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Faecal Microbiota Transplant in Ulcerative Colitis (FMTUC)

A Randomised Clinical Trial Feasibility Study

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Abstract

Aims

Restoring gut dysbiosis with faecal microbiota transplant (FMT) is of increasing interest as a therapeutic option in the management of Ulcerative colitis (UC). The aims of this thesis are to conduct a review of endpoints in UC clinical trials, to conduct a Phase II feasibility study to estimate the magnitude of treatment response to FMT in treatment-naïve patients with UC, to evaluate recruitment rates and to determine optimal study conditions and choice of endpoints for the phase III FMTUC study.

Methods

Narrative Review: The current consensus on endpoints in UC clinical trials were systematically reviewed using two indices: the Ulcerative Colitis Endoscopic Index of Severity (UCEIS) and the Ulcerative Colitis Disease Activity Index (UCDAI).

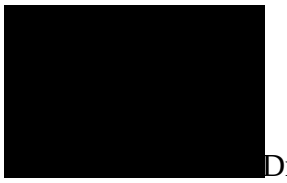
FMTUC: Treatment-naive UC subjects were randomised to i) single enema, ii) five daily enemas or iii) control group. All groups received bowel decontamination prior to the FMT interventions. Subjects were assessed using qualitative assessment tools, blood tests, 16S RNA sequencing on faecal samples for 12 weeks. Endoscopic and histological assessments were also performed at baseline and week 12. The paired primary endpoints were remission of UC and successful engraftment of donor faecal microbiota at 12 weeks. Clinical remission was defined as Mayo score ≤ 2 with an endoscopic Mayo score of 0.

Conclusions

Eighteen patients were recruited between July 2016 and February 2020 and five achieved clinical remission. Clinical remission was more frequently observed among subjects with mild-moderate disease ($P = 0.173$). No correlations between FMT dose, frequency and clinical response were observed. The 16S evaluation demonstrated the altering the faecal microbiota after the interventions. This study also showed an inverse correlation between IL-10 and the severity of UC. The narrative review highlighted the importance of urgent universal consensus on both clinical remission and validated outcome tools, such as the UCEIS. This feasibility study of FMT successfully demonstrated potential for employing this method in the management of UC.

Declarations and Statements

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Signed  Dr Maki Jitsumura

Date 05/10/2022

STATEMENT 1

This thesis is the result of my own investigations, except where otherwise stated. Where correction services have been used, the extent and nature of the correction is clearly marked in a footnote(s).

Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

Signed  Dr Maki Jitsumura

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STATEMENT 2

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

Signed  Dr Maki Jitsumura

Date 05/10/2022

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Abbreviations

16S rRNA: 16S ribosomal RNA

5-ASA: 5- aminosalicylic acid

ALP: Alkaline phosphatase

ALT: Alanine transaminase

Bp: base-pair

CD: Crohn's Disease

CDI: *Clostridioides difficile* infections

CI: Confidence Interval

CRP: C- reactive protein

ELISA: Enzyme-linked Immunosorbent Assay

ESR: Erythrocyte Sedimentation Rate

FMT: Faecal Microbiota Transplantation

GI: Gastrointestinal

Hb: Haemoglobin

HTA: Human Tissue Authority

IBD: Inflammatory Bowel Disease

ILS: Institute of Life Science, Swansea University

JAG: Joint Advisory Group on GI Endoscopy

JCR: the Joint Clinical Research Facility, Swansea Bay University Health Board

NGS: Next generation sequencing

NICE: National Institute for Health and Care Excellence

OR: Odds ratio

PCR: Polymerase Chain Reaction

RCT: Randomised Controlled Trial

SCFAs: Short-chain fatty acids

STU: Swansea Trials Unit

UC: Ulcerative Colitis

WBC: White Blood Cell

WGS: Whole genome shotgun sequencing

All scientific names of prokaryotes are written in italics in this thesis to follow the recommendations of the International Code of Nomenclature of Prokaryotes (1)

Chapter 1. Summary

Introduction

Dysbiosis of the gut microbiota may be a contributing factor in the pathogenesis of inflammatory and immunological diseases including Ulcerative Colitis (UC). Restoration of gut microbiota diversity by means of faecal microbiota transplantation (FMT) is of increasing interest as a therapeutic option in the management of UC. Several RCTs have investigated the role of FMT in UC, although there has been heterogeneity across protocols: including in disease severity, pre-FMT bowel decontamination and dose, route and frequency of FMT administration. Furthermore, each RCT defines its own clinical remission with different outcome tools, increasing variation between studies. The aims of this thesis are to conduct a review of endpoints in UC clinical trials, to conduct a Phase II feasibility study to estimate the magnitude of treatment response to FMT in treatment-naïve patients with newly diagnosed UC, to evaluate donor and patient recruitment rates and to determine optimal study conditions and choice of endpoints for the phase III FMTUC study (ISRCTN 58082603).

Methods and Analysis

Narrative Review: The current consensus on the definition of remission and the endpoints employed in clinical trials were reviewed using two common disease activity scoring systems: The Ulcerative Colitis Endoscopic Index of Severity (UCEIS) and the Ulcerative Colitis Disease Activity Index (UCDAI). Bibliographic searches from 1946 – 2016 were carried out in accordance with the PRISMA protocol using online databases (National Library of Medicine's PubMed Central Medline, OVID SP MEDLINE, OVID EMBASE, the Cochrane Library and Conference Abstracts with MESH headings ("ulcerative colitis") AND ("ulcerative colitis endoscopic index of severity" OR "UCEIS") AND ("remission") as well as ("ulcerative colitis") AND ("ulcerative colitis disease activity index") OR "UCDAI" OR "UC disease activity index" OR "Sutherland index") AND ("remission").

Phase II FMTUC Feasibility Study: Treatment-naive patients with histologically confirmed UC limited to the rectum and sigmoid (proctosigmoiditis) were recruited for this study. Subjects were randomised to i) single enema, ii) five daily enemas or iii) control group. All groups received antibiotic gut decontamination for ten days and mechanical bowel preparation 48 hours prior to the FMT interventions. Subjects were assessed at baseline, week 1, 4, 8 and 12 using qualitative assessment tools (IBDex and CUCQ-32), routine blood tests and 16S RNA sequencing on faecal samples. Mayo score as well as endoscopic and histological assessments were also performed at baseline and week 12. The paired primary endpoints were blinded assessment for endoscopic remission of UC at 12 weeks and rate of persistent microbial engraftment at 12 weeks (determined by 16S RNA sequencing). Clinical remission was defined as Mayo score ≤ 2 with an endoscopic Mayo score of 0. Secondary endpoints included qualitative assessments and mucosal cytokine profiling with IL-10.

Results

Eighteen UC patients were recruited between July 2016 and February 2020 despite recruitment challenges. Of these, five subjects achieved clinical remission. Clinical remission was more frequently observed among subjects with lower qualitative scores and mild-moderate disease at the baseline assessment, although this did not reach statistical significance ($P = 0.173$). No correlations between FMT dose, frequency and clinical response were observed in this feasibility study. A suggested minimum of 68 subjects would need to be recruited under the same conditions to demonstrate dose effect of FMT, based on a power calculation computed based on ANOVA. The 16S evaluation of the faecal samples demonstrated successful engraftment of FMT and demonstrated a similar microbiota among the FMT intervention groups, which was markedly different from the control group. As reported in previously published work, this study also showed an inverse correlation between IL-10 and the severity of UC. However, the sample size in this study was not powered to demonstrate statistical difference in these metrics. The narrative review evaluating endpoints highlighted the importance of urgent universal consensus on both clinical remission and validated outcome tools, such as the UCEIS.

Conclusion and Future Work

This feasibility study of FMT use among treatment naïve UC patients successfully demonstrated potential for employing this method in the management of UC. This study proposes that a Phase III RCT study should aim for recruitment of 100 treatment naïve UC patients to further evaluate FMT dose and frequency effects on clinical remission - which is defined as the UCEIS ≤ 1. The same study conditions can be employed with an additional intervention group of two enema doses at week 0 and 4 to study the effect on repeated FMT administration. Further studies on the correlation between IL-10 and IL-10 producing microorganisms should be included in the phase III FMTUC study.

Chapter 2. Introduction

This FMTUC project is a randomised single-blinded clinical trial feasibility study, which has been externally peer reviewed by the Joint Scientific Review Committee of Swansea Bay University Health Board. This project was funded by a Pathway to Portfolio Research and Development grant. The trial management group consists of chair, gastroenterologist, microbiologist, biochemist, Swansea Trial Unit (STU) statistician and researchers.

2.1 Objectives

The primary objective of this phase II feasibility study was to estimate the magnitude of treatment response to FMT in treatment-naive patients with newly diagnosed ulcerative colitis (UC). The secondary objectives were to define the optimal parameters for delivery of FMT, to estimate feasibility and propose the design for a large-scale multi-centre phase III FMTUC study to evaluate the efficacy of FMT in UC. Recruitment took place from July 2016 to February 2020 with 12-week post-treatment follow up.

2.2 Ulcerative Colitis (UC)

UC is defined as a chronic relapsing-remitting inflammatory bowel disease (IBD) of the large bowel (2,3). Crohn's disease (CD) is another chronic IBD and less common than UC. Although CD and UC are both IBDs, they are very different conditions in many respects.

2.2.1 Epidemiology

The incidence of UC has been rising worldwide though it is more common in industrialised countries (4). The highest annual incidence and prevalence were reported in northern Europe with 24.3 cases per 100,000 person-year and 505 cases per 100,000 respectively (5). Although UC can present at any age, the peak age of onset is in a bimodal distribution with peaks between 30 and 40 and between 50 and 80 years of age (6). Sex predominance is not observed. The recent large cohort U.K. study reported that the incidence in the UK between 2000 and 2018 was 15.7 per 100,000 people annually with the highest incidence between the age of 17 and 40 years (7).

2.2.2 The Large Bowel

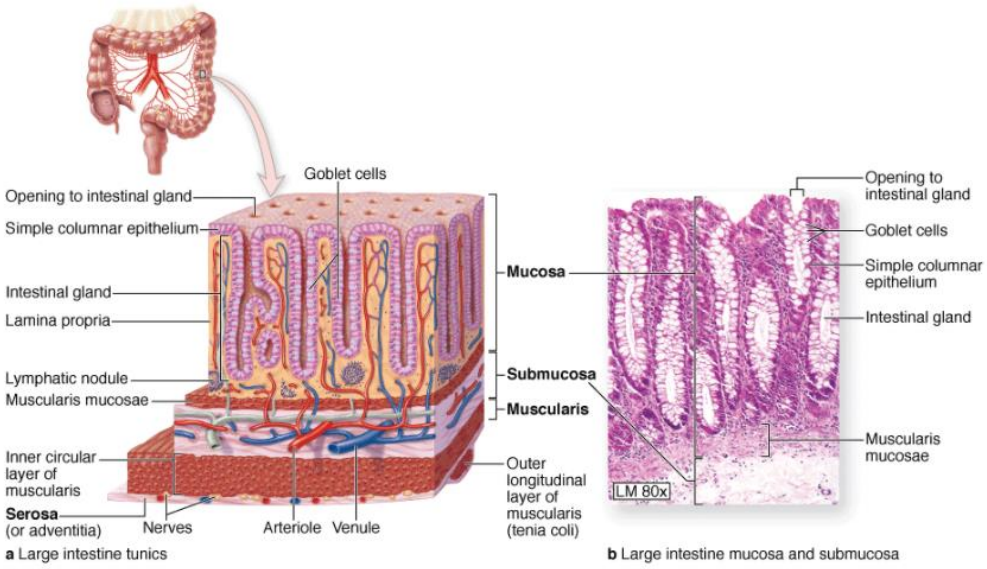


Figure 1: Histology of the normal large bowel mucosa

Source: Mescher AL: Junqueira's Basic Histology. Text and Atlas, 12th Edition (8)

The large bowel (sometimes called the colon or large intestine) is the final part of the gastrointestinal (GI) tract before the anus, and its chief functions are absorption of water and electrolytes from the luminal contents, formation of faeces and chemical digestion by gut microbes (9). It also secretes mucous, which facilitates transports of the faeces to the rectum. Figure 1 shows the anatomy and histology of normal large bowel. The mucosa of the large bowel is lined by a columnar epithelium with brush border, which acts as a biochemical and physical barrier and a link between immune cells and bacteria (10). It also consists of deep crypts and each crypt contains colonocytes, goblet cells, enteroendocrine cells and intestinal stem cells (10). Colonocytes are the predominant cells in the large bowel and carry out absorption of electrolytes, whereas goblet cells, comprising approximately 10% of all intestinal epithelial cells, provide protection from bacterial adhesion and digestive enzymes (10). Furthermore, goblet cells secrete biologically active substances, such as trefoil peptides, which facilitate innate immunity by responding to mucosal damage and promoting epithelial restitution (11). It is noteworthy that goblet cells depletion is characteristic of UC (12). Enteroendocrine cells consist of approximately 1% of the large bowel epithelium (13), which secrete hormones including vasoactive intestinal peptide (VIP). Because VIP aids to regulate colonic mucosal and epithelial integrity, loss of VIP is also suggested to be linked to developing colitis (14).

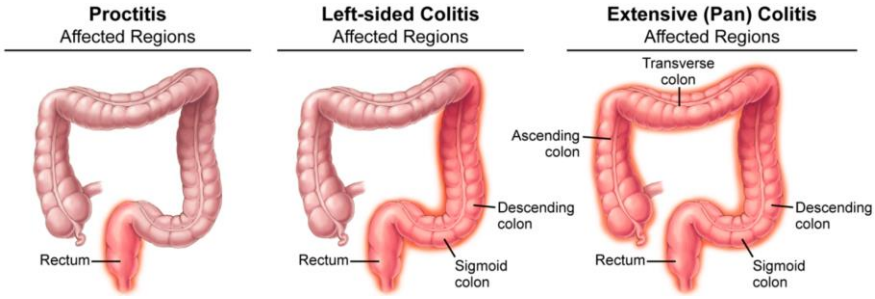


Figure 2: UC (Proctitis, Left-sided colitis and pancolitis)

Source: Kayal et al. Ulcerative Colitis: Current and Emerging Treatment Strategies (15)

Patients with UC have mucosal inflammation originating from the rectum, which can extend continuously to the proximal colon (Figure 2). The disease is limited to the mucosa of the large bowel in UC, whereas patients with CD can have full-thickness bowel wall inflammation typically originating from the terminal ileum (2). CD also shows skip-lesions unlike UC and can occur at any part of the alimentary system.

2.2.3 Clinical Presentation and Symptoms

UC is a chronic colonic inflammatory disease with unpredictable rates of relapse and remission. When patients have flare-ups, many experience diarrhoea with or without blood and mucus, rectal bleeding, faecal frequency and urgency, crampy abdominal pain often relieved by defecation, extreme fatigue and weight loss (3). When they are in acute phase, these symptoms worsen with increasing faecal frequency with more blood in stools, pyrexia (raised body temperature) and tachycardia (increased heart rate more than 90 beats per minute). These patients often require hospital admission for medical treatment and close monitoring. Failing that, the only way to manage severe UC is colectomy (removal of the large bowel) and a stoma.

UC can also affect parts of the body other than the colon and this is called extraintestinal manifestations. Extraintestinal manifestations include the musculoskeletal systems (e.g. arthritis), the ocular system (e.g. uveitis, iritis), the hepatopancreatobiliary system (e.g primary sclerosing cholangitis) and dermatological and oral systems (e.g. erythema nodosum, pyoderma gangrenosum) (16).

When patients are in remission, they often do not experience any symptoms with or without any medications. However, the disease can relapse after many years of remission without obvious cause. Some experience a long period of remission after the first presentation, whereas some may suffer from frequent attacks with hospital admissions (17). It is difficult to predict who will respond well with medical interventions or when they will relapse. The aim of UC management is to introduce remission

for as long as possible, which will be discussed more in the management section (section 2.2.6). At the time of first presentation, approximately 40% of patients have the disease confined to the rectum (proctitis), 30% present with the disease extending to the splenic flexure (left-sided colitis), and the remaining 30% present with the disease affected the whole rectum and colon (pancolitis) (3). Approximately 15% of patients are reported to progress from proctitis to pancolitis (3). A study suggests that most patients with UC achieve remission, while approximately 43% suffer a chronic intermittent or continuous course and 20-30% of patients require removal of the whole colon, colectomy, within 25 years (3).

Patients with UC are known to have an increased risk of developing colorectal cancer, and thus, they undergo surveillance colonoscopy for early detection of this (18). A recent large longitudinal cohort observation study reported a significant decline in colorectal cancer amongst patients with UC in recent years, emphasising the importance of surveillance colonoscopy (19).

2.2.4 Aetiology and Risk Factors

Although the aetiology of UC is believed to be multifactorial involving environmental factors and diet, genetic predisposition, autoimmunity and dysbiosis, the precise cause is poorly understood (Figure 3) (1,2).

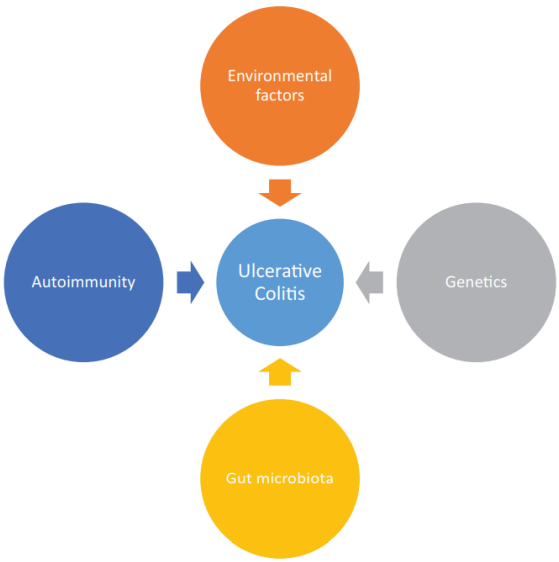


Figure 3: Multifactorial risk factors for UC

Source: Gajendran et al. A comprehensive review and update on ulcerative colitis (3)

Environmental factors and Diet

Multiple environmental factors have been suggested to be responsible for the development of UC. A recent meta-analysis, which studied environmental risk factors for UC, reported that soft drink consumption is a strong risk factor and urban living, vitamin D deficiency, contraceptive use, sucrose and meat intake and poliomyelitis vaccine are weak risk factors (20). The same study also concluded that tea consumption and previous *Helicobacter pylori* infection are highly protective. Smoking, breastfeeding, vitamin D, living near farm animals, bed sharing and access to hot water are weak protective factors against UC (20).

Antibiotic use has been also suggested to be positively linked to developing UC since antibiotics are known to alter the human gut microbiota by diminishing microbial diversity (21). Many studies have shown conflicting results and a recent large UK based case-control study as well as meta-analysis did not find any association between UC and previous antibiotic use (20,22).

Genetic predisposition

An increased incidence of developing UC has been observed amongst patients with a family history of UC (23,24). A recent systematic review and meta-analysis of seventy-one studies including 86,824 patients reported that 12% of patients with UC have a family history of IBD (24). It is also reported that the Jewish population has a higher incidence of developing UC (25). A few studies have been undertaken to identify genetic predictors of developing UC. They have shown that 71 risk loci for UC, and the TNFSF15 (TL1A) locus, in particular, have been suggested to increase the risk of developing severe UC (26–28). Nevertheless, these loci only explain 7.5% of the disease variance, and thus have little predictive value for phenotype (26,27).

Dysregulation of Immune Response and Autoimmunity

A dysregulated adaptive immune response and autoimmunity of the colonic mucosa have been proposed to be one of the pathogenic factors in UC. T cells play a pivotal role in the adaptive immune system, whose main function is to protect the hosts from pathogens (29). The most accepted theory is that the adaptive immune system over responds to antigens causing a dysregulation of the finely-tuned immune system and thus attacking the body's own cells (30). This theory is also supported by the fact that many patients with UC suffer from extraintestinal manifestations which share features of other autoimmune conditions such as primary sclerosing cholangitis and autoimmune hepatitis (31). A recent large nested case control study reported that an increased incidence of autoimmune disorders was observed in UC patients (32). Furthermore, the authors noted that an increased risk of autoimmune disorders was seen in patients with severe IBD including UC (32).

Gut microbiota and Dysbiosis

Patients with newly diagnosed UC are more likely to have a history of gastroenteritis (33). Medications such as non-steroidal anti-inflammatory drug and oral contraceptives are linked to the development of UC (34,35). In recent years, dysbiosis of the gut microbiota has been also suggested to be one of the aetiological factors (3). This is linked to alterations in colonocytes as well as mucous and epithelial barrier defects (10). Alterations in trefoil peptides from goblet cells have been reported in UC patients (36). Barrier dysfunction is believed to be the main cause of disease development since patients with active UC have fewer colonic goblet cells making the mucous barrier more permeable (37). Despite extensive research into the precise aetiology of UC, much remains unknown.

2.2.5 Diagnosis

The diagnosis of UC is made with a combination of clinical presentations, laboratory blood tests, endoscopic assessment, and histological confirmation, though histological confirmation from biopsies at the time of endoscopic assessment is the gold-standard investigation for the diagnosis. Table 1 is a list of investigations and assessments used to diagnose UC in the U.K., though there may be slight variations depending on availability and accessibility for these investigations as well as clinical context.

Table 1: List of investigations for UC

Laboratory investigations	Blood tests • Full blood count (including haemoglobin) • Inflammatory markers (C-reactive protein (CRP) and white blood cells (WBC)) Stool tests • Exclude infective causes of diarrhea (*Salmonella, Shigella, Campylobacter, Yersinia, Escherichia coli 0157:H7, C. difficile*) • Faecal calprotectin (38)
Endoscopic assessments	• Colonoscopy • Flexible sigmoidoscopy
Histology	• Biopsy of the diseased colonic mucosa
Radiological assessments	• Abdominal X-ray • Computerised tomography of abdomen and pelvis

Laboratory investigations

There is currently no single biomarker to diagnose UC, however, blood tests are used in assessing and monitoring disease activity. These tests include full blood counts, inflammatory markers and albumin. Inflammatory markers (CRP and WBC) are often elevated in active disease and used in the decision-making process especially for surgical interventions. Previously, serological markers for UC were suggested, including perinuclear anti-neutrophil cytoplasmic antibodies (pANCAs), anti-*Saccharomyces cerevisiae* antibodies (ASCA) , antibodies to outer membrane porin, anti-carbohydrate antibodies, pancreatic antibodies and serum P53 antibodies (39). However, many of these serology markers are found to be elevated in other chronic inflammatory conditions including rheumatoid arthritis and vasculitis. Furthermore, it was reported that pANCA was detected in 32% of healthy individuals (40), limiting its diagnostic value. Faecal calprotectin of stool samples also facilitates to assess severity of colonic inflammation and relapse of the disease (38). Faecal calprotectin is a calcium binding protein which is mainly found in neutrophils throughout the body, and elevated calprotectin in faeces indicates migration of neutrophils in the GI tract due to the inflammatory process (38). Infections must be excluded in patients with suspected UC with stool tests. Stool studies check for routine cultures (*Campylobacter, Salmonella, Shigella and Yersinia*), *Escherichia coli* O157:H7 and *C. difficile* (3).

Endoscopic assessments

The two main endoscopic assessment tools are colonoscopy or flexible sigmoidoscopy. Colonoscopy is used to assess the entire colon as well as the terminal ileum, whereas flexible sigmoidoscopy is used to assess mainly the left side of the colon up to the splenic flexure. Endoscopic assessment with biopsy is the gold standard for definitive diagnosis of UC as well as monitoring disease in response to medical interventions (3).

Pathognomonic findings of UC include erythema, loss of vascular pattern, erosions, contact or spontaneous bleeding, granularity and ulcerations of the colonic mucosa in a continuous distribution (Figure 4) (41). Mayo score is one of the widely used disease activity scores, which includes a component of endoscopic assessment. Photographs A-D of Figure 4 are equivalent to Mayo scores of 0-3 respectively. The colonic mucosa of UC usually has a clear demarcation between inflamed and non-inflamed mucosa, making macroscopic assessment for extent of the disease easy, however, we must be aware that macroscopically normal mucosa may still contain microscopic mucosa inflammation. A study reported a weak correlation between the extent of macroscopic and microscopic disease (42). Furthermore, several studies stated that there was a lower sensitivity for endoscopic severity grading for assessing severity when compared with histologic grading (43). This suggests that

macroscopic assessment with endoscopy in conjunction with histology assessment is key to accurate assessment of the condition.

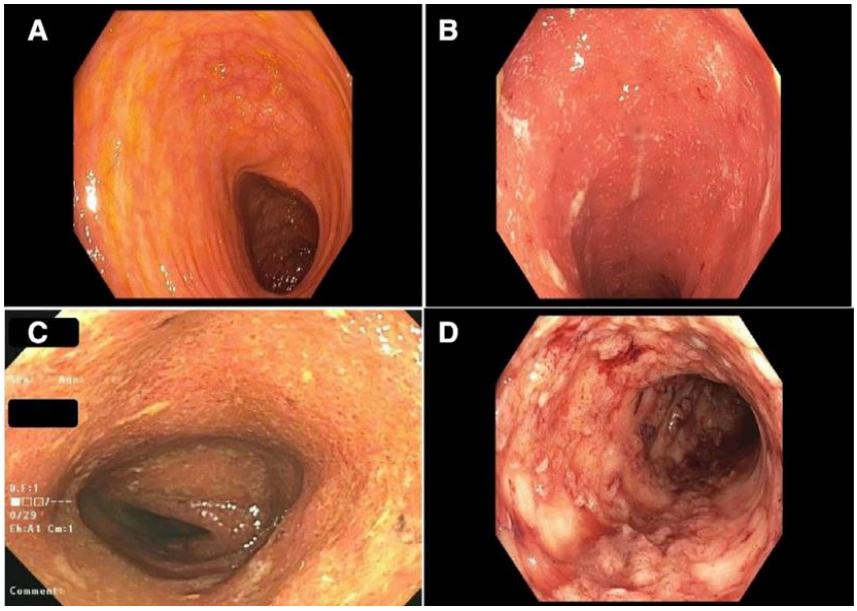


Figure 4: Endoscopic findings of UC

(A: normal mucosa, - endoscopic Mayo 0 B: Mild colitis with mucosal erythema and granularity – endoscopic Mayo 1, C: Moderate colitis with erosion, marked erythema and loss of vascular marking – endoscopic Mayo 2, D: Severe colitis with ulcers, contact bleeding – endoscopic Mayo 3) (3)

Source: Gajendran et al. A comprehensive review and update on ulcerative colitis

The risk of developing colorectal cancer amongst UC patients is proportional to disease duration (the risk of colorectal cancer is 2% and 18% after 10 and 30 years, respectively) (44). Thus, colonoscopy is not only useful to monitor UC, but also to inspect for colorectal cancer, which is particularly crucial for patients with chronic UC.

Histology

Histological abnormalities found in UC can be divided into three main categories; architectural, inflammation and epithelial abnormalities. Architectural abnormalities include surface irregularity, crypt distortion and crypt atrophy (Figure 5), whereas inflammation is signified by the presence of neutrophils. Cryptitis (neutrophils in the crypt epithelium) and crypt abscess (neutrophils in the crypt lumen) are common findings in UC. Mucin depletion and Paneth cell metaplasia are also considered to be abnormalities of the epithelium (45). Significantly decreased crypt density, crypt architectural distortion, irregular mucosal surface and diffuse mucosal inflammation without granulomas are most common findings of microscopic changes in UC (45,46).

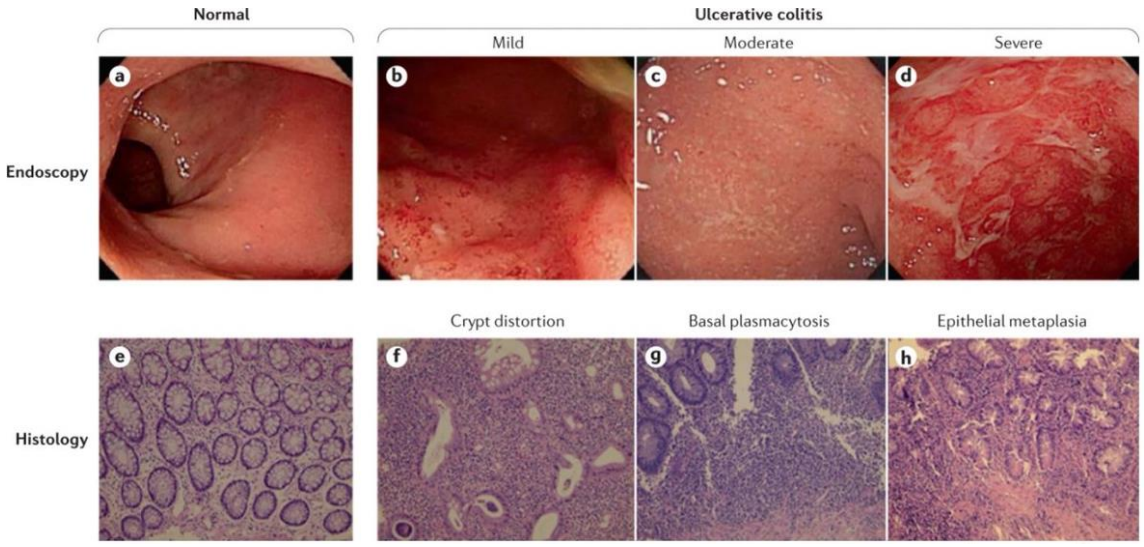


Figure 5: Histology of ulcerative colitis

Source: Kobayashi et al. Ulcerative colitis (47)

It can be difficult to distinguish between UC and acute colitis macroscopically, but the presence of crypt distortion is the hallmark of UC and acute colitis shows only acute inflammation without abnormal crypts. Quiescent disease is indicated by features of chronic injury including crypt distortion, crypt atrophy, crypt loss and Paneth cell metaplasia (48). Resolution of the crypt distortion and inflammatory infiltrate are features of histologic mucosal healing (49).

Severity of histological assessment is not well defined. Histological activity index is akin to disease qualitative indices and disease activity monitoring and endoscopic indices, no gold-standard histological activity index has been defined. The recent systematic review identified twenty-six histological indices of histological activity in UC (50), however, many lack validity, reliability, feasibility or reliability to monitor histological activity in clinical trials or real clinical practice. Of those, the biggest challenge of histological assessment is inter-observer and intra-observer variability (51). Furthermore, appropriate histology assessment relies on the quality of specimen obtained. Standardisation and optimisation of the biopsy collection and preparation is needed to minimise variability (51). Nevertheless, histological assessment is crucial as marked inflammation over time is a risk factor for colonic neoplasia in UC. Furthermore, several studies highlighted histological improvement as a prognostic indicator (51,52).

2.2.6 Current Management and Treatment

There is no curative treatment at present, thus the aims of treatment in UC are to improve quality of life, to induce steroid-free remission and to minimise the risk of colorectal cancer (Figure 6).

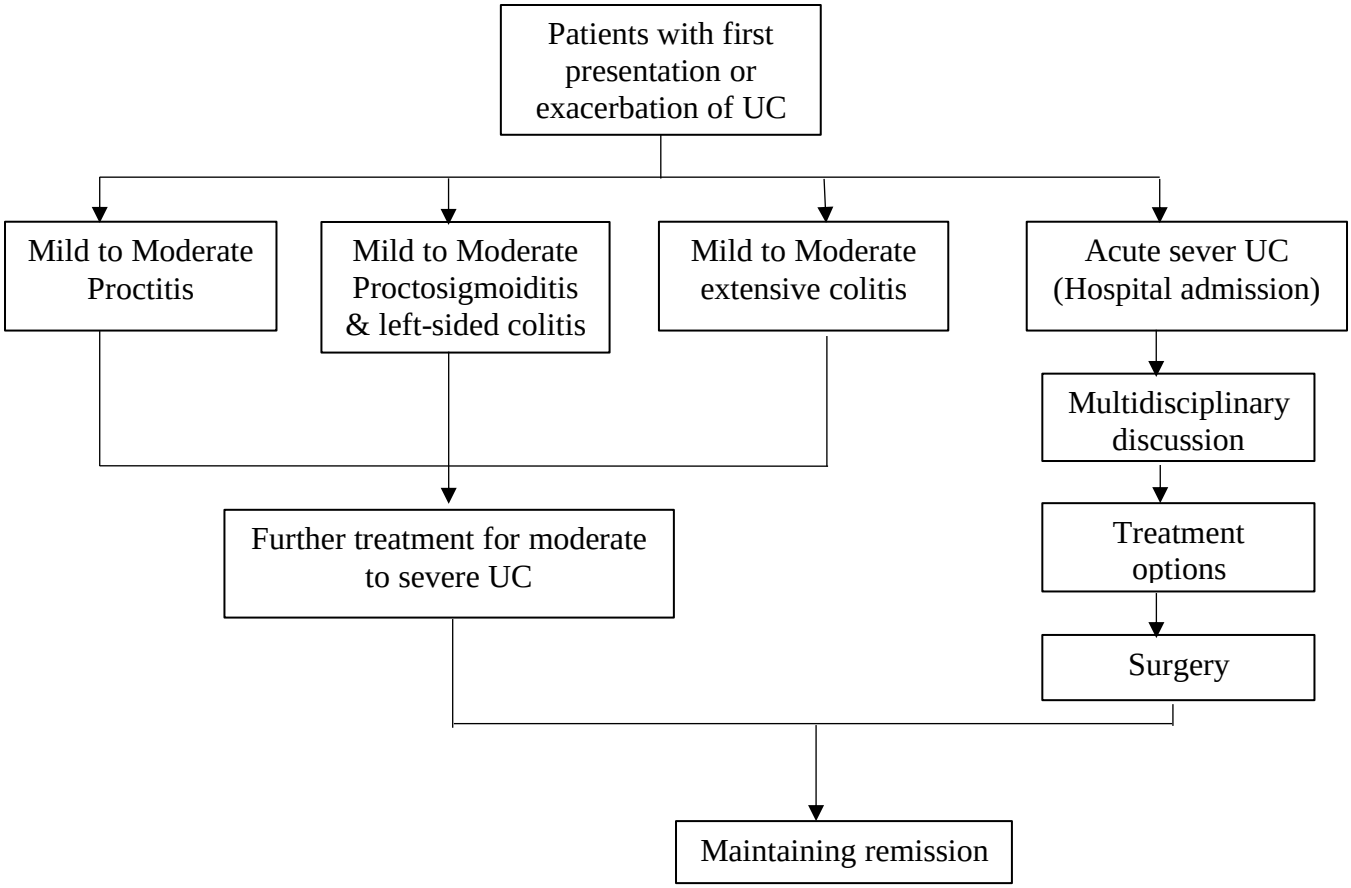


Figure 6: Overview of management to induce remission in patients with UC

At the time of initial endoscopic assessment, severity and extent of disease are evaluated based on location (proctosigmoiditis, left-sided colitis and pancolitis (Figure 2) and inflammation. The clinical severity of the disease (mild, moderate and severe) is defined based on the Truelove and Witt's severity index (Table 2) in the NICE guideline, however, erythrocyte sedimentation rate is often replaced by CRP (53).

Table 2: Truelove and Witts Severity Index

	Mild	Moderate	Severe
Bowel movements (number per day)	<4	4-6	>6
Bloods in stools	Small amount	Mild to severe	Visible blood
Pyrexia (>37.8 ℃)	No	No	Yes

Heart rate (>90 beat per minute)	No	No	Yes
Anaemia	No	No	Yes
Erythrocyte sedimentation rate (mm/hour)	<30	<30	>30

Figure 7 describes the overview of the NICE guideline on managing mild to moderate UC and extensive colitis (54). Often initial treatment is started based on this assessment before finalising with histology study.

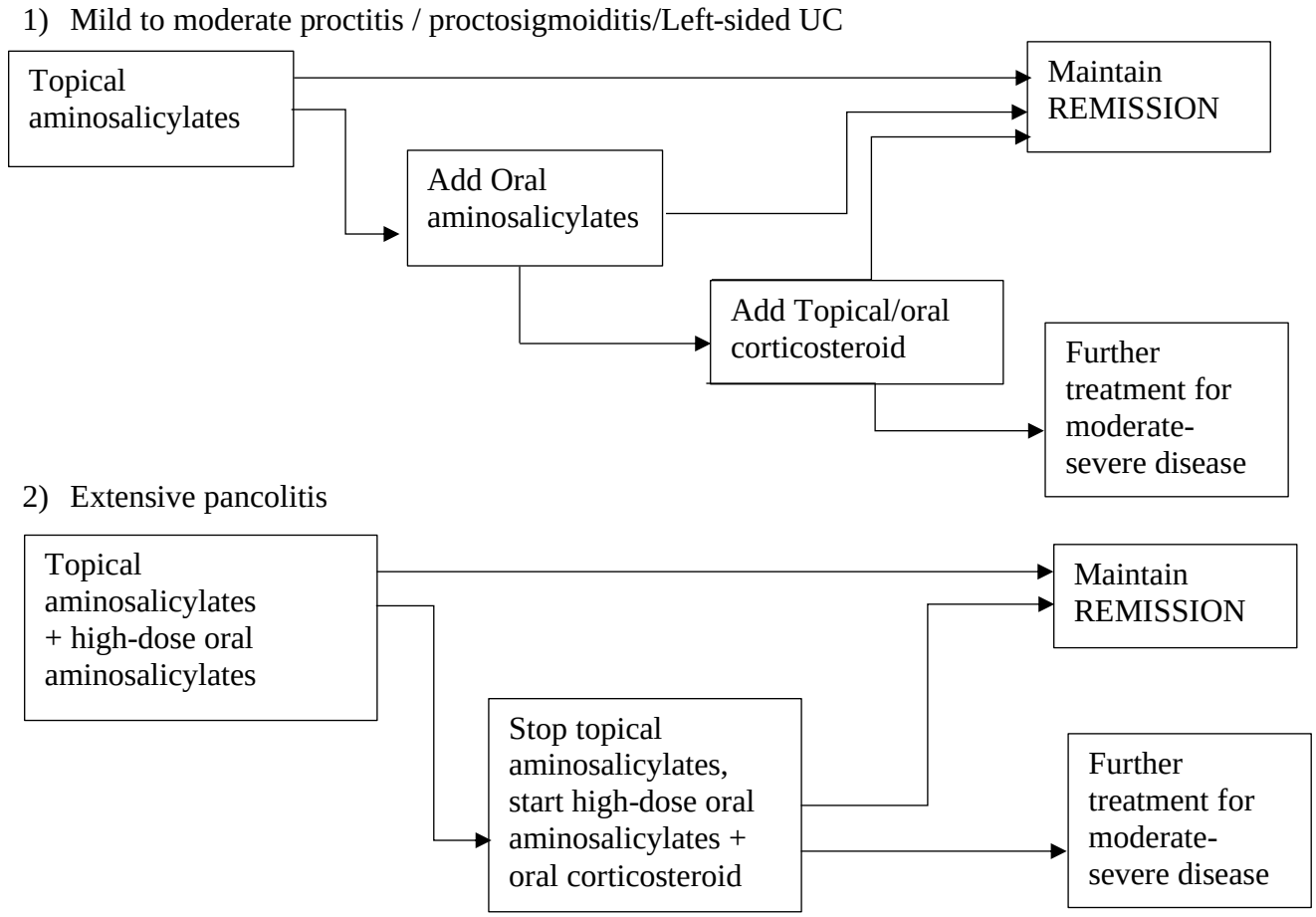


Figure 7: Management of mild to moderate proctitis, proctosigmoiditis, left-sided UC (1) and pancolitis (2)

The first line management in mild to moderate UC for proctitis, proctosigmoiditis and left-sided UC is topical aminosalicylates as either suppositories or an enema formulation (54). The NICE Guideline states that topical aminosalicylates for 4 weeks are the most effective treatment to achieve remission in patients with mild-to-moderate proctitis, proctosigmoiditis and left-sided disease (54). Patients, who do not respond to a four-week course of topical aminosalicylates, may also take oral aminosalicylates although evidence does not suggest a combination of topical and oral aminosalicylates being effective for patients with proctitis (54). Patients, who failed to achieve remission even after both topical and

oral aminosalicylates, may progress to the next step of oral or topical corticosteroids, though there is no evidence on this combination (54). Furthermore, corticosteroids are not a long-term treatment and should be tapered within 2 weeks to avoid unwanted side effects. Although aminosalicylates are usually well tolerated by patients, some may not tolerate adverse effects such as nausea, headache, vomiting, rash and abdominal pain (55).

The NICE guideline recommends biologics or Janus kinase inhibitors to manage patients with moderate to severe UC or patients who failed to respond to the aforementioned conventional therapy (56). Biologics are pharmaceutical compounds extracted or synthesized from a biological source with extremely complex structures (57). Of those biologics, the NICE guideline recommends adalimumab, infliximab and golimumab for managing moderate to severe active UC (56). These three biologics are monoclonal antibodies against TNF-alpha, which is one of the pro-inflammatory mediators, known to be raised in patients with IBD including UC (58). Vedolizumab is another biologic monoclonal antibody that the NICE guideline recommends, which targets α4β7 integrin. This integrin is expressed in white blood cells in the gut and is known to be responsible for recruiting these white blood cells to inflamed gut tissue. (59).

The failure of pharmacological therapy or refractory disease requires major colonic resectional surgery with temporary or permanent ileostomy formation (Figure 8).

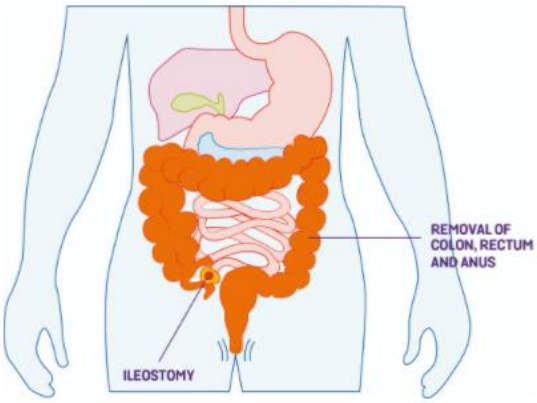


Figure 8: Subtotal colectomy with ileostomy for UC

Source: Crohn's and Colitis UK,https://www.crohnsandcolitis.org.uk/about-crohns-and-colitis/publications/surgery-for-ulcerative-colitis

(60)

2.2.7 Role of Antibiotics for Treatment of UC

Given the increasing evidence of dysbiosis being a part of pathogenesis of UC, antibiotics have been suggested for management of UC to alter dysbiosis. Previously antibiotics, including metronidazole, ciprofloxacin, tobramycin and rifaximin were shown to achieve clinical remission in RCTs. Several RCTs investigated effectiveness of metronidazole as well as ciprofloxacin in active UC, all of which found no significant difference in clinical improvement or remission (61–65). Conversely, tobramycin and rifaximin showed clinical as well as histological improvement in some studies (66,67).

Previous meta-analyses suggested adjunctive antibiotic use may be effective to achieve clinical remission (68,69), however, these studies also identified high heterogeneity due to publication bias and small study effects. Currently, the guidelines do not advise the use of antibiotic alongside standard medical treatment. Aside from uncertain efficacy, there are several reasons to avoid antibiotic use. First, no responsible bacteria have been identified to cause dysbiosis in UC, meaning empirical broad-spectrum antibiotics would be required. This could aggravate dysbiosis even further. Second, broad-spectrum antibiotics are linked with an increased risk of *Clostridioides difficile* infections (CDI). Patients with UC are at greater risk of CDI (70). Furthermore, CDI tends to be more severe when patients with UC are affected (70). Without strong evidence of antibiotics use inducing clinical remission, it is reasonable to be cautious with broad-spectrum antibiotics use.

2.3 Human Gut Microbiota

2.3.1 What is the Human Gut Microbiota?

The human gut microbiota refers to the collective genomes of micro-organisms in the human GI environment, whereas the term microbiome is defined as the community of microorganisms themselves (71). The human gut microbiota consists of a diverse biological environment comprising bacteria, viruses, phages and fungi within the faeces and in the intestinal mucosa. The number of human gut micro-organisms has been estimated to exceed one hundred trillion and most are believed to be bacteria (72). The microbiota also encodes more than three million genes, while the human genome consists of approximately 23,000 genes (72). Despite extensive sequencing and culturing studies, the complete genomic blueprint of the human gut microbiota remains undefined (73).

In the GI tract, bacteria can be categorized into anaerobic, facultative anaerobic and aerobic bacteria, of which anaerobic bacteria are the most abundant, especially in the colon (74). Most bacteria attach to the surface of the epithelial cells of the intestinal mucosa. Physiologic bacteria, which are symbiotic with the host, play key roles in nutrition and immune regulation. Physiological bacteria are usually anaerobic and include *Bifidobacterium*, *Bacteroides* and *Peptococcus* (75). Facultative aerobic bacteria, such as *Staphylococcus*, *E.coli*, *Shwanella oneidensis* and Listeria, can become harmful to the host when the balance of the gut microbiota is disturbed (76). Some other types of aerobes such as *Pseudomonas sp*. are usually scarce and do not inhabit the balanced gut microbiota long-term, but can overgrow to cause disease when the dominant gut microbiota decline due to changes in the enteral or external environments (75).

The gut virome is less well understood than bacteria, partly because of the technical difficulty in identifying viruses. Recent studies suggest that gut viruses are not only are pathogenic to the infected host cells causing gastroenteritis, but can also be symbiotic modulators of host physiology. (77).

Fungi are also a part of the gut microbiota, but their role is unclear and has been considered to be less significant as they account for less than 0.1% of the microbes present (78). Kapitan et al. recently reported that bacterial and fungal abundance in the GI tract seem to be negatively correlated (79). In other words, the imbalance of the gut bacteria is predisposed to fungal overgrowth in the GI tract. As with gut viruses, a large proportion of commensal fungi are unculturable (80) and one of the biggest limitations of characterising fungal populations with next-generation DNA sequencing technology is that fungi have different sequences and classification depending on the sexual forms of the same fungus (81). Many pathogenic fungi are pathobionts, meaning that they can be pathogenic although they are usually harmless under normal conditions. For instance, *Candida albicans* is a normal part of the gut microflora, though it can cause systemic candidiasis especially in immunosuppressed patients (82). The most common GI fungi known to date are the *Basidiomycota*, *Ascomycota* and *Zygomycota* (83,84). Hamad et al. identified 16 fungi in stool samples, of which *Galactomyces geotrichum* was the most abundant (85). Hoffmann et al. identified 66 genera of fungi, of which the *Saccharomyces* genus was the predominant one, followed by *Candida* and *Cladosporium* (83). Although the role of fungi in the human gut microbiota is not fully understood, recent studies suggest that micro-fragments of chitin, which is a substance produced by fungi and insects, have a significant immunomodulatory impact in the inflammatory process (86).

2.3.2 The Role of the Human Gut Microbiota

Although gut microbes have been studied for many years, the role of the human gut microbes have attracted much attention in recent years because numerous studies have reported their link to systemic conditions beyond infectious intestinal diseases. These studies suggest that functions of the human gut microbiota include initiating the immune response, metabolic regulation including energy metabolism, lipid and glucose homeostasis as well as protecting the mucosal barrier against pathogens (87–89). Hence, the human gut microbiota is often referred to as a hidden metabolic organ (90–93).

1) Interaction of microbes with the epithelial cells of the GI tract

The role of the GI tract is not only to digest food and absorb nutrients and electrolytes, but also to provide barriers against invading pathogens, while ensuring co-existence with the commensal microbiota. In the GI tract, there are two microbiota ecosystems; the faecal microbiota and the mucosal microbiota present in the epithelium, which is constantly altered with age, diet and lifestyle (94,95). It

is also believed that the interaction of the gut microbiota and the epithelial cells of the GI tract has a significant impact on the immune response to the host. The epithelial cells of the GI tract translate the central information to the immune cells located in the lamina propria, initiating a cascade of the innate immune response. Firstly, the innate immune system recognises microbes with pattern recognition receptors (PRRs) including toll-like receptors (TLRs) and NOD-like receptors (NLRs), and these receptors identify pathogen-associated molecular patterns (PAMPs) from microorganisms, such as peptidoglycans, lipopolysaccharides, muramyl dipeptide, lipoteichoic aid and flagellin, or danger-associated molecular patterns from damaged tissues (96). Up on activation of these PRRs, downstream signalling cascades lead to expression of different chemokines and cytokines to direct the professional immune cells. The interaction between these immune and host cells facilitates the improvement of the immune system in the human gut microbiota as well as regulating the composition of the gut microbiota in a tightly controlled fashion.

2) Metabolism regulation

The gut microbiota also promotes metabolic activities. For instance, one of the constituents of Gram-negative bacteria, lipopolysaccharides (LPSs), are known to trigger low-grade inflammation as well as the insulin resistant state through the interaction between the human gut microbiota and the innate immune system (88). Increasing evidence suggest that LPSs are partly responsible for obesity and diabetes since an increased level of circulating LPSs, also called metabolic endotoxaemia, is characteristic in diet-induced obesity and diabetes (97–99).

The gut microbiota has an ability to ferment complex carbohydrates to produce metabolites including short-chain fatty acids (SCFAs), which play a crucial role in providing healthy symbiotic state in the GI tract (100). Predominant SCFAs found in the GI tract include acetate, butyrate and propionate, which facilitate the regulation of cellular processes including gene expression, differentiation, proliferation and apoptosis (101). The most abundant SCFA in the GI tract, acetate, is used in lipogenesis and cholesterol metabolism and may be involved in the central appetite regulation (102). Butyrate is the main energy source for colonocytes and is known to have anti-inflammatory and anticancer properties by inducing apoptosis (89,101,103). Butyrate is also lipogenic. Recently, several studies suggest that it also promotes gut symbiosis by protecting host cells from potentially pathogenic bacteria through activation of β-oxidation, which provides anaerobic condition in the gut lumen (89,104). Propionate is mainly absorbed by the liver, where it regulates gluconeogenesis and satiety signalling (105). It also regulates immune cells by production of anti-microbial factors, acting as an immune regulator (106). Butyrate is predominantly produced by *Firmicutes*, whereas the production of propionate is dominated by *Bacteroidetes* (89,107).

Interactions between the gut microbiota and host cells are tightly controlled by thick mucus layers and rich SCFAs in the healthy symbiotic state (Figure 9). The production of propionate and butyrate as well as the mucous layer is reduced in the dysbiotic state, which causes further imbalances in the tightly controlled metabolic regulation. Reduced production of propionate results in the reduction in immune cells such as mucosal-associated invariant T cells and Treg, which may lead to low-grade inflammation in the GI tract (87). When activation of β-oxidation by butyrate is reduced, it allows higher oxygen to be available for Enterobacteriaceae to proliferate further. When propionate and butyrate are absent, the glucose metabolism becomes diminished, resulting in a high blood glucose state. All in all, this may lead to a leakage of PAMPs from microorganisms including LPSs causing low-grade inflammation in the GI tract (87).

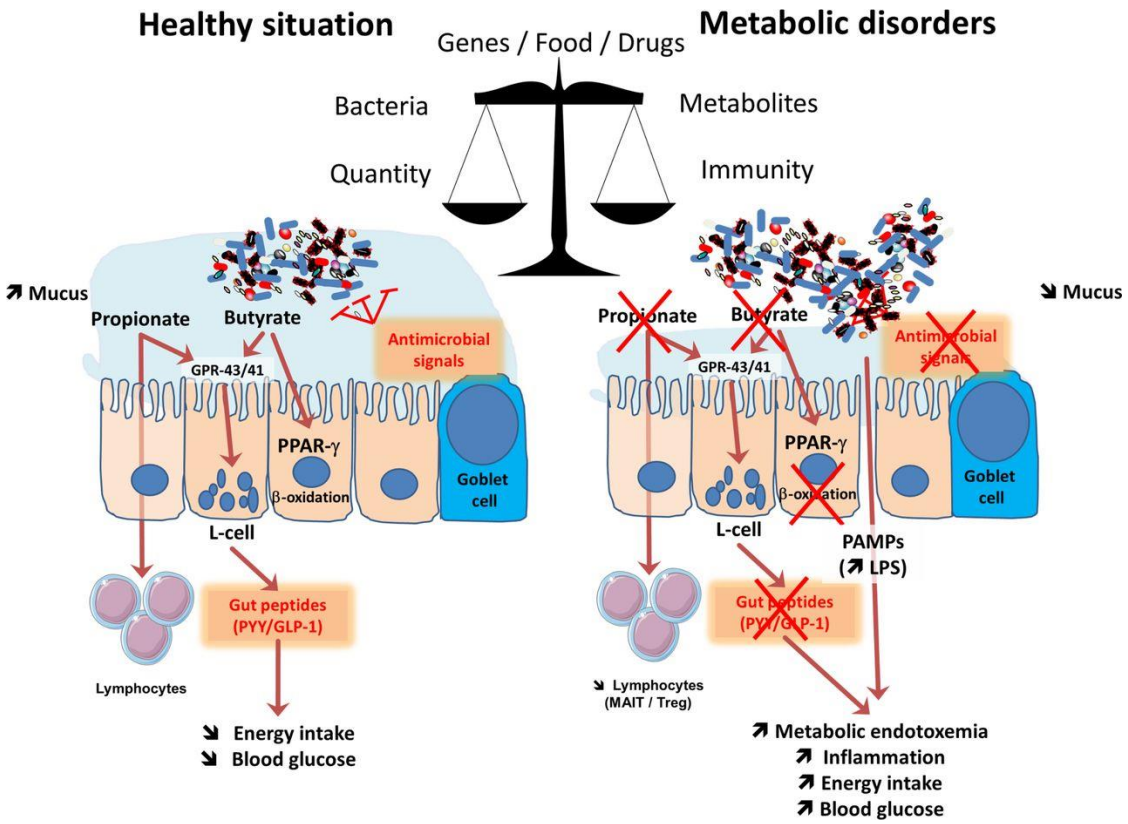


Figure 9: Microbes and host interaction: Impact of metabolism

Source: Cani, Human gut microbiota: hopes, threats and promises (87)

Secondary bile acids are another type of microbial metabolite which have been suggested to have protective functions against dysbiosis and colonic inflammation (108,109). Bile acids are a group of bioactive steroid acids predominantly found in the bile, which are subject to microbial modification transforming primary to secondary bile acids in the colon. (110). This has led to a recent increasing interest in a link between secondary bile acids and IBD including UC. Sinha et al. investigated faecal profiling of UC patients to measure the abundance of gut micro-organisms associated with secondary

bile acids production in UC and they found that *Ruminococcaceae*, which is a family of secondary bile acid producing bacteria and belongs to the phylum of *Firmicutes*, were significantly depleted in patients with UC (109). They also reported that two secondary bile acids (lithocholic acid and deoxycholic acid) as well as expression of the bile acid-inducible genes, which encode enzyme that convert primary biliary acids to secondary biliary acids, were significantly reduced in patients with UC (109). An altered bile acid pool, defined as the total amount of primary and secondary bile acids circulating in the enterohepatic circulation, has been also suggested to affect the composition of the gut microbiota contributing to the dysbiotic state (111).

The gut microbiota is also vital to the synthesis of essential vitamins, which cannot be synthesised by the host (112). Vitamins synthesised by the gut microbiota include Vitamin B12, folate, vitamin K, riboflavin, biotin, nicotinic acid, pyridoxine and thiamine (112,113). Other products of the gut microbiota have been implicated in human health. For instance, indolepropionic acid, metabolite of gut microbiota, is associated with lowering risk of type 2 diabetes and low-grade inflammation in the GI tract (114,115). Another metabolite of the gut microbiota, trimethylamine, is linked to atherosclerosis and thus cardiovascular diseases (116).

The GI cells are constantly exposed to a vast number of microbial antigens and metabolites. The human GI system is tightly regulated by fine-tuned interactions between gut microbes and the host immune system. Disruption of the gut microbiota, namely dysbiosis, has been suggested to be linked to not only CDI, but also to systemic conditions such as obesity, diabetes mellitus, liver disease, neurodegenerative diseases and IBD including UC (93,94,117). Thus, the human gut microbiota has been viewed as a potential source of novel therapeutics for these conditions.

2.4 The Gut Microbiota and Ulcerative Colitis (UC)

The pivotal role of the gut microbiota in the pathogenesis of UC has been described in many studies using several different approaches. Under normal conditions, the host's innate and adaptive immune responses prevent harmful bacterial invasion. In dysbiosis, these immune responses may be compromised. Furthermore, enterotoxins released from bacteria increase permeability of the gut mucosa. Some bacteria directly invade the intestinal epithelial cells, damaging the epithelial mucosal barrier. Antibiotics, which alter the human gut microbiota, have been shown to contribute to UC activity (118), whereas probiotics have demonstrated some efficacy in UC remission (119).

2.4.1 Bacteria and UC

The main four phyla commonly found in the human gut microbiota are *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* (29,120). Verrucomicrobia and *Fusobacteria* are also found, but less common (29,120). Although there have been many conflicting reports about the role of the gut microbiota in UC, the consensus is that the gut microbiota of UC patients has been found to be as much as 25% less diverse (68,121,122). It also appears that the gut microbiota of patients with UC has a lower abundance of *Firmicutes*, *Bacteroides* as well as *Actinobacteria* such as *Bifidobacterium* (123,124). The decrease in *Bacteroidetes* in UC mainly reflected reduction in *Bacteroides*, *Paraprevotella* and *Alloprevotella* in some studies, whereas other studies found significant increases in *Bacteroidetes* amongst UC patients (125,126). The Gram-negative, mucin-degrading, strictly anaerobic bacterium, *Akkermansia muciniphila*, which belongs to the phylum of Verrucomicrobia, is believed to utilise colonic mucin (127). *Akkermansia muciniphila* constitutes 3-5% of healthy intestinal microbiota in the colonic epithelium mucous layer and has a significant role in host-microbiota interaction through regulating inflammatory and metabolic pathways (128,129). Many studies report that reduced *Akkermansia muciniphila* abundance is linked not only to UC, but also other conditions including obesity and type 2 diabetes (128,130). Table 3 summarises the gut microorganisms, which are known to have a link with UC. Several other bacterial pathogens are suggested to be linked to UC, which include *Clostridioides difficile* (131), *Helicobacter* species (132), *Yersinia* species (133) and *Fusobacterium* species (134), though no bacteria responsible for pathogenesis of UC have been identified to date.

SCFA producing bacteria

SCFAs, such as acetate, butyrate and propionate, are believed to play an important role in healthy symbiotic state in the GI tract, and several studies report that a decreased level of SCFAs was found in the microbiota of UC patients (135,136). A recent study has shown that butyrate from *Faecalibacterium prausnitzii* not only has anti-inflammatory properties, but also provides the major nutrient for colonocytes (136), and prevents intestinal mucosa atrophy and colonocyte autophagy (137). Two key butyrate-producing *Firmicutes*, *Roseburia hominis* and *Faecalibacterium prausnitzii*, were found to be significantly less abundant in patients with active UC (138). Furthermore, Varela et al. also found that the number of *Faecalibacterium prausnitzii* was significantly increased amongst UC patients under remission (137), suggesting that *Faecalibacterium prausnitzii* might be one of the responsible bacteria leading to dysbiosis and UC.

Bacteria involved in the T-cell response

Recent studies suggest that some bacteria are involved in the differentiation of T cell subsets, causing inflammation (139,140). Both T helper cells and regulatory T cells (Treg) are different types of T cells, which play key roles in anti-inflammatory and defence mechanisms in the adaptive immune response. A study on experimental models of colitis demonstrated that the supernatant of *Faecalibacterium prausnitzii* regulates between T helper 17 (Th17) and regulatory T (Treg) cells differentiation, implying that it plays a role in the inflammatory response (141). T helper cells activate B cells to secrete macrophages and antibodies to destroy microbes as well as facilitate activation of cytotoxic T cells to kill infected target cells (142). Th17 cells, in particular, are known to maintain mucosal barriers, whereas Treg suppress immune response by inhibition of T cell proliferation and cytokine production (143). The same study also showed that butyrate produced by *Faecalibacterium prausnitzii* had anti-inflammatory effects by inhibiting the Signal Transducer and Activator of transcription3 (STAT3)/IL-17 pathway and interleukin 6 (IL-6)/signal transducer and promoting forkhead box protein P3 (Foxp3) (141). Another study suggests that *Faecalibacterium prausnitzii* stimulates IL-10 secretion and inhibits IL-12 and interferon-γ expression in the human GI tract (144). Moreover, capsular lipopolysaccharide A of *Bacteroides fragilis* was reported to be protective against inflammation in the gut through IL-10 producing CD4+ T cells (145,146). Segmented filamentous bacteria (SFB), which primarily colonise the ileum, are also reported to protect against invasion of pathogens in the gut by promoting Th17 cell differentiation (147). Other bacteria, which have anti-inflammatory or immune response modulating properties, are *Lactobacillus reuteri*, *Lactococcus lactis* and *Clostridioides* strains (148–150).

Table 3: The summary of alterations of the gut microbiota in UC

Phylum	Species/Genus/Family	Functions
Less abundant		
Firmicutes	*Faecalibacterium prausnitzii*	• Produce butyrate • Stimulate IL10 secretion • Anti-inflammatory (138,141)
	Roseburia hominis	• Produce butyrate (138) • Anti-inflammatory
	Ruminococcaceae	• Convert primary to secondary bile acids (109)
Verrucomicrobia	*Akkermansia muciniphila*	• Utilises colonic mucin • Anti-inflammatory (122,127)
Actinobacteria	*Bifidobacterium*	• Produce lactic acid and lower the pH for optimum environment for bacterial enzymes (151,152)
Bacteroidetes	*Bacteroides*	• Produce propionate to facilitate immune regulation (106)
More abundant		
Proteobacteria	*E. coli*	• Pro-inflammatory caused by adherent-invasive property (153)
Fusobacteria	*Fusobacterium varium*	Unknown (154)

2.4.2 Virome in UC

Zuo et al. characterized the mucosal virome in patients with UC (155). The virome is the collection of nucleic acid both DNA and RNA that constitute the genomes belonging to the viral community. The authors found that patients with UC showed decreased diversity of the mucosal virome compared with healthy individuals. *Caudovirales* are an order of viruses also known as the tailed bacteriophages, accounting for 95% of known phages (156). *Caudovirales* were the predominant bacteriophages in UC, but the diversity of this group of viruses was low. The dominant viruses found in mucosa of patients with UC was *Ascovirus* at the genus level and *Streptococcus phage* at the species level (155). Furthermore, the inflamed mucosa of patients with UC contained more abundant *Caudovirales* bacteriophages than non-inflamed mucosa of patients with UC or healthy control individuals. The

authors also reported a significant abundance of *Escherichia* phages and *Enterobacteria* phages in the mucosa of patients with UC.

In this study, the authors highlighted that the dysbiotic mucosal virobiota was characterized by a decreased viral diversity, which was similar finding to the reduced bacterial diversity seen in the gut microbiota. All in all, the authors suggested that alterations of the mucosal virobiota and a significant increase in *Caudovirales* bacteriophages, in particular, may contribute to disease pathogenesis (155).

2.4.3 Fungal Microbiota in UC

Candida and *Phialemonium* are present in the stomach as they survive the low pH environment. In the colonic mucosa, *Dothideomycete* sp. *Galactomyces geotrichum* and *Ustilago* sp. were most abundant (157). Sokol et al. reported that faecal samples of patients with IBD demonstrated a higher proportion of *Candida albicans*, an increased Basidiomycota/Ascomycota ratio and a decreased proportion of *Saccharomyces cerevisiae* (158). Qiu et al. recently reported an increased population of *Aspergillus*, *Sterigmatomyces, Saccharomycetales* and *Candida* amongst patients with UC, whereas *Emericella*, *Exophiala*, *Alternaria, Epicoccum*, *Acremonium, Penicillium* and *Trametes* were decreased (159), though this study was based on only 14 patients with UC. Studies examining the fungal community in patients with UC are still very limited.

2.5 Faecal Microbiota Transplant (FMT)

FMT is an infusion of faecal suspension with a full spectrum of gut microbiota from a healthy individual to restore dysbiosis of affected individuals.

The principle of FMT was described in the 4th century. Chinese medicine described “yellow soup” for a treatment of severe diarrhoea (160). The first modern FMT in humans was performed in 1958 as a treatment of pseudomembranous colitis in the United States (161). However, interest in FMT declined with growing antibiotic development in the 1960s.

The interest in FMT re-emerged following successful treatment of refractory colitis after CDI. Recently, two major multicentre studies demonstrated up to 98% success rate in treating recurrent CDI compared with conventional vancomycin therapy at 31% (162,163). In 2014, FMT use in the management of recurrent CDI was approved by NICE and FMT use was introduced into our clinical

practice. Since then, the interest in the use of FMT to restore dysbiosis for other colonic conditions has been fuelled world-wide.

Although the precise aetiology of UC is unclear and appears to be multi-factorial, many studies suggest that dysbiosis of the human gut microbiota may play an important role in pathogenesis (3,75,164). This has led a recent interest in restoring the gut microbial diversity with FMT as a therapeutic approach in the management of UC. If the colonic microbiota can be recovered, the dysbiotic state may be ameliorated, engendering complete remission of this chronic and debilitating condition without lifelong medication or the need for major surgery. The ability to demonstrate both restoring symbiosis and remission of the disease may be able to change the treatment paradigm for the condition.

2.6 The Role of Faecal Microbiota Transplant (FMT) in UC

In recent years, a few randomised clinical trials (RCTs) on FMT use in the management of UC have been published with encouraging results (25–28). The Cochrane review on FMT in UC (2018) included four randomised studies involving 277 patients (165) as shown in Table 4. It found that 37% (52/140) of UC patients achieved clinical remission at 8 weeks after FMT treatment, whereas 18% (24/137) patients in the control group achieved remission. Thus, two-fold more clinical remission was achieved following FMT than in the control subjects. The rate of serious adverse effects was similar in the FMT and control groups. Serious adverse effects of FMT included worsening of UC symptoms requiring intravenous steroid or surgical interventions. Infections with *Clostridioides difficile* and *Cytomegalovirus* were also seen. The incidence of minor side effects was also similar in both groups and included abdominal pain, bloating, flatulence, nausea, headaches and fever.

Moayyedi et al. (166) randomised 75 patients with active UC into two arms – 38 patients for weekly FMT from healthy anonymous donors and 37 patients with weekly water enema for 6 weeks. The study concluded a significant difference with 24% of the FMT group achieving clinical remission (defined as a Mayo score (167) <3 with an endoscopic Mayo score of 0) after 7 weeks compared with 5% of the control group.

Rossen et al. (168) obtained an opposing result. They randomised 50 patients with mild to moderately active UC to two arms – 23 patients with two doses of FMT from healthy donor at week 0 and 3 via the nasoduodenal route and 25 patients with autologous FMT. They used a different disease scoring system from Moayyedi et al., simple clinical colitis activity index, as the primary end point and defined clinical remission as simple clinical colitis activity index ≤2 as well as ≥1-point decrease in the Mayo

endoscopic score at week 12. They concluded that there was no statistical difference between two intervention groups.

Paramsothy et al.(169) similarly randomised 85 patients with active UC with Mayo score 4-10 into FMT and control groups. They used colonoscopic infusion as a route and enemas 5 days a week for 8 weeks. The primary outcome of the study was steroid-free clinical remission with endoscopic remission with Mayo score ≤2 as well as ≥1-point decrease in the Mayo endoscopic score at week 8. They found that 27% of patients in the FMT group achieved the primary outcome compared to 8% of patients in the control group with p = 0.021.

Costello et al.(170) conducted randomised 73 patients with active UC with Mayo score 3-10 into donor FMT and autologous FMT groups. 38 patients received donor FMT and 35 patients did autologous FMT via colonoscopy at day 1 as well as 2 enemas by day 7. The primary outcome of this study was steroid-free remission defined by a total Mayo score ≤2 and an endoscopic mayo score of ≤ 1. They concluded that 32% of patients who received donor FMT achieved the primary outcome, compared to 9% of patients with autologous FMT, which was a statistically significant difference with P = 0.02.

Since the Cochrane review in 2018, two further RCTs were published (Table 4).

Crothers et al. (171) is the first published RCT that used capsule FMT in addition to a single infusion via colonoscopy. The authors have not yet published a journal paper and only abstract is available on publication search. 15 patients with UC were included in the study - seven patients in the FMT group and eight patients in the control group. Although two patients were excluded from the study due to an absence of active inflammation at the time of endoscopic assessment, it is unclear these patients were allocated in the FMT group. The authors reported that two out of seven patients achieved clinical remission (defined as a Mayo score <3).

Sood et al. (172) randomised 61 patients with active UC. 31 and 30 patients were allocated to the FMT and placebo group respectively in this study. The intervention group received four doses (50ml each) of FMT, which was made up with 100g stool obtained from a single unrelated donor diluted with 200ml of saline. They also used Mayo score to measure clinical remission and defined it as having Mayo score ≤2 with all sub-scores ≤1 as well as being steroid free at week 48. 27/31 (87.1%) amongst the FMT intervention group and 20/30 (66.7%) amongst the control group achieved clinical remission and they did not find a statistically significant difference. This study was the longest follow-up period amongst the RCTs, which might have affected the outcome.

Table 4: Recent double-blind RCTs of FMT in UC

Author	Patients	Severity	Donor	Route	Dosage	Frequency (Number of infusions)	Fresh vs Frozen	Pre-ABx	Bowel lavage	Primary endpoint	Clinical remission (CR)	Endoscopic remission (ER)	Histologic remission (HR)	Follow up (weeks)
Moayyedi et al. (2015) (166)	75 38 FMT, 37 Control	Mild-severe (4-12)	Unrelated	Enema	50g stool in 50ml infusion	6 (weekly)	Frozen 21, Fresh : 15, Mixed : 1	No	No	CR: Mayo < 3 ER : Mayo <0	9/38 (24%) Vs 2/37 (5%)	9/38 (24%) vs 2/37 (5%)	7 FMT 1 placebo	7
Rossen et al. (2015) (168)	48 23 FMT, 25 control (autologous stool)	Mild-moderate (SCCAI 4-11)	Unrelated & related	Nasoduodenal tube infusion	Minimum 60g stool in 500ml	2 (3 weeks apart)	Fresh	No	Yes	CR and endoscopic improvement SCCAI ≤ 2 with ≥ 1 drop in Mayo endoscopic score	7/23 (30%) Vs 8/25 (32%)	No record	No record	12
Paramsorthy et al. (2017) (169)	81 41 FMT 40 controls	Mild-moderate (Mayo 4-10)	Unrelated multi-donor	Colonoscopy followed by enema	37.5g stool in 150ml saline infusion	40 (5/week for 8 weeks)	Frozen	No	Yes	Steroid free CR and endoscopic improvement, Mayo ≤ 2 With Subscore ≤ 1 & ≥ 1 drop in endoscopic score	18/41 (44%) Vs 8/40 (20%)	5/41 (12%) vs 3/40 (8%)	No record	8
Costello et al. (2017) (170)	73 38 FMT, 35 control (autologous stools)	Mild-moderate (Mayo 3-10)	Unrelated pooled donors	Colonoscopy followed by enema	NR	3 (3/week)	Frozen	No	Yes	Steroid-free CR : Mayo ≤ 2 Endoscopic score ≤ 1	19/38 (50%) vs 6/35 (17%)	21/38 (55%) vs 6/35 (17%)	No record	8
Crothers et al. (2018) (171)	15 7 FMT, 8 control	Mild-moderate (Mayo 4-10)	2 donors with high butyrate in stool	Colonoscopy and daily FMT capsule	50g stool (colonoscopy) 0.375g (capsule)	Not recorded	Not recorded	Yes	Not recorder	Measurements of stool butyrate and other SCFAs, mucosal serotonin transporter, tryptophan hydroxylase 1, CR: Mayo score < 3	2/7 (29%), 1/8 (13%)	Decrease in Mayo sub score > 1 or UCEIS > 2, 3/7 (43%) vs. 0/8 (0%)	3/6 in FMT, 0/6 in control	12
Sood et al. (2019) (173)	61 31 FMT 30 Control	Mild to moderate (Mayo 4-10)	Unrelated, single	Colonoscopy infusion	100g stool diluted in 200ml saline	1	Frozen	No	Yes	Steroid-free CR: Mayo score ≤2 and all sub-scores ≤1	27/31 (87.1%) vs 20/30 (66.7%)	Endoscopic mayo score 0, 18/31 (58.1%) vs. 8/30 (26.7%)	14/31 (45.2%) vs. 5/30 (16.7%)	48

2.7 Challenges of FMT in UC

Although the Cochrane review and meta-analysis demonstrated promising results, clinical application of FMT in management of UC requires more research. Subgroup analysis of these studies elucidated multiple issues (165,174). Although indications for the FMT interventions were similar, the treatment protocols and intervention methods differed significantly between studies. Clinical and methodological heterogeneity should not be overlooked before application of FMT in management of UC in the clinical settings.

2.7.1 Regulation of FMT

The regulation of FMT varies depending on its definition across the world. In the UK, there has been much debate like many other countries, and currently FMT is regarded as a medicinal product and regulated by the Medicines and Healthcare products Regulatory Agency (MHRA) in the U.K. (175).

In 2014, The EU Commission expressed their legal opinion on the regulation of FMT. The Commission considered faecal microbiota as a combined substance of cells and several other components from non-human origin (176). The Commission recognised that FMT contains human cells, though FMT samples are transplanted for the other components rather than the human cells. Thus, the Commission concluded that FMT is not covered by the European Human Tissue Directive 2004/23/EC (177). However, the Commission recognised the importance of setting high standards of quality and safety for FMT.

In the U.K., FMT was regulated by the Human Tissue Authority (HTA) and fell under the jurisdiction of the Human Tissue Regulation 2007 until 2015. Following the EU Commission announcement, the HTA defined FMT as a medicinal product, and FMT then fell under jurisdiction of the Human Medicines Regulations 2012 (175). Since FMT is now defined as a medicinal product and regulated by the HMRA, it can be produced and prescribed locally by a medical practitioner under a pharmacy exemption (178). Furthermore, a Specials licence became mandatory to produce and supply FMT to a third party since this change. This re-classification of FMT in the UK brought repercussions to many units since FMT became only available to intra-institution supply. This FMTUC study was also affected by this revised regulatory position and local production of FMT was the only option to continue the study until recently when the first English stool bank was established in Birmingham (178).

In the United States and Canada, FMT was unregulated initially, though the Food and Drug Administration (FDA) defined that FMT is classed as a biologic drug in 2013(179). Currently, FMT is only licensed to treat refractory CDI, but is permitted for use as an investigational product with the investigational new drug (IDA) approval after patients provide informed consent.

2.7.2 Preparation of FMT Samples

The most recent publication from the European Consensus Conference became a guideline for current FMT use in clinical practice (180), so far various methods have been used for preparation of FMT samples in different studies. Since most of the gut bacteria are anaerobes, it may be advantageous to prepare FMT under anaerobic conditions though some studies prepared FMT in normal atmospheric conditions. When the FMT is prepared in aerobic conditions, faeces are exposed to non-biological conditions, which may affect the microbial compositions. Amongst the aforementioned RCTs, the donor faeces were prepared in aerobic conditions except for Costello et al., who prepared the faecal samples under anaerobic conditions.

FMT can be in fresh or frozen forms, and Table 5 summaries key steps for the preparation of fresh and frozen FMT materials. Three RCTs have compared fresh and frozen FMT, however, these studies compared efficacy of fresh and frozen FMTs on patients with CDI rather than UC (181–183). Yet, all of the studies reported that it did not differ between the two preparations. Since the two preparations achieve similar outcomes, most of the recent studies have employed frozen FMT materials. This is because frozen form is easier to handle, transport and store. Furthermore, it is cheaper to use frozen FMTs especially when there is no available laboratory to prepare the FMT samples (184).

Table 5: Summary of general steps for the preparation of the fresh and frozen FMT materials (85)

Fresh FMT material
• Fresh stool must be used within 6 hours after evacuation or the same day • The storage and preparation should be as short as possible to protect anaerobic bacteria • The FMT sample can be stored at ambient temperature (20-30 ºC) • Anaerobic storage and processing should be applied if applicable • A minimum volume of fresh faecal sample is 30g • Stool sample must be suspended with sterile saline solution using a blender or manual effort. This is then sieved to avoid the clogging of infusion syringes and tubes • A dedicated space which is disinfected using measures that are effective against sporulating bacteria should be used • Protective gloves and facial masks should be used during preparation
Frozen FMT material
• A minimum of 30g faecal sample should be diluted with 150mL of saline solution • Glycerol should be added before freezing (10% of a final concentration) • Faecal suspension must be thawed in a warm water (37 ºC) on the day of FMT infusion. The transplant should be performed within 6 hours from thawing • Saline solution can be added for a desired suspension volume after thawing • Repeated thawing and freezing must be avoided

Fresh FMT materials

The method for preparing fresh FMT has been derived from the protocol for treating refractory CDI. Since the principal aim of FMT is to restore the gut microbiota for any conditions, it is reasonable to follow the same protocol for treating UC.

Preparation of FMT samples varies across studies, though many FMT samples are homogenized before interventions. The European consensus guidelines recommend the use of 0.9% sterile saline solution with three to five times larger volume of solvent. For instance, 50g of faecal sample should be diluted in 150 – 250mL of saline. The guidelines recommend 0.9% sterile saline as a solvent for its superior ability to preserve microbes, however, some studies reported that waster was also used successfully (185,186). After homogenisation, the solids should be strained using gauze, a tea strainer or a similar device and the suspension must be stored in a sterile container.

The challenges of fresh FMT preparation are stool handling and a short therapeutic window. Since it involves fresh faecal materials, patients may experience undesired features of fresh stools. Furthermore, fresh FMT samples should be used within 6 hours after defecation, meaning timing of FMT treatment becomes unpredictable and difficult to prepare for recipients. It may also mean that the location of patients become selective.

Frozen FMT materials

The biggest advantage of frozen FMT is that it can be stored for a much longer period of time than fresh FMT, which is crucial for stool banks. Furthermore, it is much easier to standardise the FMT protocols. All in all, the logistics of FMT interventions using a frozen form is much simpler and more practical for clinicians as well as patients.

The European consensus guidelines suggest using at least 30g of donor faeces with 150mL of 0.9% saline solution to homogenise first. The guidelines also advocate adding glycerol (10% of the final volume) to protect microbial cells from damage caused by freezing (187,188).

Similar to fresh FMT samples, frozen FMT preparation under normal air or anaerobic atmosphere achieved similar resolution rate in treatment of CDI (183). Extrapolating the result to UC may not be straightforward, however, given that pathology of UC is more complex than CDI. A significantly reduced level of the Gram-negative anaerobic bacterium *Akkermansia muciniphila* and also *Bacteroidetes* have been reported in patients with UC, which may mean that anaerobic preparation to preserve these bacteria is vital in efficacy of the FMT treatment of UC. On the other hand, Gram-positive bacteria, such as *Firmicutes,* have also been reported to be reduced in patients with UC (75). For those, anaerobic condition would not be an ideal condition. A study, which investigated resilience of *Akkermansia muciniphila* under different conditions including oxygen exposure and extreme temperatures, demonstrated a high oxygen tolerance between 4 and 37 degree up to 72 hours (189). Furthermore, many studies also propose that the dysbiotic gut microbiota is accountable rather than a single pathogen causing the condition. All in all, without identifying responsible pathogens for UC, the optimum environment to prepare FMT is unknown.

The guidelines suggest that the FMT should be stored at -80 ºC, however, some argue the extreme cold temperature may interfere with subsequent DNA extraction and microbial evaluation. Several studies assessed the effect of freezing FMT on microbial community quantifications and extracted DNA yield, and the results suggested that there was no significant difference in microbiota profiles at the phylum

and family levels amongst fresh and frozen samples after short periods of storage, but that long-term storage can cause instability of taxonomic profiles (190–192). Costello et al. examined this using different storage periods (two and six months) of frozen FMTs in 10% glycerol, which showed that the microbiota remained mostly unchanged, however, two month old sample showed a significant reduction in *aerobes* and *Lactobacilli*, and the six month old sample lost coliforms (193). This study did not find a difference in anaerobic bacteria or Bifidobacterium after 6 months. Costello et al. concluded that frozen FMTs stored in 10% glycerol are safe to use for at least 6 months without loss of viability and clinical efficacy, though their clinical outcome was a treatment for CDI.

The guidelines mention that frozen FMT samples should be thawed with 0.9% saline solution to make up a desired volume in a body temperature (37 ºC) warm water bath on the day of infusion and the samples must be used within 6 hours (180). Repeated thawing and re-freezing should be avoided.

Donor selection and screening

It is crucial to select appropriate donors to prevent adverse effects from the infused faecal material. The European guidelines for FMT recommends following the European Commission exclusion criteria for selection process of allogenic donors of human tissue transplants (180). They advocate a questionnaire for potential donors, which addresses their medical history and lifestyle to identify the potential risk factors for potential infections. Additionally, individuals with GI disorders or those on any medications which may interfere with gut microbiota are excluded. Once suitable donors for FMT are identified, the European guidelines recommend performing a series of tests of blood and stool for multiple bacterial and viral infectious conditions. In this study, the same checklist for our blood and stool tests for potential donors was used (Table 15).

Single vs multiple donors

It is yet unknown whether there is any outcome difference when patients with UC are treated with FMT samples from single donor or multiple donors. The previous RCTs (Table 4) used FMT from single as well as multiple donors. FMT samples were obtained from a stool bank in Portsmouth before the regulation change, and afterwards, from a local laboratory in this FMTUC study. In this study, if subjects received multiple doses, they could receive FMTs from different donors. All donor samples were stored for the duration of the study.

Related vs unrelated donors

Early FMT offered recipients the choice a their relative as a source of FMT samples, which was supported by early studies (194,195). The preference for related donors was attributed to their genetic and environmental circumstance resemblances. However, recent reviews report no statistically significant difference in outcome of FMT based on donor relation, though these studies are outcome of FMT for CDI (196–198). It is possible that related donors might have similar GI microbiota which might partly defeat the purpose of FMT. In recent years, the use of unrelated donors has become more popular with an increased use of stool banks. The advantages of stool bank to process FMT from unrelated donors supersedes the current evidence on benefit of the use of related subjects for FMT. This study uses unrelated donors.

2.7.3 Interventions

Route of Administration

The aforementioned double-blind RCTs (Table 4) employed different methods for the route of FMT administration; upper GI tract infusion via endoscopy, enema, infusion to the caecum via colonoscopy and capsule, though the best route of administration for FMT is unknown. A recent European consensus conference on FMT in clinical practice advocates infusion of FMT to the caecum via a colonoscopy (180). This recommendation is based on higher resolution rates of refractory CDI reported in many systematic review and meta-analyses, however, the principle is plausible for UC since FMT can be applied to the entire colon when FMT is infused from the caecum (198–200). One of the challenges of administration of FMT per rectum is that FMT can only be distributed to where the enema reaches, meaning FMT may not reach to the diseased part of the colon. Hence, some studies suggest that repeated administrations may be required to achieve the therapeutic effects. The advantages of enemas that they are much easier to handle, less invasive and cheaper than endoscopic infusion.

FMT can also be administered to the upper GI tract via endoscopy or nasogastric/nasojejunal route. Patients are required to be kept in a 45-degree upright position for at least 4 hours to avoid aspiration. An advantage of the upper GI tract route includes the much lower volume (25-50mL) needed to achieve the same effect as the lower GI route (201). The peri-interventional use of proton pump inhibitor and prokinetics were suggested with upper GI delivery, however, the use of these agents should be carefully considered since they can modify gut microbiota significantly due to the variation of acidity and electrolytes in the different part of the GI tract before reaching to the colon (202).

Since enema form was chosen as the route of administration in this study, inclusion criteria included that the disease is limited to 40 centimetres from the anal verge.

FMT dosage and frequency

Not only is the effectiveness of FMT for UC not yet known, the optimum dose and frequency of FMT infusions are also unknown. FMT success may be dependent on frequency, the number of infusions and/or dose given. Furthermore, duration of the treatment is unclear. This reflects on the significant differences in dosage, frequency and duration amongst the aforementioned RCTs.

Paramsothy et al. (169) employed a daily dose of FMT for five times a week for eight weeks within a total of 40 doses, whereas Rossen et al. (168) treated with two doses with three weeks apart. Although Paramsothy et al. appeared to demonstrate better clinical outcomes amongst the treatment group compared to their control group or Rossen's intervention group, there are other variable parameters that may have affected the results. Heterogeneity of FMT study protocols makes comparisons of these studies extremely challenging.

Some studies reported clinical remission after a single dose of FMT (203). Conversely, Paramsothy et al. (169) followed 63 participants of the intervention group for further 8 weeks after the trial period, of which 20 relapsed and required further treatment. Future studies are required to address the optimum dose, duration and frequency of FMT treatment in management of UC.

Who to treat?

Patients who were enrolled in RCTs described (Table 4) received concomitant treatments for UC including steroids and/or immunosuppressive therapy such as azathioprine, mesalamine or tumour necrosis factor antagonists. Paramsothy et al. (169) and Costello et al. (170) defined their primary points as steroid-free remission. The use of concomitant treatments can obscure the effect of FMT treatment in UC making it difficult to understand the true effect of FMT in management of UC. It is not known whether FMT is only effective alongside concomitant standard treatment. In this study, only participants who were treatment naïve were included to assess the effectiveness of FMT.

Another question is the relationship between FMT success and a degree of disease severity. Moayyedi et al. included patients with severe symptoms and demonstrated that these patients were less likely to achieve clinical remission with FMT treatments (166). Furthermore, the authors reported that newly diagnosed UC patients who were treated with FMT responded better than chronic patients, suggesting

a critical window for FMT interventions. In early disease, dysbiosis may be more receptive to reversal. Again, more studies are required to answer these unknowns.

Pre-FMT preparation of the recipients

Antibiotics

To maximise the benefit of FMT, the use of antibiotics has been proposed to cleanse the existing gut microbiota. The European guidelines advise a 3-day course of fidaxomicin and vancomycin before the FMT treatment for the treatment of CDI (180). However, there is no clear consensus on antibiotics use for patients with UC prior to the FMT treatment.

Ishikawa et al. conducted a clinical trial to study the effect of pre-FMT antibiotics treatment (fosfomycin, metronidazole and amoxicillin for 2 weeks) (204). In total 41 patients were enrolled, but 36 completed the study, of which 17 patients received antibiotics and FMT treatments and 19 patients received antibiotics alone. The authors found that the antibiotic and FMT combination therapy group showed better clinical response as well as clinical remission rates than the antibiotic monotherapy group, however, the difference was not statistically significant. The authors also observed that the proportion of *Bacteroidetes* was almost abolished two weeks post-antibiotics therapy, though it recovered after four weeks amongst clinical responders who received the combination therapy. This trend was not observed amongst antibiotic monotherapy group. Bacteria from the *Bacteroidetes* phylum have been reported to be less abundant in patients with UC (205). The authors concluded that a combination therapy of FMT and antibiotics synergistically improved the *Bacteroidetes* composition, which correlated to clinical responses.

Keshteli et al. conducted a meta-analysis study on antibiotic use as a preparation for FMT (206). This study included nine studies of 118 patients in total and concluded that antibiotic preparation before FMT likely improves clinical remission rates in patients with UC. However, limitations of this study were significant heterogeneity related to the FMT intervention protocols as well as the antibiotics used for pre-treatment. At present, there is no consensus on which antibiotics would provide the best bowel cleanse and synergistic effects with FMT interventions.

In this FMTUC study, oral formulation of vancomycin, metronidazole and rifampicin were used to achieve eradication of resident host microbiota in patients to maximise engraftment of FMT of donor microbiota in FMT.

METRONIDAZOLE

Metronidazole is a prototype nitroimidazole antimicrobial and has been widely used in GI infections for the treatment of anaerobic infections (207). The nitroimidazoles are bactericidal with toxic metabolites, which break DNA strands. Metronidazole can be administered orally, intravenously, per rectally and intravaginally, absorption of oral metronidazole is unaffected by infection and its bioavailability is 100% (207). Furthermore, food is not known to cause a significant effect on absorption. The serum half-life of metronidazole is approximately 8.2 hours (208). The common side effects of metronidazole include metallic taste, nausea, vomiting and diarrhoea (209). Metronidazole can cause more serious side effects such as neurotoxicity and optic neuropathy, though is very rare (209). Oral metronidazole was the first-line antibiotic for mild to moderate CDI until recently the NICE guideline was updated to the current protocol in January 2021 (210). Metronidazole is often used to treat complications of CD, though data is weaker for UC (211).

VANCOMYCIN

Vancomycin is a glycopeptide antibiotic and It is bactericidal by inhibiting bacterial cell wall biosynthesis (212). Vancomycin has been widely used to treat Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA). Since the recent NICE guideline update, vancomycin has become the first-line antibiotic for CDI (210). Previously, vancomycin was used to treat severe CDI or when patients were unresponsive to metronidazole treatment. Vancomycin can be administered orally or intravenously, though for CDI treatment it must be administered orally as it reaches the site of infection directly from the intestine. Vancomycin when given parenterally is known to cause nephrotoxicity, ototoxicity as well as bone marrow suppression, requiring therapeutic monitoring for safe use. However, as it is not absorbed from the gut, this is not a concern when given orally.

RIFAMPICIN

Rifampicin belongs to the rifamycin group and inhibits RNA synthesis by blocking bacterial DNA dependent RNA polymerase (213). Rifampicin is also administered orally or intravenously. When it is taken orally, it should be taken on an empty stomach as food consumption is known to cause inhibition of its absorption from the GI tract (214). Rifaximin, a derivative of rifampicin, was investigated previously to establish whether it induces remission in CD (69,215), but was not found to do so.

Bowel cleanse

As for pre-treatment antibiotics, there is no clear consensus regarding pre-treatment bowel lavage, however, it is logical to consider bowel cleanse before the FMT to maximize the engraftment of the gut microbiota from the transplanted faecal samples. The European consensus recommends that recipients should receive bowel lavage by polyethylene glycol for CDI treatment for the same reasons (180). Previous RCTs used different approaches. Rosen et al. cleansed bowel before each dose of FMT, whereas Moayyedi et al. did not administer pre-treatment bowel cleanse. Costello et al. and Paramsothy et al. used a one off bowel cleanse technique before the FMT interventions.

In this study, polyethylene glycol was also used to prepare the bowel (single dose) for maximum engraftment of FMT. Polyethylene glycol (or macrogol) is an oral liquid medication commonly used to treat constipation. Polyethylene glycol is dissolved in water and forms hydrogen bonds with water molecules, causing water retention in the faeces and increasing the osmotic pressure (216). This results in softening of the stool, allowing more frequent bowel movements.

All in all, there are many unknowns around the use of FMT in UC at present. Extensive research about FMT intervention protocols including pre-treatment, dose, route, duration and frequency of FMT is required to conclude the efficacy of FMT in management of UC. Even if the efficacy of FMT were established, the effectiveness of FMT must be further investigated to introduce FMT in the management of UC. The rationale for this feasibility study is to define the optimal parameters for delivering FMT and measuring response in a trial setting, with microbiota characterisation through 16S ribosomal RNA sequencing analysis. The data will potentially permit the design of a large scale multi-centre study aimed at establishing the efficacy of FMT in UC management.

2.8 Manufacturing FMT Samples

Regulation of FMT is changing in accordance with emerging evidence of the safety and efficacy of FMT. Currently, FMT must be manufactured in line with the guideline published by the Medicine and Healthcare Products Regulatory Agency (MHRA) in the UK.

When this feasibility clinical trial study was formalized, the MHRA was consulted to clarify the position of FMT use in UC and approved to conduct the study from the regulatory point of view. In this study, FMT samples were acquired from Wessex stool bank (Queen Alexandra Hospital, Portsmouth, UK). This manufacturer distributed FMT products to its own hospital and surrounding

hospitals mainly for treatment of CDI. The FMT products were delivered in a frozen form with a designated medical carrier company and a robust protocol was in place to receive these products. FMT from Wessex stool bank were used until January 2017, and the first six patients were treated with these FMT products. However, the manufacturer was forced to stop distributing products after January 2017 because of alterations in the regulations. Other manufactures including a laboratory in Birmingham University Hospital were contacted, but they were facing the same licensing issue at that time. Thus, the Chief Investigator of this study negotiated an exemption with the MHRA to permit usage of locally processed donor stools for the trial, which was granted after a lengthy negotiation.

Many potential premises were explored before an agreement was reached to utilize fume-cupboard within the pathology laboratory in Singleton Hospital, Swansea to process the stools.

The Standard Operating Procedure (SOP) (Appendix 1: Standard Operating Procedure (SOP) for manufacturing FMT products) for manufacturing the FMT samples were produced. This SOP was tailored based on the SOP from the Wessex stool bank to minimise discrepancy between the two different FMT manufacturing methods. Healthy potential donors from Swansea University were also screened, but many of them had previous the Epstein-Barr virus (EBV) exposure and thus did not satisfy the eligibility criteria. Thus, donors with EBV antibodies were included if recipients were also EBV positive. In total of five donors were recruited in this study.

2.9 Clinical Remission and Endpoints

Since the first disease activity outcome measurement, the Truelove and Witts Index, was developed in 1955, numerous outcome measure instruments have been developed (53). The purpose of these indices is to provide an objective measurement of disease activity by employing typical symptoms such as stool frequency and rectal bleeding. Thus, they can facilitate clinicians as well as patients to make agreed management plans for their disease conditions. However, there is no gold standard disease activity assessment tool. Many disease activity and outcomes are assessed with different assessment tools. Not only have the number of these assessment tools been growing, but also the assessed disease components have been expanding. Traditionally, disease activity has been assessed based on subjective and objective clinical symptoms. Endoscopic assessment is another dimension of the disease that has been mandated by the Food and Drug Administration (FDA) recently (217). There is much discussion whether macroscopic assessment of the bowel is enough to determine disease activity (50).

Currently, in clinical settings, disease activity is monitored with an assessment tool of the clinician's choice. This may result in diversity of disease activity assessment. Furthermore, there is no universally agreed definition of UC remission in clinical trial studies to date, despite much discussion and urge for standardisation. These discrepancies add more confusion to the assessment of clinical remission and thus endpoints in clinical trials in UC. The definitions of UC remission defined by different guidelines are summarised in Table 6.

To have better understanding of defining endpoints of this clinical trial study, a narrative review on this topic was conducted (Appendix 5; (50)). In the FMTUC feasibility study, three indices were used to assess participants disease activity during the 12-week follow up period, namely the Mayo score, the IBDex and the CUCQ-32.

Table 6: Summary of definitions - Ulcerative Colitis Remission

Guidelines	**Definition**
FDA (217)	Clinical remission • Mayo score of ≤ 2 with no individual subscore > 1 • Rectal Bleeding Subscore = 0 • Stool Frequency Subscore = 0 (At least one point decrease in Stool Frequency subscore from baseline and achieved 1 is considered) • Endoscopy subscore = (Mayo score: 0 or 1, UCDAI = 0) Clinical response • Reduction in Mayo score ≥ 3 and ≥ 30% from baseline with Rectal Bleeding subscore ≤ 1 Corticosteroid-free remission • Clinical remission in patients using oral corticosteroids at baseline who have discontinued them and are in clinical remission at the end of the study
World Gastroenterology Organisation (WGO)	Clinical remission • UCDAI ≤ 2 (2010 WGO Practice Guideline) (218) Corticosteroid-free remission • Decreasing the frequency and severity of recurrence and reliance on corticosteroids
International Organisation for the Study of IBD (IOIBD)	End points = induction of remission = mucosal healing (219) • The absence of friability, blood, erosions and ulcers in all visible segments • No mention of clinical symptoms
American College of Gastroenterology (ACG)	No clear definition (41)
British Society of Gastroenterology (BSG)	No clear definition (220)

European Crohn's and Colitis Organisation (ECCO)	Remission (221) : A complete resolution of symptoms and endoscopic mucosal healing
	• Not been a fully validated definition of remission
	• Suggest the best way forward is a combination of
	  ○ Stool Frequency ≤ 3
	  ○ No rectal bleeding
	  ○ Normal or quiescent mucosa at endoscopy
	Clinical response
	• Clinical and endoscopic response depending on the activity index
	• Generally, a decrease in the activity index > 30% plus a decrease in the rectal bleeding and endoscopic sub-scores.

2.9.1 Mayo Score

Mayo Score was developed in 1987 by Schroeder et al. as a part of their randomized clinical trial study on coated oral 5-aminosalicylic acid (5 ASA) therapy (167). Mayo score is also often called "The Disease Activity Index" or "Mayo Clinic Score". The score consists of four components: objective assessment on patient' stool patterns, rectal bleeding, endoscopic macroscopic findings and global physicians' assessment, ranging from 0 to 12 with higher scores indicating worse severity (Appendix 2). Although there is no universal gold standard disease activity scoring system, this scoring system has been widely used in clinical settings as well as clinical trial studies. This is partly because the FDA defines clinical remission as Mayo score of equal or less than 2 (217). Yet, clinical trials have used Mayo score to define their own clinical remission and clinical response. For instance, clinical trial to investigate cyclosporine enema for active UC, clinical remission was set as Mayo score of 0 and clinical response as Mayo score decrease of more than 3 from baseline (222) while a more recent study on infliximab defined clinical remission as an overall Mayo score of less than 2 points with no individual subscore of more than 1 and clinical response as an overall Mayo score decrease of more than 3 from baseline (223). This highlights the importance of careful assessment of clinical studies before applying their findings to clinical practice.

In this feasibility study, Mayo Score at the baseline and final week 12 were used to assess patients' disease severity after the interventions. The primary endpoints of this feasibility study were also defined as Mayo score ≤ 2 with an endoscopic Mayo score of 0.

2.9.2 IBDex

The IBDex was developed to reliably assess disease severity of patients with IBD in the clinical setting based on clinical assessment without any invasive procedures such as endoscopic assessment (224). Items of the questionnaire were carefully selected through a literature search to identify clinically relevant questions to assess the severity of IBD, and then finalized by an expert panel (Appendix 3). IBDex was subsequently validated on patients with IBD in multi-centre hospitals on 255 adult patients with IBD (130 with UC and 125 CD). The IBDex was also validated against the Simple Clinical Colitis Activity Index (225) and endoscopic indices, Mayo Clinical Score and Rachmilewitz Index, as well as biochemical markers (Hb, WBC, CRP, ESR and albumin). The IBDex was finally assessed with rigorous psychometric analysis to achieve responsiveness and reproducibility. Thus, IBDex was the first appropriately validated clinical disease severity index to assess severity of disease in IBD in clinical settings. IBDex was used in this study throughout the follow up; baseline, week 1, week 4, week 8 and week 12.

2.9.3 CUCQ-32

CUCQ-32 was developed to assess symptoms and disease severity subjectively by patients. It consists of 32 questions designed for IBD, which were developed by consulting patients and expert opinions as well as by reviewing previously validated questionnaires (Appendix 4).

This scoring system has been validated against the Short Form 12, EuroQoL 5D questionnaires, Simple Clinical Colitis Activity Index and the Harvey-Bradshaw Index in 205 patients (226). Psychometric analysis was conducted to assess internal consistency, assessing validity, reproducibility, and responsiveness. In this feasibility study, CUCQ-32 was also used to assess non-invasive subjective clinical assessment of the disease throughout the follow up at baseline, week 1, week 4, week 8 and week 12.

2.10 Analysis of The Microbiota

DNA-DNA hybridization has been the gold standard for identifying prokaryotic species for nearly fifty years (227). DNA-DNA hybridization measures genetic similarity of hybrid double-stranded DNA sequences. This uses the principle that a single-strand DNA or RNA of one identified sequence can base-pair to a target single-strand DNA or RNA that contains a complementary sequence and the stability of the hybrid sequence is dependent on the degree of base-pairing between them (228). DNA melting is the dissociation of the double-stranded DNA into single-stranded DNA through breaking hydrogen bonds. It requires a higher temperature to separate for double-stranded hybridized DNAs when the hybridized DNA has a higher degree of similarity (228). However, the disadvantages of DNA-DNA hybridization are very labour-intensive and time-consuming. Next-generation sequencing (NGS) technology has revolutionised DNA analysis especially for the past decade. NGS, also known as high-throughput sequencing, uses sequencing techniques to analyse multiple small fragments of DNA or RNA in parallel, shortening time to sequence significantly.

Two main NGS techniques have been utilized to study microbes in recent years; 16S ribosomal RNA (16S rRNA) and whole genomic shotgun sequencing (WGS). 16S rRNA gene sequencing utilizes the Polymerase Chain Reaction (PCR) to amplify the hypervariable regions (V) of the 16S rRNA gene whereas shotgun metagenomics sequence the whole genomic DNA of all microorganisms in a sample. The overview of these two sequencing workflows is described in Figure 10.

The choice of these approaches for microbiota study has been discussed extensively and usually determined by the nature of the studies. In general, 16S rRNA gene sequencing is suited for analysis of large numbers of samples and for identifying multiple different bacteria to the genus level, whereas shotgun metagenomics is used for detection of new microbial genes and genomes (229). WGS is known to provide detection of bacterial species with high accuracy and has potentials to discover new bacterial genes and genomes; however, it is more expensive and time-consuming process (229). WGS also allows simultaneous study of viruses, archaea and eukaryotes (230). 16S rRNA sequencing offers less functional and taxonomical resolution than WGS, however, it is better suited for a large number of samples as it is quicker to process and less expensive. WGS may also offer a superior potential for identification of strains, however, the focus of this feasibility study is characterisation of the gut microbiome instigated by FMT treatment. Thus, 16S rRNA analysis was employed to study the faecal and tissue microbiota in this feasibility study.

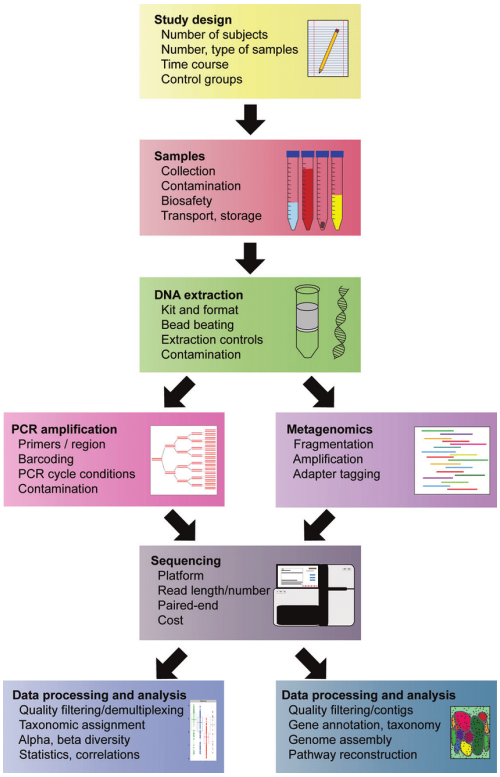


Figure 10: Workflow overview of 16S rRNA (left) and Shotgun metagenomics sequencing (231)

2.10.1 16S ribosomal RNA (16S rRNA) Gene Sequencing and Illumina MiSeq Next Gene Sequencer

16S rRNA analysis was employed for bacterial identification within faecal and bowel tissue biopsy samples. It was also used to study the changes in the gut microbiota composition after the FMT treatments and to evaluate successful engraftment of donor faecal microbiota at 12 weeks. In this study, an Illumina MiSeq next generation sequencer was used for 16S profiling. This work was carried out in the Institute of Life Science (ILS-1), Swansea University Medical School, by an experienced operator, Dr Matthew Hitchings.

16S rRNA is present in the 30S subunit of the bacterial ribosome and one of the main functions is to carry out mRNA translation. The 16S rRNA gene is present in almost all bacteria and has been highly conserved between species, making it suitable for bacterial identification. The 16S rRNA gene from distantly related bacterial lineages share similar functionalities, suggesting that it can also be reliably used as a molecular clock (232). Furthermore, the 16S rRNA is approximately 1600 base pair (bp) long, which is large enough for bioinformatics (233). The 16S rRNA contains nine hypervariable regions (V1-V9), which are species specific sequence, which is very useful to target for bacterial identification process (234). Illumina MiSeq targets hypervariable region V4, which is approximately 254 bp and contains the maximum nucleotide diversity, making the suitable region for bacterial species identification (235).

2.11 IL-10 and IL-21

Immunoassay study on IL-10 and IL-21 on bowel mucosa biopsy was conducted to investigate the effect of FMT in this study.

IL-10 is an important anti-inflammatory cytokine in the GI tract (236,237). IL-10 regulates over-stimulated immune responses including the autoimmune response (238). The link between IBD and IL-10 signalling has been studied for many years. Defective IL-10 and the loss of IL-10 signalling is suggested to be linked to spontaneous colitis in mice as well as to enterocolitis in humans (236), fuelling interest in the therapeutic use of IL-10 in IBD including UC. Moran et al. suggested that polymorphisms in the IL-10 receptor can predispose someone to develop UC (239). Mitsuyama et al. also studied IL-10 levels in 62 patients with UC, which found that an increased level of IL-10 was observed amongst patients in clinical remission (240). Several groups explored the usage of IL-10 for diagnosis as well as treatment

amongst patients with IBD without any success, and the role of IL-10 in UC has remained unclear (236,237).

Similarly, the link between IL-21 and UC has been studied. IL-21 is a pleiotropic cytokine and has potent regulatory properties in cells involving the immune system including cytotoxic T cells and natural killer cells (241). This makes a case for IL-21 being protective against viral infection as well as cancerous cells. IL-21/IL-21 receptor signalling has been also been suggested to be responsible for pathogenesis of IBD as an increase in serum level of IL-21 has been observed in patients with UC (242,243). Wang et al showed that IL-21/IL-21 receptor signalling facilitates protection against DSS-induced colitis through T_h1 suppression and T_h2 activation in mice (243). Yu et al. also demonstrated not only that IL-21 is a key regulator for inflammation of UC, but also that it regulates the proliferation and response of T follicular helper cells in UC using dextran sulphate sodium-induced IL-21 knockout mice (244). Although these *in vitro* studies are encouraging, therapeutic manipulation of IL-21/IL-21 receptor signalling in humans has not yet been achieved.

2.12 SARS-CoV-2 (COVID-19) Pandemic and FMTUC

In the UK, the onset of the SARS-CoV2 (COVID-19) pandemic meant that the NHS postponed all non-urgent clinical investigations as well as treatments including surgery in mid-March 2020, and subsequently the government directed a nationwide lockdown. This had a huge impact on healthcare systems.

Although COVID-19 was believed to transmit through the respiratory system, there was a significant concern about transmission through other routes including from faecal matter, which meant all endoscopic procedures were suspended including patients with suspected colorectal cancers during the peak of the pandemic. The impact of this varied by region in the UK. In August 2020, endoscopy lists restarted in many parts of the UK though there has been a huge backlog of patients' requiring endoscopic assessments. Patients with a suspected cancer had priority, and thus participants for this study needed to wait longer for their endoscopic assessment. Moreover, many face-to-face consultations were replaced with telephone consultation. Non-essential services and departments in the NHS including the clinical trial unit in SUMS ILS-2 were suspended during the peak of the pandemic. Two subjects were screened and received the FMT interventions just before the lockdown started, which meant those two subjects were unable to attend for follow ups including blood tests. Their face-to-face consultations were replaced with telephone

consultations and answering questionnaires. All laboratory work was also suspended at Swansea University for a period.

Before the pandemic, COVID-19 was not screened from the hosts or recipients. Two subjects (subject 17 and 18) were screened in February 2020 and did not have any symptoms suggesting COVID-19. Since the FMTUC samples were manufactured before the pandemic, the risk of COVID-19 transmission is minimised. However, future FMT production or FMTUC study protocols must consider COVID-19 and the study has faced significant delays.

Chapter 3. Narrative Review of Endpoints and Disease Activity Monitoring Tools Focusing on the UCEIS and UCDAI

During the design of this feasibility study, it became apparent that there was no universal consensus on clinical remission in UC clinical trials and thus each RCT defined its own clinical remission with different outcome tools, increasing variation between studies. Furthermore, the number of disease activity monitoring tools has been developed worldwide, despite much discussion and urge for standardisation. Disease activity has been traditionally monitored by clinical symptom scores with or without endoscopic assessment. In recent years, many indices have been proposed to measure histological, radiological, faecal or serological biomarkers, all of which claim to define and measure clinical remission more accurately. Homogeneity of clinical trial protocols may hinder advancement of new treatment in UC. This raised the question; "how do we determine clinical remission as an endpoint in UC clinical trials when we design a study?"

The current consensus on the definition of remission and the endpoints employed in clinical trials were systematically reviewed using two common disease activity scoring systems: The Ulcerative Colitis Endoscopic Index of Severity (UCEIS) and the Ulcerative Colitis Disease Activity Index (UCDAI). These two scoring systems were chosen because they were very diverse as described below.

UCEIS

The UCEIS was developed by Travis et al. in 2012 to minimise variation in endoscopic assessments and to replace existing endoscopic assessment tools such as endoscopic Mayo score (245). Endoscopic Mayo score has been a widely used in clinical trials and real clinical setting, however, it has been criticised for its crude description, leading to intra- and inter-observer variations. The authors extensively studied challenges associated with standardisation of the disease activity indices before proposing the UCEIS (246,247).

The first stage of development of the UCEIS demonstrated the significant inconsistency in endoscopic assessment among ten specialists when they scored the severity of UC using the Baron score (248) in colonoscopy videos. The greatest agreement 76% was achieved when the Baron score was high, however, only 27% and 37% agreements were obtained for normal mucosa (Baron score 0) and moderate friability (Baron score 2) respectively. The second part of the study further quantified intra- and inter-observer variations on common descriptors on endoscopic assessments as described in Table 7. For intra-observer variation, 60 repeat pair assessments of 36 different videos were scored. For inter-observer variation, 30

new investigators were randomly allocated to score 25 videos, thus each video was assessed by 10-12 investigators.

Table 7: Descriptions and intra- and inter-observer variation

Descriptor	Likert scale anchor points	Intra-observer variation (a weighted κ)	Inter-observer variation (a weighted κ)
Vascular pattern	Normal (1) Patchy loss (3) Obliterated (5)	0.61	0.42
Mucosal erythema	None (1) Light red (3) Dark red (5)	0.43	0.35
Mucosal surface (Granularity)	Normal (1) Granular (3) Nodular (5)	0.45	0.34
Mucosal oedema	None (1) Probable (3) Definite (5)	0.43	0.31
Mucous	None (1) Some (3) Lots (5)	0.47	0.40
Bleeding	None (1) Mucosal (2) Luminal mild (3) Luminal moderate (4) Luminal severe (5)	0.57	0.37
Incidental friability	None (1) Mild (2) Moderate (3) Severe (4) Very severe (5)	0.49	0.40

Contact friability	None (1) Probable (3) Definite (5)	0.34	0.30
Erosions and ulcers	None (1) Erosions (2) Superficial ulcer (3) Deep ulcer (4)	0.65	0.45
Extent of erosions or ulcers	None (1) Limited (2) Substantial (3) Extensive (4)	0.60	0.42

Both intra- and inter-observer variations demonstrated good agreement on assessments of erosions and ulcers, vascular pattern and bleeding, which were subsequently chosen for descriptors of a newly developed endoscopic assessment tool, the UCEIS (Table 8).

Table 8: The UCEIS

Descriptors	Likert Scale anchor point	Definition
Vascular pattern	0: Normal	normal vascular pattern with arborisation of capillaries clearly defined, or with blurring or patchy loss of capillary margins
	1: Patchy obliteration	
	2: Complete obliteration	Complete obliteration
Bleeding	0: None	
	1: Mucosa	some spots or streaks of coagulated blood on the surface of the mucosa
	2: Luminal mild	some free liquid blood in the lumen
	3: Luminal moderate or severe	Frank blood in the lumen ahead of endoscope or visible oozing from a haemorrhagic mucosa
Erosions and Ulcers	0: None	None
	1: Erosions	Tiny <5mm defects in the mucosa, of white

		or yellow colour with a flat edge
	2: Superficial ulcer	Larger > 5mm defect in the mucosa, which are discrete fibrin-covered ulcers in comparison with erosions, but remain superficial
	3: Deep ulcer	Deeper excavated defects in the mucosa, with a slightly raised edge

UCDAI

The UCDAI (also called UC Disease Activity Index, and Sutherland Index) was introduced by Sutherland et al. to assess efficacy of 5-aminosalicylic acid enema in the treatment of distal UC in its randomized, double-blind clinical trial in 1987 (249). The index was employed to assess the outcome of the clinical trial. The index consists of four variables to monitor the severity of UC; stool frequency, rectal bleeding, mucosal appearance, and physician's rating of disease activity (Table 9).

Table 9: The UCDAI

Variables	Score	Items
Stool frequency (SF)	0	Normal
	1	1-2 stools / day more than normal
	2	3-4 stools / day more than normal
	3	> 4 stools / day more than normal
Rectal bleeding (RB)	0	None
	1	Streaks of blood
	2	Obvious blood
	3	Mostly blood
Endoscopic appearance (EA)	0	Normal
	1	Mild friability
	2	Moderate friability
	3	Exudation, spontaneous bleeding
Physician Global Assessment (PGA)	0	Normal
	1	Mild

	2	Moderate
	3	Severe

Unlike the UCEIS, the UCDAI was developed without validation. Although the authors reported that the index incorporated many of the subscales used by other investigators, they failed to demonstrate this with statistical assessments. The authors also claimed a good correlation between the UCDAI and the physicians' global assessment by comparing with its own component of the index (P = 0.0001). Overall, the UCDAI demonstrated weak objectiveness.

Results from Bibliographic Searches

The literature research returned 37 and one 116 articles for the UCEIS and UCDAI respectively. The PRISMA flow diagrams for the UCEIS and UCDAI are show in Figure 11.

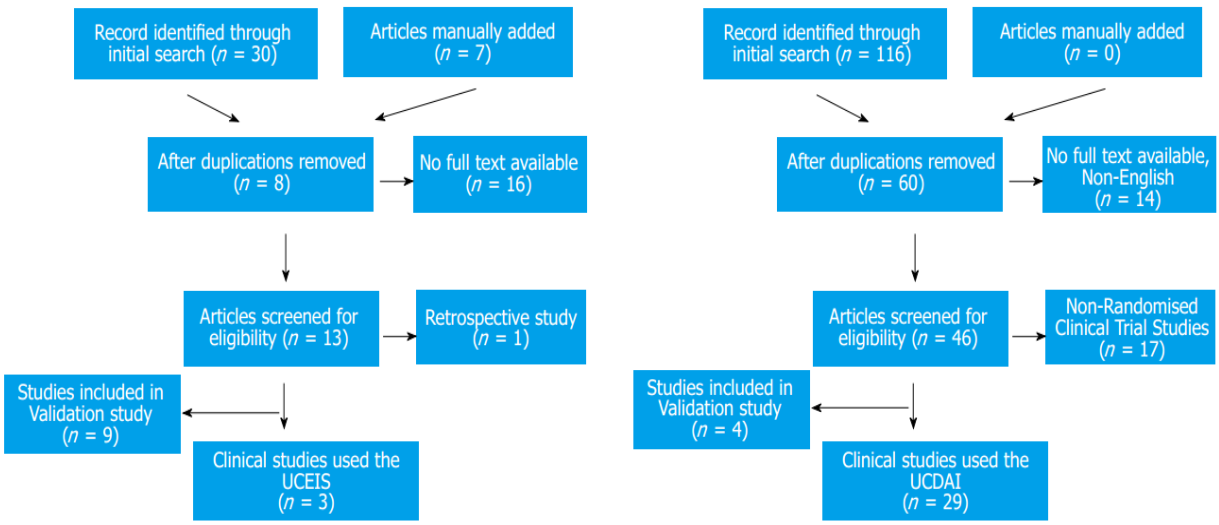


Figure 11: PRISMA flow diagrams for UCEIS (left) and UCDAI (right)

Validation of these two indices were also described in terms of validity, reproducibility, and responsiveness (Table 10).

Table 10: Validation study of UCEIS and UCDAI

	Reference	Patient number	Outcomes
UCEIS			
Validity	Corte et al. (250)	89	• Correlation between UCEIS and outcomes • The UCEIS score was directly proportional to requirement of rescue therapy • UCEIS ≥ 5 was significantly linked to requiring colectomy 18/54 (33%) patients with UCEIS ≥ 5 compared to 3/33 (9%) with UCEIS ≤ 4 • No definition of remission
	Fernandes et al. (251)	108	• Prediction of outcomes in acute severe colitis UCEIS was applied to score of the rectum and sigmoid • Segmental -UCEIS predicted to develop steroid-refractory disease and the likelihood of colectomy • Every 1 point increase in the UCEIS or Segmental-UCEIS increased the need of colectomy by 2.78 and 1.79 respectively • No definition of remission
	Arai et al. (252)	285	• The recurrence rate was directly proportional to the UCEIS score • The absence of bleeding and mucosal damage were independent factors for continued clinical remission • Mayo ≤ 1 and UCEIS ≤ 1 showed sensitivity of 68% and specificity 57% of clinical remission respectively • The expected duration of recurrence is also prolonged when UCEIS ≤ 1
	Kucharski et al. (253)	49	• Assessment of 9 endoscopic indices correlate well with clinical indices and histological Goeboes Index

			• The UCEIS showed the stronger correlation with the Geboes Index compared to UCDAI • Recommends the UCEIS for the best overall correlations with both clinical and histological indices
Responsiveness	Ikeya et al. (254)	41	• UCEIS score improvement showed closer correlation with clinical remission, colectomy-free and relapse free rates than Mayo endoscopic score • Proposed remission (score 0-1), mild (2-4), moderate (5-6), severe (7-8)
	Menasci et al. (255)	80	• Better correlation (Spearman's $r = 0.86$, $P < 0.0001$) for disease when UCEIS score ≤ 5 • Less correlation ($r = 0.48$, $P < 0.01$) when UCEIS > 5
Reliability	Travis et al. (256)		• Investigation of intra- and inter-observer consistency assessment 25 readers evaluated 28 videos including 4 duplicates to assess intra-reader reliability • The intra and inter-reader reliability ratios for the UCEIS were 0.96 and 0.88 respectively
	Feagan et al. (257)	281	• The effect of centralized review of images on inter-observer variations with patients UCDAI ≥ 2 • UCEIS showed interclass correlation coefficient of 0.83 amongst 7 central readers, which was superior to UCDAI
	Travis et al. (258)		• 40 readers evaluated 28 of 44 videos • No discrepancy between blinded and unblended readers • Intra- and inter-reader variability demonstrated moderate to substantial agreement ($\kappa = 0.47$ to 0.74 and $\kappa = 0.40$ to 0.50 respectively) • UCEIS correlated well with patient-reported symptoms - rectal bleeding, stool frequency

			and patient functional assessment (rank correlation = 0.76 to 0.82)
UCDAI			
Validity	Higgins et al. (259)	66	• Finding endpoints in disease activity indices for remission and improvement in UC • UCDAI < 2.5 for remission, which had a sensitivity and specificity of 0.82 and 0.89 • Remission in this study was defined by patients
	Poole et al. (260)	126	• Established the relationship between the UCDAI and patient reported EQ-5D • The UCDAI with or without endoscopy assessment demonstrated a good correlation with EQ-5D
	Kucharski. et al. (253)	49	• The UCDAI showed strong correlations with all 9 endoscopic indices (the coefficient in a range of 0.712 to 0.790), though compared to UCEIS, the UCDAI is less correlated with the Geboes Index
Reliability	Feagan et al. (257)	281	• The effect of centralized review of images on inter-observer variations with patients UCDAI ≥ 2 • UCDAI showed less correlation compared to UCEIS

Validity

The validity of an assessment tool refers to how accurately the tool measures the disease outcomes. This must be demonstrated by qualitative assessment and the evidence of indices measuring adequately disease activity. A correlation between an index score and objective assessment such as clinical disease activity index scores or physician global assessment of severity should also be measured. Lastly, development of these indices should also be supported by a robust systematic review of literatures.

The UCEIS is one of the extensively validated indices in many aspects. Since the UCEIS was published in 2012, four studies attempted to evaluate validity of the UCEIS, whereas three published work studied on validity of the UCDAI as summarised in Table 11.

Corte et al. studied correlations between the UCEIS and clinical outcomes on 98 patients with acute severe UC with the UCEIS score between 3 and 8 (250). The authors found that when UCEIS ≥ 5, 33% (18/54) of patients required colectomy and when UCEIS ≤ 4, only 9% (3/33) of patients required surgical interventions. When the UCEIS score was above 7 at the time of admission, almost all patients required medical therapy for severe UC, such as infliximab or ciclosporin. They concluded that patients with higher UCEIS scores more frequently required rescue therapies, surgical interventions, and hospital readmission.

Fernandes et al. also validated the UCEIS on different segments of the colon to evaluate whether the UCEIS predicted refractoriness to steroid therapy by comparing with the endoscopic Mayo score (251). The study concluded that the UCEIS was significantly more accurate at predicting clinical outcomes than the endoscopic Mayo score.

Arai et al. attempted to foresee the prognosis of 285 patients with UC, who were in clinical remission with Mayo score of ≤ 1 (252). This study demonstrated the relapse risk was direct proportional to the UCEIS score and reported that the absence of bleeding and mucosal damage were independent factors for clinical remission. It also found that the duration of relapse was also significantly prolonged in patients with lower UCEIS scores.

Kucharski et al. studied validity of multiple indices including the UCEIS and the UCDAI (253). The authors demonstrated that the UCEIS had the strongest correlations with clinical, endoscopic and histological assessments and concluded that the UCEIS was the most accurate endoscopic outcome measurement instrument of all. In contrast, the UCDAI showed moderate correlation.

Higgins et al. and Poole et al. also investigated validity of the UCDAI (260,261). As a part of the study, Higgins et al. defined that UCDAI score of 2.5 being a cut-off score for clinical remission.

Responsiveness

Responsiveness is the ability to detect changes of disease activity and severity after interventions, which is imperative in clinical trials with short follow-up periods. Two studies that evaluated the UCEIS's responsiveness were identified, whereas no study on the UCDAI's responsiveness was found.

Ikeya et al. (254) studied clinical outcomes after Tacrolimus remission induction therapy using the UCEIS and endoscopic Mayo score on 41 patients with moderate to severe UC. This study indicated that the UCEIS was able to capture characteristics of ulcers, vascular patterns or mucosal bleeding in more details than endoscopic Mayo score and concluded that the UCEIS reflected more accurately with the severity and activity of UC.

The UCEIS is traditionally scored on the most inflamed segment of the colon. Menasci et al. retrospectively compared the accuracy of the traditional method of the UCEIS score and the sum of the UCEIS scores on five colonic segments, tU, (255) using 80 UC patients. It concluded that both scoring methods showed a good correlation, though the correlation decreased when the UCEIS scores were greater than 5. Furthermore, the authors determined that tU score >20 being a risk factor for a flare within one year, whereas the traditional UCEIS score did not demonstrate a risk score.

Reliability

Despite mucosal healing becoming the goal for the management for UC, the most critical limitation of endoscopic assessment is its inherent intra- and inter-observer variations (262–264). Reliability refers to the stability of assessment and is commonly evaluated with inter- and intra-observer reliability as well as internal consistency.

The leading author of the UCEIS also investigated reliability of the score. The first study published in 2013 investigated intra- and inter-observation reliability by evaluating 28 videos using 25 readers from 14 countries (256). Four duplicated videos were purposely included to evaluate intra-observer reliability. The study found that high intra and inter-observer reliability ratios, intra-observer agreement as well as inter-observer agreement. Additionally, these observer reliabilities were compared with readers who were given clinical information at the time of the video readings, which determined no apparent bias by clinical information. Internal consistency also exhibited a good Cronbach's coefficient alpha score in this study.

To evaluate the impact of clinical information on the UCEIS, the author also undertook another study in 2015 (258). The study invited 40 readers from various countries who were experienced with endoscopic assessment. Each reader was divided into two groups (with and without clinical information) and conducted evaluation of a random 28 videos from a pool of 44 videos, which had not been used in the previous study. Furthermore, 4 videos included misleading information to ensure disparity between

endoscopic assessment and clinical information. This study proved that clinical information did not influence the UCEIS scores. Intra- and inter-observer agreements of the blinded and unblinded readers were also evaluated in this study, which showed that intra-observer agreements of the bleeding and vascular pattern components were very similar between the blinded and unblinded groups. However, erosions and ulcers components were more likely affected by endoscopists' knowledge on clinical information. The study also extended to compare the UCEIS with other indices including Mayo score, proving its superiority. The authors suggest that the UCEIS alone was sufficient for outcome measurement in clinical trials.

The only inter-observer and central reader variation study on UCDAI also referred to UCEIS (257). The authors investigated the role of centralised review of images to minimise inter-observer differences, which could lead to variations in response to placebo in UC trials in a RCT on 343 patients with UC scored UCDAI ≥ 2. Clinical remission (UCDAI = 0) was achieved by 30.0% of patients treated with mesalamine and 20.6% of those with placebo. However, when those 343 were re-assessed by seven central readers, 31% of those patients should be ineligible since their initial UCDAI score was lower than 2, resulting in changes in remission rates. The authors also extended the study to quantify the inter-observer variation on multiple indices including the UCDAI and the UCEIS. Of those indices, UCEIS demonstrated the highest interclass correlation. The authors emphasised the importance of robust methodologies to assess the disease activity in clinical trial to avoid misleading results.

How these indices are used to define remission in clinical trials

Since there is no gold-~~-~~standard outcome measurement instruments in UC, many clinical trials have employed instruments depending on its application.

Traditional disease activity monitoring instruments have been recently challenged by the FDA for their poor ~~for~~ reproducibility. The indices advocated by the FDA~~,~~, such as Mayo Score and the UCDAI, include a physician assessment component, which is highly sensitive to bias. The FDA advices that the primary endpoint should be achieved by clinical outcomes and endoscopic assessment, however, we should consider that some symptoms may show discrepancies from the actual disease activities. Nevertheless, these symptoms affect patients' quality of life. This makes the standardised assessment tool that accurately captures both qualitative and qualitative aspects of UC challenging.

In this narrative review, remission ~~and~~ endpoints were also investigated by studying the application of the

UCEIS and the UCDAI in clinical research and therapeutic trials (Table 11 and Table 12).

Three clinical research employed the UCEIS for their disease outcome measure and remission. One RCT, the ACERTIVE study, chose the UCEIS for its outcome measurement (265).

~~(42)~~ The aim of the ACERTIVE study was to evaluate potential applications of biomarkers (faecal calprotectin and neutrophil gelatinase B-associated lipocalin) as disease activity measuring instrument in patients with asymptomatic UC. ~~371 patients with asymptomatic UC, of which 366 patients were on medical therapy, were enrolled in the study.~~ The UCEIS was applied for both macroscopic and microscopic assessments and clinical remission was defined as the UCEIS ≤ 1 in this study. Lin et al. also evaluated the use of faecal calprotectin in their prospective study, in which they defined clinical remission as the UCEIS < 3 (266)~~(41)~~. Although it is one score difference, this can lead to significant variation in the clinical remission rate between studies.

~~(40)~~

Table 11: Clinical studies measured with the UCEIS

Authors	**Year**	**Type of study**	**Drug / Subject of Study**	**Entry Criteria**	**Primary endpoint**	**Secondary endpoint**	**Remission / clinical improvement**	**Length of study**
Hartman ~~et al~~et al. (267)~~(40)~~	2016	Randomised, double-blind, placebo-controlled study	AVX-470, oral	36 patients with Mayo score 5-12 and Mayo ES ≥ 2	Not set, but implies clinical response at week 4	Not set	Remission was not defined. Clinical response : Mayo reduction ≥ 3	4 weeks
Lin et al. ~~et al~~(266) ~~(41)~~	2015	Prospective, multi-centre study	Faecal calprotectin	52 patients with UC	N/A	N/A	Endoscopic remission : UCEIS < 3	N/A
Magro ~~et al~~et al. (265) ~~(42)~~ **ACERTIVE study**	2016	Cross-sectional multi-centre study	Faecal calprotectin / lipocalin	371 patients Mayo partial score <2, Montreal classification < 2			Remission : UCEIS ≤ 1 Mucosal healing : Mayo ES = 0	

~~The UCEIS was used for endoscopic assessment in a prospective study to quantify faecal calprotectin in patients with UC. (41) The authors used QB[DAH1] for faecal calprotectin measurement tools in this study. Interestingly, the authors defined endoscopic remission when the UCEIS < 3. They also concluded that faecal calprotectin and CRP were both well correlated with the UCEIS (the Spearman correlation coefficient is 0.696 and 0.581 respectively). Moreover, they concluded that when a cut-off faecal calprotectin of 191 µg/g is set, this could predict endoscopic remission and mucosal remission (UCEIS <3) with 88% sensitivity and 75% specificity. However, when UCEIS <1 clinical remission proposed by other authors (11,18) is applied, faecal calprotectin would be lower than 191 µg/g. This could lead to underestimate patients who should be treated.~~

Table 12: Randomised clinical trials measured with the UCDAI

Authors	**Year**	**Drug**	**Entry Criteria**	**Primary endpoint**	**Secondary endpoint**	**Remission / clinical improvement**	**Length of study**
Randomised Clinical Trials – To induce remission							
Mesalazine (5-ASA)							
~~Wong et al (55)~~	~~2003~~	~~Comparative study~~	~~Switch to Pentasa from Asacol~~	~~UCDAI endoscopic subscore ≥ 2 who did not respond to Asacol~~	~~Change in UCDAI~~	~~Drug safety profile and acceptability~~	~~Remission : UCDAI endoscopic subscore ≤ 1~~	~~12 weeks~~

Marteau ~~et al~~et al. (268)~~ (51)~~	2005	Pentasa (PR + PO vs PO alone)	UCDAI : 3-8	Remission at week 4	• Remission rate at week 8 • Improvement at week 4 and 8	Remission : UCDAI ≤ 1 Clinical improvement : a decrease of UCDAI ≥ 2	8 weeks
D'haens ~~et al~~et al. (269) ~~(49)~~	2006	SPD476 – MMX mesalazine	UCDAI : 4-10 + endoscopic score ≥1 PGA score ≤ 2	Remission	• Change in UCDAI, FS, histology at week 8 • Change in symptoms	Remission : UCDAI ≤ 1 (with RB 0, SF ≤ 1) at week 8	8 weeks
Sandborn ~~et al~~et al. (270) ~~(48)~~	2007	MMX Multi Matrix System mesalazine	UCDAI : 4-10 + endoscopic score ≥1 PGA score ≤ 2	Clinical/endoscopic remission at 8 weeks	• Proportion of clinical improvement • Proportion of patients as treatment failure • Change in : RB, SF, FS •	Clinical remission : UCDAI ≤ 1 Endoscopic remission : UCDAI endoscopic subscore ≤ 1 Clinical improvement : a decrease of UCDAI ≥ 3 Treatment failure : unchanged or worsened UCDAI	8 weeks
Lichtenstein ~~et al~~ et al. (271) ~~(50)~~	2007	SPD476 – MMX mesalazine OD vs BD	UCDAI : 4-10	Clinical and endoscopic remission at week 8	• Comparison of remission rate at week 8	Clinical remission : UCDAI ≤ 1 with RB/SF/EI = 0	8 weeks
Kamm ~~et al~~et al. **MEZAVANT study** ~~(52)~~ (271,272)	2007 2009	MEZAVANT MMX Mesalamine	Mild – mod UC : UCDAI 4-10 + endoscopic subscore ≥ 1, PGA ≤ 2	Clinical + Endoscopic remission at week 8	• Clinical remission • Clinical improvement • Change in UCDAI • Macroscopic changes	Clinical + endoscopic remission : UCDAI ≤ 1 + subscore RB/SF = 0, No mucosal friability + a ≥ 1 reduction in EI Clinical improvement : decrease in UCDAI ≥ 3	8 weeks
Ito ~~et a~~et al. ~~(47)~~ (273)	2010	Asacol vs Pentasa	UCDAI : 3-8 and blood stool score ≥ 1	To demonstrate Asacol over		Remission : UCDAI ≤ 2 and no blood diarrhoea Clinical improvement :	8 weeks

		Time-dependent vs pH dependent Mesalamine		Pentasa AND the decrease in UCDAI		UCDAI decreased by ≥ 2	
Hiwatashi ~~et a~~et al. (274) ~~(54)~~	2010	Mesalazine – dose study	UCDAI 6-8	Change in UCDAI at week 8	Remission, improvement, efficacy	Remission : UCDAI ≤ 1 Efficacy : decrease of UCDAI ≥ 2	8 weeks
Flourie et al. ~~et al~~ **MOTUS study** ~~(46)~~ (275)	2013	Mesalazine, Pentasa OD or BD in total of 4g/day	UCDAI : 3-8	UCDAI ≤ 1 after 8 weeks	• Complete remission (UCDAI = 0) at 8 weeks • UCDAI decreased by ≥ 2 at 8 weeks • Clinical remission at week 4,8,12 Mucosal healing at 8 weeks	Complete remission : UCDAI = 0 Endoscopic remission : UCDAI endoscopic subscore : 0 or 1 Clinical remission : UCDAI ≤ 1	12 weeks
Probert ~~et al~~et al. **PINCE study** (268)~~(25)~~	2013	Mesalazine (Pentasa) enema	UCDAI 3-8	• Remission rate (UCDAI <2) at 4 weeks	• Remission rate at 8 weeks, improvement at 2,4 and 8 • Time to cessation of RB QoL (EQ-5D)	Remission : UCDAI ≤ 1 Clinical improvement : UCDAI decreased by ≥ 2	8 weeks
Sun et al. ~~et al~~(276) ~~(53)~~	2016	Mesalazine (modified-release vs enteric-coated tablets)	UCDAI 3-8 + bloody stool score >1	• The decrease in UCDAI	• Remission rate • Efficacy rate	Remission : UCDAI ≤ 2 + bloody stool 0 Clinical improvement : a decrease of UCDAI ≥ 2	8 weeks
Suzuki ~~et al~~ et al. (277) ~~(45)~~	2016	pH dependent release mesalamine, Asacol	UCDAI : 6 – 10 Rectal bleeding score ≥ 1	• Decrease in UCDAI		Remission : UCDAI ≤ 2 Rectal bleeding score : 0 Improvement UCDAI decreased by ≥ 2	8 weeks

		Dose					
Thiazole compounds							
Mantzaris et al. (64)~~Schreiber et al (56)~~	2004 ~~2007~~	Azathioprine alone (2.2mg/kg) vs. combination with olsalazine (0.5g TID)~~Tetomilast – Thiazole compound~~	Steroid-dependent remission~~UCDAI : 4 – 11~~	Relapse rate ~~Clinical improvement : UCDAI decreased by ≥ 3 at 8 weeks~~	• Time to relapse • Time to discontinuation • Severity of relapse ~~Remission~~ • ~~Clinical improvement at week 4~~ • ~~IBDQ-32 score~~ • ~~Proportion of pts with improved Flexible Sigmoidscopy score~~ • ~~Time to clinical improvement~~ • ~~Time to remission~~	Remission : UCDAI ≤ 1 Relapse : new symptoms + UCDAI > 3 ~~Clinical improvement : UCDAI decreased by ≥ 3~~ ~~Remission : UCDAI 0-1~~	2 years~~8 weeks~~
Schreiber et al. (278)	2007	Tetomilast - Thiazole compound	UCDAI : 4 – 11	Clinical improvement : UCDAI decreased by ≥ 3 at 8 weeks	• Remission • Clinical improvement at week 4 • IBDQ-32 score • Proportion of pts with improved Flexible Sigmoidscopy score • Time to clinical improvement • Time to remission	Clinical improvement : UCDAI decreased by ≥ 3 Remission : UCDAI ≤ 1	8 weeks
~~**TNF-alpha**~~							

~~Olsen et al (57)~~	~~2009~~	~~Prospective study~~	~~Infliximab~~	~~UCDAI : 6-12~~	~~•~~	~~•~~	~~Clinical remission : UCDAI ≤ 2 and endoscopic subscore ≤ 1 Endoscopic remission : Healed mucosa ≤ 1 Partial clinical response : a decrease UCDAI ≥ 3~~	~~4 weeks~~
Steroids								
Travis et al. ~~et al~~ **CORE II study** (269) ~~(58)~~	2012	Budesonide MMX	UCDAI : 4-10	• Clinical/endoscopic remission at week 8	• Clinical improvement • Endoscopic improvement at week 8		Clinical/endoscopic Remission : UCDAI ≤ 1 + RB/SF/EI = 0 Clinical improvement : a decrease of UCDAI ≥ 3 Endoscopic improvement : a decrease of EI ≥ 1	8 weeks
Probiotics~~Probiotics~~								
Vernia ~~et al~~et al. (279) ~~(62)~~	2000	Sodium Butyrate	Mild-moderate UC	• Remission or marked improvement			Remission : UCDAI ≤ 2 Positive response : decrease of UCDAI ≥ 2	6 weeks
Mahmood ~~et al~~et al. (280) ~~(63)~~	2005	Human recombinant trefoil factor 3 enema	UCDAI >3	• Remission at week 2	• Clinical significant improvement in clinical and histological scores at 2 and 4 weeks		Remission : UCDAI ≤ 1 without RB Clinical improvement : a decrease of UCDAI >3	4 weeks
Lichtenstein ~~et al~~et al. (281)	2007	Bowman-Birk inhibitor concentrate – soy extract with high protease inhibitor activity	UCDAI : 4-10	• Remission at week 8			Remission : UCDAI ≤ 1 + no RB or SF Clinical improvement : UCDAI decrease ≥ 1	

Tursi ~~et al~~et al. (282) ~~(59)~~	2009	VSL#3 (probiotic)	UCDAI 3-8, endoscopic subscore ≥ 3	• Decrease in UCDAI of ≥ 50%	• Activity of relapsing UC • Remission • Improvement • Change in objective and subjective symtpoms	Remission : UCDAI ≤ 2	8 weeks
Sood ~~et al~~et al. (283) ~~(61)~~	2009	VSL #3 probiotic	UCDAI 3-9 with endoscopic subscore ≥ 2	• Clinical improvement at week 6	• Clinical remission	Clinical remission : UCDAI ≤ 2 Clinical improvement: a decrease UCDAI by 50%	12 weeks
Tamaki ~~et al~~et al. (201) ~~(60)~~	2016	*Bifidobacterium longum* 536 (probiotic)	UCDAI 3-9	• Change in UCDAI	• Remission • Improvement of Objective and subjective symptoms • Endoscopic improvement in Mayo subscore	Remission : UCDAI ≤ 2	8 weeks
Nicotine Therapy							
Ingram et al. (284)	2005	Nicotine enema 6mg/day	Confirmed UC with inflamed mucosa grade > 2	• Clinical remission	• Improvement in the UCDAI	Clinical remission : UCDAI EI ≤ 1 and No RB for 1 week	6 weeks
~~Olsen et al (64)~~	~~2016~~	~~Infliximab~~	~~Moderate to severe No specific index mentioned~~	~~• Intensified induction therapy to induce remission and withdraw the treatment~~		~~Remission : UCDAI ≤ 3 + endoscopic subscore ≤ 1 Relapse : UCDAI > 3 and endoscopic subscore > 1~~	~~103 month~~

Rectal Bleeding = RB ;

Stool Frequency = SF ;
Endoscopic Index / Subscore = EI ; OD = Once daily ; BD = Twice daily ; TID ; three times daily

Table 12 summarises 21 RCTs that employed the UCDAI to assess clinical outcomes, endpoint remission. ~~The m~~Most of the studies~~y that~~ (14/21) defined clinical remission as UCDAI ≤ 1, however six RCTs defined as UCDAI ≤ 2 for their clinical remission. It could interpret that some RCTs chose a higher cut-off score to achieve clinical remission to satisfy requirements of the regulatory bodies such as the FDA. This summary emphasises the variations of clinical remission in clinical trials in UC, producing heterogeneous results. This may hinder the advancement of UC management.

Conclusion

This narrative review highlighted a lack of consensus in endpoints and definition of clinical remission in UC clinical trials, resulting in significant variation across RCTs. This review selected two indices from countless indices to highlight the impact of this disparity. If all indices used in RCTs were included, the study results become almost impossible to interpret. The universal consensus on clinical remission should be sought for the advancement of UC management. However, this review showed that extensive validation and the ability to capture true disease activities were not the main criteria when a disease index was chosen in RCTs. Despite the UCEIS being extensively validated in all three aspects (validity, responsiveness and reliability), it has not been utilised in many clinical trial studies yet. Conversely, despite limited validation, the UCDAI has been used in numerous large-scaled RCTs.

The summary of definitions of UC remission proposed by the international regulatory bodies and guidelines was described in this narrative review (Table 6), which again emphasised huge variations. The confusion appears to be multifactorial, although the fundamental issue is a lack of understanding in aetiology and natural history of UC. Without clear understanding of natural history, it is also difficult to define disease remission. This has led to much discussion about what aspects of the disease reflect the severity of UC accurately. Many traditional disease monitoring instruments were first developed as a part of RCTs to monitor their disease activities. These objective assessments were criticised for inherited observer bias. Since, patients' subjective quality of life and endoscopic assessments have provided added dimensions to the monitoring of disease activities. Mucosal healing on endoscopic assessment appears to be the gold-standard for UC management, however, in recent years, the importance of microscopic activities of the colonic mucosa has been emphasised. The lack of full agreement has resulted in numerous indices, adding to the confusion.

RCTs are crucial for better understanding of the condition and the advancement of the disease management. Thus, international collaborative efforts should be sought to define clinical remission and standardisation of clinical trial protocols in UC management.

The narrative review carried out for this study was published in the World Journal of Meta-Analysis (Appendix 5).

Chapter 4. Clinical Outcomes of Faecal Microbiota Transplantation in Patients with Ulcerative Colitis

4.1 Materials and Methods

4.1.1 Trial Design

This study originally aimed to recruit thirty subjects with histologically confirmed UC, whose disease was confined to the recto-sigmoid area (defined here as within forty centimetres from the anal verge) and who were treatment naïve. Eligible patients were randomised into one of three groups with an allocation ratio of 2:2:1 as shown in Table 13 and a web-based application hosted by University of Aberdeen was used for randomisation. Groups 1 and 2 were the intervention arms and twelve subjects were aimed to be assigned to each group respectively. Group 3 was the control group, and six subjects were aimed to be randomised into this group.

Table 13: Interventions and control arms

	Group 1	Group 2	Group 3
Bowel decontamination and preparation	Yes	Yes	Yes
FMT treatment dose	1	5 consecutive doses (single treatment per day)	None
Expected number of participants	12	12	6

Intervention arms: Groups 1 and 2

Participants randomly allocated to group 1 received one single FMT treatment administered as a rectal retention enema. Participants in group 2 received a single FMT treatment on five consecutive days (total of 5 treatments) also administered by rectal retention enema.

Control arm: Group 3

Participants randomly allocated to group 3 received the pre-FMT preparation with antibiotics and bowel preparation but did not receive active FMT treatment.

4.1.2 Endpoints

Paired Primary Endpoints

- Remission of UC (mucosal healing) at 12 weeks as assessed by blinded sigmoidoscopy. Assessment defined as Mayo score ≤ 2 with an endoscopic Mayo score of 0
- Proportion of successful engraftment of donor faecal microbiota at 12 weeks in each group as analysed by 16S sequencing and longitudinal diversity index.

Secondary Endpoints

- Rate of recruitment of patients
- Disease specific scores after treatment using IBDex severity scoring index (224), CUCQ-32 severity scoring index (285) and Mayo scoring system (167)
- Histological grading of colitis severity after treatment
- Mucosal immunological response to treatment (tissue IL-10 and IL-21 by ELISA)
- Rate of development of adverse effects to FMT

4.1.3 Participant Selection

Potential participants were identified by their clinicians in clinics and endoscopy units within the Health Board. Each potential participant was screened for eligibility once referred to the research team. All subjects had to have a definitive histological diagnosis of UC before enrolment. Minimum required microscopic features included cryptitis, crypt abscesses, crypt distortion and mucin depletion in the absence of granulomata. Participants with any features not consistent with UC were excluded. A minimum period of one month from identification to screening was applied to exclude participants with acute self-limiting colitis. A written patient information sheet was provided at the time of the endoscopic diagnosis. After the formal diagnosis or the time of referral to the research team, subjects were offered a minimum of 24 hours to consider enrolment before providing written informed consent. During the screening visit, the study was fully explained, and consent was obtained if the subject satisfied all inclusion and exclusion criteria as described in Table 14.

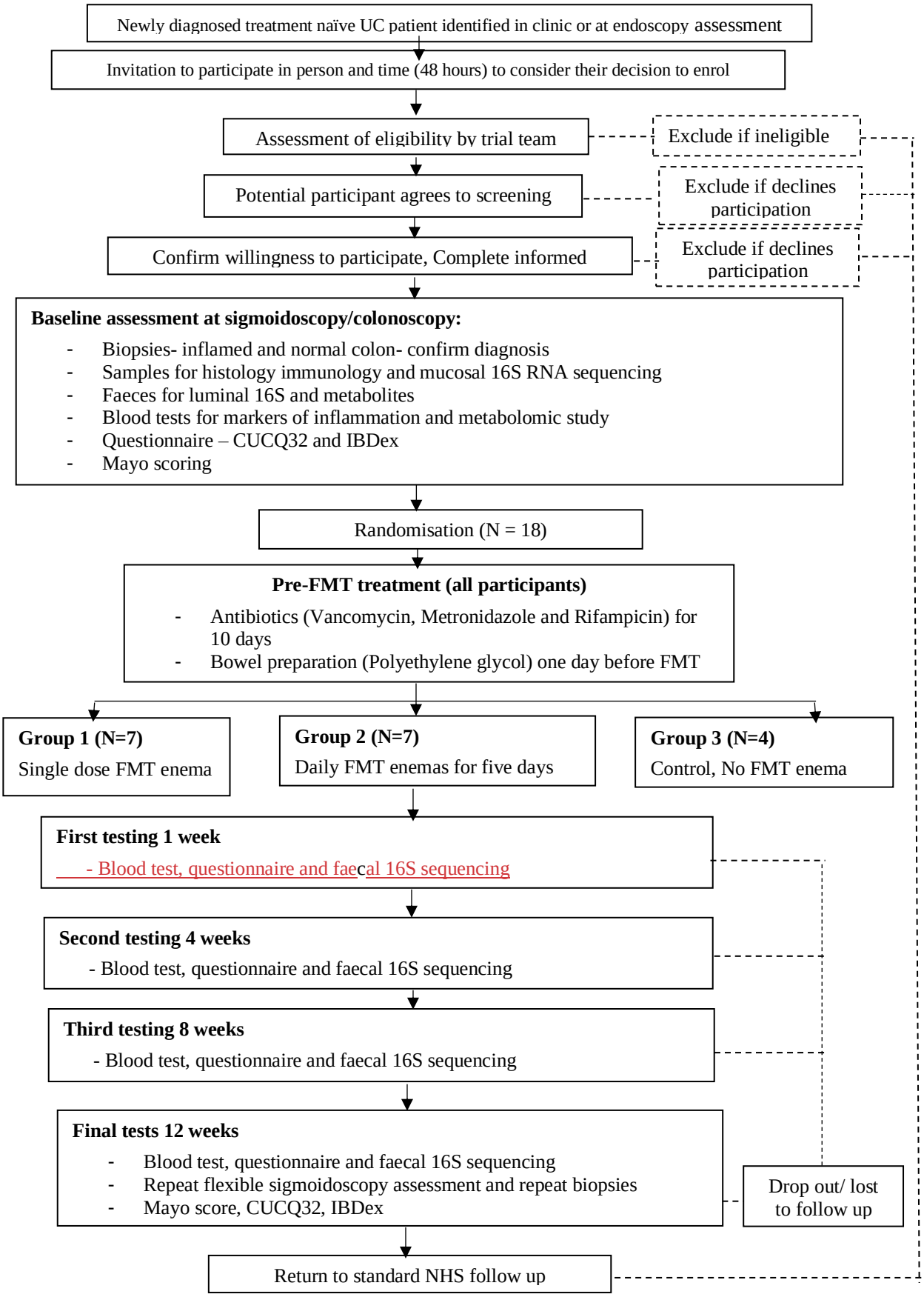


Figure 12: Study scheme flowchart

Table 14: Participant selection criteria

Inclusion Criteria
• Newly diagnosed histologically confirmed UC with inflammation limited to the rectum or recto-sigmoid (within 40cm of anal verge as measured by flexible sigmoidoscopy or colonoscopy) • Age 18 years and above • Able to give fully informed written consent • Willing to return for sequential FMT dosing and endoscopic assessments • Not in receipt of conventional medical treatment for colitis such as steroids or 5-aminosalicylic acid (5-ASA) i.e. treatment naïve
Exclusion Criteria
• Patients without a definitive diagnosis of UC (for example diagnosis of Crohn's disease or infectious colitis) • Colitis extending beyond 40cm from the anal verge • Diagnosis of acute severe colitis (defined as greater than 6 blood-stained stools per 24 hrs with one of the following: pulse rate >90/ temperature >37.8 degree/ haemoglobin <105g/L / ESR>30) • Abdominal tenderness on examination • Already commenced standard medical therapy for UC • Contraindication to oral bowel preparation • Allergy to antibiotics used in the study • Age less than 18 • Patient within a vulnerable group, defined as people who are unable to take care of him or herself, or unable to protect him or herself against significant harm or exploitation • Pregnant • Immuno-suppressed e.g. transplant patient • Known communicable disease or at least 2 weeks full recovery from infectious disease e.g. chickenpox, • Systemic autoimmunity, or atopic diseases • Previous prosthetic implant (for example metallic heart valve, joint replacement, ventricular-peritoneal shunt, cardiac stent) • Chronic pain syndromes (for example: fibromyalgia, chronic fatigue) • Neurologic, neuro-developmental or neurodegenerative disorders

- Depression (requiring therapy)
- Obesity (BMI>35)
- Malignancy
- Use of antibiotics for any indication within the past 3 months
- Foreign travel to areas of enteric disease prevalence within 3 months
- High risk sexual behavior (examples: sexual contact with anyone with HIV/HTLV/AIDS or hepatitis B/C carrier, men who have sex with men (MSM))
- Known exposure to HIV or hepatitis B/C
- Current/previous use of injected drugs or intranasal cocaine
- Tattooing, piercing, cosmetic botulinum toxin or permanent makeup within 120 days (as per Welsh Blood Transfusion guidelines)
- Recent blood transfusion, tissue / organ transplant or skin graft
- Risk factors for variant Creutzfeldt-Jakob disease e.g. blood transfusion or transplant after 1st January 1980

4.1.4 Interventions and Investigational Product

In this study, there was no fixed time frame was set from the screening, enrolling and randomisation to the time of the first FMT interventions as long as symptoms and clinical situations remained the same. This allowed participants to choose their best time to focus on the interventions. However, all participants chose their treatments within one month to control their symptoms.

All three study groups were required to complete a ten-day course of oral antibiotics (Metronidazole 400mg, vancomycin 500mg, rifampicin 150mg twice daily), ending at least forty-eight hours before the first FMT treatment. This was in order to allow the poorly absorbed vancomycin to wash out of the gastrointestinal tract. Patients therefore started the ten-day course of antibiotics twelve days before the first FMT was given. These antibiotics were chosen following the recently published guidelines on FMT in clinical practice (180) for whole gut decontamination. Additionally, all participants received bowel preparation (polyethylene glycol, 2 litres) one a day before transplantation to prepare the bowel lumen for engraftment of the FMT treatment and to minimise interference from the existing gut microbiota.

4.1.4.1 Investigational Product (FMT Samples)

Initially, all FMT samples were obtained from the Wessex stool bank (Queen Alexandra Hospital, Portsmouth). Due to the regulation change explained in chapter 2.8, all FMT samples were

manufactured in Singleton Hospital, Swansea, by Dr Andrew Cunningham, after the regulation changes.

FMT Products from Wessex Stool Bank

Wessex stool bank had two stool donors who are qualified the screening tests (Table 15), who were unrelated to and non-cohabiting with the study participants. Wessex stool bank was originally set up to provide FMT for patients with CDI. FMT products from Wessex stool bank were ordered before study subjects underwent the interventions. The products from Wessex stool bank were delivered to Swansea with a carrier specialised in delivering medical products and kept the temperature at -80°C. The pellet was resuspended and frozen in 20% glycerol and stored for up to 8 weeks at -80°C until the day of treatment.

Table 15: Stool donor checklist

TESTS
Faecal PCR
Salmonella spp.
Shigella spp. / EIEC
VTEC (E.coli)
Campylobacter coli/jejuni/lari
Yersinia enterocolitica
C.difficile Toxin B
Entamoeba histolytica
Cryptosporidium spp.
Giardia spp.
Norovirus I/II
Adenovirus
Astrovirus
Sapovirus
By culture:
Vibrio spp.
VRE
CPE
ESBL producers
Microscopy :
Cryptosporidium
Faecal concentrate for parasites
Wet prep for parasites
Serology
Acute Hepatitis A (HAV IgM)
Acute Hepatitis E
C.difficile GDH / Toxin A/B
HBs Ag

anti-HBc (IgG and IgM)
anti-HBs
HIV ELISA Type 1 and 2
HCV Ab
VDRL (Syphilis)
Rotavirus
Acid-fast stain
Cyclospora
Isospora

Manufacturing FMT Products in Singleton Hospital, Swansea

After the HMRA regulation changes, all FMT stool products were manufactured by Dr Andrew Cunningham in Singleton Hospital, Swansea, UK. The manufacturing procedure was guided by the recent international consensus guideline on FMT (180), together with strict adherence to the protocol of the Wessex stool bank to minimise the discrepancies.

Firstly, volunteers from Swansea University underwent the screening tests (Table 15) and only volunteers who were negative of these tests were selected as donors of FMT stool samples for this study. These volunteers provided their stools in a food-safe, new, clean, non-sterile, disposable plastic food storage box [http://www.sainsburys.co.uk/shop/gb/groceries/foil-food-bags-storage/sainsburys-plastic-containers-x8], which was kept in 2-5 degree straight after the void and stool samples were processed as described in Table 16 within six hours.

Table 16: FMT Sample Manufactural Steps

1. Donor sample voided by volunteer and time of voiding recorded on food safe box by way of pre-attached label.
2. Sample stored at 2-5°C until ready for preparation.
3. Sample should be processed within a maximum of 6 hours from receipt
4. Don new personal protective equipment (water resistant gown and gloves)
5. Soak a disposal plastic sieve into a Virkon bath in a bucket for 30 minutes
6. Rinse the sieve with Milli Q water thoroughly for approximately 10 minutes
7. Clear and clean work space within the fume hood as above
8. Within the fume hood weigh 1g of stool into a clean 50ml Falcon tube. Label tube with donor number and date. This should be stored at 2-5°C for pooled testing as described in SOP faecal microbiota transplant donor screening.
9. Weigh the remaining stool sample – zero balance with empty food safe box, then place box containing voided sample on balance

10. Weigh 50g of stool sample
11. The remaining will be discarded
12. Time of voiding and time of processing noted to be recorded in FMT database
13. Weigh 500ml of 0.9% saline into a clean tripoint beaker
14. Using wooden/plastic stick to help, place donor stool into the tripoint beaker with 500ml of 0.9% saline
15. Pour this into the sieve
16. Retaining contents of central mesh compartment, pour the filtrate into the tripoint beaker
17. Using a 10ml pipette divide the filtrate into 50ml aliquots in 50ml Falcon tubes
18. Centrifuge the aliquots at 1772RPM (Rotor IEC 236, 50ml inserts) for 60 mins
19. Discard supernatant and re-suspend pellet in 10% molecular grade glycerol/90% saline.
20. Label sides and lids with the donor number and date and a sequential number for each tube processed. The donor number takes the form of YEAR_XX where YEAR represents the year the donor began donating and XX is a sequential number for each new donor in that year. The date takes the form DDMMYY e.g. DonorNo_DDMMYY_SEQNo., 2015_03_040615_01, 2015_03_040615_02
21. An expiry date should be clearly marked on each tube. This is for 3 months beyond the processing date e.g. if processed on 04/06/2015 the expiry would be 04/09/2015
22. The tubes can now be frozen at -80°C
23. Dispose of the waste into an autoclave bag and place into an autoclave bin
24. Record the data for each donor aliquot onto the FMT database

Preparation of samples was carried out in a laboratory isolated room with a fume hood within category 2 environment. Before and after use all internal cabinet surfaces were decontaminated by removing visible particles and matter and then applied Virusolve. Virusolve remained in contact with the surface for 1 minute before any excess was wiped away with disposable paper towels. Furthermore, any items in category 2 laboratory were cleaned with Virusolve.

Every donated sample underwent testing as described in Table 16 and data was entered into the FMT database and each aliquot was labelled with the donor number, date of donation and an expiry date. This would provide traceability of all samples from donation to delivery and link quality control data. The freezer recording system documented fluctuations in storage temperatures or errors should they occur.

In the case of multiple treatments (group 2), all doses were obtained from the same donor to minimise variation. Appendix 1 described the standard operating procedure (SOP) for manufacturing FMT products, which was used for this study.

4.1.4.2 Administration of Investigational Product

All three study groups completed a ten-day course of oral antibiotics (vancomycin 500mg; metronidazole 400mg, rifampicin 150mg – all taken twice daily) and bowel preparation (polyethylene glycol 2 litres on the day before transplantation). The first FMT treatment dose was commenced 48 hours after the final dose of antibiotics to preserve the activity of the FMT. Frozen FMT was thawed over four hours at room temperature prior to infusion, which was subsequently diluted to 250mL with non-bacteriostatic normal saline prior to infusion. The subjects of Group 1 and 2, who received FMT treatment, were also given loperamide 4mg orally thirty minutes prior to infusion to maximise the chance of enema retention. Each participant received five doses of 50mL of enema every 15 minutes over 60 minutes. The subjects were encouraged to retain the treatment samples as long as possible (ideally more than one hour).

4.1.5 Study Setting

Recruitment took place in clinics and endoscopy units within the Swansea Bay University Health Board, Swansea. FMT was administered at the Joint Clinical Research Facility (JCRF), within ILS-2, Swansea University.

4.1.6 Randomisation

Study participants were randomised 2:2:1 by a web-based method hosted by the University of Aberdeen's Health Services Research Unit. The simple randomisation process employed in this allocation was not stratified by any factors (e.g. age, gender). We aimed to update the randomisation process based on the results of this feasibility study for potential stratifying factors in phase III.

4.1.7 Blinding

The trial statistician, the assessing independent endoscopist and the pathologist undertaking macroscopic and microscopic disease assessments were blinded to the treatment allocation.

4.1.8 Participant Timeline and Schedule of Assessment

Figure 12 and Table 17 show the follow up schedule and assessment for the trial. The study participants underwent assessment for disease activity with validated tools (CUCQ-32, IBDex and Mayo Score) alongside a full history and physical examination, which were recorded as their baseline. Baseline

biopsies of the rectum for 16S rRNA analysis and immunological studies (IL-10 and IL-21), faecal samples for 16S rRNA analysis and metabolomic profile, blood tests (renal function, liver function, full blood count, C-reactive protein, metabolomic profile) were also obtained. Furthermore, 16S rRNA analysis for the donors' faecal samples was performed at the end of the study. Subsequently, this was studied together with 16S rRNA analysis of the participant's faecal samples for the study of durability of engraftment after FMT treatments during a 12-week follow up period.

Follow-up visits took place at week 1, 4, 8 and 12 for all three study groups. Participants underwent clinical examination, blood and faecal testing including faecal microbiota profiling using the 16S rRNA analysis and metabolomic profile and completed disease activity scoring questionnaires (CUCQ-32 and IBDex) during the follow up period. At the final assessment (week 12), all subjects were also assessed with a flexible sigmoidoscopy for macroscopic assessment and biopsies. Participants who relapsed or failed to improve after FMT were offered conventional medical therapy. Study participants were instructed to inform the treating physician of any infectious symptoms or new medical conditions which developed after receiving FMT.

Table 17: Follow up schedule and assessments

	Baseline	**Week 1**	**Week 4**	**Week 8**	**Week 12**
Questionnaires					
CUCQ-32	●	●	●	●	●
IBDex	●	●	●	●	●
Mayo score	●				●
Endoscopy assessment					
Sigmoidoscopy	●				●
Rectal biopsy	●				●
Histology assessment					
Histological grading	●				●
Mucosal 16S sequencing	●				●
Mucosal IL-10	●				●
Mucosal IL-21	●				●
Blood tests					
Renal profile	●	●	●	●	●
Liver profile	●	●	●	●	●
Full blood count	●	●	●	●	●
C-reactive protein	●	●	●	●	●
Metabolomic profile	●	●	●	●	●
Faecal sample assessment					
16S sequencing	●	●	●	●	●
Metabolomic profile	●	●	●	●	●

4.1.9 Withdrawal

Participants could be withdrawn from the study if;

- they wished to terminate treatment and/or follow-up assessments
- clinical features worsened during FMT or the 12-week follow-up period
- the participant was non-compliant with the study in a manner that was either harmful to their health or interfered with the validity of the study results
- participants who withdrew their consent were able to request deletion of their study data.

4.1.10 Data Collection and Management

Data collection was performed at baseline, week 1, 4, 8 and 12 as described in Table 17. All data was to be recorded on the case report form (CRF) in an anonymized format against a unique participant number and kept in the JCR, ILS-2. Data was transferred to a computer database without patient identifiable data and analysed once all results were collected. The trial database was built in measures to assess data quality at time of input and stored securely.

4.1.11 Participant Rights and Confidentiality

The Principal Investigator is the custodian of the data. Information with regards to study participants was kept confidential and managed in accordance with the Data Protection Act, NHS Caldicott Guardian, The Research Governance Framework for Health and Social Care and Research Ethics Committee Approval. There was no patient identifiable data on the CRF and a unique participant number was allocated. The Principal Investigator held the key to the coded number of the participants only. Only the Principal Investigator had access to the patient identifiable information.

4.1.12 Statistical Analysis

Both descriptive and exploratory data analysis was performed. For each group, the number of participants approached, and/or assessed for eligibility, randomised and receiving treatment were calculated. Thus, the recruitment and retention rate along with the rate of adverse events were obtained. Descriptive statistics (mean, standard deviation, 95% confidence interval) for continuous outcomes (e.g. CUCQ-32 Score, Mayo Scoring) and raw count (n, %) for categorical outcomes (e.g. renal profile, liver profile, histological grading) were reported as per the clinical endpoints.

Statistical analysis as well as data were provided as per baseline and other follow ups (as appropriate to the outcome measure) and with respect to the three treatment arms. All the analysis and data preparation were performed using SPSS software v.28.0 (SPSS Inc., Chicago, IL, USA) as a validated statistical software for clinical trials. Statistical significance was set as P-value less than 0.05. All data

were presented as the median +/- standard deviation (SD) if the distribution was not normal. Kruskal-Wallis test was used to compare two or more samples in a non-parametric distribution.

4.1.13 Safety Measures

An adverse event (AE) is defined as any untoward medical occurrence in a patient after administration of the study intervention (FMT) that does not necessarily have to have a causal relationship with this treatment. Serious adverse events (SAEs) is any adverse experience occurring during or after FMT that results in either death, life-threatening experience or requiring inpatient hospitalisation, persistent or significant disability or incapacity. SAEs were notified to the study sponsor within twenty-four hours and to the Research Ethics Committee (REC) within fifteen days.

The common FMT related AEs are procedure related symptoms such as bloating, transient fever or abdominal discomfort as reported by previously reported studies. AEs that were expected for patients undergoing FMT, and symptoms expected from UC, were specified in the protocol and were not required to be reported as adverse events.

4.1.14 Quality Assurance

The Research and Development Quality Assurance Officer performed a Monitoring Prioritisation Assessment to assess the impact of trial participation on the rights and safety of participants and the reliability of trial results. This guided the development of procedures in the trial with respect to informed consent, confidentiality and trial monitoring. Monitoring visits to the site was made every three months during the study to ensure that all aspects of the protocol were followed except the site closure during the national pandemic. The Quality Assurance Officer also monitored the study throughout this study period. A Quality Assurance programme was also in place to ensure adherence to the study protocol. Major and minor deviations were collected.

Endoscopic assessment was carried out by one of several JAG (Join Advisory Group on GI endoscopy) accredited gastroenterologists or colorectal surgeons from hospitals of the Swansea Bay University Health Board. The study team ensured that the endoscopist performing the 12-week assessment was blinded to the intervention that the patient received. Endoscopic photographs taken at baseline and at final assessment were independently assessed by a blinded expert to provide quality assurance for this outcome measure.

Blinded histological assessment was performed by consultant pathologists who followed a standardised protocol based on Royal College of Pathologists guidelines.

4.1.15 Ethics and Dissemination

The Principal Investigator ensured that the trial was conducted in compliance with the principles of the Declaration of Helsinki (1996), and in accordance with all applicable regulatory requirements including but not limited to the Research Governance Framework, Trust and Research Office policies and procedures and any subsequent amendments. The trial was approved by the regional NHS ethics committee (REC) and Health Research Authority (HRA) or equivalent. Written informed consent was obtained from all participants. Serious adverse events were reported to the study sponsor and the regional ethics committee.

4.1.16 Clinical Trial Protocol Checklists - SPIRIT

The study protocol was published on BMJ Open under the title Protocol for faecal microbiota transplantation in ulcerative colitis (FMTUC): a randomised feasibility study (286) (Appendix 8).

SPIRIT checklist

Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) 2013 (287) is guideline to aid in improving the content and quality of clinical trial protocols (Appendix 9). These 33-item SPIRIT checklist was to ensure that the protocol provided a high-quality, transparent and comprehensive planning document for participants, sponsors, research ethics committees, peer reviewers and journals.

4.2 Clinical Results

4.2.1 Study Recruitment and Subjects' Demographics

Between July 2016 and February 2020, in total of 20 patients were recruited to the study, of which 18 patients were eventually enrolled. Although the initial target recruitment was not met, the further recruitment was forced to halt during the COVID-19 and thus the decision was made to assess the feasibility of a phase III trial. One subject One patient had busy work commitments, which meant he was unlikely to be able to attend interventions and subsequent follow up clinics, and thus he chose a conventional treatment option. The other patient did not fit the criteria. The CONSORT diagram of the FMTUC is shown in Figure 13.

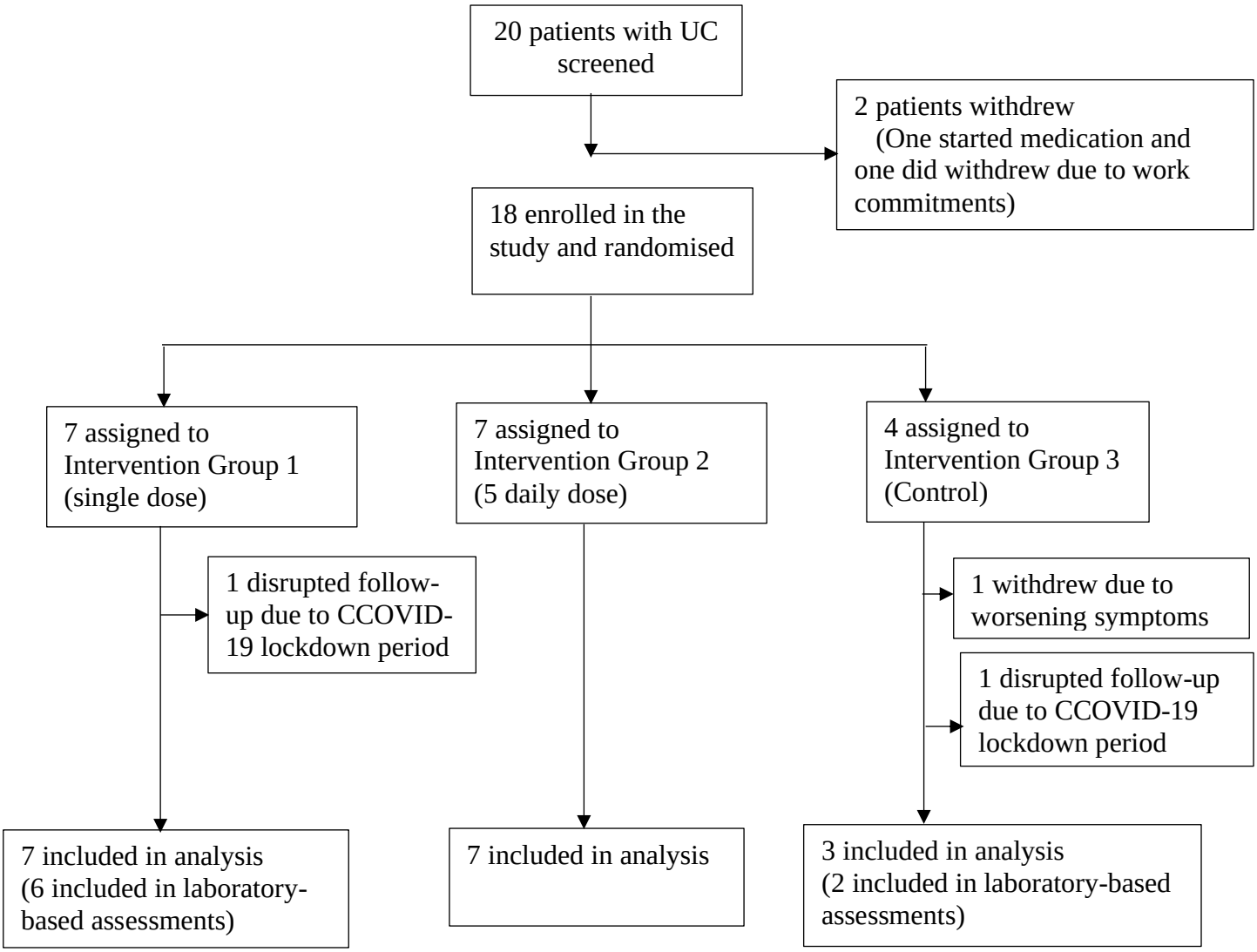


Figure 13: FMTUC CONSORT diagram

Seven patients were assigned to intervention group 1 and 2 each. Four patients were also allocated to Group 3 as shown in Table 18. The median age of the participants was 42 years old with interquartile range (IQR) of 30 - 54 years old. The median ages and IQR of each group 1-3 were 42 (34.5-50.5), 38 (28.5 – 49.5) and 49.5 (37.5-58.75) respectively. Eleven female (61.1%) and seven male (38.9%)

patients were enrolled in the study, of which five female patients were assigned to each group 1 and 2. Three male patients and one female patient were assigned to control group 3.

Two subjects' follow-ups were disrupted due to the COVID-19 lockdown. They received telephone consultations at week 1, 4, 8 and 12 instead and responded to the CUCQ-32 and the IBDex. However, they did not have blood tests during the 12-week follow up period and endoscopic assessment at week 12. Their faecal samples were also not collected at week 12, and thus their results were incomplete for our study, however, we included their data in qualitative assessments.

Table 18: Demographic information of FMTUC participants

Subject ID	DOB	Age	Sex	Study registration Date	Intervention Group
01	11/23/1965	50	F	13/07/2016	1 dose
02	03/24/1972	44	F	25/08/2016	5 doses
03	02/24/1975	42	F	14/10/2016	1 dose
04	10/06/1961	55	M	14/10/2016	5 doses
05	03/11/1992	24	M	23/11/2016	Control
06	08/03/1959	57	M	23/01/2017	Control
07	12/08/1982	36	F	13/12/2017	5 doses
08	07/25/1989	28	M	20/12/2017	1 dose
09	05/15/1976	41	M	08/01/2018	1 dose
10	13/11/1996	21	F	03/10/2018	5 doses
11	09/19/1966	51	F	20/11/2018	1 dose
12	15/05/1963	55	F	12/12/2018	1 dose
13	08/01/1954	64	M	22/03/2019	Control
14	24/11/1948	70	F	02/04/2019	5 doses
15	04/02/2001	18	M	24/07/2019	5 doses
16	07/03/1981	38	F	08/08/2019	5 doses
17	26/06/1976	42	F	19/02/2020	Control
18	13/07/1991	28	F	10/02/2020	1 dose

Baseline characteristics of participants enrolled in the FMTUC study are as summarised in Table 19.

Table 19: Baseline characteristics of FMTUC subjects

	Group 1 (Single dose) N = 7	**Group 2 (5 doses) N = 7**	**Group 3 (control) N = 4**
Age (years)	42 (34.5 – 50.5)	38 (28.5-49.5)	49.5 (37.5-58.75)
Male	2 (29%)	2 (29%)	3 (75%)
Female	5 (71%)	5 (71%)	1 (25%)
Duration of disease (months)	11 (6.5-18.5)	9 (8-11)	16.5 (9-24.5)
Disease extent			
Proctitis	4 (57%)	1 (14 %)	3 (75%)
Procto-sigmoiditis	3 (43%)	6 (86%)	1 (25%)
Total Mayo score	5 (2.5-7)	8 (5.5 – 9.5)	6 (3.5 – 8)
IBDex score	15 (11-20.5)	12 (9.5 – 24.5)	18 (5.5 – 29.75)
CUCQ-32 score	67 (55-130.5)	103 (82-127)	102 (37.5–164.25)
Haemoglobin (g/L)	128 (120.5-140.5)	130 (126-141)	142.5 (138-152)
White cell count (x10^9 cells per L)	6.3 (6.15-6.85)	6.3 (4.9-6.85)	5.6 (4.925 – 6.225)
C-reactive protein (mg/L)	5 (5-5)	5 (5-6)	5 (5 -6.25)
Albumin (g/L)	45 (44.5-46)	43 (40.5 – 46.5)	44.5 (42-47)

Data are median (IQR) or number of patients (%)

4.2.2 Qualitative Assessments of Clinical Response

4.2.2.1 Clinical Response Assessed by Mayo Score

Mayo score is derived from 4 parameters: stool frequency, rectal bleeding, physician's global assessment and endoscopic assessment (Appendix 2). Unlike other two assessment tools, the Mayo score does not consider patients' subjective views and consists of objective assessments by clinicians. Table 20 summaries the Mayo score of the study subjects at baseline and week 12.

Table 20: Mayo score total score at baseline and Week 12 and clinical remission of each subject

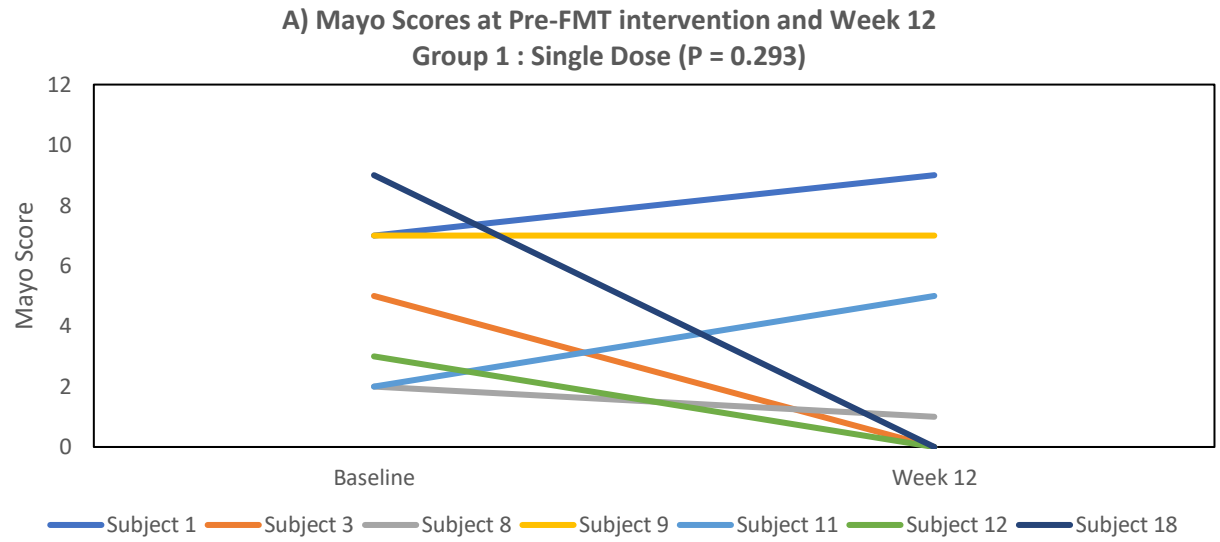
Intervention Group	**Subject ID**	**Baseline**	**Week 12**	**Clinical Remission**	**Post-trial management**
1 (1 dose)	**1**	7	9	N	Medical treatment
	3	5	0	Y	None
	8	2	1	N	None
	9	7	7	N	Medical treatment
	11	2	5	N	Medical treatment
	12	3	0	Y	None
	18	9	0	Y	None
2 (5 doses)	**2**	5	4	N	None
	4	11	6	N	Medical treatment
	7	9	1	N	None
	10	11	9	N	Medical treatment
	14	8	6	N	Medical treatment
	15	6	4	N	Medical treatment
	16	5	3	N	Medical treatment
3 (Control)	**5**	2	0	Y	None
	6	8	Withdrew	N	Withdrew, Medical treatment
	13	8	8	N	Medical treatment
	17	4	0	Y	None

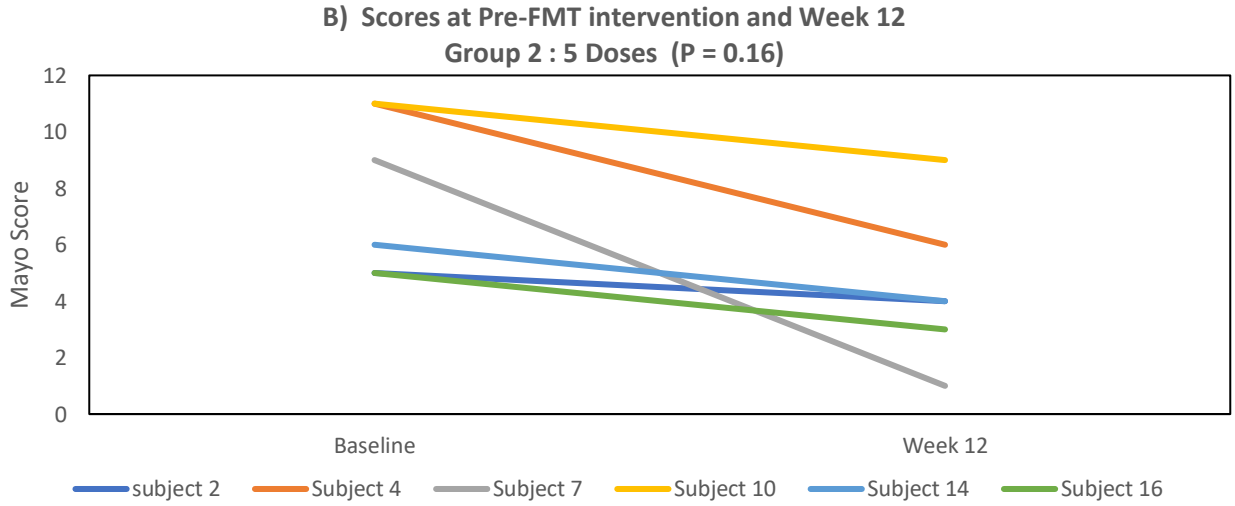
Y = Clinical remission achieved at week 12, N = Clinical remission not achieved

The median Mayo score among all subjects at the baseline assessment was 6.5 with IQR 4.25-8. This was decreased to 4 (IQR 0-6) at week 12. When this was evaluated depending on the intervention arms, the median with IQR of each group was 5 (2.5-7), 8 (5.5-10) and 6 (3.5-8) respectively. All intervention groups showed some improvement in the Mayo score, and the median Mayo score of each group 1-3 came down to 1 (0-6), 4 (3.5-6) and 4 (0-4) respectively.

Mayo score was used to determine the endpoint of this study, and clinical remission was defined as Mayo score ≤ 2 with an endoscopic Mayo score of 0. Three subjects from group 1 (single dose) achieved clinical remission, however, no subjects from group 2 (5 doses) achieved clinical remission. Two subjects from the control group also achieved clinical remission. Although clinical remission was achieved by five subjects, eight subjects experienced improvement in their clinical symptoms and required no further medical treatment after the 12-week follow up assessment. Of those eight subjects, two (one participant each from Group 1 and 2) subjects failed to achieve clinical remission by one score on the endoscopic Mayo score. In contrast, subject 2 from group 2 (5 doses) scored 4 on the Mayo score at the 12-week follow up assessment, however, this subject opted out from the conventional medical management.

The Mayo scores of all subjects before and after the interventions were compared using Wilcoxon singed ranks test, which was grouped by intervention groups (Figure 14). The Wilcoxon signed ranks test is a non-parametric statistical hypothesis test used to assess two related samples (288) and was used to assess the statistical significance of the difference in the Mayo score between pre and post interventions. The null hypothesis stated that the median difference of the Mayo score between pre- and post- intervention equalled zero. The analysis showed that all intervention groups had P values greater than 0.05, implying that the hypothesis was retained and there was no statistical difference in the Mayo score between pre and post interventions in all intervention groups. However, the sample size in this feasibility study was not powered to demonstrate statistical difference.





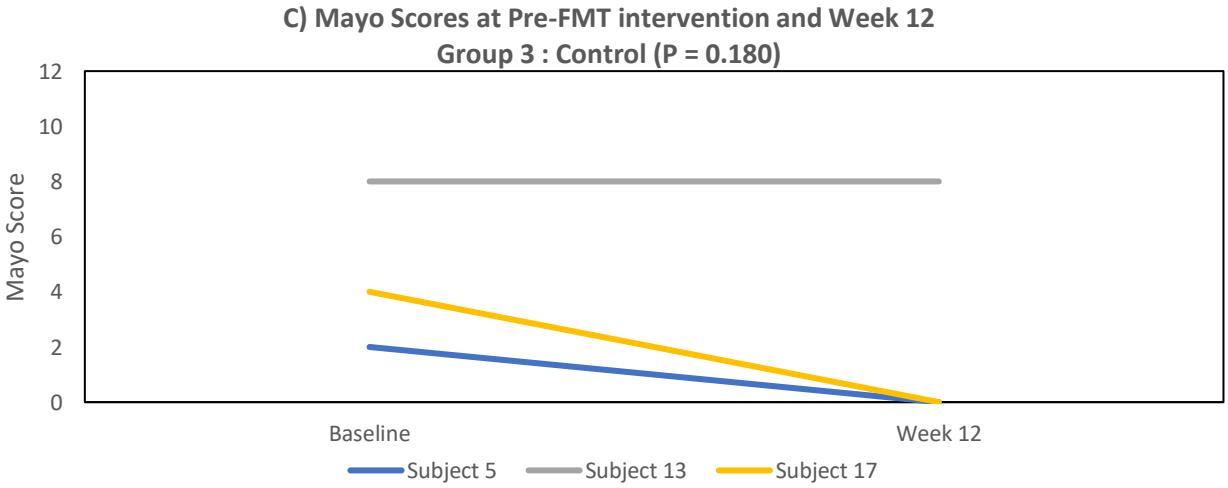


Figure 14: Mayo Scores at pre FMT interventions (baseline) and week 12 grouped by interventions A: Group 1 (Single dose), B: Group 2 (5 doses), C: Group 3 (Control)

The change in the Mayo scores of all subjects in the different treatment groups are presented in Figure 15 using Box-Whisker plot graph. The Kruskal-Wallis test was used to evaluate the change in the Mayo scores between different interventions.

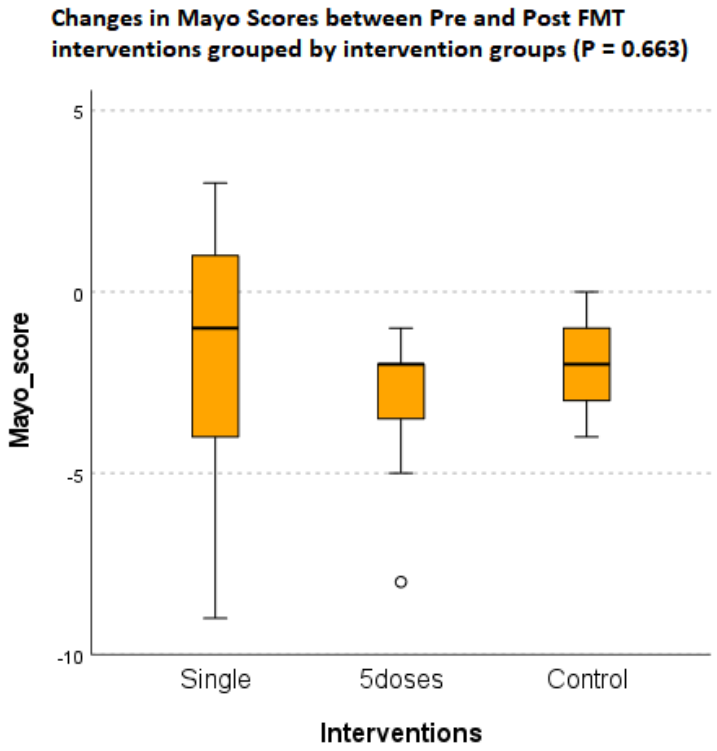


Figure 15: Box-Whisker plot of Mayo Score between the baseline screening and week 12 grouped by intervention groups.

The bold line in each box graph represents the median value (50th percentile), while the orange box contains the 25th and 27th percentiles of the data. The black upper and lower extremes denote the 5th and 95th percentiles. Values beyond these upper and lower bounds are outliners, which are marked with circles. Circle represents outlier.

This analysis showed, at 12 weeks, no statistically significant difference in change in the Mayo score between pre- and post-intervention amongst all groups ($p = 0.663$). This confirmed that there was no statistically significant difference in the Mayo score in all intervention groups between pre- and post-intervention at 12 weeks. However, the sample size was too small to draw any conclusions from this statistical analysis. The estimated sample size that would be needed for the study to have enough power to show statistically significant differences between these intervention groups was calculated using G*Power software (289). First, the effect size was calculated from chi-square obtained from the Kruskal-Wallis test using SPSS and the detailed calculation is shown in Appendix 10. This effect size was then used to calculate sample size with power of 0.8 and accepted alpha as 0.05 using G*Power.

Since Kruskal-Wallis test is a non-parametric test, the total sample size was estimated with 15% additional to the sample size computed based on ANOVA (290). This predicted the minimum of 75

subjects (23 subjects each in intervention group) would be required to provide a probability of detecting a true effect.

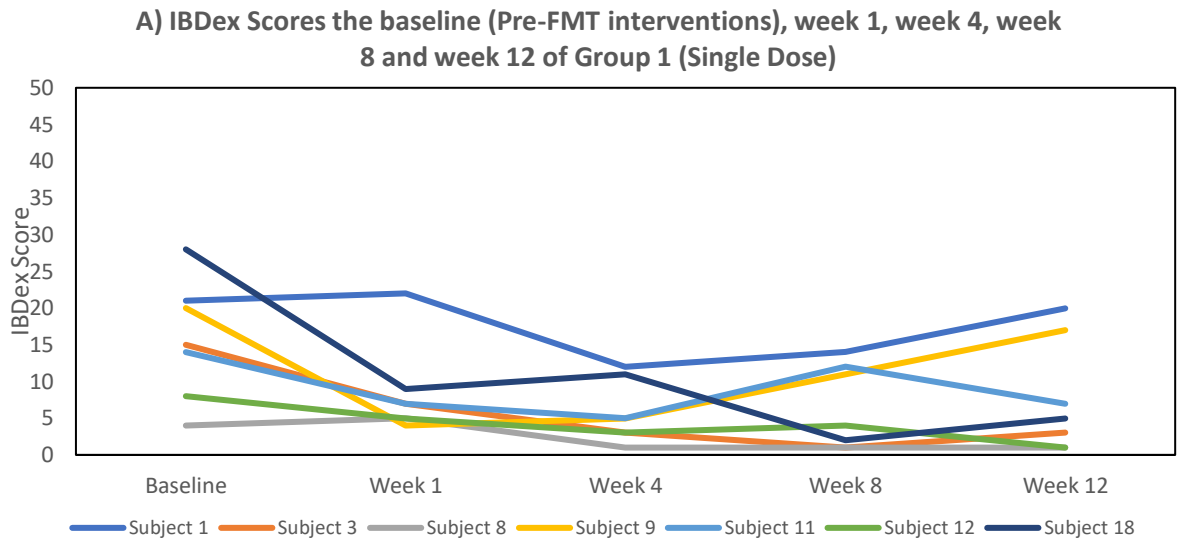
4.2.2.2 Clinical Response Assessed by IBDeX

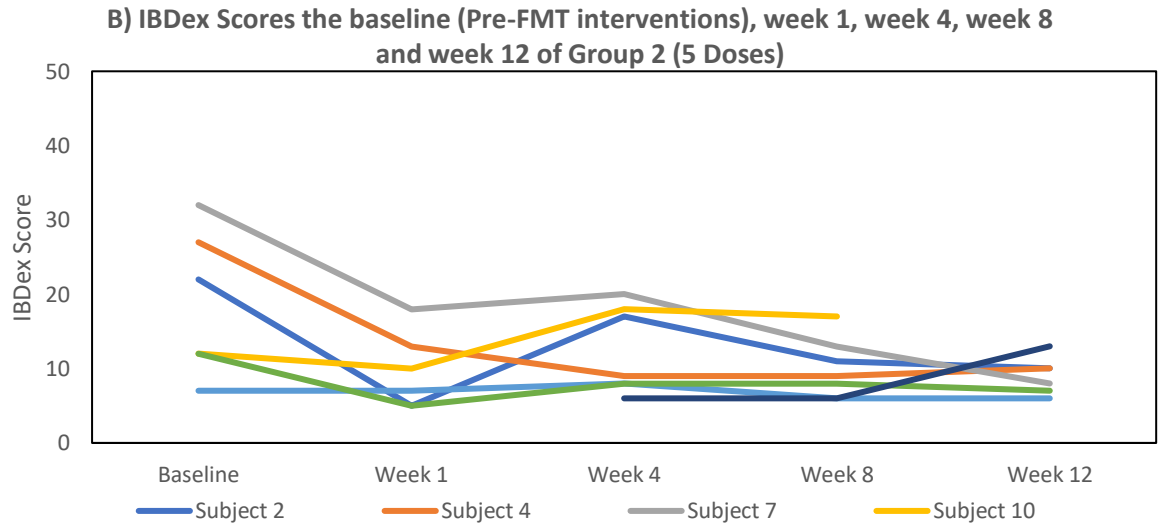
Table 21 is a summary of IBDex total scores during the 12-week follow up period. IBDex is a clinical assessment tool with a combination of subjective and objective assessments (224) (Appendix 3). The IBDex total score is the sum of 14 questions ranging from zero to more than 23. The higher the total score, the more severe the condition.

Subject 10, 16 and 17 failed to fill in the questionnaire at one of their follow up assessments. The median IBDex score of all participants at baseline screening was 14.5 with IQR of 7.25-25.75, which decreased to 7 with IQR, 3 – 10.5 at week 12. This trend was seen in all intervention groups including the control group. Furthermore, many subjects experienced significant improvement in their physical symptoms at week 1 assessment including those in the control group. This may have been attributed to the bowel cleanse, the antibiotics and/or placebo effects. However, when IBDex scores at week 1 and week 12 were compared using the Wilcoxon signed ranks test, no statistically significant difference was observed ($P = 0.222$).

Table 21: IBDex Total Scores during the 12 week follow up period

Intervention Group	**Subject ID**	**Baseline**	**Week 1**	**Week 4**	**Week 8**	**Week 12**	**Clinical remission**	**Post-trial management**
1 (Single dose)	**1**	21	22	12	14	20	N	Medical treatment
	3	15	7	3	1	3	Y	None
	8	4	5	1	1	1	N	None
	9	20	4	5	11	17	N	Medical treatment
	11	14	7	5	12	7	N	Medical treatment
	12	8	5	3	4	1	Y	None
	18	28	9	11	2	5	Y	None
2 (5 doses)	**2**	22	5	17	11	10	N	None
	4	27	13	9	9	10	N	Medical treatment
	7	32	18	20	13	8	N	None
	10	12	10	18	17	No data	N	Medical treatment
	14	7	7	8	6	6	N	Medical treatment
	15	12	5	8	8	7	N	Medical treatment
	16	6	No data	6	6	13	N	Medical treatment
3 (control)	**5**	1	1	9	1	1	Y	None
	6	32	6	29	Withdrew	Withdrew	N	Withdraw, Medical treatment
	13	29	19	22	22	12	N	Medical treatment
	17	7	No data	4	3	3	Y	None





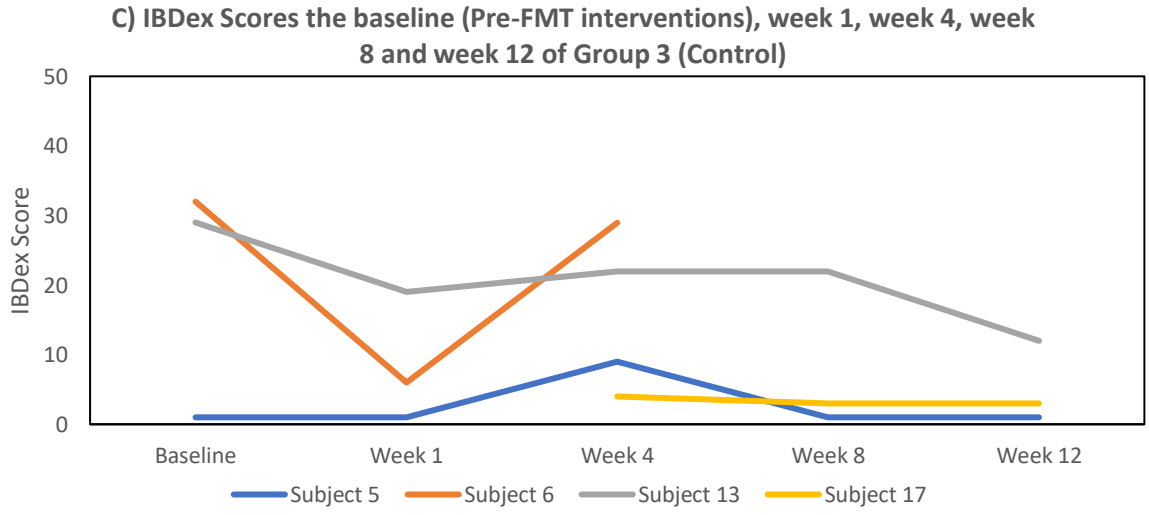


Figure 16: Changes to IBDex scores during the 12-week follow up period grouped by intervention groups

A: Group 1 (Single dose), B: Group 2 (5 doses), C: Group 3 (Control)

4.2.2.3 Clinical Response Assessed by CUCQ-32

CUCQ-32 quantifies IBD patients' quality of life from patients' perspectives (285). The sum of the CUCQ-32 ranges from 0 to 272. The higher the score is, the worse the quality of life for patients.

Table 22 is a summary of the CUCQ-32 scores grouped by the intervention arms. The raw data and answers for each CUCQ-32 question are shown in Appendix 11.

Table 22: Summary of CUCQ-32 total scores during the follow up period

Intervention Group	Subject ID	Baseline	Week 1	Week 4	Week 8	Week 12	Clinical Remission
Group 1 (1 Dose)	1	151	163	89	101	121	N
	3	110	80	35	35	27	Y
	8	47	16	17	15	14	N
	9	67	28	30	51	127	N
	11	59	15	35	97	73	N
	12	51	30	15	25	17	Y
	18	160	81	85	109	33	Y
Group 2 (5 Doses)	2	124	51	68	89	91	N
	4	130	90	39	23	21	N
	7	103	127	135	61	77	N
	10	160	62	151	169	185	N
	14	103	100	72	77	85	N
	15	61	59	49	45	40	N
	16	59	15	29	52	33	N
Group 3 (Control)	5	18	27	25	21	16	Y
	6	177	57	159	Withdrew	Withdrew	N
	13	160	177	189	144	170	N
	17	44	No Data	38	22	31	Y

The median baseline CUCQ-32 score of all subjects was 103 (59-145.75), which decreased to 40 (27-91) at the final assessment (week 12). The median score of each intervention group 1-3 at the baseline was 67 (55-130.5), 103 (82-127) and 102 (37.5-164.25) respectively, and this reduced to 33 (22-97), 77 (36.5-88) and 31 (23.5-100.5) at week 12. As seen with the IBDex score, many subjects reported significant improvement at week 1 and the median score was reduced by 44.

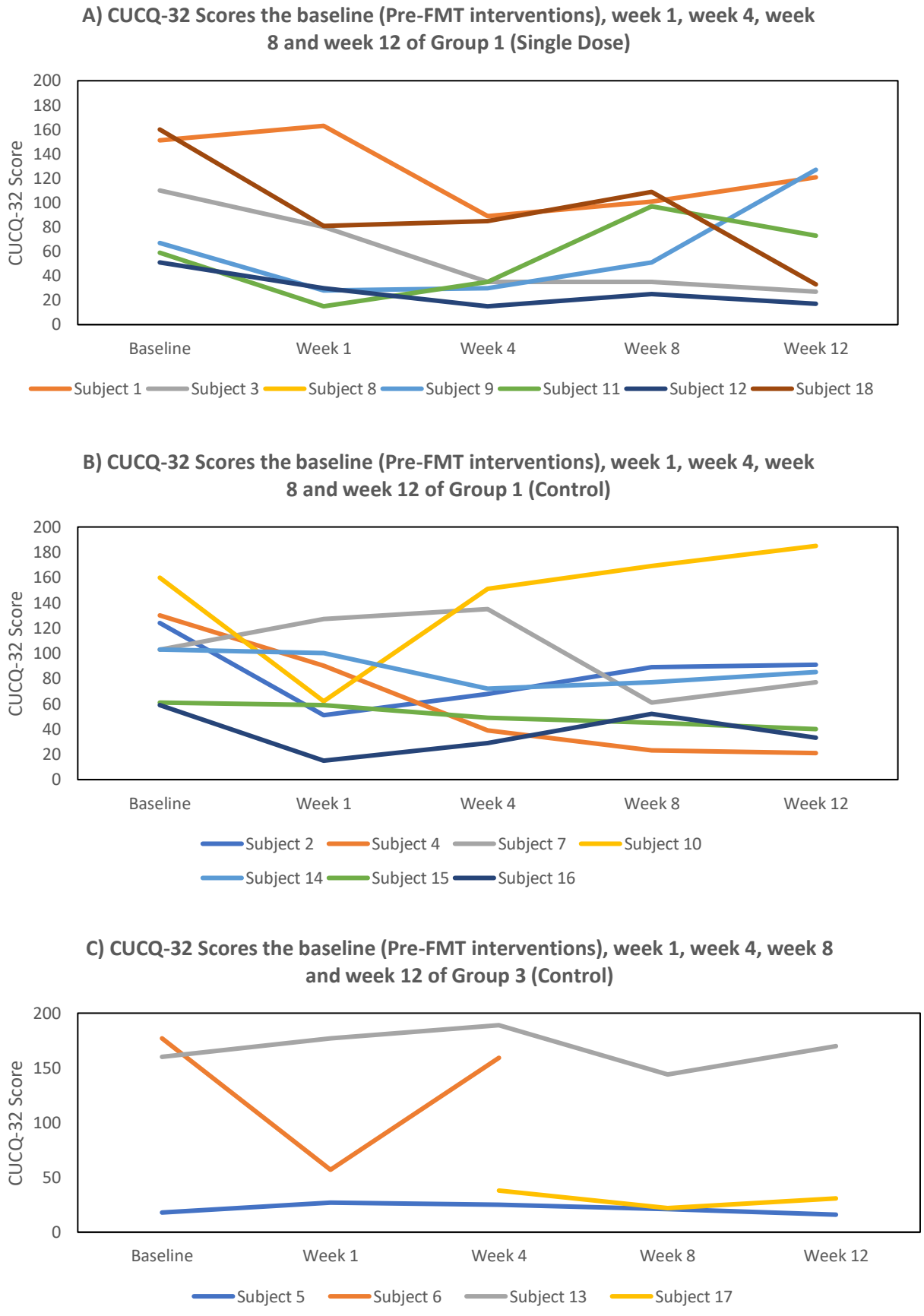


Figure 17: Changes to CUCQ-32 scores during the 12-week follow up period grouped by intervention groups

A: Group 1 (Single dose), B: Group 2 (5 doses), C: Group 3 (Control)

4.2.2.4 Overall Qualitative Response to Treatment of FMTUC

Table 23 summarises all three qualitative assessments according to the intervention groups, whereas Table 24 summarises those according to clinical response.

Table 23: Comparisons between baseline screening and week 12 of each qualitative assessment score according to intervention groups

	Mayo Score (median, IQR)		**IBDex (median, IQR)**		**CUCQ-32 (median, IQR)**		**Clinical Remission**
	Baseline	**Week 12**	**Baseline**	**Week 12**	**Baseline**	**Week 12**	
Group 1 (Single dose)	5 (2.5-7)	1 (0-6)	15 (11-20.5)	5 (2-12)	67 (55-130.5)	33 (22-97)	3/7 (43%)
Group 2 (5 doses)	8 (5.5-10)	4 (3.5-6)	12 (9.5-24.5)	9 (7.25-10)	103 (82-127)	77 (36.5-88)	0/7 (0%)
Group 3 (control)	6 (3.5-8)	4 (0-4)	18 (5.5-29.75)	3 (2-7.5)	102 (37.5-164.25)	31 (23.5-100.5)	2/4 (50%)

Table 24: Comparisons between baseline screening and week 12 of each qualitative assessment score Qualitative assessment (baseline and Week 12) according to clinical response

	Mayo (median, IQR)		**IBDex *(median, IQR)***		**CUCQ-32 *(median, IQR)***		**Duration of disease (months) *(median, IQR)***	**Extent of disease *(median, IQR)***
	Baseline	**WK12**	**Baseline**	**WK12**	**Baseline**	**WK12**		
Clinical remission	4 *(3-5)*	0 (0-0)	8 *(7-15)*	3 (1-3)	51 *(44-110)*	27 (17-31)	15 *(9-24)*	1 *(1-2)*
Non-clinical remission	7 *(5-8)*	5.5 (3.75-7.25)	20 *(12-27)*	10 (7-12.5)	103 *(61-151)*	81 (38.25-122.5)	9 *(9-12)*	2 *(1-2)*

The majority of subjects experienced improvement in their symptoms across the intervention groups (Table 23) and all three interventions improved qualitative assessment scores. Subject 10 failed to answer the IBDex at the week 12. Subject 6 also withdrew from the study for worsening symptoms at week 8, and thus subject 6's qualitative assessments were not included in the calculation. An interesting observation was made that the initial median scores of all three qualitative assessments were significantly lower amongst the cohort who achieved clinical remission (Table 24). Furthermore, marked reductions in all qualitative assessment were observed amongst the clinical remission group.

For instance, the median Mayo score at the baseline assessment among subjects who achieved clinical remission 4, whereas that of subjects who did not achieve clinical remission was 7. This may imply that clinical remission was more frequently observed among subjects with milder UC.

Three qualitative indices were further studied to evaluate correlations between these indices using the Spearman correlation, which demonstrated strong positive correlations amongst three qualitative assessments as summarised in Table 25.

Table 25: Correlation between qualitative assessments at week 12

		Mayo	IBDex	CUCQ-32
Mayo	Correlation coefficient	1.000	0.802	0.777
	P (2-tailed)		<0.001	<0.001
IBDex	Correlation coefficient	0.802	1.000	0.780
	P (2-tailed)	<0.001		<0.001
CUCQ-32	Correlation coefficient	0.777	0.780	1.000
	P (2-tailed)	<0.001	<0.001	

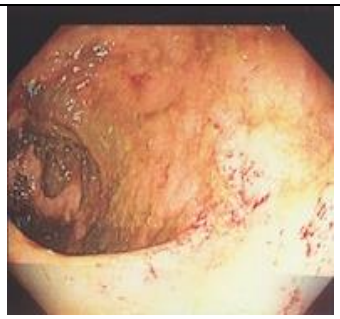
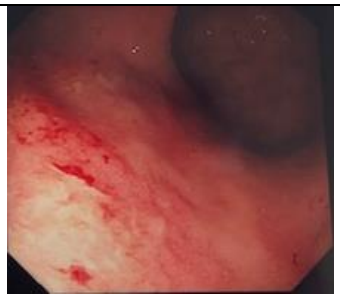
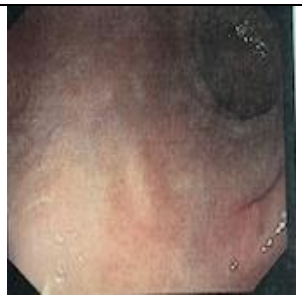
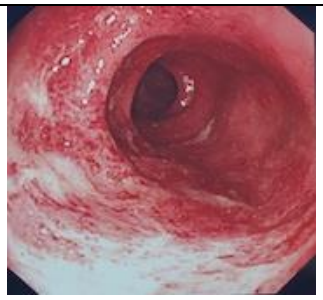
It is important to reiterate that the above observations and statistical analysis should be taken with caution as the sample size is very small in this study. Yet, some interesting observations were made. Although the median scores of qualitative assessments improved pre- and post-interventions in all intervention groups, many did not improve in a linear fashion. Many subjects reported clinical improvements at week 1 and some reported that their symptoms returned as time went on. The other key observation was the baseline median score of all qualitative assessments were lower among the cohort who achieved clinical remission. It was also observed that subjects with less extended disease were more likely to achieve clinical remission. 37.5% (3/8) of subjects with proctitis achieved clinical remission, whereas only 20% (2/10) of subjects with proctosigmoiditis achieved clinical remission. Although it was not powered for statistical significance, it raises important questions to be considered for the future. Is enema the right choice of route of FMT administration? Did the initial endoscopic assessment underestimate microscopic disease? If microscopic disease was present beyond the point where enema could physically reach, these patients might have not benefitted from FMT interventions. This could be the reason why the condition of subject 1 and 11, who initially had only proctitis, extended to more extensive left-sided colitis at the 12-week follow-up assessment after FMT interventions.

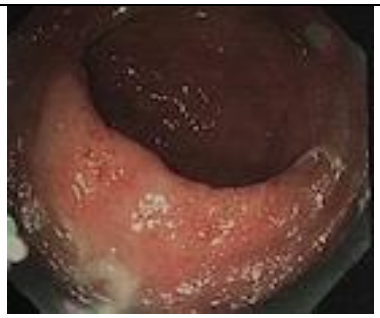
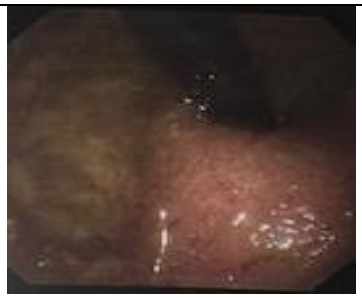
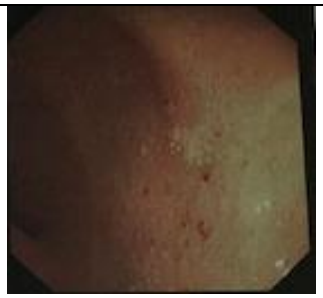
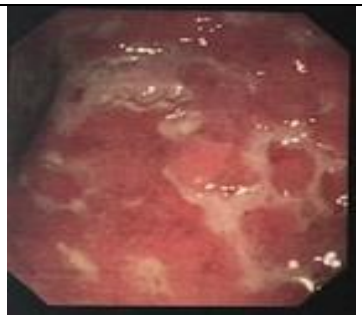
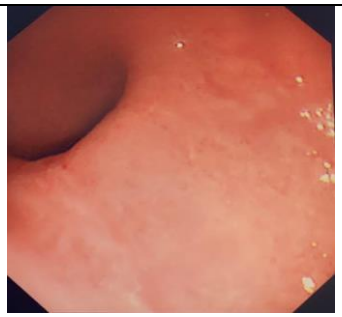
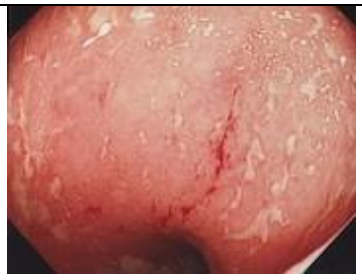
4.2.3 Endoscopic Response to Treatment of FMTUC

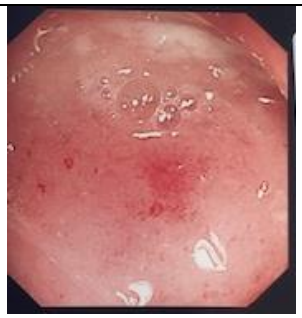
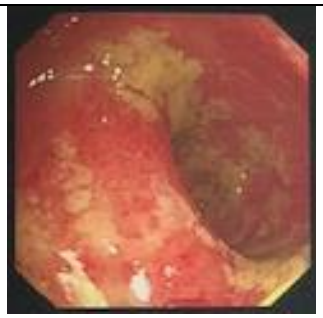
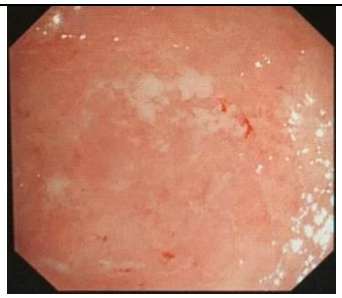
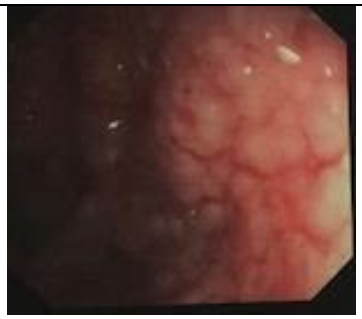
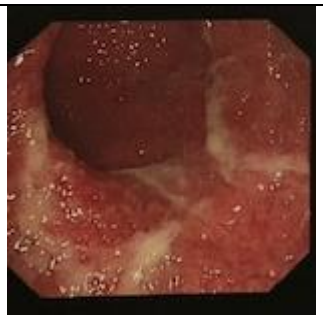
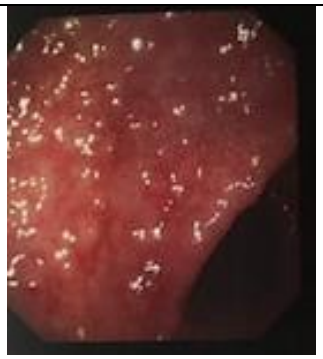
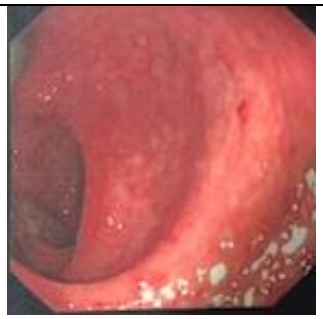
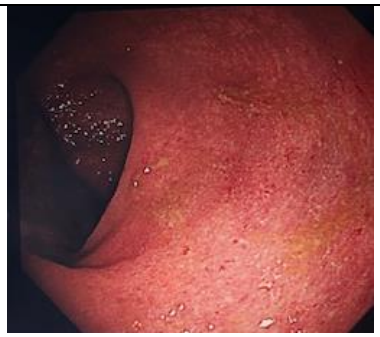
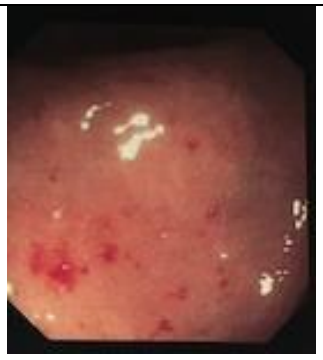
Flexible sigmoidoscopy or colonoscopy allow clinicians to assess macroscopic changes in the colonic mucosa. It also provides an assessment of the severity of UC including chronic and active inflammation as well as any other pathology including cancers.

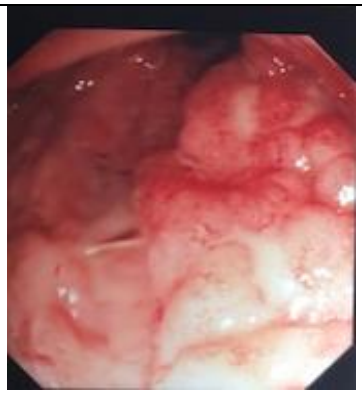
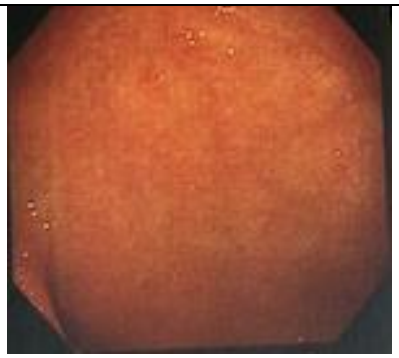
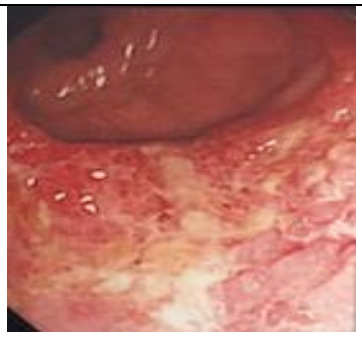
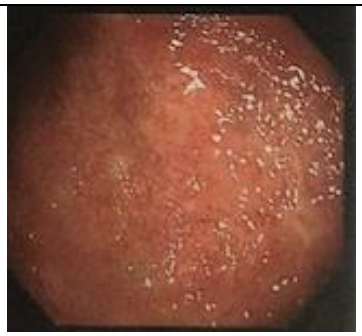
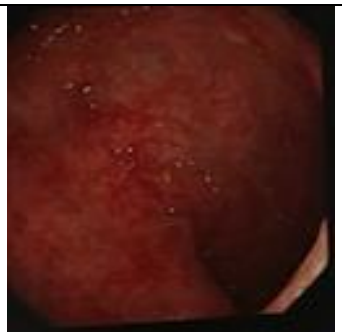
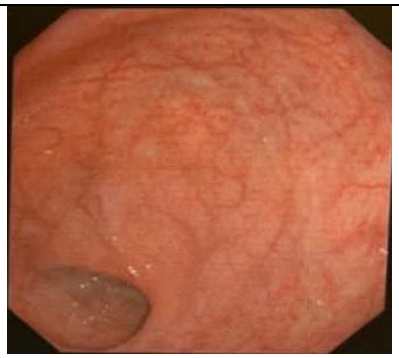
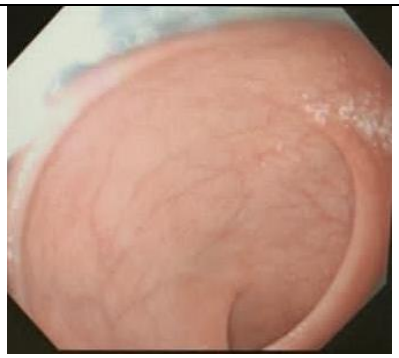
In this study, the endoscopic Mayo score was defined by endoscopists at the time of endoscopic assessments. These endoscopists were blinded from subjects' intervention group. It was not always possible to obtain high-quality photographs due to technical issues. Table 26 exhibits endoscopic Mayo scores and photographic evidence. Pre- and post-intervention photographs were not necessarily taken at the same colonic segments, however, they were taken to show macroscopic improvement or progress of the disease from the area that endoscopists felt the most representative of the disease severity.

Table 26: Endoscopic assessment of FMTUC subjects

Intervention Group	**Subject ID**	**Baseline**	**Week 12**	**Clinical Remission**
1 (Single dose)	1	 Mayo endoscopic score 1	Mayo endoscopic score 2	N
	3	 Mayo endoscopic score 1	 Mayo endoscopic score 0	Y
	8	 Mayo endoscopic score 2	Mayo endoscopic score 1	N

	9	 Mayo endoscopic score 2	Mayo endoscopic score 2	N
	11	 Mayo endoscopic score 2	 Mayo endoscopic score 2	N
	12	 Mayo endoscopic score 1	Mayo endoscopic score 0	Y
	18	 Mayo endoscopic score 2	 Mayo endoscopic score 0	Y
2 (5 doses)	2	 Mayo endoscopic score 1	Mayo endoscopic score 1	N
	4	Mayo endoscopic score 3	Mayo endoscopic score 2	N

	7	 Mayo endoscopic score 1	 Mayo endoscopic score 1	N
	10	Mayo endoscopic score 3	 Mayo endoscopic score 1	N
	14	 Mayo endoscopic score 2	 Mayo endoscopic score 1	N
	15	 Mayo endoscopic score 2	 Mayo endoscopic score 1	N
	16	 Mayo endoscopic score 1		N

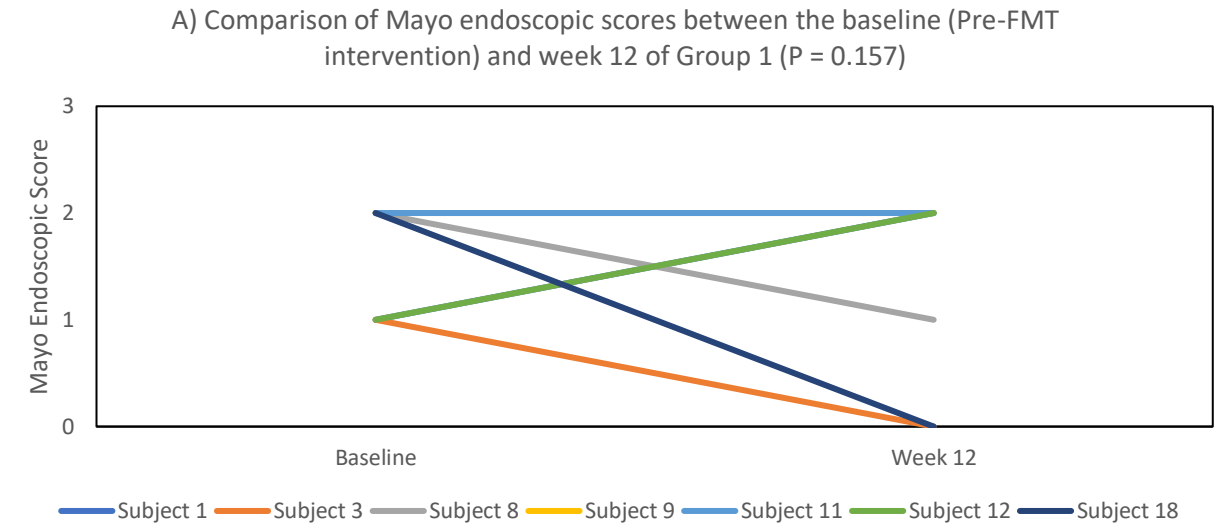
			Mayo endoscopic score 1	
3 (Control)	5	 Mayo endoscopic score 3	 Mayo endoscopic score 0	Y
	6	 Mayo endoscopic score 2	No Endoscopy	N
	13	 Mayo endoscopic score 2	 Mayo endoscopic score 2	N
	17	 Mayo endoscopic score 1	 Mayo endoscopic score 0	Y

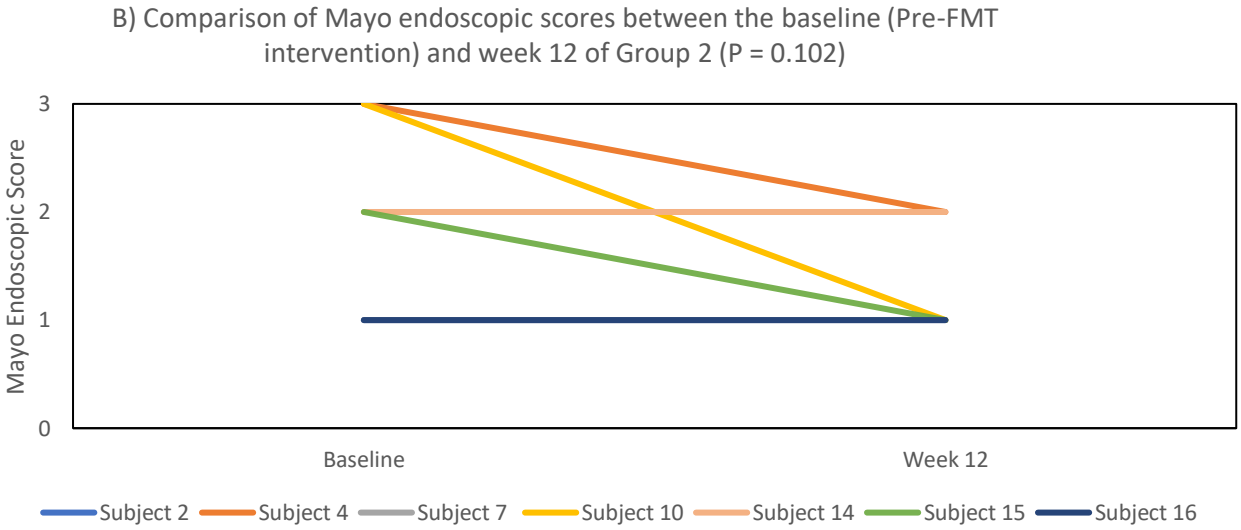
The median Mayo endoscopic scores at the baseline and week 12 were grouped by clinical remission in Table 27. Both clinical remission and non-clinical remission groups showed reductions in the median endoscopic Mayo score.

Table 27: Mayo endoscopic score grouped by clinical response

	Mayo endoscopic score (Median, IQR)	
	Baseline	**Week 12**
Clinical remission	1 (1-2)	0 (0-0)
Non-clinical remission	2 (1-2)	1 (1-2)

The endoscopic Mayo scores for subjects in all intervention groups before and after the interventions were also compared using Wilcoxon singed ranks test (Figure 18). There was no statistical difference in the endoscopic Mayo score between pre and post interventions with P value greater than 0.05, however, again, the sample size is very limited.





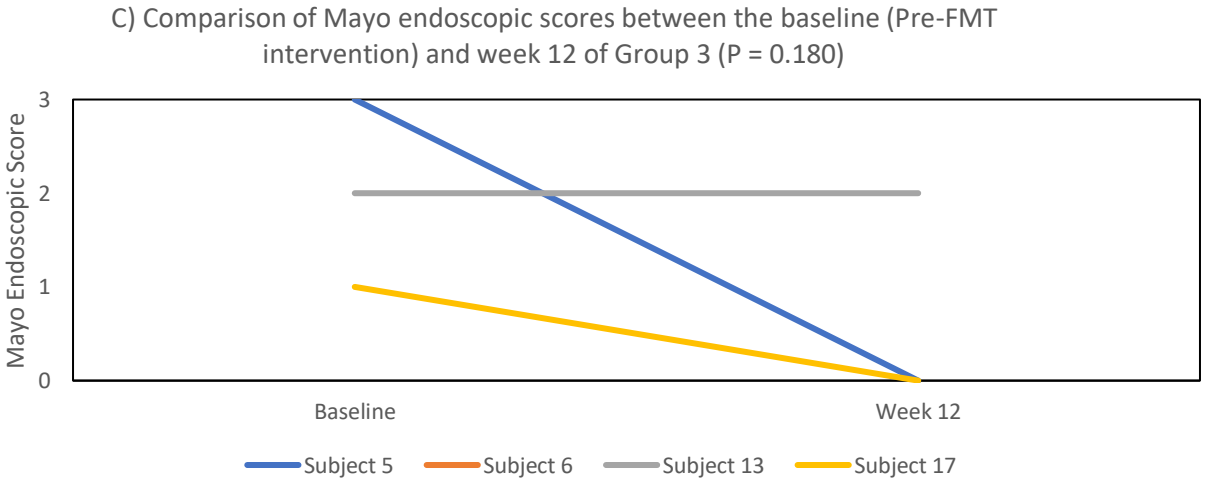


Figure 18: Comparison of the Mayo endoscopic scores between baseline (Pre-FMT intervention) and week 12 grouped by intervention groups

A: Group 1 (Single dose), B: Group 2 (5 doses), C: Group 3 (Control)

The changes in the endoscopic Mayo score of subjects depending on their intervention groups were further evaluated using the Kruskal-Wallis test (Figure 19). The statistical analysis suggested that there was no statistical difference with P = 0.679.

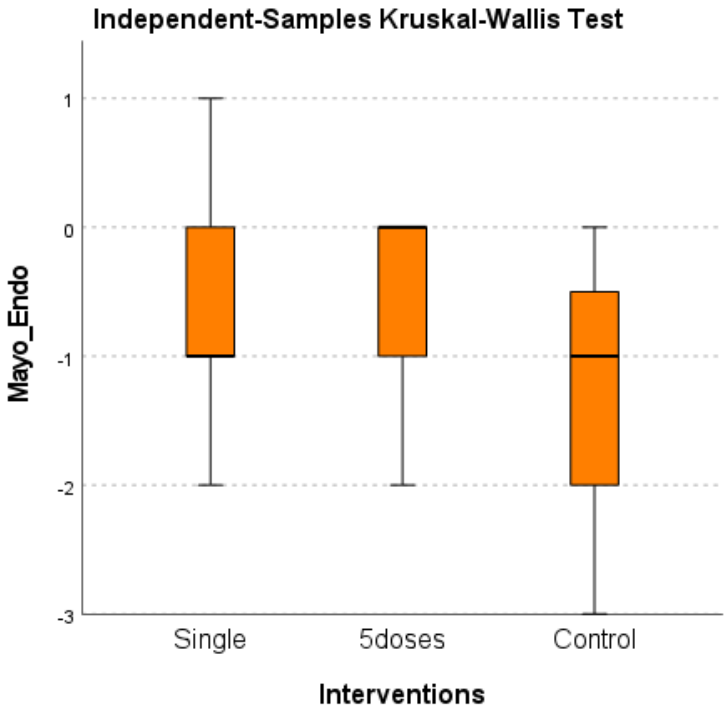


Figure 19: Box-Whisker plot of Mayo endoscopic score changes between the baseline screening and week 12 grouped by intervention groups.

The bold line in each box graph represents the median value (50th percentile), while the orange box contains the 25th and 27th percentiles of the data. The black upper and lower extremes denote the 5th and 95th percentiles.

The correlations between the endoscopic Mayo score and the three qualitative indices at week 12 were also examined with the Spearman's correlation coefficient. Overall, the analysis indicated that the endoscopic Mayo score demonstrated high correlations with the quantitative indices (Table 28), which is expected as the Mayo endoscopic score is one aspect of Mayo score. The CUCQ-32 showed a moderate correlation with the Mayo endoscopic score. This could mean that the CUCQ-32 did not capture the severity of the disease as accurate as the other indices. It is also possible that this might be type II error due to the small sample size.

Table 28: Correlation between endoscopic Mayo score and qualitative index scores

		Mayo	**IBDex**	**CUCQ-32**	**Endoscopic Mayo**
Mayo	Correlation coefficient	1.000	0.802	0.777	0.870
	P (2-tailed)		<0.01	<0.01	<0.01
IBDex	Correlation coefficient	0.802	1.000	0.780	0.713
	P (2-tailed)	<0.01		<0.01	0.002
CUCQ-32	Correlation coefficient	0.777	0.780	1.000	0.573
	P (2-tailed)	<0.001	<0.001		0.016
Endoscopic Mayo	Correlation coefficient	0.870	0.713	0.573	1.000
	P (2-tailed)	<0.001	0.002	0.016	

4.2.4 Histological Response to Treatment of FMTUC

Biopsies taken during endoscopic assessments were assessed for the presence of cryptitis, crypt abscess, granulomas, ulceration, mucin depletion, crypt loss, crypt distortion, lymphoid follicular hyperplasia, dysplasia and malignancy by a clinical consultant histopathologist. Table 29 summarises histological findings grouped by interventions.

Table 29: Histological assessments before and after FMTUC

Intervention Group	**Subject ID**	**Baseline**	**Week 12**	**Clinical Remission**
1 (Single dose)	1	Cryptitis Crypt abscess	Cryptitis Crypt abscess Crypt loss Crypt distortion	N
	3	Mucin Depletion	Crypt distortion	Y
	8	Crypt abscess Crypt distortion	Crypt distortion - Mild	N
	9	Cryptitis Crypt abscess Mucin depletion	Cryptitis Crypt distortion Ulceration	N
	11	Cryptitis Crypt abscess Mucin depletion	Crypt abscess Crypt distortion	N
	12	Cryptitis Crypt abscess	Normal	Y

		Mucin Depletion Crypt distortion		
	18	Cryptitis Crypt loss Crypt distortion	Cryptitis Crypt distortion	Y
2 (5 doses)	2	Mucin Depletion Crypt distortion	Cryptitis Crypt abscess	N
	4	Cryptitis Crypt abscess Mucin Depletion Crypt distortion	Cryptitis Crypt abscess Crypt distortion	N
	7	Cryptitis Crypt abscess Mucin depletion Crypt loss	Cryptitis Crypt distortion	N
	10	Cryptitis Crypt abscess Ulceration Mucin depletion Crypt distortion	Cryptitis Mucin depletion Crypt distortion	N
	14	Cryptitis Crypt abscess Mucin Depletion Crypt loss Crypt distortion	Marked crypt distortion Severe chronic inflammation Crypt abscess	N
	15	Cryptitis Crypt abscess	Crypt distortion Moderate inflammation Cryptitis Crypt abscess	N
	16	Mucin Depletion Crypt distortion	Mild crypt distortion	N
3 (Control)	5	Cryptitis	Crypt loss Mild crypt distortion	Y
	6	Ulceration Crypt distortion	No Data (Withdraw)	N
	13	Cryptitis Crypt abscess Mucin Depletion	Cryptitis Crypt abscess Mucin depletion	N

		Crypt distortion	Mild crypt distortion Crypt loss	
	17	Cryptitis	Normal	Y

The Nancy histological index (291) was employed to quantify these histological assessments as shown in Table 30. The Nancy histological index is one of the commonly used indices to quantify histological findings as described in Figure 20.

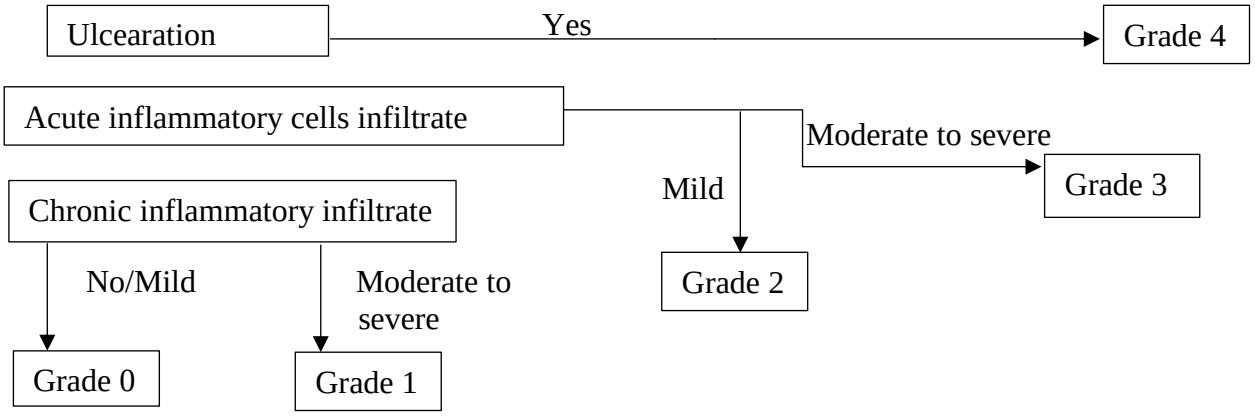


Figure 20: The Nancy histological index score

Table 30: Nancy histology index scores at baseline and week 12

Intervention Group	**Subject ID**	**Baseline**	**Week 12**	**Clinical Remission**
1 (Single dose)	1	2	3	N
	3	2	0	Y
	8	3	0	N
	9	2	3	N
	11	3	3	N
	12	3	0	Y
	18	3	0	Y
2 (5 doses)	2	3	2	N
	4	4	3	N
	7	3	2	N
	10	3	4	N
	14	2	3	N
	15	3	2	N
	16	2	1	N
3 (Control)	5	2	0	Y
	6	1	Withdrew	N
	13	2	3	N
	17	2	0	Y

The Nancy histological index scores for subjects in all three intervention groups before and after the interventions were also compared using Wilcoxon singed ranks test (Figure 21).

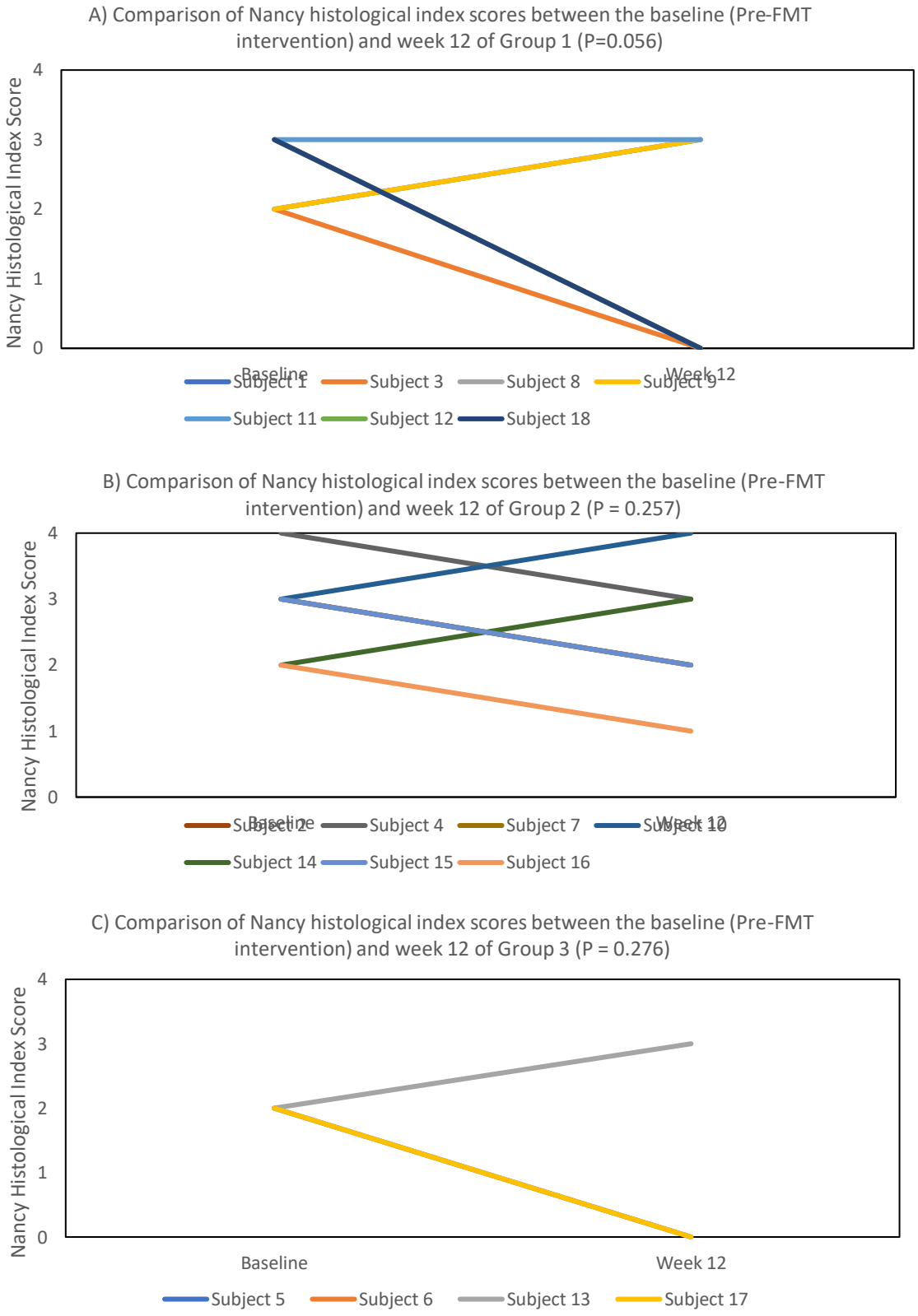


Figure 21: Comparison of the Nancy histological index scores between baseline (Pre-FMT intervention) and Week 12 grouped by intervention groups

A: Group 1 (Single dose), B: Group 2 (5 doses), C: Group 3 (Control)

There was no statistical difference in the Nancy histological index score between pre and post interventions with P value greater than 0.05.

The changes in the Nancy histological index scores for subjects in the different treatment groups were further evaluated using the Kruskal-Wallis test (Figure 22) with no statistical differences (P = 0.532).

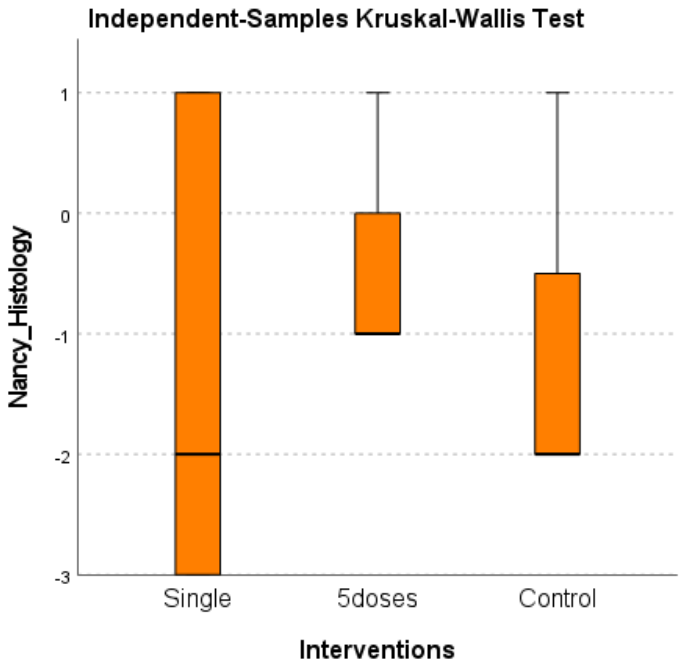


Figure 22: Box-Whisker plot of Nancy histological index score changes between the baseline screening and week 12 grouped by intervention groups.

The bold line in each box graph represents the median value (50th percentile), while the orange box contains the 25th and 27th percentiles of the data. The black upper and lower extremes denote the 5th and 95th percentiles.

The correlation of the endoscopic Mayo score and the Nancy histological index score were further studied. This showed that the correlation coefficient between the two indices of 0.854 [95% CI 0.58 – 1.00, P < 0.001], which was statistically significant and indicated a very strong correlation between the two assessments.

4.2.5 Assessments with Blood Tests after FMTUC Interventions

A set of blood results were monitored throughout the follow up period. These blood tests were to monitor participants' general health, signs of infections and inflammatory process and early detections of possible adverse effects from the interventions including antibiotics, bowel preparation and FMTs. There are no biological or serology markers to detect UC to date, although routine laboratory markers

are often used to monitor the severity of UC. C-reactive protein (CRP) is one of the most sensitive and reliable markers to monitor flare up or acute phase response in UC management.

Table 31 is a summary of the mean values (with IQR) of each blood result at the baseline and week 12, which was categorised based on the intervention groups. Raw data of all participants under Appendix 12. Reassuringly, there was no significant change in blood results during the follow up period in any intervention group to suggest worsening of the disease or significant adverse effects from the interventions.

Table 31: Summary of blood test results

	Normal range	**Baseline**			**Week 12**		
		Group 1	**Group 2**	**Group 3**	**Group 1**	**Group 2**	**Group 3**
Sodium (mmol/L)	133-146	139 (139-141)	140 (138-141.5)	139.5 (138-140)	142 (141.25-142.75)	140 (140-140.5)	143 (139-143)
Potassium (mmol/L)	3.5-5.3	4.4 (4.2-4.45)	4.2 (3.95-4.45)	4.3 (4.175-4.45)	4.25 (4.125-4.3)	4.1 (4.05-4.2)	4.4 (4.1-4.45)
Urea (mmol/L)	2.5 – 7.8	5.3 (4.475-6.2)	3.95 (3.675-5.2)	4.55 (4.05-5.65)	4.25 (4.2-4.6)	4.5 (4.15-5.35)	4.1 (4.1-4.75)
Creatinine (μmol/L)	Male 59/104 Female 45-84	70 (58.5-83.5)	59 (58-70)	81.5 (75-86)	67.5 (60.25-68.75)	74 (67-80)	86 (74.5-89.5)
Bilirubin (μmol/L)	<21	5 (4-6.5)	6 (5.5-8.5)	9.5 (7.75-11.25)	6.5 (6-7)	7 (5.5-10)	13 (8-13)
ALT (IU/L)	Male 10-60 Female 10-40	15 (13.5-18)	12 (9.5-14)	26 (21.5-33.25)	16.5 (13.75-19.25)	14 (12.5-19)	26 (22.5-34.5)
ALP (IU/L)	30-130	70 (57-73)	63 (57.5-92.5)	83.5 (72.75-90.25)	59 (51.5-77.75)	66 (48-87.5)	85 (79-89)
Albumin	35-50	45	43	44.5	44	45	46

(g/L)		(44.5-46)	(40.5-46.5)	(42-47)	(41.75-44)	(43-48.5)	(45.5-47.5)
Haemoglobin (g/L)	Male 130-170 Female 120-150	128 (120.5-140.5)	130 (126-141)	142.5 (138-152)	135 (129.5-140.75)	132 (126.5-146.5)	151 (144-151.5)
WBC ($x10^9$ cells/L)	4.0-11.0	6.3 (6.15-6.85)	6.3 (4.9-6.85)	5.6 (4.925-6.225)	6.95 (5.72-7.25)	6.6 (5.95-8.8)	6.4 (5.4-6.4)
Platelets ($x10^9$ cells/L)	150-400	217 (202.5-315)	303 (298-313)	217 (170.5-280.75)	202 (170-263.25)	284 (275-301)	269 (207.5-300)
CRP (mg/L)	<6.0	5 (5-5)	5 (5-6)	5 (5-6.25)	5 (5-5)	5 (5-5)	5 (5-5)

Chapter 5. Exploratory Mechanistic Analysis of Faecal Microbiota Transplantation in Patients with Ulcerative Colitis

5.1 Materials and Methods

The changes in the gut microbiome after the FMT interventions in patients with UC was studied with 16S rRNA analysis of their faecal samples throughout the 12-week follow up period. Colonic mucosal biopsies were also studied using 16S rRNA analysis at the baseline screening and 12 weeks after the FMT intervention, which attempted to evaluate the colonic mucosal microbiome. Furthermore, Enzyme-linked immunosorbent assay (ELISA) was employed to measure levels of IL-10 and IL-21 pre and post FMT interventions. IL-10 has anti-inflammatory properties in the GI tract, whereas IL-21 plays a regulatory role in the immune response (236,237,241). Both IL-10 and IL-21 are thought to be protective against pathogenesis of UC and this analysis was attempted to study the effect of FMT interventions on potential pathogenesis of UC. Due to the significant delay caused by COVID-19 pandemic, it was not possible to conduct metabolomic analysis for this thesis, however, metabolomic study was another aspect of the mechanistic study to investigate the modulation of the gut microbiome with FMTUC. This study would allow us to evaluate microbial metabolites, disease-related metabolites and dysregulated metabolic pathways.

5.1.1 Microbiota Analysis using 16S rRNA Profiling

A few technologies and platforms are now available to facilitate 16S rRNA sequencing though they all follow the fundamental steps as below.

1) Extraction of DNAs from samples
2) 16S rRNA amplicon library preparation
3) Sequencing
4) Bioinformatics analysis

In this study, the Illumina MiSeq next generation sequencer (Illumina, California, USA) was employed, which also follows the above key steps. This work was carried out in the Institute of Life Science (ILS-1), Swansea University Medical School, by an experienced operator, Dr Matthew Hitchings.

5.1.1.1 Extraction of DNAs from Faecal Samples

The first step of 16S sequencing is DNA extraction, which allows one to purify DNA using different techniques to separate DNA from proteins, cell membranes and other cellular components of the samples as these can interfere with DNA analysis. DNA extraction involves lysis of the cells and solubilizing DNA to remove cellular components using organic, non-organic or adsorption techniques (292). In this study, DNeasy PowerSoil Pro Kits (QIAGEN, Germany) were used to extract DNAs from faecal and tissue samples.

5.1.1.2 16S rRNA Library Preparation, Sequencing and Data Analysis with Illumina, MiSeq platform

Illumina MiSeq platform (Illumina, California, USA) offers a sequence platform for library preparation, clonal amplification, genomic DNA sequencing and data analysis in a single run and is widely employed in the microbiota analysis because of their rapid turnaround time. The work of the Illumina MiSeq consists of four main steps; library preparation, cluster generation, sequencing and data analysis (293,294) as described below. This process was carried out by Dr Matthew Hitchings who followed the MiSeq Wet Lab SOP (Appendix 13).

1) Library Preparation

In the library preparation, DNA fragments are purified for sequencing and analysis. Tagmentation is the initial step of the library preparation and performed together with the fragmentation and ligation in a single step. During tagmentation, transposomes cut the DNA into short fragments randomly and adapters are ligated to both sides of the cut points as described in Figure 23.

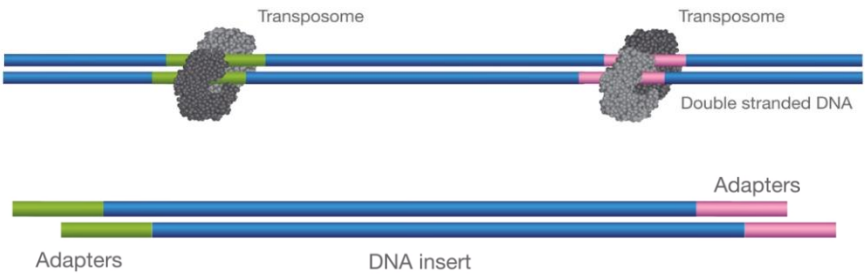


Figure 23: Tagmentation and adapter ligation

Adapters facilitate sequencing of random fragments of DNA by adding short nucleotides. This step allows for DNA fragment to attach to a flow cell and facilitate bridge-amplification at the later stage. Multiplex sequencing (barcoding of samples and generating sequencing lane of multiple DNA libraries), also takes place during this phase (295). Multiplexing permits numerous libraries to be pooled and sequenced simultaneously per sequencing run, facilitating reduced library preparation time. During the reduced cycle amplification, primer binding indices and regions complementary to the flow cell oligo are added (Figure 24). These indices are unique and enable samples identification during the DNA sequence analysis. During the analysis, up to 96 different samples can be read simultaneously (296).

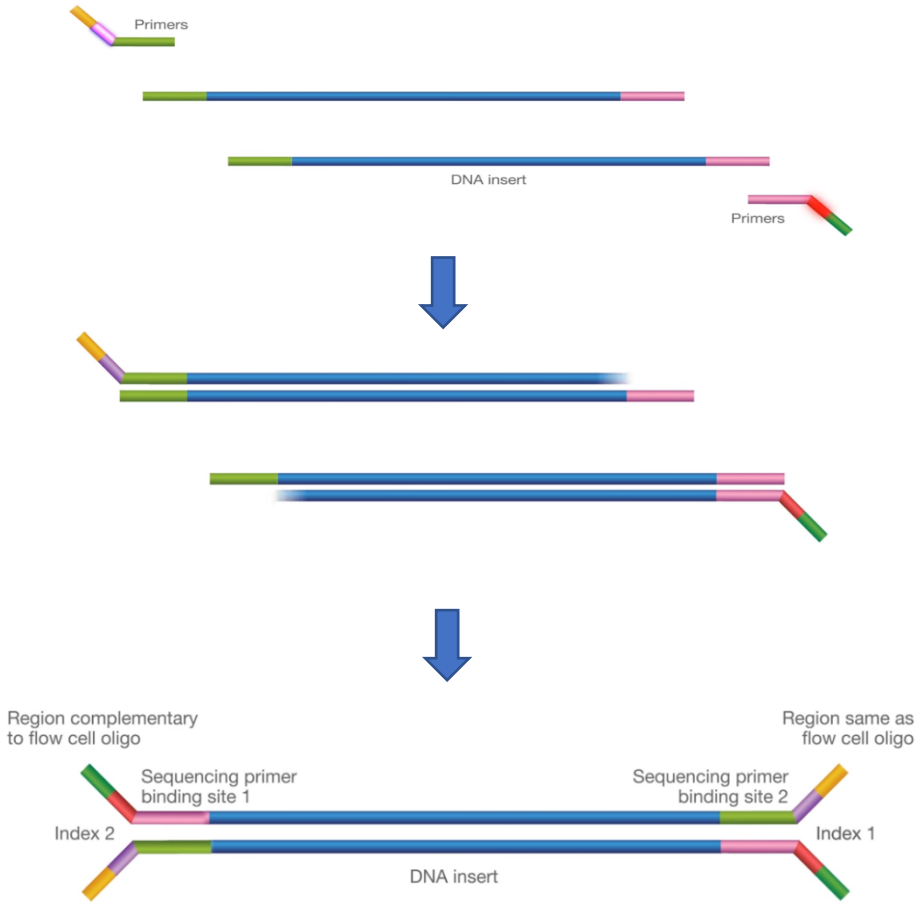


Figure 24: Reduced cycle amplification

2) Cluster Generation

Cluster generation is the next stage process where each fragment is isothermally amplified using bridge amplification technology. The flow cell surface is coated with two different types of oligonucleotides, which are complementary to adapters and enables the DNA strands to be held to the flow cell during sequencing. Cluster generation is for producing multiple copies of single-stranded DNA to intensify the signal necessary for subsequent sequencing as described in Figure 25. Firstly, the region complementary to the flow cell oligonucleotide binds to the oligonucleotide coating to the surface of the flow cell. A polymerase subsequently creates a complement of the hybridized fragment. The double-stranded molecule is then denatured, and the original strand is washed away (Figure 25, b). Secondly, the strand falls over and the top adapter attaches to the second type of oligonucleotide on the flow cell surface (Figure 25. c). A polymerase creates a complementary strand forming a double-stranded bridge. This bridge is then denatured, making two single-stranded copies of the molecule that are attached to the flow cell (Figure 25. e). This process is repeated to make multiple clusters, resulting in clonal amplification. This is vital for quality control purposes also since the forward and reverse strands must be complementary to each other. Furthermore, all the forward reads must be the same, facilitating detection of erroneous amplification and the reverse reads.

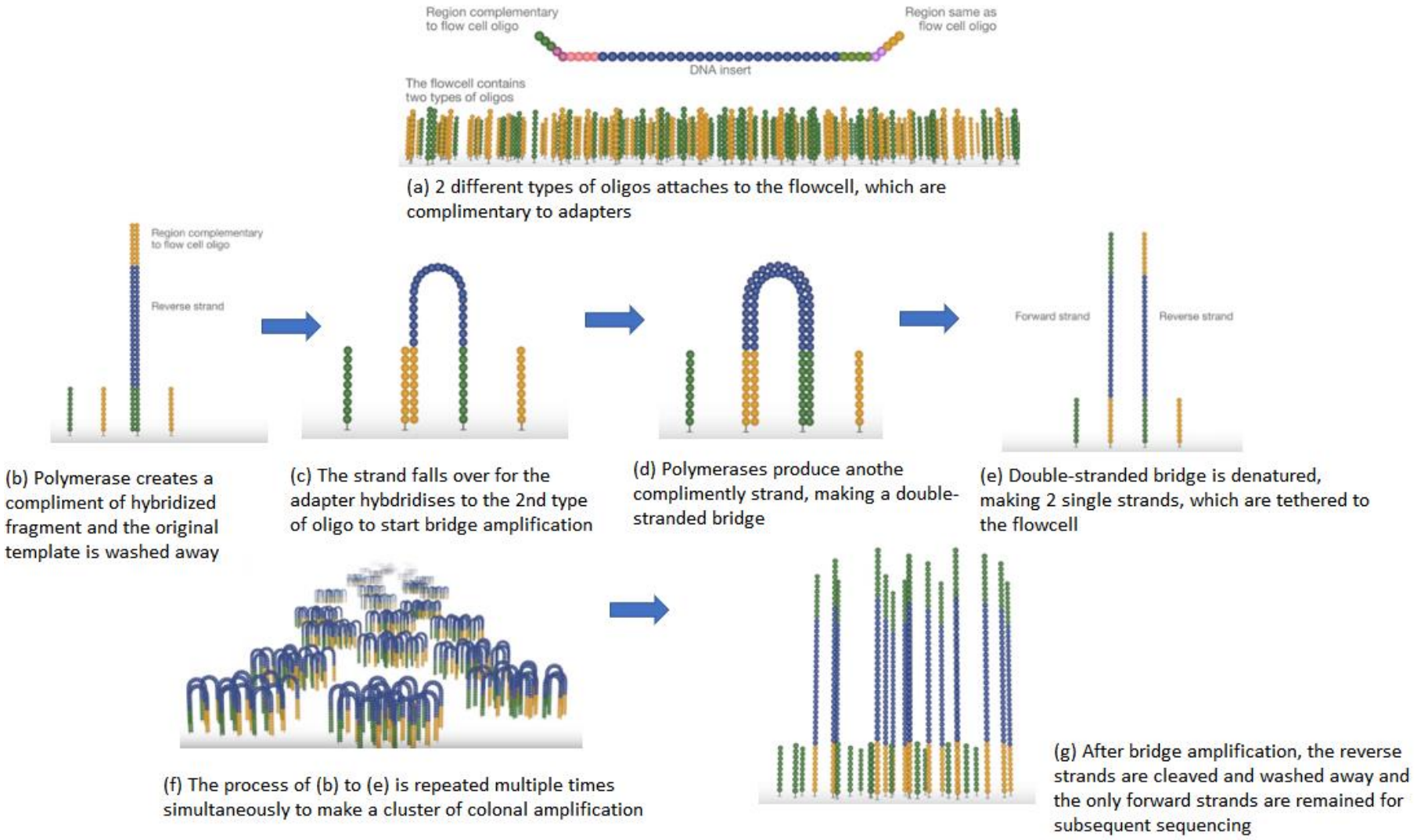
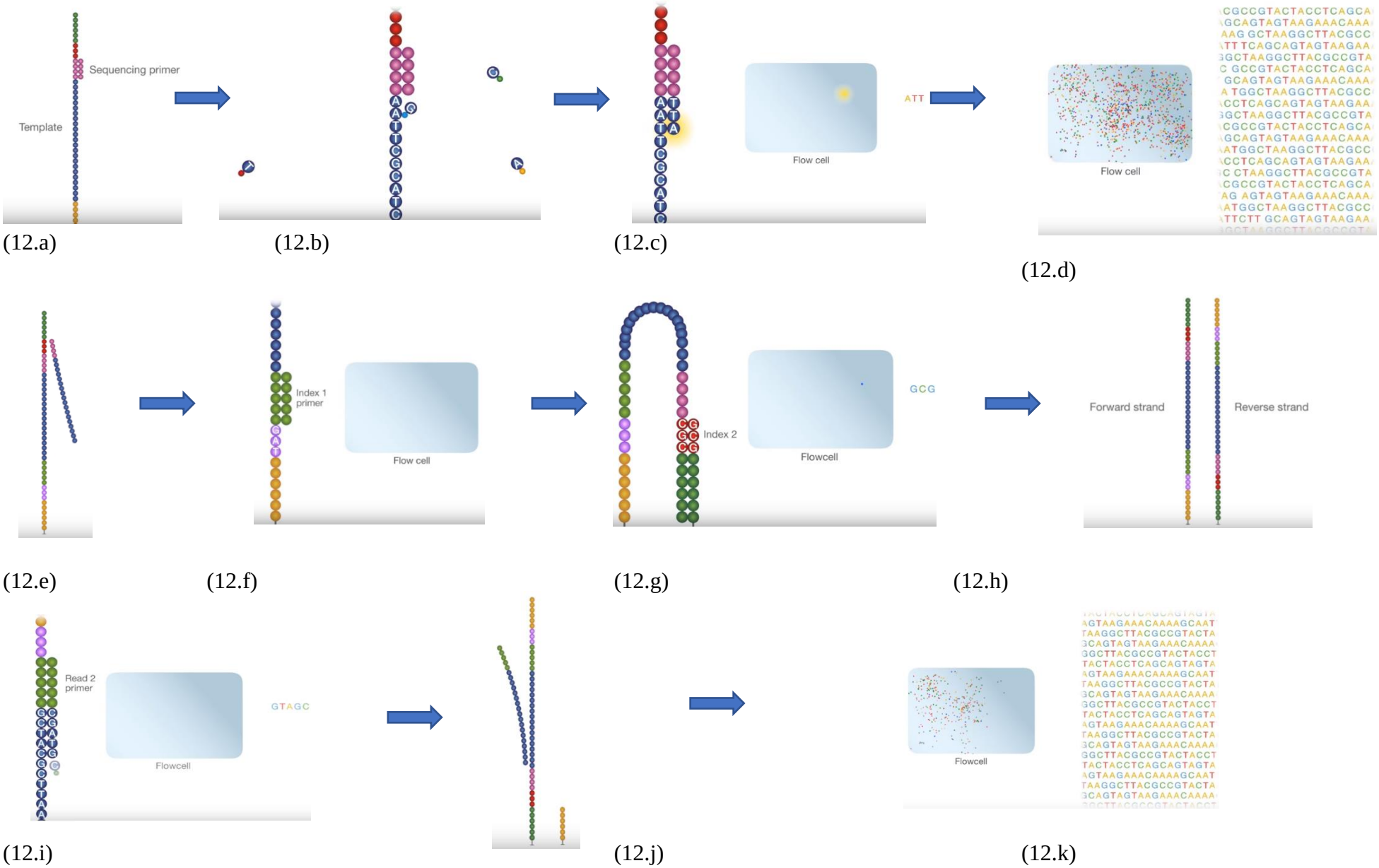


Figure 25: Cluster generation with bridge amplifications

3) Sequencing - Sequence by Synthesis (SBS)

Illumina MiSeq uses sequencing by synthesis (SBS) technology, which sequences multiple clusters in parallel as described in Figure 26 (297). After cluster generation, only forward strands remain on the flow cell. Sequencing primers subsequently bind (Figure 26.a) to the forward strands and a polymerase adds fluorescent-tagged nucleotides to the DNA strand one by one (Figure 26.b). During each SBS cycle, only one nucleotide is added based on the sequence of the template. After each addition of nucleotide, a unique fluorescent signal is emitted (Figure 26.c). The number of this cycle determines the length of read. For one cluster, all identical strands are read simultaneously, allowing multiple clusters to be sequenced in parallel (Figure 26.d). After a completion of the first read, the read product is washed away (Figure 26.e). In the next step, Index 1 primer is hybridised to the template. The read is generated in a similar manner as the first read (Figure 26.f). After the completion, the read product is washed away except 3'end of primer end. The template then falls over and binds to the second oligonucleotide of the flow cell (Figure 26.g) and Index 2 primer is read. Index 2 read product is washed away, which allows a polymerase to extend from the second flow cell oligonucleotide forming a double-stranded bridge. This is then linearized and 3' prime end is blocked (Figure 26.h). Yet again, the original forward strand is cleaved off and washed away (Figure 26.j), leaving the reverse strands only to the flow cell. This process is called paired-end sequencing.

Before the next generation sequencing method such as this SBS technology was developed, the Sanger sequencing was the mainstream DNA sequencing method for the last forty years. Although the Sanger sequencing method has its niche with studies requiring precision, the next generation sequencing has been widely accepted in human gut microbiota studies because it allows simultaneous sequencing of millions of clusters while the Sanger sequencing method only sequences a single DNA at a time, resulting in a lengthy process (298).



(12.a) (12.b) (12.c) (12.d)

(12.e) (12.f) (12.g) (12.h)

(12.i) (12.j) (12.k)

Figure 26: Illumina Sequence by Synthesis

4) Alignment and Data Analysis

The final step is data analysis of numerous reads produced in the previous steps. Sequences from the pooled sample library are separated depending on unique indices in the SBS. For each sample, the read with similar stretches of base call are locally clustered. Subsequently forward and reverse reads are paired. This creates contiguous sequences, which are aligned to the reference genome for variant identification. The paired information allows to resolution of ambiguous alignments (Figure 27).

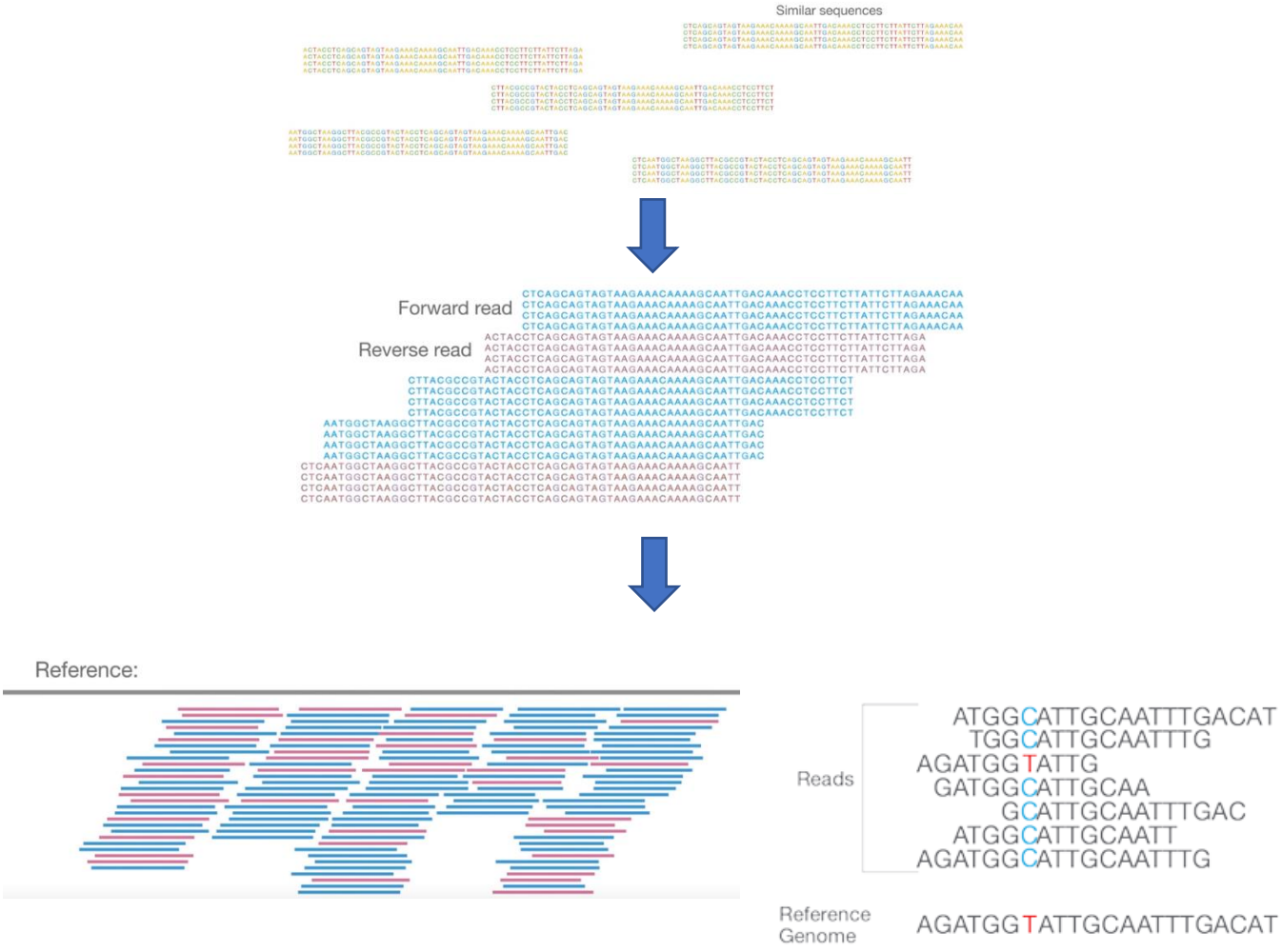


Figure 27: Illumina alignment and data analysis

5.1.1.3 PCR and PCR primer designs

The key step of library preparation is designing of amplicon primers. Primer is a strand of nucleic acid to target a starting point for DNA replication. The primer plays an important role in DNA replication since DNA polymerases can only bind to the primer and start adding nucleotides to the 3’ prime end in one direction. The primer used for our 16S rRNA gene sequencing using the Illumina MiSeq is a

synthetically designed PCR primer to facilitate 16S rRNA gene sequencing from a specific binding site, hypervariable region, V4. The sequences of the generic primer design are listed below, which allow annealing of the amplicons to the flow cell.

Generic PCR primer design:

AATGATACGGCGACCACCGAGATCTACAC <i5><pad><link><16Sf> VX.N5??
CAAGCAGAAGACGGCATACGAGAT <i7><pad><link><16Sr> VX.N7??

Generic read 1 primer design:

<pad><link><16Sf> VX.read1

Generic read 2 primer design:

<pad><link><16Sr> VX.read2

Generic index read primer design:

Reverse complement of (<pad><link><16Sr>) VX.p7_index

The sequences in the generic designs allow annealing of the amplicons to the flow-cell, but there are a few points to consider for designing PCR primers.

1) GC content and GC clamp

Primers must be specified in a 5' to 3' direction and 40-60% of each primer should be G and C bases (299). This is because G and C are stronger as their melting temperatures are higher than A and T. Thus, the last 5 bases from the 3'end of primer should be G and/or C bases, called GC clamp, which facilitates stability of the binding at the 3' end.

2) Index and Linker

The index sequences must balance the number of bases at each position. The index sequences have 8nt, <i5> and <i7>, and allows multiple libraries to be mixed up and sequenced together. With Illumina MiSeq, the software identifies these indices on each sequence read so that it can separate the reads for each sample. Link or linker is a 2-nt sequence that is non-complementary to the 16S rRNA. <16Sf> and <16Sr> is the gene specific primers that are to amplify the V4 region from the 16S rRNA gene.

3) Melting Temperature

The sequencing primers should have similar melting temperatures 65 degree so that annealing during PCR takes place for both strands simultaneously. A primer with a melting temperature much higher than the reaction's annealing temperature can cause mishybridisation, resulting in extension of the DNA sequence. If it is too low, annealing does not occur. This fine tuning of a melting temperature

can be achieved by the "pad" sequence, which is usually a 10 nucleotide (nt) sequence. The generic designs of PCR primers used in this study are listed in Appendix 14.

5.1.1.4 Bioinformatics and 16S rRNA Sequence Analysis

1) 16S Ribosomal Databases

Amongst readily available database for amplicon sequencing analysis, SILVA was chosen in this study. SILVA offers comprehensive validated database of small subunit like 16S rRNA gene sequences as well as large subunits from the bacteria, Eukarya and archaea domains (300). This database is also regularly updated. In this study, SILVA 128 was used, which was released in 2016. SILVA 128 validated 5616,941 small subunit Parc and 1922,213 small subunit Ref (301). The Parc comprise the whole SILVA database for the respective gene and the Ref represents a subset of the Parc with high-quality and nearly full length sequences (300,302). This process is done by following the basic principle of the Schloss lab, MiSeq SOP (294). The main steps of bioinformatics are pre-processing of data, taxonomic profiling and predictive metagenomics profiling as described below.

2) Processing Data with Mothur

The processing of data consists of assembly, quality control and chimera detection. This was performed with an open-source software, Mothur (https://mothur.org/wiki/miseq_sop/). During the assembly phase, paired-end reads are assembled and alignments with a high proportion of mismatches or short alignment length with low scores were discarded. Quality control included generation of unique reads, removal of adapters, and poor-quality bases. Moreover, reads with too short or poor-quality for further analysis were discarded, allowing more accurate bioinformatics. Chimeras are artifact sequences that are formed by incorrectly joined together during the amplification. There are a number of tools available to detect chimeras, but MiSeq SOP (https://mothur.org/wiki/miseq_sop/) was also used for elimination of sequencing and PCR errors. If the sequence is matched to the reference sequence with similarity of less than 97%, they were discarded.

Taxonomic profiling consists of alignment, microbial composition and diversity analysis. Before taxonomic profiling, microbiota is classified in clusters by 2 methods; phylotypes and operational taxonomic units (OTUs). Phylotypes are clustered based on the similarities with the reference database, whereas OTUs uses 97% similarity threshold. In this study, OTUs were employed for clustering. There is a few different available database such as Greengenes, Ribosomal database project, Human Microbiota project and SILVA. In this study, SILVA was used. Microbial composition and diversity analysis is performed after the taxonomic profiling. Diversity analysis is an evaluation of microbial

abundance and diversity. - α diversity means species diversity in a single ecosystem, whereas β diversity describes the diversity in microbial community between different ecosystems (303).

3) Data Presentation with Phyloseq

Following data processing using Mothur, the Phyloseq package was used to produce graphics of phylogenetic sequencing data (304). Phyloseq provide functions of import, store, analyse and graphic display phylogenetic sequencing data that has been clustered into OTUs. In other words, this project utilises many tools in R for ecology and phylogenetic analysis such as vegan as well as using advanced graphic systems to produce graphics of complex phylogenic data. Figure 28 summaries the phyloseq workflow.

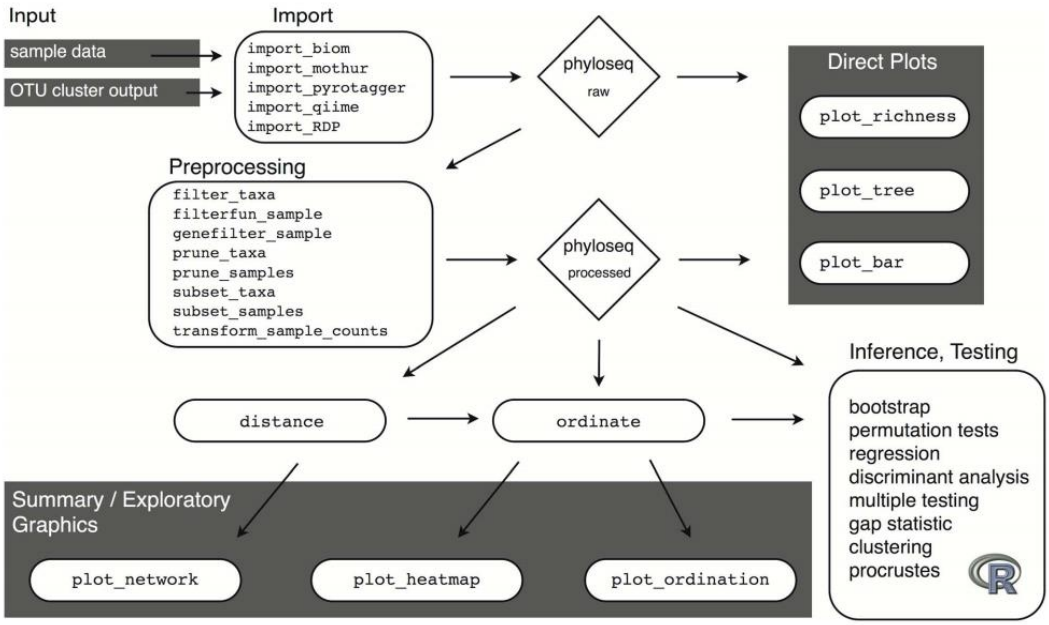


Figure 28: Analysis workflow using phyloseq

(304)

5.1.2 Measuring Colonic Mucosal Immunological Response to Treatment of FMTUC using Enzyme-linked Immunosorbent Assay (ELISA)

Enzyme-linked Immunosorbent Assay (ELISA) is an immunological assay readily used to measure proteins, antibodies, antigens and glycoproteins in biological samples. In this study, ELISA was employed to quantify IL-10 and IL-21 from colonic mucosal tissue samples. This work was carried out by an experienced technician in ILS-2, Swansea University. This work was also significantly delayed due to the COVID-19 pandemic and the regulation changes. Thus, the work needed to be conducted by a technician who had an access to the facility.

Firstly, the Introgen PARIS Kit (Waltham, USA) was used for protein extraction from samples. This kit was chosen because of its Protein And RNA Isolation System (PARIS system), which enables us to extract rare, difficult to obtain and very small samples (305). Tissues were first homogenized in cold cell disruption buffer for a cell lysate. This allows proteins and RNA to be purified directly from this lysate. Another benefit of this technology is compatibility with further ELISA applications. Secondly, for IL-10 and IL-21 ELISA protocol, R&D Systems Human IL-10 DuoSet ELISA was used (306). This is based on sandwich ELISA type, which is described in Figure 29.

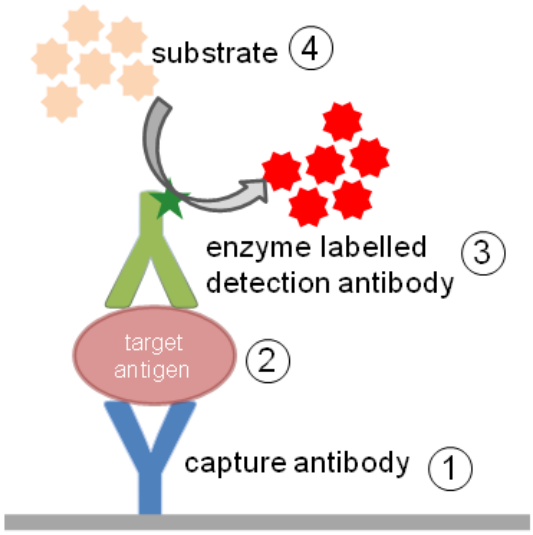


Figure 29: Sandwich ELISA method used in Human IL-10 DuoSet ELISA (R&D Systems)

1. Plastic wells are pre-coated with antibodies and sample extracted with PARIS Kit was added to the plastic wells
2. Any antigens bind to the antibodies
3. Detection antibody for IL-10 and IL-21 is added
4. Substrate is added to measure the IL-10 and IL-21

Lastly, Bradford protein assay (BIO-RAD, California, USA) was used for protein measurement. This is a colorimetric assay, which allows us to visualise the proteins of interest. Subsequently, the absorbance is measured in a spectrophotometer or microplate reader.

5.1.3 Metabolic Profiling

After faecal samples were processed for 16S rRNA analysis, it was intended to carry out metabolomic profiling at Imperial College London. However, due to the COVID-19 pandemic and contractual delays, metabolomic profiling was delayed. This part of the analysis will be carried out in future and is not included as part of this thesis.

5.2 Mechanistic Results and Analysis

The COVID-19 pandemic has caused a major delay in all the laboratory work in Swansea University across the board including this study. Full analysis of has not yet been conducted at present. The preliminary results and their analysis of 16S, which were produced before the first lockdown, were included in this thesis. These preliminary results include stool samples of donors, subject 1-16's pre-FMT and week 1 post-FMT, though the number of results decreased as follow-up week went by. At week 12, nine subjects' results were available (Table 32). The access to the full results and analysis for the completion of this thesis was attempted in multiple occasions, however, communication and accessibility to the specialist for 16S have been one of the challenges of the project and I was unable to obtain for this thesis.

Table 32: Subjects' faecal samples used for the results

	Screen	Week 1	Week 4	Week 8	Week 12
Group 1 (single dose)	1,3,8,9,11,12	1,3,8,9,11,12	1,3,8,9,11,12	1,3,8,9,11,12	1,3,9,11,12
Group 2 (5 doses)	2,4,7,10,14,15,16	2,4,7,10,14,15,16	2,4,7,10,14,15	2,4,7,10,14,15	2,4,7,14
Group 3 (control)	5,6,13	5,6,13	5,6,13	13	NONE

5.2.1 Comparison of Faecal Microbial Characterisation Between Donors and Subjects with UC using 16S rRNA

Figures 30 to 34 exhibit taxonomic bar charts of faecal samples from donors (on the first far left column) and recipient subjects during the 12-week follow up period at phylum, class, order family and genus level respectively. These taxonomic bar charts are exhibited based on relative abundance of >2%. Relative abundance represents the percentage composition of an organism relative to the total number of the organisms in the sample community.

Faecal microbiota of the donors

Due to the interruption of FMT samples in the middle of the FMTUC study after the regulation changes described chapter 4.1.4 and Appendix 1, subjects 1-6 received FMT samples manufactured by Wessex stool bank, whereas subjects 7-18 received FMT samples manufactured locally in Singleton Hospital. To minimise discrepancies, FMT was manufactured and stored by a very similar method, however, 16S analysis of the donor FMT samples revealed differences between the ones from Wessex stool bank

and those from the local laboratory, except the sample subject 3 received. The Wessex stool bank manufactured FMT from two donors, whereas the local laboratory manufactured from five donors. This illustrates the potential importance of donor stool selection, which although not currently well understood, may have a substantial impact on outcome, depending on its characteristics.

At the phylum level, dominant phyla across all donor samples were *Firmicutes* (64.5%), *Bacteroidetes* (17.7%) and *Actinobacteria* (5.5%). FMT samples from the Wessex stool bank contained approximately equal amounts of *Bacteroidetes* and *Firmicutes*, whereas FMT samples from the local laboratory contained more *Firmicutes* than any other microorganisms with relative abundance of 0.6 – 0.8. At the genus level, *Provotellaceae* were the most abundant bacteria detected in the samples from the Wessex stool bank. *Bacteroides*, which is known to have a role in immune regulation (106), was seen in approximately 20% of the samples from the Wessex stool bank, although this was not seen within the samples from the local laboratory. In samples from the local laboratory, a significant proportion of bacteria would not be classified. Furthermore, FMT samples from the local laboratory contained much less *Bacteroidetes* and more unclassified bacteria. There was also a significant difference in relative abundance at the family and genus level in the samples from the local laboratory. The samples that subject 7,8,9 and 12 received contained a considerable proportion of *Peptostreptococcaceae*, which was hardly seen in other samples. An association between *Peptostreptococcaceae* and colorectal cancer was recently reported (307,308).

Pre-intervention faecal microbiota of the subjects with UC

The faecal microbiota of the study subjects also varied individually. Previously *Proteobacteria* and *Fusobacteria* were reported to be more abundant in patients with UC (153,154), and this study supports this (Figure 30). *Actinobacteria* have been reported to be less abundant in patients with UC (151), although faecal samples from the subjects of this study were found contain more *Actinobacteria* than those from the donors. At the genus level, similarly, *Bifidobacterium* was previously reported to be less abundant in patients with UC (151), but this study showed more abundant in subjects than in donors. Although the study sample is limited in this study, it was found that the faecal microbiota of donors and study subjects with UC was notably different. *Prevotellaceae, Phascolarctobacterium* and *Veillonellaceae* were found to be less abundant, whereas *Megasphaera, Alistipes* and *Collinsella* were more abundant in subjects with UC in this study (Figure 34). Furthermore, the faecal microbiota of the subjects with UC appeared to be dominated by one type of bacteria, which varied between subjects, suggesting less diverse faecal microbiota in patients affected by UC.

Post-intervention faecal microbiota of the subjects

It was expected to see changes in the faecal microbiota after the pre-FMT bowel cleanse with antibiotics and bowel preparation. This was apparent in the subjects in the control group (subject 5, 6, 13 and 17). Furthermore, bacteria that were not detected at the baseline emerged after the antibiotics and bowel cleanse. For instance, *Verrucomicrobia* was not at all detected on subject 5's faecal sample at baseline, but became 30% of the microbiota composition after the pre-FMT optimisation intervention (Figure 30). This implies that antibiotics and bowel cleanse alter the gut microbiota for better or worse. The faecal sample of subject 6 also contained 6% of *Proteobacteria*, which was not detected in the baseline sample. Similar changes were seen amongst subjects with FMT interventions, such as subjects 3, 4 and 15, though it is unclear whether they acquired these bacteria from the FMT samples.

It is interesting to note that the faecal microbiota of subject 1, who worsened according to subjective and objective assessments, showed an increased proportion of *Proteobacteria* at the first week (from 2% to 52% of the microbiota composition) in Figure 30. The proportion of *Proteobacteria* fell later in the follow-up period, however, it remained in the faecal microbiota throughout the follow-up period. At the genus level, this *Proteobacteria* appears to be *Enterobacteria* and *Escherichia-Shigella*, which thought to have a pro-inflammatory property in the GI tract as shown in Figure 34 (153). This might be one of the reasons for worsening symptoms for subject 1, though more study is needed to determine this observation.

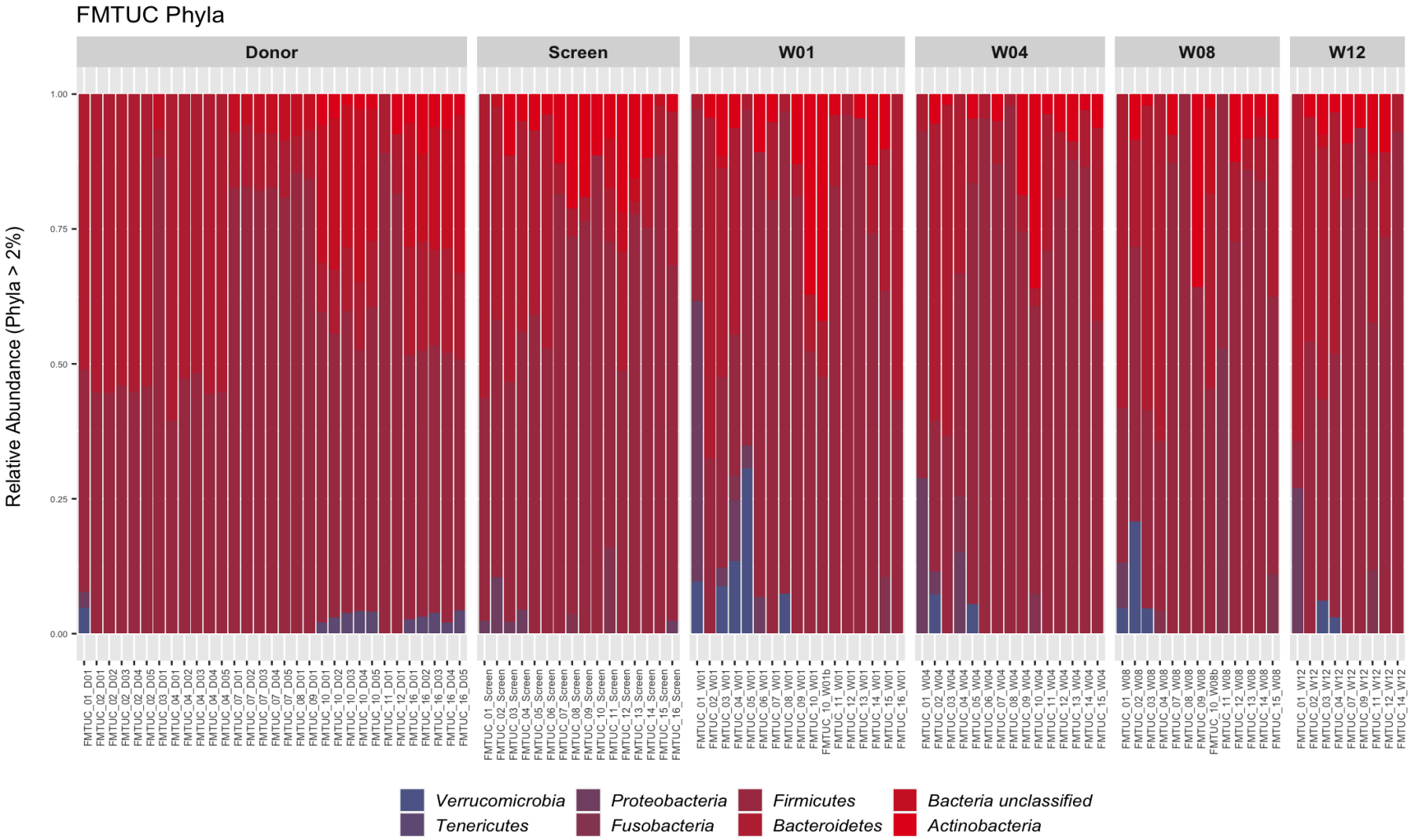


Figure 30: Taxonomic stack bar chart at phylum level of faecal samples from donors (on the left column) and subjects at the screening (pre-FMT intervention), at week 1, week 4, week 8 and week 12

Y axis is relative abundance representing the percentage of an organism relative to the total organisms within the faecal samples. X-axis is subject ID number as described FMTUC_SubjectID_(Follow-up week)

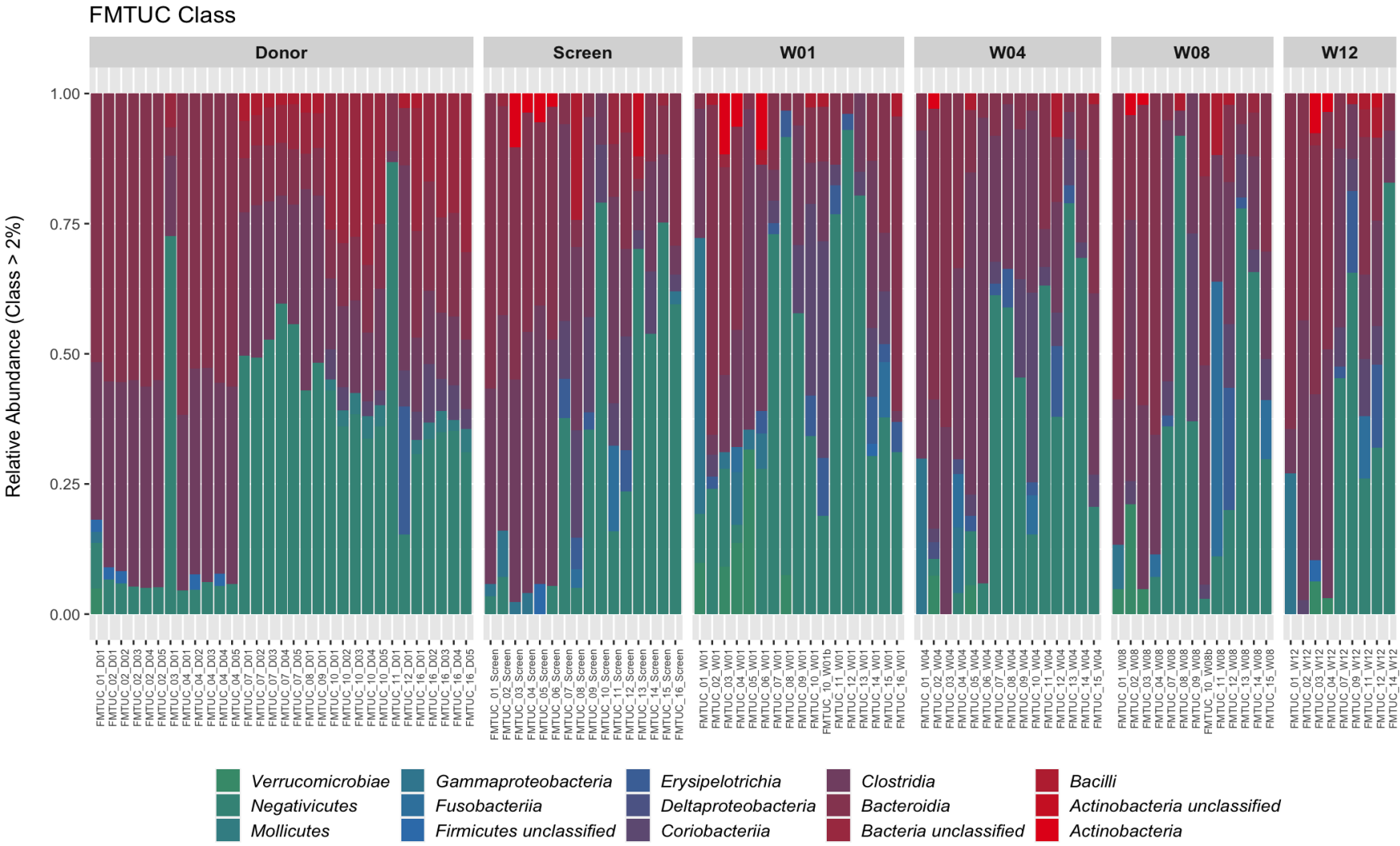


Figure 31: Taxonomic stack bar chart at class level of faecal samples from donors (on the left column) and subjects at the screening (pre-FMT intervention), at week 1, week 4, week 8 and week 12,

Y axis is relative abundance representing the percentage of an organism relative to the total organisms within the faecal samples. X-axis is subject ID number as described FMTUC_SubjectID_(Follow-up week)

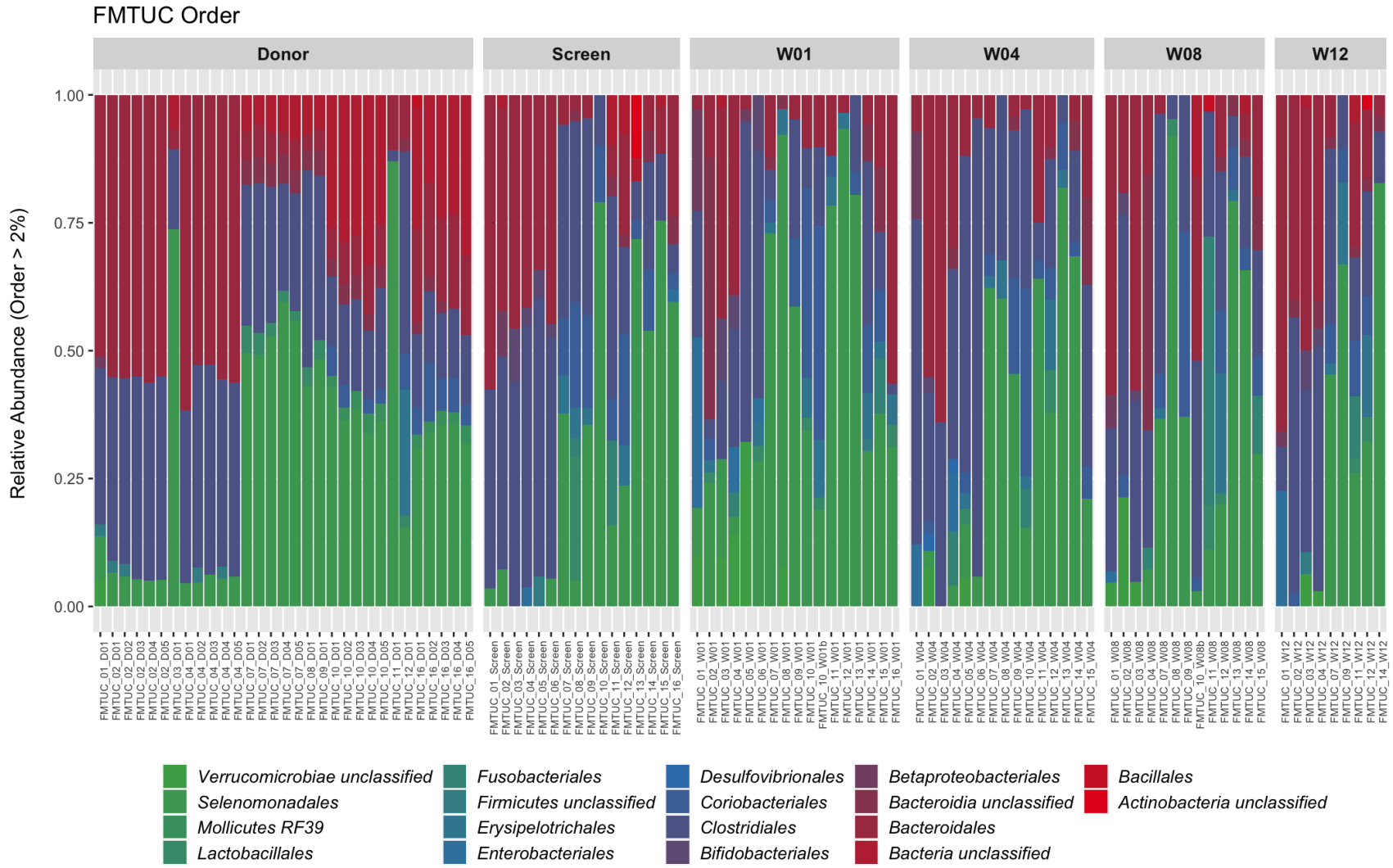


Figure 32: Taxonomic stack bar chart at order level of faecal samples from donors (on the left column) and subjects at the screening (pre-FMT intervention), at week 1, week 4, week 8 and week 12

Y axis is relative abundance representing the percentage of an organism relative to the total organisms within the faecal samples. X-axis is subject ID number as described FMTUC_SubjectID_(Follow-up week)

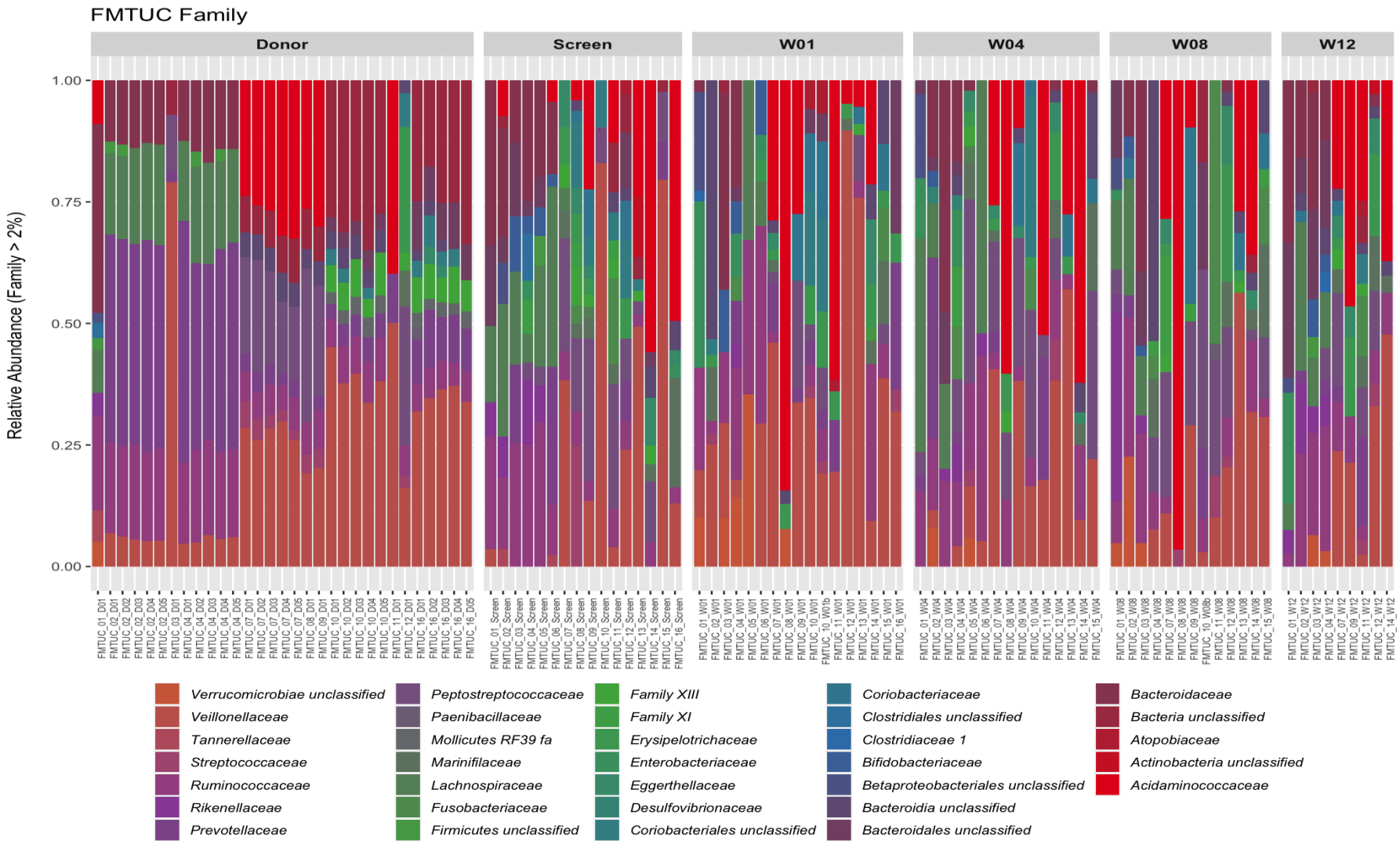


Figure 33: Taxonomic stack bar chart at family level of faecal samples from donors (on the left column) and subjects at the screening (pre-FMT intervention), at week 1, week 4, week 8 and week 12

Y axis is relative abundance representing the percentage of an organism relative to the total organisms within the faecal samples. X-axis is subject ID number as described FMTUC_SubjectID_(Follow-up week)

Figure 34: Taxonomic stack bar chart at genus level of faecal samples from donors (on the left column) and subjects at the screening (pre-FMT intervention), at week 1, week 4, week 8 and week 12

5.2.2 Comparison of Faecal Microbial Characterisation Between FMT Interventions using 16S rRNA

Figures 35-39 exhibit taxonomic stack bar charts of subjects based on relative abundance >5% of the screening faecal samples and over the 12-week follow up period, which were grouped by intervention groups (control, single dose and 5 doses). Although the number of the samples is too limited to draw any conclusions, these figures demonstrate that all subject's faecal samples were very different at the screening time as well as post-FMT interventions when samples were compared amongst the same intervention group. Furthermore, they were very different at the screening and throughout the 12-week follow up period even when they were compared from the same subject. The key observation from these graphs is between screening and week 1, the majority of taxonomic profiles changed regardless of the FMT interventions. This may be due to the bowel cleansing regime (antibiotics and mechanical bowel cleanse), but also the gut microbiome is an organic process and continuously changes with the subjects' life style, diet and general health.

Figure 40 (A-F) exhibit taxonomic stack bar charts at the phylum level of faecal samples of each subject in intervention group 1 (single dose), which were compared with their donor's sample. Figure 41 (A-E) exhibit the same from intervention group 2 (5 doses). These figures were grouped individually and allow to compare with each subject's donor taxonomic profiles throughout the 12-week follow up period with their donors. Some bacteria, which were present in the donor's taxonomic profile, but not in the recipient's, have emerged after the FMT interventions. This was seen with *Bacteroidetes* in Subject 3, unclassified bacteria in subject 10 and *Actinobacteria* in subject 11. Dominant phyla amongst the donors of Group 1 (single dose) were *Firmicutes* (55.2%) and *Bacteroidetes* (22.1%) and that of the subjects with active UC at the screen were *Firmicutes* (65.4%) and *Bacteroidetes* (11.8%). *Firmicutes* were found to be more abundant amongst the subjects with UC. Other phyla including *Actinobacteria*, *Tenericutes* and unclassified OTU were also detected in the donor and recipients' samples. After 12 weeks post-FMT one dose, *Firmicutes* were found to be more abundant with overall 82.6% relative abundance. An interesting observation was seen in subject 9. Despite subject 9 or their donor's showed *Fusobacteria*, though it emerged in week 4 after the intervention with relative abundance of 7.4%, which was not detected at week 8 though its relative abundance spiked to 97.8% at week 12. A similar phenomenon was observed in subject 4 of intervention group 2. The relative abidance of *Fusobacteria* emerged as much as 40% from non-detected previous weeks nor donors' samples. It is unclear whether this was acquired after the FMT interventions or it was manifested at week 12 due to the FMT interventions. Furthermore, it is unclear whether this has affected clinical outcomes as subject 9's Mayo score did not change though subject 4 did. *Proteobacteria* and *Actinobacteria* also emerged at week 12 in subject 1 even though these

organisms were not detected from donor FMT or at the time of screening. This reiterates how challenging it is to draw a conclusion on vast diverse microbiome and there should be more samples to make meaningful conclusions. Another observation was that some subjects did not significantly change their taxonomic profiles at the phylum level after the interventions. This was seen in subject 8, 11 and 12. Nevertheless, these subjects' taxonomic profiles have changed from screening and during the 12-week follow up, but they are not similar to their donor's microbiome. This was further analysed with subsequent bioinformatic analysis.

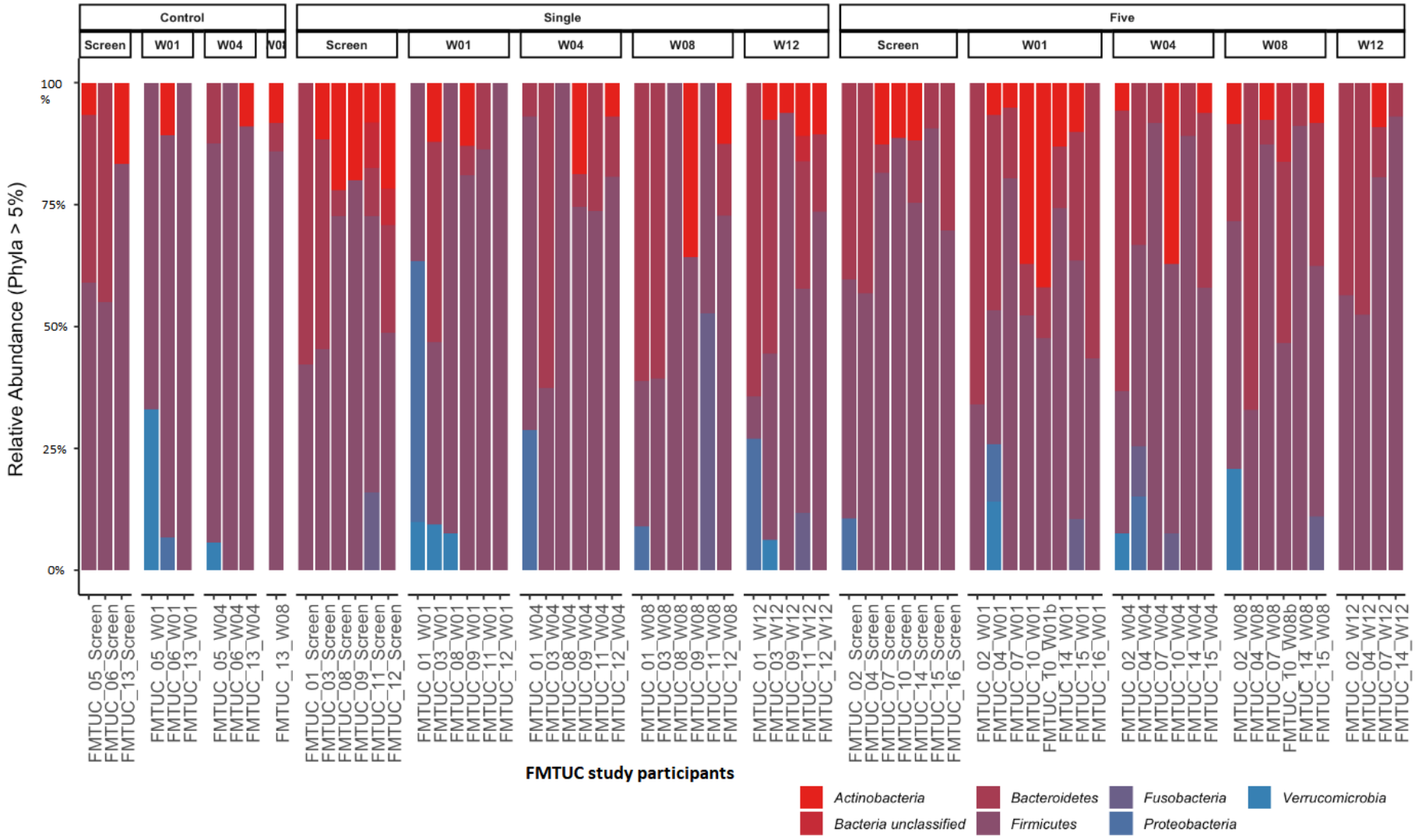


Figure 35: Taxonomic stack bar chart at phyla level of participants grouped by intervention groups; Control, FMT single dose and FMT 5 doses.

X axis: Subject ID number as described FMTUC_SubjectID_(Follow-up week), Y axis is relative abundance representing the percentage of an organism relative to the total organisms within the faecal samples.

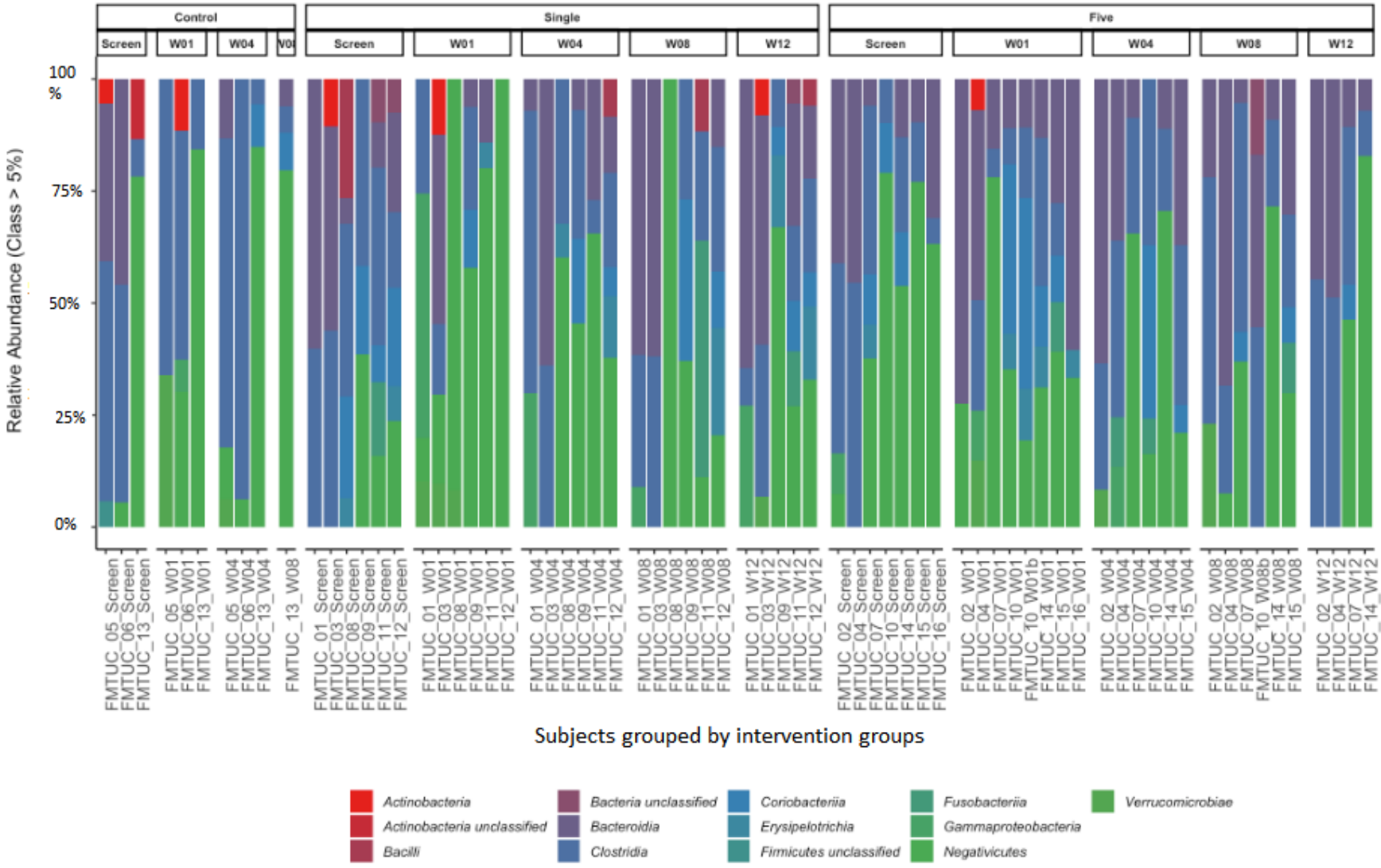


Figure 36: Taxonomic stack bar chart at class level of participants grouped by intervention groups; Control, FMT single dose and FMT 5 doses.

X axis: Subject ID number as described FMTUC_SubjectID_(Follow-up week), Y axis is relative abundance representing the percentage of an organism relative to the total organisms within the faecal samples.

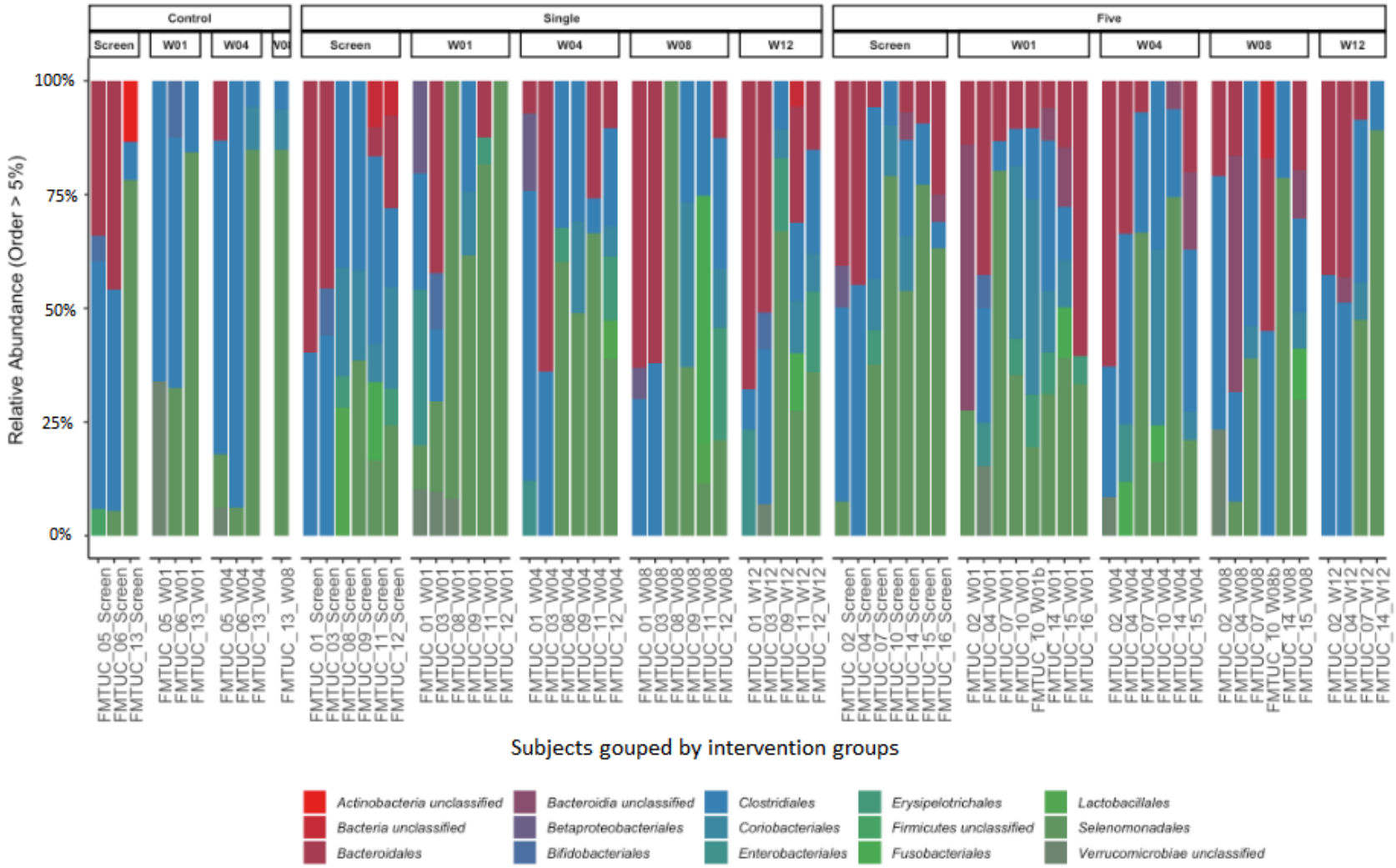


Figure 37: Taxonomic stack bar chart at order level of participants grouped by intervention groups; Control, FMT single dose and FMT 5 doses.

X axis: Subject ID number as described FMTUC_SubjectID_(Follow-up week), Y axis is relative abundance representing the percentage of an organism relative to the total organisms within the faecal samples.

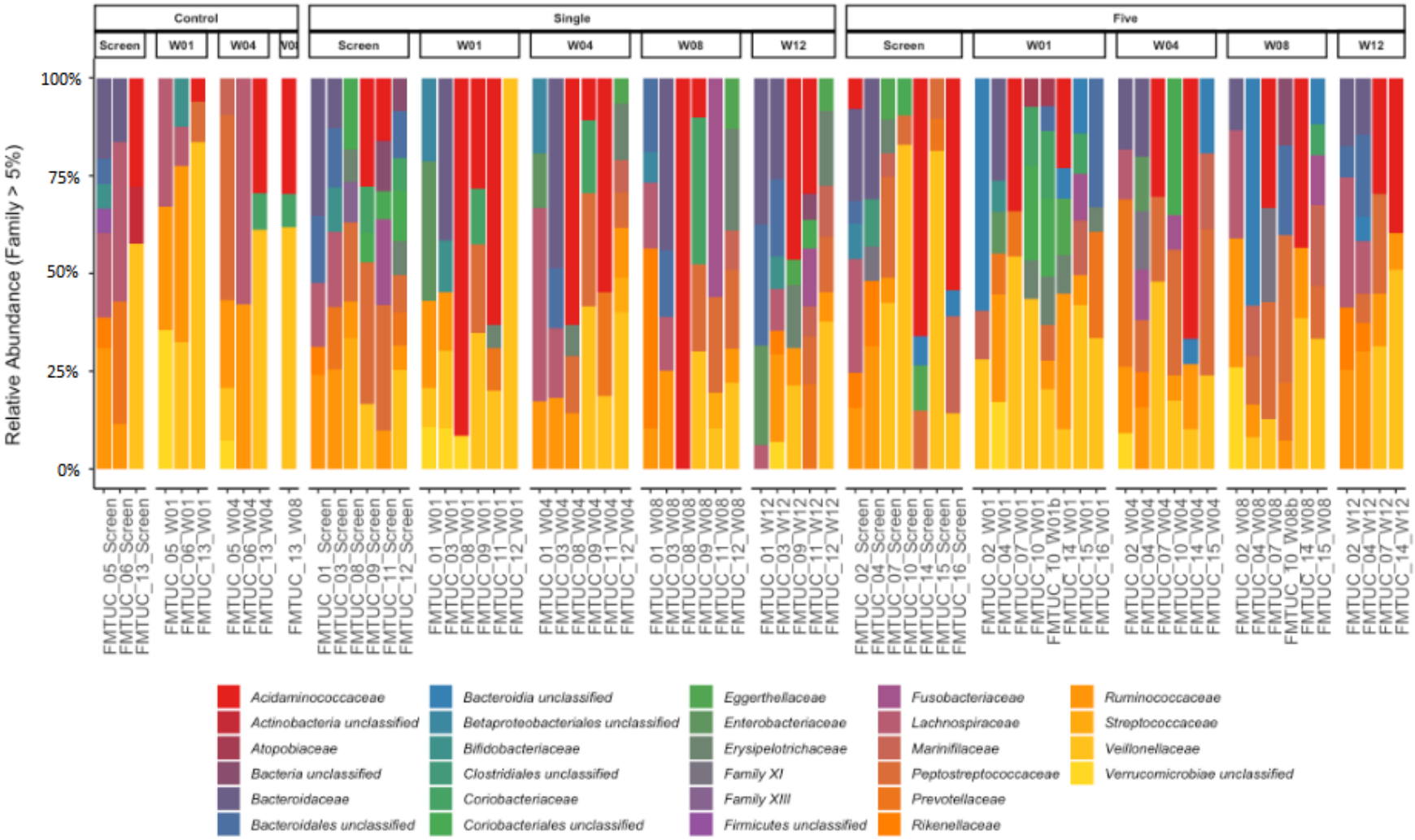


Figure 38: Taxonomic stack bar chart at family level of participants grouped by intervention groups; Control, FMT single dose and FMT 5 doses.

X axis: Subject ID number as described FMTUC_SubjectID_(Follow-up week), Y axis is relative abundance representing the percentage of an organism relative to the total organisms within the faecal samples.

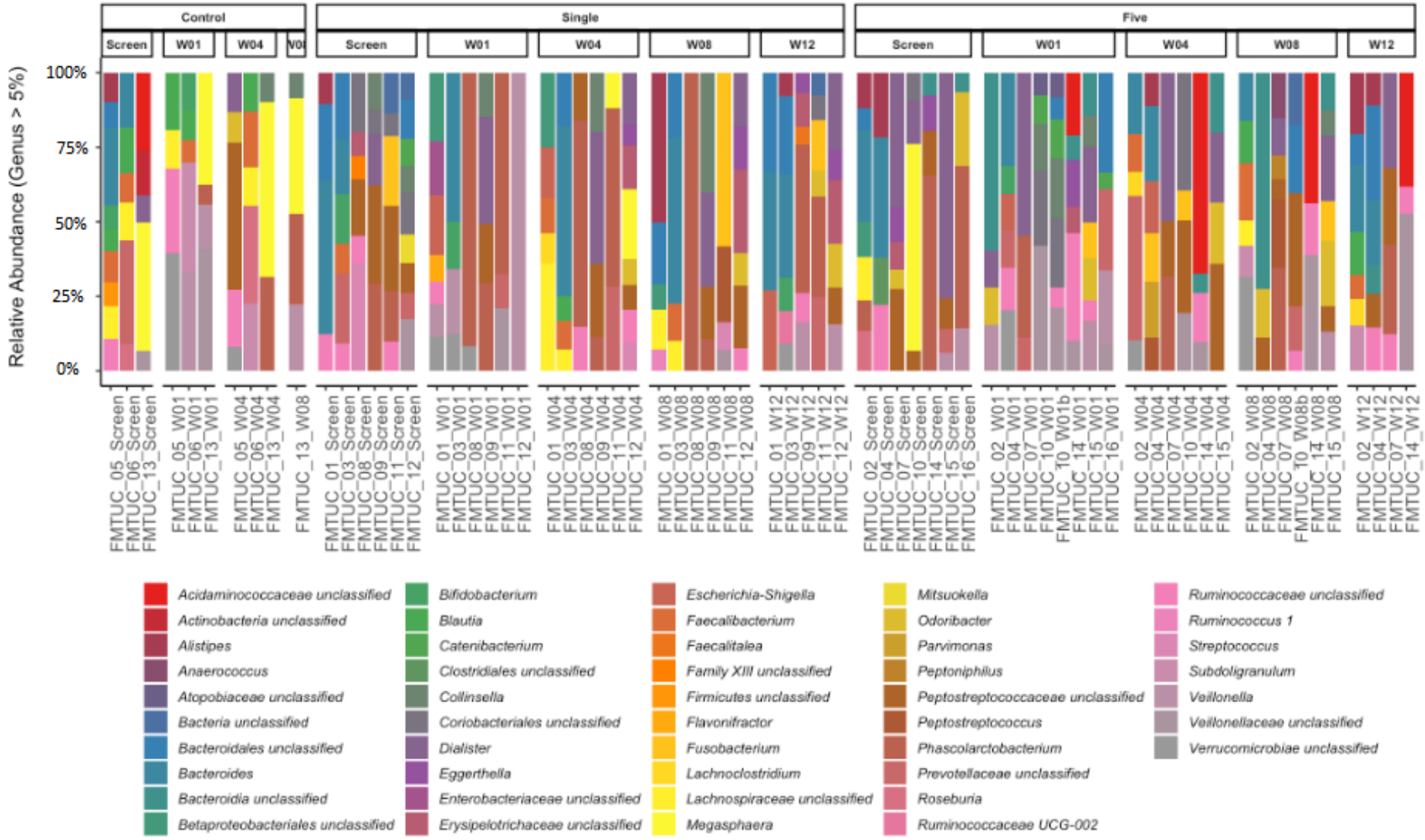


Figure 39: Taxonomic stack bar chart at genus level of participants grouped by intervention groups; Control, FMT single dose and FMT 5 doses.

X axis: Subject ID number as described FMTUC_SubjectID_(Follow-up week), Y axis is relative abundance representing the percentage of an organism relative to the total organisms within the faecal samples.

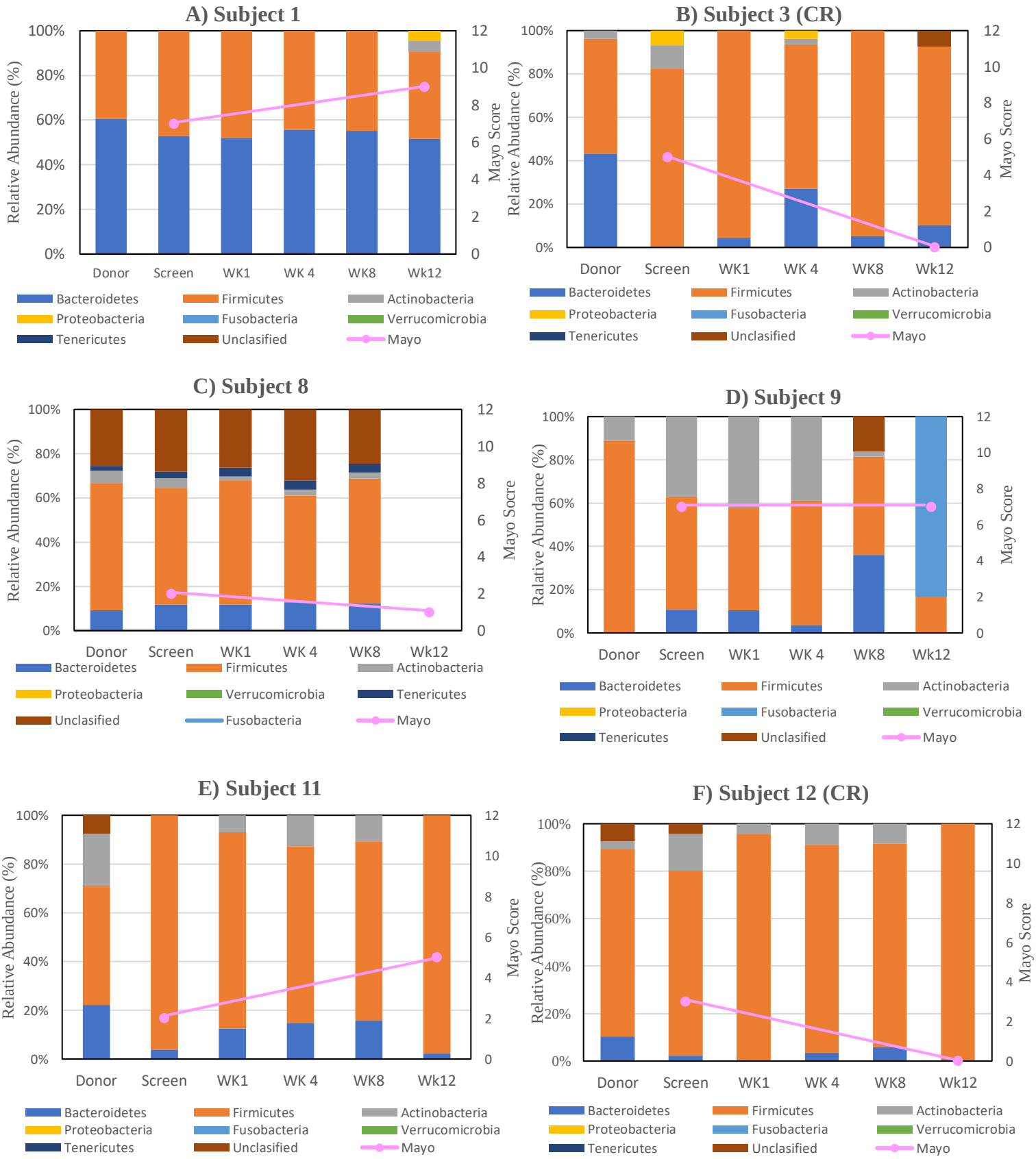


Figure 40: Taxonomic stack bar charts at the phylum level of intervention group 1 (Single dose) grouped by each subject in relation to their Mayo score.

A) Subject 1 B) Subject 3 C) Subject 8 D) Subject 9 E) Subject 11 F) Subject 12. CR = Clinical Remission achieved. X-axis: donor, screening faecal sample, week 1, week 4, week 8, week 12. Left Y axis: Relative abundance, Right Y axis: Mayo score22

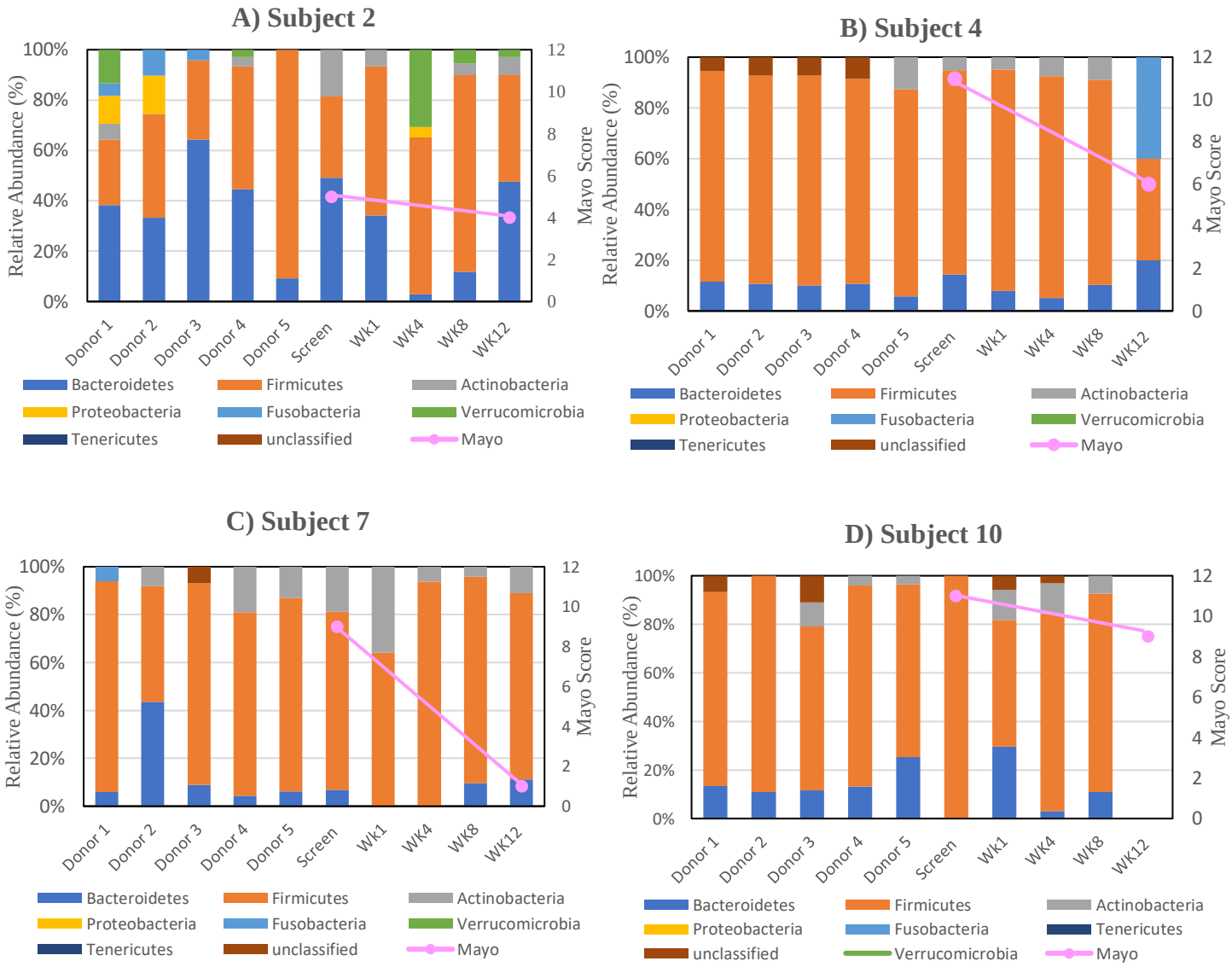


Figure 41: Taxonomic stack bar charts at phyla level of intervention group 2 (5 doses) grouped by each subject in relation to their Mayo score

A) Subject 2 B) Subject 4 C) Subject 7 D) Subject 10, X-axis: donor faecal sample 1-5, each subject's screening, 12-week follow up, Left Y-axis: Relative abundance, Right Y-axis: Mayo score

5.2.3 Differential Abundance Analysis between Intervention Groups

Figure 42 is a NMDS plot graph of all faecal samples from all subjects throughout the 12-week follow up period, which are grouped by interventions groups. Non-metric multi-dimensional scaling (NMDS) is a non-metric way to summarise multidimensional data such as multiple species and operational taxonomic units into a two-dimensional representation and allows to visualise similarity of the microbiome using specialised distance metrics. This NMDS plot suggests that faecal samples from subjects who received five doses (Intervention group 2) demonstrated closer microbiome community after the FMT interventions compared to other intervention groups. According to this NMDS plot, the microbiome of group 1 (single dose) and group 3 (control) showed more dissimilar community

after the FMT interventions than group 2. Despite group 1 (single dose) showed more diverse community, yet group 1 (single dose) and group 2 (five doses) shared the similarity in microbiome compared to group 3 (control). This may imply that the change in the gut microbiome is dose dependant, however, this needs a larger scale study.

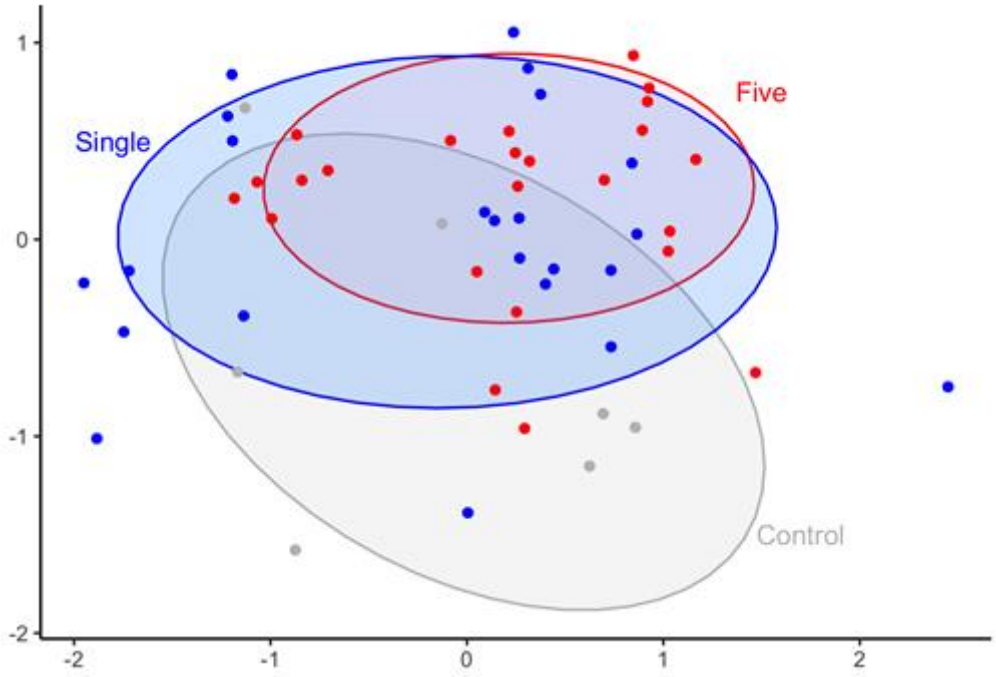


Figure 42: NMDS plot of Bray-Curtis dissimilarity of post-FMT intervention 12-week follow-up faecal samples between intervention groups.

Blue dots and oval: group 1 (Single dose), Red dots and oval: group 2 (5 doses), Grey dots and oval (control). Each dot represents each faecal sample' community. Each oval represents standard deviation of the average of NMDS sample scores. Communities that are similar to one another are located closer together. The axes and orientation of the plot are arbitrary.

5.2.4 Diversity Analysis between Intervention Groups

Species richness, evenness and diversity of ecosystems are often described with Shannon indices. Richness describes the number of species present in the community, whereas evenness is the relative abundance across the species in the community (309). Diversity, on the other hand, describes a measure of the abundance of each species as well as a measure of the number of species in the community, and it is usually described with an index such as Shannon diversity index. Figures 43-45 exhibit Shannon richness, evenness and diversity grouped by interventions respectively.

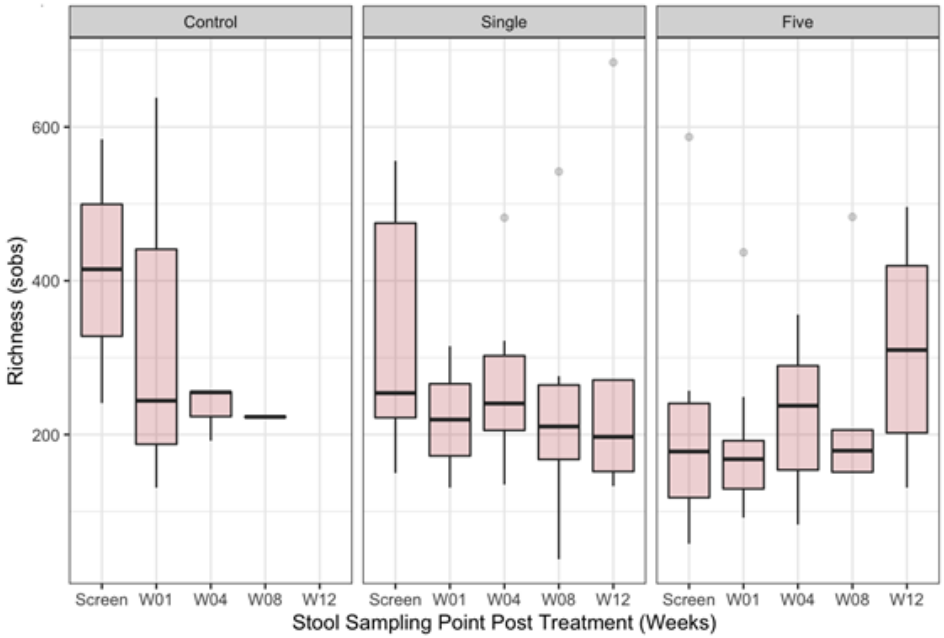


Figure 43: Boxplot of Shannon richness reflecting abundance of faecal microbiome at pre-FMT intervention (Screen) and over the 12-week follow up (W01= Week 1, W04=Week4, W08=Week 8, W12 = Week 12) grouped by intervention groups.

The greater the richness, the more organisms were found in the sample. Boxes represent the interquartile range between 25th and 75 percentiles. The horizontal line in the box is the median. Whiskers are 5th and 95th percentiles. Dots are outliers which are beyond the bounds. Y axis is Richness (Sobs), where Sobs means the total number of species observed in a sample.

Shannon richness suggests that the number of species declined after week 1 across the intervention groups, however, this decline is more noticeable in the control group. Furthermore, richness did not return to the baseline even after week 8 unlike FMT intervention groups, however, intervention group 2 (5 doses) showed increase in richness over the 12 week follow up period. This could imply that richness was declined after the bowel cleanse and pre-FMT antibiotics, but FMT infusion recovered richness of organisms in faecal samples over the period of 12 weeks.

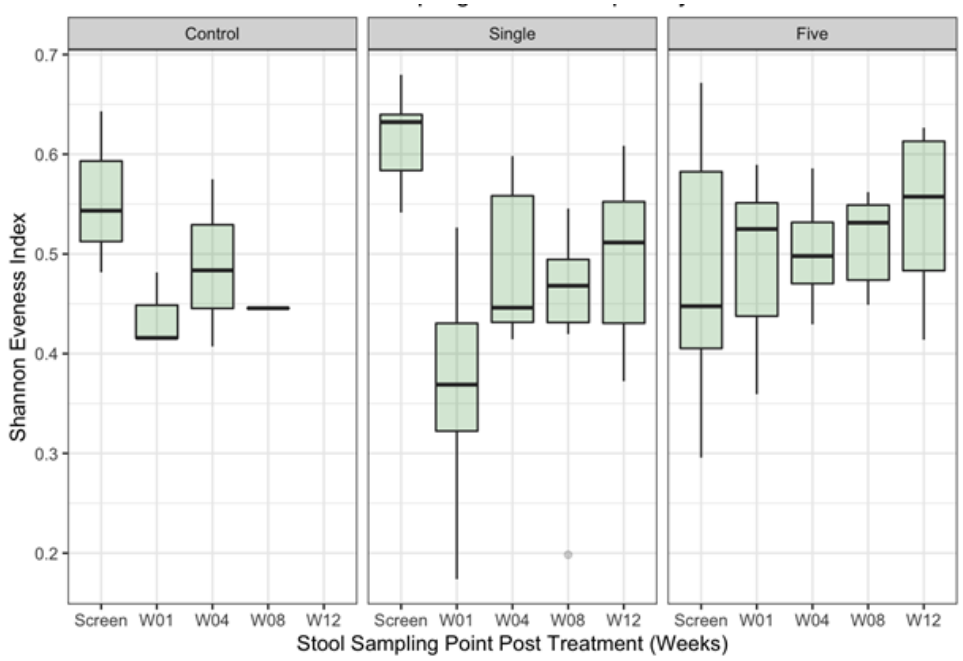


Figure 44: Boxplot of Shannon evenness index reflecting relative abundance of faecal microbiome at pre-FMT intervention (Screen) and over the 12-week follow up (W01= Week 1, W04=Week4, W08=Week 8, W12 = Week 12) grouped by intervention groups.

Y axis is Shannon evenness index and a high evenness index represents all species have similar distribution. Boxes represent the interquartile range between 25th and 75 percentiles. The horizontal line in the box is the median. Whiskers are 5th and 95th percentiles. Dots are outliers which are beyond the bounds.

Shannon evenness (Figure 44) suggests that evenness declined after week 1 amongst the control and intervention group 1 (single dose), however, it gradually returned during the follow up period in group 1 (single dose). This trend was not seen with group 2 (five doses) and evenness became higher after the FMT interventions throughout the follow-up period.

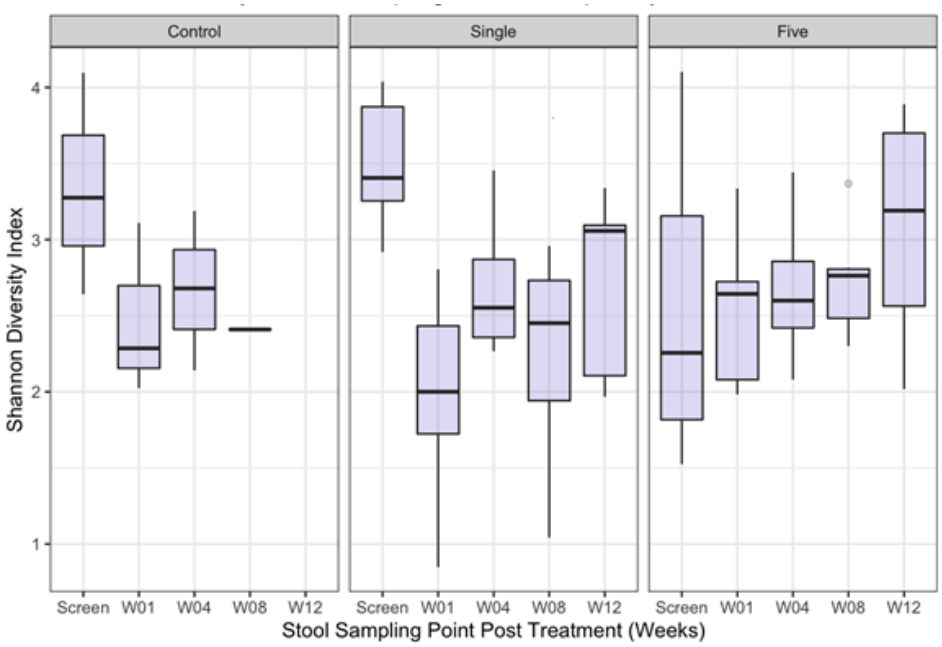


Figure 45: Boxplot of Shannon diversity index reflecting diversity of faecal microbiome at pre-FMT intervention (Screen) and over the 12-week follow up (W01= Week 1, W04=Week4, W08=Week 8, W12 = Week 12) grouped by intervention groups.

Y axis is Shannon diversity index, and a high diversity index represents a higher diversity of species in the faecal samples. Boxes represent the interquartile range between 25th and 75 percentiles. The horizontal line in the box is the median. Whiskers are 5th and 95th percentiles. Dots are outliers which are beyond the bounds.

Figure 45 shows diversity, akin to evenness, declined in the control and single dose groups, however, it gradually returned during the follow up period in group 1 (single dose). The diversity of group 2 (five daily FMT) exhibited a steady increase during the follow up period after the intervention. These alpha diversity analysis with Shannon richness, evenness and diversity demonstrated two important points. Firstly, Shannon richness, evenness and diversity declined between screening and the week 1. During this period, all subjects underwent mechanical bowel cleanse and antibiotics treatment before the FMT infusions. Although the gut microbiome reflects on other factors including diet, lifestyle and microbiome interactions with the host, this may imply that mechanical and chemical bowel cleanse can alter alpha diversity of the faecal microbiome. Secondly, subjects who received five doses showed a higher Shannon richness, evenness as well as diversity compared to subjects who received no FMT infusion or one infusion. This may imply that donor faecal microbiota was engrafted to participants in a dose dependent fashion, though the sample number is too limited in this study to draw any conclusions from this observation.

5.2.5 Differential Abundance Analysis between Clinical Remission and Non-Clinical Remission Groups

A NMDS plot was also used to assess and compare the level of similarity of the faecal microbiome amongst subjects who achieved clinical remission and ones who did not (Figure 46).

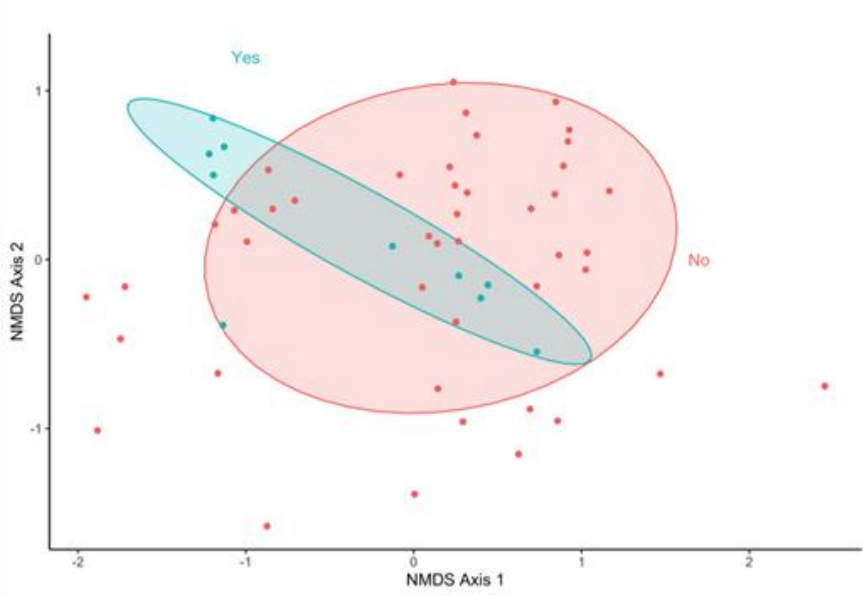


Figure 46: NMDS plot of Bray-Curtis dissimilarity of the post-FMT intervention 12-week follow-up faecal samples between clinical remission and non-clinical remission groups.

Green dots and oval are from subjects who achieved clinical remission, red dots and oval are from those who failed to achieve clinical remission. Each dot represents each faecal sample' community. Each oval represents standard deviation of the average of NMDS sample scores. Communities that are similar to one another are located closer together. The axes and orientation of the plot are arbitrary.

This NMDS plot is based on faecal samples after the FMT interventions and does not include screening faecal samples. This NMDS plot demonstrated the similarity of the faecal microbiome amongst subjects who achieved clinical remission, whereas the faecal microbiome was diverse amongst subjects who did not achieve clinical remission. Although the sample size is very small, this met statistical significance with P = 0.003. This implies that there might be microorganisms which play vital parts in clinical improvement. This was further studied to identify bacteria that were linked to clinical remission.

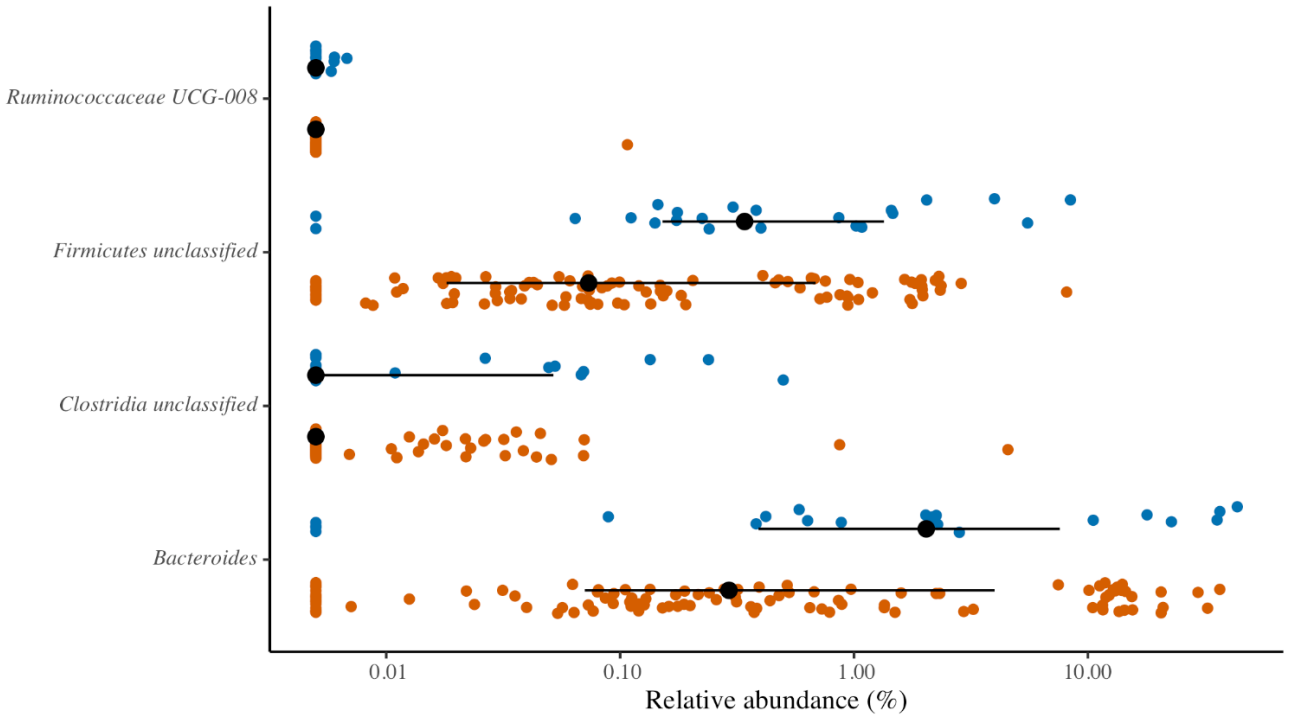


Figure 47: Scatterplots comparing taxonomic relative abundance at the phylum and genus level between clinical remission (orange dots) and non-clinical remission (blue dots) groups, which demonstrated statistical difference.

All faecal samples after the interventions are included in this analysis. The black dots are the median with the arms, which display the quartile ranges.

Figure 47 exhibits scatterplots of four taxa that demonstrated significant statistical difference between subjects with clinical remission status. At the phylum level, the abundance of *Bacteroides* was found to be less in the faecal samples of subjects with clinical remission (P = 0.0323). This finding correlates with literatures as *Bacteroides* are found less abundant amongst patients with UC previously (Table 3). At the genus level, less unclassified bacteria from the *Clostridia* and *Firmicutes* phyla were also detected amongst subjects with clinical remission (P = 0.0323). It was also found that the relative abundance of *Ruminococcaceae* UCG-008 was 0% amongst subjects without clinical remission, whereas it was 0.00000460% amongst subjects with clinical remission with P-value of 0.00287. The presence of *Ruminococcaceae* UCG-008 may be linked to clinical remission of UC, though this needs further investigation.

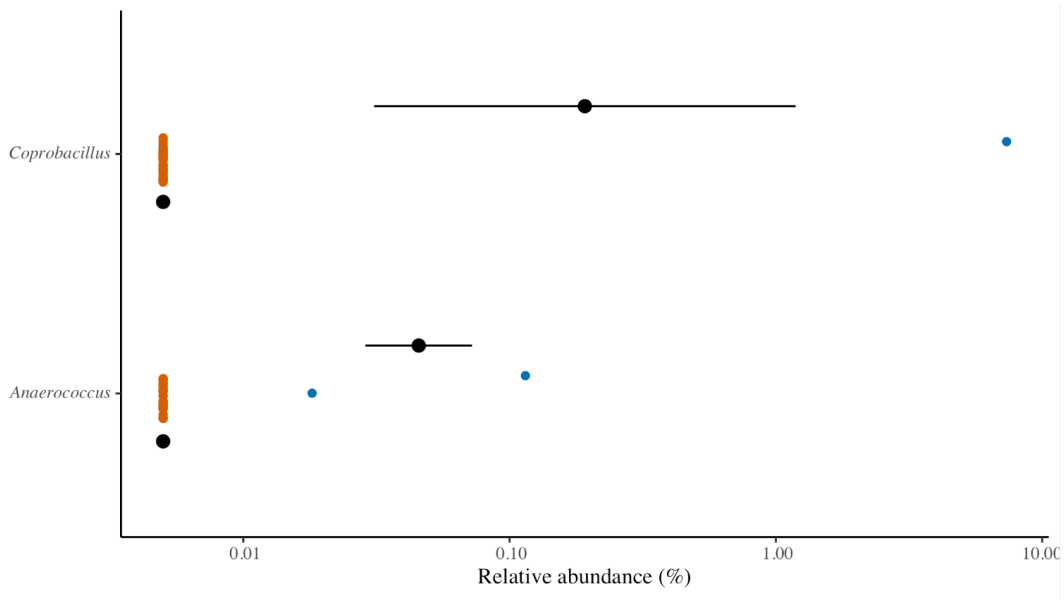


Figure 48: Scatterplots of taxonomic relative abundance with statistical difference between donors' faecal samples that have yielded subjects with clinical remission.

Orange dots represent clinical remission and blue dots are non-clinical remission. The black dots are the median with the arms, which display the quartile ranges.

The donors' faecal samples were also further studied to find any links to subsequent clinical remission. Figure 48 exhibits scatterplots of taxa that were detected in the donors' faecal samples and showed a statistically significant difference according to clinical remission status. This found that the donors' faecal samples with significant lower relative abundance of *Anaerococcus* and *Coprobacillus* were linked to successful subsequent clinical remission (P = 0.00000872 and 0.0138 respectively). Both bacteria are Gram-positive anaerobes and associated with various infections, though their link to UC has been little documented. However, they appear to be significantly linked to subject's clinical remission status and this certainly requires further studies.

5.2.6 Microbial Characterisation of Colonic Mucosal Biopsies

An attempt was made to undertake 16S rRNA analysis of the colonic tissues, however, it was not possible to amplify any meaningful products during the PCR phase despite enough DNA being extracted from the samples. This is a recognised problem when samples contain a significantly smaller ratio of bacterial to human host DNA and it is called the "off-target amplification of human DNA" (310). Although hypervariable region V3-V4 of bacterial 16S rRNA gene is commonly used for amplification because of its maximum nucleotide heterogeneity property, V3-V4 primers also align to a segment of the human mitochondrial DNA (310). Walker et al. reported that V1-V2 primers are less susceptible for off-target amplification of human DNA, however, 16S rRNA analysis of colonic tissue could not obtained in this study.

5.2.7 The Study of Inflammatory Response using IL-10 and IL-21 on Colonic Mucosal Biopsies after the FMT Interventions

Enzyme-linked immunosorbent assay (ELISA) was used to measure levels of IL-10 and IL-21 in biopsies taken from the bowel mucosa. Due to delays in the risk assessment clearance after a leak on the laboratory floor and the COVID-19 pandemic, only the preliminary analysis for IL-10 was shown in this thesis. Although IL-21 was also analysed, the yield of IL-21 was significantly lower, and many samples failed IL-21 detections.

Figures 49-51 demonstrate changes in IL-10 level, which was corrected for total protein from each sample, between pre- and post-FMT interventions and grouped by intervention. These graphs were also compared with the Mayo score on left side of the graphs to demonstrate the trend of these parameters during the follow-up period. No clear patterns were observed with respect to total protein levels.

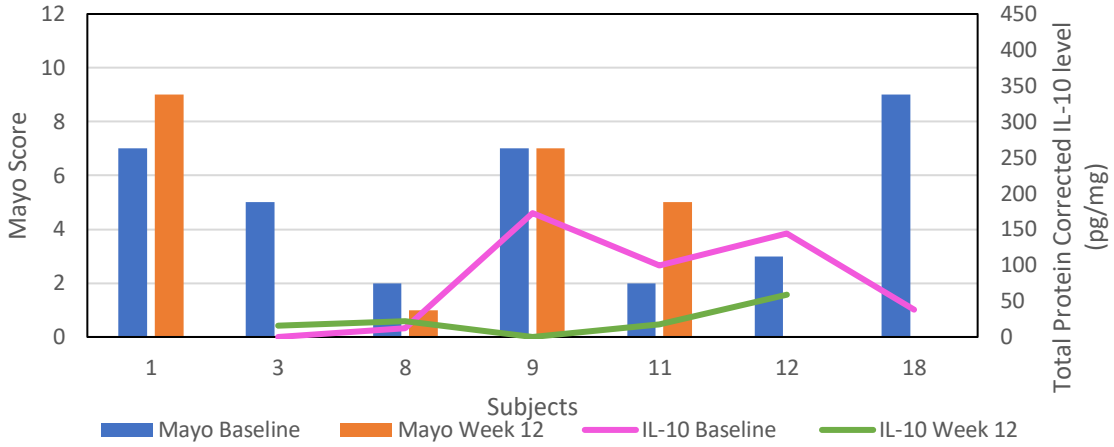


Figure 49: Total protein corrected IL-10 levels at the baseline and week 12 in relation to Mayo scores in group 1

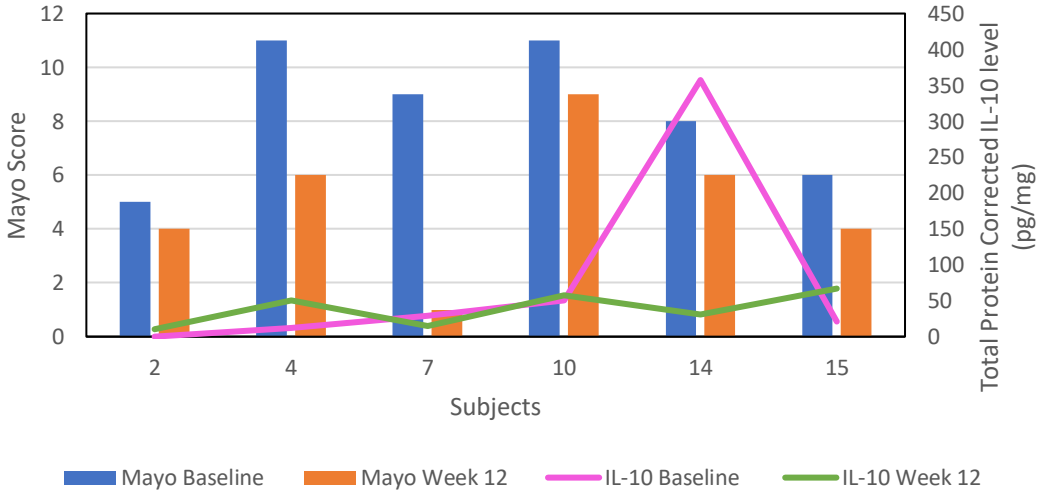


Figure 50: Total protein corrected IL-10 levels at the baseline and week 12 in relation to Mayo scores in group 2

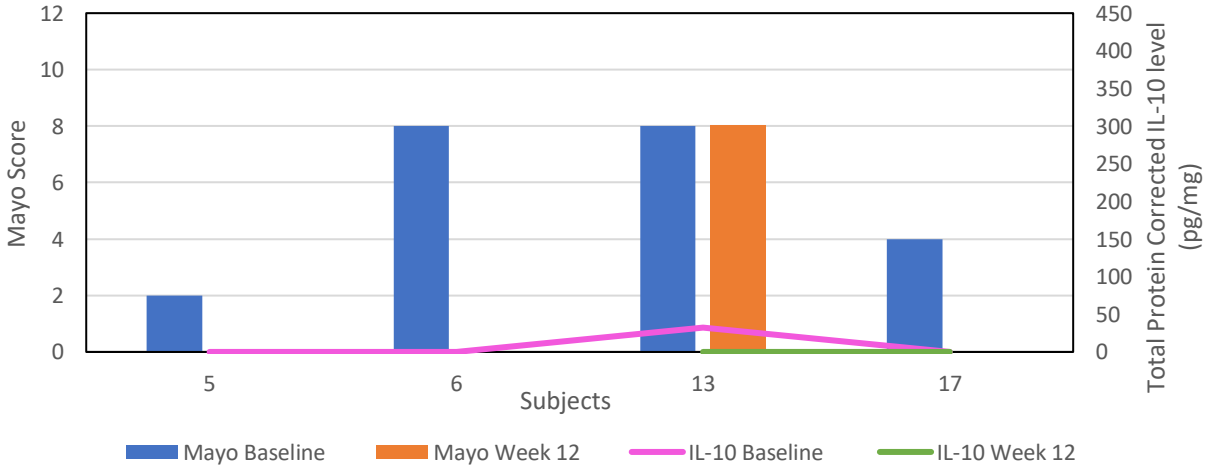


Figure 51: Total protein corrected IL-10 levels at the baseline and week 12 in relation to Mayo scores in group 3

The changes in total protein corrected IL-10 were grouped by intervention groups, which were analysed using the Kruskal-Wallis test (Figure 52). This showed no statistical difference between intervention groups with p = 0.372.

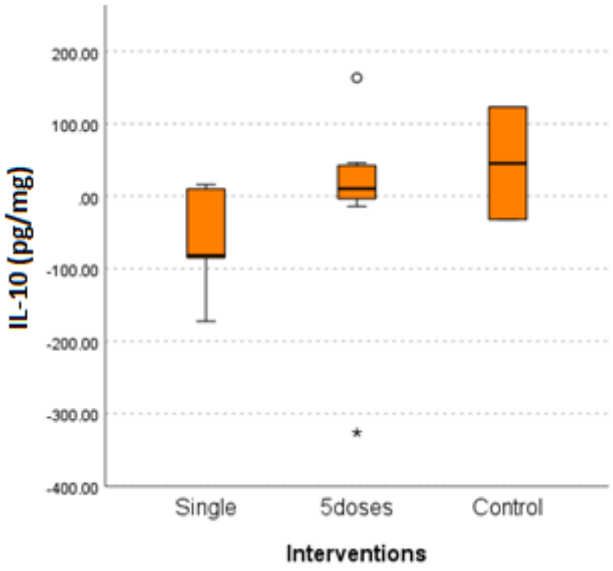


Figure 52: Box plot of total protein corrected IL-10 level changes from the baseline and week 12 FTM interventions grouped by intervention groups.

The bold line in each box graph represents the median value (50th percentile), while the orange box contains the 25th and 27th percentiles of the data. The black upper and lower extremes denote the 5th and 95th percentiles. Circle represents outlier and star represents true outlier.

Total protein corrected IL-10 was also evaluated depending on clinical remission as shown in Table 33. IL-10 is known for its anti-inflammatory properties (236), and previous published work suggested an increase in IL-10 among patients with quiescent UC (240). This feasibility study also found that more IL-10 was detected among subjects who achieved clinical remission after the interventions.

Table 33: Total protein corrected IL-10 grouped by clinical response

	Total protein corrected IL-10 (pg/mg) (median, IQR)	
	Baseline	**Week 12**
Clinical remission	0 (0-71.97)	58.91 (37.45 – 91.04)
Non-clinical remission	32.32 (16.53-135.93)	22.02 (12.57-53.93)

To ascertain if total protein corrected IL-10 at week 12 follow-up correlates with the Mayo score amongst the study subjects, a regression coefficient was derived as shown in Figure 53. This analysis suggested that total protein corrected IL-10 was correlated inversely with the Mayo score. A regression line was also derived using SPSS. Coefficient of determination (R^2) was 0.0111 (95% CI, -24.52 – 17.17) and the regression coefficient was - 0.105 with P = 0.710.

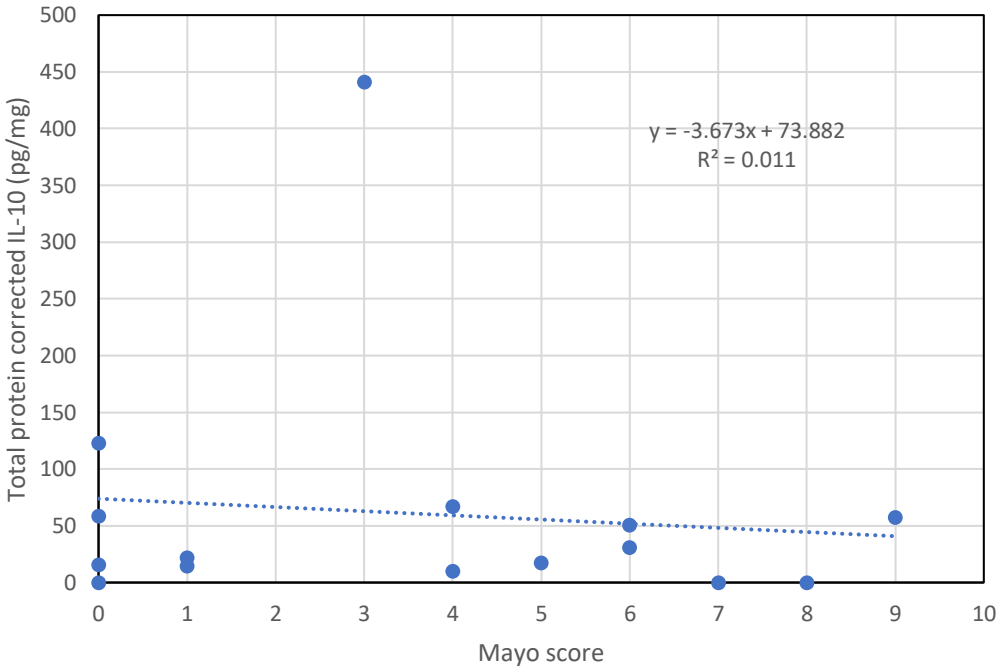


Figure 53: Total protein corrected IL-10 vs. Mayo score at week 12. Dotted line represents the line of best fit regression line. R^2 is coefficient determination.

This finding did not reach statistical significance, however, it showed a trend towards decrease in IL-10 as the severity of UC worsens. It is not possible to determine whether an increase in IL-10 level may be the results of quiescence disease or whether an increase in IL-10 leads to clinical remission. This is another aspect that requires more study.

Chapter 6. Discussion

This study is the first randomised feasibility study to investigate FMT use in treatment naïve patients with active UC to date. There are a few RCTs to evaluate FMT use in UC patients, but their patients were treated with conventional treatment at the time of the studies. Many of these studies defined one of their criteria of clinical remission as withdrawal of conventional treatment, such as steroid (169,170,173). Although it may be safer to continue treating active UC patients with conventional medications while they are treated with FMT, especially when the efficacy of FMT is yet to be known, it is difficult to tease out the defining true effects of FMT if subjects are also simultaneously treated with conventional medication. Ideally, patients with active UC should continue established treatment, not delayed by FMT interventions. This might assist recruitment as patients would not miss out on early conventional treatment in order to trial unproven FMT interventions. Furthermore, the study drop-out rate might be reduced if patients with active UC suffer fewer symptoms whilst continuing conventional treatment. In this study, however, the aim is to study the efficacy of FMT with minimal interference or artefacts. Hence treatment naïve subjects were chosen.

6.1 Endpoints of the FMTUC

6.1.1 Primary Endpoint: Clinical Remission

In total, five subjects (three from single dose FMT enema group and two from the control group) achieved clinical remission during this study period. None from intervention group 2 achieved clinical remission. There was one subject from the control group who withdrew from the study due to worsening symptoms, however, the rest of 17 subjects successfully completed their 12 weeks follow up. No adverse effects from the pre-FMT bowel cleanse and antibiotics or FMT interventions were reported during this study period.

Although five subjects achieved clinical remission in this study, eight subjects experienced clinical improvement and required no further medical treatment after the 12 weeks follow up assessment. Of those eight patients, two subjects (one subject each from Group 1 and 2) failed to achieve clinical remission due to mild inflammation with endoscopic Mayo score of 1.

The median initial Mayo score of subjects who experienced successful clinical remission was 4 (IQR 3-5), whereas that of subjects who failed clinical remission was 7 (IQR 5-8). This trend was also noted with other qualitative assessments (IBDex and CUCQ-32). It appeared that subjects with milder disease were more likely achieve clinical remission, however, this trend did not reach statistical significance. Mayo score ≤ 5 is often defined as mild-moderate disease in clinical settings. If the disease condition is defined with mild-moderate and moderate-severe, the minimum sample size of

26 would be required to demonstrate this effect. The NICE guideline on management of UC advises different treatment regimens depending on the severity and extent of the disease (Chapter 2.2.6, Figure 6 and 7). If FMT was to be effective in management of UC, FMT may be more suitable for patients with mild-moderate disease.

More marked reductions of Mayo score were also observed amongst subjects with successful clinical remission, however, this was also not statistically significant in all intervention groups. The changes in Mayo score depending on intervention groups were also studied, which again did not show statistical significance. To detect a true effect on intervention groups, a minimum of 68 subjects would be required under the same conditions.

Mucosal healing with endoscopic assessment is often regarded as the hallmark of clinical remission in many RCTs. Yet, the definition of mucosal healing is still unclear. Mucosal healing usually been assessed macroscopically with endoscopy, however, in recent years, some clinicians advocate histological assessment to define mucosal healing since some studies suggest that it provides more accurate prognostic values (311,312). In this study, endoscopic Mayo score 0 was one of the definition of clinical remission. Nine subjects, including five subjects with endoscopic Mayo score of 0, showed improved endoscopic Mayo score. Eight subjects either worsened or did not improve their endoscopic Mayo score. As with the qualitative assessments, three interventions did not show statistical difference in endoscopic Mayo scores.

Histological assessment was quantified with Nancy histology index, which showed subjects who achieved endoscopic Mayo score 0 also showed complete histological healing with Nancy histology index score of 0. Interestingly, one subject who showed complete histological response showed endoscopic Mayo score of 1. This is most likely because tissue biopsy samples were taken from healthy parts of the bowel or the biopsy tissues were insufficient for accurate diagnosis. This can be one of the issues of difficulties with defining mucosal healing merely on histological assessments.

The extent of the disease is another factor that could influence clinical response because of anatomical variations of the torturous sigmoid and difficulties in determining how far the FMT enema could reach, though this did not appear to show any significant difference in this cohort of subjects.

It is noteworthy that two subjects from the control group achieved clinical remission. Many subjects reported marked improvement in their symptoms after the bowel cleanse and antibiotics for the pre-FMT optimisation. This was reflected in changes in the faecal microbiota of subjects from the control

group. Although the faecal microbiota of subjects from intervention groups showed very different microbial communities from those of the control group, it is not yet known which microorganisms are linked to UC.

6.1.2 Primary Endpoint: Engraftment of Donor Faecal Microbiota

The16S study demonstrated successful engraftment of donor microbiota after the FMT interventions for some subjects regardless of their clinical response. The study demonstrated a significant difference between the faecal microbiota of the donors and the subjects before the FMT interventions as well as post interventions, which was shown Figure 30-34. Furthermore, marked differences in the faecal microbiota were observed amongst the donors as well as the subjects. As with the previous FMT studies, this study showed that faecal microbiota is unique and individual. As previous studies suggested (134,153), faecal microbiota of subjects contained *Proteobacteria* and *Fusobacteria*, which were no seen in that of donors. Conversely, this study showed more abundant *Actinobacteria* in faecal samples from the subjects than those from the donors, unlike previously reports (151). Faecal microbiota of the subjects with UC appeared to have one or two dominant groups of bacteria, which varied on an individual basis, implying less diverse faecal microbiota environment of patients affected by UC.

It was also noted that the faecal microbiota of stool samples from the Wessex stool bank was noticeably different from that of the local laboratory, however, no significant difference was observed in the clinical outcomes or results. Although manufacturing process of the FMT products in the local laboratory followed the protocol from the Wessex stool bank, some differences in the manufacturing and storage process might have contributed to this difference. Moreover, each donor has unique faecal microbiota, leading to the different taxonomic profiles between two stool bank sites. This also raises the question of the existence of FMT super-donors.

After the pre-FMT bowel optimisation with antibiotics and bowel cleanse, faecal microbiota was altered dramatically. Faecal microbiota also evolved throughout the 12 weeks follow up period in all groups. The faecal microbiota appears to be more diverse amongst FMT intervention groups than the control group, and FMT intervention groups showed similar profile at family and genus levels (Figure 38-39) especially at week 12. Non-metric multi-dimensional scaling (NMDS) of 16S results from faecal samples also showed a similar profile between intervention group 1 and 2 (FMT intervention groups), however, the control group was noticeably different from the intervention groups (Figure 42). Furthermore, this NMDS graph suggested that the microbiome of group 2 (5 doses) shared similarly more than that of group 1, implying that the similarity of the microbial community may be

dose dependent. Further studies required to investigate the implication of similar microbial community to clinical response. This finding implies that FMT via enema infusion successfully altered the faecal microbiota of the subjects regardless of clinical responses to these changes.

Species richness, evenness and diversity of faecal microbiota were also studied (Figures 43-45). Shannon richness graph showed declines in richness across the groups after week 1, though this was more evident in the control group without FMT infusion. In contrast, Shannon evenness and diversity exhibited a different trend. They showed sharp declines in the control group and group 1, but showed an increase in group 2 with five daily FMT enemas. This may imply that evenness and diversity are dose dependent, though the study sample is very limited in this study to draw a conclusion.

Faecal microbiota was also studied depending on clinical response, and NMDS plots grouped by a clinical remission status as shown in Figure 46. This analysis was grouped by subjects' clinical remission status. This demonstrated that the subjects who achieved clinical remission exhibited similarity in their microbiome, whereas subjects with non-clinical remission were very dissimilar. This implies that there might be responsible microorganisms or combinations thereof that lead to clinical remission. Further studies with a large number of faecal samples are required to investigate.

6.1.3 Secondary Endpoints: Qualitative, Histological and Immunological Assessments

Qualitative studies

Overall, all three qualitative assessments showed an improvement of symptoms after all interventions including the control group. Many subjects experienced significant symptom improvements after the bowel preparation and antibiotics for 10 days even before the FMT infusions. It was observed in all three index scores that the cohort who achieved clinical remission scored significantly lower at the index assessment than those who failed to achieve clinical remission, however, the sample number of this study is too small to draw any conclusions.

These three qualitative assessments show high correlations (Table 25). In a future study, one subjective assessment tool should be sufficient. IBDex may be preferable to CUCQ-32 because IBDex showed better correlation with other laboratory-based investigations, and the questions are based on the previous three days, which may make it less susceptible to bias and variability.

Macroscopic findings: Endoscopic assessments

As with qualitative assessments, the median endoscopic Mayo score at the baseline was lower amongst the subjects who achieved clinical remission. Endoscopic assessment also did not capture significant statistical difference in between FMT intervention groups.

To quantify endoscopic assessment, endoscopic Mayo score was used in this study, which scores from scale 0-4 based on the macroscopic appearances of the colorectal mucosa by endoscopists. Although endoscopic Mayo score is widely used in clinical trials as well as clinical settings, it has been criticised for its inter- and intra-rater reliability as studied in the narrative review (Chapter 3). Yet, this study exhibited good correlations with histological findings.

Endoscopic assessments on extent of the disease, direct vision of colorectal mucosa and biopsy have been a gold standard assessment for UC, however, endoscopy has its limitations. Firstly, endoscopists can misjudge the segment and length of the bowel, especially the sigmoid and rectum. A part of the bowel that the endoscopist thinks is the sigmoid can be rectum or even descending colon. This may not be too critical in the routine clinical setting, however, it might affect study comparisons if the extent of the disease is underestimated. Secondly, it is possible that even when the disease appears to be limited within the sigmoid macroscopically, the disease may be extended microscopically. The bowel segments with histologically active inflammation beyond the reach of the FMT enema infusion would not benefit from the interventions even if FMT is effective. This could be one of the reasons for some subjects worsening symptoms despite the interventions. For a future study, there should be a more robust protocol to exclude such cohort of patients or an alternative route of FMT infusion needs to be sought to ensure that the diseased mucosae can benefit from FMT.

Another learning point from this feasibility study was a lack of clear protocol on biopsy and photos. Some subjects had multiple photos, but often it was not stated clearly which part of the bowel the photos were taken from, whereas some subjects did not have any photos. Any further study should include a clear protocol on biopsies, so that sufficient biopsies are available for further 16S and immunoassay and accurate histological assessments.

Microscopic findings: Histological assessments

Histological assessment was quantified with Nancy histological index score, which showed a high correlation with endoscopic Mayo score with $r = 0.854$ [95% CI 0.58 – 1.00, $P < 0.001$]. Recently, some clinicians suggested that mucosal healing should be based on histological assessment for its better prognostic factors (291,311,313). However, in this FMTUC study, histological assessment did

not differ greatly from endoscopic assessment when this was assessed with endoscopic Mayo score. The findings of this study and the narrative review (Chapter 3) suggest that using a more robust endoscopic assessment tool, such as the ulcerative colitis endoscopic index of severity (UCEIS), would monitor more accurately severity of disease rather than replacing endoscopic assessment with another histological assessment tool to define clinical remission.

The UCEIS was developed by Travis et al. to overcome from the inherent variability of many widely used assessment tools, and it is one of the most validated tools, addressed in the narrative review of this study (50,245). The UCEIS categorises macroscopic colonic mucosa by vascular pattern, bleeding and erosions and ulcers, which are further subdivided into 2-3 point Likert scale with detailed definition. Although the UCEIS is not widely used in clinical trials, it has been validated extensively and has a great potential to capture the true severity of disease.

Immunoassay study: IL-10

Due to the COVID-19 pandemic, laboratory work including immunoassay and 16S experienced a significant delay. The preliminary results and analysis are included in this thesis. Nonetheless, a few interesting observations were made on 16S on faecal samples as well as IL-10 on tissue biopsies.

This study revealed a substantial increase in IL-10 in biopsies of subjects who achieved clinical remission and a decrease in IL-10 in those who failed to achieve remission (Table 33), though this did not reach statistical significance. Yet, this observation supports findings from previous studies. This finding was also supported when regression analysis between IL-10 and Mayo score at week 12 was evaluated (Figure 53). The regression line suggested that the level of IL-10 declined as Mayo score increased. This was not statistically significant with $R^2 = 0.0111$. There was one result, which appeared to be erroneous as it stood out from the rest of the results as highly discrepant. When this result is included, a minimum of 340 would be required to achieve power of 0.8. When this is excluded, a much smaller sample size would be required to show statistically significant results.

6.2 Challenges and Issues of the FMTUC

Recruitment

Recruitment was much slower than first expected. Between July 2016 and February 2020, 18 participants were eventually enrolled in the study. This was due to multiple reasons.

Firstly, it was very difficult to find subjects who fitted the inclusion criteria. In the real clinical setting, endoscopic assessments are usually graded into three different urgency groups depending on their symptoms and age group. Patients with suspected malignancy have the highest priority and are assessed within two weeks. Subjects with abnormal scans and/or less urgent symptoms usually have endoscopic assessments carried out within eight weeks. Many younger subjects with symptoms of UC are categorised in the least urgent group, for routine assessment, which may mean waiting up to 6 months or longer. Furthermore, many young patients are reluctant to visit hospital or general practitioners with what they consider embarrassing symptoms such as bloody diarrhoea until they become unmanageable, leading to a further delay in diagnosis. For this reason, many clinicians start conventional treatment before endoscopic assessments if they suspect UC. Even if they do not start treatment before endoscopic assessments, many clinicians start treatment at the time of endoscopic assessment when they suspect UC with macroscopic findings because these patients have been suffering from their symptoms for a long time, and through lack of knowledge of the FMTUC trial seeking participants. Another issue with the delay in endoscopic assessment is the risk of disease progression. While patients are waiting for their endoscopic assessment, the disease may progress to extend beyond the sigmoid, becoming unsuitable for the FMTUC study. The study results appear to suggest that the FMT is more effective in milder disease. Even if a subjects' disease remain within the sigmoid, disease severity may have progressed. These patients might have missed the optimum window for the FMT interventions. As a part of the FMTUC study promotion, many gastroenterologists and GI surgeons who carry out endoscopy were invited to educational meetings to discuss the protocols. They were also regularly reminded by emails and posters on each endoscopy suite about the study. Yet, many subjects with newly diagnosed with UC were referred to the study in whom conventional treatment had been already started. Despite the nature of the FMT, many participants expressed little reservation about the FMT once the rationale had been explained to them. Interestingly, many patients were more reluctant to start conventional immunosuppressant treatment, and were keen to try out a more natural method treatment like FMT. For any future study, there should be a system where suspected UC patients should have their endoscopic assessment and histology confirmation in a timely manner so that they can be recruited to the study more efficiently.

Secondly, the regulation changes around the FMT forced the study to be put on hold for almost 12 months. Although it was a slow process, the number of the participants started steadily increasing until the first disruption due to the FMT regulation changes. The FMT regulation changes meant that the FMT samples could no longer be obtained from the Wessex stool bank, necessitating the securing of an alternative supply of FMT samples.

Finally, the COVID-19 pandemic halted the FMTUC study for an uncertain period. The pandemic has been major challenge for many scientific studies as well as for the NHS, so it is unsurprising that the FMT study was also severely affected. Severe acute respiratory syndrome coronavirus (SARS-CoV-2) paralysed the healthcare from January 2020 and all resources were focussed on to this. This meant that two patients, who were in the 12-week follow up period, were unable to attend consultations or to be investigated with endoscopic assessments at week 12. Subject 17 also contracted the virus after 12 weeks. These patients' endoscopic and histological assessments were not included in the final analysis of the study. A return to recruitment without a valid method to exclude SARS-CoV-2 in the faeces of donors was also a barrier to the study recommencing.

Defining clinical remission

There is no universal consensus on clinical remission in UC as demonstrated by the narrative review (Chapter 3). In this study, clinical remission was defined as Mayo score ≤ 2 with an endoscopic Mayo score of 0. The reality is that many clinicians and researchers, define reasonable clinical remission differently, resulting in heterogeneity of the study results. In fact, even different regulatory bodies and guidelines use different criteria for UC clinical remission (Table 6). Instead of reaching to a consensus, the number of outcome measuring tools has been increasing steadily, and confusingly all claim to capture true clinical remission. The narrative review suggested that an international consensus of remission should be sought as a matter of urgency before establishing a gold standard outcome measurement. This would lead to standardisation of clinical trial protocols for advancing patient care.

The optimum parameters for delivery of FMT

FMT interventions have been explored in not only UC, but also many conditions such as liver diseases and metabolic conditions. This study's results suggested that FMT significantly alter the recipients' faecal microbiota, though the implications to clinical response are yet unclear. This study suggests that FMT may be more effective in patients with milder conditions. When the mild to moderate condition was defined as Mayo score ≤ 5, a minimum sample size of only 26 would be required to demonstrate efficacy under the same study conditions. This study also showed that FMT enema as a

choice of route was effective since the preliminary 16S results suggested successful engraftment of FMT. However, a careful selection of subjects with a clearer definition of disease extent, as well as absence of disease at a macroscopically normal segment of the proximal bowel with a histological confirmation should be required. Furthermore, some parts of the bowel, the sigmoid in particular, can be very torturous, which could prevent the FMT enema from reaching the diseased mucosa. This should be considered if FMT enema is used in clinical practice.

This study did not observe any correlations between intervention dose and clinical response, and the optimum dose and frequency remain unknown.

Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV-2)

The COVID-19 pandemic raised significant concerns with regard to the risks of FMT related to SARS-CoV-2 due to the detection of SARS-CoV-2 in stool samples in a different study (314). Furthermore, some studies suggested that prolonged infectious SARS-CoV-2 virus shedding in faecal samples may occur amongst individuals with a negative nasopharyngeal swab test (315). The FDA has advised that FMT samples donated before 1st December 2019 must be used until appropriate virus testing of donors as well as their stools become available (316). Recently, a new guideline was published to facilitate the reorganisation of FMT services in view of COVID-19 pandemic (317). This guideline advises robust laboratory testing of donors with nasopharyngeal swab, reverse transcription polymerase chain reaction (RT-PCR) test as well as molecular stool testing. It also emphasises the importance of informed consent procedures for the recipients to accept the potential risk of transmission. However, what this pandemic really highlights the fact that any transplant of human (or animal) material carries risks of potential transmission. In any future larger study of FMTUC, this should be clearly stated in the protocol, which must adhere to the standards set by the regulatory bodies.

Availability and Access to 16S results

This project required multiple specialists' assistance to complete as this project investigated the impact of FMT on subjects with active UC from clinical, endoscopic, histological and mechanistic aspects. Collaborative work involving 16S has been particularly challenging. Since the access to the laboratory or equipment to run 16S sequencing work was never granted by the internal colleague, collaborative work was inevitable. The inconsistent communication with the specialist of 16S, only partial data or results were available despite a numerous exchanges of communication throughout the project. This had been intervened by internal and external supervisors, however, very little

improvement has seen. Having robust collaborative work is vital for such studies and this has been a sharp learning curve for me to conduct future research.

6.3 Future Study: Phase III FMTUC

The number of study subjects in his trial was limited, although this feasibility study has shed a positive light on the study of FMT in UC management. Due to the COVID-19 pandemic, only preliminary 16S analysis was included in this thesis. Metabolomic studies have not been conducted yet. Once all the results and analysis are completed, this feasibility study will be published and presented at the international meetings. In due course, a large-scale phase III FMTUC study is proposed as follows though searching for reliable collaborative work and specialist to complete 16S is vital.

Design of the Phase III FMTUC Study

The phase III FMTUC study should aim to recruit 100 patients with thirty subjects in each intervention arm. An additional intervention group, which receives two FMT doses at week 0 and week 4, is proposed to be added to study the effect of repeated frequency. Two weekly doses are also suggested, as supposed to three, to see enough effect within the 12-week follow-up. However, the transplant should use the same donor samples to avoid interference of FMT engraftment from the previous intervention. The sample size is estimated by the sample size calculation from the effect analysis of different intervention arms based on Mayo score as described in section 6.1. Table 34 summarises the intervention arms and proposed treatment regimes. As the phase II feasibility study had recruitment challenges at a single dose, the study recruitment must be widened to national level.

Table 34: Proposal intervention and control arms for phase III FMTUC study

	Group 1	Group 2	Group 3	Group 4
Bowel decontamination and preparation	Yes	Yes	Yes	Yes
FMT treatment dose	1	5 consecutive days (single treatment per day)	2 (single dose at week 0 and week 4)	None
Number of participants	25	25	25	25

Inclusion and Exclusion Criteria

The inclusion and exclusion criteria (Table 14) remain the same as the feasibility study except an additional confirmation of negative mucosal active disease at 40cm from the anal verge to exclude patients with microscopic disease beyond 40cm. Two negative SARS-CoV-2 tests should be added to the inclusion criteria even if participants are asymptomatic. Exclusion criteria should also include recent SARS-CoV-2 vaccination within four weeks to avoid any potential interactions.

Endpoints

- **Paired Primary Endpoints**
    - Clinical remission of UC at week 12 as assessed by blinded endoscopy. Assessment should be defined as UCEIS ≤ 1
    - Proportion of successful engraftment of donor faecal microbiota at 12 weeks in each group as analysed by 16S sequencing and longitudinal diversity index.
- **Secondary Endpoints**
    - Subjective qualitative assessment with IBDex severity scoring index
    - Objective qualitative assessment with Mayo score
    - Histological grading of colitis after treatment at week 12
    - Mucosal immunological response to treatment with IL-10
    - Routine blood tests monitoring

Pre-FMT Optimisation

The same antibiotics and bowel preparation 10 days prior to the FMT infusion.

FMT Products

The manufacturing of FMT products should adhere to guidelines regarding SARS-CoV-2. The donors' stool samples must be negative for SARS-CoV-2. An enema form of FMT should be used as an administration route with the same procedure as this feasibility study. The storage procedure should also remain the same.

The summary of the proposed study scheme flowchart and 12-week follow up protocols are described in Figure 54 and Table 35.

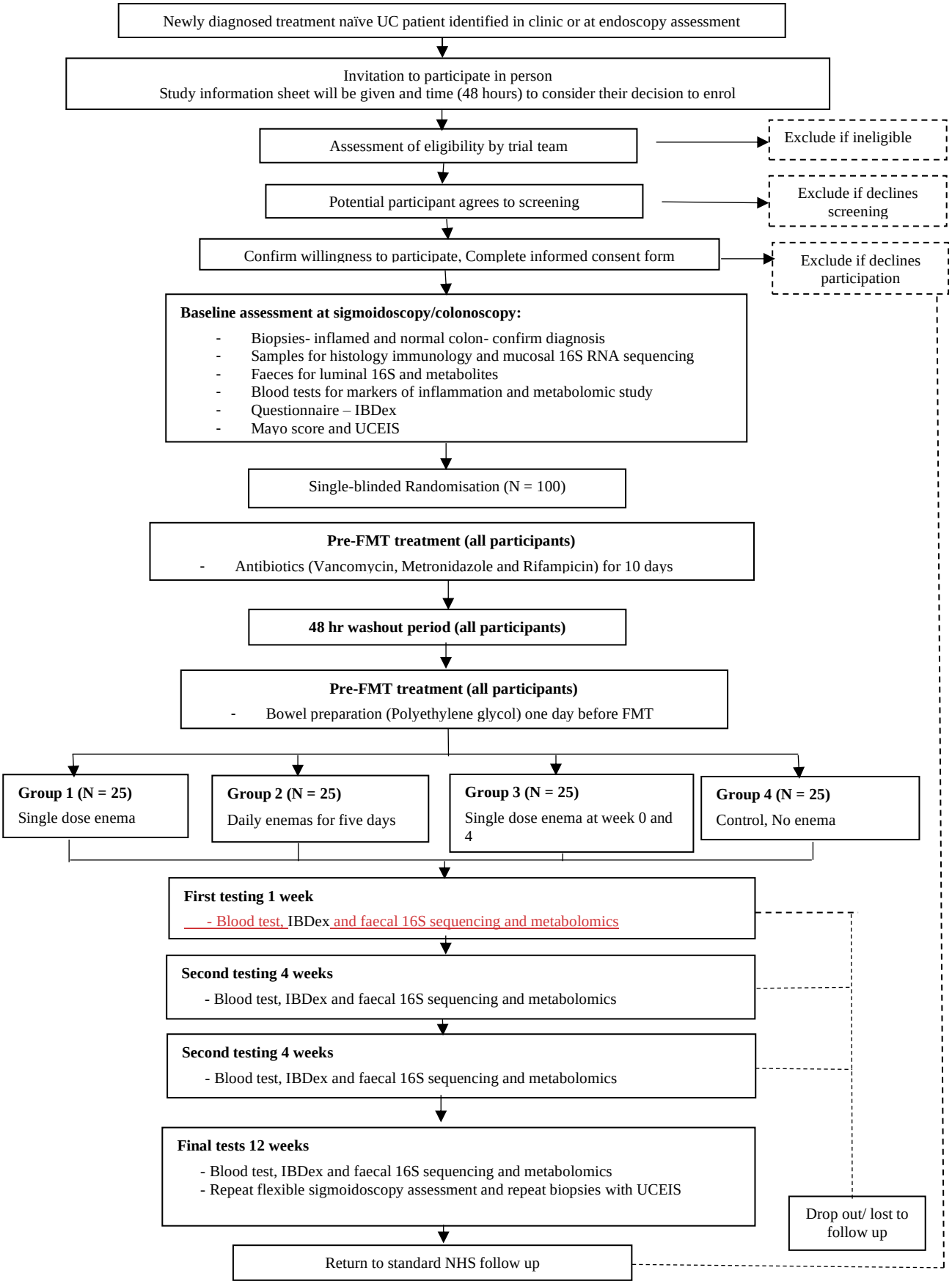


Figure 54: Proposal study scheme flowchart for Phase III FMTUC

Table 35: Summary of 12 week follow up plans

	Baseline	Week 1	Week 4	Week 8	Week 12
Questionnaires					
IBDex	●	●	●	●	●
Mayo Score	●				●
UCEIS	●				●
Endoscopy assessment					
Sigmoidoscopy	●				●
Rectal biopsy	●				●
Histology assessment					
Histological grading	●				●
Mucosal 16S sequencing	●				●
Mucosal IL-10	●				●
Blood tests					
Renal profile	●	●	●	●	●
Liver profile	●	●	●	●	●
Full blood count	●	●	●	●	●
C-reactive protein	●	●	●	●	●
Metabolomic profile	●	●	●	●	●
Faecal sample assessment					
16S sequencing	●	●	●	●	●
Metabolomic profile	●	●	●	●	●

Chapter 7. Conclusion

This feasibility study was the first study to investigate FMT use in treatment-naïve patients with UC. This thesis aimed to conduct a narrative review of endpoints in UC clinical trials, to estimate the magnitude of treatment response to FMT and to determine optimal study conditions and choice of endpoints for the phase III FMTUC. The paired primary endpoints were defined as clinical remission and successful engraftment of donor faecal microbiota at the 12-week follow up assessment. Clinical remission was defined as Mayo score ≤ 2 with an endoscopic Mayo score of 0.

In total 18 subjects were enrolled in the study between July 2016 and February 2020. These subjects were randomised into three intervention arms: single FMT enema, five daily FMT enemas and no FMT. All subjects received ten days of antibiotics and bowel cleanse 48 hours prior to the FMT interventions.
These subjects were followed up for 12 weeks using qualitative assessment (IBDex and CUCQ-32), routine blood tests and 16S study on faecal samples at week 1, 4, 8 and 12. Additionally, they underwent endoscopic and histological assessments at week 12. Their tissue biopsies were also studied using IL-10 and IL-21.

Seventeen subjects completed the 12-week follow up, of those five subjects achieved clinical remission. One subject from the control group withdrew from the study at week 4 due to worsening symptoms. No significant adverse effects from FMT were recorded during the study period. Due to the small sample size, results of qualitative, clinical and laboratory outcomes were not statistically significant, however, several interesting observations were made.

Firstly, subjects achieving clinical remission had significantly lower qualitative scores including the Mayo score at the baseline assessment. This may imply that FMT is more effective for patients with mild-moderate disease. The power calculation suggests minimum 26 subjects are required to demonstrate this effect under the same conditions if mild-moderate disease is quantified as the Mayo score ≤ 5.

Secondly, this study did not observe any correlations with FMT dose and clinical response, although this may be due to the limited sample size. Enema form as a route of FMT interventions was thought to be effective, however, negative histological confirmation at 40cm from the anal verge should be added to the inclusion criteria. Many subjects reported clinical improvement at week 1 and 4. Additional intervention group with a further FMT enema at week 4 to study the effect of repeated FMT intervention is proposed for any future study.

Thirdly, 16S study analysis on faecal samples demonstrated successful engraftment of FMT regardless of clinical outcomes. Faecal samples showed significantly different taxonomic profiles between healthy donors and study subjects. After the pre-FMT bowel optimisation, the faecal microbiota of all study subjects was altered. However, faecal microbiota in subjects who received FMT enemas altered further and resulted in very similar microbiota between subjects, which was significantly different from that of the control group. Furthermore, subjects who received five doses showed even similar microbial community than subjects received only one dose of FMT. This may imply that changes in microbial community is dose dependent. Furthermore, microbial community of subjects with successful clinical remission was similar, which was different from those without clinical remission. Further bioinformatic analysis found that the donors' faecal samples with significant lower relative abundance of *Anaerococcus* and *Coprobacillus* were linked to successful clinical remission ($P = 0.00000872$ and 0.0138 respectively). The link of *Anaerococcus* or *Coprobacillus* with UC were little documented previously, and this requires further studies.

Finally, this study observed a decreased level of IL-10 amongst subjects with severe disease, supporting previously published studies. The correlation between IL-10 and microorganisms can be investigated further in a future study.

As a part of this feasibility study, a narrative review was conducted to evaluate remission endpoints in UC clinical trials using two outcome measures - the Ulcerative Colitis Endoscopic Index of Severity (UCEIS) and Ulcerative Colitis Disease Activity Index (UCDAI). Although the UCEIS is more extensively validated outcome measuring tool than the UCDAI, the UCEIS has been employed in one RCT so far. This review also revealed significant discrepancies in definitions of clinical remission in each RCT, increasing heterogeneity across the studies. It emphasised the importance of universal consensus on clinical remission in UC and a robust protocol for UC clinical trials for the advancement of UC management.

This study proposed a protocol for the multi-centre RCT phase III FMTUC study. The Phase III FMTUC aims to recruit 100 treatment-naïve UC patients. One additional intervention group to observe the effect of repeated FMT at week 4 is also proposed. Clinical remission should be defined at a UCEIS score ≤ 1. Subjective and objective qualitative assessments can be measured with the IBDex and the Mayo score respectively. Endoscopic assessment should be monitored with the UCEIS. Histological assessment does not replace endoscopic assessment, however, it should be used for tissue diagnosis of UC. This phase III study will further study the correlation between IL-10 level and disease severity as well as IL-10 producing microorganisms.

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Chapter 9. Appendix

Appendix 1: Standard Operating Procedure (SOP) for manufacturing FMT products

CONTENTS

1. Amendment Procedure

Controlled document reference	[SOP ref]
Controlled document title	Standard Operating Procedure for Faecal Microbiota Transplant stool preparation and storage

On issue of revised or new pages each controlled document should be updated by the copyholder in the laboratory.

Amendment Number/ Date	Issue no. Discarded	Insert Issue no.	Section(s) involved	Amendment

2. ACKNOWLEDGMENTS

Author: Dr. Robert Porter.

3.Scope of Document

This document covers the collection, preparation and storage of donor faecal samples for storage as frozen, aliquoted faecal slurry for the Faecal Microbiota Transplantation (FMT).

3.1 Purposes of the examination

Clostridium difficile infection (CDI) is a major cause of mortality and morbidity. Clostridium difficile was recorded as the underlying cause of death for 10,258 patients in England and Wales between 2007 and 2011, and a contributing factor in a further 12,687 deaths. Between 2009 and 2011 91% of CDI deaths in England and Wales occurred in NHS hospitals. Most deaths occur in older people with those aged 85 and over having the highest mortality rate (1,099 per million population in 2009-11) (318). Mortality estimates have varied widely, and rates have generally reported to have increased with the widespread circulation of the 027/NAP1/BI strain, with 30 day all-cause mortality of 23-29% in an endemic setting (319–321). Mortality rates in hospitalized CDI patients from Holland, in a non-endemic setting, were recently published, with a 30 day all-cause mortality rate of 13% and a one year mortality rate of 37% (322).

A review of the economic burden of CDI in 2012 reported the cost of a single case of CDI to be between £4,577 ($6,943) and £8,843 ($13,414) at 2010 values (around £5,000 - £10,000 in 2014), with an average hospital length of stay of between 17 and 37 days (323). Patients with multiple recurrent episodes of CDI may cost the local healthcare economy significantly more than this (324,325). Faecal Microbiota Transplantation (FMT) has been shown to be highly cost-effective when compared to standard therapy with an incremental cost-effectiveness ratio of over £10,000 ($17,016) relative to oral vancomycin (326).

Recurrence occurs in around 22% of patients following a first episode of CDI (327,328). FMT has increasingly been reported as an effective treatment for recurrent CDI, with a 2011 systematic review of 317 patients across 27 case series and reports showing an overall cure rate of 92% (196). In 2013 a Dutch group reported the first randomised controlled trial of FMT for recurrent CDI, which showed an overall cure rate of 94% compared to 31% for standard therapy with high dose vancomycin (163).

Preparation of faecal slurry for FMT is time consuming. One of the largest costs for a single FMT is that of screening the donor. Fresh FMTs are time sensitive with donation, preparation, transport and procedure having to take place within a six hour window. This makes provision of fresh FMT within

a region more complex. For this reason, centres in the US have used frozen FMT. By scaling up the process, using a single stool to prepare more than one FMT, and storing faecal slurry at ultra-cold temperatures the cost per FMT to the NHS falls. It is also more practical to send a frozen sample to regional hospitals for local thawing and treatment. Frozen FMT has been reported to be as effective as fresh FMT, with stored aliquots remaining viable for at least 120 days (329,330).

A 2014 survey of UK infection specialists reported that over 90% of respondents would like access to regional guidelines, pre-screened faecal solution and expert advice to facilitate implementation, and more than two thirds of respondents would support a regional FMT referral centre (331).

3.2 Principle and method of the procedure used for examinations

The principle of the procedure is to homogenise stool from healthy donor volunteers, screened for infectious pathogens (see SOP for faecal microbiota transplantation donor screening), and reconstitute it for storing at ultra-cold (-80°C) temperatures.

3.3 Performance characteristics

N/A

3.4 Type of sample

Frozen donor stool.

4. Method

4.1 Patient preparation

Donors should be screened according to protocol for faecal microbiota transplantation donor screening.

4.2 Blood and stool screening tests

General blood testing

- Cytomegalovirus
- Epstein-Barr virus
- Hepatitis A
- HBV
- HCV
- Hepatitis E virus
- Syphilis
- HIV-1 and HIV-2
- Entamoeba histolytica
- Complete blood cell count with differential

- C-reactive protein and erythrocyte sedimentation rate
- Albumin
- Creatinine and electrolytes
- Aminotransferases, bilirubin, gamma-glutamyltransferase, alkaline phosphatase

Blood testing in specific situations

- Human T-lymphotropic virus types I and II antibodies
- Strongyloides stercoralis

General stool testing

- Detection of clostridium difficile
- Detection of enteric pathogens, including salmonella, shigella
- Campylobacter, Escherichia coli O157 H7, Yersinia, Vanocomycin-resistant enterococci, methicillin-resistant Staphylococcus aureus, Gram-negative multidrug-resistant bacteria
- Norovirus
- Antigens and/or acid fast staining for Giardia lamblia and Criptosporidium paryum
- Protozoa (including Blastocystis jominis) and helminths
- Faecal occult blood testing

Stool testing in specific situations

- Detectopm of Vibrio cholera and Listeria monocytogenes
- Antigens and/or acid fast staining for Isospora and Microsporidia
- Calprotectin
- Helicobacter pylori faecal antigen
- Rotavirus

4.3 Type of container and additives

Donors are provided with a food-safe new, clean, non-sterile, disposable plastic food storage box [http://www.sainsburys.co.uk/shop/gb/groceries/foil-food-bags-storage/sainsburys-plastic-containers-x8].

4.4 Required equipment and reagents

- Workspace with a fume hood
- Virusolve spray
- Personal protective equipment
- Wooden/Plastic toothpicks/sticks
- Empty food-safe storage box

- Pen and paper
- Baxter 0.9% normal saline 1000ml preservative free
- Disposable 800ml tripoint plastic beaker
- 10ml pipette with tips
- 50ml Falcon centrifuge tubes
- A bucket
- Tube rack
- Centrifuge
- Vortexer
- Sharpie marker pen
- Molecular grade glycerol
- Portable balance
- Dedicated ultra-cold freezer
- Plastic sieves
- Virkon
- Milli Q water

4.5 Environmental and safety controls

Preparation of samples is carried out in a laboratory isolated room with a fume hood within category 2 environment. Before and after use all internal cabinet surfaces must be decontaminated by removing visible particles and matter and then applying Virusolve. This must remain in contact with the surface for 1 minute before any excess is wiped away with disposable paper towels.

The workspace cabinet is located in a category 2 laboratory, and any items leaving this laboratory should be externally cleansed with Virusolve.

4.6 Calibration procedures (metrological traceability)

Balance and pipettes to be serviced and calibrated in accordance with manufacturer's recommendations.

The freezer will be maintained and serviced according to manufacturer's recommendations and will be monitored with a remote alarm system to ensure adequate maintenance of desired temperature.

4.7 Procedural steps

25. Donor sample voided by volunteer and time of voiding recorded on food safe box by way of pre-attached label.
26. Sample stored at 2-5°C until ready for preparation.
27. Sample should be processed within a maximum of 6 hours from receipt

28. Don new personal protective equipment (water resistant gown and gloves)
29. Soak a disposal plastic sieve into a Virkon bath in a bucket for 30 minutes
30. Rinse the sieve with Milli Q water thoroughly for approximately 10 minutes
31. Clear and clean work space within the fume hood as above
32. Within the fume hood weigh 1g of stool into a clean 50ml Falcon tube. Label tube with donor number and date. This should be stored at 2-5°C for pooled testing as described in SOP faecal microbiota transplant donor screening.
33. Weigh the remaining stool sample – zero balance with empty food safe box, then place box containing voided sample on balance
34. Weigh 50g of stool sample
35. The remaining will be discarded
36. Time of voiding and time of processing noted to be recorded in FMT database
37. Weigh 500ml of 0.9% saline into a clean tripoint beaker
38. Using wooden/plastic stick to help, place donor stool into the tripoint beaker with 500ml of 0.9% saline
39. Pour this into the sieve
40. Retaining contents of central mesh compartment, pour the filtrate into the tripoint beaker
41. Using a 10ml pipette divide the filtrate into 50ml aliquots in 50ml Falcon tubes
42. Centrifuge the aliquots at 1772RPM (Rotor IEC 236, 50ml inserts) for 60 mins
43. Discard supernatant and re-suspend pellet in 10% molecular grade glycerol/90% saline.
44. Label sides and lids with the donor number and date and a sequential number for each tube processed. The donor number takes the form of YEAR_XX where YEAR represents the year the donor began donating and XX is a sequential number for each new donor in that year. The date takes the form DDMMYY e.g. DonorNo_DDMMYY_SEQNo., 2015_03_040615_01, 2015_03_040615_02
45. An expiry date should be clearly marked on each tube. This is for 3 months beyond the processing date e.g. if processed on 04/06/2015 the expiry would be 04/09/2015
46. The tubes can now be frozen at -80°C
47. Dispose of the waste into an autoclave bag and place into an autoclave bin
48. Record the data for each donor aliquot onto the FMT database

5. Quality

5.1 Quality control procedures

Every donated sample will undergo testing as described under the Methods section in the SOP for faecal microbiota transplantation donor screening. Data will be entered into the FMT database and each aliquot will be labelled with the donor number, date of donation and an expiry date (see FMT stool preparation SOP). This will give traceability of all samples from donation to delivery and link quality control data.

Potential donors who fail to provide satisfactory screening results will have their donations destroyed. Freezer records will document fluctuations in storage temperatures or errors should they occur.

5.2 Interferences (e.g. lipaemia, drugs) and cross reactions

N/A

6. Results reporting

6.1 Principle of procedure for calculating results including, where relevant, the measurement uncertainty of measured quantity

The balance used should have an accuracy of +/- 0.1g

6.2 Biological reference intervals or clinical decision values

N/A

6.3 Reportable interval of examination results

Samples will be destroyed by autoclaving after 3 months.

6.4 Instructions for determining quantitative results when a result is not within the measurement interval

N/A

6.5 Alert/critical values

Freezer failure.

6.6 Laboratory clinical interpretation

N/A

6.7 Potential sources of variation

N/A.

Appendix 2: Mayo Severity Index

MAYO Severity Index	
	SubTotal
Stool Frequency[a] (Total number of stools / day) (3-day average[b]) **0** = Normal number of stools for this patient **1** = 1 – 2 stools/ day more than normal for this patient **2** = 3 – 4 stools / day more than normal for this patient **3** = ≥5 stools/ day more than normal for this patient	= ____
Rectal Bleeding[c] (3-day average[b]) **0** = No blood seen **1** = Streaks of blood with <50% of stools **2** = Obvious blood seen with ≥50% of stools **3** = Blood alone passed	= ____
Physician's Global Assessment (PGA)[d] **0** = Normal **1** = Mild disease **2** = Moderate disease **3** = Severe disease	= ____
Finding of Flexible Proctosigmoidoscopy **0** = Normal or inactive disease **1** = Mild disease (erythema, decreased vascular pattern, mild friability) **2** = Moderate disease (marked erythema, absent vascular pattern, friability, erosions) **3** = Severe disease (spontaneous bleeding, ulceration)	= ____
Total MAYO Score →	= ____

Appendix 3: IBDeX Scoring System

Please write the appropriate answer regarding the patient's condition in the last three days.

	Items	Responses
1.	Stool frequency	In the last 3 days, on average how many times did the patient open his/her bowels every day?
2.	Stool consistency	0. Formed 1. Semi-formed 2. Mixed liquid and solid (a mushy stool) 3. Entirely liquid
3.	Well being or energy level in the last 3 days	0. Well 1. Slightly Poorly 2. Very poorly 3. Extremely poorly/bed bound
4.	Performance status	0. No restriction of daily activities 1. Some restriction 2. Moderate restriction 3. Severe restriction
5.	Blood in the stool	0. Absent 1. Trace/small amount of blood 2. Occasional frank bleeding 3. Always frank bleeding
6.	Nocturnal diarrhoea	In the last 3 days, on average how many times did the patient have to wake up to go to the toilet per night?
7.	Abdominal pain or discomfort on a score of 0 to 10 On average for the last 3 days.	Pain score represent the points
8.	Urgency of defecation	0. None 1. Present, but patient can wait to get to toilet. 2. Present, patient needs to stop what he/she is doing and rush to toilet 3. Patient can't wait (had few incontinence accidents)
9.	Unintentional Weight loss in the last month	0. No weight loss 1. Weight loss
10.	Abdominal mass	0. None or not applicable 1. Doubtful/unsure 2. Definitely present
11.	Abdominal tenderness	0. None 1. Mild 2. Marked/moderate 3. Rebound/severe
12.	Pulse	0. Normal 1. Tachycardia
13.	Temperature	0. Normal 1. Pyrexia
14.	The presence of extra-intestinal manifestations on examination.	1 point for each: Arthralgia, arthritis, Uveitis, Erythema nodosum, Pyoderma gangrenosum, Aphthus ulcer, new perianal complication (fissure, fistula, abscess, anal stenosis, perianal ulcer)

Appendix 4: Crohn's and Ulcerative Colitis Questionnaire (CUCQ-32)

The following questions ask for your views about your bowel problem and how it has affected your life over the **last two weeks**.
The terms bowel problem or bowel condition refer to all aspects of your bowel illness and its related treatments. If you have had some bowel surgery you may wish to answer questions 1, 2, 6, 9, 24 and 26 using the "not applicable" response.
Please answer **all the questions**. If you are unsure about how to answer any question, just give the best answer you can. Do not spend too much time answering, as your first thoughts are likely to be the most accurate.

1. On how many days over the last **two weeks** have you had loose or runny bowel movements?

   ☐ days ☐ *Not Applicable*

2. On how many days in the last **two weeks** have you noticed blood in your stools?

   ☐ days ☐ *Not Applicable*

3. On how many days over the last **two weeks** have you felt tired?

   ☐ days

4. In the last **two weeks** have you felt frustrated?

   a) No, not at all
   b) Yes, some of the time
   c) Yes, most of the time
   d) Yes, all of the time

5. In the last **two weeks**, has your bowel condition prevented you from carrying out your work or other normal activities?

   a) No, not at all
   b) Yes, some of the time
   c) Yes, most of the time
   d) Yes, all of the time

6. On how many days over the last **two weeks** have you opened your bowels more than three times a day?

   ☐ days ☐ *Not Applicable*

7. On how many days over the last **two weeks** have you felt full of energy?

   ☐ days

8. In the last **two weeks** did your bowel condition prevent you from going out socially?

   a) No, not at all
   b) Yes, some of the time
   c) Yes, most of the time
   d) Yes, all of the time

9. On how many days over the last **two weeks** have your bowels opened accidentally?

☐ days ☐ *Not Applicable*

10. On how many days over the last **two weeks** have you felt generally unwell?

☐ days

11. In the last **two weeks** have you felt the need to keep close to a toilet?

a) No, not at all
b) Yes, some of the time
c) Yes, most of the time
d) Yes, all of the time

12. In the last **two weeks**, has your <u>bowel condition</u> affected your leisure or sports activities?

a) No, not at all
b) Yes, some of the time
c) Yes, most of the time
d) Yes, all of the time

13. On how many days over the last **two weeks** have you felt pain in your abdomen?

☐ days

14. On how many nights over the last **two weeks** have you been unable to sleep well (days if you are a shift worker)?

☐ nights (or days)

15. On how many nights in the last **two weeks** have you had to get up to use the toilet <u>because of your bowel condition</u> after you have gone to bed?

☐ nights

16. In the last **two weeks** have you felt depressed?

a) No, not at all
b) Yes, some of the time
c) Yes, most of the time
d) Yes, all of the time

17. In the last **two weeks** have you had to avoid attending events where there was no toilet close at hand?

a) No, not at all
b) Yes, some of the time
c) Yes, most of the time
d) Yes, all of the time

18. On how many days over the last **two weeks**, have you had a problem with large amounts of wind?

☐ days

19. On how many days over the last **two weeks** have you felt off your food?

☐ days

20. Many patients with bowel problems have worries about their illness. How often during the last **two weeks** have you felt worried?

a) No, not at all
b) Yes, some of the time
c) Yes, most of the time
d) Yes, all of the time

21. On how many days over the last **two weeks** has your abdomen felt bloated?

☐ days

22. In the last **two weeks** have you felt relaxed?

a) No, not at all
b) Yes, some of the time
c) Yes, most of the time
d) Yes, all of the time

23. In the last **two weeks** have you been embarrassed by your bowel problem?

a) No, not at all
b) Yes, some of the time
c) Yes, most of the time
d) Yes, all of the time

24. On how many days over the last **two weeks** have you wanted to go back to the toilet immediately after you thought you had emptied your bowels?

☐ days ☐ *Not Applicable*

25. In the last **two weeks** have you felt upset?

a) No, not at all
b) Yes, some of the time
c) Yes, most of the time
d) Yes, all of the time

26. On how many days over the last **two weeks** have you had to rush to the toilet?

☐ days ☐ *Not Applicable*

27. In the last **two weeks** have you felt angry as a result of your bowel problem?

a) No, not at all
b) Yes, some of the time

c) Yes, most of the time
d) Yes, all of the time

28. In the last **two weeks**, has your sex life been affected by your bowel problem?

a) No, not at all
b) Yes, some of the time
c) Yes, most of the time
d) Yes, all of the time

29. On how many days over the last **two weeks** have you felt sick?

[ ] days

30. In the last **two weeks** have you felt irritable?

a) No, not at all
b) Yes, some of the time
c) Yes, most of the time
d) Yes, all of the time

31. In the last **two weeks** have you felt lack of sympathy from others?

a) No, not at all
b) Yes, some of the time
c) Yes, most of the time
d) Yes, all of the time

32. In the last **two weeks** have you felt happy?

a) No, not at all
b) Yes, some of the time
c) Yes, most of the time
d) Yes, all of the time

Appendix 5 : Remission endpoints in ulcerative colitis : A systematic review

SYSTEMATIC REVIEWS

Remission endpoints in ulcerative colitis: A systematic review

Maki Jitsumura, Rory Frederick Kokelaar, Dean Anthony Harris
Published online: August 26, 2017

Abstract
AIM
To summarize the current consensus on the definition of remission and the endpoints employed in clinical trials.

METHODS
A bibliogragraphic search was performed from 1946 to 2016 sing online databases (National Library of Medicine's PubMed Central Medline, OVID SP MEDLINE, OVID EMBASE, the Cochrane Library and Conference Abstracts) with key words: ("ulcerative colitis") AND ("ulcerative colitis endoscopic index of severity" OR "UCEIS") AND ("remission") as well as ("ulcerative colitis") AND ("ulcerative colitis disease activity index") OR "UCDAI" OR "UC disease activity index" OR "Sutherland index") AND ("remission").

RESULTS
The search returned 37 and 116 articles for the UCEIS and UCDAI respectively. For the UCEIS, 12 articles were cited in the final analysis of which 9 validation studies have been identified. Despite the UCEIS has been more extensively validated in all three aspects (validity, responsiveness and reliability), it has been little employed to monitor disease in randomised clinical trials. For the UCDAI, 37 articles were considered for the final analysis. Although the UCDAI is only partially validated, 29 randomised clinical trials were acknowledged to use the UCDAI to determine endpoints and disease remission, though no clear protocol was identified.

CONCLUSION
Although the UCEIS has been more widely validated than the UCDAI, it has not been reflected in the monitoring of disease activity in clinical trials. Conversely, the UCDAI has been used in numerous large clinical trials to define their endpoints and disease remission, however, it is challenging to determine the best possible outcomes due to a lack of homogeneity of the clinical trial protocols. Before determining a gold standard index, international agreement on remission is urgently needed to advance patient care.

Key words: Ulcerative colitis; Remission; Ulcerative colitis endoscopic index of severity; Ulcerative disease activity index

Jitsumura M, Kokelaar RF, Harris DA. Remission endpoints in ulcerative colitis: A systematic review. *World J Meta-Anal* 2017; 5(4): 85-102 Available from: URL: http://www.wjgnet.com/2308-3840/full/v5/i4/85.htm DOI: http://dx.doi.org/10.13105/wjma.v5.i4.85

Core tip: Despite the decades of discussion, disease remission for ulcerative colitis has yet to be fully defined. Instead, numerous indices that measure a large variety of endpoints had been developed, each claiming to be accurate and informative. This systematic review aimed to summarise the issues related to the uncertain definition of disease remissions in clinical trial studies by focusing on two indices ulcerative colitis endoscopic index of severity and ulcerative disease activity index. We recommend that an international consensus of remission should be sought before establishing a gold standard outcome measurement to untangle this confusion.

INTRODUCTION

How do we determine remission as an endpoint in ulcerative colitis (UC) clinical trials when we design a study? There is no universally agreed definition of remission as an endpoint in UC clinical trials as of date, despite much discussion and urge for

the standardisation. Currently, it is chosen to reflect the purpose of the studies, rather than long-term clinical outcomes or controlling bothersome symptoms that patients often suffer from. Furthermore, a lack of homogeneity of the clinical trial protocols makes comparison of such studies more difficult to comprehend.

UC is a chronic relapsing-remitting inflammatory bowel disease, affecting mucosa of the large bowel. Patients with UC often present with debilitating symptoms such as abdominal pain and rectal bleeding. Although the aetiology of UC is believed to be multifactorial involving dysregulated immune system, intestinal mucosal disturbance and genetic predisposition, natural history of the disease is poorly understood[1]. There is no curative treatment at present, thus the aim of management is induction and maintenance of remission with immunosuppressive agents, permitting individuals to carry on their daily life. The failure of medical therapy or refractory disease often require colorectal surgery, and there is an increased risk of colorectal cancer[2].

Since the first disease activity outcome measurement was developed in 1955, the Truelove and Witts Index, numerous outcome measure instruments have been developed[3]. Not only has the number of these instruments been growing, but also the assessed disease components have been expanding. Traditionally, disease activity has been assessed by a clinical and symptom scoring system, with or without a combined endoscopic assessment. A recent review counted seventeen clinical disease activity indices which evaluate symptoms, of which eight do so without endoscopic or biomarker assessment[4]. The purpose of these disease activity indices is to provide an objective measurement of the disease activity by employing typical symptoms such as stool frequency and rectal bleeding. Endoscopic assessment is another dimension of the disease that is mandated by the Food and Drug Administration (FDA)[5] and at least thirty-one instruments were proposed[6]. Many of these endoscopic indices, such as Mayo score and ulcerative colitis disease activity index (UCDAI), evaluate the macroscopic appearance of large bowel, together with symptomatic disease activity. Recently, the prognostic potential of histological assessment in UC has been highlighted in several studies[7-9], although histological remission has yet to be proposed as a therapeutic endpoint for clinical trials or practice, twenty-six histological activity indices have been developed thus far. It is important to note that there is considerable disparity between visual endoscopic assessment and histological disease activity[9], although confusingly these terms are used interchangeably[9]. In addition to symptomatology, endoscopic, and histological scoring systems, radiological outcome instruments as well as new biochemical markers are an additional developing dimension of disease assessment[4].

In addition to these objective indices, we cannot neglect patient subjective outcome measurement tools and quality of life (QoL) questionnaires. The aim of these tools is to evaluate the patients' emotional, social or professional well-being so that their ideas, concerns, and expectations can be a part of the objective medical decision-making process. Patients often have different expectations of treatment and remission from those of physicians, and symptoms used in established scoring tools may be of relatively little concerns to some individuals. Establishing an understanding of chronicity is also important in assessing a patient's disease, especially when reconciling the long term treatment goals with a patients' concerns regarding how quickly embarrassing, troublesome and physical symptoms can be resolved with minimal side effects[10]. Furthermore, we cannot underestimate the power of the internet and smartphone use in medicine; many patients often seek online diagnosis of their symptoms before they are formally assessed by a clinician, and may already be either well informed or misguided when discussing management. Patients often use "remission" and "flare-ups" informally to describe their disease activity without reference to formal assessments of such, and thus misunderstandings may occur when discussing assessment and treatment. A few self-reporting assessment tools (smartphone apps) are available, allowing patients to monitor their disease activity on daily basis in a more objective manner[11]. Whether these patient-reported measurement instruments show a good correlation with true disease activity by other measures appears to be almost irrelevant. Many patients with asymptomatic UC do not feel the need of continuing medications in the absence of discernible symptoms, especially when they give side effects, making the negotiation more challenging for clinicians. A good rapport and the ability to reach negotiated consensus with patients is an integral skill for clinicians managing complex UC patients.

The overall picture is that there are numerous indices that measure a large variety of endpoints, each claiming to be accurate and informative regarding one or other management goal. Confusingly, these indices share similar names or are often referred to by multiple names or abbreviations, such as the UCDAI which is also referred to as the Sutherland Index. Unfortunately, no single scoring system provides comprehensive assessment of disease activity, and the majority of these indices lack robust clinical validation. Most clinical trials, from which the scoring systems are derived, choose disease outcome measures and endpoints reflecting the purpose of the studies, rather than long-term clinical outcomes or real-world symptom control for patients. Furthermore, each clinical trial defines remission differently, making comparison between different trials difficult.

The consequence of the complexity in UC outcome measurements and the huge variety in competing scoring systems is that many patients with UC may receive suboptimal therapy and poor long-term disease control.

This systematic review will reassess and summarise the current consensus on the definition of remission and the endpoints employed in clinical trials by focusing on two most validated and well-used indices, the ulcerative colitis

endoscopic index of severity (UCEIS) and UCDAI, in order to address the issues with the standardisation of clinical trial protocols.

The current target of disease remission endoscopically is mucosal healing although it has not been fully validated or no standardised definition of mucosal healing[12,13]. Yet, this appears to be the goal for many clinical practice as well as drug trials.

The recent draft guideline released by the FDA[5] states the ideal primary efficacy assessment instrument in clinical trials should consist of (1) a signs and symptoms assessment scale - best measured by a patient-reported outcome instrument. If not, an observer-reported outcome instrument; and (2) an endoscopic and histological assessment scale.

Thus, endoscopic assessment tools with comprehensive clinical symptom assessment components that come from patients would be a reasonable choice to argue remission and endpoints employed in clinical trials.

Amongst numerous endoscopic indices claiming to measure disease activity, the UCEIS is one of the most widely validated indices to date. It would be interesting to see any impact of the quality of validation for defining remission and endpoints compared with the index, such as the UCDAI, that has not been fully validated yet being widely employed in clinical trials. For these reasons, these two indices were chosen for this systematic review.

UCEIS

The UCEIS proposed by Travis *et al*[14] in 2012 is the only validated endoscopic index in ulcerative colitis to date[15]. It was developed to minimise variation in endoscopic assessment, thus it could be widely applied as a reliable outcome measure in clinical trials as well as clinical settings.

The first stage of development of the UCEIS demonstrated the significant inconsistency in endoscopic assessment amongst specialists by 10 specialists scoring the severity of UC using the Baron score[16] in colonoscopy videos. The greatest correlation was found in the "severe" level of the Baron score, demonstrating a 76% agreement, however, only 27% agreement was achieved for a normal mucosa (Baron score 0) and 37% agreement for moderate friability (Baron score 2).

The second part of the study further quantifies intra- and inter-observer variation on common descriptors on endoscopic assessments (Table 1). For intra-observer variation, 60 repeat pair assessment of 36 different videos were scored and assessed by statistics. For inter-observer variation, 30 new investigators were randomly allocated to score 25 videos, thus each video was assessed by 10-12 investigators.

Both intra- and inter-observer variation showed good agreement to assess erosions and ulcers, vascular pattern and bleeding, which were subsequently chosen for descriptors of a newly developed endoscopic assessment tool, the UCEIS (Table 2).

The authors also proposes definition of remission using the UCEIS, which is when all three descriptors were level 1 (no visible bleeding or erosions or ulceration, but some blurring or loss of capillary margins with a recognisable vascular pattern is allowed).

UCDAI

The UCDAI (also called UC Disease Activity Index, and Sutherland Index) was introduced by Sutherland *et al*[17] to assess efficacy of 5-aminosalicylic acid enema in the treatment of distal UC in its randomized, double-blind clinical trial in 1987.

The index was used for objective assessment during this drug trial and considers four variables of UC - stool frequency, rectal bleeding, mucosal appearance and physician's rating of disease activity (Table 3). Unlike the UCEIS, the UCDAI was developed without any validated study. Although the authors described that the index incorporates many of the subscales used by other investigators and demonstrated efficacy as an overall index and individual component subscale, they failed to demonstrate this with any form of statistical assessment. Furthermore, they also compared between the overall index and the physician's global assessment by this drug trial study physician and concluded that the UCDAI demonstrates good correlation with the physician's assessment ($P = 0.0001$). This conclusion fails to demonstrate objectivity although the authors' fundamental aim of designing this index was to provide objective assessment.

The UCEIS was developed based on components to minimise the variation identified in previous endoscopic assessment instruments. It has been validated from various angles at the time of designing, making it more reliable than traditional instruments. Conversely, the UCDAI was designed without any validated evidence to assess efficacy of a drug for treatment of UC. Yet, it has been widely used in numerous clinical trials for decades and even recommended by the FDA as one of the endoscopic assessment tools.

MATERIALS AND METHODS

A systematic bibliographic search was performed between 10th and 14th November 2016 of the following online databases: OVID SP MEDLINE (1946 to present), OVID EMBASE (1974 to present), National Library of Medicine's PubMed Central MEDLINE (1950 to present), the Cochrane Library, using the key heading-words strategy set below and the medial subject

heading. The bibliographies of recovered systematic review, meta-analysis, and review articles were also searched for additional articles.

Each database was searched for the following headings: (1) Ulcerative colitis endoscopic index of severity: ("ulcerative colitis") AND ("ulcerative colitis endoscopic index of severity" OR "UCEIS") AND ("remission"); and (2) Ulcerative Colitis Disease Activity Index: ("ulcerative colitis") AND ("Ulcerative colitis disease activity index" OR "UC disease activity index" OR "UCDAI" OR "Sutherland index") AND ("remission")

Non-English articles, studies pertaining to paediatric subjects, and non-human subjects were excluded. Studies presenting data of patient populations already included in other publication (duplicates) were excluded. No abstract publications without subsequent full-text published data were used. Disagreements about inclusion were resolved in a consensus meeting.

RESULTS

Ulcerative colitis endoscopic index of severity

A total of 37 articles were returned using the initial search. After applying exclusion criteria and eliminating duplication, 12 articles screened for relevance and manual search of articles referenced in the retrieved articles was performed. Nine articles were included in the final analysis for validation assessment of UCEIS and 3 articles were evaluated for the UCEIS use in clinical trials (Figure 1).

UCDAI

A total of 116 articles were returned using the initial search, which was down to 37 articles after considering exclusion criteria and duplication. Four articles were identified for the final analysis of validation assessment and 29 articles were included to evaluate defining remission and endpoints of clinical trials (Figure 2).

What do we need in outcome measurement instruments?

The definition of disease remission has not yet been validated or standardised. Inevitably, implementing clinical scoring tools based on a broad definition of remission results in inaccuracy of outcomes, and a reduction in the utility of a derived tool. The gold standard for disease activity in UC must be a diagnostic tool that truly quantifies the disease activity and can accurately assess and therefore guide future disease managements and outcome. A robust and standardised outcome measurement instrument is vital for clinical trials and establishment of medical therapy, although many instruments are not fully validated.

In this systematic review, validation of UCEIS and UCDAI studies were described by dividing into validity, reproducibility and responsiveness (Table 4).

Validity: The diagnostic and prognostic validity of an assessment tool is defined as evidence that variations in UC disease activity causally produce variations in the measurement outcomes. This must be demonstrated by qualitative assessment and evidence of indices measuring disease activity adequately and sufficient reflection of true disease. The development of these indices should be supported by a robust systematic review of literature. Statistical studies of agreement between the indices and disease activity should be assessed including sensitivity and specificity. Validity of the correlation between an index score and objective assessment score including clinical disease activity index scores or
physician global assessment of severity should be measured. Although there are many indices have been proposed the degree of validity for these indices vary, and many indices are not fully validated. In this study, UCEIS and UCDAI, one of the best validated indices and most widely used indices in drug trials respectively, were studied for their evidence of validity.

The UCEIS is one of the well validated indices in many aspects. The authors have studied difficulties in standardisation of the disease activity indices and defining remission in systematic reviews as well as reviews of literature prior to the development of the UCEIS[29,30]. The authors attempted to develop an index that minimises this variation by validating variation in endoscopic assessment of disease activity, which was described in 2.1. The study also suggested remission might be defined as no obliteration of vascular pattern, no rectal bleeding and no erosion or ulceration, although this has not been fully validated.

Since the UCEIS was published in 2012, there are nine studies attempted to validate the UCEIS, of which four studies are focusing on validity.

Corte *et al*[18] validated whether the UCEIS predicts clinical outcomes of acute severe colitis. 98 Patients with the UCEIS score from 3 to 8 were included in this study. It showed when UCEIS $\geq$ 5, 33% (18/54) of acute colitis patients required colectomy 18/54 (33%) whereas only 9% (3/33) of patients with UCEIS $\leq$ 4 required surgical interventions. When the UCEIS score is above 7 at the time of admission, almost all patients required medical therapy more than hydrocortisone, such as infliximab or ciclosporin. It concluded that the higher UCEIS score is associated with higher requirement of rescue therapy, surgical intervention and readmission.

Fernandes *et al*[19] identified patients with poor response to optimal therapy with 108 patients who are defined as acute severe colitis based on the Truelove and Witts criteria (the score $\geq$ 2). All the patients received intravenous prednisolone

40-60 mg/d, methylprednisolone 60 mg or hydrocortisone 400 mg/d. Patients who had not responded to the initial therapy within 3 d received salvage therapy, and their UCEIS scores ranged from 2 to 8. The study also divided the UCEIS scoring system to segmental bowel - rectum and sigmoid, which demonstrated a strong correlation between higher UCEIS score and unfavourable outcomes especially the UCEIS-segmental score predicted refractoriness to steroid therapy. The UCEIS was significantly better at predicting clinical outcomes than the Mayo endoscopic sub-score.

Arai *et al*[20] attempted to foresee the prognosis of patients with UC who are in clinical remission. 285 patients who are in clinical remission (partial Mayo score of ≤ 1) were included in the study. The UCEIS score of these patients with clinical remission ranged from 0 to 5, of which 92% received a UCEIS score of 2 or 3. These scores are higher than a suggested score for clinical remission. The study demonstrated the recurrence risk is direct proportional to the UCEIS score - the recurrence rate of 5.0% for UCEIS = 0, 22.4% for UCEIS = 1, 27.0% for UCEIS = 2, 35.7% for UCEIS = 3, 75% for UCEIS = 4-5. The study also highlighted the absence of bleeding and mucosal damage being independent factors for clinical remission. The duration of recurrence was also significantly prolonged in patients with lower UCEIS score. The study presented validity of the UCEIS with its predictability of clinical outcomes. Furthermore, it suggests UCEIS ≤ 1 for clinical remission based on the direct correlation between the recurrence rate and the UCEIS, which showed sensitivity of 68% and specificity 57%.

Kucharski *et al*[21] assessed correlations between 9 endoscopic indices and 11 clinical activity indices. The author also assessed correlations between those endoscopic indices and the histological Geboes index[22]. Nine endoscopic indices used are Baron score[16], Powell-Tuck Score[31], Schroeder Score[32], UCDAI, Rachmilewitz Endoscopic Index[33], LÖtberg Score[34], Lemann Endoscopic Index[35], Feagan Score[36] and UCEIS. Eleven clinical activity indices are Truelove and Witts Severity Index[3], Powell-Tuck Index[31], Schroeder Score[32], UCDAI, Rachmilewitz Index[33], Lichtiger Index[37], Seo Score[38], Walmsley Index[39], Improvement Based on Individual Symptom Scores (IBOISS)[40], Feagan Score[36] and Montreal Classification of Severity of Ulcerative Colitis[41].

The correlations between clinical and endoscopic indices were evaluated using Spearman's ranking correlation coefficient. The Rachmilewitz Index showed strong correlations with 5 clinical activity indices (UCDAI, Truelove and Witts, Schroeder Score, IBOISS and Feagan Index) with the correlation coefficient ranging in 0.710-0.788. The UCEIS also showed high correlations with the UCDAI, Schroeder Score, IBOISS and Feagan Index, the coefficient ranging from 0.722 to 0.761. When the correlations between clinical indices and the Geboes Index were assessed, all clinical indices showed low correlations, whereas all endoscopic indices showed better correlations with the histological Geboes Index. To evaluate correlations with endoscopic indices, all endoscopic indices were scored at four colonic segments right colon, transverse colon, left colon and rectum. The highest correlations were seen with the UCEIS at all four segments (the coefficient ranging from 0.434 to 0.629). The authors conclude that the UCEIS is the most effective endoscopic outcome measure instrument when considering correlations of both clinical and histological indices. In contrast, the UCDAI showed moderate correlations with rectal and transverse colonic segment with the Geboes Index with 0.651 and 0.534 respectively, though the correlations with other two segments were low with 0.428 for left colon and 0.459 for right colon.

Although the UCDAI has been widely used especially in multiple and large clinical trials, the study focused on validation of this index is much less compared to the UCEIS. The UCDAI was developed to assess the efficacy and safety of 5-aminosalicylic acid enema use for patients with UC[17]. The UCDAI claim to assess disease activity from four descriptors - stool frequency, rectal bleeding, mucosal appearance and physician's global assessment of the disease. Although the description of each scoring system is simple to understand, it cannot avoid subjectivity without clear definition of each item. In particular, physician's global assessment is far from being objective. Furthermore, the supposedly objective endoscopic assessment is scored based on severity of "friability". Yet again, this friability without clear definition cannot avoid subjectivity, meaning it is exposed to greater inter- and intra-observer variability.

Higgins *et al*[27] defined objective end points in disease activity indices including UCDAI for remission and improvement in UC. This study was conducted on 66 patients with UC and their subjective dichotomous assessment of remission and regulatory remission were compared with the UCDAI. Regulatory remission was defined as (1) no more than grade I or II changes on a Feagan endoscopic score; and (2) absence of visible rectal bleeding in this study. It suggests the cut off point for clinical remission of the UCDAI is below 2.5, offering good statistical power - sensitivity and specificity is 0.82 and 0.89 for patient defined remission and 0.92 and 0.93 for regulatory remission. Patient-defined dichotomous end points may be over-simplification, however, as it is clinically significant outcomes that determines if therapies are perceived as beneficial by patients. Regardless of physicians' objective assessment, patients with the disease are those that must agree with it in order to gain benefit in receiving therapies.

Poole *et al*[28] designed a new patient-reported disease assessment instrument, EuroQoI Five Dimensions Questionnaire (EQ-5D), for which the UCDAI was used to validate the instrument. Although validation of the UCDAI was not the aim of this study, the correlation between physician-rated and patient-rated instruments was elaborated. The study concluded that the abbreviated UCDAI (without endoscopic assessment component) and EQ-5D showed reasonable consistency when severity of the disease was measured in two randomised studies (PINCE[42] and PODIUM[43]). Goodness of fit was verified by the

mean square error for mean predicted utility score. This showed patients in remission was 0.939, 0.944 and 0.940 mean utility units for estimated-PINCE, observed-PINCE and PODIUM.

Comparison of these two very different outcome measure instruments highlighted two incomparable benefits when they are chosen for clinical trials. The UCEIS is extensively validated and development of the index is based on robust studies, whereas the UCDAI is deigned based on expert opinion on the disease. However, the UCDAI is more widely used in clinical trials. This makes the choice of an index for future clinical trial studies more difficult when the clinical benefit was considered. In this systematic review, only two indices are compared. With current inconsistent use of measurement instruments and non-standardised definition of remission, the choice is almost impossible.

Responsiveness: Responsiveness is assessed in this systematic review as the ability to detect changes after a treatment that has known efficacy.

Ikeya *et al*[23] investigated true evaluation of UC severity and outcome after Tacrolimus remission induction therapy using the UCEIS as well as Mayo endoscopic subscore (Mayo ES) with 41 patients who are known to have moderate to severe disease.

In this study, clinical remission was defined as clinical activity index (CAI) ≤ 4 and a reduction of CAI score more than 4 was defined as clinical response. On the contrary, an increase of CAI score more than 4 was defined as relapse.

After 12 wk from the treatment, 31 patients (75.6%) successfully achieved clinical remission [defined as clinical activity index (CAI) ≤ 4] and 3 patients did not respond. Overall the UCEIS and Mayo ES showed close correlations, however, when the Mayo ES was 3, there was prominent discrepancy between the two indices. The UCEIS score equivalent to Mayo ES 3 ranged from 5 to 8 pre-treatments and 3 to 7 post-treatments. This was believed to be due to a lack of ability to distinguish characteristics of ulcers, vascular patterns or bleeding with the Mayo ES. For instance, ulcers and erosions often become smaller and shallower in the early phases of mucosal healing. Since the Mayo ES does not distinguish the size and depth of ulcers, it tends to stay with the same score, meaning the Mayo ES score is 3 for all types of ulcers. Furthermore, the Mayo ES combine all those macroscopic findings of ulcers, vascular pattern and bleeding into four different overall grades. This means if there are ulcerations of any shape, the Mayo ES score becomes 3, even if vascular pattern disturbance is resolved.

The study also demonstrated significantly better relapse-free and colectomy-free rates when the UCEIS score was improved by more than 3. In addition, improvement by a UCEIS score of more than 3 was strongly associated with achieving clinical remission group (23 out of 41 patients).

Menasci *et al*[24] evaluated to see whether the global score of the sum of 5 colonic segments (rectum, sigmoid, descending, transverse, and ascending colon), abbreviated as tU score, would alter the outcome score when it is compared with the regular method of UCEIS scoring, which is to score the most inflamed colonic segment. The two scores showed a good correlation with Spearman's $r = 0.86$ and P value less than 0.0001 for less severe disease UCEIS ≤ 5. However, correlation is substantially decreased for severe disease (UCEIS > 5) with Spearman's $r = 0.48$ and $P < 0.01$. Moreover, when these two scoring methods were applied to assess patients with a flare-up at 1 year, *tU* score was more sensitive than the regular UCEIS score with area under ROC curve = 0.688 ± 0.06 *vs* 0.60 ± 0.07 and $P < 0.01$. The tU score was also significantly higher when patients with and without a flare-up at 1 year were assessed, whereas the regular UCEIS score did not differ (25.3 ± 8.2 *vs* 20.1 ± 6, $P < 0.005$). This concluded that the evaluation of disease by full colonoscopy with multiple segments may provide the more accurate method to evaluate disease activity.

Overall, the study concluded that the UCEIS confirmed better responsiveness than the UCDAI, and it is superior to describe accurate endoscopic findings in patients with severe UC. This responsiveness can be crucial in clinical trials since duration of primary endpoints in many clinical trials is approximately 12 wk[44]. Thus, indices that allow to capture small but vital improvement that reflects on disease outcome is essential to clinical trials.

Reliability: Despite mucosal healing becoming the goal for management for UC, the most critical limitation of endoscopic assessment is its inherent intra- and inter-observer variations[6,45-47]. Reliability is evaluated with inter- and intra-observer reliability as well as internal consistency. The leading author of the UCEIS led another study to investigate reliability in different aspects.

The first study published in 2013 investigated intra- and inter-observation reliability[15]. Twenty-five readers from 14 countries were recruited in this study, who evaluated 28 videos. To quantify intra-observer reliability, 4 duplicated videos were included. For inter-observer reliability, all readers were trained to ensure consistent understanding and use of the scoring system. Internal consistency was measured using the Cronbach's coefficient alpha, which was 0.863 for the overall UCEIS - bleeding 0.80, vascular pattern 0.83, and ulcers and erosions 0.79.

The study found that the intra and inter-observer reliability ratios for the UCEIS were 0.96 and 0.88 respectively. Intra-observer agreement static was calculated with kappa, which was 0.72, with individual descriptors ranging from 0.47 (for bleeding) to 0.87 (for vascular pattern). Inter-observer agreement statistic was slightly lower at 0.50, with individual descriptors ranging from 0.48 (bleeding) to 0.54 (vascular pattern). Additionally, these observer reliabilities were compared

with readers who were given clinical information at the time of the video readings, which determined no apparent bias by clinical information.

To evaluate the impact of clinical information on UCEIS scores, the author also undertook another study in 2015[26]. The study invited 40 readers from various countries who were experienced with endoscopic assessment. Each reader was divided into two groups (with and without clinical information) and conducted evaluation of a random 28 from 44 videos, which had not been used in the previous study. Furthermore, 4 videos included misleading information in order to ensure disparity between endoscopic assessment and clinical information.

This study showed there is no impact of clinical information on mean UCEIS scores. They were almost identical whether readers had knowledge of patient's clinical information and the median SD was 0.94 for blinded and 0.93 for unblinded. The SD was low for videos with severe disease.

Intra- and inter-observer agreement of the blinded and unblinded readers was also evaluated. Intra-observer agreements for bleeding and vascular pattern were very similar for the two groups, whereas that for erosions and ulcers just reached to statistical significance with kappa of 0.47 for blinded and 0.74 for unblinded.

The study also extended to compare the UCEIS with other indices and patient-reported symptom scoring systems. The full Mayo Clinic Score (MC)[32], partial MC (excluding endoscopic subscore)[48], patient-reported stool frequency and rectal bleeding subscore, patient functional assessment score and Feagan score were compared with the UCEIS as well as Feagan Score[36]. This showed the UCEIS is significantly superior to the Feagan Score including patient-reported symptom subscore. This implies that the UCEIS alone may be sufficient for outcome measurement in clinical trials.

The only inter-observer and central reader variations study on UCDAI also referred to UCEIS[25]. The authors investigated the role of central readers to minimise inter-observer variations, which may contribute to false responses to placebo in UC trials. They conducted a 10-wk randomised double-blinded placebo-controlled study on patients with UC who scored UCDAI ≥ 2.

Three hundreds and forty-three patients, who were initially assessed by site investigators, were enrolled to the randomised clinical trials. Clinical remission (UCDAI, stool frequency and bleeding scores of 0) was achieved by 30.0% of patients treated with mesalamine and 20.6% of those with placebo. However, when those 343 were re-assessed by 7 central-readers, 31% of those patients were in fact ineligible as they scored lower than 2. Furthermore, this altered the remission rate to 29.0% and 13.8% in the mesalamine and placebo groups respectively. In conclusion, this study suggests robust methodology for future clinical trials in UC to avoid misleading results.

The authors also extended the study to quantify the inter-observer variation amongst 7 central readers using UCDAI, UCEIS, Feagan score, visual analogue scale. Of those indices, UCEIS demonstrated the highest interclass correlation coefficient with 0.83 and UCDAI was 0.79. The authors concluded that this might be attributed to no friability assessment in UCEIS, which is the commonest source of disagreement between central and site readers in this study.

What is remission in UC?

Definition of remission: Remission rates can vary by more than two-fold depending on the definition of remission used for data analysis[49]. In addition to uncertainty about standardisation of disease activity measurement, disease remission has also never been conclusively defined or validated. Defining disease remission should be the fundamental starting point of studying therapeutic efficacy and disease monitoring, before standardising how to measure disease activity.

Definitions of remission in UC vary depending on users, settings and the purpose of monitoring the disease activity. The definition of remission used in clinical practice and by the patient is often different from that used in clinical trials. Remission, clinical remission, complete remission, partial remission, clinical response, mucosal healing or remission, corticosteroid-free remission, registration remission are frequently employed terms used in clinical practice by healthcare professionals and patients, although these terms are used interchangeably and variably without strict definition, including in clinical trials.

Table 5 is a summary of definitions of remissions in UC defined by large regulatory bodies and guidelines. All guidelines mention remission to manage disease, however, few guidelines explicitly define remission. The American College of Gastroenterology is no exception, though it controversially states that, "practical therapeutic end point, endoscopic demonstration of mucosal healing is not usually necessary for a patient who achieves clinical remission". The FDA recommends a primary endpoint of clinical remission, and clinical remission is defined as follows[5]: Rectal Bleeding subscore = 0: (1) Stool Frequency subscore = 0 (or stool frequency subscore 1 is considered if at least one point improvement in Stool Frequency subscore from baseline); and (2) Endoscopy subscore 0 on UCDAI.

It also describes mucosal healing should not be supported from macroscopic appearance of the mucosa through endoscopy. However, the FDA further describes that there are no criteria for histological assessment of mucosal healing due to a lack of validated gold standard histological scoring systems.

The European Crohn's and Colitis Organisation (ECCO) more realistically states in their guidelines for patients in UC that there is no fully validated definition of remission.

How indices are used in clinical trials and defining remission endpoints: Since there is no gold-standard outcome measurement instruments in UC, many clinical trials have employed instruments depending on its application. Classic disease

activity measurement instruments have been recently challenged by the FDA because of the significant effect of their subjective components affecting reproducibility. Even the traditionally promoted indices used by the FDA (Mayo Score and UCDAI) contain physician global assessment, which is highly sensitive to bias. The FDA suggests the primary endpoint should be achieved by endoscopic as well as clinical outcome, however, the difficulty in this is that these symptoms do not necessarily occur simultaneously with symptom control, especially where stool frequency and abdominal pain are considered. Nevertheless, these symptoms affect patients' quality of life. There are therefore further hurdles to overcome before standardisation of endpoint definition in UC[44].

In this systematic review, remission endpoints were investigated by studying the application of the UCEIS and UCDAI in clinical research and therapeutic trials (Tables 6 and 7). There are only three clinical research identified which applied the UCEIS for disease outcome measure and defined remission. Furthermore, only one randomised clinical trial has chosen the index for its outcome measurement instrument so far[54].

The trial was the first-in-human trial of AVX-470, which is a bovine-derived, orally-administered, anti-tumour necrosis factor (TNF) antibody, that works to intestinal mucosal tissue with minimal systematic effects. TNF is upregulated in the colonic mucosa in UC and believed to play a pathological role by loss of mucosal barrier integrity[57]. AVX-470 reduces levels of TNF protein in mice models, thus correcting immune dysregulation. In this study, the UCEIS was used to assess endoscopic response to treatment along with the total Mayo score and sub-scores.

This study successfully correlates between UCEIS scores and TNF immunohistochemistry scores at baseline. Further, it found that TNF staining was significantly reduced in proximal and distal segments of bowel, whereas the UCEIS changes were more apparent in proximal segments than distal ones. Although the study described achieving clinical remission, this was never defined.

The UCEIS was used for endoscopic assessment in a prospective study to quantify faecal calprotectin in patients with UC[55]. The authors used Quantum Blue Calprotectin High Range Rapid Test (Buhlmann laboratories AG, Schonenbuch, Switzerland) for faecal calprotectin measurement tools in this study. Interestingly, the authors defined endoscopic remission when the UCEIS < 3. They also concluded that faecal calprotectin and CRP were both well correlated with the UCEIS (the Spearman correlation coefficient is 0.696 and 0.581 respectively). Moreover, they concluded that when a cut-off faecal calprotectin of 191 □g/g is set, this could predict endoscopic remission and mucosal remission (UCEIS < 3) with 88% sensitivity and 75% specificity. However, when UCEIS < 1 clinical remission proposed by other authors[14,23] is applied, faecal calprotectin would be lower than 191 □g/g. This could lead to underestimate patients who should be treated.

The largest patient population study that used the UCEIS is a cross-sectional, multi-centre study, ACERTIVE study[56]. The aim of this study was to evaluate potential applications of biomarkers (faecal calprotectin and neutrophil gelatinase B-associated lipocalin) as disease activity measuring instrument in patients with asymptomatic UC. The UCEIS and Geboes index[22] were applied for macroscopic and microscopic assessment respectively. Nine percent of the asymptomatic patients had active disease with UCEIS > 2. Twenty-one percent of the asymptomatic patients presented with Geboes index > 3. One point fifteen percent and 5% of the patients presented with focal and diffuse basal plasmacytosis, respectively. Patients with asymptomatic disease indeed showed presence of macroscopic as well as microscopic disease. Furthermore, 50% of patients who scored a UCEIS < 2 and 15% of patients who were considered to have achieved mucosal healing (Mayo ES = 0) had diffuse basal plasmacytosis. These results support the previous published notion that macroscopic findings are not sufficient to define remission or endpoint[9].

Both biomarkers predicted mucosal healing as well as histological remission with satisfactory probability of 75%-93%. The authors proposed a cut-off figures of 150-250 □g/g for faecal calprotectin and 12 □g/g for lipocalin. This range of cut-off level for faecal calprotectin is due to the application of two faecal calprotectin measurement tools (Quantum Blue Calprotectin High Range Rapid Test and Automated Fluroimmunoassay-EliA Test) from stool samples. Although this proposed cut-off point for faecal calprotectin for clinical remission is a similar value with the Taiwan group[55], the defined remission show variance as the Taiwan group set UCEIS < 3 for remission whereas ACERTIVE study used UCEIS ≤ 1. Although it is only one score difference, this can be of significance for disease outcome. As Arai *et al*[20] concluded, the recurrence rate was directly proportional to the UCEIS score. The recurrence rate for UCEIS 1 disease is 22.4%, whereas UCEIS 2 disease increases to 27.0%. As the authors state validation of the proposed cut-off values is required before introducing them in clinical setting. Moreover, caution should be applied when introducing biomarkers especially when their intention is to replace endoscopic assessment.

Table 6 shows the summary of the randomised clinical trials that utilised the UCDAI for disease activity assessment, defining remission and endpoints. The studies were divided into introduction and maintenance of disease remission.

Most of the studies investigated the efficacy of introducing remission set clinical remission as UCDAI ≤ 1, however some studies defined as UCDAI ≤ 2 for remission. It appears that many studies have taken advantage of defining their own remission, clinical response and endpoints with the UCDAI as it is not clearly defined in previous guidelines. The studies with probiotics appear to choose higher remission cut-off point, which could interpret that it is undemanding to achieve clinical remission so that it would satisfy requirements of regulatory bodies such as the FDA. The previous validation study suggested UCDAI score < 2.5 for clinical remission[65], meaning UCDAI ≤ 2 is still within the range of remission.

Another point to note is that many studies have their own additional criteria with a specific patient-reported symptom scoring system to measure rectal bleeding and stool frequency to define remission or clinical response. This is likely attributed to the guideline published by the FDA, which encourages to assess patient-reported outcome measurement on rectal bleeding and stool frequency in addition to the macroscopic assessment with endoscopy as an endpoint for clinical trials. This is also reflected on their definition of clinical remission on Table 5.

If a more stringent primary endpoint is enforced the clinical utility of therapeutics may be harder to demonstrate, potentially limiting the number of agents available in the marketplace. Furthermore, drug development for UC faces bigger challenge due to the unknown natural history of UC, unpredictable relapse and remission patterns as well as response to medications with known efficacy. If the stringent remission and endpoint was forced by regulatory bodies, the pharmaceutical industry may choose drugs that have cheaper development cost.

Although the FDA supports the use of UCDAI for measuring primary endpoints, UCDAI has limitations. As it is highlighted in the previous section, one of the weakness is a lack of validation and vulnerability to observer bias. Adding inconsistent definition of remission and endpoint for each clinical trial hinders providing optimal management to patients with the disease.

DISCUSSION

Homogeneity of the clinical trials in UC has been discussed amongst experts for decades. Despite a desire for a single gold-standard disease activity index, the number of indices has been steadily increasing. Disease remission is yet to be fully defined, thus trial outcomes vary and limit the utility of these studies depending on the purpose of its clinical use. Most trials chose individual endpoints which are not necessarily clinically pertinent. Clinicians, on the other hand are constantly negotiating with patients to provide the best possible management for this chronic condition, regardless of an index score. This variation makes the comparison amongst clinical trials extremely difficult, hindering drug development.

So far, many review studies summarised and evaluated currently available indices on different assessments. The majority of these studies highlighted the wide variation of endpoints by different indices and emphasised the importance of having a gold-standard index to assess the efficacy of the interventions. This has led to development of more indices rather than choosing a gold-standard index, adding more choices and fuelling confusion amongst researchers and clinicians. This systematic review proposes to emphasise on a universal consensus on UC remission before developing any more indices. Futher more, this systematic review would assist scientists and clinicians to have a better understanding of confusing definitions and disease activity indices that have been used interchangeably.

This systematic review was conducted to evaluate the definition and evidence for remission endpoints in ulcerative colitis from the point of view of two particular indices. The UCDAI has been widely used in clinical studies compared to the UCEIS. Although the UCEIS has been extensively validated, only one randomised clinical trial has employed the UCEIS as their outcome measurement instrument of date. The reason may be threefold. Firstly, other traditional disease activity measurement instruments have been widely used in previous clinical trials, making comparison with those trials more straightforward, although less robust. Secondary, if clinical trials were conducted for drug development, they would more likely choose the disease activity indices as recommended by regulatory bodies such as the FDA or equivalent. Finally, the UCEIS was recently developed thus there is no surprise that the number of clinical trials using this scoring system is still low.

The other two studies that used the UCEIS are not randomised clinical trials, though they demonstrated how multiple definitions of remission used in the evaluation of biomarker, calprotectin, to monitor disease activity could alter the outcomes. Without a universal definition of remission, researchers can freely define the UCEIS score for a remission endpoint, making evaluation of calprotectin use in clinical practice very difficult.

Furthermore, regulatory bodies such as the FDA recommend measuring endpoints in terms of clinical remission with particular indices, although they still do not convey the ideal length of clinical trial to achieve a primary endpoint or the duration of clinical remission before relapsing. This diversity of clinical protocols was also emphasised in this systematic review.

One of the criticisms for traditional outcome measurement instruments has been insufficient validation. The UCEIS has designed to overcome from this problem and to take a step forward for establishing a gold standard outcome measurement instrument. Yet, this systematic review highlighted that validation is not necessarily an issue for employing an outcome measurement instrument for clinical trials. Although we focused on developing new ideal indices, a new index may not be a solution to establish a gold standard outcome measurement instrument.

A lack of understanding in aetiology and natural history of ulcerative colitis may contribute to this confusion. In order to untangle this confusion, we recommend that an international consensus of remission should be sought as a matter of urgency before establishing a gold standard outcome measurement. Once a universal consensus for remission is reached and defined, establishing a gold-standard index, which can measure true symptoms and is transferable and meaningful to clinical practice, can be determined. That would lead to standardisation of clinical trial protocols for advancing patient care.

COMMENTS

Background

The current target of disease remission endoscopically is mucosal healing although it has not been fully validated or no standardised definition of mucosal healing. Yet, this appears to be the goal for many clinical practice as well as drug trials. The recent draft guideline released by the FDA states the ideal primary efficacy assessment instrument in clinical trials should consist of (1) a signs and symptoms assessment scale - best measured by a patient-reported outcome instrument. If not, an observer-reported outcome instrument; and (2) an endoscopic and histological assessment scale. Thus, endoscopic assessment tools with comprehensive clinical symptom assessment components that come from patients would be a reasonable choice to argue remission and endpoints employed in clinical trials.

Research frontiers

The authors believe this has been mentioned everywhere in the paper that definition of ulcerative colitis endpoint has been introducing ambiguity especially when different clinical trial studies are compared. The authors also mentioned in the summary that a lack of understanding in aetiology and disease natural history may contribute to this confusion, which needs to be addressed in the future research.

Innovations and breakthroughs

The authors added to emphasize the differences from other similar studies.

Applications

The authors added “This systematic review would assist scientists and clinicians to have a better understanding of confusing definitions and disease activity indices that have been used interchangeably”.

Peer-review

This is a comprehensive review of remission endpoints in ulcerative colitis, the paper is well written and is very useful for both clinical practice and teaching purposes.

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Table 1 Descriptors and intra- and inter-observer variation

Descriptor	Likert scale anchor points	Intra-observer variation (a weighted *k*)	Inter-observer variation (a weighted *k*)
Vascular pattern	Normal (1) Patchy loss (3) Obliterated (5)	0.61	0.42
Mucosal erythema	None (1) Light red (3) Dark red (5)	0.43	0.35
Mucosal surface (Granularity)	Normal (1) Granular (3) Nodular (5)	0.45	0.34
Mucosal oedema	None (1) Probable (3) Definite (5)	0.43	0.31
Mucopus	None (1) Some (3) Lots (5)	0.47	0.4
Bleeding	None (1) Mucosal (2) Luminal mild (3) Luminal moderate (4) Luminal severe (5)	0.57	0.37
Incidental friability	None (1) Mild (2) Moderate (3) Severe (4) Very severe (5)	0.49	0.4
Contact friability	None (1) Probable (3) Definite (5)	0.34	0.3
Erosions and ulcers	None (1) Erosions (2) Superficial ulcer (3) Deep ulcer (4)	0.65	0.45
Extent of erosions or ulcers	None (1) Limited (2) Substantial (3) Extensive (4)	0.6	0.42

Table 2 The ulcerative colitis endoscopic index of severity descriptors (maximum score = 8, Scoring is based on the most severe area)

Descriptors	Likert Scale anchor point	Definition
Vascular pattern	0: Normal	Normal vascular pattern with arborisation of capillaries clearly defined, or with blurring or patchy loss of capillary margins
	1: Patchy obliteration	
	2: Complete obliteration	Complete obliteration
Bleeding	0: None	
	1: Mucosa	Some spots or streaks of coagulated blood on the surface of the mucosa
	2: Luminal mild	Some free liquid blood in the lumen
	3: Luminal moderate or severe	Frank blood in the lumen ahead of endoscope or visible oozing from a haemorrhagic mucosa
Erosions and Ulcers	0: None	None
	1: Erosions	Tiny < 5 mm defects in the mucosa, of white or yellow colour with a flat edge
	2: Superficial ulcer	Larger > 5 mm defect in the mucosa, which are discrete fibrin-covered ulcers in comparison with erosions, but remain superficial
	3: Deep ulcer	Deeper excavated defects in the mucosa, with a slightly raised edge

Table 3 Ulcerative colitis disease activity index (maximum score = 12)

Variables	Score	Items
Stool frequency	0	Normal
	1	1-2 stools/d more than normal
	2	3-4 stools/d more than normal
	3	> 4 stools/d more than normal
Rectal bleeding	0	None
	1	Streaks of blood
	2	Obvious blood
	3	Mostly blood
Endoscopic appearance	0	Normal
	1	Mild friability
	2	Moderate friability
	3	Exudation, spontaneous bleeding
Physician global assessment	0	Normal
	1	Mild
	2	Moderate
	3	Severe

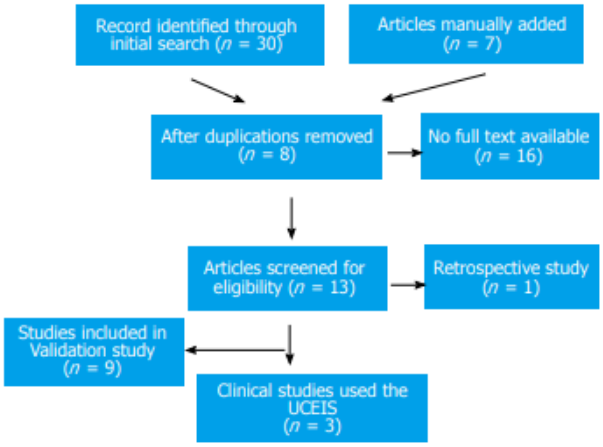

Figure 1 PRISMA flow diagram for ulcerative colitis endoscopic index of severity. UCEIS: Ulcerative colitis endoscopic index of severity.

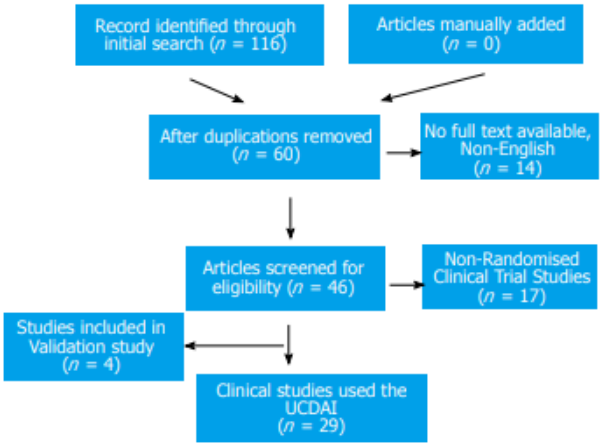

Figure 2 PRISMA flow diagram for ulcerative colitis disease activity index. UCDAI: Ulcerative colitis disease activity index.

Table 4 Validation studies of ulcerative colitis endoscopic index of severity and ul-cerative colitis disease activity index

	Ref.	Patient number	Outcomes
UCEIS			
Validity	Corte *et al*[18]	89	Correlation between UCEIS and outcomes
			The UCEIS score was directly proportional to requirement of rescue therapy
			UCEIS ≥ 5 was significantly linked to requiring colectomy 18/54 (33%) patients with UCEIS ≥ 5 compared to 3/33 (9%) with UCEIS ≤ 4
			No definition of remission
	Fernandes *et al*[19]	108	Prediction of outcomes in acute severe colitis
			UCEIS was applied to score of the rectum and sigmoid, seg-UCEIS
			Seg-UCEIS predicted to develop steroid-refractory disease and the likelihood of colectomy (seg-UCEIS = 14 had a 17 times higher risk of steroid-refractory disease and a 25 times higher risk of requiring colectomy)
			Every 1 point increase in the UCEIS or Seg-UCEIS increased the need of colectomy by 2.78 and 1.79 respectively
			Mayo score did not predict these
			No definition of remission
	Arai *et al*[20]	285	Reflection of true UC activity and remission
			The recurrence rate was directly proportional to the UCEIS score (5.0% for UCEIS = 0, 22.4% for UCEIS = 1, 27.0% for UCEIS = 2, 35.7% for UCEIS = 3, 75% for UCEIS = 4-5)
			The absence of bleeding and mucosal damage were independent factors for continued clinical remission
			UCEIS ranged from 0 to 5 when clinical remission, Mayo ≤ 1
			UCEIS ≤ 1 for clinical remission, which showed sensitivity of 68% and specificity 57%
			The expected duration of recurrence is also prolonged when UCEIS ≤ 1
	Kucharski *et al*[21]	49	Assessment of 9 endoscopic indices correlate well with (1) clinical indices; and (2) histological Geboes Index[22]
			The UCEIS showed the strongest correlation with the Geboes Index (the coefficient: 0.434 to 0.629)
			Recommends the UCEIS for the best overall correlations with both clinical and histological indices
Responsiveness	Ikeya *et al*[23]	41	The ability to detect to change after Tacrolimus remission induction treatment for moderate to severe UC
			Although Mayo endoscopic score is easy to use, it does not distinguish depth of ulcers unlike UCEIS
			Despite UCEIS score improved from 7 to 4, Mayo endoscopic score remained at 3 (severe)
			An improvement of UCEIS ≥ 3 showed close correlation with clinical remission, colectomy-free and relapse free rates
			Proposed remission (score 0-1), mild (2-4), moderate (5-6), severe (7-8)
			UCEIS 1 in remission is only from vascular pattern
	Menasci *et al*[24]	80	Comparison of the global UCEIS score from 5 segments and a traditional method of UCEIS score
			The regular method of the UCEIS is to score the most inflamed segment of the bowel
			This was compared with the sum of the score of five colonic segments
			A very good correlation (Spearman's $r = 0.86$, $P < 0.0001$) for disease with UCEIS score ≤ 5
			Less correlation ($r = 0.48$, $P < 0.01$) for disease with UCEIS > 5
Reliability	Travis *et al*[15]		Investigation of intra- and inter-observer consistency assessment
			25 readers evaluated 28 videos including 4 duplicates to assess intra-reader reliability
			The intra and inter-reader reliability ratios for the UCEIS were 0.96 and 0.88 respectively
			The USCEI revealed a strong correlation with overall assessment of severity without being influenced by knowledge of clinical information
			No definition of remission
	Feagan *et al*[25]	281	The effect of centralized review of images on inter-observer variations
			Patients with UCDAI ≥ 2 were randomised to evaluate the efficacy of delayed mesalamine treatment (4.8 g/d for 10 wk)
			UCEIS was used as a part of inter-observer agreement study and showed interclass correlation coefficient of 0.83 amongst 7 central readers, which is superior to UCDAI
	Travis *et al*[26]		Clinical information influences UCEIS score
			40 readers evaluated 28 of 44 videos
			No discrepancy between blinded and unblended readers
			Intra- and inter-reader variability demonstrated moderate to substantial agreement (κ = 0.47 to 0.74 and κ = 0.40 to 0.50 respectively)
			UCEIS correlated well with patient-reported symptoms - rectal bleeding, stool frequency and patient functional assessment (rank correlation = 0.76 to 0.82)
UCDAI			
Validity	Higgins *et al*[27]	66	Finding endpoints in disease activity indices for remission and improvement in UC
			UCDAI < 2.5 for remission, which had a sensitivity and specificity of 0.82 and 0.89
			Remission in this study was defined by patients
	Poole *et al*[28]	126	Establish the relationship between the UCDAI and patient reported EQ-5D
			The UCDAI with or without endoscopy assessment demonstrated a good correlation with EQ-5D
			Endoscopy assessment may not link with the disease activity

	Kucharski *et al*[25]	49	Assessment of 9 endoscopic indices correlate well with (1) clinical indices; and (2) histological Geboes Index (22) The UCDAI showed strong correlations with all 9 endoscopic indices (the coefficient in a range of 0.712 to 0.790) The UCDAI showed the highest correlation amongst clinical activity indices with the Geoboes Index (the Spearman's coefficient 0.478) Compared to UCEIS, the UCDAI is less correlated with the Geboes Index
Reliability	Feagan *et al*[25]	281	The effect of centralized review of images on inter-observer variations Patients with UCDAI ≥ 2 were randomised to evaluate the efficacy of delayed mesalamine treatment (4.8 g/d for 10 wk) 31% of patients with UCDAI ≥ 2 enrolled in the RCT initially were considered ineligible by the central readers Inter-observer agreement amongst 7 central readers was good (interclass correlation coefficient: 0.78)

UC: Ulcerative colitis; UCEIS: Ulcerative colitis endoscopic index of severity; Seg-UCEIS: The sum of the rectal and sigmoid segmental UCEIS score; UCDAI: Ulcerative colitis disease activity index; EQ-5D: EuroQol Five Dimensions Questionnaire; RCT: Randomised control trial.

Table 5 Definitions of remission in ulcerative colitis

Guidelines	Definition
FDA[5]	Clinical remission Mayo score of ≤ 2 with no individual subscore > 1 Rectal Bleeding subscore = 0 Stool Frequency subscore = 0 (at least one point decrease in Stool Frequency subscore from baseline and achieved 1 is considered) Endoscopy subscore = (Mayo score: 0 or 1, UCDAI = 0) Clinical response Reduction in Mayo score ≥ 3 and ≥ 30% from baseline with Rectal Bleeding subscore ≤ 1 Corticosteroid-free remission Clinical remission in patients using oral corticosteroids at baseline who have discontinued them and are in clinical remission at the end of the study
World Gastroenterology Organisation	Clinical remission UCDAI ≤ 2 (2010 World Gastroenterology Organisation Practice Guideline)[50] Corticosteroid-free remission Decreasing the frequency and severity of recurrence and reliance on corticosteroids
International Organisation for the Study of IBD	End points = induction of remission = mucosal healing[12] The absence of friability, blood, erosions and ulcers in all visible segments No mention of clinical symptoms
American College of Gastroenterology	No clear definition[51]
British Society of Gastroenterology	No clear definition[52]
European Crohn's and Colitis Organisation	Remission[53] A complete resolution of symptoms and endoscopic mucosal healing Not been a fully validated definition of remission Suggest the best way forward is a combination of Stool Frequency ≤ 3 No rectal bleeding Normal or quiescence mucosa at endoscopy Clinical response Clinical and endoscopic response depending on the activity index Generally, a decrease in the activity index > 30% plus a decrease in the rectal bleeding and endoscopic subscores

UCDAI: Ulcerative colitis disease activity index; IBD: Inflammatory bowel disease; FDA: Food and Drug Administration.

Table 6 Clinical studies measured with the ulcerative colitis endoscopic index of severity

Ref.	Year	Type of study	Drug/subject of study	Entry criteria	Primary endpoint	Secondary endpoint	Remission/clinical improvement	Length of study
Hartman *et al*[54]	2016	Randomised, double-blind, placebo-controlled study	AVX-470, oral	36 patients with Mayo score 5-12 and Mayo ES ⩾ 2	Not set, but implies clinical response at week 4	Not set	Remission was not defined. Clinical response Mayo reduction ⩾ 3	4 wk
Lin *et al*[55]	2015	Prospective, multi-centre study	Faecal calprotectin	52 patients with UC	N/A	N/A	Endoscopic remission: UCEIS < 3	N/A
Magro *et al*[56] ACERTIVE study	2016	Cross-sectional multi-centre study	Faecal calprotectin/ lipocalin	371 patients Mayo partial score < 2, montreal classification < 2			Remission: UCEIS ⩽ 1 Mucosal healing: Mayo ES = 0	

UCEIS: Ulcerative colitis endoscopic index of severity; UC: Ulcerative colitis; ES: Endoscopic subscore; N/A: Not available.

Table 7 Randomised clinical trials measured with the ulcerative colitis disease activity index

Ref.	Year	Drug	Entry criteria	Primary endpoint	Secondary endpoint	Remission/clinical improvement	Length of study
Randomised clinical trials - to induce remission Mesalazine (5-ASA)							
Marteau *et al*[59]	2005	Pentasa (PR + PO *vs* PO alone)	UCDAI: 3-8	Remission at week 4	Remission rate at week 8 Improvement at week 4 and 8	Remission: UCDAI ≤ 1 Clinical improvement: A decrease of UCDAI ≥ 2	8 wk
D'Haens *et al*[59]	2006	SPD476 - MMX mesalazine	UCDAI: 4-10 + endoscopic score ≥1 PGA score ≤ 2	Remission	Change in UCDAI, FS, histology at week 8 Change in symptoms	Remission: UCDAI ≤ 1 (with RB 0, SF ≤ 1) at week 8	8 wk
Sandborn *et al*[60]	2007	MMX Multi Matrix System mesalazine	UCDAI: 4-10 + endoscopic score ≥1 PGA score ≤ 2	Clinical/endoscopic remission at 8 wk	Proportion of clinical improvement Proportion of patients as treatment failure Change in: RB, SF, FS	Clinical remission: UCDAI ≤ 1 Endoscopic remission: UCDAI endoscopic subscore ≤ 1 Clinical improvement: A decrease of UCDAI ≥ 3 Treatment failure: Unchanged or worsened UCDAI	8 wk
Lichtenstein *et al*[61]	2007	SPD476 - MMX mesalazine OD *vs* BD	UCDAI: 4-10	Clinical and endoscopic remission at week 8	Comparison of remission rate at week 8	Clinical remission: UCDAI ≤ 1 with RB/SF/EI = 0	8 wk
Kamm *et al*[62,63] MEZAVANT study	2007 2009	MEZAVANT MMX Mesalamine	Mild - mod UC: UCDAI 4-10 + endoscopic subscore ≥ 1, PGA ≤ 2	Clinical + Endoscopic remission at week 8	Clinical remission Clinical improvement Change in UCDAI	Clinical + endoscopic remission: UCDAI ≤ 1 + subscore RB/SF = 0, No mucosal friability + a ≥ 1 reduction in EI Clinical improvement: Decrease in UCDAI ≥ 3	8 wk
Ito *et al*[64]	2010	Asacol *vs* PentasaTime-dependent *vs* pH dependent Mesalamine	UCDAI: 3-8 and blood stool score ≥ 1	To demonstrate Asacol over Pentasa AND the decrease in UCDAI	Macroscopic changes	Remission: UCDAI ≤ 2 and no blood diarrhoea Clinical improvement: UCDAI decreased by ≥ 2	8 wk
Hiwatashi *et al*[65]	2010	Mesalazine - dose study	UCDAI: 6-8	Change in UCDAI at week 8	Remission, improvement, efficacy	Remission: UCDAI ≤ 1 Efficacy: Decrease of UCDAI ≥ 2	8 wk
Flourié *et al*[66] MOTUS study	2013	Mesalazine, Pentasa OD or BD in total of 4 g/d	UCDAI: 3-8	UCDAI ≤ 1 after 8 wk	Complete remission (UCDAI = 0) at 8 wk UCDAI decreased by ≥ 2 at 8 wk Clinical remission at week 4, 8, 12 Mucosal healing at 8 wk	Complete remission: UCDAI = 0 Endoscopic remission: UCDAI endoscopic subscore: 0 or 1 Clinical remission: UCDAI ≤ 1	12 wk
Probert *et al*[67] PINCE study	2013	Mesalazine (pentasa) enema	UCDAI: 3-8	Remission rate (UCDAI < 2) at 4 wk	Remission rate at 8 wk, improvement at week 2, 4 and 8 Time to cessation of RB QoL (EQ-5D)	Remission: UCDAI ≤ 1 Clinical improvement: UCDAI decreased by ≥ 2	8 wk
Sun *et al*[68]	2016	Mesalazine (modified-release *vs* enteric-coated tablets)	UCDAI: 3-8 + bloody stool score > 1	The decrease in UCDAI	Remission rate Efficacy rate	Remission: UCDAI ≤ 2 + bloody stool 0 Clinical improvement: A decrease of UCDAI ≥ 2	8 wk
Suzuki *et al*[69]	2016	pH dependent release mesalamine, asacol dose	UCDAI: 6 - 10 Rectal bleeding score ≥ 1	Decrease in UCDAI		Remission: UCDAI ≤ 2 Rectal bleeding score: 0 Improvement UCDAI decreased by ≥ 2	8 wk
Thiazole compounds							
Mantzaris *et al*[70]	2004	Azathioprine alone (2.2 mg/kg) *vs* combination with olsalazine (0.5 g TID)	Steroid-dependent remission	Relapse rate	Time to relapse Time to discontinuation Severity of relapse	Remission: UCDAI ≤ 1 Relapse: New symptoms + UCDAI > 3	2 yr

Schreiber *et al*[70]	2007	Tetomilast - Thiazole compound	UCDAI: 4-11	Clinical improvement: UCDAI decreased by ≥ 3 at 8 wk	Remission Clinical improvement at week 4 IBDQ-32 score Proportion of pts with improved Flexible Sigmoidscopy score Time to clinical improvement Time to remission	Clinical improvement: UCDAI decreased by ≥ 3 Remission: UCDAI ≤ 1	8 wk
Steroids							
Travis *et al*[71] CORE II study	2012	Budesonide MMX	UCDAI: 4-10	Clinical/endoscopic remission at week 8	Clinical improvement Endoscopic improvement at week 8	Clinical/endoscopic Remission: UCDAI ≤ 1 + RB/SF/EI = 0 Clinical improvement: A decrease of UCDAI ≥ 3 Endoscopic improvement: A decrease of EI ≥ 1	8 wk
Probiotics							
Vernia *et al*[72]	2000	Sodium Butyrate	Mild-moderate UC	Remission or marked improvement		Remission: UCDAI ≤ 2 Positive response: Decrease of UCDAI ≥ 2	6 wk
Mahmood *et al*[73]	2005	Human recombinant trefoil factor 3 enema	UCDAI: >3	Remission at week 2	Clinical significant improvement in clinical and histological scores at 2 and 4 wk	Remission: UCDAI ≤ 1 without RB Clinical improvement: A decrease of UCDAI >3	4 wk
Lichtenstein *et al*[74]	2007	Bowman-Birk inhibitor concentrate - soy extract with high protease inhibitor activity	UCDAI: 4-10	Remission at week 8		Remission: UCDAI ≤ 1 + no RB or SF Clinical improvement: UCDAI decrease ≥ 1	
Tursi *et al*[75]	2009	VSL #3 (probiotic)	UCDAI 3-8, endoscopic subscore ≥ 3	Decrease in UCDAI of ≥ 50%	Activity of relapsing UC Remission Improvement Change in objective and subjective symptoms	Remission: UCDAI ≤ 2	8 wk
Sood *et al*[76]	2009	VSL #3 probiotic	UCDAI 3-9 with endoscopic subscore ≥ 2	Clinical improvement at week 6	Clinical remission	Clinical remission: UCDAI ≤ 2 Clinical improvement: A decrease UCDAI by 50%	12 wk
Tamaki *et al*[77]	2016	Bifidobacterium longum 536 (probiotic)	UCDAI 3-9	Change in UCDAI	Remission Improvement of Objective and subjective symptoms Endoscopic improvement in Mayo subscore	Remission: UCDAI ≤ 2	8 wk
Helminth therapy Garg *et al*[78]	2014	Helminth Trichuris suis ova	UCDAI of ≥ 4	Clinical improvement	Clinical remission	Clinical improvement: Decrease in the UCDAI of ≥ 4 Clinical remission: UCDAI of ≤ 2	12 wk
Nicotine therapy							
Ingram *et al*[79]	2005	Nicotine enema 6 mg/d	Confirmed UC with inflamed mucosa grade > 2	Clinical remission	Improvement in the UCDAI	Clinical remission: UCDAI EI ≤ 1 and No RB for 1 wk	6 wk
Randomised clinical trials - to maintain remission							
Lichtenstein *et al*[80-82] and Zakko *et al*[83]	2010 2012 2015 2016	Mesalamine granules 1.5 g/d, OD	Previously achieved remission with steroids for > 1 mo and < 12 mo	Percentage of patients relapse-free at 6 mo	Mean changes from baseline at month 6	Relapse: UCDAI RB ≥ 1 and EI ≥ 2 Remission: UCDAI RB = 0, EI < 2	6 mo
Bokemeyer *et al*[43] and Dignass *et al*[84]	2009 2011	Mesalazine, Pentasa OD or BD in total of 2 g/d	Clinical remission: UCDAI < 2	To demonstrate OD is not inferior to BD	Time to relapse between 2 groups UC-DAI total and subscores between 2 groups	Remain in remission UCDAI ≤ 2	12 mo

RB: Rectal bleeding; SF: Stool frequency; EI: Endoscopic index/subscore; OD: Once daily; BD: Twice daily; TID: Three times daily; UCDAI: Ulcerative colitis disease activity index; QoL: Quality of life.

Appendix 6 : PRISMA Checklist

Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	

Section and Topic	Item #	Checklist item	Location where item is reported
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	
Study characteristics	17	Cite each included study and present its characteristics.	
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	
	23b	Discuss any limitations of the evidence included in the review.	
	23c	Discuss any limitations of the review processes used.	
	23d	Discuss implications of the results for practice, policy, and future research.	
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	

Section and Topic	Item #	Checklist item	Location where item is reported
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	
Competing interests	26	Declare any competing interests of review authors.	
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	

From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. doi: 10.1136/bmj.n71

For more information, visit: http://www.prisma-statement.org/

Appendix 7: AMSTAR 2 Checklist

AMSTAR 2: a critical appraisal tool for systematic reviews that include randomised or non-randomised studies of healthcare interventions, or both

1. Did the research questions and inclusion criteria for the review include the components of PICO?

For Yes:
- ☐ Population
- ☐ Intervention
- ☐ Comparator group
- ☐ Outcome

Optional (recommended)
- ☐ Timeframe for follow-up

☐ Yes
☐ No

2. Did the report of the review contain an explicit statement that the review methods were established prior to the conduct of the review and did the report justify any significant deviations from the protocol?

For Partial Yes:
The authors state that they had a written protocol or guide that included ALL the following:
- ☐ review question(s)
- ☐ a search strategy
- ☐ inclusion/exclusion criteria
- ☐ a risk of bias assessment

For Yes:
As for partial yes, plus the protocol should be registered and should also have specified:
- ☐ a meta-analysis/synthesis plan, if appropriate, *and*
- ☐ a plan for investigating causes of heterogeneity
- ☐ justification for any deviations from the protocol

☐ Yes
☐ Partial Yes
☐ No

3. Did the review authors explain their selection of the study designs for inclusion in the review?

For Yes, the review should satisfy ONE of the following:
- ☐ *Explanation for* including only RCTs
- ☐ OR *Explanation for* including only NRSI
- ☐ OR *Explanation for* including both RCTs and NRSI

☐ Yes
☐ No

4. Did the review authors use a comprehensive literature search strategy?

For Partial Yes (all the following):
- ☐ searched at least 2 databases (relevant to research question)
- ☐ provided key word and/or search strategy
- ☐ justified publication restrictions (e.g. language)

For Yes, should also have (all the following):
- ☐ searched the reference lists / bibliographies of included studies
- ☐ searched trial/study registries
- ☐ included/consulted content experts in the field
- ☐ where relevant, searched for grey literature
- ☐ conducted search within 24 months of completion of the review

☐ Yes
☐ Partial Yes
☐ No

5. Did the review authors perform study selection in duplicate?

For Yes, either ONE of the following:
- ☐ at least two reviewers independently agreed on selection of eligible studies and achieved consensus on which studies to include
- ☐ OR two reviewers selected a sample of eligible studies and achieved good agreement (at least 80 percent), with the remainder selected by one reviewer.

☐ Yes
☐ No

6. Did the review authors perform data extraction in duplicate?

For Yes, either ONE of the following:
- ☐ at least two reviewers achieved consensus on which data to extract from included studies
- ☐ OR two reviewers extracted data from a sample of eligible studies and achieved good agreement (at least 80 percent), with the remainder extracted by one reviewer.

☐ Yes
☐ No

7. Did the review authors provide a list of excluded studies and justify the exclusions?

For Partial Yes:
- ☐ provided a list of all potentially relevant studies that were read in full-text form but excluded from the review

For Yes, must also have:
- ☐ Justified the exclusion from the review of each potentially relevant study

☐ Yes
☐ Partial Yes
☐ No

8. Did the review authors describe the included studies in adequate detail?

For Partial Yes (ALL the following):
- ☐ described populations
- ☐ described interventions
- ☐ described comparators
- ☐ described outcomes
- ☐ described research designs

For Yes, should also have ALL the following:
- ☐ described population in detail
- ☐ described intervention in detail (including doses where relevant)
- ☐ described comparator in detail (including doses where relevant)
- ☐ described study's setting
- ☐ timeframe for follow-up

☐ Yes
☐ Partial Yes
☐ No

9. Did the review authors use a satisfactory technique for assessing the risk of bias (RoB) in individual studies that were included in the review?

RCTs

For Partial Yes, must have assessed RoB from
- ☐ unconcealed allocation, *and*
- ☐ lack of blinding of patients and assessors when assessing outcomes (unnecessary for objective outcomes such as all-cause mortality)

For Yes, must also have assessed RoB from:
- ☐ allocation sequence that was not truly random, *and*
- ☐ selection of the reported result from among multiple measurements or analyses of a specified outcome

☐ Yes
☐ Partial Yes
☐ No
☐ Includes only NRSI

NRSI

For Partial Yes, must have assessed RoB:
- ☐ from confounding, *and*
- ☐ from selection bias

For Yes, must also have assessed RoB:
- ☐ methods used to ascertain exposures and outcomes, *and*
- ☐ selection of the reported result from among multiple measurements or analyses of a specified outcome

☐ Yes
☐ Partial Yes
☐ No
☐ Includes only RCTs

10. Did the review authors report on the sources of funding for the studies included in the review?

For Yes
- ☐ Must have reported on the sources of funding for individual studies included in the review. Note: Reporting that the reviewers looked for this information but it was not reported by study authors also qualifies

☐ Yes
☐ No

11. If meta-analysis was performed did the review authors use appropriate methods for statistical combination of results?

RCTs

For Yes:

- ☐ The authors justified combining the data in a meta-analysis
  - ☐ AND they used an appropriate weighted technique to combine study results and adjusted for heterogeneity if present.
  - ☐ AND investigated the causes of any heterogeneity

☐ Yes
☐ No
☐ No meta-analysis conducted

For NRSI

For Yes:

- ☐ The authors justified combining the data in a meta-analysis
  - ☐ AND they used an appropriate weighted technique to combine study results, adjusting for heterogeneity if present
  - ☐ AND they statistically combined effect estimates from NRSI that were adjusted for confounding, rather than combining raw data, or justified combining raw data when adjusted effect estimates were not available
  - ☐ AND they reported separate summary estimates for RCTs and NRSI separately when both were included in the review

☐ Yes
☐ No
☐ No meta-analysis conducted

12. If meta-analysis was performed, did the review authors assess the potential impact of RoB in individual studies on the results of the meta-analysis or other evidence synthesis?

For Yes:

- ☐ included only low risk of bias RCTs
- ☐ OR, if the pooled estimate was based on RCTs and/or NRSI at variable RoB, the authors performed analyses to investigate possible impact of RoB on summary estimates of effect.

☐ Yes
☐ No
☐ No meta-analysis conducted

13. Did the review authors account for RoB in individual studies when interpreting/ discussing the results of the review?

For Yes:

- ☐ included only low risk of bias RCTs
- ☐ OR, if RCTs with moderate or high RoB, or NRSI were included the review provided a discussion of the likely impact of RoB on the results

☐ Yes
☐ No

14. Did the review authors provide a satisfactory explanation for, and discussion of, any heterogeneity observed in the results of the review?

For Yes:

- ☐ There was no significant heterogeneity in the results
- ☐ OR if heterogeneity was present the authors performed an investigation of sources of any heterogeneity in the results and discussed the impact of this on the results of the review

☐ Yes
☐ No

15. If they performed quantitative synthesis did the review authors carry out an adequate investigation of publication bias (small study bias) and discuss its likely impact on the results of the review?

For Yes:

- ☐ performed graphical or statistical tests for publication bias and discussed the likelihood and magnitude of impact of publication bias

☐ Yes
☐ No
☐ No meta-analysis conducted

16. Did the review authors report any potential sources of conflict of interest, including any funding they received for conducting the review?

For Yes:

- ☐ The authors reported no competing interests OR
- ☐ The authors described their funding sources and how they managed potential conflicts of interest

☐ Yes
☐ No

Appendix 8: Protocol for faecal microbiota transplantation in ulcerative colitis : a randomised feasibility study (BMJ)

BMJ Open

Protocol for faecal microbiota transplantation in ulcerative colitis (FMTUC): a randomised feasibility study

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ABSTRACT

Background The interaction of the gut microbiota with the human host is implicated in the pathogenesis of inflammatory and immunological diseases including ulcerative colitis (UC). Faecal microbiota transplantation (FMT) as a method of restoring gut microbial diversity is of increasing interest as a therapeutic approach in the management of UC. The current literature lacks consensus about the dose of FMT, route of administration and duration of response.

Methods and analysis This single-blinded randomised trial will explore the feasibility of FMT in 30 treatment-naïve patients with histologically confirmed distal UC limited to the recto-sigmoid region (up to 40 cm from the anal verge). This study aims to estimate the magnitude of treatment response to FMT under controlled conditions. The intervention (FMT) will be administered by rectal retention enema. It will test the feasibility of randomising patients to: (i) single FMT dose, (ii) five daily FMT doses or (iii) control (no FMT dose). All groups will receive standard antibiotic gut decontamination and bowel preparation before FMT. Recruitment will take place over a 24-month period with a 12-week patient follow-up. Trial objectives include evaluation of the magnitude of treatment response to FMT, investigation of the clinical value of metabolic phenotyping for predicting the clinical response to FMT and testing the recruitment rate of donors and patients for a study in FMT. This feasibility trial will enable an estimate of number of patients needed, help determine optimal study conditions and inform the choice of endpoints for a future definitive phase III study.

Ethics and dissemination The trial is approved by the regional ethics committee and is sponsored by Abertawe Bro Morgannwg University's Health Board. Written informed consent from all patients will be obtained. Serious adverse events will be reported to the sponsor. Trial results will be disseminated via peer review publication and shared with trial participants.

Trial registration number ISRCTN 58082603; Pre-results.

Strengths and limitations of this study

- This is one of the first trials to have a homogeneous study group of newly diagnosed and treatment naïve patients with ulcerative colitis (UC).
- The trial will not only show the efficacy of faecal microbiota transplantation (FMT) treatment by rectal administration, but also help to define the optimal number of doses of FMT for treatment of UC.
- Metabolomic analysis will demonstrate mechanism of action of FMT in treatment responders.
- Patient's reported quality of life measures will be reported.
- This study is limited by a short (12 weeks) follow-up period.

INTRODUCTION

Ulcerative colitis (UC) is a chronic relapsing-remitting mucosal inflammatory bowel disease (IBD). Clinical features include rectal bleeding, diarrhoea, faecal urgency, fatigue and weight loss. The aetiology of UC is believed to be multifactorial involving immune dysregulation, mucosal disruption and genetic predisposition, though the precise cause is poorly understood.[1]

There is no curative treatment at present; thus the aim of current management is induction and maintenance of remission with immunosuppressive agents. Failure of medical therapy or refractory disease may require major resectional surgery with temporary or permanent ostomy formation. UC is also a recognised risk factor for colorectal cancer requiring lifelong surveillance.[2] However, it is uncertain how to predict which group of patients will respond to medical therapy.

The human gut microbiota consists of a diverse biological environment comprising bacteria, viruses and fungi within the gut

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lumen and lining mucosa. The biodiversity of the gut microbiota is a dynamic process and is known to be affected by age, diet and lifestyle.[3 4] It has been referred as a hidden metabolic organ through its major role is as a driver of metabolic and immunological communications and the regulation of the immunological processes within the intestinal mucosa.[5–7] Disruption of the gut microbiota, also called dysbiosis, has been suggested to be responsible for not only intestinal pathology such as *Clostridium difficile* infection, but also for systemic conditions such as obesity, diabetes mellitus and IBD including UC.[3 8] The role of gut microbiota with host–microbiome interactions are likely to be a key driver in the pathogenesis of UC.[9 10] Antibiotics, which alter the human gut microbiome, have been shown to contribute to UC activity,[11] whereas probiotics have been implicated in UC remission.[12] The gut microbiota of patients with UC lacks diversity[13 14] and *Bacteroidetes* and *Firmicutes* are found in significantly less amounts in the microbiota of patients with UC.[13 15] Furthermore, reduced amounts of bacterial producers of short-chain fatty acids (SCFA) (butyrate, propionate and acetate) are found in the microbiota of patients with UC.[16 17] These SCFAs are products of starch fermentation from gut bacteria and are believed to have anti-inflammatory properties. Moreover, recent studies have shown that butyrate produced from *Faecalibacterium prausnitzii* not only has anti-inflammatory properties, but also provides the major nutrient for colonocytes,[18] and prevents intestinal mucosa atrophy and colonocyte autophagy.[19] A number of studies demonstrate that the butyrate producer *F. prausnitzii* was less abundant in patients with UC.[20–22] Moreover, recent studies have suggested that not only living bacteria may be responsible inflammatory process of UC, but also bacterial specific components and structures, antimicrobial compounds and metabolites produced by bacteria may contribute to the gut microenvironment and thus its inflammatory process.[23] Understanding of a critical role of secondary metabolites has also been highlighted recently by Buffie *et al* recently as they have indicated that certain species may inhibit *C. difficile* with their secondary metabolites, including secondary bile acids by *Clostridium scindens*.[24 25] Although the role of fungi in the human microbiome has not yet been fully understood, recent studies suggest microfragments of chitin, which is a substance produced by fungi and insects, display a significant immunomodulatory impact in the inflammatory process.[26 27] This suggests that not only viable common gut anaerobic micro-organisms, but also products and particles from other micro-organisms may be responsible for dysregulation of the immune response. Despite extensive studies, no single pathogen has been identified as responsible for the pathogenesis of UC. The current consensus is that the loss of certain bacterial strains with immunomodulatory as well as mucosal regulatory functions leads to gut dysbiosis, resulting in the pathogenesis of UC. Faecal microbiota transplantation (FMT) is an infusion of a faecal suspension from a healthy individual (donor) to restore the dysbiosis of affected individuals (recipient). Since the approval of FMT in the management of recurrent *C. difficile* infections in 2014 by the National Institute for Health and Care Excellence (NICE), FMT has been of increasing interest as a therapeutic approach in the management of UC. If we can successfully and durably alter the colonic microbiota,[28] it may be possible to achieve complete remission of this chronic debilitating disease without the use of lifelong immunosuppression or the need for major gastrointestinal surgery. The ability to induce remission and establish the microbiological basis for this would change the treatment paradigm for UC. Recent years have seen several randomised clinical studies emerging to investigate FMT in the management of UC with encouraging results.[14 29–31] Despite these studies, many unknown aspects remain in the clinical application of FMT in UC, such as the optimum dose, route of administration and frequency of treatments. Equally it is not known whether FMT is effective as a first line treatment in drug-naïve patients. To study the optimum parameters for delivering FMT in UC and estimating the clinical response, this randomised feasibility trial was designed.

The objectives of this feasibility study include evaluation of the magnitude of treatment response to FMT, investigation of the functional metabolic changes associated with FMT using a metabolic phenotyping methodology and testing the recruitment rate of donors and patients. Furthermore, we aim to measure the duration of clinical response with microbiome identification through 16S rRNA sequencing and metabolomic analysis. This will facilitate the design of a definitive multicentred study to confirm the efficacy of FMT as a first-line treatment option in UC.

Primary objectives

The primary objective of this phase II study is to estimate the magnitude of the treatment response to FMT in treatment naïve patients with UC.

Secondary objectives

- Determine the recruitment rate of donors and participants for a study of FMT.
- Determine the optimal study conditions and choice of endpoints for phase III study to include dosage and frequency of FMT treatments.
- Establish how many participants would be required for phase III to demonstrate the efficacy of FMT in the treatment of UC.

METHODS

This is a single-blinded interventional randomised feasibility study to estimate the magnitude of the treatment response to FMT in newly diagnosed patients with distal UC who are treatment naïve. Recruitment is proposed over a 2-year period with a 12-week post-treatment follow-up period. This feasibility trial will help determine the recruitment rate of donors and participants, define the optimal study conditions and choice of endpoints for

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Table 1 Intervention arms (groups 1 and 2) and control arm (group 3)

	Group 1	Group 2	Group 3
Bowel decontamination and preparation	Yes	Yes	Yes
FMT treatment dose	1	Five consecutive days (single treatment per day)	None
Number of participants	12	12	6

FMT, faecal microbiota transplantation.

a phase III definitive study. It will also allow us to establish how many participants would be required at phase III to demonstrate the efficacy of FMT in the treatment of UC.

Trial design

We aim to recruit 30 subjects with histologically confirmed UC, whose disease is confined to the recto-sigmoid area (defined here as within 40 cm from the anal verge) and who are treatment naïve. Participants will be randomly assigned to study groups through a web-based application hosted by University of Aberdeen.

Eligible patients will be randomised into one of three groups with an allocation ratio of 2:2:1 as shown in table 1. Groups 1 and 2 are the intervention arms and 12 subjects will be assigned to each group respectively. Group 3 is the control arm and six subjects will be randomised into this group.

Intervention arms: groups 1 and 2

Participants randomly allocated to group 1 will receive one single FMT treatment administered as a rectal retention enema. Participants in group 2 will receive a single FMT treatment on five consecutive days (total of five treatments) also administered by rectal retention enema.

Control arm: group 3

Participants randomly allocated to group 3 will receive the pre-FMT preparation with antibiotics and bowel preparation but will not receive active FMT treatment.

Endpoints

Paired primary endpoints

- Remission of UC (mucosal healing) at 12 weeks as assessed by blinded sigmoidoscopy. Assessment defined as Mayo score ≤2 with an endoscopic Mayo score of 0
- Proportion of successful engraftment of donor faecal microbiota at 12 weeks in each group as analysed by 16S sequencing and longitudinal diversity index

Secondary endpoints

- Rate of recruitment of patients
- Disease specific scores after treatment using IBDex severity scoring index,[32] Crohns and Ulcerative Colitis Questionnaire (CUCQ)-32 severity scoring index[33] and Mayo scoring system[34]
- Histological grading of colitis severity after treatment
- Mucosal immunological response to treatment (tissue IL-10 and IL-21 by ELISA)
- Rate of development of adverse effects to FMT

Participant selection

Potential participants will be identified by their usual clinicians in clinics and endoscopy units within the Health Board. Each potential participant will be screened for eligibility once he or she is referred to the research team. All subjects must have a definitive histological diagnosis of UC before enrolment as made by a gastrointestinal pathologist with a special interest in colitis. The minimum required microscopic features include cryptitis, crypt abcesses, crypt distortion and mucin depletion in the absence of granulomata. Participants with any features not consistent with UC will be excluded. A minimum time period of 1 month from identification to screening will exclude participants with acute self-limiting colitis.

A written patient information sheet will be provided and participants will be offered a minimum of 24 hours to consider enrolment before providing written informed consent.

During the screening visit, the study will be fully explained, and consent will be obtained if the subject satisfies all inclusion and exclusion criteria (box 1).

Interventions and investigational products

All three study groups will complete a 10-day course of oral antibiotics (Metronidazole 400 mg, vancomycin 500 mg, rifampicin 150 mg twice daily), which should be completed at least 48 hours before the first FMT treatment. This will allow the poorly absorbed vancomycin to wash out of the gastrointestinal tract. Patients should therefore start the 10-day course of antibiotics 12 days before the first FMT is given. These antibiotics were chosen following the recently published guidelines on FMT in clinical practice[35] towards whole gut decontamination. Additionally, all participants will receive bowel preparation (polyethylene glycol, 2 L) on the day before transplantation to prepare the lumen for engraftment of the FMT treatment and to minimise interference from the existing gut microbiota.

Investigational product

The investigational product is donated faecal material from healthy volunteers who are unrelated and non-cohabiting to the study participants. The FMT products are obtained either from Wessex stool bank or material that has been locally processed using the identical FMT preparation technique by a physician for the purposes of the research trial. The pellet is resuspended and frozen in 20% glycerol and stored for up to 8 weeks at −80°C until the day of treatment. Donors are screened for infections in accordance with current best practice[35] (table 2). In the case of multiple treatments (group 2) all doses

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Box 1 Participant selection criteria

Inclusion criteria

- Newly diagnosed histologically confirmed ulcerative colitis (UC) with inflammation limited to the rectum or recto-sigmoid (within 40 cm of anal verge as measured by flexible sigmoidoscopy).
- Age 18 years and older.
- Able to give full informed written consent.
- Willing to return for sequential faecal microbiota transplantation dosing and endoscopic assessment.
- Not in receipt of conventional medical treatment for colitis such as steroids or 5-aminosalicylic acid, that is, treatment naïve.

Exclusion criteria

- Patients without a definitive diagnosis of UC (for example, diagnosis of Crohn's disease or infectious colitis).
- Colitis extending beyond 40 cm from the anal verge.
- Diagnosis of acute severe colitis (defined as greater than six blood-stained stools per 24 hours with one of the following: pulse rate>90/ temperature>37.8° /haemoglobin < 105 g/L / erythrocyte sedimentation rate>30).
- Abdominal tenderness on examination.
- Already commenced standard medical therapy for UC.
- Contraindication to oral bowel preparation.
- Allergy to study antibiotics.
- Age less than 18.
- Patient is within a vulnerable group, defined as people who are unable to take care of him or herself, or unable to protect him or herself against significant harm or exploitation.
- Pregnant.
- Immunosuppressed for example, transplant patient.
- Known communicable disease or at least 2 weeks full recovery from infectious disease for example, chickenpox.
- Systemic autoimmunity, or atopic diseases.
- Previous prosthetic implant (for example, metallic heart valve, joint replacement, ventricular-peritoneal shunt, cardiac stent),
- Chronic pain syndromes (for example, fibromyalgia, chronic fatigue).
- Neurologic, neurodevelopmental or neurodegenerative disorders.
- Depression (requiring therapy).
- Obesity (body mass index>35).
- Malignancy.
- Use of antibiotics for any indication within the past 3 months.
- Foreign travel to areas of enteric disease prevalence within 3 months.
- High-risk sexual behaviour (examples: sexual contact with anyone with HIV/human T-lymphocyte virus/AIDS or hepatitis B/C carrier, men who have sex with men).
- Known exposure to HIV or hepatitis B/C.
- Current/previous use of injected drugs or intranasal cocaine.
- Tattooing, piercing, cosmetic botulinum toxin or permanent makeup within 120 days (as per Welsh blood transfusion guidelines).
- Recent blood transfusion, tissue/organ transplant or skin graft.
- Risk factors for variant Creutzfeldt-Jakob disease, for example, blood transfusion or transplant after 1 January 1980.

are obtained from the same donor to minimise variation. Faecal microbiota of the donated faecal samples is studied using 16S rRNA analysis. This will be used as a reference for the effect of FMT treatments, evaluation of magnitude of treatment response to FMT and durability of engraftment after FMT.

Table 2 Infectious disease screening

Blood tests	► Cytomegalovirus. ► Epstein-Barr virus. ► Hepatitis A virus. ► Hepatitis B virus. ► Hepatitis C virus. ► Hepatitis E virus. ► Syphilis. ► HIV-1 and HIV-2. ► *Entamoeba histolytica.* ► Human T-lymphotropic virus types I and II antibodies. ► *Strongyloides stercoralis.*
Faecal tests	► Detection of *C. difficile.* ► Detection of enteric pathogens, including *Salmonella, Shigella.* ► *Campylobacter, Escherichia coli O157 H7, Yersinia*, Vancomycin-resistant enterococci, methicillin-resistant *Staphylococcus aureus*, Gram-negative mutidrug-resistant bacteria. ► Norovirus. ► Antigens and/or acid fast staining for *Giardia sp* and *Cryptosporidium sp.* ► Protozoa (including *Blastocystis hominis*) and helminths.

Administration of investigational product

All three study groups will complete a 10-day course of oral antibiotics (vancomycin 500 mg; metronidazole 400 mg, rifampicin 150 mg—all taken twice daily) and bowel preparation (polyethylene glycol 2 L on the day before transplantation). The first FMT treatment dose will be commenced 48 hours after the final dose of antibiotics to preserve the activity of the FMT. Frozen FMT will be thawed over 4 hours at room temperature prior to infusion, which will subsequently be diluted to 250 mL with non-bacteriostatic normal saline prior to infusion. The subjects of groups 1 and 2, who receive FMT treatment, will also be given loperamide 4 mg orally 30 min prior to administration to maximise the chance of enema retention. Each participant receives 50 mL of enema every 15 min over 60 min. The subjects will be encouraged to retain the treatment samples as long as possible (ideally more than 1 hour).

Study setting

Recruitment will take place from clinics and endoscopy units within the Abertawe Bro Morgannwg University Health Board, Swansea. FMT will be administered at the Joint Clinical Research Facility within Swansea University.

Randomisation

Study participants will be randomised 2:2:1 by a web-based method hosted by the University of Aberdeen's Health Services Research Unit. The simple randomisation process employed in this allocation was not stratified by any factors (eg, age, gender). We aim to update the

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randomisation process based on the results of this feasibility study for potential stratifying factors in phase III.

Blinding

The trial statistician, the assessing independent endoscopist and the pathologist undertaking macroscopic and microscopic disease assessments will be blinded to the treatment allocation.

Participant timeline and schedule of assessment

Figure 1 and table 3 show the follow-up schedule and assessment for the trial. At baseline the study participants will undergo assessment for disease activity with validated tools (CUCQ-32, IBDex and Mayo Score) alongside a full history and physical examination. Baseline biopsies of the rectum for 16S rRNA analysis and immunological

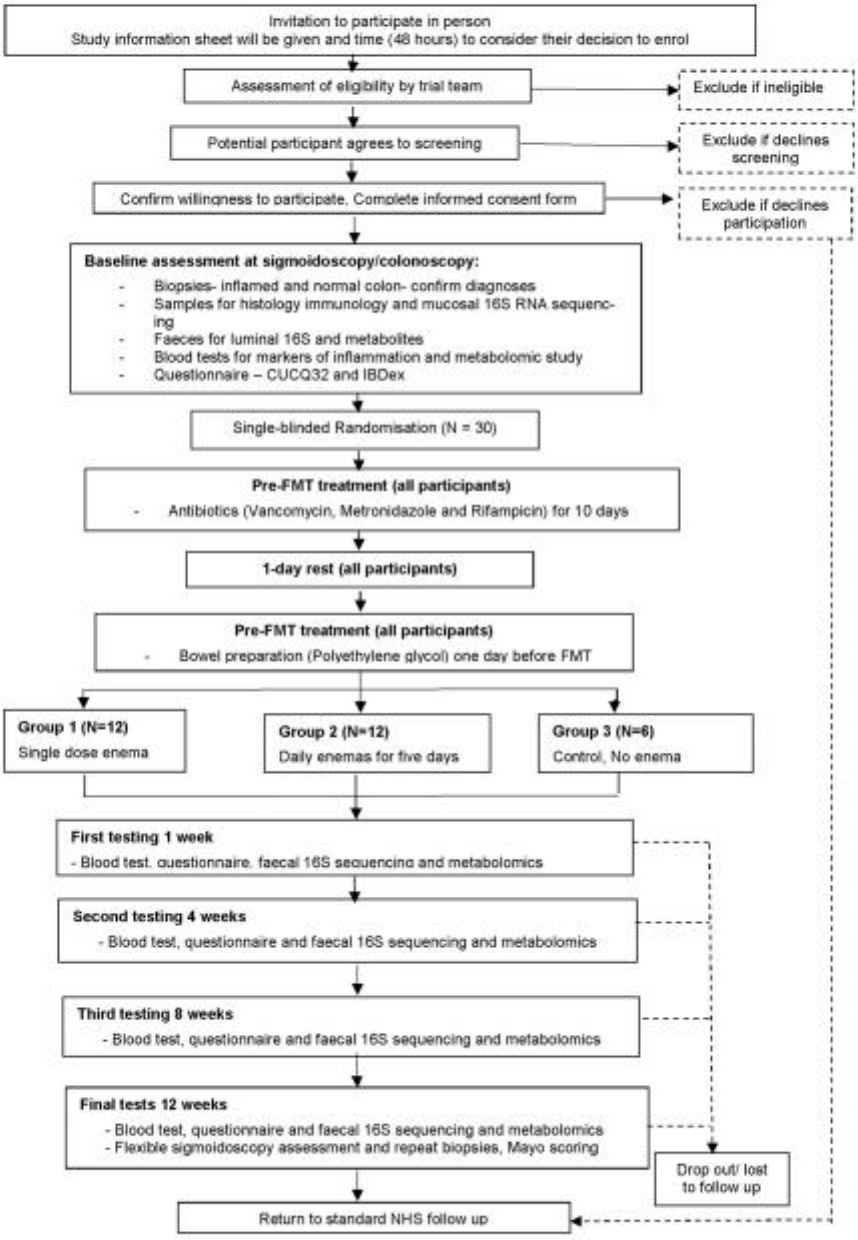

Figure 1 Study scheme flowchart.

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Table 3 Follow-up schedule and assessments

	Baseline	Week 1	Week 4	Week 8	Week 12
Questionnaires					
CUCQ-32	•	•	•	•	•
IBDex	•	•	•	•	•
Mayo score	•				•
Endoscopy assessment					
Sigmoidoscopy	•				•
Rectal biopsy	•				•
Histology assessment					
Histological grading	•				•
Mucosal 16S sequencing	•				•
Mucosal IL-10	•				•
Mucosal IL-21	•				•
Blood tests					
Renal profile	•	•	•	•	•
Liver profile	•	•	•	•	•
Full blood count	•	•	•	•	•
C-reactive protein	•	•	•	•	•
Metabolomic profile	•	•	•	•	•
Faecal sample assessment					
16S sequencing	•	•	•	•	•
Metabolomic profile	•	•	•	•	•

studies (IL-10 and IL-21), faecal samples for 16S rRNA analysis and metabolomic profile, blood tests (renal function, liver function, full blood count, C-reactive protein, metabolomic profile) will be obtained. Furthermore, 16S rRNA analysis for the donors' faecal samples is performed. Subsequently, this will be studied together with 16S rRNA analysis of the participant's faecal samples for the study of durability of engraftment after FMT treatments during a 12-week follow-up period.

Follow-up visits will take place at week 1, 4, 8 and 12 for all the three study groups. Participants will undergo clinical examination, blood and faecal testing to include faecal microbiota profiling using the 16S rRNA analysis and metabolomic profile and complete disease activity scoring questionnaires (CUCQ-32 and IBDex) at baseline and thereafter at 1 week. At the final assessment (week 12), all subjects will also undertake a repeat flexible sigmoidoscopy for macroscopic assessment and biopsies for degree of inflammation or confirmation of remission. Participants who relapse or fail to improve after FMT will be offered conventional medical therapy.

Study participants will be instructed to inform the treating physician of any infectious symptom or new medical condition that develops after receiving FMT and a patient registry will be maintained.

Withdrawal

Participants may be withdrawn from the study if

- They wish to terminate treatment and/or follow-up assessments.
- Clinical features worsen during FMT or the 12 week follow-up period.
- The participant is non-compliant with the study in a manner that is either harmful to their health or interferes with the validity of the study results.
- Participants who withdraw their consent may not wish for their data to be used—if this is the case then it will be deleted.

Data collection and management

Data collection will be performed at baseline, week 1, 4, 8 and 12 as described in table 3. All data is to be recorded on the case report form (CRF) in an anonymised format against a unique participant number.

Data will be transferred to a computer database without patient identifiable data and analysed once all results have been collected. The trial database will have built in measures to assess data quality at time of input and stored securely.

Metabolic profiling

We will use both untargeted (1 hour NMR) and targeted quantitative approaches such as high-performance liquid chromatography-mass spectrometry to analyse a panel of gut microbial cometabolites involved in cell signalling, namely SCFAs, bile acids, indoles and cresols and branch

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chain amino acids. This will include a novel eicosanoid assay[36] for precision measurement of pro and anti-inflammatory regulators and the use of a bile acid assay.[37] Metabolome data will be analysed by several multivariate ordinations including principal component analyses, non-metric multidimensional scaling Kruskal-Wallis independent tests, and multivariate analysis of variance with Bonferroni correction. We will create receiver operating curves for both multivariate models and individual metabolites for key clinical outcomes. Metabolic reaction networks of metabolites found differentially expressed between different transplants will be created using the MetaboNetworks software.[38]

Participant rights and confidentiality

The chief investigator will be the custodian of the data. Information with regards to study participants will be kept confidential and managed in accordance with the Data Protection Act, National Health Service Caldicott Guardian, The Research Governance Framework for Health and Social Care and Research Ethics Committee Approval.

There will be no patient identifiable data on the CRF and a unique participant number will be allocated. The principal investigator will hold the key to the coded number of the participants only. Only the principal investigator will have access to the patient identifiable information.

Statistical analysis

Both descriptive and exploratory data analysis will be performed. For each group, we will calculate the number of participants approached and/or assessed for eligibility, randomised and received the treatment. Thus, we will calculate the recruitment and retention rate along with the rate of adverse events. Descriptive statistics (mean, SD, 95% CI) for continuous outcomes (eg, CUCQ-32 Score, Mayo score) and raw count (n, %) for categorical outcomes (eg, renal profile, liver profile, histological grading) will be reported as per the clinical endpoints.

All these summary statistics will be provided as per baseline and other follow-ups (as appropriate to the outcome measure) and with respect to the three treatment arms. All the analysis and data preparation will be performed using SPSS v.22.0 as a validated statistical software for clinical trials.

Safety measures

An adverse event (AE) is defined as any untoward medical occurrence in a patient after administration of the study intervention (FMT) that does not necessarily have to have a causal relationship with this treatment. Serious adverse event (SAE) is any adverse experience occurring during or after FMT that results in either death, life-threatening experience or requiring inpatient hospitalisation, persistent or significant disability or incapacity. SAEs will be notified to the study sponsor within 24 hours and to the Research Ethics Committee (REC) within 15 days. AEs that are expected for patients undergoing FMT, and symptoms expected from UC, are specified in the protocol and will not require to be reported as adverse events. FMT-related AEs are procedure-related symptoms such as bloating, transient fever or abdominal discomfort as reported by previously reported studies.

Quality assurance

The research and development quality assurance officer has performed a monitoring prioritisation assessment to assess the impact of trial participation on the rights and safety of participants and the reliability of trial results. This has guided the development of procedures in the trial with respect to informed consent, confidentiality and trial monitoring. Monitoring visits to the site will be made every 3 months during the study to ensure that all aspects of the protocol are followed. The quality assurance officer will also monitor the study after the first participant has been recruited. The monitoring visit timeframe can be changed depending on the monitoring findings. A quality assurance programme is also in place to ensure adherence to the study protocol. Major and minor deviations will be collected.

Endosocopy: One of several JAG accredited gastroenterologists or colorectal surgeons from hospitals of the Abertawe Bro Morgannwg University's Health Board will perform the sigmoidoscopy assessment at baseline and week 12. The study team will ensure that the endoscopist performing the 12-week assessment is blinded to the intervention that the patient has received. Endoscopic photographs taken at baseline and at final assessment will be independently assessed by a blinded expert to provide quality assurance for this outcome measure.

Pathology: A standardised protocol based on RCPath guidelines will be used for histological assessment of the disease as per standard of care by consultant pathologists.

Patient and public involvement

Patients with UC were surveyed during the trial design stage to ascertain willingness to participate in the trial as described. All seven patients approached indicated by return of questionnaire their willingness to be recruited into the trial.

The investigators will invite IBD-specific charitable organisations and their patient representatives to help disseminate the findings of the feasibility trial and to design phase III.

Ethics and dissemination

The chief investigator will ensure that the trial is conducted in compliance with the principles of the Declaration of Helsinki (1996), and in accordance with all applicable regulatory requirements including but not limited to the Research Governance Framework, Trust and Research Office policies and procedures and any subsequent amendments. Written informed consent will be obtained from all participants. SAEs will be reported to the study sponsor and the regional ethics committee.

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Table 4 Uncertainties for the optimal application of faecal microbiota transplantation (FMT) in ulcerative colitis (UC)

Human gut microbiota	► Responsible pathogens and their roles. ► Microbiome profiling techniques.
FMT preparations	► Frozen versus fresh. ► Donor screening protocol. ► Preparation methodology.
Donors	► Related versus unrelated. ► Single donor versus multiple donors.
Pre-medications/preparation	► Bowel preparation. ► Antibiotics versus non-antibiotics.
FMT in clinical application	► Dose. ► Administration routes. ► FMT alone versus with other traditional medications. ► Durability of engraftment. ► Who to treat—active, remission, refractory. ► Adverse effects. ► Long-term effects and safety. ► Long-term effects after transplant.
Clinical remission	► How to assess clinical response. ► How to define clinical remission. ► When to stop FMT treatment. ► Maintenance dose required for remission. ► Postremission dietary modification.

Trial results will be disseminated through oral presentations at national conferences and through peer-reviewed publication, which will include named members of the Trial Management Group (TMG) who meet the three criteria of scholarship (design, execution, analysis and/or interpretation of the data), authorship (drafting, reviewing and revision of the manuscript) and approval (approving the manuscript to be published). Participants in the study will be given a copy of the results and a final report will be written by the TMG for the funding body and the REC. Results will be used to aid in the development of a definitive phase III trial.

DISCUSSION

A recently published systematic review on the usage of FMT in IBD concluded that overall 36% of patients with UC achieved clinical remission (a total of 41 studies and four randomised controlled trials (RCTs)).[39] Meta-analysis, which included 4 RCTs (a total of 140 individuals), demonstrated that FMT was significantly linked to clinical remission with a pooled OR of 2.89, 95% CI of 1.36 to 6.13 and p-value of 0.016.

The number of FMT studies with high methodological quality has increased of late, yet the optimal conditions for durable FMT engraftment and maximal remission are presently unclear for UC. Table 4 summarises the current knowledge gaps in the application of FMT in UC.

Current studies are difficult to interpret as there is no universally agreed definition of remission as an endpoint in UC clinical trials to date.[40] Furthermore, a lack of homogeneity of clinical trial protocols makes comparison of such studies more difficult to comprehend and these clinical trials are no exception. Moreover, different clinical trials use different patient groups, donors, treatment dose, routes, frequency and pretreatment medications. These multiple variables make the comparison of studies very challenging, although all studies appear to demonstrate promising results for the usage of FMT in active UC. Finally, and most importantly, patients recruited in published RCTs had been on previous conventional medical therapy until given the FMT treatment if not being assigned to further medical treatment. This makes the interpretation of the magnitude of treatment response to FMT very difficult.

Although the efficacy of FMT in UC appears to be promising, more clarity is required around optimal treatment conditions through a rigorous study. This study will estimate the efficacy of rectally administered FMT in treatment naïve patients towards the design of a definitive trial. This phase II study allows us not only to estimate the magnitude of treatment response to FMT in UC, but also to determine the changes and durability of engraftment of the gut microbiota after FMT treatment. Furthermore, we will study the dose response by comparing one dose only and five daily doses towards establishing the optimum dosage of rectally administered FMT treatment for UC. There is a fundamental lack of mechanistic data to support the use of FMT in clinical practice. Bacteria represent a diverse and highly active chemical engine that creates a suite of biologically active small molecules through secondary metabolism. The critical function of these target metabolites in the initiation and maintenance of systemic inflammation remains poorly defined and this trial will provide a detailed insight into the role

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of the gut microbiome in UC therapy that have the potential to stratify care in the future and improve the precision of this intervention.

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Contributors DAH and ADR are responsible for the idea for the trial. MJ, ALC, ADR, MDH, SI, JK, APD, PER, TSW, GJJ, JGW and DAH have drafted and the manuscript and/or provided critical revision. ADR, MDH, SI, JK, APD, PER, TSW, GJJ, JGW and DAH have made substantial contributions to the conception and design of the work and subsequent protocol revisions. MJ, ALC, ADR, MDH, SI, JK, APD, PER TSW, GJJ, JGW and DAH all agree to be accountable for all aspects of work ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Competing interests None declared.

Patient consent Not required.

Ethics approval Research Ethics Committee (Wales REC6) REC reference 15/WA/0262.

Provenance and peer review Not commissioned; externally peer reviewed.

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Appendix 9; SPIRIT Checklist for Protocol for faecal microbiota transplantation in ulcerative colitis: a randomised feasibility study

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	6
	2b	All items from the World Health Organization Trial Registration Data Set	
Protocol version	3	Date and version identifier	6
Funding	4	Sources and types of financial, material, and other support	6
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	4
	5b	Name and contact information for the trial sponsor	3
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	6

	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	_____
Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	____7_____
	6b	Explanation for choice of comparators	____7-9_____
Objectives	7	Specific objectives or hypotheses	____9_____
Trial design	8	Description of trial design including type of trial (e.g., parallel group, crossover, factorial, single group), allocation ratio, and framework (e.g., superiority, equivalence, noninferiority, exploratory)	____10_____
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (e.g., community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	____10_____
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (e.g., surgeons, psychotherapists)	____13-15_____
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	___11, Table 1

	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (e.g., drug dose change in response to harms, participant request, or improving/worsening disease)	____12_____
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (e.g., drug tablet return, laboratory tests)	____18_____
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	____14_____
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (e.g., systolic blood pressure), analysis metric (e.g., change from baseline, final value, time to event), method of aggregation (e.g., median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	____13_____
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	____17. Table 4, Figure 1_____
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	____13_____
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	___13_____

Methods: Assignment of interventions (for controlled trials)

Allocation:

Sequence generation	16a	Method of generating the allocation sequence (e.g., computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (e.g., blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	___17_____
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (e.g., central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	____17__
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	____17__
Blinding (masking)	17a	Who will be blinded after assignment to interventions (e.g., trial participants, care providers, outcome assessors, data analysts), and how	_____17____
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	____17_____

Methods: Data collection, management, and analysis

Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (e.g., duplicate measurements, training of assessors) and a description of study instruments (e.g., questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	_19
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	__19___

Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (e.g., double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	__19_____
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	_19_____
	20b	Methods for any additional analyses (e.g., subgroup and adjusted analyses)	N/A_____
	20c	Definition of analysis population relating to protocol non-adherence (e.g., as randomised analysis), and any statistical methods to handle missing data (e.g., multiple imputation)	___N/A _____
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	_19_____
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	___n/a__
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	___20_____
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	_20_____

Ethics and dissemination

Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	21_____
Protocol amendments	25	Plans for communicating important protocol modifications (e.g., changes to eligibility criteria, outcomes, analyses) to relevant parties (e.g., investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	n/a_____
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	5,13, 21,_____
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	n/a_____
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	19_____
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	n/a_____
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	n/a
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	N/A_____
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (e.g., via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	21_____
	31b	Authorship eligibility guidelines and any intended use of professional writers	21

	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	N/A __
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	__N/A_____
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	__N/A____

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

Appendix 10: Calculation of effect size and eta squared to estimate sample size

Kruskal-Wallis test is a non-parametric test comparing three or more groups, which produces chi-squared values with (the number of group – 1) degree of freedom. This chi-squared value can subsequently be transformed to F value. From this F value, partial eta squared (which is the same as eta squared in Kruskal-Wallis test) can be obtained (332).

$F(dfn, dfd) = Chi^2 / (k-1)$

dfn = numerator degrees of freedom

dfd = denominator degrees of freedom

k = number of groups

$\eta^2 = (F \times dfn) / (F \times dfn + dfd)$

Chi^2 of changes in Mayo score amongst three intervention groups was calculated using SPSS, which gave $Chi^2 = 8.294$.

$F(3-1, 17-3) = 8.294 / (3-1) = 4.147$

$\eta^2 = (4.147 \times (3-1)) / (4.147 \times (3-1) + (17-3)) = 0.372$

The effect size is 0.372.

Appendix 11: CUCQ-32 questions and answers of all participants

Table 36: CUCQ-32 Question 1 "On how many days over the last two weeks have you had loose or runny bowel movements?"

Intervention Group	Subject ID	Baseline (Days)	Week 1 (Days)	Week 4 (Days)	Week 8 (Days)	Week 12 (Days)
Control	5	0	3	1	0	0
	6	6	7	8	Withdraw	Withdraw
	13	7	10	14	6	10
	17	2	No data	1	1	1
1 Dose	1	14	14	14	14	14
	3	3	8	2	0	0
	8	4	3	0	0	0
	9	0	2	1	3	8
	11	10	1	2	10	10
	12	2	3	0	1	0
	18	1	6	3	0	0
5 Doses	2	14	10	14	14	14
	4	0	14	3	1	0
	7	8	9	10	6	14
	10	14	2	14	14	No data
	14	14	10	8	10	6
	15	14	12	10	4	6
	16	14	9	10	14	10

Table 37: Question 2 “On how many days in the last two weeks have you noticed blood in your stools?”

Intervention Group	**Subject ID**	**Baseline (Days)**	**Week 1 (Days)**	**Week 4 (Days)**	**Week 8 (Days)**	**Week 12 (Days)**
Control	5	0	0	0	0	0
	6	3	0	14	Withdraw	Withdraw
	13	7	14	14	14	14
	17	0	No Data	1	1	1
1 Dose	1	14	14	14	14	14
	3	14	1	5	0	0
	8	4	0	2	0	0
	9	14	5	3	5	14
	11	12	3	0	14	14
	12	5	0	0	1	0
	18	7	14	14	0	0
5 Doses	2	4	0	4	10	14
	4	14	7	14	1	14
	7	3	1	8	3	2
	10	14	6	14	14	No data
	14	12	14	10	10	12
	15	14	10	7	4	4
	16	10	0	0	14	0

Table 38: Question 3 "On how many days over the last two weeks have you felt tired?"

Intervention Group	**Subject ID**	**Baseline (Days)**	**Week 1 (Days)**	**Week 4 (Days)**	**Week 8 (Days)**	**Week 12 (Days)**
Control	5	3	2	0	0	0
	6	6	8	10	Withdraw	Withdraw
	13	7	14	14	14	14
	17	7	No data	0	3	0
1 Dose	1	14	14	8	14	14
	3	14	7	3	3	5
	8	14	0	2	0	0
	9	14	4	3	7	14
	11	7	4	0	10	10
	12	14	9	4	0	3
	18	7	14	14	14	14
5 Doses	2	14	14	14	10	10
	4	14	7	2	14	0
	7	10	10	8	2	1
	10	14	2	14	14	No Data
	14	0	6	2	12	3
	15	5	10	7	3	5
	16	2	2	0	6	4

Table 39: Question 4 “In the last two weeks have you felt frustrated?”

Intervention Group	Subject ID	Baseline	Week 1	Week 4	Week 8	Week 12
1	5	a	a	a	a	a
	6	c	c	c	Withdraw	Withdraw
	13	c	d	d	b	c
	17	b	No Data	b	a	a
2	1	b	b	b	b	c
	3	b	b	a	a	a
	8	b	a	a	a	a
	9	c	a	a	b	b
	11	b	a	a	b	b
	12	b	a	a	a	a
	18	No answer	a	b	a	a
3	2	b	b	c	b	b
	4	c	b	a	a	a
	7	b	b	c	b	b
	10	c	b	b	c	No Data
	14	b	d	b	a	b
	15	a	b	b	b	b
	16	b	b	b	b	c

Table 40 : Question 5 “In the last two weeks, has your bowel condition prevented you from carrying out your work or other normal activities?”

Intervention Group	Subject ID	Baseline	Week 1	Week 4	Week 8	Week 12
1	5	a	a	a	a	a
	6	c	b	c	Withdraw	Withdraw
	13	c	d	b	b	c
	17	a	No Data	a	a	a
2	1	b	b	b	b	b
	3	b	a	a	a	a
	8	a	a	a	a	a
	9	b	a	a	a	b
	11	a	a	a	a	a
	12	b	a	a	a	a
	18	d	a	a	a	a
3	2	c	a	a	a	b
	4	c	b	b	a	a
	7	b	c	c	b	a
	10	b	b	b	b	No Data
	14	a	a	a	a	a
	15	a	a	a	a	a
	16	a	a	a	a	a

Table 41 : Question 6 "On how many days over the last two weeks have you opened your bowels more than three times a day?"

Intervention Group	**Subject ID**	**Baseline (Days)**	**Week 1 (Days)**	**Week 4 (Days)**	**Week 8 (Days)**	**Week 12 (Days)**
1	5	0	2	0	1	0
	6	14	7	14	Withdraw	Withdraw
	13	7	14	9	9	14
	17	0	No Data	2	0	2
2	1	4	14	7	14	14
	3	3	5	0	0	0
	8	0	0	2	1	0
	9	7	2	5	6	10
	11	3	0	1	7	2
	12	0	0	0	0	0
	18	2	2	7	0	6
3	2	14	3	14	14	14
	4	14	9	4	2	2
	7	8	10	14	4	2
	10	14	2	14	14	No data
	14	14	12	6	6	12
	15	4	3	3	4	2
	16	0	0	0	2	2

Table 42: Question 7 "On how many days over the last two weeks have you felt full of energy?"

Intervention Group	Subject ID	Baseline (Days)	Week 1 (Days)	Week 4 (Days)	Week 8 (Days)	Week 12 (Days)
1	5	6	7	14	12	10
	6	4	0	0	Withdraw	Withdraw
	13	0	0	0	0	0
	17	5	No Data	12	2	14
2	1	0	1	0	0	0
	3	1	5	8	14	6
	8	0	5	6	10	10
	9	0	4	12	0	0
	11	0	0	7	0	0
	12	0	0	0	0	2
	18	0	1	0	0	0
3	2	0	0	0	0	0
	4	0	0	0	0	8
	7	3	2	0	5	8
	10	0	3	0	5	No Data
	14	12	7	7	12	3
	15	5	3	3	3	4
	16	0	0	0	2	0

Table 43: Question 8 "In the last two weeks did your bowel condition prevent you from going out socially?"

Intervention Group	Subject ID	Baseline	Week 1	Week 4	Week 8	Week 12
1	5	a	a	a	a	a
	6	b	a	c	Withdraw	Withdraw
	13	d	d	c	d	c
	17	a	No Data	a	a	a
2	1	b	a	a	a	b
	3	c	b	a	a	a
	8	a	a	a	a	a
	9	a	a	a	b	a
	11	b	a	a	a	a
	12	a	a	a	a	a
	18	No data	a	a	a	a
3	2	d	a	a	a	a
	4	c	b	b	a	a
	7	b	b	c	b	a
	10	b	b	b	b	No Data
	14	b	a	a	a	a
	15	a	a	a	a	a
	16	a	No Answer	a	a	a

Table 44: Question 9 "On how many days over the last two weeks have your bowels opened accidentally?"

Intervention Group	**Subject ID**	**Baseline (Days)**	**Week 1 (Days)**	**Week 4 (Days)**	**Week 8 (Days)**	**Week 12 (Days)**
1	5	0	0	0	0	0
	6	3	0	0	Withdraw	Withdraw
	13	4	4	3	3	2
	17	0	No Data	0	0	0
2	1	0	0	0	0	0
	3	0	1	0	0	0
	8	0	0	0	0	0
	9	3	1	1	3	5
	11	0	0	0	0	0
	12	1	0	1	0	0
	18	3	0	0	6	0
3	2	1	0	0	1	0
	4	0	0	2	0	1
	7	1	2	4	0	1
	10	14	3	2	11	No Data
	14	2	1	1	0	1
	15	0	0	1	0	0
	16	0	0	1	0	0

Table 45: Question 10 "On how many days over the last two weeks have you felt generally unwell?"

Intervention Group	Subject ID	Baseline (Days)	Week 1 (Days)	Week 4 (Days)	Week 8 (Days)	Week 12 (Days)
1	5	0	1	1	1	0
	6	0	5	10	Withdraw	Withdraw
	13	4	14	14	14	10
	17	2	No Data	1	2	0
2	1	14	14	5	0	4
	3	10	7	2	2	2
	8	3	0	0	0	0
	9	0	0	0	0	5
	11	0	0	0	7	7
	12	0	0	0	0	0
	18	12	1	4	7	0
3	2	0	0	0	1	4
	4	5	7	0	0	0
	7	6	10	8	0	0
	10	7	2	10	7	No Data
	14	0	3	0	0	0
	15	4	4	2	1	2
	16	0	2	0	2	0

Table 46: Question 11 "In the last two weeks have you felt the need to keep close to a toilet?"

Intervention Group	Subject ID	Baseline	Week 1	Week 4	Week 8	Week 12
1	5	a	b	a	a	a
	6	c	a	c	Withdraw	Withdraw
	13	c	c	c	c	c
	17	a	No Data	b	a	a
2	1	b	b	b	c	c
	3	b	b	a	a	a
	8	b	a	a	a	a
	9	c	a	a	a	b
	11	b	b	b	b	b
	12	b	b	a	a	a
	18	d	b	b	b	a
3	2	d	a	a	d	b
	4	c	b	b	a	b
	7	c	c	c	b	b
	10	d	c	c	d	No Data
	14	a	b	b	a	c
	15	a	b	a	a	b
	16	a	a	a	a	a

Table 47: Question 12 "In the last two weeks, has your bowel condition affected your leisure or sports activities?"

Intervention Group	Subject ID	Baseline	Week 1	Week 4	Week 8	Week 12
1	5	a	a	a	a	a
	6	b	a	c	Withdraw	Withdraw
	13	c	c	c	c	c
	17	a	No Data	b	a	a
2	1	b	a	a	b	b
	3	b	a	a	a	a
	8	a	a	a	a	a
	9	b	a	a	b	b
	11	a	a	a	a	a
	12	a	a	a	a	a
	18	c	b	a	a	a
3	2	c	a	a	b	b
	4	c	b	b	a	a
	7	b	c	c	b	a
	10	c	b	b	b	No Data
	14	a	a	b	a	a
	15	a	a	a	a	a
	16	a	a	a	a	a

Table 48: Question 13 "On how many days over the last two weeks have you felt pain in your abdomen?"

Intervention Group	**Subject ID**	**Baseline (Days)**	**Week 1 (Days)**	**Week 4 (Days)**	**Week 8 (Days)**	**Week 12 (Days)**
1	5	0	1	1	0	0
	6	14	4	14	Withdraw	Withdraw
	13	7	6	14	8	8
	17	4	No Data	2	1	1
2	1	14	14	14	14	10
	3	6	3	2	0	0
	8	2	0	1	0	0
	9	0	0	0	0	6
	11	4	0	1	7	4
	12	2	1	0	0	0
	18	14	2	2	0	0
3	2	10	3	2	1	2
	4	7	7	0	0	0
	7	4	7	4	0	1
	10	0	2	10	0	No Data
	14	0	3	0	1	0
	15	3	2	2	2	2
	16	1	2	2	6	2

Table 49: Question 14 "On how many nights over the last two weeks have you been unable to sleep well (days if you are a shift worker)?"

Intervention Group	Subject ID	Baseline (Days)	Week 1 (Days)	Week 4 (Days)	Week 8 (Days)	Week 12 (Days)
1	5	0	0	0	0	0
	6	14	4	14	Withdraw	Withdraw
	13	7	14	14	5	14
	17	0	No Data	0	0	0
2	1	6	14	3	0	14
	3	6	1	5	2	3
	8	2	0	0	0	0
	9	0	0	0	0	5
	11	2	0	0	7	0
	12	7	3	3	5	0
	18	14	0	4	0	0
3	2	0	0	0	2	0
	4	7	5	0	0	0
	7	4	6	10	2	0
	10	0	0	0	7	No Data
	14	2	14	0	12	0
	15	0	0	0	0	0
	16	6	0	4	0	0

Table 50: Question 15 "On how many nights in the last two weeks have you had to get up to use the toilet because of your bowel condition after you have gone to bed?"

Intervention Group	**Subject ID**	**Baseline (Days)**	**Week 1 (Days)**	**Week 4 (Days)**	**Week 8 (Days)**	**Week 12 (Days)**
1	5	0	0	0	0	0
	6	14	4	14	Withdraw	Withdraw
	13	7	7	9	5	8
	17	0	No Data	1	0	0
2	1	7	3	0	1	2
	3	3	3	0	0	1
	8	1	1	0	1	0
	9	0	0	0	0	2
	11	0	0	0	0	0
	12	1	0	0	0	0
	18	12	0	4	2	0
3	2	7	0	0	0	0
	4	10	5	0	2	2
	7	4	10	10	2	2
	10	6	0	4	7	No Data
	14	3	2	4	0	10
	15	0	0	0	0	0
	16	0	0	0	0	0

Table 51: Question 16 "In the last two weeks have you felt depressed?"

Intervention Group	Subject ID	Baseline	Week 1	Week 4	Week 8	Week 12
1	5	a	a	a	a	a
	6	b	a	b	Withdraw	Withdraw
	13	b	c	c	d	b
	17	b	No Data	b	a	a
2	1	b	b	B	b	b
	3	b	a	a	a	a
	8	a	a	a	a	a
	9	a	a	a	a	b
	11	a	a	a	a	b
	12	b	a	a	a	a
	18	b	b	b	b	a
3	2	b	a	a	a	a
	4	b	a	a	a	a
	7	b	b	c	b	b
	10	c	b	b	c	No Data
	14	a	a	b	a	a
	15	a	a	a	a	a
	16	a	b	a	a	b

Table 52: Question 17 "In the last two weeks have you had to avoid attending events where there was no toilet close at hand?"

Intervention Group	Subject ID	Baseline	Week 1	Week 4	Week 8	Week 12
1	5	a	a	a	a	a
	6	c	a	d	Withdraw	Withdraw
	13	b	d	d	d	c
	17	a	No Data	a	a	a
2	1	a	a	a	a	a
	3	a	a	a	a	a
	8	a	a	a	a	a
	9	b	a	a	a	b
	11	a	a	a	a	a
	12	a	a	a	a	a
	18	d	a	No Data	a	a
3	2	c	a	a	a	a
	4	b	b	a	a	a
	7	a	b	a	a	a
	10	b	a	b	b	No Data
	14	b	a	a	a	a
	15	a	a	a	a	a
	16	a	a	a	a	a

Table 53: Question 18 "On how many days over the last two weeks, have you had a problem with large amounts of wind?"

Intervention Group	**Subject ID**	**Baseline (Days)**	**Week 1 (Days)**	**Week 4 (Days)**	**Week 8 (Days)**	**Week 12 (Days)**
1	5	0	0	0	0	0
	6	14	0	1	Withdraw	Withdraw
	13	14	8	10	8	10
	17	5	No Data	0	0	0
2	1	14	14	4	7	5
	3	10	10	3	3	5
	8	3	0	1	0	0
	9	0	2	3	9	11
	11	2	1	1	7	4
	12	2	0	0	1	3
	18	14	14	5	14	2
3	2	11	0	0	0	4
	4	14	4	2	0	0
	7	10	6	8	6	14
	10	7	2	4	14	No Data
	14	10	10	10	2	14
	15	0	2	0	2	0
	16	10	0	1	0	2

Table 54: Question 19 "On how many days over the last two weeks have you felt off your food?"

Intervention Group	Subject ID	Baseline (Days)	Week 1 (Days)	Week 4 (Days)	Week 8 (Days)	Week 12 (Days)
1	5	2	0	0	0	0
	6	7	0	4	Withdraw	Withdraw
	13	14	0	5	0	0
	17	2	No Data	1	2	0
2	1	14	3	0	0	3
	3	2	7	0	2	0
	8	0	0	0	0	0
	9	0	0	0	0	0
	11	0	0	0	7	2
	12	0	0	0	0	0
	18	0	0	0	0	0
3	2	3	8	2	3	0
	4	14	0	0	0	0
	7	0	8	6	2	3
	10	2	4	7	7	No Data
	14	0	0	0	0	0
	15	0	0	0	0	0
	16	0	0	0	0	0

Table 55: Question 20 "Many patients with bowel problems have worries about their illness. How often during the last two weeks have you felt worried?"

Intervention Group	Subject ID	Baseline	Week 1	Week 4	Week 8	Week 12
1	5	b	b	b	b	b
	6	c	c	d	Withdraw	Withdraw
	13	c	b	c	b	b
	17	b	No Data	b	a	b
2	1	c	d	b	b	c
	3	b	a	a	a	a
	8	b	a	a	a	a
	9	b	b	a	a	b
	11	b	b	b	b	b
	12	b	b	b	b	a
	18	d	a	b	b	a
3	2	b	b	b	b	b
	4	b	a	a	a	b
	7	c	b	c	b	b
	10	b	d	b	b	No Data
	14	b	b	b	a	b
	15	b	b	b	b	b
	16	b	b	b	b	a

Table 56: Question 21 "On how many days over the last two weeks has your abdomen felt bloated?"

Intervention Group	Subject ID	Baseline (Days)	Week 1 (Days)	Week 4 (Days)	Week 8 (Days)	Week 12 (Days)
1	5	0	0	0	0	0
	6	14	4	3	Withdraw	Withdraw
	13	14	14	14	14	10
	17	4	No Data	4	2	0
2	1	7	14	4	7	3
	3	14	3	0	2	1
	8	0	2	0	0	0
	9	3	2	0	0	12
	11	5	2	2	7	4
	12	3	5	3	4	3
	18	14	14	No Data	14	0
3	2	4	0	0	3	4
	4	0	4	0	0	0
	7	8	8	8	5	8
	10	14	6	12	14	No Data
	14	3	5	0	No Data	No Data
	15	0	2	3	3	3
	16	4	0	2	4	2

Table 57: Question 22 "In the last two weeks have you felt relaxed?"

Intervention Group	Subject ID	Baseline	Week 1	Week 4	Week 8	Week 12
1	5	c	c	c	c	c
	6	b	c	d	Withdraw	Withdraw
	13	b	a	b	b	b
	17	c	No Data	b	c	c
2	1	a	b	b	a	a
	3	b	b	c	d	c
	8	c	c	c	b	c
	9	b	c	c	b	b
	11	b	b	c	b	b
	12	b	b	b	b	d
	18	a	b	b	c	c
3	2	b	c	c	b	d
	4	b	b	b	b	b
	7	b	b	a	b	b
	10	a	b	b	b	No Data
	14	c	c	c	c	c
	15	c	c	c	c	c
	16	b	b	b	b	b

Table 58: Question 23 "In the last two weeks have you been embarrassed by your bowel problem?"

Intervention Group	Subject ID	Baseline	Week 1	Week 4	Week 8	Week 12
1	5	c	b	b	b	b
	6	d	a	a	Withdraw	Withdraw
	13	d	d	d	d	c
	17	b	No Data	a	a	a
2	1	b	c	b	d	c
	3	b	b	a	a	a
	8	b	a	a	a	a
	9	b	a	a	a	b
	11	b	b	a	b	b
	12	a	a	a	a	a
	18	d	a	a	a	a
3	2	c	a	b	b	b
	4	d	b	b	b	b
	7	b	b	b	b	b
	10	b	b	b	b	No Data
	14	b	a	a	a	b
	15	a	b	b	b	c
	16	b	a	a	a	c

Table 59: Question 24 "On how many days over the last two weeks have you wanted to go back to the toilet immediately after you thought you had emptied your bowels?"

Intervention Group	**Subject ID**	**Baseline (Days)**	**Week 1 (Days)**	**Week 4 (Days)**	**Week 8 (Days)**	**Week 12 (Days)**
1	5	0	1	0	0	0
	6	8	0	3	Withdraw	Withdraw
	13	14	14	4	8	10
	17	1	No Data	2	1	6
2	1	10	10	2	1	3
	3	2	2	0	0	0
	8	2	0	0	0	0
	9	5	1	0	4	12
	11	2	0	1	1	2
	12	2	2	0	1	0
	18	10	1	5	14	3
3	2	7	3	3	10	8
	4	6	0	2	0	0
	7	8	6	1	5	3
	10	14	3	7	10	No Data
	14	7	4	4	4	5
	15	3	2	2	1	1
	16	1	0	0	0	0

Table 60: Question 25 "In the last two weeks have you felt upset?"

Intervention Group	Subject ID	Baseline	Week 1	Week 4	Week 8	Week 12
1	5	a	a	b	b	a
	6	c	a	c	Withdraw	Withdraw
	13	b	c	c	b	b
	17	a	No Data	a	a	a
2	1	c	b	b	a	b
	3	b	a	a	a	a
	8	a	a	a	a	a
	9	a	a	a	b	b
	11	a	a	a	b	b
	12	b	a	a	a	a
	18	c	b	c	a	a
3	2	b	a	b	b	a
	4	a	a	a	a	b
	7	b	b	c	b	b
	10	d	b	b	b	No Data
	14	a	a	a	a	a
	15	a	a	a	a	a
	16	b	b	b	b	b

Table 61: Question 26 "On how many days over the last two weeks have you had to rush to the toilet?"

Intervention Group	**Subject ID**	**Baseline (Days)**	**Week 1 (Days)**	**Week 4 (Days)**	**Week 8 (Days)**	**Week 12 (Days)**
1	5	0	1	0	0	0
	6	14	0	7	Withdraw	Withdraw
	13	14	14	10	10	14
	17	2	No Data	1	0	1
2	1	3	5	1	1	3
	3	3	2	0	0	0
	8	1	1	0	0	0
	9	6	0	1	5	9
	11	2	1	1	3	2
	12	3	2	0	0	0
	18	14	2	5	15	3
3	2	11	3	2	6	4
	4	6	4	4	0	0
	7	8	6	8	5	6
	10	14	5	14	14	No Data
	14	12	9	10	3	8
	15	0	1	2	1	1
	16	2	0	1	2	2

Table 62: Question 27 "In the last two weeks have you felt angry as a result of your bowel problem?"

Intervention Group	Subject ID	Baseline	Week 1	Week 4	Week 8	Week 12
1	5	a	a	a	a	a
	6	c	a	b	Withdraw	Withdraw
	13	d	b	c	c	b
	17	a	No Data	a	a	a
2	1	b	b	b	a	b
	3	b	a	a	a	a
	8	b	a	a	a	a
	9	c	a	a	b	b
	11	b	a	b	b	b
	12	a	a	a	a	a
	18	b	a	a	a	a
3	2	a	a	b	c	a
	4	a	a	a	a	a
	7	b	b	c	b	b
	10	b	b	b	b	No Data
	14	a	b	a	a	b
	15	b	b	b	b	b
	16	a	a	a	a	a

Table 63: Question 28 "In the last two weeks, has your sex life been affected by your bowel problem?"

Intervention Group	Subject ID	Baseline	Week 1	Week 4	Week 8	Week 12
1	5	a	a	a	a	a
	6	c	c	c	Withdraw	Withdraw
	13	c	c	d	d	d
	17	a	No Data	a	a	a
2	1	c	b	b	c	b
	3	b	a	a	a	a
	8	b	a	a	a	a
	9	b	a	a	b	b
	11	a	a	a	a	a
	12	a	a	a	a	a
	18	c	b	c	c	b
3	2	b	a	b	a	a
	4	b	b	b	a	a
	7	b	b	c	b	b
	10	b	a	b	b	No Data
	14	a	a	a	a	a
	15	a	a	a	a	a
	16	b	b	a	b	b

Table 64: Question 29 "On how many days over the last two weeks have you felt sick?"

Intervention Group	**Subject ID**	**Baseline (Days)**	**Week 1 (Days)**	**Week 4 (Days)**	**Week 8 (Days)**	**Week 12 (Days)**
1	5	0	1	1	0	0
	6	14	3	14	Withdraw	Withdraw
	13	5	0	7	0	7
	17	1	No Data	2	2	0
2	1	0	0	0	0	1
	3	2	7	0	2	0
	8	0	0	0	0	0
	9	0	0	0	0	0
	11	0	0	0	0	2
	12	0	0	0	0	0
	18	4	3	6	0	0
3	2	0	0	0	0	0
	4	0	0	0	0	0
	7	2	4	4	0	0
	10	2	2	3	2	No Data
	14	0	3	0	0	0
	15	0	0	0	0	0
	16	0	0	0	0	0

Table 65: Question 30 "In the last two weeks have you felt irritable?"

Intervention Group	Subject ID	Baseline	Week 1	Week 4	Week 8	Week 12
1	5	a	a	a	1	a
	6	d	c	c	Withdraw	Withdraw
	13	c	c	c	b	b
	17	b	No data	a	b	a
2	1	b	b	b	b	b
	3	c	b	a	a	a
	8	b	a	a	a	a
	9	b	a	a	b	b
	11	b	a	b	b	b
	12	b	b	b	b	b
	18	c	a	b	b	a
3	2	b	b	b	b	b
	4	a	b	q	a	a
	7	b	b	c	a	b
	10	d	b	b	c	No Data
	14	b	b	a	b	b
	15	b	b	b	b	b
	16	b	b	b	b	b

Table 66: Question 31 "In the last two weeks have you felt lack of sympathy from others?"

Intervention Group	Subject ID	Baseline	Week 1	Week 4	Week 8	Week 12
1	5	a	a	a	a	a
	6	a	a	a	Withdraw	Withdraw
	13	a	a	b	a	a
	17	a	No data	a	a	a
2	1	b	a	b	b	b
	3	b	b	b	a	a
	8	a	a	a	a	a
	9	a	a	a	a	a
	11	a	a	a	a	a
	12	a	a	a	a	a
	18	a	a	a	a	a
3	2	b	a	b	a	b
	4	a	a	a	a	a
	7	a	b	b	a	b
	10	b	b	b	b	No Data
	14	a	a	a	a	a
	15	b	b	b	b	a
	16	a	b	b	b	b

Table 67: Question 32 "In the last two weeks have you felt happy?"

Intervention Group	Subject ID	Baseline	Week 1	Week 4	Week 8	Week 12
1	5	c	c	c	c	c
	6	b	c	c	Withdraw	Withdraw
	13	b	b	c	b	c
	17	c	No Data	c	c	c
2	1	a	b	b	a	a
	3	b	a	c	c	c
	8	c	c	c	c	c
	9	b	c	a	b	b
	11	c	c	c	b	b
	12	b	b	c	c	c
	18	a	b	b	c	c
3	2	c	c	c	c	c
	4	b	a	b	a	b
	7	b	b	a	b	b
	10	b	c	c	b	No Data
	14	c	c	c	c	c
	15	d	c	c	c	d
	16	c	b	c	c	c

Appendix 12 : Routine Blood results of all participants

Table 68: Subject 1 blood test results

	Baseline	Week 1	Week 4	Week 8	Week 12
Sodium	139	141	143	140	142
Potassium	4.1	4.1	4.1	4.2	4.3
Urea	Missing data	6.1	6.3	5.2	4.3
Creatinine	52	45	52	51	56
Bilirubin	6	4	6	4	3.0
ALT	14	15	9	12	10
ALP	73	67	73	80	82
Albumin	45	43	42	42	41
Haemoglobin	128	133	130	131	131
WBC	6.2	6.1	7.6	6.7	7.3
Platelets	181	182	206	194	162
CRP	5	1	1	1	8

Table 69: Subject 2 blood test results

	Baseline	Week 1	Week 4	Week 8	Week 12
Sodium	138	136	137	139	140
Potassium	4.6	3.9	4.2	3.8	4.2
Urea	4.0	2.9	3.8	2.9	5.5
Creatinine	58	52	58	66	65
Bilirubin	3	3	4	5	7
ALT	8	10	10	10	14
ALP	69	55	71	62	52
Albumin	42	42	44	43	40
Haemoglobin	106	110	115	115	120
WBC	4.8	5.0	4.3	5.8	5.4
Platelets	346	172	213	298	287
CRP	5	6	39	5	5

Table 70: Subject 3 blood test results

	Baseline	Week 1	Week 4	Week 8	Week 12
Sodium	139	137	139	140	141
Potassium	4.4	4.1	4.4	4.4	4.5
Urea	3.1	3.3	3.4	2.9	3.7
Creatinine	70	60	60	59	67
Bilirubin	10	4	9	10	6
ALT	13	23	17	17	16
ALP	61	52	55	57	51
Albumin	47	44	45	45	44
Haemoglobin	117	123	119	126	129
WBC	7.3	7.2	7.6	7.1	7.1
Platelets	217	210	203	206	210
CRP	5	5	5	5	5

Table 71: Subject 4 blood test results

	Baseline	Week 1	Week 4	Week 8	Week 12
Sodium	140	140	141	141	140
Potassium	4.4	4.4	4.7	4.1	4.2
Urea	3.6	3.7	3.9	4.7	3.8
Creatinine	69	80	79	77	83
Bilirubin	10	8	14	13	16
ALT	15	21	20	20	23
ALP	116	99	101	103	100
Albumin	44	44	44	45	47
Haemoglobin	142	139	140	149	158
WBC	9.2	7.1	7.5	8.0	12
Platelets	312	280	247	279	267
CRP	7	18	5	5	5

Table 72: Subject 5 blood test results

	Baseline	Week 1	Week 4	Week 8	Week 12
Sodium	139	141	140	143	143
Potassium	4.1	4.2	4	4.1	3.8
Urea	4.2	5	3.8	4.8	4.1
Creatinine	79	70	84	87	86
Bilirubin	9	9	7	8	13
ALT	17	29	59	27	43
ALP	78	79	89	87	93
Albumin	50	51	46	48	49
Haemoglobin	145	146	143	147	152
WBC	6.6	10	12.4	8.4	6.4
Platelets	346	296	271	324	331
CRP	5	5	32	5	5

Table 73: Subject 6 blood test results

	Baseline	Week 1	Week 4	Week 8	Week 12
Sodium	140	141	142	Withdraw	Withdraw
Potassium	4.6	4.3	4.7		
Urea	7.9	5.6	6		
Creatinine	92	81	87		
Bilirubin	10	8	7		
ALT	46	57	38		
ALP	57	46	56		
Albumin	46	44	45		
Haemoglobin	173	161	173		
WBC	6.1	5.3	5.7		
Platelets	259	302	252		
CRP	5	7	5		

Table 74: Subject 7 blood test results

	Baseline	Week 1	Week 4	Week 8	Week 12
Sodium	138	139	141	140	141
Potassium	3.8	4.2	4.6	4.0	4.2
Urea	Missing data	4.2	5.5	7.0	5.9
Creatinine	58	5.8	67	77	69
Bilirubin	5	65	6	8	4
ALT	12	3	11	13	13
ALP	53	18	46	50	44
Albumin	4	44	44	41	45
Haemoglobin	140	139	Missing data	128	136
WBC	6.3	4.9	Missing data	5.8	6.6
Platelets	300	266	Missing data	259	282
CRP	7	5	Missing data	5	5

Table 75: Subject 8 blood test results

	Baseline	Week 1	Week 4	Week 8	Week 12
Sodium	142	141	142	143	143
Potassium	4.5	4.1	4.3	4.4	4.1
Urea	5.3	4.8	3.9	6.3	4.2
Creatinine	88	84	93	89	86
Bilirubin	4	5	6	6	7
ALT	20	44	25	37	28
ALP	70	69	72	58	65
Albumin	50	48	53	50	53
Haemoglobin	146	147	157	151	159
WBC	6.3	5.9	6.5	5.5	6.8
Platelets	296	291	260	257	281
CRP	5	10	5	5	5

Table 76: Subject 9 blood test results

	Baseline	Week 1	Week 4	Week 8	Week 12
Sodium	138	143	141	142	142
Potassium	4.4	3.9	4.2	3.9	4.3
Urea	4.2	5.8	4.7	5.5	4.2
Creatinine	92	75	72	73	68
Bilirubin	5	6	8	8	6
ALT	125	20	29	16	20
ALP	53	55	57	56	53
Albumin	41	44	48	47	44
Haemoglobin	147	140	151	149	141
WBC	6.8	4.9	5.8	5.5	5.3
Platelets	192	146	167	143	150
CRP	5	5	5	5	5

Table 77: Subject 10 blood test results

	Baseline	Week 1	Week 4	Week 8	Week 12
Sodium	141	142	142	140	140
Potassium	4.5	4.3	4.3	4.3	4.0
Urea	3.9	3.4	4.4	5.0	3.4
Creatinine	52	54	59	55	62
Bilirubin	6	3	3	7	7
ALT	7	7	9	8	8
ALP	63	46	64	66	75
Albumin	43	40	43	43	45
Haemoglobin	123	111	118	117	126
WBC	7.2	5.4	4.2	6.2	9.3
Platelets	296	277	317	320	287
CRP	5	20	5	5	7

Table 78: Subject 11 blood test results

	Baseline	Week 1	Week 4	Week 8	Week 12
Sodium	139	142	142	142	139
Potassium	4.5	4.6	4.4	4.4	4.2
Urea	7.1	6.4	5.4	4.9	4.7
Creatinine	79	77	70	79	69
Bilirubin	7	3	4	5	7
ALT	12	13	14	12	13
ALP	80	69	74	80	83
Albumin	45	39	39	41	40
Haemoglobin	111	105	102	103	105
WBC	6.9	6.3	7.5	7.6	7.8
Platelets	475	421	480	439	443
CRP	5	5	5	7	5

Table 79: Subject 12 blood test results

	Baseline	Week 1	Week 4	Week 8	Week 12
Sodium	143	142	143	145	144
Potassium	3.9	4.0	4.2	3.9	4.1
Urea	6.5	5.9	6.2	5.9	6.5
Creatinine	52	48	54	55	58
Bilirubin	4	7	5	4	7
ALT	15	16	15	18	17
ALP	46	42	43	49	45
Albumin	45	45	44	44	44
Haemoglobin	135	144	142	140	140
WBC	6.1	4.5	4.3	5.0	4.5
Platelets	213	184	202	204	194
CRP	5	5	5	5	5

Table 80: Subject 13 blood test results

	Baseline	Week 1	Week 4	Week 8	Week 12
Sodium	140	141	140	140	143
Potassium	4.2	4.3	4.2	4.5	4.4
Urea	4.9	4.8	4.8	5.2	5.4
Creatinine	84	101	84	92	93
Bilirubin	15	9	15	11	13
ALT	23	29	32	24	26
ALP	89	80	84	86	85
Albumin	43	45	41	44	45
Haemoglobin	140	148	143	152	151
WBC	4.4	4.4	5.2	5.3	4.4
Platelets	157	146	192	146	146
CRP	5	5	5	5	5

Table 81: Subject 14 blood test results

	Baseline	Week 1	Week 4	Week 8	Week 12
Sodium	142	138	141	142	142
Potassium	4.2	4.2	4.4	4.2	4.1
Urea	6.1	5.0	5.1	5.7	5.2
Creatinine	79	76	75	82	77
Bilirubin	7	4	7	9	7
ALT	11	16	18	14	15
ALP	62	58	59	61	66
Albumin	39	39	43	40	41
Haemoglobin	129	129	126	123	127
WBC	4.7	5.3	6.0	5.9	5.9
Platelets	314	337	327	305	315
CRP	5	5	5	5	5

Table 82: Subject 15 blood test results

	Baseline	Week 1	Week 4	Week 8	Week 12
Sodium	142	140	142	140	140
Potassium	4.1	4.4	4.6	4.4	4.1
Urea	3.2	4.2	3.1	3.6	4.5
Creatinine	71	82	67	67	74
Bilirubin	6	7	5	6	3
ALT	13	13	13	9	12
ALP	131	118	107	102	109
Albumin	49	49	49	46	51
Haemoglobin	130	136	123	124	132
WBC	6.5	5.8	4.8	5.8	8.3
Platelets	303	286	324	320	328
CRP	5	5	5	5	5

Table 83: Subject 16 blood test results

	Baseline	Week 1	Week 4	Week 8	Week 12
Sodium	138	141	139	141	140
Potassium	3.8	4.0	3.7	4	3.7
Urea	5.6	4.4	Missing data	5.5	4.5
Creatinine	59	54	58	62	84
Bilirubin	16	8	11	14	13
ALT	20	41	29	27	30
ALP	48	45	43	40	44
Albumin	50	47	48	46	50
Haemoglobin	150	149	152	154	157
WBC	5.0	3.9	5.4	5.8	6
Platelets	264	255	281	251	268
CRP	5	5	5	5	5

Table 84: Subject 17 blood test results

	Baseline	Week 1	Week 4	Week 8	Week 12
Sodium	135	No test performed due to Covid			135
Potassium	4.4				4.5
Urea	3.6				4.1
Creatinine	63				63
Bilirubin	4				3.0
ALT	29				19
ALP	94				73
Albumin	39				46
Haemoglobin	132				137
WBC	5.1				6.4
Platelets	175				269
CRP	10				5

Table 85: Subject 18 blood test results

	Baseline	Week 1	Week 4	Week 8	Week 12
Sodium	140	No test performed due to Covid			
Potassium	4.3				
Urea	5.3				
Creatinine	65				
Bilirubin	4				
ALT	16				
ALP	73				
Albumin	44				
Haemoglobin	124				
WBC	5.2				
Platelets	334				
CRP	5				

Appendix 13: MiSeq Wet Lab SOP

1) Introduction and Workflow

1.1) Introduction

- The Purpose of this protocol is to define the steps for the preparation and sequencing of 16S rRNA gene sequence libraries using the Illumina MiSeq sequencing platform, as described in the paper Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform by Kozich et al.
- The Illumina MiSeq Sequencer can produce 2 x 250bp (or 2 x 300bp with v3 chemistry) paired-end reads and up to 8.5 Gb of data in a single run. Dual indexing of library samples allows up to 384 samples to be run simultaneously. The instrument is capable of producing in excess of 24 million reads. However for low diversity runs about 12 million reads can be expected. A wide range of applications is possible including 16S analysis, metagenomics, genome sequencing, transcriptomics, and RNA sequencing.
- Our lab typically sequences the V4 region of the 16S rRNA gene. Its short length (~250bp) allows for fully overlapping forward and reverse reads, which, in combination with our curaton pipeline, results in the lowest error rates.
- There are several steps in preparing samples for sequencing on the MiSeq. Broadly, these include library generation and indexing, quality control, normalization, quantification and pooling, sequencing, run quality assessment, and data export. An overview of each step and more detailed protocols are below.

1.2) 16S Prep Workflow

This section is an overview of the steps involved in library preparation.

For a more detailed description of the methods, see Section 5 below.

1 Extracted DNA should be arrayed in 96 well plate format, preferably with two wells on each plate open for controls.

2 Samples are PCR amplified with Schloss lab indices. Each plate should contain a negative control (water) and a positive control (mock community)

3 A subset of 12-24 samples from each plate undergoes electrophoresis on a 1% agarose gel to ensure amplification proceeded normally.

4 Library clean up and normalization is performed using the Ampure XP bead clean up followed by Qubit Quantification.

5 Samples from each plate are pooled into single wells (i.e. 1 well/plate).

6 (Optional) To assess the quality of the library, a 1 % agarose gel electrophoresis run is perfromed

7 (Optional) If the post-PCR gel suggests contaminant DNA from leftover indices/primer-dimer, an

additional gel purification of the pooled plates is recommended. This often improves the quality of the sequencing run.

8 Each pooled plate is quantified using a Qubit fluorometer.

9 Plates are pooled to equal concentration into a single well (i.e. 1 well per run)

10 The pooled library enters the Sequencing Workflow.

1.3) Sequencing Workflow

This section is an overview of the steps involved in initiating a sequencing run.

For a more detailed description of the methods, see Section 5 below.

1 A Sample Plate is created for each plate using Illumina Experiment Manager. Sample Plates are then used to create a Sample Sheet. This sheet serves as the set of run parameters and indexing scheme used by the MiSeq for the run. The sample sheet is then transferred to the MiSeq via flash drive.

2 The reagent cartridge is thawed in a water bath per the MiSeq System Guide.

3 Unless otherwise specified, dilution and loading will follow the steps outlined in the document: Preparing DNA Libraries for Sequencing on the MiSeq a. Pooled library and PhiX control are denatured and diluted. b. Diluted library and PhiX are pooled (10-15% PhiX, 85-90% Library). c. Sequencing primers and library/PhiX are loaded into the reagent cartridge. d. MiSeq flow cell is washed.

4 The sample sheet, flow cell, reagent cartridge, PR2 bottle, and an empty waste bottle are loaded onto the MiSeq, and the run is initiated. A 500 cycle run takes approx. 44 hours.

5 The run is monitored using Illumina Sequence Analysis Viewer.

6 Upon completions of the run, fastq files are transferred to HPC Wales for data processing

7 A post run wash is performed, followed by a standby wash if the machine will be idle for a week or more. a. Between sequencing runs we recommend a bleach wash which is part of the post run wash. - 0.5% Tween20 in reservoir bottle per usual. - 6 mL of 0.5% Tween20 to each cartridge well except 17. - Mix 870uL of ultrapure H2O AND 30uL of 6% Bleach to a 1.5 mL tube. - Note: This is to be made fresh for each wash. - Mix 50uL of the bleach/water solution above and 950uL of ultrapure H2O in the MiSeq wash tube. - Place the MiSeq wash tube in cartridge well 17. Load the cartridge into the MiSeq. - Start a post run wash on the MiSeq with “template line wash” selected.

2) Safety and Waste Disposal

- The SUMS ILS1 Hygiene Plan should be followed at all times.
- Standard PPE (nitrile gloves, safety glasses, and lab coat) should be used at all times.

• Each reagent cartridge contains a small amount of formamide and must be disposed of in an appropriate container following the run. Liquid waste from a run must also be disposed of as hazardous due to the formamide content.

3) Consumables

MiSeq Reagent Kits v3 (600 cycle)	MS-102-3003
MiSeq Service	Internal
Filter Tips	ILS_Stores
HotStar HiFidelity Polymerase Kit	202602
96 well plates	ILS_Stores
Qubit® dsDNA HS Assay Kit	10606433
Qubit® Assay Tubes	12037609
AMPure XP Beads	10453438
16 PCR Amplicon Primers	NA

4) Detailed Method(s)

4.1) Published Protocols

• The following methods and references are used in the workflows above.

◦ MiSeq System Guide

◦ Preparing DNA Libraries for Sequencing on the MiSeq

◦ **HotStar HiFidelity Polymerase Kit**

◦ Ampure XP Beads

4.2) Initial Set up

1 Reconstitute indexed primers and sequencing primers to 100 uM. See Appendix D for primer design.

2 Prepare 100ul 10 uM aliquots of indexed primers. Do not dilute sequencing primers.

3 Array aliquots of indexed primers into four 96 well plates using the following scheme: a. A701 – A712 with A501 – A508 b. A701 – A712 with B501 – B508 c. B701 – B712 with B501 – B508 d. B701 – B712 with A501 – A508

Note: These primer plates can be stored at -20°C and used for subsequent runs.

4 Extract template DNA and array in 96 well format leaving two wells open. (One for a negative water control and another for the positive Mock Community control)

5 Using Illumina Experiment Manager, create a sample plate for each 96 well plate of template.

Choose indexes that correspond to one of the four index pair plates above. See Appendix A for instruction on creating a custom assay in IEM.

6 Using Illumina Experiment Manager, create a sample sheet for the run. Ensure that index choices are compatible with one another and there is sufficient diversity in the index reads so as to activate both light channels every cycle. Note: Primer plate A has insufficient diversity to be run alone. If sequencing 96 or fewer samples, choose plate B, C, or D.

4.3) PCR

Note: These steps may be performed using an epMotion or similar automated pipetting system.

1 Dispense 20 ul of Accuprime Pfx Supermix into each well of a new 96 well plate (enzyme, buffer and H_20) mix).

2 Using a multichannel pipette, transfer 1 ul of template DNA per well to the corresponding well on the PCR plate.

3 Using a multichannel pipette, transfer 2 ul of each paired set of index primers from the primer plate to the corresponding well on the PCR plate. Be sure to follow the layout chosen in the sample sheet.

4 Add 1 ul of PCR grade H_2O to the negative control

5 Repeat for up to four 96 well plates. Seal plates, vortex briefly and spin down contents.

6 Place in thermocycler.

Use the following program:

95°C 2:00

--------30 cycles--------

95°C 00:20

55°C 00:15

72°C 5:00

72°C 10:00

4°C Hold

4.4) Gel Electrophoresis

1 1 or 2 random rows of 12 should be selected from each PCR plate and run on a gel to confirm success of the PCR. (Alternatively, all samples can be run on a single E-Gel)

2 Use 2 ul of sample, 4 ul of loading dye in a 1% agarose gel.

3 Run at 100v for 30 minutes alongside a 1kb+ ladder.

4 Photograph gel under UV. Check to be sure there is a band for every well.

4.5) Cleanup

1) Use AmpureXP beads at a ratio of 1:1, mix with samples at 1800 rpm for 2 mins in a 1 ml midi plate (Note: Make sure beads are at room temp and are fully suspended in a homogenous mixture)

2) Incubate mixed plate at room temperature for 5 mins before placing plate on a magnetic stand for 2 mins or as long as it takes for the beads to bind to the magnet

3) Remove buffer and discard taking care not to suspend the beads. With the plate still on magnetic stand, wash beads with 200 ul of 80% ethanol (Do not re-suspend the beads). Remove ethanol and repeat.

4) Remove all traces of ethanol and leave plate dry at RT for 5 mins. Do not allow the beads to over dry.

5) Resuspend the beads in 22ul of 10mM Tris ph 8.0 or ddH_20, shake at 1800 rpm for 2 mins, incubate at RT for 5 mins followed by incubation on the magnetic plate stand for 2 mins.

6) Transfer 20 ul of eluted DNA to a clean PCR plate keeping the well order. Care should be taken not to suspend the beads and not to transfer ANY beads to the clean plate.

7) The cleaned DNA can be checked on a 1% gel to assess clean up. Any remaining primers-dimers or indices can be removed by gel purification.

4.6) Quantification and Normalisation

1) Cleaned DNA should be quantified using the Qubit HS/BR assay using 2ul of DNA as input material.

2) Quantities should be converted to molarity (nM) and diluted to 4nM.

3) Equamolar libraries should be pooled in equal concentration (2ul of each library) into clean PCR tubes and subsequently combined into a single 1.5ml tube). This is the PAL (pooled amplicon library)

4.7) Sequencing

1 Remove a 600 cycle reagent cartridge from the -20°C freezer. Place in room temperature water bath for one hour. Place HT1 buffer tube in 4°C fridge. While reagent cartridge is thawing, perform steps 2-6.

2 Prepare fresh 0.2N NaOH.

3 To a 1.5ml tube, add 5 ul of the PAL and 5 ul of 0.2N NaOH. To a separate tube add 2 ul PhiX, 3 ul PCR grade water, and 5 ul of 0.2N NaOH. Pipette to mix. Note: NaOH concentration on the flow cell must remain under 0.001N. Adjusting the concentration of the NaOH used to denature the DNA to 0.1N may be necessary if library concentration is 1nM or below.[^1]

4 Allow the tubes to incubate at room temperature for 5 minutes. Immediately add 990 ul of ice-cold HT1 to the library tube, and 990 ul HT1 to the PhiX tube. Note: the resulting 20pM PhiX can

be frozen and used for subsequent runs.

5 Use HT1 to further dilute both the library and PhiX to 10pM for a v3 kit. Can load up to 10pM for a v3 kit. See example below: a. (1.45 nM library x 10 ul) + (0.2N NaOH x 10 ul) + 980 ul HT1 = 14.5pM Lib, 0.002N NaOH b. (14.5pM lib x 275.86 ul) + 724.14 ul HT1 = 4.0pM lib, 0.00055N NaOH c. [(10nM PhiX x 2 ul) + 3 ul H2O] + (0.2N NaOH x 5 ul) + 990 ul HT1 = 20pM PhiX, 0.001N NaOH d. (20pM PhiX x 200 ul) + 800 ul HT1 = 4.0pM PhiX, 0.0002N NaOH e. (4.0pM Lib x 900 ul) + (4.0pM PhiX x 100 ul) = solution loaded f. Solution loaded is 4.0pM overall with a 3.6pM Library concentration, 0.4pM PhiX concentration, and 0.000515N NaOH

6 For a 15% PhiX run, combine 850 ul of 4.0pM Library and 150 ul PhiX in a final tube. Vortex.

7 When the reagent cartridge has thawed, dry bottom with paper towel. Invert the cartridge repeatedly to check each well is thawed. This also serves to mix the reagents. Place in hood.

8 Using a clean 1000 ul pipette tip, break the foil covering wells 12, 13, 14, and 17 of the reagent cartridge.

9 Load 600 ul of the final Libary/PhiX solution into well 17 on the reagent cartridge.

10 Place 3 ul of the 100 uM Read 1 Sequencing Primer(s) into a clean PCR tube. Repeat in separate tubes for the Index Primer(s) and Read 2 Sequencing Primer(s).

11 Use an extra long 100 ul tip and pipetter transfer the 3 ul of Read 1 Sequencing Primer to the bottom of well 12 and pipette to mix. Repeat this process spiking the Index Primer into well 13 and the Read 2 Sequencing Primer into well 14.

12 Set reagent cartridge aside. Unbox flow cell and PR2 bottle.

13 Thoroughly rinse the flow cell with Milli-Q water. Carefully dry by blotting with lint free wipes (Kimwipes). Give special attention to the edges and points of intersection between the glass and plastic.

14 Wet a new wipe with 100% alcohol and wipe the glass on both sides avoiding the rubber intake ports.

15 Visually inspect the flow cell to ensure there are no blemishes, particles, or fibers on the glass.

16 Transfer reagent cartridge, flow cell, PR2 bottle, and flash drive with the sample sheet to the MiSeq.

17 Copy Sample Sheet from the flash drive to the "Sample Sheets" folder on the desktop of the MiSeq.

18 Follow on screen instructions to load the flow cell, reagent cartridge, and PR2 bottle. Empty and replace the waste bottle.

19 Ensure the machine recognizes the correct sample sheet and the run parameters are correct.

20 Wait for the MiSeq to perform its pre-run checks, and press start.

4.8) Run Monitoring

1 The run should be monitored periodically using Illumina Sequence Analysis Viewer.

2 Ideal parameters for a 90% 16S run:a. Cluster density 700-800k/mm2 for v2 kitsb. Cluster density 1000-1100k/mm2 for v3 kitsc. >85% clusters passing filterd. 15% aligned (amount of PhiX)e. No spikes in corrected intensity plotf. All indices identified following index readsg. Final >Q30 score of >70%

4.9) Final Steps

1 Perform a post run wash on the MiSeq. We recommend the bleach wash.

2 Dispose of liquid waste in appropriate hazardous jug and reagent cartridge in hazardous bucket.

3 When MiSeq Reporter finishes, copy the fastq files from the output folder to the run folder on the HPC Wales.

4 Perform maintenance or standby wash if required.

5 Check data to confirm they are of sufficient quality and quantity.

Appendix 14: Primer design

Overall design considerations

- The sequencing primers must have a melting temperature near 65°C. This can be achieved by altering the pad sequence
- The index sequences must balance the number of bases at each position. The index sequences listed here have a 25% ATGC composition at each site. If you are going to cherry pick indices from the list, make sure that you have even representation.

Generic PCR primer design:

AATGATACGGCGACCACCGAGATCTACAC <i5><pad><link><16Sf> VX.N5??

CAAGCAGAAGACGGCATACGAGAT <i7><pad><link><16Sr> VX.N7??

Generic read 1 primer design

<pad><link><16Sf> VX.read1

Generic read 2 primer design

<pad><link><16Sr> VX.read2

Generic index read primer design

Reverse complement of (<pad><link><16Sr>) VX.p7_index

The listed sequences in the generic design, above, are the adapter sequences to allow annealing of the amplicons to the flow cell. The i5 and i7 sequences are the 8-nt index sequences. The pad is a 10-nt sequence to boost the sequencing primer melting temperatures. The link is a 2-nt sequence that is anti-complementary to the known sequences. The 16Sf and 16Sr are the gene specific primer sequences. Primers are purchased from IDT with no special purification. This system should work for any other region of the 16S rRNA gene or any other gene. The only thing to change would be the 16Sf/16Sr sequences and confirm that when combined with the pad sequence that the melting temperature is near 65°C.

16Sf

V3: CCTACGGGAGGCAGCAG

V4: GTGCCAGCMGCCGCGGTAA

16Sr

V4: GGACTACHVGGGTWTCTAAT

V5: CCCGTCAATTCMTTTRAGT

Link:

V4f: GT

V4r: CC

V3f: GG

V5r: GG

Pad:

Forward: TATGGTAATT

Reverse: AGTCAGTCAG

i5

SA501 ATCGTACG

SA502 ACTATCTG

SA503 TAGCGAGT

SA504 CTGCGTGT

SA505 TCATCGAG

SA506 CGTGAGTG

SA507 GGATATCT

SA508 GACACCGT

SB501 CTACTATA

SB502 CGTTACTA

SB503 AGAGTCAC

SB504 TACGAGAC

SB505 ACGTCTCG

SB506 TCGACGAG

SB507 GATCGTGT

SB508 GTCAGATA

SC501 ACGACGTG

SC502 ATATACAC

SC503 CGTCGCTA

SC504 CTAGAGCT

SC505 GCTCTAGT

SC506 GACACTGA

SC507 TGCGTACG

SC508 TAGTGTAG

SD501 AAGCAGCA

SD502 ACGCGTGA

SD503 CGATCTAC

SD504 TGCGTCAC

SD505 GTCTAGTG

SD506 CTAGTATG

SD507 GATAGCGT

SD508 TCTACACT

i7

SA701 AACTCTCG

SA702 ACTATGTC

SA703 AGTAGCGT

SA704 CAGTGAGT

SA705 CGTACTCA

SA706 CTACGCAG

SA707 GGAGACTA

SA708 GTCGCTCG

SA709 GTCGTAGT

SA710 TAGCAGAC

SA711 TCATAGAC

SA712 TCGCTATA

SB701 AAGTCGAG

SB702 ATACTTCG

SB703 AGCTGCTA

SB704 CATAGAGA

SB705 CGTAGATC

SB706 CTCGTTAC

SB707 GCGCACGT

SB708 GGTACTAT

SB709 GTATACGC

SB710 TACGAGCA

SB711 TCAGCGTT

SB712 TCGCTACG

SC701 ACCTACTG

SC702 AGCGCTAT

SC703 AGTCTAGA

SC704 CATGAGGA

SC705 CTAGCTCG

SC706 CTCTAGAG

SC707 GAGCTCAT

SC708 GGTATGCT
SC709 GTATGACG
SC710 TAGACTGA
SC711 TCACGATG
SC712 TCGAGCTC
SD701 ACCTAGTA
SD702 ACGTACGT
SD703 ATATCGCG
SD704 CACGATAG
SD705 CGTATCGC
SD706 CTGCGACT
SD707 GCTGTAAC
SD708 GGACGTTA
SD709 GGTCGTAG
SD710 TAAGTCTC
SD711 TACACAGT
SD712 TTGACGCA

Primers used to amplify 1536 samples using the V4 region. If you only want 384 then use a subset of the listed primers (e.g. all of the v4.SA5* and v4.SB5* and v4.SA7* and v4.SB7* primers):

v4.SA501
AATGATACGGCGACCACCGAGATCTACACATCGTACGTATGGTAATTGTGTGCCAGCM
GCCGCGGTAA
v4.SA502
AATGATACGGCGACCACCGAGATCTACACACTATCTGTATGGTAATTGTGTGCCAGCM
GCCGCGGTAA
v4.SA503
AATGATACGGCGACCACCGAGATCTACACTAGCGAGTTATGGTAATTGTGTGCCAGCM
GCCGCGGTAA
v4.SA504
AATGATACGGCGACCACCGAGATCTACACCTGCGTGTTATGGTAATTGTGTGCCAGCM
GCCGCGGTAA
v4.SA505
AATGATACGGCGACCACCGAGATCTACACTCATCGAGTATGGTAATTGTGTGCCAGCM
GCCGCGGTAA
v4.SA506

AATGATACGGCGACCACCGAGATCTACACCGTGAGTGTATGGTAATTGTGTGCCAGCM
GCCGCGGTAA
v4.SA507
AATGATACGGCGACCACCGAGATCTACACGGATATCTTATGGTAATTGTGTGCCAGCM
GCCGCGGTAA
v4.SA508
AATGATACGGCGACCACCGAGATCTACACGACACCGTTATGGTAATTGTGTGCCAGCM
GCCGCGGTAA
v4.SB501
AATGATACGGCGACCACCGAGATCTACACCTACTATATATGGTAATTGTGTGCCAGCM
GCCGCGGTAA
v4.SB502
AATGATACGGCGACCACCGAGATCTACACCGTTACTATATGGTAATTGTGTGCCAGCM
GCCGCGGTAA
v4.SB503
AATGATACGGCGACCACCGAGATCTACACAGAGTCACTATGGTAATTGTGTGCCAGCM
GCCGCGGTAA
v4.SB504
AATGATACGGCGACCACCGAGATCTACACTACGAGACTATGGTAATTGTGTGCCAGCM
GCCGCGGTAA
v4.SB505
AATGATACGGCGACCACCGAGATCTACACACGTCTCGTATGGTAATTGTGTGCCAGCM
GCCGCGGTAA
v4.SB506
AATGATACGGCGACCACCGAGATCTACACTCGACGAGTATGGTAATTGTGTGCCAGCM
GCCGCGGTAA
v4.SB507
AATGATACGGCGACCACCGAGATCTACACGATCGTGTTATGGTAATTGTGTGCCAGCM
GCCGCGGTAA
v4.SB508
AATGATACGGCGACCACCGAGATCTACACGTCAGATATATGGTAATTGTGTGCCAGCM
GCCGCGGTAA
v4.SC501
AATGATACGGCGACCACCGAGATCTACACACGACGTGTATGGTAATTGTGTGCCAGCM
GCCGCGGTAA v4.SC502

AATGATACGGCGACCACCGAGATCTACACATATACACTATGGTAATTGTGTGCCAGCM
GCCGCGGTAA v4.SC503
AATGATACGGCGACCACCGAGATCTACACCGTCGCTATATGGTAATTGTGTGCCAGCM
GCCGCGGTAA v4.SC504
AATGATACGGCGACCACCGAGATCTACACCTAGAGCTTATGGTAATTGTGTGCCAGCM
GCCGCGGTAA v4.SC505
AATGATACGGCGACCACCGAGATCTACACGCTCTAGTTATGGTAATTGTGTGCCAGCM
GCCGCGGTAA v4.SC506
AATGATACGGCGACCACCGAGATCTACACGACACTGATATGGTAATTGTGTGCCAGCM
GCCGCGGTAA v4.SC507
AATGATACGGCGACCACCGAGATCTACACTGCGTACGTATGGTAATTGTGTGCCAGCM
GCCGCGGTAA v4.SC508
AATGATACGGCGACCACCGAGATCTACACTAGTGTAGTATGGTAATTGTGTGCCAGCM
GCCGCGGTAA v4.SD501
AATGATACGGCGACCACCGAGATCTACACAAGCAGCATATGGTAATTGTGTGCCAGCM
GCCGCGGTAA v4.SD502
AATGATACGGCGACCACCGAGATCTACACACGCGTGATATGGTAATTGTGTGCCAGCM
GCCGCGGTAA v4.SD503
AATGATACGGCGACCACCGAGATCTACACCGATCTACTATGGTAATTGTGTGCCAGCM
GCCGCGGTAA v4.SD504
AATGATACGGCGACCACCGAGATCTACACTGCGTCACTATGGTAATTGTGTGCCAGCM
GCCGCGGTAA v4.SD505
AATGATACGGCGACCACCGAGATCTACACGTCTAGTGTATGGTAATTGTGTGCCAGCM
GCCGCGGTAA v4.SD506
AATGATACGGCGACCACCGAGATCTACACCTAGTATGTATGGTAATTGTGTGCCAGCM
GCCGCGGTAA v4.SD507
AATGATACGGCGACCACCGAGATCTACACGATAGCGTTATGGTAATTGTGTGCCAGCM
GCCGCGGTAA v4.SD508
AATGATACGGCGACCACCGAGATCTACACTCTACACTTATGGTAATTGTGTGCCAGCM
GCCGCGGTAA
v4.SA701
CAAGCAGAAGACGGCATACGAGATAACTCTCGAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT
v4.SA702
CAAGCAGAAGACGGCATACGAGATACTATGTCAGTCAGTCAGCCGGACTACHVGGGT

WTCTAAT

v4.SA703

CAAGCAGAAGACGGCATACGAGATAGTAGCGTAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT

v4.SA704

CAAGCAGAAGACGGCATACGAGATCAGTGAGTAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT

v4.SA705

CAAGCAGAAGACGGCATACGAGATCGTACTCAAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT

v4.SA706

CAAGCAGAAGACGGCATACGAGATCTACGCAGAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT

v4.SA707

CAAGCAGAAGACGGCATACGAGATGGAGACTAAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT

v4.SA708

CAAGCAGAAGACGGCATACGAGATGTCGCTCGAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT

v4.SA709

CAAGCAGAAGACGGCATACGAGATGTCGTAGTAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT

v4.SA710

CAAGCAGAAGACGGCATACGAGATTAGCAGACAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT

v4.SA711

CAAGCAGAAGACGGCATACGAGATTCATAGACAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT

v4.SA712

CAAGCAGAAGACGGCATACGAGATTCGCTATAAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT

v4.SB701

CAAGCAGAAGACGGCATACGAGATAAGTCGAGAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT

v4.SB702

CAAGCAGAAGACGGCATACGAGATATACTTCGAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT
v4.SB703
CAAGCAGAAGACGGCATACGAGATAGCTGCTAAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT
v4.SB704
CAAGCAGAAGACGGCATACGAGATCATAGAGAAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT
v4.SB705
CAAGCAGAAGACGGCATACGAGATCGTAGATCAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT
v4.SB706
CAAGCAGAAGACGGCATACGAGATCTCGTTACAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT
v4.SB707
CAAGCAGAAGACGGCATACGAGATGCGCACGTAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT
v4.SB708
CAAGCAGAAGACGGCATACGAGATGGTACTATAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT
v4.SB709
CAAGCAGAAGACGGCATACGAGATGTATACGCAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT
v4.SB710
CAAGCAGAAGACGGCATACGAGATTACGAGCAAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT
v4.SB711
CAAGCAGAAGACGGCATACGAGATTCAGCGTTAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT
v4.SB712
CAAGCAGAAGACGGCATACGAGATTCGCTACGAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT
v4.SC701
CAAGCAGAAGACGGCATACGAGATACCTACTGAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT v4.SC702

CAAGCAGAAGACGGCATACGAGATAGCGCTATAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT v4.SC703
CAAGCAGAAGACGGCATACGAGATAGTCTAGAAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT v4.SC704
CAAGCAGAAGACGGCATACGAGATCATGAGGAAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT v4.SC705
CAAGCAGAAGACGGCATACGAGATCTAGCTCGAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT v4.SC706
CAAGCAGAAGACGGCATACGAGATCTCTAGAGAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT v4.SC707
CAAGCAGAAGACGGCATACGAGATGAGCTCATAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT v4.SC708
CAAGCAGAAGACGGCATACGAGATGGTATGCTAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT v4.SC709
CAAGCAGAAGACGGCATACGAGATGTATGACGAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT v4.SC710
CAAGCAGAAGACGGCATACGAGATTAGACTGAAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT v4.SC711
CAAGCAGAAGACGGCATACGAGATTCACGATGAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT v4.SC712
CAAGCAGAAGACGGCATACGAGATTCGAGCTCAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT v4.SD701
CAAGCAGAAGACGGCATACGAGATACCTAGTAAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT v4.SD702
CAAGCAGAAGACGGCATACGAGATACGTACGTAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT v4.SD703
CAAGCAGAAGACGGCATACGAGATATATCGCGAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT v4.SD704
CAAGCAGAAGACGGCATACGAGATCACGATAGAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT v4.SD705
CAAGCAGAAGACGGCATACGAGATCGTATCGCAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT v4.SD706
CAAGCAGAAGACGGCATACGAGATCTGCGACTAGTCAGTCAGCCGGACTACHVGGGT
WTCTAAT v4.SD707
CAAGCAGAAGACGGCATACGAGATGCTGTAACAGTCAGTCAGCCGGACTACHVGGGT

WTCTAAT v4.SD708

CAAGCAGAAGACGGCATACGAGATGGACGTTAAGTCAGTCAGCCGGACTACHVGGGT

WTCTAAT v4.SD709

CAAGCAGAAGACGGCATACGAGATGGTCGTAGAGTCAGTCAGCCGGACTACHVGGGT

WTCTAAT v4.SD710

CAAGCAGAAGACGGCATACGAGATTAAGTCTCAGTCAGTCAGCCGGACTACHVGGGT

WTCTAAT v4.SD711

CAAGCAGAAGACGGCATACGAGATTACACAGTAGTCAGTCAGCCGGACTACHVGGGT

WTCTAAT v4.SD712

CAAGCAGAAGACGGCATACGAGATTTGACGCAAGTCAGTCAGCCGGACTACHVGGGT

WTCTAAT

Read 1 primer for V4 region

TATGGTAATTGTGTGCCAGCMGCCGCGGTAA

Read 2 primer for V4 region

AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT

Index primer for V4 region

ATTAGAWACCCBDGTAGTCCGGCTGACTGACT