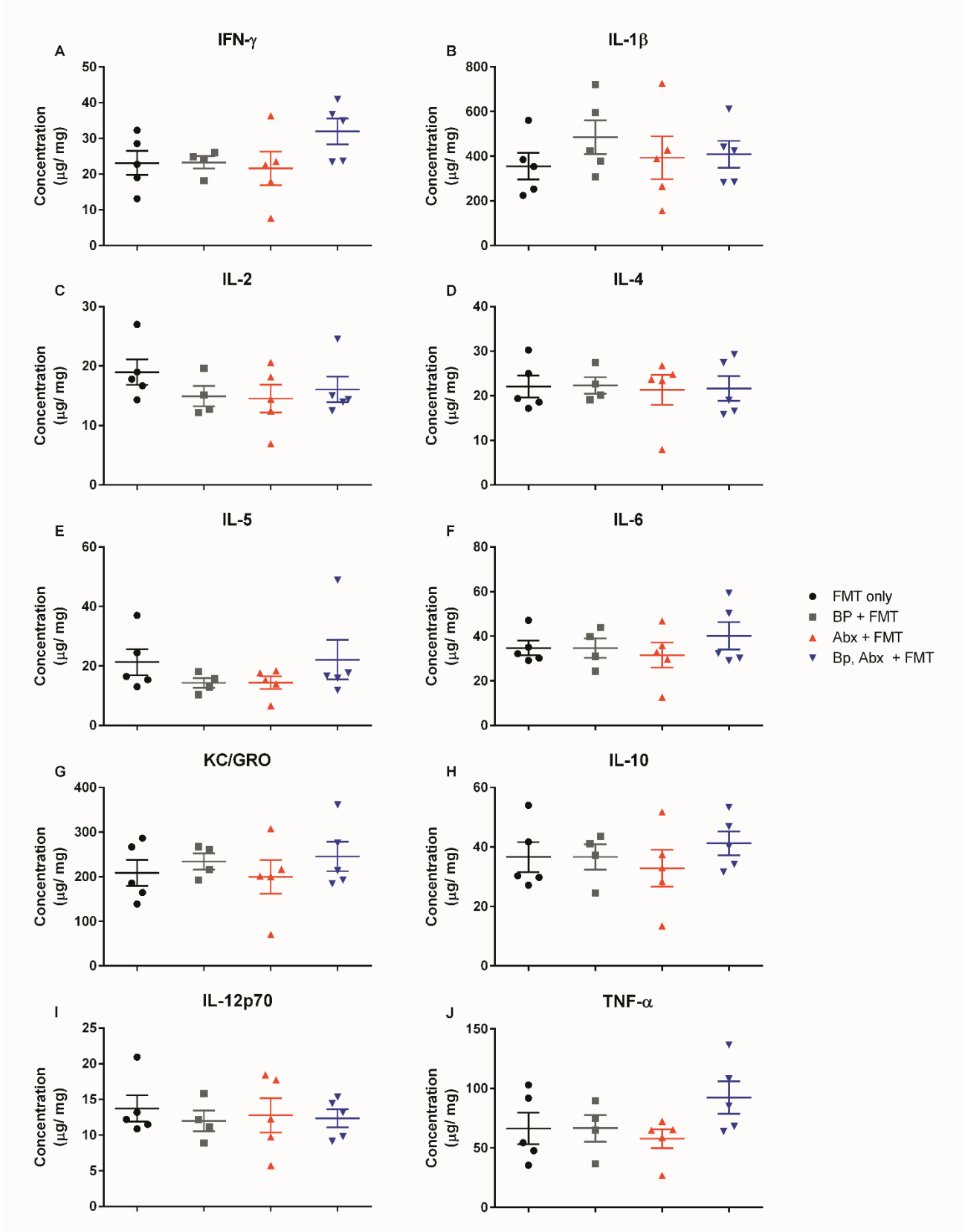


Figure A4-3: Serum cytokines measured using Meso Scale Discovery