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Host-microbiota interactions underlying functional gastrointestinal disorders and the impact of gold kiwifruit consumption

A thesis presented in partial fulfilment of the requirements for the degree of

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Abstract

The microbial ecosystem exists in a mutualistic relationship with its host, contributing to a healthy gastrointestinal tract. Growing evidence supports the role of microbial-immune interactions in functional gastrointestinal disorders (FGIDs), including irritable bowel syndrome (IBS). However, these mechanisms are poorly understood. It has been hypothesised that taxonomic and gene abundance in the faecal microbiota and gene expression of peripheral blood mononuclear cells (PBMCs) could discriminate FGID subtypes (functional constipation (FC), IBS constipation, functional diarrhoea (FD), IBS diarrhoea) from each other and healthy subjects (controls). A second hypothesis was that consuming two gold kiwifruit daily for four weeks had a different effect on the microbial composition and gene abundance than psyllium in constipation predominant FGID subjects or controls. A systems biology approach was used to address these hypotheses.

Different microbial compositional and gene abundance profiles were associated with constipation and/or diarrhoea, particularly facultative anaerobes and obligate fermenters, and genes related to tyrosine metabolism, secretion systems and micronutrient utilisation. Differentially PBMC expressed immunoglobulin variable domain genes were shared among FGIDs, except for IBS constipation. Increased expression levels of interferon-induced genes and those linked to the complement system and platelet functions characterised the immune signature of functional constipation. Increased expression levels of immunoglobulin variable domain associated with immunoglobulin E/G receptor-mediated pathways characterised the immune signature of IBS diarrhoea and FD. Further analyses showed that computationally selected microbial, immune gene and symptomatic variables were associated with constipation or diarrhoea predominant FGIDs, and that symptoms remain the best way to discriminate among FGIDs or controls than PBMC genes or microbial taxa except for FC, which was best discriminated from other FGIDs or controls by selected PBMC genes. *Eggerthella* and *Bacteroides* were the only genera that differed between subjects consuming gold kiwifruit or psyllium or between each intervention compared to pre-intervention levels, regardless of the digestive health status of the subjects. This PhD thesis presents novel insights into the host-microbiota interactions underlying FGIDs and the microbiota responses to daily consumption of two gold kiwifruit over four weeks in constipation predominant FGID subjects. The knowledge generated can be used for future research on food-based treatments supporting gastrointestinal health and comfort.

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List of abbreviations

ANOSIM: Analysis Of Similarities

AUC: Area Under Curve

BCFAs: Branched-Chain Fatty Acids

BCR: B Cell Receptor

CgA: Chromogranin A

CIM: Clustered Image Maps

COMFORT: Christchurch IBS cohort to investigate mechanisms for gut relief, and improved transit

CSBM: Complete Spontaneous Bowel Movements

DEG: Differently Expressed Gene

DIABLO: Data Integration Analysis for Biomarker discovery using Latent cOmponents

FC: Functional Constipation

FCERI: IgE Receptor

FCGR: IgG Fc region Receptor

FD: Functional Diarrhoea

FGIDs: Functional GI Disorders

FODMAPs: Fermentable Oligo-, Di-, Monosaccharides And Polyols

GI: Gastrointestinal

GO: Gene Ontology

GSEA: Gene Set Enrichment Analysis

GSRS: Gastrointestinal Symptom Rating Scale

GPRs: G-Protein-coupled Receptors

HADS: Hospital Anxiety and Depression Scale

IBD: Inflammatory Bowel Diseases

IBS: Irritable Bowel Syndrome

IBS-D: IBS-Diarrhoea

IBS-C: IBS-Constipation

IBS-M: IBS-Mixed

IBS-U: IBS-Unclassified

IFN: Interferon

IG: Immunoglobulin

IL: Interleukin

LogCPM: Log Counts Per Million

LogFC: Log Fold Change

LPS: Lipopolysaccharide

MAPK: Mitogen-Activated Protein Kinase

PBMCs: Peripheral Blood Mononuclear Cells

PCA: Principle Coordinate Analysis

PI-IBS: Post-Infectious IBS

PLS-DA: Partial Least Squares Discriminant Analysis

PSYKI: Psyllium and Kiwifruit translation (study)

PRO: Patient-Reported Outcomes

PROMIS: Patient-Reported Outcomes Measurement Information System and Hospital

ROC: Receiver Operating Characteristic

ROS: Reactive Oxygen Species

SAGIS: Structured Assessment of Gastrointestinal Symptoms Scale

sPLS: Sparse Partial Least Squares

SRB: Sulphate-Reducing Bacteria

TGF: Transforming Growing Factor

TJ: Tight Junction

TLR: Toll-like Receptor

TNF: Tumor Necrosis Factor

Chapter 1

Review of literature

Part of this literature review has been published in *Frontiers in Cellular and Infection Microbiology*; "Caterina Carco, Wayne Young, Richard B. Gearry, Nicholas J. Talley, Warren C. McNabb, Nicole C. Roy, Increasing evidence that Irritable Bowel Syndrome and Functional Gastrointestinal Disorders have a microbial pathogenesis. *Frontiers in Cellular and Infection Microbiology*, 2020 Sep 9;10:468. doi: 10.3389/fcimb.2020.00468. PMID: 33014892; PMCID: PMC7509092."

1.1 Introduction

In the human body, there are about 39 trillion microbial cells, the majority of which inhabit the gastrointestinal (GI) tract, forming a dynamic ecological environment collectively known as the microbiota [1]. The microbiota encompasses up to 500 transient and indigenous species, including bacteria, viruses, fungi and protozoa, and comprise up to 20 million genes [2].

The microbial ecosystem exists in a mutualistic relationship with its host and plays a crucial role in maintaining a healthy GI tract. The GI microbiota exerts important functions, such as the extraction of energy from nutrients, metabolism of xenobiotics, modulation of motility, and maintaining the integrity of the epithelial barrier [3, 4]. Therefore, the GI microbiota contributes to the beneficial effects of food beyond the provision of nutrients [5].

Disruptions in the GI microbiota (dysbiosis) [6] have been associated with many abnormal GI functions. It is now accepted that its composition and function potentially contribute to functional GI disorders (FGIDs) [7]. These conditions are classified by GI symptoms related to any combination of motility disturbance, visceral hypersensitivity, and alterations of central nervous system processing, immunity and GI microbiota [8]. Irritable bowel syndrome (IBS) is the most common and best known of these disorders [9], characterised by abdominal pain associated with altered bowel movements and often bloating in the absence of morphological changes [7].

A growing body of evidence supports the role of microbial-immune interactions in the phenotype of IBS (reviewed by Ford & Talley [10]) and FGIDs, although most of the evidence comes from studies with IBS participants. However, the mechanisms responsible for FGIDs are poorly understood and there is a lack of consensus on the role of the GI microbiota and how changes to it relate to these conditions.

The role of GI microbiota in IBS is supported by the amelioration of symptoms by certain probiotics, prebiotics and selective diets [11-13]. Subjects with FGIDs often report that food plays a critical role in symptom development and diet is known to play an important role in modulating immune competence. Therefore, future nutritional solutions could be used to beneficially modify the GI microbiota and improve digestive functions in subjects with FGIDs, as well as in healthy subjects.

However, the way to achieve this is still unknown, and additional studies are required to understand better the underlying microbiota-mediated immune pathways and to establish whether diet can directly affect these pathways, thereby GI and overall health.

In this respect, subjects with FGIDs represent a model of an unhealthy GI tract to study digestive comfort and function in an otherwise healthy population. The characterisation of the main microbiota-mediated immune pathways associated with GI and non-GI symptoms will further our understanding of the mechanisms underlying FGIDs.

This review of literature summarises the current knowledge regarding the relationship between microbial and immunological mechanisms, and diet in the pathophysiology of FGIDs. The nomenclature of microbial taxonomy has undergone changes and reclassifications over time. However, for the purpose of my research during my PhD, I have utilised the names that were in use when I started.

1.2 Functional gastrointestinal disorders

Functional GI disorders are a highly prevalent group of disorders diagnosed solely by symptom-based criteria [13], including symptom severity and frequency (sporadic, daily) and faecal characteristics [14]. These conditions include IBS, functional constipation (FC) and functional diarrhoea (FD). The predominant FGID, IBS, is classified into mutually exclusive categories according to Rome IV criteria, depending on their predominant bowel habit: diarrhoea predominant (IBS-D), constipation predominant (IBS-C), mixed diarrhoea/constipation (IBS-M) and unclassified (IBS-U). Rome IV criteria provide parameters for diagnosing of IBS based on abdominal pain and altered bowel habits in the absence of specific pathology [8]. However, symptoms like bloating, the passage of mucus and incomplete rectal evacuation, which are common and troublesome symptoms in people with FGIDs, are not included in the Rome IV criteria [15].

Irritable bowel syndrome is a multifactorial condition characterised by chronic and relapsing abdominal pain and altered bowel habits, while FC and FD feature a significant change in bowel habits but not abdominal pain in the absence of alternative pathology. The symptoms of IBS can overlap with those of other FGIDs, and it has been estimated that up to a third of patients with FGIDs have features of more than one, suggesting a

common underlying aetiology [16]. Irritable bowel syndrome is likely to result from genetic, environmental and dietary factors, and not a single aetiological cause.

Subjects with IBS can be further classified as sporadic (nonspecific), post-infectious (PI) or inflammatory bowel disease (IBD)-associated IBS. Post-infectious IBS occurs after an episode of infectious gastroenteritis [17], and IBD-associated IBS indicates IBS-like symptoms in patients with clinically quiescent IBD [18].

FGIDs are also described as "disorders of gut-brain interaction", as they can be classified as disorders that span both the GI tract and the neurological systems (Figure 1.1). For this reason, FGIDs has been renamed as disorders of gut–brain interaction [8, 19]. Subjects with FGIDs have high rates of psychological comorbidity [20] and treatments aimed at stress and anxiety (e.g. hypnotherapy [21], cognitive behavioural therapy [22], exercise [23] and antidepressants [24]) can be effective.

Several proposed pathophysiological mechanisms for FGIDs are based on altered neuro-gastroenterology, including changes in GI motility and visceral afferent hypersensitivity. Visceral hypersensitivity tends to be more strongly associated with IBS than with FC or FD, although many subjects with FC report abdominal pain [25]. IBS-C subjects report a shorter colonic transit time [26] and more severe symptoms of constipation compared to FC [27].

Several conditions feature symptoms which may be confused with IBS. Clinical overlap between IBS and other IBS-like disorders has been proposed. The overlap between IBS and functional dyspepsia and gastroesophageal reflux disease is characterised by early satiety, postprandial fullness, epigastric pain, heartburn, and regurgitation often associated with more severe symptomatology [28, 29]. Irritable bowel syndrome is also commonly associated with non-GI symptoms seen in other disorders, including fibromyalgia, chronic fatigue and temporomandibular joint disorder [30]. Irritable bowel syndrome was also observed in 33% of subjects reporting sleep disturbance [31] and 48% of subjects with bladder pain [32].

Although IBS is not fatal and seldomly requires hospitalisation, it is amongst the most frequent reasons for presentation to primary care. This condition leads to increased costs through consultations with health care practitioners, investigations for GI and non-GI disorders and subsequent treatments. Overall, it is estimated that more than 40% of people worldwide are affected by FGIDs [33]. Irritable bowel syndrome affects 11% of the

global adult population [7, 34], with a higher prevalence (60–75%) in women than men, especially for IBS-C [35]. Sex hormones have been postulated to be responsible for this gender difference, because of their involvement in the stress response, colonic motility, epithelial barrier function, immune activation and several regulatory mechanisms of the gut-brain axis [36]. Sex hormones can also directly affect colonic microbiota composition and metabolism through the oestrogen receptor β, as demonstrated in an animal study [37].

The severity of abdominal pain and the unpredictability of bowel function are the major factors lowering the quality of life of people with FGIDs. The subjects report quality of life scores close to or lower than subjects with rheumatoid arthritis and dialysis-dependent kidney failure [38, 39]. Regardless, research into FGIDs has been relatively underfunded. Understanding the aetiology and pathophysiology promises an opportunity to develop new, effective and personalised treatments in addition to biomarkers for diagnosis, determining severity and treatment response.

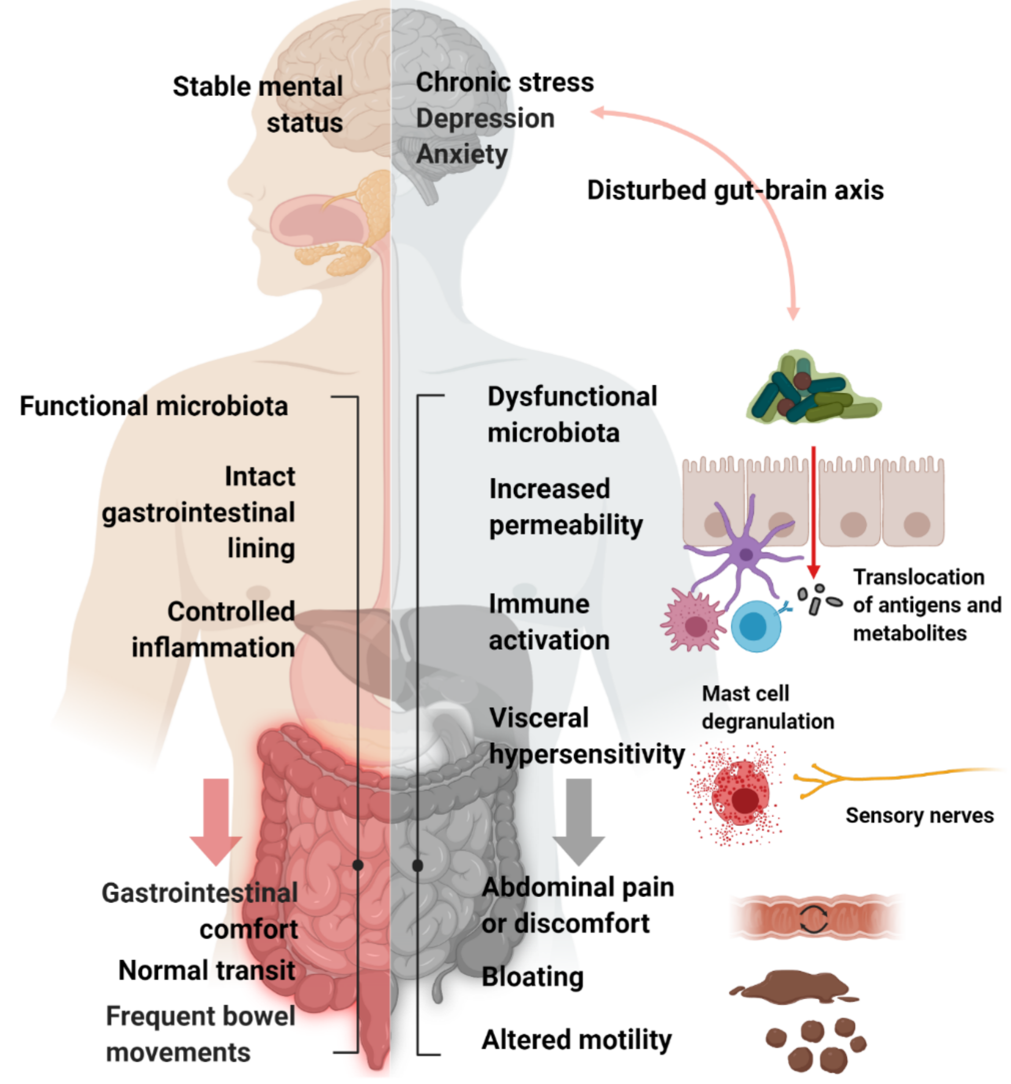


Figure 1. 1: Schematic representation of IBS pathophysiology.

Psychological, physiological and neuro-gastroenterological factors are thought to be involved in the generation of IBS symptoms, including bloating, abdominal pain and altered motility. Created with *BioRender.com*.

1.2.1 Post-infectious irritable bowel syndrome

Between 20-30% of IBS subjects report the onset of GI symptoms after an episode of infectious gastroenteritis. If the diarrhoea predominant phenotype persists despite clearance of the causative pathogen, this sub-group can be defined as PI-IBS [40]. Several studies confirm the link between bacterial infection and continuous abnormal bowel habits [41-43]. The predictability of this association may be influenced by parameters that

characterise IBS, the time of onset and the nature of the pathogen involved. Indeed, the PI-IBS subtype has been indicated as a consequence of bacterial (*Campylobacter jejuni* [41, 44, 45], *Clostridium difficile* [46, 47], *Salmonella* [42], *Shigella* [41, 48], *Escherichia coli* [49]), viral (Norovirus [50]) or parasitic (*Giardia duodenalis* [51]) infections (Figure 1.2). Whether different pathogenic species pose an equal risk of inducing PI-IBS remains under investigation. Viral gastroenteritis seems to be the most common cause [52] and more transitory if compared to the bacterial form [53]. On the other hand, *Campylobacter* spp. have been indicated as the most common bacterial cause of PI-IBS worldwide, with association rates between PI-IBS and *Campylobacter* greater than 20% [54].

The mechanisms underlying the generation of PI-IBS are not fully understood. It is evident that bacterial or viral infections compromise GI barrier integrity and enhance chronic inflammation, which can detrimentally affect the GI microbiota [55]. Microbiota composition in subjects with PI-IBS differs from both IBS subjects and controls [60]. It is characterised by an increased relative abundance of the Bacteroidetes phylum (usually decreased in IBS) and decreased relative abundance of the Firmicutes phylum, including the Clostridia class [56]. Moreover, it has been demonstrated that lamina propria T cell counts ($CD4^+$ or $CD8^+$) increased in PI-IBS, even when the infecting pathogen was no longer detectable [57].

From this, it can be speculated that the initial infection may have decreased the abundance of the beneficial bacterial species and altered the microbial environment, leading to a prolonged immune response [58]. However, whether the prolonged immune response is attributable to the pathogen or commensal bacteria remains to be determined.

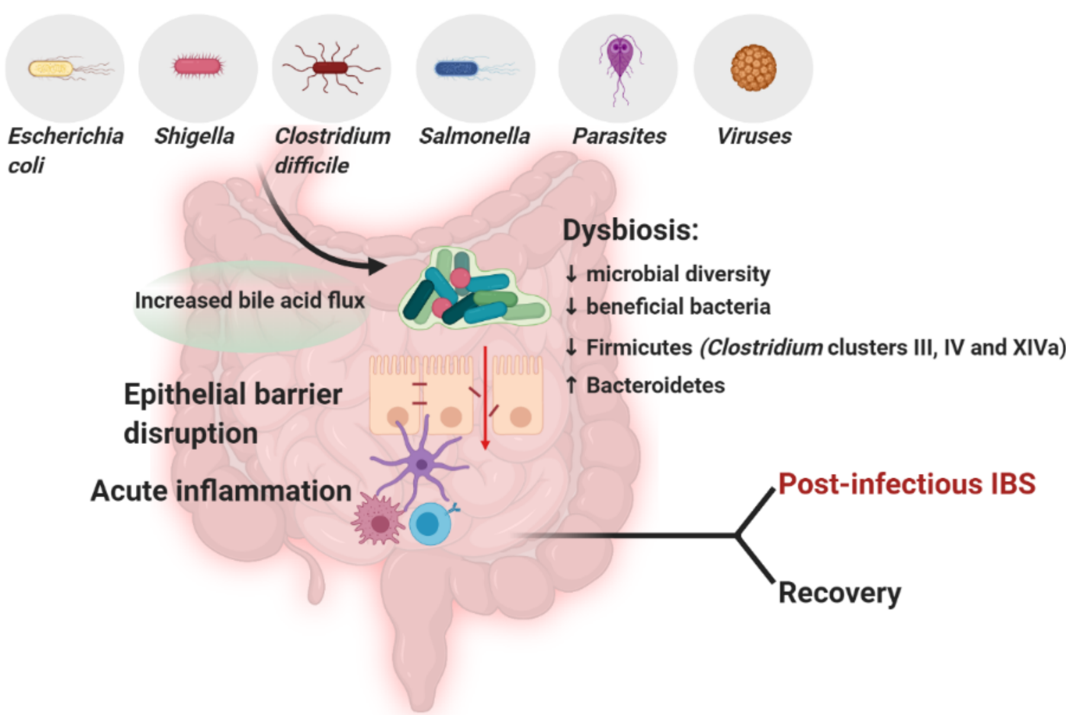


Figure 1. 2: Dysbiosis in post-infectious IBS.

In subjects with post-infectious IBS, the infection of certain pathogens, such as *Clostridiodes difficile* [48, 49], *Salmonella* [44], *Shigella* [43, 50] or *Escherichia coli* [51], compromises the integrity of the epithelial barrier, triggers inflammation and decreases microbial diversity and the abundance of beneficial bacteria, detrimentally affecting GI microbiota composition [60]. The microbiota composition in post-infectious IBS subjects differs from both IBS subjects and controls, featuring an increase in Bacteroidetes phylum, which is usually decreased IBS, and decreased Firmicutes phylum, particularly from the Clostridia class [56]. Created with *BioRender.com*.

1.3 A microbial signature in health and functional gastrointestinal disorders

The human GI tract is the organ with the largest surface area (250–400 m^2) in the human body and separates the host from external molecules, such as antigens from microbes and nutrients [59]. The resident microbiota has co-evolved with the human host over thousands of years to form a complex and mutually beneficial relationship [60]. The most abundant phyla in the healthy GI tract are Firmicutes and Bacteroidetes, but Actinobacteria, Proteobacteria, Verrucomicrobia and the less represented Fusobacteria, Tenericutes, Spirochaetes and Cyanobacteria are also present [61, 62]. Furthermore, the

microbial composition changes across the different regions of the GI tract, with a predominance of Firmicutes phylum in the proximal colon and Bacteroidetes phylum in the distal colon [63].

The health-associated patterns of microbial colonisation of the GI tract are difficult to define, as different individuals can harbour functional and distinctive variants of microbial composition, reflecting early-life events such as mode of delivery, type of feeding and gender [64]. However, a "healthy" microbial signature is generally characterised by a prevalence of Firmicutes phylum and Bacteroidetes phylum and a general lack of Proteobacteria phylum [65].

Despite inconsistencies between studies, some differences between a healthy and an IBS-related faecal microbiota have been observed. At the phylum level, a higher [66-69] or lower [70, 71] Firmicutes:Bacteroides ratio and differences in Actinobacteria and Proteobacteria prevalence have been observed in IBS [72].

At the genus level, IBS subjects generally have increased *Ruminococcus* [66, 73-76], *Clostridium, Coprococcus* and *Blautia* and reduced *Faecalibacterium* genus relative abundances [66, 77]. These bacteria are thought to have a prominent role in carbohydrate metabolism in the colon.

Other alterations have been generally described in IBS, including an increased relative abundances of pathobionts, such as *Veillonella* genus [69, 73, 78], *Enterobacteriaceae* family and *Bacteroides* genus or a decrease in *Prevotella* genus [66] and *Desulfovibrionaceae* family [79]. The *Desulfovibrionaceae* family include sulphur-reducing bacteria that compete with methanogens for hydrogen in the human colon [80]. Overall, the relative differential abundance of taxa from the Bacteroidetes phylum and *Ruminococcaceae* and *Lachnospiraceae* families have been reported across studies [66-68, 76].

Previous studies showed that methanogen relative abundance, exhaled methane level and symptom severity were negatively correlated with microbial richness, suggesting methane may contribute to slower intestinal motility and constipation [67, 81, 82]. In addition, an increase in faecal *Methanobrevibacter smithii* abundance and breath methane concentration [83] and a positive association between *Methanobrevibacter* abundance and faecal firmness [84] have been reported in IBS-C subjects.

It was demonstrated that methane decreases colonic transit time by increasing the amplitude of contraction, decreasing peristalsis, and contributing to constipation [85].

Methane is not inert, so it can interfere with the neuromuscular function of the GI tract, leading to a reduced propagation of the peristaltic movement [86]. The elevated breath methane production in IBS-C subjects could reflect the outgrowth of slow-growing microbes, which are advantaged in conditions of slowed colonic transit and are resistant to the lack of water that characterise firmer faeces [87]. However, another study did not observe an association between breath methane production and constipation or colonic transit, although they reported an association between breath methane production and changes in faecal microbiota composition [88].

Other findings linked decreased levels of methanogens in faeces to excess abdominal gases in IBS, suggesting that IBS subjects may lack some functions for hydrogen removal [70, 71]. In addition, hydrogen accumulation has been linked to bloating and abdominal pain [89]. Hydrogen sulphide alternatively derives from the activity of sulphur-reducing bacteria and has been shown to modulate peripheral pain-related signals, and colonic motility [90].

1.3.1 Microbial signatures in irritable bowel syndrome subtypes

Several studies report discrepancies in faecal microbiota profiles between the IBS subtypes. For example, some studies report no differences in the composition of the microbial community between IBS-C and IBS-D [91], while other studies associated different IBS subtypes with an unique microbial signature (Table 1.1).

IBS-C usually features a higher abundance of Firmicutes and a selective reduction in the abundance of lactate-producing and utilising bacteria, such as *Bifidobacterium* genus and *Eubacterium hallii*/*Anaerostipes caccae*, respectively [92]. IBS-D, compared to IBS-C, is characterised by an overall reduction in microbial diversity, an increase in potentially detrimental bacteria, such as those from the Proteobacteria phylum, and lower numbers of Actinobacteria and Bacteroidetes phyla [73, 77].

Decreased relative abundances of the *Bifidobacterium* genus in both faecal [66, 73, 93, 94] and mucosal samples [93, 94], and *Lactobacillus* genus in faecal samples [73] have been described in IBS-D, although some studies reported the opposite findings [69, 72,

78, 95]. The reduction of *Bifidobacterium* and *Lactobacillus* genera is noteworthy because of their capacity to exert bactericidal effects against pathogens and promote immune tolerance by producing metabolites, including lactate and acetate.

Acetate, butyrate and propionate, known as short-chain fatty acids (SCFAs) [96], represent the end-products of fermentation of non-digestible polysaccharides by the ileal and colonic microbiota [97]. They are directly associated with host-microbial interactions through nutritional, regulatory and immunomodulatory functions.

The relative abundance of SCFA-producers (e.g., the Clostridiales order, the *Bifidobacterium* genus, the *Ruminococccaceae* family, and the *Erysipelotrichaceae* family) have been reported to be increased [66] or decreased [71] in IBS-related microbiota profiles. Altered levels of SCFAs in faeces were associated with a different distribution of Clostridiales order, and faecal consistency in both IBS-C and IBS-D subjects [98]. *In vitro* studies demonstrated that SCFAs could lower the colonic pH [99]. Taxa from the *Lachnospiraceae* family have been shown to be resistant to lower pH values, but not from the Bacteroidetes phylum.

Instillation of boluses of SCFAs at high concentrations in the ileum increased ileal motility and abdominal pain in humans [100] or promoted visceral hypersensitivity in a rat model [101]. These observations may be relevant since abnormal levels of SCFAs, visceral hypersensitivity and dysmotility are often observed in those with IBS. On the other hand, the low relative abundance of butyrate-producing bacteria observed in IBS-C [92], IBS-D and IBS-M [71], could decrease the butyrate availability and therefore reduce the potential health benefit from this SCFA, including anti-inflammatory effects, colonic defence barrier and decrease in oxidative stress [102].

Discrepancies in the relative abundance of beneficial bacteria and SCFA-producers may be explained by several factors, including differences in diet, study size, the predominance of IBS subtypes, IBS severity, as well as DNA extraction methods, analytic techniques or primers used for amplicon generation.

The relative abundance of specific genera positively correlated with IBS symptom severity. The composition linked to the IBS-D subtype is the most different from "normal" in terms of composition and is associated with the most severe symptomatology [67]. The immune profile associated with IBS-D has also been reported as different from

the other subtypes and positively correlated with pain severity, dissatisfaction with bowel habits and overall GI symptoms [103].

The differences in microbial composition between IBS and healthy subjects and within IBS subtypes raise questions regarding which microbes are associated with IBS and whether qualitative (dysbiosis) or quantitative (e.g., bacterial overgrowth) changes occur first in IBS aetiology. The usefulness of describing the microbiota at higher taxonomic levels may be limited since this may not provide enough differentiating information between taxa. Current techniques relying on 16S ribosomal RNA analysis may also overlook potential pathogens, such as colonic spirochetes, due to the incompatibility of standard primers [104].

The involvement of the GI microbiota in IBS pathophysiology is also supported by clinical evidence. In a randomised, double-blind, placebo-controlled study, Rifaximin, a non-systemic antibiotic for treating IBS-D [105], showed a largely transient effect across a broad range of faecal microbes, such as *Peptostreptococcaceae*, *Verrucomicrobiaceae* and *Enterobacteriaceae* families [106].

Faecal microbiota transplantation to restore the colonic microbiota of IBS subjects to a healthy status showed positive outcomes depending on the mode of delivery [107-109]. Despite this, other studies demonstrated no relationship between post-transplantation microbial diversity and clinical improvements in IBS [110-112].

Finally, the majority of the studies on IBS analysed only a single microbial colonic niche [68, 70, 73-75, 77-79, 92] because of the convenience of analysing the microbiota in faecal samples rather than mucosa-associated contents (Figure 1.3). The faecal- and mucosal-associated microbiota have been reported to be structurally distinct but highly correlated [67] and at times have been described as able to discriminate between IBS-D subjects and controls [95], to discriminate only the subjects with severe IBS [67], or to not discriminate IBS subjects from controls [113, 114]. Another study showed that the composition of the colonic-associated mucosal microbiota could also separate patients with FC from controls with 94% accuracy [88].

Table 1. 1: Main differences in faecal microbiota composition between IBS subtypes. F/B ratio, Firmicutes:Bacteroidetes ratio [115]. The direction of the arrows indicates increased (↑) or decreased (↓) relative abundance in comparison to controls.

	IBS-C	IBS-D	IBS-M
Phylum	↑ Firmicutes ↑ Actinobacteria	↑ F/B ratio ↑ Proteobacteria ↓ Bacteroidetes ↓ Actinobacteria	↑ F/B ratio
Class	↑ Clostridia		
Order	↑ Clostridiales ↑ Coriobacteriales		
Family	↑ *Incertae Sedis* XIII ↑ *Lachnospiraceae* ↑ *Ruminococcaceae* ↑ *Rhodospirillaceae* ↑ *Coriobacteriaceae*	↓ *Erysipelotrichaceae* ↓ *Ruminococcaceae* ↓ *Porphyromonadaceae* ↓ *Ruminococcaceae* ↓ *Unknown Clostridiales* ↓ *Methanobacteriaceae* ↓ *Incertae sedis* XIII	↓ *Erysipelotrichaceae* ↓ *Ruminococcaceae* ↓ *Incertae sedis* XIII ↓ *Eubacteriaceae*
Genus	↓ *Roseburia* ↓ *Bifidobacterium*	↓ *Bifidobacterium* ↓ *Lactobacillus*	
Species	↓ *Eubacterium rectale* ↓ *Eubacterium hallii* ↓ *Anaerostipes caccae* ↑ *Methanobrevibacter smithii*		

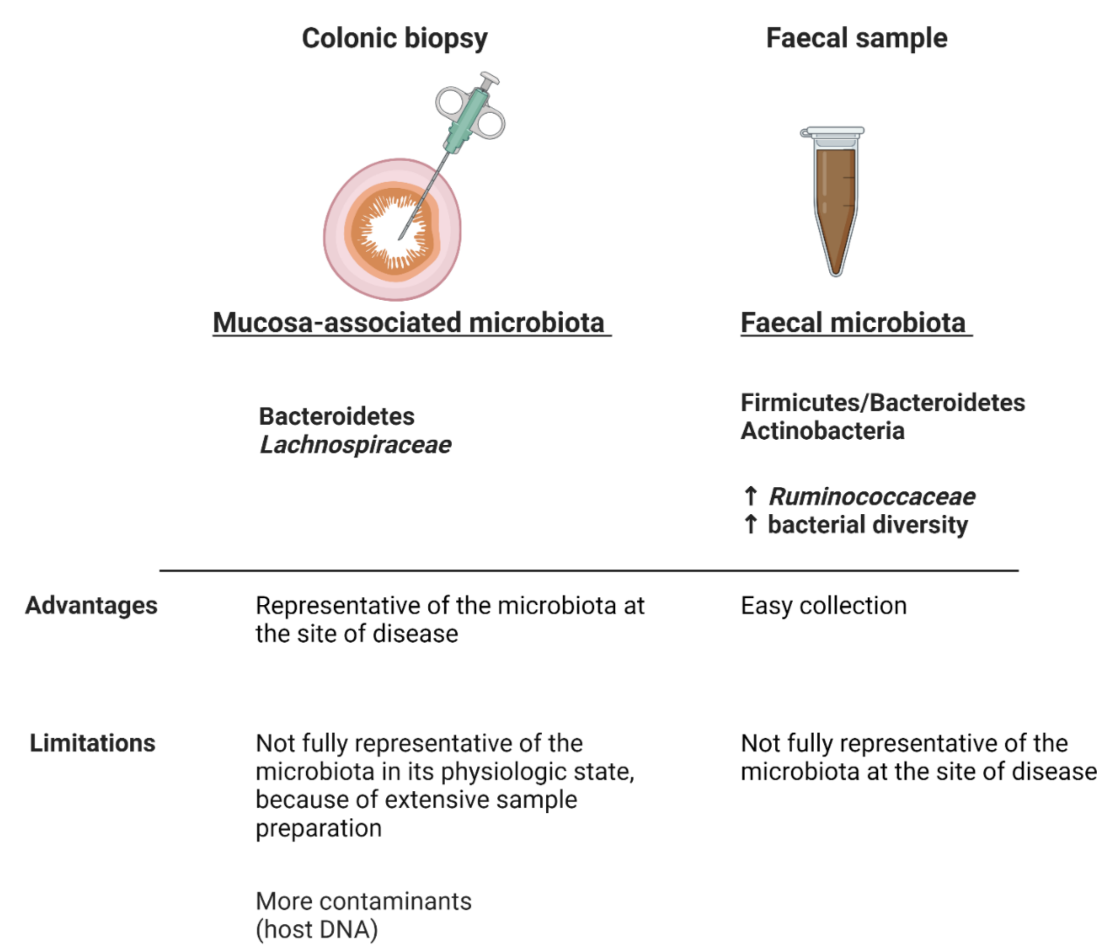


Figure 1. 3: Comparison between mucosa-associated and faecal microbiota.

Faecal and colonic mucosal-associated microbiota can interact with the immune system and be involved in FGID symptomatology [91]. The faecal microbiota is not fully representative of the mucosal-associated microbiota at the site of the disease. Taxonomic and diversity differences between faecal and colonic mucosal microbiota highlight the importance of comparing the microbial composition in both niches when analysing the role of the GI microbiota in FGIDs. The colonic mucosa-associated microbiota seems to be predominantly characterised by the Bacteroidetes phylum [67, 116] and the *Lachnospiraceae* family [113]. In contrast, the faecal microbiota by the Firmicutes phylum, the Actinobacteria phylum [67, 116], a higher relative abundance of the *Ruminococcaceae* family [113], and a higher microbial diversity compared to the colonic mucosa-associated microbiota [116]. Microbial abnormalities in IBS subjects have been reported to be more pronounced in faecal samples than in colonic mucosal samples and the separation between mucosal and faecal microbiota composition was more distinct in IBS subjects than in controls [116]. Whether IBS symptomatology is associated with taxonomic differences in the faecal and/or mucosal microbiota remain to be determined. Created with *BioRender.com*.

1.3.2 Microbial modulation of immunity and homeostasis

Several studies highlight the immunological and regulatory effects of microbially-derived molecules, such as SCFAs, in modulating inflammatory responses through different signalling pathways. For instance, butyrate can act as an inhibitor of histone deacetylases, regulatory proteins acting on the epigenome through chromatin-remodelling changes [117]. Alternatively, SCFAs can interact with G-protein-coupled receptor (GPR)41, GPR109A and GPR43, which are abundantly expressed on small intestinal and colonic epithelial cells, monocytes, and neutrophils, to decrease pro-inflammatory cytokine [118, 119]. GPR109A, a receptor for niacin, is bound by butyrate in the colon, promoting regulatory T cell differentiation, interleukin (IL)-10 and IL-18 expression in the colonic epithelium [120]. IL-18 can promote epithelial restoration and inflammation recession [121].

On the other hand, SCFAs can mediate protective immunity in some conditions. For example, SCFA activation of GPR41 and GPR43 on intestinal epithelial cells has been shown to cause rapid production of pro-inflammatory chemokines and cytokines [122] (Figure 1.4). In addition, SCFAs are well known for modulating immune cell chemotaxis, phagocytosis, reactive oxygen species (ROS) release and reduction of NF-κB activity. The three major SCFAs were shown in order of potency being butyrate>propionate>acetate [123]. In particular, butyrate can inhibit the production of pro-inflammatory IL-8 and tumour necrosis factor (TNF) by macrophages *in vitro* [124] and *in vivo* [125].

Despite the potential relevance of abnormal levels of colonic SCFAs in IBS pathophysiology, findings are inconsistent and often conflicting between studies. A recent meta-analysis identified an overall reduction of butyrate and propionate in faecal samples of IBS-C subjects and higher levels of butyrate in faecal samples of IBS-D subjects compared to controls [126].

These findings support the role of the colonic microbiota in modulating the host's immune responses. However, this relationship is likely to exist in a mutual feedback loop, where the immune systems can also shape the composition of the microbiota. Several observations support this hypothesis. For example, in mice, the absence of the myeloid differentiation primary response 88, an adapter protein involved in toll-like receptor (TLR) signalling, leads to the overgrowth of taxa from the Bacteroidetes phylum [127].

In addition, the risk of developing IBS after an episode of gastroenteritis [57] suggests that the activation of the immune system by infectious triggers including microbes, viruses or parasites, could impact the composition and function of the microbial community.

Further evidence of these mutual microbe-immune interactions is an increased abundance of the pro-inflammatory bacterial protein flagellin [128] and flagellin-producing taxa (Clostridia class) in IBS subjects [129, 130]. In addition, the mucin-degrader *Ruminococcus torques* produce flagellin proteins [136] and is also frequently associated with IBS [131].

Different species of commensals have been reported to induce specific effects on the host immune responses. For example, *Bacteroides fragilis* was demonstrated to have a protective role by inducing the proliferation of IL-10-producing-regulatory T cells through the expression of the surface factor polysaccharide A [132]. Similarly, bacterial strains from the *Lachnospiraceae* and *Ruminococcaceae*, families from a healthy human faecal sample were demonstrated to increase the number and function of colonic regulatory T cells in colonised rodents [133].

Several species from the Clostridia class can generate biologically active catecholamines. The association of germ-free mice with *Clostridium* species with abundant β-glucuronidase activity, increased free active catecholamines [134]. These findings suggest a crucial role for the Clostridia class in GI immune homeostasis, and potentially in IBS pathophysiology.

1.3.2.1 Microbiota-immune interactions between the gastrointestinal tract and the central nervous system

The high co-morbidity between FGIDs and stress-related symptoms and the sensitivity of the microbiota to stress exposure represent further evidence of the involvement of the gut-brain axis in FGIDs [135]. Maes *et al.* were the first to demonstrate that psychological stress in humans induces inflammatory responses with increased production of the pro-inflammatory cytokines interferon (IFN)-γ, TNF and IL-6 [136]. In addition, stress-induced mediators, such as the corticotropin-releasing factor, increased macromolecular permeability in the healthy human colon via corticotropin-releasing factor receptors on subepithelial mast cells [137]. Animal models of stress-related disorders showed critical

changes in faecal [138] and mucosal [139] microbial composition, caecal metabolite concentration [140], ileal immune gene expression, and serum cytokine concentration [138, 140].

Microbiota-immune interactions in the GI tract and the central nervous system can also be affected by the availability of the essential amino acid tryptophan [141] and its metabolites (indole, indolic acid derivatives, skatole, and tryptamine).

The hydroxylation of L-tryptophan by the tryptophan hydroxylase 1 enzyme, expressed in enterochromaffin cells in the small and large intestines, generates the majority of serotonin in the body [142, 143]. In IBS, increased tryptophan metabolism was associated with low-grade inflammation and microbiota alterations [144]. A link between the microbiota and tryptophan metabolism was confirmed in germ-free mice exhibiting abnormal levels of the neurotransmitter serotonin in the colon but not in the small intestine [145]. In addition, mucosal biopsies from subjects with IBS showed reduced mRNA expression levels of tryptophan hydroxylase 1 [146]. Therefore, dysregulation of the tryptophan pathway, which affects mood and cognition, colonic motility, and visceral hypersensitivity [147], may be involved in IBS pathogenesis.

Similarly, reduced serotonin reuptake and impaired serotonin release were reported in IBS-D and IBS-C subjects, respectively [148]. Tegaserod, which is used to treat IBS-C, and alosetron, which is used to treat IBS-D, respectively stimulate and block the serotonin 5HT4 and 5HT3 receptors [149].

Although inflammation may underlie many pathways involved in FGID symptom generation, including visceral hypersensitivity [150], abdominal pain [151] and increased permeability [137], the mechanisms behind the connection between stress, inflammation and colonic mucosal barrier function are still largely unknown.

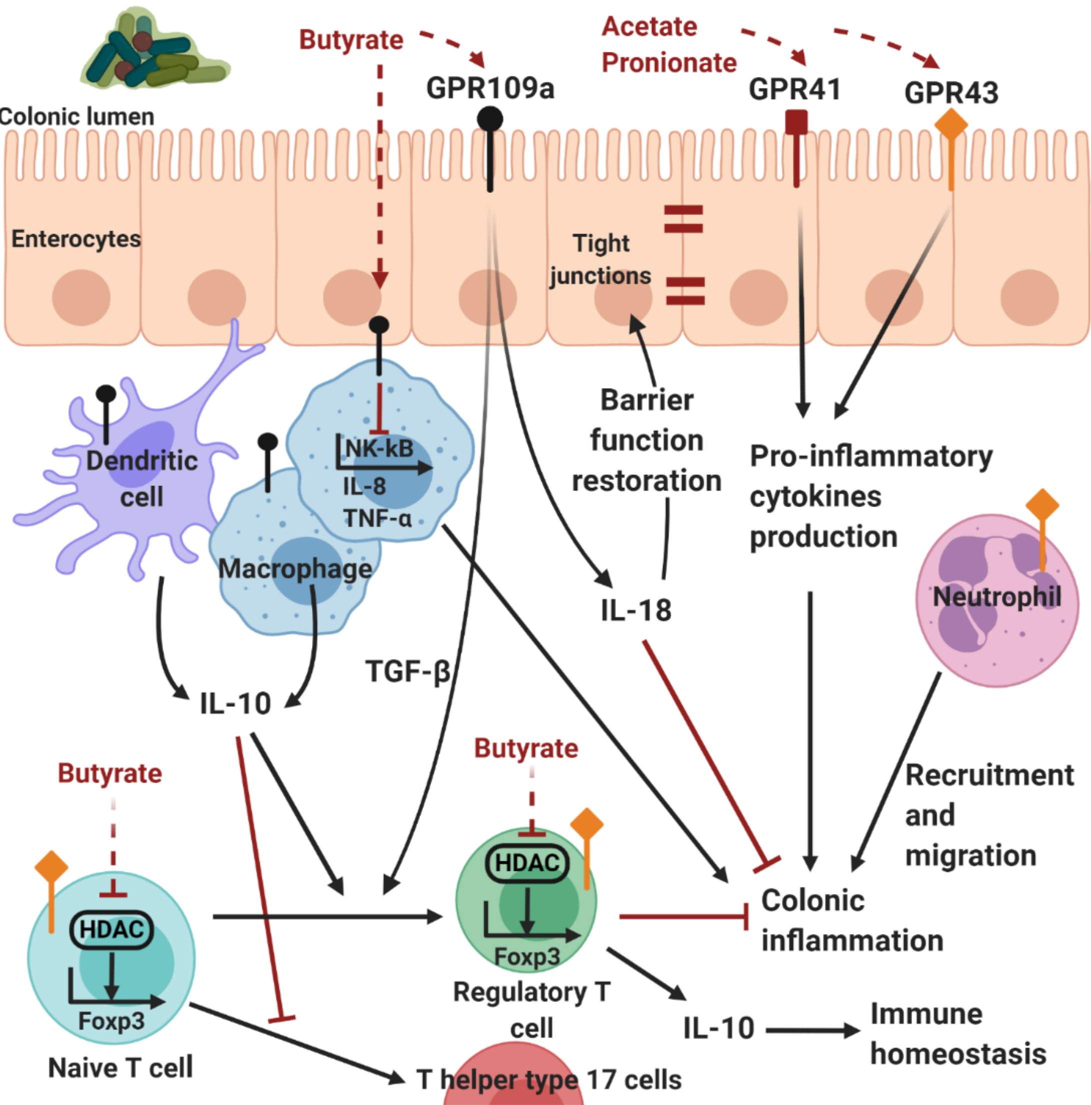


Figure 1. 4: Host-microbe interactions mediated by SCFAs.

G-protein-coupled receptors expressed on small intestinal and colonic epithelial and immune cells are activated by SCFAs. Acetate and propionate are the most efficient agonists for GPR43 and GPR43, followed by butyrate and other SCFAs [122]. Propionate binds GPR43 on colonic regulatory T cells to inhibit histone deacetylase function and enhance FOXP3 expression, thereby promoting regulatory T cell differentiation and IL-10 production. Although acetate is a potent GPR43 ligand and mediates colonic regulatory T-cell accumulation, it is unclear whether this is through this receptor [122]. Butyrate has similar effects by either stimulating dendritic cells and macrophages to produce IL-10 or directly acting on naive T cells, inhibiting the activity of histone deacetylases on the Foxp3 gene, inducing naive CD4+ T cells differentiation and regulatory T cell expansion [122]. Butyrate can induce the production of TGF-β and the cytoprotective IL-18 by the enterocytes through the activation of GPR109A. In addition, butyrate can inhibit NF-κB signalling, reducing the expression of pro-inflammatory IL-8 and TNF [122]. On the other hand, SCFAs can mediate protective immunity, activating GPR41 and GPR43 on small intestinal and colonic epithelial cells and producing pro-inflammatory chemokines and cytokines [122]. Therefore, SCFAs contribute to the maintenance of intestinal homeostasis through multiple mechanisms. Created with *BioRender.com*.

1.3.3 Microbial regulation of epithelial barrier function

In a healthy GI tract, the mucosal barrier prevents direct contact between the microbiota and the host. Together with the mucus layer, the mucosal barrier represents a "shield" against pathogens. The mucosal barrier also includes the mucosal immune system and the enteric nervous system [152].

Mucins are highly glycosylated macromolecule components of the mucus barrier. They represent an alternative substrate to dietary polysaccharides for mucin-degrading bacteria, such as *Ruminococcus torques* and *Akkermansia muciniphila* [153]. An abnormal increase in these species (such as through dietary restriction) may reduce mucus layer thickness, possibly contributing to impaired mucus barrier function, increased pathogen susceptibility and inflammatory conditions [154]. An altered relative abundance of mucin-degraders may otherwise reflect changes in mucus shedding in subjects with IBS-D, resulting in mucous discharge in their faeces.

The metabolism of sulphated mucins by mucin-degrading bacteria is a source of sulphate, which can be subsequently reduced to hydrogen sulphide [155]. High concentrations of hydrogen sulphide have been demonstrated to induce oxidative stress, impair cellular respiration and adenosine triphosphate production [156] and inhibit butyrate oxidation by colonocytes *in vivo* [157] and *in vitro* [158]. The inhibition of butyrate oxidation means colonocytes are therefore deprived of their main sources of energy. Oxidative stress and energy starvation may result in colonocyte death, weakening of the epithelial barrier and direct contact of commensals with the mucosal immune system [157]. Therefore, increased levels of hydrogen sulphide, in conjunction with increased microbial nitric oxygen production and decreased mucosal sulphide detoxification, have been shown to damage the colonic epithelium and contribute to mucosal inflammation [159].

The GI microbiota can also directly control epithelial permeability by upregulating tight junction (TJ) proteins in normal and pathological conditions [160-162]. Given this crucial role played by the commensals in the maintenance of epithelial barrier integrity, alterations in this community may be relevant for the increased small intestinal and colonic permeability often seen in IBS-D [163, 164]. In particular, biopsies from subjects with IBS-D showed a reduced expression of occludin [165] and claudin-1 in the colonic mucosa [166] and a disrupted apical junctional complex integrity in the jejunal mucosa [167].

Alterations of TJ proteins in IBS have also been associated with visceral hypersensitivity, abdominal pain [166, 168] and mast cell activation [167]. The increased small intestinal and colonic permeability may result in the translocation of bacteria and their products through the barrier, influencing local and systemic immune responses and contributing to the low-grade inflammation in IBS [152]. Pro-inflammatory cytokines such as IFN-γ, TNF, IL-4, IL-12 and IL-1β contribute to TJ disruption and increased paracellular permeability [169]. Hypersensitivity and symptom severity have been observed to be increased in IBS-D subjects with increased small intestinal and colonic permeability compared to controls and IBS-D subjects with normal permeability [170].

A subtype-specific increase of mucosal mast cell mediators, such as serine proteases and tryptases, in IBS-D subjects may be responsible for the observed increased colonic permeability [171, 172]. In addition, an *in vitro* study demonstrated that plasma lipopolysaccharides (LPS) and tryptase levels were increased in IBS-D but not in IBS-C [173]. The same study also showed an increased permeability when intestinal epithelial cells (Caco-2) were exposed to plasma from IBS-D and IBS-C subjects, with a higher effect for IBS-D than IBS-C. In addition, IBS-D subjects show distinctive transcription patterns regarding epithelial permeability, mast cell activity and gene and protein expression of TJ. In particular, zonula occludens mRNA expression has been observed to be inversely correlated with the mRNA expression of tryptase [174]. *In vitro* studies with Caco-2 monolayers [168], murine tissues incubated with colonic [175] or faecal [176] supernatants from IBS subjects support the correlation observed between decreased epithelial barrier function, zonula occludens-1 mRNA expression, inflammation and pain severity.

Colonic permeability in IBS may be ameliorated by the positive effect exerted by lactic-acid bacteria on TJ proteins. Indeed, a probiotic cocktail including *Streptococcus thermophilus, Lactobacillus* spp. and *Bifidobacterium longum* was shown to improve colonic barrier function in IBS-D subjects [177]. Probiotics are live microorganisms that may be beneficial for conditions featuring dysbiosis, such as IBS. Recent systematic reviews and meta-analyses reported contrasting results [178, 179], but suggest that probiotics as a class, have limited but beneficial effect over placebo on IBS symptoms, such as bloating and flatulence [178].

In conclusion, increased small intestinal and colonic permeability, which seems to be a prevalent feature of IBS-D, may trigger low-grade small intestinal, colonic and systemic

inflammation and may correlate with symptom severity. The molecular mechanisms responsible for increased small intestinal and colonic permeability in FGIDs are still poorly understood, but represent potential therapeutic and discriminating targets for IBS-D from other IBS subtypes and health.

1.4 Biomarkers to discriminate functional gastrointestinal disorders.

Understanding the mechanisms underlying host-microbial interactions and symptoms pathophysiology will likely improve the current knowledge of pathways involved and the predictive value of FGID biomarkers. Biomarkers can be measured in blood, faecal, urine or breath samples to discriminate FGID from other GI disorders or health potentially, and more importantly within the IBS subtypes, and to characterise improvements in well-being and quality of life. A true mechanistic biomarker could be defined as "one that exhibits accuracy, reproducibility, sensitivity, specificity and patient acceptability" [180], and that would make it possible to identify IBS rather than to rule out other organic diseases [181]. Sensitivity and specificity are measures of how well a marker correctly identifies true positive cases and true negative cases, respectively, and the combination of sensitivity and specificity of a biomarker can be expressed with a diagnostic value. A biomarker with a diagnostic value of 0.8 would be usually considered having good power in distinguishing subjects with and without a certain condition, correctly classifying 80% of the cases [182].

General features that differ in IBS versus healthy subjects include microbial composition, immune profile, GI motor and sensory function, pain perception, serotonin metabolism, and the expression of genes involved in immune activation [183]. Differences in the concentrations of faecal bile acids and lipids have also been shown to discriminate IBS-D from IBS-C. In particular, reduced total and primary faecal bile acids and increased faecal lithocholic acid were indicators of decreased faecal weight, frequency and consistency [184]. Fasting serum C4 (7a-hydroxy-4-cholesten-3-one), and fibroblast growth factor 19 showed good specificity to exclude the diagnosis of bile acid diarrhoea in IBS-D and FD [185].

In 2009, Lembo *et al.* reported 10 "first-generation" serum biomarkers with high specificity (88%), although the sensitivity was poor (50%) [186]. However, reflecting the complex pathophysiology, the utility increased when the panel was expanded to 34 serological and gene expression markers to discriminate IBS from controls [35]. Subsequently, other studies combined plasma and faecal biomarkers associated with different parameters of GI function, to reflect the multifactorial nature of IBS [187]. As a result, a novel multi-domain non-invasive biomarker panel was identified and validated,

which could discriminate IBS from health with high sensitivity (88.1%) and specificity (86.5%), and could be correlated with GI symptom severity in IBS and the general population [187]. This panel included plasma cytokine levels, such as IL-1β, IL-6, IL12p70, and TNF, as markers of systemic immune activation, faecal chromogranin A (CgA), as an indicator of the colonic neuroendocrine cell activity, faecal human β-defensin 2, as an indicator of immune protection against microbes, calprotectin, as an indicator of colorectal inflammation via neutrophil migration to the mucosa, and caproate, a product of microbial fermentation of non-digested oligosaccharides in the colon.

1.4.1 Toward an immune signature in functional gastrointestinal disorders

Some molecules of the immune system measured in faeces or blood could also be putative biomarkers. For example, faecal CgA plays a role in pain regulation and antimicrobial activity, and its faecal levels have been negatively correlated with colonic transit time in IBS subjects [188]. In addition, the faecal granin profile of IBS subjects was negatively associated with the microbiota α-diversity and positively associated with the genus *Bacteroides* [189]. Despite this, granins are not considered useful biomarkers for IBS because they lack specificity and discriminatory power.

Calprotectin, a protein released by neutrophils during GI inflammation, can be easily measured in faecal samples as it is resistant to degradation in the colon, it can be considered a non-invasive marker of low-grade inflammation in the GI tract. In a prospective study, faecal calprotectin was elevated in one-third of all patients across IBS subtypes [190]. A recent study demonstrated that differences in faecal calprotectin concentrations in children discriminated IBS from health and between IBS subtypes. In particular, faecal calprotectin concentration was highest in IBS-D, followed by IBS-M and IBS-C [191]. Although faecal calprotectin is primarily used to distinguish IBS from IBD [192, 193], it may effectively discriminate IBS from health and between IBS subtypes in combination with other plasma and faecal biomarkers [194].

Serine proteases (e.g., tryptases) are released by colonic mast cells and microbes. They have been reported to be elevated in IBS-D [195, 196]. These proteases are thought to play a role in several pathways involved in IBS symptom generation, such as the stimulation of colonic nerves through the protease-activated receptor-2, leading to

abdominal pain [197, 198]. Proteases also contribute to colonic mucosal inflammation [196], affecting smooth muscle motility and increasing paracellular permeability [196].

TLRs are a family of receptors on both epithelial and immune cells in those tissues exposed to the external environment, such as the lungs and GI tract [199]. TLRs bind to conserved microbial molecular patterns, and their activation induces the expression of pro- and anti-inflammatory cytokines and chemokines [200]. Increased levels of TLRs 4/5 [199, 201] or decreased levels of TLRs 7/8 [202, 203] were reported in IBS. A differential expression of TLRs in IBS subjects according to the disease subtype has also been reported [204].

In addition, it has been demonstrated that TLR activation can have consequences on colonic motility, through the activation of neuroendocrine mechanisms [201, 205] or interactions with the sulphide system. For example, TLR2 or TLR4 deficiency affected colonic free sulphide levels and sulphide-reducing-bacteria number [206], and $Tlr4^{-/-}$ mice showed a decreased amplitude and frequency of colonic contractions [207].

Consistent with these observations, differences in pro- and anti-inflammatory cytokine concentrations in the colonic mucosa or blood have also been associated with IBS [103, 208]. Increased concentration of pro-inflammatory IL-1β, IL-6, IL-8, TNF and IFN-γ [103, 209-215], and decreased concentration of the anti-inflammatory IL-10 [103, 216] in serum, plasma or colonic biopsies of IBS patients were reported. However, these changes were inconsistent between studies [217, 218].

Differences in the count and the activation rate of immune cell populations, particularly mast cells but also macrophages, lymphocytes and eosinophils, have also been reported in IBS [219, 220]. Mediators produced by these cells (nitric oxide, histamine and proteases) are likely to play a role in IBS symptom generation (Figure 1.5). Prostaglandin D_2 and leukotriene C4, D4 and E4, the main eicosanoids produced by human mast cells [221], have been demonstrated to increase the activity of afferent nerves [222] and enteric neurons, enhancing the epithelial response to inflammatory mediators [223]. In addition, lamina propria $CD3^+$, $CD4^+$ and $CD8^+$ T cells and activated macrophages have been observed in subjects with PI-IBS [57].

Finally, immune activation of peripheral CD4+ T-cells did not correlate with GI or psychological symptoms in IBS-D subjects [224]. In contrast, increased pro-

inflammatory cytokine release in IBS-D was associated with GI symptoms and anxiety in another study [225].

1.4.1.1 Mast cells

The role of mast cells in the pathology of FGIDs remains undefined. Despite this, the number and activation rate of mucosal mast cells were reported to be higher in IBS-D subjects than in controls and correlated with abdominal pain severity and frequency [151, 226]. Another study reported no difference in mast cell count, but the percentage of degranulated mast cells was increased in IBS-D subjects compared to controls [227]. Jakate et al. observed that 70% of patients with FD screened in their study had increased colonic levels of mast cells than the control group. This increased number of mast cells in the mucosa of subjects with chronic FD has been defined as mastocytic enterocolitis [228].

Psychological factors also seem to contribute to higher numbers of colonic mast cells in FGID subjects with high depression and fatigue scores [229]. In addition to the number of colonic mast cells, an augmented activity of colonic mast cells in proximity to sensory nerves is likely to play a role in IBS symptom development. These findings are supported by the fact that the mast cell stabiliser ketotifen improves the symptom score in subjects with IBS [150]. These findings advance the understanding of the role of immunity in FGIDs, but the diagnostic value of these biomarkers remains uncertain [230].

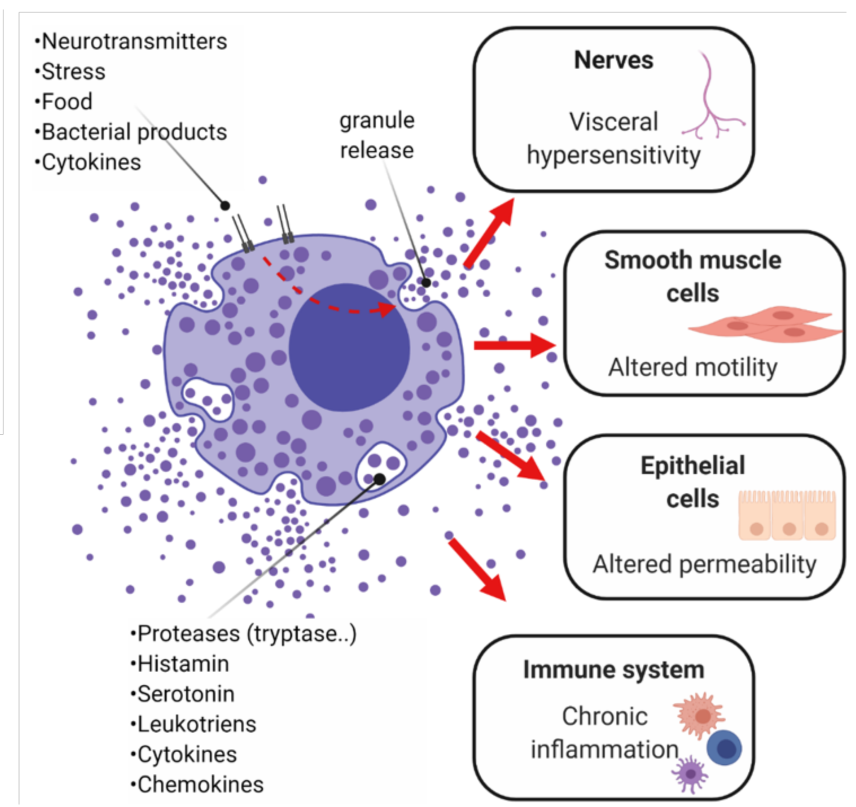


Figure 1. 5: Potential role of mast cells in IBS and chronic low-grade inflammation.

Mast cells are thought to play a role in the onset of abdominal pain, as well as diarrhoea or constipation. These symptoms are modulated by the mediators released by activated mast cells of the colonic mucosa, which stimulate other immune cells, perpetuate chronic inflammation and alter secretion and peristalsis, resulting in abnormal permeability and motility. A possible mechanism connecting stress and GI symptoms is the interaction between mast cells and the brain, which is mediated by extrinsic sensory nerves [231]. The mast cell-enteric nervous system axis combines the ability of the enteric nervous system to integrate paracrine signals with the specialised functions of the enteric mast cells. Mast cells, located close to nerve fibres, are thought to trigger pain signals. The mediator histamine sensitises the nociceptor transient receptor potential channel V1 on the peripheral nerve terminal of nociceptive submucosal neurons, resulting in visceral hypersensitivity [232]. Studies on rectal biopsies from IBS subjects demonstrated that the histamine H1 receptor-mediated stimulation of the nociceptor transient receptor potential channel V1 was potentiated in IBS subjects but not controls [233]. Proteases degranulated by mast cells may also destroy various epithelial gap junctional proteins (e.g., zonula occludens), leading to impairments in epithelial barrier function. Alterations in motility also seem to be linked to mast cells' degranulation. In particular, the stimulation of prostanoid receptors P2X on smooth muscle cells generates the excitatory potential responsible for contraction, impacting on smooth muscle contractility [234]. Created with *BioRender.com.*

1.5 The link between dietary components and functional gastrointestinal disorders

A growing body of evidence supports the role of dietary macronutrients (carbohydrates, proteins and lipids) in modifying the GI microbiota, leading to impacts on host metabolic and immune markers [235] (Figure 1.6). Several molecules, either coming directly from food, endogenous secretion or released by commensals are likely to influence the activity of the immune system [236].

Diet has been recognised to be involved in the predisposition or exacerbation of IBS. Up to 65% of subjects with IBS report that foods play a crucial role in their symptoms [237]. Three mechanisms have been proposed to explain the dietary intolerances in subjects with IBS; hypersensitivity to specific foods; hypersensitivity to food chemicals and luminal distension.

Firstly, food hypersensitivity may involve immunoglobulin (Ig) E-mediated (atopic) or non-IgE-mediated (non-atopic) reactions. Acute-phase IgE-mediated hypersensitivity results in the activation of mast cells, eosinophils, and other immune cells and the release of molecules (histamine, leukotrienes) involved in GI symptom generation [238].

Previous studies have not observed increased levels of IgE in IBS subjects [239] and serum IgE has not correlated with IBS symptom severity [240], rectal eosinophilia [241], colonic mast cell or eosinophil activation in IBS subjects [242]. Another study showed that more than 50% of IBS subjects responded to specific foods, characterised by eosinophil activation but was not associated with increased IgE levels [243]. Therefore, although atopic reactions to specific foods are common in IBS subjects, the association with IBS pathogenesis is not supported in the literature, and IgE-mediated food hypersensitivity in IBS is rare [244].

There is increasing evidence that IgG-mediated food hyperreactivity may play a role in IBS symptom generation, but results remain contradictory. Recent studies found elevated food-specific IgG levels in IBS subjects in comparison to healthy subjects [239, 245, 246]. In a randomised controlled trial, IBS subjects excluded foods that increased IgG levels from their diet. After three months, the dietary exclusion resulted in a reduction of symptom severity, suggesting that food elimination based on Ig levels may be promising

for reducing IBS symptoms [247]. The 87% of IBS subjects from this study reported symptomatic reactions to yeast, but previous studies with a similar number of participants observed lower percentages (5% and 12% [248, 249]) of IBS patients indicating yeast as an offending food. This finding suggests that increased levels of IgG to a specific food may not be necessarily linked to IBS symptom generation.

Other findings showed that no differences were found in IgG levels between IBS subjects and controls, and the association of low or high IgG levels in IBS subjects with symptomatology severity was inconsistent [250]. This finding supports that the increased IgG production is more likely to reflect a physiological response to diet rather than a pathological reaction, and that IgG-mediated hypersensitivity to yeast or other foods in IBS is unlikely.

Secondly, food bioactive chemicals, such as salicylates (contained, for example, in almonds, apples, and berries), can trigger a non-specific antigen-induced pseudo-allergic hypersensitivity reaction in IBS subjects, causing the release of cysteinyl leukotrienes [251]. These molecules are pro-inflammatory lipid mediators deriving from arachidonic acid, which increase smooth muscle contraction and vascular permeability [251], resulting in nausea, bloating, diarrhoea or visceral hypersensitivity.

Although salicylate sensitivity was suggested to affect 2–7 % of IBD subjects [251], there is a lack of concrete evidence linking salicylate sensitivity to FGIDs. In a survey with 643 IBS subjects, 12% reported their symptoms to be associated with the combined use of analgesics, including salicylate aspirin [252]. However, the study also showed that these subjects were intolerant to many foods, which could be associated with the reported symptoms.

The third mechanism involves a category of nutrients defined as fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAPs), which are short-chain, soluble, carbohydrates rapidly fermented by the colonic microbiota. Fermentable carbohydrates (e.g. fibre) are the most investigated dietary category in the context of IBS [253] because of their ability to influence the microbiota composition. Primary fibre-fermenters include the Clostridia class, the genus *Roseburia* and the species *Ruminococcus bromii* and *Eubacterium rectale* [254].

Soluble fibre supplementation may ameliorate constipation in IBS, as fibre increases faecal bulk with water and fermentation by-products (gas and SCFAs) [237]. However,

the fermentative properties of FODMAPs have been linked to luminal distension and IBS symptom generation (abdominal pain and bloating) [255] (Figure 1.6). In addition, although diets low in FODMAPs seem to allow for an adequate nutrient intake [256], they may decrease the absolute and relative microbial load and diversity, potentially leading to detrimental effects on the colonic environment and microbiota [257].

1.5.1 Fibre

Dietary fibre can act as a prebiotic, promoting the growth of beneficial bacteria (e.g., *Lactobacillus* and *Bifidobacterium* genera) and increasing SCFA and mucus production [258], which are important in the maintenance of homeostasis [259]. Human studies demonstrate that a diet enriched in prebiotics (fructooligosaccharides, galactooligosaccharides, xylooligosaccharides and arabinooligosaccharides) increases microbial gene richness and supports the growth of specific bacteria genera [260]. On the other hand, a low intake of prebiotic sources was demonstrated to reduce the total bacterial load [257].

For example, fructooligosaccharides and inulin, a fructooligosaccharide with longer fructose chains, seem to stimulate *B. longum* expansion [261], which is also associated with increased butyrate concentration [262, 263] and IBS symptom amelioration [261]. Co-culture experiments demonstrated that increased butyrate concentration results from bacterial cross-feeding, where inulin/fructooligosaccharide degradation-products, lactate and acetate, are converted into butyrate [262, 264]. In a study including mixed IBS subtypes, administration of a mixture of galactooligosaccharides led to a reduction of IBS symptoms, which was associated with decreased faecal levels of *Clostridium perfringens* and *Bacteroides* and *Prevotella* species, along with increased faecal levels of *Bifidobacterium* species [265]. However, other human studies investigating oligofructose [266] or fructooligosaccharides [267] reported no IBS symptom improvement.

Other important prebiotic effects and selective antimicrobial activity have been attributed to polyphenols [268]. Dietary polyphenols are organic chemicals in fruits, vegetables, cereals, tea, coffee and wine [269]. The colonic microbiota is responsible for converting dietary polyphenols into bioactive phenolic metabolites. The beneficial effects of polyphenol-derived metabolites include anti-inflammatory, antioxidant, anticarcinogenic, antiadipogenic, antidiabetic and neuroprotective effect [270]. Microbial

species involved in the hydrolysis of polyphenols include *Bacteroides distasonis, Bacteroides uniformis, Bacteroides ovatus, Enterococcus casseliflavus, Eubacterium cellulosolvens, Lachnospiraceae CG19-1*, *Eubacterium ramulus* [271], and members of the family *Coriobacteriaceae* [272].

Members of the *Gordonibacter* genus can convert ellagic acid (a dietary phenolic compound present in several fruits) to the anti-inflammatory, cardioprotective, and anticancer metabolite urolithins [273]. In particular, urolithins can affect the inflammatory functions of neutrophils, inhibiting IL-8 and extracellular matrix-degrading enzyme metallopeptidase-9 production [274].

1.5.2 Fats

Host lipid metabolism is often associated with changes in colonic microbial composition, particularly with the *Erysipelotrichaceae* and *Coriobacteriaceae* families [275]. Some members of the *Coriobacteriaceae* family are thought to be involved in metabolic disorders and FGIDs and are considered fat-induced pathobionts (i.e., potentially pathogenic symbionts of the microbiota) [276].

Diets enriched in saturated fats of animal origin have been associated with low-grade colonic inflammation through the activation of TLR-dependent signalling by microbial factors [277, 278]. The relative abundance of the *Erysipelotrichaceae* family has been linked to diets high in fats [279] and colonic inflammation. Some members of this bacterial family are coated with IgA and, therefore, are highly immunogenic [280]. However, it is unclear if the *Erysipelotrichaceae* family may play a causative role in colonic inflammation or if their relative abundance reflects more the dietary status of the host.

1.5.3 Proteins

A high intake of dietary protein, specifically animal-based proteins, has been linked to IBS through multiple mechanisms [281]. Excessive microbial fermentation of protein releases toxic end-products, such as ammonia, phenols, branched-chain fatty acids, and hydrogen sulphide (Figure 1.6).

Hydrogen sulphide is produced by the colonic microbiota mostly through the degradation of the dietary sulphur-containing amino acid cysteine [282]. Sulphate-reducing bacteria, such as the *Desulphovibrio* genus are mostly involved in colonic sulphur metabolism, but *Streptococcus*, *Fusobacterium*, *Salmonella*, *Enterobacter*, and *Helicobacter* genera also produce hydrogen sulphide from L-cysteine through cysteine desulphydrase activity [283]. *Akkermansia muciniphila* uses hydrogen sulphide for cysteine production, potentially limiting hydrogen sulphide concentrations and damaging to the mucus layer [284]. Hydrogen sulphide exerts a protective effect when produced from endogenous metabolism [285], but it can be deleterious for the colonic mucous barrier integrity when generated at high concentrations by colonic microbes [286].

High hydrogen sulphide levels were associated with IBS or IBD [287], and *Clostridium* spp. have long been considered major producers of ammonia from protein fermentation [288], which can impair the function of the colonic barrier [289] and stimulate the release of pro-inflammatory cytokines [290]. These observations may explain why many IBS subjects report that foods rich in animal protein (meat, fish and eggs), induce or worsen GI symptoms [291].

1.5.4 Vitamins

Vitamins are naturally occurring organic compounds required for diverse bodily functions, including the immune system [292]. Vitamins can be classified as either water- or fat-soluble. The water-soluble vitamins, including vitamins from group B (B_1, B_2, B_3, B_5, B_6, B_7, B_9 and B_{12}) and vitamin C, are readily absorbed by enterocytes by either diffusion or carrier-dependent active transport. Fat-soluble vitamins (A, D, E, and K), which are dissolved within fat droplets, must be broken down by lipases and combined with bile salts in the duodenum to facilitate absorption by the enterocytes [293]. Over 95% of dietary vitamins are absorbed in the small intestine, except cobalamin (B_{12}), which is absorbed in the distal ileum. The distal ileum also absorbs bile acids, which are required for the absorption of fat and fat-soluble vitamins [294].

Vitamins must be obtained from the diet, as humans do not sufficiently synthesise them; however, the colonic microbiota can also contribute to vitamin synthesis. For example, up to half of the daily vitamin K requirement is provided by the colonic microbiota [295]. Although daily nutrient intake in IBS subjects is similar to the general population, a higher

daily intake of foods containing vitamin E, folate, iron, vitamin C and dietary fibre, as well as a lower intake of foods containing vitamin A, vitamin D, cobalamin, riboflavin, calcium and potassium was observed in IBS subjects [296]. These differences could result from the dietary restrictions followed by IBS subjects and reflect a tendency toward a lower intake of meat and dairy products. Some bacteria cannot synthesise B vitamins for their growth and must acquire them from the host diet or other commensals. This observation suggests that colonic microbiota composition and function may affect host B vitamin usage [297].

A previous study reported an association between a low dietary intake of vitamin B_6 and IBS symptom severity [298]. Similarly, the administration of a dietary integrator containing vitamins from the B group (B_1, B_2, B_6) improved abdominal pain and abdominal distension to regulate bowel movements in an IBS cohort with mixed subtypes [299].

Other studies suggested a link between a deficiency in vitamin D and IBS symptoms [300, 301]. Vitamin D is a crucial immune-modulator, anti-inflammatory, and anti-microbial agent, explaining its association with IBS [302]. Depression, which initiates or aggravates IBS symptoms, is more common in vitamin D deficiency [303]. Moreover, vitamin D nuclear receptors seem to modulate colonic motility, antimicrobial peptide release and colonic inflammation, as several immune cells express vitamin D nuclear receptors [304].

Other studies showed that vitamin D nuclear receptors might play a role in maintaining a functional microbiota [305] and that vitamin D deficiency affects the transcription of cathelicidin and defensin β-4, leading to dysbiosis [306].

The most common reason for vitamin D deficiency in IBS subjects could be attributable to inadequate sun exposure, as subjects with IBS often tend to spend less time outdoors because of their condition [307]. Alternatively, IBS subjects may suffer from malabsorption of dietary vitamin D, as impaired bile acid circulation can lead to malabsorption of fat-soluble vitamins, such as vitamin D [308]. Although the exact role of vitamin D deficiency in IBS has not yet been elucidated, vitamin D supplementation improved IBS symptoms and quality of life in a double-blind, randomised, placebo-controlled study [309], reduced symptom severity in IBS-D subjects [310], as well as in adolescents with IBS [311].

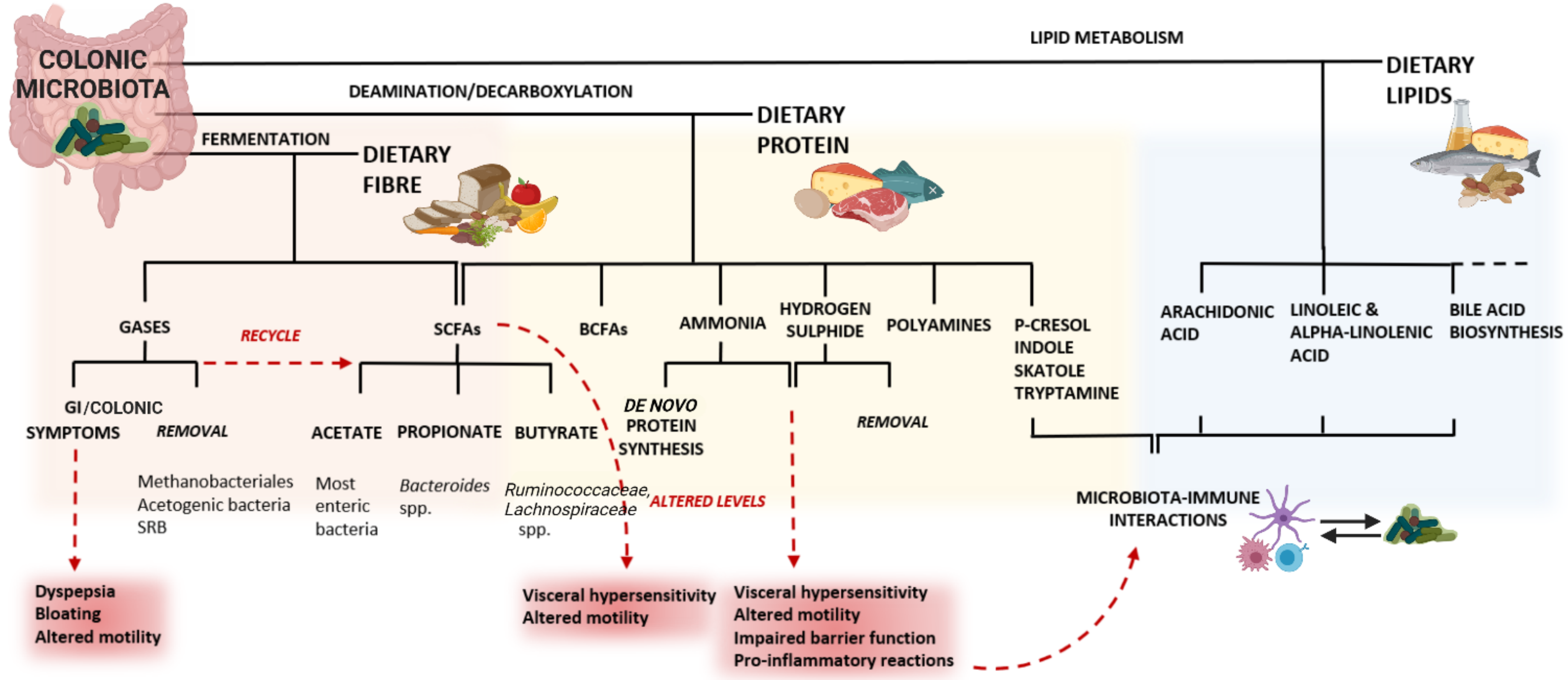


Figure 1. 6: The consequences of diet on a dysbiotic microbiota may lead to altered levels of these metabolites, resulting in GI symptoms.

In the colon, the fermentation of dietary fibre results in changes in the microbiota composition, supporting the growth of beneficial bacteria. Consequently, the microbiota generates gases, SCFAs and other metabolites. The microbial metabolism of lipids entering the colon is involved in several important pathways for the host. The families *Erysipelotrichaceae* and *Coriobacteriaceae* also play an important role in converting cholesterol-derived metabolites, such as bile salts and steroids [275]. Altered bile acid metabolism has been associated with chronic inflammation in the colon [312] and microbiota-derived bile acid metabolites can potentially affect both host metabolism and immune responses [313]. The microbiota-mediated protein metabolism is largely affected by the proteolytic activity of amino acid-fermenting bacteria, mainly species from *Clostridia*, *Peptostreptococcus*, *Bacteroides*, *Propionibacterium*, *Fusobacterium*, *Streptococcus*, *Lactobacillus*, *Veillonella* genera, and species *Selenomonas ruminantium* and *Megasphaera elsdenii* [314]. The microbial catabolism of amino acids occurs mostly through deamination and decarboxylation [315] and

can generate immuno-modulatory molecules and neurotransmitters (like catecholamines) that affect the immune and nervous systems. For example, the microbial glutamate decarboxylases convert glutamate into γ-aminobutyric acid, which has immunomodulatory effects in the GI tract [316]. Histamine, derived from the bacterial decarboxylation of L-histidine, can inhibit the release of pro-inflammatory cytokines via the histamine type 2 receptor on epithelial cells [317]. Hydrogen sulphide is thought to be responsible for an increased visceral hypersensitivity related to colonic distension, altered colonic motility [318] and other deleterious effects on the colonic epithelium [157]. SRB: sulphate-reducing bacteria; BCFAs: branched-chain fatty acids. Created with *BioRender.com.*

1.6 Effects of kiwifruit on gastrointestinal parameters and microbiota

The concept of "functional food" is becoming increasingly popular among healthy consumers and subjects with constipation predominant FGIDs (IBS-C or FC), seeking digestive benefits [319]. Many nutritional compounds have been investigated for their ability to promote GI health [320-324], but regular consumption of kiwifruit has been shown to be particularly effective at improving digestive comfort through digestive and immune functions [325] and modulating the colonic microbiota composition (Figure 1.7).

Currently, the most cultivated kiwifruit varieties are *Actinidia deliciosa* (green kiwifruit "Hayward") and gold kiwifruit *Actinidia chinensis* (ZESPRI® SunGold) [326]. The nutritional composition of green and gold kiwifruit is similar (Table 1.2), except for a lower enzymatic activity of actinidin (26 % vs. 100 % activity) [327], half the content of fibre (3.0 g/100 vs. 1.4 g/100) and lower pectic polysaccharides in the gold variety. However, twice the amount of vitamin C (161.3 mg/100 g vs. 92.7 mg/100 g) and higher levels of hemicellulosic polysaccharides are present in the gold kiwifruit compared to green kiwifruit [328, 329].

The characteristics that make kiwifruit a good candidate for providing digestive benefits include its content of potassium, vitamin K, E, folate, the presence of the actinidin enzyme, high level of vitamin C, bioactive elements, such as antioxidants, carotenoids and polyphenols [330], as well as a high amount of fibre [331]. The fibre in kiwifruit includes oligosaccharides of various chain lengths, pectic polysaccharides, cellulose and hemicellulose [332].

The physicochemical properties of green and gold kiwifruit suggest it would be a beneficial food for sustaining digestive health and immune function in subjects with constipation predominant FGIDs and healthy subjects [333]. The effects of green and gold kiwifruit on digestive functions are likely to be modulated by its water retention capacity associated with the fibre content [334], resulting in changes in faecal consistency and softness, reduced transit time and abdominal discomfort [335]. Many interventions, either with green or gold kiwifruit, have modified GI physiological and microbial parameters in different population groups, as well as improved FC and IBS-C (Table 1.3)

Table 1. 2: Comparison between the nutritional values of the green and the gold kiwifruit [353].

Nutrients for 100 g	Green kiwifruit *Actinidia deliciosa* ("Hayward")	Gold kiwifruit *Actinidia chinensis* (ZESPRI® SunGold)
Total carbohydrate	14.66 g	15.79 g
Fibre	3.0 g	1.4 g
Sugars	8.99 g	12.30 g
Fructose	4.35 g	5.80 g
Galactose	0.17 g	0.00 g
Glucose (dextrose)	4.11 g	5.28 g
Lactose	0.00 g	0.00 g
Maltose	0.19 g	0.00 g
Starch	0.00 g	0.12 g
Sucrose	0.15 g	1.22 g
Total protein	1.14 g	1.02 g
Total fat	0.52 g	0.28 g
Saturated fatty acids	0.029 g	0.065 g
Monounsaturated fatty acids	0.047 g	0.023 g
Polyunsaturated fatty acids	0.287 g	0.111 g
Vitamin A	87.00 IU	23.00 IU
Vitamin B1	0.027 mg	0.000 mg
Vitamin B2	0.025 mg	0.074 mg
Vitamin B3	0.341 mg	0.231 mg
Vitamin B5	0.183 mg	0.120 mg
Vitamin B6	0.063 mg	0.079 mg
Vitamin B9	25.00 mcg	31.00 mcg
Vitamin B12	0.00 mcg	0.08 mcg
Vitamin C	92.7 mg	161.3 mg
Vitamin D	0.00 mcg	0.00 mcg
Vitamin E	1.46 mg	1.40 mg
Vitamin K	40.3 mcg	6.1 mcg
Calcium	34.00 mg	17.00 mg
Copper	0.130 mg	0.151 mg
Iron	0.31 mg	0.21 mg
Magnesium	17.00 mg	12.00 mg
Manganese	0.098 mg	0.048 mg
Phosphorus	34.00 mg	25.00 mg
Potassium	312.00 mg	315.00 mg
Water	83.07 g	82.44 g

Table 1. 3: Summary of findings from human studies with green or gold kiwifruit for digestive health.

Study	**Population group**	**Intervention**	**Effect on GI or microbial parameters**
Weir *et al.* [336]	FC vs. baseline	Randomised, double-blind, placebo-controlled clinical trial; N= 58; green kiwifruit extract for 3 weeks	Increased bowel movements; decreased constipation, bloating, abdominal gas and flatulence.
Chan *et al.* [337]	FC vs. healthy controls	Prospective observational study; N= 53; 2 green kiwifruit per day for 4 weeks	Decreased transit time and laxative use in FC, no differences in healthy controls.
Chang *et al.* [338]	IBS-C vs. healthy controls	Randomised study with capsule control; N= 41; 2 green kiwifruit per day for 4 weeks	Increased bowel frequency; decreased transit time in IBS-C.
Cunillera *et al.*[339]	FC vs. baseline	Open, non-controlled and non-randomised longitudinal study; N= 46; 3 green kiwifruit per day for 3 weeks	Increased bowel movements, faecal softness and bulk.
Rush *et al.* [340]	Healthy elderly prone to constipation vs. baseline	Randomised crossover study, no control; N= 38; 1 green kiwifruit per 30 kg of body weight for 3 weeks	Increased laxation, faecal softness and bulk.
Udani and Bloom [341]	FC vs. baseline	Randomised, double-blind, placebo-controlled, parallel-group study; N= 138; Green kiwifruit powder (5.5 g/day) for 4 weeks	Increased bowel frequency; Reduced abdominal pain and flatulence.
Ansell *et al.* [342]	FC vs. healthy controls	Randomised, double-blind, placebo-controlled, crossover study; N= 28; Gold kiwifruit "Zesy002" powder (2400 mg/day) in capsules for 4 weeks; Green kiwifruit powder (high dose: 2400 mg/day or low dose: 600 mg/day) in capsules for 4 weeks	Gold and green kiwifruit increased bowel movements in healthy controls. No differences in FC.
Eady *et al*. [327]	Mild FC vs. baseline	Randomised, single-blind, crossover study N= 32; 3 gold kiwifruit "Zesy002" per day for 4 weeks	Increased spontaneous bowel movements and faecal softness; decreased abdominal pain, constipation, and indigestion.
Eady *et al*. [343]	IBS-C vs. healthy controls	Randomised cross-over study N= 38; 3 gold kiwifruit "Zesy002" per day for 4 weeks	Increased bowel frequency; decreased GI symptoms in IBS-C and healthy controls
Blatchford *et al.* [344]	FC vs. healthy controls	Randomised cross-over; N= 28; Gold kiwifruit "Zesy002" powder (2400 mg/day) in capsules for 4 weeks; Green kiwifruit powder (high dose: 2400 mg/day or low dose: 600 mg/day) in capsules for 4 weeks	Gold kiwifruit increased Clostridiales in healthy controls and increased *Faecalibacterium prausnitzii* in FC; Green kiwifruit increased *Dorea* spp. in FC.

Wilson *et al.* [345]	Prediabetes vs. baseline	N= 24; 2 gold kiwifruit per day for over 12 weeks	Increased uncharacterised members of the *Coriobacteriaceae* family.

1.6.1 The gastrointestinal microbiota

Previous data from human and *in vitro* studies suggest that green and gold kiwifruit can affect the growth of specific bacterial groups, microbial diversity and metabolite profiles, mostly of the lower GI microbiota. For example, in human studies (Table 1.3), consuming a green kiwifruit-derived supplement over 4 weeks increased the abundance of *Dorea* spp. from 0 to 4% in subjects with FC [344]. In contrast, a gold kiwifruit-derived supplement increased the abundance of Clostridiales in controls and the abundance of *Faecalibacterium prausnitzii* in FC subjects [344]. Increased abundance in *F. prausnitzii* could have been attributable to the presence of pectin and uronic acids in the gold kiwifruit, which are fundamental substrates for the growth of this bacterium [346]. This result is particularly relevant since the increase in butyrogenic bacteria (*F. prausnitzii, Ruminococcaceae*) could positively affect FC subjects.

Another study demonstrated that gold kiwifruit supplementation in subjects with prediabetes increased the relative abundance of taxa from the *Coriobacteriaceae* family [345]. Members of the *Coriobacteriaceae* family are involved in the hydrolysis of polyphenols, which are abundant in green and gold kiwifruit [347]. The polyphenol and fibre content of kiwifruit seems to affect colonic microbial metabolism and correlate with lactate production, as demonstrated in an *in vitro* study with different cultivars of kiwifruit [348]. This study showed that when the human faecal microbiota was used to ferment green kiwifruit, the amount of fibre and polyphenols correlated with lactate concentration.

More *in vitro* studies demonstrated that the green and gold kiwifruit increase the relative abundance of members of the *Bifidobacterium* genus and *Ruminococcaceae* family and decrease the relative abundance of members of the *Bacteroides* genus [348, 349]. In addition, green and gold kiwifruit promoted the colonisation and adhesion of *Bacteroides-Prevotella-Porphyromonas* groups and *Bifidobacterium* spp. to Caco-2 cells in an *in vitro* model of gastric-ileal digestion followed by faecal fermentation [350]. Moreover, increased microbial glycosidase activity, especially related to the breakdown of kiwifruit oligosaccharides, and increased SCFAs generation were observed [350]. A previous *in vitro* study confirmed that digested gold kiwifruit increased the relative abundance of *Bacteroides* spp., *Parabacteroides* spp. and *Bifidobacterium* spp. after 48 hours of fermentation [351].

In vivo studies showed that green kiwifruit skin or flesh increased the relative abundance of *Lachnospiraceae* and *Lactobacillus* spp. in rats [352] and that pigs fed freeze-dried green kiwifruit or green kiwifruit fibre diets had increased *Bacteroides* genus, decreased *Enterobacteriaceae* family and *E. coli*, as well as a greater ratio of *Lactobacillus* to *Enterobacteriaceae* in comparison to pigs fed a control diet [353]. However, metagenomic analyses of the taxonomic and functional capability of the microbiota following a green or gold kiwifruit intervention are needed, as the above-mentioned studies relied on 16S ribosomal RNA analysis.

1.6.2 Motility and epithelial barrier function

Previous research on pigs [354, 355] and humans (Table 1.3) demonstrated that consuming green kiwifruit increases water retention and faecal bulking, likely due to the pectic fraction of the kiwifruit, which is entirely fermented by the colonic microbiota [356, 357], increasing the production of SCFAs. The increased concentration of SCFAs may enhance colonic motility and increase faecal bulking [358]. Effects of green or gold kiwifruit consumption also support colonic lubrication and peristalsis [359].

A reduced colonic transit time is mostly associated with the actions of actinidin [360], as this enzyme favours laxation through the stimulation of colonic receptors [361]. The polyphenols in green kiwifruit could also increase bile acid secretion, which decreases water reabsorption and softens faecal bulk, stimulating colonic motility and increasing urgency [362].

Data from *in vitro* experiments [363] suggest that the fibre in the green kiwifruit is likely to improve colonic epithelial barrier integrity by increasing microbial production of butyrate. In rats, the consumption of gold kiwifruit increased colonic goblet cell numbers per crypt after 6 weeks of treatment [364], and the consumption of green and gold kiwifruit improved barrier function by stimulating the expression of the mucin genes *muc2* and *muc3* [364, 365]. Furthermore, the increased expression of these genes was demonstrated to inhibit the adherence of pathogens to the colonic epithelium in an *in vitro* study [366].

Several components and properties of green or gold kiwifruit, such as the high fibre content, actinidin action and water-holding capacity, have been suggested to be

responsible for affecting colonic mucin production and increasing the mucous layer [367]. Considering the previous data about mucin gene expression, goblet cell numbers, and digesta mucin content in animal studies [368], it is plausible that the consumption of green kiwifruit or gold kiwifruit could affect colonic mucin production and enhance colonic barrier function in humans.

On the other hand, the proteolytic activity of actinidin in green kiwifruit has been associated with the disruption of tight junctions and increased colonic permeability, thus contributing to allergic responses to kiwifruit [369, 370]. The protease-dependent disruption of occludins, increased permeability, and reduced transepithelial resistance were demonstrated *in vivo* and *in vitro* [369, 371]. Gold kiwifruit was considered less allergenic than green kiwifruit because it contains lower levels of actinidin [372].

Overall, several human studies demonstrated that the daily consumption of green or gold kiwifruit is a safe and effective natural laxative for FC or IBS-C subjects (Table 1.3). However, direct comparisons between studies and the application of results to the larger healthy population are not always possible, because of the differences in the size of the cohorts, duration of the intervention, and screening questionnaires used. In addition, dietary, GI habit questionnaires, socioeconomic status and "quality of life" questionnaires are not always considered but are important for holistically evaluating the efficacy of kiwifruit consumption on the digestive function of subjects with FGIDs.

1.6.3 Immunity

The effects of green or gold kiwifruit on the immune system have been investigated in several *in vivo*, *in vitro* and *ex vivo* studies (Table 1.4). Decreased levels of pro-inflammatory cytokines [373-375], nitric oxide [374] and reactive oxygen species, and a decreased inflammatory response [375] were observed after treatments with green or gold kiwifruit. Gamma-aminobutyric acid, which can be generated by the decarboxylation of glutamic acid by the host or the colonic microbiota [376], was shown to increase in concentration after the microbial fermentation of both kiwifruit cultivars (green kiwifruit > gold kiwifruit) [349]. Fermented green or gold kiwifruit were incubated with Caco-2

cells and, after 3 hours, decreased gene expression of pro-inflammatory TNF was observed as an effect of both kiwifruit cultivars [349].

Different results were observed in mice studies, where pro-inflammatory cytokine [343, 377, 378], nitric oxide [377], and immune cell functions, including phagocytic [379] and natural killer cell activity, were promoted after treatments with green or gold kiwifruit. The ovalbumin-specific antibody isotypes, typical of both a T-helper 1 (IgG2b and 2c) and a T-helper 2 (IgG1) immune response, were produced after that mice were fed gold kiwifruit puree [380]. Increased neutrophil chemotaxis was also observed in young man after a four-week intervention with two gold kiwifruit/day [381]. These results suggested that the green and gold kiwifruit could enhance both innate and adaptive immune responses.

The different modulation of the immune response by green or gold kiwifruit was attributed to several compounds. Among these, the enzyme actinidin is thought to modulate inflammation and abdominal pain through proteolytic activation of the protease activated receptor 2 and 4 [382]. These receptors were shown to be involved in anti-inflammatory responses in a mouse model of colitis [383] and in the reduction of visceral hypersensitivity in mice [384].

Actinidin cleaves the protein kiwellin into KiTH and kissper, a thaumatin-like protein identified in green kiwifruit [385, 386]. The biological functions of kiwellin and KiTH are poorly understood. However, kissper has been suggested to have anti-inflammatory properties. Among these, there is the induction of anti-inflammatory transforming growing factor (TGF)-β secretion, and the reduction of NF-kB activation and TNF expression. These effects were demonstrated in Caco-2 cells and *ex vivo* LPS-stimulated colonic mucosa from Crohn's disease subjects [375]. Moreover, the pre-incubation and culture of intestinal explants from celiac disease subjects with kissper decreased the levels of transglutaminase 2, human leukocyte antigen- DR isotype and cyclooxygenase 2 [387]. Kissper also has antifungal activity [388].

Vitamin C and E, folate and potassium, which are beneficial for maintaining an anti-inflammatory environment and the function of epithelial and endothelial cells [389, 390], are present in high levels in green and gold kiwifruit. Vitamin C accumulates in phagocytic cells and can enhance chemotaxis, phagocytosis, generation of reactive oxygen species and bactericidal activity [381, 391-393]. Similarly, vitamin E enhances

cell-mediated and humoral immunity, lymphocyte proliferation, Ig levels, antibody responses and natural killer cell activity [394] and indirectly modulates T cell function by affecting inflammatory mediators generated from other immune cells [395]. Colonocytes likely need high intracellular levels of vitamin C and E to reduce inflammation, as it was previously shown that other specialised epithelial cells, such as keratinocytes, need vitamin C and E to limit inflammation caused by UV damage [396].

Finally, vitamin C has strong antioxidant properties, scavenging free radicals and nitrogen species and regenerating other small antioxidant molecules from their respective radicals [397]. The antioxidant properties of vitamins C and E could help maintain the functionality and structural integrity of immune cells, therefore enhancing immunity [398].

Table 1. 4: Summary of findings from human, *in vitro* and *in vivo* studies investigating the effect of the green or gold kiwifruit on the immune status.

	Study	**Experimental group/ model**	**Intervention/ treatment**	**Effect on immune parameters**
Human studies	Eady *et al*. [343]	IBS-C vs. healthy controls	Randomised cross-over study N= 38; 3 gold kiwifruit "Zesy002" per day for 4 weeks	Increased TNF
	Bøhn *et al.* [399]	Healthy smokers vs. healthy controls	N= 102 3 kiwifruits (variety not defined)/day for 8 weeks	Upregulation of blood cell expression of genes involved in the regulation of cellular stress defence, such as DNA repair, apoptosis and hypoxia
	Bozonet *et al.* [381]	Healthy subjects vs. baseline	N= 14 2 gold kiwifruits/day for 4 weeks	Increased neutrophil chemotaxis and oxidant generation
	Brevik *et al.* [400]	Healthy subjects eating 1 kiwifruit vs. healthy subjects eating 2 kiwifruits	N= 24 1 or 2 gold kiwifruits/day for 4 weeks	Decreased purine oxidation in lymphocyte DNA and whole blood platelet aggregation after 1 kiwifruit/day; decreased pyrimidine oxidation after 2 kiwifruits/day
In vitro studies	D'Eliseo *et al.* [373]	LPS-stimulated THP-1 monocytes	Green kiwifruit peel extract	Decreased production of IL-6, IL-1β, TNF, HMGB1 and granzyme B
	Russo *et al.* [387]	Intestinal culture explant from celiac disease patients and healthy controls	Kissper preincubation and culture for 24 hours	Decreased transglutaminase 2, Human Leukocyte Antigen- DR isotype and cyclooxygenase 2
	Ciacci *et al.* [375]	Caco-2 cells; *Ex vivo* LPS-stimulated colonic mucosa from subjects with Chron's disease	Fresh fruit or raw kiwi fruit extracts	Decreased inflammatory response, reactive oxygen species levels, TNF and NF-kB activation in both Caco-2 cells and the colonic mucosa; increased TGF-β1
	Edmunds *et al.* [401]	*Ex vivo* explants of primary macrophages and intestinal	Aqueous and ethyl acetate extracts of gold kiwifruit or green kiwifruit	Green and gold kiwifruit extracts reduced nitric oxide and cytokine secretion by both models

		epithelial cells from C57BL/5J and *Il10*$^{-/-}$ mice; RAW 264.7		
	Paturi *et al.* [402]	HT-29 cells	In *vitro* gastro-ileal digestion and colonic fermentation of gold and green kiwifruit	Soluble bacteria-free fermented green and gold kiwifruit increased IL-7 secretion; Increased IL-7 secretion after incubation of HT-29 cells with fermented green kiwifruit, but not fermented gold kiwifruit, in the presence of IFN-γ
In vivo studies	Bentley-Hewitt *et al.* [377]	Rats	Diet with 20% fresh pureed green or gold kiwifruit	Increased expression of inflammation-related genes *NOS2* and *TNF* in the duodenum of aspirin-treated rats fed green kiwifruit; No effects in inflammation-related genes in rats fed gold kiwifruit
	Qu *et al.* [374]	Obese mice	Gold kiwifruit seed oil for 12 weeks	Decreased gene expression of TNF, IL-6, IL-1β, cyclooxygenase 2, and inducible nitric oxide
	Edmunds *et al.* [403]	*Il10*$^{-/-}$ mice	Aqueous and ethyl acetate extracts of gold kiwifruit or green kiwifruit	Results from an *in vitro* study [401] were not confirmed *in vivo*
	Hunter *et al.* [380]	Mice	Gold kiwifruit puree or gold kiwifruit juice for 20 days	Increased total Ig and IgG in mice fed gold kiwifruit puree
	Mahmoud *et al.* [404]	Mice	Green kiwifruit juice for 8 days	Increased nuclear factor erythroid 2–related factor 2; decreased NF-*κ*B
	Ma *et al.* [379]	Mice	Green kiwifruit extract for 30 days	Increased levels of IgA, IgG and IgM, phagocytic activity and lymphocytes transformation;
	Shu *et al.* [405]	Mice undergoing vaccination for Cholera or diphtheria/tetanus	Diet with an extract of combined green and gold kiwifruit for 11 days or 29 days	Increased IFN-γ production, natural killer cell activity and mucosal and serum antibody response to the vaccines

	Iwasawa *et al.* [378]	Mice	Green and gold kiwifruit juice for 8 days	Increased cytokine production and neutrophil accumulation; decreased urinary oxidative stress markers in mice fed gold and green kiwifruit juice

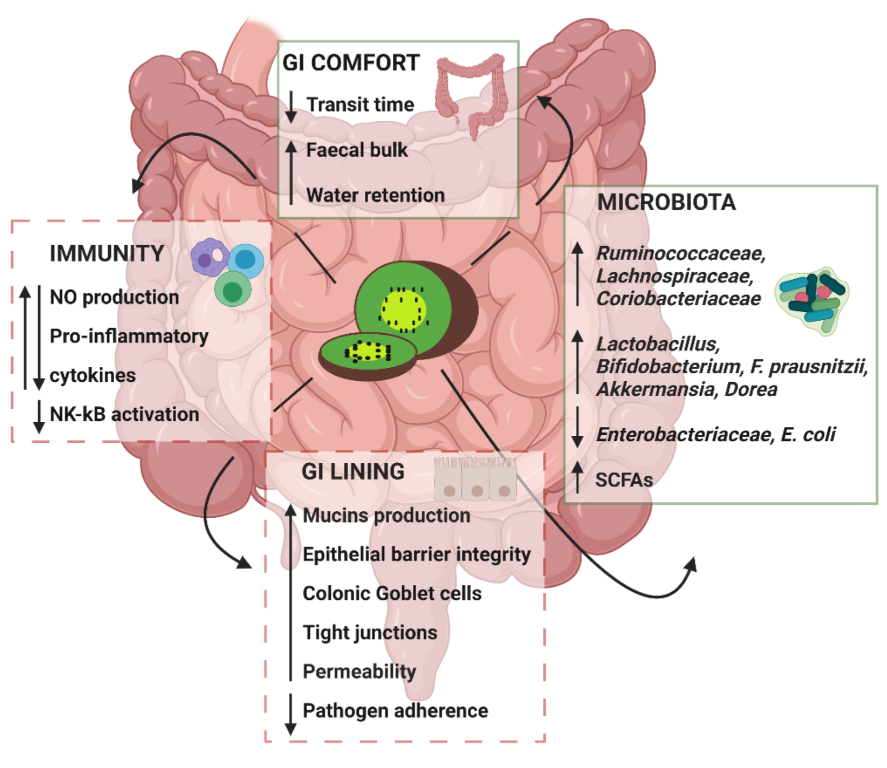


Figure 1. 7: Overview of the possible beneficial activities of green kiwifruit in the GI tract.
Several consequences of green kiwifruit consumption have been proven in previous studies (green: solid line), but other mechanisms are less studied (orange dashed line). Created with *BioRender.com.*

1.7 PhD dissertation overview

It is evident from the literature review that the microbiota-host interplay in the GI tract needs to be investigated in the context of a dynamic community interacting with different cell types, with local and systemic effects. The research reviewed in Chapter 1 sought to detail the current evidence of these complex interactions and their role in the pathophysiology of FGIDs. However, the mechanisms responsible for FGIDs are still poorly understood, and many questions remain about the exact role of the GI microbiota and how changes to it relate to these conditions. Defining the relationship between FGIDs, the colonic microbiota, and immunity is an important research gap, and priority should be given to a systems biology approach.

The sequencing technologies and the bioinformatics tools to analyse large datasets have been adapted for human microbiota and immune cell gene expression analyses, but the methodology has not been standardised yet. For the microbiota, differences related to the study design, sample collection, DNA extraction methods (G'NOME, QIAGEN, PROMEGA), analysis methods (16S ribosomal RNA sequencing and whole metagenome sequencing) and the large inter-individual differences in microbiota composition make it difficult to compare and generalise findings from different groups directly.

The '**C**hristchurch IBS c**o**hort to investigate **m**echanisms **fo**r gut **r**elief, and improved **t**ransit' (COMFORT) cohort with 315 participants with FGIDs provides an opportunity to study the key microbiota-immune-mediated pathways responsible for GI comfort and function. The distinctive trait of the COMFORT cohort is the large recruitment of volunteers (healthy controls, FGID participants), where biological samples (faeces, blood, biopsies) for targeted and untargeted biochemical analyses have been collected in combination with a range of GI and non-GI symptoms and dietary data. A systems biology approach integrating multiple datasets rather than analysing *omics* data and clinical questionnaires alone will generate a better mechanistic insight into the pathophysiology of FGIDs.

The difficulty in discriminating between FGID subtypes represents an additional gap. It is not always possible to confidently discriminate IBS-C from FC, or IBS-D from FD, as the symptoms often overlap between different FGID subtypes, and the Rome IV criteria are exclusively based on symptom reports. However, the most important evidence is that in all FGID subtypes, changes in microbiota composition have been reported. This finding

suggests that these differences may be used to discriminate between FGID or IBS subtypes.

Low-grade inflammation and immune activation have been hypothesised to play a role in the pathophysiology of FGIDs, affecting visceral sensitivity and mucosal barrier function. However, *in vitro* experiments measuring immune alterations in the blood or the colonic mucosa of IBS subjects have yielded conflicting results [406].

Gene expression analyses using biopsy samples from IBS subjects have been performed by others [407], but studies investigating peripheral blood mononuclear cells (PBMCs) gene expression patterns in FGIDs are lacking [408]. Peripheral blood samples are more accessible and less invasive sources of cells than biopsies, and gene expression profiling of PBMCs is a validated tool to provide information and explore the pathogenetic background of a condition at the systemic level.

Previous dietary interventions with different foods or supplements attempted to modify the GI microbiota composition and function and improve the immune status. In this regard, regular consumption of green or gold kiwifruit has been demonstrated to affect faecal microbiota composition, digestive function and immune function. However, these studies relied on 16S ribosomal RNA analysis, which does not provide information on the functional capability of the microbiota. Therefore, metagenomic analyses of the taxonomic and functional capability of the faecal microbiota (as a proxy of the colonic microbiota) following a kiwifruit intervention are needed.

Although several studies and data support the role of the microbiota-immune interplay in the pathophysiology of FGIDs, it is important to consider the methodological challenges to effectively investigate the GI microbiota and gaps in the field that need to be filled before causation is established. Technological advances provide a robust framework to identify and fill some of these gaps more effectively.

This PhD dissertation addresses some of the research questions deriving from the gaps found in the literature as follows:

1. Does the combination of taxonomic and gene abundance analyses of the faecal microbiota allow more accurate discrimination within FGID subtypes and between FGID subtypes and healthy subjects (controls) than taxonomic data alone?

2. What are the differences in PBMC gene expression within FGID subtypes and between FGID subtypes and controls?
3. Can an integrative correlative analysis of microbial taxonomic with PBMC gene expression and GI/non-GI symptom data provide insight into the microbial-host interactions underlying FGIDs?
4. Do the faecal microbiota composition and functional potential change after daily consumption of two gold kiwifruit *Actinidia chinensis* (ZESPRI® SunGold) for four weeks in constipation predominant FGID subjects and controls?
5. Do the microbiota compositional profiles of constipation predominant FGID subjects or controls correlate to GI and non-GI symptoms after the intervention with either gold kiwifruit or psyllium?

1.7.1 Main hypotheses and aims

The main hypothesis of the research presented in this PhD thesis dissertation is that the combined analysis of the faecal microbiota composition, gene abundance, and PBMC transcriptome profile, along with the integration of these biological datasets with GI and non-GI symptoms, provide a more accurate discrimination of FGIDs within subtypes or FGIDs from controls than the analysis of each dataset alone.

An additional hypothesis was that changes in the faecal microbiota composition and gene abundance profiles occurring in constipation predominant FGIDs (IBS-C or FC) and controls differ after consuming two gold kiwifruit or a fibre-match intervention (psyllium) daily for four weeks, and these changes are associated with GI and non-GI symptoms.

A systems biology approach was used in this PhD dissertation to determine the changes in the faecal microbial and systemic immune signatures in FGID subjects. The first aim was to characterise the faecal microbial signature (as a proxy of the microbiota present in the colonic lumen) and the systemic immune signature (as a proxy of the systemic immune interactions and signalling network) of subjects with FGIDs (IBS-C, IBS-D, FC, FD) and controls, to gain mechanistic insights into the microbiota-immune-mediated pathways underlying FGIDs.

The second aim was to integrate the faecal microbiota taxonomic composition and PBMC gene expression datasets with GI and non-GI symptoms from subjects with FGIDs and controls to identify potential relationships between variables and better understand the host-microbe interactions underlying the phenotype of constipation or diarrhoea predominant FGIDs.

The third aim was to evaluate a classification model of FGIDs by analysing the receiver operating characteristic (ROC) curve and area under the curve (AUC) of selected microbial taxa, PBMC genes and GI and non-GI symptoms to distinguish between FGID subtypes and controls or within FGID subtypes.

The final aim was to determine the effect of the daily consumption for four weeks of two gold kiwifruit or psyllium (fibre-matched) on microbial composition and gene abundance profiles, and their relationships to GI and non-GI symptoms, in subjects with constipation predominant FGIDs (IBS-C or FC) and controls.

Table 1. 5: Overview of the thesis structure.

	Chapter 1	**Literature review**
Research chapters	Chapter 2	**The faecal microbiota in FGIDs** • Characterisation of the faecal microbiota taxonomic composition and predicted gene function in FGID subjects and controls. • Determination of the faecal microbiota signature of the FC and FD subtypes. • Determination of the faecal microbiota signature of the IBS-C and IBS-D subtypes.
	Chapter 3	**Peripheral blood immune profile in FGIDs** • Characterisation of the gene expression profile of PBMCs from FGID subjects and controls. • Functional enrichment analysis to identify related gene groups and explore the functional associations between genes likely to influence a mutual biological purpose. • Determination of the immune gene expression signature of the FC and FD subtypes. • Determination of the immune gene expression signature of the IBS-C and IBS-D subtypes.
	Chapter 4	**Microbial-host interactions in FGIDs** • Integrative analysis of microbial taxonomic composition with PBMC gene expression data and GI and non-GI symptoms. • Evaluation of a classification model of FGIDs by analysing the receiver operating characteristic (ROC) curve and area under the curve (AUC) of selected microbial composition, PBMC genes and GI and non-GI symptoms.
	Chapter 5	**The gold kiwifruit and psyllium interventions in FC and IBS-C subjects** • Characterisation of the faecal microbiota taxonomic composition and predicted gene function in constipation predominant subjects and controls fed the gold kiwifruit/psyllium intervention for four weeks.
	Chapter 6	**General discussion**

Chapter 2

Characterisation of the taxonomic composition and gene abundances of the faecal microbiota in subjects with functional gastrointestinal disorders

A journal article titled "Identification of a faecal microbiota signature in functional gastrointestinal disorders" (Caterina Carco, Wayne Young, Phoebe E. Heenan, Richard B. Gearry , Elizabeth Forbes-Blom, Jacqueline I. Keenan, Nicholas J. Talley, Warren C. McNabb, Nicole C. Roy) will be submitted to the *American Journal of Gastroenterology* in 2023.

2.1 Abstract

It is now well accepted that alterations in the colonic microbiota play a role in functional gastrointestinal disorders (FGIDs), including irritable bowel syndrome (IBS) (IBS-D: predominantly diarrhoea; IBS-C: predominantly constipation) and their functional counterparts (FC: functional constipation; FD: functional diarrhoea). However, the exact mechanisms are still unclear. Chapter 2 aims to characterise the taxonomic composition and gene abundance of the faecal microbiota (as a proxy of colonic microbiota) of FGID subjects and healthy subjects (controls) to gain mechanistic insights into the microbial interactions underlying these disorders. Microbial DNA was extracted from 274 faecal samples (72 IBS-C or FC, 73 IBS-D or FD and 129 controls) and analysed by shotgun sequencing. Odds ratios were used to rank the relative enrichment or underrepresentation of microbial taxa. Gene abundances were analysed using the pairwise negative binomial generalised log-linear models with a quasi-likelihood test.

Changes in the relative abundance of facultative anaerobes (proteobacteria) and obligate fermenters (hydrogen-consuming taxa and butyrate producers), and an increased abundance of genes related to secretion systems and micronutrient competition were detected in both diarrhoea (FD+IBS-D) and constipation (FC+IBS-C). The constipation phenotype (FC+IBS-C), compared to controls, was characterised by an increased relative abundance of facultative anaerobes over obligate fermenters, along with increased relative abundance of genes related to virulence and micronutrient competition. The diarrhoea phenotype (FD+IBS-D), compared to controls, was characterised by a lower relative abundance of lactic acid bacteria and differential relative abundance of genes involved in nitrogen fixation, and lactose fermentation. Overall, distinct microbiota profiles associated with constipation or diarrhoea phenotypes were identified. When each FGID subtype was compared to controls, a distinctive microbial signature discriminated FC from controls and IBS-C, suggesting FC as a distinct condition rather than part of the IBS-C spectrum. Despite FD and IBS-D had similar taxonomic signatures in comparison to controls, the microbial functional changes discriminating the diarrhoea group (IBS-D + FD) from controls were related to IBS-D rather than FD.

2.2 Introduction

The crucial role of the gastrointestinal (GI) microbiota in health and disease is increasingly recognised [409]. The resident microbes in the GI tract have several implications for the absorption and metabolism of nutrients, maintenance of structural integrity of the mucosa, immunomodulation, and protection against pathogens [3, 4]. These processes underlie GI functions in health and disease.

It is well accepted that the composition and function of the colonic microbiota potentially contribute to the pathophysiology of functional gastrointestinal disorders (FGIDs), including irritable bowel syndrome (IBS) (predominantly diarrhoea, constipation, or a mixed subtype) and its functional counterparts (functional constipation and functional diarrhoea) [11-13]. These disorders feature chronic abdominal discomfort with changes in bowel habits [14].

The clinical phenotype of FGIDs is associated with early childhood events, somatisation, improper diets, and psychological, hereditary and environmental factors [7]. In addition, these parameters can affect the composition and function of the colonic microbiota along with many GI functions such as motility, sensitivity and mucosal permeability, contributing to the pathophysiology of FGIDs [7].

The main differences between a healthy and an IBS-related faecal microbiota are changes in Firmicutes:Bacteroides ratio [66-71], differences in the prevalence of Actinobacteria and Proteobacteria phyla [72], increased relative abundances of the *Enterobacteriaceae* family*, Bacteroides* genus, *Ruminococcus* genus [66, 73-76], and reduced *Prevotella* [66] and *Faecalibacterium* genera [66, 77]. Other alterations reported in IBS include an increased relative abundance of potential pathobionts, such as the *Veillonella* genus [69, 73, 78], and differences in the abundance of hydrogen using microbes [80]. These differences include a decreased relative abundance of the *Desulphovibrionaceae* family [79] and an increased relative abundance of the *Methanobrevibacter* genus [67, 81, 82].

Few studies have reported data on the dysbiosis of the faecal microbiota between IBS subtypes and between IBS and functional counterparts. Some studies reported no differences in the composition of the microbial community between IBS-C and IBS-D [91]. Other studies associated different IBS subtypes with a unique microbial signature [115].

A higher relative abundance of taxa from the Firmicutes and Proteobacteria phyla [410], the Clostridia class [411], and potential pathogens (*Pseudomonas*, *Acinetobacter, Arcobacter* genera) [410], along with a lower relative abundance of the genera *Bacteroides* and *Bifidobacterium* and the species *Eubacterium rectale* and *Faecalibacterium prausnitzii* [412] were reported in FD in comparison to controls. However, the GI and psychosomatic symptoms in IBS-D and FD often overlap [413].

Similarly, IBS-C and FC are often referred to as a single syndrome varying along one clinical dimension, as they differ only by the presence of abdominal pain [414]. Studies investigating the dysbiosis in FC reported an increased relative abundance of taxa from the *Firmicutes* phylum [415], the Gamma-Proteobacteria class [416], and the *Enterobacteriaceae* family [416, 417], including potential pathogens (*Escherichia coli* [417], *Staphylococcus aureus* [417], *Veillonella* genus [416]) compared to controls. The relative abundance of taxa from the *Bacteroidetes* phylum [415] and the genera *Prevotella, Clostridium*, *Bifidobacterium*, *Lactobacillus* and *Bacteroides* have been reported as higher [415, 416, 418] or lower [417, 419]. However, no studies directly compared the differences in the faecal microbiota composition and function between IBS-C and FC, and no biological biomarkers discriminate these conditions.

Only a few studies have investigated the integrated taxonomic and gene abundance in FGIDs [420-422]. Differences in the relative abundance of the Proteobacteria and Actinobacteria phyla, and the families *Acidaminococcaceae, Sutterellaceae,* and *Desulphovibrionaceae*, along with an increased abundance of genes related to virulence factors, antibiotic resistance [420-422], and vitamins and cofactors [420-422] were reported in subjects with different IBS subtypes.

The inconsistencies among studies in the faecal microbiota profiles of IBS subtypes and their functional counterparts have contributed to the lack of consensus about the role of the microbiota and how changes to it relate to IBS symptoms. Moreover, identifying a signature is difficult, due to the complexity, instability, and inter-individual variability of the faecal microbiota. Diet, study size, IBS subtype predominance, different analytical techniques and DNA extraction methods may also contribute to this lack of consistency.

The direct sequencing of all microbial DNA from faecal samples (metagenomics) allows the investigation of the composition and functional potential of the microbiota [423], providing data on community structure and diversity, new taxa and genes, and predicting

the metabolic pathways encoded in the community. This information may help to identify potential pathways and markers of dysbiosis, and discriminate FGIDs within subtypes and from controls.

Chapter 2 aims to characterise the microbial taxonomic composition and gene abundance of the faecal microbiota (as a proxy of colonic, luminal microbiota) of subjects with FGIDs and healthy subjects recruited in the **C**hristchurch IBS c**o**hort to investigate **m**echanisms **fo**r gut **r**elief, and improved **t**ransit' (COMFORT) cohort to gain mechanistic insights into the complex microbial interactions underlying these disorders. My contribution was to perform DNA extraction from faecal samples, conduct bioinformatics and statistical analyses, interpret the datasets, and write the research chapter.

2.3 Hypothesis and aims

The main hypothesis of this analysis was that the faecal microbiota of subjects with constipation predominant (IBS-C+FC) or diarrhoea predominant FGIDs (IBS-D+FD) could be discriminated from controls, according to differences in composition and predictive function.

An additional hypothesis was that the individual FGID subgroups (IBS-C, IBS-D, FC, FD) could be discriminated from controls and from each other, according to differences in composition and predictive function of the faecal microbiota.

The aim was to determine the microbial composition and gene abundance of faecal samples obtained from subjects diagnosed and clustered in different FGID subgroups (IBS-C, IBS-D, FC, FD) and controls from the COMFORT cohort to generate new insights concerning potential microbiota-mediated pathways underlying FGIDs. Shotgun metagenomic sequencing of faecal samples (as a proxy of the microbiota present in the colonic lumen) was undertaken, and microbial taxonomic and functional gene abundance analysis was conducted using the R programme. The microbiota data was visualised for all FGID groups (FC, IBS-C, FD, IBS-D) and controls. Several comparisons were made:

1. Subjects were grouped according to the bowel movement pattern subtypes (constipation phenotype: IBS-C+FC; diarrhoea phenotype: IBS-D+FD) and each phenotype was compared to controls.
2. Subjects with IBS-D were compared to controls.
3. Subjects with FD were compared to controls.
4. Subjects with IBS-C were compared to controls.
5. Subjects with FC were compared to controls.

Subjects with IBS-M and IBS-U were excluded from the statistical analyses to focus on the common and exclusive microbial taxonomic and functional differences among subjects characterised by diarrhoea or constipation only.

2.4 Materials and Methods

2.4.1 Study population

The participants of the COMFORT cohort were continuously recruited since July 2016 by former PhD Fellow Phoebe Heenan at the Christchurch Public Hospital gastroenterology day ward and the Southern Cross Hospital Southern Endoscopy Centre in Christchurch, New Zealand. The description of the COMFORT cohort has been published in [424] and summarised here.

Participants were recruited by referral by their gastroenterologist (colonoscopy subgroup) and the general public who responded to advertisements. The subgroup recruited from the general public did not undergo colonoscopy but provided the questionnaire data and biological samples. All participants were required to understand, date, and sign an informed consent sheet if they agreed to participate. No procedures were started without the written informed consent of the participant. Ethical approval was obtained in August 2017 from the University of Otago Human Ethics Committee (Health) (Reference H16/094) [424].

A total of 349 participants were enrolled before December 2018. However, not all participants could provide all questionnaire data or biological samples as planned. As a result, 315 participants completed the study: 220 from either Christchurch Public Hospital or Southern Cross Hospital and 95 from the general public (Figure 2.1).

The eligibility of participants was assessed using the Rome IV criteria according to the results of the Modified Hunter New England Survey questionnaire [424], which allows the classification of participants as cases (IBS, FC, or FD) or controls (Figure 2.2). In total, the cohort involved 129 controls and 186 cases. The cases were classified as 57 diarrhoea predominant IBS (IBS-D), 30 constipation predominant IBS (IBS-C), 41 mixed-phenotype IBS (IBS-M), 42 FC, and 16 FD (Table 2.1).

The average age of the participants (18-70-year-old inclusive) was 53.4 years for the cases and 54.4 years for the controls. Cases (80.1%) and controls (55.8%) were predominantly female (Table 2.1). Participants with GI organic or systemic diseases, GI symptoms (e.g., blood in the faeces, nocturnal symptoms), unexplained weight loss, and anaemia were

excluded from the study. The presence of other FGIDs, like functional dyspepsia and functional distension, was not evaluated.

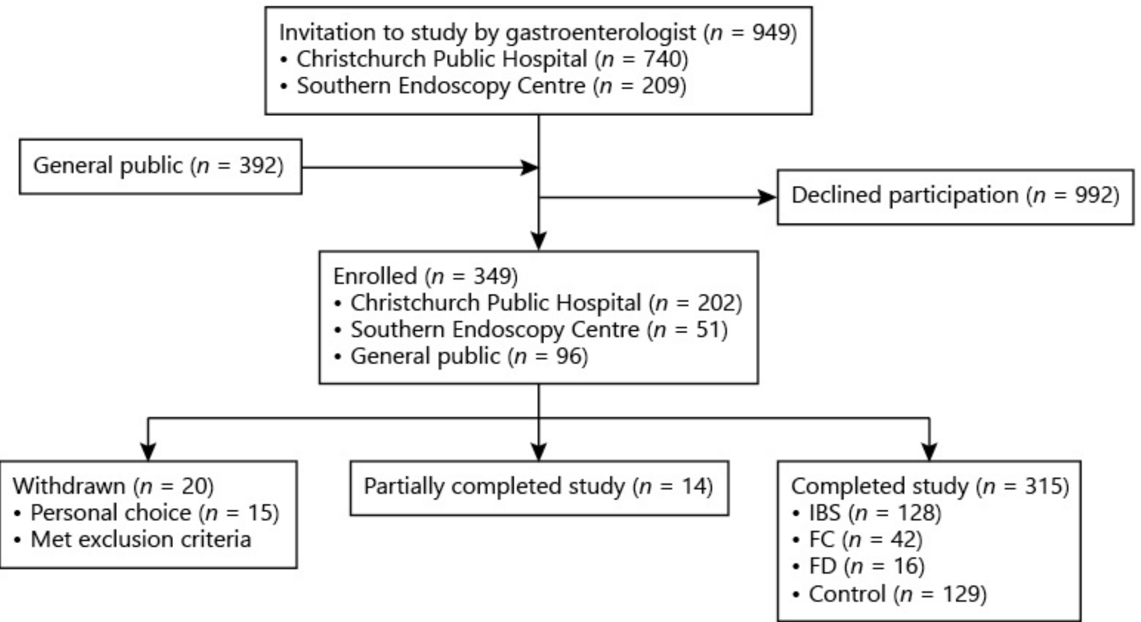


Figure 2. 1: Workflow of the recruitment of participants of the COMFORT cohort.
IBS, irritable bowel syndrome, FC, functional constipation; FD, functional diarrhoea (Figure from Heenan *et al* [424], reproduced with permission).

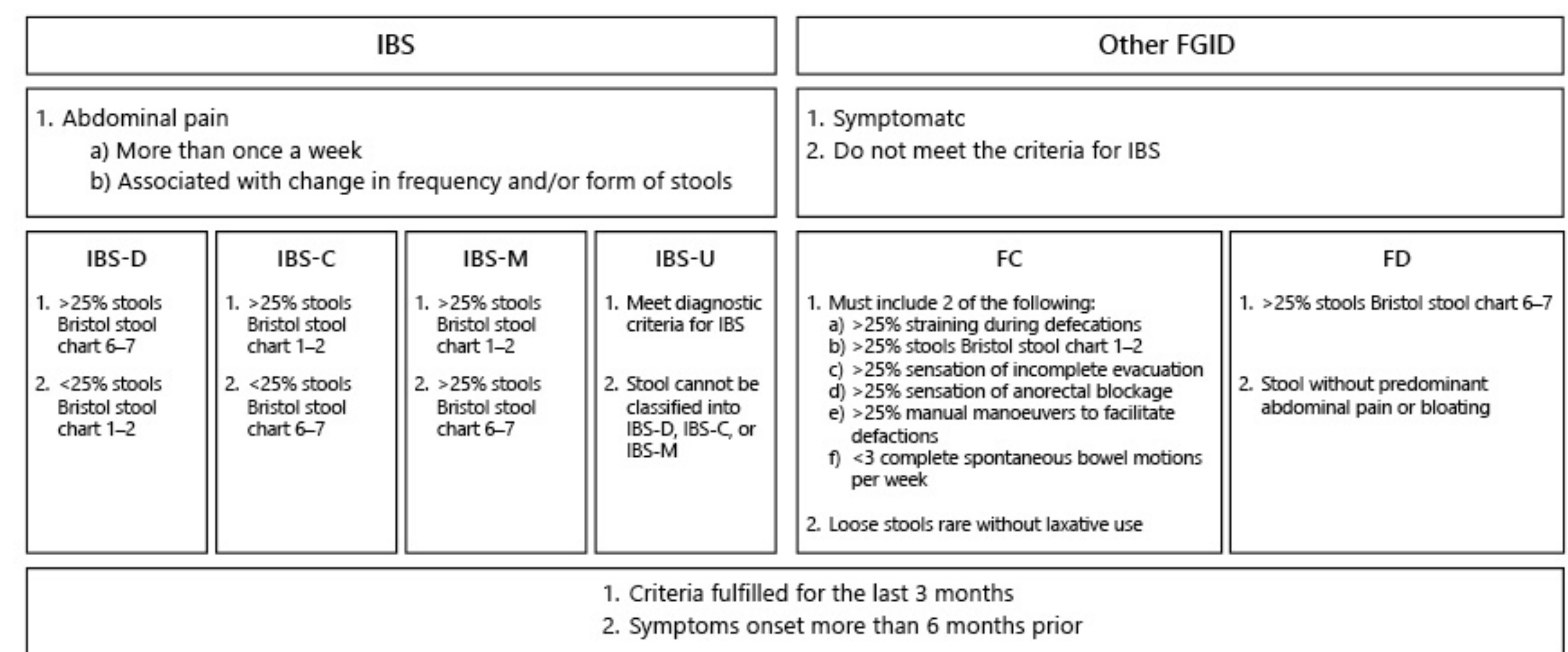


Figure 2. 2: The bowel patterns classified by the Rome IV criteria for the diagnosis of FGIDs [15].

The Bristol stool scale is a diagnostic medical tool designed to classify human faeces into seven categories. Categories 1 and 2 indicate constipation, Categories 3 and 4 indicate the ideal faeces, and Categories 5, 6 and 7 indicate diarrhoea [15]. FGIDs, functional gastrointestinal disorders; FC, functional constipation; FD, functional diarrhoea; IBS, irritable bowel syndrome; IBS-D, diarrhoea predominant irritable bowel syndrome; IBS-C, constipation predominant irritable bowel syndrome; IBS-M, irritable bowel syndrome-mixed phenotype predominant; IBS-U, irritable bowel syndrome-undefined phenotype (Figure from Heenan *et al.* [424], reproduced with permission).

Table 2. 1: Characteristics of the participants recruited in the COMFORT cohort, including sex, age, education level and personal habits. FGIDs, functional gastrointestinal disorders; FC, functional constipation; FD, functional diarrhoea; IBS, irritable bowel syndrome; IBS-D, diarrhoea predominant irritable bowel syndrome; IBS-C, constipation predominant irritable bowel syndrome; IBS-M, irritable bowel syndrome-mixed phenotype predominant; MET, Metabolic Equivalent of Task (Table from Heenan et al. [424], reproduced with permission).

	IBS				Other FGIDs			Controls
	IBS-D	IBS-C	IBS-M	total	FC	FD	total	
Participants	57	30	41	128	42	16	58	129
Male/female	13/44	2/28	7/34	22/106	12/30	3/13	15/43	57/72
Average age ± SD, years	52.8±12	51.1±14	49.7±13	51.5±13	57.7±10	57.8±10	57.7±10	54.4±16
Education level achieved								
Postgraduate	7 (18.4%)	4 (13.8%)	7 (20.0%)	18 (17.6%)	9 (22.0%)	5 (33.3%)	14 (25.0%)	37 (29.6%)
Tertiary	11 (28.9%)	15 (51.7%)	17 (48.6%)	43 (42.2%)	15 (36.6%)	7 (46.7%)	22 (39.3%)	59 (47.2%)
Secondary	11 (28.9%)	4 (13.8%)	4 (11.4%)	19 (18.6%)	11 (26.8%)	2 (13.3%)	13 (23.2%)	15 (12.0%)
Primary	9 (23.7%)	6 (20.7%)	7 (20.0%)	22 (21.6%)	6 (14.6%)	1 (6.7%)	7 (12.5%)	14 (11.2%)
Currently drink alcohol	44 (80.0%)	23 (74.2%)	33 (80.5%)	100 (78.7%)	34 (81.0%)	12 (85.7%)	46 (82.1%)	102 (79.1%)
Currently smoking	2 (7.7%)	2 (15.4%)	1 (6.3%)	5 (3.9%)	1 (7.1%)	2 (33.3%)	3 (5.4%)	5 (12.2%)
Past smoking	26 (46.4%)	13 (43.3%)	16 (39.0%)	55 (43.3%)	14 (3.3%)	6 (42.9%)	20 (35.7)	41 (31.8%)
Average hours of exercise per week (% participants)								
Walking	366.0 (77.2%)	652.4 (80.0%)	411.7 (74.4%)	452.6 (77.0%)	472.7 (81.0%)	501.4 (50.0%)	477.7 (73.2%)	431.2 (82.0%)
Moderate	225.0 (68.4%)	228.8 (76.7%)	228.8 (77.0%)	227.2 (73.0%)	230.0 (50.0%)	146.7 (64.3%)	205.8 (53.6%)	161.1 (80.0%)
Vigorous	146.8 (30.0%)	97.9 (30.0%)	71.4 (35.9%)	109.4 (31.7%)	94.7 (21.4%)	85.8 (21.4%)	95.3 (21.4%)	96.7 (46.5%)
Average MET	18,620.7	42,114.3	26,733.6	26,725.6	12,545.5	28,294.8	23,950.1	29,586.8

2.4.2 Biological samples

2.4.2.1 DNA extraction from faecal samples

Participants were asked to collect a faecal sample at home and store the faecal sample at - 20°Cand then deliver the samples to the study centre within 24 hours of collection in the provided faeces collection kit with ice packs. Former PhD Fellow Phoebe Heenan processed the faecal samples at the University of Otago, Department of Medicine, Gastroenterology Research, Christchurch. Two samples of 1 g of faecal material were snap-frozen in liquid nitrogen after the samples arrived and stored at -80˚C until they were sent to AgResearch in Palmerston North for extraction.

Extraction of DNA was carried out using the Macherey-Nagel NucleoSpin Soil kit (Macherey-Nagel, Auckland, New Zealand) according to the manufacturer's instructions with some modifications. First, approximately 100-200 mg of the sample were transferred to a tube containing ceramic beads, and the weight was recorded. Next, 700 µL of buffer SL2 and 150 µl of Enhancer SX were added to each tube to lyse the material. The tubes were then briefly vortexed and held for 20 min at room temperature. Next, the samples were homogenised for 4 min using the Mini Bead-beater 96 (Biospec, Gisborne, New Zealand).

After the bead-beating, the foam caused by the detergent was reduced by centrifuging the samples at 11,000 x g for 2 min at room temperature. Finally, the clear supernatants were transferred to a new collection tube to improve DNA yield before precipitation. The particulate material was precipitated by adding 150 µL of lysis buffer SL3, vortexing for 5 seconds and incubating for 5 min at 4˚C. After the precipitation, the tube was centrifuged at 11,000 x g for 3 min at room temperature, and 700 µL of clear supernatant was loaded into a new collection tube with a NucleoSpin Inhibitor Removal Column filter. After centrifuging the tubes at 11,000 x g for 1 min at room temperature, the column was discarded, and 250 µL of binding buffer SB was added to the flow-through to adjust binding conditions. Next, the sample was mixed by inversion, and 550 µL was loaded into a new collection tube with a NucleoSpin Soil Column. The filter binds the microbial DNA. The sample was then centrifuged at 11,000 x g for 1 min at room temperature, and the flow-through containing host DNA was discarded.

Five consecutive washes of the silica membrane were followed by centrifuging at 11,000 x g for 30 seconds at room temperature after each wash. Next, 500 µl of the binding buffer SB was used for the first wash, 550 µL of the wash buffer SW1 was used for the second wash, and 700 µL of the wash buffer SW2 was used for the remaining washes. After the last wash, the column was washed by centrifuging at 11,000 x g for 2 min at room temperature.

The NucleoSpin Soil Column was placed into a new microcentrifuge tube, and 30 µL of elution buffer SE was added to the columns and incubated at room temperature for 1 min. The microbial DNA was then eluted by centrifuging at 11,000 x g for 1 min at room temperature. The microbial DNA was quantitated using the NanoDrop analyser (Thermofisher Scientific, Waltham, USA) and checked for fragmentation induced by bead beating by visualising 2 µL of DNA on a 1-2% agarose gel. DNA was stored at -80˚C. DNA samples met the sequencing criteria from Teagasc; the amount of DNA (0.2ng/µl in 5 µl) and DNA quality (260/280 ratio > 1.5).

2.4.3 Shotgun metagenomic sequencing

Standard procedures were used for shotgun metagenomic sequencing. After extraction, metagenomic DNA samples were paired-end shotgun sequenced by Dr Fiona Crispie at Teagasc, Food Research Centre, Moorepark, Ireland. The DNA shotgun libraries were prepared using the Illumina Nextera XT kit, as described in the manufacturer's instructions with the following change. Tagmentation time was increased from 5 to 7 min. Following index PCR and clean up, the sizes of the fragments of all samples were assessed by running on an Agilent bioanalyser using an Agilent High Sensitivity Kit and quantified using a Qubit High Sensitivity Kit. The libraries were then pooled equi-molar and the final concentration was determined using the Kapa Library Quantification Kit for Illumina. The final library was then denatured and sequenced on an Illumina NextSeq 550 using the NextSeq 500/550 High Output Kit v2.5 (300 Cycles) as outlined in the manufacturer's instructions.

2.4.4 Data acquisition and statistical analysis

The data acquisition and statistical analysis workflow used to identify differences in the taxonomic and functional categories that may underlie the phenotypic variations in FGIDs is described in Figure 2.3. Data were analysed with Human Microbiome Project pipelines [425]. Mr Paul Maclean, Bioinformatician at AgResearch, carried out sequence processing. This step included merging paired sequences using PEAR version 0.9.6 [426] and detecting and removing host sequences with the human genome (Human GRCh38) as a reference, using the BBMAP package version 38.22-0 with the bbduk.sh function. In addition, Metaxa2 version 2.1.3a [427] was used to identify small-subunit ribosomal DNA, and taxonomic classifications were determined using the Silva 128 database [428]. Next, sequences were aligned against the NCBI non-redundant protein reference database using the "blastx" function of DIAMOND version 0.9.22 [429]. Finally, the DIAMOND alignment files were assigned with putative gene functions using MEGAN version 6 ultimate edition [430] against the Kyoto Encyclopedia of Genes and Genomes (KEGG) and SEED (http://pubseed.theseed.org/) databases. Both annotation schemes contain categories of metabolic functions organised in multiple hierarchical levels. KEGG analysis maps enzymes onto known metabolic pathways while SEED is a constantly updated integration of genomic data with a genome database.

Analyses were carried out to characterise the faecal microbiota composition and gene abundance in subjects grouped as described in Section 3.1. Participants were grouped according to their bowel movement patterns (constipation phenotype: IBS-C+FC and diarrhoea phenotype: IBS-D+FD) or IBS subtypes (IBS-C, IBS-D) or functional subtypes (FC, FD). These groups were compared to controls or between each other. Subjects with IBS-M and IBS-U were excluded from the statistical analyses to focus on the common and exclusive microbial taxonomic and functional differences among subjects characterised by diarrhoea or constipation only. Alpha microbial diversity was calculated in Qiime 1.8 (Quantitative Insights Into Microbial Ecology) using the alpha_rarefaction.py script, rarefied to 1M reads (-e 1000000). Analysis of variance was used to identify significant differences in microbial diversity.

2.4.4.1 MetagenomeSeq

The R package "metagenomeSeq" [431], specifically designed for sparse high-throughput sequencing analyses, was used to identify taxa or gene abundance that were differentially abundant between groups, accounting for the effects of both normalisation and undersampling of microbial communities. The strength of this package is a normalisation method that avoids errors in measurements across taxonomic features. This software is also appropriate for sparse data sets for which the zero-inflated Gaussian mixture model is applied (i.e., probability distributions that allow for frequent zero-valued observations).

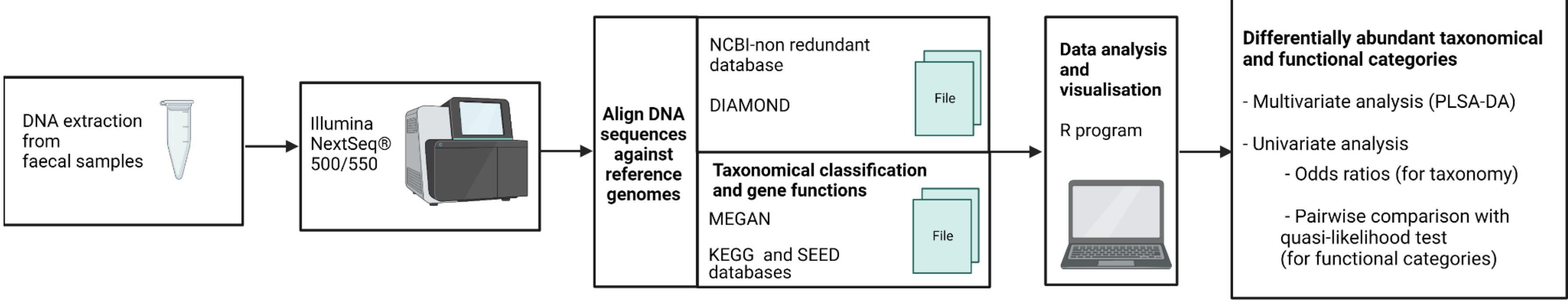


Figure 2. 3: Overview of the workflow for microbiota taxonomic and functional analysis.

Created with *BioRender.com.*

2.4.4.2 MRexperiment function

The MRexperiment function inside the "metagenomeSeq" package was used to create an object of class "MRexperiment", storing metagenomic count data and metadata info. After preprocessing and annotation, counts, feature information, and phenotype data were loaded into MRexperiment objects using the function "loadPhenoData", which considers data as a list. Next, files of counts (i.e., genus, species) were analysed using the function "loadMeta". Finally, the MRexperiment objects were used for subsequent normalisation, statistical tests, and visualisations. The normalisation, which is required due to varying depths of coverage across samples, was performed using the function "cumNorm". This normalisation method calculates scaling factors equal to the sum of counts up to a particular quantile.

The data were separated into different taxa levels (Table 2.2) and normalised across samples to minimise redundancy (duplicate data) and remove taxa that had less than 50 counts in total across all samples. MRexperiment objects for each taxonomic level were created, repeated samples were removed and only taxa with more than 50 counts in total across all samples were kept. MRexperiment objects were also created for each SEED and KEGG category. The categories were separated into different taxa levels and normalised across samples.

Table 2. 2: Hierarchy of the 8 major taxonomic ranks in the biological classification of microorganisms.

Taxonomy	Classification example
Domain	Bacteria
Kingdom	Bacteria
Phylum	Firmicutes
Class	Bacilli
Order	Lactobacillales
Family	*Streptococcaceae*
Genus	*Streptococcus*
Species	*Streptococcus mutans*

2.4.4.3 Multivariate and univariate analyses

Partial least squares discriminant analysis (PLS-DA), a supervised multivariate model, was used to identify the key genera that were responsible for the differential microbiota structure using the "plsda" function in the R package "mixOmics" (http://www.mixOmics.org). The statistical significance of differences in microbial taxonomic composition and gene abundances between groups was evaluated using the multivariate analysis of similarities (ADONIS).

Odds ratios and pairwise comparisons are univariate analyses which were used to detect differentially abundant microbial taxa and functional categories, respectively. Odds ratios were performed quantifying the association strength between two events by assessing if the microbial counts (of all counts) were in greater proportion in a group. Fisher's exact test on actual counts was used to create a 2x2 contingency table and calculate p-values, odds ratios, and confidence intervals. The function "fitDO" calculates the proportion of counts for each organism in the community and returns a data frame of p-values, odds ratios, lower and upper confidence limits for every row of a matrix. An odds ratio of one indicated that the community DNA had the same proportion of hits to a given category as the comparison data set; an odds ratio greater than one indicated underrepresentation in the FGID group (more hits to the control group than expected), whereas an odds ratio less than one indicated enrichment in the FGID group (fewer hits to the control group than expected). Results with FDR< 0.05 were considered significant. Only results with FDR< 0.05 are presented below.

Univariate analysis for differentially abundant functional categories was performed to evaluate changes in microbial functionality with pairwise negative binomial generalised log-linear models with a quasi-likelihood test. The datasets were filtered to remove rare features and subsetted to analyse different functional levels, restricted to FGIDs or IBS only. A quasi-likelihood test was applied to count data using the function "glmQLFit" provided in the edgeR package, and a pairwise statistical test was used to compare groups. Pairwise comparisons are statistical tests analysing multiple populations' means in pairs to ascertain whether they differ significantly. Pairwise comparisons were conducted for a given coefficient or a contrast, where the coefficient or the contrast indicates which comparisons to be tested (i.e., IBS-C vs. controls, IBS-D vs. controls or IBS-C vs. IBS-D). The edgeR package shows log2 fold change (FC) values by default. Results with

FDR< 0.05 were considered significant. The KEGG and SEED databases were used to identify high-level functions from large-scale molecular datasets generated by high-throughput genome sequencing. Only results with FDR< 0.05 are presented below.

2.5 Results

2.5.1 Faecal microbiota diversity

Alpha microbial diversity (the richness and evenness of the microbial community) was measured using the Chao 1 index, an estimator based on total richness. Analysis of variance was used to identify significant differences in microbial diversity between groups.

No significant differences in alpha microbial diversity were detected between the IBS-D, IBS-C, FD and FC groups, and controls (Figure 2.4 A, B). When subjects were grouped according to the bowel movement pattern subtypes (constipation phenotype: IBS-C/FC and diarrhoea phenotype: IBS-D/FD) and compared to controls, the alpha microbial diversity was increased in subjects with constipation ($p = 0.013$) but not in subjects with diarrhoea in comparison to controls (Figure 2.4 C).

2.5.2 Taxonomic analysis of the faecal microbiota

The mean number of reads per sample was 5,936,025, with a minimum of 1,393,848, a maximum of 34,399,091 and a standard deviation of 3,367,564. Univariate analysis of the composition of the faecal microbiota among participants of the COMFORT cohort revealed that it was highly variable with no differences between controls and FGIDs (Figures 2.5 A, B and 2.6). Multivariate analyses, such as PLS-DA, did not show apparent distinct taxonomic compositions between FGID and control groups (R2=0.035; p= 0.16) (Figure 2.7 A) or between IBS subtypes and controls (R2=0.015; p= 0.38) (Figure 2.7 B).

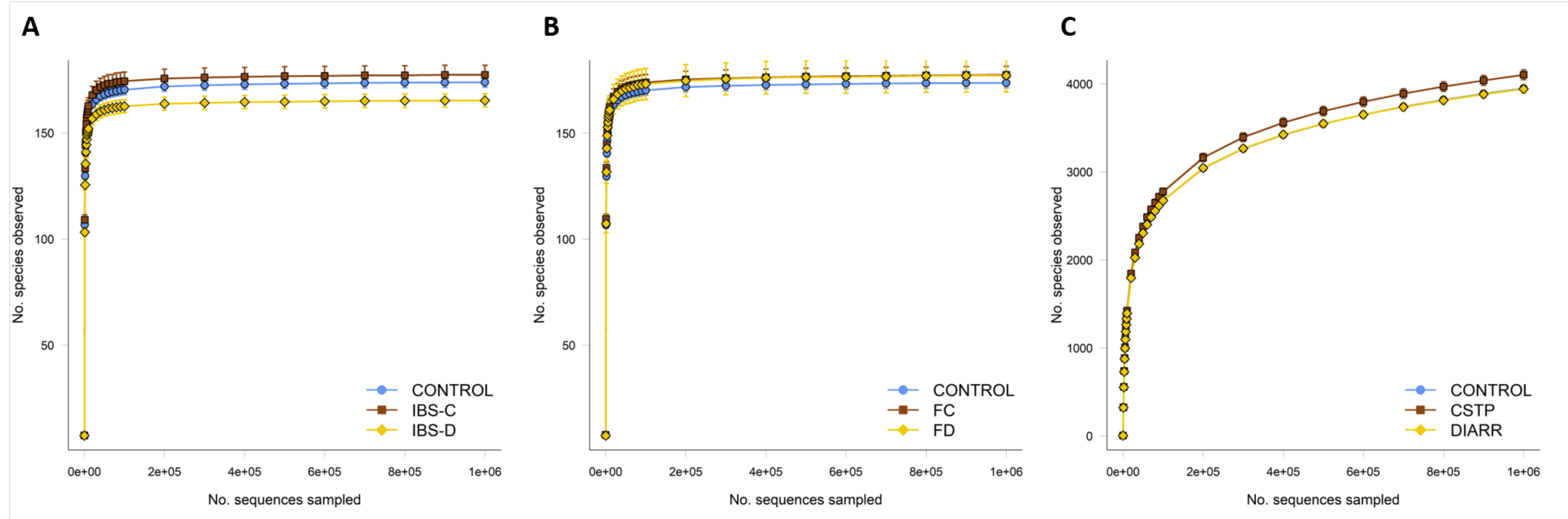
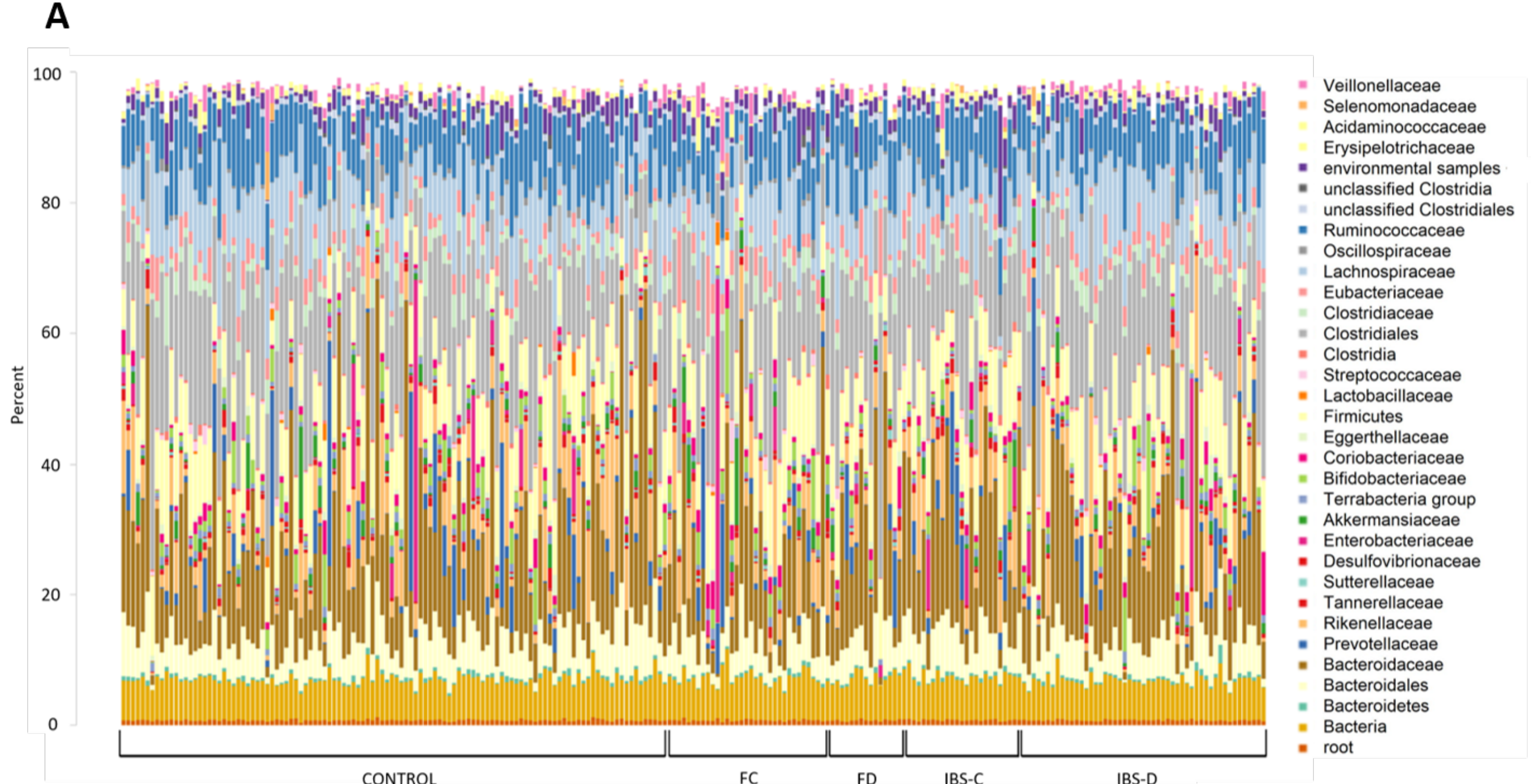


Figure 2. 4: Microbial diversity analysis.

Rarefaction curves showing richness in the faecal microbiota of subjects with IBS (**A**), FC or FD (**B**), or subjects grouped according to the bowel movement pattern subtypes (**C**). Different colours represent different FGID groups. The rarefaction curve shows the expected number of distinct taxa observed in a random subsample of a given size from a larger pool of taxa, to estimate the species richness of a community. The rarefaction curve is generated by plotting the number of observed species on the Y-axis against the number of sampled sequences on the X-axis. As the number of sampled sequences increases, the number of observed species also increases. When more sequences are sampled, the chances of observing new species decrease, and the curve eventually reaches a plateau, indicating that most of the diversity in the community has been detected. A steep curve quickly reaching a plateau indicates a low diversity community, while a sloping curve not reaching a plateau indicates a high diversity community that has not been fully sampled. The error bars represent the standard deviation and indicate the variability in the number of observed species at each level of sampling effort, showing the uncertainty in the estimated number of observed species. DIARR: diarrhoea (IBS-D+FD), CSTP: constipation (IBS-C+FC).


A
Percent
100
80
60
40
20
0
CONTROL
FC
FD
IBS-C
IBS-D
Veillonellaceae
Selenomonadaceae
Acidaminococcaceae
Erysipelotrichaceae
environmental samples
unclassified Clostridia
unclassified Clostridiales
Ruminococcaceae
Oscillospiraceae
Lachnospiraceae
Eubacteriaceae
Clostridiaceae
Clostridiales
Clostridia
Streptococcaceae
Lactobacillaceae
Firmicutes
Eggerthellaceae
Coriobacteriaceae
Bifidobacteriaceae
Terrabacteria group
Akkermansiaceae
Enterobacteriaceae
Desulfovibrionaceae
Sutterellaceae
Tannerellaceae
Rikenellaceae
Prevotellaceae
Bacteroidaceae
Bacteroidales
Bacteroidetes
Bacteria
root

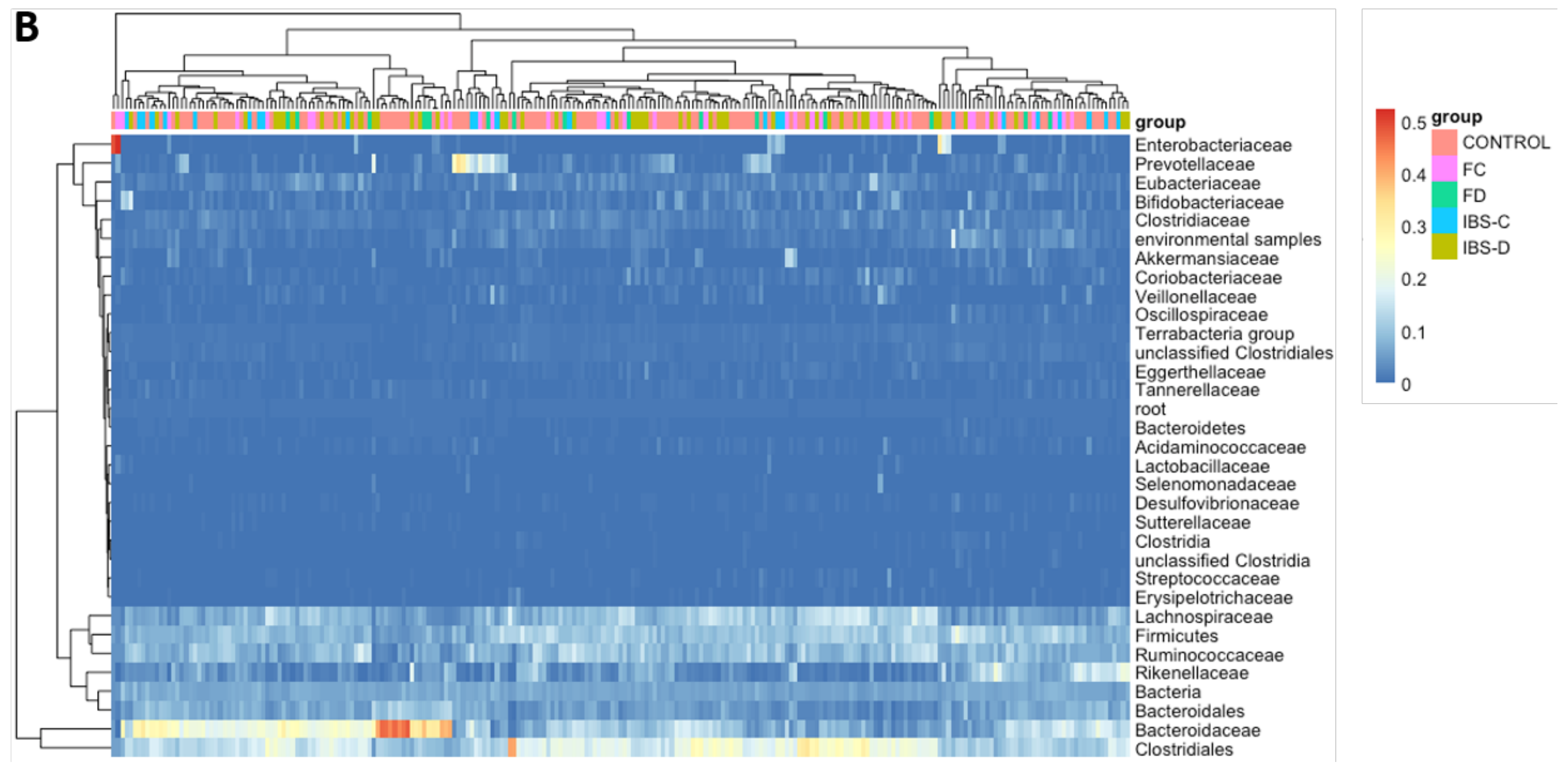


Figure 2. 5: Faecal microbiota composition of controls and participants with FGIDs (FC, FD, IBS-C, and IBS-D) up to the family level visualised as a stacked barplot (A) or heatmap (B). In **A**, each colour represents a different microbial taxon and each vertical column represents one individual. In **B**, the heatmap shows the preferential occurrence of taxa up to the family level across participants. The dendrograms cluster taxa and FGID subtypes with similar occurrence patterns across participants, based on their Euclidean distance. The colour scale represents the proportion of each taxa within a sample. The relative abundance of each taxa was normalised to 1 to adjust the patterns of all taxa.

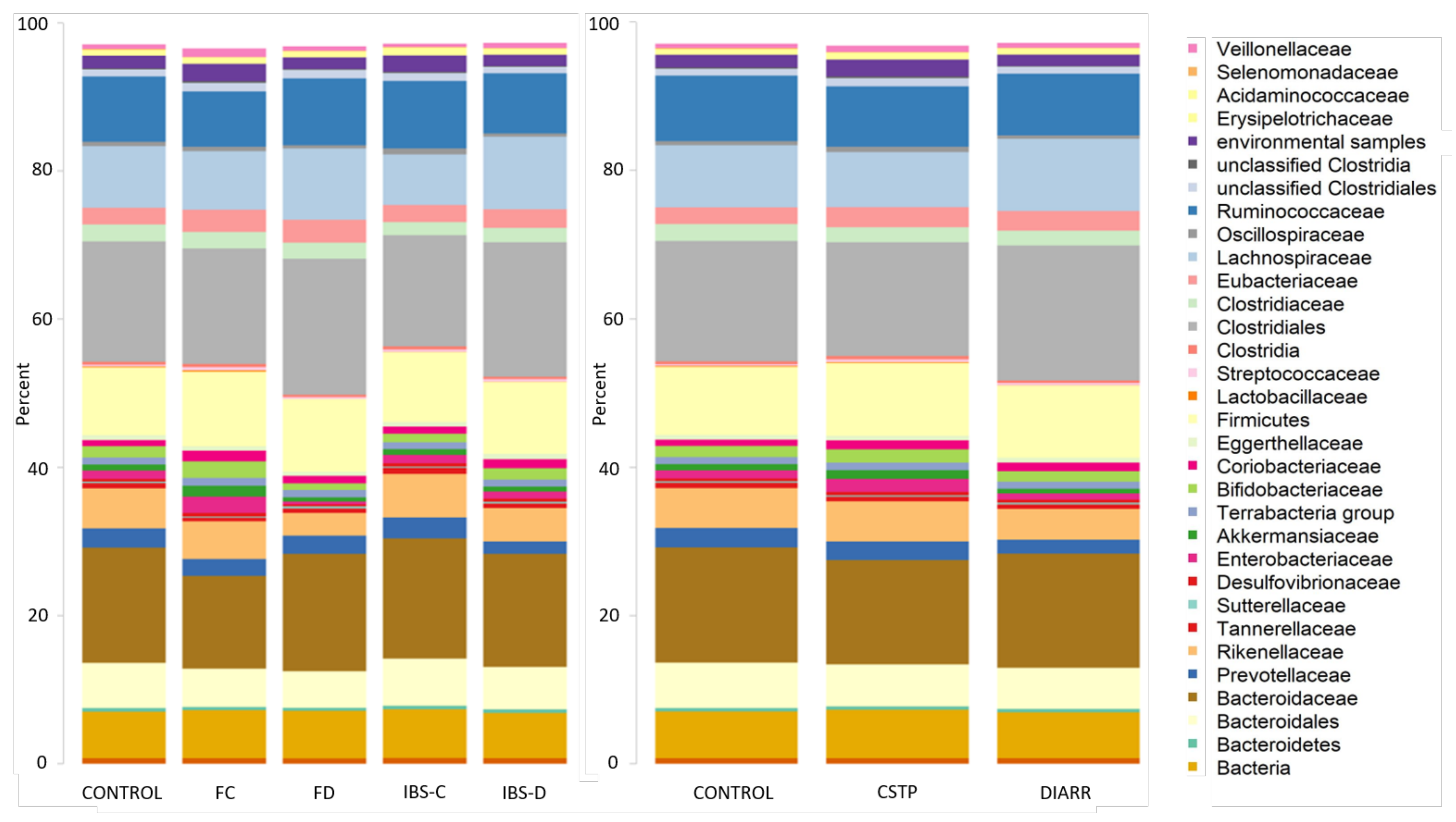


Figure 2. 6: Bar plot of taxonomic composition means of the faecal microbiota of (left) subjects with FC, FD, IBS-C, IBS-D, and controls and (right) subjects with constipation (IBS-C+FC), diarrhoea (IBS-D+FD) and controls. Profiles were visualised individually or grouped according to the symptom phenotype.

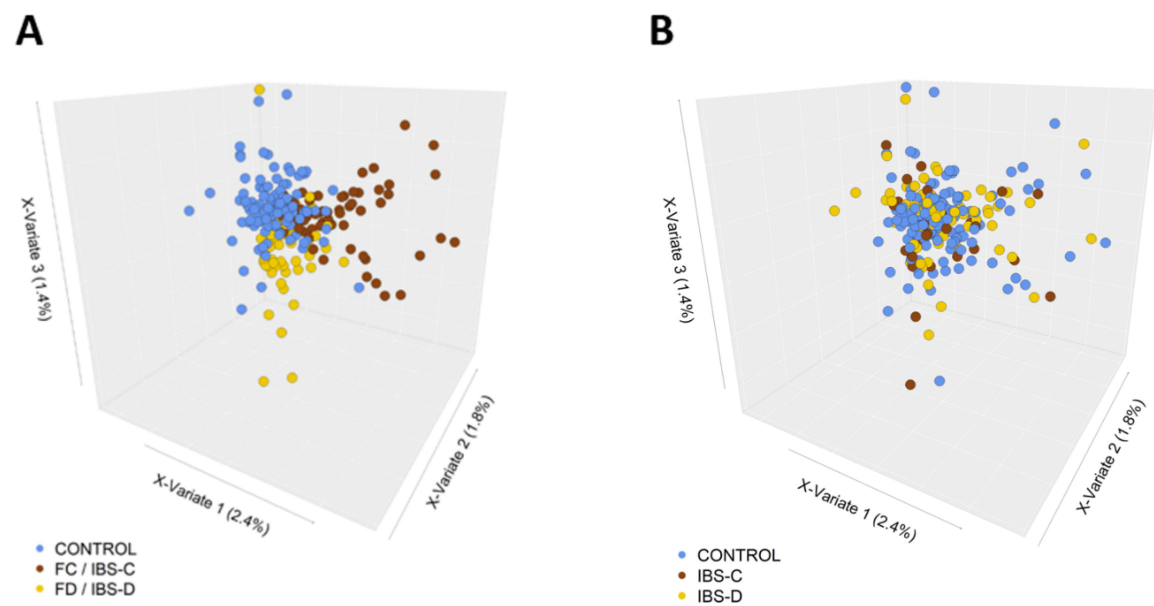


Figure 2. 7: PLS-DA score plots of the taxonomic composition of the faecal microbiota of subjects with constipation (FC+ IBS-C), diarrhoea (FD+ IBS-D) and controls (A), and subjects with IBS-C, IBS-D, and controls (B).

PLS-DA was used to identify potential compositional differences between groups in the microbial community at the species level. Each point represents a sample, and points with the same colour belong to the same group. Analysis of differences among similarities (ADONIS) was used to evaluate differences between groups. For IBS-D+FD and IBS-C+FC: R2=0.035; *p*= 0.16. For IBS-D and IBS-C: R2=0.015; *p*= 0.38). R2: proportion of variance (range 0 to 1). It indicates the amount of variability in the data, where 0= grouping variables explain none of the variance in the data, and 1= grouping variables explain all the variance in the data.

2.5.2.1 Diarrhoea predominant functional gastrointestinal disorders

Based on the odds ratio analysis, participants with diarrhoea predominant FGIDs (IBS-D+FD) were characterised by a lower relative abundance of taxa from the phyla Tenericutes and Candidatus Melainabacteria, along with a higher abundance of taxa from the phyla Firmicutes, including the Mollicutes class, and Lentisphaerae (Appendix Table A).

2.5.2.1.1 Proteobacteria and pathobionts

Taxa from the Proteobacteria α-, β- and δ- classes (e.g., *Rhodospirillaceae, Sutterellaceae, Pasteurellaceae* and *Desulphovibrionaceae* families) showed a higher abundance in diarrhoea predominant FGIDs compared to controls. Several genera belonging to these families are pathobionts (e.g., *Enterobacter*, *Klebsiella,* and *Haemophilus* genera) and were over-represented in diarrhoea predominant FGIDs (Table 2.3, Appendix Table A).

The relative abundance of the *Peptostreptococcaceae* family, which includes many potentially pathogenic anaerobic taxa, and of the pathobiont *Clostridioides difficile*, was higher in diarrhoea predominant FGIDs than in controls (Table 2.3, Appendix Table A).

2.5.2.1.2 Lactic acid bacteria

Among the lactic acid bacteria, the families *Bifidobacteriaceae* and *Lactobacillaceae*, including the genera *Bifidobacterium* and *Lactobacillus*, were under-represented. In contrast, the families *Streptococcaceae* and *Enterococcaceae,* including the *Streptococcus* and *Enterococcus* genera, were over-represented in diarrhoea predominant FGIDs compared to controls (Table 2.6, Appendix Table A).

2.5.2.1.3 Short-chain fatty acid producers

Several taxa directly or indirectly involved in SCFA production had different relative abundances in diarrhoea predominant FGIDs compared to controls (Table 2.5). These include several butyrate producers from the Clostridia class, particularly from the

Lachnospiraceae family, whose relative abundances were higher in those with diarrhoea predominant FGIDs compared to controls, but not in those with constipation predominant FGIDs (Appendix Table A; Table 2.5). The relative abundance of butyrate producers through amino acid fermentation (e.g., *Porphyromonadaceae* (genus *Porpyromonas*), *Odoribacteriaceae* (genus *Odoribacter*), and *Rikenellaceae* (genus *Alistipes*)), was lower in diarrhoea predominant FGIDs than in controls (Appendix Table A). In contrast, the relative abundance of taxa that possessed enzymes involved in propionate production (e.g., *Phascolarctobacterium* and *Megamonas* genera and many species from the *Blautia* genus) was higher in diarrhoea predominant FGIDs than in controls (Table 2.5; Appendix Table A).

The relative abundance of many succinate users from the family *Veillonellaceae* was higher in diarrhoea predominant FGIDs than in controls (Table 2.5). Conversely, the relative abundance of known succinate consumers from the families *Odoribacteraceae* and *Clostridaceae* was lower in diarrhoea predominant FGIDs than in controls (Appendix Table A). At lower taxonomic levels, succinate-producing species such as *Prevotella clara, Bacteroides vulgatus* and *Blautia wexlerae* were enriched in faecal samples of subjects with diarrhoea predominant FGIDs, which was accompanied by a lower abundance of succinate-consuming species *Phascolarctobacterium succinatutens, Bacteroides thetaiotaomicron, Ruminococcus bromii* and genus *Odoribacter* (Appendix Table A). Correspondingly, the ratio of specific succinate producers to consumers (*Prevotellaceae+Veillonellaceae/Odoribacteraceae+Clostridaceae*) was higher in diarrhoea predominant FGIDs than in controls (Appendix Table A).

A lower relative abundance of many species of acetate producers from the *Bacteroides*, *Bifidobacterium*, *Prevotella, Ruminococcus* and *Clostridium* genera was observed in diarrhoea predominant FGIDs compared to controls (Table 2.5; Appendix Tables A, B, C).

2.5.2.1.4 Hydrogen-metabolising bacteria

The relative abundance of the sulphate-reducing genera *Bilophila* and *Desulphovibrio* was higher and lower, respectively, in diarrhoea predominant FGIDs, compared to controls (Table 2.4). Conversely, the relative abundance of their competitors for

hydrogen, *Blautia* and *Methanobrevibacter* genera, was higher and lower, respectively, in diarrhoea predominant FGIDs compared to controls (Table 2.4; Appendix Table A).

2.5.2.1.5 Individual analysis of the IBS-D and FD subtypes

When the subtypes were individually compared to controls, the significant changes in the relative abundance of some hydrogen-metabolising taxa were attributable to IBS-D rather than FD (Figure 2.8 B). For example, the relative abundance of taxa from the Euryarchaeota phylum, including the *Methanobrevibacter* genus, was decreased in IBS-D but increased in FD compared to controls. The relative abundance of sulphate-reducing bacteria from the *Desulphovibrionaceae* family, including *Bilophila,* was increased in IBS-D but decreased in FD, compared to controls (Table 2.4; Figure 2.8 B; Appendix Tables B, C).

The relative abundance of many butyrate-producing genera (*Anaerotruncus, Catenibacterium, Coprobacillus* and *Roseburia*) was higher in FD than in IBS-D (Appendix Tables B, C). Conversely, the increased relative abundances of propionate producers, including the Negativicutes class and its members *Phascolarctobacterium* and *Megamonas* genera*,* were associated with IBS-D rather than FD (Table 2.5).

An increased relative abundance of the *Haemophilus* genus (commensal and pathogenic species) was exclusively detected in FD, while that of *Enterobacter* and *Klebsiella* genera was exclusively detected in IBS-D. Compared to controls, an increased relative abundance of the *Peptostreptococcaceae* family, including the potential pathogen *C. difficile*, was detected in both FD and IBS-D subtypes (Table 2.3; Figure 2.8 B; Appendix Tables B, C).

Table 2. 3: Relative abundance of pathobionts and potential pathogens across FGIDs at the genus and species level compared to controls.

Odds ratios (OR) were used to rank the relative enrichment or underrepresentation of microbial taxa, where OR <1 indicated enrichment in the FGID group, and OR >1 indicated underrepresentation in the FGID group.

	IBS-C+FC		**IBS-D+FD**		**IBS-C**		**FC**		**IBS-D**		**FD**	
Genus	**OR**	**FDR**	**OR**	**FDR**	**OR**	**FDR**	**OR**	**FDR**	**OR**	**FDR**	**OR**	**FDR**
Klebsiella	0.24	<0.0001	0.08	0	0.39	<0.0001	0.18	<0.0001	0.06	0	0.66	<0.01
Salmonella	0.38	<0.0001	-	-	-	-	0.22	<0.0001	-	-	-	-
Shigella	0.41	<0.0001	1.41	<0.001	-	-	0.29	<0.0001	-	-	-	-
Enterobacter	0	<0.0001	0	<0.0001	-	-	0	<0.0001	0	<0.0001	-	-
Haemophilus	3.31	<0.0001	0.47	<0.0001	-	-	1.90	<0.0001	-	-	0.17	<0.0001
Escherichia	0.74	<0.0001	2.19	0	1.41	<0.0001	0.54	0	2.00	0	3.07	<0.0001
Species												
Clostridioides difficile	-	-	0.49	<0.0001	-	-	-	-	0.5	<0.0001	0.51	<0.0001
Escherichia coli	0.66	<0.0001	2.00	<0.0001	1.22	<0.0001	0.5	<0.0001	1.79	<0.0001	3.42	<0.0001
Klebsiella pneumoniae	0.42	<0.0001	0.49	<0.0001	0.41	<0.0001	0.43	<0.0001	0.38	<0.0001	-	-
Salmonella enterica	0.41	<0.01	-	-	-	-	0.24	<0.0001	-	-	-	-
Shigella sonnei	0.48	<0.0001	1.44	<0.01	-	-	0.36	<0.0001	-	-	-	-

Table 2. 4: Relative abundance of hydrogenotrophic microbes across FGIDs at the genus level compared to controls.

Odds ratios (OR) were used to rank the relative enrichment or underrepresentation of microbial taxa, where OR <1 indicated enrichment in the FGID group, and OR >1 indicated underrepresentation in the FGID group.

		IBS-C+FC		**IBS-D+FD**		**IBS-C**		**FC**		**IBS-D**		**FD**	
Function	**Genus**	**OR**	**FDR**	**OR**	**FDR**	**OR**	**FDR**	**OR**	**FDR**	**OR**	**FDR**	**OR**	**FDR**
Sulphate reduction	*Bilophila*	0.72	<0.0001	0.76	<0.0001	0.71	<0.0001	0.72	<0.0001	0.64	<0.0001	1.86	<0.0001
	Desulphovibrio	0.75	<0.0001	1.62	<0.0001	0.68	<0.0001	0.81	<0.0001	1.84	<0.0001	1.16	<0.01
Methanogenesis	*Methanobrevibacter*	0.77	<0.0001	1.29	<0.0001	-	-	0.68	<0.0001	2.2	<0.0001	0.55	<0.0001
Acetogenesis	*Blautia*	1.05	<0.0001	0.75	0	1.08	<0.0001	1.02	<0.01	0.76	0	0.74	<0.0001

Table 2. 5: Relative gene abundance of microbial butyrate, propionate, and acetate producers in FGID subtypes compared to controls. The arrows indicate increased (↑) or decreased (↓) relative abundance of the taxa in comparison to controls in each subtype with a false discovery rate (FDR) < 0.05. Odd ratios were used to rank the relative enrichment or underrepresentation of microbial taxa, where OR <1 indicated enrichment in the FGID group, and OR >1 indicated underrepresentation in the FGID group.

			FGID groups vs controls					
	Producers		**IBS-D**	**FD**	**IBS-D+FD**	**IBS-C**	**FC**	**IBS-C+FC**
Butyrate	Clostridia class	*Anaerotruncus colihominis*	-	-	↑	-	-	↓
		Faecalibacterium prausnitzii	↓	↓	↓	↑	↓	↓
		Eubacterium rectale	↑	↓	↑	↓	↓	↓
		Eubacterium ventriosum	↑	-	↑	↓	-	↓
		Subdoligranulum variabile	-	-	-	-	↓	↓
		Oscillospiraceae	-	↓	↓	-	↑	↑
	Lachnospiraceae family	*Butyrivibrio crossotus*	↓	-	↓	↓	↑	↑
		Clostridium symbiosum	-	-	↑	-	↓	↓
		Coprococcus eutactus	↑	↑	↑	↓	↑	↓
		Roseburia intestinalis	↑	↓	-	↓	↓	↓
		Fusicatenibacter saccharivorans	↑	-	↑	↓	-	↓
		Anaerostipes spp.	↑	-	↑	↓	-	↓
Propionate	Succinate pathway	Bacteroidetes phylum	↓	↓	↓	↑	↓	↓
		Negativicutes class	↑	↓	↑	-	↑	↑
		Prevotellaceae family	↓	↓	↓	↑	↓	↑
		*Veillonella*ceae family	↑	↑	↑	↓	↑	↑
		Phascolarctobacterium	↑	↓	↑	↑	↓	-
		Megamonas	↑	↓	↑	-	↓	↓
		Prevotella clara	↑	-	↑	-	↓	-
		Bacteroides vulgatus	↑	-	↑	↑	↓	↓
		Blautia wexlerae	↑	↑	↑	↓	↑	-
		Dialister spp.	↑	↑	↑	↑	↑	↓
	Acrylate pathway	*Megasphaera elsdenii*	-	-	-	-	↑	↑
		Coprococcus catus	-	↓	-	↓	-	↓

	Propanediol pathway	*Salmonella* spp.	-	-	-	-	↑	↑
		Roseburia inulinivorans	-	↑	-	-	↓	↓
		Blautia spp.	↑	↑	↑	↓	↓	↓
Acetate	from pyruvate via acetyl-CoA	most enteric bacteria, e.g., *Akkermansia muciniphila*	↓	↓	↓	↓	↑	↑
		Bacteroides spp.	↓	↓	↓	↑	↓	↓
		Bifidobacterium spp.	↓	↓	↓	↓	↑	↑
		Prevotella spp.	↓	↓	↓	↑	↓	↓
		Ruminococcus spp.	↓	↓	↓	-	↓	↓
	Wood-Ljungdahl pathway	*Clostridium*	↓	↓	↓	↓	↓	↓
		Streptococcus spp.	↑	-	↑	↑	-	↑

2.5.2.2 Constipation predominant functional gastrointestinal disorders

When constipation predominant FGIDs (IBS-C+FC) were analysed as a group, the relative abundances of Verrucomicrobia, Euryarchaeota, and Spirochaetes phyla were increased, compared to controls. At the same time, the relative abundance of Tenericutes and Candidatus Melainabacteria phyla decreased (Appendix Table D).

2.5.2.2.1 Proteobacteria and pathobionts

The relative abundance of Proteobacteria from the δ- and γ- classes was higher in constipation predominant FGIDs than in controls. Families belonging to these classes (*Succinivibrionaceae* and *Enterobacteriaceae*), and many potential pathobionts (*Succinatimonas* genus, *Enterobacter* genus, *Escherichia coli*, *Klebsiella* spp., *Salmonella* spp. and *Shigella* spp.), had a higher relative abundance in subjects with constipation predominant FGIDs compared to controls (Table 2.3; Appendix Table D).

2.5.2.2.2 Lactic acid bacteria

The relative abundance of lactic acid bacteria from the *Lactobacillaceae*, *Bifidobacteriaceae*, *Streptococcaceae* and *Enterococcaceae* families, including the *Bifidobacterium*, *Lactobacillus, Streptococcus* and *Enterococcus* genera, was overall higher in constipation predominant FGIDs than controls (Table 2.6; Appendix Table D).

2.5.2.2.3 Short-chain fatty acid producers

Many obligate fermenters from the Clostridia and Bacteroidia classes, and the *Lachnospiraceae* and *Clostridiaceae* families, had a lower abundance in constipation predominant FGIDs compared to controls (Table 2.5; Appendix Table D).

The relative abundance of many succinate producers from the *Prevotellaceae* and *Veillonellaceae* families, including the species *Prevotella xylaniphila, Bacteroides caccae* and *Megasphaera elsdenii*, was higher in constipation predominant FGIDs than in controls (Table 2.5; Appendix Table D). Conversely, the relative abundance of many succinate consumers from the *Odoribacteraceae* and *Clostridaceae* families, including

the species *Phascolarctobacterium succinatutens, B. thetaiotaomicron, Dialister succinatiphilus, R. bromii* and *Odoribacter*, was lower in constipation predominant FGIDs, than in controls (Appendix Table D). Thus, the ratio of specific succinate producers to consumers (*Prevotellaceae+Veillonellaceae/Odoribacteraceae+Clostridaceae*) was higher in constipation predominant FGIDs than in controls (Appendix Table D).

2.5.2.2.4 Hydrogen-metabolising bacteria

The relative abundance of the sulphate-reducing genera *Bilophila* and *Desulphovibrio*, and methanogens, such as the *Methanobrevibacter* genus, was higher in constipation predominant FGIDs, compared to controls. Conversely, the relative abundance of their competitor for hydrogen, the *Blautia* genus, was lower in constipation predominant FGIDs compared to controls (Table 2.4; Appendix Table D).

2.5.2.2.5 Individual analysis of the IBS-C and FC subtypes

When the subtypes FC and IBS-C were individually compared to controls, many taxonomic differences were attributable to changes in FC rather than in IBS-C, compared to controls (Figure 2.8 A). For example, the relative abundance of taxa from the Tenericutes and Candidatus Melainabacteria phyla was decreased in FC but increased in IBS-C compared to controls (Appendix Table E, F).

Many potential pathobionts (e.g., *Desulphovibrio* genus, *Enterobacter* genus, *Klebsiella pneumoniae*, *E. coli, Salmonella enterica* and *Shigella sonnei*) were over-represented in FC but not in IBS-C, compared to controls (Table 2.3; Figure 2.8 A; Appendix Table E, F).

The relative abundances of lactic acid bacteria from the *Lactobacillaceae* and *Bifidobacteriaceae* families, including the *Lactobacillus* genus, and the *Bifidobacterium* spp. (*B. adolescentis* and *B. longum*), were increased in FC but decreased in IBS-C compared to controls (Table 2.6; Figure 2.8 A; Appendix Table E, F). The increased abundance of the *Enterococcaceae* family and the *Enterococcus genus* was specific to FC (Table 2.3; Figure 2.8 A; Appendix Table E, F).

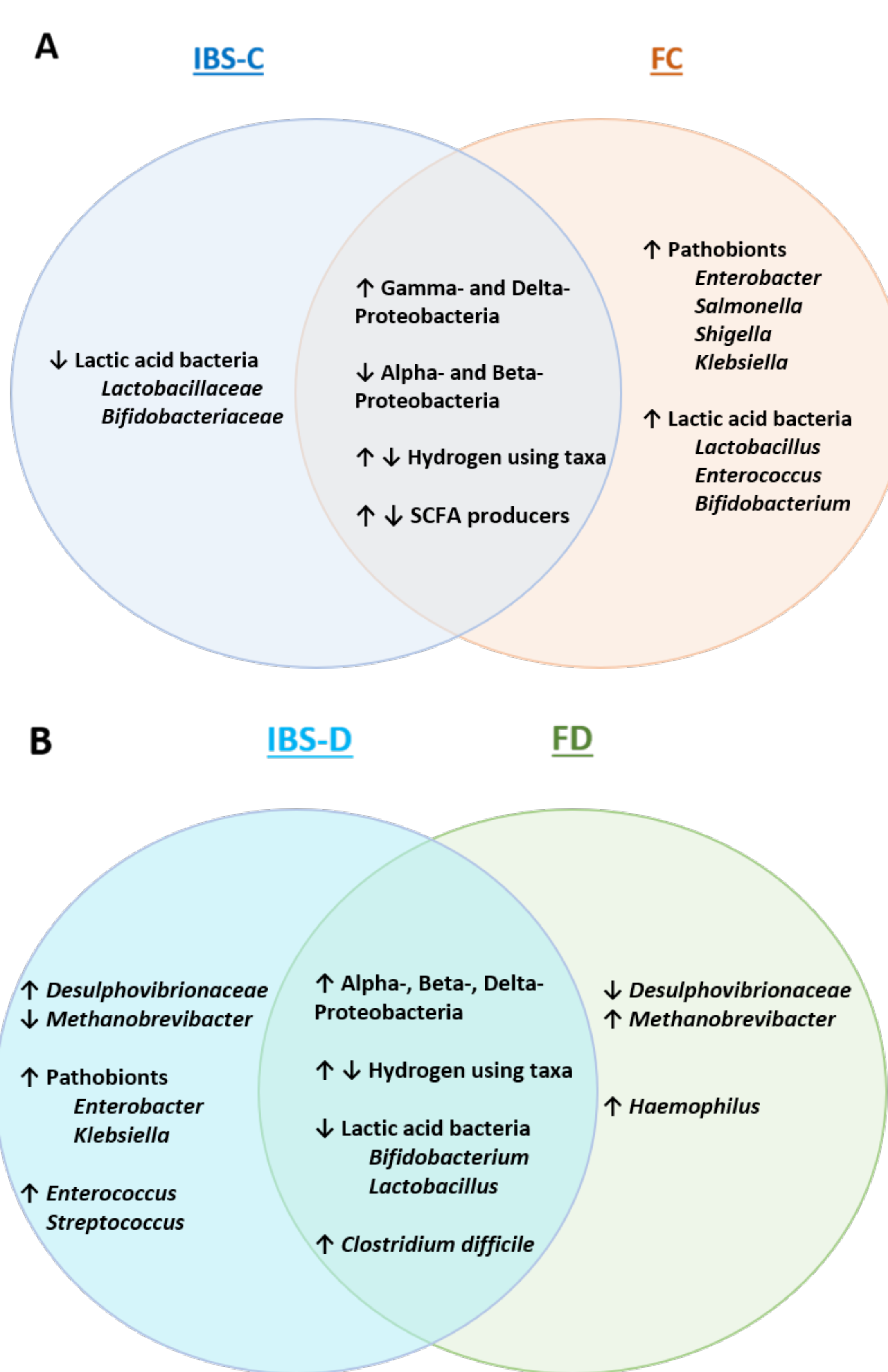
The relative abundance of the species *F. prausnitzii* was lower in FC than in IBS-C or controls (Appendix Table E, F). The relative abundance of obligate fermenters from the Bacteroidia class (including the genera *Bacteroides* and *Parabacteroides*), the families *Tannerellaceae* and *Prevotellaceae* (including the genera *Prevotella* and *Paraprevotella*), and the family *Rikenellaceae* (including the genus *Alistipes*) were decreased in FC but increased in IBS-C compared to controls (Appendix Table E, F).

The most relevant taxa whose relative abundances were commonly and exclusively different among FGIDs when each subtype was compared to controls are shown in Figure 2.8 C.

Table 2. 6: Relative abundance of lactic acid bacteria across FGIDs at the family, genus and species level compared to controls.

Odds ratios (OR) were used to rank the relative enrichment or underrepresentation of microbial taxa, where OR <1 indicated enrichment in the FGID group, and OR >1 indicated underrepresentation in the FGID group.

	IBS-C+FC		**IBS-D+FD**		**IBS-C**		**FC**		**IBS-D**		**FD**	
Family	**OR**	**FDR**	**OR**	**FDR**	**OR**	**FDR**	**OR**	**FDR**	**OR**	**FDR**	**OR**	**FDR**
Enterococcaceae	0.01	0	0.04	<0.0001	-	-	0.01	0	-	-	-	-
Bifidobacteriaceae	0.85	<0.0001	1.16	<0.0001	1.31	<0.0001	0.68	0	1.03	<0.0001	1.75	0
Lactobacillaceae	0.73	<0.0001	3.59	0	2.11	<0.0001	0.49	<0.0001	-	-	4.7	<0.0001
Streptococcaceae	-	-	0.8	<0.0001	0.7	<0.0001	0.55	0	0.7	<0.0001	-	-
Genus												
Enterococcus	0.01	0	0.04	<0.0001	-	-	0.01	0	0.03	<0.0001	-	-
Bifidobacterium	0.80	<0.0001	1.13	<0.0001	0.3	<0.0001	0.62	<0.0001	1.02	<0.001	1.67	0
Lactobacillus	0.71	<0.0001	3.42	<0.0001	2.13	<0.0001	0.48	<0.0001	3.19	<0.0001	4.45	<0.0001
Streptococcus	0.6	<0.0001	0.7	<0.0001	0.7	<0.0001	0.5	<0.0001	0.7	<0.0001	-	-
Species												
Enterococcus faecalis	0	0	-	-	-	-	0	<0.0001	-	-	-	-
B. adolescentis	0.74	<0.0001	1.22	<0.0001	1.19	<0.0001	0.58	<0.0001	1.10	<0.001	1.88	<0.0001
B. longum	0.84	<0.0001	1.05	<0.05	1.26	<0.0001	0.68	<0.0001	-	-	1.4	<0.0001
B. animalis	0.45	<0.0001	0.6	<0.0001	0.47	<0.0001	0.44	<0.0001	-	-	0.23	<0.0001
B. bifidum	-	-	1.52	<0.0001	-	-	0.54	<0.0001	1.17	<0.05	-	-


A
IBS-C
FC
↓ Lactic acid bacteria
Lactobacillaceae
Bifidobacteriaceae
↑ Gamma- and Delta-Proteobacteria
↓ Alpha- and Beta-Proteobacteria
↑ ↓ Hydrogen using taxa
↑ ↓ SCFA producers
↑ Pathobionts
Enterobacter
Salmonella
Shigella
Klebsiella
↑ Lactic acid bacteria
Lactobacillus
Enterococcus
Bifidobacterium
B
IBS-D
FD
↑ Desulphovibrionaceae
↓ Methanobrevibacter
↑ Pathobionts
Enterobacter
Klebsiella
↑ Enterococcus
Streptococcus
↑ Alpha-, Beta-, Delta-Proteobacteria
↑ ↓ Hydrogen using taxa
↓ Lactic acid bacteria
Bifidobacterium
Lactobacillus
↑ Clostridium difficile
↓ Desulphovibrionaceae
↑ Methanobrevibacter
↑ Haemophilus

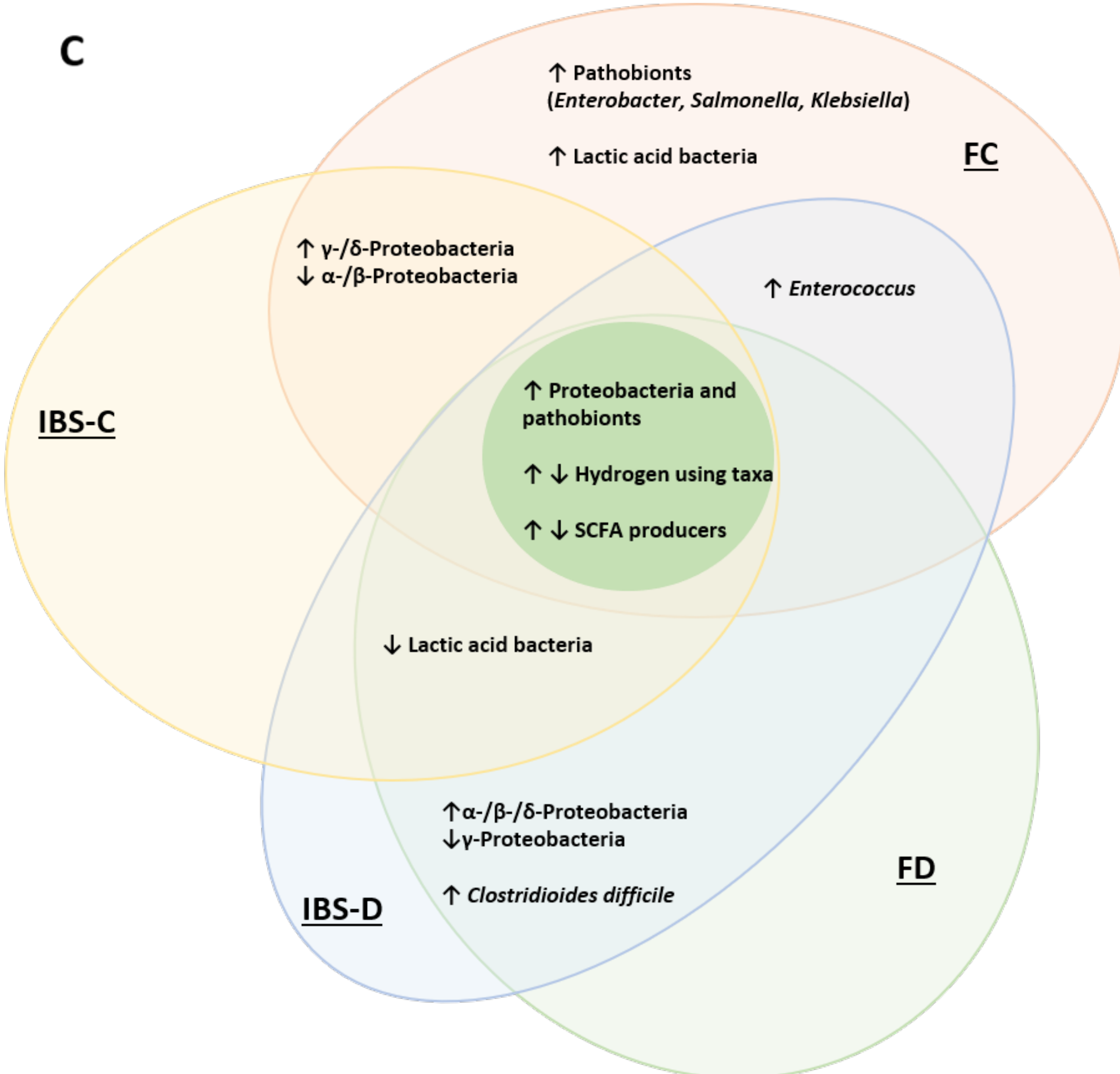


Figure 2. 8: Venn diagram.

Venn diagram shows microbes whose relative abundance was commonly and exclusively different in constipation predominant subtypes (IBS-C+FC) compared to controls (**A**), diarrhoea predominant subtypes (IBS-D+FD) (**B**), or among all the FGID subtypes (**C**), compared to controls. SCFAs: short-chain fatty acids. The arrows indicate increased (↑) or decreased (↓) relative abundance in comparison to controls with a false discovery rate (FDR) < 0.05.

2.5.3 Functional analysis of the faecal microbiota

The PLS-DA plots of the relative abundance of genes assigned to predicted functions from the faecal microbiota show a separation between FGID groups and controls (R2=0.017; *p*= 0.008) (Figure 2.9 A) but not between IBS subtypes and controls (R2=0.015; *p*= 0.38) (Figure 2.9 B). In addition, negative binomial generalised linear models with a quasi-likelihood test and pairwise statistical test were performed to detect the relative enrichment or underrepresentation of genes or groups of genes annotated to functional categories in diarrhoea- or constipation predominant FGIDs (IBS-D+FD or IBS-C+FC), and IBS (IBS-D or IBS-C), compared to controls and each other. Only significant results with FDR< 0.05 are presented below.

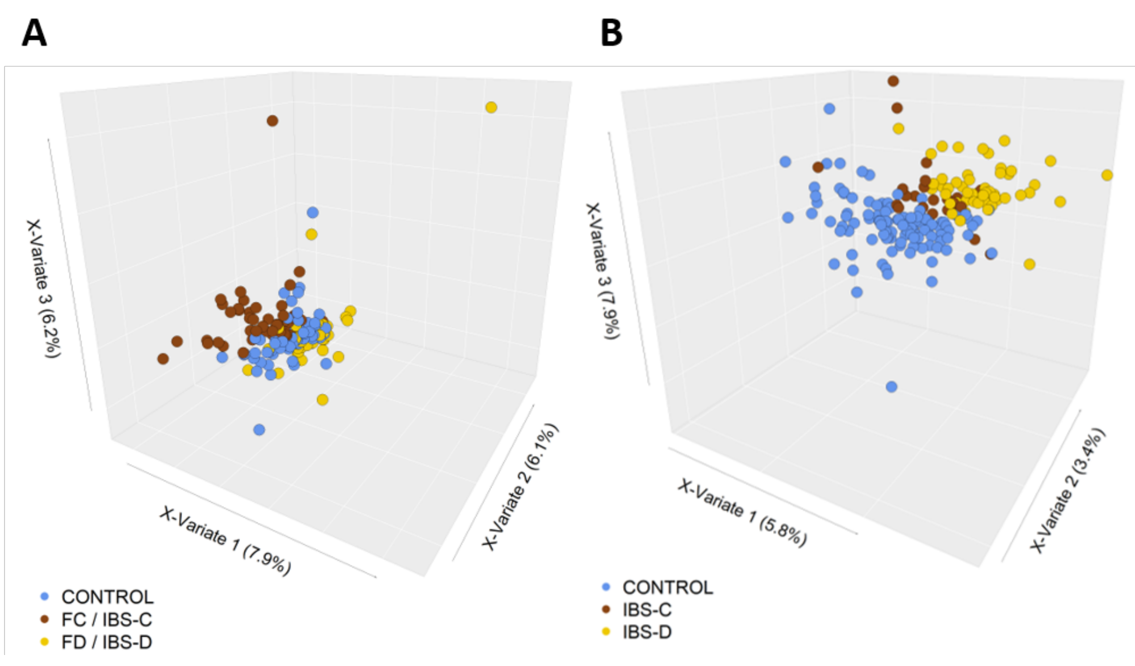


Figure 2. 9: PLS-DA score plots of the gene abundances of the faecal microbiota (A) of subjects with constipation (FC+IBS-C), diarrhoea (FD+IBS-D) and controls, and (B) subjects with IBS-C, IBS-D, and controls.

PLS-DA was used to identify predicted functional differences in the microbial community between groups at the enzyme level (KEGG level 4) in FGIDs and IBS. Each point represents a sample, and points with the same colour belong to the same group. Analysis of differences among similarities (ADONIS) was used to evaluate differences between groups. For IBS-D+FD and IBS-C+FC: R2=0.017; *p*= 0.008. For IBS-D and IBS-C: R2=0.015; *p*= 0.38). R2: proportion of variance (range 0 to 1). It indicates the amount of variability

in the data, where 0= grouping variables explain none of the variance in the data, and 1= grouping variables explain all the variance in the data.

2.5.3.1 Diarrhoea predominant functional gastrointestinal disorders

The abundances of genes annotated using SEED and KEGG functional categories differed between diarrhoea predominant FGIDs (IBS-D+FD) and controls (Appendix Table G), and between IBS-D or FD and controls (Appendix Tables H, I).

2.5.3.1.1 Carbohydrate and protein metabolism

When diarrhoea predominant FGIDs (IBS-D+FD) were analysed as a group and compared to controls, a higher relative abundance of genes related to the metabolism of 2-ketogluconate (SEED, logFC= 0.96; logCPM=1.45; FDR< 0.05) characterised subjects with diarrhoea predominant FGIDs (Appendix Table G). The relative abundance of genes related to galactose metabolism was higher in diarrhoea predominant FGIDs than in controls. These genes include the β-galactosidase 3 enzyme (SEED, logFC= 0.96; logCPM=3.7; FDR< 0.0001), which catalyses the first step of lactose fermentation in the colon, the galactose-6-phosphate isomerase, LacB subunit (SEED, logFC= 0.81; logCPM=2; FDR< 0.05) and the tagatose-1-phosphate kinase TagK (SEED, logFC= 0.90; logCPM=3.6; FDR< 0.05) (Appendix Table G).

When the subtypes were individually compared to controls, the relative abundance of genes related to the metabolism of 2-ketogluconate (SEED, logFC= 1.20; logCPM=1.34; FDR< 0.01) was detected in IBS-D subjects, but not in FD subjects, compared to controls (Appendix Tables H, I).

Concerning protein metabolism, the KEGG analysis showed the relative abundance of genes related to tyrosine metabolism (KEGG, logFC=0.70; logCPM=4.6; FDR< 0.05) was higher in IBS-D subjects, but not in FD subjects, compared to controls (Appendix Tables H, I).

2.5.3.1.2 Energy metabolism, cofactors acquisition and utilisation

An increased relative abundance of genes related to nitrogen fixation (SEED, logFC= 0.51; logCPM=3.07; FDR< 0.05) was observed in IBS-D subjects but not in FD subjects, compared to controls (Appendix Tables H, I). The relative abundance of genes associated with cobalt-specific energy-coupling factor transporters, particularly three out of four

genes belonging to the vitamin B_{12} biosynthetic operon *cbiMNQO*, was higher in IBS-D subjects, but not in FD subjects, compared to controls (Appendix Tables H, I). The cobalt-specific transport system includes the transmembrane unit *cbiM* (SEED, logFC=0.51; logCPM=3.4; FDR< 0.05), the T unit *cbiQ* (SEED, logFC=0.56; logCPM=3; FDR< 0.05) and the ABC ATPase *cbiO* (SEED, logFC=0.45; logCPM=3.9; FDR< 0.05).

Genes involved in salmochelin-mediated iron acquisition (SEED, logFC=-3.66; logCPM=2.6; FDR< 0.01) had a lower relative abundance in IBS-D subjects, but not in FD subjects, compared to controls (Appendix Tables H, I). These genes include the IroD trilactone hydrolase (SEED, logFC=-3.81; logCPM=1.45; FDR< 0.01), the IroB glycosyltransferase (SEED, logFC=-3.60; logCPM=1.56; FDR< 0.01) and the ATP-binding cassette responsible for the export of salmochelin/enterobactin IroC (KEGG, logFC=-4.43; logCPM=3; FDR< 0.001).

2.5.3.1.3 Transposable elements and antimicrobial resistance

When diarrhoea predominant FGIDs (IBS-D+FD) were analysed as a group and compared to controls, the relative gene abundance of proteins related to the transfer of the incompatibility plasmid F (IncF) was lower in diarrhoea predominant FGIDs compared to controls (Table 2.7). When the subtypes were individually compared to controls, a lower relative abundance of genes associated with the type IV secretion (SEED, logFC=-1.86; logCPM=5.54; FDR< 0.05) (Appendix Table H), including proteins related to the conjugative transfer of the IncF plasmid was observed in IBS-D subjects, but not in FD subjects, compared to controls (Table 2.7).

The relative abundances of genes related to microbial antibiotic resistance and copper homeostasis were higher in IBS-D subjects, but not in FD subjects, compared to controls (Table 2.7).

Table 2. 7: Relative abundance of microbial genes associated with plasmid transfer, stress resistance and copper homeostasis in IBS-D and FD, compared to controls.

The analysis was conducted using the SEED database at level 3 and the KEGG database at level 4 (enzymes and proteins). Differentially abundant functional categories between groups were identified using a pairwise statistical analysis with a quasi-likelihood test. A positive logFC indicates higher abundance in the FGID/IBS group, regardless of subtypes, and a negative logFC indicates higher abundance in the control group.

Functional category	IBS-D+FD vs. controls		IBS-D vs. controls	
IncF plasmid conjugative transfer pilus assembly protein	**logFC**	**FDR**	**logFC**	**FDR**
TraC	-2.37	< 0.01	-2.71	< 0.05
TraH	-	-	-3.08	< 0.01
TraU	-	-	-2.26	< 0.05
IncF plasmid conjugative transfer protein				
TraG	-2.18	< 0.05	-2.98	< 0.01
TraI	-	-	-2.54	< 0.05
TraN	-1.70	< 0.05	-2.40	< 0.05
Resistance to antibiotics				
Bacitracin stress response	0.48	< 0.05	-	-
Hygromycin-B 4-O-kinase	2.46	< 0.001	2.67	< 0.001
Copper sensing and homeostasis				
Heavy metal sensor histidine kinase	-	-	0.90	< 0.05
Multicopper oxidase	1.16	< 0.05	2.17	< 0.0001

2.5.3.2 Constipation predominant functional gastrointestinal disorders

The relative abundances of genes annotated using SEED and KEGG functional categories differed between constipation predominant FGIDs (IBS-C+FC) and controls (Appendix Table L), and between IBS-C or FC and controls (Appendix Tables M, N).

2.5.3.2.1 Metabolism of tyrosine, aromatic compounds, and carbohydrates

When constipation predominant FGIDs were analysed as a group and compared to controls, the relative abundance of genes involved in tyrosine metabolism and the 4-hydroxyphenylacetic acid catabolic pathway was higher in constipation predominant FGIDs (Table 2.8, Appendix Table L). However, when the subtypes were individually compared to controls, these differences were attributable to significant changes in FC rather than in IBS-C (Table 2.8, Appendix Table L, M, N).

The relative abundance of genes related to the metabolism of tyrosine, and the catabolism of aromatic amines and aromatic amino acid (tyrosine and phenylalanine) degradation products, including 4-hydroxyphenylacetic acid and phenylacetyl-CoA, was higher in FC, but not in IBS-C, compared to controls (Table 2.8, Appendix Tables N, M).

Similarly, an increased relative abundance of genes related to the utilisation of 2-ketogluconate (SEED, logFC= 1.24; logCPM=1.3; FDR< 0.01) was observed in FC but not in IBS-C or controls (Appendix Tables M, N).

Table 2. 8: Relative abundance of genes involved in tyrosine and aromatic compounds metabolism in FC and IBS-C, compared to controls. The analysis was conducted using the SEED database at level 2 and the KEGG database at level 3 (pathways). Differentially abundant functional categories between groups were identified by performing a pairwise statistical analysis with a quasi-likelihood test. A positive logFC indicates higher abundance in the FGID/IBS group, regardless of subtypes, and a negative logFC indicates higher abundance in the control group.

	IBS-C+FC vs. controls		**FC vs. controls**	
Functional category	**logFC**	**FDR**	**logFC**	**FDR**
Tyrosine metabolism	0.27	< 0.05	0.97	< 0.01

4-hydroxyphenylacetic acid catabolic pathway	1.19	< 0.01	1.19	< 0.01
Phenylacetyl-CoA catabolic pathway	-	-	1.94	< 0.05
o Phenylacetate-coenzyme A ligase (PaaF)	-	-	1.66	< 0.05
o Ring-opening aldehyde dehydrogenase (PaaN)	-	-	2.22	< 0.05
Aromatic amin catabolism	-	-	2.0	< 0.05
o Primary-amine oxidase	-	-	2.30	< 0.05
o Monoamine oxidase	-	-	2.32	< 0.05

2.5.3.2.2 Micronutrient metabolism and transport

Genes related to the metabolism and transport of micronutrients (copper and iron) discriminated subjects with constipation predominant FGIDs from controls (Table 2.9, Appendix Tables L, M). Genes related to copper homeostasis, copper sensitivity, and efflux were over-represented in FC but not in IBS-C compared to controls (Table 2.9). Genes related to the siderophore yersiniabactin synthesis were over-represented in FC compared to controls. In contrast, genes related to the siderophore salmochelin/enterobactin export were underrepresented in IBS-C and FC subtypes compared to controls (Table 2.9).

Table 2. 9: Relative abundance of genes involved in micronutrient metabolism in FC and IBS-C, compared to controls. The analysis was conducted using the SEED database at Levels 2 (pathway) or 3 (enzymes) and the KEGG database at Levels 3 (pathway) or 4 (enzymes). Differentially abundant functional categories between groups were identified by performing a pairwise statistical analysis with a quasi-likelihood test. A positive logFC indicates higher abundance in the FGID/IBS group, regardless of subtypes, and a negative logFC indicates higher abundance in the control group.

Iron metabolism		**FC vs. control**		**IBS-C vs. control**		**IBS-C+FC vs. control**	
Type of siderophore	**Protein**	**logFC**	**FDR**	**logFC**	**FDR**	**logFC**	**FDR**
Yersiniabactin (chelators)	K04786 Yersiniabactin synthase	-	-	-	-	1.83	< 0.001
	K04785 Yersiniabactin synthetase	2.96	< 0.0001	-	-	-	-
Salmochelin/ enterobactin (exporters)	ATP-binding cassette salmochelin/enterobactin exporter	-3.28	< 0.05	-6.69	< 0.01	-2.53	< 0.001
Copper metabolism							
Multicopper oxidase		2.30	< 0.0001	1.71	< 0.05	1.61	< 0.0001
Copper homeostasis		0.86	< 0.05	-	-	-	-
Copper sensor kinase CusS		0.95	< 0.01	-	-	-	-
P-type copper transporter		0.35	< 0.05	-	-	-	-
Micronutrient transport							
Energy-coupling factor transporters		0.38	< 0.05	-	-	0.31	< 0.05

2.5.3.2.3 Secretion system and transposable elements

An increased relative gene abundance of the type IV secretion system of the incompatibility group I1 plasmids (IncI1) was observed in subjects with constipation predominant FGIDs compared to controls (SEED, logFC=2.36; logCPM=3.4; FDR= 0.0028) (Appendix Table L). However, when the subtypes IBS-C and FC were individually compared to controls, differences related to the type IV secretion system of IncI1 plasmids were attributable to significant changes in FC rather than in IBS-C (SEED, logFC=2.86; logCPM=3.6; FDR< 0.001) (Appendix Table M-N).

The relative gene abundance of components of type II, IV and VI of the bacterial secretion systems was also higher in subjects with FC, but not in subjects with IBS-C, compared to controls (Table 2.10). Conversely, a decreased relative gene abundance of the type IV secretion (SEED, logFC=-2.83; logCPM=5.5; FDR< 0.05), including conjugative proteins involved in the transfer of the IncF plasmid, was observed in IBS-C but not in FC, compared to controls (Table 2.10).

2.5.3.2.4 Virulence and social behaviour

The relative abundance of genes related to bacterial virulence factors discriminated FC but not IBS-C from controls. These include a higher relative gene abundance of the outer-membrane pili, α-fimbriae (SEED, logFC=2.50; logCPM=2.3; FDR< 0.05), including the α-fimbriae usher protein (SEED, logFC=2.47; logCPM=1.5; FDR< 0.05). The relative gene abundance of the transcriptional regulator for hemolysin was also higher than controls in subjects with FC but not in subjects with IBS-C (KEGG, logFC=0.99; logCPM=2.1; FDR< 0.05) (Appendix Tables N, M).

Other virulence factors whose relative gene abundances were higher in FC but not IBS-C compared to controls were proteins related to biofilm remodelling and quorum sensing, including autoinducers and other chemical signals (Table 2.10). A lower relative gene abundance of proteins inhibiting peptidoglycan hydrolysis, such as the interacting proteins LrgA (SEED, logFC=-0.18; logCPM=6.1; FDR< 0.05) and LrgB (SEED, logFC=-0.22; logCPM=7.3; FDR< 0.01), along with proteins involved in peptidoglycan biosynthesis, such as the penicillin-binding proteins (KEGG, logFC=-0.13; logCPM=9.1;

FDR< 0.05), suggests augmented peptidoglycan hydrolysis rather than synthesis in FC but not in IBS-C, compared to controls (Appendix Tables N, M).

The relative gene abundance of the enzyme ethanolamine ammonia-lyase (SEED, logFC=1.49; logCPM=1.2; FDR= 0.0011), involved in bacterial anaerobic respiration, was increased in subjects with constipation predominant FGIDs (IBS-C+ FC) compared to controls (Appendix Table L).

Table 2. 10: The relative abundance of genes associated with microbial secretion systems, virulence, and social behaviours in FC and IBS-C, compared to controls.

Social behaviour refers to the ways bacteria interact and communicate in the context of a community, as opposed to behaving as individual cells. Predicted genes were detected using the SEED database at level 3 and the KEGG database at level 4 (enzymes and proteins). Differentially abundant functional categories between groups were identified by performing a pairwise statistical analysis with a quasi-likelihood test. A positive logFC indicates higher abundance in the FGID/IBS group, regardless of subtypes, and a negative logFC indicates higher abundance in the control group.

			FC vs. control		**IBS-C vs. control**	
	Protein	**Function**	**logFC**	**FDR**	**logFC**	**FDR**
Type II secretion system	cpaC	Outer membrane channel for pilus assembly	1.93	<0.05	-	-
	cpaE	Pilus assembly protein	1.77	<0.05	-	-
Type III secretion system	Protein U	Inner membrane protein	1.31	<0.05		
Type IV secretion system	VirB4	ATPase	2.53	< 0.0001	-	-
	VirB11	ATPase	2.17	< 0.01	-	-
Type VI secretion system	VgrG	Tip spike-like protein	1.40	<0.05	-	-
IncF plasmid transfer	TraC	Pilus assembly protein	-	-	-4.16	< 0.05
	TraI	DNA-nicking protein	-	-	-4.23	< 0.05
	TraP	Inner membrane protein	-	-	-3.98	< 0.05
Intracellular multiplication protein	IcmB		1.78	< 0.01	-	-
Social behaviour	Antigen 43	Biofilm formation	2.54	< 0.01	-	-
	Pga A		2.28	< 0.05	-	-
	Pga B		1.63	< 0.05	-	-
	Lsr operon transcriptional repressor (LsrR)	Biofilm formation and quorum sensing	1.84	< 0.05	-	-
	Autoinducer-2 kinase	Quorum sensing	0.76	< 0.05	-	-
	Autoinducer-2 permease protein		0.92	< 0.05	-	-

2.6 Discussion

Chapter 2 reported the taxonomic composition and predicted functional genes of the faecal microbiota of subjects with FGIDs and healthy subjects recruited in the COMFORT cohort. The findings confirmed the hypothesis that the faecal microbiota of subjects with FGIDs could be discriminated according to differences in microbial composition from controls and among FGID subtypes. The most significant signature related to FGIDs (compared to controls) was characterised by an increased relative abundance of members of the Firmicutes and Proteobacteria phyla, and members of the Negativicutes class, along with a decreased relative abundance of members of the Bacteroidetes phylum. Differences in the relative abundance of genera involved in colonic hydrogen metabolism, including *Bilophila*, *Desulphovibrio, Blautia* and *Methanobrevibacter*, were also observed across FGID subtypes compared to controls. Along with these taxonomic differences, relative abundances of some genes involved in antibiotic resistance, stress response and type IV secretion system also differed across FGID subtypes compared to controls.

2.6.1 Microbial hydrogen metabolism in constipation and diarrhoea

The findings here show that the major hydrogen-consuming organisms in the human colon, which use hydrogen to reduce carbon dioxide to methane (*Methanobacteriaceae* family) or sulphur to hydrogen sulphide (*Desulphovibrio, Bilophila Streptococcus, Enterobacter*, and *Salmonella* genera) [432, 433] differentiated constipation and diarrhoea phenotypes.

Increased relative abundance of taxa from the *Methanobacteriaceae* family, and taxa involved in hydrogen sulphide production (*Desulphovibrio, Bilophila Streptococcus, Enterobacter*, and *Salmonella* genera) were found in subjects with constipation, compared to controls. The increased abundance of taxa from the *Methanobacteriaceae* family could reflect their "slow-growing" nature, resistance to the lack of water in firmer faeces, and advantages in conditions of slowed colonic transit [87]. Similarly, consequent changes in composition could result from resistance to water stress of other species.

Other studies reported an increased relative abundance of the *Methanobrevibacter* genus in IBS-C [83] and FC [434] and a link between higher exhaled methane levels, slower

motility and faecal firmness [67, 81-84, 435]. Moreover, hydrogen removal through methane production alters colonic fermentation and likely peristalsis [82].

Conversely, in subjects with diarrhoea, an increased relative abundance of taxa from the *Desulphovibrionaceae* family and *Blautia* genus and a decreased abundance of taxa from the *Methanobacteriaceae* family were observed. This result suggests competition between them for hydrogen disposal [436] or the fitness of a family that prefers a colonic environment characterised by slow transit [87].

The amount of hydrogen in the colon can be influenced by changes in the composition of the microbiota and differences in the hydrogen disposal pathways, such as acetogenesis, methanogenesis or sulphate reduction [88, 437]. Decreased faecal levels of methanogens have been linked to excess abdominal gas in IBS patients, suggesting they may lack normal hydrogen removal function [70, 71]. In addition, common symptoms of IBS (bloating and abdominal pain) have been associated with hydrogen accumulation in the colon [89]. Hydrogen sulphide produced by sulphur-reducing bacteria was also reported to play a role in abdominal pain and motility disorders with dysbiosis [90].

2.6.2 Microbial fermentation in constipation and diarrhoea

The relative abundance of butyrate-producing bacteria from the Clostridia class, including *F. prausnitzii,* was lower in subjects with constipation predominant FGIDs compared to controls. Previous research showed a lower abundance of obligate anaerobes from the *Lachnospiraceae* family (Clostridia class) in IBS-C [73, 438], confirming the results from the current study. Conversely, many butyrate producers from the Clostridia class and the *Lachnospiraceae* family showed a higher relative abundance in subjects with diarrhoea predominant FGIDs compared to controls. In this regard, a lower or higher faecal butyrate concentration was reported in subjects with IBS-C and IBS-D, respectively [439].

Butyrate producers (e.g., *Faecalibacterium*, *Roseburia*, and *Coprococcus* genera) have been positively associated with fast colonic transit [88], presumably because of the ability of butyrate to induce serotonin release and stimulate the cholinergic pathways [440, 441]. A lower relative abundance of butyrate-producing bacteria from the Clostridia class (including *F. prausnitzii*), along with a higher abundance of the *Enterococcus* and

Staphylococcus genera were detected during chronic inflammation [442, 443]. Interestingly, the relative abundance of *Enterococcus* and *Staphylococcus* was also higher in subjects with constipation predominant FGIDs or FC from the current study compared to controls. This result suggests that the Clostridia class could help maintain immune homeostasis in the colon, exerting anti-inflammatory effects through butyrate and promoting regulatory T cell accumulation [444].

The relative abundance of succinate-producing bacteria from the genus *Veillonella* was lower in IBS-C, while it was increased in other FGID subtypes. This finding contrasts with studies reporting a higher abundance of this genus in IBS-C [73, 445]. Despite this, the ratio of succinate producers to consumers was higher in all FGID groups compared to controls.

Studies report that increased levels of succinate in faecal samples were associated with a higher relative abundance of succinate-producing taxa from the *Prevotellaceae* and *Veillonellaceae* families, and a lower relative abundance of succinate-consuming taxa from the *Odoribacteraceae* and *Clostridaceae* families [446]. High colonic levels of succinate have been associated with the metabolic regulation of immune cells, inflammation and dysbiosis [446-448]. Ferreyra *et al.* showed that succinate enhanced *C. difficile* expansion in mice after antibiotic treatment, and that loss of succinate pathways conferred a competitive disadvantage to *C. difficile* [449]. It is also worth noting that the co-culture of *E. coli* with *E. faecalis*, another succinate-producer, increased *E. coli* virulence gene expression [450]. Therefore, an unbalanced ratio between succinate-producing and succinate-consuming taxa may affect commensal-pathogen interactions in constipation and diarrhoea.

An increased relative abundance of genes related to utilising 2-ketogluconate was observed in subjects with FC and IBS-D but not in subjects with IBS-C or FD. The organic acid 2-ketogluconate derives from the ketogluconate metabolism, a precursor step to the Entner-Doudoroff pathway, which is an alternative pathway to glycolysis [451].

Due to the low energy yield of the Entner–Doudoroff pathway, bacteria that use this pathway for glucose metabolism are mostly gram-negative aerobes and facultative anaerobes, with the ability to use other non-glycolytic pathways requiring less energy input for creating ATP. In contrast, obligate anaerobes mainly use glycolysis [452]. The

increased gene abundance of 2-ketogluconate may thus reflect the observed increased relative abundance of facultative anaerobes in FC (members of the *Enterobacteriaceae* family) and IBS-D (Proteobacteria phylum, *Enterobacter* genus, *Klebsiella* genus and *Haemophilus* spp.).

Therefore, the increased relative abundance of genes related to utilising 2- ketogluconate may result from a dysbiotic environment in subjects with FC and IBS-D, where bacteria require alternative metabolic pathways to gain advantage and derive energy from diverse nutrient sources.

2.6.3 Microbial protein metabolism in constipation and diarrhoea

The relative abundance of genes related to tyrosine metabolism was increased in FC and IBS-D, but not in IBS-C or FD, compared to controls. In addition, an increased relative gene abundance of the catabolism of aromatic amines and aromatic amino acid degradation products, including 4-hydroxyphenylacetic acid and phenylacetyl-CoA, discriminated subjects with constipation predominant FGIDs from controls and subjects with FC from subjects with IBS-C or controls.

Phenylacetic acid and 4-hydroxyphenylacetic acid are the end-products of phenylalanine and tyrosine oxidative metabolism by colonic bacteria, and 4-hydroxyphenylacetic acid is also the direct precursor of p-cresol [453]. The production of cresols from tyrosine has been attributed to various anaerobic taxa, including species from the *Enterobacteriaceae, Bifidobacteriaceae, and Lactobacillaceae* families [454]. The relative abundance of members of these families was also increased in FC subjects compared to controls, suggesting a potential increase in cresols and other bioactive substances, such as phenolic compounds, in these subjects. The longer transit time in FC would enhance the colonic absorption of these aromatic amino acid-derived metabolites, which could act as signalling molecules at local and systemic levels [455]. High concentrations of p-cresol were associated with cytotoxicity, oxidative stress, and DNA damage to colonocytes [456]. Moreover, aromatic amino acid biosynthesis pathways were shown to be the main metabolic pathways induced in stress-induced IBS rats [457].

These findings suggest a potential role for aromatic amino acid-derived metabolites in the gut-brain axis interactions underlying the pathophysiology of FGIDs, particularly FC.

2.6.4 Constipation predominant FGIDs

2.6.4.1 Bacteroidia and Proteobacteria classes

The most robust bacterial pattern in subjects with constipation was a reduction of strictly anaerobic members of the Clostridia and Bacteroidia classes, including the genus *Bacteroides*, and an expansion of facultative anaerobic taxa from the *Enterobacteriaceae* family and Proteobacteria phylum (δ and γ class). The *Enterobacteriaceae* family represents approximately 0.1% of the colonic microbiota in healthy subjects [458], but their prevalence increased in disease states [459-462]. This family encompasses many gram-negative pathogenic species potentially involved in inflammation and associated with FGIDs. The ability of pathogenic species to thrive in the colonic environment is high when the bacterial community is unstable due to increased levels of taxa from the Proteobacteria phylum [463].

Many pathobionts from the Gammaproteobacteria class (*Enterobacter* genus*, Klebsiella pneumoniae*, *E. coli*, *S. enterica* and *S. sonnei*) were over-represented when constipation predominant FGIDs were analysed as a single group. However, when FC and IBS-C were individually compared to controls, their differences were attributable to changes in FC rather than in IBS-C.

Increased taxa from the Proteobacteria phylum, particularly facultative anaerobic from the *Enterobacteriaceae* family, are thought to be implicated in the pathophysiology of many colonic conditions [459-462], including pathogen-induced colonic inflammation in mice [464-469], Crohn's disease [470-472], necrotising enterocolitis in preterm infants [473], IBS-C [79, 92] and IBS-D [77, 474]. In addition, the abundance of *E. coli* has been positively correlated with IBS-like symptoms [66] and bacterial overgrowth [475]. Antibiotics targeting *E. coli*, such as rifaximin, reduced these symptoms, supporting a potential role of *E. coli* in FGID [476].

The compositional changes in the faecal microbiota of subjects with constipation agree with a dysbiotic environment characterised by a reduction of strictly anaerobic members of the Bacteroidia and Clostridia classes that dominate a healthy colonic microbiota [477] and a concomitant outgrowth of facultative anaerobic bacteria [464-466] belonging to the Gammaproteobacteria and Bacilli classes [464, 478]. Laxatives have been shown to

normalise these dysbiotic changes, suggesting they might be secondary to constipation rather than a cause [417].

2.6.4.2 Microbial anaerobic respiration genes

An increased relative abundance of taxa and genes involved in anaerobic respiration and a decreased relative abundance of obligate fermenters from the Clostridia and Bacteroidia classes, characterised the subjects with constipation predominant FGIDs (IBS-C+ FC) compared to controls.

These include facultative anaerobes and opportunistic bacteria from the *Enterobacteriaceae* family (*Escherichia* spp. and *Salmonella* spp.) and microbial genes encoding the enzyme ethanolamine ammonia-lyase. This enzyme catalyses the first step in ethanolamine utilisation [479], a compound that gram-negative bacteria and facultative anaerobes can be used as a sole source of carbon and/or nitrogen [480]. As an alternative nitrogen source, the ability to use ethanolamine would confer a competitive advantage to these bacteria, and has been described as a virulence factor during host-pathogen interactions [479].

In addition, the breakdown of ethanolamine could contribute to immune modulation and evasion via the production of acetate, as it interacts with a G-protein coupled receptor 43, affecting innate immunity [481].

It is possible that in the dysbiotic colonic environment of subjects with constipation predominant FGIDs, especially FC, facultative anaerobes and opportunistic bacteria could gain a growth advantage because of their ability to use ethanolamine as a sole carbon source, outcompeting anaerobic fermenters (Clostridia and Bacteroidia classes) [482, 483]. This ability would reduce competition for nutritional resources in an environment deprived of nutrients with fermentative properties, such as in IBS, where the intake of foods with highly fermentable substrates is often restricted to manage GI symptoms.

2.6.4.3 Microbial virulence genes

Taxa from the *Enterobacteriaceae* family can express virulence factors responsible for invasion, colonisation, and persistence in the colon. These factors include adhesins, hemolysins, plasmids, biofilm formation and secretion systems, transporting proteins, plasmids and toxins across the bacterial membranes [484-486].

Along with an expansion of taxa from the *Enterobacteriaceae* family, subjects with constipation had higher abundance of genes related to virulence factors. These include bacterial α-fimbriae, type IV pilus assembly proteins and genes related to the type IV secretion system for bacterial intracellular multiplication, biofilm formation and the conjugative transfer of IncI1 plasmid type. However, when FC and IBS-C were individually compared to controls, their relative abundances were increased in FC, but not in IBS-C.

Virulence genes (adhesins, toxins, iron acquisition, antimicrobial resistance and secretion systems [487]) from 89 different *E. coli* were reported in IBS subjects with mixed phenotypes. The most prevalent virulence genes detected in *E. coli* encode type II, IV and VI secretion system proteins, long polar fimbriae and iron acquisition [488]. In addition, several genes encoding for antibiotic resistance were reported in IBS subjects with unspecified subtype [489].

A higher relative gene abundance of the interacting proteins LrgA and LrgB, which inhibit peptidoglycan hydrolysis by murein hydrolases, and a lower relative gene abundance of penicillin-binding proteins, involved in peptidoglycan biosynthesis, were also detected in FC but not IBS-C in comparison to controls. The higher relative gene abundance of trans-envelope complexes such as pili, fimbriae, and secretion systems in subjects with FC, suggests that this augmented peptidoglycan hydrolysis may be due to peptidoglycan remodelling for the insertion of trans-envelope complexes.

Notably, a higher relative gene abundance of proteins linked to the expression of autoinducers was observed in FC compared to controls. Autoinducers are chemical signals produced during quorum sensing, a type of cell-to-cell communication that the bacteria use to exchange information and increase their fitness in adaptation to changes in the environment. Several physical-chemical environmental parameters and quorum sensing regulate the expression of virulence factors and secretion systems [490-492]. Therefore, it is plausible that the increased relative abundance of genes associated with

virulence factors and secretion systems, especially in FC, could be due to quorum sensing through an increased expression of autoinducers.

2.6.4.4 Microbial micronutrient metabolism genes

In the current study, the relative abundance of different siderophores (high-affinity ferric iron chelators transporting iron across cell membranes) differed in constipation predominant FGIDs compared to controls. In particular, the relative abundance of genes involved in the synthesis of the siderophore yersiniabactin increased, while those involved in the export of the siderophore salmochelin/enterobactin were lower. However, when the subtypes IBS-C and FC were individually compared to controls, these differences were attributable to changes in FC rather than in IBS-C.

These results were supported by a higher relative abundance of taxa from the Bacilli class, *E. coli,* and *Klebsiella* genus in subjects with FC, as yersiniabactin biosynthetic clusters are found in many pathogenic enteric Gram-negative bacteria [493, 494].

During colonic inflammation, the host's innate defence secretes lipocalin-2, which binds and sequesters the iron-scavenging siderophore enterobactin, preventing bacterial iron acquisition to limit bacterial growth [495, 496]. Some pathogens avoid lipocalin-2 binding by making alternative siderophores, such as yersiniabactin. Unlike enterobactin, yersiniabactin is not sequestered by the human host [497], allowing pathogens to scavenge ferric iron and evade the host metal-withholding response [498]. Therefore, the ability to produce yersiniabactin and its association with virulence would give a selective advantage to enteric pathogens [499].

Siderophores can also sequester extracellular copper as a protective mechanism [500], as the antimicrobial properties of copper are exploited by the human host to kill bacteria [501]. A higher relative abundance of genes involved in copper sensitivity, efflux and detox, such as the multicopper oxidase enzyme, was observed in FC but not in IBS-C. The enzyme multicopper oxidase can oxidise enterobactin in the presence of copper, preventing the generation of toxic ions at the expense of the iron scavenging activity of the siderophore [500]. This observation suggests a decreased export and scavenging activity of enterobactin in subjects with FC, resulting from the oxidation of the siderophore by the multicopper oxidase when the concentration of intracellular copper is

high. In addition, the decreased relative abundance of genes related to enterobactin/salmochelin-mediated iron acquisition and the concomitant ability to express lipocalin-resistant siderophores observed in subjects with FC may enhance the competitive advantage of resident and pathogenic taxa from the *Enterobacteriaceae* family and production of energetically costly virulence factors.

2.6.5 Diarrhoea predominant FGIDs

2.6.5.1 Proteobacteria and Clostridia classes, pathobionts and lactic acid bacteria

The microbial signature specific to diarrhoea predominant FGIDs, compared to controls, included a higher relative abundance of facultative anaerobic taxa from the α-, β- and δ-Proteobacteria classes, along with a higher abundance of strictly anaerobic members of the Clostridia class (*Clostridium* spp.). A lower relative abundance of protective species from the *Bifidobacterium* genus, Bacilli and Bacteroidia classes (*Bifidobacterium* spp., *Lactobacillus* spp., and *Bacteroides fragilis)* was also detected.

These findings agree with the association of a Clostridia-rich microbiota with excessive bile acid excretion in subjects with IBS-D reported in a previous study [502] and the COMFORT study [503]. Among the Clostridia class, the increased relative abundance of *C. difficile* in diarrhoea agrees with other studies in subjects with PI-IBS [46, 504], in subjects with IBS (undefined phenotype) without risk factors [505], and in subjects with diarrhoea co-infected with a pathogen [506]. Thus, disturbances caused by diarrhoeal events may increase the susceptibility to *C. difficile* colonisation and exacerbate symptoms in IBS-D [506, 505], depending on strain toxigenicity.

A higher relative abundance of pathobionts discriminated FD from IBS-D. The genus *Haemophilus* (commensal and pathogenic species) was exclusively detected in FD, while increased counts of *Enterobacter* and *Klebsiella* genera were exclusively detected in IBS-D. In diarrhoea, higher pathogenic (Clostridia class: *C. difficile* and other pathobionts) and lower protective species (Bacilli and Bacteroidia classes: *Bifidobacterium* spp., *Lactobacillus* spp., *Clostridium* spp., and *B. fragilis*) abundances may create a "pathogenic community", inducing or worsening symptoms.

2.6.5.2 Microbial lactose fermentation

Consistent with published studies [237, 507, 508], the relative abundance of lactose and galactose degradation genes was higher in subjects with diarrhoea. This result includes a higher relative gene abundance of the β-galactosidase 3 enzyme, which catalyses the first step of lactose fermentation in the colon. Lactose intolerance has been associated with IBS-D [509]. The malabsorption of lactose in the small intestine leads to the fermentation of undigested lactose by the colonic microbiota, increasing the osmotic trapping of water and the production of SCFAs, hydrogen, carbon dioxide and methane. The imbalance between the removal and production rate of lactose and intermediate metabolites in the colon might be a key factor in developing diarrhoea symptoms [510].

2.6.5.3 Microbial nitrogen fixation

The analysis conducted here showed novel observations regarding microbial nitrogen fixation in IBS-D. The relative abundances of nitrogen-fixing bacteria (Clostridiales order and the *Klebsiella*, *Enterobacter* and *Streptococcus* genera) and genes related to nitrogen fixation and enzyme nitrogenase activity (nitrogen reduction to ammonia) were higher in IBS-D but not in FD, compared to controls. These findings are supported by a new pathway identified in bacteria that used nitrogenase during anaerobic growth to generate methane from carbon dioxide by methane-metabolising archaea or aerobic methanotrophic bacteria [511].

2.6.6 Strengths and limitations

The characterisation of the faecal microbial community of the participants recruited in the COMFORT cohort has several strengths. A well-defined study population generated 300 samples with rigorous inclusion/exclusion criteria, a clinical interview by a gastroenterologist, and questionnaires of GI symptoms, demographic, and psychological variables. Enrolment processes ensured the completion of the questionnaires, efficient return of biological samples, and close timing of data collection.

The present analyses have some limitations. The limitations in DNA extraction and sequencing protocols among studies may lead to erroneous and disparate estimates of

taxonomic diversity. Detecting specific microbial DNA can identify the corresponding taxa but only predict pathway activity. Many transcriptional silencing and repression events occur in bacteria [512, 513], negatively controlling gene expression. Another limitation relates to the KEGG functional database, which is unspecific to bacteria. Bacteria and other microorganisms can express similar genes but with different functions. The KEGG database categorises these genes under the same functional category, which can be overcome by investigating the individual genes/enzymes rather than the broad categories.

Shotgun metagenomics may be insufficient to detect low abundance taxa or achieve the separation of individual bacterial strains. Moreover, many microbial functional genes had low abundance or small differential expression levels due to the complexity and diversity of the microbial communities, variability in sampling and technical noise, functional redundancy, and environmental factors. Despite microbial genes with low abundance or small differential expression levels that may still be biologically relevant in the context of a community, the factors mentioned above should be considered when analysing and interpreting metagenomic data.

Other limitations relate to the collection and microbial structure of faecal samples [412]. Firstly, the impossibility to collect faecal samples into a nucleic acid stabiliser may have affected the composition of the faecal microbiota. Freezing immediately the samples at -80°C was not possible, as participants collected their samples at home and kept at 4°C for up to 24 hours. Although this protocol is sub-optimal, it is the recommended process to preserve microbial DNA for faecal samples not chemically preserved or immediately frozen.

Secondly, sample homogenisation is critical to reducing the intraindividual variation to detect each component [514]. Wu et al. reported that 35% of low-abundance taxa (~0.2–0.4% of the microbiota in one sample), could not be found in its replicate [515]. Faecal microbiota is a proxy of the microbiota at the site of effects and may bias estimates as differences in microbial composition between segments, lumen, mucosa and faeces have been reported [516-518]. Such information may be relevant for the pathogenesis of bloating symptoms in IBS because of the overlap with small intestinal bacterial overgrowth.

2.7 Conclusion

Metagenomic analysis of the faecal microbiota in FGID subjects identified common features and different microbiota profiles associated with constipation or diarrhoea compared to controls. In both constipation and diarrhoea, changes in the abundance of proteobacteria, hydrogen-consuming taxa and butyrate producers, along with an increased abundance of genes related to secretion systems and copper homeostasis were observed. These changes might provide an adaptive advantage for opportunistic bacteria to adhere and compete for host resources, altering the composition of the microbiota and potentially contributing to a pathogen-driven immune response at the subclinical level.

An increased relative abundance of microbial genes related to tyrosine metabolism was observed in FC and IBS-D. Aromatic amino acid-derived metabolites may play a role in gut-brain axis interactions in FGIDs. Despite FD and IBS-D had similar taxonomic signatures in comparison to controls, the microbial functional changes discriminating the diarrhoea group (IBS-D + FD) from controls were related to IBS-D rather than FD.

A distinctive microbial signature discriminated FC from IBS-C, suggesting FC as a distinct condition rather than part of the IBS-C spectrum. Specific features of FC included an increased relative abundance of facultative anaerobes over obligate fermenters, along with increased relative abundance of genes related to virulence and micronutrient competition.

Chapter 3

Characterisation of the gene expression profile of peripheral blood mononuclear cells in subjects with functional gastrointestinal disorders and healthy subjects

A journal article titled “Peripheral blood mononuclear cell gene expression signature in functional gastrointestinal disorders” (Caterina Carco, Wayne Young, Jane Mullaney, Phoebe E. Heenan, Richard B. Gearry , Elizabeth Forbes-Blom, Jacqueline I. Keenan, Nicholas J. Talley, Warren C. McNabb, Nicole C. Roy) will be submitted to the *American Journal of Gastroenterology* in 2023.

3.1 Abstract

The unavoidable interaction between the upper and lower gastrointestinal (GI) microbiota and the immune system was proposed to be involved in the low-grade chronic inflammation often observed in subjects with functional gastrointestinal disorders (FGIDs), including irritable bowel syndrome (IBS). Chapter 3 aims to identify differences in the RNA expression of peripheral blood mononuclear cells (PBMCs) collected from FGID subjects and healthy subjects (controls) to identify a potential immune signature of the FGID subtypes.

Total RNA was extracted from PBMCs isolated from 274 FGID subjects and controls (72 subjects with IBS constipation or functional constipation, 73 subjects with IBS diarrhoea or functional diarrhoea and 129 controls) and analysed by RNA-sequencing. Differentially expressed genes (DEGs) between FGID subjects and controls were determined using negative binomial generalised log-linear models with a quasi-likelihood F-test. Over-represented pathways of DEGs were identified performing functional enrichment analyses with the REACTOME browser.

Increased expression levels of immunoglobulin light chains and various innate, humoral, adaptive immune and haemostatic processes discriminated IBS-D, FD and FC, but not IBS-C, from controls. Increased expression levels of immunoglobulin variable domain associated with immunoglobulin E/G receptor-mediated pathways (phagocytosis and intracellular signalling) characterised the immune signature of IBS diarrhoea and FD. A higher number of DEGs characterised the immune signature of FC in comparison to other FGID subtypes and controls. These included increased expression levels of interferon (IFN)-induced genes and those linked to the complement system and platelet functions. In summary, these findings provided potential evidence of subclinical immune abnormalities at the systemic level in FGID subjects and highlighted a distinctive immune signature in FC.

3.2 Introduction

A growing body of evidence supports the role of microbe-immune interactions in the pathophysiology of irritable bowel syndrome (IBS) and functional gastrointestinal disorders (FGIDs) [519, 520]. Low-grade chronic inflammation seems to be involved in most mechanisms underlying symptom generation in FGIDs, including visceral hypersensitivity [150], abdominal pain [151] and increased colonic permeability [137]. The dysbiosis theory, reviewed by Tamboli *et al.*, proposed that an imbalance between putative "harmful" and "protective" bacterial species may promote chronic colonic inflammation [521]. Many studies suggest that a bloom of taxa from the *Enterobacteriaceae* family is associated with increased colonic inflammation. For example, their abundance was increased in faecal samples of a mouse model of inflammatory bowel diseases, and their transfer to other animals exacerbated colonic inflammation [478, 522]. In addition, the adherent-invasive *Escherichia coli* isolated from the colonic mucosa of Crohn's disease subjects exacerbated colonic inflammation when administrated to colitic mice [523]. These studies suggest that the mechanisms leading to dysbiosis might favour the colonisation of potential pathogens from the *Enterobacteriaceae* family, such as *E. coli*, exacerbating inflammation and interfering with its resolution. The changes in the faecal microbiota in subjects with FGIDs, as a proxy of colonic microbiota, discussed in Chapter 2, suggest that the affected taxa may be involved in the onset of colonic inflammation or the exacerbation of a pre-existing inflamed environment. This pathogen-driven subclinical immune response may be due to an adaptive advantage of pathogenic and opportunistic microbes to spread fitness, virulence and antibiotic-resistance determinants and to adhere and compete for host resources.

Previous research revealed the presence of a subclinical immune activation in the colonic mucosa or at the systemic level (plasma and serum) in subjects with FGIDs [524]. Overall, the currently available data reported increased levels of pro-inflammatory interleukins (ILs) (e.g., IL-1β, IL-6 and IL-8) and tumour necrosis factor (TNF) in plasma and serum samples of IBS subjects regardless of the subtype [210, 525]. Mast cell activation [167] and increased frequency of circulating cytotoxic CD8+ T cells [526] have been reported in IBS-D subjects. Elevated counts of circulating total T lymphocytes CD3+, CD4+ T-helper cells, CD25+ regulatory T cells and increased spontaneous

proliferation of lymphocytes were found in subjects with FC [417]. In addition, elevated titres of circulating antibodies to bacterial flagellin in IBS subjects (subtype not mentioned) [128] and to *E. coli* and *Staphylococcus aureus* in FC subjects were reported [417]. Other studies demonstrated that mucosal or luminal immune mediators from subjects with different IBS subtypes, but not controls, stimulated an abnormal immune response in enteric and sensory nerves and impaired colonic barrier function in rodents [527, 528] , human colonic tissue [529] or cellular models [168].

Despite this evidence, existing data about low-grade inflammation in subjects with FGIDs are conflicting and expressed as average numbers, probably reflecting symptom heterogeneity. Gender, age, geographic differences, genetic predisposition, diet and differences in the colonic microbiota are likely to affect the immune response.

Moreover, there is limited data regarding peripheral blood mononuclear cell (PBMC) gene expression patterns in FGIDs. Increased levels of pro-inflammatory ILs were detected in *ex-vivo* resting or stimulated PBMCs collected from IBS-D subjects [530]. Similarly, PBMCs from subjects with IBS-C showed increased *E. coli* lipopolysaccharide (LPS)-induced IL-1β levels compared with controls [531]. Gupta *et al*. attempted to identify PBMC gene expression inflammatory profiles correlating with altered functional activity in the salience network of the brain in subjects with IBS [408]. Regions of the salience network, including the mid-cingulate cortex and mid and superior temporal gyrus have been positively correlated with PBMC pro-inflammatory genes (IL6, APOL2) in IBS but negatively correlated with PBMC anti-inflammatory genes (KRT8, APOA4) in controls. However, the study involved only 16 IBS subjects, which were all females and with unspecified IBS subtypes.

Gene expression of PBMC provides a useful insight into the pathophysiological mechanisms of disease, as they play a leading role in immune activation. PBMCs are comprised of lymphocytes (70–90%), monocytes (10–20%), dendritic cells (1–2%) [532] and a trace amount of circulating stem cells including platelets and erythroid progenitors (0.1-0.2%) [533]. PBMCs provide a less invasive alternative to biopsies [534] and a more representative cell pool compared to mouse models or human cell lines, which have limitations in predicting *in vivo* responses, restricting their clinical translation. There is a movement of leukocyte subsets to and from the gastrointestinal (GI) tract at a steady state, suggesting that PBMCs can reflect the molecular events occurring in the GI tract [535].

Chapter 3 aims to identify differences in the RNA expression of PBMCs collected from subjects with FGIDs and healthy subjects (controls) and characterise their immune signatures by characterising their RNA expression profiles. My contribution was performing RNA extraction of the PBMC samples, conducting bioinformatics and statistical analyses, data interpretation, and writing the research chapter and journal article.

3.3 Hypothesis and aims

The primary hypothesis of the research presented in this Chapter was that the individual FGID subgroups (IBS-C, IBS-D, FC, or FD) could be discriminated from controls, according to differences in their PBMC transcriptomes.

An additional hypothesis was that participants with constipation predominant (IBS-C+FC) or diarrhoea predominant FGIDs (IBS-D+FD) could be discriminated from controls, according to differences in their PBMC transcriptomes.

The aim was to analyse the RNA gene expression profile of PBMC samples obtained from 274 subjects diagnosed and clustered in different FGID subgroups (IBS-C, IBS-D, FC, FD) and controls from the '**C**hristchurch IBS c**o**hort to investigate **m**echanisms **fo**r gut **r**elief, and improved **t**ransit' (COMFORT) cohort to characterise their immune signature and to identify potential differences in the expression of PBMC genes.

RNA-sequencing of PBMC samples was undertaken and differences in the PBMC gene expression were determined using R programming. The RNA gene expression data was visualised for all FGID groups (FC, IBS-C, FD, IBS-D) and the control group. The following comparisons were made:

1. Subjects with IBS-D were compared to controls.
2. Subjects with FD were compared to controls.
3. Subjects with IBS-C were compared to controls.
4. Subjects with FC were compared to controls.
5. Subjects were grouped according to the bowel movement pattern subtypes (constipation phenotype: IBS-C+FC; diarrhoea phenotype: IBS-D+FD), and each phenotype was compared to controls.

Subjects with IBS-M and IBS-U were excluded from the statistical analyses to focus on the common and exclusive differences in the PBMC transcriptome among subjects characterised by diarrhoea or constipation.

3.4 Materials and Methods

3.4.1 Isolation of peripheral blood mononuclear cells

Samples isolated from 274 participants of the COMFORT cohort (129 controls, 57 IBS-D, 30 IBS-C, 42 FC, and 16 FD) (see Chapter 2, section 4.1) were initially processed by Dr Phoebe Heenan, former PhD candidate at the University of Otago, Department of Medicine, Gastroenterology Research, Christchurch. PBMCs were extracted from 18 ml of lithium heparin-treated blood as per protocol (Figure 3.1), one-third of which was stored in a solution of 90% foetal bovine serum in the gaseous phase of liquid nitrogen and two-thirds were stored in RNAlater cell protect at −80°C until analysis. Samples were then sent on dry ice to AgResearch in Palmerston North.

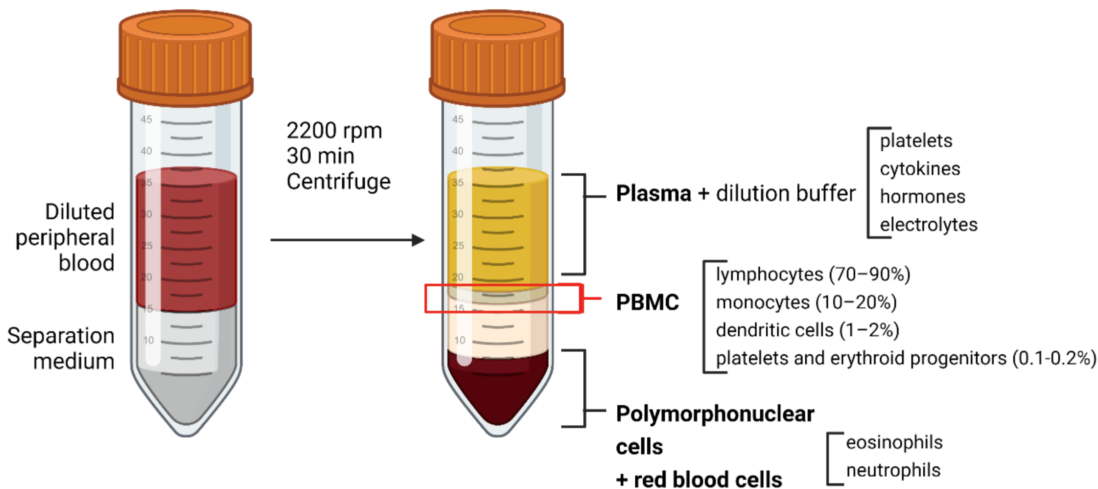


Figure 3. 1: Isolation of PBMCs.

Peripheral blood was layered over the Ficoll-Paque and, following centrifugation, the blood components were separated into plasma, lymphocytes, monocytes, platelets, and granulocytes, erythrocytes The PBMC pool was then extracted and centrifuged to remove platelets. The residual cells represent the PBMC population. Created with BioRender.com.

3.4.2 Purification of RNA from RNAprotect stabilised peripheral blood mononuclear cells

Total RNA was extracted from total PBMCs using the RNAeasy Extraction kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RNAprotect stabilised PBMCs were re-suspended by vortexing and then centrifuged for 5 minutes at 5000 x g, and the supernatants were completely removed by pipetting. After loosening the pellets, 350 µl or 600 µl Buffer RLT Plus, were added to the pellets for cell numbers less than 5 x 10^6 or 5 x 10^6 – 1 x 10^7, respectively. The pellets were dissolved completely by vortexing for 1 minute. The lysates were homogenised for 20 seconds and transferred to a genomic DNA eliminator spin column placed in a 2 ml collection tube. The samples were centrifuged for 30 seconds at 8000 x g until all liquid had passed through the membrane. Then one volume of 70% ethanol was added to the flow-throughs, according to the volume of the flow-through (the volume may be less than expected, due to loss during homogenisation and DNA removal). Up to 700 µl of the sample, including any precipitate that may have formed, were transferred to an RNeasy spin column, placed in a new collection tube, centrifuged for 15 seconds at 8000 x g, and the flow-through discarded. Next, 700 µl of buffer RW1 were added to the RNeasy spin column, centrifuged for 15 seconds at 8000 x g to wash the spin column membrane, and the flow-through was discarded. Five hundred µl of buffer RPE, were added to the RNeasy spin column, centrifuged for 15 seconds at 8000 x g, and the flow-through discarded. Finally, the spin column membrane was washed, and 500 µl of buffer RPE was centrifuged for 2 minutes at 8000 x g to dry the membrane and remove any residual ethanol. Next, the RNeasy spin column was placed in a new collection tube and centrifuged at full speed for 1 minute to eliminate any possible carryover of the buffer RPE or residual flow-through of the RNeasy spin column. Thirty µl of RNase-free water were added directly to the spin column membrane and centrifuged for 1 minute at 8000 x g to elute the DNA.

3.4.3 Quantitative and qualitative analysis of RNA from peripheral blood mononuclear cells

The RNA concentration was quantitated using the NanoDrop analyser. RNA quantitation and quality were determined using an Agilent 2100 Bioanalyser with the Agilent RNA

6000 Nano kit (Agilent, CA, United States), according to the manufacturer's manuals. Before starting, the assay equipment was set and the Bioanalyser electrodes were decontaminated using 350 µl of RNaseZAP solution and 350 µl of RNase-free water to avoid the degradation of RNA samples. After setting up the Chip Priming Station, the gel and the gel-dye mix were prepared, and 9 µl of the gel-dye mix, 5 µl of the Nano marker and 1 µl of the ladder were pipetted into the designated wells of the Chip Priming Station. Finally, 1 µl of each sample was pipetted into the corresponding well. The chip was then inserted in the Agilent 2100 Bioanalyser for reading, and the results were obtained starting the 2100 Expert Software. RNA integrity numbers ranged from 2 to 10 with an average score of 8.3 (numbers equal to or greater than 8, with 10 being excellent).

3.4.4 Acquisition of gene expression data

RNA libraries were created for RNA sequencing by oligo(dT) and fragmenting it by adaptive focused acoustic energy. A cDNA library of nucleotide sequences was built with millions of short DNA reads generated in a paired-end orientation. Sequencing was performed with the Illumina NovaSeq6000 sequencing platform (Novogene Co., Ltd., Beijing). The Illumina platform generates millions of relatively short sequences ("reads") representing fragments of the original RNA molecules. Data output in fastq file format contain information about sequences and quality (Figure 3.2). The recommended Phred quality score of 25 was used as a cut off and host sequences were identified by mapping against the human GRCh38 genome.

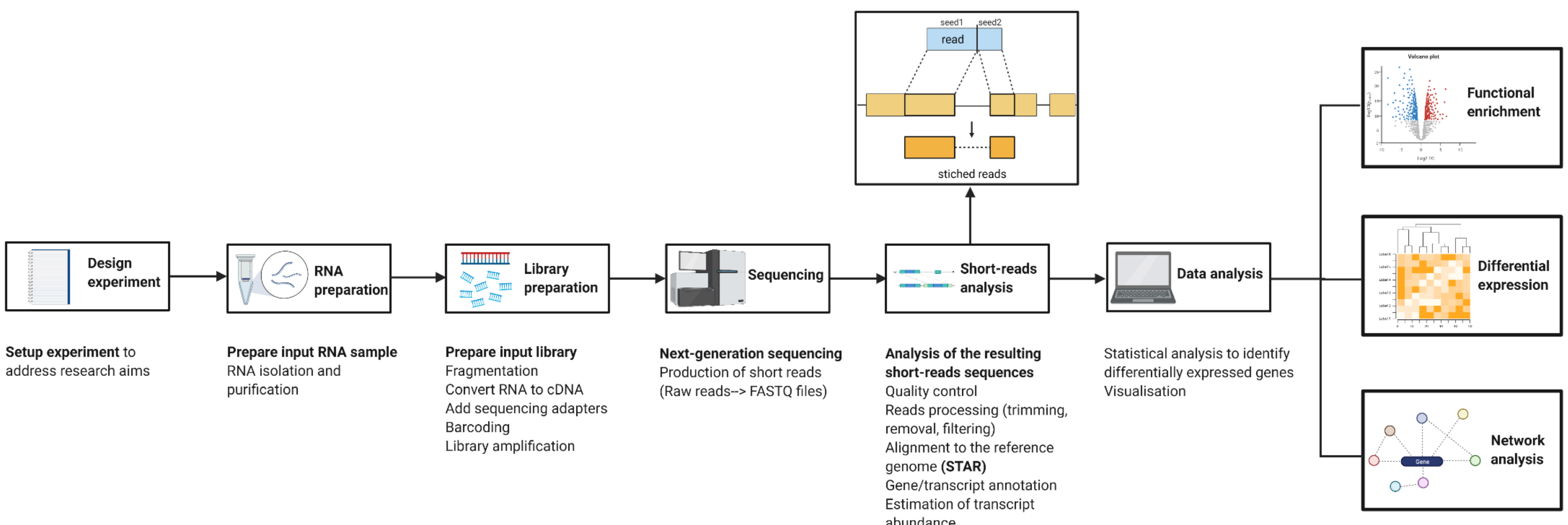


Figure 3. 2: The RNA-sequencing workflow.

RNA sequencing was a multi-step process reflecting the actively expressed sequences in PBMCs. This technique examined the quantity and sequences of RNA in PBMCs using Next-Generation Sequencing. Transcriptional patterns encoded within the RNA were analysed in different ways. Created with *Biorender.com*.

3.4.5 Statistical analysis of gene expression data

Mapping large sets of high-throughput sequencing reads to a reference genome is one of the foundational steps in RNA-sequencing data analysis. The Spliced Transcripts Alignment to a Reference (STAR) [536] software package was used to enable a highly accurate and ultra-fast alignment of RNA-sequencing reads to a reference genome (Human GRCh38). The reads were quality-filtered and trimmed using Trimmomatic. Dr Wayne Young carried out the sequence processing.

Statistical analyses were carried out to identify differentially expressed genes (DEGs) between controls and subjects with FGIDs using a negative binomial generalised log-linear model with a quasi-likelihood F-test, using the function "glmQLFit" provided in the edgeR package. This test is preferred to the likelihood ratio test as it reflects the uncertainty in estimating the negative binomial dispersion for each gene in the likelihood ratio hypothesis test framework and provides more robust and reliable error rate control. The edgeR package shows log2 fold change (FC) values by default. A two-group comparison with permutation analysis for differential expression of DEGs was performed to provide an estimate of the FDR for each transcript comparison. Transcripts with an FDR < 0.05 were considered significantly differentially expressed. The two-group comparisons were conducted for a given coefficient, indicating which comparisons to be tested (IBS-C, FC, IBS-D or FD vs control) to find genes different between any of the chosen groups).

The two-group comparisons with the quasi-likelihood F-test were performed comparing individual IBS subtypes (IBS-C or IBS-D) or functional subtypes (FC or FD) to controls, or comparing subjects grouped according to the bowel movement pattern subtypes (constipation phenotype: IBS-C and FC and diarrhoea phenotype: IBS-D and FD) to controls. Subjects with IBS-M and IBS-U were excluded from the statistical analyses to focus on the common and exclusive PBMC gene expression differences among subjects characterised by diarrhoea or constipation.

3.4.5.1 Functional enrichment analysis and protein-protein interaction network construction

The REACTOME pathway browser version 3.7 [537] was used to identify related gene groups based on their function and scores to explore the functional associations between DEGs likely to influence a mutual biological purpose. These databases also allowed the visual representation of biological pathways while making the source data available in a computationally accessible format. REACTOME cross-references information from numerous sources (e.g., NCBI, Ensembl, UniProt, KEGG, ChEBI, PubMed and Gene Ontology (GO)) [538], including experimental data, computational prediction methods and public text collections.

An over-representation analysis was performed, which is a statistical (hypergeometric distribution) test that determined whether certain pathways were over-represented (enriched) among the submitted DEGs. A binomial test produced a probability score, which answered the question “Does my list contain more proteins for pathway X than would be expected by chance?”. The probability score was corrected for FDR using the Benjamini-Hochberg method. The pathways were declared significant at FDR less than 0.05 and gene number higher or equal to 2 as recommended [539, 540].

3.5 Results

3.5.1 Diarrhoea predominant irritable bowel syndrome

In subjects with IBS-D, 28 genes were significantly differentially expressed (FC > 1.5, FDR< 0.05) in comparison to controls. Among these genes, 17 genes had increased expression levels and 11 genes had decreased expression levels (Figure 3.3 A). After DEGs with very low expression were removed, 10 genes were significantly differentially expressed, 6 genes had increased expression levels, and 4 genes had decreased expression levels (Table 3.1).

3.5.1.1 Functional enrichment analysis of DEGs using the REACTOME pathway database

Six out of 10 DEGs in PBMCs of subjects IBS-D were included in the REACTOME pathway analysis, revealing that 60 enriched pathways were linked to at least one of them (Table 3.2). Twenty-five pathways were significantly associated with DEGs (Table 3.2).

Two genes related to different immunoglobulin (Ig) variable domains (*IGLV3-27* and *IGLV2-18*) were associated with "cell-vascular wall interactions" and several pathways related to disease and immune activation in IBS-D (Table 3.2). These include "complement cascade", "binding and uptake of ligands by scavenger receptors" and "classical antibody-mediated complement activation" (Table 3.2). Moreover, *IGLV3-27* and *IGLV2-18* were also associated with "activation of the IgG Fc region receptor (FCGR)" and related inflammatory pathways ("phagocytosis" and "IL-10 synthesis") and enriched pathways related to signalling through the high-affinity IgE receptor (FCERI) (Table 3.2). These pathways included "NF-kB activation", "mitogen-activated protein kinase (MAPK) activation cascade activation" and "calcium mobilisation" (Table 3.2).

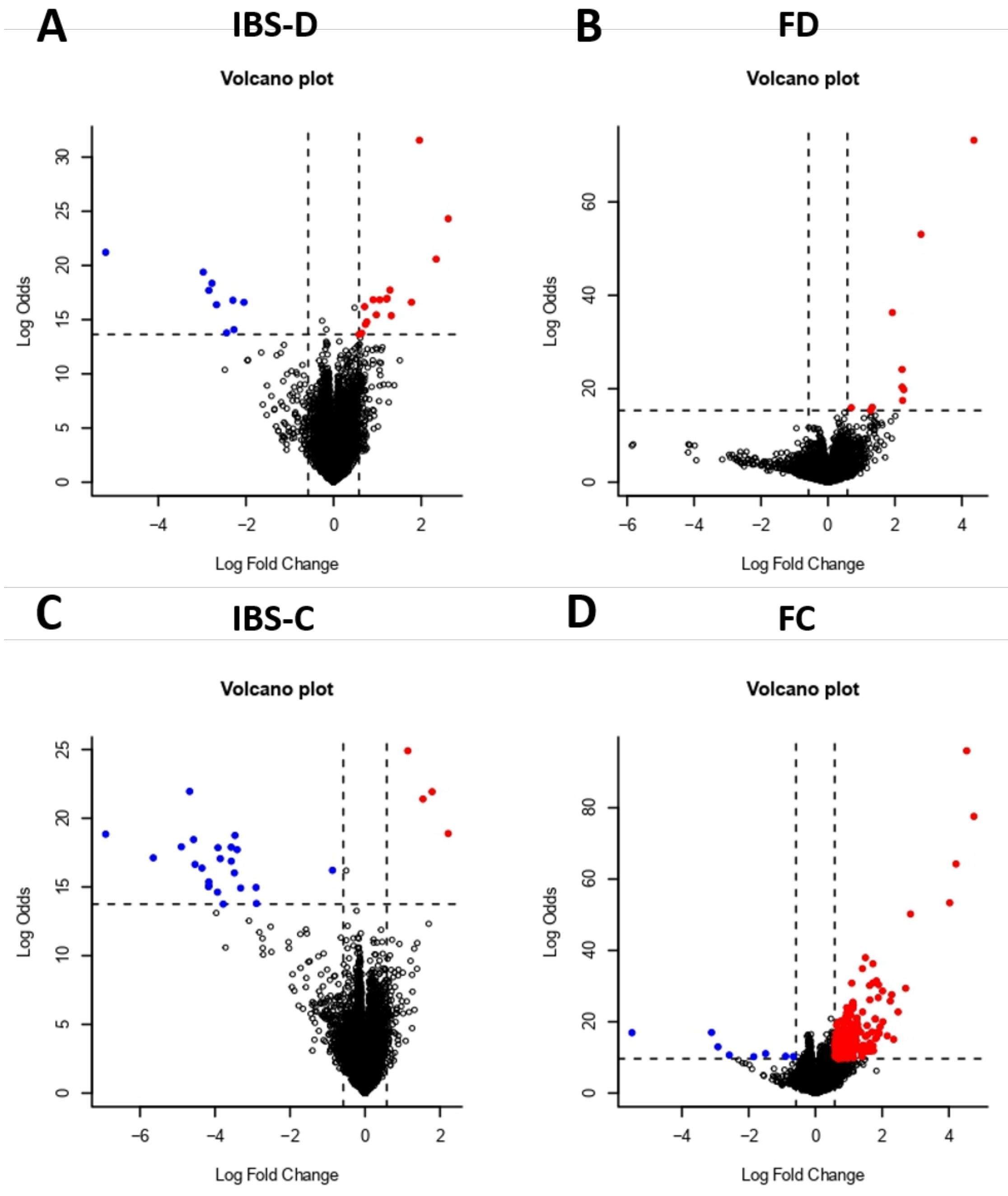


Figure 3. 3: Volcano plots of gene expression in PBMCs in subjects with IBS-D (A), FD (B), IBS-C (C) and FC (D) compared to controls.

The cut-off value for significantly DEGs was set at a log2 fold change (FC) value of |0.58|, which represents an FC of 1.5 (vertical lines). The colour red represents DEGs with increased expression levels, and the colour blue represents DEGs with decreased expression levels. The log odds (y-axes) refer to the negative logarithm of the FDR, represented as -log10(FDR). This transformation allows better visualisation of statistical significance, which was set at an FDR < 0.05 (horizontal line), with highly significant genes having lower FDR and, therefore, higher negative log odds values.

Table 3. 1: List of 10 DEGs with significantly increased or decreased expression (FDR< 0.05) in PBMCs of subjects with IBS-D, compared to controls, using a quasi-likelihood F-test.

Positive or negative log fold change (logFC) shows higher or lower expression levels, respectively. LogCPM (log counts per million) shows the expression level.

Ensembl	Gene name	Gene description	logFC	logCPM	FDR
ENSG00000211658	*IGLV3-27*	immunoglobulin lambda variable 3-27	1.96	1.16	5.63E-06
ENSG00000230202	*AL450405.1*	ribosomial protein L29 (RPL29) pseudogene	1.32	2.89	0.021
ENSG00000211664	*IGLV2-18*	immunoglobulin lambda variable 2-18	1.29	1.81	0.010
ENSG00000109272	*PF4V1*	platelet factor 4 variant 1	1.21	0.93	0.012
ENSG00000229769	*TRBV10-2*	T cell receptor beta variable 10-2	1.05	0.71	0.012
ENSG00000103740	*ACSBG1*	acyl-CoA synthetase bubblegum family member 1	0.48	1.10	0.014
ENSG00000052723	*SIKE1*	suppressor of IKBKE 1	-0.17	5.16	0.041
ENSG00000175087	*PDIK1L*	PDLIM1 interacting kinase 1 like	-0.26	3.72	0.028
ENSG00000241755	*IGKV1-9*	immunoglobulin kappa variable 1-9	-2.05	4.64	0.012
ENSG00000108379	*WNT3*	Wnt family member 3	-2.78	1.09	0.009

Table 3. 2: Pathways enriched from the network of DEGs in PBMCs from subjects with IBS-D, compared to controls, identified using the REACTOME database.

Pathway enrichment was determined with an over-representation analysis with a hypergeometric distribution test. The Benjamini-Hochberg FDR was significant at < 0.05. Entities found represents the number of mapped identifiers that match the pathway for the selected molecular type. Entities total represents the total number of identifiers in the pathway for the selected molecular type. The number of submitted entities does not always match those found because of the redundancy of gene names. Pathways involving only one gene were not considered.

Pathway name	**Submitted entities found**	**#Entities found**	**#Entities total**	**Entities FDR**
Cell surface interactions at the vascular wall	*IGLV2-18, IGLV3-27, PF4V1*	4	246	9.04E-04
Classical antibody-mediated complement activation	*IGLV2-18, IGLV3-27*	3	95	9.04E-04
Scavenging of haem from plasma	*IGLV2-18, IGLV3-27*	3	99	9.04E-04
FCGR activation	*IGLV2-18, IGLV3-27*	3	101	9.04E-04
Role of LAT2/NTAL/LAB on Ca^{+2} mobilisation	*IGLV2-18, IGLV3-27*	3	102	9.04E-04
Creation of C4 and C2 activators	*IGLV2-18, IGLV3-27*	3	103	9.04E-04
Initial triggering of complement	*IGLV2-18, IGLV3-27*	3	111	9.04E-04
Role of phospholipids in phagocytosis	*IGLV2-18, IGLV3-27*	3	114	9.04E-04
FCERI-mediated Ca^{+2} mobilisation	*IGLV2-18, IGLV3-27*	3	117	9.04E-04
FCERI-mediated MAPK activation	*IGLV2-18, IGLV3-27*	3	119	9.04E-04
FCGR3A-mediated IL-10 synthesis	*IGLV2-18, IGLV3-27*	3	128	9.04E-04
Binding and uptake of ligands by scavenger receptors	*IGLV2-18, IGLV3-27*	3	129	9.04E-04
Regulation of complement cascade	*IGLV2-18, IGLV3-27*	3	135	9.04E-04
Complement cascade	*IGLV2-18, IGLV3-27*	3	146	9.04E-04
FCGR3A-mediated phagocytosis	*IGLV2-18, IGLV3-27*	3	149	9.04E-04
Leishmania phagocytosis	*IGLV2-18, IGLV3-27*	3	149	9.04E-04
Parasite infection	*IGLV2-18, IGLV3-27*	3	149	9.04E-04
Regulation of actin dynamics for phagocytic cup formation	*IGLV2-18, IGLV3-27*	3	150	9.04E-04
FCERI-mediated NF-kB activation	*IGLV2-18, IGLV3-27*	3	167	9.04E-04
Anti-inflammatory response favouring Leishmania parasite infection	*IGLV2-18, IGLV3-27*	3	168	9.04E-04

FCGR-dependent phagocytosis	*IGLV2-18, IGLV3-27*	3	175	0.001018
FCERI signalling	*IGLV2-18, IGLV3-27*	3	218	0.001924
Immunoregulatory interactions between a lymphoid and a non-lymphoid cell	*IGLV2-18, IGLV3-27*	3	297	0.004669
Haemostasis	*IGLV2-18, IGLV3-27, PF4V1*	4	726	0.006939
Innate Immune System	*SIKE1, IGLV3-27, IGLV2-18*	4	1201	0.024242

3.5.2 Functional diarrhoea

In subjects with FD, 10 DEGs were significantly differentially expressed (FC > 1.5, FDR< 0.05) in comparison to controls, and all the genes had increased expression levels (Figure 3.3 B). After DEGs with low expression were removed, 9 genes were significantly differentially expressed. The DEGs that scored the lowest FDR value were mostly related to the different Ig variable domains (Table 3.3).

3.5.2.1 Functional enrichment analysis of DEGs using the REACTOME pathway database

Seven out of 9 DEGs in PBMCs of subjects with FD were included in the REACTOME pathway analysis, where 57 pathways were linked to at least one of them. Thirty-seven pathways were significantly associated with DEGs (Table 3.4).

A four-gene cluster related to the Ig variable domains (*IGKV2-30, IGLV3-27, IGKV1D-16* and *IGHV3-23*) was enriched in pathways related to the complement system, such as "regulation of complement cascade", "classical antibody-mediated complement activation", "creation of C4 and C2 activators" and "initial triggering of complement" (Table 3.4).

This cluster was also associated with "FCGR activation" and related inflammatory pathways ("phagocytosis" and "IL-10 synthesis") and enriched pathways related to signalling through the FCERI (Table 3.4). These pathways included "NF-kB activation", "MAPK cascade activation" and "calcium mobilisation" (Table 3.4).

In addition, *IGKV2-30, IGLV3-27, IGKV1D-16* and *IGHV3-23* were linked to bacterial and parasitic mechanisms of evasion of the macrophage-mediated immune response, such as "parasite infection" "anti-inflammatory response favouring Leishmania parasite infection" and "Leishmania phagocytosis" (Table 3.4). Finally, this cluster was enriched in pathways related to "B cell receptor (BCR) signalling" ("antigen activates BCR leading to generation of second messengers" and "CD22 mediated BCR regulation") and "haemostasis" (Table 3.4).

Table 3. 3: List of 9 DEGs with significantly increased or decreased levels (FDR< 0.05) in PBMCs from subjects with FD, compared to controls, using a quasi-likelihood F-test.

Positive or negative log fold change (logFC) indicates higher or lower expression levels, respectively. LogCPM (log counts per million) indicates the expression level.

Ensembl	**Gene name**	**Gene description**	**logFC**	**logCPM**	**FDR**
ENSG00000243238	*IGKV2-30*	immunoglobulin kappa variable 2-30	4.36	4.92	1.64E-18
ENSG00000211949	*IGHV3-23*	immunoglobulin heavy variable 3-23	2.78	6.09	9.48E-13
ENSG00000167034	*NKX3-1*	NK3 homeobox 1	1.92	1.00	6.93E-08
ENSG00000211658	*IGLV3-27*	immunoglobulin lambda variable 3-27	2.21	0.86	0.00024
ENSG00000241244	*IGKV1D-16*	immunoglobulin kappa variable 1D-16	2.22	0.86	0.003
ENSG00000106714	*CNTNAP3*	contactin associated protein family member 3	2.26	0.79	0.003
ENSG00000257335	*MGAM*	maltase-glucoamylase	1.32	4.92	0.031
ENSG00000196083	*IL1RAP*	interleukin 1 receptor accessory protein	0.70	4.42	0.031
ENSG00000073150	*PANX2*	pannexin 2	1.27	1.66	0.042

Table 3. 4: Pathways enriched from the network of DEGs in PBMCs from subjects with FD, compared controls, identified using the REACTOME database. Pathway enrichment was determined with an over-representation analysis with a hypergeometric distribution test. The Benjamini-Hochberg FDR was significant at < 0.05. Entities found represents the number of mapped identifiers that match the pathway for the selected molecular type. Entities total represents the total number of identifiers in the pathway for the selected molecular type. The number of submitted entities does not always match those found because of the redundancy of gene names. Pathways involving only 1 gene were not considered.

Pathway name	**Submitted entities found**	**#Entities found**	**#Entities total**	**Entities FDR**
Classical antibody-mediated complement activation	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	95	2.67E-07
Scavenging of haem from plasma	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	99	2.67E-07
FCGR activation	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	101	2.67E-07
Role of LAT2/NTAL/LAB on Ca^{+2} mobilisation	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	102	2.67E-07
Creation of C4 and C2 activators	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	103	2.67E-07
Initial triggering of complement	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	111	2.97E-07
Role of phospholipids in phagocytosis	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	114	2.97E-07
FCERI mediated Ca^{+2} mobilisation	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	117	2.97E-07
FCERI mediated MAPK activation	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	119	2.97E-07
FCGR3A-mediated IL10 synthesis	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	128	3.56E-07
Binding and uptake of ligands by scavenger receptors	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	129	3.70E-07
Regulation of complement cascade	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	135	3.70E-07
Complement cascade	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	146	4.52E-07
Leishmania phagocytosis	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	149	4.52E-07
FCGR3A-mediated phagocytosis	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	149	4.52E-07
Parasite infection	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	149	4.52E-07
Regulation of actin dynamics for phagocytic cup formation	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	150	4.67E-07
FCERI mediated NF-kB activation	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	167	7.93E-07
FCGR-dependent phagocytosis	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	175	8.42E-07
CD22 mediated B cell receptor regulation	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	4	70	8.42E-07
FCERI signalling	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	218	1.96E-06

Antigen activates B cell receptor leading to generation of second messengers	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	4	95	2.82E-06
Cell surface interactions at the vascular wall	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	246	3.54E-06
Leishmania parasite growth and survival	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	259	4.56E-06
Anti-inflammatory response favouring Leishmania parasite infection	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	259	4.56E-06
Immunoregulatory interactions between a lymphoid and a non-lymphoid cell	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	297	8.89E-06
Leishmania infection	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	345	1.60E-05
Potential therapeutics for SARS	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	4	164	1.60E-05
B cell receptor signalling	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	4	176	1.60E-05
Haemostasis	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	726	3.20E-04
Innate immune system	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23, MGAM*	6	1201	3.56E-04
Vesicle-mediated transport	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	762	4.02E-04
SARS-CoV Infections	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	4	536	0.001
Adaptive immune system	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	958	0.001
Immune system	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23, MGAM, IL1RAP*	7	2261	0.002
Infectious disease	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	1404	0.006
Disease	*IGKV2-30, IGLV3-27, IGKV1D-16, IGHV3-23*	5	2224	0.043

3.5.3 Constipation predominant irritable bowel syndrome

In subjects with IBS-C, 28 DEGs were significantly differentially expressed (FC > 1.5, FDR< 0.05) in comparison to controls. The majority of the DEGs (24 genes) had lower expression levels, while only four genes had higher expression levels in comparison to controls (Figure 3.3 C). After DEGs with low expression were removed, there were 10 genes significantly differentially expressed, and all had decreased expression levels (Table 3.5).

3.5.3.1 Functional enrichment analysis of DEGs using the REACTOME pathway database

Seven out of 10 DEGs were included in the REACTOME pathway analysis in IBS-C, revealing that 59 enriched pathways were linked to at least one. The genes *KDM5D* and *UTY* were the only cluster associated with the pathway "histone demethylases demethylating histones" significantly (Table 3.6).

Table 3. 5: List of 10 DEGs with significantly increased or decreased levels (FDR< 0.05) in PBMCs of subjects with IBS-C, compared to controls, using a quasi-likelihood F-test. Positive or negative log fold change (logFC) indicated higher or lower expression levels. LogCPM (log counts per million) indicated the expression level.

Ensembl	Gene name	Gene description	logFC	logCPM	FDR
ENSG00000122176	*FMOD*	fibromodulin	-6.92	1.96	0.0058
ENSG00000162896	*PIGR*	polymeric immunoglobulin receptor	-4.90	1.26	0.007
ENSG00000012817	*KDM5D*	lysine demethylase 5D	-4.53	3.66	0.011
ENSG00000099725	*PRKY*	protein kinase Y-linked (pseudogene)	-4.35	3.43	0.012
ENSG00000183878	*UTY*	ubiquitously transcribed tetratricopeptide repeat containing, Y-linked	-4.17	3.39	0.020
ENSG00000108379	*WNT3*	Wnt family member 3	-3.32	1.09	0.023
ENSG00000131002	*TXLNGY*	taxilin gamma pseudogene, Y-linked	-4.16	3.83	0.023
ENSG00000114374	*USP9Y*	ubiquitin specific peptidase 9 Y-linked	-4.17	3.88	0.023
ENSG00000067646	*ZFY*	zinc finger protein Y-linked	-3.93	3.12	0.027
ENSG00000198692	*EIF1AY*	eukaryotic translation initiation factor 1A Y-linked	-3.78	3.47	0.046

Table 3. 6: Pathways enriched from the uploaded network of DEGs in PBMCs from subjects with IBS-C, compared to controls, identified using the REACTOME database. Pathway enrichment was determined with an over-representation analysis with a hypergeometric distribution test. Benjamini-Hochberg FDR was significant at < 0.05. Entities found represents the number of mapped identifiers that match the pathway for the selected molecular type. Entities total represents the total number of identifiers in the pathway for the selected molecular type. The number of submitted entities does not always match those found because of the redundancy of gene names. Pathways involving only 1 gene were not considered.

Pathway name	Submitted entities found	#Entities found	#Entities total	Entities FDR
Histone demethylases demethylating histones	*KDM5D, UTY*	2	26	0.013471
Chromatin modifying enzymes	*KDM5D, UTY*	2	240	0.09274
Chromatin organisation	*KDM5D, UTY*	2	240	0.09274
Metabolism of proteins	*EIF1AY, USP9Y*	2	1950	0.517126
Signal transduction	*USP9Y, WNT3*	2	2599	0.688973

3.5.4 Functional constipation

In subjects with FC, 450 DEGs were significantly differentially expressed (FC > 1.5, FDR< 0.05) in comparison to controls (Appendix Table O). Among these genes, 337 genes had higher expression levels, and 113 genes had lower expression levels (Figure 3.3 D). After DEGs with low expression were removed, there were 347 genes significantly differentially expressed, 241 genes had increased expression levels, and 106 genes had decreased expression levels. Table 3.7 shows the 25 most significant DEGs in FC.

3.5.4.1 Functional enrichment analysis of DEGs using the REACTOME pathway database

Two hundred and seventy-five out of 347 DEGs were included in the REACTOME pathway analysis (Table 3.8). The analysis showed that 959 enriched pathways were linked to at least one DEG. Fourteen pathways were significantly enriched in the gene list (Table 3.8).

The biological pathways "IFN signalling" (FDR< 0.0001) and "IFN-α and -β signalling" (FDR< 0.0001) were enriched in the gene network in FC (Table 3.8). Other biological pathways enriched in the network included innate and cell-mediated immune responses, such as "cytokine signalling in the immune system" (FDR< 0.01) and "transcriptional regulation of granulopoiesis" (FDR< 0.01) (Table 3.8).

Hematopoietic processes associated with the network of upregulated genes included "megakaryocyte differentiation" and "platelet function by the transcription factor RUNX1" (FDR< 0.01) (Table 3.8). Megakaryocytic promoters regulated by the *RUNX1-CBFB* gene complex included other genes with increased expression levels in FC, such as *ITGA2B* (logFC = 1.12; FDR< 0.001), *GP1BA* (logFC = 0.71; FDR< 0.05) and *THBS1* (logFC = 1.55; FDR< 0.001). The *RUNX1-CBFB* gene complex also stimulates the transcription of the *PF4* gene (logFC = 0.92; FDR< 0.01) (Appendix Table O).

Many genes encoding for molecules contained in platelet α granules and platelet receptors involved in platelet activation had higher expression levels in subjects with FC or subjects with constipation (IBS-C + FC) compared to controls (Table 3.9). These include pro-inflammatory chemokines (*CXCL5, CXCL7* and *CCL2*), various coagulation factors from

the glycoprotein 1b-5-9 (*GP1B-GP5-GP9*) complex, acting as a ligand for the Von Willebrand factor (*VWF*) (Table 3.9). The expression level of the protease inhibitor *SERPING1*, involved in hematopoietic processes and the complement system, was increased two-fold in FC compared to controls (logFC = 1.72, FDR< 0.0001) (Table 3.7, 9).

A cluster of 9 genes was associated with the pathway "DNA damage/telomere stress induced senescence" (FDR< 0.05) (Table 3.8). This cluster included 6 upregulated histone genes (*HIST1H2BC, HIST1H2BJ, HIST1H2AC, HIST1H2BG, HIST2H2BE, HIST1H1C*), a cyclin-dependent kinase inhibitor (*CDKN1A*), and 2 downregulated genes, a histone chaperone gene (*ASF1A*) and a telomere specific gene (*TERF1*).

Table 3. 7: List of the 25 most significantly DEGs with increased or decreased levels (FDR< 0.05) in PBMCs from subjects with FC, compared to controls, using a quasi-likelihood F-test. Positive log fold change (logFC) indicated higher expression levels. LogCPM: log counts per million, indicating the expression level.

Ensembl	Gene name	Gene description	logFC	logCPM	FDR
ENSG00000165949	*IFI27*	interferon α inducible protein 27	4.75	1.85	3.95E-20
ENSG00000230021	*AL669831.3*	septin 14 (SEPT14) pseudogene	4.53	2.35	2.29E-25
ENSG00000108691	*CCL2*	C-C motif chemokine ligand 2	4.21	2.84	2.71E-16
ENSG00000251546	*IGKV1D-39*	immunoglobulin kappa variable 1D-39	2.85	3.16	2.73E-12
ENSG00000158578	*ALAS*	5'-aminolevulinate synthase 2	2.48	4.08	9.00E-05
ENSG00000143416	*SELENBP1*	selenium binding protein 1	2.02	1.59	4.12E-04
ENSG00000244734	*HBB*	haemoglobin subunit beta	1.91	11.68	2.04E-03
ENSG00000242371	*IGKV1-39*	immunoglobulin kappa variable 1-39	1.89	1.70	1.05E-06
ENSG00000004939	*SLC4A1*	solute carrier family 4 member 1	1.89	3.42	1.96E-03
ENSG00000109272	*PF4V1*	platelet factor 4 variant 1	1.88	1.12	9.11E-06
ENSG00000088827	*SIGLEC1*	sialic acid binding Ig like lectin 1	1.83	4.79	6.67E-07
ENSG00000167680	*SEMA6B*	semaphorin 6B	1.80	1.62	2.83E-04
ENSG00000134321	*RSAD2*	radical S-adenosyl methionine domain containing 2	1.72	4.88	8.49E-07
ENSG00000149131	*SERPING1*	serpin family G member 1	1.72	3.57	3.07E-08
ENSG00000058335	*RASGRF1*	Ras protein specific guanine nucleotide releasing factor 1	1.68	1.09	1.24E-02
ENSG00000206172	*HBA1*	haemoglobin subunit alpha 1	1.66	4.41	1.07E-02
ENSG00000115155	*OTOF*	otoferlin	1.63	0.82	1.33E-05
ENSG00000211950	*IGHV1-24*	immunoglobulin heavy variable 1-24	1.63	1.74	1.14E-06
ENSG00000188536	*HBA2*	haemoglobin subunit alpha 2	1.55	8.43	1.61E-02
ENSG00000137801	*THBS1*	thrombospondin 1	1.55	5.91	7.04E-04
ENSG00000224858	*RPL29P11*	ribosomal protein L29 pseudogene 11	1.54	1.06	2.09E-02
ENSG00000187608	*ISG15*	ISG15 ubiquitin like modifier	1.50	6.04	1.09E-08
ENSG00000119508	*NR4A3*	nuclear receptor subfamily 4 group A member 3	1.49	1.09	1.75E-02
ENSG00000137198	*GMPR*	guanosine monophosphate reductase	1.41	2.60	6.92E-08
ENSG00000133742	*CA1*	carbonic anhydrase 1	1.40	1.15	3.82E-02

Table 3. 8: Fourteen pathways enriched from the network of DEGs from subjects with FC, compared to controls, identified with the REACTOME database. Pathway enrichment was determined with an over-representation analysis with a hypergeometric distribution test. Benjamini-Hochberg FDR was significant at < 0.05. Entities found represents the number of mapped identifiers that match the pathway for the selected molecular type. Entities total represents the total number of identifiers in the pathway for the selected molecular type. The number of submitted entities does not always match those found because of the redundancy of gene names. Pathways involving only 1 gene were not considered.

Pathway name	Submitted entities found	#Entities found	#Entities total	Entities FDR
Interferon α/β signalling	*IRF7, USP18, ISG20, IFI35, ISG15, IFI27, RSAD2, XAF1, OAS3, OASL, MX1, IFITM3, IFIT2, IFI6, MX2, IFIT3, SOCS1, IFIT1*	34	190	1.21E-11
Interferon signalling	*IRF7, USP18, ISG20, IFI35, ISG15, IFI27, RSAD2, HERC5, XAF1, OAS3, OASL, MX1, IFITM3, IFIT2, IFI6, EIF2AK2, MT2A, MX2, IFIT3, TRIM58, KPNA3, SOCS1, IFIT1, PML*	43	397	3.06E-08
RUNX1 regulates genes involved in megakaryocyte differentiation and platelet function	*HIST1H2BC, HIST1H2AC, PF4, THBS1, ITGA2B, HIST1H2BJ, NR4A3, HIST1H2BG, GP1BA, MYL9, HIST2H2BE, HIST1H1C*	18	78	1.55E-07
Formation of fibrin clot (clotting cascade)	*PF4, PF4V1, SERPING1, VWF, THBD, GP1BA, PROS1, PPBP, GP9*	10	43	8.16E-04
Cytokine signalling in the immune system	*IRF7, USP18, ISG15, MAP3K7, XAF1, CA1, OAS3, RALA, IFITM3, IFIT2, IRS1, CXCL10, EIF2AK2, MT2A, SOCS1, PML, ISG20, IFI35, IGHG1, IFI27, RSAD2, TNFRSF12A, HERC5, BCL2L1, IL1RN, MX1, OASL, CSF1, IFI6, MX2, IFIT3, TRIM58, KPNA3, CCL2, IFIT1, TAL1, CDKN1A*	66	1107	0.002
Transcriptional regulation by RUNX1	*HIST1H2BC, HIST1H2AC, PF4, THBS1, ITGA2B, HIST1H2BJ, NR4A3, HIST1H2BG, GP1BA, MYL9, HIST2H2BE, HIST1H1C, CLDN5, TP73, RNF2, PML, TAL1*	24	261	0.003
Platelet activation, signalling and aggregation	*PF4, SERPING1, SELP, THBS1, ITGA2B, EGF, CLEC1B, GNG11, PROS1, ITGB3, ABHD6, GP9, GP6, LGALS3BP, VWF, MPIG6B, SPARC, CLU, GP1BA, PPBP, CDC37L1*	25	293	0.005
Platelet degranulation	*LGALS3BP, PF4, SERPING1, VWF, SELP, THBS1, ITGA2B, EGF, SPARC, CLU, PROS1, PPBP, ITGB3, CDC37L1*	16	141	0.005
Transcriptional regulation of granulopoiesis	*HIST1H2BC, HIST1H2BJ, HIST1H2AC, HIST1H2BG, E2F1, KLF5, HIST2H2BE, HIST1H1C, TAL1, PML, CDKN1A*	11	71	0.005
Response to elevated platelet cytosolic Ca^{2+}	*LGALS3BP, PF4, SERPING1, VWF, SELP, THBS1, ITGA2B, EGF, SPARC, CLU, PROS1, PPBP, ITGB3, CDC37L1*	16	148	0.007

Haemostasis	*PF4, SERPING1, SELP, THBS1, TUBB2A, ITGA2B, PRKAR2B, EGF, PF4V1, IGKV1D-39, CLEC1B, TUBB4B, GNG11, PROS1, ITGB3, ABHD6, GP9, EHD3, GP6, TUBB1, LGALS3BP, HBB, VWF, IGLV2-8, IGHV3-33, THBD, MPIG6B, IGKV1-39, SPARC, CLU, GP1BA, PPBP, ESAM, CDC37L1, HBD*	48	803	0.0135
DNA damage/telomere stress induced senescence	*HIST1H2BC, HIST1H2BJ, HIST1H2AC, ASF1A, HIST1H2BG, TERF1, HIST2H2BE, HIST1H1C, CDKN1A*	10	71	0.017
Antiviral mechanism by IFN-stimulated genes	*USP18, ISG15, EIF2AK2, HERC5, MX2, KPNA3, IFIT1, OAS3, OASL, MX1*	11	94	0.038
Prefoldin mediated transfer of substrate to CCT/TriC	*CCT4, TUBB1, VBP1, TUBB4B, CCT6A, TUBB2A*	6	29	0.040

Table 3. 9: Proteins and molecules in the platelet α granules and platelet receptors that DEGs with higher expression levels in PBMCs that discriminated subjects with FC or subjects with constipation (IBS-C + FC) from controls.

A positive log fold change (logFC) means higher expression levels.

Proteins and molecules and gene targets in the platelet α granule pathways				**FC vs Controls**		**IBS-C + FC vs Controls**	
Functions	**Secreted factor**	**Gene**	**Target cells**	**logFC**	**FDR**	**logFC**	**FDR**
Adhesion and signalling molecule	P-selectin	*SELP*	Polymorphonuclear cells, monocytes, lymphocytes	0.68	0.003	0.48	0.03
	Thrombospondin 1	*THBS1*	Macrophages, dendritic cells, Tregs	1.55	0.0007	1.10	0.01
	Von Willebrand factor	*VWF*	Macrophages, lymphocytes	0.64	0.040	-	-
	TREM like transcript-1	*TREML1*	Neutrophils, macrophages, endothelial cells	0.99	0.003	0.71	0.03
Chemokines	Platelet factor-4	*CXCL4 (PF4)*	Polymorphonuclear cells, monocytes, macrophages	0.92	0.007	0.71	0.0005
	Chemokine (C-X-C motif) ligand 7	*CXCL7 (PPBP)*	Polymorphonuclear cells	0.82	0.01	-	-
	Chemokine (C-X-C motif) ligand 5	*CXCL5*	Polymorphonuclear cells	1.14	0.002	0.99	0.002
	Chemokine (C-C motif) ligand 2	*CCL2*	Monocytes, T cells, B cells (40), natural killer lymphocytes, basophils, macrophages, dendritic cells, myeloid-derived suppressor cells, neutrophils	4.21	2.71E-16	3.40	1.58E-13
Platelets receptors				**FC vs Controls**		**IBS-C + FC vs Controls**	
Class	**Receptors (family)**	**Ligand**	**Function**	**logFC**	**FDR**	**logFC**	**FDR**
Glycoprotein	*GP1BA-GP9* complex	VWF, thrombin, P-selectin, αMβ2, Mac-1	Initiation of platelet recruitment	0.71 and 0.96	0.01 and 0.005	0.50 and -	- and 0.03
	GP6	Collagen, laminin	Platelet aggregation	0.76	0.02	0.55	-
C-type lectin-like	*CLEC1B*	Podoplanin, rhodocytin	Platelet aggregation	0.66	0.04	-	-
Tetraspanin	*PEAR1*	FCER1α	Aggregation, stabilisation of aggregates	0.90	0.003	0.67	0.02

3.5.5 Constipation predominant FGIDs and diarrhoea predominant FGIDs

When constipation predominant FGIDs (IBS-C and FC) or diarrhoea predominant FGIDs (IBS-D and FD) were compared to controls, many DEGs and associated biological pathways highlighted specific immune signatures related to these phenotypes (Figure 3.4).

In subjects with constipation predominant FGIDs (IBS-C and FC), the PBMCs RNA expression analysis revealed that 106 DEGs were significantly differentially expressed (FC> 1.5, FDR< 0.05) compared to controls: 93 genes had higher expression levels, and 13 genes had lower expression levels (Appendix Table P).

The functional enrichment analysis with the REACTOME database revealed that 12 pathways were significantly associated with DEGs (Table 3.10). Among these, pathways related to IFN α/β signalling, megakaryocyte differentiation and platelet activation, signalling, degranulation, and aggregation were associated with IFN-induced genes, IFN-regulatory factors and other genes involved in haemostasis (Figure 3.4 B, Table 3.10).

In subjects with diarrhoea predominant FGIDs (IBS-D and FD), the PBMCs RNA expression analysis revealed that 11 DEGs were significantly differentially expressed (FC> 1.5, FDR< 0.05) compared to controls. Six of these genes had higher expression levels, and 5 had lower expression levels (Appendix Table Q). The functional enrichment analysis with the REACTOME database revealed that 31 pathways were significantly associated with the DEG dataset (Table 3.10). A four-gene cluster related to the Ig variable domains (*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23*) was enriched in pathways related to the complement system, such as “complement cascade”, “classical antibody-mediated complement activation”, “creation of C4 and C2 activators” and “initial triggering of complement” (Table 3.10).

This cluster was also associated with “FCGR activation” and related inflammatory pathways (“phagocytosis” and “IL-10 synthesis”) and enriched pathways related to signalling through the FCERI. These pathways included “NF-kB activation”, “MAPK cascade activation”, and “calcium mobilisation” (Table 3.10). In addition, *IGKV2-30, IGLV2-18, IGLV3-27* and *IGHV3-23* were linked to pathways related to “B cell receptor (BCR) signalling” (“antigen activates BCR leading to generation of second messengers” and “CD22 mediated BCR regulation”), and “haemostasis” (Table 3.10).

The constipation and diarrhoea groups shared only 3 DEGs (*UBA1, IGKV1-9* and *PF4V1*) (Figure 3.4 A). “Haemostasis” and “Complement cascade” were the pathways enriched in the constipation and diarrhoea groups although they were associated with different DEG clusters (Table 3.10, Figure 3.4 B).

3.5.5.1 Gene sets with overlapping or differential expression

Potential markers differentiating between subtypes were identified to stratify the FGID phenotypes based on gene expression patterns. Venn diagrams showed DEGs that were commonly and exclusively different among FGIDs, when each subtype was compared to controls (Figure 3.5 A). No DEGs were shared when each FGID subtype was compared to controls (Figure 3.5 A).

Conversely, IBS-D and FD commonly showed a higher expression of the Ig variable domain *IGLV3-37* compared to controls (Figure 3.5 A). Higher expression levels of genes linked to B cell receptor signalling were specific to FD (*IGKV2-30, IGKV1D*-16 and *IGHV3-23*) (Table 3.4, Figure 3.5 B).

Increased expression levels of cytokine and chemokine signalling genes and IFN signalling genes (IFN α/β signalling and the antiviral mechanism by IFN-stimulated genes) (Table 3.8, Figure 3.5 A), genes involved in “platelet activation, signalling, aggregation, and degranulation”, “megakaryocyte differentiation” and genes linked to “DNA damage/telomere stress induced senescence” were specific to FC (Table 3.8, Figure 3.5 B). Different gene clusters of Ig variable domains were associated with pathways mediated by FcE and FcG receptors in IBS-D and FD but not in FC (Tables 3.2 and 4, Figure 3.5 B).

Overall, genes commonly differentially expressed across FGIDs included Ig variable domains (Table 3.11, Figure 3.5 B), and pathways commonly enriched across FGIDs were related to innate and adaptive immunity and complement system activation (Figure 3.5 B). Many DEGs across FGIDs encoding for novel transcripts, long noncoding RNA or pseudogenes were not included because they lacked functional annotations or were not mapped into pathways.

Table 3. 10: Pathways enriched from the network of DEGs from subjects with constipation predominant FGIDs (IBS-C and FC) and diarrhoea predominant FGIDs (IBS-D and FD), compared to controls.

Pathway enrichment was determined with an over-representation analysis with a hypergeometric distribution test. Benjamini-Hochberg FDR was significant at < 0.05. Entities found represents the number of mapped identifiers that match the pathway for the selected molecular type. Entities total represents the total number of identifiers in the pathway for the selected molecular type. The number of submitted entities does not always match those found because of the redundancy of gene names. Pathways involving only 1 gene were not considered.

Constipation predominant FGIDs (IBS-C and FC)				
Pathway name	**Submitted entities found**	**#Entities found**	**#Entities total**	**Entities FDR**
Interferon α/β signalling	*USP18, IFIT2, ISG15, IFI6, IFI27, RSAD2, IFIT3, OAS3, OASL*	17	190	2.84E-07
Interferon signalling	*USP18, IFIT2, ISG15, IFI6, IFI27, RSAD2, IFIT3, OAS3, OASL, MT2A, TRIM58*	21	397	1.26E-05
Platelet degranulation	*LGALS3BP, SPARC, CLU, PF4, SERPING1, SELP, THBS1, ITGB3, ITGA2B*	11	141	4.17E-04
Response to elevated platelet cytosolic Ca^{2+}	*LGALS3BP, SPARC, CLU, PF4, SERPING1, SELP, THBS1, ITGB3, ITGA2B*	11	148	4.42E-04
Haemostasis	*PF4, SERPING1, SELP, THBS1, ITGA2B, PF4V1, IGKV1D-39, ITGB3, GP9, EHD3, TUBB1, LGALS3BP, HBB, IGLV2-8, IGHV3-33, MPIG6B, IGKV1-39, SPARC, CLU, IGKV1D-12, ESAM*	27	803	4.42E-04
Platelet activation, signalling and aggregation	*LGALS3BP, PF4, SERPING1, SELP, MPIG6B, THBS1, ITGA2B, SPARC, CLU, ITGB3, GP9*	14	293	0.002
RUNX1 regulates genes involved in megakaryocyte differentiation and platelet function	*PF4, THBS1, ITGA2B*	7	78	0.005
Formation of fibrin clot (clotting cascade)	*PF4V1, PF4, SERPING1, GP9*	5	43	0.015
Regulation of complement cascade	*IGKV1D-12, CLU, IGKV1D-39, SERPING1, IGLV2-8, IGHV3-33, C1QC, IGKV1-39*	8	139	0.025
Cytokine signalling in immune system	*USP18, ISG15, IFI27, RSAD2, OAS3, OASL, CSF1, IFIT2, IFI6, IRS1, MT2A, IFIT3, TRIM58, CCL2, TAL1*	27	1107	0.044
Complement cascade	*IGKV1D-12, CLU, IGKV1D-39, SERPING1, IGLV2-8, IGHV3-33, C1QC, IGKV1-39*	8	156	0.044

Non-integrin membrane-ECM interactions	*ITGB5, AGRN, ITGB3, THBS1*	5	61	0.049
Diarrhoea predominant FGIDs (IBS-D and FD)				
Classical antibody-mediated complement activation	*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23*	5	97	5.22E-05
FCGR activation	*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23*	5	103	5.22E-05
Scavenging of haem from plasma	*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23*	5	106	5.22E-05
Role of LAT2/NTAL/LAB on Ca^{+2} mobilisation	*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23*	5	107	5.22E-05
Creation of C4 and C2 activators	*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23*	5	111	5.22E-05
Initial triggering of complement	*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23*	5	120	5.70E-05
FCERI mediated MAPK activation	*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23*	5	124	5.70E-05
Role of phospholipids in phagocytosis	*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23*	5	129	5.70E-05
FCERI mediated Ca^{+2} mobilisation	*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23*	5	129	5.70E-05
Regulation of complement cascade	*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23*	5	139	7.26E-05
FCGR3A-mediated IL10 synthesis	*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23*	5	141	7.78E-05
Cell surface interactions at the vascular wall	*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23, PF4V1*	6	257	8.13E-05
Complement cascade	*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23*	5	156	8.13E-05
FCGR3A-mediated phagocytosis	*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23*	5	157	8.13E-05
Leishmania phagocytosis	*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23*	5	157	8.13E-05
Parasite infection	*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23*	5	157	8.13E-05
Regulation of actin dynamics for phagocytic cup formation	*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23*	5	158	8.38E-05
Binding and uptake of ligands by scavenger receptors	*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23*	5	168	8.98E-05
FCERI mediated NF-kB activation	*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23*	5	175	1.09E-04
FCGR-dependent phagocytosis	*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23*	5	193	1.73E-04
FCERI signalling	*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23*	5	235	4.37E-04
Anti-inflammatory response favouring Leishmania parasite infection	*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23*	5	297	9.67E-04

Leishmania parasite growth and survival	*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23*	5	297	9.67E-04
Immunoregulatory interactions between a lymphoid and a non-lymphoid cell	*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23*	5	316	0.001
CD22 mediated BCR regulation	*IGKV2-30, IGLV3-27, IGHV3-23*	3	72	0.001
Haemostasis	*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23, PF4V1*	7	803	0.003
Antigen activates BCR leading to generation of second messengers	*IGKV2-30, IGLV3-27, IGHV3-23*	3	103	0.0037
Leishmania infection	*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23*	5	406	0.004
Potential therapeutics for SARS	*IGKV2-30, IGLV3-27, IGHV3-23*	3	168	0.009
Signalling by the BCR	*IGKV2-30, IGLV3-27, IGHV3-23*	3	189	0.013
Adaptive immune system	*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23, UBA1*	6	1012	0.031

Table 3. 11: Different Ig variable and constant domains whose gene expression levels in PBMCs were differentially expressed in subjects with IBS-D, IBS-C, FC and FD, or subjects with diarrhoea predominant FGIDs (IBS-D+FD) or constipation predominant FGIDs (IBS-C+FC) in comparison to controls. Differentially expressed Ig domains were identified by performing a two-group comparison with quasi-likelihood F-test.

Ig light or heavy variable domain	logFC	FDR	Condition
Light λ (*IGLV2-18*)	1.29	< 0.05	IBS-D
Light k (*IGKV1-9*)	-2.05	< 0.05	
Light κ (*IGKV2-30*)	4.36	< 0.0001	FD
Light κ (*IGKV1D-16*)	2.21	< 0.01	
Light λ (*IGLV3-27*)	2.21	< 0.001	
Heavy (*IGHV3-23*)	2.78	< 0.0001	
Light κ (*IGKV2-30*)	2.22	< 0.0001	IBS-D+FD
Light λ (*IGLV2-18*)	1.14	< 0.01	
Light λ (*IGLV3-27*)	2.01	< 0.0001	
Heavy (*IGHV3-23*)	1.21	< 0.001	
Light κ (*IGKV1D-39*)	2.85	< 0.0001	FC
Light κ (*IGKV1-39*)	1.89	< 0.0001	
Light λ (*IGLV2-8*)	0.94	< 0.01	
Heavy (*IGHV3-33*)	0.78	< 0.01	
Heavy (*IGHV1-24*)	1.62	< 0.0001	
Heavy (*IGHV1-69D*)	0.81	< 0.05	
Heavy (*IGHV6-1*)	0.73	< 0.05	
Light κ (*IGKV1D-39*)	2.16	< 0.0001	IBS-C+FC
Light κ (*IGKV1-39*)	1.33	< 0.001	
Light λ (*IGLV2-8*)	0.73	< 0.05	
Heavy (*IGHV3-33*)	0.60	< 0.05	
Heavy (*IGHV1-24*)	1.55	< 0.001	
Heavy (*IGHV6-1*)	0.70	< 0.05	

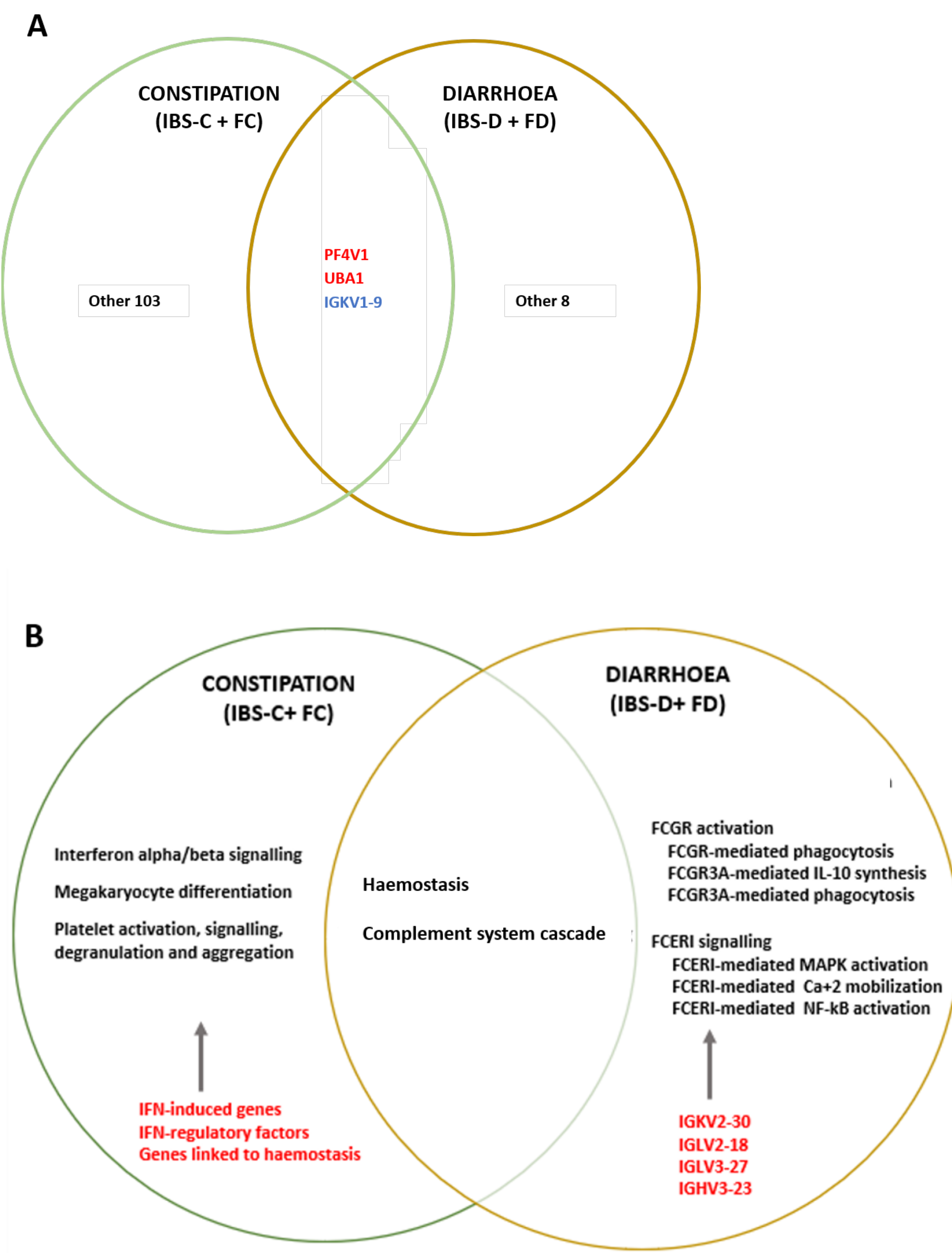


Figure 3. 4: Nineteen DEGs and associated enriched pathways in PBMCs discriminating among FGIDs compared to controls.

(**A**) The Venn diagram shows genes commonly differentially expressed among FGIDs when constipation predominant FGIDs (IBS-C and FC) and diarrhoea predominant FGIDs (IBS-D and FD) were compared to controls. The red colour represents genes with increased expression levels. The blue colour represents genes with lower expression levels. (**B**) Venn diagram shows the pathways enriched in the gene network, which were common and exclusively differentially expressed among FGIDs when constipation predominant FGIDs and diarrhoea predominant FGIDs were compared to controls. FCGR: IgG Fc region receptor; FcGR3A: low affinity IIIa receptor for the Fc Fragment of IgG; FCERI: IgE Fc region receptor.

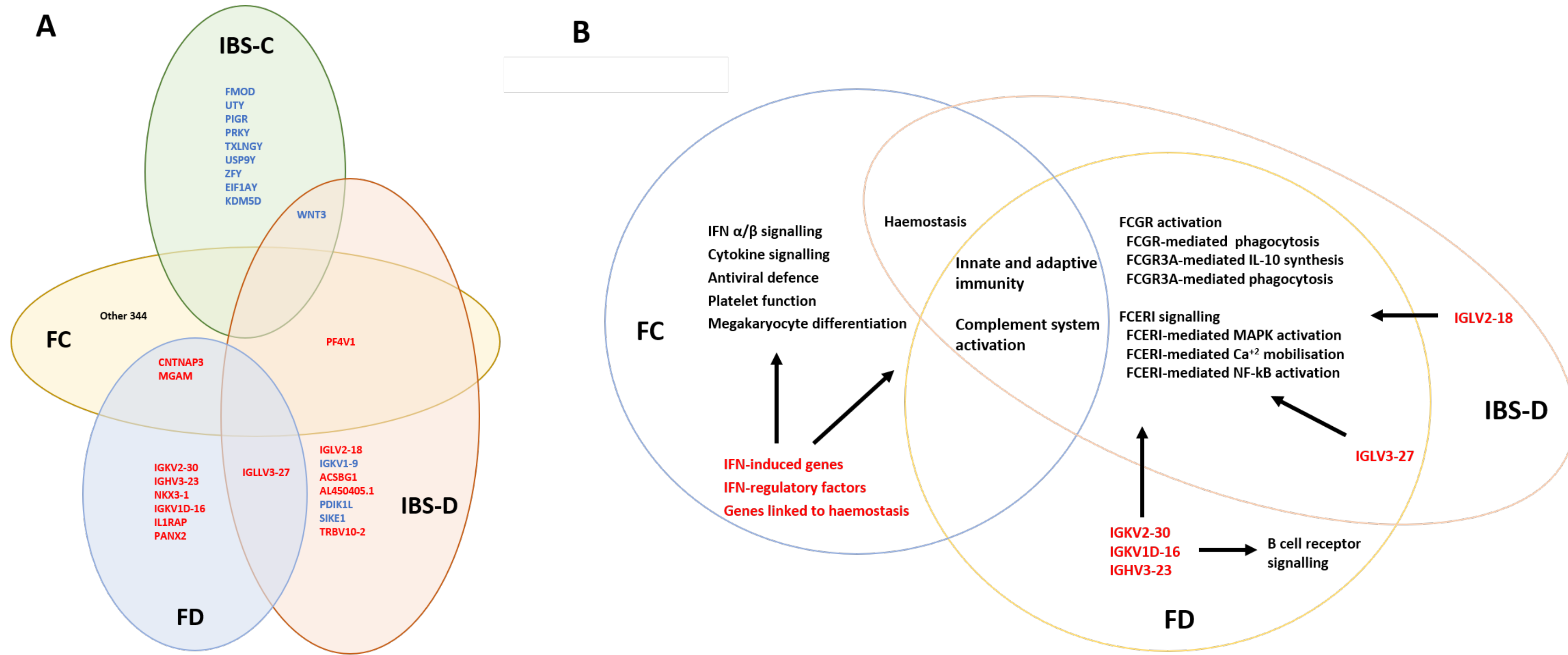


Figure 3. 5: Venn diagram of DEGs (A) and biological pathways enriched in the DEG network (B) of PBMCs discriminating among FGIDs from controls. In (A) the red colour represents genes with increased expression levels. The blue colour represents genes with lower expression levels. In (B) arrows indicate the biological processes enriched in the DEG network that are potentially affected by the changes in gene expression in each FGID subtype. IBS-C was not included in the Venn diagram, as it did not have enough DEGs to reach statistical significance.

3.6 Discussion

The analysis described in Chapter 3 identified for the first time many overlapping or uniquely DEGs in PBMCs among FGID subtypes, compared to controls, of the subjects recruited in the COMFORT cohort. These DEGs reflected changes in innate, humoral, adaptive immune, and haemostatic processes representative of phagocyte-mediated mechanisms. There were more DEGs in constipation predominant FGIDs (IBS-C and FC) compared to diarrhoea predominant FGIDs (IBS-D and FD).

3.6.1 The PBMC gene expression signature in diarrhoea and constipation

An increased PBMC gene expression of Ig variable domains, mostly Ig light chains, was detected in IBS-D, FD and FC, compared to controls. However, a corresponding increased gene expression of Ig heavy chains was not observed. Plasma cells normally produce some excess light chains that do not combine with heavy chains, referred to as free light chains [541].

Evidence of the relationship of free light chain RNA levels in PBMCs to disease activity has been reported in various inflammatory and autoimmune diseases (systemic lupus erythematosus [542], rheumatoid arthritis [543], atopic dermatitis [544], asthma [545], rhinitis [546], food allergy [547] and inflammatory bowel diseases [548]). Free light chains may have a higher binding affinity with monocytes [549] to support antigen presentation and mast cell degranulation hence promoting immune responses [550]. Nevertheless, the antigen-binding ability of free light chains has been considered non-specific, inducing self-reactive or unnecessary immunological responses [551].

Redegeld *et al*. demonstrated that free light chains could elicit immediate hypersensitivity responses in sensitised mice [552]. Other studies demonstrated that Ig free light chains could inhibit neutrophil apoptosis [553] and stimulate neutrophil activation *in vitro* [554], potentially contributing to chronic inflammation. However, free light chains may also function as anti-inflammatory molecules [555] or convert self-reactive antibodies into innocuous antibodies [556].

Finally, different types of Ig variable domains from the PBMC DEG network of subjects with diarrhoea predominant (*IGKV2-30, IGLV2-18, IGLV3-27, IGHV3-23*) or

constipation predominant (*IGHV3-33, IGKV1-39, IGKV1D-39, IGLV2-8*) FGIDs were associated with the pathway "complement system". The increased gene expression of Ig variable domains, particularly Ig light chains, across the FGID subtypes is a novel finding. The involvement of Ig variable domains with many immune processes, including antigen presentation, complement system triggering, mast cell degranulation and hypersensitivity responses may suggest a role for these proteins in immune pathophysiological mechanisms in FGIDs.

3.6.2 The PBMC gene expression signature in diarrhoea

Among the DEGs identified in diarrhoea predominant FGIDs (IBS-D+FD) compared to controls, Ig light chain genes were associated with pathways related to the activation of the complement system and signalling through IgG and IgE receptors, including molecular functions "phagocytosis", "IL-10 synthesis", "NF-kB activation", "MAPK cascade activation" and "Ca^{+2} mobilisation".

Circulating IgE and IgG can modulate *de novo* synthesis and degranulation of inflammatory mediators by mast cells and basophils, which express FCERI [557] and the low affinity IIIa receptor for the Fc Fragment of IgG (FcGR3A) on their surfaces [558]. Subjects with frequent and severe symptoms of IBS often report an increased prevalence of dietary allergies, food intolerance and atopic diseases [559, 560], which is inconsistently supported by increased serum IgE levels typical of these conditions or diseases [561]. Despite this, the immune response to food antigens might trigger the low-grade inflammation associated with IBS [247]. This hypothesis is supported by a clinical case of an individual with severe asthma and IBS where an anti-IgE monoclonal antibody therapy led to an almost complete resolution of IBS symptoms [562]. These findings suggest that IgE-mediated allergic processes might trigger IBS symptoms in predisposed subjects.

Furthermore, mast cells are involved in IgE receptor signalling, professional phagocytic cells in IgG receptor signalling, and FcGR3-expressing macrophages were shown to accumulate in inflamed colonic tissues. These observations suggest that these cell types may play a role in immune abnormalities at the subclinical level in subjects with diarrhoea

predominant FGIDs. However, additional single-cell RNA sequencing analyses may be required to confirm these observations.

3.6.3 The PBMC gene expression signature in constipation

Among the DEGs in constipation predominant FGIDs (IBS-C+FC) compared to controls, IFN-induced genes and IFN-regulatory factors were associated with IFN α/β signalling pathways. DEGs linked to haemostasis were enriched with pathways related to megakaryocyte differentiation, platelet activation, signalling, degranulation, and aggregation. However, when IBS-C and FC were individually compared to controls, the association between these pathways and the DEG network were attributable to FC rather than IBS-C. The IBS-C group did not include enough genes to reach statistical significance.

3.6.3.1 Complement system

Many genes encoding for components of the classical pathway of the complement system, such as the protease C1 inhibitor *SERPING1* and other proteins (*CLU, C1QB, C1QC, C2*), showed increased expression levels in FC subjects, compared to controls. In particular, the expression of *SERPING1* was increased twice as much as the controls. It was associated with the pathways “regulation of complement cascade“ and “platelet activation, signalling and aggregation”, supporting its pleiotropic role targeting all the coagulation, fibrinolytic and complement systems [563].

During inflammation, levels of SERPING1 protein in the blood rise approximately two-fold to moderate the response of the complement system [564]. *SERPING1* mainly regulates and prevents the spontaneous activation of the complement system, but it can also prevent the binding of bacterial endotoxins to macrophages and suppress leukocyte rolling and migration [565].

These findings suggest that the complement system may be in a potential state of subclinical activation in subjects with constipation predominant FGIDs, particularly in subjects with FC.

3.6.3.2 IFN α/β signalling

Many upregulated genes included in the pathway "IFN α/β signalling" were IFN-regulatory and IFN-stimulated factors, including *ISG15, IRF7*, *TNFRSF12A* and *CXCL10*. Increased gene expression of Ig variable domains, mostly Ig light chains, was observed in subjects with FC. Elevated free Ig light chain serum levels associated with IFN-dependent proteins (TNFRSF1A and CXCL10), were also reported in systemic autoimmune diseases [566].

IFN-α and IFN-β trigger a transcriptional cascade of IFN-stimulated genes, activating antiviral factors (*OASL, OAS3*). The expression of IFN-regulatory factors and IFN-stimulated genes usually increases following infection [567, 568], driving immunomodulatory, antiviral [569], antiproliferative [570], and antibacterial actions [568] in the GI tract [571] and the body [572].

In particular, *ISG15* has immunomodulatory functions, including neutrophil chemotaxis [573], recruitment of IL1β-producing CD8α+ dendritic cells [574], and release of IFN-γ [575] or IL-10 [576] from lymphocytes. Other studies [577] have shown an enhanced expression of *ISG15* mRNA and a dysregulated IFN α/β signalling in active inflammatory bowel diseases and murine colitis. An increased expression of IFN-γ, but not IFN α/β, was observed in the colonic mucosa of IBS subjects of mixed subtypes [215]. These observations in the literature support the findings obtained here and suggest that the constitutive and induced production of IFN α/β plays a crucial role in GI homeostasis and inflammation.

3.6.3.3 Neutrophils and phagocytes

Genes encoding for IFN-α/β-stimulated pro-inflammatory chemokines involved in neutrophil and monocyte recruitment (*CXCL4, CXCL4V1, CXCL5, CXCL7, CXCL10, CCL2, CCL8*), and cytokines secreted by neutrophils (colony-stimulating factor 1, *CSF1*) had increased expression levels in FC, in comparison to controls. In addition, genes involved in the transcriptional regulation of granulopoiesis (*HIST1H2BC, HIST1H2BJ, HIST1H2AC, HIST1H2BG, E2F1, KLF5, HIST2H2BE, HIST1H1C, TAL1, PML, CDKN1A*), for the production of neutrophils, eosinophils and basophils, had also increased expression levels in FC, in comparison to controls.

Increased expression levels of genes related to immune cell differentiation may increase circulating granulocytes and monocytes/macrophages in subjects with FC. This hypothesis agrees with the increased peripheral blood neutrophil to lymphocyte ratio reported in subjects with IBS-C [578] and colonic mast cell numbers in patients with severe FC [579].

Supporting this, treating adult mice with an antibody targeting the CSF1 receptor resulted in macrophage depletion and colonic dysmotility, either colonic hyperactivity or increased colonic transit time [580]. Another study demonstrated that the initial phase of monocyte/macrophage maturation was more advanced in subjects with IBS compared to controls, but they became more alike after LPS stimulation, suggesting that subjects with IBS may have a higher immune activation state [581].

These findings suggest that an increased gene expression of IFN-regulatory and IFN-stimulated factors in FC may affect the activity and the recruitment of neutrophils and monocytes/macrophages, suggesting a potential a role for these cell types in the pathophysiology of FGIDs.

3.6.3.4 Platelet function

The DEG network of PBMCs in FC, compared to controls showed increased expression levels of genes and over-representation of pathways related to megakaryocyte differentiation and haemostasis (platelet signalling, activation, aggregation, and degranulation). Many genes encoding for molecules contained in platelet α granules and surface markers of platelet activation had higher expression levels in FC compared to controls. Platelet activation genes included P-selectin (*SELP*), platelet factor 4 (*PF4*), glycoproteins (*LAMP-3, ITGA2B- ITGB3* complex) and glycoprotein receptors (*GP1BA-GP9* complex), and *VWF,* a ligand for various coagulation factors.

These results contradict a study reporting blood coagulation factor deficiency and hypo-coagulation in children with dysbiosis and FC [582]. A link between the VWF protein and inflammation has been confirmed in literature [583]: VWF can recruit leukocytes via direct leukocyte binding or by recruiting platelets to attract leukocytes [583]. When activated by TNF, IFN or bacterial LPS, platelets form aggregates with leukocytes creating bridges between leukocytes and endothelium, largely mediated by the protein P-

selectin [584, 585]. Qasim *et al*. showed increased surface expression of P-selectin, in mostly IBS females with the IBS-D subtype (90%) [586]. The same study observed an increased gene expression of platelet activation markers and reduced glycoprotein reactivity following platelet stimulation in IBS-D [586], likely due to chronic low-level platelet activation and degranulation. Other studies reported alteration of the platelet transcriptome in viral [587] and bacterial infections [588]. The role of platelets in FGIDs remains largely unknown [589].

3.6.4 Strengths and limitations

PBMCs provide a more representative cellular pool compared to *ex vivo* samples or human cell lines, which may have limitations in predicting *in vivo* responses. However, PBMC gene expression analysis does not identify the immune pathways affected in FGIDs because it is impossible to discriminate between the cause and the consequence.

The REACTOME database offers a valuable framework for categorising the complex biological processes of the immune system, providing categories for the interconnected network of various cells, molecules, and processes. However, like any classification system, it has limitations and can be considered oversimplified in some contexts. For example, GO categories are hierarchical, with broader categories encompassing more specific subcategories. While this hierarchy allows for a level of granularity, these categories may not adequately represent all the complex aspects of an immune response, and may not fully capture the dynamic nature of these interactions. Despite this, GO categories are valuable tools for summarising the intricacy of the immune system, and could be used as a starting point for deeper investigations. Although PBMCs provide a valuable insight into the immune status at the systemic level, they may not be entirely suitable for investigating low-grade inflammation in FGIDs. This is due to the fact that PBMCs may not fully reflect the potential colonic sub-clinical inflammation in FGID subjects, which could be hard to detect at the systemic level. Despite this, PBMCs can provide insights into the potential pathways affected and lay the groundwork for more mechanistic studies.

Transcriptomics rarely identify genes with large impacts on adaptive responses [590], as changes in RNA abundance are an unreliable indicator of protein activity [590],

particularly during cell differentiation or adaptation/response to specific stimuli. Several studies describe a moderate concordance of transcriptome and proteome [591-593], reflecting a temporal delay between processes. Moreover, long intergenic non-coding RNAs can affect post-transcriptional mRNA processing, protein translation regulation and post-translation modifications [594].

In addition, the transcriptome profile of PBMCs cannot be attributed to specific immune cell sub-populations because PBMCs are a heterogeneous population of myeloid and lymphoid cells [595]. Single-cell transcriptomics, or fluorescent activated cell-sorting of different PBMC immune cell subtypes prior to RNA sequencing would give more useful information on the role of specific immune populations in FGIDs.

Moreover, limited to one time point, this analysis did not allow comparing changes in temporal response in gene expression. Finally, except for FC, the DEG networks detected in IBS-D, IBS-C and FD included many DEGs with low abundance or small differential expression levels. This result may depend on the number and nature of DEGs in the gene set, the number of participants, and the type of sample (PBMCs). Even though DEGs with low abundance or small differential expression levels may be biologically relevant, results should be interpreted cautiously.

3.7 Conclusion

One of the major conclusions derived from the present gene expression of PBMCs of FGID subjects and control subjects is that increased Ig light chains and various innate, humoral, adaptive immune, and haemostatic processes discriminated levels of Ig light chains and various innate, humoral, adaptive immune, and haemostatic processes discriminated IBS-D, FD and FC from controls. Among these processes, there were pathways related to the activation of the complement system and the coagulation cascade. These pathways were, however, linked to different DEGs across the subtypes. Different gene clusters of Ig variable domains were associated with pathways mediated by IgE and IgG receptors, including "phagocytosis", "IL-10 synthesis", "NF-kB activation", and "MAPK cascade activation", in IBS-D and FD, but not in FC.

A higher number of DEGs characterised the immune signature of PBMCs in subjects with FC in comparison to the other FGID subtypes: increased expression levels of IFN-induced genes and those linked to the complement system and platelet functions. This finding suggested a distinctive immune signature in FC.

In summary, these findings provide potential evidence of subclinical immune abnormalities at the systemic level in subjects with FGIDs and, particularly, with FC.

Chapter 4

Correlations between faecal microbial composition and gene abundance, immune gene expression and symptoms of subjects with functional gastrointestinal disorders and healthy subjects

4.1 Abstract

The aetiology of functional gastrointestinal disorders (FGIDs), including irritable bowel syndrome (IBS) is multifactorial. There is increasing evidence that environmental factors, dysfunctional colonic microbiota, and immune dysregulation contribute to the pathophysiology of gastrointestinal (GI) symptoms, including pain and bloating associated with dysmotility and non-GI (psychological) symptoms. Chapter 4 aims to integrate GI and non-GI symptoms (reported in the thesis of former PhD student Dr Phoebe Heenan) with biological data (faecal microbiota composition and peripheral blood mononuclear cells (PBMCs) reported in Chapters 1 and 2, respectively) to provide insight into immune-microbial interactions specific for the constipation and diarrhoea phenotypes.

Shotgun metagenomic sequencing datasets of faecal microbial DNA, mature PBMC RNA, and GI and non-GI symptoms (Structured Assessment of Gastrointestinal Symptoms, Patient-Reported Outcomes Measurement Information System and Hospital Anxiety and Depression Scale) from 274 FGID subjects and controls (72 subjects with IBS constipation or functional constipation, 73 subjects with IBS diarrhoea or functional diarrhoea and 129 controls) were correlated using canonical correlation analysis and integrative analysis with Data Integration Analysis for Biomarker discovery using Latent cOmponents (DIABLO). The receiver operating characteristic curve (ROC) and area under the curve (AUC) analyses were performed to evaluate the predictor performance of the classification model of FGIDs. Three "biomarker" panels including selected variables from each dataset were computationally generated to maximise the value of the correlations.

Symptoms from the constipation phenotype, including epigastric pain, constipation, abdominal pain, and bloating, were strongly positively associated ($0.84 > r > 0.74$) with the *Oscillospiraceae* family and negatively associated with the PBMC gene *RPS4Y* (a Y-chromosome–encoded minor histocompatibility antigen). The diarrhoea phenotype was strongly positively associated ($0.8 > r > 0.62$) with the *Peptostreptococcaceae* family and PBMC genes involved in neutrophil degranulation and cellular apoptosis. The ROC and AUC analyses showed that GI and non-GI symptoms remain the best way to discriminate among FGIDs or controls than PBMC genes or microbial taxa except for FC, which was best discriminated from other FGIDs or controls by PBMC genes.

4.2 Introduction

The aetiology of functional gastrointestinal disorders (FGIDs) is multifactorial. Several environmental factors, psychosocial stressors, abnormal colonic motility and visceral hypersensitivity, dysregulation of the immune function and microbiota-immune dysfunctions contribute to FGID pathophysiology [582, 596, 597]. An imbalance between putative harmful and protective bacterial species may promote chronic colonic inflammation [521], which seems to be involved in most mechanisms underlying symptom generation in FGIDs, including visceral hypersensitivity [150], abdominal pain [151] and increased colonic permeability [137].

Adverse food reactions may also play a key role in FGIDs, as up to 65% of subjects with irritable bowel syndrome (IBS) report that their symptoms worsen after ingesting specific foods [598, 599]. Previous studies suggested that postprandial symptoms in IBS may depend on the presence of immunoglobulin (Ig)G and IgE [239, 247], indicating that immune factors may contribute to the generation or worsening of gastrointestinal (GI) symptoms. This observation was confirmed by other studies investigating the association between colonic transit, inflammation, visceral hypersensitivity, or faecal concentrations of inflammation biomarkers like granins [600], calprotectin [601] or mucosal mast cells [226]. In addition, alterations in the colonic microbiota reflect the host lifestyle and diet and affect blood levels of biomarkers in FGIDs.

Symptom-based criteria remain the best way to diagnose and discriminate between FGID subtypes or between FGIDs and controls [13]. Few studies investigated the potential associations between the faecal microbiota composition (proxy of colonic microbiota), GI and non-GI symptoms [67], 709] or peripheral blood mononuclear cell (PBMC) gene expression in FGIDs [224, 408, 602], and commonly used approaches consider each biological dataset independently.

The '**C**hristchurch IBS c**o**hort to investigate **m**echanisms **fo**r gut **r**elief, and improved **t**ransit' (COMFORT) cohort was designed to characterise subjects with FGIDs and healthy subjects using demographic, dietary, GI symptom, non-GI symptom (psychological), biological (faecal microbiota composition and gene abundance, plasma/faecal metabolites), and peripheral blood mononuclear cell (PBMC) gene expression data. Integrating datasets from different sources using a systems biology

approach provides an opportunity to identify relationships between them and improve the understanding of the mechanisms potentially underlying the FGID phenotype. This approach has been used to understand better several diseases, such as inflammatory bowel diseases [603], fibromyalgia [604], and Huntington's disease [605], but seldomly used for FGIDs. One study used an integrative approach between *omics* data types and clinical features to predict IBS symptom improvement and exacerbation in 60 female subjects, based on a multi-modal neuro-psychosocial signature [606]. Results showed that after 3 and 12 months, subjects without symptom improvement paid more attention to painful visceral stimuli for longer.

The analysis reported in Chapter 4 aimed to correlate symptom severity reported in the thesis of former PhD student Dr Phoebe Heenan, faecal microbiota composition, gene abundances and PBMC gene expression reported in Chapters 1 and 2, respectively, and integrate them to provide an insight into immune-microbe interactions in FGID subjects and controls. The integrative analysis did not include the microbial gene abundance dataset to keep data visualisation manageable. Diet and metabolite datasets were also not used in this analysis as they were part of former PhD students Dr Phoebe Heenan (diet) and Dr Shanalee James (metabolites). My contribution was to conduct correlative and integrative analyses, interpret the findings and write the research chapter.

4.3 Hypotheses and Aims

The primary hypothesis of the research presented in this Chapter is the integrative analysis of a combination of computationally selected variables from different datasets (faecal microbiota composition, PBMC transcriptome and GI and non-GI symptoms) from subjects with constipation and diarrhoea predominant FGIDs and controls identify potential relationships between these datasets.

A secondary hypothesis is that a combination of computationally selected variables from different datasets (faecal microbiota composition, PBMC transcriptome and GI and non-GI symptoms) have good diagnostic power to discriminate within FGIDs or FGIDs from controls.

Faecal microbiota taxonomic composition and PBMC transcriptome datasets were chosen as a proxy of the microbiota in the colonic lumen and the immune interaction networks and integrated with the GI and non-GI symptom dataset as a proxy of the phenotype of FGID and control subjects. Identifying potential relationships between variables from datasets of different origins may provide insight into the immune-microbiota interactions underlying the pathophysiology of FGIDs.

A receiver operating characteristic (ROC) curve and area under the curve (AUC) were calculated for selected variables from each dataset (faecal microbiota composition, PBMC transcriptome and GI and non-GI symptoms) using one-vs-all comparisons to evaluate their ability to discriminate FGIDs within subtypes or from controls.

The symptom-based categorisation of FGID subjects was performed using the Structured Assessment of Gastrointestinal Symptoms (SAGIS), the Patient-Reported Outcomes Measurement Information System (PROMIS) and Hospital Anxiety and Depression Scale (HADS) questionnaires. These datasets were reported in Dr Heenan's PhD thesis [424]. All analyses were performed regardless of the digestive health status of the participants.

4.4 Materials and Methods

4.4.1 Study population

The recruitment process, participant eligibility and characteristics are described in Chapter 2, section 4.1.

4.4.1.1 Questionnaires

The participants were asked to complete weekly questionnaires, regarding their general, physical and psychological health, as described in Phoebe Heenan's published paper [424] and summarised in Table 4.1. The Modified Hunter New England Survey included Rome IV questionnaires [607] to classify participants as controls, cases, and sub-phenotypes. HADS was used to evaluating their psychological status [608]. In addition, SAGIS [609] evaluated the severity of GI symptoms, and PROMIS [610] evaluated GI symptoms and emotional distress. The questionnaires were labelled and posted to each participant. The participants completed the SAGIS questionnaire on day 1 and the Modified Hunter New England Survey and PROMIS questionnaire on day 3. On day 4 of the study, the participants collected faecal and urine samples at home and then met with the study team to collect blood and breath samples (colonic tissue biopsies were obtained from a subset of participants). Days 1-4 were consecutive days.

Table 4. 1: Schedule of COMFORT cohort variables and visits related to the current study.

<table>
<tr><th></th><th></th><th>Pre-enrolment</th><th>Day 1</th><th>Day 2</th><th>Day 3</th><th>Day 4</th><th>Colonoscopy</th></tr>
<tr><td rowspan="3">Recruitment</td><td>Advertisements</td><td>x</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Referral to Gastroenterologist</td><td>x</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Written informed consent</td><td>x</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td rowspan="3">Modified Hunter New England Survey</td><td>Rome IV</td><td rowspan="3"></td><td rowspan="3">x</td><td rowspan="3"></td><td rowspan="3"></td><td rowspan="3"></td><td rowspan="3"></td></tr>
<tr><td>Mental and physical quality of life</td></tr>
<tr><td>Medical history</td></tr>
</table>

	Hospital Anxiety and Depression Scale						
	Demographics						
SAGIS questionnaire (GI symptom severity)			x				
PROMIS questionnaire (GI and emotional distress)					x		

4.4.2 Dataset integration analysis for biomarker discovery using latent components

Three different datasets (faecal microbiota composition, PBMC transcriptome and GI and non-GI symptoms) were chosen for integration. The faecal microbiota taxonomic composition dataset obtained in Chapter 2, was chosen as a proxy of the microbiota in the colonic lumen; the PBMC transcriptome dataset obtained in Chapter 3, as a proxy of the immune interactions; the GI and non-GI symptom dataset, obtained from Dr Heenan's PhD thesis [424], as a proxy of the phenotype of FGID subjects and controls. The integrative analysis did not include the microbial gene abundance dataset to keep data visualisation manageable. Diet and metabolite datasets were also not used in this analysis as they were part of former PhD students Dr Phoebe Heenan (diet) and Dr Shanalee James (metabolites).

The data were normalised across samples to minimise redundancy (duplicate data), and samples from participants that did not have PBMC, symptoms or microbial data were removed. The microbiota composition, PBMC gene expression and symptoms datasets, regardless of the digestive health status of the participants, were integrated with the R package 'mixOmics' (Version 4.0.4) (Figure 4.1), using **D**ata **I**ntegration **A**nalysis for **B**iomarker discovery using **L**atent c**O**mponents (DIABLO) [611]. The main output from DIABLO is a set of Panels, including a list of selected variables from each nominated dataset associated with each panel using a component-based integrative tuning process to maximise the correlation or covariance between variables to form a specific panel.

DIABLO was used to explore correlations between microbiota composition, PBMC gene expression and GI and non-GI symptom datasets, using highly correlated parameters to best discriminate within constipation predominant and diarrhoea predominant subtypes

and from controls. The optimal number of variables from each dataset was selected using the tune.block.splsda' function, to maximise the correlation or covariance between variables.

The Circos plot was used to visualise correlations between different datasets within each panel's variables. The splsda function does not generate probability values for the canonical correlation analysis, so the r values provide only information on the strength of the correlation, not the significance. The cut-off was set according to the number of correlations displayed in the Circos plot, starting from the more stringent (0.7, "strong" correlation) cut-off. If the Circos plot did not display strong correlations, then the level of stringency was decreased to 0.5 ("moderate" correlation) but not lower. The PlotLoadings function was used to visualise the loading weights of each selected variable in each panel.

An over-representation analysis was performed (as described in Chapter 3) to investigate whether certain biological pathways were over-represented (enriched) in the list of PBMC genes selected by the DIABLO tuning process and associated with each panel. The probability score was corrected for FDR using the Benjamini-Hochberg method. The pathways were declared significant at FDR less than 0.05 and gene number superior or equal to 2 as recommended [539, 540].

4.4.3 Predictive performance of classification model of functional gastrointestinal disorders

The ROC and AUC were calculated using one-vs-all comparisons to evaluate the predictor performance of a classification model to distinguish between FGIDs and controls or within FGID subtypes (Figure 4.1). The one-vs-all comparisons method evaluates multi-groups models by comparing each group against all the others simultaneously. This process reduces the multi-group classification output into a binary classification.

The ROC curve is a probability curve summarising true and false positive rates. A ROC AUC plot for each dataset (using the function 'auroc') indicates the ability of the model to distinguish between FGIDs and controls or within FGID subtypes. The AUC values of 0.5 or less correspond to classification no better than chance, between 0.6 to 0.7

correspond to a poor classification, between 0.7 to 0.8 correspond to a fair classification, between 0.8 to 0.9 correspond to a good classification, and 1.0 correspond to perfect classification.

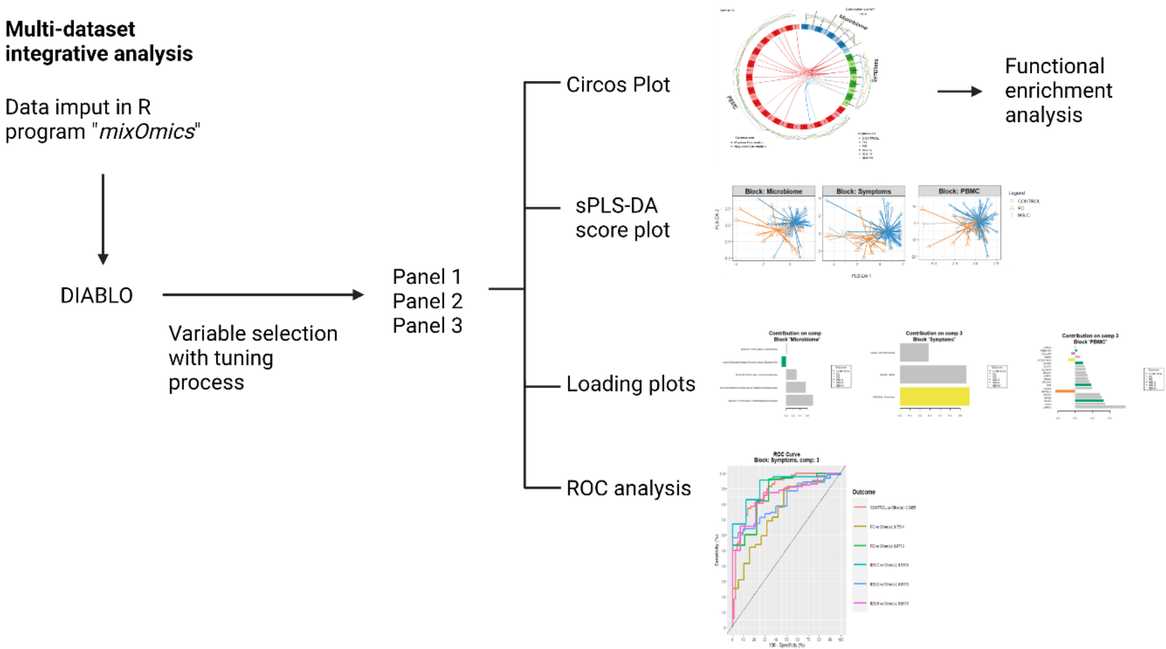


Figure 4. 1: Correlation and integrative analysis workflow of multi-datasets.

The R package 'mixOmics' was used to conduct statistical analyses and visualise data. Different visualisation tools were used to display DIABLO results and to assess relationships between the selected variables. The CircosPlot displayed the variables from different datasets selected by DIABLO on a circle, with links between datasets, indicating strong positive or negative correlations. The sparse PLS-DA score plots combining the variables for each dataset from each panel were plotted to identify which variables better discriminate within FC, IBS-C and control groups or FD, IBS-D and control groups. Loading plots visualised the loading weights of each selected variable for each panel. An over-representation analysis was performed to investigate whether certain biological pathways were over-represented in the list of PBMC genes selected by the tuning process and associated with microbial or symptomatic variables. Finally, the receiver operating characteristic (ROC) curve and area under the curve (AUC) were calculated using one-vs-all comparisons to evaluate the predictor performance of the classification model of FGIDs. Created with *Biorender.com*.

4.5 Results

4.5.1 N-integration across multiple data sets with data integration analysis for biomarker discovery using latent components

4.5.1.1 Projection to Latent Structures models and variable selection

The tuning process in DIABLO generated three 'biomarker' Panels (Table 4.2). Panel 1 included three selected microbial components, 10 selected PBMC genes, and five selected symptoms. Panel 2 included 50 selected microbial components, 100 selected PBMC genes, and five selected symptoms. Panel 3 included five selected microbial components, 20 selected PBMC genes were, and three selected symptoms. The selected variables by DIABLO in the tuning process were integrated and presented as Circos plots.

4.5.1.2 Dataset integration

The Circos plot diagrams represent the variables from different datasets with the strongest correlations (Figures 4.2 A and B). The Circos plot in Figure 4.2 A showed the variables from Panel 1 and 3 combined selected by DIABLO, which scored the strongest correlations (r> 0.6).

The *Oscillospirillaceae* family had the strongest negative correlation with the PBMC gene *RPS4Y1* (r= -0.83) and the strongest positive correlations with SAGIS_Constipation (r= 0.84) or SAGIS_Epigastric pain (r= 0.83). The PBMC gene *RPS4Y1* was also very strongly negatively correlated with SAGIS_Epigastric pain (r= -0.81) (Figures 4.2 A and C). The *Oscillospirillaceae* family was positively correlated with PROMIS_Constipation (r= 0.74), PROMIS_Belly pain (r= 0.76) and PROMIS_Bloating symptoms (r= 0.74) (Figure 4.2 A) (Appendix Table R).

The diarrhoea symptom (PROMIS_Diarrhoea and SAGIS_IBS-D) strongly correlated to 10 PBMC genes (Figures 4.2 A). Five genes were correlated to PROMIS_Diarrhoea only (*APAF1*, r= 0.73; *SIRPA*, r= 0.73; *LILRB3*, r= 0.73; *SLC6A6*, r= 0.71; *MEGF9*, r= 0.74), and 5 PBMC genes were correlated to both PROMIS_Diarrhoea (*LRRK2*, r= 0.80; *MNDA*, r= 0.77; *IGSF6,* r= 0.78; *TMEM154*, r= 0.73; *ACSL1,* r= 0.67) and SAGIS_IBS-

D (*LRRK2*, r= 0.76; *MNDA*, r= 0.73; *IGSF6,* r= 0.74; *TMEM154*, r= 0.72; *ACSL1,* r= 0.67) (Figure 4.2 A) (Appendix Table R).

The *Peptostreptococcaceae* family showed a positive correlation with diarrhoea (PROMIS_Diarrhoea) (r= 0.62) and a cluster of PBMC genes (*LRRK2*, r= 0.66; *APAF1*, r= 0.57; *SIRPA*, r= 0.56; *MNDA*, r= 0.59; *LILRB3*, r= 0.58; *SLC6A6*, r= 0.63; *MEGF9*, r= 0.59; *IGSF6*, r= 0.59; *LYST*, r= 0.58; *SULF2*, r= 0.54 and *TMEM154* r= 0.51). *LRRK2* was the PBMC gene that scored the strongest positive correlations with PROMIS_Diarrhoea (r= 0.80) and the *Peptostreptococcaceae* family (r= 0.66) (Figure 4.2 A) (Appendix Table R).

The functional enrichment analysis was performed to investigate whether certain biological pathways were over-represented in the list of PBMC genes selected by the tuning process and associated with the *Peptostreptococcaceae* family and the diarrhoea predominant FGIDs (Table 4.3). This analysis with the REACTOME pathway browser showed that some PBMC genes were linked to the pathway “innate immune system” (*APAF1, SIRPA, MNDA, LILRB3*; FDR= 0.008), “neutrophil degranulation” (*APAF1, SIRPA, MNDA, LILRB3*; FDR= 0.0003), and “apoptosis” (*APAF1*) (Table 4.3).

No strong correlations (r=0.7) were identified in the Circos plot using Panel 2 variables. However, when the cut-off was set at r= 0.5, the Circos plot showed a moderate positive association between PROMIS_Constipation and the PBMC genes *PNISR* (r= 0.60), *ARGLU1* (r= 0.52), *CCNL1* (r= 0.63) and *SRRM2* (r= 0.66) (Figure 4.2 B; Appendix Table R). These genes also showed a moderate positive association with microbial taxa from the Terrabacteria group (Table 4.4) and the Actinobacteria phylum (Coriobacteriia class and Coriobacteriales order) (Table 4.4).

4.5.1.3 Loading plots

The contribution of each selected variable for Panels 1, 2 and 3 is represented in a pyramid bar plot in increasing order of importance (according to the absolute value of their coefficients) from bottom to top (Figures 4.3 A, B, C). Each bar length corresponds to the loading weight (contribution) of the variable, and the colour corresponds to the group in which the feature is contributing the most (Figures 4.3 A, B, C). This graphical output

shows better the molecular signature of FGID subtypes. Only the variables with the highest loading weight are described in the text.

The loading weights in Panel 1 showed that the *Oscillospirillaceae* family and the symptoms from the constipated phenotype (SAGIS_Epigastric pain and SAGIS_Constipation,) had the highest loading weights in IBS-C, while the PBMC gene *RPS4Y1* had the highest loading weight in controls (Figures 4.3 A).

The loading weights in Panel 2 showed that Unclassified Coriobacteriia and the gene *SRRM2* had the highest loading weight in FC, while the symptom of constipation (PROMIS) had a high loading weight in IBS-C (Figure 4.3 B).

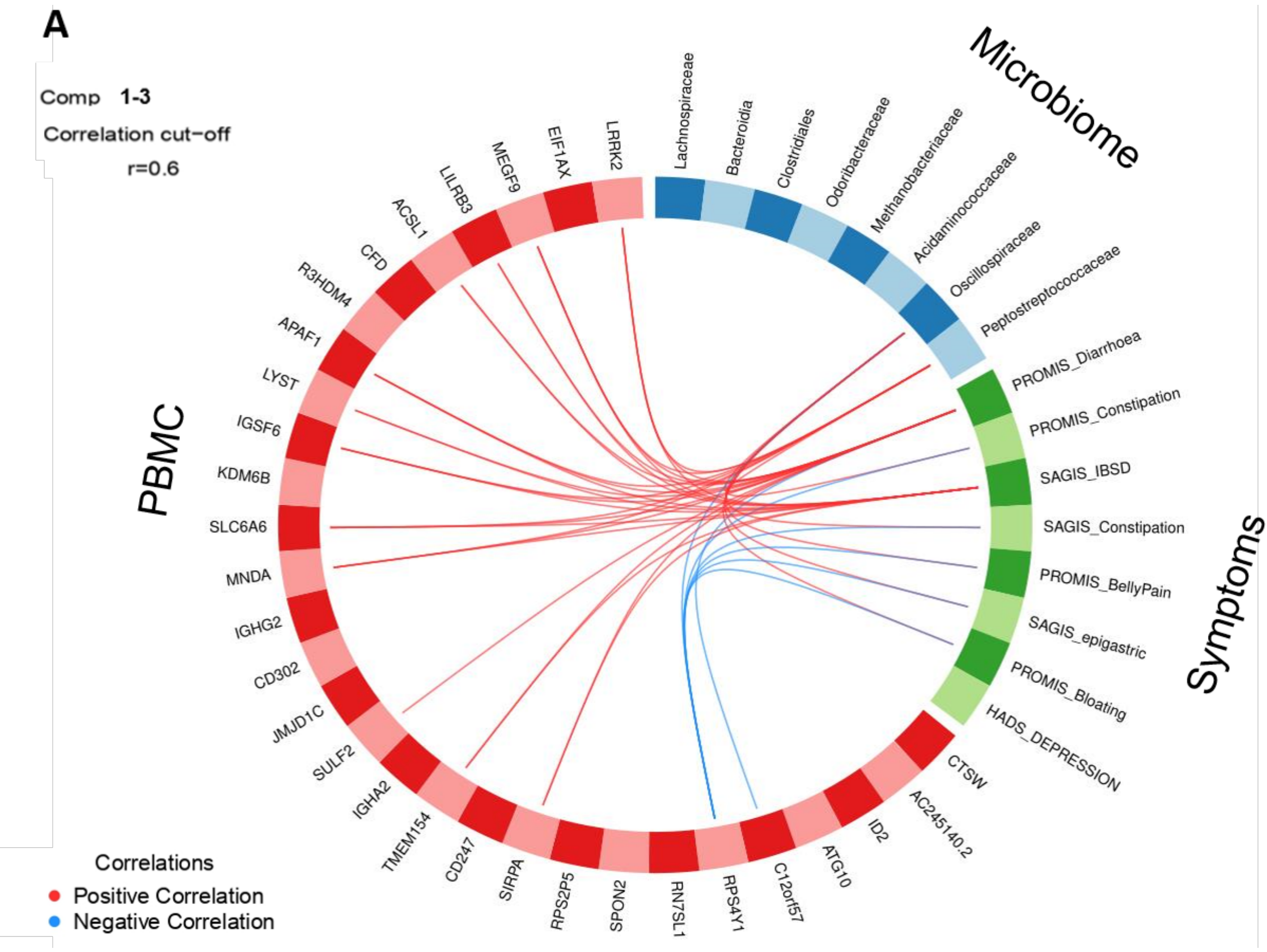
The loading weights of Panel 3 showed that the family *Peptostreptococcaceae*, the PBMC gene *LRRK2*, and the symptom SAGIS_IBS-D had the highest loading weight in FD (Figure 4.3 C). PROMIS_Diarrhoea had the highest loading weight in IBS-M (Figure 4.3 C).

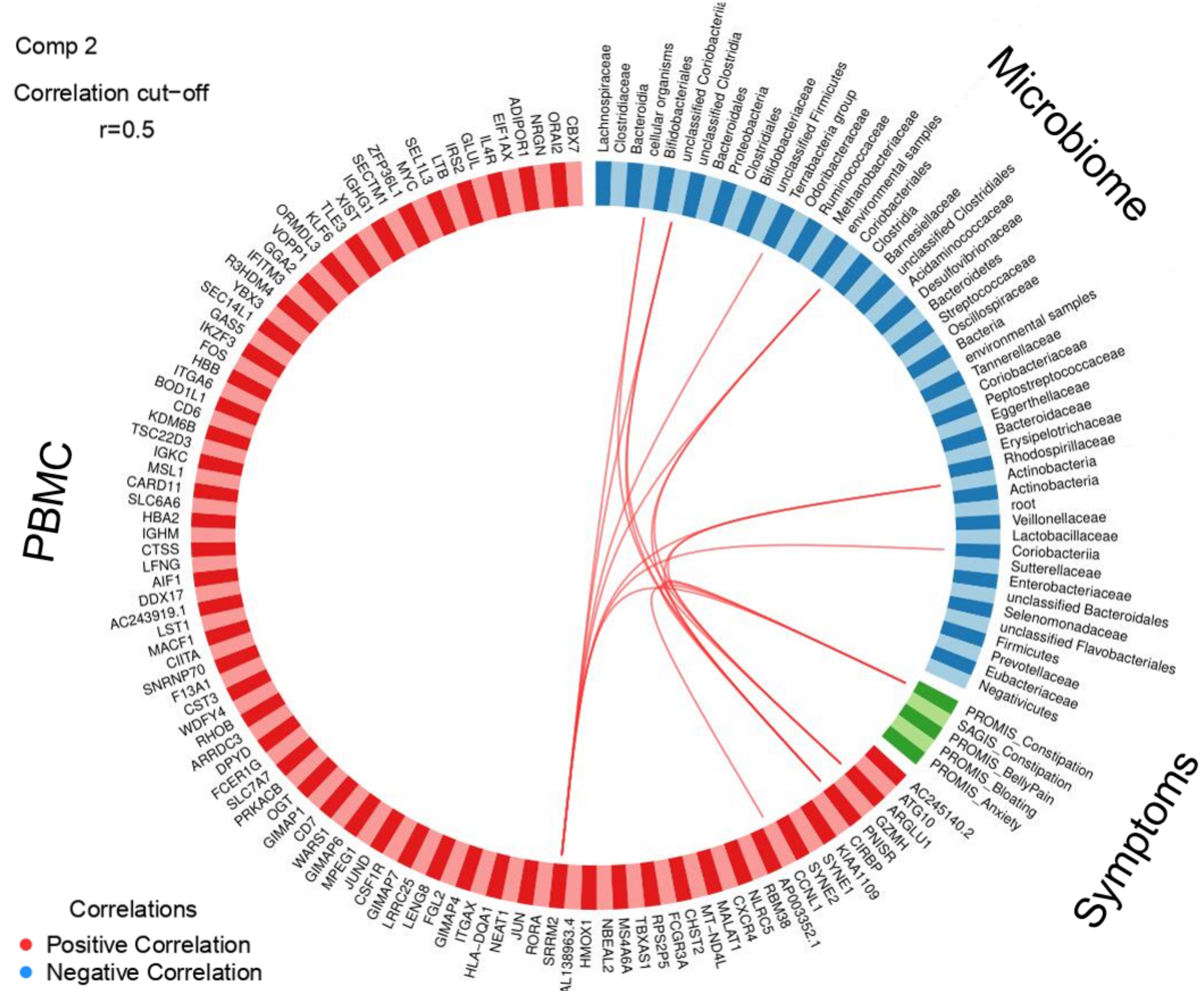
None of the Panels included symptoms contributing to IBS-D or FC (Figures 4.3 A, B, C). PBMC genes moderately contributed to IBS-D (KDM6B from Panel 2 and SPON2 from Panel 1) and IBS-M (AC245140.2 from Panel 3) (Figures 4.3 A, B, C).

Table 4. 2: Panels with the variables selected from faecal microbiota composition, PBMC gene expression and symptom datasets of subjects with FGIDs (IBS-C, IBS-D, IBS-M, FC, and FD) and controls.

Variables in each Panel were selected using the 'tune.block.splsda' function by integrating microbiota, PBMC gene and symptom datasets using DIABLO.

Variable	**Panel 1**	**Panel 2**	**Panel 3**
Microbiota taxa	*Oscillospiraceae* *Odoribacteraceae* *Acidaminococcaceae*	Coriobacteriia, Unclassified *Flavobacteriales*, Terrabacteria group, Clostridia, *Lachnospiraceae*, Bifidobacteriales, Bacteria superkingdom, Clostridiales, Actinobacteria, Clostridiales, *Tannerellaceae*, *Acidaminococcaceae*, Proteobacteria, *Eggerthellaceae*, *Methanobacteriaceae*, *Eubacteriaceae*, *Bacteroidaceae*, *Veillonellaceae*, *Clostridiaceae*, *Lactobacillaceae*, *Rhodospirillaceae*, *Enterobacteriaceae*, *Barnesiellaceae*, Firmicutes, *Erysipelotrichaceae*, *Prevotellaceae*, Bacteroidales, *Sutterellaceae*, Bacteroidia, *Peptostreptococcaceae, Ruminococcaceae*, Negativicutes, *Oscillospiraceae, Odoribacteraceae*, Bacteroidetes, *Streptococcaceae, Selenomonadaceae, Unclassified Bacteroidales, Desulphovibrionaceae*	*Peptostreptococcaceae* *Methanobacteriaceae* *Lachnospiraceae* Bacteroidia Clostridiales
PBMC genes	*RPS4Y1* *KDM6B* *SPON2* *CD247* *ACSL1* *RPS2P5* *CTSW* *R3HDM4* *ID2* *EIF1AX*	*SRRM2, NLRC5, SYNE2, IGHG1, ADIPOR1, FCGR3A, CCNL1, SYNE1, NBEAL2, RBM38, CHST2, WDFY4, KIAA1109, AC245140.2, PNISR, TSC22D3, HBB, CD7, NRGN, LENG8, ATG10, WARS1, YBX3, SEL1L3, GIMAP4, KDM6B, F13A1, RPS2P5, PRKACB, TBXAS1, GIMAP1, AL138963.4, MALAT1, GIMAP6, VOPP1, ARGLU1, IGKC, HMOX1, LRRC25, NEAT1, JUN, IKZF3, KLF6, HBA2, FOS, IRS2, CSF1R, ORMDL3, CBX7, GIMAP7, R3HDM4, JUND, MACF1, CIITA, CIRBP, RORA, OGT, TLE3, FGL2, MSL1, MS4A6A, DDX17, CXCR4, LTB, ORAI2, LFNG, BOD1L1, LST1, ZFP36L1, GGA2, HLA-DQA1, CST3, MPEG1, AC243919.1, AIF1, GAS5, RHOB, FCER1G, IL4R, SLC7A7, GLUL, GZMH, MYC, SLC6A6, DPYD, ARRDC3, CARD11, IFITM3, ITGA6, XIST, AP003352.1, IGHM, SEC14L1, CTSS, EIF1AX, MT-ND4L, ITGAX, SNRNP70, CD6, SECTM1*	*LRRK2, LYST, IGHA, IGHG2, APAF1, RN7SL1, CD302, CFD, JMJD1C, MNDA, SIRPA, MEGF9, SLC6A6, SULF2, LILRB3, AC245140.2, IGSF, C12orf57, TMEM154, ATG10*
GI and non- GI symptoms	SAGIS_Epigastric SAGIS_Constipation PROMIS_BellyPain PROMIS_Bloating PROMIS_Constipation	PROMIS_Constipation PROMIS_BellyPain SAGIS_Constipation PROMIS_Bloating PROMIS_Anxiety	PROMIS_Diarrhoea SAGIS_IBSD HADS_Depression


A
Comp 1-3
Correlation cut-off
r=0.6
Microbiome
PBMC
Symptoms
Lachnospiraceae
Bacteroidia
Clostridiales
Odoribacteraceae
Methanobacteriaceae
Acidaminococcaceae
Oscillospiraceae
Peptostreptococcaceae
PROMIS_Diarrhoea
PROMIS_Constipation
SAGIS_IBSD
SAGIS_Constipation
PROMIS_BellyPain
SAGIS_epigastric
PROMIS_Bloating
HADS_DEPRESSION
CTSW
AC245140.2
ID2
ATG10
C12orf57
RPS4Y1
RN7SL1
SPON2
RPS2P5
SIRPA
CD247
TMEM154
IGHA2
SULF2
JMJD1C
CD302
IGHG2
MNDA
SLC6A6
KDM6B
IGSF6
LYST
APAF1
R3HDM4
CFD
ACSL1
LILRB3
MEGF9
EIF1AX
LRRK2
Correlations
Positive Correlation
Negative Correlation



Comp 2
Correlation cut−off
r=0.5
Microbiome
PBMC
Symptoms
Correlations
Positive Correlation
Negative Correlation
Lachnospiraceae
Clostridiaceae
Bacteroidia
cellular organisms
Bifidobacteriales
unclassified Clostridia
Bacteroidales
Proteobacteria
Clostridiales
Bifidobacteriaceae
unclassified Firmicutes
Terrabacteria group
Odoribacteraceae
Ruminococcaceae
Methanobacteriaceae
environmental samples
Coriobacteriales
Clostridia
Barnesiellaceae
unclassified Clostridiales
Acidaminococcaceae
Desulfovibrionaceae
Bacteroidetes
Streptococcaceae
Oscillospiraceae
Bacteria
environmental samples
Tannerellaceae
Coriobacteriaceae
Peptostreptococcaceae
Eggerthellaceae
Bacteroidaceae
Erysipelotrichaceae
Rhodospirillaceae
Actinobacteria
Actinobacteria
root
Veillonellaceae
Lactobacillaceae
Coriobacteriia
Sutterellaceae
Enterobacteriaceae
unclassified Bacteroidales
Selenomonadaceae
unclassified Flavobacteriales
Firmicutes
Prevotellaceae
Eubacteriaceae
Negativicutes
PROMIS_Constipation
SAGIS_Constipation
PROMIS_BellyPain
PROMIS_Bloating
PROMIS_Anxiety
AC245140.2
ATG10
ARGLU1
GZMH
PNISR
CIRBP
KIAA1109
SYNE1
SYNE2
CCNL1
AP003352.1
RBM38
NLRC5
CXCR4
MALAT1
MT−ND4L
CHST2
FCGR3A
RPS2P5
TBXAS1
MS4A6A
NBEAL2
HMOX1
AL138963.4
SRRM2
RORA
JUN
NEAT1
HLA−DQA1
ITGAX
GIMAP4
FGL2
LENG8
LRRC25
GIMAP7
CSF1R
JUND
MPEG1
GIMAP6
WARS1
CD7
GIMAP1
OGT
PRKACB
SLC7A7
FCER1G
DPYD
ARRDC3
RHOB
WDFY4
CST3
F13A1
SNRNP70
CIITA
MACF1
LST1
AC243919.1
DDX17
AIF1
LFNG
CTSS
IGHM
HBA2
SLC6A6
CARD11
MSL1
IGKC
TSC22D3
KDM6B
CD6
BOD1L1
ITGA6
HBB
FOS
IKZF3
GAS5
SEC14L1
YBX3
R3HDM4
IFITM3
GGA2
VOPP1
ORMDL3
KLF6
TLE3
XIST
IGHG1
SECTM1
ZFP36L1
MYC
SEL1L3
LTB
IRS2
GLUL
IL4R
EIF1AX
ADIPOR1
NRGN
ORAI2
CBX7

Figure 4. 2: Outputs representing the variables selected by DIABLO from different datasets with the strongest correlations, regardless of the digestive health status of the participants.

(A) Circos plot showing correlations between variables from the microbiota composition, PBMC gene and symptom datasets related to Panels 1 and 3 combined (cut-off= 0.6). The most relevant information was found correlating variables from Panels 1 and 3, combining them in the same Circos plot. **(B)** Circos plot showing correlations between variables from the microbiota composition, PBMC gene and symptom datasets related to Panel 2 (cut-off= 0.5). Each sector indicates microbiota taxa (blue), PBMC genes (red), symptoms (green), and lines with either positive (red) or negative (blue) correlation.

Table 4. 3: REACTOME pathways enriched in PBMC gene cluster positively correlated with the *Peptostreptococcaceae* family (0.66 > r > 0.5) in subjects with FGID and controls.

The enrichment in the REACTOME pathways was determined with an over-representation analysis with a hypergeometric distribution test. The Benjamini-Hochberg method was used to correct the FDR, which was declared significant at < 0.05. Entities found represents the number of mapped identifiers that match the pathway for the selected molecular type. Entities total represents the total number of identifiers in the pathway for the selected molecular type. The number of submitted entities does not always match those found because of the redundancy of gene names. APAF1: Apoptotic Peptidase Activating Factor 1; SIRPA: Signal Regulatory Protein A; MNDA: Myeloid Cell Nuclear Differentiation Antigen; LILRB3: Leukocyte Immunoglobulin Like Receptor B3; SLC6A6: Solute Carrier Family 6 Member 6.

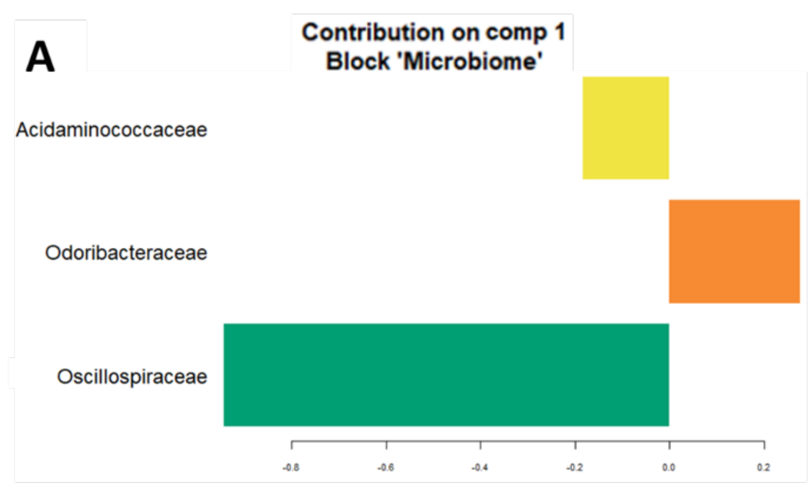
Pathway name	Submitted entities found	#Entities found	#Entities total	Entities FDR
Neutrophil degranulation	*APAF1; SIRPA; MNDA; LILRB3*	5	480	0.000
Signal regulatory protein family interactions	*SIRPA*	2	18	0.001
TP53 regulates the transcription of caspase activators and caspases	*APAF1*	2	20	0.001
Transcriptional regulation by E2F6	*APAF1*	2	46	0.004
Innate immune system	*APAF1; SIRPA; MNDA; LILRB3*	5	1334	0.008
TP53 regulates the transcription of cell death genes	*APAF1*	2	83	0.009
Cell-cell communication	*SIRPA*	2	133	0.017
SMAC(DIABLO)-mediated dissociation of IAP: caspase complexes	*APAF1*	1	7	0.017
SMAC (DIABLO) binds to IAPs	*APAF1*	1	7	0.017
Activation of caspases through apoptosome-mediated cleavage	*APAF1*	1	8	0.017
SMAC, XIAP-regulated apoptotic response	*APAF1*	1	8	0.017
PTK6 promotes HIF1A stabilisation	*LRRK2*	1	9	0.018
Formation of apoptosome	*APAF1*	1	13	0.018
Regulation of the apoptosome activity	*APAF1*	1	13	0.018
Cytochrome c-mediated apoptotic response	*APAF1*	1	15	0.021
Apoptotic factor-mediated response	*APAF1*	1	23	0.032
Immune system	*APAF1; SIRPA; MNDA; LILRB3*	5	2684	0.044
Na+/Cl- dependent neurotransmitter transporters	*SLC6A6*	1	51	0.044
DAP12 interactions	*SIRPA*	1	53	0.044
Transcriptional regulation by TP53	*APAF1*	2	487	0.044

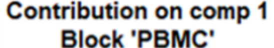
Intrinsic pathway for apoptosis	*APAF1*	1	64	0.044

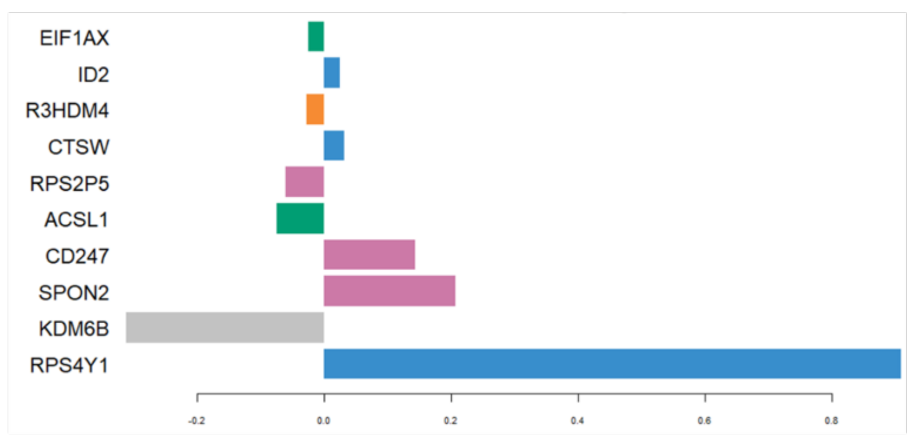
Table 4. 4: Canonical partial least squares correlation between microbial composition and PBMC gene variables selected from Panel 2 resulting from the Circos plot output across subjects with FGIDs (IBS-C, IBS-D, IBS-M, FC, and FD) and controls.

The cut-off value for positive or negative correlations was set at 0.5. r= correlation coefficient.

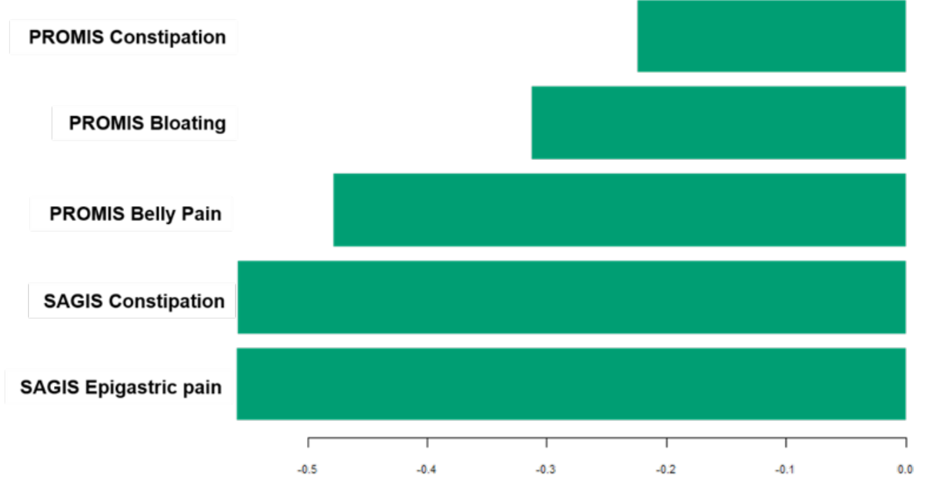
PBMC genes	Terrabacteria group	Actinobacteria	Coriobacteriia	Coriobacteriales
ARGLU1	-	r= 0.51	r= 0.50	r= 0.51
SRRM2	r= 0.58	r= 0.52	r= 0.53	r= 0.52
CCNL1	r= 0.58	-	r= 0.50	-
PNISR	r= 0.51	r= 0.53	r= 0.52	r= 0.52

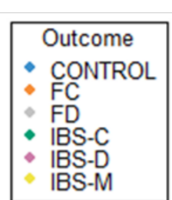


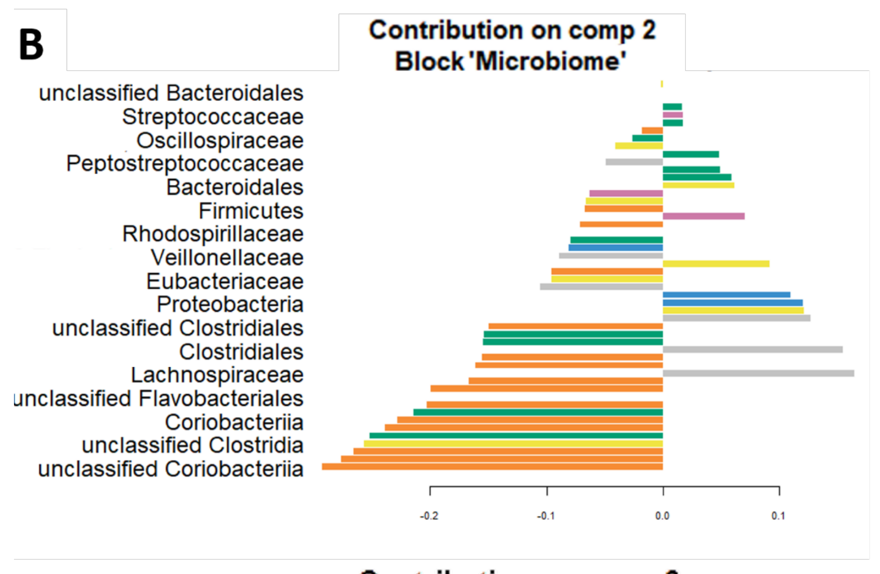




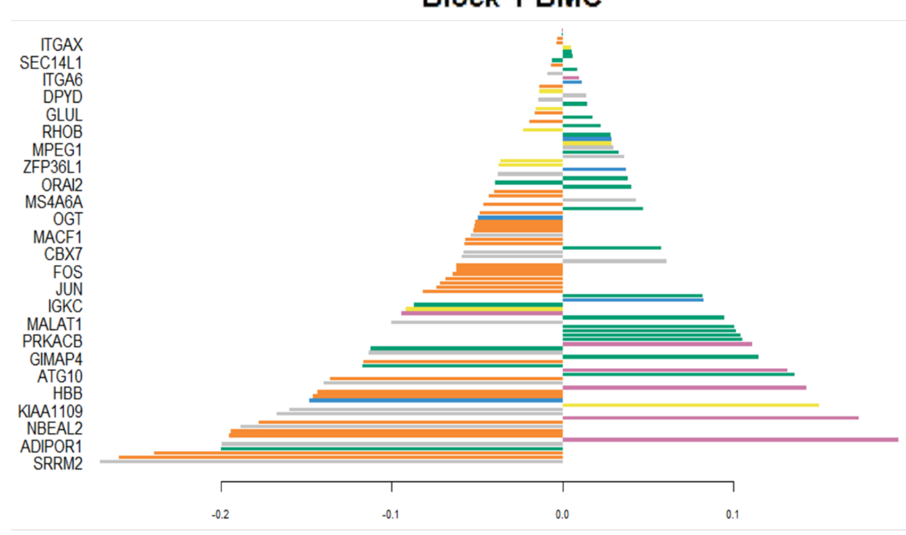
Contribution on comp 1
Block 'Symptoms'

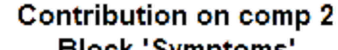


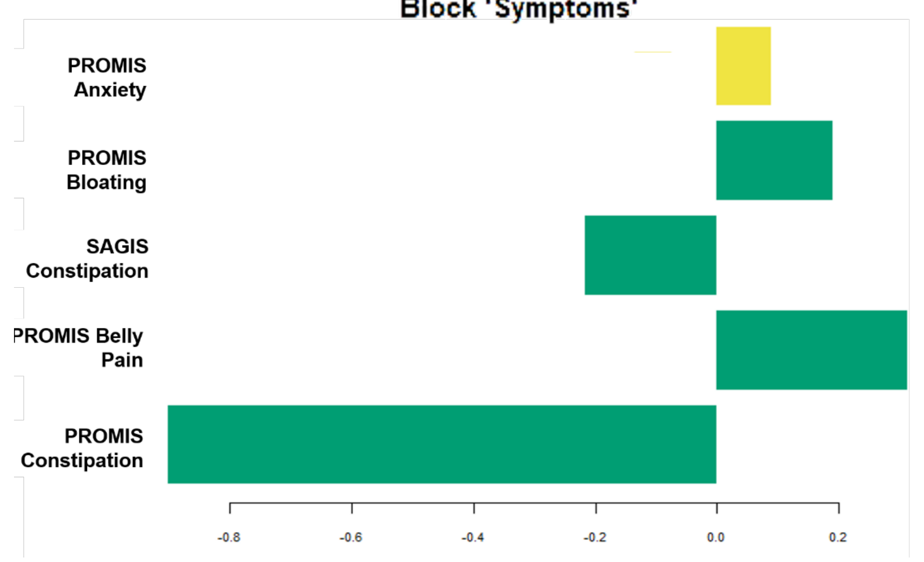


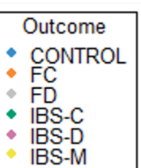


Contribution on comp 2
Block 'PBMC'









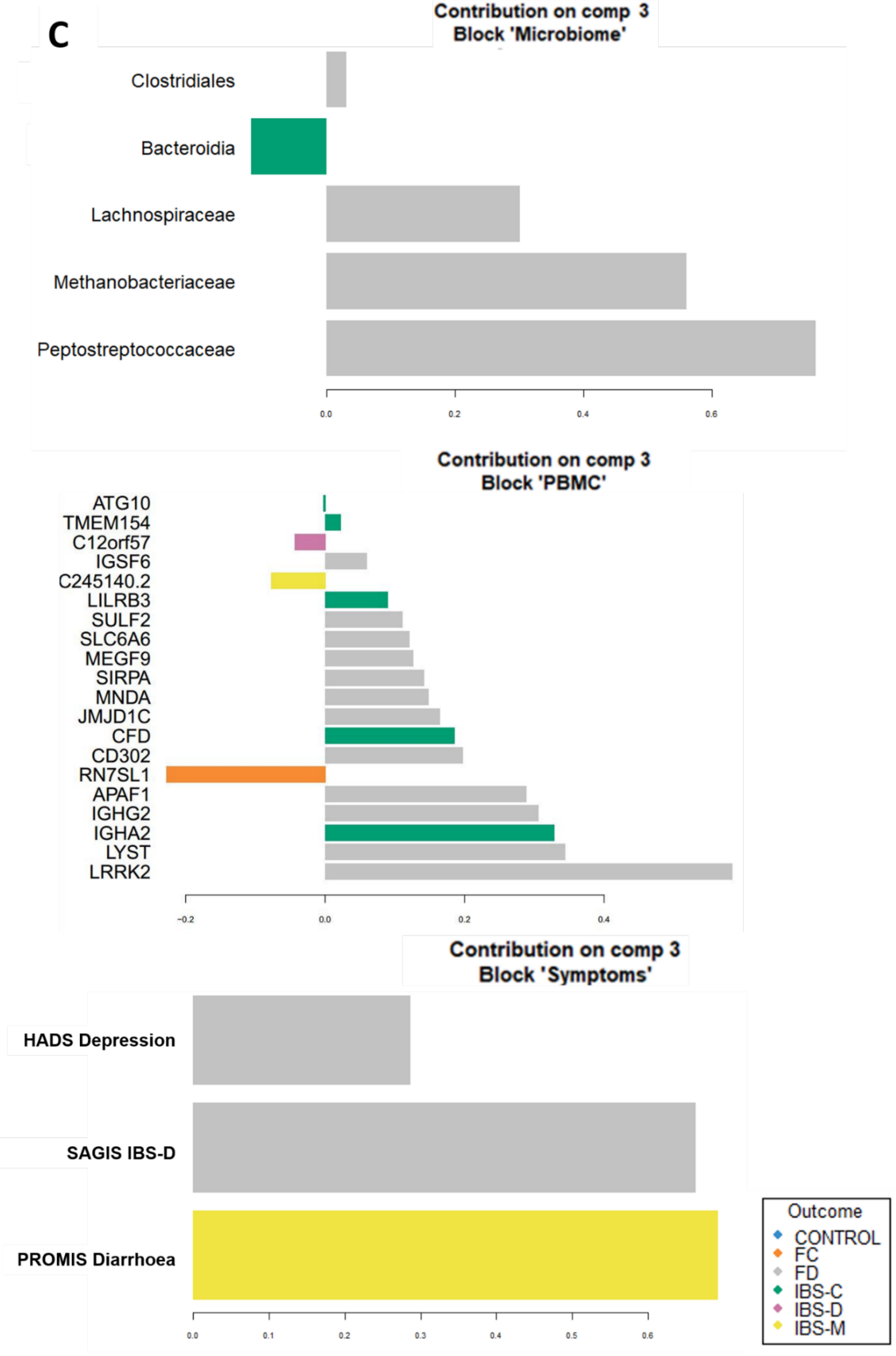

C
Contribution on comp 3
Block 'Microbiome'
Clostridiales
Bacteroidia
Lachnospiraceae
Methanobacteriaceae
Peptostreptococcaceae
0.0
0.2
0.4
0.6
Contribution on comp 3
Block 'PBMC'
ATG10
TMEM154
C12orf57
IGSF6
C245140.2
LILRB3
SULF2
SLC6A6
MEGF9
SIRPA
MNDA
JMJD1C
CFD
CD302
RN7SL1
APAF1
IGHG2
IGHA2
LYST
LRRK2
-0.2
0.0
0.2
0.4
Contribution on comp 3
Block 'Symptoms'
HADS Depression
SAGIS IBS-D
PROMIS Diarrhoea
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Outcome
CONTROL
FC
FD
IBS-C
IBS-D
IBS-M

Figure 4. 3: Loading plots showing the loading weights of each DIABLO-selected variable (faecal microbiota composition, PBMC genes and symptoms) in Panels 1 (A), 3 (B) and 2 (C) across subjects with FGIDs (IBS-C, IBS-D, IBS-M, FC, and FD) and controls.

The loading plot represents faecal microbiota composition, PBMC genes or symptoms selected in Panels (comp) 1, 2 and 3. Bar length indicates the loading coefficient weight of the selected variable, ranked by importance, bottom to top. The bar colour indicates the group (FGIDs or control) in which the mean expression value is the highest or the lowest for that variable. Values of negative loadings mean that the variable is related in the opposite direction from the group. Blue: control; green: IBS-C; orange: FC; grey: FD; pink: IBS-D; yellow: IBS-M.

4.5.2 Establishment of classification model of functional gastrointestinal disorders

4.5.2.1 Sparse partial least square discriminant analysis

Sample plots combining the variables from the three Panels for each dataset suggested that symptoms could discriminate among subjects with FC and IBS-C or from controls (Figure 4.4 A) and among subjects with FD and IBS-D or from controls (Figure 4.4 B). A less clear separation was visually observed between subjects with constipation and controls (Figure 4.4 A) or between IBS-D, FD and controls (Figure 4.4 B) based on microbial taxa or PBMC genes than symptoms.

4.5.2.2 Receiver operating characteristic curve

A ROC AUC was calculated for each dataset for Panels 1, 2 and 3 using one-vs-all comparisons to evaluate their ability to discriminate FGIDs within subtypes or from controls. Only correlations with an r superior to 0.8 are described for Panels 1, Panel 2 (Table 4.5) and Panel 3 (Table 4.5 and Figure 4.5). ROC curves (Figure 4.5) were represented only for Panel 3, as it included the AUC values with the highest diagnostic power.

The symptoms selected in Panel 1 ("SAGIS_Epigastric pain", "SAGIS_Constipation", "PROMIS_BellyPain", "PROMIS_Bloating" and "PROMIS_Constipation") had good diagnostic power to discriminate IBS-C (AUC= 0.86; *P*= 5.97E-04), IBS-M (AUC= 0.84; *P*= 2.14E-05) or controls (AUC= 0.86; *P*= 1.19E-13) from the other FGID and/or control groups (Table 4.5).

The PBMC genes selected in Panel 2 (cytokine signalling, haem signalling and repression of WNT target genes) had good diagnostic power to discriminate subjects with FC (AUC= 0.80; *P*= 2.18E-05) or IBS-C (AUC= 0.90; *P*= 1.58E-04) from the other FGID and control groups (Table 4.5). The symptoms selected in Panel 2 ("PROMIS_Constipation", "PROMIS_BellyPain", "SAGIS_Constipation", "PROMIS_Bloating", and "PROMIS_Anxiety") had good diagnostic power to discriminate IBS-C (AUC= 0.86; *P*= 6.17E-04) or IBS-M (AUC= 0.85; *P*= 1.95E-05) from the other FGID and control groups (Table 4.5). Similarly, these symptom variables

had good diagnostic power also to discriminate controls (AUC= 0.86; *P*= 1.15E-13) from the FGID groups (Table 4.5).

The symptoms selected in Panel 3 ("PROMIS_Diarrhoea", "SAGIS_IBS-D", and "HADS_Depression") had good diagnostic power (AUC= 0.80) to discriminate within FGIDs (but not FC) or FGIDs from controls (Table 4.5 and Figure 4.5 C). The PBMC genes (innate immunity, neutrophil degranulation, signalling and apoptosis) selected in Panel 3 had good diagnostic power to discriminate FC (AUC= 0.80; *P*= 2.920e-05), FD (AUC= 0.81; *P*= 1.72E-03) or IBS-C (AUC= 0.89; *P*= 1.53E-04) from other FGID and control groups (Table 4.5 and Figure 4.5 B).

Finally, IBS-C was the only subtype that could be discriminated with good diagnostic accuracy from other FGID or control groups by the microbial taxa selected in Panel 3 (AUC= 0.80; *P*= 0.004) (Table 4.5 and Figure 4.5).

Table 4. 5: Classification of FGID subtypes or controls compared to others based on the area under the curve (AUC) of the receiver operating characteristic curve (ROC).

AUC <0.70, low diagnostic accuracy; AUC 0.70–0.90, moderate diagnostic accuracy; AUC ≥0.90, high diagnostic accuracy and AUC=1, 100% accuracy. Values above 0.80 are highlighted in yellow. Others[1] means other FGIDs and controls.

One-vs-all comparison	AUC	p-value	AUC	p-value	AUC	p-value
	Microbiota Panel 1		**Symptom Panel 1**		**PBMC Panel 1**	
CONTROL vs Others (all FGIDs)	0.6421	0.003	0.8607	1.19E-13	0.7022	3.21E-05
FC vs Others[1]	0.5443	0.53	0.6598	2.52E-02	0.6483	3.79E-02
FD vs Others[1]	0.5852	0.39	0.5146	8.84E-01	0.7235	2.51E-02
IBS-C vs Others[1]	0.6567	0.14	0.8619	5.97E-04	0.7481	1.86E-02
IBS-D vs Others[1]	0.4744	0.71	0.6857	7.89E-03	0.4939	9.30E-01
IBS-M vs Others[1]	0.7701	0.00093	0.8465	2.14E-05	0.5859	2.92E-01
	Microbiota Panel 2		**Symptom Panel 2**		**PBMC Panel 2**	
CONTROL vs Others (all FGIDs)	0.6091	0.025	0.8609	1.15E-13	0.700	3.90E-05
FC vs Others[1]	0.7668	0.00019	0.7167	2.41E-03	0.8032	2.18E-05
FD vs Others[1]	0.5246	0.805	0.5865	3.86E-01	0.7151	3.11E-02
IBS-C vs Others[1]	0.6409	0.182	0.861	6.17E-04	0.8983	1.58E-04
IBS-D vs Others[1]	0.6328	0.06	0.732	9.03E-04	0.6209	8.36E-02
IBS-M vs Others[1]	0.6574	0.054	0.8482	1.95E-05	0.6217	1.36E-01
	Microbiota Panel 3		**Symptom Panel 3**		**PBMC Panel 3**	
CONTROL vs Others (all FGIDs)	0.6226	0.0117	0.8855	2.22E-15	0.6998	3.97E-05
FC vs Others[1]	0.7775	0.0001	0.7514	4.31E-04	0.7985	2.92E-05
FD vs Others[1]	0.665	0.1	0.8713	1.98E-04	0.8129	1.72E-03
IBS-C vs Others[1]	0.805	0.004	0.9198	6.85E-05	0.8993	1.53E-04
IBS-D vs Others[1]	0.6311	0.06	0.8176	5.50E-06	0.6373	4.95E-02
IBS-M vs Others[1]	0.6607	0.05	0.8516	1.62E-05	0.6217	1.36E-01

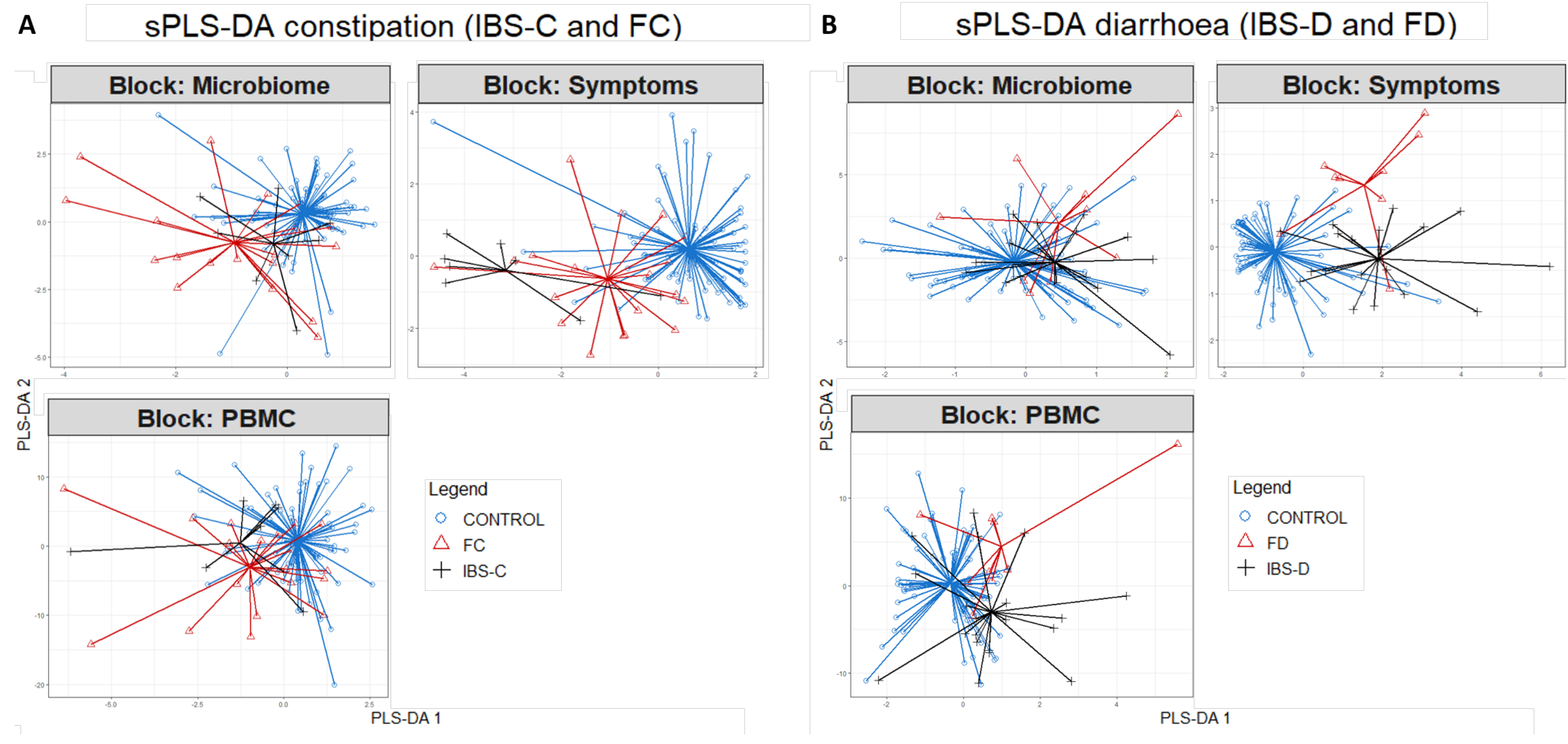


Figure 4. 4: Sparse Partial Least Square Discriminant Analysis (sPLS-DA) score plots of microbial taxonomic composition, symptoms and PBMC gene expression of subjects with constipation (FC and IBS-C) (A) or subjects with diarrhoea (FD and IBS-D) (B) compared to controls.

The arrow plot displays the group membership of each sample. Coloured lines lead from each group centroid towards each sample to aid in the visualisation of groups. Symbols indicate different FGID subtypes or controls: blue lines and circles (controls), orange lines and triangles (FC or FD), and grey lines and crosses (IBS-C or IBS-D). The axis labels indicate the amount of variation explained per component.

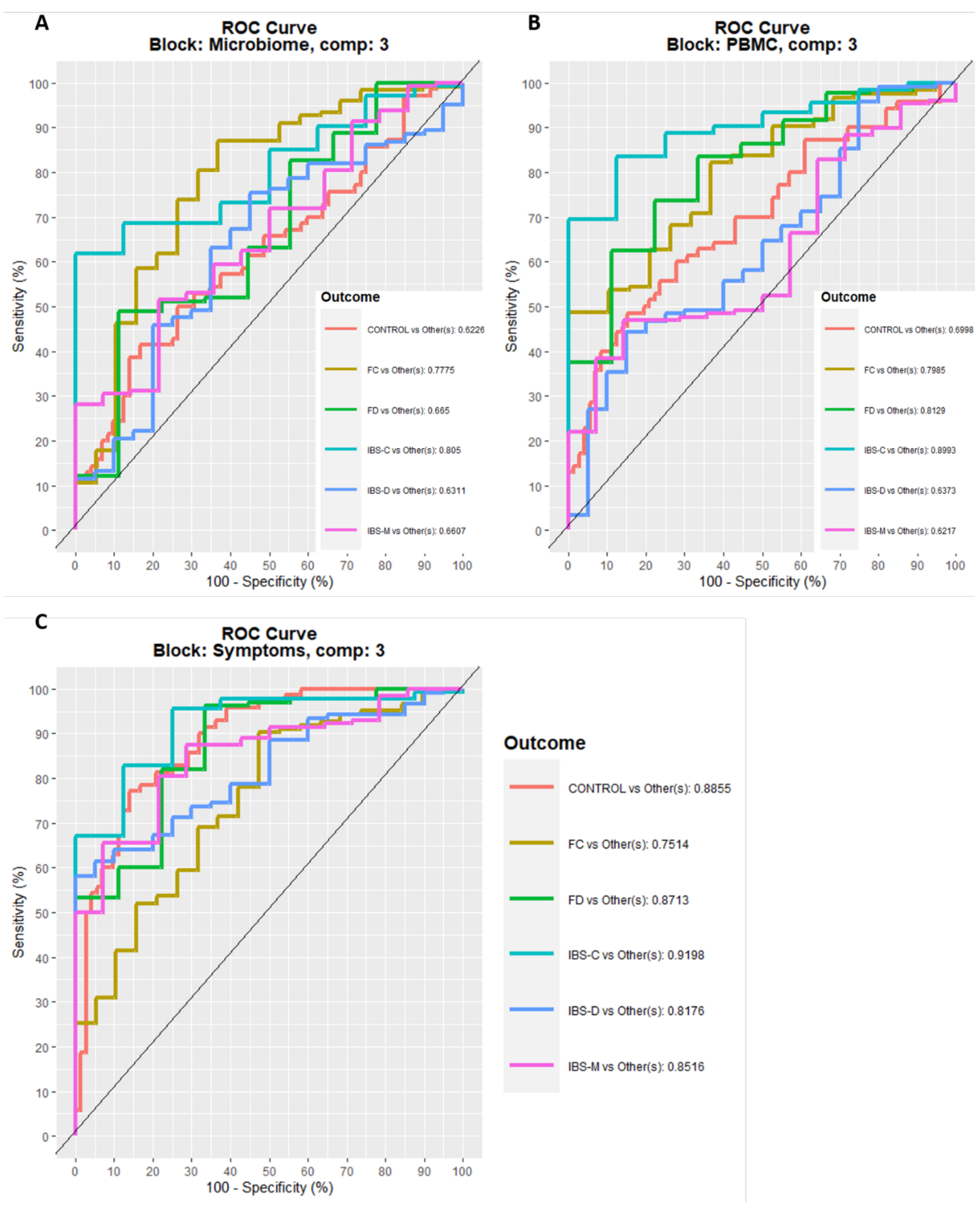


Figure 4. 5: The area under the curve (AUC) of the receiver operating characteristic curve (ROC) of subjects with FGIDs and controls, using selected values from Panel 3.

ROC is a probability curve, and AUC is the degree of separability, showing how much the model can distinguish FGIDs within subtypes or from controls. The higher the AUC, the better the model distinguishes FGIDs within subtypes or from controls. 100% diagnostic power: sensitivity= 1, specificity=1, AUC 1. ROC curves following or above the diagonal indicate a random guess or better than a random guess, respectively. AUC <0.70, low diagnostic accuracy; AUC 0.70–0.90, moderate diagnostic accuracy; AUC ≥0.90, high diagnostic accuracy; AUC 1, 100% accuracy. Others mean other FGIDs and controls.

4.6 Discussion

In this Chapter, microbial data (generated in Chapter 2) and PBMC gene expression data (generated in Chapter 3) were integrated with GI and non-GI symptoms (generated in Phoebe Heenan's thesis) using DIABLO. The integrative analysis showed potential relationships between these variables in FGIDs subjects and controls and provided insight into the immune-microbiota interactions underlying constipation and diarrhoea phenotypes beyond the information obtained from the individual datasets.

The analysis of ROC curve and AUC showed that a combination of GI and non-GI symptoms was the most accurate way to discriminate among IBS-D, IBS-C, IBS-M and FD or controls than selected PBMC genes or microbial taxa. On the other hand, FC was best discriminated from other FGIDs or controls by selected PBMC genes involved in cytokine, haem, and WNT signalling. IBS-C was the only subtype that could also be discriminated with good diagnostic accuracy from other FGID subtypes or controls by selected microbial taxa.

4.6.1 Integration of microbiota, immune gene expression and symptoms in constipation predominant functional gastrointestinal disorders

Symptoms of constipation predominant FGIDs (IBS-C+FC), including constipation, epigastric pain, abdominal pain, and bloating, showed a strong positive correlation with the family *Oscillospiraceae*, and a strong negative correlation with the PBMC gene *RPS4Y1*. Studies have reported that the genera *Oscillibacter* [612] and *Oscillospira* [613] were negatively correlated with defecation. In particular, the *Oscillospira* genus was positively associated with constipation and slow colonic transit times and negatively associated with Bristol stool scale [614]. In the same study, the confidence for this association was second only to that with *Methanobrevibacter*, a genus known to be linked to longer colonic transit times [615]. Therefore, *Oscillospira* species probably grow slowly and may play a role in aggravating constipation [84], explaining why this genus was strongly positively linked to methane production in women with chronic constipation [88].

Taxa from the *Oscillospira* genus produce all kinds of short-chain fatty acids (SCFAs), but especially butyrate [616], which *in vivo* inhibit smooth muscle contractility and fluid

transit in the colon [617], contributing to constipation. In addition, SCFAs also increased active sodium and chloride absorption, making the faeces dry and hard [618].

Results from Chapter 2 showed that the relative abundance of the *Oscillospiraceae* family was increased in subjects with constipation predominant FGIDs compared to controls. The expression of the PBMC *RPS4Y1* gene, which was very strongly negatively correlated with the *Oscillospiraceae* family, showed a decreasing trend in all FGID groups compared to controls. This gene is a Y-chromosome–encoded minor histocompatibility (H-Y) antigen. Here, more than one-third of PBMC genes with lower expression levels in subjects with IBS-C were Y-linked and many were considered biomarkers of the male gender in a study on Y-chromosome genes [619]. Among these PBMC genes, the *USP9Y*, *DDX3Y*, *UTY* and *TMSB4Y* genes encode H-Y antigens [620]. Therefore, the lower expression levels of these genes in IBS-C may simply reflect the predominance of females (28 females and 2 males) in this group of the COMFORT cohort. Interestingly, the relative abundance of the *Oscillospira* genus was previously reported to be higher in females than in males [613]. Therefore, the expression of the PBMC *RPS4Y1* gene was likely to be gender-related rather than related to IBS-C.

4.6.2 Integration of microbiota, immune gene expression and symptoms in diarrhoea predominant functional gastrointestinal disorders

The symptoms of subjects with diarrhoea predominant FGIDs (IBS-D and FD) were positively associated with the family *Peptostreptococcaceae* and PBMC genes involved in innate immunity, neutrophil degranulation, and cellular apoptosis. In particular, the PBMC *LRRK2* gene had the strongest correlations with diarrhoea symptoms and the *Peptostreptococcaceae* family.

Studies with gain and loss of function suggested the involvement of the *LRRK2* gene in the host response to pathogens [621]. For example, the siRNA knockdown of the *LRRK2* gene in RAW macrophage cells demonstrated that reduced LRRK2 protein was associated with impaired clearance of *Salmonella typhimuriu*m.

Although it is still unclear whether high *LRRK2* gene expression in immune cells is protective or detrimental, *LRRK2 gene* expression in PBMCs was reported to be high under homeostatic conditions and increased upon stimulation. This observation suggests

that the *LRRK2* gene might play a regulatory role in PBMC effector functions and may be relevant here, particularly because LRRK2 has been reported to modulate inflammatory responses to pathogens [621].

The strong positive association between the PBMC *LRRK2* gene and the *Peptostreptococcaceae* family, which includes many potentially pathogenic anaerobic taxa (including *C. difficile*), suggests a potential role for *LRRK2* expression in the dysbiotic environment of FGIDs. In Chapter 2 *C. difficile* was detected in FD and IBS-D samples. Another study showed that subjects with *C. difficile* infection had a predominance of taxa from the *Peptostreptococcaceae* family [622], likely associated with changes in the microbiota composition, including the rise of potentially pathogenic anaerobic taxa from *Peptostreptococcaceae* and *Enterobacteriaceae* families. The increased abundance of these families and facultative anaerobes might increase the susceptibility to *C. difficile* colonisation and immune activation [623].

Other PBMC genes (innate immunity (*LILRB3*), neutrophil degranulation (*MNDA*) and cellular apoptosis (*APAF*)) were positively associated with the diarrhoea predominant FGIDs and the *Peptostreptococcaceae* family showed increased expression in FD but not in IBS-D. Apoptosis may be delayed, induced or enhanced by different microorganisms, according to their immune evasion strategies and the health status of the host [624]. The observed increased expression of genes which induce neutrophil apoptosis and suppress neutrophil degranulation may be the consequence of a prolonged neutrophil stimulation by the microbiota. For example, increased blood neutrophils were observed after *C. difficile* infection in animal models and humans [625, 626].

These results suggest a potential interplay between microorganisms and PBMC genes involved in the modulation of neutrophil activity. In addition, calprotectin, produced by activated neutrophils, discriminated IBS from inflammatory bowel diseases [192, 193], which supports the role of neutrophils in FGIDs. In particular, in another study, faecal calprotectin concentration (not measured here) was reported to be highest in IBS-D, followed by IBS-M and IBS-C [191].

4.6.3 Establishment of a classification model for functional gastrointestinal disorders

The analysis of the ROC curve and AUC variables showed that subjects with constipation predominant FGIDs (IBS-C and FC) could be discriminated accurately from other diarrhoea predominant FGIDs and controls by combining PBMC genes associated with neutrophil degranulation, innate immune system and apoptosis (Panel 3) or PBMC genes associated with cytokine signalling (*CSF1R, JUN, IGHG1, IL4R, MYC, ITGAX, F13A1, HMOX1, RORA, IFITM3, IRS2, FOS*), haem signalling (*HMOX1, HBB, HBA2, RORA*) and WNT signalling (*TLE3, MYC*) (Panel 2). Increased expression levels of *IGHG1, IFITM3, HBB*, and *TLE3* genes and cytokine and chemokine signalling genes were specific to FC, as reported in Chapter 3.

In particular, the PBMC genes selected in Panel 2 better discriminated FC from other FGIDs and controls than selected GI and non-GI symptoms or microbial taxa. IBS-C was the only subtype discriminated accurately from other FGIDs and controls combining taxa from *Peptostreptococcaceae*, *Methanobacteriaceae*, *Lachnospiraceae* families and taxa from the Bacteroidia class and Clostridiales order. These taxa are directly or indirectly involved in SCFA production or methane production in the colon, and their relative abundance was reported to be altered in subjects with IBS-C in Chapter 2 and other studies [83, 67, 627].

IBS-C and IBS-M could be discriminated accurately from other FGIDs and controls by the symptoms selected in Panel 1 (constipation, belly pain, bloating and epigastric pain), Panel 2 (constipation, belly pain, bloating and anxiety) and Panel 3 (diarrhoea and depression). Conversely, subjects with diarrhoea predominant FGIDs (IBS-D and FD) could be discriminated accurately from other FGIDs and controls by the symptoms selected in Panel 3 (diarrhoea and depression).

Except for FC, combining GI and non-GI symptoms remains the most accurate way to discriminate among different FGID subtypes or from controls than combining PBMC genes or microbial taxa. These findings confirmed that FC might have a distinctive immune signature underlying its pathophysiology.

Combining datasets from microbiome, immune system, and symptom profiles can be highly beneficial for the translation of research findings into clinical practice and

personalised medicine. This would allow the potential discovery of predictive biomarkers correlating with disease severity or treatment response, for more personalised treatment strategies based on a patient's unique combination of microbiome, immune, and symptom data. The integration of datasets over time can help to understand how the microbiome, immune system, and symptoms change during the course of a disease, enabling the identification of early warning signs for individuals at risk and allowing preventive interventions. Finally, machine learning can be applied to integrated datasets to identify complex patterns that may not be detected through traditional analyses.

4.6.4 Strengths and limitations

The strength of the analyses conducted in Chapter 4 lies in the ability of DIABLO to select correlated and relevant variables to maximise the correlation between selected datasets [628]. However, a limitation of this analysis is that correlation does not equal causation, so results should be interpreted cautiously, and more research would need to be carried on before deducing a cause-and-effect relationship between two variables. In addition, removing samples from participants that did not have PBMC, symptom or microbial data (from 273 to 142 participants) reduced the sample size and, thus, the power of analysis [629].

Another limitation is that DIABLO was primarily developed for *omics* datasets, so further validations are needed for categorical and nominal variables, such as symptoms. Data heterogeneity is another limitation in combining datasets from different biological sources [630], as studies with multiple datasets include data that differ in type, scale and distribution, with often thousands of variables [631].

Furthermore, the correlation analysis was conducted on the entire dataset (subjects with FGIDs and controls), regardless of the digestive health status. This choice was made to maximise the sample size and power for this analysis. Further analyses should include more participants in each FGID subtype group to perform a correlation analysis specific to FGID subjects.

4.7 Conclusion

The correlative and integrative analyses provided information that could not be obtained from the individual dataset analysis in Chapters 2 and 3. These findings allow insight into the relationships between faecal microbes and immune cells specific for constipation and diarrhoea phenotypes.

Some symptoms from the constipation phenotype, including epigastric pain, constipation, abdominal pain, and bloating, were positively associated with the family *Oscillospiraceae* and negatively associated with the PBMC gene *RPS4Y*. On the other hand, the diarrhoea phenotype was strongly positively associated with the family *Peptostreptococcaceae* and PBMC genes, such as *LRRK2*, involved in neutrophil degranulation and cellular apoptosis.

The ROC analysis revealed that subjects with FC could be discriminated from other FGID and control groups by selected PBMC genes involved in cytokine, haem and WNT signalling with better diagnostic accuracy than selected GI and non-GI symptoms or microbial taxa. In addition, IBS-C was the only subtype that could be discriminated with good diagnostic accuracy from other FGID subtypes or controls by selected microbial taxa.

Except for FC, selected GI and non-GI symptoms remain the most accurate way to discriminate among different FGID subtypes or controls than selected PBMC genes or microbial taxa.

Chapter 5

The effect of the daily consumption of two gold kiwifruit on the faecal microbiota composition and gene abundance in healthy subjects or subjects with constipation

A journal article titled "The effect of the daily consumption of two gold kiwifruit on the faecal microbiota composition and gene abundance in healthy subjects or subjects with constipation" (Caterina Carco, Wayne Young, Simone B. Bayer, Phoebe E. Heenan, Richard B. Gearry, Lynley N. Drummond, Warren C. McNabb, Nicole C. Roy) will be submitted to *Frontiers in Nutrition* in 2023.

5.1 Abstract

Constipation predominant functional gastrointestinal disorders (FGIDs) affect up to 14 % of the world's population. The different factors contributing to the development of constipation may also lead to perturbed colonic microbiota composition and functionality. There is evidence that consuming certain foods, such as green and gold kiwifruit, might affect colonic microbiota composition and function and improve constipation. Seventy-three subjects with constipation predominant FGIDs (functional constipation; irritable bowel syndrome constipation) or healthy controls (controls) were enrolled in a 16-week randomised, single-blind, cross-over study. Of these, 56 subjects completed the study, receiving either gold kiwifruit (2/day) or psyllium (fibre-matched, 2.5g/day, as a positive control) for four weeks, followed by a four-week washout before crossing over and a two-week follow-up period after the end of the intervention. DNA extracted from faecal samples collected from the participants was analysed by shotgun sequencing. Analysis of similarities was used to identify differences in microbial taxonomic composition and functional potential between the intervention with kiwifruit or psyllium. In addition, the faecal microbial data were correlated with gastrointestinal (GI) and non-GI symptoms recorded during the study.

Eggerthella and *Bacteroides* were the only genera that differed between subjects consuming gold kiwifruit or psyllium, respectively, or between each intervention compared to pre-intervention levels, regardless of the digestive health status of the subjects**.** Many microbial functional genes were differentially abundant after the intervention with kiwifruit or psyllium, in comparison to baseline, and between the two interventions. These included genes involved in carnitine metabolism and vitamin K2 biosynthesis in those fed gold kiwifruit, or genes involved in complex polysaccharide metabolism and genetic transcription and translation, in those fed psyllium. A greater number of canonical correlations between symptoms and microbial taxa was observed in constipation predominant FGID subjects compared to controls, and particularly the opposite association of *Phascolarctobacterium* and *Dialister* with GI (diarrhoea, reflux, nausea, epigastric pain) and non-GI symptoms (anxiety and depression). Although the intervention with psyllium resulted in greater effects on gene abundances than gold kiwifruit, both interventions resulted in changes in microbial gene abundances potentially associated with health benefits.

5.2 Introduction

Many subjects suffering from functional gastrointestinal disorders (FGIDs) often experience changes in faecal frequency or the consistency characteristic of constipation [632]. Functional constipation is highly prevalent, with up to 14 % of the general population reporting related symptoms [633]. It is characterised by having at least two of six symptoms (straining, lumpy/hard faeces, incomplete evacuation, sensation of anorectal obstruction, need for manual manoeuvres and fewer than three spontaneous bowel movements per week that are infrequent and hard to pass). No symptoms reliably separate irritable bowel syndrome constipation (IBS-C) from functional constipation (FC); visceral hypersensitivity is more strongly associated with IBS-C than FC, and delayed colonic transit is more common in FC [634]. Symptoms related to these conditions often impact the quality of life of subjects with constipation predominant FGIDs [635]. Many factors contribute to the development of constipation, including age, gender, diet, lifestyle, stress, use of certain medications, and psychological and neurological conditions [636]. These factors may cause imbalances in the colonic microbial composition and function. Consequently, microbial dysbiosis has been observed in subjects with constipation predominant FGIDs in comparison to healthy subjects (reviewed by Ohkusa *et al*. [637], Chapter 2).

Consuming certain foods improves constipation symptoms and affects the composition and function of the colonic microbiota, and changes can be observed 24 hours after consumption [638]. For example, fibre supplements, such as psyllium, are commonly used to treat constipation, and their efficacy has been clinically proven [322-324, 639, 640], ameliorating defecation frequency and faecal consistency compared to placebo or laxative therapies [641]. However, the use of psyllium may result in increased gas or bloating, and its taste or texture may limit its consumption [642]. Other remedies, such as laxatives, increased water intake and exercise, have limited clinical evidence for their effectiveness and poor long-term efficacy [641]. In addition, although some treatments are effective for IBS-C and FC, such as prosecretory and prokinetic agents, other treatments are specific to IBS-C (antidepressants, antispasmodics, cognitive behaviour therapy) or FC (prucalopride, biofeedback). Among prokinetic agents, Tegaserod was withdrawn in 2007 due to an association with serious adverse cardiovascular effects and reintroduced in 2019 for use in restricted to low-risk populations [643].

Consequently, natural, food-based, effective and clinically proven alternatives are needed. Kiwifruit has recently been recognised as a food for constipation management in clinical studies [336-341]. Kiwifruit contains polyphenolics and nondigestible carbohydrates such as pectic, hemicellulosic, and cellulosic polysaccharides. These compounds can be degraded by members of the colonic microbiota, potentially resulting in beneficial health effects. Consumption of green kiwifruit in subjects with constipation predominant FGIDs (FC or IBS-C) showed improvements in bowel frequency, faecal softness, and a reduction in constipation symptoms [350, 377], transit time, and other gastrointestinal (GI) symptoms [336-342], compared to healthy subjects. The beneficial effects of green kiwifruit on motility have mostly been attributed to its soluble fibre content, which has a high water-holding capacity and viscosity that increase faecal bulking and softening [337]. Other effects of green kiwifruit included modulating the relative abundance in microbial composition by affecting the growth of specific microbial groups and improving aspects of the immune function [373, 375, 377-379, 387, 401-405]. Increased faecal short-chain fatty acid (SCFA) production was linked to the levels of complex carbohydrates in green kiwifruit [350]. Further digestive benefits of green kiwifruit may be related to the activity of actinidin, a proteolytic enzyme potentially inducing laxative effects, and to a high amount of polyphenols [341].

On the other hand, there is limited evidence for gold kiwifruit as a food for constipation management in clinical studies. The gold-fleshed kiwifruit, compared to the green variety, has half the total fibre (1.5 vs 3 g/100 g), lower activity of actinidin (26 *v*s. 100 %), higher amount of vitamin C (160 *vs*. 85 mg/100 g) but the same amount of soluble fibre, other vitamins, phytochemicals and minerals [644]. A four-week intervention with three gold-fleshed kiwifruit daily improved GI comfort and bowel movements in subjects with constipation predominant FGIDs [327]. A similar intervention with two gold-fleshed kiwifruit per day showed improved GI symptoms and bowel movements in participants with constipation predominant (FC or IBS-C) [645]. A randomised cross-over study investigated the effect of consuming capsules containing two gold kiwifruit-derived powder daily for 4 weeks on the faecal microbiota; it increased the relative abundance of taxa from the Clostridiales order in healthy controls and the abundance of the *Faecalibacterium prausnitzii* species in FC [344]. Another study reported that consuming two gold-fleshed kiwifruit daily over 12 weeks by people with prediabetes increased the

relative abundance of uncharacterised members of the bacterial family *Coriobacteriaceae* [345].

Fibre-based interventions suggest that the sustained, habitual consumption of green kiwifruit could restore the faecal microbiota of subjects with FGIDs to a composition more similar to controls, supporting its beneficial effects on bowel movements and GI symptoms. However, no studies investigated the effect of the gold-fleshed kiwifruit on microbial structure and gene abundances in FC and IBS-C and their correlations to bowel movements and GI symptoms.

The Psyllium and Kiwifruit translation (PSYKI) clinical study was undertaken to understand the sustained impact of consuming daily two gold kiwifruit (Zespri® SunGold *Actinidia chinensis* "Zesy002") or psyllium (fibre-matched positive control) over four weeks on the GI comfort of subjects with either constipation predominant FGIDs (FC or IBS-C classified by Rome IV) or healthy participants. The primary outcome was the feasibility of incorporating methods developed for the "Christchurch IBS cohort to investigate mechanisms for gut relief and improved transit" (COMFORT) study detailed in Chapter 1, an observational study into GI symptoms and underlying biological signatures, into this intervention study. Secondary outcomes included alterations in GI symptoms and bowel movements during the intervention. Other outcomes were subjective assessments of the anxiety and depression status and measurements of underlying biological signatures correlating with bowel movement and GI symptoms. The biological signatures included the faecal microbial composition and gene abundance, faecal and plasma metabolomes, faecal organic acids and bile acids, and plasma neurotransmitters.

The PSYKI study was designed by Professor Richard Gearry, Professor Nicole Roy and Dr Simone Bayer of the University of Otago as part of the Healthy Digestion Priority Research programme co-led by Professors Roy and Gearry. The clinical phase and analysis of all clinical data (GI and non-GI symptoms) were carried out by Dr Bayer. Dr Diana Cabrera, Dr Karl Fraser and Dr Shanalee James conducted the laboratory and data analyses of metabolomes and bile acids. Dr Janine Cooney and her team conducted laboratory and data analyses of neurotransmitters and organic acids.

My contribution to the PSYKI study was to carry out bioinformatic and statistical analyses of the faecal microbiota composition and gene abundances, by extracting faecal

microbial DNA for shotgun metagenomic sequencing. My contribution also included the correlation of faecal microbiota data with bowel movement, and GI and non-GI symptom datasets, writing the research chapter and journal article.

5.3 Hypothesis and Aims

The main aim of the secondary exploratory analyses of the PSYKI study reported in Chapter 5 was to investigate the effects of consuming two gold kiwifruit or psyllium daily over four weeks on faecal microbial composition and gene abundances, and their relationships to GI and non-GI symptoms, in those with constipation predominant FGIDs (IBS-C or FC) and healthy subjects (controls).

Psyllium (Ispaghula) was selected as a control intervention as it is a well-studied and well-tolerated soluble fibre supplement for FGIDs [322-324, 639, 640]. Therefore, psyllium was dose-matched for fibre content in lieu of a true placebo in the PSYKY study. The fibre content of a whole fresh gold kiwifruit is thought to contribute to digestive comfort, therefore, a simple control material as a placebo could not have adequately matched the gold kiwifruit.

Here, the first hypothesis was that the faecal microbiota composition and gene abundance profiles in subjects with constipation predominant FGIDs will move closer towards those of controls during the interventions.

A second hypothesis was that the changes in the faecal microbiota composition and gene abundance profiles are different between psyllium intervention and the gold kiwifruit intervention, and that this difference can be used to better understand the observed underlying aetiology of GI and non-GI symptoms in subjects with constipation predominant FGIDs.

To test these hypotheses:

1. The microbiota compositional and gene abundance profiles were compared between interventions in subjects with the constipation predominant FGIDs and controls.
2. The microbiota compositional and gene abundance profiles of each intervention group were compared to pre-intervention levels in constipation predominant FGIDs and controls.
3. The microbiota compositional profiles of constipation predominant FGID subjects or controls were correlated to GI and non-GI symptoms after the intervention with either gold kiwifruit or psyllium.

5.4 Materials and Methods

5.4.1 Study population and design

Dr Simone Bayer, a Postdoctoral Fellow at the University of Otago, conducted the PSYKI study. The description of the PSYKI study and clinical outcomes have been published [645] and summarised here. The PSYKI study was developed from the previous COMFORT observational study, which used a multi-*omics* approach (metabolome and microbiome) of biological samples (breath, blood, urine, and faeces), symptoms recorded as patient recorded outcomes (PRO), regarding digestive health and mood, and dietary information to investigate biological markers [424]. The primary outcome was to assess whether the comprehensive evaluation undertaken in the COMFORT study could be translated into a clinical trial. During the PSYKI study recruitment, the design was accepted by the participants, so the recruitment was expanded to provide more reliable outcomes on efficacy and safety. The protocol was approved by the NZ Human Disability and Ethics committee (18/STH/154) and prospectively registered with the Australian NZ Clinical Trial Registry (12618001286235).

The target population of the study was the adult population (ages 18 – 65). Seventy-three participants were recruited from the COMFORT cohort and the general population. Among these were 36 subjects with constipation predominant FGIDs (17 with FC, 19 with IBS-C) and 37 controls. Of these, 56 subjects completed the study (mean age 38.2 years, 85.7 % female, 80.3 % NZ European).

The FC participants were selected according to the Rome IV diagnostic criteria [15] (Chapter 2, section 4.1). Exclusion criteria were significant GI disorders other than IBS-C and FC, such as inflammatory bowel diseases, diverticulitis, coeliac disease, chronic diseases (cardiovascular disease or cancer), neurological conditions (multiple sclerosis, spinal cord injury or stroke), and kiwifruit allergy.

The study was a single-blinded, positive-controlled, randomised, cross-over (Figure 5.1): two-week lead-in phase, four-week intervention phase, four-week washout phase, four-week intervention phase and two-week follow-up phase. The total duration was 16 weeks.

After a two-week lead-in period, the participants were divided into two blocks and randomised in equal numbers by pulling numbers out of a container to the order of intervention. After informed consent, participants were randomly given either two gold

kiwifruit (*Actinidia chinensis* SunGold®, Zespri® International Ltd., fibre ~2.5g) to be eaten without the skin or 1.5 level teaspoons of psyllium (BonVit® Orange, BonVit®, Australia, fibre ~2.5g) per day for four weeks. The psyllium preparation was given in two doses of 10 g, matching the kiwifruit's fibre content as a control intervention.

After a four-week washout phase, the participants were crossed over and received the other intervention. At the end of the intervention, there was a two-week follow-up period. The participants also filled out questionnaires about their clinical symptoms, recorded as PRO, their bowel movements, demographic variables, mental and physical health, and general well-being, as described for the COMFORT cohort [645] and summarised in Figure 5.1. At baseline, the modified Hunter New England Health Survey (modHNES), including the hospital anxiety and depression scale (HADS), assesses general physical and mental health in the last four weeks, health questions like diabetes, lifestyle questions, mental health during the last seven days, and demographic data, and the Economic Living Standard Index short form (ELSISF), assessing the standard of living and socio-economic class, were used. At baseline and weekly, the following questionnaires were used: the Structured Assessment of GI Symptoms (SAGIS), assessing information on GI symptoms, the GI Symptom Rating Scale (GSRS), assessing symptom severity; and the Patient Reported Outcomes Measurement Information System (PROMIS), assessing GI symptoms, bowel movements, pain, bloating, reflux, anxiety, and depression in the last seven days. Daily: the Bowel Habit Diary, assessing the frequency of bowel movements, the level of straining and the Bristol stool scale form was used. At the end of the study, the Food and Symptoms Times (FAST), which assesses dietary and GI symptom changes, was used [645].

5.4.2 Sample collection and analysis

Participants provided blood, urine and faecal samples before and after each intervention. The collection of faecal samples, DNA extraction from them, shotgun sequencing of metagenomic DNA samples, all data acquisition steps, and statistical analyses were also carried out as described in Chapter 2, apart from the variations described below.

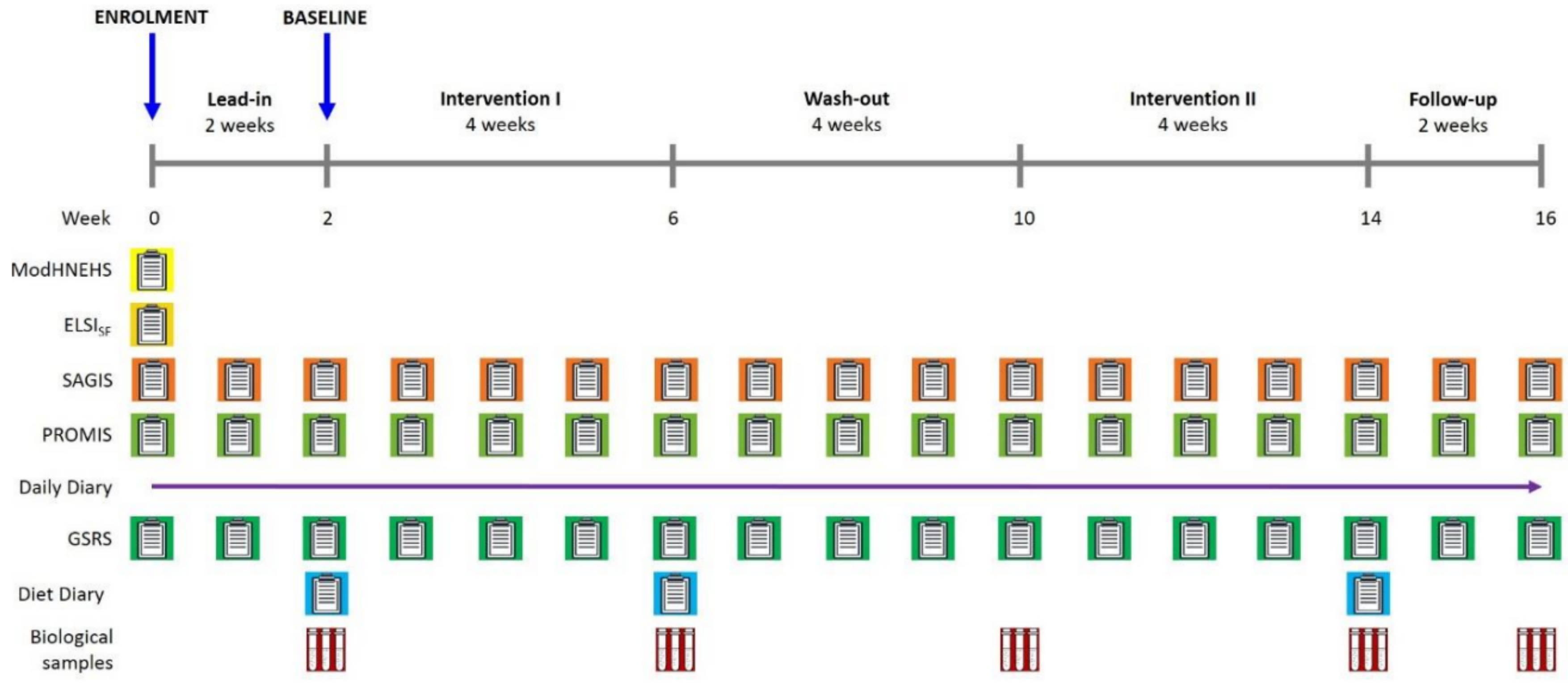


Figure 5. 1: Overview of the cross-over study of the total duration of 16 weeks.

Biological samples (faeces, urine and blood) were provided at all other time points (weeks 2, 6, 10, 14, 16). Participants filled out clinical questionnaires weekly and daily before and after the intervention. ModHNES: modified Hunter New England Health Survey;HADS: hospital anxiety and depression scale; ELSISF: Economic Living Standard Index short form; SAGIS: Structured Assessment of GI Symptoms; GSRS:GI Symptom Rating Scale; PROMIS:Patient Reported Outcomes Measurement Information System; FAST: Food and Symptoms Times.

5.4.3 Data analyses

The multivariate analysis of similarities (ANOSIM) was performed to identify differences in microbial taxonomic composition and gene abundances between interventions with kiwifruit and psyllium in subjects with constipation predominant FGIDs and healthy controls and compared to pre-intervention levels. The univariate analysis MaAsLin2 (Microbiome Multivariable Associations with Linear Models) was used to identify differences in groups with adjustments for other covariates [646], with health status, pre-intervention levels, gender, cross-over sequence, and treatment as fixed effects and participant identification as a random effect. Results with FDR< 0.05 were considered significant. The complete GI and non-GI symptom datasets are presented in [645].

5.4.3.1 Clustered Image Maps

Clustered Image Maps (CIM), also called "clustered correlation" or "heatmaps" [647], were created to visualise data results from the Pearson correlation between biological and symptom datasets from subjects with constipation predominant FGIDs (FC and IBS-C) and controls. This type of representation is based on hierarchical clustering operating on the rows and columns of a real-valued similarity matrix. This figure was graphically represented as a two-dimensional coloured image. Each entry of the matrix was coloured based on its value, and where the rows and columns were reordered according to the hierarchical clustering. In addition, dendrograms (tree diagrams) illustrating the arrangement of the clusters produced by the hierarchical clustering were added to the left side and the top of the image. The colour in the heatmap indicates the nature of the correlation between subsets of variables (positive, negative, strong or weak), while the dendrogram indicates the proximity between correlated variables. Positive (red) or negative (blue) correlation index (r) was set at > 0.5. Rectangles or squares of the same colour correspond to long branches of the dendrograms. CIM is implemented in the R package "mixOmics", dedicated to the integrative analysis of 'omics' data. In particular, "mixOmics" integrates two different data types to appropriately model the relationships between the two types of variables [628].

5.4.3.2 Functions for interfacing with other software packages

Once a co-expression network was generated, the network was imported into the network analysis tool Cytoscape_v3.8.1 [648]. The resulting Excel table contains the interactors (i.e., taxa exhibiting a strong correlation) in two columns, and their interaction strength (e.g., co-expression score). Using tools within Cytoscape, the network was analysed to identify nodes (i.e., taxa) that are "hubs", meaning these interactors have large numbers of co-expression partners.

5.5 Results

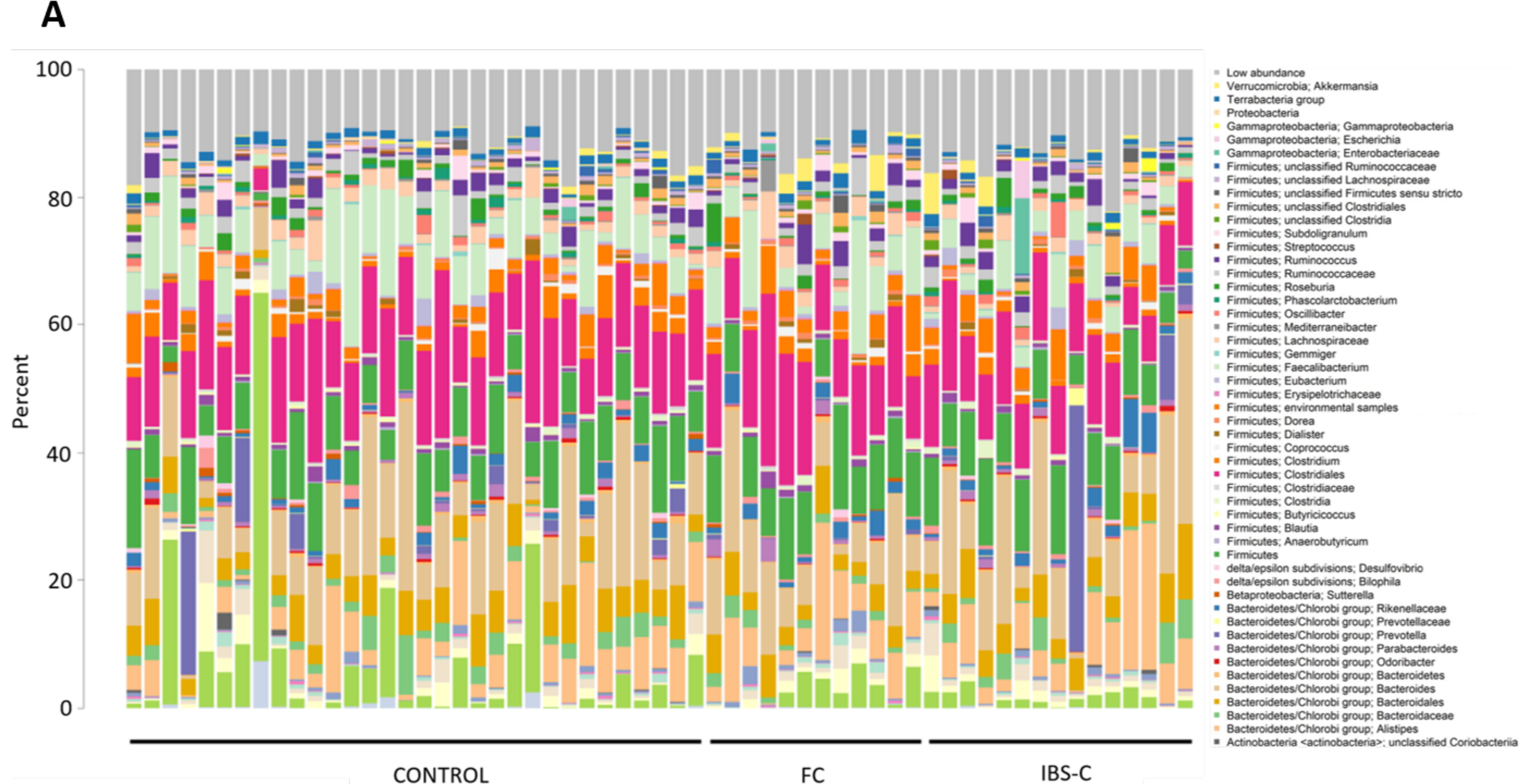
5.5.1 Overview of the taxonomic and functional analysis of the faecal microbiota

At pre-intervention levels, the taxonomic composition of the faecal microbiota among all subjects was highly variable. There were no detectable differences at the phylum, family and genus levels between controls and subjects with constipation predominant FGIDs (Figure 5.2 A, B). The microbial composition remained highly variable throughout the gold kiwifruit and psyllium interventions, regardless of whether the subjects were healthy or diagnosed with FGIDs (Figure 5.3 A). The most dominant phyla were the Firmicutes, Bacteriodetes, Actinobacteria and Proteobacteria (Figure 5.3 A).

The microbial gene abundance had less variation than microbial composition throughout the gold kiwifruit and psyllium interventions, despite no obvious differences on cursory examination (Figure 5.3 B). Over one-third of the gene count was related to the microbial functional categories of carbohydrate metabolism, genetic information processing and signalling and cellular processes, and amino acid metabolism (Figure 5.3 B).

5.5.2 Multivariate analyses

Principle coordinate analysis (PCA) of microbial taxonomic composition (Figure 5.4) and gene abundance (Figure 5.5) showed no differences between the subjects with FGIDs and controls or between intervention phases for all subjects, regardless of their digestive health status However, using a supervised method, such as the partial least squares discriminant analysis (PLS-DA), significant differences in the microbial taxonomic composition (anosim P=0.019) (Figure 5.6) and gene abundance (anosim P=0.013) (Figure 5.7) were observed between psyllium and kiwifruit interventions in all the subjects, regardless of the digestive health status.


A
100
80
60
40
20
0
Percent
CONTROL
FC
IBS-C
Low abundance
Verrucomicrobia; Akkermansia
Terrabacteria group
Proteobacteria
Gammaproteobacteria; Gammaproteobacteria
Gammaproteobacteria; Escherichia
Gammaproteobacteria; Enterobacteriaceae
Firmicutes; unclassified Ruminococcaceae
Firmicutes; unclassified Lachnospiraceae
Firmicutes; unclassified Firmicutes sensu stricto
Firmicutes; unclassified Clostridiales
Firmicutes; unclassified Clostridia
Firmicutes; Subdoligranulum
Firmicutes; Streptococcus
Firmicutes; Ruminococcus
Firmicutes; Ruminococcaceae
Firmicutes; Roseburia
Firmicutes; Phascolarctobacterium
Firmicutes; Oscillibacter
Firmicutes; Mediterraneibacter
Firmicutes; Lachnospiraceae
Firmicutes; Gemmiger
Firmicutes; Faecalibacterium
Firmicutes; Eubacterium
Firmicutes; Erysipelotrichaceae
Firmicutes; environmental samples
Firmicutes; Dorea
Firmicutes; Dialister
Firmicutes; Coprococcus
Firmicutes; Clostridium
Firmicutes; Clostridiales
Firmicutes; Clostridiaceae
Firmicutes; Clostridia
Firmicutes; Butyricicoccus
Firmicutes; Blautia
Firmicutes; Anaerobutyricum
Firmicutes
delta/epsilon subdivisions; Desulfovibrio
delta/epsilon subdivisions; Bilophila
Betaproteobacteria; Sutterella
Bacteroidetes/Chlorobi group; Rikenellaceae
Bacteroidetes/Chlorobi group; Prevotellaceae
Bacteroidetes/Chlorobi group; Prevotella
Bacteroidetes/Chlorobi group; Parabacteroides
Bacteroidetes/Chlorobi group; Odoribacter
Bacteroidetes/Chlorobi group; Bacteroidetes
Bacteroidetes/Chlorobi group; Bacteroides
Bacteroidetes/Chlorobi group; Bacteroidales
Bacteroidetes/Chlorobi group; Bacteroidaceae
Bacteroidetes/Chlorobi group; Alistipes
Actinobacteria <actinobacteria>; unclassified Coriobacteriia

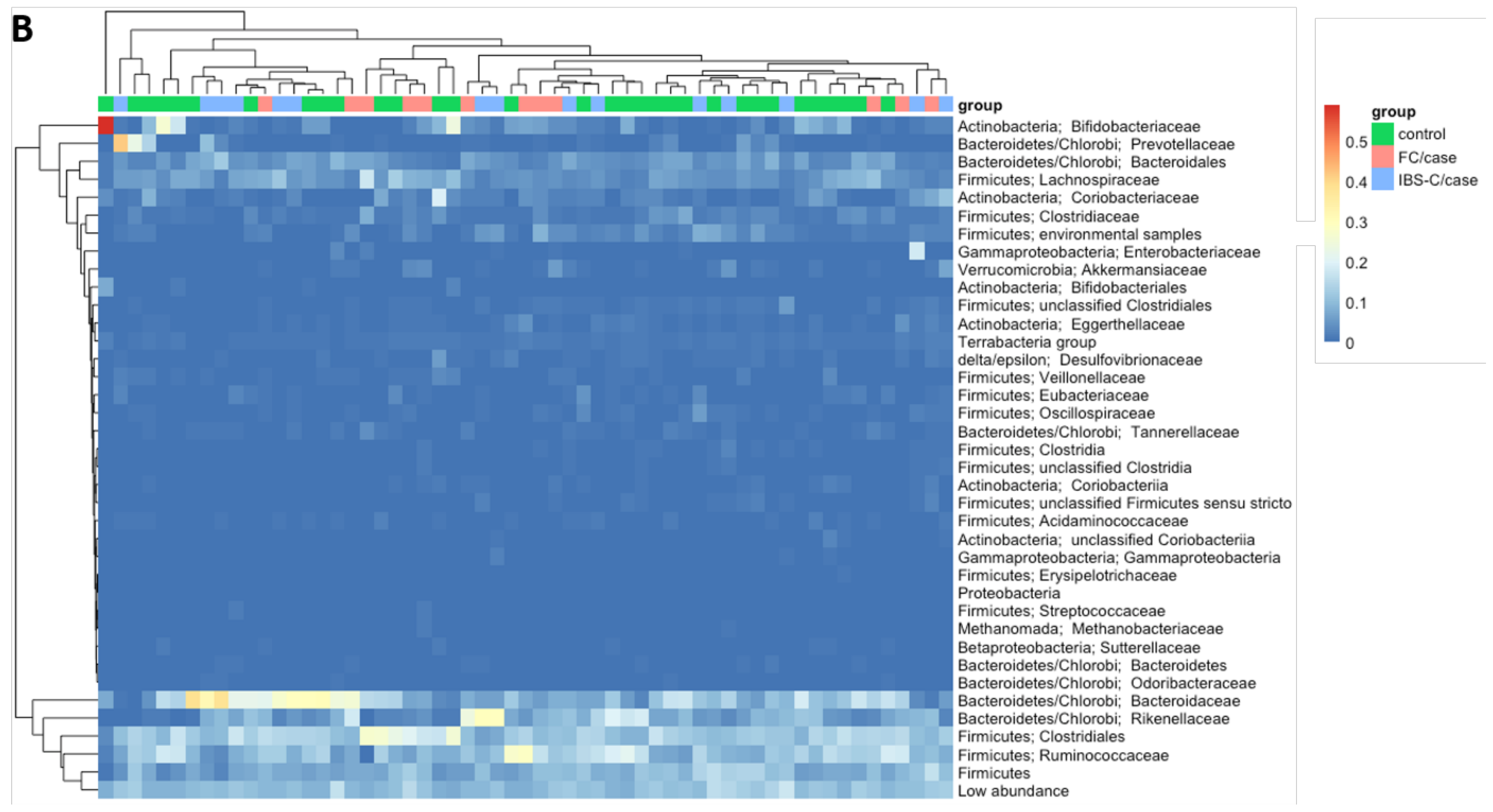
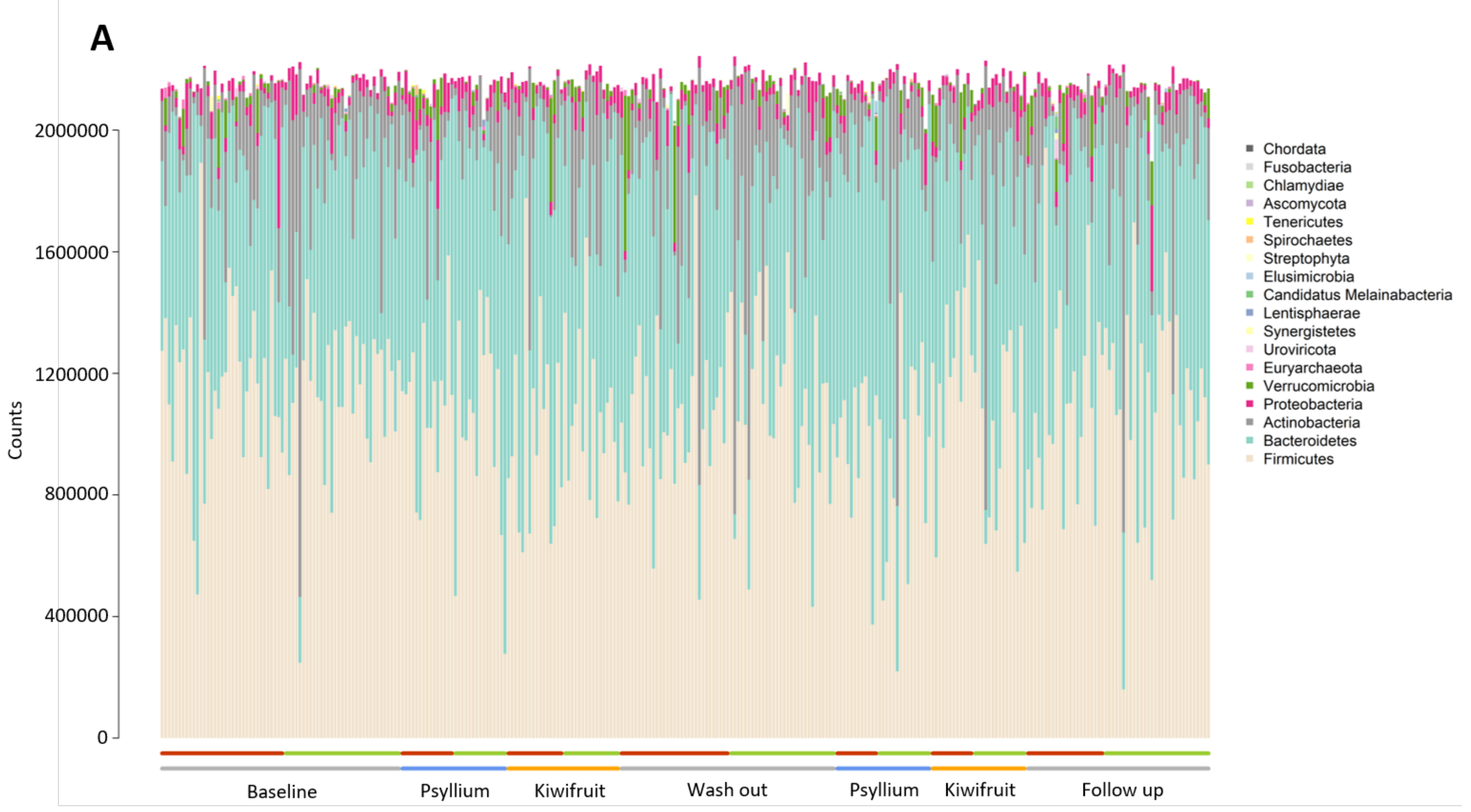


Figure 5. 2: Taxonomic composition of the faecal microbiota of subjects with constipation predominant FGIDs (IBS-C or FC) and controls at baseline, visualised as a stacked barplot (A) or heatmap (B). In **A**, colours represent genes related to the corresponding microbial category **up to the genus level**. Bars along the X-axis indicate each subject group, and the Y-axis represents relative microbial abundance**.** In **B**, the heatmap shows the preferential occurrence of taxa up to the family level across subjects. The dendrograms cluster taxa and FGID subtypes with similar occurrence patterns across subjects, based on their Euclidean distance. The colour scale represents the proportion of each taxa within a sample. The relative abundance of each taxa was normalised to 1 to adjust the patterns of all taxa.


A
Counts
2000000
1600000
1200000
800000
400000
0
Chordata
Fusobacteria
Chlamydiae
Ascomycota
Tenericutes
Spirochaetes
Streptophyta
Elusimicrobia
Candidatus Melainabacteria
Lentisphaerae
Synergistetes
Uroviricota
Euryarchaeota
Verrucomicrobia
Proteobacteria
Actinobacteria
Bacteroidetes
Firmicutes
Baseline
Psyllium
Kiwifruit
Wash out
Psyllium
Kiwifruit
Follow up

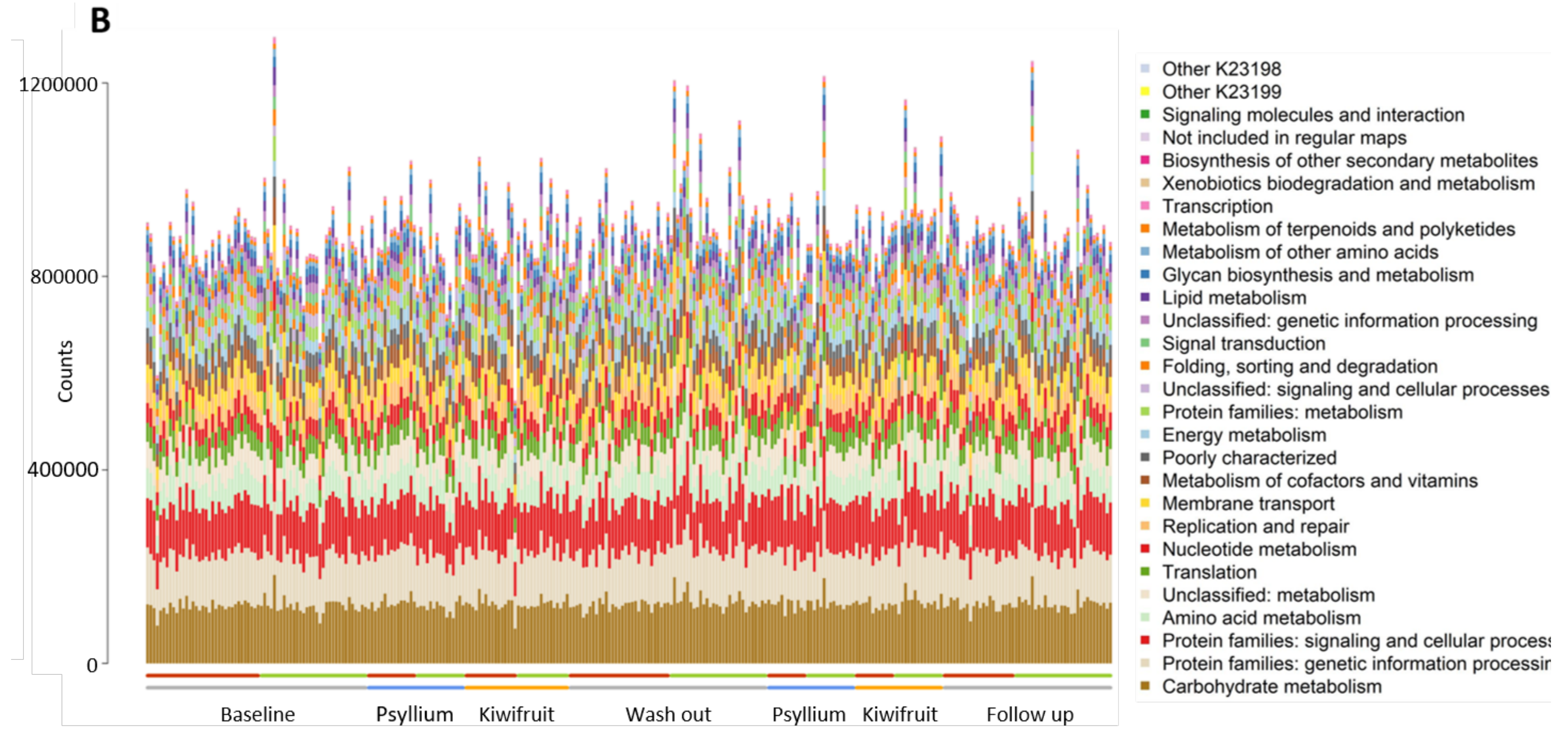


Figure 5. 3: (A) Taxonomic composition at the phylum level and (B) gene abundance at KEGG level 2 of the faecal microbiota of subjects with constipation predominant FGIDs (IBS-C or FC) and controls consuming two gold kiwifruit a day or a fibre-matched active control, psyllium for four weeks.

In (**A**), each colour represents a different bacterial taxon at the phylum level. In (**B**), colours represent genes related to the corresponding microbial category. Bars along the X axis indicate sample information, bars along the Y axis indicate microbial gene counts; Constipation predominant FGIDs (red), Control (green), Baseline/Wash out/Follow Up period (grey), Psyllium (blue), Kiwifruit (orange).

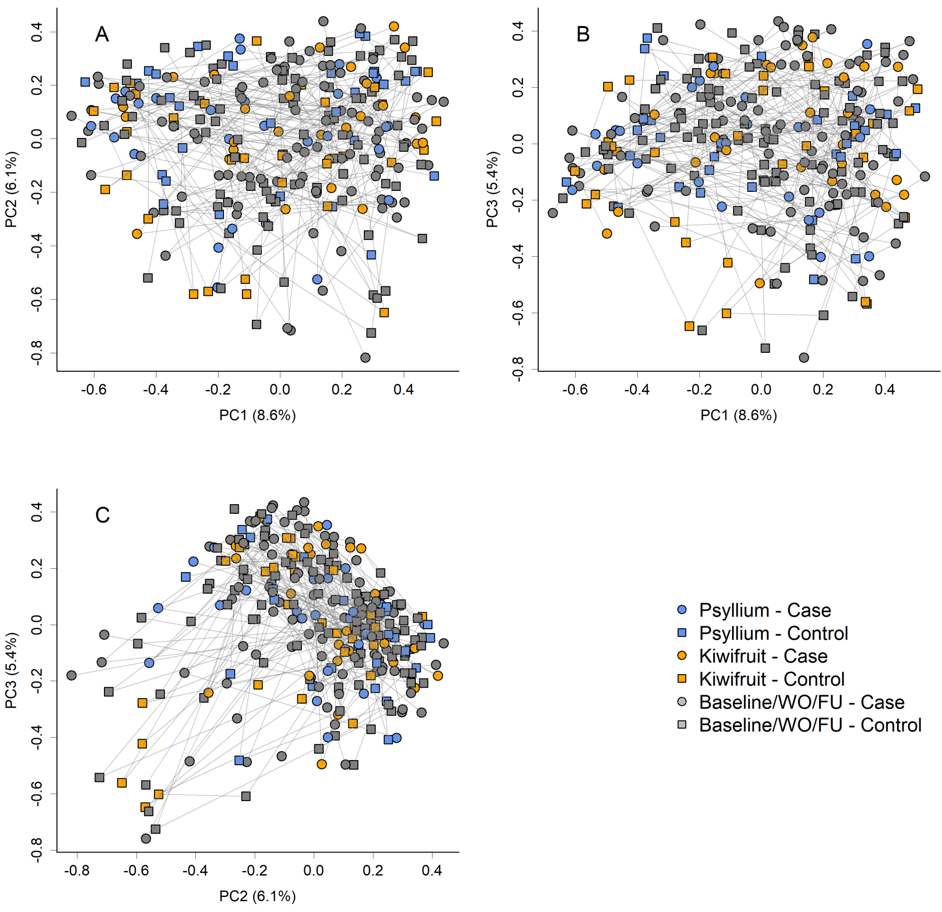


Figure 5. 4: Principal component analysis (PCA) of the microbial taxonomic composition at the genus level of subjects with constipation predominant FGIDs (IBS-C or FC) or controls consuming two gold kiwifruit daily or a fibre-matched active control, psyllium, for four weeks.

The PCA score plots (**A**) PC1 vs PC2, (**B**) PC1 vs PC3, and (**C**) PC2 vs PC3 show high variation between participants and within the same individual, as no distinct sample clustering was observed between participants, type of intervention, or between intervention phases, regardless of the digestive health status of the participants. The percentage values associated with the axes refer to the proportion of total variance in the data explained by each principal component, which identifies the most important components for explaining the variation in the data. Shapes indicate subjects with constipation predominant FGIDs (IBS-C or FC, Circle) or controls (Square). Colours mean the intervention with psyllium (blue) or kiwifruit (orange). The colour grey indicated baseline, washout (WO) or follow-up (FU) phases. Lines join samples from the same participant.

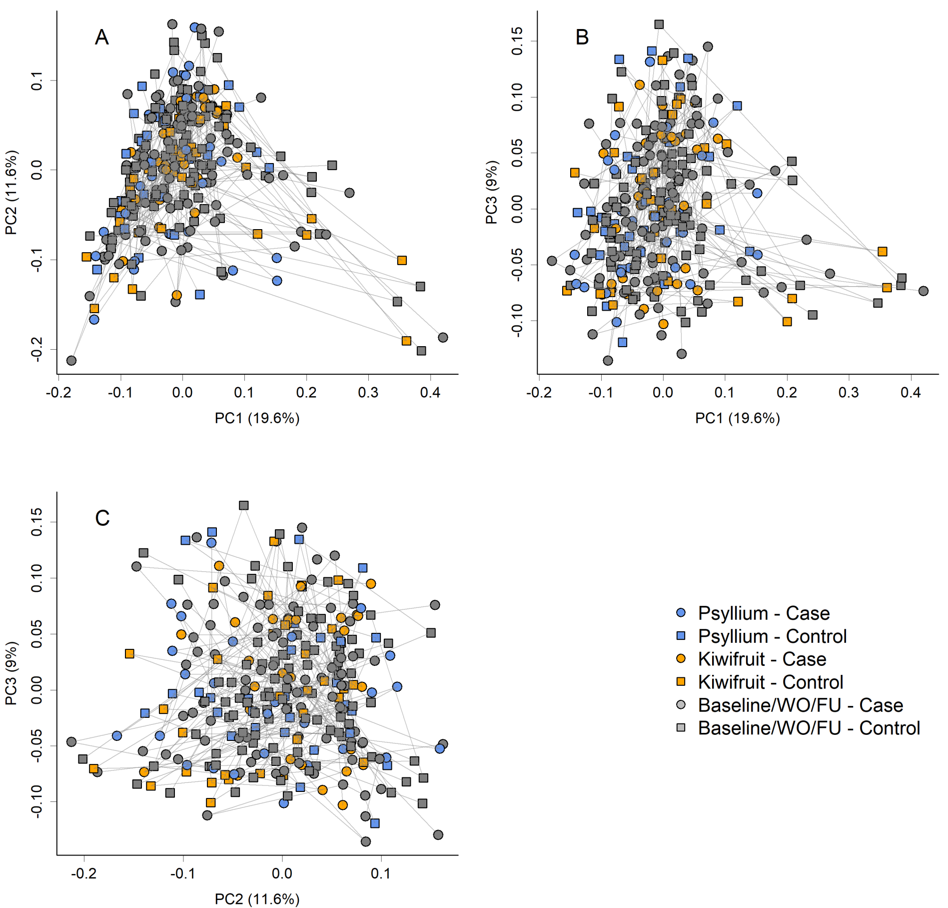


Figure 5. 5: Principal component analysis (PCA) of the microbial gene at the highest functional level (KEGG level 4) of subjects with constipation predominant FGIDs (IBS-C or FC) or controls consuming two gold kiwifruit a day or a fibre-matched active control psyllium, for four weeks.
The PCA scores plots (**A**) PC1 vs PC2, (**B**) PC1 vs PC3, and (**C**) PC2 vs PC3 show high variation between participants and within the same individual, as no distinct sample clustering was observed between participants, type of intervention, or between intervention phases, regardless of the digestive health status of the participants. The percentage values associated with the axes refer to the proportion of total variance in the data explained by each principal component, which identifies the most important components for explaining the variation in the data. Shapes indicate subjects with constipation predominant FGIDs (IBS-C or FC, Circle) or controls (Square). Colours mean the intervention with psyllium (blue) or kiwifruit (orange). The colour grey indicated baseline, washout (WO) or follow up (FU) phases. Lines join samples from the same participant.

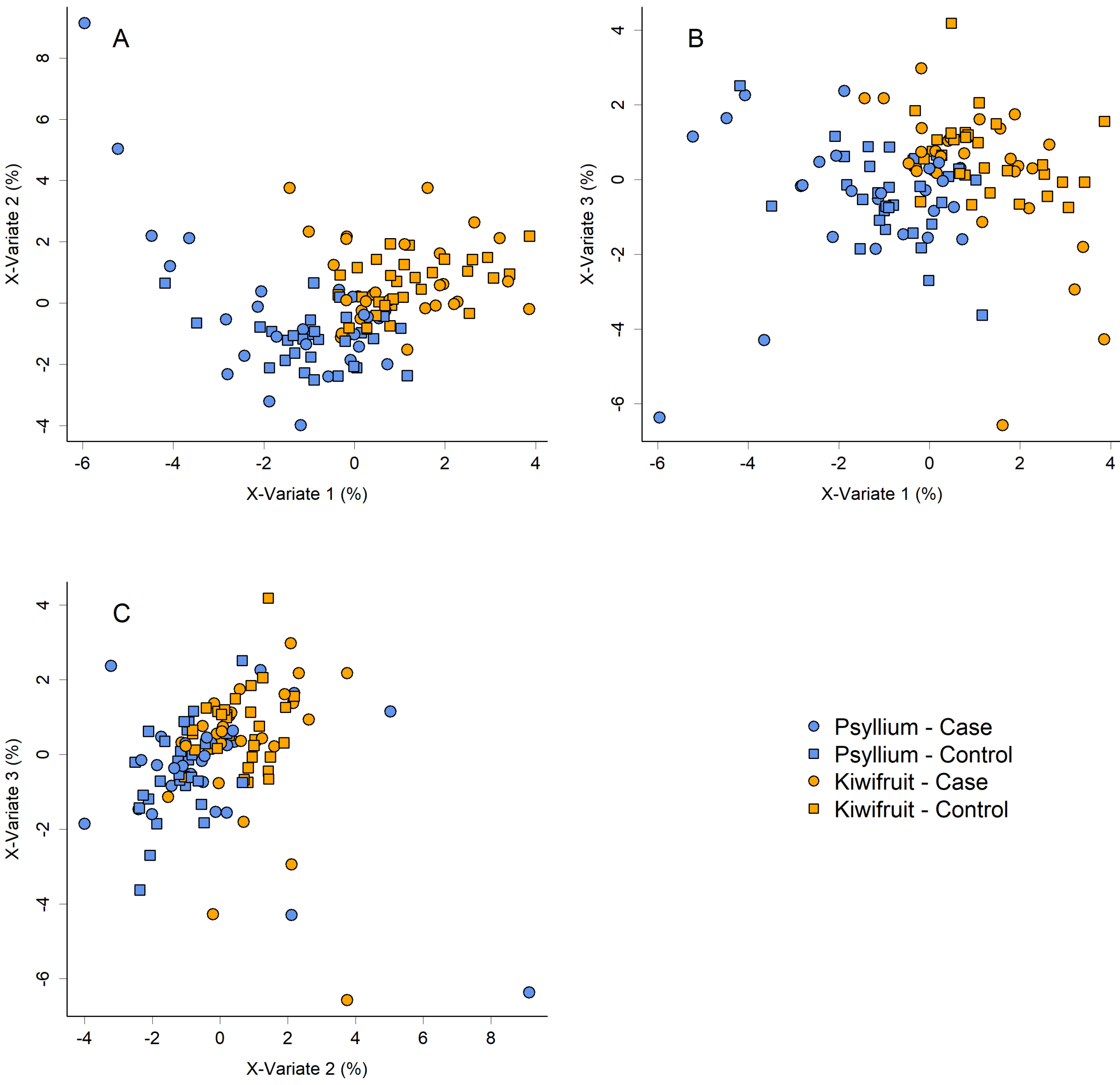


Figure 5. 6: Partial least squares discriminant analysis (PLS-DA) of the microbial taxonomic composition at the genus level of subjects with constipation predominant FGIDs (IBS-C or FC) or controls consuming two gold kiwifruit a day or a fibre-matched positive control, psyllium treatment for four weeks.

Shapes indicate subjects with constipation predominant FGIDs (IBS-C or FC, Circle) or Controls (Square). Colour means the intervention with psyllium (blue) or kiwifruit (orange). The graph shows differences in the microbial taxonomic composition for all the participants between the psyllium and kiwifruit interventions, but not between subjects with constipation predominant FGIDs and controls.

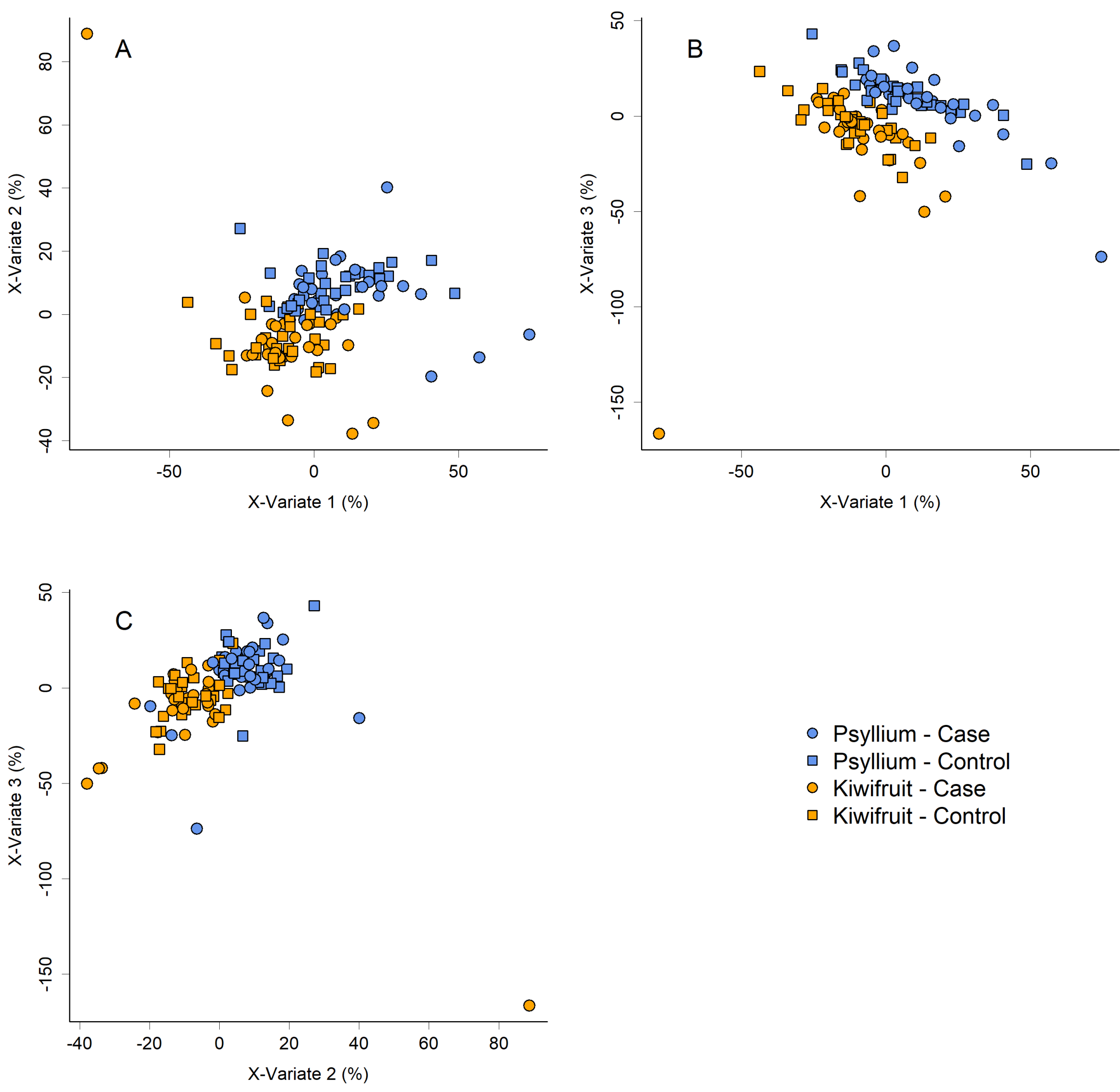


Figure 5. 7: PLSDA of the microbial gene functions at the highest functional level (KEGG level 4) of subjects with constipation predominant FGIDs (IBS-C or FC) or controls consuming two gold kiwifruit a day or a fibre-matched positive control, psyllium treatment for four weeks.

Shapes indicate subjects with constipation predominant FGIDs (IBS-C or FC, Circle) or controls (Square). Colour means the intervention with psyllium (blue) or kiwifruit (orange). The graph shows differences in microbial gene abundances for all the participants between the psyllium and kiwifruit interventions but not between subjects with constipation predominant FGIDs and controls.

5.5.3 Univariate analyses of microbial taxonomic composition

The relative abundance of two microbial families (*Bacteroidaceae* and *Eggerthellaceae*) and related genera (*Bacteroides* and *Eggerthella*) significantly changed between kiwifruit and psyllium interventions in all subjects (constipation predominant FGIDs or controls). The relative abundance of taxa from the family *Bacteroidaceae*, including the genus *Bacteroides*, was significantly lower in all subjects consuming gold kiwifruit compared to those consuming psyllium (family, mean: 30.9 vs 21.2, FDR=0.02; genus, mean: 32.7 vs 22.5, FDR=0.02) (Figure 5.8 A) (Appendix Table S). The relative abundance of taxa from the family *Eggerthellaceae*, including the genus *Eggerthella*, was significantly higher in subjects consuming gold kiwifruit compared to those consuming psyllium (family, mean: 2.47 vs 1.43, FDR=0.02; genus, mean: 0.95 vs 0.33, FDR=0.0004) (Figure 5.8 D) (Appendix Table S).

The relative abundance of taxa from the *Bacteroides* genus was higher in subjects consuming psyllium but not gold kiwifruit, compared to pre-intervention levels (mean: 33.80 vs 22.63 at baseline; FDR=0.008) (Figure 5.8 B, C) (Appendix Table T). The relative abundance of taxa from the *Eggerthella* genus was higher in subjects consuming gold kiwifruit but not psyllium, compared to pre-intervention levels (mean: 0.96 vs 0.46 at baseline; P=0.04) (Figure 5.8 E, F) (Appendix Table T).

5.5.4 Univariate analyses of microbial gene functions

While only two families and genera differed between the psyllium and kiwifruit interventions, a greater number of microbial functional genes were differentially abundant after the intervention with psyllium or kiwifruit, in comparison to baseline and between interventions (Table 5.1).

Genes involved in vitamin K2 biosynthesis (O-succinylbenzoate synthase, menaquinone-specific isochorismate synthases) (KEGG level 4) and genes involved in carnitine, betaine and crotonobetaine metabolism (carnitine operon) (SEED level 2 and 3) were relatively more abundant in all subjects (regardless of their digestive health status) fed gold kiwifruit compared to subjects fed psyllium and compared to their pre-intervention levels (Table 5.1, Figure 5.9).

Other microbial functional genes were differentially abundant after the intervention with kiwifruit in comparison to the baseline. These categories were “electron accepting reactions” (SEED level 3), “genetic information processing”, “folding, sorting and degradation” (KEGG level 2), “protein export” (KEGG level 3) and enzymes involved in carbohydrate metabolism (acyl-CoA synthetase and glycerate 2-kinase) and cellular signalling (putative metabolite transport protein) (KEGG level 4) (Table 5.1).

On the other hand, genes involved in the metabolism of bacterial capsular polysaccharides (3-deoxy-D-glycero-D-galacto-nononate 9-phosphate synthase and heparan-α-glucosaminide N-acetyltransferase) and complex carbohydrates (endo-1,4β-xylanase and formyltetrahydrofolate deformylase), were relatively more abundant in all subjects (regardless of their digestive health status) fed psyllium compared to subjects fed gold kiwifruit and compared to their pre-intervention levels (Table 5.1).

Genes involved in genetic transcription (sigma-B regulation protein RsbQ and AraC family transcriptional regulator) (KEGG level 4) and genes involved in the signal transduction system for the tolerance to colicin E2 and CreBC (SEED level 3) were also more abundant in all subjects (regardless of their digestive health status) fed psyllium compared to subjects fed gold kiwifruit and compared to their pre-intervention levels (Table 5.1).

Many microbial functional genes (74 pathways between KEGG level 3 and SEED level 2 combined and 529 enzymes/proteins between KEGG level 4 and SEED level 3 combined) were differentially abundant after the intervention with psyllium in comparison to the baseline. These genes encode numerous enzymes and processes involved in metabolising carbohydrates, glycans, amino acids and lipids, membrane transport, transcription and translation (KEGG levels 2, 3 and 4) (Appendix Table U). Microbial gene functions from SEED levels 1, 2 and 3 were also differentially abundant after the intervention with psyllium in comparison to the baseline. These genes encoded many enzymes and processes involved in “metabolism” and “secondary metabolism”, “membrane transport”, “cell envelope”, “stress response”, “DNA processing”, and “protein processing” (Appendix Table U).

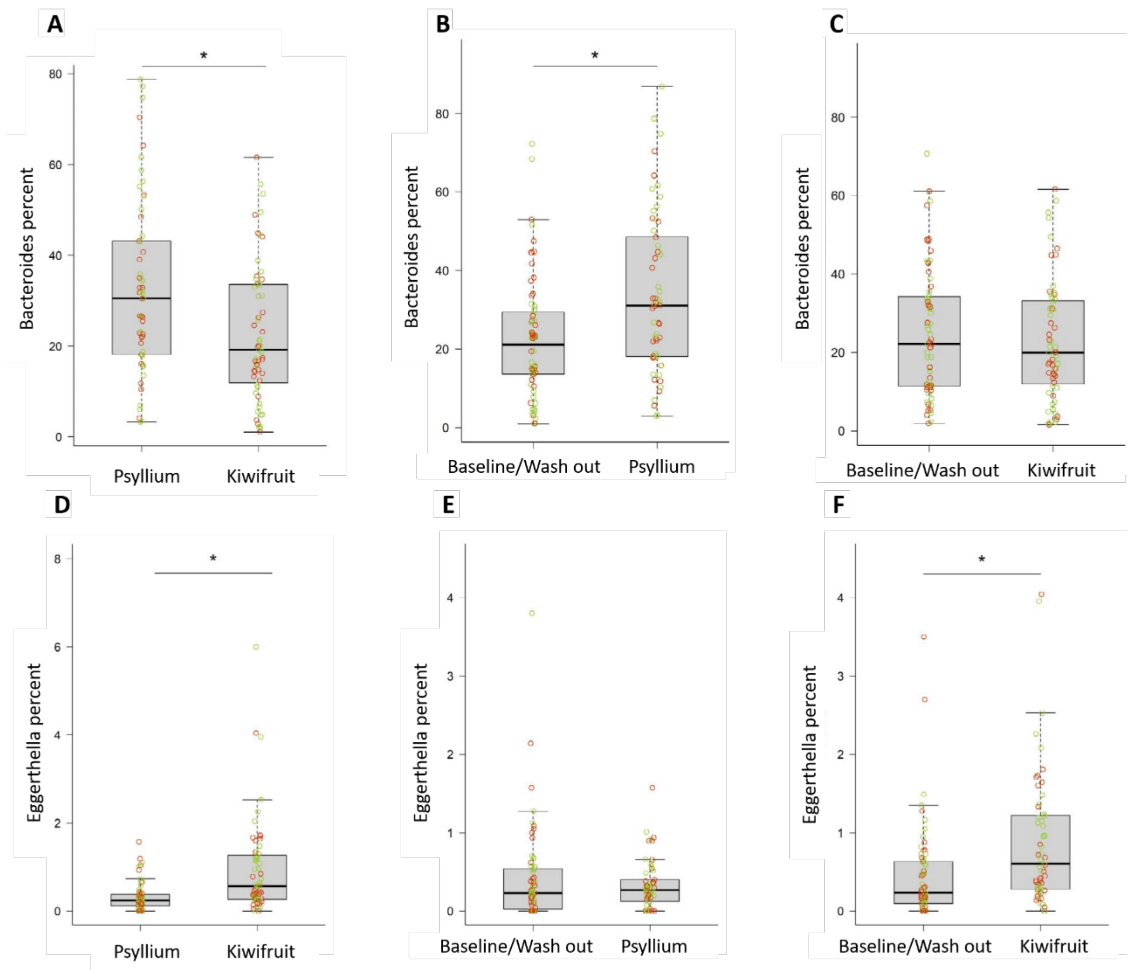


Figure 5. 8: Box plots showing the relative abundance of genera *Bacteroides* and *Eggerthella* in faecal samples of all subjects (constipation predominant FGIDs and controls combined).

(**A, D**) Box plots showing the relative abundance of genera *Bacteroides* and *Eggerthella* in faecal samples of all subjects (constipation predominant FGIDs and controls combined) fed gold kiwifruit compared to subjects fed psyllium. (**B, C**) Box plots of the relative abundance of the genera *Bacteroides* and (**E, F**) *Eggerthella* in faecal samples of all subjects (constipation predominant FGIDs and controls combined) fed gold kiwifruit or psyllium compared to pre-intervention levels (Baseline/washout combined). Midline represents the median and upper and lower limits of the box showing the third and first quartile (i.e., 75th and 25th percentile), respectively and whiskers indicate 1.5 times the interquartile range. Circles represent individual data points, coloured according to case status; Red – constipation predominant FGIDs, Green – Control. Asterix (*) indicates a significant difference at FDR<0.05 between-group comparisons.

Table 5. 1: Microbial functional categories based on KEGG and SEED databases with significantly different relative abundances in faecal samples of all subjects (constipation predominant FGIDs and controls combined) consuming gold kiwifruit or psyllium in comparison to the other intervention and pre-intervention levels (baseline/washout combined).

In the column "Kiwifruit vs Psyllium", the coefficient indicates a fold change, with a positive value indicating higher abundance in the kiwifruit group and a negative value indicating higher abundance in the psyllium group. In the columns "Kiwifruit/ Psyllium vs pre-intervention", the coefficient fold change, with a positive value indicating higher abundance at baseline and a negative value indicating higher abundance after the intervention. The coefficient and false discovery rate (FDR) were declared significant at values less than 0.05 and only those values are shown. Hyphens represent FDR> 0.05.

		Kiwifruit vs Psyllium		**Kiwifruit vs pre-intervention**		**Psyllium vs pre-intervention**	
Level	**Category**	Coefficient	FDR	Coefficient	FDR	Coefficient	FDR
KEGG 4	Menaquinone-specific isochorismate synthase [EC:5.4.4.2]	0.31	0.013	-0.26	0.005	-	-
	O-succinylbenzoate synthase [EC:4.2.1.113]	0.28	0.013	-0.20	0.039	-	-
	3-deoxy-D-glycero-D-galacto-nononate 9-phosphate synthase [EC:2.5.1.132]	-0.47	0.013	-	-	-0.44	0.001
	Salicylate biosynthesis isochorismate synthase [EC:5.4.4.2]	0.35	0.013	-	-	-	-
	Sigma-B regulation protein RsbQ	-0.39	0.018	-	-	-0.39	0.009
	Heparan-α-glucosaminide N-acetyltransferase [EC:2.3.1.78]	-0.41	0.022	-	-	-0.42	0.003
	Electron transfer flavoprotein-quinone oxidoreductase [EC:1.5.5.-]	0.22	0.022	-	-	-	-
	Porphyrinogen peroxidase [EC:1.11.1.-]	-0.34	0.022	-	-	-0.35	0.003
	Endo-1,4β-xylanase [EC:3.2.1.8]	-0.22	0.022	-	-	-0.23	0.004
	5-oxoprolinase (ATP-hydrolysing) subunit C [EC:3.5.2.9]	0.29	0.022	-	-	-	-
	AraC family transcriptional regulator	-0.27	0.022	-	-	-0.29	0.005
	Carnitine-CoA ligase [EC:6.2.1.48]	0.28	0.025	-	-	-	-
	ATP-binding cassette, subfamily C, bacterial CydD	0.25	0.025	-	-	-	-
	Formyltetrahydrofolate deformylase [EC:3.5.1.10]	-0.27	0.026	-	-	-0.23	0.01
	Protoporphyrin/coproporphyrin ferrochelatase [EC:4.99.1.1 4.99.1.9]	0.23	0.028	-	-	-	-
	23S rRNA (adenine2030-N6)-methyltransferase [EC:2.1.1.266]	-0.38	0.028	-	-	-0.33	0.005
	Formate dehydrogenase major subunit [EC:1.17.1.9]	0.17	0.045	-	-	-	-

SEED 2	Betaine, crotonobetaine, L-carnitine (trimethyl ammonium compounds)	0.24	0.012	-0.18	0.014	-	-
SEED 3	Carnitine operon	0.26	0.014	-0.20	0.009	-	-
	Tolerance to colicin E2 and CreBC signal transduction system	-0.23	0.024	-	-	-0.21	0.007
KEGG 2	Folding, sorting and degradation	-	-	-0.013	0.003	-	-
	Unclassified: genetic information processing	-	-	0.03	0.007	-	-
KEGG 3	Protein export	-	-	-0.012	0.04	-	-
KEGG 4	Acyl-CoA synthetase	-	-	-0.29	0.02	-	-
	MFS transporter, putative metabolite transport protein	-	-	-0.19	0.02	-	-
	Glycerate 2-kinase	-	-	0.07	0.02	-	-

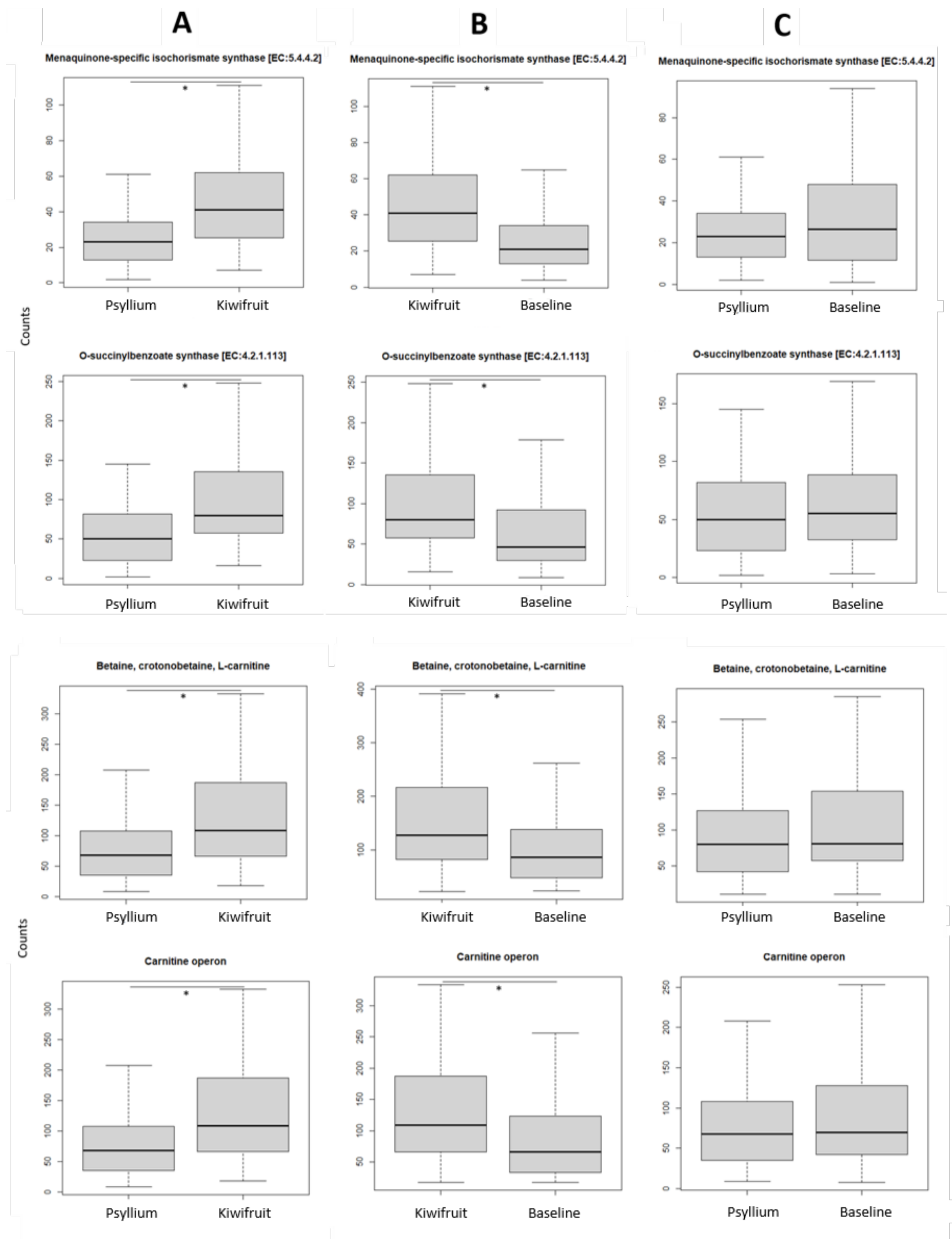


Figure 5. 9: Box plots showing the relative abundance of the microbial gene categories that were relatively more abundant in all subjects consuming two gold kiwifruit a day compared to those consuming psyllium over four weeks and compared to their pre-intervention levels.

(**A**) Four microbial gene categories (menaquinone-specific isochorismate synthases, carnitine, betaine and crotonobetaine metabolism, carnitine operon) were relatively more abundant in faecal samples of all subjects (constipation predominant FGIDs and controls combined) fed gold kiwifruit compared to subjects fed psyllium. These categories were relatively more abundant after the intervention with gold kiwifruit (**B**)

but not psyllium (**C**) compared to their pre-intervention levels (baseline/washout combined). Midline represents the median and upper and lower limits of the box showing the third and first quartile (i.e., 75th and 25th percentile), respectively and whiskers indicate 1.5 times the interquartile range. Asterix (*) indicates a significant difference at a false discovery rate (FDR) less than 0.05.

5.5.5 Canonical correlation analysis of faecal microbiota with gastrointestinal and non-gastrointestinal symptom outcomes datasets

Although no differences in microbial taxonomic composition were observed between subjects with constipation predominant FGIDs and controls after the intervention with gold kiwifruit or psyllium, some differences in microbiota composition may be associated with the symptoms reported by the subjects. The heatmap in Figure 5.10 represents data from the canonical correlation analysis between the faecal microbiota composition and symptoms of subjects with constipation predominant FGIDs consuming gold kiwifruit or psyllium. The heatmap representing the same parameters in controls consuming gold kiwifruit or psyllium is not shown.

There were no attempts to correlate symptoms with the microbial gene abundance dataset, as results from Chapter 4 showed weak associations between these parameters.

The relative abundance of the genera *Phascolarctobacterium* (r ranged between 0.51 and 0.59) and *Dialister* (r ranged between -0.52 and -0.72) were, respectively, positively and negatively associated with diarrhoea, pain, reflux, nausea, epigastric pain, anxiety and depression in subjects with constipation predominant FGIDs (Figure 5.11 A) but not in controls (Figure 5.11 B), (Appendix Table V).

The relative abundance of the genus *Subdoligranulum* was negatively correlated to total bowel movements and spontaneous bowel movements in faecal samples of subjects with constipation predominant FGIDs (r ranged from -0.67 to -68) (Figure 5.11 A), and controls (r ranged from -0.51 to -52) (Figure 5.11 B) (Appendix Table V).

The relative abundance of the genus *Oscillibacter* was negatively correlated to total bowel movements (r= -0.60) and spontaneous bowel movements (r= -0.61) in faecal samples of subjects with constipation predominant FGIDs (Figure 5.11 A) and to complete bowel movements (r= -0.52) in faecal samples of controls (Figure 5.11 B) (Appendix Table V).

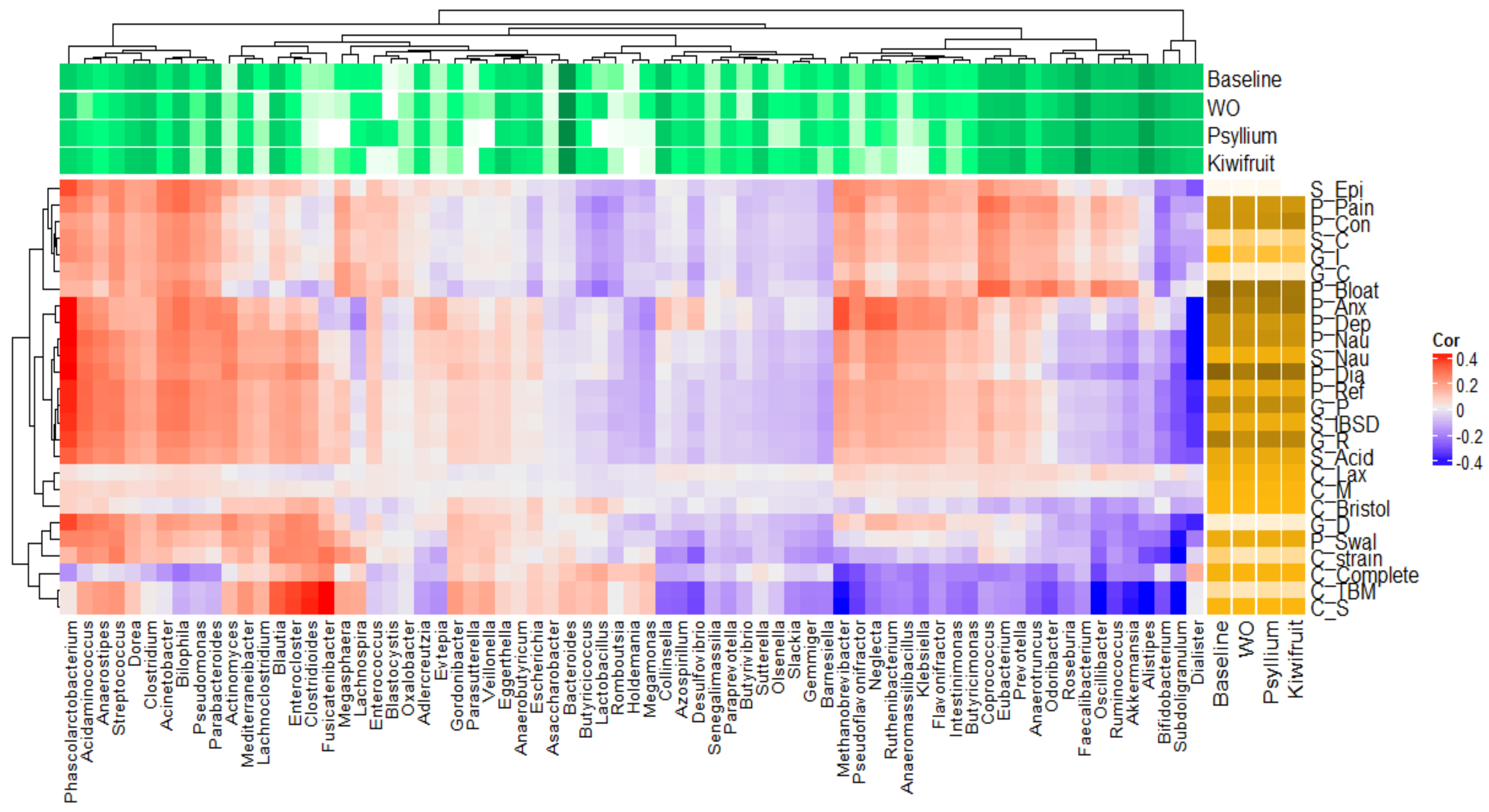
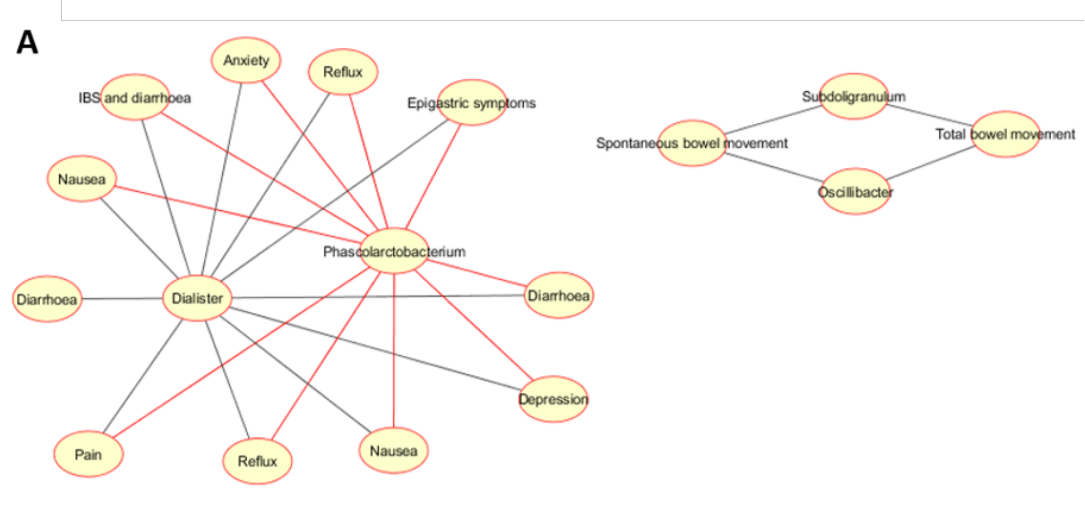


Figure 5. 10: Heatmap of the correlations between the faecal microbiota composition at the genus level and patient recorded gastrointestinal and non-gastrointestinal symptoms (patient reported outcomes, PRO) of subjects with constipation predominant FGIDs (IBS-C or FC) consuming two gold kiwifruit or psyllium daily over four weeks.

Green and yellow colours are the mean relative genus abundances and PRO scores, respectively. Positive (red) or negative (blue) correlation index (r) at > 0.5 are shown. PRO labels; G_D = GSRS Diarrhoea; G_I = GSRS Indigestion; G_C = GSRS Constipation; G_P = GSRS Pain; G_R = GSRS Reflux; S_Epi = SAGIS Epigastric symptoms; S_IBSD = SAGIS IBS and diarrhoea; S_Acid = SAGIS Acid reflux; S_Nau = SAGIS Nausea; S_C = SAGIS Constipation; P_Dep = PROMIS Depression; P_Anx = PROMIS Anxiety; P_Pain = PROMIS Pain; P_Con = PROMIS Constipation; P_Dia = PROMIS Diarrhoea; P_Swal = PROMIS Swallowing difficulties; P_Bloat = PROMIS Bloating; P_Nau = PROMIS Nausea; P_Ref = PROMIS Reflux; C_TBM = CSBM Total bowel movement; C_Complete = CSBM Complete bowel movement; C_S = CSBM Spontaneous bowel movement; C_M = CSBM Manual evacuation techniques; C_Lax = CSBM Laxative use; C-strain = CSBM Straining; C_Bristol = CSBM Bristol stool index.



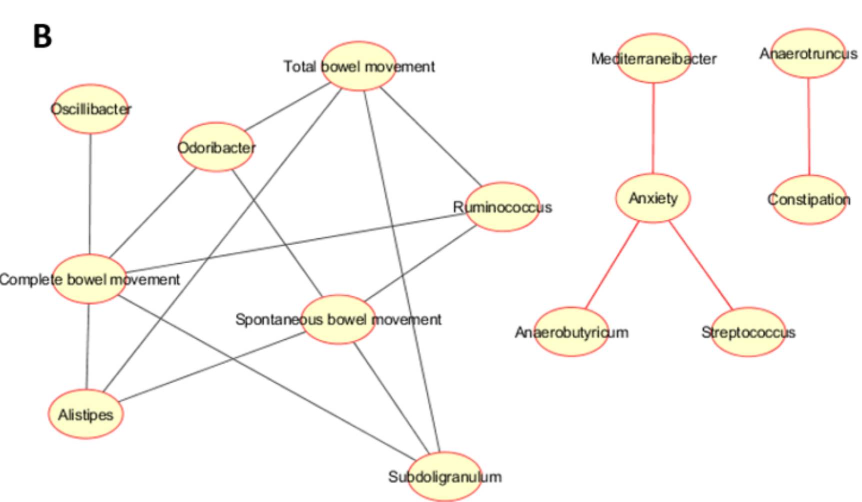


Figure 5. 11: Canonical partial least squares correlations between the faecal microbiota composition and recorded gastrointestinal symptoms in subjects with constipation predominant FGIDs (A) or controls (B), consuming two gold kiwifruit or psyllium daily over four weeks.

Red lines indicate positive correlations and grey lines indicate negative correlations. Cut-off values for positive or negative correlations were set at 0.5 and -0.5, respectively. PRO labels; P_Anx = PROMIS Anxiety; P_Con = PROMIS Constipation; C_TBM = CSBM Total bowel movement; C_Complete = CSBM Complete bowel movement; C_S = CSBM Spontaneous bowel movement; G_D = GSRS Diarrhoea; G_P = GSRS Pain; G_R = GSRS Reflux; S_Epi = SAGIS epigastric symptoms; S_IBSD = SAGIS IBS and diarrhoea; S_Nau = SAGIS Nausea; P_Dep = PROMIS Depression; P_Dia = PROMIS Diarrhoea; P_Nau = PROMIS Nausea; P_Ref = PROMIS Reflux.

5.6 Discussion

This study is the first to report the effect of a sustained, habitual consumption of two gold kiwifruit on the microbial taxonomic composition and gene abundances in faecal samples of subjects with constipation predominant FGIDs (IBS-C or FC). Some differences in the faecal microbial community composition and gene abundance profiles were identified after the two dietary interventions.

The primary differences compared to baseline were the increased relative abundance of taxa from the *Bacteroides* genus after the psyllium intervention and taxa from the *Eggerthella* genus after the kiwifruit intervention in all subjects, independent of the digestive health status. The daily consumption of psyllium for four weeks resulted in more differences in the microbial gene abundance profiles in all subjects (constipation predominant FGIDs and controls combined) in comparison to kiwifruit. However, contrary to the stated hypothesis, there was no difference in composition and gene abundance in faecal samples from subjects with constipation predominant FGIDs and controls fed gold kiwifruit or psyllium.

5.6.1 Microbial taxonomic composition

Following the two dietary interventions, the primary differences compared to baseline were the increased relative abundance of taxa from the *Bacteroides* genus after the psyllium intervention, and taxa from the *Eggerthella* genus after the kiwifruit intervention in all subjects, independent of the digestive health status. This finding agrees with the reported increased relative abundance of taxa from the *Bacteroides* genus and Bacteroidetes phylum in faecal samples of healthy subjects consuming diets high in dietary fibre in numerous studies [649-651]. Conversely, the fermentation of green kiwifruit digesta with human faecal microbiota for 18 hours decreased the relative abundance of *Bacteroides* [349].

A previous study showed differences between the healthy and the FC groups, after consuming a gold kiwifruit-derived supplement for four weeks, which increased the abundance of Clostridiales in healthy subjects and the abundance of *F. prausnitzii* in FC subjects [344]. In contrast, the current study showed no differences in the microbial composition between healthy and constipated subjects after the two interventions.

On the other hand, a higher relative abundance of taxa from the *Eggerthella* genus was also reported after consuming non-starch polysaccharides by obese males for 10 weeks [652] or red wine by healthy subjects for 20 days [653]. Red wine and kiwifruit are rich in polyphenols, and members of the *Coriobacteriaceae* family, including the genus *Eggerthella*, can produce bioactive metabolites from hydrolysis of several dietary polyphenols [654]. This observation is corroborated by the increased relative abundance of the family *Coriobacteriaceae* in subjects with prediabetes consuming gold kiwifruit for 2 weeks compared to baseline [345]. The new family *Eggerthellaceae* was previously considered bacterial genera within the *Coriobacteriaceae* family [655]. Therefore, bacterial species described as polyphenol transformers have been regrouped within the Eggerthellales order.

Many polyphenols in gold kiwifruit, such as chlorogenic acid, catechin, epigallocatechin, and quercetin, demonstrated antioxidant and antibacterial properties against pathogenic bacteria (*Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus*) *in vitro* [656] as well as improve IBS symptoms [362, 657].

Furthermore, the numerical (non-significant) decrease in the relative abundance of *E. coli* in all subjects (regardless of their digestive status) on the kiwifruit intervention provides some support for a link to the polyphenol content of gold kiwifruit (Appendix Table T). Therefore, it is plausible that consuming the whole gold kiwifruit (as opposed to without the peel here), which has a greater polyphenol and flavonoid content in the peel than in the flesh (12.8 mg/g vs. 2.7 mg/g) [656] could provide more marked effects on pathogenic bacteria.

5.6.2 Microbial gene abundances

5.6.2.1 Carbohydrate metabolism

Increased abundance of microbial genes involved in metabolising bacterial capsular polysaccharides (e.g., 3-deoxy-D-glycero-D-galacto-nononate 9-phosphate synthase) and complex carbohydrates (e.g., heparan-α-glucosaminide N-acetyltransferase) in all subjects (regardless of their digestive health status) consuming psyllium compared to those consuming kiwifruit agreed with the increased relative abundance of taxa from the *Bacteroides* genus. The gene encoding for the 3-deoxy-D-glycero-D-galacto-nononate 9-

phosphate synthase is involved in the biosynthesis of the sialic acid 3-deoxy-D-glycero-D-galacto-non-2-ulopyranosonate, which is abundant in capsular polysaccharides of bacteria that belong to the *Bacteroides* genus [658]. Heparan-α-glucosaminide N-acetyltransferase is involved in the degradation of glycosaminoglycans, high-priority carbon sources for species from the *Bacteroides* genus, such as *Bacteroides thetaiotaomicron* [659].

The *Bacteroides* genus is the most abundant in the colonic microbiota (~30%) and it harbours a broad saccharolytic potential, with some strains able to metabolise several different complex glycans [660]. In this regard, the utilisation hierarchy for *Bacteroides thetaiotaomicron* showed that the host glycosaminoglycans heparin and heparan sulphate are high-priority carbohydrates for them [661]. The Bacteroidetes phylum includes the major glycan degraders of the colonic microbiota [659], as up to 20% of their genome is dedicated to metabolising complex glycan in the form of genes primarily encoding carbohydrate-active enzymes. These results suggest that the digestion-resistant carbohydrates contained in psyllium may affect a larger part of the microbial community than kiwifruit.

5.6.2.2 Carnitine metabolism

The consumption of gold kiwifruit increased the relative abundance of genes involved in the carnitine, betaine and crotonobetaine metabolism, including those from the carnitine operon, in all subjects (regardless of their digestive health status) compared to those consuming psyllium. Carnitine can act as an osmoprotectant and/or osmolyte that bacteria can use to protect against osmotic stress [662]. It maintains a high fibre fermentation ability by the colonic microbiota and regulates levels of short-chain fatty acids (SCFAs) in the cytosol and mitochondria [663], contributing to energy maintenance for the host. *De novo* carnitine synthesis has not been confirmed in microbial species. Therefore uptake of carnitine and its immediate precursors is critical [664]. Members of the *Enterobacteriaceae* family, such as *Salmonella* and *Proteus* genera*,* and *E. coli*, can metabolise carnitine via crotonobetaine to γ-butyrobetaine in the presence of carbon and nitrogen sources during anaerobic growth [662, 665], and use carnitine in an aerobic or anaerobic environments for cellular functions (e.g., electron acceptor). In this regard, the increased relative abundance of genes related to the microbial SEED function "electron-

accepting reactions" in all subjects consuming gold kiwifruit supports this role of carnitine.

The increased relative abundance of genes involved in carnitine metabolism could result from an increased intake of vitamin C associated with consuming gold kiwifruit in all subjects. Vitamin C is an important co-factor for many enzymes in various biological functions, including L-carnitine biosynthesis [666].

5.6.2.3 Vitamin K metabolism

Many bacteria reported as menaquinone producers were observed to have a higher relative abundance in subjects with constipation predominant FGIDs in Chapter 2. These include the genera *Veillonella* and *Enterobacter*, and the species *Lactococcus lactis*, *Enterococcus faecalis*, *E. coli* and *Enterococcus faecium.* However, consuming gold kiwifruit did not change their abundances, which may have contributed to the increased expression of genes involved in vitamin K2 biosynthesis (e.g., O-succinylbenzoate synthase and menaquinone-specific isochorismate synthase).

Vitamin K quinones (menaquinones) are consumed in the diet and produced by the colonic microbiota. These quinones can be synthesised via the classical menaquinone pathway or the futalosine pathway [667]. The menaquinone-specific isochorismate synthase generates isochorismate from chorismite, a key intermediate for menaquinone ring biosynthesis in the first step of the classical pathway. Vitamin K quinone electron carriers in bacterial respiration act as a reversible redox component of the electron transfer chain and play a role in bacterial sporulation and cytochrome formation in some Gram-positive bacteria [668]. Reduced menaquinones exhibit antioxidant properties and can play a role in protecting cellular membranes from lipid oxidation [669].

5.6.3 Correlation between faecal microbiota composition and constipation predominant FGID symptoms

The link between the faecal microbiota composition, GI symptoms and non-GI symptoms was highlighted by the canonical correlation analysis for subjects with constipation predominant FGIDs and control subjects consuming gold kiwifruit or psyllium. These

associations differed between subjects with constipation predominant FGIDs and controls, regardless of the intervention they consumed. As expected, there were more significant correlations between microbial composition with GI and non-GI symptoms in subjects with constipation predominant FGIDs than in controls.

Members of the *Veillonellaceae* family, such as *Phascolarctobacterium* and *Dialister* genera, were positively and negatively associated with GI and non-GI symptoms, including diarrhoea, pain, reflux, nausea, epigastric pain, anxiety and depression. Conversely, these genera showed opposing correlations to inflammatory markers in psoriasis [670] and major depressive disorders [671].

The opportunistic pathogenic genus *Oscillibacter* was negatively associated with total and spontaneous bowel movements in subjects with constipation predominant FGIDs. These results are consistent with *Oscillibacter*-like organisms being considered slow-growing microbes and their negative association with mean faecal frequency [672] and positive association with slow colonic transit time [673], constipation [674] and harder faeces [675]. In addition, *Oscillibacter* produces valeric acid as its main metabolic end product, a homolog of neurotransmitter γ-aminobutyric acid, which abundance negatively associates with depression [671, 676]. These findings support the link between the colonic microbiota and health, including mental health.

Although the relative abundance of *Oscillibacter* and *Dialister* genera was increased in subjects with constipation predominant FGIDs in Chapter 2, it did not differ after the intervention phase compared to baseline and between the two interventions. Therefore, the observed symptom improvement after the intervention with kiwifruit does not seem to be specifically linked to the relative abundance of the genera *Oscillibacter* and *Dialister,* nor the increased relative abundance of the genus *Eggerthella.*

Given the fact that the two interventions mostly affected microbial predicted functions rather than microbial composition, it is plausible that the correlation between microbial functional categories and symptoms might show more associations.

5.6.4 Strengths and limitations

The major strength was the cross-over design of the PSYKI study, as it limited the effect of inter-individual variation in the microbiota, which is physiologically observed in any individual. Moreover, the participants did not alter their diet during the washout phase and only took rescue medicine in an emergency.

The major limitation was the inability to blind subjects to the food interventions they were receiving. Therefore, some bias may have introduced preconceptions related to the effect of kiwifruit and/or psyllium on constipation. A further limitation of this study is that data analysis was performed using a "complete-dataset" analysis instead of a "per-protocol" or "intention-to-treat" analysis, which may have increased attrition bias. In addition, excluding participants with missing samples or incomplete questionnaires in one or both groups may have reduced the sample size and, thus, study power [629].

Part of the study design was that the participants consumed the kiwifruit without the skin, limiting any effects observed to unpeeled fruit. However, this allowed more translatable results, as people do not usually eat the skin [677]. Despite this, studies have shown that whole-fruit consumption can have different effects on biological and clinical parameters compared to the consumption of the flesh [343, 656]. For example, the consumption of whole gold kiwifruit (including the skin) increases fibre, vitamin E, and folate contents by 50%, 32% and 34%, respectively, and 30% of the total phenolic fraction comes from the skin [678]. Therefore, the gold kiwifruit skin has a higher content of phenolics and flavonoids and may result in greater antioxidant and antibacterial activity than the flesh [656].

Other limitations relate to the collection and microbial structure of faecal samples [412]. Firstly, the inability to collect faecal samples into a nucleic acid stabiliser may have affected the composition of the faecal microbiota. Freezing immediately the samples at -80°C was not possible, as participants collected their samples at home and kept at -20°C for up to 24 hours. Although this protocol is sub-optimal, it is the recommended process to preserve microbial DNA for faecal samples not chemically preserved or immediately frozen. Secondly, sample homogenisation is critical to reducing the intraindividual variation to detect each component [514]. Faecal microbiota is a proxy of the microbiota at the site of effects and may bias estimates as differences in microbial composition between segments, lumen, mucosa and faeces have been reported [516-518]. Such

information may be relevant for the evaluation of symptoms like nausea and diarrhoea in IBS because of the overlap with small intestinal bacterial overgrowth.

5.7 Conclusion

This study is the first to assess the impact of consuming two gold kiwifruit daily on microbial composition and functional potential and (GI/non-GI) symptom parameters in subjects with constipation predominant FGIDs and controls. The daily consumption of two gold kiwifruit for four weeks increased the relative abundance of the *Eggerthellaceae* family and the *Eggerthella* genus, while the intervention with psyllium increased the *Bacteriodaceae* family and the *Bacteroides* genus in all subjects (constipation predominant FGIDs and controls combined).

Despite only two taxa differences between the intervention with psyllium and kiwifruit, a greater number of microbial functional genes were differentially abundant after the intervention with kiwifruit or psyllium, in comparison to baseline, and between the two interventions. These included many microbial genes involved in the metabolism of carnitine and vitamin K2 biosynthesis in those fed gold kiwifruit, or genes involved in complex polysaccharide metabolism and genetic transcription and translation, in those fed psyllium. Finally, a greater number of canonical correlations between symptoms and microbial taxa was observed in constipation predominant FGID subjects compared to controls, and particularly the opposite association of *Phascolarctobacterium* and *Dialister* with GI (diarrhoea, reflux, nausea, epigastric pain) and non-GI symptoms (anxiety and depression).

Overall, the differences in microbial composition and gene abundances suggest potential health benefits for gold kiwifruit beyond the positive effects of laxation, which were comparable to psyllium. Further studies are required to replicate these findings with the whole gold kiwifruit (with the skin) to gain a deeper insight into the mechanisms of action that may underlie these results. These studies will help to confirm and grow the evidence for the gold kiwifruit as a food-based treatment for relieving constipation and GI symptoms in subjects with constipation-predominant FGIDs, and for the support of GI health in the general population.

Chapter 6

General discussion

6.1 Background

It is now accepted that disruption in composition and/or function of the colonic microbiota contributes to functional gastrointestinal (GI) disorders (FGIDs), including irritable bowel syndrome (IBS) (IBS-D: predominantly diarrhoea; IBS-C: predominantly constipation) and their functional counterparts (FC: functional constipation; FD: functional diarrhoea) [7]. Alterations in the colonic microbiota can reflect host lifestyle, diet, microbiota-mediated immune responses and related blood biomarkers. However, there are limited reports of associations between faecal microbiota, blood or tissue immune cell expression and dysregulated gut-brain axis in FGIDs [224, 408, 602] and few specific and sensitive biological markers of GI symptom severity [679].

Understanding the role of the colonic microbiota in immune-microbe interactions in FGIDs is challenged by the multifactorial nature of these conditions and the complexity and inter-individual variability of the colonic microbiota composition. Integrating taxonomic and functional changes in the faecal microbiota and immune cell gene expression changes with GI and non-GI symptoms might be an alternative approach to identify signatures to better discriminate these disorders within subtypes or from controls.

The main hypotheses tested in this PhD dissertation were:

The combined analysis of the faecal microbiota composition, gene abundance, and peripheral blood mononuclear cell (PBMC) transcriptome profile, along with the integration of these biological datasets with GI and non-GI symptoms, provide more accurate discrimination of FGIDs within subtypes or FGIDs from healthy controls (controls) than the analysis of each dataset alone.

Changes in the faecal microbiota compositional and gene abundance profiles occurring in constipation predominant FGIDs (IBS-C or FC) and controls are different after consuming two gold kiwifruit or a fibre-match intervention (psyllium) daily for four weeks, and these changes are associated with GI and non-GI symptoms.

The main aims of this PhD dissertation were:

Characterise faecal microbial signatures in subjects with different FGID subtypes and controls to predict microbiota-mediated pathways underlying these disorders (Chapter 2).

Characterise PBMC gene expression patterns in subjects with different FGID subtypes and controls to determine whether subtype-specific profiles are linked to microbial changes (Chapter 3).

Integrate the microbiota taxonomic composition, immune gene expression and GI and non-GI symptom datasets from subjects with FGIDs and controls to identify potential relationships between variables and better understand the host-microbe interactions underlying the phenotype of constipation or diarrhoea predominant FGIDs (Chapter 4).

Evaluate a classification model of FGIDs by analysing the receiver operating characteristic (ROC) curve and area under the curve (AUC) of selected microbial composition, PBMC genes and GI and non-GI symptoms to distinguish between FGID subtypes and controls or within FGID subtypes. (Chapter 4).

Determine the effect of the daily consumption for four weeks of two gold kiwifruit or psyllium, which are known to relieve constipation symptoms, on the microbial taxonomic and gene abundance profiles of subjects with constipation predominant FGIDs (IBS-C or FC) and controls (Chapter 5).

The investigation of the immune-microbe interactions underlying FGID pathophysiology in Chapters 2, 3 and 4 relied on data obtained from the '**C**hristchurch IBS C**o**hort to Investigate **M**echanisms **Fo**r Gut **R**elief and Improved **T**ransit' (COMFORT) cohort. The data from 315 controls and participants with FGIDs were analysed as part of this PhD dissertation. Shotgun metagenomic sequencing of faecal DNA samples and mRNA-sequencing of PBMC samples were undertaken. Differences in the microbiota (taxonomic and gene abundance) and PBMC gene expression were determined using the R programme in Chapters 2 and 3.

In Chapter 4, GI and non-GI symptom data were correlated and integrated with biological datasets (microbial composition and PBMC gene expression) using **D**ata **I**ntegration **A**nalysis for **B**iomarker discovery using **L**atent c**O**mponents (DIABLO). The analysis of the ROC curves and AUC were carried out with selected biological and symptom

variables in Chapter 4 using one-vs-all comparisons to evaluate the predictor performance of a proposed classification model of FGIDs.

Finally, in Chapter 5, 36 subjects with constipation predominant FGIDs and 37 controls completed the PSYKI study, a 16-week randomised, single-blind, cross-over study, where they received either two gold kiwifruits (2 /day) or psyllium (fibre-matched active control, 2.5g/day) for four weeks. The correlation of the microbiota taxonomic composition to GI and non-GI symptoms in subjects with constipation predominant FGIDs and controls was also performed.

6.2 Specific commonly altered microbial and immune features in functional gastrointestinal disorders

The analysis conducted in Chapter 2 revealed that some microbial taxa and gene abundances were commonly altered among FGID subtypes compared to controls (Figure 6.1). These changes included:

- Increased or decreased relative abundance of taxa involved in hydrogen-consuming and short-chain fatty acid (SCFA) production.
- Increased relative abundance of taxa from the Proteobacteria phylum and pathobionts.
- Higher relative abundance of microbial genes involved in 2-ketogluconate and tyrosine metabolism, secretion systems, and micronutrient (iron and copper) utilisation.

Some microbial differences detected in the faecal samples of FGID subjects agreed with previous findings reported in the literature, including increased relative abundance of opportunistic bacteria from the Proteobacteria phylum [77, 79, 92, 474] and increased or decreased relative abundance of taxa involved in cross-feeding interactions, including hydrogen [83, 434] and SCFA metabolism [93, 448].

The increased relative abundance of hydrogen sulphide-producing microbes (*Desulphovibrionaceae* family) in FGIDs agrees with published studies that show that hydrogen sulphide accumulation plays a role in abdominal pain and motility disorders

with dysbiosis [90]. In addition, changes in the relative abundance of hydrogen-consuming microbes or hydrogen disposal pathways (acetogenesis, methanogenesis or sulphate reduction) can affect gas accumulation in the colon [88, 437], which has been associated with common symptoms of IBS (bloating and abdominal pain) [89].

Conversely, the relative abundance of taxa from the *Methanobacteriaceae* family responsible for methane production was increased in subjects with constipation and decreased in subjects with diarrhoea compared to controls. These findings agree with other studies reporting an increased relative abundance of taxa from the *Methanobrevibacter* genus in IBS-C [83] and FC [434] and a correlation between higher exhaled methane levels, slower motility and faecal firmness [67, 81-84, 435]. Decreased faecal relative abundances of methanogens have been linked to excess abdominal gas in IBS-D subjects, suggesting their microbiota may lack a normal hydrogen removal function [70, 71]. Despite this, no changes in the relative abundance of genes involved in methane or sulphur metabolism were observed in subjects with constipation or diarrhoea. The findings from Chapter 2 and published studies suggest either competition between these taxa for hydrogen disposal [436] or greater fitness of some taxa that prefers a colonic environment characterised by slow transit [87].

Similarly, the PBMC gene expression signatures of subjects with FGIDs suggest the existence of common immune pathways underlying FGIDs (Figure 6.1). Overall, genes commonly differently expressed or enriched across FGIDs included immunoglobulin (Ig) variable domains and pathways related to innate and adaptive immunity and complement system activation, respectively.

6.3 The microbial and immune signatures of the diarrhoea phenotype

The diarrhoea predominant subtypes (FD+IBS-D), compared to controls, were associated with a lower relative abundance of lactic acid bacteria and differences in the abundance of genes involved in nitrogen fixation, and lactose fermentation. Overall, diarrhoea predominant subtypes mostly showed a similar microbial taxonomic signature but a dissimilar functional signature, as the gene abundance differences were attributable to

significant changes in IBS-D rather than FD. A reason for this difference may be the lower number of subjects with FD (16) in comparison to 57 subjects with IBS-D.

A higher relative abundance of the *Peptostreptococcaceae* family, including many pathogenic anaerobic taxa [622] and the potential pathogen *Clostridioides difficile,* was detected in samples of both IBS-D and FD subjects (Figure 6.1). The role of different strains of *C. difficile* on the pathogenesis of IBS is unknown but their presence may exacerbate existing symptoms due to their toxigenic nature [505]. Furthermore, the interactions of the immune system and microbiota can restrict the ability of *C. difficile* to expand in the colon, protecting against its effects [680].

A higher relative abundance of pathogenic (*C. difficile* and other pathobionts) and lower abundance of protective protective (*Bifidobacterium spp.*, *Lactobacillus spp.*, *Clostridium* spp., and *B. fragilis)* species may create a pathogenic community, inducing or worsening symptoms in diarrhoea. In the current study, the RNA abundance of PBMC genes indicative of the activity of *C. difficile*, such as *IL-22* [681], *CXCL5* [682], *IL-8* [683] and *IL-23*, which recruit neutrophils to the colon [681], was unchanged in IBS-D and FD, compared to controls. These observations suggest that the higher abundance of C. *difficile* in subjects with IBS-D and FD did not result in active infection but might result from microbial dysbiosis.

The integration of the faecal microbiota composition, PBMC gene expression and symptoms showed a strong positive correlation between the family *Peptostreptococcaceae*, PBMC genes involved in innate immunity, neutrophil degranulation and cellular apoptosis (the leucine-rich repeat kinase 2 *LRRK2* scored the strongest positive correlations), and GI symptoms in the diarrhoea predominant group (Figure 6.1). These findings may suggest an interplay between taxa from this family and PBMC genes involved in the modulation of neutrophil activity. This hypothesis is supported by a study showing that the faecal concentration of calprotectin (not measured here), produced by activated neutrophils [192, 193], was highest in IBS-D, followed by IBS-M and IBS-C [191].

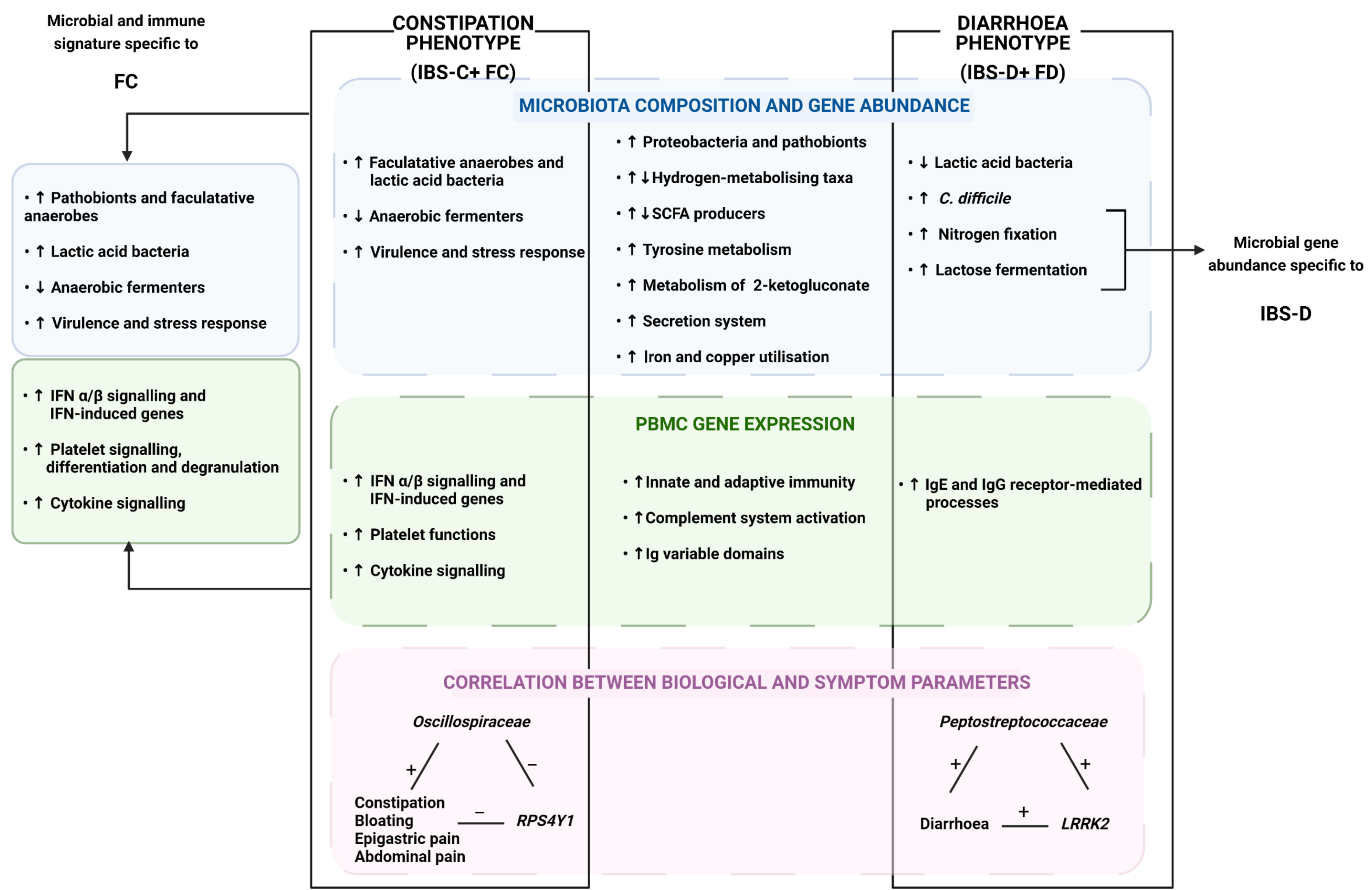

Microbial and immune signature specific to
FC
• ↑ Pathobionts and faculatative anaerobes
• ↑ Lactic acid bacteria
• ↓ Anaerobic fermenters
• ↑ Virulence and stress response
• ↑ IFN α/β signalling and IFN-induced genes
• ↑ Platelet signalling, differentiation and degranulation
• ↑ Cytokine signalling
CONSTIPATION PHENOTYPE
(IBS-C+ FC)
DIARRHOEA PHENOTYPE
(IBS-D+ FD)
MICROBIOTA COMPOSITION AND GENE ABUNDANCE
• ↑ Faculatative anaerobes and lactic acid bacteria
• ↓ Anaerobic fermenters
• ↑ Virulence and stress response
• ↑ Proteobacteria and pathobionts
• ↑↓ Hydrogen-metabolising taxa
• ↑↓ SCFA producers
• ↑ Tyrosine metabolism
• ↑ Metabolism of 2-ketogluconate
• ↑ Secretion system
• ↑ Iron and copper utilisation
• ↓ Lactic acid bacteria
• ↑ C. difficile
• ↑ Nitrogen fixation
• ↑ Lactose fermentation
Microbial gene abundance specific to
IBS-D
PBMC GENE EXPRESSION
• ↑ IFN α/β signalling and IFN-induced genes
• ↑ Platelet functions
• ↑ Cytokine signalling
• ↑ Innate and adaptive immunity
• ↑ Complement system activation
• ↑ Ig variable domains
• ↑ IgE and IgG receptor-mediated processes
CORRELATION BETWEEN BIOLOGICAL AND SYMPTOM PARAMETERS
Oscillospiraceae
+
–
Constipation
Bloating
Epigastric pain
Abdominal pain
–
RPS4Y1
Peptostreptococcaceae
+
+
Diarrhoea
+
LRRK2

Figure 6. 1: Overview of the most relevant findings obtained in this PhD thesis dissertation underlying FGID pathophysiology.

Chapter 2 (blue box) identified common and distinct microbiota profiles associated with constipation or diarrhoea phenotypes. When each FGID subtype was compared to controls, a distinctive microbial signature discriminated FC from controls and from IBS-C, suggesting FC as a distinct condition rather than part of the IBS-C spectrum. This finding was supported by findings obtained in Chapter 3 (green box), where the PBMC gene expression analysis showed that an increased expression of haemostatic and IFN-induced genes characterised FC, but not IBS-C, from controls. Differentially expressed Ig variable domains were shared among constipation or diarrhoea phenotypes. Increased expression levels of Ig variable domains associated with IgE/IgG receptor-mediated pathways characterised the immune signature of the diarrhoea phenotype. The integrative analysis conducted in Chapter 4 (pink box), revealed that symptoms related to diarrhoea were strongly positively associated with the *Peptostreptococcaceae* family and PBMC genes involved in neutrophil degranulation and cellular apoptosis, where the LRRK2 gene scored the strongest positive correlation. Symptoms of constipation (constipation, epigastric pain, abdominal pain, and bloating) were strongly positively associated with the family *Oscillospiraceae* family and negatively associated with the PBMC gene *RPS4Y.* Lines between variables indicate positive (+) or negative (-) correlations. *RPS4Y:* Y-chromosome–encoded minor histocompatibility antigen; *LRRK2:* leucine rich repeat kinase 2.

6.4 Functional constipation had a distinctive microbial and immune signature

In agreement with the literature [417, 684], the constipation phenotype (FC+IBS-C), compared to controls, was associated with a higher relative abundance of gram-negative aerobes, facultative anaerobes, and opportunistic bacteria from the γ- and δ-Proteobacteria classes (*Enterobacteriaceae* family*, Salmonella enterica* and *Escherichia coli*), along with a lower relative abundance of butyrate-producers and obligate fermenters (Bacteroidia and Clostridia classes). This finding was supported by an increased relative abundance of genes involved in gaining energy from alternative sources (2-ketogluconate and ethanolamine), virulence, plasmids, and micronutrient metabolism (Figure 6.1). However, when the subtypes were individually compared to controls, most gene abundance differences were attributable to significant changes in subjects with FC (n= 42) rather than those with IBS-C (n= 30).

These changes might provide an adaptive advantage to facultative anaerobes and opportunistic bacteria to adhere and compete for host resources. For example, species from the *Enterobacteriaceae* family (*S. enterica* and *E. coli*) can use ethanolamine as an alternative nitrogen source during anaerobiosis [482, 483], and many gram-negative aerobes and facultative anaerobes, can use non-glycolytic pathways for creating ATP (2-ketogluconate metabolism). In contrast, obligate anaerobes mainly use glycolysis [452].

Moreover, the increased abundance of microbial genes involved in the synthesis of iron chelators and copper detoxification in FC suggests the involvement of the host immune response to starve pathogens of essential metals or kill bacteria with the antimicrobial properties of copper.

These observations highlight a potential dysbiotic colonic environment in subject with FC, where the ability of opportunistic bacteria and facultative anaerobes to use alternative carbon sources would confer a competitive growth advantage on obligate fermenters and favour the spread of fitness, virulence, and reassorted plasmid-encoded genes between pathogens and commensals [685].

The relative abundance of mucin-degrading bacteria [686], including *E. coli, Akkermansia muciniphila* and taxa from the Clostridia class (e.g., *Oscillospiraceae* family, *Lachnoclostridium* genus and *Ruminococcus torques*) was increased in FC

subjects in the current study. An abnormal increase in their abundance may reduce the mucus layer thickness of the colonic epithelium, possibly contributing to impaired barrier function, increased pathogen susceptibility and inflammation [154].

This distinctive microbial signature in subjects with FC was supported by the data presented in Chapter 3 (Figure 6.1), where differentially expressed PBMC genes in subjects with FC reflected the microbiota-mediated immune activation in FC. These genes (e.g., Ig light chain genes, IFN-induced genes, IFN-regulatory factors and other genes) were implicated in pathways related to interferon IFN α/β signalling, cytokine release, complement system activation and platelet function. It is possible that pathogenic bacteria secretion systems, injecting bacterial products into the host's cells (e.g., flagellin), may trigger the IFN α/β pathways, and the increased expression of IFN-induced PBMC genes. Elevated titres of circulating antibodies to bacterial products and pathogenic bacteria have been previously detected in subjects with IBS (subtype not mentioned) [128] and FC [417].

In addition, many genes encoding for molecules contained in platelet α granules and surface markers of platelet activation had higher expression levels in FC than controls. The enrichment in many pathways linked to haemostasis could be explained by platelets forming aggregates with leukocytes in peripheral blood, especially during inflammatory states [687, 688]. Alternatively, platelets, although anucleate, contain a complex transcriptome of mRNA, miRNA, long noncoding RNA, pre-mRNA, and circular RNA [689], which can be translated into proteins that influence platelet functional responses. In this study, despite the platelets being removed during isolation, they may have contaminated the PBMC pool.

The immune signature detected in FC may reflect a potential pathogen-driven subclinical immune activation involving bacterial secretion systems, iron and copper metabolism, virulence, and immune system evasion. These observations validate treating IBS-C and FC as distinct disorders [690-694]. This finding is supported by previous evidence suggesting different pathophysiologic mechanisms in FC and IBS-C, which are not expected to be parts of a single continuum [695, 696]. However, as no symptoms reliably separate IBS-C from FC, other studies suggested that IBS-C and FC may be parts of a spectrum, with differences based on symptom severity, such as the absence or presence

of abdominal pain [697, 698]. The 2016 update to Rome IV improved the separation between FC and IBS-C, introducing higher thresholds for abdominal pain in IBS-C [699].

The integration of the microbiota taxonomic composition with PBMC genes and symptoms showed that there was a strong positive correlation between the family *Oscillospiraceae*, the PBMC gene ribosomal protein S4 Y-Linked 1 (*RPS4Y*) and constipation symptoms (constipation, bloating, epigastric pain and abdominal pain) in subjects with constipation predominant FGIDs (Figure 6.1). Results from Chapter 2 showed that the relative abundance of the *Oscillospiraceae* family was increased in subjects with constipation predominant FGIDs, but not in subjects with diarrhoea predominant FGIDs, compared to controls. Genera belonging to this family, such as *Oscillibacter* [612] and *Oscillospira* [613], have been negatively correlated with defecation frequency. In particular, the *Oscillospira* genus was positively associated with constipation and slow colonic transit times and negatively associated with Bristol stool scale [614]. The slow-growing nature of *Oscillospira* species could help explain why their relative abundances have been strongly positively linked to constipation symptoms in the current study and methane production in women with FC [88].

As reported in the literature [84], these findings suggested that the overgrowth of slow-growing bacteria, such as methanogens and members of the *Oscillospiraceae* family, may play a role in aggravating constipation in subjects with constipation, especially FC. Overall, there was a distinctive microbial and immune signature discriminating FC from IBS-C and FC from controls, suggesting that IBS-C and FC may be different disorders rather than parts of a spectrum. Furthermore, integrating these datasets with GI symptoms supports a potential role for the *Oscillospiraceae* family in constipation predominant subjects.

6.5 Symptoms most accurately discriminate functional gastrointestinal disorders subtypes, except functional constipation

An integrative analysis was performed to explore correlations between microbiota composition, PBMC gene expression and GI and non-GI symptom datasets, using highly correlated parameters to best discriminate within constipation predominant and diarrhoea

predominant subtypes and from controls. A list of variables from each dataset (microbiota composition, PBMC gene and symptom datasets) were computationally selected into three "biomarker" Panels by DIABLO to maximise the value of the correlations between variables.

The ROC curve and AUC analyses were used to determine which variable selection from microbial, PBMC and symptoms datasets best discriminated each subtype from others and from controls. The analyses compared one FGID subtype to all other groups, or the control group to all the FGID subtypes.

The analysis of the ROC curve and AUC showed that subjects with IBS-C and FC can be discriminated accurately from those without by selected PBMC genes involved in cytokine, haem and WNT signalling, or selected PBMC genes involved in neutrophil degranulation, innate immune system and apoptosis.

In particular, a combination of selected PBMC genes discriminated FC from those without with better diagnostic accuracy than selected GI and non-GI symptoms or microbial taxa. In addition, IBS-C was the only subtype that could be discriminated accurately from those without by a combination of selected taxa from the *Peptostreptococcaceae*, *Methanobacteriaceae*, *Lachnospiraceae* families, the Bacteroidia class and Clostridiales order.

IBS-C and IBS-M were accurately discriminated from those without using different combinations of selected symptoms ((constipation, belly pain, bloating and epigastric pain), (constipation, belly pain, bloating and anxiety) or (diarrhoea and depression)). Conversely, subjects with IBS-D and FD were accurately discriminated from those without only one symptom combination (diarrhoea and depression).

These findings agree with IBS-C subjects having more frequent belly pain, epigastric pain and bloating than IBS-D subjects [700]. In addition, depression levels were reported to be higher in IBS-C, IBS-D and IBS-M, and anxiety levels to be higher in IBS-C and IBS-M [701].

Except for FC, combining selected GI and non-GI symptoms remains the most accurate way to discriminate among FGID subtypes or FGID subtypes from controls than selected PBMC genes or microbial taxa. These findings confirmed that FC might have a distinctive immune signature underlying its pathophysiology.

6.6 Microbial taxonomic and functional changes after gold kiwifruit or psyllium intervention

The composition and functional potential analysis of the faecal microbiota of FGID and control subjects of the PSYKI study showed no differences in the microbial taxonomic composition or gene abundance between FGID subjects and controls. Not many taxonomic differences were detected between subjects consuming gold kiwifruit or psyllium or between each intervention compared to their pre-intervention levels, regardless of the digestive health status of the subjects.

Only the relative abundance of taxa from the genus *Bacteroides* increased after the intervention with psyllium. The *Bacteroides* genus harbours harbour a broad saccharolytic potential [660]. Subjects consuming psyllium also showed a higher abundance of microbial genes involved in the complex carbohydrate and capsular polysaccharide metabolism, genetic transcription and translation, and signal transduction compared to their pre-intervention levels or subjects consuming kiwifruit. Since *Bacteroides* is one of the most abundant genera in the microbial community, these results suggest that psyllium can significantly impact on the microbial community. The abundance of *Bacteroides* was reported to increase with diets high in fibre [649-651].

However, fibre content cannot explain the increased abundance of this genus after the intervention with psyllium but not after the intervention with gold kiwifruit, as the two interventions were fibre-matched. A possible explanation is that psyllium and gold kiwifruit contain different fibre types. Psyllium contains 70% of soluble fibre and 30% of insoluble fibre, while the gold kiwifruit contains 30% of soluble fibre (pectic polysaccharides) and 70% of insoluble fibre (cellulose/hemicellulose) [325, 702]. In comparison to insoluble fibre, soluble fibre can be easily accessed and fermented by the colonic microbiota. Conversely, most insoluble dietary fibres, such as cellulose and hemi-cellulose, are generally poorly fermented by colonic bacteria but increase faecal bulk and GI transit time, likely reducing the time available for colonic bacterial fermentation [703].

In contrast, the relative abundance of taxa from the *Eggerthella* genus increased with the kiwifruit intervention, similar to when subjects consumed dietary polyphenols [654]. Furthermore, the main polyphenols in the gold kiwifruit were recently shown to exert

antioxidant [704, 705] and antibacterial properties against many pathogenic bacteria, including *E. coli* [656, 705]. Kiwifruit consumption also increased the abundance of microbial genes involved in carnitine metabolism and vitamin K2 biosynthesis.

Despite only two taxa differences between the intervention with psyllium and kiwifruit, a greater number of microbial functional genes were differentially abundant after the intervention with psyllium or kiwifruit, in comparison to baseline, and between the two interventions. Many taxonomic differences could have been at the strain level and, therefore, not detectable using shotgun metagenomic sequencing.

Considering that improvements in GI symptom parameters were observed in the PSYKI study [645], the faecal microbiota may not be the main parameter driving or associated with symptom relief with interventions in subjects with constipation. Alternatively, excluding participants with missing samples or incomplete questionnaires from one or both groups may have reduced the sample size and thus study power, as significant results were only obtained when constipated subjects and controls were analysed, regardless of their digestive health status. The sample size could have been not large enough to detect differences in microbial composition but enough to detect differences in GI/non-GI symptoms.

Integrating symptoms with microbial signatures from subjects with constipation or controls of the PSYKI study confirmed some of the findings observed in the multi-dataset integrative analysis (Chapter 4) and provided new information. In particular, the relative abundance of the genus *Oscillibacter* was negatively correlated to bowel movements in subjects with constipation predominant FGIDs and controls.

On the other hand, symptoms reported by the participants of the PSYKI study, including diarrhoea, pain, reflux, nausea, epigastric pain, anxiety and depression, were positively associated with the genus *Phascolarctobacterium* and negatively associated with the genus *Dialister* in subjects with constipation predominant FGIDs but not in controls. This finding is novel.

6.7 Strengths and limitations

The current study has several strengths. The COMFORT cohort was a well-defined study of FGID and controls, which generated over 300 samples with rigorous inclusion/exclusion criteria, a clinical interview by a gastroenterologist, and questionnaires of GI and non-GI symptoms, dietary information, demographic information, and psychological variables. Enrolment processes ensured the completion of the questionnaires, efficient return of biological samples, and close timing of data collection. In addition, the COMFORT cohort included dietary assessment and characterisation of microbial and immune signatures of FGIDs other than IBS, which has been mostly ignored in other cohorts.

Shotgun metagenomic analyses of microbial DNA extracted from faecal samples allowed the investigation of the composition and functional potential of the microbiota. These analyses provided data on community structure and diversity, new taxa and genes, and insights into the metabolic pathways potentially encoded in the microbial community of subjects with FGIDs. This knowledge helped to identify potential pathways and markers of dysbiosis discriminating within FGID subtypes and between FGIDs and controls. These data could not be gained using a 16S ribosomal RNA analysis, which does not provide information on the functional capability of the colonic microbiota.

Moreover, using PBMCs in the RNA gene expression analysis was an advantage, providing a more representative cellular pool compared to *ex vivo* models or human cell lines, which may have limitations in predicting *in vivo* responses, thus representing the complexity of FGIDs.

An additional strength was the cross-over design of the PSYKI study, as it limited the effect of inter-individual variation in the faecal microbiota, which is physiologically observed in any individual. Finally, other studies only investigated changes in the microbiota composition after the kiwifruit (gold or green) intervention, while the current study analysed the microbiota composition and gene abundance changes.

6.7.1 Limitations due to study design and inclusion and exclusion factors

The COMFORT study was a human observational study broadly comparable with other IBS cohorts and the general population, relatively small once uncontrolled diets, living conditions, ethnicity and cultural background are considered and limited to measurement

at a one-time point. Another limitation is the exclusion of participants unable to restrict laxative use during the week before sample collection. Other limitations included a cohort predominantly composed of female participants, reducing the generalisability of results.

For the PSYKI study, the inability to blind the subjects to the food intervention might have biased the results due to potential preconceptions about the effects of kiwifruit and/or psyllium on constipation. Another limitation was that subjects consumed the kiwifruit without the skin. However, other studies have shown that whole-fruit consumption can have different effects on biological and clinical parameters compared to the consumption of the flesh only [343, 656]. In particular, phenolics and flavonoids are more abundant in the peel than in the flesh, resulting in greater antioxidant and antibacterial properties against many pathogenic bacteria, including *E. coli* [656]. This finding can be particularly relevant for the results reported in Chapter 5, as the abundance of bacteria with a potential role in FGIDs, including *Eggerthella* and *E. coli,* could have been more strongly affected if the fruit had been consumed with the skin.

6.7.2 Limitations related to sample collection

Faecal samples were collected to analyse the microbiota composition and gene abundances. However, faecal samples are a proxy for the colonic microbiota and are not fully representative of the microbiota at the site of disease, and do not provide information about the localisation of the microbiota differences (e.g., ascending vs. descending colon, luminal vs. associated to the colonic epithelium, small intestine vs. colon). Moreover, the overlap with other clinical conditions in IBS, such as bacterial overgrowth in the small intestine, may affect the composition of the faecal microbiota, potentially introducing some bias.

In addition, the impossibility to collect faecal samples into a nucleic acid stabiliser may have affected the composition of the faecal microbiota. Freezing immediately the samples at -80°C was not possible, as participants collected their samples at home and kept at 4°C for up to 24 hours. This is the recommended process to preserve microbial DNA for faecal samples not chemically preserved or immediately frozen.

6.7.3 Limitations related to laboratory and data analytical procedures

Limitations in DNA extraction and sequencing protocols may affect the representation of different species in the extracted DNA samples, possibly leading to erroneous estimates of taxonomic diversity. For example, the faecal microbiota is unequally distributed within a faecal sample, and the homogenisation of the sample can perturb faecal biostructure and reduce the intraindividual variation in the detection of each faecal microbiota component [514]. Moreover, microbial samples can be highly heterogeneous, with high variation in microbial composition between subjects and over time, making the identification of patterns and associations in metagenomic data challenging. The variability from individual lab members processing samples may affect the accuracy and reliability of experimental results. Several factors contribute to this variability, including different skills or protocol adherence of lab members, sample handling and lab environmental conditions. Despite the significance of this variability depends on the specific experiments conducted, it is worth to be mentioned as a potential limitation, as PBMC samples were processed in different labs. In addition, there are some limitations regarding metagenomic analyses, such as potential errors in taxonomy and annotations, as taxonomic classifications are always changing and updating. Databases can be incomplete or biased towards certain microbial taxa, making the reads often "unclassified" at any taxonomic level. This can lead to inaccurate taxonomic assignments and functional predictions. The main underlying reason is that the current classification has been limited to pure cultures, although most microorganisms cannot yet be cultured in laboratory settings and remain uncharacterised [706].

Similarly, differences in other low-abundance taxa or individual bacterial strains may not be detected, as non-stringent similarity thresholds can result in distinct subspecies being assigned to the same strain. This observation would have been relevant for discriminating different *E. coli* strains, including several potentially pathogenic strains with different virulence genes. Low gene count in metagenomic data can be caused by various factors, including low sequencing depth, poor quality of sequencing reads, incomplete reference databases, low abundance of certain microbial taxa, and the complexity of microbial communities. For these reasons, the statistical power and metagenomic data interpretation

can be challenging, requiring careful statistical analyses to avoid false positive and false negative results.

Other potential limitations include that data pre-processing steps, quality filtering, adapter trimming, and read merging, can introduce biases and errors in the data. Moreover, the accuracy and reliability of the results can be affected by the choice of software and computational parameters used. Finally, result interpretation can be subjective and affected by the assumptions and biases of the analysis method chosen.

Although PBMCs provide a valuable insight into the immune status at the systemic level, they may not be entirely suitable for investigating low-grade inflammation in FGIDs. This is due to the fact that PBMCs may not fully reflect a potential sub-clinical inflammation in the colon, which could be hard to detect at the systemic level.

The main limitation of microbial and PBMC gene abundance data is that they cannot predict protein levels in many cases, reflecting the differential activity of mechanisms involved in the temporal delay between transcription and protein production. These processes include mRNA maturation, export, and translation [591-593]. Furthermore, transcriptional silencing and repression in bacteria [512, 513], which are ways to control gene expression [707, 708], may also affect the analysis of microbial functionality. In addition, microbes can also interfere with the expression of PBMC microRNAs to modify immune signalling, autophagy, or apoptotic machinery, evading the defence mechanisms of host cells.

6.8 Future perspectives

Metagenomic data provides comprehensive information regarding the structure and functional potential of the microbial community, as shown in this PhD dissertation. However, metagenomic data analysis is complicated by the presence of closely related microbes with similar genomes, and it is insufficient to detect low-abundance taxa or separate of individual bacterial strains. In addition, quantifying strain-level variation is challenging, as very few species genomes have been sequenced.

Various approaches and analytical pipelines were created to overcome these problems [709]. For example, metagenomic assembly is a computational process aiming at reconstructing genes and genomes from metagenomic mixtures after deep sequencing [709, 710]. MetaSPAdes [711], Ray Meta [712], and MEGAHIT [713] are the most popular genome assembly algorithms. While a sequencing depth of 5-10 million reads per sample is usually sufficient for taxonomic profiling and functional annotation in human faecal metagenomic samples, new studies showed that deep sequencing (>20 million reads per sample) would be required to detect low-abundant taxa (<0.1% abundance) and to identify novel strains [714, 715]. Future studies using these algorithms would be needed to identify the involvement of specific bacterial strains, providing more resolution at the species level.

Although some bacteriophages and viruses showed differential abundance between FGIDs and controls in the current study, the DNA extraction protocol, sample preparation method, and bioinformatics pipelines were not optimised for these microorganisms. As a result, there are limited data about the faecal viral composition in subjects with FGIDs; some studies have reported viral clusters with differential abundance between IBS subjects and controls [716, 717]. These findings indicate the need for future research.

The next steps could include single-cell transcriptomics on PBMCs, or FACS-sorting different PBMC immune cell subtypes prior to RNA sequencing, to analyse their transcriptomes individually and better understand the role of specific immune cell subtypes in FGIDs. Other analyses could include the transcriptome of immune cells from mucosal samples instead of PBMCs, and extending the analyses to the immune cells not found in the buffy coat, such as granulocytes and platelets.

Future advancements would be required regarding the integration of multiple datasets. While informative, the analysis using the ROC curve and AUC used in this research is a single dataset analysis and does not integrate multiple datasets. Future analyses are needed to integrate microbial and immune data with other biological datasets of the COMFORT cohort that have been generated by former PhD Students in the wider programme: Dr Phoebe Heenan (diet, symptoms, SCFAs, colonic tissue gene expression), Dr Shanalee James (faecal and plasma metabolome, faecal bile acids and plasma amino acids) and other researchers (plasma neurotransmitters). This integrative analysis would allow the identification of additional correlations and increase the accuracy of FGID discrimination within subtypes or compared to controls.

Correlations between biological parameters and symptoms have been identified in the current study, validating findings from other studies [88, 613, 614, 698, 622]. A challenge for future research is determining the causal relationships in such interactions. This research could be achieved by manipulating the microbiota with germ-free, gnotobiotic, xenografted and murine disease models.

As subjects in the PSYKI study consumed the kiwifruit without the skin, further studies are required to test the benefits of the whole kiwifruit (with the skin). Providing standard meals to the participants for the two weeks prior to sample collection would help elucidate the effect of food on FGID symptoms and the microbiota in subjects with FGIDs. However, intervention studies like this may be quite expensive.

Moreover, combining microbial data with symptoms and physiological measurements, such as transit time and gas volume in various segments of the GI tract, could give further insight into how transit time can affect microbial composition and the host phenotype.

Finally, replicating a long-term intervention beyond a month to a year with longitudinal sampling would elucidate if the effects observed in the PSYKI study would remain. This data would grow the evidence for the gold kiwifruit as a food-based treatment to relieve constipation and GI and non-GI symptoms.

6.9 Concluding remarks

The research in this PhD dissertation highlighted the value of using a systems biology approach to gain mechanistic insights into the microbiota-immune-mediated pathways underlying the phenotype of FGIDs. The characterisation of the faecal microbiota and the PBMC transcriptome from subjects with FGIDs, and the integration of these datasets with GI- and non-GI-symptoms emphasised the potential interactions and mechanisms that may be important in the pathophysiology of FGIDs. These findings advanced the current knowledge in the field and provided future directions for continuing research on FGIDs. The research also highlighted the value of using a systems biology approach to gain mechanistic insights into the microbiota-immune-mediated pathways underlying the phenotype of FGIDs. Finally, as diet plays an important role in modulating microbiota and immune competence, these findings could lay the groundwork for developing nutritional approaches with clinically validated efficacy applicable to the general population.

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Appendix

Data Tables are provided electronically.

Table A. Chapter 2, paragraph 2.5.2.1. Differentially abundant taxa at the phylum, class, order, family, genus, and species level in subjects with diarrhoea predominant FGIDs (IBS-D+FD) compared to controls. Odd ratios were used to rank the relative enrichment or underrepresentation of microbial taxa. OR greater than one indicated enrichment in the control group, OR less than one indicated underrepresentation in the control group. Significance was set at FDR< 0.05.

Table B. Chapter 2, paragraph 2.5.2.1.5. Differentially abundant taxa at the phylum, class, order, family, genus, and species level in subjects with IBS-D compared to controls. Odd ratios were used to rank the relative enrichment or underrepresentation of microbial taxa. OR greater than one indicated enrichment in the control group, OR less than one indicated underrepresentation in the control group. Significance was set at FDR< 0.05.

Table C. Chapter 2, paragraph 2.5.2.1.5. Differentially abundant taxa at the phylum, class, order, family, genus, and species level in subjects with FD compared to controls. Odd ratios were used to rank the relative enrichment or underrepresentation of microbial taxa. OR greater than one indicated enrichment in the control group, OR less than one indicated underrepresentation in the control group. Significance was set at FDR< 0.05.

Table D. Chapter 2, paragraph 2.5.2.2. Differentially abundant taxa at the phylum, class, order, family, genus, and species level in subjects with constipation predominant FGIDs (IBS-C+FC) compared to controls. Odd ratios were used to rank the relative enrichment or underrepresentation of microbial taxa. OR greater than one indicated enrichment in the control group, OR less than one indicated underrepresentation in the control group. Significance was set at FDR< 0.05.

Table E. Chapter 2, paragraph 2.5.2.2.5. Differentially abundant taxa at the phylum, class, order, family, genus, and species level in subjects with IBS-C compared to controls. Odd ratios were used to rank the relative enrichment or underrepresentation of microbial taxa. OR greater than one indicated enrichment in the control group, OR less than one indicated underrepresentation in the control group. Significance was set at FDR< 0.05.

Table F. Chapter 2, paragraph 2.5.2.2.5. Differentially abundant taxa at the phylum, class, order, family, genus, and species level in subjects with FC compared to controls. Odd ratios were used to rank the relative enrichment or underrepresentation of microbial taxa. OR greater than one indicated enrichment in the control group, OR less than one indicated underrepresentation in the control group. Significance was set at FDR< 0.05.

Table G. Chapter 2, paragraph 2.5.3.1. Relative abundances of genes annotated using SEED and KEGG functional categories that differed between subjects with diarrhoea predominant FGIDs (IBS-D+FD) compared to controls. Differentially abundant functional categories between groups were identified by performing a pairwise statistical analysis with a quasi-likelihood test. Positive logFC indicates higher abundance in the FGIDs (IBS-D+FD) group and negative logFC indicates higher abundance in the control group. Significance was set at FDR< 0.05.

Table H. Chapter 2, paragraph 2.5.3.1. Relative abundances of genes annotated using SEED and KEGG functional categories that differed between subjects with IBS-D compared to controls. Differentially abundant functional categories between groups were identified by performing a pairwise statistical analysis with a quasi-likelihood test. Positive logFC indicates higher abundance in the IBS-D group and negative logFC indicates higher abundance in the control group. Significance was set at FDR< 0.05.

Table I. Chapter 2, paragraph 2.5.3.1. Relative abundances of genes annotated using SEED and KEGG functional categories that differed between subjects with FD compared to controls. Differentially abundant functional categories between groups were identified by performing a pairwise statistical analysis with a quasi-likelihood test. Positive logFC indicates higher abundance in the FD group and negative logFC indicates higher abundance in the control group. Significance was set at FDR< 0.05.

Table L. Chapter 2, paragraph 2.5.3.2. Relative abundances of genes annotated using SEED and KEGG functional categories that differed between subjects with constipation predominant FGIDs (IBS-C+FC) compared to controls. Differentially abundant functional categories between groups were identified by performing a pairwise statistical analysis with a quasi-likelihood test. Positive logFC indicates higher abundance in the FGIDs (IBS-C+FC) group and negative logFC indicates higher abundance in the control group. Significance was set at FDR< 0.05.

Table M. Chapter 2, paragraph 2.5.3.2. Relative abundances of genes annotated using SEED and KEGG functional categories that differed between subjects with IBS-C compared to controls. Differentially abundant functional categories between groups were identified by performing a pairwise statistical analysis

with a quasi-likelihood test. Positive logFC indicates higher abundance in the IBS-C group and negative logFC indicates higher abundance in the control group. Significance was set at FDR< 0.05.

Table N. Chapter 2, paragraph 2.5.3.2. Relative abundances of genes annotated using SEED and KEGG functional categories that differed between subjects with FC compared to controls. Differentially abundant functional categories between groups were identified by performing a pairwise statistical analysis with a quasi-likelihood test. Positive logFC indicates higher abundance in the FC group and negative logFC indicates higher abundance in the control group. Significance was set at FDR< 0.05.

Table O. Chapter 3, paragraph 3.5.4. List of the DEGs sorted by logFC in subjects with FC compared to controls. Differentially expressed genes between controls and subjects with FC were identified using a two-group comparison with a quasi-likelihood F-test. Transcripts with an FDR< 0.05 were considered significantly differentially expressed. LogFC: log fold change. A positive logFC indicates higher expression/abundance in the FC group and negative logFC means higher expression/abundance in the control group. logCPM: log counts per million, indicating the expression level.

Table P. Chapter 3, paragraph 3.5.5. List of the DEGs sorted by logFC in subjects with constipation predominant FGIDs (IBS-C+ FC) compared to controls. Differentially expressed genes between controls and subjects with constipation predominant FGIDs were identified using a two-group comparison with a quasi-likelihood F-test. Transcripts with an FDR< 0.05 were considered significantly differentially expressed. LogFC: log fold change. A positive logFC indicates higher expression/abundance in the constipation predominant FGID group and negative logFC means higher expression/abundance in the control group. logCPM: log counts per million, indicating the expression level.

Table Q. Chapter 3, paragraph 3.5.5. List of the DEGs sorted by logFC in subjects with diarrhoea predominant FGIDs (IBS-D+ FD) compared to controls. Differentially expressed genes between controls and subjects with diarrhoea predominant FGIDs were identified using a two-group comparison with a quasi-likelihood F-test. Transcripts with an FDR< 0.05 were considered significantly differentially expressed. LogFC: log fold change. A positive logFC indicates higher expression/abundance in the constipation predominant FGID group and negative logFC means higher expression/abundance in the control group. logCPM: log counts per million, indicating the expression level.

Table R. Chapter 4, paragraph 4.5.1.2. Correlations between variables from the faecal microbiota composition, PBMC genes and symptom datasets related to Panels 1 and 3 combined (cut-off value for

positive or negative correlations = 0.6), and Panel 2 (cut-off value for positive or negative correlations = 0.5). r= correlation coefficient.

Table S. Chapter 5, paragraph 5.5.3. Relative abundance of taxa at the family and genus level that had a differential relative abundance in subjects on the gold kiwifruit intervention compared to those on the psyllium intervention, or in subjects consuming psyllium compared to those consuming gold kiwifruit.

Table T. Chapter 5, paragraph 5.5.3. Relative abundance of taxa at the family and genus level that had a differential relative abundance in subjects on the gold kiwifruit or psyllium intervention compared to pre-intervention levels (baseline/washout combined).

Table U. Chapter 5, paragraph 5.5.4. Relative abundance of microbial gene functions (KEGG levels 2, 3 and 4; SEED levels 1, 2 and 3) that were differentially abundant after the intervention with psyllium compared to pre-intervention levels (baseline/washout combined).

Table V. Chapter 5, paragraph 5.5.5. Canonical partial least squares correlations between the faecal microbiome composition and recorded GI symptoms in subjects with constipation predominant FGIDs or controls consuming two gold kiwifruit or psyllium daily over four weeks.

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