Understanding host and microbial metabolites in functional gut disorders

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Abstract

Interactions between diet, host, and the gut microbiome can result in beneficial or detrimental effects on human health. Functional gut disorders (FGDs) are an example of the negative effects of these interactions. However, an understanding of the mechanisms behind FGDs remains unknown. Metabolomics is a powerful tool to understand possible mechanisms. It was hypothesised that key metabolite groups in faecal samples would differ between or within FGD subtypes and healthy controls reflective of mechanistic perturbations. This PhD aimed to characterise the metabolite profile of FGD individuals (functional constipation (FC), IBS-constipation (IBS-C), functional diarrhoea (FD), IBS-diarrhoea (IBS-D), IBS-mixed (IBS-M)) and healthy controls. The concentration of faecal bile acids and plasma amino acids were quantified to ascertain changes in known metabolites associated with FGDs. The faecal metabolome was characterised to potentially identify wider perturbations, and this was then integrated with dietary intake, plasma metabolome abundance and faecal microbiota composition for a systems biology analysis.

Constipation (FC + IBS-C) and diarrhoea (FD + IBS-D) were combined to determine differences between healthy controls and disease states. Faecal bile acid concentrations differed between all FGD participants and healthy controls. In the combined analysis of the diarrhoea (FD+IBS-D) and constipation (FC+IBS-C) groups, the diarrhoea group had a higher concentration of bile acids than the constipation group or healthy control group. The concentration of plasma amino acids did not differ between FGD participants and healthy controls. Furthermore, analysis of key amino acid groups showed that only the concentration of branched chain amino acids were different between all subtypes and healthy controls.

Characterisation of faecal polar, semi-polar and lipid metabolites showed a differential relative abundance of some polar and semi-polar metabolites (e.g., riboflavin, nicotinic acid) between diarrhoea and healthy control groups, and constipation and healthy control groups. Substantial changes in the abundance of some lipids (e.g., ceramides, triglycerides) were evident between constipation and healthy control groups, and diarrhoea and healthy control groups.

Integration of the faecal metabolome with other datasets (faecal microbiome, plasma metabolome, dietary intake) showed the faecal metabolome and microbiome separated healthy controls from constipation or diarrhoea. Additionally, differential positive and negative correlations were observed between faecal lipids (triglycerides and diglycerides) and microbial species (*Firmicutes* and *Bacteroidetes*).

This PhD thesis presents novel insights into the metabolite signature characterising FGD participants and healthy controls and provides directions for future research.

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Abbreviations

5-HT	Serotonin
ACTH	Adrenocorticotropic Hormone
ANOVA	Analysis Of Variance
ANS	Autonomic Nervous System
BAM	Bile Acid Malabsorption
BCAA	Branch Chain Amino Acid
BSH	Bile Salt Hydrolase
CA	Cholic Acid
CDCA	Cheno-Deoxycholic Acid
Cer	Ceramide
Che	Cholesterol Ester
CI	Confidence Interval
CNS	Central Nervous System
COMFORT	Christchurch Ibs Cohort To Investigate Mechanisms For Gut Relief And Improved Transit
CRH	Corticotrophin Releasing Hormone
CV-ANOVA	Cross Validated ANOVA
d4-CA	Deuterated (D4) Cholic Acid (internal standard)
DG	Diglyceride
DGDG	Digalactosyldiacylglycerol
EAA	Essential Amino Acid
EDTA	Ethylenediaminetetraacetic Acid
ELSISF	Economic Living Standard Index Short Form
ENS	Enteric Nervous System
ESI	Electrospray Ionisation
FAST	Food And Symptoms Times Diary
FC	Functional Constipation
FD	Functional Diarrhoea
FDR	False Discovery Rate
FGD	Functional Gut Disorder
FGF15	Fibroblast Growth Factor 15
FGF19	Fibroblast Growth Factor 19
FODMAP	Fermentable Oligo-Di Mono Saccharide And Polyols
FXR	Farnesoid X Receptor
GABA	γ-aminobutyric Acid
GBA	Gut-Brain Axis
GCA	Glyco-Cholic Acid
GCDCA	Glyco-Cheno-Deoxycholic Acid
GC-MS	Gas Chromatography-Mass Spectrometry
GDCA	Glyco-Deoxycholic Acid
GF	Germ Free

GHDCA	Glyco-Hyo-Deoxycholic Acid
GLCA	Glyco-Litho Cholic Acid
GUDCA	Glyco-Urso-Deoxycholic Acid
HADS	Hospital Anxiety And Depression Scale
HC	Healthy Control
HCA	Hyo-Cholic Acid
HDCA	Hyo-Deoxycholic Acid
HMDB	Human Metabolomics Database
HPA	Hypothalamic Pituitary Adrenal Axis
HVN	High Value Nutrition
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
IBS-A	Irritable Bowel Syndrome – Alternating
IBS-C	Irritable Bowel Syndrome -Constipation
IBS-D	Irritable Bowel Syndrome – Diarrhoea
IBS-M	Irritable Bowel Syndrome – Mixed
IBS-U	Irritable Bowel Syndrome - Undefined
IL-10	Interleukin-10
IL-17	Interleukin-17
ILA	Iso-Lithocholic Acid
LCA	Litho-Cholic Acid
LC-MS	Liquid Chromatography-Mass Spectrometry
LiH	Lithium Heparin
LNAA	Large Neutral Amino Acid
LogFC	Log Fold Change
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
LPG	Lysophosphatidylglycerol
LRT	Likelihood Ratio Testing
MG	Monoglyceride
MGDG	Monogalactosyldiacylglycerol
ModHNEHS	Modified Hunter New England Health Survey
MRM	Multiple Reaction Monitoring
MS/MS	Tandem Mass Spectrometry
NEAA	Non-Essential Amino Acid
NMR	Nuclear Magnetic Resonance Spectrometry
OAHFA	(O-Acyl)-Ω-Hydroxy Fatty Acids
OTU	Operational Taxonomic Unit
PBMC	Peripheral Blood Mononuclear Cells
PC	Phosphatidylcholine
PCA	Principle Component Analysis
PE	Phosphatidylethanolamine
PEt	Phosphatidylethanol
PG	Phosphatidylglycerol

PI	Phosphatidylinositol
PLS-DA	Partial Least Squares-Discriminant Analysis
PROMIS-ED	Patient-Reported Outcomes Measurement Information System-Emotional Distress
PROMIS-GI	Patient-Reported Outcomes Measurement Information System-Gastrointestinal
PS	Phosphatidylserine
SAGIS	Structured Assessment Of Gastrointestinal Symptoms Scale
SCFA	Short Chain Fatty Acid
SERT	Serotonin Reuptake Transporter
SF12	Short Form 12 Health Survey
SiE	Sitosteryl Ester
SM	Sphingomyelin
So	Sphingoshine
SQDG	Sulfoquinovosyl Diacylglycerol
StE	Stigmasteryl Ester
Taurine	Taurine
TCA	Tauro-Cholic Acid
TCDCA	Tauro-Cheno-Deoxycholic Acid
TDCA	Tauro-Deoxycholic Acid
TG	Triglyceride
TGF-β1	Transforming Growth Factor
THDCA	Tauro-Hyo-Deoxycholic Acid
TLCA	Tauro-Litho Cholic Acid
TNF-α	Tumour Necrosis Factor-A
TUDCA	Tauro-Urso-Deoxycholic Acid
TαMCA	Tauro-Alpha-Muricholic Acid
TβMCA	Tauro-Beta-Muricholic Acid
UC	Ulcerative Colitis
UDCA	Urso-Deoxycholic Acid
UPLC	Ultra-Performance Liquid Chromatography
VIP	Variable Importance In Projection
ZyE	Zymosteryl
βMCA	Beta-Muricholic Acid

Chapter One
Review of literature

Sections of this chapter have been published: see Appendix for full paper

James, S. C., Fraser, K., Young, W., McNabb, W. C. & Roy, N. C. (2020). Gut microbial metabolites and biochemical pathways involved in irritable bowel syndrome: effects of diet and nutrition on the microbiome. *The Journal of Nutrition*, *150*(5), 1012-1021.

Chapter One

1.1. Introduction

Functional gut disorders (FGDs) cause pain, discomfort and can debilitate a person's quality of life [1]. However, despite a high prevalence rate worldwide [2], the cause of FGDs remains relatively unknown based on a lack of physical abnormalities and the multiple phenotypes [3, 4]. Additionally, the functional nature results in symptoms that vary widely between people, and within people between days, making diagnosis and treatment options difficult [5].

There is currently no cure for FGDs; rather, treatment options are focused solely on symptom relief [4] which is due in part to the significant lack of understanding surrounding what causes FGDs. Based on the lack of physical gut disturbances and issues linked to symptom-based diagnoses, biochemical and mechanistic features must instead be utilised for diagnosis and treatment [6-8]. Alterations in the gut microbiome, immune dysfunction, visceral hypersensitivity, genetic predisposition, structural and molecular mucosal alterations, nerve sensitivity, immune dysregulation, host and microbial metabolism, gut dysmotility (e.g., faecal transit), and environmental factors are important to understanding FGDs (**Figure 1-1**) [4]. In many cases, symptoms of an FGD coincide with other psychiatric conditions, for example, depression and anxiety [4]. The multi-factorial nature of FGDs means that a combination of processes likely contributes to symptom onset and severity.

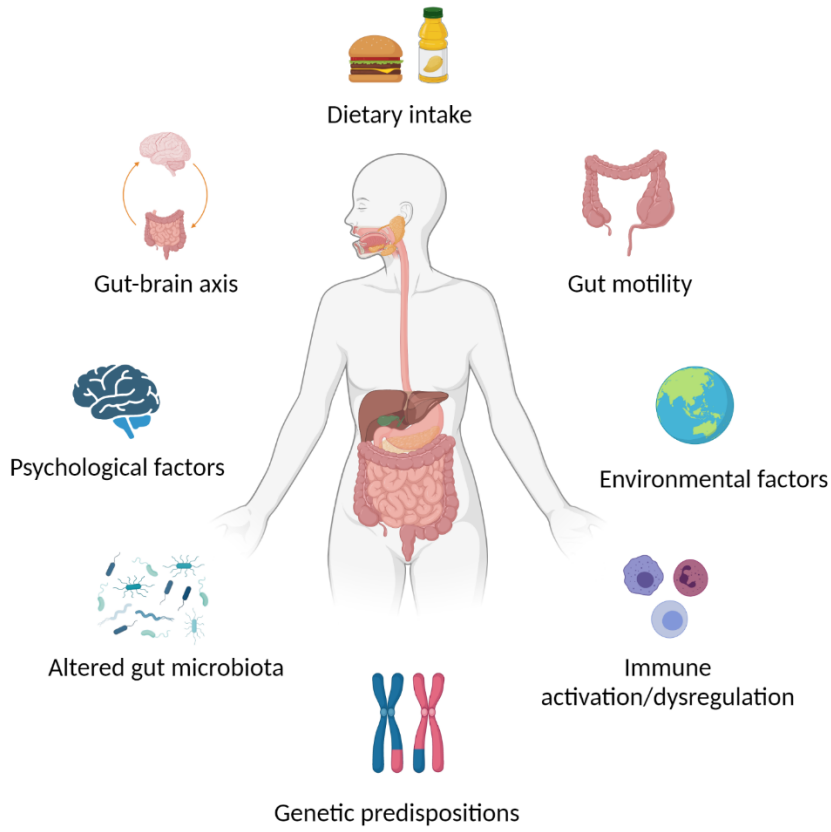


Figure 1-1: Factors influencing functional gut disorders. Figure created using BioRender.

Further adding to the complexity of FGDs is dietary intake. Between 60-89% of individuals who have an FGD believe that diet exacerbates their symptoms [9-14]. Therefore, most individuals seek to modify their dietary intake or consume foods with validated health benefits to help alleviate symptoms [15]. Hence, an understanding of the mechanisms of FGDs and dietary-based prevention or amelioration is essential.

The European Food Safety Authority has recognised FGDs as a relevant model of gut comfort and function that shows variation from the norm, and that applies to those with FGDs and the general population [16]. Scientifically validated biomarkers linked to the mechanisms and phenotypes of FGDs are first required to understand how dietary interventions could alleviate common symptoms and determine the efficiency

of interventions. Even with the increasing research focused on FGDs, reliable biomarkers are still absent.

The aim of this literature review is to explore the main aspects that contribute to a functioning "healthy" gut and an "unhealthy" gut commonly associated with FGDs. The secondary aim is to highlight the importance of dietary intake, the gut microbiome, interactions with host features, and the consequent production of metabolites in FGDs. A summary of the current research investigating biomarkers linked to FGDs concludes this review.

1.2. A 'healthy' gut

The food we consume, its interaction with the host, the gut microbiome, and the consequential production of metabolites can affect normal gut function. The gut is an 'organ' consisting of a highly complex arrangement of host and microbial cells [17]. Understanding these relationships and how beneficial or detrimental effects can arise is crucial to gaining insight into nutritionally driven health solutions. The concept of a 'healthy' gut is difficult to define as merely the absence of disease is not enough to differentiate a 'healthy' from an 'unhealthy' gut. Hence, studying the composition of microbiota and metabolites could allow for discrimination between 'unhealthy' and 'healthy' individuals [18].

Of late, even those that do not experience symptoms are increasingly recognising the importance of the gut for their wellbeing [19]. What constitutes a 'healthy' gut remains poorly defined. However, it has been broadly classified to include i) effective digestion of food and absorption of nutrients, ii) absence of gut illness, iii) normal and stable gut microbial composition, iv) effective activation and responses of the immune system, and v) status of wellbeing [19, 20].

At each stage of digestion, from the mouth to the colon, there are specialised features to ensure efficient digestion, absorption of nutrients, and excretion of waste products. For instance, gastric secretions in the stomach aid in the breakdown of food following mastication, while at the same time, mucus is secreted by the gastric epithelial cells to protect the stomach from these acidic substances [21]. In addition, there are physiological compartments, barrier layers, secretions, and tight junctions within the gut, all efficiently designed to optimise breakdown of food, nutrient absorption, and protection from pathogens.

The mutualistic relationship between the trillions of gut bacterial cells and the host has led to some considering it a 'metabolic organ' [17, 22]. The gut microbiome is defined as all microorganisms (e.g., bacteria, viruses) and their metagenomes (the collection of genes from the microbiota), combined with the surrounding environmental factors [23]. Exploration and understanding of the human gut microbiome have heightened in recent years, highlighting the growing recognition of how critical microbes are for human health [24-26]. The metagenome encodes many key metabolic processes the human body cannot carry out on its own [27, 28]. For instance, the gut metagenome contributes genes that code for enzymes to aid in the breakdown of food and production of essential compounds for energy [26, 29]. Recent studies investigating gut microbial perturbations have shown links to health issues such as obesity, autism, and depression [30-33].

There is a growing awareness that poor quality of life, inadequate nutrition, antibiotic use [17], and everyday stress are taking a toll on health and wellbeing, which in turn has the potential to alter the micro-environment of the gut [25, 34]. Alterations to host processes, nutritional intake, and microbial abundance can profoundly affect the

consequential production of metabolites, metabolic pathways, and successive reactions.

1.3. Functional gut disorders as a model of gut dysfunction

Approximately 40% of all referrals made to gastroenterologists are patients presenting with FGD symptoms [3]. FGDs are classified into five categories, irritable bowel syndrome (IBS; includes IBS-constipation, IBS-diarrhoea, IBS-mixed, IBS-alternating, IBS-undefined (IBS-C, IBS-D, IBS-M, IBS-A, IBS-U)), functional constipation, functional diarrhoea, functional abdominal bloating/distention, unspecific FGD, and opioid-induced constipation [3]. Significant overlap exists between the disorders, with all classified using a version of the Rome Criteria designed by gastroenterologists to classify symptoms [3]. The Rome Criteria originated in 1989 to understand FGDs and has developed through the years from Rome Criteria I to Rome Criteria IV [35]. The latter was developed in collaboration with 126 experts from 26 countries [3]. Whilst the Rome Criteria has benefits, it lacks reliability as it is based on subjective symptom-based criteria of questionnaire responses rather than biological evidence [20, 36] compounded further by the heterogeneous nature of IBS [37, 38]. Additionally, the Rome Criteria sometimes fails to differentiate IBS from more organic diseases like inflammatory bowel diseases (IBD) that have similar symptom characteristics but are more severe [36].

The difference between IBS groups and their functional counterparts is pain, as this is a feature of IBS only [3]. IBS is the most prevalent FGD characterised by abdominal and bowel pain, bloating, changes in stool frequency, and form [39, 40]. It is common worldwide, with around 11% of people being affected in 2017 and up to 15% affected in Asian populations [20, 39-41]. IBS is prevalent in all age groups from childhood to the elderly, though is most common in people between 35-50 years of age [40]. Based

on its high prevalence, most research has focused predominantly on IBS rather than its functional counterparts.

In addition to the Rome Criteria IV, a more robust diagnosis method is needed to identify individuals with IBS and rule out those who have colon cancer, IBD, coeliac disease, or other FGDs. Clark *et al.,* stated that "the successful identification of biomarkers is critical to progressing our understanding of IBS and addressing the unmet therapeutic needs of this debilitating condition" [42, 43]. In addition, a better understanding of the mechanisms and perturbations behind what causes IBS will improve robust diagnoses and symptom prevention mechanisms.

1.4. The gut microbiome

Central to an understanding of gut health is the gut microbiome. The human body is home to many regionally distinct microbiota, for example, the skin, saliva, hair, mouth, and gut [44]. The gut microbiota performs important functions linked to nutrient metabolism, maintaining the structural integrity of the mucosa, and immune protection [45]. At a metabolic level, the production of short-chain fatty acids (SCFAs), some vitamins, amino acids, as well as the biotransformation of bile acids can be attributed to the gut microbiota [18, 45]. Therefore, understanding both the composition of the resident microbes and the metabolic processes they perform, or influence has been a central focus of understanding perturbations linked to IBS [46-50].

The composition of the gut microbiota varies from person to person due to both intrinsic and extrinsic factors, as microbial interactions are complex and multi-directional [51]. From birth, the infant becomes inoculated with a microbiota, with colonisation initially depending, for example, on the method of delivery and feeding

type (breastfeeding versus formula milk) [52, 53]. Microbial colonisation during this time is thought to be critical for the development and maturation of the gut [53].

Analysis of the gut microbiota across approximately 3,400 individuals showed 148 lifestyle and clinical factors that correlated positively and negatively with microbial diversity [54]. In addition, the intake of certain food groups, lifestyle, activity, and bowel health were broad categories of factors linked to microbial diversity [54].

The community nature of the gut microbiota means every microorganism occupies a specific niche that is constrained by the surrounding microbial community [55]. This network results in an equilibrium of the system, whereby the mutualistic and competitive features present cause some microbes to occur in high abundance and others in low abundance [55, 56]. The genera that make up the major and minor members of the core microbiome vary at different body sites, and both members are important [57]. Population-level analysis combining multiple large-scale cohorts (Belgian Flemish Gut Flora Project *n*=1,106; Dutch LifeLines-DEEP study *n*= 1,135; and others) showed that 14 genera form the core microbiota of 3,948 human samples. The authors, however, hypothesised there were remaining genera undiscovered [57]. The major core, which forms the 'housekeeping' core, is key to metabolic functioning as it possesses many genes found only in the microbiome [55]. These genes allow for the modification and production of metabolites and other compounds, highlighting the importance of the functional capability of the gut microbiome [58].

Bacteroidetes and *Firmicutes* are the two phyla that dominate the gut environment together, being around 85-90% of the total bacterial gut population, and with people generally having inverse relationships in relative abundances of the two [45, 54, 55, 59-62]. *Bacteroidetes* show the largest individual variation [63] and are associated

with an increased diversity of other phyla compared to *Firmicutes* [54]. *Actinobacteria*, *Proteobacteria*, *Tenericutes, Euryarchaeota, Lentisphaerae, Cyanobacteria, Fusobacteria* and *Verrucomicrobia* represent other key phyla but are present in lower abundances [45, 54, 63, 64]. *Faecalibacterium prausnitzii, Roseburia intestinalis,* and *Bacteroides uniformis* are the three major species found in the core microbiome of most individuals [59, 64, 65].

1.4.1. The gut microbiome in IBS

FGDs, and more specifically IBS, are often characterised by a perturbed microbiota that could be either cause or effect of the syndrome (Summary of studies **Table 1-1**) [66, 67]. Multiple studies report an increased relative abundance of *Firmicutes* and decreased relative abundance in *Bacteroidetes* in faecal samples of IBS patients compared to healthy controls [68, 69]. Rajilić-Stojanović *et al.,* analysed the faecal samples of healthy controls and IBS subtypes, finding differences between IBS and controls, but not within subtypes [70]. The relative abundance of *Bacteroidetes*, *Actinobacteria* and *Bifidobacterium* were decreased in IBS individuals regardless of subtype, with a corresponding increase in *Firmicutes* [70]. Analysis of the faecal microbiota using 454 pyrosequencing in children with IBS (Rome Criteria III) and age-matched healthy controls showed higher relative abundances of *Haemophilus* and *Dorea* in those with IBS [71]. There was also a corresponding reduction in *Eubacterium* and *Anaerovorax* relative abundances in children with IBS [71].

Table 1-1: Studies investigating microbial variation in IBS and healthy controls.

Author/date	Diagnostic criteria	IBS/HC	Sample matrix	Analytical method	*Bacteroidetes/ Firmicutes* ratio in IBS	Other phyla, class, order in IBS compared to HC	Measure of α-diversity	α-diversity result
Jeffery *et al.,* 2012 [69]	Rome III	15 IBS-D, 10 IBS-C, 12 IBS-A, 20 HC	faecal	16S rRNA V4 region, 454 pyrosequencing	↑*Firmicutes* ↓ *Bacteroidetes*	↑ *Actinobacteria*	Faith's phylogenetic diversity	Lower diversity in IBS
Tap *et al.,* 2017 [72]	Rome III	43 IBS-D, 18 IBS-C, 43 IBS-M, 2 IBS-U - exploratory set, 14 IBS-D, 3 IBS-C, 9 IBS-M, 1 IBS-U - validation set, 39 HC	59 mucosal biopsies, 252 faecal	16S rRNA V5-V6 region, 454 pyrosequencing	Ratio not investigated	↑ *Costridiale*s in IBS-C	Number of OTUs	No diversity difference
Labus *et al.,* 2017 [73]	Rome III	10 IBS-D, 11 IBS-C, 1 IBS-A, 5 IBS-M, 2 IBS-U, 23 HC	faecal	16S rRNA V3-V5 region, 454 pyrosequencing	↑*Firmicutes* ↓*Bacteroidetes*	↑*Clostridia, Lactobacillales* ↓*Bacteroidia, Bacteroidales, Parabacteroides*	Faith's phylogenetic diversity	Higher diversity in IBS

Zeber-Lubecka *et al.,* 2016 [74]	Rome III	31 IBS-D, 11 IBS-C, 30 IBS-M, 30 HC	faecal	16S rRNA V2-4-8, V3-6, 7-9, PGM platform	↑*Firmicutes* IBS-C, ↑ *Bacteroidetes* HC, IBS-D, IBS-M	↑ *Clostridia* in IBS-C	Chao1 richness estimator - species richness Simpson index - community diversity	Higher diversity in IBS No diversity difference
Pozuelo *et al.,* 2015 [75]	Rome III	54 IBS-D, 32 IBS-C, 27 IBS-M, 66 HC	faecal	16S rRNA V4 region, Illumina MiSeq platform	↑ *Bacteroidetes* ↓ *Firmicutes*	↓ *Tenericutes*, *Erysipelotrichaeceae, Ruminococcaceae*	Chao1 richness estimator	Lower diversity in IBS
Ringel-Kulka *et al.,* 2016 [76]	Rome III	21 IBS-D, 21 IBS-C, 14 IBS-M, 20 HC	faecal	16S rRNA V1-V2 region, 454 pyrosequencing platform	No ratio difference	↑*Actinobacteria* in IBS-C, ↑*Lactobacillus, Streptococcus*	Not investigated	NA
Rajilić-Stojanović *et al.,* 2011 [70]	Rome II	25 IBS-D, 18 IBS-C, 19 IBS-A, 46 HC	faecal	16S rRNA sequencing qPCR	Ratio not investigated		Not investigated	NA

Jeffery *et al.,* 2020 [77]	Rome IV	21 IBS-D, 30 IBS-C, 29 IBS-M, 65 HC	faecal, urine (metabolic profiling)	16S rRNA sequencing, Illumina HiSeq 2000 platform	Ratio not investigated	↑*Ruminococcus gnavus, Lachnospiracea spp.* ↓*Barnesiella intestinihominis, Coprococcus catus*	Undisclosed	Lower diversity in IBS
Saulnier *et al.,* 2011 [71]	Rome III (paediatric)	1 IBS-D, 13 IBS-C, 7 IBS-U, 22 HC	faecal	16S rRNA V1-V3, V3-V5 region, 454 pyrosequencing platform	Ratio not investigated	↑*Gammaproteobacteria, Haemophilus, Dorea* ↓*Eubacterium, Anaerovorax*	Undisclosed	Higher diversity in IBS
Chung *et al.,* 2015 [78]	Rome III	14 IBS-D, 7 IBS-C, 7 IBS-M, 19 HC	mucosa biopsies, faecal	16S rRNA V1-V3 region, Illumina MiSeq platform	↑ IBS-D compared to HC	↑*Veillonellaceae, Prevotellaceae* ↓*Mycobacteriaceae, Neisseriaceae*	Not investigated	NA
Durban *et al.,* 2012 [79]	Rome II	13 IBS-D, 3 IBS-C, 9 HC	mucosa biopsies, faecal	16S rRNA V1-V2 region, 454 pyrosequencing platform	Ratio not investigated		Shannon index & Chao1 richness	Lower diversity in IBS

							estimator ACE index	
Hugerth *et al.,* 2020 [80]	Rome IV	8 IBS-C, 4 IBS-D, 12 IBS-M, 5 IBS-U, 149 HC	mucosa biopsies, faecal	16S rRNA, Illumina MiSeq platform	Ratio not investigated		Chao1 richness estimator & Shannon's entropy	No difference between mucosa and faecal samples of IBS compared to HCs
Carrol *et al.,* 2011 [81]	Rome III	16 IBS-D, 21 HC	faecal, mucosa biopsies	16S rRNA T-RFLP	Ratio not investigated	↑*Lactobacillus spp.*	Shannon-Weiner index	Lower diversity in IBS in faecal, no difference in the mucosa

Footnote 1: Overview of analytical methods, Firmicutes/Bacteroidetes ratio and diversity measures. Abbreviations: HC (healthy controls); IBS (irritable bowel syndrome); IBS-C (irritable bowel syndrome – constipation); IBS-D (irritable bowel syndrome – diarrhoea); IBS-M (irritable bowel syndrome – mixed); IBS-A (irritable bowel syndrome – alternating); IBS-U (irritable bowel syndrome – undefined).

Alpha diversity is the diversity of microbes found within a specific sample [55, 56]. Inconsistent trends in alpha diversity are evident, with studies reporting greater diversity [73], lower diversity [75], and no differences [72]. However, different diversity indexes are utilised across studies, for example, Simpson index, Chao1 index, number of OTUs and Shannon index [82]. Studies that used multiple indexes showed different conclusions for alpha diversity dependent on which index was used [74, 82] and the interpretation of results by users.

The 454 pyrosequencing of the faecal microbiota of healthy controls and IBS individuals showed an increased relative abundance of *Firmicutes*, *Actinobacteria* and *Bifidobacteriaceae*, and decreased relative abundance of *Bacteroidetes* and *Alistipes* in individuals with IBS [69]. Alpha diversity of faecal samples was decreased in IBS compared to healthy controls [69], in contrast to other studies which have found no differences [72].

IBS individuals could be separated into three main clusters irrelevant of subtype or health status [69], consistent with a subsequent analysis where microbial taxonomy separated participants into a healthy control, IBS-like healthy control, and IBS groups [73]. The three groups were identified from principal coordinate analysis of faecal microbial operational taxonomic units (OTUs), showing an IBS-like healthy control group that consisted of individuals with microbial profiles similar to healthy controls [73]. In addition, *Firmicutes* relative abundance was highest in the IBS group, with decreasing abundance in the IBS-like healthy control and healthy control groups [73]. The inverse relationship was evident for *Bacteroidetes* [73]. *Actinobacteria, Proteobacteria* and *Cyanobacteria* did not differ between the groups [73]. The identification of three separate groups suggests a need to understand IBS at an individual level rather than grouping all IBS patients together and for other variables to be considered.

Analysis by Pozuelo *et al.,* observed a reduction in the relative abundance of *Firmicutes* in IBS individuals with a corresponding increase in *Bacteroidetes* [75]. Distance-based redundancy analysis separated IBS individuals and subtypes into separate clusters from healthy controls [75]. The separation into clusters was achieved based on a small percentage of the data, where, for example, one OTU at the species level from the *Lachnobacterium* genus was responsible for the separation between IBS subjects and healthy controls [75]. However, this taxon was detected in only a small number of healthy control samples (22%) and IBS samples (1.7%) [75]. Overall, alpha diversity was lower in IBS individuals compared to healthy controls. Within subtypes, IBS-D was characterised by a lower microbial diversity, compared to IBS-C and IBS-M groups which harboured diversity similar to healthy controls [75].

In contrast, Ringel-Kulka *et al.,* using 454 pyrosequencing, analysed the faecal microbiota and removed OTUs that were absent in at least 75% of the samples to remove low prevalence taxa, resulting in analysis of the 'core' microbiome consisting of 46 species [76]. Linear discriminant analysis of the 46 species separated the data between healthy controls, IBS participants, and within IBS subtypes [76]. Additionally, individuals were separated into another three primary outcomes, based on the presence or absence of bloating (IBS with bloating, IBS without bloating, and healthy controls), with separation into clusters achieved [76].

Microbial analysis using 454 pyrosequencing of faecal and colonic mucosal samples in one study showed variation in the dominant phyla, where *Firmicutes* and *Actinobacteria* were prevalent at higher abundance in the faecal microbiome, compared to *Bacteroidetes* and *Proteobacteria* enriched in the mucosa [72]. Nevertheless, there was still a strong link between the colonic mucosa and faecal microbiome of individuals [72]. Furthermore, variation was observed within IBS subtypes where, for example, *Firmicutes* dominated

IBS-C, compared to *Bacteroidetes* in IBS-D, IBS-M, and healthy controls in one study [74]. These variations correspond to the functionality of the microbiome where amino acid, glutamate and oxidative phosphorylation metabolism were reduced in IBS-C individuals, and methane and pyruvate metabolism increased compared to healthy controls and other IBS groups [74]. This finding highlights why investigating microbial taxonomy and analysing the microbiota of all IBS individuals as one group can lead to discrepancies.

Recent investigation linking bacterial metagenomic data and metabolic information provides an example of the field progressing to better understand IBS aetiology. Jeffery *et al.,* analysed healthy controls and IBS individuals using shotgun metagenomic sequencing, showing that microbial gene abundance separated IBS and controls, but with significant overlap, with the same evident between subtypes [77]. However, pathway analysis showed differences in the IBS group compared to healthy controls, with amino acid pathways increased and sulphur metabolism pathways decreased in individuals with IBS [77].

Evidence of interindividual variation of the gut microbiota and the extent of unknown microbial taxonomy suggests that understanding disease phenotypes based on strict classification into subtypes may not be feasible or effective. The progression of shotgun metagenomic sequencing combined with other omics technologies provides a more comprehensive overview of the gut microbiome to elucidate its role in health and perturbed gut states.

1.5. Metabolites produced in the gut

Metabolites are the products of biological pathways and enzymatic processes. They can be measured to determine the function of a particular tissue, organ or system and assist in

distinguishing between disease phenotypes by providing a functional readout of gut processes [18]. Metabolites produced in the gut can be of diet, microbial or host origin, or a combination of all, and can be absorbed across the intestinal barrier into the blood where they enter host circulation, having both beneficial and detrimental effects [44]. Dietary intake is a strong determinant for not just the gut microbial community that exists but also the consequent production of microbial metabolites [44]. For example, an increase in dietary fibre may increase the concentration of fermentation end products (e.g., SCFAs), thereby altering the gut microenvironment and thus the abundance of other microbial taxa, metabolic production, and host processes [44]. Furthermore, the gut environment is constantly changing due to pH fluctuations, stomach secretions, faecal passage, and dietary substrates, which impacts the microbial community and metabolites produced [44]. In addition, the production of metabolites by the gut microbiome (e.g., bacteriocins) can then influence the abundance of other bacterial species [83].

SCFAs, vitamins, bile acids, lipids, tryptophan metabolites, polyamines, neurotransmitters, and amino acids can be produced or modified by the host or the gut microbiota [52]. Compared to normal homeostatic levels, excess or insufficient production of these metabolites could signal disruptions to pathways important to gut and overall health [18]. Many metabolites are signalling molecules both with actions localised in the gut and elsewhere in the body. For example, a reduction in the SCFA butyrate may alter energy supply to colonocytes, transepithelial fluid transport, mucosal inflammation, oxidative stress, gut tissue development, and immune modulation [84]. Additionally, a disruption to bile acid production or recycling could impact cholesterol absorption from the body, gut secretions, and food digestion [84]. Furthermore, cross-feeding, where the metabolic by-product from one microbe acts as the substrate for another microbe, is an

important process that can dictate successive processes, underlining the complexity of the gut [42].

1.5.1. Known metabolites important to FGDs

Alterations to metabolite production in the gut by either host, microbiota or as a result of their interactions may be linked to IBS symptoms [70]. In addition, these metabolites can change the luminal environment through various mechanisms and could thus be important in understanding perturbations.

1.5.1.1. *Bile acids*

Bile acid profiles are affected by diet, host characteristics, age and life stages, and the resident microbiota with recent research showing bile acid metabolism may be linked to IBS [37, 85-89]. There is evidence for variation in the plasma concentration of primary and secondary bile acids in IBS participants [88]. Primary bile acids are produced in the liver from cholesterol via the enzyme cholesterol 7-α-hydroxylase to produce 7-α-hydroxy-4-cholesten-3-one (C4), which is then converted into the two primary bile acids: chenodeoxycholic acid (CDCA) and cholic acid (CA) (**Figure 1-2**). These bile acids are conjugated to either taurine or glycine, stored in the gallbladder, secreted into the gut lumen following digestion and then unconjugated from taurine and glycine by bacterial bile salt hydrolase (BSH) enzymes [89, 90]. Most bile acids are reabsorbed and recycled via hepatic circulation (95%), which is controlled by fibroblast growth factor 15 (FGF15) and the bile acid receptor farnesoid X receptor (FXR) [91]. Five percent of bile acids escape this process and undergo modification by microorganisms with the 7α-dehydroxylase enzyme, resulting in secondary bile acids with altered structures that may interact differently with cellular receptors, potentially having impacts on the functionality of metabolites [89]. It is unknown if bile acid levels fluctuate due to differences or

disruptions in the ileal epithelial transporter, FGF15 molecules, precursor mechanisms in the liver or microbial modification of the metabolites.

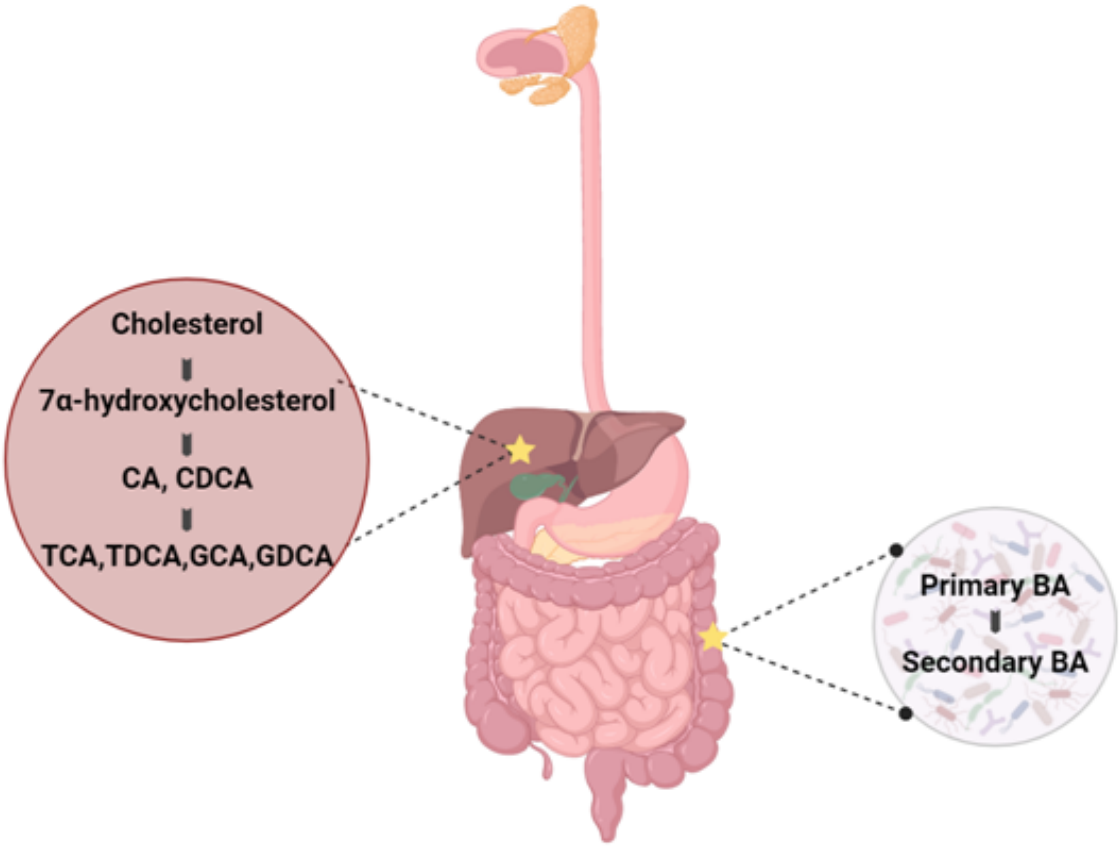


Figure 1-2: Production of bile acids in the liver from cholesterol, followed by storage in the gallbladder. Following food intake, bile acids are excreted into the gut lumen, where they act as detergent molecules to aid in the absorption of nutrients. Next, they are converted to secondary bile acids in the large intestine due to microbes possessing the bile salt hydrolase enzyme. Abbreviations: chenodeoxycholic acid (CDCA), cholic acid (CA), glyco-chenodeoxycholic acid (GDCA), glyco-cholic acid (GCA), tauro-chenodeoxycholic acid (TDCA), tauro-cholic acid (TCA). Figure created using BioRender.

A meta-analysis of studies reporting on IBS-D symptoms showed that increased bile acid malabsorption (BAM) was evident in 16.9%-35.3% of the individuals diagnosed with IBS-D [92]. Increased BAM is linked to diarrhoea, where defective bile acid recycling or overproduction may increase colonic bile acid levels leading to the onset of laxation [91, 93, 94]. Conversely, a reduction in bile acid levels in the colonic mucosa may have the opposite effect causing constipation and slowing colonic transit. In a study by Sadik *et al.,* increased BAM levels positively associated with accelerated colonic transit in patients

with chronic diarrhoea [94]. However, not all bile acids have the same effect on the gut. Unlike CDCA and CA that are predominantly recycled, the secondary bile acid, lithocholic acid (LCA), is poorly reabsorbed and instead passed through to the colon for further modifications by bacteria and then excretion [95]. The action of CDCA may be facilitated by activation of intracellular secretory channels, increased mucosal permeability, or decreased fluid and electrolyte absorption [96].

Differences in faecal and serum bile acid concentrations were observed in individuals with IBS-C and IBS-D, showing links to visceral pain and colonic transit [93, 97, 98]. In the faeces of IBS-D individuals, the concentration of primary bile acids was higher and secondary bile acids lower compared to healthy controls, suggesting increased BAM and the inability of bile acids to be modified by the gut microbiota [99]. In addition, positive correlations were evident between concentrations of C4 and FGF19, faecal weight, and total bile acids in IBS-D individuals suggesting an increase in bile acid production to counteract bile acids lost in faecal samples. Interestingly, the composition of the faecal microbiota in IBS-D individuals was characterised by reduced relative abundances of *Bifidobacterium* and *Clostridium leptum;* bacteria that possess the BSH enzyme involved in bile acid transformation [99].

David *et al.,* investigated how dietary intake over five days influenced the gut microbiota and its metabolites [32]. In this study, they showed that an animal-based compared to a plant-based diet increased the abundance of bile acids in faecal samples, which they surmised was due to higher levels of cholesterol (a precursor of bile acids) in individuals consuming animal-based diets. Consequently, based on the relationship between dietary patterns, metabolism, microbial enzymatic activities, host epithelial transporters, hepatic portal circulation and metabolism, bile acid fluctuations could provide valuable insights into understanding the mechanisms contributing to the onset and severity of IBS.

1.5.1.2. *Short-chain fatty acids (SCFAs)*

Carbohydrates, for example, sugars, starches, and fibre that escape digestion in the stomach and small intestine enter the large intestine where they are fermented by the gut microbiota, producing SCFAs [18, 28, 100]. Acetate, propionate, and butyrate are the primary SCFAs produced in the gut [68]. Approximately 80-90% of SCFAs produced in the colon are used by the body, with the rest excreted in faeces [100].

Many bacteria can produce SCFAs, including butyrate [101]. Some of the most common butyrate producers include *Fecalibacterium prausnitzii, Roseburia spp., Eubacterium rectale,* and *Eubacterium hallii* [102-104]. Butyrate is produced via pathways utilising lactate, acetate, sugars, and amino acids that may be by-products produced by other bacteria [103]. Of the three pathways producing propionate, the succinate pathway is the most common and performed predominantly by *Bacteroides spp.* and *Veillonella spp*. [103]. Acetate pathways are more widespread, produced from a range of fermented carbohydrates and microbes [103, 105]. Colonocytes predominantly use butyrate for energy, whilst hepatocytes can use propionate leading to gluconeogenesis, and acetate passes into peripheral circulation and is utilised throughout the body [105, 106]. Acetate and propionate are linked to the regulation of glucose homeostasis and fatty acid metabolism in the liver and stimulation of energy and appetite regulation, suggesting that relative proportions of specific SCFAs could be more important than total abundance [105].

Alterations in the gut microbial composition and SCFA (butyrate and propionate) concentration have been observed in IBS compared to healthy controls [42, 107]. Lower butyrate concentration in faecal samples of IBS participants could indicate disrupted energy supply to colonocytes with consequences for IBS symptoms [42]. However, a different study reported no difference in faecal acetate, propionate, butyrate, and lactate

between controls and IBS participants, although total SCFA abundance was lower in the IBS-C subtype compared to other subtypes (IBS-D, IBS-M) [76]. Tana *et al.,* showed higher SCFA concentrations in faecal samples of IBS participants along with an increased relative abundance of *Veillonella* and *Lactobacillus* [107]. This is a consistent observation as *Lactobacillus* are prominent producers of lactic and acetic acids, whilst *Veillonella* transforms lactic acid to acetic and propionic acids [107]. Additionally, there was a positive correlation between faecal SCFA concentration and symptom severity, signifying a possible association between metabolite production and gut discomfort [107].

The relationship between SCFAs and IBS is inconsistent in the literature, as there is evidence for both higher and lower faecal SCFA levels in IBS [70, 108, 109]. A potential explanation for this variation is the functional redundancy of a microbial community where if one species is reduced in abundance, another species may fill the vacated niche, potentially contributing the same metabolites (e.g., SCFA) to the system. Additionally, the inconsistency in methodological analysis of SCFA analysis may be another potential source of variation, where for example the time between sample collection and processing can affect the continued fermentation and thus concentration of SCFAs. Consequently, understanding the interaction between dietary patterns, SCFA concentration, host functions, and gut microbial activity, including species abundance, could be relevant to successfully elucidating a possible link to IBS [70, 107].

1.5.1.3. *Vitamins*

Perturbations in circulatory vitamin concentrations have been linked to IBS [110]. Vitamins are obtained directly from dietary intake or are biosynthesised in the body. However, sufficient quantities required for the effective functioning of cellular processes may not be met by dietary intake and the host alone [111, 112]. Some species of the

human gut microbiota, for example, lactic acid bacteria, can synthesise folate, thiamine, biotin, vitamin K, nicotinic acid, pantothenic acid, pyridoxine, and riboflavin, which may be utilised by the host [18, 111-115]. These vitamins can have essential roles within the body, for example, folate, which is vital in DNA replication [111]. David *et al.,* noted that subjects consuming animal-based dietary patterns had an increased abundance of microbes with vitamin synthesising genes compared to those on plant-based dietary patterns, highlighting the potential role of diet, gut microbiota, and metabolome relationships [32].

Vitamin B_6 (pyridoxine) has been linked to inflammatory conditions and, therefore, could be important in IBS [110, 116]. In a study investigating the dietary intake of 17 individuals with IBS, a low vitamin B_6 concentration in plasma correlated to a high IBS symptom score [110]. Vitamin B_6-producing pathways are found in species from *Actinobacteria*, *Bacteroidetes* and *Proteobacteria* phyla, with less synthesising capabilities found in *Firmicutes* [112].

Magnúsdóttir *et al*., investigated the B-vitamin-producing capacity of 256 gut microbial genomes, found 40-65% contained biosynthetic pathways necessary to synthesise eight key vitamins (biotin, cobalamin, folate, niacin, pantothenate, pyridoxine, riboflavin, and thiamin) [117]. Riboflavin, an activator of mucosal-associated invariant T cells and essential in cellular metabolism as a precursor to flavin adenine dinucleotide and flavin mononucleotide, had synthesising genes present in 166 of the 256 genomes [111, 118]. Niacin synthesis was the second most prevalent pathway, present in 162 genomes [117]. The biosynthesis of riboflavin pathways was found predominantly in *Bacteroidetes*, *Proteobacteria* and *Fusobacteria*, with less found in *Firmicutes* and no pathways in *Actinobacteria*, in contrast to niacin pathways which were uniformly distributed across the five phyla [117]. Differences in vitamin synthesising capability across different taxa

raise the potential for vitamin production to vary between healthy individuals and those with IBS [117]. Interestingly, *Firmicutes*, often found in high abundance in IBS compared to healthy individuals, was the only phylum analysed that did not possess all eight vitamin synthesis pathways [68, 69, 117, 119]. However, mathematical modelling indicated the gut microbiota could only produce four of the eight vitamins in concentrations that could have clinical relevance [117]. These estimates are based solely on computational modelling, and therefore, investigating the rate and source of both host and microbial vitamin production are required as the presence of vitamin-synthesising genes does not necessarily correlate to clinical outcomes. Additionally, investigating the absorption of host and microbially produced vitamins using methods such as stable isotope probing are needed. A more in-depth understanding of host and microbial interactions could help to elucidate further roles for vitamins in IBS and the clinical significance of microbially produced vitamins.

1.5.1.4. *Amino acids*

It is estimated that approximately 5 to 10% of dietary protein consumed is passed unabsorbed into the colon, whereby the gut microbiota can metabolise the protein into amino acids and other derivatives [44]. Amino acids can be grouped into branched-chain amino acids (BCAAs; leucine, isoleucine, valine), non-essential amino acids (NEAAs; alanine, asparagine, aspartate, glutamate, glycine, serine, tyrosine), essential amino acids (EAAs; arginine, cysteine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine), and large neutral amino acids (LNAAs; phenylalanine, tyrosine, tryptophan, leucine, isoleucine, valine) (**Figure 1-3**). EAAs cannot be synthesised by the body and instead need to be obtained exogenously through the diet. The amino acid groups rather than the individual amino acids can have important

implications for health and disease. For example, LNAAs, which can be precursors for neurotransmitters of the brain and gut.

Tryptophan is an essential amino acid obtained from dietary intake and is the precursor for serotonin. Therefore, it has been hypothesised that tryptophan may be an important amino acid in IBS due to the relative importance of serotonin (5-HT) in FGDs [120]. However, the examination of circulatory plasma tryptophan concentration in IBS individuals showed no difference to healthy controls [120]. Two competitive pathways, the kynurenine and serotonin pathways, metabolise tryptophan into either the vitamin niacinamide or the neurotransmitters 5-HT and melatonin [120, 121]. While tryptophan levels may not differ, the balance between the kynurenine pathway compared to the serotonin pathway may be important because of the different biological functions of the resulting metabolites [120-123].

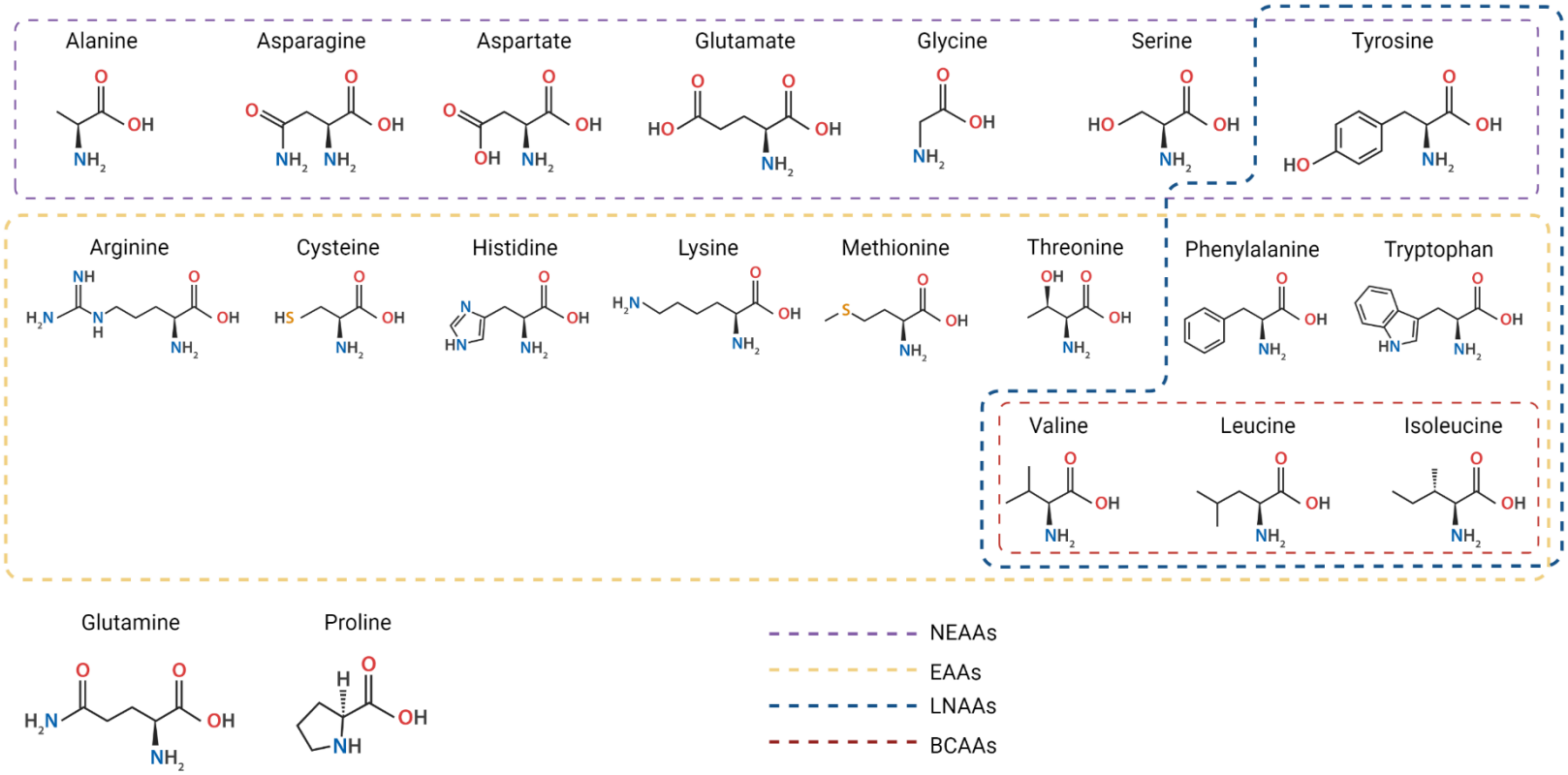


Figure 1-3: Twenty amino acids and associated groupings. Abbreviations: non-essential amino acids (NEAAs), essential amino acids (EAAs), large neutral amino acids (LNAAs), branch chain amino acids (BCAAs). Figure created using BioRender.

Investigation of urinary metabolites between individuals with IBS, ulcerative colitis (UC), and healthy controls found histidine, lysine, glutamine, proline, and glutamic acid concentrations varied between IBS and UC participants, but not from healthy controls [124]. Ornithine, a metabolite of the urea cycle, was the only amino acid that varied between IBS and healthy controls with a lower concentration in IBS [124]. However, a dietary analysis was not completed in this study, and thus it is impossible to determine if concentrations are attributable to protein consumption. Glutamine is involved in the energy supply to the epithelial cells of the gut, and consequently, a depletion could be crucial in IBS symptomology. When given as an oral supplement (5g three times a day), glutamine reduced IBS symptom severity in individuals with diarrhoea-predominant, post-infectious IBS [125]. In general, understanding the role of amino acid metabolism in IBS requires further research to investigate their clinical relevance.

1.5.1.5. *Neurotransmitters*

The neurotransmitter 5-HT can be produced in the gut and affects neuronal signals in the brain, highlighting its importance in gut-brain responses [126]. Ninety-five percent of 5-HT is produced by the enterochromaffin cells of the gut epithelium, while the other 5% is produced in serotonergic neurons [126-128]. 5-HT in the gut is assumed to activate neurons linked to pain, sensitivity, and reflexes via enterochromaffin and enteroendocrine cells [129, 130]. An overproduction of 5-HT can lead to overactivation of nerve sensing mechanisms causing increased hypersensitivity [127]. The biological activity of 5-HT is terminated by serotonin reuptake transport (SERT), the recycling mechanism for 5-HT in the body [126, 130]. Polymorphisms in SERT may influence IBS, although studies investigating the possible association between 5-HT, the SERT gene, and IBS subtypes have had varying results [128]. Atkinson *et al.,* found a lack of 5-HT uptake was associated with IBS-D symptoms due to the deletion of a base deletion [131], while others

concluded there was no relationship between the SERT polymorphism and IBS [132, 133].

In one study, colonic mucosa enterochromaffin cell counts and concentrations of 5-HT were shown to be higher in those with IBS compared to healthy controls [126]. However, such differences in enterochromaffin cells were not consistently observed in the literature [134, 135]. Supporting this finding, the authors noted the severity of visceral pain and hypersensitivity felt by IBS participants correlated to 5-HT release [126].

Dopamine and γ-aminobutyric acid (GABA) are key neurotransmitters which may also be implicated in IBS. Dopamine, a neurotransmitter of the catecholamine family, was linked to depression and anxiety [136] and has been found at lower concentrations in individuals with IBS compared to healthy controls [124]. Additionally, GABA, which exerts important anti-inflammatory effects, was reduced in IBS-D individuals compared to healthy controls [137].

1.5.1.6. *Inflammatory molecules*

Cytokines are signalling molecules that regulate inflammatory responses [138]. Tumour necrosis factor-α (TNF-α), a pro-inflammatory molecule, and the anti-inflammatory cytokines interleukin 10 (IL-10) and transforming growth factor β_1 (TGF-β_1) may have important roles in IBS [138, 139]. Polymorphisms in the genetic components encoding these cytokines may increase or decrease concentration, causing disruptions to inflammatory responses [138]. Gonsalkorale *et al.,* found an association between IBS symptoms and reduced IL-10 levels compared to healthy controls in peripheral blood samples [138]. A meta-analysis of nine studies showed gender differences in blood TNF-α and IL-10 concentrations in patients with IBS [128, 140]. However, in general, the importance and relevance of inflammatory molecules in IBS remains unclear.

1.6. Diet in functional gut disorders

As most individuals with FGDs note that diet exacerbates or alleviates symptoms, it is not surprising that people seek to exclude or include certain foods or whole food groups from their diet [9, 10]. Coffee, spices, cabbage, onions, alcohol, along with lactose and carbohydrates, are common foods or food components thought to exacerbate symptoms [10, 141, 142]. There are multiple diets (current standard dietary therapy, habitual diet, sham diet, reduced resistant protein diet, low capsaicin diet, fermentable oligo-di monosaccharide and polyols (FODMAP) diet, gluten-free diet etc.) that have been shown to be beneficial to those with IBS [9, 11, 143]. Literature reports show fibre and lactose were studied heavily in the past millennium, with probiotics taking over in the 2000s, and more recently, attention turning to a low FODMAP diet [9]. A diet low in FODMAPs lowers carbohydrate components of the diet assumed to be poorly absorbed and highly fermentable, thereby decreasing hydrogen gas production and associated hypersensitivity that may be linked to IBS [143-149].

However, caution must be taken in adopting dietary regimes for FGDs as they can potentially disrupt the gut microbial community and production of beneficial metabolites. In a study of 36,448 individuals from France (dietary data, Rome III Diagnostic Criteria), 1,870 people diagnosed with IBS had different food consumption patterns compared to healthy controls [150]. Reduced intake of protein and micronutrients (e.g., vitamins) was characteristic of IBS individuals, attributed to lower intakes of milk, yoghurt, and fruit [150]. The study's findings are consistent with previous results where people with FGDs were reported to exclude lactose intake [151, 152].

Evidence for how the removal of whole food groups can affect the gut microbial composition and successive production of metabolites has been shown in studies comparing predominantly animal- or plant-based dietary patterns in cohorts of healthy

adults. An animal-based diet increased the relative abundance of *Alisipes, Bilophila and Bacteroides* and decreased proportions of microbes known to degrade plant compounds (e.g., *Roseburia, Eubacterium rectale,* and *Ruminococcus bromii*) [32]. This study concluded that a predominantly animal-based dietary intake rapidly altered microbial composition within one day, but the population returned to its original composition within two days following the withdrawal of the diet [32]. Alterations to bile acid and SCFA profiles were observed with both diets [32], emphasising that dietary intake has the potential to affect the host and microbial metabolomes. Additionally, the microbial transcriptome in those consuming a predominantly animal-based compared to a predominantly plant-based dietary pattern showed increased expression of microbial genes involved in key metabolic pathways, for example, vitamin biosynthesis [32].

A similar study comparing the microbiota of children from Italy and Africa found that Italian children who consumed more protein in their dietary intake, had higher relative abundances of *Alistipes* and *Bacteroides* [153, 154]. In contrast, African children consuming more legumes and vegetables had higher counts of *Prevotella* and *Succinivibrio* microbes capable of degrading fibre and polysaccharides [154]. Fibre intake between the two groups of children showed positive correlations to faecal SCFAs, highlighting metabolite production from the lower gut microbiota [154].

1.6.1. Microbial production of gases

Hydrogen gas, a product of microbial carbohydrate fermentation, is produced by numerous members of the gut microbiota [155, 156]. Hydrogen is further used through cross-feeding to produce methane, hydrogen sulfide, (or sulphide) and acetate by methanotrophic, sulphate-reducing and acetogenic bacteria, respectively [157]. These molecules are produced solely from the gut microbiota and re-absorbed into the body [155]. An excess of hydrogen gas can cause discomfort for healthy and IBS individuals.

Firmicutes are the primary hydrogen producers of the gut [155] and are often found in higher quantities in IBS patients with a corresponding decrease in *Bacteroidetes*, which may explain the common bloating and discomfort symptoms in IBS. This hypothesis is supported by the higher concentrations of breath hydrogen in IBS individuals compared to healthy controls [158, 159]. King *et al.,* and Dear *et al*., both noted that reducing consumption of foods known to promote hydrogen production decreased symptoms of IBS [159, 160]. Additionally, an increase in breath methane concentration was linked to a decrease in gut motility that is evident in IBS-C patients [161].

1.6.2. Fermentable oligo-di monosaccharide and polyols (FODMAPs)

FODMAPS are fermentable oligosaccharides (e.g., wheat; fructo-oligosaccharides), disaccharides (e.g., cheese; lactose), monosaccharides (e.g., honey; fructose) and polyols (e.g., certain fruits; sorbitol) that are assumed to be poorly digested and easily fermented. There is evidence that dietary regimens which exclude or reduce FODMAPS alleviate the pain and distension associated with IBS symptoms [11, 162]. The association between reduced IBS symptoms and a low dietary FODMAP intake is primarily based on symptom improvement as the outcome measure, which can be subjective, rather than biochemical or mechanistic alterations [11, 146, 147, 163, 164].

McIntosh *et al.,* used breath tests to measure volatile metabolites of microbial fermentation where IBS participants were randomised to either a low or high FODMAP dietary intervention and then given a Kristalose® sachet (used as a lactulose supplement for constipation) [165]. Results showed an increase in breath hydrogen concentration in the high FODMAP group compared to the low FODMAP group from baseline over the 21 days. In this study, methane concentration showed no variation, suggesting a low FODMAP diet may not differentially alter microbial gas production [165]. Both groups had similar baseline urine metabolite profiles, but following dietary intervention, three

metabolites (histamine, azelaic acid, and p-hydroxybenzoic) showed differences [165]. Urinary histamine, an immune response molecule, was three times higher in concentration (0.0008 μmol/mmol compared to 0.0085 μmol/mmol) in the high compared to low FODMAP interventions, in line with other findings that histamine is linked to hypersensitivity and immune activation [166, 167].

Analysis of a low FODMAP dietary intake compared to normal dietary guidelines often given to IBS patients for four weeks showed a similar decrease in symptom severity [163]. IBS dietary guidelines were focused mainly on the timing of meals, eating regular meals, avoidance of large meals and reducing the intake of fat, caffeine, cabbage, beans, and onions [163]. Further investigation into potential side-effects of a FODMAP dietary regimen is required, as removing key food groups could present unfavourable conditions within the gut ecosystem and the wider body. FODMAPs are often used as prebiotic supplements [168].

Consequently, the widespread movement for their reduction to reduce the symptoms of FGDs is paradoxical, considering the beneficial effects of prebiotics are mediated by microbial fermentation, yet the adverse effects of FODMAPS are also mediated by microbial fermentation. This paradox is consistent with findings where *Bifidobacterium,* was reduced after consumption of a 4-week low FODMAP diet (concentration 7.4 $\log_{10}$ cells/g faeces) compared to normal dietary intake (concentration 8.2 $\log_{10}$ cells/g faeces) [164, 168]. Furthermore, SCFAs are produced from the fermentation of FODMAPs by the gut microbiota [168, 169]. Thus, although evidence suggests a low FODMAP diet is warranted in IBS, further studies must aim to better understand the impact of FODMAP reduction in the dietary pattern of healthy individuals compared to those with IBS.

1.6.3. Probiotics

Probiotics, or foods with added beneficial bacteria, have been investigated extensively for their ability to alleviate IBS symptoms, with the majority based on outcome measures of abdominal pain, bloating and IBS symptoms [170-173]. Two interventions showed improvement in symptoms following consumption of probiotics, where metabolic or microbial features were also recorded as outcome measures [174, 175]. IBS-D participants given 100 g of probiotic yoghurt each day (7 $\log_{10}$ cfu/g of *Lactobacillus fermentum* ATCC 14931 and 7 $\log_{10}$ cfu/g *Lactobacillus plantarum* ATCC 14917) for four weeks showed beneficial changes to symptom scores, abdominal pain, and quality of life together with a reduction in faecal calprotectin from baseline [174]. Faecal calprotectin is a marker of inflammation, prevalent at increased concentrations in IBD.

Yoon *et al.,* gave participants either a multi-strain probiotic capsule (*Bifidobacterium bifidum* (KCTC12200BP)*, B. lactis* (KCTC11904BP)*, B. longum* (KCTC12200BP)*, Lactobacillus acidophilus* (KCTC11906BP)*, L. rhamnosus* (KCTC12202BP), and *Streptococcus thermophilus* (KCTC11870BP) total 5 x 10^9 viable cells) or a placebo daily for four weeks [175]. Abdominal pain and bloating were reduced in the probiotic group compared to the placebo group, although there was no difference in faecal form or frequency in either group compared to at baseline [175]. Measurement of the faecal microbiota showed three probiotic species (*B. lactis*, 6.09 to 7.57 $\log_{10}$ cells/g faeces*; L. rhamnosus*, 2.80 to 5.05 $\log_{10}$ cells/g faeces*; S.thermophilus*, 4.81 to 5.35 $\log_{10}$ cells/g faeces) were increased following the intervention [175]. These findings show that modification and disturbances to the gut microbiome following probiotic intervention may be instrumental in understanding the underlying mechanisms linked to IBS.

1.6.4. High fibre foods

There is an increasing awareness that some commonly consumed foods may reduce the symptoms and prevalence of IBS. Prunes, psyllium husk, wholegrain powders, and kiwifruit, which are all characterised by high dietary fibre content, have been investigated for their ability to beneficially alter IBS constipation symptoms [176-180]. The soluble component of dietary fibre, for example fructans and inulin, are utilised by the gut microbiota as energy sources, promoting the growth of some beneficial bacteria, for example, *Lactobacillus and Bifidobacteria* [176, 181, 182]. Insoluble fibre, for example cellulose, is utilised less by the gut microbiota but is essential as it increases gut transit time by passing through the colon undissolved [182].

One example, kiwifruit is a high fibre food that has been recommended to individuals with IBS-C for many years. The high vitamin C content, actinidin (a unique protease abundant in kiwifruit), and amino acids (glutathione, arginine, and GABA) coupled with a high-water swelling capacity may contribute to alleviating constipation symptoms [176]. Consumption of two green kiwifruit compared to two placebo capsules (glucose powder) per day for 4-weeks decreased colonic transit time and increased weekly defecation in the kiwifruit consuming participants [180].

Prunes are another example that have also been shown to be effective in decreasing colonic transit and increasing faecal consistency to treat chronic constipation [177]. Forty participants with chronic constipation were given either prunes or psyllium (11 g twice daily) for 3-weeks as part of a randomised cross-over study [177]. Both interventions improved complete spontaneous bowel movement compared to baseline, but consumption of prunes decreased colonic transit time compared to psyllium [177]. Prunes also resulted in softer faecal compared to psyllium, with both interventions improving straining when trying to pass faecal matter compared to baseline. The improvement in

symptoms from these studies highlights the relevance of using dietary interventions to understand better, mechanisms behind FGDs and their use in alleviating prevalence.

1.7. Other factors that contribute to IBS

The gut-brain axis (GBA) is an important for the effective functioning of the gut [183]. Additionally, the gut-brain link acts as a bi-directional signalling mechanism from the gut to the central nervous system (CNS) and vice-versa, affecting mood and cognitive function (**Figure 1-4**) [183, 184]. The CNS, immune system, enteric nervous system (ENS), hypothalamic pituitary adrenal (HPA) axis and autonomic nervous system (ANS) are established pathways of communication in the GBA that may influence bodily functions [185, 186].

IBS is often referred to as a disorder of not just the gut, but also the brain, believed to be affected by both 'brain to gut' and 'gut to brain' pathways [187, 188]. Gut-brain interactions in IBS could be cause or effect of a perturbed gut microbiota [189], altered pain and stressor sensitivity [185], a perturbed GBA, or likely a combination of these factors.

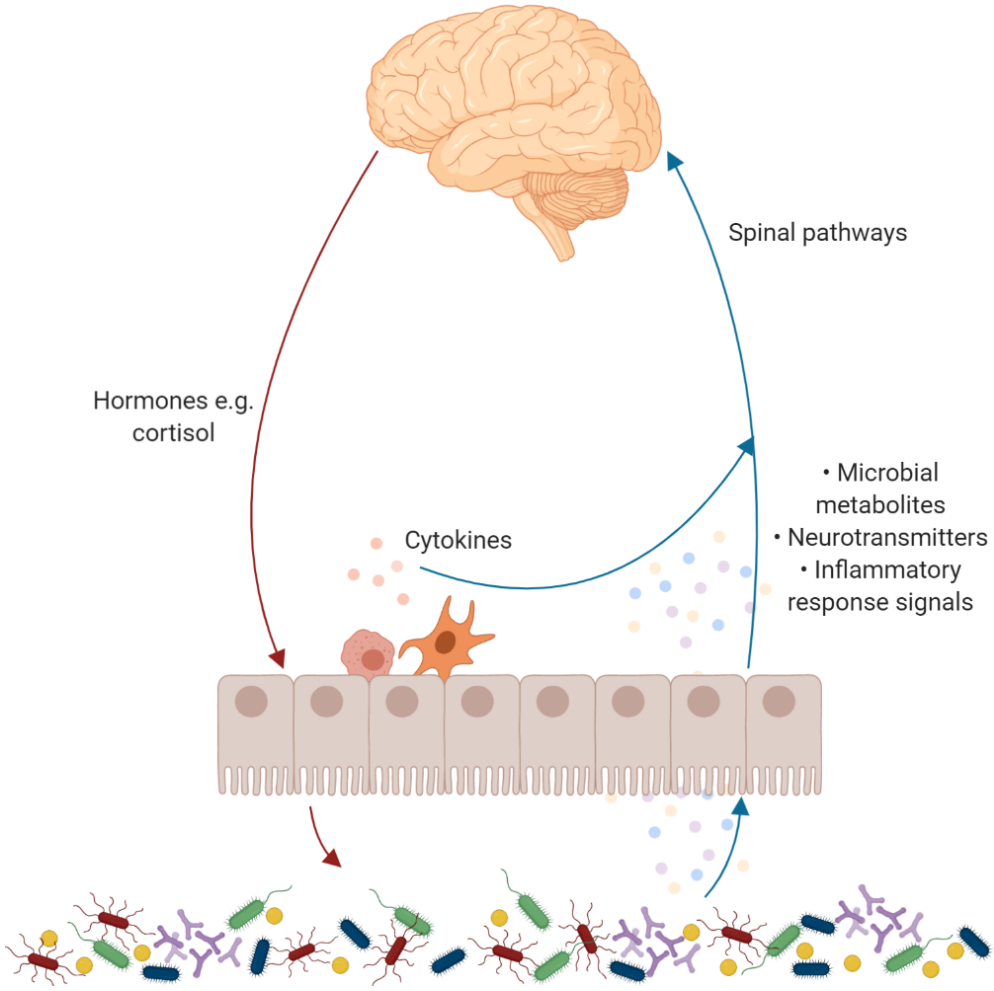


Figure 1-4: 'Brain to gut' and 'gut to brain' signalling in the gut-brain axis (GBA). Figure created using BioRender.

1.7.1. Neurological components

Visceral sensitivity, the increased sensitivity within the gut, is postulated to play a significant role in IBS, and evidence has shown that colonic distension is associated with increasing gut symptom severity. In the event of visceral pain, bacterial compounds, neurotransmitters, and activation of immune responses can transmit signals from the gut to the brain via the GBA [191]. Serotonin production, for example, can affect the epithelial layer of the gut that is linked to nerve endings in cortical and limbic regions of the brain. In turn, this can influence cognitive and emotional responses and trigger the release of neurotransmitters and an inflammatory response [191, 192, 193].

Biopsychosocial refers to the biological, psychological, and social interactions that may occur in health-related conditions that are not solely medical-based [194]. For example, symptoms of anxiety and depression are evident in individuals suffering from IBS [195, 196]. In addition, studies have shown the relevance of anxiety and depression in individuals before and after they develop gut symptoms, and that anxiety and depression were predictors of developing a FGD in the future [187, 188].

Individuals with IBS may have a lower pain threshold and increased anxiety as a side-effect of the visceral pain and sensitivity associated with IBS [197]. Whilst it is generally believed visceral sensitivity is linked to psychological factors, Simren *et al.,* showed there might be a neurological basis for the increased sensitivity in IBS patients by analysing colonic distension in 1,144 individuals, using different methods across multiple cohorts from different countries [198]. Different distension methods were used that included ballooning of different regions of the gut (e.g., rectum, descending colon, gastric fundus), whilst controlling for psychological distress to determine if there were consistent results which could be indicative of a neurochemical stimulus [198]. Results showed that visceral sensitivity contributed to the development of IBS gut symptom severity [198]. Additionally, controlling for anxiety and depression did not alter the correlation between visceral sensitivity and gut symptom severity in IBS, suggesting that while psychological factors are important in IBS, this is not the sole cause of IBS symptoms [198].

Stress is evident in individuals suffering from IBS [199]. Kano *et al.,* investigated pain perception in a cohort of IBS-D and healthy control individuals and showed a reduction in colorectal distension tolerance in IBS individuals [190]. ANS activity, measured using an electrocardiogram, showed a dampened sympathovagal balance (the state of equilibrium between the sympathetic and parasympathetic components of the ANS [200]), in IBS-D individuals compared to healthy controls during rectum distension [190].

Higher thresholds of distention were evident in healthy controls compared to IBS individuals. The ANS controls the body's unconscious response, potentially indicating a dampened ANS response in IBS, with these findings consistent across multiple studies [190, 201, 202].

The main evidence for a gut microbiota related GBA link comes from germ-free (GF) rodent studies where different behaviour and stress responses have been observed compared to those of conventional mice [192, 203]. In addition, manipulation of the gut microbiota using antibiotics or probiotics has been shown to alter behavioural, stress, and pain responses [186, 204].

In mouse models, *Ruminococcaceae* and *Lachnospiraceae*, members of the Clostridia class, stimulated the release of serotonin by the gut enterochromaffin cells [205]. Bailey *et al.,* investigated the response of stress on mice colonised with *Citrobacter rodentium*, a pathogenic microbe that disrupts the brush border of the gut epithelium, similar to *Escherichia coli* in humans [206]. When mice were assigned to prolonged stress conditions, *C. rodentium* abundance increased, which the authors hypothesised was linked to a reduction in other commensal beneficial bacteria [206].

Whilst it remains unknown why a stressor induces a change in the microbial composition, other studies have shown similar results where inflammation caused by a stressor reduces the relative abundance of certain microbial species, thereby allowing other species to fill the ecological niche [206-208]. However, there are many limitations of a GF mouse model, including, but not limited to, the aseptic birth conditions of these mice, their different brain biochemistry, and abnormal behaviour of GF mothers compared to normal counterparts [184].

1.7.2. Neurotransmitters and inflammatory molecules

Metabolites and neurotransmitters produced in the gut and the brain reach the systemic blood circulation via the blood-brain barrier (**Figure 1-5**) [209]. In adults, this barrier consists of a 12-18 m^2 surface available for exchange and absorption of metabolites and neurotransmitters [209].

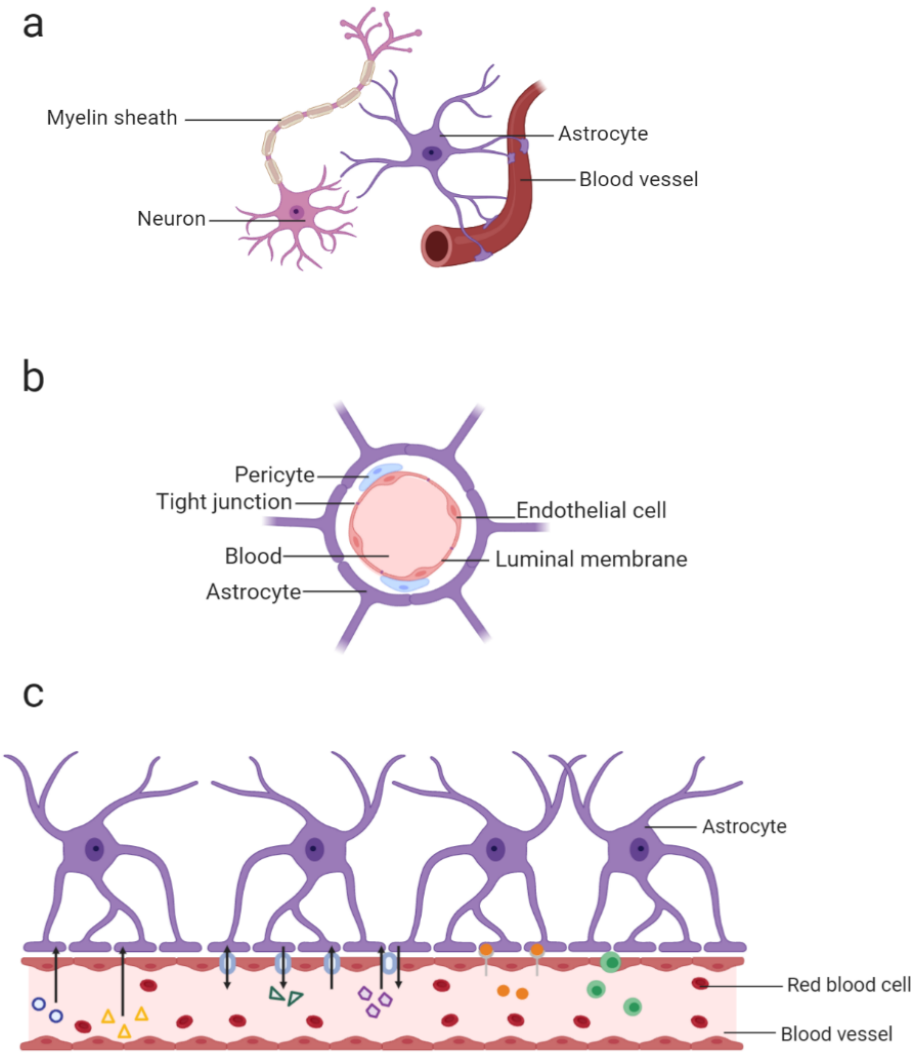


Figure 1-5: a) Blood-brain barrier in contact with neuronal astrocytes that uptake nutrients and metabolites and pass these to neurons. b) Cross-section of the blood-brain barrier in contact with astrocyte endfeet that attach to the membrane and endothelial cells for direct transfer of metabolites. c) Passive diffusion of lipid-soluble non-polar metabolites and diffusion of dissolved gases through the epithelial cells across the membrane (blue and yellow symbol). Solute carriers transport other metabolites, for example, amino acids and glucose, via passive or active transport (green and purple symbol). Receptor-mediated binding

transports specific macromolecules, for example, proteins and cytokines (orange symbol). Leukocytes cross the epithelium and membrane. Figure created using BioRender.

A decrease in circulatory plasma tryptophan concentration, leading to downregulation of the catabolism pathways, has been shown to impact mood, depression, and behaviour [210-212]. The catabolism of tryptophan is via two metabolic pathways: the kynurenine pathway producing neuroprotective kynurenine and neurotoxic quinolinic, or the serotonin pathway producing serotonin. These compounds are neurochemical compounds that can affect the CNS and the ENS [211].

A link between faecal microbial composition and circulatory plasma serotonin concentration can be found in GF mice, characterised by increased levels of plasma tryptophan in GF mice compared to conventional mice, with a reversal to normal concentration following microbial colonisation [211, 213, 214]. The interaction between tryptophan, the microbiota, and the production of tryptophan catabolites may be part of the signalling via the GBA. Several bacterial species, for example, *E. coli, Clostridium spp*., and *Peptostreptococcus spp*. convert tryptophan into indolic compounds, for example, indoleacryclic acid, which diminishes inflammatory responses and promotes neurogenesis [215-217]. Therefore, it may reduce neurochemical signals transmitted to the CNS from an inflammatory response in the gut.

Corticotropin-releasing hormone (CRH), a brain-derived hormone, is released in response to a stressful stimulus causing secretion of adrenocorticotropic hormone (ACTH), triggering the subsequent release of cortisol [218-220]. Cortisol affects other regions of the brain and sympathetic and parasympathetic responses throughout the body [218-220]. Inflammatory cytokines produced in the gut can exert a bottom-up effect on the HPA [219]. As evidence for the bi-directional capability of the GBA, cortisol can trigger the

production of inflammatory cytokines in response to a stressful stimulus. Conversely, inflammatory cytokines may cause activation of the HPA axis and the subsequent release of cortisol in the brain. Increased concentrations of serum TNF-α and interleukin-17, and decreased concentration in IL-10 have been shown in IBS individuals compared to healthy controls [219]. Analysis by Scully *et al.,* showed similar results in a female cohort, characterised by increased circulatory plasma concentration of interleukin-1β, interleukin-6, interleukin-8, and TNF-α in IBS participants compared to healthy controls [221]. An increase in inflammatory cytokine levels is consistent with increased CRH release, evidence of overactivation of the HPA axis [221] and increased depression and anxiety [222]. Additionally, CRH stimulates colonic motility [218], with evidence showing exaggerated colonic contractions and a greater ACTH response in a group of IBS males compared to healthy control males over the first hour following intravenous administration with CRH [190, 218].

1.8. Biomarkers of IBS

Biomarkers are defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention" [223]. When considering diagnostic biomarker panels, high specificity and sensitivity are important for effective application in the clinical setting [224]. Sensitivity refers to the true positive value where the biomarker(s) must have a high chance of selecting the true positives, and that false positives are not selected. Conversely, specificity refers to the true negative value, where there is selection for the true negatives and no selection of the false positives.

Multiple mechanisms are implicated in the pathology of IBS with links to diet, host physiology and metabolism and their interactions with the gut microbiome and microbial-derived metabolites. Hence, biomarkers that incorporate an understanding of the wider

system are necessary for effective diagnosis. Ideally, biomarkers also need to be easy to measure and cost-effective in a clinical setting [37].

Studies in humans have reported measures of biomarkers in serum and breath in IBS and 'healthy' participants [225]. The results showed a high specificity (88%) but only a sensitivity of 50% for differentiating IBS from 'non-IBS' [37, 226]. The lack of sensitivity is likely due to these biomarkers being related to IBD rather than IBS [225, 226].

Jones *et al.,* used these 10 biomarkers and combined them with another 24 markers of gene expression and psychological components (listed in **Table 1-2**) [20]. When all 34 markers were grouped with an additional four psychological measures, the specificity (80%) and sensitivity (93%) were higher (**Table 1-2)**. However, feasibility issues can arise with the difficulty of measuring 38 markers routinely for clinical diagnosis [20, 225].

Research conducted by Mujagic *et al.,* investigated common IBS biomarkers (listed in **Table 1-2**) in a large sample group, which led to high specificity (86.5%) and high sensitivity (88.1%) [224]. Of the 15 biomarkers they measured in plasma and faecal samples, eight showed concentration differences between IBS and healthy controls [224]. A similar analysis of metabolites in serum samples found conflicting results with no differences between IBS and healthy controls, highlighting a need for further analysis to determine effectiveness [85].

Analysis of 16 compounds in the breath of IBS participants and healthy controls showed a specificity of 73.3% and a sensitivity of 89.4% (listed in **Table 1-2**)[225, 227]. Thus, this study presented compelling evidence with good sensitivity and specificity and minimally invasive sampling. Analysis of 16 compounds, however, can be challenging

for clinical diagnosis, and it is difficult to determine what breath metabolites (alkanes) may be linked to metabolic pathways in the gut.

Camilleri *et al.,* measured total faecal bile acid abundance and colonic transit [86]. They found that bile acids were different between healthy controls and IBS participants, as well as allowing differentiation between those with IBS-C and IBS-D with a specificity of 75-90% and 60% sensitivity [85, 86]. These findings demonstrated positive correlations between bile acids, colonic transit and IBS subtypes [86].

These studies show that there are some biomarkers with good selectivity and sensitivity for differentiating IBS subtypes, IBS and healthy controls. However, these studies fail to investigate the mechanisms that cause the symptoms of IBS. Diagnosis and therapy should go hand in hand. Hence, understanding the mechanisms underlying IBS and how these can be modulated to ameliorate symptoms is necessary.

Table 1-2: Studies investigating biomarker panels in IBS

Biomarkers	Sample type	Sample cohort	Sample size	Sensitivity	Specificity	Reference
Interleukin-1B Growth related oncogene-α Brain-derived neurotrophic factor Anti-Saccharomyces cerevisiae antibody Antibody against bacterial flagellin (CBir1) Antihuman tissue transglutaminase Tumour necrosis factor-like weak inducer of apoptosis Antineutrophil cytoplasmic antibody Tissue inhibitor of metalloproteinase-1 Neutrophil gelatinase-associated lipocalin	Serum	IBS, IBD, coeliac disease, HC	IBS *n*= 876, IBD *n*=398, coeliac disease *n*=57, HC *n*=235	50%	88%	[226]
10 original biomarkers above from [226] and 24 more added biomarkers: Histamine Prostaglandin E2 (PGE2) Tryptase Serotonin Substance P	Serum	IBS, HC	IBS *n*=168, HC *n*=76	81%	64%	[20]

Interleukin-1 Interleukin-10 Interleukin-6 Interleukin-8 Tumour necrosis factor-like weak inducer of apoptosis 14 gene expression markers (CBFA2T2, CCDC147, HSD17B11, LDLR, MAP6D1, MICALL1, RAB7L1, RNF26, RRP7A, SUSD4, SH3BGRL3, VIPR1, WEE1, ZNF326).						
Interleukin-1B Interleukin 6 Interleukin-12p70 Tumor necrosis factor-like weak inducer of apoptosis Chromogranin A Human beta-defensin 2 Calprotectin Caproate	Faecal & plasma	IBS, HC	IBS *n*=196, HC *n*=160	88.1%	86.5%	[224]
Butane N-hexane Tetradecanol $C_{11}H_{24}$	Breath	IBS, HC	IBS *n*=170, HC *n*=153	89.4%	73.3%	[227]

6-Methyloctadecane						
1,4-Cyclohexandiene						
Unknown volatile organic compound						
Methylcyclohexane						
2-Undecene						
N-Heptane						
Aziridine (Ethylenimine)						
$C_{17}H_{36}$						
Benzyl-oleate						
6,10-dimethyl-5,9-undecadien-2-one						
1-Ethyl-2methyl-cyclohexane						

Abbreviations: HC (healthy controls); IBS (irritable bowel syndrome); IBD (inflammatory bowel disease)

1.9. Conclusion

Although FGDs have been heavily studied in the past decade, the underlying cause still remains unknown, as do the best options for prevention or amelioration. Understanding why some individuals develop FGDs and others do not is a challenge, compounded by the lack of physical disturbance to the gut. Various studies have assessed plasma, faecal, or urinary metabolite abundance in FGD participants; however, this is usually in a small cohort group or based on analysis of few known metabolites. Furthermore, whilst the characterisation of the gut microbiota composition has been studied, the characterisation of the faecal metabolome has only recently been attempted. Understanding concentration differences of key groups of metabolites is needed to provide evidence of mechanistic differences and molecular targets for biomarker development to aid with diagnosis. Comparatively, untargeted characterisation of the faecal metabolome provides an in-depth analysis of the metabolites associated with host-microbial interactions that may be occurring in the gut and thereby advancing the understanding of FGDs.

1.10. Scientific aims and

The overall aim of this thesis was to quantify key metabolites and characterise the faecal metabolome of individuals with FGDs and healthy controls. Specifically, I aimed to address the following research questions:

1. *Do faecal bile acid concentrations differ between individuals with FGDs, IBS subtypes, and healthy controls that could be reflective of differential processes in FGDs?*

   Prior research has highlighted the importance of bile acids in FGDs, especially diarrhoea and BAM. However, previous studies have been limited to small sample sizes or the analysis of only a few bile acids which is not reflective of the variability in populations or bile acid diversity. The aim of **Chapter 3** was to quantify the

concentration of faecal bile acids in a large FGD cohort that may reflect a perturbation in metabolic processes. To assess bile acid concentrations in faecal samples, I utilised liquid chromatography-mass spectrometry (LC-MS) with 23 bile acid standards.

2. *Do plasma amino acid concentrations differ between individuals with FGDs, IBS subtypes, and healthy controls that could be reflective of differential processes in FGDs?*

Analysis of all amino acids metabolites has not previously been conducted in a FGD cohort. Aside from being building blocks of protein, amino acids have other potentially important physiological effects, as do the subsequent metabolites produced from amino acids. The aim of **Chapter 4** was to quantify the concentration of plasma amino acids in a FGD cohort that may reflect a perturbation in metabolic processes detectable at systemic level. To assess amino acid concentrations, I utilised ultra-performance liquid chromatography (UPLC) with 20 available amino acid standards.

3. *Does the faecal metabolome of those with constipation (FC + IBS-C) or diarrhoea (FD + IBS-D) symptoms differ from healthy controls and are these differences linked to perturbed metabolic pathways and mechanistic processes in the gut, specific to phenotype?*

It is only recently that untargeted characterisation of the faecal metabolome has been carried out in FGD cohorts. However, the current studies have focused on polar and semi-polar metabolites and did not include lipid metabolites. The aim of **Chapter 5** was to characterise the faecal metabolome using mass spectrometry technologies to highlight pathways and processes that differ between constipation (FC + IBS-C) and healthy controls or diarrhoea (FD + IBS-D), each in comparison to healthy controls.

In my analysis, three untargeted analytical streams were utilised to detect polar, semi-polar and lipid metabolites.

4. *Does the integration of available data from the wider research programme provide further insight into the pathways and processes occurring in individuals with constipation (FC + IBS-C) or diarrhoea (FD + IBS-D) that differentiate each phenotype from healthy controls?*

In health research, systems biology attempts to understand system-wide perturbation that cause disorders or disease. The interaction between dietary intake, the gut microbiome, host processes and the consequent production of host-microbial metabolites in FGDs have been investigated only in a few studies, and systems biology analysis of FGDs is limited. The aim of **Chapter 6** was to integrate the data of dietary intake, the faecal metabolome, faecal microbiome, and plasma metabolome to highlight pathways and processes that differ between constipation (FC + IBS-C) and healthy controls or diarrhoea (FD + IBS-D), each compared to healthy controls.

Chapter Two
Cohort Introduction

Chapter Two

2.1. Introduction

Chapter Two is designed to provide an overview of the wider research programme that the research presented in this PhD sits within.

2.2. Programme overview

This PhD thesis forms part of the Digestive Health Programme within the High-Value Nutrition (HVN), National Science Challenge. HVN is a collaboration between the science community, government, and the NZ Food and Beverage industry to bring together a multi-disciplinary approach to better understand the human body for developing scientifically validated, high-value foods beneficial to health. HVN consists of four priority research programmes: Metabolic Health, Immune Health, Infant Health, and Digestive Health. All programmes aim to take a systems nutrition approach to study the food and health relationships supported by Science of Food and Consumer Insights research platforms.

The Digestive Health Priority Research Programme investigates the link between diet, the microbiome, and the host to better understand metabolic processes and pathways that differ between FGDs and healthy controls, and within FGD subtypes. A cohort was established to recruit participants with FGDs and healthy participants as part of this programme and named The Christchurch IBS cOhort to investigate Mechanisms FOr gut Relief and improved Transit (COMFORT) cohort (**Figure 2-1**). From there, food and beverage interventions are investigated, targeting the perturbed pathways between FGDs and healthy controls to generate scientifically validated digestive health benefits for the New Zealand food and beverage export market. The data analysis is broadly categorised into dietary records, clinical symptom data, and biological data; the latter includes the gut

microbiome, plasma, and faecal metabolomes, known metabolites of importance (bile acids, SCFAs, neurotransmitters), and gut physiological measurements (physiome).

The Digestive Health research priority programme supported three PhD thesis candidates using the COMFORT cohort resources. Dr Phoebe Heenan (awarded PhD in August 2021) focused on the association of diet and acute gastrointestinal symptoms in irritable bowel syndrome. PhD Candidate Caterina Carco studied the characterisation of the gut microbiome and blood immune cell transcriptome.

This PhD thesis is a chemistry-based approach that combines mass-spectrometry (MS) metabolomics and analytical chemistry to investigate metabolites from key pathways important to perturbed mechanisms underlying FGDs and compare them to those in healthy participants. **Chapters 3** and **4** (analytical chemistry), and **5** (untargeted metabolomics) are based on data collected from the participants recruited in the COMFORT cohort. These chapters aimed to quantify known molecules (bile acids and amino acids) involved in metabolic processes underlying FGDs and characterise the faecal metabolome to generate new insights into FGD processes. **Chapter 6** aims to integrate these datasets from **Chapter 5** with the plasma metabolome and faecal microbiome using a systems biology approach.

Chapters 3 and **4** are aimed at quantifying concentrations of known metabolites and comparing them across all FGD groups, and between the two main phenotypic groups (constipation (FC + IBS-C) and diarrhoea (FD + IBS-D)) and the healthy control group. **Chapters 5** and **6** are aimed to identify potential metabolites and pathways that differ between constipation or diarrhoea compared to healthy controls toward a systems-based understanding of FGDs. **Chapters 5** and **6** do not compare between all FGD groups as

the ultimate aim of the wider programme is to shift the phenotype of those with constipation or diarrhoea back towards that of healthy individuals.

Information regarding study design, exclusion and inclusion criteria, sample collection information, and all other relevant details are provided here and will not be replicated in successive chapters.

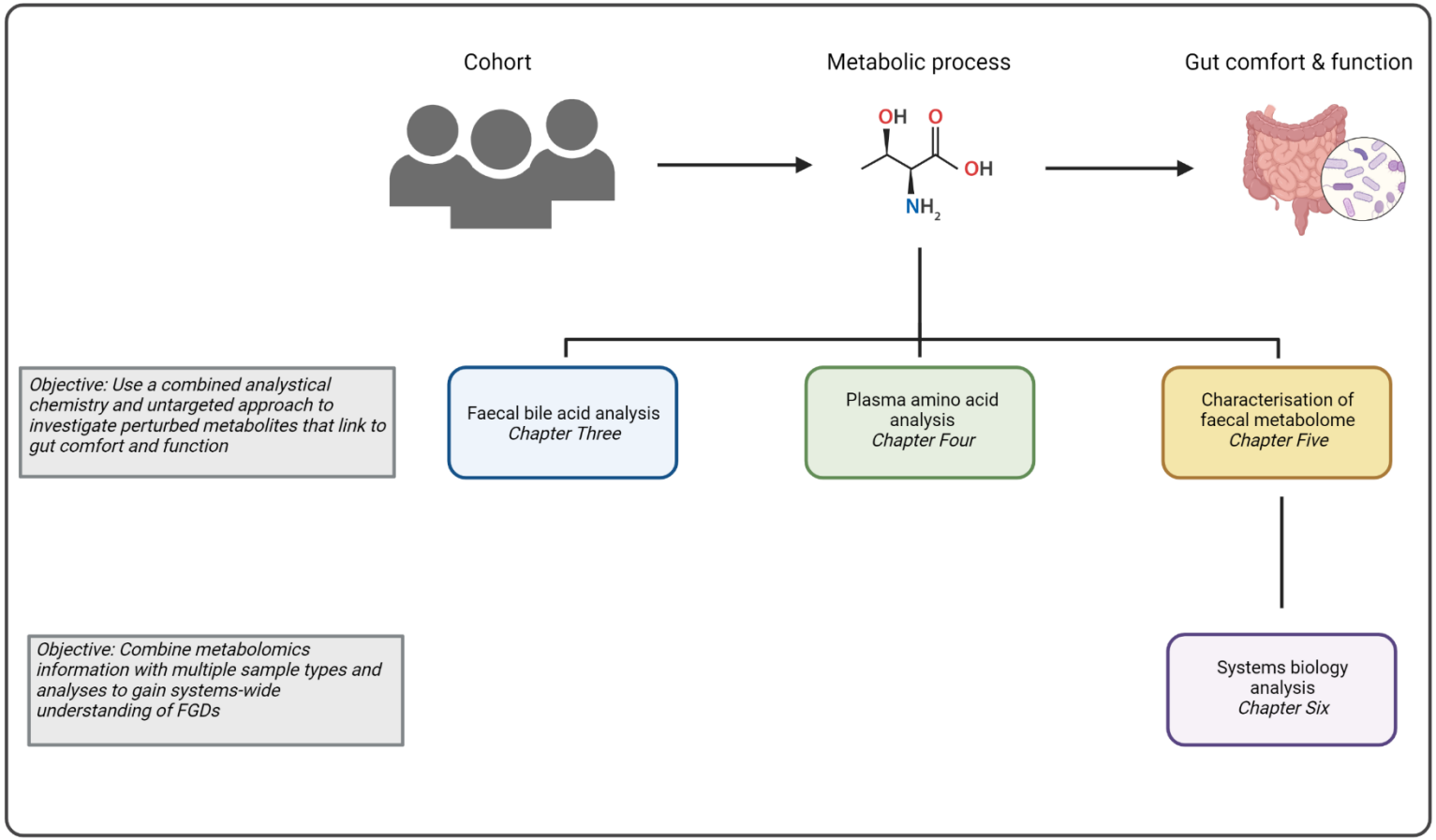


Figure 2-1: Schematic briefly outlines this PhD thesis, showing chapters relating to COMFORT cohort and systems biology analyses of host-microbial interactions underlying FGDs. Abbreviation: COMFORT (The Christchurch IBS cOhort to investigate Mechanisms FOr gut Relief and improved Transit) cohort.

2.3. The COMFORT cohort

The clinical study was conducted following the protocol, International Conference on Harmonisation guidelines, applicable national and local requirements, and the ethical principles that have their origin in the Declaration of Helsinki. The study was approved by the University of Otago Human Ethics Committee (Health) (Ref. # H16/094).

The COMFORT cohort was established as a core resource of the Digestive Health Priority Research Programme [228]. Participants were recruited between 2016 and 2018 by PhD candidate Phoebe Heenan and staff under the leadership of Professor Richard Gearry, Department of Medicine of the University of Otago in Christchurch, New Zealand. Faecal, urine, and plasma samples were collected alongside clinical, dietary, and demographic information. Study centres were the Department of Gastroenterology, Christchurch Hospital, Christchurch, New Zealand and the Southern Endoscopy Centre, Caledonian Road, Christchurch, New Zealand.

Participants were those with a known FGD (cases) or without (controls) aged 17-70 years. Healthy controls were individuals with a known history of family colonic cancer, polyps or screening for polyps undergoing colonoscopy as part of regular bowel screening. Exclusion criteria were an inability to give consent, pregnancy, other known gut disorders (e.g., IBD, colorectal cancer, diverticulitis), previous bowel resection, coeliac disease, other known diseases (e.g., hepatitis), or less than five days between obtaining written consent and commencing preparation for colonoscopy. Participants were able to withdraw from the study for any reason and at any time.

Potential participants were sent patient information sheets and consent forms prior to meeting with study personnel. In addition, the following questionnaires were collected as part of the study:

- ROME IV Questionnaire
- Modified Hunter New England Health Survey (ModHNEHS)
- Economic Living Standard Index Short Form (ELSISF)
- Structured Assessment of Gastrointestinal Symptoms Scale (SAGIS)
- Hospital Anxiety and Depression scale (HADS)
- Short Form 12 Health Survey (SF12)
- Patient-Reported Outcomes Measurement Information System- Gastrointestinal (PROMIS-GI) and PROMIS-emotional distress (PROMIS-ED).
- Food and Symptoms Times (FAST) diary

At baseline, blood was drawn in sequential order of 6 x 6mL lithium heparin (LiH) vacutainer, 3 x 4mL ethylenediaminetetraacetic acid (EDTA) vacutainer, and 1 x 10mL untreated vacutainer. Samples were transported to the laboratory and spun within 60 min at 2000 *x g* for 5 min at room temperature before being aliquoted into 1.7mL Eppendorf tubes.

- The sample in the EDTA vacutainer was aliquoted into 3 x 500µL aliquots and 2 x 1.5mL biobank.

Dietary information was recorded for three consecutive days (including one day of the weekend). Dietary intake was converted to nutrient group intakes for each meal by trained clinicians. On the fourth day of the diet diary, SAGIS, PROMIS-GI, and PROMIS-ED questionnaires were filled in, and urine and faecal samples were collected at home. The faecal sample was stored in home freezers and urine sample in home refrigerators. Both samples were transported within 24 hours on ice to the study centre, where all biological samples were aliquoted and stored at -80°C until distributed to other locations for analysis. Faecal samples for metabolomic analyses were freeze-dried prior to analysis.

Chapter Three
Analysis of faecal bile acids in participants with functional gut disorders

Sections of this chapter have been published: see Appendix for full paper

James, S.C.; Fraser, K.; Young, W.; Heenan, P.E.; Gearry, R.B.; Keenan, J.I.; Talley, N.J.; Joyce, S.A.; McNabb, W.C.; Roy, N.C. Concentrations of fecal bile acids in participants with Functional Gut Disorders and healthy controls. *Metabolites* 2021, *11*, 612.

Abstract

Bile acids are metabolites involved in nutrient absorption and signalling, with levels influenced by dietary intake, metabolic processes, and the gut microbiome. This chapter aimed to quantify 23 bile acids in faecal samples to ascertain if concentrations differed between healthy participants and those with FGDs. Faecal bile acids were measured using LC-MS in 250 participants (Rome IV: IBS-C, IBS-D, IBS-M, FC, FD (FC n = 35, FD n = 13, IBS-C n = 24, IBS-D n = 52, IBS-M n = 29, and healthy control n = 97)). Some faecal bile acid concentrations, predominantly primary bile acids, were significantly different between all FGD participants and healthy controls (CDCA p = 0.011, CA p = 0.003) and between combined constipation (FC + IBS-C) and diarrhoea (FD + IBS-D) groups (CDCA p = 0.001, CA p = 0.0002). Comparison of bile acids between all functional groups showed four metabolites were significantly different, although analysis of combined constipation (FC + IBS-C) and diarrhoea (FD + IBS-D) groups showed that 10 metabolites were significantly different. The bile acid profiles of FD individuals were similar to those with IBS-D, and likewise, those with FC were similar to IBS-C. Individuals with a diarrhoea phenotype had higher concentrations of bile acids compared to those with constipation. Bile acid metabolites distinguish between individuals with FGDs and healthy controls but are similar in constipation (or diarrhoea), whether classified as IBS or not.

Chapter Three

3.1. Introduction

Bile acids are chemical detergents aiding in the digestion and absorption of nutrients [229] and have a regulatory role in the circulatory system impacting lipid, glucose, nutrient, and energy homeostasis [229]. They also mediate interactions between the host and microbiome via cellular receptors (e.g., FXR, G-coupled protein receptors, vitamin D receptor) [89, 230].

Bile acids are synthesised in hepatocytes from cholesterol via the classic and alternative pathways, producing the primary bile acids CA and CDCA [89]. Primary bile acids are conjugated to either glycine or taurine and stored in the gallbladder [89, 231]. Bile acids are excreted from the gallbladder into the small intestinal lumen with the bile flow and unconjugated in the colon by microbial bile salt hydrolase enzymes [232], then modified to secondary bile acids, deoxycholic acid and LCA by microbial dehydroxylase and dehydrogenase enzymes [89, 233]. Some bile acids can be toxic to host, and microbiota in excess quantities, and hence the regulation of their concentration and metabolism within the hepatic portal system is tightly controlled [229]. Most bile acids are recycled via the enterohepatic circulation multiple times a day; however, approximately 5% of bile acids escape this process and are further modified bacterially before excretion through faeces [229].

The microbial conversion of primary to secondary bile acids can be a multi-step rate-limiting process. Only microbial species possessing the bile salt hydrolase enzyme (for example, some members of the *Bacteroides, Clostridium, Lactobacillus,* and *Bifidobacterium* genera) can deconjugate primary bile acids [232, 234]. Disturbances to the gut microbiota composition can affect bile acid deconjugation and modification [232].

Furthermore, the gut microbiome converts secondary bile acids to bacterially modified or 'tertiary' bile acids [234].

Thus, the interaction between bile acids, the gut microbiome, and host metabolism is an important homeostatic metabolic process [233]. There is increasing evidence that alterations to bile acid metabolism may be associated with clinical disease, including FGDs such as IBS. BAM has been associated with IBS-D and is characterised by increased colonic transit and bowel movements, mucus production, and greater epithelial permeability [93, 98, 235]. Studies have shown increased concentrations of specific primary and secondary bile acids in the plasma and faeces of individuals with IBS-D compared to IBS-C and healthy controls [91, 93, 97, 99]. However, these studies are often limited to a smaller cohort size that does not incorporate multiple FGDs or a smaller bile acid panel.

Recently, it was shown that a major subgroup with IBS-D have BAM, and that those in the severe BAM group had a gut microbial shift that correlated with changes in the faecal metabolome and diet [77]. Another study demonstrated that 25% of IBS-D individuals had increased faecal bile acid concentrations [236] compared to healthy controls. Additionally, those with high faecal bile acids had increased relative abundance of *Clostridia*, and elevated expression of the 7α-hydroxylase gene, the primary enzyme converting cholesterol into bile acids [236].

It was hypothesised that faecal bile acid concentrations would differ between IBS subtypes, functional groups, and healthy controls, reflecting a perturbation in the metabolic processing of bile acids in FGDs. Within IBS subtypes, bile acids will have a higher faecal concentration in individuals presenting with diarrhoea (IBS-D+FD) rather than constipation (IBS-C+FC) based on the available research showing a link between

diarrhoea and faecal bile acids. To test these hypotheses, this analysis aimed to quantify 23 bile acids implicated in multiple conversion steps and available as chemical standards in faecal samples collected from a cohort of individuals with FGDs and healthy controls.

3.2. Methods

3.2.1. Participants

Information on recruitment, inclusion, exclusion, and sample collection methodology was as described in **Chapter 2**. Two hundred and fifty faecal samples were analysed from the COMFORT cohort.

3.2.2. Standards and reagents

Deuterated-cholic acid (d_4-CA), bile acids (CA, CDCA, LCA, TCA, UDCA, taurine, βMCA, TαMCA, TβMCA, TLCA, TCDCA) and formic acid were purchased from Sigma-Aldrich Chemicals Co. (St Louis, MO, USA). All other bile acid standards (GCDCA, GCA, GDCA, GHDCA, GLCA, GUDCA, HCA, HDCA, ILA, TDCA, THDCA, TUDCA) were purchased from Steraloids Inc. (Newport, RI, USA). **Table 3-1** outlines abbreviations and full, common names. Acetonitrile (ACN) and MeOH of optima LC-MS grade quality were purchased from Thermo Fisher Scientific (Auckland, New Zealand).

3.2.3. Sample extraction

Extraction methods followed those previously described by Joyce *et al*., with minor modifications [234]. Briefly, 100mg of freeze-dried faecal samples were spiked with 100ng of d_4-CA and extracted with 700µL ice-cold 50% MeOH in Eppendorf tubes pre-filled with 4mm ceramic beads. The mixture was homogenised for six 30 sec intervals (QIAGEN TissueLyser II, QIAGEN, Hilden, Germany) and incubated at -20°C for 30 min and then centrifuged at 10,000 *x g* for 25 min. Next, 450µL of the extract was

transferred to a fresh tube and dried under nitrogen at 45°C. One mL of ice-cold ACN containing 5% formic acid was added to each tube, and the sample briefly vortexed and agitated for 1 hr gently at room temp. The mixture was centrifuged at 10,000 *x g* for 10 min, and the resulting supernatant was transferred to Eppendorf tubes and dried under nitrogen at 45°C. The residual extract was dissolved in 150µL of 50% MeOH, centrifuged at 10,000 *x g* for 5 min and transferred to glass vials for chromatographic analysis.

The analysis was completed on a SCIEX LCMS/MS QTRAP® 6500+ system coupled to an ExionLC™ (SCIEX, Victoria, Australia). 1µL of the sample was injected into a Waters Aquity UPLC® column (Massachusetts, USA) maintained at 50°C with a flow rate of 300µL/min. The mobile phase, solvent A, consisted of 10mM ammonium formate in H_20 and solvent B, 10mM ammonium formate, 5% ACN/95% MeOH. Gradient elution was as follows; 50% B held for 2 min then increased to 87% B at 13.5 min, 99% B at 18 min, returning to 50% B at 19 min and held until 21 min for re-equilibration.

Mass spectral detection was performed in negative electrospray ionisation mode using multiple reaction monitoring (MRM) for 23 bile acid compounds and the internal standard using electrospray ionisation. Standards for all target compounds were run prior to sample analysis to optimise MRM conditions and separation of compounds. The source voltage was set to -4500V, with a source temperature of 550°C. Data was captured using Analyst® (V1.6) software and processed on MultiQuant (V3.0.2) SCIEX software. Bile acid concentrations were generated from standard curves of standard injections for all 23 bile acids and the deuterated internal standard (d_4-CA). Concentrations of bile acids were corrected to the dry weight of faecal matter and are presented as µg/mg of a dried faecal sample. K-nearest neighbour was employed to input any missing values in the data using MetaboAnalyst (V4.0) [237, 238].

Table 3-1: Bile acids analysed using UPLC-MS with the corresponding acronym.

Name	Acronym
Beta-muricholic acid	βMCA
Cheno-deoxycholic acid	CDCA
Cholic acid	CA
Deuterated (d4) cholic acid (IS)	d4-CA
Glyco-cheno-deoxycholic acid	GCDCA
Glyco-cholic acid	GCA
Glyco-deoxycholic acid	GDCA
Glyco-hyo-deoxycholic acid	GHDCA
Glyco-litho cholic acid	GLCA
Glyco-urso-deoxycholic acid	GUDCA
Hyo-cholic acid	HCA
Hyo-deoxycholic acid	HDCA
Iso-lithocholic acid	ILA
Litho-cholic acid	LCA
Taurine	Taurine
Tauro-alpha-muricholic acid	TαMCA
Tauro-beta-muricholic acid	TβMCA
Tauro-cheno-deoxycholic acid	TCDCA
Tauro-cholic acid	TCA
Tauro-deoxycholic acid	TDCA
Tauro-hyo-deoxycholic acid	THDCA
Tauro-litho cholic acid	TLCA
Tauro-urso-deoxycholic acid	TUDCA
Urso-deoxycholic acid	UDCA

Bile acids with the corresponding acronym

3.2.4. Data processing and statistical analysis

Residual plots and Shapiro-Wilk tests showed the data was unevenly distribution, and thus the data were log-transformed. The R statistical package (V3.6.1) was used for individual metabolite analyses and heatmap visualisations. ANOVA was used to compare means, with a probability (*p*) less than 0.05 deemed statistically significant. If a metabolite was significantly different, pairwise mean comparisons were used to compare differences between participant groups. Bile acid metabolite distributions were visualised using notched box plots, with the boundaries of the notches showing a 95% confidence interval (CI). Metaboanalyst (V4.0) [237] was used for hierarchical clustering analysis. Basic nutritional data were analysed using ANOVA to compare group differences in three-day dietary intake. Faecal dry weight was calculated relative to wet weight.

3.3. Results

A total of 259 faecal samples were analysed; however, there was incomplete metadata for nine participants, leaving 250 participants in the final analyses. Two participants were taking cholesterol-lowering medication. Symptom questionnaires based on the Rome Criteria IV classified these 250 participants as FC *n=35,* FD *n=13,* IBS-C *n=24*, IBS-D *n=52,* IBS-M *n=29,* and healthy control *n=97*.

Of the 23 bile acid metabolites measured, THDCA, βMCA, TβMCA, TαMCA, GDDCA, UDCA and TUDCA were below the limit of detection. Therefore 16 bile acids were quantified and included in the further analyses.

Table 3-2 shows the sex and age of the participants in the COMFORT cohort. A gender effect ($p = 0.000103$) was observed between the subtypes as there was a larger proportion of females in all groups compared to males. Age ($p = 0.128$) did not significantly differ between the groups. The mean faecal dry weight percentage for all groups is presented in

Table 3-2. The mean faecal dry weight was significantly different within the cohort (p = 0.013), where FC and IBS-C had a higher dry weight (%) compared to FD and IBS-D. The dry weight (%) of faecal samples from controls was between that of constipation (FC and IBS-C) and diarrhoea groups (FD and IBS-D), while IBS-M was higher than all other groups. Pairwise comparison showed no significant difference between the FC and IBS-C groups or the FD and IBS-D groups (**Table 3-2**).

Analysis of three-day dietary information showed no significant difference in reported fibre (p = 0.848) or fat (p = 0.401) intake by the participants of the cohort (**Figure 3-1**).

Table 3-2: Characteristics of the participants of the COMFORT cohort faecal bile acid analysis.

	Control	IBS-C	FC	IBS-D	FD	IBS-M	*p* value
Female (male) *n*	52 (45)	23 (1)	25 (10)	40 (12)	11 (2)	24 (5)	0.0001
Age (mean)	54.4	53.5	59.1	52.8	58.4	50.5	0.128
Faecal mean dry weight (%)	27.25	31.11	30.16	25.18	26.10	31.35	0.013

Abbreviations: Healthy control (control), functional constipation (FC), IBS-constipation (IBS-C), functional diarrhoea (FD), IBS-diarrhoea (IBS-D), IBS-mixed (IBS-M). p value for female (male) is the significance between gender.

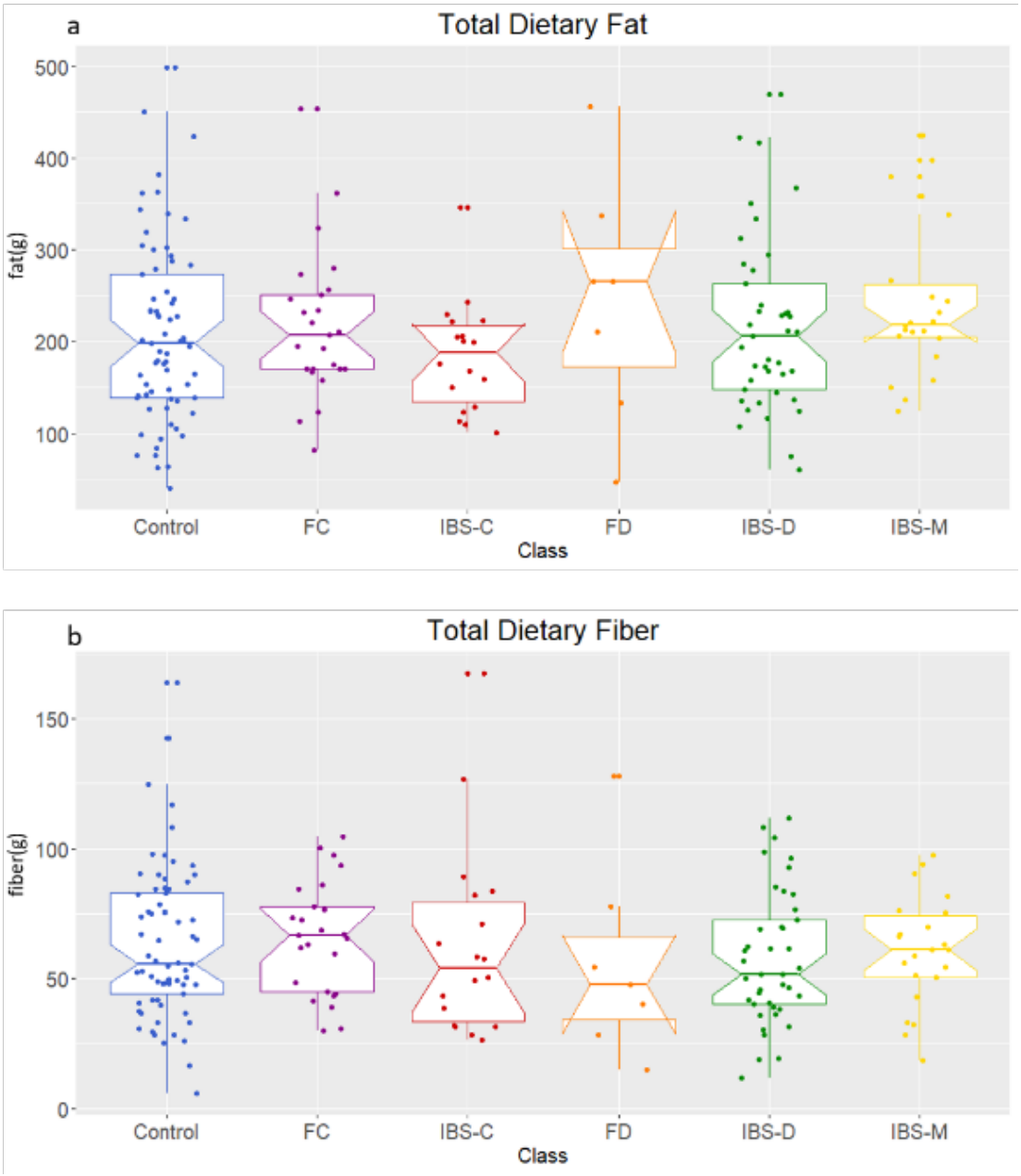


Figure 3-1: Dietary intake of (**a**) fat and (**b**) fibre over three-day period recorded using diet diaries for each participant. Boxplots show median (centre line), 25th and 75th percentile (top and bottom of boxes, respectively), with whiskers representing 1.5 times the inter-quartile range, and boundaries of notches show 95% confidence interval (CI). Abbreviations: healthy control (control), functional constipation (FC), IBS-constipation (IBS-C), functional diarrhoea (FD), IBS-diarrhoea (IBS-D), IBS-mixed (IBS-M).

3.3.1. Faecal bile acid concentrations between healthy control, IBS subtypes, and functional groups

Univariate analysis showed that four bile acid metabolites (CDCA $p = 0.011$, CA $p = 0.003$, GCA $p = 0.048$, Taurine $p = 0.038$) were significantly different in faecal concentration between groups **(Figure 3-2, Table 3-3)**. As shown in **Figure 3-2**, further pairwise comparisons were performed for significant metabolites and showed no significant difference between FC and IBS-C groups or FD and IBS-D groups. However, there were significant differences between IBS-C and IBS-D in the faecal concentration of all four metabolites. The differences between healthy control and FC groups were only significantly different for CA. IBS-D and healthy control groups were significant for CA and CDCA. IBS-C and healthy control groups were significant between GCA and taurine. The concentrations of the primary bile acid CDCA were similar between the constipation and diarrhoea groups, respectively **(Table 3-4)**. Two metabolites were significantly higher in males than females (CDCA $p = 0.009$, HDCA $p = 0.030$).

Hierarchical clustering analysis for group averages showed IBS-D and FD, IBS-C and FC, and healthy controls and IBS-M clustered together **(Figure 3-3)**. In addition, FD and IBS-D groups clustered separately from the other two groups. FD and IBS-D participants had increased faecal concentrations of bile acids, whilst FC and IBS-C participants had decreased concentration compared to both IBS-M and healthy control groups, which were characterised by variable concentrations of bile acids.

Table 3-3: Significance probability (*p*) values for bile acid metabolites between healthy control, constipation (FC + IBS-C), and diarrhoea (FD + IBS-D) groups.

	Group *p*-value	Gender *p*-value	Group x Gender *p*-value
CA	0.0002 ***	0.168	0.092
CDCA	0.001 ***	0.034	0.152
GCA	0.006 **	0.488	0.613
GDCA	0.030 *	0.286	0.016 *
GHDCA	0.015 *	0.646	0.214
GLCA	0.295	0.765	0.25
GUDCA	0.138	0.205	0.848
HCA	0.025 *	0.277	0.14
HDCA	0.16	0.045	0.003 *
ILA	0.893	0.821	0.632
LCA	0.581	0.539	0.316
Taurine	0.018 *	0.402	0.768
TCA	0.098	0.157	0.134
TCDCA	0.021 *	0.32	0.456
TDCA	0.018 *	0.445	0.083
TLCA	0.007 **	0.348	0.653

Statistical significance denoted as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). Abbreviations: cholic acid (CA); chenodeoxycholic acid (CDCA); glyco-hyo-deoxycholic acid (GHDCA); glyco-urso-deoxycholic acid (GUDCA); glyco-deoxycholic acid (GDCA); hyo-deoxycholic acid (HDCA); hyo-cholic acid (HCA); glyco-cholic acid (GCA); iso-lithocholic acid (ILA); glyco-litho cholic acid (GLCA); lithocholic acid (LCA); tauro-lithocholic acid (TLCA); tauro-cheno-deoxycholic acid (TCDCA); tauro-deoxycholic acid (TDCA); tauro-cholic acid (TCA).

Table 3-4: Mean concentration values of groups for faecal bile acid metabolites.

	Control	IBS-C	FC	IBS-D	FD	IBS-M
CA	56.16 ± 255.46	12.44 ± 23.53	11.16 ± 30.87	58.79 ± 172.97	160.13 ± 513.21	37.52 ± 99.60
CDCA	29.65 ± 102.48	9.31 ± 15.35	11.61 ± 23.37	31.92 ± 67.42	32.39 ± 70.86	22.83 ± 50.26
GCA	199.35 ± 317.56	68.1 ± 86.16	118.23 ± 222.78	221.83 ± 346.59	177.95 ± 169.83	166.30 ± 353.11
GDCA	110.41 ± 167.88	53.49 ± 54.29	126.42 ± 371.25	119.12 ± 130.64	91.47 ± 88.84	92.10 ± 112.37
GHDCA	0.72 ± 3.61	0.29 ± 0.56	0.21 ± 0.26	0.34 ± 0.31	0.40 ± 0.49	0.24 ± 0.24
GLCA	0.18 ± 0.18	0.17 ± 0.13	0.21 ± 0.21	0.20 ± 0.15	2.01 ± 6.22	0.16 ± 0.11
GUDCA	0.81 ± 3.88	0.32 ± 0.57	0.47 ± 1.26	0.40 ± 0.38	0.47 ± 0.58	0.28 ± 0.25
HCA	6.77 ± 13.63	2.26 ± 1.97	3.43 ± 3.87	5.62 ± 6.66	9.87 ± 21.42	5.21 ± 7.40
HDCA	90.55 ± 101.77	92.99 ± 96.97	87.33 ± 77.63	122.15 ± 135.87	84.13 ± 88.98	71.12 ± 71.11
ILA	31.51 ± 20.67	30.91 ± 18.1	31.25 ± 17.92	33.17 ± 23.27	28.97 ± 15.79	35.01 ± 22.27
LCA	548.75 ± 336.88	483.52 ± 270.23	481.59 ± 271.46	618.36 ± 426.56	451.99 ± 189.5	513.83 ± 289.08
Taurine	5.56 ± 18.91	4.65 ± 18.29	7.29 ± 23.0	17.85 ± 46.93	16.59 ± 29.4	1.84 ± 4.07
TCA	4.14 ± 7.82	2.06 ± 3.1	2.10 ± 4.87	6.47 ± 20.76	2.70 ± 4.0	6.55 ± 28.14
TCDCA	3.35 ± 10.5	1.19 ± 2.48	4.65 ± 14.37	6.35 ± 19.67	7.28 ± 12.12	3.30 ± 8.97
TDCA	4.84 ± 12.5	0.64 ± 0.68	2.27 ± 6.24	3.54 ± 11.74	5.34 ± 5.89	3.26 ± 10.22
TLCA	0.94 ± 4.46	0.26 ± 0.77	0.35 ± 0.8	1.69 ± 6.10	2.17 ± 3.82	0.76 ± 3.06

Values presented as mean (µg/mg) ± standard deviation. Abbreviations: Healthy control (control), functional constipation (FC), IBS-constipation (IBS-C), functional diarrhoea (FD), IBS-diarrhoea (IBS-D), IBS-mixed (IBS-M). Cholic acid (CA); chenodeoxycholic acid (CDCA); glyco-hyo-deoxycholic acid (GHDCA); glyco-urso-deoxycholic acid (GUDCA); glyco-deoxycholic acid (GDCA); hyo-deoxycholic acid (HDCA); hyo-cholic acid (HCA); glyco-cholic acid (GCA); iso-lithocholic acid (ILA); glyco-litho cholic acid (GLCA); lithocholic acid (LCA); tauro-lithocholic acid (TLCA); tauro-cheno-deoxycholic acid (TCDCA); tauro-deoxycholic acid (TDCA); tauro-cholic acid (TCA).

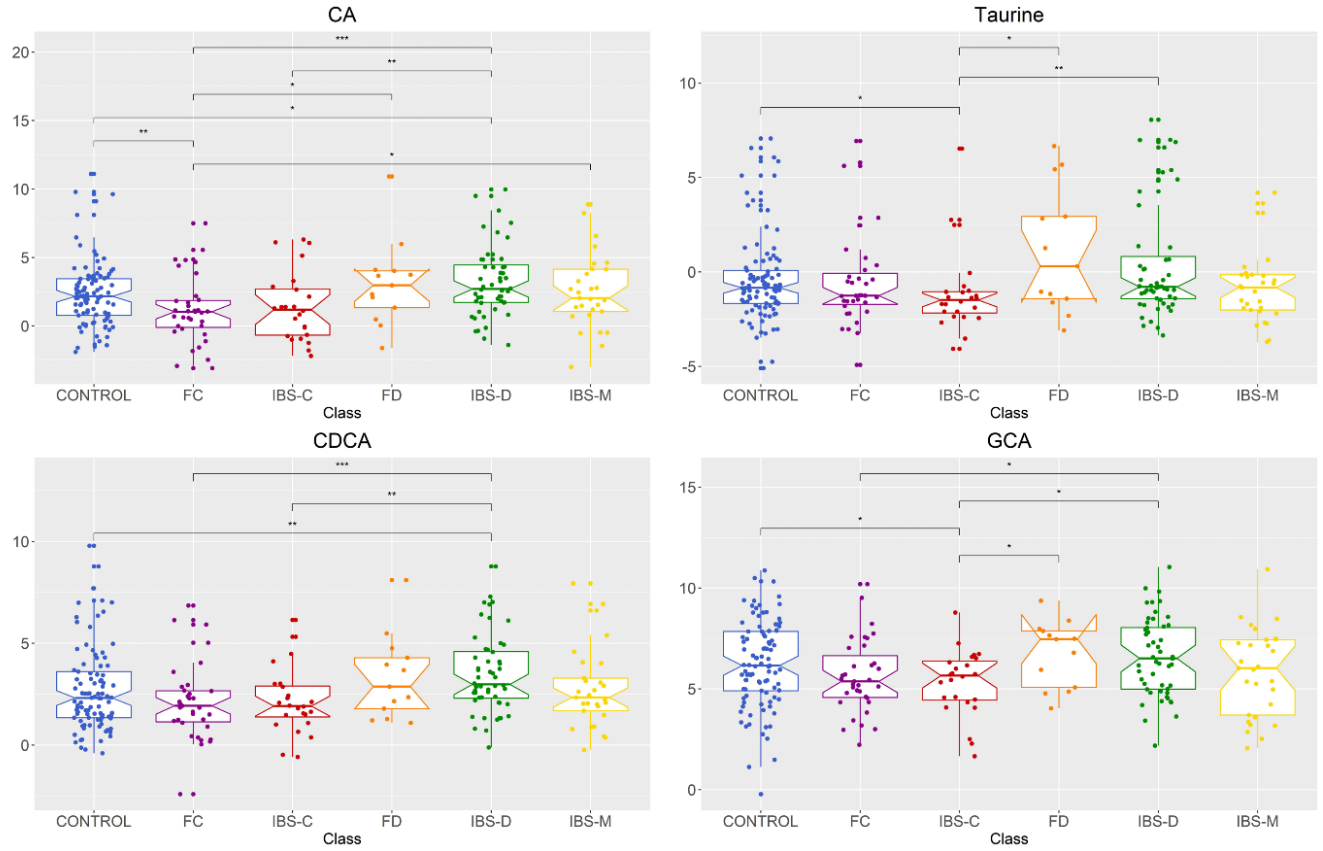


Figure 3-2: Bile acid metabolite distributions between healthy control, IBS subtype, and functional groups for metabolites with significantly different abundances between groups. Data presented as logged values of µg/mg of faecal dried weight. Boxplots show median (centre line), 25th and 75th percentile (top and bottom of boxes, respectively), with whiskers representing 1.5 times the inter-quartile range, and boundaries of notches show 95% confidence interval (CI). Statistical significance denoted as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). Abbreviations: healthy control (control), functional constipation (FC), IBS-constipation (IBS-C), functional diarrhoea (FD), IBS-diarrhoea (IBS-D), IBS-mixed (IBS-M), cholic acid (CA), chenodeoxycholic acid (CDCA), glycocholic acid (GCA).

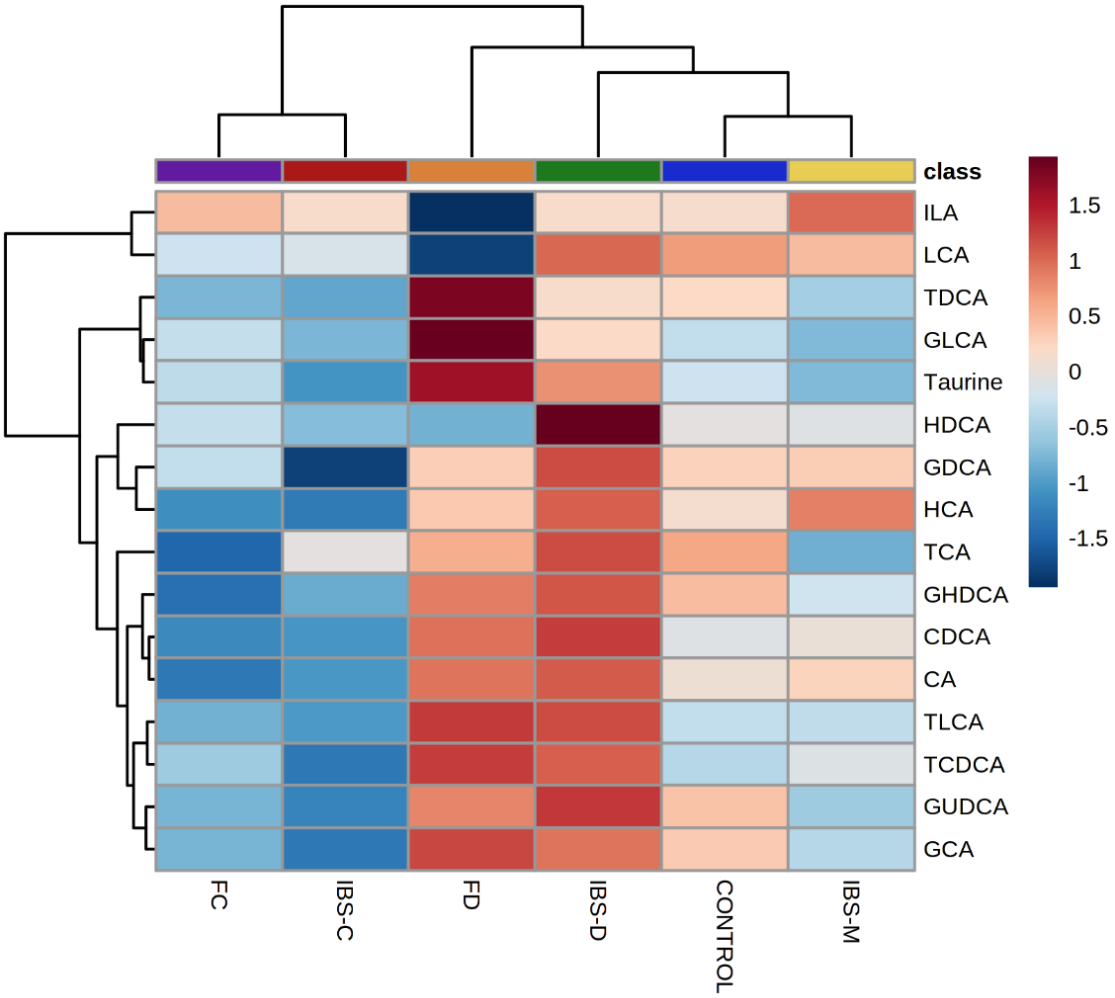


Figure 3-3: Hierarchical clustering analysis for mean faecal bile acid values between healthy control, IBS subtype, and functional groups. Data presented as z score of logged values of µg/mg. Colour ribbon beneath the upper dendrogram identifies groups; healthy control—blue, IBS-C—red, IBS-D—green, IBS-M—yellow, FC—purple, FD—orange. Abbreviations: healthy control (control), functional constipation (FC), IBS-constipation (IBS-C), functional diarrhoea (FD), IBS-diarrhoea (IBS-D), IBS-mixed (IBS-M).

3.3.2. Faecal bile acid concentrations between healthy control and combined groups

As shown in **Table 3-4**, **Figure 3-2,** and **Figure 3-3**, FC and IBS-C and FD and IBS-D groups, respectively, have similar faecal bile acid profiles and faecal dry weight percentage. Therefore, the datasets from the FC and IBS-C groups were merged into a combined constipation group. Similarly, the datasets of the FD and IBS-D groups were combined into a diarrhoea group. Both groups were used to determine if concentration differences in faecal bile acid metabolites could be discerned between healthy controls and those exhibiting constipation or diarrhoea symptoms. Additionally, there is uncertainty around the symptoms being experienced by IBS-M participants at the time of faecal sample collection. Thus, further analyses were performed without the IBS-M group.

The subsequent ANOVA analysis showed that the faecal concentration of 10 of the 16 measurable bile acids, CA ($p = 0.0002$), CDCA ($p = 0.001$), GHDCA ($p = 0.015$), GDCA ($p = 0.030$), HCA ($p = 0.025$), GCA ($p = 0.006$), Taurine ($p = 0.018$), TLCA ($p = 0.007$), TCDCA ($p = 0.021$), TDCA ($p = 0.018$) were significantly different between healthy control, constipation, and diarrhoea groups **(Figure 3-4)**. Post hoc analysis using the Wilcoxon test depicted as significance bars on boxplots showed that all 10 bile acids were significantly higher in the diarrhoea group compared to the constipation group.

Univariate analysis of the faecal concentration of total primary bile acids (sum of CA and CDCA) **(Figure 3-5)** was significant, and further pairwise mean comparisons showed there were significant differences between all three groups (healthy controls, constipation, and diarrhoea). The constipation group was significantly lower than healthy controls and the diarrhoea group, and the diarrhoea group was significantly higher than healthy controls and constipation.

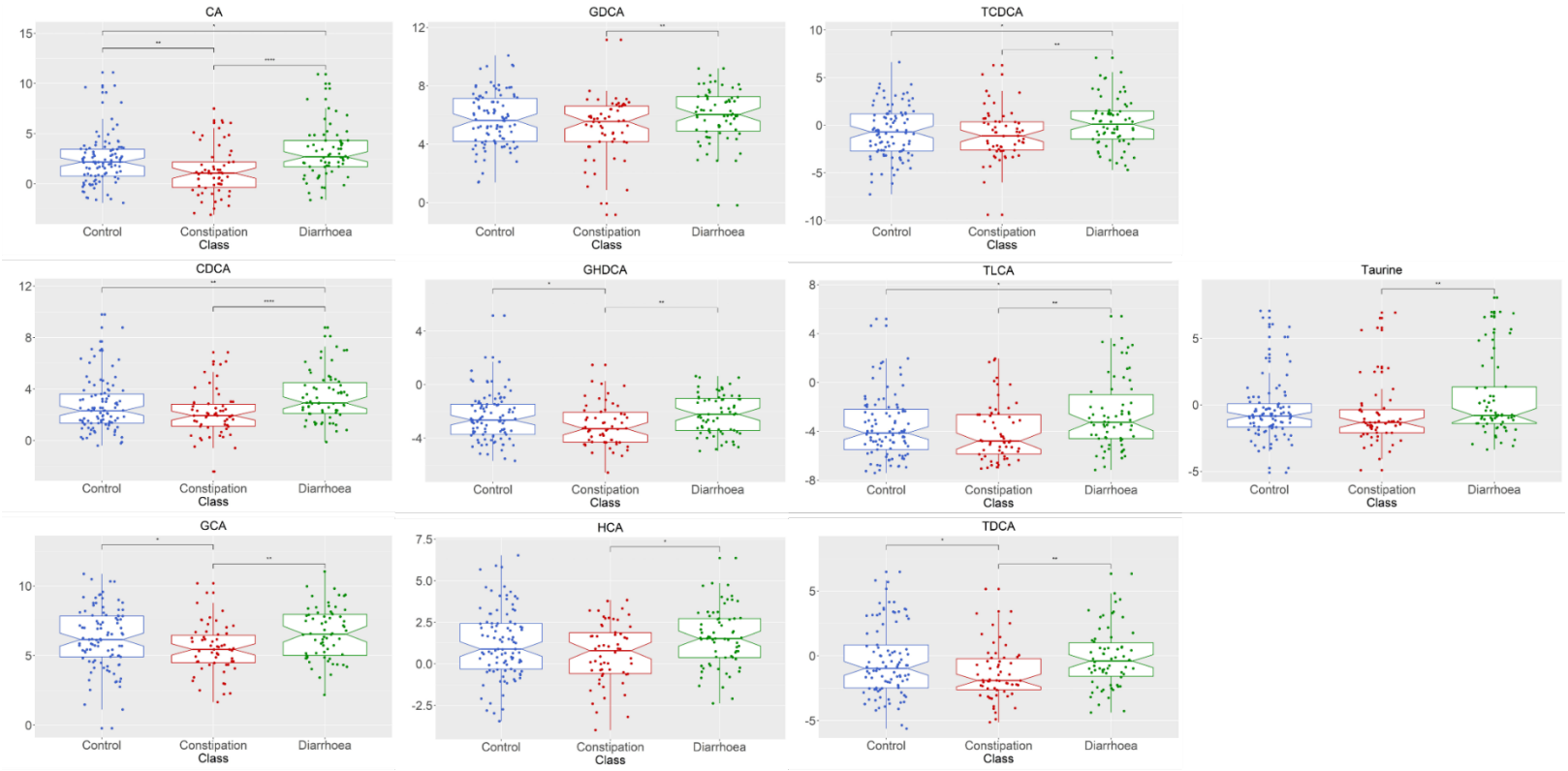


Figure 3-4: Bile acid metabolite distributions between healthy control, constipation (FC + IBS-C), and diarrhoea (FD + IBS-D) group for metabolites with significantly different abundances between groups. Data presented as logged values of dried faecal weight µg/mg. Boxplots show median (centre line), 25th and 75th percentile (top and bottom of boxes, respectively), with whiskers representing 1.5 times the inter-quartile range, and boundaries of notches show 95% confidence interval (CI). Statistical significance denoted as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****). Abbreviations: cholic acid (CA); chenodeoxycholic acid (CDCA); glyco-cholic acid (GCA); glyco-deoxycholic acid (GDCA); glyco-hyo-deoxycholic acid (GHDCA); hyo-cholic acid (HCA); glyco-litho cholic acid (GLCA); tauro-cheno-deoxycholic acid (TCDCA); tauro-lithocholic acid (TLCA); tauro-deoxycholic acid (TDCA).

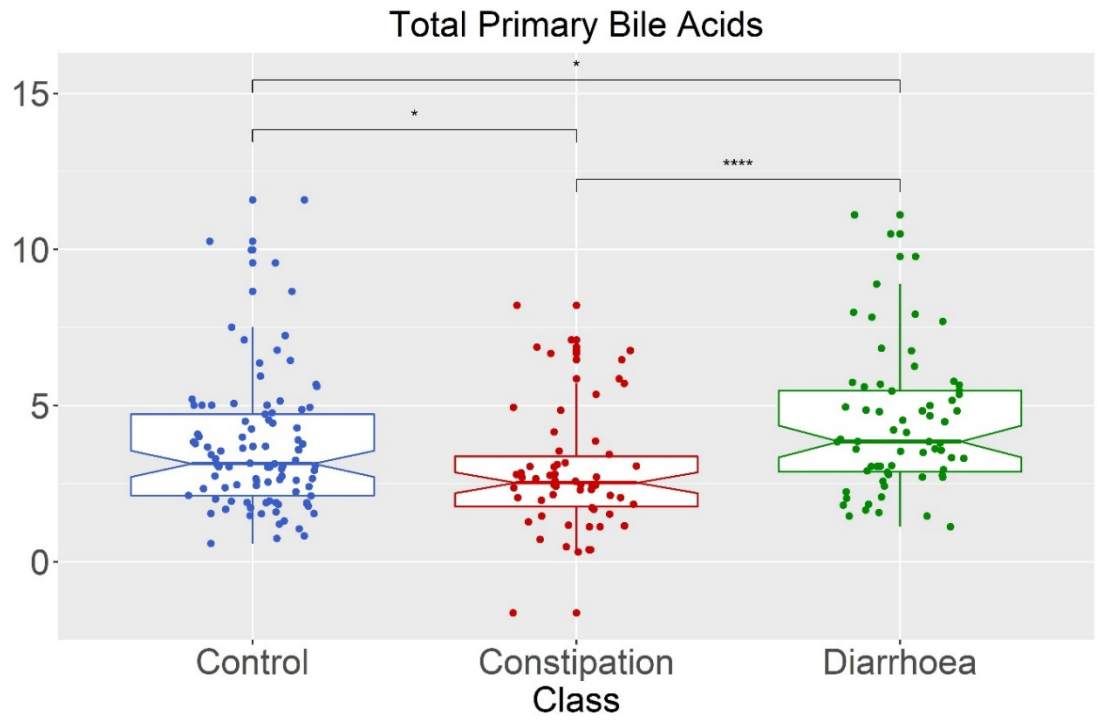


Figure 3-5: Total concentrations of faecal primary bile acids (sum of chenodeoxycholic acid (CDCA) and cholic acid (CA)) for healthy control, constipation (FC + IBS-C), and diarrhoea (FD + IBS-D) groups. Data presented as logged values of dried faecal weight µg/mg. Boxplots show median (centre line), 25th and 75th percentile (top and bottom of boxes, respectively), with whiskers representing 1.5 times the inter-quartile range, and boundaries of notches show 95% confidential interval (CI). Statistical significance denoted as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

Hierarchical clustering analysis for mean values of the faecal concentration of 16 bile acids **(Figure 3-6)** showed the constipation group clustered separately from healthy control and diarrhoea groups, which were clustered together. The heatmap highlighted a lower faecal concentration of all but one bile acid in the constipation group than the diarrhoea group.

When investigating gender, two bile acids (GDCA $p = 0.016$, HDCA $p = 0.003$) were significantly higher in males compared to females.

Pathway visualisation of bile acid metabolites **(Figure 3-7)** summarised the significant differences ($p < 0.05$) between the constipation group, diarrhoea group, and healthy control group. Reduced concentration of some faecal bile acid metabolites (CA, GCA, TCA, TCDA, TLCA, GHDCA) was observed in the constipation group compared to the control group. Similarly, the diarrhoea group was characterised by an increased concentration in some bile acid metabolites (CA, CDCA, GCDCA, TCDCA) compared to healthy control and the constipation groups (CA, CDCA, GCA, TCA, GCDCA, TCDCA, GDCA, TDCA, TLDA, GHDCA, HCA).

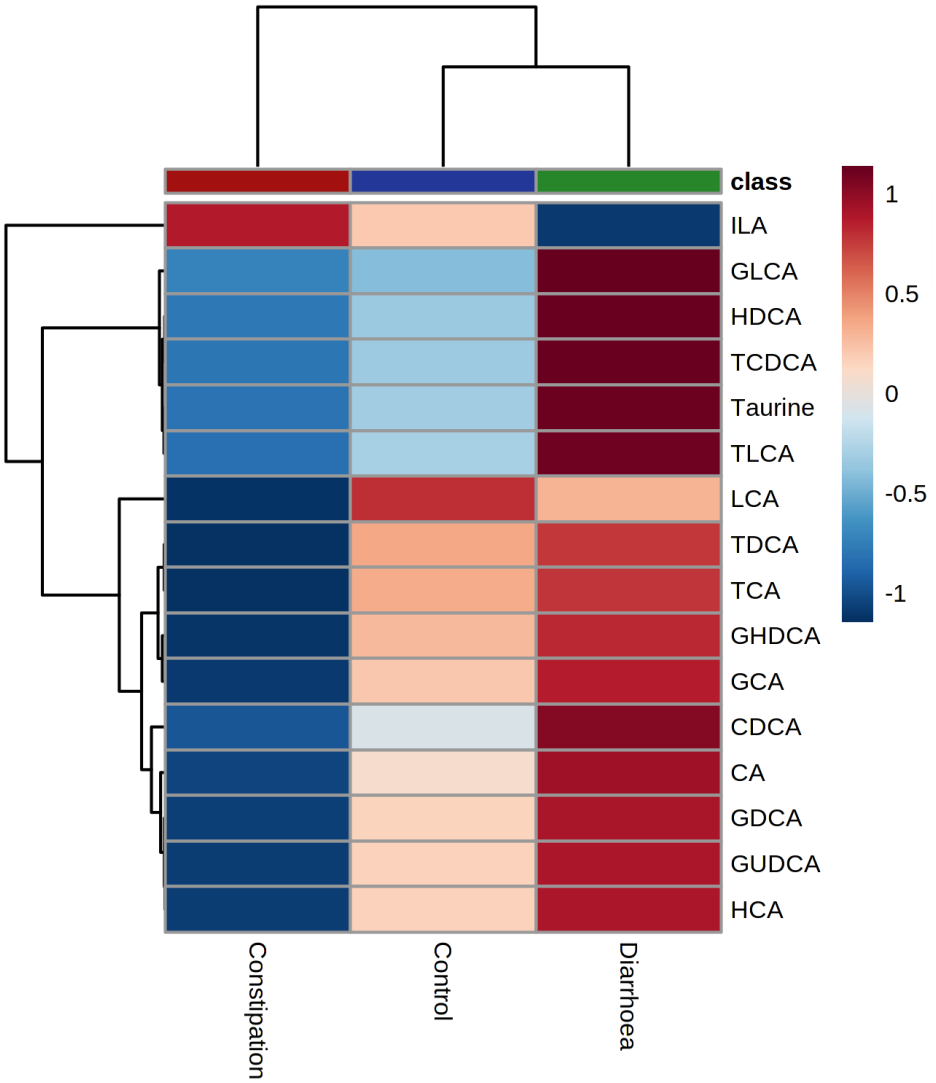


Figure 3-6: Hierarchical clustering analysis for mean faecal bile acid values between healthy control, constipation (FC + IBS-C), and diarrhoea (FD + IBS-D) groups. Data presented as z score of logged values of µg/mg. Colour ribbon beneath the upper dendrogram identifies group; healthy control – blue, constipation phenotype - red, diarrhoea phenotype – green.

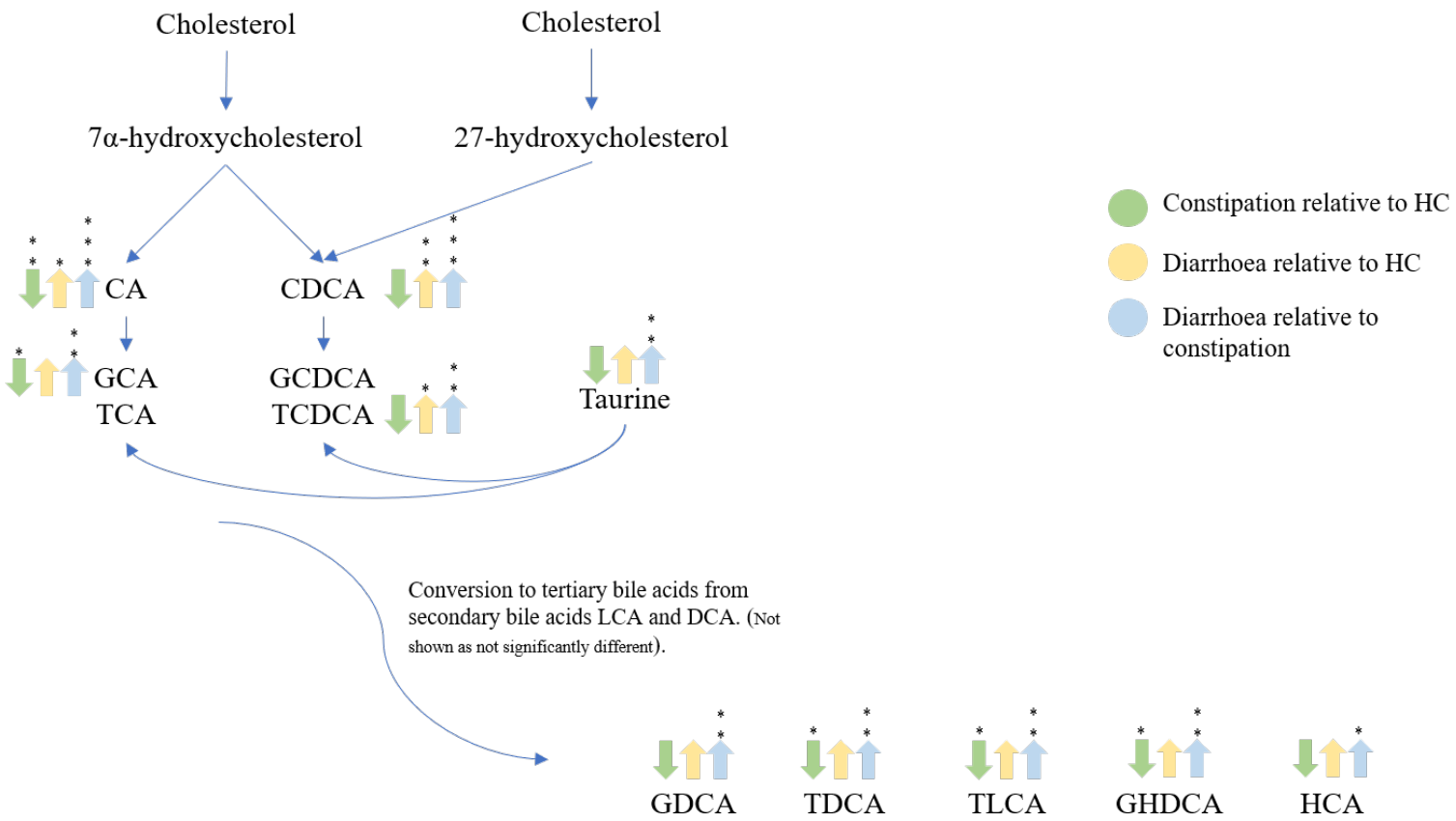


Figure 3-7: Bile acid pathway visualisation showing significant increased or decreased concentrations in faecal samples for healthy control, constipation (FC + IBS-C), and diarrhoea (FD + IBS-D) groups. Arrows depict if the concentration was either up or down relative to the described colour in the legend. Statistical significance denoted as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). Abbreviation: deoxycholic acid (DCA).

3.4. Discussion

This study reports the quantification of 16 bile acids in faecal samples from a cohort of participants across functional lower gut disorders. Quantitative analysis of 23 bile acids (including primary bile acids CA and CDCA) in faecal samples of the participants from the COMFORT cohort revealed that 16 bile acids were above detectable levels. The data showed that faecal concentrations of specific bile acids differed between individuals with FGDs and healthy controls. Individuals in the diarrhoea group were, in general, characterised by increased faecal excretion of bile acid metabolites (CA, CDCA, GCA, TCDCA, GDCA, TDCA, TLDA, GHDCA, HCA) compared to that of individuals in the constipation group, IBS-M and healthy controls. Individuals with functional diarrhoea and constipation had similar bile acid concentration profiles to IBS-D and IBS-C, respectively.

The COMFORT cohort was predominantly female with similar age distributions between the phenotypes, reflective of worldwide rates of FGDs. Analysis of fat and fibre intake, both of which could impact bile acid production, recorded as part of three-day dietary diaries, showed no difference between the groups suggesting that differences in bile acid excretion were independent of diet and instead indicative of perturbed host or microbial mechanisms.

Faecal bile acids promote laxation [97, 239]. The faecal concentration of CDCA and CA was higher in the combined diarrhoea group compared to the combined constipation and healthy control groups, consistent with the findings of others [91, 97]. The constipation group was characterised by a reduction in CA compared with healthy controls, unlike CDCA, where there was no difference in concentration. CDCA is produced from both primary and alternative pathways, while CA is produced solely via the primary pathway.

This result suggests a possible dysfunction in the primary pathway in individuals with constipation.

These findings suggest one of three mechanisms may be occurring. Either individuals with diarrhoea (IBS-D+FD) have perturbed biosynthesis or feedback regulating mechanisms, and therefore the known laxative effects of bile acids result in decreased colonic transit time and increased diarrhoea. Alternatively, decreased colonic transit time could have reduced bile acid re-absorption from the luminal compartment into hepatic circulation, resulting in increased faecal bile acid concentrations in individuals with diarrhoea, as reported here. Others have suggested that a cyclic process might occur where decreased re-absorption in the large intestine in participants with diarrhoea initiates feedback mechanisms resulting in continuous production of bile acids [93].

Previous studies support the finding that faecal and plasma bile acid concentrations differ within IBS subtypes [93, 97, 99]. However, they do not report concentrations per mg/g of bile acids but rather are focused on concentration differences compared to other groups. Results show an increased concentration of faecal bile acids in those with IBS-D and FD, and a proportion of these individuals may have undiagnosed BAM [240], either as cause or effect of diarrhoea itself. The IBS-C and FC group was characterised by reduced faecal bile acids, which could be linked to decreased faecal output and increased colonic transit, as previously described in other studies [91, 98]. The findings suggest that FC and IBS-C or FD and IBS-D are functionally similar regarding bile acid metabolism.

Similarly, to the findings reported here studies have noted concentration differences in specific bile acids between healthy controls and IBS subtypes [98], although others have not [93]. Shin *et al.,* found no difference in total faecal bile acids but reduced proportions of the primary bile acid CDCA and secondary bile acid deoxycholic acid in IBS-C and

healthy control individuals [91]. In contrast, Dior *et al.,* showed an increase in primary, but not secondary, faecal bile acids [97].

In the present study, the analysis of the 16 bile acids using hierarchical clustering and other supervised statistical tools (for example, PLS-DA) could not reliably differentiate IBS participants within subtypes and from healthy participants according to their groupings based on the ROME IV criteria. Inherent variability and the difficulty with defining what makes a person 'healthy' could explain the lack of definitive clusters [241]. Classifying healthy participants based on responses to questionnaires means standardisation can be difficult, ultimately highlighting the need for objectively measured scientifically validated biomarkers. Additionally, the functional basis of IBS exacerbates this, as even a healthy individual will experience gut ailments at certain times due to diet, stress, and other lifestyle factors.

Primary bile acids (CA and CDCA), either measured separately or as a total combined concentration, could be accurately measured to distinguish between IBS subtypes. Although the concentration of other bile acids was altered, CA and CDCA were most different within the IBS subtypes. Additionally, when functional groups and IBS were combined into constipation or diarrhoea groups, these same differences were observed, suggesting the functional outcomes were similar between IBS and relevant functional groups.

The measurement of the primary bile acids CA and CDCA provides information at the start of the bile acid pathway where under-activation or over-activation of one pathway could increase or decrease shuttling through downstream bile acids. The relative concentrations of glycine and taurine conjugated compounds (GCA, TCA, GCDCA and TCDCA) can provide a downstream view of the bile acid pathway. Measurement of

glycine in faecal samples was not performed in this study. However, concentrations of taurine were different between healthy controls and IBS subtypes, perhaps highlighting differences in conjugation potential and suggesting that further analysis should include glycine. This analysis will make inferences about changes occurring downstream in the pathway and is likely important for a better understanding of, if and how these metabolites are involved in FGDs. The combination of the analysis of primary bile acids in faecal samples with the analysis of predominantly 'tertiary' bile acids and microbial community changes would be necessary to advance the knowledge of the role of bile acids in FGDs.

The strengths of the analysis and data reported here are the quantitative LC-MS method used to quantify the 23 bile acids rather than total bile acids in faecal samples from the COMFORT cohort representing the FGD spectrum. The sample size of the FD group was small in comparison to other groups. However, when combined with IBS-D participants, the group size was comparable to the other groups. The quantification of total bile acids in faecal samples is a proven method to diagnose BAM [240]. However, measuring total bile acids may provide limited insights into the physiological responses and mechanisms underlying FGDs as the total will not equate to 100% of bile acids present [97]. Furthermore, considering the extensive microbial modification and epimerisation results in a diverse range of bile acids and derived metabolites, obtaining standards to quantify all possible bile acids remains elusive. The data for total primary bile acids (CA and CDCA) reported here were accurately measured using internal standards.

There are also some limitations of this study relating to sample collection, dietary records, and sample analysis. Bile acids are metabolites that are influenced by dietary intake, host and microbial metabolism, and gut transit, and it was expected that some of these factors would impact the findings. Variations could arise as active recycling mechanisms will differ naturally between individuals. Additionally, the homogeneity of the samples could

alter the concentration of bile acids. The home collection kit brings some potential sources of variation, such as differences in how long participants kept their sample out of the freezer or travel time on ice to the laboratory. The accuracy of the diet dataset relies on the participants accurately recording their dietary intake or when bowel movements were performed after food consumption.

3.5. Conclusion

In conclusion, this study shows that IBS subtypes combined with their respective functional groups have different faecal bile acid profiles compared to the healthy control group. Measuring faecal bile acid concentrations could not differentiate between functional groups and the respective IBS subtypes. Individuals in the diarrhoea group showed increased faecal bile acid excretion compared to individuals in the constipation group and healthy controls, suggesting a perturbed bile acid metabolism from that of a normal healthy gut. More specifically, concentration differences in primary bile acids in faecal samples could be used to distinguish between the constipation group and healthy controls or between the diarrhoea group and healthy controls. Host-microbial metabolism results in a diverse range of bile acids and derived metabolites. Considering the microbial community and the physiological changes in the large intestine of these participants would help further advance the knowledge of the role of bile acids in FGDs.

Chapter Four
Analysis of circulatory plasma amino acids in participants with functional gut disorders

Abstract

The concentration of circulating amino acids are important in several biochemical pathways as precursors to biological processes that have been previously linked to FGDs e.g., tryptophan metabolism or conversion of histidine to histamine. The concentration of circulating amino acids can be influenced by dietary protein consumption, metabolic host processes, and the gut microbiome. This chapter aimed to quantify 20 amino acids to ascertain if circulating plasma amino acid concentrations differed between healthy participants and those with FGDs. Plasma amino acids were measured using UPLC in 205 participants (Rome IV: IBS-C, IBS-D, IBS-M, FC, FD (FC *n=26,* FD *n=8,* IBS-C *n=21*, IBS-D *n=42,* IBS-M *n=25,* and control *n=73*)). Individual plasma amino acid concentrations were not significantly different between all functional groups and healthy controls. Analysis of BCAAs, LNAAs, EAAs, and NEAAs showed that only BCAAs were significantly different between all functional groups and healthy controls. Analysis of individual amino acids and the amino acid groups between diarrhoea (FD + IBS-D), constipation (FC + IBS-C), and healthy control groups showed no significant differences. Circulating amino acid concentrations did not highlight mechanistic differences between individuals with FGDs and healthy controls.

Chapter Four

4.1. Introduction

Amino acids are important circulating metabolites that are precursors to several crucial metabolites and pathways in FGDs [44]. The importance of amino acids is often not the metabolite itself, but the associated conversion to other metabolites with important biochemical functions, e.g., neurotransmission [120, 242]. Most amino acids originate from the breakdown of dietary protein in the gut and the subsequent absorption from the small intestine. However, 5 to 10% escapes this process passing through into the large intestine as whole protein or peptides (small chains of amino acids) where it can either serve as a microbial substrate, be assimilated by the microbiome or excreted undigested [44].

The assimilation of protein can have differential impacts, notably because of how these macronutrients are metabolised to a different extent by the gut microbiome to produce metabolites [243]. The extent of microbial protein fermentation is dependent on substrate availability and environmental constraints, for example., carbohydrate fermentation [243].

The amino acid concentration profile has been previously reported to differ in diabetes, obesity, IBD, and metabolic dysfunction [245, 246]. However, limited data is available for a possible role in FGDs [122, 124]. It has been previously shown that in UC, amino acid gene pathways were increased in inflamed areas of tissue compared to non-inflamed tissue, suggesting the use of different metabolic pathways in inflammation [247]. For example, glutamine is important in gut permeability and function [248], and histidine as the precursor to histamine has potential importance in immune system function [249].

Additionally, metabolites derived from amino acid metabolism, for example, amines, sulphuric metabolites, phenols, and indoles can in excess quantities, exert several deleterious effects such as inflammation of the gut mucosa [243, 250]. Neurotransmitters, for example, GABA, norepinephrine, dopamine, and serotonin, are also produced from amino acid degradation of tryptophan, tyrosine, and phenylalanine, though some quantities can enter the systemic circulation directly [251].

Neurotransmitters derived from amino acids are thought to be important in FGDs [250]. Serotonin, for example, is postulated to be important in IBS, with previous research showing links to visceral sensitivity [134, 211, 252]. In addition, Clarke *et al*., showed that the activity of indoleamine 2,3-dioxygenase, an immune-responsive enzyme responsible for tryptophan degradation along the kynurenine pathway, is increased in IBS patients relative to healthy controls [122].

This analysis aimed to test whether plasma amino acid concentrations differed in a cohort of individuals with FGDs compared to healthy controls. It was hypothesised that plasma amino acid concentrations would differ between IBS subtypes, those with FGDs, and healthy controls, reflecting a perturbation in circulating amino acid concentrations in participants with impaired digestive function compared to healthy controls. Therefore, to test this hypothesis, the study aimed to quantify 20 amino acids in plasma samples collected from the COMFORT cohort.

4.2. Methods

4.2.1. Participants

Information on recruitment, inclusion, exclusion, and sample collection methodology was as described in **Chapter 2**. Two hundred and five EDTA treated plasma samples were analysed from the COMFORT cohort.

4.2.2. Sample extraction

A Tungstate precipitation protocol to analyse free amino acids in plasma samples was carried out [253]. Briefly, 160µL of 0.04M sulphuric acid containing 15µM L-Nor-Valine as an internal standard for data quantification and analyte recovery was aliquoted into Eppendorf tubes. First, 20µL of plasma sample was added, vortexed and held on ice for 2 min. Next, 20µL of 10% sodium tungstate was added, mixed instantly, and held on ice for 3 min. Samples were vortexed immediately before centrifuging at 4°C, 14,000 *x g* for 10 min. For fluorescent derivatisation, 70µL of 0.2M borate buffer (1.24g boric acid in 100mL pH 8.8, adjusted with fresh 5M NaOH) was aliquoted into small glass tubes. Next, 10µL of the supernatant from samples, QCs and standards was added, vortexed, and then 10µL of AccQ-tag reagent (2.8mg/mL in dry acetonitrile) was added and vortexed instantly. Finally, the solution was transferred to UPLC vials, capped, and heated for 10 min at 55°C to ensure reaction completion. The UPLC analysis of the sample was completed on a Thermo Scientific Dionex Ultimate 3000 (Thermo Scientific, Dornierstrasse, Germany). Samples were injected onto a Kinetex (Phenomenex, Auckland, New Zealand) 1.7µm C18 x 2.1mm column, preceded by a Krudkatcher™ filter (Phenomenex, Auckland, New Zealand) at 45°C. The mobile phase buffer (80mM sodium acetate, 3mM triethylamine, 2.67µM disodium calcium ethylenediaminetetraacetic acid, pH 6.43 obtained with addition of orthophosphoric acid) was run with acetonitrile gradient from 2 to 17% (balance water) over a 24 min run time for detection of 20 amino acid compounds.

4.2.3. Data processing and statistical analysis

Data was captured using Chromeleon 7.1 software (Thermo Scientific, Auckland). Standard curves were formulated for each compound within the physiological range of human plasma. Concentrations of amino acids are presented as µmol/L of plasma. Data

is presented as means ± SEM. The R statistical package (V3.6.1) was used for individual amino acid analysis and Metaboanalyst (V4.0) for hierarchical clustering analysis (Ward's Method clustering type). Residual plots and the Shapiro-Wilk test were employed to determine normality. ANOVA was used to compare means, with $p < 0.05$ deemed statistically significant. If a metabolite was significantly different, pairwise mean comparisons were used to compare differences between participant groups.

4.3. Results

A total of 205 plasma samples were analysed. However, there was incomplete metadata for 10 participants. Therefore 195 samples were included in the final analyses. Symptom questionnaires based on the Rome Criteria IV clustered participants as FC *n=26,* FD *n=8,* IBS-C *n=21*, IBS-D *n=42,* IBS-M *n=25,* and healthy control *n=73*. Sample characteristics for all groups are presented in **Table 4-1**.

Analysis of three-day dietary information showed no significant difference in protein intake between any of the groups ($p = 0.693$) **(Figure 4-1**). There was a significant difference in protein intake between gender ($p = 0.05$) and age ($p = 0.03$). However, the values were representative of normal protein consumption.

Table 4-1: Mean concentration values of groups for circulating plasma amino acid metabolites.

Groups	Healthy control	IBS-C	FC	IBS-D	FD	IBS-M
Age (mean, years)	54.8	51.6	59.5	53.7	62.8	51.0
Female count	38	20	18	34	7	20
Male count	35	1	8	8	1	5
Alanine	403.1	379.5	419.1	426.6	400.2	387.4
Arginine	69.7	68.1	70.2	73.6	74.6	70.1
Asparagine	58.1	59.3	54.3	57.6	53.0	57.3
Aspartic Acid	5.7	6.1	6.2	5.9	5.8	5.9
Glutamic Acid	54.3	42.6	55.8	56.1	40.6	49.7
Glutamine	584.9	580.5	606.9	604.6	603.7	588.1
Glycine	249.6	254.5	249.4	270.3	307.3	261.0
Histidine	59.7	58.9	57.2	62.2	57.9	62.0
HydroxyProline	13.0	11.5	11.3	12.7	14.0	13.2
Isoleucine	81.9	61.7	76.2	78.2	64.1	78.6
Leucine	146.1	109.6	138.6	137.9	119.1	143.2
Lysine	96.0	87.4	96.7	93.9	94.3	98.1
Methionine	31.6	27.8	32.2	30.6	28.3	31.9
Phenylalanine	67.0	60.6	66.5	66.7	60.2	65.8
Proline	281.2	242.0	293.8	279.2	300.2	271.3
Serine	115.3	116.6	114.5	117.9	123.7	114.9
Threonine	114.1	117.5	117.2	117.1	111.9	119.7
Tryptophan	47.8	45.2	47.4	45.4	46.4	44.6
Tyrosine	73.8	65.4	81.7	75.9	66.6	74.8
Valine	257.5	215.5	250.2	251.6	218.6	249.3
LNAA	673.4	557.9	660.3	655.7	575.0	656.2
BCAA	485.5	386.8	464.9	467.8	401.8	471.0
EAA	970.7	852.3	951.9	957.1	875.5	963.5
NEAA	959.9	924.0	980.8	1010.5	997.2	951.2

Values presented as μmol/L of plasma. Abbreviations: Healthy control (control), functional constipation (FC), IBS-constipation (IBS-C), functional diarrhoea (FD), IBS-diarrhoea (IBS-D), IBS-mixed (IBS-M).

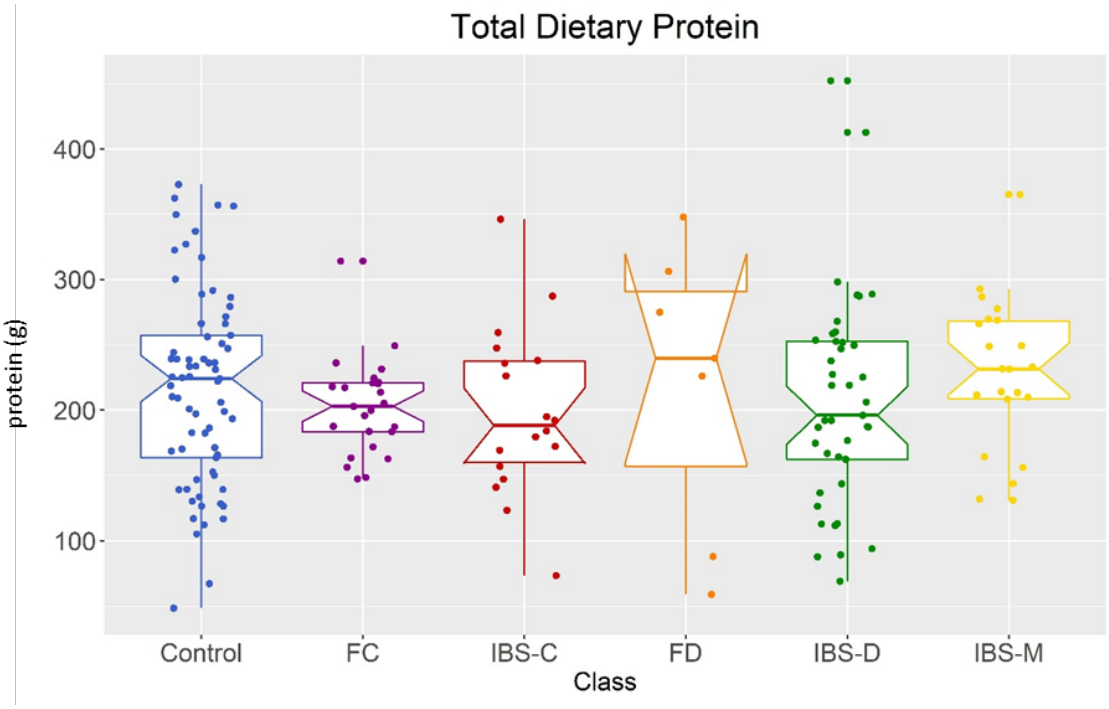


Figure 4-1: Dietary intake of total protein consumption over a three-day period recording using diet diaries for each participant. Boxplots show median (centre line), 25th and 75th percentile (top and bottom of boxes, respectively), with whiskers representing 1.5 times the inter-quartile range, and boundaries of notches show 95% confidence interval (CI). Abbreviations: Healthy control (control), functional constipation (FC), IBS-constipation (IBS-C), functional diarrhoea (FD), IBS-diarrhoea (IBS-D), IBS-mixed (IBS-M).

4.3.1. Plasma amino acid concentrations between healthy control, IBS subtypes and functional groups

Univariate analyses showed no statistical differences between groups for any of the circulating plasma concentrations of 23 amino acids analysed (**Table 4-2**). The plasma concentration of four amino acids (proline, isoleucine, leucine, phenylalanine) was significantly different between groups when gender was accounted for as a fixed effect. Phenylalanine was significantly different between groups with age as a fixed effect.

BCAA concentrations differed significantly between the cohort groups ($p = 0.05$) (**Figure 4-2**). Individual pairwise comparisons were significant between controls and IBS-C, FC and IBS-C, IBS-C and IBS-D, and IBS-C and IBS-M. There was no significant difference in LNAA, NEAA or EAA concentrations.

Hierarchical heatmap clustering analysis of mean circulating plasma amino acid concentrations for each participant group (**Figure 4-3**) showed FD and IBS-C groups clustered together and separated from the other groups. IBS-D and IBS-M groups were most similar, and control and FC groups formed a cluster.

Table 4-2: Significance probability (*p*) values for circulating plasma amino acid metabolites in healthy control, IBS subtype (IBS-C, IBS-D, IBS-M), and functional constipation and diarrhoea groups (FC, FD).

	Group *p*-value	Gender *p*-value	Age *p*-value	Group x gender *p* value	Group x age *p*-value
Aspartic acid	0.91	0.042 *	0.482	0.12	0.612
Alanine	0.487	0.131	0.0003 ***	0.208	0.245
Arginine	0.697	0.192	0.52	0.483	0.821
Asparagine	0.478	0.607	0.65	0.439	0.36
Glutamic acid	0.238	0.007 **	0.005 **	0.793	0.449
Glutamine	0.656	0.017 *	0.0008 ***	0.611	0.281
Glycine	0.322	0.003 **	0.024 *	0.454	0.897
Histidine	0.376	0.040 *	0.608	0.638	0.882
Hydroxy-Proline	0.598	0.008 **	0.178	0.216	0.758
Isoleucine	0.307	0.001 ***	0.952	0.044 *	0.457
Leucine	0.274	0.0006 ***	0.322	0.043 *	0.723
Lysine	0.889	0.051	0.083	0.67	0.356
Methionine	0.694	0.013 *	0.141	0.158	0.094
Phenylalanine	0.638	0.019 *	0.406	0.029 *	0.040 *
Proline	0.736	0.031 *	0.207	0.049 *	0.557
Serine	0.774	0.352	0.389	0.243	0.12
Threonine	0.378	0.019 *	0.195	0.568	0.064
Tryptophan	0.95	0.001	0.459	0.086	0.501
Tyrosine	0.214	0.065	0.002 **	0.141	0.162
Valine	0.225	0.0001 ***	0.183	0.112	0.205

Statistical significance denoted as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

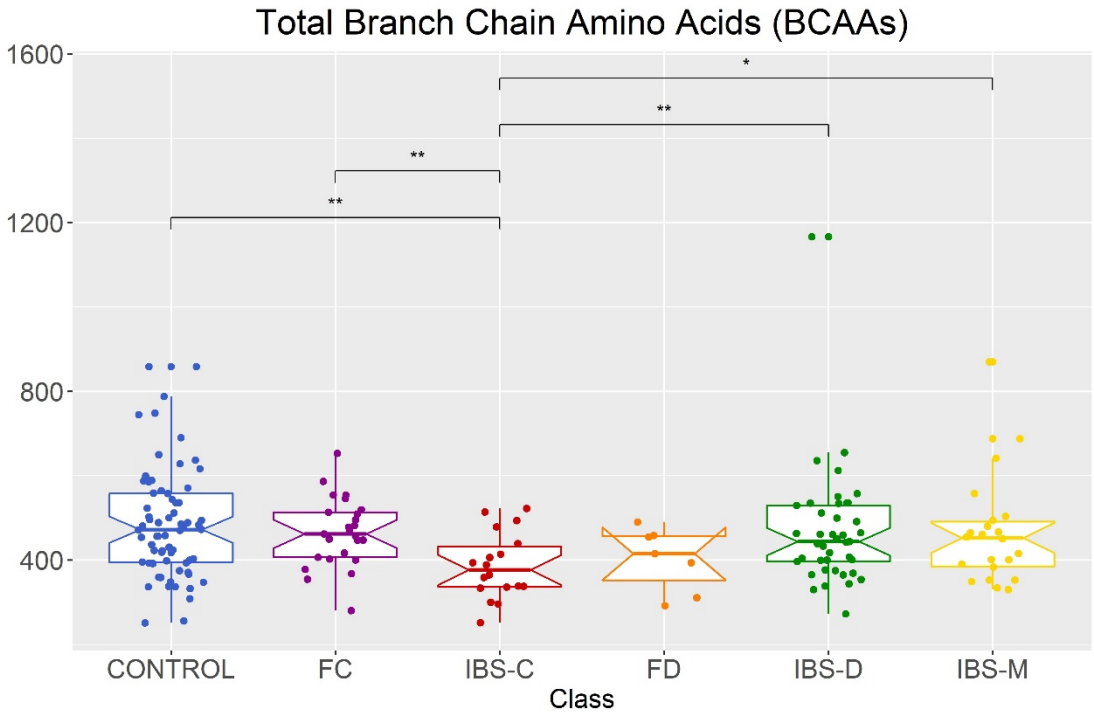


Figure 4-2 Total sum of branch chain amino acids (BCAAs) between healthy control, IBS subtypes, and functional groups. Boxplots show median (centre line), 25th and 75th percentile (top and bottom of boxes, respectively), with whiskers representing 1.5 times the inter-quartile range, and boundaries of notches show 95% confidence interval (CI). Abbreviations: healthy control (control), functional constipation (FC), IBS-constipation (IBS-C), functional diarrhoea (FD), IBS-diarrhoea (IBS-D), IBS-mixed (IBS-M).

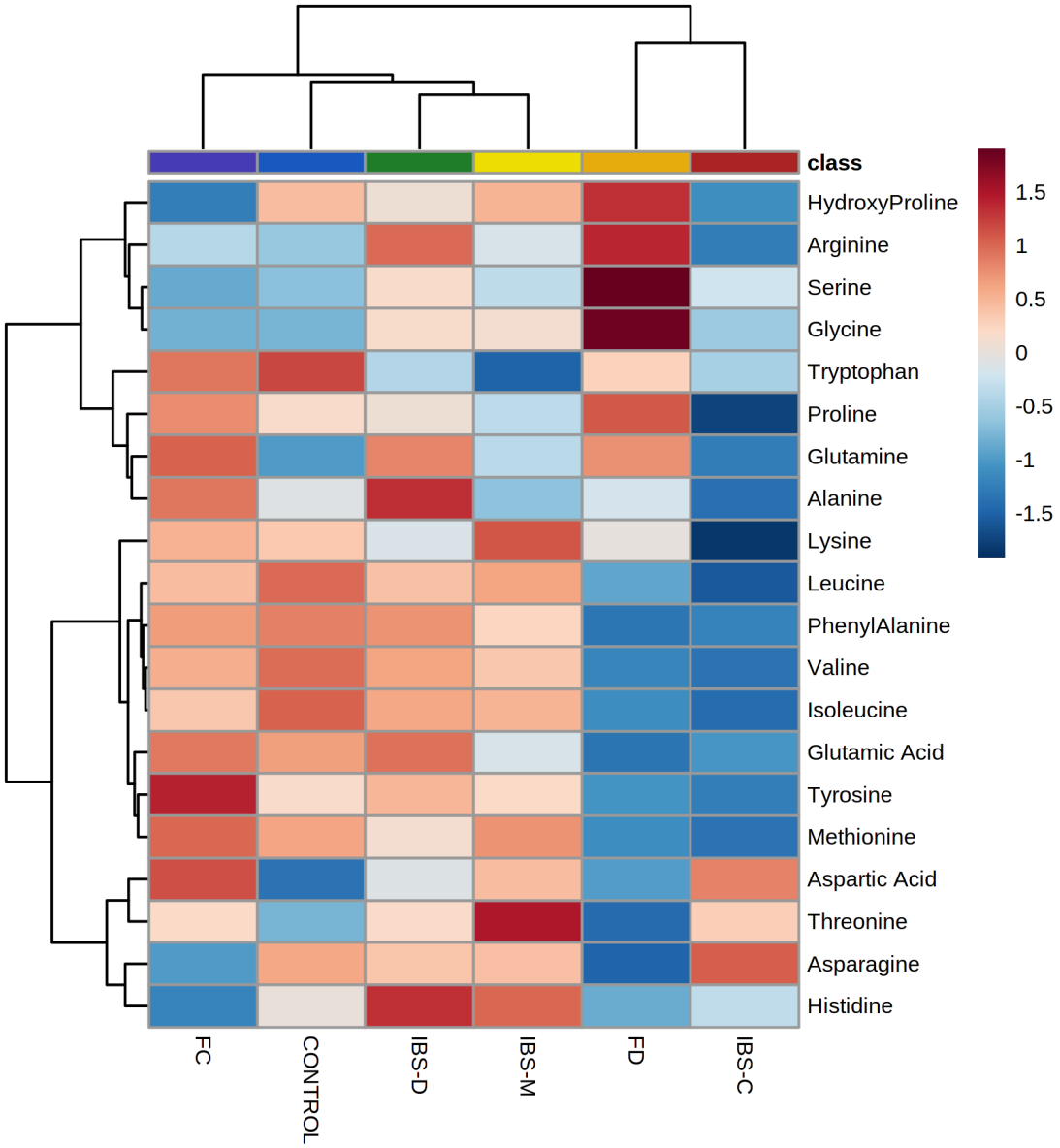


Figure 4-3: Hierarchical clustering analysis for mean plasma amino acid values between healthy control, IBS subtypes, and functional groups. Data presented as z score of logged values of µg/mg. The colour ribbon beneath the upper dendrogram identifies groups; control – blue, IBS-C - red, IBS-D – green, IBS-M – yellow, FC – purple, FD – orange. Abbreviations: healthy control (control), functional constipation (FC), IBS-constipation (IBS-C), functional diarrhoea (FD), IBS-diarrhoea (IBS-D), IBS-mixed (IBS-M).

4.3.2. Plasma amino acid concentrations between healthy controls and combined groups

The datasets from the FC and IBS-C groups were merged into a constipation group. Similarly, the datasets of the FD and IBS-D groups were grouped as a diarrhoea group. Both groups were used to determine if the concentration differences in plasma amino acids could be discerned between healthy controls and those exhibiting constipation or diarrhoea symptoms. Analyses were carried out without the IBS-M group due to the uncertainty around the symptoms experienced by these participants at the time of faecal sample collection.

Univariate analysis showed that circulatory plasma amino acid concentrations were not significantly different between healthy control and the diarrhoea groups, or healthy control group and constipation groups (**Table 4-3**). Fifteen amino acids (aspartic acid, glutamic acid, hydroxy-proline, glycine, glutamine, threonine, proline, tyrosine, valine, methionine, isoleucine, leucine, phenylalanine, lysine, tryptophan) were significant when gender was classified as a correlative factor. Six (glutamic acid, glycine, glutamine, alanine, tyrosine, ornithine) were significant when age was a correlative factor.

Four amino acids (aspartic acid, isoleucine, leucine, tryptophan) were significant between groups when gender was accounted for as a fixed effect and phenylalanine where age was a fixed effect (**Table 4-3**). However, the univariate analysis of circulating plasma concentrations of BCAAs, NEAAs, EAAs, and LNAAs was not significantly different between the groups (**Figure 4-4**). Additionally, heatmap analysis showed that constipation and diarrhoea groups were more closely clustered together compared to healthy controls (**Figure 4-5**).

Table 4-3: Significance probability (*p*) values for circulating plasma amino acid metabolites in healthy control, constipation (FC + IBS-C), and diarrhoea (FD + IBS-D) groups.

	Group *p*-value	Gender *p*-value	Age *p*-value	Group x gender *p* value	Group x age *p* value
Aspartic acid	0.788	0.001 **	0.545	0.037 *	0.344
Alanine	0.485	0.083	0.0003 ***	0.081	0.13
Arginine	0.559	0.214	0.596	0.387	0.906
Asparagine	0.998	0.816	0.966	0.527	0.122
Glutamic acid	0.602	0.004 **	0.008 **	0.739	0.968
Glutamine	0.478	0.012 *	0.0006 ***	0.453	0.336
Glycine	0.257	0.003 **	0.011 *	0.355	0.943
Histidine	0.485	0.064	0.377	0.68	0.794
Hydroxy-Proline	0.426	0.011*	0.172	0.26	0.693
Isoleucine	0.307	0.001 ***	0.952	0.044 *	0.457
Leucine	0.269	0.0006 ***	0.988	0.048 *	0.383
Lysine	0.765	0.043 *	0.069	0.637	0.738
Methionine	0.722	0.009 **	0.157	0.209	0.214
Phenylalanine	0.863	0.017 *	0.558	0.409	0.010 **
Proline	0.925	0.021 *	0.137	0.108	0.312
Serine	0.796	0.293	0.403	0.808	0.189
Threonine	0.3	0.033 *	0.308	0.346	0.125
Tryptophan	0.788	0.001 **	0.545	0.037 *	0.344
Tyrosine	0.964	0.032 *	0.003 **	0.246	0.099
Valine	0.353	0.00008 ***	0.216	0.069	0.127

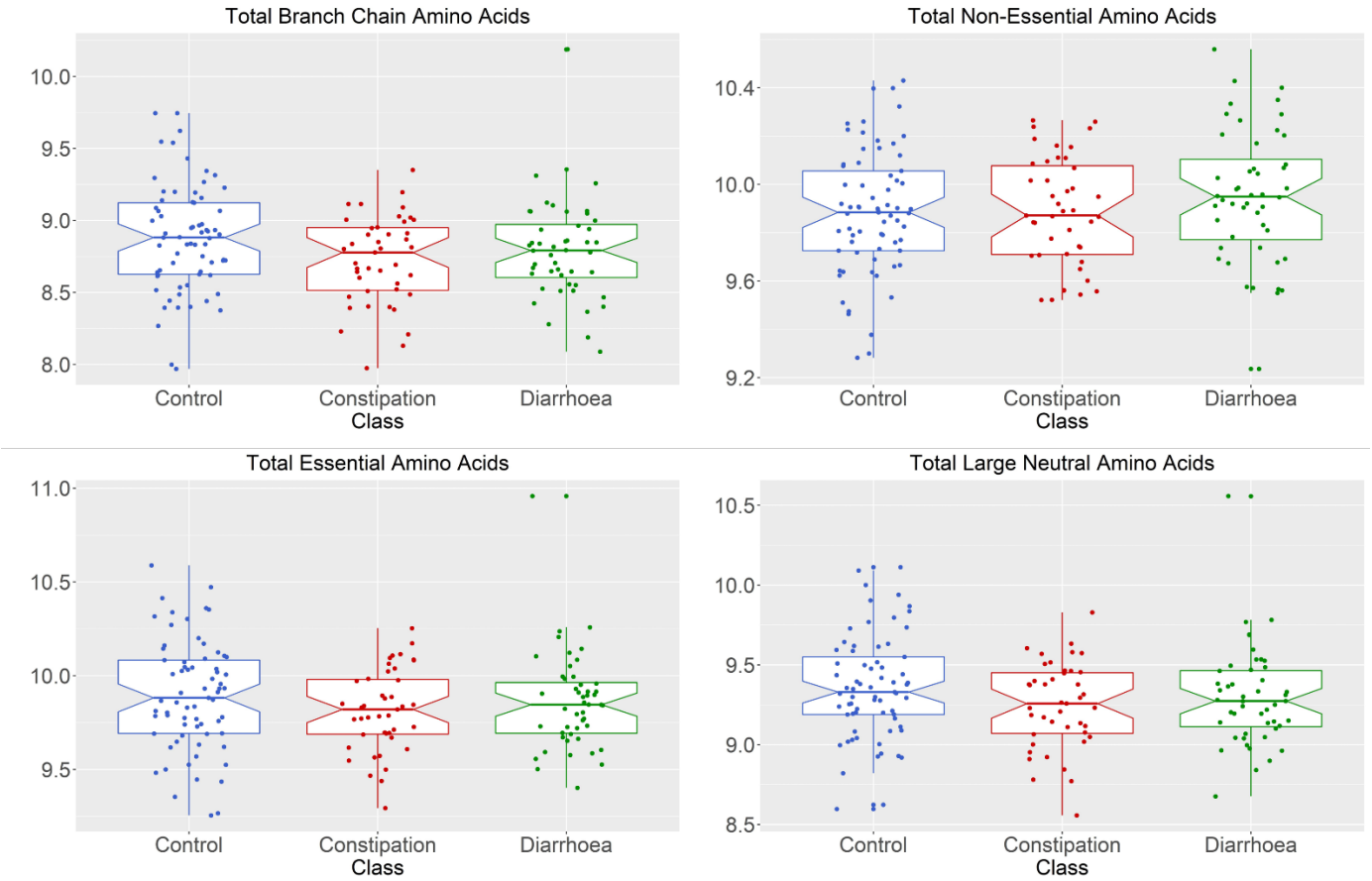


Figure 4-4: Total sum of branch chain amino acids (BCAAs), non-essential amino acids (NEAAs), essential amino acids (EAAs), and large neutral amino acids (LNAAs) between healthy control, constipation (FC + IBS-C), and diarrhoea (FD + IBS-D) groups. Data presented as logged values of µg/mg. Boxplots show median (centre line), 25th and 75th percentile (top and bottom of boxes, respectively), with whiskers representing 1.5 times the inter-quartile range, and boundaries of notches show 95% confidential interval (CI). Statistical significance denoted as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

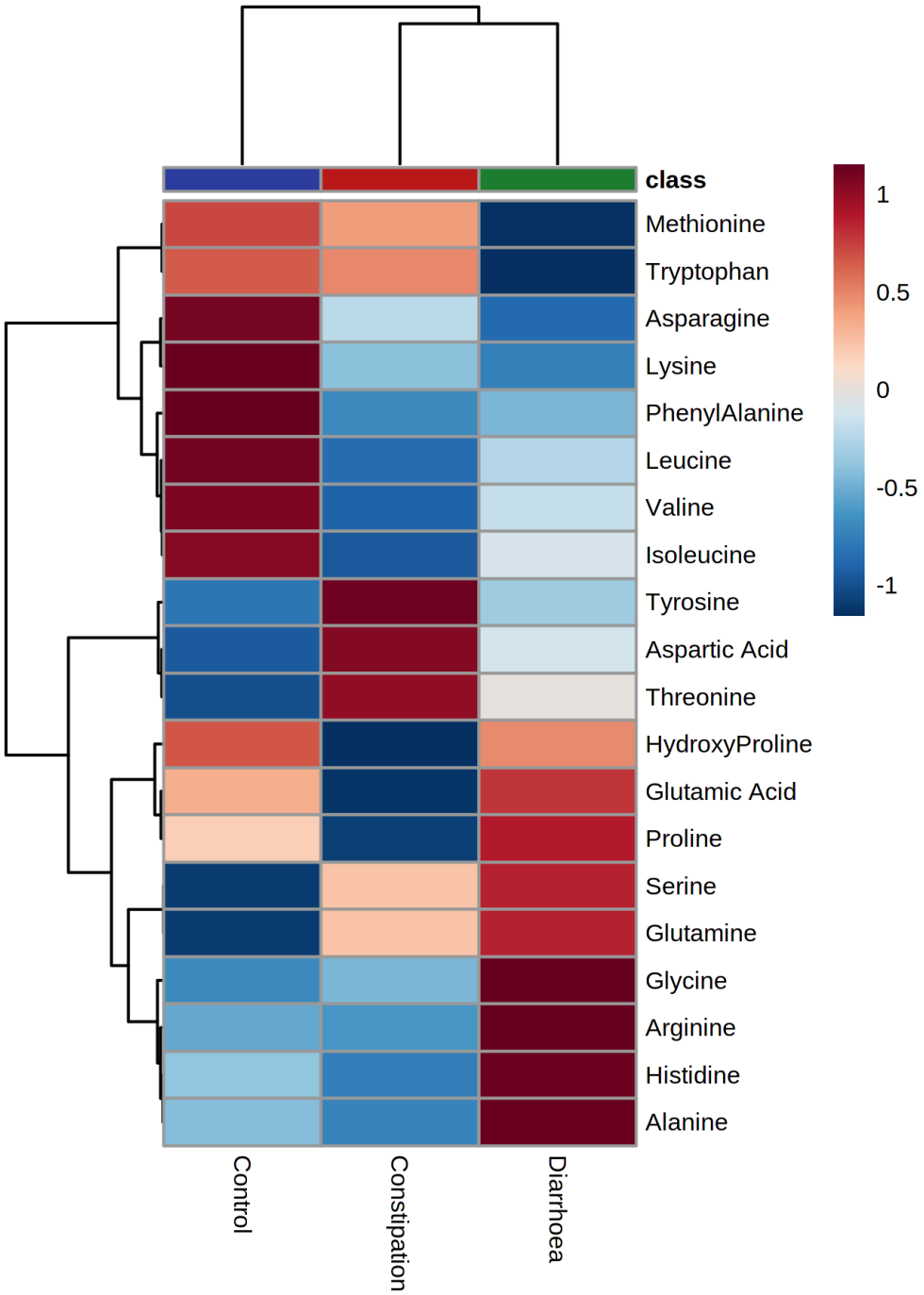


Figure 4-5: Hierarchical clustering analysis for mean values of plasma amino acids between healthy control, constipation (FC + IBS-C), and diarrhoea (FD + IBS-D) groups. Data presented as z score of logged values of µg/mg. The colour ribbon beneath the upper dendrogram identifies group; healthy control – blue, constipation phenotype - red, diarrhoea phenotype – green.

4.4. Discussion

This study is the first to report the circulating plasma concentration of 20 amino acids in participants with FGDs and healthy controls recruited to be part of the COMFORT cohort. The concentration of all amino acids was similar to those reported in the literature for healthy volunteers. Contrary to the stated hypothesis, the data showed minimal differences in concentration of individual amino acids and NEAAs, LNAAs, and EAAs between healthy controls or FGDs subtypes. Only BCAAs differed between groups, but this difference was no longer apparent when the IBS subtypes and functional counterparts were combined into constipation and diarrhoea groups.

All participants' groups had similar estimated protein intakes. However, age and gender were strong determinants for differing amino acid profiles among groups, as reported in other studies [244, 253]. The results reported here showed increased protein consumption in males compared to females, as expected in the general population.

Tryptophan is a precursor to serotonin thought to play a critical role in IBS symptomology because of disruptions in motility, sensitivity and secretion, and the GBA modulation [120]. Here, there were no differences in circulating plasma tryptophan concentration between IBS subtypes, functional groups, or healthy controls, in line with similar findings from other studies [122, 123, 254]. Clarke *et al*., reported, however, higher circulating plasma concentration of kynurenine, a metabolite derived from tryptophan catabolism, and a higher ratio of tryptophan to kynurenine in IBS participants compared to healthy controls [122, 254]. Kynurenine is the dominant pathway for tryptophan metabolism [122], and thus an increased ratio of tryptophan to kynurenine could suggest a decreased production of serotonin from tryptophan. Thus, it could be plausible that the systemic concentration of tryptophan may be less important than the pathways it is metabolised through, though further investigations are required to confirm or negate this. However,

the metabolites of tryptophan metabolism were not measured here, and it would be warranted to quantify their concentrations in the participants of the COMFORT cohort.

The lack of difference in plasma glutamine concentration in the systemic circulation between any groups, functional groups, and respective IBS counterparts disagrees with the literature. Glutamine is important in gut permeability and function [248, 255] as shown by destruction of membrane components of gut epithelium and altered tight junction distribution and increased inflammation of the epithelial layer *in vitro*. These effects have also been reported in FGD individuals [248, 256]. Perfusion with glutamine, given to individuals with FGDs, resulted in decreased concentrations of the pro-inflammatory cytokines interleukin-6 and interleukin-8 compared to healthy controls and could be a modulator for inflammatory disorders of the gut [257]. A similar study where IBS-D participants were given an oral glutamine dose (15g/day for 8 weeks) showed a reduction in symptom severity, Bristol Stool Scale score, intestinal permeability, and bowel movement compared to the placebo group [258].

Some amino acids play an important role in maintaining cellular pathways and mechanisms. For example, histidine, as the precursor to histamine, could also be important in inflammatory conditions of the gut. The mechanisms of action of histamine in the gut are linked to ion secretion, motility, and gastric acid production [259]. However, the results here showed no differences in plasma histamine concentration between any of the groups.

The lack of difference in dietary protein intake and circulatory plasma amino acid concentrations here indicated the mechanisms involved in FGD might be not linked to their appearance in plasma. Additionally, it suggests no perturbations to host, or microbial processes involved in the assimilation and metabolism of dietary protein into peptides

and amino acids. There is, however, extensive knowledge suggesting amino acid metabolites are important to processes linked to inflammation [260] and thus FGDs. Therefore, the downstream processes might be perturbed, or that measurement of circulating amino acids does not provide a conclusive representation of the amino acid pool.

It has previously been shown that IBS individuals have higher concentrations of histidine, lysine and glutamine in urine samples compared to patients with UC [124]. Additionally, differences have been found in plasma and urine amino acid concentrations between UC and healthy controls [124, 261]. Thus, the physical irregularities of UC compared to an FGD may account for the differential amino acid profiles.

There are some limitations relating to sample collection, dietary records and sample analysis. There is potential variation in sample collection and aliquots, which could alter the results of this study. Amino acids are influenced by dietary intake, host, and microbial metabolism, and these factors were expected to impact the findings. Therefore, the accuracy of the diet dataset relies on the participants accurately recording their dietary intake. Additionally, there are significant limitations in measuring circulating plasma metabolites that may not reflect processes or perturbations localised to the gut mucosa in FGDs. For example, homocysteine and cysteine could not be measured, and thus it was not possible to present data as a combined group of sulphur-amino acids.

4.5. Conclusion

In conclusion, this study showed minor differences in the plasma circulating plasma amino acid profile, mainly for BCAAs, between healthy controls, IBS subtypes, and functional groups. However, BCAAs were similar when combined into constipation or diarrhoea groups. The lack of differences in dietary protein intake suggests there was no

perturbation in protein catabolism or amino acid concentration between the individuals with FGDs and healthy controls. Instead, metabolites in the downstream pathways of some amino acids might be involved in regulating mechanisms involved in FGDs.

Chapter Five
Characterisation of the faecal metabolome in participants with functional gut disorders

Abstract

There is evidence of perturbed microbial and host processes in the gut of individuals with FGDs compared to healthy controls. The faecal metabolome provides insight into the metabolic processes localised to the gut. This chapter aimed to profile the faecal metabolome of individuals with constipation (FC + IBS-C) and diarrhoea (FD + IBS-D) symptoms relative to healthy controls (identified using the Rome Criteria IV). The faecal metabolome was measured using multi-modal LC-MS technologies in 252 samples (n = 97 healthy controls, n = 58 constipation (FC + IBS-C), n = 66 diarrhoea (FD + IBS-D)). Discriminant analysis separated patients with constipation from healthy controls and diarrhoea from healthy controls. The relative abundances of lipids, particularly ceramides, diglycerides, and triglycerides, varied significantly ($p < 0.05$) between healthy controls and constipation, and between healthy controls and diarrhoea. Upregulated or downregulated lipid pathways were evident when comparing the participants with constipation compared to healthy controls and those with diarrhoea compared to healthy controls. Key polar and semi-polar metabolites (e.g., bile acids, homovanillic acid, riboflavin, nicotinic acid) were differentially abundant between all three groups. The faecal metabolome, particularly lipids, showed perturbations between constipation, diarrhoea, and healthy control groups that may reflect processes and mechanisms linked to FGDs.

Chapter Five

5.1. Introduction

Dietary intolerances, visceral hypersensitivity, immune activation, dysmotility, impaired mucosa structure in the gut, and dysregulated GBA [4]) are postulated to be of importance in understanding processes and mechanisms that cause FGDs. Therefore, it is postulated that a better understanding of the interactions between dietary intake, the gut microbiome, and the host would provide insights into the underlying cause of FGDs. However, there are constraints to investigating gut tissue or contents, primarily due to the difficulty and invasive nature of obtaining samples [262, 263].

The faecal metabolome is an alternative sample type shown to provide a readout reflective of host-microbial metabolic processes, primarily of the lower gut [264]. However, studies analysing the faecal metabolome are still infrequent and mostly focused on determining the efficacy of intervention-based studies in FGDs [265]. While factors such as sex, age, and ethnicity can influence a person's metabolic phenotype, metabolites resulting from the interactions between dietary intake, host digestion and absorption, and microbial metabolism are crucial to understanding FGDs [266].

A recent analysis by Jeffery *et al*., provides the most comprehensive faecal metabolome analysis to date [77]. In their study, discriminant analyses of the urine and faecal metabolomes showed that a combined IBS group (IBS-C, IBS-D, IBS-M) could be distinguished from a healthy control group, but not between IBS subtypes [77]. Of the 128 metabolites they identified in the faecal metabolome, 77 were less abundant in IBS participants compared to healthy controls [77]. Specific metabolites from multiple classes, for example, amino acids, fatty acids, adenosine, inosine, and purine metabolites, were important in differentiation between IBS and healthy control groups [77].

Grouping the IBS subtypes has benefits, but it does not accommodate a better understanding of the potential gut mechanisms, either dietary, microbial, or host-derived, contributing to the disorders' symptoms. For example, constipation and diarrhoea phenotypes, most frequently associated with FGDs, are functionally different. However, less is known about what metabolic pathways and processes distinguish them.

These findings support the hypothesis for this chapter that the faecal metabolome of individuals with constipation (FC + IBS-C) or diarrhoea (FD + IBS-D) symptoms would differ from healthy controls and highlight mechanistic differences linked to perturbed metabolic pathways and processes in the gut, specific to phenotype. Thus, the aim of the analysis was to characterise the faecal metabolome using MS to better understand the metabolic pathways and processes that differ between healthy controls compared to individuals with constipation (FC + IBS-C) or diarrhoea (FD + IBS-D) symptoms.

5.2. Methods

5.2.1. Participants

Information on recruitment, inclusion, exclusion, and sample collection methodology was as described in **Chapter 2**. A total of 252 faecal samples were analysed from the COMFORT Cohort.

5.2.2. Standards and reagents

d_4-alanine, d_2-tyrosine, d_5-tryptophan, and d_{10}-leucine were purchased from Cambridge Isotope Laboratories, Inc (Tewksbury, MA) as standards for MS performance. Avanti® Polar Lipids, Inc. 1-palmitoyl-d_{31}-2-oleoyl-sn-glycero-3-phosphate (PE, sodium salt) (16:0 D31/18:1)), ammonium formate and formic acid were purchased from Sigma Aldrich (Auckland, New Zealand). Acetonitrile, methanol, methyl tert-butyl ether, and

chloroform of optima LC-MS grade were purchased from Thermo Fisher Scientific (Auckland, New Zealand).

5.2.3. Sample extraction

Samples were freeze-dried under vacuum and 50mg transferred to 2.0mL Eppendorf tubes with a ceramic bead and homogenised for 1 min using a QIAGEN TissueLyser II. Next, 400µL of 75% MeOH/MilliQ H20 was added, and tubes vortexed for 30 s. Samples were sonicated for 2 min, then transferred onto ice for 10 min. Next, 1mL of MTBE was added to tubes and samples agitated on a shaker for 1 hr at 4°C, 450rpm. Next, 250µL of MilliQ water was added, samples vortexed for 30 s and left to rest for 10 min. Next, tubes were centrifuged at 14,000 *x g* for 25 min at 4°C. Next, 850µL of the upper lipid phase was transferred to a new tube and 300µL of MilliQ water was added to the remaining extract and then centrifuged for a further 20 min (14,000 *x g*, 4°C). Finally, the remaining polar phase was transferred to a new tube, centrifuged for a further 20 min, and then 300µL aliquots were taken into two different tubes for polar and semi-polar analyses. Finally, all tubes were evaporated under nitrogen.

5.2.4. General mass spectrometry analytical parameters

The metabolomic analysis was conducted on a Thermo Fisher Accela 1250 UHPLC pump system (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a PAL auto-sampler (CTC Analytics AG., Zwingen, Switzerland) and a Q-Exactive Orbitrap with electrospray ionisation. Positive and negative mass calibrations using Pierce™ LTQ electrospray ionisation (ESI) (Positive and Negative Ion Calibration Solutions (Thermo Fisher Scientific, Waltham, MA, USA)) of the Orbitrap system were completed prior to sample analysis and after every 100 samples by direct infusion. Samples were cooled at 4°C in

the autosampler until sample injection. Source parameters were 3500 V, a capillary temperature of 275°C, sheath, auxiliary and sweep gas were 40,10 and 5 respectively.

5.2.4.1. *Lipidomic analysis*

Samples for lipidomic analysis were reconstituted in 500µL of a 2:1 $CHCl_3$:MeOH containing PE(16:0 D_{31}/18:1) internal standard at 10µg/mL concentration and vortexed until all material was redissolved. Samples were centrifuged at 12,000 *x g* for 12 min at 4°C, and 100µL of the solution was transferred to a glass vial insert. Next, 5µL of the sample was injected into a 2µL injection loop and eluted on an Acquity CSH™ C18 column 1.7µm, 2.1µm x 100mm (Waters, Milford, MA, USA) maintained at 65°C with a flow rate of 600µL/min. Solvent A was 60% acetonitrile in water with 10mM ammonium formate and 0.1% formic acid. Solvent B was 90% iso-propanol in acetonitrile with 10mM ammonium formate and 0.1% formic acid. Gradient elution started at 15% B, increasing to 30% B at 2 min, 48% B at 2.50 min, 82% B at 11 min, then 99% B at 11.50, maintained until 14.10 min, then reduced to 15% B and held there for 3 min for equilibration before next sample injection. Mass spectral detection was performed in positive and negative ionisation modes with ESI over 15 min and a mass range from 200 to 2000 *m/z*.

5.2.4.2. *Polar metabolic analysis*

The faecal samples for polar metabolic analysis (hydrophilic interaction liquid chromatography, HILIC) were reconstituted in 200µL of a 50:50 acetonitrile/H_20 solution then vortexed until all material dissolved. Samples were centrifuged at 12,000 *x g* for 12 min at 4°C, and 100µL of the solution was transferred to a glass vial insert. Next, a volume of 5µL of the sample was injected into a 2µL injection loop and eluted on SeQuant® ZIC®-pHILIC column (100 x 2.1mm x 5µm, PEEK coated, Merck KGaA, Darmstadt,

Germany), attached to a SeQuant® ZIC®-pHILIC Guard (20 x 2.1mm, PEEK coated, Merck KGaA, Darmstadt, Germany), and a KrudKatcher™ ULTRA HPLC In-Line Filter (0.004in x 0.5µm, Torrance, CA, USA). A 250µL/min flow rate was maintained with solvent A 0.1% formic acid in acetonitrile and solvent B 16mM ammonium formate in water. Gradient elution started at 3% B, increasing to 30% B at 12 min, 90% B at 14.50 min and held there till 18.50 min where it returned to 3% B until 24 min for equilibration before next sample injection. Mass spectral detection was performed in positive and negative ionisation modes with ESI over 19 min, with a mass range from 55 to 825 *m/z*.

5.2.4.3. *Semi-polar metabolic analysis*

The faecal samples for semi-polar metabolite (C18) analysis were reconstituted in 200µL of a 90:10 H_20/acetonitrile solution then vortexed until all material dissolved. Samples were centrifuged at 12,000 *x g* for 12 min at 4°C, and 100µL of the solution was transferred to a glass vial insert. A volume of 5µL of the sample was injected into a 2µL injection loop and eluted on a Hypersil GOLD column (2.1mm x 100mm x 1.9µm, Thermo Fisher Scientific, Waltham, MA, USA) with a 400µL/min flow rate. Solvent A was 0.1% formic acid in the water, and solvent B was 0.1% formic acid in acetonitrile. Gradient elution started at 0% B going to 100% B at 11 min held for 3 min and then back to 0% B at 14 min and held there for 2 min for equilibration before next sample injection. Mass spectral detection was performed in positive and negative ionisation modes with ESI over 14 min, with a mass range from 80 to 1200 *m/z*.

5.2.5. Data processing and statistical analysis

The general metabolomic analytical workflow is summarised in **Figure 5-1**. Raw files were converted to mzML format using MS Convert (ProteoWizard) [267]. Peak detection and alignment were completed using XCMS as part of the Bioconductor package for R

statistical software (R version 3.6.1) [268] and batch correction using Workflow 4 Metabolomics (Version 4.0) [269]. In-house libraries were used for the annotation of polar and semi-polar metabolites. MS Dial (version 4.6) [270] was used to annotate metabolites using tandem MS/MS data that was collected from polar and semi-polar analytical streams. Human metabolome database (HMDB) [271] was used to assist identification of metabolic features selected from partial least squares – discriminant analysis (PLS-DA) models based on their variable importance in projection (VIP) score.

PLS-DA models were analysed using SIMCA (16.0.1). The quality of the PLS-DA models generated was measured using R2X, R2Y, and Q2, with values closest to one signifying a better model [272]. Cross-validated ANOVA (CV-ANOVA) was used to test the significance of the PLS-DA models [272].

In the analytical streams where duplicate measures of the same peak were detected in both positive and negative modes, the negative mode was removed as a lower intensity peak most frequently characterises it. This procedure was completed to reduce the effect of false correlations for the same metabolite.

Lipid metabolites were annotated using Lipid Search™ software (Thermo Fisher Scientific). The reliability of lipid annotations were based on the m-score, a scoring algorithm that matches the ion peaks of the MS spectrum, with higher scores depicting greater reliability. Similarly, matches are based on a grading score from A to D, with an A grade depicting the highest reliability through to D reflecting lowest reliability. For quality control no identifications were included for analysis that were based off a D grading score or an m-score below 8. The average m-score and grade score for each lipid class is highlighted in **Table 5.1.** BioPAN [273] as part of Lipid Maps® Lipidomic Gateway was used to highlight lipid pathways of importance. Metaboanalyst (version 5.0)

[238] was used for hierarchical clustering analysis. ANOVA was used to compare metabolites from the hierarchical clustering analysis. ANOVA considers only the variation of a single variable at a time, so it was more suitable for reduced datasets, such as those used for the hierarchical clustering analysis. Comparatively, likelihood ratio testing (LRT) (from the edgeR package) for R accounts for variation of the entire dataset, so is more suitable for datasets with large numbers of variables and was thus used to test for significant metabolites from the whole dataset [274]. Multiple testing adjustment was performed using the false discovery rate (FDR) method, with an adjusted $p < 0.05$ deemed statistically significant.

Venn diagrams were created using Venny (2.1.0). Metscape (version 3.1) [275] as part of Cytoscape were used for pathway mapping.

As discussed in **Chapter 2**, statistical analyses were only made between the constipation and healthy control group or diarrhoea and healthy control group to identify the variables that differ between individuals with altered bowel movement (constipation or diarrhoea) and healthy individuals. However, to provide an overview of how all the groups compare, methods such as hierarchical clustering analyses were utilised.

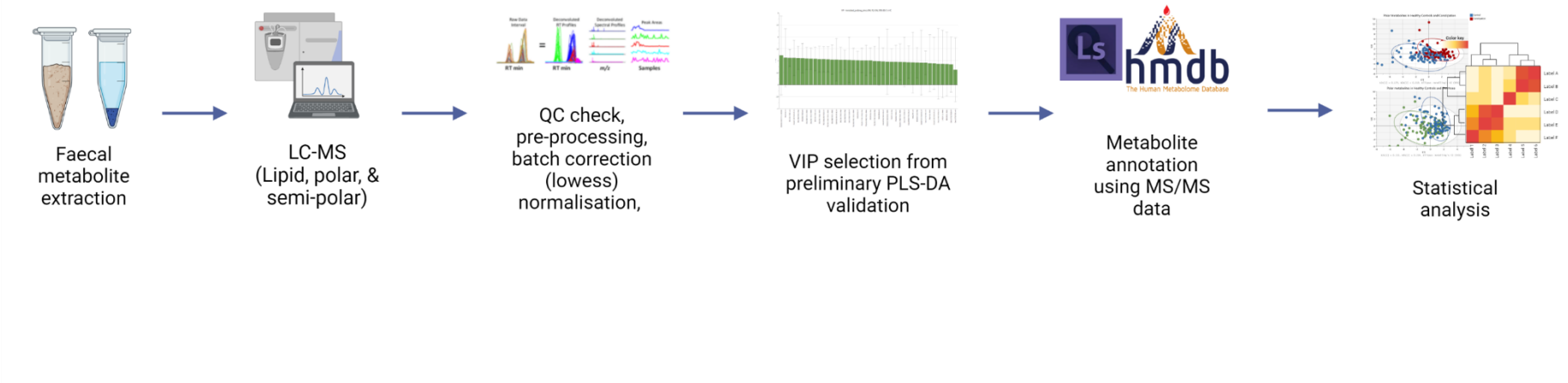


Figure 5-1: General metabolomic analytical and data processing workflow. Figure created using BioRender.

5.3. Results

A list of all annotated metabolites is presented in **Appendix Table 1**. A total of 252 faecal samples were analysed. Symptom questionnaires based on the Rome Criteria IV clustered participants as constipation (FC + IBS-C, *n*=58), diarrhoea (FD + IBS-D, *n*=66) and healthy control (*n*=97).

5.3.1. Lipidomic

The lipidomic analysis found 421 annotated lipids belonging to 32 different lipid classes (**Table 5-1**). Unsupervised principal component analysis (PCA) could not distinguish the faecal lipidome of the participants with constipation from healthy controls or diarrhoea from healthy controls. However, PLS-DA models based on the top 5% VIP of lipids showed the lipid profile of the individuals with constipation or diarrhoea were distinct from that of the healthy controls, albeit with an overlap of the confidence intervals (**Figure 5-2**). Both models were significant in separating the constipation or diarrhoea phenotypes from healthy controls, though the models were characterised by weak Q2 values for the constipation and healthy control comparison (CV-ANOVA p = 7.82e-05, R2X = 0.442, R2Y = 0.205, Q2 = 0.143), and the diarrhoea and healthy control comparison (CV-ANOVA p = 0.00089, R2X = 0.409, R2Y = 0.261, Q2 = 0.12)).

The loadings plot of the PLS-DA models showed that lipids belonging to the ceramides, diglycerides, and triglycerides class were important in the separation between constipation or diarrhoea groups when compared against healthy controls (**Figure 5-3**). Ceramides were associated positively or negatively with constipation or diarrhoea groups, but not with the healthy control group. The opposite relationship was observed for triglycerides and diglycerides.

Table 5-1: Lipid groups annotated from faecal sample analysis with corresponding common abbreviation and number of lipids detected from each group.

Lipid Group	Lipid Abbreviation	Lipids in Group	m-score	Grade
Ceramide	Cer	74	34	A
Cholesterol ester	Che	4	13	B
Diglyceride	DG	102	29	A
Digalactosyldiacylglycerol	DGDG	7	40	A
Lysophosphatidylcholine	LPC	3	31	A
Lysophosphatidylethanolamine	LPE	3	29	A
Lysophosphatidylglycerol	LPG	1	25	B
Monoglyceride	MG	9	9	C
Monogalactosyldiacylglycerol	MGDG	11	30	A
(*O*-Acyl)-ω-hydroxy fatty acids	OAHFA	17	15	A
Phosphatidylcholine	PC	8	40	B
Phosphatidylethanolamine	PE	13	39	A
Phospahtidylethanol	PEt	5	9	C
Phosphatidylglycerol	PG	17	22	B
Phosphatidylinositol	PI	4	87	B
Phosphatidylserine	PS	3	11	C
Sitosteryl ester	SiE	1	8	B
Sphingomyelin	SM	6	17	C
Sphingoshine	So	1	22	B
Sulfoquinovosyl diacylglycerol	SQDG	1	28	A
Stigmasteryl ester	StE	1	8	B
Triglyceride	TG	128	59	A
Zymosteryl	ZyE	2	8	B

Figure 5-2: PLS-DA of lipid metabolites between healthy control and constipation (FC + IBS-C) groups (CV-ANOVA p = 7.82e-05, R2X = 0.442, R2Y = 0.205, Q2 = 0.143), and healthy control and diarrhoea (FD + IBS-D) groups (CV-ANOVA p = 0.00089, R2X = 0.409, R2Y = 0.261, Q2 = 0.12).

Figure 5-3: Loadings plot of diarrhoea (FD + IBS-D) and healthy control groups, and constipation (FC + IBS-C) and healthy control groups. Plot shows the lipid contribution to the model separation and differences between the two case phenotypes compared to healthy controls.

Of the lipids selected during the PLS-DA VIP refinement, 39 lipids were important for PLS-DA separation between the lipidome of participants with diarrhoea and healthy controls, and 37 lipids between participants with constipation and healthy controls. Notably, only five lipids (four ceramides and a digalactosyldiacylglycerol) were commonly shared between the diarrhoea and constipation models **(Figure 5-4).**

The LRT analysis showed that the abundance of eight lipids was significantly different between individuals with diarrhoea and healthy controls (**Table 5-2**). These lipids belonged to triglyceride, phosphatidylglycerol, diglyceride, (*O*-Acyl)-ω-hydroxy fatty acids, monogalactosyldiacylglycerol, and zymosteryl ester lipid groups. Except for two triglycerides and a monogalactosyldiacylglycerol, the remaining five lipids were more abundant in healthy controls compared to participants with diarrhoea.

The abundance of 18 lipids was significantly different between constipation and healthy control groups, with 15 more abundant in healthy controls. The abundance of the other three lipids belonging to the ceramide and monoglycerides classes was higher in the constipation group.

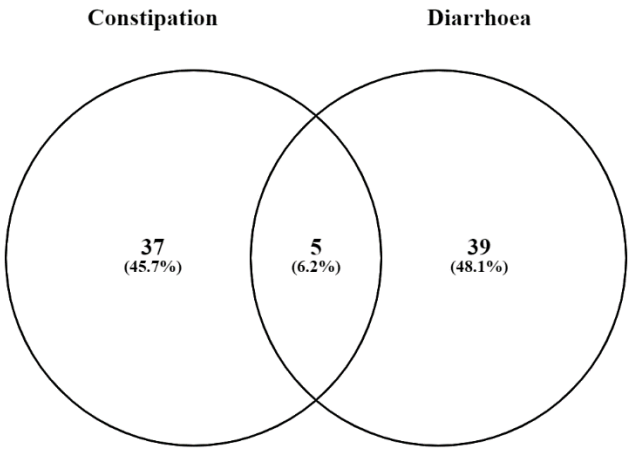


Figure 5-4: Venn diagram of top 5% of lipids that contributed to the separation of lipids in constipation (FC + IBS-C) compared to healthy controls and diarrhoea (FD + IBS-D) compared to healthy controls based on VIP plots (**Figure 5-2**).

Table 5-2: Lipids with significantly different abundances (LRT FDR<0.05) between constipation and healthy controls (Constipation-HC), and diarrhoea and healthy controls (Diarrhoea-HC).

LRT coefficients	Lipid metabolite	logFC
Constipation-HC	Cer(d18:0/12:0)+H	1.42
Constipation-HC	Cer(d18:1/12:0)+H	1.08
Constipation-HC	DG(18:0/18:1)+NH4	-1.07
Constipation-HC	DG(18:1/22:0)+NH4	-1.69
Constipation-HC	DG(18:3/18:2)+H	-1.20
Constipation-HC	DG(20:0/18:1)+NH4	-1.28
Constipation-HC	DG(22:0/18:2)+NH4	-1.09
Constipation-HC	MG(20:2)+NH4	1.18
Constipation-HC	MGDG(16:3/18:3)+HCOO	-1.25
Constipation-HC	MGDG(18:3/18:3)+HCOO	-1.13
Constipation-HC	PEt(19:1/18:1)+H	-1.09
Constipation-HC	PG(12:0/14:0)-H	-1.15
Constipation-HC	SM(d18:1/16:0)+HCOO	-1.12
Constipation-HC	SM(d36:1)+H	-1.06
Constipation-HC	SM(d36:4)+H	-1.06
Constipation-HC	TG(18:1/12:0/18:1)+NH4	-0.96
Constipation-HC	TG(4:0/14:0/18:3)+NH4	-1.12
Constipation-HC	TG(4:0/18:2/18:2)+NH4	-2.36
Diarrhoea-HC	DG(15:0/18:1)+NH4	-1.20
Diarrhoea-HC	MGDG(16:0/18:3)+HCOO	1.10
Diarrhoea-HC	OAHFA(41:2)-H	-1.18
Diarrhoea-HC	PG(12:0/14:0)-H	-1.99
Diarrhoea-HC	PG(16:0/12:0)-H	-1.14
Diarrhoea-HC	TG(16:0/16:0/16:0)+NH4	1.30
Diarrhoea-HC	TG(18:0/16:0/16:0)+NH4	1.38
Diarrhoea-HC	ZyE(22:2)+NH4	-1.01

A negative log fold change (logFC) value indicates higher abundance in the healthy control compared to constipation (FC + IBS-C) or diarrhoea (FD + IBS-D) groups. Conversely, a positive logFC value indicates higher abundance in constipation or diarrhoea groups compared to the control group. Abbreviations; HC – healthy control, FC – fold change. Lipid abbreviations defined in Table 5-1. Common lipid nomenclature: Lipid metabolite followed by total carbon number:number of double bonds followed by MS ion fragmentation.

Hierarchical clustering (of the group means using the top 50 significantly different metabolites determined by ANOVA) showed that the diarrhoea group was distinct from constipation and healthy control groups, which were more similar (**Figure 5-5**). Average *z-scores* showed that the diarrhoea and constipation groups differed. Triglyceride levels, for example, were increased in the diarrhoea group, compared to ceramides and (*O*-Acyl)-ω-hydroxy fatty acids, with the opposite trend observed in the constipation group. The relative concentration of these lipids in healthy controls ranged between the diarrhoea and constipation groups. Lipids from the same lipid class did not always follow the same pattern of abundance; for example, shorter chain fatty acid ceramides (carbon-chain lengths ~C15-C18) were higher in relative abundance in the constipation group compared to the diarrhoea group. Conversely, longer chain fatty acids ceramides (carbon chain lengths ~C18-C24) were higher in relative abundance in the diarrhoea group compared to the constipation group.

Lipid Maps® BioPAN software highlighted correlations between lipid classes which differed between the networks of participants with diarrhoea and healthy controls, and those with constipation and healthy controls (**Figure 5-6**). In the constipation and healthy control comparison, ceramides and sphingomyelin belonging to the wider sphingolipid class were metabolically active, compared to inactive in the diarrhoea group based on BioPAN mapping. The correlation strength of the lipid classes differed between the phenotypic groups. In constipation, diglycerides were positively correlated to phosphatidylcholine (0.014), compared to diarrhoea, where there was a negative correlation (-0.006). In the constipation group, dihydroceramides were negatively correlated to ceramides (-3.051), as were ceramides to sphingomyelin (-1.629), compared to the equivalent positive correlations in the diarrhoea group (0.572; 0.16).

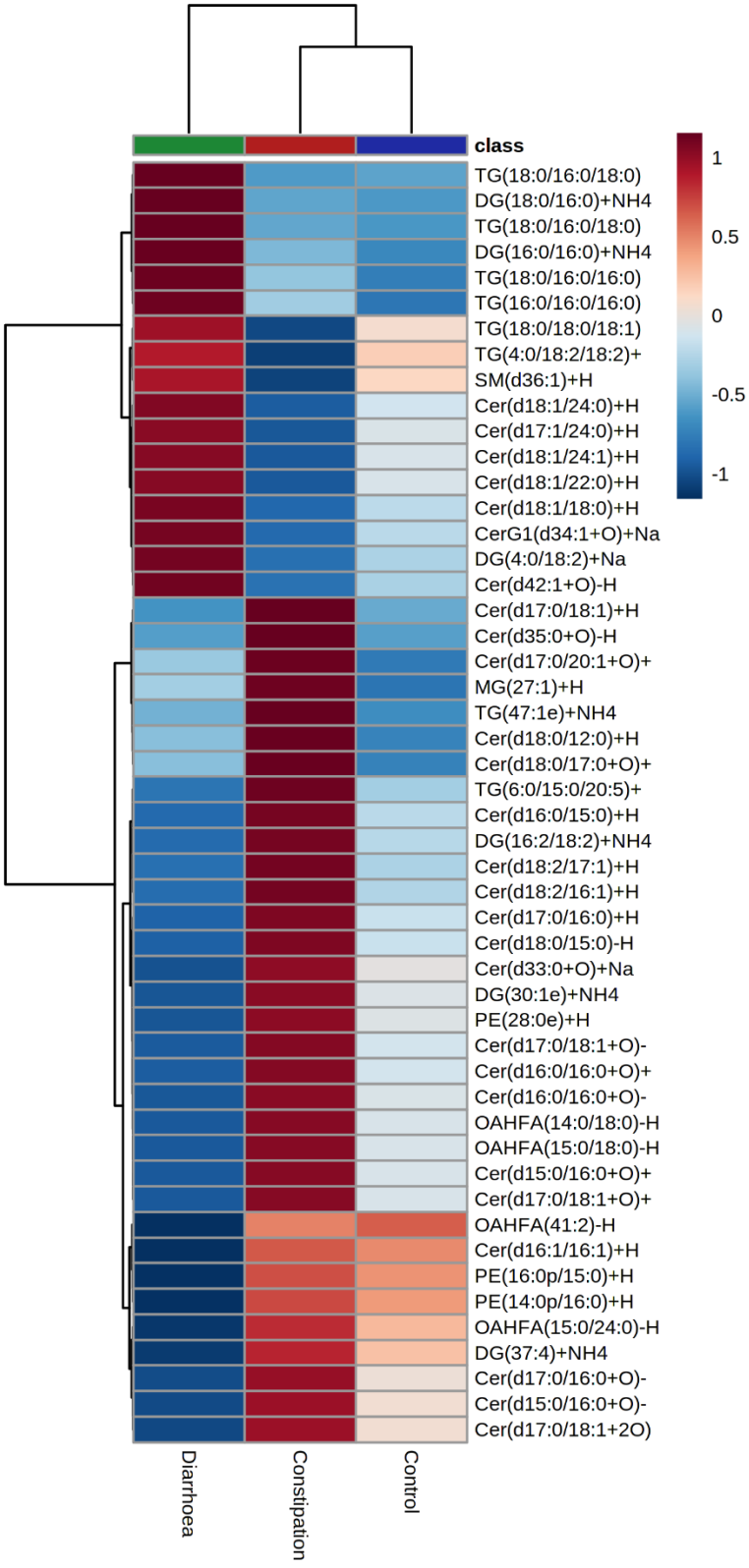


Figure 5-5: Hierarchical clustering analysis of the group means for the top 50 ANOVA lipid metabolites between healthy control, constipation (FC + IBS-C), and diarrhoea (FD + IBS-D) groups. Data presented as z-score. Colour ribbon beneath the upper dendrogram identifies group; healthy control – blue, constipation group – red, diarrhoea group – green.

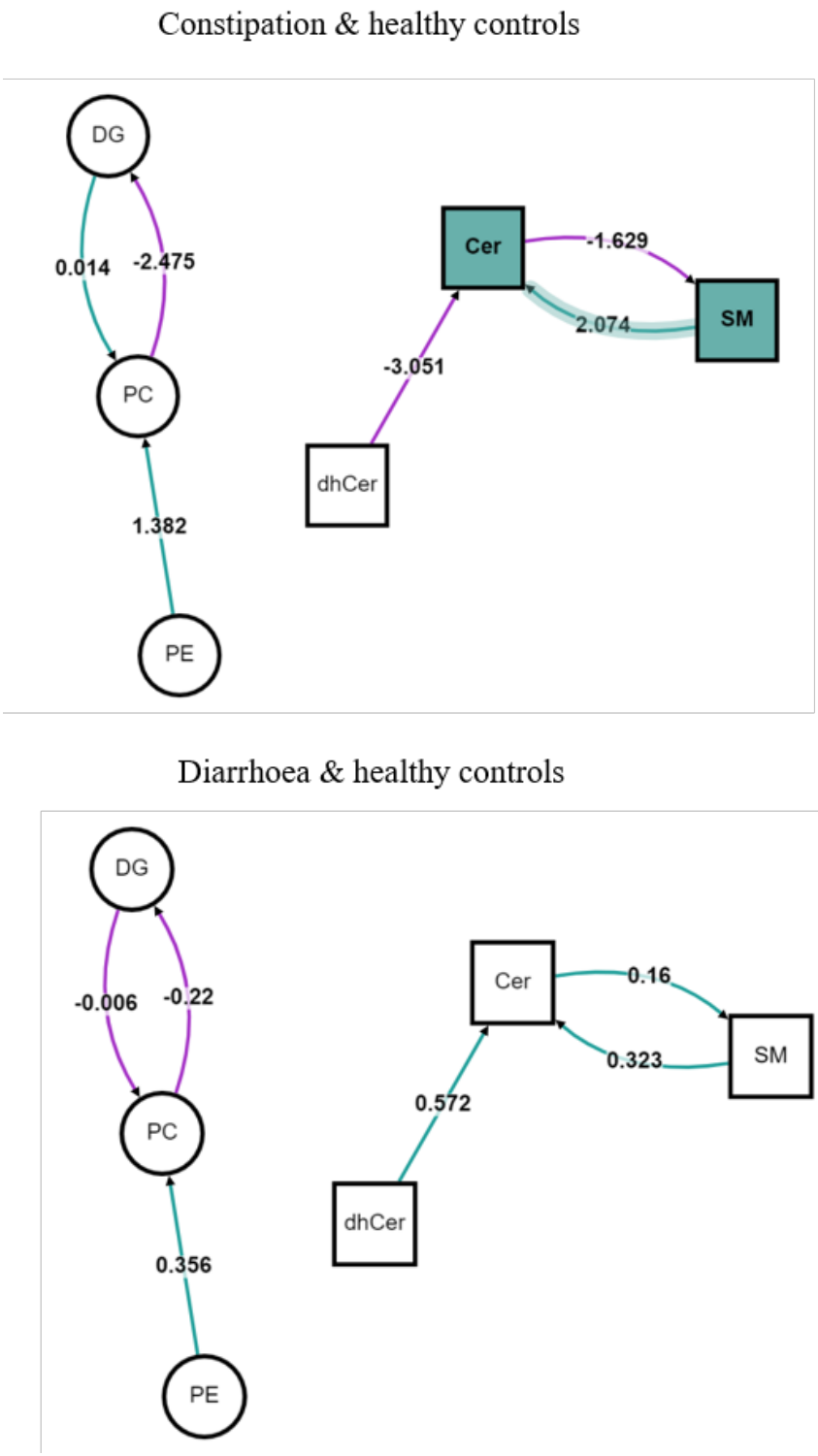


Figure 5-6: Correlation pathway analysis of lipid classes between constipation (FC + IBS-C) and healthy control groups, and diarrhoea (FD + IBS-D) and healthy control groups. Node shape depicts lipid group (circle – glycerolipids or glycerophospholipids or square – sphingolipids). The green colouring of the node depicts an active state. No colouring shows no active state. Negative correlations are shown with purple arrows and positive with green arrows. Abbreviations: DG: diglyceride, PC: phosphatidylcholine, PE: phosphatidylethanolamine, dhCer: dihydroceramide, Cer: ceramide, SM: sphingomyelin.

5.3.2. Polar metabolite

Six hundred and ninety-four metabolites were measured in the polar analysis of the faecal samples with 79 metabolites annotated using a combination of in-house libraries, MS/MS data, and HMDB. The VIP lists from PLS-DA analysis were used as a focal point to search the HMDB database to annotate metabolites that contributed to model separation.

Unsupervised PCA could not distinguish polar metabolite profiles between participants with constipation or diarrhoea from the profile of healthy controls. Similar to the lipid results, PLS-DA analysis based on the top 5% VIP polar metabolites showed separation between constipation and healthy control groups or diarrhoea and healthy control groups, albeit with an overlap in confidence intervals (**Figure 5-7**). The CV-ANOVA of both models was significant for constipation and healthy control groups (CV-ANOVA p = 4.27e-7, R2X = 0.466, R2Y = 0.397, Q2 = 0.221), and for diarrhoea and healthy control groups (CV-ANOVA p = 4.37e-5, R2X = 0.376, R2Y = 0.259, Q2 = 0.154).

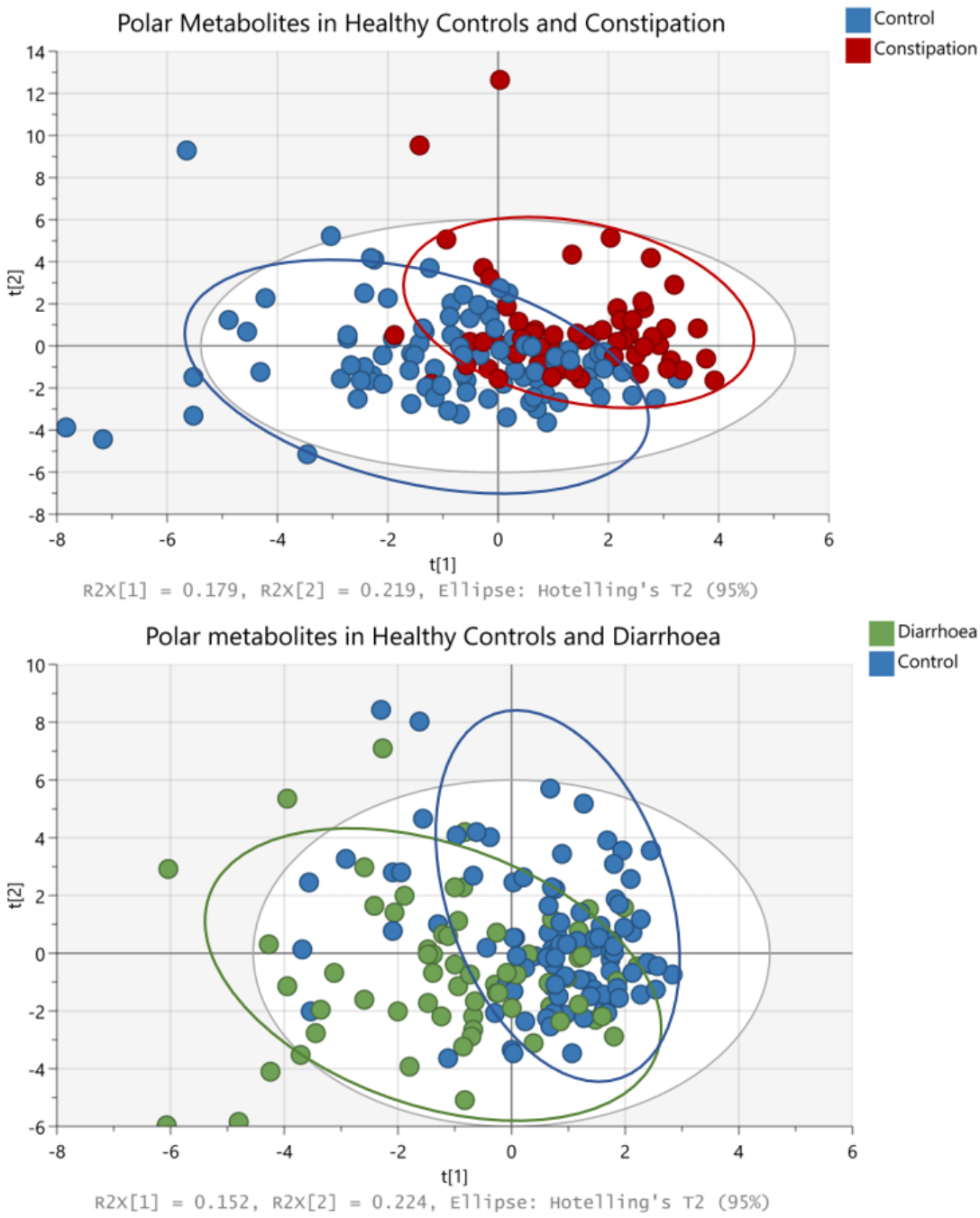


Figure 5-7: PLS-DA of polar metabolites between healthy control and constipation (FC+IBS-C) groups (CV-ANOVA p = 4.27e-07, R2X = 0.466, R2Y = 0.397, Q2 = 0.221), and healthy control and diarrhoea (FD + IBS-D) groups (CV-ANOVA p = 4.37e-05, R2X = 0.376, R2Y = 0.259, Q2 = 0.154).

The LRT analysis showed that 64 metabolites were significant between diarrhoea and healthy control groups, of which eight metabolites were annotated (**Table 5-3**). Gabapentin was significantly lower in the healthy control group than the diarrhoea group. On the other hand, creatinine, guanosine, 4-methyl-5-thiazoleethanol, 2-piperidone, metformin, N-acetylputrescine, and phenylacetaldehyde were present at a higher relative abundance in the diarrhoea group compared to the healthy control group.

Forty-five metabolites were significant between constipation and healthy control groups. Of these significant metabolites, isonipecotic acid had a higher relative abundance in the healthy control group than the constipation group. Conversely, metformin was more abundant in constipation compared to healthy controls.

Hierarchical clustering (of the group means using the top 50 significantly different metabolites determined by ANOVA) showed that the metabolite profiles of participants with diarrhoea and healthy controls were more similar compared to that of individuals with constipation (**Figure 5-8**). Of the top 50 metabolites that accounted for the differentiation, only four were annotated: cytarabine, D-Ala-D-Ala, metformin, and N-acetylpurescine. Visually, it can be inferred that most metabolites had a higher relative abundance in the diarrhoea group. However, some metabolites differed, higher in constipation and healthy control groups; though, these were un-annotated. The VIP plots show 47 metabolites contributed to the separation between the participants with diarrhoea and healthy controls, and 38 metabolites between the participants with constipation and healthy controls. Of these metabolites, ten were commonly shared between the two groups; however, only one metabolite, L-tyrosine, was annotated.

Table 5-3: Annotated polar metabolites with significantly different abundances (LRT FDR<0.05) between constipation and healthy controls (Constipation-HC), and diarrhoea and healthy controls (Diarrhoea-HC).

LRT coefficients	Polar metabolites	logFC
Constipation-HC	Isonipecotic Acid	-0.79
Constipation-HC	Metformin	1.29
Diarrhoea-HC	2-Piperidone	0.76
Diarrhoea-HC	4-methyl-5-thiazoleethanol	1.20
Diarrhoea-HC	Creatinine	0.89
Diarrhoea-HC	Gabapentin	-2.77
Diarrhoea-HC	Guanosine	0.70
Diarrhoea-HC	Metformin	2.06
Diarrhoea-HC	n-Acetylputrescine	1.52
Diarrhoea-HC	Phenylacetaldehyde	1.00

A negative log fold change (logFC) value indicates higher abundance in the healthy control compared to constipation (FC + IBS-C) or diarrhoea (FD + IBS-D) groups. Conversely, a positive logFC value indicates higher abundance in constipation or diarrhoea groups compared to the control group. Abbreviations; HC – healthy control, FC – fold change.

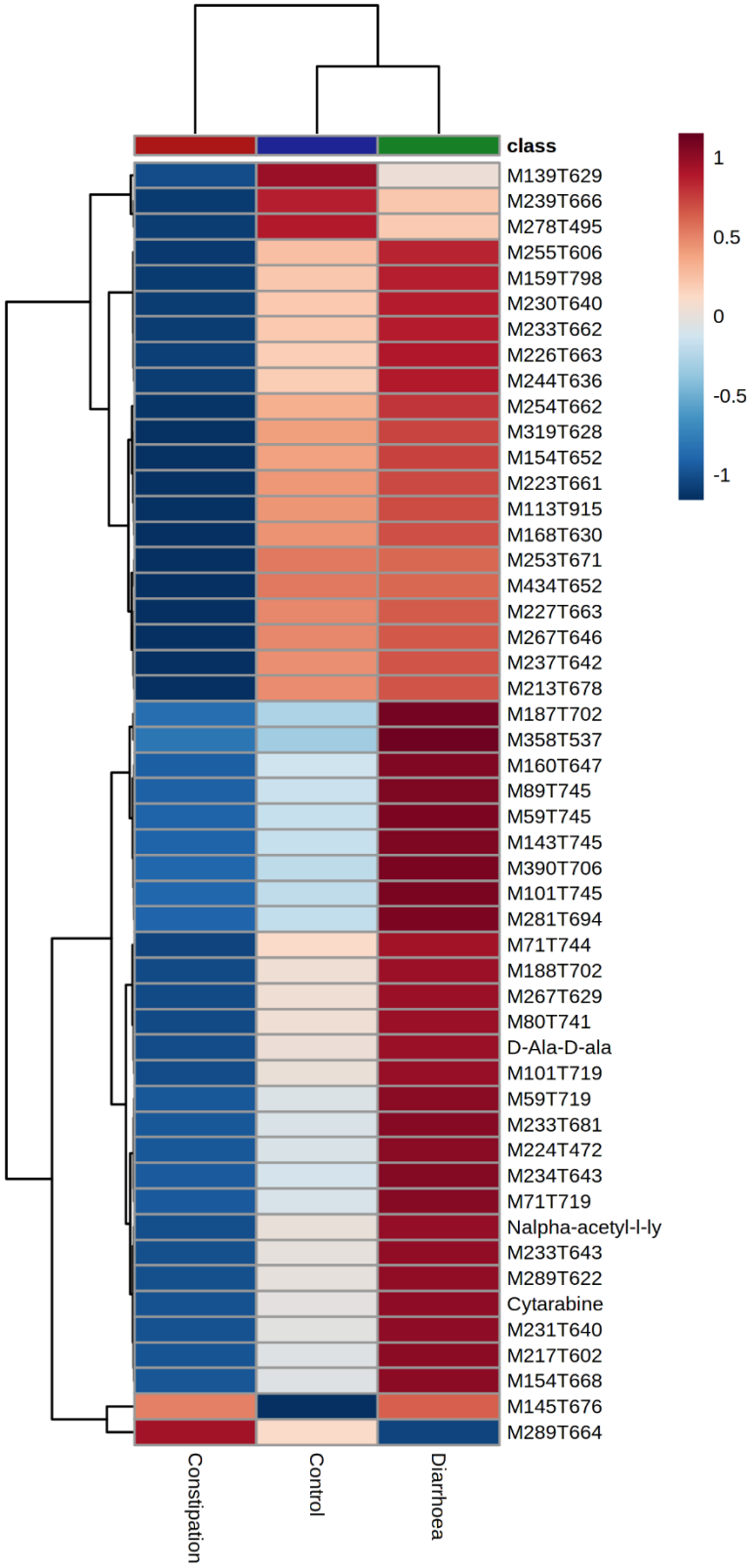


Figure 5-8: Hierarchical clustering analysis of the group means for the top 50 ANOVA polar metabolites (annotated and unannotated) between healthy control, constipation (FC + IBS-C), and diarrhoea (FD + IBS-D) groups. Data presented as z-score. Colour ribbon beneath the upper dendrogram identifies group; healthy control – blue, constipation group – red, diarrhoea group – green.

5.3.3. Semi-polar metabolite

One thousand two hundred and three metabolites were detected in the semi-polar faecal analysis, with 141 metabolites annotated from libraries, MS/MS data, and VIP lists to search HMDB. Unsupervised PCA analysis could not distinguish between groups. PLS-DA analysis based on the top 5% VIP semi-polar metabolites showed separation between constipation and healthy control groups, and diarrhoea and healthy control groups, albeit with an overlap in PLS-DA models (**Figure 5-9**). PLS-DA analysis of the semi-polar profile of the constipation and healthy control groups showed greater separation compared to the profile of the diarrhoea and healthy control groups (constipation and healthy controls: CV-ANOVA p = 6.66e-08, R2X = 0.537, R2Y = 0.297, Q2 = 0.226; diarrhoea and healthy controls: CV-ANOVA p = 6.98e-07, R2X = 0.245, R2Y = 0.361, Q2 = 0.209).

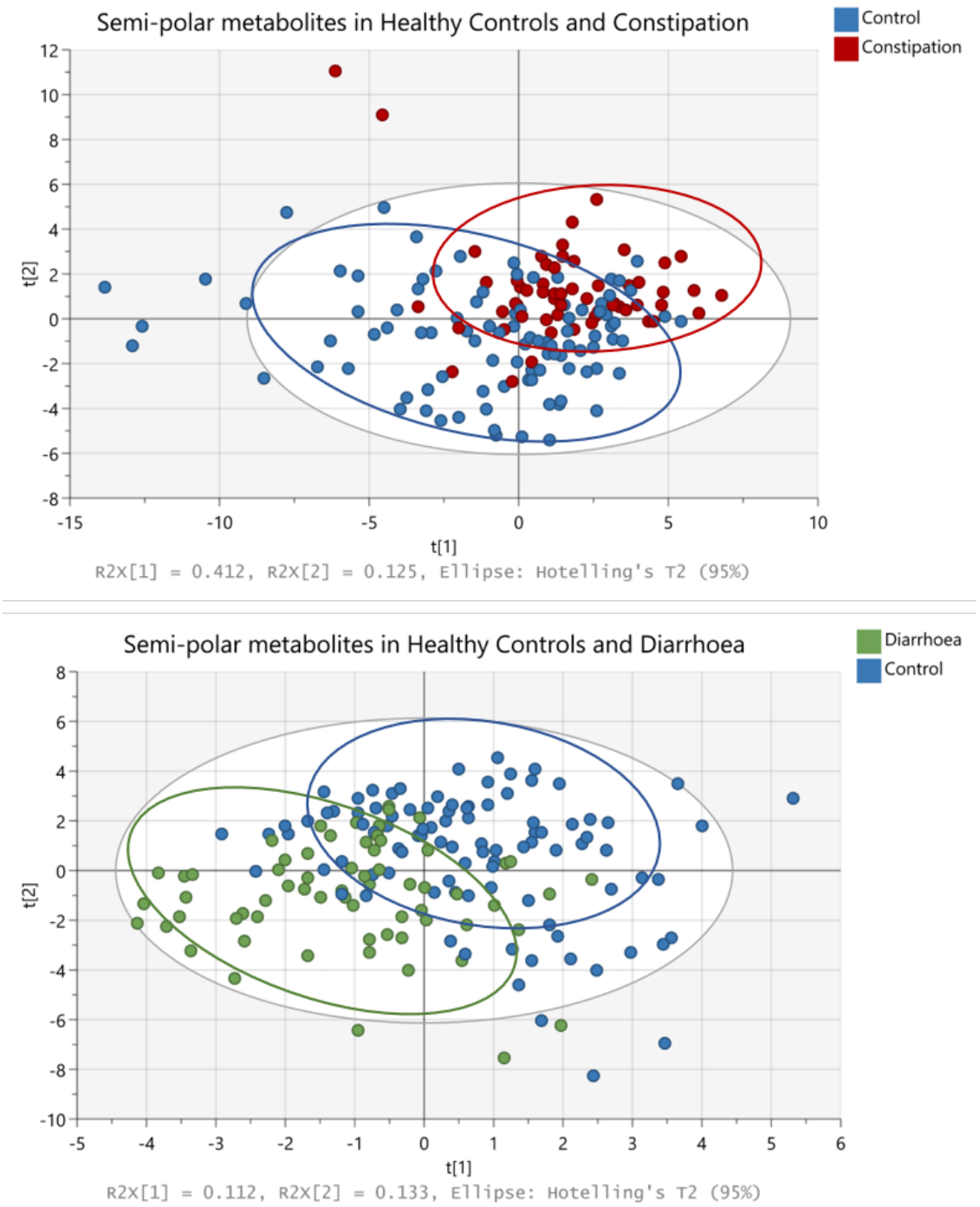


Figure 5-9: PLS-DA of semi-polar metabolites between healthy control and constipation (FC + IBS-C) groups (CV-ANOVA p = 6.66e-08, R2X = 0.537, R2Y = 0.297, Q2 = 0.226), and healthy control and diarrhoea (FD + IBS-D) groups (CV-ANOVA p = 6.98e-07, R2X = 0.245, R2Y = 0.361, Q2 = 0.209).

The LRT analysis showed 103 metabolites were significant between participants with constipation and healthy controls, of which 15 metabolites were annotated (**Table 5-4**). Of the significant metabolites, gabapentin, alanylphenylalanine, metoprolol, homovanillic acid, N-omega-acetylhistamine, cholic acid, glycocholic acid, and taurocholic acid had negative logFC values. Thus, these metabolites were present in higher abundance in the healthy control group compared to the constipation group. 5-aminonaphthalene-2-sulfonic acid, metformin, N-acetyl-L-tyrosine, creatinine, paracetamol, and N-acetyl mesalazine had a higher relative abundance in the participants with constipation compared to healthy controls.

One hundred and forty-seven metabolites were significant between the participants with diarrhoea and healthy controls in LRT analysis (**Table 5-4**), of which 20 were annotated metabolites. Gabapentin, coumaroylquinic acid, and alanylphenylalanine were present at higher abundance in the healthy control group compared to the diarrhoea group. The remaining 17 metabolites were higher in the diarrhoea group, including homovanillic acid, creatinine, metformin, metoprolol, paracetamol, quinoic acid, succinic acid, and bile acids (cholic acid, tauro-deoxycholic acid, taurocholic acid).

Hierarchical clustering (of the group means using the top 50 significantly different metabolites determined by ANOVA) showed that healthy controls and participants with constipation clustered more similarly than those with diarrhoea (**Figure 5-10**). Tyrosine, azelaic acid, pimelic acid, homovanillic acid, riboflavin, nicotinic acid, and spermidine had different relative abundances between participants with diarrhoea, participants with constipation, and healthy controls. Riboflavin and nicotinic acid were more abundant in the healthy control group than constipation and diarrhoea groups. Of the VIP metabolites that contributed to the separation of each PLS-DA model, only five metabolites were

commonly shared between constipation and diarrhoea groups, of which nicotinic acid and D-ala-D-ala were annotated.

Table 5-4: Annotated semi-polar metabolites with significantly different abundances (LRT FDR<0.05) between constipation and healthy controls (Constipation-HC), and diarrhoea and healthy controls (Diarrhoea-HC).

LRT coefficients	Metabolite	LogFC
Constipation-HC	5-Aminonaphthalene-2-sulfonic acid	0.97
Constipation-HC	Alanylphenylalanine	-1.06
Constipation-HC	Cholic acid	-0.92
Constipation-HC	Creatinine	0.98
Constipation-HC	Gabapentin	-1.01
Constipation-HC	Glycocholic acid	-0.90
Constipation-HC	Homovanillic acid	-1.01
Constipation-HC	Metformin	0.93
Constipation-HC	Metoprolol	-1.02
Constipation-HC	N-acetyl mesalazine	2.17
Constipation-HC	N-acetyl-L-tyrosine	1.29
Constipation-HC	N-omega-acetylhistamine	-1.04
Constipation-HC	Paracetamol	1.59
Constipation-HC	Taurocholic acid	-1.12
Diarrhoea-HC	2-Isopropylmalic acid	0.87
Diarrhoea-HC	2-Piperidone	1.30
Diarrhoea-HC	3 4 5-triOME benzoic acid	1.31
Diarrhoea-HC	3-(4-Hydroxyphenyl)propionic acid	1.05
Diarrhoea-HC	Alanylphenylalanine	-0.81
Diarrhoea-HC	Cholic acid	0.95
Diarrhoea-HC	Coumaroylquinic acid	-0.98
Diarrhoea-HC	Creatinine	1.15
Diarrhoea-HC	Gabapentin	-2.97
Diarrhoea-HC	Homovanillic acid	2.14
Diarrhoea-HC	Metformin	1.57
Diarrhoea-HC	Metoprolol	1.55
Diarrhoea-HC	Paracetamol	2.19
Diarrhoea-HC	Phenylacetaldehyde	1.30
Diarrhoea-HC	Quinoic acid	1.23
Diarrhoea-HC	Succinic acid	0.79
Diarrhoea-HC	Taurine	0.96
Diarrhoea-HC	Tauro-deoxycholic acid	1.63
Diarrhoea-HC	Taurocholic acid	0.92
Diarrhoea-HC	Tyramine	1.02

A negative log fold change (logFC) value indicates higher abundance in the healthy control compared to constipation (FC + IBS-C) or diarrhoea (FD + IBS-D) groups. Conversely, a positive logFC value indicates higher abundance in constipation or diarrhoea groups compared to the control group. Abbreviations; HC – healthy control, FC – fold change.

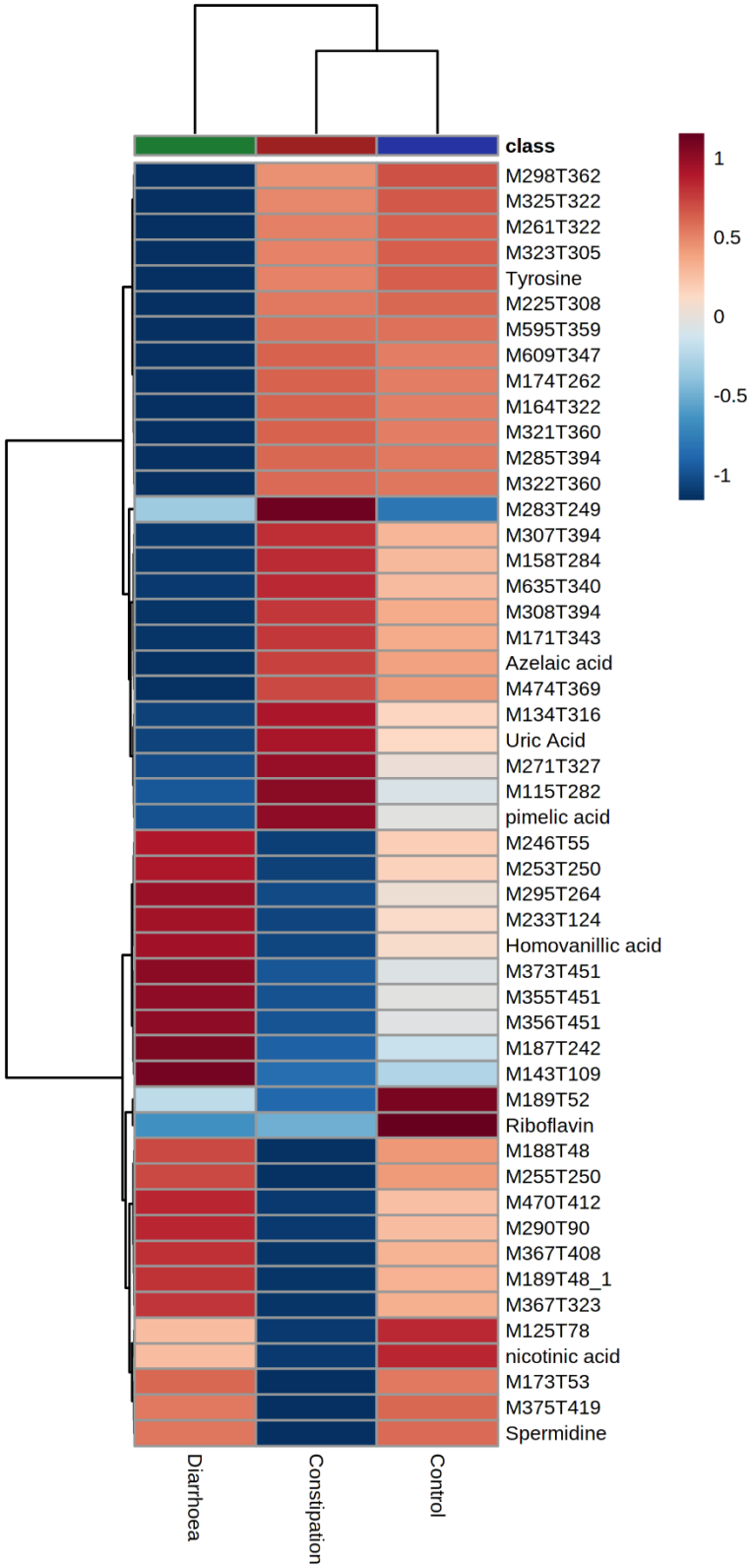


Figure 5-10: Hierarchical clustering analysis of the group means for the top 50 ANOVA semi-polar (annotated and unannotated) metabolites between healthy control, constipation (FC + IBS-C), and diarrhoea (FD + IBS-D) groups. Data presented as z-score. The colour ribbon beneath the upper dendrogram identifies group; healthy control – blue, constipation group – red, diarrhoea group – green.

5.3.4. All metabolites combined

Polar and semi-polar metabolite datasets were combined to analyse possible links between faecal metabolites reflective of variations in metabolic pathways between the groups. Metscape (Cytoscape) was used to visualise pathway correlations with a correlation cut off greater than 0.5 or less than -0.5.

There was no significant difference between constipation and healthy controls or diarrhoea and healthy controls in correlation between annotated metabolites (data not shown). As there was no difference in correlations, the polar and semi-polar datasets for all participants were combined to produce a pathway map using Metscape highlighting how key metabolic hubs link together regardless of gut health status (**Figure 5-11**).

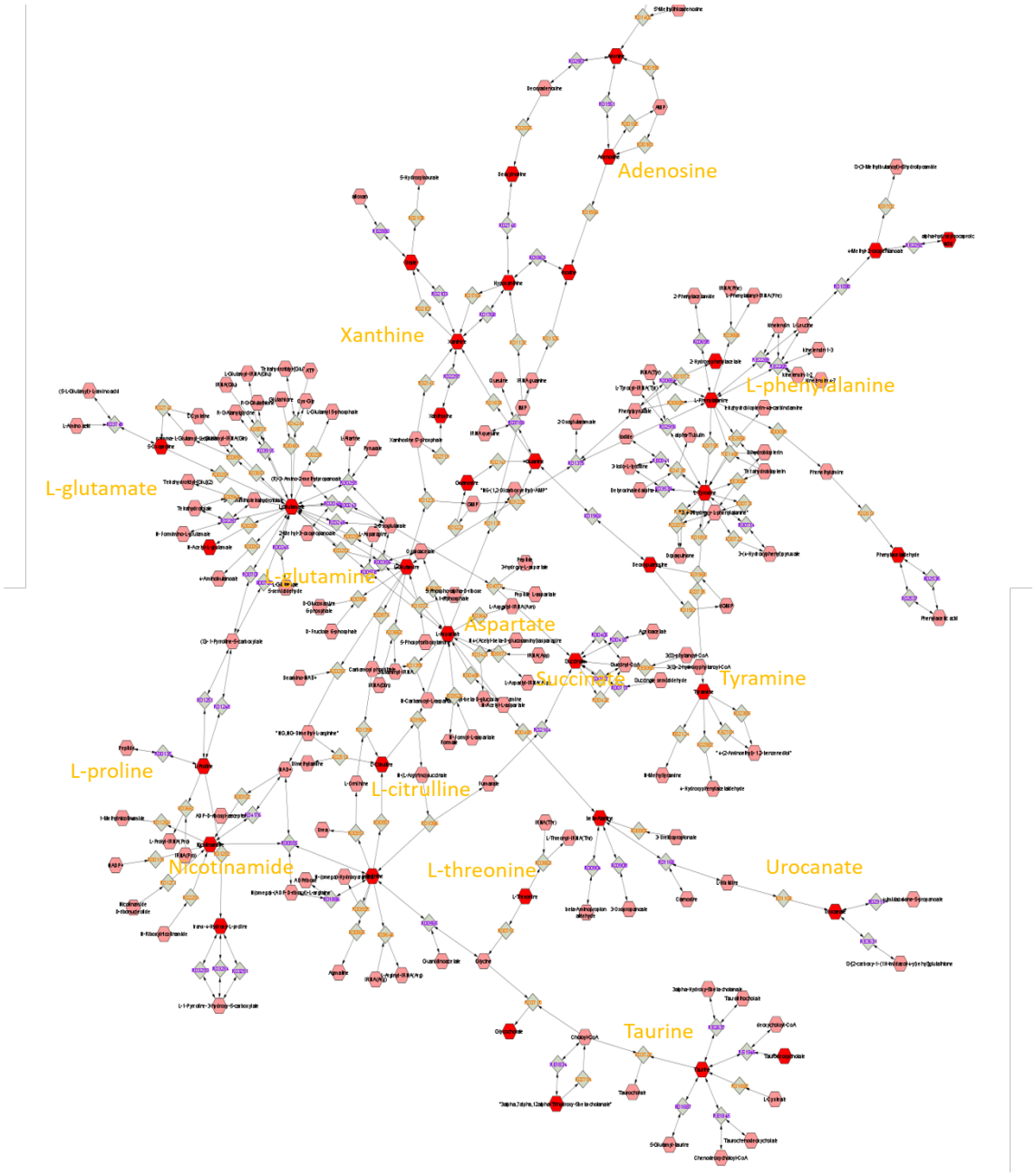


Figure 5-11: Pathway mapping of key metabolic nodes detected as part of the polar and semi-polar metabolic analysis of constipation (FC + IBS-C), diarrhoea (FD + IBS-D), and healthy control groups combined. Bright red hexagons are detected metabolites in the analysis. Pink hexagons are metabolites that are linked to these inputs, highlighting the reaction chain. Arrows depict the direction of the reaction. Diamonds are reactions and associated enzymes. Key metabolic nodes important to basic gut processes and health are highlighted in yellow.

5.4. Discussion

This chapter highlights the benefits of using an untargeted approach to investigate metabolic features important to understanding perturbations in the gut of individuals with FGDs compared to healthy individuals. The characterisation of the faecal metabolome showed differences between individuals with constipation (FC + IBS-C) or diarrhoea (FD + IBS-D) from healthy controls reflective of processes localised to the gut. Lipid metabolites were primarily important to group differentiation, albeit some differences in semi-polar and polar metabolites were noted.

Certain annotated metabolites that contributed to group differentiation have been associated with gut ailments in a previous study [276]. Discriminatory analysis showed partial differentiation of constipation or diarrhoea groups from healthy controls. The dissimilarity in the three analyses conducted (VIP metabolites, LRT analysis and logFC) highlights the variability and complexity that may reflect biochemical pathways and regulatory metabolites distinguishing constipation or diarrhoea from healthy controls.

Analysis of the faecal lipidome showed key classes were differentially abundant, particularly ceramides, triglycerides, and diglycerides, between constipation or diarrhoea groups. Jeffery *et al.,* have previously shown that faecal glycerophospholipids and oligopeptides were important for the differentiation of IBS and healthy participants [77]. Increased concentrations of ceramides, glycosphingolipids, diglycerides, and triglycerides have also been reported in mucosal biopsies and plasma samples of individuals with IBS compared to healthy controls [277, 278].

In the current study, triglycerides were more abundant in the participants with diarrhoea. This finding agrees with existing literature, where elevated faecal triglycerides have been linked to bile acid malabsorption and diarrhoea conditions via the FXR receptor [279].

The FXR receptor is crucial to the production of primary bile acids and regulates lipid and glucose homeostasis via a range of mechanisms which include increasing triglyceride hydrolysis [280, 281]. FXR-deficient mice showed hypertriglyceridemia and impaired bile acid homeostasis [281, 282], and individuals with hypertriglyceridemia have shown disruptions to ileal bile acid reabsorption [281, 283]. Thus, possible disruptions to the FXR receptor or bile acid hepatic circulation commonly associated with diarrhoea predominant conditions could be linked to increased triglyceride concentrations in faecal samples. Concomitant to the increased abundance of triglycerides reported here, increased faecal bile acids were observed in participants with diarrhoea compared to participants with constipation or healthy controls in **Chapter 3**. These findings support an association between bile acid and triglyceride metabolism in FGDs.

Ceramides with longer fatty-acid sidechains (carbon chain lengths ~C18-C24) were less abundant in the constipation group, while ceramides with shorter fatty-acid sidechains (carbon chain lengths ~C15-C18) were more abundant. Opposite changes were found in the diarrhoea group. Lipid correlation analysis using BioPAN highlighted increased sphingolipid metabolism in the constipation group compared to the healthy control group. Ceramides are waxy lipids that have been associated with pain sensitivity, cell toxicity, inflammation, and various diseases such as metabolic disorders, Alzheimer's disease, insulin resistance, and IBD [277, 278]. Kajander *et al*., showed that the abundance of lipids in the ceramide and sphingomyelin pathways were increased in IBS participants (all subtypes combined) compared to healthy controls [277]. In individuals with myalgic encephalomyelitis or chronic fatigue syndrome (often associated with IBS), changes in the gut microbiome were associated with increased lipopolysaccharide concentrations [277]. This increase may trigger sphingomyelinases that, when hydrolysed, form ceramides [278, 284] that contribute to oxidative stress and gut barrier dysfunction [277].

Furthermore, the lipotoxicity of ceramides could degrade other key lipid structures that culminate to influence IBS symptoms [277]. Additionally, there is evidence to suggest bacterial pathogens can manipulate the structural and signalling properties of ceramides to promote pathogenic bacterial colonisation [285], highlighting a further possible link to FGDs.

However, not all ceramides or all concentrations of ceramides may be toxic. Sphingolipids, the wider group encompassing ceramides, are important in cell membrane structure and signalling [286]. For example, ceramides are suggested to induce cell apoptosis in response to stressors such as radiation or chemotherapy, acting as ligands that bind to and regulate enzyme activity and many other intracellular functions [286]. Therefore, only during other stressors, such as those commonly associated with FGDs, may ceramides compound to increase lipotoxicity.

Odd sidechain ceramides were present at higher abundance in the constipation group compared to even sidechain ceramides in the diarrhoea group. Published studies have shown odd sidechain fatty acids are not a human metabolic product and are instead obtained from the diet or through microbial modification, with potential use as a biomarker of dietary fibre [287]. The differences in sidechains found here, together with evidence supporting the importance of ceramides in other diseases [288, 289] highlight a need for further investigation. The lack of literature regarding the role of lipids in FGDs and their importance to gut tissue integrity and metabolism suggests the importance of lipids may be overlooked and further quantitative analysis warranted.

Significant differences in faecal polar and semi-polar metabolite abundances were detected between constipation or diarrhoea groups compared to healthy controls. Discriminant analysis of polar and semi-polar metabolites showed that constipation and

healthy control groups had visually greater separation than the diarrhoea group. Although hierarchical clustering analysis and comparison of fold changes showed distinct differences in polar and semi-polar metabolites between groups, correlation analysis did not. Differentially abundant metabolites in faecal samples included, but were not limited to bile acids, homovanillic acid, riboflavin, and nicotinic acid.

Four related bile acids, cholic acid, glycocholic acid, tauro-deoxycholic acid, and taurocholic acid, were more abundant in relative concentration in the diarrhoea group compared to the healthy control and constipation groups. These results were similar to those reported in **Chapter 3,** where bile acids and their metabolites were quantified.

Homovanillic acid, metabolised from dopamine and linked to neurological disorders, including epilepsy, Parkinson's disease, and major depression [290] was higher in healthy controls compared to the constipation group. The role of homovanillic acid is linked to dopamine; however, it has also been found in beer and olives and could therefore be a dietary intake by-product.

Hierarchical clustering showed that the relative abundance of riboflavin (vitamin B2) was higher in healthy controls compared to participants with constipation or diarrhoea. Studies have shown that riboflavin supplementation has anti-inflammatory, antioxidant and microbiome-modulatory effects and, in a Crohn's disease cohort, reduced circulating serum inflammatory metabolites [291]. There are two possible mechanisms of action; either riboflavin directly alleviates inflammation via redox mediating functions or alters the gut microbiome [291]. A reduction in riboflavin could have flow-on effects to reduced flavin adenine dinucleotide and flavin mononucleotide, important to many redox reactions in the body [292].

Riboflavin can be produced in *vivo,* predominantly by lactic-acid bacteria [293], which may suggest that the gut microbiota perturbations associated with FGDs could impact the riboflavin concentrations in the gut lumen and consequently in faeces. Analysis of riboflavin supplementation in healthy adults was shown to increase the faecal relative abundance of *Faecalibacterium prausnitzii,* a beneficial microbe [294]. This increase was additional to the increased *Roseburia* species and decreased *E. coli* relative abundances*,* indicating the potential use of riboflavin as a beneficial gut microbiome modulator [294]. This result may suggest a decreased relative abundance of riboflavin in faecal samples of constipation or diarrhoea groups could be linked to perturbed microbial and host metabolism, and thus contribute to or reflect FGD symptomology.

Similarly, nicotinic acid (also known as niacin, vitamin B3) was important in hierarchical separation, found at lower abundance in constipation or diarrhoea groups compared to the healthy control group. The GPR109A cell receptor is activated by niacin and butyrate, promoting regulatory T-cell differentiation and anti-inflammatory cytokines, for example, IL-10 and interleukin-18 [295, 296]. Faecal butyrate concentration is frequently shown to be lower in FGDs, linked to a reduction in the relative abundance of butyrate-producing microbes [75]; as also observed in the participants of the COMFORT cohort ([297] a collaborator of this project) however, there is less evidence for the possible role of niacin. A potential reduction in either butyrate, niacin, or both due to dietary intake or microbial production could be linked to inflammation in the gut via a downregulated production of anti-inflammatory cytokines.

Certain compounds in the faecal samples that were significant originated from consumption of exogenous drugs in a few people rather than biochemical differences linked to FGDs. Gabapentin, used to treat nerve pain, migraine, and headaches were detected in both semi-polar and polar streams in four individuals (control (three)

constipation (one)). Mesalazine (detected as N-acetyl mesalazine), often administered to individuals with IBD, was detected in one participant belonging to the constipation phenotype. Metformin, a treatment for type-2 diabetes, was present in six individuals detected in both semi-polar and polar streams. Of the six individuals who had detectable metformin levels, only one individual was a healthy control. The consumption of metformin was recorded by individuals on patient questionnaires. Gabapentin and mezalazine were not documented possibly suggesting a failure to accurately record medication. However, it may be that the detected metabolite is present in other medications and therefore further investigation is required.

Additionally, paracetamol was detected in the polar stream, present at a higher relative concentration in the diarrhoea and constipation groups than the healthy control group. The detection of these metabolites in only a few individuals validates the use of an untargeted approach as a functional readout of metabolites derived from consumption and endogenous metabolism. However, it can be considered a misleading result that the concentrations of these metabolites were different between the groups when this was only observed in a few individuals.

Dissimilar to other studies, the faecal metabolome of the constipation group was compared separately to the diarrhoea group. Although FGDs are an encompassing disorder, it is postulated that the mechanisms of each FGD subtype would differ based on the phenotypic aetiologies. The dissimilarity in VIP metabolites important to separating the PLS-DA plots, LRT analysis, and fold changes highlights the variability and complexity between phenotypic symptoms that may reflect different biochemical processes between these groups. Similar to microbiome-based studies, this heterogeneity makes comparisons and inferences between findings difficult due to the lack of standardisation in analytical methods and statistical approaches [266].

A metabolomics approach utilising three analytical streams is a strength of the research presented. Here, it provided comprehensive coverage of polar, semi-polar, and lipid metabolites and insights into changes in biochemical processes in the gut of individuals with FGDs. However, there are some limitations as the results are limited to metabolites that can be accurately annotated using libraries and databases based on mass and retention time. Annotation was only possible for a small number of the polar and semi-polar metabolites from the hierarchical clustering analysis, VIP lists, and FDR significant metabolites. Only 11% of polar and semi-polar metabolites could be annotated, compared to 20% of lipids. Although some detected features were isotopes and not representative of unique metabolites, the low annotation percentages highlight the complexity of the faecal metabolome and the origins of the metabolites (dietary, host, and/or microbial sources), making annotation using primarily human databases difficult. The importance of the lipidome in this study, where annotations were more achievable, suggests that there are likely important unknown compounds in the polar and semi-polar metabolomes, which are currently unidentifiable and would be important to understand FGDs. Future advances in databases that include microbial metabolites will exponentially increase the understanding of host-microbial interactions in FGDs. Another limitation is that the faecal metabolome of participants with IBS-C and FC or IBS-D and FD were combined into constipation or diarrhoea groups. This grouping removes the possibility of understanding biochemical differences between the functional and IBS subtypes.

5.5. Conclusion

In conclusion, the faecal metabolome offers insights into metabolites and pathways that could be important in FGDs. Polar and semi-polar analyses found specific metabolites (e.g., bile acids and B-vitamins) in faeces with higher or lower relative abundance between constipation or diarrhoea groups. The most significant variation was observed in

the faecal lipidome, where ceramides and other lipids differed between groups and could indicate perturbed metabolic processes in the gut of the participants with constipation or diarrhoea. Further mechanistic insights of FGDs could be obtained by integrating the faecal metabolome with the faecal microbiome and plasma metabolome of individuals with constipation symptoms (FC + IBS-C) or diarrhoea symptoms (FD + IBS-D), each compared to healthy controls.

Chapter Six
Towards a systems biology view of functional gut disorders

Abstract

Systems biology aims to investigate the relationship between components of a biological system. Here, a systems biology approach was postulated to provide new insights of the complex interactions between dietary intake, host and microbial metabolites, and the gut microbiome in FGDs using information generated in the wider COMFORT cohort study. Data integration, statistical analyses, correlation analyses, and pathway network analyses were performed on 149 individuals (n = 62 healthy controls, n = 40 constipation (FC + IBS-C), n = 47 diarrhoea (FD + IBS-D)). Discriminant analysis using both the faecal and plasma metabolomes enabled the separation of patients with constipation (FC + IBS-C) or diarrhoea (FD + IBS-D), each from healthy controls. Correlation analysis showed positive and negative correlations (above 0.5 or below -0.5) between faecal lipids and plasma lipids for all participants, regardless of grouping. Combined analysis of the faecal metabolome and faecal microbiome showed a separation of the constipation group from the healthy control group, and the diarrhoea group from the healthy control group. Positive correlations were evident between the relative abundance of *Firmicutes* and faecal lipids, whilst that of *Bacteroidetes* was primarily negatively correlated to faecal lipids. Procrustes analysis showed significant concordance between data types (e.g., faecal and plasma metabolomes, faecal metabolome, and faecal microbiome) for the same participant. However, there were no differences in the level of agreement between constipation, diarrhoea, or healthy control groups. The utilisation of a systems biology approach showed important correlations between multiple different data types. However, the precise metabolic or microbial perturbations between individuals with constipation and diarrhoea that may reflect FGD symptoms remain to be elucidated and will require a more advanced systems biology analysis.

Chapter Six

6.1. Introduction

The multi-factorial nature of IBS is postulated to involve nervous, immune, microbial, digestive, and environmental factors, all of which interact continuously as a complex system [298]. What can be surmised from the past decade of research is that not one singular component alone can be attributed as the sole cause behind IBS pathophysiology.

Systems biology is increasingly being utilised to understand the biological network in disease conditions [299-305]. Though originally proposed over 20 years ago [299], utilising systems biology to understand syndromes or disease has only recently been adopted [305-307]. This approach focuses on shifting away from the reductionist approach and instead towards understanding the whole system to consider a comprehensive view of disease phenotypes [299-301, 308]. This approach is necessary when symptoms of the same syndrome can differ between people and even within the same person over a day (e.g., IBS-M). With the increasing accessibility and feasibility of high throughput omics, combining these technologies has been proposed as the best approach for a better understanding of the heterogeneous and multi-factorial pathology behind FGDs [298].

In 2015, Mayer *et al.,* published a comprehensive review outlining how brain-gut interactions may have interacted to cause the common comorbidities of IBS [298]. It is accepted that the relationship between diet, the microbiome, and host are constantly adapting based on both extrinsic and intrinsic constraints [55, 309]. Composition alterations to gut integrity and associated genetic functions of the microbiota have been linked to IBS [46, 69, 72, 81]. Additionally, there is evidence to show alterations to the gut microbiome further influence visceral sensitivity, epithelial permeability, immune system activities, and the GBA [4]. For example, an overabundance of certain bacterial

species attached to the mucosal layer can cause low-grade inflammation, with metabolites produced either as a cause or consequence of this interaction [4].

Dietary intake adds another level of complexity and can impact host-microbe interactions and the production of subsequent metabolites, contributing to or reflecting IBS pathophysiology [310]. In healthy individuals, the gut microbiome remains relatively stable over time, with only dramatic dietary changes causing a noticeable shift in composition. However, in the context of IBS, a low FODMAP diet, probiotic consumption, a gluten-free diet, and fibre supplementation are some of the common interventions employed to reduce symptoms [310] that may consequently modify subspecies level or genetic biodiversity and result in functional variation [310] impacting host physiology.

Recent progress has been made combining multiple data types such as dietary intake, microbiome, and metabolome datasets [77, 276, 310, 311], or brain pathways and faecal metabolites [307] to build networks. Consistent between these studies is the importance of the faecal microbiome and metabolites showing distinctive differences between IBS and healthy control individuals [77, 276, 310, 312]. For example, one study showed that 155 metabolites and 54 microbial species accounted for the differentiation between these groups [276]. The metabolites were the main driver of the separation, but the inclusion of microbial abundance was necessary to understand separation [276]. Twenty-two pathways of functional importance were predicted to differ between IBS and healthy controls that included increased amino acid metabolism, epithelial cell death and epithelial cell apoptosis [276]. In another study, Tap *et al.,* showed the importance of dietary intake as an influence on microbial subspecies abundance, hydrogen gas production, and symptom severity in IBS [310]. These studies highlighted the potential

for understanding the interactions in a multi-factorial disease like FGDs that has not been achieved with the reductionist approach utilised in most studies.

Here, a systems biology approach was postulated to provide new insights into the complex interactions between dietary intake, host and microbial metabolites, and the gut microbiome in FGDs using information generated in the COMFORT cohort study. Thus, the aim of this chapter was to integrate dietary intake, the faecal metabolome, faecal microbiome, and plasma metabolome from healthy controls compared to individuals with constipation (FC + IBS-C) or diarrhoea (FD + IBS-D) symptoms (**Figure 6-1)**. Comparisons were not made between datasets that were not collected as part of this thesis. A secondary aim was to compare within an individual the similarity or dissimilarity across datasets. Together the analyses of these datasets could provide better insights into the potential mechanisms behind FGDs.

6.2. Methods

Data from 149 participants was analysed. Faecal DNA was extracted for shotgun metagenomic sequencing, and the microbial taxonomy data (**Appendix Table 2**) was analysed by fellow PhD Candidate Caterina Carco (Carco 2021, unpublished). One of my PhD supervisors, Dr Karl Fraser, analysed the plasma metabolome (Personal comment, **Appendix Table 3**). The plasma metabolome was measured using the same instrumentation and data processing techniques as outlined in **Chapter 5** for the faecal metabolome. Dietary intake was collected as described in **Chapter 2** by Dr Phoebe Heenan, a former PhD candidate who studied the association of diet and acute gut symptoms in IBS participants of the COMFORT cohort (**Appendix Table 4**) [297]. These data sources were solely used to integrate using a systems biology approach in this chapter.

6.2.1. Data processing and statistical analysis

Data integration, statistical analyses, correlation analyses, and pathway networking were performed on 149 individuals (n = 62 healthy controls, n = 40 constipation (FC + IBS-C), n = 47 diarrhoea (FD + IBS-D)). The general analytical workflow is summarised in **Figure 6-1**. PLS-DA models were completed using SIMCA (16.0.1) using data from the faecal and plasma metabolomes, and faecal microbiome. The quality of the PLS-DA models generated was measured using R2X, R2Y, and Q2, with values closest to one signifying the goodness of fit [272]. CV-ANOVA was used to test the significance of the PLS-DA models [272].

Microbial abundance or metabolite features were selected based on the VIP of the PLS-DA models. These features were then used to further refine the PLS-DA models to

understand the variability and complexity of the data and mitigate false discovery of an overfitting model [272].

Metaboanalyst (version 5.0) [238] was used to generate volcano scatter plots to highlight those metabolites with fold changes superior to 2, which were also significantly different ($p < 0.05$ raw). R studio was utilised for Procrustes rotation analysis (Vegan package) to understand the agreement between all paired data points for the same participant.

The ComplexHeatmap package for R was used to visualise correlations between all paired data generated by the MixOmics package for R. Positive and negative correlations above 0.5 or below -0.5 were visualised. Correlation outputs generated as part of the complex heatmap analysis were converted into visual network pathway correlations in Cytoscape. $p < 0.05$ was deemed statistically significant for all analyses.

As discussed in **Chapter 2**, statistical analyses were only made between the constipation and healthy control group or diarrhoea and healthy control group to identify the variables that differ between individuals with altered bowel movement (constipation or diarrhoea) and healthy individuals. However, to provide an overview of how all the groups compare, methods such as partial least square's correlation analysis correlation analyses were utilised.

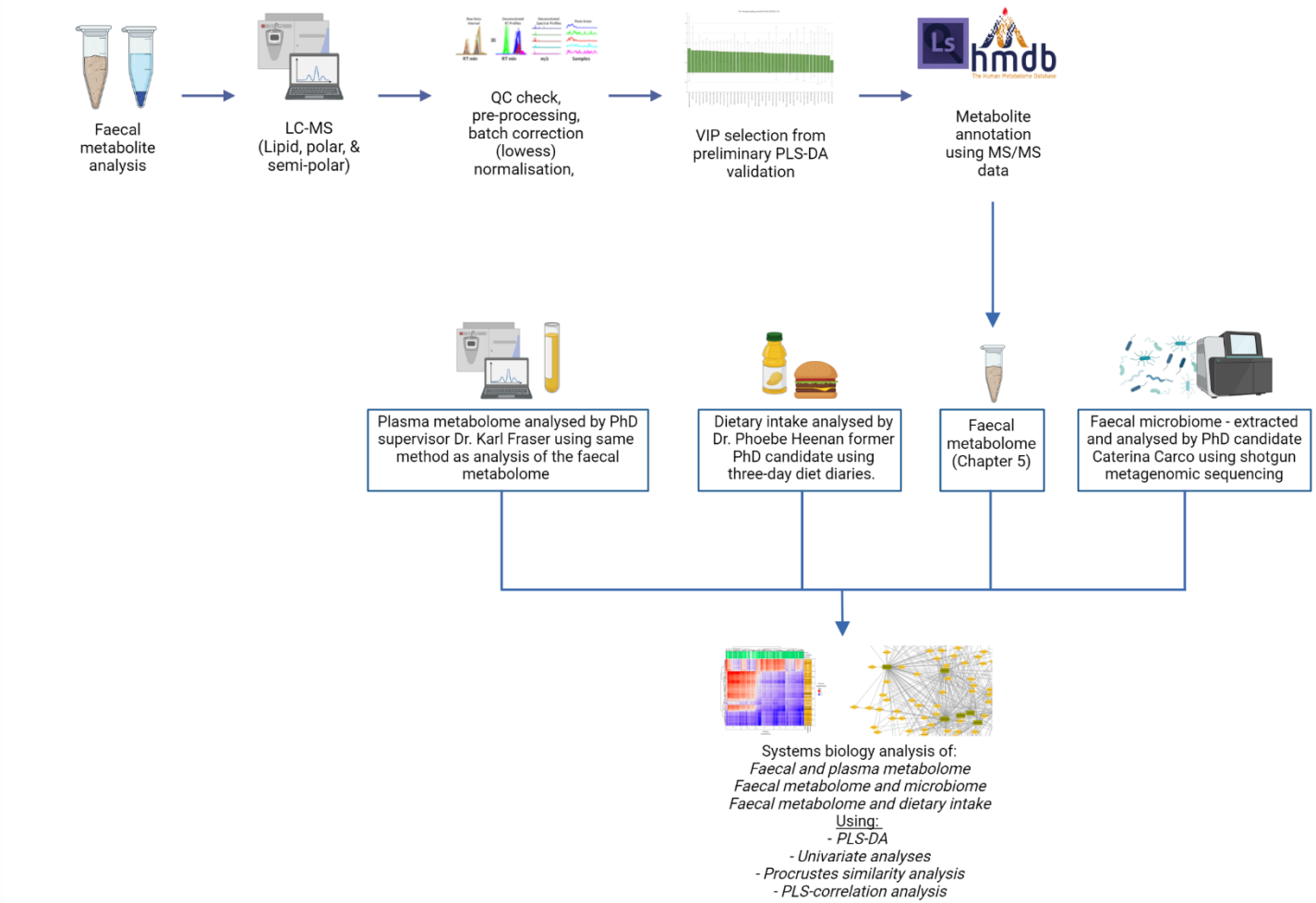


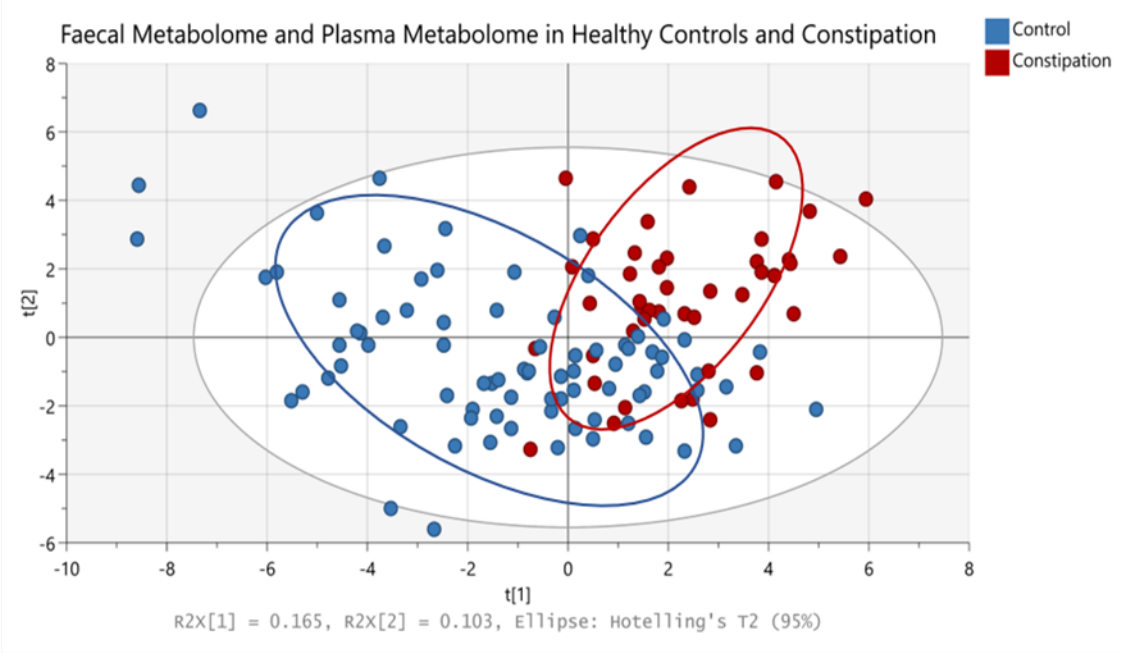
Figure 6-1: General metabolomic analytical and data processing workflow including the final step using a systems biology approach. Figure created using BioRender.

6.3. Results

6.3.1. Faecal and plasma metabolomes

Datasets of the faecal and plasma metabolomes were merged to investigate the differences that may be evident between the constipation group or diarrhoea group, each compared to the healthy control group.

Five hundred and eighty-four metabolites from the faecal metabolome and 648 metabolites from the plasma metabolome were analysed. PLS-DA based on the top 10% of VIP score metabolites showed that the combined plasma and faecal metabolomes of individuals with constipation were distinct from healthy controls, and diarrhoea from healthy controls, were also distinct although with an overlap of the 95% confidence intervals (**Figure 6-2**). Both models were significant in separating the constipation group or diarrhoea group from the healthy control group (constipation and healthy control comparison: CV-ANOVA $p = 6.71e^{-5}$, R2X = 0.267, R2Y = 0.447, Q2 = 0.282; diarrhoea and healthy control comparison: CV-ANOVA $p = 1.21e^{-12}$, R2X = 0.286, R2Y = 0.508, Q2 = 0.382).



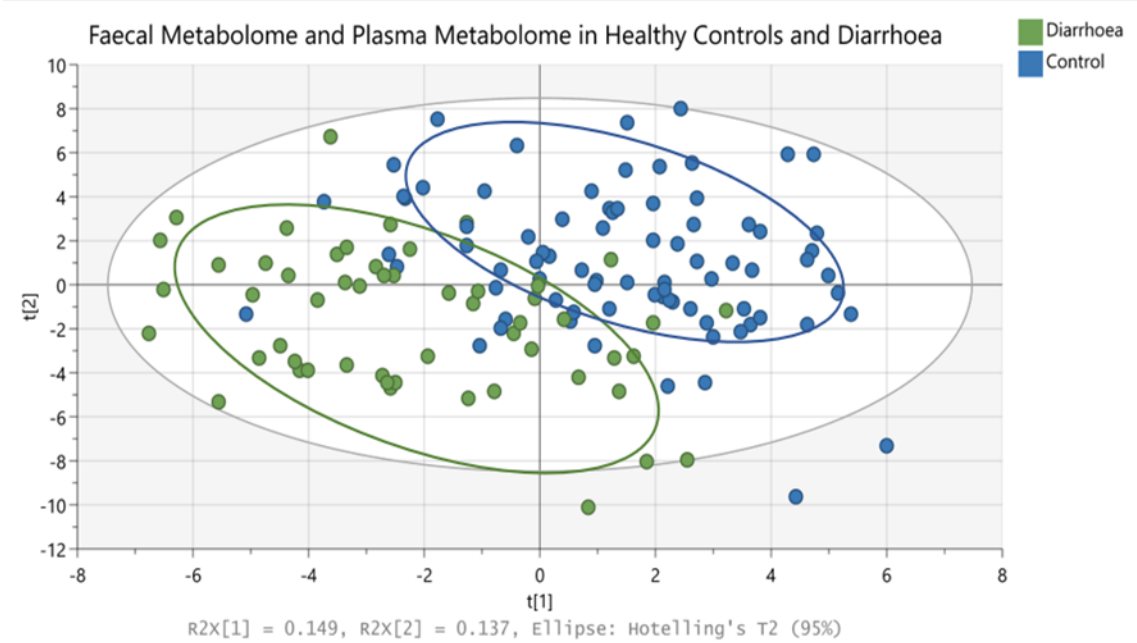


Figure 6-2: PLS-DA of combined metabolites of the faecal and plasma metabolomes between healthy control and constipation (FC + IBS-C) groups (CV-ANOVA $p = 6.71e^{-5}$, R2X = 0.267, R2Y = 0.447, Q2 = 0.282), and healthy control and diarrhoea (FD + IBS-D) groups (CV-ANOVA $p = 1.21e^{-12}$, R2X = 0.286, R2Y = 0.508, Q2 = 0.382).

Procrustes rotation analysis showed significant agreement between an individual's plasma and faecal metabolomes for all participants ($p = 0.013$), regardless of the groups they belonged to (**Figure 6-3**).

Univariate and fold change analyses of the constipation group compared to the healthy control group showed that nine metabolites were significantly different with a corresponding fold change above the threshold of 2.0 (**Figure 6-4**). Five metabolites were significantly decreased, and four significantly increased in the constipation group. The five metabolites with decreased abundance were all faecal metabolites: homovanillic acid, cholic acid, triglyceride, diglyceride, and 3,4,5-triOME benzoic acid. Of the increased metabolites, three were faecal lipids: triglycerides (2) and ceramide (1) and one plasma metabolite: salicylic acid.

Conversely, comparison of the diarrhoea and healthy control groups showed only one significant metabolite. Phenyl acetic acid from the plasma metabolome was increased in the diarrhoea group (**Figure 6-5**).

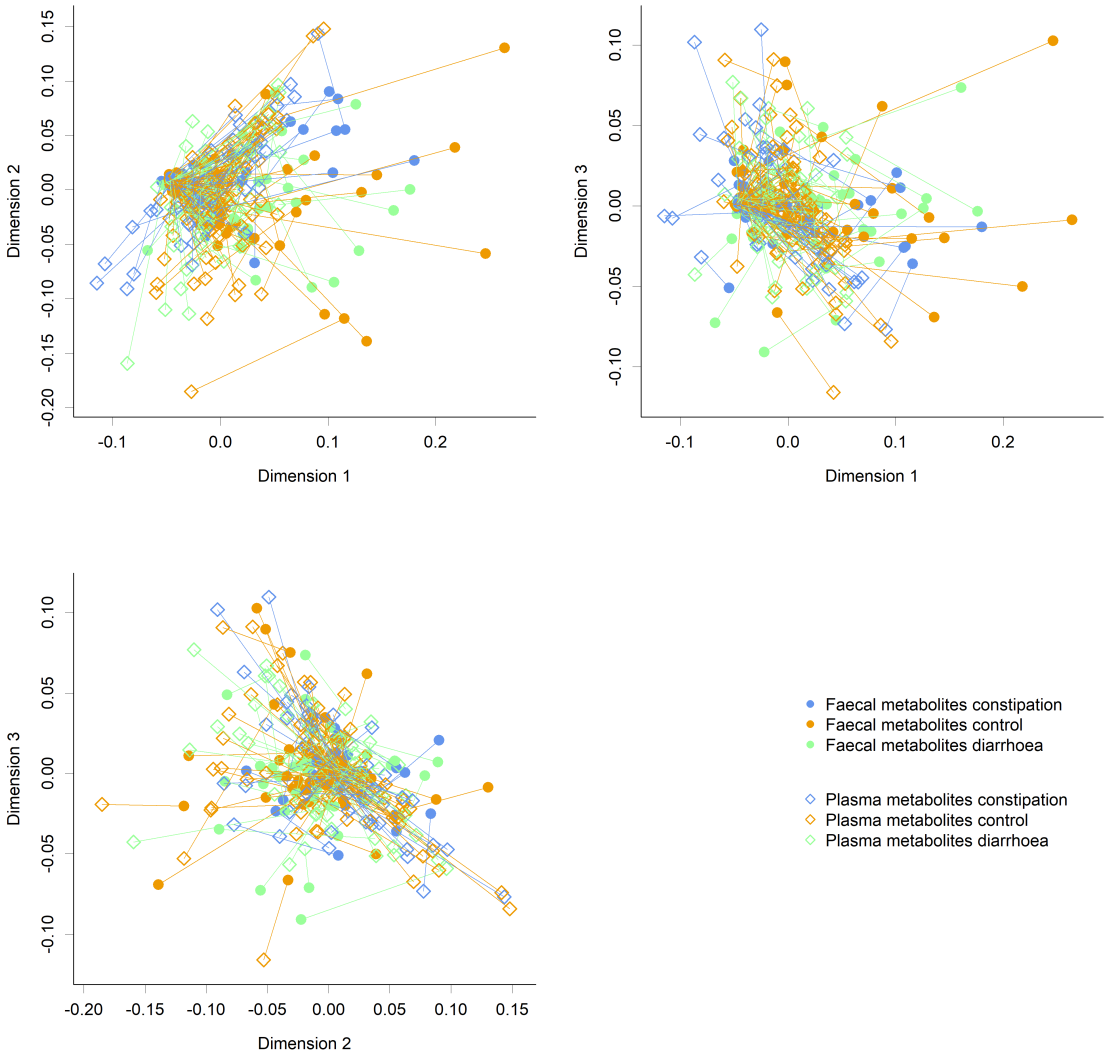


Figure 6-3: Procrustes rotation analysis of the faecal and plasma metabolomes of participants with constipation (FC + IBS-C), diarrhoea (FD + IBS-D), or healthy controls. Circles show ordination of faecal metabolites, and diamonds show ordination of plasma metabolites. Lines join the faecal and plasma metabolites for the same participant, with similarity (shorter) and dissimilarity (longer) shown by the length of the line. Constipation – blue, healthy controls – orange, diarrhoea – green. Significance shows the similarity of data points for the same participant $p = 0.013$.

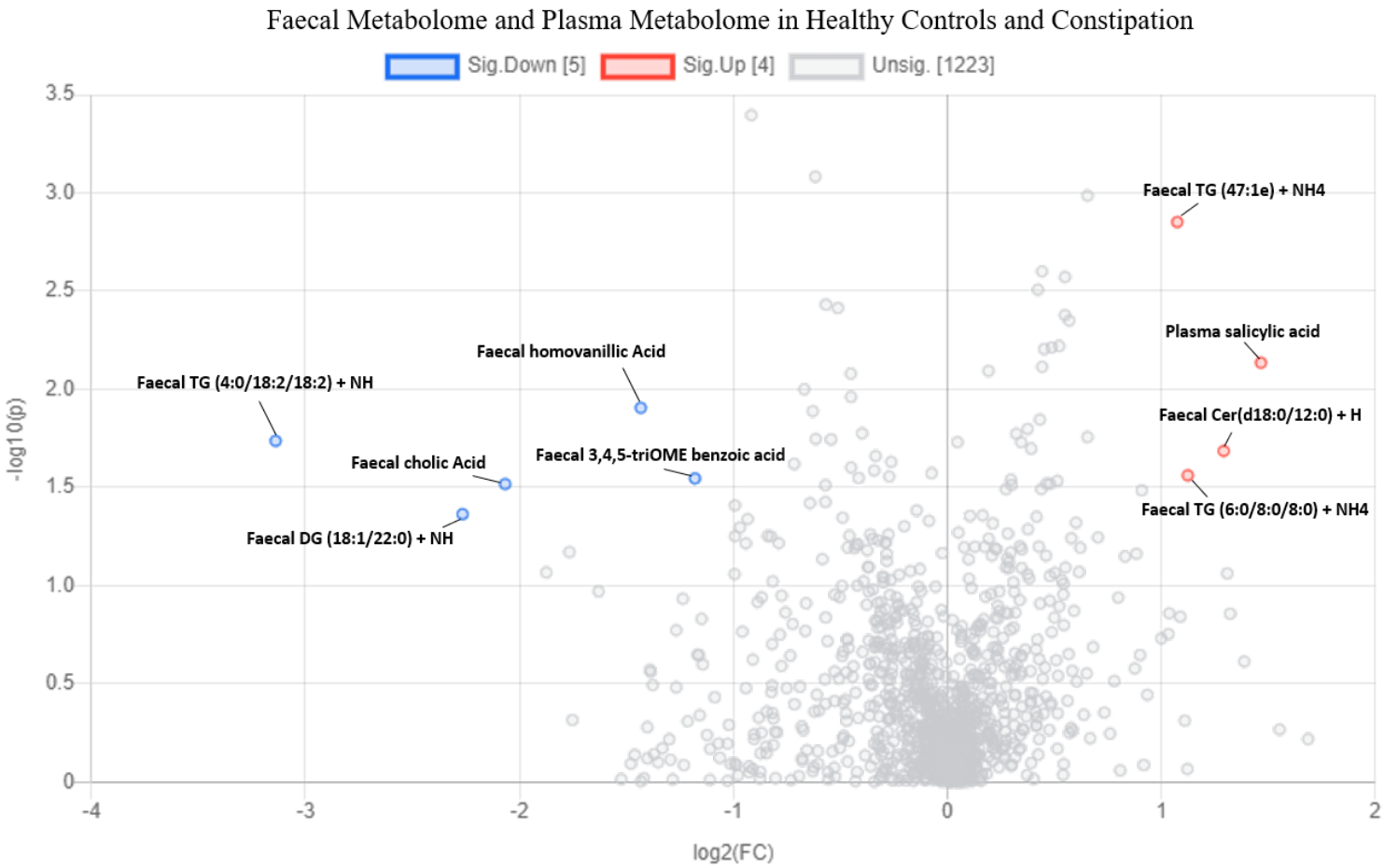


Figure 6-4: Volcano plot indicating fold change and statistical significance of metabolites from the faecal and plasma metabolomes in healthy control and constipation (FC + IBS-C) groups. Each dot represents a metabolite. Metabolites increased in healthy controls are coloured in red, and decreased variables are coloured in blue. Fold change is presented as log2FC. *P* values ($p < 0.05$) are presented as -log10. Dots in grey are non-significant metabolites.

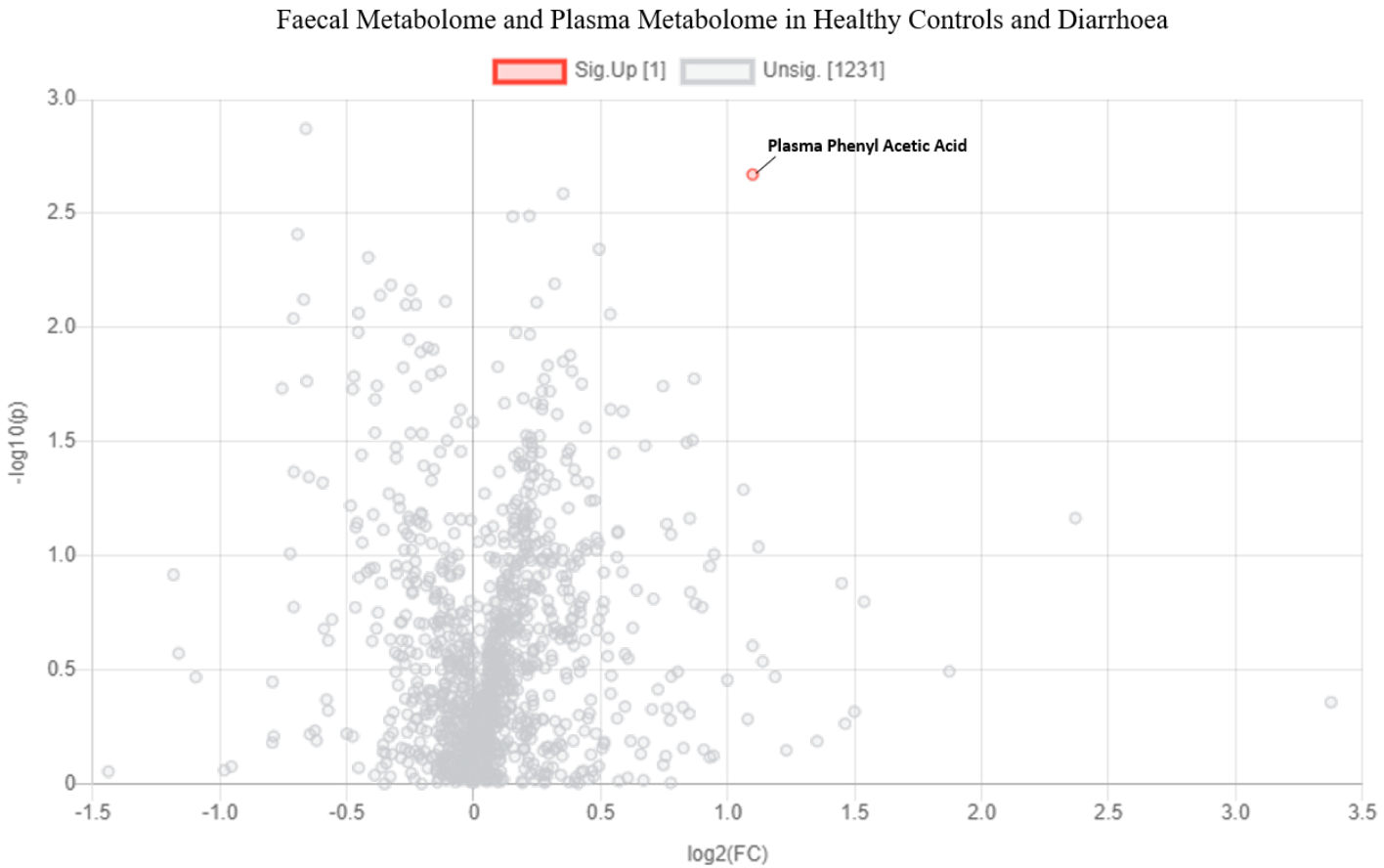


Figure 6-5: Volcano plot indicating fold change and statistical significance of the faecal and plasma metabolites in healthy control and diarrhoea (FD + IBS-D) groups. Each dot represents a metabolite. Metabolites increased in healthy controls are coloured in red, and decreased variables are coloured in blue. Fold change is presented as log2FC. *P* values ($p < 0.05$) are presented as -log10. Dots in grey are non-significant metabolites

Partial least square's correlation analysis between the faecal and plasma metabolomes for all participants regardless of health status revealed significant correlations in the lipidomes (**Figure 6-6, Appendix Figure 1** & **2**). The plasma metabolome, presented along the x-axis and the faecal metabolome presented along the y-axis of **Figure 6-6** were those metabolites corresponding to a correlation score cut-off above 0.5 or below -0.5. There were no correlations meeting these cut-offs for polar or semi-polar metabolites from either the faecal or plasma metabolome. Of the faecal lipidome with correlations above or below 0.5 and -0.5 respectively, triglycerides and diglycerides were predominant, accounting for 78 and 41 respectively of the 139 lipids that correlated between sample types. The remaining correlations in the faecal metabolome were between the lipids: lysophosphatidylethanolamine (n = 1), monoglyceride (n = 2), phosphatidylcholine (n = 6), phospahtidylethanol (n = 3), phosphatidylglycerol (n = 4), phosphatidylinositol (n = 1) and phosphatidylserine (n = 3).

Comparatively, the plasma metabolome had a greater diversity of correlated lipid species; ceramide (n = 8), cholesterol ester (n = 6), diglyceride (n = 18), phosphatidylcholine (n = 77), phosphatidylethanolamine (n = 15), phosphatidylinositol (n = 2), plasmenyl PE (n = 4), plasmenyl PC (n = 3), phosphatidylserine (n = 4), sphingomyelin (n = 40), sterol (n = 1), triglyceride (n = 67) and zymosteryl (n = 3). Positive correlations, as shown in red, were predominantly between faecal and plasma triglycerides and diglycerides. The area of strong negative correlations in the bottom right of **Figure 6-6** shows correlations predominantly between lipid groups phosphatidylinositol, phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin from the plasma metabolome, and triglycerides and diglycerides from the faecal metabolome. The upper and right colour bars in **Figure 6-6** show no difference in the mean relative abundance of metabolites, regardless of sample type, between constipation, diarrhoea, or healthy control groups.

Due to the number of correlations at 0.5 cut-off in **Figure 6-6**, a higher cut-off at 0.7 was selected to highlight the key relationships between the plasma and faecal metabolomes (**Figure 6-7**). Correlations meeting these cut-offs were due to faecal lipid species: diglycerides (*n* = 17), phosphatidylcholine (*n* = 2), phospahtidylethanol (*n* = 2), phosphatidylglycerol (*n* = 3), triglyceride (*n* = 26) and plasma lipids species belonging to cholesterol ester (*n* = 1), phosphatidylcholine (*n* = 25), phosphatidylethanolamine (*n* = 5), phosphatidylinositol (*n* = 1), plasmenyl PE (*n* = 3), plasmenyl PC (*n* = 2), sphingomyelin (*n* = 16), triglyceride (*n* = 2) groups. Strong positive correlations remained for the triglycerides and two phosphatidylethanolamine lipids between the plasma and faecal metabolomes. The remaining plasma lipids were negatively correlated to the faecal lipids. Again, the upper and right colour bars in **Figure 6-7** showed no difference in relative abundance of metabolites between the groups.

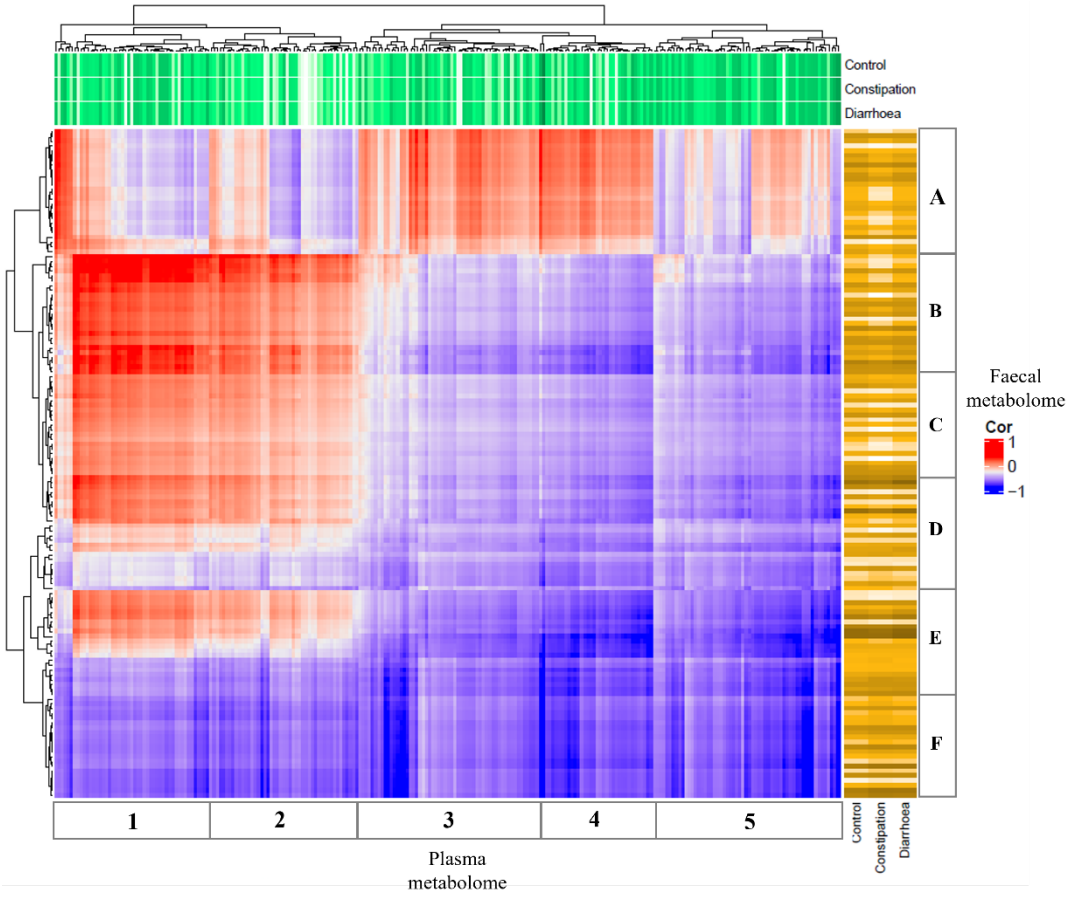


Figure 6-6: Canonical partial least squares correlation analysis shown as heatmap between the plasma metabolome (x-axis, 1-5) and faecal metabolome (y-axis, A-F) of participants with constipation (FC + IBS-C), diarrhoea (FD + IBS-D), or healthy controls. Labelled boxes along the axis correspond to the metabolite list shown in **Table 6-1** (plasma metabolome, x-axis) and **Table 6-2** (faecal metabolome, y-axis). Heatmap colour indicates canonical correlation scores. Only correlations above 0.5 or below -0.5 are shown. Positive correlations are coloured red and negative correlations are coloured blue. The upper (green) and right (yellow) colour bars indicate the mean relative abundance of metabolites according to constipation, diarrhoea, or healthy control group, with the darker shade indicating higher abundance.

Table 6-1: List of metabolites corresponding to the plasma metabolome (x-axis) as shown in complex heatmap **Figure 6-6**.

Plasma metabolome				
1	**2**	**3**	**4**	**5**
PS.39.1..H	TG.18.1.18.1.18.2..Na	PS.39.2..H	PC.34.2..H	PC.16.0.18.1..HCOO
PS.40.1...M.H..	PE.16.0.18.2..H	PS.38.2...M.H..	SM.d39.1..H	PC.16.0.16.1..HCOO
PC.36.4..H	TG.18.1.12.0.14.0..Na	PC.38.5..H	SM.d18.1.24.3..H	PC.34.4..H
PC.16.0.18.2..HCOO	TG.57.4...M.NH4..	ST.37.2...M.H..	Plasmenyl.PE.36.2...M.H..	PC.35.1..H
dMePE.16.0.18.2..H	TG.16.0.18.1.18.2..NH4	Cer.d17.1.24.0..H	SM.d40.1..H	PE.16.0.22.6..H
PE.18.1.18.2..H	TG.17.0.18.1.18.2..Na	PC.35.3..H	SM.d18.1.25.3..H	PC.40.5..H
TG.18.1.18.1.18.2..NH4	TG.60.2...M.NH4..	DG.18.0.16.0..Na	SM.d41.1..H.1	PC.40.5..H.1
TG.55.4...M.NH4..	TG.15.0.18.1.18.2..Na	Cer.d18.2.24.0..H	Plasmenyl.PE.34.2...M.H..	PC.38.3..H.1
TG.20.1.18.1.18.1..NH4	TG.16.0.18.2.18.3..Na	PE.34.2...M.H..	PC.34.0..H	PE.16.0.20.4..H
TG.59.4...M.NH4..	TG.16.0.14.0.18.3..Na	Cer.d18.1.22.0..H	SM.d40.4..H	ChE.20.3..NH4
PE.18.0.18.2..H	TG.16.0.12.0.18.3..NH4	PC.38.3..H	SM.d38.1..H	ZyE.20.4..NH4
DG.18.1.18.2..NH4	TG.57.5...M.NH4..	PC.18.0.20.3..Na	PC.32.0e..H	ZyE.18.2..NH4
DG.18.1.18.2..Na	TG.16.0.18.1.18.3..Na	PE.18.0.18.1..Na	PC.36.2p..H.1	PC.37.5..H
TG.60.4...M.NH4..	TG.16.0.12.0.18.1..Na	Cer.d18.1.24.0..H	PC.34.1e..H	PC.16.0.18.3..Na
TG.18.0.18.1.18.2..NH4	TG.18.0.18.1.18.1..Na	PC.38.2..H	PC.34.2p..H	SM.d36.0..H
PE.18.0.18.1..H	TG.16.1.12.0.18.1..Na	PI.18.0.20.4..NH4	SM.31.1...M..	PE.18.0p.20.4..H
TG.58.5...M.NH4..	PE.18.0.18.2..H	PC.35.2..H	SM.d34.0..H	PE.16.0p.20.4..H
TG.58.4...M.NH4..	TG.16.0e.18.1.18.1..NH4	PE.40.2...M.H..	PC.42.4p..H	ChE.16.0..NH4
TG.19.1.18.1.18.1..NH4	PE.36.1...M.H..	PC.33.2..H	SM.32.2...M..	PC.16.0.20.5..Na
TG.15.0.18.1.18.2..NH4	TG.16.0.18.1.18.2..Na	SM.d34.1..HCOO	SM.d33.1..H	PC.36.5..H.1
TG.16.0.14.0.18.2..NH4	DG.32.1...M.NH4..	CerG2.d18.1.16.0..H	Plasmenyl.PC.30.0...M.H..	PC.36.5..H
TG.59.2...M.NH4..	TG.59.5...M.NH4..	ZyE.18.1..NH4	PC.40.6e..H	PC.16.0.20.4..Na
DG.32.2...M.NH4..	TG.19.1.18.0.18.1..NH4	Plasmenyl.PC.38.4...M.H..	PE.16.0p.22.6..H	PC.34.4..H.1
TG.17.0.18.1.18.1..NH4	TG.58.2...M.NH4..	PC.38.6e..H	SM.32.0...M..	PC.33.0..H
DG.36.1...M.NH4..	DG.38.5...M.NH4..	SM.d44.5..H	SM.d42.5..H	PC.38.5..H.2
TG.57.3...M.NH4..	TG.56.1...M.NH4..	PC.39.6..H	SM.d38.2..H	ChE.18.1..NH4
TG.16.1.17.0.18.1..NH4	TG.56.2...M.NH4..	PI.38.6...M.H..	SM.d32.1..H	PC.38.4..H.1
TG.58.3...M.NH4..	TG.16.0.16.1.18.1..Na	PC.38.6e..H.1	SM.d34.4..H	ChE.16.1..NH4
TG.16.0.16.0.17.0..NH4	SM.45.2...M..	SM.d42.2..H	SM.d34.1..H	PC.40.4..H
TG.18.0.16.0.16.0..NH4	TG.16.0e.16.0.18.1..NH4	PC.38.7..H	SM.d18.1.18.3..H	PC.32.1..H
TG.16.0.17.0.18.1..NH4	TG.18.0e.16.0.18.1..NH4	PC.38.6p..H	PC.34.2e..H.1	PC.35.4..H
DG.18.1.18.1..NH4	TG.18.3.18.2.18.2..Na	CerG1.d42.1..H	SM.d18.2.18.3..H	PC.16.1p.20.2..Na

DG.42.2...M.NH4..	TG.62.5...M.NH4..	CerG1.d40.1..H	SM.d34.2..H	PC.18.2.20.4..Na
TG.18.0.16.0.18.1..NH4	DG.30.0...M.NH4..	SM.d42.1..H	PC.32.1e..H	PC.38.4e..H.1
TG.16.0.14.0.18.1..NH4	TG.62.6...M.NH4..	PC.40.7..H	SM.d42.3..H	PC.30.0..H
TG.16.1.16.1.18.1..NH4	TG.61.6...M.NH4..	PC.34.2e..H	PC.15.0.18.1..HCOO	PC.38.6..H
TG.15.0.16.0.18.2..NH4	TG.16.0.14.0.18.2..Na	SM.d18.1.26.3..H		PC.16.0.22.6..Na
TG.17.0.18.1.18.1..Na	TG.18.0.16.0.22.0..NH4	PC.40.6p..H		PC.38.6..H.1
TG.18.0.17.0.18.1..NH4	TG.18.1.18.1.20.4..Na	PC.36.2e..H		PC.16.0e.22.5..Na
DG.40.1...M.NH4..	DG.38.0...M.NH4..	ChE.22.6..NH4		PC.40.5e..H
TG.16.0.16.0.18.1..NH4	TG.16.1.16.1.18.1..Na	PC.37.6..H		Plasmenyl.PE.34.1...M.H..
DG.16.0.18.1..Na	TG.61.5...M.NH4..	SM.40.0...M..		SM.d38.4..H
DG.16.0.18.1..NH4	TG.18.0.16.0.18.1..Na	CerP.42.5...M.H..		PC.36.4e..H
TG.16.0.16.0.18.2..NH4	DG.34.3...M.NH4..	SM.d32.1..HCOO		PC.38.4p..H
TG.14.0.18.2.18.2..Na	TG.16.0.18.1.20.4..Na.1	Plasmenyl.PE.40.6...M.H..		PC.34.3..H
TG.16.1.17.0.18.1..Na	DG.40.0...M.NH4..	SM.30.1...M..		SM.d36.1..H
TG.58.1...M.NH4..	DG.16.0.16.0..NH4	SM.d35.1..H		PC.36.1..H
DG.18.1.18.1..Na		SM.d34.2..HCOO		PC.38.4..H
TG.16.0.18.1.18.1..NH4		SM.37.2...M..		PC.33.1..H
		SM.d40.2..H		PC.32.0..H
		PC.31.0..H		ChE.20.4..NH4
		PC.36.6..H		PC.38.4p..H.1
		PC.18.0.22.6..Na		SM.d36.2..H
		PC.40.6..H		PC.36.4p..H
		PC.32.2..H		Plasmenyl.PC.32.1...M.H..
		SM.39.2...M..		PC.38.5..H.1
		SM.37.1...M..		PC.36.4..H.1
		PC.36.2..H		PC.34.1..H

Column corresponds to the labelled box along the x-axis in **Figure 6-6**. Metabolites are listed in the order they appear along the axis. Abbreviations of lipids are outlined in **Table 5-1**.

Table 6-2: List of metabolites corresponding to the faecal metabolome (y-axis) as shown in complex heatmap **Figure 6-6**.

Faecal metabolome					
A	**B**	**C**	**D**	**E**	**F**
TG.8.0.10.0.10.0..Na	TG.18.3.18.2.21.6..Na	TG.16.0.18.1.24.0..NH4	TG.16.0.18.1.18.1..NH4	TG.18.1.18.2.23.0..NH4	TG.4.0.14.1.18.3..Na
TG.8.0.10.0.10.0..NH4	PG.46.0..H	TG.20.1.18.2.22.1..NH4	TG.18.1.18.1.18.2..NH4	TG.18.2.18.2.23.0..NH4	PG.28.0.18.3..H
TG.18.1.12.0.12.0..NH4	TG.57.10..Na	TG.18.1.18.2.22.1..NH4	TG.18.0.18.1.18.1..NH4	DG.18.2.18.2..Na	PEt.30.2p..Na
TG.16.0.12.0.18.1..Na	DG.18.1.18.1..Na	TG.18.0.18.0.18.1..Na	TG.16.1.16.1.18.1..Na	TG.14.0.18.2.18.2..NH4	PG.28.0.18.2..H
DG.16.0.12.0..Na	DG.18.3.18.2..H	DG.14.0.18.2..NH4	MG.34.1..Na	DG.18.2.18.2..H	TG.57.12..Na
TG.4.0.16.0.18.1..NH4	DG.18.1.18.3..H	TG.20.5.18.2.18.2..H	TG.20.1.18.1.18.2..Na	DG.18.2.18.2..NH4	TG.20.1.18.3.18.3..NH4
TG.10.0.12.0.18.1..NH4	MG.20.4..H	TG.24.3.18.2.18.2..H	TG.16.0.17.1.18.1..NH4	TG.18.2.18.2.18.2..H	TG.16.1.18.2.18.2..NH4
TG.10.0.12.0.12.0..NH4	DG.16.0.18.1..Na	TG.16.0.18.1.22.0..NH4	TG.18.1.18.1.18.1..NH4	TG.16.0.18.2.18.2..NH4	DG.18.3.18.3..Na
TG.18.1.12.0.14.0..NH4	PEt.19.1.18.1..H	TG.20.0.18.1.18.1..NH4	TG.18.0.18.1.18.1..Na	TG.18.1.18.2.18.2..NH4	TG.18.3.18.2.18.3..Na
TG.16.0.12.0.14.0..NH4	DG.34.2p..H	TG.18.1.18.1.22.0..Na	DG.20.1.18.2..NH4	TG.18.2.18.2.18.2..NH4	TG.16.1.18.3.18.3..NH4
TG.12.0.14.0.14.0..NH4	TG.18.0.16.0.18.1..NH4	TG.17.0.18.1.18.1..NH4	TG.16.0.18.2.18.2..Na	TG.15.0.18.2.18.2..NH4	DG.18.3.18.3..NH4
DG.12.0.12.0..Na	DG.18.0.18.1..NH4	TG.18.1.18.1.24.0..Na	LPE.18.2..H	TG.18.4.18.1.21.6..Na	DG.18.3.18.3..H
TG.8.0.18.1.18.1..NH4	TG.18.0.18.0.18.1..NH4	TG.18.1.18.2.24.0..NH4	PC.34.2..H	PG.28.0.18.1..H	DG.34.2p..Na
TG.6.0.18.1.18.1..NH4	TG.25.0.16.0.18.1..NH4	TG.19.0.18.1.18.2..NH4	PI.16.0.18.2..H	TG.18.2.17.1.18.2..NH4	DG.34.3p..Na
TG.10.0.18.1.18.1..NH4	DG.18.1.18.1..H	TG.18.1.18.1.23.0..NH4	TG.20.1.18.2.18.2..NH4	PC.36.5..H	TG.18.3.18.2.18.2..NH4
TG.16.0.12.0.18.1..NH4	DG.18.1.18.1..NH4	TG.18.1.18.1.21.0..NH4	TG.16.1.16.1.18.1..NH4	TG.16.1.18.2.18.2..Na	DG.32.1p..Na
DG.10.0.12.0..Na	DG.18.0.18.1..Na	TG.16.1.18.1.18.1..Na	PC.19.1.15.0..H	DG.16.1.18.2..Na	TG.16.2.18.2.18.2..NH4
DG.12.0.12.0..NH4	DG.16.0.18.1..NH4	TG.28.0.18.1.18.1..NH4	PS.39.1..H	TG.18.3.18.3.18.3..Na	TG.18.3.18.3.18.3..H
DG.10.0.12.0..NH4	TG.16.0.16.0.18.2..NH4	TG.17.0.18.1.18.2..NH4	PC.18.1.18.2..Na	DG.16.1.18.2..NH4	DG.34.4p..Na
DG.18.1.12.0..Na	DG.32.0p..Na	TG.18.1.18.2.22.0..NH4	PC.36.3..H	PEt.14.0e.16.2..Na	TG.18.3.18.3.18.3..NH4
TG.18.0.12.0.14.0..NH4	DG.16.0.18.2..Na	TG.20.0.18.1.18.2..NH4	PS.39.2..H	DG.18.3.18.2..Na	TG.18.3.18.2.18.3..NH4
DG.16.0.12.0..NH4	TG.12.0p.8.0.16.2..H		PS.39.3..H	DG.18.3.18.2..NH4	
DG.36.4p..H	DG.18.1.18.2..NH4		PC.36.4..H		
TG.18.1.12.0.18.1..Na	DG.16.0.18.2..NH4		DG.34.4p..H		
TG.18.1.12.0.18.1..NH4	DG.18.1.18.2..Na				
DG.6.0.12.0..Na					

Column corresponds to the labelled box along the y-axis in **Figure 6-6**. Metabolites are listed in the order they appear along the axis. Abbreviations of lipids are outlined in **Table 5-1**.

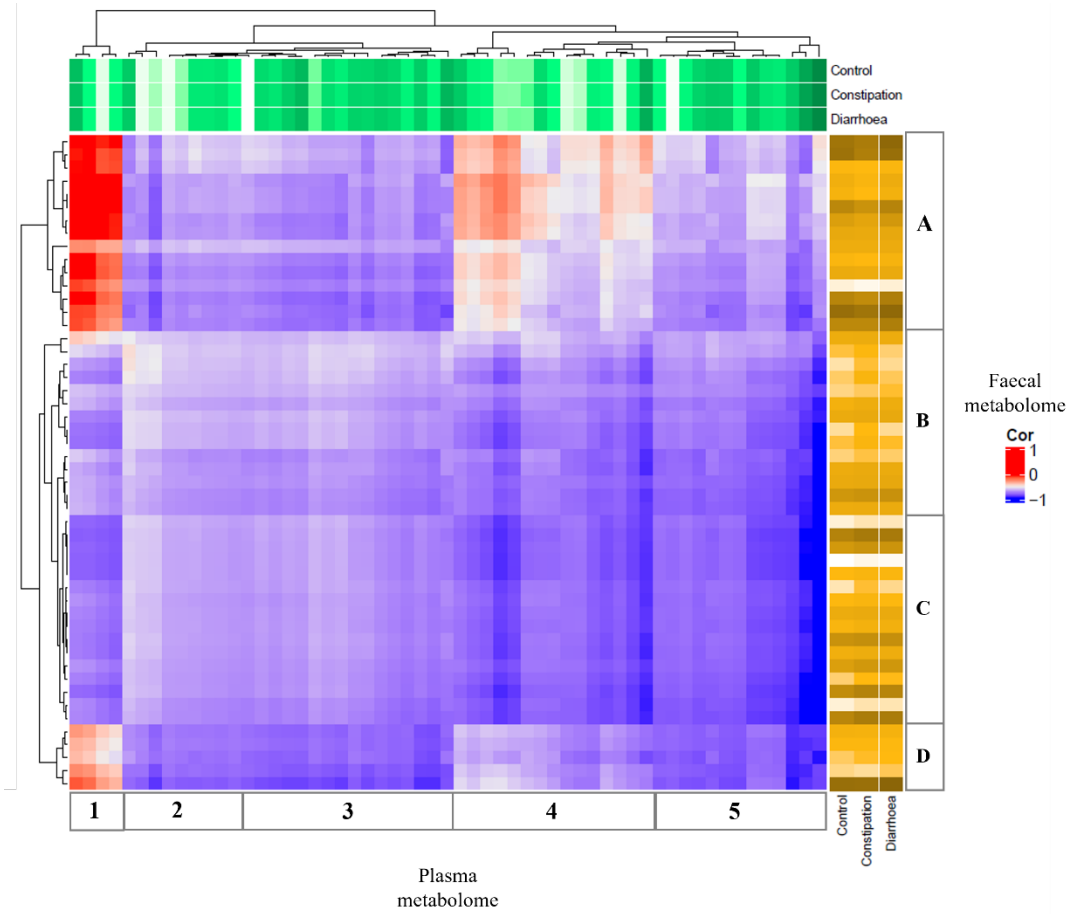


Figure 6-7: Canonical partial least squares correlation analysis shown as heatmap between the plasma metabolome (x-axis, 1-5) and faecal metabolome (y-axis, A-D) of participants with constipation (FC + IBS-C), diarrhoea (FD + IBS-D), or healthy controls. Labelled boxes along the axis correspond to the metabolite list shown in **Table 6-3** (plasma metabolome, x-axis) and **Table 6-4** (faecal metabolome, y-axis). Heatmap colour indicates canonical correlation scores. Only correlations above 0.7 or below -0.7 are shown. Positive correlations are coloured red and negative correlations are coloured blue. The upper (green) and right (yellow) colour bars indicate the mean relative abundance of metabolites according to constipation, diarrhoea, or healthy control groups, with the darker shade indicating higher abundance.

Table 6-3: List of metabolites corresponding to the plasma metabolome (x-axis) as shown in complex heatmap **Figure 6-7**.

Plasma metabolome				
1	**2**	**3**	**4**	**5**
TG.16.0.18.1.18.1..NH4	ChE.20.4..NH4	SM.31.1...M..	PC.38.3..H	SM.d40.4..H
TG.14.0.18.2.18.2..Na	Plasmenyl.PC.30.0...M.H	PC.34.2P..H	PC.18.0.20.3..Na	Plasmenyl.PE.34.1...M.H..
PE.36.1...M.H..	Plasmenyl.PC.32.1...M.H	SM.d34.0..H	PE.18.0.18.1..Na	PC.32.0e..H
PE.18.0.18.2..H	SM.32.0...M..	SM.d18.1.18.3..H	PE.34.2...M.H..	SM.d38.1..H
	SM.d34.4..H	SM.d34.1..H	Cer.d18.1.22.0..H	PC.38.5..H.1
	PC.34.1e..H	SM.d18.2.18.3..H	PE.16.0.20.4..H	SM.d36.1..H
	SM.d38.2..H	SM.d34.2..H	PC.40.5..H	PC.34.3..H
	SM.d32.1..H	PC.34.2e..H.1	PC.40.4..H	PC.36.1..H
	PC.40.5e..H	SM.d36.2..H	Plasmenyl.PE.34.2...M.H..	PC.33.1..H
		PC.36.4P..H	Plasmenyl.PE.36.2...M.H..	PC.38.4..H
		PC.36.4e..H	PC.34.0..H	PC.32.0..H
		PC.38.4P..H	Cer.d18.1.24.0..H	PC.34.1..H
		SM.d38.4..H	PI.18.0.20.4..NH4	PC.34.2..H
		SM.d42.3..H	PC.38.2..H	
		PC.32.1e..H	PC.36.2..H	
		PC.36.4..H.1		

Column corresponds to the labelled box along the x-axis in **Figure 6-7**. Metabolites are listed in the order they appear along the axis. Abbreviations of lipids are outlined in **Table 5-1**.

Table 6-4: List of metabolites corresponding to the faecal metabolome (y-axis) as shown in complex heatmap **Figure 6-7**.

Faecal metabolome			
A	**B**	**C**	**D**
TG.18.1.18.1.18.2..NH4	PC.36.4..H	DG.32.1p..Na	TG.18.4.18.1.21.6..Na
TG.18.1.18.1.18.1..NH4	PC.36.5..H	TG.18.3.18.3.18.3..NH4	PG.28.0.18.1..H
TG.18.0.18.1.18.1..Na	TG.4.0.14.1.18.3..Na	TG.16.2.18.2.18.2..NH4	TG.18.2.17.1.18.2..NH4
DG.32.0p..Na	PG.28.0.18.3..H	TG.18.3.18.3.18.3..H	TG.15.0.18.2.18.2..NH4
TG.12.0p.8.0.16.2..H	TG.16.1.18.2.18.2..Na	DG.34.4p..Na	TG.18.2.18.2.18.2..NH4
DG.18.1.18.2..NH4	TG.18.3.18.3.18.3..Na	TG.16.1.18.3.18.3..NH4	
DG.16.0.18.2..NH4	PEt.30.2p..Na	TG.20.1.18.3.18.3..NH4	
DG.18.1.18.2..Na	PG.28.0.18.2..H	DG.18.3.18.3..Na	
TG.16.1.16.1.18.1..NH4	TG.57.12..Na	TG.18.3.18.2.18.3..Na	
TG.14.0.18.2.18.2..NH4	DG.16.1.18.2..Na	DG.18.3.18.3..NH4	
DG.18.2.18.2..H	DG.16.1.18.2..NH4	DG.18.3.18.3..H	
TG.18.2.18.2.18.2..H	PEt.14.0e.16.2..Na	TG.16.1.18.2.18.2..NH4	
DG.18.2.18.2..NH4	DG.18.3.18.2..NH4	DG.34.2p..Na	
TG.18.1.18.2.18.2..NH4	DG.18.3.18.2..Na	TG.18.3.18.2.18.3..NH4	
TG.16.0.18.2.18.2..NH4		DG.34.3p..Na	
		TG.18.3.18.2.18.2..NH4	

Column corresponds to the labelled box along the x-axis in **Figure 6-7**. Metabolites are listed in the order they appear along the axis. Abbreviations of lipids are outlined in **Table 5-1**.

6.3.2. Faecal metabolome and faecal microbiome

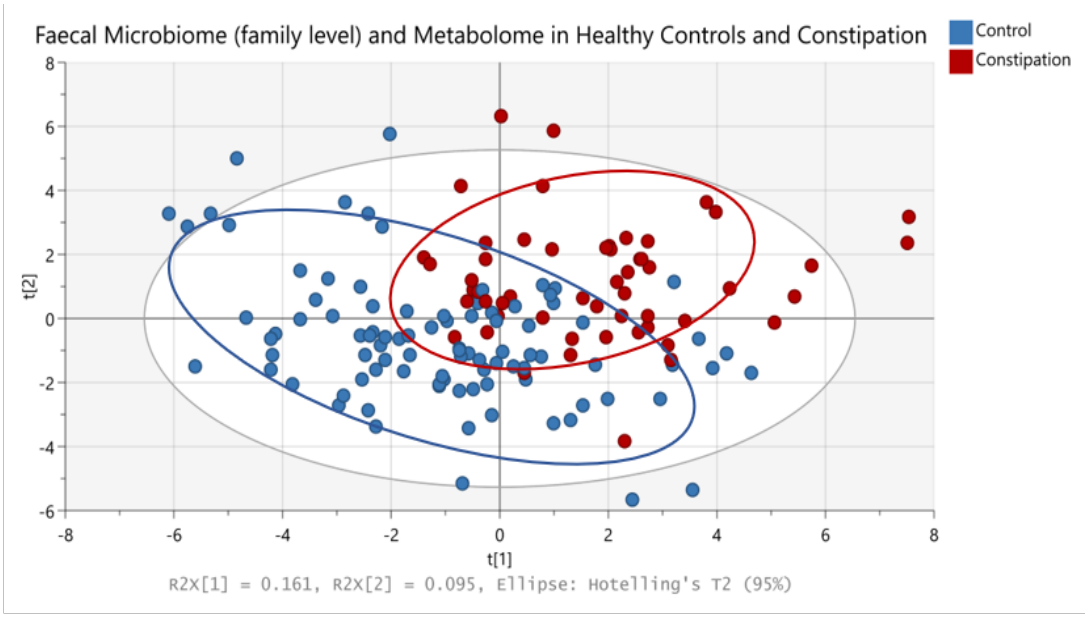
Five hundred and eighty-four metabolites from the faecal metabolome were analysed with 198 annotated microbial abundance features. PLS-DA of the top 10% VIP scores of the faecal metabolome and faecal microbiome (family level) showed differentiation between constipation and healthy controls (**Figure 6-8**) (CV-ANOVA p = 1.58e^{-11}, R2X = 0.363, R2Y = 0.503, Q2 = 0.336), and diarrhoea and healthy controls (CV-ANOVA p = 2.93.e^{-15}, R2X = 0.255, R2Y = 0.495, Q2 = 0.376). Although the PLS-DA models were significant, there was an overlap of the 95% confidence intervals. PLS-DA of the diarrhoea and healthy control groups were better separated, with fewer outliers identified compared to the constipation and healthy control groups.

Procrustes analysis of the faecal metabolome and microbiome (at the microbial family level) of all participants showed agreement between sample types (p = 9.99e^{-5}). However, there were no differences in the level of agreement between constipation, diarrhoea, or healthy control groups (**Figure 6-9**).

Univariate and fold-change analyses of the faecal metabolome and microbiome revealed ten features significantly different between the diarrhoea and healthy control groups (**Figure 6-10**). Two triglycerides were significantly decreased in the healthy control group compared to the diarrhoea group, while the remaining eight features were all microbial and higher in abundance in healthy controls compared to participants with diarrhoea (*Euryarchaeota, Tenericutes, Tenericutes Mollicutes, Eukaryota, Firmicutes Selenomonadaceae, Blastocystidae, Stramenopiles,* and *Cyanobacteria Melainabacteria*).

Univariate and fold-change analyses of the faecal metabolome and microbiome showed nine features were significantly different between constipation and healthy control groups

(**Figure 6-11**). Homovanillic acid and a diglyceride were significantly higher in healthy controls compared to participants with constipation. The relative abundance of the remaining seven features (metabolites: two ceramides, monoglyceride; microbial: *Proteobacteria Aeromonadale, Proteobacteria Succinivibrionaceae, Firmicutes Enteroccaceae, PVC Group Lentispaerae*) were significantly lower in the healthy control group compared to the constipation group.



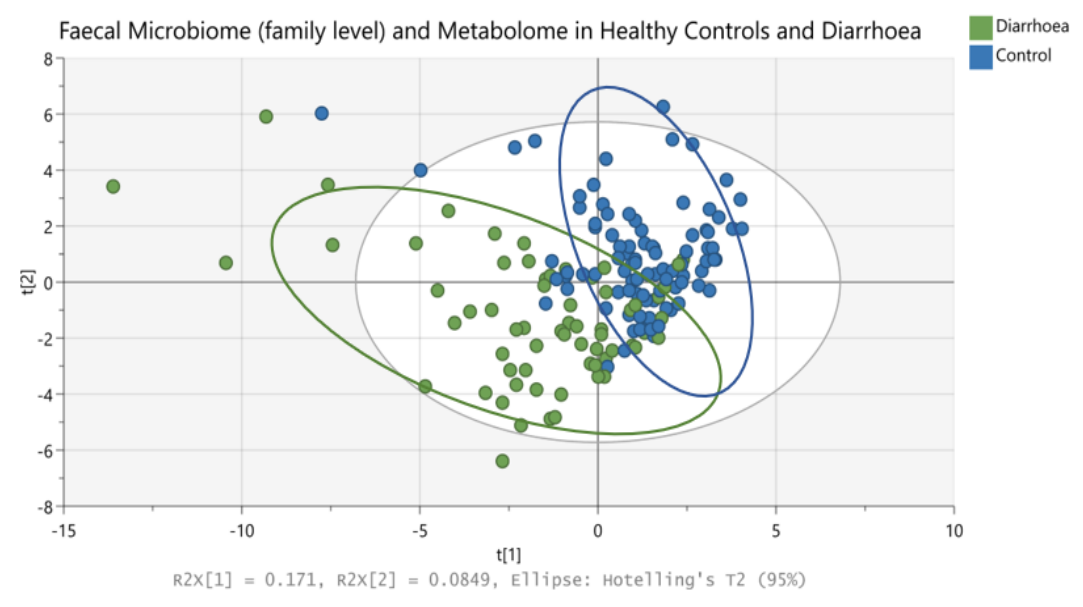


Figure 6-8: PLS-DA of combined faecal metabolome and faecal microbiome between healthy control and constipation (FC + IBS-C) groups (CV-ANOVA p = 1.58e-11, R2X = 0.363, R2Y = 0.503, Q2 = 0.336), and healthy control and diarrhoea (FD + IBS-D) groups (CV-ANOVA p = 2.93.e-15, R2X = 0.255, R2Y = 0.495, Q2 = 0.376).

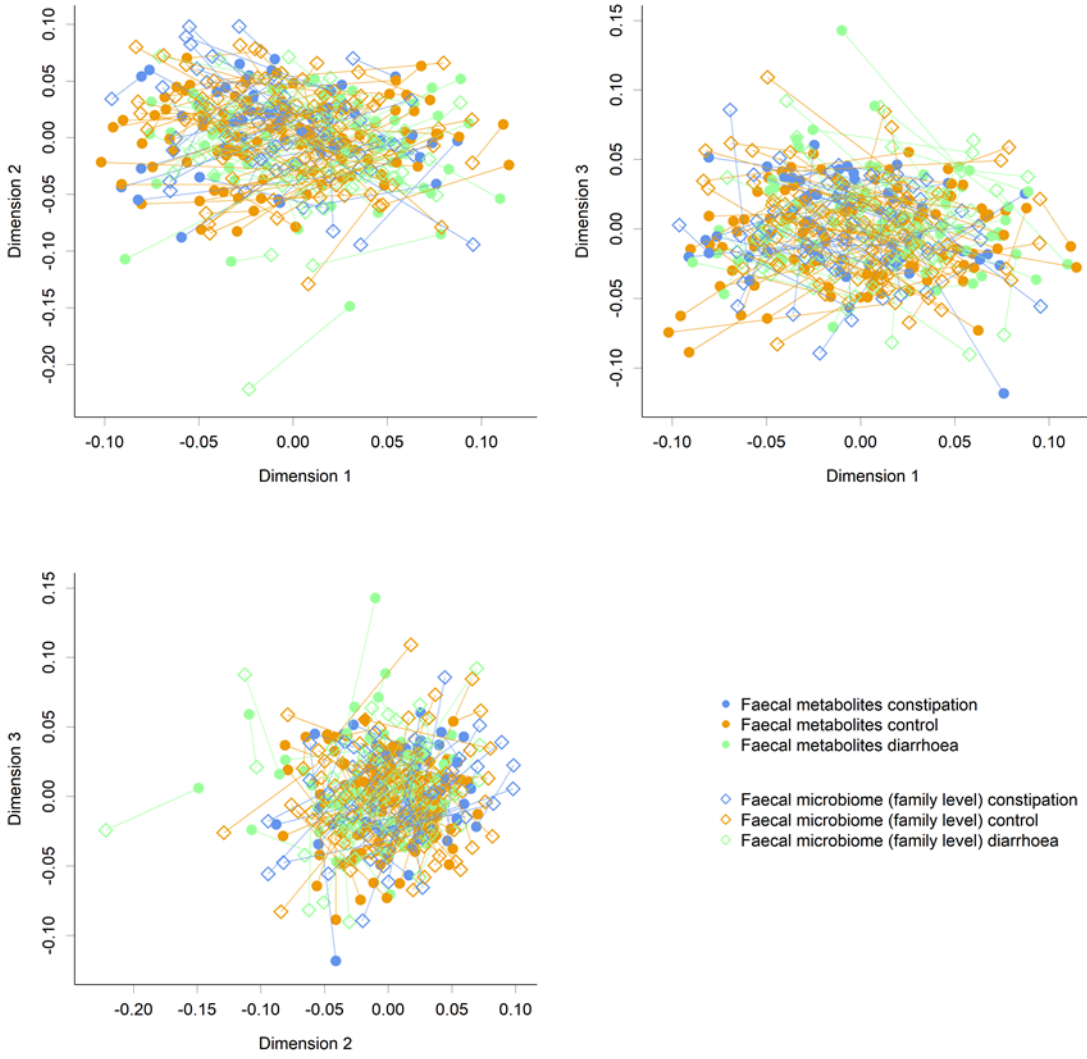


Figure 6-9: Procrustes rotation analysis of the faecal metabolome and faecal microbiome of participants with constipation (FC + IBS-C), diarrhoea (FD + IBS-D), or healthy controls. Circles show ordination of faecal metabolites, and diamonds show ordination of the faecal microbiome. Lines join the faecal metabolite and faecal microbiome for the same participant, with similarity (shorter) and dissimilarity (longer) shown by the length of the line. Constipation – blue, healthy control – orange, diarrhoea – green. Significance shows the similarity of data points for the same participant $p = 9.99e^{-5}$.

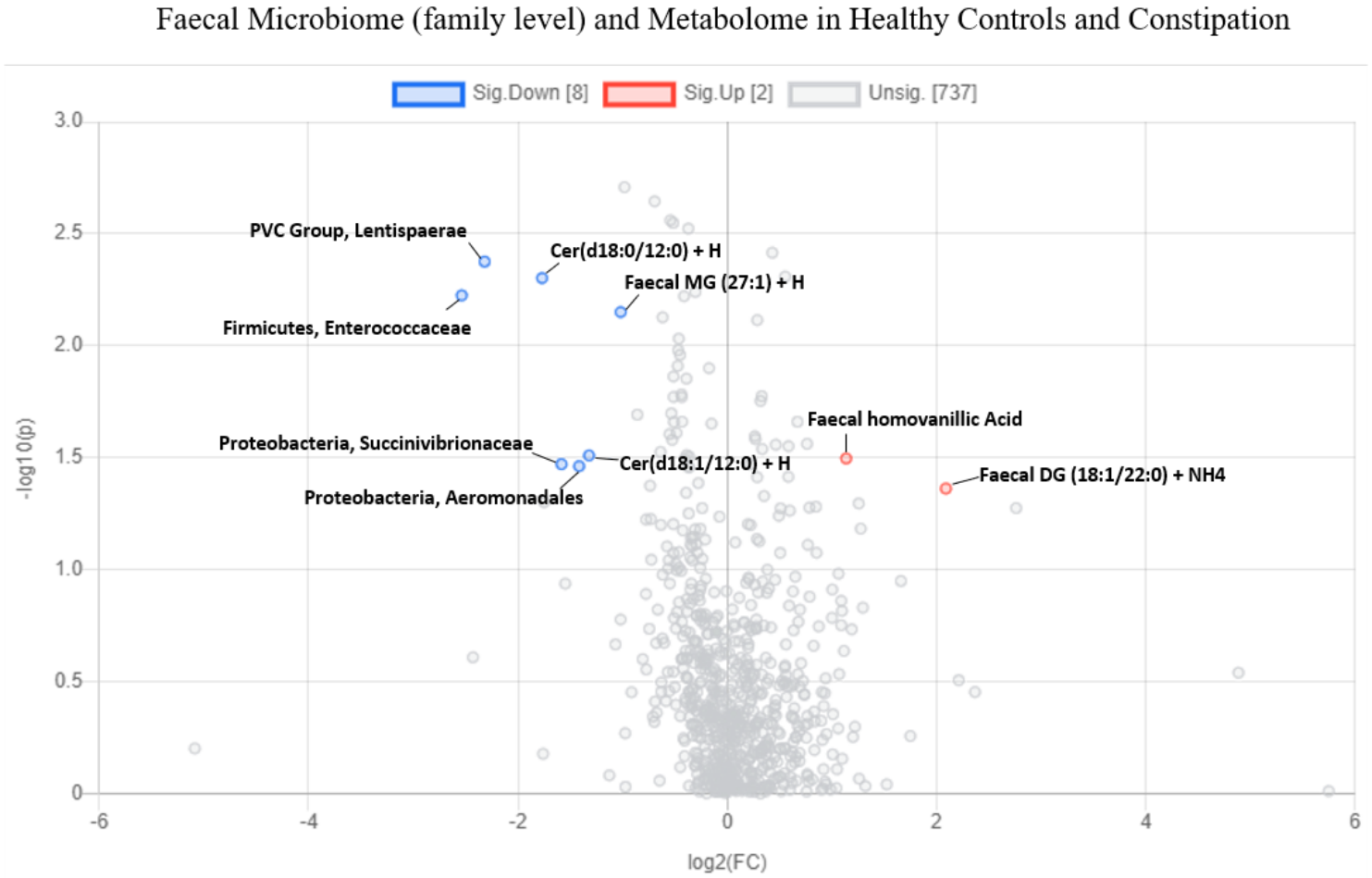


Figure 6-10: Volcano plot indicating fold change and statistical significance of metabolites from the faecal metabolome and faecal microbiome in healthy control and constipation groups. Each dot represents a metabolite or microbial feature. Features increased in healthy controls are coloured in red and decreased are coloured in blue. Fold change is presented as log2FC. *P* values ($p < 0.05$) are presented as -log10. Dots in grey are non-significant metabolites

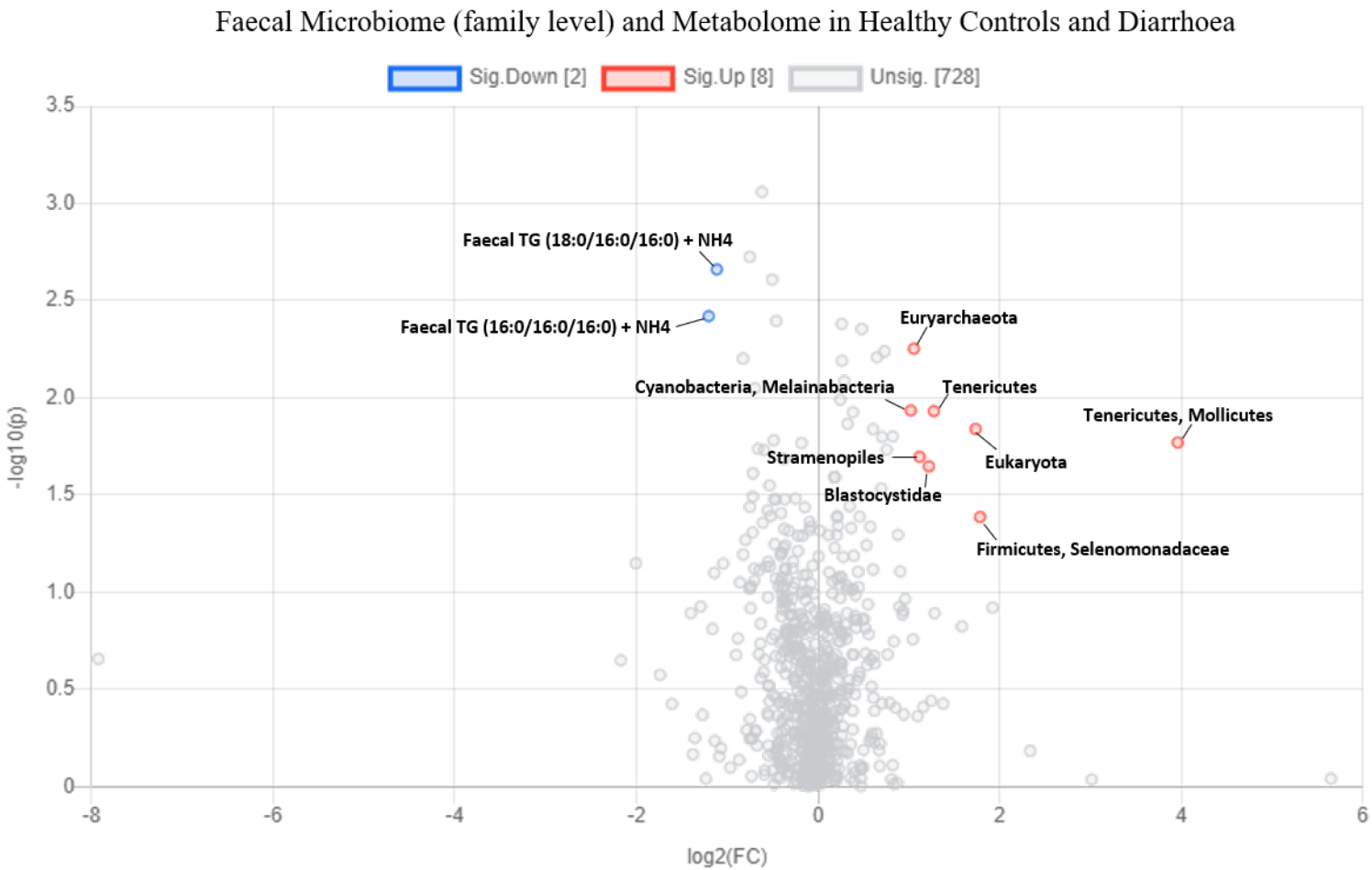


Figure 6-11: Volcano plot indicating fold change and statistical significance of metabolites from the faecal metabolome and faecal microbiome in healthy control and diarrhoea groups. Each dot represents a metabolite or microbial feature. Features increased in healthy controls are coloured in red and decreased are coloured in blue. Fold change is presented as log2FC. *P* values ($p < 0.05$) are presented as -log10. Dots in grey are non-significant metabolites.

Partial least square's correlation analysis of the faecal metabolome (x-axis) and faecal microbiome (y-axis) revealed correlations between the faecal lipidome and microbial taxa (**Figure 6-12**). There were no correlations above 0.5 or below -0.5 between faecal polar and semi-polar metabolite abundances to microbial taxonomic abundance. In general, the *Firmicutes* phylum was characterised by strong positive correlations (0.5 to 1, indicated in red) with the faecal lipids shown. *Clostridiales,* belonging to the *Firmicutes* phylum were notably different, with a strong positive correlation to some lipids and a strong negative correlation to others. Conversely, *Bacteroidetes* showed strong negative correlations (-0.5 to -1, indicated in blue) to faecal lipids.

Diglycerides and triglycerides accounted for 38 and 67, respectively of the 123 faecal lipids that were correlated with the faecal microbiome (above 0.5 or below -0.5). There were minimal correlations between other faecal lipid classes phosphatidylserine, phosphatidylcholine, phosphatidylinositol**,** phosphatidylglycerol, phospahtidylethanol, monoglyceride, and microbial species. Microbial taxa correlated to faecal lipids were predominantly those belonging to the *Firmicutes* and *Bacteroidetes* phylum. Additionally, the relative abundance of all bacteria, all *Firmicutes* and all *Bacteroidetes* correlated to faecal lipids. There was no difference in the mean relative abundance of metabolites or microbial taxa between constipation, diarrhoea and healthy control groups as indicated by the upper and right colour bars.

The correlations were plotted as networks, which showed microbial species as the central hubs connected to many faecal lipids (**Appendix figure 3** & **4**). The node "Bacteria", representing sequences that could not be classified at a lower level, was central to the pathway, branching into two hubs that were grouped around *Bacteroidetes* and *Firmicutes.*

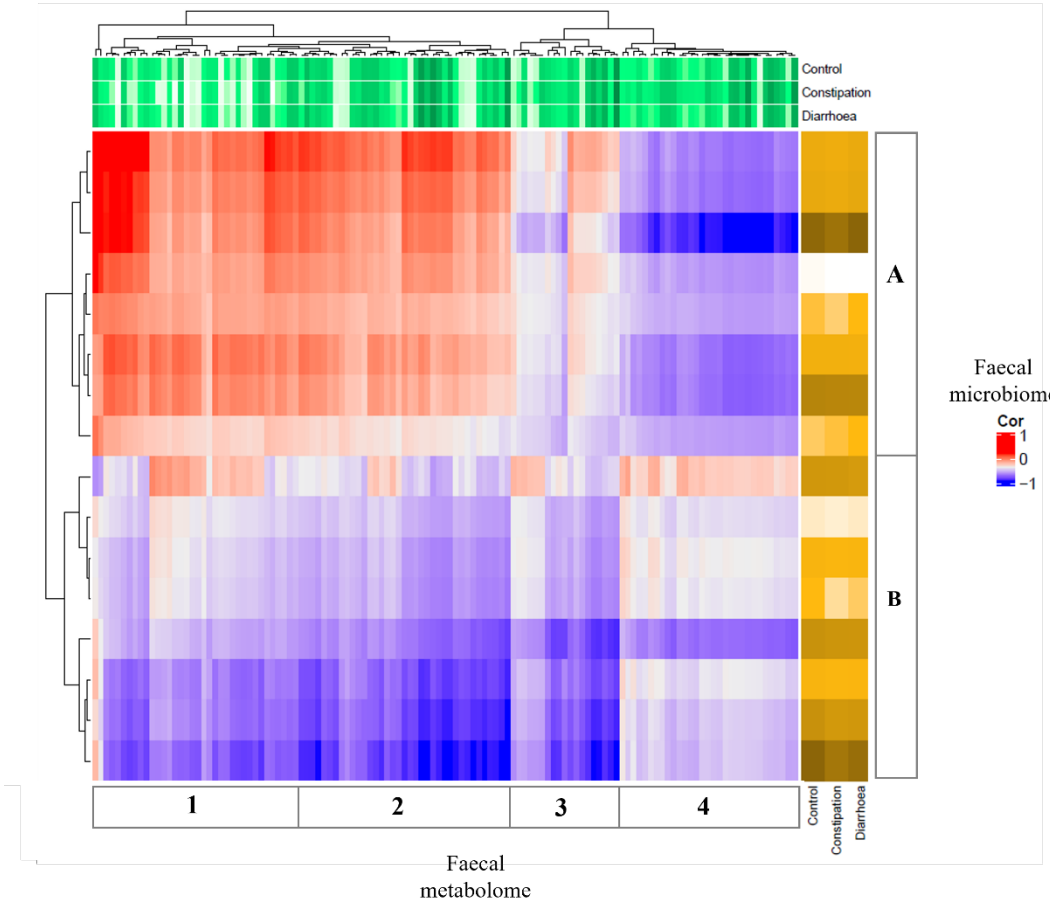


Figure 6-12: Canonical partial least squares correlation analysis shown as heatmap between the faecal metabolome (x-axis, 1-4) and faecal microbiome (y-axis, A-B) of participants with constipation (FC + IBS-C), diarrhoea (FD + IBS-D), or healthy controls. Labelled boxes along the axis correspond to the metabolite and microbial lists shown in **Table 6-5** (faecal metabolome, x-axis) and **Table 6-6** (faecal microbiome, y-axis). Heatmap colour indicates canonical correlation scores. Only correlations above 0.5 or below -0.5 are shown. Positive correlations are coloured red and negative correlations are coloured blue. The upper (green) and right (yellow) colour bars indicate the mean relative abundance of metabolites according to constipation, diarrhoea, or healthy control groups, with the darker shade indicating higher abundance.

Table 6-5: List of metabolites corresponding to the faecal metabolome (x-axis) as shown in complex heatmap **Figure 6-12**.

Faecal metabolome			
1	**2**	**3**	**4**
DG.36.4p..H	DG.18.1.18.1..H	PS.39.3..H	StE.18.2..NH4
TG.18.1.12.0.18.1..NH4	DG.18.1.18.1..NH4	PC.36.4..H	PC.36.5..H
TG.4.0.14.0.18.3..NH4	DG.18.0.18.1..NH4	PC.18.1.18.2..Na	DG.34.4p..H
TG.18.3.18.2.21.6..Na	MG.34.1..Na	PS.39.2..H	TG.18.3.18.3.18.3..Na
TG.59.11..H	TG.18.1.18.2.22.1..NH4	PC.36.3..H	TG.16.1.18.2.18.2..Na
DG.18.1.18.1..Na	TG.20.1.18.2.22.1..NH4	TG.16.1.16.1.18.1..NH4	TG.4.0.14.1.18.3..Na
TG.57.10..Na	TG.24.3.18.2.18.2..H	TG.18.4.18.1.21.6..Na	PG.28.0.18.3..H
PG.46.0..H	TG.25.0.16.0.18.1..NH4	PG.28.0.18.1..H	PEt.30.2p..Na
DG.14.0.18.2..NH4	TG.18.0.18.0.18.1..Na	TG.18.2.17.1.18.2..NH4	PEt.14.0e.16.2..Na
DG.16.0.18.1..Na	TG.20.5.18.2.18.2..H	DG.16.0.18.2..Na	DG.18.3.18.2..Na
TG.26.0.18.1.18.1..NH4	DG.34.2p..H	TG.20.1.18.2.18.2..NH4	TG.57.12..Na
DG.18.1.22.0..NH4	TG.16.0.16.0.18.2..NH4	TG.18.2.18.2.18.2..H	PG.28.0.18.2..H
DG.20.1.18.1..Na	TG.18.1.18.2.22.0..NH4	DG.18.2.18.2..Na	DG.34.2p..Na
DG.20.1.18.1..NH4	TG.20.0.18.1.18.2..NH4	DG.18.2.18.2..NH4	DG.16.1.18.2..Na
DG.20.0.18.1..NH4	TG.17.0.18.1.18.2..NH4	DG.18.2.18.2..H	TG.16.1.18.3.18.3..NH4
TG.18.1.18.1.24.0..NH4	TG.18.1.18.2.24.0..NH4	TG.16.0.18.2.18.2..NH4	DG.16.1.18.2..NH4
TG.25.0.18.1.18.1..NH4	TG.19.0.18.1.18.2..NH4	TG.15.0.18.2.18.2..NH4	TG.18.3.18.2.18.3..Na
TG.18.1.18.1.21.0..NH4	DG.20.1.18.2..NH4	TG.18.2.18.2.18.2..NH4	TG.20.1.18.3.18.3..NH4
TG.18.1.18.1.24.0..Na	DG.18.0.18.1..Na		DG.32.1p..Na
PE.18.0p..H	TG.16.1.16.1.18.1..Na		TG.16.2.18.2.18.2..NH4
TG.16.1.18.1.18.1..Na	DG.16.0.18.1..NH4		DG.18.3.18.3..NH4
TG.16.0.18.1.24.0..NH4	TG.18.1.18.1.18.1..NH4		DG.18.3.18.3..H
TG.18.0.17.0.18.1..NH4	TG.18.0.18.1.18.1..NH4		TG.18.3.18.3.18.3..NH4
DG.22.0.18.2..NH4	TG.18.1.18.1.18.2..NH4		DG.34.4p..Na
TG.18.1.18.1.23.0..NH4	TG.16.0.18.1.18.1..NH4		TG.18.3.18.3.18.3..H
TG.17.0.18.1.18.1..NH4	TG.12.0p.8.0.16.2..H		TG.16.1.18.2.18.2..NH4
TG.18.1.18.1.22.0..Na	DG.18.1.18.2..NH4		TG.18.3.18.2.18.3..NH4
TG.28.0.18.1.18.1..NH4	TG.16.0.17.1.18.1..NH4		DG.18.3.18.2..NH4
TG.16.0.18.1.22.0..NH4	TG.20.1.18.1.18.2..Na		DG.18.3.18.3..Na
TG.20.0.18.1.18.1..NH4	TG.18.1.18.2.23.0..NH4		DG.34.3p..Na
MG.20.4..H	TG.18.2.18.2.23.0..NH4		TG.18.3.18.2.18.2..NH4
PEt.19.1.18.1..H	TG.18.0.18.1.18.1..Na		

DG.18.1.18.3..H	DG.18.1.18.2..Na		
DG.18.3.18.2..H	DG.32.0p..Na		
TG.18.0.16.0.18.1..NH4	DG.16.0.18.2..NH4		
TG.18.0.18.0.18.1..NH4	TG.14.0.18.2.18.2..NH4		
	TG.18.1.18.2.18.2..NH4		

Column corresponds to the labelled box along the x-axis in **Figure 6-12**. Metabolites are listed in the order they appear along the axis. Abbreviations of lipids are outlined in **Table 5-1**.

Table 6-6: List of microbial features corresponding to the faecal microbiome (y-axis) as shown in complex heatmap **Figure 6-12**.

Faecal microbiome	
A	**B**
Firmicutes.Blautia.	Bacteroidetes.Alistipes.
Firmicutes.Lachnospiraceae.	Firmicutes.Flavonifractor.
Firmicutes.Clostridiales.	Bacteroidetes.
Firmicutes.Eubacteriaceae.	Bacteroidetes.Bacteroidia.
Firmicutes.Erysipelotrichaceae.	Bacteria.
Terrabacteria.group.	Bacteroidetes.Bacteroidaceae.
Firmicutes.	Bacteroidetes.Bacteroidales.
Firmicutes.Lachnoclostridium.	Bacteroidetes.Bacteroides.

Column corresponds to the labelled box along the y-axis in **Figure 6-12**. Microbial features are listed in the order they appear along the axis.

6.3.3. Faecal metabolome and dietary intake

Five hundred and eighty-four faecal metabolites were analysed together with 46 common nutrient constituents. Procrustes rotation was used to analyse the pairing of metabolome data and dietary intake for each participant. Procrustes of the faecal metabolome and three-day dietary data recorded as part of participant reported dietary dietaries were not significant (**Appendix figure 5**). The Procrustes rotation separately comparing metabolites against macronutrients (e.g., protein, fibre, carbohydrate) and micronutrients (e.g., iron, zinc, vitamins) were only significant ($p = 0.044$) for micronutrients (**Figure 6-13** & **Figure 6-14**). Thus, there was agreement of an individual's micronutrient profile and their faecal metabolome, however again this was regardless of grouping.

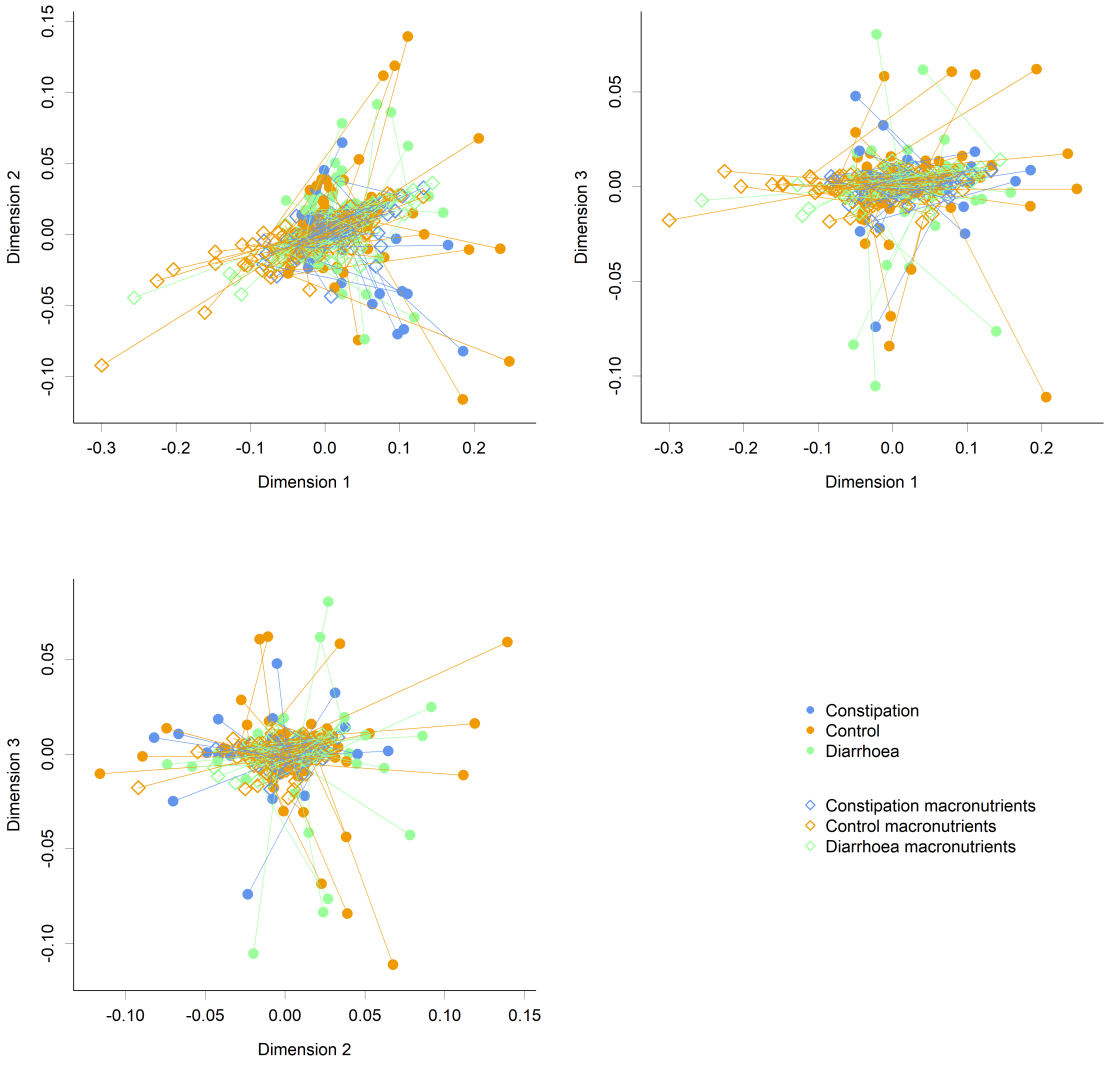


Figure 6-13: Procrustes rotation analysis of the faecal metabolome and dietary macronutrients of participants with constipation (FC + IBS-C), diarrhoea (FD + IBS-D), or healthy controls. Circles show ordination of faecal metabolites, and diamonds show ordination of dietary macronutrients. Lines join the faecal metabolite and dietary macronutrients for the same participant, with similarity (shorter) and dissimilarity (longer) shown by the length of the line. Constipation – blue, control – orange, diarrhoea – green. Significance shows the similarity of data points for the same participant $p = 0.668$.

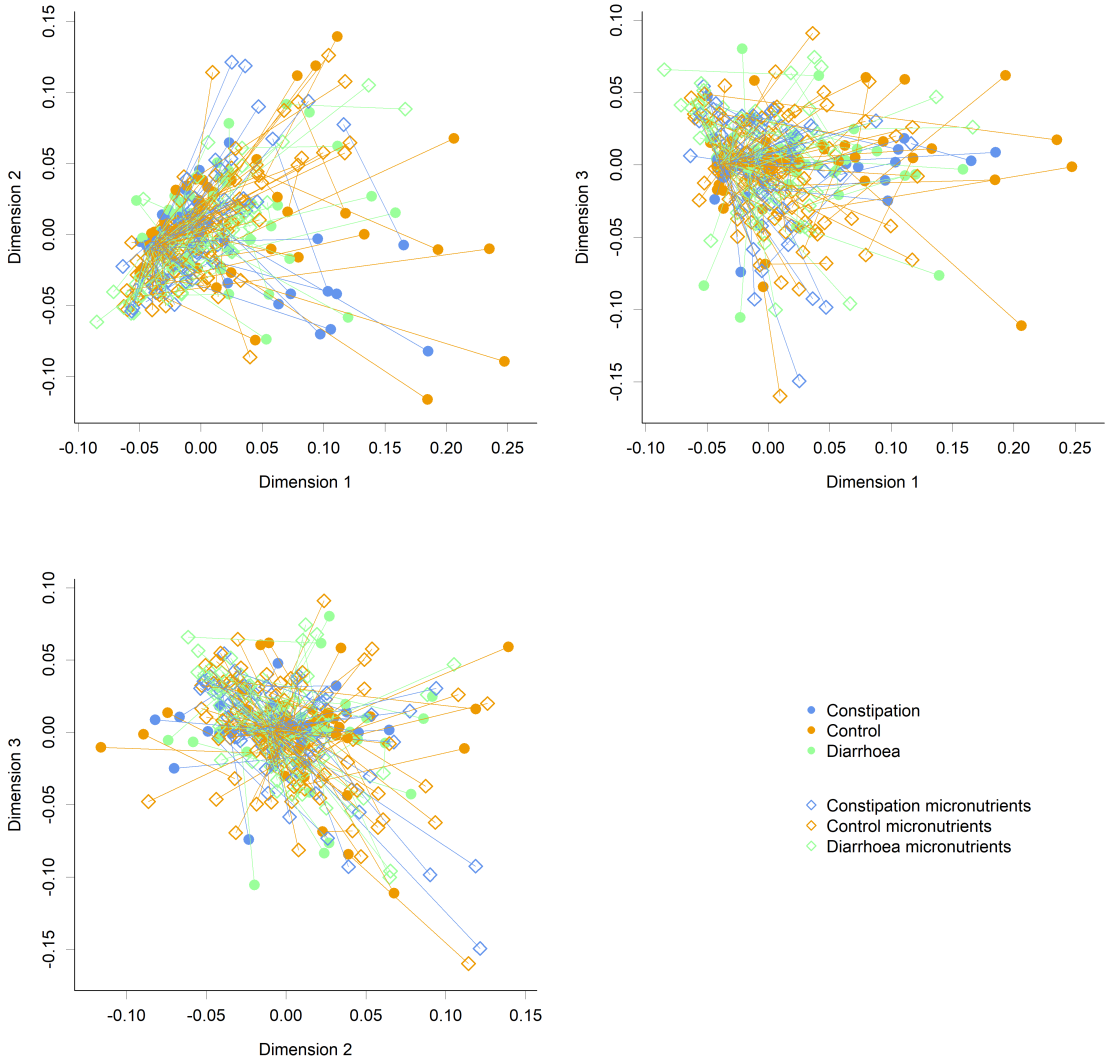


Figure 6-14: Procrustes rotation analysis of the faecal metabolome and dietary micronutrients of participants with constipation (FC + IBS-C), diarrhoea (FD + IBS-D), and healthy controls. Circles show ordination of faecal metabolites, and diamonds show ordination of dietary micronutrients. Lines join the faecal metabolite and dietary micronutrients for the same participant, with similarity (shorter) and dissimilarity (longer) shown by the length of the line. Constipation – blue, control – orange, diarrhoea – green. Significance shows the similarity of data points for the same participant $p = 0.044$.

Partial least square's correlation analysis revealed correlations between the faecal metabolome (x-axis) and dietary constituents (y-axis) for all participants (**Figure 6-15**). Correlation analysis showed no difference in the mean relative abundance of faecal metabolites or dietary intake between constipation or diarrhoea groups, each compared to the healthy control groups as indicated by the upper and right colour bars. The correlations between dietary constituents, macronutrients, and micronutrients were correlated primarily to faecal triglycerides and diglycerides.

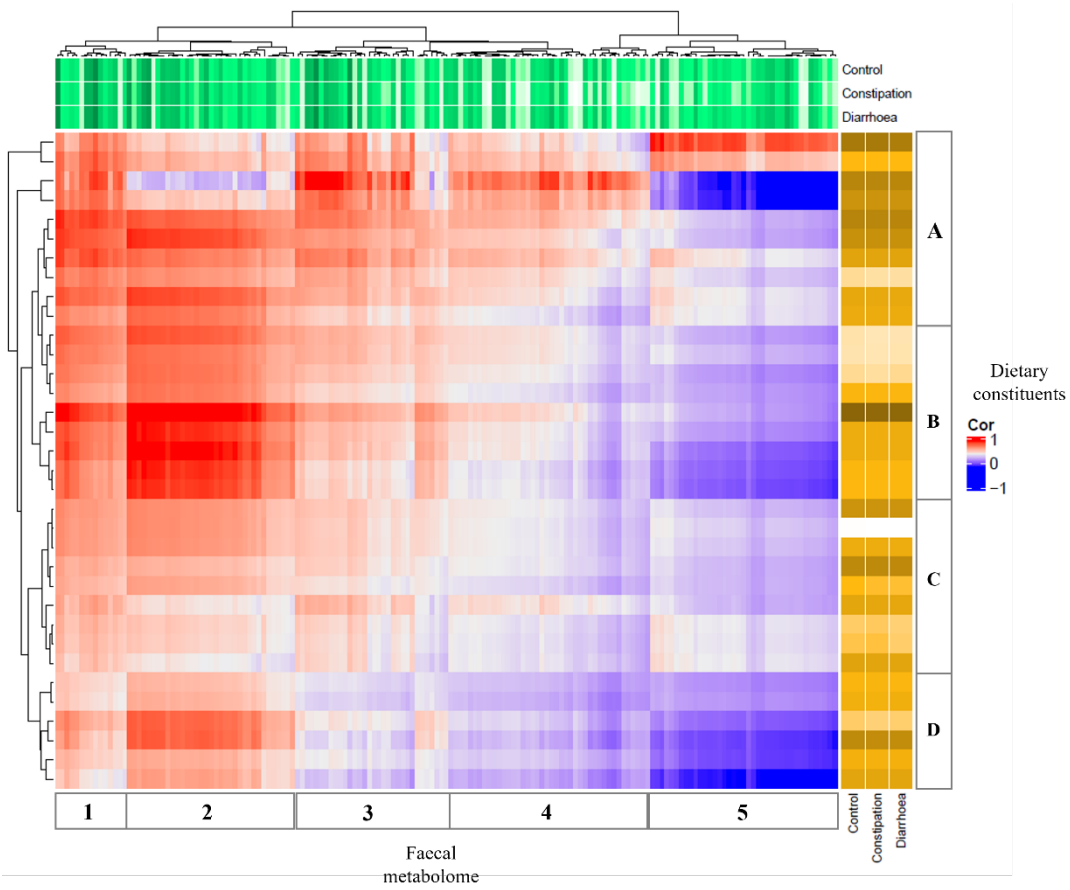


Figure 6-15: Canonical partial least squares correlation analysis shown as heatmap between the faecal metabolome (x-axis, 1-5) and dietary constituents (y-axis, A-D) of participants with constipation (FC + IBS-C), diarrhoea (FD + IBS-D), or healthy controls. Labelled boxes along the axis correspond to the metabolite and dietary constituent lists shown in **Table 6-7** (faecal metabolome, x-axis) and **Table 6-8** (dietary constituents, y-axis). Heatmap colour indicates canonical correlation scores. Only correlations above 0.5 or below -0.5 are shown. Positive correlations are coloured red and negative correlations are coloured blue. The upper (green) and right (yellow) colour bars indicate the mean relative abundance of metabolites according to constipation, diarrhoea, or healthy control group, with the darker shade indicating higher abundance.

Table 6-7: List of metabolites corresponding to the faecal metabolome (x-axis) as shown in complex heatmap **Figure 6-15**.

Faecal metabolome				
1	**2**	**3**	**4**	**5**
TG.18.2.18.2.18.2..NH4	TG.18.3.18.2.18.2..NH4	TG.18.0.18.1.18.1..Na	TG.20.5.18.2.18.2..H	TG.18.1.12.0.18.1..NH4
TG.15.0.18.2.18.2..NH4	DG.34.3p..Na	TG.18.1.18.2.23.0..NH4	TG.18.1.18.2.22.0..NH4	TG.18.1.12.0.18.1..Na
TG.18.2.17.1.18.2..NH4	DG.18.3.18.2..Na	TG.16.0.18.1.18.1..NH4	TG.18.0.18.0.18.1..Na	DG.36.4p..H
TG.18.4.18.1.21.6..Na	TG.18.3.18.2.18.3..NH4	TG.18.0.18.1.18.1..NH4	TG.18.1.18.2.24.0..NH4	DG.6.0.12.0..Na
PG.28.0.18.1..H	TG.18.3.18.3.18.3..NH4	TG.18.1.18.1.18.1..NH4	TG.18.0.16.0.18.1..NH4	DG.18.1.14.0..NH4
TG.18.2.18.2.18.2..H	TG.18.3.18.3.18.3..H	TG.12.0p.8.0.16.2..H	TG.20.1.18.2.22.1..NH4	DG.18.1.14.0..Na
TG.16.0.18.2.18.2..NH4	DG.34.4p..Na	DG.18.1.18.2..NH4	TG.18.1.18.2.22.1..NH4	TG.16.0.14.0.18.1..NH4
DG.18.2.18.2..NH4	TG.16.2.18.2.18.2..NH4	DG.16.0.18.2..NH4	TG.25.0.16.0.18.1..NH4	TG.10.0.18.1.18.1..NH4
TG.18.1.18.2.18.2..NH4	PEt.14.0e.16.2..Na	DG.18.1.18.2..Na	TG.28.0.18.1.18.1..NH4	DG.18.1.12.0..Na
DG.18.2.18.2..H	TG.16.1.18.2.18.2..NH4	DG.32.0p..Na	TG.20.0.18.1.18.1..NH4	DG.8.0.12.0..Na
TG.14.0.18.2.18.2..NH4	DG.18.3.18.2..NH4	TG.16.0.17.1.18.1..NH4	TG.18.0.18.0.18.1..NH4	DG.16.0.12.0..NH4
TG.16.1.16.1.18.1..NH4	DG.34.2p..Na	TG.18.1.18.1.18.2..NH4	TG.20.0.18.1.18.2..NH4	DG.10.0.12.0..NH4
DG.18.2.18.2..Na	DG.18.3.18.3..Na	TG.20.1.18.1.18.2..Na	TG.18.1.18.1.23.0..NH4	DG.12.0.12.0..NH4
TG.18.2.18.2.23.0..NH4	TG.20.1.18.3.18.3..NH4	TG.16.0.16.0.18.2..NH4	TG.17.0.18.1.18.1..NH4	DG.10.0.12.0..Na
TG.20.1.18.2.18.2..NH4	DG.32.1p..Na	TG.16.1.16.1.18.1..Na	TG.18.1.18.1.21.0..NH4	TG.16.0.14.0.16.0..NH4
	DG.18.3.18.3..H	DG.16.0.18.2..Na	TG.18.1.18.1.22.0..Na	TG.18.0.12.0.14.0..NH4
	DG.18.3.18.3..NH4	TG.16.1.18.1.18.1..Na	TG.18.1.18.1.24.0..Na	TG.16.0.12.0.18.1..NH4
	TG.16.1.18.3.18.3..NH4	TG.17.0.18.1.18.2..NH4	DG.34.2p..H	TG.6.0.18.1.18.1..NH4
	TG.18.3.18.2.18.3..Na	DG.20.1.18.2..NH4	TG.16.0.18.1.22.0..NH4	TG.8.0.18.1.18.1..NH4
	DG.16.1.18.2..NH4	TG.19.0.18.1.18.2..NH4	DG.16.0.18.1..Na	DG.12.0.12.0..Na
	PG.28.0.18.2..H	MG.34.1..Na	DG.18.0.18.1..NH4	DG.8.0.12.0..NH4
	TG.57.12..Na	TG.24.3.18.2.18.2..H	DG.18.1.18.1..NH4	DG.18.1.12.0..NH4
	DG.16.1.18.2..Na	DG.16.0.18.1..NH4	DG.18.1.18.1..H	DG.12.0.14.0..Na
	PEt.30.2p..Na	DG.18.0.18.1..Na	TG.16.0.16.0.18.1..NH4	TG.10.0.12.0.14.0..NH4
	TG.16.1.18.2.18.2..Na	DG.14.0.18.2..NH4	TG.16.0.18.1.24.0..NH4	TG.16.0.12.0.14.0..NH4
	TG.18.3.18.3.18.3..Na	PI.16.0.18.2..H	PEt.19.1.18.1..H	TG.12.0.14.0.14.0..NH4
	PG.28.0.18.3..H	PS.39.1..H	TG.25.0.18.1.18.1..NH4	TG.18.1.12.0.14.0..NH4
	TG.4.0.14.1.18.3..Na	PC.34.2..H	TG.18.0.17.0.18.1..NH4	TG.10.0.12.0.18.1..NH4
	DG.34.4p..H	StE.18.2..NH4	TG.18.1.18.1.24.0..NH4	TG.10.0.12.0.12.0..NH4
	PC.36.3..H	TG.16.0.18.2.18.2..Na	TG.57.10..Na	TG.4.0.16.0.18.1..NH4
	PC.36.4..H	LPE.18.2..H	MG.20.4..H	DG.16.0.12.0..Na
	PS.39.2..H	PC.19.1.15.0..H	PG.46.0..H	TG.16.0.12.0.18.1..Na

	PS.39.3..H PC.18.1.18.2..Na PC.36.5..H		DG.18.1.18.1..Na TG.18.3.18.2.21.6..Na TG.59.11..H TG.4.0.14.0.18.3..NH4 DG.18.1.18.3..H DG.18.3.18.2..H DG.22.0.18.2..NH4 DG.20.0.18.1..NH4 DG.18.1.22.0..NH4 DG.20.1.18.1..Na	TG.4.0.12.0.18.1..NH4 TG.8.0.10.0.10.0..NH4 TG.18.1.12.0.12.0..NH4 TG.6.0.8.0.12.0..NH4 TG.8.0.10.0.10.0..Na TG.4.0.16.0.16.0..NH4 TG.16.0.12.0.14.0..Na

Column corresponds to the labelled box along the x-axis in **Figure 6-15**. Metabolites are listed in the order they appear along the axis. Abbreviations of lipids are outlined in **Table 5-1**.

Table 6-8 List of metabolites corresponding to dietary constituents (y-axis) as shown in the complex heatmap **Figure 6-15**.

Dietary constituents			
A	**B**	**C**	**D**
Manganese	Zinc	Calcium	Sucrose
Fibre	Vitamin E	Riboflavin	Selenium
Beta-carotene equivalent	Polyunsaturated fatty acid	Sugar	Niacin equivalent from tryptophan
Vitamin A	Total niacin equivalent	Water	Sodium
Potassium	Calculated energy	Niacin	Iodine
Phosphorus	Total protein	Glucose	Cholesterol
Magnesium	Fat	Fructose	
Iron	Saturated fatty acids	Folate	
Cholesterol available	Saturated monounsaturated fatty acids		
Starch			

Column corresponds to the labelled box along the x-axis in **Figure 6-15**. Dietary constituents are listed in the order they appear along the axis.

6.4. Discussion

It was hypothesised that analysis of combined omics datasets from the wider research programme would provide further insight into differences between individuals with constipation (FC + IBS-C) and healthy controls, or diarrhoea (FD + IBS-D) and healthy controls. Discriminant analysis showed that the combined analysis of the faecal microbiome and faecal metabolome, compared to the combined plasma and faecal metabolomes, resulted in better differentiation of the constipation group from the healthy control group, and the diarrhoea group from the healthy control group. This finding was similar to those by Ahluwalia *et al.,* [276] and supports the conclusions from **Chapter 5** where the faecal metabolome was central to distinguishing between unhealthy and healthy states.

The analysis of the faecal metabolome has been somewhat neglected in attempts to understand FGDs, with research predominantly focused on investigating the faecal microbiome. Additionally, the importance of lipids in gut function has been overlooked, with prior research focused on the analysis of polar and semi-polar metabolites [313]. In this study, there were more correlations between faecal lipids than polar and semi-polar metabolites when comparing the faecal metabolome, plasma metabolome, faecal microbiome, and dietary intake, highlighting the potential importance of the faecal lipidome. However, all the correlation analyses showed no significant difference in the relative abundance of microbial or metabolic features between the constipation, diarrhoea, or healthy control groups.

Similarly, the Procrustes rotation analysis showed the faecal metabolome was concordant with the faecal microbiome, plasma metabolome, and dietary micronutrient profile within an individual regardless of grouping (constipation, diarrhoea, or healthy control) This observation suggests that a personalised approach [314], rather than grouping individuals

based on subjective clinical parameters (Rome IV), might be a better alternative to understand FGDs.

6.4.1. Faecal and plasma metabolomes

Across all the groups, triglycerides and diglycerides were the two main lipid classes from the faecal metabolome that showed positive and negative correlations with plasma sphingomyelins, phosphatidylcholines, triglycerides, ceramides, and cholesterol esters. Both triglycerides and diglycerides are the predominant lipid by-products of dietary intake [315], thus explaining why they are predominant in faecal samples. Dietary lipids are predominantly broken down into either triglycerides, cholesterol esters, phospholipids, diglycerides and then further into fatty acids [315]. These fatty acids are absorbed both passively and actively across the apical cell membrane into enterocytes where they are re-synthesised locally into triglycerides or transported via systemic circulation to the liver for re-synthesis [315]. However, during transport to the liver, fatty acids can be taken-up and modified by other organs [315], which likely increases the variability of lipid species present in plasma, as shown in the results here. The observed correlations therefore between plasma and faecal lipids might simply reflect normal lipid biochemistry, although further investigations are needed to confirm or negate this.

However, certain plasma lipid species detected and correlated with faecal lipids, for example, ceramides, sphingomyelin, and phosphatidylcholines have bioactive properties that have previously been linked to inflammatory conditions such as IBD [316]. Interestingly, bile acids are crucial to the process of lipid absorption aiding in lipolysis due to their hydrophilic and hydrophobic properties [315]. Thus, based on the results of this chapter and **Chapter 3**, further analyses investigating a link between bile acid and both plasma and faecal lipid concentrations might be warranted.

The univariate analysis highlighted that the faecal metabolome consisted of more differentially abundant metabolites than the plasma metabolome when comparing the healthy control group to either diarrhoea or constipated groups. Nine metabolites were differentially abundant in the analysis of diarrhoea and healthy controls, compared to only one metabolite between constipation and healthy controls. This finding highlighted that the faecal metabolome is likely a better indicator of perturbed processes that may be linked to FGD symptoms though diet, microbiome, age and sex may still be confounding factors.

Research investigating the plasma metabolome has focused on the investigation of known metabolites for biomarker identification (e.g., citrulline, tryptophan) [123, 317]. Prior metabolomic analyses of samples from FGD participants have been limited to the faecal metabolome and urinary metabolome [77, 318]. Comparison of the faecal and urinary metabolomes of IBS (all subtypes) and healthy control participants by Jeffery *et al.,* showed that both metabolomes could distinguish between these two groups [77]. However, they noted that discriminatory metabolites in urine were associated with diet and medication, and therefore, not representative of symptomology between healthy controls and IBS individuals [77], compared to the faecal metabolome.

6.4.2. Faecal metabolome and microbiome

The analysis of the faecal metabolome and faecal microbiome showed correlations between the relative abundance of faecal lipids and the relative abundance of taxa belonging to the *Firmicutes* and *Bacteroidetes* phylum, regardless of group. Triglycerides and diglycerides were again the main lipid classes detected by the correlation analysis, highlighting again the importance of lipids. There was however no difference in the mean relative abundance of faecal metabolites and microbial composition across constipation, diarrhoea, or healthy control groups reflective of differential correlations. The Procrustes

rotation analyses showed concordance between the faecal microbiome and metabolome of an individual, however there were no differences evident between the groups that would highlight links to symptomology. Both the discriminant and univariate analysis of the faecal metabolome and microbiome separated the constipation group from healthy controls, and the diarrhoea group from healthy controls. Both microbial and metabolic features were important to this separation and thus combining these omics technologies for future insights into FGDs should not be overlooked.

The interactions between faecal lipids and gut microbial abundance have only recently been recognised for their importance in a range of disease conditions [313]. The findings of this chapter contribute further to this, highlighting the importance of understanding the interaction between the gut microbiota and faecal and plasma lipids. Other authors have shown the importance of faecal polar and semi-polar metabolites and microbial taxa abundance in FGDs [77, 276]. However, they did not measure the faecal lipidome.

As discussed in **Chapter 5,** lipids have important biochemical functions. There is evidence to suggest important positive correlations between ceramides and *Bacteroides* [319]. However, a correlation between ceramides and *Bacteroides* was not observed here. As discussed in **Chapter 1**, numerous studies have reported a perturbed ratio of *Bacteroidetes/Firmicutes* in IBS. Correlation analysis showed *Firmicutes* was predominantly characterised by positive correlations to lipids while negative correlations to lipids were obtained with *Bacteroidetes*.

Clostridiales, belonging to the *Firmicutes* phylum was the exception to the positively associated correlations between lipids and microbial abundance in this chapter. *Clostridium* have been associated with beneficial effects to gut health, with potential use as a probiotic [320] and reduced abundance has been observed in IBS individuals (all

subtypes) compared to healthy controls [276]. Further investigation of the relative abundance of *Clostridiales* and related gene abundances between constipation, diarrhoea, and healthy control groups is ongoing as part of the PhD thesis of Caterina Carco.

The relationship between faecal lipids and the gut microbiome is ill-defined. Evidence however suggests an important link between plasma lipids and the gut microbiome, although the mechanisms of this are not well understood. Studies have highlighted the importance of the gut microbiome in host lipid metabolism, especially fatty acids, as important signalling molecules and building blocks [321]. The absence of the gut microbiota decreased the synthesis and abundance of fatty acids and the accumulation of lipids and fat in the body of GF mice [321-323], and GF obese mice [324, 325]. Additionally, differences in gut microbial composition have been shown in mice fed a diet containing equal fat content, but with fat from different sources, and those different microbial colonies had downstream effects linked to inflammation, e.g., increased toll-like receptor activation [326].

As shown in this chapter and **Chapter 5**, lipids are important to potentially understanding FGDs, however, elucidating if lipids originate from host, microbial, dietary or co-metabolic products is more elusive [313]. Analysis of the determinants that influence the serum metabolome in a cohort of healthy individuals highlighted diet and the gut microbiome were the most influential contributors to metabolite profiles, including lipids, and in some instances accounted for over 50% of the variation between people [327]. Johnson *et al.,* have previously shown certain quantities of sphingolipids are generated in the gut by *Bacteroides* species that have serine palmitoyltransfease and that these lipids are utilised by the host [319]. Furthermore, it has been shown in mice models that these bacterial species with sphingolipid producing capabilities also play key roles in processing dietary sphinganine [328]. Similarly, another example, are microbial species

that possess cholesterol dehydrogenases shown to reduce small intestinal cholesterol concentrations by conversion to coprostanol [329], thus providing further evidence of the link between gut microbial species and lipids. Although links between the gut microbiota and lipids are evident, mechanistic efforts to understand this relationship are infrequent. Approaches such as Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway identification or stable isotope tracing to understand how microbial species mediate lipid absorption, modification, and metabolism will provide new insights into potential links between the gut microbiota and lipids in health. A better ability to distinguish the origin of lipids and understand microbial lipid processing will provide evidence for their possible role in FGDs.

6.4.3. Faecal metabolome and dietary intake

The Procrustes rotation of the micronutrient data and faecal metabolome showed concordance between data points of the same person, though again this did not appear to be influenced by grouping. Correlations were evident between dietary constituents and the faecal metabolome, though there was no differentiation of correlations between for example, macronutrients or micronutrients and the faecal metabolome. Additionally, there were no differences in mean relative abundance between the groups (constipation, diarrhoea, or healthy controls) indicative of differential correlation strength. Diet is known to alter the gut microbiome, and the sequential production of metabolites in healthy individuals are highly personalised and correlated [309]. However, the extent of this is not well characterised in FGDs. Furthermore, macronutrient and micronutrient intakes ignore other chemicals, microbial-accessible-carbohydrates, preservatives, and additives present in food [309, 330] which are potentially relevant to understanding the role of food in FGDs. Johnson *et al.,* have previously noted that dietary intake often determines the microbial abundance of a faecal sample obtained the day after [309]. Thus,

on the basis this would also be true for faecal metabolites, the averaging of dietary intake across multiple days (three-days in this study) might present limitations to understanding diet and metabolite interactions.

6.4.4. Limitations, novelty, and future directions

The analysis undertaken in this chapter aimed to combine omics datasets. It provides evidence that future analyses combining multiple datasets are needed. Using more advanced computational tools to better understand system wide perturbations may provide insight into host, microbe, and dietary interaction.

The research in this chapter is novel within the field of FGDs and has only recently been implemented in other disease or disorder conditions. Studies that have previously combined analyses of the faecal metabolome and microbiome in FGDs have solely utilised univariate and discriminant analyses [77, 276]. Thus, making further inferences from the correlations results of this chapter based on other similar studies is limited. The importance of the faecal microbiome, as a proxy of lipid metabolism in the gut has only recently been identified [313].

Although omics technologies and computational tools are increasingly common, understanding the results of these high-dimensional datasets as meaningful findings that relates to system-level thinking is still difficult. For example, whilst it is known the microbiome assimilates dietary constituents into metabolites that are circulated throughout the body and appear in multiple different metabolomes, the mechanisms through which these processes occur is less understood, especially lipids [315]. There are other fields where multi-omics technologies and computational tools are being utilised and these tools have provided a better understanding of system wide perturbations related to disease conditions [331-333].

The integration of the plasma metabolome with the faecal microbiome may highlight further evidence of perturbed system-wide processes. Although the combined analysis of the faecal metabolome and plasma metabolome did not lead to mechanistic insights, it did provide areas for future research and highlighted again the importance of the lipidome in both healthy and FGD individuals. A link between the gut microbiota and circulating plasma lipids is evident in metabolic disorders with the use of GF mice [334] and thus a possible role for this relationship in FGDs must not be excluded from future research. For example, analysis of the gut microbiome and serum metabolome in individuals with colorectal cancer or adenoma showed gut microbial differences that were evident in alterations to the serum metabolome compared to healthy controls [335]. Additionally, previous integration of plasma and faecal metabolome datasets has highlighted specific metabolic fingerprints in a cohort of individuals with insulin resistance [336] and in children with autism spectrum disorder [337]. Furthermore, bile acids provide further evidence for important links between the gut microbiota, plasma metabolites and lipids, where bile acids are modified by bacterial BSH enzymes and linked to lipid absorption.

The faecal metabolome and microbiome are central to proxy molecular signatures in processes occurring in the gut that might help to better understanding FGDs. However, inclusion of other metabolomes, dietary information and utilising systems biology approaches are necessary. For example, understanding the origin of triglycerides and diglycerides that were positive and negatively correlated to the faecal microbiome is necessary to make further inferences about the relationship between lipids and the gut microbiome. Additionally, the importance of lipids as energy stores, structural components, and in signalling pathways means potential perturbations could have wide-ranging consequences. Lipidomic systems biology approaches [338] that attempt to understand the diversity of lipids, host and microbial absorption of dietary lipids, host and

microbial produced lipids, and their fate throughout the body may highlight further the role of lipids in health and disease.

As mentioned, the collection of the plasma samples on the day of recruitment in the clinic, compared to at-home faecal sample collection on a consecutive day, is a limitation of accurately combining the faecal and plasma metabolome. Limitations related to the plasma metabolome are the same as those outlined in **Chapter 5** for the faecal metabolome. Dietary intake is reliant on cohort participants completing questionnaires accurately and the time in relation to meal consumption that biological samples was obtained. There are also limitations related to grouping dietary intake into strict categories of macronutrient or micronutrient intake.

The systems biology approach used is limited to statistical analyses that only included data from **Chapter 5**. Therefore, there are limitations of not being able to make inferences about individual datasets or other combined datasets that would provide further insights. Despite these limitations, the current chapter provides evidence of the benefit of working towards a systems biology approach to combine multiple omics technologies to understand FGDs.

6.5. Conclusion

In conclusion, discriminant and univariate analyses separated constipation from healthy controls, or diarrhoea from healthy controls. The faecal microbiome and metabolome were the most robust combined analyses to separate groups based on discriminant and univariate analyses, highlighting the importance of both theses omics datasets in FGDs. Correlation analyses showed a link between diet, microbiome, and metabolome datasets; however, this was regardless of either healthy control or FGD grouping. Correlations between an individual's faecal and plasma metabolomes, faecal metabolome and

microbiome, or faecal metabolome and dietary intake showed that lipids were primarily important. Similarly, *Firmicutes* and *Bacteroidetes* showed distinct positive and negative correlations, respectively, to the faecal lipidome. This chapter highlights that links between omics datasets are evident, but that future analyses are required.

Chapter Seven
General Discussion

Chapter Seven

7.1. Thesis discussion

FGDs are highly prevalent with associated economic, medical, and psychological burdens [3, 339, 340]. Dietary intake, the gut microbiome, and host mechanisms are hypothesised to be where perturbations occur and thus understanding the interaction between all three constituents is central to a better understanding of FGDs [50]. Metabolites are evidence of reactions and biochemical processes [341] and thus are a powerful tool to contribute towards a systems-level understanding of disease conditions. Metabolites are the by-products and end-products of an individual's environment, providing evidence of the interaction between the microbiome and the host. The multi-factorial nature and heterogeneity make understanding FGDs particularly difficult, as does the reliance on symptom-based questionnaires [340], thus there is a need to examine objective measures such as metabolites, concurrently with other indices.

The overall aim of this PhD project was to utilise MS-based approaches to investigate metabolites that may distinguish biochemical mechanisms perturbed in individuals with FGDs from healthy controls and within FGD subtypes. This PhD sits within the HVN Digestive Health priority research programme, which aims to better understand FGDs and determine the effectiveness of dietary interventions on improving FGDs symptoms, and the underlying physiological and molecular responses. **Chapter 3** and **Chapter 4** utilised analytical chemistry to investigate targeted groups of metabolites (bile acids and amino acids) known to be important mechanistically for differentiating between healthy and FGD states. In **Chapter 5**, untargeted MS methods were used to characterise metabolites of the faecal metabolome which are reflective of localised gut processes and could inform further investigations. Finally, **Chapter 6** integrated the faecal metabolome with other datasets generated by PhD candidates and researchers in the wider programme

(faecal microbiome, plasma metabolome and dietary intake) to move toward a systems biology view of important factors underlying FGDs.

In **Chapters 3** and **4**, comparisons were made between all FGD groups, and combined groups of constipation (FC + IBS-C) and diarrhoea (FD + IBS-D) relative to healthy controls. In **Chapters 5** and **6**, the aim was to understand potential mechanistic differences in the gut between constipation (FC + IBS-C) and healthy controls, or diarrhoea (FD + IBS-D) and healthy controls to highlight key areas for future research.

Bile acids are an important group of metabolites that link dietary intake, gut microbial modifications, and host processes, and have been shown to be important in FGD conditions, especially in individuals with diarrhoea [91-93, 236]. **Chapter 3** showed that the concentration of faecal bile acids was perturbed between FGDs and healthy controls. Furthermore, the analysis showed that diarrhoea conditions were associated with increased bile acid excretion compared to healthy controls and those with constipation. These findings are in agreement with other authors who have shown that BAM could underlie cases of diarrhoea [91, 93]. In addition, the current study was the first to quantify the faecal bile acid profile in IBS-C and IBS-D, and their functional counterparts. No differences in bile acid concentrations were evident between functional constipation and IBS-C, or functional diarrhoea and IBS-D. Therefore, bile acids could not differentiate between the presence or absence of pain in conditions with the same symptoms.

Within the COMFORT cohort, differences in quantitative concentration of bile acids in faecal samples of the participants highlighted the value of bile acids to better understand FGDs, and their potential use as a biomarker to classify individuals without sole reliance on the Rome Criteria. The latter aspect will require validation in distinct cohorts of FGDs and healthy participants. Additionally, bile acids could be utilised to monitor the efficacy

of dietary interventions that aimed to ameliorate symptoms in participants with FGDs. Future studies investigating other bile acid targets, for example, the FXR receptor and serum C4, could highlight the origin of bile acid perturbations. Furthermore, as bile acids are produced from cholesterol and secreted following dietary intake, their link to dietary consumption may be important and warrants further investigation. Additionally, dietary consumption can increase or decrease the abundance of microbial species with the BSH enzyme. Thus, understanding further the link between diet, microbiome and bile acid metabolism is necessary.

Amino acids, metabolites linked to dietary intake and microbial processing, can have important biochemical effects throughout the body. Most amino acids are primarily obtained through dietary intake, and a certain quantity comes from endogenous host origin or are produced by the gut microbiome [342]. However, studies have only reported a few amino acids as part of wider biomarker panels or detected some as part of an untargeted approach [43, 77, 124, 276, 307]. Based on this, it was hypothesised that plasma amino acid concentrations might differ between FGD subtypes and healthy controls reflective of altered dietary intake of amino acids or perturbed microbial production, host utilisation, or host endogenous origin.

The findings from **Chapter 4** highlighted that individual amino acid concentrations did not differ between any of the FGD subtypes or in the combined constipation (FC + IBS-C) and diarrhoea (FD + IBS-D) groups when these combined groups were compared to healthy controls. The analysis of amino acid groups (e.g., BCAA, NEAA) also showed similar results with only the BCAA group being significantly different. The lack of difference in circulating plasma amino acid concentration suggests they might not reflect differences in diet-host-microbial interactions between individuals with FGDs and healthy controls. However, it is important to consider that a limitation of this analysis was

that the measurement of circulating plasma amino acids in a single sample is a snapshot and thus not representative of the flux of amino acids in the body.

The faecal metabolome compared to circulatory fluids provides more direct evidence of biochemical reactions in the gut [264] in response to changes in dietary intake, and host and microbial processes. Therefore, it was hypothesised that the faecal metabolome would highlight biochemical processes that may distinguish individuals with constipation (FC + IBS-C) from healthy controls, and diarrhoea (FD + IBS-D) from healthy controls. In addition, that this data may help identify metabolic hubs highlighting altered biochemical mechanisms which could be investigated using quantitative analytical chemistry.

The untargeted MS approach, combining analysis of semi-polar, polar, and lipid metabolites, used in **Chapter 5** provided broad coverage of the faecal metabolome. The findings showed differences in the faecal lipidome between constipation and healthy control groups, and between diarrhoea and healthy control groups, reflecting lipid perturbations across multiple lipid classes. Ceramides, for example, showed distinct differences between constipation or diarrhoea groups relative to healthy controls. Recent evidence has highlighted the importance of ceramides in conditions of inflammation and showed strong links to microbial species, many of which either have beneficial or detrimental effects to gut health [277, 278, 286, 288]. The analysis of polar and semi-polar metabolites highlighted differentially abundant metabolites between constipation and healthy controls, and diarrhoea and healthy controls; however, these differences did not reflect perturbations to pathways or metabolic hubs, though this may be a limitation of microbial metabolite annotation. Similar to others [77, 290, 291], the abundance of riboflavin, nicotinic acid, and homovanillic acid differed between individuals with FGDs compared to healthy controls.

Although widely studied, understanding of the causative mechanisms behind FGDs remains fragmented partly due to the reductionist approach of investigating singular components separately. Systems biology approaches are increasingly utilised to understand the interactions between multiple aspects of a biological system and how this may contribute to the onset and severity of disorders [306]. Other datasets from fellow collaborators working on the COMFORT cohort study (PhD candidate Caterina Carco, Dr Phoebe Heenan (former PhD candidate), Dr Karl Fraser) were used to investigate the relationship between the microbiome, dietary intake, and the plasma and faecal metabolomes of participants with constipation compared to healthy controls, and participants with diarrhoea compared to healthy controls using a systems biology approach. **Chapter 6** demonstrated that the faecal lipidome was important to explain the correlations between the plasma metabolome, faecal microbiome and dietary intake regardless of grouping.

The importance of faecal lipids in **Chapters 5** and **6** highlighted that lipids could be a new area to investigate for advancing the understanding of FGDs, and their association with diet and the microbiome. As lipids can arise from the breakdown of dietary intake, microbial metabolism, microbial cell lysis, mucin degradation, and/or host metabolism, the inability to classify the origin of lipids makes elucidating mechanisms difficult. A better characterisation of the origin of lipids, e.g., microbial, host, dietary, or the product of these interactions is necessary to advance the understanding of FGDs. Utilising a targeted analytical approach to investigate faecal lipids would highlight quantitative differences between FGD and healthy control groups.

The quantification of bile acids and characterisation of the faecal metabolome carried out here are more comprehensive compared to other studies where smaller cohorts were used

or lipidomic analyses were excluded [77, 91, 93, 236], both which are central to the findings reported in this thesis.

7.2. Limitations

As dietary intake is linked to bile acids, amino acids, and the faecal metabolome, the variation in dietary consumption between individuals makes comparisons difficult. In this study, diet data were recorded as nutrient-based constituents. This classification does not account for the bioavailability or microbial assimilation of nutrients. Additionally, the reliance on participants to accurately record their dietary intake does present a substantial limitation when trying to accurately identify metabolites of dietary origin. Although the use of at-home collection kits is common in many cohorts, it might alter the variability of bacterial composition [343]. Sample collection and handling that is conducted within clinics will help to reduce potential sources of variation, however, the timely defaecation in individuals with varying bowel movements (several bowel motions a day in diarrhoea to once a week in constipation) might reduce the number of faecal samples collected and render the logistics of the study impracticable.

Within FGDs, the grouping of participants into defined groups based on symptom-based questionnaires might present limitations to understanding the biology that may contribute to symptomology. The multi-factorial nature of FGDs, combined with the complexity and influence of lifestyle, ethnicity, sex, age, psychology, homeostatic bodily functions etc., presents limitations to understanding FGDs as what is important in one individual, may not be the same for another individual. Based on the variability of FGD symptoms between people and within the same individual, a more personalised approach might be the best option for future research. As previously shown, the relationship between dietary intake and microbial abundance is highly personalised in healthy individuals [309], and it can therefore be postulated that this would also be evident in people with FGDs. The

personalised approach should include longitudinal multi-omics to understand how the metabolome and microbiome respond over time.

Further correlations and comparisons could be made from the data collected in this thesis. For example, in **Chapters 3** and **4**, comparisons were made across all FGDs, and between constipation, diarrhoea and healthy control groups. However, in **Chapters 5** and **6**, comparisons were only made between constipation (FC + IBS-C) and healthy control groups, or between diarrhoea (FD + IBS-D) and healthy control groups. This choice is because I focused on analysing constipation and diarrhoea groups separately from the healthy control group, to initially understand better what differs between a healthy and 'unhealthy' gut. Ongoing analyses within the wider programme will extend the analysis to compare constipation and diarrhoea groups or even within IBS subtypes. The findings from these studies would be crucial for developing a more personalised nutritional intervention for ameliorating FGD symptoms. **Figure 7-1** shows a lack of similarity between the possible comparisons of the annotated VIP faecal metabolites that were important to the PLS-DA separation of participant groups in **Chapter 5**. This figure highlights why separate analyses are required, as what is important in one group was not the same for other groups.

The combined analysis of female and male participants within the FGD groups is another limitation of this study. Bile acids, amino acids and lipids have previously been shown to differ between sexes [344-347] and thus not analysing the effect of sex between the groups may present potential biases to the results of this study. However, in this cohort investigating the influence of sex between the FGD groups would not have been statistically feasible based on the low proportion of male versus female participants. The high proportion of females compared to males in this study is representative of a true FGD population where females are more likely to have FGDs compared to males. The

influence of sex would be an important aspect to investigate in a large FGD population, however recruitment and trial design would need to be focused on obtaining an equal distribution of male and female participants for such analyses to be feasible.

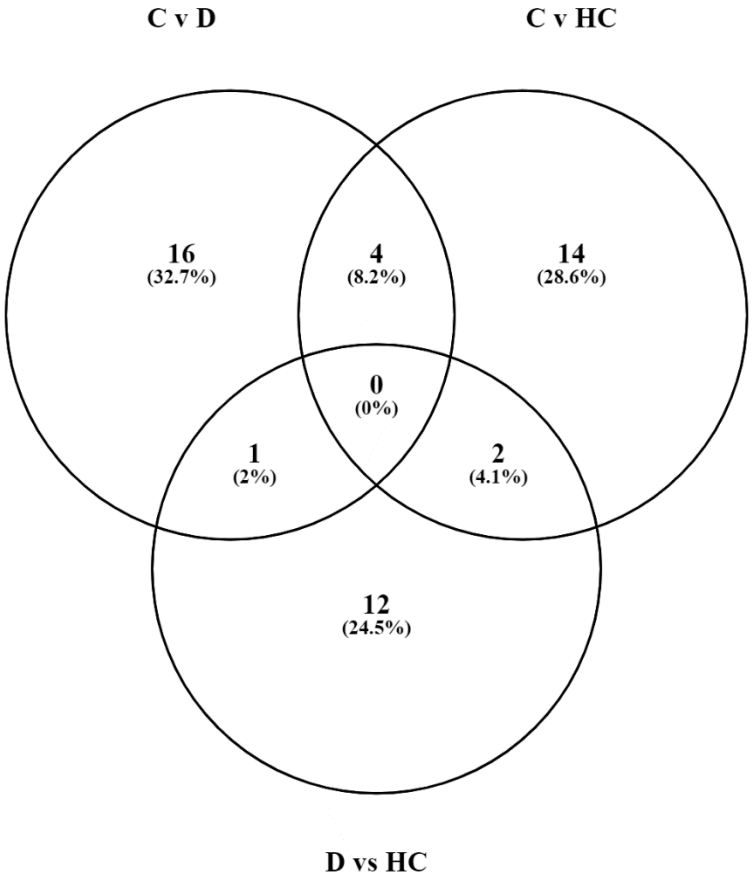


Figure 7-1: Venn diagram highlights possible comparisons that could be made and similarities and dissimilarities between each based on annotated faecal metabolites (**Chapter 5**). Abbreviations: constipation (FC + IBS-C): C, diarrhoea (FD + IBS-D): D, healthy control: HC.

Databases such as HMDB are valuable tools for annotating metabolic features. However, these are limited to annotating human-based metabolites. As reported in this thesis, microbial metabolites likely account for a substantial proportion of faecal metabolites detected but not annotated. This unannotated metabolome represents potentially important metabolites that may provide further insight into pathways underlying FGDs.

It is well established that the gut microbiome influences host health in many ways, such as the production of SCFAs. However, further insights into the mutualistic relationship remain limited without annotation of bacterial metabolites. Even with the extensive multi-omics technologies available, the annotation of unknown metabolites is a significant limitation hindering the progress of this field. Identifying and developing a human microbiome-related metabolite library would advance understanding the relationship between diet, the gut microbiome, and the host, and its overall influence on health.

Whilst LC-MS technologies are desirable for broad coverage detection of compounds, the use of further technologies such as nuclear magnetic resonance (NMR) or gas chromatography-mass spectrometry (GC-MS) might elucidate further metabolites of importance. For example, future analyses that combine NMR for the detection of high concentration peaks, and GC-MS for low molecular weight volatile molecules or molecules that do not ionise well by LC-MS, might provide further pathway linkages. However, NMR and GC-MS are unable to detect lipids and thus in this study, LC-MS was the preferential technology to use.

7.3. Future directions

Over the past few years, new methods such as dried blood spots have rapidly advanced the collection of samples that can be utilised, both at home and within clinical settings. The use of these less invasive technologies will increase the ease of sample collection and thus make the possibility of longitudinal studies more feasible.

Additional technologies, for example, the Atmo Gas Capsule (Atmo Biosciences, Victoria, Australia), provides an accurate gas concentration profile and knowledge of fermentation events from various locations in the gut. Many of these technologies are coupled to app-based software that delivers a real-time output of physiological measures.

Further links to artificial intelligence will provide individuals with personalised information (e.g., probiotics and prebiotics) to benefit their gut health. The progression from manual documentation using symptom-based questionnaires to accurate real-time recordings will significantly progress understanding FGDs. Additionally, utilising this information coupled with dietary intake, gut microbial abundance, and multiple metabolomes will provide insight into system-wide metabolic and physiological perturbations of FGDs.

7.4. Conclusion

Finally, this PhD thesis has highlighted the value of measuring targeted metabolites (bile acids) to advance the knowledge of FGDs and possible biomarkers. Characterisation of the faecal metabolome and systems biology analyses of various datasets (dietary intake, plasma metabolome, faecal microbiome) emphasised the interactions and mechanisms, particularly in faecal lipids that may be important in FGDs. This research has advanced the current knowledge and provided future directions for continuing research in FGDs.

References

References

1. Drossman, D.A., *Functional gastrointestinal disorders: history, pathophysiology, clinical features, and Rome IV.* Gastroenterology, 2016. **150**(6): p. 1262-1279. e2.
2. Sperber, A.D., et al., *Worldwide Prevalence and Burden of Functional Gastrointestinal Disorders, Results of Rome Foundation Global Study.* Gastroenterology, 2021. **160**(1): p. 99-114 e3.
3. Lacy, B.E., F. Mearin, L. Chang, W.D. Chey, A.J. Lembo, M. Simren, and R. Spiller, *Bowel disorders.* Gastroenterology, 2016. **150**(6): p. 1393-1407. e5.
4. Enck, P., et al., *Irritable bowel syndrome.* Nat Rev Dis Primers, 2016. **2**: p. 16014.
5. Sperber, A.D., et al., *The global prevalence of IBS in adults remains elusive due to the heterogeneity of studies: a Rome Foundation working team literature review.* Gut, 2017. **66**(6): p. 1075-1082.
6. Viennois, E., Y. Zhao, and D. Merlin, *Biomarkers of IBD: from classical laboratory tools to personalized medicine.* Inflammatory bowel diseases, 2015. **21**(10): p. 2467-2474.
7. Iskandar, H.N. and M.A. Ciorba, *Biomarkers in Inflammatory Bowel Disease: Current Practices and Recent Advances.* Translational Research, 2012. **159**(4): p. 313-325.
8. Lewis, J.D., *The utility of biomarkers in the diagnosis and therapy of inflammatory bowel disease.* Gastroenterology, 2011. **140**(6): p. 1817-1826. e2.
9. Tuck, C.J. and S.J. Vanner, *Dietary therapies for functional bowel symptoms: Recent advances, challenges, and future directions.* Neurogastroenterology and Motility, 2018. **30**(1).
10. Hayes, P., C. Corish, E. O'mahony, and E. Quigley, *A dietary survey of patients with irritable bowel syndrome.* Journal of human nutrition and dietetics, 2014. **27**(s2): p. 36-47.
11. Halmos, E.P., V.A. Power, S.J. Shepherd, P.R. Gibson, and J.G. Muir, *A diet low in FODMAPs reduces symptoms of irritable bowel syndrome.* Gastroenterology, 2014. **146**(1): p. 67-75. e5.
12. Halpert, A., C.B. Dalton, O. Palsson, C. Morris, Y. Hu, S. Bangdiwala, J. Hankins, N. Norton, and D. Drossman, *What patients know about irritable bowel syndrome (IBS) and what they would like to know. National Survey on Patient Educational Needs in IBS and development and validation of the Patient Educational Needs Questionnaire (PEQ).* The American journal of gastroenterology, 2007. **102**(9): p. 1972.
13. Böhn, L., S. Störsrud, H. Törnblom, U. Bengtsson, and M. Simrén, *Self-reported food-related gastrointestinal symptoms in IBS are common and associated with more severe symptoms and reduced quality of life.* The American journal of gastroenterology, 2013. **108**(5): p. 634.
14. Monsbakken, K.W., P.O. Vandvik, and P.G. Farup, *Perceived food intolerance in subjects with irritable bowel syndrome – etiology, prevalence and consequences.* European Journal Of Clinical Nutrition, 2005. **60**: p. 667.
15. Rej, A., I. Aziz, H. Tornblom, D.S. Sanders, and M. Simren, *The role of diet in irritable bowel syndrome: implications for dietary advice.* J Intern Med, 2019. **286**(5): p. 490-502.
16. EFSA Panel on Dietetic Products, N. and Allergies, *Guidance on the scientific requirements for health claims related to gut and immune function.* EFSA Journal, 2011. **9**(4): p. 1984.
17. Guinane, C.M. and P.D. Cotter, *Role of the gut microbiota in health and chronic gastrointestinal disease: understanding a hidden metabolic organ.* Therapeutic advances in gastroenterology, 2013. **6**(4): p. 295-308.
18. Vernocchi, P., F. Del Chierico, and L. Putignani, *Gut microbiota profiling: metabolomics based approach to unravel compounds affecting human health.* Frontiers in Microbiology, 2016. **7**: p. 1144.
19. Bischoff, S.C., *'Gut health': a new objective in medicine?* BMC medicine, 2011. **9**(1): p. 24.

20. Jones, M., W. Chey, S. Singh, H. Gong, R. Shringarpure, N. Hoe, E. Chuang, and N. Talley, *A biomarker panel and psychological morbidity differentiates the irritable bowel syndrome from health and provides novel pathophysiological leads.* Alimentary pharmacology & therapeutics, 2014. **39**(4): p. 426-437.
21. Schubert, M.L. and D.A. Peura, *Control of Gastric Acid Secretion in Health and Disease.* Gastroenterology, 2008. **134**(7): p. 1842-1860.
22. O'Hara, A.M. and F. Shanahan, *The gut flora as a forgotten organ.* EMBO reports, 2006. **7**(7): p. 688-693.
23. Marchesi, J.R. and J. Ravel, *The vocabulary of microbiome research: a proposal*. 2015, BioMed Central.
24. Hugon, P., J.-C. Lagier, P. Colson, F. Bittar, and D. Raoult, *Repertoire of human gut microbes.* Microbial pathogenesis, 2017. **106**: p. 103-112.
25. Jeffery, I.B. and P.W. O'Toole, *Diet-microbiota interactions and their implications for healthy living.* Nutrients, 2013. **5**(1): p. 234-52.
26. Round, J.L. and S.K. Mazmanian, *The gut microbiota shapes intestinal immune responses during health and disease.* Nature Reviews Immunology, 2009. **9**: p. 313.
27. Rowland, I., G. Gibson, A. Heinken, K. Scott, J. Swann, I. Thiele, and K. Tuohy, *Gut microbiota functions: metabolism of nutrients and other food components.* European Journal of Nutrition, 2017: p. 1-24.
28. Kau, A.L., P.P. Ahern, N.W. Griffin, A.L. Goodman, and J.I. Gordon, *Human nutrition, the gut microbiome and the immune system.* Nature, 2011. **474**(7351): p. 327.
29. Man, A.L., N. Gicheva, and C. Nicoletti, *The impact of ageing on the intestinal epithelial barrier and immune system.* Cellular immunology, 2014. **289**(1): p. 112-118.
30. Ley, R.E., P.J. Turnbaugh, S. Klein, and J.I. Gordon, *Microbial ecology: human gut microbes associated with obesity.* Nature, 2006. **444**(7122): p. 1022-1023.
31. Malinen, E., L. Krogius-Kurikka, A. Lyra, J. Nikkilä, A. Jääskeläinen, T. Rinttilä, T. Vilpponen-Salmela, A.J. von Wright, and A. Palva, *Association of symptoms with gastrointestinal microbiota in irritable bowel syndrome.* World journal of gastroenterology: WJG, 2010. **16**(36): p. 4532.
32. David, L.A., C.F. Maurice, R.N. Carmody, D.B. Gootenberg, J.E. Button, B.E. Wolfe, A.V. Ling, A.S. Devlin, Y. Varma, and M.A. Fischbach, *Diet rapidly and reproducibly alters the human gut microbiome.* Nature, 2014. **505**(7484): p. 559-563.
33. Huerta-Franco, M.-R., M. Vargas-Luna, P. Tienda, I. Delgadillo-Holtfort, M. Balleza-Ordaz, and C. Flores-Hernandez, *Effects of occupational stress on the gastrointestinal tract.* World journal of gastrointestinal pathophysiology, 2013. **4**(4): p. 108.
34. Larauche, M., A. Mulak, and Y. Taché, *Stress and visceral pain: from animal models to clinical therapies.* Experimental neurology, 2012. **233**(1): p. 49-67.
35. Thompson, W.G., *The road to rome.* Gastroenterology, 2006. **130**(5): p. 1552-6.
36. Ministro, P. and D. Martins, *Fecal biomarkers in inflammatory bowel disease: how, when and why?* Expert Review of Gastroenterology & Hepatology, 2017. **11**(4): p. 317-328.
37. Camilleri, M., H. Halawi, and I. Oduyebo, *Biomarkers as a diagnostic tool for irritable bowel syndrome: where are we?* Expert Review of Gastroenterology & Hepatology, 2017. **11**(4): p. 303-316.
38. Stern, E.K. and D.M. Brenner, *Gut Microbiota-Based Therapies for Irritable Bowel Syndrome.* Clinical And Translational Gastroenterology, 2018. **9**: p. e134.
39. Harris, L.A. and N. Baffy, *Modulation of the gut microbiota: a focus on treatments for irritable bowel syndrome.* Postgraduate medicine, 2017. **129**(8): p. 872-888.
40. Canavan, C., J. West, and T. Card, *The epidemiology of irritable bowel syndrome.* Clinical epidemiology, 2014. **6**: p. 71.
41. Wang, Y.T., H.Y. Lim, D. Tai, T.L. Krishnamoorthy, T. Tan, S. Barbier, and J. Thumboo, *The impact of irritable bowel syndrome on health-related quality of life: a Singapore perspective.* BMC gastroenterology, 2012. **12**(1): p. 104.
42. Farup, P.G., K. Rudi, and K. Hestad, *Faecal short-chain fatty acids-a diagnostic biomarker for irritable bowel syndrome?* BMC gastroenterology, 2016. **16**(1): p. 51.

43. Clarke, G., E.M. Quigley, J.F. Cryan, and T.G. Dinan, *Irritable bowel syndrome: towards biomarker identification.* Trends in Molecular Medicine, 2009. **15**(10): p. 478-489.
44. Krautkramer, K.A., J. Fan, and F. Backhed, *Gut microbial metabolites as multi-kingdom intermediates.* Nat Rev Microbiol, 2021. **19**(2): p. 77-94.
45. Rinninella, E., P. Raoul, M. Cintoni, F. Franceschi, G.A.D. Miggiano, A. Gasbarrini, and M.C. Mele, *What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases.* Microorganisms, 2019. **7**(1).
46. Chong, P.P., V.K. Chin, C.Y. Looi, W.F. Wong, P. Madhavan, and V.C. Yong, *The Microbiome and Irritable Bowel Syndrome - A Review on the Pathophysiology, Current Research and Future Therapy.* Front Microbiol, 2019. **10**: p. 1136.
47. Menees, S. and W. Chey, *The gut microbiome and irritable bowel syndrome.* F1000Res, 2018. **7**.
48. Pittayanon, R., J.T. Lau, Y. Yuan, G.I. Leontiadis, F. Tse, M. Surette, and P. Moayyedi, *Gut Microbiota in Patients With Irritable Bowel Syndrome-A Systematic Review.* Gastroenterology, 2019. **157**(1): p. 97-108.
49. Andrews, C.N., S. Sidani, and J.K. Marshall, *Clinical Management of the Microbiome in Irritable Bowel Syndrome.* J Can Assoc Gastroenterol, 2021. **4**(1): p. 36-43.
50. Pimentel, M. and A. Lembo, *Microbiome and Its Role in Irritable Bowel Syndrome.* Dig Dis Sci, 2020. **65**(3): p. 829-839.
51. Schmidt, T.S., J. Raes, and P. Bork, *The human gut microbiome: from association to modulation.* Cell, 2018. **172**(6): p. 1198-1215.
52. Nicholson, J.K., E. Holmes, J. Kinross, R. Burcelin, G. Gibson, W. Jia, and S. Pettersson, *Host-gut microbiota metabolic interactions.* Science, 2012: p. 1223813.
53. Houghteling, P.D. and W.A. Walker, *Why is initial bacterial colonization of the intestine important to the infant's and child's health?* Journal of pediatric gastroenterology and nutrition, 2015. **60**(3): p. 294-307.
54. Manor, O., C.L. Dai, S.A. Kornilov, B. Smith, N.D. Price, J.C. Lovejoy, S.M. Gibbons, and A.T. Magis, *Health and disease markers correlate with gut microbiome composition across thousands of people.* Nat Commun, 2020. **11**(1): p. 5206.
55. Huttenhower, C., D. Gevers, R. Knight, S. Abubucker, J.H. Badger, A.T. Chinwalla, H.H. Creasy, A.M. Earl, M.G. FitzGerald, and R.S. Fulton, *Structure, function and diversity of the healthy human microbiome.* Nature, 2012. **486**(7402): p. 207.
56. Arumugam, M., J. Raes, E. Pelletier, D. Le Paslier, T. Yamada, D.R. Mende, G.R. Fernandes, J. Tap, T. Bruls, and J.-M. Batto, *Enterotypes of the human gut microbiome.* Nature, 2011. **473**(7346): p. 174.
57. Falony, G., M. Joossens, S. Vieira-Silva, J. Wang, Y. Darzi, K. Faust, A. Kurilshikov, M.J. Bonder, M. Valles-Colomer, and D. Vandeputte, *Population-level analysis of gut microbiome variation.* Science, 2016. **352**(6285): p. 560-564.
58. Wu, G.D., C. Compher, E.Z. Chen, S.A. Smith, R.D. Shah, K. Bittinger, C. Chehoud, L.G. Albenberg, L. Nessel, and E. Gilroy, *Comparative metabolomics in vegans and omnivores reveal constraints on diet-dependent gut microbiota metabolite production.* Gut, 2014: p. gutjnl-2014-308209.
59. Qin, J., R. Li, J. Raes, M. Arumugam, K.S. Burgdorf, C. Manichanh, T. Nielsen, N. Pons, F. Levenez, and T. Yamada, *A human gut microbial gene catalogue established by metagenomic sequencing.* Nature, 2010. **464**(7285): p. 59-65.
60. Le Chatelier, E., et al., *Richness of human gut microbiome correlates with metabolic markers.* Nature, 2013. **500**: p. 541.
61. Hold, G.L., S.E. Pryde, V.J. Russell, E. Furrie, and H.J. Flint, *Assessment of microbial diversity in human colonic samples by 16S rDNA sequence analysis.* FEMS microbiology ecology, 2002. **39**(1): p. 33-39.
62. Turnbaugh, P.J., R.E. Ley, M. Hamady, C.M. Fraser-Liggett, R. Knight, and J.I. Gordon, *The human microbiome project.* Nature, 2007. **449**(7164): p. 804.
63. Eckburg, P.B., E.M. Bik, C.N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, S.R. Gill, K.E. Nelson, and D.A. Relman, *Diversity of the human intestinal microbial flora.* Science, 2005. **308**(5728): p. 1635-1638.

64. Lozupone, C.A., J.I. Stombaugh, J.I. Gordon, J.K. Jansson, and R. Knight, *Diversity, stability and resilience of the human gut microbiota.* Nature, 2012. **489**(7415): p. 220.
65. Mueller, S., K. Saunier, C. Hanisch, E. Norin, L. Alm, T. Midtvedt, A. Cresci, S. Silvi, C. Orpianesi, and M.C. Verdenelli, *Differences in fecal microbiota in different European study populations in relation to age, gender, and country: a cross-sectional study.* Applied and environmental microbiology, 2006. **72**(2): p. 1027-1033.
66. Vandeputte, D., G. Falony, S. Vieira-Silva, R.Y. Tito, M. Joossens, and J. Raes, *Stool consistency is strongly associated with gut microbiota richness and composition, enterotypes and bacterial growth rates.* Gut, 2016. **65**(1): p. 57-62.
67. Gorkiewicz, G., G.G. Thallinger, S. Trajanoski, S. Lackner, G. Stocker, T. Hinterleitner, C. Gülly, and C. Högenauer, *Alterations in the colonic microbiota in response to osmotic diarrhea.* PLoS One, 2013. **8**(2): p. e55817.
68. Mayer, E.A., T. Savidge, and R.J. Shulman, *Brain–gut microbiome interactions and functional bowel disorders.* Gastroenterology, 2014. **146**(6): p. 1500-1512.
69. Jeffery, I.B., P.W. O'Toole, L. Öhman, M.J. Claesson, J. Deane, E.M. Quigley, and M. Simrén, *An irritable bowel syndrome subtype defined by species-specific alterations in faecal microbiota.* Gut, 2012. **61**(7): p. 997-1006.
70. Rajilić–Stojanović, M., E. Biagi, H.G. Heilig, K. Kajander, R.A. Kekkonen, S. Tims, and W.M. de Vos, *Global and deep molecular analysis of microbiota signatures in fecal samples from patients with irritable bowel syndrome.* Gastroenterology, 2011. **141**(5): p. 1792-1801.
71. Saulnier, D.M., K. Riehle, T.A. Mistretta, M.A. Diaz, D. Mandal, S. Raza, E.M. Weidler, X. Qin, C. Coarfa, and A. Milosavljevic, *Gastrointestinal microbiome signatures of pediatric patients with irritable bowel syndrome.* Gastroenterology, 2011. **141**(5): p. 1782-1791.
72. Tap, J., M. Derrien, H. Törnblom, R. Brazeilles, S. Cools-Portier, J. Doré, S. Störsrud, B. Le Nevé, L. Öhman, and M. Simrén, *Identification of an intestinal microbiota signature associated with severity of irritable bowel syndrome.* Gastroenterology, 2017. **152**(1): p. 111-123. e8.
73. Labus, J.S., E.B. Hollister, J. Jacobs, K. Kirbach, N. Oezguen, A. Gupta, J. Acosta, R.A. Luna, K. Aagaard, and J. Versalovic, *Differences in gut microbial composition correlate with regional brain volumes in irritable bowel syndrome.* Microbiome, 2017. **5**(1): p. 49.
74. Zeber-Lubecka, N., M. Kulecka, F. Ambrozkiewicz, A. Paziewska, K. Goryca, J. Karczmarski, T. Rubel, W. Wojtowicz, P. Mlynarz, and L. Marczak, *Limited prolonged effects of rifaximin treatment on irritable bowel syndrome-related differences in the fecal microbiome and metabolome.* Gut Microbes, 2016. **7**(5): p. 397-413.
75. Pozuelo, M., S. Panda, A. Santiago, S. Mendez, A. Accarino, J. Santos, F. Guarner, F. Azpiroz, and C. Manichanh, *Reduction of butyrate- and methane-producing microorganisms in patients with Irritable Bowel Syndrome.* Sci Rep, 2015. **5**: p. 12693.
76. Ringel-Kulka, T., C.H. Choi, D. Temas, A. Kim, D.M. Maier, K. Scott, J.A. Galanko, and Y. Ringel, *Altered colonic bacterial fermentation as a potential pathophysiological factor in irritable bowel syndrome.* The American journal of gastroenterology, 2015. **110**(9): p. 1339.
77. Jeffery, I.B., et al., *Differences in Fecal Microbiomes and Metabolomes of People With vs Without Irritable Bowel Syndrome and Bile Acid Malabsorption.* Gastroenterology, 2020. **158**(4): p. 1016-1028 e8.
78. Chung, C.S., P.F. Chang, C.H. Liao, T.H. Lee, Y. Chen, Y.C. Lee, M.S. Wu, H.P. Wang, and Y.H. Ni, *Differences of microbiota in small bowel and faeces between irritable bowel syndrome patients and healthy subjects.* Scand J Gastroenterol, 2016. **51**(4): p. 410-9.
79. Durban, A., J.J. Abellan, N. Jimenez-Hernandez, P. Salgado, M. Ponce, J. Ponce, V. Garrigues, A. Latorre, and A. Moya, *Structural alterations of faecal and mucosa-associated bacterial communities in irritable bowel syndrome.* Environ Microbiol Rep, 2012. **4**(2): p. 242-7.

80. Hugerth, L.W., A. Andreasson, N.J. Talley, A.M. Forsberg, L. Kjellstrom, P.T. Schmidt, L. Agreus, and L. Engstrand, *No distinct microbiome signature of irritable bowel syndrome found in a Swedish random population.* Gut, 2020. **69**(6): p. 1076-1084.
81. Carroll, I.M., T. Ringel-Kulka, T.O. Keku, Y.-H. Chang, C.D. Packey, R.B. Sartor, and Y. Ringel, *Molecular analysis of the luminal-and mucosal-associated intestinal microbiota in diarrhea-predominant irritable bowel syndrome.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 2011. **301**(5): p. G799-G807.
82. Duan, R., S. Zhu, B. Wang, and L. Duan, *Alterations of Gut Microbiota in Patients With Irritable Bowel Syndrome Based on 16S rRNA-Targeted Sequencing: A Systematic Review.* Clin Transl Gastroenterol, 2019. **10**(2): p. e00012.
83. Ruan, W., M.A. Engevik, J.K. Spinler, and J. Versalovic, *Healthy Human Gastrointestinal Microbiome: Composition and Function After a Decade of Exploration.* Dig Dis Sci, 2020. **65**(3): p. 695-705.
84. Canani, R.B., M. Di Costanzo, L. Leone, M. Pedata, R. Meli, and A. Calignano, *Potential beneficial effects of butyrate in intestinal and extraintestinal diseases.* World journal of gastroenterology: WJG, 2011. **17**(12): p. 1519.
85. Camilleri, M., *What's new in functional and motility disorders in the lower GI tract?* Malta Medical Journal, 2017. **29**(2): p. 3-13.
86. Camilleri, M., A. Shin, I. Busciglio, P. Carlson, A. Acosta, A.E. Bharucha, D. Burton, J. Lamsam, A. Lueke, and L.J. Donato, *Validating biomarkers of treatable mechanisms in irritable bowel syndrome.* Neurogastroenterology & Motility, 2014. **26**(12): p. 1677-1685.
87. Camilleri, M., I. Oduyebo, and H. Halawi, *Chemical and molecular factors in irritable bowel syndrome: Current knowledge, challenges, and unanswered questions.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 2016. **311**(5): p. G777-G784.
88. Long, S.L., C.G.M. Gahan, and S.A. Joyce, *Interactions between gut bacteria and bile in health and disease.* Mol Aspects Med, 2017. **56**: p. 54-65.
89. Joyce, S.A. and C.G.M. Gahan, *Bile Acid Modifications at the Microbe-Host Interface: Potential for Nutraceutical and Pharmaceutical Interventions in Host Health.* Annual Review of Food Science and Technology, 2016. **7**: p. 313-333.
90. Zheng, X., et al., *Bile acid is a significant host factor shaping the gut microbiome of diet-induced obese mice.* BMC Biology, 2017. **15**(1).
91. Shin, A., M. Camilleri, P. Vijayvargiya, I. Busciglio, D. Burton, M. Ryks, D. Rhoten, A. Lueke, A. Saenger, and A. Girtman, *Bowel functions, fecal unconjugated primary and secondary bile acids, and colonic transit in patients with irritable bowel syndrome.* Clinical Gastroenterology and Hepatology, 2013. **11**(10): p. 1270-1275. e1.
92. Slattery, S.A., O. Niaz, Q. Aziz, A.C. Ford, and A.D. Farmer, *Systematic review with meta-analysis: the prevalence of bile acid malabsorption in the irritable bowel syndrome with diarrhoea.* Aliment Pharmacol Ther, 2015. **42**(1): p. 3-11.
93. Wong, B.S., M. Camilleri, P. Carlson, S. McKinzie, I. Busciglio, O. Bondar, R.B. Dyer, J. Lamsam, and A.R. Zinsmeister, *Increased bile acid biosynthesis is associated with irritable bowel syndrome with diarrhea.* Clinical Gastroenterology and Hepatology, 2012. **10**(9): p. 1009-1015. e3.
94. Sadik, R., H. Abrahamsson, K.-A. Ung, and P.-O. Stotzer, *Accelerated regional bowel transit and overweight shown in idiopathic bile acid malabsorption.* The American journal of gastroenterology, 2004. **99**(4): p. 711.
95. Makishima, M., T.T. Lu, W. Xie, G.K. Whitfield, H. Domoto, R.M. Evans, M.R. Haussler, and D.J. Mangelsdorf, *Vitamin D receptor as an intestinal bile acid sensor.* Science, 2002. **296**(5571): p. 1313-1316.
96. Odunsi–Shiyanbade, S.T., M. Camilleri, S. McKinzie, D. Burton, P. Carlson, I.A. Busciglio, J. Lamsam, R. Singh, and A.R. Zinsmeister, *Effects of chenodeoxycholate and a bile acid sequestrant, colesevelam, on intestinal transit and bowel function.* Clinical Gastroenterology and Hepatology, 2010. **8**(2): p. 159-165. e5.

97. Dior, M., et al., *Interplay between bile acid metabolism and microbiota in irritable bowel syndrome.* Neurogastroenterology and Motility, 2016. **28**(9): p. 1330-1340.
98. Vijayvargiya, P., I. Busciglio, D. Burton, L. Donato, A. Lueke, and M. Camilleri, *Bile Acid Deficiency in a Subgroup of Patients With Irritable Bowel Syndrome With Constipation Based on Biomarkers in Serum and Fecal Samples.* Clinical Gastroenterology and Hepatology, 2017.
99. Duboc, H., D. Rainteau, S. Rajca, L. Humbert, D. Farabos, M. Maubert, V. Grondin, P. Jouet, D. Bouhassira, and P. Seksik, *Increase in fecal primary bile acids and dysbiosis in patients with diarrhea-predominant irritable bowel syndrome.* Neurogastroenterology & Motility, 2012. **24**(6): p. 513.
100. Huda-Faujan, N., A. Abdulamir, A. Fatimah, O.M. Anas, M. Shuhaimi, A. Yazid, and Y. Loong, *The impact of the level of the intestinal short chain fatty acids in inflammatory bowel disease patients versus healthy subjects.* The open biochemistry journal, 2010. **4**: p. 53.
101. Furusawa, Y., et al., *Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells.* Nature, 2013. **504**(7480): p. 446-50.
102. Vital, M., A. Karch, and D.H. Pieper, *Colonic Butyrate-Producing Communities in Humans: an Overview Using Omics Data.* mSystems, 2017. **2**(6): p. e00130-17.
103. Barbara, G., C. Feinle-Bisset, U.C. Ghoshal, J. Santos, S.J. Vanner, N. Vergnolle, E.G. Zoetendal, and E.M. Quigley, *The intestinal microenvironment and functional gastrointestinal disorders.* Gastroenterology, 2016. **150**(6): p. 1305-1318. e8.
104. Louis, P., P. Young, G. Holtrop, and H.J. Flint, *Diversity of human colonic butyrate-producing bacteria revealed by analysis of the butyryl-CoA: acetate CoA-transferase gene.* Environmental microbiology, 2010. **12**(2): p. 304-314.
105. Morrison, D.J. and T. Preston, *Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism.* Gut microbes, 2016. **7**(3): p. 189-200.
106. Lin, H.V., A. Frassetto, E.J. Kowalik Jr, A.R. Nawrocki, M.M. Lu, J.R. Kosinski, J.A. Hubert, D. Szeto, X. Yao, and G. Forrest, *Butyrate and propionate protect against diet-induced obesity and regulate gut hormones via free fatty acid receptor 3-independent mechanisms.* PloS one, 2012. **7**(4): p. e35240.
107. Tana, C., Y. Umesaki, A. Imaoka, T. Handa, M. Kanazawa, and S. Fukudo, *Altered profiles of intestinal microbiota and organic acids may be the origin of symptoms in irritable bowel syndrome.* Neurogastroenterology & Motility, 2010. **22**(5): p. 512.
108. Mortensen, P., J. Andersen, S. Arffmann, and E. Krag, *Short-chain fatty acids and the irritable bowel syndrome: the effect of wheat bran.* Scandinavian journal of gastroenterology, 1987. **22**(2): p. 185-192.
109. Treem, W.R., N. Ahsan, G. Kastoff, and J.S. Hyams, *Fecal short-chain fatty acids in patients with diarrhea-predominant irritable bowel syndrome: in vitro studies of carbohydrate fermentation.* Journal of pediatric gastroenterology and nutrition, 1996. **23**(3): p. 280-286.
110. Ligaarden, S.C. and P.G. Farup, *Low intake of vitamin B6 is associated with irritable bowel syndrome symptoms.* Nutrition research, 2011. **31**(5): p. 356-361.
111. LeBlanc, J.G., C. Milani, G.S. de Giori, F. Sesma, D. van Sinderen, and M. Ventura, *Bacteria as vitamin suppliers to their host: a gut microbiota perspective.* Current opinion in biotechnology, 2013. **24**(2): p. 160-168.
112. Yoshii, K., K. Hosomi, K. Sawane, and J. Kunisawa, *Metabolism of Dietary and Microbial Vitamin B Family in the Regulation of Host Immunity.* Frontiers in Nutrition, 2019. **6**: p. 48.
113. O'Connor, E., E. Barrett, G. Fitzgerald, C. Hill, C. Stanton, and R. Ross, *Production of vitamins, exopolysaccharides and bacteriocins by probiotic bacteria.* Probiotic Dairy Products, 2005: p. 167-194.
114. Hill, M., *Intestinal flora and endogenous vitamin synthesis.* European journal of cancer prevention: the official journal of the European Cancer Prevention Organisation (ECP), 1997. **6**: p. S43-5.

115. Said, H.M. and Z.M. Mohammed, *Intestinal absorption of water-soluble vitamins: an update.* Current opinion in gastroenterology, 2006. **22**(2): p. 140-146.
116. Saibeni, S., M. Cattaneo, M. Vecchi, M.L. Zighetti, A. Lecchi, R. Lombardi, G. Meucci, L. Spina, and R. De Franchis, *Low vitamin B6 plasma levels, a risk factor for thrombosis, in inflammatory bowel disease: role of inflammation and correlation with acute phase reactants.* The American journal of gastroenterology, 2003. **98**(1): p. 112-117.
117. Magnúsdóttir, S., D. Ravcheev, V. de Crécy-Lagard, and I. Thiele, *Systematic genome assessment of B-vitamin biosynthesis suggests co-operation among gut microbes.* Frontiers in genetics, 2015. **6**: p. 148.
118. Kjer-Nielsen, L., O. Patel, A.J. Corbett, J. Le Nours, B. Meehan, L. Liu, M. Bhati, Z. Chen, L. Kostenko, and R. Reantragoon, *MR1 presents microbial vitamin B metabolites to MAIT cells.* Nature, 2012. **491**(7426): p. 717.
119. Krogius-Kurikka, L., A. Lyra, E. Malinen, J. Aarnikunnas, J. Tuimala, L. Paulin, H. Mäkivuokko, K. Kajander, and A. Palva, *Microbial community analysis reveals high level phylogenetic alterations in the overall gastrointestinal microbiota of diarrhoea-predominant irritable bowel syndrome sufferers.* BMC gastroenterology, 2009. **9**(1): p. 95.
120. Berstad, A., J. Raa, and J. Valeur, *Tryptophan: 'essential' for the pathogenesis of irritable bowel syndrome?* Scandinavian journal of gastroenterology, 2014. **49**(12): p. 1493-1498.
121. Heitkemper, M.M., C.J. Han, M.E. Jarrett, H. Gu, D. Djukovic, R.J. Shulman, D. Raftery, W.A. Henderson, and K.C. Cain, *Serum tryptophan metabolite levels during sleep in patients with and without irritable bowel syndrome (IBS).* Biological research for nursing, 2016. **18**(2): p. 193-198.
122. Clarke, G., P. Fitzgerald, J.F. Cryan, E.M. Cassidy, E.M. Quigley, and T.G. Dinan, *Tryptophan degradation in irritable bowel syndrome: evidence of indoleamine 2, 3-dioxygenase activation in a male cohort.* BMC gastroenterology, 2009. **9**(1): p. 6.
123. Clarke, G., D.P. McKernan, G. Gaszner, E.M. Quigley, J.F. Cryan, and T.G. Dinan, *A Distinct Profile of Tryptophan Metabolism along the Kynurenine Pathway Downstream of Toll-Like Receptor Activation in Irritable Bowel Syndrome.* Front Pharmacol, 2012. **3**: p. 90.
124. Keshteli, A.H., K.L. Madsen, R. Mandal, G.E. Boeckxstaens, P. Bercik, G. De Palma, D.E. Reed, D. Wishart, S. Vanner, and L.A. Dieleman, *Comparison of the metabolomic profiles of irritable bowel syndrome patients with ulcerative colitis patients and healthy controls: new insights into pathophysiology and potential biomarkers.* Alimentary pharmacology & therapeutics, 2019.
125. Zhou, Q., M.L. Verne, J.Z. Fields, J.J. Lefante, S. Basra, H. Salameh, and G.N. Verne, *Randomised placebo-controlled trial of dietary glutamine supplements for postinfectious irritable bowel syndrome.* Gut, 2018: p. gutjnl-2017-315136.
126. Cremon, C., et al., *Intestinal Serotonin Release, Sensory Neuron Activation, and Abdominal Pain in Irritable Bowel Syndrome.* The American Journal Of Gastroenterology, 2011. **106**: p. 1290.
127. Yeo, A., P. Boyd, S. Lumsden, T. Saunders, A. Handley, M. Stubbins, A. Knaggs, S. Asquith, I. Taylor, and B. Bahari, *Association between a functional polymorphism in the serotonin transporter gene and diarrhoea predominant irritable bowel syndrome in women.* Gut, 2004. **53**(10): p. 1452-1458.
128. Makker, J., S. Chilimuri, and J.N. Bella, *Genetic epidemiology of irritable bowel syndrome.* World Journal of Gastroenterology: WJG, 2015. **21**(40): p. 11353.
129. Gershon, M.D. and J. Tack, *The serotonin signaling system: from basic understanding to drug development for functional GI disorders.* Gastroenterology, 2007. **132**(1): p. 397-414.
130. Martin, C.R., V. Osadchiy, A. Kalani, and E.A. Mayer, *The Brain-Gut-Microbiome Axis.* Cellular and Molecular Gastroenterology and Hepatology, 2018. **6**(2): p. 133-148.
131. Atkinson, W., S. Lockhart, P.J. Whorwell, B. Keevil, and L.A. Houghton, *Altered 5-hydroxytryptamine signaling in patients with constipation-and diarrhea-predominant irritable bowel syndrome.* Gastroenterology, 2006. **130**(1): p. 34-43.

132. Pata, C., M.E. Erdal, E. Derici, A. Yazar, A. Kanık, and O. Ulu, *Serotonin transporter gene polymorphism in irritable bowel syndrome.* American Journal Of Gastroenterology, 2002. **97**: p. 1780.
133. Lee, D.Y., H. Park, W.H. Kim, S.I. Lee, Y.J. Seo, and Y.C. Choi, *Serotonin transporter gene polymorphism in healthy adults and patients with irritable bowel syndrome.* The Korean journal of gastroenterology= Taehan Sohwagi Hakhoe chi, 2004. **43**(1): p. 18-22.
134. Faure, C., N. Patey, C. Gauthier, E.M. Brooks, and G.M. Mawe, *Serotonin signaling is altered in irritable bowel syndrome with diarrhea but not in functional dyspepsia in pediatric age patients.* Gastroenterology, 2010. **139**(1): p. 249-258.
135. Coates, M.D., C.R. Mahoney, D.R. Linden, J.E. Sampson, J. Chen, H. Blaszyk, M.D. Crowell, K.A. Sharkey, M.D. Gershon, and G.M. Mawe, *Molecular defects in mucosal serotonin content and decreased serotonin reuptake transporter in ulcerative colitis and irritable bowel syndrome 1.* Gastroenterology, 2004. **126**(7): p. 1657-1664.
136. Dunlop, B.W. and C.B. Nemeroff, *The role of dopamine in the pathophysiology of depression.* Archives of general psychiatry, 2007. **64**(3): p. 327-337.
137. Aggarwal, S., V. Ahuja, and J. Paul, *Dysregulation of GABAergic Signalling Contributes in the Pathogenesis of Diarrhea-predominant Irritable Bowel Syndrome.* Journal of neurogastroenterology and motility, 2018. **24**(3): p. 422.
138. Gonsalkorale, W., C. Perrey, V. Pravica, P. Whorwell, and I. Hutchinson, *Interleukin 10 genotypes in irritable bowel syndrome: evidence for an inflammatory component?* Gut, 2003. **52**(1): p. 91-93.
139. Komuro, H., N. Sato, A. Sasaki, N. Suzuki, M. Kano, Y. Tanaka, Y. Yamaguchi-Kabata, M. Kanazawa, H. Warita, and M. Aoki, *Corticotropin-releasing hormone receptor 2 gene variants in irritable bowel syndrome.* PloS one, 2016. **11**(1): p. e0147817.
140. Bashashati, M., N. Rezaei, A. Shafieyoun, D. McKernan, L. Chang, L. Öhman, E.M. Quigley, M. Schmulson, K. Sharkey, and M. Simrén, *Cytokine imbalance in irritable bowel syndrome: a systematic review and meta-analysis.* Neurogastroenterology & Motility, 2014. **26**(7): p. 1036-1048.
141. Williams, E.A., X. Nai, and B.M. Corfe, *Dietary intakes in people with irritable bowel syndrome.* BMC gastroenterology, 2011. **11**(1): p. 9.
142. Lea, R. and P.J. Whorwell, *The role of food intolerance in irritable bowel syndrome.* Gastroenterology Clinics, 2005. **34**(2): p. 247-255.
143. Roest, R.d., B. Dobbs, B. Chapman, B. Batman, L. O'brien, J. Leeper, C. Hebblethwaite, and R. Gearry, *The low FODMAP diet improves gastrointestinal symptoms in patients with irritable bowel syndrome: a prospective study.* International journal of clinical practice, 2013. **67**(9): p. 895-903.
144. Serra, J., F. Azpiroz, and J. Malagelada, *Impaired transit and tolerance of intestinal gas in the irritable bowel syndrome.* Gut, 2001. **48**(1): p. 14-19.
145. Ong, D.K., S.B. Mitchell, J.S. Barrett, S.J. Shepherd, P.M. Irving, J.R. Biesiekierski, S. Smith, P.R. Gibson, and J.G. Muir, *Manipulation of dietary short chain carbohydrates alters the pattern of gas production and genesis of symptoms in irritable bowel syndrome.* Journal of gastroenterology and hepatology, 2010. **25**(8): p. 1366-1373.
146. Shepherd, S.J. and P.R. Gibson, *Fructose Malabsorption and Symptoms of Irritable Bowel Syndrome: Guidelines for Effective Dietary Management.* Journal of the American Dietetic Association, 2006. **106**(10): p. 1631-1639.
147. Staudacher, H.M., et al., *A Diet Low in FODMAPs Reduces Symptoms in Patients With Irritable Bowel Syndrome and A Probiotic Restores Bifidobacterium Species: A Randomized Controlled Trial.* Gastroenterology, 2017. **153**(4): p. 936-947.
148. Krogsgaard, L., M. Lyngesen, and P. Bytzer, *Systematic review: quality of trials on the symptomatic effects of the low FODMAP diet for irritable bowel syndrome.* Alimentary pharmacology & therapeutics, 2017.
149. Murray, K., V. Wilkinson-Smith, C. Hoad, C. Costigan, E. Cox, C. Lam, L. Marciani, P. Gowland, and R.C. Spiller, *Differential Effects of FODMAPs (Fermentable Oligo-, Di-, Mono-Saccharides and Polyols) on Small and Large Intestinal Contents in Healthy Subjects Shown by MRI.* The American Journal Of Gastroenterology, 2013. **109**: p. 110.

150. Torres, M.J., J.-M. Sabate, M. Bouchoucha, C. Buscail, S. Hercberg, and C. Julia, *Food consumption and dietary intakes in 36,448 adults and their association with irritable bowel syndrome: Nutrinet-Santé study.* Therapeutic advances in gastroenterology, 2018. **11**: p. 1756283X17746625.
151. Deng, Y., B. Misselwitz, N. Dai, and M. Fox, *Lactose intolerance in adults: biological mechanism and dietary management.* Nutrients, 2015. **7**(9): p. 8020-8035.
152. Yang, J., Y. Deng, H. Chu, Y. Cong, J. Zhao, D. Pohl, B. Misselwitz, M. Fried, N. Dai, and M. Fox, *Prevalence and presentation of lactose intolerance and effects on dairy product intake in healthy subjects and patients with irritable bowel syndrome.* Clinical gastroenterology and hepatology, 2013. **11**(3): p. 262-268. e1.
153. Singh, R.K., H.-W. Chang, D. Yan, K.M. Lee, D. Ucmak, K. Wong, M. Abrouk, B. Farahnik, M. Nakamura, and T.H. Zhu, *Influence of diet on the gut microbiome and implications for human health.* Journal of translational medicine, 2017. **15**(1): p. 73.
154. De Filippo, C., D. Cavalieri, M. Di Paola, M. Ramazzotti, J.B. Poullet, S. Massart, S. Collini, G. Pieraccini, and P. Lionetti, *Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(33): p. 14691-14696.
155. Carbonero, F., A.C. Benefiel, and H.R. Gaskins, *Contributions of the microbial hydrogen economy to colonic homeostasis.* Nature Reviews Gastroenterology & Hepatology, 2012. **9**(9): p. 504.
156. Louis, P. and H.J. Flint, *Formation of propionate and butyrate by the human colonic microbiota.* Environmental microbiology, 2017. **19**(1): p. 29-41.
157. Smith, N.W., P.R. Shorten, E.H. Altermann, N.C. Roy, and W.C. McNabb, *Hydrogen cross-feeders of the human gastrointestinal tract.* Gut microbes, 2018: p. 1-19.
158. Kumar, S., A. Misra, and U.C. Ghoshal, *Patients with irritable bowel syndrome exhale more hydrogen than healthy subjects in fasting state.* Journal of neurogastroenterology and motility, 2010. **16**(3): p. 299.
159. King, T., M. Elia, and J. Hunter, *Abnormal colonic fermentation in irritable bowel syndrome.* The Lancet, 1998. **352**(9135): p. 1187-1189.
160. Dear, K.L., M. Elia, and J.O. Hunter, *Do interventions which reduce colonic bacterial fermentation improve symptoms of irritable bowel syndrome?* Digestive diseases and sciences, 2005. **50**(4): p. 758-766.
161. Pimentel, M., A.G. Mayer, S. Park, E.J. Chow, A. Hasan, and Y. Kong, *Methane production during lactulose breath test is associated with gastrointestinal disease presentation.* Digestive diseases and sciences, 2003. **48**(1): p. 86-92.
162. Gibson, P. and S. Shepherd, *Personal view: food for thought–western lifestyle and susceptibility to Crohn's disease. The FODMAP hypothesis.* Alimentary pharmacology & therapeutics, 2005. **21**(12): p. 1399-1409.
163. Böhn, L., S. Störsrud, T. Liljebo, L. Collin, P. Lindfors, H. Törnblom, and M. Simrén, *Diet Low in FODMAPs Reduces Symptoms of Irritable Bowel Syndrome as Well as Traditional Dietary Advice: A Randomized Controlled Trial.* Gastroenterology, 2015. **149**(6): p. 1399-1407.e2.
164. Staudacher, H.M., M.C. Lomer, J.L. Anderson, J.S. Barrett, J.G. Muir, P.M. Irving, and K. Whelan, *Fermentable Carbohydrate Restriction Reduces Luminal Bifidobacteria and Gastrointestinal Symptoms in Patients with Irritable Bowel Syndrome–4.* The Journal of nutrition, 2012. **142**(8): p. 1510-1518.
165. McIntosh, K., D.E. Reed, T. Schneider, F. Dang, A.H. Keshteli, G. De Palma, K. Madsen, P. Bercik, and S. Vanner, *FODMAPs alter symptoms and the metabolome of patients with IBS: a randomised controlled trial.* Gut, 2017. **66**(7): p. 1241-1251.
166. Camilleri, M., K. Lasch, and W. Zhou, *Irritable bowel syndrome: methods, mechanisms, and pathophysiology. The confluence of increased permeability, inflammation, and pain in irritable bowel syndrome.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 2012. **303**(7): p. G775-G785.

167. Barbara, G., B. Wang, V. Stanghellini, R. De Giorgio, C. Cremon, G. Di Nardo, M. Trevisani, B. Campi, P. Geppetti, and M. Tonini, *Mast cell-dependent excitation of visceral-nociceptive sensory neurons in irritable bowel syndrome.* Gastroenterology, 2007. **132**(1): p. 26-37.
168. Halmos, E.P., C.T. Christophersen, A.R. Bird, S.J. Shepherd, P.R. Gibson, and J.G. Muir, *Diets that differ in their FODMAP content alter the colonic luminal microenvironment.* Gut, 2015. **64**(1): p. 93-100.
169. Zhang, Y., Z.F. Ma, H. Zhang, B. Pan, Y. Li, H.A. Majid, and Y.Y. Lee, *Low fermentable oligosaccharides, disaccharides, monosaccharides, and polypols diet and irritable bowel syndrome in Asia.* JGH Open, 2019. **3**(2): p. 173-178.
170. Ducrotte, P., P. Sawant, and V. Jayanthi, *Clinical trial: Lactobacillus plantarum 299v (DSM 9843) improves symptoms of irritable bowel syndrome.* World J Gastroenterol, 2012. **18**(30): p. 4012-8.
171. Pineton de Chambrun, G., C. Neut, A. Chau, M. Cazaubiel, F. Pelerin, P. Justen, and P. Desreumaux, *A randomized clinical trial of Saccharomyces cerevisiae versus placebo in the irritable bowel syndrome.* Dig Liver Dis, 2015. **47**(2): p. 119-24.
172. Jafari, E., H. Vahedi, S. Merat, S. Momtahen, and A. Riahi, *Therapeutic effects, tolerability and safety of a multi-strain probiotic in Iranian adults with irritable bowel syndrome and bloating.* Arch Iran Med, 2014. **17**(7): p. 466-70.
173. Lorenzo-Zuniga, V., E. Llop, C. Suarez, B. Alvarez, L. Abreu, J. Espadaler, and J. Serra, *I.31, a new combination of probiotics, improves irritable bowel syndrome-related quality of life.* World J Gastroenterol, 2014. **20**(26): p. 8709-16.
174. Noorbakhsh, H., M. Yavarmanesh, S.A. Mortazavi, P. Adibi, and A.A. Moazzami, *Metabolomics analysis revealed metabolic changes in patients with diarrhea-predominant irritable bowel syndrome and metabolic responses to a synbiotic yogurt intervention.* European journal of nutrition, 2018: p. 1-11.
175. Yoon, J.S., W. Sohn, O.Y. Lee, S.P. Lee, K.N. Lee, D.W. Jun, H.L. Lee, B.C. Yoon, H.S. Choi, and W.S. Chung, *Effect of multispecies probiotics on irritable bowel syndrome: a randomized, double-blind, placebo-controlled trial.* Journal of gastroenterology and hepatology, 2014. **29**(1): p. 52-59.
176. Bayer, S.B., R.B. Gearry, and L.N. Drummond, *Putative mechanisms of kiwifruit on maintenance of normal gastrointestinal function.* Critical reviews in food science and nutrition, 2017: p. 1-21.
177. Attaluri, A., R. Donahoe, J. Valestin, K. Brown, and S.S.C. Rao, *Randomised clinical trial: dried plums (prunes) vs. psyllium for constipation.* Alimentary pharmacology & therapeutics, 2011. **33**(7): p. 822-828.
178. Cheskin, L., A. Mitola, M. Ridoré, S. Kolge, K. Hwang, and B. Clark, *A naturalistic, controlled, crossover trial of plum juice versus psyllium versus control for improving bowel function.* Internet J Nutr Wellness, 2009. **7**(2).
179. Woo, H.-I., S.H. Kwak, Y. Lee, J.H. Choi, Y.M. Cho, and A.-S. Om, *A controlled, randomized, double-blind trial to evaluate the effect of vegetables and whole grain powder that is rich in dietary fibers on bowel functions and defecation in constipated young adults.* Journal of cancer prevention, 2015. **20**(1): p. 64.
180. Chang, C.-C., Y.-T. Lin, Y.-T. Lu, Y.-S. Liu, and J.-F. Liu, *Kiwifruit improves bowel function in patients with irritable bowel syndrome with constipation.* Asia Pacific journal of clinical nutrition, 2010. **19**(4): p. 451-457.
181. Brownlee, I.A., *The physiological roles of dietary fibre.* Food Hydrocolloids, 2011. **25**(2): p. 238-250.
182. Holscher, H.D., *Dietary fiber and prebiotics and the gastrointestinal microbiota.* Gut Microbes, 2017. **8**(2): p. 172-184.
183. Foster, J.A., L. Rinaman, and J.F. Cryan, *Stress & the gut-brain axis: regulation by the microbiome.* Neurobiology of stress, 2017. **7**: p. 124-136.
184. Mayer, E.A., K. Tillisch, and A. Gupta, *Gut/brain axis and the microbiota.* J Clin Invest, 2015. **125**(3): p. 926-38.

185. Carabotti, M., A. Scirocco, M.A. Maselli, and C. Severi, *The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems.* Annals of gastroenterology: quarterly publication of the Hellenic Society of Gastroenterology, 2015. **28**(2): p. 203.
186. Foster, J.A. and K.-A.M. Neufeld, *Gut–brain axis: how the microbiome influences anxiety and depression.* Trends in neurosciences, 2013. **36**(5): p. 305-312.
187. Koloski, N.A., M. Jones, J. Kalantar, M. Weltman, J. Zaguirre, and N. Talley, *The brain–gut pathway in functional gastrointestinal disorders is bidirectional: a 12-year prospective population-based study.* Gut, 2012. **61**(9): p. 1284-1290.
188. Jones, M.P., J. Tack, L. Van Oudenhove, M.M. Walker, G. Holtmann, N.A. Koloski, and N.J. Talley, *Mood and Anxiety Disorders Precede Development of Functional Gastrointestinal Disorders in Patients but Not in the Population.* Clin Gastroenterol Hepatol, 2017. **15**(7): p. 1014-1020 e4.
189. Raskov, H., J. Burcharth, H.C. Pommergaard, and J. Rosenberg, *Irritable bowel syndrome, the microbiota and the gut-brain axis.* Gut Microbes, 2016. **7**(5): p. 365-83.
190. Kano, M., et al., *Parasympathetic activity correlates with subjective and brain responses to rectal distension in healthy subjects but not in non-constipated patients with irritable bowel syndrome.* Sci Rep, 2019. **9**(1): p. 7358.
191. Moloney, R.D., A.C. Johnson, S.M. O'Mahony, T.G. Dinan, B. Greenwood-Van Meerveld, and J.F. Cryan, *Stress and the Microbiota-Gut-Brain Axis in Visceral Pain: Relevance to Irritable Bowel Syndrome.* CNS Neurosci Ther, 2016. **22**(2): p. 102-17.
192. Collins, S.M., *A role for the gut microbiota in IBS.* Nat Rev Gastroenterol Hepatol, 2014. **11**(8): p. 497-505.
193. Knowles, S.R., E.A. Nelson, and E.A. Palombo, *Investigating the role of perceived stress on bacterial flora activity and salivary cortisol secretion: a possible mechanism underlying susceptibility to illness.* Biol Psychol, 2008. **77**(2): p. 132-7.
194. Van Oudenhove, L., M.D. Crowell, D.A. Drossman, A.D. Halpert, L. Keefer, J.M. Lackner, T.B. Murphy, B.D. Naliboff, and R.L. Levy, *Biopsychosocial Aspects of Functional Gastrointestinal Disorders.* Gastroenterology, 2016.
195. Stasi, C., et al., *The complex interplay between gastrointestinal and psychiatric symptoms in irritable bowel syndrome: A longitudinal assessment.* J Gastroenterol Hepatol, 2019. **34**(4): p. 713-719.
196. Chitkara, D.K., M.A. van Tilburg, N. Blois-Martin, and W.E. Whitehead, *Early life risk factors that contribute to irritable bowel syndrome in adults: a systematic review.* Am J Gastroenterol, 2008. **103**(3): p. 765-74; quiz 775.
197. Mayer, E.A., J. Labus, Q. Aziz, I. Tracey, L. Kilpatrick, S. Elsenbruch, P. Schweinhardt, L. Van Oudenhove, and D. Borsook, *Role of brain imaging in disorders of brain-gut interaction: a Rome Working Team Report.* Gut, 2019. **68**(9): p. 1701-1715.
198. Simren, M., H. Tornblom, O.S. Palsson, M.A.L. van Tilburg, L. Van Oudenhove, J. Tack, and W.E. Whitehead, *Visceral hypersensitivity is associated with GI symptom severity in functional GI disorders: consistent findings from five different patient cohorts.* Gut, 2018. **67**(2): p. 255-262.
199. Chang, L., *The role of stress on physiologic responses and clinical symptoms in irritable bowel syndrome.* Gastroenterology, 2011. **140**(3): p. 761-5.
200. Goldberger, J.J., *Sympathovagal balance: how should we measure it?* Am J Physiol, 1999. **276**(4): p. H1273-80.
201. Cheng, P., W. Shih, M. Alberto, A.P. Presson, A. Licudine, E.A. Mayer, B.D. Naliboff, and L. Chang, *Autonomic response to a visceral stressor is dysregulated in irritable bowel syndrome and correlates with duration of disease.* Neurogastroenterol Motil, 2013. **25**(10): p. e650-9.
202. Ng, C., A. Malcolm, R. Hansen, and J. Kellow, *Feeding and colonic distension provoke altered autonomic responses in irritable bowel syndrome.* Scand J Gastroenterol, 2007. **42**(4): p. 441-6.

203. Neufeld, K.M., N. Kang, J. Bienenstock, and J.A. Foster, *Reduced anxiety-like behavior and central neurochemical change in germ-free mice.* Neurogastroenterol Motil, 2011. **23**(3): p. 255-64, e119.
204. Bercik, P., E. Denou, J. Collins, W. Jackson, J. Lu, J. Jury, Y. Deng, P. Blennerhassett, J. Macri, and K.D. McCoy, *The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice.* Gastroenterology, 2011. **141**(2): p. 599-609. e3.
205. Yano, J.M., K. Yu, G.P. Donaldson, G.G. Shastri, P. Ann, L. Ma, C.R. Nagler, R.F. Ismagilov, S.K. Mazmanian, and E.Y. Hsiao, *Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis.* Cell, 2015. **161**(2): p. 264-276.
206. Bailey, M.T., S.E. Dowd, N.M. Parry, J.D. Galley, D.B. Schauer, and M. Lyte, *Stressor exposure disrupts commensal microbial populations in the intestines and leads to increased colonization by Citrobacter rodentium.* Infect Immun, 2010. **78**(4): p. 1509-19.
207. Lupp, C., M.L. Robertson, M.E. Wickham, I. Sekirov, O.L. Champion, E.C. Gaynor, and B.B. Finlay, *Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae.* Cell Host Microbe, 2007. **2**(3): p. 204.
208. Bailey, M.T., S.E. Dowd, J.D. Galley, A.R. Hufnagle, R.G. Allen, and M. Lyte, *Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation.* Brain Behav Immun, 2011. **25**(3): p. 397-407.
209. Abbott, N.J., A.A. Patabendige, D.E. Dolman, S.R. Yusof, and D.J. Begley, *Structure and function of the blood-brain barrier.* Neurobiol Dis, 2010. **37**(1): p. 13-25.
210. Young, S.N., *Acute tryptophan depletion in humans: a review of theoretical, practical and ethical aspects.* J Psychiatry Neurosci, 2013. **38**(5): p. 294-305.
211. O'Mahony, S.M., G. Clarke, Y.E. Borre, T.G. Dinan, and J.F. Cryan, *Serotonin, tryptophan metabolism and the brain-gut-microbiome axis.* Behav Brain Res, 2015. **277**: p. 32-48.
212. Browne, C.A., G. Clarke, T.G. Dinan, and J.F. Cryan, *An effective dietary method for chronic tryptophan depletion in two mouse strains illuminates a role for 5-HT in nesting behaviour.* Neuropharmacology, 2012. **62**(5-6): p. 1903-15.
213. Waclawikova, B. and S. El Aidy, *Role of Microbiota and Tryptophan Metabolites in the Remote Effect of Intestinal Inflammation on Brain and Depression.* Pharmaceuticals (Basel), 2018. **11**(3).
214. Lukic, I., D. Getselter, O. Koren, and E. Elliott, *Role of Tryptophan in Microbiota-Induced Depressive-Like Behavior: Evidence From Tryptophan Depletion Study.* Front Behav Neurosci, 2019. **13**: p. 123.
215. Wlodarska, M., et al., *Indoleacrylic Acid Produced by Commensal Peptostreptococcus Species Suppresses Inflammation.* Cell Host Microbe, 2017. **22**(1): p. 25-37 e6.
216. Roager, H.M. and T.R. Licht, *Microbial tryptophan catabolites in health and disease.* Nat Commun, 2018. **9**(1): p. 3294.
217. Wei, G.Z., et al., *Tryptophan-metabolizing gut microbes regulate adult neurogenesis via the aryl hydrocarbon receptor.* Proc Natl Acad Sci U S A, 2021. **118**(27).
218. Fukudo, S., *Role of corticotropin-releasing hormone in irritable bowel syndrome and intestinal inflammation.* J Gastroenterol, 2007. **42 Suppl 17**: p. 48-51.
219. Choghakhori, R., A. Abbasnezhad, A. Hasanvand, and R. Amani, *Inflammatory cytokines and oxidative stress biomarkers in irritable bowel syndrome: Association with digestive symptoms and quality of life.* Cytokine, 2017. **93**: p. 34-43.
220. Kennedy, P.J., J.F. Cryan, T.G. Dinan, and G. Clarke, *Irritable bowel syndrome: a microbiome-gut-brain axis disorder?* World journal of gastroenterology: WJG, 2014. **20**(39): p. 14105.
221. Scully, P., D.P. McKernan, J. Keohane, D. Groeger, F. Shanahan, T.G. Dinan, and E.M. Quigley, *Plasma cytokine profiles in females with irritable bowel syndrome and extra-intestinal co-morbidity.* Am J Gastroenterol, 2010. **105**(10): p. 2235-43.

222. Simen, B.B., C.H. Duman, A.A. Simen, and R.S. Duman, *TNFalpha signaling in depression and anxiety: behavioral consequences of individual receptor targeting.* Biol Psychiatry, 2006. **59**(9): p. 775-85.
223. Group, B.D.W., A.J. Atkinson Jr, W.A. Colburn, V.G. DeGruttola, D.L. DeMets, G.J. Downing, D.F. Hoth, J.A. Oates, C.C. Peck, and R.T. Schooley, *Biomarkers and surrogate endpoints: preferred definitions and conceptual framework.* Clinical pharmacology & therapeutics, 2001. **69**(3): p. 89-95.
224. Mujagic, Z., E.F. Tigchelaar, A. Zhernakova, T. Ludwig, J. Ramiro-Garcia, A. Baranska, M.A. Swertz, A.A. Masclee, C. Wijmenga, and F.J. Van Schooten, *A novel biomarker panel for irritable bowel syndrome and the application in the general population.* Sci Rep, 2016. **6**: p. 26420.
225. Kim, J.H., E. Lin, and M. Pimentel, *Biomarkers of irritable bowel syndrome.* Journal of neurogastroenterology and motility, 2017. **23**(1): p. 20-26.
226. Lembo, A.J., B. Neri, J. Tolley, D. Barken, S. Carroll, and H. Pan, *Use of serum biomarkers in a diagnostic test for irritable bowel syndrome.* Alimentary Pharmacology and Therapeutics, 2009. **29**(8): p. 834-842.
227. Baranska, A., Z. Mujagic, A. Smolinska, J. Dallinga, D. Jonkers, E. Tigchelaar, J. Dekens, A. Zhernakova, T. Ludwig, and A. Masclee, *Volatile organic compounds in breath as markers for irritable bowel syndrome: a metabolomic approach.* Alimentary pharmacology & therapeutics, 2016. **44**(1): p. 45-56.
228. Heenan, P., et al., *Cohort Profile: The Christchurch IBS cOhort to investigate Mechanisms FOr gut Relief and improved Transit (COMFORT).* Inflammatory Intestinal Diseases, 2020. **5**(3): p. 132-143.
229. Zhou, H. and P.B. Hylemon, *Bile acids are nutrient signaling hormones.* Steroids, 2014. **86**: p. 62-8.
230. Molinaro, A., A. Wahlstrom, and H.U. Marschall, *Role of Bile Acids in Metabolic Control.* Trends Endocrinol Metab, 2018. **29**(1): p. 31-41.
231. Oduyebo, I. and M. Camilleri, *Bile acid disease: the emerging epidemic.* Curr Opin Gastroenterol, 2017. **33**(3): p. 189-195.
232. Molinero, N., L. Ruiz, B. Sanchez, A. Margolles, and S. Delgado, *Intestinal Bacteria Interplay With Bile and Cholesterol Metabolism: Implications on Host Physiology.* Front Physiol, 2019. **10**: p. 185.
233. Wahlstrom, A., S.I. Sayin, H.U. Marschall, and F. Backhed, *Intestinal Crosstalk between Bile Acids and Microbiota and Its Impact on Host Metabolism.* Cell Metab, 2016. **24**(1): p. 41-50.
234. Joyce, S.A., J. MacSharry, P.G. Casey, M. Kinsella, E.F. Murphy, F. Shanahan, C. Hill, and C.G. Gahan, *Regulation of host weight gain and lipid metabolism by bacterial bile acid modification in the gut.* Proceedings of the National Academy of Sciences, 2014. **111**(20): p. 7421-7426.
235. Camilleri, M., *Bile Acid diarrhea: prevalence, pathogenesis, and therapy.* Gut Liver, 2015. **9**(3): p. 332-9.
236. Zhao, L., W. Yang, Y. Chen, F. Huang, L. Lu, C. Lin, T. Huang, Z. Ning, L. Zhai, and L.L. Zhong, *A Clostridia-rich microbiota enhances bile acid excretion in diarrhea-predominant irritable bowel syndrome.* The Journal of clinical investigation, 2019. **130**(1).
237. Chong, J., O. Soufan, C. Li, I. Caraus, S. Li, G. Bourque, D.S. Wishart, and J. Xia, *MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis.* Nucleic Acids Res, 2018. **46**(W1): p. W486-W494.
238. Chong, J., D.S. Wishart, and J. Xia, *Using MetaboAnalyst 4.0 for Comprehensive and Integrative Metabolomics Data Analysis.* Curr Protoc Bioinformatics, 2019. **68**(1): p. e86.
239. Rao, A.S., et al., *Chenodeoxycholate in females with irritable bowel syndrome-constipation: a pharmacodynamic and pharmacogenetic analysis.* Gastroenterology, 2010. **139**(5): p. 1549-58, 1558 e1.

240. Walters, J.R. and S.S. Pattni, *Managing bile acid diarrhoea.* Therap Adv Gastroenterol, 2010. **3**(6): p. 349-57.
241. Pimentel, M., N.J. Talley, E.M. Quigley, A. Hani, A. Sharara, and V. Mahachai, *Report from the multinational irritable bowel syndrome initiative 2012.* Gastroenterology, 2013. **144**(7): p. e1-5.
242. Dalangin, R., A. Kim, and R.E. Campbell, *The Role of Amino Acids in Neurotransmission and Fluorescent Tools for Their Detection.* Int J Mol Sci, 2020. **21**(17).
243. Oliphant, K. and E. Allen-Vercoe, *Macronutrient metabolism by the human gut microbiome: major fermentation by-products and their impact on host health.* Microbiome, 2019. **7**(1): p. 91.
244. Canfield, C.-A. and P.C. Bradshaw, *Amino acids in the regulation of aging and aging-related diseases.* Translational Medicine of Aging, 2019. **3**: p. 70-89.
245. De Preter, V. and K. Verbeke, *Metabolomics as a diagnostic tool in gastroenterology.* World journal of gastrointestinal pharmacology and therapeutics, 2013. **4**(4): p. 97.
246. Solon-Biet, S.M., et al., *Branched chain amino acids impact health and lifespan indirectly via amino acid balance and appetite control.* Nat Metab, 2019. **1**(5): p. 532-545.
247. Davenport, M., J. Poles, J.M. Leung, M.J. Wolff, W.M. Abidi, T. Ullman, L. Mayer, I. Cho, and P. Loke, *Metabolic alterations to the mucosal microbiota in inflammatory bowel disease.* Inflamm Bowel Dis, 2014. **20**(4): p. 723-31.
248. Marc Rhoads, J. and G. Wu, *Glutamine, arginine, and leucine signaling in the intestine.* Amino Acids, 2009. **37**(1): p. 111-122.
249. Branco, A.C.C.C., F.S.Y. Yoshikawa, A.J. Pietrobon, and M.N. Sato, *Role of Histamine in Modulating the Immune Response and Inflammation.* Mediators of Inflammation, 2018. **2018**: p. 9524075.
250. Portune, K.J., M. Beaumont, A.-M. Davila, D. Tomé, F. Blachier, and Y. Sanz, *Gut microbiota role in dietary protein metabolism and health-related outcomes: the two sides of the coin.* Trends in Food Science & Technology, 2016. **57**: p. 213-232.
251. Markus, C.R., *Dietary amino acids and brain serotonin function; implications for stress-related affective changes.* Neuromolecular Med, 2008. **10**(4): p. 247-58.
252. Stasi, C., M. Bellini, G. Bassotti, C. Blandizzi, and S. Milani, *Serotonin receptors and their role in the pathophysiology and therapy of irritable bowel syndrome.* Techniques in Coloproctology, 2014. **18**(7): p. 613-621.
253. Milan, A.M., R.F. D'Souza, S. Pundir, C.A. Pileggi, M.P.G. Barnett, J.F. Markworth, D. Cameron-Smith, and C. Mitchell, *Older adults have delayed amino acid absorption after a high protein mixed breakfast meal.* The journal of nutrition, health & aging, 2015. **19**(8): p. 839-845.
254. Fitzgerald, P., M. Cassidy Eugene, G. Clarke, P. Scully, S. Barry, M.M. Quigley Eamonn, F. Shanahan, J. Cryan, and G. Dinan Timothy, *Tryptophan catabolism in females with irritable bowel syndrome: relationship to interferon-gamma, severity of symptoms and psychiatric co-morbidity.* Neurogastroenterol Motil, 2008. **20**(12): p. 1291-7.
255. Wang, W.W., S.Y. Qiao, and D.F. Li, *Amino acids and gut function.* Amino Acids, 2009. **37**(1): p. 105-110.
256. Li, N., P. Lewis, D. Samuelson, K. Liboni, and J. Neu, *Glutamine regulates Caco-2 cell tight junction proteins.* Am J Physiol Gastrointest Liver Physiol, 2004. **287**(3): p. G726-33.
257. Coëffier, M.s., O. Miralles-Barrachina, F. Le Pessot, O. Lalaude, M. Daveau, A. Lavoinne, E. Lerebours, and P. Déchelotte, *Influence of glutamine on cytokine production by human gut in vitro.* Cytokine, 2001. **13**(3): p. 148-154.
258. Zhou, Q., M.L. Verne, J.Z. Fields, J.J. Lefante, S. Basra, H. Salameh, and G.N. Verne, *Randomised placebo-controlled trial of dietary glutamine supplements for postinfectious irritable bowel syndrome.* Gut, 2019. **68**(6): p. 996-1002.
259. Fabisiak, A., J. Włodarczyk, N. Fabisiak, M. Storr, and J. Fichna, *Targeting Histamine Receptors in Irritable Bowel Syndrome: A Critical Appraisal.* Journal of neurogastroenterology and motility, 2017. **23**(3): p. 341-348.

260. He, F., C. Wu, P. Li, N. Li, D. Zhang, Q. Zhu, W. Ren, and Y. Peng, *Functions and Signaling Pathways of Amino Acids in Intestinal Inflammation.* Biomed Res Int, 2018. **2018**: p. 9171905.
261. Stephens, N.S., J. Siffledeen, X. Su, T.B. Murdoch, R.N. Fedorak, and C.M. Slupsky, *Urinary NMR metabolomic profiles discriminate inflammatory bowel disease from healthy.* J Crohns Colitis, 2013. **7**(2): p. e42-8.
262. Chang, L., M. Adeyemo, I. Karagiannidis, E.J. Videlock, C. Bowe, W. Shih, A.P. Presson, P.-Q. Yuan, G. Cortina, and H. Gong, *Serum and colonic mucosal immune markers in irritable bowel syndrome.* The American journal of gastroenterology, 2012. **107**(2): p. 262.
263. Sundin, J., et al., *Evidence of altered mucosa-associated and fecal microbiota composition in patients with Irritable Bowel Syndrome.* Sci Rep, 2020. **10**(1): p. 593.
264. Zierer, J., M.A. Jackson, G. Kastenmüller, M. Mangino, T. Long, A. Telenti, R.P. Mohney, K.S. Small, J.T. Bell, and C.J. Steves, *The fecal metabolome as a functional readout of the gut microbiome.* Nature genetics, 2018: p. 1.
265. Rossi, M., R. Aggio, H.M. Staudacher, M.C. Lomer, J.O. Lindsay, P. Irving, C. Probert, and K. Whelan, *Volatile Organic Compounds in Feces Associate With Response to Dietary Intervention in Patients With Irritable Bowel Syndrome.* Clin Gastroenterol Hepatol, 2018. **16**(3): p. 385-391 e1.
266. Bennet, S.M., A.H. Keshteli, P. Bercik, K.L. Madsen, D. Reed, and S.J. Vanner, *Application of metabolomics to the study of irritable bowel syndrome.* Neurogastroenterol Motil, 2020. **32**(6): p. e13884.
267. Chambers, M.C., et al., *A cross-platform toolkit for mass spectrometry and proteomics.* Nat Biotechnol, 2012. **30**(10): p. 918-20.
268. Smith, C.A., E.J. Want, G. O'Maille, R. Abagyan, and G. Siuzdak, *XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification.* Anal Chem, 2006. **78**(3): p. 779-87.
269. Giacomoni, F., et al., *Workflow4Metabolomics: a collaborative research infrastructure for computational metabolomics.* Bioinformatics, 2015. **31**(9): p. 1493-5.
270. Tsugawa, H., T. Cajka, T. Kind, Y. Ma, B. Higgins, K. Ikeda, M. Kanazawa, J. VanderGheynst, O. Fiehn, and M. Arita, *MS-DIAL: data-independent MS/MS deconvolution for comprehensive metabolome analysis.* Nat Methods, 2015. **12**(6): p. 523-6.
271. Wishart, D.S., et al., *HMDB 4.0: the human metabolome database for 2018.* Nucleic Acids Res, 2018. **46**(D1): p. D608-D617.
272. Bennouna, D., F. Tourniaire, T. Durand, J.-M. Galano, F. Fine, K. Fraser, S. Benatia, C. Rosique, C. Pau, and C. Couturier, *The Brassica napus (oilseed rape) seeds bioactive health effects are modulated by agronomical traits as assessed by a multi-scale omics approach in the metabolically impaired ob-mouse.* Food Chemistry: Molecular Sciences, 2021. **2**: p. 100011.
273. Gaud, C., C.S. B, A. Nguyen, M. Fedorova, Z. Ni, V.B. O'Donnell, M.J.O. Wakelam, S. Andrews, and A.F. Lopez-Clavijo, *BioPAN: a web-based tool to explore mammalian lipidome metabolic pathways on LIPID MAPS.* F1000Res, 2021. **10**: p. 4.
274. Streiner, D.L., *Best (but oft-forgotten) practices: the multiple problems of multiplicity-whether and how to correct for many statistical tests.* Am J Clin Nutr, 2015. **102**(4): p. 721-8.
275. Karnovsky, A., et al., *Metscape 2 bioinformatics tool for the analysis and visualization of metabolomics and gene expression data.* Bioinformatics, 2012. **28**(3): p. 373-80.
276. Ahluwalia, B., C. Iribarren, M.K. Magnusson, J. Sundin, E. Clevers, O. Savolainen, A.B. Ross, H. Tornblom, M. Simren, and L. Ohman, *A Distinct Faecal Microbiota and Metabolite Profile Linked to Bowel Habits in Patients with Irritable Bowel Syndrome.* Cells, 2021. **10**(6).
277. Kajander, K., E. Myllyluoma, S. Kyronpalo, M. Rasmussen, P. Sipponen, I. Mattila, T. Seppanen-Laakso, H. Vapaatalo, M. Oresic, and R. Korpela, *Elevated pro-inflammatory*

and lipotoxic mucosal lipids characterise irritable bowel syndrome. World J Gastroenterol, 2009. **15**(48): p. 6068-74.
278. Nagy-Szakal, D., et al., *Insights into myalgic encephalomyelitis/chronic fatigue syndrome phenotypes through comprehensive metabolomics.* Sci Rep, 2018. **8**(1): p. 10056.
279. Sagar, N.M., M. McFarlane, C. Nwokolo, K.D. Bardhan, and R.P. Arasaradnam, *Mechanisms of triglyceride metabolism in patients with bile acid diarrhea.* World J Gastroenterol, 2016. **22**(30): p. 6757-63.
280. Stofan, M. and G.L. Guo, *Bile Acids and FXR: Novel Targets for Liver Diseases.* Front Med (Lausanne), 2020. **7**: p. 544.
281. Jiao, Y., Y. Lu, and X.Y. Li, *Farnesoid X receptor: a master regulator of hepatic triglyceride and glucose homeostasis.* Acta Pharmacol Sin, 2015. **36**(1): p. 44-50.
282. Sinal, C.J., M. Tohkin, M. Miyata, J.M. Ward, G. Lambert, and F.J. Gonzalez, *Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis.* Cell, 2000. **102**(6): p. 731-44.
283. Angelin, B., K.S. Hershon, and J.D. Brunzell, *Bile acid metabolism in hereditary forms of hypertriglyceridemia: evidence for an increased synthesis rate in monogenic familial hypertriglyceridemia.* Proc Natl Acad Sci U S A, 1987. **84**(15): p. 5434-8.
284. Giloteaux, L., J.K. Goodrich, W.A. Walters, S.M. Levine, R.E. Ley, and M.R. Hanson, *Reduced diversity and altered composition of the gut microbiome in individuals with myalgic encephalomyelitis/chronic fatigue syndrome.* Microbiome, 2016. **4**(1): p. 30.
285. Rolando, M. and C. Buchrieser, *A Comprehensive Review on the Manipulation of the Sphingolipid Pathway by Pathogenic Bacteria.* Front Cell Dev Biol, 2019. **7**: p. 168.
286. Breslow, D.K. and J.S. Weissman, *Membranes in balance: mechanisms of sphingolipid homeostasis.* Mol Cell, 2010. **40**(2): p. 267-79.
287. Weitkunat, K., S. Schumann, D. Nickel, S. Hornemann, K.J. Petzke, M.B. Schulze, A.F. Pfeiffer, and S. Klaus, *Odd-chain fatty acids as a biomarker for dietary fiber intake: a novel pathway for endogenous production from propionate.* Am J Clin Nutr, 2017. **105**(6): p. 1544-1551.
288. Liu, J., et al., *Ceramides and their interactive effects with trimethylamine-N-oxide metabolites on risk of gestational diabetes: A nested case-control study.* Diabetes Res Clin Pract, 2021. **171**: p. 108606.
289. Bryan, P.F., C. Karla, M.T. Edgar Alejandro, E.P. Sara Elva, F. Gemma, and C. Luz, *Sphingolipids as Mediators in the Crosstalk between Microbiota and Intestinal Cells: Implications for Inflammatory Bowel Disease.* Mediators Inflamm, 2016. **2016**: p. 9890141.
290. Thakur, P. and B. Nehru, *Anti-inflammatory properties rather than anti-oxidant capability is the major mechanism of neuroprotection by sodium salicylate in a chronic rotenone model of Parkinson's disease.* Neuroscience, 2013. **231**: p. 420-31.
291. von Martels, J.Z.H., et al., *Riboflavin Supplementation in Patients with Crohn's Disease [the RISE-UP study].* J Crohns Colitis, 2020. **14**(5): p. 595-607.
292. Averianova, L.A., L.A. Balabanova, O.M. Son, A.B. Podvolotskaya, and L.A. Tekutyeva, *Production of Vitamin B2 (Riboflavin) by Microorganisms: An Overview.* Front Bioeng Biotechnol, 2020. **8**: p. 570828.
293. Mohedano, M.L., et al., *Real-Time Detection of Riboflavin Production by Lactobacillus plantarum Strains and Tracking of Their Gastrointestinal Survival and Functionality in vitro and in vivo Using mCherry Labeling.* Front Microbiol, 2019. **10**: p. 1748.
294. Steinert, R.E., M. Sadaghian Sadabad, H.J. Harmsen, and P. Weber, *The prebiotic concept and human health: a changing landscape with riboflavin as a novel prebiotic candidate?* Eur J Clin Nutr, 2016. **70**(12): p. 1461.
295. Singh, N., et al., *Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis.* Immunity, 2014. **40**(1): p. 128-39.
296. Carco, C., W. Young, R.B. Gearry, N.J. Talley, W.C. McNabb, and N.C. Roy, *Increasing Evidence That Irritable Bowel Syndrome and Functional Gastrointestinal Disorders Have a Microbial Pathogenesis.* Front Cell Infect Microbiol, 2020. **10**: p. 468.

297. Heenan, P., *The Colonic Luminal Microenvironment and Symptoms of Irritable Bowel Syndrome*. PhD thesis, 2021.
298. Mayer, E.A., J.S. Labus, K. Tillisch, S.W. Cole, and P. Baldi, *Towards a systems view of IBS.* Nature Reviews Gastroenterology and Hepatology, 2015. **12**(10): p. 592.
299. Ideker, T., T. Galitski, and L. Hood, *A NEW APPROACH TO DECODING LIFE: Systems Biology.* Annual Review of Genomics and Human Genetics, 2001. **2**(1): p. 343-372.
300. Hood, L., J.R. Heath, M.E. Phelps, and B. Lin, *Systems biology and new technologies enable predictive and preventative medicine.* Science, 2004. **306**(5696): p. 640-3.
301. Weston, A.D. and L. Hood, *Systems biology, proteomics, and the future of health care: toward predictive, preventative, and personalized medicine.* J Proteome Res, 2004. **3**(2): p. 179-96.
302. Noor, E., S. Cherkaoui, and U. Sauer, *Biological insights through omics data integration.* Current Opinion in Systems Biology, 2019. **15**: p. 39-47.
303. Kitano, H., *Systems Biology: A Brief Overview.* Science, 2002. **295**(5560): p. 1662-1664.
304. Kitano, H., *Computational systems biology.* Nature, 2002. **420**: p. 206.
305. Yang, X., *Multitissue Multiomics Systems Biology to Dissect Complex Diseases.* Trends Mol Med, 2020. **26**(8): p. 718-728.
306. Joshi, A., M. Rienks, K. Theofilatos, and M. Mayr, *Systems biology in cardiovascular disease: a multiomics approach.* Nat Rev Cardiol, 2021. **18**(5): p. 313-330.
307. Osadchiy, V., E.A. Mayer, K. Gao, J.S. Labus, B. Naliboff, K. Tillisch, L. Chang, J.P. Jacobs, E.Y. Hsiao, and A. Gupta, *Analysis of brain networks and fecal metabolites reveals brain-gut alterations in premenopausal females with irritable bowel syndrome.* Transl Psychiatry, 2020. **10**(1): p. 367.
308. Breitling, R., *What is systems biology?* Front Physiol, 2010. **1**: p. 9.
309. Johnson, A.J., et al., *Daily Sampling Reveals Personalized Diet-Microbiome Associations in Humans.* Cell Host Microbe, 2019. **25**(6): p. 789-802 e5.
310. Tap, J., S. Storsrud, B. Le Neve, A. Cotillard, N. Pons, J. Dore, L. Ohman, H. Tornblom, M. Derrien, and M. Simren, *Diet and gut microbiome interactions of relevance for symptoms in irritable bowel syndrome.* Microbiome, 2021. **9**(1): p. 74.
311. Polster, A., L. Ohman, J. Tap, M. Derrien, B. Le Neve, J. Sundin, H. Tornblom, M. Cvijovic, and M. Simren, *A novel stepwise integrative analysis pipeline reveals distinct microbiota-host interactions and link to symptoms in irritable bowel syndrome.* Sci Rep, 2021. **11**(1): p. 5521.
312. Shankar, V., N.V. Reo, and O. Paliy, *Simultaneous fecal microbial and metabolite profiling enables accurate classification of pediatric irritable bowel syndrome.* Microbiome, 2015. **3**: p. 73.
313. Lamichhane, S., P. Sen, M.A. Alves, H.C. Ribeiro, P. Raunioniemi, T. Hyotylainen, and M. Oresic, *Linking Gut Microbiome and Lipid Metabolism: Moving beyond Associations.* Metabolites, 2021. **11**(1).
314. Masid, M. and V. Hatzimanikatis, *Quantitative modeling of human metabolism: A call for a community effort.* Current Opinion in Systems Biology, 2021. **26**: p. 109-115.
315. Ko, C.W., J. Qu, D.D. Black, and P. Tso, *Regulation of intestinal lipid metabolism: current concepts and relevance to disease.* Nat Rev Gastroenterol Hepatol, 2020. **17**(3): p. 169-183.
316. Alhouayek, M., H. Ameraoui, and G.G. Muccioli, *Bioactive lipids in inflammatory bowel diseases - From pathophysiological alterations to therapeutic opportunities.* Biochim Biophys Acta Mol Cell Biol Lipids, 2021. **1866**(2): p. 158854.
317. Mujagic, Z., et al., *Biomarkers for visceral hypersensitivity in patients with irritable bowel syndrome.* Neurogastroenterology and Motility, 2017. **29**(12).
318. Liu, T., X. Gu, L.X. Li, M. Li, B. Li, X. Cui, and X.L. Zuo, *Microbial and metabolomic profiles in correlation with depression and anxiety co-morbidities in diarrhoea-predominant IBS patients.* BMC Microbiol, 2020. **20**(1): p. 168.
319. Johnson, E.L., S.L. Heaver, J.L. Waters, B.I. Kim, A. Bretin, A.L. Goodman, A.T. Gewirtz, T.S. Worgall, and R.E. Ley, *Sphingolipids produced by gut bacteria enter host metabolic pathways impacting ceramide levels.* Nat Commun, 2020. **11**(1): p. 2471.

320. Guo, P., K. Zhang, X. Ma, and P. He, *Clostridium species as probiotics: potentials and challenges.* J Anim Sci Biotechnol, 2020. **11**: p. 24.
321. Kindt, A., et al., *The gut microbiota promotes hepatic fatty acid desaturation and elongation in mice.* Nat Commun, 2018. **9**(1): p. 3760.
322. Kimura, I., et al., *The gut microbiota suppresses insulin-mediated fat accumulation via the short-chain fatty acid receptor GPR43.* Nat Commun, 2013. **4**: p. 1829.
323. Backhed, F., J.K. Manchester, C.F. Semenkovich, and J.I. Gordon, *Mechanisms underlying the resistance to diet-induced obesity in germ-free mice.* Proc Natl Acad Sci U S A, 2007. **104**(3): p. 979-84.
324. Backhed, F., H. Ding, T. Wang, L.V. Hooper, G.Y. Koh, A. Nagy, C.F. Semenkovich, and J.I. Gordon, *The gut microbiota as an environmental factor that regulates fat storage.* Proc Natl Acad Sci U S A, 2004. **101**(44): p. 15718-23.
325. Crovesy, L., D. Masterson, and E.L. Rosado, *Profile of the gut microbiota of adults with obesity: a systematic review.* Eur J Clin Nutr, 2020. **74**(9): p. 1251-1262.
326. Caesar, R., V. Tremaroli, P. Kovatcheva-Datchary, P.D. Cani, and F. Backhed, *Crosstalk between Gut Microbiota and Dietary Lipids Aggravates WAT Inflammation through TLR Signaling.* Cell Metab, 2015. **22**(4): p. 658-68.
327. Bar, N., et al., *A reference map of potential determinants for the human serum metabolome.* Nature, 2020. **588**(7836): p. 135-140.
328. Lee, M.T., H.H. Le, and E.L. Johnson, *Dietary sphinganine is selectively assimilated by members of the mammalian gut microbiome.* J Lipid Res, 2021. **62**: p. 100034.
329. Kenny, D.J., et al., *Cholesterol Metabolism by Uncultured Human Gut Bacteria Influences Host Cholesterol Level.* Cell Host Microbe, 2020. **28**(2): p. 245-257 e6.
330. Schwieterman, M.L., et al., *Strawberry flavor: diverse chemical compositions, a seasonal influence, and effects on sensory perception.* PLoS One, 2014. **9**(2): p. e88446.
331. van Best, N., U. Rolle-Kampczyk, F.G. Schaap, M. Basic, S.W.M. Olde Damink, A. Bleich, P.H.M. Savelkoul, M. von Bergen, J. Penders, and M.W. Hornef, *Bile acids drive the newborn's gut microbiota maturation.* Nat Commun, 2020. **11**(1): p. 3692.
332. Metwaly, A., et al., *Integrated microbiota and metabolite profiles link Crohn's disease to sulfur metabolism.* Nat Commun, 2020. **11**(1): p. 4322.
333. Bowerman, K.L., et al., *Disease-associated gut microbiome and metabolome changes in patients with chronic obstructive pulmonary disease.* Nat Commun, 2020. **11**(1): p. 5886.
334. Yu, Y., F. Raka, and K. Adeli, *The Role of the Gut Microbiota in Lipid and Lipoprotein Metabolism.* J Clin Med, 2019. **8**(12).
335. Chen, F., X. Dai, C.C. Zhou, K.X. Li, Y.J. Zhang, X.Y. Lou, Y.M. Zhu, Y.L. Sun, B.X. Peng, and W. Cui, *Integrated analysis of the faecal metagenome and serum metabolome reveals the role of gut microbiome-associated metabolites in the detection of colorectal cancer and adenoma.* Gut, 2021.
336. Palomino-Schatzlein, M., et al., *Combining metabolic profiling of plasma and faeces as a fingerprint of insulin resistance in obesity.* Clin Nutr, 2020. **39**(7): p. 2292-2300.
337. Needham, B.D., et al., *Plasma and Fecal Metabolite Profiles in Autism Spectrum Disorder.* Biol Psychiatry, 2021. **89**(5): p. 451-462.
338. Alves, M.A., S. Lamichhane, A. Dickens, A. McGlinchey, H.C. Ribeiro, P. Sen, F. Wei, T. Hyotylainen, and M. Oresic, *Systems biology approaches to study lipidomes in health and disease.* Biochim Biophys Acta Mol Cell Biol Lipids, 2021. **1866**(2): p. 158857.
339. Oka, P., H. Parr, B. Barberio, C.J. Black, E.V. Savarino, and A.C. Ford, *Global prevalence of irritable bowel syndrome according to Rome III or IV criteria: a systematic review and meta-analysis.* Lancet Gastroenterol Hepatol, 2020. **5**(10): p. 908-917.
340. Black, C.J. and A.C. Ford, *Global burden of irritable bowel syndrome: trends, predictions and risk factors.* Nat Rev Gastroenterol Hepatol, 2020. **17**(8): p. 473-486.
341. Fiehn, O., *Metabolomics—the link between genotypes and phenotypes.* Functional genomics, 2002: p. 155-171.
342. Lin, R., W. Liu, M. Piao, and H. Zhu, *A review of the relationship between the gut microbiota and amino acid metabolism.* Amino Acids, 2017. **49**(12): p. 2083-2090.

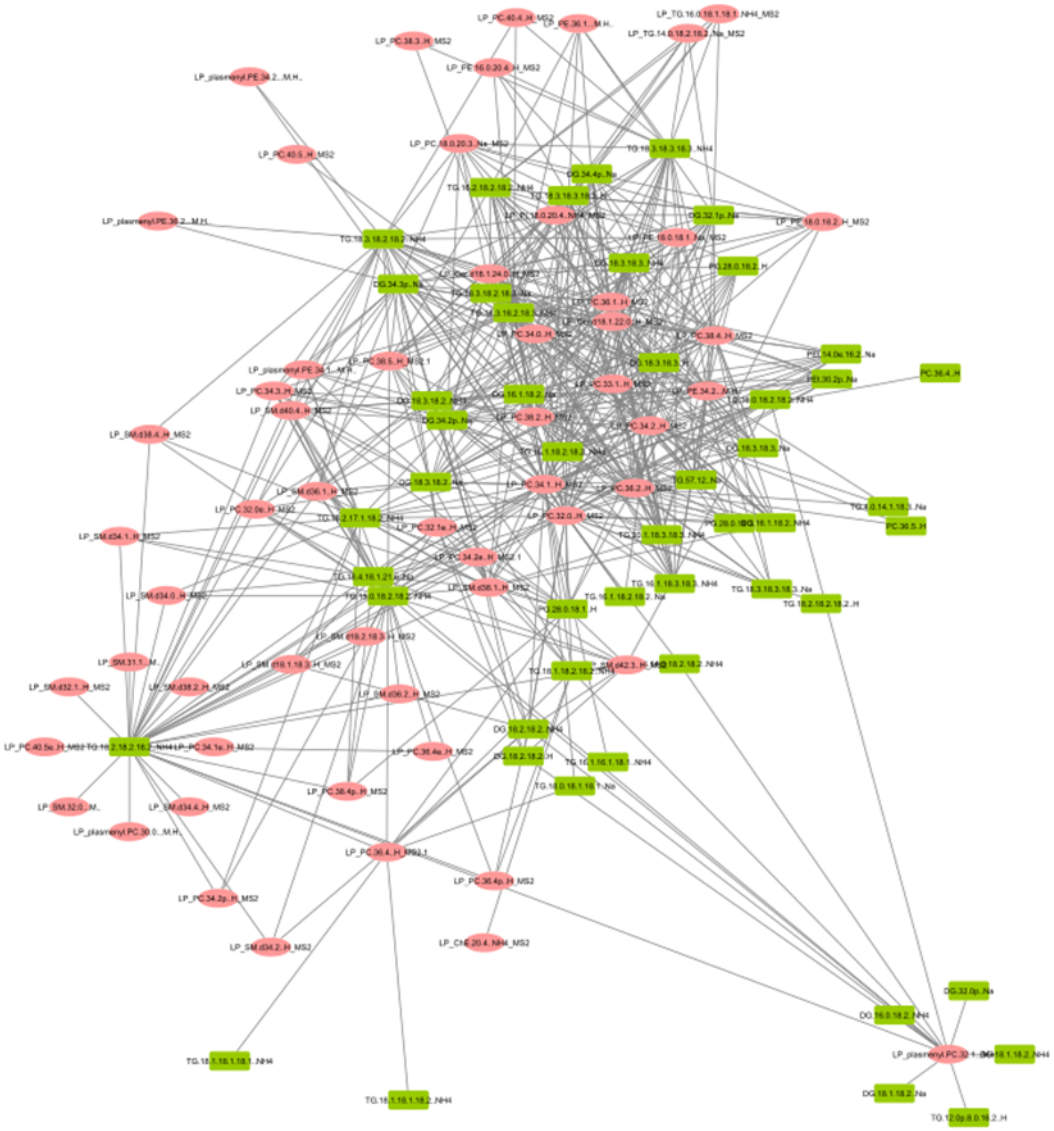
343. Jones, J., S.N. Reinke, A. Ali, D.J. Palmer, and C.T. Christophersen, *Fecal sample collection methods and time of day impact microbiome composition and short chain fatty acid concentrations.* Sci Rep, 2021. **11**(1): p. 13964.
344. Cui, M., Trimigno, A., Aru, V., Rasmussen, M. A., Khakimov, B., & Engelsen, S. B. (2021). Influence of Age, Sex, and Diet on the Human Fecal Metabolome Investigated by 1H NMR Spectroscopy. *Journal of Proteome Research*, 20(7): p, 3642-3653.
345, Barupal, D. K., Zhang, Y., Fan, S., Hazen, S. L., Tang, W. W., Cajka, T., ... & Fiehn, O. (2019). The circulating lipidome is largely defined by sex descriptors in the goldn, genebank and the adni studies. *BioRxiv*, 731448.
346. Andraos, S., Lange, K., Clifford, S. A., Jones, B., Thorstensen, E. B., Wake, M., ... & O'Sullivan, J. M. (2021). Population epidemiology and concordance for plasma amino acids and precursors in 11–12-year-old children and their parents. *Scientific reports*, 11(1): p. 1-11.
347. Sheng, L., Jena, P. K., Liu, H. X., Kalanetra, K. M., Gonzalez, F. J., French, S. W., ... & Wan, Y. J. Y. (2017). Gender differences in bile acids and microbiota in relationship with gender dissimilarity in steatosis induced by diet and FXR inactivation. *Scientific reports*, 7(1): p. 1-12.

Appendices

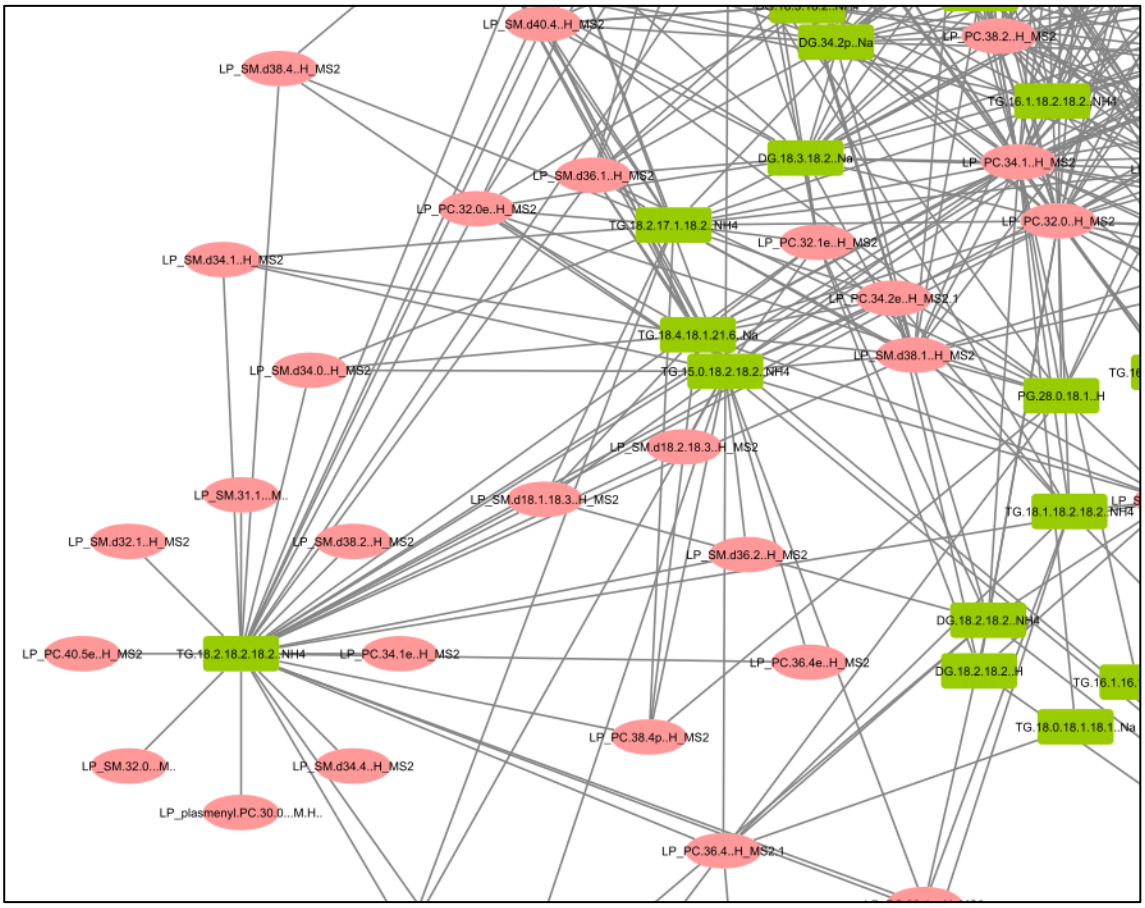
Appendix tables

Datasets of the faecal metabolome, plasma metabolome, faecal microbiome and dietary intake provided electronically.

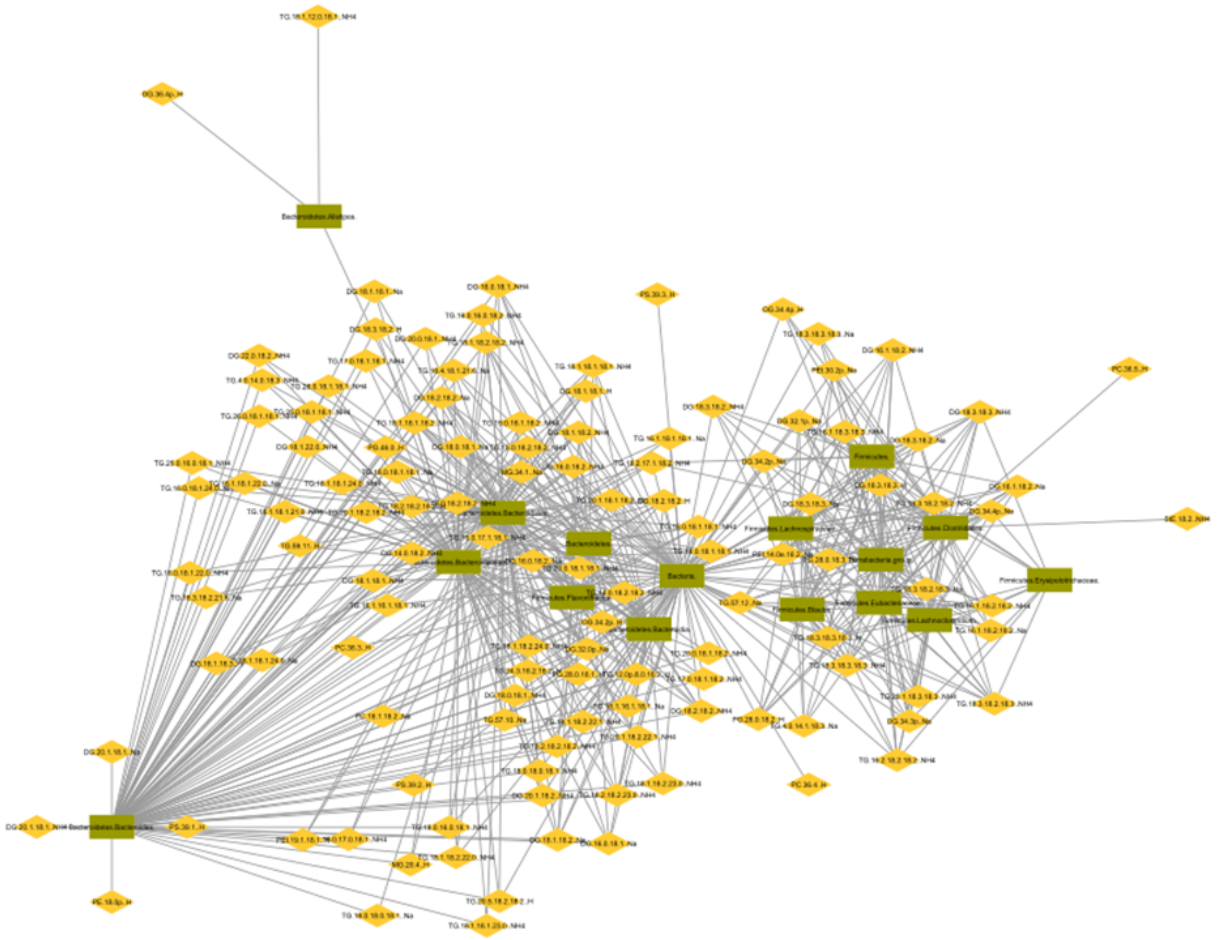
Appendix figures



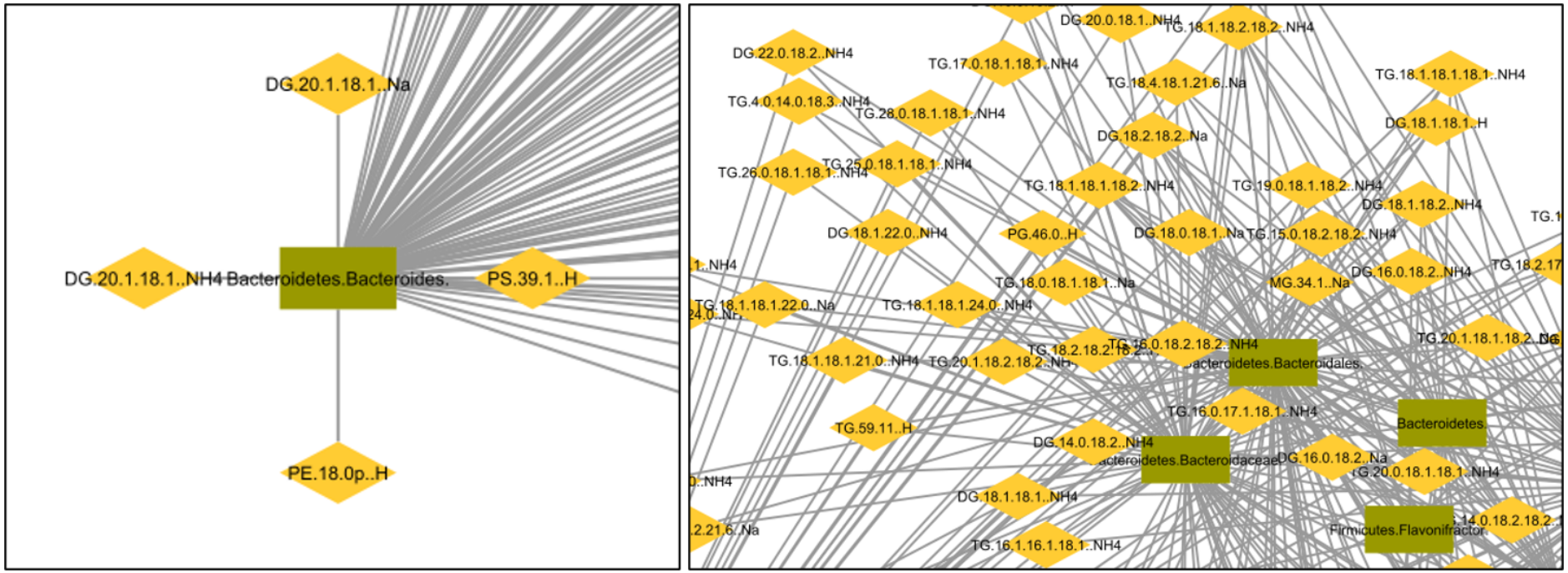
Appendix figure 1: Canonical partial least squares correlation analysis shown as pathway correlation network between the faecal metabolome (green rectangle) and plasma metabolome (pink oval) of participants with constipation, diarrhoea and healthy controls. Only correlations above 0.7 or below -0.7 are shown.



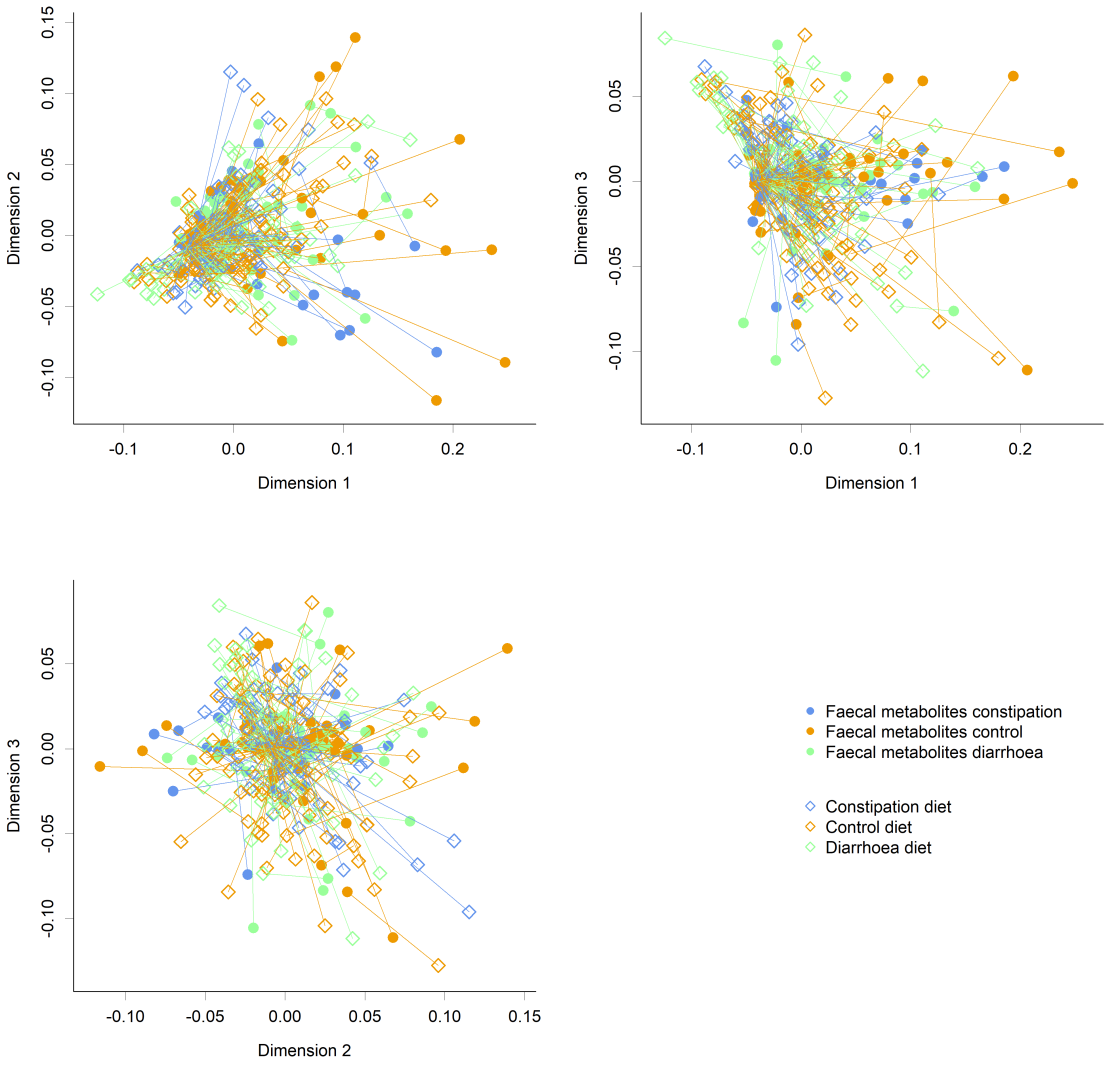
Appendix figure 2: Close-up depiction of canonical partial least squares correlation analysis shown as pathway correlation network between the faecal metabolome (green rectangle) and plasma metabolome (pink oval) of participants with constipation, diarrhoea and healthy controls. Only correlations above 0.7 or below -0.7 are shown.



Appendix figure 3: Canonical partial least squares correlation analysis shown as pathway correlation network between the faecal metabolome (yellow diamond) and faecal microbiome (green rectangle) for all participants. Only correlations above 0.5 or below -0.5 are shown.



Appendix figure 4: Close-up depiction of canonical partial least squares correlation analysis shown as pathway correlation network between the faecal metabolome (yellow diamond) and faecal microbiome (green rectangle) for all participants. Only correlations above 0.5 or below -0.5 are shown.



Appendix figure 5: Procrustes rotation analysis of the faecal metabolome and dietary intake of participants with constipation (FC + IBS-C), diarrhoea (FD + IBS-D) or healthy controls. Circles show ordination of faecal metabolites, and diamonds show ordination of dietary constituents. Lines join the faecal metabolite and dietary constituent for the same participant, with similarity (shorter) and dissimilarity (longer) shown by the length of the line. Constipation – blue, healthy controls – orange, diarrhoea – green. Significance shows the similarity of data points for the same participant $p = 0.079$.

Appendix published papers

DRC 16

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STATEMENT OF CONTRIBUTION
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James, S. C., Fraser, K., Young, W., McNabb, W. C., & Roy, N. C. (2020). Gut microbial metabolites and biochemical pathways involved in irritable bowel syndrome: effects of diet and nutrition on the microbiome. The Journal of nutrition, 150(5), 1012-1021.

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The Journal of Nutrition
Critical Review

Gut Microbial Metabolites and Biochemical Pathways Involved in Irritable Bowel Syndrome: Effects of Diet and Nutrition on the Microbiome

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ABSTRACT

The food we consume and its interactions with the host and their gut microbiota affect normal gut function and health. Functional gut disorders (FGDs), including irritable bowel syndrome (IBS), can result from negative effects of these interactions, leading to a reduced quality of life. Certain foods exacerbate or reduce the severity and prevalence of FGD symptoms. IBS can be used as a model of perturbation from normal gut function with which to study the impact of foods and diets on the severity and symptoms of FGDs and understand how critical processes and biochemical mechanisms contribute to this impact. Analyzing the complex interactions between food, host, and microbial metabolites gives insights into the pathways and processes occurring in the gut which contribute to FGDs. The following review is a critical discussion of the literature regarding metabolic pathways and dietary interventions relevant to FGDs. Many metabolites, for example bile acids, SCFAs, vitamins, amino acids, and neurotransmitters, can be altered by dietary intake, and could be valuable for identifying perturbations in metabolic pathways that distinguish a "normal, healthy" gut from a "dysfunctional, unhealthy" gut. Dietary interventions for reducing symptoms of FGDs are becoming more prevalent, but studies investigating the underlying mechanisms linked to host, microbiome, and metabolite interactions are less common. Therefore, we aim to evaluate the recent literature to assist with further progression of research in this field. *J Nutr* 2020;150:1012–1021.

Keywords: irritable bowel syndrome, functional gut disorder, gut microbiota, metabolites, diet

Introduction

The human gut is integral to well-being, with interactions between the diet, gut, and the resident microbiota resulting in beneficial or detrimental health effects. Functional gut disorders (FGDs) are characterized by bloating, pain, and stool inconsistency (1). Irritable bowel syndrome (IBS) is the most widespread example of an FGD with ~11% of the population diagnosed worldwide, although there are variations in reported rates between geographical regions, in part as a result of language barriers that affect interpretation and communication of symptoms, and partly owing to the criteria used for diagnoses (2). IBS is broadly classified into 3 subtypes: constipation-predominant IBS (IBS-C), diarrhea-predominant IBS (IBS-D), and mixed constipation and diarrhea IBS (IBS-M) (1). The mechanisms behind the onset of IBS symptoms remain unknown, and what differentiates a "normal, healthy" gut from a "dysfunctional, unhealthy" gut is difficult to define. Investigation of the gut microbial community and interactions between host and microbial metabolites may advance our understanding of mechanisms that differ between healthy individuals and those with IBS (3).

Metabolites are the products of biological pathways and enzymatic processes. Importantly, they can be measured using minimally invasive procedures to reflect the function of a tissue, organ, or system, and assist in distinguishing between disease phenotypes (3, 4). Many metabolites are signaling

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Abbreviations used: ATCC, American Type Culture Collection; BA, bile acid; BAM, bile acid malabsorption; BSH, bile salt hydrolase; CA, cholic acid; CDCA, chenodeoxycholic acid; C4, 7-α-hydroxy-4-cholesten-3-one; FGD, functional gut disorder; FGF, fibroblast growth factor; FODMAP, fermentable oligo-di-monosaccharide and polyol; GABA, γ-aminobutyric acid; IBS, irritable bowel syndrome; IBS-C, constipation-predominant irritable bowel syndrome; IBS-D, diarrhea-predominant irritable bowel syndrome; IBS-M, mixed constipation and diarrhea irritable bowel syndrome; SERT, serotonin reuptake transporter; TGF-β_1, transforming growth factor β_1; UC, ulcerative colitis; 5-HT, serotonin.

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molecules that influence biological functions throughout the body. Alterations to metabolite production in the gut from either host, microbiota, or their interactions may link to FGD symptoms (5).

SCFAs, vitamins, bile acids (BAs), lipids, neurotransmitters, and amino acids are metabolites that can be produced or modified by the host or the gut microbiota (6). Excess or insufficient production of these metabolites, compared with normal homeostatic amounts, could signal disruptions to pathways important to gut and overall health (3). Metabolites produced can be used or further modified by the host or microorganisms, highlighting the complexity of the gut environment and the requirement for comprehensive measurement of these metabolites.

The European Food Safety Authority has recognized IBS as a relevant model of gut comfort and function that shows variation from the norm and applies to the general population (7). Because diet and nutrition are popular as interventions for alleviating FGDs, and because people with IBS are motivated to find solutions (including by modifying their food consumption), investigating the responses to dietary changes in this context is a useful approach for understanding mechanisms behind FGDs.

Metabolites Linked to IBS

BAs

BA profiles are affected by diet, host characteristics, age and life stages, and the resident microbiota, with recent research showing BA metabolism may be linked to IBS (8–13). There is evidence for variation in the concentration of primary and secondary BAs in plasma in IBS participants (12). Primary BAs are produced in the liver from cholesterol via the enzyme cholesterol 7-α-hydroxylase, to produce 7-α-hydroxy-4-cholesten-3-one (C4), which is then converted into the 2 primary Bas: chenodeoxycholic acid (CDCA) and cholic acid (CA) (**Figure 1**). These BAs are conjugated to either taurine or glycine, stored in the gallbladder, secreted into the gut lumen after digestion, and then unconjugated from taurine and glycine by bacterial bile salt hydrolase (BSH) enzymes (13, 14). Most BAs (95%) are reabsorbed and recycled via the hepatic circulation, which is controlled by fibroblast growth factor 15 (FGF15) and the BA receptor farnesoid X receptor (15). Five percent of BAs escape this process and undergo modification by microorganisms with the 7α-dehdroxylase enzyme, resulting in secondary BAs with altered structures that may interact differently with cellular receptors, potentially having impacts on the functionality of metabolites (13). It is unknown if BA concentrations fluctuate owing to differences or disruptions in the ileal epithelial transporter, FGF15 molecules, precursor mechanisms in the liver, or microbial modification of the metabolites.

A meta-analysis of studies reporting on IBS-D symptoms showed that BA malabsorption (BAM) was evident in 16.9–35.3% of the individuals diagnosed with IBS-D (16). BAM is linked to diarrhea, where defective BA recycling or overproduction may increase colonic BA concentrations, leading to the onset of laxation (15, 17, 18). Conversely, a reduction in BA concentrations in the colonic mucosa may have the opposite effect, causing constipation and slowing colonic transit. In a study by Sadik et al. (18), BAM was positively associated with accelerated colonic transit in patients with chronic diarrhea (18). However, not all BAs have the same effect on the gut. Unlike CDCA and CA, which are predominantly

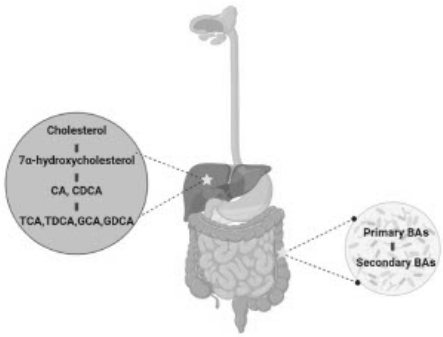

FIGURE 1 BA production and processes within the body. BAs are produced in the liver from cholesterol, followed by their storage in the gallbladder. After food intake, BAs are excreted out into the gut lumen, where they act as detergent molecules to aid in the absorption of nutrients. In the large intestine, they are converted to secondary BAs owing to the action of microbes possessing the bile salt hydrolase enzyme. Created with BioRender. BA, bile acid; CA, cholic acid; CDCA, chenodeoxycholic acid; GCA, glyco-cholic acid; GDCA, glyco-chenodeoxycholic acid; TCA, tauro-cholic acid; TDCA, tauro-chenodeoxycholic acid.

recycled, the secondary BA lithocholic acid is poorly reabsorbed, instead passing through to the colon for further modification by bacteria and then excretion (19). The action of CDCA may be facilitated by activation of intracellular secretory channels, increased mucosal permeability, or decreased fluid and electrolyte absorption (20).

Differences in fecal and serum BA concentrations are observed in individuals with IBS-C and IBS-D and may correlate with visceral pain and colonic transit (17, 21, 22). In the feces of IBS-D individuals, primary BAs were higher and secondary BAs lower in concentration than in healthy controls, suggesting BAM and the inability of BAs to be modified by the gut microbiota (23). Positive correlations were evident between concentrations of C4 and FGF19, stool weight, and total BAs in IBS-D individuals, suggesting an increase of BA production to counteract BAs lost in fecal samples. Interestingly, the relative abundance of the fecal microbiota in IBS-D individuals was characterized by reduced concentrations of *Bifidobacterium* (<1 $\log_{10}$ difference) and *Clostridium leptum* (>1 $\log_{10}$ difference), bacteria possessing BSH enzymes involved in BA transformation (23).

David et al. (24) investigated how dietary intake over 5 d influenced the gut microbiota and metabolites. In this study, they showed that an animal-based diet, compared with a plant-based diet, increased the abundance of BAs in fecal samples, which they surmised was due to higher amounts of cholesterol (a precursor of BAs) in animal-based diets. Consequently, based on the relation between dietary patterns, BA metabolism, microbial enzymatic activities, host epithelium, hepatic portal circulation, and metabolism, BA fluctuations could provide valuable insight into understanding the mechanisms contributing to the onset and severity of IBS.

SCFAs

Carbohydrates that escape digestion in the stomach are passed intact to the small and large intestines where the gut microbiota

ferments them into SCFAs (3, 25, 26). Acetate, propionate, and butyrate are the primary SCFAs produced in the gut (27). Approximately 80–90% of SCFAs produced in the colon are used by the body, with the rest excreted in feces (26).

Many bacteria can produce SCFAs, including butyrate. Some of the most common butyrate producers include *Fecalibacterium prausnitzii*, *Roseburia* spp., *Eubacterium rectale*, and *Eubacterium hallii* (28–30). Butyrate is produced via pathways utilizing lactate, acetate, sugars, and amino acids that may be by-products produced by other bacteria (29). Of the 3 pathways producing propionate, the succinate pathway is the most common and performed predominantly by *Bacteroides* spp. and *Veillonella* spp. (29). Acetate production pathways are more widespread, produced from a range of fermented carbohydrates and by a range of microbes (29, 31). Colonocytes predominantly use butyrate for energy, whereas propionate is utilized by the liver in gluconeogenesis and acetate circulates throughout the body (31, 32). Acetate and propionate are linked to the regulation of glucose homeostasis, fatty acid concentrations in the liver, and stimulating energy and appetite regulation, suggesting that relative proportions of specific SCFAs could be more important than total abundance (31).

Alterations in microbial composition and butyrate and propionate concentrations are evident in individuals with IBS compared with healthy controls (33, 34). Lower butyrate concentrations in IBS could indicate a disrupted energy supply to large intestinal colonocytes with consequences for IBS symptoms (34). A different study reported no difference in fecal acetate, propionate, butyrate, and lactate between controls and IBS participants, although total SCFA abundance was lower in the IBS-C subtype than in other subtypes (IBS-D, IBS-M) (35). Tana et al. (33) showed higher SCFA concentrations in fecal samples of IBS participants along with an increased relative abundance of *Veillonella* and *Lactobacillus*, a consistent observation because *Lactobacillus* prominently produces lactic and acetic acids, whereas *Veillonella* transforms lactic acid to acetic acid and propionic acid (33). There was a positive correlation between fecal SCFA concentration and symptom severity, signifying a possible association between metabolite production and gut discomfort (33).

The relation between SCFAs and IBS is inconsistent in the literature, because there is evidence for both higher and lower SCFA concentrations in IBS (5, 36, 37). A potential explanation for this variation is the functional redundancy of a microbial community where if one species is reduced in abundance, another species may fill the vacated niche, potentially contributing the same metabolites (e.g., SCFAs) to the system. Consequently, understanding the interaction between dietary patterns, SCFA concentration, host functions, and gut microbial activity, including species abundance, could be relevant to successfully elucidating a possible link to IBS (5, 33).

Vitamins

Perturbations in vitamin concentrations have been linked to IBS (38). Vitamins are obtained directly from dietary intake or are biosynthesized in the body. However, sufficient quantities required for the effective functioning of cellular processes may not be met by dietary intake and the host alone (39, 40). Some species of the human gut microbiota, for example lactic acid bacteria, can synthesize folate, thiamin, biotin, vitamin K, nicotinic acid, pantothenic acid, pyridoxine, and riboflavin, which may be utilized by the host (3, 39–42). These vitamins can have essential roles within the body, for example folate, which is vital in DNA replication (39). David et al. (24) noted subjects consuming animal-based dietary patterns had increased microbes with vitamin-synthesizing genes compared with those on plant-based dietary patterns, highlighting the potential role of diet, microbiota, and metabolome relations.

Vitamin B-6 (pyridoxine) has been linked to inflammatory conditions and, therefore, could be important in IBS (38, 43). In a study investigating the dietary intake of 17 individuals with IBS, a low vitamin B-6 concentration correlated to a high IBS symptom score (38). Consistent with other B-vitamins, vitamin B-6-producing pathways can be found in species from Actinobacteria, Bacteroidetes, and Proteobacteria phyla, with fewer synthesizing capabilities found in Firmicutes (40).

Magnúsdóttir et al. (44) investigated the B-vitamin-producing capacity of 256 gut microbial genomes, finding 40–65% encoded for biosynthetic pathways necessary to synthesize 8 key vitamins (biotin, cobalamin, folate, niacin, pantothenate, pyridoxine, riboflavin, and thiamin) (44). Riboflavin, an activator of mucosal-associated invariant T cells and essential in cellular metabolism as a precursor to FAD and FMN, had synthesizing genes present in 166 of the 256 annotations (39, 45). Niacin synthesis was the second most prevalent pathway, present in 162 gene annotations (44). The biosynthesis of riboflavin was found predominantly in Bacteroidetes, Proteobacteria, and Fusobacteria, with low amounts in Firmicutes and no gene pathways in Actinobacteria, in contrast to niacin pathways which were uniformly distributed over the 5 phyla (44). Differences in vitamin-synthesizing capability across different taxa raise the potential for vitamin production to vary between healthy individuals and those with IBS (44). Interestingly, Firmicutes, often found in high abundance in IBS compared with healthy individuals, was the only phylum analyzed without all 8 vitamin synthesis pathways (27, 44, 46, 47). However, mathematical modeling indicated the gut microbiota could only produce 4 of the 8 vitamins in concentrations that could have clinical relevance (44). These estimates are based solely on computational modeling and, therefore, investigating the rate and source of both host and microbial vitamin production is required because the presence of vitamin-synthesizing genes does not necessarily correlate to clinical outcomes. In addition, investigating the absorption of host- and microbially produced vitamins using methods such as stable isotope probing is needed. A more in-depth understanding of host and microbial interactions could help to elucidate further roles for vitamins in IBS, and the clinical significance of microbially produced vitamins.

Amino acids

Tryptophan is an essential amino acid obtained from dietary patterns and is an important precursor for serotonin. Therefore, it has been hypothesized that tryptophan may be an important amino acid in IBS, owing to the relative importance of serotonin (5-HT) in FGDs (48). However, an examination of plasma tryptophan concentration in IBS individuals showed no difference to healthy controls (48). Two competitive pathways, the kynurenine and serotonin pathways, metabolize tryptophan into either the vitamin niacinamide or the neurotransmitters 5-HT/melatonin (48, 49). Although tryptophan concentrations may not differ, the balance between the kynurenine pathway and the serotonin pathway may be important because of the different biological functions of the resulting metabolites (48–51).

Investigation of urinary metabolites between individuals with IBS, ulcerative colitis (UC), and healthy controls found histidine, lysine, glutamine, proline, and glutamic acid concentrations varied between IBS and UC participants, but not from healthy controls (52). Ornithine, a metabolite of the urea cycle, was the only amino acid that varied between IBS and healthy controls, with a lower concentration in IBS participants (52). However, a dietary analysis was not completed in this study. Glutamine is involved in energy supply to the epithelial cells of the gut and consequently a depletion could be crucial in IBS symptomology. When given as an oral supplement (5 g 3 times/d), glutamine reduced IBS symptom severity in individuals with postinfectious IBS-D (53). In general, understanding the possible role of amino acid metabolism in IBS requires further research to investigate their clinical relevance.

Neurotransmitters

The neurotransmitter 5-HT is produced in the gut and affects neuronal signals in the brain, highlighting its importance in gut–brain responses (54). Ninety-five percent of 5-HT is produced by the enterochromaffin cells of the gut epithelium, whereas the other 5% is produced in serotonergic neurons (54–56). 5-HT in the gut is assumed to activate neurons linked to pain, sensitivity, and reflexes via enterochromaffin and enteroendocrine cells (57, 58). The biological activity of 5-HT is terminated by serotonin reuptake transporter (SERT), the recycling mechanism for 5-HT in the body (54, 58). An overproduction of 5-HT can lead to overactivation of nerve sensing mechanisms, causing increased hypersensitivity (55). It is possible that polymorphisms in SERT may influence IBS, although studies investigating the possible association between 5-HT, the SERT gene, and IBS subtypes have had varying results (56). Atkinson et al. (59) found a lack of 5-HT uptake was associated with IBS-D symptoms owing to the deletion of a base fragment (59), whereas others concluded there was no relation between the SERT polymorphism and IBS (60, 61).

In the gut of individuals with IBS, enterochromaffin cell counts and concentrations of 5-HT were higher than in healthy controls (54). However, such differences in enterochromaffin cells are not consistently observed in the literature (62, 63). Mast cell concentration was increased in the mucosal layer of the IBS group, suggesting activation of the immune system as a causal factor in the pain and discomfort associated with IBS. Supporting this finding, the authors noted the severity of visceral pain and hypersensitivity felt by IBS participants correlated to 5-HT release (54).

Dopamine and γ-aminobutyric acid (GABA) are key neurotransmitters which may be implicated in IBS. Dopamine, a neurotransmitter of the catecholamine family, is linked to depression and anxiety (64) and has been found at lower concentrations in individuals with IBS than in healthy controls (52). In addition, GABA, which exerts important anti-inflammatory effects, was reduced in IBS-D individuals compared with healthy controls (65).

Inflammatory molecules

Cytokines are metabolites linked to inflammatory responses (66). TNF-α, a proinflammatory molecule, and the anti-inflammatory cytokines IL-10 and transforming growth factor β_1 (TGF-β_1), have potential importance in IBS (66, 67). Polymorphisms in the genetic components encoding these cytokines may increase or decrease in concentration, causing disruptions to inflammatory responses (66). Gonsalkorale et al. (66) found an association between IBS symptoms and reduced IL-10 concentrations compared with healthy controls. Another investigation showed that the concentrations of IL-β_1, IL-6, and TNF-α were higher in IBS-D participants than in healthy controls (65). A meta-analysis of 9 studies showed gender differences in TNF-α and IL-10 blood concentrations in patients with IBS (56, 68). However, results from another meta-analysis showed no correlation between TGF-β_1 and IBS (69). In general, the importance and relevance of inflammatory molecules in IBS remain unclear but plausible.

Putative biomarkers of FGDs

A biomarker is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention" (70). When considering diagnostic biomarker panels, both specificity and sensitivity are important for application in the clinical setting (71). Sensitivity refers to the true positive value where the biomarkers must accurately select for the true positives while ensuring false negatives are not selected (72). Conversely, specificity refers to the accurate selection of the true negative value, and no selection of false positives (72). Ideally, biomarkers need to be easy to measure and cost-effective in a clinical setting (8).

Studies have reported panels of metabolites in different biological matrixes between IBS and non-IBS participants with varying degrees of specificity and sensitivity (Table 1). In most cases, a high sensitivity and specificity is achieved by measuring a range of metabolic markers, and therefore the applicability for an effective and efficient diagnosis, although appropriate for research settings, would be limited in clinical settings. Current biomarker panels fail to address the underlying biochemical mechanisms and pathways that cause IBS, and therefore although diagnosis may be possible, effective treatment options remain elusive. Although biomarker panels are moving toward understanding IBS, there remains scope for significant improvement.

Impact of Dietary Intake on IBS

Between 60% and 89% of individuals with an FGD found that dietary patterns exacerbate their symptoms, resulting in individuals excluding or including certain foods or even whole food groups from or into their diet (76–82). Caution must be taken in adopting dietary regimens for IBS because they can disrupt how and why specific metabolites are produced. In a study of 36,448 individuals from France (dietary data, Rome III Diagnostic Criteria), 1870 people diagnosed with IBS had different food consumption patterns than healthy controls (83). Reduced intake of protein and micronutrients (e.g., vitamins) was characteristic of IBS individuals, attributed to lower intakes of milk, yogurt, and fruit (83). The study's findings are consistent with previous results where people believed lactose intolerance was a contributing factor to their symptoms (84, 85).

Evidence for how the removal of whole food groups can affect microbial composition and successive metabolites has been shown in studies comparing predominantly animal- or plant-based dietary patterns in healthy cohorts. An animal-based diet increased the relative abundance of *Alisipes*, *Bilophila*, and *Bacteroides* and decreased proportions of microbes known to degrade plant compounds (e.g., *Roseburia*, *E. rectale*, and

TABLE 1 Biomarker panels for discrimination of IBS[1]

Biomarkers	Sample type	Sample cohort	Sample size	Sensitivity	Specificity	Reference
1. IL-1B 2. Growth related oncogene-α 3. Brain-derived neurotrophic factor 4. Anti-*Saccharomyces cerevisiae* antibody 5. Antibody against bacterial flagellin (CBir1) 6. Antihuman tissue transglutaminase 7. TNF-like weak inducer of apoptosis 8. Antineutrophil cytoplasmic antibody 9. Tissue inhibitor of metalloproteinase-1 10. Neutrophil gelatinase-associated lipocalin	Serum	IBS, IBD, celiac disease, HC	IBS, $n = 876$; IBD, $n = 398$; celiac disease, $n = 57$; HC, $n = 235$	50%	88%	Lembo et al. (73)
Ten original biomarkers from Lembo et al. (73) and 24 additional biomarkers: 1. Histamine 2. PGE_2 3. Tryptase 4. Serotonin 5. Substance P 6. IL-1 7. IL-10 8. IL-6 9. IL-8 10. TNF-like weak inducer of apoptosis 11. 14 gene expression markers (*CBFA2T2, CCDC147, HSD17B11, LDLR, MAP6D1, MICALL1, RAB7L1, RNF26, RRP7A, SUSD4, SH3BGRL3, VIPR1, WEE1, ZNF326*).	Serum	IBS, HC	IBS, $n = 168$; HC, $n = 76$	81%	64%	(74)
1. IL-1B 2. IL-6 3. IL-12p70 4. TNF-like weak inducer of apoptosis 5. Chromogranin A 6. Human β-defensin 2 7. Calprotectin 8. Caproate	Fecal and plasma	IBS, HC	IBS, $n = 196$; HC, $n = 160$	88.1%	86.5%	(71)
1. Butane 2. N-hexane 3. Tetradecanol 4. $C_{11}H_{24}$ 5. 6-Methyloctadecane 6. 1,4-Cyclohexandiene 7. Unknown volatile organic compound 8. Methylcyclohexane 9. 2-Undecene 10. N-Heptane 11. Aziridine (ethylenimine) 12. $C_{17}H_{36}$ 13. Benzyl-oleate 14. 6,10-Dimethyl-5,9-undecadien-2-one 15. 1-Ethyl-2-methyl-cyclohexane	Breath	IBS, HC	IBS, $n = 170$; HC, $n = 153$	89.4%	73.3%	(75)

[1]HC, healthy control; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome.

Ruminococcus bromii) (24). This study concluded a predominantly animal-based dietary intake rapidly altered microbial composition within 1 d, but the population returned to its original composition within 2 d after the withdrawal of the diet (24). Alterations to BA and SCFA profiles were observed with both diets (24), emphasizing dietary intake has the potential to affect the host and microbial metabolome. In addition, the microbial transcriptome in those consuming a predominantly animal-based compared with a predominantly plant-based dietary pattern showed increased expression of microbial genes involved in key metabolic pathways, for example, vitamin biosynthesis (24). A similar study comparing the microbiota of children from Italy and Africa found that Italian children who consumed more protein in their dietary intake had higher concentrations of *Alisipes* and *Bacteroides* (86, 87). In contrast, African children consuming more legumes and vegetables had

higher counts of *Prevotella* and *Succinivibrio* microbes capable of degrading fiber and polysaccharides (87). Fiber intake between the 2 groups of children showed positive correlations to fecal SCFAs, highlighting metabolite production from the lower gut microbiota (87).

Microbial production of gases

Hydrogen gas, a product of microbial carbohydrate fermentation, is produced by numerous members of the gut microbiota (88, 89). Hydrogen is further used through cross-feeding to produce methane, hydrogen sulfide, and acetate by methanotrophic, sulfate-reducing, and acetogenic bacteria, respectively (90). These molecules are produced solely from the gut microbiota and reabsorbed into the body (88). An excess of hydrogen gas can cause discomfort for healthy and IBS individuals. Firmicutes are the primary hydrogen producers of the gut (88) and are often found in higher quantities in IBS patients with a corresponding decrease in Bacteroidetes, which may explain the common bloating and discomfort symptoms in IBS. This hypothesis is supported by the higher concentrations of breath hydrogen in IBS individuals than in healthy controls (91, 92). King et al. (92) and Dear et al. (93) both noted reducing consumption of foods known to promote hydrogen production decreased symptoms of IBS (92, 93). In addition, an increase in methane concentration is linked to a decrease in gut motility that is evident in IBS-C patients (94).

Fermentable oligo-di-monosaccharides and polyols

Fermentable oligo-di-monosaccharides and polyols (FODMAPs) are low-fermentable oligosaccharides (e.g., wheat, fructo-oligosaccharides), disaccharides (e.g., cheese, lactose), monosaccharides (e.g., honey, fructose), and polyols (e.g., certain fruits, sorbitol) that are assumed to be poorly digested and easily fermented. There is evidence that dietary regimens which exclude or reduce FODMAPs alleviate the pain and distension associated with IBS symptoms (78, 95). The association between reduced IBS symptoms and a low dietary FODMAP intake is well defined but primarily based on symptom improvement as the outcome measure, which can be subjective, rather than biochemical or mechanistic alterations (78, 96–99). Analysis by McIntosh et al. (100), where IBS participants were randomly assigned to either a low- or a high-FODMAP dietary intervention and then given a Kristalose® sachet, predominantly used as a lactulose supplement for constipation, used breath tests to measure volatile metabolites of microbial fermentation (100). Results showed an increase in hydrogen concentration in the high-FODMAP group compared with the low-FODMAP group from baseline over the 21-d period. In this study, methane concentration showed no variation, suggesting a low-FODMAP diet may not differentially alter microbial gas production (100). Both groups had similar baseline urine metabolite profiles, but after dietary intervention, 3 metabolites (histamine, azelaic acid, and p-hydroxybenzoic acid) showed variable differences (100). Urinary histamine, an immune response molecule, was higher in concentration (0.0085 μmol/mmol compared with 0.0008 μmol/mmol) in the high- than in the low-FODMAP intervention (100), in line with other findings that histamine is linked to hypersensitivity and immune activation (101, 102).

Dietary patterns, for example, normal dietary guidelines often given to IBS patients, are different to a low-FODMAP dietary regime because they involve the removal of specific foods, rather than whole food groups. Analysis of a low-FODMAP dietary intake compared with normal dietary guidelines often given to IBS patients for 4 wk showed a similar decrease in symptom severity (96). IBS dietary guidelines were focused mainly around the timing of meals, eating regular meals, avoidance of large meals, and reducing the intake of fat, caffeine, cabbage, beans, and onions (96). Further investigation into potential side-effects of a FODMAP dietary regime is required, because the removal of key food groups could present unfavorable conditions within the gut ecosystem and to the wider body. FODMAPs are often used as prebiotic supplements (103). Consequently, the widespread movement for their removal to reduce the symptoms of FGDs is paradoxical, considering the beneficial effects of prebiotics are mediated by microbial fermentation, yet the adverse effects of FODMAPs are also mediated by microbial fermentation. This is consistent with findings where *Bifidobacteria*, a butyrate-producing bacterium, was reduced after consumption of a 4-wk low-FODMAP diet (concentration 7.4 $\log_{10}$ cells/g feces) compared with normal dietary intake (concentration 8.2 $\log_{10}$ cells/g feces) (98, 103). Furthermore, SCFAs known to benefit the gut environment through a variety of mechanisms are produced from the fermentation of FODMAPs by the gut microbiota (103, 104). Although there is evidence to suggest a low-FODMAP diet is warranted in IBS, it is crucial that further studies aim to better understand the impact of FODMAP removal in the dietary pattern of healthy individuals compared with those with IBS.

Probiotics

Probiotics or foods with added beneficial bacteria have been investigated extensively for their ability to alleviate IBS symptoms, with the majority based on outcome measures of abdominal pain, bloating, and IBS symptoms (105–108). Two interventions showed improvement in symptoms after consumption of probiotics, where metabolic or microbial features were also recorded as outcome measures (109, 110). IBS-D participants given 100 g probiotic yogurt each day {7 $\log_{10}$ *Lactobacillus fermentum*[American Type Culture Collection (ATCC) 14931] CFU per gram and 7 $\log_{10}$ *Lactobacillus plantarum* (ATCC 14917) CFU per gram} for 4 wk showed beneficial changes to symptom scores, abdominal pain, and quality of life together with a reduction in fecal calprotectin from baseline (109). Fecal calprotectin is a marker of inflammation, prevalent at increased concentrations in inflammatory bowel disease. Yoon et al. (110) gave participants either a multistrain probiotic capsule [*Bifidobacterium bifidum* (KCTC12200BP), *Bifidobacterium lactis* (KCTC11904BP), *Bifidobacterium longum* (KCTC12200BP), *Lactobacillus acidophilus* (KCTC11906BP), *Lactobacillus rhamnosus* (KCTC12202BP), and *Streptococcus thermophilus* (KCTC11870BP); total 5×10^9 viable cells] or a placebo daily for 4 wk (110). Abdominal pain and bloating were both reduced in the probiotic group compared with the placebo group, although there was no difference in stool form or frequency in either group (110). Measurement of fecal microbiota showed 3 (*B. lactis*: 6.09 $\log_{10}$ cells/g feces to 7.57 $\log_{10}$ cells/g feces; *L. rhamnosus*: 2.80 $\log_{10}$ cells/g feces to 5.05 $\log_{10}$ cells/g feces; and *S. thermophilus*: 4.81 $\log_{10}$ cells/g feces to 5.35 $\log_{10}$ cells/g feces) probiotic species were increased after the intervention (110). These findings show modification and disturbances to the gut microbiome may be instrumental in understanding the underlying mechanisms linked to IBS.

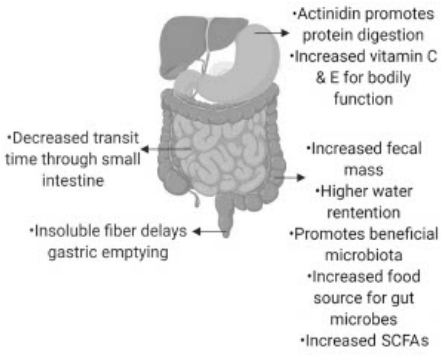

FIGURE 2 Potential beneficial effects of kiwifruit on healthy digestive progresses and on alleviating symptoms, including constipation, associated with IBS. Created with BioRender. IBS, irritable bowel syndrome.

High-fiber foods

There is an increasing awareness that some commonly consumed foods may reduce the symptoms and prevalence of IBS. Prunes, psyllium husk, wholegrain powders, and kiwifruit, which are all characterized by high dietary fiber content, have been investigated for their ability to beneficially alter IBS constipation symptoms (111–115). The soluble components of dietary fiber, for example fructans and inulin, are utilized by the gut microbiota as energy sources, promoting the growth of some beneficial bacteria, for example, *Lactobacillus* and *Bifidobacteria* (111, 116, 117). Insoluble fiber, for example, cellulose, is utilized less by the gut microbiota but is essential because it increases gut transit time by passing through the colon undissolved (117). Kiwifruit has a high nutritional value and for many years has been recommended to individuals with IBS-C (Figure 2). The high vitamin C content, actinidin (a unique protease abundant in kiwifruit), and amino acids (glutathione, arginine, and GABA) coupled with a high water-swelling capacity may be responsible for alleviating constipation symptoms (111). The effect of Hayward green kiwifruit (*Actinida deliciosa* var.) on individuals with IBS-C showed differences in symptom measures after the intervention (115). Consumption of 2 kiwifruit compared with 2 placebo capsules (glucose powder) per day showed decreased colonic transit time and increased weekly defecation in the kiwifruit-consuming participants (115). Prunes have also been shown to be effective in decreasing colonic transit and increasing stool consistency to treat chronic constipation (112). Forty participants with chronic constipation were given either prunes or psyllium (11 g twice daily) as part of a randomized crossover study (112). Both interventions improved complete spontaneous bowel movement compared with baseline, but consumption of prunes decreased colonic transit time compared with psyllium (112). Prunes also resulted in softer stool than did psyllium, with both interventions improving straining when trying to pass fecal matter compared with baseline. The improvement in symptoms from these studies highlights the relevance of using dietary interventions to understand better the mechanisms behind FGDs and their use in alleviating prevalence.

Conclusions

The underlying mechanisms governing the interaction between dietary patterns, the gut microbiota, and the host are still unclear in IBS. New evidence suggests that research and clinical practices should move away from solely relying on symptoms as a diagnostic and results-based tool. Understanding variations and fluctuations in the concentrations of host- or microbial-derived metabolites that can be used to infer processes contributing to the symptoms and severity of IBS will provide important new insights for FGD research.

In this review, we have discussed 2 main themes, the first being critical metabolites linked to IBS, and the second being studies analyzing dietary interventions to reduce the symptoms and severity of IBS. There is increasing literature focused on the clinical aspect (including dietary solutions) of reducing IBS symptoms, but analyses that further investigate the mechanisms behind the success of interventions are less common. The metabolites discussed in this review are a few key metabolic groups potentially important in understanding IBS. A decrease or increase in production of these metabolites could theoretically disrupt metabolic processes throughout the body; however, further investigation is required. Data from the literature suggest that understanding the biochemical pathways and respective metabolic products will help to identify metabolic biomarkers that could be indicative of a "dysfunctional, unhealthy" gut.

There is overwhelming evidence to suggest the microbiome is involved in FGDs, although whether this is causative or correlative needs further investigation. Less research is focused on investigating the microbially produced metabolites, including those that could be utilized by other microbes in cross-feeding reactions or that could affect localized regions of the gut or be distributed throughout the body. Because metabolites are evidence that a process or pathway has occurred, measuring their fluctuation in individuals with FGDs and related interventions will give further insight into how dietary intake is linked to IBS. Specific metabolites, for example, SCFAs, have been extensively researched; however, the possible role of BAs, vitamins, neurotransmitters, and inflammatory molecules deserves more attention in FGDs because they can have metabolic properties that are directly or indirectly linked to symptoms of IBS.

Dietary intake undoubtedly plays a role in the severity of FGDs; however, future research needs to supplement clinical studies that aim to determine the underlying mechanisms. Defining an improvement in, for example, colonic transit time does little in improving our understanding of why some individuals develop FGDs when others do not, and how we can alleviate the prevalence of FGDs worldwide. For advancements to be made, investigations that undertake dietary interventions should be followed by a thorough analysis of the gut microbiota and both host and microbial metabolites. Critically, this will accommodate not just a better understanding of the epidemiology of FGDs but also recommendations for dietary intakes to alleviate symptoms. Dietary guidelines based on studies that lack mechanistic evidence may result in the adoption of dietary regimens that lead to beneficial outcomes, but equally may be detrimental after long-term adherence owing to unintentional impacts on other biological mechanisms.

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References

1. Lacy BE, Mearin F, Chang L, Chey WD, Lembo AJ, Simren M, Spiller R. Bowel disorders. Gastroenterology 2016;150(6):1393–1407.e5.
2. Sperber AD, Dumitrascu D, Fukudo S, Gerson C, Ghoshal UC, Gwee KA, Hungin APS, Kang JY, Minhu C, Schmulson M, et al. The global prevalence of IBS in adults remains elusive due to the heterogeneity of studies: a Rome Foundation working team literature review. Gut 2017;66(6):1075–82.
3. Vernocchi P, Del Chierico F, Putignani L. Gut microbiota profiling: metabolomics based approach to unravel compounds affecting human health. Front Microbiol 2016;7:1144.
4. De Preter V, Verbeke K. Metabolomics as a diagnostic tool in gastroenterology. World J Gastrointest Pharmacol Ther 2013;4(4): 97–107.
5. Rajilić–Stojanović M, Biagi E, Heilig HG, Kajander K, Kekkonen RA, Tims S, de Vos WM. Global and deep molecular analysis of microbiota signatures in fecal samples from patients with irritable bowel syndrome. Gastroenterology 2011;141(5):1792–801.
6. Nicholson JK, Holmes E, Kinross J, Burcelin R, Gibson G, Jia W, Pettersson S. Host-gut microbiota metabolic interactions. Science 2012;336(6086):1262–7.
7. EFSA Panel on Dietetic Products, Nutrition and Allergies. Guidance on the scientific requirements for health claims related to gut and immune function. EFSA J 2011;9(4):1984.
8. Camilleri M, Halawi H, Oduyebo I. Biomarkers as a diagnostic tool for irritable bowel syndrome: where are we? Expert Rev Gastroenterol Hepatol 2017;11(4):303–16.
9. Camilleri M. What's new in functional and motility disorders in the lower GI tract? Malta Med J 2017;29(2):3–13.
10. Camilleri M, Shin A, Busciglio I, Carlson P, Acosta A, Bharucha AE, Burton D, Lamsam J, Lueke A, Donato LJ, et al. Validating biomarkers of treatable mechanisms in irritable bowel syndrome. Neurogastroenterol Motil 2014;26(12):1677–85.
11. Camilleri M, Oduyebo I, Halawi H. Chemical and molecular factors in irritable bowel syndrome: current knowledge, challenges, and unanswered questions. Am J Physiol Gastrointest Liver Physiol 2016;311(5):G777–84.
12. Long SL, Gahan CGM, Joyce SA. Interactions between gut bacteria and bile in health and disease. Mol Aspects Med 2017;56:54–65.
13. Joyce SA, Gahan CGM. Bile acid modifications at the microbe-host interface: potential for nutraceutical and pharmaceutical interventions in host health. Annu Rev Food Sci Technol 2016;7:313–33.
14. Zheng X, Huang F, Zhao A, Lei S, Zhang Y, Xie G, Chen T, Qu C, Rajani C, Dong B, et al. Bile acid is a significant host factor shaping the gut microbiome of diet-induced obese mice. BMC Biol 2017;15(1):120.
15. Shin A, Camilleri M, Vijayvargiya P, Busciglio I, Burton D, Ryks M, Rhoten D, Lueke A, Saenger A, Girtman A. Bowel functions, fecal unconjugated primary and secondary bile acids, and colonic transit in patients with irritable bowel syndrome. Clin Gastroenterol Hepatol 2013;11(10):1270–5.e1.
16. Slattery SA, Niaz O, Aziz Q, Ford AC, Farmer AD. Systematic review with meta-analysis: the prevalence of bile acid malabsorption in the irritable bowel syndrome with diarrhoea. Aliment Pharmacol Ther 2015;42(1):3–11.
17. Wong BS, Camilleri M, Carlson P, McKinzie S, Busciglio I, Bondar O, Dyer RB, Lamsam J, Zinsmeister AR. Increased bile acid biosynthesis is associated with irritable bowel syndrome with diarrhea. Clin Gastroenterol Hepatol 2012;10(9):1009–15.e3.
18. Sadik R, Abrahamsson H, Ung K-A, Stotzer P-O. Accelerated regional bowel transit and overweight shown in idiopathic bile acid malabsorption. Am J Gastroenterol 2004;99(4):711–8.
19. Makishima M, Lu TT, Xie W, Whitfield GK, Domoto H, Evans RM, Haussler MR, Mangelsdorf DJ. Vitamin D receptor as an intestinal bile acid sensor. Science 2002;296(5571):1313–6.
20. Odunsi-Shiyanbade ST, Camilleri M, McKinzie S, Burton D, Carlson P, Busciglio IA, Lamsam J, Singh R, Zinsmeister AR. Effects of chenodeoxycholate and a bile acid sequestrant, colesevelam, on intestinal transit and bowel function. Clin Gastroenterol Hepatol 2010;8(2):159–65.e5.
21. Dior M, Delagrèverie H, Duboc H, Jouet P, Coffin B, Brot L, Humbert L, Trugnan G, Seksik P, Sokol H, et al. Interplay between bile acid metabolism and microbiota in irritable bowel syndrome. Neurogastroenterol Motil 2016;28(9):1330–40.
22. Vijayvargiya P, Busciglio I, Burton D, Donato L, Lueke A, Camilleri M. Bile acid deficiency in a subgroup of patients with irritable bowel syndrome with constipation based on biomarkers in serum and fecal samples. Clin Gastroenterol Hepatol 2018;16(4): 522–7.
23. Duboc H, Rainteau D, Rajca S, Humbert L, Farabos D, Maubert M, Grondin V, Jouet P, Bouhassira D, Seksik P, et al. Increase in fecal primary bile acids and dysbiosis in patients with diarrhea-predominant irritable bowel syndrome. Neurogastroenterol Motil 2012;24(6): 513–e247.
24. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling AV, Devlin AS, Varma Y, Fischbach MA. Diet rapidly and reproducibly alters the human gut microbiome. Nature 2014;505(7484):559–63.
25. Kau AL, Ahern PP, Griffin NW, Goodman AL, Gordon JI. Human nutrition, the gut microbiome and the immune system. Nature 2011;474(7351):327–36.
26. Huda-Faujan N, Abdulamir A, Fatimah A, Anas OM, Shuhaimi M, Yazid A, Loong Y. The impact of the level of the intestinal short chain fatty acids in inflammatory bowel disease patients versus healthy subjects. Open Biochem J 2010;4:53.
27. Mayer EA, Savidge T, Shulman RJ. Brain–gut microbiome interactions and functional bowel disorders. Gastroenterology 2014;146(6): 1500–12.
28. Vital M, Karch A, Pieper DH. Colonic butyrate-producing communities in humans: an overview using omics data. mSystems 2017;2(6):e00130–17.
29. Barbara G, Feinle-Bisset C, Ghoshal UC, Santos J, Vanner SJ, Vergnolle N, Zoetendal EG, Quigley EM. The intestinal microenvironment and functional gastrointestinal disorders. Gastroenterology 2016;150(6):1305–18.e8.
30. Louis P, Young P, Holtrop G, Flint HJ. Diversity of human colonic butyrate-producing bacteria revealed by analysis of the butyryl-CoA:acetate CoA-transferase gene. Environ Microbiol 2010;12(2):304–14.
31. Morrison DJ, Preston T. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. Gut Microbes 2016;7(3):189–200.
32. Lin HV, Frassetto A, Kowalik EJ, Jr, Nawrocki AR, Lu MM, Kosinski JR, Hubert JA, Szeto D, Yao X, Forrest G. Butyrate and propionate protect against diet-induced obesity and regulate gut hormones via free fatty acid receptor 3-independent mechanisms. PLoS One 2012;7(4):e35240.
33. Tana C, Umesaki Y, Imaoka A, Handa T, Kanazawa M, Fukudo S. Altered profiles of intestinal microbiota and organic acids may be the origin of symptoms in irritable bowel syndrome. Neurogastroenterol Motil 2010;22(5):512–e115.
34. Farup PG, Rudi K, Hestad K. Faecal short-chain fatty acids—a diagnostic biomarker for irritable bowel syndrome? BMC Gastroenterol 2016;16(1):51.
35. Ringel-Kulka T, Choi CH, Temas D, Kim A, Maier DM, Scott K, Galanko JA, Ringel Y. Altered colonic bacterial fermentation as a potential pathophysiological factor in irritable bowel syndrome. Am J Gastroenterol 2015;110(9):1339–46.
36. Mortensen P, Andersen J, Arffmann S, Krag E. Short-chain fatty acids and the irritable bowel syndrome: the effect of wheat bran. Scand J Gastroenterol 1987;22(2):185–92.
37. Treem WR, Ahsan N, Kastoff G, Hyams JS. Fecal short-chain fatty acids in patients with diarrhea-predominant irritable bowel syndrome: in vitro studies of carbohydrate fermentation. J Pediatr Gastroenterol Nutr 1996;23(3):280–6.
38. Ligaarden SC, Farup PG. Low intake of vitamin B_6 is associated with irritable bowel syndrome symptoms. Nutr Res 2011;31(5): 356–61.

39. LeBlanc JG, Milani C, de Giori GS, Sesma F, van Sinderen D, Ventura M. Bacteria as vitamin suppliers to their host: a gut microbiota perspective. Curr Opin Biotechnol 2013;24(2):160–8.
40. Yoshii K, Hosomi K, Sawane K, Kunisawa J. Metabolism of dietary and microbial vitamin B family in the regulation of host immunity. Front Nutr 2019;6:48.
41. O'Connor E, Barrett E, Fitzgerald G, Hill C, Stanton C, Ross R. Production of vitamins, exopolysaccharides and bacteriocins by probiotic bacteria. In: Tamime A , editor. Probiotic dairy products. Oxford: Blackwell; 2005. pp. 167–94.
42. Hill M. Intestinal flora and endogenous vitamin synthesis. Eur J Cancer Prev 1997;6(Suppl 1):S43–5.
43. Saibeni S, Cattaneo M, Vecchi M, Zighetti ML, Lecchi A, Lombardi R, Meucci G, Spina L, De Franchis R. Low vitamin B6 plasma levels, a risk factor for thrombosis, in inflammatory bowel disease: role of inflammation and correlation with acute phase reactants. Am J Gastroenterol 2003;98(1):112–7.
44. Magnúsdóttir S, Ravcheev D, de Crécy-Lagard V, Thiele I. Systematic genome assessment of B-vitamin biosynthesis suggests co-operation among gut microbes. Front Genet 2015;6:148.
45. Kjer-Nielsen L, Patel O, Corbett AJ, Le Nours J, Meehan B, Liu L, Bhati M, Chen Z, Kostenko L, Reantragoon R. MR1 presents microbial vitamin B metabolites to MAIT cells. Nature 2012;491(7426):717–23.
46. Jeffery IB, O'Toole PW, Öhman L, Claesson MJ, Deane J, Quigley EM, Simrén M. An irritable bowel syndrome subtype defined by species-specific alterations in faecal microbiota. Gut 2012;61(7):997–1006.
47. Krogius-Kurikka L, Lyra A, Malinen E, Aarnikunnas J, Tuimala J, Paulin L, Mäkivuokko H, Kajander K, Palva A. Microbial community analysis reveals high level phylogenetic alterations in the overall gastrointestinal microbiota of diarrhoea-predominant irritable bowel syndrome sufferers. BMC Gastroenterol 2009;9(1):95.
48. Berstad A, Raa J, Valeur J. Tryptophan: 'essential' for the pathogenesis of irritable bowel syndrome? Scand J Gastroenterol 2014;49(12):1493–8.
49. Heitkemper MM, Han CJ, Jarrett ME, Gu H, Djukovic D, Shulman RJ, Raftery D, Henderson WA, Cain KC. Serum tryptophan metabolite levels during sleep in patients with and without irritable bowel syndrome (IBS). Biol Res Nurs 2016;18(2):193–8.
50. Clarke G, Fitzgerald P, Cryan JF, Cassidy EM, Quigley EM, Dinan TG. Tryptophan degradation in irritable bowel syndrome: evidence of indoleamine 2,3-dioxygenase activation in a male cohort. BMC Gastroenterol 2009;9(1):6.
51. Clarke G, McKernan DP, Gaszner G, Quigley EM, Cryan JF, Dinan TG. A distinct profile of tryptophan metabolism along the kynurenine pathway downstream of toll-like receptor activation in irritable bowel syndrome. Front Pharmacol 2012;3:90.
52. Keshteli AH, Madsen KL, Mandal R, Boeckxstaens GE, Bercik P, De Palma G, Reed DE, Wishart D, Vanner S, Dieleman LA. Comparison of the metabolomic profiles of irritable bowel syndrome patients with ulcerative colitis patients and healthy controls: new insights into pathophysiology and potential biomarkers. Aliment Pharmacol Ther 2019;49(6):723–32.
53. Zhou Q, Verne ML, Fields JZ, Lefante JJ, Basra S, Salameh H, Verne GN. Randomised placebo-controlled trial of dietary glutamine supplements for postinfectious irritable bowel syndrome. Gut 2019;68(6):996–1002.
54. Cremon C, Carini G, Wang B, Vasina V, Cogliandro RF, De Giorgio R, Stanghellini V, Grundy D, Tonini M, De Ponti F, et al. Intestinal serotonin release, sensory neuron activation, and abdominal pain in irritable bowel syndrome. Am J Gastroenterol 2011;106(6):1290–8.
55. Yeo A, Boyd P, Lumsden S, Saunders T, Handley A, Stubbins M, Knaggs A, Asquith S, Taylor I, Bahari B. Association between a functional polymorphism in the serotonin transporter gene and diarrhoea predominant irritable bowel syndrome in women. Gut 2004;53(10):1452–8.
56. Makker J, Chilimuri S, Bella JN. Genetic epidemiology of irritable bowel syndrome. World J Gastroenterol 2015;21(40):11353–61.
57. Gershon MD, Tack J. The serotonin signaling system: from basic understanding to drug development for functional GI disorders. Gastroenterology 2007;132(1):397–414.
58. Martin CR, Osadchiy V, Kalani A, Mayer EA. The brain-gut-microbiome axis. Cell Mol Gastroenterol Hepatol 2018;6(2):133–48.
59. Atkinson W, Lockhart S, Whorwell PJ, Keevil B, Houghton LA. Altered 5-hydroxytryptamine signaling in patients with constipation- and diarrhea-predominant irritable bowel syndrome. Gastroenterology 2006;130(1):34–43.
60. Pata C, Erdal ME, Derici E, Yazar A, Kanık A, Ulu O. Serotonin transporter gene polymorphism in irritable bowel syndrome. Am J Gastroenterol 2002;97(7):1780–4.
61. Lee DY, Park H, Kim WH, Lee SI, Seo YJ, Choi YC. Serotonin transporter gene polymorphism in healthy adults and patients with irritable bowel syndrome. Korean J Gastroenterol 2014;64(1):18–22.
62. Faure C, Patey N, Gauthier C, Brooks EM, Mawe GM. Serotonin signaling is altered in irritable bowel syndrome with diarrhea but not in functional dyspepsia in pediatric age patients. Gastroenterology 2010;139(1):249–58.
63. Coates MD, Mahoney CR, Linden DR, Sampson JE, Chen J, Blaszyk H, Crowell MD, Sharkey KA, Gershon MD, Mawe GM, et al. Molecular defects in mucosal serotonin content and decreased serotonin reuptake transporter in ulcerative colitis and irritable bowel syndrome. Gastroenterology 2004;126(7):1657–64.
64. Dunlop BW, Nemeroff CB. The role of dopamine in the pathophysiology of depression. Arch Gen Psychiatry 2007;64(3):327–37.
65. Aggarwal S, Ahuja V, Paul J. Dysregulation of GABAergic signalling contributes in the pathogenesis of diarrhea-predominant irritable bowel syndrome. J Neurogastroenterol Motil 2018;24(3):422.
66. Gonsalkorale W, Perrey C, Pravica V, Whorwell P, Hutchinson I. Interleukin 10 genotypes in irritable bowel syndrome: evidence for an inflammatory component? Gut 2003;52(1):91–3.
67. Komuro H, Sato N, Sasaki A, Suzuki N, Kano M, Tanaka Y, Yamaguchi-Kabata Y, Kanazawa M, Warita H, Aoki M, et al. Corticotropin-releasing hormone receptor 2 gene variants in irritable bowel syndrome. PLoS One 2016;11(1):e0147817.
68. Bashashati M, Rezaei N, Shafieyoun A, McKernan DP, Chang L, Öhman L, Quigley EM, Schmulson M, Sharkey KA, Simrén M. Cytokine imbalance in irritable bowel syndrome: a systematic review and meta-analysis. Neurogastroenterol Motil 2014;26(7):1036–48.
69. Bashashati M, Rezaei N, Bashashati H, Shafieyoun A, Daryani NE, Sharkey KA, Storr M. Cytokine gene polymorphisms are associated with irritable bowel syndrome: a systematic review and meta-analysis. Neurogastroenterol Motil 2012;24(12):1102–e566.
70. Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther 2001;69(3):89–95.
71. Mujagic Z, Tigchelaar EF, Zhernakova A, Ludwig T, Ramiro-Garcia J, Baranska A, Swertz MA, Masclee AAM, Wijmenga C, Van Schooten FJ, et al. A novel biomarker panel for irritable bowel syndrome and the application in the general population. Sci Rep 2016;6:26420.
72. Parikh R, Mathai A, Parikh S, Chandra Sekhar G, Thomas R. Understanding and using sensitivity, specificity and predictive values. Indian J Ophthalmol 2008;56(1):45–50.
73. Lembo AJ, Neri B, Tolley J, Barken D, Carroll S, Pan H. Use of serum biomarkers in a diagnostic test for irritable bowel syndrome. Aliment Pharmacol Ther 2009;29(8):834–42.
74. Jones M, Chey W, Singh S, Gong H, Shringarpure R, Hoe N, Chuang E, Talley N. A biomarker panel and psychological morbidity differentiates the irritable bowel syndrome from health and provides novel pathophysiological leads. Aliment Pharmacol Ther 2014;39(4):426–37.
75. Baranska A, Mujagic Z, Smolinska A, Dallinga J, Jonkers D, Tigchelaar E, Dekens J, Zhernakova A, Ludwig T, Masclee A, et al. Volatile organic compounds in breath as markers for irritable bowel syndrome: a metabolomic approach. Aliment Pharmacol Ther 2016;44(1):45–56.
76. Tuck CJ, Vanner SJ. Dietary therapies for functional bowel symptoms: recent advances, challenges, and future directions. Neurogastroenterol Motil 2018;30(1):e13238.
77. Hayes P, Corish C, O'Mahony E, Quigley E. A dietary survey of patients with irritable bowel syndrome. J Hum Nutr Diet 2014;27(s2):36–47.
78. Halmos EP, Power VA, Shepherd SJ, Gibson PR, Muir JG. A diet low in FODMAPs reduces symptoms of irritable bowel syndrome. Gastroenterology 2014;146(1):67–75.e5.

79. Halpert A, Dalton C, Palsson O, Morris C, Hu Y, Bangdiwala S, Hankins J, Norton N, Drossman D. What patients know about irritable bowel syndrome (IBS) and what they would like to know. National Survey on Patient Educational Needs in IBS and development and validation of the Patient Educational Needs Questionnaire (PEQ). Am J Gastroenterol 2007;102(9):1972–82.
80. Böhn L, Störsrud S, Törnblom H, Bengtsson U, Simrén M. Self-reported food-related gastrointestinal symptoms in IBS are common and associated with more severe symptoms and reduced quality of life. Am J Gastroenterol 2013;108(5):634–41.
81. Monsbakken KW, Vandvik PO, Farup PG. Perceived food intolerance in subjects with irritable bowel syndrome – etiology, prevalence and consequences. Eur J Clin Nutr 2006;60:667–72.
82. Barrett JS, Gibson PR. Fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs) and nonallergic food intolerance: FODMAPs or food chemicals? Therap Adv Gastroenterol 2012;5(4):261–8.
83. Torres MJ, Sabate J-M, Bouchoucha M, Buscail C, Hercberg S, Julia C. Food consumption and dietary intakes in 36,448 adults and their association with irritable bowel syndrome: Nutrinet-Santé study. Therap Adv Gastroenterol 2018;11:1756283X17746625.
84. Deng Y, Misselwitz B, Dai N, Fox M. Lactose intolerance in adults: biological mechanism and dietary management. Nutrients 2015;7(9):8020–35.
85. Yang J, Deng Y, Chu H, Cong Y, Zhao J, Pohl D, Misselwitz B, Fried M, Dai N, Fox M. Prevalence and presentation of lactose intolerance and effects on dairy product intake in healthy subjects and patients with irritable bowel syndrome. Clin Gastroenterol Hepatol 2013;11(3):262–8.e1.
86. Singh RK, Chang H-W, Yan D, Lee KM, Ucmak D, Wong K, Abrouk M, Farahnik B, Nakamura M, Zhu TH, et al. Influence of diet on the gut microbiome and implications for human health. J Transl Med 2017;15(1):73.
87. De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, Collini S, Pieraccini G, Lionetti P. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci U S A 2010;107(33):14691–6.
88. Carbonero F, Benefiel AC, Gaskins HR. Contributions of the microbial hydrogen economy to colonic homeostasis. Nat Rev Gastroenterol Hepatol 2012;9(9):504–18.
89. Louis P, Flint HJ. Formation of propionate and butyrate by the human colonic microbiota. Environ Microbiol 2017;19(1):29–41.
90. Smith NW, Shorten PR, Altermann EH, Roy NC, McNabb WC. Hydrogen cross-feeders of the human gastrointestinal tract. Gut Microbes 2019;10(3):270–88.
91. Kumar S, Misra A, Ghoshal UC. Patients with irritable bowel syndrome exhale more hydrogen than healthy subjects in fasting state. J Neurogastroenterol Motil 2010;16(3):299–305.
92. King T, Elia M, Hunter J. Abnormal colonic fermentation in irritable bowel syndrome. Lancet 1998;352(9135):1187–9.
93. Dear KL, Elia M, Hunter JO. Do interventions which reduce colonic bacterial fermentation improve symptoms of irritable bowel syndrome? Dig Dis Sci 2005;50(4):758–66.
94. Pimentel M, Mayer AG, Park S, Chow EJ, Hasan A, Kong Y. Methane production during lactulose breath test is associated with gastrointestinal disease presentation. Dig Dis Sci 2003;48(1):86–92.
95. Gibson P, Shepherd S. Personal view: food for thought – Western lifestyle and susceptibility to Crohn's disease. The FODMAP hypothesis. Aliment Pharmacol Ther 2005;21(12):1399–409.
96. Böhn L, Störsrud S, Liljebo T, Collin L, Lindfors P, Törnblom H, Simrén M. Diet low in FODMAPs reduces symptoms of irritable bowel syndrome as well as traditional dietary advice: a randomized controlled trial. Gastroenterology 2015;149(6):1399–407.e2.
97. Staudacher HM, Lomer MCE, Farquharson FM, Louis P, Fava F, Franciosi E, Scholz M, Tuohy KM, Lindsay JO, Irving PM, et al. A diet low in FODMAPs reduces symptoms in patients with irritable bowel syndrome and a probiotic restores bifidobacterium species: a randomized controlled trial. Gastroenterology 2017;153(4):936–47.
98. Staudacher HM, Lomer MC, Anderson JL, Barrett JS, Muir JG, Irving PM, Whelan K. Fermentable carbohydrate restriction reduces luminal bifidobacteria and gastrointestinal symptoms in patients with irritable bowel syndrome. J Nutr 2012;142(8):1510–18.
99. Shepherd SJ, Gibson PR. Fructose malabsorption and symptoms of irritable bowel syndrome: guidelines for effective dietary management. J Am Diet Assoc 2006;106(10):1631–9.
100. McIntosh K, Reed DE, Schneider T, Dang F, Keshteli AH, De Palma G, Madsen K, Bercik P, Vanner S. FODMAPs alter symptoms and the metabolome of patients with IBS: a randomised controlled trial. Gut 2017;66(7):1241–51.
101. Camilleri M, Lasch K, Zhou W. Irritable bowel syndrome: methods, mechanisms, and pathophysiology. The confluence of increased permeability, inflammation, and pain in irritable bowel syndrome. Am J Physiol Gastrointest Liver Physiol 2012;303(7):G775–85.
102. Barbara G, Wang B, Stanghellini V, De Giorgio R, Cremon C, Di Nardo G, Trevisani M, Campi B, Geppetti P, Tonini M. Mast cell-dependent excitation of visceral-nociceptive sensory neurons in irritable bowel syndrome. Gastroenterology 2007;132(1):26–37.
103. Halmos EP, Christophersen CT, Bird AR, Shepherd SJ, Gibson PR, Muir JG. Diets that differ in their FODMAP content alter the colonic luminal microenvironment. Gut 2015;64(1):93–100.
104. Zhang Y, Ma ZF, Zhang H, Pan B, Li Y, Majid HA, Lee YY. Low fermentable oligosaccharides, disaccharides, monosaccharides, and polypols diet and irritable bowel syndrome in Asia. JGH Open 2019;3(2):173–8.
105. Ducrotte P, Sawant P, Jayanthi V. Clinical trial: *Lactobacillus plantarum* 299v (DSM 9843) improves symptoms of irritable bowel syndrome. World J Gastroenterol 2012;18(30):4012–8.
106. Pineton de Chambrun G, Neut C, Chau A, Cazaubiel M, Pelerin F, Justen P, Desreumaux P. A randomized clinical trial of *Saccharomyces cerevisiae* versus placebo in the irritable bowel syndrome. Dig Liver Dis 2015;47(2):119–24.
107. Jafari E, Vahedi H, Merat S, Momtahen S, Riahi A. Therapeutic effects, tolerability and safety of a multi-strain probiotic in Iranian adults with irritable bowel syndrome and bloating. Arch Iran Med 2014;17(7):466–70.
108. Lorenzo-Zuniga V, Llop E, Suarez C, Alvarez B, Abreu L, Espadaler J, Serra J. I.31, a new combination of probiotics, improves irritable bowel syndrome-related quality of life. World J Gastroenterol 2014;20(26):8709–16.
109. Noorbakhsh H, Yavarmanesh M, Mortazavi SA, Adibi P, Moazzami AA. Metabolomics analysis revealed metabolic changes in patients with diarrhea-predominant irritable bowel syndrome and metabolic responses to a synbiotic yogurt intervention. Eur J Nutr 2019;58(8):3109–19.
110. Yoon JS, Sohn W, Lee OY, Lee SP, Lee KN, Jun DW, Lee HL, Yoon BC, Choi HS, Chung WS, et al. Effect of multispecies probiotics on irritable bowel syndrome: a randomized, double-blind, placebo-controlled trial. J Gastroenterol Hepatol 2014;29(1):52–9.
111. Bayer SB, Gearry RB, Drummond LN. Putative mechanisms of kiwifruit on maintenance of normal gastrointestinal function. Crit Rev Food Sci Nutr 2018;58(14):2432–52.
112. Attaluri A, Donahoe R, Valestin J, Brown K, Rao SSC. Randomised clinical trial: dried plums (prunes) vs. psyllium for constipation. Aliment Pharmacol Ther 2011;33(7):822–8.
113. Cheskin L, Mitola A, Ridoré M, Kolge S, Hwang K, Clark B. A naturalistic, controlled, crossover trial of plum juice versus psyllium versus control for improving bowel function. Internet J Nutr Wellness 2009;7(2):5447.
114. Woo H-I, Kwak SH, Lee Y, Choi JH, Cho YM, Om A-S. A controlled, randomized, double-blind trial to evaluate the effect of vegetables and whole grain powder that is rich in dietary fibers on bowel functions and defecation in constipated young adults. J Cancer Prev 2015;20(1):64–9.
115. Chang C-C, Lin Y-T, Lu Y-T, Liu Y-S, Liu J-F. Kiwifruit improves bowel function in patients with irritable bowel syndrome with constipation. Asia Pac J Clin Nutr 2010;19(4):451–7.
116. Brownlee IA. The physiological roles of dietary fibre. Food Hydrocolloids 2011;25(2):238–50.
117. Holscher HD. Dietary fiber and prebiotics and the gastrointestinal microbiota. Gut Microbes 2017;8(2):172–84.

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Concentrations of Fecal Bile Acids in Participants with Functional Gut Disorders and Healthy Controls

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Abstract: Bile acids are metabolites involved in nutrient absorption and signaling with levels influenced by dietary intake, metabolic processes, and the gut microbiome. We aimed to quantify 23 bile acids in fecal samples to ascertain if concentrations differed between healthy participants and those with functional gut disorders. Fecal bile acids were measured using liquid chromatography-mass spectrometry (LC-MS) in the COMFORT (The Christchurch IBS cohort to investigate mechanisms for gut relief and improved transit) cohort of 250 participants with Rome IV IBS (IBS-constipation (C), IBS-diarrhea (D), IBS-mixed (M)), functional gut disorders (functional constipation (FC), functional diarrhea (FD)) and healthy controls (FC $n = 35$, FD $n = 13$, IBS-C $n = 24$, IBS-D $n = 52$, IBS-M $n = 29$, and control $n = 97$). Dietary information was recorded to ascertain three-day dietary intake before fecal samples were collected. Fecal bile acid concentrations, predominantly primary bile acids, were significantly different between all functional gut disorder participants and healthy controls (CDCA $p = 0.011$, CA $p = 0.003$) and between constipation (FC + IBS-C) and diarrhea (FD + IBS-D) groups (CDCA $p = 0.001$, CA $p = 0.0002$). Comparison of bile acids between all functional groups showed four metabolites were significantly different, although analysis of combined groups (FC + IBS-C vs. FD + IBS-D) showed that 10 metabolites were significantly different. The bile acid profiles of FD individuals were similar to those with IBS-D, and likewise, those with FC were similar to IBS-C. Individuals with a diarrhea phenotype (FD + IBS-D) had higher concentrations of bile acids compared to those with constipation (FC + IBS-C). Bile acid metabolites distinguish between individuals with functional gut disorders and healthy controls but are similar in constipation (or diarrhea) whether classified as IBS or not.

Keywords: bile acids; irritable bowel syndrome; functional gut disorder; liquid chromatography-mass spectrometry

1. Introduction

Bile acids are chemical detergents aiding in the digestion and absorption of nutrients [1] and have a regulatory role in the circulatory system impacting lipid, glucose, nutrient, and energy homeostasis [1]. They also mediate interactions between the host

and microbiome via cellular receptors (e.g., farnesoid X receptor (FXR), G-coupled protein receptors, vitamin D receptor) [2,3].

Bile acids are synthesized in hepatocytes from cholesterol via the classic and alternative pathways, producing the primary bile acids cholic (CA) and chenodeoxycholic acid (CDCA) [2]. Primary bile acids are conjugated to either glycine or taurine and stored in the gallbladder [2,4]. Bile acids are excreted from the gallbladder into the small intestinal lumen with the bile flow and are unconjugated in the colon by microbial bile salt hydrolase enzymes [5], then modified to secondary bile acids, deoxycholic acid and lithocholic acid (LCA), by microbial dehydroxylase and dehydrogenase enzymes [2,6]. Some bile acids can be toxic to host and microbiota in excess quantities, and hence the regulation of their concentration and metabolism within the hepatic portal system is tightly controlled [1]. Most bile acids are recycled via the enterohepatic circulation multiple times a day; however, approximately 5% of bile acids escape this process and are further modified bacterially before excretion through feces [1].

The microbial conversion of primary to secondary bile acids can be a multi-step rate-limiting process. Only microbial species possessing the bile salt hydrolase enzyme (for example, some members of the *Bacteroides*, *Clostridium*, *Lactobacillus* and *Bifidobacterium* genera) can deconjugate primary bile acids [5,7]. Disturbances to the gut microbiota composition can affect bile acid deconjugation and modification [5]. Furthermore, the gut microbiome converts secondary bile acids to bacterially modified or 'tertiary' bile acids [7].

Thus, the interaction between bile acids, the gut microbiome, and host metabolism is an important homeostatic metabolic process [6]. There is increasing evidence that alterations to bile acid metabolism may be associated with clinical disease, including functional gastrointestinal disorders such as IBS. Bile acid malabsorption (BAM) was associated with IBS-diarrhea and is characterized by increased colonic transit and bowel movements, mucus production, and greater epithelial permeability [8–10]. Studies showed increased concentrations of specific primary and secondary fecal bile acids in the plasma and feces of individuals with IBS-D compared to IBS-constipation (IBS-C) and healthy controls [10–13], however these studies are often limited to either a smaller cohort size that does not incorporate multiple functional gut disorders or a smaller bile acid panel. Recently, it was shown that a major subgroup with IBS-D have BAM, and that those in the severe BAM group had a gut microbial shift that correlated with changes in the fecal metabolome and diet [14]. Another study demonstrated that 25% of IBS-D individuals had increased fecal bile acid concentrations [15] compared to healthy controls. Additionally, those with high fecal bile acids had increased relative abundance of *Clostridia*, and elevated expression of the 7α-hydroxylase (hdhA) gene, the primary enzyme converting cholesterol into bile acids [15].

We sought here to report a comprehensive panel of bile acid metabolites across the functional gut disorder spectrum (IBS-C, IBS-D, IBS-M, FC, and FD). We hypothesized that fecal bile acid concentrations will differ between IBS subtypes, functional groups, and healthy controls reflecting a perturbation in the metabolic processing of bile acids in functional gut disorders. Within IBS subtypes, bile acids will have a higher fecal concentration in individuals presenting with diarrhea (IBS-D + FD) rather than constipation (IBS-C + FC) based on the available research showing a link between diarrhea and fecal bile acids. To test these hypotheses, this analysis aimed to quantify 23 bile acids that are implicated in multiple different conversion steps and available as chemical standards in fecal samples collected from a cohort of individuals with functional gut disorders (FC, FD, IBS-C, IBS-D, IBS-M) and healthy controls.

2. Results

A total of 259 fecal samples were analyzed; however, there was incomplete metadata for 9 participants, leaving 250 participants in the final analyses. Two participants were taking cholesterol-lowering medication. Symptom questionnaires based on the Rome

Criteria IV classified these 250 participants as FC $n = 35$, FD $n = 13$, IBS-C $n = 24$, IBS-D mboxemphn = 52, IBS-M $n = 29$, and healthy control $n = 97$.

Table 1 outlines bile acid abbreviations and full, common names. Of the 23 bile acid metabolites measured THDCA, βMCA, TβMCA, TαMCA, GDDCA, UDCA, and TUDCA were below the limit of detection. Therefore 16 bile acids were quantified and included in the further analyses.

Table 1. Bile acids analyzed using UPLC-MS with the corresponding acronym.

Name	Acronym
Beta-muricholic acid	βMCA
Cheno-deoxycholic acid	CDCA
Cholic acid	CA
Deuterated (d4) cholic acid (IS)	d4-CA
Glyco-cheno-deoxycholic acid	GCDCA
Glyco-cholic acid	GCA
Glyco-deoxycholic acid	GDCA
Glyco-hyo-deoxycholic acid	GHDCA
Glyco-litho cholic acid	GLCA
Glyco-urso-deoxycholic acid	GUDCA
Hyo-cholic acid	HCA
Hyo-deoxycholic acid	HDCA
Iso-lithocholic acid	ILA
Litho-cholic acid	LCA
Taurine	Taurine
Tauro-alpha-muricholic acid	TαMCA
Tauro-beta-muricholic acid	TβMCA
Tauro-cheno-deoxycholic acid	TCDCA
Tauro-cholic acid	TCA
Tauro-deoxycholic acid	TDCA
Tauro-hyo-deoxycholic acid	THDCA
Tauro-litho cholic acid	TLCA
Tauro-urso-deoxycholic acid	TUDCA
Urso-deoxycholic acid	UDCA

Bile acids with corresponding acronym.

Table 2 shows the sex and age of the participants in the COMFORT cohort. A gender effect ($p = 0.000103$) was observed between the subtypes as there was a larger proportion of females in all groups compared to males. Age ($p = 0.128$) did not significantly differ between the groups. The average fecal dry weight percentage for all groups is presented in Table 2. Average fecal dry weight was significantly different within the cohort ($p = 0.013$), where FC and IBS-C had a higher dry weight (%) compared to FD and IBS-D. The dry weight (%) of fecal samples from controls was between that of constipation (FC and IBS-C) and diarrhea groups (FD and IBS-D) while IBS-M was higher than all other groups. Pairwise comparison showed no significant difference between the FC and IBS-C groups or the FD and IBS-D groups (Table 2).

Table 2. Characteristics of the participants of the COMFORT cohort used for the bile acid analyses, including average fecal dry weight percentage by subtypes.

	Control	IBS-C	FC	IBS-D	FD	IBS-M	*p*-Value
Female (male) *n*	52 (45)	23 (1)	25 (10)	40 (12)	11 (2)	24 (5)	0.0001
Age (mean)	54.4	53.5	59.1	52.8	58.4	50.5	0.128
Fecal average dry weight (%)	27.25	31.11	30.16	25.18	26.10	31.35	0.013

Abbreviations: Healthy control (control), functional constipation (FC), IBS-constipation (IBS-C), functional diarrhea (FD), IBS-diarrhea (IBS-D), IBS-mixed (IBS-M). *p* value for female (male) is significance between gender.

Analysis of 3-day dietary information showed no significant difference in reported fiber ($p = 0.848$) or fat ($p = 0.401$) intake by the participants of the cohort (Figure 1).

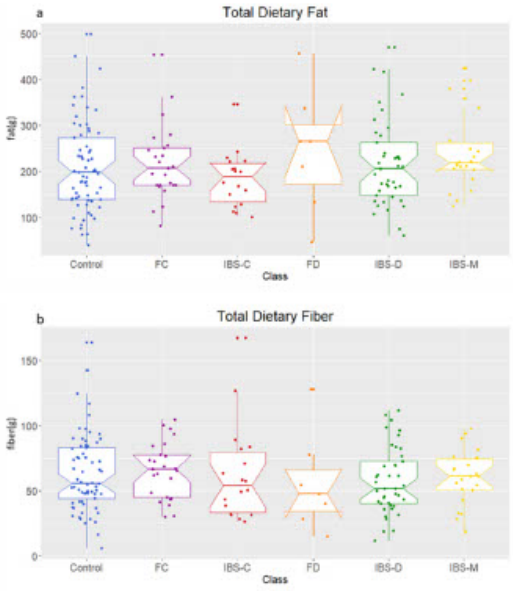

Figure 1. Dietary intake of (a) fat and (b) fiber over 3-day period recorded using diet diaries for each participant. Healthy control (control), functional constipation (FC), IBS-constipation (IBS-C), functional diarrhea (FD), IBS-diarrhea (IBS-D), IBS-mixed (IBS-M). Boxplots show median (center line), 25th and 75th percentile (top and bottom of boxes, respectively), with whiskers representing 1.5 times the inter-quartile range, and boundaries of notches show 95% confidence interval (CI).

2.1. Comparison of Fecal Bile Acid Concentrations between Healthy Control, IBS Subtypes, FC, and FD Groups

Univariate analysis showed that four bile acid metabolites (CDCA $p = 0.011$, CA $p = 0.003$, GCA $p = 0.048$, taurine $p = 0.038$) were significantly different in fecal concentration between groups (Figure 2, Table A1). Data of all the 16 available bile acids analyzed are shown in Table A2. As shown in Figure 2, further pairwise comparisons were performed for significant metabolites and showed no significant difference between FC and IBS-C groups or FD and IBS-D groups. However, there were significant differences between IBS-C and IBS-D in the fecal concentration of all four metabolites. The differences between healthy control and FC groups were only significantly different for CA. IBS-D and healthy control groups were significant for CA and CDCA. IBS-C and healthy control groups were significant between GCA and taurine. The concentrations of the primary bile acid CDCA was similar between constipation (IBS-C + FC) and diarrhea (IBS-D + FD) groups, respectively (Table A2). Two metabolites were significantly higher in males than females (CDCA $p = 0.009$, HDCA $p = 0.030$).

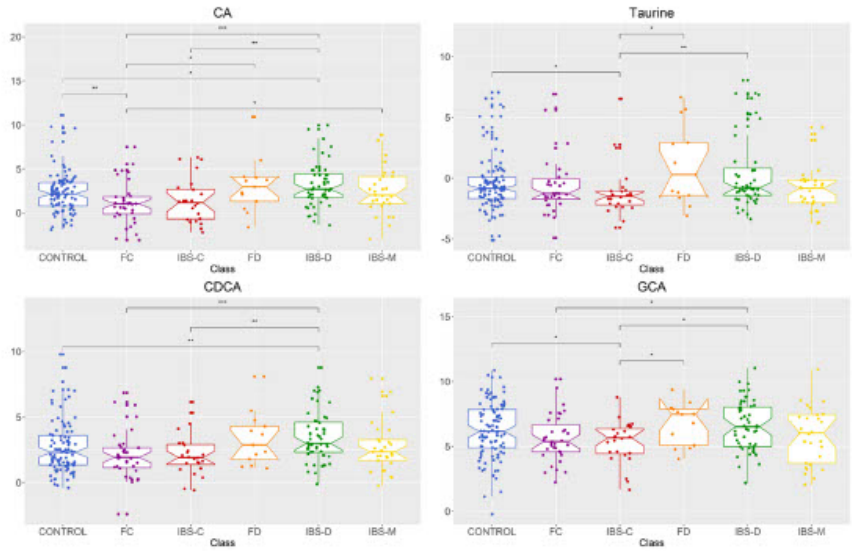

Figure 2. Bile acid metabolite distributions among healthy control, IBS subtypes, and functional gut disorders groups for metabolites with significantly different abundances between groups. Data presented as logged values of µg/mg of fecal dried weight. Healthy control (control), functional constipation (FC), IBS-constipation (IBS-C), functional diarrhea (FD), IBS-diarrhea (IBS-D), IBS-mixed (IBS-M). Boxplots show median (center line), 25th and 75th percentile (top and bottom of boxes, respectively), with whiskers representing 1.5 times the inter-quartile range, and boundaries of notches show 95% confidence interval (CI). Statistical significance denoted as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). Abbreviations: cholic acid (CA), chenodeoxycholic acid (CDCA), glycol-cholic acid (GCA).

Hierarchical clustering analysis for group averages showed IBS-D and FD, IBS-C and FC, and healthy controls and IBS-M clustered together (Figure 3). In addition, FD and IBS-D groups clustered separately from the other two groups. FD and IBS-D participants had increased fecal concentrations of bile acids, whilst FC and IBS-C had decreased concentration compared to both IBS-M and healthy controls which were characterized by variable concentrations of bile acids.

2.2. Bile Acid Concentrations Compared between Healthy Control and Combined Functional Groups

As shown in Table 2, Figures 2 and 3, FC and IBS-C and FD and IBS-D, respectively, have similar fecal bile acid profiles and fecal dry weight percentage. Therefore, the datasets from the FC and IBS-C groups were merged into a combined constipation group. Similarly, the datasets of the FD and IBS-D groups were grouped as a combined diarrhea group. Both groups were used to determine if concentration differences in fecal bile acid metabolites could be discerned between healthy controls and those exhibiting constipation or diarrhea symptoms. Additionally, there is uncertainty around the symptoms being experienced by IBS-M participants at the time of fecal sample collection. Thus, further analyses were performed without the IBS-M group.

The subsequent ANOVA analysis showed that the fecal concentration of 10 of the 16 measurable bile acids, CA ($p = 0.0002$), CDCA ($p = 0.001$), GHDCA ($p = 0.015$), GDCA ($p = 0.030$), HCA ($p = 0.025$), GCA ($p = 0.006$), taurine ($p = 0.018$), TLCA ($p = 0.007$), TCDCA ($p = 0.021$), and TDCA ($p = 0.018$) were significantly different between healthy control, constipation (FC + IBS-C), and diarrhea (FD + IBS-D) groups (Figure 4). Post hoc analysis

using the Wilcoxon test depicted as significance bars on boxplots showed that all ten bile acids were significantly higher in the diarrhea group compared to the constipation group.

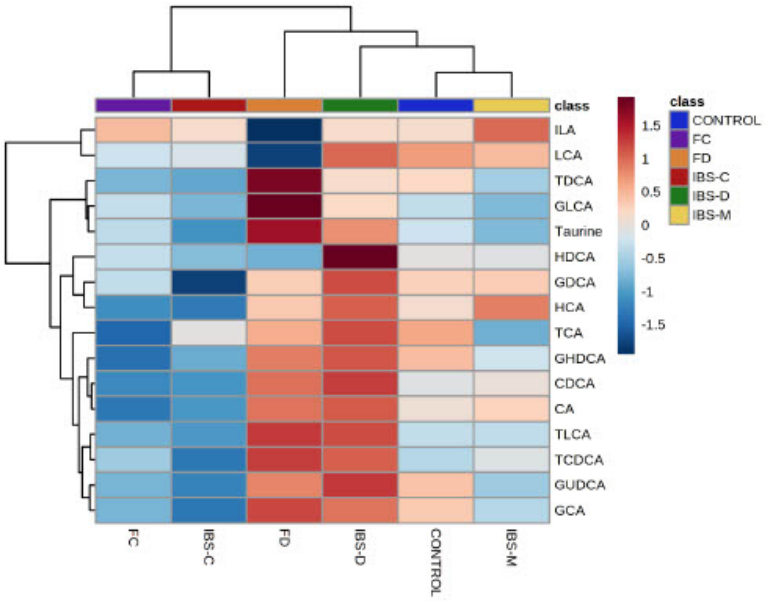

Figure 3. Hierarchical clustering analysis for average values of groups. Healthy control (control), functional constipation (FC), IBS-constipation (IBS-C), functional diarrhea (FD), IBS-diarrhea (IBS-D), IBS-mixed (IBS-M). Data presented as z score of logged values of µg/mg. Color ribbon beneath upper dendrogram identifies groups; healthy control—blue, IBS-C—red, IBS-D—green, IBS-M—yellow, FC—purple, FD—orange.

Univariate analysis showed that the fecal concentration of total primary bile acids (sum of CA and CDCA) (Figure 5) was significant, and further pairwise mean comparisons showed there were significant differences between all three groups (healthy controls, constipation (FC + IBS-C), and diarrhea (FD + IBS-D)). Constipation (FC + IBS-C) was significantly lower than healthy controls and diarrhea (FD + IBS-D), and diarrhea (FD + IBS-D) significantly higher than healthy controls and constipation (FC + IBS-C).

Hierarchical clustering analysis for average values of the fecal concentration of 16 bile acids (Figure 6) showed that the constipation (FC + IBS-C) group clustered separately from healthy controls and diarrhea (FD + IBS-D) groups which were clustered together. The heatmap highlighted a lower fecal concentration of all but one bile acid in the constipation (FC + IBS-C) group than in the diarrhea (FD + IBS-D) group.

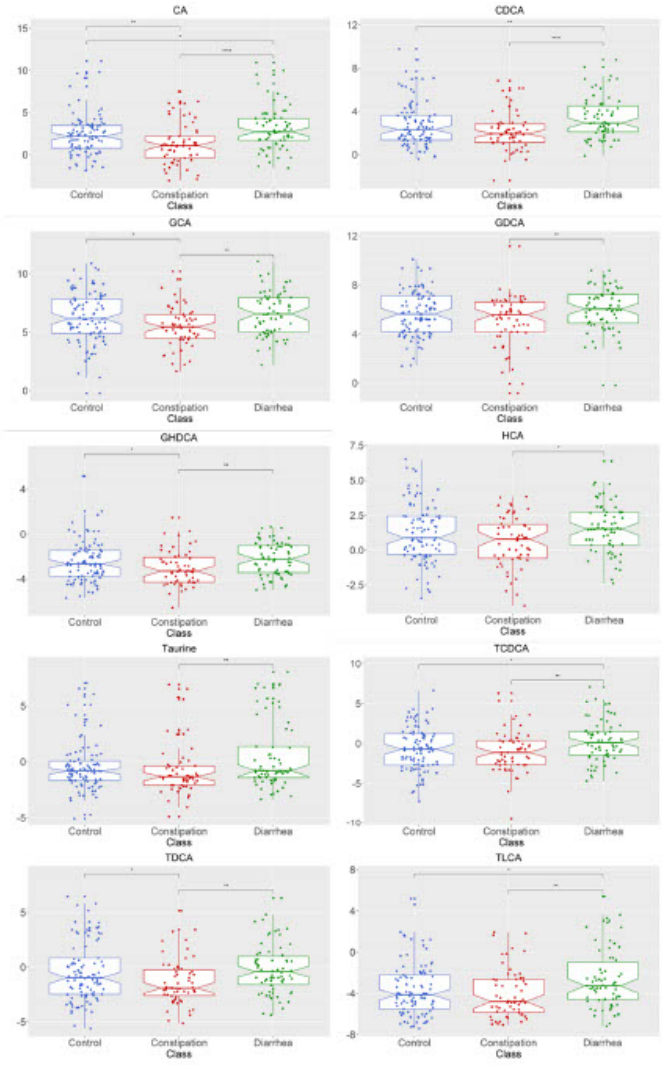

Figure 4. Bile acid metabolite distributions between healthy control, constipation (FC + IBS-C) group, and diarrhea (FD + IBS-D) group for metabolites with significantly different abundances between groups. Data presented as logged values of µg/mg. Boxplots show median (center line), 25th and 75th percentile (top and bottom of boxes, respectively), with whiskers representing 1.5 times the inter-quartile range, and boundaries of notches show 95% confidence interval (CI). Statistical significance denoted as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.0001$ (****). Abbreviations: cholic acid (CA); chenodeoxycholic acid (CDCA); glyco-cholic acid (GCA); glyco-deoxycholic acid (GDCA); glyco-hyo-deoxycholic acid (GHDCA); hyo-cholic acid (HCA); tauro-cheno-deoxycholic acid (TCDCA); tauro-deoxycholic acid (TDCA); tauro-lithocholic acid (TLCA). Constipation group is defined as individuals with both functional constipation and IBS-C. Diarrhea group is defined as individuals with both functional diarrhea and IBS-D.

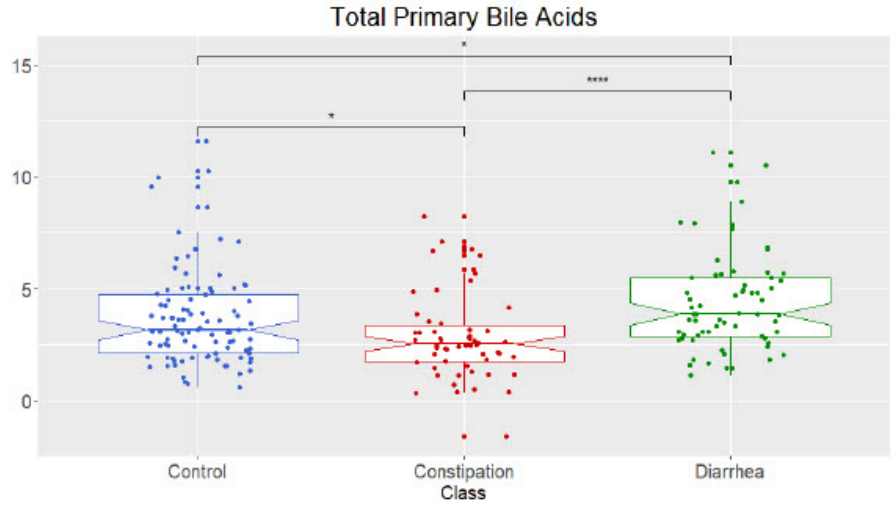

Figure 5. Total concentrations of fecal primary bile acids (sum of chenodeoxycholic acid (CDCA) and cholic acid (CA)) for healthy control and combined groups. Data presented as logged values of µg/mg of dried weight. Healthy control, constipation (FC + IBS-C) phenotype, diarrhea (FD + IBS-D) phenotype. Boxplots show median (center line), 25th and 75th percentile (top and bottom of boxes, respectively), with whiskers representing 1.5 times the inter-quartile range, and boundaries of notches show 95% confidential interval (CI). Statistical significance denoted as $p < 0.05$ (*), $p < 0.001$ (****). Constipation group is defined as individuals with both FC and IBS-C. Diarrhea group is defined as individuals with both FD and IBS-D.

When investigating gender, two bile acids (GDCA $p = 0.016$, HDCA $p = 0.003$) were significantly higher in males compared to females.

Pathway visualization of bile acid metabolites (Figure 7) summarized the significant differences ($p < 0.05$) between the constipation group (FC + IBS-C), diarrhea group (FD + IBS-D), and healthy control group. Reduced concentration of some fecal bile acid metabolites (CA, GCA, TCA, TCDA, TLCA, GHDCA) was observed in the constipation group (FC + IBS-C) compared to the control group. Similarly, the diarrhea group (FD + IBS-D) was characterized by an increased concentration in some bile acid metabolites (CA, CDCA, GCDCA, TCDCA) compared to the healthy control and the constipation group (FC + IBS-C) (CA, CDCA, GCA, TCA, GCDCA, TCDCA, GDCA, TDCA, TLDA, GHDCA, HCA).

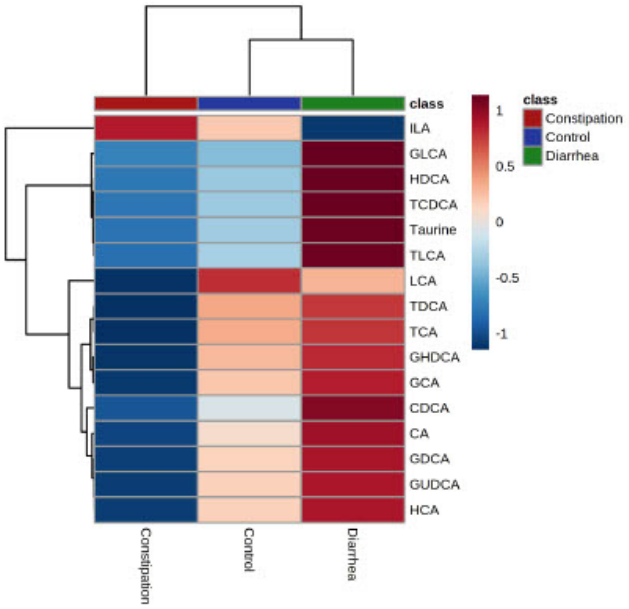

Figure 6. Hierarchical clustering analysis for average values of groups healthy control, constipation (FC + IBS-C) group, and diarrhea (FD + IBS-D) group. Data presented as z score of logged values of µg/mg. Color ribbon beneath upper dendrogram identifies group; healthy control—blue, constipation phenotype—red, diarrhea phenotype—green. Constipation group is defined as individuals with both functional constipation and IBS-C. Diarrhea group is defined as individuals with both functional diarrhea and IBS-D.

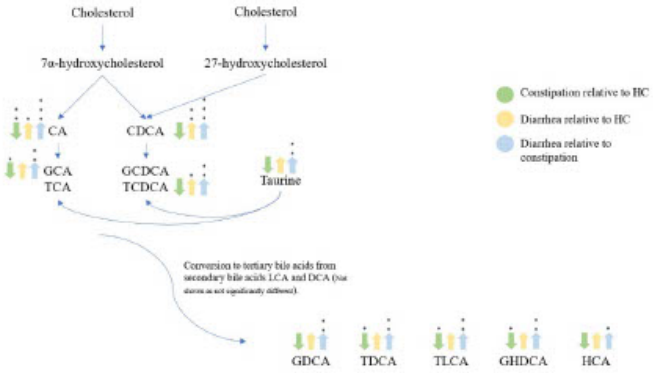

Figure 7. Bile acid pathway visualization showing significant increased or decreased concentrations in fecal samples for healthy control, constipation (FC + IBS-C), and diarrhea (FD + IBS-D) groups. Arrows depict whether the concentration is either up or down relative to the described color in the legend. Statistical significance denoted as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). Abbreviation: deoxycholic acid (DCA).

3. Discussion

This study reports the quantification of 16 bile acids in fecal samples from a cohort of participants across functional lower gut disorders. Quantitative analysis of 23 bile acids (including primary bile acids CA and CDCA) in fecal samples of the participants from this cohort was conducted and revealed that 16 bile acids were above detectable levels. The data showed that fecal concentrations of specific bile acids differed between individuals with functional gut disorders and healthy controls. Individuals with diarrhea (FD + IBS-D) were, in general, characterized by increased fecal excretion of bile acid metabolites (CA, CDCA, GCA, TCDCA, GDCA, TDCA, TLDA, GHDCA, HCA) compared to that of individuals with constipation (FC + IBS-C), IBS-M, and healthy controls. Individuals with functional diarrhea and constipation had similar bile acid concentration profiles to IBS-D and IBS-C, respectively.

The COMFORT cohort was predominantly female with similar age distributions between the phenotypes, reflective of worldwide rates of functional gut disorders. Analysis of fat and fiber intake, both of which could impact bile acid production, recorded as part of 3-day dietary diaries, showed no difference between the groups suggesting that differences in bile acid excretion were independent of diet and instead indicative of perturbed host or microbial mechanisms.

Fecal bile acids promote laxation [13,16]. The fecal concentration of CDCA and CA was higher in the combined diarrhea group (IBS-D + FD) compared to the combined constipation (IBS-C + FC) and healthy control groups, consistent with the findings of others [11,13]. The constipation group (IBS-C + FC) was characterized by a reduction in CA compared with healthy controls, unlike CDCA, where there was no difference in concentration. CDCA is produced from both primary and alternative pathways, while CA is produced solely via the primary pathway. This result suggests a possible dysfunction in the primary pathway in individuals with constipation (IBS-C + FC).

These findings suggest one of three mechanisms may be occurring. Either diarrhea (IBS-D + FD) and constipation (IBS-C + FC) individuals have perturbed biosynthesis or feedback regulating mechanisms, and therefore the known laxative effects of bile acids result in decreased colonic transit time and increased diarrhea. Alternatively, decreased colonic transit time could have reduced bile acid re-absorption from the luminal compartment into hepatic circulation, resulting in increased fecal bile acid concentrations in individuals with diarrhea as reported here. Others have suggested [10] that a cyclic process might occur where decreased re-absorption in the large intestine in participants with diarrhea initiates feedback mechanisms resulting in continuous production of bile acids.

Previous studies support the finding that fecal and plasma bile acid concentrations differ within IBS subtypes [10,12,13]. However, they do not report values for concentration per mg/g of bile acids, but rather are focused on concentration differences compared to other groups. Our results show an increased concentration of fecal bile acids in those with IBS-D and FD, and a proportion of these individuals may have undiagnosed BAM [17], either as a cause or effect of diarrhea itself. The IBS-C and FC group was characterized by reduced fecal bile acids which could be linked to decreased fecal output and increased colonic transit, as previously described in other studies [8,11]. The findings suggest that FC and IBS-C or FD and IBS-D are functionally similar regarding bile acid metabolism.

Similarly to the findings reported here, previous studies have noted concentration differences in specific bile acids between healthy controls and IBS subtypes [8], although others have not [10]. Shin et al. [11] found no difference in total fecal bile acids, but reduced proportions of the primary bile acid CDCA and secondary bile acid deoxycholic acid in IBS-C and healthy control individuals. In contrast, Dior et al. [13] showed an increase in primary, but not secondary, fecal bile acids.

In the present study, the analysis of the 16 bile acids using hierarchical clustering and other supervised statistical tools (for example, partial least squares-discriminant analysis) could not reliably differentiate IBS participants within subtypes and from healthy participants according to their groupings based on the ROME IV criteria. Inherent variability and

the difficulty with defining what makes a person 'healthy' could explain the lack of definitive clusters [18]. Classifying healthy participants based on responses to questionnaires means standardization can be difficult, ultimately highlighting the need for objectively measured scientifically validated biomarkers. Additionally, the functional basis of IBS exacerbates this, as even a healthy individual will experience gut ailments at certain times due to diet, stress, and other lifestyle factors.

Primary bile acids (CA and CDCA), either measured separately or as a total combined concentration, could be accurately measured to distinguish between IBS subtypes. Although the concentration of other bile acids was altered, CA and CDCA were most different within the IBS subtypes. Additionally, when functional groups and IBS were combined into constipation or diarrhea groups, these same differences were observed, suggesting that the functional outcomes are similar between IBS and relevant functional groups.

The measurement of the primary bile acids, CA and CDCA, provides information at the start of the bile acid pathway where under-activation or over-activation of one pathway could increase or decrease shuttling through downstream bile acids. The relative concentrations of glycine and taurine conjugated compounds (GCA, TCA, GCDCA, and TCDCA) can provide a downstream view of the bile acid pathway. Measurement of glycine was not performed in this study. However, concentrations of taurine were different between healthy controls and IBS subtypes, perhaps highlighting differences in conjugation potential and suggesting that further analysis should include glycine. This analysis will make inferences about changes occurring downstream in the pathway and is likely important for a better understanding of, if and how these metabolites are involved in functional gut disorders. The combination of the analysis of primary bile acids in fecal samples with the analysis of predominantly 'tertiary' bile acids and microbial community changes would be necessary to advance the knowledge of the role of bile acids in functional gut disorders.

The strengths of the analysis and data reported here are the quantitative LCMS method used to quantify the 23 bile acids, rather than total bile acids in stool samples from the COMFORT cohort representing the functional gut disorder spectrum. The sample size of the FD group was small in comparison to other groups. However, when combined with IBS-D participants, the group size was comparable to the other groups. The quantification of total bile acids in fecal samples is a proven method to diagnose BAM [17]. However, measuring total bile acids may provide limited insights into the physiological responses and mechanisms underlying functional gut disorders as the total will not equate to 100% of bile acids present [13]. Furthermore, considering the extensive microbial modification and epimerization results in a diverse range of bile acids and derived metabolites, obtaining standards to quantify all possible bile acids remains elusive. The data for total primary bile acids (CA and CDCA) reported here were accurately measured using internal standards.

There are also some limitations of this study relating to sample collection, dietary records, and sample analysis. Bile acids are metabolites that are influenced by dietary intake, host and microbial metabolism, and gut transit, and it was expected that some of these factors would impact the findings. Variations could arise as active recycling mechanisms will differ naturally between individuals. Additionally, the homogeneity of the samples could alter the concentration of bile acids. The home collection kit brings some potential sources of variation, such as differences in how long participants kept their sample out of the freezer or travel time on ice to the laboratory. The accuracy of the diet dataset relies on the participants accurately recording their dietary intake or when after food consumption bowel movements were performed.

4. Materials and Methods

4.1. Participants

Two hundred and fifty-nine individuals from Canterbury, New Zealand, were recruited to participate in The Christchurch IBS cohort to investigate mechanisms for gut relief and improved transit (COMFORT cohort. Universal trial number: U1111-1216-6662) cohort as previously described [19]. Cases were individuals with IBS, or a functional lower

gut disorder diagnosis defined by the Rome Criteria IV (including Bristol stool score to identify subtypes; FC, FD, IBS-C, IBS-D, and IBS-M) undergoing colonoscopy for symptom investigation or surveillance aged 18–70 years. Healthy controls were asymptomatic individuals undergoing colonoscopy for surveillance due to a family history of colorectal cancer, personal history, or screening of colorectal cancer or polyps aged 18–70 years. Individuals that were pregnant or had a known organic disorder (inflammatory bowel disease, colorectal cancer, diverticulitis), previous bowel resection, and coeliac disease were excluded from the study. The study was approved by the University of Otago Human Ethics Committee (Ref. # H16/094).

4.2. Diet Record and Sample Collection

Dietary records were kept for three sequential days (including one day of the weekend) before fecal collection [19]. Fecal samples were collected using at-home kits by participants, stored at 4 °C and transferred to the research facility within 24 h, where specimens were snap-frozen in liquid nitrogen and stored at −80 °C. Samples were transported to AgResearch, Palmerston North, New Zealand, on dry ice for bile acid analysis. Samples were freeze-dried and stored at −80 °C prior to extraction.

4.3. Standards and Reagents

Deuterated-cholic acid (d4-CA), bile acids (CA, CDCA, LCA, TCA, UDCA, taurine, βMCA, TαMCA, TβMCA, TLCA, TCDCA), and formic acid were purchased from Sigma-Aldrich Chemicals Co. (St Louis, MO, USA). All other bile acid standards (GCDCA, GCA, GDCA, GHDCA, GLCA, GUDCA, HCA, HDCA, ILA, TDCA, THDCA, TUDCA) were purchased from Steraloids Inc. (Newport, RI, USA). Acetonitrile (ACN) and methanol (MeOH) of Optima LC-MS grade quality were purchased from Thermo Fisher Scientific (Auckland, New Zealand).

4.4. Sample Extraction

Extraction methods followed those previously described by Joyce et al. [7] with minor modifications. Briefly, 100 mg of freeze-dried fecal samples were spiked with 100 ng of d4-CA and extracted with 700 µL ice-cold 50% MeOH in Eppendorf tubes pre-filled with 4 mm ceramic beads. The mixture was homogenized for six 30 s intervals (QIAGEN TissueLyser II, QIAGEN, Hilden, Germany) and incubated at −20 °C for 30 min and then centrifuged at 10,000× *g* for 25 min. Furthermore, 450 µL of the extract was transferred to a fresh tube and dried under nitrogen at 45 °C. One milliliter of ice-cold ACN containing 5% formic acid was added to each tube, and the sample briefly vortexed and agitated for 1 h gently at room temperature. The mixture was centrifuged at 10,000× *g* for 10 min and the resulting supernatant transferred to Eppendorf tubes and dried under nitrogen at 45 °C. The residual extract was dissolved in 150 µL of 50% MeOH, centrifuged at 10,000× *g* for 5 min and transferred to glass vials for chromatographic analysis.

The analysis was completed on a SCIEX LCMS/MS QTRAP 6500+ system coupled to an ExionLC (SCIEX, Victoria, Australia). Furthermore, 1 µL of the sample was injected into a Waters Aquity Ultra Performance Liquid Chromatography (UPLC) column (Massachusetts, USA) maintained at 50 °C with a flow rate of 300 µL/min. The mobile phase, solvent A, consisted of 10 mM ammonium formate in H_2O and solvent B, 10 mM ammonium formate, 5% ACN/95% MeOH. Gradient elution was as follows; 50% B held for 2 min then increased to 87% B at 13.5 min, 99% B at 18 min, returning to 50% B at 19 min and held until 21 min for re-equilibration.

Mass spectral detection was performed in negative electrospray ionization mode using multiple reaction monitoring (MRM) for 23 bile acid compounds and the internal standard using electrospray ionization. Standards for all target compounds were run prior to sample analysis to optimize MRM conditions and separation of compounds. The source voltage was set to −4500 V, with a source temperature of 550 °C. Data was captured using Analyst (V1.6) software and processed on MultiQuant (V3.0.2) SCIEX software. Bile acid

concentrations were generated from standard curves of standard injections for all 23 bile acids and the deuterated internal standard (d4-CA). Concentrations of bile acids were corrected to dry weight of fecal matter and are presented as µg/mg of dried fecal sample. K-nearest neighbor (KNN) was employed to input any missing values in the data using MetaboAnalyst (V4.0) [20,21].

4.5. Statistical Analyses

Residual plots and the Shapiro–Wilk test were employed to determine normality, showing uneven distribution, and thus the data were log-transformed. R statistical package (V3.6.1) was used for individual metabolite analyses and heatmap visualizations. ANOVA was used to compare means, with a probability (p) less than 0.05 deemed statistically significant. If a metabolite was significantly different, pairwise mean comparisons were used to compare differences between participant groups. Bile acid metabolite distributions were visualized using notched box plots, with the boundaries of the notches showing 95% confidence interval (CI). Metaboanalyst (V4.0) [20] was used for hierarchical clustering analysis (Ward's Method clustering type). Basic nutritional data were analyzed using ANOVA to compare group differences in three-day dietary intake. Fecal dry weight was calculated relative to wet weight.

5. Conclusions

In conclusion, this study shows that IBS subtypes combined with their respective functional groups have different fecal bile acid profiles compared to the healthy control group. Measuring fecal bile acid concentrations could not differentiate between functional groups and the respective IBS subtypes. Individuals with diarrhea (IBS-D + FD) showed increased fecal bile acid excretion compared to individuals with constipation (IBS-C + FC) and healthy controls, suggestive of a perturbed bile acid metabolism from that of a normal healthy gut. More specifically, concentration differences in primary bile acids in stool samples could be used to distinguish between constipation (IBS-C + FC) and healthy controls or between diarrhea (IBS-D + FD) and healthy controls. Host-microbial metabolism results in a diverse range of bile acids and derived metabolites. This study shows that bile acids have the potential to be utilized as biomarkers in the clinical setting. Although bile acid concentrations were not distinguishable between functional diarrhea and IBS-D, the study showed that diarrhea conditions are associated with increased bile acid excretion. Others showed that bile acid malabsorption could underlie many cases of diarrhea [10,11]. Therefore, understanding not just the total concentration of bile acids in the feces but also the relative concentrations may lead to more targeted use of established and novel bile acid sequestrants. Considering the microbial community and the physiological changes in the large intestine of these participants would help further advance the knowledge of the role of bile acids in functional gut disorders.

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Appendix A

Table A1. Significance probability (p) values for bile acid metabolites between healthy control, constipation (FC + IBS-C) group and diarrhea (FD + IBS-D) group.

	Group p-Value	Gender p-Value	Group × Gender p-Value
CA	0.0002 ***	0.168	0.092
CDCA	0.001 ***	0.034	0.152
GHDCA	0.015 *	0.646	0.214
GUDCA	0.138	0.205	0.848
GDCA	0.030 *	0.286	0.016 *
HDCA	0.160	0.045	0.003 *
HCA	0.025 *	0.277	0.140
GCA	0.006 **	0.488	0.613
ILA	0.893	0.821	0.632
GLCA	0.295	0.765	0.250
Taurine	0.018 *	0.402	0.768
LCA	0.581	0.539	0.316
TLCA	0.007 **	0.348	0.653
TCDCA	0.021 *	0.320	0.456
TDCA	0.018 *	0.445	0.083
TCA	0.098	0.157	0.134

Statistical significance denoted as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). Abbreviations: cholic acid (CA); chenodeoxycholic acid (CDCA); glyco-hyo-deoxycholic acid (GHDCA); glyco-urso-deoxycholic acid (GUDCA); glyco-deoxycholic acid (GDCA); hyo-deoxycholic acid (HDCA); hyo-cholic acid (HCA); glyco-cholic acid (GCA); iso-lithocholic acid (ILA); glyco-litho cholic acid (GLCA); lithocholic acid (LCA); tauro-lithocholic acid (TLCA); tauro-cheno-deoxycholic acid (TCDCA); tauro-deoxycholic acid (TDCA); tauro-cholic acid (TCA). Constipation group is defined as individuals with both functional constipation and IBS-C. Diarrhea group is defined as individuals with both functional diarrhea and IBS-D.

Table A2. Average concentration values of groups for bile acid metabolites.

	Control	IBS-C	FC	IBS-D	FD	IBS-M
GHDCA	0.72 ± 3.61	0.29 ± 0.56	0.21 ± 0.26	0.34 ± 0.31	0.40 ± 0.49	0.24 ± 0.24
GUDCA	0.81 ± 3.88	0.32 ± 0.57	0.47 ± 1.26	0.40 ± 0.38	0.47 ± 0.58	0.28 ± 0.25
GDCA	110.41 ± 167.88	53.49 ± 54.29	126.42 ± 371.25	119.12 ± 130.64	91.47 ± 88.84	92.10 ± 112.37
HDCA	90.55 ± 101.77	92.99 ± 96.97	87.33 ± 77.63	122.15 ± 135.87	84.13 ± 88.98	71.12 ± 71.11
HCA	6.77 ± 13.63	2.26 ± 1.97	3.43 ± 3.87	5.62 ± 6.66	9.87 ± 21.42	5.21 ± 7.40
GCA	199.35 ± 317.56	68.1 ± 86.16	118.23 ± 222.78	221.83 ± 346.59	177.95 ± 169.83	166.30 ± 353.11
ILA	31.51 ± 20.67	30.91 ± 18.1	31.25 ± 17.92	33.17 ± 23.27	28.97 ± 15.79	35.01 ± 22.27
GLCA	0.18 ± 0.18	0.17 ± 0.13	0.21 ± 0.21	0.20 ± 0.15	2.01 ± 6.22	0.16 ± 0.11
Taurine	5.56 ± 18.91	4.65 ± 18.29	7.29 ± 23.0	17.85 ± 46.93	16.59 ± 29.4	1.84 ± 4.07
LCA	548.75 ± 336.88	483.52 ± 270.23	481.59 ± 271.46	618.36 ± 426.56	451.99 ± 189.5	513.83 ± 289.08
CDCA	29.65 ± 102.48	9.31 ± 15.35	11.61 ± 23.37	31.92 ± 67.42	32.39 ± 70.86	22.83 ± 50.26

Table A2. *Cont.*

	Control	IBS-C	FC	IBS-D	FD	IBS-M
CA	56.16 ± 255.46	12.44 ± 23.53	11.16 ± 30.87	58.79 ± 172.97	160.13 ± 513.21	37.52 ± 99.60
TLCA	0.94 ± 4.46	0.26 ± 0.77	0.35 ± 0.8	1.69 ± 6.10	2.17 ± 3.82	0.76 ± 3.06
TCDCA	3.35 ± 10.5	1.19 ± 2.48	4.65 ± 14.37	6.35 ± 19.67	7.28 ± 12.12	3.30 ± 8.97
TDCA	4.84 ± 12.5	0.64 ± 0.68	2.27 ± 6.24	3.54 ± 11.74	5.34 ± 5.89	3.26 ± 10.22
TCA	4.14 ± 7.82	2.06 ± 3.1	2.10 ± 4.87	6.47 ± 20.76	2.70 ± 4.0	6.55 ± 28.14

Values presented as mean (μg/mg) ± standard deviation. Abbreviations: Healthy control (control), functional constipation (FC), IBS-constipation (IBS-C), functional diarrhea (FD), IBS-diarrhea (IBS-D), IBS-mixed (IBS-M). Cholic acid (CA); chenodeoxycholic acid (CDCA); glyco-hyo-deoxycholic acid (GHDCA); glyco-urso-deoxycholic acid (GUDCA); glyco-deoxycholic acid (GDCA); hyo-deoxycholic acid (HDCA); hyo-cholic acid (HCA); glyco-cholic acid (GCA); iso-lithocholic acid (ILA); glyco-litho cholic acid (GLCA); lithocholic acid (LCA); tauro-lithocholic acid (TLCA); tauro-cheno-deoxycholic acid (TCDCA); tauro-deoxycholic acid (TDCA); tauro-cholic acid (TCA).

References

1. Zhou, H.; Hylemon, P.B. Bile acids are nutrient signaling hormones. *Steroids* **2014**, *86*, 62–68. [CrossRef]
2. Joyce, S.A.; Gahan, C.G.M. Bile Acid Modifications at the Microbe-Host Interface: Potential for Nutraceutical and Pharmaceutical Interventions in Host Health. *Annu. Rev. Food Sci. Technol.* **2016**, *7*, 313–333. [CrossRef] [PubMed]
3. Molinaro, A.; Wahlstrom, A.; Marschall, H.U. Role of Bile Acids in Metabolic Control. *Trends Endocrinol. Metab.* **2018**, *29*, 31–41. [CrossRef]
4. Oduyebo, I.; Camilleri, M. Bile acid disease: The emerging epidemic. *Curr. Opin. Gastroenterol.* **2017**, *33*, 189–195. [CrossRef] [PubMed]
5. Molinero, N.; Ruiz, L.; Sanchez, B.; Margolles, A.; Delgado, S. Intestinal Bacteria Interplay with Bile and Cholesterol Metabolism: Implications on Host Physiology. *Front. Physiol.* **2019**, *10*, 185. [CrossRef] [PubMed]
6. Wahlstrom, A.; Sayin, S.I.; Marschall, H.U.; Backhed, F. Intestinal Crosstalk between Bile Acids and Microbiota and Its Impact on Host Metabolism. *Cell Metab.* **2016**, *24*, 41–50. [CrossRef]
7. Joyce, S.A.; MacSharry, J.; Casey, P.G.; Kinsella, M.; Murphy, E.F.; Shanahan, F.; Hill, C.; Gahan, C.G. Regulation of host weight gain and lipid metabolism by bacterial bile acid modification in the gut. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 7421–7426. [CrossRef]
8. Vijayvargiya, P.; Busciglio, I.; Burton, D.; Donato, L.; Lueke, A.; Camilleri, M. Bile Acid Deficiency in a Subgroup of Patients with Irritable Bowel Syndrome with Constipation Based on Biomarkers in Serum and Fecal Samples. *Clin. Gastroenterol. Hepatol.* **2018**, *16*, 522–527. [CrossRef]
9. Camilleri, M. Bile Acid diarrhea: Prevalence, pathogenesis, and therapy. *Gut Liver* **2015**, *9*, 332–339. [CrossRef]
10. Wong, B.S.; Camilleri, M.; Carlson, P.; McKinzie, S.; Busciglio, I.; Bondar, O.; Dyer, R.B.; Lamsam, J.; Zinsmeister, A.R. Increased bile acid biosynthesis is associated with irritable bowel syndrome with diarrhea. *Clin. Gastroenterol. Hepatol.* **2012**, *10*, 1009–1015.e1003. [CrossRef]
11. Shin, A.; Camilleri, M.; Vijayvargiya, P.; Busciglio, I.; Burton, D.; Ryks, M.; Rhoten, D.; Lueke, A.; Saenger, A.; Girtman, A. Bowel functions, fecal unconjugated primary and secondary bile acids, and colonic transit in patients with irritable bowel syndrome. *Clin. Gastroenterol. Hepatol.* **2013**, *11*, 1270–1275.e1271. [CrossRef]
12. Duboc, H.; Rainteau, D.; Rajca, S.; Humbert, L.; Farabos, D.; Maubert, M.; Grondin, V.; Jouet, P.; Bouhassira, D.; Seksik, P. Increase in fecal primary bile acids and dysbiosis in patients with diarrhea-predominant irritable bowel syndrome. *Neurogastroenterol. Motil.* **2012**, *24*, 513. [CrossRef] [PubMed]
13. Dior, M.; Delagrèverie, H.; Duboc, H.; Jouet, P.; Coffin, B.; Brot, L.; Humbert, L.; Trugnan, G.; Seksik, P.; Sokol, H.; et al. Interplay between bile acid metabolism and microbiota in irritable bowel syndrome. *Neurogastroenterol. Motil.* **2016**, *28*, 1330–1340. [CrossRef] [PubMed]
14. Jeffery, I.B.; Das, A.; O'Herlihy, E.; Coughlan, S.; Cisek, K.; Moore, M.; Bradley, F.; Carty, T.; Pradhan, M.; Dwibedi, C.; et al. Differences in Fecal Microbiomes and Metabolomes of People With vs Without Irritable Bowel Syndrome and Bile Acid Malabsorption. *Gastroenterology* **2020**, *158*, 1016–1028.e1018. [CrossRef] [PubMed]
15. Zhao, L.; Yang, W.; Chen, Y.; Huang, F.; Lu, L.; Lin, C.; Huang, T.; Ning, Z.; Zhai, L.; Zhong, L.L. A Clostridia-rich microbiota enhances bile acid excretion in diarrhea-predominant irritable bowel syndrome. *J. Clin. Investig.* **2019**, *130*, 438–450. [CrossRef] [PubMed]
16. Rao, A.S.; Wong, B.S.; Camilleri, M.; Odunsi-Shiyanbade, S.T.; McKinzie, S.; Ryks, M.; Burton, D.; Carlson, P.; Lamsam, J.; Singh, R.; et al. Chenodeoxycholate in females with irritable bowel syndrome-constipation: A pharmacodynamic and pharmacogenetic analysis. *Gastroenterology* **2010**, *139*, 1549–1558.e1541. [CrossRef] [PubMed]
17. Walters, J.R.; Pattni, S.S. Managing bile acid diarrhoea. *Ther. Adv. Gastroenterol.* **2010**, *3*, 349–357. [CrossRef]
18. Pimentel, M.; Talley, N.J.; Quigley, E.M.; Hani, A.; Sharara, A.; Mahachai, V. Report from the multinational irritable bowel syndrome initiative 2012. *Gastroenterology* **2013**, *144*, e1–e5. [CrossRef]

19. Heenan, P.; Creemers, R.H.; Sharma, S.; Keenan, J.; Bayer, S.; Young, W.; Cooney, J.; Armstrong, K.; Fraser, K.; Skidmore, P.M.; et al. Cohort Profile: The Christchurch IBS cOhort to investigate Mechanisms for gut Relief and improved Transit (COMFORT). *Inflamm. Intest. Dis.* **2020**, *5*, 132–143. [CrossRef]
20. Chong, J.; Soufan, O.; Li, C.; Caraus, I.; Li, S.; Bourque, G.; Wishart, D.S.; Xia, J. MetaboAnalyst 4.0: Towards more transparent and integrative metabolomics analysis. *Nucleic Acids Res.* **2018**, *46*, W486–W494. [CrossRef]
21. Chong, J.; Wishart, D.S.; Xia, J. Using MetaboAnalyst 4.0 for Comprehensive and Integrative Metabolomics Data Analysis. *Curr. Protoc. Bioinform.* **2019**, *68*, e86. [CrossRef] [PubMed]