Faecal Microbiota Transplantation for the Treatment of Active Ulcerative Colitis

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5. Samuel P Costello, Adrian Chung, Jane M Andrews, Robert J Fraser. **Fecal microbiota transplant for *Clostridium difficile* colitis-induced toxic megacolon**. *American Journal of Gastroenterology* 2015; 110: 775–777.

Articles 1, 3 and 5 are published in American journals and the original American English spelling has been retained in these manuscripts in this thesis.

Abbreviations

5-ASA	5-Aminosalicylate
16S rRNA	16S Ribosomal RNA
BMI	Body Mass Index
CDI	*Clostridium difficile* Infection
cfu	Colony-Forming Units
CI	Confidence Interval
CRP	C-Reactive Protein
FACS	Fluorescence-Activated Cell Sorting
FDR	False Discovery Rate
FMT	Faecal Microbiota Transplantation
HIV	Human Immunodeficiency Virus
IBD	Inflammatory Bowel Disease
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IQR	Interquartile Range
LPMC	Lamina Propria Mononuclear Cell
NK	Natural Killer
OTU	Operational Taxonomic Unit
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction

rCDI	Recurrent *Clostridium difficile* Infection
RCT	Randomised Controlled Trial
RNA	Ribonucleic Acid
SCCAI	Simple Clinical Colitis Activity Index
SCFA	Short-Chain Fatty Acid
TNF	Tumour Necrosis Factor
UC	Ulcerative colitis

Abstract

Introduction

Ulcerative colitis (UC) is an inflammatory bowel disease that has high rates of persistent or relapsing symptoms despite available therapies. Many of these therapies also have the potential for unacceptable side effects including allergy, intolerance, serious infection and malignancy due to long-term immunosuppression. It is for these reasons that new therapies for UC are required; particularly therapies that target novel pathways and do not suppress the immune system. Faecal microbiota transplantation (FMT) has demonstrated efficacy in the treatment of recurrent and refractory *Clostridium difficile* infection (CDI) and has been proposed as a novel therapy for UC.

Aims

The aims of this thesis were to:

1. establish a stool bank of screened donor stool containing viable organisms
2. assess the efficacy and safety of FMT for the induction of remission of UC
3. explore the mechanisms by which FMT may alter the disease process of UC.

Methods

Methods of stool donor recruitment and screening as well as anaerobic stool processing were developed and optimised. The viability of culturable organisms was validated after 6 months of frozen storage. A double-blind randomised controlled trial of a short duration of FMT using anaerobically prepared stool for the induction of remission of mild to moderate UC was undertaken with clinical and endoscopic remission assessed at 8 weeks and 12 months. Exploratory immunological, microbiological and metabolomic analyses were undertaken. A systematic review and meta-analysis was undertaken to assess the broader evidence for FMT as therapy for the induction of remission of UC.

Results

A stool bank of anaerobically prepared donor stool was established; 14 (31%) of 44 respondents to donor recruitment questionnaires were eligible. Bacterial viability was similar to baseline at both 2 and 6 months in specimens stored with saline and 10%

glycerol and at 2 months in stool stored only in saline, but was reduced by >1 log at 6 months for aerobes, coliforms and lactobacilli in saline alone. In patients undergoing FMT with stool frozen for 2–10 months in 10% glycerol, the cure rate for rCDI was 88% after a single FMT.

In mild to moderate active UC, clinical and endoscopic remission was achieved in 12 of the 38 participants (32%) who received pooled donor FMT, compared with 3 of the 35 (9%) who received autologous FMT (odds ratio [OR] 5.0 [95% CI 1.2–20.1]; P = 0.03). A number of bacterial species were associated with the observed donor FMT treatment effect. Neither lamina propria mononuclear cell populations nor short-chain fatty acid levels were associated with the donor FMT treatment effect.

Meta-analysis of randomised controlled trials of FMT for UC demonstrated that clinical remission was achieved in 39 of 140 (28%) patients in the donor FMT groups, compared with 13 of 137 (9%) patients in the placebo groups (OR 3.67 [95% CI 1.82–7.39]; $P < 0.01$].

Conclusions

Establishing a bank of anaerobically prepared frozen donor stool facilitates the delivery of FMT for clinical and clinical trial purposes. Anaerobic stool processing with normal saline and glycerol results in viability of bacteria in frozen storage for 6 months. Donor FMT is an effective therapy for the induction of remission of UC. Further research is required to assess the efficacy and safety of FMT as maintenance therapy for UC and to establish the mechanism of treatment effect.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and, where applicable, any partner institution responsible for the joint-award of this degree.

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Chapter 1: Overview

Ulcerative colitis (UC) is an incurable, relapsing–remitting inflammatory bowel disease (IBD) characterised by colonic mucosal inflammation that has significant morbidity. UC affects approximately 40,000 Australians at a cost to Australia of 1.38 billion dollars per year.[1,2] Approximately 25% of those affected with UC have onset by 18 years of age and have life-long disease. Despite available therapies, UC has an unacceptably high rate of persistent or relapsing disease activity[3] characterised by bloody diarrhoea, anaemia, weight loss and abdominal pain. UC is associated with a risk of colectomy[4] and an increased risk of colorectal cancer relative to the general population.[5] Current treatments also have the potential for unacceptable side effects including allergy, intolerance, serious infection and malignancy due to long-term immunosuppression.[6] It is for these reasons that new therapies for UC are required, particularly therapies that target novel pathways and are not immune suppressing.

Faecal microbiota transplantation (FMT) involves the transfer of faecal material from a healthy individual to a person with disease with the aim of treating that disease. At present, FMT is predominantly used to treat *Clostridium difficile* infection (CDI) where its use is supported by society guidelines[7,8] and evidence of efficacy from multiple randomised controlled trials (RCTs).[9-12] There is strong evidence for the involvement of gut microbiota in UC pathogenesis[13-15] and evidence that manipulating the microbiome improves symptoms.[16] FMT is proposed to treat UC by modifying the colonic ecosystem; however, the potential biochemical and immune mechanisms by which this may occur are unknown. Human stool comprises multiple components including food; organisms such as bacteria, protozoa, archaea, fungi and viruses including phages, as well as metabolic products of these organisms; and abundant human cells and products secreted into the gut lumen, such as bile acids.[17] Each of these components may play a role in a therapeutic effect of FMT.

At the beginning of this thesis in 2013, there were anecdotal case reports indicating that UC may be successfully treated by FMT[18-21]; however, there were no RCTs evaluating this therapy.[22] Despite this paucity of data, UC online forums documented that many patients with UC were resorting to non-medical (independent) FMT.[23] It was also known that FMT was offered to people with UC by doctors in Australia.[18] Moreover, a

survey from a respected IBD centre found that those with UC will consider FMT and were eager for it to become available.[24] Thus, FMT was occurring in an unregulated fashion, despite the lack of high-level efficacy data. Thus, a clinical trial was planned to test the efficacy and safety of FMT for UC.

A stool bank of screened donor stool was required to conduct the proposed study of FMT in UC. On review of the blood donation literature and in consultation with infectious disease specialists, donor screening protocols were devised. Methods of donor recruitment as well as anaerobic stool processing were developed to establish a stool bank. This study is presented in Chapter 3 (Costello et al., "Establishing a fecal microbiota transplant service for the treatment of *Clostridium difficile* infection", manuscript published in *Clinical Infectious Diseases*, 2016 [impact factor (IF) 2017 9.117]).

The viability of bacteria frozen for the purposes of FMT was not known. A study was therefore undertaken to evaluate the viability of culturable organisms from stool processed under anaerobic conditions and frozen for up to 6 months, using different preparatory methods. The viability of frozen bacteria within the stool bank was important to ascertain prior to interpreting the subsequent analysis of microbiome samples from donors and recipients in the RCT of FMT for UC in Chapter 5. To gauge the clinical efficacy of stool frozen with 10% glycerol, we assessed efficacy of stool frozen for more than 6 months in treating patients with CDI, an indication for which the efficacy of FMT using fresh or short-term frozen stool was known. This study is presented in Chapter 4 (Costello et al., "Faecal microbiota transplant for recurrent *Clostridium difficile* infection using longer-term frozen stool is effective: Clinical efficacy and bacterial viability data", manuscript published in *Alimentary Pharmacology & Therapeutics*, 2015 [IF 2016 7.286]).

During the first year of the trial, high-level evidence emerged for the use of FMT for the treatment of CDI. In this context, the stool bank was used to treat patients with CDI in South Australia. A notable case was the first reported treatment of toxic megacolon using FMT in the literature. This case demonstrated the utility of having rapid access to screened stool for emergency treatment and is presented in Appendix 1 (Costello et al., "Fecal microbiota transplant for *Clostridium difficile* colitis-induced toxic megacolon",

manuscript published in *American Journal of Gastroenterology*, 2015 [IF 2015 10.383]).

A multi-centre RCT of FMT for the treatment of mild to moderately active ulcerative colitis (FIRST-UC) was undertaken. The aims of the study were to demonstrate efficacy and safety of FMT therapy as well as explore the mechanism of action with microbiome, metabolomic and mucosal immune analysis. This study is presented in Chapter 5 (Costello et al., "Effect of fecal microbiota transplantation on 8-week remission in patients with ulcerative colitis: A randomized clinical trial", manuscript published in *Journal of American Medical Association*, 2019 [IF 2017 47.661]).

During the course of the thesis, evidence for FMT for the induction of remission of UC emerged from other studies; therefore, a systematic review with meta-analysis was undertaken to give context to the FIRST-UC study. This study is presented in Chapter 6 (Costello et al., "Systematic review with meta-analysis: Faecal microbiota transplantation for the induction of remission for active ulcerative colitis", manuscript published in *Alimentary Pharmacology & Therapeutics*, 2017 [IF 2017 7.357]).

Finally, a discussion is presented summarising and integrating the findings of the thesis (Chapter 7). The place of FMT in the management of UC is discussed. Possible mechanisms of action of FMT for UC are explored and the relevance of these findings to the development of microbial-based therapies for UC are discussed.

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Chapter 2: Introduction

2.1 Definitions

Ulcerative colitis is a relapsing and remitting IBD characterised by superficial colonic mucosal inflammation that extends proximally from the rectum in a contiguous manner. FMT is the transfer of faecal material from a healthy individual to a person with disease with the aim of treating that disease.

2.2 Gut Microbiome: The "Normal" Gut Microbiome

The gut microbiota consists of all the organisms that live within the human gut and the gut microbiome consists of the genes of these microorganisms. The gastrointestinal microbiota alone contains approximately 3.9×10^{13} organisms, meaning that there are similar numbers of microorganisms in the gut as there are human cells in the human body.[1] The first gut microbes are acquired in utero[2], and thereafter environmental exposures shape the development of the gut ecosystem. An infant's microbiome is dependent on mode of birth, breast feeding and weaning, maternal diet, antibiotic exposure and interaction with its environment.[3,4] There is a large amount of flux in the composition of an infant's microbiome; however, after about 3 years of age, it is relatively stable.[5] In adult life, the microbiome broadly remains stable, but can be altered by persistent changes in diet, lifestyle or environment, the development of disease or treatments such as medications or surgery.[6]

Bacteria are commonly classified phylogenetically into taxonomic groupings of phylum, family, genus, species and strain, where phyla are broad groupings of genetically similar bacteria and strains contain genetically and phenotypically identical or almost identical organisms.[7] The human gut microbiota is dominated by two main phyla of bacteria, *Firmicutes* and *Bacteriodetes*, which together account for 90% of gut bacteria. Viruses (including phages), fungi, protozoa and archaea are also important elements of the human gut microbiome.[8] Many of these microorganisms have co-evolved with humans and perform essential functions for their host.[8] An important example of this is the short-chain fatty acid (SCFA) butyrate, which is derived from bacterial metabolism of fibre in the colon and is the primary energy source of colonocytes.[9] Some gut microbiota live in close association with the epithelium and

have a role in regulating local and distant immune function.[10] Others regulate gut barrier functions and provide protection against pathogenic organisms such as vancomycin-resistant enterococci by competitive inhibition.[11]

There is large variation in the composition of the gut microbiome between individuals as measured by phylogenetic beta diversity. There are axial (mucosal to luminal) gradients and longitudinal (proximal to distal) gradients in composition within the gut of an individual.[12] Many of the organisms that reside in the human gut live in a symbiotic relationship with their human host and perform important functions for health. Although the composition of the organisms in the gut may vary widely between individuals, there is remarkable similarity in the functional pathways in the microbiome between individuals.[13] Many of the critical metabolic pathways have redundancy within the microbiota such that a number of organisms can perform the same metabolic function.[14] The metabolic capacity of the gut microbiome is much greater than that of the human host and some metabolic activities such as bile acid metabolism are shared between human and microbial cells.[15]

2.3 Analysis of the Microbiome and Its Products

There are a number of techniques that have been deployed to assess the gut microbiome. The original method of determining the composition of the microbiome was bacterial culture. Many species and strains of bacteria have been identified by this method; however, with the advent of molecular methods of profiling bacterial populations, it was realised that the majority of bacteria had not been cultured. This was predominantly because the majority of the gut microbiota are obligatory anaerobes with very specific growth conditions such that they were not able to be grown in vitro. In recent years, new bacterial culture techniques have been developed and many previously "unculturable" bacteria have now been cultured.[16]

Mapping the composition of the microbiome with 16S ribosomal RNA (16S rRNA) sequencing is the most common technique used to identify bacterial groups within stool. This technique uses a conserved portion of bacterial RNA to determine the relative genetic similarity of organisms within a population. This process is dependent on having a reference library of the genome of organisms with which to compare.

Sequencing with 16S rRNA has the ability to identify phyla or species of bacteria, but the resolution is not typically precise enough to identify organisms at the strain level.

Metagenomic sequencing facilitates sequencing of the entire genomes of the organisms within a sample, and organisms can be identified using computational tools. Therefore, with metagenomic sequencing, identification of individual strains of bacteria is possible and an assessment of the functional potential of the microbiome can be made. However, to determine the expressed function of the microbiome, other techniques are required. Metatranscriptomics assays the proportion of a microbial metagenome that is being expressed at a specific point in time through RNA transcripts.[17] Proteomic and metabolomic analysis typically use mass spectroscopy or gas chromatography to measure the relative abundance of proteins and metabolites, such as SCFAs, produced by a microbiome.[18] These analyses give the greatest insight into the function of a microbiome in a particular environment.

2.4 Ulcerative Colitis

2.4.1 Clinical presentation

UC typically presents with bloody diarrhoea with associated urgency, tenesmus, mucus discharge, fatigue and lower abdominal pain.[19] Symptoms may vary depending on disease severity and disease extent. Fever and weight loss can accompany severe disease. Patients with proctitis (disease limited to the rectum) may present with urgency and tenesmus, while patients with pancolitis may be more likely to present with bloody diarrhoea and abdominal pain. UC is accompanied by extra-intestinal manifestations in approximately a third of patients. These include arthritis, venous thromboembolism, metabolic bone disease, uveitis, scleritis and skin disease (e.g., pyoderma gangrenosum and erythema nodosum).[20,21] Primary sclerosing cholangitis is a cholestatic hepatobiliary disorder that occurs in approximately 5% of patients with UC and carries significant risk of colon cancer as well as cholangiocarcinoma and liver transplantation.[22]

Approximately 25% of those affected with UC have onset by 18 years and life-long persistence, with 15% of patients requiring colectomy.[23] UC is associated with a significantly increased risk of colorectal cancer.[24]

2.4.2 Diagnosis

The diagnosis of UC is based on a composite assessment, incorporating clinical, endoscopic, histological and radiological factors.[25,26] A diagnosis is established using colonoscopy, with consistent features including continuous and confluent rectal and colonic mucosal friability, spontaneous bleeding and ulceration.[25] Mucosal biopsy features of UC include changes of chronicity, basal plasmacytosis, cryptitis and crypt abscess formation. At colonoscopy, the differential diagnosis includes Crohn's disease or infection.

2.4.3 Natural history of ulcerative colitis

The natural history of UC is one of relapsing and remitting disease, although some patients experience an unremitting disease course from the outset.[27] Proximal extension occurs in around 30% of patients with UC, and around 10% will undergo colectomy within 10 years of diagnosis.[28-30]

2.4.4 Epidemiology

The peak age of incidence of UC is between 20 and 30 years of age[31] and there is no sex predominance.[31-33] The incidence and prevalence of UC have been increasing over time in Australia and elsewhere around the world, particularly in the last 3 decades.[34,35] The incidence in Australia has been reported at 7.33 per 100,000[36], and prevalence rates are as high as 505 per 100,000 people in northern Europe.[37] There has also been a rapid increase in the incidence of IBD in developing countries, where changing environmental conditions, urbanisation and the adoption of a Western lifestyle have been implicated.[36,38] Accordingly, epidemiologic studies have demonstrated that the risk of developing UC in migrants from low-incidence areas is dependent on the age at which migration to the high-incidence area occurs; a younger age of immigration is associated with a higher risk of developing UC after arrival.[39] The children of migrants from low-incidence to high-incidence countries assume a similar risk of UC to non-immigrants.[40] These observations support the notion that environmental factors are prime drivers of disease pathogenesis.

2.4.5 Pathogenesis of ulcerative colitis

The aetiology of UC is complex and many elements are thought to play a role in the pathogenesis.[41,42] Two intimately related factors thought to contribute include an abnormal composition and function of the microbiome and immune dysregulation.[43] Important components of the interaction between the immune system and the intestinal microbiota involve epithelial barrier function, microbial handling, and regulation of adaptive and innate immunity.[41] Host genetics play a role; however, environmental factors appear to be a larger determinant of disease risk.[41,42] Environmental exposures in the first few years of life play an important role in disease susceptibility; these include infant formula feeding and antibiotic exposure in utero.[44,45] Other environmental exposures that influence both susceptibility and the natural history of UC later in life include dietary intake, enteric infections, medications and lifestyle factors such as stress and smoking.[46]

2.4.6 Evidence of microbial involvement in the pathogenesis of ulcerative colitis

UC is a mucosal disease occurring at the colonic interface between the luminal contents, including the microbiota, and the mucosal immune system. Apart from anatomical proximity, there are many lines of evidence that the faecal microbiome is pivotal to inflammatory activity in UC: Diversion of the faecal stream away from the colon, by creating a temporary ileostomy, can improve colonic inflammation[47]; antibiotics have been shown to improve UC[48], and many of the genetic risk alleles associated with UC are related to microbes and mucosal defence.[49] Additionally, UC-like inflammation cannot be induced in germ-free animals.[50]

However, bacteria are critical for colonic health as their metabolic products, such as the SCFA butyrate, are a source of enterocyte nutrition and have anti-inflammatory effects.[51] Thus, patients with ileostomy and subsequent diversion of luminal contents develop “diversion colitis”[47] that is ameliorated by the delivery of butyrate. Rather than the traditional pathogen model that has successfully described infectious disease since Pasteur and Lister[52], UC may represent a loss of function within the gut ecosystem. The relative paucity of some important colonic bacteria has been associated with UC, including *Faecalibacterium prausnitzii* (a butyrate producer with independent anti-inflammatory properties); sulphate-reducing bacteria; and mucosa-associated

Akkermansia muciniphila, *Ruminococcus torques* and *Ruminococcus gnavus* (potential roles in mucus barrier integrity).[53,54] It has been demonstrated that the microbiome in UC, both active and remission, is less diverse than that of healthy subjects.[53,55] This loss of diversity is predominantly attributable to a decrease in gram-positive *Firmicutes*, especially *Clostridium* clusters IV and XIV; meanwhile, some species in the *Proteobacteriae* group, particularly *E. Coli* and *Enterobacteriaceae*, are relatively overabundant.[54,56] Increased gram-negative bacteria such as *E. coli* can be associated with increased lipopolysaccharide (LPS), a component of their cell wall, which triggers inflammation via the innate immune pathway.[55] It is unclear whether these alterations initiate or result from the inflammatory process.

There has also been increasing recognition of altered function of the microbiome in patients with UC through the study of metabolomics.[57] There is evidence that there is a failure of butyrate oxidation in the colonocytes of patients with UC.[9,58,59] Butyrate oxidation in human colonocytes has been demonstrated to be inhibited by high levels of nitric oxide that result from high levels of nitrite and sulphides.[60] This leads to an energy deficiency state within the colonocyte with an associated loss of critical functions such as maintenance of the mucosal barrier by mucus and tight-junction production. Barrier loss leads to immune activation and the resultant inflammation and hyperaemia can further promote invasive oxygen-tolerant bacteria.[61]

Taken together, these observations lead to the hypothesis that "dysbiosis", or perturbation of the faecal microbiota, plays a causal role in UC, and thus modifying the microbiome may promote mucosal healing. Little detail is currently understood of the presumed bidirectional relationship between the metabolic and immunologic functions of the microbiome and its possible role in UC.

2.4.7 Mucosal immune and barrier functions in ulcerative colitis

The gastrointestinal tract contains a potent immune system that is separated from the luminal microorganisms by barriers of the mucous layer and the epithelium.[62] The innate immune system provides a further non-specific defence against invading organisms and is aided by the adaptive immune cells that provide specific responses to antigens. Current evidence from human studies indicates that the mucosal inflammatory infiltrate in UC consists of a complex mixture of innate and adaptive immune cells and

their products.[63] However, little is clearly understood regarding the mechanism of this immune response. The hypothesis with the most traction is that UC is characterised by a natural killer (NK) T cell-driven, IL-13 and IL-5 dependent, T_H2 mediated immune response initiated by a loss of immune tolerance to colonic microbiota.[63] This is proposed to initiate a cascade of cytokine and chemokine secretion, causing an influx of granulocytes including eosinophils and neutrophils as well as plasma B cells. However, much of these data are derived from animal models and a limited number of human studies. Several studies indicate alterations in the immune cell types involved in immune tolerance, such as regulatory T cells, where numbers are consistently decreased in blood but increased in inflamed colonic mucosa in people with UC.[64] The phenotype and function of dendritic cells, dedicated antigen-presenting cells that link innate and adaptive immunity, are also altered in patients with UC in flare, compared with healthy controls, with gut-resident dendritic cells having increased expression of the microbial sensors TLR4 and TLR2 in UC.[65] The resultant immune cell infiltrate is complex and relatively undefined in diseased regions of UC patients, and likely differs according to current disease with symptom severity and therefore within and between patients.[63] Longitudinal investigations of immune function in humans with UC in flare or remission have not been previously reported.

2.4.8 Conventional management of ulcerative colitis

The management of UC involves both induction therapy (to induce remission) and maintenance therapy (to prevent further flares).[66] The goal of treatment is maintenance of remission without steroids.[66] Ideally remission should be both clinical (absent symptoms, patient feels well and normal quality of life) and endoscopic (absent inflammation, thus no ongoing damage or increased risk for colectomy or colorectal cancer). Targets for remission include resolution of clinical symptoms, defined as cessation of rectal bleeding and diarrhoea, and endoscopic healing, which is commonly defined as an endoscopic Mayo score of 0 or 1.[67] Mucosal healing at colonoscopy has been shown to greatly improve long-term clinical remission, decrease risk of colectomy and limit corticosteroid use.[68]

Induction therapy is usually high-dose oral 5-aminosalicylic acid compounds (5-ASAs) with or without topical 5-ASAs via enema or suppository. More severe flares require systemic corticosteroids (tapered over time and discontinued). Prolonged steroids are

ineffective at preventing flares and are associated with a myriad of complications not limited to infection, such as osteoporosis, obesity, diabetes, poor healing, thinning skin, mood changes and insomnia. As thiopurines take 8–12 weeks to be effective, they have no role as induction agents. Severe flares of UC unresponsive to steroids require medical rescue therapy (cyclosporin or infliximab) or urgent colectomy.[69]

Maintenance therapy choice in UC is determined by disease extent, severity, frequency of flares and past treatment history. The mainstays of maintenance therapy are 5-ASAs used orally or topically, and thiopurines for patients with repeated flares despite 5-ASAs.[66] In recent years, new biological agents have demonstrated efficacy and have been funded by the Australian Pharmaceutical Benefits Scheme for the maintenance of remission in UC. These are the anti-tumour necrosis factor α (anti-TNFα) agents infliximab[70], adalimumab[71] and golimumab[72]and the anti-integrin agent vedolizumab.[73] However, these newer agents are expensive and have incomplete efficacy, with induction of remission gains over placebo ranging from 7% for adalimumab to 20% for infliximab in registration studies.[70-73]

2.4.9 Current unmet need

Current treatments are hampered by incomplete efficacy and have the potential for unacceptable side effects including allergy, intolerance, serious infection and malignancy due to long-term immunosuppression.[74] The current treatments for UC are inadequate to maintain long-term remission in a significant proportion of patients.[66] Many patients have chronic or relapsing inflammation of the colon, leading to work and personal impairment at a cost to the Australian community estimated to be 1.3 billion dollars per year[75], and up to 10% require colectomy during the course of their disease despite current therapies.[76,77] A meta-analysis of oral 5-ASA trials found an unacceptably high relapse rate of 61.5% by 2 years in patients initially in remission with these agents.[78] Thiopurines and anti-TNF agents induce systemic immunosuppression, reducing the incidence and severity of flares, but at the cost of increased risk of serious infections and malignancy, particularly lymphoma.[74] Patients in whom mucosal inflammation cannot be controlled fully eventually require total colectomy, which entails surgical risks, such as infection and wound breakdown, and has a mortality rate of approximately 1% in high volume centres.[79] Colectomy is considered “curative”, especially if patients have an ileostomy created; however, it frequently leads to

significant short- and long-term complications. In addition, when an ileal–anal pouch is fashioned (to avoid stoma), up to 50% of patients develop pouchitis by 4 years post surgery.[80] This documented therapeutic gap requires that new therapies are developed, particularly those that mediate a clinical benefit via novel pathways ideally without the risks of immunosuppression.

2.4.10 Gut microbial manipulation

Given the strong evidence for colonic microbial dysbiosis in UC pathogenesis, the gastrointestinal administration of microorganisms has been proposed as therapy. Probiotics are live microorganisms that indend to provide beneficial health effects when administered in adequate amounts.[81] While some putative probiotics modulate gut metabolic function or gut immune cells in vitro[82,83], they are many and varied and do not act as a uniform class of agents. For example, studies have indicated that some probiotics have modest efficacy in UC, with *E. coli* Nissle 1917 equally effective as 5-ASA for maintaining remission[84] and VSL#3, a cocktail of eight different bacteria, better than placebo in inducing remission at 12 weeks.[85] VSL#3 has shown both therapeutic and prophylactic efficacy for patients with pouchitis.[86] However, there are also several negative studies (many never reaching full publication) and the outcomes for probiotics are generally regarded to be modest and inconsistent.[81] This may be due to variable actions of different bacterial species tested and limitations of preparations. Probiotics provide a very low number and diversity of species in contrast to the vast human gut microbiota. Thus, probiotics may be unable to compete effectively against the complex interactions of an established and adapted indigenous gut microbial community.[87]

2.4.11 Faecal microbiota transplantation

FMT involves the transfer of stool from a healthy individual to a person with disease with the aim of treating the disease. FMT can be described as “the ultimate probiotic” as it provides an entire ecosystem, with a much greater number and diversity of strains than any available probiotic. FMT is a therapy with a long tradition in human and veterinary medicine. FMT for animals is referred to as transfaunation and has been used to treat ruminal acidosis in cattle for centuries.[88] The first reports of FMT or “yellow soup” being used to treat diarrhoea and other human gastrointestinal ailments come

from 4th century China.[89] The Bedouin people have a long tradition of using camel faeces as a treatment for dysentery, and this practice was adopted by German soldiers stationed in north Africa during World War 2.[90] Human FMT was first reported in the Western literature in 1958 for the curative treatment of four critically ill patients with pseudomembranous colitis.[91]

In the past decade, there has been heightened interest in FMT, predominantly driven by increasing rates and virulence of CDI. At present, FMT is predominantly used to treat recurrent or refractory CDI (rCDI), where there is evidence of efficacy from multiple RCTs[92] and its use is supported by society guidelines.[93,94] FMT successfully treats rCDI in >90% of cases, compared with cure rates of 26–30% with the previous standard of care, vancomycin.[87,95-97] Cost–benefit analyses have demonstrated FMT to be more cost effective than traditional antibiotic therapy for rCDI in the Australian setting with savings of over $4,000 per patient treated.[98] FMT for the treatment of rCDI also has an impressive short-term safety record, with very few side effects directly attributable to FMT.[92] The long-term safety of FMT has not been well studied as the vast majority of FMT reported in the literature has been performed in the last 5–10 years.

The success of FMT in the treatment of rCDI has inspired research into the use of FMT for other disorders associated with dysbiosis. There are case reports of the use of FMT to treat a wide range of ailments including autism, Crohn's disease, irritable bowel syndrome and constipation.[87,99-101] At the commencement of this thesis, there were case reports and small case series of patients with active UC receiving FMT in the medical literature. A systematic review in 2012 included 18 reported cases: symptoms resolved in 12 subjects, reduced in four and worsened in two; endoscopically, UC resolved in 12 patients, deteriorated in two and persisted in two.[102]

2.5 Research Question

2.5.1 Rationale

Current therapies for ulcerative colitis are limited by incomplete efficacy, with most having rates of sustained disease remission of 30% or less. Many of these therapies are hampered by intolerance as well as side effects of infection and malignancy.

2.5.2 Aim

The aims of this thesis were to assess the efficacy and safety of donor FMT to induce remission in active UC and develop methodologies to deliver this therapy.

2.5.3 Research objectives

The objectives of this research were to:

1. develop methods to establish a stool bank for the reliable delivery of FMT
2. optimise stool-processing methods to maximise bacterial viability during storage of stool
3. evaluate the efficacy and safety of FMT as a therapy for active ulcerative colitis
4. explore the possible mechanisms by which FMT may have a therapeutic effect in UC.

2.5.4 Research process

Several studies were conducted to address the overarching aim set out for this thesis. The research performed may be broadly divided into two inter-related workstreams along with a systematic review and meta-analysis:

- **Project 1**. The first workstream set out to develop a stool bank capable of delivering reliable and safe FMT.
- **Project 2**. The second workstream involved evaluation of FMT as a therapy for UC.

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Chapter 3: Methods for Establishing a Stool Bank

3.1 Background

FMT is a proven and potentially life-saving therapy for rCDI. Emerging data have also demonstrated efficacy of FMT for other indications including UC. Establishing a stool bank is fundamental to delivering safe and reliable FMT.

Frozen stool banking gives clinicians rapid access to thoroughly screened donor stool when needed, without the ethical and logistical problems associated with patient-selected donors. Frozen stool banking facilitates timely, safe and effective access to FMT. Although frozen and fresh stool are equally efficacious for treatment of rCDI, frozen stool banking offers a number of advantages. Stool banking allows time for the application of rigorous screening protocols, enhancing the safety of FMT and allowing FMT to be delivered on demand. Moreover, stool banking allows for cost efficacy in manufacture and ensures anonymity for the donor.

Despite the advantages, a number of technical, logistical and regulatory issues have hampered the development of FMT capability at most hospitals. This manuscript describes the methods used to establish the first public FMT service in Australia using a stool bank of pre-screened donor stool, including details regarding donor recruitment and screening, stool preparation and delivery of the FMT.

Presented in this chapter is the manuscript published in *Clinical Infectious Diseases* (2016, Vol. 62, pp. 908–914).

3.2 Specific Author Contributions

Title of paper	Establishing a fecal microbiota transplant service for the treatment of *Clostridium difficile* infection
Publication status	Published
Publication details	*Clinical Infectious Diseases* 2016;62:908-14 Impact factor: 9.1

Name of principal author (candidate)	Samuel Paul Costello		
Contribution to the paper	Conception and design of the project Development of stool processing techniques Data acquisition and management Analysis and interpretation of research data Drafting and revision of article		
Overall percentage (%)	60%		
Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	18/11/2015

Co-author contributions

By signing the Statement of Authorship, each author certifies that:

1. the candidate's stated contribution to the publication is accurate (as detailed above);
2. permission is granted for the candidate to include the publication in the thesis; and
3. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of co-author	Emily C Tucker		
Contribution to the paper 10%	Development of stool processing techniques Analysis and interpretation of research data Drafting and revision of article		
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3.3 Manuscript—Establishing a Fecal Microbiota Transplant Service for the Treatment of *Clostridium difficile* Infection

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Running title: Establishing an FMT Service

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Summary: Whilst FMT is a highly effective therapy for rCDI, numerous technical, logistical and regulatory issues have hampered development of FMT capability at many sites. We describe how to establish such a service using a frozen stool bank.

Abstract

Recurrent or refractory CDI has become an increasing problem in the past decade. FMT is a highly efficacious treatment for rCDI; however, a number of technical, logistical and regulatory issues have hampered the development of FMT capability at many hospitals. The development of a frozen stool bank of screened donor stool is an important step in the standardization of the procedure. This gives clinicians rapid access to thoroughly screened donor stool when needed, without the ethical and logistical problems associated with patient-selected donors. We describe the practicalities of establishing such a service using a stool bank of pre-screened donor stool, including details regarding donor recruitment and screening, stool preparation and delivery of the FMT.

Introduction

Clostridium difficile is the most common cause of health care associated diarrhea, and its incidence has been increasing in the last decade[1]. The recent emergence of hypervirulent strains has also lead to increased morbidity and mortality associated with the infection[2]. A major impediment to the successful treatment of patients with *C. difficile* infection CDI has been the 25–30% rate of relapse following antibiotic therapy[3]. In those patients who do relapse, further antibiotic treatments give diminishing rates of cure, in that after a second recurrence the chance of further recurrence increases to 60% and it is even greater for subsequent recurrences[3].

The problem of recurrent CDU (rCDI) is thought to result from an underlying deficiency of the microbiome as well as, in some patients, defective antibody mediated immunity[4]. Patients with CDI usually have a depleted commensal flora with reduced diversity of organisms[5]. This is commonly caused by previous exposure to antibiotics or is a manifestation of other conditions such as UC[6]. Traditional antibiotic therapy for CDI can perpetuate this dysbiosis, thus leaving an ecological void into which *C. difficile* emerges and proliferates[7]. FMT is thought to be effective as it replenishes the colonic microbial diversity by providing a new and diverse microbiome that occupies the niche into which *C. difficile* would otherwise multiply[5].

FMT is by far the most successful treatment for rCDI, with primary cure rates between 81% and 94%[8-12]. A randomized control trial of FMT delivered via duodenal infusion for rCDI demonstrated symptom resolution in 81% of patients receiving a single FMT, compared with only 31% receiving vancomycin and 23% receiving vancomycin with bowel lavage. This study was stopped early after an interim analysis revealed the clear superiority of FMT over vancomycin therapy[10]. It is therefore now incumbent upon hospitals to establish FMT services so that rCDI can be effectively managed with this therapy. However, from a large institutional viewpoint, there are technical and logistical issues in establishing such a non-standardized, non-drug therapy with appropriate safety and governance.

Establishing an FMT service

An FMT service should be run in a standardized and efficient manner that allows safe and effective treatment to be delivered in a timely fashion. This is important because

patients with rCDI are often unwell, elderly patients with co-morbidities that make prompt treatment critical. The development of a frozen stool bank is the optimal way to standardize the FMT process and allow stool to be available on demand[11]. This is particularly important in cases where delayed treatment could result in colectomy[13]. A frozen stool bank also allows fecal donors to be recruited and thoroughly screened ahead of time, in a methodical manner, without time pressure. Donor stool banks can be established at individual treatment centers or stool can be shipped from a centralized stool bank such as the not-for-profit organization "open-biome" in the United States[14].

Historically, patients undergoing FMT would often select their own stool donor from family or friends and this known donor would then undergo screening prior to giving their stool donation. This creates delays with therapy, and moreover there are a number of potential problems with this approach. These include the possibility of coercion of donors and also ethical and confidentiality concerns regarding screening known donors in the event that disease is found in a donor or transmitted to the recipient. Stringent exclusion criteria can be more easily and dispassionately applied to volunteer donors from the community than recipient-directed donors as there are a greater number of potential candidates and no perceived personal obligation between recipients and donors. There is also evidence from blood transfusion safety analyses that recipient-directed donors are more likely to test positive for infectious disease than unrelated volunteer donors[15]. Depending on stool weight, up to eight treatments can be produced from each stool donation, and donors to the frozen stool bank can give multiple samples over a short period of time, making the process more economical[16]. Lastly, stool contains viable bacteria after 6 months of frozen storage at -80°C[11], and in case series, frozen stool appears to be as effective as fresh stool for treating patients with rCDI[11, 16, 17]. For these reasons, units such as ours have moved to only using pre-screened, unrelated donors who are anonymous to the recipient.

Common problems associated with establishing an FMT service

A major difficulty in establishing FMT services around the world has been regulatory restriction and uncertainties surrounding the practice[18]. This has, in part, occurred because of concern regarding potential risk to patients and the non-standardized nature of donor feces. To date, the short-term risk of FMT for rCDI is documented to be very

low and there have not been any directly attributable long-term side effects reported, although there are currently little long-term data[9, 19]. Despite randomized control trial efficacy data, the United States Food and Drug Administration (FDA) have implemented a policy of "enforcement discretion" regarding FMT only for rCDI after initially imposing a moratorium on the procedure[20]. There has also been difficulty classifying FMT as a therapeutic "drug" or tissue[21]. In the authors' opinion, feces for FMT would be better classified as a bodily tissue donation in a similar way that blood and blood donation are regarded. Stool is derived from human donors, and is not a "standard product" as are manufactured drugs. In Australia[22] and many European countries[23], the development of FMT services has been left to local health administrations rather than national organizations such as the FDA in the United States. Therefore, there remains a need for consensus guidelines to achieve greater standardization of the procedure.

Funding for FMT services can also be problematic with most third-party payers not rebating or covering the costs of individual FMT treatments or service establishment and delivery. Our FMT service has been established as a linked benefit from a clinical trial examining FMT in UC from research funds. However, for long-term sustainability, clinical funding from the health service will be needed.

Recruitment and screening of donors

Donor screening is expensive and time consuming, and therefore recruiting donors who are more likely to pass screening is advantageous. We found young, healthy donors by advertising at a nearby university. There is currently no evidence that donor characteristics or "enterotypes" predict the success of FMT treatment for CDI and so donor screening focuses on risk reduction rather than increasing the therapeutic effect.

Donor screening has a high exclusion rate with rates of donor eligibility as low as 10% in an Australian study where donors were sought by advertisement to the general community[24]. We found that even when targeting young, apparently healthy university students, only 14 (31%) of 44 respondents were eligible donors after completing screening (Figure 3.1). When screening potential stool donors, it is advisable to take the medical history and physical examination prior to stool and then

blood screening, because the majority of candidates are excluded on history and stool testing, thus avoiding the additional costs of blood testing (Table 3.1).

The risk of infection transmission is minimized with a thorough history for known exposures or risk factors as well as stool and blood screening. A number of atopic, autoimmune, psychiatric, malignant and neurological diseases are associated with gut dysbiosis, and so donors with these conditions are excluded[7]. Transmission of an obese phenotype has been demonstrated in animal studies[25], and the possible transmission of obesity has been reported in a single human case report [26]. Also, increased insulin sensitivity has been demonstrated in obese subjects following duodenal infusion of feces from lean donors[27]. Given these findings, elements of the metabolic syndrome should also be donor exclusions. Our screening protocol has evolved and is adapted from previous guidelines[10, 12, 28] (Table 3.1). Screening guidelines should reflect the risk of diseases applicable to the local population as this may vary depending on the geographical location of donor recruitment.

Processing stool (Figure 3.2)

Once screening is completed, stool should be collected from an individual donor within 1 month[28]. Alternatively, screening can be undertaken both before and after a period of donation to ensure that all stool collected and frozen between the two dates is safe[17]. We give donors a clean opaque plastic bag that can be opened over a toilet to collect the stool and then sealed with a cable tie and placed in a larger zip-lock bag. Donors have the option of donating on site or taking the bag home with a cooler box and an ice pack so it can be delivered within 1 hour of defecation. Stool can be stored for up to 8 hours at 4°C without significant impact on bacterial survival, but viability declines at room temperature or at 4°C for more than 8 hours[29].

Approximately 50g of stool is required for each treatment[8]. Fresh stool (25%) should be blended with normal saline (65%) and pharmaceutical grade glycerol (10%). This ratio maximizes the amount of stool in suspension without being too viscous for delivery via the biopsy channel of a colonoscope or nasoduodenal tube. Glycerol is used to retain bacterial viability in the frozen stool preparations[11]. The entire donor stool can be placed in the blender to make as many 200mL aliquots as possible with a minimum of 50g stool content. Once blending is complete, the stool mixture should be

aliquoted into individual cryo-tolerant pots and immediately frozen at -80°C. We use 250mL pots filled with 200mL of stool suspension as the liquid expands on freezing. In our experience the number of aliquots obtained from a single stool can vary from insufficient for a single preparation to eight aliquots. After 57 individual stool donations from 14 donors, we found a wide range in donor stool size from 7g to 436g with a median of 105g (IQR 51–220g). The mean stool weight was significantly greater for male donors (172g, CI 213–122g) than female donors (92g, CI 118–67g; $P = 0.006$).

We conduct the blending in an anaerobic chamber to reduce operator exposure, and because it has the theoretical advantage of preventing oxygen exposure to obligate anaerobic bacteria during the blending process. However, there is evidence that processing and freezing stool under aerobic conditions is also clinically effective[17, 30] and can be conducted without a fume hood as exposure risk is probably less than for colonoscopy (given the rigorous stool screening)[7, 30]. Blending for 1 minute produces a suspension with sufficiently small particle size for it to be easily drawn into a catheter tip syringe and flushed down the biopsy channel of a colonoscope with the cap removed. There is no need to strain the blended suspension for colonoscopic or enema delivery. We have conducted more than 80 FMT procedures using colonoscopic delivery of blended stool without filtration with no instances of colonoscope channel or syringe blockage. Many butyrate-producing colonic bacteria require fiber as a substrate[31] and so there are also theoretical reasons for not removing fibrous material from the suspension. However, if delivery is via a nasoduodenal or nasogastric tube, filtering is required to prevent tube blockage.

If a blender or an autoclave is not available, the FMT suspension can be prepared by combining stool, saline and glycerol in the collection bag and manually agitating the contents. Alternatively, stool can be mixed directly in the plastic storage container with a spatula[30] or shaken in a bottle of normal saline[32]. Whilst these methods are simple, they can result in a suspension with large unsuspended particles that can block the syringe and so filtering the suspension is often required.

Stool donors should be given an identification number that is marked onto each pot of stool suspension with the date the sample was produced. This identification number should be recorded in a secure donor document along with contact details and screening

results so that the donor is de-identified to the recipient but can be traced in the event of illness developing in the recipient.

Cleaning equipment

Cleaning the equipment between donor stool processing is important to minimize the risk of cross contamination. We use a blender that has a stainless steel container (Waring SS515) with Teflon seals and a stainless steel lid, both of which can be sterilized with an autoclave as this is the best infection control practice[33]. The blender container and spoon must be cleaned to remove all residue prior to autoclaving. We use an enzymatic wash and then detergent wash followed by a water rinse and then autoclave both at 121°C for 20 minutes. The container and spoon are then autoclaved again immediately prior to next use. Given donor stool is screened for potential pathogens it is likely to be safer than stool encountered during routine colonoscopy. Therefore, we believe endoscope cleaning following FMT should follow standard protocols.

The equipment required to establish such a service with a frozen stool bank is listed in Table 3.2.

Patient selection

The decision to proceed with FMT should be made on an individual patient basis; however, there are three main factors that influence the decision: the number of CDI recurrences, the severity of the episode and whether the disease is refractory to antimicrobial therapy[28]. More than two relapses of CDI following antimicrobial therapy gives <35% chance that subsequent antimicrobial therapy alone will be successful. In these patients, FMT offers a much higher chance of success[9, 10]. A severe infection with CDI resulting in shock or requiring supportive care in hospital in which recurrence of CDI could be life threatening is another indication, as is moderate disease not responding to antimicrobial therapy for at least 1 week[28]. Gastrointestinal perforation is an absolute contraindication and anaphylactic food allergy a relative contraindication. Severe immunosuppression has previously been regarded as a contraindication; however, FMT in patients with at least moderate immunosuppression appears safe[34]. Patients with toxic megacolon should be offered subtotal colectomy in the first instance, and FMT via colonoscopy is contraindicated in these patients. In those

refusing surgery, FMT via the upper gastrointestinal route can be cautiously considered and has resulted in cure[13].

Consent

There have been two deaths directly attributable to FMT, with both of these patients developing aspiration pneumonia. The first patient aspirated during sedated endoscopic FMT delivery to the duodenum[35], and the other aspirated during the anaesthetic for colonoscopic FMT[34]. One other death following FMT occurred because of toxic megacolon and sepsis[36]; however, this may have been attributable to the recurrence of the underlying CDI or a gastrostomy tube leak and not the FMT. There have been no other directly attributable long-term side effects of FMT in over 600 cases in the literature[19]. There is a paucity of long-term data[37], and so the possibility of as yet unknown long-term risk needs to be factored into any screening protocol and discussed with patients when consenting patients for FMT. A cohort study of patients who had received FMT for rCDI found 4 of 77 patients developed new autoimmune disease during the follow-up period of 3–68 months[37]. This study had no control group and thus no association between FMT and the development of autoimmune disease could be made.

Route of delivery of FMT

FMT can be delivered directly to the colon via colonoscopy or retention enema, or alternatively into the upper gastrointestinal tract via nasogastric tube, nasoduodenal tube or duodenoscopy. Another potential delivery method of FMT is the use of enteric-coated or lyophilized capsules that contain stool[38] or synthetic stool made of multiple different bacterial strains. These have shown success in small case series and phase I/II trials and similar preparations, although not widely available, are under commercial development[39].

The only randomized control trial comparing methods of delivery had 10 patients receive FMT via nasogastric tube and 10 patients receive FMT via colonoscopy for rCDI[17]. Resolution of diarrhea was achieved in 6/10 in the nasogastric tube delivery group, compared with 8/10 in the colonoscopic delivery group. There was no significant difference between groups although the numbers in this study were small. In a systematic review of case series, cure rates varied depending on the site of infusion;

when FMT was infused into the stomach, duodenum/jejunum, caecum/ascending colon and rectum the rates of cure were 81%, 86%, 93% and 84%, respectively[40]. Colonoscopy appears to have a higher cure rate than other methods; however, it is difficult to compare case series with heterogeneous populations. Colonoscopy does have the advantage of assessing the degree of inflammation and assessing other pathology that may be present; however, it is resource intensive and costly, and for this reason enema delivery is a reasonable alternative[18]. The upper gastrointestinal route carries the risk of fever and abdominal cramping[8, 10], whereas colonoscopic delivery carries the theoretical but unreported risk of colonic perforation. The FMT is delivered down the biopsy channel of the colonoscope into the caecum by removing the cap from the biopsy channel and opposing the catheter tip syringe.

Patient preparation and follow-up (Figure 3.3)

The standard approach has been to give 5–10 days of oral vancomycin (250mg QID), ceasing 36–72 hours prior to the procedure[12]; however, there are no data comparing no antimicrobial preparation or various different antimicrobial preparations. FMT via the duodenal route does not require bowel lavage, whereas colonoscopic delivery usually does[17]. We give the patient 4mg loperamide 3 hours prior to the procedure and have them lie on their right side at the point of delivery and for 1 hour following the procedure to aid with retention of the FMT. Symptoms of diarrhea and cramping often improve quite rapidly, and thus there is no routine need for further stool testing. However, if diarrhea persists for longer than 1 week, then repeat *C. difficile* toxin PCR should be done and FMT repeated if positive.

Conclusion

FMT is the most efficacious treatment available for the increasing problem of rCDI. This necessitates that health care services develop the capability to deliver FMT safely and reliably. A stool bank of pre-screened frozen aliquots from healthy volunteers is the most practical, ethical and cost-effective approach. The practical steps outlined here should assist other facilities to establish an FMT capability.

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Conflict of interest

The authors have no conflicts of interest to declare.

Prof JM Andrews has been on advisory boards, educational meetings, steering committees and on speakers panels for AbbVie and Janssen. She has also received research support and speakers fees from each company.

Table 3.1: Donor screening criteria

Medical interview (exclusions)
Age: <18 or >65
Antimicrobial therapy or probiotics in the past 3 months
Active medical illness or symptoms
Any medications
International travel in last 6 months to areas at high risk of traveler's diarrhea
High-risk sexual activity (unprotected sex in last 1 month outside of a monogamous relationship, men who have sex with men, sex for drugs or money)
Illicit drug use
Tattoo or body piercing within 6 months
Known HIV or viral hepatitis exposure in the last 12 months
Incarceration or a history of incarceration
Family history of colorectal carcinoma involving two or more first-degree relatives
Household members with active gastrointestinal infection
Medical history and examination (exclusions)
Any gastrointestinal disorder
Obesity (BMI >30),hypertension, type 2 diabetes and dyslipidaemia
Malnutrition (BMI <18)
Autoimmune disease
Atopic disease
Depression
Infection with HIV, syphilis, hepatitis B or C
Malignancy
Chronic pain syndromes, neurologic or neurodevelopmental disorders
Blood screening
Full blood count
Electrolytes, urea and creatinine
Liver function tests
Human T cell lymphotropic virus 1 and 2 serology
Epstein Barr Virus IgM and IgG
Cytomegalovirus IgM and IgG
Syphilis (rapid plasma reagin)
Strongyloides stercoralis, *Entamoeba histolytica*, *Helicobacter pylori* serology
Hepatitis A virus IgM
Hepatitis B surface antigen, core antibody, hepatitis C virus antibody
HIV type 1 and 2 antibody and p24 antigen
Antinuclear antibody
Fasting lipids and blood sugar level

C-reactive protein and erythrocyte sedimentation rate
Stool screening
Microscopy and culture
Rotavirus, norovirus and adenovirus PCR
Clostridium difficle toxin PCR
Eggs, cysts and parasites (including *Cryptosporidium* spp., *Giardia* spp., *Dientamoeba fragilis* and *Entamoeba histolytica* PCR)
Vancomycin-resistant enterococcus screen

Table 3.2: Equipment

Equipment required
Opaque plastic bag (to collect stool), cable tie
Blender with autoclaveable container
Stainless steel spoon
Normal saline
Pharmaceutical grade glycerol
Safe work bench on which to blend stool (fume hood or anaerobic chamber ideally)
-80°C freezer
Cryo-tolerant screw top containers (250 mL)
Catheter tip syringes (60 mL)
Personal protective equipment: gloves, gown, face shield
Autoclave

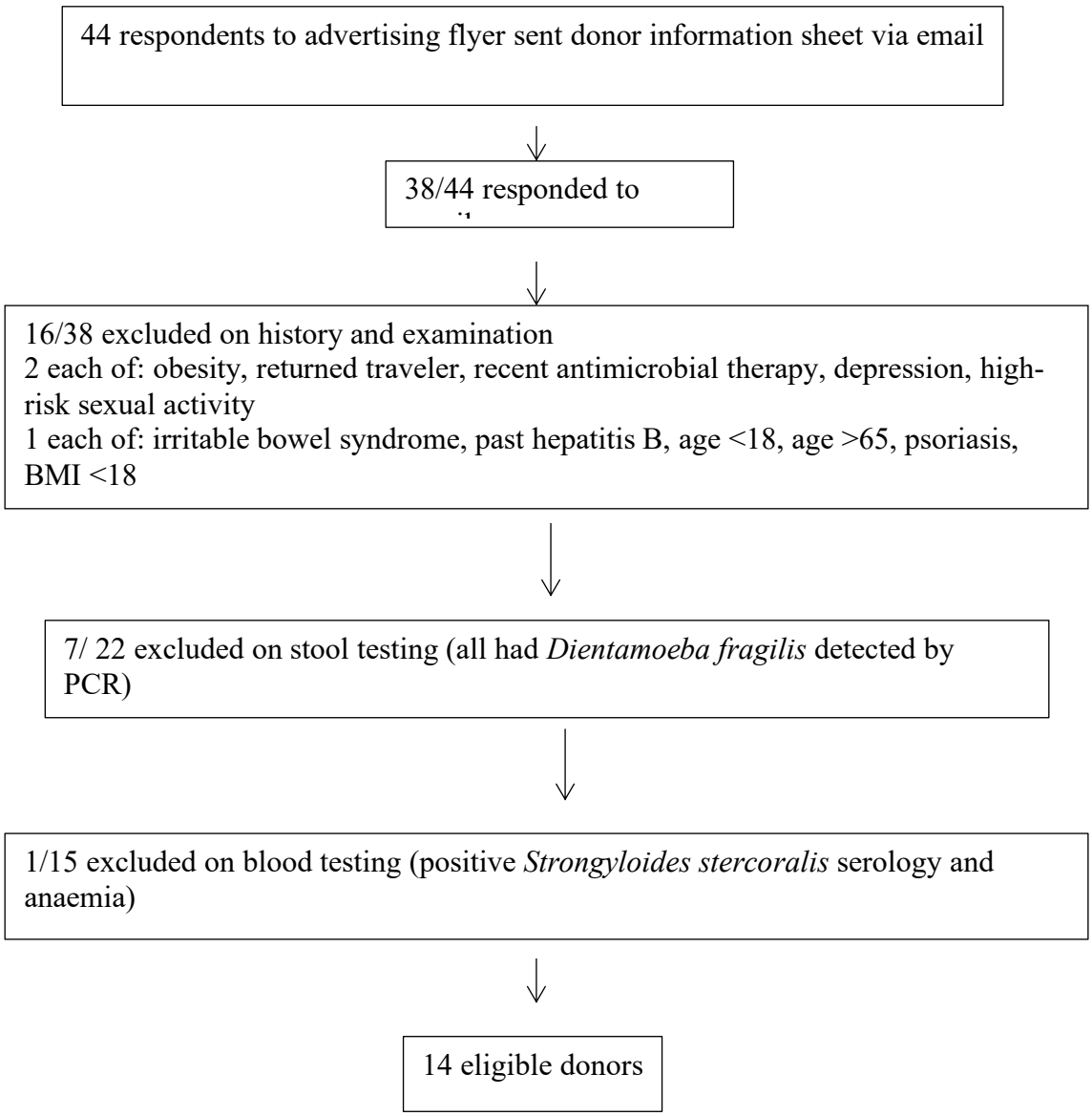

Figure 3.1: Donor recruitment

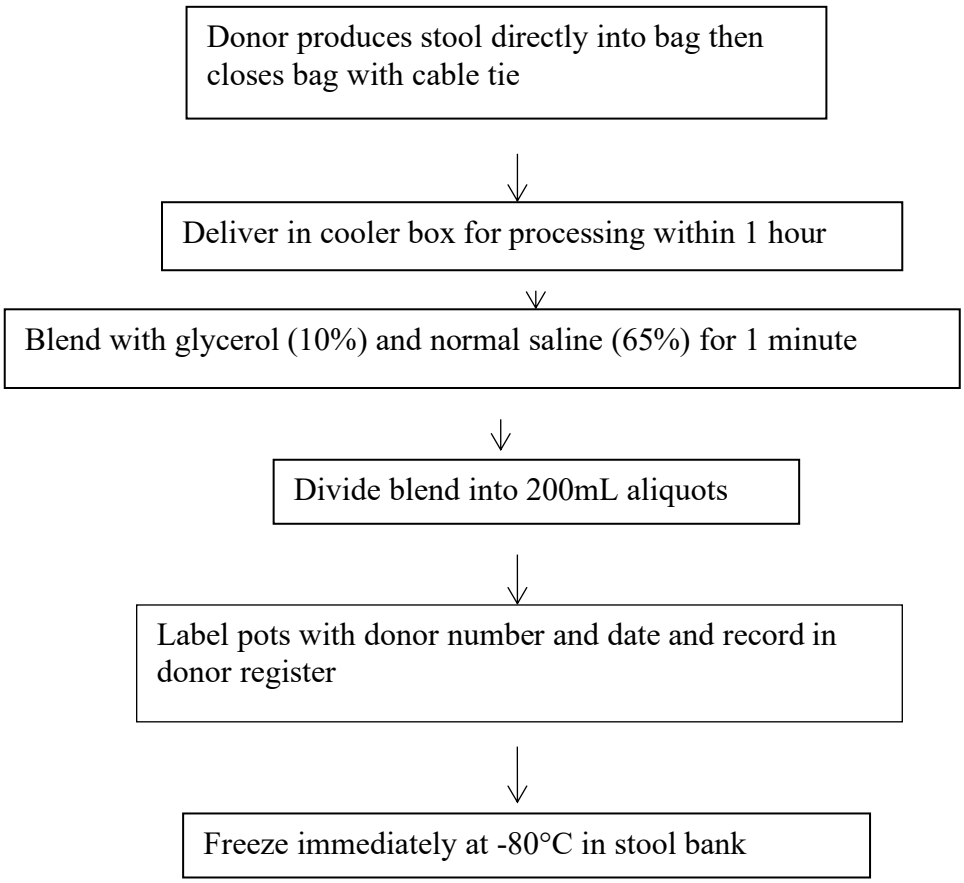

Figure 3.2: Stool preparation

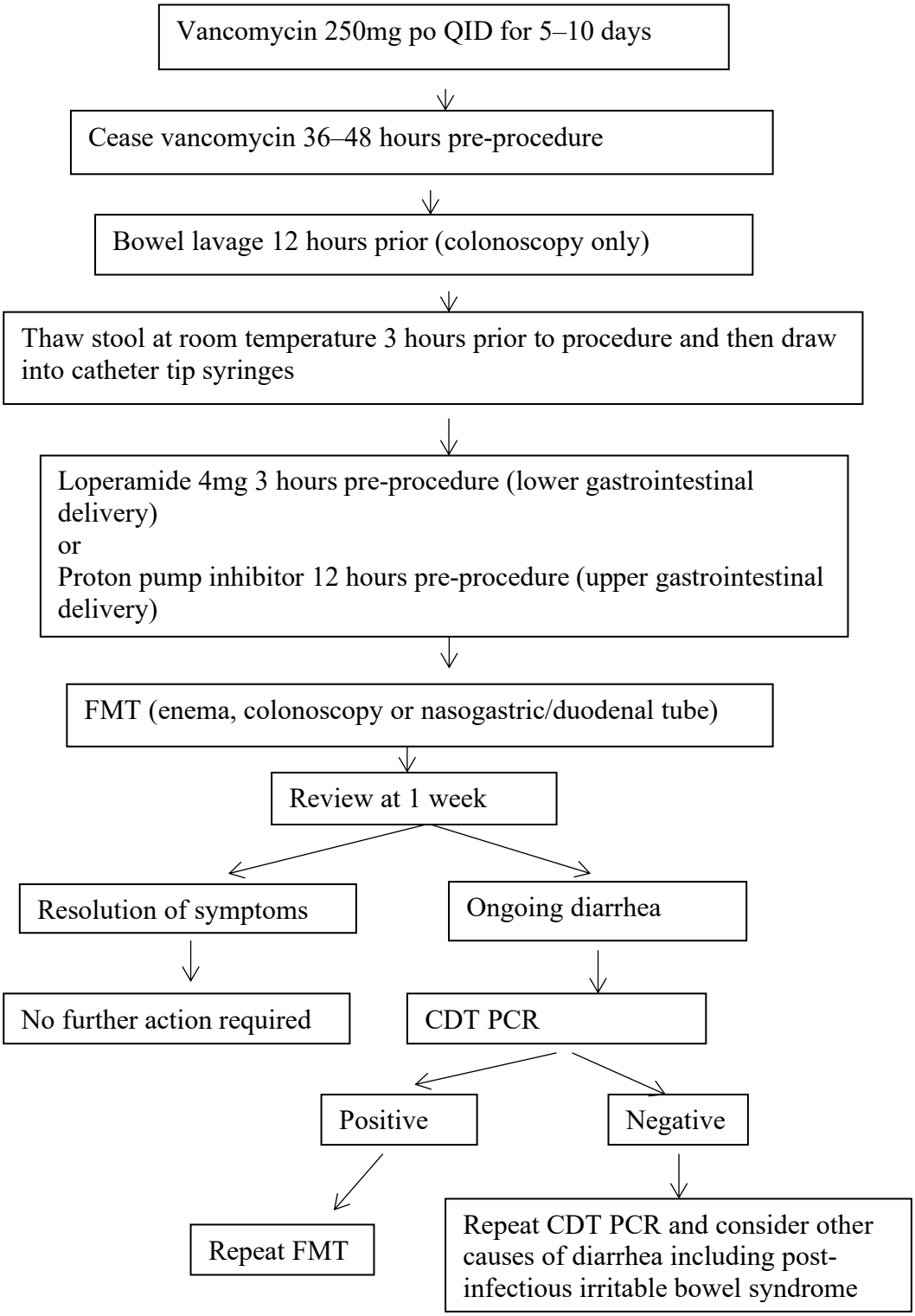

Figure 3.3: Patient preparation and follow-up

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Chapter 4: Validating Stool Bank Storage Methods

4.1 Background

In Chapter 3 of this thesis, the methods detailing the establishment of a stool bank for the reliable delivery of FMT were outlined. Critical to the efficient and reliable delivery of FMT from a stool bank is the ability to store stool frozen for prolonged time periods.

The storage methods for stool, including the components of the emulsion, had not been validated to ensure viability of the frozen organisms. It had previously been demonstrated that FMT using fresh stool and stool frozen for less than 2 months was effective treatment for rCDI; however, the efficacy of using stool stored for prolonged periods was not known.

This manuscript therefore had two parts. The first part assessed bacterial viability in stool frozen in saline with and without glycerol for up to 6 months. In the second part, a retrospective analysis of the treatment efficacy of FMT for the treatment of CDI where stool had been frozen for more than 2 months was undertaken.

Presented in this chapter is the manuscript published in *Alimentary Pharmacology & Therapeutics* (2015, Vol. 42, pp. 1011–1018).

4.2 Specific Author Contributions

Title of paper	Faecal microbiota transplant for recurrent *Clostridium difficile* Infection using long-term frozen stool is effective: Clinical efficacy and bacterial viability data
Publication status	Published
Publication details	*Alimentary Pharmacology & Therapeutics* 2015;42:1011-8 Impact factor: 7.3

Name of principal author (candidate)	Samuel Paul Costello		
Contribution to the paper	Conception and design of the project Data acquisition and management Analysis and interpretation of research data Drafting and revision of article		
Overall percentage (%)	50%		
Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	18/5/2015

Co-author contributions

By signing the Statement of Authorship, each author certifies that:

1. the candidate's stated contribution to the publication is accurate (as detailed above);
2. permission is granted for the candidate to include the publication in the thesis; and
3. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of co-author	Michael Conlon		
Contribution to the paper 15%	Conception and design of the project Analysis and interpretation of research data Drafting and revision of article		
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Signature		Date	18/5/2015

4.3 Manuscript—Faecal Microbiota Transplant for Recurrent *Clostridium difficile* Infection Using Longer-Term Frozen Stool is Effective: Clinical Efficacy and Bacterial Viability Data

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Running title: Frozen donor stool bacterial viability for *C. difficile* FMT

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Key words: faecal microbiota transplant, *Clostridium difficile*, frozen, microbiome, bacterial viability

Abstract

Background: Faecal microbial transplantation (FMT) for recurrent *Clostridium difficile* infection rCDI is greatly facilitated by frozen stool banks. However, the effect of frozen storage of stool for more than 2 months on the viability of stool bacteria is unknown and the efficacy of FMT is not clear.

Aim: To evaluate the viability of bacteria in stool frozen for up to 6 months, and the clinical efficacy of FMT with stool frozen for 2–10 months, for the treatment of rCDI.

Methods: Viability of six representative groups of faecal bacteria after 2 and 6 months of storage at -80°C, in normal saline or 10% glycerol, was assessed by culture on plate media. The clinical outcomes of 16 consecutive patients with rCDI treated with aliquots of stool frozen in 10% glycerol and stored for 2–10 months were also examined.

Results: Viability was similar to baseline at both 2 and 6 months in specimens stored in 10% glycerol and at 2 months in stool stored in saline, but was reduced by >1 log at 6 months for aerobes ($P < 0.01$), total coliforms ($P < 0.01$) and lactobacilli ($P < 0.01$) in saline. In patients undergoing FMT with stool frozen for 2–10 months in 10% glycerol, the cure rate for rCDI was 88% with one FMT and 100% after repeat FMT in those who relapsed.

Conclusions: Stool for FMT to treat rCDI can be safely stored frozen in 10% glycerol for at least 6 months without loss of clinical efficacy or viability in the six bacterial groups tested.

Introduction

Clostridium difficile is a spore-forming gram-positive bacillus that is a common cause of health care associated diarrhoea, particularly in elderly or debilitated patients[1-3]. CDI is thought to result from a diminished indigenous colonic flora, particularly after the use of broad spectrum antibiotics, that allows *C. difficile* to proliferate into the resultant ecological void[4-6]. rCDI is an increasing problem and hypervirulent strains have emerged[7-9] resulting in increased morbidity and mortality[10]. FMT has become the standard of care for patients with rCDI as a result of randomized control trial evidence of its superiority to traditional antibiotic therapy alone[11]. It is therefore now incumbent upon hospitals to establish FMT services so that rCDI can be effectively managed with this new therapy.

Whilst many facilities use fresh stool from donors known to the recipients, there are a number of issues making this practice problematic. First, there are ethical concerns regarding coercion as well as confidentiality concerns in screening known donors in the event that pre-existing undeclared disease is found in a donor or transmitted to the recipient. There is also evidence from blood transfusion safety analyses that recipient-directed donors are more likely to test positive for infectious disease than unrelated volunteer donors[12]. Stringent exclusion criteria can be more easily and dispassionately applied to volunteer donors from the community than recipient-directed donors as there are a greater number of potential candidates and no perceived personal obligation between donors, recipients and healthcare workers. Lastly, donor recruitment and testing is labor intensive and costly and by using a frozen stool bank with anonymous volunteers the process becomes more economical. Up to six treatments can be produced from each stool donation and suitable donors can give multiple samples over a short period of time.

Treating FMT donation in a similar way to blood banking, with pre-screened anonynmized donors, addresses many of the issues with fresh and/or recipient-directed donors. The development of a frozen stool bank is the most efficient and reliable way to standardize the donor stool processing and screening and allows stool to be available for the clinician to use on demand[13]. The precise elements of donor stool that determine the success of FMT are not known. A determinant of the success of a frozen stool bank may be the viability of the bacteria within the frozen stool specimens. There have also been

suggestions that success may be attributable to non-toxigenic clostridial spores in the donor samples[14]. There is evidence that FMT using stool frozen for less than 2 months is clinically as effective as fresh samples for rCDI[15] and the successful use of stool frozen for up to 5 months has been reported[16, 17]. However, the concurrent clinical efficacy and bacterial viability of stool frozen for periods substantially greater than 2 months has not been reported to date.

Glycerol is commonly used as a cryoprotective agent for frozen faecal samples[13, 15]; however, it is not known for how long stool can be frozen and continue to deliver viable bacteria. We therefore examined the viability of six culturable bacterial populations within stool stored for 2 and 6 months in two different storage media: normal saline (0.9% sodium chloride) and a normal saline and 10% glycerol mix. In addition, we reviewed our prospectively maintained FMT for rCDI database for patients who received stool that had been in frozen storage in 10% glycerol for >2 months to assess its clinical effectiveness.

Methods

Volunteer stool donors were sought by advertisement at the University of Adelaide. Donors were thoroughly screened with history, examination, and blood and stool testing similar to published guidelines[18]. Stool collected for clinical use and stored in our stool bank was processed using a blender under anaerobic conditions where stool, saline and glycerol were mixed in a 2:7:1 ratio.

Stool bacterial viability study

Stool samples collected from four volunteers were immediately processed aseptically under anaerobic conditions in an anaerobic chamber (Bactron IV Work Station, Shel Lab, Cornelius, Oregon, USA) in a nitrogen (85%) + hydrogen (10%) + carbon dioxide (5%) atmosphere. A normal saline stool suspension and a 10% glycerol stool suspension were prepared. To create the normal saline suspensions, 30g of stool from each volunteer was mixed and homogenised into suspension with of 120mL normal saline and then divided into three 50mL aliquots (ratio stool:saline = 1:4). To create the 10% glycerol suspensions, 20g of stool was mixed with 70mL normal saline and 10mL glycerol and divided into two 50mL aliquots (ratio stool:glycerol:saline = 2:1:7).

Two normal saline suspension aliquots and two 10% glycerol suspension aliquots from each of the four volunteers were then frozen immediately at -80°C. One sample of each storage medium was stored for 2 months and the other for 6 months.

A single saline suspension aliquot was then used to inoculate the plates at time zero within 2 hours of collection. Homogenised samples were serially diluted from 10^{-2} to 10^{-9} with sterile pre-reduced buffered peptone (20g/L buffered peptone water, 0.5% cysteine HCL and 0.1% Tween 80), and 100µL aliquots were plated onto the following media: chromogenic coliform agar (Oxoid, Basingstoke, UK) for total aerobes, total coliforms and *Escherichia coli*; Columbia blood agar (Oxoid, Basingstoke, UK) with 5% defibrinated horse blood for total anaerobes; Rogosa agar (Oxoid, Basingstoke, UK) for lactobacilli; and bifidus-blood agar (Oxoid, Basingstoke, UK) for bifidobacteria. We chose to examine the viability of anaerobes and aerobes as these collectively cover the breadth of the bacteria present in stool. We also chose to examine the commonly cultured bacteria, *E coli* and coliforms, that are of relevance to human health[19]. Lactobacilli and bifidobacteria were assessed because these are often used and promoted as probiotics and increases in their stool numbers are generally regarded as beneficial[20, 21].

The dilutions were inoculated onto agar using the spread plate method and incubated at 37°C. Plates were incubated for 1–5 days depending on the media, and all plates were incubated anaerobically within heat sealed plastic pouches containing Anaerogen Compact sachets (Oxoid, Basingstoke, UK), except for the cultures of aerobic bacteria, which were incubated aerobically. The numbers of colonies characteristic of each bacterial group were visually counted and the concentration calculated as colony forming units (cfu) per gram of wet weight. Typical morphology included raised copper colonies on bifidus-blood agar, identified as bifidobacteria, and purple and pink colonies on chromogenic agar identified as *E.coli* and coliforms, respectively. All colonies were counted for both Rogosa and Columbia agars.

At 2 months, a normal saline suspension aliquot and a 10% glycerol aliquot from each participant were thawed at room temperature for 180 minutes before inoculating and incubating the plates as per the technique described above. Identical thawing and inoculating techniques were again repeated at 6 months.

Clinical use of FMT

FMT was performed for rCDI at three tertiary referral hospitals in South Australia from August 2013 until February 2015. Only stool stored in normal saline with 10% glycerol suspension and frozen in a stool bank at -80°C was used for this purpose. FMT was delivered by colonoscopy to the caecum in all but one patient, who had FMT instilled into the jejunum by push enteroscopy as this patient had toxic megacolon[22] and colonoscopy was therefore unable to be performed safely.

Our prospectively collected database of FMT for patients with rCDI was interrogated for patients who had received stool that had been in frozen storage for more than 2 months at the time of use. The database contains patient demographic details, history of CDI, and FMT delivery and outcome. Patients were reviewed clinically or contacted via telephone to confirm resolution of symptoms and report side effects 3 to 14 months after FMT. In those with ongoing or recurrent diarrhoea, a stool sample was interrogated for the presence of the *C. difficile* toxin gene by PCR. Patients with a positive PCR test were treated with repeat FMT. Primary cure was defined as resolution of diarrhoea and/or absence of *C. difficile* toxin in stool as measured by PCR after at least 3 months follow-up following the first FMT treatment. Secondary cure was defined as this outcome being achieved after a second FMT treatment. Failure was defined as persistent diarrhoea and/or ongoing *C. difficile* detected in stools.

Statistics

Microbiology

For each of the four individual stool donors, absolute bacterial counts (cfu/mL) over each set of six plates were calculated. These counts were log10 transformed to obtain data normality and the four means of the six transformed measurements were used as the response variable. The four stool samples were used as the random component of the mixed model analyses, and storage and time were the fixed effects. A >1 log decrease in cfu/mL over time was considered a relevant decrease in bacterial viability for the purposes of FMT. The P-values used for indicating significances between means were adjusted using the Bonferroni correction for multiple comparisons. Viability was calculated using 10^(baseline mean - mean over time), that is, the log difference between the fresh stool culture (baseline) and the frozen sample. The percentage

viability represents the log count of the frozen sample/log count of the fresh sample multiplied by 100. All statistical analyses were performed using the R statistical package.

Clinical

Data were expressed as a median with an interquartile range.

Results

Microbiological outcomes

After storage in 10% glycerol, there was no significant effect of time on bifidobacteria, *E. coli*, total coliforms, lactobacilli, total anaerobic bacteria or total aerobes (log10 counts) and no bacterial group had a mean >1 log reduction in colony forming unit per millilitre (cfu/mL) counts after 2 months or 6 months storage with +10% glycerol (Table 4.1).

After storage in normal saline, none of the tested bacteria had a statistically significant or mean >1 log reduction in cfu/mL counts at 2 months, compared with baseline. However, there was a significant (>1 log) decrease in lactobacilli ($P < 0.01$), aerobes ($P < 0.01$) and total coliforms($P < 0.01$) at 6 months, compared with baseline (Table 4.2). At 6 months, there was also a non-significant trend towards a reduction in *E. coli* but no significant reduction in anaerobic bacteria or bifidobacteria.

After storage in normal saline, all cultures showed at least some decrease in bacteria numbers (Figure 4.1), whereas after storage in 10% glycerol, falls were minor and non-significant or counts showed a minor non-significant rise (anaerobes). Storage of stool in normal saline suspension resulted in a reduction in all cultured bacterial species, compared with 10% glycerol suspension with only total coliforms (-1.02 log, $P < 0.01$) and lactobacilli (-0.92 log, $P < 0.01$) species reaching statistical significance at 6 months.

Clinical outcomes

A total of 20 instances of primary FMT were performed in 20 discrete patients, from June 2013 to December 2014, and 16 patients received stool aliquots that had been in

storage for >2 months. Four patients received stool that had been in storage for <2 months.

The median age of the 16 patients receiving stool frozen for >2 months at the time of the FMT was 69 years (IQR 43–77). The median number of rCDI episodes prior to FMT was three (IQR 2–4). Fifteen subjects responded clinically to the initial FMT, one relapsed after 1 week following new antibiotic exposure and one subject was a primary non-responder. Both of these patients were cured with a second FMT treatment (88% primary cure and 100% secondary cure). There were no patients in whom more than 2 FMTs were required and no cases where FMT failed to cure rCDI.

Stool used for the 16 episodes of primary FMT was stored for a median of 227 days (8 months; IQR 170–272 days). There were no complications from FMT. Stool used to treat both cases of recurrence had been frozen for >2 months.

There were four patients with rCDI who received FMT with stool that had been in storage <2 months; three patients had primary cure and one had recurrence during the follow-up period. This recurrence was treated successfully with a second FMT procedure using stool also frozen for <2 months.

Discussion

Here we show that stool frozen in 10% glycerol for 2 and 6 months at -80°C contains a high number of viable and culturable bacteria. Furthermore, stool that has been frozen in 10% glycerol for 2–10 months is highly efficacious at treating rCDI. Previous studies have demonstrated the effectiveness of frozen stool for the treatment of rCDI[15-17]; however, the use of stool stored for a prolonged period of >2 months has not been specifically assessed. Studies by Satokari et al. and Youngster et al. have included stool frozen for up to 16 weeks and 5 months, respectively, to treat rCDI, but these studies did not indicate what proportion of their samples were frozen for a prolonged period and therefore are difficult to interpret in this context[16, 17]. The rate of primary cure of 88% in our cohort is comparable to other studies using fresh stool[11, 23] or stool frozen for <2 months[15].

These data are important as they inform stool preparation and storage practices and also support the proposition that healthy donors can be pre-screened and stool stored for

many months, ready for prompt and convenient use, thus avoiding many practical issues that make the use of fresh stool difficult.

Our data indicate that culturable organisms are viable for at least 6 months in frozen storage at -80°C using a 10% glycerol suspension. Viable organisms are important as donor FMT increases colonic bacterial diversity in the recipient with engraftment of donor bacteria[24]. This may be the mechanism by which resistance to *C. difficile* recurrence is conferred. Moreover, our data show that stool kept in frozen storage for over 2 months in 10% glycerol is clinically effective at treating rCDI. This has important implications for stool banks, allowing longer storage of stool than has previously been recommended[15]. Longer storage allows for less frequent donor recruitment and screening and furthermore enables the use of frozen stool that would have otherwise been discarded on the basis of current recommendations. Frequent blood and stool testing of donors is a burden on donors and also adds costs to FMT services that are not currently covered by most third-party payer schemes. The ability to store stool in a frozen stool bank for prolonged periods of time thus makes the development of a stool bank less resource intensive and more economical. Donors can therefore produce multiple samples at the time of screening and have them stored for a longer period of time.

Storage in various glycerol solutions has been demonstrated to enhance the viability of a range of bacteria in frozen storage[25-28]. Acha et al. found that after 1 year of frozen storage of infant and calf stool in 10% glycerol broth at -70°C, *E. coli* isolates were viable and in similar proportion to fresh samples[26]. Bonten et al. found that bacteria recovered from rectal swabs stored in phosphate buffered glycerol saline at -20°C for 4 weeks had reduced viability of a number of cultured bacteria, compared with fresh specimens, but significantly better viability than storage with Cary-Blair media and Amies media[27]. The reduced viability of organisms stored in phosphate buffered glycerol saline in that study might have in part been due to the storage temperature of -20°C rather than -80°C, because it has been demonstrated that lower temperatures enhance bacterial survival[26]. Cryopreserving media, such as Cary-Blair media, skim milk and Amies media, have been used to store stool for the purpose of later analysis[29-31]; however, some of these media are less effective than 10% glycerol solutions at retaining microbial viability and others are unsuitable to be administered into a patient's gastrointestinal tract as they are untested on humans.

Most FMT frozen stool banks use normal saline + 10% glycerol as a storage medium at -80°C [15-17]; however, there are no data on the viability of stool bacteria stored in this way for the purpose of FMT. Our study demonstrated a high viability of six culturable bacterial groups after 6 months of frozen storage in 10% glycerol solution. At 6 months, the viability of many of the cultured organisms was reduced in saline, compared with the 10% glycerol solution, with total coliforms (-1.02 log, $P < 0.01$) and lactobacillus (-0.92 log, $P < 0.01$) species reaching statistical significance, indicating that glycerol has a cryoprotective effect for these stool bacteria in storage. Anaerobes and lactobacilli groups had non-significant rises in their populations after 2 months' storage in 10% glycerol and bifidobacteria had non-significant rises after 2 and 6 months' storage in 10% glycerol solution (Table 4.1). This is likely to be artifactual and may partially represent statistical variation or possibly a small growth-enhancing effect of glycerol on those bacteria.

Different species of bacteria vary in their susceptibility to damage by freezing and thawing. Haines et al. found that a single freeze–thaw cycle killed 98% of *Saccharomyces cerevisiae*, but only 5% of *Staphylococcus aureus*[32]. We demonstrated that bifidobacteria had better survival in frozen storage at 6 months, compared with total coliforms and *E. coli*, particularly in the saline-only solution ($P < 0.01$) (Table 4.2). The different rates of viability for different bacterial species in frozen storage may have clinical implications for long-term FMT storage and warrants further investigation. All of the tested species were within 1 log of the baseline levels after 2 months of storage in saline alone. This suggests that stool frozen for less than 2 months in saline alone may have a sufficiently diverse viable population for use in FMT although this has not been confirmed in vivo.

Limitations

Few species of colonic flora are readily culturable with conventional techniques and so these data represent only a fraction of the bacteria in the samples that we have tested. The six groups of bacteria tested, however, represent a diverse range of bacteria and each group contains a number of distinct species. Also, these culture studies demonstrate that the six bacterial groups that we did culture at baseline remained viable after 2 and 6 months of storage with 10% glycerol. PCR-based sequencing techniques

are not useful in testing the viability of bacteria after frozen storage as they are unable to differentiate between viable and non-viable bacteria.

Only 16 patients in this study received stool that was frozen for 2 months or more and a larger sample size would be necessary to confirm that there is no difference in clinical efficacy of stool frozen for prolonged periods, compared with stool <2 months old. The success in all 16 patients does, however, suggest that the bacterial viability data that we describe are relevant clinically and that any negative effect on clinical efficacy is small. Only the 10% glycerol storage media was tested clinically in this study as this is the current standard and is highly effective at treating rCDI. Given this, there is not sufficient clinical equipoise to justify a trial of less viable FMT storage media such as normal saline alone.

Conclusion

This study demonstrates that a selected group of culturable bacteria are viable in stool stored at -80°C in 10% glycerol for up to 6 months and in normal saline for up to 2 months. Moreover, stool stored in 10% glycerol for more than 2 months is effective in vivo at treating rCDI. Longer storage times for frozen stool will allow greater flexibility in the recruitment of donors and the processing of donor stool and reduce the cost of frequent screening of donors. This has important practical implications for the maintenance and cost effectiveness of frozen stool banks used for FMT as a treatment for rCDI. Moreover, it avoids the potential practical and ethical issues surrounding recipient-directed donations.

Authorship statement

All authors have contributed to and approve the final version of the article. Dr SP Costello was the primary author of the manuscript and performed the stool processing and culture. He also established the FMT service for rCDI and performed all FMT procedures. Dr MA Conlon conceived the stool culture studies and edited the manuscript. Ms M Vuaran performed stool culture studies and assisted in interpretation of culture results and analysis. Prof I Roberts-Thomson assisted with the establishment of the FMT service and edited the manuscript. Prof JM Andrews assisted with the establishment of the FMT service and was the primary editor of the manuscript and is the guarantor of the article.

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The authors have no conflicts of interest to declare.

Prof JM Andrews has been on advisory boards, educational meetings, steering committees and on speakers panels for AbbVie and Janssen. She has also received research support and speakers fees from each company.

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Table 4.1: Culture results from faecal aliquots frozen in 10% glycerol

	Baseline (fresh) log10 cfu/mL	2 months log10 cfu/mL %baseline	6 months log10 cfu/ml %baseline	Log drop after 6 months' storage	*P* value
Bifidobacteria	8.38±0.18	8.53±0.11 145%	8.49±0.11 132%	+0.11	0.51
Aerobes	6.98±0.37	6.95±0.53 91%	6.63±0.37 44%	-0.35	0.28
Total coliforms	7.3±0.3	6.82±0.54 33%	7.11±0.14 65%	-0.19	0.54
Escherichia coli	6.15±0.48	5.97±0.7 66%	5.8±0.5 45%	-0.35	0.81
Anaerobes	9.62±0.1	9.8±0.3 151%	9.67±0.03 112%	+0.05	0.81
Lactobacilli	7.86±0.4	8.14±0.4 191%	7.78±0.31 83%	-0.08	0.76

Data are shown as the log10 (means±sem) for each bacteria stored in 10% glycerol solution. cfu = colony forming units.

Table 4.2: Culture results from faecal aliquots frozen in normal saline

	Baseline (fresh) log10 cfu/mL	2 months log10 cfu/mL %baseline	6 month log10 cfu/mL %baseline	Log drop after 6 months' storage	*P* value
Bifidobacteria	8.38±0.18	8.29±0.17 83%	8.02±0.13 45%	-0.36	0.07
Aerobes	6.98±0.37	6.67±0.3 48%	5.97±0.33 10%	-1.01	<0.01
Total coliforms	7.3±0.3	6.48±0.21 15%	5.9±0.18 4%	-1.40	<0.01
Escherichia coli	6.15±0.48	5.27±0.54 13%	5.19 11%	-0.96	0.11
Anaerobes	9.62±0.1	9.56±0.09 87%	9.23 41%	-0.39	0.13
Lactobacilli	7.86±0.4	7.25±0.49 25%	6.86±0.5 10%	-1.0	<0.01

Data are shown as the log10 (means±sem) for each bacteria stored in normal saline. cfu = colony forming units.

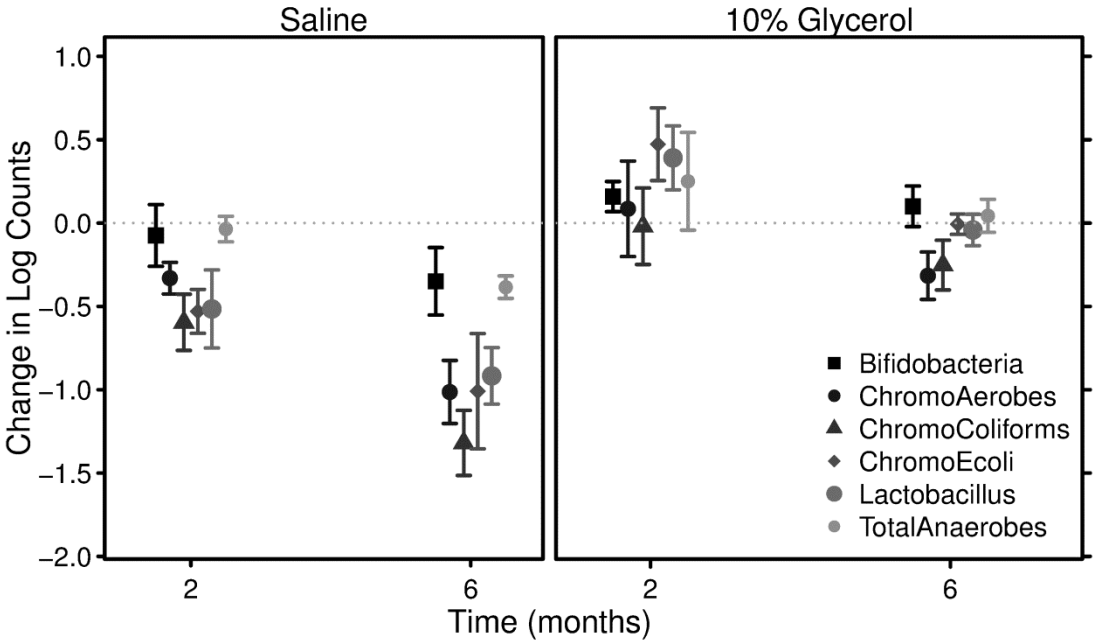

Figure 4.1: Changes in bacterial culture colony counts relative to baseline (0.0) after -80°C storage in saline and10% glycerol

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Chapter 5: Faecal Microbiota Transplantation for the Induction of Remission of Ulcerative Colitis

5.1 Background

UC is a chronic IBD that occurs at the interface between the colonic microbiota and the mucosal immune system. Although there is growing evidence implicating the colonic microbiome in UC pathogenesis[4,5], most currently available therapies target the immune response rather than the luminal microbial environment.[6] These therapies are limited by incomplete efficacy as well as intolerance and side effects, many of which result from immunosuppression. Hence, new therapies are needed for UC and microbiota-based therapies offer potential advantages over immunosuppressive medications.

This RCT (FIRST-UC study) evaluated the use of FMT for the induction of remission of mild to moderately active UC. Patients were randomised to receive either anaerobically prepared pooled donor stool or autologous FMT via colonoscopy. They were then reassessed at 8 weeks for clinical and endoscopic remission of UC. Patients underwent exploratory faecal microbiome and metabolome analyses as well as peripheral blood and mucosal mononuclear cell population analysis prior to and following FMT with the aim of understanding the mechanisms by which FMT may have a clinical effect.

During the course of this trial, the results of three other studies assessing FMT for UC emerged. These studies had relatively intensive FMT protocols and used aerobic processing methods for donor stool. Most colonic bacteria are obligate anaerobes, and are extremely oxygen sensitive; thus, they may be diminished or eliminated when stool is processed under aerobic conditions. High-intensity treatment FMT regimes may not be suitable for real-world practice. Unique features of this study were the use of anaerobic stool processing and a short-duration and low-intensity FMT treatment regimen over a 1-week period.

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5.2 Specific Author Contributions

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Name of principal author (candidate)	Samuel Paul Costello		
Contribution to the paper	Conception and design of the project Data acquisition and management Donor and patient recruitment and treatment Analysis and interpretation of research data Drafting and revision of article		
Overall percentage (%)	50%		
Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-author contributions

By signing the Statement of Authorship, each author certifies that:

1. the candidate's stated contribution to the publication is accurate (as detailed above);
2. permission is granted for the candidate to include the publication in the thesis; and
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5.3 Manuscript—Effect of Fecal Microbiota Transplantation on 8-Week Remission in Patients with Ulcerative Colitis: A Randomized Clinical Trial

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Key points

Question: Can a short duration of FMT using anaerobically prepared pooled stool suspension induce remission in active UC?

Findings: In this randomized clinical trial that included 73 adults with mild to moderately active UC, the proportion achieving steroid-free remission at 8 weeks was 32% with donor FMT vs 9% with autologous FMT—a significant difference.

Meaning: Anaerobically prepared fecal microbiota transplantation may be effective in treating UC, but further research is needed to assess longer-term efficacy and safety.

Abstract

Importance: High-intensity, aerobically prepared FMT has demonstrated efficacy in treating active UC. FMT protocols involving anaerobic stool processing methods may enhance microbial viability and allow efficacy with a lower treatment intensity.

Objective: To assess the efficacy of a short duration of FMT therapy to induce remission in UC using anaerobically prepared stool.

Design, setting and participants: A total of 73 adults with mild to moderately active UC were enrolled in a multicenter, randomized, double-blind clinical trial in three Australian tertiary referral centers between June 2013 and June 2016, with 12-month follow-up until June 2017.

Intervention: Patients were randomized to receive either anaerobically prepared pooled donor FMT (n = 38) or autologous FMT (n = 35) via colonoscopy followed by two enemas over 7 days. Open-label therapy was offered to autologous FMT participants at 8 weeks and they were followed up for 12 months.

Main outcome and measures: The primary outcome was steroid-free remission of UC, defined as a total Mayo score of ≤2 with an endoscopic Mayo score of 1 or less at week 8. Total Mayo score ranges from 0 to 12 (0 = no disease and 12 = most severe disease). Steroid-free remission of UC was reassessed at 12 months. Secondary clinical outcomes included adverse events.

Results: Among 73 patients who were randomized (mean age, 39 years; women, 33 [45%]), 69 [95%] completed the trial. The primary outcome was achieved in 12 of the 38 participants (32%) receiving pooled donor FMT, compared with 3 of the 35 (9%) receiving autologous FMT (difference 23% [95% CI 4–42%]; OR 5.0 [95% CI 1.2–20.1]; $P = 0.03$). Five of the 12 participants (42%) who achieved the primary endpoint at week 8 following donor FMT maintained remission at 12 months. There were three serious adverse events in the donor FMT group and two in the autologous FMT group.

Conclusions and relevance: In this preliminary study of adults with mild to moderate UC, 1-week treatment with anaerobically prepared donor FMT, compared with autologous FMT, resulted in a higher likelihood of remission at 8 weeks. Further research is needed to assess longer-term maintenance of remission and safety.

Trial registration: anzctr.org.au identifier: ACTRN12613000236796

Background

UC is a chronic IBD characterized by colonic mucosal inflammation occurring at the interface between the luminal contents and the mucosal immune system. UC is increasingly common worldwide and has a high rate of persistent or relapsing symptoms[1] characterized by bloody diarrhea, anemia, and abdominal pain. UC is associated with a risk of colectomy[2] and an increased risk of colorectal cancer relative to the general population.[3] Although there is growing evidence implicating the colonic microbiome in UC pathogenesis[4,5], most therapies target the immune response rather than the luminal microbial environment.[6]

In studies conducted since 2013, FMT was an extremely effective treatment for rCDI.[7-10] This has encouraged research examining FMT as a potential therapy for other diseases possibly influenced by the microbiome. FMT is proposed to treat UC by modifying the colonic ecosystem; however, the potential biochemical and/or immune mechanisms by which this may occur are unknown. FMT has demonstrated variable efficacy in treating active UC in three randomized clinical trials using aerobically prepared stool suspensions with relatively high treatment intensities.[11-13]

Most colonic bacteria and archaea are obligate anaerobes, and are extremely oxygen sensitive; thus, they may be diminished or eliminated when stool is processed under aerobic conditions.[14] If oxygen-sensitive organisms or their metabolites contribute to the clinical effect of FMT, preserving their viability may enhance the clinical effect. The objective of this study was to investigate whether using anaerobically prepared stool with a lower treatment burden would be effective at inducing remission in active UC.

Methods

Study design, setting, and patients

A randomized, double-blind clinical trial of FMT that enrolled 73 patients with active UC was conducted between June 2013 and June 2016 at three Australian centers. Participants were followed for 12 months until June 2017. All participants were 18 years of age or older and gave written informed consent. The ethics committee at each site approved the protocols. The full protocol appears in Appendix 2B.

Eligible patients had active UC with a total Mayo score[15] of 3 to 10 points and an endoscopic subscore of ≥2. The total Mayo score is a composite of clinical and endoscopic markers and ranges from 0 to 12 (0 = no disease and 12 = most severe disease). Patients were excluded if they had severe disease defined by either a total Mayo score of 11 to 12 or Truelove and Witts criteria[16] (passing >6 bloody stools/ day plus one or more of the following: temperature >37.8°C, pulse >90bpm, hemoglobin <10.5g/dL and erythrocyte sedimentation rate >30mm/hour). Other exclusion criteria were previous colonic surgery, gastrointestinal infection, pregnancy, anticoagulant therapy and current use of antibiotics or probiotics.

Stable dosing of UC maintenance therapy was required prior to enrollment: 4 weeks for 5-ASA, 6 weeks for thiopurines and methotrexate, and 8 weeks for biological agents. Patients could enroll taking an oral dose of prednisolone ≤25mg, with a mandatory taper of 5mg per week. Participants unable to cease oral prednisolone by week 8 were considered FMT non-responders.

Patient screening included total Mayo score comprised of symptom and sigmoidoscopy assessment. Stool was collected for autologous FMT, fecal calprotectin, microbiota and metabolome analysis, and infective screening (microscopy, culture, and *C. difficile* toxin mRNA). Baseline Simple Clinical Colitis Activity Index score (range, 0–19, 0 = no symptoms, and 19 = most severe symptoms),[17] medical history, demographic details, a survey of patient perception and acceptability of FMT, and a 3-day diet diary including a weighed record of all food and fluid consumed for 2 weekdays and 1 weekend day were recorded. Blood was taken for complete blood examination, electrolytes, and liver function; C-reactive protein; and peripheral blood mononuclear cell populations.

Donor selection and stool processing

Donors were sought by advertisement. Strict criteria applied to potential donors to minimize risks of disease transmission as previously described[18] (Table S5.1 in Supplement 2). Potential stool donors sequentially underwent a screening questionnaire, medical interview, and examination followed by blood and stool testing; 76 potential donors were screened, with 19 (25%) fulfilling the screening strategy. Stool was pooled and blended from three to four donors at 16 collection time points, producing 16 distinct

batches. Each stool batch provided treatment for one to seven participants. Treatment batches consisted of pooled stool (25%) blended with normal saline (65%) and glycerol (10%) under anaerobic conditions, and aliquoted into three containers for each recipient and frozen immediately at -80°C. The container for colonoscopic delivery contained 50g of stool in 200mL and the two containers for enema delivery contained 25g of stool in 100mL. Autologous stool containers had identical ratios and volumes of stool, saline, and glycerol; however, they were processed under aerobic conditions.

Randomization

Accrued participants were randomized 1:1 using a computer-generated simple randomization algorithm (http://www.random.org) to receive either pooled donor stool FMT (dFMT) or autologous FMT (aFMT). The randomization and blinding procedure was conducted by nursing staff who were not present at FMT administration. The randomization record was kept in a separate document to the patient record and other study data such that participants and clinicians performing the procedures and assessing the primary and secondary endpoints were blinded to the therapy received.

Interventions

Participants received 3L polyethylene glycol bowel preparation the evening before and loperamide, 2mg orally, immediately prior to colonoscopy. At colonoscopy, 200mL of fecal suspension of either donor stool or autologous stool was delivered into the right colon. Two further 100mL aliquots of the same fecal suspension were administered by enema in the following 7 days. The total weight of stool administered over the three FMT procedures was 100g. Recipient stool samples were collected at baseline (week 0) and weeks 4, 8, and 52 for microbiome, metabolome, and fecal calprotectin assessment. Biopsies were taken at colonoscopy at weeks 0 and 8 for lamina propria mononuclear cell (LPMC) analysis.

At the week 8 colonoscopy, following an assessment of the primary and secondary endpoints of remission, un-blinding of randomization occurred, and autologous FMT participants received open-label donor FMT induction by colonoscopy followed by two donor FMT enemas over the following 7 days. The same IBD specialized gastroenterologist performed and assessed both colonoscopies for each patient. Participants who did not undergo the week 8 assessment, required rescue therapy, or

were unable to wean oral steroids were considered to have not achieved the primary outcome of steroid-free remission.

Outcomes

Primary outcome

The primary outcome was steroid-free remission of UC as defined as a total Mayo score of ≤2 (range, 0–12) with an endoscopic Mayo score of ≤1 (range, 0–3) at week 8.

Secondary outcomes

There were several secondary outcome measures. Clinical response (measured by a ≥3 point reduction in total Mayo score at week 8 and 12 months), clinical remission (measured by a Simple Clinical Colitis Activity Index ≤2 at week 8 and 12 months), and endoscopic remission (measured by a Mayo score of <1 at week 8 and 12 months) were compared for participants receiving donor FMT with those receiving autologous FMT. Patients' perception and acceptability of FMT were assessed using a written questionnaire completed by patients prior to enrollment and at 12 months (details appear in Supplement 2). Adverse events were assessed at week 8 and 12 months by patient survey.

Changes from baseline in peripheral blood and colonic LPMC populations (assessed by flow cytometry) following FMT were evaluated at week 8, stratified by both change in total Mayo score following FMT and randomization. LPMCs were isolated enzymatically from left colonic biopsies and peripheral blood mononuclear cells isolated from blood by density gradient centrifugation as previously described[19,20] and processed immediately for analysis of immune cell populations by flow cytometry (methods are detailed in Supplement 2).

Changes in fecal-associated microbiota following FMT (at 8 weeks and 12 months) were assessed by 16S rRNA sequencing, stratified by both change in total Mayo score following FMT and randomization. The durability of engraftment of these species acquired following donor FMT was assessed by quantifying these species at 12 months. The V4 hypervariable region of the 16S rRNA gene was amplified and raw sequencing data processed into operational taxonomic units at 97% similarity in stool samples from

individual donors, pooled stool batches, and FMT recipients taken at weeks 0, 4, 8, and 52 (methods are detailed in Supplement 2).

Fecal SCFA analyses were not a pre-specified secondary endpoint; however, SCFA levels were assessed during microbiome analysis. These were performed via the tube filtration method using high-performance gas chromatography as previously described.[21]

Sample size

Sample size was calculated using a *Z* test with pooled variance for the difference of two independent proportions. The estimated remission rate in the aFMT group was set at 26% and the remission rate in the dFMT group at 60% (based on case series[22]). With 64 patients, there would be 80% power to detect a 34% difference between groups. Type 1 error was set at 5% (2-sided).

Statistical analysis

Baseline demographic, medication, and dietary factors are presented using means (standard deviations) or frequencies (percentages) as appropriate, unless otherwise stated. Baseline levels of butyrate and dietary fiber were compared between donors and UC participants using non-parametric Mann-Whitney-Wilcox tests. Nutrient intake was analyzed using FoodWorks 9 software package (Xyris).

The primary analysis compared steroid-free remission of UC at week 8 between treatment groups using a Fisher's exact test. Individuals were analyzed in the group to which they were allocated (intention to treat). A post hoc linear mixed effects logistic regression was performed, estimating the effect of treatment (fixed effect) on remission. Non-nested random intercepts were included to account for batch effects (individuals receiving the same donor mix) and site effects (treating institution). Secondary dichotomous clinical outcomes were also compared using Fisher's exact tests and identical mixed effects logistic regression models. Change in total Mayo score (week 8 minus week 0) was assessed using linear mixed effects regression with randomization, baseline score, and steroid use as fixed effects and non-nested random intercepts per batch and site, as above. Individuals missing the week 8 Mayo assessment were assumed missing at random, imputed using multiple multivariate fully conditional

imputation by chained equations (100 imputations, 20 iterations each). In addition to the variables used in the mixed effects regressions (baseline Mayo score, randomized allocation, use of steroids, donor mix, and treating institution), patient characteristics (sex, age at diagnosis, and age at study entry), disease characteristics (extent of disease and baseline endoscopic Mayo score), and medication use (oral 5-ASA, topical 5-ASA, immunomodulatory drugs, and biologic drugs) were included in the imputation.

Assessment of treatment effect on immunological markers was also assessed using linear mixed effects regressions with week 8 values as outcome, treatment group and baseline values as fixed effects. Random intercepts were included for each group of individuals receiving the same donor mix (batch effects), and post hoc non-nested random intercepts were included for each treating institution (site effects). Treatment effect models on immunological markers were extended to include change in Mayo score (week 8 minus week 0) as a fixed effect. The estimate of treatment effect on calprotectin and SCFAs, which underwent an extra assessment at week 4, was similarly modelled, but with both week 4 and week 8 assessments as outcome. Logistic mixed effects regressions were used to assess associations with microbiome diversity and zero-inflated negative binomial mixed effects regressions used to assess associations with microbiome abundance. Organisms defined as being associated with donor FMT were those for which the change was statistically significant at both week 4 and week 8 with a *P* value <.01. The details of SCFA and microbiome models are presented in Supplement 2.

Interactions between baseline factors and week 8 Mayo score were assessed by including a pairwise interaction between the factor and treatment allocation as a fixed effect in the mixed effects regression models with Mayo score as outcome. Similarly, associations between week 8 Mayo scores and change in SCFA were assessed by including, as fixed effects, the estimated change in SCFAs (see Supplement 2 for details). Associations between baseline total Mayo scores and both baseline SCFAs and immunological measures were assessed using linear regressions with Mayo scores as outcome, adjusting for oral steroid use. In these models, individuals missing week 8 Mayo score were excluded from the analyses and the calprotectin, SFCA measures, and immunological markers were log transformed. Because of the small number of individuals missing baseline covariate data (at most n = 6), these missing values were imputed using cohort means.

For all linear models, visual inspections of residual and (for mixed effects) random effect distributions were performed. A two-tailed *P* value <.05 was considered significant. No adjustment for multiple testing was performed as all secondary analyses were considered exploratory. Analyses were performed in R version (3.5.0) using *lme4*, *mice* and *glmmTMB* packages (R Foundation for Statistical Computing).

Results

Between June 2013 and June 2016, 133 patients were assessed for eligibility; 73 were randomized, 38 to the dFMT group and 35 to the aFMT group. Three participants withdrew from the dFMT group and one from the aFMT group, leaving 69 participants who completed the week 8 assessment (Figure 5.1). Baseline patient demographics, clinical data, and measures of disease activity and inflammation appeared well balanced between the two treatment groups (Table 5.1).

Primary outcome

The primary endpoint of steroid-free remission was achieved in more participants who received donor compared with autologous FMT (12/38 [32%] vs 3/35 [9%]; difference 23% [95% CI 4–42%], OR 5.0 [95% CI 1.2–20.1]; *P* = .03) (Table 5.2).

The mean total Mayo score decreased in both groups at week 8 (aFMT, -1.2 [95% CI -1.9 to -0.5] and dFMT, -3.5 [95% CI -4.3 to -2.7]). The change in total Mayo score for each participant is represented in Figure 5.2.

Secondary outcomes

8 weeks

Clinical response was also observed in more participants receiving donor FMT than autologous FMT (21/38 [55%] vs 8/35 [23%]; difference 32% [95% CI 10–54%], OR 4.3 [95% CI 1.5–11.9]; *P* = 0.007), as was clinical remission (18/38 [47%] vs 6/35 [17%]; difference 30% [95% CI 7–51%], OR 4.5 [95% CI 1.5–13.5]; *P* = 0.01) (Table 5.2). Steroid-free endoscopic remission occurred in 4 of the 38 participants (11%) receiving donor FMT vs 0 out of 35 (0%) receiving autologous FMT (difference 11% [95% CI -1% to 27%]; *P* = 0.12) (Table 5.2). At 8 weeks, 34 of 35 participants (97%) in the autologous FMT group received donor FMT.

12 months

At 12 months, 72 of 73 participants had received donor FMT, 69 of 73 (95%) were contactable, and 9 of 69 (13%) had undergone colectomy. Flexible sigmoidoscopy was performed on 26 of 38 patient (68%) randomized to the dFMT group, and 11 of 26 (42%) were in clinical and endoscopic remission. Five of the 12 participants (42%) who achieved the primary endpoint of steroid-free remission at week 8 following donor FMT maintained remission at 12 months (Table S5.2 in Supplement 2).

Patient acceptability

Prior to FMT, 65 of 69 participants (94%) thought that 1-week induction therapy with donor FMT would be acceptable to patients with UC, compared with 57 of 60 (95%) 12 months following FMT (Tables S5.3 and S5.4 in Supplement 2).

Immune analysis

Lamina propria B cell (β = 0.46 [95% CI 0.06–0.87]; *P* = 0.03) and dendritic cell (β = 0.43 [95% CI0, 0.04–0.82]; *P* = 0.03) populations were positively associated with total Mayo score at baseline. Conversely, NK cells (β = -0.50 [95% CI -0.91 to -0.09]; *P* = 0.02) were negatively associated with total Mayo score at baseline. However, donor FMT and donor FMT adjusted for total Mayo score were not significantly associated with change in any lamina propria cell populations at week 8 (Table S5.5 in Supplement 2).

Microbial diversity, abundance, and durability

At baseline, blended donor stool showed the most microbial diversity (measured by operational taxonomic units), followed by individual donor stool and UC stool of patients with UC. Diversity increased following donor FMT, compared with autologous FMT, at weeks 4 and 8 (Figure 5.3 and Table S5.6 in Supplement 2). There was no significant association between change in total Mayo score following donor FMT and baseline diversity (β = 0.6 [95% CI -4.8 to 5.9]; *P* = 0.84) nor change in diversity at week 8 (β = -20.3 [95% CI -50.7 to 11.2]; *P* = 0.23).

The 10 bacteria and the archaea *Methanobrevibacter smithii*, the increased abundance of which were most strongly associated with donor FMT at weeks 4 and 8, were all

anaerobic (Table S5.7 in Supplement 2). The abundance of these organisms remained relatively stable from week 4 to week 8; however, by 12 months there was variability in abundance of many of these organisms (Table S5.8 in Supplement 2). Increased abundance of *Anaerofilum pentosovorans* and *Bacteroides coprophilus* species was strongly associated with disease improvement following donor FMT (Table S5.9 in Supplement 2).

Other outcomes

Metabolome

Change from baseline in-stool concentrations of butyrate and other SCFAs was not significantly different between treatment groups at week 4 or 8 (Table S5.10 in Supplement 2). Stool SCFA concentrations were not associated with any observed donor FMT treatment effect (Table S5.11 in Supplement 2).

Post hoc outcomes

We did not detect any interactions of age at diagnosis or randomization, disease duration, disease distribution, sex, medication use (other than oral steroid), and macronutrient intake with the change in total Mayo score following donor FMT (Table S5.12 in Supplement 2).

Adverse events

Week 8

There were three serious adverse events in the dFMT group (worsening colitis, *C. difficile* colitis requiring colectomy, and pneumonia) and two serious adverse events in the aFMT group (both worsening colitis).

Three participants developed new anemia (aFMT, 2; dFMT, 1), two mild elevation in alkaline phosphatase (aFMT, 0; dFMT, 2), and four mild elevations of alanine aminotransferase (aFMT, 3; dFMT, 1). Overall, there were no significant differences from baseline in serum creatinine, alanine aminotransferase, alkaline phosphatase, bilirubin, and hemoglobin at week 8 between donor and autologous FMT groups (Table S5.13 in Supplement 2).

12 months

At least one adverse event was reported by 31 of 61 (51%) of participants who completed the questionnaire; 13 reported worsening colitis and nine of these underwent colectomy. There were eight reported infections and five immune-related diseases (two new cases of psoriatic arthritis and one each of enteropathic arthritis, Crohn's disease, and allergy to infliximab) that developed in the 12-month follow-up period. During this time, 13 participants reported weight gain, 8 weight loss, and 40 weight unchanged (Table S5.14 in Supplement 2).

Discussion

The main finding of this study was that a three-dose, 1-week induction course of donor FMT was more likely to induce clinical and endoscopic remission in participants with active UC at week 8 compared with autologous FMT. The study also showed a significant difference in favor of donor FMT for the secondary endpoints of clinical remission and clinical response.

Important differences between this study and previous trials of FMT for UC are the short duration and low intensity of the induction regime. Paramsothy et al.[13] demonstrated efficacy of donor FMT over placebo with an intensive regime that involved a single colonoscopic delivery of FMT to the right colon followed by enemas 5 days per week for 8 weeks. This is a high treatment burden that would likely limit applicability to practice. The other studies did not use colonoscopic delivery; Moayyedi et al.[12] demonstrated efficacy of donor FMT over placebo using a weekly FMT enema for 7 weeks and Rossen et al.[11] reported no significant difference between donor FMT and autologous FMT using a nasoduodenal infusion of FMT at weeks 0 and 6. In addition to being efficacious, the low-intensity regime was also considered acceptable to most participants; of the surveyed participants who received the short induction course of FMT over 1 week in this study, 95% found it to be acceptable therapy for UC.

A unique feature of this study was the use of anaerobic stool processing, a method that has been previously demonstrated to preserve viable anaerobes.[23] Previous FMT studies[11-13] used aerobic stool processing methods; however, it has been demonstrated that many obligate anaerobes such as *F. prausnitzi* are lost with aerobic stool processing but are preserved with anaerobic stool processing.[14] All of the organisms positively

associated with the observed treatment response in this study were anaerobes (mostly obligate anaerobes). Preservation of donor-derived anaerobes may explain the similar clinical effect seen with this low-intensity treatment study when compared with other protocols with more intensive regimes.[12,13] The use of pooled stool increased the diversity of microbes in each aliquot and this may also have increased the chance that donor FMT contained organisms with the potential to correct a functional deficit in the microbiome of people with active UC. Sequencing analysis indicated that the abundance of organisms, that changed significantly from baseline to week 4, remained stable to week 8; however, abundances varied by 12 months. This pattern paralleled the observed treatment effect.

To our knowledge, this is the first study to assess bacterial metabolites as well as mucosal and blood immune cell populations following FMT in UC. These are exploratory (hypothesis-generating) analyses conducted to explore potential mechanistic effects of FMT. There was no correlation between stool butyrate concentrations and either donor FMT effect or disease activity of UC. There was a significant association between mucosal immune populations and disease activity; however, there was no significant correlation between mucosal immune populations and donor FMT. It is plausible that the treatment effect of donor FMT resulted from the acquisition of metabolic functional capacity from donor microorganisms and was not driven by a primary immunological effect; however, further dedicated studies are required to validate these findings.

Limitations

This study has several limitations. First, the 12-month data are limited by the crossover design, being open-label, and incomplete ascertainment, and therefore are observational only. Second, there was a significant loss of follow-up at 12 months compared with 8 weeks. Third, because of both power limitations and the risk of type 1 error, secondary outcome and subgroup analyses should be considered exploratory. Fourth, central video reading of colonoscopy was not undertaken; however, autologous stool is a more effective blind to the endoscopist and preferable to water-based placebo stool used in previous trials.[12,13] Fifth, there was not a pre-specified antibiotic “washout period” prior to study entry. It is therefore possible that some participants took antibiotics prior to the trial and this might have biased the initial microbiome assessment. Sixth, stool handling

was not under completely anaerobic conditions outside of the anaerobic chamber. However, the processing methods used in this study have been demonstrated to preserve the viability of anaerobic organisms.[23] Seventh, the study was not powered to assess safety and thus further larger studies are required to assess this.

Conclusions

In this preliminary study of adults with mild to moderate ulcerative colitis, 1-week treatment with anaerobically prepared donor FMT, compared with autologous FMT, resulted in a higher likelihood of remission at 8 weeks. Further research is needed to assess longer-term maintenance of remission and safety.

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Concept and design: Costello, Andrews, Hughes, Conlon, Roberts-Thomson.

Acquisition, analysis, or interpretation of data: All authors.

Drafting of the manuscript: Costello, Vincent, Hughes, Blatchford, Andrews.

Critical revision of the manuscript: All authors.

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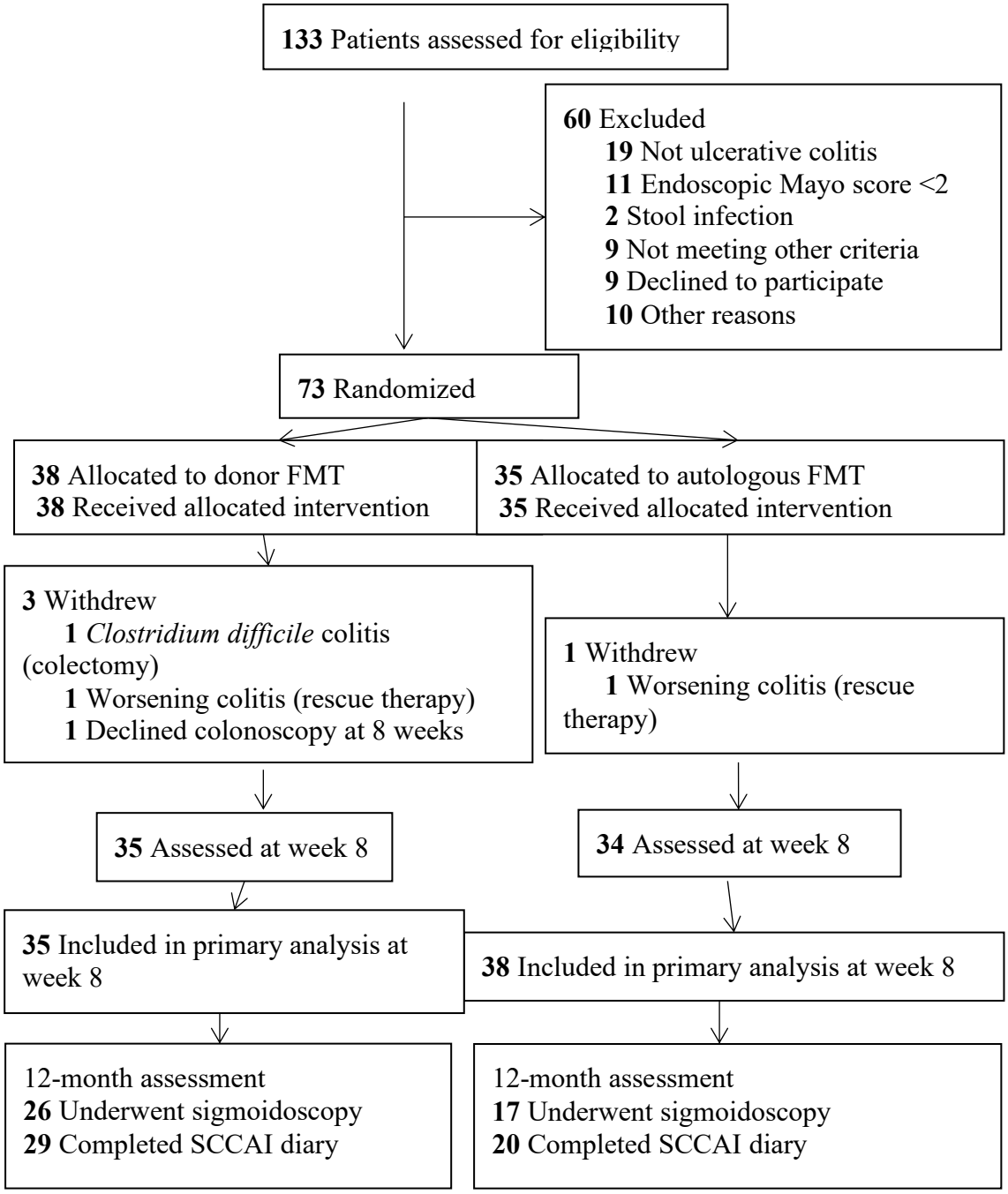

Figure 5.1: Flow of patients in randomized clinical trial of FMT for UC

Abbreviations: SCCAI, Simple Clinical Colitis Activity Index.

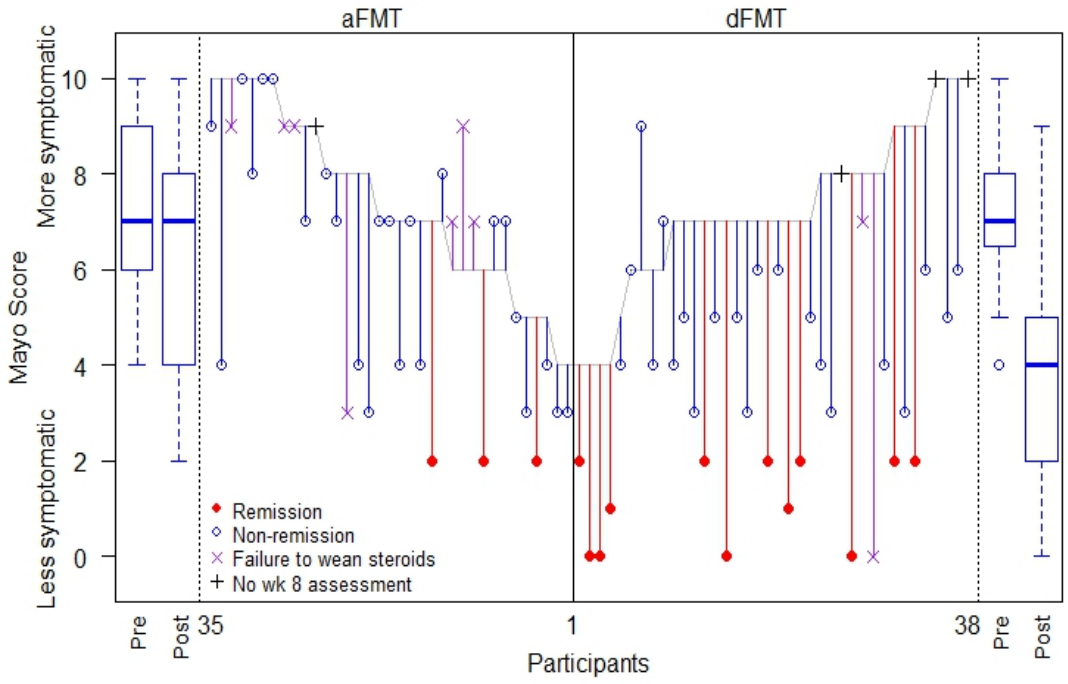

Figure 5.2: Parallel line plot depicting change in total Mayo score for individual patients

For each participant a line starts at their baseline total Mayo score and finishes at their week 8 Mayo score. Boxplots of baseline (pre) and week 8 (post) Mayo scores per treatment group present the median and interquartile range (IQR = 25th to 75th percentiles) with whisker length equal to 1.5 IQR.

Abbreviations: aFMT, autologous fecal microbiota transplantation; dFMT, donor fecal microbiota transplantation.

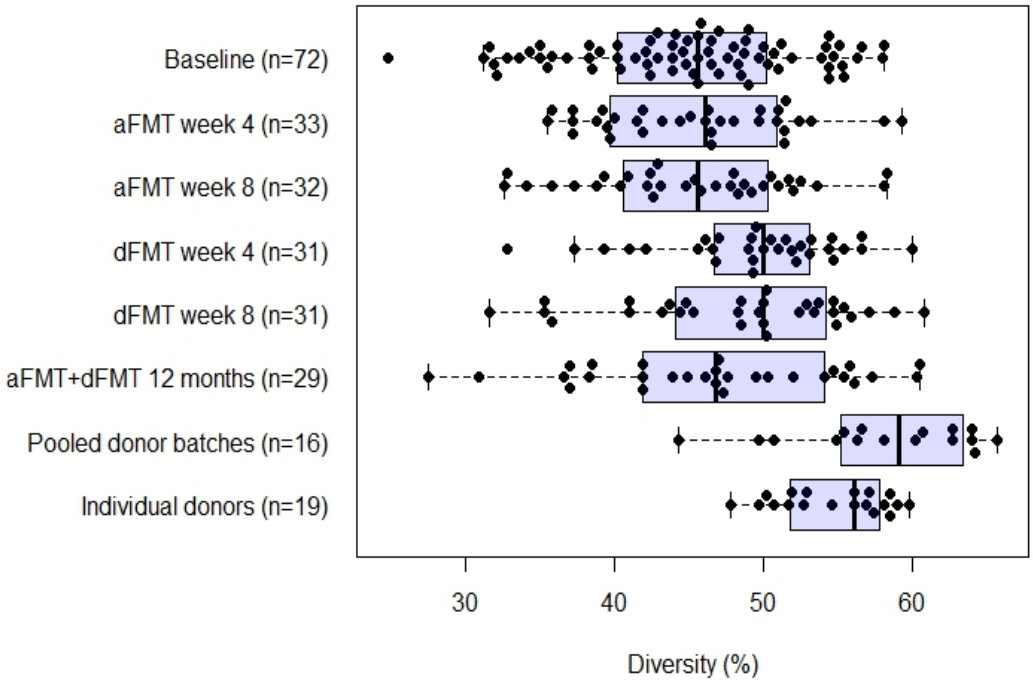

Figure 5.3: Colonic bacterial diversity in UC patients

Data are shown at baseline, 4 and 8 weeks after either donor or autologous FMT; combined groups at 12 months; donors and donor stool mixes. Diversity was assessed as the percentage of the total number of identified species.

Abbreviations: aFMT, autologous fecal microbiota transplantation; dFMT, donor fecal microbiota transplantation.

Table 5.1: Baseline characteristics of the study groups

	Donor FMT (n = 38)	Autologous FMT (n = 35)
Female participants	18 (47)	15 (43)
Male participants	20 (53)	20 (57)
Age at diagnosis, median (IQR)	30.5 (22–48)	29 (21–39)
Age at randomization, median (IQR)	38.5 (28–52)	35 (25–46)
Duration of disease (years), median (IQR)	4.9 (1.6–9.6)	5.8 (2.4–11)
Left-sided disease extent[a]	23 (61)	22 (63)
Total Mayo score[b], mean (SD)	7.2 (1.7)	7.4 (1.9)
Medication, no. (%)		
Oral steroids	8 (21)	11 (31)
5-ASA oral	33 (87)	24 (69)
5-ASA topical	11 (29)	7 (20)
Immunomodulator[c]	14 (37)	15 (43)
Biologics[d]	3 (8)	4 (11)
Inflammatory markers, median (IQR)		
CRP (mg/L)	2.8 (1.3–7.2)	2.3 (0.8–10)
WBC count ($\times 10^9$/L)	6.2 (5.3–7.3)	7.9 (6.1–8.9)
Fecal calprotectin (mg/kg)	566.5 (372.5–2687.5)	774 (221–1768)
Diet[e], mean (SD)		
Protein (g)	97 (38)	109 (42)
Carbohydrate (g)	230 (70)	221 (102)
Total fat (g)	76 (33)	86 (34)
Saturated fat (g)	29 (16)	32 (15)
Sugars (g)	90 (36)	103 (74)
Starch (g)	139 (56)	115 (54)
Fiber (g)	19 (8)	21 (8)
Calcium (mg)	700 (467)	718 (447)
Iron (g)	11.1 (6.5)	10.8 (4.4)
Energy (kj)	8742 (2574)	9049 (3111)
Sulphate (mg)	1768 (2110)	2073 (3191)

Abbreviations: IQR, interquartile range; SD, standard deviation; 5-ASA, 5-aminosalicylate; CRP, C-reactive protein; WBC, white blood cell; g, grams; mg, milligrams; kg, kilograms; kj, kilojoules; L, litres.

N (%) unless otherwise specified.

[a] Left-sided disease extent defined as disease not extending proximal to the splenic flexure.

[b] Total Mayo score is a composite of clinical and endoscopic parameters. It ranges from 0 to 12; clinical remission ≤2; mild disease 3–6, moderate disease 7–10, severe disease 11–12.

[c] Immunomodulators were either azathioprine or 6-mercaptopurine.

[d] Biologics were either infliximab or vedolizumab.

[e] Dietary information was acquired via 3-day diet diary conducted prior to patient receiving fecal microbiota transplantation.

Table 5.2: Outcome measures comparing donor FMT with autologous FMT at week 8

	Donor FMT (n = 38)	Autologous FMT (n = 35)	Absolute percentage gain over autologous FMT[a] (%) [95% CI]	Mixed effects odds ratio [95% CI]	*P* value[b]
	No. (%)				
Primary outcome[c]					
Steroid-free remission of UC at week 8[d]	12/38 (32)	3 /35 (9)	23 [4 to 42]	5.0 [1.2–20.1]	.03
Secondary outcomes[c]					
Clinical response[e]	21/38 (55)	8/35 (23)	32 [10 to 54]	4.3 [1.5–11.9]	.007
Clinical remission[f]	18/38 (47)	6/35 (17)	30 [7 to 51]	4.5 [1.5–13.5]	.01
Endoscopic remission[g]	4/38 (11)	0/35 (0)	11 [-1 to 27]	h	.12
Other outcomes					
Mean change in total Mayo score from week 0 to week 8 (SD)	-1.2 (2.1)	-3.5 (2.5)	-33 [-48 to -17]	-2.4 [-3.5 to -1.2]	<.001

[a] Absolute percentage gain refers to donor FMT over autologous FMT.

[b] *P* value applies to odds ratio.

[c] The primary and secondary outcomes at week 8 between treatment groups were assessed on an intention to treat basis using a Fisher's exact test. A post hoc logistic mixed effects analysis was performed estimating the effect of treatment (fixed effect) on remission. Non-nested random intercepts were included to account for batch effects (individuals receiving the same donor mix) and site effects (treating institution).

[d]Steroid-free remission was defined as a total Mayo score of ≤2 (range 0–12) with an endoscopic Mayo score of ≤1 (range 0–3).

[e]Clinical response was measured by a ≥3 point reduction in total Mayo score at week 8.

[f]Clinical remission was measured by a Simple Clinical Colitis Activity Index score ≤2 at week 8.

[g]Endoscopic remission was measured by a Mayo score of < 1 at week 8.

[h]Unable to calculate odds ratio for endoscopic remission.

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5.5 Supplement 2

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Tables

Table S5.1: Donor screening criteria

Medical interview (exclusions)
Age: <18 or >65
Antimicrobial therapy or probiotics in the past 6 months
Active medical illness or symptoms
Any medications (other than oral contraceptive pill)
International travel in last 1 months to areas at high risk of traveler's diarrhea
High-risk sexual activity (unprotected sex in last 1 month outside of a monogamous relationship)
Illicit drug use
Known HIV or viral hepatitis exposure in the last 12 months
Incarceration or a history of incarceration
Medical history and examination (exclusions)
Any gastrointestinal disorder
Obesity (BMI >30), hypertension, type 2 diabetes and dyslipidaemia
Malnutrition (BMI <18)
Autoimmune disease
Atopic disease
Depression
Infection with HIV, syphilis, hepatitis B or C
Malignancy
Chronic pain syndromes, neurologic or neurodevelopmental disorders
Blood screening
Full blood count
Electrolytes, urea and creatinine
Liver function tests
Human T cell lymphotropic virus 1 and 2 serology
Epstein Barr virus IgM and IgG
Cytomegalovirus IgM and IgG
Syphilis (rapid plasma reagin)
Strongyloides stercoralis, *Entamoeba histolytica*, *Helicobacter pylori* serology
Hepatitis A virus IgM
Hepatitis B surface antigen and core antibody, hepatitis C virus antibody
HIV PCR
Fasting lipids and blood sugar level
C-reactive protein and erythrocyte sedimentation rate
Stool screening
Microscopy and culture

Clostridium difficle toxin PCR

Egg, cysts and parasites (including *Cryptosporidium* spp., *Giardia* spp., *Dientamoeba fragilis* and *Entamoeba histolytica* PCR)

Table S5.2: Twelve-month clinical follow-up of UC patients

Remission definition	Randomized group	Remission 12-month assessment	Remission and no UC symptoms since donor FMT
		Number (%)	
Clinical and endoscopic remission[a]	dFMT	11/26 (42)	4/26 (7)
	aFMT[b]	10/17 (58)	5/17 (29)
	Combined	21/43 (49)	9/43 (21)
Clinical remission[c]	dFMT	18/29 (62)	5/29 (17)
	aFMT[b]	9/20 (45)	4/20 (20)
	Combined	27/49 (55)	9/49 (18)
Endoscopic remission[d]	dFMT	4/26 (15)	1/26 (4)
	aFMT[b]	4/17 (23)	3/17 (18)
	Combined	8/43 (19)	4/43 (9)
Clinical and endoscopic remission at week 8 in dFMT group (n = 12)[a]		5/12 (42)	3/12 (25)

[a] Clinical and endoscopic remission was defined as a total Mayo score ≤2 and endoscopic Mayo score ≤1.

[b] Because of FMT patients crossing over at 8 weeks, 72 of 73 study patients received donor FMT after the 8-week time point.

[c] Clinical remission was defined as a Simple Clinical Colitis Activity Index score ≤2.

[d] Endoscopic remission was defined as an endoscopic Mayo score equal to 0.

Table S5.3: Patient survey of perception and acceptability of FMT prior to undergoing FMT

Question	Number (% of responders)					No response
	Impossible	Not likely	Unsure	Quite likely	Very likely	
Do you believe that FMT is likely to help with your symptoms? (n = 69)	0 (0)	0(0)	25 (36)	36 (52)	8 (12)	4
Do you consider that FMT is likely to be safe? (n = 69)	0(0)	0(0)	10 (14)	45 (65)	14 (20)	4
Do you consider that 5-ASA medication (e.g. sulfasalazine, mesalazine) is likely to be safe? (n = 69)	6 (9)	7 (10)	18 (26)	26 (38)	12 (17)	4
Do you consider that steroid medication (e.g. prednisolone) is likely to be safe? (n = 69)	9 (13)	33 (48)	13 (19)	12 (17)	2 (3)	4
Do you consider that thiopurine medication (e.g. azathioprine/ 6-mercaptopurine) is likely to be safe? (n = 68)	3 (4)	31 (46)	22 (32)	10 (15)	2 (3)	5
Do you consider that methotrexate medication is likely to be safe? (n = 67)	3 (4)	20 (30)	41 (61)	3 (4)	0(0)	6
Do you consider that anti-TNF medication (e.g. infliximab/adalimumab) is likely to be safe? (n = 68)	6 (9)	12 (18)	45 (66)	5 (7)	0(0)	5
Do you consider that surgical removal of the colon is likely to be safe? (n = 69)	3 (4)	32 (46)	24 (35)	10 (14)	0(0)	4

Table S5.3: Patient survey of perception and acceptability of FMT prior to undergoing FMT (continued)

Question	Number (% of responders)			No response
	Yes	No	Unsure	
Do you believe FMT as carried out in this study would be seen as acceptable by the general Australian population? (n = 66)	29 (44)	9 (14)	28 (42)	7
Do you believe FMT as carried out in this study would be seen as acceptable by patients with ulcerative colitis? (n = 68)	65 (96)	0 (0)	3 (4)	5
Do you have any cultural or religious concerns about receiving fecal material from another person? (n = 68)	0 (0)	65 (96)	3 (4)	5
Do you have any concerns about discussion FMT with friends or family? (n = 63)	19 (30)	44 (70)	0(0)	10

Table S5.4: Patient survey of perception and acceptability of FMT 12 months after donor FMT (continued)

Question	Number (% of responders)					No response
	Not at all	Yes (at all)	Yes a little	Yes a lot	Unsure	
Do you believe that FMT helped with your symptoms at least temporarily? (n = 61)	17 (28)	38 (62)	17 (28)	21 (34)	6 (10)	12
	Increased	Decreased	The same	Unsure	Not applicable	No response
Has your medication requirement decreased or increased in the 12 months since FMT? (n = 60)	10 (17)	18 (30)	30 (50)	2 (3)	0(0)	13
Has the amount of steroid medication changed in the 12 months post FMT compared to the 12 months prior? (n = 60)	7 (12)	25 (42)	12 (20)	2 (3)	14 (23)	13
	Impossible	Not likely	Unsure	Quite likely	Very likely	No response
Do you consider that FMT is likely to be safe? (n = 60)	0	0	12 (20)	19 (32)	29 (48)	13

Table S5.4: Patient survey of perception and acceptability of FMT 12-months after donor FMT (continued)

Question	Number (% of responders)			No response
	Yes	No	Unsure	
Do you believe FMT as carried out in this study would be seen as acceptable by the general Australian population? (n = 59)	30 (52)	8 (14)	21 (36)	14
Do you believe FMT as carried out in this study would be acceptable to patients with ulcerative colitis? (n = 60)	57 (95)	0	3 (5)	13
Do you have any cultural or religious concerns about receiving fecal material from another person? If yes, what are your concerns? (n = 57)	1 (2)	56 (98)		16
Do you have any concerns about discussing FMT with friends or family? (n = 60)	5 (8)	55 (92)		13
Have you required hospitalization in the 12 months after FMT? (n = 61)	18 (30)	43 (70)		12
Did you require surgery (colectomy) for your ulcerative colitis since your FMT? (n = 69)	9 (13)	60 (87)		4

Table S5.5: Correlation of immune cell populations with baseline total Mayo score, change in total Mayo score, and donor FMT treatment effect

Immune cell population	Flow cytometry marker	Baseline total Mayo Score		Mayo change from baseline to week 8		Donor FMT treatment		Donor FMT adjusted for total Mayo score	
		Est [95% CI]	*P* value	Est [95% CI]	*P* value	Est [95% CI]	*P* value	Est [95% CI]	*P* value
Lamina propria mononuclear cells									
γδ T cell	CD3+ gamma delta T+	-0.17 [-0.65 to 0.31]	.48	-0.3 [-1 to 0.41]	.42	-0.51 [-1.2 to 0.19]	.16	-0.49 [-1.2 to 0.27]	.21
Natural killer cell	CD19/CD20- CD16/CD56 +	-0.5 [-0.91 to -0.099]	.02	-0.39 [-0.84 to 0.05]	.11	0.022 [-0.74 to 0.78]	.95	-0.25 [-1.1 to 0.57]	.55
Natural killer T cell	CD3+ NKT+	-0.21 [-0.66 to 0.25]	.36	-0.43 [-1 to 0.15]	.18	-0.43 [-1.1 to 0.23]	.2	-0.47 [-1.2 to 0.26]	.21
Memory T cell	CD3+ve CD45RO+ve	0.34 [-0.16 to 0.83]	.18	0.18 [-0.61 to 0.97]	.66	-0.21 [-0.65 to 0.23]	.35	0.05 [-0.4 to 0.5]	.83
B cells	CD19+/CD20+ CD45RO-	0.46 [0.057 to 0.87]	.03	0.67 [0.13 to 1.2]	.03	-0.053 [-0.82 to 0.71]	.89	0.37 [-0.35 to 1.1]	.31
Macrophage	Lineage- HLA-DR+ CD33+ SSC+	0.26 [-0.26 to 0.77]	.33	-0.00032 [-0.61 to 0.61]	1	-0.36 [-0.9 to 0.19]	.20	-0.22 [-0.84 to 0.41]	.49
Dendritic	Lineage- HLA-DR+ CD11c+ CD33+ve	0.43 [0.042 to 0.82]	.03	0.36 [-0.08 to 0.81]	.13	-0.14 [-0.76 to 0.47]	.64	0.24 [-0.41to 0.9]	.46
Helper T cell	cd4 scc+	0.11 [-0.34 to 0.57]	.62	-0.8 [-1.4 to -0.19]	.03	-0.17 [-0.63 to 0.29]	.47	-0.31 [-0.8 to 0.18]	.22
Cytotoxic T cell	cd8 scc+	-0.28 [-0.75 to 0.19]	.24	-0.62 [-1.2 to -0.026]	.08	-0.32 [-1.2 to 0.54]	.46	-0.37 [-1.3 to 0.53]	.42

Immune cell population	Flow cytometry marker	Baseline total Mayo Score		Mayo change from baseline to week 8		Donor FMT treatment		Donor FMT adjusted for total Mayo score	
		Est [95% CI]	*P* value	Est [95% CI]	*P* value	Est [95% CI]	*P* value	Est [95% CI]	*P* value
$T_{REGULATORY}$ cell	cd4 scc+ CD25+ FOXP3+	0.45 [-0.13 to 1]	.13	1.1 [0.27 to 2]	.03	-0.21 [-0.73 to 0.3]	.41	-0.056 [-0.59 to 0.48]	.84
Peripheral blood mononuclear cells									
Gut-homing T_{HELPER} cell (blood)	CD4+ CD8- CD45RO+ β_7+	-0.057 [-0.45 to 0.34]	.78	0.01 [-0.57 to 0.59]	.97	0.47 [0.053 to 0.88]	.03	0.45 [0.0088 to 0.89]	.05
Gut-homing $T_{REGULATORY}$ cell (blood)	CD4+ CD8- CD45RO+ β_7+ CD25+ FOXP3+	0.029 [-0.7 to 0.76]	.94	0.41 [-0.58 to 1.4]	.44	-0.12 [-0.6 to 0.36]	.61	-0.056 [-0.56 to 0.45]	.83

Table S5.6: Microbial diversity comparisons

Comparison of diversity (number of operational taxonomic units)	Odds ratio (95% CI)	*P* value
Baseline UC patients vs individual stool donors	0.65 (0.53 to 0.80)	<.001
Pooled donor stool vs individual donor stool	1.89 (1.44 to 2.48)	<.001
UC patients week 4 dFMT vs aFMT	1.35 (1.11 to 1.64)	.002
UC patients week 8 dFMT vs aFMT	1.31 (1.08 to 1.60)	.006
UC patients at 12 months following open-label donor FMT vs baseline	1.17 (1.10 to 1.24)	<.001
UC patients at 4 weeks following aFMT vs baseline	0.92 (0.89 to 0.96)	<.001
UC patients at 8 weeks following aFMT vs baseline	0.94 (0.90 to 0.98)	.001
UC patients 12 months aFMT vs dFMT	0.98 (0.80 to 1.20)	.82

Table S5.7: Organisms associated with a change in abundance following donor FMT as compared to autologous FMT at weeks 4 and 8 (cut off $P \leq .01$ at weeks 4 and 8)

Species	Family	Phylum	Week 4 log change abundance β [95% CI]	Week 4 *P* value	Week 8 log change abundance β [95% CI]	Week 8 *P* value
Association with increased abundance following donor FMT						
Peptococcus niger	*Peptococcaceae 1*	*Firmicutes*	4.95 [3.18 to 6.73]	<.001	4.6 [2.86 to 6.34]	<.001
Faecalicoccus pleomorphus	*Erysipelotrichaceae*	*Firmicutes*	3.77 [2.17 to 5.37]	<.001	3.07 [1.47 to 4.68]	<.001
Olsenella sp.	*Coriobacteriaceae*	*Actinobacteria*	3.07 [1.96 to 4.17]	<.001	2.41 [1.33 to 3.49]	<.001
Acidaminococcus intestini	*Acidaminococcaceae*	*Firmicutes*	1.76 [0.73 to 2.8]	<.001	2.27 [1.23 to 3.31]	<.001
Senegalimassilia anaerobia	*Coriobacteriaceae*	*Actinobacteria*	1.9 [0.88 to 2.92]	<.001	2.03 [1.02 to 3.04]	<.001
Prevotella copri	*Prevotellaceae*	*Bacteroidetes*	2.16 [1.01 to 3.32]	<.001	2.03 [0.86 to 3.2]	<.001
Methanobrevibacter smithii	*Methanobacteriaceae*	*Euryarchaeota*	1.78 [0.57 to 3]	.004	1.65 [0.44 to 2.86]	.008
Clostridium methylpentosum	*Ruminococcaceae*	*Firmicutes*	2.03 [0.95 to 3.11]	<.001	1.57 [0.49 to 2.66]	.004
Alistipes indistinctus	*Rikenellaceae*	*Bacteroidetes*	1.58 [0.67 to 2.5]	<.001	1.49 [0.58 to 2.4]	.001
Slackia isoflavoniconvertens	*Coriobacteriaceae*	*Actinobacteria*	1.44 [0.55 to 2.32]	.002	1.44 [0.54 to 2.33]	.002
Odoribacter splanchnicus strain	*Porphyromonadaceae*	*Bacteroidetes*	1.18 [0.38 to 1.97]	.004	1.07 [0.26 to 1.87]	.009
Association with reduced abundance following donor FMT						
Anaerostipes caccae	*Lachnospiraceae*	*Firmicutes*	-2.78 [-4.36 to -1.21]	<.001	-2.53 [-4.23 to -0.84]	.003
Gordonibacter pamelaeae	*Coriobacteriaceae*	*Actinobacteria*	-1.46 [-2.37 to-0.54]	.002	-1.7 [-2.65 to -0.76]	<.001
Clostridium aldenense	*Lachnospiraceae*	*Firmicutes*	-1.38 [-2.31 to-0.45]	.004	-1.4 [-2.36 to -0.44]	.004

Table S5.8: Log change from baseline abundance following donor FMT at weeks 4, 8 and 12 months in the species listed in Table S5.7

Species	Family	Phylum	Week 4 log change abundance [95% CI]	Week 4 *P* value	Week 8 log change abundance [95% CI]	Week 8 *P* value	12-month log change abundance [95% CI]	12-month *P* value
Positive associations (increase in species following donor FMT)								
Peptococcus niger	*Peptococcaceae*	*Firmicutes*	4.05 [2.76 to 5.34]	<.001	3.79 [2.57 to 5]	<0.001	4.05 [2.49 to 5.6]	<.001
Faecalicoccus pleomorphus	*Erysipelotrichaceae*	*Firmicutes*	3.22 [2.07 to 4.38]	<.001	2.37 [1.23 to 3.5]	<0.001	1.93 [0.48 to 3.39]	.009
Olsenella sp.	*Coriobacteriaceae*	*Actinobacteria*	2.17 [1.38 to 2.96]	<.001	1.59 [0.81 to 2.36]	<0.001	1.22 [0.24 to 2.19]	.01
Acidaminococcus intestini	*Acidaminococcaceae*	*Firmicutes*	1.06 [0.34 to 1.79]	.004	1.1 [0.38 to 1.83]	0.003	1.19 [0.24 to 2.15]	.01
Senegalimassilia anaerobia	*Coriobacteriaceae*	*Actinobacteria*	1.62 [0.9 to 2.34]	<.001	1.69 [0.95 to 2.42]	<0.001	0.71 [-0.21 to 1.64]	.13
Prevotella copri	*Prevotellaceae*	*Bacteroidetes*	1.69 [0.88 to 2.51]	<.001	2.08 [1.26 to 2.91]	<0.001	1.99 [0.89 to 3.1]	<.001
Methanobrevibacter smithii	*Methanobacteriaceae*	*Euryarchaeota*	1.32 [0.46 to 2.17]	.002	1.03 [0.18 to 1.88]	0.02	0.46 [-0.67 to 1.58]	.43
Clostridium methylpentosum	*Ruminococcaceae*	*Firmicutes*	0.87 [0.1 to 1.64]	.03	0.83 [0.05 to 1.61]	0.04	1.14 [0.15 to 2.12]	.02
Alistipes indistinctus	*Rikenellaceae*	*Bacteroidetes*	0.93 [0.29 to 1.58]	.004	0.68 [0.04 to 1.31]	0.04	1.29 [0.45 to 2.12]	.002
Slackia isoflavoniconvertens	*Coriobacteriaceae*	*Actinobacteria*	0.8 [0.17 to 1.42]	.01	0.79 [0.15 to 1.43]	0.01	0.73 [-0.13 to 1.59]	.10

Species	Family	Phylum	Week 4 log change abundance [95% CI]	Week 4 *P* value	Week 8 log change abundance [95% CI]	Week 8 *P* value	12-month log change abundance [95% CI]	12-month *P* value
Odoribacter splanchnicus	*Porphyromonadaceae*	*Bacteroidetes*	0.29 [-0.27 to 0.85]	.31	0.52 [-0.04 to 1.08]	0.07	0.91 [0.19 to 1.63]	.01
Negative associations (decrease in species following donor FMT)								
Anaerostipes caccae	*Lachnospiraceae*	*Firmicutes*	-2.24 [-3.47 to -1.01]	<.001	-2.43 [-3.74 to -1.11]	<0.001	1.98 [0.69 to 3.26]	.003
Gordonibacter pamelaeae	*Coriobacteriaceae*	*Actinobacteria*	-0.99 [-1.65 to -0.33]	.003	-1.39 [-2.08 to -0.7]	<0.001	-0.28 [-1.18 to 0.62]	.54
Clostridium aldenense	*Lachnospiraceae*	*Firmicutes*	-0.9 [-1.59 to -0.21]	.01	-1.15 [-1.86 to -0.44]	0.002	1.01 [0.21 to 1.82]	.01

Table S5.9: Organisms whose change in abundance (a) was associated with change in total Mayo score and (b) differed by treatment

Species	Family	Phylum	Total Mayo score change[a] [95% CI]	*P* value	Treatment difference log change[b] [95% CI]	*P* value
Species associated with Mayo score decrease (disease improvement)						
Anaerofilum pentosovorans	*Ruminococcaceae*	*Firmicutes*	-1.08 [-1.51 to -0.64]	<.001	1.41 [0.51 to 2.32]	.002
Bacteroides coprophilus	*Bacteroidaceae*	*Bacteroidetes*	-0.89 [-1.23 to -0.55]	<.001	2.84 [0.14 to 5.53]	.04
Clostridium methylpentosum	*Ruminococcaceae*	*Firmicutes*	-0.63 [-1.1 to -0.15]	.01	1.84 [0.97 to 2.72]	<.001
Acidaminococcus intestini	*Acidaminococcaceae*	*Firmicutes*	-0.55 [-1.01 to -0.08]	.03	1.93 [1.14 to 2.73]	<.001
Senegalimassilia anaerobia	*Coriobacteriaceae*	*Actinobacteria*	-0.51 [-1.01 to -0.01]	.05	1.84 [0.97 to 2.72]	<.001
Species associated with Mayo score increase (disease deterioration)						
Fusicatenibacter saccharivorans[c]	*Lachnospiraceae*	*Firmicutes*	0.58 [0.07 to 1.09]	.03	-0.67 [-1.11 to -0.23]	.003
Paraprevotella xylaniphila[d]	*Prevotellaceae*	*Bacteroidetes*	0.5 [0.11 to 0.89]	.02	0.83 [0.04 to 1.63]	.04

[a] Total Mayo change was defined as the change in total Mayo score per standard deviation in log abundance of organism (cut off $p \leq .05$).

[b] Treatment difference log change was defined as organisms associated with a change in abundance following donor FMT as compared to autologous FMT at weeks 4 and 8 (cut off $p \leq 0.05$).

[c] Treatment caused *Fusicatenibacter saccharivorans* to decrease and thereby was associated with a higher Mayo score.

[d] Only *Paraprevotella xylaniphila* was associated in the incorrect direction, i.e., it increased after treatment and was positively associated with Mayo score change.

Table S5.10: Change in SCFA levels from baseline at weeks 4 and 8 in donor and autologous FMT groups

Short chain fatty acid	Autologous FMT		Donor FMT		Treatment effect *P* value
	Week 4 vs 0 % baseline [95% CI]	Week 8 vs 0 % baseline [95% CI]	Week 4 vs 0 % baseline [95% CI]	Week 8 vs 0 % baseline [95% CI]	
Acetate	114.0 [89.6 to 145.1]	88.8 [70.0 to 112.5]	98.5 [77.7 to 124.8]	107.4 [85.3 to 135.0]	.75
Propionate	126.7 [96.6 to 166.0]	104.2 [79.8 to 136.1]	130.1 [98.4 to 171.9]	147.8 [112.5 to 194.2]	.34
Butyrate	134.1 [99.3 to 181.0]	99.0 [73.7 to 132.9]	86.4 [64.3 to 116.1]	97.8 [73.5 to 130.2]	.47
Iso-butyrate	142.3 [108.2 to 187.1]	107.7 [82.2 to 140.9]	93.7 [70.9 to 123.9]	115.0 [87.6 to 150.9]	.11
Valerate	90.3 [64.3 to 126.9]	81.6 [58.4 to 114.2]	119.3 [85.5 to 166.6]	142.9 [103.2 to 197.8]	.41
Iso-valerate	136.8 [102.2 to 182.9]	95.8 [72.0 to 127.6]	93.7 [69.5 to 126.3]	113.1 [84.5 to 151.3]	.46
Caproate	108.7 [79.8 to 148.1]	89.3 [65.9 to 121.1]	125.9 [91.8 to 172.7]	111.8 [82.1 to152.3]	.51

Table S5.11: Associations between total Mayo score at baseline and change in Mayo score with SCFA levels (at baseline and change respectively)

	Baseline Mayo		Mayo change	
	Est [95% CI]	*P* value	Est [95% CI]	*P* value
Acetate	-0.015 [-0.45 to 0.42]	.95	-0.23 [-1.3 to 0.83]	.67
Propionate	-0.0092 [-0.36 to 0.35]	.96	-0.19 [-0.98 to 0.6]	.64
Butyrate	-0.036 [-0.38 to 0.3]	.83	-0.14 [-1 to 0.75]	.75
Iso-butyrate	0.024 [-0.35 to 0.39]	.90	-0.42 [-1.3 to 0.5]	.38
Valerate	-0.078 [-0.42 to 0.26]	.65	-0.39 [-1.3 to 0.55]	.42
Iso-valerate	0.027 [-0.34 to 0.4]	.88	-0.48 [-1.3 to 0.37]	.27
Caproate	-0.13 [-0.57 to 0.31]	.55	-0.48 [-1.6 to 0.65]	.41

Table S5.12: The mean change in Mayo score for the two treatment groups for each baseline factor, and the linear mixed effects regression estimated *P* value for the pairwise interaction[a]

		Mayo score change		Interaction
		Autologous FMT	Donor FMT	LME *P* value
Sex	Male	-1.2 (2.0)	-3.4 (2.6)	.79
	Female	-1.2 (2.4)	-3.7 (2.4)	
Age at diagnosis (years)	Younger	-1.4 (2.1)	-3.6 (2.5)	.77
	Older	-1.1 (2.3)	-3.4 (2.6)	
Age at randomization (years)	Younger	-1.9 (2.0)	-3.8 (2.4)	.12
	Older	-0.5 (2.1)	-3.3 (2.7)	
Duration of disease (years)	Shorter	-1.6 (1.7)	-3.2 (2.9)	.1
	Longer	-0.9 (2.5)	-3.8 (2.1)	
Disease extent	Pancolitis	-0.8 (2.0)	-3.7 (2.5)	.34
	Left-sided	-1.5 (2.2)	-3.4 (2.6)	
Oral steroids	No	-1.6 (1.9)	-3.1 (2.3)	.01
	Yes	-0.5 (2.5)	-5.7 (2.5)	
5-ASA oral	No	-1.3 (2.4)	-2.2 (1.7)	.34
	Yes	-1.2 (2.1)	-3.7 (2.6)	
5-ASA topical	No	-1.2 (2.2)	-3.5 (2.5)	.99
	Yes	-1.4 (1.8)	-3.7 (2.7)	
Immunomodulator	No	-1.5 (2.1)	-3.5 (2.9)	.61
	Yes	-0.9 (2.2)	-3.5 (1.9)	
Biologics	No	-1.1 (2.0)	-3.5 (2.6)	.97
	Yes	-2.0 (3.2)	-4.0 (1.0)	
CRP (mg/L)	Low	-1.5 (1.9)	-3.4 (2.1)	.35
	High	-0.9 (2.4)	-3.6 (2.9)	
WBC ($\times 10^9$/L)	Low	-1.7 (2.0)	-3.6 (2.2)	.97
	High	-1.0 (2.2)	-3.3 (3.1)	
Calprotectin (mg kg)	Low	-1.4 (1.9)	-3.2 (2.4)	.23
	High	-1.1 (2.3)	-3.9 (2.7)	
Protein (g)	Low	-1.0 (1.9)	-3.5 (2.8)	.25
	High	-1.4 (2.4)	-3.6 (2.2)	
Carbohydrate (g)	Low	-1.2 (2.3)	-3.4 (3.0)	.49
	High	-1.3 (1.9)	-3.6 (2.0)	
Total fat (g)	Low	-1.1 (2.4)	-3.5 (2.8)	.43
	High	-1.3 (1.9)	-3.6 (2.2)	
Saturated fat (g)	Low	-1.4 (2.6)	-3.6 (2.8)	.26

		Mayo score change		Interaction
		Autologous FMT	Donor FMT	LME *P* value
	High	-1.1 (1.7)	-3.4 (2.2)	
Sugars (g)	Low	-1.4 (2.4)	-3.8 (3.1)	.91
	High	-1.1 (1.8)	-3.2 (1.9)	
Starch (g)	Low	-0.7 (1.9)	-3.9 (3.0)	.47
	High	-1.9 (2.2)	-3.2 (2.1)	
Fiber (g)	Low	-1.1 (1.8)	-3.5 (2.8)	.63
	High	-1.3 (2.4)	-3.6 (2.2)	
Calcium (mg)	Low	-1.1 (1.7)	-3.3 (2.7)	.16
	High	-1.4 (2.6)	-3.7 (2.4)	
Iron (g)	Low	-0.9 (1.4)	-3.3 (2.8)	.87
	High	-1.5 (2.7)	-3.7 (2.2)	
Energy (kj)	Low	-1.4 (2.2)	-3.2 (3.1)	.25
	High	-1.1 (2.1)	-3.8 (1.9)	
Emulsifier	Low	-0.8 (1.9)	-3.7 (3.0)	.45
	High	-1.9 (2.3)	-3.3 (1.9)	
Sulphate	Low	-1.4 (2.2)	-4.1 (3.0)	.38
	High	-1.0 (2.1)	-2.9 (1.8)	

Abbreviations: LME, linear mixed effects; CRP, C-reactive protein; WBC, white blood cell.

[a] For presentation of means (SD), continuous predictors are divided by their population median scores.

Table S5.13: Mean blood measures at baseline and week 8 and the comparison in the change over time between treatment groups

	Mean (%)				
	Autologous FMT		Donor FMT		
	Week 0	Week 8	Week 0	Week 8	*P* value
Haemoglobin (g/L)	142.1 (17.6)	141 (21.6)	137.2 (16.9)	138.1 (15.7)	.55
Creatinine (umol/L)	74.9 (18.1)	75.9 (18.2)	74.2 (14.5)	75.3 (14.9)	.52
Bilirubin (umol/L)	14.7 (9.3)	13.4 (8)	13.9 (7.2)	13.9 (6)	.43
Alkaline phosphatase (U/L)	76.8 (29.2)	80.7 (59.3)	80.8 (26.3)	84.8 (35.7)	.72
Alanine aminotransferase (U/L)	23.7 (9)	30 (19.7)	25.1 (13.3)	32.6 (43.5)	.73
White blood cells (×10*9/L)	7.7 (2.4)	7.2 (2.6)	6.6 (2.3)	6.2 (1.9)	.42
Neutrophils (×10*9/L)	6.5 (8.7)	6.5 (10.9)	4.2 (1.8)	3.9 (1.7)	.54
C-reactive protein (mg/L)	6.8 (8.5)	7.4 (10.4)	6.5 (8.3)	5 (8.3)	.38

Table S5.14: Twelve-month adverse events

Adverse effects	Number (%) (n = 61)
Worsening colitis	13 (21)
Colectomy	9 (15)
No colectomy	4 (7)
Weight gain	13 (21)
Weight loss	8 (13)
Fecal incontinence	2 (3)
Infections	
Influenza	2 (3)
Clostridium difficile infection	2 (3)
Sinusitis	1 (2)
Pneumonia	1 (2)
Wisdom tooth infection	1 (2)
Respiratory virus	1 (2)
Immune related	
Psoriatic arthritis	2 (3)
Crohn's disease	1 (2)
Enteropathic arthritis	1 (2)
Allergic reaction to infliximab	1 (2)
Dermatitis	1 (2)
Back pain	1 (2)
Skin petechiae	1 (2)
Urinary hesitancy	1 (2)
Asthma	1 (2)
Diverticulitis	1 (2)
Oesophageal dysmotility	1 (2)

Table S5.15: Fecal calprotectin level relative to baseline at week 4 and week 8 (log transformed)

		% of baseline fecal calprotectin [95% CI]	*P* value
Donor FMT	Week 4	47.0 [23.3, 94.6]	.03
	Week 8	44.1 [22.4, 87.2]	.02
Placebo FMT	Week 4	81.8 [41.2, 162.2]	.56
	Week 8	35.5 [18.3, 69.1]	.002

Table S5.16: Baseline and week 8 data for patients randomized to autologous FMT

			Visit 1 (week 0)		Visit 2 (week 8)		Week 8					
Study participant	Sex	Disease extent	Left endoscopic Mayo score	Total Mayo score	Left endoscopic Mayo score	Total Mayo score	Primary end point	Clinical remission	Clinical response	Endoscopic remission	Medications (study entry)	Colectomy by week 8
1	Male	Pancolitis	2	6	2	7	No	No	No	No	Prednisolone, mesalazine	No
4	Female	Pancolitis	2	9	2	9	No	No	No	No	Prednisolone, 6-mercaptopurine	No
6	Female	Pancolitis	3	8	3	8	No	No	No	No	Mesalazine, azathioprine	No
7	Male	Left-sided	2	7	2	7	No	No	No	No	Prednisolone, mesalazine	No
9	Female	Pancolitis	2	9	2	9	No	No	No	No	Prednisolone, mesalazine, methotrexate	No
10	Female	Left-sided	2	5	2	5	No	No	No	No	Budesonide	No
11	Male	Pancolitis	2	7	2	7	No	No	No	No	Sulfasalazine	No
14	Female	Left-sided	2	7	1	4	No	No	Yes	No	Sulfasalazine, mesalazine (topical), azathioprine	No
19	Male	Left-sided	2	4	1	3	No	No	No	No	Azathioprine	No
21	Female	Left-sided	2	6	2	9	No	No	No	No	Prednisolone, mesalazine, azathioprine	No
22	Male	Left-sided	2	7	2	7	No	No	No	No	Mesalazine, mesalazine (topical)	No

			Visit 1 (week 0)		Visit 2 (week 8)		Week 8					
Study participant	Sex	Disease extent	Left endoscopic Mayo score	Total Mayo score	Left endoscopic Mayo score	Total Mayo score	Primary end point	Clinical remission	Clinical response	Endoscopic remission	Medications (study entry)	Colectomy by week 8
23	Male	Pancolitis	2	8	2	7	No	No	No	No	Mesalazine	No
25	Female	Left-sided	2	9	2	7	No	No	No	No	Mesalazine, mesalazine (topical)	No
27	Male	Pancolitis	2	6	1	7	No	No	No	No	Mesalazine, mesalazine (topical), 6-mercapropurine	No
28	Male	Left-sided	3	10	2	9	No	No	No	No	Azathioprine	No
30	Male	Left-sided	2	6	1	2	Yes	Yes	Yes	No	Mesalazine, mesalazine (topical)	No
35	Male	Left-sided	2	6	2	7	No	No	No	No	Budesonide, topical steroid	No
37	Male	Pancolitis	2	7	2	4	No	Yes	Yes	No	Mesalazine	No
38	Female	Pancolitis	2	9	n/a	9	No	No	No	No	Azathioprine	No
39	Female	Left-sided	2	8	1	3	No	No	No	No	Prednisolone, mesalazine	No
43	Female	Pancolitis	3	10	2	4	No	Yes	Yes	No	Azathioprine, infliximab	No
44	Male	Left-sided	3	10	3	9	No	No	No	No	Prednisolone, 6-mercaptopurine	No
45	Male	Left-sided	3	8	2	4	No	No	Yes	No	Mesalazine	No
46	Male	Pancolitis	2	5	2	3	No	Yes	No	No	Mesalazine	No

			Visit 1 (week 0)		Visit 2 (week 8)		Week 8					
Study participant	Sex	Disease extent	Left endoscopic Mayo score	Total Mayo score	Left endoscopic Mayo score	Total Mayo score	Primary end point	Clinical remission	Clinical response	Endoscopic remission	Medications (study entry)	Colectomy by week 8
49	Female	Left-sided	2	5	0	2	Yes	No	Yes	No	Mesalazine, azathioprine, infliximab	No
51	Male	Pancolitis	2	5	2	4	No	Yes	No	No	Prednisolone	No
52	Male	Left-sided	3	10	3	10	No	No	No	No	Mesalazine, mesalazine (topical)	No
55	Female	Left-sided	3	10	3	8	No	No	No	No	Mesalazine, mesalazine (topical), azathioprine	No
57	Female	Left-sided	3	10	3	10	No	No	No	No	Mesalazine	No
59	Female	Pancolitis	2	6	3	7	No	No	No	No	Prednisolone	No
61	Male	Left-sided	2	7	1	2	Yes	Yes	Yes	No	Nil	No
62	Male	Left-sided	2	4	1	3	No	No	No	No	Mesalazine	No
64	Male	Left-sided	2	7	2	8	No	No	No	No	Mesalazine, budesonide azathioprine vedolizumab	No
70	Male	Left-sided	2	8	1	3	No	No	Yes	No	Prednisolone mesalazine	No
72	Female	Left-sided	3	10	3	10	No	No	No	No	Mesalazine, Budesonide azathioprine vedolizumab	No

Table S5.17: Baseline and week 8 data for patients randomized to donor FMT

			Visit 1 (week 0)		Visit 2 (week 8)			Week 8				
Study participant	Sex	Disease extent	Left endoscopic Mayo score	Total Mayo score	Left endoscopic Mayo score	Total Mayo score	Primary end point	Clinical remission	Clinical response	Endoscopic remission	Medications (study entry)	Colectomy by week 8
2	Male	Left-sided	2	7	1	4	No	Yes	Yes	Yes	Sulfasalzine, azathioprine	No
3	Male	Pancolitis	2	7	1	5	No	No	No	Yes	Mesalazine, mesalazine (topical), methotrexate	No
5	Female	Left-sided	3	8	1	4	No	Yes	Yes	Yes	Mesalazine, azathioprine	No
8	Male	Left-sided	2	7	1	3	No	Yes	Yes	Yes	Sulfasalazine	No
12	Female	Pancolitis	3	10	n/a	10	No	No	No	No	Prednisolone, sulfasalazine, mesalazine (topical), azathioprine	Yes
13	Male	Pancolitis	2	8	1	3	No	Yes	Yes	Yes	Mesalazine, azathioprine	No
15	Female	Left-sided	2	7	1	2	Yes	No	Yes	Yes	Mesalazine, mesalazine (topical)	No
16	Male	Left-sided	2	7	2	5	No	Yes	No	No	Mesalazine, mesalazine (topical)	No
17	Female	Left-sided	2	8	n/a	8	No	No	No	No	Mesalazine, mesalazine (topical)	No
18	Male	Pancolitis	2	7	0	0	Yes	Yes	Yes	Yes	Mesalazine	No

			Visit 1 (week 0)		Visit 2 (week 8)					Week 8		
Study participant	Sex	Disease extent	Left endoscopic Mayo score	Total Mayo score	Left endoscopic Mayo score	Total Mayo score	Primary end point	Clinical remission	Clinical response	Endoscopic remission	Medications (study entry)	Colectomy by week 8
20	Female	Left-sided	2	5	1	4	No	No	No	No	Mesalazine	No
24	Male	Pancolitis	2	8	0	0	Yes	Yes	Yes	Yes	Mesalazine	No
26	Female	Left-sided	3	9	1	2	Yes	Yes	Yes	No	Prednisolone, sulfasalazine	No
29	Male	Pancolitis	2	6	2	6	No	No	No	No	Nil	No
31	Female	Left-sided	3	7	2	5	No	Yes	No	No	Mesalazine (topical), methotrexate	No
32	Male	Left-sided	2	6	2	9	No	No	No	No	Sulfasalazine	No
33	Male	Left-sided	2	7	1	3	No	No	Yes	No	Mesalazine, mesalazine (topical)	No
34	Female	Pancolitis	2	7	2	6	No	No	No	No	Mesalazine	No
36	Male	Left-sided	2	8	2	7	No	No	No	No	Mesalazine, 6-mercaptopurine	No
40	Female	Left-sided	2	8	0	0	No	No	No	No	Prednisolone, sulfasalazine, mesalazine (topical)	No
41	Male	Pancolitis	2	4	1	2	Yes	No	No	No	Mesalazine	No
42	Female	Left-sided	3	9	1	3	No	Yes	Yes	No	Sulfasalazine, mesalazine (topical)	No
47	Male	Left-sided	2	4	0	0	Yes	Yes	Yes	Yes	Sulfasalazine	No
48	Male	Pancolitis	3	9	1	2	Yes	No	Yes	No	Prednisolone, mesalazine,	No

			Visit 1 (week 0)		Visit 2 (week 8)				Week 8			
Study participant	Sex	Disease extent	Left endoscopic Mayo score	Total Mayo score	Left endoscopic Mayo score	Total Mayo score	Primary end point	Clinical remission	Clinical response	Endoscopic remission	Medications (study entry)	Colectomy by week 8
											Azathioprine	
50	Male	Left-sided	1	4	0	0	Yes	Yes	Yes	Yes	Mesalazine, azathioprine	No
53	Female	Left-sided	2	7	1	2	Yes	Yes	Yes	No	Mesalazine, mesalazine (topical), vedolizumab	No
54	Female	Left-sided	2	6	1	4	No	No	No	No	Mesalazine, azathioprine	No
56	Male	Pancolitis	2	7	2	6	No	No	No	No	Mesalazine, azathioprine	No
58	Female	Left-sided	3	9	2	6	No	No	Yes	No	Nil	No
60	Female	Pancolitis	3	10	2	5	No	No	Yes	No	Prednisolone, mesalazine, azathioprine	No
63	Female	Left-sided	2	6	2	7	No	No	No	No	Mesalazine, mesalazine (topical)	No
65	Male	Pancolitis	3	10	2	6	No	Yes	Yes	No	Mesalazine	No
66	Male	Left-sided	2	7	1	1	Yes	Yes	Yes	No	Prednisolone, mesalazine, 6-mercaptopurine	No
67	Male	Pancolitis	2	7	1	2	Yes	Yes	Yes	No	Mesalazine, mesalazine (topical)	No
68	Female	Left-sided	3	8	2	4	No	Yes	Yes	No	Infliximab	No
69	Female	Left-sided	3	10	n/a	10	No	No	No	No	Prednisolone	No

			Visit 1 (week 0)		Visit 2 (week 8)				Week 8			
Study participant	Sex	Disease extent	Left endoscopic Mayo score	Total Mayo score	Left endoscopic Mayo score	Total Mayo score	Primary end point	Clinical remission	Clinical response	Endoscopic remission	Medications (study entry)	Colectomy by week 8
71	Female	Pancolitis	2	4	1	1	Yes	Yes	Yes	No	Mesalazine, azathioprine, infliximab	No
73	Male	Pancolitis	2	7	2	5	No	No	No	No	Mesalazine	No

Table S5.18: Twelve-month data for patients randomized to autologous FMT

Study participant	Sex	Left endoscopic Mayo	Total Mayo score	Clinical and endoscopic remission	Clinical remission	Endoscopic remission	Medications (12 months)	Months taking corticosteroid	Symptom-free for 12 months	Colectomy by 12 months
1	Male	n/a	n/a	n/a	n/a	n/a	Unknown	Unknown	No	No
4	Female	n/a	n/a	n/a	n/a	n/a	Nil	0	No	Yes
6	Female	0	1	Yes	Yes	Yes	Azathioprine	0	Yes	No
7	Male	2	9	No	No	No	Prednisolone, mesalazine	11	No	No
9	Female	n/a	n/a	n/a	No	n/a	Prednisolone, mesalazine	12	No	No
10	Female	1	1	Yes	Yes	No	Mesalazine	0	Yes	No
11	Male	n/a	n/a	n/a	n/a	n/a	Infliximab, methotrexate	2	No	Yes
14	Female	n/a	n/a	n/a	n/a	n/a	Unknown	Unknown	Yes	No
19	Male	1	2	Yes	Yes	No	Azathioprine	3	No	No
21	Female	3	7	No	No	No	Prednisolone, mesalazine, azathioprine	6	No	No
22	Male	n/a	n/a	n/a	No	n/a	Mesalazine, mesalazine (topical)	0	Yes	No
23	Male	n/a	n/a	n/a	n/a	n/a	Unknown	Unknown	No	Yes
25	Female	1	2	Yes	Yes	No	Mesalazine	0	No	No
27	Male	1	2	Yes	Yes	No	Mesalazine, 6-mercapropurine	3	No	No
28	Male	n/a	n/a	n/a	n/a	n/a	Nil	0	No	No

Study participant	Sex	Left endoscopic Mayo	Total Mayo score	Clinical and endoscopic remission	Clinical remission	Endoscopic remission	Medications (12 months)	Months taking corticosteroid	Symptom-free for 12 months	Colectomy by 12 months
30	Male	2	5	No	Yes	No	Mesalazine, mesalazine (topical)	0	No	No
35	Male	1	2	Yes	Yes	No	Budesonide	0	Yes	No
37	Male	n/a	n/a	n/a	n/a	n/a	Unknown	Unknown	No	No
38	Female	n/a	n/a	n/a	n/a	n/a	Unknown	Unknown	No	No
39	Female	1	2	Yes	Yes	No	Azathioprine	3	No	No
43	Female	2	7	No	No	No	Azathioprine (100mg)	0	No	No
44	Male	n/a	n/a	n/a	n/a	n/a	Nil	0	No	Yes
45	Male	0	0	Yes	Yes	Yes	Azathioprine	0	Yes	No
46	Male	2	7	No	No	No	Mesalazine, vedolizumab	3	No	No
49	Female	0	2	Yes	n/a	Yes	Unknown	Unknown	No	No
51	Male	2	7	No	No	No	Infliximab	0	No	No
52	Male	n/a	n/a	n/a	No	n/a	mesalazine (topical)	0	No	No
55	Female	n/a	n/a	n/a	No	n/a	Mesalazine, infliximab	0	No	No
57	Female	3	9	No	No	No	Mesalazine	2	No	No
59	Female	n/a	n/a	n/a	n/a	n/a	Unknown	Unknown	No	Yes
61	Male	n/a	n/a	n/a	n/a	n/a	Unknown	Unknown	No	No
62	62	n/a	n/a	n/a	No	n/a	Prednisolone, mesalazine, azathioprine, infliximab	3	No	No

Study participant	Sex	Left endoscopic Mayo	Total Mayo score	Clinical and endoscopic remission	Clinical remission	Endoscopic remission	Medications (12 months)	Months taking corticosteroid	Symptom-free for 12 months	Colectomy by 12 months
64	64	n/a	n/a	n/a	n/a	n/a	Nil	0	No	Yes
70	70	0	0	Yes	n/a	Yes	Nil	0	Yes	No
72	72	n/a	n/a	n/a	n/a	n/a	Mesalazine, azathioprine, infliximab	0	Yes	No

Table S5.19: Twelve-month data for patients randomized to donor fecal microbiota transplantation

Study Participant	Sex	Left endoscopic Mayo	Total Mayo score	Clinical and endoscopic remission	Clinical remission	Endoscopic remission	Medications (12 months)	Months taking corticosteroid	Symptom-free for 12 months	Colectomy by 12 months
2	Male	0	0	Yes	Yes	Yes	Azathioprine	0	Yes	No
3	Male	n/a	n/a	n/a	n/a	n/a	Mesalazine, Infliximab	3	No	No
5	Female	2	7	No	No	No	Mesalazine, Mesalazine (topical), Azathioprine	2	No	No
8	Male	2	4	No	No	No	Sulfasalazine, Infliximab	4	No	No
12	Female	n/a	n/a	n/a	n/a	n/a	Prednisolone, Sulfasalazine, Mesalazine (topical)	10	No	Yes
13	Male	n/a	n/a	n/a	Yes	n/a	Mesalazine, Azathioprine	0	No	No
15	Female	n/a	n/a	n/a	No	n/a	Mesalazine	0	No	No
16	Male	3	10	No	No	No	Unknown	Unknown	No	No
17	Female	n/a	n/a	n/a	n/a	n/a	Mesalazine (topical)	8	No	Yes
18	Male	1	2	Yes	Yes	No	Mesalazine	0	Yes	No
20	Female	0	0	Yes	Yes	Yes	Mesalazine (topical)	0	No	No
24	Male	2	5	No	No	No	Mesalazine	0	Yes	No
26	Female	1	3	No	No	No	Prednisolone, Mesalazine	10	No	No
29	Male	n/a	n/a	n/a	n/a	n/a	Vedolizumab	4	No	No

Study Participant	Sex	Left endoscopic Mayo	Total Mayo score	Clinical and endoscopic remission	Clinical remission	Endoscopic remission	Medications (12 months)	Months taking corticosteroid	Symptom-free for 12 months	Colectomy by 12 months
31	Female	2	4	No	Yes	No	Mesalazine (topical), methotrexate	0	Yes	No
32	Male	1	6	No	No	No	Sulfasalazine, vedolizumab	Unknown	No	No
33	Male	n/a	n/a	n/a	n/a	n/a	Mesalazine, mesalazine (topical)	1	No	No
34	Female	2	3	No	Yes	No	Mesalazine	Unknown	No	No
36	Male	n/a	n/a	n/a	No	n/a	Prednisolone, mesalazine. 6-mercaptopurine, vedolizumab	12	No	No
40	Female	n/a	n/a	n/a	Yes	n/a	Sulfasalazine	2	No	No
41	Male	1	2	Yes	Yes	No	Mesalazine	0	Yes	No
42	Female	3	7	No	Yes	No	Sulfasalazine	0	No	No
47	Male	1	2	Yes	Yes	No	Sulfasalazine	0	No	No
48	Male	1	1	Yes	Yes	No	Azathioprine	6	No	No
50	Male	n/a	n/a	n/a	Yes	n/a	Unknown	Unknown	No	No
53	Female	n/a	n/a	n/a	n/a	n/a	Nil	0	No	Yes
54	Female	0	0	Yes	Yes	Yes	Mesalazine, azathioprine	2	No	No
56	Male	1	3	No	No	No	Unknown	Unknown	No	No
58	Female	1	4	No	Yes	No	Unknown	Unknown	No	No
60	Female	0	0	Yes	No	Yes	Mesalazine, azathioprine	2	No	No

Study Participant	Sex	Left endoscopic Mayo	Total Mayo score	Clinical and endoscopic remission	Clinical remission	Endoscopic remission	Medications (12 months)	Months taking corticosteroid	Symptom-free for 12 months	Colectomy by 12 months
63	Female	1	1	Yes	No	No	Mesalazine, mesalazine (topical)	Unknown	No	No
65	Male	2	6	No	Yes	No	Mesalazine	0	No	No
66	Male	1	3	No	No	No	Mesalazine, 6-mercaptopurine	0	No	No
67	Male	2	7	No	No	No	Mesalazine, adalimumab	2	No	No
68	Female	1	2	Yes	Yes	No	Infliximab	0	No	No
69	Female	n/a	n/a	n/a	Yes	n/a	Vedolizumab	4	No	No
71	Female	1	2	Yes	Yes	No	Infliximab	0	Yes	No
73	Male	2	6	No	No	No	Mesalazine	1	Yes	No

Table S5.20: Change due to treatment in butyrate-producing species and genera

Species	Family	Phylum	Treatment difference log change abundance week 4 [95% CI]	Week 4 *P* value	Treatment difference log change abundance week 8 [95% CI]	Week 8 *P* value
Anaerostipes caccae	*Lachnospiraceae*	*Firmicutes*	-2.78 [-4.36 to -1.21]	.0005	-2.53 [-4.23 to -0.84]	.003
Butyricicoccus pullicaecorum	*Ruminococcaceae*	*Firmicutes*	0.95 [-0.13 to 2.03]	.09	-0.45 [-1.55 to 0.65]	.42
Roseburia inulinivorans	*Lachnospiraceae*	*Firmicutes*	0.54 [-0.41 to 1.48]	.27	-0.36 [-1.3 to 0.59]	.46
Anaerostipes butyraticus	*Lachnospiraceae*	*Firmicutes*	-1.26 [-4 to 1.47]	.37	-5.11 [-8.12 to -2.1]	<.001
Roseburia. intestinalis	*Lachnospiraceae*	*Firmicutes*	-0.3 [-1.02 to 0.41]	.4	-0.27 [-0.98 to 0.44]	.46
Faecalibacterium prausnitzii	*Ruminococcaceae*	*Firmicutes*	0.16 [-0.22 to 0.54]	.41	-0.06 [-0.45 to 0.32]	.74
Anaerostipes sp.	*Lachnospiraceae*	*Firmicutes*	-0.12 [-0.59 to 0.35]	.62	-0.13 [-0.6 to 0.35]	.60

Figures

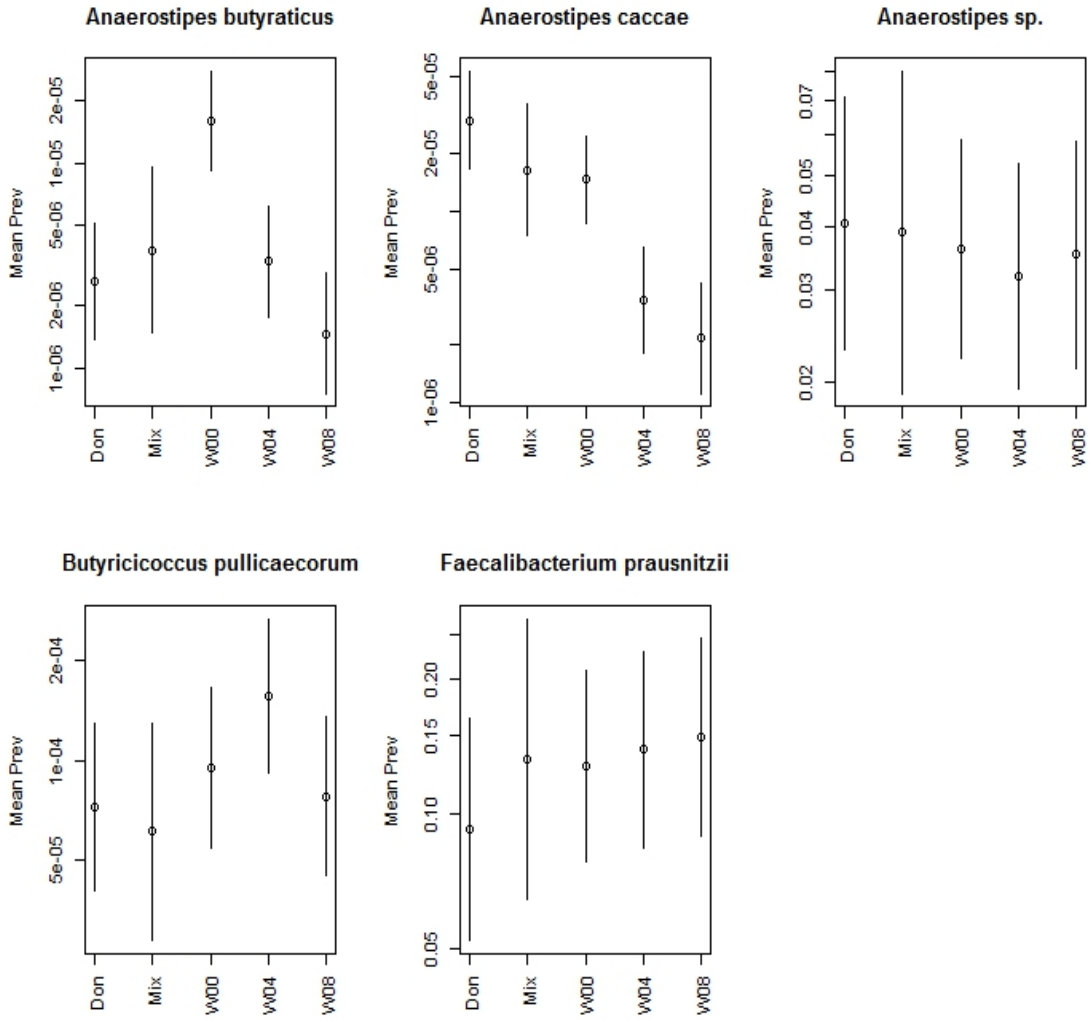

Figure S5.1: Butyrate-producing bacteria prevalence in donors (individual and pooled) and patients prior to, then 4 and 8 weeks after, donor FMT

Supplementary methods

Bacterial analysis methods

There were 228 fecal samples available from 72 patients enrolled in the study and 72 fecal samples available from donors (53 individual donor and 19 pooled batches). Stool from patients and individual donors was frozen without additive at -80°C. Stool swabs were stored for up to 8 weeks at -20°C prior to transfer to -80°C. Stool from the donor batches was frozen at -80°C with 65% saline and 10% glycerol.

We extracted bacterial DNA from the samples using the MoBio PowerMag Microbial DNA Isolation kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's protocol. All stool samples were extracted and processed in duplicate. Amplicon library preparation was performed using a modified dual-index PCR approach.[1] The first-step primers (515F, 806R), which were modified by the inclusion of a phaser to increase heterogeneity in the sequencing run,[2] amplified the V4-V5 hypervariable region of the 16S rRNA gene and the second set (i5, i7) added the indexed barcodes to enable multiplexing of our large number of samples.[1] The library was pooled at equi-molar concentrations and run on an Illumina HiSeq2500 Rapid instrument using 2 × 250 bp paired-end chemistry (Ramaciotti Centre for Genomics, University of New South Wales). The median number of reads per sample was 143k (thousand) (IQR, 111k-196k). Samples with total read count <10k were excluded.

Bioinformatics

Raw sequencing data were processed using a combination of both in-house and open-source software. The bioinformatic pipeline utilised USEARCH algorithms,[3] which included merging, quality-filtering, partitioning/de-replicating and clustering into operational taxonomic units (OTUs) at 97% similarity. Representative sequences from each OTU were classified in two ways: via the RDP Naïve Bayesian Classifier and by finding the closest match in a set of curated reference sequences (RDP 16S Training Set + RefSeq 16S).[4] The use of two independent classification techniques improves confidence in the taxonomic assignments.

Flow cytometry

LPMC isolation: Colonic mucosal biopsies were incubated twice in Hepes buffered HBSS supplemented with 1mM EDTA and 1mM DTT (Sigma) for 10 minutes at 37°C under slow rotation, with the suspension strained (100μM) between incubations. Residual tissue was incubated in Hepes buffered Ca^{2+}/Mg^{2+} free HBSS for 10 minutes at 37°C under slow rotation and strained (100μM). Residual tissue was minced and incubated in complete media (RPMI 1640 [Gibco, Germany] supplemented with fetal calf serum, glutamax and penicillin/streptomycin, Collagenase D [1mg/mL, Roche], DNAse1 [0.5mg/mL, Sigma] and Dispase [3mg/mL, Roche]) 20 minutes twice, with supernatant removal from centrifugation (300*g*, 5 minutes) after each incubation. Residual suspensions were sequentially strained (100μM followed by 40μM), with the supernatant centrifuged (300*g*, 5min), resuspended, and stained with trypan blue to determine viability and cell number as previously described.[5-7]

Cell staining: 0.5×10^6 F_c blocked cells (BD Biosciences, NSW, Australia) were stained for viability (FVD eFlour450, eBioscience) and the following anti-human monoclonal antibody panels (BD Bioscience unless otherwise stated): (a) HLADR-APC, CD11C-FITC, Lin (CD3, CD14, CD16, CD19, CD34, CD56 all APC-Cy7, CD33-PerCP Cy5.5); (b) CD3-APC, CD45RO-PerCP Cy5.5, CD19-APC Cy7, CD20-APC Cy7, CD16-PE, CD56-PE, Vα24jα-FITC (eBioscience); and (c) CD3-APC, CD8-FITC, CD45RO-PerCP Cy5.5, γδT-PE (eBioscience). For T_{REG}, cells were stained with CD4-APC Cy7, CD8-PE, CD45RO PerCP Cy5.5, CD25 PE Cy7, β7-FITC, followed by fixation and permeabilization (Transcription buffer staining set, eBioscience) and staining with FOXP3-APC (eBioscience). The following gating strategy was used to identify cell populations: macrophages (lin-ve/HLADR/CD33+ve), dendritic cells (lin – ve HLADR+/CD33+/CD11c+), T_{HELPER} (CD4+ CD8-), $T_{CYTOTOXIC}$ (CD8+ CD4-), $T_{REGULATORY}$ (CD4+/CD8-/CD25+/FOXP3+), B (CD3-, CD19+ CD20+), natural killer (CD3-/CD16+/CD56+/CD45RO-), natural killer T (CD3+/NKT+), γδ T (CD3+/γδT+) in LPMCs; and gut-homing T_{HELPER} (CD4+/CD8-/CD45RO+/β_7+) and gut-homing $T_{REGULATORY}$ (CD4+/CD8-/CD45RO+/β_7+/CD25+/FOXP3+) in PBMCs. Twenty thousand events/tube were analyzed on a FACSCanto II (BD Biosciences) and proportions of live singlets were determined using FlowJo (Tree Star, OR, USA) as previously described.[5-7]

Statistical analysis

Microbiome diversity

Microbiome diversity was defined as the fraction of unique species present at an assessment out of all species present at any analysis in any sample. Logistic mixed effects regressions were used to compare between treatment groups with donor stool and stool mix samples. Outcome was the presence of a species in a particular sample. Fixed effects included sample origin (donor vs mix vs treated patient vs untreated patient) and total sample count (log transformed). Three non-nested random effects were included: patient identifier, donor batch, and the microbiome species identifier. To assess the effect of treatment, a separate model was contrasted with only post-baseline samples included as outcome. This model was identical to the previous except that the fixed effects were baseline prevalence (logit transformed), treatment allocation, assessment time (week 4 vs week 8), the pairwise treatment–assessment time interaction, and total sample count (log transformed).

Associations between both baseline diversity and change in diversity, and change in Mayo score were assessed as before (re-associations with baseline factors). A two-stage approach was taken. First, the mean diversity was estimated using the logistic mixed effects models previously described in this section. These diversity estimates were then included in the models of total Mayo score as fixed effects.

Microbiome abundance

Associations between changes in biome species abundance with total Mayo score were modelled in a similar manner. For each sample, the mean proportion of total counts was calculated, and subsequently for individuals with samples at both week 4 and 8 averaged to estimate baseline and post randomization prevalence estimates. The change in prevalence was then included in linear mixed effects models of total Mayo score. A false discovery rate (FDR) analysis was performed to provide evidence of associations beyond what would be expected because of multiple testing, with the FDR being compared with the same analysis repeated, but with outcome (total Mayo score) permuted between individuals.

The change in abundance by treatment group and assessment time were assessed using a negative binomial mixed effects regression for each microbiome species. Fixed effects included treatment allocation, assessment time (baseline, week 4, week 8, and 12-months) and their pairwise interaction. Nested random intercepts per patient and assessment were included in the model, with total sample count (log transformed) included as an offset. Due to the large variation in abundance across species, from highly abundant to mostly absent, a zero-inflation term was included in the model and Akaike's information criteria was used to determine whether this improved model fit per species.

Fecal short chain fatty acids and calprotectin

The estimate of treatment effect on calprotectin and SCFAs, which had an extra assessment at week 4, was similarly modelled but with both week 4 and week 8 assessments as outcome. Baseline values, treatment group, assessment time (week 4 vs week 8), and the pairwise interaction between time and treatment were included as fixed effects. In addition to the batch and site random intercepts, within-individual random intercepts were included nested within site. After inspection of the distribution of the residuals, these analyses were performed on log-transformed calprotectin, SFCA measures, and immunological markers, with results converted back to the original scale.

Associations between estimated change in SCFAs and week 8 Mayo score were assessed by including the estimated change in SCFAs as a fixed effect in the mixed effects regression models with week 8 Mayo score as outcome. Individual level SCFA change scores were estimated using linear mixed effects regressions, adjusting for baseline levels and treatment, with random intercepts per batch, individual, and site, with individual level effects nested within site.

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Name: DOB: Date:

Patient perception of faecal transplantation for ulcerative colitis questionnaire

Prior to faecal transplantation

Please circle the most appropriate answer

1. Do you believe that faecal transplantation is likely to help with your symptoms?

Impossible Not likely Unsure Quite likely Very likely

2. Have you considered faecal transplantation for ulcerative colitis previously?

Yes I have considered it I have heard of it, but not considered it I have never heard of it before

3.1 Do you consider that faecal transplantation is likely to be safe?

Impossible Not likely Unsure Quite likely Very likely

Please explain why

3.2 Do you consider that 5-ASA medication (e.g. sulphasalazine, mesalazine) is likely to be safe?

Impossible Not likely Unsure Quite likely Very likely

3.3 Do you consider that steroid medication (e.g., prednisolone) is likely to be safe?

Impossible Not likely Unsure Quite likely Very likely

3.4 Do you consider that thiopurine medication (e.g. azathioprine/ 6-MP) is likely to be safe?

Impossible Not likely Unsure Quite likely Very likely

3.5 Do you consider that methotrexate medication is likely to be safe?

Impossible Not likely Unsure Quite likely Very likely

3.6 Do you consider that anti-TNF medication (e.g., infliximab (Remicade)/adalimumab (Humira)) is likely to be safe?

Impossible Not likely Unsure Quite likely Very likely

3.7 Do you consider that surgical removal of the colon is likely to be safe?

Impossible Not likely Unsure Quite likely Very likely

4. Do you believe faecal transplantation as carried out in this study would be seen as acceptable by

1. The general Australian population? Yes No Unsure
2. Patients with ulcerative colitis? Yes No Unsure

5. Do you have any cultural or religious concerns about receiving faecal material from another person?

Yes No Unsure

If yes, what are your concerns?

6. How would you compare faecal transplantation to traditional medical treatments of ulcerative colitis?

- How do you compare the acceptability of these treatments?

7. How would you compare faecal transplantation to other treatments such as probiotics?

- How do you compare the acceptability of these treatments

8. Do you have any concerns about discussing faecal transplant with friends or family?

If so why?

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Name: DOB: Date:

Patient perception of faecal transplantation for ulcerative colitis questionnaire

12 months post faecal transplantation

Please circle the most appropriate answer

1. Do you believe that faecal transplantation helped with your symptoms at least temporarily?

Not at all Yes a little Yes a lot Unsure

If you had symptom improvement how long did this last?

2. Has your medication requirement decreased or increased in the 12 months since faecal transplant?

Decreased Increased The same

What are you now taking?

For how many months were you taking steroid (eg prednisolone) in the 12 months after faecal transplant?

Has the amount of steroid medication changed in the 12 months post faecal transplant compared to the 12 months prior?

Increased Decreased Stayed the same

3. How many flares of disease did you have in the 12 months after faecal transplant?

If you had flares of disease, for how many months were you symptomatic in the 12 months after faecal transplant?

Have you required hospitalisation in the 12 months after faecal transplant?

Yes (how many times:) No

4. Did you require surgery (colectomy) for your ulcerative colitis since your faecal transplant

Yes (date:) No

4. Do you consider that faecal transplantation is likely to be safe?

Impossible Not likely Unsure Quite likely Very likely

5. How would you compare faecal transplantation to traditional medical treatments of ulcerative colitis?

- How do you compare the acceptability of these treatments?
- How do you compare the effectiveness of these treatments?

6. How would you compare faecal transplantation to other treatments such as probiotics?

- How do you compare the acceptability of these treatments?
- How do you compare the effectiveness of these treatments?

7. Do you believe faecal transplantation as carried out in this study would be seen as acceptable by:

- the general Australian population Yes No Unsure
- patients with ulcerative colitis? Yes No Unsure

8. Do you have any cultural or religious concerns about receiving faecal material from another person? If yes, what are your concerns?

9 . Do you have any concerns about discussing faecal transplant with friends or family?

If so why?

10. If you had your time in the study again would you like any aspects of the faecal transplant process to be done differently?

If yes please elaborate

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Chapter 6: Systematic Review and Meta-Analysis: Faecal Microbiota Transplantation for the Induction of Remission of Ulcerative Colitis

6.1 Background

During the course of the FIRST-UC study (detailed in Chapter 5), evidence emerged from a number of other studies assessing FMT for the induction of remission for active UC. Each of these trials used different stool processing as well as FMT timing and delivery methods. A systematic review with meta-analysis was undertaken to give context to the FIRST-UC study

This systematic review of the literature assessed cohort studies as well as RCTs of FMT for the induction of remission of UC. The meta-analysis was restricted to the RCTs only.

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Name of principal author (candidate)	Samuel Paul Costello		
Contribution to the paper	Conception and design of the project Data acquisition and management Analysis and interpretation of research data Drafting and revision of article		
Overall percentage (%)	50%		
Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	10/3/2017

Co-author contributions

By signing the Statement of Authorship, each author certifies that:

1. the candidate's stated contribution to the publication is accurate (as detailed above);
2. permission is granted for the candidate to include the publication in the thesis; and
3. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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6.2 Manuscript—Systematic Review with Meta-Analysis: Faecal Microbiota Transplantation for the Induction of Remission for Active Ulcerative Colitis

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Running head: Systematic review with meta-analysis: FMT for UC

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Abstract

Background: FMT is emerging as a novel therapy for UC. Interpretation of efficacy of FMT for UC is complicated by differences among studies in blinding, FMT administration procedures, intensity of therapy and donor stool processing methods.

Aim: To determine whether FMT is effective and safe for the induction of remission in active UC.

Methods: Medline (Ovid), Embase and the Cochrane Library were searched from inception through February 2017. Original studies reporting remission rates following FMT for active UC were included. All study designs were included in the systematic review and a meta-analysis performed including only RCTs.

Results: There were 14 cohort studies and four RCTs, which used markedly different protocols. In the meta-analysis of RCTs, clinical remission was achieved in 39 of 140 (28%) patients in the donor FMT groups, compared with 13 of 137 (9%) patients in the placebo groups (OR 3.67 [95% CI 1.82–7.39]; $P < 0.01$). Clinical response was achieved in 69 of 140 (49%) donor FMT patients, compared with 38 of 137 (28%) placebo patients (OR 2.48 [95% CI 1.18–5.21]; $P = 0.02$). In cohort studies, 39 of 168 (24%; 95% CI 11–40%) achieved clinical remission.

Conclusions: Despite variation in processes, FMT appears to be effective for induction of remission in UC, with no major short-term safety signals. Further studies are needed to better define dose frequency and preparation methods and explore its feasibility, efficacy and safety as a maintenance agent.

Introduction

UC is a chronic, relapsing and remitting, inflammatory disease of the colon occurring at the interface between the luminal contents and the mucosal immune system. There is increasing evidence implicating the colonic microbiome in the pathogenesis of UC with luminal bacterial antigens contributing to immune cell activation[1]. Many of the genetic risk alleles associated with UC relate to immunological handling of microbes and mucosal defence[2]. The microbiome in UC, both active and remission, is less diverse than that of healthy subjects[3]. This loss of diversity is predominantly attributable to a decrease in gram-positive *Firmicutes*, especially *Clostridium* clusters IV and XIV, whereas some species in the *Proteobacteriae* group, particularly *E. Coli* and *Enterobacteriaceae* are relatively overabundant[4]. However, the relative paucity of many other bacteria has also been implicated in IBD, including *Faecalibacterium prausnitzii* (a butyrate producer with independent anti-inflammatory properties); sulphate-reducing bacteria; and mucosal-associated *Akkermansia muciniphila*, *Ruminococcus torques* and *Ruminococcus gnavus*, which have possible roles in mucus barrier integrity[3, 5]. Colonic enterocyte health is also fundamentally dependent on microbial metabolites with butyrate, a product of anaerobic bacterial fermentation of undigested dietary carbohydrates, being a primary source of enterocyte nutrition with anti-inflammatory effects[6].

Despite evidence implicating microbial factors in the pathogenesis of UC, most medical therapies target the immune response without modifying the luminal microbial environment. Whilst effective, these "immunocentric" therapies have incomplete efficacy and are sometimes limited by side effects including allergy, intolerance, serious infection, increased risk of malignancy, drug antibody formation and cost[7].

In recent years, FMT has been proven to be extremely effective for treatment of recurrent *Clostridium difficile* infection (rCDI)[8-14]. This has encouraged research in FMT as a potential therapy for other microbial-related diseases such as UC. FMT is proposed as a means of altering the disease process by modifying the colonic ecosystem and thus the potential biochemical and antigenic drivers of the disease.

Since the first case of FMT for the treatment of UC was described by Justin Bennet in 1989[15], there have been a number of case reports, case series and, more recently, RCTs.

In order to assimilate these data, we conducted a systematic review and meta-analysis to assess the efficacy and safety of FMT for induction of remission in active UC.

Methods

Search strategy

A systematic search and retrieval of records was performed in accordance with the MOOSE, PRISMA and Cochrane guidelines[16-18].

We searched Medline (1948–February 2017), EMBASE (1948–February 2017) and the Cochrane Inflammatory Bowel Disease/Functional Bowel Disorders review group specialist trials register without language restriction from inception through February 2017. Abstracts from major meetings in gastroenterology and IBD were searched manually and assessed for relevance. These included the Digestive Disease Week (2010–2016), Congress of the European Crohn's and Colitis Organisation (2006–2017) and United European Gastroenterology Week (2010–2016). Bibliographies of review articles and meta-analyses[19-21] were searched in order to identify additional studies. Searching was limited to publications with human subjects. Articles in English and Mandarin were reviewed and other languages excluded.

An initial database search using the key words "fecal", "faecal", "microbiota", "microflora", "feces", "faeces", "stool", "fecal flora" and "faecal flora" was performed. Subsequently, each key word was combined with each of the following variations on transplant: "transplant", "enema", "donor", "infusion", "transfusion", "implant" "implantation" and "instillation". These terms were searched in combination and "bacteriotherapy" searched for individually. The results were combined with terms for UC by using the Boolean term "AND" "ulcerative colitis" "inflammatory bowel disease", "colitis", "IBD" or "UC". This strategy was used both as Medical Subject Headings (MeSH) terms if available and as free text. Variations of root word were also searched alone or in combination. Two authors independently reviewed all articles other than articles in Mandarin, which were reviewed by WS alone, and then discussed together with SPC.

Study selection

Eligible studies were RCTs and cohort studies that investigated FMT as induction of remission for active UC in both paediatric and adult patients with clearly described measures of remission.

Studies were excluded if enrolled patients did not have clearly defined active disease, there was no definition of remission, clinical endpoints were not reported, or the study included only patients with comorbid infections such as CDI. Studies that included patients with concomitant infections were considered only if the data from patients with UC and without infection could be clearly delineated from those with infection.

The Cochrane risk of bias tool[18] was used to assess for bias in RCTs (Supplementary Table 6.1. Data were extracted into Microsoft Excel for Mac 2011 (Microsoft, Redmont, USA). Risk of bias of the cohort studies was assessed using a modified Newcastle–Ottawa scale for cohort studies. The scale was adjusted for use in cohort studies without a control group. This was adjusted to a 6-point scale after questions that assessed for control groups were removed (Supplementary Table 6.2).

Two investigators (SPC and WS) independently completed the Cochrane risk of bias tool for RCTs and the inter-investigator agreement was 100% (Cohen's kappa = 1.0). The inter- investigator agreement on meeting the inclusion criteria for the observational studies was 88% (Cohen's kappa = 0.75). Consensus was reached by combined review of any studies (n = 4) where there had been initial discrepancy. Only RCTs were included in the meta-analysis to provide a pooled remission and response rate for FMT for induction of remission in UC.

Data extraction

Full-text articles and conference abstracts that met inclusion criteria were reviewed by SPC and WS to identify study characteristics such as study type, patient characteristics (Table 6.1), FMT processing methods and delivery protocols (Table 6.4), as well as clinical remission and response rates and definitions (Table 6.2 and Table 6.3).

Statistical analysis

For RCTs, a random effects model was used to assess the pooled estimate of remission, clinical response and endoscopic remission in the meta-analysis using Review Manager 5.4. (Nordic Cochrane Centre, Copenhagen, Denmark 2014). A random effects model was used because of the heterogeneous study designs. Statistical heterogeneity for each meta-analysis was assessed using the Cochran Q test (×2) and I^2 method. The I^2 method was used to assess the degree of heterogeneity with scores of 0–39, 40–59 and 60–100 representing low, moderate and substantial heterogeneity, respectively.

Results

Search results

Figure 6.1 (PRISMA flow diagram) shows the selection methodology of the included articles.

The initial search revealed 2072 potential articles. After screening titles and abstracts, 44 studies remained after duplicates were removed. After review of full-text articles and abstracts, 18/44 were eligible for inclusion, consisting of four RCTs and 14 cohort studies.

Cohort studies

There were 14 cohort studies[22-35], which included a total of 168 patients (Tables 6.1 and 6.2). The studies were reported from 2013 until 2017. Three of 14 studies included both UC and Crohn's disease patients[30, 31, 36], with only the data for UC patients included here. Both paediatric and adult patients were included in the analysis (age range 4.6 to 70 years); follow-up varied between 1 and 72 months. All patients had active UC; however, the severity of disease varied from mild to severe among studies.

Of 168 patients, FMT was administered via the upper gastrointestinal tract (nasogastric/nasojejunal tube or endoscopic duodenal infusion) in 22 (13%), via the lower gastrointestinal tract (enema, colonoscopy or rectal tube) in 125 (74%) and via both routes in 21 (12%). The stool weight used in each dose was reported in 10/14 cohort studies, ranging from 8 to 250g. The number of faecal infusions given ranged from one to six. Seven studies (50%) used fresh donor stool, and four (29%) used a

frozen stool protocol; in the remaining three (21%) studies, it was unclear whether fresh or frozen FMT was administered (Table 6.1). There were no cohort studies that reported the use of anaerobic stool processing techniques.

In all cohort studies, patients remained on their regular IBD medications during FMT therapy, except for a single study where medications other than 5-ASA were ceased[22]; 5/14 (36%) cohort studies used pre-treatment with antibiotics prior to FMT. In a single study, half of the patients had a prebiotic (pectin) administered with the FMT[34].

Of the 168 patients, 93 (55%; 95% CI 36.7–71.7%) achieved a clinical response, of whom 39 (24%; 95% CI 11–40%) achieved clinical remission (both outcomes variably defined as per Table 6.2). Six (4%) patients deteriorated and there was no clinical change in 68 (40%). Endoscopic remission was only assessed in 7/14 (50%) studies and was achieved in 16/56 patients (29%) (Table 6.2).

Treatment success was numerically higher in the five studies reporting antibiotic pre-treatment[22, 29, 30, 34, 37], with 39/58 patients (67%) demonstrating clinical response and 19/58 (32%) clinical remission. Clinical remission was achieved in 24/107 (22%) patients using fresh stool, compared with 12/40 (30%) using frozen stool. Clinical response rates were 12/19 (63%) for upper gastrointestinal delivery, compared with 64/121 (53%) for lower gastrointestinal delivery. Clinical remission rates were 4/19 (21%) for upper gastrointestinal delivery and 25/121 (19%) for lower gastrointestinal delivery.

Randomised controlled trials

Four placebo-controlled RCTs were identified, reporting on a total of 277 patients with UC enrolled between 2011 and 2016, of whom 140 (51%) received donor FMT and 137 (49%) received placebo[38-41]. Two RCTs used autologous stool as placebo[39, 41] and two used water (with and without discolourant)[38, 40]. The RCTs ranged in size from 50 to 81 patients. All were randomised in a 1:1 ratio of FMT:placebo.

The studies were heterogeneous in design with inclusion criteria, route of FMT administration and follow-up periods varying among studies. Each of the four RCTs included patients with active UC, and the majority of enrolled patients had mild to moderate disease. Rossen et al.[39] included patients with SCCAI values of 4–11.

Moayyedi et al. included patients with a total Mayo score >4, which also included patients with severe disease[38]. Paramsothy et al. and Costello et al.[40, 41] enrolled patients with total Mayo scores of 4–10. All studies required endoscopic evidence of active disease for inclusion; three studies required an endoscopic Mayo score ≥1, with the study of Costello et al. requiring an endoscopic Mayo score ≥2[41]. FMT was delivered via colonoscopy in the two Australian studies[40, 41] and via enema[38] and nasoduodenal tube[39] in one each of the other studies. Patient follow-up periods varied from 7 to 12 weeks.

Pooled remission and response rates

The endpoints reported in each RCT varied. Definitions used in each study are shown in Table 6.3.

Overall, remission was achieved in 39/140 patients (28%) in donor FMT recipients, compared with 13/137 (9%) in placebo groups (OR 3.67, 95% CI 1.82–7.39; $P < 0.01$) (Figure 6.2), with no statistically significant heterogeneity between studies ($I^2 = 0\%$). There were no clinical features consistently associated with remission in the donor FMT groups. Moayyedi et al. found that patients who had UC for less than 1 year were more likely to enter remission following donor FMT than those with UC for longer[38]. However, UC duration prior to FMT was not associated with remission in two other studies[40,41].

The Rossen et al. study was terminated early because of an interim futility analysis as it was powered to detect a treatment effect of 70%. This study showed no significant difference between donor and placebo groups on intention to treat analysis (30.4% v. 20%; $P = 0.51$). The other three studies each demonstrated statistically significant benefit of donor FMT over placebo for induction of remission in UC.

Clinical response was achieved in 69/140 patients (49%) who received donor FMT, compared with 38/137 (28%) who received placebo (OR 2.48, 95% CI 1.18–5.21; $P = 0.02$) (Figure 6.3) with moderate heterogeneity between studies ($I^2 = 52\%$). Endoscopic remission, defined as an endoscopic Mayo = 0, was achieved in 20/140 (14%) who received donor FMT, compared with 7/137 (5%) who received placebo (OR 2.69, 95% CI 1.07–6.74; $P = 0.04$) (Figure 6.4), with no statistically significant heterogeneity between studies ($I^2 = 0\%$).

Stool weight and delivery

The weight of stool per treatment and the method of delivery varied between trials. All RCTs used a polyethylene glycol-based bowel preparation prior to colonoscopic or nasoduodenal delivery, but not prior to enema delivery. The Costello et al. and Paramsothy et al. studies used colonoscopic delivery of 50g and 37.5g stool per treatment, respectively, and then followed this with two further 25g enemas and 39 further 37.5g enemas, respectively[40, 41]. Rossen et al. used nasoduodenal delivery at weeks 0 and 3 (median 120g stool per treatment) and Moayyedi et al. used a weekly enema for 6 weeks (8.3g per treatment).

Stool processing

The method of stool processing varied between trials (Table 6.4). Paramsothy et al. and Costello et al. used pooled (3–7 and 3–4 donors, respectively) frozen donor stool from de-identified, unrelated healthy volunteers[40, 41]. Rossen et al. used fresh stool from single known or de-identified donors and Moayyedi et al. used fresh and frozen stool from single anonymous donors[38, 39]. Costello et al. processed donor stool anaerobically and the other three studies processed stool aerobically. Paramosthy et al. and Costello et al. used 10% glycerol as a cryoprotective agent during freezing; Moayyedi et al. used water alone.

Safety

Adverse events were monitored and reported in each study. Overall, FMT was well tolerated. The most common serious adverse event reported was worsening colitis, which occurred in 3/140 patients in donor arms (with one colectomy) and 4/137 patients in placebo arms. There were three cases of small bowel Crohn's disease subsequently reported and two cases of *C. difficile* colitis, with one requiring colectomy, in donor arms. In placebo arms, there was one case each of Crohn's disease, primary cytomegalovirus infection and cervical cancer (unrelated). Two studies reported adverse events more extensively, with 50/63 (79%) in donor arms and 49/65 (75%) in placebo arms having at least one adverse event[39, 40]. The most common adverse events were self-limiting gastrointestinal complaints. There were no significant differences between number or type of adverse event between donor and placebo groups in either study.

Microbiome changes

Rossen et al. showed that compared with donors, pre-FMT UC patients had significantly lower abundance of members of *Clostridium* clusters IV, XIVa and XVIII and higher abundance of *Bacteroidetes*, bacilli, *Proteobacteria* and *Clostridium* clusters IX and XI[39]. The stool microbial diversity of UC patients and donors did not differ at baseline; however, the Shannon diversity index for responders increased significantly following FMT. Redundancy analysis showed that the microbiota composition of responders in the donor FMT group shifted from overlap with non-responders at baseline to healthy donors at week 12. This shift was mainly explained by regain of *Clostridium* clusters IV, XIVa and XVIII and reduction in *Bacteroidetes*[39]. Moayyedi et al. found an enrichment for the family *Lachnospiraceae* and the genus *Ruminococcus* in a particular donor who was associated with most cases (7/9, 78%) of remission[38]. When similarity was compared between the active cohort after FMT and their respective donors, there was a statistically significant effect of the active therapy group being more similar to their donor than to a control faecal sample.

Paramsothy et al. found that those patients who went on to achieve remission had a greater diversity at both baseline and following FMT. In their study, achieving remission following FMT was associated with gains in *Ruminococcus*, *Clostridium* clusters IV and XVIII, *Barnesiella* spp., *Blautia* spp., *Dorea* spp. and *Parabacteroides* spp. Conversely, the presence of *Fusobacterium* spp and *Sutterella* spp following FMT was associated with no remission (Table 6.5).

Discussion

In the meta-analysis of four published RCTs performed to date, FMT was significantly more effective than placebo for induction of remission of active UC, achieving clinical remission in 28% of patients with mild to moderate UC (OR 3.67, 95% CI 1.82–7.39; $P < 0.01$) (Figure 6.2)[38-41]. This effect appears to be robust as it is seen despite variation in stool processing and delivery methods between the trials. In addition, no major short-term safety signals were observed.

Each trial used different stool delivery protocols and donor stool processing methods, which makes it difficult to recommend a particular protocol or technique over the other. This also makes interpretation of the pooled results of the studies more difficult. There

are likely to be aspects of stool processing and delivery that influence the efficacy of FMT, and further research is required to optimise the methods and treatment protocols of FMT in UC. Paramsothy et al. and Costello et al. used pooled donor stool. This provided an increased diversity of microorganisms in the stool suspension than that of a single donor[40]. Pooling stool may result in a greater chance that an important element of stool will be transmitted to the recipient. However, it is not clear whether this theoretical benefit translates to improved efficacy, and using pooled stool requires blending and does add some logistical complexity to the manufacturing process[42].

The four RCTs had different intensities of FMT delivery, and this has implications for the real-world feasibility of use of FMT for UC (Table 6.4). Paramsothy et al. used the most intensive protocol of 40 FMT treatments, delivering 187.5g of stool per week over the 8-week period[40]. This study achieved similar remission rates to the Moayyedi et al. and Costello et al. studies that used much lower treatment intensities, which may be more practical outside of the trial setting[38, 41]. Moayyedi et al. delivered only 8.3g of stool per week via enema and Costello et al. delivered 100g of stool in three treatments in the first week and then no further stool for the remaining 7 weeks. Rossen et al. used a low-intensity protocol of two large stool weight (median 120g) FMT deliveries via nasoduodenal tube; however, they did not demonstrate a significant difference in remission rates over placebo[39]. It appears that a short duration of therapy may be sufficient for remission induction in some patients. These studies do not however provide data on the longevity of the treatment response, long-term safety or possible ability to maintain remission with interval dosing.

In cohorts of patients who have undergone FMT to treat CDI, the choice of donor does not have an important impact on the efficacy of therapy[43]. Similarly, it seems that neither anaerobic or aerobic stool preparation, nor fresh or frozen stool, significantly influences the efficacy of FMT for CDI[10, 44]. It seems that stool from any healthy person processed under many different conditions is likely to be effective in treating CDI. However, the same may not be true for UC. *C. difficile* is an oxygen-tolerant spore-forming clostridial species, and its important ecological competitors may be similarly hardy species or other components in the stool, such as bile acids, viruses and prebiotic substrates, which may contribute to the treatment effect[45]. It is unclear which elements of faeces are important to its therapeutic effect in UC; however, they may be different to those that are important in treating CDI. The benefit, if any, of anaerobic preparation of

faeces for FMT in UC is uncertain; however, it may be hypothesised that bacterial susceptibility to oxygen exposure may influence bacterial viability and therefore bacterial engraftment into the colonic ecosystem[46]. For example, *Faecalibacterium prausnitzii*, a bacterium frequently associated with colonic health, is an extremely oxygen-sensitive bacterium[47].

The choice of placebo agent in FMT studies may have an influence on trial results. A water placebo is likely to be hypotonic and recognizably different to faeces to both the administrator and recipient of FMT, and therefore has the capacity to bias results (Supplementary Table 6.2). To attempt to mitigate this issue, Moayyedi et al. had a person not involved in the assessment of patients deliver the FMT enemas[38] and Paramsothsy et al. used a fluid discolourant and odorant[40]. Autologous stool is likely to represent a better placebo than water; however, through the process of faecal processing and freezing, the recipient's own stool composition will also be altered. A recent FMT study for the treatment of CDI had a high placebo response and there is the possibility that processing and storage of the autologous stool could have influenced the placebo response[11]. The Rossen and Paramsothy studies had central reading of the colonoscopy images, which minimises bias in the blinding of outcome; the other RCT did not use central reading of colonoscopy images[38-41].

The optimal route of FMT delivery in UC, whether via the upper or lower gastrointestinal tract, is uncertain, although it is likely to influence the success of the procedure. In favour of lower gastrointestinal delivery of FMT in UC, the three positive RCTs to date employed lower gastrointestinal delivery techniques, and upper gastrointestinal delivery was used in the only negative RCT identified. Further, systematic review of FMT for the treatment of CDI demonstrated that colonoscopic delivery has higher rates of success than other delivery methods for this indication[48]. However, the rate of clinical remission in the UC cohort and RCT studies combined is similar between upper gastrointestinal delivery (11/42 [26%]) and lower gastrointestinal delivery (63/252 [25%]). This may include the delivery of FMT or rationally designed microbial therapeutics via capsule as research and development into these technologies is already underway[49-51]. In addition to efficacy, patient acceptability of any delivery method is important to the success of FMT therapy. Clearly, further research is needed to elucidate the optimal route of FMT administration.

Stool is a non-standardized product with stool composition differing between individual donors and even from sample to sample from the same donor[52]. It is possible that certain donors may provide stool that is more or less efficacious than others and this could affect individual trial results. For instance, Moayyedi et al. found that the majority of the treatment effect observed related to one particular "super donor"[38]. This particular donor had a relative enrichment of the genera *Lachnospiraceae* and *Ruminococcus*. There are observable changes in bacterial phyla following FMT (Table 6.5); however, the 16S rRNA-based analysis is limited as it does not provide strain level detail or functional information. Despite these limitations, there are patterns to the microbial changes associated with response to FMT that seem to be common among the studies. Increases in *Clostridium* clusters IV and XVIII were observed in those who responded in two RCTs[39, 40], and *Bacteroidetes* including *Sutterela* and *Fusobacterium* spp. have been associated with non-response to FMT[40]. Paramsothy et al. found significant changes in the microbiota at both week 4 and week 8 following FMT, with phylogenic diversity increased from UC patient baseline and similar to individual donors. This raises the possibility that the FMT infusions prior to week 4 were sufficient to induce remission[40]. In support of this proposition, Costello el al. demonstrated similar efficacy of FMT with a shorter duration and intensity of treatment[41].

Prolonged frozen storage will increase the *Firmicutes* to *Bacteroidetes* ratio as some of the gram-negative species are more fragile and deplete during frozen storage[53]. There may therefore be a theoretical benefit to frozen over fresh stool if elements of the *Firmicutes* phyla are relatively beneficial, compared with elements of the *Bacteroidetes*. Patients in the Rossen et al. study that did not demonstrate efficacy were treated with fresh stool[39] and the majority of the patients who achieved remission in the other RCTs were treated with frozen stool[38, 40, 41]. There was also a higher rate of remission reported in the cohort studies using frozen stool (30%), compared with. fresh stool (22%).

Given the success of FMT as an agent to induce remission in UC, there is now a need to investigate the mechanisms by which this effect is achieved. There are shifts in the microbial composition following FMT with increased diversity as well as more specific changes in bacterial phyla, particularly in responders[40]. These changes in microbial composition could result in functional alterations in luminal and epithelial metabolic and biochemical processes as well as mucosal immune responses to the microbiota. Butyrate production and oxidation are dependent on luminal bacterial metabolic

processes as are sulphate and nitrogen metabolic pathways[54, 55]. One small open-label study has attempted to potentiate this effect by combining the prebiotic pectin with FMT in half of their cohort, demonstrating an improvement in Mayo scores in the group receiving pectin[34]. There may be other elements of the stool, such as phages, fungi, bile acids, proteins and other bacterial products, that convey some of the observed therapeutic effect. Research into the mechanisms that lead to FMT effecting remission in UC should aid the development of rationally designed microbial therapies. In the interim, there is a need for studies to further investigate the different methods of FMT preparation and delivery as well as the role of FMT in the maintenance of remission in UC. There is also a need to develop infrastructure to deliver this therapy to patients efficiently.

Conclusion

FMT is an effective and safe therapy for the induction of remission of UC, compared with placebo. However, there are insufficient data on the long-term efficacy and safety of FMT for UC at present. There is a need for further research to refine the techniques of stool preparation and delivery for FMT, and to understand the microbial and immunological effects of FMT and its relationship to the pathogenesis of UC.

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Authorship statement

All authors have contributed to and approve the final version of the article. Dr SP Costello was the primary author of the manuscript and performed the literature search with systematic review and meta-analysis and wrote the manuscript. Dr W Soo performed a systematic review of all articles and edited the manuscript. Dr RV Bryant assisted with the design of the systematic review and edited the manuscript. Dr V Jairath assisted with the design of the systematic review and edited the manuscript. Prof AL Hart assisted with the design of the systematic review and edited the manuscript. Prof JM Andrews assisted with the design of the FMT service and was the primary editor of the manuscript.

Statement of interests

Dr SP Costello has received scientific advisory board fees from Merck Sharpe and Dohme; and travel support for conference attendance from Shire pharmaceuticals.

Dr W Soo has received no funds from the pharmaceutical industry.

Dr RV Bryant has received speaking honoraria from Shire, Takeda and Janssen pharmaceuticals; and travel support for conference attendance from Janseen and Takeda pharmaceuticals.

Dr V Jairath has received scientific advisory board fees from AbbVie, Sandoz, Ferring and Janssen; speaker's fees from Takeda and Ferring; and travel support for conference attendance from Vifor pharmaceuticals.

Prof AL Hart has served as consultant, advisory board member or speaker for AbbVie, Atlantic, Bristol-Myers Squibb, Celltrion, Falk, Ferring, Janssen, MSD, Napp Pharmaceuticals, Pfizer, Pharmacosmos, Shire and Takeda. She also serves on the Global Steering Committee for Genentech.

Prof JM Andrews has served as a speaker, a consultant and an advisory board member for AbbVie, Abbott, Allergan, Celgene and Ferring, Hospira, Janssen, Pfizer, MSD, Shire and Takeda, and has received research funding from AbbVie, Abbott, Ferring, Janssen, MSD, Shire and Takeda.

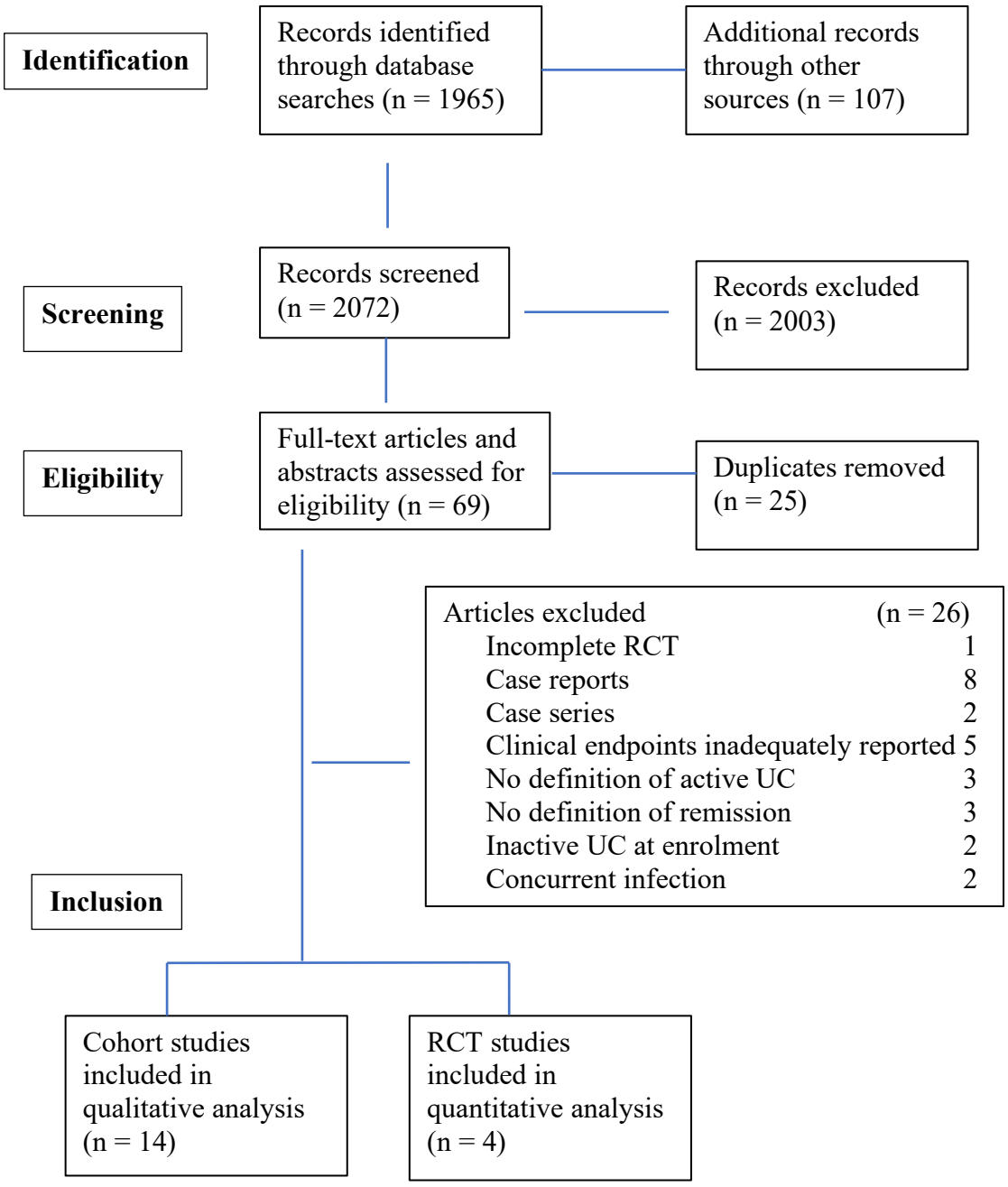

Figure 6.1: PRISMA flow diagram: meta-analysis of FMT studies in UC

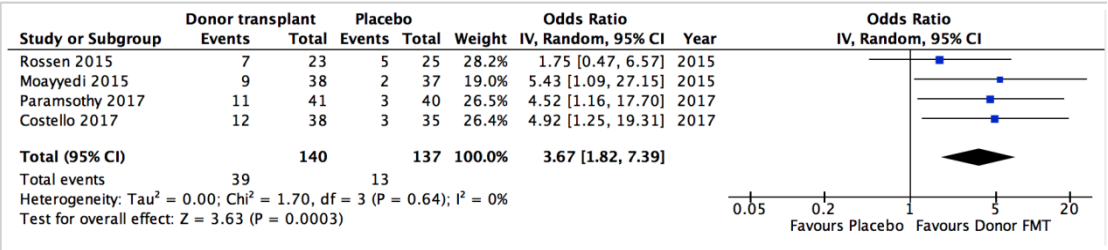

Figure 6.2: Forest plot for remission in RCTs of FMT for UC

Remission was variably defined as per Table 6.3.

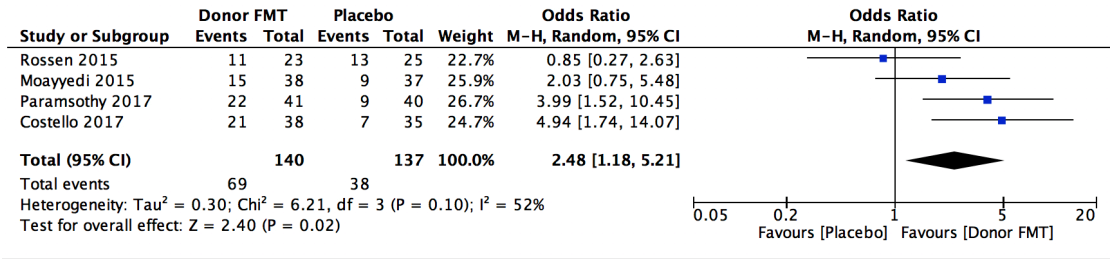

Figure 6.3: Forest plot for clinical response in RCTs of FMT for UC

Clinical response was variably defined as per Table 6.3.

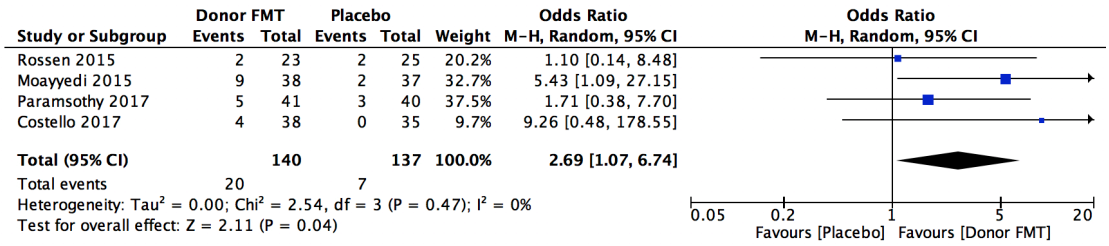

Figure 6.4: Forest plot for endoscopic remission (endoscopic Mayo = 0) in RCTs of FMT for UC

Table 6.1: Cohort studies of FMT for UC: Protocol descriptions

Author	n	Disease severity	UC medication	Bowel preparation	Pre-antibiotic	Pre-medication	FMT route	FMT (n)	FMT dosage per treatment (g)	Fresh or frozen
Angelberger et al. (2013)	5	Mayo 8–11	5-ASA (other medication ceased)	PEG	Metronidazole 5-10 days	Pantoprazole, loperamide	NJ, enema	3	NJ 17–25g/250 mL Enema 6–22g/100 mL	Fresh
Kump et al. (2013)	6	Mayo 8–11	5-ASA, Azathioprine, Methotrexate, Infliximab, Prednisolone	PEG	NR	NR	Colonoscopy	1	100–150g/200–350mL saline, total 300–500mL infused	Frozen
Kunde et al. (2013)	10	PUCAI 15–65	5-ASA, Thiopurines, Steroids	None	None	None	Enema	5	70–113g/250mL saline	Fresh
Cui et al. (2015)	15	Montreal S2 and S3	Mesalamine. Thiopurine, Steroid	None	None	Metoclopramide, esomeprazole	Endoscopy (distal duodenum)	1-2	NR	Frozen
Damman et al. (2015)	7	UCDAI 3–10	5-ASA	PEG	None	None	Colonoscopy	1	58–72g stool in 175–290 mL	Fresh
Ren et al. (2015)	7	Mayo >6	Mesalazine (others ceased 1 week prior)	NR	None		Endoscopic duodenal infusion and/or colonoscopy	1-3	Colonoscopy 200–300 mL Gastro 100–200 mL	Fresh
Scaldaferri et al. (2015)	8	Mayo ≥4 Endoscopic Mayo	Not specified – stable for ≥2 weeks	NR	NR	NR	colonoscopy	3	200 cc	NR

		≥1								
Suskind et al. (2015)	4	PUCAI 20–55	Azathioprine, Mesalamine, VSL#3	PEG	Rifaximin	Omeprazole	Nasogastric	1	8g in 30mL infused	NR
Wei et al. (2015)	11	Mayo 2–10	Mesalazine, Gatifolxacin,Norfloxacin, Gentamicin	PEG	Vancomycin 500mg bd 3 days	None	Colonoscopy	1	44g in 300mL infused	Frozen
Goyal et al. (2016)	9	PUCAI 12.5–32.5	NR	NR	NR	NR	Duodenoscopy, colonoscopy	NR	NR	NR
Vermeire et al. (2016)	8	Mayo 3	5-ASA, azathioprine, steroid, Infliximab	PEG	None	None	3 Nasojejunal, 5 rectal tube	2	100g/200mL saline	Frozen
Nishida et al. (2016)	41	Mayo 3–9	Steroids, immunomodulators, tacrolimus, anti-TNF	PEG	None	None	Colonoscopy	1	150–200g	Fresh
Wei et al. (2016)	20	Mayo 2–10	NR	PEG	Vancomycin 500mg bd 3 days	None	Colonoscopy	1	60g/500mL saline.	Fresh
Ishikawa et al. (2017)	18	Lichtiger's Clinical Activity Index ≥5 or endo Mayo ≥1	5-ASA, Steroids, immunomodulators, tacrolimus, anti-TNF, apheresis	PEG	Amoxicillin (1500mg/day), fosfomycin (3000mg/ day) and metronidazole (750mg/day)	None	Colonoscopy	1	150-250g	Fresh

Legend: FMT = faecal microbiota transplantation; UC = ulcerative colitis; n = number of patients; NR = not reported; PEG = polyethylene glycol; 5-ASA = 5-aminosalicylic acid; TNF = tumor necrosis factor.

Table 6.2: Cohort studies of FMT for UC: Efficacy measures

Author	Follow-up (months)	Def of response	Def of clinical remission	Clinical response	Deterioration	No change	Clinical remission	Endoscopic remission
Angelberger et al. (2013)	12	Mayo score reduced ≥3, bleeding subscore ≤1	Mayo ≤2	1	2	2	0	0
Kump et al. (2013)	12	Mayo score reduced ≥3	Mayo ≤2	2	1	3	0	0
Kunde et al. (2013)	6	PUCAI decrease by >15	PUCAI <10	6	0	3	3	NR
Cui et al. (2015)	4 to 72	NR	Montreal S0	12	0	2	4	4
Damman et al. (2015)	3	UCDAI decrease by ≥3 (week 4)	UCDAI ≤2 with no individual subscore ≥1	1	0	6	1	1
Ren et al. (2015)	1 to 7	Reduction in Mayo score more than 30% or ≥3	Mayo ≤2, no subscore >1	7	0	0	7	7
Scaldaferri et al. (2015)	3	Mayo reduced by ≥2	Mayo ≤ 2, no subscore > 1	6	2	0	2	2
Suskind et al. (2015)	3	NR	PUCAI < 10	0	0	4	0	NR
Wei et al. (2015)	1	IBDQ increase >16	IBDQ >170, Mayo <2	11	0	0	6	NR
Goyal et al. (2016)	6	PUCAI reduce by >15	PCDAI <10 or normalisation of calprotectin/lactoferrin	7 (1 month)	0	2	1	NR
Vermeire et al. (2016)	24	NR	Mayo endoscopic subscore 0/1	2	0	6	2	2

Nishida et al. (2016)	2	Mayo score reduce by ≥3	Mayo ≤2	11	0	30	0	NR
Wei et al. (2016)	3	30% reduction in Mayo or >16 point IBDQ improvement	Mayo ≤2	13	0	7	7	NR
Ishikawa et al. (2017)	1	Reduction in CAI ≥3 and CAI <10	CAI <+3	14	1	3	6	NR

Legend: Total Mayo score (0–12) with endoscopic Mayo score (0–3); PUCAI = Paediatric Ulcerative Colitis Activity Index (score 0–85); UCDAI = Ulcerative Colitis Disease Activitiy Index (score 0–12); IBDQ = Inflammatory Bowel Disease Questionnaire (score 32–224); CAI = Lichtiger's Colitis Activity Index (range 0–16); NR = not recorded.

Table 6.3: Study characteristics of RCTs of FMT for UC

	Rossen et al.[39]	Moayyedi et al.[38]	Paramsothy et al.[40]	Costello et al.[41]
Patients enrolled (donor/placebo)	50 (23/25)	75 (38/37)	81 (41/40)	73 (38/35)
Completed primary endpoint	37	70		69
Placebo	Autologous stool	Water	Discoloured and odoured water	Autologous stool in saline
Endpoint (weeks)	12 weeks	6 weeks	8 weeks	8 weeks
Disease severity	SCCAI 4–11 + endoscopic subscore ≥1	Mayo ≥4 with endoscopic subscore ≥1	Mayo 4–10	Mayo 3–10 with endoscopic subscore ≥2
Mean Mayo enrollment (donor vs placebo)	NR	8.24 vs 7.86	8 vs 8 (median)	7.40 vs 7.24
Mean Mayo at endpoint (donor vs placebo)	NR	6.09 vs 6.34	?	6.20 vs 4.00
Concomitant medication	Stable dose thiopurines, mesalamine, corticosteroids	Stable dose glucocorticoid, 5-ASA, thiopurine, anti-TNF	Stable dose 5-ASA, immune-modulator; tapering prednisolone	Stable dose immuno-modulator, 5-ASA, biologic; tapering prednisolone
Definition remission	SCCAI ≤2 with ≥1 point improvement on the combined Mayo endoscopic Mayo score of sigmoid and rectum	Total Mayo <3 with endoscopic Mayo = 0	Total Mayo ≤2 with subscores of ≤1 for rectal bleeding, stool frequency and endoscopic appearance; and a ≥1 point reduction in endoscopic subscore	Total Mayo ≤ 2 with endoscopic Mayo ≤ 1
Definition clinical remission	SCCAI ≤2	NR	Combined Mayo score of ≤1 for both rectal bleeding + stool frequency	SCCAI ≤2
Definition clinical response	≥1.5 point reduction in SCCAI	≥3 point reduction in Mayo score	≥3 point reduction in Mayo score ***or*** ≥50% reduction from baseline in combined rectal bleeding plus stool frequency subscores	≥3 point reduction in Mayo score
Remission induction donor FMT vs placebo	7/23 (30%) vs 5/25 (20%)	9/38 (24%) vs 2/37(5%)	11/41 (27%) vs 3/40 (8%)	12/38 (32%) vs 3/35 (9%)

	$P = 0.51$	$P = 0.03$	$P = 0.02$	$P < 0.01$
Clinical remission induction donor FMT vs placebo	7/23 (30%) vs 8/25 (32%) $P = 1.0$	NR	18/41 (44%) vs 8/40 (20%) $P = 0.02$	19/38 (50%) vs 6/35 (17%) $P < 0.01$
Clinical response donor FMT vs placebo	11/23 (48%) vs 13/25 (52%) $P = 0.58$	15/38 (39%) vs 9/37 (24%) $P = 0.16$	22/41 (54%) vs 9/40 (23%) $P < 0.01$	21/38 (55%) vs 7/35 (20%) $P < 0.01$
Adverse events Worsening colitis	Nil	1 placebo arm	1 placebo, 2 donor including one colectomy (3 in open-label phase)	2 placebo, 1 donor
Other serious adverse effects	2 placebo arm, 2 donor arm: small bowel Crohn's, abdominal pain	2 placebo arm, 3 donor arm: 2 Crohn's, 1 *C. difficile*	Nil	Donor arm: *C. difficile* requiring colectomy, pneumonia

Legend: Total Mayo score (0-12) with endoscopic Mayo score (0-3); FMT = faecal microbiota transplantation; UC = ulcerative colitis; SCCAI = Simple Clinical Colitis Index (score 0–19)56; NR = not recorded.

Table 6.4: Summary of donor stool delivery and processing methods in RCTs of FMT for UC

	Rossen et al. 2015[39]	Moayyedi et al. 2015[38]	Paramsothy et al. 2017[39]	Costello et al. 2017[40]
FMT route	Nasoduodenal	Enema weekly	Colonoscopy (×1) and enema (×39)	Colonoscopy (×1) and enema (×2)
FMT treatments during trial	2	6	40	3
Preparation fresh/frozen	Fresh	Fresh + Frozen	Frozen	Frozen
Stool processing oxygen exposure	Aerobic	Aerobic	Aerobic	Anaerobic
FMT stool weight (g)	Median 120g (85–g 208g)	8.3g	37.5g	Colon 50g, Enema 25g
Stool weight week 1	120g	8.3g	187.5g	100g
Average stool weight per week of trial	30g/w	8.3g/w	187.5g/w	12.5g/w
Dilutant	Saline (500mL)	Water (50mL)	Saline 97.5mL (65%)	Saline (65%)
Stool additive during preparation	Nil	Nil	Glycerol 15mL (10%)	Glycerol (10%)
Stool donor relationship to recipient	Anonymous	Anonymous	Anonymous	Anonymous
Donor stool	Single donor	Single donor	Pooled (3–7 donors)	Pooled (3–4 donors)

Legend: FMT = faecal microbiota transplantation; UC = ulcerative colitis; g = grams; w = week.

Table 6.5: Summary of microbiome analysis of RCTs of FMT for UC

Study and sequencing type	Bacterial changes in UC patients relative to donors	Bacterial changes associated with response	Bacterial changes associated with non-response
Rossen et al. 2015[39] Phylogenetic microarray (HITChip) analysis of 16S ribosomal RNA	↑Bacteroidetes, bacilli, Proteobacteria and *Clostridium* clusters IX and XI ↓*Clostridium* clusters IV, XIVa and XVIII No difference in diversity	↑Diversity in responders post FMT ↑Clostridial clusters IV, XIVa and XVIII ↓*Bacteroidetes*	No change in diversity post FMT
Moayyedi et al. 2015[38] 16S ribosomal RNA sequencing		Change in microbiota closer to donor profile Lachnospiraceae, *Ruminococcus*	
Paramsothy et al. 2017[40] 16S ribosomal RNA sequencing		*Clostridium* cluster IV, XVIII, *Ruminococcus*, *Barnesiella* spp., *Blautia* spp., *Dorea* spp., *Parabacteroides* spp.	*Fusobacterium* spp. and *Sutterella* spp.

Legend: FMT = faecal microbiota transplantation; UC = ulcerative colitis.

Supplementary Table 6.1: Cochrane collaboration's tool for assessing the risk of bias: RCTs of FMT for UC

	Rossen et al.[39]	Moayyedi et al.[38]	Paramsothy et al.[40]	Costello et al.[41]
Random sequence generation	+	+	+	+
Allocation concealment	+	+	+	+
Blinding of participants and personnel	+	?	?	+
Blinding of outcome assessment	+	?	+	?
Incomplete outcome data	+	+	+	+
Selective reporting	+	+	+	+
Other bias	+	+	+	+

Legend: + = low risk of bias; ? = unclear risk of bias; - = high risk of bias.

Supplementary Table 6.2: Modified Newcastle–Ottawa scale for assessing cohort studies: faecal microbiota transplant for ulcerative colitis

Quality scale	Accepted criteria	Angleberger (2013)[22]	Kump (2013)[23]	Kunde (2013)[24]	Cui (2013)[25]	Damman (2015)[26]	Ren (2015)[27]	Scaldaferri (2015)[28]	Suskind (2015)[29]	Wei (2015)[30]	Goyal (2016)[31]	Vermeire (2016)[32]	Nishida (2016)[33]	Wei (2016)[34]	Ishikawa (2016)[35]
Selection representative of the exposed cohort	Representation of ulcerative colitis population with sex, age and disease severity	0	0	1	0	0	0	0	0	0	0	0	1	0	1
Ascertainment of FMT exposure	Record of procedure, structured interview	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Demonstration that outcome of interest was not present at the start of the study	Evidence of disease activity by standard disease activity scores prior to FMT	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Assessment of outcome	Record of remission using standard disease activity	1	1	1	1	1	1	1	1	1	1	1	1	1	1

	scores														
Follow-up enough for outcome to occur	At least 6 weeks	1	1	1	1	1	1	1	1	0	1	1	1	1	0
Adequacy of follow-up of cohorts	Follow-up of complete cohort	1	1	1	0	1	0	0	1	1	1	1	1	1	1
Total		5	5	6	4	5	4	4	5	4	5	5	6	5	5

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Chapter 7: Discussion

The body of work presented in this thesis explores FMT as a therapy for UC. During the course of this thesis, FMT has emerged from being a speculative proposition to an evidence-based therapy for the induction of remission of UC. FMT is now ready for large scale (phase 3) registration studies and evaluation as an agent for the maintenance of remission of UC.

7.1 Key Outcomes, Significance and Limitations

7.1.1 Frozen stool banking is critical to the reliable and timely delivery of faecal microbiota transplantation

The stool bank established in Chapters 3 and 4 successfully facilitated the FMT in UC study conducted in Chapter 5. We were able to conduct three FMT procedures in a week using the same mixture of multi-donor stool in 73 patients. The banking of frozen stool allowed screening and stool donation to occur prior to a patient enrolling in the study. The stool bank also had a linked benefit of providing screened donor stool to treat South Australian patients with rCDI. This has resulted in the successful treatment of over 90 patients to date, many of whom would have otherwise required colectomy to cure their persistent infection. We were able to perform the first reported case worldwide of FMT for *C. difficile* induced toxic megacolon in a woman who, having declined surgery, would have otherwise succumbed to her infection (Appendix 1).[1] This case demonstrated one of the major advantages that a frozen stool bank offers over ad hoc or patient-directed fresh stool donation—the rapid availability of thoroughly screened donor stool on demand. This patient deteriorated rapidly on Christmas eve, and finding and successfully screening a donor in time would not have been possible without access to pre-screened frozen stool aliquots.

7.1.2 Anaerobic stool processing and glycerol storage media preserve bacterial viability

The anaerobic stool processing methods developed and described in Chapters 3 and 4 added an important dimension to the FMT study conducted in Chapter 5. It is known that the majority of the bacteria and archaea that inhabit the colon are obligate

anaerobes, and it has been demonstrated that important species such as *Faecalibacterium prausnitzii* are lost with aerobic stool processing and preserved with anaerobic stool processing.[2] The culture studies in Chapter 4 demonstrated that the viability of a number of representative organisms was maintained to at least 6 months by using stool blended with normal saline and glycerol under anaerobic conditions. The three previously published FMT studies in UC used aerobic stool processing methods; therefore, potentially therapeutic organisms might have been lost by the time the stool was administered to patients in these studies. It is possible that the therapeutic effect seen in Chapter 5 (23% gain of donor FMT over placebo) was similar to that in previous positive trials despite a shorter duration and lower intensity of treatment because of the enhanced potency of anaerobically prepared stool.

7.1.3 Short-duration and low-intensity faecal microbiota transplantation is effective at inducing remission in mild to moderately active ulcerative colitis and is acceptable to patients

The study in Chapter 5 demonstrated that a three-dose, 1-week induction course of donor FMT was more likely to induce clinical and endoscopic remission in participants with active UC at week 8 than autologous FMT. The study also showed a significant difference in favour of donor FMT for the secondary end points of clinical remission and clinical response.

This study used a rigorous composite primary end point of corticosteroid-free clinical and endoscopic remission and achieved a 23% gain of donor FMT over placebo. The meta-analysis in Chapter 6 demonstrated a 19% gain of donor FMT over placebo across all four RCTs. These results compare favourably with the most effective therapies currently available for UC. In the ACT-1 study, the gain for infliximab (combined 5mg/kg and 10mg/kg groups) over placebo was 20%.[3] In the Ultra 2 study, the gain for adalimumab over placebo was 7%[4]; in the PURSUIT study, the gain for golimumab over placebo was 11%[5], and in the GENIMI 1 study, the gain for vedolizumab over placebo was 12%.[6] All of these biologic agents are expensive and the anti-TNF therapies have potential side effects of serious infection or malignancy, which limit their use. FMT is an effective therapy acting via a novel pathway and may compliment standard therapies.

Moayyedi et al. demonstrated efficacy of donor FMT over placebo with weekly enemas for 6 weeks[7] and Paramsothy et al. demonstrated efficacy of donor FMT over placebo with an intensive regime that involved enemas 5 days per week for 8 weeks.[8] This is a relatively high treatment burden that may limit its applicability in routine practice. The short induction regime of a single FMT delivered via colonoscopy followed by two FMT enemas over 1 week using anaerobically prepared stool suspension is likely to be more acceptable in "real-world" practice.

7.1.4 Patients believe faecal microbiota transplantation to be an acceptable therapy for ulcerative colitis

Patient acceptance of any new therapy is crucial to its effectiveness in the clinical setting. This was explored in Chapter 5 and supplement. Prior to undergoing FMT therapy, 96% of patients believed that FMT as carried out in this study would be acceptable to people with UC. Importantly, 95% of surveyed patients who received the short induction course in the study in Chapter 5 found it to be an acceptable therapy. The majority (92%) had no concerns about discussing their FMT treatment with friends and family, and 98% had no cultural or religious concerns with FMT. Moreover, 62% of patients believed that FMT helped their symptoms and 80% of patients considered it likely to be safe, with 20% of patients unsure of its safety. This patient feedback indicates that the overall patient experience of FMT therapy was favourable.

7.1.5 Induction faecal microbiota transplantation therapy is not sufficient to maintain remission in the majority of ulcerative colitis patients

The study in Chapter 5 was the first to assess efficacy and safety out to 1 year following donor FMT. These 12-month data have a number of limitations. First, they were "open-label" and are without a control group. Second, some patients had changes in therapy during the period of follow-up. Despite these limitations, some patients appeared to achieve sustained remission following donor FMT without additional medical therapy. Of the 12 patients who achieved clinical and endoscopic remission in the donor FMT arm, 5 (42%) were in clinical and endoscopic remission at 12 months and 3/12 reported no symptoms in the intervening 12-month period. At 12 months, 21 of 49 (49%) patients who underwent colonoscopy were in clinical and endoscopic remission; 9 of these 21 (43%) patients reported no symptoms since receiving donor FMT. At 12

months, 27 of 49 (55%) of patients were in clinical remission and 8 of 43 (19%) were in endoscopic remission.

7.1.6 No short-term safety signal for faecal microbiota transplantation for ulcerative colitis; larger studies with longer follow-up required

There were very few side effects noted during the induction phase of the study in Chapter 5. There were three serious adverse events in the dFMT group (worsening colitis, CDI requiring colectomy and pneumonia) and two in the aFMT group (both worsening colitis), and none were obviously attributable to FMT. Similarly, the induction phases of the other three RCTs analysed in Chapter 6 showed low rates of adverse effects. A recent Cochrane review meta-analysis addressed serious adverse events in the induction phase of these studies (7–12 weeks) and found no significant difference between the donor FMT and control groups; 10/140 (7%) in the FMT group, compared with 7/137 (5%) in the control group (RR 1.40, 95% CI 0.55–3.58, $I^2 = 0\%$).[9]

The study of FMT in UC in this thesis was the first to assess adverse effects at 12 months. Of the 61 patients who completed the 12-month adverse effects questionnaire, 31 (51%) reported at least one adverse event. Many of these adverse events would be expected in the usual course of UC, such as 13 patients reporting worsening colitis. Nine patients underwent colectomy during the 12-month follow-up (including one patient within 8 weeks of donor FMT). Colectomy was an expected outcome for many patients in this study because they often had disease refractory to available medical therapy. Of note, maintenance therapy with biological agents was not available to many of the patients in this study as they were only funded by the PBS for this indication in 2015. There were eight reported infections, and these appeared to be unrelated to FMT; however, there were two cases of CDI, one of which resulted in colectomy 7 weeks following donor FMT. UC is a risk factor for CDI; however, CDI in this context is notable given the high efficacy of FMT in treating CDI in patients with IBD.[10]

There were five immune-related diseases (two new cases of psoriatic arthritis and one each of enteropathic arthritis, Crohn's disease and allergy to infliximab) that developed in the 12-month follow-up period. Enteropathic arthritis occurs in up to 14% of individuals with UC[11]; thus, the development of this condition in this cohort is not unexpected with or without FMT. It is not uncommon for cases of putative UC to be

subsequently diagnosed as Crohn's disease, and it is unclear whether the patient who developed Crohn's disease had an incorrect diagnosis entering the study or whether he developed new Crohn's disease following donor FMT.

There were two cases of psoriatic arthritis that developed in the 12 months of follow-up. Psoriatic arthritis is a T cell-mediated disease in which pathogenic T cells produce IL-17 in response to IL-23.[12] Patients with psoriatic arthritis have been noted to have dysbiosis with low levels of *Akkermansia mucinophilia* species and *Ruminococcus* genera, compared with controls.[13] In a mouse model, segmented filamentous bacteria induced autoimmune arthritis through the ability to specifically promote differentiation of the Th17 subset[14]. The gut microbiota can influence local and systemic immune responses via alterations in intestinal permeability, molecular mimicry and activation of effector immune cells.[15] It is therefore possible that alterations in the gut microbiome may contribute to the direction of differentiation of naïve T cells into specific effector T cells that drive the pathogenesis of psoriatic arthritis.

During the 12 months of follow-up, 13 patients reported weight gain, 8 weight loss and 40 unchanged weight (Supplementary Table S5.14). There is evidence from studies in mice that an obese phenotype can be transmitted by FMT from an obese individual to a germ-free mouse, resulting in weight gain in that animal.[16] There is a single case report of obesity occurring in a human recipient of donor FMT for CDI; however, it was not clear from this case report that donor FMT was responsible for the weight gain observed.[17] In the study in chapter 5, donors all had a normal BMI (18–25); therefore, any change in weight post FMT was unlikely due to acquiring the phenotype from an individual with an abnormal BMI.

The 8-week adverse events data did not demonstrate any safety signal for donor FMT; however, the study was not powered to assess this secondary end point. In addition, 8 weeks' follow-up is not sufficient time to manifest many potential adverse events. The 12-month safety data give a longer view but have the limitation of being "open-label". Without a control group, it is not possible to assign causality from donor FMT to any adverse effects as UC patients are at risk of developing many of the observed conditions irrespective of therapy.

7.1.7 Lamina propria mononuclear cell populations correlate with disease severity but not with donor faecal microbiota transplantation effect

The immunological changes that occur in UC in humans are not well characterised or well understood. UC is known to be associated with damage to the mucosal barrier, allowing the luminal microbiota to trigger a sustained inflammatory response. Previous studies have suggested that UC is associated with a possible atypical type 2 helper T cell immune response driven by NK T cells (potentially linked with a loss of immune tolerance) with an altered profile of regulatory T cells and microbial sensors on dendritic cells.[18-21] However, these data have many limitations in that they derive from animal models or from small or poorly characterised cohorts of human subjects.

This was the first study to assess mucosal and blood immune cell populations prior to and following donor FMT for UC. As far as we are aware, this was also the first study to assess mucosal and blood mononuclear cell populations in relation to disease activity (total Mayo score) in UC. Previous studies have compared UC patients with healthy controls only. The exploratory studies from Chapter 5 demonstrate an association of a number of mucosal immune cell populations and total Mayo score. Lamina propria B cell (β = 0.46 [95% CI 0.06 to -0.87]; *P* = 0.03) and dendritic cell (β = 0.43 [95% CI 0.04 to -0.82]; *P* = 0.03) populations were positively associated with total Mayo score at baseline. Conversely, lamina propria NK cells (β = -0.50 [95% CI -0.91 to -0.09]; *P* = 0.02) were negatively associated with total Mayo score at baseline. Lamina propria B cell (β = 0.67 [95% CI 0.13 to -1.2]; *P* = 0.03) and T regulatory cell (β = 1.1 [95% CI 0.27 to -2.0]; *P* = 0.03) populations were positively associated with change in total Mayo score from baseline to week 8. Conversely, helper T cells (β = -0.8 [95% CI, -1.4 to -0.19]; *P* = 0.03) were negatively associated with change in total Mayo score from baseline to week 8. The association between disease severity at baseline and LPMC populations as well as change in disease severity and LPMC populations are novel findings.

Fuss et al. previously compared samples from 15 patients with severe UC with six controls without colitis by using Student *t* tests without adjustment for patient-related factors. They found increased numbers of nonclassical NK T cells (i.e., NK T cells that do not bear an invariant T cell receptor) that produce markedly increased amounts of IL-13.[20] In Chapter 5 of this thesis, there was no association between the entire NK T

cell population or the nonclassical NK T cells and total Mayo score at baseline. The analyses in this thesis included 10 LPMC populations and two peripheral blood mononuclear cell populations that were compared with disease severity (total Mayo score). Samples were taken from a greater number of patients (67) than previous studies[20,22], and statistical modelling that adjusted for multiple potential biases (e.g., patient age and steroid use) was used, making the data here more robust.

There is evidence for a loss of immunoregulation to luminal antigens in UC. Peripheral blood lymphocytes and LPMCs respond vigorously to antigens from sonicated autologous bacteria but not bacteria from healthy stool donors.[23] There is also evidence that regulatory T cells (CD4+CD25+FOXP3+) may not adequately counteract the inflammatory response in UC.[24] In previous studies, CD4+LAP+ T cells have been noted to be increased in UC relative to controls; however, FOXP3+ regulatory T cells were not increased.[22] We therefore hypothesised that donor FMT may induce a therapeutic response by stimulating T regulatory cell activity. However, donor FMT was not significantly associated with change in regulatory T cell numbers or any other lamina propria cell populations at week 8. Similarly, donor FMT adjusted for total Mayo score was not significantly associated with change in any lamina propria cell populations at week 8. If the therapeutic effect of donor FMT was primarily driven via stimulating a particular immune population, then a correlation with donor FMT and/or donor FMT adjusted for total Mayo score would be expected. The pattern that we have observed of correlations between various cell populations and both baseline total Mayo score and change in total Mayo score from baseline but not FMT treatment effect (adjusted and unadjusted for total Mayo score) suggests that the immune populations studied are not the primary driver of the donor FMT effect. However, the immunological analyses in this study were exploratory and had limitations of analysing the proportions of mononuclear cells in the lamina propria without assessing their behaviour. It is therefore possible that changes in the activity of mononuclear cells may have occurred following donor FMT without significant change in the proportion of mononuclear cells.

7.1.8 Butyrate levels were not associated with disease severity or donor faecal microbiota transplantation effect

Another exploratory hypothesis in this thesis was that donor FMT may have a therapeutic effect by increasing colonic butyrate levels. Butyrate, a product of colonic bacterial fermentation of dietary fibre, is the primary energy source of colonocytes.[25] Further, butyrate is essential for the maintenance of the colonic mucosal barrier and inhibits colonic inflammation, carcinogenesis and oxidative stress.[26] Despite the known beneficial effects of butyrate on colonocyte health, butyrate delivered via enema has failed to demonstrate therapeutic efficacy[27-29] or improve inflammatory and oxidative stress parameters in human subjects with UC.[30,31] In Chapter 5, we did not find any association between butyrate or other SCFA levels in stool, and UC disease activity or donor FMT therapy. Similarly, butyrate-producing species and genera such as *Faecalibacterium prausnitzii*, *Rosburia* spp. and *Anaerostipes* spp. did not increase following donor FMT. These exploratory data suggest that the therapeutic effect of donor FMT is not mediated via increasing butyrate production by the microbiome. It is possible, however, that butyrate production did increase following donor FMT but did not result in an increased butyrate concentration being measured as it was used at an increased rate by healing enterocytes with recovery of their metabolic capacity.

7.2 Future Research Directions

Based on the work performed in this thesis, future research directions regarding FMT as a therapy for UC are presented below.

There are two major issues that need to be resolved before FMT can become a realistic treatment option for patients with UC in Australia. The first is evidence of efficacy and safety of FMT as a maintenance therapy and the second is regulatory change to allow distribution of stool from stool banks.

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7.2.1 Maintenance faecal microbiota transplantation for ulcerative colitis and further safety data are required

There are currently no published trials examining FMT maintenance therapy in UC. However, there is a single trial published in abstract form for which limited details are available. In this single-blind randomised placebo-controlled trial, 78 Indian patients underwent induction therapy with multi-session FMT (0, 2, 6, 10, 14, 18 and 22 weeks). Forty-three of 78 patients treated with induction FMT achieved clinical remission; 22 of these were randomly assigned to receive FMT and 21 received placebo colonoscopically every 8 weeks. The primary outcome was achieved in 19 of 22 (86.4%) participants allocated to FMT versus 14 of 21 (66.7%) patients allocated to placebo (P = 0.126). The secondary end point of histological remission [12 of 22 (54.5%) with FMT versus 3 of 21 (14.3%) with placebo; P = 0.006] was achieved in a significantly higher number of patients with donor FMT than placebo. The results from this abstract are encouraging and the full-trial publication is awaited.

Further trials of maintenance therapy are required to establish efficacy and safety of FMT as maintenance therapy. In the published abstract, FMT was delivered via colonoscopy every 8 weeks; this demonstrates proof of concept but is not deliverable in real-world practice. Therefore, an important question to answer with regard to maintenance therapy is the optimal delivery method. In the outpatient setting, a therapy would need to be delivered via capsule or enema to be feasible for patients. Other important questions to answer are dose and dose interval of both induction and maintenance therapy. Dose-finding maintenance trials of FMT for UC will be important to determine dosing interval and quantity. They may also shed light on the mechanisms responsible for the therapeutic effect of FMT. These trials will be better placed than induction studies to monitor a number of elements (such as bacterial strains or their products) that may be driving the therapeutic response and correlate their levels with disease activity. Repeated sampling over time with associated meta-data would give more power to potentially elucidate complex interactions that may be at play.

In addition to maintenance data, further studies with larger patient numbers and long follow-up periods are required to assess the long-term safety of FMT for UC. In future, if FMT is prescribed for the treatment of UC outside of the clinical trial setting, then a

dedicated registry, such as has been established in the United States of America for FMT for CDI[32], should be established and would provide additional safety data.

7.2.2 Regulatory change and funding of faecal microbiota transplantation are required

Delivering FMT for UC in clinical practice in Australia would require nationwide access to screened stool aliquots. This would require both regulatory change, Australian Register of Therapeutic Goods, and funding from the Medicare benefits schedule or other government agencies to ensure equity of access.

Frozen stool banking facilities that provide timely, safe and effective access to FMT are highly desirable. OpenBiome in the United States of America is a good model.[33] At present, there is both under- and over-regulation of FMT in Australia. On one hand, FMT may be delivered in the local care setting without being subject to agreed standards for screening and manufacturing practice. Medical practitioners are able to compound and administer an unregistered therapeutic product to a patient under their care with appropriate consent. Conversely, supply of FMT aliquots from stool banks that adhere to rigorous production standards is not currently permissible. Current legislation prohibits the wider distribution of therapeutic products that are not certified according to Good Manufacturing Practice (GMP) protocols. The need for GMP certification, as currently interpreted, represents a significant barrier to the distribution of screened stool aliquots from stool banks. Beyond stringent screening for potentially transmissible conditions, a GMP requirement for standardisation of donor faecal product is not achievable as stool composition varies widely. As a consequence, access to safe and screened FMT therapy for CDI in Australia is inequitable. Developing a regulatory framework that focuses on achievable screening and safe manufacturing practice without product standardisation would facilitate this.

Unlike most pharmaceutical therapies, FMT is not currently funded by most third-party payers, and this presents problems with equity of access to this therapy. Given the strong evidence for FMT as a treatment of rCDI, funding should be provided within government health budgets. As indications for FMT in other diseases such as UC evolve, funding should also include equitable patient access for these additional indications.

7.2.3 Does faecal microbiota transplantation act via altering the metabolic capacity of the recipient microbiota?

The observation of no association of donor FMT treatment effect with colonic lamina propria immune populations or luminal butyrate levels suggests that FMT may be reducing inflammation via an alternative mechanism. One possible explanation is that FMT has a therapeutic effect in UC by resolving a metabolic deficit within the colonic microbiota.

There is evidence that there is a failure of butyrate oxidation in the colonocytes of patients with UC.[34-36] This leads to an energy deficiency state within the colonocyte with an associated loss of critical functions such as maintenance of the mucosal barrier by mucus and tight-junction production. Butyrate oxidation in human colonocytes has been demonstrated to be inhibited by high levels of nitric oxide.[36] Nitric oxide is produced by bacterial reduction of dietary nitrate (potentiated by sulphide) as well as by inflammatory immune cells.[37]

It is plausible that the treatment effect of donor FMT results from the acquisition of metabolic functional capacity from donor microorganisms. A number of organisms that increased following donor FMT (table S5.8) have been demonstrated to be involved in nitrogen, sulphur and hydrogen metabolism in vitro. Nitric oxide, a product of bacterial reduction of nitrate as well as lamina propria inflammatory cells, has been proposed as an important injurious agent in UC pathogenesis. High levels of nitric oxide can inhibit the ability of enterocytes to utilise butyrate[38], and sulphides in the colon are able to inhibit the reduction of nitric oxide.[39,40] *Peptococcus niger*, *Acidaminococcus intestini* and *Odoribacter splanchnicus* can all reduce nitric oxide to nitrous oxide and nitrogen. *M. smithii* has an important role reducing sulphide in the colon. *M. smithii* and the reductive acetogenic bacteria species, *Clostridium methylpentosum*, also utilise hydrogen and carbon dioxide and may optimise colonic fermentation.[41]

The two organisms most strongly associated with donor FMT treatment effect (table S5.9), *Anaerofilum pentosovorans* and *Bacteroides coprophilus*, do not reduce sulphate to the injurious sulphide; however, their ability to reduce nitric oxide (theoretically beneficial) is not known.[42,43]

The major limitation of the microbiome analysis in Chapter 5 is that 16S rRNA sequencing was used to identify candidate organisms associated with donor FMT effect. 16S rRNA sequencing can characterise bacteria phylogenetically in terms of their genera and can assign species grouping for some organisms. However, 16S rRNA sequencing does not have the resolution to accurately identify strains. It is therefore difficult to make inferences about the metabolic potential of organisms identified using this technique. Two different strains from the same species can have different metabolic potential and therefore disease potentiating or ameliorating potential. Metagenomic sequencing was prohibitively expensive when this study was designed. However, the cost of metagenomic sequencing has fallen dramatically in the last few years and, as a result, it is far more accessible to researchers.

Future studies should use a combination of metagenomic sequencing and culture based techniques to identify candidate metabolic pathways and corresponding organisms that may be involved in the disease process or the FMT treatment effect. This data could then be used to select candidates for rationally designed microbial therapeutics that could replace donor FMT. In addition to using metagenomics to survey the changing metabolic potential of the microbiome post FMT, more extensive metabolomics would also be informative. In this study, there was no association with SCFA levels and the donor FMT effect; however, other metabolites may be important. Given the proposed role that sulphites and nitrite/nitric oxide may play in UC pathogenesis[37], measuring these metabolic products would be informative in terms of supporting or refuting the evidence from the colonocyte culture models as to the role these pathways play in UC pathogenesis. It may also be helpful in exploring the therapeutic benefit of donor FMT.

7.2.4 Faecal microbiota transplantation as a tool to discover disease mechanism and microbial therapy

Uncovering the mechanism of action of donor FMT would pave the way for the development of rationally designed microbial therapeutics. It seems likely that the demonstrated effect of donor FMT is mediated by microbes or microbial products and that these elements could be refined and delivered in a standardised therapy. This would have a number of advantages over donor FMT. First, a standardised product is likely to provide a more predictable therapeutic and safety profile than donor FMT and would be more easily evaluated by regulatory agencies. Second, reducing the therapy to include

only the necessary active constituents would enhance safety by reducing the risk of disease transmission due to failed screening of a donor. It is known from blood donor safety analysis that screening failures do rarely occur for known diseases and historically occurred for hepatitis C before the virus was characterised.[44,45] These infectious risks are present for FMT but would be absent for defined microbial consortia derived from pure isolates. Third, defined strains or consortia of organisms with known metabolic potential would potentially allow tailoring of therapy to the specific microbial "deficit" identified in a prospective patient's gut microbiota. Theoretically, these advantages of rationally designed microbial therapy should lead to gains in both efficacy and safety.

There are currently efforts underway to develop microbial therapies for UC. Data from this thesis have been licensed by the University of Adelaide to the UK company Microbiotica for the purpose of developing a rationally designed microbial therapy. Seres Therapeutics presented data of a phase 1b study on a consortia of spore-forming gram-positive bacteria (SER-287) in abstract form in 2018. The trial included 58 adults with mild to moderate UC assigned to one of three treatment arms or placebo. The treatment arms included 6 days of vancomycin pre-treatment followed by 8 weeks of SER-287, either daily or weekly, and placebo pre-treatment followed by weekly SER-287. Remission was defined as a total modified Mayo score ≤2 and endoscopic subscore ≤1. Microbiome engraftment was also assessed. Remission occurred in 6/15 (40%) receiving vancomycin pre-treatment followed by SER-287 daily, 2/15 (13%) receiving placebo pre-treatment followed by SER-287 daily, 3/17 (17%) receiving vancomycin pre-treatment followed by SER-287 weekly and 0/11 (0%) receiving placebo. Engraftment of SER-287 bacteria was greatest in the vancomycin pre-treatment groups followed by the SER-287 treatment group, compared with other SER-287 treatment or placebo groups. This trial had small numbers and participants were divided into four study populations. Therefore, it was not adequately powered to assess efficacy or safety; however, the results are interesting and larger phase III studies are awaited.

An interesting finding from this study was the possible greater efficacy of the vancomycin pre-treatment arm than the placebo pre-treatment arm. It appeared that by reducing luminal gram-positive populations with vancomycin, both the therapeutic effect and the engraftment of the bacterial treatment were enhanced. None of the four RCTs of FMT for UC assessed in Chapter 6 used antibiotic pre-treatment. However, all

of the studies other than Moayyedi et al. used bowel preparation with polyethylene glycol, which would have the effect of reducing the colonic bacterial load prior to FMT. The systematic review conducted in Chapter 6 included 14 cohort studies of FMT for UC, five of which used antibiotic pre-treatment. The rate of clinical remission with FMT was 19/57 (33%) in the studies using antibiotic pre-treatment, compared with 20/111 (18%) without antibiotic pre-treatment. Antibiotic pre-treatment perhaps offered some advantage despite heterogeneity in study design between the groups. Donor FMT contains a large number of phages, which may provide a similar effect to antibiotic pre-treatment by reducing recipient colonic microbial populations. Phages may not be present or be present in far smaller numbers in defined microbial consortia such as SER-287, and therefore antibiotic pre-treatment may provide a greater benefit in this setting than in the setting of donor FMT.

7.2.5 Other indications for faecal microbiota transplantation

This thesis has demonstrated the utility of using FMT as a therapy, a tool to learn about disease pathogenesis and a means to develop new microbial therapies. In these respects, FMT offers huge potential. There are recent trials of FMT published in irritable bowel syndrome[46,47], hepatic encephalopathy[48] and autism[49], and a number are underway in a variety of other conditions.

There are theoretical reasons why FMT may be of benefit to patients with diseases where there is a metabolic "lesion" or deficiency within the gut microbiome. Traditional medical therapies have consisted of molecules that knock out certain unwanted physiological processes such as a bacterial enzyme or inflammatory cytokines. Almost all current medications from antibiotics to new anti-TNF biological drugs operate in this way. This model is highly successful where there is a single pathogen or isolated pathway that can be targeted. However, many common chronic diseases are associated with reduction in diversity and metabolic function within the microbiome.[50] If loss of these functions performed by the microbiome results in disease, then replenishing these metabolic pathways would be a logical solution. Knocking out downstream physiological pathways with drugs in this context should only be expected to yield modest results. FMT and microbial therapies offer the possibility of ecological restoration and replenishment of lost metabolic function and, in this respect, involve a paradigm shift in the way medicines are conceived.

7.3 Conclusion

This thesis has demonstrated that FMT is an effective therapy for the indication of remission of active UC. This opens up the possibility for rationally designed microbial therapeutics as novel therapies for the disease. New therapies for UC are needed because those currently available are limited by incomplete efficacy and adverse effects including serious infections and malignancy. Further trials of FMT in UC assessing its role in maintenance therapy and its long-term safety are needed before FMT can become a standard therapy for UC.

7.4 References

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Appendix 1: Fecal Microbiota Transplant for *Clostridium difficile* Colitis-Induced Toxic Megacolon

Background

Toxic megacolon is a severe and potentially fatal complication of *Clostridium difficile* (CD) induced colitis that occurs in up to 5% of cases. It results in acute colonic distension and is accompanied by major systemic disturbance, including shock. The standard treatment for toxic megacolon is subtotal colectomy. This manuscript describes the first case of faecal microbiota transplantation (FMT) as an effective treatment for toxic megacolon without surgery reported in the literature. This case demonstrates the life-saving potential of having access to pre-screened donor stool, frozen and available on demand.

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Name of Principal Author (Candidate)	Samuel Paul Costello		
Contribution to the Paper	Screening, preparation and supply of stool Data acquisition and management Analysis and interpretation of research data Drafting and revision of article		
Overall percentage (%)	50%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Fecal Microbiota Transplant for *Clostridium difficile* Colitis-Induced Toxic Megacolon

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Running head: Fecal microbiota transplant for toxic megacolon

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To the editor:

Toxic megacolon (TMC) is a severe and potentially fatal complication of colonic inflammation in which acute colonic distension >6 cm is accompanied by major systemic disturbance(1). It occurs as a complication in up to 5% of cases of *Clostridium difficile* (CD) induced colitis(2-4). The standard treatment for TMC is subtotal colectomy(5). Here we report the first case of fecal microbiota transplant (FMT) as an effective treatment for TMC without surgery.

A 69-year-old woman was transferred to our institution from a renal dialysis centre with fever, abdominal pain and 4 days of profuse watery diarrhoea. Three weeks prior she had received oral vancomycin for a severe episode of CD colitis following a course of

oral flucloxacillin for cellulitis. Her past medical history included haemodialysis for end-stage renal failure secondary to polycystic kidney disease, polymyalgia rheumatica, cardiomyopathy, hypertension and post-polio syndrome.

On admission she was febrile and tachycardic. Her abdomen was soft with bowel sounds present but there was diffuse tenderness over the lower abdomen without guarding. CT abdomen showed marked wall thickening throughout the colon without dilatation. Flexible sigmoidoscopy showed pseudomembranous colitis of the rectum and sigmoid colon (image 1). CD toxin was positive by PCR. Despite oral vancomycin (250mg 6 hourly) her condition deteriorated over the following 3 days with ongoing fever, frequent diarrhea and diffuse abdominal pain. On day 4 her bowels did not open and she developed shock (blood pressure 76/30 mmHg, pulse 110 bpm). She was transferred to the intensive care unit for fluid resuscitation and intravenous metronidazole was commenced in addition to oral vancomycin.

Following extensive discussion with medical and surgical staff she declined subtotal colectomy. An abdominal x-ray the following morning demonstrated dilatation of the cecum and ascending colon to 9 cm consistent with TMC (image 2). She remained febrile and became drowsy. The patient and her relatives consented to FMT understanding that the procedure would be investigational and not standard therapy for TMC. Then, 160mL of thawed fecal suspension was administered via a push enteroscope positioned in the jejunum, as colonic delivery was thought to be too high risk. The donor stool had been collected 5 months earlier and stored at -112°F in a 10% glycerol suspension. The material was thawed 3 hours prior to the procedure.

Following FMT, the patient became afebrile within 24 hours. Her abdominal pain resolved and inflammatory markers improved. On day 7 post FMT, the patient developed left lower lobe pneumonia that was treated successfully with 12 days of antibiotic therapy. She was discharged from hospital and subsequently had no further reported CD colitis within 12 months of follow-up.

To our knowledge this is the first case report of successful treatment of TMC due to CD with FMT and antibiotics without surgery. Although a previous case was reported(6), the patient received FMT following decompression surgery and it is possible the improvement partially reflected mechanical factors. Our patient declined surgery and

FMT was conducted as a “last ditch” measure. The prompt and durable response to FMT at 12 months suggests that in some cases FMT can reverse even severe disruption to colonic physiology associated with TMC in CD colitis. However, FMT cannot be recommended until further data are available and should be currently considered only where surgery is declined or unavailable.

For patients who develop recurrent, antibiotic refractory CD colitis, FMT has been shown to offer the best chance of cure with success in 81–94% of cases(7, 8). As such it is becoming standard treatment for recurrent CD colitis(9). However FMT remains an investigational therapy and should be considered within the framework of relevant policies such as those set out by the FDA. Although FMT should not replace surgery in the majority of patients with TMC, the outcome reported here suggests that the role of FMT in CD colitis may expand to encompass later stage disease particularly for patients who decline surgery or have unacceptable surgical risk.

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Specific author contributions

Dr SP Costello established the fecal microbiota transplant service and stool bank in South Australia and provided the stool and technical instruction on the faecal transplant procedure. Dr Costello is the author of the letter.

Dr A Chung performed the push enteroscopy that delivered the faecal transplant in the jejunum. Dr Chung edited the letter.

Professor JM Andrews helped establish the fecal microbiota transplant service and edited the letter.

Professor RJ Fraser was clinically responsible for the patient and the decision to proceed with fecal microbiota transplant. He performed the final edit of the letter and is the guarantor.

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Potential competing interests

None

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Appendix 2A: Protocol (May 2013)

TITLE: Faecal microbiota transplantation (FMT) for the treatment of active ulcerative colitis (UC)

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1. Objectives of Study

Primary

- To determine whether faecal microbiota transplantation (FMT) improves clinical and inflammatory outcomes in patients with active ulcerative colitis (UC).

Secondary

- To determine whether any clinical change is accompanied by an alteration in the faecal- and/or mucosa-associated microbiome of UC patients prior to and following FMT.
- Assessment of alteration and durability of change in the recipient microbiota after FMT in UC patients.
- To examine the mucosal immune changes induced by FMT and to examine whether they are influenced by changes in microbiome and/or disease activity.
- To examine the durability of clinical response/improvement after initial response to FMT in subjects with active UC.
- To establish patient satisfaction with FMT as a therapy for UC.

2. Background and Significance

UC is a chronic inflammatory bowel disease (IBD) that is characterised by recurring episodes of inflammation primarily involving the mucosal layer and occasionally the submucosa of the colon. Inflammation usually originates in the rectum and progresses in a contiguous fashion proximally. Although the aetiology of UC remains unclear, several factors are believed to play a role in its development and progression, including host genotype, immune disequilibrium and the composition of microbial communities resident in the gastrointestinal tract.

There is strong evidence for the involvement of microbes in the development of UC. IBD is associated with changes in the diversity of the gut microbiota, and although alterations in the abundance of specific bacterial species have often been identified, there remains no specific organism that is reliably associated with the condition[1]. There also appear to be changes in the functional activity of the microbiome, with changes in gene expression as well as protein production in the microbes of patients with IBD[1]. It

is unclear whether the altered microbiota is a result of, or initiates, the inflammatory process in humans. There is some evidence, however, that an altered microbiota develops prior to the onset of colitis in an animal model of interleukin (IL)-10 knockout mice[2].

Intestinal flora and their metabolic products play a critical role in maintaining the health of the colon. Patients who undergo ileostomy and have subsequent diversion of the luminal contents from the colon often develop a "diversion" colitis. The distal colonocytes in this instance are deprived of short-chain fatty acids (SCFAs) such as butyrate, a product of anaerobic bacterial fermentation of undigested dietary carbohydrates[3]. Yet, animal models of IBD also require bacteria within the colon for inflammation to develop[4]. It has been observed that altering the bacterial and nutrient colonic milieu by diverting the faecal stream using ileostomy reduces the recurrence of Crohn's disease in the colon[5]. Further supporting the notion that bacterial antigens contribute to, or drive, the autoimmune injury to the bowel, is evidence that antibiotics have some therapeutic efficacy in UC. In a recent systematic review, antibiotic therapy for UC was significantly associated with remission[6]. Antibiotic therapy gave a statistically significant relative risk reduction for active disease of 0.64 (CI 0.43–0.96). However, while bacteria are necessary to develop IBD, with germ-free animals unable to be induced to develop IBD, bacterial absence (or change in abundance/mix) can also lead to inflammation.

Probiotics have as yet demonstrated only limited therapeutic efficacy in UC[7]. In vitro studies have demonstrated that probiotic bacteria are able to modulate gut immune cells[8,9], while in vivo in a German study, *E.coli* Nissle 1917 was equivalent in efficacy to mesalamine for maintenance of remission of UC[10]. Additionally, a randomised trial of 77 patients with UC found that VSL#3, a cocktail of eight different bacteria, was more effective than placebo in improving symptoms and inducing remission at 12 weeks[11]. For patients with pouchitis, trials with VSL#3 have shown both therapeutic and prophylactic efficacy[12]. The outcomes in probiotic studies, however, have often been inconsistent and modest. This may be due to the variable actions of the different bacterial species that have been tested as well as the general limitations of most probiotic preparations. These probiotics provide a comparatively low number and diversity of bacterial species in comparison with the vast human gut microbiota. For this reason, some probiotic bacterial strains may not be able to compete effectively against

the complex interactions of an established and adapted indigenous gut microbial community[13].

FMT has been described as "the ultimate probiotic" as it provides an entire microbiome to the recipient. This therapy delivers a much greater number and diversity of bacteria than any current commercially available preparation. FMT was first reported in humans by Eiseman et al. in 1958 in the treatment of four patients with pseudomembranous colitis[14]. Three of the four patients were described as terminally or critically ill requiring vasopressor support and all were successfully cured. Over the subsequent years, there have been case reports and case series describing FMT predominantly for *Clostridium difficile* colitis but also for treating IBD, irritable bowel syndrome and constipation[13,15,16]. In the past decade, there has been a heightened interest in the use of this therapy, predominantly driven by increasing rates of recurrent *C. difficile* infection. During this time *C. difficile* has become more frequent, more severe and more refractory to standard treatment as well as more likely to relapse[17]. Standard treatment with metronidazole or vancomycin alters the normal gut flora that would usually provide colonisation resistance against *C. difficile* infection. For this reason, after successful initial therapy, up to 35% of patients will experience a symptomatic recurrence after ceasing antibiotics[18]. A subset of patients will have multiple recurrences and subsequent relapses occur in 45–65% of patients who have relapsed one or more times[19,20]. For patients with recurrent *C. difficile* colitis, FMT offers the greatest chance of cure of any therapy, with success in 87–100% of cases[13,21-25]. This impressive success rate is presumably due to the ability of the transplanted bacteria to recolonise/occupy the missing components/niches of the normal intestinal microbiota, thus removing the microbial niche that *C. difficile* would otherwise exploit.

FMT for UC was first reported in the literature by a gastroenterologist, Dr Justin D Bennet, from Kansas City, who described the results of a faecal transplant he received for his own disease[26]. Dr Bennet had continuous active, severe UC for 7 years, confirmed endoscopically and histologically, that was refractory to standard therapy. Dr Bennet described receiving antibiotics to "sterilise" his bowel prior to retention stool enemas. At the time of publication in 1989, he had been symptom and medication free for the first time in 11 years, at 6 months post FMT.

Borody et al. described case reports of six patients (three men and three women aged 25–53 years) with UC for at least 5 years who were treated with FMT[16]. All patients had suffered severe, recurrent symptoms and UC had been confirmed on colonoscopy and histological examination. Faecal flora donors were healthy adults who were extensively screened for parasites and bacterial pathogens. Patients were prepared with oral antibiotics and oral polyethylene glycol lavage.

Faecal suspensions were administered as retention enemas and the process repeated daily for 5 days. By 1 week post FMT, some symptoms of UC had improved. Complete reversal of symptoms was achieved in all patients by 4 months post FMT, by which time all other UC medications had been ceased. At 1 to 13 years post FMT, and without any UC medication, there was no clinical, colonoscopic or histologic evidence of UC in any patient. The authors concluded that colonic infusion of donor human intestinal flora can reverse UC in selected patients, and that these results support the concept of abnormal bowel flora or even a specific, albeit unidentified, bacterial pathogen causing UC. However, caution is needed when interpreting their data as this centre is known to have undertaken a large number of these treatments and it is uncertain why only six are reported. There is no comment in this paper as to the number of patients at their facility in whom this technique was attempted and if there were any patients in whom the treatment failed; moreover, this is open-label treatment, which is now an insufficient standard of proof when evaluating novel therapies. Hence, randomised placebo-controlled trials are needed to rigorously examine the efficacy of this proposed "alternative" therapy.

An anticipated concern in the medical community regarding FMT has been patient acceptance. This has been an assumption based on little evidence. To look at this question of patient willingness to undergo FMT, Kahn et al. performed a qualitative study to explore the attitudes and concerns of patients and parents of children with UC regarding FMT as a potential treatment[27]. They conducted six focus groups at a clinic in Chicago, Illinois and participants were asked about their perceptions of and interest in FMT as a treatment for UC. Sessions were recorded, transcribed and reviewed to identify domains, themes and major concepts. The focus groups included 15 adult patients and seven parents of children with colitis. The study identified five major domains pertaining to FMT: impressions of treatment, benefits, risks, potential mechanisms and social concerns. All but one participant expressed interest in FMT and

several wished it were already available. Participants compared FMT to probiotics, felt it was “natural”, was easier than current therapies and would be safe with donor screening. Although initial distaste and the “yuck factor” were uniformly mentioned, these concerns were outweighed by perceived benefits. The study concluded that given adequate supporting research, donor selection and screening, adult patients and parents of children with UC will consider FMT and are eager for it to become available.

FMT for UC is currently undertaken at a private gastroenterology clinic in Sydney and case reports of success from this clinic are reported in the literature[16]. There is also evidence from UC online forums that patients are conducting FMT for UC outside of the health care setting[28,29]. This is occurring in an unregulated fashion, with only very limited evidence of efficacy from seven case reports in the literature. These occurrences underline the need for more robust scientific evidence in this area and a randomised controlled study of efficacy.

2.1 Standard of care for ulcerative colitis

The management of UC involves both maintenance medication and medication used to control flares of the disease. The goal of maintenance therapy in UC is to maintain steroid-free remission, clinically and endoscopically. This requires regular clinical assessment including history, physical examination and at times colonoscopic examination. Other tools of assessment include blood (e.g., C-reactive protein [CRP], WCC) and stool (calprotectin) testing for inflammatory markers and imaging including MRI, CT or ultrasound.

The choice of maintenance treatment in UC is determined by disease extent, disease course (frequency of flares), failure of previous maintenance treatment, severity of the most recent flare, treatment used for inducing remission during the most recent flare, safety of maintenance treatment and cancer prevention. The mainstay of maintenance medication is the 5-aminosalicylic acid (5-ASA) compounds such as mesalazine and sulphasalazine[30,31]. These compounds are commonly taken orally in formulations that predominantly deliver the active 5-ASA component to the colon. Alternatively, or in addition, mesalazine preparations can be delivered topically via enema or suppository if the disease only involves the left side of the colon (although it is only PBS funded for topical therapy during a flare and not for maintenance of remission—even though it also

works in this setting). The majority of patients can be managed with maintenance 5-ASA compounds most of the time. For patients who have repeated flares of disease on 5-ASA maintenance therapy (one or more flares in a year needing steroids), thiopurine medication such as azathioprine or 6-mercapropurine should be used[32]. These medications induce systemic immunosuppression, and reduce the incidence and severity of flares of colitis, but also slightly increase the risk of some infections and malignancy. Anti-TNF agents such as infliximab and adalimumab have been shown to have benefit in maintaining remission in UC[33] (and are licensed for this indication by the TGA); however, these agents are very expensive and not funded by the pharmaceutical benefits scheme in Australia, and so are not readily available. The anti-TNF agents also give an increased risk of infection, particularly latent TB reactivation.

Mild flares of UC can be managed with higher doses of oral 5-ASA compounds or the addition of topical 5-ASAs given via enema or suppository. More severe flares are usually managed with a course of systemic corticosteroid. These can be given intravenously in acute, severe disease or orally in less severe flares. The steroids should then be tapered over time and discontinued. There is no indication for long-term steroid use in UC, and prolonged steroid use is associated with a number of complications including infection, osteoporosis, obesity, diabetes, poor wound healing, thinning skin, mood changes and insomnia. Severe flares of UC not responsive to steroids may respond to rescue therapy with the addition of either cyclosporin or anti-TNF therapy.

Patients in whom colonic inflammation cannot be controlled adequately frequently undergo total colectomy. This may be done electively (for refractory disease) or emergently in acute fulminant colitis. Colectomy entails surgical risk that is higher in the emergent setting; this risk includes infection, wound breakdown and mortality. Colectomy is considered “curative” for UC especially if an ileostomy stoma is created; however, it frequently also leads to complications both short- and long-term. In addition, in patients in whom an ileal–anal pouch is fashioned, up to 50% will subsequently develop pouchitis at 4 years post surgery[34].

3. Specific Safety Considerations

A recent review article assessed all cases of FMT in the literature prior to 2011[13]. A total of 239 patients had undergone FMT. The authors did not find any serious adverse

events related to the procedure. Some studies reported patient deaths due to the underlying disease, where the patient has not responded to the FMT. In one study, in which donor faeces were instilled via a nasogastric tube, a patient died of peritonitis. This patient was undergoing peritoneal dialysis for end-stage renal failure at the time and was septic with severe *C. difficile* colitis. Her condition remained unchanged immediately post transplantation; however, on the third day, she developed peritonitis. Although considered more likely the result of peritoneal dialysis, the nasogastric tube insertion could not be discounted to have been contributory[35]. One patient in a study by Silverman et al. developed irritable bowel symptoms following FMT[25].

Following this literature review in 2011, there have been four further cohort studies in the literature of patients who have undergone faecal transplant for *C. difficile* colitis[21,22,36,37]. A total of 216 patients who underwent FMT via colonoscopy were included in these four studies with no immediate adverse effects from FMT noted.

There is a potential to transmit infection via contaminated donor stool. The donor stool will therefore undergo microscopy and culture for potential bacterial pathogens, and microscopy for ova, cysts and parasites, as well as viral studies and *C. difficile* toxin analysis.

Blood testing to exclude HIV, hepatitis B and C, and syphilis will be undertaken.

Changes in faecal microbiota have been found in patients with a number of gastrointestinal and extra-intestinal diseases. Changes in the microbiome of patients with IBD and irritable bowel syndrome are well documented in the literature. There have also been associations between various bowel flora and obesity and the metabolic syndrome[38]. The association has not been documented as causal and it appears probably related to the diet consumed by these subjects. It would, however, be prudent to exclude donors with the metabolic syndrome from the study.

In an audit of 16,318 colonoscopies performed in Northern California from 1994 to 2002, Levin et al. found serious complications occurred in 5.0 of 1000 procedures[39]. The major risk of colonoscopy, bowel perforation, occurred in 0.09% of colonoscopies in that study. Other risks include dehydration from bowel preparation, over-sedation, aspiration, bleeding and splenic laceration. This patient group will however be undergoing regular colonoscopies for their UC and will be familiar with these risks.

Risks from standard therapies they may be offered for active disease (e.g., steroids, immunomodulators and colectomy) are also substantial, thus risks from colonoscopy for FMT are relative.

4. Ethical Considerations

UC is a chronic, debilitating disease with a near-normal life expectancy[40]. Current therapies are inadequate and the disease continues to have an unacceptably high rate of chronic relapsing symptoms. This is underlined by evidence that up to 30% of patients will require colectomy after 25 years of disease[41]. For this reason, it is important for the medical community to rigorously examine potential new therapies that may benefit this group of patients.

A small number of case reports of successful treatment of UC with FMT have been reported in the literature[16,26]. However, the findings of these case reports have never been tested in a randomised controlled trial (RCT). Despite this very limited evidence, there is a clinic in Australia offering UC patients FMT as a therapy[16]. There is also evidence from online forums that patients are undertaking this therapy without medical supervision[28,29]. Despite the minimal evidence in the literature, there is a willingness among sufferers of UC to try this potential therapy[27]. We believe an RCT in this area is necessary to gather evidence for or against the effectiveness of FMT as a treatment for UC. A positive result will avail UC sufferers of a new therapy and a negative one will help discourage the use of an unproven, invasive therapy. Stool analysis of faecal transplant success may also fast-track development of tailored probiotic medicines.

Donors will be anonymous and so will not be known to the recipient. This avoids any apportion of blame towards a known donor should a complication or treatment failure arise during the trial.

Colonoscopy will be used to deliver the initial stool transplantation and to assess the colon during follow-up. This is an invasive procedure that carries some risk. Most of the recent studies of FMT for *C. difficile* have used colonoscopic delivery[13,22,23] as it allows assessment of the underlying disease and allows the donor bacteria to contact the entire colon. Patients with symptomatic UC ordinarily undergo examination with colonoscopy as part of the assessment of disease activity to help guide treatment. The initial colonoscopy in this trial will therefore not be an additional procedure. However, the

colonoscopic examinations at week 8 and at 12 months may be additional procedures depending on the state of the patient's disease and symptoms.

The colonoscopic examinations will involve biopsy of the mucosa for analysis of microbiota as well as immune function and histopathology. The majority of these biopsies will be additional to that which the patient would ordinarily receive outside of the trial. These biopsies will be critical to detect any changes in the mucosal-associated microbiota or immune changes associated with the FMT. The risk of biopsy of the mucosa is small with the major risk being bleeding. Biopsies can be safely performed on a single antiplatelet agent[42]. Patients on duel antiplatelet therapy or anticoagulant medication (e.g., warfarin or heparin) will be excluded from the study.

As FMT has only been performed in large numbers in the past decade, there may be unknown long-term risks. However, there have been no reports of major complications of faecal transplant in the literature to date.

Taking blood may cause short-term pain or discomfort and patients will be informed about this before entering the trial. The volume of blood taken is not extreme and will not cause side effects. If patients are of the view that blood sampling is too painful, they may withdraw from the study at any time. Blood tests as well as answering questionnaires will involve an increased time burden and patients will be informed about this before the trial begins. It is not anticipated that the FMT procedure will cause any adverse reactions, but participants will be provided with information about supports they can contact should they experience any distress in relation to the study.

Before taking part in the study, informed written consent will be obtained from patients. The researchers will ensure that the patient is given full and adequate verbal and written information about the nature, purpose, possible risk and benefit of the trial. They will be given sufficient time to consider the information, to ask questions and to seek advice prior to being asked whether they wish to participate in the study. Participants will also be assured their participation in the trial is absolutely voluntary. All treatment decisions are at the discretion of the usual treating physician, and will not be altered by the trial. The participation is strictly confidential, and the identity of subjects will not be disclosed to other medical or research staff unless subjects agree.

Once subjects have been enrolled in this study, they will be given a study participant code, and only study investigators will have access to their name and personal details. We intend to summarise the results in a manuscript and to submit it for publication in a peer reviewed journal. Therefore, all information gathered from this study will be published in a form that does not allow patient identification. We will not provide any feedback with regard to individual microbiota composition or immunologic function.

Our proposed study has the support of the director of the Royal Adelaide Hospital Department of Gastroenterology and Hepatology, Richard Holloway, as well as the head of Endoscopic Services, Mark Schoeman. The head of the IBD Service, Jane Andrews, will be the lead supervisor of the study. The Royal Adelaide Hospital has a large cohort of approximately 800 patients with IBD and a strong record of successful clinical research. The proposed study also has the support of the head of gastroenterology at the Queen Elizabeth Hospital, Ian Roberts-Thomson. The Queen Elizabeth Hospital has a cohort of approximately 300 patients with IBD. The study supervisors all have extensive experience in medical research as well as experience in supervising PhD students.

The Commonwealth Scientific and Industrial Research Organisation (CSIRO) in Adelaide has broad experience in human gut flora and microbial analysis. This is an area of focus for its recent research. The Nerve Gut Research Laboratory at the Royal Adelaide Hospital is a leader in the field of research into the neuro-immunological and neuro-endocrine processes of the human gut.

5. Study Design

This study is an 8-week randomised placebo-controlled trial with a 44-week open-label extension.

Randomisation

Patients will be randomly allocated into the following groups:

- Group 1: Patients receive previously frozen pooled **donor** stool via colonoscopic insertion into the right colon.

- Group 2: Patients receive previously frozen **own** stool via colonoscopic insertion into the right colon.

Open-label therapy from 8 weeks

Patients who are randomly assigned to the placebo group who do not have a clinically relevant response (achieving remission, having a drop in Mayo score by ≥3 or achieving an endoscopic subscore of 0–1) by week 8 will then cross over to receive active donor FMT at the 8 week colonoscopy. The FMT will be conducted in an identical manner to Group 1 with FMT followed by two enemas on day 3 or 4 and one on day 6 or 7.

5.1 Recruitment

Patients will be recruited from IBD clinics at the Royal Adelaide and Queen Elizabeth Hospitals. Patients on the mailing list for the Royal Adelaide Hospital will be contacted about the trial through the quarterly newsletter. Gastroenterologists in Adelaide will be informed about the trial though a presentation at the South Australian Gut Club and an email to the South Australian Gut Club members. The trial will be listed on the Australian and New Zealand clinical trials registry as well as the Gastroenterology Society of Australia website.

We will enrol 70 patients with 35 patients in each arm of the trial.

5.2 Inclusion criteria

Patients with the following characteristics will be included:

1. mild to moderate active UC (total Mayo score 3 to 10)
2. endoscopic subscore of 2 or greater (to ensure symptoms are due to UC, not post-inflammatory irritable bowel syndrome)
3. patients aged 18 to 75 years with established diagnosis of UC.

5.3 Exclusion criteria

Patients with any of the following characteristics will be excluded:

1. severe UC (Mayo score 11–12 or Truelove and Witts criteria)
2. more than 25mg of prednisolone per day (or equivalent steroid)

3. previous colonic surgery
4. active gastrointestinal infection
5. pregnancy
6. anticoagulant therapy or duel antiplatelet therapy (e.g., aspirin and clopidogrel)
7. current use of antibiotics
8. anti-TNF therapy.

Activity of disease will be defined by the Mayo score. This scoring system has 3 points each for stool frequency, rectal bleeding, endoscopic findings and physician's global assessment, giving a total score out of 12.

A score of:

- 0, 1 or 2 indicates inactive disease and exclusion from the trial
- 3 to 10 allows inclusion in the trial. Subjects will need an endoscopic subscore of at least 2 for inclusion to prove active disease (0 = normal mucosa and 1 = erythema only; most studies start with 2)
- 11–12 indicates severe disease and these patients will be excluded from the trial.

Similarly, any patient who fulfils Truelove and Witts criteria for severe colitis will be excluded while they meet these criteria. Truelove and Witts is defined as >6 bloody bowel motions per day plus one or more of the following: haemoglobin <10.5g/dL, ESR >30mm/hr, pulse rate >90 beats per minute and temperature over 37.5°C.

5.4 Medication prior to enrolment

Stable dosing of UC maintenance therapy is required prior to enrolment:

1. 5-ASA stable dosing for at least 4 weeks
2. thiopurines and methotrexate stable dosing for at least 6 weeks
3. biological agents stable dosing for at least 8 weeks
4. option to enrol on an oral dose of prednisolone ≤25mg, with a mandatory taper of 5mg per week.

6. Outcome Measures

6.1 Primary outcome

The primary outcome will be steroid-free remission of UC at week 8 defined as:

1. total Mayo score of ≤2 *AND*
2. Mayo endoscopic score of ≤1.

6.2 Secondary outcomes

Secondary outcomes will comprise:

1. clinical response (≥3 point reduction in total Mayo score at week 8 and 1 year)
2. clinical remission (Simple Clinical Colitis Activity Index (SCCAI) ≤2 at week 8 and 1 year)[43]
3. endoscopic remission (Mayo <1 at week 8 and 1 year)
4. safety (assessed at week 8 and 1 year)
5. changes in mucosal- and faecal-associated microbiota following FMT assessed by 16S ribosomal RNA sequencing, stratified by:

    i. change in total Mayo score following FMT
    ii. randomisation
    iii. durability of engraftment of donor microbiome following FMT
    iv. changes in peripheral blood and colonic lamina propria mononuclear cell populations (assessed by FACS) following FMT
    v. patient perception and palatability.

Disease activity measures of symptoms score (SCCAI), and endoscopic and histologic gradings, as well as records of hospitalisation, corticosteroid requirement, periods of symptom flares and colectomy rate will be recorded at the 1-year mark as part of the open-label observation period from 8 weeks to 1 year.

7. Patient Participation

7.1 Recruitment

Participants will be recruited from:

- Royal Adelaide Hospital (RAH) and The Queen Elizabeth Hospital (TQEH) gastroenterology IBD databases and newsletters
- gastroenterology in- or out-patient encounter(s) at the RAH and TQEH (and Flinders Medical Centre after relevant approvals) by referral from their clinicians and by searching OPD letters.

Patients on clinical databases who have previously consented to being contacted regarding research studies will receive information about the study in the RAH IBD Service regular newsletter and may also be contacted by telephone, and if no answer is obtained a letter will be sent. All other patients will be contacted via a letter or by their treating clinician in whichever way the clinician feels is most appropriate to the particular patient.

Regarding the use of letters for contact, subjects who have already consented to be contacted regarding research (on database) will receive a letter signed by A/Prof Andrews (at RAH) or Prof Roberts-Thomson (at TQEH) on behalf of the study investigators.

The initial invitation letter will include an opt-out slip for subjects not wishing to be contacted further. Subjects not opting out or responding within 4 weeks after invitation will be contacted up to a further three times by two different methods (phone, SMS, email or letter) to ascertain whether they wish to participate or not. Demographic details of non-responders will be recorded to enable a full description of the sources of possible bias. All who agree to participate will be subsequently screened to ensure they fulfil inclusion criteria.

Donors will be recruited with a flyer advertisement on notice boards at the RAH and TQEH as well as the Adelaide University Medical School and Adelaide University campus.

7.2 Withdrawal criteria

Patients may withdraw from the study at any time. We will ask for their reasons for statistical purposes; however, they will not be obliged to provide this information. Withdrawal from the study will not affect ongoing standard medical care in any way. Their clinicians will be informed of their participation in the study. We will ask patients

to notify us of any changes in their treatment during the course of the study and, if necessary, we will seek their permission to verify this with their treating clinician.

8. Ulcerative Colitis Patient Assessment

8.1 The week prior to enrolment

1. The patient should have the opportunity to read the patient information sheet, discuss the trial with family or friends and ask questions of the investigators prior to signing trial consent.

2. Patient questionnaire regarding perception and expectation of faecal transplant prior to procedure.

3. Detailed history of UC:

- date of diagnosis
- extent of disease
- medication use—current and prior
- previous surgery
- previous hospitalisation
- comorbid disease
- current symptoms
- extra-articular manifestations.

4. Stool collected for:

- infection screen: microscopy culture + sensitivity, *Clostridium difficile* toxin (5g)
- possible re-administration for placebo arm subjects (50g)
- microbiome analysis: 6 × 0.25g stool in Eppendorf tubes, 2 × 5g stool in larger brown stool pots.

For collection and processing methods see section 10.

5. Disease activity assessment:

- faecal calprotectin

- CRP, ESR, FBC, U+E, LFTs
- symptom severity (SCCAI) at screening and one day prior to FMT
- flexible sigmoidoscopy:

    i. total Mayo score
    ii. disease extent (≥10cm of disease required)
    iii. biopsy for light microscopy and histopathology to exclude CMV inclusions.

8.2 Randomisation

Randomisation will be conducted once the patient satisfies the inclusion and exclusion criteria and has consented to enter the study. This should occur within 1–7 days prior to the first faecal transplant, which will be delivered via colonoscopy.

Prior to randomisation, three aliquots of pooled donor stool suspension from a single batch and three aliquots of the donor's own stool suspension will each be placed in clear plastic bags in the -80°C freezer at the endoscopy unit. All stool aliquots will be in identical yellow-topped 250mL cryo-safe containers. These will include 1 × 200mL suspension for colonoscopic delivery and 2 × 100mL suspensions for enema delivery.

Donor stool pots will be labelled on the lid with:

1. batch number
2. date of manufacture of batch.

Patient's own stool will be labelled on the lid with:

1. patient ID consisting of initials and study number (e.g., AB-1)
2. date of patient stool donation.

Randomisation to be conducted by hospital clinical trial nursing staff using www.random.org.

1. Cardboard circular caps with patient ID and either "Transplant" or "Save" and either "Colon" or "Enema" are then placed on the pots containing donor or patient's own stool depending on randomisation.

2. “Transplant” caps are placed on the pots to be given at and in the week following the first colonoscopy.
3. “Save” pots will be saved and the cap removed following the 8 week colonoscopy. If these contain donor stool, they will be given to the patient at the 8 week colonoscopy and in the subsequent week.
4. “Colon” pots will be delivered at colonoscopy and the “Enema” pots delivered via enema in the following week.
5. The randomisation document for the use by study nursing staff is listed on the next page.

Faecal transplant for active ulcerative colitis trial

Protocol for randomisation of FMT

1–7 days prior to faecal transplant use the random number generator http://www.random.org/

Into the "true random number generator" box on the right of the screen set the minimum to 1 and maximum to 2

Select: Generate

1 = Donor faecal transplant

2 = Placebo faecal transplant (patient's own stool)

Go to the -80°C Freezer.

1. For Donor Faecal transplant stick the "Transplant" disks on the top of the donor stool yellow pots and the "Save" disks on the placebo pots. Record the batch number in the transplant record book.
2. For Placebo Faecal transplant stick the "Save" disks on the donor stool yellow pots and the "Save" disks on the donor pots. The donor stool will then be saved to transplant at 8 weeks at the open-label crossover.

The **donor stool** pots are labelled with a Batch number and date (e.g., Batch 3, 2/7/13).

The **patient's own stool** (placebo) is labelled with patient study number.

8.3 Week 1 of trial

8.3.1 Day prior to colonoscopy

1. Patient to take a light breakfast and then to fast from solids.
2. Maintain high fluid intake throughout the day.
3. Take three sachets of Colonlytely bowel preparation (polyethylene glycol) in 3L of water.

8.3.2 Morning of colonoscopy

1. Randomised faecal aliquot labelled “Transplant” and “Colon” to be removed from the -80°C freezer and thawed at room temperature for 3.5 hours prior to delivery.
2. Patient to receive loperamide 2mg orally prior to colonoscopy.
3. SCCAI score diary to be collected.
4. Mayo symptom scores to be taken (endoscopic score to be added at colonoscopy).
5. Biopsy posts should be pre-labelled with the site and number of biopsies required.
6. Consent should be obtained for this procedure on a standard CALHN consent form in addition to the study consent form that has previously been signed.
7. While inserting cannula, take 60mL of blood:

    i. 50mL into 6 × heparin tubes (green and black top) for peripheral blood mononuclear cell flow cytometry (to be taken to Dr Hughes at Nerve Gut Laboratory)
    ii. 5mL into a small EDTA (purple tube) (to be sent to clinical laboratory)
    iii. 5mL into a GEL (white top) for electrolytes and liver function, C-reactive protein (to be sent to SA pathology laboratory).

8.3.3 At colonoscopy

1. Assess disease severity (using endoscopic Mayo score at point of maximum inflammation) and disease extent.
2. Biopsies should be taken on colonoscope insertion*:

i. left-sided biopsies: 11 total; 6 in RPMI media, 2 into RNA later (microbiome), 1 RNA later (PCR), 1 formalin (histopath), 1 PFA (IHC)
ii. right-sided biopsies: 9 biopsies; 6 in RPMI media, 2 into RNA later (microbiome), 1 formalin (histopath).

3. An attempt should be made to remove any residual fluid or faecal material during colonoscope insertion with suction and washing if required.
4. Once at caecum patient should be rolled onto the right lateral position and randomised faecal suspension delivered into the right colon. If caecum cannot be reached then delivery of faecal suspension into the right colon beyond the hepatic flexure is acceptable.
5. Patient should then remain on their right side for 1 hour following procedure.
6. Following 1 hour the patient should be assessed for any adverse effects and if well sat up and offered food and drink prior to discharge.

*Biopsies at each colonoscopy in more detail:

- 2 biopsies will be taken from both the recto-sigmoid region and ascending colon-caecal region of the colon for microbiota analysis. Each in 2.5mL RNA later.
- 1 biopsy from each region will be taken for histopathology analysis into formalin.
- 1 biopsy from left colon for immunohistochemistry (formalin) and 1 from the left colon for PCR (cytokines, transcription factors) (RNA later).
- 4 biopsies will be taken from the left and 4 from the right colon for flow cytometry (FACS) analysis (RPMI complete media), processed the same day as colonoscopy.
- 2 biopsies from the left colon and 2 from the right for supernatant release for cytokines/mast cell mediators (RPMI complete media).
- This will amount to 9 biopsies in the right colon and 11 in the left colon.

8.3.4 Enemas

Two enemas of 100mL faecal suspension will be delivered by a gastroenterologist at the clinic in the week following colonoscopy (days 2–4, days 5–7).

Patient should:

1. take 2mg of loperamide prior to enema
2. lay on left lateral position for enema insertion
3. roll from the left lateral to prone position then right lateral and then back to left lateral position following enema insertion. This is to encourage proximal distribution of the enema
4. attempt to hold the enema for 1 hour.

8.4 Week 4 assessment

1. Stool collection for faecal calprotectin level and microbiome analysis.
2. Stool to be collected in sterile blue bags that are placed over the toilet.
3. Patient to zip tie blue bag closed and place blue bag in clear zip-lock bag.
4. Deliver to CSIRO laboratory within 1 hour.
5. Bag to be opened and stool processed under anaerobic conditions in anaerobic chamber:

    i. microbiome analysis: 6 × 0.25g stool in Eppendorf tubes; 2 × 5g stool in larger brown stool pots.

8.5 Week 8 assessment

8.5.1 Two days prior to colonoscopy

1. Stool collection for faecal calprotectin level and microbiome analysis.
2. Stool to be collected in sterile blue bags that are placed over the toilet.
3. Patient to zip tie blue bag closed and place blue bag in clear zip-lock bag.
4. Deliver to CSIRO laboratory within 1 hour.
5. Bag to be opened and stool processed under anaerobic conditions in anaerobic chamber:

    i. microbiome analysis: 6 × 0.25g stool in Eppendorf tubes; 2 × 5g stool in larger brown stool pots.

8.5.2 One day prior to colonoscopy

1. Patient to take a light breakfast and then to fast from solids.

2. Maintain high fluid intake throughout the day.
3. Take three sachets of Colonlytely bowel preparation (polyethylene glycol) in 3L of water.

8.5.3 Morning of colonoscopy

1. Randomised faecal aliquot labelled “Save” and “Colon” to be removed from the -80°C freezer and thawed at room temperature for 3.5 hours prior to delivery.
2. Patient to receive loperamide 2mg orally prior to colonoscopy.
3. SCCAI score diary to be collected.
4. Mayo symptom scores to be taken (endoscopic score to be added at colonoscopy).
5. Adverse events since randomisation recorded.
6. Biopsy pots should be pre-labelled with the site and number of biopsies required.
7. Consent should be obtained for this procedure on a standard CALHN consent form in addition to the study consent form that has previously been signed.
8. While inserting cannula, take 60mL of blood:

    i. 50 mL into 6 × heparin tubes (green and black top) for peripheral blood mononuclear cell flow cytometry (to be taken to Dr Hughes at Nerve Gut Laboratory)
    ii. 5mL into a small EDTA (purple tube) (to be sent to clinical laboratory)
    iii. 5mL into a GEL (white top) for electrolytes and liver function, C-reactive protein (to be sent to clinical laboratory).

8.5.4 At colonoscopy

1. Assess disease severity using endoscopic Mayo score at point of maximum inflammation and disease extent.
2. Biopsies should be taken on colonoscope insertion*.
3. Left-sided biopsies: 11 total; 6 in RPMI media, 2 into RNA later (microbiome), 1 RNA later (PCR), 1 formalin (histopath), 1 PFA (IHC).
4. Right-sided biopsies: 9 biopsies; 6 in RPMI media, 2 into RNA later (microbiome), 1 formalin (histopath).

5. An attempt should be made to remove any residual fluid or faecal material during colonoscope insertion with suction and washing if required.
6. Once at caecum (and disease severity has been assessed and recorded), the cardboard "Save" cap should be removed from the pot to reveal the contents of the faecal pot:

    i. If this is labelled as the patient's own stool it should be discarded and the colonoscope withdrawn.
    ii. If this is labelled as donor stool then the patient should be rolled onto the right lateral position and the un-blinded faecal suspension delivered into the right colon.

7. If caecum cannot be reached then delivery of faecal suspension into the right colon beyond the hepatic flexure is acceptable.
8. Patient should then remain on their right side for 1 hour following procedure.
9. Following 1 hour the patient should be assessed for any adverse effects and offered food and drink prior to discharge.
10. Patient to be informed about randomisation. If they were initially randomised to placebo/autologous FMT then they will require two further donor FMTs via enema.

*Biopsies at each colonoscopy in more detail:

- 2 biopsies will be taken from both the recto-sigmoid region and ascending colon-caecal region of the colon for microbiota analysis. Each in 2.5mL RNA later.
- 1 biopsy from each region will be taken for histopathology analysis into formalin.
- 1 biopsy from left colon for immunohistochemistry (formalin) and 1 from the left colon for PCR (cytokines, transcription factors) (RNA later).
- 4 biopsies will be taken from the left and 4 from the right colon for flow cytometry (FACS) analysis (RPMI complete media), processed the day of colonoscopy.
- 2 biopsies from the left colon and 2 from the right for supernatant release for cytokines / mast cell mediators (RPMI complete media).
- This will amount to 9 biopsies in the right colon and 11 in the left colon.

8.5.5 Enemas (patients randomised to placebo/autologous FMT)

Two enemas of 100mL faecal suspension will be delivered by a medical practitioner at the clinic in the week following colonoscopy (days 2–4, days 5–7).

1. Patient to take 2mg of loperamide prior to enema.
2. Lay on left lateral position for enema insertion.
3. Roll into prone positions, right lateral and then back to left lateral position following enema insertion.
4. Patient should attempt to hold the enema for 1 hour.

8.6 One-year assessment

Patient will be posted or emailed:

1. SCCAI symptoms score
2. patient questionnaire regarding experience of faecal transplant prior to procedure and adverse events
3. invitation to undergo disease activity assessment.

Patients who do not return forms within 2 weeks will be contacted via telephone.

8.6.1 Two days prior to flexible sigmoidoscopy

1. Stool collection for faecal calprotectin level and microbiome analysis.
2. Stool to be collected in sterile blue bags that are placed over the toilet.
3. Patient to zip tie blue bag closed and place blue bag in clear zip-lock bag.
4. Deliver to CSIRO laboratory within 1 hour.
5. Bag to be opened and stool processed under anaerobic conditions in anaerobic chamber:

    i. microbiome analysis: 6 × 0.25g stool in Eppindorf tubes; 2 × 5g stool in larger brown stool pots
    ii. faecal calprotectin.

8.6.2 Flexible sigmoidoscopy

1. Mayo symptom scores to be taken (endoscopic score to be added at colonoscopy).
2. Adverse events since randomisation recorded.
3. Biopsy pots should be pre-labelled with the site and number of biopsies required.
4. Consent should be obtained for this procedure on a standard consent form in addition to the study consent form that has previously been signed.
5. While inserting cannula, take 60mL of blood:

    i. 50mL into 6 × heparin tubes (green and black top) for peripheral blood mononuclear cell flow cytometry (to be taken to Dr Hughes at Nerve Gut Laboratory)
    ii. 5mL into a small EDTA (purple tube) (to be sent to clinical laboratory)
    iii. 5mL into a GEL (white top) for electrolytes and liver function, C-reactive protein (to be sent to clinical laboratory).

6. Assess disease severity using endoscopic Mayo score at point of maximum inflammation.
7. Biopsies should be taken on the left side only.
8. Left-sided biopsies: 11 total; 6 in RPMI media, 2 into RNA later (microbiome), 1 RNA later (PCR), 1 formalin (histopath), 1 PFA (IHC).
9. Following 1 hour the patient should be assessed for any adverse effects and offered food and drink prior to discharge.

8.6.3 Care during the follow-up period

During the trial, subjects will be treated to the standard of care for UC. This involves a fixed maintenance medication as prescribed/advised by their own physician. Patients will enter this trial because of a flare, and all therapy they are on at entry will be continued except for the steroid taper as described.

A subject who experiences a flare of their disease during the study will be treated with standard therapy as if they were not in the study. This will include increasing their oral 5-ASA and/or adding a topical enema or suppository therapy. Systemic steroid therapy

may also be used. Steroid use will be quantified during the study and steroid requirement over the 12 month period will be another secondary endpoint. Once patients are commenced on steroid it will be tapered as explained above.

If a subject deteriorates on steroid therapy, they may require escalation of their medical therapy or surgery. Escalation of medical therapy may involve increasing the steroid dose temporarily. Patients who are naïve to thiopurine therapy may benefit from the addition of a thiopurine. Thiopurines can take up to 12 weeks to reach their therapeutic effect and so "rescue therapy" may be needed in the intervening period. Rescue therapy involves the addition of cyclosporine or an anti-TNF agent such as infliximab (if available through compassionate access) in the short term. Rescue therapy will be continued for 6 to 12 weeks to allow the thiopurine medication to reach its full effect.

Patients who have a severe flare of UC that does not respond to intravenous steroid medication within 3 to 5 days are unlikely to improve and should be assessed for surgical colectomy[44], as would be the case in routine care.

9. Stool Donor Recruitment and Screening

9.1 Donor recruitment

Posters will be placed on noticeboards on the University of Adelaide campus. These will detail that we are recruiting stool donors and the posters will have the contact details of Dr Costello and Dr Andrews.

9.2 Donor screening

Potential donors will be sent the donor information sheet via email or post.

Donors who consent will undergo a four-stage screening process with medical history, physical examination, blood testing and stool testing with the aim of reducing the risk of disease transmission from donor to recipient.

9.2.1 Medical history

Inclusion of patients who:

1. are 18 to 65 years of age

2. have not received antibiotic therapy for the past 6 months
3. have not had unprotected sexual intercourse in the last 1 month outside of a long-term monogamous relationship
4. have not travelled outside of Australia for past 1 month.

Inclusion of patients who have no active medical problems or a history of:

1. inflammatory bowel disease
2. irritable bowel syndrome
3. colonic polyps
4. bowel cancer
5. any other gastrointestinal disorder
6. obesity
7. high blood pressure
8. diabetes
9. heart disease
10. stroke
11. major depression
12. infection with hepatitis B or C, HIV or syphilis
13. autoimmune disease (e.g., rheumatoid arthritis, SLE).

9.2.2 Physical examination

Cardiovascular and gastrointestinal examination.

Height and weight: BMI <18 and >30 is an exclusion.

9.2.3 Blood testing

- full blood count (anaemia, WCC >12.5 are exclusions)
- electrolytes, urea and creatinine (renal impairment eGFR <60 is an exclusion)
- liver function tests (abnormal LFTs are exclusions)
- human T cell lymphotropic virus 1 and 2 serology (positive serology is an exclusion)
- Epstein Barr virus IgM and IgG (positive IgM is exclusion)
- cytomegalovirus IgM and IgG (positive IgM is exclusion)

- syphilis (positive rapid plasma regain is an exclusion)
- *Strongyloides stercoralis*, *Entamoeba histolytica* (positive serology is an exclusion)
- toxoplasma serology (positive serology is an exclusion)
- hepatitis A virus IgM (positive serology is an exclusion)
- hepatitis B PCR (positive PCR is an exclusion)
- hepatitis C PCR (positive PCR is an exclusion)
- HIV PCR (positive PCR is an exclusion)
- fasting lipids and blood sugar level (total cholesterol >4.0mmol/L, LDL >2.5 mmol/L, triglycerides >2.0mmol/L, HDL <1.0mmol/L are exclusions)
- C-reactive protein (>8 exclusion)

9.2.4 Stool testing

- microscopy and culture
- *Clostridium difficle* toxin PCR
- egg, cysts and parasites (including *Cryptosporidium* spp., *Giardia* spp. and *Entamoeba histolytica* PCR)

10. Stool Collection and Processing

Once donors have passed all the screening requirements they are eligible to donate for 1 month. To donate stool beyond this time will require repeat screening.

10.1 Stool collection

1. Stool collected in sterile blue bags that are placed over the toilet.
2. Stool donor to zip tie blue bag closed and place blue bag in clear zip-lock bag.
3. Stool donor to produce stool at CSIRO or deliver to CSIRO laboratory in esky within 1 hour of defecation.
4. 4–6 stool donors will be asked to provide stool on each collection day.

10.2 Stool processing

10.2.1 Donor stool processing

Setting up

Ensure anaerobic chamber is primed with gas and is anaerobic.

See instructions on setting up anaerobic chamber.

Set-up

Blender case as well as spatulas, glass beaker and glass measuring cylinder to be autoclaved within 24 hours of commencing stool processing (ideally the night prior):

1. weigh stool (empty clear and blue bag weight = 47g)
2. saline (mL) = 2.6 × total stool weight (g).
3. glycerol (mL) = 0.4 × total stool weight (g).
4. sterile 200mL yellow pots (number) = total stool weight/50 (rounded up)
5. transfer these minimum amounts into the anaerobic chamber.

Equipment

Blender (cylinder and base)
Stainless steel spatulas (autoclaved)
Glass beaker (autoclaved)
Glass measuring cylinder (autoclaved)
8 × Eppendorf tubes labelled:

- donor number
- date
- tube number
- F = fresh. G = Glycerol

Note pad, pen and scissors

Scientific weigh scales

Prior to blending

1. Add 0.25g of stool to each of 6 × labelled, capped Eppendorf tubes.
2. Add 5g of stool to 2 × larger brown pots.
3. Record weight of stool in note pad.

Blending process

1. Stool from four donors will be pooled and blended with normal saline and sterile pharmaceutical grade glycerol (in the ratio 25% stool, 65% saline, 10% glycerol).
2. The number of donors to be pooled will be limited to four to reduce the risk of transmissible disease from a single donor.
3. Blend on low power for 20 seconds and then high power for a further 20 seconds.
4. Aliquot the stool suspension into the sterile yellow pots (Colonoscopy, 200mL; Enema, 100mL) and label with batch number and date.
5. Each batch consists of 1 × 200mL pot and 2 × 100mL pot.
6. Each recipient will receive the same batch (same blend of donor stool from single day donation) for each of their three faecal transplants.
7. Multiple such batches can be produced from each donor stool blend.
8. Half fill a further 2 × Eppendorf tubes with blended stool mix.
9. Transfer the stool suspensions and tubes directly into -80 degree freezer.

10.2.2 Documentation and tracing of donors

1. Each stool donor will be recorded in the secure and confidential study "stool donor register" document. This will include:

    i. donor name
    ii. date of birth
    iii. address and contact details
    iv. result of screening history, physical examination and blood and stool tests.

2. Each stool donor will be assigned a donor number.

3. Each stool aliquot will be numbered and recorded in the secure and confidential faecal transplant aliquot document that will list the four stool donors who contributed to each aliquot. In this way any possible transmission of infection can be traced.
4. A small amount of each individual donation will be set aside and frozen individually. This will allow repeat testing and tracing of each individual donation in the future in the event of possible transmission of infection.

10.2.3 Ulcerative colitis patient stool processing

1. Each subject potentially suitable for the study, will also be asked to donate a stool sample of their own.
2. A small portion of the stool will be set aside to undergo faecal-associated microbiota analysis.
3. 50g of the remainder will be mixed with 20mL sterile pharmaceutical grade glycerol and 130mL saline and placed into frozen storage at -80°C. This stool will then be used to transplant those subjects randomised to receive "placebo" with their own stool. In this way the FMT will remain blinded to both the subject and colonoscopist.

10.2.4 Cleaning equipment

Blender case, stainless steel implements and glassware should all be cleaned following stool processing in the order listed below:

1. rinsed with water in the sink
2. washed with detergent and water
3. rinsed with water
4. washed with enzymatic wash
5. rinsed with water
6. autoclaved.

11. Analysis and Reporting of Results

All of the outlined techniques are well established and have been used in previous studies.

Analysis of stool microbiota and microbiota metabolites will mainly be conducted at CSIRO Animal, Food and Health research laboratories in Adelaide under the guidance of Dr Michael Conlon. Some analyses may be outsourced to other laboratories, but under the broad direction of Dr Conlon in consultation with Dr Costello and other collaborators. The abundance and/or activities of faecal and mucosal (biopsy)-associated microbes will be analysed using molecular methods. This is expected to include the use of QPCR for a range of bacterial targets but may also include deep sequencing of microbial DNA for an in-depth analysis of microbial population changes. Isolation (culture) of bacteria from stool samples may be considered to further understanding of metabolic changes occurring in bacteria of IBD patients compared with healthy controls. Stool will be analysed for SCFAs, ammonia, phenols, cresols and bile acids using a range of methods established at CSIRO where sufficient material is available. Other metabolites may also be measured.

Gut mucosal immunological analysis with be performed with Dr Patrick Hughes at the Nerve Gut Research Laboratory.

Blood sampling

A total of 60mL will be taken at each time point and will be used for further experiments outlined below.

Isolation of PBMC and LPMC cells

Peripheral blood mononuclear cells (PBMCs) are isolated from whole blood via density gradient centrifugation. Lamina propria mononuclear cells (LPMCs) are isolated from colonic biopsies via collagenase digestion and density gradient centrifugation. Cells will be stored under liquid nitrogen until further analysed.

PBMCs and biopsy tissue will be used for flow cytometry and cell sorting.

PBMCs and LPMCs are surface stained using monoclonal antibodies against specific immune cell subsets (e.g., T memory cells CD45(RO); T helper cells (CD4); cytoxic T cells (CD8); B cells (CD19); natural killer cells (6B11), monocytes (CD14); and the integrins **a**4, **b**7 and CCR9). PBMCs and LPMCs will be surface stained, permeabilised and stained with anti-cytokine or opioid antibodies to detect intracellular cytokine

content/opioid content (e.g., TNF-**a**, IL-1**b**, **b**-endorphin), as well as transcription factor content (e.g., FOXP3).

12. Statistical Analysis

Patient information will be de-identified and the results of microbiota, immune analysis and clinical scores will be recorded in an Excel spread sheet. This data will then be imported into the R program for statistical analysis. Statistical analysis will be conducted in collaboration with the University of Adelaide department of statistics.

12.1 Primary outcome power analysis

The study is powered to detect a significant difference in the primary outcome of inducing remission at 8 weeks post FMT with 32 patients in each arm. This was calculated using a *Z* test with pooled variance for the difference of two independent proportions. The significance level was set at 5% and the power at 80%. The estimated remission rate in the placebo group was 26.4% and the minimum clinically relevant remission rate we are powered to detect is 60%.

Comparisons between treatment groups of the primary and secondary dichotomous outcomes will be assessed using Fisher's exact tests with an intention to treat analysis.

The placebo remission rate is difficult to predict because of the heterogeneous nature of previous studies that investigated induction of remission in UC. Our placebo remission rate was derived from the active UC trials 1 and 2[45] (ACT-1 and ACT-2). The ACT-1 and ACT-2 trials were randomised, double-blind, placebo-controlled studies that evaluated the efficacy of IV infliximab 5 or 10mg/kg IV infusion for induction and maintenance treatment in adults with UC. The clinical response rate in those patients in the ACT-2 trial who were not steroid dependent was 26.4%. These patients had moderate to severe colitis with a Mayo score of 6 to 12 on enrolment and so had more severe disease on average than our patients. Response was defined as at least a 3-point reduction and 30% reduction in the Mayo score to determine clinical response at week 8. Another trial of patients with mild to moderate ulcerative colitis[46] found a remission rate at 8 weeks with oral mesalamine 2.4g daily of 22%. Many of our patients will be taking an oral aminosalicilate compound and some a concomitant steroid. The remission rate in this case would be expected to be higher than 22%.

12.2. Safety

The analysis of serious adverse effects at week 8 will be by Fischer's exact test. Assessment of treatment on the change in serum creatinine, ALT, ALP, bilirubin and haemoglobin will be assessed using linear mixed effects regression with week 8 values as the outcome. Adverse effects at 1 year will be recorded; however, there will not be a comparator group.

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Appendix 2B: Final Protocol

TITLE: Faecal microbiota transplantation (FMT) for the treatment of active ulcerative colitis (UC)

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1. Objectives of Study

Primary

- To determine whether faecal microbiota transplantation (FMT) improves clinical and inflammatory outcomes in patients with active ulcerative colitis (UC).

Secondary

- To determine whether any clinical change is accompanied by an alteration in the faecal- and/or mucosal-associated microbiome of UC patients prior to and following FMT.
- Assessment of alteration and durability of change in the recipient microbiota after FMT in UC patients.
- To examine the mucosal immune changes induced by FMT and to examine whether they are influenced by changes in microbiome and/or disease activity.
- To examine the durability of clinical response/improvement after initial response to FMT in subjects with active UC.
- To establish patient satisfaction with FMT as a therapy for UC.

2. Background and Significance

UC is a chronic inflammatory bowel disease (IBD) that is characterised by recurring episodes of inflammation primarily involving the mucosal layer and occasionally the submucosa of the colon. Inflammation usually originates in the rectum and progresses in a contiguous fashion proximally. Although the aetiology of UC remains unclear, several factors are believed to play a role in its development and progression, including host genotype, immune disequilibrium and the composition of microbial communities resident in the gastrointestinal tract.

There is strong evidence for the involvement of microbes in the development of UC. IBD is associated with changes in the diversity of the gut microbiota, and although alterations in the abundance of specific bacterial species have often been identified, there remains no specific organism that is reliably associated with the condition[1]. There also appear to be changes in the functional activity of the microbiome, with changes in gene expression as well as protein production in the microbes of patients with IBD[1]. It

is unclear whether the altered microbiota is a result of, or initiates, the inflammatory process in humans. There is some evidence, however, that an altered microbiota develops prior to the onset of colitis in an animal model of interleukin (IL)-10 knockout mice[2].

Intestinal flora and their metabolic products play a critical role in maintaining the health of the colon. Patients who undergo ileostomy and have subsequent diversion of the luminal contents from the colon often develop a "diversion" colitis. The distal colonocytes in this instance are deprived of short-chain fatty acids (SCFAs) such as butyrate, a product of anaerobic bacterial fermentation of undigested dietary carbohydrates[3]. Yet, animal models of IBD also require bacteria within the colon for inflammation to develop[4]. It has been observed that altering the bacterial and nutrient colonic milieu by diverting the faecal stream using ileostomy reduces the recurrence of Crohn's disease in the colon[5]. Further supporting the notion that bacterial antigens contribute to, or drive, the autoimmune injury to the bowel, is evidence that antibiotics have some therapeutic efficacy in UC. In a recent systematic review, antibiotic therapy for UC was significantly associated with remission[6]. Antibiotic therapy gave a statistically significant relative risk reduction for active disease of 0.64 (CI 0.43–0.96). However, while bacteria are necessary to develop IBD, with germ-free animals unable to be induced to develop IBD, bacterial absence (or change in abundance/mix) can also lead to inflammation.

Probiotics have as yet demonstrated only limited therapeutic efficacy in UC[7]. In vitro studies have demonstrated that probiotic bacteria are able to modulate gut immune cells[8,9], while in vivo in a German study, *E. coli* Nissle 1917 was equivalent in efficacy to mesalamine for maintenance of remission of UC[10]. Additionally, a randomised trial of 77 patients with UC found that VSL#3, a cocktail of eight different bacteria, was more effective than placebo in improving symptoms and inducing remission at 12 weeks[11]. For patients with pouchitis, trials with VSL#3 have shown both therapeutic and prophylactic efficacy[12]. The outcomes in probiotic studies, however, have often been inconsistent and modest. This may be due to the variable actions of the different bacterial species that have been tested as well as the general limitations of most probiotic preparations. These probiotics provide a comparatively low number and diversity of bacterial species in comparison with the vast human gut microbiota. For this reason, some probiotic bacterial strains may not be able to compete effectively against

the complex interactions of an established and adapted indigenous gut microbial community[13].

FMT has been described as “the ultimate probiotic” as it provides an entire microbiome to the recipient. This therapy delivers a much greater number and diversity of bacteria than any current commercially available preparation. FMT was first reported in humans by Eiseman et al. in 1958 in the treatment of four patients with pseudomembranous colitis[14]. Three of the four patients were described as terminally or critically ill requiring vasopressor support and all were successfully cured. Over the subsequent years, there have been case reports and case series describing FMT predominantly for *Clostridium difficile* colitis but also for treating IBD, irritable bowel syndrome and constipation[13,15,16]. In the past decade, there has been a heightened interest in the use of this therapy, predominantly driven by increasing rates of recurrent *C. difficile* infection. During this time, *C. difficile* has become more frequent, more severe and more refractory to standard treatment as well as more likely to relapse[17]. Standard treatment with metronidazole or vancomycin alters the normal gut flora that would usually provide colonisation resistance against *C. difficile* infection. For this reason, after successful initial therapy, up to 35% of patients will experience a symptomatic recurrence after ceasing antibiotics[18]. A subset of patients will have multiple recurrences, and subsequent relapses occur in 45–65% of patients who have relapsed one or more times[19,20]. For patients with recurrent *C. difficile* colitis, FMT offers the greatest chance of cure of any therapy, with success in 87–100% of cases[13,21-25]. This impressive success rate is presumably due to the ability of the transplanted bacteria to recolonise/occupy the missing components/niches of the normal intestinal microbiota, thus removing the microbial niche that *C. difficile* would otherwise exploit.

FMT for UC was first reported in the literature by a gastroenterologist, Dr Justin D Bennet, from Kansas City, who described the results of a faecal transplant he received for his own disease[26]. Dr Bennet had continuous active, severe UC for 7 years, confirmed endoscopically and histologically, that was refractory to standard therapy. Dr Bennet described receiving antibiotics to “sterilise” his bowel prior to retention stool enemas. At the time of publication in 1989, he had been symptom and medication free for the first time in 11 years, at 6 months post FMT.

Borody et al. described case reports of six patients (three men and three women aged 25–53 years) with UC for at least 5 years who were treated with FMT[16]. All patients had suffered severe, recurrent symptoms and UC had been confirmed on colonoscopy and histological examination. Faecal flora donors were healthy adults who were extensively screened for parasites and bacterial pathogens. Patients were prepared with oral antibiotics and oral polyethylene glycol lavage.

Faecal suspensions were administered as retention enemas and the process repeated daily for 5 days. By 1 week post FMT, some symptoms of UC had improved. Complete reversal of symptoms was achieved in all patients by 4 months post FMT, by which time all other UC medications had been ceased. At 1 to 13 years post FMT, and without any UC medication, there was no clinical, colonoscopic or histologic evidence of UC in any patient. The authors concluded that colonic infusion of donor human intestinal flora can reverse UC in selected patients, and that these results support the concept of abnormal bowel flora or even a specific, albeit unidentified, bacterial pathogen causing UC. However, caution is needed when interpreting their data as this centre is known to have undertaken a large number of these treatments and it is uncertain why only six are reported. There is no comment in this paper as to the number of patients at their facility in whom this technique was attempted and if there were any patients in whom the treatment failed; moreover, this is open-label treatment, which is now an insufficient standard of proof when evaluating novel therapies. Hence, randomised placebo-controlled trials are needed to rigorously examine the efficacy of this proposed "alternative" therapy.

An anticipated concern in the medical community regarding FMT has been patient acceptance. This has been an assumption based on little evidence. To look at this question of patient willingness to undergo FMT, Kahn et al. performed a qualitative study to explore the attitudes and concerns of patients and parents of children with UC regarding FMT as a potential treatment[27]. They conducted six focus groups at a clinic in Chicago, Illinois and participants were asked about their perceptions of and interest in FMT as a treatment for UC. Sessions were recorded, transcribed and reviewed to identify domains, themes and major concepts. The focus groups included 15 adult patients and seven parents of children with colitis. The study identified five major domains pertaining to FMT: impressions of treatment, benefits, risks, potential mechanisms and social concerns. All but one participant expressed interest in FMT and

several wished it were already available. Participants compared FMT to probiotics, felt it was “natural”, was easier than current therapies and would be safe with donor screening. Although initial distaste and the “yuck factor” were uniformly mentioned, these concerns were outweighed by perceived benefits. The study concluded that given adequate supporting research, donor selection and screening, adult patients and parents of children with UC will consider FMT and are eager for it to become available.

FMT for UC is currently undertaken at a private gastroenterology clinic in Sydney and case reports of success from this clinic are reported in the literature[16]. There is also evidence from UC online forums that patients are conducting FMT for UC outside of the health care setting[28,29]. This is occurring in an unregulated fashion, with only very limited evidence of efficacy from seven case reports in the literature. These occurrences underline the need for more robust scientific evidence in this area and a randomised controlled study of efficacy.

2.1 Standard of care for ulcerative colitis

The management of UC involves both maintenance medication and medication used to control flares of the disease. The goal of maintenance therapy in UC is to maintain steroid-free remission, clinically and endoscopically. This requires regular clinical assessment including history, physical examination and at times colonoscopic examination. Other tools of assessment include blood (e.g., C-reactive protein [CRP] and WCC) and stool (calprotectin) testing for inflammatory markers, and imaging including MRI, CT or ultrasound.

The choice of maintenance treatment in UC is determined by disease extent, disease course (frequency of flares), failure of previous maintenance treatment, severity of the most recent flare, treatment used for inducing remission during the most recent flare, safety of maintenance treatment and cancer prevention. The mainstay of maintenance medication is the 5-aminosalicylic acid (5-ASA) compounds such as mesalazine and sulphasalazine[30,31]. These compounds are commonly taken orally in formulations that predominantly deliver the active 5-ASA component to the colon. Alternatively, or in addition, mesalazine preparations can be delivered topically via enema or suppository if the disease only involves the left side of the colon (although it is only PBS funded for topical therapy during a flare and not for maintenance of remission—even though it also

works in this setting). The majority of patients can be managed with maintenance 5-ASA compounds most of the time. For patients who have repeated flares of disease on 5-ASA maintenance therapy (one or more flares in a year needing steroids), thiopurine medication such as azathioprine or 6-mercapropurine should be used[32]. These medications induce systemic immunosuppression, and reduce the incidence and severity of flares of colitis, but also slightly increase the risk of some infections and malignancy. Anti-TNF agents such as infliximab and adalimumab have been shown to have benefit in maintaining remission in UC[33] (and are licensed for this indication by the TGA); however, these agents are very expensive. The anti-TNF agents also give an increased risk of infection, particularly latent TB reactivation.

Mild flares of UC can be managed with higher doses of oral 5-ASA compounds or the addition of topical 5-ASAs given via enema or suppository. More severe flares are usually managed with a course of systemic corticosteroid. These can be given intravenously in acute, severe disease or orally in less severe flares. The steroids should then be tapered over time and discontinued. There is no indication for long-term steroid use in UC, and prolonged steroid use is associated with a number of complications including infection, osteoporosis, obesity, diabetes, poor wound healing, thinning skin, mood changes and insomnia. Severe flares of UC not responsive to steroids may respond to rescue therapy with the addition of either cyclosporin or anti-TNF therapy.

Patients in whom colonic inflammation cannot be controlled adequately frequently undergo total colectomy. This may be done electively (for refractory disease) or emergently in acute fulminant colitis. Colectomy entails surgical risk that is higher in the emergent setting; this risk includes infection, wound breakdown and mortality. Colectomy is considered “curative” for UC especially if an ileostomy stoma is created; however, it frequently also leads to complications both short- and long-term. In addition, in patients in whom an ileal–anal pouch is fashioned, up to 50% will subsequently develop pouchitis at 4 years post surgery[34].

3. Specific Safety Considerations

A recent review article assessed all cases of FMT in the literature prior to 2011[13]. A total of 239 patients had undergone FMT. The authors did not find any serious adverse events related to the procedure. Some studies reported patient deaths due to the

underlying disease, where the patient has not responded to the FMT. In one study, in which donor faeces were instilled via a nasogastric tube, a patient died of peritonitis. This patient was undergoing peritoneal dialysis for end-stage renal failure at the time and was septic with severe *C. difficile* colitis. Her condition remained unchanged immediately post transplantation; however, on the third day, she developed peritonitis. Although considered more likely the result of peritoneal dialysis, the nasogastric tube insertion could not be discounted to have been contributory[35]. One patient in a study by Silverman et al. developed irritable bowel symptoms following FMT[25].

Following this literature review in 2011, there have been four further cohort studies in the literature of patients who have undergone faecal transplant for *C. difficile* colitis[21,22,36,37]. A total of 216 patients who underwent FMT via colonoscopy were included in these four studies with no immediate adverse effects from FMT noted.

There is a potential to transmit infection via contaminated donor stool. The donor stool will therefore undergo microscopy and culture for potential bacterial pathogens, and microscopy for ova, cysts and parasites, as well as viral studies and *C. difficile* toxin analysis.

Blood testing to exclude HIV, hepatitis B and C, and syphilis will be undertaken.

Changes in faecal microbiota have been found in patients with a number of gastrointestinal and extra-intestinal diseases. Changes in the microbiome of patients with IBD and irritable bowel syndrome are well documented in the literature. There have also been associations between various bowel flora and obesity and the metabolic syndrome[38]. The association has not been documented as causal and it appears probably related to the diet consumed by these subjects. It would, however, be prudent to exclude donors with the metabolic syndrome from the study.

In an audit of 16,318 colonoscopies performed in Northern California from 1994 to 2002, Levin et al. found serious complications occurred in 5.0 of 1000 procedures[39]. The major risk of colonoscopy, bowel perforation, occurred in 0.09% of colonoscopies in that study. Other risks include dehydration from bowel preparation, over-sedation, aspiration, bleeding and splenic laceration. This patient group will however be undergoing regular colonoscopies for their UC and will be familiar with these risks. Risks from standard therapies they may be offered for active disease (e.g., steroids,

immunomodulators and colectomy) are also substantial, thus risks from colonoscopy for FMT are relative.

4. Ethical Considerations

UC is a chronic, debilitating disease with a near-normal life expectancy[40]. Current therapies are inadequate and the disease continues to have an unacceptably high rate of chronic relapsing symptoms. This is underlined by evidence that up to 30% of patients will require colectomy after 25 years of disease[41]. For this reason, it is important for the medical community to rigorously examine potential new therapies that may benefit this group of patients.

A small number of case reports of successful treatment of UC with FMT have been reported in the literature[16,26]. However, the findings of these case reports have never been tested in a randomised controlled trial (RCT). Despite this very limited evidence, there is a clinic in Australia offering UC patients FMT as a therapy[16]. There is also evidence from online forums that patients are undertaking this therapy without medical supervision[28,29]. Despite the minimal evidence in the literature, there is a willingness among sufferers of UC to try this potential therapy[27]. We believe an RCT in this area is necessary to gather evidence for or against the effectiveness of FMT as a treatment for UC. A positive result will avail UC sufferers of a new therapy and a negative one will help discourage the use of an unproven, invasive therapy. Stool analysis of faecal transplant success may also fast-track development of tailored probiotic medicines.

Donors will be anonymous and so will not be known to the recipient. This avoids any apportion of blame towards a known donor should a complication or treatment failure arise during the trial.

Colonoscopy will be used to deliver the initial stool transplantation and to assess the colon during follow-up. This is an invasive procedure that carries some risk. Most of the recent studies of FMT for *C. difficile* have used colonoscopic delivery[13,22,23] as it allows assessment of the underlying disease and allows the donor bacteria to contact the entire colon. Patients with symptomatic UC ordinarily undergo examination with colonoscopy as part of the assessment of disease activity to help guide treatment. The initial colonoscopy in this trial will therefore not be an additional procedure. However,

colonoscopic examinations at week 8 and at 12 months may be additional procedures depending on the state of the patient's disease and symptoms.

The colonoscopic examinations will involve biopsy of the mucosa for analysis of microbiota as well as immune function and histopathology. The majority of these biopsies will be additional to that which the patient would ordinarily receive outside of the trial. These biopsies will be critical to detect any changes in the mucosal-associated microbiota or immune changes associated with the FMT. The risk of biopsy of the mucosa is small with the major risk being bleeding. Biopsies can be safely performed on a single antiplatelet agent[42]. Patients on duel antiplatelet therapy or anticoagulant medication (e.g., warfarin or heparin) will be excluded from the study.

As FMT has only been performed in large numbers in the past decade, there may be unknown long-term risks. However, there have been no reports of major complications of faecal transplant in the literature to date.

Taking blood may cause short-term pain or discomfort and patients will be informed about this before entering the trial. The volume of blood taken is not extreme and will not cause side effects. If patients are of the view that blood sampling is too painful they may withdraw from the study at any time. Blood tests as well as answering questionnaires will involve an increased time burden and patients will be informed about this before the trial begins. It is not anticipated that the FMT procedure will cause any adverse reactions, but participants will be provided with information about supports they can contact should they experience any distress in relation to the study.

Before taking part in the study, informed written consent will be obtained from patients. The researchers will ensure that the patient is given full and adequate verbal and written information about the nature, purpose, possible risk and benefit of the trial. They will be given sufficient time to consider the information, to ask questions and to seek advice prior to being asked whether they wish to participate in the study. Participants will also be assured their participation in the trial is absolutely voluntary. All treatment decisions are at the discretion of the usual treating physician, and will not be altered by the trial. The participation is strictly confidential, and the identity of subjects will not be disclosed to other medical or research staff unless subjects agree.

Once subjects have been enrolled in this study, they will be given a study participant code, and only study investigators will have access to their name and personal details. We intend to summarise the results in a manuscript and to submit it for publication in a peer reviewed journal. Therefore, all information gathered from this study will be published in a form that does not allow patient identification. We will not provide any feedback with regard to individual microbiota composition or immunologic function.

Our proposed study has the support of the director of the Royal Adelaide Hospital Department of Gastroenterology and Hepatology, Richard Holloway, as well as the head of Endoscopic Services, Mark Schoeman. The head of the IBD Service, Jane Andrews, will be the lead supervisor of the study. The Royal Adelaide Hospital has a large cohort of approximately 800 patients with IBD and a strong record of successful clinical research. The proposed study also has the support of the head of gastroenterology at the Queen Elizabeth Hospital, Ian Roberts-Thomson. The Queen Elizabeth Hospital has a cohort of approximately 300 patients with IBD. The study supervisors all have extensive experience in medical research as well as experience in supervising PhD students.

The Commonwealth Scientific and Industrial Research Organisation (CSIRO) in Adelaide has broad experience in human gut flora and microbial analysis. This is an area of focus for its recent research. The Nerve Gut Research Laboratory at the Royal Adelaide Hospital is a leader in the field of research into the neuro-immunological and neuro-endocrine processes of the human gut.

5. Study Design

This study is an 8-week randomised placebo-controlled trial with a 44-week open-label extension.

Randomisation

Patients will be randomly allocated into the following groups:

- Group 1: Patients receive previously frozen pooled **donor** stool via colonoscopic insertion into the right colon.

- Group 2: Patients receive previously frozen **own** stool via colonoscopic insertion into the right colon.

Open-label therapy from 8 weeks

Patients who are randomly assigned to the placebo group by week 8 will then be offered active donor FMT at the week 8 colonoscopy. The FMT will be conducted in an identical manner to Group 1 with FMT followed by two enemas on day 3 or 4 and one on day 6 or 7.

5.1 Recruitment

Patients will be recruited from IBD clinics at the Royal Adelaide and Queen Elizabeth Hospitals. Patients on the mailing list for the Royal Adelaide Hospital will be contacted about the trial through the quarterly newsletter. Gastroenterologists in Adelaide will be informed about the trial though a presentation at the South Australian Gut Club and an email to the South Australian Gut Club members. The trial will be listed on the Australian and New Zealand clinical trials registry as well as the Gastroenterology Society of Australia website.

We will enrol 70 patients with 35 patients in each arm of the trial.

5.2 Inclusion criteria

Patients with the following characteristics will be included:

1. mild to moderate active UC (total Mayo score 3 to 10)
2. endoscopic subscore of 2 or greater (to ensure symptoms are due to UC, not post-inflammatory irritable bowel syndrome)
3. patients aged 18 to 75 years with established diagnosis of UC.

5.3 Exclusion criteria

Patients with any of the following characteristics will be excluded:

1. severe UC (Mayo score 11–12 or Truelove and Witts criteria)
2. more than 25mg of prednisolone per day (or equivalent steroid)
3. previous colonic surgery

4. active gastrointestinal infection
5. pregnancy
6. anticoagulant therapy or duel antiplatelet therapy (e.g., aspirin and clopidogrel)
7. current use of antibiotics.

Activity of disease will be defined by the Mayo score. This scoring system has 3 points each for stool frequency, rectal bleeding, endoscopic findings and physician's global assessment, giving a total score out of 12.

A score of:

- 0, 1 or 2 indicates inactive disease and exclusion from the trial
- 3 to 10 allows inclusion in the trial. Subjects will need an endoscopic subscore of at least 2 for inclusion to prove active disease. (0 = normal mucosa and 1 = erythema only—most studies start with 2)
- 11–12 indicates severe disease and these patients will be excluded from the trial.

Similarly, any patient who fulfils Truelove and Witts criteria for severe colitis will be excluded while they meet these criteria. Truelove and Witts is defined as >6 bloody bowel motions per day plus one or more of the following: haemoglobin <10.5g/dL, ESR >30mm/hr, pulse rate >90 beats per minute and temperature over 37.5°C.

5.4 Medication prior to enrolment

Stable dosing of UC maintenance therapy is required prior to enrolment:

1. 5-ASA stable dosing for at least 4 weeks
2. thiopurines and methotrexate stable dosing for at least 6 weeks
3. biological agents stable dosing for at least 8 weeks
4. option to enrol on an oral dose of prednisolone ≤25mg, with a mandatory taper of 5mg per week.

6. Outcome Measures

6.1 Primary outcome

The primary outcome will be steroid-free remission of UC at week 8 defined as:

1. total Mayo score of ≤2 *AND*
2. Mayo endoscopic score of ≤1.

6.2 Secondary outcomes

Secondary outcomes will comprise:

1. clinical response (≥3 point reduction in total Mayo score at week 8 and 1 year)
2. clinical remission (Simple Clinical Colitis Activity Index (SCCAI) ≤2 at week 8 and 1 year)[43]
3. endoscopic remission (Mayo <1 at week 8 and 1 year)
4. safety (assessed at week 8 and 1 year)
5. changes in mucosal- and faecal-associated microbiota following FMT assessed by 16S ribosomal RNA sequencing, stratified by:

    i. change in total Mayo score following FMT
    ii. randomisation
    iii. durability of engraftment of donor microbiome following FMT
    iv. changes in peripheral blood and colonic lamina propria mononuclear cell populations (assessed by FACS) following FMT
    v. patient perception and palatability.

Disease activity measures of symptoms score (SCCAI), endoscopic and histologic grading, and records of hospitalisation, corticosteroid requirement, periods of symptom flares and colectomy rate will be recorded at the 1-year mark as part of the open-label observation period from 8 weeks to 1 year.

7. Patient Participation

7.1 Recruitment

Participants will be recruited from:

- Royal Adelaide Hospital (RAH), The Queen Elizabeth Hospital (TQEH) and Fiona Stanley Hosptial (FSH) Gastroenterology IBD databases and newsletters
- gastroenterology in- or out-patient encounter(s) at the RAH, TQEH and FSH by referral from their clinicians and by searching OPD letters.

Patients on clinical databases who have previously consented to being contacted regarding research studies will receive information about the study in the RAH IBD Service regular newsletter and may also be contacted by telephone, and if no answer is obtained a letter will be sent. All other patients will be contacted via a letter or by their treating clinician in whichever way the clinician feels is most appropriate to the particular patient.

Regarding the use of letters for contact, subjects who have already consented to be contacted regarding research (on database) will receive a letter signed by A/Prof Andrews (at RAH), Prof Roberts-Thomson (at TQEH) or Dr Waters (at FSH) on behalf of the study investigators.

The initial invitation letter will include an opt-out slip for subjects not wishing to be contacted further. Subjects not opting out or responding within 4 weeks after invitation will be contacted up to a further three times by two different methods (phone, SMS, email or letter) to ascertain whether they wish to participate or not. Demographic details of non-responders will be recorded to enable a full description of the sources of possible bias. All who agree to participate will be subsequently screened to ensure they fulfil inclusion criteria.

Donors will be recruited with a flyer advertisement on notice boards at Adelaide University Medical School and Adelaide University campus.

7.2 Withdrawal criteria

Patients may withdraw from the study at any time. We will ask for their reasons for statistical purposes; however, they will not be obliged to provide this information. Withdrawal from the study will not affect ongoing standard medical care in any way. Their clinicians will be informed of their participation in the study. We will ask patients to notify us of any changes in their treatment during the course of the study and, if necessary, we will seek their permission to verify this with their treating clinician.

8. Ulcerative Colitis Patient Assessment

8.1 The week prior to enrolment

1. The patient should have the opportunity to read the patient information sheet, discuss the trial with family or friends and ask questions of the investigators prior to signing trial consent.

2. Patient questionnaire regarding perception and expectation of faecal transplant prior to procedure.

3. Detailed history of UC:

- date of diagnosis
- extent of disease
- medication use—current and prior
- previous surgery
- previous hospitalisation
- comorbid disease
- current symptoms
- extra-articular manifestations.

4. Stool collected for:

- infection screen: microscopy culture + sensitivity, *Clostridium difficile* toxin (5g)
- possible re-administration for placebo arm subjects (50g)
- microbiome analysis: 6 × 0.25g stool in Eppendorf tubes, 2 × 5g stool in larger brown stool pots.

For collection and processing methods see section 10.

5. Disease activity assessment:

- faecal calprotectin
- CRP, ESR, FBC, U+E, LFTs
- symptom severity (SCCAI) at screening and one day prior to FMT

- flexible sigmoidoscopy:

    i. total Mayo score
    ii. disease extent (≥10cm of disease required)
    iii. biopsy for light microscopy and histopathology to exclude CMV inclusions.

8.2 Randomisation

Randomisation will be conducted once the patient satisfies the inclusion and exclusion criteria and has consented to enter the study. This should occur within 1–7 days prior to the first faecal transplant, which will be delivered via colonoscopy.

Prior to randomisation, three aliquots of pooled donor stool suspension from a single batch and three aliquots of the donor's own stool suspension will each be placed in clear plastic bags in the -80°C freezer at the endoscopy unit. All stool aliquots will be in identical yellow-topped 250mL cryo-safe containers. These will include 1 × 200mL suspension for colonoscopic delivery and 2 × 100mL suspensions for enema delivery.

Donor stool pots will be labelled on the lid with:

1. batch number
2. date of manufacture of batch.

Patient's own stool will be labelled on the lid with:

1. patient ID consisting of initials and study number (e.g., AB-1)
2. date of patient stool donation.

Randomisation to be conducted by hospital clinical trial nursing staff using www.random.org.

1. Cardboard circular caps with patient ID and either "Transplant" or "Save" and either "Colon" or "Enema" are then placed on the pots containing donor or patient's own stool depending on randomisation.
2. "Transplant" caps are placed on the pots to be given at and in the week following the first colonoscopy.

3. “Save” pots will be saved and the cap removed following the 8 week colonoscopy. If these contain donor stool, they will be given to the patient at the 8 week colonoscopy and in the subsequent week.
4. “Colon” pots will be delivered at colonoscopy and the “Enema” pots delivered via enema in the following week.
5. The randomisation document for use by study nursing staff is listed on the next page.

Faecal transplant for active ulcerative colitis trial

Protocol for randomisation of FMT

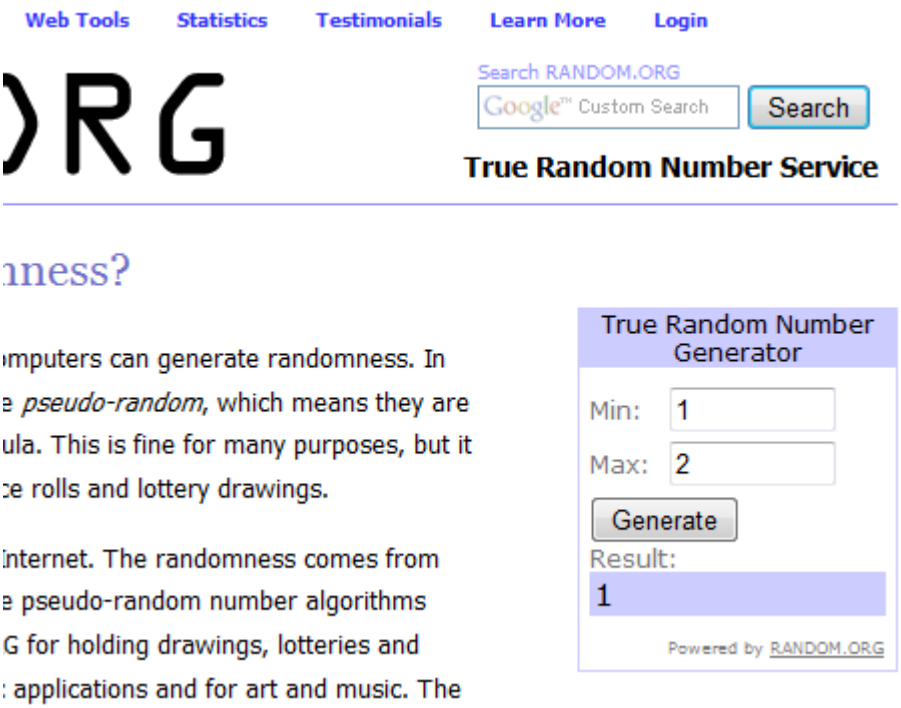
1–7 days prior to faecal transplant use the random number generator http://www.random.org/

Into the "true random number generator" box on the right of the screen set the minimum to 1 and maximum to 2

Select: Generate

1 = Donor faecal transplant

2 = Placebo faecal transplant (patient's own stool)



Go to the -80°C Freezer.

1. For Donor Faecal transplant stick the "Transplant" disks on the top of the donor stool yellow pots and the "Save" disks on the placebo pots. Record the batch number in the transplant record book.
2. For Placebo Faecal transplant stick the "Save" disks on the donor stool yellow pots and the "Save" disks on the donor pots. The donor stool will then be saved to transplant at 8 weeks at the open-label crossover.

The **donor stool** pots are labelled with a Batch number and date (e.g., Batch 3, 2/7/13).

The **patient's own stool** (placebo) is labelled with patient study number.

8.3 Week 1 of trial

8.3.1 Day prior to colonoscopy

1. Patient to take a light breakfast and then to fast from solids.
2. Maintain high fluid intake throughout the day.
3. Take three sachets of Colonlytely bowel preparation (polyethylene glycol) in 3L of water.

8.3.2 Morning of colonoscopy

1. Randomised faecal aliquot labelled “Transplant” and “Colon” to be removed from the -80°C freezer and thawed at room temperature for 3.5 hours prior to delivery.
2. Patient to receive loperamide 2mg orally prior to colonoscopy.
3. SCCAI score diary to be collected.
4. Mayo symptom scores to be taken (endoscopic score to be added at colonoscopy).
5. Biopsy posts should be pre-labelled with the site and number of biopsies required.
6. Consent should be obtained for this procedure on a standard CALHN consent form in addition to the study consent form that has previously been signed.
7. While inserting cannula, take 60mL of blood:

    i. 50mL into 6 × heparin tubes (green and black top) for peripheral blood mononuclear cell flow cytometry (to be taken to Dr Hughes at Nerve Gut Laboratory)
    ii. 5mL into a small EDTA (purple tube) (to be sent to clinical laboratory)
    iii. 5mL into a GEL (white top) for electrolytes and liver function, C-reactive protein (to be sent to SA pathology laboratory).

8.3.3 At colonoscopy

1. Assess disease severity (using endoscopic Mayo score at point of maximum inflammation) and disease extent.
2. Biopsies should be taken on colonoscope insertion*:

i. left-sided biopsies: 11 total; 6 in RPMI media, 2 into RNA later (microbiome), 1 RNA later (PCR), 1 formalin (histopath), 1 PFA (IHC).
ii. right-sided biopsies: 9 biopsies; 6 in RPMI media, 2 into RNA later (microbiome), 1 formalin (histopath).

3. An attempt should be made to remove any residual fluid or faecal material during colonoscope insertion with suction and washing if required.
4. Once at caecum, patient should be rolled onto the right lateral position and randomised faecal suspension delivered into the right colon. If caecum cannot be reached, then delivery of faecal suspension into the right colon beyond the hepatic flexure is acceptable.
5. Patient should then remain on their right side for 1 hour following procedure.
6. Following 1 hour, the patient should be assessed for any adverse effects and if well sat up and offered food and drink prior to discharge.

*Biopsies at each colonoscopy in more detail:

- 2 biopsies will be taken from both the recto-sigmoid region and ascending colon-caecal region of the colon for microbiota analysis. Each in 2.5mL RNA later.
- 1 biopsy from each region will be taken for histopathology analysis into formalin.
- 1 biopsy from left colon for immunohistochemistry (formalin) and 1 from the left colon for PCR (cytokines, transcription factors) (RNA later).
- 4 biopsies will be taken from the left and 4 from the right colon for flow cytometry (FACS) analysis (RPMI complete media), processed the same day as colonoscopy.
- 2 biopsies from the left colon and 2 from the right for supernatant release for cytokines/mast cell mediators (RPMI complete media).
- This will amount to 9 biopsies in the right colon and 11 in the left colon.

8.3.4 Enemas

Two enemas of 100mL faecal suspension will be delivered by a gastroenterologist at the clinic in the week following colonoscopy (days 2–4, days 5–7).

Patient should:

1. take 2mg of loperamide prior to enema
2. lay on left lateral position for enema insertion
3. roll from the left lateral to prone position then right lateral and then back to left lateral position following enema insertion. This is to encourage proximal distribution of the enema
4. attempt to hold the enema for 1 hour.

8.4 Week 4 assessment

1. Stool collection for faecal calprotectin level and microbiome analysis.
2. Stool to be collected in sterile blue bags that are placed over the toilet.
3. Patient to zip tie blue bag closed and place blue bag in clear zip-lock bag.
4. Deliver to CSIRO laboratory within 1 hour.
5. Bag to be opened and stool processed under anaerobic conditions in anaerobic chamber:

    i. microbiome analysis: 6 × 0.25g stool in Eppendorf tubes, 2 × 5g stool in larger brown stool pots.

8.5 Week 8 assessment

8.5.1 Two days prior to colonoscopy

1. Stool collection for faecal calprotectin level and microbiome analysis.
2. Stool to be collected in sterile blue bags that are placed over the toilet.
3. Patient to zip tie blue bag closed and place blue bag in clear zip-lock bag.
4. Deliver to CSIRO laboratory within 1 hour.
5. Bag to be opened and stool processed under anaerobic conditions in anaerobic chamber:

    i. microbiome analysis: 6 × 0.25g stool in Eppendorf tubes, 2 × 5g stool in larger brown stool pots.

8.5.2 One day prior to colonoscopy

1. Patient to take a light breakfast and then to fast from solids.

2. Maintain high fluid intake throughout the day.
3. Take three sachets of Colonlytely bowel preparation (polyethylene glycol) in 3L of water.

8.5.3 Morning of colonoscopy

1. Randomised faecal aliquot labelled “Save” and “Colon” to be removed from the -80°C freezer and thawed at room temperature for 3.5 hours prior to delivery.
2. Patient to receive loperamide 2mg orally prior to colonoscopy.
3. SCCAI score diary to be collected.
4. Mayo symptom scores to be taken (endoscopic score to be added at colonoscopy).
5. Adverse events since randomisation recorded.
6. Biopsy pots should be pre-labelled with the site and number of biopsies required.
7. Consent should be obtained for this procedure on a standard CALHN consent form in addition to the study consent form that has previously been signed.
8. While inserting cannula, take 60mL of blood:

    i. 50mL into 6 × heparin tubes (green and black top) for peripheral blood mononuclear cell flow cytometry (to be taken to Dr Hughes at Nerve Gut Laboratory)
    ii. 5mL into a small EDTA (purple tube) (to be sent to clinical laboratory)
    iii. 5mL into a GEL (white top) for electrolytes and liver function, C-reactive protein (to be sent to clinical laboratory).

8.5.4 At colonoscopy

1. Assess disease severity using endoscopic Mayo score at point of maximum inflammation and disease extent.
2. Biopsies should be taken on colonoscope insertion*.
3. Left-sided biopsies: 11 total; 6 in RPMI media, 2 into RNA later (microbiome), 1 RNA later (PCR), 1 formalin (histopath), 1 PFA (IHC).
4. Right-sided biopsies: 9 biopsies; 6 in RPMI media, 2 into RNA later (microbiome), 1 formalin (histopath).

5. An attempt should be made to remove any residual fluid or faecal material during colonoscope insertion with suction and washing if required.
6. Once at caecum (and disease severity has been assessed and recorded), the cardboard "Save" cap should be removed from the pot to reveal the contents of the faecal pot:

    i. If this is labelled as the patient's own stool, it should be discarded and the colonoscope withdrawn.
    ii. If this is labelled as donor stool, then the patient should be rolled onto the right lateral position and the un-blinded faecal suspension delivered into the right colon.

7. If caecum cannot be reached then delivery of faecal suspension into the right colon beyond the hepatic flexure is acceptable.
8. Patient should then remain on their right side for 1 hour following procedure.
9. Following 1 hour the patient should be assessed for any adverse effects and offered food and drink prior to discharge.
10. Patient to be informed about randomisation. If they were initially randomised to placebo/autologous FMT then they will require two further donor FMTs via enema.

*Biopsies at each colonoscopy in more detail:

- 2 biopsies will be taken from both the recto-sigmoid region and ascending colon-caecal region of the colon for microbiota analysis. Each in 2.5mL RNA later.
- 1 biopsy from each region will be taken for histopathology analysis into formalin.
- 1 biopsy from left colon for immunohistochemistry (formalin) and 1 from the left colon for PCR (cytokines, transcription factors) (RNA later).
- 4 biopsies will be taken from the left and 4 from the right colon for flow cytometry (FACS) analysis (RPMI complete media), processed the day of colonoscopy.
- 2 biopsies from the left colon and 2 from the right for supernatant release for cytokines/mast cell mediators (RPMI complete media).
- This will amount to 9 biopsies in the right colon and 11 in the left colon.

8.5.5 Enemas (patients randomised to placebo/autologous FMT)

Two enemas of 100mL faecal suspension will be delivered by a medical practitioner at the clinic in the week following colonoscopy (days 2–4, days 5–7).

1. Patient to take 2mg of loperamide prior to enema.
2. Lay on left lateral position for enema insertion.
3. Roll into prone positions, right lateral and then back to left lateral position following enema insertion.
4. Patient should attempt to hold the enema for 1 hour.

8.6 One-year assessment

Patient will be posted or emailed:

1. SCCAI symptoms score
2. patient questionnaire regarding experience of faecal transplant prior to procedure and adverse events
3. invitation to undergo disease activity assessment.

Patients who do not return forms within 2 weeks will be contacted via telephone.

8.6.1 Two days prior to flexible sigmoidoscopy

1. Stool collection for faecal calprotectin level and microbiome analysis.
2. Stool to be collected in sterile blue bags that are placed over the toilet.
3. Patient to zip tie blue bag closed and place blue bag in clear zip-lock bag.
4. Deliver to CSIRO laboratory within 1 hour.
5. Bag to be opened and stool processed under anaerobic conditions in anaerobic chamber:

    i. microbiome analysis: 6 × 0.25g stool in Eppindorf tubes; 2 × 5g stool in larger brown stool pots
    ii. faecal calprotectin.

8.6.2 Flexible sigmoidoscopy

1. Mayo symptom scores to be taken (endoscopic score to be added at colonoscopy).
2. Adverse events since randomisation recorded.
3. Biopsy pots should be pre-labelled with the site and number of biopsies required.
4. Consent should be obtained for this procedure on a standard consent form in addition to the study consent form that has previously been signed.
5. While inserting cannula, take 60mL of blood:

    i. 50 mL into 6 × heparin tubes (green and black top) for peripheral blood mononuclear cell flow cytometry (to be taken to Dr Hughes at Nerve Gut Laboratory)
    ii. 5mL into a small EDTA (purple tube) (to be sent to clinical laboratory)
    iii. 5mL into a GEL (white top) for electrolytes and liver function, C-reactive protein (to be sent to clinical laboratory).

6. Assess disease severity using endoscopic Mayo score at point of maximum inflammation.
7. Biopsies should be taken on the left side only.
8. Left-sided biopsies: 11 total; 6 in RPMI media, 2 into RNA later (microbiome), 1 RNA later (PCR), 1 formalin (histopath), 1 PFA (IHC).
9. Following 1 hour the patient should be assessed for any adverse effects and offered food and drink prior to discharge.

8.6.3 Care during the follow-up period

During the trial, subjects will be treated to the standard of care for UC. This involves a fixed maintenance medication as prescribed/advised by their own physician. Patients will enter this trial because of a flare, and all therapy they are on at entry will be continued except for the steroid taper as described.

A subject who experiences a flare of their disease during the study will be treated with standard therapy as if they were not in the study. This will include increasing their oral 5-ASA and/or adding a topical enema or suppository therapy. Systemic steroid therapy

may also be used. Steroid use will be quantified during the study and steroid requirement over the 12-month period will be another secondary endpoint. Once patients are commenced on steroid, it will be tapered as explained above.

If a subject deteriorates on steroid therapy, they may require escalation of their medical therapy or surgery. Escalation of medical therapy may involve increasing the steroid dose temporarily. Patients who are naïve to thiopurine therapy may benefit from the addition of a thiopurine. Thiopurines can take up to 12 weeks to reach their therapeutic effect and so "rescue therapy" may be needed in the intervening period. Rescue therapy involves the addition of cyclosporine or an anti-TNF agent such as infliximab (if available through compassionate access) in the short term. Rescue therapy will be continued for 6 to 12 weeks to allow the thiopurine medication to reach its full effect.

Patients who have a severe flare of UC that does not respond to intravenous steroid medication within 3 to 5 days are unlikely to improve and should be assessed for surgical colectomy[44], as would be the case in routine care.

9. Stool Donor Recruitment and Screening

9.1 Donor recruitment

Posters will be placed on noticeboards on the University of Adelaide campus. These will detail that we are recruiting stool donors and the posters will have the contact details of Dr Costello and Dr Andrews.

9.2 Donor screening

Potential donors will be sent the donor information sheet via email or post.

Donors who consent will undergo a four-stage screening process with medical history, physical examination, blood testing and stool testing with the aim of reducing the risk of disease transmission from donor to recipient.

9.2.1 Medical history

Exclusion of patients with:

- age: <18 or >65

- antimicrobial therapy or probiotics in the past 3 months
- active medical illness or symptoms
- any medications (other than oral contraceptive pill)
- international travel in last 6 months to areas at high risk of traveller's diarrhoea
- high-risk sexual activity (unprotected sex in last 1 month outside of a monogamous relationship, men who have sex with men, sex for drugs or money)
- illicit drug use
- tattoo or body piercing within 6 months
- known HIV or viral hepatitis exposure in the last 12 months
- incarceration or a history of incarceration
- family history of colorectal carcinoma involving two or more first-degree relatives
- household members with active gastrointestinal infection.

Inclusion of patients who have no active medical problems or a history of:

- inflammatory bowel disease
- irritable bowel syndrome
- colonic polyps
- bowel cancer
- any other gastrointestinal disorder
- obesity
- high blood pressure
- diabetes
- heart disease
- stroke
- major depression
- infection with hepatitis B or C, HIV or syphilis
- autoimmune disease (e.g., rheumatoid arthritis, SLE).

9.2.2 Physical examination

Cardiovascular and gastrointestinal examination.

Height and weight: BMI <18 and >30 is an exclusion.

9.2.3 Blood testing

- full blood count (anaemia, WCC >12.5 are exclusions)
- electrolytes, urea and creatinine (renal impairment eGFR <60 is an exclusion)
- liver function tests (abnormal LFTs are exclusions)
- human T cell lymphotropic virus 1 and 2 serology (positive serology is an exclusion)
- Epstein Barr virus IgM and IgG (positive IgM is exclusion)
- cytomegalovirus IgM and IgG (positive IgM is exclusion)
- syphilis (positive rapid plasma regain is an exclusion)
- *Strongyloides stercoralis*, *Entamoeba histolytica* (positive serology is an exclusion)
- toxoplasma serology (positive serology is an exclusion)
- hepatitis A virus IgM (positive serology is an exclusion)
- hepatitis B PCR (positive PCR is an exclusion)
- hepatitis C PCR (positive PCR is an exclusion)
- HIV PCR (positive PCR is an exclusion)
- fasting lipids and blood sugar level (total cholesterol >4.0mmol/L, LDL >2.5 mmol/L, triglycerides >2.0mmol/L, HDL <1.0mmol/L are exclusions)
- C-reactive protein (>8 exclusion)
- ANA (>1/160 is an exclusion)
- helicobacter serology (positive serology is an exclusion)

9.2.4 Stool testing

- microscopy and culture
- *Clostridium difficle* toxin PCR
- egg, cysts and parasites (including *Cryptosporidium* spp., *Giardia* spp. and *Entamoeba histolytica* PCR)
- rotavirus, norovirus and adenovirus PCR

10. Stool Collection and Processing

Once donors have passed all the screening requirements they are eligible to donate for 1 month. To donate stool beyond this time will require repeat screening.

10.1 Stool collection

1. Stool collected in sterile blue bags that are placed over the toilet.
2. Stool donor to zip tie blue bag closed and place blue bag in clear zip-lock bag.
3. Stool donor to produce stool at CSIRO or deliver to CSIRO laboratory in esky within 1 hour of defecation.
4. 4–6 stool donors will be asked to provide stool on each collection day.

10.2 Stool processing

10.2.1 Donor stool processing

Setting up

Ensure anaerobic chamber is primed with gas and is anaerobic.

See instructions on setting up anaerobic chamber.

Set-up

Blender case as well as spatulas, glass beaker and glass measuring cylinder to be autoclaved within 24 hours of commencing stool processing (ideally the night prior):

1. weigh stool (empty clear and blue bag weight = 47g)
2. saline (mL) = 2.6 × total stool weight (g)
3. glycerol (mL) = 0.4 × total stool weight (g)
4. sterile 200mL yellow pots (number) = total stool weight/50 (rounded up)
5. transfer these minimum amounts into the anaerobic chamber.

Equipment

Blender (cylinder and base)
Stainless steel spatulas (autoclaved)
Glass beaker (autoclaved)
Glass measuring cylinder (autoclaved)
8 × Eppendorf tubes labelled:

- donor number
- date

- tube number
- F = fresh. G = Glycerol

Note pad, pen and scissors

Scientific weigh scales

Prior to blending

1. Add 0.25g of stool to each of 6 labelled, capped Eppendorf tubes.
2. Add 5g of stool to 2 × larger brown pots.
3. Record weight of stool in note pad.

Blending process

1. Stool from four donors will be pooled and blended with normal saline and sterile pharmaceutical grade glycerol (in the ratio 25% stool, 65% saline, 10% glycerol).
2. The number of donors to be pooled will be limited to four to reduce the risk of transmissible disease from a single donor.
3. Blend on low power for 20 seconds and then high power for a further 20 seconds.
4. Aliquot the stool suspension into the sterile yellow pots (Colonoscopy, 200mL; Enema, 100mL) and label with batch number and date.
5. Each batch consists of 1 × 200mL pot and 2 × 100mL pot.
6. Each recipient will receive the same batch (same blend of donor stool from single day donation) for each of their three faecal transplants.
7. Multiple such batches can be produced from each donor stool blend.
8. Half fill a further 2 × Eppendorf tubes with blended stool mix.
9. Transfer the stool suspensions and tubes directly into -80 degree freezer.

10.2.2 Documentation and tracing of donors

1. Each stool donor will be recorded in the secure and confidential study “stool donor register” document. This will include:

    i. donor’s name

ii. date of birth
iii. address and contact details
iv. result of screening history, physical examination, and blood and stool tests.

2. Each stool donor will be assigned a donor number.
3. Each stool aliquot will be numbered and recorded in the secure and confidential faecal transplant aliquot document that will list the four stool donors who contributed to each aliquot. In this way any possible transmission of infection can be traced.
4. A small amount of each individual donation will be set aside and frozen individually. This will allow repeat testing and tracing of each individual donation in the future in the event of possible transmission of infection.

10.2.3 Ulcerative colitis patient stool processing

1. Each subject potentially suitable for the study will also be asked to donate a stool sample of their own.
2. A small portion of the stool will be set aside to undergo faecal-associated microbiota analysis.
3. 50g of the remainder will be mixed with 20mL sterile pharmaceutical grade glycerol and 130mL saline and placed into frozen storage at -80°C. This stool will then be used to transplant those subjects randomised to receive "placebo" with their own stool. In this way the FMT will remain blinded to both the subject and colonoscopist.

10.2.4 Cleaning equipment

Blender case, stainless steel implements and glassware should all be cleaned following stool processing in the order listed below:

1. rinsed with water in the sink
2. washed with detergent and water
3. rinsed with water
4. washed with enzymatic wash
5. rinsed with water

6. autoclaved.

11. Analysis and Reporting of Results

All of the outlined techniques are well established and have been used in previous studies.

Analysis of stool microbiota and microbiota metabolites will mainly be conducted at CSIRO Animal, Food and Health research laboratories in Adelaide under the guidance of Dr Michael Conlon. Some analyses may be outsourced to other laboratories, but under the broad direction of Dr Conlon in consultation with Dr Costello and other collaborators. The abundance and/or activities of faecal and mucosal (biopsy)-associated microbes will be analysed using molecular methods. This will include the use of 16S ribosomal RNA sequencing. Isolation (culture) of bacteria from stool samples may be considered to further understanding of metabolic changes occurring in bacteria of IBD patients compared with healthy controls. Stool will be analysed for SCFAs using a range of methods established at CSIRO, where sufficient material is available. Other metabolites may also be measured.

11.1 Bacterial analysis

Bacterial DNA will be extracted from the samples using the MoBio PowerMag Microbial DNA Isolation kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's protocol. All stool samples will be extracted and processed in duplicate. Amplicon library preparation will be performed using a modified dual-index PCR approach. The V4-V5 hypervariable region of the 16S rRNA gene will be amplified using first-step primers (515F, 806R), modified by the inclusion of a phaser, and the indexed barcodes added to the second set (i5, i7) enable multiplexing of the large number of samples[45]. The library will be pooled at equi-molar concentrations and run on an Illumina HiSeq2500 Rapid instrument using 2 × 250 base pair paired-end chemistry (Ramaciotti Centre for Genomics, University of New South Wales).

11.2 Bioinformatics

Raw sequencing data will be processed using a combination of both in-house and open-source software. The bioinformatic pipeline will utilise USEARCH algorithms[46], which

include merging, quality-filtering, partitioning/de-replicating and clustering into operational taxonomic units (OTUs) at 97% similarity. Representative sequences from each OTU will be classified in two ways: via the RDP Naïve Bayesian Classifier and by finding the closest match in a set of curated reference sequences (RDP 16S Training Set + RefSeq 16S)[47]. The use of two independent classification techniques improves confidence in the taxonomic assignments.

11.3 Immunological analysis via flow cytometry

Gut mucosal immunological analysis with be performed with Dr Patrick Hughes at the Nerve Gut Research Laboratory.

Blood sampling

A total of 60mL will be taken at each time point, and will be used for further experiments outlined below.

Isolation of PBMC and LPMC cells

Peripheral blood mononuclear cells (PBMCs) are isolated from whole blood via density gradient centrifugation. Lamina propria mononuclear cells (LPMCs) are isolated from colonic biopsies via collagenase digestion and density gradient centrifugation. Cells will be stored under liquid nitrogen until further analysed.

PBMC cells and biopsy tissue will be used for the following.

LPMC isolation: Colonic mucosal biopsies will be incubated twice in Hepes buffered HBSS supplemented with 1mM EDTA and 1mM DTT (Sigma) for 10min at 37°C under slow rotation, with the suspension strained (100µM) between incubations. Residual tissue will be incubated in Hepes buffered Ca^{2+}/Mg^{2+} free HBSS for 10min at 37°C under slow rotation and strained (100µM). Residual tissue will be minced and incubated in complete media (RPMI 1640 [Gibco, Germany] supplemented with foetal calf serum, glutamax and penicillin/streptomycin, Collagenase D [1mg/mL; Roche, NSW, Australia], DNAse1 [0.5mg/mL; Sigma] and Dispase [3mg/mL; Roche]) for 20min twice, with supernatant removal from centrifugation (300*g*, 5min) after each incubation. Residual suspensions will be sequentially strained (100µM followed by

40μM), with the supernatant centrifuged (300*g*, 5min), resuspended and stained with trypan blue to determine viability and cell number as previously described[48-50].

Cell staining: 0.5 × 10^6 F_c blocked (BD Biosciences, NSW, Australia) cells will be stained for viability (FVD eFlour450, eBioscience) and the following anti-human monoclonal antibody panels (BD Bioscience unless otherwise stated): (a) HLADR-APC, CD11C-FITC, Lin (CD3, CD14, CD16, CD19, CD34, CD56 all APC-Cy7, CD33-PerCP Cy5.5); (b) CD3-APC, CD45RO-PerCP Cy5.5, CD19-APC Cy7, CD20-APC Cy7, CD16-PE, CD56-PE, Vα24jα-FITC (eBioscience), c) CD3-APC, CD8-FITC, CD45RO-PerCP Cy5.5, γδT-PE (eBioscience). For T_{REG}, cells will be stained with CD4-APC Cy7, CD8-PE, CD45RO PerCP Cy5.5, CD25 PE Cy7, β7-FITC, followed by fixation and permeabilisation (Transcription buffer staining set, eBioscience) and staining with FOXP3-APC (eBioscience). The following gating strategy will be used to identify cell populations: macrophages (lin-ve/HLADR/CD33+ve), dendritic cells (lin – ve HLADR+/CD33+/CD11c+), T_{HELPER} (CD4+ CD8-), $T_{CYTOTOXIC}$ (CD8+ CD4-), $T_{REGULATORY}$ (CD4+/CD8-/CD25+/FOXP3+), B (CD3-, CD19+ CD20+), natural killer (CD3-/CD16+/CD56+/CD45RO-), natural killer T (CD3+/NKT+), γδ T (CD3+/γδT+) in LPMCs; and gut-homing T_{HELPER} (CD4+/CD8-/CD45RO+/β_7+) and gut-homing $T_{REGULATORY}$ (CD4+/CD8-/CD45RO+/β_7+/CD25+/FOXP3+) in PBMCs.

12. Statistical Analysis

Patient information will be de-identified and the results of microbiota, immune analysis and clinical scores will be recorded in an Excel spread sheet. This data will then be imported into the R program for statistical analysis. Statistical analysis will be conducted in collaboration with the University of Adelaide department of statistics.

12.1 Primary outcome power analysis

The study is powered to detect a significant difference in the primary outcome of inducing remission at 8 weeks post FMT with 32 patients in each arm. This was calculated using a *Z* test with pooled variance for the difference of two independent proportions. The significance level was set at 5% and the power at 80%. The estimated remission rate in the placebo group was 26.4% and the minimum clinically relevant remission rate we are powered to detect is 60%.

The placebo remission rate is difficult to predict because of the heterogeneous nature of previous studies that investigated induction of remission in UC. Our placebo remission rate was derived from the active UC trials 1 and 2[51] (ACT-1 and ACT-2). The ACT-1 and ACT-2 trials were randomised, double-blind placebo-controlled studies that evaluated the efficacy of IV infliximab 5 or 10mg/kg IV infusion for induction and maintenance treatment in adults with UC. The clinical response rate in those patients in the ACT-2 trial who were not steroid dependent was 26.4%. These patients had moderate to severe colitis with a Mayo score of 6 to 12 on enrolment and so had more severe disease on average than our patients. Response was defined as at least a 3-point reduction and 30% reduction in the Mayo score to determine clinical response at week 8. Another trial of patients with mild to moderate UC[52] found a remission rate at 8 weeks with oral mesalamine 2.4g daily of 22%. Many of our patients will be taking an oral aminosalicilate compound and some a concomitant steroid. The remission rate in this case would be expected to be higher than 22%.

12.2 Clinical outcomes

Comparisons between treatment groups of the primary and secondary dichotomous outcomes will be assessed using Fisher's exact tests, with individuals analysed in the group to which they are allocated (intention to treat). Assessment of treatment on the change in total Mayo score will be assessed using linear mixed effects regression with week 8 total Mayo score as outcome and adjusting for baseline total Mayo score and steroid use at either time point. A random intercept will be included for each group of individuals receiving the same donor mix. Associations between baseline factors and change in total Mayo score will be assessed in a similar manner, with treatment group also adjusted for as a fixed effect covariate. To assess the effect of oral steroid use at either time point, a mixed effects regression will be constructed with total Mayo score (at either assessment) as outcome with oral steroid use, assessment time and the treatment–assessment time pairwise interaction as fixed effects. Two non-nested random intercepts will be included, one for correlations due to treatment batch effects, the other to account for observations within the same patient. The random effects will be non-nested as the treatment batch effects are only present at week 8.

12.3 Safety

As with the clinical outcomes, the comparison between treatment groups and occurrence of SAEs will be assessed using a Fischer's exact test. Assessment of treatment on the change in serum creatinine, ALT, ALP, bilirubin and haemoglobin will be assessed using linear mixed effects regressions with week 8 values as outcome. Fixed effects covariates include treatment group and baseline values with a random intercept to account for within-batch correlations.

12.4. Inflammatory markers

The models used to assess the differences due to treatment in white blood cell count, neutrophil count and C-reactive protein will be the same as those used to assess the safety blood markers (see above). The exception being calprotectin, which has an extra assessment at week 4. This model extends the mixed effects regressions with assessment time (week 4 v week 8) and the pairwise interaction with treatment as additional fixed effects. As before, random effect intercepts will be included for each individual and each treatment batch, with individual effects nested with batch. After inspection of the residual distribution, these analyses will be performed on the change in log transformed calprotectin, with results being converted back to the original scale.

12.5 Microbiome: Diversity

Diversity will be defined as the fraction of unique species present at an assessment out of all species present at any analysis in any sample. Therefore, logistic mixed effects regressions will be used to compare between treatment groups with donor stool and stool mix samples. Outcome will be the presence of a species in a particular sample. Fixed effects will include sample origin (donor v mix v treated patient v untreated patient) and total sample count (log transformed). Three non-nested random effects will be included: patient identifier, donor batch and the microbiome species identifier. To assess the effect of treatment, a separate model will be contrasted with only post baseline samples included as outcome. This model will be identical to the previous, except that the fixed effects will be baseline prevalence (logit transformed), treatment allocation, assessment time (week 4 v week 8), the pairwise treatment–assessment time interaction and total sample count (log transformed). Associations between both baseline diversity and change in diversity, and change in total Mayo score will be

assessed as before (re Clinical outcomes). A two-stage approach will be taken. First, the mean diversity will be estimated using the logistic mixed effects models previously described in this section. These diversity estimates will then be included in the models of total Mayo score as fixed effects.

12.6 Microbiome: Abundance v total Mayo score

Associations between changes in biome species abundance with change in total Mayo score will be modelled in a similar manner. For each sample, the mean proportion of total counts will be calculated, and subsequently for individuals, with samples at both week 4 and 8 averaged to estimate baseline and post randomisation prevalence estimates. The change in prevalence will then be included in linear mixed effects models of total Mayo score (re Clinical outcomes). A false discovery rate (FDR) analysis will be performed to provide evidence of associations beyond what would be expected because of multiple testing, with the FDR being compared with the same analysis repeated, but with outcome (total Mayo score) permuted between individuals.

12.7 Microbiome: Abundance v treatment

The change in prevalence by treatment group and assessment time will be assessed using a negative binomial mixed effects regression for each microbiome species. Fixed effects include treatment allocation, assessment time (baseline, week 4, week 8 and 12 months) and their pairwise interaction. Nested random intercepts per patient and assessment will be included in the model, with total sample count (log transformed) included as an offset.

12.8 Metabolome

Baseline levels of butyrate and dietary fibre will be compared between donors and UC patients using non-parametric Mann-Whitney-Wilcox tests. The effect of treatment on these and other SCFAs will be assessed using linear mixed effects regressions. Fixed effects include assessment time (week 4 v 8), treatment group and baseline SCFA abundance, with two nested random intercepts at the donor batch and patient levels. After examination of residual distributions, all SFCA variables will be log transformed and results reported as percentages of baseline scores. Associations between baseline total Mayo scores and SCFAs will be assessed using linear regressions, adjusting for

oral steroid use, with baseline SCFA levels log transformed. Associations between change in total Mayo scores and change in SCFAs will be performed in the same two-stage approach. SCFA change levels will be estimated per individual using linear mixed effects regressions, adjusting for baseline levels and treatment, with individual random effects nested within batch. Patient level estimates of SCFA change will be entered into linear mixed effects regressions of total Mayo score as a fixed effect using the same methodology described above (re Clinical outcomes).

12.9 Immune system

The models used to assess associations between immunological measures and total Mayo score both at baseline and for post-treatment change will be the same as those used for SCFAs (see Metabolome above). With baseline and week 8 assessments for the immunological data, the difference in log transformed values will be included in the mixed effects regression of total Mayo score.

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Appendix 2C: Summary of Changes

Page numbers apply to the final protocol:

1. Title page. Oliver Waters added as investigator at Fiona Stanley Hospital in Western Australia. Fiona Stanley Hospital was added as a third site for the trial in 2015.
2. Pages 271. Under “Standard of care”. Deleted “Anti TNF agents not funded by the pharmaceutical benefits scheme in Australia and so, are not readily available”. Funding became available for anti-TNF agents in 2014 (after commencement of the study).
3. Page 276. Under “Open-label therapy”. Changed such that all who are randomly assigned to the placebo group by week 8 will then be offered active donor FMT at the 8 week colonoscopy. Previously only those who do not have a clinically relevant response (achieving remission, having a drop in Mayo score by ≥3 or achieving a endoscopic subscore of 0–1) would be offered donor FMT. This change was made as patient 4 was enrolled and as such all patients in the placebo arm were offered donor FMT at week 8.
4. Page 276. Under “Exclusion criteria”. Anti-TNF therapy removed as exclusion criteria. This therapy became funded for ulcerative colitis in 2014 and at this time we allowed patients on this medication to enter the trial.
5. Page 276. Under “Recruitment”. Fiona Stanley Hospital in Western Australia added as a study site in 2015 and Dr Oliver Waters added as an investigator at that site.
6. Page 276. Under “Recruitment”. Donor recruitment flyers were not placed on the hospital grounds and only the university. The university population were considered more suitable to be stool donors.
7. Page 277. Under “Medication prior to enrolment”. Biological agents dosing stable for at least 8 weeks. This change was made when biological agents were no longer an exclusion.
8. Page 292. Under “Medical history”. Exclusion criteria broadened in 2015 to keep up with latest screening practices internationally and on advice from local experts.

9. Page 293. Under “Blood testing”. ANA and helicobacter serology added in 2015.
10. Page 293. Under “Stool testing”. Viral studies added in 2015.
11. Page 297. Under “11. Analysis and Reporting of Results”. More detailed plan for bacterial and immunological analysis added.
12. Page 300. Under “12.2 Clinical outcomes”. The statistical plan for clinical outcomes is expanded beyond using Fischer’s exact test alone to test the primary and secondary clinical endpoints. Factors affecting remission will be analysed using linear mixed effects regression.
13. Page 301. Under “12.4 Inflammatory markers”. Statistical plan for analysis of inflammatory markers included with linear mixed effects regression.
14. Page 301. Under “12.5-12.7 Microbiome”. Statistical plan for the analysis of diversity, abundance v total Mayo score and abundance v treatment added.
15. Page 302. Under “12.8 Metabolome”. Statistical analysis of stool short-chain fatty acids and dietary fibre intake. Again, linear mixed effects regression analysis was proposed.
16. Page 303. Under “12.9 Immune system”. Statistical analysis of immune cell populations was added. Again linear mixed effects regression analysis was proposed to compare immune populations to total Mayo score.