# *Investigation of the Gut Microbiome and Host Metabolome in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome*

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## Abstract

**Background:** Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is a devastating, complex chronic disease with ambiguous aetiology and pathophysiology. There are neither confirmed specific biomarker/s nor clearly defined biological mechanism/s currently available that enable routine, accurate, and efficient diagnosis. ME/CFS individuals suffer from a myriad of debilitating symptoms including profound fatigue and post-exertional malaise and are often affected by comorbid fibromyalgia (FM) and irritable bowel syndrome (IBS). The heterogeneous, multisystemic nature of the condition and numerous diverse comorbidities is a major point of confusion. It is unclear whether these varying presentations in ME/CFS are phenotypes of the same underlying mechanisms or a combination of different diseases and their pathophysiology. The challenge in distinguishing between ME/CFS cases and grappling with the variability in clinical practice and research often means that individuals are confronted with years without a proper diagnosis and adequate health care. To address these issues, this project focused on the gut microbiome, host metabolome, and its relationship (gut microbe-derived metabolites). Overall differences in the gut microbiome and host metabolome have been observed between ME/CFS and non-ME/CFS cohorts; however, not to the level of detail presented here. This thesis also sought to objectively address and explore the bearing of the FM and IBS comorbidities in ME/CFS within this framework.

**Material and Methods:** 40 ME/CFS and 43 non-ME/CFS participants were recruited. All participants provided faecal and urine samples with questionnaire responses for Part One of the study. A selection of these participants (28 ME/CFS and 28 non-ME/CFS) provided a blood plasma and urine sample for Part Two. Participant metadata was used to form the comparative groups of interest described and discussed throughout this thesis: A) ME/CFS overall versus Control, B) ME/CFS with FM versus ME/CFS without FM, C) ME/CFS with IBS versus ME/CFS without IBS, and D) age-matched ME/CFS with IBS versus ME/CFS without IBS. All biological samples underwent a $^{1}$H-NMR and LC-MS/MS targeted metabolomic analytical workflow to characterise the host metabolome with polar metabolites. The faecal samples were used to provide insight into the gut microbiome with the usage of culture microbiology with MALDI-TOF MS, and 16S rRNA gene amplicon sequencing. PICRUSt2 and MiMeDB pipelines were used with the 16S rRNA sequences and culture data outcomes, respectively, to investigate the metabolic functionality and metabolite profiles of the gut microbiome. Across all datasets, univariate and multivariate statistics were used to evaluate the comparative groups.

**Results and Conclusions:** Investigating the heterogeneity and intricate host-microbiome relationship in ME/CFS requires a multi-omic and multi-disciplinary approach. There is value in using complementary approaches and techniques including expanding coverage and data capabilities. The comparisons across the datasets indicate that due consideration needs to be given to the presence or absence of FM and IBS comorbidities in ME/CFS. Overall, there are nuanced and subtle differences and similarities in the patterns and features that emerge from the gut microbiome and host metabolome findings. This may have far-reaching implications for disease pathophysiology and mechanisms, and the downstream establishment of preventative and management options. While the findings from this project only display a snapshot in time and cannot prove causality or prognosis; it offers an objective framework and pragmatic workflow for approaching the multifaceted issues in ME/CFS.

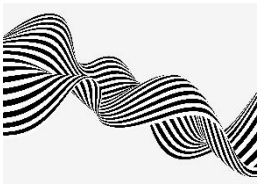
**Key Words and Phrases:** ME/CFS, fibromyalgia, irritable bowel syndrome, comorbidities, disease heterogeneity, gut, gut microbiome, host metabolomics, complementary approaches and techniques

## Doctor of Philosophy and Ethics Declaration

"I, Amber Jaa-Kwee, declare that the PhD thesis entitled *Investigation of the Gut Microbiome and Host Metabolome in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome* is no more than 80,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references, and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

"I have conducted my research in alignment with the Australian Code for the Responsible Conduct of Research and Victoria University's Higher Degree by Research Policy and Procedures".

"All research procedures reported in the thesis were approved by the St Vincent's Hospital (Melbourne) Human Research Ethics Committee (Reference HREC/18/SVHM127)."



29th February 2024

## Dedication

This thesis is dedicated to Dr Donald Peter Lewis

25 July 1946 - 29 July 2019

## Acknowledgements

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## Preface

My PhD started in 2017 with a different project plan and course of action from what is displayed in this thesis. The revised concept of this project was planned in the months leading up to the start of 2020. The course of this project has had to adapt to accommodate the changes due to major issues with supervision, laboratory facilities, resource availability, and the logistics of recruiting participants. Most of the issues experienced were largely unrelated to the COVID-19 pandemic and restrictions. The COVID-19 restrictions in Melbourne, Victoria did cause some disruption and delay with my PhD progress; however, they were less extensive than the non-COVID-19 issues encountered.

While aspects of the project changed and evolved, the core interest of disease heterogeneity, the gut, its microbiome, and the relationship with host metabolism and biochemistry in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) remained. The recruitment of participants, collection of samples and questionnaire information, and initial processing and storage of biological samples were completed before the supervision changes and moving to another laboratory group were necessary. The faecal culture microbiology with MALDI-TOF MS, DNA extraction, and 16S rRNA Illumina MiSeq wet laboratory work was completed as originally intended. The metabolomics experiments were arranged with the new supervisors and laboratory group. The metabolomics work was completed with the samples that had already been collected and stored. The extent and level of knowledge and skill to process and analyse these specific, large, and complex dataset types were not accommodated, nor anticipated, at the start of my PhD before the changes happened. Particularly for the 16S rRNA component, time was invested much later in my PhD course to learn some bioinformatic approaches and acquire tools, for example, Linux, high-performance computing, QIIME2, and R/R Studio, to be able to handle these datasets.

The outcome of managing and resolving my various challenges during my PhD candidacy has led to a project of significant and satisfying researcher and personal development involving work with more breadth and applicability when leaning into the space of chronic health conditions and diseases, and the host metabolome and gut microbiome relationship. Lastly, the discussion of post-COVID-19 pandemic health issues and the comparisons to ME/CFS is now a topic. The focus of this thesis is ME/CFS; however, the findings and discussion of this thesis, to an extent could have some applicability to this conversation.

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## List of Common Abbreviations

| | |
|---|---|
| AJK | Amber Jaa-Kwee |
| 16S rRNA/ 16S | 16S rRNA gene amplicon sequencing |
| bp | Base pairs |
| CCC | 2003 Canadian Consensus Criteria for ME/CFS (case definition) |
| CD or CDs | Case definition or Case definitions |
| DAA | Differential abundance analysis |
| DESeq2 | Differential gene expression analysis Version 2 |
| FM | Fibromyalgia |
| GI | Gastrointestinal |
| IBS | Irritable bowel syndrome |
| LCMS | Liquid chromatography-mass spectrometry |
| LEfSe | Linear discriminant analysis (LDA) effect size |
| MALDI-TOF MS or MALDI-TOF | Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry |
| ME/CFS | Myalgic Encephalomyelitis/Chronic Fatigue Syndrome |
| MiMeDB | The Human Microbial Metabolome Database |
| NMR | Nuclear magnetic resonance (spectroscopy) |
| PBQC | Pooled biological quality control |
| PBS | Phosphate-buffered saline |
| PCA | Principal component analysis |
| PCR | Polymerase chain reaction |
| PICRUSt2 | Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 |
| PLS-DA | Partial least squares-discriminant analysis |
| VIP | Variable Importance in the Projection |
| VP | Volcano plot |

# 1 CHAPTER ONE: Introduction and Literature Review

## 1.1 Executive Summary

Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is a neglected, debilitating long-term chronic disease affecting several millions of people worldwide. There is evidence for the multisystemic pathophysiological abnormalities and the biological basis for ME/CFS; however, aetiology and precise disease mechanisms remain elusive. An incomplete understanding of this disease, compounded by the lack of a universally accepted case definition or measurable biomarker/s, impedes the availability of diagnostic or therapeutic options specific to ME/CFS. In many ways, ME/CFS is a victim of its complexity. ME/CFS individuals suffer from a myriad of diverse symptoms and comorbidities ME/CFS is a heterogeneous condition and an objective framework and common language are needed. It is unclear whether these varying presentations in ME/CFS are subgroups and phenotypes of the same underlying mechanisms or a combination of different diseases and their pathophysiology. While overall differences in the gut microbiome and host metabolome are observed between ME/CFS and non-ME/CFS cohorts, and in other chronic diseases, the microbial metabolic potential and functionality of the gut microbiome have rich potential to further insight into ME/CFS. This project and thesis aimed to focus on exploring the gut microbiome, host metabolome, and its relationship (gut microbe-derived metabolites) using complementary analytical platforms to evaluate the role and bearing of the FM and IBS comorbidities in ME/CFS.

## 1.2 ME/CFS Background

ME/CFS, also more recently named Systemic Exertion Intolerance Disease (SEID), is a complex, extremely debilitating, and burdensome disease. The debate about the nomenclature and whether ME, CFS, and SEID are distinct entities is still unresolved and ongoing (Campagne et al., 2022; Huth et al., 2020; Jason et al., 2015, 2016; Lim & Son, 2020). Acknowledging that this condition is much more involved and serious than "chronic fatigue", the common umbrella term "ME/CFS" was used for this study. There are no approved biomarkers, diagnostic tests, curative, or therapeutic options for ME/CFS owing to its ambiguous aetiology, pathogenesis, and pathophysiology. ME/CFS is associated with significant personal loss and economic burden with health-related quality of life measures that are on par with, if not worse than, conditions such as multiple sclerosis, rheumatoid arthritis, cancer, type-2 diabetes, and stroke (Hvidberg et al., 2015; Mirin et al., 2020). This medical crisis is accompanied by an appalling and remarkably low level of research funding and support (Mirin et al., 2020, 2022). A study completed by Mirin and colleagues in 2020 found that the disease burden of ME/CFS in the United States is double that of HIV/AIDS and over half that of breast cancer and that the United States National Institutes of Health (NIH) funding for ME/CFS would need to increase roughly 14-fold to be commensurate with disease burden (Mirin et al., 2020). A more recent study revised this figure to be up to a 40-fold increase given the impact of the pandemic, inflation, and rising costs (Mirin et al., 2022).

Anyone can get ME/CFS (or the complications of ME/CFS if not formally diagnosed) at any point in time and it does not discriminate (Ghali et al., 2022; Nacul et al., 2020). Studies have found that ME/CFS affects approximately three to four times as many adult females as males with sex differences found in the pathophysiology of ME/CFS; however, ME/CFS is not a "women's disease" and there are paediatric cases (Bested & Marshall, 2015; Rivera et al., 2019; Valdez et al., 2019). Remission or a full recovery to pre-ME/CFS functioning is rare with reported recovery rates ranging from 0% to 8% (Ghali et al., 2022). There is a wide spectrum of severity ranging from mild to very severe with up to 75% of patients unable to work and an estimated 25-29% being consistently housebound or bedbound (Bateman et al., 2021; Mirin et al., 2020). The economic impact of ME/CFS has been estimated to range from $36 billion to $51 billion (USD) annually in medical expenses (direct costs) and lost income (indirect costs); these figures are conservative and expected to rise (Jason & Mirin, 2021; Mirin et al., 2022). Estimates of ME/CFS prevalence vary depending on the case definition used and it has been

difficult to get an accurate epidemiological representation (Lim et al., 2020). Although there is the likely possibility of many undiagnosed or misdiagnosed cases, figures say that ME/CFS affects “approximately 1% of the population, 17 to 24 million people worldwide” (Bested & Marshall, 2015; Lim & Son, 2020). These numbers and figures aside, ME/CFS is a horrific disease and a terrifying reality for one too many (Dafoe et al., 2021). A complicated clinical presentation and chequered historical backdrop, including the indefinite terminologies and aetiology, have contributed to a general lack of understanding, awareness, and support, and at times, sheer empathy, and humanity. Medical education programs rarely cover ME/CFS and guidance for health care providers is often outdated and inappropriate with numerous instances reported where the legitimacy of ME/CFS has been doubted and patients have been harmed (Bateman et al., 2021; Mirin et al., 2020). Research, education, and advocacy efforts have somewhat overcome the stigma and improved the support, perception, and recognition of ME/CFS; however, it is nowhere near the amount that individuals, their caregivers, friends, and family, require or rightly deserve.

## 1.3 ME/CFS Symptom Presentation

ME/CFS is commonly described as a chronic and multifaceted disorder that has lasted for at least six months and cannot be explained by any other underlying medical condition. It is now generally accepted that ME/CFS is characterised by profound, persistent, recurring fatigue that is not improved by rest, and post-exertional malaise (PEM). PEM is defined as an exacerbation or worsening of some or all baseline ME/CFS symptoms and a further reduction in function and capacity following any physical, cognitive, emotional, or social activities, events, stress, or challenges that were normally previously tolerated (Bateman et al., 2021; Ghali et al., 2021; Stussman et al., 2020). The PEM experience is often described as a “crash”, “relapse” or “payback” and the smallest amount and simplest of activities can result in an event. Common signs and symptoms of ME/CFS apart from the mental and physical fatigue include but are not limited to, muscle and/or joint pain, muscle weakness, cognitive dysfunction, “brain fog”, sleep dysfunction, mood changes, headache, immune system dysfunction, sore throat, nausea and dizziness. Multiple intolerances, hypersensitivities, and sensitivities, include issues with hot and cold temperatures, chemicals, drugs/medication, light, noise, touch, exercise, and orthostatic regulation. Many ME/CFS individuals also report gastrointestinal (GI) symptoms and problems including abdominal pain, bloating, diarrhoea, constipation, irritable bowel, intestinal discomfort, food intolerances, a changed appetite, and weight changes (Aaron et al., 2000; Carruthers et al., 2003; König et al., 2022; Lakhan & Kirchgessner, 2010; Lupo et al., 2021; Maes et al., 2014; Martín et al., 2023; Varesi et al., 2021).

Not only are there a plethora of symptoms that affect multiple organs and body systems, but they show differently with varying severity, frequency, patterns, progression, and duration among and within ME/CFS individuals. Symptoms can present at inopportune times and be both debilitating and embarrassing (Corbitt et al., 2018). Any of the symptoms can vary in the timing and chronological sequence that they appear ranging from suddenly to more gradually over weeks and months (Bateman et al., 2021). Disease onset is generally described as “acute”, “sudden”, or “gradual” with individuals often reporting a bout of a viral infectious illness or stressful/major life event before they become unwell (Chu et al., 2019). However, Chu et al. (2019) found that many studies did not adequately define the timeframe of onset and that further investigation of aetiological triggers (infectious and non-infectious), predisposing factors, the course of ME/CFS, and its contribution to the heterogeneity observed in ME/CFS was warranted (Chu et al., 2019). In addition to this often-marked variability in presentation, the way ME/CFS

manifests in everyone may change with time (Chu et al., 2019; Nacul et al., 2020). In some studies, female sex, increased age, and lower socioeconomic status have been found to predict poor prognosis; however, the inconsistent nature of both population sampling and diagnostic criteria usage has led to vague results (Bakken et al., 2014; Kidd et al., 2016; Nacul et al., 2020; Vyas et al., 2022). Besides the worsening of baseline symptoms, new or non-typical symptoms could emerge in some ME/CFS patients, and these symptoms can also differ from those usually experienced by individuals after exposure to a PEM event or trigger (Ghali et al., 2021). Therefore, inter- and intra-individual phenotypic variations contribute toward the categorisation of different subtype trajectories of ME/CFS that may differ in pathogenesis and prognosis (Ghali et al., 2022; Jason et al., 2005; Lacasa et al., 2023; Maclachlan et al., 2017; Nacul et al., 2020; W. P. Tate et al., 2023).

Symptoms are not specific to ME/CFS and can occur in other chronic conditions and healthy populations. However, the post-exertional worsening of function and the constellation of symptoms seen in ME/CFS are distinctive (Bateman et al., 2021; Stussman et al., 2020). PEM onset is unpredictable since it can be immediate or delayed by several hours or days. This onset delay can help distinguish ME/CFS from other diseases, in particular, multiple sclerosis and systemic lupus erythematosus, that manifest with severe fatigue and malaise but without the delayed onset (Ghali et al., 2021). It also has an unpredictable course and duration because the intensity and duration of PEM are unexpectedly disproportionate to the magnitude of the event that initiated it (Bateman et al., 2021; Ghali et al., 2021; Stussman et al., 2020). For the mildly affected individuals, working a few hours a day can result in PEM, whereas for the most severely ill, the most basic activities of daily living will be sufficient (Bateman et al., 2021). Recovery from a PEM event is also varied, unpredictable, and prolonged and may take days weeks, months, or longer. Intriguingly, in healthy and other unwell populations, physical exercise, and cognitive behavioural therapy (CBT) are shown to improve the symptoms of fatigue, sleep, pain, cognition, and mood and are often encouraged and implemented as prescribed therapy (Bateman et al., 2021; Dauwan et al., 2021; Ehde et al., 2014; Geneen et al., 2017; Lenzen et al., 2020; Nakao et al., 2021; Schuch et al., 2016). By stark contrast, ME/CFS patients experience PEM where exercise and CBT activities can exacerbate symptoms rather than improve them (Stussman et al., 2020). Graded exercise therapy (GET) and CBT in a biopsychosocial model and context were investigated in the highly controversial, dubious, and potentially unethical PACE trial. My study and views support their (any PACE-trial-like or endorsed, GET and CBT protocols) removal from guidelines and practices for managing

ME/CFS as the distress, harm, and risks outweigh any minimal benefits. The consideration of PEM in diagnostic protocols is critical in the absence of any other means to term ME/CFS as a distinct entity.

## 1.4 ME/CFS Name and Case Definitions

As early as the 19th century, reasonably reliable medical records have been available that describe a multisystemic and debilitating disease of unknown origin causing chronic and severe fatigue which prevents individuals from carrying out normal levels of daily activities (Brurberg et al., 2014; Lim & Son, 2020; Prins et al., 2006; Varesi et al., 2021). ME/CFS has long been associated with discrete outbreaks and sporadic incidents of viral-like illness; however, the causality of ME/CFS has not been verified (Hanson, 2023; Rasa et al., 2018). It is often taken for granted that a disease has a clear-cut definition with established aetiology, identifiable biomarkers with known underlying biological mechanisms let alone consistent differentials, efficient and reliable diagnostic and management protocols, and even one name that everyone uses. Acknowledging the historical trajectory of ME/CFS is imperative because its events go hand in hand with the various names and case definitions (CDs) which emphasise different clinical features and characteristics through the lens of different clinicians' and researchers' perspectives (Brurberg et al., 2014; Christley et al., 2012; Lim & Son, 2020). Figure 1.4.1 is from a more recent review of ME/CFS CDs; it shows the developmental timeline of the 25 that have been developed and published in English to date (Lim & Son, 2020).

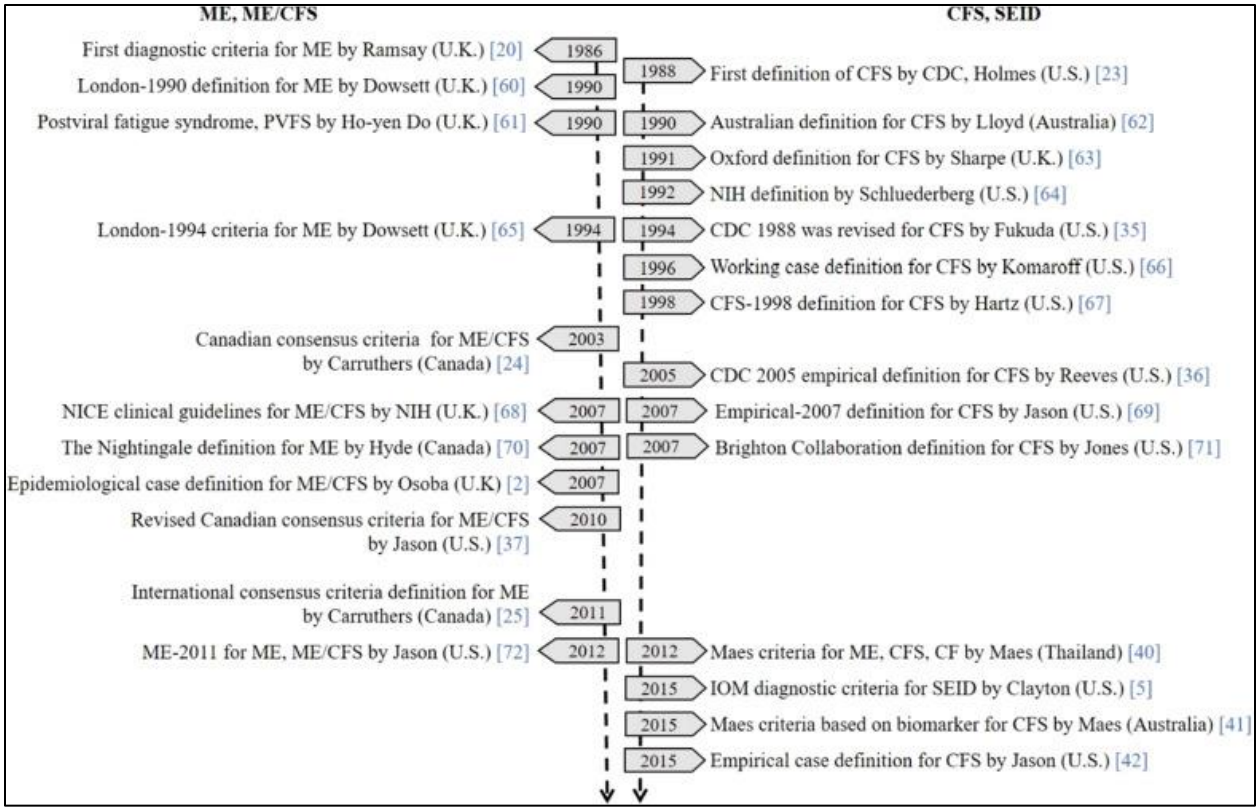



**Figure 1.4.1** Figure 1 from Lim and Son's 2020 review shows the timeline and development of ME/CFS case definitions and terminologies (Lim & Son, 2020).

The initially distinct view of ME and CFS was later reformulated to ME/CFS and then SEID (Lim & Son, 2020). The first "ME" case definition was developed in 1986 by Dr Melvin Ramsay following outbreaks in the United Kingdom that described cases featuring predominantly neurological symptoms and an alarming level of chronicity (MEpedia, 2023b; Ramsay, 1965; Ramsey et al., 1988). A series of outbreaks led to the proposal that the condition was linked to a viral infection which altered the name for a period to "chronic Epstein-Barr virus syndrome" in 1982 and "post-viral fatigue syndrome" in 1985 (Institute of Medicine, 2015; Lim & Son, 2020). In 1988, "CFS" was proposed by the Centers for Disease Control and Prevention (CDC) in the United States to describe the outbreak of cases that were similar, if not identical to, the ones observed earlier in the UK, and numerous sporadic cases (Brurberg et al., 2014; Fukuda et al., 1994; G. P. Holmes et al., 1988). It was deemed by the CDC that there was insufficient evidence to link the viral origins of the illness and that the CFS terminology would more inclusively describe the symptom complex including psychological symptoms (Institute of Medicine, 2015; Lim & Son, 2020). The name "ME/CFS" was coined in 2003 by the Canadian

Consensus Criteria (CCC) to encapsulate the clinical features of both “ME” (neuroinflammation) and “CFS” and highlight the level of complexity that goes beyond the often misjudged and overlooked “chronic fatigue” (Carruthers et al., 2003). The name of “ME/CFS” itself although commonly used and widely accepted, remains controversial with views that oppose the combined term as they consider them to be separate diagnostic entities (Jason et al., 2016; Twisk, 2014). To some extent, these arguments are redundant and moot without confirmed aetiology and pathophysiology. More recently in 2015, SEID was recommended by the National Academy of Medicine, formerly the Institute of Medicine (IOM), as a new name for the condition. The IOM authors offered SEID with the hope that the new name would be the first step towards a widespread change in public and clinician attitudes in line with current literature and understanding of the patient experience with the disorder/disease (Clayton, 2015; Lancet, 2015). Overall, the terminologies can be linked to the aetiologies that have been suggested for this condition: ME by a viral infection, ME/CFS by a neuroinflammatory disorder, CFS with unknown cause, and SEID by multisystemic effects (Lim & Son, 2020).

The “what is in a name(?)” debate and issues are not to be ignored in ME/CFS (Bested & Marshall, 2015). However, the more concerning matter lies in the CDs or diagnostic criteria associated with these terminologies. The use of differing definitions has a profound impact on epidemiological estimates of prevalence hence the blanket caution needed when viewing them (Brurberg et al., 2014; Christley et al., 2012; Lim & Son, 2020). Eight CDs get cited the most (in descending order): Fukuda, Holmes, Oxford, CCC, International Consensus Criteria (ICC), Australian, Ramsay, and SEID; they can also be categorised by name into ME (ICC and Ramsay), ME/CFS (CCC), CFS (Holmes, Australian, Oxford, Fukuda), and SEID (Lim & Son, 2020). These definitions have largely been developed based on expert opinion and perspectives which have continuously changed and differed rather than empirically (Conroy et al., 2022). The issue is not so much the comparability of which is the best of the case definitions but rather that they are being used and relied on to establish and determine a diagnosis. A CD is meant to perform the essential role of ensuring that illnesses are systematically identified, diagnosed, and classified with clear key signs and symptoms (Christley et al., 2012). Unfortunately, in ME/CFS, the CDs have created a great deal of confusion as the differing definitions place inconsistent emphasis on symptoms and features (Christley et al., 2012; Lim & Son, 2020). It begs the question of whether ME/CFS cases are appropriately or inappropriately diagnosed with criteria that are arguably either too broad or narrow, too inclusive or exclusive, and where the line should be drawn for research and clinical practice (Brurberg et al., 2014; Lim & Son, 2020).

Figure 1.4.2 shows a comparison of the symptoms and scope of the eight CDs from Lim and Son's (2020) review.

Across the board, cognitive impairment is the core symptom that is commonly intersected in the eight definitions (Lim & Son, 2020). The five symptoms, fatigue, cognitive impairment, PEM, sleep disorder, and orthostatic intolerance overlap with the four categories of ME, ME/CFS, CFS, and SEID; these symptoms are considered core signs by the SEID definition (Lim & Son, 2020). In general, CFS and SEID definitions focused on cognitive impairment and fatigue, whereas ME and ME/CFS further emphasised muscle disturbance with neuro-autonomic symptoms such as sensitivity to food, chemicals, or light (Lim & Son, 2020). As shown in Figure 1.4.2, there are other symptoms experienced that encompass the ME/CFS condition which is only a testament to the innate complexity and heterogeneity of the condition and the challenge of its conceptualisation. It is likely that all CDs capture conditions with different or multifactorial pathogenesis and varying prognosis (Brurberg et al., 2014). There is some criticism that the Oxford or Fukuda criteria are too broad and not specific enough to discriminate between generalised chronic fatigue and ME/CFS fatigue which specifically involves PEM (Brown et al., 2013; Institute of Medicine, 2015; Jason et al., 2004, 2014; Nacul et al., 2019). This distinction is important as PEM, also referred to as post-exertional symptom exacerbation, is considered a cardinal sign that characterises ME/CFS. For this reason, the CCC, ICC, and SEID definitions are preferred (Chu et al., 2018). While the optional presence of PEM by other CDs may not be as critical in a direct clinician-patient setting where vital clinical judgements should not be substituted in providing adequate care, it is for research studies (Brurberg et al., 2014; Conroy et al., 2022). Research requires uniform and reproducible criteria, suitable for unambiguous definitions of the target population (Brurberg et al., 2014). Clinical practice requires research-based approaches but ultimately focuses on relieving patient uncertainty and providing care (Brurberg et al., 2014). Irrespective of the setting, the inconsistent use of CDs is problematic and hinders an efficient diagnostic process and the comparability of findings. In addition to this challenge with the diagnostic criteria, the lack of clinical expertise and clinician confidence in ME/CFS often delays and makes the process of receiving a diagnosis and care an utterly resource-exhaustive, overwhelming, and unpleasant experience (Pheby et al., 2020, 2021).

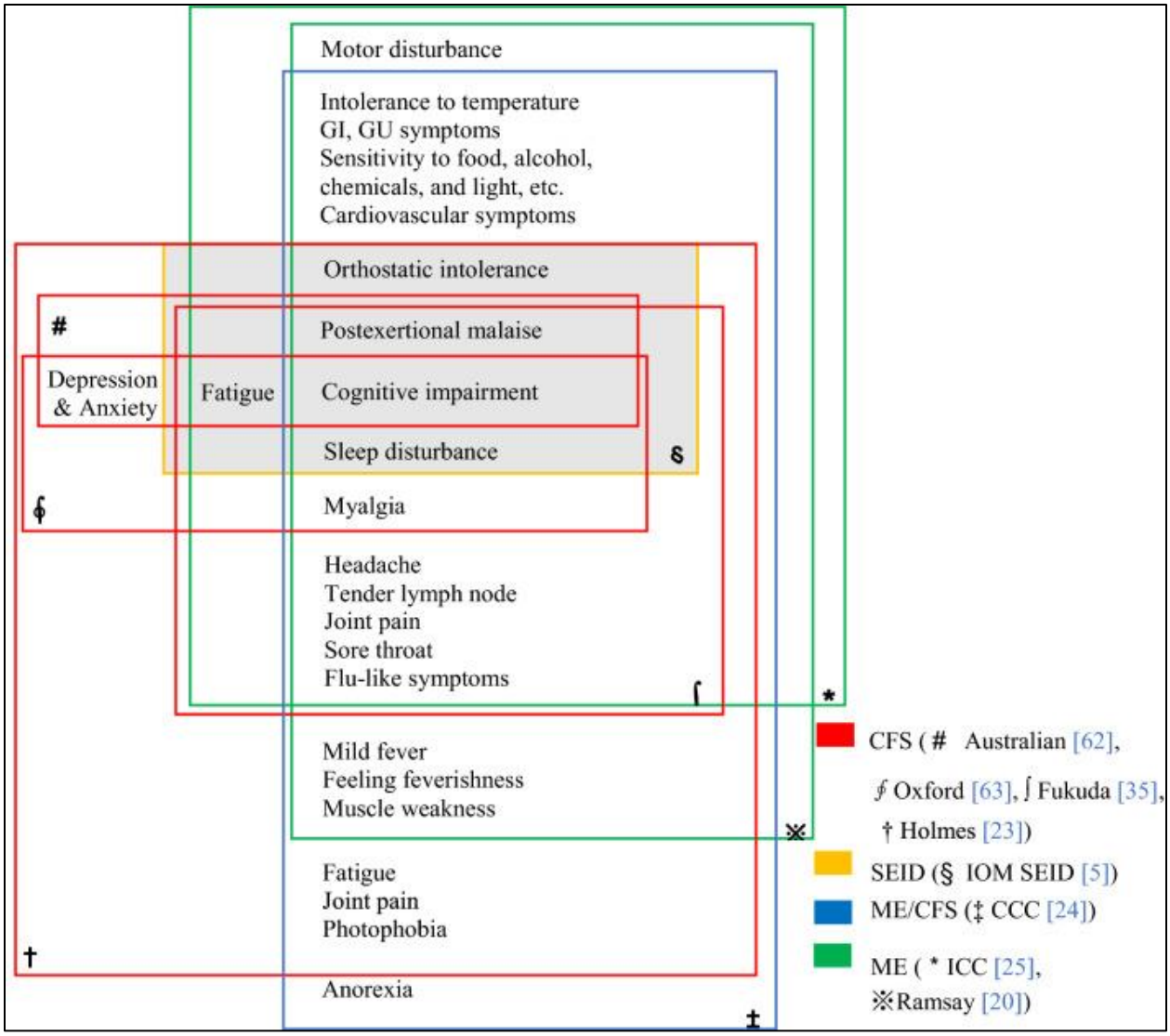



**Figure 1.4.2** Figure 2 from Lim and Son's 2020 review showing the scope of ME/CFS by case definitions (CFS, chronic fatigue syndrome, IOM, Institute of Medicine, SEID, systemic exertion intolerance disorder, ME, myalgic encephalomyelitis, CCC Canadian Consensus Criteria, ICC International Consensus Criteria, GI gastrointestinal, GU, genito-urinary symptoms) (Lim & Son, 2020).

## 1.5 ME/CFS Comorbidities

There are several comorbidities associated with ME/CFS and they can also complicate diagnosis, prognosis, treatment, and research (Ghali et al., 2022; Nacul et al., 2020). Although the rationale for diagnosing ME/CFS has shifted its focus to identifying positive signs and symptoms rather than being a diagnosis of exclusion, the last resort, or "wastebasket", clinical presentation across patients and time may mimic the presentation of other conditions (Clayton, 2015; Grach et al., 2023; Institute of Medicine, 2015). Symptoms of ME/CFS can present similarly and overlap with several medical and psychiatric conditions, and patients can have both ME/CFS and other diseases (Bateman et al., 2021). Two different studies of ME/CFS patients that fit the Fukuda definition found that 84% of 960 and 97% of 150 participants suffered from at least one comorbidity (Bateman et al., 2015; Chu et al., 2019). Chu et al. (2019) also stated that "multiple comorbidities are the rule rather than the exception". Another study of 1757 ME/CFS patients that met both the Fukuda definition and CCC found that over 80% of its cohort presented comorbidities (Castro-Marrero, Faro, et al., 2017). Irrespective of the CD used, comorbidities can substantially influence and contribute to the individual's disease progress, symptom burden, and functional limitations (Bateman et al., 2015; Castro-Marrero, Faro, et al., 2017; Chu et al., 2019; Ghali et al., 2022). Beyond this aspect of the ME/CFS manifold, comorbidities may reduce life expectancy, and increase morbidity and mortality; however, findings on this are inconclusive (Castro-Marrero, Sáez-Francàs, et al., 2017; McManimen et al., 2016; Mirin et al., 2020; E. Roberts et al., 2016). It is often said that deaths from ME/CFS are very rare and rarely recorded on death certificates (MEpedia, 2023a). Considering the complex nature and numerous challenges surrounding ME/CFS to date, these evaluations would be better revised with a more objective and better understanding of comorbidities in ME/CFS, that is, in the context of revealing and understanding the biological underpinnings of the disease.

This thesis studied participants who were diagnosed according to the CCC and focused on FM and IBS comorbidities in the ME/CFS cohort. The CCC recognises several comorbid entities including FM and IBS (Carruthers et al., 2003). Further to the issues described above with comorbidities in ME/CFS, comorbidities also raise the possibility of different pathophysiological processes that cannot be confined to any one organ (Lakhan & Kirchgessner, 2010; Natelson, 2019). This in turn could mean different effects and outcomes for diagnostic, preventative, treatment, and management options. Currently, pharmacological and non-pharmacological treatment options for ME/CFS are focused on symptom alleviation and management but they

are non-specific and a bit of trial and error (Bateman et al., 2021; Bested & Marshall, 2015; Grach et al., 2023). Treatment of comorbidities can positively affect a patient's quality of life and improve the severity of symptoms (Bateman et al., 2021; Leong et al., 2022). However, patient-specific care is essential, as inter- and intra-individual variability means that management strategies may dynamically benefit or not benefit to varying degrees (Bateman et al., 2021; Bested & Marshall, 2015; Castro-Marrero, Sáez-Francàs, et al., 2017). For example, exercise and other pain treatment modalities may help the FM, but it can worsen ME/CFS (Bateman et al., 2021; Bested & Marshall, 2015). Joint and muscle pain and chronic pain that are akin to FM, a chronic musculoskeletal pain disorder, are commonly reported in ME/CFS (Barhorst et al., 2022). Currently, ME/CFS and FM are complex syndromes that are governed by their respective diagnostic criteria(s) and management direction but show many similarities (Mckay et al., 2021). It has been estimated that between 20% and 70% of patients with FM meet the criteria for ME/CFS and conversely, 35% to 70% of those with ME/CFS, also have FM (Aaron et al., 2000). A recent meta-analysis found a 47.3% clinical overlap between ME/CFS and FM (Ramírez-Morales et al., 2022). Like ME/CFS, FM also faces the challenge of heterogeneity, multifactorial clinical presentation, disability, and unclear characterisation and pathophysiology (Barhorst et al., 2022; Dizner-Golab et al., 2023; Ramírez-Morales et al., 2022).

The mystery of ME/CFS and FM origins has contributed at times to the (one) functional somatic disorders and cognitive behavioural psychiatric model viewpoint which is now discouraged and proven as unfounded (Eccles et al., 2021; Geraghty et al., 2019; Wessely et al., 1999; Wessely & White, 2004). These observations and the shared symptomology have led the debates and contrasting views on whether ME/CFS and FM manifestations are of the same spectrum disorder (single syndrome unitary hypothesis), extensions of one another, or entirely separate clinical entities with distinct disease mechanisms and a degree of misdiagnosis (Abbi & Natelson, 2013; Natelson, 2019; Natelson et al., 2019; Wessely & White, 2004). ME/CFS with and without FM, and pain in ME/CFS have been investigated with a variety of modalities (from clinical observations, physical assessments, and questionnaires to biomolecular/omic-based experiments). While symptomology is similar and overlapping, pathophysiological, biochemical, and molecular findings show evidence for both its distinction and non-distinction. No study so far has unanimously addressed and confirmed either side of this argument (Aaron et al., 2000; Barhorst et al., 2022; Campen et al., 2020b, 2020a, 2021; Conroy et al., 2022; Das et al., 2022; Eccles et al., 2021; Faro et al., 2014; Gerwyn & Maes, 2017; Ghali et al., 2021; Martín et al., 2023; Mckay et al., 2021; McManimen & Jason, 2017; Meeus et al., 2016; Monden et al., 2022;

Morris et al., 2013; Nepotchatykh et al., 2023; Schutzer et al., 2023; van Eeden et al., 2023). While the somatisation and psychiatric stance have been debunked, the involvement and association of pain and FM comorbidity in ME/CFS pathophysiology requires further investigation.

In addition to FM, IBS also appears frequently in ME/CFS. Interestingly, the CCC states the following in their primer about comorbidities in ME/CFS: “Comorbid entities may occur in the setting of ME/CFS. Others such as IBS may precede the development of ME/CFS by many years, but then become associated with it. The same holds true for migraines and depression. Their association is thus looser between the symptoms within the syndrome. ME/CFS and fibromyalgia syndrome often closely connect and should be considered to be “overlap syndromes” (Carruthers et al., 2003). This statement, following the notion that “there are differences, then there are differences,” suggests that comorbidities, such as FM and IBS, may have different roles in their association or connection with ME/CFS. This could point toward different pathophysiological processes depending on the comorbidity and therefore illuminate varying outcomes in the individual and their experiences with ME/CFS (Natelson et al., 2019). It is noteworthy that Conroy et al. (2022) in their evaluation of ME/CFS case diagnostic criteria that pain was found not to be an independent factor (therefore, pain may not be a discrete symptom category but a manifestation of other symptoms), but that gastrointestinal (GI) distress including irritable bowel problems appeared to be a unique and revealing symptom category (Conroy et al., 2022). IBS is a prevalent chronic functional disorder of the GI tract that also has an unclear pathophysiology and challenges with its heterogeneity and dynamic nature. Generally, it is a disorder of gut-brain interaction that manifests as recurrent episodes of abdominal pain, with altered bowel habits in the absence of another obvious causative organic disease (Holtmann et al., 2016; Tarar et al., 2023). IBS is often associated with other medically unexplained conditions like ME/CFS and FM (Aaron et al., 2000; Berstad et al., 2020; Tarar et al., 2023). Like ME/CFS and FM, IBS currently does not have any reliable, confirmed, or validated diagnostic biomarkers and largely relies on symptom presentation, exclusion of other reasons, and the Rome diagnostic criteria (Camilleri, 2021; J. H. Kim et al., 2017; Nakov et al., 2022; Shin & Kashyap, 2023).

In ME/CFS, GI disturbances and related symptoms are often reported and there is a very frequent comorbidity with IBS (Aaron et al., 2000; König et al., 2022; Nagy-Szakal et al., 2017; Varesi et al., 2021). GI symptoms are recognised under the CCC and ICC CDs (Du Preez et al., 2018). Not surprisingly, 35-90% of ME/CFS individuals have IBS compared to 10-20% of the

general population (Nagy-Szakal et al., 2018). FM, independent of ME/CFS as a diagnostic entity, also frequently describes GI symptoms and reports of IBS comorbidity (Garofalo et al., 2023; Minerbi & Fitzcharles, 2020; Tarar et al., 2023). In a meta-analysis of FM-diagnosed individuals according to the American College of Rheumatology criteria for FM, 1.78% of the general population especially women are suffering from FM; however, it was significantly higher (12.90%) among those with IBS (Heidari et al., 2017; Tarar et al., 2023). The early study by Aaron et al. (2000), found that lifetime rates of IBS were particularly striking in their patient groups (ME/CFS 92% and FM 77%). Natelson et al. (2019) reported that IBS and FM were comorbid with ME/CFS in 67% and 54% of their study participants, respectively (Natelson et al., 2019). The high incidence of these conditions after infection, including GI infections, and after the use of antibiotics, has led to the hypothesis that gut dysbiosis and altered gut permeability may play a role in their pathogenesis (Berstad et al., 2020; Garofalo et al., 2023; König et al., 2022; Tarar et al., 2023; Varesi et al., 2021; L. Wang et al., 2020). The “dysbiotic march” hypothesis has been postulated whereby the “march” starts with IBS early in life and proceeds to a systemic disease with chronic fatigue, FM, and possibly ME (ME/CFS) later in life (Berstad et al., 2020). However, exactly how the gut and gut microbiome are involved in symptomology, disease pathogenesis, development and progression (within a chronic condition as per its current governing diagnostic entity, and between the comorbidities) remains underexplored and unresolved. Regardless of the specific diagnostic criteria used, the frequent coexistence and overlap of features among ME/CFS, FM, and IBS has led to thoughts that underlying disease mechanisms, particularly those of the gastrointestinal tract, gut microbiome, host metabolism, immune, autonomic and enteric nervous systems, and broader gut-brain-axis, are similar. Understanding these intricate mechanisms would not only help elucidate differential and similar pathophysiology and pathways in the overlap or kaleidoscopic presentations but also pave the way for the development of therapies that simultaneously address multiple symptoms and concerns.

Whether it is the symptomology, nomenclature, case definitions, comorbidities, multisystemic individual presentation, patient and clinician perspectives, timing, disease progression, biological mechanisms and their processes, or a combination of all these issues, ME/CFS is a colossally complex, heterogeneous disease. One way of describing the challenge that is faced in ME/CFS is that the disease is like a jigsaw puzzle (or jigsaw puzzles) with an undetermined number of, but probably several, pieces that can unpredictably change in shape, colour, and picture. Despite the unfavourable historical developments and many ongoing hurdles in the

ME/CFS clinical and research space, recent studies and technologies have mercifully debunked the psychological/psychiatric misnomer and enabled meaningful discoveries into the biological basis and diagnostic potential for ME/CFS (Maksoud et al., 2023; Rivera et al., 2019; Sweetman et al., 2019; W. P. Tate et al., 2023). Several markers have been suggested as potential candidates but there is no consensus on a biomarker for ME/CFS (Maksoud et al., 2023). Studies across a wide range of disciplines have pointed towards a loss of normal homeostasis, and a suite of alterations and abnormalities in multiple organ and body systems (Komaroff & Lipkin, 2023; Nacul et al., 2020). It is not known whether these changes occur before the onset of the disease or as its consequence (the chicken or the egg(?) dilemma) and whether stratification or subsets of ME/CFS can be described from this (Nacul et al., 2020; Noor et al., 2021; Renz-Polster et al., 2022).

Nacul et al. (2020) proposed the natural history framework of ME/CFS whereby tentative classification of disease stages supports better understanding, research, and healthcare interventions for ME/CFS (Nacul et al., 2020). These interconnected stages which are roughly marked by an increase in the degree of homeostatic dysfunction and irreversibility are predisposition and trigger, prodromal, early, established, and long-term, advanced and complicated disease (Nacul et al., 2020). Viral infections have long been considered the main trigger of disease onset; however, other factors including physical or emotional trauma, genetic profile, environmental exposures, and the nature of the host response have also been proposed as contributing or predisposing components for disease origin, risk, manifestation, and chronicity (Deumer et al., 2021; Nacul et al., 2020; Rivera et al., 2019; Varesi et al., 2021). These different “origins” or “starting points” of ME/CFS are thought to be involved in the initiation or inducement of several body-wide pathological cascades or interconnected spirals of aberrant homeostasis with similar outcomes (Missailidis et al., 2019; Nacul et al., 2020). However, as individuals move between the different hypothetical stages of ME/CFS, different molecular and system abnormalities may be encountered (Nacul et al., 2020). At the pathobiological or pathophysiological level, ME/CFS is no less complex (Renz-Polster et al., 2022). Several studies report evidence comprising, but not limited to, immunological, inflammatory, neurological, metabolic, mitochondrial, microbiome, gastrointestinal, circulatory, endocrinological, autonomic, infectious, oxidative-reductive (redox), and muscular abnormalities (Deumer et al., 2021; Komaroff, 2019; Komaroff & Lipkin, 2023; Missailidis et al., 2019; Nacul et al., 2020). A few hypotheses based on these biological underpinnings of ME/CFS have been proposed; however, it is possible and likely that all these threads are entangled in the

progression (recovery and deterioration) over time, upstream and downstream picture of ME/CFS (Missailidis et al., 2019; Nacul et al., 2020; Renz-Polster et al., 2022).

## 1.6 ME/CFS Gut Microbiome and Host Metabolome

The human microbiome comprises approximately 100 trillion microorganisms, most of them bacteria, but also viruses, eukaryotes (like fungi, protozoa, and yeasts), and archaea that exist primarily in the gastrointestinal tract (gut) (Ogunrinola et al., 2020; Shreiner et al., 2015; Vijay & Valdes, 2021). The collection of all intestinal microbial genes in an individual represents a genetic repertoire that is more than one order of magnitude higher than the human genome (Y. Chen et al., 2021; Fan & Pedersen, 2020). Microorganisms essentially function as "chemical factories" and the number and diversity of metabolites generated by the human gut microbiome are impressively large (Wishart, 2019). Hippocrates was said to have claimed that "All disease begins in the gut", and while the statement is not an all-encompassing truth, the evidence so far has revealed that the gut and its microbiome have an important role in health and disease (Hatton et al., 2018; Lyon, 2018).

The gut microbiome and gastrointestinal system have received considerable public and scientific attention (Afzaal et al., 2022; De Vos et al., 2022; K. Hou et al., 2022; König et al., 2022; Rackaityte & Lynch, 2020; Valdes et al., 2018; Vijay & Valdes, 2021). Virtually all body sites are colonised by microbes suggesting different types of crosstalk with our organs (De Vos et al., 2022). The gut microbiome is now considered one of the key elements or most significant sites in contributing to the regulation and maintenance of host health; it is now best thought of as a virtual or hidden organ of the body (De Vos et al., 2022; K. Hou et al., 2022; Valdes et al., 2018). Nowadays, the role of the gut and gut microbiome is documented in many conditions and diseases that have gastrointestinal, extra-intestinal, and wider systemic manifestations including obesity, type 2 diabetes, some cancers, Alzheimer's disease, Parkinson's disease, autism spectrum disorder, asthma, allergy, anxiety, depression, hypertension, celiac disease, inflammatory bowel disease, FM, IBS, and ME/CFS. In ME/CFS, the gut and gut microbiome, and its association with changes in host metabolism and responses, are becoming increasingly recognised as a potential gateway for future research and predictive, diagnostic, and treatment avenues (Armstrong et al., 2017; Borody et al., 2012; Briese et al., 2023; Che et al., 2022; Conroy et al., 2022; Frémont et al., 2013; Germain et al., 2017, 2018, 2020; Giloteaux, Goodrich, et al., 2016; Giloteaux, Hanson, et al., 2016; Guo, Che, et al., 2023; G. He et al., 2023; S.-Y. Hsieh et al., 2023; M. L. Jackson et al., 2015; Keating et al., 2019; Kenyon et al., 2019; Kitami et al., 2020; König et al., 2022; Lakhan & Kirchgessner, 2010; Lupo et al., 2021; Mandarano et al., 2018; Martín et al., 2023; Nagy-Szakal et al., 2017, 2018; Navaneetharaja et

al., 2016; Naviaux et al., 2016; Newberry et al., 2018; Seton et al., 2023; Sheedy et al., 2010; Shukla et al., 2015; Sullivan et al., 2009; Varesi et al., 2021; Wallis et al., 2016, 2017; Xiong et al., 2023).

In a state of eubiosis, the gut microbiome acts in favour of health and provides a myriad of essential and positive functions for human life (Afzaal et al., 2022; Bull & Plummer, 2014). Without the gut microbiome, humans are unable to do many things – host metabolism and sustaining life would look very different (Wishart, 2019). It is involved in and responsible for several vital processes that influence or impact host metabolic pathways, physiology, and systems. This includes an assortment of roles such as circadian rhythmicity, changing insulin resistance and affecting its secretion, host digestion and nutrient metabolism, energy metabolism, cellular metabolism and signalling, xenobiotic and drug metabolism, maintenance of the structural integrity of the gut mucosal barrier, controlling the proliferation and differentiation of epithelial cells, immunomodulation, protection against pathogens and infection, and influencing brain-intestinal communication (Bull & Plummer, 2014; De Vos et al., 2022; K. Hou et al., 2022; Jandhyala et al., 2015; Lotti et al., 2023; Maciel-Fiuza et al., 2023; Valdes et al., 2018). Although the establishment of an ideal model remains ongoing and important research, it is generally accepted that in healthy conditions, the gut microbiome exhibits stability, resilience, and symbiotic interactions with the host (K. Hou et al., 2022; McBurney et al., 2019; Rinninella et al., 2019). However, the gut and gut microbiome can also be, or become, potentially harmful with deleterious consequences. A very considerable amount of evidence has linked gut dysbiosis and intestinal dysfunction (permeability, aberrations, disturbances, or deviations in microbial communities and the GI system) to a variety of diseases and detrimental outcomes, including ME/CFS (Afzaal et al., 2022; De Vos et al., 2022; K. Hou et al., 2022; König et al., 2022; Maciel-Fiuza et al., 2023; Varesi et al., 2021).

This thesis recognises that the “gut dysbiosis” terminology needs to be used and viewed with caution, not taken out of context or extended beyond what the data can and cannot show when precise relationships (causation, response, associations, contribution to disease) between the gut, microbiome, and disease, especially in ME/CFS, remains unresolved (Brüssow, 2020; Duvallet et al., 2017; König et al., 2022). Interestingly, while the symptoms of GI disturbance have been recognised under the CCC and ICC, these case definitions do not specifically describe gut dysbiosis or alterations in the gut microbiome as further investigation is required (Du Preez et al., 2018). It does not help that a specific and consistent microbial signature has

not been found with contradictory changes in the overall composition (direction of microbial abundance being higher or lower) and the diversity of the gut microbiome reported (Du Preez et al., 2018; König et al., 2022). This aspect of gut dysbiosis has been hypothesised to contribute to microbial shifts that can result in the growth and expansion of pathogenic microbes and the production of factors toxic or harmful to host cells (Newberry et al., 2018). If not for pathogenic microbes, the collective commensal bacterial populations could shift to an increase or overgrowth of pro-inflammatory, harmful, opportunistic features and a decrease or undergrowth in beneficial, protective, and anti-inflammatory features (Carding et al., 2015; Frémont et al., 2013; Giloteaux, Goodrich, et al., 2016; Jandhyala et al., 2015; Sweetman et al., 2019). Of course, depending on the gut microbiota composition, effects on the immune system, and other functions of host health that it is involved in can differ or be positively and negatively affected (Varesi et al., 2021).

The gut microbiome research area continues to grow with increasing capability for the further unveiling of intricate disease mechanisms that are required to accompany these findings to human translation and application which is the goal and end point (Puschhof & Elinav, 2023). Recent and rapidly developing advances in techniques and technologies in the field continue to progress the understanding of what exactly the gut microbiome means (Y. Chen et al., 2021; König et al., 2022; J. Liu et al., 2022; Maciel-Fiuza et al., 2023; Puschhof & Elinav, 2023; Varesi et al., 2021). Currently, it is generally thought that homoeostatic stability, and therefore, health and disease status, are involved with changes in the microbial composition and imbalances of the gut microbiome. However, in most cases, it is unclear whether dysbiosis or changes in the microbiome are a cause or consequence of disease and intestinal insults, or both, and whether its manipulation can help control or even treat the condition (Frémont et al., 2013; G. He et al., 2023; McBurney et al., 2019; Talapko et al., 2022). Again, it is another “chicken or the egg(?)” dilemma. Further, the overall metabolic potential of the gut microbiome, the intricacies or interplay between the gut microbiome and host metabolism under different conditions remain largely underexplored (Dey & Ray Chaudhuri, 2023; Montenegro et al., 2023; Schröder, 2022; Visconti et al., 2019). While it is widely accepted that gut dysbiosis and gut dysfunction are closely associated with or linked to disease, it is becoming apparent that the functionality, relationship or interaction between the gut microbiome and host metabolome, beyond its composition and taxonomy, warrants further and more consideration (Agus et al., 2021; M. X. Chen et al., 2019; Y. Chen et al., 2021; Dey & Ray Chaudhuri, 2023; Guo, Che, et al., 2023; Lamichhane et al., 2018; J. Liu et al., 2022; Ma et al., 2022; Maciel-Fiuza et al., 2023; Puschhof

& Elinav, 2023; Visconti et al., 2019; Wilmanski et al., 2021; Xiong et al., 2023; Y. Zhang et al., 2023). These interactions are highly dynamic and complex (Lamichhane et al., 2018). Metabolites of the gut microbiome, measured via metabolomics in a workflow combined with metataxonomics, can provide another element of insight into the metabolic activity and structures of the microbes and the role of the gut in ME/CFS.

The genetic complexity and non-redundant genes of the gut microbiome allow the microbes to produce many enzymes and metabolites that their human hosts cannot produce (Puig-Castellví et al., 2023; Wishart, 2019). The gut microbiome can interact with the host by producing metabolites, representing intermediates or end products of microbial metabolism (Agus et al., 2021; Lamichhane et al., 2018). These metabolites can be derived de novo by the gut microbes, the transformation of ingested materials, or those generated by the host and biochemically modified by gut microbes (Agus et al., 2021; Lamichhane et al., 2018; Y. Zhang et al., 2023). While metabolites produced by the host and microbiota play a crucial role in the body, most of them are produced by the microbiota and hosts in the digestive tract (Moya & Ferrer, 2016; Y. Zhang et al., 2023). Further, microbiota-derived compounds can vary more among individuals in concentration compared to host-derived metabolites (Agus et al., 2021; Ma et al., 2022; Y. Zhang et al., 2023). It is thought that host-derived metabolites in circulation are maintained with relative consistency between individuals (Puig-Castellví et al., 2023; Y. Zhang et al., 2023). Beyond their key role in physiological signalling and homeostasis within the GI system, gut microbial metabolites appear to have actions that influence other organs and systems (Wishart, 2019). Metabolomics offers the opportunity to simultaneously explore the various axes (kidney, lung, brain, liver, etc.) connected with the gut that may reveal potential mechanisms and targets with multisystemic implications and treatment benefits (Wishart, 2019). Previous ME/CFS gut microbiome studies have suggested a possible link between dysbiosis and disease pathogenesis and pathophysiology; however, accompanying metabolomic information about its association with disrupted metabolism is not always presented simultaneously (Du Preez et al., 2018; König et al., 2022; Newberry et al., 2018; Varesi et al., 2021). Similarly, other ME/CFS studies have revealed insightful metabolic abnormalities that signal or point towards a potentially important association or involvement of gut issues, symptoms, and the microbiome; however, they do not always directly address or mention these topics in their discussions (Glass et al., 2023; Hoel et al., 2021; Maya et al., 2023; Missailidis et al., 2021; Nkiliza et al., 2021). For the most part, studies do mention or discuss the gut with their findings; however, the accompanying gut microbiome information is not always presented concurrently (Che et al.,

2022; Germain et al., 2017, 2018, 2020, 2022; Giloteaux et al., 2023; Mandarano et al., 2020; McGregor et al., 2019; Naviaux et al., 2016). A consistent metabolomic signature for ME/CFS is also yet to be discovered (Huth et al., 2020; Maksoud et al., 2023; Taccori et al., 2023).

It can be appreciated that these studies (and any research investigation) are major undertakings and commitments with slightly different focus points, and resources are not unlimited. If nothing else, they highlight that there is a lot of ground to cover as far as ME/CFS biomedical insights go. Studies that have looked at the gut microbiome and host metabolome, or at least the gut microbiology and host metabolism but not to an “omic” extent, have shown that the dual investigation provides the opportunity to characterise the microbes and further understand the microbial function and its metabolic output in ME/CFS. However, they do not prove causality and raise other unanswered questions in ME/CFS about heterogeneity including comorbidities (Armstrong et al., 2017; Guo, Che, et al., 2023; Kitami et al., 2020; Lupo et al., 2021; Maksoud et al., 2023; Nagy-Szakal et al., 2017, 2018; Sheedy et al., 2009; Shukla et al., 2015; Xiong et al., 2023). Moreover, beyond the collective multi-dimensional challenges prevalent in ME/CFS research that accentuate its lack of reproducibility and direct comparability, heterogeneity, and overall complexity, such as the case definitions, relatively limited sample sizes in the majority of studies, a multitude of involved symptoms and comorbidities, and varying degrees of disease presentation, frequency (acute versus chronic), severity and duration (long-term versus short-term), there exists the issue of plausible yet undefined subgroups/subtypes, individual-related factors, and likely multifaceted pathogenesis and pathophysiology. Additionally, study design and data harmonisation elements such as the selection and usage of different biospecimen types, and experimental, data, and analytical approaches, need to be taken into consideration.

## 1.7 Thesis Aims and Objectives

There is currently no standard diagnostic test or biomarker/s for ME/CFS where the clarity and simplicity in the pursuit of finding these urgently needed tools is hampered by comorbidities and its many phenotypic presentations. As the critical role of the gut microbiome, host metabolism, and its relationship seems likely to be involved in ME/CFS in a substantial way, this project was undertaken within this framework to contribute to the understanding of some of the factors that could underpin pathophysiology and heterogeneity. Following this overarching aim, this project was also undertaken with the interest of exploring and utilising different biospecimen types and complementary analytical techniques and approaches. Together, this project aspired to investigate ME/CFS with the appreciation and awareness that tackling a multifaceted issue would necessitate a holistic, multidisciplinary, and multi-omic approach.

Currently, most ME/CFS studies report findings from ME/CFS versus Control cohorts. Given that ME/CFS is potentially made up of different subgroups of individuals that are yet to be fully defined, I chose in this thesis to compare a ME/CFS overall group with a Control (non-ME/CFS) group, as well as the ME/CFS group alone according to their FM and IBS comorbidity determined by the questionnaire metadata. So far, some studies have looked at ME/CFS with and without IBS or FM, but even more rarely with both. Recently, Nepotchatykh et al. (2023) and Schutzer et al. (2023) performed molecular and proteomic-based investigations with ME/CFS samples that were also grouped by whether there was comorbid FM or not; however, neither of these insightful studies ascertained IBS profiles. Similarly, ME/CFS with or without IBS has been explored in a few biomolecular-based studies; however, without factoring in FM comorbidity in their data analyses (Che et al., 2022; Giloteaux et al., 2023; Guo, Che, et al., 2023; Nagy-Szakal et al., 2017). One study on ME/CFS proteomic profiles looked at outcomes that considered ME/CFS cases, controls, ME/CFS cases with IBS, ME/CFS cases without IBS, ME/CFS cases with FM, ME/CFS cases without FM (Milivojevic et al., 2020). In light of this, the following four comparative groups of interest feature throughout this thesis unless otherwise stated (Section 2.1): Comparison A compares the ME/CFS overall and Control groups, Comparison B compares the ME/CFS with FM and ME/CFS without FM groups (ME/CFS +/- FM), Comparison C compares the ME/CFS with IBS and ME/CFS without IBS groups (ME/CFS +/- IBS), and Comparison D compares the age-matched ME/CFS with IBS and ME/CFS without IBS groups (ME/CFS +/- IBS**).

# 2 CHAPTER TWO: Methodology

## 2.1 Analytical Approaches, Platforms, and Technologies

Composite data from the gut microbiome and the host metabolome currently provides the most powerful evidence that can demonstrate the closest associations with healthy and diseased states (M. X. Chen et al., 2019; Jandhyala et al., 2015; Wishart et al., 2023). A theme that emerges from the microbiome and metabolomic fields, and in other omic and scientific areas, are the applications and utility of different approaches, platforms, and technologies that enable the work. This applies to processes and activities further upstream as well as downstream of the overall research design and directive. No single approach, technique, or method is without some shortcomings; everything has its advantages and disadvantages for their respective reasons. While the findings and outcomes of a study are essential, it is just as important to acknowledge and evaluate the benefits and limitations of any methodology used and the details of how it got there.

### 2.1.1 Gut Microbiome with Culture-Based and Culture-Free Methods

This study used culture with matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF MS or MALDI-TOF) and 16S rRNA gene amplicon sequencing (16S rRNA) for bacterial identification in participant faecal matter. Previous ME/CFS studies have used these methods, but both have not been applied to the same participant cohort within the same study (Du Preez et al., 2018). These methods were chosen to have a culture and molecular-based complementary approach to characterising the gut microbiota. They were also selected given supervision, laboratory facilities, and resources available at the time of commencing the study. The same laboratory that supported the culture MALDI-TOF gut microbiology work in previous ME/CFS studies was used for the work presented in this thesis (Armstrong et al., 2017; Sheedy et al., 2009; Wallis et al., 2016, 2018). I used a similar protocol for my experiments with some modifications. Instead of the glucose-saline buffer that was used in the previous studies to prepare the faecal samples for culture, I opted for a standard phosphate-buffered saline (PBS) diluent medium. Given the interest in enumerating faecal bacteria from samples and comparing the differences in the microbial abundance between a ME/CFS and non-ME/CFS cohort, it was not apparent to me why having glucose, a nutrient and energy source, in the dilution medium would be a suitable choice for this work. Glucose is the

classical carbon source that is used to investigate the transport, metabolism, and regulation of nutrients in bacteria (Jahreis et al., 2008). While these studies, including my own, also have an interest in the gut microbes (bacteria) and their metabolites with host metabolism, enumerating bacteria as they are in a sample at “baseline” without giving them an energy source is a different topic from looking at microorganisms and their capacity and cellular physiology to utilise a nutrient such as glucose (Jahreis et al., 2008). Addressing these topics regarding gut microbes would require different experimental designs and apparatus. Instead, PBS offers a non-toxic, nutrient-free environment to suspend the faecal samples and transfer them to agar plates for culture, counting, and MALDI-TOF identification. The dilution series used in previous work was not a strict or straightforward preparation as would be expected for standard plate counting; I elected to use a 10-fold series that made calculations less onerous. Added to this, from observation of the previous experimental process of this technique, I used a motorised homogenising instrument to achieve a seamless mixture before completing the dilution series and spread plating the samples onto agar. These steps replaced the manual mixing of the faecal samples with the diluent using wooden sticks which did not always blend the sample well or make it an easy material to work with, and streak plating (Y. H. Hsieh et al., 2016; Santiago et al., 2014; Tarazona Carrillo et al., 2023). Aside from these details with the experimental design and details of the culture MALDI-TOF MS work, the platform does offer a reliable and relatively simple way of identifying microorganisms (Franco-Duarte et al., 2019; T. Y. Hou et al., 2019; Rahi & Vaishampayan, 2020; Rychert, 2019). Further, in terms of the gut microbiome, culture with MALDI-TOF MS offers insight, added knowledge of microbes, and an extension of the known gut repertoire where molecular and bioinformatic/computationally based approaches fall short (L. Chen et al., 2023; Lagier et al., 2018; Yada et al., 2023).

Traditionally, bacteria have been identified and characterised by microbiological methods based on morphological and biochemical attributes of the isolates which initially relied on culture (Franco-Duarte et al., 2019). The utility of MALDI-TOF has presented laboratories with a tool to reduce its reliance on, and in some cases, an attractive and favourable replacement of traditional methods for identifying microorganisms following culture (Franco-Duarte et al., 2019; A. Gupta et al., 2023; T. Y. Hou et al., 2019; Rychert, 2019; Singhal et al., 2015). Its capability has allowed for a much more accurate and specific, faster, and cost-effective identification of microbes from cultivated specimens and in some cases, directly from medical specimens with minimal sample preparation needs (this is more common in routine monitoring clinical microbiology laboratory settings) (Elbehiry et al., 2022; T. Y. Hou et al., 2019; Rahi &

Vaishampayan, 2020; Rychert, 2019). MALDI-TOF MS is an analytical technique in which particles are ionised, separated according to their mass-to-charge ratio, and measured by determining the time it takes for the ions to travel to a detector at the end of a time-of-flight tube. The use of MALDI-TOF has allowed for the rebirth and renewed interest in culture techniques in microbiology, hence the term “culturomics” (Lagier et al., 2018; Yadav et al., 2023). Generally, culturomics is a high-throughput approach that implements MALDI-TOF in its workflow and offers a window to improve and optimise the identification and study of microbes that were previously considered challenging to examine, unculturable with traditional culturing methodologies or remain uncultured (Lagier et al., 2018; Yada et al., 2023). The MALDI-TOF platform can identify gram-positive, gram-negative, aerobic, and anaerobic bacteria as well as mycobacteria, yeast, and moulds, typically at the genus and species level (T. Y. Hou et al., 2019; Rychert, 2019). Aside from the features that are not in the MALDI-TOF reference database that enable microbe identifications to be made, the platform does offer an advantageous opportunity to characterise microbes that are closely related to each other at the genus and species level (Elbehiry et al., 2022; T. Y. Hou et al., 2019; Rychert, 2019; Topić Popović et al., 2023). The resolution and accuracy of microbe identification at the species level are typically as good or better than traditional or molecular methods for microorganism identification (Elbehiry et al., 2022; Lagier et al., 2018; Rychert, 2019). While molecular-based techniques have provided record insights into bacterial identification and typing, there is still a valuable place for MALDI-TOF culture-based techniques in gut microbiota investigations (Franco-Duarte et al., 2019; Lagier et al., 2018).

In the context of profiling the “overall” gut microbiota, using culture with MALDI-TOF MS is still a workload-intensive and time-consuming process given the need for culture on several types of agar media and the sheer number of microbes which makes it difficult to study various isolates on an agar plate (Armstrong et al., 2017; L. Chen et al., 2023; Franco-Duarte et al., 2019; Jandhyala et al., 2015; Lagier et al., 2018; Sheedy et al., 2009; Wallis et al., 2016, 2018). Isolation, identification, and enumeration of the vast majority of gastrointestinal microorganisms using culture-based techniques is not only an arduous and time-consuming process, but it is also insensitive and offers less comprehensive coverage of features by comparison to 16S rRNA-based sequencing (Jandhyala et al., 2015). For this reason, molecular-based, culture-independent techniques have been preferred and used in most gut microbiome studies, not to mention the unprecedented insights into bacterial identification and typing that the “molecular biology age” of sequencing has provided (Franco-Duarte et al., 2019; Jandhyala et al., 2015;

Lau et al., 2016). Although culture and MALDI-TOF by comparison to culture-free molecular approaches are not as frequently used to study the “entire” gut microbiota, the benefits that culture coupled with MALDI-TOF MS does provide is something that cannot be overlooked (L. Chen et al., 2023; S. Gupta et al., 2019; Jandhyala et al., 2015; Lagier et al., 2018; Yada et al., 2023). 16S amplicon sequencing offers many advantages and is a mainstay of sequence-based bacterial analysis; however, the platform does struggle to provide taxonomic resolution at the species and strain level because of short read lengths (Johnson et al., 2019; Lagier et al., 2018; Peterson et al., 2021). Further, culture-based approaches also allow for the determination of viable microbes in a community whereas the majority of standard molecular methods do not, or cannot, distinguish between DNA material obtained from alive or dead cells (Bellali et al., 2021; Emerson et al., 2017; Kallastu et al., 2023; Lagier et al., 2018; Lau et al., 2016). In some cases, the culture-based approach may also discover less abundant bacteria that may be missed by depth bias or insufficient sequencing depth in culture-independent studies (Lagier et al., 2018; Lau et al., 2016; Yadav et al., 2023). Outside of characterising the overall gut microbiome, culture with MALDI-TOF can be used to study the gut and model various conditions in vivo (Yadav et al., 2023). Some argue that a dead or alive microbe does not matter, but information regarding microbe viability may provide more information to capture the diversity of microbes, and their functional capacity and metabolic state in an environment (Bellali et al., 2021; Emerson et al., 2017; Wutkowska et al., 2019). Lastly, information at the species and strain level of resolution deepens the understanding and interpretation of the microbes which may exhibit high variability in their activity and interactions with the host despite being similar in genomic content, for example, pathogenicity, antibiotic resistance, clinic outcomes and therapeutic efficacy, response to environmental stress (B. D. Anderson & Bisanz, 2023; Carrow et al., 2020; V. K. Gupta et al., 2020; Johnson et al., 2019; P. Liu et al., 2022; Park et al., 2022; Peterson et al., 2021; Shetty et al., 2022; W. Xu et al., 2023; J. Yang et al., 2020).

Since its advent, culture-independent, molecular-based next-generation sequencing (NGS) approaches have revolutionised the sciences and study of complex microbial communities, including that of the gastrointestinal system. NGS has enabled major advanced investigations and discoveries of the gut microbiome in many diseases with exceptional resolution and throughput (Jovel et al., 2016; Satam et al., 2023; Tang et al., 2020). High throughput next-generation sequencing methods have compared large cohorts in record time, allowed for the parallel sequencing of millions of DNA fragments (multiplexing), improved sensitivity compared to culture, detected uncultured bacteria, facilitated sophisticated analyses, and deeper and

wider scope of understanding the microbiome from its taxonomy, genomic structure, function, and dynamics (Jovel et al., 2016; Lagier et al., 2018; Satam et al., 2023). In recent times, an overall improved cost-effectiveness supported by the advances in scalability, robotics, liquid handling, sample collection and processing, data analysis, bioinformatics, and IT infrastructure have made, and will continue to make, NGS workflows more accessible and widespread in several fields and studies including agricultural, environmental, medical research, and routine clinical practice (Satam et al., 2023). Metataxonomics (16S ribosomal RNA, 16S rRNA gene amplicon sequencing) and whole shotgun metagenomic sequencing (metagenomics) are the two main NGS approaches using extracted DNA samples for analysing the microbiome (Durazzi et al., 2021; Jovel et al., 2016). NGS of the 16S rRNA gene is one of the most widely used culture-independent applications to investigate the microbiota at any given body site in research (S. Gupta et al., 2019; Peterson et al., 2021). While the NGS downstream data analytical area has become more accessible and easier to navigate, data generated by both 16S rRNA and metagenomics still requires extensive computer hardware resources and computational analytical methods (bioinformatic sequence processing and data analyses) compared to culture methodologies which can be an initial barrier and steep learning curve for those unfamiliar with the workflow requirements and tools available (Jovel et al., 2016; Y. Kim, 2023; Larson et al., 2023; Szopinska-Tokov et al., 2023). However, all in all, the wide availability of several options from free up-to-date open-source, and commercially based sequencing and bioinformatic service providers for all microbiome needs makes the future and application of NGS in research developments a promising and well-supported one.

The choice between metataxonomics (16S) and metagenomics can be made on the comparison of costs and needs; both provide a wealth of microbiome data and information (Jovel et al., 2016; Usyk et al., 2023). 16S rRNA amplicon gene sequencing generally involves PCR to target and amplify portions of the hypervariable regions (V1-V9) of the bacterial 16S rRNA gene, followed by sequencing and comparison to known databases for identification (Kameoka et al., 2021). Profiling microbial communities using 16S sequencing is a relatively straightforward and cost-effective method to profile the taxonomic composition of a microbial community by comparison to metagenomics (Peterson et al., 2021; Usyk et al., 2023). 16S sequencing does come with the caveat of sequence artifacts from PCR errors, and primer bias where the selection of primers targeting regions of the 16S gene can influence the resulting microbial composition and taxonomic representation of the microbiome (Abellan-Schneyder et al., 2021; Peterson et al., 2021; Usyk et al., 2023). Another major limitation of 16S sequencing is that the

shorter length of the amplicon sequences usually limits the taxonomic classification of the microbial community to the genus level (J. Yang et al., 2020). Shotgun metagenomics indiscriminately sequences all of the DNA material in a sample, and therefore typically requires more sequenced reads per sample to find unique taxonomic identifiers (Peterson et al., 2021). This potentially means that non-microbial reads could interfere with and eclipse the microbiome outcome; this is particularly relevant in human microbiome investigations where samples such as faeces may contain human DNA (Tomofuji et al., 2023). Metagenomics can reveal more about the gut microbiota at a deeper and wider level with improved detection of low abundant taxa and a strain-level of identification (Durazzi et al., 2021; Jovel et al., 2016; Peterson et al., 2021). Metagenomic sequencing can also simultaneously study archaea, viruses, virophages, and eukaryotes (Jovel et al., 2016). Although shotgun sequencing information yields more information on many genes rather than only one, the need for increased sequencing depth carries a higher cost (Peterson et al., 2021; Usyk et al., 2023). Overall, when weighing up project-specific circumstances, costs, and benefits, utilisation of the 16S rRNA gene offers a suitable and comprehensive method to profile the gut microbiome despite its limitations (Durazzi et al., 2021; Jovel et al., 2016; Peterson et al., 2021; Usyk et al., 2023). Both molecular-based sequencing methods have been adopted in ME/CFS microbiome research, but this study opted for 16S sequencing. This came at the trade-off of collecting data at deeper taxonomic resolution and about the functional capacity of the microbe which the 16S sequencing information does not provide but shotgun metagenomics does. However, computational tools, such as PICRUSt2, MicFunPred, Piphillin, and Tax4Fun2 are available to leverage the 16S data to predict and estimate the functional capacity of the microbe metagenome (Douglas et al., 2020; Mongad et al., 2021; Wemheuer et al., 2020). PICRUSt2 was applied to the 16S data in this study as the pipeline was expected to perform well for gut microbiome data and also because other studies and available computational support (Douglas et al., 2020; M. H. Kim et al., 2020; Vänni et al., 2021). Analysing and visualising PICRUSt2 outputs has also been a challenging task; however, previously STAMP software and more recently, ggpicrust2 a R package, have been developed to support researchers in getting the most out of their 16S rRNA data (Parks et al., 2014; C. Yang et al., 2023).

### 2.1.2 Host Metabolomics with LCMS and NMR

LCMS and NMR were used as independent but complementary platforms in a targeted metabolomics workflow to assay polar metabolites typically part of primary metabolism from faecal, urine, and blood plasma samples. LCMS and NMR have been used in previous ME/CFS metabolomic studies; however, both platforms have also not been applied to the same participant cohort and samples within the same study (Huth et al., 2020). Metabolomics seeks to comprehensively and systematically identify and measure all metabolites (small, low-molecular-weight molecules less than 1500 Da) in a given organism or biological sample (Clish, 2015; Qiu et al., 2023). The field has steadily grown over the past few decades and shows no signs of diminishing (Edison et al., 2021). The most common analytical platforms for metabolomics are nuclear magnetic resonance spectroscopy (NMR), gas chromatography-mass spectrometry (GCMS), and liquid chromatography-mass spectrometry (LCMS) (Edison et al., 2021; X. Liu & Locasale, 2017; Wishart et al., 2022). At present, no single method or instrument can fulfil the mission of capturing and covering the entire metabolome (Qiu et al., 2023; Roca et al., 2021). An important part (or the true extent) of the human metabolome remains uncovered although continued improvements in analytical chemistry and bioinformatics have considerably increased the number of measurable metabolites (Roca et al., 2021; Wishart et al., 2018).

While LCMS methods are becoming increasingly popular, accounting for more than 70% of published metabolomics studies to date, there are considerable benefits and advantages to NMR-based methods for metabolomic studies (Edison et al., 2021; Nagana Gowda & Raftery, 2023; Wishart et al., 2022). Recent data shows that a record number of NMR-based metabolomic publications have been published in 2021; NMR continues to grow and offer a lot to the research community (Nagana Gowda & Raftery, 2023; Wishart et al., 2022). It is now widely recognised that using more than one platform expands the metabolomic data output, broadens coverage and the scope of understanding the metabolome in any given context, system, or sample (Bustamam et al., 2021; Gathungu et al., 2020; González-Domínguez et al., 2017; H. Lau et al., 2022; Letertre et al., 2020; Moosmang et al., 2019; Nkobole & Prinsloo, 2021; Qiu et al., 2023; Roca et al., 2021). Metabolomics can be categorised into targeted and untargeted approaches. Generally, untargeted metabolomics reveals a broad view of previously known and unknown metabolic information and conversely, targeted highlights analysing a set of metabolites which tends to be more defined, hypothesis-driven, sensitive, and reproducible relative to an untargeted approach (Letertre et al., 2020; Qiu et al., 2023; L. D. Roberts et al.,

2012). Further, different biospecimen types provide different but complementary, metabolic information, and insights about various processes occurring and phenotypes presenting (Smith et al., 2020). In the biomedical sciences, a variety of sample types are of interest because they each provide useful information about the organs or tissues that produce them. A summary of the advantages and disadvantages of LCMS and NMR, and different biospecimen types are provided below.

*Advantages of LCMS:*

- Initial set-up costs and ongoing instrument maintenance can be more attractive than NMR
- Generally, MS has had more success than NMR in the clinical chemistry laboratory because of its overall lower costs, its smaller instrumental footprint, and its ability to measure low-concentration metabolites that are more frequently used in diagnostic assays
- Although destructive and the sample cannot be recovered after analysis, only a small amount of sample material is required
- High sensitivity where low abundant metabolites and nanomolar concentrations can be detected
- Generally, LCMS has higher coverage and number of detectable metabolites in a sample

*Disadvantages of LCMS:*

- Higher cost per sample as some of the required standards, consumables, and equipment parts can be more expensive; however, advancements in analytical technologies have made overheads more cost-effective and reduced the environmental impact of solvent consumption
- Sample separation and preparation needs are generally more demanding to enhance detection or analysis; although some NMR workflows can also be just as demanding to prepare if there are many samples without automation assistance
- Chromatographic and ionsiation techniques to separate and prepare compounds for detection can be cumbersome and time-consuming when run on the instrument
- The intensity of the MS line is often not correlated with metabolite concentrations as the ionisation efficiency is also a determining factor
- Quantitation is more difficult to achieve with the use of labelled internal standards

- Destructive to sample and sample cannot be recovered after analysis which can be an issue if samples are rare, difficult to obtain, need to be measured repeatedly, or if an in vivo, metabolic flux or metabolic imaging study design is required
- LCMS is limited to detecting compounds that readily ionise, which is further diminished by ion suppression common to complex, heterogenous mixtures (biological samples)
- Reproducibility is a challenge in LCMS in comparison to NMR although the development of newer approaches and technologies has contributed to improvements in reproducible data
- Inter- and intra-variability in LCMS comes from its technical limitations – batch effects, different instruments even if the same model, signal drifts, performance of the same instrument over time
- Harmonisation of disparate datasets, protocols pre- and post-instrument analysis is more challenging to achieve in and between laboratories in comparison to NMR

*Advantages of NMR:*

- Lower cost per sample not including the personnel, equipment, and maintenance, but consumables required for sample preparation are often less expensive
- Advances in magnet shielding, electronics, and cryo-technology are making NMR instruments smaller, cheaper, easier to maintain, and more compatible with a wider range of environments beyond large university departments
- Non-destructive and non-invasive, samples remain intact after analysis and can be recovered, stored, and used for reanalysis using NMR or at a later time with other methods such as MS
- NMR does not involve harsh sample treatment before analysis where metabolites can be fragile or sensitive to ionisation voltage used by MS analysis; sample preparation is relatively simple
- Unbiased by comparison to LCMS as no chromatographic separation of compounds before ionisation and detection is required
- Easily quantifiable so both relative and absolute metabolite concentrations can be obtained – NMR is inherently quantitative as the signal intensity is directly proportional to the metabolite concentrations and number of nuclei in the molecule
- Requires little to no sample preparation and does not need chemical derivatisation
- The gold standard for the identification of novel compounds

- NMR is highly amenable to metabolic flux and metabolic imaging studies making it ideal for investigating living cells, tissues, and organs
- Easily automatable and exceptionally reproducible, making automated high-throughput metabolomics studies much more feasible and reliable with NMR compared to LCMS or GCMS
- Amenable to detecting and characterising compounds that can be challenging for LCMS, such as sugars, organic acids, alcohols, polyols, and other highly polar compounds
- The possibility of detecting non-ionic compounds, such as sugars and alcohols, without disrupting their structure
- Reproducibility among different instruments, and laboratories, and the robustness of NMR measurements means that the development of standard procedures has become progressively easier, especially for clinical application

*Disadvantages of NMR:*

- Relative lower signal-to-noise ratio, compared to other analytical techniques – microprobes and cryoprobes are some strategies being used to enhance sensitivity in NMR
- Lower sensitivity by comparison to mass spectrometry
- Initial set-up costs can be greater and sometimes prohibitive for equipment and ongoing maintenance is also expensive; larger space for the instrumentation and non-vibrational floors and isolation from magnetic and radio frequency are required
- Instrument cost often scales exponentially with the field strength of the NMR magnet – the “sweet spot” for metabolomics to have field strength, resolution, and cost-effectiveness is the 600 MHz spectrometer
- Unstable helium supplies and/or high delivery costs where self-recycling systems can be advantageous
- $^{1}$H NMR spectra for metabolomics require consistent solvent suppression and flat baseline; many biological matrices are water-based, and it is essential that pulse sequences suppress the solvent signal to allow for better detection of lower abundant compounds and increased sensitivity
- All $^{1}$H NMR spectra in metabolomics suffer from considerable signal overlap since sample preparation is minimal; each metabolite often leads to several signals in the spectrum, and many metabolites can be detected which makes the accurate and efficient deconvolution of spectra onerous

- Many NMR applications have traditionally been processed manually and data analysis is inherent to data acquisition; however, more computational tools and resources are becoming available to support NMR-based metabolomics in a more user-friendly way – even though I had to manually process and deconvolve all my spectra, this point led me to seek advice from the Wishart lab group. I sought their advice so that I could get the most information and metabolites assigned out of my already acquired NMR spectra, and where it was possible to implement, improve, and build from the existing NMR metabolomic procedures used by my laboratory group that require review and updating with what is now currently available.

(Cui et al., 2018; A. H. Emwas et al., 2015, 2019; A. H. M. Emwas, 2015; Ghosh et al., 2021; Hajjar et al., 2023; Moco, 2022; Nagana Gowda & Raftery, 2021, 2023; Plumb et al., 2023; Wishart et al., 2022)

*Advantages of Faeces:*

- Non-invasive sample collection that can be repeated relatively easily if required (this depends on the individual and sometimes faecal samples are not that easy to collect)
- A reflection of the metabolic interplay between the host and its gut microbiome
- Directly associated with the gut and may reflect changes in metabolism very early through its transit in the gut
- Bacterial biomass with a complex composition that can provide rich information about the gut microbiome and host metabolism

*Disadvantages of Faeces:*

- Considerations for sample collection (anaerobic and cool temperatures) can be logistically difficult as aerobic conditions and room temperature might quickly change the faecal metabolome
- Water content bias requires dry weight measurements, lyophilisation, or freeze drying of samples; each of these normalisation approaches also has its pros and cons for the detection of metabolites
- Homogenisation is necessary; too much can perturb the faecal biostructure, and too little yields an inadequate representation of the sample

- The great variability in material content and characteristics makes it difficult to standardise the collection process, sample preparation, and analysis including the analytical platform
- The sample material is complicated to study and more complicated in the wet laboratory to handle since its composition is heterogeneous, multi-component, rich in macromolecules, particles of undigested food, and bacterial biomass of living and dead bacteria
- Apart from the perceived grossness with the sample, variability from an individual with feeding patterns, bowel activity, and other “gut health” factors that can make passing and collecting a faecal sample an unpredictable and difficult experience

*Advantages of Urine:*

- Non-invasive sample collection that can be repeated relatively easily if required
- Provides a historical overview of the global metabolic events
- Profiles provide an overall measure of the metabolic phenotype including diet and gut microbiota metabolic activity; capture a wide range of metabolites and metabolic processes occurring throughout the body
- Changes in urine metabolite concentrations can be greater than the changes seen in protein levels

*Disadvantages of Urine:*

- Large variability depending on the collection time caused by the circadian rhythm regulating the energy metabolism and gut microbiome metabolism, also due to a difference in hydration status, physical activity, and feeding state
- 24-hour sampling is preferred to eliminate the day-time variability in metabolic profiles; however, this is not always feasible or practical
- Unlike blood, where metabolite concentration is tightly maintained, urine concentration can vary drastically from sample to sample requiring appropriate pre-analytical normalisation of urinary metabolomic data
- A complex sample matrix including a large number of salts can lead to challenges in separating and identifying metabolites (shifts in retention times, matrix effects, many overlapping signals or features with urine being a biological waste material, and collection of biological by-products)

*Advantages of Blood Plasma:*

- Most metabolites in the blood reflect endogenous metabolites; hence, the joint metabolomics analysis of blood and urine could provide complementary data reflecting the state of the whole system at a defined time point
- Blood metabolic profiles are dynamic and vary continuously in response to changes in gene expression or changes induced by exogenous metabolites such as those provided by nutrients or drugs
- Provides the opportunity to study disruptions or impairments in homeostatic function
- There is better reproducibility in plasma due to the absence of the blood-clotting step when separating the two main components (plasma and serum) of whole blood

*Disadvantages of Blood Plasma:*

- Slightly more invasive sample collection than urine and faeces
- The choice of collection tube for plasma is critical depending on the analytical platform used
- The risk of highly dynamic and pronounced changes of the metabolome in the sample tube after blood drawing

(Bouatra et al., 2013; A. H. M. Emwas, 2015; Erben et al., 2021; Karu et al., 2018; Matysik et al., 2016; Smith et al., 2020; Tang et al., 2020; Yin et al., 2015; Zhgun & Ilyina, 2020)

## 2.2 Recruitment and Participant Selection Criteria

Participants who were at least 18 years old were recruited for the ME/CFS and control groups between March 2019 and November 2019. The project was reviewed and approved by the St Vincent's Hospital (Melbourne) Human Research Ethics Committee (Reference HREC/18/SVHM127). All participants were invited to join the study through word-of-mouth advertising, and assistance from the CFS Discovery Clinic, in Melbourne, Australia. Figure 2.2.1 outlines the selection criteria that were used. A power analysis calculation was initially conducted using the power analysis module in Statistica (Version 12.0) to determine a target sample size. At least 35 participants in each group (ME/CFS, fibromyalgia, and control) were required to achieve a power of 80% with a 0.01 significance level while detecting a mean difference of 12.26 with a standard deviation of 4.6. The parameters for the power calculation were based on raw data from McGregor et al. (2016) reflecting changes in acetate and the serum metabolome. Acetate is a short-chain fatty acid metabolite that was found to be involved in widespread pain development and ME/CFS (McGregor et al., 2016). The target sample size also considered other small-scale ME/CFS, and fibromyalgia (FM) research projects published at the time, and the availability of project funding, time, and resources (Armstrong et al., 2017; Germain et al., 2018; Nagy-Szakal et al., 2017; Slim et al., 2015; Wallis et al., 2018).

Due to practical and logistical factors, data and biological samples were collected across two parts. Part One and Part Two collected different biological sample types. Figure 2.2.2 provides an overview of the steps undertaken to collect participant data and samples that were used across the various analyses and experiments that appear in this thesis. Part One and Part Two formed the two overall ME/CFS and Control cohorts of interest; Comparison A in Table 2.2.1 and Table 2.2.2 provides details of these cohorts. Herein, a total of 40 ME/CFS and 43 Control participants represented Part One, and 28 ME/CFS and 28 Control participants represented Part Two. Efforts were made to closely age- and sex-match the control and patient group volunteers; however, the closure of the CFS Discovery Clinic prioritised the recruitment of those who could be involved. All the ME/CFS patients conformed to the ME/CFS Canadian Consensus (diagnostic) Criteria (Carruthers et al., 2003). To ensure that an adequate number of participants were recruited in the available timeframe, some of the control group volunteers were household members and/or relatives of someone diagnosed with ME/CFS and/or FM.

We invite you to participate if you are at least 18 years of age and meet at least one of the following criteria:

• Are currently experiencing symptoms or have unresolved symptoms of ME/CFS and/or FM

OR

• Are currently experiencing symptoms or have unresolved symptoms of chronic fatigue and/or pain

Alternatively, we invite you to participate as a healthy volunteer if you are at least 18 years of age and meet the following criteria:

• Do not have ME/CFS and/or FM

• Have not ever been given a diagnosis or suspected of having ME/CFS and/or FM

• Have not experienced symptoms of ME/CFS and/or FM; or chronic fatigue and/or pain in the last 6 months

• Have not been affected by severe, unexplained (including undiagnosed) chronic fatigue and/or pain for at least 6 months

• Are generally healthy

**Figure 2.2.1** Selection criteria for participants to be recruited and included in the study

All participants (n = 89) were required to complete Part One involving a faecal and urine sample collection, and completion of the questionnaire from a time point (Section 2.4.1). A subset of this cohort was formed from those who could complete Part Two (n = 60 of the 89). Part Two was optional depending on participant attendance at the designated collection room and it involved the collection of a blood sample and a second urine sample (Section 2.4.2). Participants were excluded from Part One (n = 3) and Part Two (n = 3) if they had incomplete questionnaires or experimental data. During the initial analyses of the questionnaire responses, control group participants were also excluded from Part One (n = 3) and Part Two (n = 1) if they had recorded that they had a FM or IBS diagnosis but were otherwise considered to be non-ME/CFS diagnosed. FM and IBS are ME/CFS comorbidities of interest in this study; however, there were not enough participant numbers to include those who had FM and/or IBS but not ME/CFS. Section 2.3 provides details on how the questionnaire metadata was used to select and form the FM and IBS subgroups for Parts One and Two (Comparison B, C, and D in Table 2.2.1 and Table 2.2.2) after the exclusions were made.

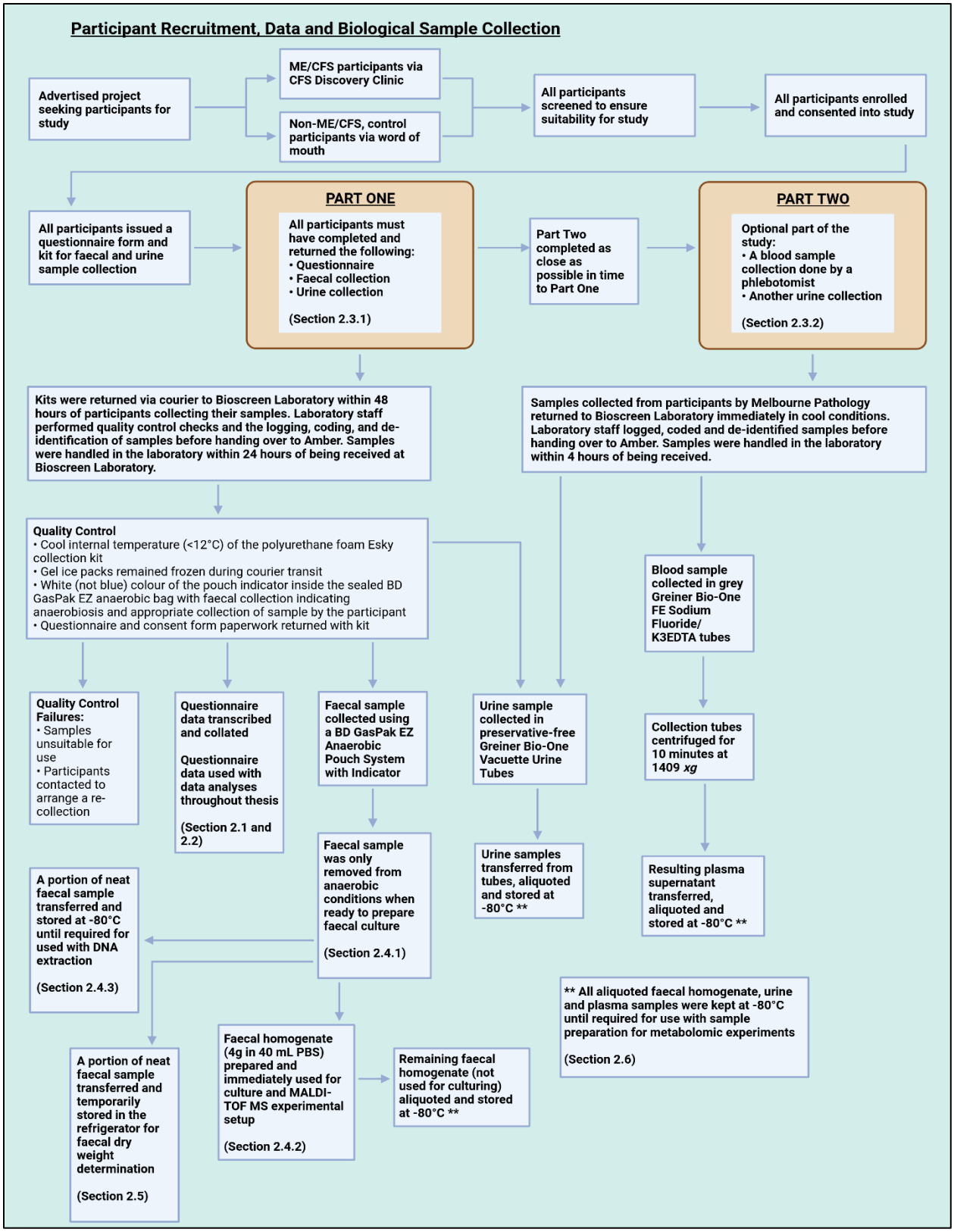



**Figure 2.2.2** Overview of the steps undertaken with participant recruitment, data, and biological sample collection. Created with BioRender.com.

**Table 2.2.1** Part One Age and Sex Baseline Characteristics

| | | | Comparison A | | | Comparison B | | |
|---|---|---|---|---|---|---|---|---|
| | | | ME/CFS | Control | | ME/CFS with FM | ME/CFS without FM | |
| | Sex | Age | n = 40 | n = 43 | P-value | n = 19 | n = 21 | P-value |
| Part One | Male and Female | Mean ± SD | 43 ± 13 | 43 ± 17 | 0.5115 | 45 ± 12 | 41 ± 13 | 0.1469 |
| | | Median (Range) | 41 (21-75) | 37 (21-80) | | 44 (21-75) | 39 (22-69) | |
| | | | n = 33 | n = 20 | P-value | n = 17 | n = 16 | P-value |
| | Female-only | Mean ± SD | 44 ± 13 | 43 ± 18 | 0.4683 | 46 ± 13 | 41 ± 14 | 0.2005 |
| | | Median (Range) | 41 (21-75) | 34.5 (23-80) | | 44 (21-75) | 39 (22-69) | |

| | | | Comparison C | | | Comparison D | | |
|---|---|---|---|---|---|---|---|---|
| | | | ME/CFS with IBS | ME/CFS without IBS | | ME/CFS with IBS age-matched | ME/CFS without IBS age-matched | |
| | Sex | Age | n = 20 | n = 20 | P-value | n = 14 | n = 14 | P-value |
| Part One | Male and Female | Mean ± SD | 49 ± 12 | 37 ± 10 | 0.0028 | 44 ± 6 | 40 ± 7 | 0.0761 |
| | | Median (Range) | 45 (29-75) | 36.5 (21-58) | | 44 (33-55) | 39 (32-53) | |
| | | | n = 17 | n = 16 | P-value | INTENTIONALLY BLANK Not enough participant numbers to age-match with only females | | |
| | Female-only | Mean ± SD | 49 ± 13 | 38 ± 11 | 0.0182 | | | |
| | | Median (Range) | 46 (29-75) | 37.5 (21-58) | | | | |

**Table 2.2.2** Part Two Age and Sex Baseline Characteristics

| | | | Comparison A | | | Comparison B | | |
|---|---|---|---|---|---|---|---|---|
| | | | ME/CFS | Control | | ME/CFS with FM | ME/CFS without FM | |
| | Sex | Age | n = 28 | n = 28 | P-value | n = 13 | n = 15 | P-value |
| Part Two | Male and Female | Mean ± SD | 43 ± 13 | 42 ± 19 | 0.3212 | 44 ± 14 | 42 ± 12 | 0.5186 |
| | | Median (Range) | 40.5 (21-75) | 34 (21-80) | | 42 (21-75) | 40 (26-69) | |
| | | | n = 22 | n = 17 | P-value | n = 12 | n = 10 | P-value |
| | Female | Mean ± SD | 44 ± 14 | 44 ± 19 | 0.5144 | 45 ± 14 | 43 ± 13 | 0.5975 |
| | | Median (Range) | 41 (21-75) | 34 (23-80) | | 43 (21-75) | 39.5 (26-69) | |

| | | | Comparison C | | | Comparison D | | |
|---|---|---|---|---|---|---|---|---|
| | | | ME/CFS with IBS | ME/CFS without IBS | | ME/CFS with IBS age-matched | ME/CFS without IBS age-matched | |
| | Sex | Age | n = 14 | n = 14 | P-value | n = 10 | n = 10 | P-value |
| Part Two | Male and Female | Mean ± SD | 49 ± 13 | 36 ± 9 | 0.0067 | 43 ± 6 | 41 ± 7 | 0.4046 |
| | | Median (Range) | 44 (33-75) | 35.5 (21-53) | | 41.5 (33-54) | 39.5 (32-53) | |
| | | | n = 12 | n = 10 | P-value | INTENTIONALLY BLANK Not enough participant numbers to age-match with only females | | |
| | Female | Mean ± SD | 49 ± 14 | 37 ± 11 | 0.0477 | | | |
| | | Median (Range) | 43 (33-75) | 36.5 (21-53) | | | | |

## 2.3 Participant Characteristics and Subgroups

The participants completed the questionnaires with Part One (Section 2.4.1). The questions collected a variety of participant information which provided insight into their medical history, demographics, symptomology, and other diagnoses/comorbidities (Appendix 1 provides a copy of the questionnaires used). The questionnaires were paper-based, and raw responses were manually collated, tabulated, and adjusted to correct for any scaling differences in Microsoft Excel. Analyses of the questionnaire responses in R Studio determined subgroups of interest based on sex, age, and other points of interest (Section 2.3.1). Significance for all tests used in Section 2.3.1 was defined as p-value < 0.05. These metadata parameters and outcomes informed the decision for this thesis to focus on the four comparative groups in Part One and Part Two using male and female participants (Appendix 2 shows the metadata used for this thesis). These comparisons are A) ME/CFS overall versus Control, B) ME/CFS with FM versus ME/CFS without FM (ME/CFS +/- FM), C) ME/CFS with IBS versus ME/CFS without IBS (ME/CFS +/- IBS), and D) age-matched ME/CFS with IBS versus ME/CFS without IBS (ME/CFS +/- IBS**). Table 2.3.1 and Table 2.3.2 summarises the age and sex baseline characteristics with the number of participants used for these comparisons in Part One and Part Two, respectively.

### 2.3.1 Analyses of Questionnaire Metadata

It was anticipated that there would be a higher proportion of female participants consistent with community and clinical prevalence rates in ME/CFS and FM patients (Buchwald & Garrity, 1994; Carruthers et al., 2003; Heidari et al., 2017). In Part One, there were 33 female and 7 male ME/CFS participants, and 20 female and 23 male Control participants. In Part Two, there were 22 female and 6 male ME/CFS participants, and 17 female and 11 male Control participants. There was a significant difference between the proportion of females and males in the ME/CFS overall and Control group cohorts in Part One; two-tailed Fisher's exact test, p = 0.0012. However, there was no significant difference between the sex, and ME/CFS overall or Control group variables in Part Two; two-tailed Fisher's exact test, p = 0.2448. These outcomes indicated that analyses of experimental outcomes would need to take into consideration the potential influence of the sex differences. In this thesis, Comparisons A to D in Part One and Part Two used both the male and female participants given the already limited sample size. This

study acknowledges the sex disparity, however, there was a lack of power to adequately test for sex disparities owing to the difficulty in obtaining participant, especially male, numbers.
The metadata regarding diagnostic conditions was used to select and assign the subgroups of interest for further data investigations with the experimental data outcomes. The participants were asked whether they had been diagnosed to have that condition with “yes” or “no”. Two-tailed Fisher’s exact tests were used to determine whether there was a significant association between the ME/CFS or Control groups, and the listed diagnostic condition. Table 2.3.1 (Part One) and Table 2.3.2 (Part Two) displays the percentages of the ME/CFS and Control group participants that had responded “yes” to having that diagnostic condition, and the p-value determining the statistical association between the two variables. A statistically significant difference was identified for a number of these diagnostic conditions (Table 2.3.1 and Table 2.3.2 p-value < 0.0001 highlighted in red, and p-value < 0.05 highlighted in green). The p-value outcomes for Fisher’s exact tests confirmed that this thesis had the basis to delve further into the interest in investigating the gut microbiology, host metabolomics, and subgroupings of the other diagnostic conditions/comorbidities in ME/CFS. The total numbers from these responses were also used to assign the FM and IBS subgroups within the ME/CFS cohorts (Comparisons B and C in Table 2.3.1 and Table 2.3.2) in addition to Comparison A which compares the ME/CFS overall and Control group.

The investigation of the overlapping and comorbid conditions in ME/CFS required a stepwise methodical approach. This thesis focuses on fibromyalgia (FM) and irritable bowel syndrome (IBS) as these comorbidities and their symptoms are frequently discussed in the context of ME/CFS (Aaron et al., 2000, 2001; Abbi & Natelson, 2013; Martín et al., 2023; Nacul et al., 2020; Natelson, 2019; Natelson et al., 2019; Petersen et al., 2020). This was also a factor that was taken into consideration when choosing to assess these comorbidities. Table 2.2.1 (Part One) and Table 2.2.2 (Part Two) shows the age and sex baseline characteristics of the four comparisons that are central to this thesis. Two-tailed Mann-Whitney U tests were conducted to determine whether there was a difference due to age between the groups of Comparisons A, B, and C in Part One and Part Two. The test results indicated for Part One and Two, that there was no significant difference in age between ME/CFS and Control groups (Comparison A), and ME/CFS with FM and ME/CFS without FM groups (Comparison B); however, there was a statistically significant difference in age between the ME/CFS with IBS, and ME/CFS without IBS groups (Comparison C). This difference was determined with a p-value of 0.0028 (all), and 0.0182 (female-only) in Part One, and 0.0067 (all), and 0.0477 (female-only) in Part Two. To

address the issue of age potentially being a confounding variable for Comparison C, Comparison D was formed (Bakken et al., 2014; Hornig et al., 2015; Kidd et al., 2016a). Comparison D in Part One and Two, comprises the ME/CFS with IBS and ME/CFS without IBS male and female participants that have been age-matched and selected. Based on the age Z-scores of the overall ME/CFS group, those that had a value below or above 1.0 standard deviation away from the mean were removed. This study acknowledges the limitations of this approach due to the sample size of a small-scale project, however, this age-matched Comparison D grouping was an attempt to address the potential age-confounding variable in IBS. The participant numbers were re-totalled, and a two-tailed Mann-Whitney U test was conducted on the new grouping (Part One and Part Two, Comparison D). The test results indicated that there was no significant difference due to age for the matched ME/CFS with IBS and ME/CFS without IBS groups; p-values of 0.0761 and 0.4046 for Part One and Two, respectively. This age-match approach was only applied to the group with male and female (all) participants as there were not enough numbers for an age-matched, female-only participant subgrouping (Table 2.2.1 and Table 2.2.2).

**Table 2.3.1** Part One Summary of Diagnostic Conditions

| Diagnostic Condition | Part One | | | | | |
|---|---|---|---|---|---|---|
| | All Participants (n = 83) | | | Female-only Participants (n = 53) | | |
| | ME/CFS (n = 40) | Control (n = 43) | | ME/CFS (n = 33) | Control (n = 20) | |
| | Percentage of YES responses from ME/CFS group | Percentage of YES responses from Control group | p-value † | Percentage of YES responses from ME/CFS group | Percentage of YES responses from Control group | p-value † |
| ME/CFS | 100% | 0% | < 0.0001 | 100% | 0% | < 0.0001 |
| Postural orthostatic tachycardia syndrome | 78% | 0% | < 0.0001 | 82% | 0% | < 0.0001 |
| Food intolerance/s | 78% | 14% | < 0.0001 | 82% | 25% | < 0.0001 |
| Irritable bowel syndrome | 50% | 0% | < 0.0001 | 52% | 0% | < 0.0001 |
| Fibrositis or fibromyalgia | 48% | 0% | < 0.0001 | 52% | 0% | < 0.0001 |
| Trouble eating/drinking milk/dairy products | 65% | 12% | < 0.0001 | 67% | 25% | 0.0047 |
| Tension headaches | 40% | 5% | 0.0001 | 42% | 5% | 0.0040 |
| Anxiety or other mental disorder | 43% | 7% | 0.0002 | 45% | 10% | 0.0137 |
| Sinus problems | 40% | 7% | 0.0005 | 42% | 5% | 0.0040 |
| Dermatitis or any other skin problem | 40% | 7% | 0.0005 | 39% | 10% | 0.0284 |
| Tinnitus or ringing in the ears | 35% | 5% | 0.0006 | 39% | 5% | 0.0087 |
| Multiple chemical sensitivity | 23% | 0% | 0.0008 | 21% | 0% | 0.0365 |
| Clinical depression | 38% | 7% | 0.0010 | 42% | 10% | 0.0150 |
| Low blood pressure | 28% | 2% | 0.0012 | 33% | 5% | 0.0197 |
| Food allergy/allergies | 48% | 14% | 0.0016 | 48% | 20% | 0.0461 |
| Joint hyperflexibility/mobility | 20% | 0% | 0.0020 | 24% | 0% | 0.0187 |
| Gastritis | 20% | 0% | 0.0020 | 21% | 0% | 0.0365 |
| Glandular fever | 43% | 12% | 0.0024 | 42% | 5% | 0.0040 |
| Trouble eating or drinking sugar | 25% | 2% | 0.0028 | 27% | 5% | 0.0697 |
| Low blood sugar or hypoglycaemia | 13% | 0% | 0.0227 | 15% | 0% | 0.1438 |
| Allergies | 43% | 19% | 0.0302 | 45% | 30% | 0.3859 |
| Migraine with aura | 20% | 5% | 0.0438 | 21% | 0% | 0.0365 |
| Migraine without aura | 28% | 9% | 0.0452 | 33% | 10% | 0.0978 |
| Gall bladder problems | 10% | 0% | 0.0497 | 9% | 0% | 0.2816 |
| Streptococcal throat | 10% | 0% | 0.0497 | 6% | 0% | 0.5210 |
| Kidney infections | 15% | 2% | 0.0520 | 18% | 0% | 0.0722 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Physical/sexual/emotional abuse | 15% | 2% | 0.0520 | 18% | 0% | 0.0722 |
| Urinary tract infection/cystitis | 30% | 12% | 0.0561 | 36% | 20% | 0.2370 |
| Eye conditions with or without vision loss | 18% | 5% | 0.0815 | 21% | 5% | 0.2336 |
| Yeast disease or candidiasis | 18% | 5% | 0.0815 | 18% | 10% | 0.6947 |
| Cluster headaches | 13% | 2% | 0.1013 | 15% | 0% | 0.1438 |
| Stomach or gastric ulcers | 8% | 0% | 0.1075 | 6% | 0% | 0.5210 |
| Pneumonia | 8% | 0% | 0.1075 | 3% | 0% | 1.0000 |
| Thyroid problems | 20% | 7% | 0.1089 | 21% | 10% | 0.4556 |
| Cold sores, herpes labialis | 30% | 14% | 0.1098 | 27% | 15% | 0.4996 |
| Bronchitis | 23% | 9% | 0.1335 | 24% | 0% | 0.0187 |
| Fractured bone | 23% | 9% | 0.1335 | 24% | 10% | 0.2863 |
| Autoimmune disease | 15% | 5% | 0.1465 | 15% | 10% | 0.6974 |
| Genital infections | 10% | 2% | 0.1912 | 9% | 0% | 0.2816 |
| Anaemia | 10% | 2% | 0.1912 | 9% | 0% | 0.2816 |
| Cancers | 10% | 2% | 0.1912 | 12% | 0% | 0.2848 |
| Other gastrointestinal disorder | 10% | 2% | 0.1912 | 12% | 5% | 0.6388 |
| Slipped disk or other back problems | 20% | 9% | 0.2173 | 21% | 10% | 0.4556 |
| Eczema | 20% | 9% | 0.2173 | 18% | 10% | 0.6947 |
| Ehlers-Danlos syndrome | 5% | 0% | 0.2292 | 6% | 0% | 0.5210 |
| Colitis/Crohn's disease | 5% | 0% | 0.2292 | 6% | 0% | 0.5210 |
| Yellow jaundice or hepatitis | 5% | 0% | 0.2292 | 6% | 0% | 0.5210 |
| Psychiatric illness | 5% | 0% | 0.2292 | 6% | 0% | 0.5210 |
| Hormone disorder | 5% | 0% | 0.2292 | 3% | 0% | 1.0000 |
| Injury to head or neck | 13% | 5% | 0.2538 | 9% | 0% | 0.2816 |
| Iron deficiency anaemia | 18% | 9% | 0.3401 | 21% | 15% | 0.7248 |
| A genetic (inherited) disease | 8% | 2% | 0.3481 | 9% | 5% | 1.0000 |
| Asthma | 20% | 12% | 0.3709 | 18% | 5% | 0.2333 |
| Ear infection | 20% | 12% | 0.3709 | 21% | 5% | 0.2336 |
| Arthritis or rheumatism | 10% | 5% | 0.4219 | 12% | 5% | 0.6388 |
| Concussion | 13% | 7% | 0.4729 | 9% | 0% | 0.2816 |
| Psoriasis | 13% | 7% | 0.4729 | 15% | 5% | 0.3904 |
| Type 2 diabetes | 3% | 0% | 0.4819 | 3% | 0% | 1.0000 |
| Carpal tunnel syndrome | 3% | 0% | 0.4819 | 3% | 0% | 1.0000 |
| Malaria | 3% | 0% | 0.4819 | 3% | 0% | 1.0000 |
| Disease of the hair or scalp including hair loss | 3% | 0% | 0.4819 | 3% | 0% | 1.0000 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Any other blood disorder | 3% | 0% | 0.4819 | 3% | 0% | 1.0000 |
| Suicide attempts | 3% | 0% | 0.4819 | 3% | 0% | 1.0000 |
| Drug abuse | 3% | 0% | 0.4819 | 3% | 0% | 1.0000 |
| Alcohol abuse | 3% | 0% | 0.4819 | 3% | 0% | 1.0000 |
| Heart valve disease | 0% | 5% | 0.4946 | 0% | 10% | 0.1379 |
| A stroke | 0% | 5% | 0.4946 | 0% | 10% | 0.1379 |
| Rheumatic fever | 0% | 5% | 0.4946 | 0% | 5% | 0.3774 |
| Sexual problems | 5% | 2% | 0.6069 | 6% | 0% | 0.5210 |
| Haemochromatosis | 5% | 2% | 0.6069 | 3% | 5% | 1.0000 |
| Hayfever | 28% | 21% | 0.6090 | 30% | 20% | 0.5274 |
| Gall bladder removed | 8% | 5% | 0.6685 | 9% | 5% | 1.0000 |
| Eye disease/s or disorder/s | 8% | 5% | 0.6685 | 9% | 10% | 1.0000 |
| Your appendix removed | 5% | 9% | 0.6770 | 3% | 10% | 0.5492 |
| High blood pressure | 15% | 19% | 0.7728 | 15% | 10% | 0.6974 |
| Other neurological disorder | 0% | 2% | 1.0000 | 0% | 5% | 0.3774 |
| Type 1 diabetes | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Gout | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Rheumatoid arthritis | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Epilepsy | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Leukaemia | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Lymphoma | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Lupus erythematosus | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Sjogren's syndrome | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Heart attack/myocardial infarct | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Glaucoma | 3% | 2% | 1.0000 | 3% | 5% | 1.0000 |
| Loss of hearing | 8% | 7% | 1.0000 | 9% | 5% | 1.0000 |
| Meniere's disease | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Tuberculosis | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Duodenal ulcers | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Cirrhosis of the liver | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Splenectomy | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Subfertility | 3% | 2% | 1.0000 | 3% | 0% | 1.0000 |
| Radiation therapy | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Prosthetic valve/joint or implants | 3% | 2% | 1.0000 | 3% | 0% | 1.0000 |
| Scarlet fever | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Sarcoidosis | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Premenstrual syndrome * | * | * | * | 24% | 0% | 0.0187 |
| Endometriosis * | * | * | * | 3% | 15% | 0.1450 |
| Hysterectomy * | * | * | * | 3% | 10% | 0.5492 |
| Hormone replacement therapy * | * | * | * | 9% | 10% | 1.0000 |
| Hyperogestrogenaemia (high oestrogen) * | * | * | * | 0% | 0% | 1.0000 |

* Female Only

† Two-tailed Fisher's exact test, $p < 0.05$

**Table 2.3.2** Part Two Summary of Diagnostic Conditions

| Diagnostic Condition | Part Two | | | | | |
|---|---|---|---|---|---|---|
| | All Participants (n = 56) | | | Female-only Participants (n = 39) | | |
| | ME/CFS (n = 28) | Control (n = 28) | | ME/CFS (n = 22) | Control (n = 17) | |
| | Percentage of YES responses from ME/CFS group | Percentage of YES responses from Control group | p-value † | Percentage of YES responses from ME/CFS group | Percentage of YES responses from Control group | p-value † |
| ME/CFS | 100% | 0% | < 0.0001 | 100% | 0% | < 0.0001 |
| Postural orthostatic tachycardia syndrome | 82% | 0% | < 0.0001 | 86% | 0% | < 0.0001 |
| Food intolerance/s | 75% | 11% | < 0.0001 | 82% | 18% | < 0.0001 |
| Irritable bowel syndrome | 50% | 0% | < 0.0001 | 55% | 0% | 0.0002 |
| Trouble eating/drinking milk/dairy products | 71% | 14% | < 0.0001 | 77% | 24% | 0.0013 |
| Fibrositis or fibromyalgia | 46% | 0% | < 0.0001 | 55% | 0% | 0.0002 |
| Tension headaches | 36% | 4% | 0.0052 | 41% | 0% | 0.0025 |
| Sinus problems | 36% | 4% | 0.0052 | 36% | 0% | 0.0056 |
| Dermatitis or any other skin problem | 39% | 7% | 0.0095 | 41% | 12% | 0.0733 |
| Low blood pressure | 25% | 0% | 0.0102 | 32% | 0% | 0.0124 |
| Clinical depression | 32% | 4% | 0.0116 | 41% | 6% | 0.0240 |
| Anxiety or other mental disorder | 36% | 7% | 0.0200 | 41% | 12% | 0.0733 |
| Joint hyperflexibility/mobility | 21% | 0% | 0.0232 | 27% | 0% | 0.0267 |
| Bronchitis | 29% | 4% | 0.0248 | 32% | 0% | 0.0124 |
| Food allergy/allergies | 43% | 14% | 0.0366 | 45% | 12% | 0.0365 |
| Tinnitus or ringing in the ears | 32% | 7% | 0.0403 | 36% | 6% | 0.0518 |
| Ear infection | 18% | 0% | 0.0515 | 18% | 0% | 0.1179 |
| Gastritis | 18% | 0% | 0.0515 | 18% | 0% | 0.1179 |
| Multiple chemical sensitivity | 18% | 0% | 0.0515 | 14% | 0% | 0.2429 |
| Glandular fever | 36% | 11% | 0.0550 | 32% | 0% | 0.0124 |
| Trouble eating or drinking sugar | 21% | 4% | 0.1012 | 27% | 6% | 0.1125 |
| Yeast disease or candidiasis | 14% | 0% | 0.1115 | 18% | 0% | 0.1179 |
| Streptococcal throat | 14% | 0% | 0.1115 | 9% | 0% | 0.4953 |
| Urinary tract infection/cystitis | 29% | 11% | 0.1771 | 36% | 12% | 0.1395 |
| Kidney infections | 18% | 4% | 0.1927 | 23% | 0% | 0.0565 |
| Migraine with aura | 18% | 4% | 0.1927 | 18% | 0% | 0.1179 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Low blood sugar or hypoglycaemia | 11% | 0% | 0.2364 | 14% | 0% | 0.2429 |
| Stomach or gastric ulcers | 11% | 0% | 0.2364 | 9% | 0% | 0.4953 |
| Anaemia | 11% | 0% | 0.2364 | 9% | 0% | 0.4953 |
| Iron deficiency anaemia | 21% | 7% | 0.2516 | 27% | 12% | 0.4260 |
| Asthma | 25% | 11% | 0.2955 | 23% | 6% | 0.2056 |
| Cold sores, herpes labialis | 29% | 14% | 0.3290 | 27% | 12% | 0.4260 |
| Cluster headaches | 14% | 4% | 0.3516 | 18% | 0% | 0.1179 |
| Your appendix removed | 4% | 14% | 0.3516 | 0% | 12% | 0.1835 |
| Psoriasis | 14% | 4% | 0.3516 | 18% | 6% | 0.3634 |
| Eye conditions with or without vision loss | 14% | 4% | 0.3516 | 18% | 6% | 0.3634 |
| Allergies | 32% | 18% | 0.3550 | 36% | 24% | 0.4940 |
| Migraine without aura | 18% | 7% | 0.4216 | 23% | 6% | 0.2056 |
| Concussion | 18% | 7% | 0.4216 | 14% | 0% | 0.2429 |
| Thyroid problems | 18% | 7% | 0.4216 | 18% | 12% | 0.6790 |
| Slipped disk or other back problems | 21% | 11% | 0.4688 | 23% | 12% | 0.4383 |
| Heart valve disease | 0% | 7% | 0.4909 | 0% | 12% | 0.1835 |
| A stroke | 0% | 7% | 0.4909 | 0% | 12% | 0.1835 |
| Cancers | 7% | 0% | 0.4909 | 9% | 0% | 0.4953 |
| A genetic (inherited) disease | 7% | 0% | 0.4909 | 9% | 0% | 0.4953 |
| Colitis/Crohn's disease | 7% | 0% | 0.4909 | 9% | 0% | 0.4953 |
| Yellow jaundice or hepatitis | 7% | 0% | 0.4909 | 9% | 0% | 0.4953 |
| Psychiatric illness | 7% | 0% | 0.4909 | 9% | 0% | 0.4953 |
| Pneumonia | 7% | 0% | 0.4909 | 5% | 0% | 1.0000 |
| Eczema | 25% | 14% | 0.5027 | 23% | 12% | 0.4383 |
| Physical/sexual/emotional abuse | 11% | 4% | 0.6110 | 14% | 0% | 0.2429 |
| Genital infections | 11% | 4% | 0.6110 | 9% | 0% | 0.4953 |
| Autoimmune disease | 11% | 4% | 0.6110 | 9% | 6% | 1.0000 |
| Fractured bone | 14% | 7% | 0.6695 | 14% | 0% | 0.2429 |
| High blood pressure | 11% | 18% | 0.7049 | 9% | 12% | 1.0000 |
| Rheumatic fever | 0% | 4% | 1.0000 | 0% | 6% | 0.4359 |
| Glaucoma | 0% | 4% | 1.0000 | 0% | 6% | 0.4359 |
| Injury to head or neck | 11% | 7% | 1.0000 | 9% | 0% | 0.4953 |
| Loss of hearing | 7% | 4% | 1.0000 | 9% | 0% | 0.4953 |
| Sexual problems | 7% | 4% | 1.0000 | 9% | 0% | 0.4953 |
| Hayfever | 25% | 21% | 1.0000 | 27% | 18% | 0.7042 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Type 1 diabetes | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Type 2 diabetes | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Gout | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Rheumatoid arthritis | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Epilepsy | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Leukaemia | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Lymphoma | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Lupus erythematosus | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Sjogren's syndrome | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Ehlers-Danlos syndrome | 4% | 0% | 1.0000 | 5% | 0% | 1.0000 |
| Hormone disorder | 4% | 0% | 1.0000 | 5% | 0% | 1.0000 |
| Heart attack/myocardial infarct | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Other neurological disorder | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Carpal tunnel syndrome | 4% | 0% | 1.0000 | 5% | 0% | 1.0000 |
| Meniere's disease | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Tuberculosis | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Malaria | 4% | 0% | 1.0000 | 5% | 0% | 1.0000 |
| Duodenal ulcers | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Cirrhosis of the liver | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Gall bladder problems | 4% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Gall bladder removed | 4% | 4% | 1.0000 | 5% | 6% | 1.0000 |
| Splenectomy | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Other gastrointestinal disorder | 4% | 0% | 1.0000 | 5% | 0% | 1.0000 |
| Arthritis or rheumatism | 7% | 7% | 1.0000 | 9% | 6% | 1.0000 |
| Disease of the hair or scalp including hair loss | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Haemochromatosis | 7% | 4% | 1.0000 | 5% | 6% | 1.0000 |
| Any other blood disorder | 4% | 0% | 1.0000 | 5% | 0% | 1.0000 |
| Subfertility | 4% | 4% | 1.0000 | 5% | 0% | 1.0000 |
| Suicide attempts | 4% | 0% | 1.0000 | 5% | 0% | 1.0000 |
| Drug abuse | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Alcohol abuse | 4% | 0% | 1.0000 | 5% | 0% | 1.0000 |
| Radiation therapy | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Prosthetic valve/joint or implants | 0% | 4% | 1.0000 | 0% | 0% | 1.0000 |
| Scarlet fever | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |
| Sarcoidosis | 0% | 0% | 1.0000 | 0% | 0% | 1.0000 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Eye disease/s or disorder/s | 7% | 7% | 1.0000 | 9% | 12% | 1.0000 |
| Premenstrual syndrome * | * | * | * | 27% | 0% | 0.0267 |
| Endometriosis * | * | * | * | 5% | 12% | 0.5703 |
| Hysterectomy * | * | * | * | 5% | 12% | 0.5703 |
| Hormone replacement therapy * | * | * | * | 14% | 6% | 0.6180 |
| Hyperogestrogenaemia (high oestrogen) * | * | * | * | 0% | 0% | 1.0000 |

* Female Only

† Two-tailed Fisher's exact test, $p < 0.05$

### 2.3.2 Other Participant Cohort Characteristics

Brief analyses were conducted to characterise the socio-economic demographic and activity profiles of the overall ME/CFS and Control group cohort recruited for this study. These questionnaire responses were not a primary focus for this thesis; however, the study did ask some questions to be able to give some indication of the participants' demographics, health status, and activity profile. The questionnaire for the study was based on forms previously used by McGregor et al., Bennett et al., Dr John Whiting, and Dr Don Lewis (materials obtained via personal communications). A copy of the questionnaire can be viewed electronically via Appendix 1. Table 2.3.3 summarises actions and changes that were undertaken in response to health status. Participants were asked to respond "yes" or "no" to these questions regarding if they had to take certain actions or make changes due to their health status. Table 2.3.4 tabulates the difficulties experienced in completing several everyday activities. Participants were asked to score on a scale of "no difficult" to "very difficult" the experience they had in doing the activities over the last seven days. The activities were individually examined, and responses were also tallied to give an overall score of all the activities combined. Table 2.3.3 and Table 2.3.4 showed a statistically significant difference when the overall ME/CFS (n = 40) and Control (n = 43) group participants were compared across several measures.

The responses to the Bell's Disability scale also indicated that 100% of the control and 16% of the ME/CFS participants were 50 or above on the scale, respectively. Conversely, the scale indicated that none of the control and 84% of the ME/CFS participants were 40 or below on the scale. The association between ME/CFS and Bell's disability scale is statistically significant (two-tailed Fisher's exact test, p-value < 0.0001 grouping patients either above or below 50 on the scale). In brief, 100 on the Bell's Disability scale is fully recovered, normal activity with no symptoms, 50 is able to do 4-5 hours a day of work or similar activity at home with daily rests required and symptoms are mostly moderate, 0 is bedridden constantly and unable to care for self. The responses to the scale were according to the participant in the last 7 days at the time of their sample collection and questionnaire completion for Part One.

**Table 2.3.3** Socio-demographic characteristics of all ME/CFS and Control participants (Part One)

| Actions due to health status | Part One (Overall Participant Cohort n = 83) | | | | p-value † |
|---|---|---|---|---|---|
| | **ME/CFS (n = 40)** | | **Control (n = 43)** | | |
| | YES responses from ME/CFS group | | YES responses from Control group | | |
| | # | % | # | % | |
| Change employment/occupation | 35 | 87.5 | 0 | 0 | < 0.0001 |
| Seek financial assistance | 30 | 75 | 0 | 0 | < 0.0001 |
| Change living arrangements | 20 | 50 | 0 | 0 | < 0.0001 |
| Seek assistance or make arrangments for extra care | 31 | 77.5 | 1 | 2.33 | < 0.0001 |
| † Two-tailed Fisher's exact test, p < 0.05 | | | | | |

**Table 2.3.4** Difficulty in completing activities due to health condition.

| Difficulty experienced in completing these activities | Part One (Overall Participant Cohort n = 83) | | | | p-value † |
|---|---|---|---|---|---|
| | **ME/CFS (n = 40)** | | **Control (n = 43)** | | |
| | Mean | Std. Dev. | Mean | Std. Dev. | |
| Overall of all these activities | 5.36 | 2.28 | 0.33 | 0.81 | < 0.0001 |
| Brush or comb hair | 0.24 | 0.27 | 0.02 | 0.04 | < 0.0001 |
| Walk continuously for 20 minutes | 0.74 | 0.30 | 0.03 | 0.11 | < 0.0001 |
| Prepare a homemade meal | 0.65 | 0.31 | 0.04 | 0.11 | < 0.0001 |
| Vacuum, scrub or sweep floors | 0.75 | 0.29 | 0.04 | 0.11 | < 0.0001 |
| Lift and carry a bag full of groceries | 0.61 | 0.29 | 0.04 | 0.12 | < 0.0001 |
| Climb one flight of stairs | 0.57 | 0.33 | 0.04 | 0.12 | < 0.0001 |
| Change bed sheets | 0.66 | 0.32 | 0.03 | 0.11 | < 0.0001 |
| Sit in a chair for 45 minutes | 0.42 | 0.35 | 0.06 | 0.14 | < 0.0001 |
| Go shopping for groceries | 0.72 | 0.3 | 0.03 | 0.10 | < 0.0001 |
| † t-test, p < 0.05 | | | | | |

## 2.4 Data and Biological Sample Collection from Participants

### 2.4.1 Part One – questionnaires, faecal and urine sample collection

The home collection kit came as a polyurethane foam insulated container including gel freeze ice packs, sample collection components, the participant information and consent form, and the questionnaires. Contents from this kit formed Part One of the study. Participants could peruse the materials first to ensure that they were familiar with all the requirements. Participants were asked to observe the following requests:

- Complete the consent form and Self-Report questionnaire before collecting samples.
- Complete the Symptom Questionnaire and collect the faecal and urine samples as close as possible to each other in time as this component was time sensitive.
- Ideally, collect the faecal and urine samples at the same time point, but no more than 12 hours apart from each other.
- Document their diet, medications, and/or supplements in the questionnaire although not required to cease or change anything for the project.
- Be adequately hydrated (water) to facilitate an easier collection experience and be able to produce sufficient sample quantities.
- Female participants were asked to refrain from collecting samples during menstruation to avoid cross-contamination with the different sample types.
- Promptly return their completed collection kit without delay (time and temperature sensitivity) to Bioscreen Laboratory

Urine samples were collected using a 70 mL specimen container, a Greiner Bio-one urine transfer device (part reference: 450251), and 10 mL urine preservative-free Greiner Bio-One Vacuette tubes (part reference: 455007). Faecal samples were collected using a 70 mL specimen container with a hole drilled into the lid (to aid anaerobiosis), and BD GasPak™ EZ Anaerobe Gas Generating Pouch System with Indicator (part reference: 260683). The kits arrived at the laboratory no more than 48 hours after paperwork completion and sample collection. If samples had been collected the night before, samples were kept cool in the fridge until they went to the post office. Frequent communication and check-ins with the participants during recruitment and sample collection, and postal tracking aided compliance.

Upon receiving the kit, Bioscreen laboratory staff performed quality control checks and completed the administrative requirements for the samples, questionnaires, and paperwork. Returned kits were checked to ensure that they had a cool internal temperature (<12°C), gel packs remained frozen during transit, the faecal sample collection bag was properly sealed indicating anaerobiosis, and all documentation had been completed. If the quality control checks failed, the samples were discarded, participants were contacted to inform them of the outcome, and arrangements were made for a re-collection and an update of the time-sensitive questionnaire responses. The administrative requirements also involved the staff member logging, coding, and de-identifying the samples, questionnaires, and paperwork before handing them over to AJK. AJK handled coded samples that were in random order during all sample preparation and experimental procedures; these measures kept AJK unbiased and from drawing early conclusions. The participant codes and metadata were matched once experimental outcomes were ready for data analysis.

The questionnaires were manually transcribed, tabulated, and used for data analysis (Section 2.3). The faecal samples were prepared into a homogenate (Section 2.5.1) and immediately used for the faecal culture and MALDI-TOF MS experiment (Section 2.5.2). A portion of the neat faecal sample was transferred and stored in a clean polypropylene plastic tube at -80°C for future DNA extraction and molecular-based experiments (Section 2.5.3). Section 2.5 details the gut microbiology experimental workflow used with the faecal samples. Another portion of the neat faecal sample was transferred into a polypropylene plastic tube and temporarily (no more than 24 hours) stored in the refrigerator for faecal dry weight determination (Section 2.7). The urine sample and remainder of the faecal homogenate were aliquoted and stored in clean polypropylene plastic cryogenic tubes at -80°C for metabolomic experiments (Section 2.8). All this sample processing and preparation was completed within 24 hours of the kit being returned and received at the laboratory.

### 2.4.2 Part Two – blood plasma and urine sample collection

Part Two was optional for participants (Section 2.1). Participants were asked to be adequately hydrated (water) to facilitate an easier collection experience and be able to produce sufficient sample quantities. Female participants were asked to refrain from collecting during menstruation to avoid sample cross-contamination. At the Melbourne Pathology collection room, participants were asked to provide a blood sample and a urine sample from the same time point (no more than 30 minutes apart from each other). Whole blood was drawn and collected into two grey 4 mL FE Sodium Fluoride/K3EDTA Greiner Bio-One Vacuette tubes (part reference: 454033) by a phlebotomist. Participants collected their urine into a 70 mL specimen container. These blood and urine samples were immediately taken to the Bioscreen laboratory in cool conditions.

Like the process for Part One, laboratory staff completed the administrative requirements for the samples (including blinding) before handing them over to AJK (Section 2.4.1). These coded samples were handled within four hours of being collected and returned to the laboratory. The blood collection tubes were centrifuged for 10 minutes at 1450 × g. Following centrifugation, the resulting plasma supernatant was transferred, apportioned into clean polypropylene plastic cryogenic tubes, and stored at -80°C. The urine sample was first transferred from the container into 10 mL urine preservative-free Greiner Bio-One Vacuette tubes (part reference: 455007) then apportioned into clean polypropylene plastic cryogenic tubes, and stored at -80°C. The blood plasma and urine sample aliquots were used for metabolomic experiments (Section 2.8).

## 2.5 Gut Microbiology Experiments

Two approaches, a culture-based, and a culture-free/molecular-based, were used to analyse the gut microbiome in the faecal samples (Section 2.4.1). The faecal homogenate was first prepared by AJK to set up the culture and matrix-assisted laser desorption ionization time-of-flight Mass Spectrometry (MALDI-TOF MS) experiment (Sections 2.5.1 and 2.5.2). The counting and reading of the agar plates, MALDI-TOF MS instrument loading, and final bacterial identification reporting were conducted by a nominated Bioscreen laboratory scientist. This staff member completed this work for all participant samples, maintaining consistency in plate counting and reading, and reporting of outcomes. This staff member was not involved in handling the return of the collection kits to the laboratory and was blinded from identified participant information (Section 2.4.1). A portion of the neat faecal sample was used for 16S rRNA gene amplicon sequencing. Section 2.5.3 details the molecular-based work that was involved in this technique; this work was completed by AJK across Bioscreen and Victoria University laboratories.

### 2.5.1 Faecal Homogenate Preparation

An anaerobic bio-safety cabinet was not available. Oxygen exposure was minimised by preparing the faecal homogenate and setting up the culture and MALDI-TOF experiment in < 45 minutes. The faecal sample was not removed from the BD GasPak™ EZ Anaerobe Gas Generating Pouch System with Indicator (part reference: 260683) zip-lock bag until the homogenate was ready to be made. Four grams of the neat sample were immediately transferred to 40 mL of freshly prepared phosphate-buffered saline (PBS) in a compatible IKA Ultra Turrax tube. The PBS was prepared using the standard recipe for PBS tablets (Sigma Life Science P4417) and autoclaved less than 24 hours before use. The same PBS was used as the diluent for the serial dilutions used in the culture and MALDI-TOF MS preparation. The neat sample was homogenised using an IKA Ultra Turrax® tube disperser workstation system and centrifuged at 15 × g for 10 minutes to make a homogenate that could be pipetted without blockage (very low centrifuge speed intended to prevent potential sample damage and disturbing the microbes). The remaining homogenate material that was not used for the faecal culture and MALDI-TOF MS experiment was immediately aliquoted and stored in cryogenic tubes at -80°C; these were used in the metabolomics experiments (Section 2.8).

### 2.5.2 Faecal Culture (Microbiology) and MALDI-TOF MS

This methodology was adapted from protocols previously described (Armstrong et al., 2017; Wallis et al., 2016); see also Section 2.1. The PBS and 4 g in 40 mL faecal homogenate prepared in Section 2.5.1 were immediately used to set up eleven 1:10 serial dilutions (undiluted to $10^{-11}$) for the culture and MALDI-TOF MS experiment. The first step of the dilution series (lowest dilution, most concentrated) was prepared by transferring 1 mL of the faecal homogenate to 9 mL of PBS diluent. Subsequent steps of the dilution series were prepared by transferring 1 mL of the sample mixture to 9 mL of PBS diluent. The sample tubes were gently inverted three times and vortexed (two seconds) to mix in between each transfer.

A variety of whole standard-sized (90 mm diameter) agar-based medium plates were used to enumerate bacteria. Plates were purchased from Edwards, Australia, a specialist microbiology media supplier. Anaerobic plates were pre-reduced in anaerobic jars 24 hours before use; anaerobic plates were spread and prepared for incubation first before the aerobic plates to minimise oxygen exposure. One whole plate was spread with 100 µL for each respective dilution step being assessed. Plates were spread as soon as the 100 µL was transferred onto the agar preventing the sample from unevenly drying and setting in one spot. Dilutions $10^{-6}$ to $10^{-11}$ inclusive were transferred on pre-reduced Columbia horse blood agar for anaerobic incubation. Dilutions $10^{-4}$ to $10^{-9}$ inclusive were transferred onto pre-reduced Raka-Ray with cycloheximide agar for anaerobic incubation. Dilutions $10^{-1}$ to $10^{-7}$ inclusive were transferred onto Columbia horse blood agar, chromogenic medium agar, and Columbia colistin and nalidixic acid horse blood agar for aerobic incubation. Dilutions $10^{-1}$ and $10^{-2}$ were transferred onto chloramphenicol-gentamicin selective Sabouraud dextrose agar for aerobic incubation. All anaerobic plates were incubated for 4 days (96 hours) using the BD Gas Pak™ EZ Anaerobe Container System with Indicator (part reference: 260001). All aerobic plates were incubated for 2 days (48 hours).

A stereomicroscope was used to examine aerobic and anaerobic culture plates before bacterial identification. Colonies from each agar medium were microscopically examined and the colony/viable counts were quantified for each plate. Similar morphotypes were sub-cultured onto Columbia horse blood agar in case it was required for re-identification by MALDI-TOF MS. Counts were performed and calculated depending on the dilution factor plated. While plates were being counted and examined, index bacterial colonies were transferred to a target

polished steel plate (MSP 96, Bruker Daltonics, Germany). Each of the samples on the target plate was subjected to protein extraction with 1 µL of 70% formic acid (Sigma) and left to air dry. The target plate was then overlaid with 1 µL of matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid [HCCA] in a mixture of 47.5% ultra-pure water, 2.5% trifluoroacetic acid, and 50% acetonitrile) and left to air dry again. The prepared and dried target plate was analysed using a Microflex MALDI-TOF mass spectrometer (Bruker Daltonics, Germany) equipped with a 60 Hz nitrogen laser. Spectra were recorded in the positive linear mode for the mass range of 2,000-20,000 Da at maximum laser frequency. The raw spectra were automatically analysed without user intervention using the default settings of the MALDI Biotyper 3.0 software package (Bruker, Daltonics, Germany). The software contained a proprietary peptide database that can detect up to approximately five thousand species. The classification results from the software were assigned to the bacteria counted providing the final reporting outcome of the faecal bacteria identified and quantified. Bacterial counts via culture MALDI-TOF MS were corrected to faecal sample dry weight (Section 2.7) before results were used for further statistical analyses and visualisation (Section 2.6.5) and utilising MiMeDB (Section 2.6.8).

### 2.5.3 DNA Extraction for 16S rRNA Illumina Sequencing

Faecal bacterial communities were also profiled by 16S rRNA gene amplicon sequencing on the Illumina MiSeq platform. This required a 16S rRNA library to be constructed with DNA sample extracts and instrument-specific adapters attached so that it could be sequenced on the platform. Microbial genomic DNA extracts were prepared using the portion of the neat faecal sample that had been stored at -80°C immediately after the homogenate preparation and set-up were completed for the culture and MALDI-TOF MS experiment (Section 2.5.1 and Section 2.5.2, respectively). DNA extractions were performed in batches of 12 samples using a QIAcube instrument (Qiagen). The QIAcube automated the manual spin-column-based aspect of compatible sample preparation kits for up to 12 samples in a single run. The Qiagen QIAamp® Fast DNA Stool Mini Kit (Category Number 51604) was used with the QIAcube according to the manufacturer's standard instructions. The DNA extractions were stored at -20°C until all samples were ready for DNA quantification and quality checking (Section 2.5.4). This step was conducted before using the DNA extracts for library preparation and MiSeq instrument loading (Section 2.5.5).

### 2.5.4 DNA Quantification and Quality Check Before 16S rRNA Illumina Sequencing

DNA concentrations were measured fluorometrically on a Qubit® 1.0 fluorometer device using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Q32851). The fluorescence readouts determined the quantity of DNA from each sample that was used for preparing the sequencing library (Section 2.5.5). The Illumina protocol required 2.5 µL of microbial DNA (5 ng/µL) from each sample to be used as the template for amplicon PCR (Illumina, 2013). The fluorescence readouts were used to normalise the samples to this specified volume and concentration. Samples with concentrations above 5 ng/µL were diluted with 10 mM Tris pH 8.5. For samples with concentrations below 5 ng/µL, a maximum 5 µL cut-off was used to maximise the concentration but not compromise the total volume of the final pooled library. This was a minor deviation from the Illumina protocol (Illumina, 2013). DNA quality and presence were also determined by 0.8% agarose gel electrophoresis in a 1X TAE (Tris-acetate-EDTA) buffer. The gel was visualised on a BioRad ChemiDoc™ MP imaging instrument using SYBR® Safe DNA Gel Stain (Thermo Fisher Scientific, S33102), Blue/Orange Loading Dye 6X (G190A, Promega), and 100 bp DNA ladder (G210A, Promega). PCR-grade water was used as a blank control.

### 2.5.5 Library Preparation for 16S rRNA Gene Amplification and Illumina Sequencing

A sequencing library was prepared according to a standard Illumina protocol (Part #15044223 Rev B), targeting the variable V3 and V4 regions of the 16S rRNA gene (Illumina 2013). The full-length primers suggested by the protocol were ordered from IDT® Integrated DNA Technologies using standard IUPAC nucleotide nomenclature (Klindworth et al., 2013). The full-length primers included the Illumina adapter overhang nucleotide sequences and the gene-specific sequences. The amplicon forward primer targeting this region used was 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3'. The amplicon reverse primer targeting this region used was = 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'.

The amplicon (first stage) polymerase chain reaction (PCR) reaction was set up according to the composition in Table 2.5.1. PCR on a BioRad T100 Thermal Cycler was performed with these conditions: one cycle of initial denaturation at 95°C for 3 minutes, followed by 25 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 30 seconds, a final extension at 72°C for 5 minutes, and then hold at 4°C. The PCR product size after amplicon PCR was checked by 2% agarose gel electrophoresis in a 1X TAE buffer and was determined to be approximately 550 base pairs (bp). The same approach as detailed in Section 2.5.4 was used to visualise the agarose gel.

**Table 2.5.1** Composition of the amplicon (first stage) PCR reaction per sample

| **Component** | **Volume (µL)** |
|---|---|
| Microbial DNA (Section 2.5.4) | 2.5 |
| Amplicon PCR Forward Primer 1 µM | 5 |
| Amplicon PCR Reverse Primer 1 µM | 5 |
| 2 X KAPA HiFi HotStart Ready Mix (Kapa Biosystems, Inc., Reference KK2602 07958935001) | 12.5 |
| TOTAL | 25 |

The PCR products were cleaned and purified using Agencourt AMPure XP Reagent magnetic beads (Beckman Coulter Genomics, Part Number A63881). The magnetic beads purified the amplicon product away from free primers and primer dimer species. The products from the

amplicon (first) PCR stage were used as the template for indexing (second) PCR. To be able to sequence the amplicon, dual indices and Illumina sequencing adapters were attached using the Illumina Nextera® XT indexes (Nextera XT Index Kit V2 Set A, Reference 15052163). The indexing (second) PCR reaction was set up according to the composition in Table 2.5.2. Indexing PCR on a BioRad T100 Thermal Cycle was performed with these conditions: one cycle of initial denaturation at 95°C for 3 minutes, followed by eight cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 30 seconds, a final extension at 72°C for 5 minutes, and then hold at 4°C. The PCR products after indexing were cleaned again using the AMPure XP magnetic beads before fluorometric quantification was conducted to construct the final pooled library. The PCR product size after index PCR was checked by 2% agarose gel electrophoresis in a 1X TAE buffer and the average library size was determined conservatively to be ~580 bp. The same approach as detailed in Section 2.5.4 was used to visualise the agarose gel.

**Table 2.5.2** Composition of the index (second stage) PCR reaction per sample

| **Component** | **Volume (µL)** |
|---|---|
| DNA (product from amplicon first-stage PCR) | 5 |
| Nextera XT Index Primer 1 (N7xx) | 5 |
| Nextera XT Index Primer 2 (S5xx) | 5 |
| 2 X KAPA HiFi HotStart Ready Mix (Kapa Biosystems, Inc., Reference KK2602 07958935001) | 25 |
| PCR-grade water | 10 |
| TOTAL | 50 |

The libraries (amplicons from the individual samples with unique adapters attached) were quantified fluorometrically ahead of normalising and pooling the libraries for MiSeq loading. The DNA concentrations from each library were measured on a Qubit® 1.0 fluorometer device using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Q32851). A few of the libraries were below the 4 nM concentration that the Illumina protocol suggested. As a workaround, instead of normalising the final libraries to 4 nM before pooling, they were normalised to 5 nM with 10 mM Tris pH 8.5. The respective volumes from each library were transferred to make a pooled library stock that was adjusted with the cumulative total volume of 10 mM Tris pH 8.5 diluent (all indexed amplicon samples together in one 1.5 mL microcentrifuge tube). To ensure that there was a sufficient volume of the library to transfer from the completed second PCR

clean-up step, the total volume of each normalised individual library stock was capped at 11 µL. For libraries that were below 5 nM, a maximum of 11 µL was transferred to the pooled library stock.

Quantification of the pooled library using quantitative polymerase chase reaction (qPCR) was done using a commercial kit (KAPA Library Quantification Kit, Illumina Platforms, KK4824). Following the manufacturer's instructions, dilution series were made from the pooled library and run in parallel with the standards provided in the kit. A set of dilutions was made from the pooled library stock with 10 mM Tris pH 8.5 diluent (1:1,000, 1:10,000, 1:100,000) and compared against six standards (respective concentrations of 20, 2, 0.2, 0.02, 0.002, and 0.0002 pM). PCR-grade water was used as a no-template control. All samples and standards were run in triplicates. The BioRad CFX96 Real-Time System was programmed to run qPCR with the following conditions: one cycle of initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing/extension/data acquisition at 60°C for 45 seconds, then hold at 4°C. The KAPA Library Quantification Data Analysis template was used to calculate the concentration of the undiluted pooled library with the quantitation cycle (Cq) values from the qPCR results. The concentration of the undiluted pooled library was determined by qPCR to be 3.5 nM.

The pooled (sample) library was spiked with 10% PhiX Control, a proprietary-generated control library (Illumina, Reference FC-110-3001). The libraries were denatured, diluted, and combined before they were loaded onto the MiSeq for sequencing (Illumina, 2013, 2019). The MiSeq was loaded with 600 µL of the combined library that had a final overall concentration of 8 pM. The details of the denaturing and dilution calculations can be found in Appendix 3. The final prepared library was sequenced in a 2 x 300 bp paired-end run on the MiSeq with a 600-cycle proprietary reagent kit (Illumina MiSeq Reagent Kit v3, Reference MS-102-3003). The MiSeq instrument was programmed to operate according to standard Illumina settings.

### 2.5.6 Bioinformatic Processing of 16S rRNA Sequences with QIIME2

After the MiSeq sequencing run was completed, the instrument generated FASTQ files containing demultiplexed raw sequencing data of the 16S rRNA gene amplicons. The demultiplexing step separated the sequenced data from the PhiX and the indexes that uniquely tagged the different samples. The FASTQ files initially required translation into a result format that is compatible and usable for downstream analytical and visualisation requirements (Hall & Beiko, 2018; Rai et al., 2021). There are several commonly used bioinformatic platforms and computational tools, including Quantitative Insights into Microbial Ecology Version 2 (QIIME2), to process FASTQ files; however, there is no single gold-standard option for this task (Kang et al., 2021; Marizzoni et al., 2020; Prodan et al., 2020). QIIME2 is a widely used next-generation microbiome bioinformatics platform that is extensible, free, open source, and community-developed; it enables the processing and analysis of raw sequencing data to provide insight into the microbial environment of interest (Bolyen et al., 2019; Estaki et al., 2020; Hall & Beiko, 2018; Rai et al., 2021). The choice to use QIIME2 was also based on its frequent use in 16S rRNA amplicon data analyses, other gut microbiome studies, and the local assistance that was available (Bolyen et al., 2019; Estaki et al., 2020; Giloteaux, Goodrich, et al., 2016; Rai et al., 2021; Simpson et al., 2021).

The FASTQ files (forward and reverse sequences) were quality filtered, trimmed, denoised, and merged using QIIME2 (Version 2021.8) and other computational tools that are part of the platform (q2-plugins) (Bolyen et al., 2019). These processes, along with taxonomic assignment and generation of a phylogenetic tree, also performed in QIIME2, produced the final representative amplicon sequencing variants (ASVs) and outputs that were used downstream in R. QIIME2 was installed and operated in a high-performance computing Conda environment via a Linux workstation (Ubuntu 20.04.4 LTS). The script that was used to run QIIME2 can be found in Appendix 4. Briefly, the demultiplexed forward and reverse reads (FASTQ files) were imported and the primers were removed using the q2-cutadapt plugin (Martin, 2011). DADA2, as a q2-plugin, was used for quality-score-based filtering, denoising, and merging of the input sequences (Callahan et al., 2016). DADA2 is a software package that models and corrects Illumina-sequence amplicon errors (Callahan et al., 2016). It was also used to construct the feature table which contained the counts of each unique sequence of each sample. Since DADA2 was used, “ASV” instead of Operational Taxonomic Units or OTUs is a more fitting terminology to describe the processed sequences (Chiarello et al., 2022). These DADA2

parameters were used after the primers were trimmed from the sequences: –p-trunc-len-f 270 –p-trunc-len-r 220 –p-max-ee-f 3 –p-max-ee-r 3. Taxonomy was then assigned using the sklearn feature-classifier q2-plugin against a trained SILVA (version 138) classifier (Bokulich et al., 2018; Pedregosa et al., 2011; Quast et al., 2013; Robeson et al., 2020). ASVs that were observed fewer than two times (singletons) were removed from the dataset before exporting outputs required for statistical analyses and visualisation (Section 2.6.1) and running PICRUSt2 (Section 2.6.3).

## 2.6 Visualisation and Analyses of Gut Microbiology Data

### 2.6.1 Phyloseq and R Studio with 16S rRNA Data

There are several approaches and opinions on 16S rRNA amplicon sequencing processing and data analysis which can make workflows complicated (Allaband et al., 2019; Cameron et al., 2021; Gloor et al., 2017; McMurdie & Holmes, 2014; Nearing et al., 2022; Schmidt et al., 2022; Weiss et al., 2017). A major reason for this is because of the differences in library sizes and how the sequencing instruments fundamentally operate; therefore, these challenges come with the territory of using next-generation sequencing techniques. The approach taken in this study was informed by a combination of workflows from other laboratory groups, common practices in this area, and awareness of the inherent compositional nature of 16S rRNA gut microbiome datasets. If the wrong assumptions about the data are made, this can have inappropriate consequences with normalisation and data analysis strategies, thereby affecting downstream outcomes and interpretation (Cameron et al., 2021; Gloor et al., 2017; Schmidt et al., 2022). As such, both the non-rarefied and rarefied 16S rRNA Phyloseq objects were used based on the data characteristics and normalisation caveats associated with each metric and analysis undertaken (Sections 2.6.2, 2.6.3, 2.6.3).

Library sizes (i.e., the total number of sequencing reads within a sample) often vary over several ranges of magnitude, even within a single sequencing run, and the data contains many zeros with a non-gaussian distribution. The disparity in library sizes between samples does not represent actual differences in microbial communities and means that libraries cannot be directly compared. Further, microbiome datasets collected by next-generation sequencing of 16S rRNA amplicons are compositional because they have an arbitrary total imposed by the instrument. To mitigate some of these issues, data are often normalised by various computational processes before downstream analysis. Rarefaction is a widely used normalisation tool for amplicon sequencing data to allow for sample comparison without associated bias from differences in library size; it is accomplished by reducing the number of observations to a size threshold shared among the samples of interest (Cameron et al., 2021; Weiss et al., 2017). However, rarefaction is the subject of considerable debate and statistical criticism as it introduces artificial variation to the data by omitting a random subset of observed sequences and potentially also necessitates discarding samples with library sizes deemed too small (Cameron et al., 2021; Gloor et al., 2017; McMurdie & Holmes, 2014). While rarefying

data is not an ideal normalisation method, it is accepted practice for data analytical approaches, particularly for alpha diversity measures (Cameron et al., 2021; Weiss et al., 2017; Willis, 2019).

The performance of beta diversity measures and differential abundance analyses (Section 2.6.3) are affected by rarefaction to varying extents (Cameron et al., 2021; Nearing et al., 2022; Weiss et al., 2017; Wong et al., 2016). Although rarefying and data normalisation can be problematic, it is important to keep in mind that there are no perfect models of microbiome data, and therefore, a selection of analytical approaches should be used (Cameron et al., 2021; Nearing et al., 2022; Wong et al., 2016). Further, alternatives to rarefying have only recently been sufficiently developed (Weiss et al., 2017). The authors of the UniFrac method have suggested that rarefying more clearly clusters samples according to the biological origin than other normalisation techniques do for metrics based on presence or absence (i.e., unweighted UniFrac) (Wong et al., 2016); therefore, this metric is often calculated from rarefied data. As weighted UniFrac takes into abundance of different taxa, it is not as sensitive to rarefaction and the common practice uses non-rarefied data transformed to relative abundance (Gloor & Reid, 2016; Wong et al., 2016). However, it is argued that the use of relative abundances to represent compositional data is also problematic as it is constrained by the simplex (sum to 1) and is not free-floating in the Euclidean space (Gloor & Reid, 2016; Weiss et al., 2017). Various transformations for compositional data analyses have been considered to address this issue and often, the centred log-ratio transformation (CLR) of non-rarefied data is used (Gloor et al., 2017; Gloor & Reid, 2016). The CLR transformation was introduced by Aitchison in 1986; its values are scale-invariant meaning that the same ratio is expected to be obtained in a sample with few read counts or an identical sample with many read counts (Gloor et al., 2017; Gloor & Reid, 2016). In light of these analytical considerations, this thesis has explored weighted and unweighted Unifrac and Aitchison distances to measure beta diversity between the comparative groups of interest.

In R Studio (Version 4.2.0 and 4.2.1), Phyloseq (version 1.42.0) was used to create an object (a type of electronic data format for working with large amounts of data in R). This object contained the participant metadata of the Part One cohort (Sections 2.1 and 2.3) and the outputs from QIIME2 (SILVA taxonomy classification table, DADA2 feature table with the representative ASVs, and phylogenetic tree as described in Section 2.5.6). Phyloseq, with other R packages in R Studio, was used to handle, store, conduct and visualise statistical analyses (McMurdie & Holmes, 2013). The R scripts used can be found in Appendix 5. The object was filtered to

remove all non-bacterial sequences (mitochondria or chloroplasts), as well as ASVs that were unassigned at the phylum level. This final filtered Phyloseq object contained 5894 ASVs (features) and was used for all subsequent analyses related to the 16S rRNA dataset (Sections 2.6.2, 2.6.3, and 2.6.3). Figure 2.6.1 and Figure 2.6.2 shows an overview of the ASV and sequencing read counts from the non-rarefied object. Rarefaction without replacement was performed on the filtered Phyloseq object with the lowest sequencing depth, 7044 sequences per sample (Cameron et al., 2021; Willis, 2019). The rarefied data retained 3958 ASVs. Figure 2.6.3 (individual samples from Part One) and Figure 2.6.4 (ME/CFS overall vs Control group Part One) show the rarefaction curves of the non-rarefied data. Figure 2.6.5 (individual samples from Part One) and Figure 2.6.6 (ME/CFS overall vs Control group Part One) show the rarefaction curves of the rarefied data. Both the rarefied and non-rarefied data in their respective Phyloseq objects to reflect Comparisons A, B, C, and D (Part One) were used with the analyses in Sections 2.6.2, 2.6.3, and 2.6.4.

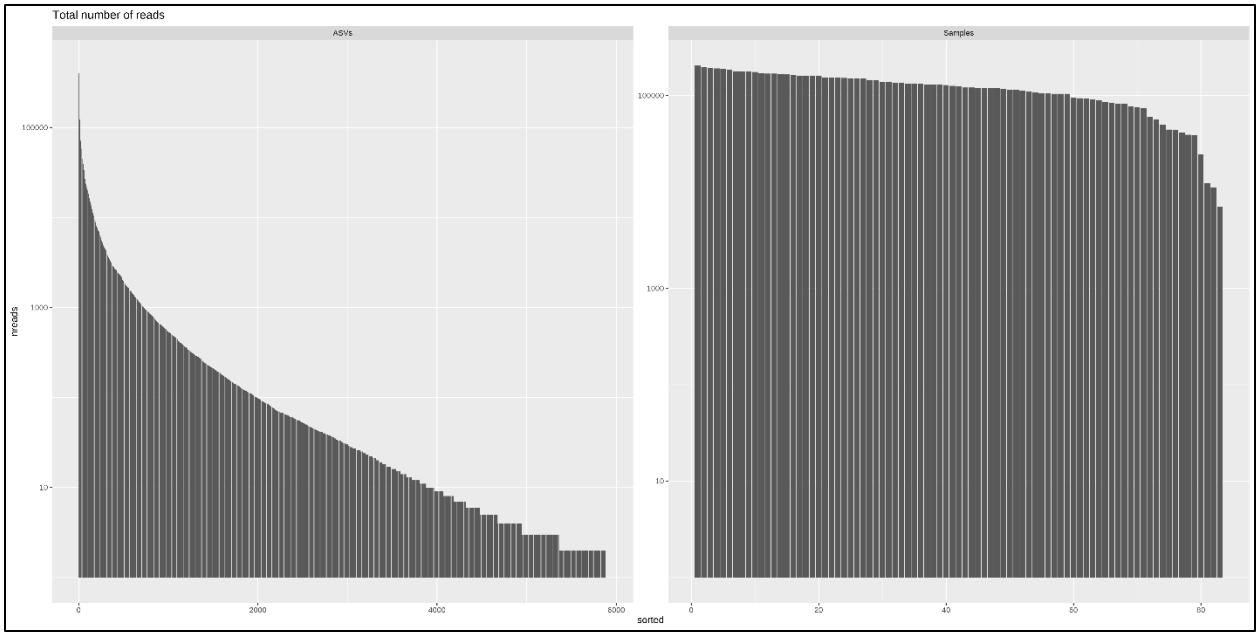



**Figure 2.6.1** Overview of the number of reads per Amplicon Sequence Variant (ASV), and per all the samples in Part One

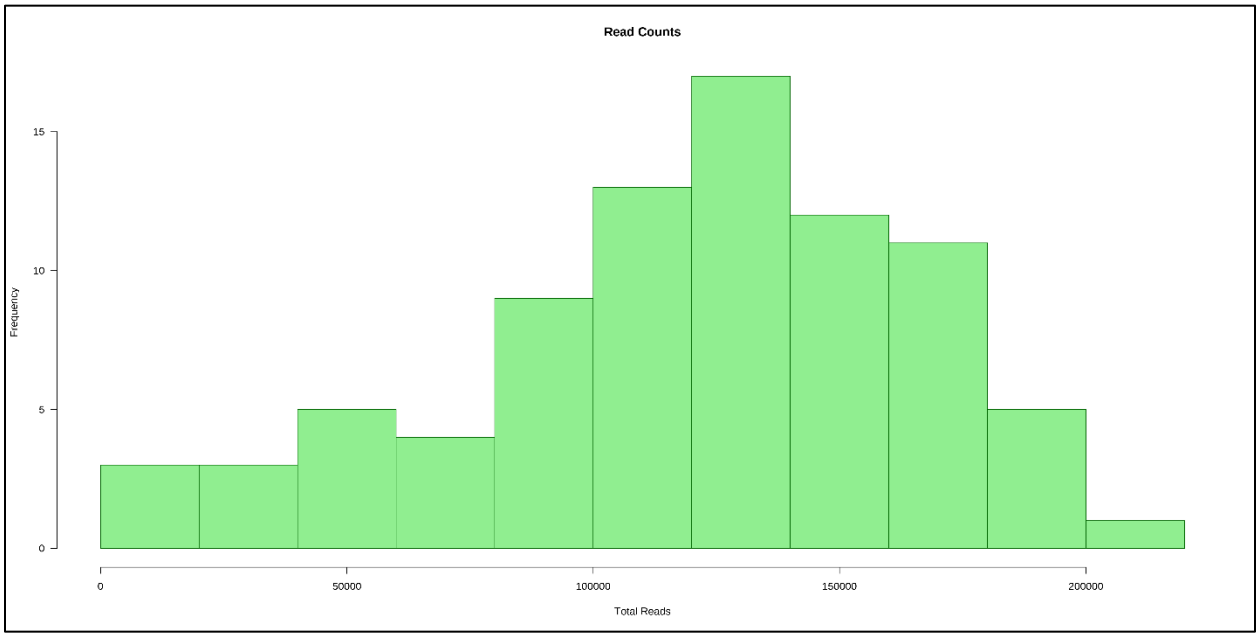



**Figure 2.6.2** Histogram of the distribution of the total read count from all the samples in Part One

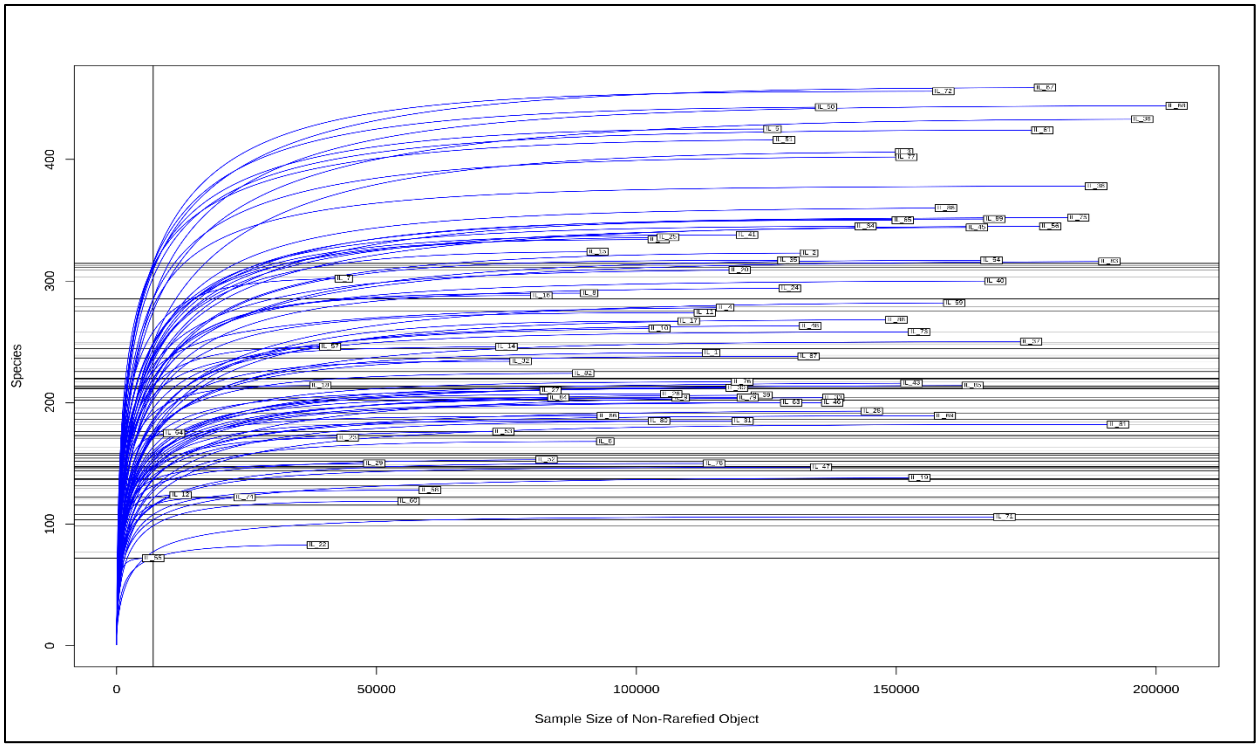



**Figure 2.6.3** Rarefaction curves for all samples in the Part One dataset (n = 83) before rarefaction

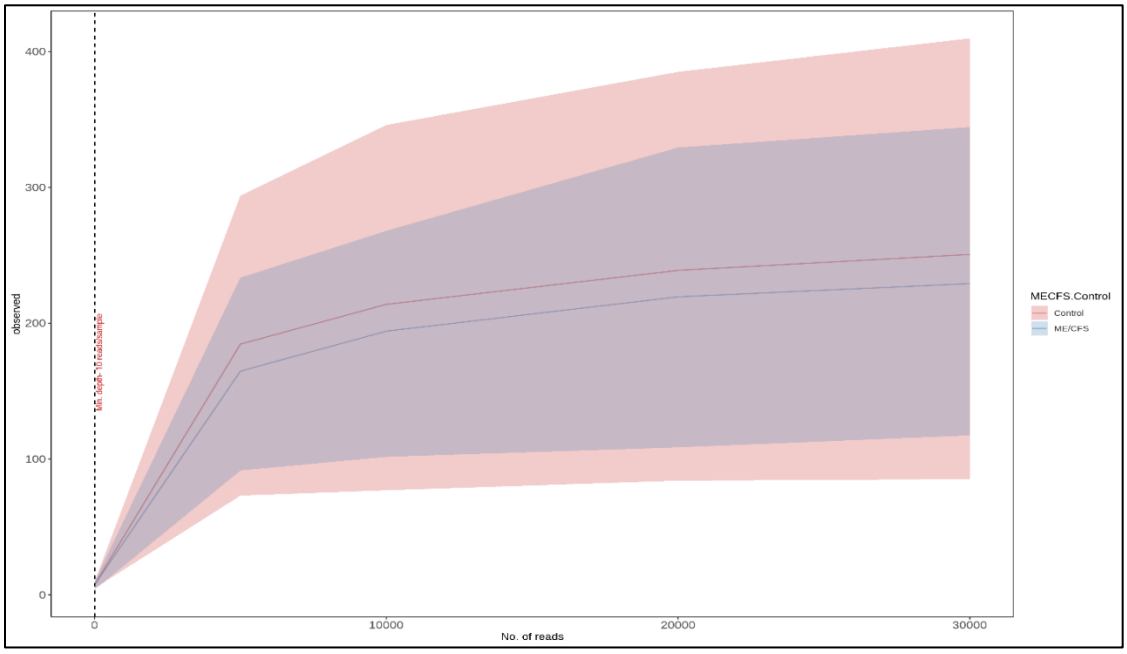



**Figure 2.6.4** Rarefaction curves of ME/CFS overall (n = 40) vs. Control (n = 43) groups in Part One prior to rarefaction

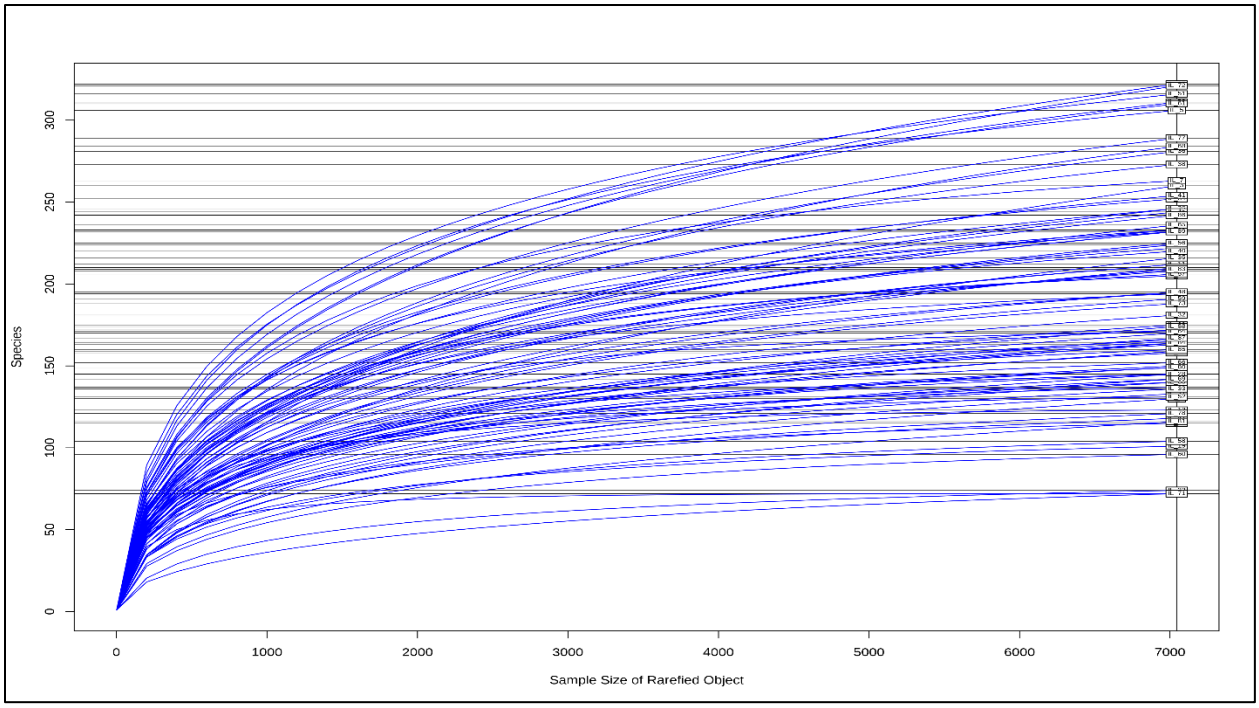



**Figure 2.6.5** Rarefaction curves for all samples in the Part One dataset (n = 83) rarefied to even sequencing depth without replacement

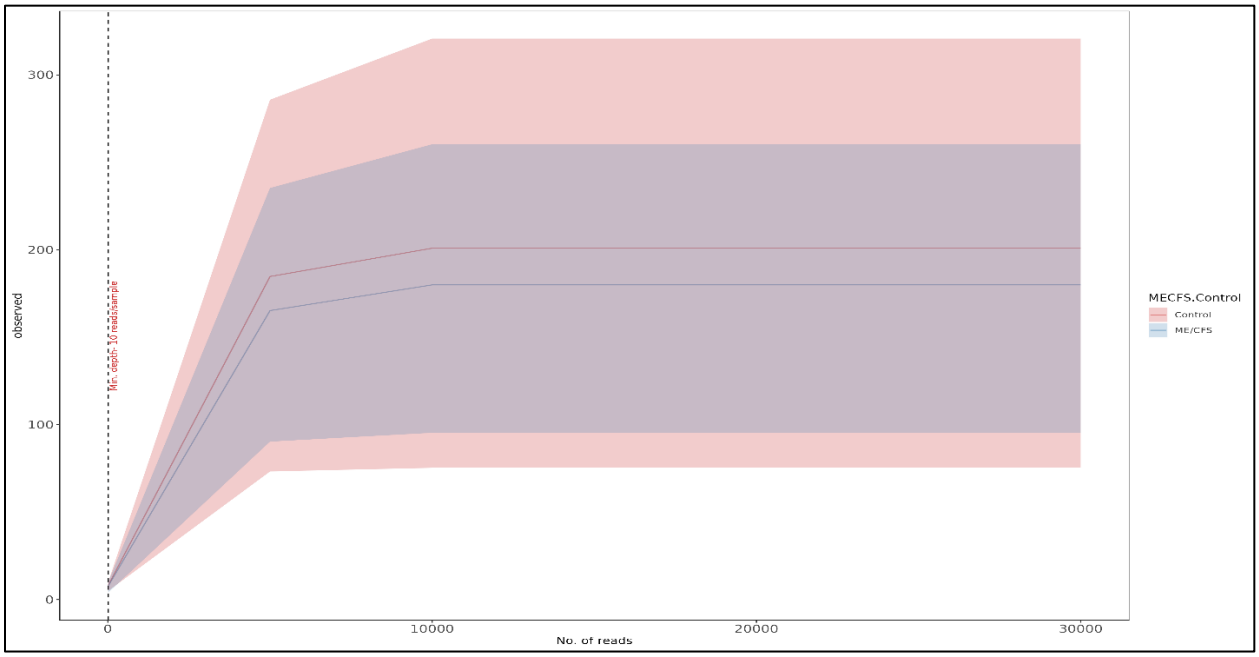



**Figure 2.6.6** Rarefaction curves of ME/CFS overall (n = 40) vs. Control (n = 43) groups in Part One rarefied to even sequencing depth without replacement

### 2.6.2 Composition and Diversity Analysis with 16S rRNA Data

The following compositional and diversity analyses were applied to Comparisons A, B, C, and D (Part One) using Phyloseq and other R packages in R Studio.

#### 2.6.2.1 Alpha Diversity

Alpha diversity is the local diversity, that is the diversity *within* a community (sample). Using the rarefied data, the following metrics were calculated at the ASV level: ACE, Chao1, Fisher, Inverse Simpson, Observed ASVs, Shannon, and Simpson. Alpha diversity comparisons among multiple groups were done using a Kruskal-Wallis test, while the Wilcoxon rank-sum test was used for comparing two groups. Uncorrected p-values were reported and $p < 0.05$ was considered statistically significant.

#### 2.6.2.2 Beta Diversity

Beta diversity is the diversity *between* communities (samples). Principal coordinates analysis (PCoA) ordination plots of Unweighted and Weighted Unifrac distances were used to compare beta diversity between the comparative groups of interest at the ASV, family, genus, and species taxonomic levels. Unweighted Unifrac was assessed using rarefied data. Weighted Unifrac was assessed using non-rarefied data that had been transformed using the "compositional" function in the Microbiome R package (version 1.23.1); this scaled the ASV counts to relative abundance (Lahti & Shetty, 2019). In addition, principal component analysis (PCA) ordination of the Aitchison distance, a compositionally aware distance metric, was also used to compare beta diversity between the comparative groups of interest at the ASV, family, genus, and species taxonomic levels using the non-rarefied data object. The non-rarefied data was first transformed using the centred-log ratio (CLR) transform function in the microbiome R package (version 1.23.1) (Lahti & Shetty, 2019). Comparisons of all beta diversity metrics between groups were performed using a permutational multivariate analysis of variance (PERMANOVA), as implemented as the "Adonis2" function in the vegan R-package (version 2.6.4) with 1000 permutations (M. J. Anderson, 2017; Oksanen J, 2022). Permutational multivariate analysis of dispersion (PERMDISP) was used to check for the homogeneity of group dispersion values; this was tested also using 1000 permutations using the "betadisper"

function in the vegan R package (M. J. Anderson, 2017; Oksanen J, 2022). For both tests, comparisons were considered significantly different if Pr(F) was < 0.05 (uncorrected).

#### 2.6.2.3 Composition

The composition and distribution (absolute, relative abundance, and CLR-transformed abundance) of the 16S data were visualised at the phylum level using non-rarefied and rarefied data. Phyloseq, Ggplot2 (version 3.4.4), and the “taxa_distribution” function from MicrobiomeUtilities (version 1.00.17) R packages were used to construct these visualisations in R Studio (Lahti & Shetty, 2019; Wickham, 2016).

### 2.6.3 Differential Abundance Analysis with 16S rRNA Data

Differential gene expression analysis Version 2 (DESeq2) and Linear discriminant analysis (LDA) effect size (LEfSe) were used to find microbial features differentially represented between the comparative groups of interest (Love et al., 2014; Segata et al., 2011). DESeq2 and LEfSe were performed using MicrobiomeAnalyst 2.0, a web-based server providing a variety of microbiome data analytical tools (Lu et al., 2023). Non-rarefied and rarefied ASV raw/absolute counts, metadata, and taxonomic and phylogenetic information were used as the inputs in the "Marker Data Profiling" module of MicrobiomeAnalyst. Non-rarefied ASV counts were used for DESeq2 and LEfSe as it has been shown that rarefaction may result in high rates of false positives in tests for differential abundance (McMurdie & Holmes, 2014). However, there is some disagreement with LEfSe and whether read count tables should be rarefied to correct for different read depths across samples (Nearing et al., 2022; Segata et al., 2011; Wallen, 2021; Weiss et al., 2017). As such, read count tables are often rarefied before running LEfSe; this test has also been performed with rarefied data (Nearing et al., 2022).

These required inputs for MicrobiomeAnalyst were prepared prior in R Studio by exporting components of the Phyloseq object in csv or nwk format. At the data filtering step in the module, the default settings were used; "low count filter" was set to a minimum count of 4 and 20% prevalence in samples, and the "low variance filter" was set to remove features at 10% based on the inter-quantile range. This filtering step was to remove potentially uninformative features before applying the differential abundance tests thereby improving downstream outcomes (J. Chong et al., 2020; Nearing et al., 2022). DESeq2 was performed with the default settings in the module using filtered absolute, non-rarefied ASV count data. For LEfSe only, the non-rarefied and rarefied count data was scaled using total sum scaling (TSS) without any other data transformations applied at the data normalisation step in the module following filtering. The csv files of the DESeq2 results were exported from MicrobiomeAnalyst and used in R Studio to create volcano plots with a fold change threshold set at 0.5 and an uncorrected p-value set at 0.05. The following conditions were used for LEfSe with the filtered and transformed data (non-rarefied and rarefied): the alpha level for the Kruskal-Wallis rank sum test among classes was set at 0.05 (uncorrected p-value) and the threshold for logarithmic LDA score for discriminative features was set at 2.0. The bar plot function from the module was used for visualising the LEfSe outputs.

### 2.6.4 PICRUSt2 Software with 16S rRNA Gene Sequences

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) is a bioinformatics software platform designed to predict the functional potential of a bacterial community based on marker gene sequencing profiles (Douglas et al., 2020). PICRUSt2 was used to obtain insight into the potential functional capabilities of the faecal bacterial communities identified from 16S rRNA gene sequencing. The standard PICRUSt2 (Version 2.5.0) algorithm was run in a high-performance computing environment using the filtered feature table and representative 16S rRNA sequencing outputs obtained from QIIME2 (Section 2.5.6). Total-frequency-based filtering was applied to remove singletons (bacterial identifications found only by one sequencing read in one sample) to reduce any noise from the PICRUSt2 calculations. To retain all samples from Part One of the study for PICRUSt2 analyses, no further filtering was applied.

Three reference gene family databases were considered when running the PICRUSt2 pipeline at the ASV level: the Kyoto Encyclopaedia of Genes and Genomes (KEGG) orthologs (KOs), Enzyme Commission numbers (ECs), and MetaCyc pathway abundances (PWYs). The PICRUSt2 outputs are tabulated estimates of the functional abundances of the microbial communities. The outputs (KOs, ECs, PWYs) were analysed using ggpicrust2 R package to identify significant features of interest among the comparative groups of interest (Part One Comparisons A, B, C, and D). The ANOVA-Like Differential Expression 2 (ALDEx2) function in ggpicrust2 was used to test whether abundances of the predicted KO, EC, and PWY outputs differed among Comparisons A, B, C, and D; $p < 0.01$ was considered significant for this test. Ggpicrust2 is a recently released seamless and intuitive R software package designed to facilitate the analysis and interpretation of PICRUSt2 outputs (C. Yang et al., 2023). The standard ggpicrust2 workflow was followed and its packaged features were utilised to perform pathway name/description annotations, differential abundance testing (ALDEX2), and visualisation (C. Yang et al., 2023).

### 2.6.5 Phyloseq and R Studio with Culture MALDI-TOF MS Data

Phyloseq was used to create an object for the downstream analyses of the faecal culture MALDI-TOF data. This object contained the participant metadata (Sections 2.1 and 2.3) and the MALDI-TOF data (Section 2.5.2). The MALDI-TOF data provided bacterial count (corrected to its faecal dry weight) and identification at the species taxonomic level. This data in their respective Phyloseq objects to reflect Comparisons A, B, C, and D (Part One) were used with the analyses in Sections 2.6.6, 2.6.7, and 2.6.8. The analytical workflow is alike the one used for the 16S rRNA data. The R scripts used can be found in Appendix 5.

### 2.6.6 Composition and Diversity Analysis with Culture MALDI-TOF MS Data

#### 2.6.6.1 Alpha Diversity

The following metrics were calculated at the species level: Observed species, Inverse Simpson, Chao1, Shannon, and Simpson. Alpha diversity comparisons among multiple groups were done using a Kruskal-Wallis test, while the Wilcoxon rank-sum test was used for comparing two groups. Uncorrected p-values were reported and $p < 0.05$ was considered statistically significant.

#### 2.6.6.2 Beta Diversity

Principal coordinates analysis (PCoA) ordination plots of Bray-Curtis and Jaccard distances were used to compare beta diversity between the comparative groups of interest at the species taxonomic level. Bray-Curtis and Jaccard metrics were assessed using data that had been transformed using the “transform_sample_counts” function in the Phyloseq package; this scaled the count data to relative abundance. Before the data was visualised using PCoA ordination, the data already scaled to relative abundance underwent a Hellinger transformation using the function that is a part of the MicrobiotaProcess R package (version 1.13.2.994) (S. Xu et al., 2023). This final data object was visualised using the PCoA function implemented in the MicrobiotaProcess R package (version 1.13.2.994) (S. Xu et al., 2023). Outside of the MicrobiotaProcess R package, the Hellinger transformation was performed by applying a square root to the “transform_sample_counts” calculation. Comparisons of all beta diversity metrics between groups were performed using a permutational multivariate analysis of variance

(PERMANOVA), as implemented as the “Adonis2” function in the vegan R-package (version 2.6.4) with 1000 permutations (M. J. Anderson, 2017; Oksanen J, 2022). Permutational multivariate analysis of dispersion (PERMDISP) was used to check for the homogeneity of group dispersion values; this was tested also using 1000 permutations using the “betadisper” function in the vegan R package (M. J. Anderson, 2017; Oksanen J, 2022). For both tests, comparisons were considered significantly different if Pr(F) was < 0.05 (uncorrected).

#### 2.6.6.3 Composition

The composition and distribution of the MALDI-TOF data were visualised at the genus level using relative abundance values. Phyloseq and Ggplot2 (version 3.4.4) R packages were used to construct these visualisations in R Studio (Wickham, 2016).

### 2.6.7 Differential Abundance Analysis with Culture MALDI-TOF MS Data

Linear discriminant analysis (LDA) effect size (LEfSe) was used to find microbial features from the culture MALDI-TOF data that were differentially represented between the comparative groups of interest (Love et al., 2014; Segata et al., 2011). LEfSe was performed using MicrobiomeAnalyst 2.0 (Lu et al., 2023). Raw/absolute counts, metadata, and taxonomic information were used as the inputs in the “Marker Data Profiling” module of MicrobiomeAnalyst. These required inputs for MicrobiomeAnalyst were prepared prior in R Studio by exporting components of the Phyloseq object in csv format. At the data filtering step in the module, the default settings were used; “low count filter” was set to a minimum count of 4 and 20% prevalence in samples, and the “low variance filter” was set to remove features at 10% based on the inter-quantile range. This filtering step was to remove potentially uninformative features before applying the differential abundance tests thereby improving downstream outcomes (J. Chong et al., 2020; Nearing et al., 2022). For LEfSe, the count data was scaled using total sum scaling (TSS) without any other data transformations applied at the data normalisation step in the module following filtering. The following conditions were used for LEfSe with the filtered and transformed data: the alpha-level for the Kruskal-Wallis rank sum test among classes was set at 0.05 (uncorrected p-value) and the threshold for logarithmic LDA score for discriminative features was set at 2.0. The bar plot function from the module was used for visualising the LEfSe outputs.

### 2.6.8 MiMeDB with Culture MALDI-TOF MS Data

The Human Microbial Metabolome Database (MiMeDB) (https:mimedb.org) is a comprehensive multi-omic, microbiome resource that connects: (i) microbes to microbial genomes; (ii) microbial genomes to microbial metabolites; (iii) microbial metabolites to the human exposome and (iv) all of these 'omes' to human health (Wishart et al., 2023). This thesis utilised MiMeDB with the faecal culture MALDI-TOF dataset on the premise that it is not the microbes themselves that lead to specific health effects, but rather the chemicals (metabolites) they produce (Wishart et al., 2023). Particularly, this study used the information output from MiMeDB to provide further insight into the potential connections between the metabolites and other associated health conditions. MiMeDB is in its infancy and this study was an opportunity for the Wishart Lab members to test the database based on user input and feedback, and for this project to explore the culture MALDI-TOF MS data further. Input data was provided by AJK to the Wishart Lab so they could generate output information using the backend server of the MiMeDB platform as the web-based interface is not ready for handling multiple queries at once.

For Comparisons A, B, and C in Part One (Table 2.2.1), the bacterial count table (corrected to faecal dry weight and scaled to relative abundance) with the corresponding list of the faecal bacteria (species taxonomic level) identified were exported as Excel files from their respective R Phyloseq objects (Section 2.6.5). In Excel, the relative abundance values from each of the microbes identified were summed for each comparative group of interest. For example, Comparison A (Part One) compares 43 ME/CFS and 40 Control participants. The relative abundance values for one of the microbes (e.g., Bacteroides vulgatus) identified were summed for the ME/CFS group, followed by the Control group. This calculation was applied to all microbes identified via MALDI-TOF. The difference in relative abundance between the two groups for each of the microbes (direction: ME/CFS minus Control) was calculated and these values were used to select the microbes (features of interest) that would be put through MiMeDB. The same calculations were applied to Comparisons B and C (Part One). The subtraction direction for Comparison B was ME/CFS with FM minus ME/CFS without FM, and for Comparison C, it was ME/CFS with IBS minus ME/CFS without IBS. A difference of $\geq \pm 0.5$ between the total relative abundance values of the comparisons was used to filter and select the features of interest. This selection was then used as the input information for MiMeDB with the assumption that the microbes with larger differences were more likely to be of interest.

## 2.7 Faecal Dry Weight Determination

A portion of the neat faecal sample was used to determine its dry weight (Section 2.4.1). Approximately 300 milligrams of faecal sample were transferred into a pre-weighed disposable borosilicate glass culture tube 12 x 75 mm (part reference: Kimble 73500-1275). The combined mass of the sample and glass tube was recorded before it was placed into a pre-heated centrifugal concentrator capable of removing water from samples. The sample was dried out until a constant total weight of the sample and glass tube was achieved. All samples were monitored and achieved their constant total weight over 72 hours. The dry weight and percentages of the dry weight and water content of the samples were calculated based on the mass values recorded pre-drying and post-drying. Appendix 2 contains the faecal dry weight data for the participants as part of the study metadata.

## 2.8 LCMS and NMR Metabolomics Experiments

Targeted liquid-chromatography mass spectrometry (LCMS) and nuclear magnetic resonance (NMR) spectroscopy analytical techniques were used to assay biological samples for polar metabolites/small molecules. The faecal homogenate, urine, and blood plasma aliquots that were prepared during sample collection were kept in -80°C conditions until they were required for the metabolomics experiments (Sections 2.8.1 and 2.8.2). Figure 2.2.2 summarises the steps that were undertaken during sample collection before these experiments were conducted. Table 2.8.1 below outlines the batches of the samples used, and the corresponding section in this thesis detailing their sample preparation. The aliquots were thawed on ice and prepared simultaneously for metabolomic experiments in batches according to the sample type and analytical platform (LCMS or NMR). Part One and Two urine samples were prepared for LCMS analysis and run on the instrument at the same time. All sample preparation was done in cool conditions with samples and materials kept on ice throughout.

**Table 2.8.1** Details of the biological sample aliquots used for metabolomics sample preparation

| Sample Type (Aliquot) | Aliquot Preparation Details (refer to the corresponding section) | Part One or Two or Project | LCMS Metabolomic Sample Preparation (refer to the corresponding section) | NMR Metabolomic Sample Preparation (refer to the corresponding section) |
|---|---|---|---|---|
| Faecal Homogenate | 2.5.1 | One | 2.8.1.1 | 2.8.2.1 |
| Urine | 2.4.1 | One | 2.8.1.2 | 2.8.2.2 |
| Urine | 2.4.2 | Two | 2.8.1.2 | 2.8.2.2 |
| Blood Plasma | 2.4.2 | Two | 2.8.1.3 | 2.8.2.3 |

### 2.8.1 LCMS Sample Preparation and Data Acquisition

Samples were prepared according to polar metabolite extraction protocols advised by Metabolomics Australia, Bio21 Institute (MA), and transported to the MA facility on dry ice for instrument loading. Internal standards were prepared and provided by MA. Eppendorf-branded 1.5mL microcentrifuge tubes (Eppendorf Tubes ® 3810X 0030 125.150) were used. Metabolites were extracted from their respective sample types (Sections 2.8.1.1, 2.8.1.2, 2.8.1.3) and measured according to the details in Section 2.8.1.4.

#### 2.8.1.1 Faecal Homogenate

200 µL of a faecal homogenate was added to 800 µL of ice-cold extraction solvent with internal standards (100% methanol with 3 µM 13C6-Sorbitol, 3 µM 13C6-Leucine, and 3 µM 13C5,15N1-Valine) in a 1.5 mL microcentrifuge tube. The microcentrifuge tube was mixed on a dry block shaking incubator (temperature constantly maintained at 4°C) at 1200 rpm for 20 minutes to homogenise the sample. A pipette tip was used to break through the top layer and redisperse the upper part of the sample in the microcentrifuge tube before returning to the dry block shaking incubator for another 10 minutes; the sample had to be evenly homogenised before proceeding. Following homogenisation, the samples were centrifuged at 14, 100 × g for 10 minutes at 4°C to pellet precipitated proteins and cell debris. The supernatant was collected into a new 1.5 mL microcentrifuge tube and stored at -80°C until the instrument was ready for analysis. 20 µL of the supernatant was used from each sample to prepare the pooled biological quality control (PBQC).

#### 2.8.1.2 Urine

20 µL of urine sample was added to 180 µL of ice-cold extraction solvent with internal standards (acetonitrile:methanol: water 1:1:1 with 3 µM 13C6-Sorbitol, 3 µM 13C6-Leucine, and 3 µM 13C5,15N1-Valine) in a 1.5 mL microcentrifuge tube. The microcentrifuge tube was vortexed for 20 seconds before being put into an ultrasonic bath for 5 minutes. The bath water in the sonicator was pre-chilled with ice and replaced to maintain a cold temperature throughout. Following sonication, the microcentrifuge tubes were centrifuged at 14, 100 × g for 10 minutes at 4°C to pellet precipitated proteins and cell debris. The supernatant was collected into a new

1.5 mL microcentrifuge tube and stored at -80°C until the instrument was ready for analysis. 10 µL of the supernatant was used from each sample to prepare the PBQC.

#### 2.8.1.3 Blood Plasma

20 µL of plasma sample was added to 180 µL of ice-cold extraction solvent with internal standards (acetonitrile:methanol: water 40:40:20 with 3 µM 13C6-Sorbitol, 3 µM 13C6-Leucine, and 3 µM 13C5,15N1-Valine) in a 1.5 mL microcentrifuge tube. The microcentrifuge tube was vortexed for 20 seconds before being put into an ultrasonic bath for 5 minutes. The bath water in the sonicator was pre-chilled with ice and replaced to maintain a cold temperature throughout. Following sonication, the microcentrifuge tubes were incubated on a dry block shaking incubator (temperature constantly maintained at 4°C) for 10 minutes. The microcentrifuge tubes were then centrifuged at 14, 100 × g for 10 minutes at 0°C to pellet precipitated protein and cell debris. The supernatant was collected into a new 1.5 mL microcentrifuge tube. 10 µL of the supernatant was used from each sample to prepare the PBQC.

#### 2.8.1.4 LCMS Analysis of Polar Metabolites

Polar metabolite analysis of the extracted samples, PBQCs, and 13 mixtures of authentic standard mixes was performed on an Agilent 6545B series quadrupole time-of-flight mass spectrometer (QTOF-MS, Agilent Technologies) using a hydrophilic column (ZIC-pHILIC) as described previously (Kong et al., 2021). The PBQC was analysed as quality control in set intervals between the extracted samples; the PBQC was run with every fifth sample. Data analysis and the generation of targeted data matrices were done using MassHunter Quantitative Analysis Software (Version B.09.00, Agilent Technologies) based on the retention time and molecular masses matching an authentic standard as described previously (Sumner et al., 2007).

### 2.8.2 NMR Sample Preparation and Data Acquisition

Polar metabolites were extracted from their respective sample types and quantified using NMR. All biological samples underwent a similar sample preparation process involving the addition of 297 µL of the biological sample material, 363 µL NMR buffer, vortex mixing, and centrifugation before transferring to NMR tubes for analysis. The plasma samples were prepared using a liquid-liquid extraction technique (Sheedy et al., 2010). The faecal homogenate and urine samples did not use this technique (Beckonert et al., 2007; Dona et al., 2014). Pooled biological quality control samples were prepared for NMR. For each sample set and type, 200 µL from four control samples were pooled in a 1.5 mL microcentrifuge tube. From this PBQC stock, a 297 µL aliquot of sample was taken, prepared, and treated the same way for their respective sample type. Two replicates from the PQBC stock were prepared and analysed. The spectrometer parameters and settings used to acquire NMR data are detailed in Section 2.8.2.4.

The NMR buffer, per sample, contained 297 µL 200 mM sodium phosphate in deuterium oxide (pH 7.4), and 66 µL of deuterium oxide containing 5 mM 3-(trimethylsilyl)-1-propanesulfonic acid-d6 sodium salt (DSS) and 0.2% (w/v) sodium azide. The NMR buffer stock was freshly prepared and used within 12 hours; 363 µL of NMR buffer was transferred into a 1.5 mL microcentrifuge tube. 297 µL of the biological sample (faecal homogenate, urine, or blood plasma) was added to the tube with the buffer after it was handled according to sample-specific preparation requirements (Section 2.8.2.1, 2.8.2.2, 2.8.2.3). This made a total of 660 µL of the sample. These samples were kept at -80°C until the NMR instruments were available for analysis (Section 2.8.2.4). The samples were thawed on ice, mixed by vortex, centrifuged at 16, 000 × g for 1 minute, and 550 µL of the supernatant was transferred into a new 177.8 x 4.97 mm, 0.38 mm wall thickness, 507-grade NMR tube for NMR analysis.

#### 2.8.2.1 Faecal Homogenate

The faecal homogenate was prepared according to the steps in Section 2.8.2; 297 µL of faecal homogenate sample was added to 363 µL of NMR buffer in a 1.5 mL microcentrifuge tube and mixed by vortexing.

#### 2.8.2.2 Urine

Part One and Part Two urine samples were prepared as separate batches for NMR analysis. The urine samples were filtered using a 0.2 µM, 25 mm diameter syringe filter unit then dispensed into a 1.5 mL microcentrifuge tube, and stored at minus 80°C. The filtered urine samples were thawed on ice and prepared for NMR analysis according to the steps in Section 2.8.2; 297 µL of filtered urine sample was added to 363 µL of NMR buffer in a 1.5 mL microcentrifuge tube and mixed by vortexing.

#### 2.8.2.3 Blood Plasma

200 µL of plasma sample was added to 200 µL of ice-cold deuterated chloroform and 200 µL of ice-cold deuterated methanol in a 1.5 mL microcentrifuge tube. The sample tubes were mixed by vortexing and then left to sit on ice for 15 minutes. The samples were then centrifuged at 16, 000 × g at 4°C for 10 minutes to produce a biphasic mixture with an upper hydrophilic phase of water/deuterated methanol containing polar metabolites, and a lower phase of deuterated chloroform containing lipophilic compounds. The upper phase of the sample was prepared according to the steps in Section 2.8.2; 297 µL of the sample from the top hydrophilic layer was added to 363 µL of NMR buffer in a 1.5 mL microcentrifuge tube and mixed by vortexing.

#### 2.8.2.4 NMR Spectroscopy of Polar Metabolites

Two NMR instruments operating at different frequencies were used due to instrument availability. Part One faecal homogenate and urine samples were measured on a Bruker 600 MHz spectrometer. Part Two urine and blood plasma samples were measured on a Bruker 700 MHz spectrometer. Both spectrometers were equipped with a 5 mm triple resonance cryoprobe and set to a constant temperature of 298 K. Standard one-dimensional (1D) $^{1}$H spectra were acquired for all prepared biological samples using 1D Carr-Purcell-Meiboom-Gill (CPMG) pulse

sequence with presaturation for solvent suppression and $T_2$ relaxation filtering. The CPMG presat pulse sequence had the form –RD-90°-(*t*-180°-*t*)$_n$-ACQ. The RD (relaxation delay) was 4 s, *t* (spin-echo delay) was 300 us, *n* (number of loops) was 128 and 180° is a 180° RF (radiofrequency) pulse, and ACQ is the data acquisition period depending on the sample type. The 90° pulse width was calibrated, and the receiver gain was optimised. The faecal homogenate spectra were collected over 65, 536 time domain points and 128 scans. The blood plasma spectra were collected over 73, 728 time domain points and 32 scans. The urine samples were collected over 65, 536 time domain points and 64 scans. All spectra were collected with a spectral width of 20 ppm. Water suppression was achieved by presaturation of the water signal during RD (4 s).

Using the Chenomx NMR Suite Professional Version 6.0 software package, the free induction decay was multiplied by 0.3 Hz exponential line broadening and zero-filled before Fourier transformation (all biological sample types). Spectra were manually phased, baseline corrected, and referenced to DSS with a chemical shift of 0 ppm. The compound libraries in the Chenomx software were used to identify and quantify metabolites in the NMR 1D $^1$H spectra based on their characteristic chemical shifts using 0.5 mM DSS as an internal chemical shift reference. Reference lists of NMR-measured metabolites in the urine, blood, and faecal samples were provided by the Wishart Lab; these lists were used to guide the selection of metabolites from the compound library (B. Lee and D. Wishart, personal communication, January 2023). A total of 36 faecal, 26 urine, and 47 blood plasma metabolite features per sample assayed were identified from the NMR experiments. 2D $^1$H TOCSY experiments for each sample type were also collected using WATERGATE solvent suppression and a DIPSI-2 mixing sequence with 60 ms mixing time and 4096 t2 and 1024 t1 data points. States-TPPI was used for quadrature detection along the indirect dimension and the 2D NMR data was processed using TOPSPIN 1.3. These 2D $^1$H TOCSY spectra with NMRFAM-Sparky were used to confirm the identities of metabolites observed in the 1D.

## 2.9 Analyses of Metabolomics Data

### 2.9.1 LCMS Data Analysis

Following Section 2.8.1.4, Metabolomics Australia (MA) provided Excel data matrices of the faecal, urine, and blood plasma samples that were used for downstream analyses. Each data matrix output contained the relative concentrations (peak area) of metabolites assayed from the participant and pooled biological control (PBQC) samples. The LCMS method used by MA could not differentiate between stereoisomers and metabolites eluting in the same region (retention time) with similar mass. However, initial inspection of the data matrices indicated otherwise with similar relative concentration values assigned (0 to 2% difference) suggesting that these features were “duplicates” of the metabolite. Clarification and advice were sought and given by MA on this. To ensure that the data matrices were reflective of the LCMS methodology used, each metabolite was assigned its molecular mass and sorted accordingly. HMDB (hmdb.ca) was used to acquire the mass and structural information on the metabolites that assisted with the consolidation of the data matrices. This process highlighted metabolite features that were potentially ambiguous in the data matrix. For metabolites that were initially listed but were stereoisomers of each other, the features were merged, and the average of their relative concentrations was taken. For example, the original matrix listed D- and L- aspartic acid, and their relative concentration values for all the samples were nearly identical to each other (0 to 2% difference). In the refined matrix, this metabolite feature was considered as one (aspartic acid), and its relative concentration was taken from the D- and L-aspartic acid. For non-stereoisomer metabolites that had the same mass, but different relative concentrations, these were reported independently as their retention times were different. The data matrices were consolidated accordingly and subsequently used for data analyses with MetaboAnalyst (Section 2.9.3). A total of 137 faecal, 147 urine, and 128 blood plasma metabolite features per sample assayed were identified from the LCMS experiments.

### 2.9.2 NMR Data Analysis

Following the assignment of metabolites, NMR data matrices of the faecal, urine, and blood plasma samples were generated and exported from Chenomx as Excel files. These data matrices were subsequently used for the data analyses with MetaboAnalyst (Section 2.9.3).

### 2.9.3 MetaboAnalyst for Metabolomics Data Analysis

Consolidated LCMS (Section 2.9.1) and NMR (Section 2.9.2) metabolomic data matrices were analysed with MetaboAnalyst 5.0. MetaboAnalyst (https://www.metaboanalyst.ca/) is a well-maintained, open-source analytical tool provided by the Xia lab (Pang et al., 2021). CSV-formatted files were prepared using Microsoft Excel according to the stipulated format (samples in rows, unpaired). In Excel, participant metadata was assigned to the LCMS and NMR data matrices in the second column following the first column which contained the sample labels. LCMS and NMR data matrices for the faecal samples were corrected to their sample dry weight in Excel. For the urine samples, the LCMS and NMR PBQCs were averaged and merged into one entity in Excel; this was used in the normalisation section of the MetaboAnalyst module. Initially, data matrices for all sample types were analysed in MetaboAnalyst with the PBQC samples; close clustering of these quality control samples indicated that the differences observed between the groups were attributable to biological factors. In total, eight CSV files were prepared to reflect the metadata groupings of Comparisons A, B, C, and D using the LCMS and NMR data matrices (Appendix 8 and Appendix 10). HMDB (https://hmdb.ca/) was searched to obtain information about the differential metabolites post-acquisition and data analysis.

The final CSV-Excel files were uploaded into the MetaboAnalyst "Statistical Analysis One Factor" module as "concentrations". At the data integrity check step, missing values were detected, and default settings for this were accepted i.e., missing values were replaced by 1/5 of the minimum positive values of their corresponding variables. Following this, sample normalisation, data transformation, and scaling options were selected. This data pre-treatment step was performed to achieve a data matrix with a more Gaussian-type distribution. For the faecal (as already done in Excel) and plasma samples, no sample normalisation was selected. For the urine samples, normalisation by a reference sample was selected (averaged PBQCs). All samples were log-transformed (base 10) and auto-scaled (mean-centred and divided by the standard deviation of each variable). From this point, univariate (volcano plot (VP)) and multivariate (unsupervised principal component analysis (PCA) and supervised partial least-squares discriminant (PLS-DA)) analyses were conducted. Multivariate and univariate analyses were used to determine whether metabolites measured by LCMS and NMR were significantly different from the comparative groups of interest. All plots for the metabolomic results present

the LCMS outcomes first, followed by NMR outcomes in sections that are organised according to Comparisons A to D.

PCA, presented as a 2D scores plot with ellipses representing the 95% confidence interval of the group clustering, was performed to search for trends by visual inspection in group separation and to identify potential outliers. PLS-DA was performed to maximise the separation of the groups observed and assess the extent of the top 15 different metabolites that may reveal potential features and pathophysiological states of interest. PLS-DA was presented as a 2D scores plot with ellipses representing a 95% confidence interval of the group clustering, and Variable Importance in the Projection (VIP) score results > 1.0 from Component 1. Separation of the PLS-DA plots was inspected visually, and the VIP plots show the rank of the top 15 different metabolites identified by PLS-DA according to the VIP score on the x-axes. The most discriminating metabolites in the VIP plots are shown in descending order of their coefficient scores and the coloured boxes indicate whether the metabolite relative concentration is increased (red/high) or decreased (blue/low). For both the PCA and PLS-DA 2D score plots, samples in the comparative group of interest are represented by the coloured and shaped markers denoted in the legend. Further, the separation of the ellipses in the 2D score plots was described as overlapping, very partial, partial, distinguished, or complete.

Univariate statistical analysis, presented as volcano plots, was used to focus on the independent changes in metabolite levels and detect more strictly the significant differences in the metabolome profiles. All volcano plots followed the same direction: Comparison A Control minus ME/CFS, Comparison B ME/CFS with FM minus ME/CFS without FM, Comparison C ME/CFS with IBS minus ME/CFS without IBS, and Comparison D age-matched ME/CFS with IBS minus age-matched ME/CFS without IBS. For LCMS volcano plot analyses, a fold change (FC) threshold of 1.5, along with raw and FDR-adjusted p-values of 0.05, to identify significant differences was applied. However, an exception was made for the Part Two LCMS plasma analysis comparison of age-matched ME/CFS with IBS and ME/CFS without IBS groups (Figure 5.1.25) as these parameters did not yield any data points; instead, a FC threshold of 1.0 with a raw p-value of 0.1 was used. For NMR volcano plot analyses, a FC threshold of 1.0, along with raw and FDR-adjusted p-values of 0.1, to identify significant differences was applied. However, an exception was made for the Part One NMR faecal analysis comparison of ME/CFS overall and Control groups (Figure 4.1.1) where a FC threshold of 1.0 with a raw and FDR-adjusted p-value of 0.05 was used instead.

# 3 CHAPTER THREE: Gut Microbiome (Part One)

The plausible involvement or implications of the gut microbiome emerge from its critical role in several complex functions that are closely juxtaposed. The gut-brain axis, germ-free mouse models, and observations from antibiotic, probiotic, diet, faecal transplantation, and other gut-mediated or involved therapeutic interventions provide further compelling support for the integral role of the gut microbiome in the disease (Carding et al., 2015; König et al., 2022; Varesi et al., 2021). It has been hypothesised that gut dysbiosis or disturbed alterations in the composition, capacity, and function of the gut microbiome may contribute to the development, exacerbation, and perpetuation of the disease. Indeed, it has been proposed that ME/CFS patients with IBS might encompass a distinct subgroup based on microbiome and metabolic profiling (Maes et al., 2014; Nagy-Szakal et al., 2017). If it is not IBS per se, GI symptoms, and dysregulation might constitute a potential subtype in ME/CFS (Aaron et al., 2001; Frémont et al., 2013; Germain et al., 2018, 2020; Giloteaux, Goodrich, et al., 2016; Guo, Che, et al., 2023; Milivojevic et al., 2020; Nagy-Szakal et al., 2017, 2018; Shukla et al., 2015; Xiong et al., 2023). However, while there is a considerable amount of evidence for the gut in ME/CFS, findings remain limited, inconsistent, and unresolved (Du Preez et al., 2018).

Research has not elucidated any definite gut-related mechanistic, pathophysiological, unique subgroupings/categories, cause-effect, or before-during-after relationships in ME/CFS (G. He et al., 2023; Newberry et al., 2018; Varesi et al., 2021). Pursuing the role of the gut, and gut dysbiosis in disease considers not only its composition but also its diversity, functionality, and capacity to impart influence, respond, and interact with the host. In ME/CFS, many studies have found disturbances and alterations in the taxonomic composition and community structure of the gut microbiota from their cohorts; however, these datasets are not necessarily “first stop” or “final destination” answers (König et al., 2022; Olesen & Alm, 2016; Proal & Marshall, 2018; Varesi et al., 2021). It is becoming more and more suggested and indicated that the complex link between dysbiosis and host responses is a representation of increased intestinal hyperpermeability and bacterial translocation (Varesi et al., 2021). A “leaky gut” has been observed via the unusual and unbalanced measurements of various pro-inflammatory, immune-mediated markers and/or metabolites (König et al., 2022; Navaneetharaja et al., 2016; Seton et al., 2023; Varesi et al., 2021). Under a “healthy” or “normal” gut composition, passage through the gut barrier should not be possible, and the gut microbiome would be in favour of regulation, defence, protection, and repair (De Vos et al., 2022; König et al., 2022). This aligns with the

broader concept that the chronicity, perturbations, and breakdown of homeostasis in ME/CFS are accompanied by presentations of affected and/or triggered stress, and systemic immune and inflammatory responses.

This chapter focused on the gut microbiome characterised by the faecal samples from Part One. This included the overall microbial composition, alpha and beta diversity measures, and differential abundance analyses from the 16S rRNA sequencing and culture MALDI-TOF MS datasets. A similar data analytical workflow was applied to both methodological platforms; therefore, a discussion of the results and findings is presented here simultaneously. Generally, the 16S rRNA outcomes are displayed or discussed first, followed by the culture MALDI-TOF MS outcomes. PICRUSt2 and MiMeDB results will be presented here; however, also be discussed more in the context of Chapter 4. Subsequent chapters (Chapters 4 and 5) of this thesis will look at the metabolomic profiles from Part One and Part Two of the study. These metabolomic measurements may provide further insight into the metabolic functionality of the gut microbiome, and differences and similarities among the assigned comparative participant groups (Comparison A-D). The faecal and urine metabolomic outcomes from Part One are presented in Chapter 4, and the plasma and urine from Part Two are in Chapter 5. Part One looked at 40 ME/CFS and 43 Control participants which included males and females (Section 2.1). Table 2.2.1 shows the baseline characteristics of the four comparative groups (Comparison A-D). Comparison A compares the ME/CFS overall and Control groups, Comparison B compares the ME/CFS +/- FM groups, Comparison C compares the ME/CFS +/- IBS groups, and Comparison D compares the age-matched ME/CFS +/- IBS** groups.

## 3.1 Overall Microbial Composition

From the non-rarefied 16S rRNA data (Section 3.1.1), absolute abundance (Figure 3.1.1) and relative abundance (Figure 3.1.2) values showed that Bacteroidota (or its synonym Bacteroidetes) and Firmicutes overall were the most highly represented phyla across the dataset. These are two major important phyla of bacteria and together they comprise up to about 90% of the total human gut community (König et al., 2022; Stojanov et al., 2020). A visual comparison between the ME/CFS overall and Control groups (Comparison A) showed that both Bacteroidetes and Firmicutes phyla appeared to be reduced in the ME/CFS overall group (Figure 3.1.1 and Figure 3.1.2). The decrease in Bacteroidetes was also observed in some other ME/CFS studies (König et al., 2022; Raijmakers et al., 2020); however, others have found

an increased or comparable abundance between their ME/CFS and non-ME/CFS cases (Frémont et al., 2013; Giloteaux, Goodrich, et al., 2016; König et al., 2022; Lupo et al., 2021; Varesi et al., 2021). The decreasing trend in Firmicutes appeared to be consistent with some of the other ME/CFS studies (Frémont et al., 2013; Giloteaux, Goodrich, et al., 2016; König et al., 2022; Lupo et al., 2021; Varesi et al., 2021). Where the data has taken a "traditional" approach to handling abundance values, it appeared that phyla Patescibacteria, Proteobacteria, and Verrucomicrobiota were different with the shifts in Bacteroidetes and Firmicutes between the ME/CFS overall and Control groups (Figure 3.1.1 and Figure 3.1.2). However, the other phyla features, Actinobacteriota, Campilobacterota, Cyanobacteria, Desulfobacterota, Fusobacteriota, and Synergiosta, were more obscure to visually discriminate between the two groups (Figure 3.1.1 and Figure 3.1.2). Instead, the centred-log ratio (CLR) transformed non-rarefied data, a "newer" compositional approach to data analyses, displayed a more obvious and apparent comparison in abundance across all 11 of the bacteria phyla features (Figure 3.1.3). Note, that the negative CLR values from the transformation indicated a low/lower microbial abundance (Figure 3.1.3).

The CLR data transformation was applied to the non-rarefied dataset to address the compositional bias and issue inherent in 16S rRNA gene amplicon sequencing data (Gloor et al., 2017). It has been said that applying the CLR transformation brings new perspectives and "opens up" the data (Mullineaux et al., 2021). Interestingly, while the decrease in Bacteroidetes was observed in the ME/CFS overall group, Firmicutes appeared to be increased from the CLR-transformed data (Figure 3.1.3). Although the CLR transformation was not applied to them, an increase in Firmicutes has also been observed by other ME/CFS studies (Giloteaux, Hanson, et al., 2016; König et al., 2022; Newberry et al., 2018; Raijmakers et al., 2020). Similarly, different observations in abundance being higher or lower for all the other phyla can be made depending on whether the data is in its absolute abundance values (Figure 3.1.1), relative abundance values (Figure 3.1.2), or CLR-transformed state (Figure 3.1.3). This indicates that the approach to data analysis should be considered when describing shifts and trends in the gut microbiome. The phylum features of the non-rarefied data converted to relative abundance values were visualised with distribution plots (Figure 3.1.4, Figure 3.1.5, Figure 3.1.6, and Figure 3.1.7). These plots provided a general overview of data when grouped according to ME/CFS overall versus the Control group (Comparison A), or with and without the FM and IBS comorbidities (Comparison C-D). The pattern of each line which represents a bacterial phylum all varied slightly according to the comparison involved. Comparison C and D, where D is the ME/CFS

age-matched cohort for the IBS comorbidity, showed a more similar pattern to each other than the comparison involving FM (Comparison B). Noticeably, the Synergistota phylum did not appear in the ME/CFS - FM (Figure 3.1.5), ME/CFS - IBS (Figure 3.1.6), and age-matched ME/CFS - IBS (Figure 3.1.7) groups. It is not possible to make direct comparisons and account for every microbial taxonomic feature with the results from this thesis and other studies given the differences in cohorts, platforms, study designs, and the variety of data analytical approaches used. Despite the discrepancies across study outcomes, the data presented here supports the overall evidence that suggests alterations in the gut microbiota composition exist between ME/CFS and non-ME/CFS cohorts. It also provides some indication of microbial alterations within ME/CFS, with or without the FM, or IBS comorbidity.

Rarefied 16S rRNA data was also used to characterise the gut microbiome (Section 3.1.2). Compared to the non-rarefied faceted box plots, the rarefied box plots of absolute abundance (Figure 3.1.8) and relative abundance (Figure 3.1.9) values showed that the sequences for Campilobacterota were lost from rarefying the data; however, all of the other phylum features were retained. It was apparent in the rarefied 16S rRNA data that the Bacteroidota (or its synonym Bacteroidetes) and Firmicutes phylum features were the most dominant phyla regardless of the disease state. It was also observed in the rarefied data that these two predominant phyla were reduced in the ME/CFS overall compared to the Control group (Comparison A). From visual inspection of Figure 3.1.8 and Figure 3.1.9, Actinobacteriota, Proteobacteria, and Verrucomicrobiota at the phylum level were different between the ME/CFS overall and Control groups (Comparison A). Actinobacteriota was decreased in this ME/CFS group which was inconsistent with other studies that identified it; decreased in the Giloteaux, Hanson et al. (2016) ME/CFS cohort yet increased in the Raijmakers et al. (2020) ME/CFS cohort. Proteobacteria was more abundant in the ME/CFS group compared to Controls which aligned with the findings from Giloteaux, Goodrich et al. (2016) but conflicted with Giloteaux, Hanson et al. (2016). Like the non-rarefied data, all comparisons, Comparison A-D, were visualised with distribution plots using rarefied data (Figure 3.1.10, Figure 3.1.11, Figure 3.1.12, and Figure 3.1.13). The pattern of each line which represents a bacterial phylum varied slightly according to the respective comparison and group involved. Comparison C and D, where D is the age-matched cohort for the IBS comorbidity, showed a more similar pattern to each other than the comparison involving FM (Comparison B). Again, the data here does not contribute to finding a consistent microbial signature for dysbiosis and alterations in ME/CFS. Instead, it

reiterates the issues with heterogeneity of the ME/CFS cohort, study designs, and analytical approaches used.

Across all of the comparisons for the culture MALDI-TOF dataset (Section 3.1.3), Bacteroides and related genera at the genus level were the most abundant in all samples (Figure 3.1.14, Figure 3.1.15, Figure 3.1.16, and Figure 3.1.17). Armstrong et al. (2017) and Wallis et al. (2018) also identified that Bacteroides at the genus level were a major fraction of the gut microbiota findings. However, as the same culturing methods and data approaches were not used (Section 2.1.1), direct comparisons to these studies were not possible. Other high relative abundance genera observed in all samples from this study included Bifidobacterium and related genera, Eubacterium and related genera, Prevotella, and Escherichia. At an initial glance, there does not appear to be any standout differences or patterns between the ME/CFS overall and the Control group (Comparison A) that could suggest dysbiosis; in fact, each sample regardless of its assigned cohort appears unique (Figure 3.1.14). However, when the Control group is removed from the comparisons, some differences in the ME/CFS group depending on FM or IBS comorbidity, become more apparent, indicating some bearing of the comorbidities in ME/CFS (Comparison B-D). However, further comparisons of the groups were examined with alpha and beta diversity metrics and differential abundance analysis testing.

### 3.1.1 Non-Rarefied Taxonomic Composition (16S rRNA)

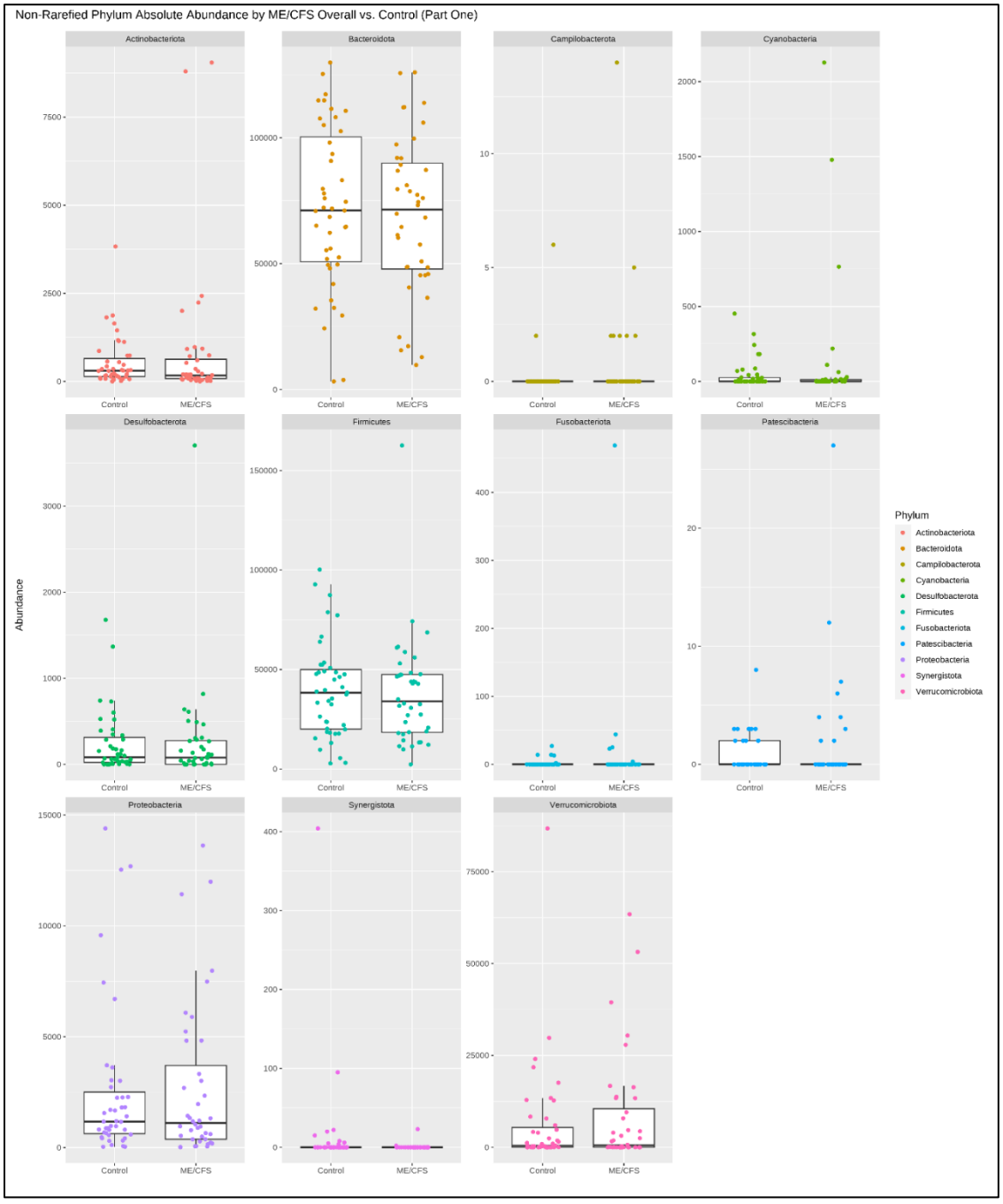



**Figure 3.1.1** Overview of taxonomy and absolute abundance values at the phylum level from the non-rarefied Part One dataset (ME/CFS overall (n = 40) vs. Control (n = 43))

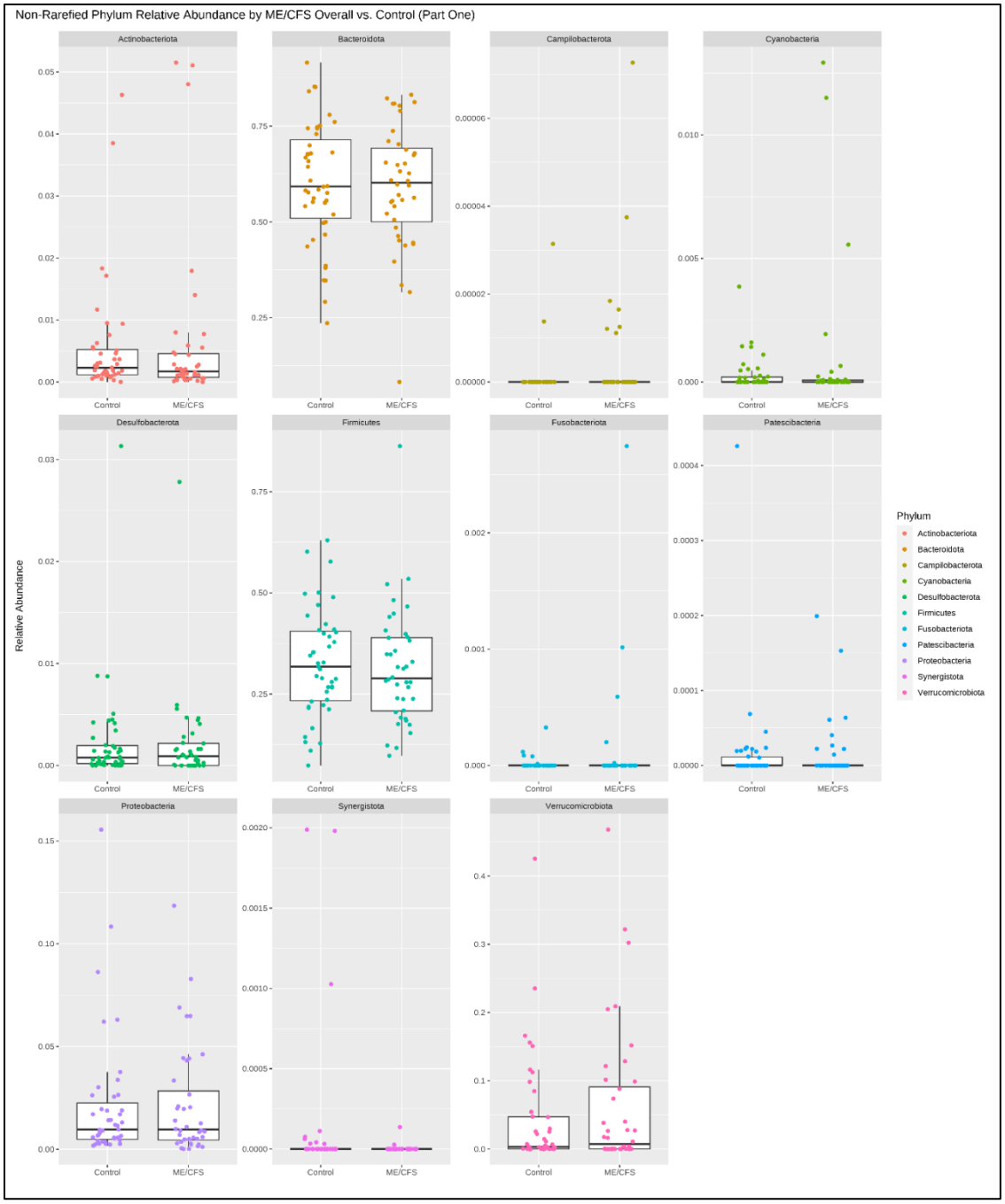



**Figure 3.1.2** Overview of the taxonomy and relative abundance values at the phylum level from the non-rarefied Part One dataset (ME/CFS overall (n = 40) vs. Control (n = 43))


**Figure 3.1.3** Overview of the taxonomy and centred-log ratio (CLR) transformed abundance values at the phylum level from the non-rarefied Part One dataset (ME/CFS overall (n = 40) vs. Control (n = 43))

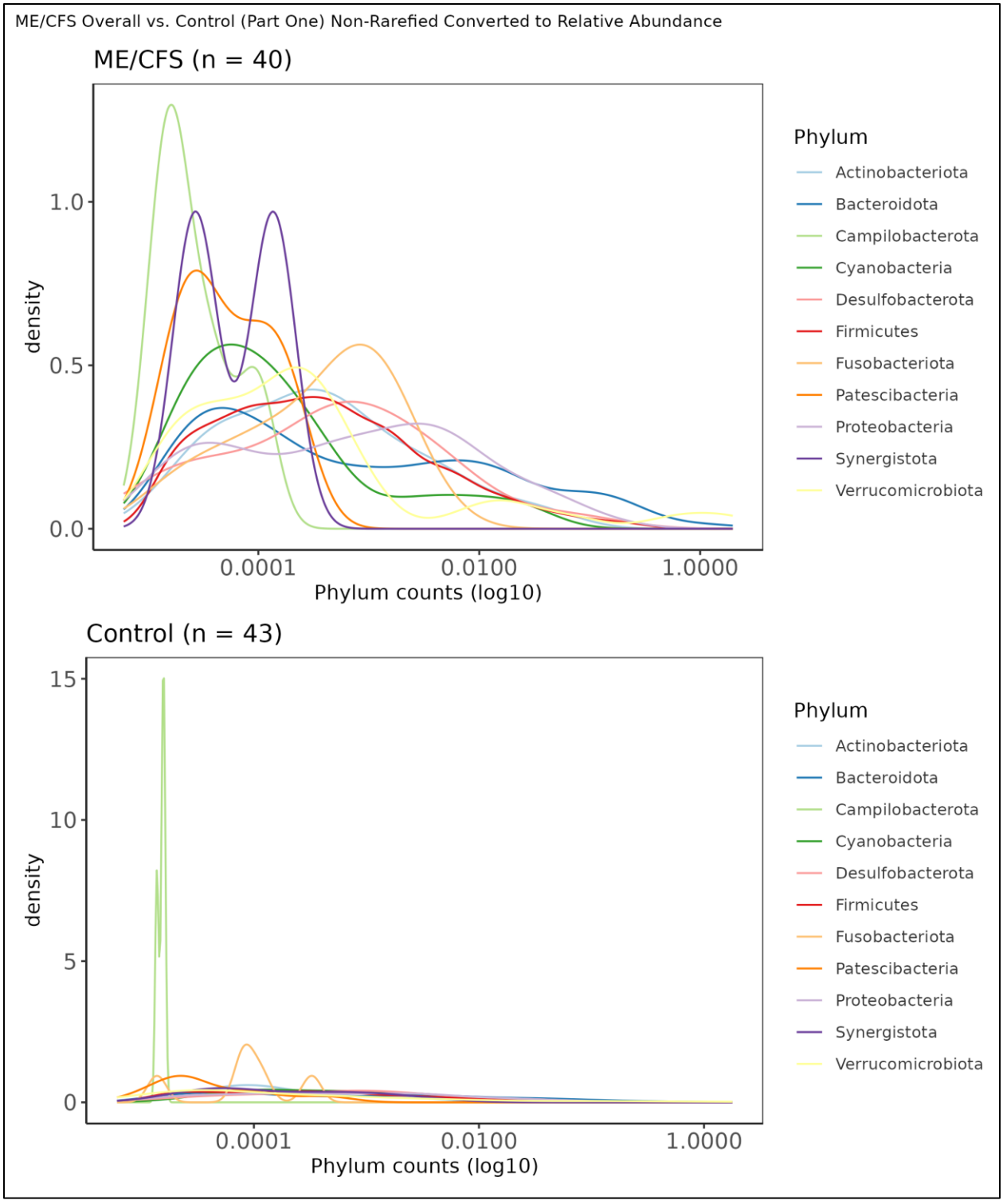



**Figure 3.1.4** Overview of the distribution of the taxonomy at the phylum level from the ME/CFS overall (n = 40) and Control (n = 43) Part One Comparison A, non-rarefied, converted to relative abundance dataset

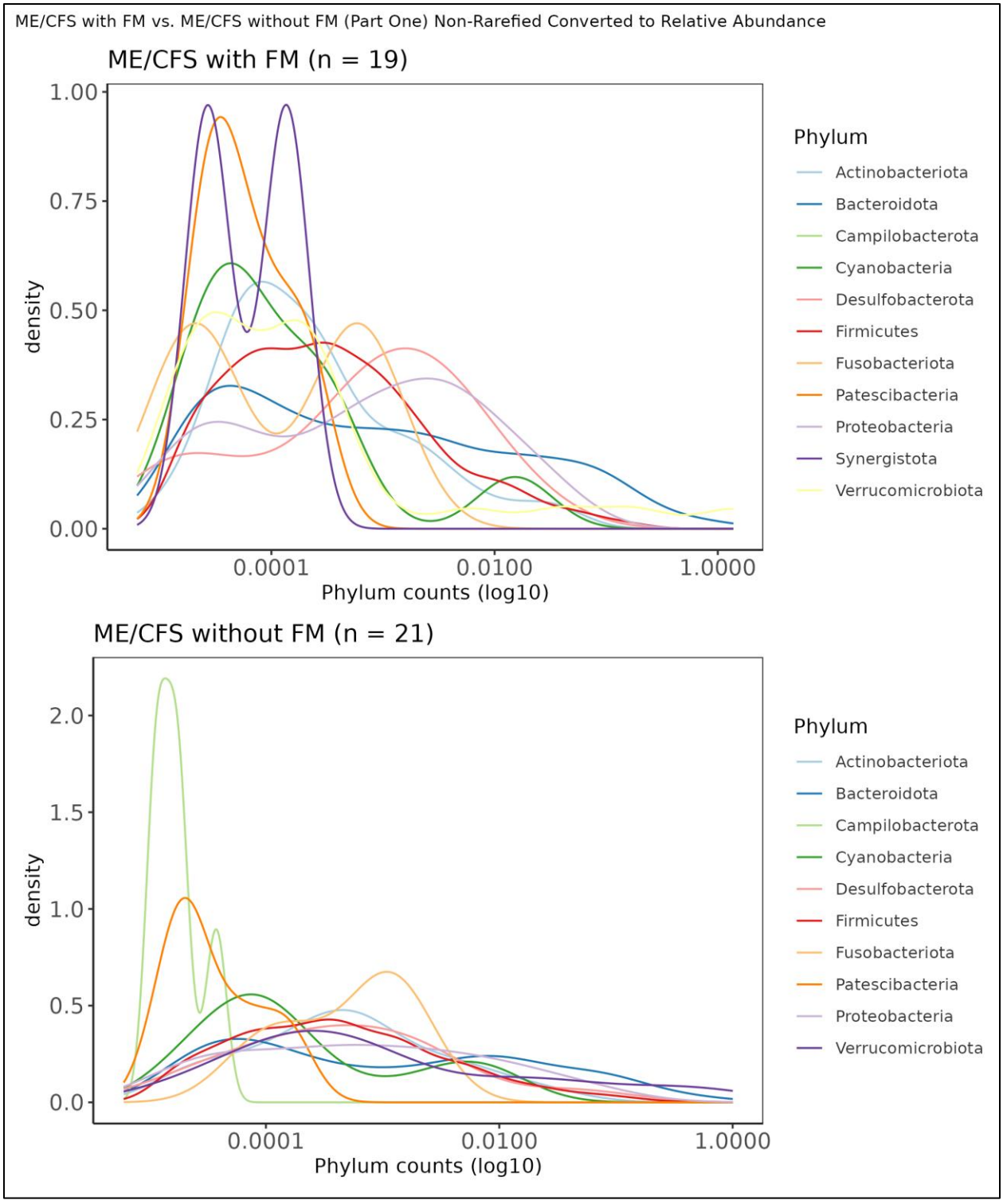



**Figure 3.1.5** Overview of the distribution of the taxonomy at the phylum level from the ME/CFS with FM (n = 19) and ME/CFS without FM (n = 21) Part One Comparison B, non-rarefied, converted to relative abundance dataset

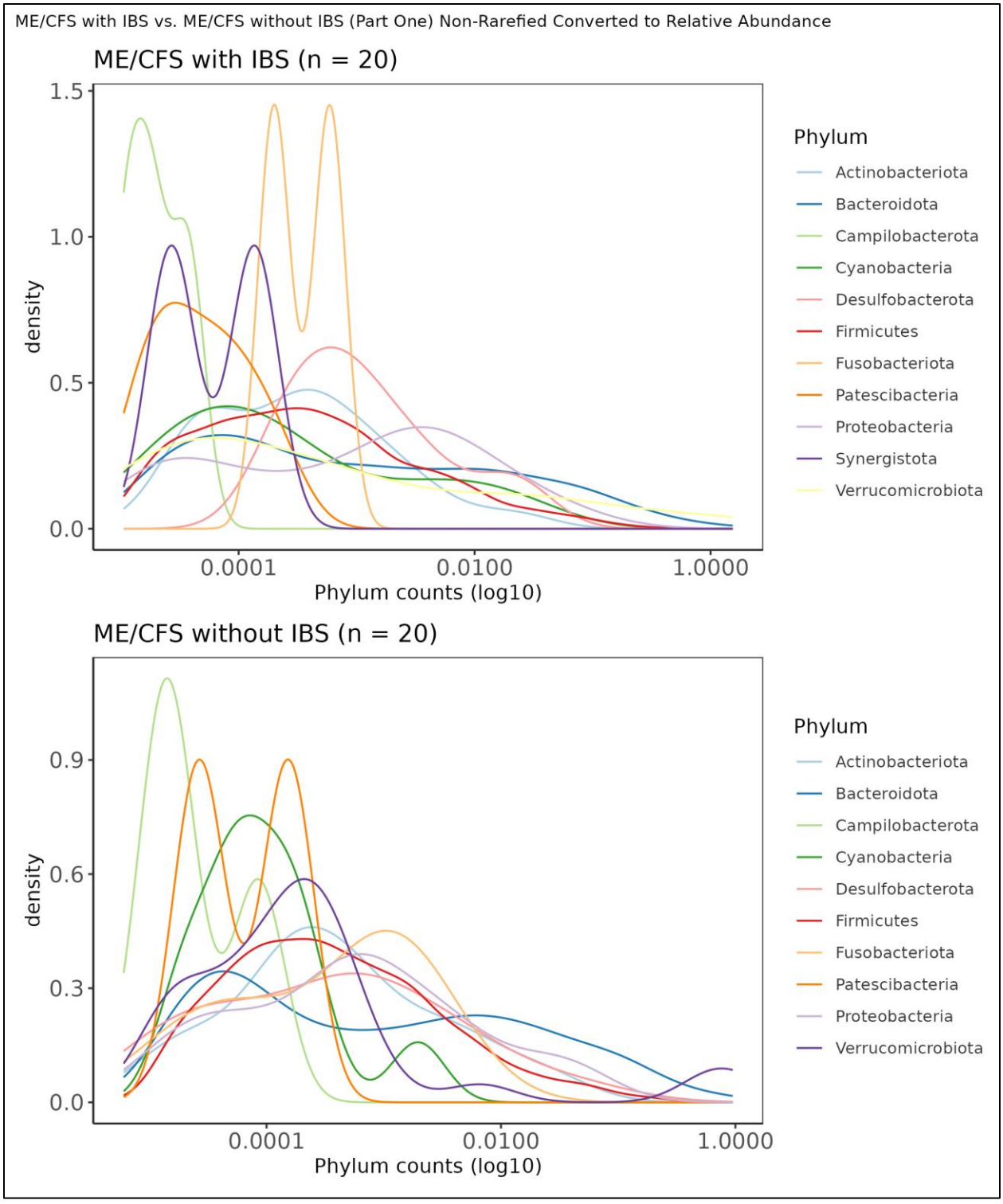



**Figure 3.1.6** Overview of the distribution of the taxonomy at the phylum level from the ME/CFS with IBS (n = 20) and ME/CFS without IBS (n = 20) Part One Comparison C, non-rarefied, converted to relative abundance dataset

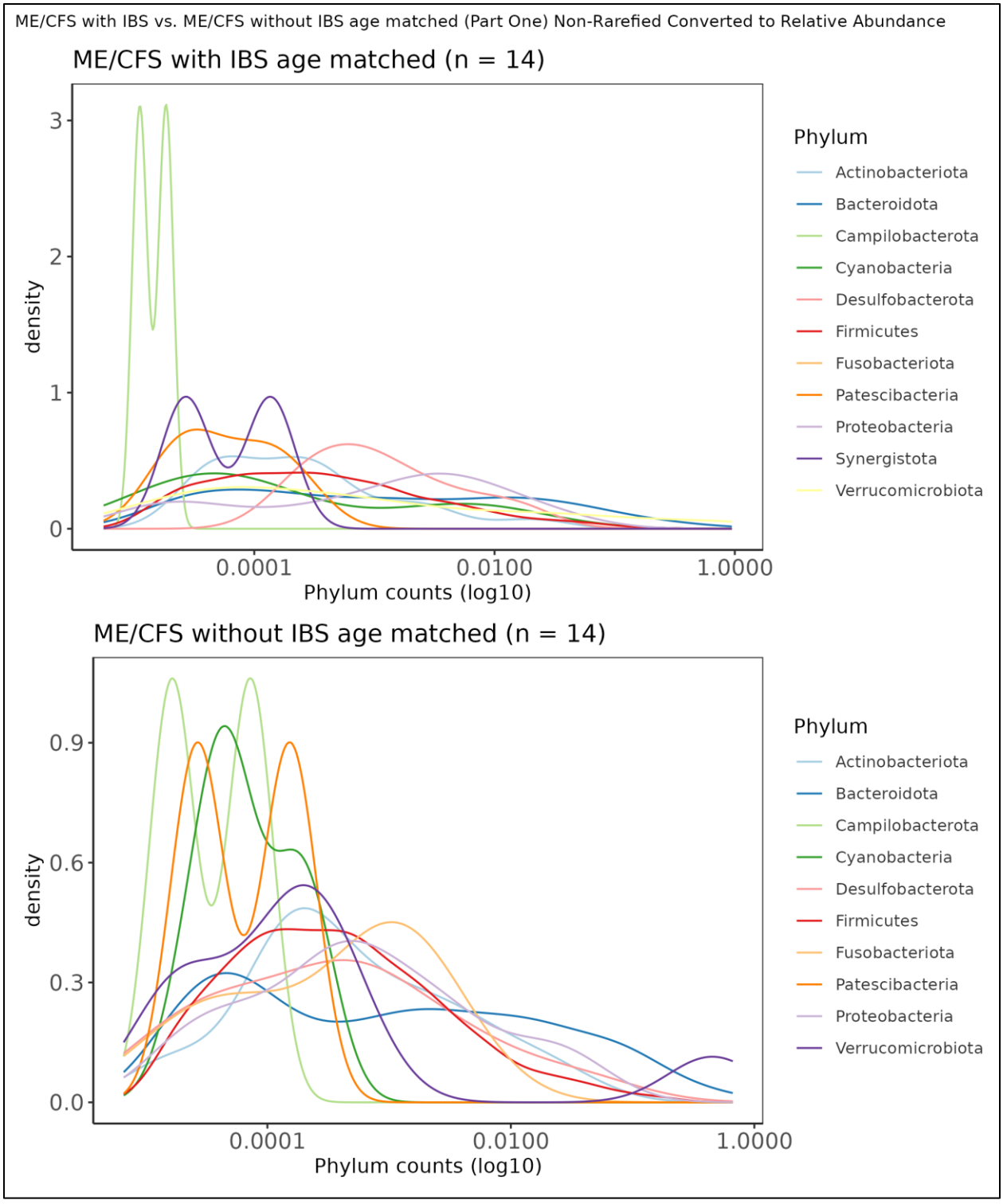



**Figure 3.1.7** Overview of the distribution of the taxonomy at the phylum level from the age-matched ME/CFS with IBS (n = 14) and ME/CFS without IBS (n = 14) Comparison D, Part One non-rarefied, converted to relative abundance dataset

### 3.1.2 Rarefied Taxonomic Composition (16S rRNA)

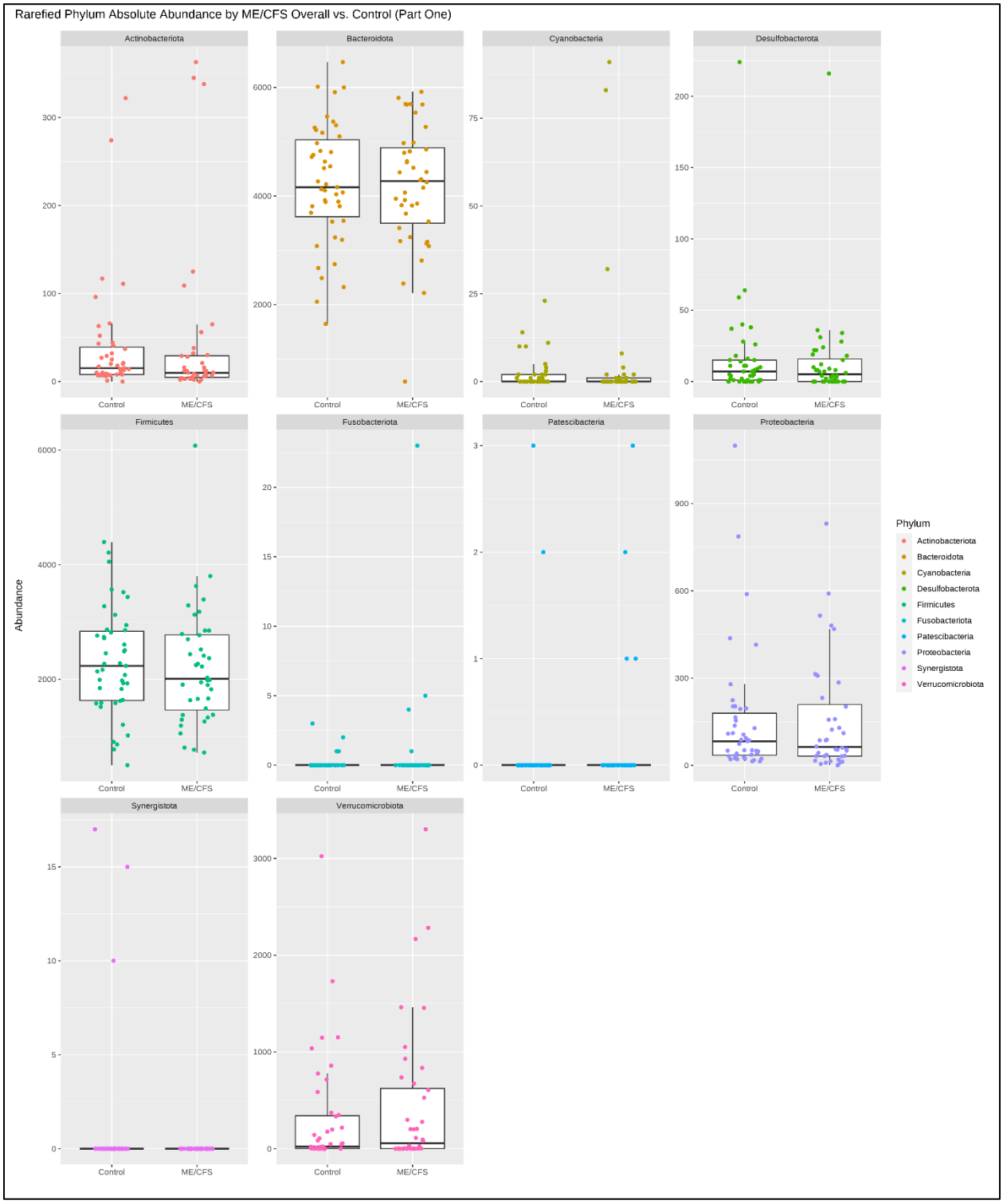



**Figure 3.1.8** Overview of taxonomy and absolute abundance values at the phylum level from the rarefied Part One dataset (ME/CFS overall (n = 40) vs. Control (n = 43))

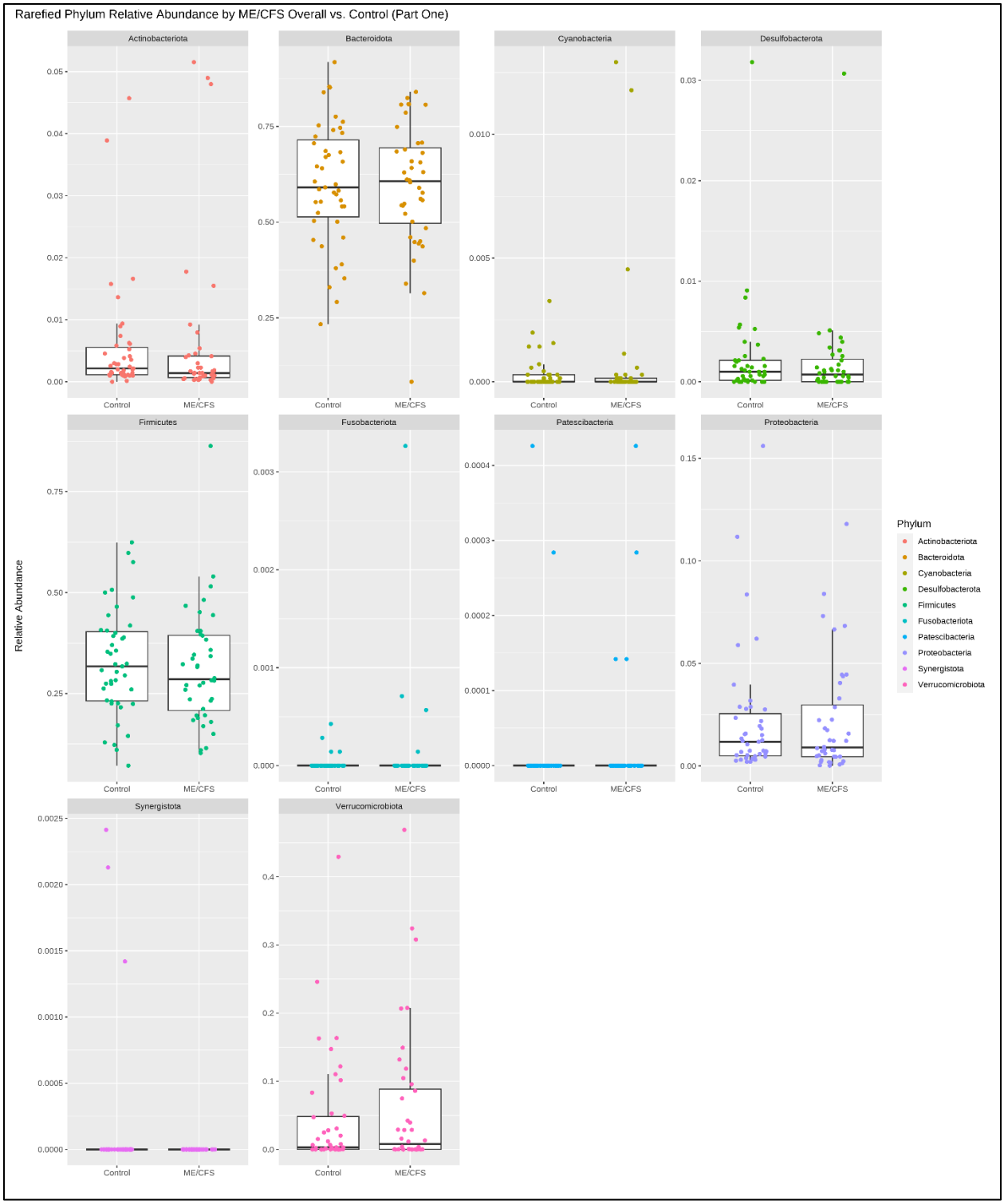



**Figure 3.1.9** Overview of taxonomy and relative abundance values at the phylum level from the rarefied Part One dataset (ME/CFS overall (n = 40) vs. Control (n = 43))

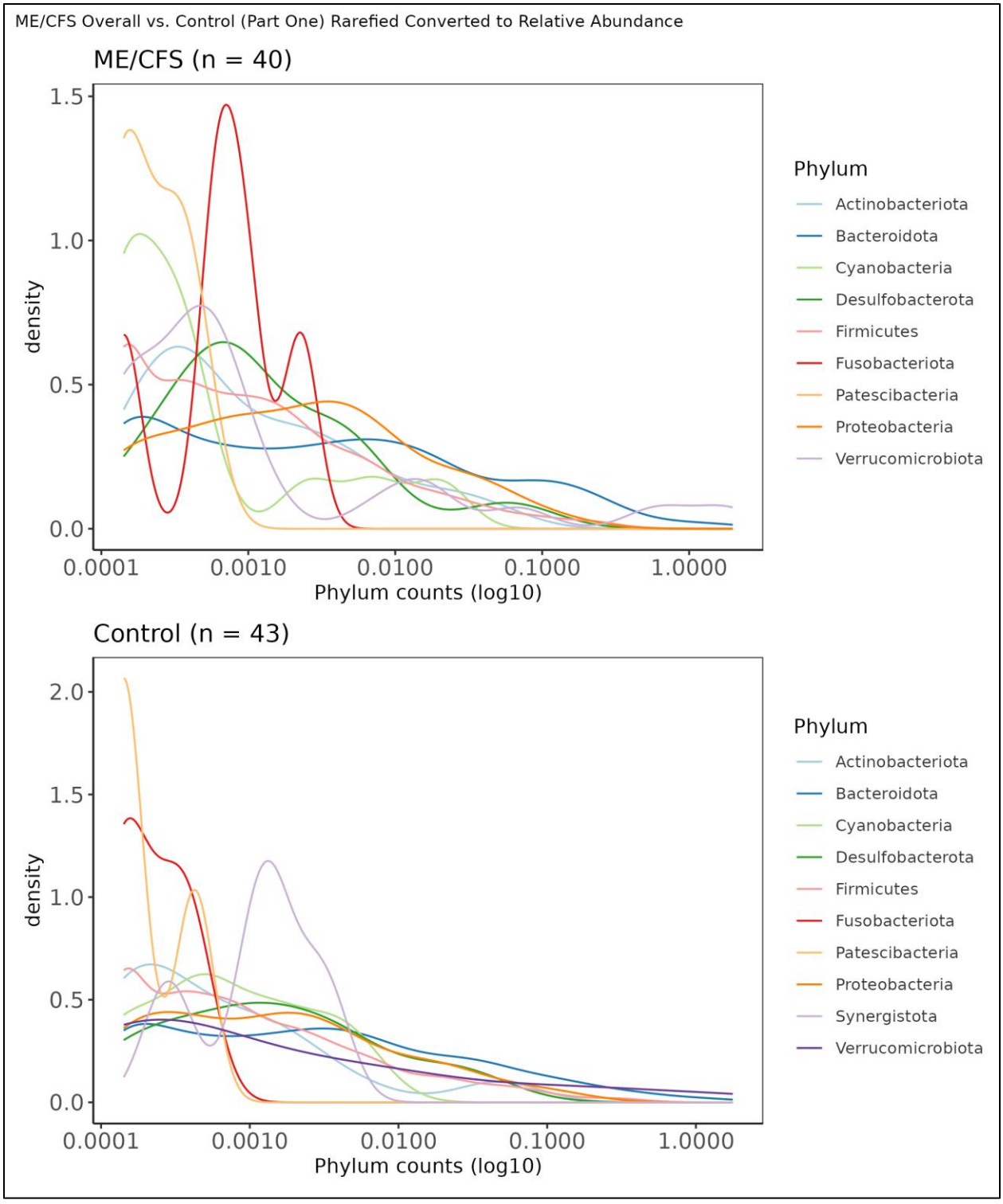



**Figure 3.1.10** Overview of the distribution of the taxonomy at the phylum level from the ME/CFS overall (n = 40) and Control (n = 43) Part One rarefied, converted to relative abundance dataset

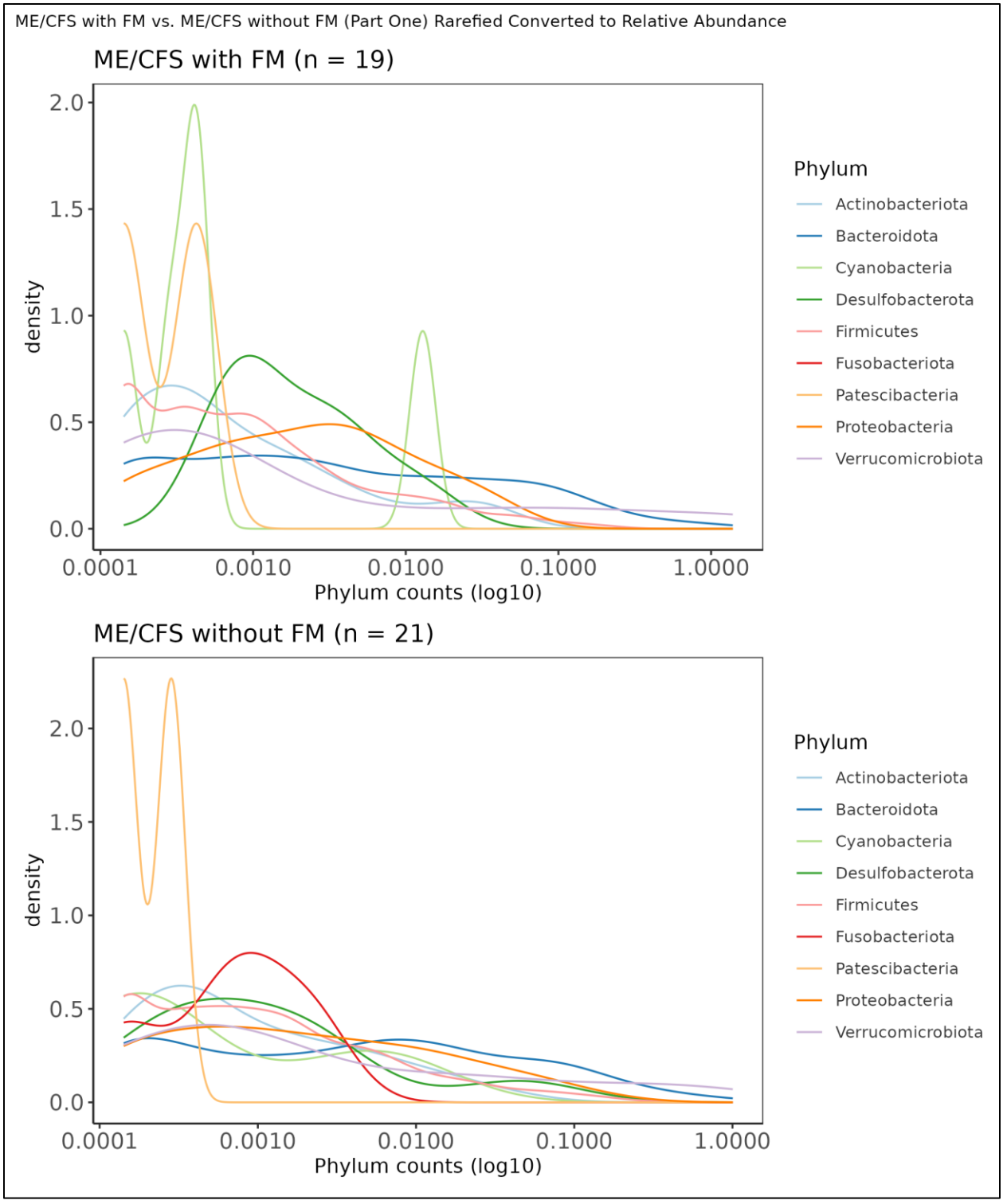



**Figure 3.1.11** Overview of the distribution of the taxonomy at the phylum level from the ME/CFS with FM (n = 19) and ME/CFS without FM (n = 21) Part One rarefied, converted to relative abundance dataset

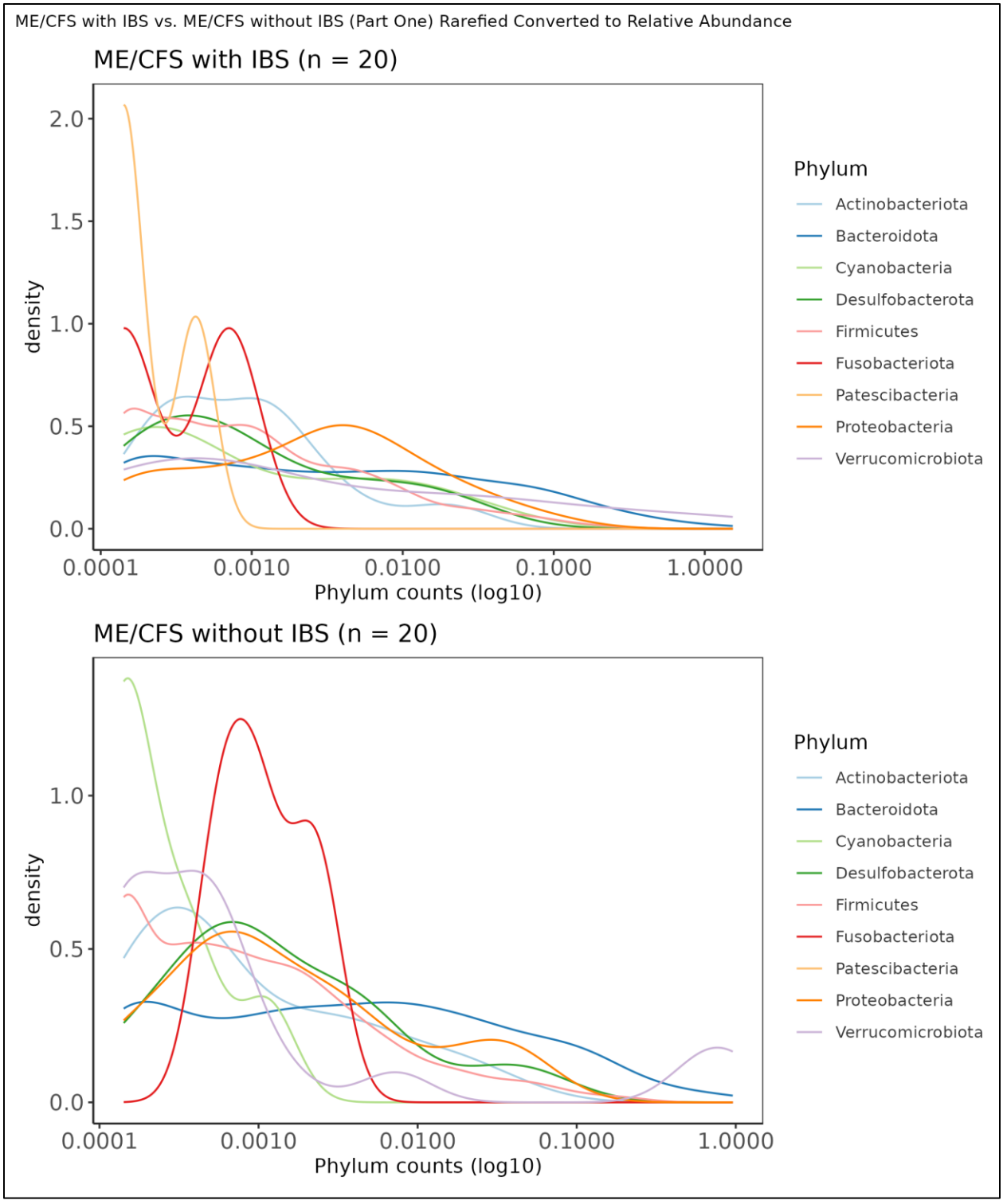



**Figure 3.1.12** Overview of the distribution of the taxonomy at the phylum level from the ME/CFS with IBS (n = 20) and ME/CFS without IBS (n = 20) Part One rarefied, converted to relative abundance dataset

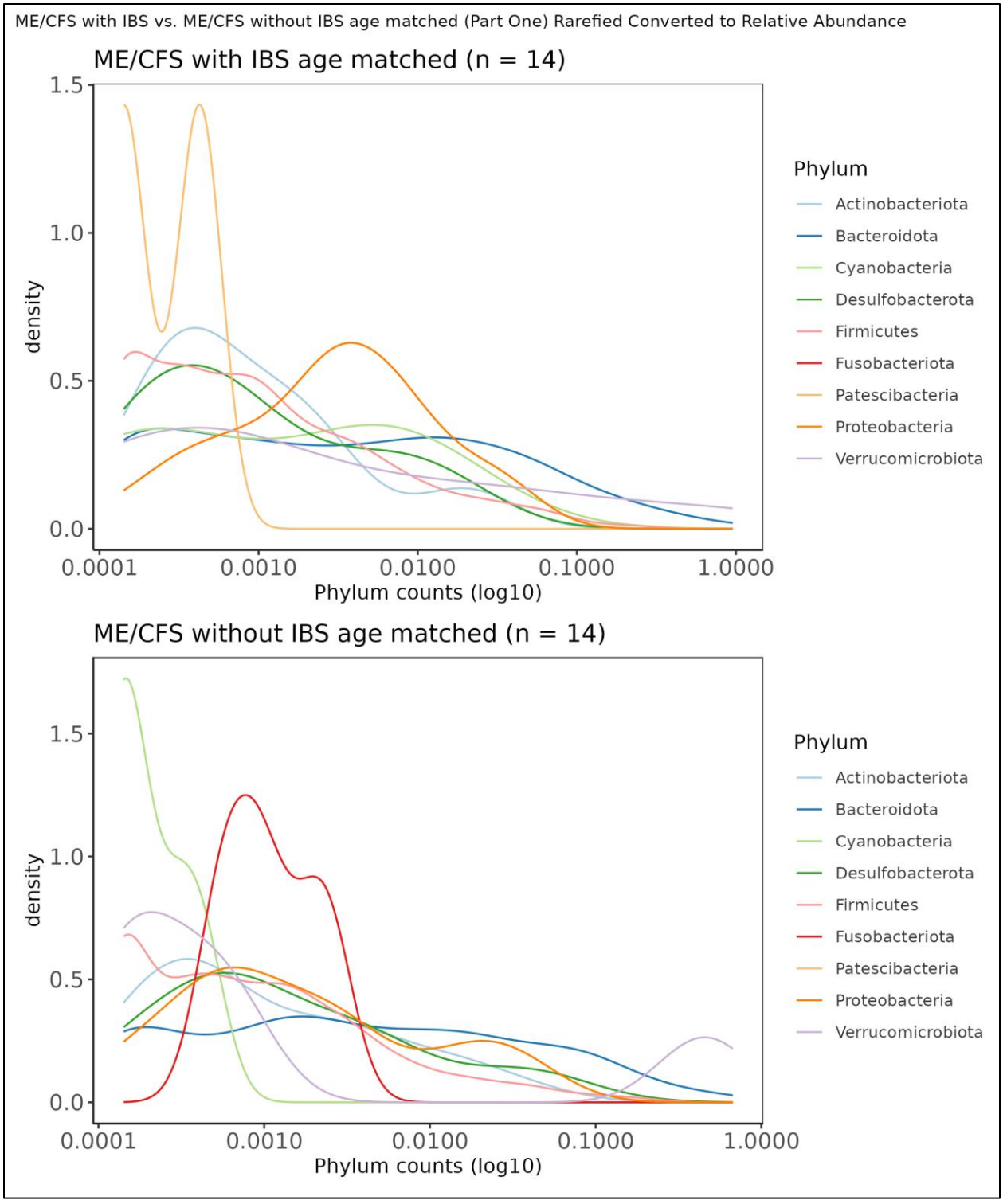



**Figure 3.1.13** Overview of the distribution of the taxonomy at the phylum level from the age-matched ME/CFS with IBS (n = 14) and ME/CFS without IBS (n = 14) Part One rarefied, converted to relative abundance dataset

### 3.1.3 Data Composition (Culture MALDI-TOF)

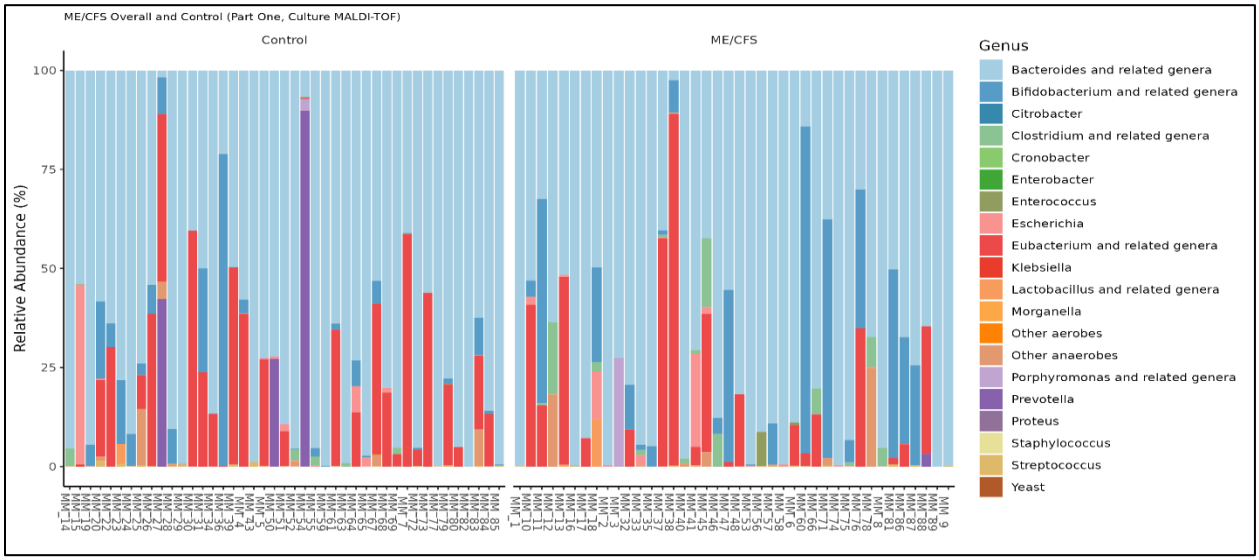



**Figure 3.1.14** Stacked bar plot showing the relative abundance at the genus level from the culture MALDI-TOF dataset in the ME/CFS overall (n = 40) and Control (n = 43) Part One participants.

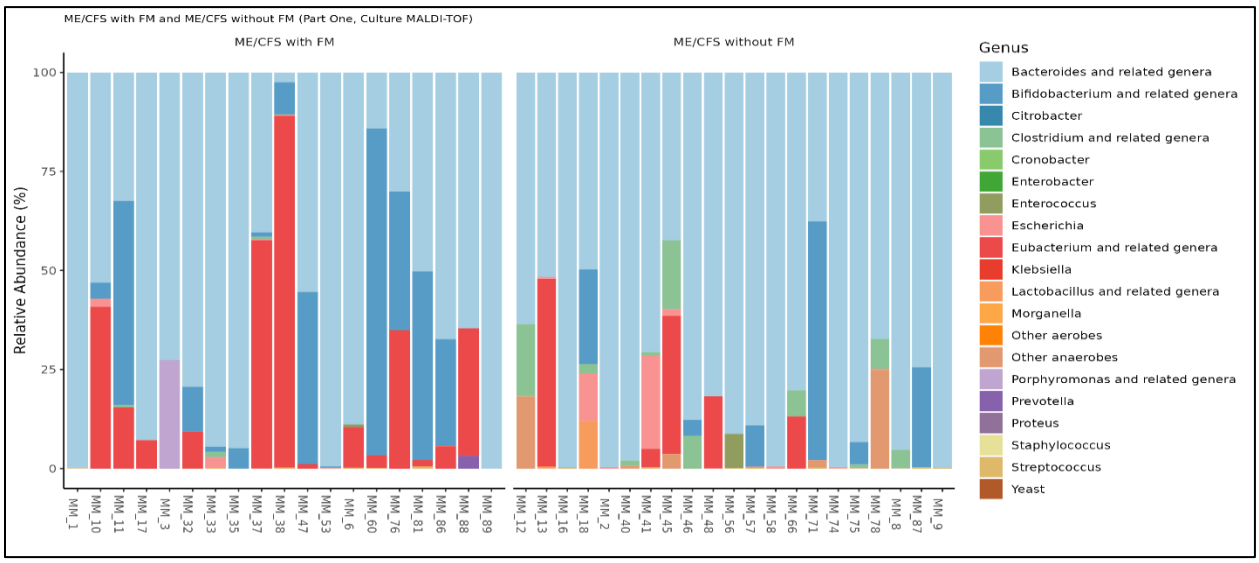



**Figure 3.1.15** Stacked bar plot showing the relative abundance at the genus level from the culture MALDI-TOF dataset in ME/CFS with FM (n = 19) and ME/CFS without FM (n = 21) Part One participants.

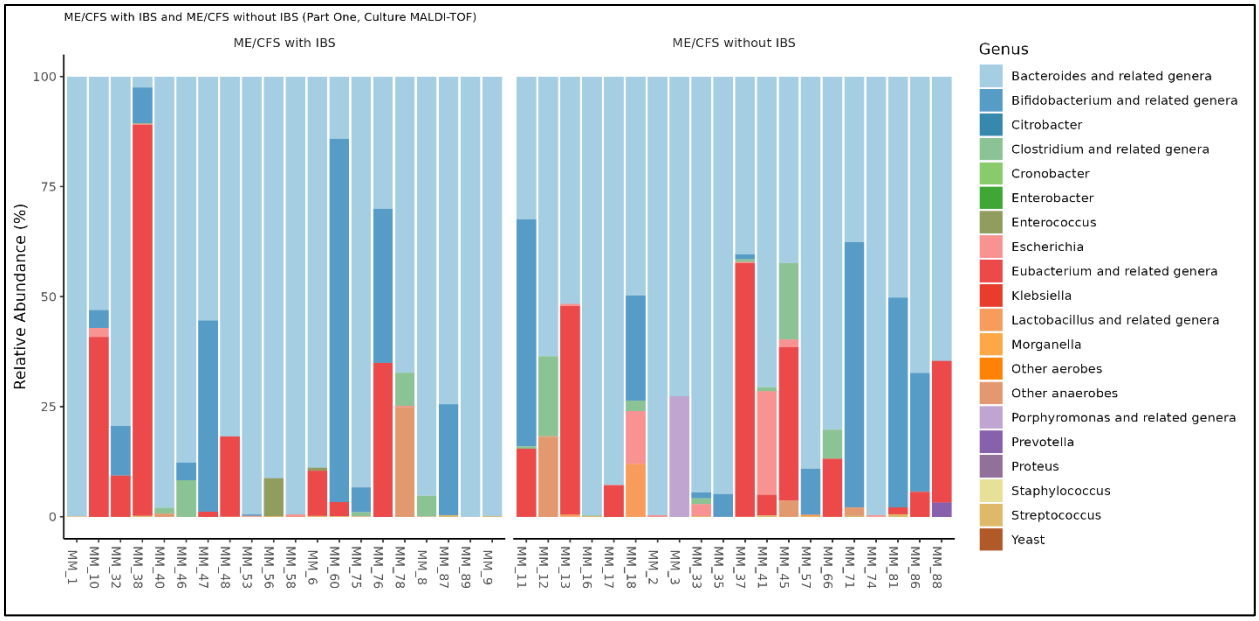



**Figure 3.1.16** Stacked bar plot showing the relative abundance at the genus level from the culture MALDI-TOF dataset in ME/CFS with IBS (n = 20) and ME/CFS without IBS (n = 20) Part One participants.

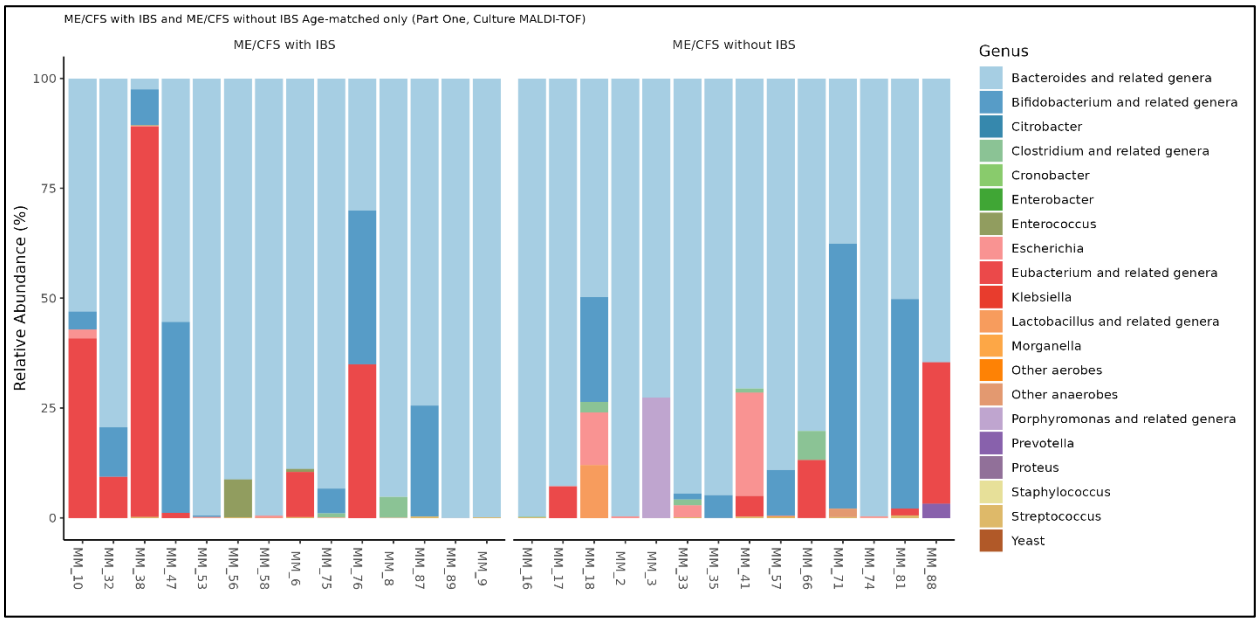



**Figure 3.1.17** Stacked bar plot showing the relative abundance at the genus level from the culture MALDI-TOF dataset in age-matched ME/CFS with IBS (n = 14) and ME/CFS without IBS (n = 14) Part One participants.

## 3.2 Alpha Diversity

Bacterial alpha diversity metrics were calculated from the rarefied 16S rRNA data at the ASV level for all comparisons, Comparison A-D (Figure 3.2.1, Figure 3.2.2, Figure 3.2.3, and Figure 3.2.4, respectively). Although the ME/CFS overall group had a lower number across all alpha diversity metrics, univariate analyses between each comparative group (Comparison A-D) were not significantly different (Table 3.2.1). Alpha diversity findings throughout ME/CFS gut microbiome studies are inconsistent although the general message is that it is reduced or lower compared to non-ME/CFS cohorts (König et al., 2022; Varesi et al., 2021). Some studies have found differences in their cohorts for alpha diversity (Frémont et al., 2013; Giloteaux, Goodrich, et al., 2016; Giloteaux, Hanson, et al., 2016; Guo, Che, et al., 2023; Lupo et al., 2021; Xiong et al., 2023). In some cases, alpha diversity metrics were not extensively presented in their publication which is possibly a missed opportunity for using the data (Armstrong et al., 2017; Nagy-Szakal et al., 2017; Raijmakers et al., 2020; Sheedy et al., 2009; Shukla et al., 2015; Wallis et al., 2018). In other cases, there was no evidence of differences (Seton et al., 2023).

Bacterial alpha diversity metrics were calculated at the species level from the culture MALDI-TOF MS data that had been corrected to its faecal sample dry weight for Comparison A-D (Figure 3.2.5, Figure 3.2.6, Figure 3.2.7, and Figure 3.2.8). The alpha diversity metrics were compared in univariate analyses between each comparative group involved in Comparison A-D (Table 3.2.2). In Comparison A, the Chao 1 index and observed species were significantly different with lower numbers presented in the ME/CFS overall group versus the Control group. There were no statistically significant differences calculated in Comparison B which evaluated the presence or absence of FM comorbidity in ME/CFS. Interestingly, there were statistically significant differences observed in Comparisons C and D for some of the alpha diversity metrics. For Comparison C (ME/CFS +/- IBS), it was the Shannon index, although the Inverse Simpson and Simpson metrics approached a 0.05 alpha significance level. While Comparison D with the ME/CFS +/- IBS groups that have been age-matched were compared to each other throughout this thesis, it was noted that when these groups were compared with the Control group, a statistically significant difference was observed for Chao1 and observed number of species. To some extent, these observations in ME/CFS +/- IBS or age-matched IBS** (Comparison C and D) provide some insight into the IBS comorbidity, and how the overall ME/CFS cohort could be characteristically influenced and distinguished by it.

### 3.2.1 Alpha Diversity (16S rRNA)

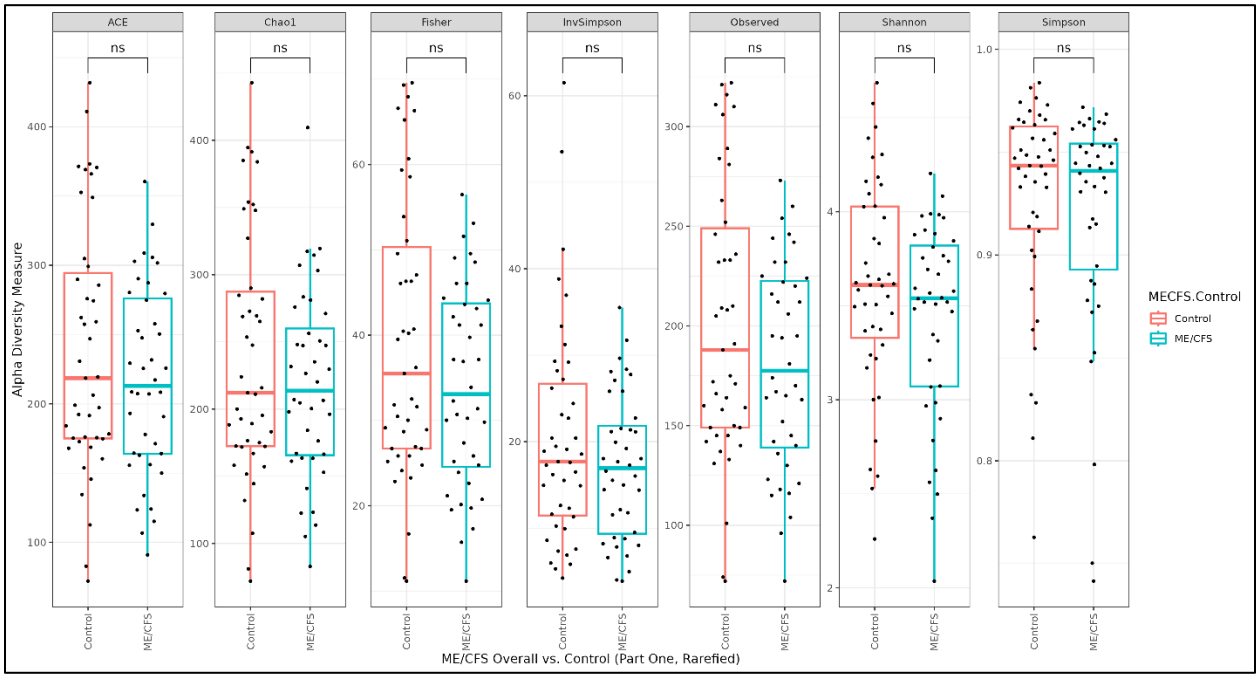



**Figure 3.2.1** Box plots of the alpha diversity from ME/CFS overall and Control groups using different parameters on rarefied data at the ASV level.

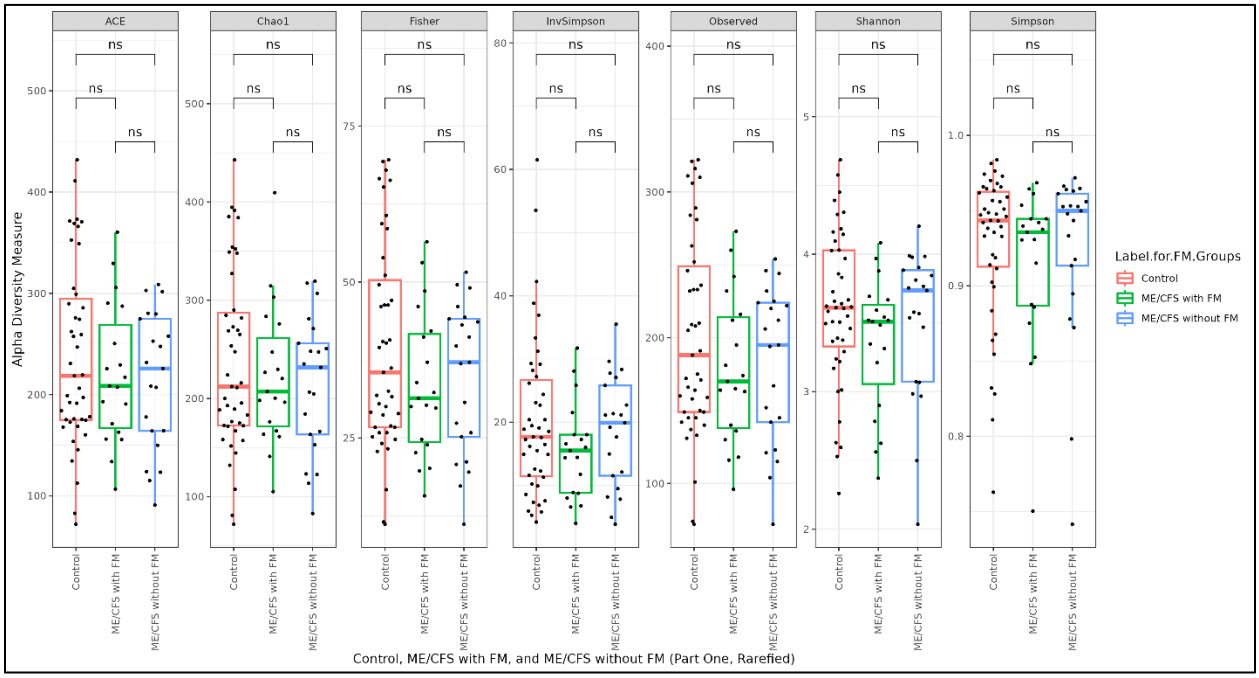



**Figure 3.2.2** Box plots of the alpha diversity from Control, ME/CFS with FM and ME/CFS without FM groups using different parameters on rarefied data at the ASV level.

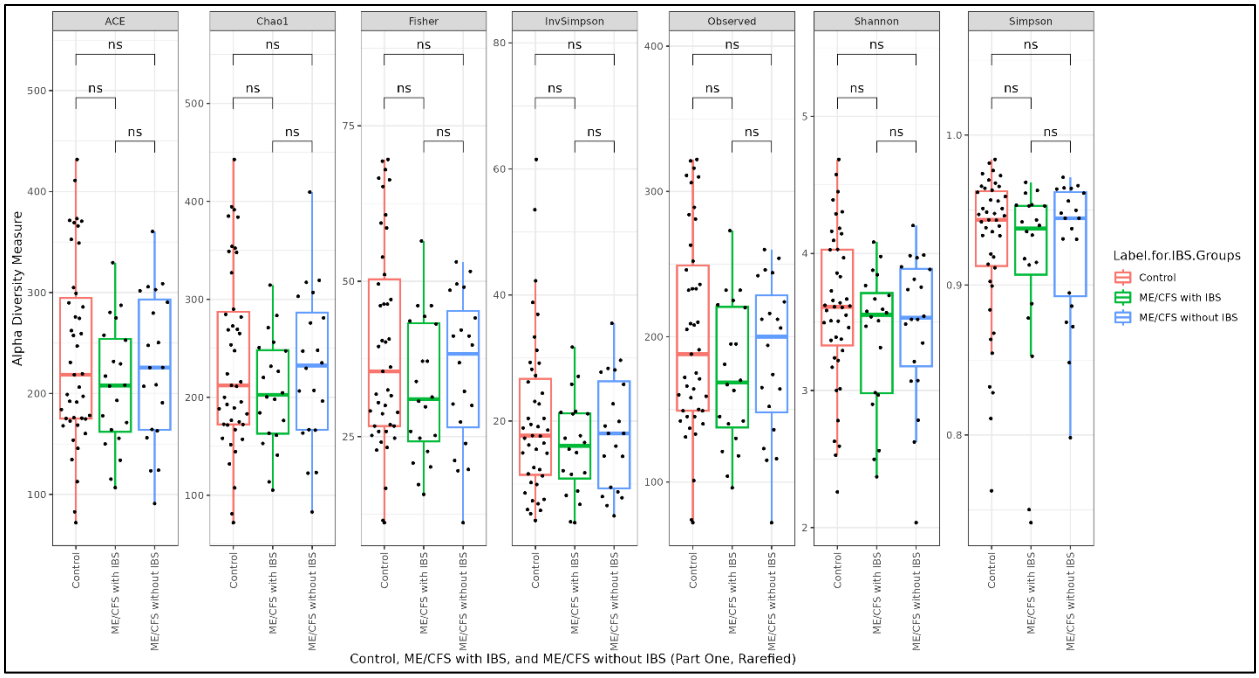



**Figure 3.2.3** Box plots of the alpha diversity from Control, ME/CFS with IBS and ME/CFS without IBS groups using different parameters on rarefied data at the ASV level.

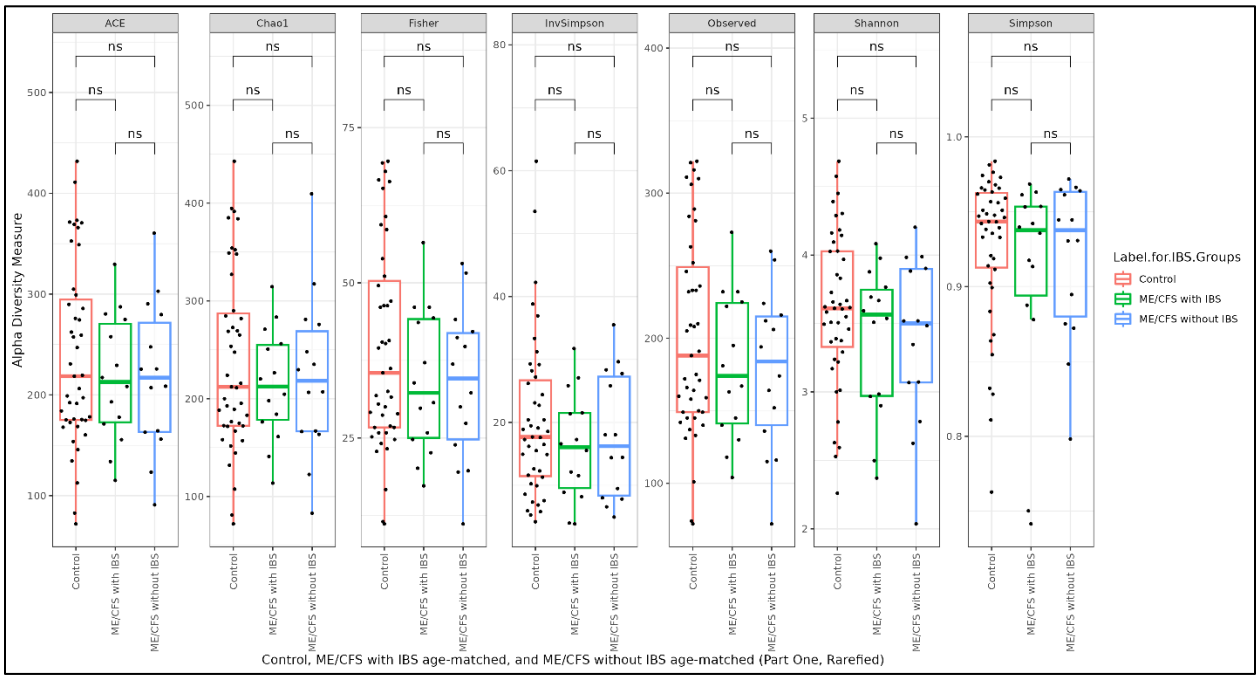



**Figure 3.2.4** Box plots of the alpha diversity from Control, age-matched ME/CFS with IBS and age-matched ME/CFS without IBS groups using different parameters on rarefied data at the ASV level.

**Table 3.2.1** Comparison of alpha diversity metrics from rarefied data at the ASV level

| Part One | Alpha Diversity Metric | | | | | | |
|---|---|---|---|---|---|---|---|
| | ACE | Chao1 | Fisher | Inv Simpson | Observed | Shannon | Simpson |
| All participants (n = 83) | 228 ± 81 | 228 ± 82 | 37 ± 15 | 19 ± 11 | 191 ± 62 | 3.5 ± 0.6 | 0.92 ± 0.05 |
| ME/CFS overall (n = 40) | 217 ± 68 | 218 ± 70 | 34 ± 12 | 17 ± 8 | 180 ± 52 | 3.4 ± 0.5 | 0.92 ± 0.05 |
| Control (n = 43) | 238 ± 90 | 238 ± 92 | 39 ± 17 | 20 ± 13 | 201 ± 69 | 3.6 ± 0.6 | 0.93 ± 0.05 |
| *p-value* † | 0.3961 | 0.4659 | 0.2085 | 0.4063 | 0.2085 | 0.2285 | 0.4063 |
| ME/CFS with FM (n = 19) | 220 ± 69 | 222 ± 72 | 34 ± 12 | 15 ± 8 | 178 ± 51 | 3.5 ± 0.5 | 0.91 ± 0.05 |
| ME/CFS without FM (n = 21) | 214 ± 68 | 214 ± 70 | 35 ± 12 | 19 ± 9 | 182 ± 54 | 3.5 ± 0.6 | 0.92 ± 0.06 |
| *p-value* † | 0.9147 | 1 | 0.7349 | 0.2257 | 0.7349 | 0.145 | 0.2257 |
| p-value ‡ | 0.6884 | 0.7499 | 0.432 | 0.332 | 0.432 | 0.203 | 0.332 |
| ME/CFS with IBS (n = 20) | 207 ± 61 | 205 ± 56 | 33 ± 11 | 16 ± 8 | 174 ± 50 | 3.4 ± 0.5 | 0.91 ± 0.07 |
| ME/CFS without IBS (n = 20) | 226 ± 74 | 231 ± 81 | 36 ± 12 | 18 ± 9 | 186 ± 54 | 3.5 ± 0.5 | 0.93 ± 0.05 |
| *p-value* † | 0.3983 | 0.2648 | 0.4407 | 0.4777 | 0.4407 | 0.5117 | 0.4777 |
| p-value ‡ | 0.4924 | 0.4724 | 0.3177 | 0.5576 | 0.3177 | 0.4045 | 0.5576 |
| ME/CFS with IBS age matched (n = 14) | 217 ± 63 | 214 ± 57 | 34 ± 12 | 16 ± 9 | 181 ± 51 | 3.4 ± 0.6 | 0.91 ± 0.07 |
| ME/CFS without IBS age matched (n = 14) | 218 ± 75 | 222 ± 84 | 34 ± 13 | 18 ± 10 | 178 ± 55 | 3.4 ± 0.6 | 0.92 ± 0.05 |
| *p-value* † | 0.982 | 0.8388 | 0.8722 | 0.7345 | 0.8722 | 0.9459 | 0.7345 |
| p-value ‡ | 0.7462 | 0.825 | 0.49 | 0.5996 | 0.49 | 0.4327 | 0.5996 |

Values are shown as mean ± standard deviation

Alpha diversity comparisons of two groups was done using a Wilcoxon rank sum test †

Alpha diversity comparisons among multiple groups (Control, ME/CFS with Subgrouping of Interest, ME/CFS without Subgrouping of Interest) was done using a Kruskall-Wallis test ‡

### 3.2.2 Alpha Diversity (Culture MALDI-TOF)

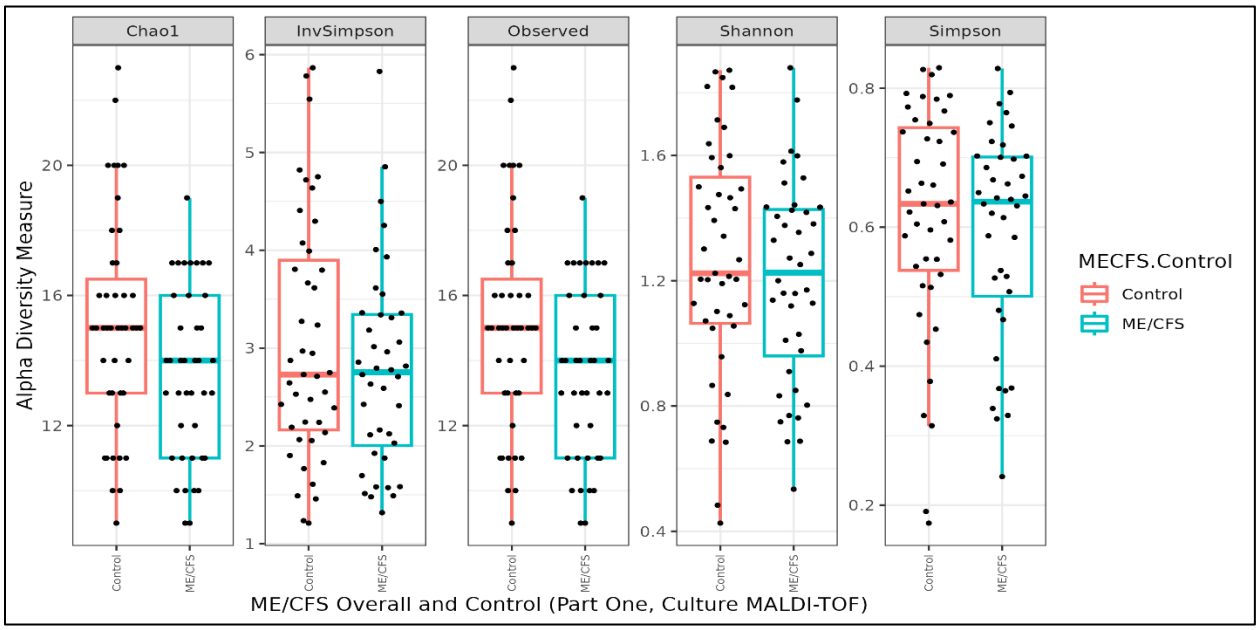



**Figure 3.2.5** Box plots of the alpha diversity from ME/CFS overall and Control groups using different parameters on the culture MALDI-TOF data at the species level.

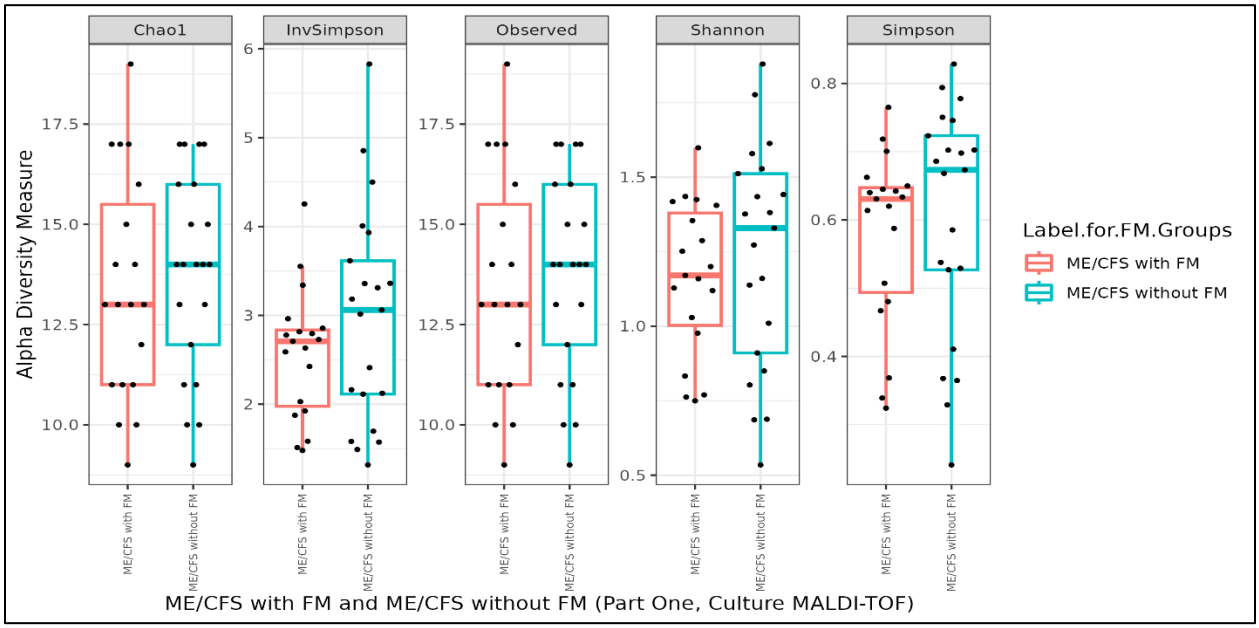



**Figure 3.2.6** Box plots of the alpha diversity from ME/CFS with FM and ME/CFS without FM groups using different parameters on the culture MALDI-TOF data at the species level.

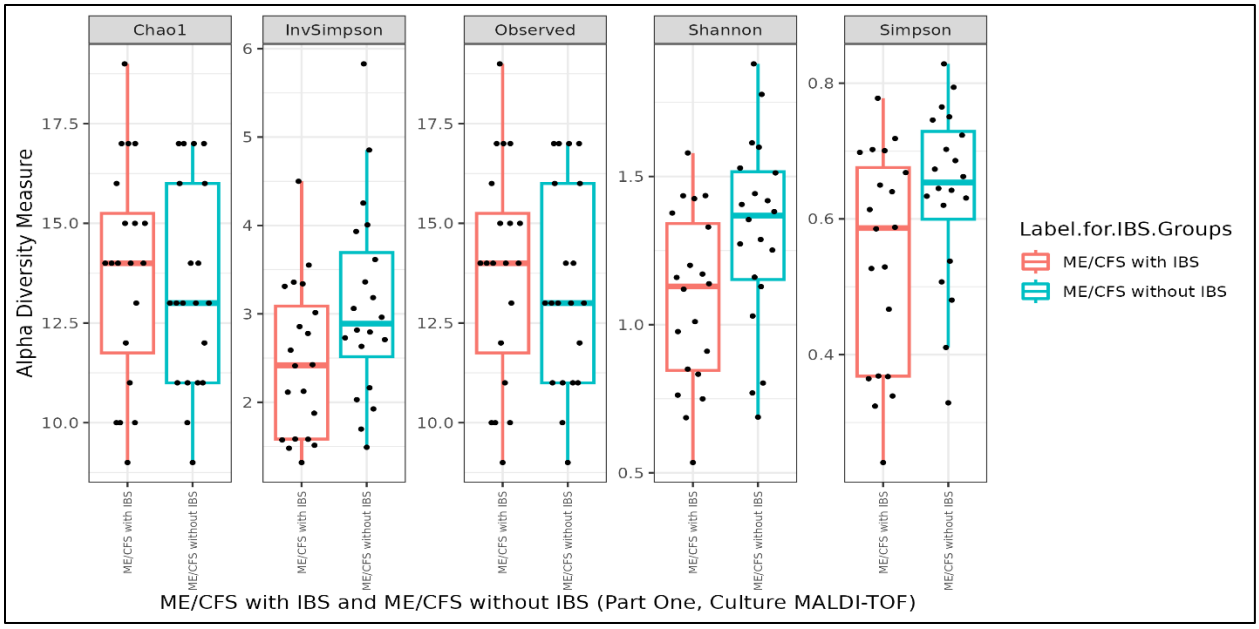



**Figure 3.2.7** Box plots of the alpha diversity from ME/CFS with IBS and ME/CFS without IBS groups using different parameters on the culture MALDI-TOF data at the species level.

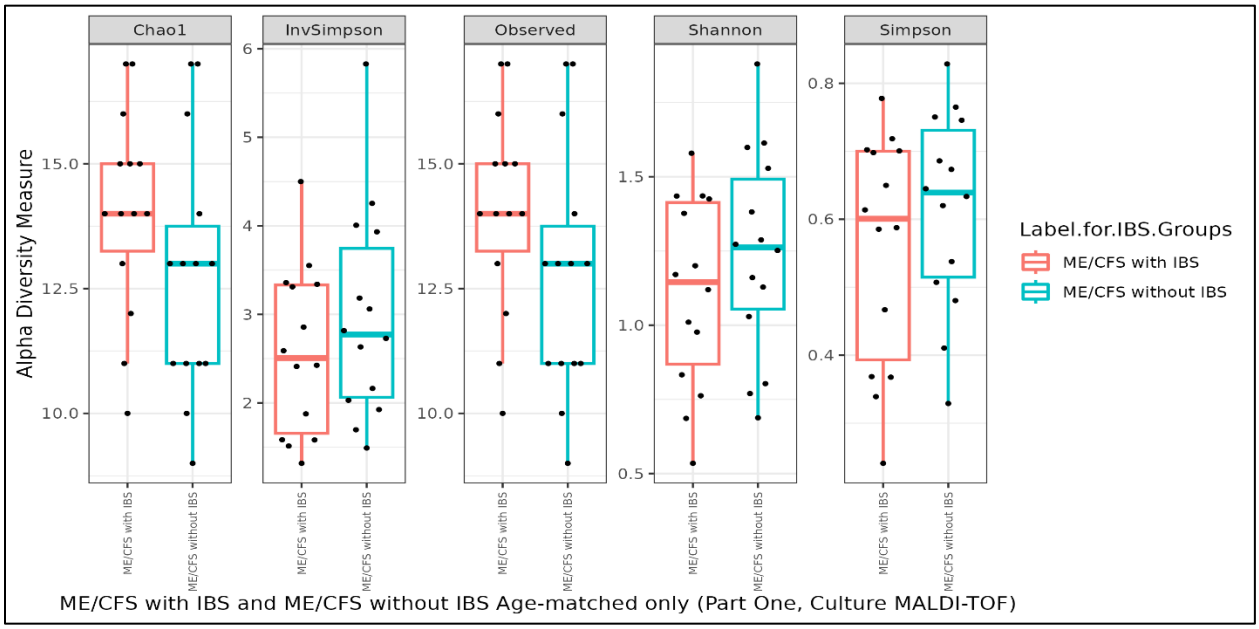



**Figure 3.2.8** Box plots of the alpha diversity from age-matched ME/CFS with IBS and age-matched ME/CFS without IBS groups using different parameters on the culture MALDI-TOF data at the species level.

**Table 3.2.2** Comparison of alpha diversity metrics from faecal culture MALDI-TOF data at the species level

| Part One | Alpha Diversity Metric | | | | |
|---|---|---|---|---|---|
| | **Chao1** | **Inv Simpson** | **Observed** | **Shannon** | **Simpson** |
| All participants (n = 83) | 14.4 ± 3.1 | 2.9 ± 1.2 | 14.4 ± 3.1 | 1.2 ± 0.4 | 0.6 ± 0.2 |
| ME/CFS overall (n = 40) | 13.6 ± 2.6 | 2.8 ± 1.0 | 13.6 ± 2.6 | 1.2 ± 0.3 | 0.6 ± 0.2 |
| Control (n = 43) | 15.1 ± 3.3 | 3.1 ± 1.3 | 15.1 ± 3.3 | 1.3 ± 0.4 | 0.6 ± 0.2 |
| *p-value* † | 0.04556 * | 0.4379 | 0.04556 * | 0.4012 | 0.4379 |
| ME/CFS with FM (n = 19) | 13.4 ± 2.8 | 2.6 ± 0.7 | 13.4 ± 2.8 | 1.2 ± 0.3 | 0.6 ± 0.1 |
| ME/CFS without FM (n = 21) | 13.8 ± 2.5 | 3.0 ± 1.2 | 13.8 ± 2.5 | 1.2 ± 0.4 | 0.6 ± 0.2 |
| *p-value* † | 0.5847 | 0.2701 | 0.5847 | 0.3902 | 0.2701 |
| p-value ‡ | 0.1197 | 0.4636 | 0.1197 | 0.4805 | 0.4636 |
| ME/CFS with IBS (n = 20) | 13.8 ± 2.8 | 2.5 ± 0.9 | 13.8 ± 2.8 | 1.1 ± 0.3 | 0.5 ± 0.2 |
| ME/CFS without IBS (n = 20) | 13.4 ± 2.5 | 3.1 ± 1.1 | 13.4 ± 2.5 | 1.3 ± 0.3 | 0.6 ± 0.1 |
| *p-value* † | 0.5759 | 0.05589 | 0.5759 | 0.02447 * | 0.05589 |
| p-value ‡ | 0.1216 | 0.1362 | 0.1216 | 0.06243 | 0.1362 |
| ME/CFS with IBS age matched (n = 14) | 14.1 ± 2.1 | 2.6 ± 1.0 | 14.1 ± 2.1 | 1.1 ± 0.3 | 0.6 ± 0.2 |
| ME/CFS without IBS age matched (n = 14) | 12.8 ± 2.5 | 3.0 ± 1.2 | 12.8 ± 2.5 | 1.2 ± 0.4 | 0.6 ± 0.2 |
| *p-value* † | 0.1198 | 0.4544 | 0.1198 | 0.3287 | 0.4544 |
| p-value ‡ | 0.04924 * | 0.5402 | 0.04924 * | 0.3402 | 0.5402 |

Values are shown as mean ± standard deviation

Alpha diversity comparisons of two groups was done using a Wilcoxon rank sum test †

Alpha diversity comparisons among multiple groups (Control, ME/CFS with Subgrouping of Interest, ME/CFS without Subgrouping of Interest) was done using a Kruskall-Wallis test ‡

* p-value < 0.05

## 3.3 Beta Diversity

Two approaches to beta diversity were calculated for the 16S rRNA data (Section 2.6.2.2) at the amplicon sequence variant (ASV), family, genus, and species levels. Briefly, the first approach was unweighted and weighted Unifrac metrics that were ordinated using principal coordinate analysis (PCoA) (Figure 3.3.1, Figure 3.3.2, Figure 3.3.3, and Figure 3.3.4 for Comparison A-D, respectively in Section 3.3.1). The second approach was Aitchison distances that were ordinated using principal component analysis (PCA) (Figure 3.3.5, Figure 3.3.6, Figure 3.3.7, and Figure 3.3.8 for Comparison A-D, respectively in Section 3.3.2). Beta diversity differences/dissimilarities were observed in this study. For similar reasons stated in the previous sections with overall composition and alpha diversity, other ME/CFS studies have inconsistently found differences in their beta diversity calculations. However, the beta diversity findings provide support for the overall gut dysbiosis hypothesis in ME/CFS.

Each plot in Comparison A-D of the 16S rRNA data, by visual inspection, showed that the samples could be clustered together differently depending on how it was defined with their FM or IBS comorbidity. The majority of the ellipsoids appeared to be broad clusters. However; tighter clusters were observed in Figure 3.3.2 H, Figure 3.3.3 H, and Figure 3.3.4 H which signalled a possible association or commonality due to the IBS comorbidity. Table 3.3.1 and Table 3.3.2 summarise the PERMANOVA and PERMDISP analyses that tested and assessed beta diversity statistical differences. Both approaches showed a statistically significant difference between the ME/CFS overall and Control groups (Comparison A) at all levels. Neither approach showed any differences in ME/CFS +/- FM (Comparison B). Both approaches showed statistically significant differences in ME/CFS +/- IBS or age-matched IBS** (Comparison C and D) at some levels. This indicated that beta diversity differences are associated with IBS comorbidity and that some distinction of these samples could be achieved out of the entire cohort.

Similarly, two approaches were used to calculate beta diversity for the culture MALDI-TOF data (Section 2.6.6.2). Briefly, the first approach used the Bray Curtis metric, and the second approach used the Jaccard metric. Both approaches were ordinated using PCoA; plots are presented in the figures together with the Bray Curtis metric outcomes first, followed by the Jaccard metric outcomes. Figure 3.3.9, Figure 3.3.10, Figure 3.3.11, and Figure 3.3.12 present the beta diversity outcomes for Comparison A-D, respectively in Section 3.3.3. Unlike the 16S

rRNA data stratifications with IBS and FM in Comparisons C-D where IBS appeared to be significantly different, and FM not significantly different, the culture MALDI-TOF statistical analyses revealed the opposite (Table 3.3.3). Table 3.3.3 summarises the PERMANOVA AND PERMDISP analyses; while it shows that there was a significant difference between the ME/CFS +/- FM groups in beta diversity (PERMANOVA), the PERMDISP outcomes indicated that the groups do not have similar dispersion. In other words, the significant difference in PERMDSIP of the ME/CFS +/- FM groups signified that there might be dispersion or variability in these groups which needs to be considered further with the PERMANOVA outcomes.

### 3.3.1 Unifrac Beta Diversity (16S rRNA)

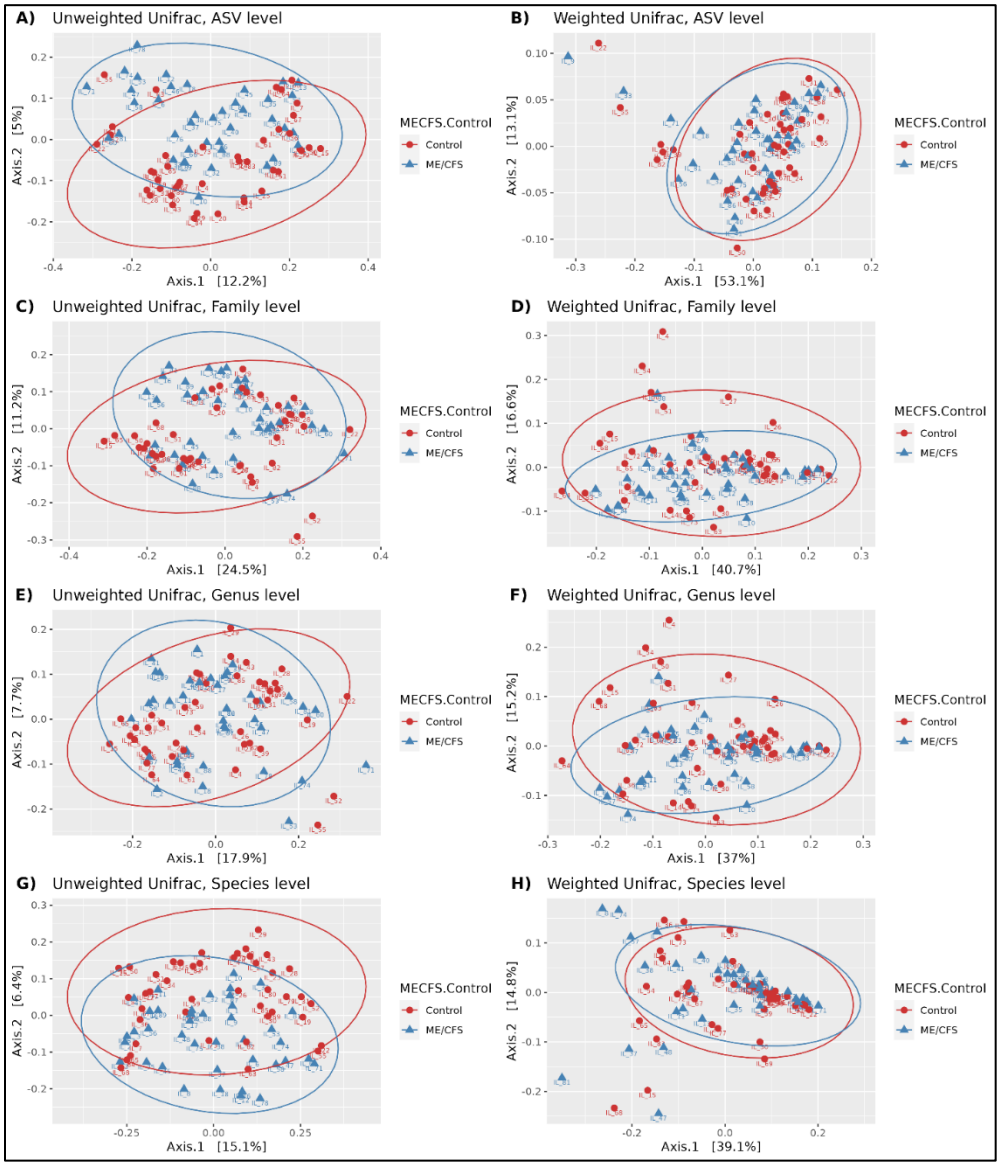



**Figure 3.3.1** PCoAs based on the unweighted and weighted Unifrac distances showing the beta diversity between the ME/CFS overall and Control groups at the ASV, family, genus, and species taxonomic levels. The ellipses represent 95% confidence intervals for each group.

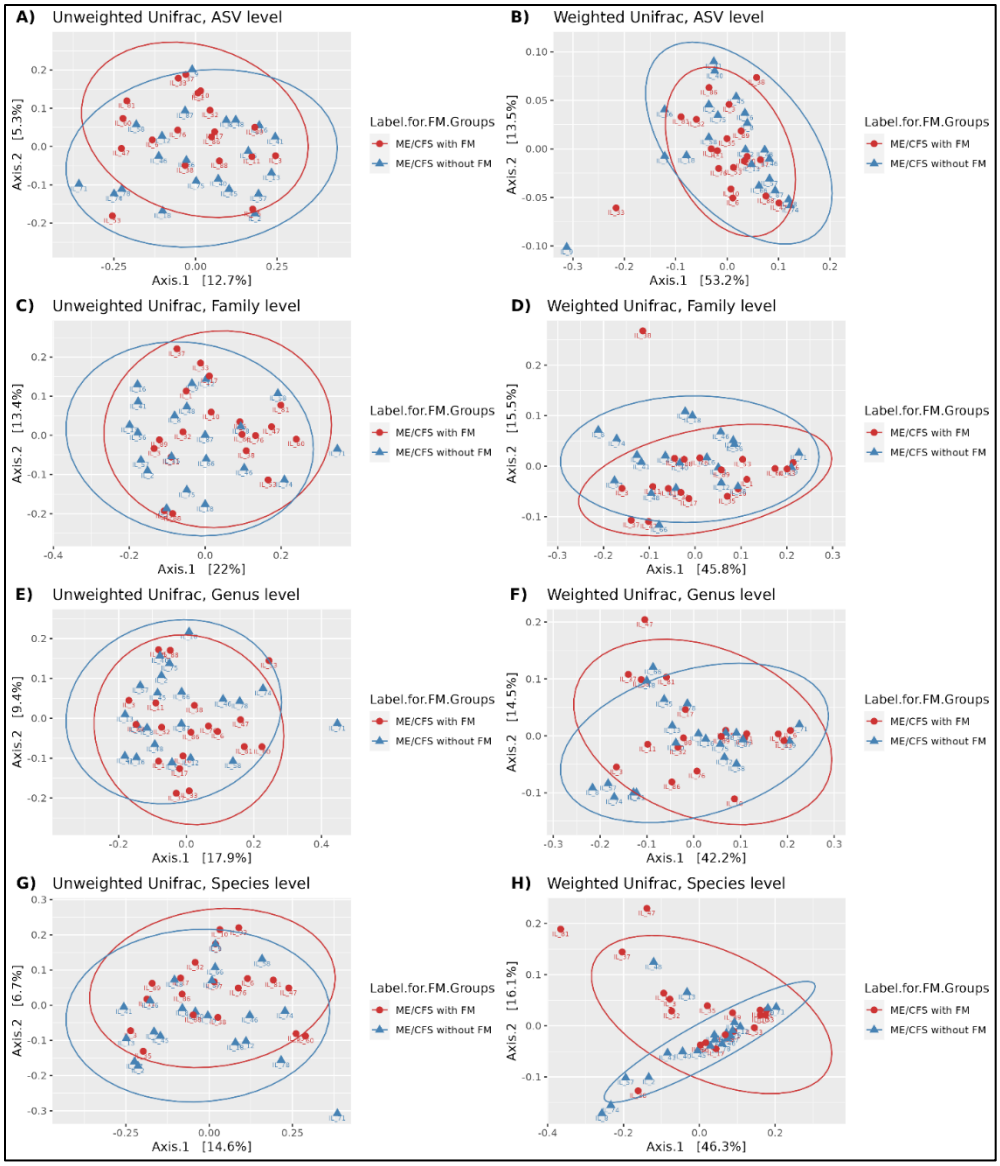



**Figure 3.3.2** PCoAs based on the unweighted and weighted Unifrac distances showing the beta diversity between the ME/CFS with FM and ME/CFS without FM groups at the ASV, family, genus, and species taxonomic levels. The ellipses represent 95% confidence intervals for each group.

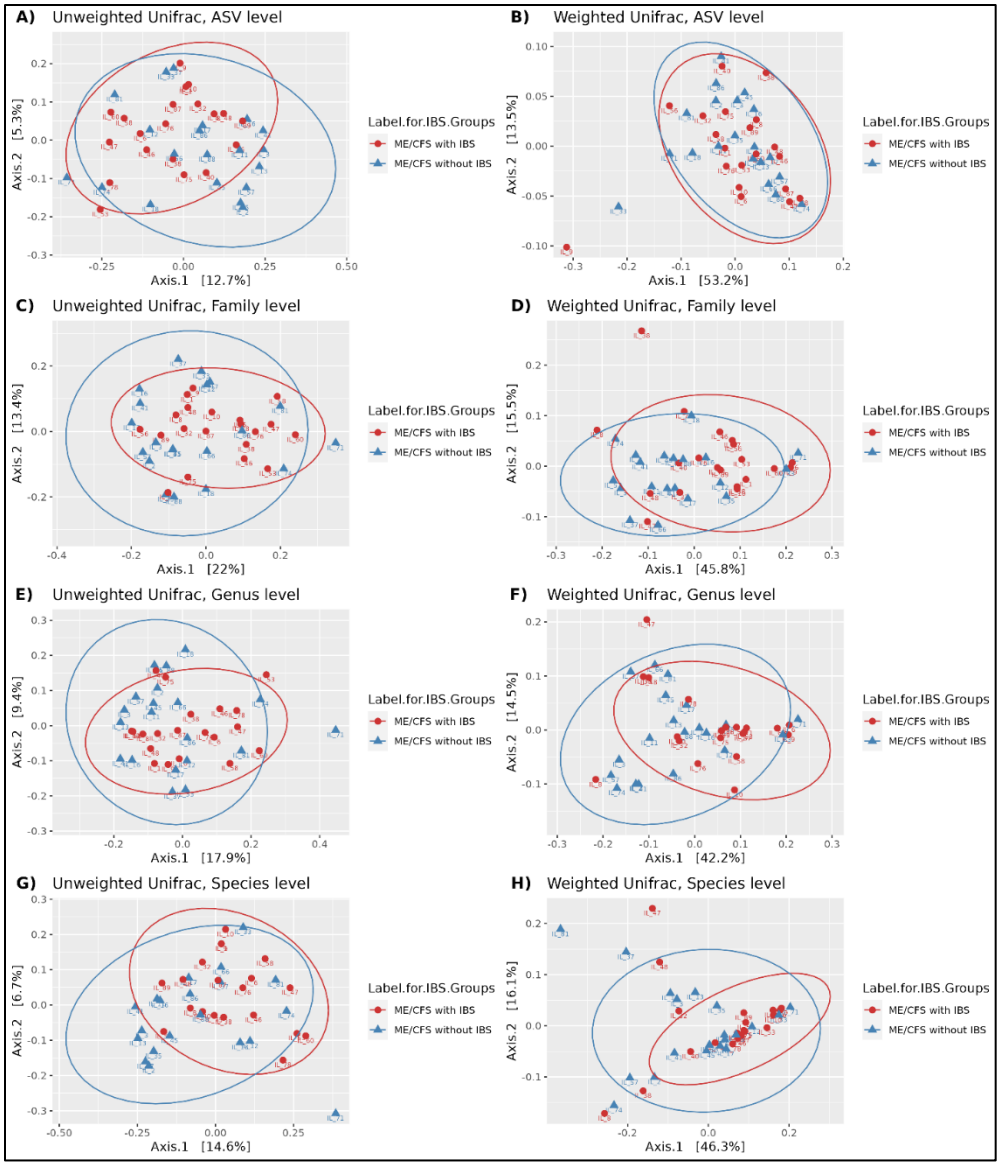



**Figure 3.3.3** PCoAs based on the unweighted and weighted Unifrac distances showing the beta diversity between the ME/CFS with IBS and ME/CFS without IBS groups at the ASV, family, genus, and species taxonomic levels. The ellipses represent 95% confidence intervals for each group.

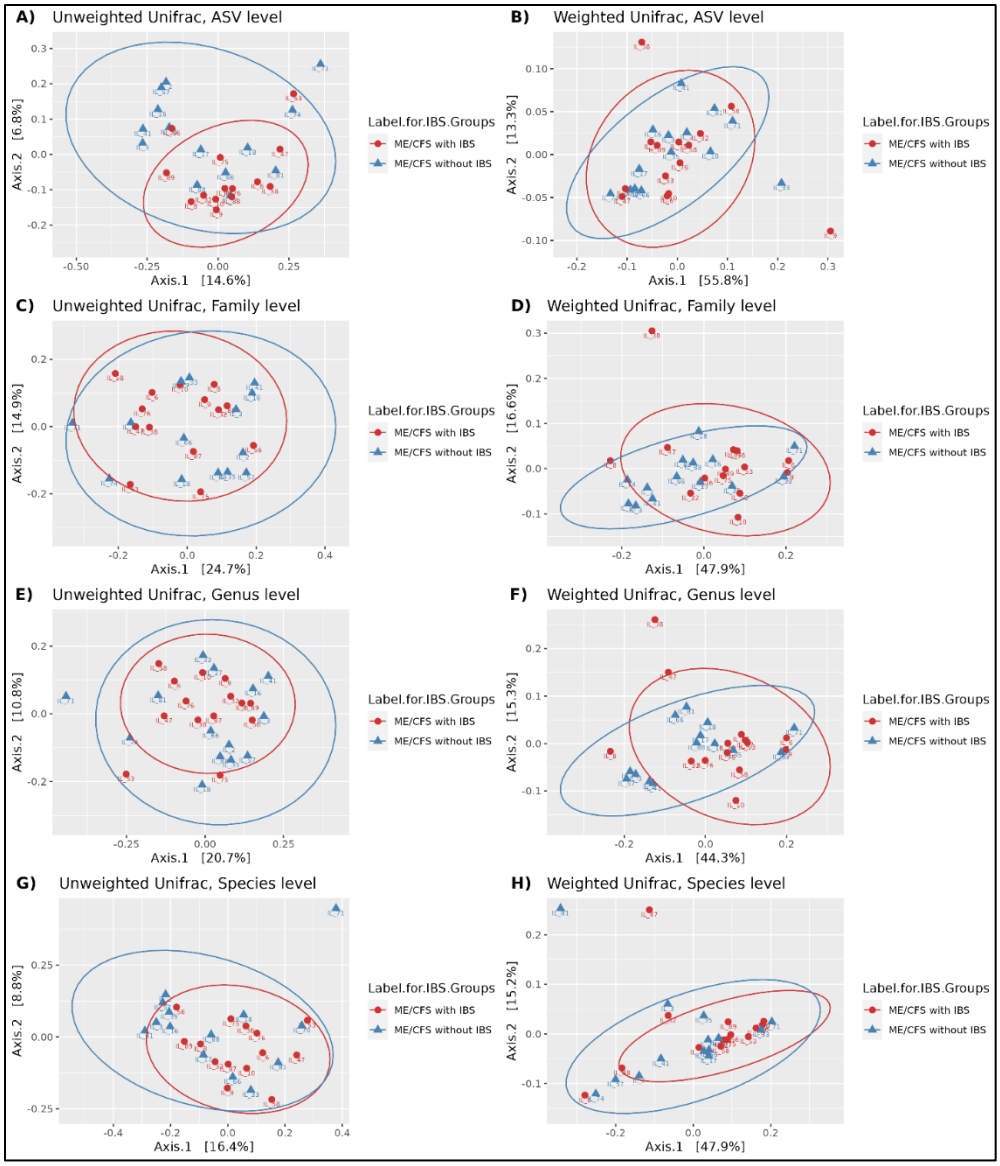



**Figure 3.3.4** PCoAs based on the unweighted and weighted Unifrac distances showing the beta diversity between the Age-matched ME/CFS with IBS and ME/CFS without IBS groups at the ASV, family, genus, and species taxonomic levels. The ellipses represent 95% confidence intervals for each group.

**Table 3.3.1** PERMANOVA and PERMDISP statistical analyses of unweighted and weighted Unifrac beta diversity metrics using the 16S rRNA data for the comparative groups of interest at the ASV, family, genus, and species levels

| PART ONE | PERMANOVA | | | PERMDISP | |
|---|---|---|---|---|---|
| | R2 | Pseudo-F | Pr(>F) | F | Pr(>F) |
| **ME/CFS overall vs. Control** | | | | | |
| ***Unweighted UniFrac*** | | | | | |
| ASV level | 0.02418 | 2.0069 | 0.000999 *** | 1.4477 | 0.2228 |
| Family level | 0.02709 | 2.2552 | 0.01399 * | 0.0097 | 0.9271 |
| Genus level | 0.02808 | 2.3403 | 0.003996 ** | 0.2641 | 0.6364 |
| Species level | 0.02616 | 2.176 | 0.000999 *** | 0.3712 | 0.5894 |
| ***Weighted UniFrac*** | | | | | |
| ASV level | 0.00896 | 0.7321 | 0.5894 | 0.0388 | 0.8541 |
| Family level | 0.01391 | 1.1423 | 0.3017 | 1.4576 | 0.2288 |
| Genus level | 0.01634 | 1.3452 | 0.2048 | 0.8625 | 0.3497 |
| Species level | 0.01649 | 1.3579 | 0.1938 | 0.0034 | 0.9441 |
| **ME/CFS with FM vs. ME/CFS without FM** | | | | | |
| ***Unweighted UniFrac*** | | | | | |
| ASV level | 0.02499 | 0.9741 | 0.5165 | 2.5195 | 0.1219 |
| Family level | 0.02738 | 1.0696 | 0.3786 | 3.1689 | 0.08392 |
| Genus level | 0.02407 | 0.9374 | 0.5345 | 1.1814 | 0.2987 |
| Species level | 0.02829 | 1.1064 | 0.2687 | 1.1911 | 0.3177 |
| ***Weighted UniFrac*** | | | | | |
| ASV level | -0.00037 | -0.0139 | 1 | 1.1556 | 0.3287 |
| Family level | 0.02147 | 0.8338 | 0.4765 | 0.0277 | 0.8741 |
| Genus level | 0.01912 | 0.7405 | 0.5864 | 0.1189 | 0.7283 |
| Species level | 0.03116 | 1.222 | 0.2657 | 0.0014 | 0.96 |
| **ME/CFS with IBS vs. ME/CFS without IBS** | | | | | |
| ***Unweighted UniFrac*** | | | | | |
| ASV level | 0.03344 | 1.3147 | 0.05195 | 1.2041 | 0.3057 |
| Family level | 0.02791 | 1.0909 | 0.3586 | 0.2128 | 0.6593 |
| Genus level | 0.02905 | 1.1371 | 0.2757 | 0.1402 | 0.7263 |
| Species level | 0.04111 | 1.6292 | 0.01698 * | 0.0792 | 0.8132 |
| ***Weighted UniFrac*** | | | | | |
| ASV level | 0.00836 | 0.3204 | 0.9211 | 0.0008 | 0.981 |
| Family level | 0.06338 | 2.5715 | 0.04196 * | 0.0003 | 0.988 |
| Genus level | 0.06073 | 2.4569 | 0.03996 * | 0.0078 | 0.9401 |
| Species level | 0.0439 | 1.7446 | 0.1289 | 0.2159 | 0.6633 |
| **Age-matched ME/CFS with IBS vs. ME/CFS without IBS** | | | | | |
| ***Unweighted UniFrac*** | | | | | |
| ASV level | 0.04415 | 1.201 | 0.1069 | 3.1536 | 0.08591 |
| Family level | 0.03684 | 0.9945 | 0.4286 | 2.4496 | 0.1289 |
| Genus level | 0.03998 | 1.0827 | 0.3277 | 1.503 | 0.2507 |
| Species level | 0.05028 | 1.3766 | 0.06494 | 0.5941 | 0.4655 |
| ***Weighted UniFrac*** | | | | | |
| ASV level | 0.0112 | 0.2944 | 0.9301 | 0.0494 | 0.8272 |
| Family level | 0.0511 | 1.4002 | 0.1898 | 0.2991 | 0.5944 |
| Genus level | 0.05162 | 1.4152 | 0.1748 | 0.4008 | 0.5425 |
| Species level | 0.03854 | 1.0422 | 0.3307 | 0.2237 | 0.6074 |
| 1000 permutations used for all tests | | | | | |
| Significance Codes (Pr(>F)): 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1 | | | | | |
| All Pr(>F) values are uncorrected P-values | | | | | |

### 3.3.2 Aitchison Beta Diversity (16S rRNA)

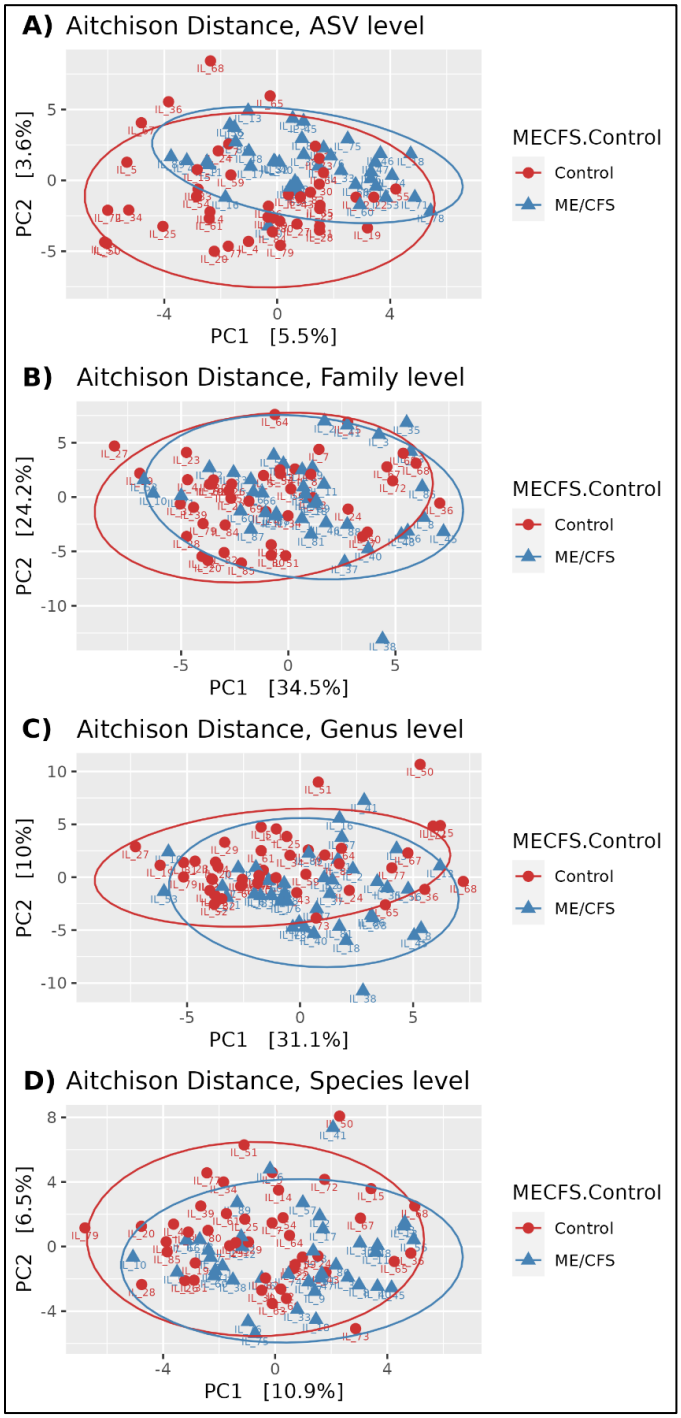



**Figure 3.3.5** PCAs based on the Aitchison distances showing the beta diversity between the ME/CFS overall and Control groups at the ASV, family, genus, and species taxonomic levels. The ellipses represent 95% confidence intervals for each group.

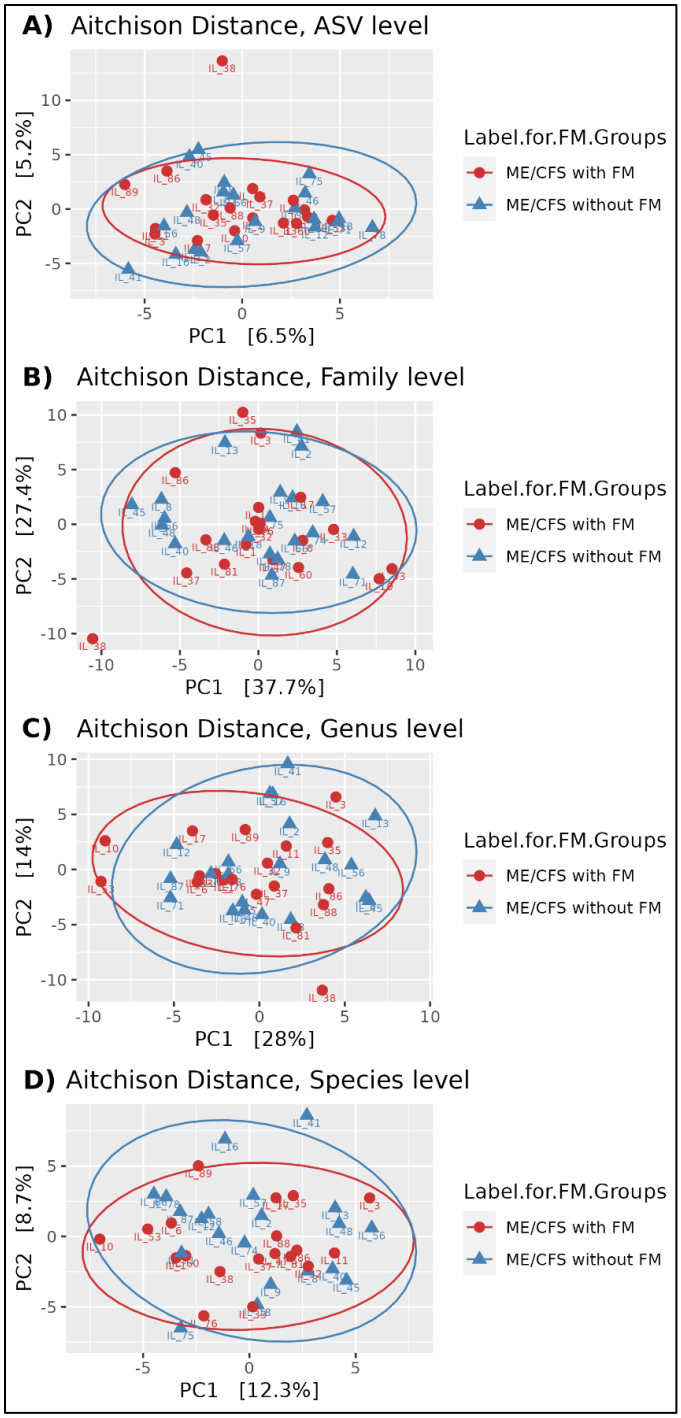



**Figure 3.3.6** PCAs based on the Aitchison distances showing the beta diversity between the ME/CFS with FM and ME/CFS without FM groups at the ASV, family, genus, and species taxonomic levels. The ellipses represent 95% confidence intervals for each group.

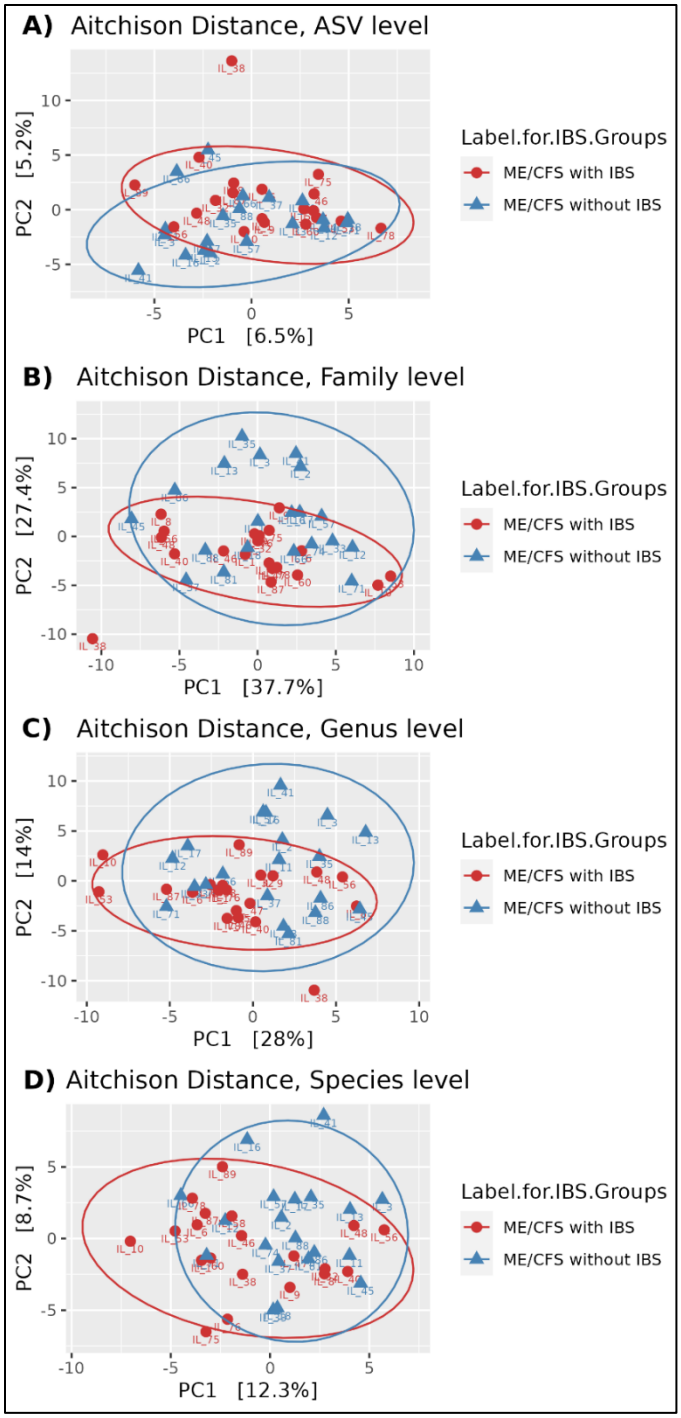



**Figure 3.3.7** PCAs based on the Aitchison distances showing the beta diversity between the ME/CFS with IBS and ME/CFS without IBS groups at the ASV, family, genus, and species taxonomic levels. The ellipses represent 95% confidence intervals for each group.

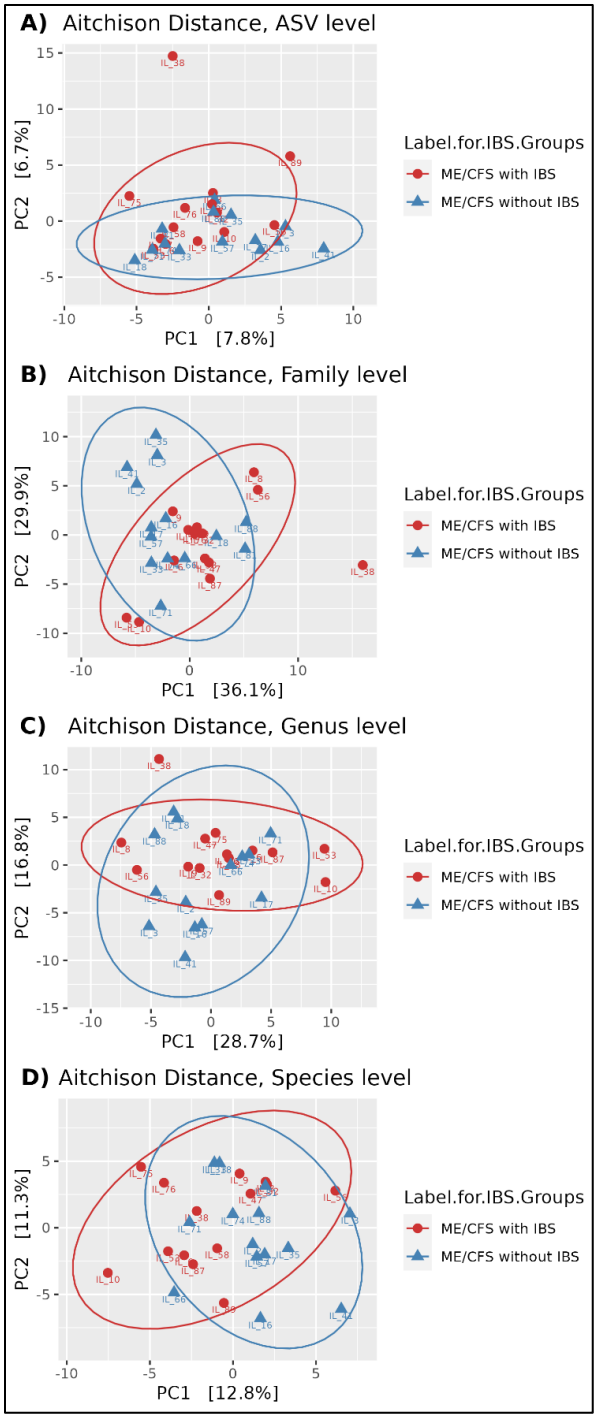



**Figure 3.3.8** PCAs based on the Aitchison distances showing the beta diversity between the Age-matched ME/CFS with IBS and ME/CFS without IBS groups at the ASV, family, genus, and species taxonomic levels. The ellipses represent 95% confidence intervals for each group.

**Table 3.3.2** PERMANOVA and PERMDISP statistical analyses of the Aitchison Distance beta diversity metric using the 16S rRNA data for the comparative groups of interest at the ASV, family, genus, and species levels

| PART ONE | PERMANOVA | | | PERMDISP | |
|---|---|---|---|---|---|
| ***Aitchison Distance*** | **R2** | **Pseudo-F** | **Pr(>F)** | **F** | **Pr(>F)** |
| **ME/CFS overall vs. Control** | | | | | |
| ASV level | 0.02045 | 1.6913 | 0.000999 *** | 0.3234 | 0.5654 |
| Family level | 0.03028 | 2.5293 | 0.04595 * | 0.5981 | 0.4426 |
| Genus level | 0.03648 | 3.0664 | 0.00999 ** | 0.2138 | 1.5277 |
| Species level | 0.02429 | 2.0162 | 0.004955 ** | 0.6383 | 0.4296 |
| **ME/CFS with FM vs. ME/CFS without FM** | | | | | |
| ASV level | 0.02548 | 0.9936 | 0.4895 | 0.0112 | 0.9271 |
| Family level | 0.01561 | 0.6024 | 0.7073 | 0.1436 | 0.6823 |
| Genus level | 0.02439 | 0.9501 | 0.4575 | 0.1695 | 0.6673 |
| Species level | 0.02523 | 0.9835 | 0.4915 | 0.0432 | 0.8392 |
| **ME/CFS with IBS vs. ME/CFS without IBS** | | | | | |
| ASV level | 0.02905 | 1.1371 | 0.05594 | 0.0528 | 0.8232 |
| Family level | 0.07549 | 3.103 | 0.01299 * | 1.3559 | 0.2418 |
| Genus level | 0.04779 | 1.907 | 0.04795 * | 0.9468 | 0.3556 |
| Species level | 0.03941 | 1.5591 | 0.01898 * | 0.1831 | 0.6813 |
| **Age-matched ME/CFS with IBS vs. ME/CFS without IBS** | | | | | |
| ASV level | 0.04108 | 1.1138 | 0.06494 | 0.0342 | 0.8531 |
| Family level | 0.08769 | 2.499 | 0.02997 * | 0.0346 | 0.8611 |
| Genus level | 0.04945 | 1.3527 | 0.1718 | 0.0036 | 0.95 |
| Species level | 0.0458 | 1.248 | 0.1139 | 0.394 | 0.5405 |
| 1000 permutations used for all tests | | | | | |
| Significance Codes (Pr(>F)): 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1 | | | | | |
| All Pr(>F) values are uncorrected P-values | | | | | |

### 3.3.3 Bray Curtis and Jaccard Beta Diversity (Culture MALDI-TOF)

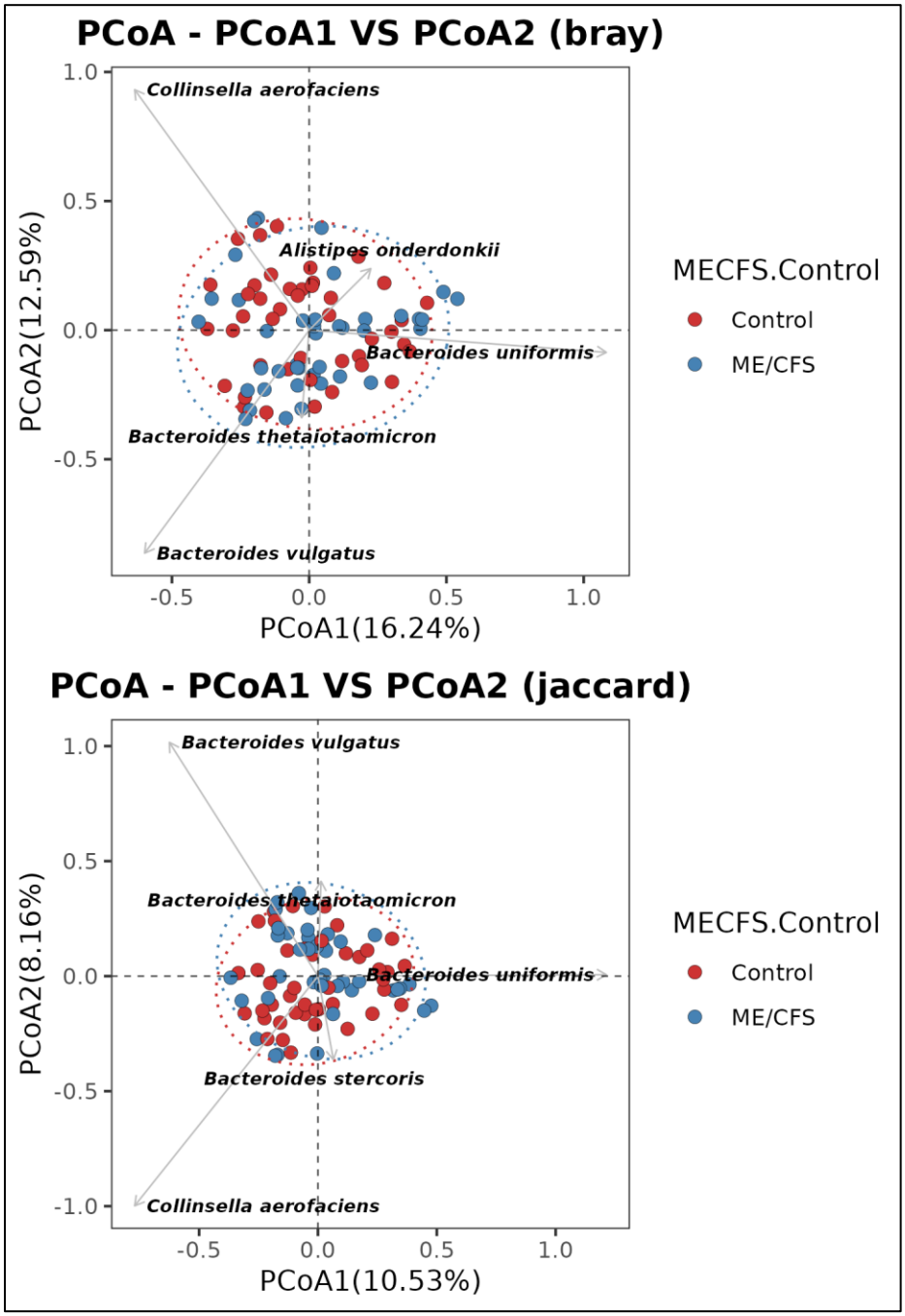



**Figure 3.3.9** PCoAs comparing the ME/CFS overall and Control groups based on the Bray-Curtis and Jaccard beta diversity metrics using the culture MALDI-TOF data at the species level. The ellipses represent 95% confidence intervals for each group. PCoA plots are annotated with the species feature of interest.

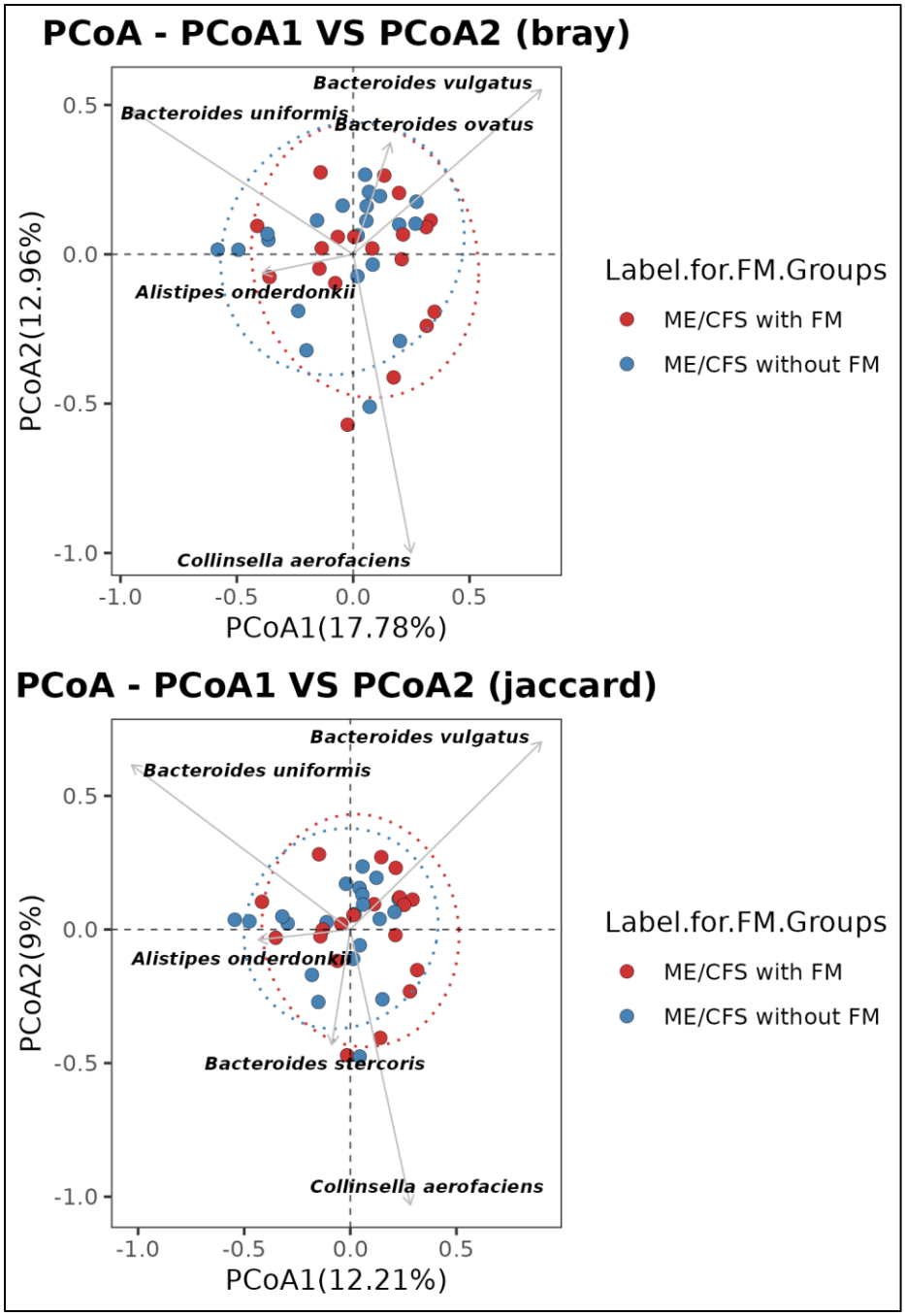



**Figure 3.3.10** PCoAs comparing the ME/CFS with FM and ME/CFS without FM groups based on the Bray-Curtis and Jaccard beta diversity metrics using the culture MALDI-TOF data at the species level. The ellipses represent 95% confidence intervals for each group. PCoA plots are annotated with the species feature of interest.

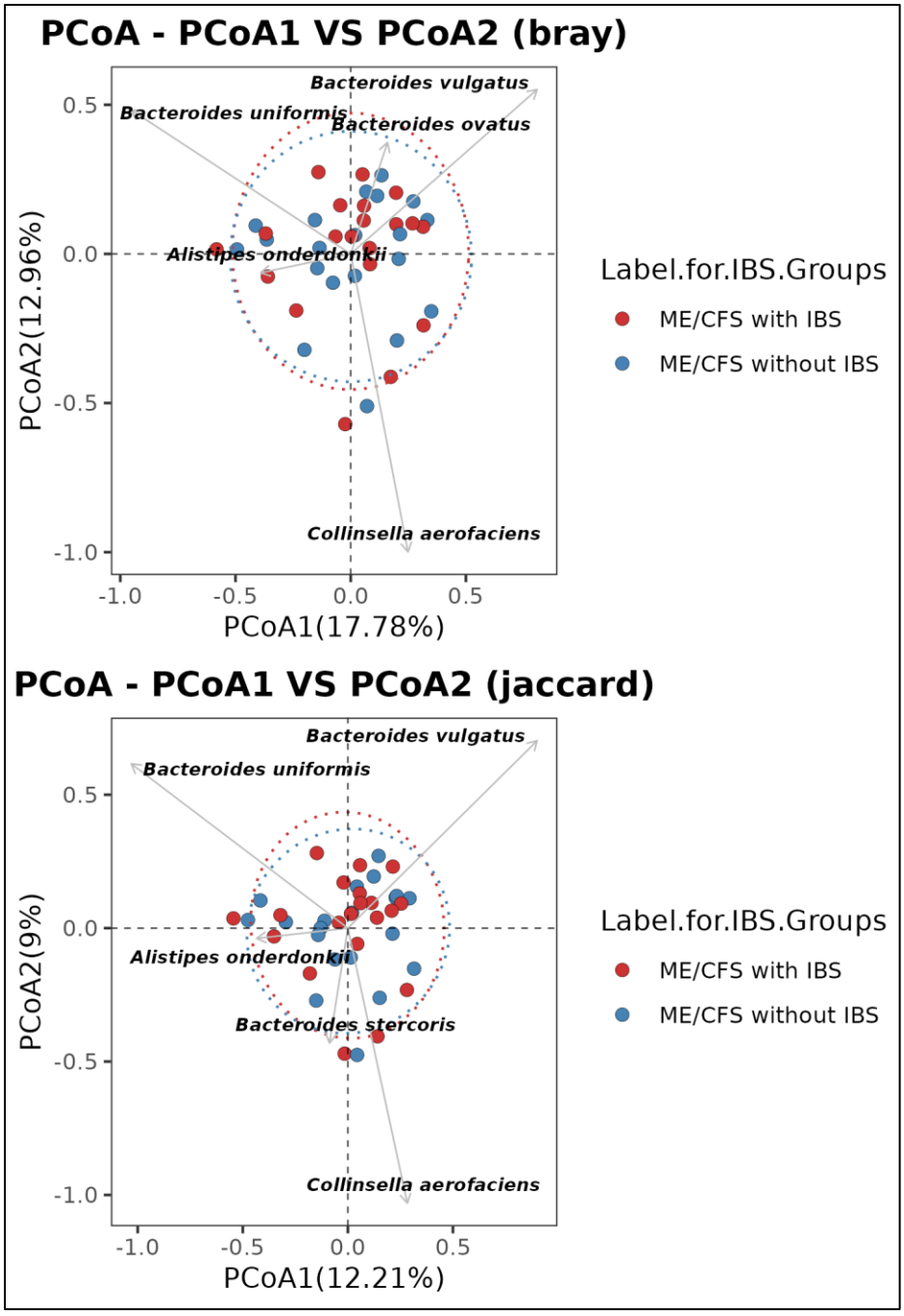



**Figure 3.3.11** PCoAs comparing the ME/CFS with IBS and ME/CFS without IBS groups based on the Bray-Curtis and Jaccard beta diversity metrics using the culture MALDI-TOF data at the species level. The ellipses represent 95% confidence intervals for each group. PCoA plots are annotated with the species feature of interest.

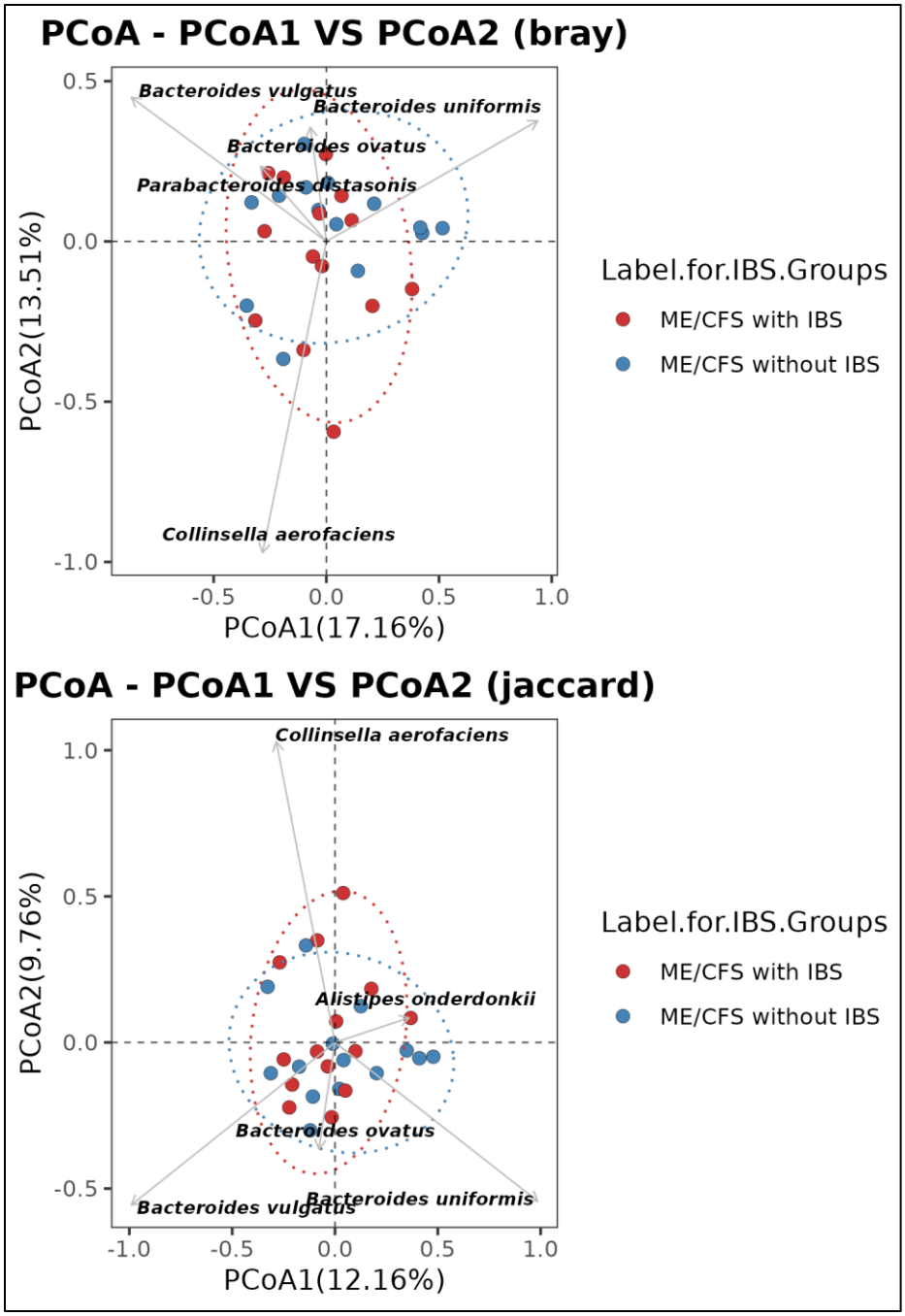



**Figure 3.3.12** PCoAs comparing the Age-matched ME/CFS with IBS and ME/CFS without IBS groups based on the Bray-Curtis and Jaccard beta diversity metrics using the culture MALDI-TOF data at the species level. The ellipses represent 95% confidence intervals for each group. PCoA plots are annotated with the species feature of interest.

**Table 3.3.3** PERMANOVA and PERMDISP statistical analyses of the Bray-Curtis and Jaccard beta diversity metrics using the faecal culture MALDI-TOF data for the comparative groups of interest at the species level.

| PART ONE | PERMANOVA | | | PERMDISP | |
|---|---|---|---|---|---|
| **Species level** | **R2** | **Pseudo-F** | **Pr(>F)** | **F** | **Pr(>F)** |
| **ME/CFS overall vs. Control** | | | | | |
| Bray-Curtis | 0.01318 | 1.0815 | 0.3666 | 1.9208 | 0.1838 |
| Jaccard | 0.01326 | 1.0887 | 0.3187 | 1.8468 | 0.1948 |
| **ME/CFS with FM vs. ME/CFS without FM** | | | | | |
| Bray-Curtis | 0.04745 | 1.893 | 0.01998 * | 5.3997 | 0.02498 * |
| Jaccard | 0.03996 | 1.5816 | 0.01998 * | 5.5725 | 0.02398 * |
| **ME/CFS with IBS vs. ME/CFS without IBS** | | | | | |
| Bray-Curtis | 0.01527 | 0.5893 | 0.8781 | 0.1355 | 0.7313 |
| Jaccard | 0.01798 | 0.6959 | 0.9321 | 0.1332 | 0.7323 |
| **Age-matched ME/CFS with IBS vs. ME/CFS without IBS** | | | | | |
| Bray-Curtis | 0.03202 | 0.86 | 0.5934 | 0.2087 | 0.6494 |
| Jaccard | 0.03411 | 0.918 | 0.5844 | 0.2038 | 0.6543 |
| 1000 permutations used for all tests | | | | | |
| Significance Codes (Pr(>F)): 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1 | | | | | |
| All Pr(>F) values are uncorrected P-values | | | | | |

## 3.4 Differential Abundance Analysis

The alpha and beta diversity measures from the 16S rRNA and culture MALDI-TOF data revealed some variability in the gut microbiota between the ME/CFS overall compared to a non-ME/CFS cohort, and within the ME/CFS cohort depending on the FM or IBS comorbidity. The presence and absence of different comorbidities demonstrate different gut microbiota profiles that may be a display of gut dysbiosis, although it does not prove or confirm it. The diversity metrics, PERMANOVA, and PERMDISP results suggested that ME/CFS with or without IBS warrants further consideration as far as subgrouping and stratifying ME/CFS individuals go. Given that IBS has been associated with gut microbiota changes, it is necessary to account for it so that it does not detract, obscure, or confound but instead enhances the overall understanding of ME/CFS (Ghaffari et al., 2022; Guo, Che, et al., 2023; Nagy-Szakal et al., 2017; Napolitano et al., 2023; Xiong et al., 2023). This is not to say that FM is not an important issue in ME/CFS, but each comorbidity may play a different and unique role in the disease and capability to distinguish and disentangle the heterogeneity via distinct subgrouping or subtyping.

Even though all gut microbiome studies, including this one, have not reproduced or represented consistent findings for the composition and diversity of a "ME/CFS gut microbiota profile", they all observe alterations that generally and broadly support the gut dysbiosis and microbiome hypothesis in ME/CFS. Beta diversity was evaluated by some of the ME/CFS studies, all with

varying extents of significance which could likely be due to the selection of diversity metrics and ordination, study design, sequencing platform, read numbers, normalisation, and other data analytical handling considerations (Giloteaux, Goodrich, et al., 2016; Guo, Che, et al., 2023; Lupo et al., 2021; Nagy-Szakal et al., 2017; Xiong et al., 2023). The other consideration is that more participants are required for the cohort sizes and the inherent complexity and heterogeneity of the disease that underpins much of the discrepancies and difficulties in ME/CFS research (Du Preez et al., 2018; König et al., 2022; Varesi et al., 2021). Aside from these limitations and considerations, differential abundance analysis tests were performed with the datasets to provide further insight into the gut microbiome of the cohort involved in this study.

Linear discriminant analysis (LDA) of effect size (LEfSe) and Differential gene expression analysis (DESeq2) were used to perform differential abundance analysis (DAA) testing from the 16S rRNA data. These approaches were used to determine the taxonomic features that most likely explained the differences between the comparative groups of interest, irrespective of whether there was a statistically significant difference observed in the alpha and beta diversity measures. The outcomes from these analyses are shown in Section 3.4.1. All tests were performed at the A) family B) genus and C) species taxonomic level. DESeq2 was performed with non-rarefied data and LEfSe was performed with non-rarefied and rarefied data (Section 2.6.3); therefore three figures per comparative group of interest are displayed for Comparison A-D. Figure 3.4.1, Figure 3.4.2, and Figure 3.4.3 reflect the outcomes from Comparison A (ME/CFS overall versus Control). Figure 3.4.4, Figure 3.4.5, and Figure 3.4.6 reflect the outcomes from Comparison B (ME/CFS +/- FM). Figure 3.4.7, Figure 3.4.8, and Figure 3.4.9 reflect the outcomes from Comparison C (ME/CFS +/- IBS). Figure 3.4.10, Figure 3.4.11, and Figure 3.4.12 reflect the outcomes from Comparison D (age-matched ME/CFS +/- IBS**).

Linear discriminant analysis (LDA) of effect size (LEfSe) was used to perform DAA from the culture MALDI-TOF dataset. This approach was used to determine the species (features) that most likely explained the differences between the comparative groups of interest, irrespective of whether there was a statistically significant difference observed in the diversity measures. The outcomes from the analyses are shown in Section 3.4.2. The test was performed with a p-value significance threshold of 0.05 (A) and 0.1 (B) and the outcomes are presented in the same figure. Figure 3.4.13, Figure 3.4.14, Figure 3.4.15, and Figure 3.4.16 represent the outcomes from Comparison A-D, respectively. ***Continued just before the start of Section 3.5****

### 3.4.1 Differential Abundance Analysis (16S rRNA)

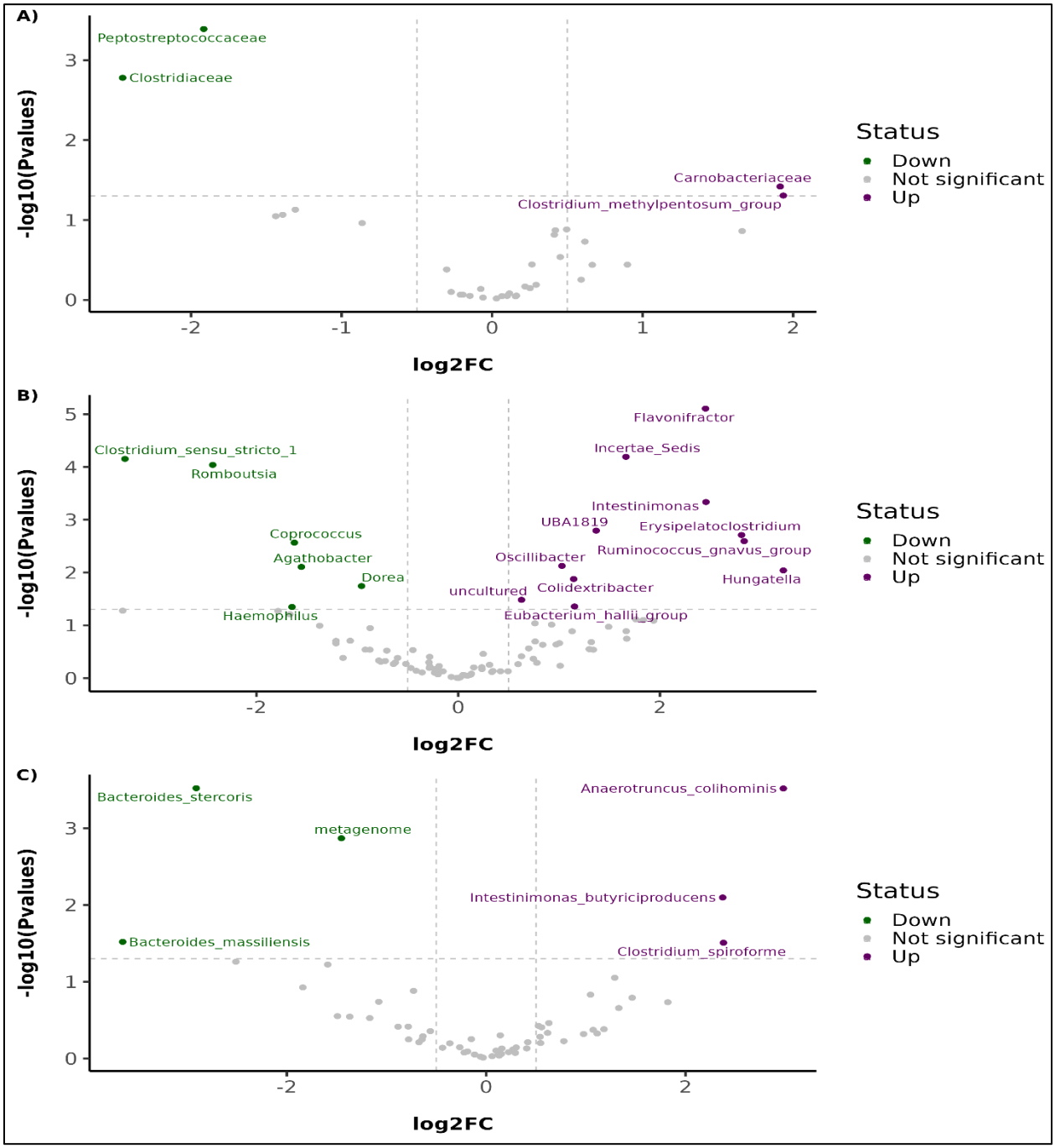



**Figure 3.4.1** DESeq2 at the A) family B) genus and C) species taxonomic level between ME/CFS overall and Control groups (Part One) from non-rarefied data. Purple circles represent features with increased expression in the ME/CFS group. Green circles represent features with decreased expression in the ME/CFS group. Grey circles represent non-significant features (log2FC threshold 0.5 and uncorrected p-values ≥ 0.05).

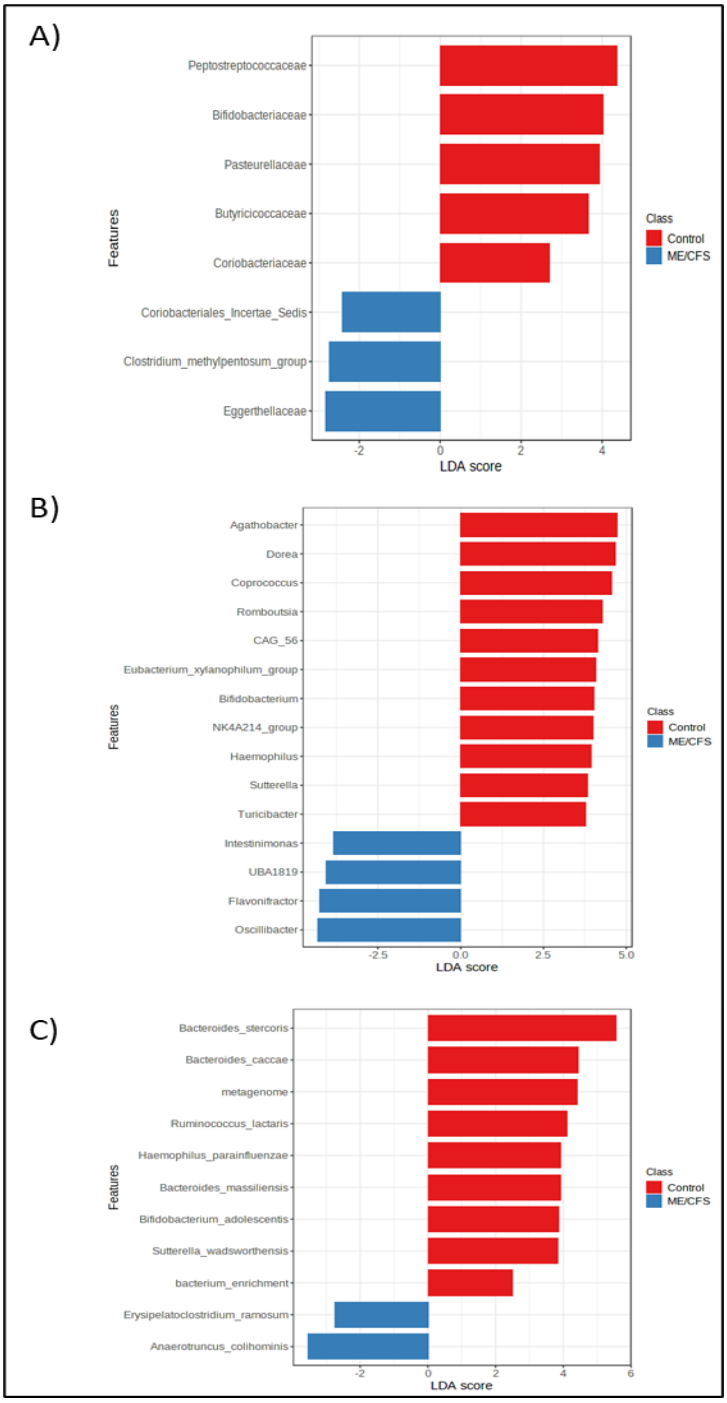



**Figure 3.4.2** LEfSe at the A) family B) genus and C) species taxonomic level from non-rarefied data between ME/CFS overall and Control groups (Part One). The horizontal bars represent the effect size for each taxon. The length of the bar represents the log10-transformed LDA score indicated by vertical dotted lines. The red bars represent the significant ASVs of the Control group and the blue bars of the ME/CFS group. Non-adjusted p-values 0.05 and (log) LDA score >2 were used as the threshold values for significance.

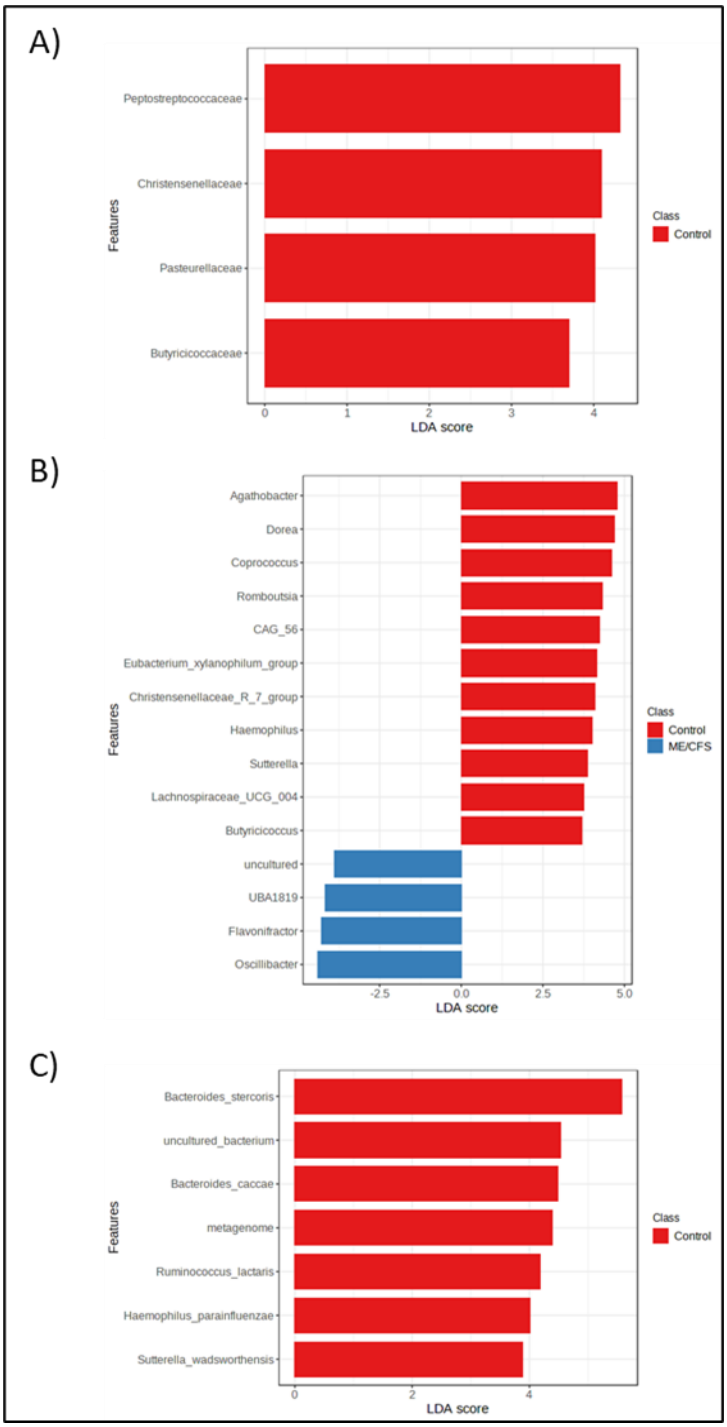



**Figure 3.4.3** LEfSe at the A) family B) genus and C) species taxonomic level from rarefied data between ME/CFS overall and Control groups (Part One). The horizontal bars represent the effect size for each taxon. The length of the bar represents the log10-transformed LDA score indicated by vertical dotted lines. The red bars represent the significant ASVs of the Control group and the blue bars of the ME/CFS group. Non-adjusted p-values 0.05 and (log) LDA score >2 were used as the threshold values for significance.

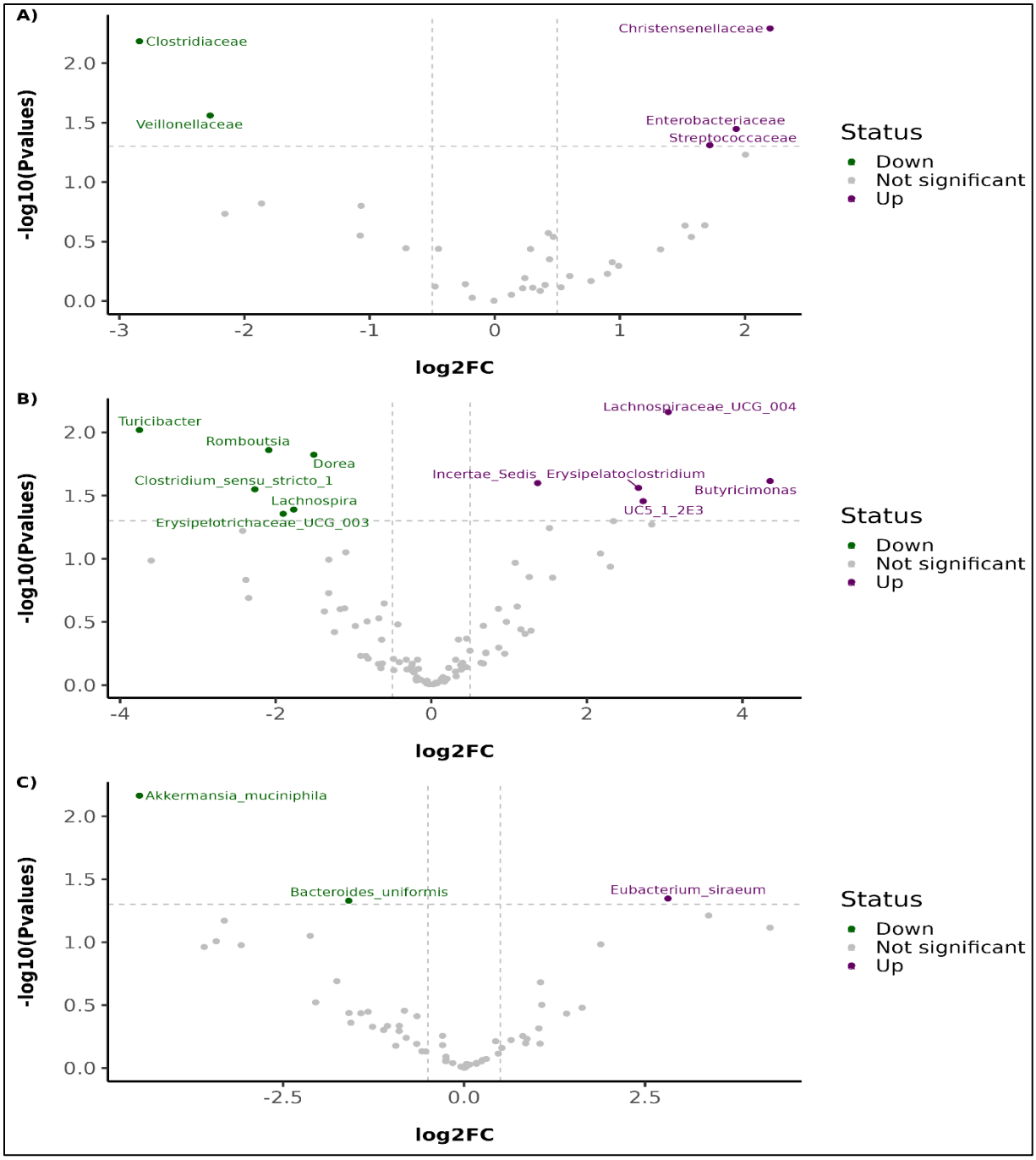



**Figure 3.4.4** DESeq2 at the A) family B) genus and C) species taxonomic level between ME/CFS with FM and ME/CFS without FM groups (Part One) from non-rarefied data. Purple circles represent features with increased expression in the ME/CFS without FM group. Green circles represent features with decreased expression in the ME/CFS without FM group. Grey circles represent non-significant features (log2FC threshold 0.5 and uncorrected p-values ≥ 0.05).

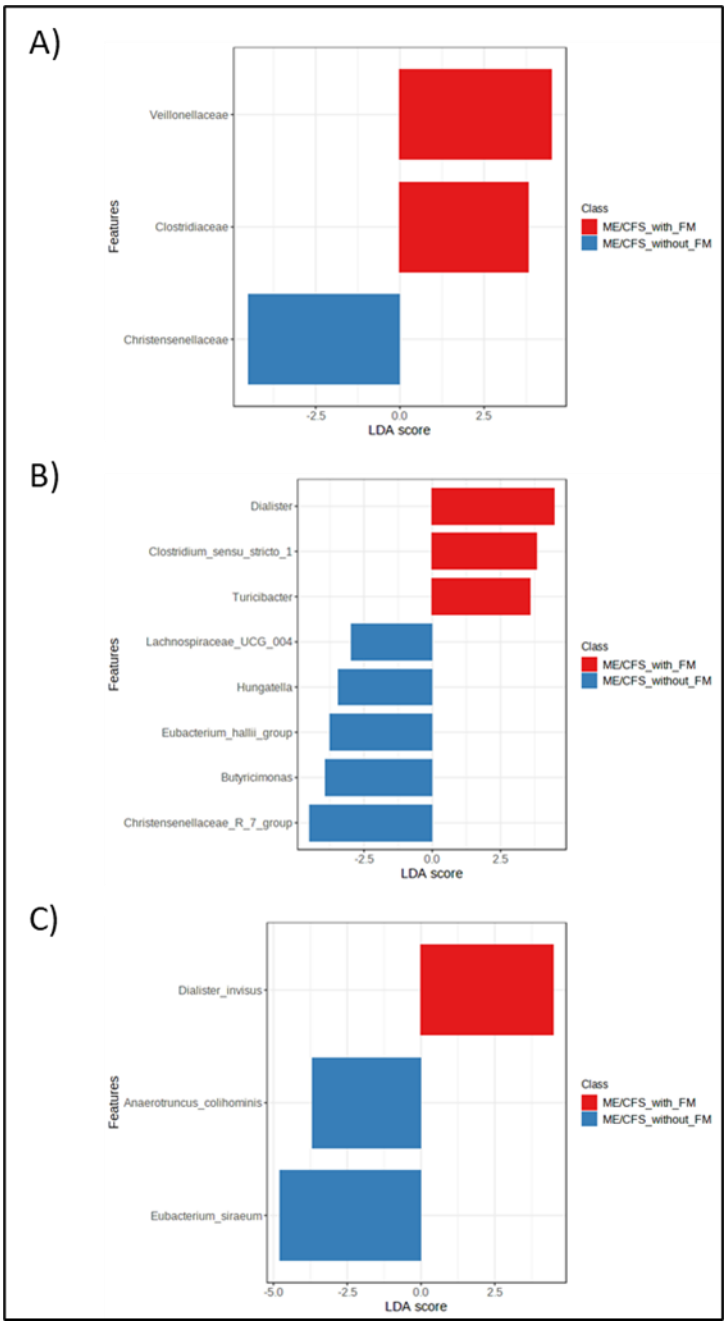



**Figure 3.4.5** LEfSe at the A) family B) genus and C) species taxonomic level from non-rarefied data between ME/CFS with FM and ME/CFS without FM groups (Part One). The horizontal bars represent the effect size for each taxon. The length of the bar represents the log10-transformed LDA score indicated by vertical dotted lines. The red bars represent the significant ASVs of the ME/CFS with FM group and the blue bars of the ME/CFS without FM group. Non-adjusted p-values 0.05 and (log) LDA score >2 were used as the threshold values for significance.

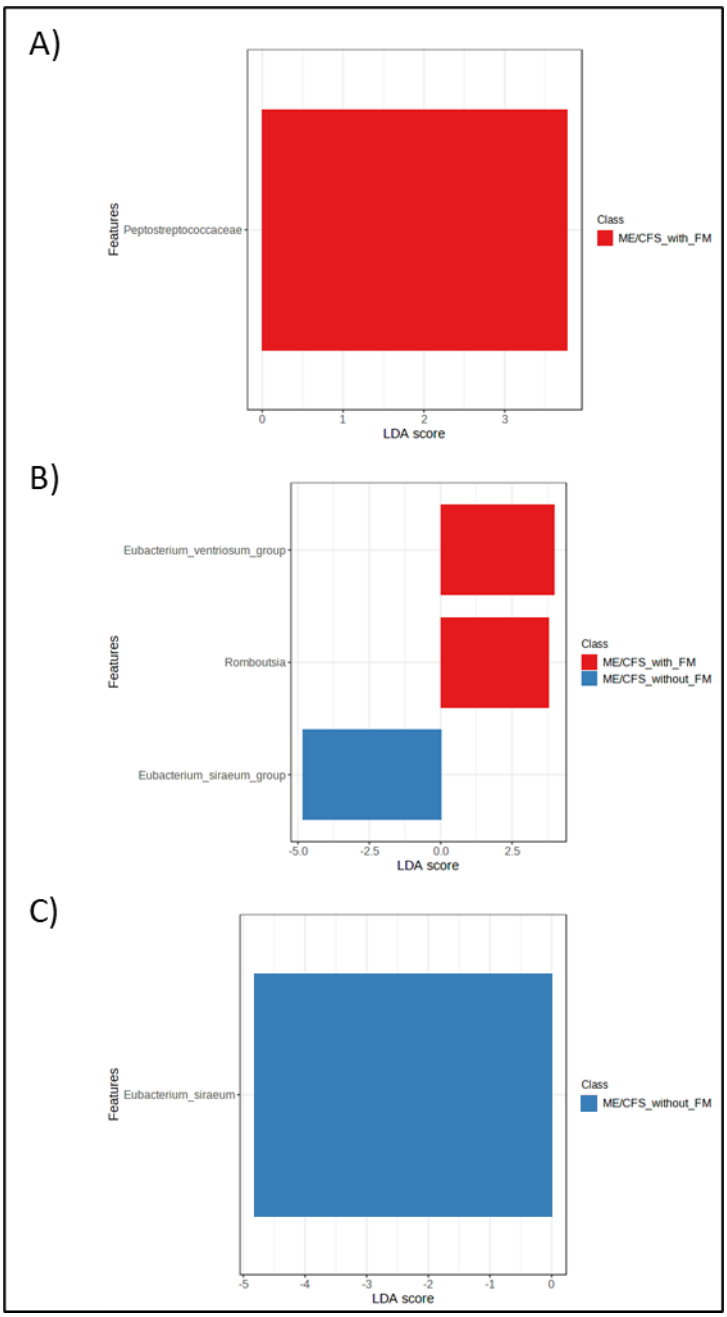



**Figure 3.4.6** LEfSe at the A) family B) genus and C) species taxonomic level from rarefied data between ME/CFS with FM and ME/CFS without FM groups (Part One). The horizontal bars represent the effect size for each taxon. The length of the bar represents the log10-transformed LDA score indicated by vertical dotted lines. The red bars represent the significant ASVs of the ME/CFS with FM group and the blue bars of the ME/CFS without FM group. Non-adjusted p-values 0.05 and (log) LDA score >2 were used as the threshold values for significance.

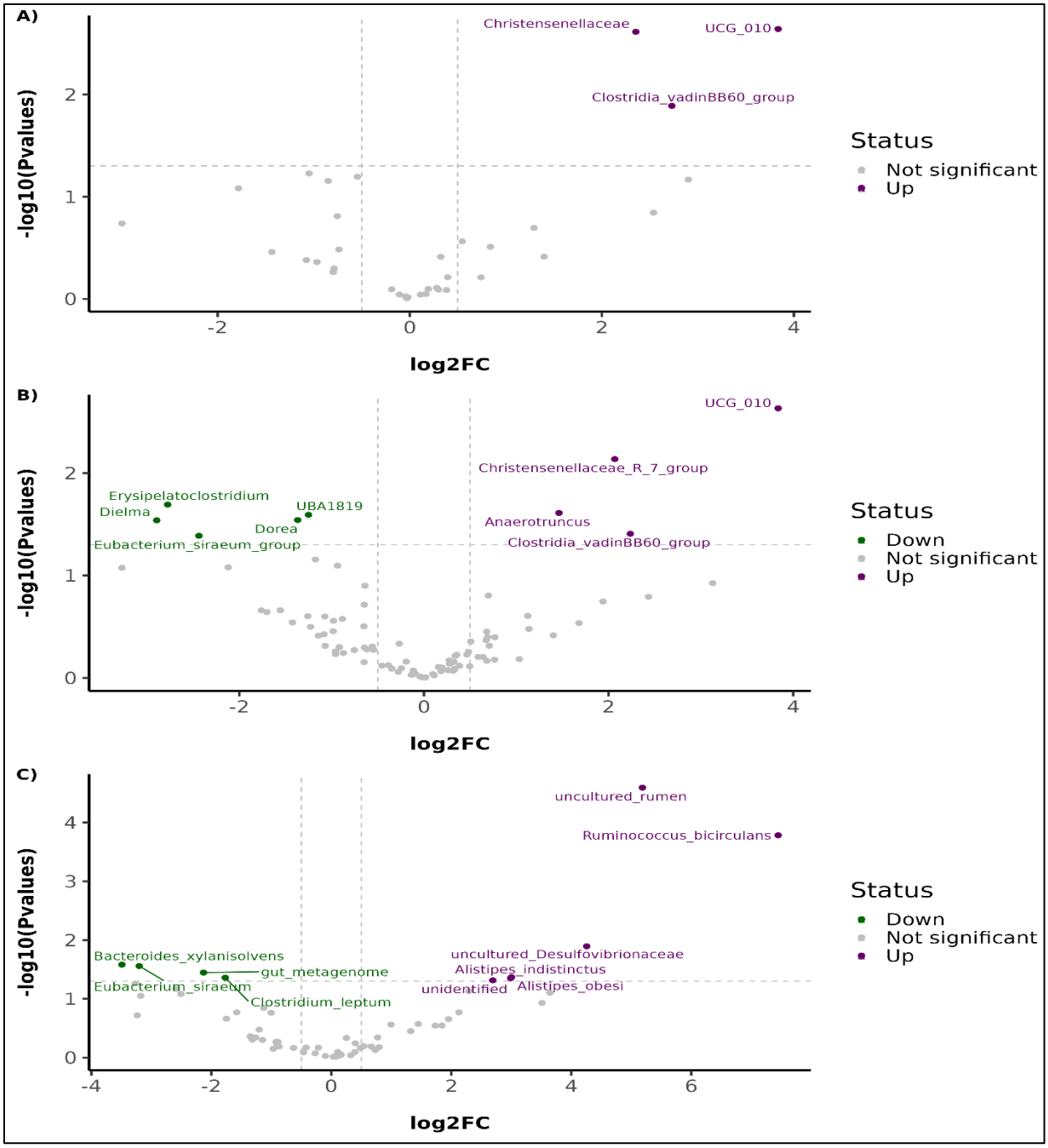



**Figure 3.4.7** DESeq2 at the A) family B) genus and C) species taxonomic level between ME/CFS with IBS and ME/CFS without IBS groups (Part One) from non-rarefied data. Purple circles represent features with increased expression in the ME/CFS without IBS group. Green circles represent features with decreased expression in the ME/CFS without IBS group. Grey circles represent non-significant features (log2FC threshold 0.5 and uncorrected p-values ≥ 0.05).

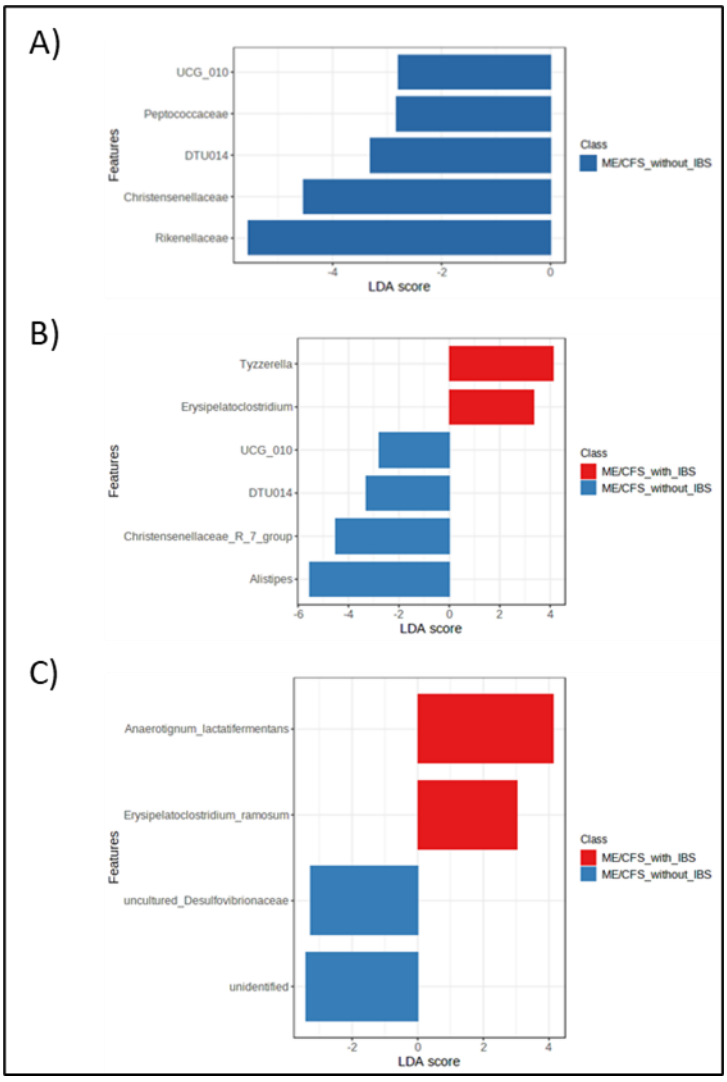



**Figure 3.4.8** LEfSe at the A) family B) genus and C) species taxonomic level from non-rarefied between ME/CFS with IBS and ME/CFS without IBS groups (Part One). The horizontal bars represent the effect size for each taxon. The length of the bar represents the log10-transformed LDA score indicated by vertical dotted lines. The red bars represent the significant ASVs of the ME/CFS with IBS group and the blue bars of the ME/CFS without IBS group. Non-adjusted p-values 0.05 and (log) LDA score >2 were used as the threshold values for significance.

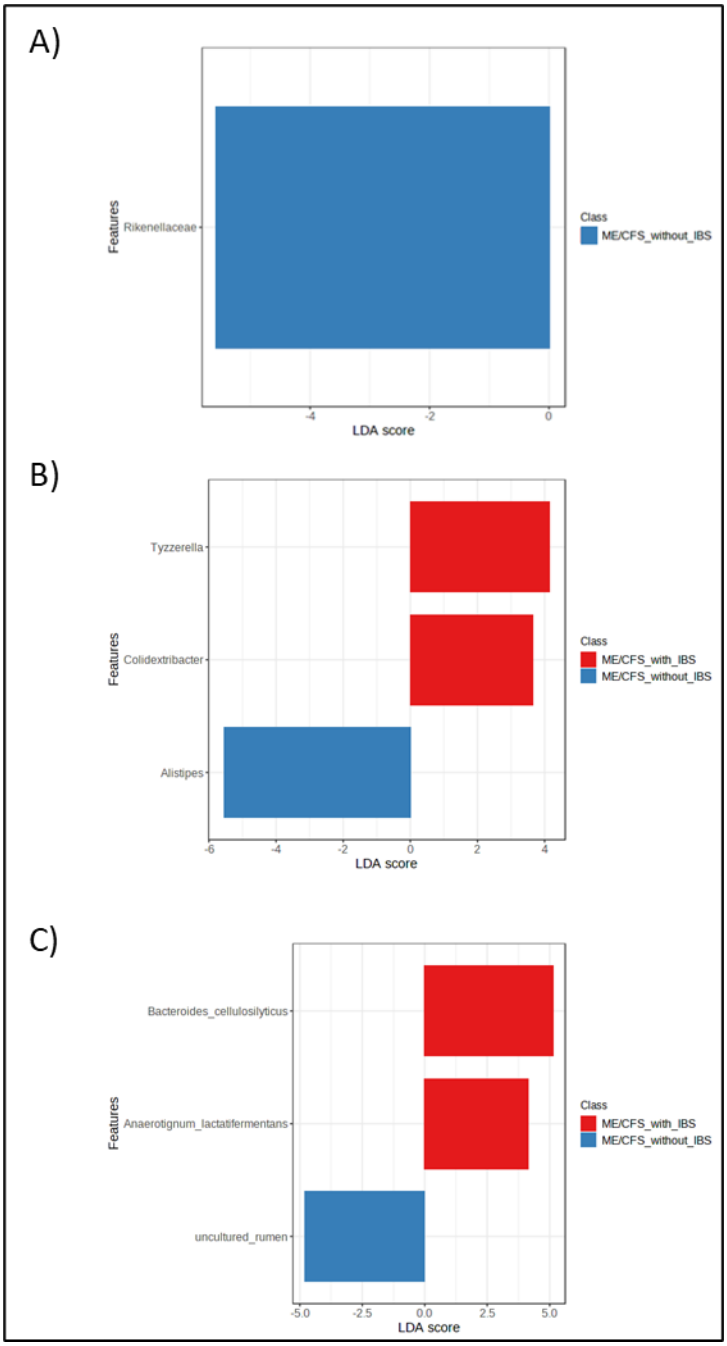



**Figure 3.4.9** LEfSe at the A) family B) genus and C) species taxonomic level from rarefied data between ME/CFS with IBS and ME/CFS without IBS groups (Part One). The horizontal bars represent the effect size for each taxon. The length of the bar represents the log10-transformed LDA score indicated by vertical dotted lines. The red bars represent the significant ASVs of the ME/CFS with IBS group and the blue bars of the ME/CFS without IBS group. Non-adjusted p-values 0.05 and (log) LDA score >2 were used as the threshold values for significance.

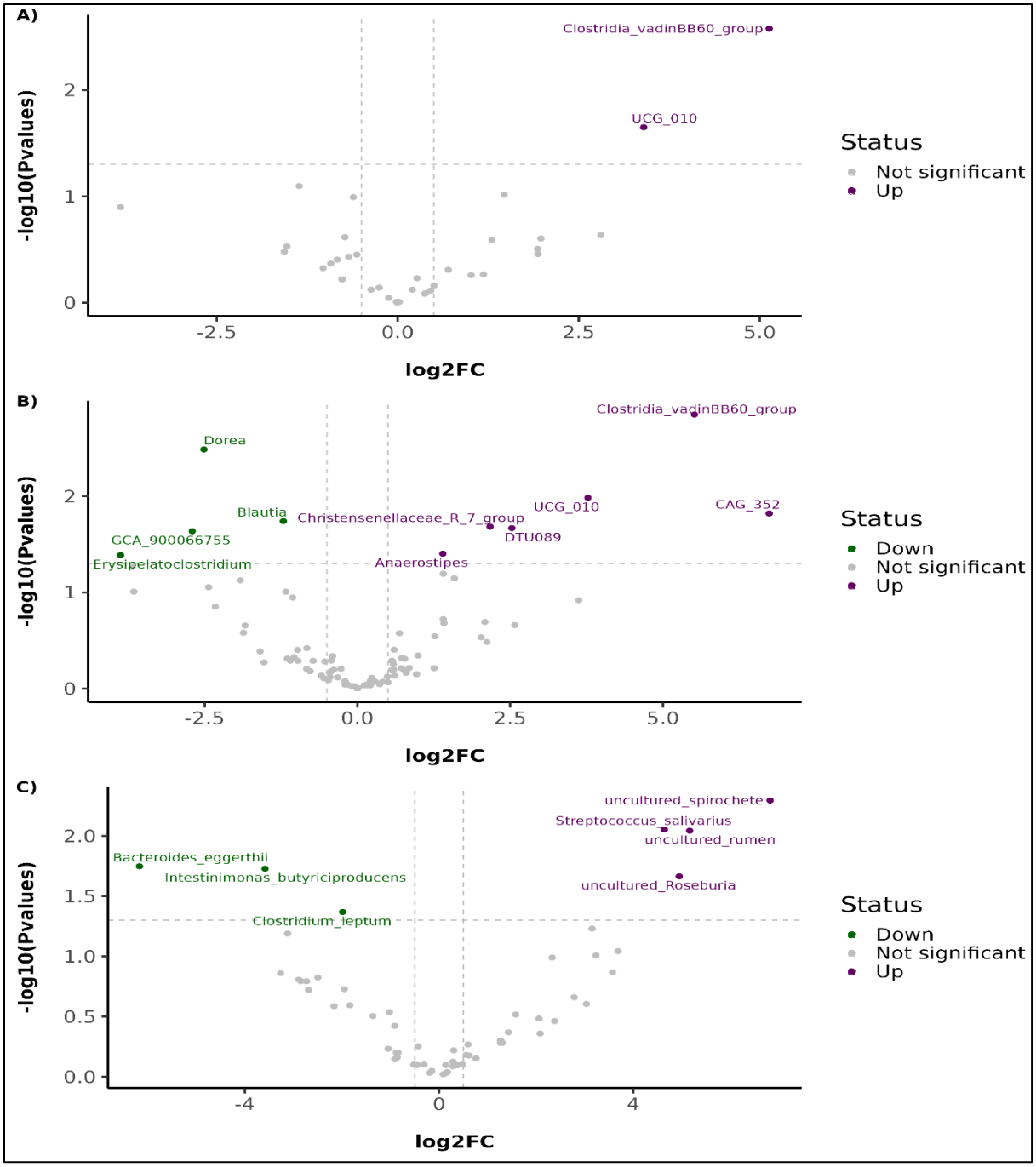



**Figure 3.4.10** DESeq2 at the A) family B) genus and C) species taxonomic level between age-matched ME/CFS with IBS and ME/CFS without IBS groups (Part One) from non-rarefied data. Purple circles represent features with increased expression in the age-matched ME/CFS without IBS group. Green circles represent features with decreased expression in the age-matched ME/CFS without IBS group. Grey circles represent non-significant features (log2FC threshold 0.5 and uncorrected p-values ≥ 0.05).

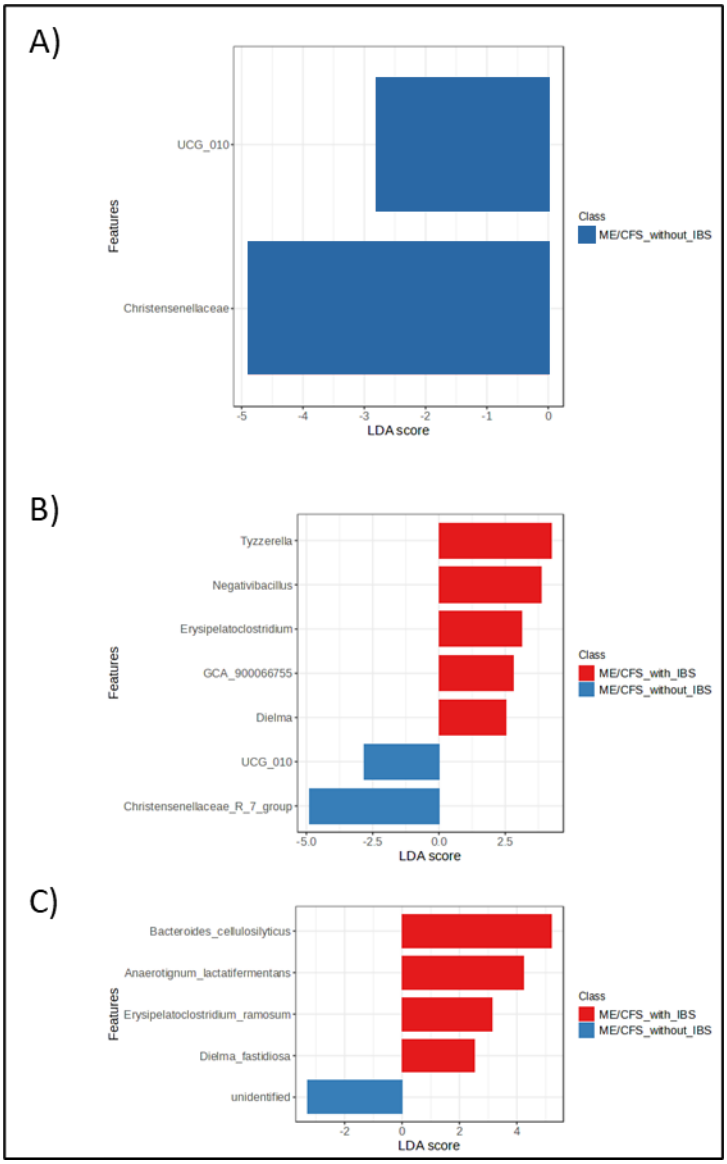



**Figure 3.4.11** LEfSe at the A) family B) genus and C) species taxonomic level from non-rarefied data between age-matched ME/CFS with IBS and ME/CFS without IBS groups (Part One). The horizontal bars represent the effect size for each taxon. The length of the bar represents the log10-transformed LDA score indicated by vertical dotted lines. The red bars represent the significant ASVs of the age-matched ME/CFS with IBS group and the blue bars of the age-matched ME/CFS without IBS group. Non-adjusted p-values 0.05 and (log) LDA score >2 were used as the threshold values for significance.

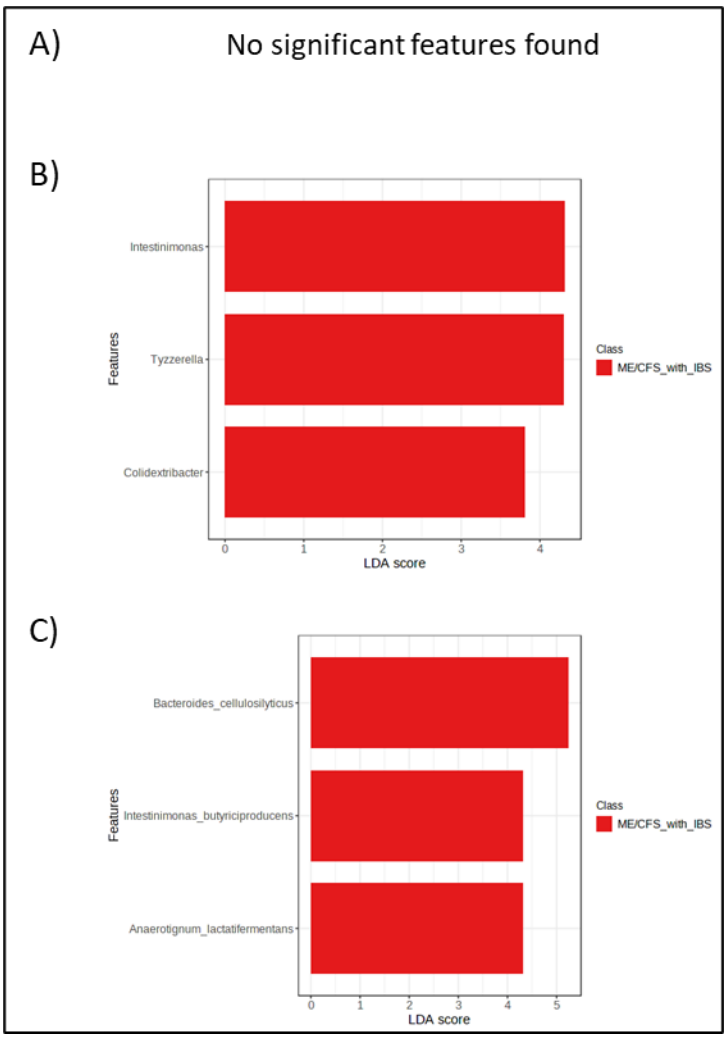



**Figure 3.4.12** LEfSe at the A) family B) genus and C) species taxonomic level from rarefied data between age-matched ME/CFS with IBS and ME/CFS without IBS groups (Part One). The horizontal bars represent the effect size for each taxon. The length of the bar represents the log10-transformed LDA score indicated by vertical dotted lines. The red bars represent the significant ASVs of the age-matched ME/CFS with IBS group and the blue bars of the age-matched ME/CFS without IBS group. Non-adjusted p-values 0.05 and (log) LDA score >2 were used as the threshold values for significance.

### 3.4.2 Differential Abundance Analysis (Culture MALDI-TOF)

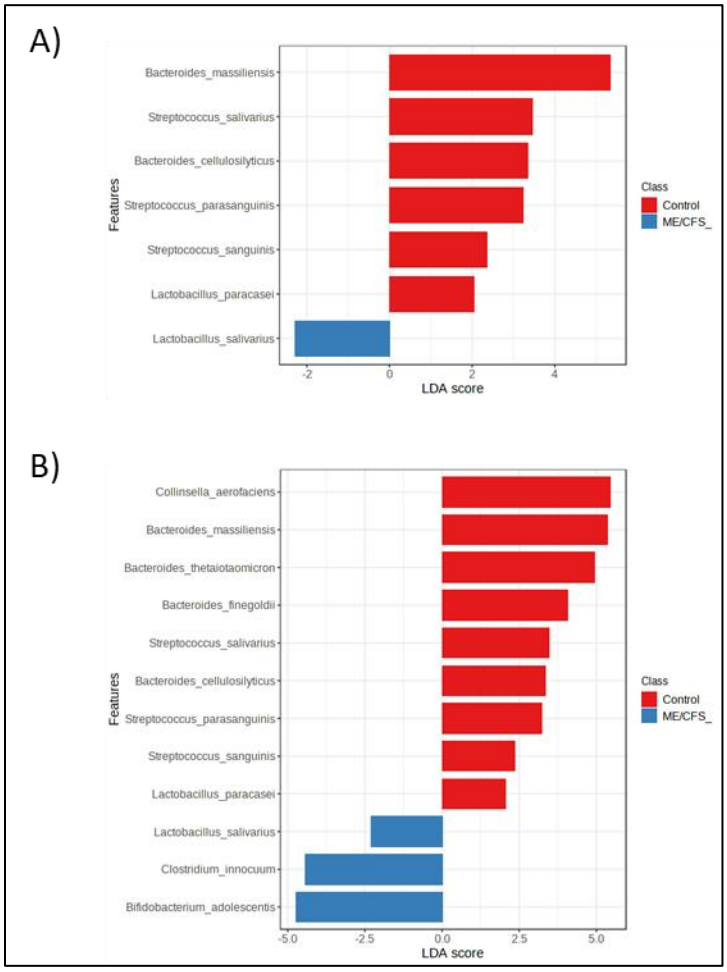



**Figure 3.4.13** LEfSe at the species taxonomic level from faecal culture MALDI-TOF data between ME/CFS overall and Control groups (Part One). Plot A) and Plot B) show the significant features with a non-adjusted p-value threshold of 0.05 and 0.1, respectively. The horizontal bars represent the effect size for each taxon. The length of the bar represents the log10-transformed LDA score indicated by vertical dotted lines. The red bars represent the significant ASVs of the Control group and the blue bars of the ME/CFS group. A (log) LDA score >2 was used as the threshold for significance for both plots.

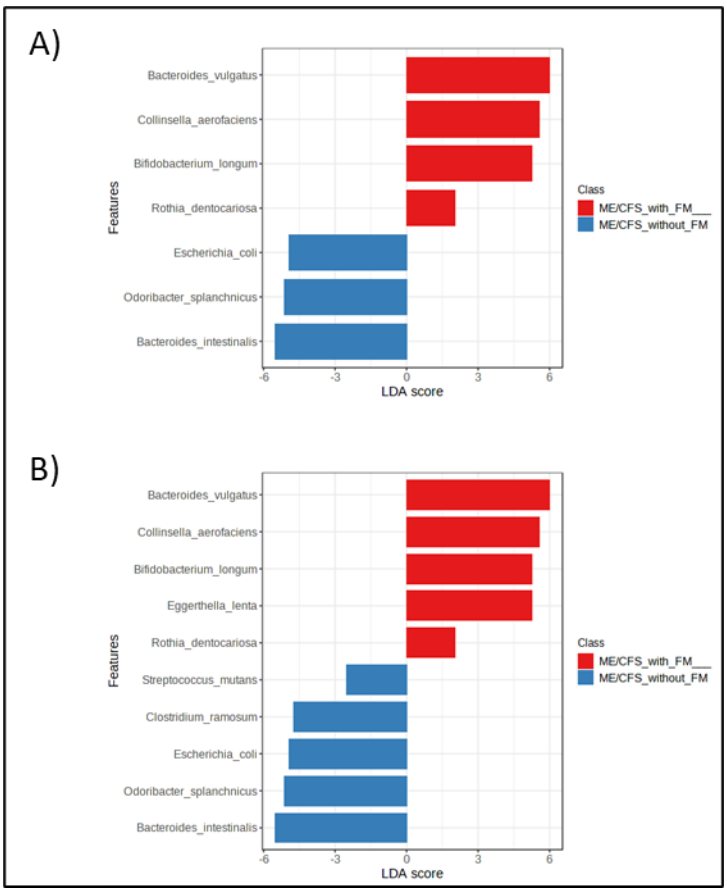



**Figure 3.4.14** LEfSe at the species taxonomic level from faecal culture MALDI-TOF data between ME/CFS with FM and ME/CFS without FM groups (Part One). Plot A) and Plot B) show the significant features with a non-adjusted p-value threshold of 0.05 and 0.1, respectively. The horizontal bars represent the effect size for each taxon. The length of the bar represents the log10-transformed LDA score indicated by vertical dotted lines. The red bars represent the significant ASVs of the Control group and the blue bars of the ME/CFS group. A (log) LDA score >2 was used as the threshold for significance for both plots.

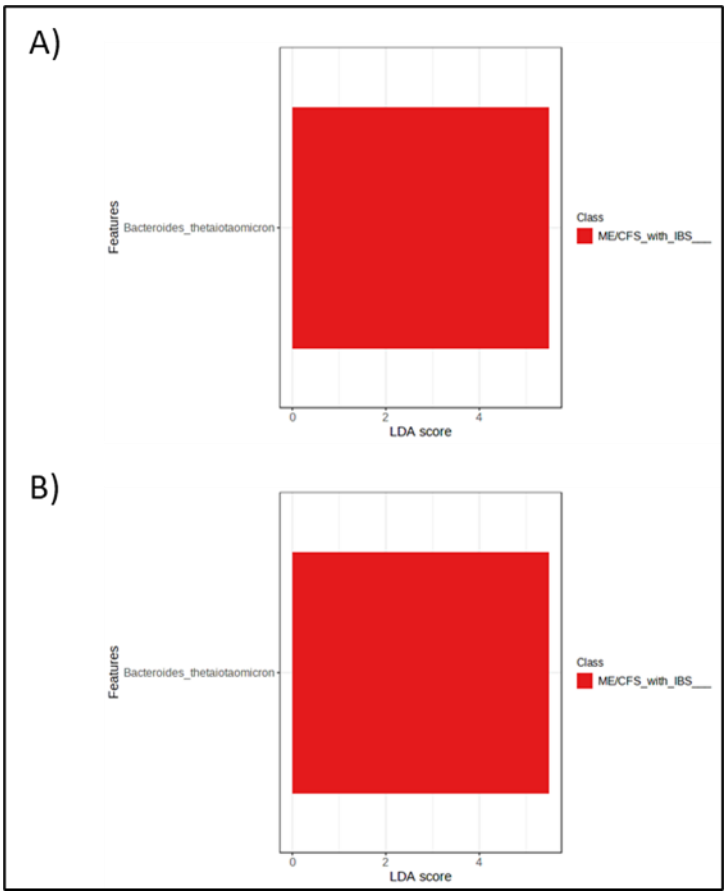



**Figure 3.4.15** LEfSe at the species taxonomic level from faecal culture MALDI-TOF data between ME/CFS with IBS and ME/CFS without IBS groups (Part One). Plot A) and Plot B) show the significant features with a non-adjusted p-value threshold of 0.05 and 0.1, respectively. The horizontal bars represent the effect size for each taxon. The length of the bar represents the log10-transformed LDA score indicated by vertical dotted lines. The red bars represent the significant ASVs of the Control group and the blue bars of the ME/CFS group. A (log) LDA score >2 was used as the threshold for significance for both plots.

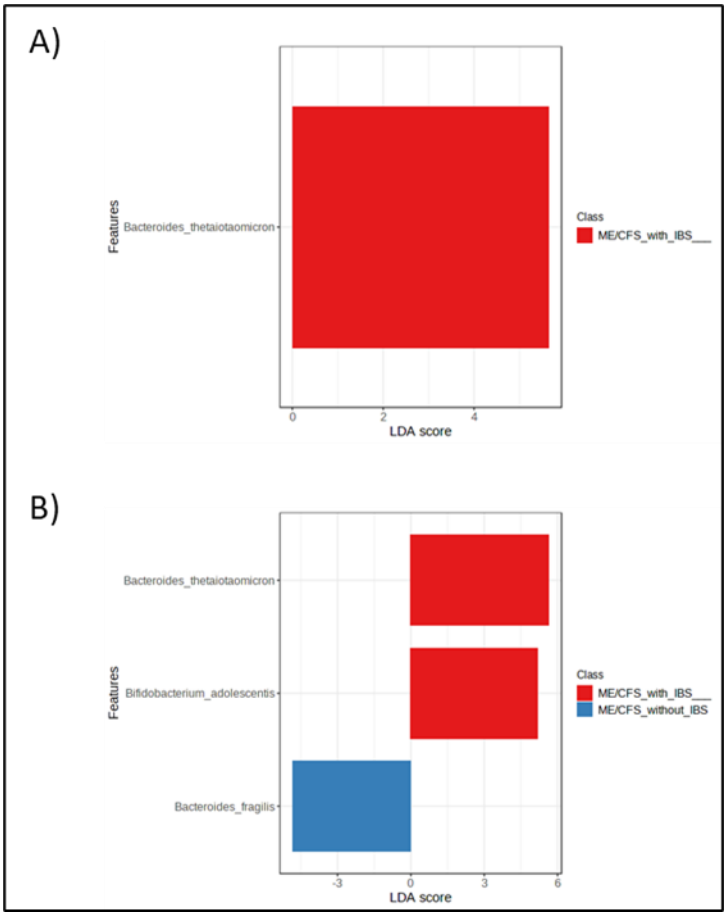



**Figure 3.4.16** LEfSe at the species taxonomic level from faecal culture MALDI-TOF data between age-matched ME/CFS with IBS and ME/CFS without IBS groups (Part One). Plot A) and Plot B) show the significant features with a non-adjusted p-value threshold of 0.05 and 0.1, respectively. The horizontal bars represent the effect size for each taxon. The length of the bar represents the log10-transformed LDA score indicated by vertical dotted lines. The red bars represent the significant ASVs of the Control group and the blue bars of the ME/CFS group. A (log) LDA score >2 was used as the threshold for significance for both plots.

***Continued from the beginning of Section 3.4****

The DAA results showed that an assortment of taxonomic features from both 16S rRNA and culture MALDI-TOF are differentially abundant between the comparative groups in Comparison A-D. The intention of using DAA in microbiome data analysis is to help identify microbial features for further biological validation and biomarker discovery based on observed count data (Cappellato et al., 2022; Nearing et al., 2022; L. Yang & Chen, 2022). The use of DAA is now commonplace in microbiome data analysis; although only DESeq2 and LEfSe were used in this thesis, there are 14 (several) DAA testing methods (Nearing et al., 2022). The use of DAA is currently problematic given that microbiome data has special characteristics and challenges such as its compositionality, normalisation, constraints imposed by sequencing depth between samples, zero inflation, and computational burden (Cappellato et al., 2022; Weiss et al., 2017; L. Yang & Chen, 2022). It is one of the more controversial areas of microbiome data analysis. Over the past decade, these DAA methods have been developed to address the issues in microbiome data and differ mainly where compositional effects and zero inflation are involved (L. Yang & Chen, 2022). No single method or approach to DAA has been able to circumvent or mitigate all of the difficulties that the complexity of the microbiome sequencing data poses. The full technical and analytical details of the drawbacks that each DAA approach poses are detailed elsewhere in the literature (Cappellato et al., 2022; Lin & Peddada, 2020; Nearing et al., 2022; Weiss et al., 2017; L. Yang & Chen, 2022). It is therefore recommended that researchers use a consensus approach based on DAA methods to help biological interpretations (Nearing et al., 2022). However, the concern and issue are that the different DAA tools could sometimes produce quite discordant results, opening up the possibility of bias and cherry-picking misleading results (Nearing et al., 2022; L. Yang & Chen, 2022). This inconsistency was also observed in the DAA results covered in this thesis and a “consensus” of microbial candidates remains vague.

There is a lack of consistent known biological truth and a priori information which makes DAA validation difficult (Cappellato et al., 2022; L. Yang & Chen, 2022). Combined with the DAA data issues, and its scope for errors/false discovery, this makes it difficult to wholly rely on the DAA results as features that have been identified as potentially “dysbiotic”. These data performance considerations are important to be mindful of when attempting to interpret its biological meaning. It also became apparent that the DAA results may not be the most efficient, practical, or biologically realistic basis to evaluate “gut dysbiosis” in the cohort. While the results do show

some taxonomic features that may be of interest, DAA only identifies microbial features that are differentially abundant based on count data. The compositional nature of the data can sometimes make it look like all the other taxa are changing even if the absolute abundances remain constant (Morton et al., 2019). While the vast majority of the dominant human gut microbiota belong to a few phyla, low abundant microbes should not be overlooked (Puig-Castellví et al., 2023). Low-abundant microbes can still exert major influence and functionality in its community and DAA may not necessarily identify this (Puig-Castellví et al., 2023). By this token, higher-abundance microbes do not necessarily mean more metabolic and enzymatic activity, and therefore increased metabolite production/concentration as a direct result. Perhaps this widespread assumption in research has made identifying microbial consortia elusive and challenging (Moya & Ferrer, 2016).

The microbial features are identified in an individual fashion and DAA does not reveal much as far as the overall impact on and outlook of the gut microbiome – it risks being a “stamp collecting” undertaking. The gut microbiome is often said to exhibit balance, stability, resilience, and maintenance of homeostasis. The DAA results cannot provide any indication of whether these microbial features that have been identified with the respective comparative groups in Comparison A-D are involved with disease (or repair) processes and presentation. With this, functionally redundant taxa may be identified as differentially abundant. Further, the metabolic pool and functional potential of the gut microbiome often outweigh the sheer number of microbes (Visconti et al., 2019; Wilmanski et al., 2021). Most microbial metabolic processes occur in the context of communities, not that of a single microorganism feature or pure cultures in isolation (Bowman & Ducklow, 2015; Turnbaugh & Gordon, 2008). With microbial communities in an environment as complex and dynamic as the human gut microbiome, it is therefore preferable at a conceptual and practical level to consider the functionality and metabolic structure of a community over its taxonomic structure (Bowman & Ducklow, 2015). Therefore, this thesis generally takes a metabolic/metabolomic-centric approach in the attempt to describe microbial communities versus a “one-by-one” exploration of each DAA-microbe-identified feature to its metabolites and metabolic pathways approach. That is, where host metabolomic outcomes in this study indicate potential gut dysbiosis linked to certain microbes/microbial features, the associations to the DAA test outcomes and taxonomy will then be made in light of current literature.

## 3.5 Gut Microbiome Discussion

### 3.5.1 Gut Microbiome Variations in Taxonomic Structure

The study revealed some differences in the composition, alpha, and beta diversity (taxonomic structures) of the gut microbiota among the comparative groups in Comparison A-D. The two applications broadened the coverage and taxonomic levels of the gut microbiome characterised using the faecal samples. As far as I am aware, no other ME/CFS study has used the two methods or considered as many data analytical approaches concurrently. The lack of consistency and direct comparability with other ME/CFS studies remains a widespread issue in finding any definitive dysbiosis taxonomic signatures. Nonetheless, the study did contribute to the overall understanding that there are alterations in the gut microbiota taxonomic structures across a range of taxa levels. The results also provided some demonstration that the FM and IBS comorbidities are relevant when attempting to understand some aspects of the heterogeneity in ME/CFS. It appeared that there may be some involvement of the IBS comorbidity in ME/CFS that warrants further consideration. Although subtle, these results provided some initial indication that looking beyond the usual ME/CFS versus Control/non-ME/CFS was likely warranted, intricate, and involved. It became obvious from the DAA outcomes that a more robust approach via metabolomic and functional-based measurements would be required. Exactly defined gut microbiomes in health and ME/CFS remain elusive and tricky to define, and many combinations of gut commensal taxa could be considered “balanced” (Rinninella et al., 2019; Wilmanski et al., 2021). Gut microbiota composition is highly variable, and it is often difficult to ascertain whether a change is beneficial, detrimental, or of any consequence (Rinninella et al., 2019). While further definition of “gut dysbiosis” was involved in this study with metabolomics, the taxonomic findings from this chapter still provide valuable information that should be taken into consideration.

The alpha and beta diversity metrics showed that ME/CFS is potentially associated with gut dysbiosis, and there may be some involvement of IBS in ME/CFS. Similar observations and commentary have been expressed by previous ME/CFS studies (Giloteaux, Goodrich, et al., 2016; Guo, Che, et al., 2023; König et al., 2022; Nagy-Szakal et al., 2017). IBS, regardless of ME/CFS, has been associated with changes in the taxonomic structure of the gut microbiome. FM irrespective of ME/CFS has been too, although this study did not observe its taxonomic structure to be a point of distinction as a unique subgroup in the ME/CFS cohort. It is important

to clarify that the taxonomic results from this study do not suggest that FM in ME/CFS is devoid of any dysbiosis or microbial alterations and that such changes are exclusive to cases where ME/CFS is accompanied by IBS. Significantly lower alpha diversity and varied beta diversity have generally been associated with IBS (P. P. Chong et al., 2019; Ghaffari et al., 2022; Napolitano et al., 2023). Generally, higher alpha diversity and stability in beta diversity are often thought to reflect a healthier gut microbiome (Wilmanski et al., 2021). Studies in IBS have shown various shifts in taxonomic composition including both higher and lower ratios of Firmicutes/Bacteroidetes. The F/B ratio is widely accepted to have an important influence on maintaining normal intestinal homeostasis; any increased or decreased ratio is regarded as dysbiosis (Ghaffari et al., 2022; Stojanov et al., 2020). Other microbial patterns in various IBS studies have shown a range of differences in microbial features such as an increase in E.Coli, Dorea, Enterobacteriaceae, Clostridia, Clostridiales, and Prevotella, while Bifidobacterium and Lactobacillus were decreased compared to controls (Napolitano et al., 2023). However, evidence of a consistent IBS taxonomic signature also remains conflicting, heterogeneous, and unclear. It is also unclear from the study and in the literature whether the presentation of IBS is a cause or consequence of microbial alterations, and whether IBS and/or gut dysbiosis is an optional, partial, or an entire explanation for ME/CFS pathogenesis and pathophysiology. Indeed, IBS has been observed and linked with many other gastrointestinal and extraintestinal pathologies including FM and chronic pain. Despite this vagueness across both the ME/CFS and IBS diagnostic entities, it can be reasonably thought that the shifts and differences, even slight disturbances, in gut taxonomic structure contribute to, or are associated with, the ME/CFS symptom presentation, and underlying pathophysiology involving inflammation, immune responses, stress, intestinal permeability, and microbial translocation across the mucosa (Ghaffari et al., 2022; Napolitano et al., 2023; Varesi et al., 2021).

### 3.5.2 Gut Microbiome Variations in Metabolic and Functional Structure

Compositional changes do not always translate directly into functional changes in the gut microbiome (Moya & Ferrer, 2016). Functional redundancy and metabolic plasticity are concepts of the gut microbiome that shape its dynamics and functioning, and how it interacts and works with the host and environment in health and disease. It is a paradoxical concept but while there is considerable inter-individual and intra-individual heterogeneity in gut microbiome composition, the collective gene composition and functional capacity of the gut microbiome tends to show greater preservation across individuals (Rinninella et al., 2019; Tian et al., 2020;

Wilmanski et al., 2021). In other words, the taxonomic variability indicates a high degree of functional redundancy across human gut microbes, giving rise to a myriad of taxonomic compositions that result in comparable and interchangeable microbiome functioning (L. Tian et al., 2020; Wilmanski et al., 2021). Such functional redundancy has been hypothesised to underlie the stability, resistance, and resilience of the human microbiome up to a point even though it is continuously exposed and responding to various external challenges and interactions with the host (Fassarella et al., 2021; Tian et al., 2020). High microbial diversity is thought to result in an increased level of functional redundancy which has a role in stabilising microbiota functions during perturbations and thus supporting resilience (Fassarella et al., 2021; Moya & Ferrer, 2016). While many microbiome functions remain well conserved due to the functional redundancy among microbial species, the existence of individual variations has been found to rely on strain-specific functions (Puig-Castellví et al., 2023). On the other hand, metabolic plasticity refers to the ability of the microorganism, or a microbial community, to adapt and change its metabolic processes in response to environmental changes or stressors. The plasticity implies that similar microbiome compositions may have significantly different functionalities (Mayneris-Perxachs & Fernández-Real, 2020). While the exact mechanisms and degree of expression of these concepts between different states and fluctuations of “health” and “unhealthy” are not fully understood, metabolomics can play a pivotal role in characterising the endpoints of these processes and reflect a close metabolic phenotype.

Metagenomics and metabolomics with metataxanomics/taxonomic investigations are suitably positioned to provide a more comprehensive and insightful understanding of the complex gut microbiome-host interactions and dysbiosis (Mayneris-Perxachs & Fernández-Real, 2020; Wilmanski et al., 2021). In the situation that the microbiota changes, due to “unhealthy” perturbations, changes, stress, or exposures that it cannot protect or restore itself from, a state of dysbiosis is acquired (Fassarella et al., 2021; Moya & Ferrer, 2016). The transition to a different state is thought to be a breach of the functional redundancy, resiliency, stability, and the harmonious coexistence of the microbes, and their interactions with each other which then affect the interplay with the host. However, it is not possible to capture the entire landscape of this with only a time point, nor the different stages of ME/CFS progression or severity which come into consideration of the microbiome’s robustness and capacity to perform, recover, and respond over time. Interestingly, the recent study from Xiong et al. (2023) observed that their long-term patients returned to a gut microbiome composition more similar to the healthy controls but had more severe clinical symptoms and metabolic dysbiosis (Xiong et al., 2023). While this

study cannot look beyond the associations from a snapshot in time, the consideration of the stability and resiliency of the gut microbiome should be factored in with the context of ME/CFS. PICRUSt2 can be used to study the functional redundancy in the gut microbiome by predicting the functional potential of the community and identifying the functions that are performed by multiple taxonomic features. It provides the flexibility for marker gene metagenome inference. PICRUSt2 was used with the acknowledgement that it is an amplicon-based functional prediction that relies on the existing reference genomes. More importantly, PICRUSt2 cannot provide resolution to distinguish strain-specific functionality; it can only differentiate taxa to the degree they differ at the amplified marker gene sequence (Douglas et al., 2020). MiMeDB contains consolidated information from the taxonomy of several microbes and the metabolites they produce. It can help with understanding the potential functional redundancy by providing a summary of the multiple databases that it draws from. MiMeDB houses extensive data about the metabolites generated by the microbes including their various reactions, bioactivities, processes, and pathways. The work with MiMeDB is by no means complete but an offering of how more insightful understanding to the microbes, host metabolism, and their connection might be achieved with related information drawn from one source (Wishart et al., 2023). Section 3.6 shows the outcomes of PICRUSt2 and MiMeDB and is considered with the faecal and urine metabolome in Chapter 4.

### 3.5.3 Gut Microbiome Study Considerations

#### 3.5.3.1 Next Generation Sequencing (NGS)

This study has followed a standard 16S rRNA gene amplicon sequencing (16S analysis) workflow using Illumina, QIIME2, and R packages. The use of 16S rRNA has provided insight into the gut microbiome of the study cohorts. 16S analysis has been a mainstay of bacterial analysis for decades and has played an important role in efforts to identify and compare bacteria diversity and communities from complex microbiomes such as the human gut (Bharti & Grimm, 2021; Johnson et al., 2019). It is now one of the most widely used applications to investigate the microbiome at any given body site in research with many experimental and analytical amplicon sequencing protocols targeting the 16S rRNA gene (S. Gupta et al., 2019; Kameoka et al., 2021; Satam et al., 2023). However, there are no gold standard protocols and the standardisation of various applications in the workflow is an issue (Bharti & Grimm, 2021; Kameoka et al., 2021). There are many applications (sequencing chemistry, bioinformatic, and

analytical) and platforms (for example, Illumina, Oxford Nanopore, Pacific Biosciences) both within 16S analysis itself and in the wider scheme of NGS (Marizzoni et al., 2020; Satam et al., 2023). While options are plentiful and accessible, there are general concerns and discussions in the field that inconsistent and poor practices with all components involved in the NGS workflow might lead to inconsistent, non-comparable, and misleading outcomes (Bharti & Grimm, 2021). There is no doubt that NGS-based technologies have made possible several research discoveries and advancements, but there are analytical, experimental, and computational challenges and limitations that need to be considered when interpreting outcomes and planning future projects.

One of the most controversial issues in 16S analysis protocol variations is the selection of the hypervariable region(s) to target (Kameoka et al., 2021). The 16S rRNA gene is conserved in most bacteria and archaea and is approximately 1500 bp long with nine different hypervariable regions (Kameoka et al., 2021). Various universal primers targeting the partial sequences in hypervariable regions have been developed for microbiome analysis (Kameoka et al., 2021). This project only considered the V3 and V4 regions as adopted by the official Illumina protocol. These regions are widely used in gut microbiome studies; however, studies have shown that this primer-pair selection causes bias, amplification artifacts and has a deviating composition compared to other regions (Kameoka et al., 2021; Wensel et al., 2022). Several studies have also demonstrated that primer selection matters for different environments, such as the soil, oral, vaginal, and skin microbiomes, and different conditions such as autism spectrum disorder and colorectal cancer where the gut microbiome is of interest (Fadeev et al., 2021; Kameoka et al., 2021; Na et al., 2023; Osman et al., 2018; Palkova et al., 2021; Wasimuddin et al., 2020). Choice and design of primer pairs is an ongoing effort and perhaps future ME/CFS studies would benefit from looking at a different primer set from the standard Illumina protocol (Kameoka et al., 2021). Another consideration of the study design is workflows and protocols that can provide better species and strain resolution. The culture MALDI-TOF work presented here provided a valuable complement and contribution to the species information. However, a concession is that it was much more labour-intensive than 16S in the wet laboratory and did not have as much of an offering as far as data analysis, and other publications using similar approaches for their work. Future datasets could benefit from NGS applications, such as sequencing the full 16S gene, shotgun metagenomic sequencing, and RNA sequencing, that now can provide this taxonomic resolution and differentiation (Satam et al., 2023; Wensel et al.,

2022). Further, neither of the methods used in this study could achieve strain-level resolution; these NGS offerings are better geared to provide this offering (Wensel et al., 2022).

#### 3.5.3.2 Faecal Samples as Proxies

This thesis, as the majority of ME/CFS microbiome studies have, focused on the faecal bacterial component of the gut. However, other microorganisms such as archaea, eukaryotes and viruses in the gut, and communities of other microorganisms at different sites like the oral microbiome, could also be possibly involved in ME/CFS (Briese et al., 2023; Giloteaux, Hanson, et al., 2016; K. Hou et al., 2022; S.-Y. Hsieh et al., 2023; Lupo et al., 2021; Mandarano et al., 2018; Navaneetharaja et al., 2016; Newberry et al., 2018). While faecal samples are frequently used, they are only proxies to characterise the gut microbiome (Tang et al., 2020). Faecal samples are usually used with sequencing technologies for gut microbiome investigations as the specimens are naturally collected, non-invasive, and can be sampled repeatedly (Tang et al., 2020). However, it has become apparent that faecal samples cannot accurately reflect the entire composition of the intestinal microbiome and therefore provide only a partial view of the microbial diversity (Levitan et al., 2023; Tang et al., 2020). Physiological changes in different areas of the small intestine and colon, including chemical and nutritional gradients and isolated host immune activity, are thought to affect the composition of bacterial communities (Levitan et al., 2023; S. Sun et al., 2021; Tang et al., 2020). Thus, different biospecimen types may be needed to sample microorganisms residing in different niches or to reflect different anatomical and physiological conditions (Rinninella et al., 2019; Sun et al., 2021). There are other sample types or sampling approaches that can be considered, including rectal swabs, mucosal tissue biopsies, and luminal brushings.

Further, it is not only the biochemical and molecular considerations of the gut microbiome. The more physical and external anatomy of the intestinal region in dysbiosis requires further understanding of transit times, motility, mucosal and structural changes. All approaches have their unique pros and cons depending on what information about the gastrointestinal system is required (S. Sun et al., 2021; Tang et al., 2020). Investigations are ongoing to find improved alternatives to faecal samples with NGS capabilities for several clinical applications in the gastroenterological field (Levitan et al., 2023). While faecal samples are still highly useful specimen types for microbiome and metabolomic studies, it is also important to consider their collection methods. There are a few options available with varying degrees of sophistication for

faecal collection systems which aid with convenience, preservation, storage, and transport between the collection point and arrival at the laboratory (Z. Wang et al., 2018). While standard and mainstream practice currently remains with faecal samples and a supplied container that are kept in cold or frozen conditions, new approaches to tackle gut microbiome investigations may at some point be of further interest and importance in ME/CFS research and diagnostic applications.

#### 3.5.3.3 Other Study Considerations

The overarching limitation and difficulty in the development of validated and routinely used diagnostic biomarkers, treatment, and management protocols for ME/CFS is that it is endlessly multifactorial and heterogeneous in many dimensions. This may mean that a single biomarker that can capture all ME/CFS cases with the accuracy, efficiency, and reliability required of a clinically useful test cannot be guaranteed. FM and IBS within their respective current diagnostic directives also have the challenge of plausible subtypes that have yet to be fully defined. Whether these subtypes also exist and are important within the sphere of ME/CFS and its comorbidities is also unclear. According to the Rome IV criteria, IBS can be subclassified into four types based on the main clinical presentation: diarrhoea-predominant IBS, constipation-dominant IBS, mixed IBS, and unclassified IBS, (Napolitano et al., 2023). This study did not capture any further details of these comorbidities. No specific or consistent signatures have been found for the IBS subtypes, nor with the severity of its symptoms (Ghaffari et al., 2022). Different studies have investigated changes in gut microbiome composition and microbial-associated metabolites and their implications in different IBS subtypes (Ghaffari et al., 2022; Napolitano et al., 2023). Diet and therapeutic modulation of the gut microbiome have shown that it may improve clinical outcomes in some IBS patients which further leans into the subtype categorisation of patients, and the need for personalised interventions. Therefore, in the context of ME/CFS, the use of therapeutic and gut modulatory tools, such as diets, prebiotics, probiotics, synbiotics, postbiotics, and faecal transplants, need to be expanded to establish their role and utility in the future (Ghaffari et al., 2022; Napolitano et al., 2023).

Differences in taxonomic composition can be explained by several extrinsic and intrinsic factors. Other than highlighting their FM and IBS comorbidity with some control for the age variable attempted, this study did not investigate the many other factors that could serve as possible explanations for the shifts in taxa. Section 6.2.1 details the challenges and limitations of the

study overall, ultimately highlighting the need for studies with more data points over time and for individuals to be their own control (longitudinal). However, it is worth mentioning here that in any gut microbiome (and metabolomic) investigation, there are considerations for issues such as other host characteristics and comorbidities, environmental changes, living conditions, lifestyle, medication and supplements, diet, nutrition, feeding patterns, BMI, bowel activity, and exercise. Studies have extensively looked at a number of these variables and shown that the dynamic gut microbiome is influenced and impacted as either a cause or consequence (Moya & Ferrer, 2016; Puig-Castellví et al., 2023; Rinninella et al., 2019; Wilmanski et al., 2021). It is not uncommon for individuals, whether they are ME/CFS patients or not to partake in a range of interventions from prescription medication, non-prescription medication, supplements, and special diets, that impact or influence their gut microbiome and host metabolism. It is almost impossible to absolutely "level the playing field" for studies if any participants, patients, controls, or otherwise, are to be involved in a study. Seton et al. (2023) provide a good example of this where their recruitment for matched healthy-household controls to account for environmental confounders was challenging because of other confounders such as age and sex (Seton et al., 2023). Asking participants to cease or change any of their routines specifically for the study (especially where antibiotics, probiotics, and antivirals are concerned) is a whole other caveat of consideration for a study design, ethics, and recruitment (Armstrong et al., 2017; Du Preez et al., 2018; Sheedy et al., 2009; Shukla et al., 2015; Wallis et al., 2018). Study participants were not asked to change anything especially for the project but were asked to record any of these details in their questionnaire responses (see Section 2.1). Apart from the priority of completing recruitment for this project before the recruitment site, CFS Discovery, closed permanently, many participants said they would be reluctant to participate if they had to change anything. One also must accept the limitations of a one-off sample time-point whether or not any changes were made for study participation. Nonetheless, any observations made from this study are mindful of these factors as potential confounders for the gut microbiome and host metabolome.

## 3.6 Functional Predictions from Gut Microbiome

### 3.6.1 PICRUSt2 (16S rRNA)

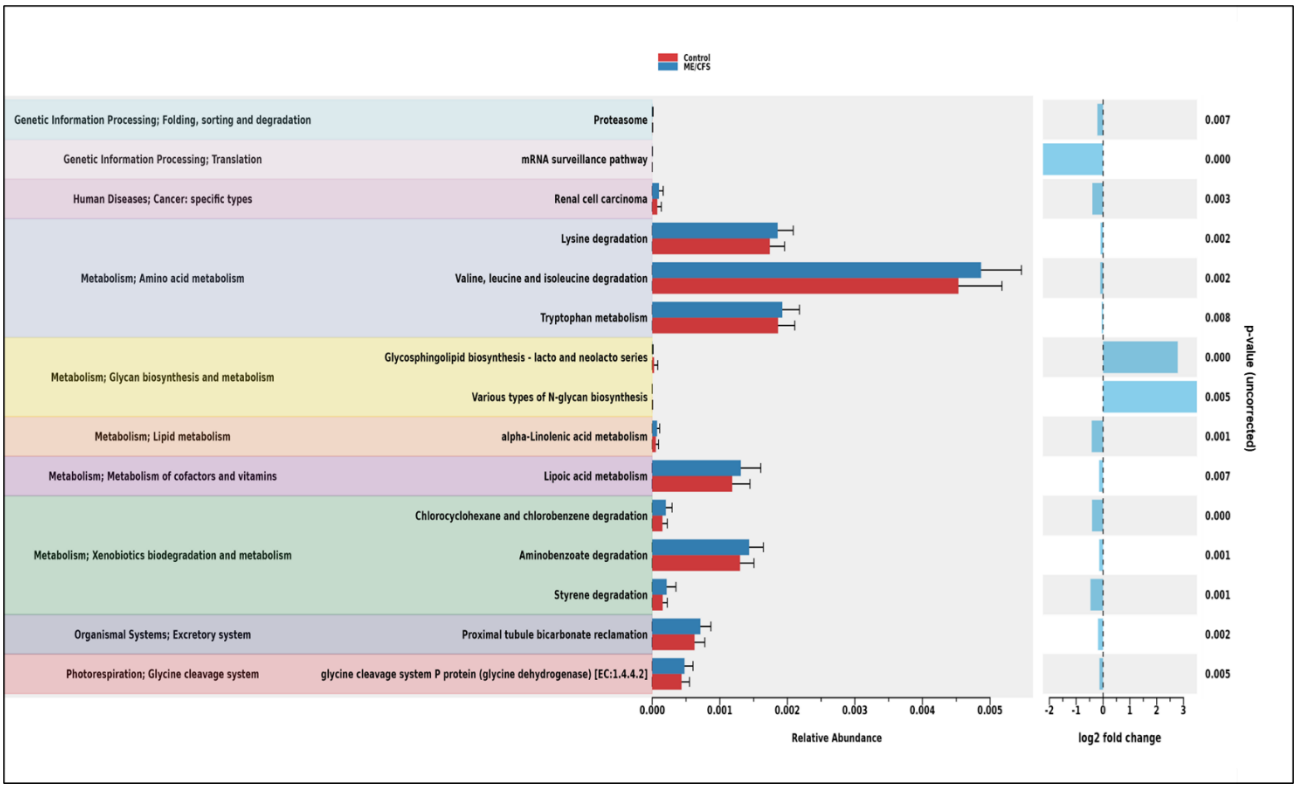



**Figure 3.6.1** Prediction of different KEGG pathways between ME/CFS and Control groups using PICRUSt2 and ALDEx2 analysis and visualised using ggpicrust2 R package. Left-hand side bar plots display the relative abundance of each predicted KEGG pathway. Right-hand side bar plots display the log2 fold change of the p-values calculated using Wilcoxon rank-sum test (uncorrected) from ALDEx2 results; only p-values ≤ 0.01 are displayed. Positive direction of log2 gold change bar presents increased expression in Control group. Negative direction of log2 fold change bar represents increased expression in the ME/CFS group.

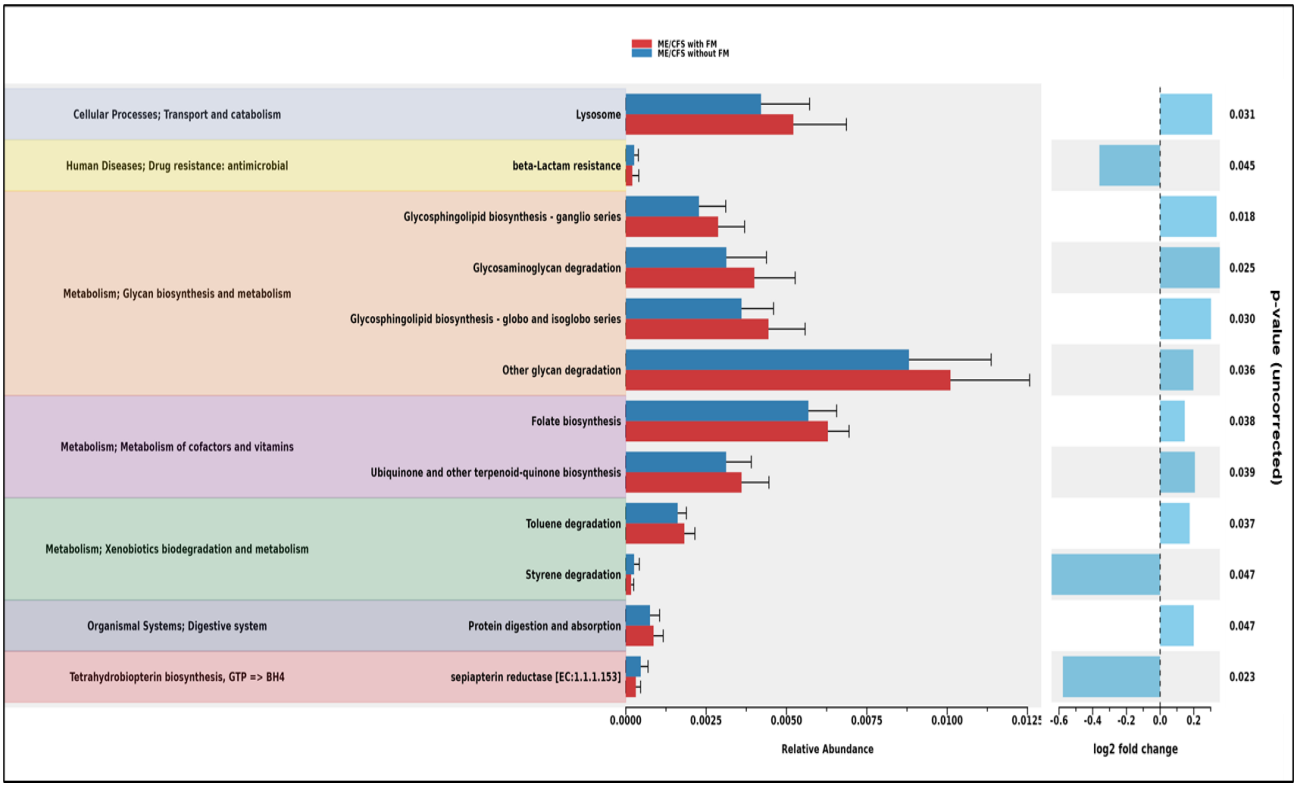



**Figure 3.6.2** Prediction of different KEGG pathways between ME/CFS with FM and ME/CFS without FM groups using PICRUSt2 and ALDEx2 analysis and visualised using ggpicrust2 R package. Left-hand side bar plots display the relative abundance of each predicted KEGG pathway. Right-hand side bar plots display the log2 fold change of the p-values calculated using Wilcoxon rank-sum test (uncorrected) from ALDEx2 results; only p-values ≤ 0.05 are displayed. Positive direction of log2 fold change bar represents increased expression in ME/CFS with FM group. Negative direction of log2 fold change bar represents increased expression in ME/CFS without FM group.

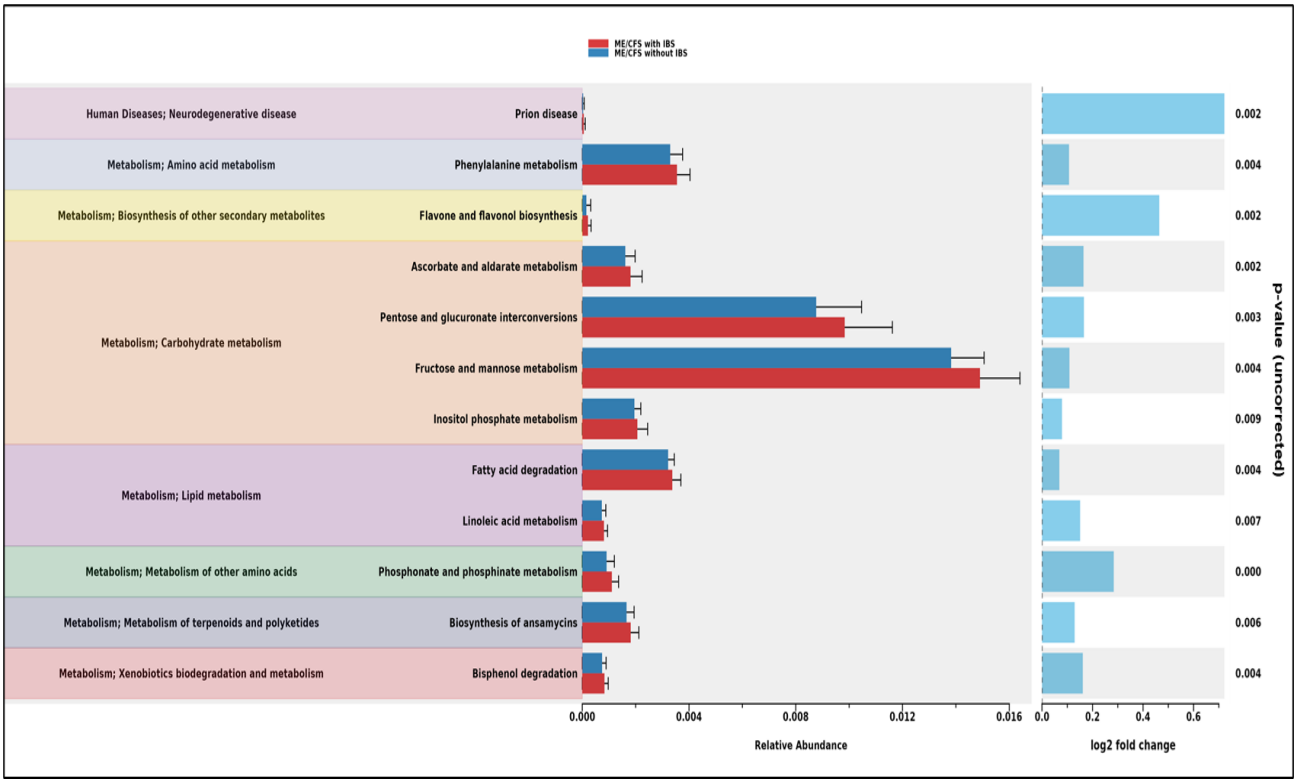



**Figure 3.6.3** Prediction of different KEGG pathways between ME/CFS with IBS and ME/CFS without IBS groups using PICRUSt2 and ALDEx2 analysis and visualised using ggpicrust2 R package. Left-hand side bar plots display the relative abundance of each predicted KEGG pathway. Right-hand side bar plots display the log2 fold change of the p-values calculated using Wilcoxon rank-sum test (uncorrected) from ALDEx2 results; only p-values ≤ 0.01 are displayed. Positive direction of log2 fold change bar represents increased expression in ME/CFS with IBS group.

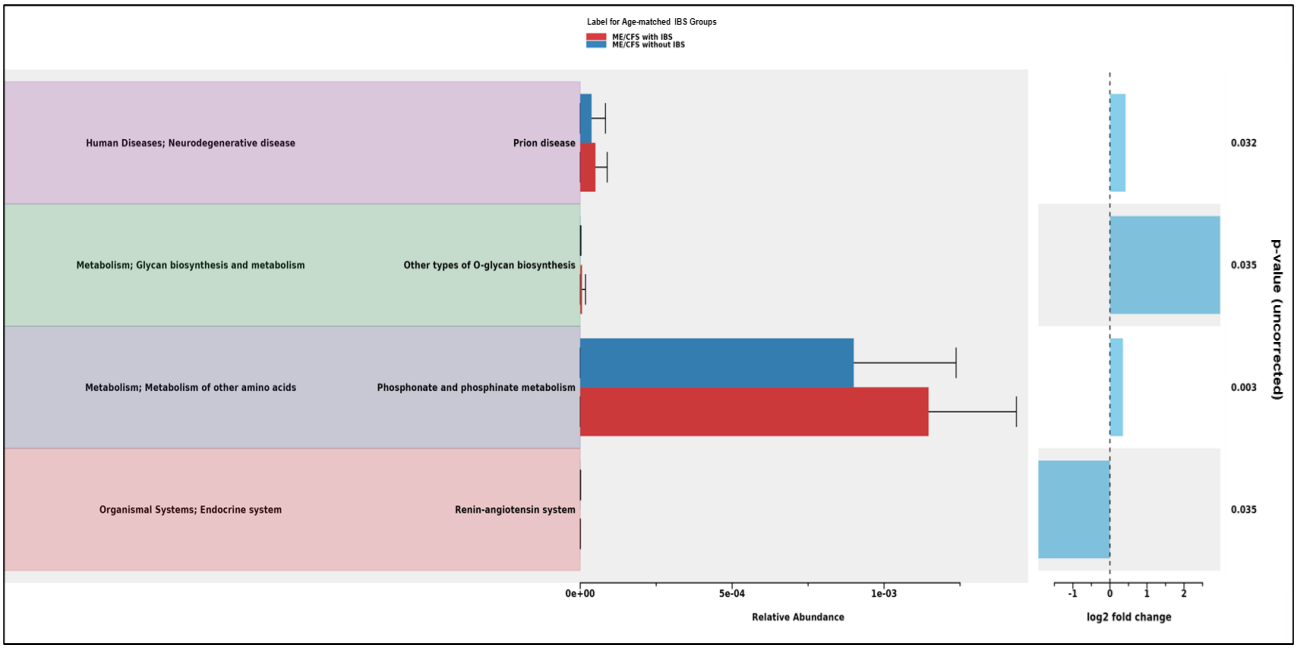



**Figure 3.6.4** Prediction of different KEGG pathways between age-matched ME/CFS with IBS and ME/CFS without IBS groups using PICRUSt2 and ALDEx2 analysis and visualised using ggpicrust2 R package. Left-hand side bar plots display the relative abundance of each predicted KEGG pathway. Right-hand side bar plots display the log2 fold change of the p-values calculated using Wilcoxon rank-sum test (uncorrected) from ALDEx2 results; only p-values ≤ 0.05 are displayed. Positive direction of log2 fold change bar represents increased expression in ME/CFS with IBS group. Negative direction of log2 fold change bar represents increased expression in ME/CFS without IBS group.

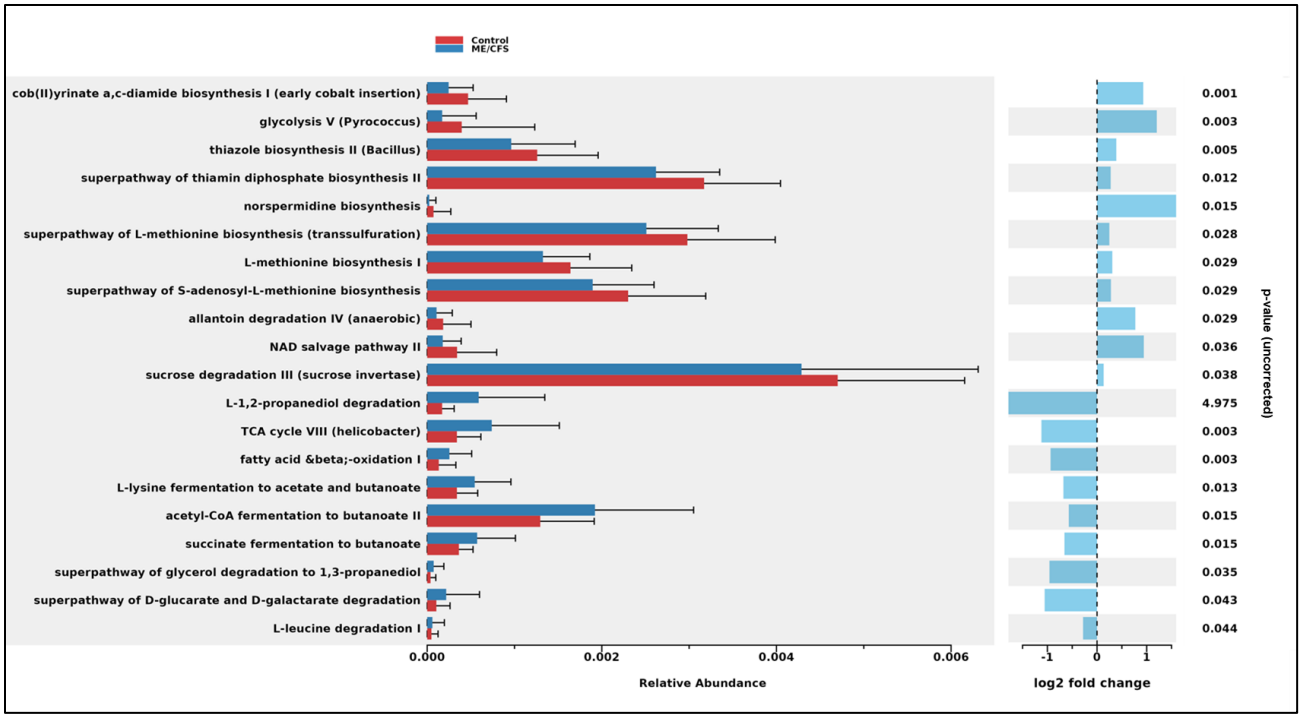



**Figure 3.6.5** Prediction of different MetaCyc pathways between ME/CFS and Control groups using PICRUSt2 and ALDEx2 analysis and visualised using ggpicrust2 R package. Left-hand side bar plots display the relative abundance of each predicted MetaCyc pathway. Right-hand side bar plots display the log2 fold change of the p-values calculated using Wilcoxon rank-sum test (uncorrected) from ALDEx2 results; positive direction of log2FC bar represents increased expression in the Control group. Only the top 20 features with p-values ≤ 0.05 are displayed.

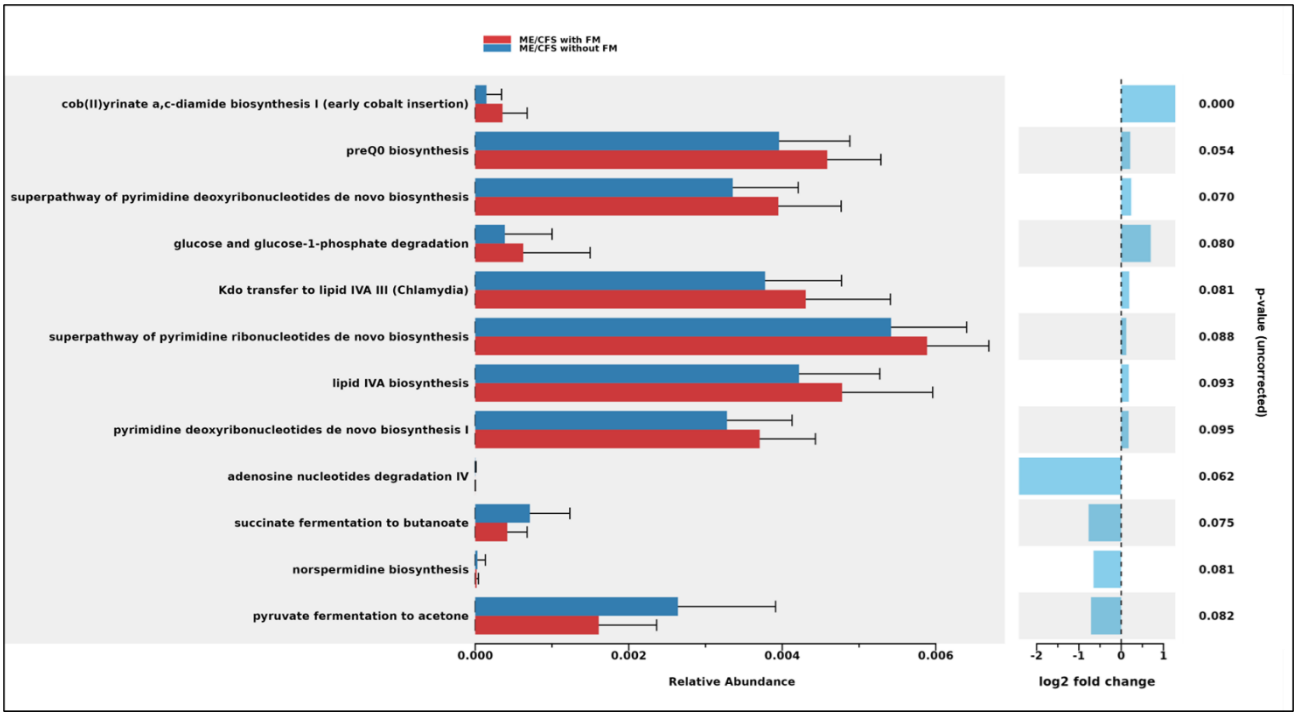



**Figure 3.6.6** Prediction of different MetaCyc pathways between ME/CFS with FM and ME/CFS without FM groups using PICRUSt2 and ALDEx2 analysis and visualised using ggpicrust2 R package. Left-hand side bar plots display the relative abundance of each predicted MetaCyc pathway. Right-hand side bar plots display the log2 fold change of the p-values calculated using Wilcoxon rank-sum test (uncorrected) from ALDEx2 results; positive direction of log2FC bar represents increased expression in the ME/CFS with FM group. Only the features with p-values ≤ 0.10 are displayed.

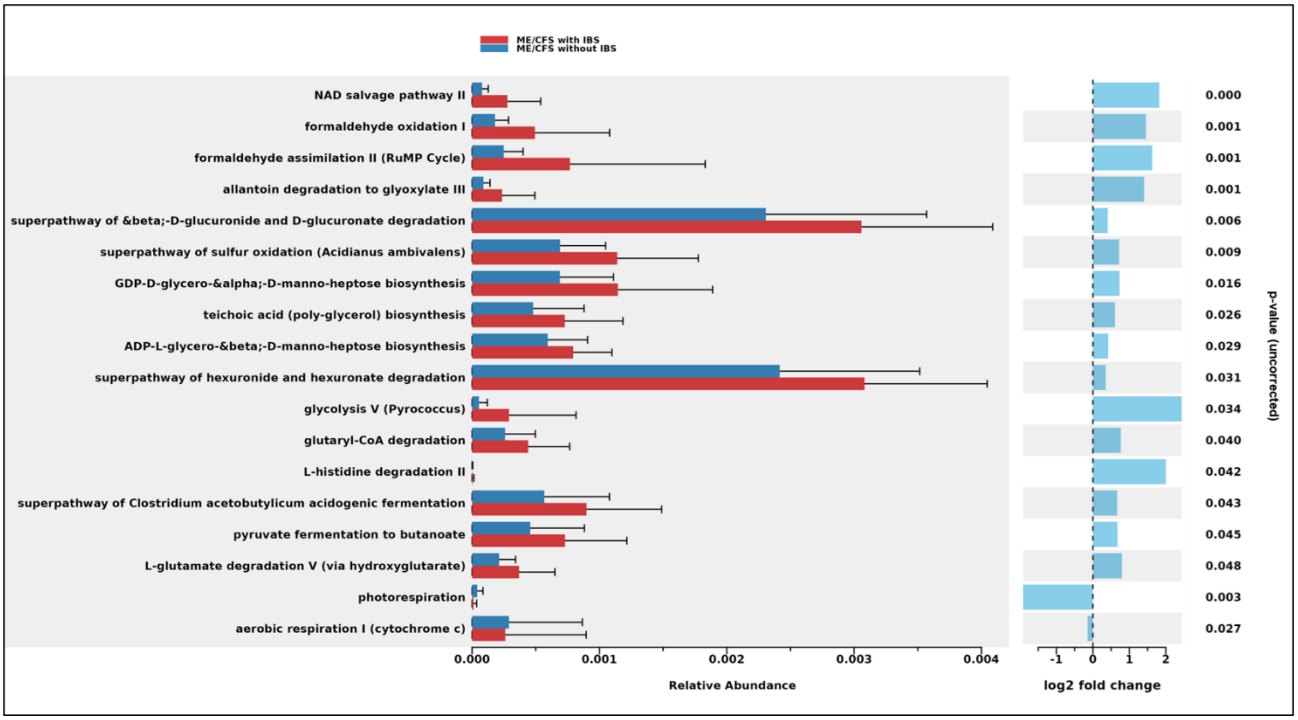



**Figure 3.6.7** Prediction of different MetaCyc pathways between ME/CFS with IBS and ME/CFS without IBS groups using PICRUSt2 and ALDEx2 analysis and visualised using ggpicrust2 R package. Left-hand side bar plots display the relative abundance of each predicted MetaCyc pathway. Right-hand side bar plots display the log2 fold change of the p-values calculated using Wilcoxon rank-sum test (uncorrected) from ALDEx2 results; positive direction of log2FC bar represents increased expression in the ME/CFS with IBS group. Only the features with p-values ≤ 0.05 are displayed.

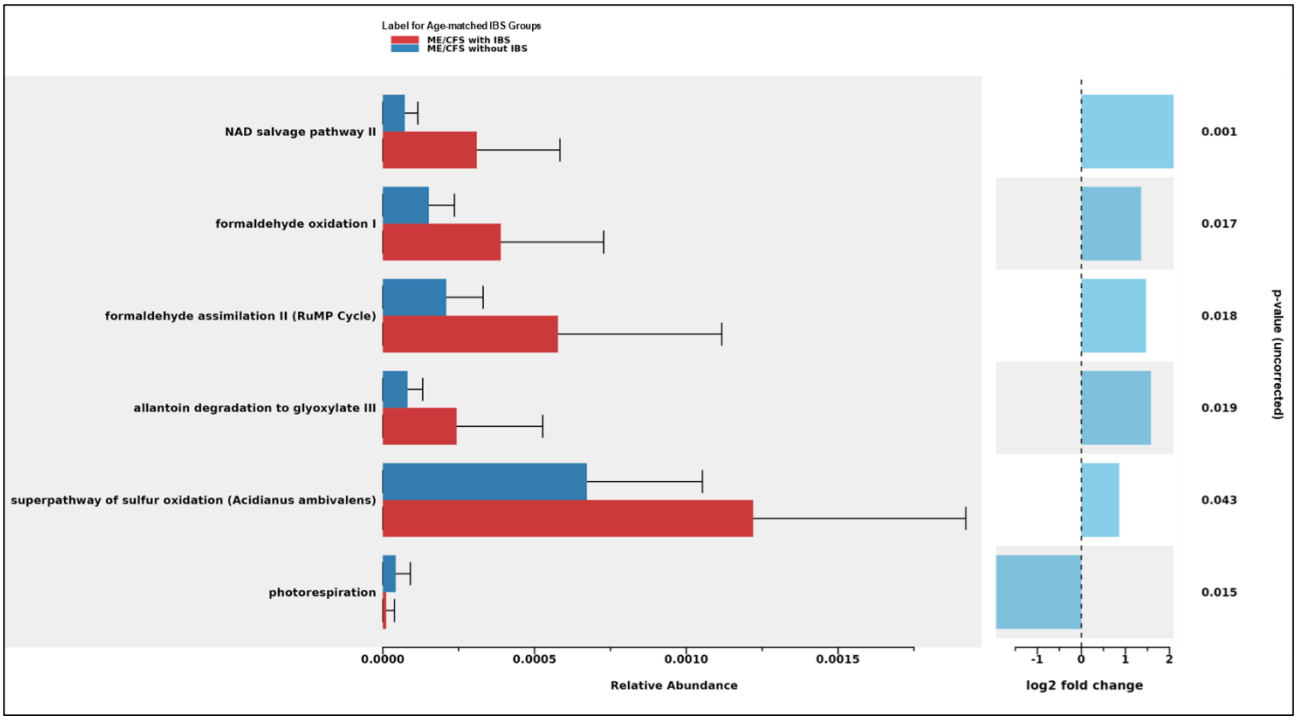



**Figure 3.6.8** Prediction of different MetaCyc pathways between age-matched ME/CFS with IBS and ME/CFS without IBS groups using PICRUSt2 and ALDEx2 analysis and visualised using ggpicrust2 R package. Left-hand side bar plots display the relative abundance of each predicted MetaCyc pathway. Right-hand side bar plots display the log2 fold change of the p-values calculated using Wilcoxon rank-sum test (uncorrected) from ALDEx2 results; positive direction of log2FC bar represents increased expression in the age-matched ME/CFS with IBS group. Only the features with p-values ≤ 0.05 are displayed.

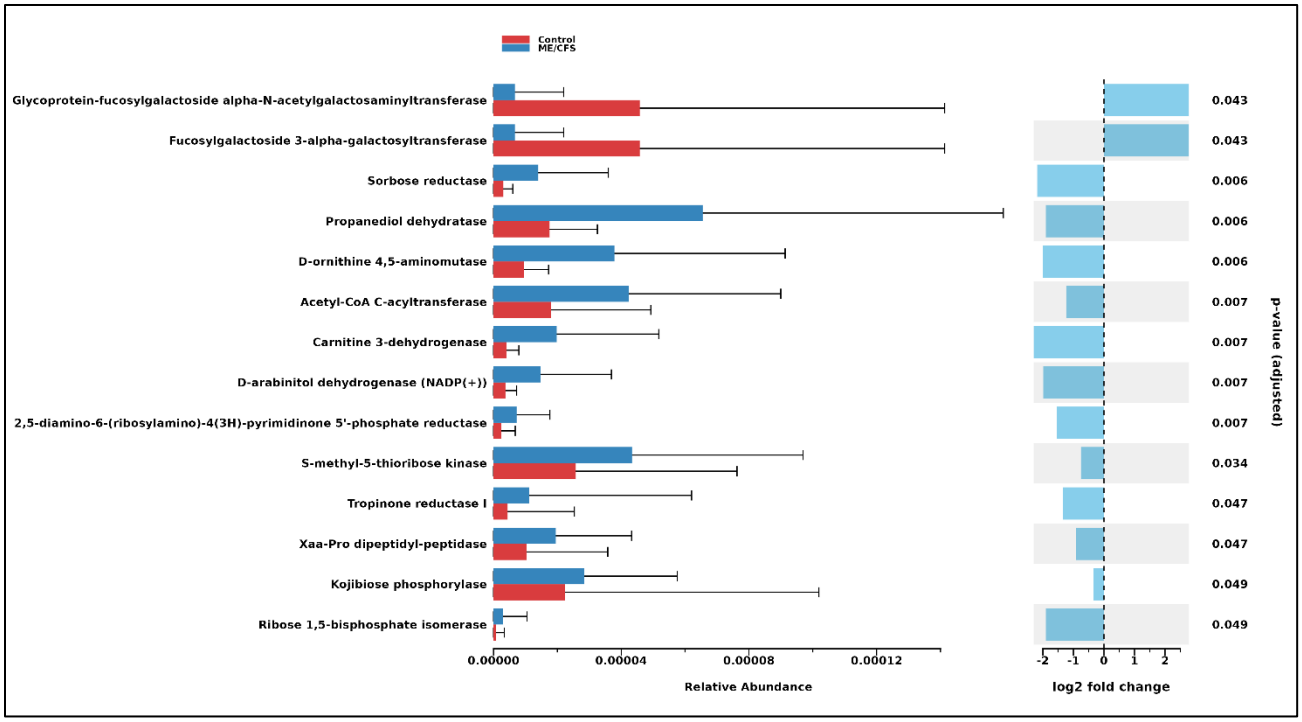



**Figure 3.6.9** Prediction of different Enzyme Classification (EC) gene families between ME/CFS and Control groups using PICRUSt2 and ALDEx2 analysis and visualised using ggpicrust2 R package. Left-hand side bar plots display the relative abundance of each predicted EC gene family. Right-hand side bar plots display the log2 fold change of the p-values calculated using Wilcoxon rank-sum test (adjusted) from ALDEx2 results; positive direction of log2FC bar represents increased expression in the Control group. Only FDR-adjusted p-values ≤ 0.05 are displayed.

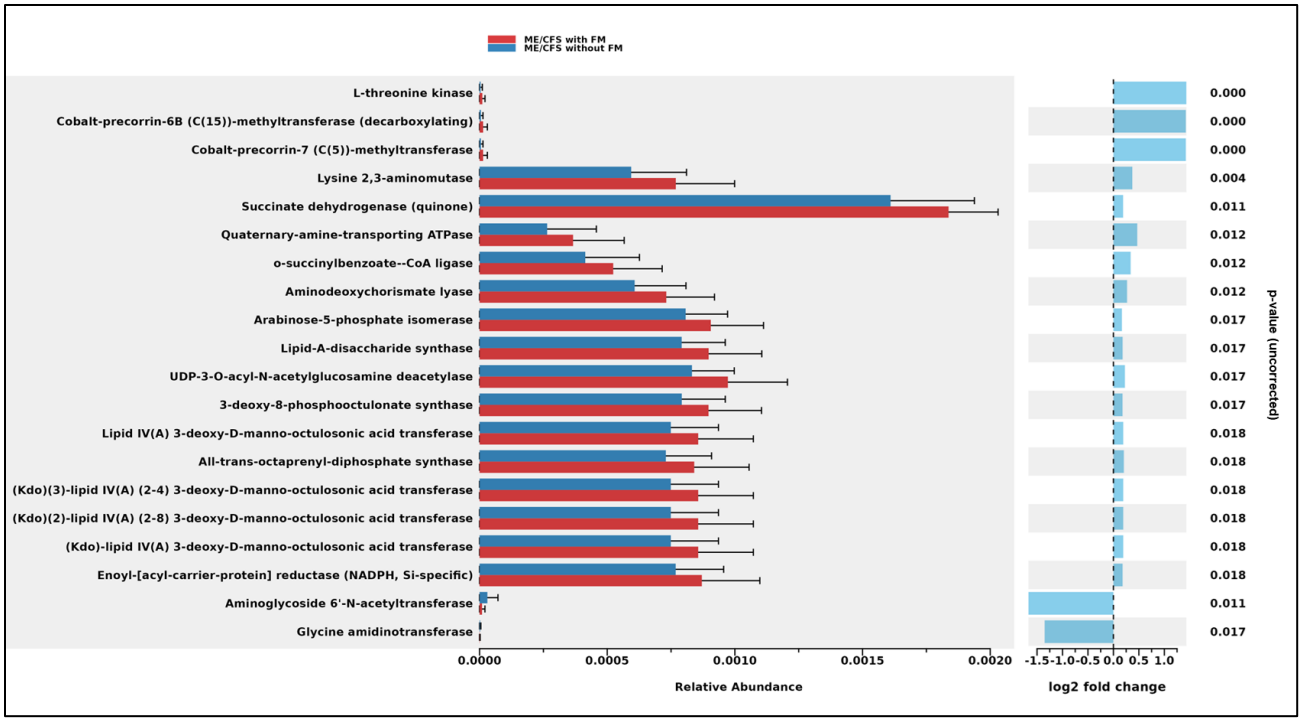



**Figure 3.6.10** Prediction of different Enzyme Classification (EC) gene families between ME/CFS with FM and ME/CFS without FM groups using PICRUSt2 and ALDEx2 analysis and visualised using ggpicrust2 R package. Left-hand side bar plots display the relative abundance of each predicted EC gene family. Right-hand side bar plots display the log2 fold change of the p-values calculated using Wilcoxon rank-sum test (uncorrected) from ALDEx2 results; positive direction of log2FC bar represents increased expression in the ME/CFS with FM group. Only the top 20 features with p-values ≤ 0.05 are displayed.

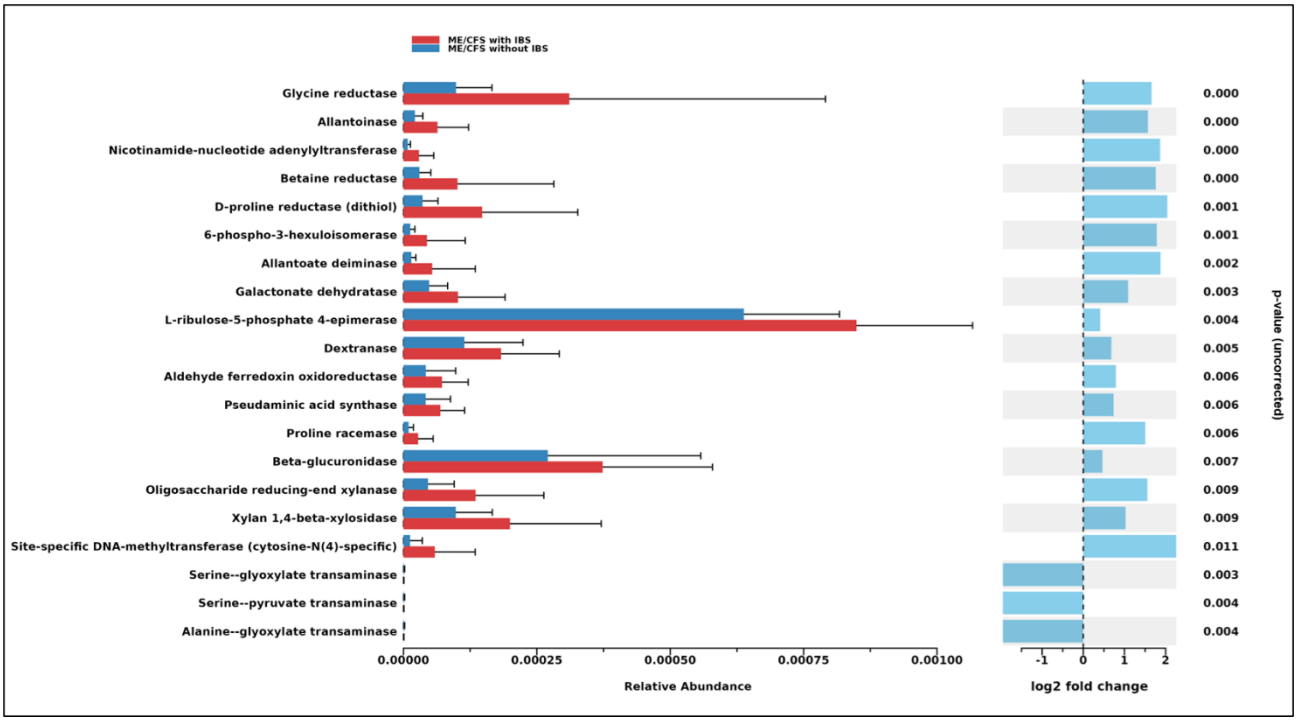



**Figure 3.6.11** Prediction of different Enzyme Classification (EC) gene families between ME/CFS with IBS and ME/CFS without IBS groups using PICRUSt2 and ALDEx2 analysis and visualised using ggpicrust2 R package. Left-hand side bar plots display the relative abundance of each predicted EC gene family. Right-hand side bar plots display the log2 fold change of the p-values calculated using Wilcoxon rank-sum test (uncorrected) from ALDEx2 results; positive direction of log2FC bar represents increased expression in the ME/CFS with IBS group. Only the top 20 features with p-values ≤ 0.05 are displayed.

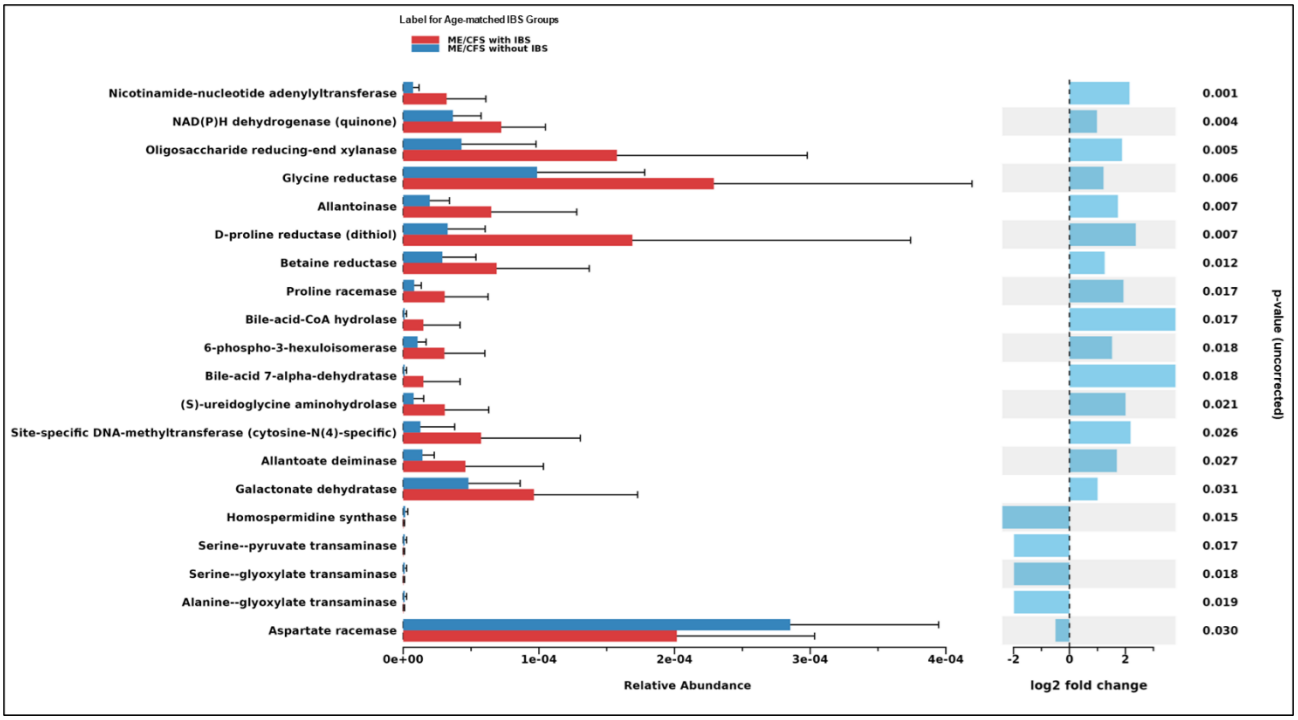



**Figure 3.6.12** Prediction of different Enzyme Classification (EC) gene families between age-matched ME/CFS with IBS and ME/CFS without IBS groups using PICRUSt2 and ALDEx2 analysis and visualised using ggpicrust2 R package. Left-hand side bar plots display the relative abundance of each predicted EC gene family. Right-hand side bar plots display the log2 fold change of the p-values calculated using Wilcoxon rank-sum test (uncorrected) from ALDEx2 results; positive direction of log2FC bar represents increased expression in the age-matched ME/CFS with IBS group. Only the top 20 features with p-values ≤ 0.05 are displayed.

### 3.6.2 MiMeDB (Culture MALDI-TOF)

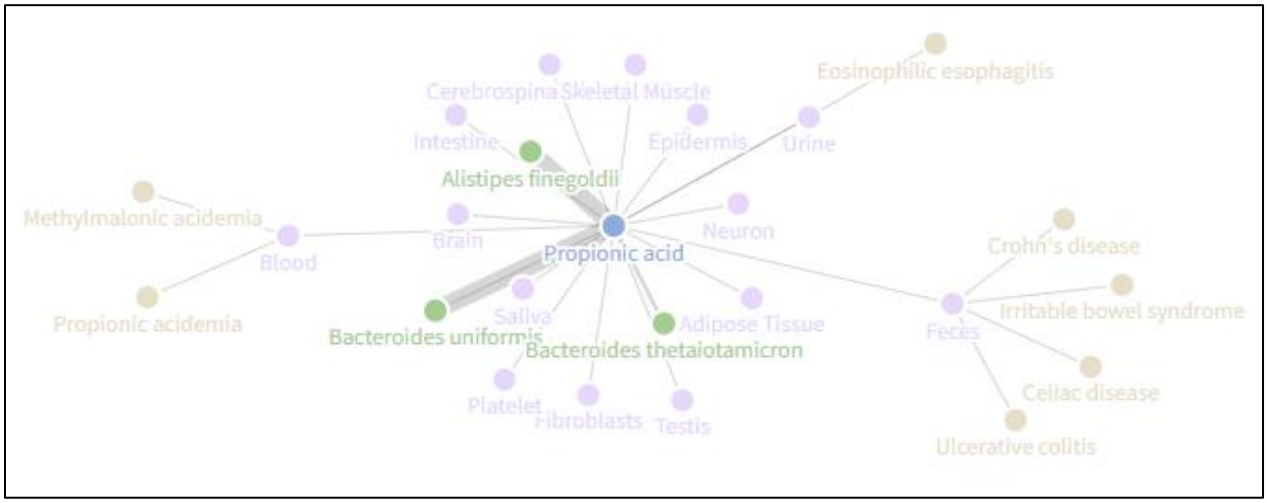



**Figure 3.6.13** Interactive network visualisation using the Flourish tool of the microbe, disease, and host biospecimen connections with metabolite Propionic Acid (each represented by a differently coloured node). The larger width of the links/edges represents a more important connection between the nodes of interest. The graphic is interactive and can be viewed at the following link provided by the Wishart Lab: https://public.flourish.studio/visualisation/15853980/

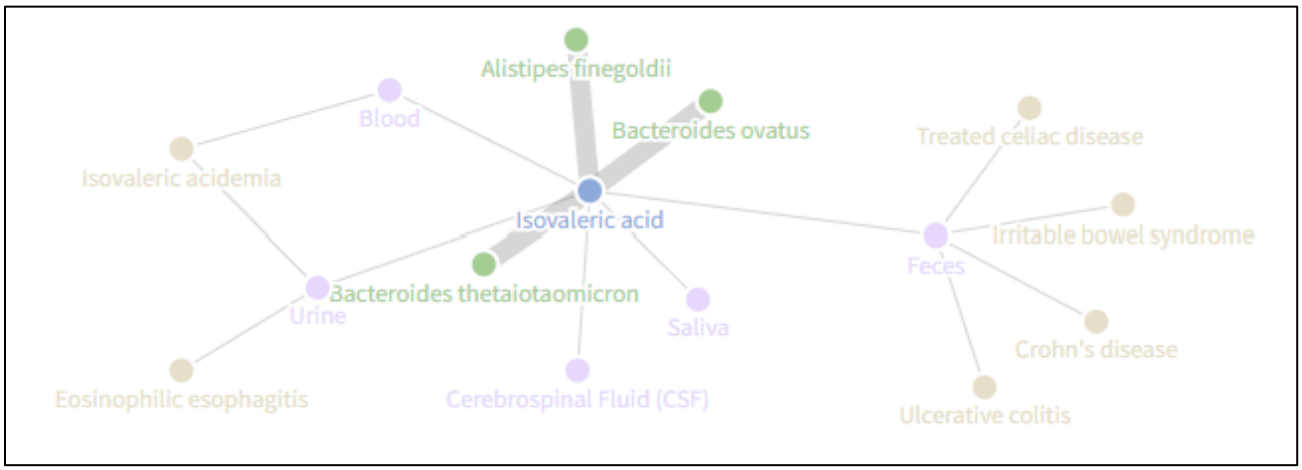



**Figure 3.6.14** Interactive network visualisation using the Flourish tool of the microbe, disease, and host biospecimen connections with metabolite Isovaleric Acid (each represented by a differently coloured node). The larger width of the links/edges represents a more important connection between the nodes of interest. The graphic is interactive and can be viewed at the following link provided by the Wishart Lab: https://public.flourish.studio/visualisation/16057354/

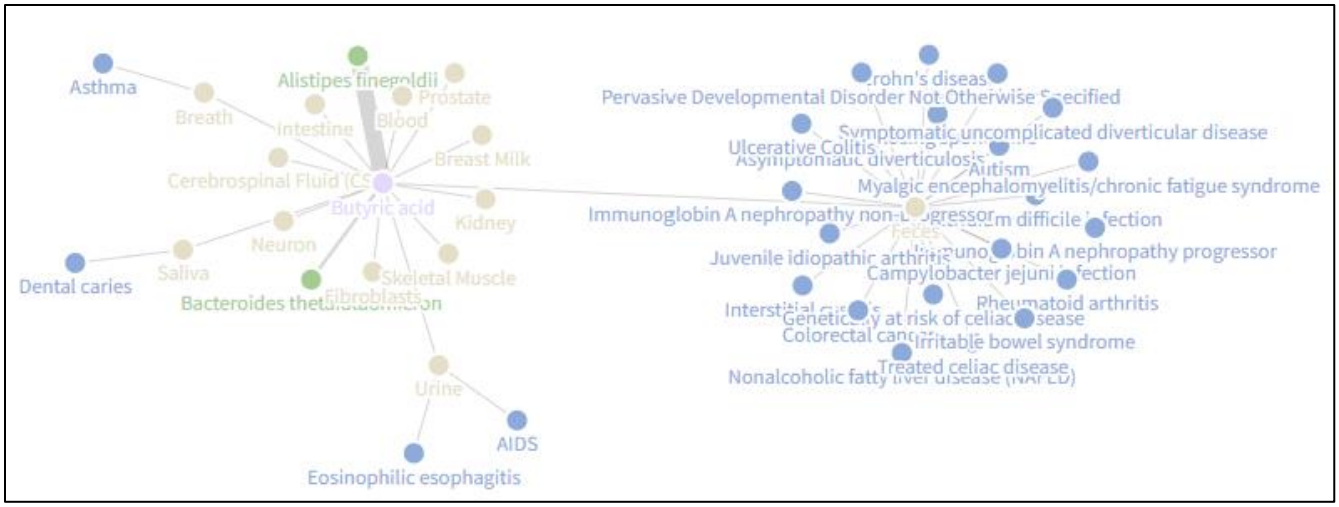



**Figure 3.6.15** Interactive network visualisation using the Flourish tool of the microbe, disease, and host biospecimen connections with metabolite Butyric Acid (each represented by a differently coloured node). The larger width of the links/edges represents a more important connection between the nodes of interest. The graphic is interactive and can be viewed at the following link provided by the Wishart Lab: https://public.flourish.studio/visualisation/16057533/

# 4 CHAPTER FOUR: Faecal and Urine Host Metabolome (Part One)

Complex diseases like ME/CFS are time-consuming, difficult, and expensive to diagnose and monitor (Naviaux et al., 2016). Currently, there are no approved laboratory tests sensitive and specific for ME/CFS although metabolomic studies so far have provided evidence of disturbed, irregular metabolic processes and pathophysiology (Huth et al., 2020; Maksoud et al., 2023; Taccori et al., 2023). Over the past few years, one of the most striking aspects of metabolomics has been its potential for differentiating disease subtypes via screening of systemic metabolome (metabolite) alterations (Qiu et al., 2023). This study is mainly concerned with the various endogenous metabolites (host-derived, microbe-derived, and host-microbial-co-metabolites) in human urine, blood plasma, and faeces(Lamichhane et al., 2018; Wishart, 2019). Metabolomics with its continually improving analytical technologies, platforms, and workflow approaches, offers the possibility for the tandem advancements of elucidating disease pathophysiology, reliable biomarkers, and the development of novel diagnostics and therapeutics. Metabolomics with a precision medicine scope and edict has much to offer to ME/CFS research and clinical applications.

Common endogenous metabolites include but are not limited to, cholesterol, lipids, amino acids, bile acids, short peptides, nucleic acids, sugars, alcohols, fatty acids, and organic acids. Endogenous metabolites are poised to provide a unique metabolic reflection of a healthy or diseased state and the metabolic pathways involved. Metabolomics (or the metabolome) is a useful, sensitive probe of an individual's phenotype and is fundamentally different from the genome (Qiu et al., 2023; Wishart, 2019). The metabolome represents both the downstream outputs of the genome, transcriptome, or proteome, and the upstream input from the environment (Qiu et al., 2023). Although the endogenous metabolites are largely conserved, individual metabolomes are not invariant and they are constantly very sensitive to several internal and external variables (Wishart, 2019). Metabolic phenotypes can provide a more timely, accurate snapshot of what is happening in a participant and their current health and functional state.

Beyond the role of cellular functioning and energetics/energy metabolism, metabolomics has revealed that metabolites have much more varied, specific, and important roles (Qiu et al., 2023; Wishart, 2019). It is therefore generally hypothesised that disease pathophysiology is closely underpinned or governed by a constellation of interrelated metabolic changes,

abnormalities, and dysfunction. Of particular interest in this study is the host's interplay or bidirectional connection with gut physiology and the gut microbiome. The gut microbiome is made up of trillions of bacteria and other microbial cells that co-exist with other human cells. The gut (lower GI tract, especially the small and large intestine) or GI tract is the organ system that is most central to metabolism (Wishart, 2019). Endogenous metabolites are derived from the metabolism of the microbes in the gut microbiome or the human host. However, most of these metabolites are produced in the gut by the microbiota and these metabolites may modulate and significantly affect host metabolism (Y. Zhang et al., 2023). The metabolic activity of the gut microbiome is essential in maintaining host homeostasis and health (Visconti et al., 2019). Without a gut microbiome, humans are essentially unable to survive and perform necessary tasks, hence the common calling of "forgotten, hidden metabolic organ", "the additional organ", and "the second brain and endocrine system" among other similar phrases. Although the presence of the microbiota is vital, variations in its composition (gut dysbiosis, dysfunction, disturbances) induce metabolic shifts that may result in changes in host phenotype, and vice versa (Turnbaugh et al., 2006; Visconti et al., 2019). Interestingly, where changes may not be observed or obvious in the composition of the gut microbiome from metataxonomic investigations, using other omic approaches including metabolomics, can provide information regarding the metabolically active microbes (Visconti et al., 2019; Whon et al., 2021; Wishart, 2019; Wishart et al., 2023). Monitoring taxonomic and metagenomic profiles can help with marking certain microbiome perturbations; however, small molecular-weight microbial products have the potential to result in more reproducible signals that translate readily across vastly diverse cohorts and disease states (Wilmanski et al., 2021).

Metabolomics is an extremely useful tool to understand the complex metabolic interactions between gut microbes and the host (Lamichhane et al., 2018). However, before metabolomics can be used for any urgently needed routine monitoring and testing needs in ME/CFS, the disease requires clarification of the comorbidities and heterogeneity. This chapter (Chapter 4) focused on the faecal and urine metabolome from Part One of the study. Metabolomic outcomes from plasma and urine samples in Part Two are in Chapter 5. Both chapters utilised a LCMS and NMR metabolomics workflow. As NMR is much less sensitive than LCMS, leading to much-reduced metabolic coverage, it can be reasonably assumed that metabolites measured by both platforms are present at relatively high concentrations (Dona et al., 2016; Karu et al., 2018). Part One looked at 40 ME/CFS overall and 43 Control participants which included males and females (Section 2.1). Table 2.2.1 shows the baseline characteristics of all four comparative

groups of interest (Comparison A-D). Comparison A compares the ME/CFS overall and Control groups, Comparison B compares the ME/CFS +/- FM groups, Comparison C compares the ME/CFS +/- IBS groups, and Comparison D compares the age-matched ME/CFS +/- IBS** groups. Results relevant to faecal metabolomics are in Section 4.1. Results relevant to urinary metabolomics are in Section 4.2. Lastly, Section 4.3 covers the discussion for the Part One metabolomic outcomes from the faeces and urine, both independently and when compared to each other.

## 4.1 Faecal Metabolomics Results

Globally, LCMS detected 137 and NMR detected 36 faecal metabolite features from all samples (Appendix 8). 99 significantly different metabolite features in total from LCMS and NMR were highlighted from the volcano plots (VPs) and PLS-DA VIP plots across all the comparisons, Comparison A-D (Table 4.1.1). These significantly different metabolites, categorised according to the HMDB database, included bile acids, alcohols and derivatives (12.12%), amino acids, peptides and analogues (24.24%), purines and purine derivatives (3.03%), other metabolites (15.15%), alcohols (3.03%), carboxylic acids and derivatives (11.11%), fatty acyls and acids (21.21%), sugar, carbohydrates and carbohydrate conjugates (8.08%), and pyrimidines and pyrimidine derivatives (2.02%). There were eight significant differential faecal metabolite features (nicotinic acid, malonic acid, glycine, xanthine, glutamic acid, propionic acid, butyric acid, and fumaric acid) that were measured by both analytical platforms, LCMS and NMR (Table 4.1.1). The relative concentrations found for these shared metabolites were consistent in their direction as either up/higher/red or down/lower/blue from the VP and PLS-DA analyses (Appendix 9). The individual plots from the VP, PCA, and PLS-DA analyses for the faecal metabolomic outcomes in Comparisons A-D are shown in Sections 0 (ME/CFS overall vs. Control), 4.1.2 (ME/CFS +/- FM), 4.1.3 (ME/CFS +/- IBS), and 4.1.4 (age-matched ME/CFS +/- IBS**), respectively.

**Table 4.1.1** LCMS and NMR Faecal Metabolome (Part One) Significant Features and Results

| | Faecal Metabolite | Comparative Group (A-D) | Group with significant elevation in relative concentration | LCMS or NMR |
|---|---|---|---|---|
| Bile acids, alcohols and derivatives | 3-Oxocholic acid | A | ME/CFS | LCMS |
| | Allocholic acid/Cholic acid | A | ME/CFS | LCMS |
| | Allocholic acid* | | | |
| | Cholic acid* | | | |
| | Deoxycholic/Hyodeoxycholic/Ursodeoxycholic | C, D | ME/CFS + IBS, ME/CFS + IBS** | LCMS |
| | Deoxycholic acid* | | | |
| | Hyodeoxycholic acid* | | | |
| | Ursodeoxycholic acid* | | | |
| | 7a-Hydroxy-3-oxo-5b-cholanoic/Nutriacholic | D | ME/CFS + IBS** | LCMS |
| | 7a-Hydroxy-3-oxo-5b-cholanoic acid* | | | |
| | Nutriacholic acid* | | | |
| | 3b-Hydroxy-5-cholenoic acid* | C | ME/CFS - IBS | LCMS |
| Amino acids, peptides and analogues | Glycine ‡ | A | ME/CFS | LCMS, NMR |
| | Serine | A, B, C | ME/CFS, ME/CFS - FM, ME/CFS - IBS | NMR |
| | Glutamine | B | ME/CFS - FM | NMR |
| | Proline | B | ME/CFS - FM | NMR |
| | Glutamate (aka. Glutamic acid) ‡ | B, D | ME/CFS - FM, ME/CFS + IBS ** | NMR, LCMS |
| | Tyrosine | D | ME/CFS + IBS** | NMR |
| | Histidine | B | ME/CFS + FM | NMR |
| | Methionine | B | ME/CFS - FM | NMR |
| | Lysine | C, D | ME/CFS + IBS, ME/CFS + IBS** | NMR |
| | Phenylalanine | D | ME/CFS + IBS** | NMR |
| | Taurine | A, C | ME/CFS, ME/CFS + IBS | NMR |
| | Beta-Leucine/L-Alloisoleucine/L-Isoleucine | A | ME/CFS | LCMS |
| | L-Leucine/L-Norleucine | A | ME/CFS | LCMS |
| | L-Alloisoleucine* | | | |
| | L-Isoleucine* | | | |
| | L-Valine | A | ME/CFS | LCMS |
| | L-Leucine* | | | |
| | Pyroglutamic acid | A | ME/CFS | LCMS |
| | Beta-Leucine* | | | |
| | N-Alpha-acetyllysine | D | ME/CFS + IBS** | LCMS |
| | N6-Acetyl-L-lysine | C, D | ME/CFS + IBS, ME/CFS + IBS** | LCMS |
| | Citrulline | D | ME/CFS + IBS** | LCMS |

| | | | | |
|---|---|---|---|---|
| | Ornithine | D | ME/CFS + IBS** | LCMS |
| | L-Norleucine* | | | |
| Purines and purine derivatives | Adenine | B | ME/CFS + FM | LCMS |
| | Xanthine ‡ | A, C, D | Control, ME/CFS + IBS, ME/CFS + IBS** | NMR, LCMS |
| | Hypoxanthine | A, C, D | Control, ME/CFS + IBS, ME/CFS + IBS** | NMR |
| Other metabolites | Homogentisic acid/Vanillic acid | C | ME/CFS + IBS | LCMS |
| | Homogentisic acid* | | | |
| | Alpha-Tocopherol | B | ME/CFS - FM | LCMS |
| | Beta-Glycerophosphoric acid/Glycerol 3-phosphate | A | Control | LCMS |
| | Beta-Glycerophosphoric acid* | | | |
| | Glycerol 3-phosphate* | | | |
| | Oxoglutaric acid | A | ME/CFS | LCMS |
| | Acetone | B, C, D | ME/CFS - FM, ME/CFS - IBS, ME/CFS - IBS** | NMR |
| | Vanillic acid* | | | |
| | 4-Hydroxyphenylpyruvic acid | A | ME/CFS | LCMS |
| | Guanosine | A, B | Control, ME/CFS + FM | LCMS |
| | 2-Pyrocatechuic acid | B, C | ME/CFS + FM, ME/CFS + IBS | LCMS |
| | Trimethylamine | A, C, D | Control, ME/CFS + IBS, ME/CFS + IBS** | NMR |
| | 3-Methylphenylacetic acid | D | ME/CFS + IBS** | LCMS |
| | Nicotinic acid (aka. Nicotinate) ‡ | A, B, C | Control, ME/CFS + FM, ME/CFS + IBS | LCMS, NMR |
| Alcohols | Ethanolamine | B | ME/CFS - FM | NMR |
| | Ethanol | A | ME/CFS | NMR |
| | Methanol | B, C, D | ME/CFS + FM, ME/CFS + IBS, ME/CFS + IBS** | NMR |
| Carboxylic acid and derivatives | L-Malic acid/Malic acid | C | ME/CFS + IBS | LCMS |
| | Succinate | A, B, C | ME/CFS, ME/CFS + FM, ME/CFS - IBS | NMR |
| | Malonic acid (aka. Malonate) ‡ | A, B, C, D | ME/CFS, ME/CFS - FM, ME/CFS + IBS, ME/CFS + IBS** | LCMS, NMR |
| | Oxalic acid | B | ME/CFS + FM | LCMS |
| | Succinic anhydride | B | ME/CFS + FM | LCMS |
| | Fumaric acid (aka. Fumarate) ‡ | C, D | ME/CFS + IBS, ME/CFS + IBS** | LCMS, NMR |
| | Phenylacetate | A, B | ME/CFS, ME/CFS - FM | NMR |
| | 3.4-Dihydroxyhydrocinnamic acid | A | Control | LCMS |
| | Hydrocinnamic acid | B | ME/CFS + FM | LCMS |
| | Formate | A | ME/CFS | NMR |
| | Citric acid | B | ME/CFS + FM | LCMS |
| Fatty acyls and acids | Butyric acid (aka. Butyrate) ‡ | A, D | Control, ME/CFS + IBS** | LCMS, NMR |
| | Acetate | A, C, D | Control, ME/CFS + IBS, ME/CFS + IBS** | NMR |
| | Isovalerate | A, B | ME/CFS, ME/CFS - FM | NMR |
| | Valerate | B | ME/CFS - FM | NMR |
| | Propionic acid (aka. Propionate) ‡ | C, D | ME/CFS + IBS, ME/CFS + IBS** | LCMS, NMR |
| | Pelargonic acid (aka. 1-nonanoic acid) | B | ME/CFS - FM | LCMS |
| | Adipic acid* | | | |

| | | | | |
|---|---|---|---|---|
| | 3-Methyladipic acid/Pimelic acid | C | ME/CFS - IBS | LCMS |
| | 3-Methyladipic acid* | | | |
| | Pimelic acid* | | | |
| | 12-Hydroxydodecanoic acid | C, D | ME/CFS + IBS, ME/CFS + IBS** | LCMS |
| | Suberic acid | C | ME/CFS - IBS | LCMS |
| | Pentadecanoic acid | B | ME/CFS - FM | LCMS |
| | Heptadecanoic acid | B | ME/CFS - FM | LCMS |
| | Myristic acid | B | ME/CFS - FM | LCMS |
| | Octadecanedioic acid | C, D | ME/CFS + IBS, ME/CFS + IBS** | LCMS |
| | Linoleic acid | C | ME/CFS + IBS | LCMS |
| | 2-Methylglutaric acid/Adipic acid | B | ME/CFS - FM | LCMS |
| | 2-Methylglutaric acid* | | | |
| | 3-Methyl-2-oxovaleric acid | A | ME/CFS | LCMS |
| | Erucic acid | B | ME/CFS - FM | LCMS |
| Sugar; carbohydrates and carbohydrate conjugates | Fructose 6-phosphate/Glucose 6-phosphate | A | Control | LCMS |
| | Fructose 6-phosphate* | | | |
| | Glucose 6-phosphate* | | | |
| | N-Acetylneuraminic acid | A | ME/CFS | LCMS |
| | D-Glucose | D | ME/CFS + IBS** | LCMS |
| | Glyceric acid | B | ME/CFS + FM | LCMS |
| | Galacturonic acid | D | ME/CFS + IBS** | LCMS |
| | Glycerol | C, D | ME/CFS + IBS, ME/CFS + IBS** | LCMS |
| Pyrimidines and pyrimidine derivatives | Uracil | A, C, D | Control, ME/CFS + IBS, ME/CFS + IBS** | NMR |
| | Orotic acid | A | ME/CFS | LCMS |
| NOTE | * From LCMS consolidated metabolite feature<br><br>**Comparative Group (A-D)**<br>A) ME/CFS overall vs. Control [ME/CFS, Control]<br>B) ME/CFS with FM vs. ME/CFS without FM [ME/CFS + FM, ME/CFS - FM]<br>C) ME/CFS with IBS vs. ME/CFS without IBS [ME/CFS + IBS, ME/CFS - IBS]<br>D) ME/CFS with IBS vs. ME/CFS without IBS (age-matched) [ME/CFS + IBS**, ME/CFS - IBS**]<br>‡ "Duplicate" metabolite assayed by LCMS and NMR | | Refer to **Appendix 9** for electronic version of this table and raw data input | |

## 4.1 Faecal Metabolomics Results continued…

The significantly differential faecal metabolite features were elevated in their relative concentrations depending on how the samples were grouped with their ME/CFS, FM, or IBS metadata that comprised Comparison A-D (Table 4.1.1). Not every single faecal metabolite appeared in all the comparisons when observing the significant differences between the involved groups. A total of 17, 19, 6, and 9 metabolite features were found only to be significantly different in Comparison A (ME/CFS overall vs. Control), B (ME/CFS +/- FM), C (ME/CFS +/- IBS), and D (age-matched ME/CFS +/- IBS**), respectively; for example, 3-Oxocholic acid was only differential and elevated in the ME/CFS overall group from Comparison A. The metabolites, for example, xanthine and propionic acid, that were highlighted in the ME/CFS +/- IBS and ME/CFS +/- IBS** groups (Comparison C and D), were always consistently lower or higher in their relative concentration providing some indication of IBS involvement where an attempt to mitigate the age variable has been made. Methanol and 2-Pyrocatechuic acid were consistently elevated in ME/CFS + either FM, IBS, or IBS** (Comparison B-D). Acetone was consistently elevated in ME/CFS - either FM, IBS, or IBS** (Comparison B-D).

VPs of the LCMS faecal data matrix highlighted 17 metabolites (12 down and 5 up) in the Control vs. ME/CFS (Figure 4.1.1), 6 metabolites (5 down and 1 up) in the ME/CFS +/- FM (Figure 4.1.9), 4 metabolites (1 down and 3 up) in the ME/CFS +/- IBS (Figure 4.1.17), and 6 metabolites (all up) in the ME/CFS +/- IBS** (Figure 4.1.25) comparisons that were significantly different. VPs of the NMR faecal data matrix highlighted 8 metabolites (4 down and 4 up) in the Control vs. ME/CFS (Figure 4.1.2), 3 metabolites (2 down and 1 up) in the ME/CFS +/- FM (Figure 4.1.10), 6 metabolites (all up) in the ME/CFS +/- IBS (Figure 4.1.18), and 8 metabolites (all up) in the ME/CFS +/- IBS** (Figure 4.1.26) comparisons that were significantly different. The metabolites from the VPs that were also found to be significantly different by PLS-DA VIP analyses reflected and supported the same pattern as either high or low in relative concentration (Appendix 9). The PCAs from the LCMS and NMR data matrices did not show a clear separation between the groups for all comparisons (Table 4.1.2). Although the PCAs did not discriminate between the groups, the clusters extended in different directions. Tighter clusters identified by the ellipsoid shape were observed from LCMS and NMR ME/CFS + IBS (Figure 4.1.19, Figure 4.1.20), and LCMS and NMR ME/CFS + IBS** (Figure 4.1.27, Figure 4.1.28). Further, the ellipsoid from the NMR ME/CFS + IBS** group also resided within the broader ME/CFS - IBS** ellipsoid (Figure 4.1.28); this inner group may represent a subset of a

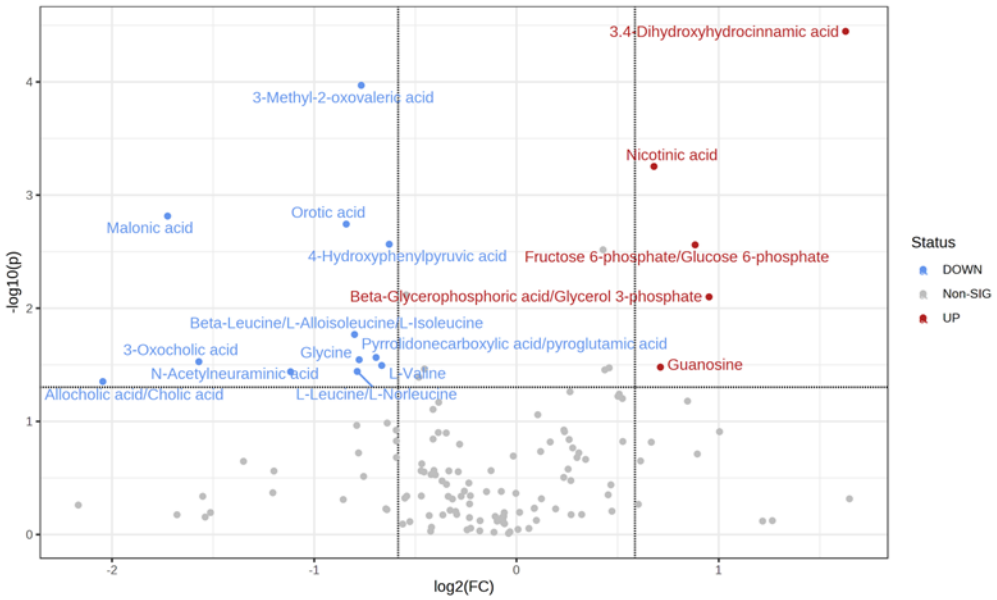
more specific subgroup within the broader category of ME/CFS. The PLS-DAs demonstrated improved clustering and separation for Comparison A-D (Table 4.1.2). The PLS-DA for the LCMS data matrix found a partial to distinguished separation between the ME/CFS +/- FM (Figure 4.1.13) and ME/CFS +/- IBS (Figure 4.1.21) comparisons. A partial to distinguished separation for LCMS (Figure 4.1.29) and NMR (Figure 4.1.31) was also observed between the ME/CFS +/- IBS** groups.

**Table 4.1.2** Summary of the total variance from the first two components of the PCA and PLS-DA analyses (LCMS and NMR Faecal Metabolome (Part One))

| **Faeces Part One** | | **PCA** | | | | **PLSDA** | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | % of the variance | | | Separation | % of the variance | | | Separation |
| | | PC1 | PC2 | Total† | | Comp. 1 | Comp. 2 | Total‡ | |
| A) ME/CFS vs. Control | LCMS | 18 | 15.8 | 33.8 | Overlapping | 9.5 | 12.2 | 21.7 | Partial |
| | NMR | 47.3 | 12.2 | 59.5 | Overlapping | 11.7 | 4.1 | 15.8 | Partial |
| B) ME/CFS +/- FM | LCMS | 20.1 | 15 | 35.1 | Overlapping | 13.1 | 5.8 | 18.9 | Partial-Distinguished |
| | NMR | 54.7 | 9 | 63.7 | Overlapping | 45.5 | 16.5 | 62 | Partial |
| C) ME/CFS +/- IBS | LCMS | 20.1 | 15 | 35.1 | Overlapping | 14.1 | 10 | 24.1 | Partial-Distinguished |
| | NMR | 54.7 | 9 | 63.7 | Overlapping | 41.4 | 21.6 | 63 | Partial |
| D) ME/CFS +/- IBS** | LCMS | 21.6 | 16.5 | 38.1 | Overlapping | 19.4 | 6.8 | 26.2 | Partial-Distinguished |
| | NMR | 55.1 | 9.8 | 64.9 | Overlapping | 51.1 | 12.1 | 63.2 | Partial-Distinguished |
| † PC1 and PC2 added together | | | | | | | | | |
| ‡ Component 1 and Component 2 added together | | | | | | | | | |
| Partial-Distinguished to complete separation between groupings | | | | | | | | | |

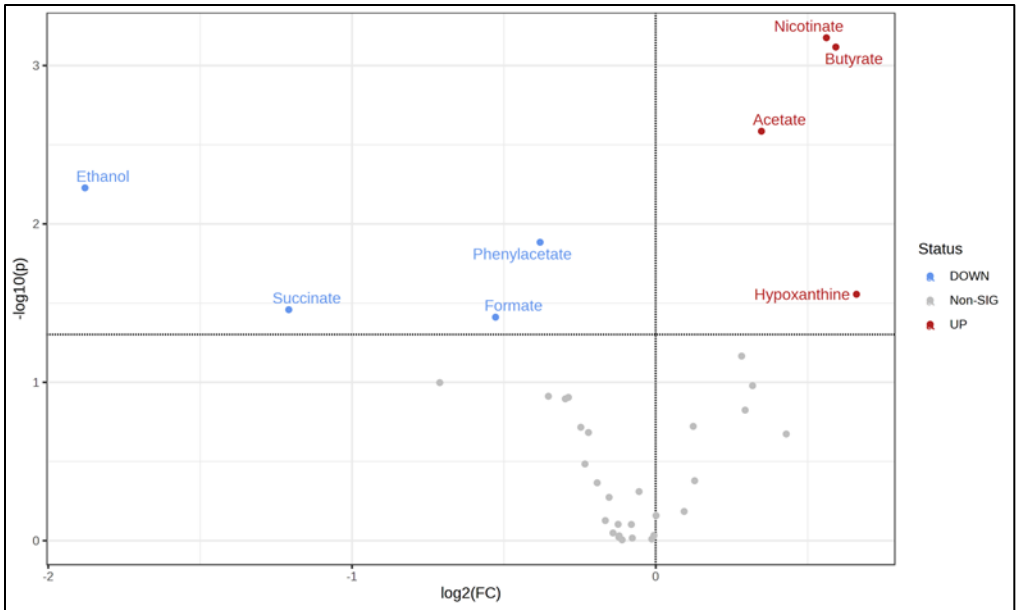
### 4.1.1 Faecal Comparison A) ME/CFS Overall vs. Control Groups

#### 4.1.1.1 Univariate Analysis




| | FC | log2(FC) | p | -log10(p) | q |
|---|---|---|---|---|---|
| 3.4-Dihydroxyhydrocinnamic acid | 3.0892 | 1.6272 | 3.57E-05 | 4.4469 | 0.0048955 |
| 3-Methyl-2-oxovaleric acid | 0.5874 | -0.7676 | 0.00010725 | 3.9696 | 0.007347 |
| Nicotinic acid | 1.6018 | 0.6797 | 0.00055855 | 3.2529 | 0.025507 |
| Malonic acid | 0.30247 | -1.7251 | 0.0015333 | 2.8144 | 0.049458 |
| Orotic acid | 0.55793 | -0.84186 | 0.001805 | 2.7435 | 0.049458 |
| 4-Hydroxyphenylpyruvic acid | 0.64646 | -0.62937 | 0.0027198 | 2.5655 | NS |
| Fructose 6-phosphate/Glucose 6-phosphate | 1.8445 | 0.88325 | 0.0027549 | 2.5599 | NS |
| Beta-Glycerophosphoric acid/Glycerol 3-phosphate | 1.9347 | 0.95211 | 0.0079416 | 2.1001 | NS |
| Beta-Leucine/L-Alloisoleucine/L-Isoleucine | 0.57402 | -0.80083 | 0.017062 | 1.768 | NS |
| Pyrrolidonecarboxylic acid/pyroglutamic acid | 0.61798 | -0.69437 | 0.027228 | 1.565 | NS |
| Glycine | 0.5832 | -0.77795 | 0.028516 | 1.5449 | NS |
| 3-Oxocholic acid | 0.33665 | -1.5707 | 0.029671 | 1.5277 | NS |
| L-Valine | 0.6301 | -0.66634 | 0.032056 | 1.4941 | NS |
| Guanosine | 1.6377 | 0.7117 | 0.033159 | 1.4794 | NS |
| L-Leucine/L-Norleucine | 0.57932 | -0.78756 | 0.036192 | 1.4414 | NS |
| N-Acetylneuraminic acid | 0.46103 | -1.1171 | 0.0364 | 1.4389 | NS |
| Allocholic acid/Cholic acid | 0.24223 | -2.0455 | 0.044435 | 1.3523 | NS |

**Figure 4.1.1** Volcano plot of LCMS Faecal Part 1 ME/CFS overall vs Control groups using test outcomes from the raw p-values. Metabolites with significantly higher (blue) or lower (red) concentrations in the ME/CFS cohort are highlighted (FC threshold 1.5 and uncorrected p-values ≤ 0.05) with non-significant metabolites represented in grey. The q-values of significant FDR-adjusted metabolites are shown (NS = q-value ≥ 0.05).




| | FC | log2(FC) | p | -log10(p) | q |
|---|---|---|---|---|---|
| Nicotinate | 1.4758 | 0.56153 | 0.00066762 | 3.1755 | 0.01376 |
| Butyrate | 1.5079 | 0.59254 | 0.00076447 | 3.1166 | 0.01376 |
| Acetate | 1.2725 | 0.34768 | 0.0025979 | 2.5854 | 0.031175 |
| Ethanol | 0.27186 | -1.8791 | 0.005921 | 2.2276 | NS |
| Phenylacetate | 0.76796 | -0.38089 | 0.01306 | 1.8841 | NS |
| Hypoxanthine | 1.5807 | 0.66056 | 0.027803 | 1.5559 | NS |
| Succinate | 0.43279 | -1.2083 | 0.034799 | 1.4584 | NS |
| Formate | 0.69372 | -0.52757 | 0.038797 | 1.4112 | NS |

**Figure 4.1.2** Volcano plot of NMR Faecal Part 1 ME/CFS overall vs Control groups using test outcomes from the raw p-values. Metabolites with significantly higher (blue) or lower (red) concentrations in the ME/CFS cohort are highlighted (FC threshold 1.0 and uncorrected p-values ≤ 0.05) with non-significant metabolites represented in grey. The q-values of significant FDR-adjusted metabolites are shown (NS = q-value ≥ 0.05).

### 4.1.1.2 Multivariate Analysis (PCA)

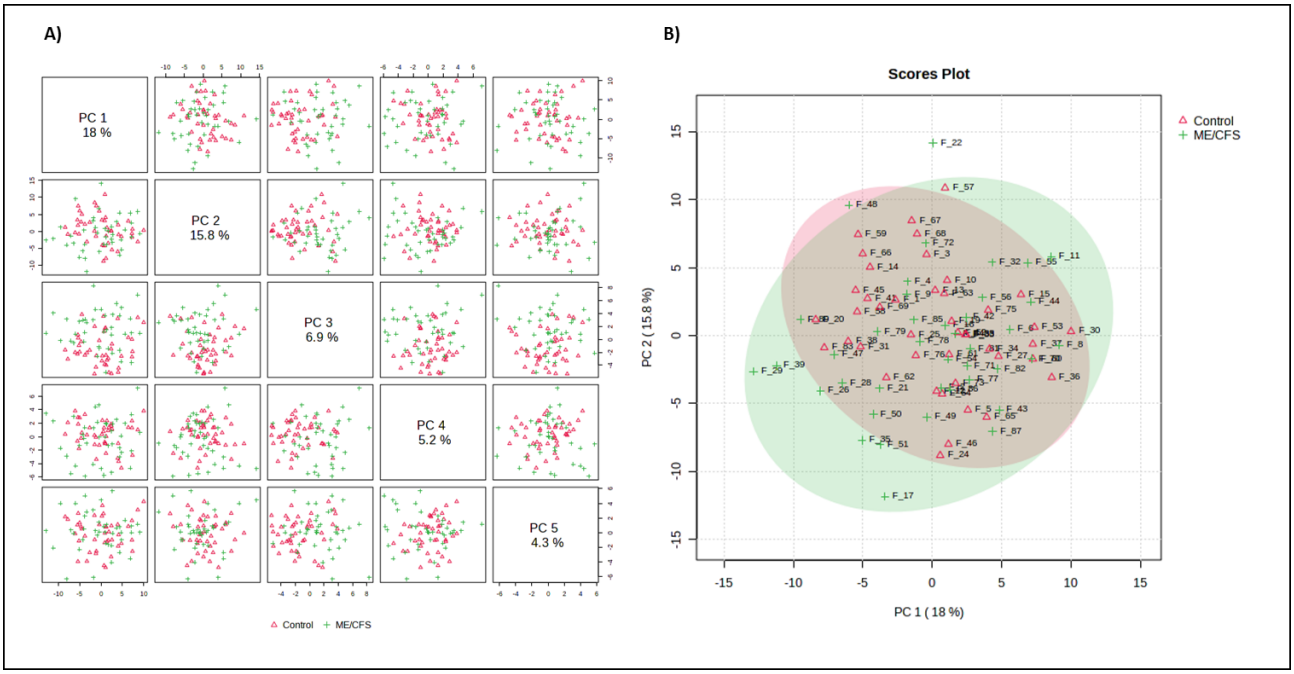



**Figure 4.1.3** PCA plots A) overview B) PC1 and PC2 only of LCMS Faecal Part 1 ME/CFS overall vs. Control groups

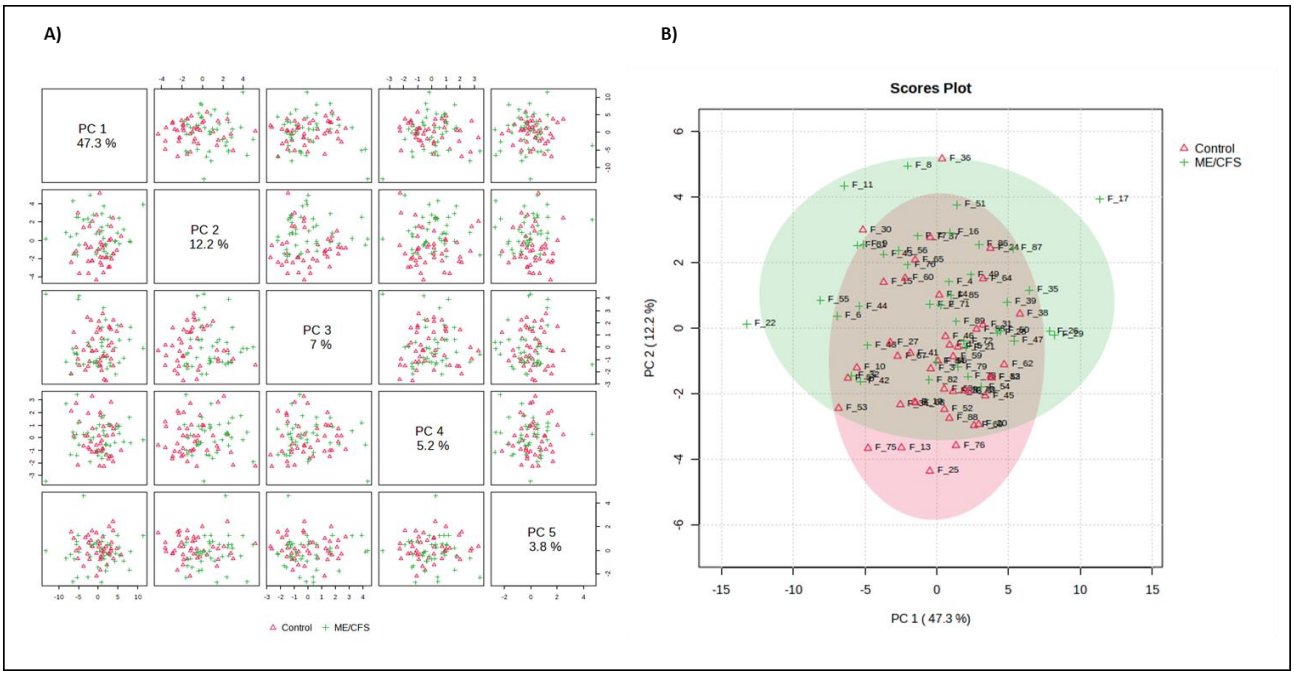



**Figure 4.1.4** PCA plots A) overview B) PC1 and PC2 only of NMR Faecal Part 1 ME/CFS overall vs. Control groups

### 4.1.1.3 Multivariate Analysis (PLS-DA)

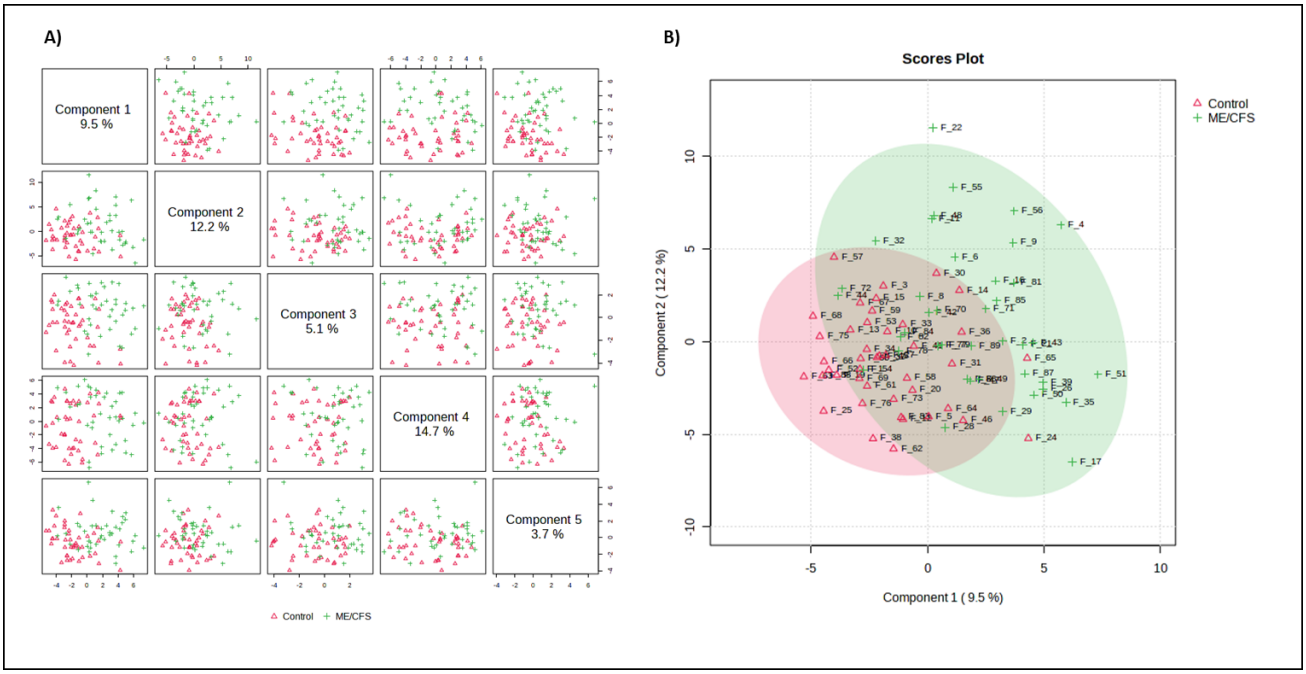



**Figure 4.1.5** PLSDA A) overview B) components 1 and 2 only of LCMS Faecal Part 1 ME/CFS overall vs. Control groups

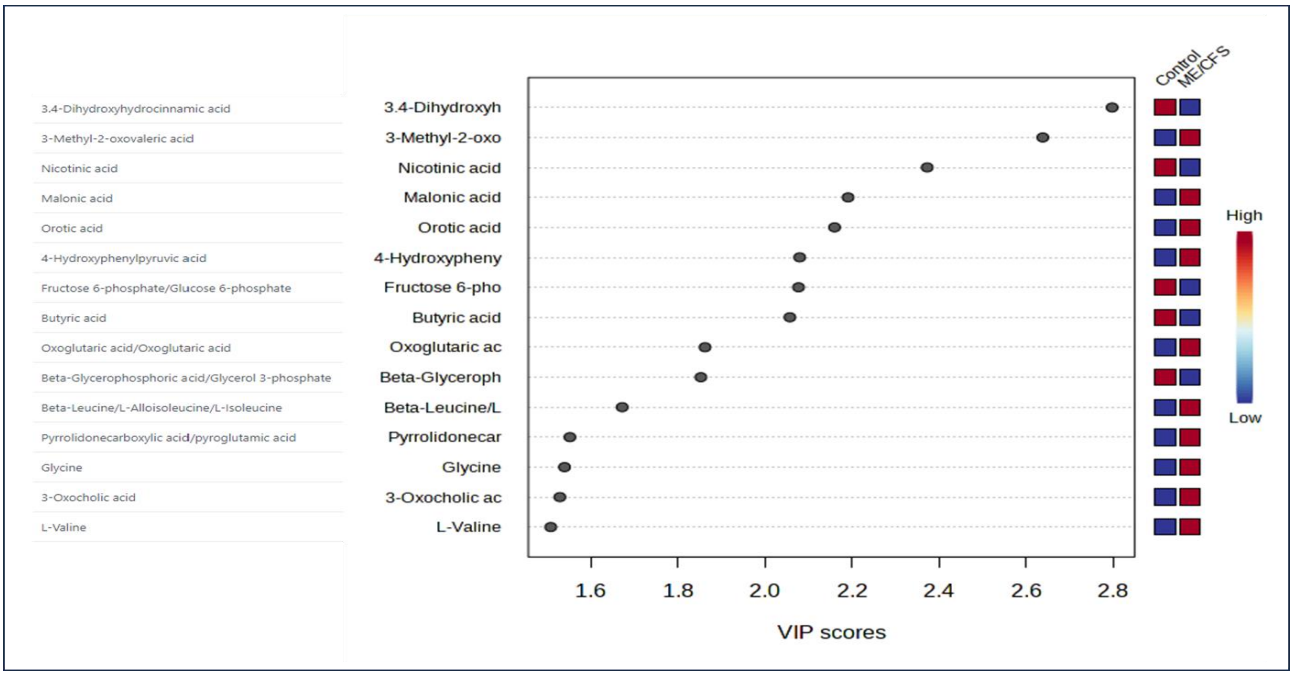



**Figure 4.1.6** VIP scores of corresponding PLSDA for LCMS Faecal Part 1 ME/CFS overall vs. Control groups

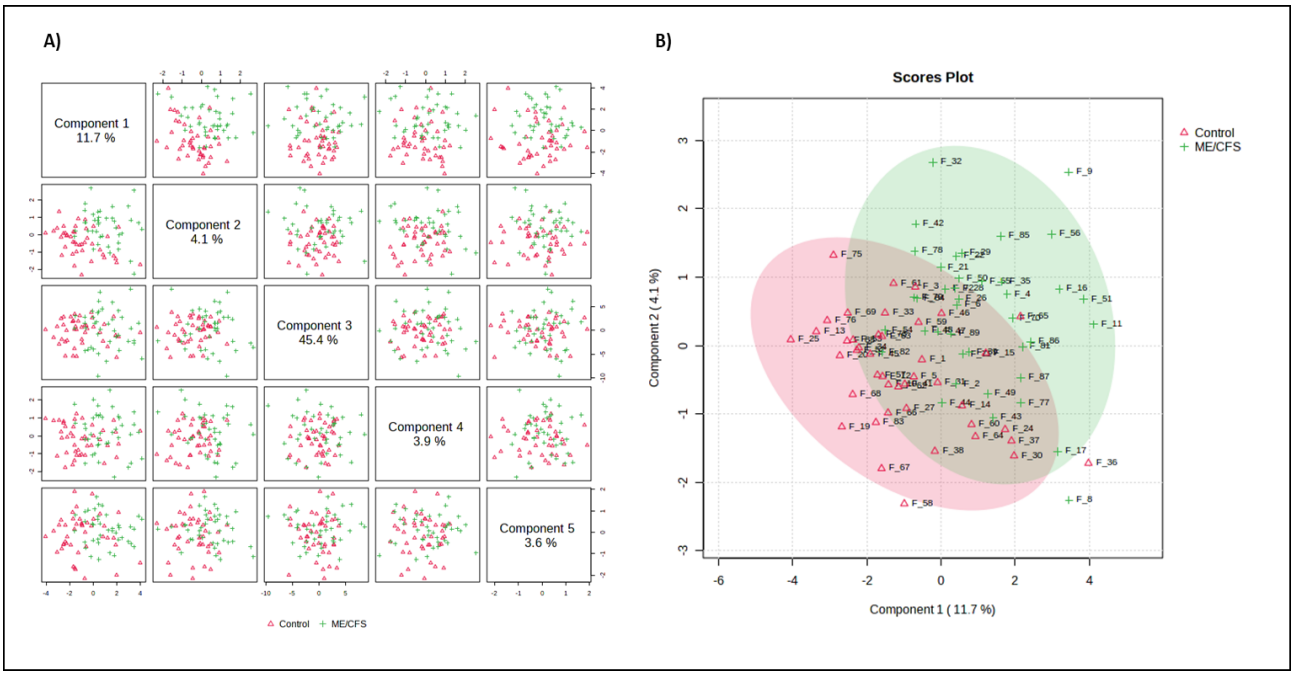



**Figure 4.1.7** PLSDA A) overview B) components 1 and 2 only of NMR Faecal Part 1 ME/CFS overall vs. Control groups

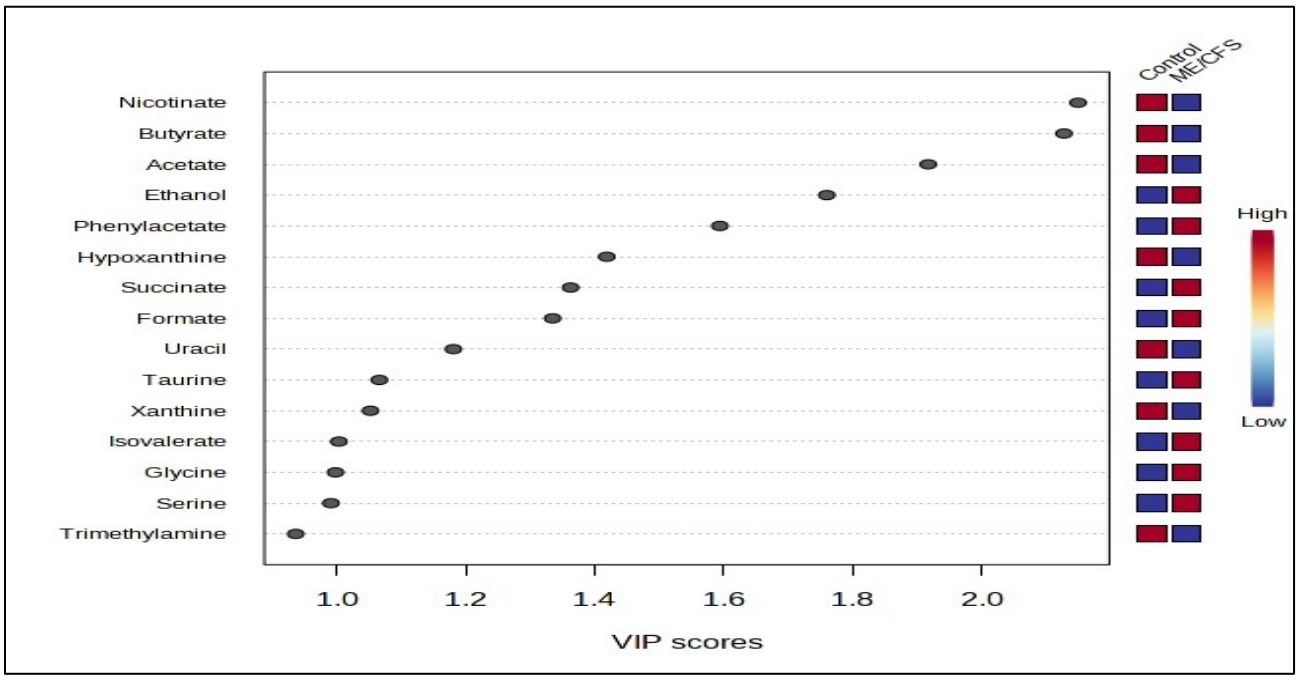
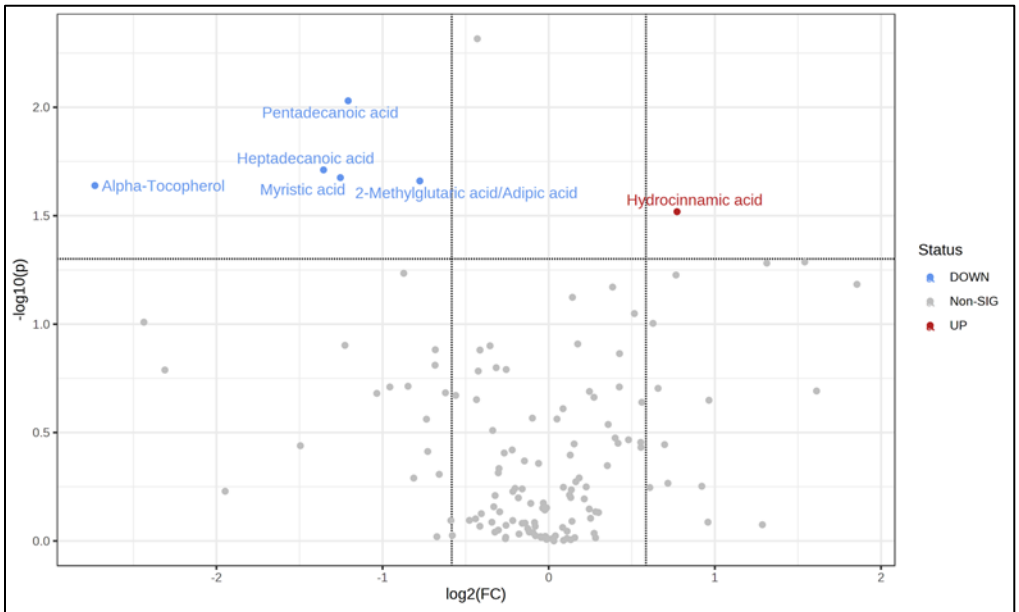



**Figure 4.1.8** VIP scores of corresponding PLSDA of NMR Faecal Part 1 ME/CFS overall vs. Control groups

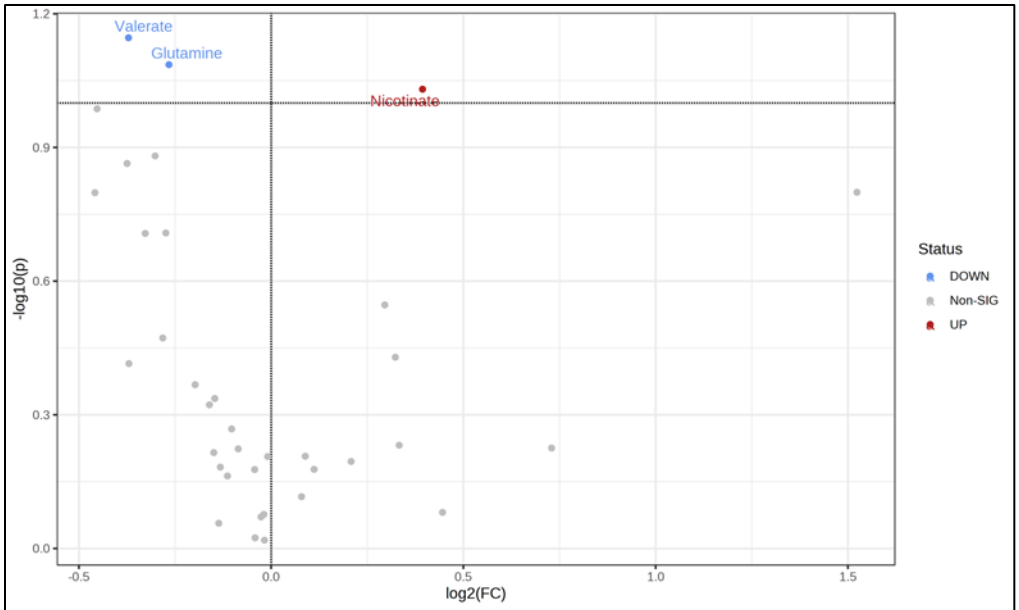
### 4.1.2 Faecal Comparison B) ME/CFS with FM vs. ME/CFS without FM

#### 4.1.2.1 Univariate Analysis




| | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|
| Pentadecanoic acid | 0.43315 | -1.2071 | 0.0093315 | 2.03 |
| Heptadecanoic acid | 0.39083 | -1.3554 | 0.01944 | 1.7113 |
| Myristic acid | 0.4193 | -1.2539 | 0.021103 | 1.6757 |
| 2-Methylglutaric acid/Adipic acid | 0.58403 | -0.77588 | 0.021865 | 1.6603 |
| Alpha-Tocopherol | 0.15058 | -2.7314 | 0.022961 | 1.639 |
| Hydrocinnamic acid | 1.708 | 0.77233 | 0.030302 | 1.5185 |

**Figure 4.1.9** Volcano plot of LCMS Faecal Part 1 ME/CFS with FM versus ME/CFS without FM. Metabolites with significantly higher (red) or lower (blue) concentrations in the ME/CFS with FM cohort are highlighted (FC threshold 1.5 and uncorrected p-values ≤ 0.05) with non-significant metabolites represented in grey.




| | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|
| Valerate | 0.77325 | -0.371 | 0.071434 | 1.1461 |
| Glutamine | 0.83174 | -0.2658 | 0.082085 | 1.0857 |
| Nicotinate | 1.3138 | 0.39379 | 0.093168 | 1.0307 |

**Figure 4.1.10** Volcano plot of NMR Faecal Part 1 ME/CFS with FM versus ME/CFS without FM. Metabolites with significantly higher (red) or lower (blue) concentrations in the ME/CFS with FM cohort are highlighted (FC threshold 1.0 and uncorrected p-values ≤ 0.10) with non-significant metabolites represented in grey.

### 4.1.2.2 Multivariate Analysis (PCA)

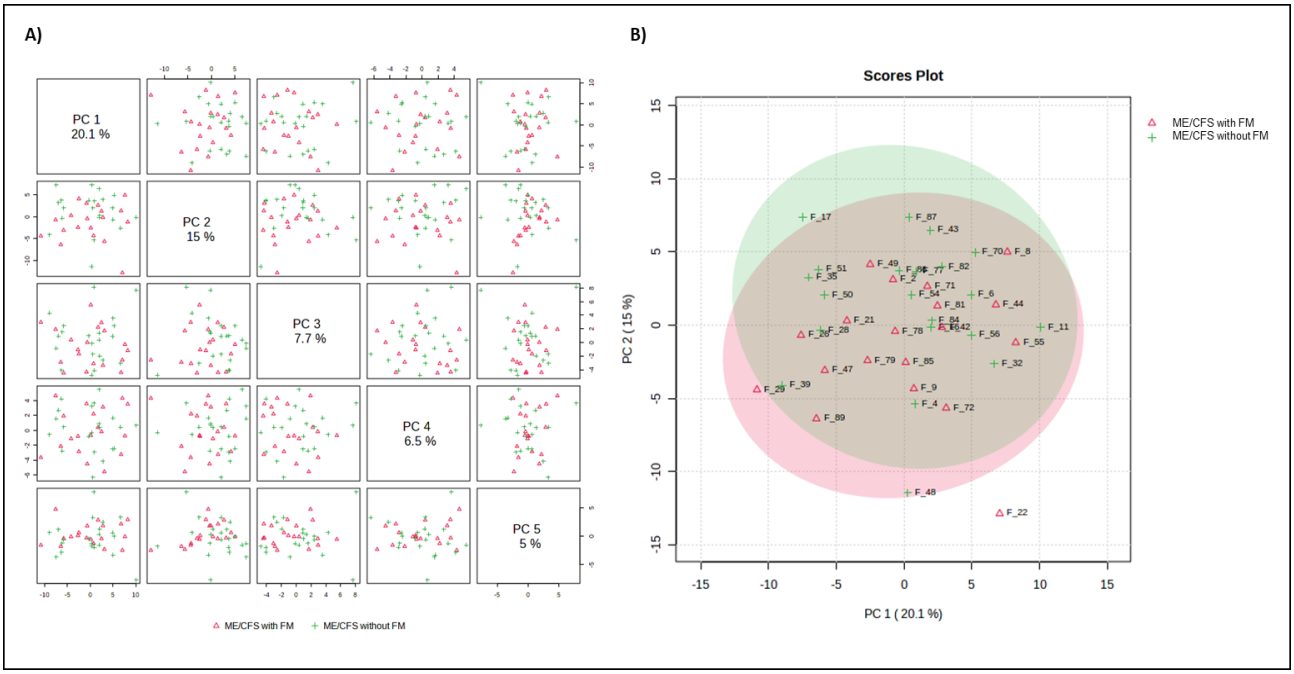



**Figure 4.1.11** PCA plots A) overview B) PC1 and PC2 only of LCMS Faecal Part 1 ME/CFS with FM versus ME/CFS without FM

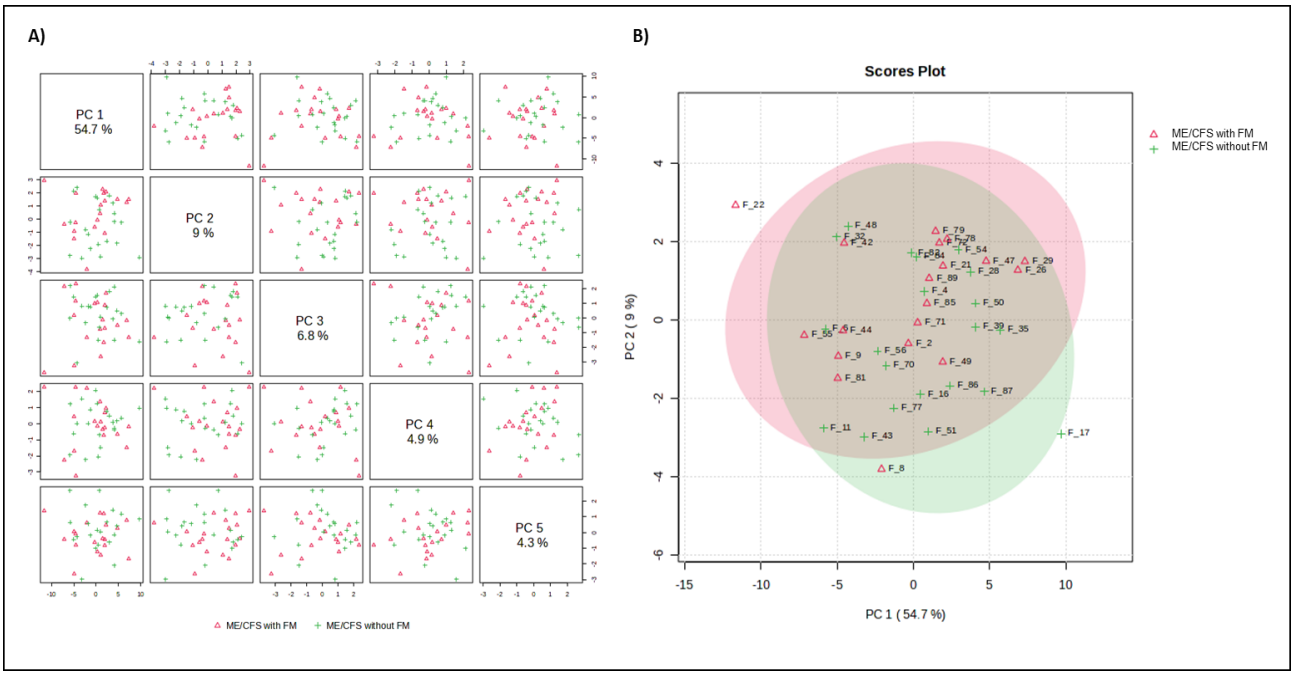



**Figure 4.1.12** PCA plots A) overview B) PC1 and PC2 only of NMR Faecal Part 1 ME/CFS with FM versus ME/CFS without FM

### 4.1.2.3 Multivariate Analysis (PLS-DA)

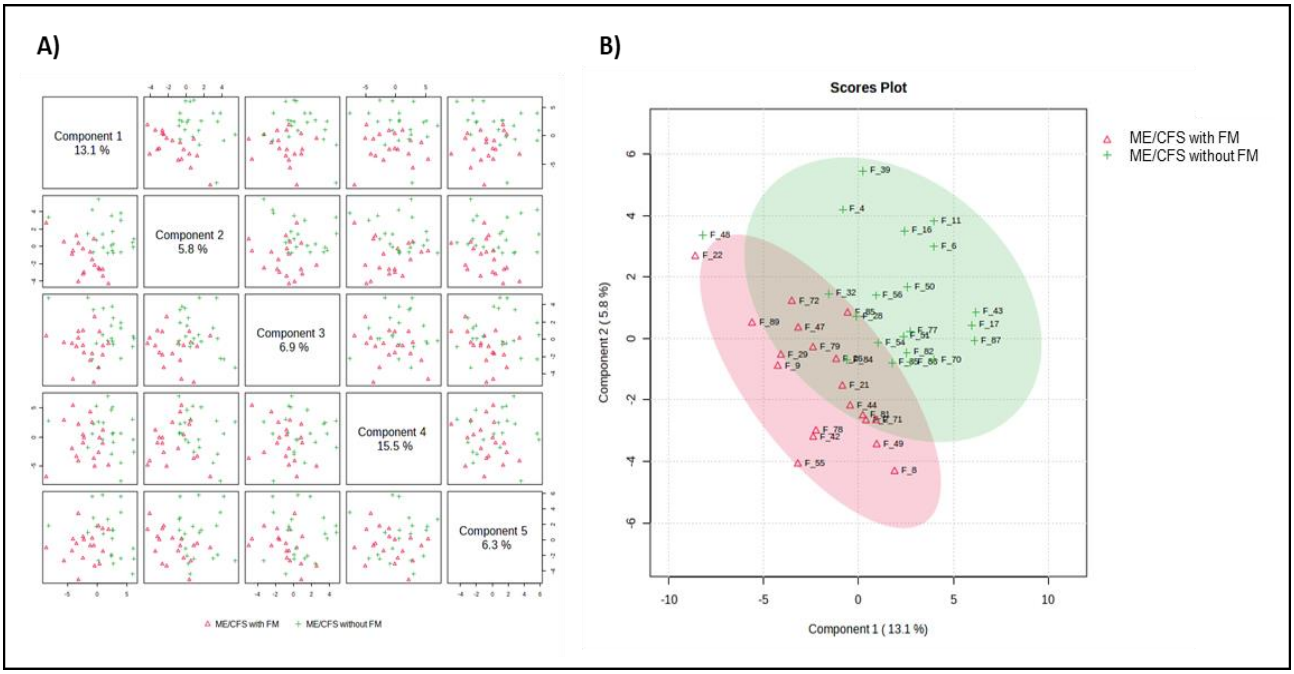



**Figure 4.1.13** PLSDA A) overview B) components 1 and 2 only of LCMS Faecal Part 1 ME/CFS with FM versus ME/CFS without FM

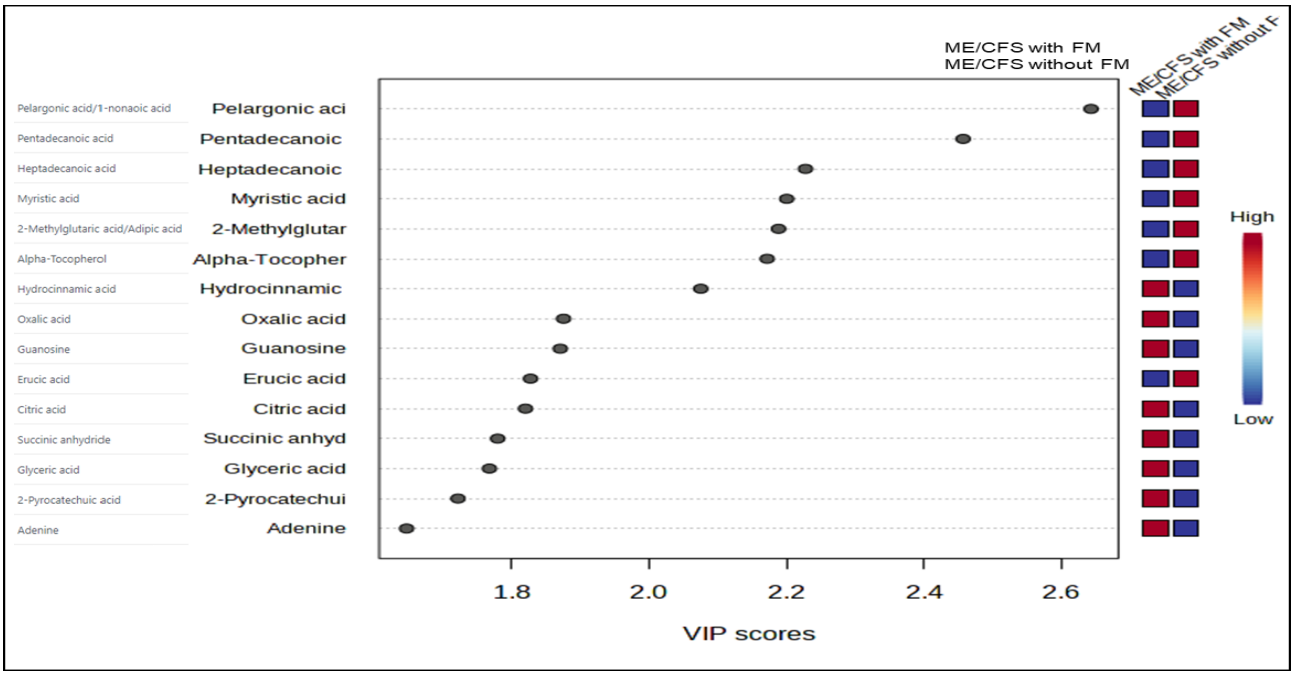



**Figure 4.1.14** VIP scores of corresponding PLSDA for LCMS Faecal Part 1 ME/CFS with FM versus ME/CFS without FM

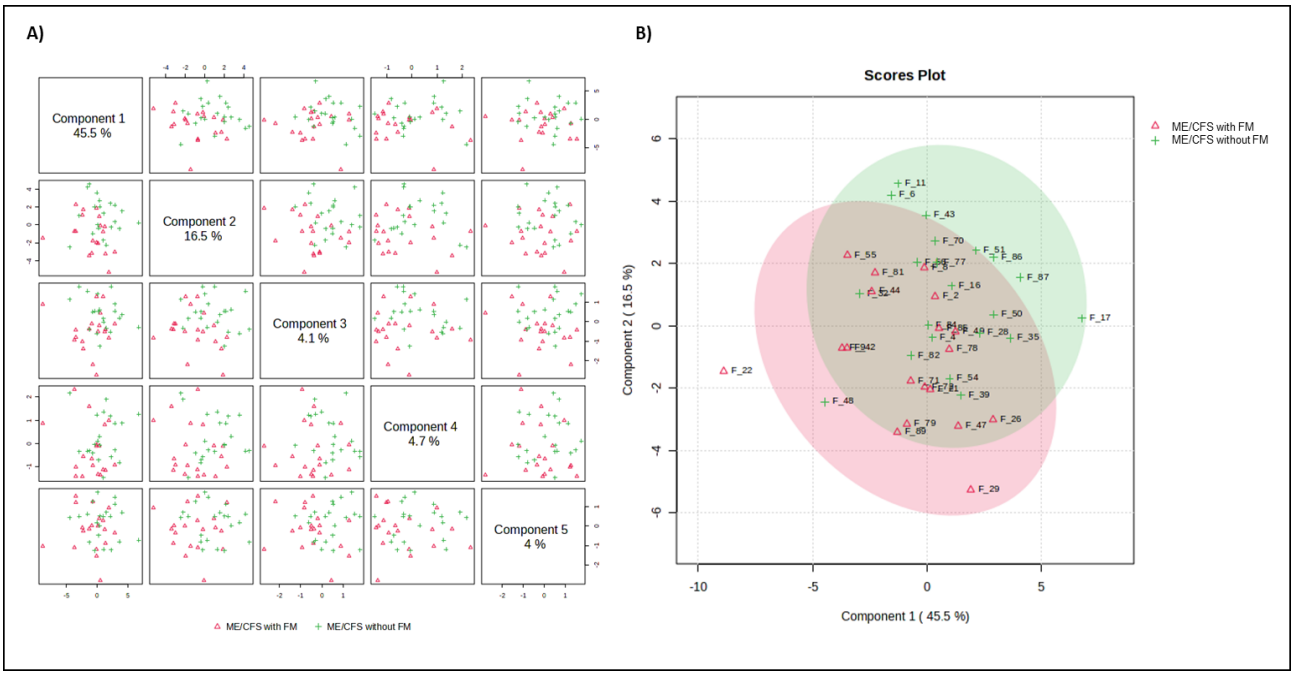



**Figure 4.1.15** PLSDA A) overview B) components 1 and 2 only of NMR Faecal Part 1 ME/CFS with FM versus ME/CFS without FM

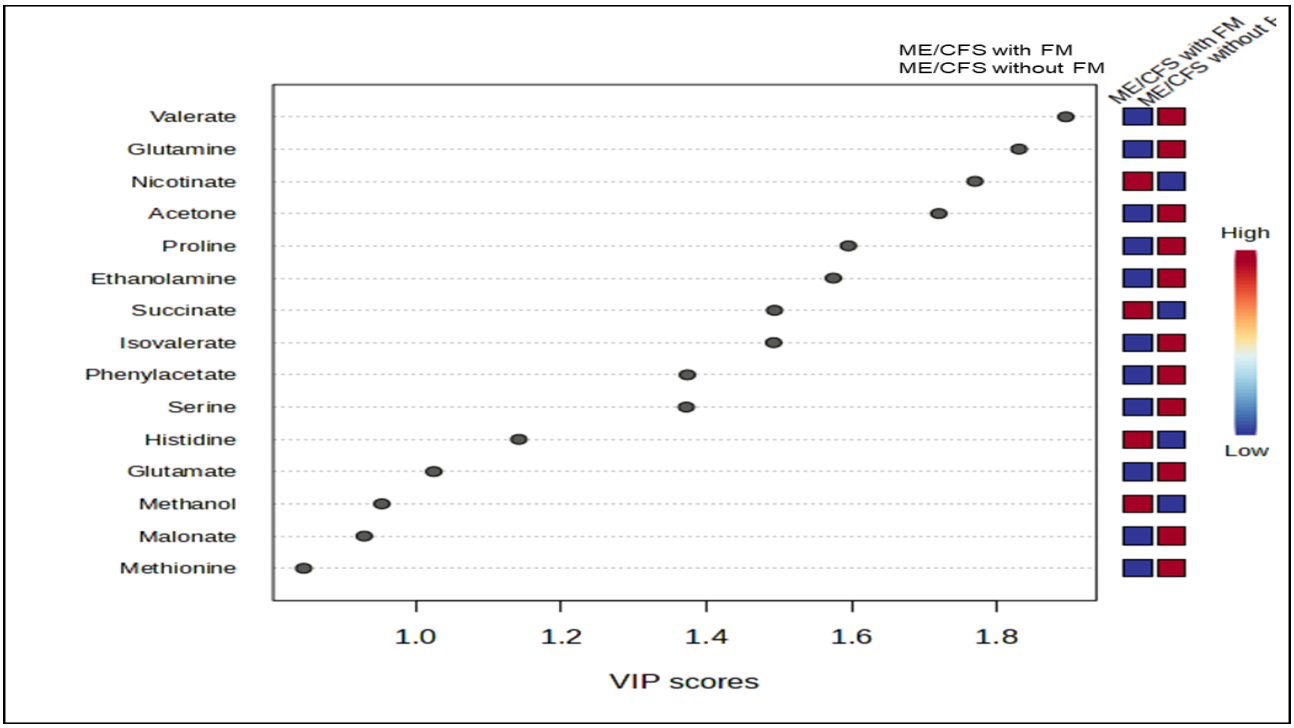
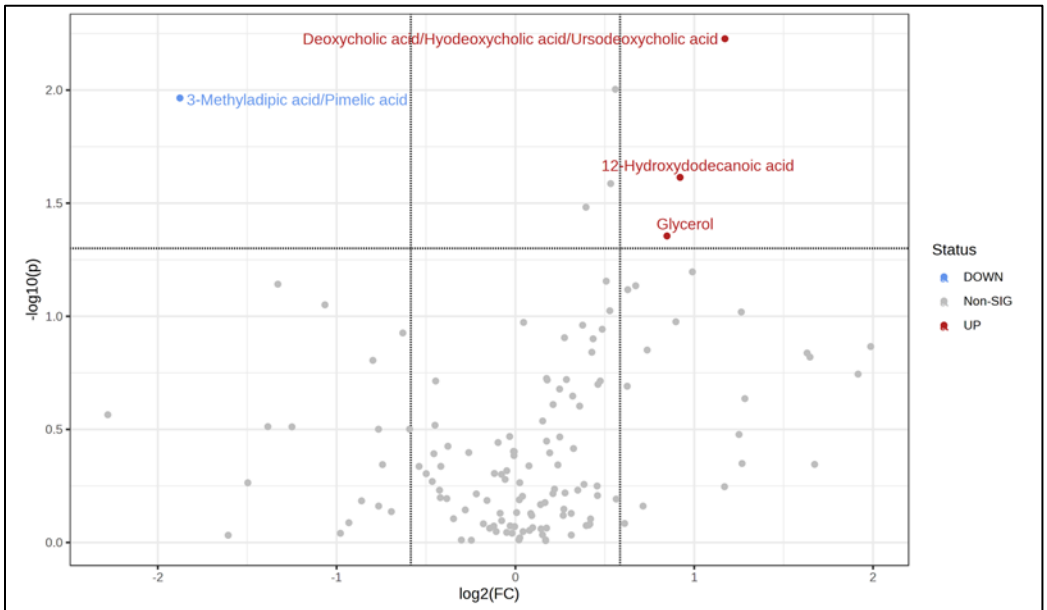



**Figure 4.1.16** VIP scores of corresponding PLSDA for NMR Faecal Part 1 ME/CFS with FM versus ME/CFS without FM

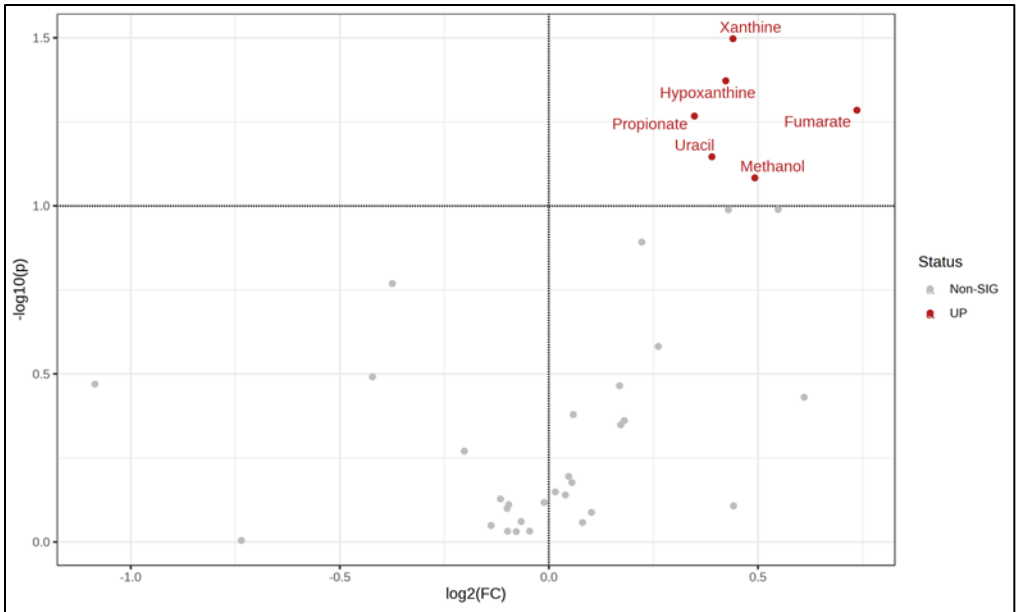
### 4.1.3 Faecal Comparison C) ME/CFS with IBS vs. ME/CFS without IBS

#### 4.1.3.1 Univariate Analysis




| | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|
| Deoxycholic acid/Hyodeoxycholic acid/Ursodeoxycholic acid | 2.2527 | 1.1716 | 0.00593 | 2.227 |
| 3-Methyladipic acid/Pimelic acid | 0.27238 | -1.8763 | 0.010839 | 1.965 |
| 12-Hydroxydodecanoic acid | 1.8931 | 0.92074 | 0.024307 | 1.6143 |
| Glycerol | 1.7996 | 0.84764 | 0.044136 | 1.3552 |

**Figure 4.1.17** Volcano plot of LCMS Faecal Part 1 ME/CFS with IBS versus ME/CFS without IBS. Metabolites with significantly higher (red) or lower (blue) concentrations in the ME/CFS with IBS cohort are highlighted (FC threshold 1.5 and uncorrected p-values ≤ 0.05) with non-significant metabolites represented in grey.




| | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|
| Xanthine | 1.3568 | 0.44024 | 0.031831 | 1.4972 |
| Hypoxanthine | 1.3407 | 0.42295 | 0.042459 | 1.372 |
| Fumarate | 1.6664 | 0.73675 | 0.051922 | 1.2846 |
| Propionate | 1.2728 | 0.34799 | 0.054073 | 1.267 |
| Uracil | 1.3102 | 0.38983 | 0.071443 | 1.146 |
| Methanol | 1.4069 | 0.49249 | 0.082552 | 1.0833 |

**Figure 4.1.18** Volcano plot of NMR Faecal Part ME/CFS with IBS versus ME/CFS without IBS. Metabolites with significantly higher (red) or lower (blue) concentrations in the ME/CFS with IBS cohort are highlighted (FC threshold 1.0 and uncorrected p-values ≤ 0.10) with non-significant metabolites represented in grey.

### 4.1.3.2 Multivariate Analysis (PCA)

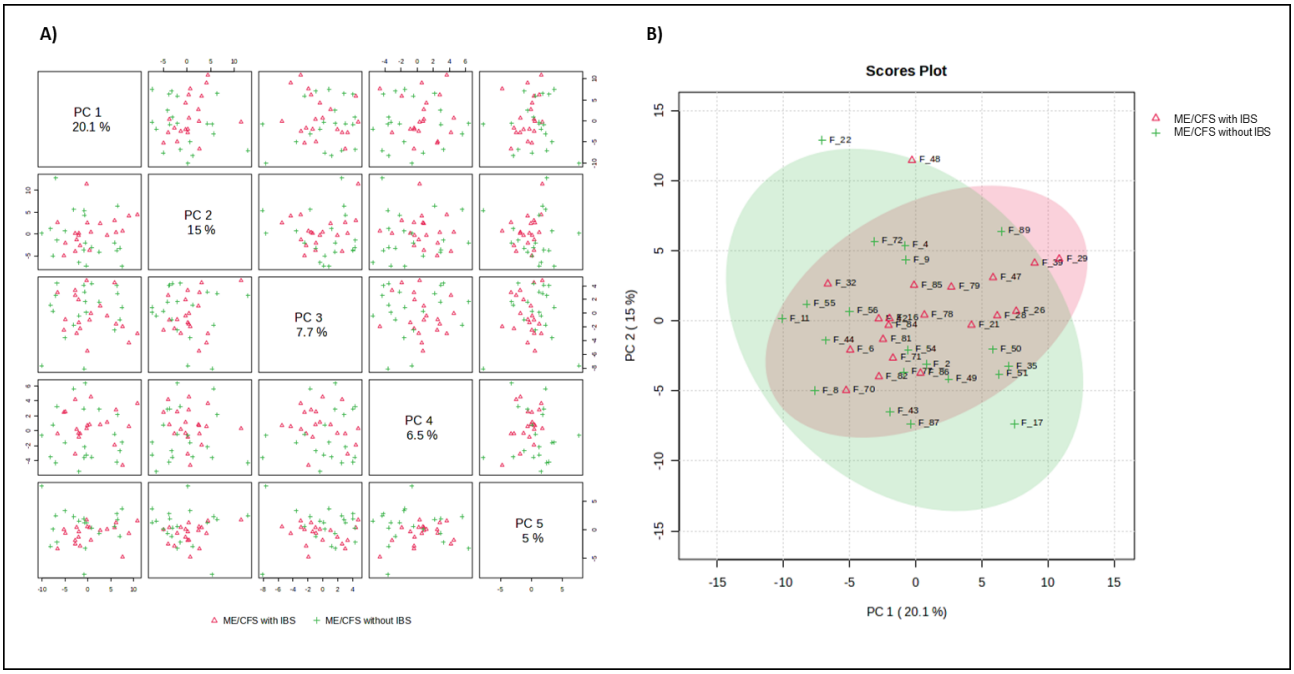



**Figure 4.1.19** PCA plots A) overview B) PC1 and PC2 only of LCMS Faecal Part 1 ME/CFS with IBS versus ME/CFS without IBS

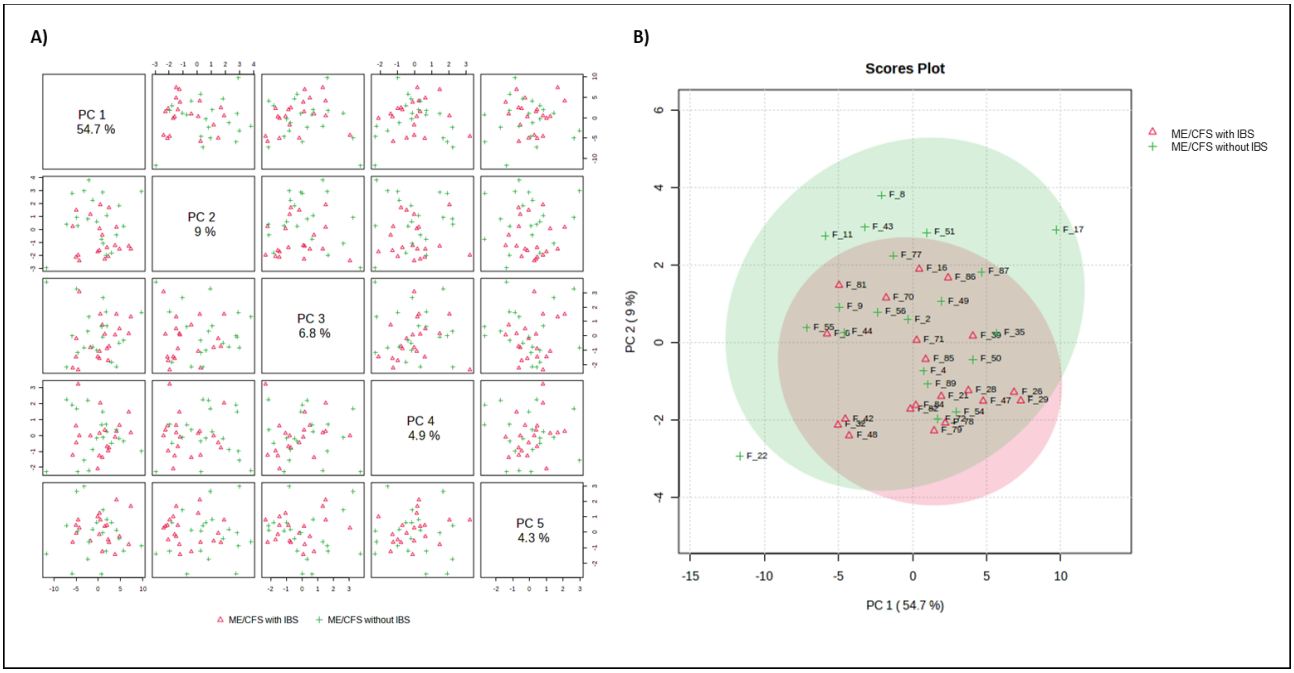



**Figure 4.1.20** PCA plots A) overview B) PC1 and PC2 only of NMR Faecal Part 1 ME/CFS with IBS versus ME/CFS without IBS

### 4.1.3.3 Multivariate Analysis (PLS-DA)

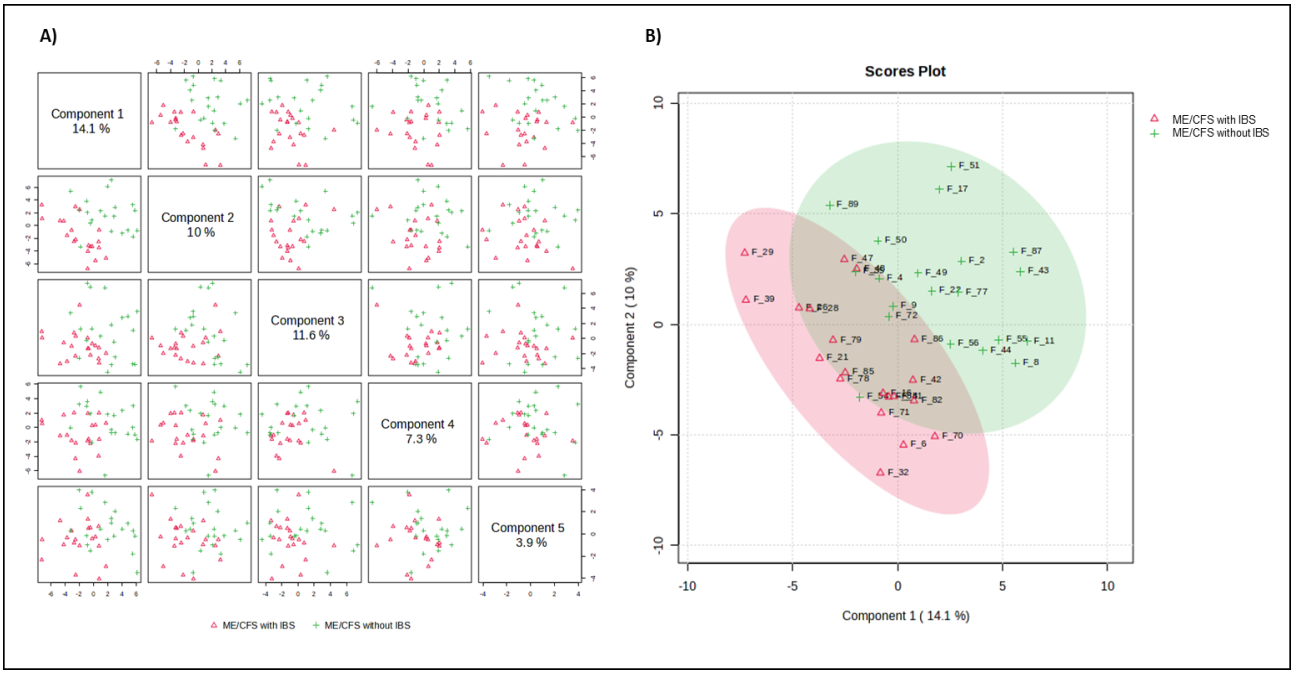



**Figure 4.1.21** PLSDA A) overview B) components 1 and 2 only of LCMS Faecal Part 1 ME/CFS with IBS versus ME/CFS without IBS

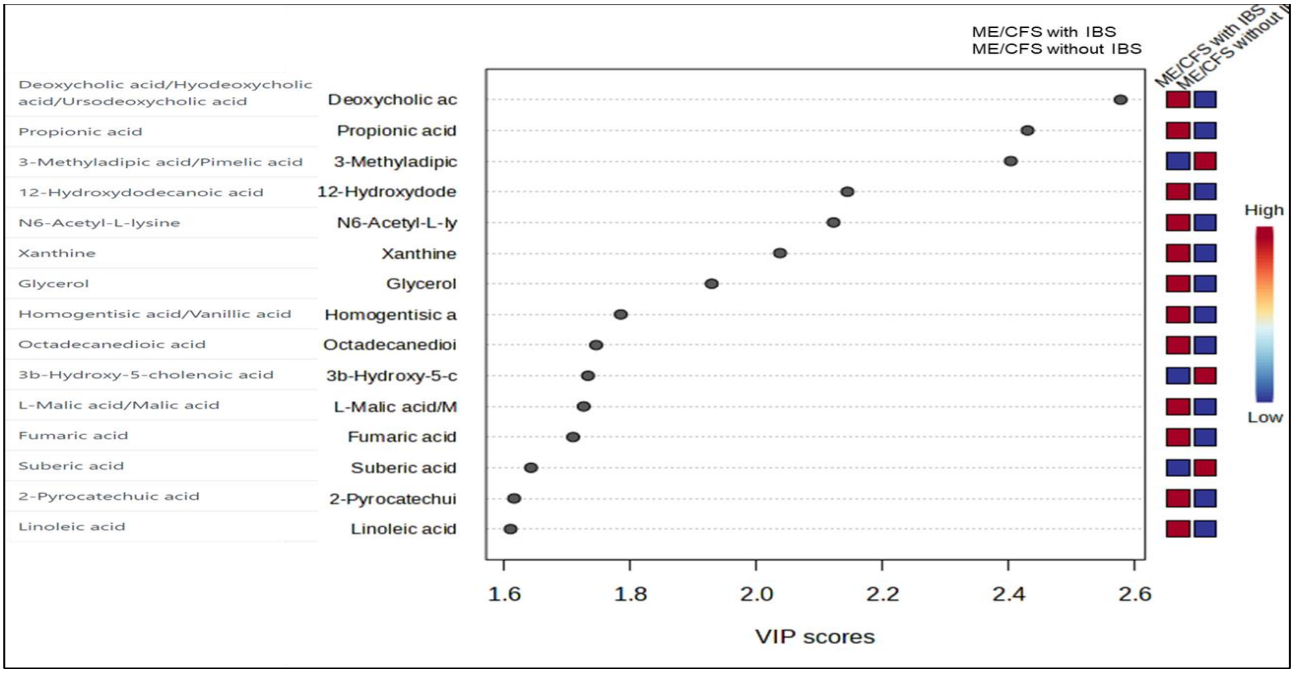



**Figure 4.1.22** VIP scores of corresponding PLSDA for LCMS Faecal Part 1 ME/CFS with IBS versus ME/CFS without IBS

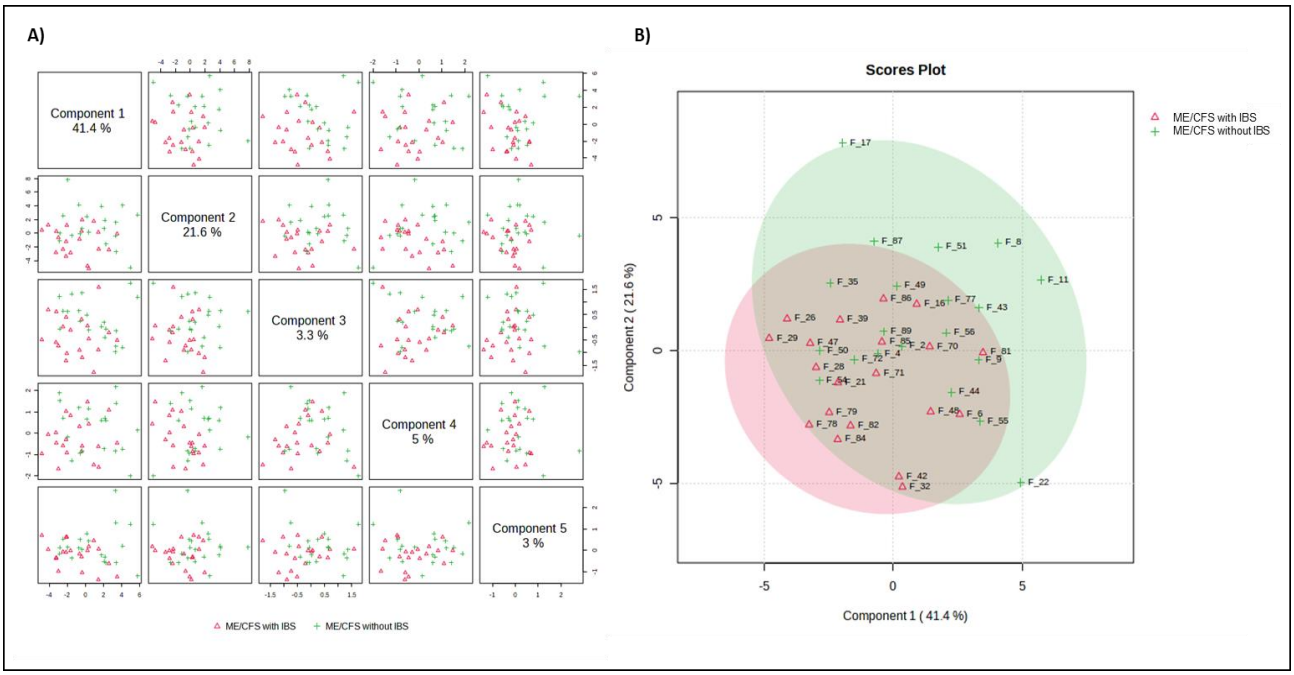



**Figure 4.1.23** PLSDA A) overview B) components 1 and 2 only of NMR Faecal Part 1 ME/CFS with IBS versus ME/CFS without IBS

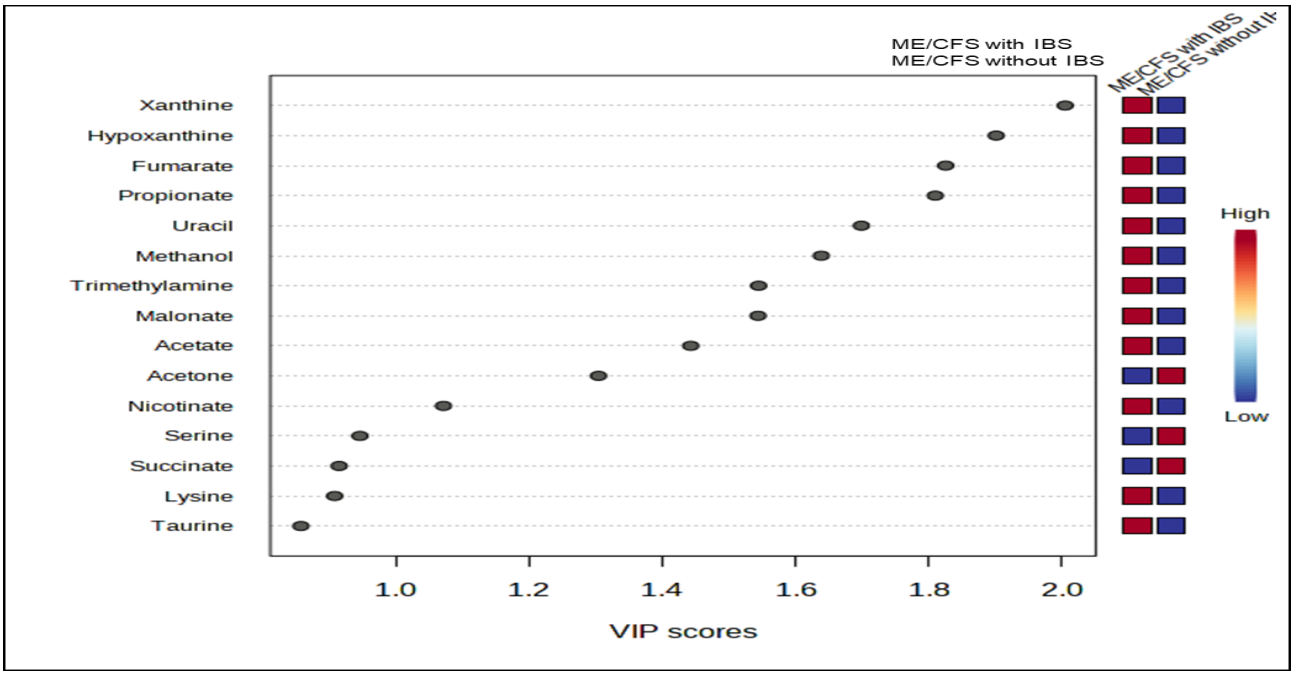
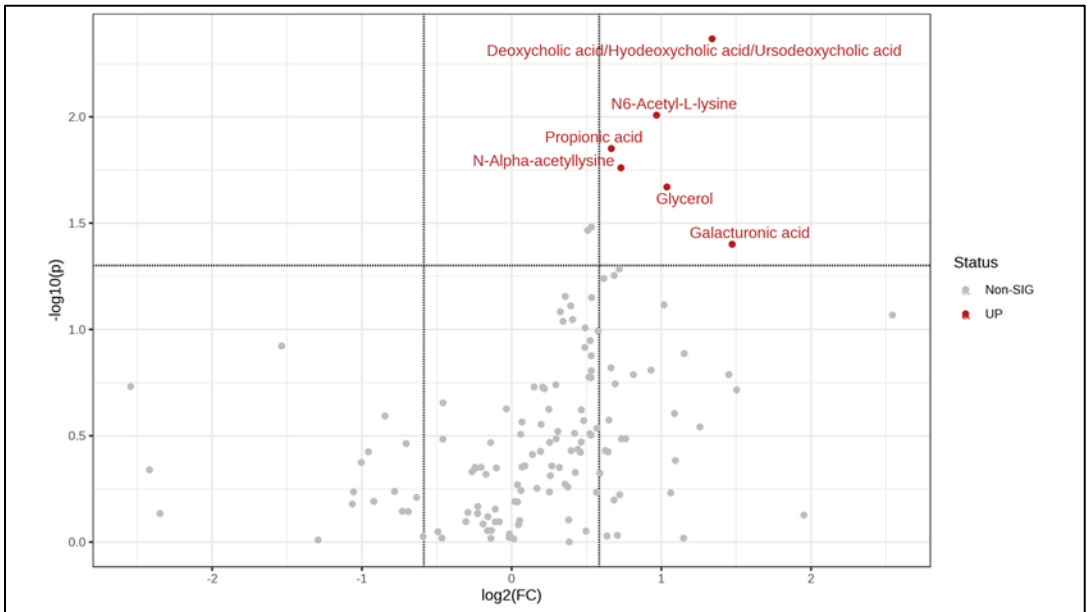



**Figure 4.1.24** VIP scores of corresponding PLSDA for NMR Faecal Part 1 ME/CFS with IBS versus ME/CFS without IBS

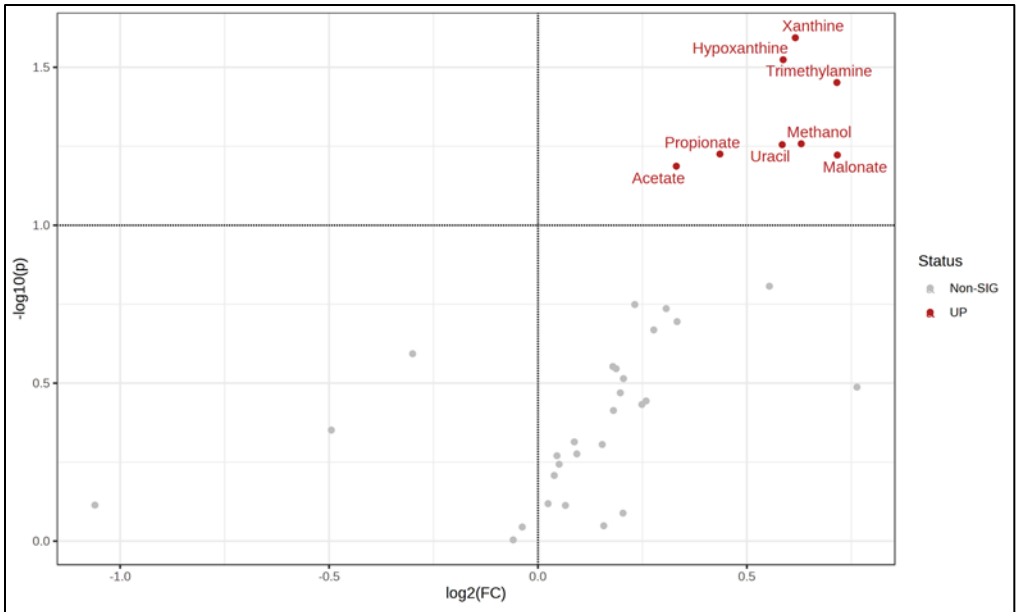
### 4.1.4 Faecal Comparison D) Age-matched ME/CFS with IBS vs. ME/CFS without IBS

#### 4.1.4.1 Univariate Analysis




| | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|
| Deoxycholic acid/Hyodeoxycholic acid/Ursodeoxycholic acid | 2.5301 | 1.3392 | 0.004301 | 2.3665 |
| N6-Acetyl-L-lysine | 1.9562 | 0.96805 | 0.009829 | 2.0075 |
| Propionic acid | 1.5868 | 0.6661 | 0.014112 | 1.8504 |
| N-Alpha-acetyllysine | 1.659 | 0.73035 | 0.017377 | 1.76 |
| Glycerol | 2.0524 | 1.0373 | 0.021385 | 1.6699 |
| Galacturonic acid | 2.7768 | 1.4734 | 0.03977 | 1.4004 |

**Figure 4.1.25** Volcano plot of LCMS Faecal Part 1 age-matched ME/CFS with IBS versus ME/CFS without IBS. Metabolites with significantly higher (red) or lower (blue) concentrations in the age-matched ME/CFS with IBS cohort are highlighted (FC threshold 1.5 and uncorrected p-values ≤ 0.05) with non-significant metabolites represented in grey.




| | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|
| Xanthine | 1.5326 | 0.61594 | 0.025498 | 1.5935 |
| Hypoxanthine | 1.502 | 0.58686 | 0.029926 | 1.524 |
| Trimethylamine | 1.642 | 0.71542 | 0.035336 | 1.4518 |
| Methanol | 1.5477 | 0.63011 | 0.05522 | 1.2579 |
| Uracil | 1.4996 | 0.58457 | 0.055571 | 1.2552 |
| Propionate | 1.3523 | 0.43546 | 0.059496 | 1.2255 |
| Malonate | 1.6432 | 0.71647 | 0.059948 | 1.2222 |
| Acetate | 1.258 | 0.33113 | 0.065022 | 1.1869 |

**Figure 4.1.26** Volcano plot of NMR Faecal Part 1 age-matched ME/CFS with IBS versus ME/CFS without IBS. Metabolites with significantly higher (red) or lower (blue) concentrations in the age-matched ME/CFS with IBS cohort are highlighted (FC threshold 1.0 and uncorrected p-values ≤ 0.10) with non-significant metabolites represented in grey.

### 4.1.4.2 Multivariate Analysis (PCA)

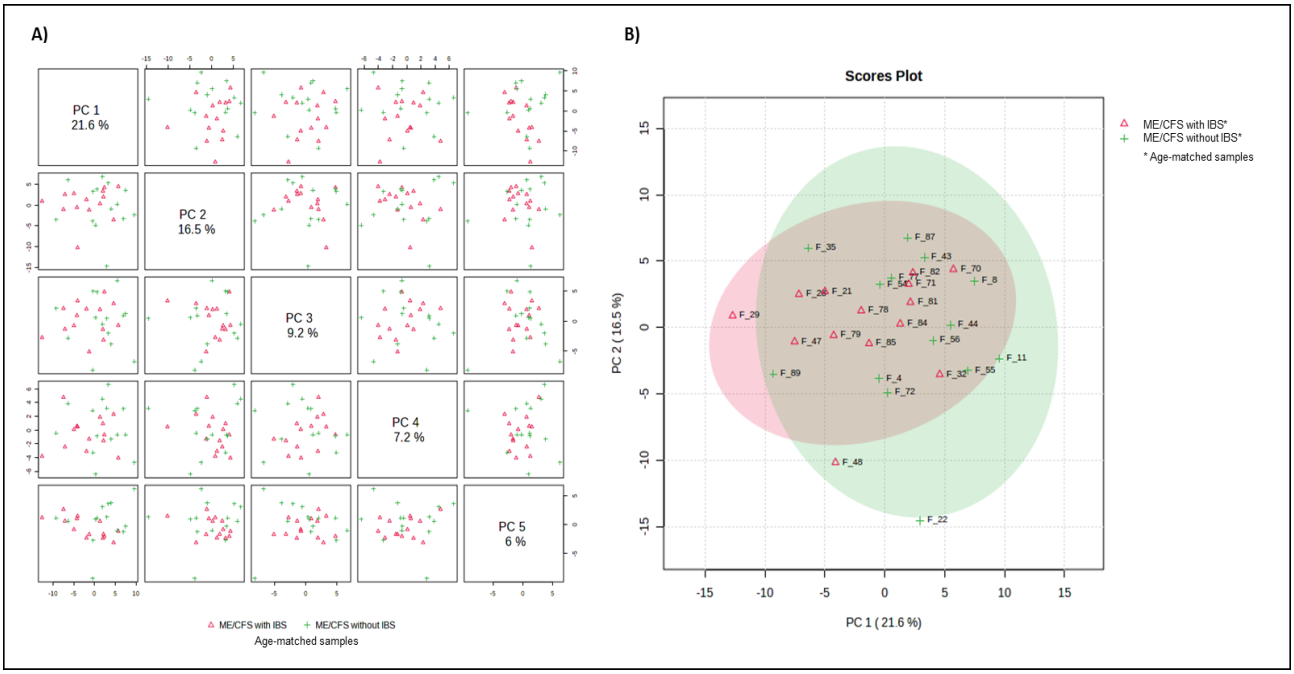



**Figure 4.1.27** PCA plots A) overview B) PC1 and PC2 only of LCMS Faecal Part 1 age-matched ME/CFS with IBS versus ME/CFS without IBS

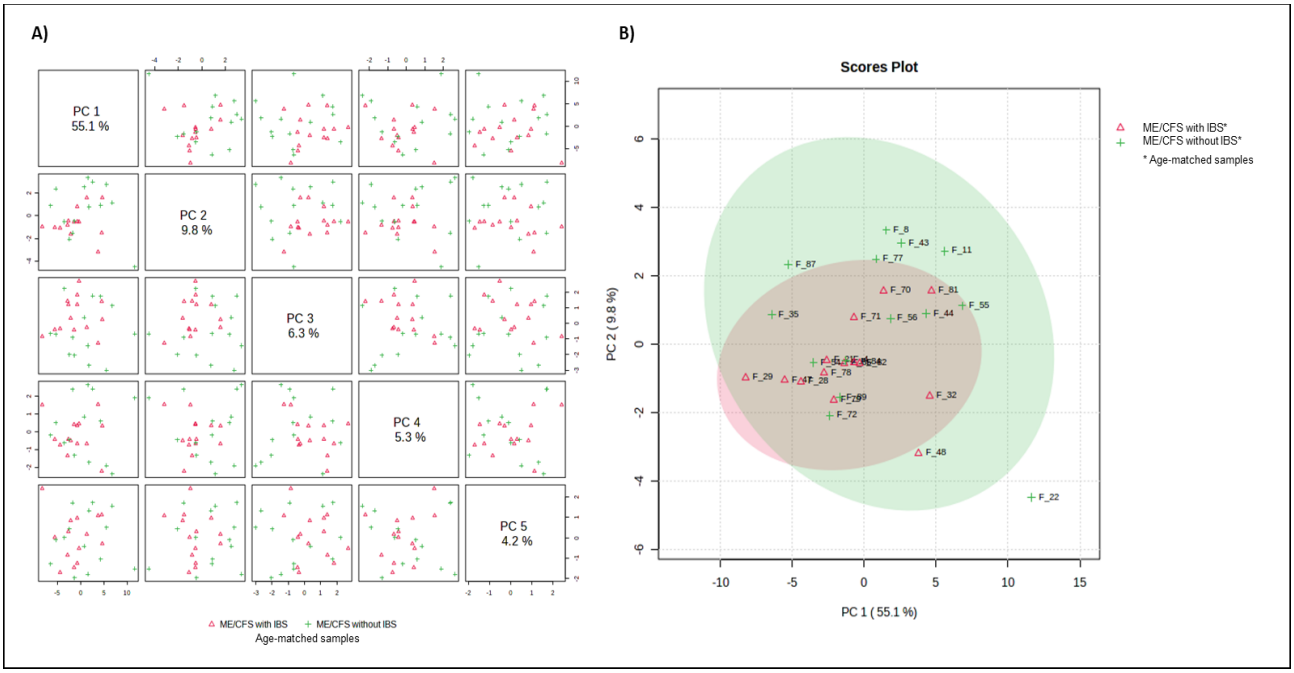



**Figure 4.1.28** PCA plots A) overview B) PC1 and PC2 only of NMR Faecal Part 1 age-matched ME/CFS with IBS versus ME/CFS without IBS

### 4.1.4.3 Multivariate Analysis (PLS-DA)

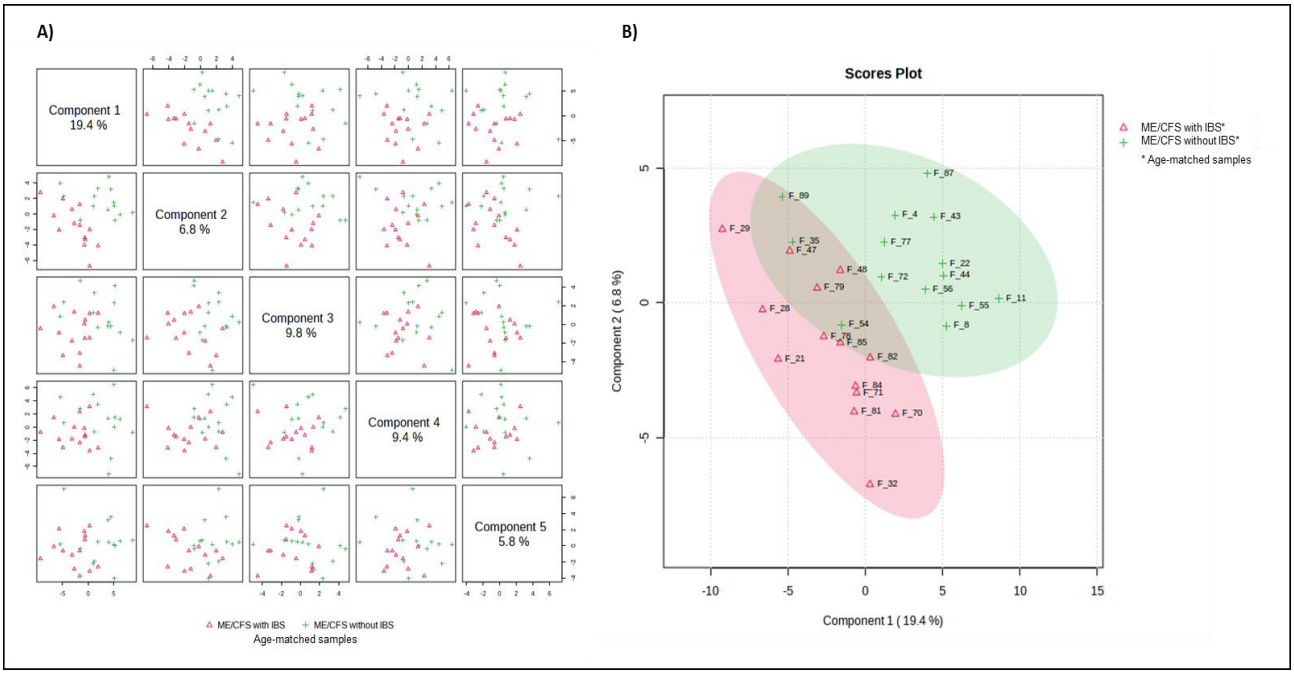



**Figure 4.1.29** PLSDA A) overview B) components 1 and 2 only of LCMS Faecal Part 1 age-matched ME/CFS with IBS versus ME/CFS without IBS

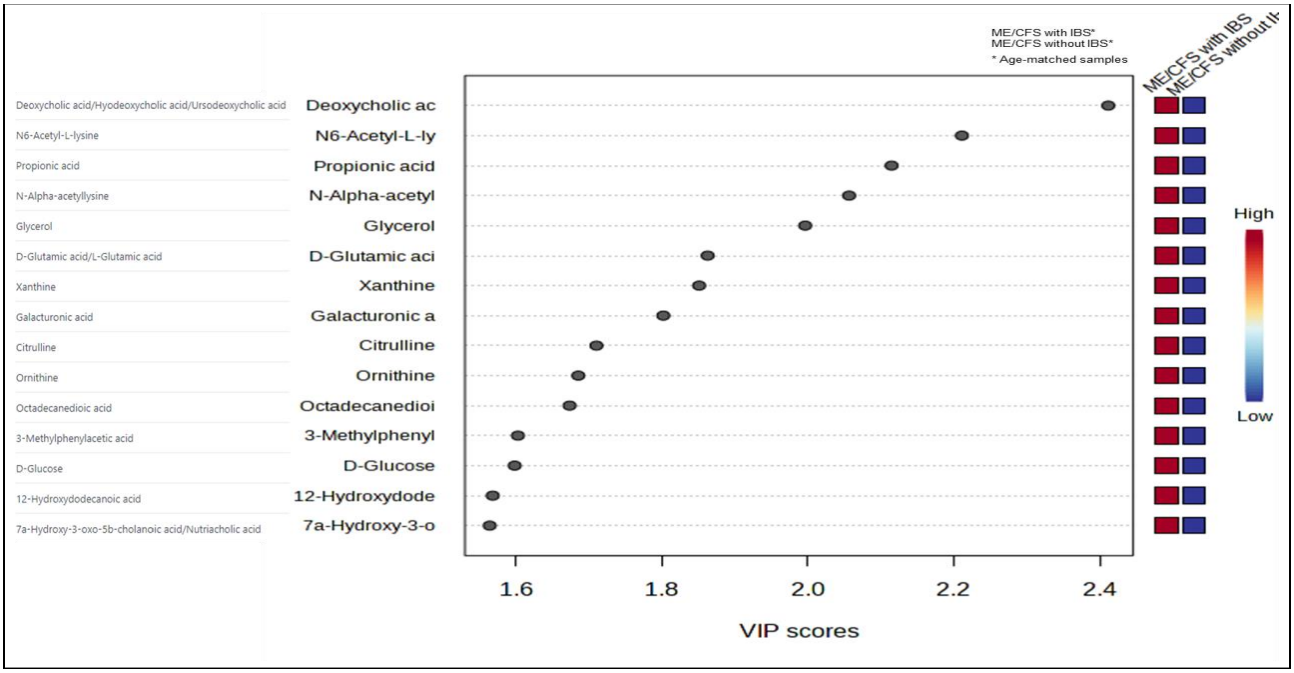



**Figure 4.1.30** VIP scores of corresponding PLSDA for LCMS Faecal Part 1 age-matched ME/CFS with IBS versus ME/CFS without IBS

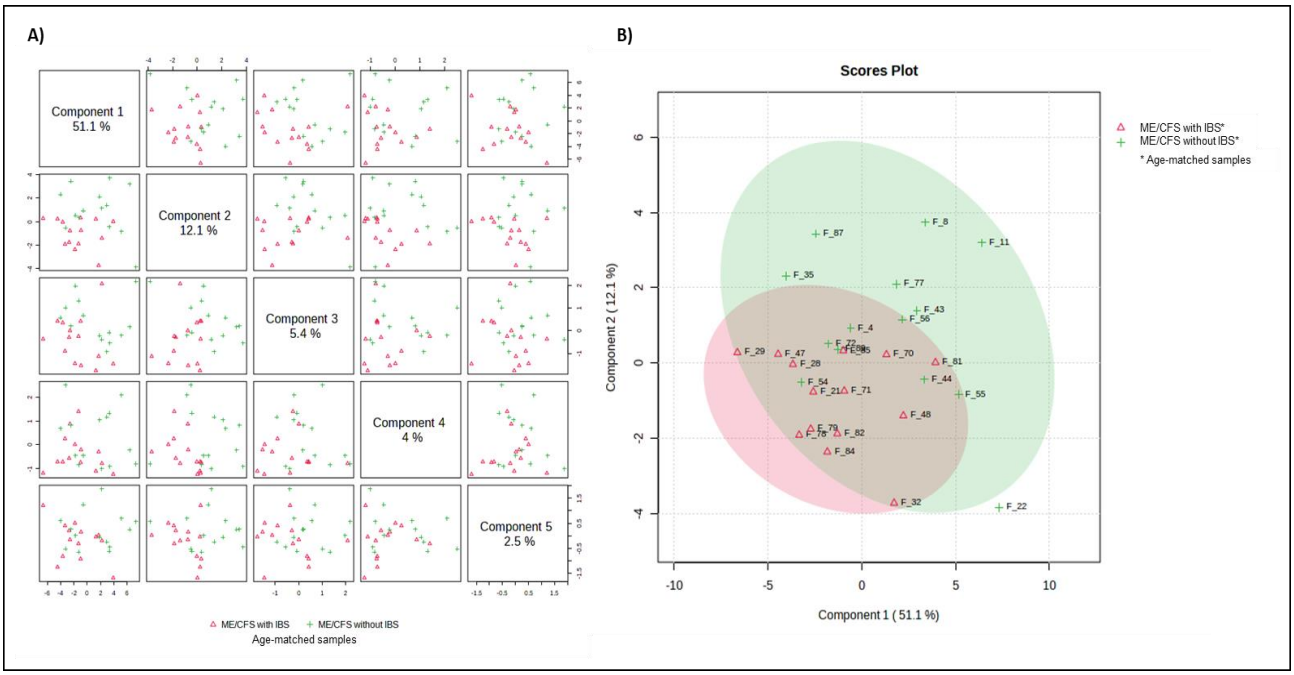



**Figure 4.1.31** PLSDA A) overview B) components 1 and 2 only of NMR Faecal Part 1 age-matched ME/CFS with IBS versus ME/CFS without IBS

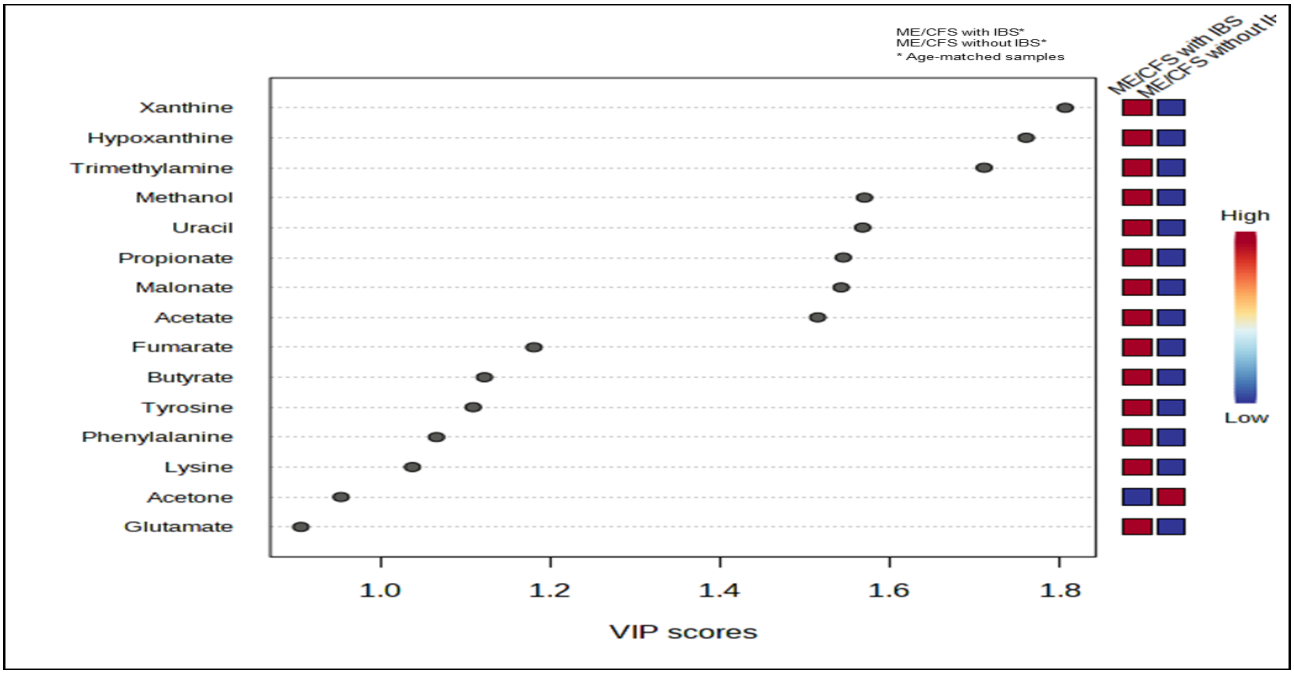



**Figure 4.1.32** VIP scores of corresponding PLSDA for NMR Faecal Part 1 age-matched ME/CFS with IBS versus ME/CFS without IBS

## 4.2 Urine Metabolomics Results

Globally, LCMS detected 147 and NMR detected 26 urine metabolite features from all samples (Appendix 8). 90 significantly different metabolite features in total from LCMS and NMR were highlighted from the volcano plots (VPs) and PLS-DA VIP plots across all the comparisons, Comparison A-D (Table 4.2.1). These significantly different metabolites, categorised according to the HMDB database, included amino acids, peptides and analogues (27.78%), sugar, carbohydrates and carbohydrate conjugates (14.44%), carboxylic acids and derivatives (16.67%), bile acids and derivatives (2.22%), fatty acyls, fatty acids and conjugates (10.00%), benzenoids (6.67%), other metabolites (12.22%), alcohols (2.22%), and organic acids and derivatives (7.78%). There were five differential urine metabolite features (glycine, valine, lactic acid, malonic acid, and citric acid) that were measured by both analytical platforms, LCMS and NMR (Table 4.2.1). The relative concentrations found for these shared metabolites were consistent in their direction as either up/higher/red or down/lower/blue from the VP and PLS-DA analyses (Appendix 9). The individual plots from the VP, PCA, and PLS-DA analyses for the urine metabolomic outcomes in Comparisons A-D are shown in Sections 4.2.1 (ME/CFS overall vs. Control), 4.2.2 (ME/CFS +/- FM), 4.2.3 (ME/CFS +/- IBS), and 4.2.4 (age-matched ME/CFS +/- IBS**), respectively.

**Table 4.2.1** LCMS and NMR Urine Metabolome (Part One) Significant Features and Results

| | Urine Part One Metabolite | Comparative Group (A-D) | Group with significant elevation in relative concentration | LCMS or NMR |
|---|---|---|---|---|
| Amino acids, peptides and analogues | Glycine ‡ | B, D | ME/CFS - FM, ME/CFS + IBS** | NMR, LCMS |
| | Glutamic acid (Glutamate) | A | ME/CFS | LCMS |
| | Cysteine | A, B, C, D | Control, ME/CFS - FM, ME/CFS + IBS, ME/CFS + IBS** | NMR |
| | Alanine | A, C | Control, ME/CFS - IBS | NMR |
| | Histidine | A, B, C, D | ME/CFS, ME/CFS + FM, ME/CFS + IBS, ME/CFS + IBS** | NMR |
| | *L-Threonine** | *B, D* | *ME/CFS - FM, ME/CFS + IBS** | LCMS |
| | L-Phenylalanine | C | ME/CFS - IBS | LCMS |
| | Taurine | C, D | ME/CFS - IBS, ME/CFS - IBS** | NMR |
| | Valine (aka. L-Valine) ‡ | A, B, C, D | Control, ME/CFS + FM, ME/CFS - IBS, ME/CFS - IBS** | NMR, LCMS |
| | 3-Aminoisobutanoic acid/D-Alpha-aminobutyric acid | B | ME/CFS - FM | LCMS |
| | L-Allothreonine/L-Threonine | B, D | ME/CFS - FM, ME/CFS + IBS** | LCMS |
| | Creatinine | A, C, D | Control, ME/CFS - IBS, ME/CFS - IBS** | NMR |
| | L-Cystathionine | A | Control | LCMS |
| | Guanidoacetate | B, C, D | ME/CFS - FM, ME/CFS + IBS, ME/CFS + IBS** | NMR |
| | Creatine | B, C | ME/CFS - FM, ME/CFS + IBS | NMR |
| | Dimethylglycine | B | ME/CFS - FM | LCMS |
| | *3-Aminoisobutanoic acid** | *B* | ME/CFS - FM | LCMS |
| | *D-Alpha-aminobutyric acid** | *B* | ME/CFS - FM | LCMS |
| | N6-Acetyl-L-lysine | B | ME/CFS - FM | LCMS |
| | 5-Hydroxylysine/5-Hydroxylysine | B | ME/CFS - FM | LCMS |
| | *L-Allothreonine** | *B, D* | ME/CFS - FM, ME/CFS + IBS** | LCMS |
| | Isobutyrylglycine | C, D | ME/CFS - IBS, ME/CFS - IBS** | LCMS |
| | Phenylacetylglycine | D | ME/CFS + IBS** | LCMS |
| | Alanylglycine | B | ME/CFS - FM | LCMS |
| | Carnosine | B | ME/CFS - FM | LCMS |
| Sugar; carbohydrates and carbohydrate conjugates | 1.5-Anhydrosorbitol | B | ME/CFS + FM | LCMS |
| | Dihydroxyacetone/D-Lactic acid/Glyceraldehyde | C, D | ME/CFS - IBS, ME/CFS - IBS** | LCMS, NMR |
| | *Dihydroxyacetone** | *C* | *ME/CFS - IBS* | LCMS |
| | *Glyceraldehyde** | *C* | *ME/CFS - IBS* | LCMS |
| | Glucose | A, B, C, D | ME/CFS, ME/CFS + FM, ME/CFS - IBS, ME/CFS - IBS** | NMR |
| | Galactitol/Mannitol/Sorbitol | C, D | ME/CFS + IBS, ME/CFS + IBS** | LCMS |
| | Gluconic acid | A | Control | LCMS |
| | Threonic acid | C, D | ME/CFS + IBS, ME/CFS + IBS** | LCMS |
| | Glyceric acid | D | ME/CFS + IBS** | LCMS |
| | *Galactitol** | *C, D* | *ME/CFS + IBS, ME/CFS + IBS** | LCMS |

| | | | | |
|---|---|---|---|---|
| | *Mannitol** | *C, D* | *ME/CFS + IBS, ME/CFS + IBS*** | LCMS |
| | *Sorbitol** | *C, D* | *ME/CFS + IBS, ME/CFS + IBS*** | LCMS |
| | Glycerol | A | ME/CFS | LCMS |
| Carboxylic acids and derivatives | Glycolate | B, D | ME/CFS - FM, ME/CFS + IBS** | NMR |
| | Succinate | A, B | ME/CFS, ME/CFS + FM | NMR |
| | Oxalic acid | A | ME/CFS | LCMS |
| | Malonate (aka. Malonic acid) ‡ | A, B, C, D | Control, ME/CFS - FM, ME/CFS - IBS, ME/CFS - IBS** | NMR, LCMS |
| | Dimethylmalonic acid/Ethylmalonic acid | B | ME/CFS - FM | LCMS |
| | *Dimethylmalonic acid** | *B* | *ME/CFS - FM* | LCMS |
| | Fumarate | C, D | ME/CFS - IBS, ME/CFS - IBS** | NMR |
| | Indoleacetic acid | A | ME/CFS | LCMS |
| | 2-Indolecarboxylic acid | D | ME/CFS + IBS** | LCMS |
| | p-Hydroxyphenylacetic acid | B, C | ME/CFS - FM, ME/CFS + IBS | LCMS |
| | Formate | D | ME/CFS + IBS** | NMR |
| | cis-Aconitic acid | A | ME/CFS | LCMS |
| | Citrate (aka. Citric acid) * ‡ | A, B | ME/CFS, ME/CFS + FM | NMR, LCMS |
| | *D-threo-Isocitric acid** | *B* | *ME/CFS + FM* | LCMS |
| | *Isocitric acid** | *B* | *ME/CFS + FM* | LCMS |
| Bile acids and derivatives | Glycocholic acid | A | ME/CFS | LCMS |
| | Deoxycholic acid glycine conjugate | D | ME/CFS + IBS** | LCMS |
| Fatty acyls; fatty acids and conjugates | *Ethylmalonic acid** | *B* | *ME/CFS - FM* | LCMS |
| | Myristic acid | A | ME/CFS | LCMS |
| | Oleic acid | A | ME/CFS | LCMS |
| | Stearic acid | A | ME/CFS | LCMS |
| | Palmitic acid | A | ME/CFS | LCMS |
| | Caprylic acid | A | ME/CFS | LCMS |
| | Caproic acid | A | ME/CFS | LCMS |
| | Adipic acid | A | ME/CFS | LCMS |
| | Succinic acid semialdehyde (MH2) | A | ME/CFS | LCMS |
| Benzenoids | Hippurate | A, C, D | Control, ME/CFS + IBS, ME/CFS + IBS** | NMR |
| | Phenol | C | ME/CFS + IBS | LCMS |
| | Pyrocatechol | C | ME/CFS + IBS | LCMS |
| | Vanillin | B | ME/CFS + FM | LCMS |
| | 4-Hydroxyphenylpyruvic acid | A | Control | LCMS |
| | 2.6-Dihydroxybenzoic acid | A, C, D | Control, ME/CFS + IBS, ME/CFS + IBS** | LCMS |
| Other metabolites | Maleate | A | ME/CFS | NMR |
| | Loperamide | A, B | Control, ME/CFS + FM | LCMS |
| | Sumiki's acid/5-(Hydroxymethyl)furoic acid | B | ME/CFS + FM | LCMS |
| | Beta-Glycerophosphoric acid/Glycerol 3-phosphate | A | Control | LCMS |
| | *Beta-Glycerophosphoric acid** | *A* | *Control* | LCMS |
| | *Glycerol 3-phosphate** | *A* | *Control* | LCMS |
| | Phosphoric acid | A, C, D | Control, ME/CFS - IBS, ME/CFS - IBS** | LCMS |

| | | | | |
|---|---|---|---|---|
| | Dimethylamine | A, B, C, D | Control, ME/CFS + FM, ME/CFS - IBS, ME/CFS - IBS** | NMR |
| | 7-Methylguanine | A, B | ME/CFS, ME/CFS + FM | LCMS |
| | Kynurenic acid | C | ME/CFS - IBS | LCMS |
| | Trimethylamine N-oxide | C, D | ME/CFS + IBS, ME/CFS + IBS** | NMR |
| Alcohols | Ethanolamine | A | Control | NMR |
| | Methanol | A, B | Control, ME/CFS + FM | NMR |
| Organic acids and derivatives | *D-Lactic acid (aka. Lactate) * ‡* | *C, D* | *ME/CFS - IBS, ME/CFS - IBS*** | LCMS, NMR |
| | Hydroxypropionic acid | A | ME/CFS | LCMS |
| | Oxoglutaric acid/Oxoglutaric acid | C, D | ME/CFS - IBS, ME/CFS - IBS** | LCMS |
| | O-Phosphoethanolamine | A, C, D | Control, ME/CFS - IBS, ME/CFS - IBS** | LCMS |
| | Acetate | A, B, C, D | ME/CFS, ME/CFS + FM, ME/CFS + IBS, ME/CFS + IBS** | NMR |
| | 2-Ketobutyric acid | A | ME/CFS | LCMS |
| | Urea | B | ME/CFS + FM | NMR |
| NOTE | * From LCMS consolidated metabolite feature<br>**Comparative Group (A-D)**<br>A) ME/CFS overall vs. Control [ME/CFS, Control]<br>B) ME/CFS with FM vs. ME/CFS without FM [ME/CFS + FM, ME/CFS - FM]<br>C) ME/CFS with IBS vs. ME/CFS without IBS [ME/CFS + IBS, ME/CFS - IBS]<br>D) ME/CFS with IBS vs. ME/CFS without IBS (age-matched) [ME/CFS + IBS**, ME/CFS - IBS**]<br>‡ "Duplicate" metabolite assayed by LCMS and NMR<br>Refer to **Appendix 9** for electronic version of this table and raw data input | | | |

## 4.2 Urine Metabolomics Results continued…

The significantly differential urine metabolite features were elevated in their relative concentrations depending on how the samples were grouped with their ME/CFS, FM, or IBS metadata that comprised Comparison A-D (Table 4.2.1). Not every single urine metabolite appeared in all the comparisons when observing the significant differences between the involved groups. A total of 24, 17, 6, and 5 metabolite features were found only to be significantly different in Comparison A (ME/CFS overall vs. Control), B (ME/CFS +/- FM), C (ME/CFS +/- IBS), and D (age-matched ME/CFS +/- IBS**), respectively; for example, succinic acid semialdehyde (MH2) and glutamic acid were only differential and elevated in the ME/CFS overall group from Comparison A. The metabolites, for example, isobutyrylglycine and threonic acid, that were highlighted in the ME/CFS +/- IBS and ME/CFS +/- IBS** groups (Comparison C and D), were always consistently lower or higher in their relative concentration providing some indication of IBS involvement where an attempt to mitigate the age variable has been made. Histidine and acetate were consistently elevated in the ME/CFS overall (Comparison A) and the ME/CFS + either FM, IBS, or IBS** (Comparison B-D) groups.

VPs of the LCMS urine data matrix highlighted 22 metabolites (15 down and 7 up) in the Control vs. ME/CFS (Figure 4.2.1), 6 metabolites (3 down and 3 up) in the ME/CFS +/- FM (Figure 4.2.9), 4 metabolites (1 down 3 up) in the ME/CFS +/- IBS (Figure 4.2.17), and 1 metabolite (down only) in the ME/CFS +/- IBS** (Figure 4.2.25) comparisons that were significantly different. VPs of the NMR urine data matrix highlighted 5 metabolites (2 down and 3 up) in the Control vs. ME/CFS (Figure 4.2.2), 2 metabolites (1 down and 1 up) in the ME/CFS +/- FM (Figure 4.2.10), 6 metabolites (2 down and 4 up) in the ME/CFS +/- IBS (Figure 4.2.18), and 4 metabolites (all up) in the ME/CFS +/- IBS** (Figure 4.2.26) comparisons that were significantly different. The metabolites from the VPs that were also found to be significantly different by PLS-DA VIP analyses reflected and supported the same pattern as either high or low in relative concentration (Appendix 9). The PCAs from the LCMS and NMR data matrices did not show a clear separation between the groups for all comparisons (Table 4.2.2). Although the PCAs did not discriminate the groups, the clusters for these comparisons extended in different directions: LCMS ME/CFS vs. Control (Figure 4.2.3), LCMS and NMR ME/CFS +/- FM (Figure 4.2.11, Figure 4.2.12), NMR ME/CFS +/- IBS (Figure 4.2.20), and LCMS ME/CFS +/- IBS** (Figure 4.2.27). Tighter clusters that resided within a broader cluster observed by ellipsoid shape and size were noted for LCMS and NMR ME/CFS vs. Control (Figure 4.2.3 and Figure 4.2.4), NMR

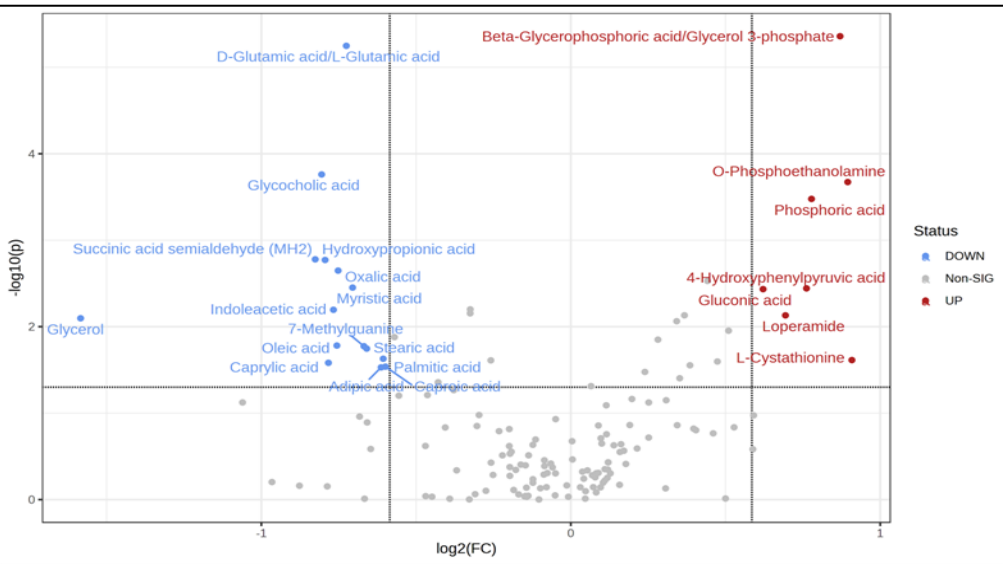
ME/CFS +/- FM (Figure 4.2.12), LCMS ME/CFS +/- IBS (Figure 4.2.19), and LCMS ME/CFS +/- IBS** (Figure 4.2.27). These patterns in the PCAs of urine samples may reflect a subset or specific trait from a wider cohort. The PLS-DAs demonstrated improved clustering and separation for Comparison A-D (Table 4.2.2). The PLS-DA for the LCMS data matrix found a partial to distinguished separation between the ME/CFS vs. Control (Figure 4.2.5), and ME/CFS +/- FM (Figure 4.2.13). A partial to distinguished separation for LCMS (Figure 4.2.21) and NMR (Figure 4.2.23) was also observed between the ME/CFS +/- IBS groups. Similarly, a distinguished separation for LCMS (Figure 4.2.29), and a partial to distinguished separation for NMR (Figure 4.2.31), for ME/CFS +/- IBS** groups was also observed.

**Table 4.2.2** Summary of the total variance from the first two components of the PCA and PLS-DA analyses (LCMS and NMR Urine Metabolome (Part One))

| Urine Part One | | PCA | | | | PLSDA | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | % of the variance | | | Separation | % of the variance | | | Separation |
| | | PC1 | PC2 | Total† | | Comp. 1 | Comp. 2 | Total‡ | |
| A) ME/CFS vs. Control | LCMS | 15.3 | 9.2 | 24.5 | Overlapping | 11 | 8.3 | 19.3 | Partial-Distinguished |
| | NMR | 13.9 | 10.6 | 24.5 | Overlapping | 8 | 8 | 16 | Overlapping-Partial |
| B) ME/CFS +/- FM | LCMS | 16.1 | 12.3 | 28.4 | Overlapping | 11.4 | 7.7 | 19.1 | Partial-Distinguished |
| | NMR | 17.6 | 14.4 | 32 | Overlapping | 10.5 | 11.5 | 22 | Partial |
| C) ME/CFS +/- IBS | LCMS | 16.1 | 12.3 | 28.4 | Overlapping | 7.6 | 11.1 | 18.7 | Partial-Distinguished |
| | NMR | 17.6 | 14.4 | 32 | Overlapping | 13.3 | 10.4 | 23.7 | Partial-Distinguished |
| D) ME/CFS +/- IBS** | LCMS | 18.8 | 14.3 | 33.1 | Overlapping | 9.8 | 12.9 | 22.7 | Distinguished |
| | NMR | 17.2 | 12.7 | 29.9 | Overlapping | 11.7 | 11 | 22.7 | Partial-Distinguished |
| † PC1 and PC2 added together | | | | | | | | | |
| ‡ Component 1 and Component 2 added together | | | | | | | | | |
| Partial-Distinguished to complete separation between groupings | | | | | | | | | |

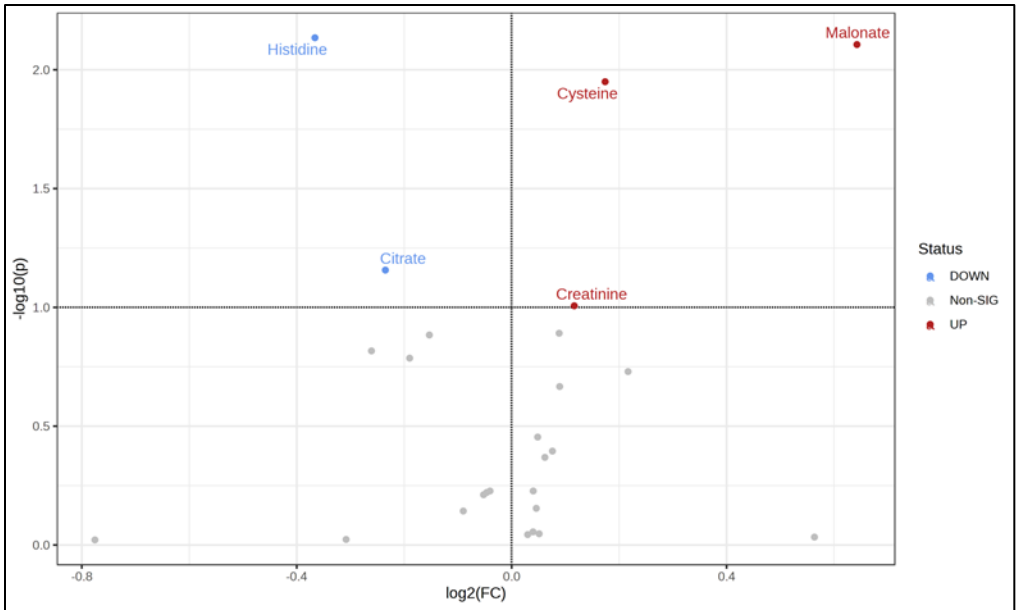
### 4.2.1 Urine Comparison A) ME/CFS Overall vs. Control Group

#### 4.2.1.1 Univariate Analysis




| | FC | log2(FC) | p | -log10(p) | q |
|---|---|---|---|---|---|
| Beta-Glycerophosphoric acid/Glycerol 3-phosphate | 1.8278 | 0.87011 | 4.38E-06 | 5.3589 | 0.00041546 |
| D-Glutamic acid/L-Glutamic acid | 0.60484 | -0.72537 | 5.65E-06 | 5.2478 | 0.00041546 |
| Glycocholic acid | 0.57229 | -0.80518 | 0.00017362 | 3.7604 | 0.007811 |
| O-Phosphoethanolamine | 1.8595 | 0.89488 | 0.00021254 | 3.6726 | 0.007811 |
| Phosphoric acid | 1.7146 | 0.77786 | 0.00033321 | 3.4773 | 0.0097963 |
| Succinic acid semialdehyde (MH2) | 0.56414 | -0.82588 | 0.0016651 | 2.7786 | 0.035551 |
| Hydroxypropionic acid | 0.57675 | -0.79397 | 0.0016929 | 2.7714 | 0.035551 |
| Oxalic acid | 0.59383 | -0.75189 | 0.0022475 | 2.6483 | 0.041298 |
| Myristic acid | 0.6134 | -0.70509 | 0.0035338 | 2.4518 | 0.045102 |
| 4-Hydroxyphenylpyruvic acid | 1.6954 | 0.76161 | 0.0036078 | 2.4428 | 0.045102 |
| Gluconic acid | 1.5383 | 0.62136 | 0.0036818 | 2.4339 | 0.045102 |
| Indoleacetic acid | 0.58764 | -0.767 | 0.0063808 | 2.1951 | NS |
| Loperamide | 1.6174 | 0.69369 | 0.0074015 | 2.1307 | NS |
| Glycerol | 0.33354 | -1.5841 | 0.0079999 | 2.0969 | NS |
| Oleic acid | 0.59215 | -0.75596 | 0.016542 | 1.7814 | NS |
| 7-Methylguanine | 0.6291 | -0.66864 | 0.016819 | 1.7742 | NS |
| Stearic acid | 0.63309 | -0.65952 | 0.017938 | 1.7462 | NS |
| Palmitic acid | 0.65696 | -0.60612 | 0.023498 | 1.629 | NS |
| L-Cystathionine | 1.8768 | 0.9083 | 0.024353 | 1.6135 | NS |
| Caprylic acid | 0.58107 | -0.78323 | 0.026196 | 1.5818 | NS |
| Caproic acid | 0.66017 | -0.59908 | 0.028998 | 1.5376 | NS |
| Adipic acid | 0.65385 | -0.61297 | 0.029575 | 1.5291 | NS |

**Figure 4.2.1** Volcano plot of LCMS Urine Part 1 ME/CFS overall vs Control groups using test outcomes from the raw p-values. Metabolites with significantly higher (blue) or lower (red) concentrations in the ME/CFS cohort are highlighted (FC threshold 1.5 and uncorrected p-values ≤ 0.05) with non-significant metabolites represented in grey. The q-values of significant FDR-adjusted metabolites are shown (NS = q-value ≥ 0.05).




| | FC | log2(FC) | p | -log10(p) | q |
|---|---|---|---|---|---|
| Histidine | 0.77586 | -0.36613 | 0.0073343 | 2.1346 | 0.097302 |
| Malonate | 1.5612 | 0.64265 | 0.0078316 | 2.1061 | 0.097302 |
| Cysteine | 1.1282 | 0.174 | 0.011227 | 1.9497 | 0.097302 |
| Citrate | 0.84969 | -0.23499 | 0.069681 | 1.1569 | NS |
| Creatinine | 1.0841 | 0.1165 | 0.098464 | 1.0067 | NS |

**Figure 4.2.2** Volcano plot of NMR Urine Part 1 ME/CFS overall vs Control groups using test outcomes from the raw p-values. Metabolites with significantly higher (blue) or lower (red) concentrations in the ME/CFS cohort are highlighted (FC threshold 1.0 and uncorrected p-values ≤ 0.10) with non-significant metabolites represented in grey. The q-values of significant FDR-adjusted metabolites are shown (NS = q-value ≥ 0.10).

### 4.2.1.2 Multivariate Analysis (PCA)

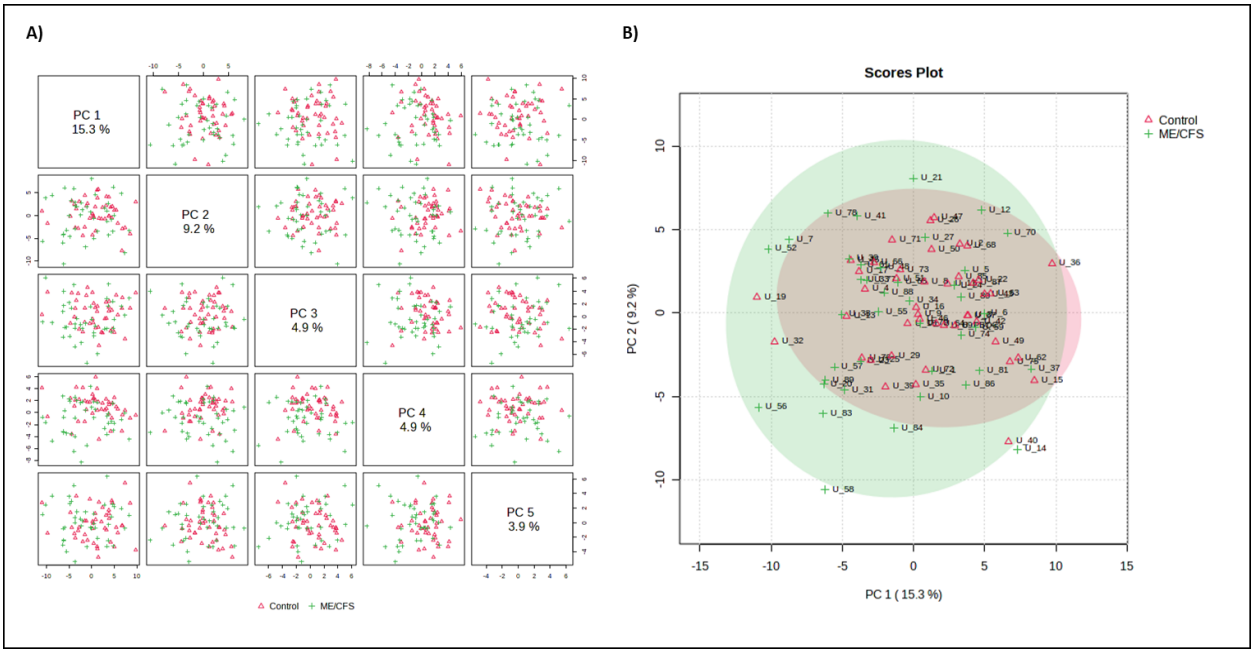



**Figure 4.2.3** PCA plots A) overview B) PC1 and PC2 only of LCMS Urine Part 1 ME/CFS overall vs. Control groups

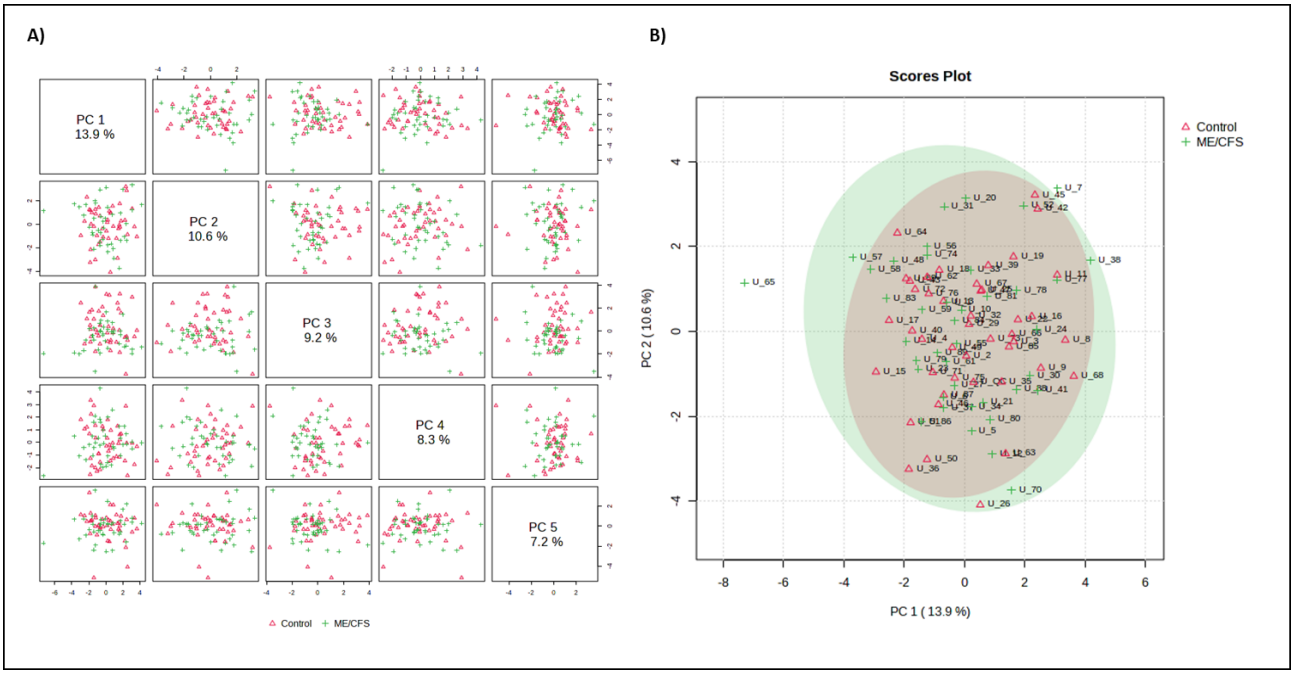



**Figure 4.2.4** PCA plots A) overview B) PC1 and PC2 only of NMR Urine Part 1 ME/CFS overall vs. Control groups

### 4.2.1.3 Multivariate Analysis (PLS-DA)

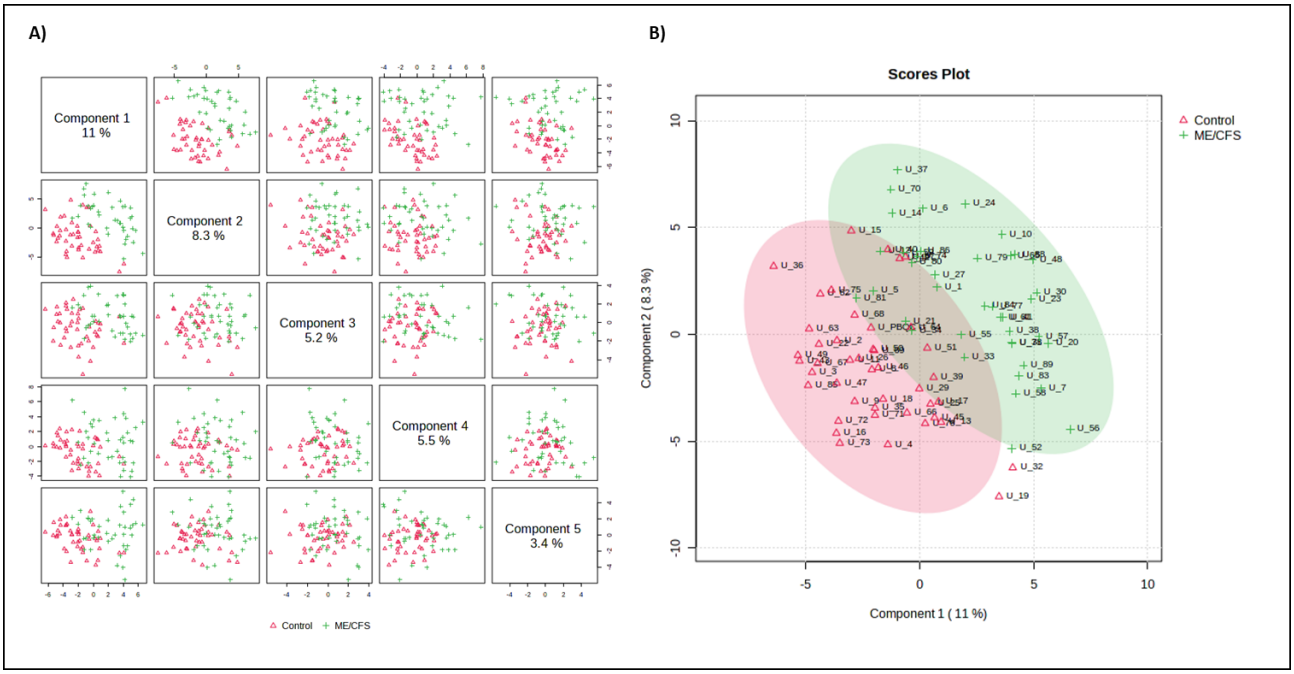



**Figure 4.2.5** PLSDA A) overview B) components 1 and 2 only of LCMS Urine Part 1 ME/CFS overall vs. Control groups

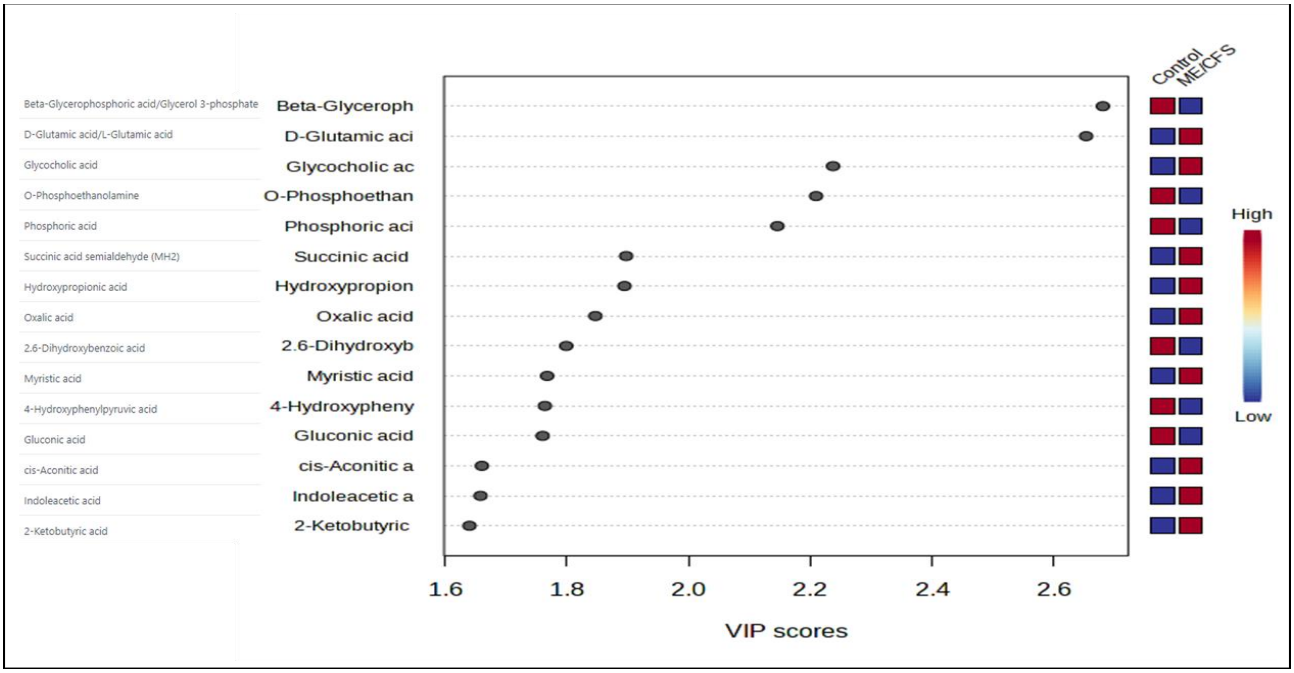



**Figure 4.2.6** VIP scores of corresponding PLSDA for LCMS Urine Part 1 ME/CFS overall vs. Control groups

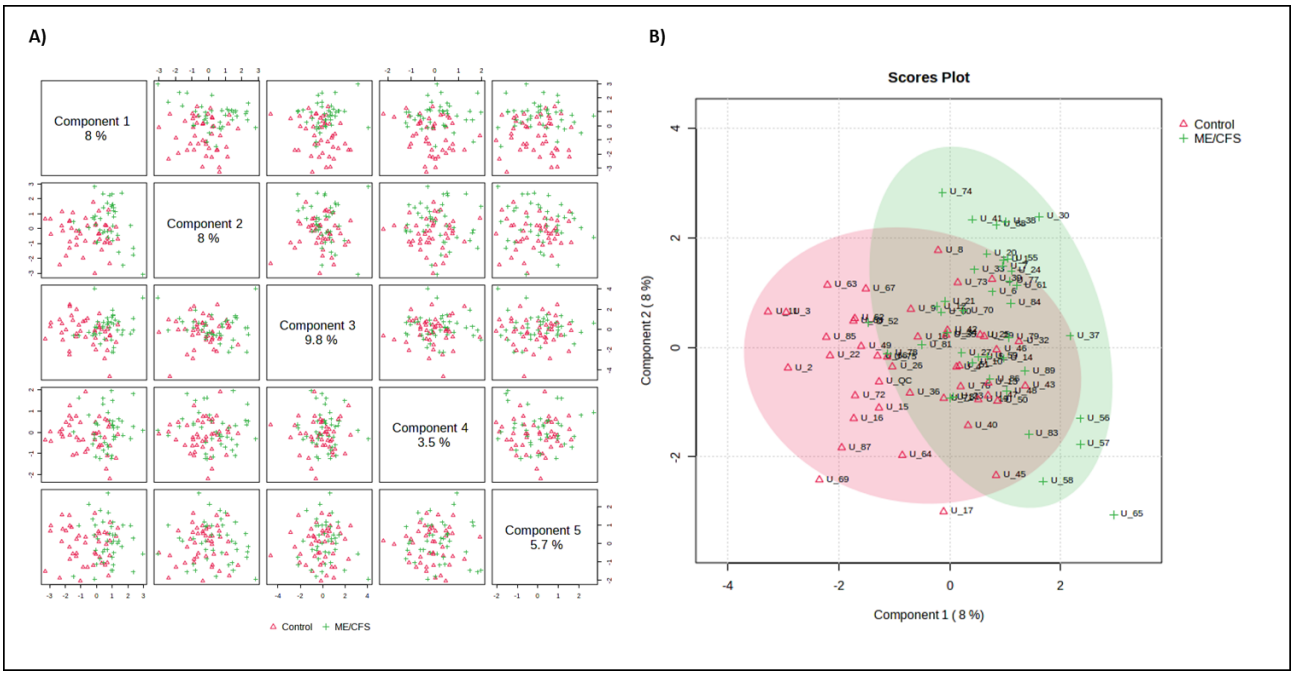



**Figure 4.2.7** PLSDA A) overview B) components 1 and 2 only of NMR Urine Part 1 ME/CFS overall vs. Control groups

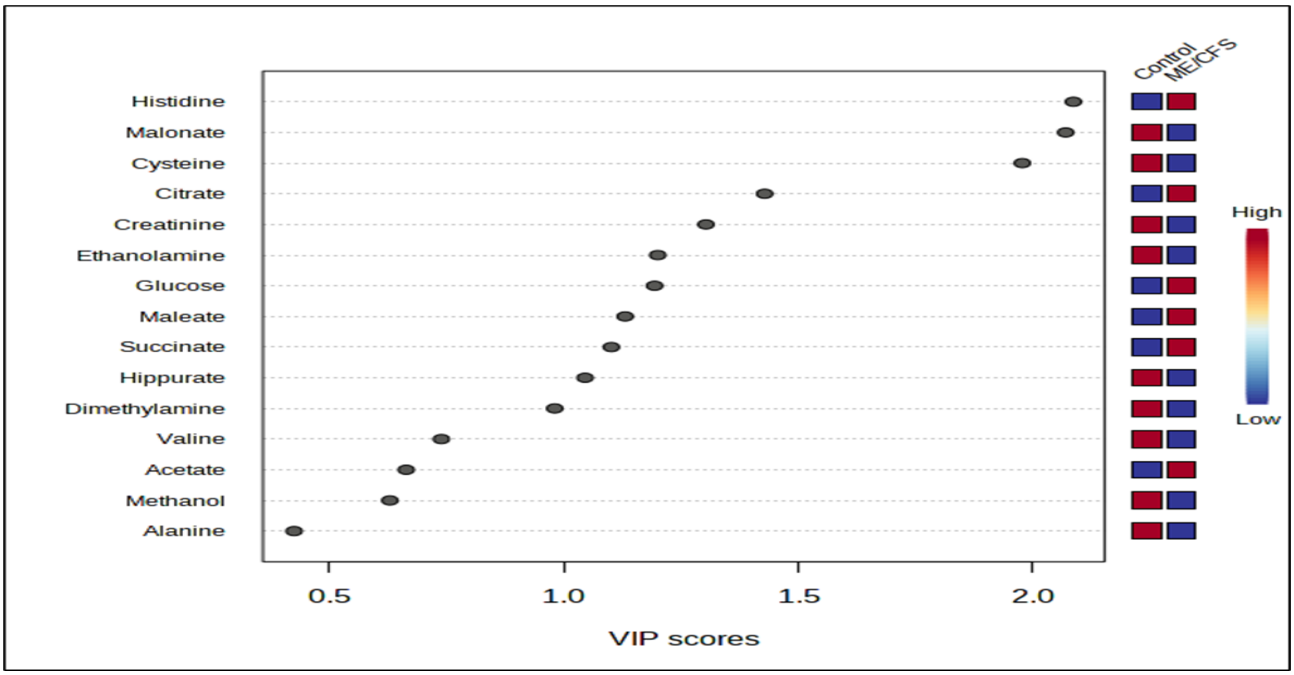
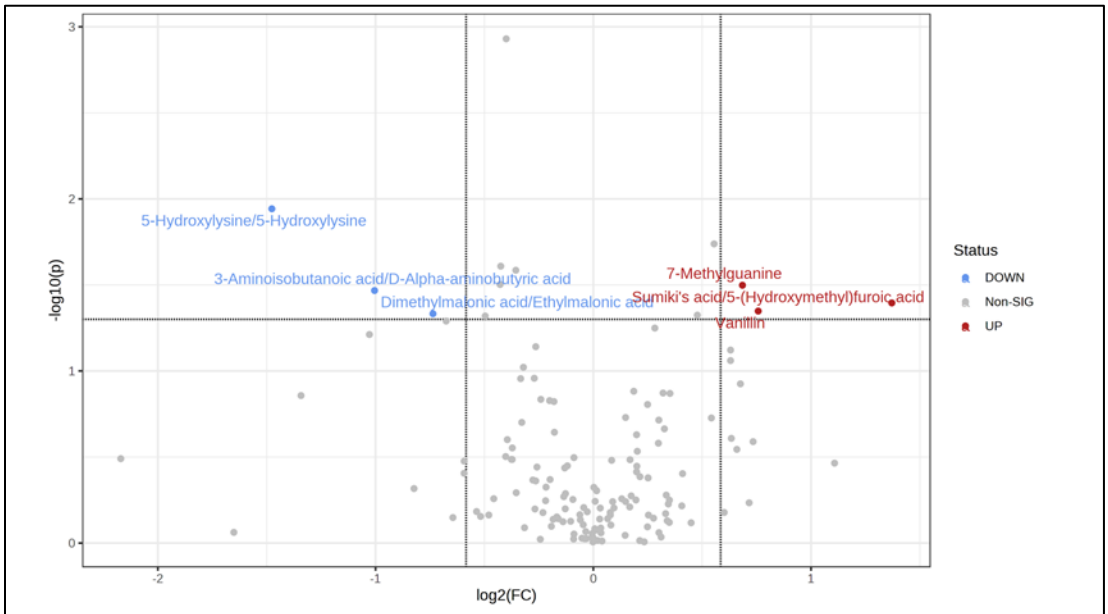



**Figure 4.2.8** VIP scores of corresponding PLSDA for NMR Urine Part 1 ME/CFS overall vs. Control groups

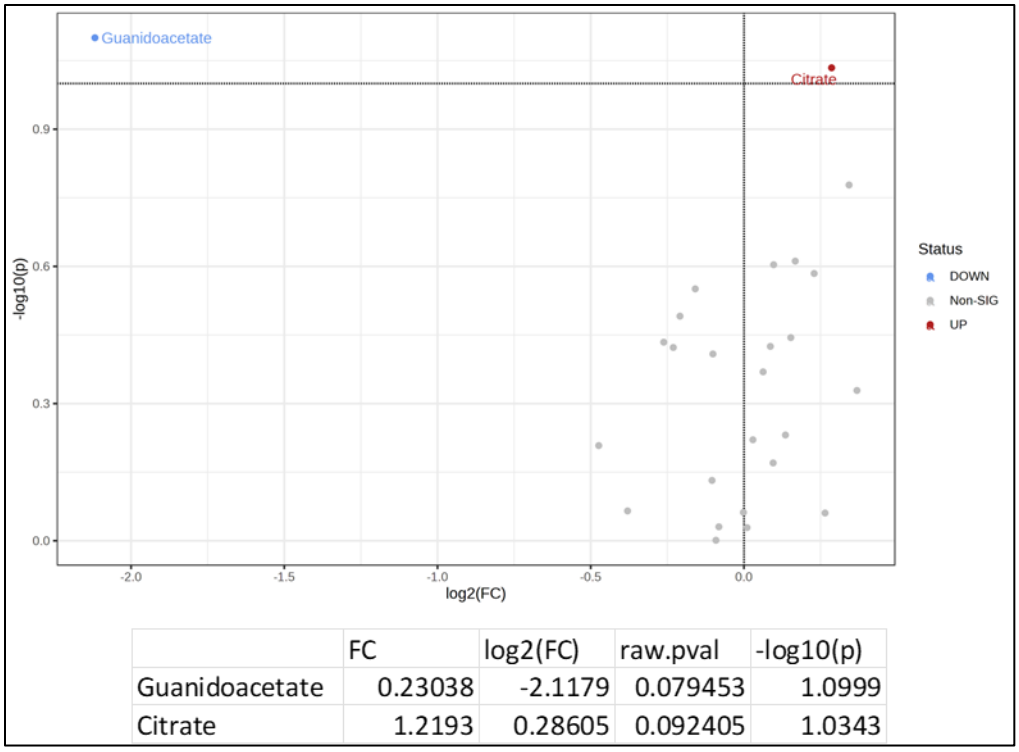
### 4.2.2 Urine Comparison B) ME/CFS with FM vs. ME/CFS without FM

#### 4.2.2.1 Univariate Analysis




| | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|
| 5-Hydroxylysine/5-Hydroxylysine | 0.3594 | -1.4764 | 0.011418 | 1.9424 |
| 7-Methylguanine | 1.6077 | 0.68499 | 0.031756 | 1.4982 |
| 3-Aminoisobutanoic acid/D-Alpha-aminobutyric acid | 0.49834 | -1.0048 | 0.034081 | 1.4675 |
| Sumiki's acid/5-(Hydroxymethyl)furoic acid | 2.5865 | 1.371 | 0.040241 | 1.3953 |
| Vanillin | 1.6904 | 0.7574 | 0.04486 | 1.3481 |
| Dimethylmalonic acid/Ethylmalonic acid | 0.60022 | -0.73643 | 0.046495 | 1.3326 |

**Figure 4.2.9** Volcano plot of LCMS Urine Part 1 ME/CFS with FM versus ME/CFS without FM. Metabolites with significantly higher (red) or lower (blue) concentrations in the ME/CFS with FM cohort are highlighted (FC threshold 1.5 and uncorrected p-values ≤ 0.05) with non-significant metabolites represented in grey.




| | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|
| Guanidoacetate | 0.23038 | -2.1179 | 0.079453 | 1.0999 |
| Citrate | 1.2193 | 0.28605 | 0.092405 | 1.0343 |

**Figure 4.2.10** Volcano plot of NMR Urine Part 1 ME/CFS with FM versus ME/CFS without FM. Metabolites with significantly higher (red) or lower (blue) concentrations in the ME/CFS with FM cohort are highlighted (FC threshold 1.0 and uncorrected p-values ≤ 0.10) with non-significant metabolites represented in grey.

### 4.2.2.2 Multivariate Analysis (PCA)

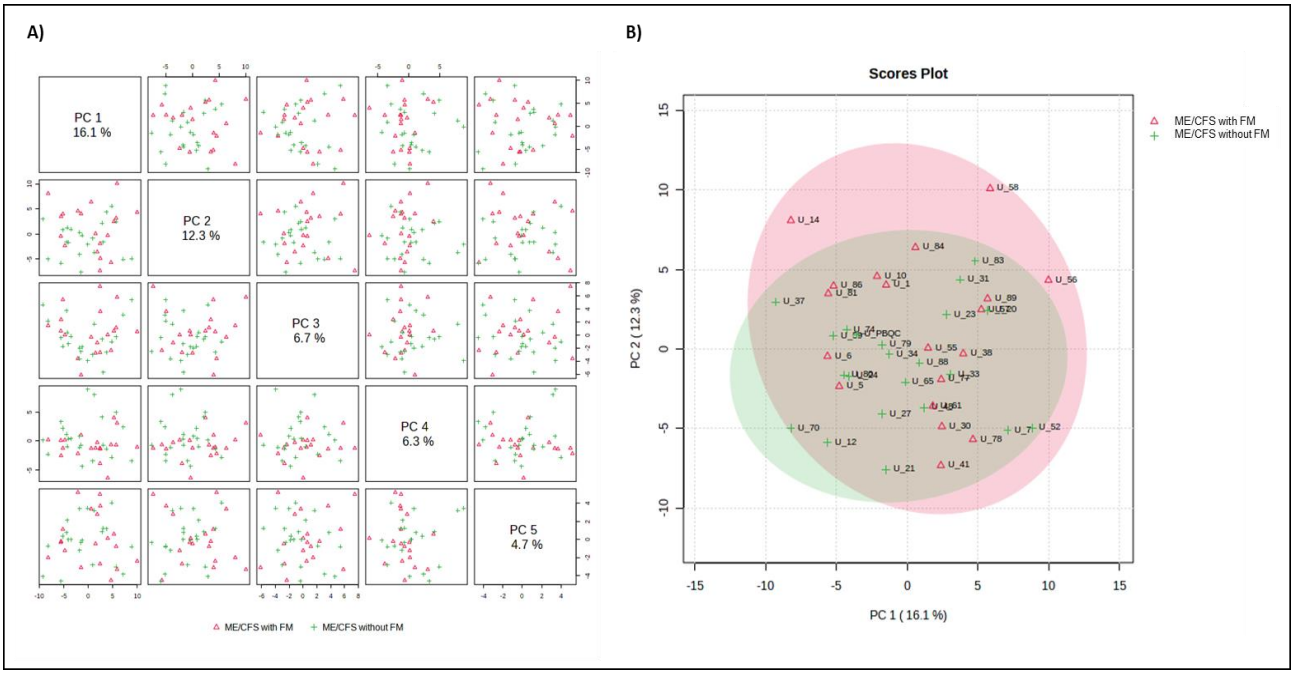



**Figure 4.2.11** PCA plots A) overview B) PC1 and PC2 only of LCMS Urine Part 1 ME/CFS with FM versus ME/CFS without FM

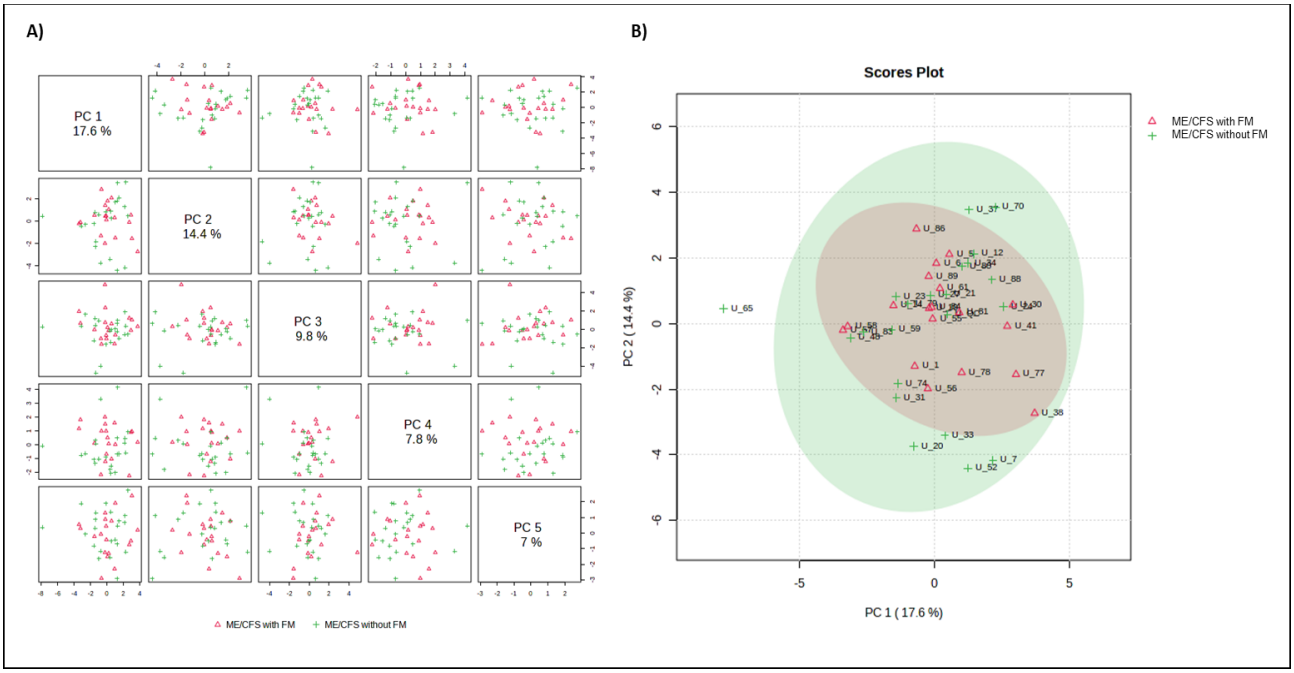



**Figure 4.2.12** PCA plots A) overview B) PC1 and PC2 only of NMR Urine Part 1 ME/CFS with FM versus ME/CFS without FM

### 4.2.2.3 Multivariate Analysis (PLS-DA)

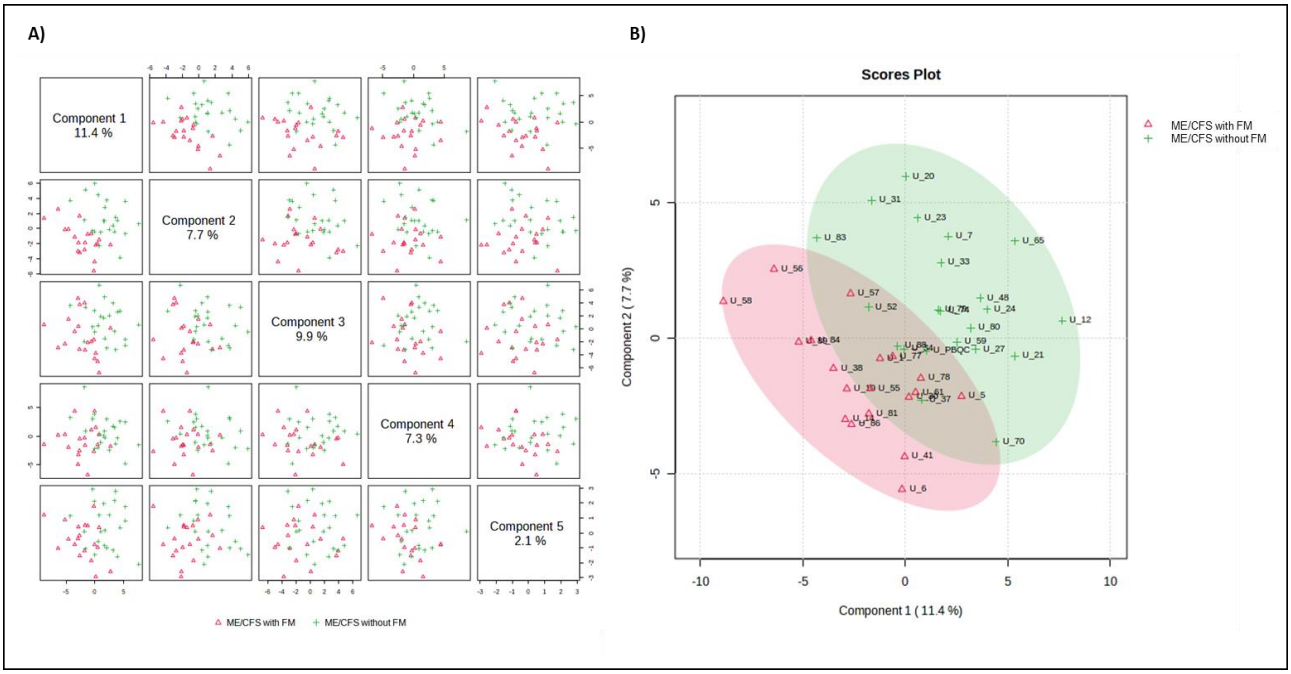



**Figure 4.2.13** PLSDA A) overview B) components 1 and 2 only of LCMS Urine Part 1 ME/CFS with FM versus ME/CFS without FM

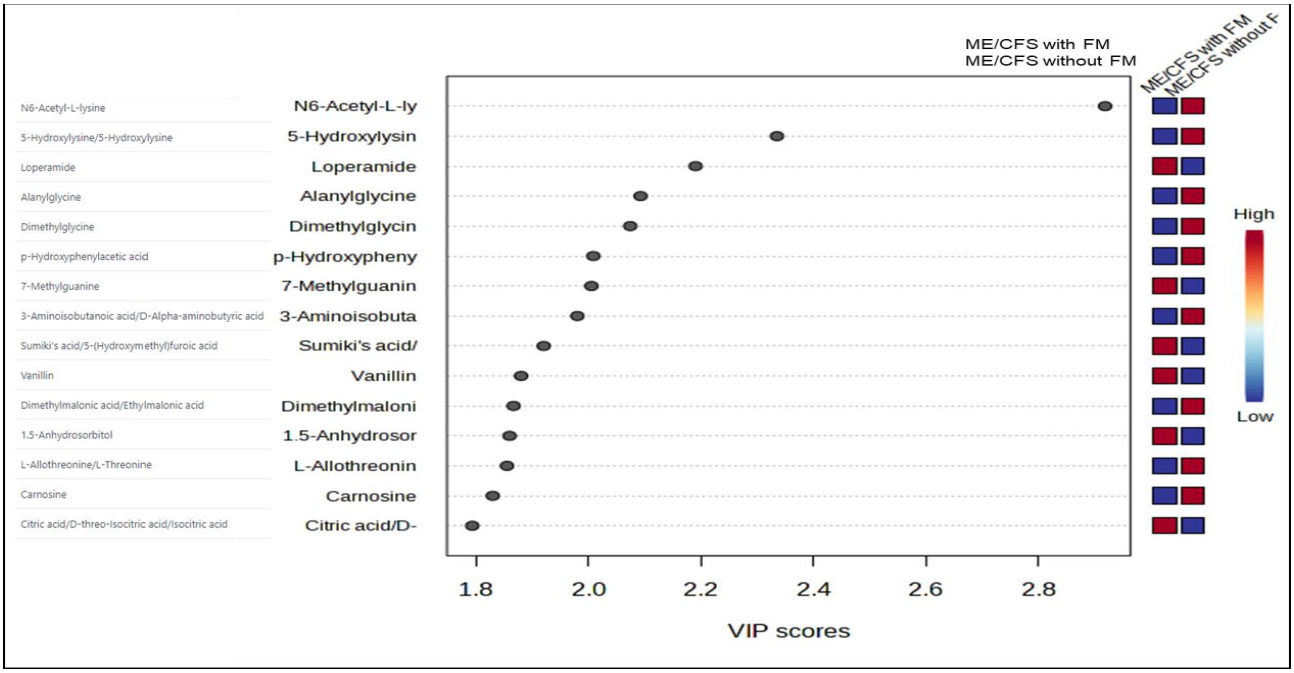



**Figure 4.2.14** VIP scores of corresponding PLSDA for LCMS Urine Part 1 ME/CFS with FM versus ME/CFS without FM

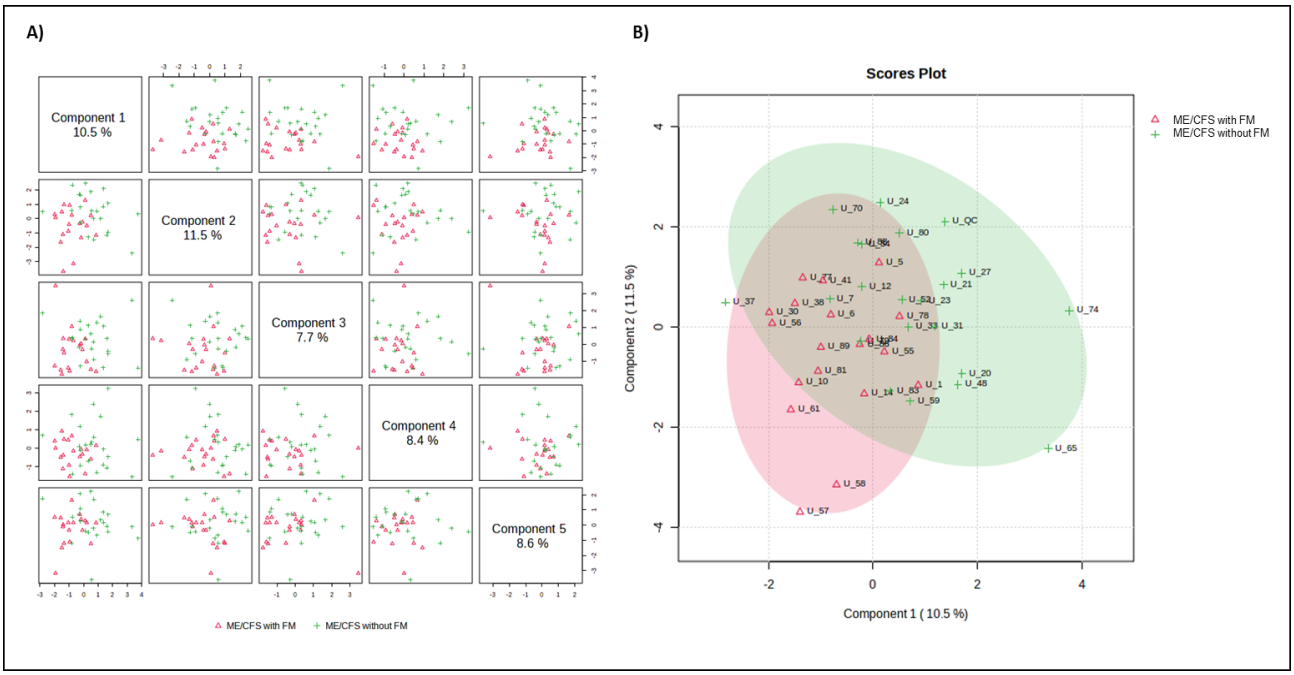



**Figure 4.2.15** PLSDA A) overview B) components 1 and 2 only of NMR Urine Part 1 ME/CFS with FM versus ME/CFS without FM

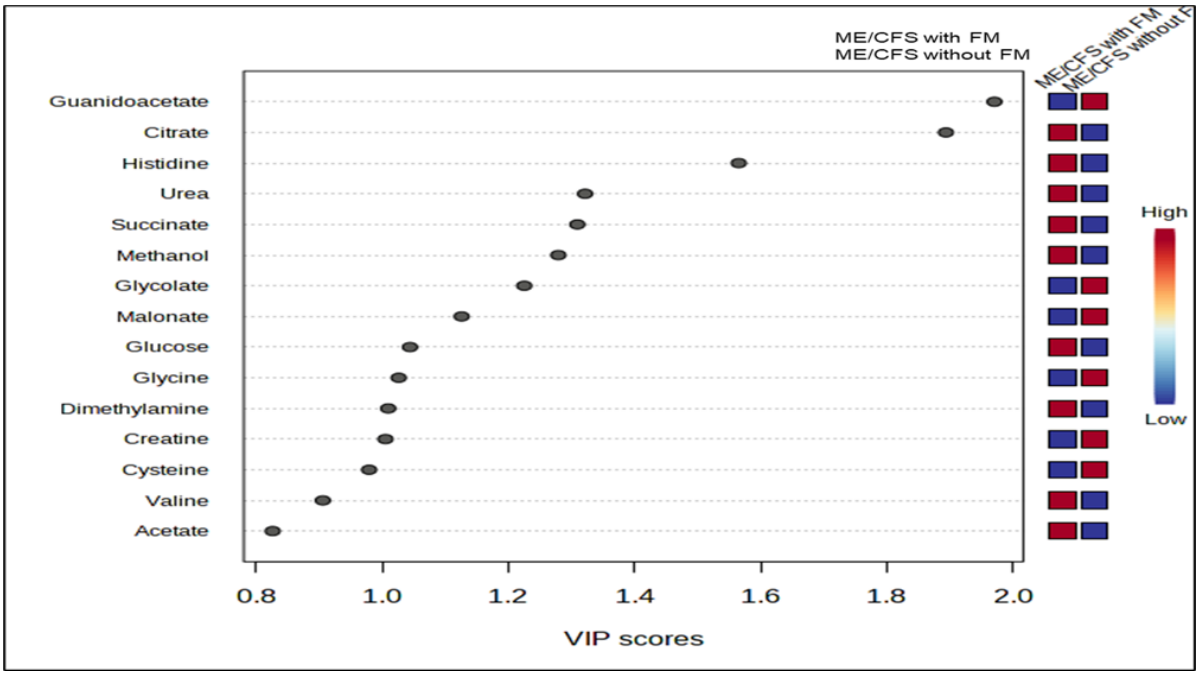
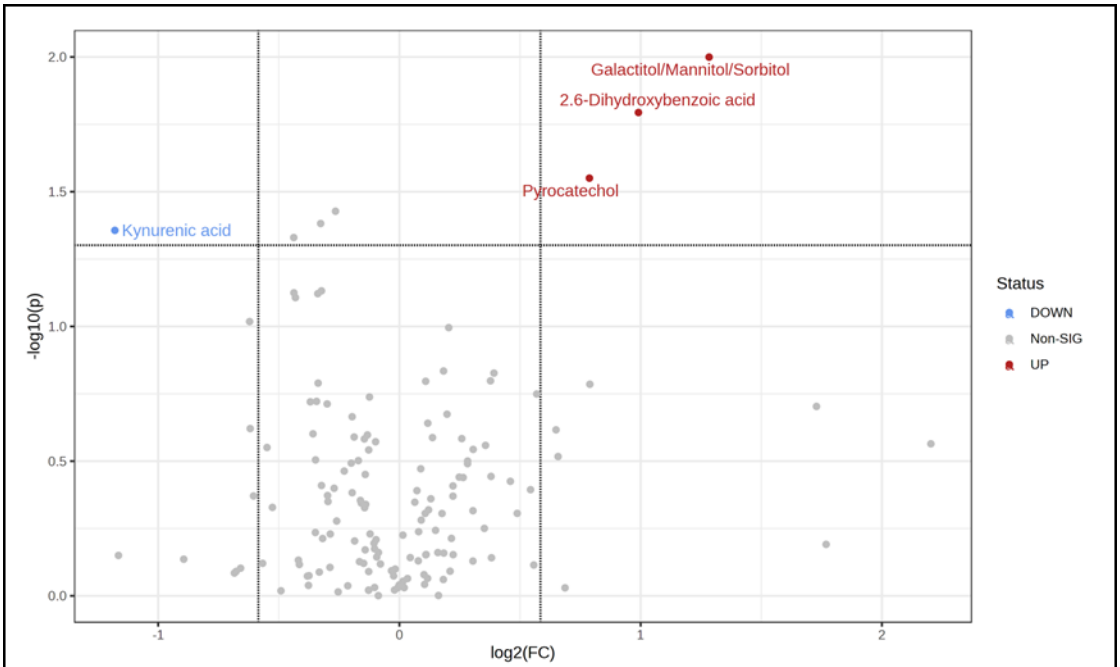



**Figure 4.2.16** VIP scores of corresponding PLSDA for NMR Urine Part 1 ME/CFS with FM versus ME/CFS without FM

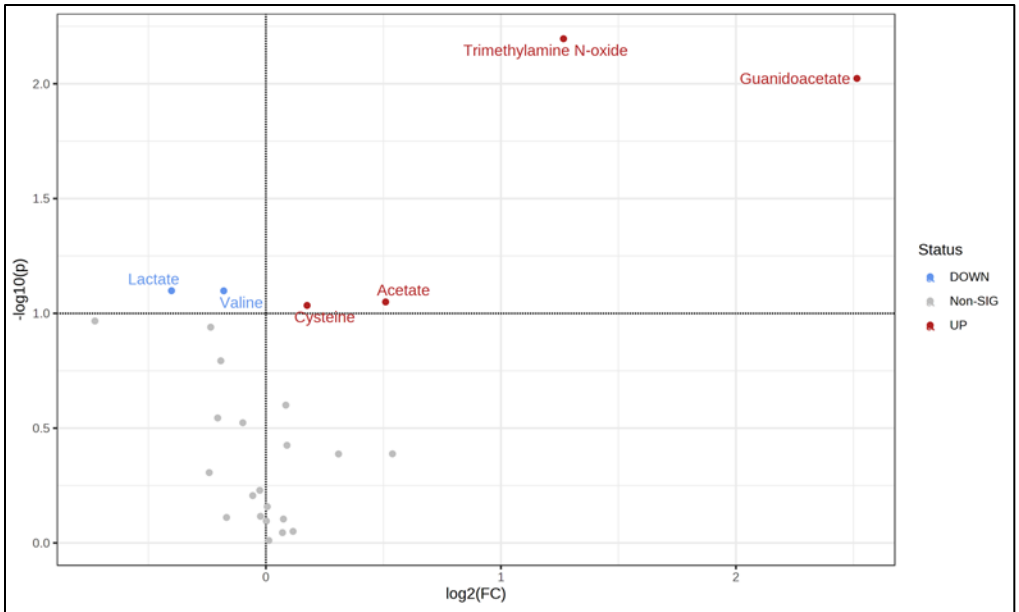
### 4.2.3 Urine Comparison C) ME/CFS with IBS vs. ME/CFS without IBS

#### 4.2.3.1 Univariate Analysis




| | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|
| Galactitol/Mannitol/Sorbitol | 2.4346 | 1.2837 | 0.010007 | 1.9997 |
| 2.6-Dihydroxybenzoic acid | 1.9873 | 0.99078 | 0.016066 | 1.7941 |
| Pyrocatechol | 1.7262 | 0.78756 | 0.028159 | 1.5504 |
| Kynurenic acid | 0.4415 | -1.1795 | 0.043998 | 1.3566 |

**Figure 4.2.17** Volcano plot of LCMS Urine Part 1 ME/CFS with IBS versus ME/CFS without IBS. Metabolites with significantly higher (red) or lower (blue) concentrations in the ME/CFS with IBS cohort are highlighted (FC threshold 1.5 and uncorrected p-values ≤ 0.05) with non-significant metabolites represented in grey.




| | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|
| Trimethylamine N-oxide | 2.4054 | 1.2663 | 0.006367 | 2.1961 |
| Guanidoacetate | 5.7154 | 2.5149 | 0.009476 | 2.0234 |
| Lactate | 0.75698 | -0.40167 | 0.079688 | 1.0986 |
| Valine | 0.88296 | -0.17957 | 0.079783 | 1.0981 |
| Acetate | 1.4234 | 0.50937 | 0.089189 | 1.0497 |
| Cysteine | 1.1291 | 0.17518 | 0.092353 | 1.0345 |

**Figure 4.2.18** Volcano plot of NMR Urine Part 1 ME/CFS with IBS versus ME/CFS without IBS. Metabolites with significantly higher (red) or lower (blue) concentrations in the ME/CFS with IBS cohort are highlighted (FC threshold 1.0 and uncorrected p-values ≤ 0.10) with non-significant metabolites represented in grey.

### 4.2.3.2 Multivariate Analysis (PCA)

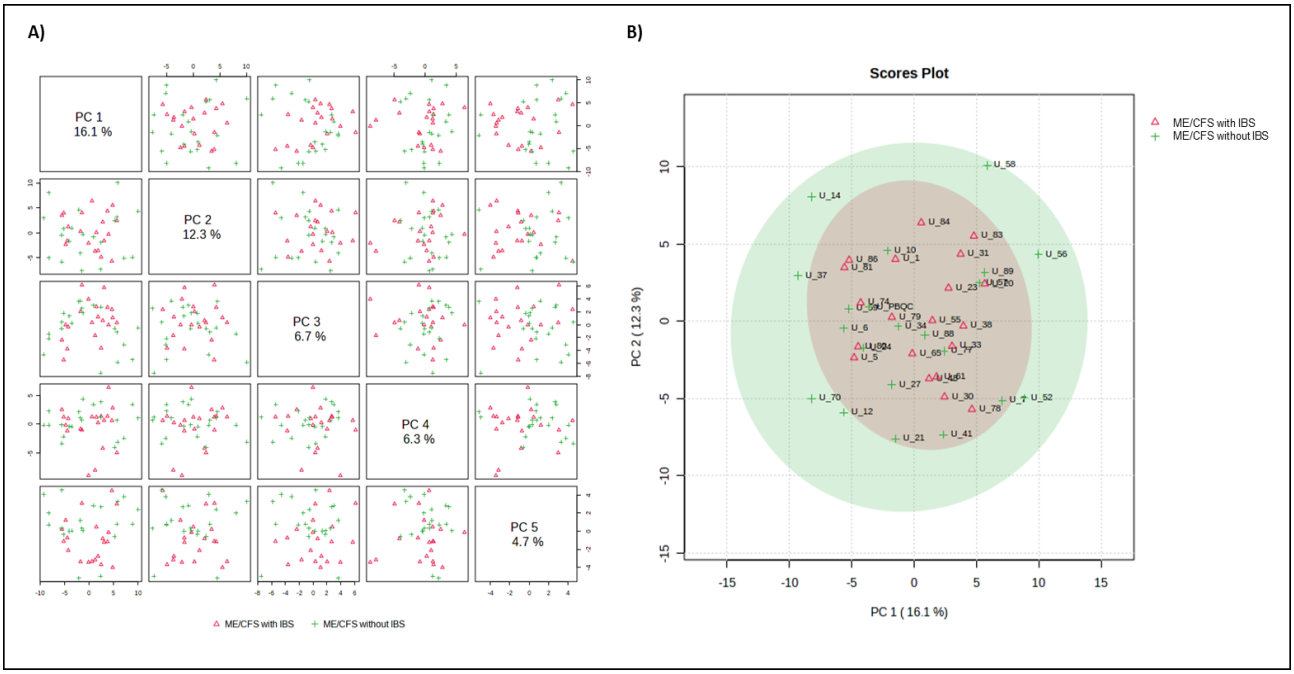



**Figure 4.2.19** PCA plots A) overview B) PC1 and PC2 only of LCMS Urine Part 1 ME/CFS with IBS versus ME/CFS without IBS

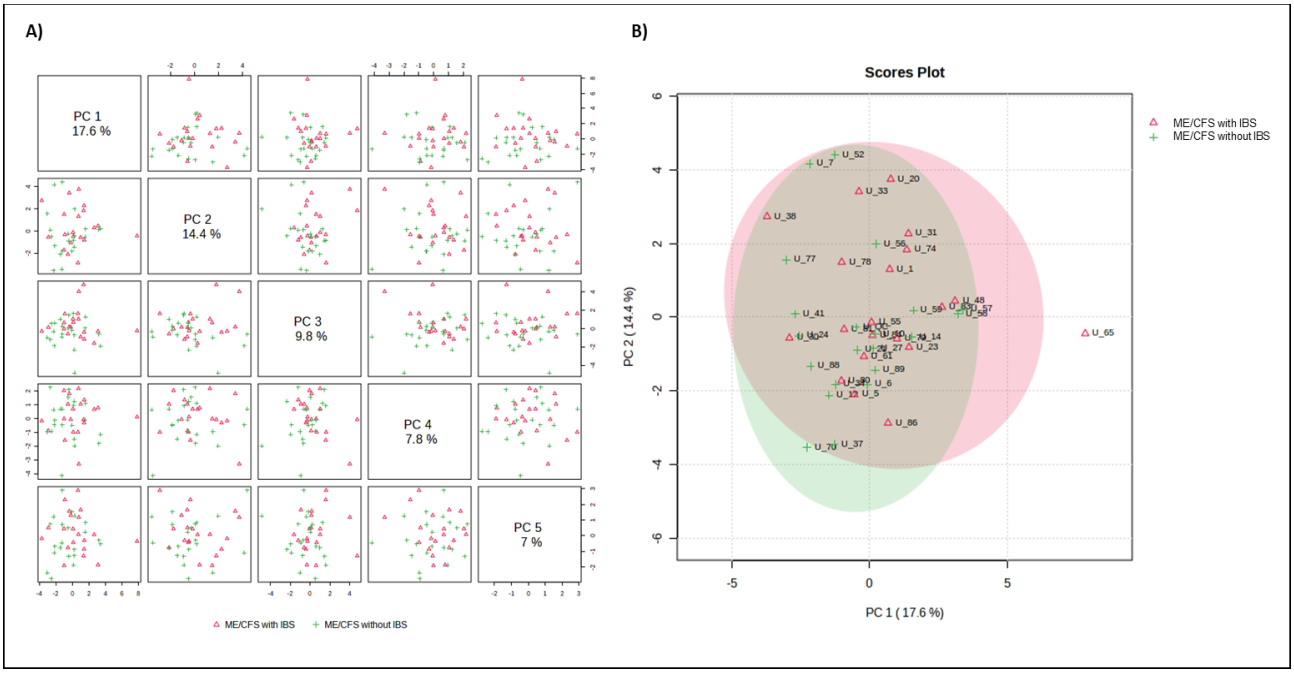



**Figure 4.2.20** PCA plots A) overview B) PC1 and PC2 only of NMR Urine Part 1 ME/CFS with IBS versus ME/CFS without IBS

### 4.2.3.3 Multivariate Analysis (PLS-DA)

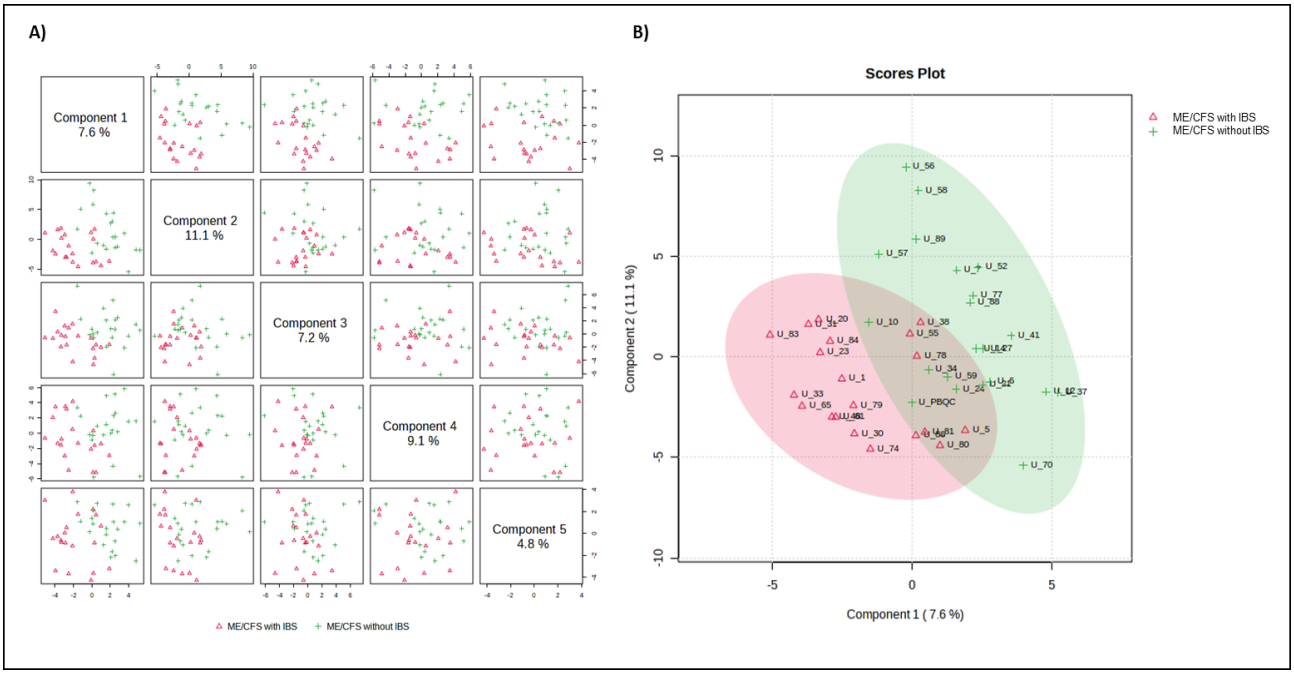



**Figure 4.2.21** PLSDA A) overview B) components 1 and 2 only of LCMS Urine Part 1 ME/CFS with IBS versus ME/CFS without IBS

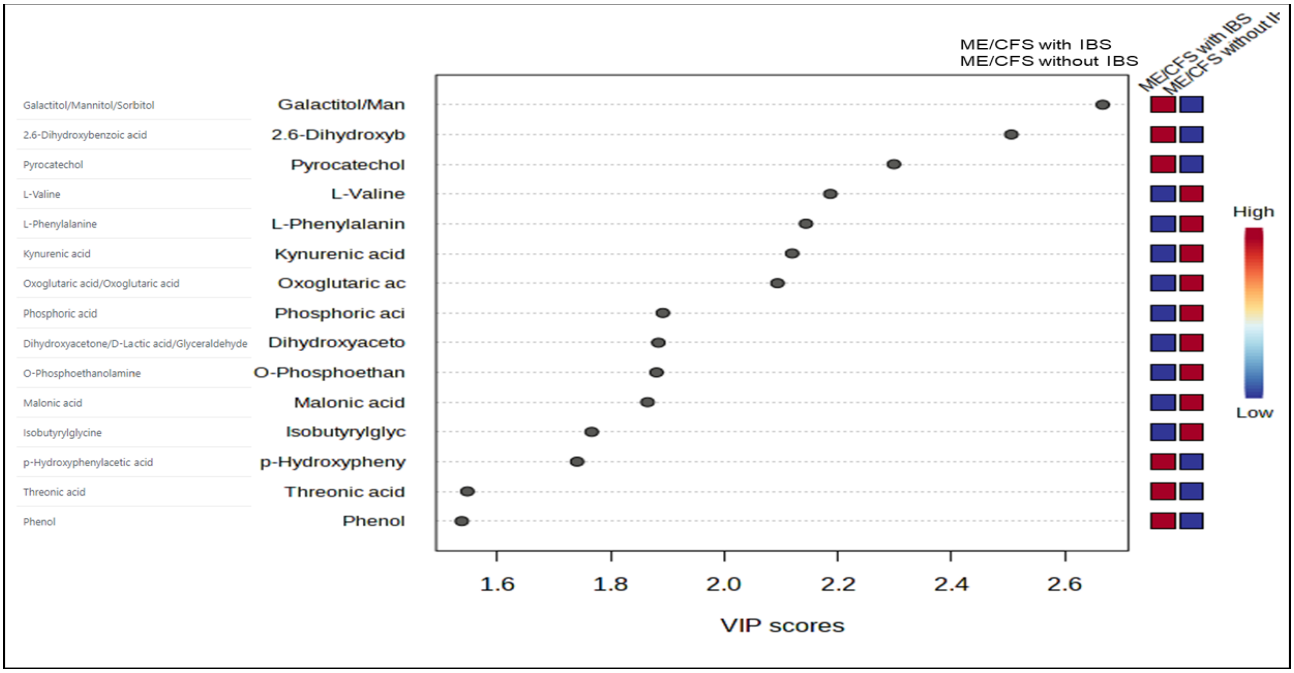



**Figure 4.2.22** VIP scores of corresponding PLSDA for LCMS Urine Part 1 ME/CFS with IBS versus ME/CFS without IBS

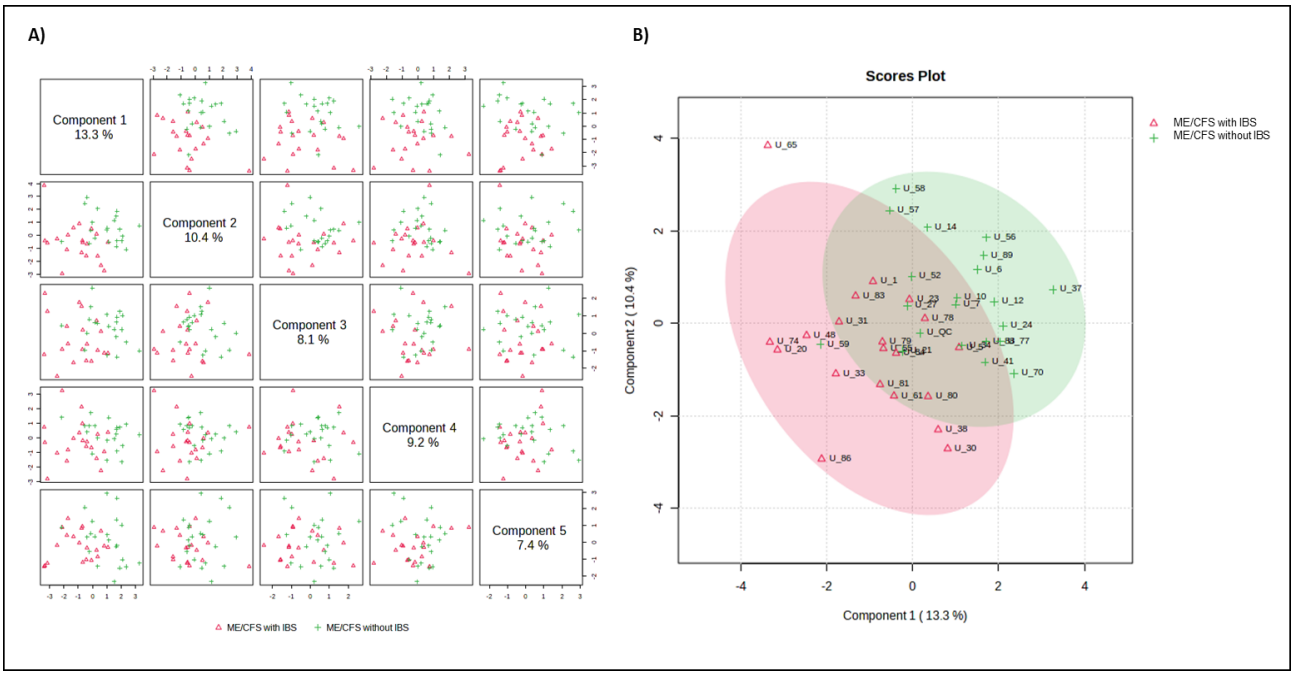



**Figure 4.2.23** PLSDA A) overview B) components 1 and 2 only of NMR Urine Part 1 ME/CFS with IBS versus ME/CFS without IBS

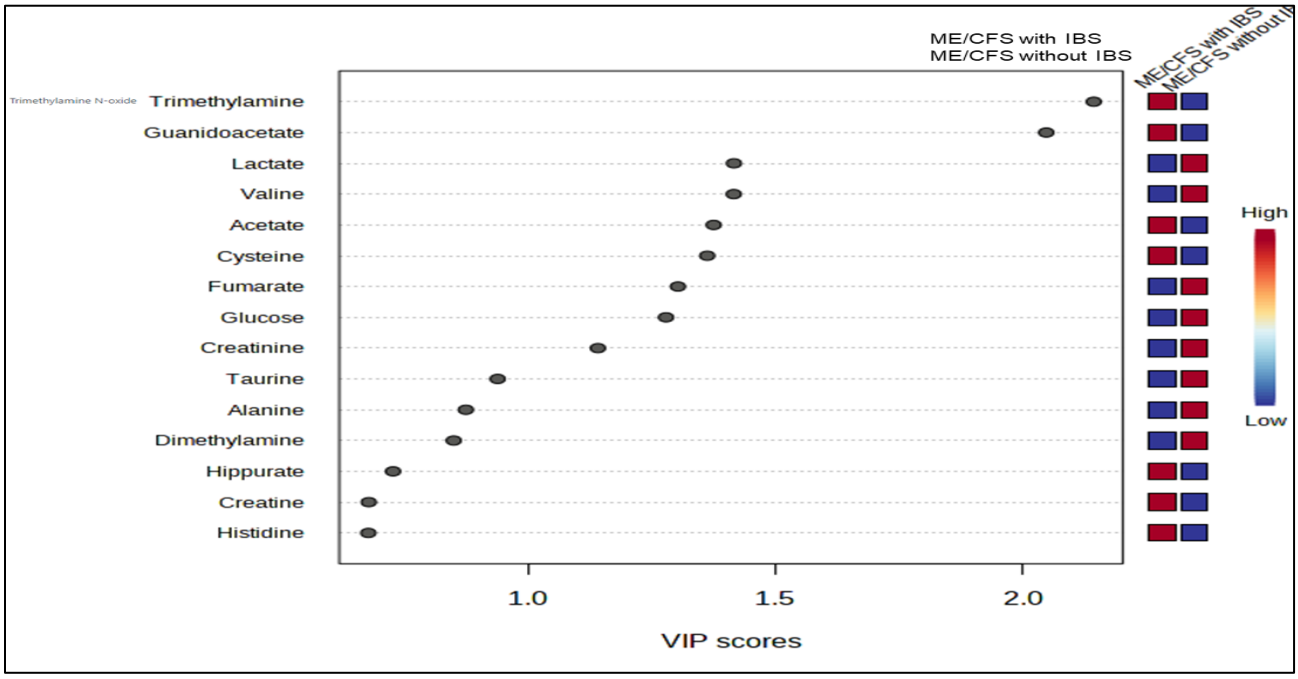
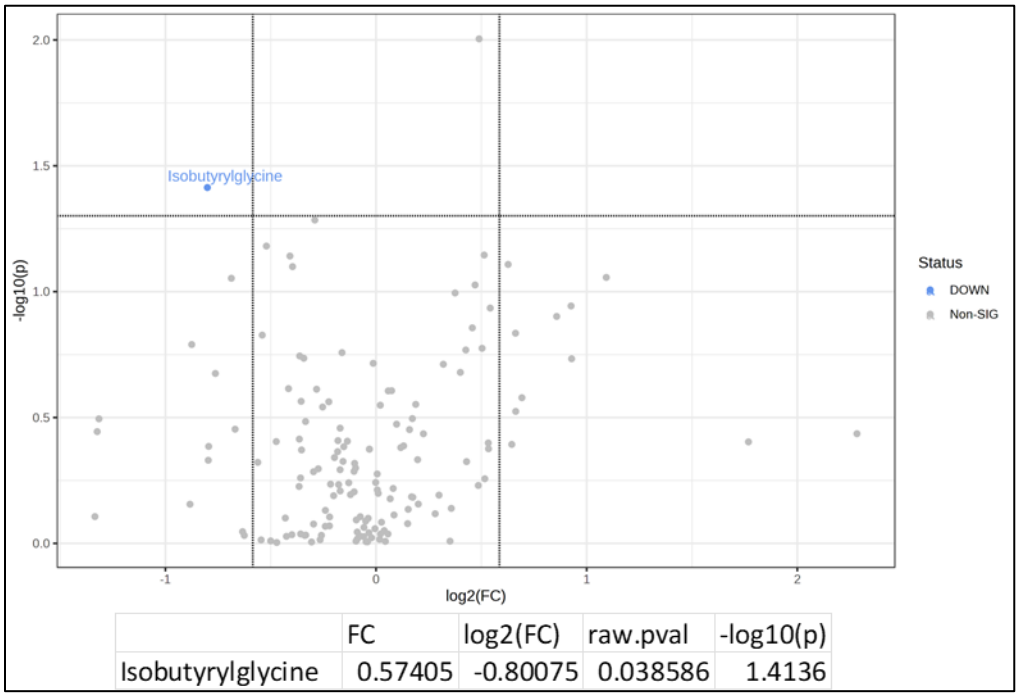



**Figure 4.2.24** VIP scores of corresponding PLSDA for NMR Urine Part 1 ME/CFS with IBS versus ME/CFS without IBS

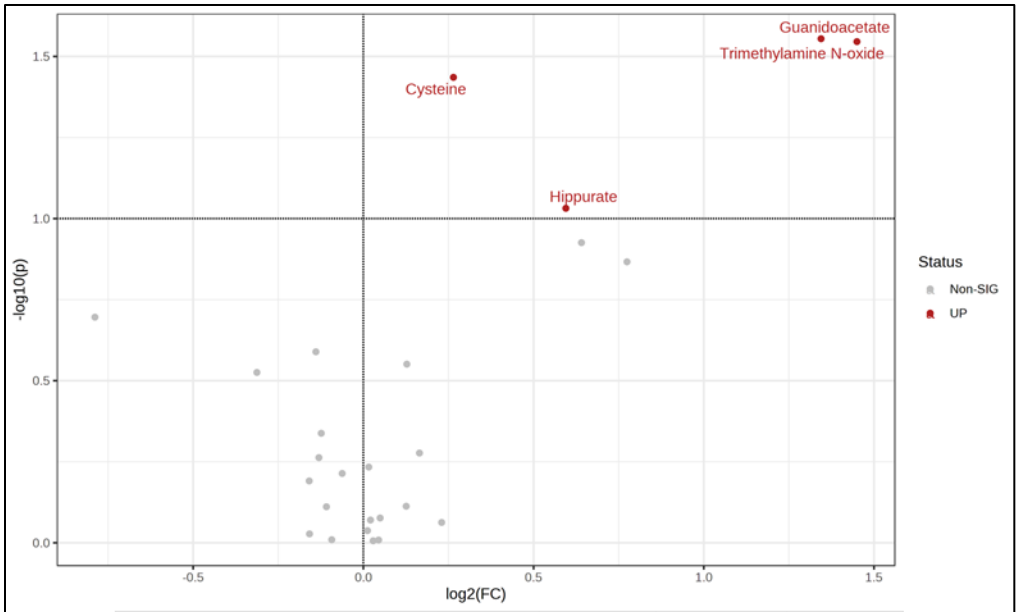
### 4.2.4 Urine Comparison D) Age-matched ME/CFS with IBS vs. ME/CFS without IBS

#### 4.2.4.1 Univariate Analysis




| | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|
| Isobutyrylglycine | 0.57405 | -0.80075 | 0.038586 | 1.4136 |

**Figure 4.2.25** Volcano plot of LCMS Urine Part 1 age-matched ME/CFS with IBS versus ME/CFS without IBS. Metabolites with significantly higher (red) or lower (blue) concentrations in the age-matched ME/CFS with IBS cohort are highlighted (FC threshold 1.5 and uncorrected p-values ≤ 0.05) with non-significant metabolites represented in grey.




| | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|
| Guanidoacetate | 2.5393 | 1.3444 | 0.027889 | 1.5546 |
| Trimethylamine N-oxide | 2.732 | 1.4499 | 0.02846 | 1.5458 |
| Cysteine | 1.2013 | 0.26461 | 0.036673 | 1.4356 |
| Hippurate | 1.5103 | 0.59482 | 0.092967 | 1.0317 |

**Figure 4.2.26** Volcano plot of NMR Urine Part 1 age-matched ME/CFS with IBS versus ME/CFS without IBS. Metabolites with significantly higher (red) or lower (blue) concentrations in the age-matched ME/CFS with IBS cohort are highlighted (FC threshold 1.0 and uncorrected p-values ≤ 0.10) with non-significant metabolites represented in grey.

### 4.2.4.2 Multivariate Analysis (PCA)

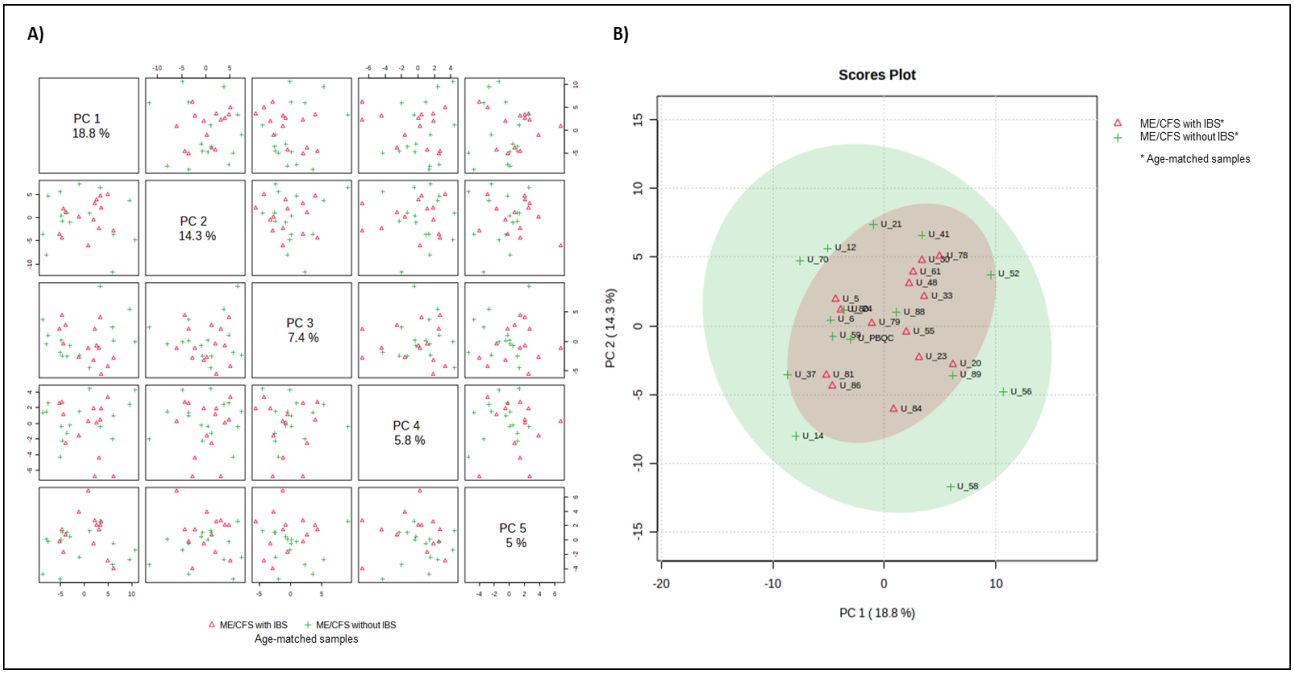



**Figure 4.2.27** PCA plots A) overview B) PC1 and PC2 only of LCMS Urine Part 1 age-matched ME/CFS with IBS versus ME/CFS without IBS

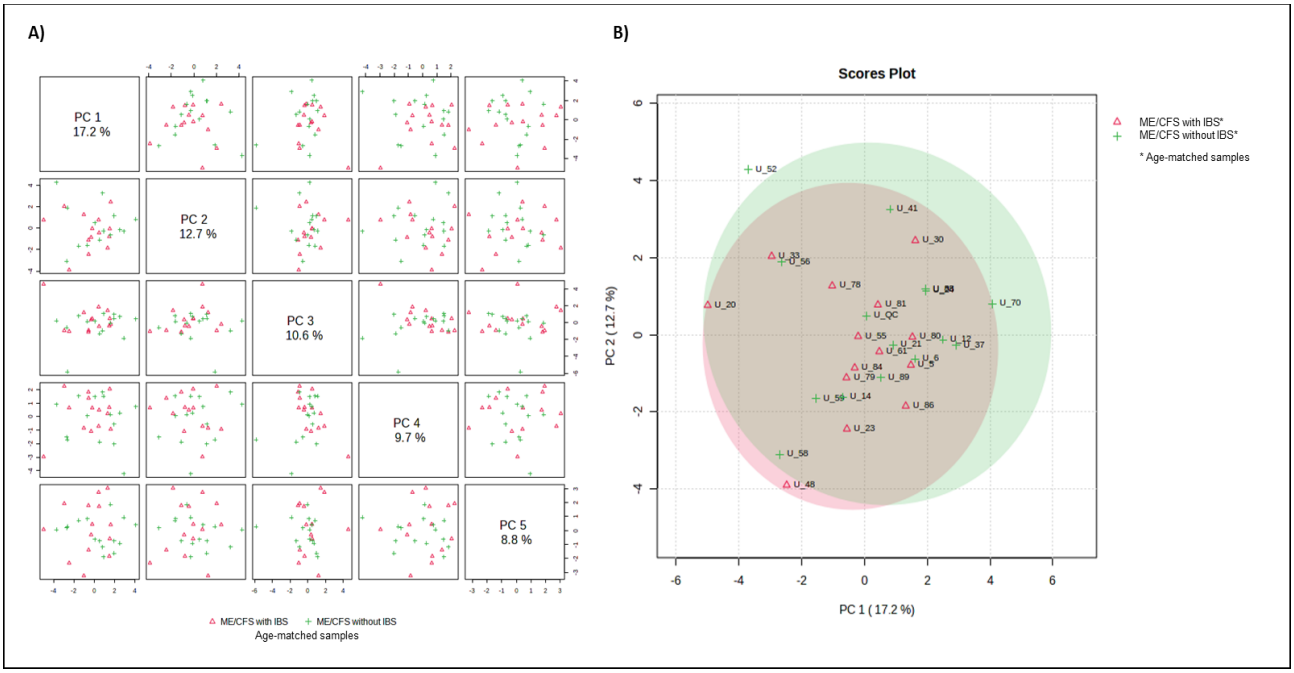



**Figure 4.2.28** PCA plots A) overview B) PC1 and PC2 only of NMR Urine Part 1 age-matched ME/CFS with IBS versus ME/CFS without IBS

### 4.2.4.3 Multivariate Analysis (PLS-DA)

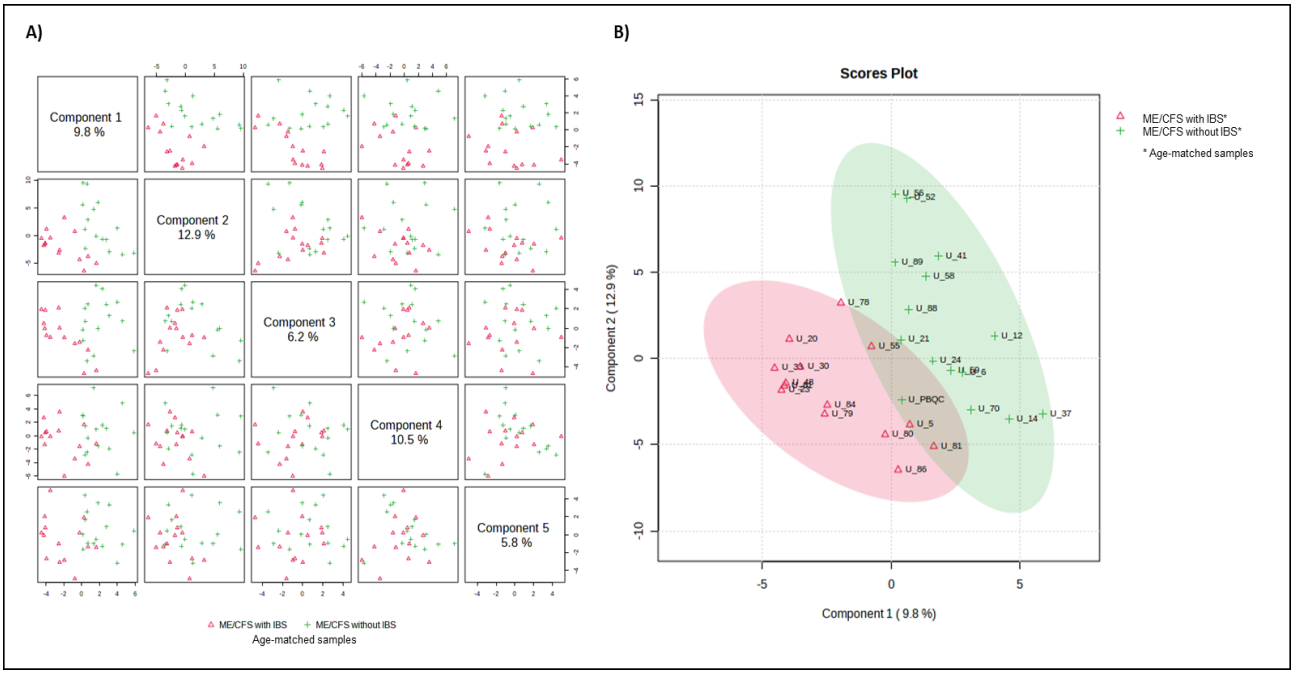



**Figure 4.2.29** PLSDA A) overview B) components 1 and 2 only of LCMS Urine Part 1 age-matched ME/CFS with IBS versus ME/CFS without IBS

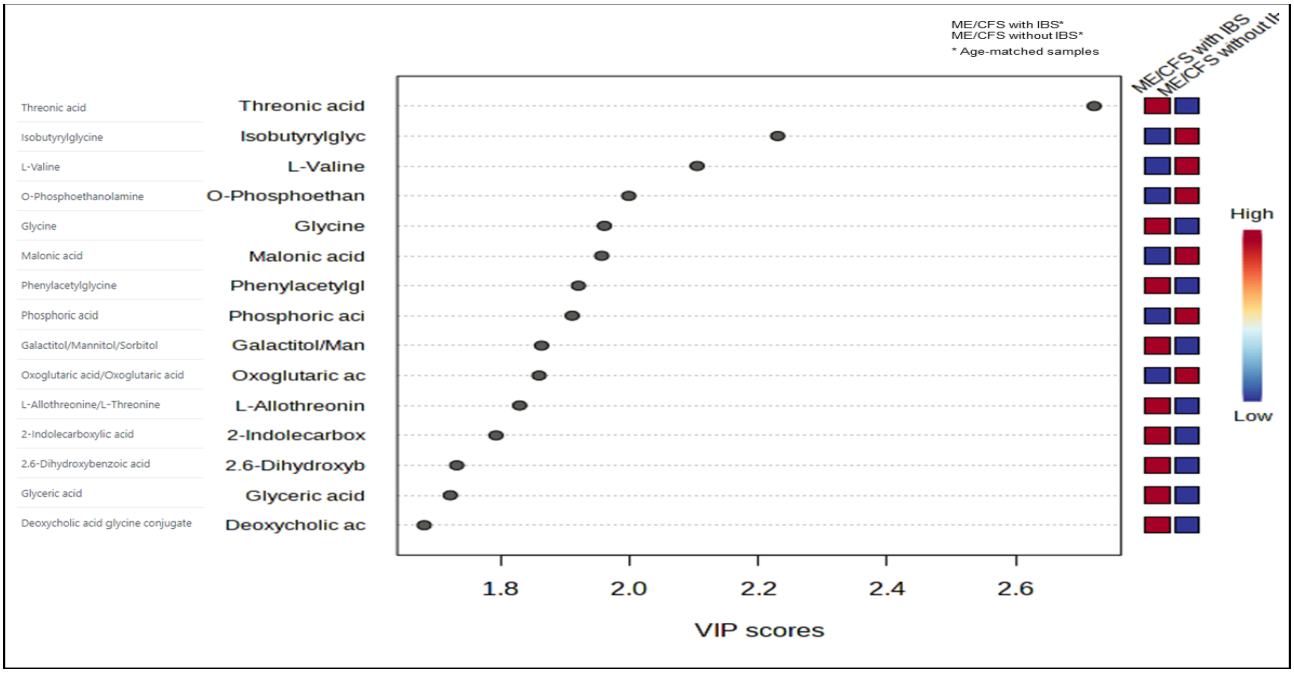



**Figure 4.2.30** VIP scores of corresponding PLSDA for LCMS Urine Part 1 age-matched ME/CFS with IBS versus ME/CFS without IBS

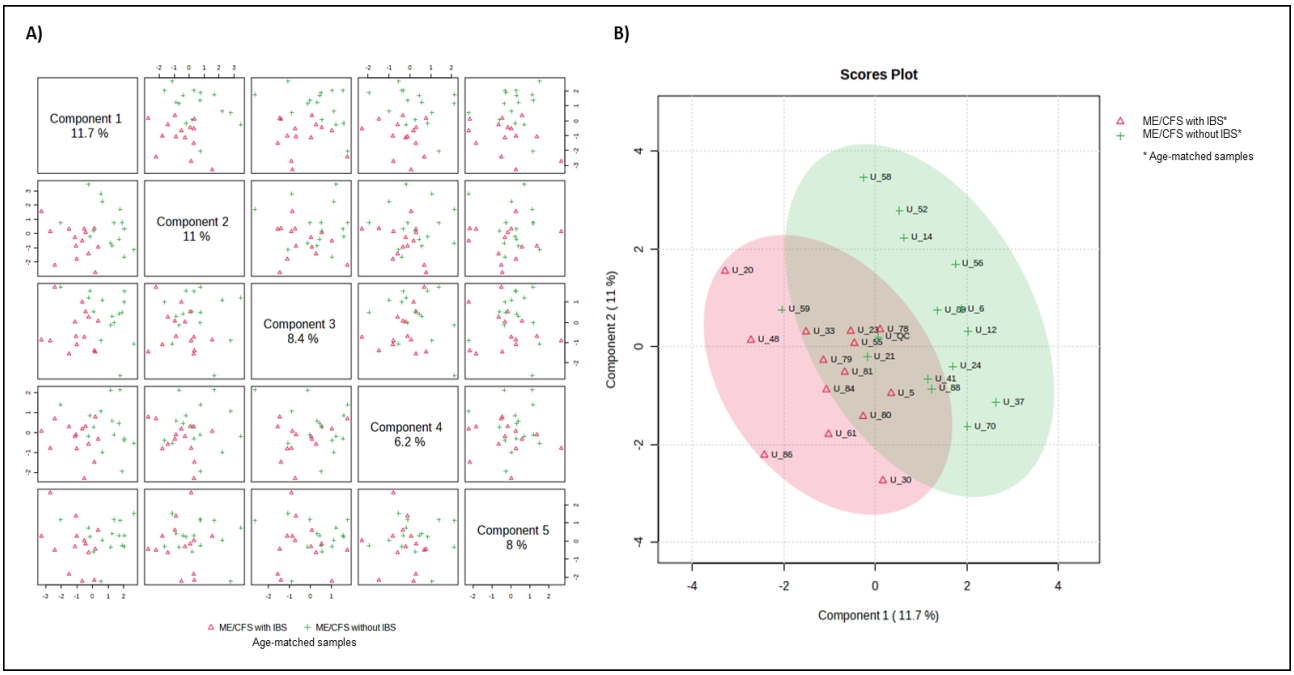



**Figure 4.2.31** PLSDA A) overview B) components 1 and 2 only of NMR Urine Part 1 age-matched ME/CFS with IBS versus ME/CFS without IBS

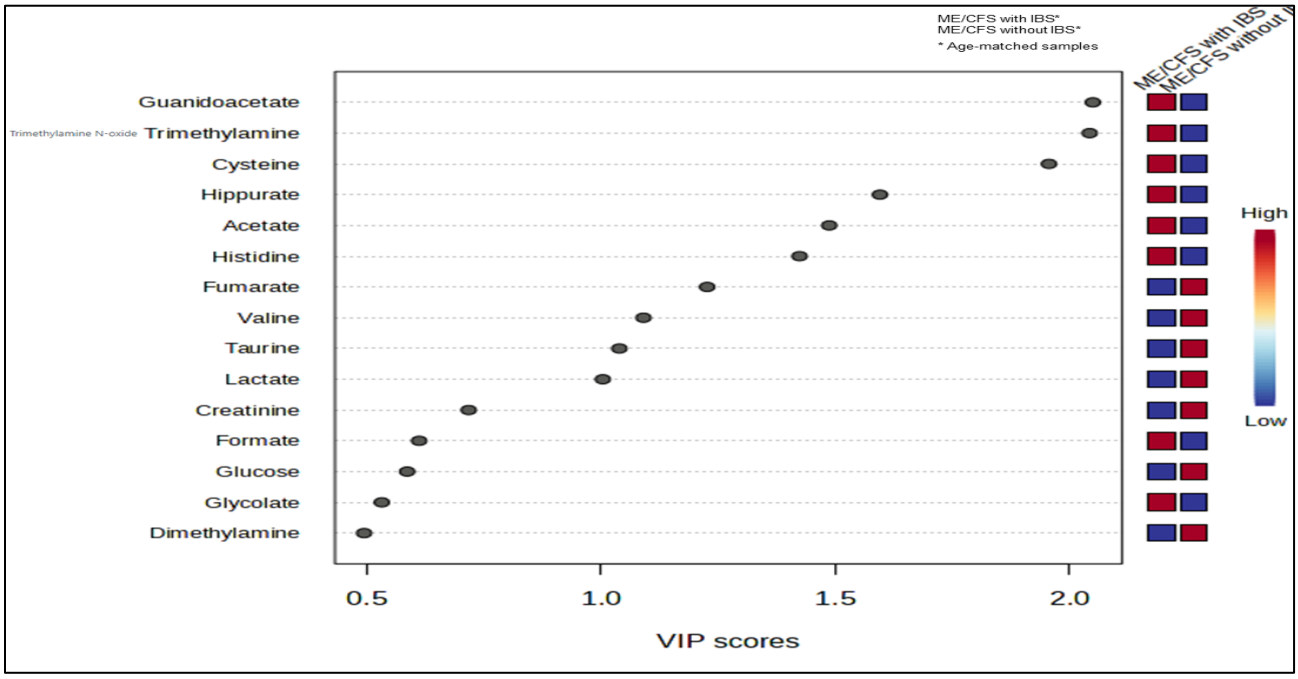



**Figure 4.2.32** VIP scores of corresponding PLSDA for NMR Urine Part 1 age-matched ME/CFS with IBS versus ME/CFS without IBS

## 4.3 Faeces and Urine Metabolomics Discussion

### 4.3.1 Previous Metabolomic Studies in ME/CFS

This chapter presents the faecal and urinary metabolomic outcomes with polar metabolites from Part One. Chapter 5 presents the plasma and urine outcomes from Part Two. Due to the similarity of many discussion points between the two chapters, they will mostly be covered here. To date, there have been a growing number of ME/CFS metabolomic studies published although inconsistences and lack of reproducibility remain an issue. Like the gut microbiome studies, no certain pathophysiological mechanisms or disease signature(s) via the use of metabolomics has been found (Huth et al., 2020; Maksoud et al., 2023; Taccori et al., 2023; Yamano et al., 2021). Again, heterogeneity across the board from the complexity of ME/CFS, the individuality of the participants, participant selection, sample types assayed, platforms used, and other analytical methodological variations, make it difficult to validate and draw consistent observations and fair comparisons. On this note, it is worth noting and clarifying that metabolomics is not a "contributor to the pathogenesis of ME/CFS" as the systematic review from Huth et al. (2020) have implied in their conclusions on the state of evidence for metabolomic dysregulation in ME/CFS (Huth et al., 2020). Instead, metabolomics should be viewed as an attractive field and domain capable of providing more unified and system-wide perspectives of the biology and processes at play in ME/CFS. While inconsistent in their specific details, metabolomics in ME/CFS has demonstrated the similarity of overall potential molecular pathophysiological changes, disturbances, and biological issues.

Human blood (plasma and serum) and urinary metabolomics have been the most used sample types in ME/CFS studies. The publications by Glass et al. (2023), Germain et al. (2022), Huth et al. (2023), Taccori et al. (2023) and Yamano et al. (2021) provide a more extensive list of these ME/CFS studies from several labs across the world. Already, without considering any of the other potential factors, there is a potential source of discrepancy among studies, including the work from this study. In metabolomics research blood plasma and serum have been considered to possess similar compositions and properties; their perceived equivalence has resulted in researchers choosing arbitrarily between the two for analysis (L. Liu et al., 2010). They are both used to evaluate various biochemical parameters in medical science and in the study and diagnosis of disease. Currently, there is no consensus on which blood sample matrix type is preferable for metabolomics. However, differences in metabolite profiles have been shown

between the two (Huang et al., 2022; L. Liu et al., 2010; X. Liu et al., 2018). This caveat is something to be mindful of when evaluating blood metabolomic outcomes and planning any future studies.

Metabolomics with faeces and other materials like cerebrospinal fluid and saliva have been much less common in ME/CFS. While faecal material has widely been used with the gut microbiome studies in ME/CFS with shotgun metagenomics and 16S rRNA sequencing work, they do not appear much in metabolomics. Even where a multi-omic focused study design has been implemented to investigate the gut microbiome-host metabolome relationship, the faeces do not necessarily go through a metabolomic platform and workflow. A search of "faecal/stool metabolomics in ME/CFS" yields studies from Armstrong et al. (2017) and Lupo et al. (2021), and one thesis by Daniel Vipond (2018) (Vipond, 2018). Guo et al. (2023) also performed some faecal metabolomics with their cohort; however, only short chain fatty acids were profiled. For the faecal metabolomic data outcome alone, this study contributes to ME/CFS research. So far in ME/CFS, any NMR-based metabolomics work is mainly by Armstrong, McGregor, and colleagues from Melbourne, Australia (Armstrong et al., 2012, 2015, 2017; McGregor et al., 2016, 2019). Other groups have produced metabolomics work that are MS-based. As far as I am aware, this is the first ME/CFS study that has utilised both the LCMS and NMR platforms for metabolomics in urine, plasma, and faeces (Huth et al., 2020; Taccori et al., 2023; Yamano et al., 2021). Using both platforms has allowed the coverage of metabolites to be broadened compared to using a NMR-only approach and provided some confirmation of what is observed from the LCMS outcomes.

While it is not necessarily the fairest comparison, as the details of the methodologies involved are not the same as other ME/CFS metabolomic studies, the number of LCMS metabolites is comparable to targeted metabolomic profiles (not untargeted), and NMR metabolites for faeces and blood is an improvement as far as total number of metabolites that were obtained from the respective 1D participant sample and 2D confirmation spectra. See Section 2.1.2 and 2.8.2.4 regarding improvements and modifications sought for NMR work; instead of 24 faecal, 30 urine, and 29 blood metabolites, this study found 36, 26, and 47, respectively. The challenges of NMR metabolomics with urine are acknowledged, and future work with current raw spectra and any other NMR-based assays would look to improve these outcomes (Bouatra et al., 2013; A. H. Emwas et al., 2015). Further, the assay of two biospecimen types from the same time-point in Part One and Two of this study is a contribution. Many studies only utilised or considered one

biospecimen type for their metabolomics analysis, except for the work by Armstrong and colleagues, although they only used NMR-based metabolomics. The remainder of the discussion considers the biospecimen types separately and together in terms of what they could explain in the context of ME/CFS, the FM and IBS comorbidities, and the gut microbiome-host metabolome relationship.

### 4.3.2 Variations in Faecal Metabolome

The faecal metabolome provides a functional readout of microbial activity and can be used as an intermediate phenotype mediating host-microbiome interactions (Zierer et al., 2018). Faecal metabolomics has increasingly gained attention and shown promising results in characterising microbial metabolic functions and the gastrointestinal system (Karu et al., 2018; Lamichhane et al., 2018). The faecal metabolites were characterised by LCMS and NMR from ME/CFS and non-ME/CFS participants in Part One. The results revealed alterations in these metabolites across the comparative groups of interest (Comparison A-D). Some metabolites could explain the difference between the overall ME/CFS versus Control groups (Comparison A). With the same LCMS and NMR data matrices; Comparison C-D looked at the FM and IBS comorbidities in the ME/CFS cohort without the non-ME/CFS participants involved. The presence or absence of these comorbidities in the ME/CFS cohort revealed several patterns in the faecal metabolites that suggest potential heterogeneity in relevant metabolic activity, processes, and host interplay.

There were faecal metabolite features that were highlighted across several of the comparisons which showed inconsistent trends in their relative concentration depending on the comorbidity grouping. There were eight metabolites, for example, nicotinic acid and butyric acid, that were elevated in both the Control group from Comparison A and in the groups from Comparison C-D which are ME/CFS participants overall that have been defined according to their FM and IBS metadata. Glutamate showed a contrasting trend in the presence or absence of FM and IBS comorbidities where it was elevated in ME/CFS - FM (Comparison B) and ME/CFS + IBS** (Comparison D). Further, metabolites elevated in the ME/CFS overall group of Comparison A and C-D (ME/CFS only participants) revealed another notable variation in trend. For example, succinate was elevated in the ME/CFS overall group (Comparison A) but when looking at Comparison C-D with the ME/CFS participants, it was elevated in ME/CFS - FM (Comparison B), and ME/CFS + IBS and ME/CFS + IBS** (Comparison C and D). All these incongruent or

interesting trends and patterns indicated that faecal metabolite characteristics may be different depending on the FM or IBS comorbidity and how participants are grouped.

There were some more intriguing observations where metabolites were significantly elevated in both the Control group from Comparison A, and at least one of the groups from Comparison B-D in the ME/CFS cohort. Regardless of the comparative group and the direction of the relative concentration being elevated, the metabolites and categories of metabolites identified have often been cited and discussed in the literature at the intersection of the gut microbiome and many health issues and chronic diseases. This includes short chain fatty acids, organic acids, branched chain amino acids, and bile acids (Agus et al., 2021; Fassarella et al., 2021). In addition to the previous ME/CFS microbiome studies, these findings provided further evidence and support of the gut microbiome and comorbidities having a critical role in ME/CFS. Further, the usefulness of faecal metabolomics with taxonomic and predicted functional findings has been demonstrated in the attempt to understand (the remainder of this section) what may be going on in ME/CFS heterogeneity and pathophysiology.

ME/CFS research follows the general research trend that has seen more urine and plasma metabolomics studies than faecal ones. Armstrong et al. (2017), using NMR absolute concentration values, identified that valerate and isovalerate were increased and lactate was decreased in ME/CFS patients. When using relative concentration values, they found that valerate and isovalerate were again increased along with an increase of butyrate in their ME/CFS group (Armstrong et al., 2017). This study observed the same trend with isovalerate in the overall ME/CFS group; however, valerate was only elevated in the ME/CFS - FM. By contrast, with LCMS and NMR, butyrate in this study showed that relative concentration trends were increased in the Control (decreased in the overall ME/CFS) group from Comparison A and ME/CFS + IBS** from Comparison D. A comparison on NMR relative concentrations is difficult to draw as calculations were based on a total of 24 (Armstrong et al. 2017) and 36 faecal metabolites (this study). Lupo et al. (2021) profiled a subgroup of their cohort revealed high levels of glutamic acid and arginiosuccinic acid, together with a decrease of alpha-tocopherol in ME/CFS. This selection of participants was five ME/CFS individuals which were found to belong to the same cluster at a family taxonomic level, and five of their matched relatives as a control. Arginiosuccinic acid did not appear in any of the faecal metabolomic assays, and glutamic acid and alpha tocopherol only appeared elevated in certain comparisons.

More recently, Guo et al. (2023) used a GCMS metabolomics workflow that measured faecal short chain fatty acids (SCFAs), acetate, butyrate, and propionate. Where a ME/CFS overall versus Control group comparison is considered, their study and results here (LCMS and NMR with the same trends for butyrate and propionate) present similar observations with the acetate and butyrate levels being lower in ME/CFS, and propionate not being significantly different. However, where a stratification of the IBS comorbidity is compared in the ME/CFS cohort, their study and this study showed differences in trends across all three SCFAs. Instead of ME/CFS + IBS being reduced, this study observed an increased trend in relative concentration. That is, ME/CFS + IBS from this study was observed to be lower in trend for these faecal SCFAs, albeit with some difference in study and data approaches. While these trends were observed from the VIP analyses, they weren't by the VP (Section 4.1.3) indicating that changes in concentrations, not just trends need to be considered. As mentioned in Section 4.3.4, Comparison B-D requires the additional consideration of the non-ME/CFS cohort as a third group with univariate analyses to determine if these findings are associated with ME/CFS overall or dependent on IBS comorbidity. The importance of propionate being flagged as important only in Comparison C and D with IBS stratified is of mention too. Further, these SCFAs did not appear as an important metabolite when the ME/CFS cohort for Comparison B was stratified according to FM. Independently co-published in the same issue as Guo and colleagues, Xiong and colleagues presented a multi-omics study of gut microbiome-host interactions in ME/CFS patients (Xiong et al., 2023). Although they did not perform faecal metabolomics, their shotgun metagenomic analyses observed a reduced genomic functional capacity for butyrate production in the ME/CFS microbiome of their early-stage patients. Despite the differences with ME/CFS study designs that have looked at faecal metabolites in some way, and some of their overall findings, the results presented here support and align with the theme of subtle but not trivial heterogeneity, gut microbiome disturbances, functional consequences of dysbiosis, and pathophysiological issues in ME/CFS.

Short chain fatty acids, of which acetate, propionate, and butyrate are the most abundant, are important metabolites in maintaining intestinal homeostasis (Venegas et al., 2019). Propionic acid is likely to be associated with Bacteroides uniformis, Alistipes finegoldii, and Bacteroides thetaiotaomicron (Figure 3.6.13). Butyric acid is likely to be associated with Alistipes finegoldii, and Bacteroides thetaiotaomicron (Figure 3.6.15). As the main end products of colonic bacterial fermentation, they participate in the maintenance of intestinal mucosa integrity, improve glucose and lipid metabolism, control energy expenditure, and regulate the immune system and

inflammatory responses (Agus et al., 2021; Venegas et al., 2019). Figure 3.6.3 and Figure 3.6.4 with PICRUSt2 provide some indication of this. Acetate and butyric acid showed a decrease in the ME/CFS overall group (Comparison A); however, an increased trend was also observed with ME/CFS + IBS/IBS** (Comparison C, D). Propionate was not observed as important in Comparison A; however, it came up as elevated in both ME/CFS + IBS/IBS** for Comparison C and D. Faecal SCFA deficiencies and alterations in the proportions of these metabolites have been observed in several IBS, (and different types of IBS) cases (Jiang et al., 2022; Q. Sun et al., 2019). Conversely, faecal SCFAs have also reported significantly higher levels of acetate, propionate, and total SCFAs compared to controls (Jiang et al., 2022; Q. Sun et al., 2019). Further investigation of these metabolite concentrations, ratios, IBS subtypes, gastrointestinal symptoms, and diet need to be considered. Just as there are no “good” or “bad” bacteria, there are probably no “good” or “bad” metabolites but rather how they exist with each other determines health and disease outcomes. Bacteroides thetaiotaomicron (Figure 3.4.15) are able to switch their metabolism to utilise host-derived glycans when dietary polysaccharides are scarce, showing a high metabolic flexibility, which also contributes to their persistence in the gut (Fassarella et al., 2021).

The theme of bile acids was identified in this study. As far as I am aware, other than the small study by Lupo et al. (2021) and the thesis by (Vipond, 2018) with small cohort numbers that did not stratify these comorbidities, faecal bile acids and derivatives have not been extensively measured in any ME/CFS study using faecal metabolomics. Other studies have discussed bile acid metabolism in their findings; however, they did not use faecal metabolomics. Plasma metabolomics has often been the sample type to discuss anything related to possible issues with bile acid metabolism (Germain et al., 2017, 2020, 2022; Nagy-Szakal et al., 2018; Naviaux et al., 2016; Xiong et al., 2023). The findings here present some updated perspective with a panel assayed from the LCMS faecal matrix, and loose corroboration of these studies to date and metabolic dysregulation in ME/CFS. Indeed, these metabolites observed would not have been possible if only NMR was used. Of all the metabolite categories presented, ME/CFS with or without FM did not appear elevated for any of the bile acids. Bile acids are steroid compounds which have a primary function for lipid and vitamin digestion, absorption and excretion; however, are also heavily involved in gut-microbiome host interplay (Agus et al., 2021; Collins et al., 2022). They are synthesised from cholesterol by the liver in a multi-enzyme process. 95% of them are reabsorbed actively from the terminal ileum and are recycled in the liver via enterohepatic circulation. Hence, decreased enterohepatic circulation could be an

indication of bile acid malabsorption that manifests as GI symptoms. Primary bile acids are also transformed into secondary bile acids and deconjugated by gut microbiota (Agus et al., 2021). They can either be passively reabsorbed to re-enter the circulation bile acid pool or excreted in the faeces (Agus et al., 2021). Not surprisingly, not only was there a difference between ME/CFS overall and Control, but observations were also made where IBS have been considered. Across all the bile acid metabolites identified as significantly different, the stratification with FM did not highlight any importance in the ME/CFS cohort. Bile acids in GI-related conditions, symptoms, and IBS has been extensively discussed. Individuals with IBS have reported altered microbial profiles and modified bile acid profiles (Collins et al., 2022; Min et al., 2022). Like SCFAs, and the need to consider IBS subtypes, the type of IBS has observed a variety of trends in faecal bile acids. A significant increase in faecal primary bile acid and a corresponding decrease in secondary bile acid has been observed in IBS with predominant diarrhoea (Min et al., 2022).

### 4.3.3 Variations in Urine Metabolome

There were urine metabolite features that were highlighted across several of the comparisons which showed inconsistent trends in their relative concentration depending on the comorbidity grouping. There were 12 metabolites, for example, alanine and cysteine that were elevated in both the Control group from Comparison A and in the groups from Comparison C-D which are ME/CFS participants overall that have been assigned according to their FM and IBS metadata. Creatine, p-Hydroxyphenylacetic acid, glycine, threonine, allothreonine, glycolate, and guanidoacetate showed a contrasting trend in the presence or absence of FM and IBS comorbidities where it was elevated in its relative concentration, i.e. if the metabolite was elevated in ME/CFS - FM (Comparison B), it was elevated in ME/CFS + IBS and/or IBS** (Comparison C and D). Further, metabolites elevated in the ME/CFS group of Comparison A and C-D (ME/CFS only participants) revealed another notable variation in trend. For example, glucose was elevated in the ME/CFS overall group (Comparison A) but when looking at Comparison C-D with the ME/CFS participants, it was elevated in ME/CFS + FM (Comparison B), and ME/CFS - IBS and ME/CFS - IBS** (Comparison C and D). All these incongruent or interesting trends and patterns indicated that urine metabolite characteristics may be different depending on the FM or IBS comorbidity and how participants are grouped.

I acknowledge that further discussion of the remaining faecal metabolites and the urine and metabolites is by no means complete. Indeed, further analyses, particularly where a pathway overview is given, would be beneficial (see Section 4.3.5). While IBS comorbidity appears to be a potential subgroup of ME/CFS, the heterogeneity from FM should not be overlooked. There are several metabolites where Comparison B ME/CFS +/- FM has been the only comparison to highlight a significantly different or important metabolite. Although likely to be several differences and inconsistencies with other ME/CFS studies using the same sample matrix, this study will overlap with the observations that reflect a breakdown and dysfunction in overall host metabolism including energy, carbohydrate, lipid, amino acid, and redox metabolism that is closely governed by the relationship with the gut microbiome.

### 4.3.4 Metabolites in Faeces and Urine

Up until this point faeces and urine in Part One have been considered separately with respect to their sample-specific metabolomic profile. The following significantly differential metabolites appeared in both specimen types (Appendix 9): 4-Hydroxyphenylpyruvic acid, acetate, adipic acid, Beta-Glycerophosphoric acid, Glycerol 3-phosphate, citric acid, glucose, ethanolamine, formate, furmaric acid, glutamic acid, glyceric acid, glycerol, glycine, histidine, malonic acid, methanol, myristic acid, N6-Acetyl-L-lysine, oxalic acid, oxoglutaric acid, succinate, and taurine. Of these metabolites across the two sample types, only adipic acid, ethanolamine, formate, glutamic acid, glyceric acid, glycerol, glycine, myristic acid, N6-Acetyl-L-lysine, oxalic acid, oxoglutaric acid were highlighted as significant but in different comparative groups. Although these metabolites were highlighted in different comparative groups, the faecal, and urine sample types provide a different insight into the relative concentration trend of their respective grouping.

Beta-Glycerophosphoric acid, Glycerol 3-phosphate, citric acid, histidine, methanol, and succinate, appeared in the same comparative groups between the sample types with a consistent trend of their relative concentration. However, 4-Hydroxyphenylpyruvic acid, acetate, glucose, fumaric acid, and taurine showed an opposite trend. These metabolites did not mirror each other in the relative concentration patterns of faeces and urine. For example, fumarate assayed by NMR for Comparison D was elevated in the faecal ME/CFS + IBS** group; however, it was elevated in the urinary ME/CFS - IBS** group. Lastly, malonic acid, which was observed across all comparisons, Comparison A-D, was both consistent and inconsistent between the sample types. Malonic acid, assayed by LCMS and NMR for Comparison A was

elevated in the Control and ME/CFS groups, for urine and faeces, respectively. The opposite trend was also observed in Comparison C and D where malonic acid was elevated in the urinary ME/CFS - IBS/IBS** groups but it was instead elevated in the faecal ME/CFS + IBS/IBS** groups. However, for Comparison B, malonic acid was consistently elevated in the ME/CFS - FM groups in both sample types.

The different and varied trends found when considering the faecal and urine metabolome results alone, and metabolites across both biospecimen types indicated that altered metabolic patterns associated with ME/CFS are subtle yet complex and detailed. Literature relevant to the specific pairings of faeces and urine, and plasma and urine in Part Two are becoming more available across a wide range of chronic disease topics. Indeed (gut microbiome related to) metabolomic studies across any pairing of sample type, or more than two sample types are as well (usually blood, faeces, and urine). The need for the different profiling patterns of different metabolic samples in analyses make it a matter of concern for which type of sample is most closely associated with gut microbes and disease (Zhao et al., 2022). However, regardless of the combination of sample looked at, these studies all appear to draw similar conclusions that further hypothesise the complex intricacies of the gut and host relationships. They all also reiterate that caution should be taken when attempting to make any inferences between the microbiome and disease association from any of the metabolomic data (the specific biospecimen type that data comes from is important). It is tempting to speculate from these paired sample metabolomic studies that these metabolites reflect a compensatory, adapted, unstable and/or inefficient physiological and biochemical system associated with the availability of metabolites, changes (but various and different changes, hence the array of trends and phenotypic presentations) in the gut microbiota, intermediate processes, immune modulation, and impaired intestinal barrier function (Armstrong et al., 2017; Deng et al., 2023; Hill et al., 2023; Jain et al., 2019; Kolho et al., 2017; Lee et al., 2021; McGregor et al., 2019; Z. Tian et al., 2020; X. J. Xu et al., 2021; Zhou et al., 2023). Variations in trends in these biospecimen types may reflect differences in homeostatic control that regulate absorption, transport, transformation, degradation, and excretion of these metabolites. Metabolites that are produced by the gut bacteria may experience complex intermediate processes, such as intestinal epithelial absorption, enterohepatic circulation, and liver absorption and transformation, when transporting from intestinal tract to the blood stream (Deng et al., 2023).

The metabolites that are measured by both biospecimen types require further analyses to remove the “noise” from the other metabolites. Given that each metabolite has shown differences in their patterns across each sample medium, it is assumed that each of these features reflects a different metabolic and biological situation. Further analyses would also require the Control participants, or at least a matched selection of them, to be compared with the groups from Comparison B-D to evaluate what is attributable to a comorbidity rather than ME/CFS, and vice versa. This would be a similar set up to the univariate analyses by Guo et al. (2023). Certainly, some data visualisations of these metabolites of interest and their absolute and relative concentrations across comparative groups and sample types will assist with deciphering their biological meaning. However, so far, malonic acid, is a “low hanging fruit” to make some commentary on, as it has appeared in all comparisons (A-D), and in both faecal and urine samples. Malonic acid is a dicarboxylic acid and participates in several enzymatic reactions in humans, it is involved in fatty acid biosynthesis (HMDB000691). It has been associated with several diseases and symptoms linked to the inborn metabolic disorder malonyl-CoA decarboxylase deficiency (for example, hypoglycaemia, malonic aciduria, renal dysplasia), vitamin B12 deficiency, and ethylmalonic encephalopathy (Ambati et al., 2017; J. Zhang et al., 2021). It has been attributed to inducing mitochondrial dysfunction by inhibiting succinate dehydrogenase therefore promoting the generation of superoxide radicals, apoptosis, and secondary excitotoxicity (J. Zhang et al., 2021). However, it has also been shown to exert anti-inflammatory effects and may protect against inflammatory reactions caused by the activation of microglia, which can lead to neurodegeneration and cause diseases such as Alzheimer's and Parkinson’s disease (Lee et al., 2021; Zhou et al., 2023). The malonic acid observations from this study indicate that the metabolite may have a variety of biological activities, perhaps at different levels or host circumstances, and how different systems utilise and excrete it. Further analysis with malonic acid, and its related metabolites, methylmalonic acid, and ethylmalonic acid across the samples with due consideration for medication and diet intake (some of this data is available from participant questionnaire data) is required before its role in ME/CFS can be established.

### 4.3.5 Other Metabolomics Study Considerations

These reflections apply to this chapter and Chapter 5. Section 3.5.3.3 is also relevant here for the metabolomics work, The LCMS and NMR datasets throughout this thesis were considered separately. That is, the same analytical workflow with MetaboAnalyst was applied then the most

significant or important outcomes were taken and subsequently put together into their respective summary tables. Another analytical workflow consideration with the metabolomic datasets would be to join the LCMS and NMR outcomes first of each respective sample type before using MetaboAnalyst. Instead of two separate data matrices put into MetaboAnalyst side-by-side, there is a “global metabolomic” one for urine, plasma, and faeces. In the first instance a normalisation and data scaling measure would be applied before sorting the combined dataset to identify and consolidate any metabolites measured by both platforms. This is made with the awareness that LCMS and NMR are inherently different in their quantitative characteristic, and it is potentially an “apple and pear” situation. Ideally, there would be no major discrepancies in the relative concentrations; however, this approach would be useful to investigate this. While the metabolites that appeared in both LCMS and NMR followed the same direction in their relative concentrations, a concession of this thesis is that a comparison of their relative concentrations was not more carefully considered. As it stands, this thesis has observed that dual-platform metabolites follow the same trends in concentration, but it is unclear whether concentrations recorded are consistent as well. A closer look in of these dual-platform metabolites with univariate-based statistics is worthwhile.

The focus of the metabolomics work in this thesis was placed more so on overall trends and patterns. The summary tables of each sample datasets represent the most significantly important and different metabolites across the comparative groups of interest, with a mention of which group was elevated. These tables are a combination of the outcomes from the volcano plots (univariate analysis) and the PLS-DA (multivariate analysis). It is worth mentioning that some metabolite features were seen multivariately but not univariately, and vice versa. The metabolomics appendix files contain a summary of the results that have investigated this as part of the process in constructing the final summary tables. Although the results were similar in trend, it is not advisable to use the other as a means of validation – this thesis did not go any further than the seeing of any similarities or differences in the results. Saccenti et al. (2014) provide a more in-depth explanation of the univariate and multivariate data analysis of metabolomics data; however, they are different in terms of their assumptions, considerations, and offerings of the data (Saccenti et al., 2014). In general, multivariate methods focus on the relations between metabolites and their orchestrated or complementary behaviour in relation to biological processes, and univariate methods on independent changes in metabolite levels (Saccenti et al., 2014). It is common in the literature to see both modalities used to unveil information from metabolomic data with common practice dictating that both should be used

because they show different things. This is pertinent to situations like ME/CFS and this study where it is not known (or completely known) beforehand how the biological phenomenon studies express itself in (relations between) metabolite levels (Saccenti et al., 2014).

Only these univariate and multivariate analyses were used in this thesis. With the raw metabolomics (and gut microbiome) data that is available for this study, several other analyses could be considered and done in the future. In the first instance, it would be to look at the performance and validation of the PLS-DA. No doubt the small numbers of the project will have some impact on this. Other approaches with the data that could be considered include various predictive machine learning analyses, biomarker analysis based on ROC curves, pathway analysis, ChemRICH analysis, pathway enrichment and topological analysis, and Pearson correlations between the biological sample types from the same part of the study. Beyond the metabolomics realm, there is the opportunity to perform further investigations with the gut microbiome data. This includes options for a more specifically integrated meta-analysis, multi-omic take with clustering, correlational analyses, network analyses, and heat maps (Huang et al., 2022; Karczewski & Snyder, 2018; Krassowski et al., 2020; Muller et al., 2022; Y. Yang et al., 2019). This is made with the caution that any combination or attempt to integrate and combine omic data is challenging and has its limitations (Krassowski et al., 2020; Muller et al., 2022). More than anything else, doing these further analyses with the data available from this study does not mean that it can predict or establish causality in ME/CFS.

All metabolomic datasets from this thesis (and most ME/CFS studies) are based on relative or at best, semi-quantitative absolute measures that have been normalised and scaled accordingly. For both LCMS and NMR, future projects would highly benefit from the use of methods that involve absolute quantitation. This would involve the use of standards with known concentrations during sample preparation, which are then used to create a standard curve during downstream processing and analysis of spectra. While there may be some extra work and cost involved, and optimisation may sacrifice some sensitivity and coverage, there are advantages. This includes having a known concentration that is comparable and robust over time, amenable to establishing reference ranges, and the next step of deriving accurate and more meaningful and versatile biological insights. Larger organisations like Metabolon, TMIC, Nightingale Health, and Biocrates, and smaller ones like Metabolomics Australia and in-house university metabolomic groups have kits and workflows that facilitate this with varying degrees of arrangements, services and products offered between them and the enquiring

partner/client/research group. There is also the possibility to couple the LCMS (and GCMS) and NMR platforms from one sample tube and acquisition. Along with the consideration of absolute measurements comes the opportunity for better metabolomic practices in general including sample collection and handling, metabolite quenching and extraction procedures, reproducibility and repeatability, spectra processing and annotations, workflow efficiency, automation where possible, collaboration, and usage of data from other groups. Leveraging the experience, expertise and offerings of these companies or specific laboratory groups in both the metabolomics and gut microbiome fields offers a capacity to standardise and expand research interests and capabilities both within and between research groups. Further, while metabolomics has been a useful research tool and is increasingly recognised as “clinical metabolomics” in medicine, this may not translate practically and seamlessly to a clinical setting with routine laboratory testing environments. The fact that metabolomics is advantageously used to probe complex host systems does mean that sample collection and storage considerations need to ensure that it does not introduce high variability, interferences with instrumentation, or degradation of metabolites (Karu et al., 2018; Lamichhane et al., 2018; Le Gouellec et al., 2023; Smith et al., 2020).

# 5 CHAPTER FIVE: Plasma and Urine Host Metabolome (Part Two)

The background and introduction and most of the discussion points presented in Chapter 4 is relevant to this chapter. This chapter (Chapter 5) focused on the plasma and urine metabolome from Part Two. Chapter 4 focused on the faecal and urine metabolome from Part One. Part Two looked at 28 ME/CFS and 28 Control participants which included males and females (Section 2.1). Table 2.2.2 shows the baseline characteristics of the four comparative groups of interest (Comparison A-D). Comparison A compares the ME/CFS overall and Control groups, Comparison B compares the ME/CFS +/- FM groups, Comparison C compares the ME/CFS +/- IBS groups, and Comparison D compares the age-matched ME/CFS +/- IBS** groups. Results relevant to plasma metabolomics are in Section 5.1. Results relevant to urinary metabolomics (Part Two) are in Section 5.2. Lastly, Section 5.3 covers the discussion for the Part Two metabolomic outcomes from the plasma and urine, both independently and when compared to each other.

## 5.1 Plasma Metabolomics Results

Globally, LCMS detected 128 and NMR detected 47 plasma metabolite features from all samples (Appendix 10). 82 significantly different metabolite features in total from LCMS and NMR were highlighted from the volcano plots (VPs) and PLS-DA VIP plots across all the comparisons, Comparison A-D (Table 5.1.1). These significantly different metabolites, categorised according to the HMDB database, included bile acids and derivatives (9.76%), amino acids, peptides and analogues (29.27%), fatty acyls and acids (17.07%), sugar and sugar derivatives (8.54%), carboxylic acids and derivatives (7.32%), purines and purine derivatives (6.10%), other metabolites (14.63%), and organic acids (7.32%). There were seven significant differential plasma features (hypoxanthine, alanine, 2-Hydroxy-3-methylbutyric acid aka. 2-Hydroxyisovalerate, 3-Hydroxyisovaleric acid, arginine, 3-Methyl-2-oxovaleric acid, and tryptophan) that were measured by both analytical platforms, LCMS and NMR (Table 5.1.1). The relative concentrations found for these shared metabolites were consistent in their direction as either up/higher/red or down/lower/blue from the VP and PLS-DA analyses (Appendix 11). The individual plots from the VP, PCA, and PLS-DA analyses for plasma Comparisons A-D are shown in Sections 5.1.1 (ME/CFS overall vs. Control), 5.1.2 (ME/CFS +/- FM), 5.1.3 (ME/CFS +/- IBS), and 5.1.4 (age-matched ME/CFS +/- IBS**), respectively.

**Table 5.1.1** LCMS and NMR Plasma Metabolome (Part Two) Significant Features and Results

| | Plasma Part Two Metabolite | Comparative Group (A-D) | Group with significant elevation in relative concentration | LCMS or NMR |
|---|---|---|---|---|
| Bile acid and derivatives | Deoxycholic acid glycine conjugate | A | Control | LCMS |
| | 7a-Hydroxy-3-oxo-5b-cholanoic acid/Nutriacholic acid | B | ME/CFS - FM | LCMS |
| | 7a-Hydroxy-3-oxo-5b-cholanoic acid | | | |
| | Nutriacholic acid | | | |
| | Allocholic acid/Cholic acid | C, D | ME/CFS - IBS, ME/CFS - IBS** | LCMS |
| | Allocholic acid | | | |
| | Cholic acid | | | |
| | Glycocholic acid | C, D | ME/CFS - IBS, ME/CFS - IBS** | LCMS |
| Amino acids, peptides and analogues | Beta-Alanine | A | Control | LCMS |
| | Alpha-N-Phenylacetyl-L-glutamine | A, B | ME/CFS, ME/CFS - FM | LCMS |
| | D-Aspartic acid/L-Aspartic acid | A | Control | LCMS |
| | D-Alanine/L-Alanine/Sarcosine (aka. Alanine) ‡ | A, C, D | Control, ME/CFS + IBS, ME/CFS + IBS** | LCMS, NMR |
| | Sarcosine | A, B | Control, ME/CFS + FM | NMR |
| | Phenylalanine | A, B, C | Control, ME/CFS + FM, ME/CFS + IBS | NMR |
| | Ornithine | A, B, C, D | Control, ME/CFS + FM, ME/CFS + IBS, ME/CFS + IBS** | NMR |
| | Tyrosine | A, C, D | Control, ME/CFS + IBS, ME/CFS + IBS** | NMR |
| | Arginine (aka. L-Arginine) ‡ | A, C | Control, ME/CFS + IBS | NMR, LCMS |
| | Creatine | A, B | Control, ME/CFS + FM | NMR |
| | Betaine | A, B | Control, ME/CFS - FM | NMR |
| | L-Proline | B | ME/CFS - FM | LCMS |
| | Histidine | B, C | ME/CFS + FM, ME/CFS + IBS | NMR |
| | Carnitine | B | ME/CFS + FM | NMR |
| | Glutamate | B, D | ME/CFS + FM, ME/CFS - IBS** | NMR |
| | Threonine | B | ME/CFS + FM | NMR |
| | L-Tryptophan (aka. Tryptophan) ‡ | C, D | ME/CFS + IBS, ME/CFS + IBS** | LCMS, NMR |
| | 3-Methylhistidine | C, D | ME/CFS - IBS, ME/CFS - IBS** | LCMS |
| | Glutamine | C, D | ME/CFS + IBS, ME/CFS + IBS** | NMR |
| | Lysine | C | ME/CFS + IBS | NMR |
| | Glycine | C | ME/CFS - IBS | NMR |
| | Acetylglycine | D | ME/CFS - IBS** | LCMS |
| | Methylcysteine | D | ME/CFS + IBS** | LCMS |
| | Serine | D | ME/CFS - IBS** | NMR |
| Fatty acyls and | Arachidic acid/Phytanic acid | A | Control | LCMS |
| | Arachidic acid | | | |
| | Phytanic acid | | | |

| | | | | |
|---|---|---|---|---|
| | Capric acid | A | Control | LCMS |
| | Alpha-Linolenic acid | A | Control | LCMS |
| | Octadecanedioic acid | A | Control | LCMS |
| | Tetradecanedioic acid | A | Control | LCMS |
| | Acetate | A | Control | NMR |
| | 2-Hydroxy-2-methylbutyric acid/2-Hydroxy-3-methylbutyric acid/3-Hydroxyisovaleric acid | D | ME/CFS - IBS** | LCMS |
| | 2-Hydroxy-2-methylbutyric acid | | | |
| | 2-Hydroxy-3-methylbutyric acid (aka. 2-Hydroxyisovalerate) ‡ | D | ME/CFS - IBS** | LCMS, NMR |
| | 3-Hydroxyisovaleric acid (aka. 3-Hydroxyisovalerate) ‡ | A, D | Control, ME/CFS - IBS** | LCMS, NMR |
| | Hydroxyoctanoic acid | B | ME/CFS + FM | LCMS |
| | Hexadecanedioic acid | C, D | ME/CFS - IBS, ME/CFS - IBS** | LCMS |
| Sugar and sugar derivatives | Fructose 6-phosphate/Glucose 1-phosphate | A, B | Control, ME/CFS + FM | LCMS |
| | Fructose 6-phosphate | | | |
| | Glucose 1-phosphate | | | |
| | myo-Inositol/myo-Inositol | A, C | Control, ME/CFS + IBS | LCMS |
| | Sucrose | A, C | Control, ME/CFS + IBS | LCMS |
| | Gluconic acid | B | ME/CFS + FM | LCMS |
| | 1.5-Anhydrosorbitol | D | ME/CFS - IBS** | LCMS |
| Carboxylic acids and derivatives | Formate | A, B | Control, ME/CFS + FM | NMR |
| | Succinate | A, D | Control, ME/CFS - IBS** | NMR |
| | Malonate | B | ME/CFS + FM | NMR |
| | 2.3-Dihydroxybutanedioic acid | C | ME/CFS + IBS | LCMS |
| | Galactaric acid | C, D | ME/CFS + IBS, ME/CFS + IBS** | LCMS |
| | cis-Aconitic acid | C | ME/CFS + IBS | LCMS |
| Purines and purine | Adenine | A | ME/CFS | LCMS |
| | Hypoxanthine ‡ | A, B | Control, ME/CFS + FM | LCMS, NMR |
| | Adenosine monophosphate | B | ME/CFS + FM | LCMS |
| | 9-Methyluric acid | B | ME/CFS + FM | LCMS |
| | Inosine | D | ME/CFS - IBS** | LCMS |
| Other metabolites | Alpha-Tocopherol | A, B | ME/CFS, ME/CFS - FM | LCMS |
| | Hippuric acid | A, D | Control, ME/CFS + IBS** | LCMS |
| | Propylene glycol | A | Control | NMR |
| | Phthalic acid | B | ME/CFS - FM | LCMS |
| | Phosphoric acid | B | ME/CFS + FM | LCMS |
| | Dimethyl sulfone | B | ME/CFS + FM | NMR |
| | Methanol | B, C, D | ME/CFS + FM, ME/CFS + IBS, ME/CFS + IBS** | NMR |
| | Trigonelline | C, D | ME/CFS + IBS, ME/CFS + IBS** | LCMS |
| | 4-Hydroxyhippuric acid | C, D | ME/CFS + IBS, ME/CFS + IBS** | LCMS |
| | D-Glucuronic acid | C | ME/CFS + IBS | LCMS |
| | Choline | C, D | ME/CFS + IBS, ME/CFS + IBS** | NMR |
| | Isopropanol | D | ME/CFS - IBS** | NMR |
| Or ga | Pyruvate | A, B, C, D | Control, ME/CFS + FM, ME/CFS - IBS, ME/CFS - IBS** | NMR |

| | | | | |
|---|---|---|---|---|
| | 3-Hydroxybutyrate | A, C | Control, ME/CFS + IBS | NMR |
| | Glyceric acid | B | ME/CFS - FM | LCMS |
| | 3-Methyl-2-oxovaleric acid (aka. 3-Methyl-2-oxovalerate) ‡ | B | ME/CFS - FM | LCMS, NMR |
| | Lactate | C, D | ME/CFS - IBS, ME/CFS - IBS** | NMR |
| | Acetoacetate | D | ME/CFS - IBS** | NMR |
| NOTE | **Comparative Group (A-D)**<br>A) ME/CFS overall vs. Control [ME/CFS, Control]<br>B) ME/CFS with FM vs. ME/CFS without FM [ME/CFS + FM, ME/CFS - FM]<br>C) ME/CFS with IBS vs. ME/CFS without IBS [ME/CFS + IBS, ME/CFS - IBS]<br>D) ME/CFS with IBS vs. ME/CFS without IBS (age-matched) [ME/CFS + IBS**, ME/CFS - IBS**]<br>‡ "Duplicate" metabolite assayed by LCMS and NMR<br>Refer to **Appendix 11** for the electronic version of this table and raw data input | | | |

## 5.1 Plasma Metabolomics Results continued…

The significantly differential plasma metabolite features were elevated in their relative concentrations depending on how the samples were grouped with their ME/CFS, FM, or IBS metadata that comprised Comparison A-D (Table 5.1.1). Not every single plasma metabolite appeared in all of the comparisons when observing the significant differences between the involved groups. A total of 11, 14, 5, and 9 metabolite features were found only to be significantly different in Comparison A (ME/CFS overall vs. Control), B (ME/CFS +/- FM), C (ME/CFS +/- IBS), and D (age-matched ME/CFS +/- IBS**), respectively; for example, Beta-Alanine and aspartic acid were only differential and elevated in the Control group from Comparison A. The metabolites, for example, glutamine and glycocholic acid, that were highlighted in the ME/CFS +/- IBS and ME/CFS +/- IBS** groups (Comparison C and D), were always consistently lower or higher in their relative concentration providing some indication of IBS involvement where an attempt to mitigate the age variable has been made. Histidine and methanol were consistently elevated in ME/CFS + either FM, IBS, or IBS** (Comparison B-D).

VPs of the LCMS plasma data matrix highlighted 6 metabolites (2 down and 4 up) in the Control vs. ME/CFS (Figure 5.1.1), 1 metabolite (down) in the ME/CFS +/- FM (Figure 5.1.9), 2 metabolites (both up) in the ME/CFS +/- IBS (Figure 5.1.17), and 2 metabolites (both up) in the ME/CFS +/- IBS** (Figure 5.1.25) comparisons that were significantly different. VPs of the NMR plasma data matrix highlighted 9 metabolites (all up) in the Control vs. ME/CFS (Figure 5.1.2), 3 metabolites (all up) in the ME/CFS +/- FM (Figure 5.1.10), 8 metabolites (2 down and 6 up) in the ME/CFS +/- IBS (Figure 5.1.18), and 4 metabolites (3 down and 1 up) in the ME/CFS +/- IBS** (Figure 5.1.26) comparisons that were significantly different. The metabolites from the VPs that were also found to be significantly different by PLS-DA VIP analyses reflected and supported the same pattern as either high or low in relative concentration (Appendix 11). The PCAs from the LCMS and NMR data matrices did not show a clear separation between the groups for all comparisons (Table 5.1.2). Although the PCAs did not discriminate the groups, the clusters extended in different directions and tighter clusters identified by the ellipsoid shape were observed from NMR ME/CFS + FM (Figure 5.1.12), and LCMS and NMR ME/CFS + IBS (Figure 5.1.19, Figure 5.1.20) and ME/CFS + IBS** (Figure 5.1.27, Figure 5.1.28). The PLS-DAs demonstrated improved clustering and separation for Comparison A-D (Table 5.1.2). The PLS-DA for the LCMS data matrix found a distinguished separation between the ME/CFS +/- FM (Figure 5.1.13) and ME/CFS +/- IBS (Figure 5.1.21) comparisons. A complete to distinguished

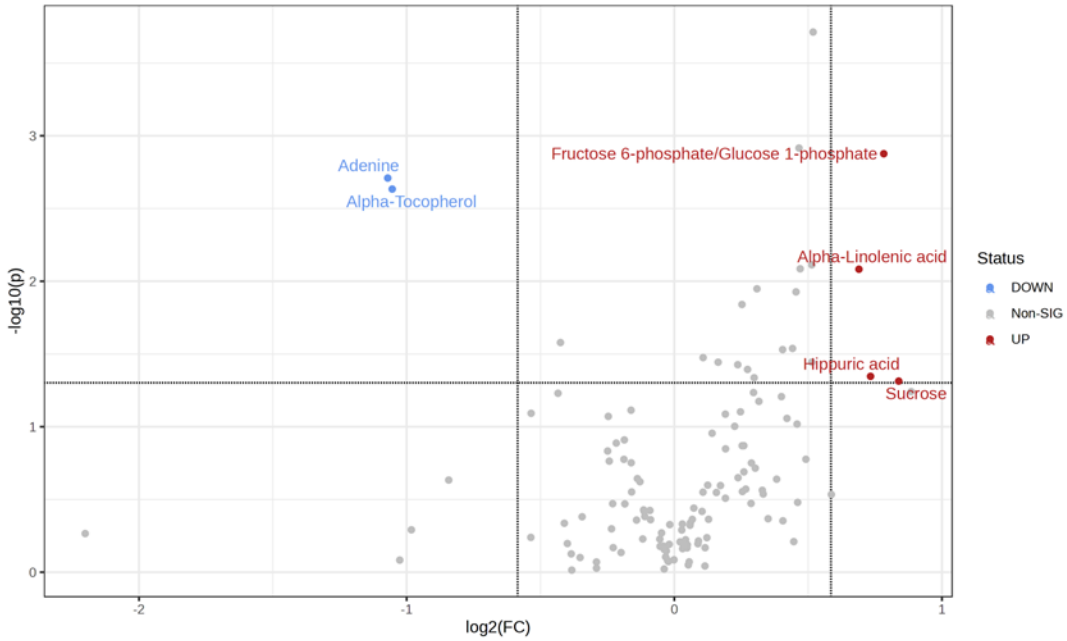
separation for LCMS (Figure 5.1.29) and NMR (Figure 5.1.31), respectively, was also observed between the ME/CFS +/- IBS** groups.

**Table 5.1.2** Summary of the total variance from the first two components of the PCA and PLS-DA analyses (LCMS and NMR Plasma Metabolome (Part Two))

| Plasma Part Two | | PCA | | | | PLSDA | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | % of the variance | | | Separation | % of the variance | | | Separation |
| | | PC1 | PC2 | Total† | | Comp. 1 | Comp. 2 | Total‡ | |
| A) ME/CFS vs. Control | LCMS | 11.3 | 10.1 | 21.4 | Overlapping | 8.2 | 6 | 14.2 | Partial |
| | NMR | 29 | 11.5 | 40.5 | Overlapping | 26.7 | 7.5 | 34.2 | Very partial |
| B) ME/CFS +/- FM | LCMS | 13.1 | 11.9 | 25 | Overlapping | 6.8 | 7.6 | 14.4 | Distinguished |
| | NMR | 24.7 | 14.7 | 39.4 | Overlapping | 14 | 16.2 | 30.2 | Very partial |
| C) ME/CFS +/- IBS | LCMS | 13.1 | 11.9 | 25 | Overlapping | 7.4 | 7.6 | 15 | Distinguished |
| | NMR | 24.7 | 14.7 | 39.4 | Overlapping | 14.2 | 18 | 32.2 | Very partial |
| D) ME/CFS +/- IBS** | LCMS | 15.2 | 12.8 | 28 | Overlapping | 7.3 | 10.7 | 18 | Complete |
| | NMR | 22.8 | 17.4 | 40.2 | Overlapping | 9 | 6.2 | 15.2 | Distinguished |
| † PC1 and PC2 added together | | | | | | | | | |
| ‡ Component 1 and Component 2 added together | | | | | | | | | |
| Distinguished to complete separation between groupings | | | | | | | | | |

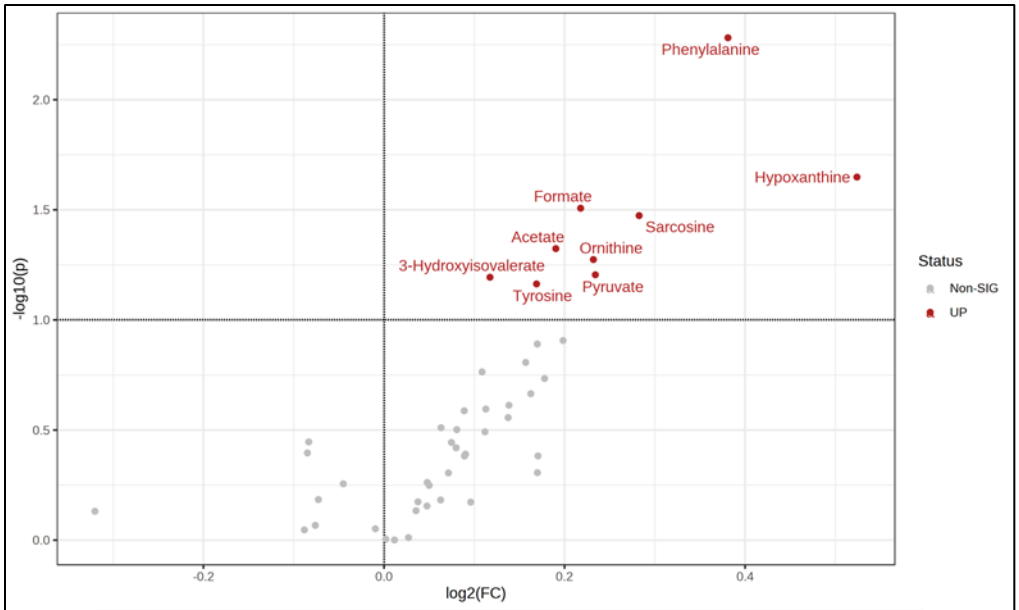
### 5.1.1 Plasma Comparison A) ME/CFS Overall vs. Control Group

#### 5.1.1.1 Univariate Analysis




| | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|
| Fructose 6-phosphate/Glucose 1-phosphate | 1.7201 | 0.78253 | 0.0013289 | 2.8765 |
| Adenine | 0.47613 | -1.0706 | 0.0019523 | 2.7095 |
| Alpha-Tocopherol | 0.48166 | -1.0539 | 0.0023251 | 2.6336 |
| Alpha-Linolenic acid | 1.6131 | 0.68982 | 0.008264 | 2.0828 |
| Hippuric acid | 1.6622 | 0.73312 | 0.045035 | 1.3465 |
| Sucrose | 1.7881 | 0.83841 | 0.048552 | 1.3138 |

**Figure 5.1.1** Volcano plot of LCMS Plasma Part 2 ME/CFS overall vs Control groups. Metabolites with significantly higher (blue) or lower (red) concentrations in the ME/CFS cohort are highlighted (FC threshold 1.5 and uncorrected p-values ≤ 0.05) with non-significant metabolites represented in grey.




| | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|
| Phenylalanine | 1.3023 | 0.38104 | 0.005232 | 2.2813 |
| Hypoxanthine | 1.4378 | 0.52391 | 0.022451 | 1.6488 |
| Formate | 1.1629 | 0.21778 | 0.031094 | 1.5073 |
| Sarcosine | 1.2164 | 0.28261 | 0.033596 | 1.4737 |
| Acetate | 1.141 | 0.19034 | 0.047433 | 1.3239 |
| Ornithine | 1.1744 | 0.23193 | 0.053181 | 1.2742 |
| Pyruvate | 1.1762 | 0.23411 | 0.062377 | 1.205 |
| 3-Hydroxyisovalerate | 1.0848 | 0.11738 | 0.063996 | 1.1938 |
| Tyrosine | 1.1243 | 0.16897 | 0.068646 | 1.1634 |

**Figure 5.1.2** Volcano plot of NMR Plasma Part 2 ME/CFS overall vs Control groups. Metabolites with significantly higher (blue) or lower (red) concentrations in the ME/CFS cohort are highlighted (FC threshold 1.0 and uncorrected p-values ≤ 0.10) with non-significant metabolites represented in grey.

### 5.1.1.2 Multivariate Analysis (PCA)

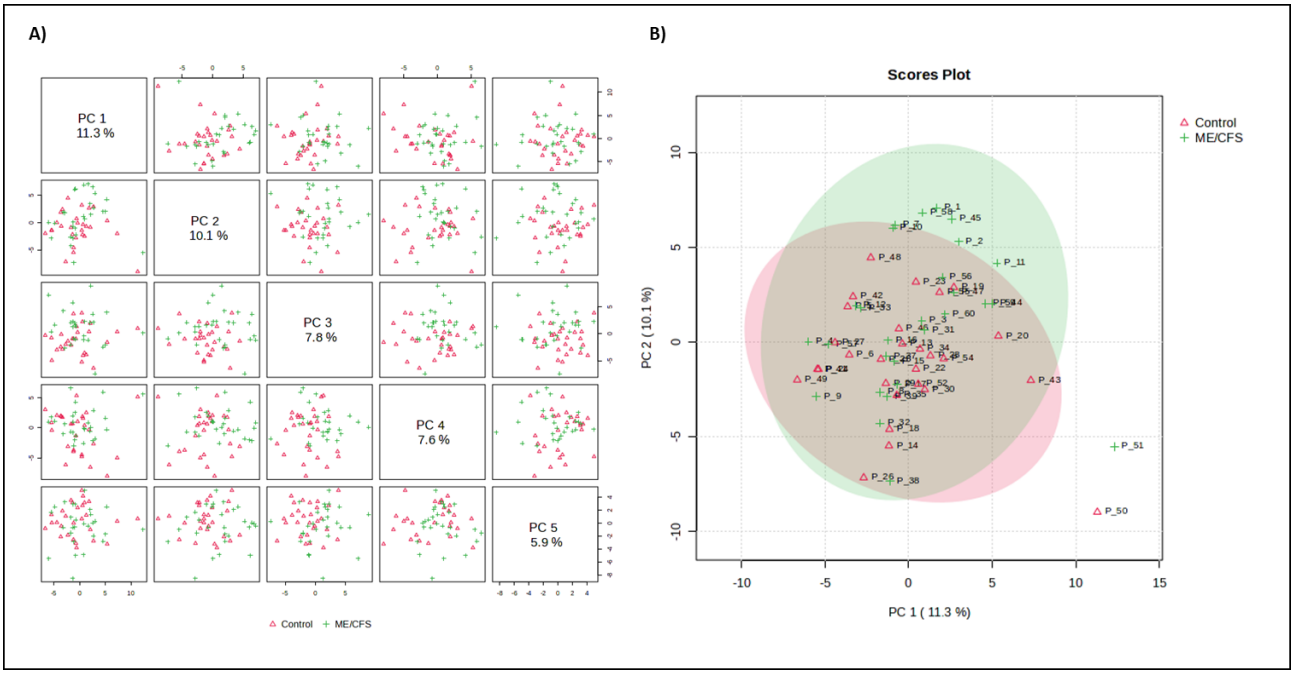



**Figure 5.1.3** PCA plots A) overview B) PC1 and PC2 only of LCMS Plasma Part 2 ME/CFS overall vs. Control groups

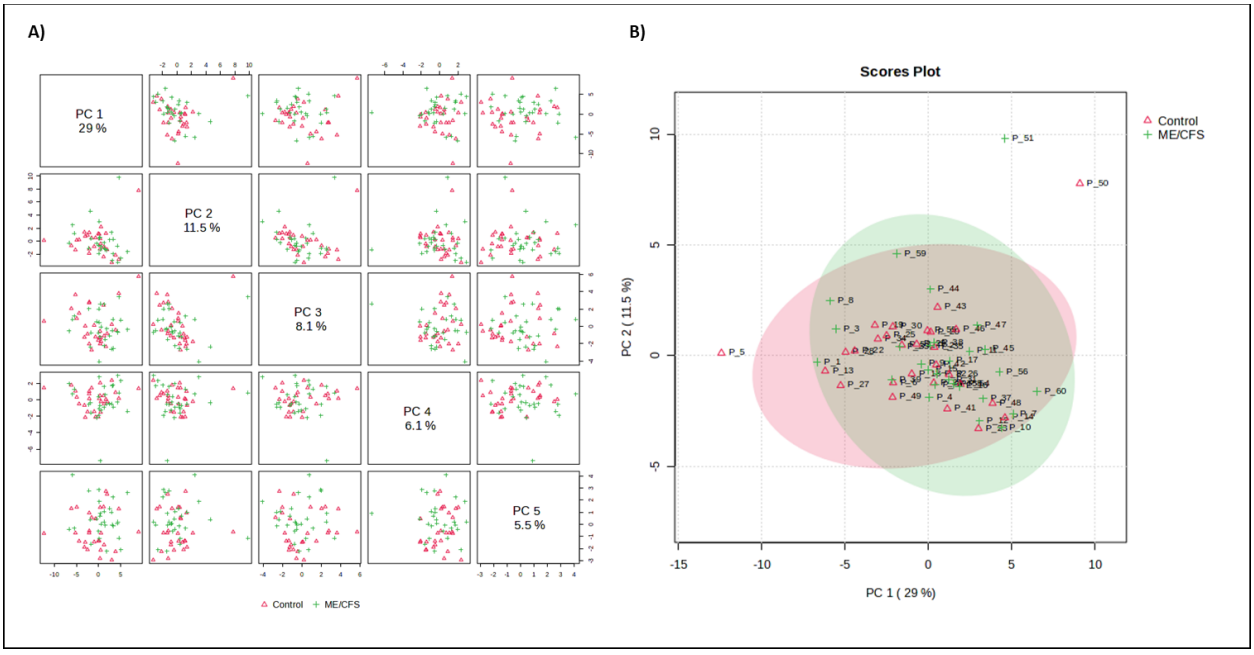



**Figure 5.1.4** PCA plots A) overview B) PC1 and PC2 only of NMR Plasma Part 2 ME/CFS overall vs. Control groups

### 5.1.1.3 Multivariate Analysis (PLS-DA)

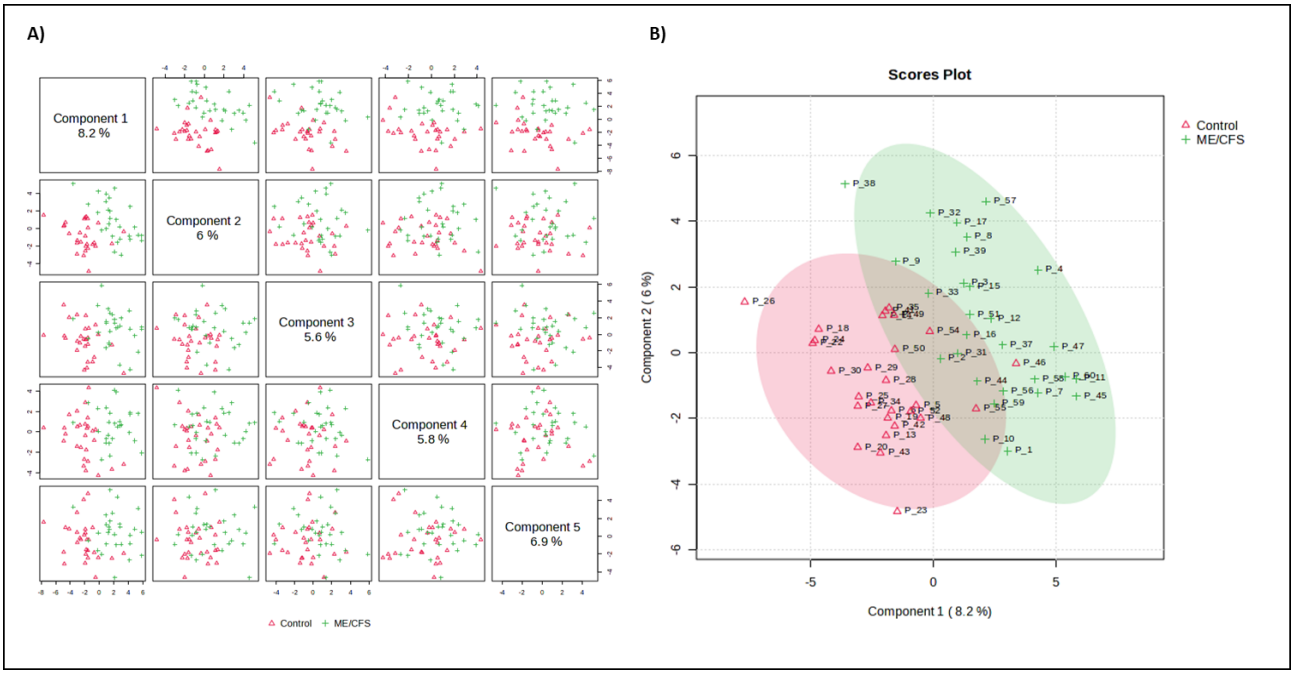



**Figure 5.1.5** PLSDA A) overview B) components 1 and 2 only of LCMS Plasma Part 2 ME/CFS overall vs. Control groups

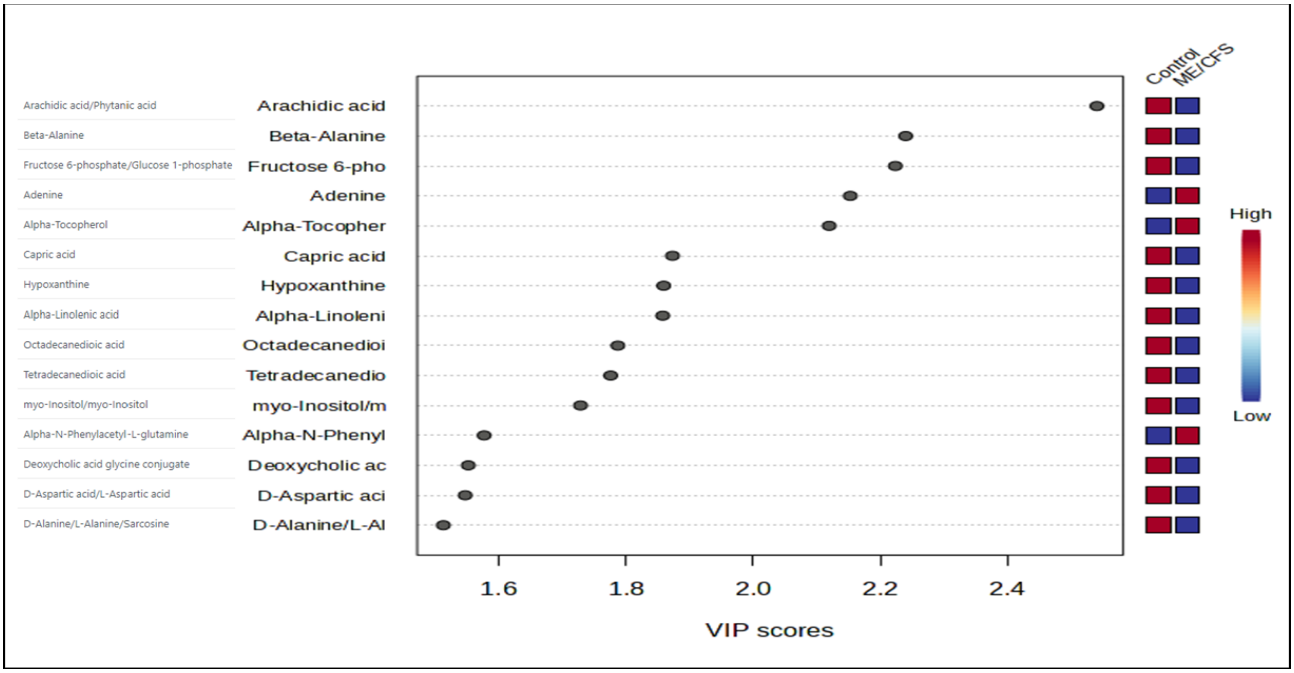



**Figure 5.1.6** VIP scores of corresponding PLSDA for LCMS Plasma Part 2 ME/CFS overall vs. Control groups

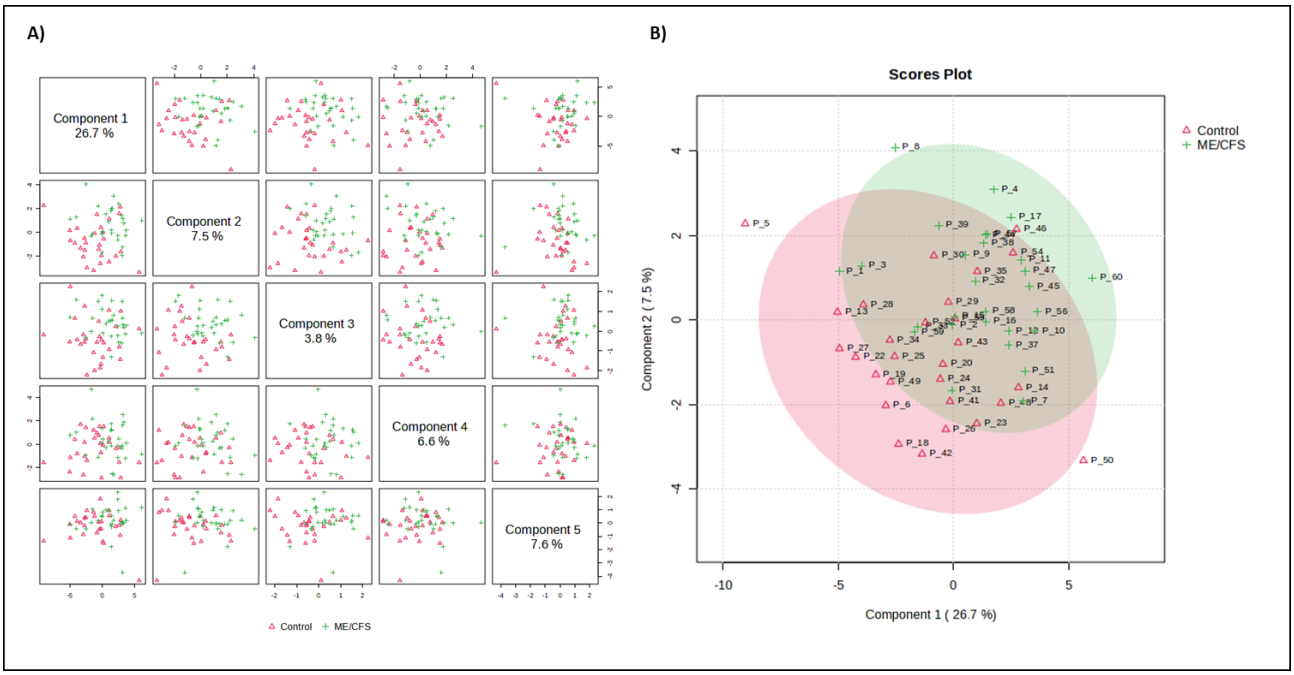



**Figure 5.1.7** PLSDA A) overview B) components 1 and 2 only of NMR Plasma Part 2 ME/CFS overall vs. Control groups

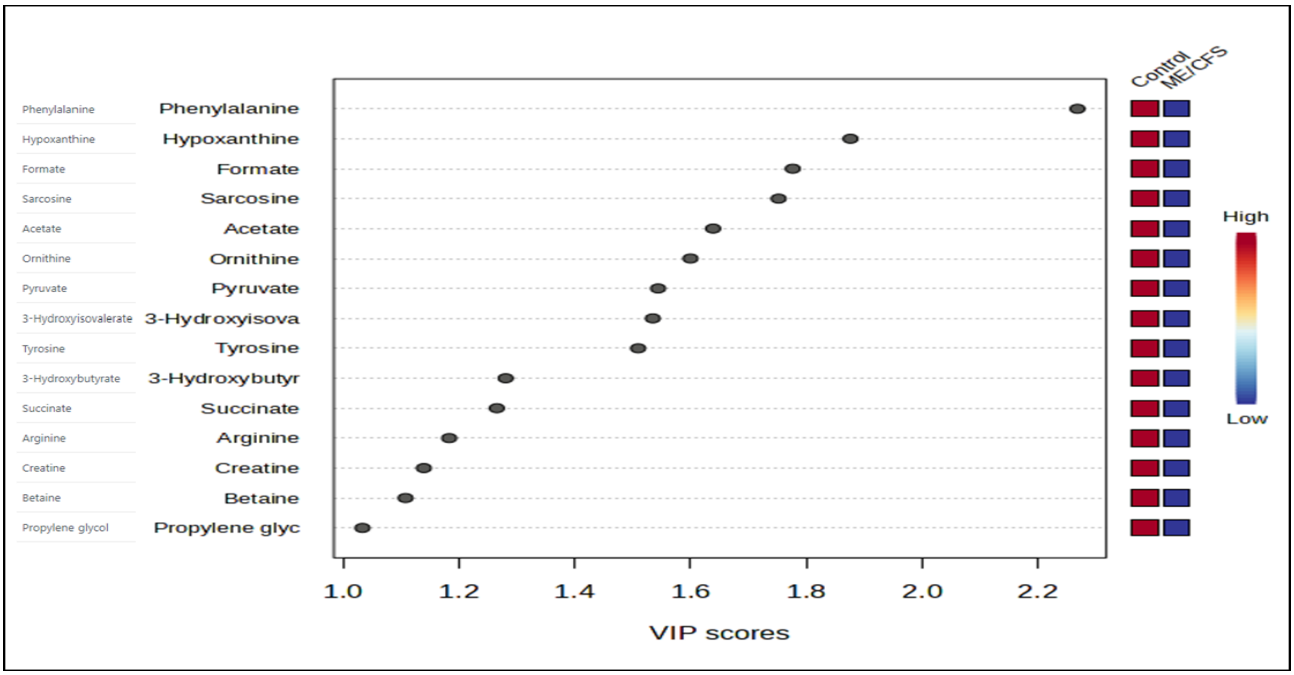
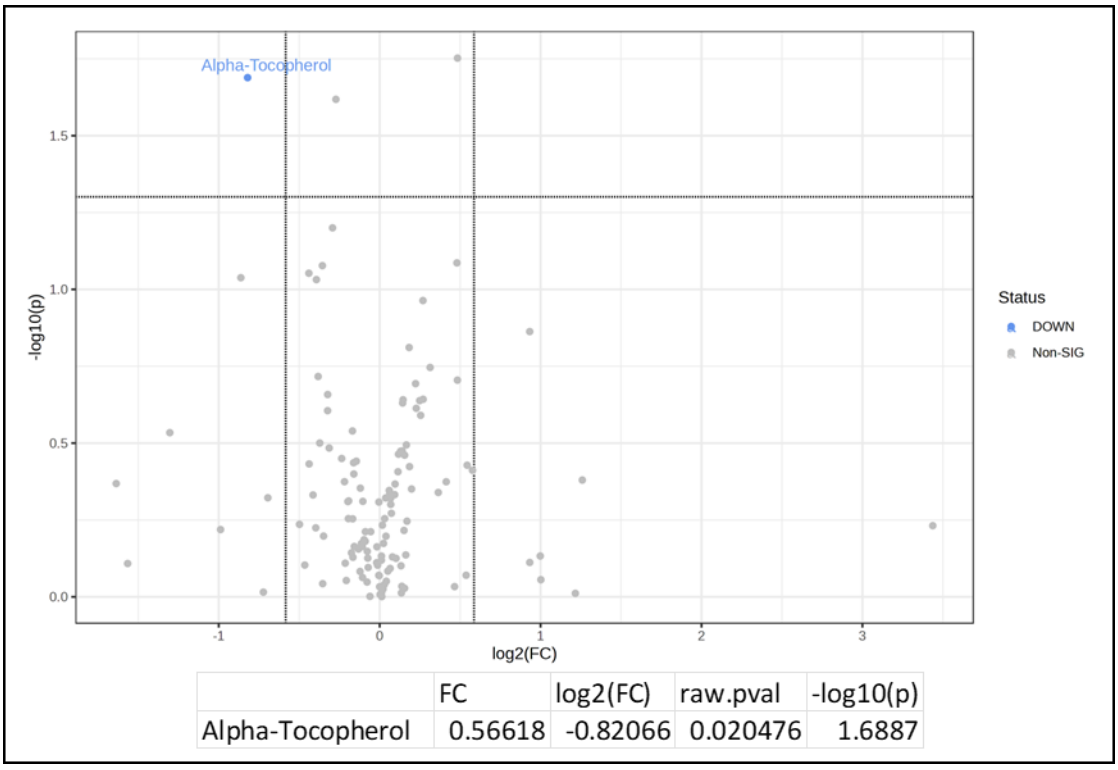



**Figure 5.1.8** VIP scores of corresponding PLSDA for NMR Plasma Part 2 ME/CFS overall vs. Control groups

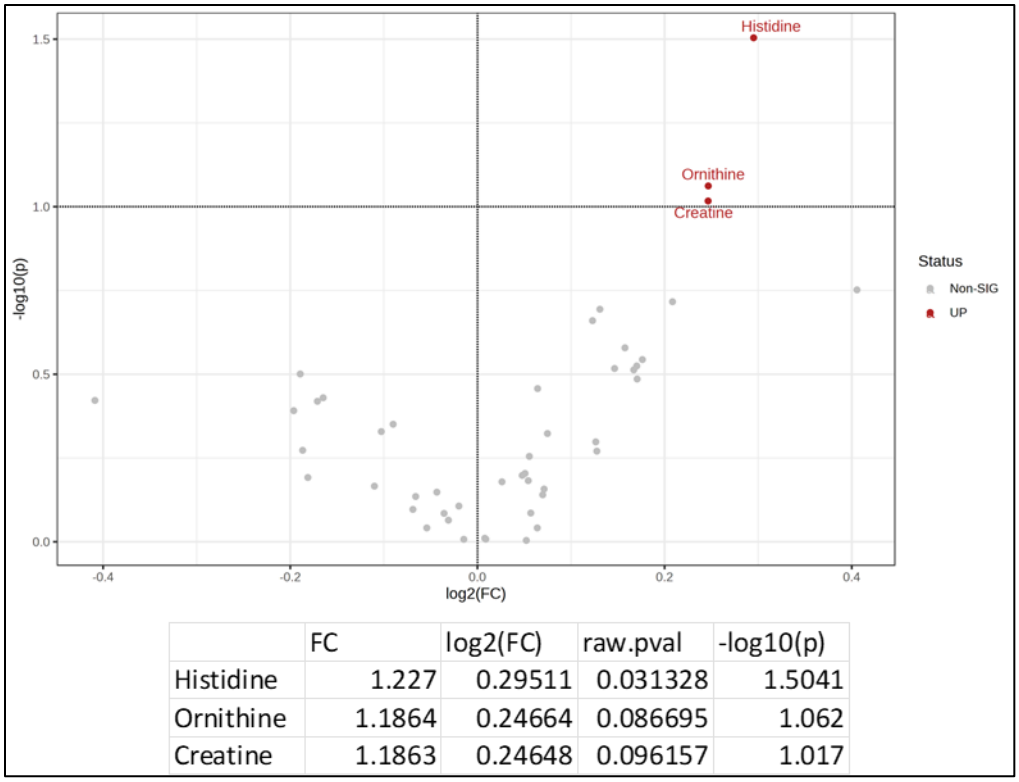
### 5.1.2 Plasma Comparison B) ME/CFS with FM vs. ME/CFS without FM

#### 5.1.2.1 Univariate Analysis




| | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|
| Alpha-Tocopherol | 0.56618 | -0.82066 | 0.020476 | 1.6887 |

**Figure 5.1.9** Volcano plot of LCMS Plasma Part 2 ME/CFS with FM versus ME/CFS without FM. Metabolites with significantly higher (red) or lower (blue) concentrations in the ME/CFS with FM cohort are highlighted (FC threshold 1.5 and uncorrected p-values ≤ 0.05) with non-significant metabolites represented in grey.




| | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|
| Histidine | 1.227 | 0.29511 | 0.031328 | 1.5041 |
| Ornithine | 1.1864 | 0.24664 | 0.086695 | 1.062 |
| Creatine | 1.1863 | 0.24648 | 0.096157 | 1.017 |

**Figure 5.1.10** Volcano plot of NMR Plasma Part 2 ME/CFS with FM versus ME/CFS without FM. Metabolites with significantly higher (red) or lower (blue) concentrations in the ME/CFS with FM cohort are highlighted (FC threshold 1.0 and uncorrected p-values ≤ 0.10) with non-significant metabolites represented in grey.

### 5.1.2.2 Multivariate Analysis (PCA)

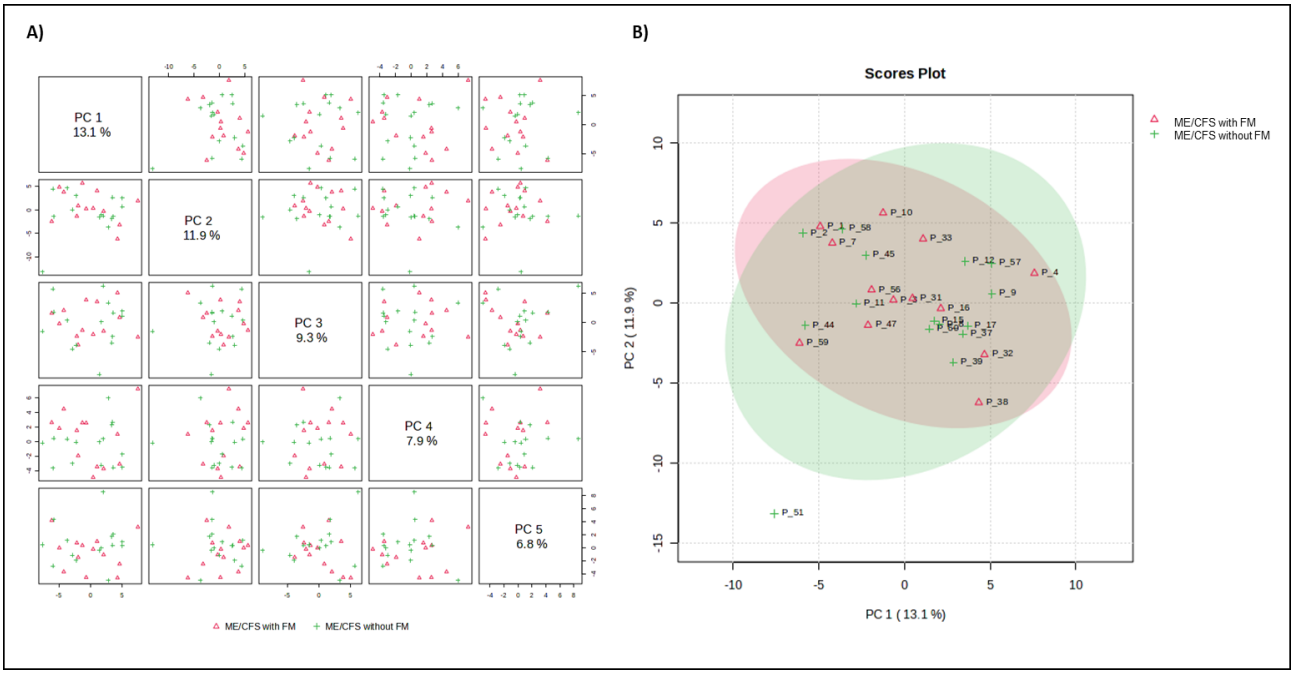



**Figure 5.1.11** PCA plots A) overview B) PC1 and PC2 only of LCMS Plasma Part 2 ME/CFS with FM versus ME/CFS without FM

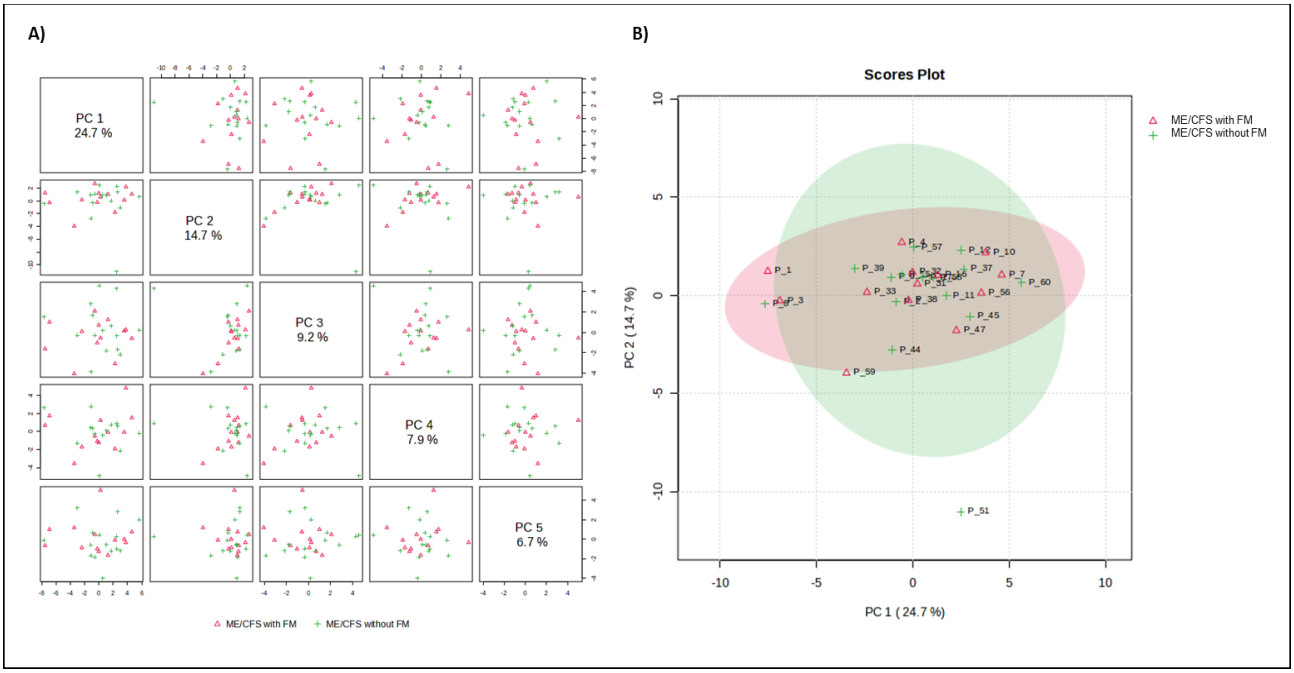



**Figure 5.1.12** PCA plots A) overview B) PC1 and PC2 only of NMR Plasma Part 2 ME/CFS with FM versus ME/CFS without FM

### 5.1.2.3 Multivariate Analysis (PLS-DA)

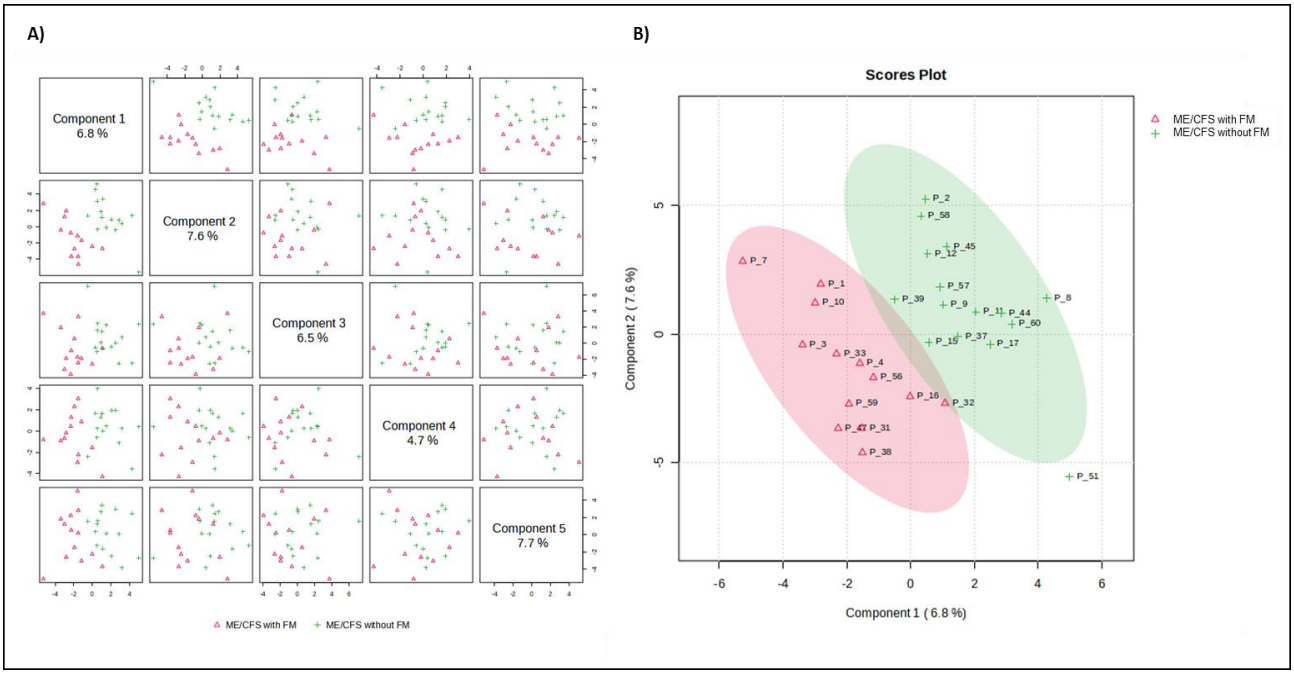



**Figure 5.1.13** PLSDA A) overview B) components 1 and 2 only of LCMS Plasma Part 2 ME/CFS with FM versus ME/CFS without FM

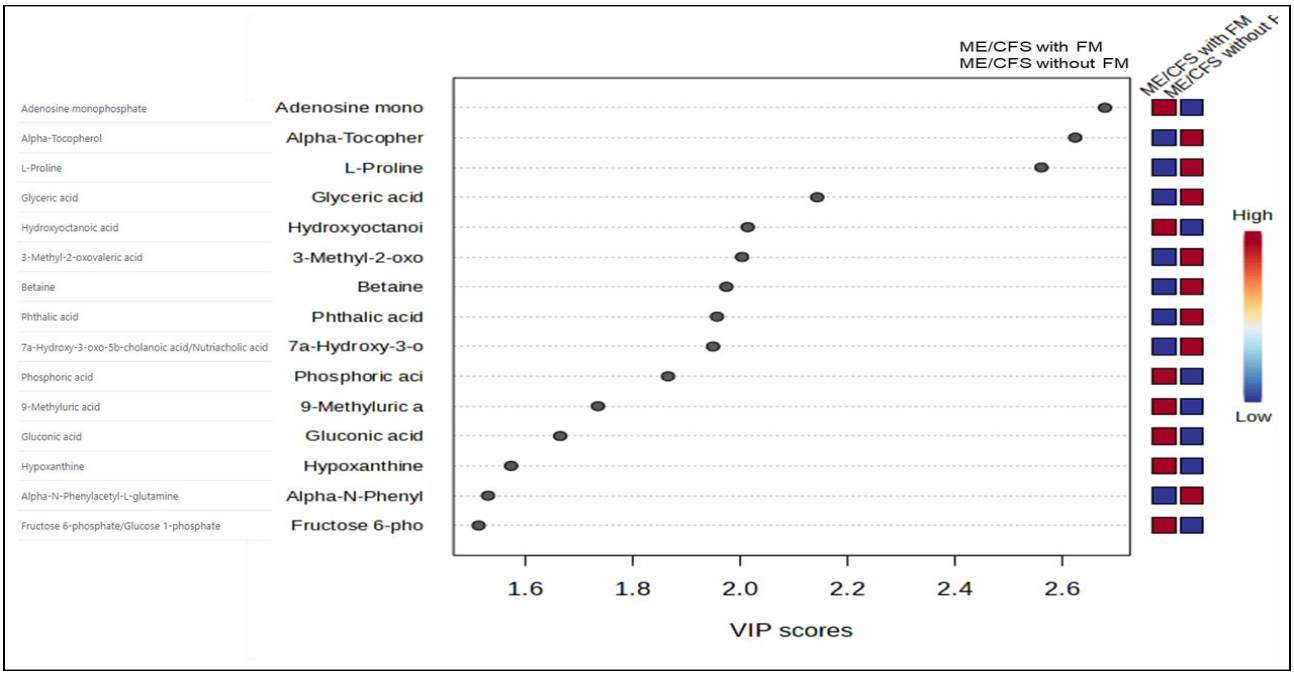



**Figure 5.1.14** VIP scores of corresponding PLSDA for LCMS Plasma Part 2 ME/CFS with FM versus ME/CFS without FM

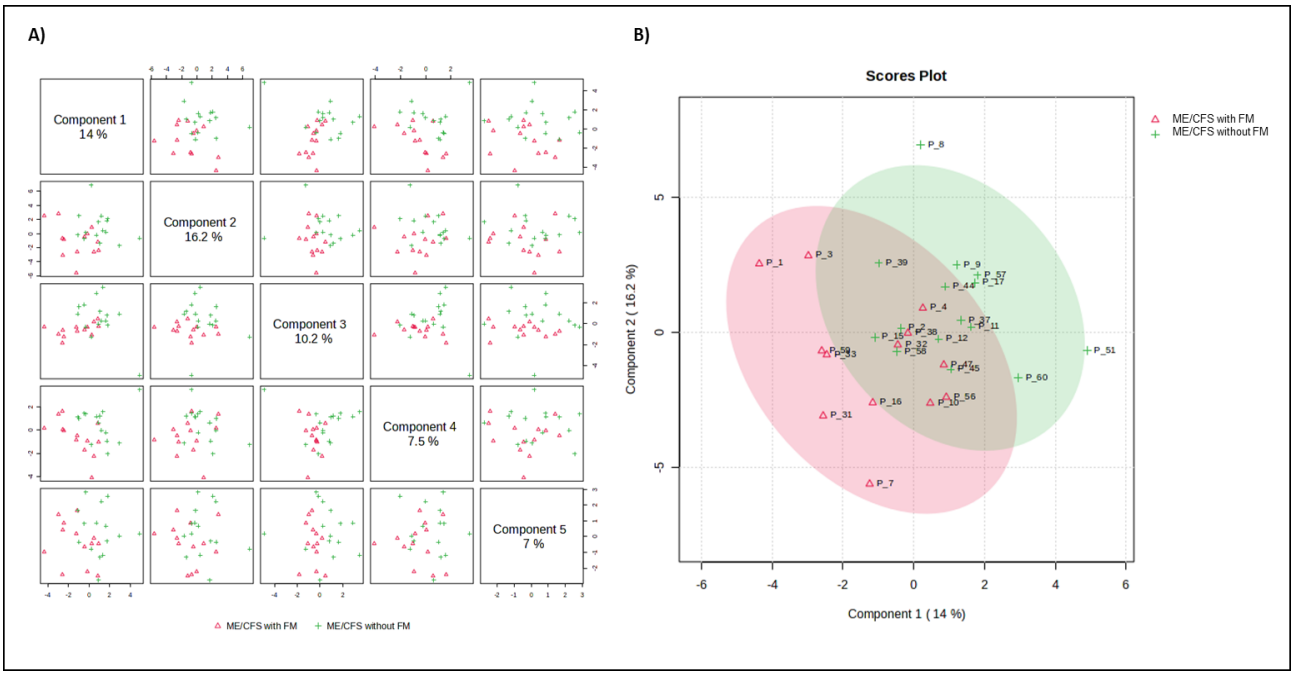



**Figure 5.1.15** PLSDA A) overview B) components 1 and 2 only of NMR Plasma Part 2 ME/CFS with FM versus ME/CFS without FM

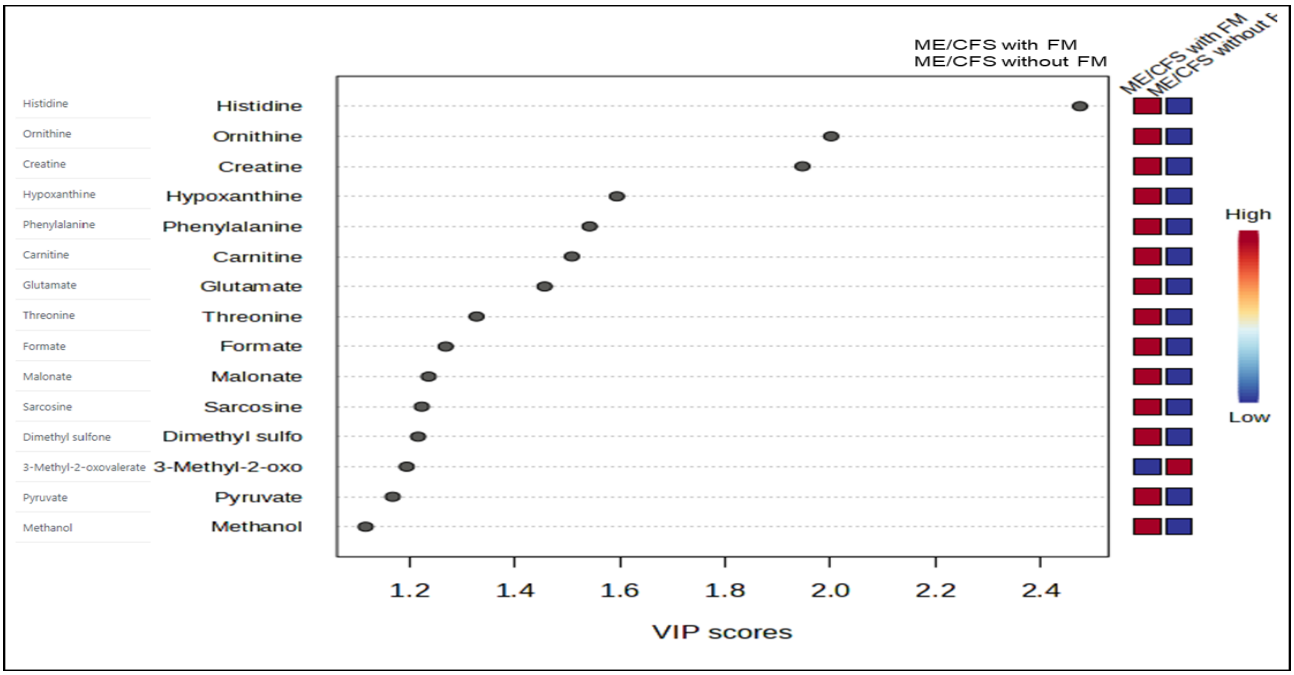
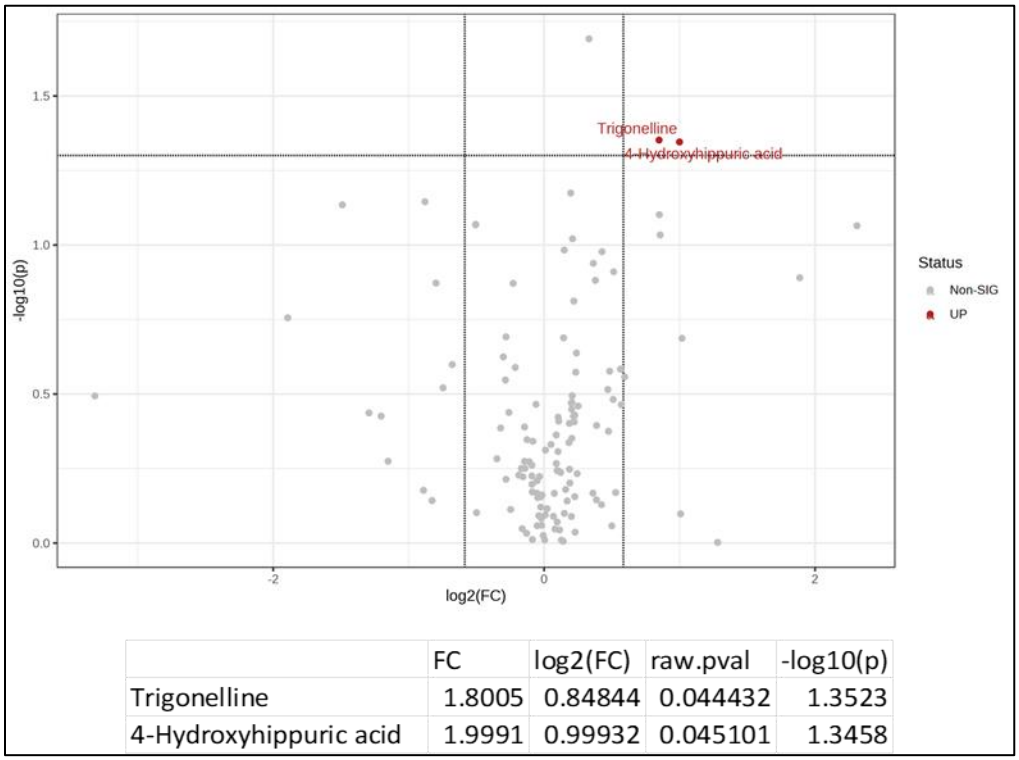



**Figure 5.1.16** VIP scores of corresponding PLSDA for NMR Plasma Part 2 ME/CFS with FM versus ME/CFS without FM

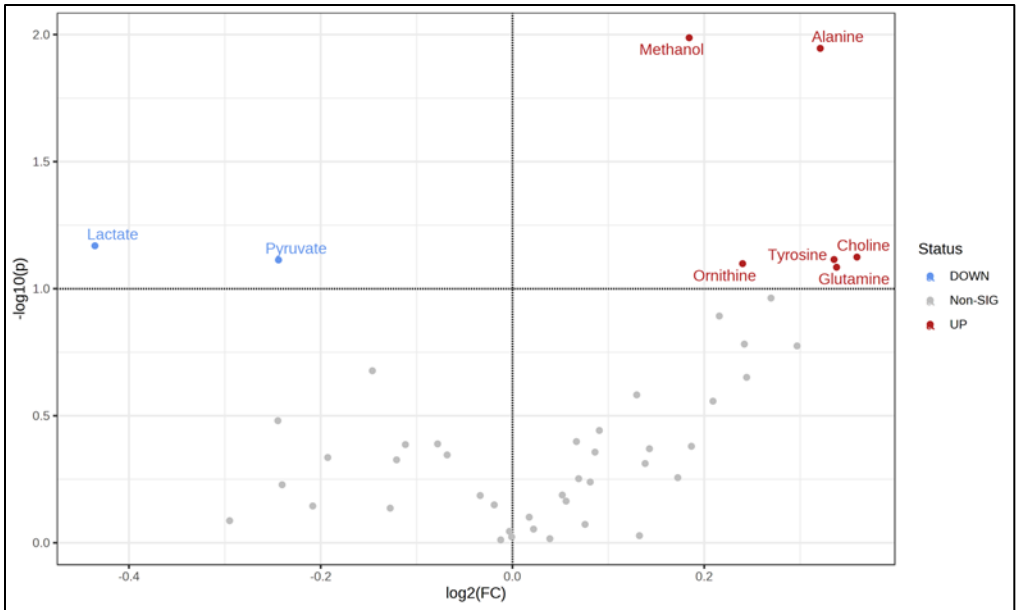
### 5.1.3 Plasma Comparison C) ME/CFS with IBS vs. ME/CFS without IBS

#### 5.1.3.1 Univariate Analysis




| | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|
| Trigonelline | 1.8005 | 0.84844 | 0.044432 | 1.3523 |
| 4-Hydroxyhippuric acid | 1.9991 | 0.99932 | 0.045101 | 1.3458 |

**Figure 5.1.17** Volcano plot of LCMS Plasma Part 2 ME/CFS with IBS versus ME/CFS without IBS. Metabolites with significantly higher (red) or lower (blue) concentrations in the ME/CFS with IBS cohort are highlighted (FC threshold 1.5 and uncorrected p-values ≤ 0.05) with non-significant metabolites represented in grey.




| | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|
| Methanol | 1.1363 | 0.18429 | 0.010295 | 1.9874 |
| Alanine | 1.2492 | 0.32098 | 0.01133 | 1.9458 |
| Lactate | 0.73946 | -0.43546 | 0.067787 | 1.1689 |
| Choline | 1.2828 | 0.35926 | 0.075104 | 1.1243 |
| Tyrosine | 1.2617 | 0.33538 | 0.076781 | 1.1147 |
| Pyruvate | 0.84439 | -0.24401 | 0.077084 | 1.113 |
| Ornithine | 1.181 | 0.24002 | 0.079698 | 1.0986 |
| Glutamine | 1.264 | 0.33802 | 0.082326 | 1.0845 |

**Figure 5.1.18** Volcano plot of NMR Plasma Part 2 ME/CFS with IBS versus ME/CFS without IBS. Metabolites with significantly higher (red) or lower (blue) concentrations in the ME/CFS with IBS cohort are highlighted (FC threshold 1.0 and uncorrected p-values ≤ 0.10) with non-significant metabolites represented in grey.

### 5.1.3.2 Multivariate Analysis (PCA)

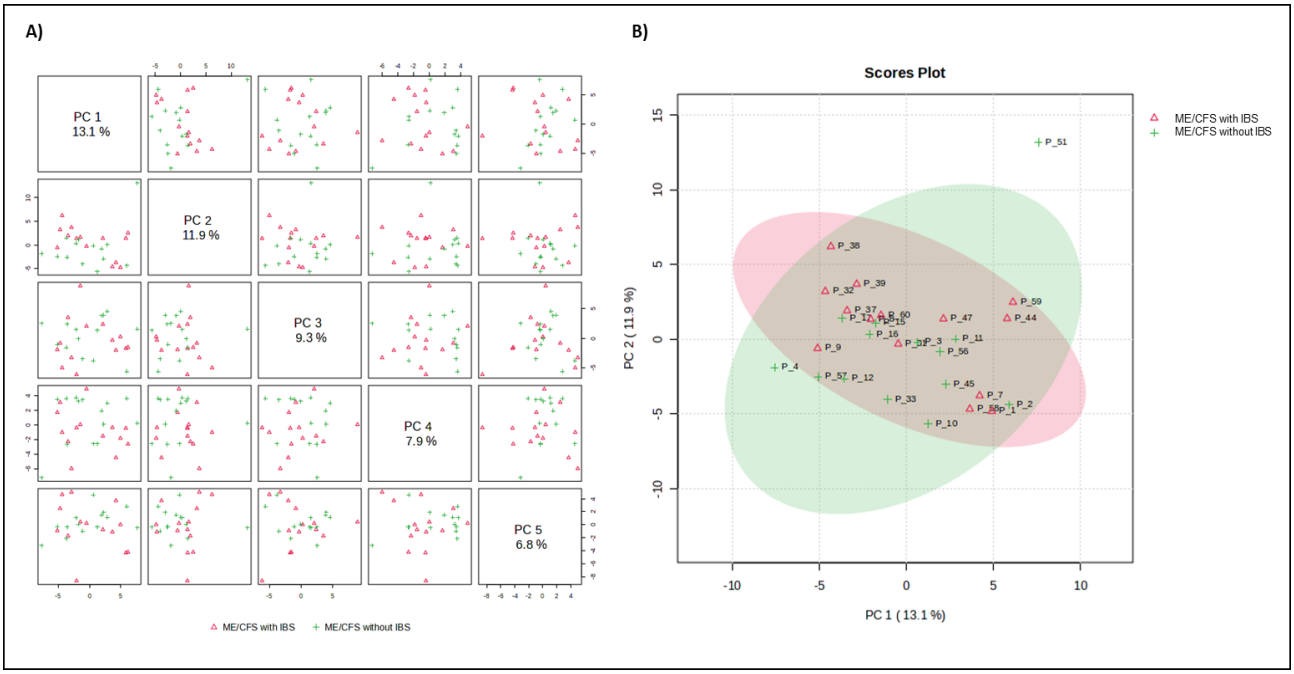



**Figure 5.1.19** PCA plots A) overview B) PC1 and PC2 only of LCMS Plasma Part 2 ME/CFS with IBS versus ME/CFS without IBS

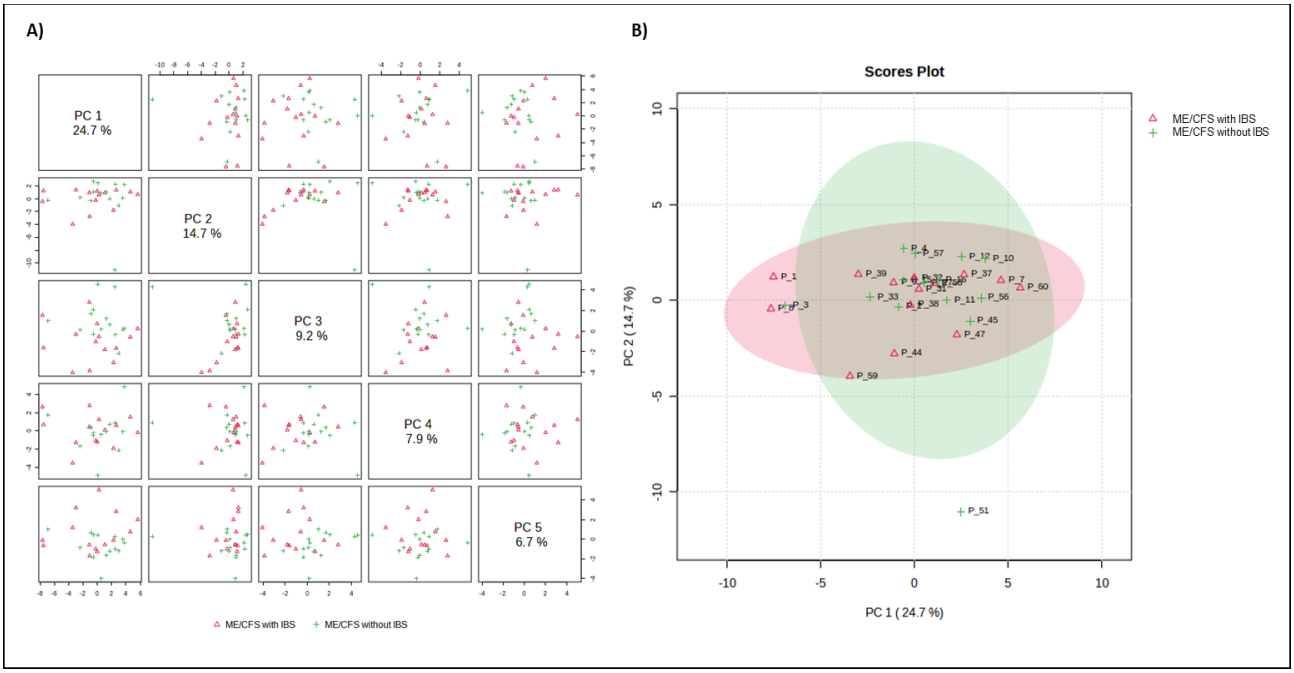



**Figure 5.1.20** PCA plots A) overview B) PC1 and PC2 only of NMR Plasma Part 2 ME/CFS with IBS versus ME/CFS without IBS

### 5.1.3.3 Multivariate Analysis (PLS-DA)

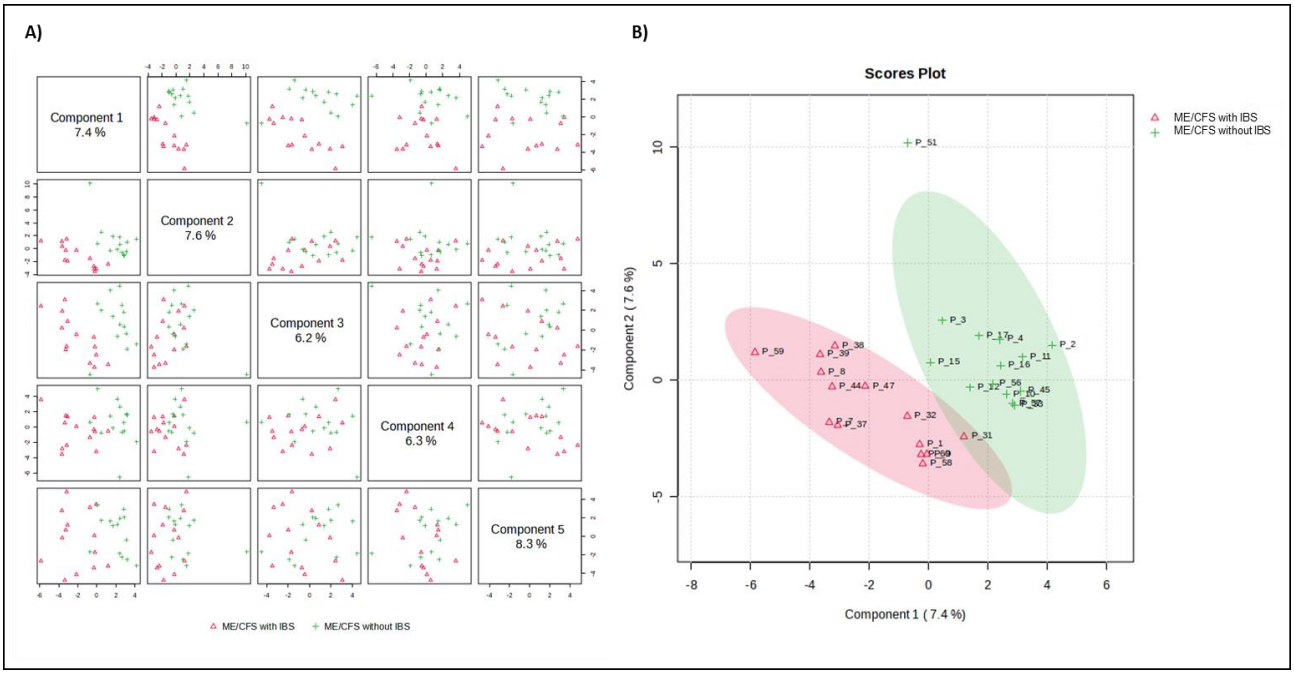



**Figure 5.1.21** PLSDA A) overview B) components 1 and 2 only of LCMS Plasma Part 2 ME/CFS with IBS versus ME/CFS without IBS

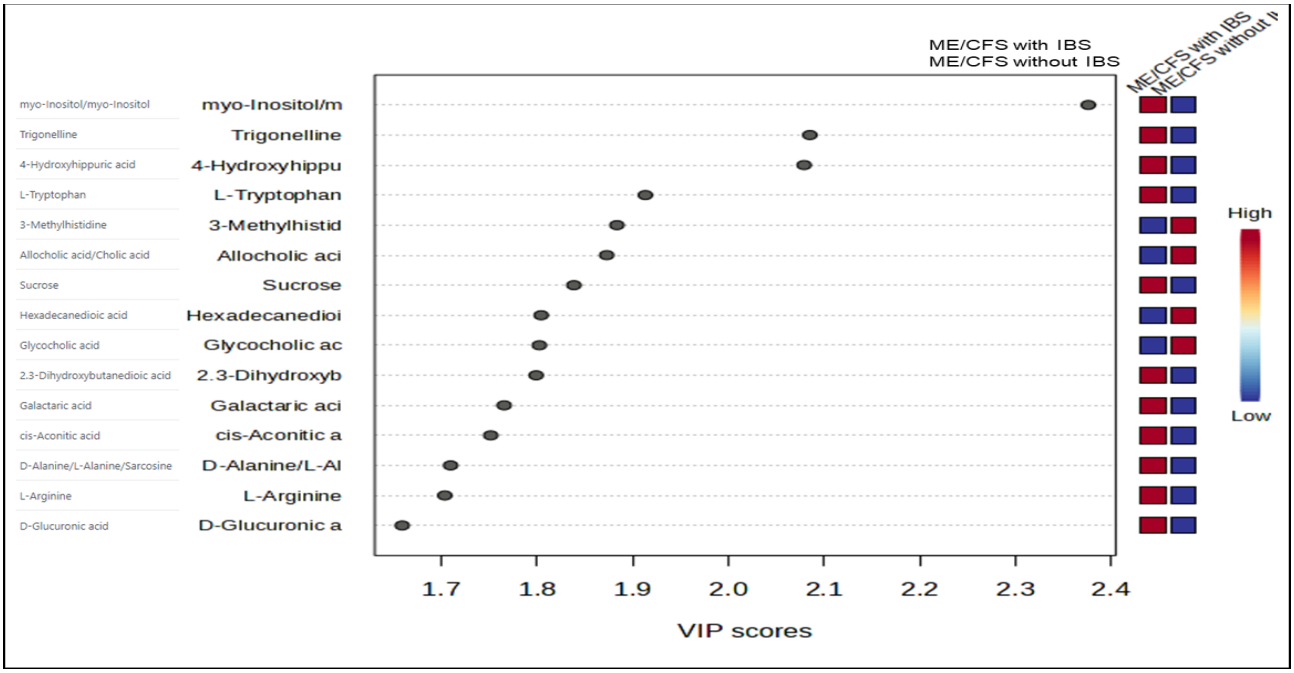



**Figure 5.1.22** VIP scores of corresponding PLSDA for LCMS Plasma Part 2 ME/CFS with IBS versus ME/CFS without IBS

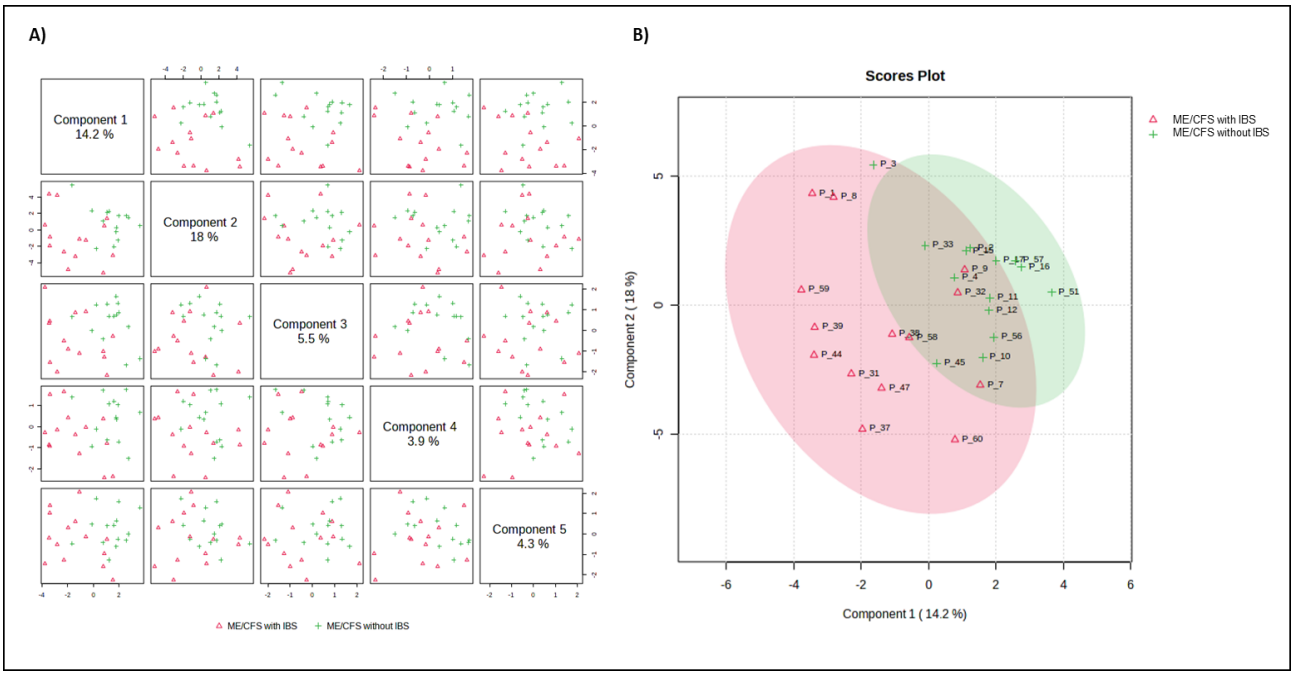



**Figure 5.1.23** PLSDA A) overview B) components 1 and 2 only of NMR Plasma Part 2 ME/CFS with IBS versus ME/CFS without IBS

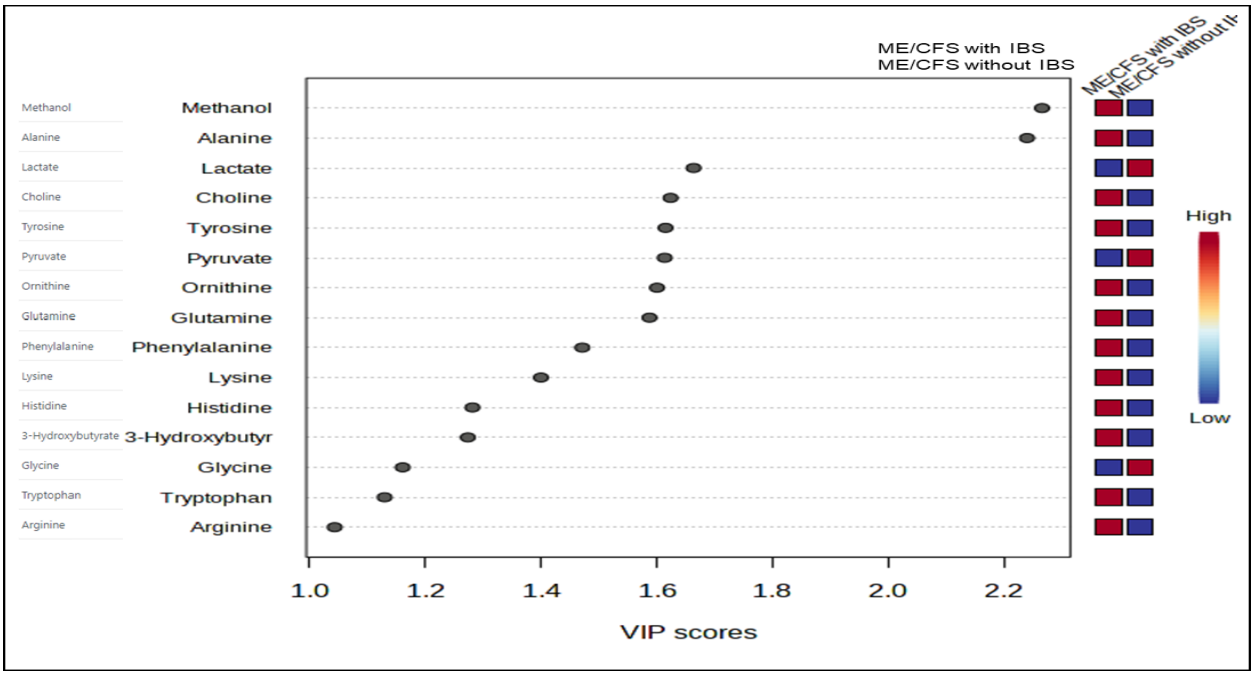
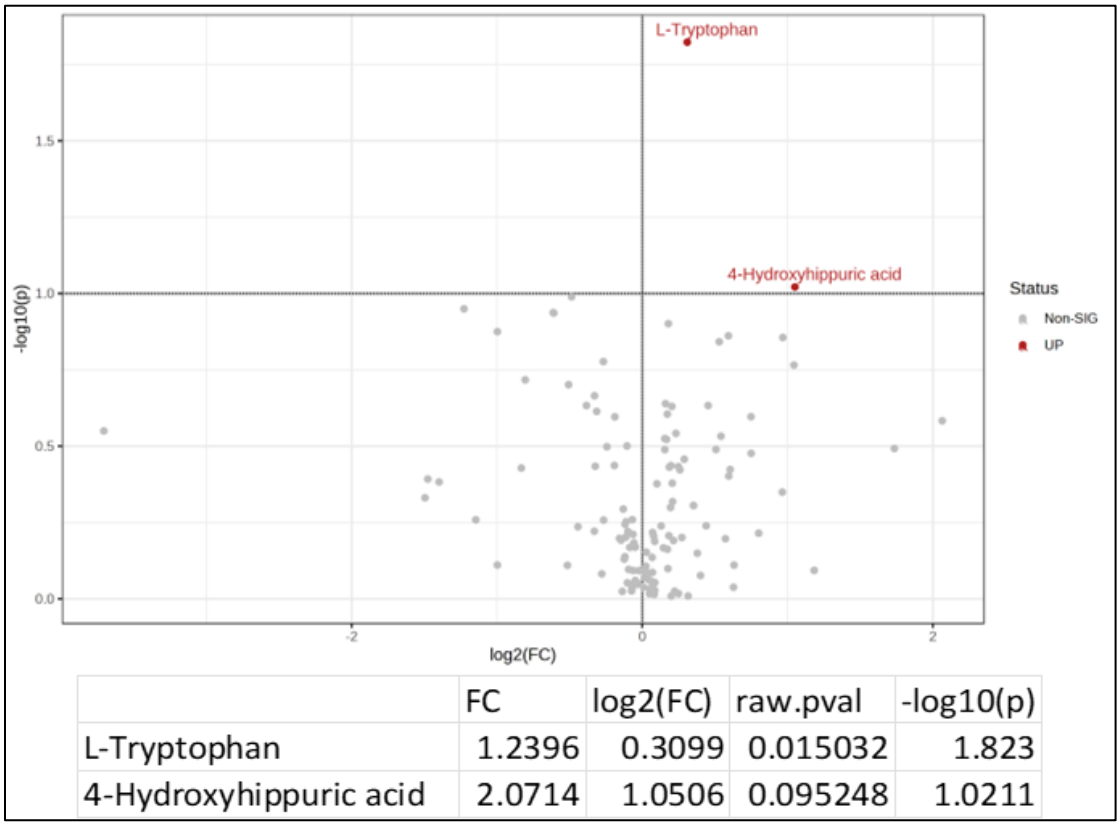



**Figure 5.1.24** VIP scores of corresponding PLSDA for NMR Plasma Part 2 ME/CFS with IBS versus ME/CFS without IBS

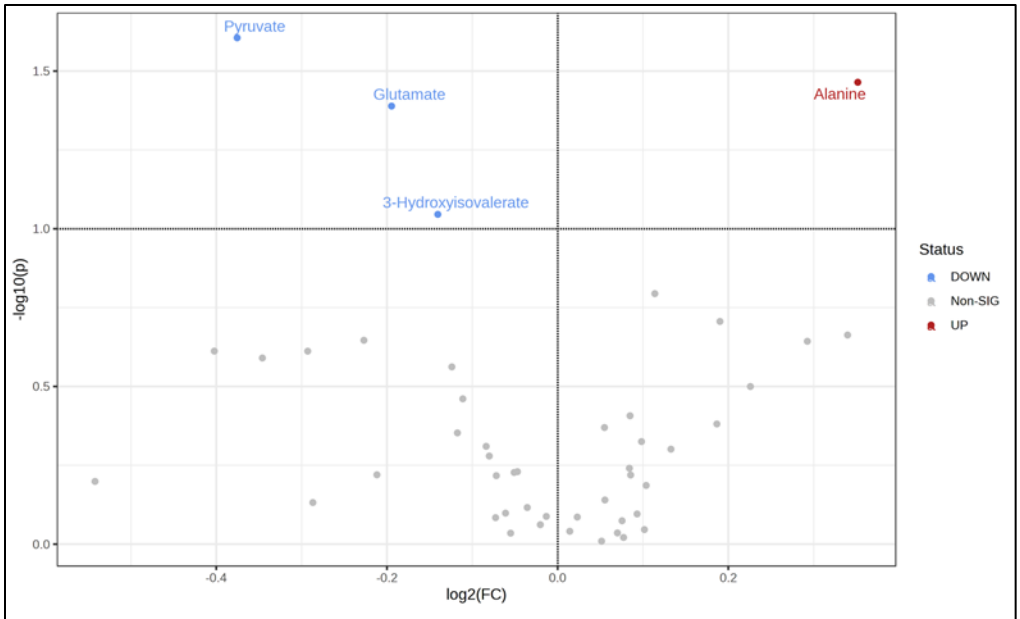
### 5.1.4 Plasma Comparison D) Age-matched ME/CFS with IBS vs. ME/CFS without IBS

#### 5.1.4.1 Univariate Analysis




| | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|
| L-Tryptophan | 1.2396 | 0.3099 | 0.015032 | 1.823 |
| 4-Hydroxyhippuric acid | 2.0714 | 1.0506 | 0.095248 | 1.0211 |

**Figure 5.1.25** Volcano plot of LCMS Plasma Part 2 age-matched ME/CFS with IBS versus ME/CFS without IBS. Metabolites with significantly higher (red) or lower (blue) concentrations in the age-matched ME/CFS with IBS cohort are highlighted (FC threshold 1.0 and uncorrected p-values ≤ 0.10) with non-significant metabolites represented in grey.




| | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|
| Pyruvate | 0.77083 | -0.37551 | 0.02481 | 1.6054 |
| Alanine | 1.2761 | 0.3517 | 0.034319 | 1.4645 |
| Glutamate | 0.87382 | -0.19458 | 0.040834 | 1.389 |
| 3-Hydroxyisovalerate | 0.90728 | -0.14039 | 0.090026 | 1.0456 |

**Figure 5.1.26** Volcano plot of NMR Plasma Part 2 age-matched ME/CFS with IBS versus ME/CFS without IBS. Metabolites with significantly higher (red) or lower (blue) concentrations in the age-matched ME/CFS with IBS cohort are highlighted (FC threshold 1.0 and uncorrected p-values ≤ 0.10) with non-significant metabolites represented in grey.

### 5.1.4.2 Multivariate Analysis (PCA)

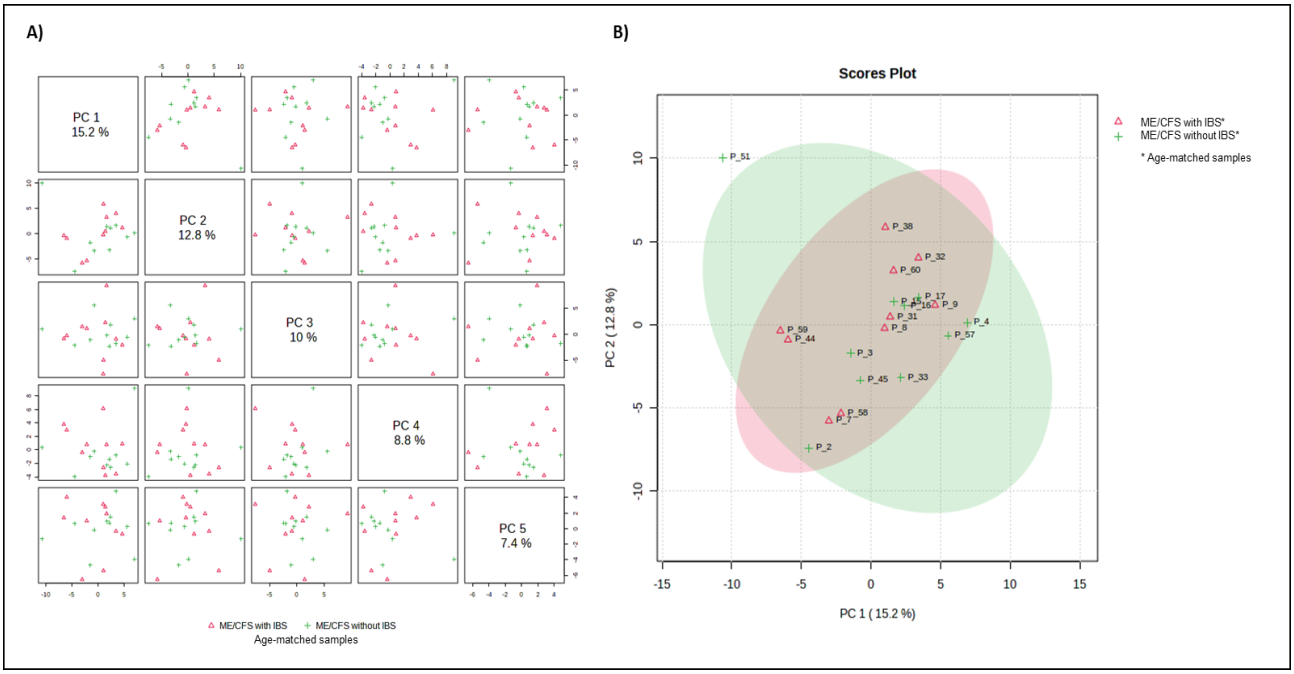



**Figure 5.1.27** PCA plots A) overview B) PC1 and PC2 only of LCMS Plasma Part 2 age-matched ME/CFS with IBS versus ME/CFS without IBS

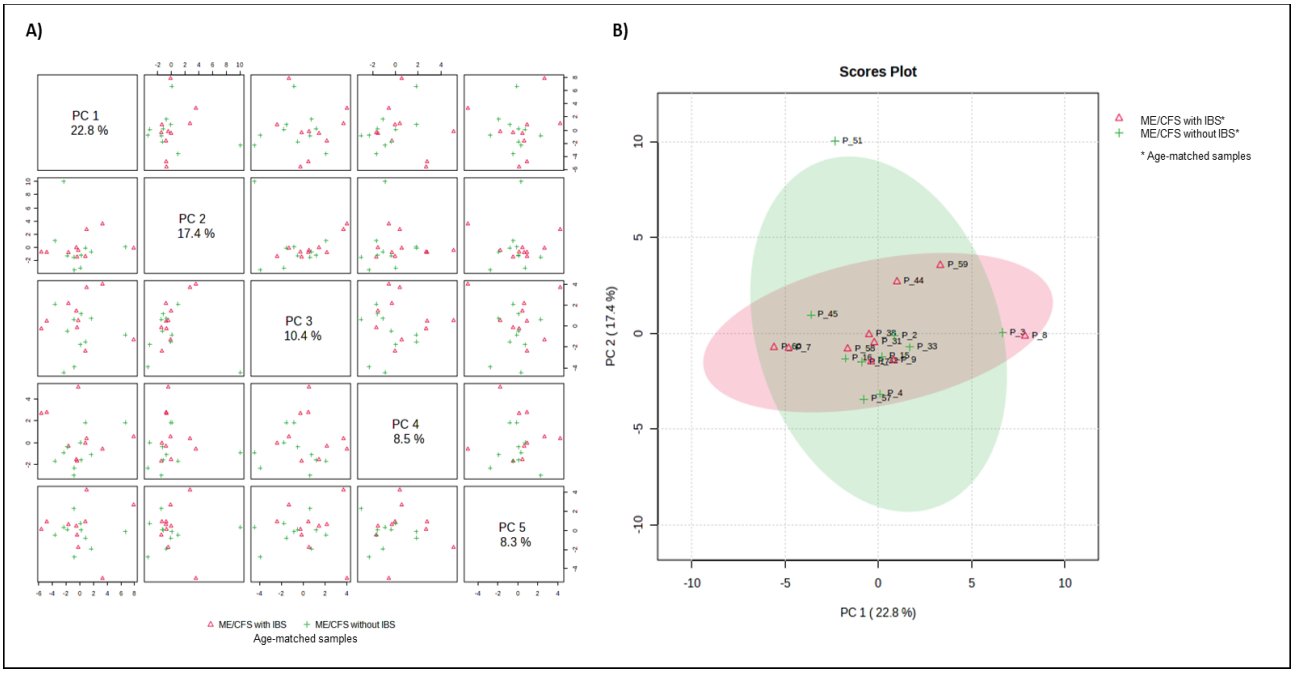



**Figure 5.1.28** PCA plots A) overview B) PC1 and PC2 only of NMR Plasma Part 2 age-matched ME/CFS with IBS versus ME/CFS without IBS

### 5.1.4.3 Multivariate Analysis (PLS-DA)

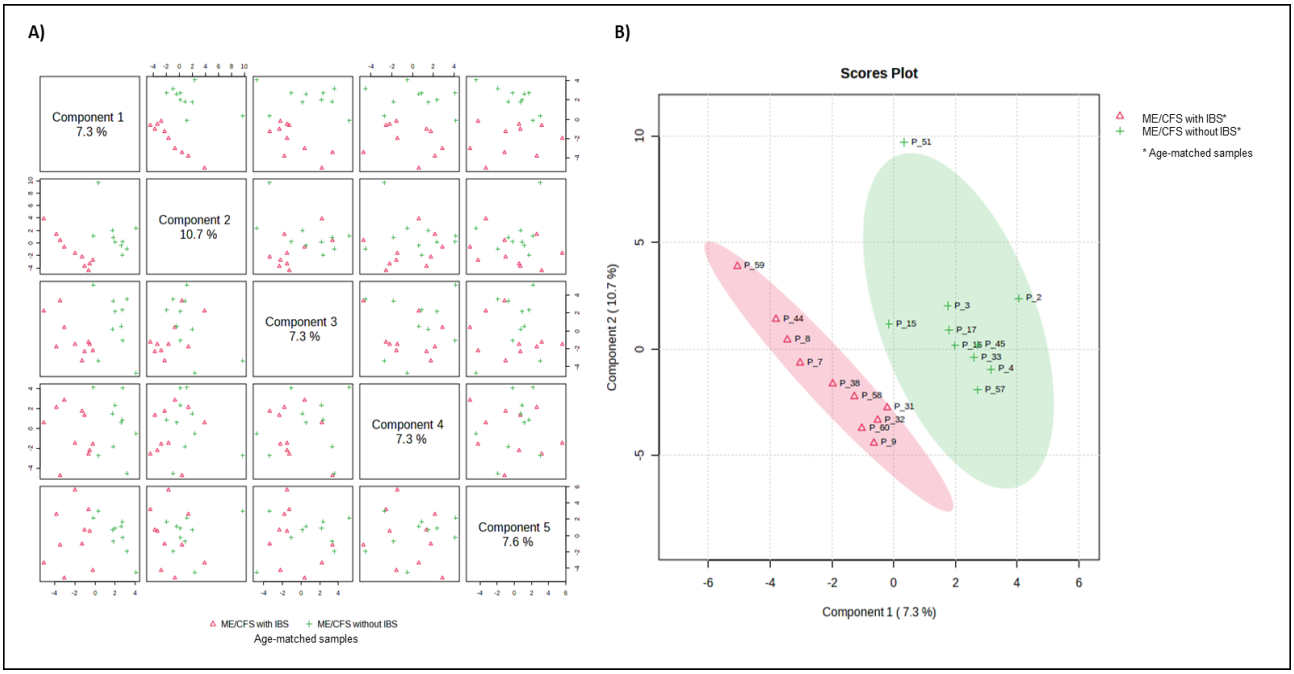



**Figure 5.1.29** PLSDA A) overview B) components 1 and 2 only of LCMS Plasma Part 2 age-matched ME/CFS with IBS versus ME/CFS without IBS

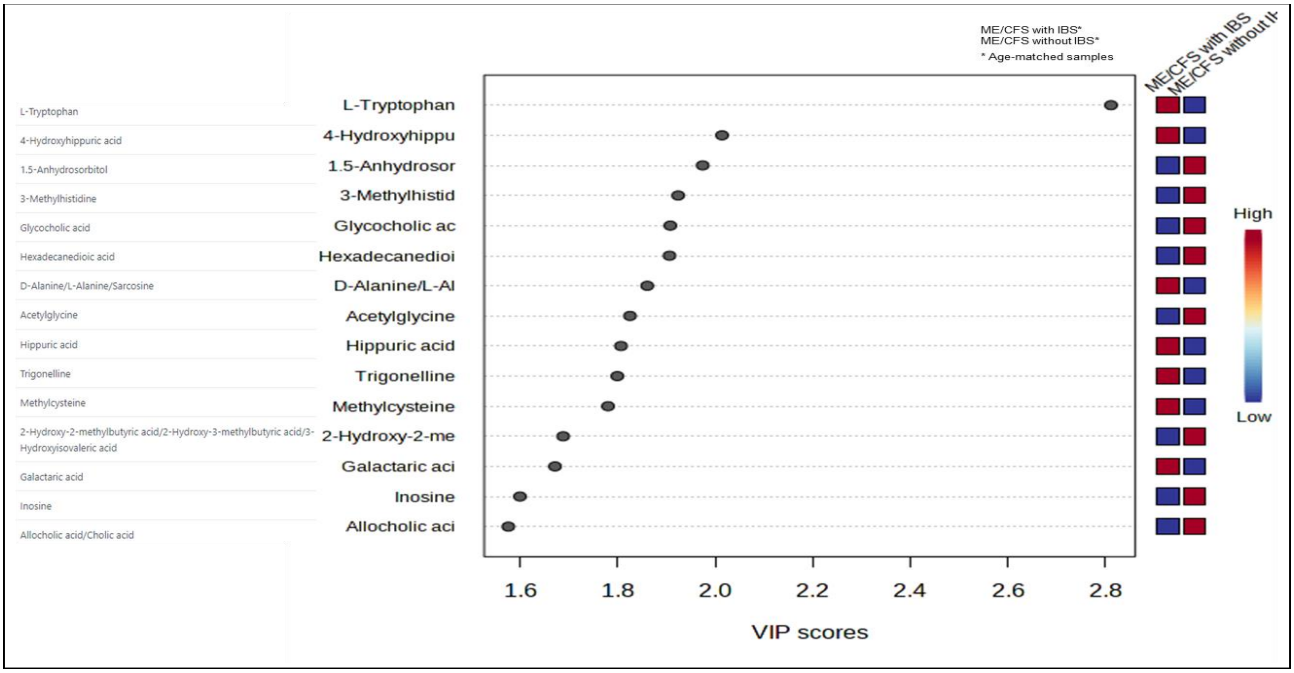



**Figure 5.1.30** VIP scores of corresponding PLSDA for LCMS Plasma Part 2 age-matched ME/CFS with IBS versus ME/CFS without IBS

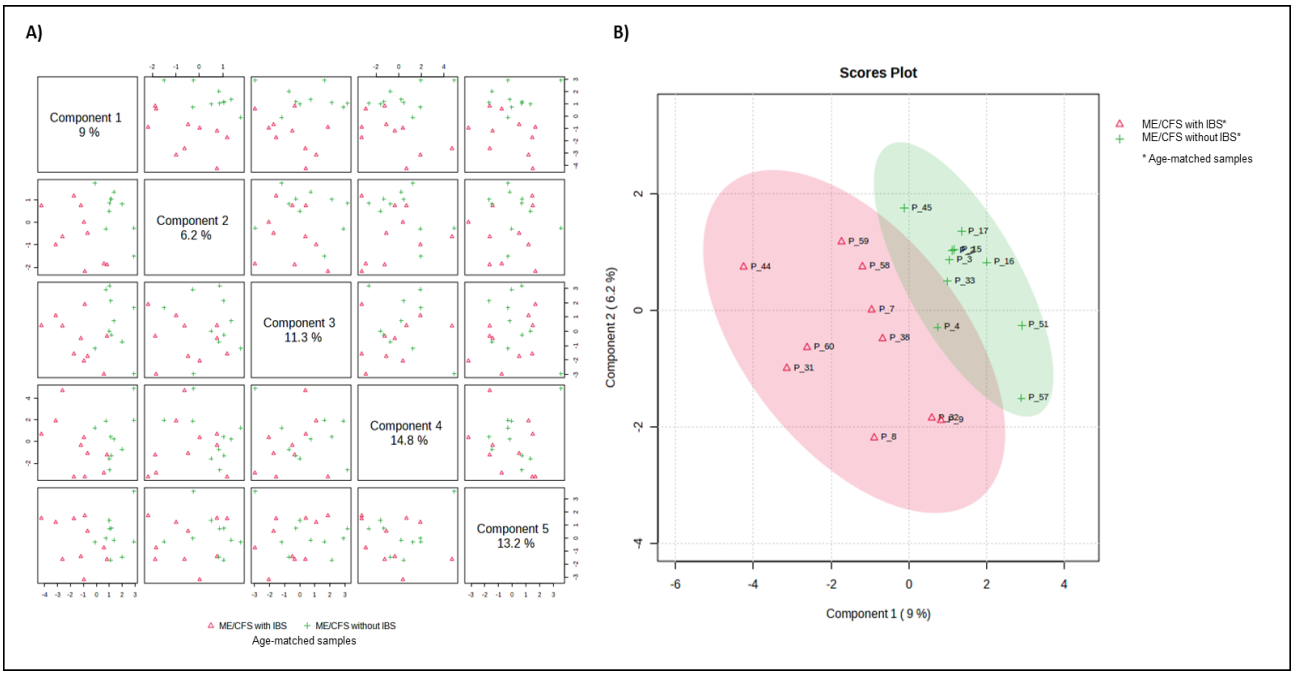



**Figure 5.1.31** PLSDA A) overview B) components 1 and 2 only of NMR Plasma Part 2 age-matched ME/CFS with IBS versus ME/CFS without IBS

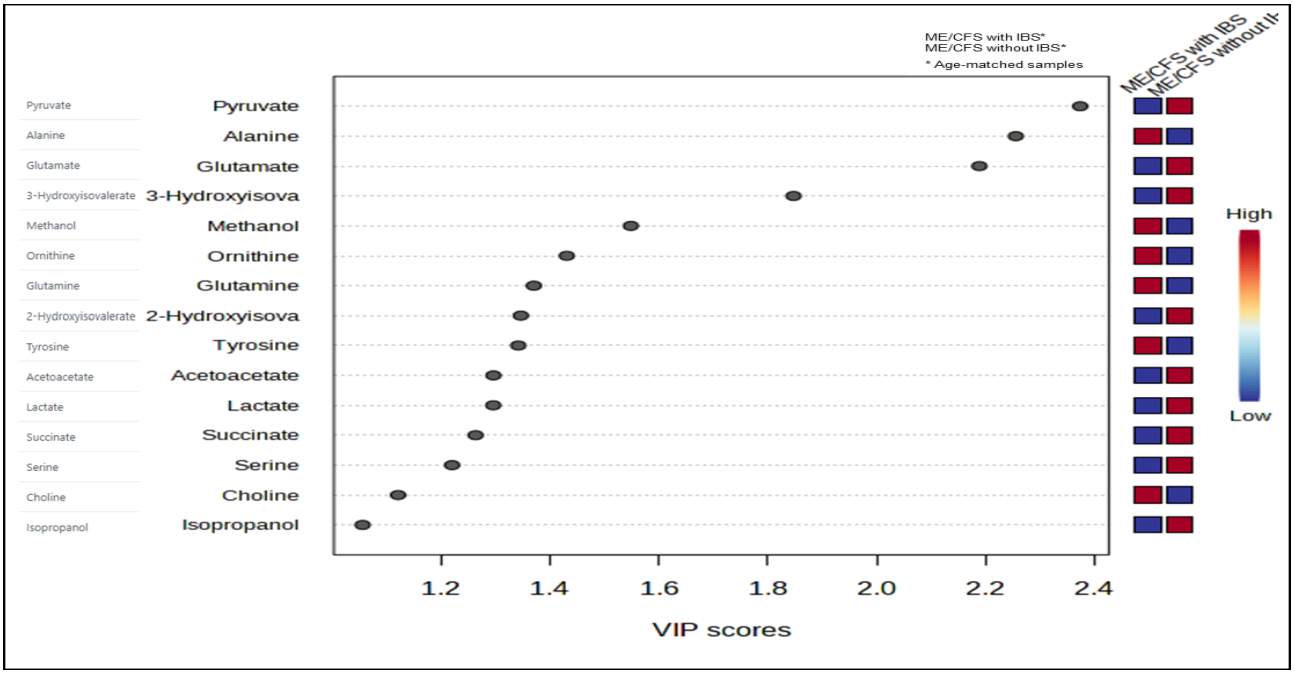



**Figure 5.1.32** VIP scores of corresponding PLSDA for NMR Plasma Part 2 age-matched ME/CFS with IBS versus ME/CFS without IBS

## 5.2 Urine Metabolomics Results

Globally, LCMS detected 147 and NMR detected 26 urine metabolite features from all samples (Appendix 10). 83 significantly different metabolite features in total from LCMS and NMR were highlighted from the volcano plot (VPs) and PLS-DA VIP plots across all the comparisons, Comparison A-D (Table 5.2.1). These significantly different metabolites, categorised according to the HMDB database, included amino acids, peptides and analogues (31.33%), fatty acyls and acids (9.64%), carboxylic acids and derivatives (12.05%), sugar, carbohydrates and carbohydrate conjugates (10.84%), other metabolites (6.02%), purine and purine derivatives (1.20%), organic acids and derivatives (10.84%), benzenoids (12.05%), organic nitrogen compounds (3.61%), and alcohols and polyols (2.41%). There were two significant differential urine features (valine and lactic acid) that were measured by both analytical platforms, LCMS and NMR (Table 5.2.1). The relative concentrations found for these shared metabolites were consistent as either up/higher/red or down/lower/blue from the VP and PLS-DA analyses (Appendix 11). The individual plots from the VP, PCA, and PLS-DA analyses for the urine metabolomic outcomes in Comparisons A-D are shown in Sections 5.2.1 (ME/CFS overall vs. Control), 5.2.2 (ME/CFS +/- FM), 5.2.3 (ME/CFS +/- IBS), and 5.2.4 (age-matched ME/CFS +/- IBS**), respectively.

**Table 5.2.1** LCMS and NMR Urine Metabolome (Part Two) Significant Features and Results

| | Urine Part Two Metabolite | Comparative Group (A-D) | Group with significant elevation in relative concentration | LCMS or NMR |
|---|---|---|---|---|
| Amino acids, peptides and analogues | L-Cystathionine | A | Control | LCMS |
| | Phenylacetylglycine | A | Control | LCMS |
| | D-Glutamic acid/L-Glutamic acid | A | ME/CFS | LCMS |
| | Alanine | A | Control | NMR |
| | Creatine | A, B, C, D | Control, ME/CFS + FM, ME/CFS + IBS, ME/CFS + IBS** | NMR |
| | Histidine | A, C, D | ME/CFS, ME/CFS + IBS, ME/CFS + IBS** | NMR |
| | Valine (aka. L-Valine) ‡ | A, B, C, D | Control, ME/CFS - FM, ME/CFS - IBS, ME/CFS - IBS** | NMR, LCMS |
| | Creatinine | A, C, D | ME/CFS, ME/CFS - IBS, ME/CFS - IBS** | NMR |
| | Glycine | A, C, D | Control, ME/CFS + IBS, ME/CFS + IBS** | NMR |
| | Alanylglycine | B | ME/CFS - FM | LCMS |
| | Dimethylglycine | B | ME/CFS - FM | LCMS |
| | L-Tyrosine | B | ME/CFS - FM | LCMS |
| | Citrulline | B | ME/CFS - FM | LCMS |
| | D-Lysine/L-Lysine | B | ME/CFS - FM | LCMS |
| | Cysteine | B, C, D | ME/CFS - FM, ME/CFS + IBS, ME/CFS + IBS** | NMR |
| | Isobutyrylglycine | C, D | ME/CFS - IBS, ME/CFS - IBS** | LCMS |
| | Beta-Alanine | C, D | ME/CFS - IBS, ME/CFS - IBS** | LCMS |
| | 3-Methylhistidine | C, D | ME/CFS - IBS, ME/CFS - IBS** | LCMS |
| | Guanidoacetate | C, D | ME/CFS + IBS, ME/CFS + IBS** | NMR |
| | Urea | C, D | ME/CFS - IBS, ME/CFS - IBS** | NMR |
| | Taurine | C, D | ME/CFS - IBS, ME/CFS - IBS** | NMR |
| | Beta-Leucine/L-Alloisoleucine/L-Isoleucine | D | ME/CFS - IBS** | LCMS |
| | Beta-Leucine | | | |
| | L-Alloisoleucine | | | |
| | L-Isoleucine | | | |
| | 4-Hydroxyproline | D | ME/CFS - IBS** | LCMS |
| Fatty acyls and acids | Adipic acid | A | ME/CFS | LCMS |
| | Acetate | A, B, C, D | ME/CFS, ME/CFS + FM, ME/CFS + IBS, ME/CFS + IBS** | NMR |
| | Formate | A, B, C | Control, ME/CFS - FM, ME/CFS - IBS | NMR |
| | Succinic acid semialdehyde (MH2) | B | ME/CFS + FM | LCMS |
| | 2-Isopropylmalic acid | C, D | ME/CFS + IBS, ME/CFS + IBS** | LCMS |
| | Stearic acid | C | ME/CFS + IBS | LCMS |
| | 3-Methylglutaconic acid | C | ME/CFS + IBS | LCMS |
| Carboxylic acid and derivatives | Dimethylmalonic acid/Ethylmalonic acid | A | ME/CFS | LCMS |
| | Ethylmalonic acid | | | |

| | | | | |
|---|---|---|---|---|
| | Dimethylmalonic acid | | | |
| | 2-Indolecarboxylic acid | A | Control | LCMS |
| | 3-Phenylbutyric acid | A, C, D | Control, ME/CFS + IBS, ME/CFS + IBS** | LCMS |
| | Isocitrate | A, B | ME/CFS, ME/CFS + FM | NMR |
| | Succinate | A, B, D | ME/CFS, ME/CFS - FM, ME/CFS + IBS** | NMR |
| | Citrate | A | Control | NMR |
| | Maleate | B | ME/CFS + FM | NMR |
| | Fumarate | B, C | ME/CFS + FM, ME/CFS + IBS | NMR |
| | Malonate | B | ME/CFS - FM | NMR |
| Sugar, carbohydrates and carbohydrate conjugates | Gluconic acid | A | Control | LCMS |
| | Lactulose/Sucrose/Turanose | A | Control | LCMS |
| | Lactulose | | | |
| | Sucrose | | | |
| | Turanose | | | |
| | 1.5-Anhydrosorbitol | B | ME/CFS + FM | LCMS |
| | Dihydroxyacetone* | B | ME/CFS + FM | LCMS |
| | Glyceraldehyde* | B | ME/CFS + FM | LCMS |
| | Glucose | D | ME/CFS - IBS** | NMR |
| Other metabolites | L-Gulonolactone | B | ME/CFS + FM | LCMS |
| | Loperamide | B | ME/CFS + FM | LCMS |
| | Kynurenic acid | C | ME/CFS - IBS | LCMS |
| | Trigonelline* | C | ME/CFS + IBS | LCMS |
| | Deoxycytidine | D | ME/CFS + IBS** | LCMS |
| # | 7-Methylguanine | C, D | ME/CFS - IBS, ME/CFS - IBS** | LCMS |
| Organic acids and derivatives | (S)-3-Hydroxyisobutyric acid/Alpha-Hydroxyisobutyric acid | A | ME/CFS | LCMS |
| | (S)-3-Hydroxyisobutyric acid | | | |
| | Alpha-Hydroxyisobutyric acid | | | |
| | 2-Ketobutyric acid | A | ME/CFS | LCMS |
| | Pyruvic acid | B | ME/CFS - FM | LCMS |
| | Hydroxypropionic acid | B | ME/CFS + FM | LCMS |
| | D-Lactic acid (aka. Lactate) * ‡ | B | ME/CFS + FM | LCMS, NMR |
| | Oxoglutaric acid/Oxoglutaric acid | B, C | ME/CFS + FM, ME/CFS - IBS | LCMS |
| | Indoxyl sulfate | C | ME/CFS - IBS | LCMS |
| Benzenoids | Pyrocatechol | A, C, D | Control, ME/CFS + IBS, ME/CFS + IBS** | LCMS |
| | 2.6-Dihydroxybenzoic acid | A, C, D | Control, ME/CFS + IBS, ME/CFS + IBS** | LCMS |
| | 4-Hydroxyphenylpyruvic acid | A | Control | LCMS |
| | p-Hydroxymandelic acid | A | Control | LCMS |
| | Hippurate | A, B, C, D | Control, ME/CFS - FM, ME/CFS + IBS, ME/CFS + IBS** | NMR |
| | 2.3.4-Trihydroxybenzoic acid | B | ME/CFS + FM | LCMS |
| | 4-Hydroxyhippuric acid | C, D | ME/CFS + IBS, ME/CFS + IBS** | LCMS |
| | p-Aminobenzoic acid* | C | ME/CFS + IBS | LCMS |
| | Vanillylmandelic acid | D | ME/CFS - IBS** | LCMS |

| | | | | |
|---|---|---|---|---|
| | Benzoic acid | D | ME/CFS + IBS** | LCMS |
| Organic nitrogen compounds | Dimethylamine | A, B, C, D | Control, ME/CFS + FM, ME/CFS - IBS, ME/CFS - IBS** | NMR |
| | Trimethylamine N-oxide | A, B | Control, ME/CFS - FM | NMR |
| | Ethanolamine | A, B, D | ME/CFS, ME/CFS - FM, ME/CFS + IBS** | NMR |
| ## | myo-Inositol/myo-Inositol | B | ME/CFS - FM | LCMS |
| | Methanol | C | ME/CFS + IBS | NMR |
| NOTE | # Purine and purine derivatives<br>## Alcohols and polyols<br>* From LCMS consolidated metabolite feature<br>**Comparative Group (A-D)**<br>A) ME/CFS overall vs. Control [ME/CFS, Control]<br>B) ME/CFS with FM vs. ME/CFS without FM [ME/CFS + FM, ME/CFS - FM]<br>C) ME/CFS with IBS vs. ME/CFS without IBS [ME/CFS + IBS, ME/CFS - IBS]<br>D) ME/CFS with IBS vs. ME/CFS without IBS (age-matched) [ME/CFS + IBS**, ME/CFS - IBS**]<br>‡ "Duplicate" metabolite assayed by LCMS and NMR<br>Refer to **Appendix 11** for the electronic version of this table and raw data input | | | |

## 5.2 Urine Metabolomics Results continued…

The significantly differential urine metabolite features were elevated in their relative concentrations depending on how the samples were grouped with their ME/CFS, FM, or IBS metadata that comprised Comparison A-D (Table 5.2.1). A total of 14, 18, 7, and 6 metabolite features were found only to be significantly different in Comparison A (ME/CFS overall vs. Control), B (ME/CFS +/- FM), C (ME/CFS +/- IBS), and D (age-matched ME/CFS +/- IBS**), respectively; for example, Alanine was only differential and elevated in the Control group from Comparison A. The metabolites, for example, Taurine and Dimethylamine, that were highlighted in the ME/CFS +/- IBS and ME/CFS +/- IBS** groups (Comparison C and D), were always consistently lower or higher in their relative concentration providing some indication of IBS involvement where an attempt to mitigate the age variable has been made. Acetate and fumarate were consistently elevated in ME/CFS + either FM, IBS, or IBS** (Comparison B-D).

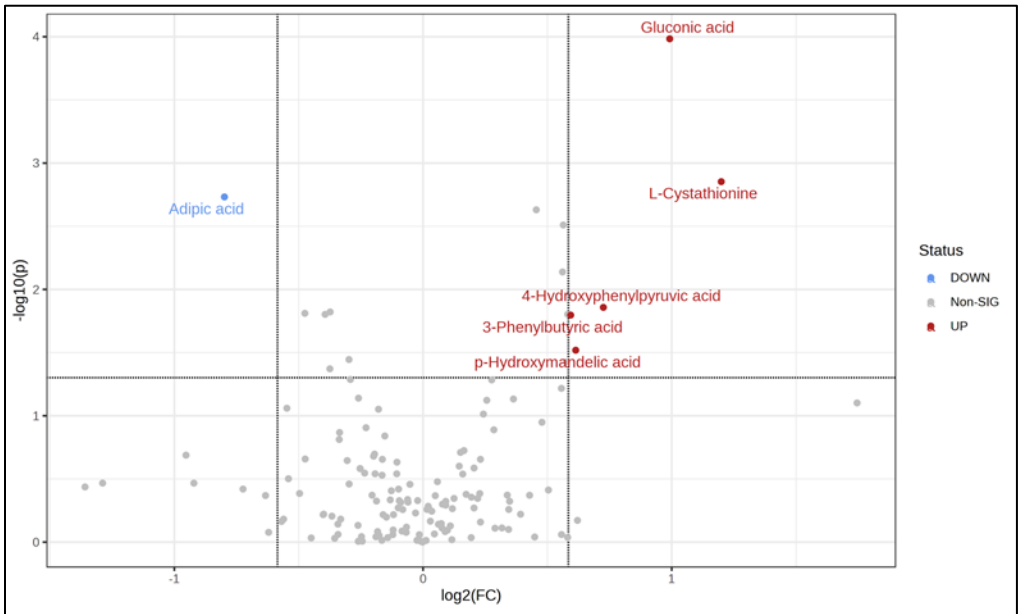
VPs of the LCMS urine data matrix highlighted 6 metabolites (1 down and 5 up) in the Control vs. ME/CFS (Figure 5.2.1), 2 metabolites (both up) in the ME/CFS +/- FM (Figure 5.2.9), 8 metabolites (4 down and 4 up) in the ME/CFS +/- IBS (Figure 5.2.17), and 2 metabolites (1 down and 1 up) in the ME/CFS +/- IBS** (Figure 5.2.25) comparisons that were significantly different. VPs of the NMR urine data matrix highlighted 4 metabolites (3 down and 1 up) in the Control vs. ME/CFS (Figure 5.2.2), 3 metabolites (2 down and 1 up) in the ME/CFS +/- FM (Figure 5.2.10), 5 metabolites (3 down and 2 up) in the ME/CFS +/- IBS (Figure 5.2.18), and 1 metabolite (up) in the ME/CFS +/- IBS** (Figure 5.2.26) comparisons that were significantly different. The metabolites from the VPs that were also found to be significantly different by PLS-DA VIP analyses reflected and supported the same pattern as either high or low in relative concentration (Appendix 11). The PCAs from the LCMS and NMR data matrices did not show a clear separation between the groups for all comparisons (Table 5.2.2). Although the PCAs did not discriminate the groups, the clusters extended in different directions and tighter clusters identified by the ellipsoid shape were observed from LCMS ME/CFS + FM (Figure 5.2.11) and LCMS ME/CFS + IBS** (Figure 5.2.27). The PLS-DAs demonstrated improved clustering and separation for Comparison A-D (Table 5.2.2). The PLS-DA for the LCMS data matrix found a distinguished separation between the ME/CFS +/- FM (Figure 5.2.13) and ME/CFS +/- IBS (Figure 5.2.21) comparisons. A complete to distinguished separation for LCMS (Figure 5.2.29) and NMR (Figure 5.2.31), respectively, was also observed between the ME/CFS +/- IBS** groups.

**Table 5.2.2** Summary of the total variance from the first two components of the PCA and PLS-DA analyses (LCMS and NMR Urine Metabolome (Part Two))

| Urine Part Two | | PCA | | | | PLSDA | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | % of the variance | | | Separation | % of the variance | | | Separation |
| | | PC1 | PC2 | Total† | | Comp. 1 | Comp. 2 | Total‡ | |
| A) ME/CFS vs. Control | LCMS | 16.1 | 9.8 | 25.9 | Overlapping | 6.8 | 10 | 16.8 | Partial |
| | NMR | 16 | 13.6 | 29.6 | Overlapping | 7.3 | 11.5 | 18.8 | Very partial |
| B) ME/CFS +/- FM | LCMS | 15.5 | 11.4 | 26.9 | Overlapping | 8.7 | 9.6 | 18.3 | Distinguished |
| | NMR | 18.8 | 15.5 | 34.3 | Overlapping | 14.2 | 10.9 | 25.1 | Very partial |
| C) ME/CFS +/- IBS | LCMS | 15.5 | 11.4 | 26.9 | Overlapping | 6.9 | 9.8 | 16.7 | Distinguished |
| | NMR | 18.8 | 15.5 | 34.3 | Overlapping | 13.9 | 9.8 | 23.7 | Partial |
| D) ME/CFS +/- IBS** | LCMS | 16.8 | 14.8 | 31.6 | Overlapping | 5.3 | 10.9 | 16.2 | Complete |
| | NMR | 18.3 | 15.5 | 33.8 | Overlapping | 11.1 | 9.9 | 21 | Distinguished |
| † PC1 and PC2 added together | | | | | | | | | |
| ‡ Component 1 and Component 2 added together | | | | | | | | | |
| Distinguished to complete separation between groupings | | | | | | | | | |

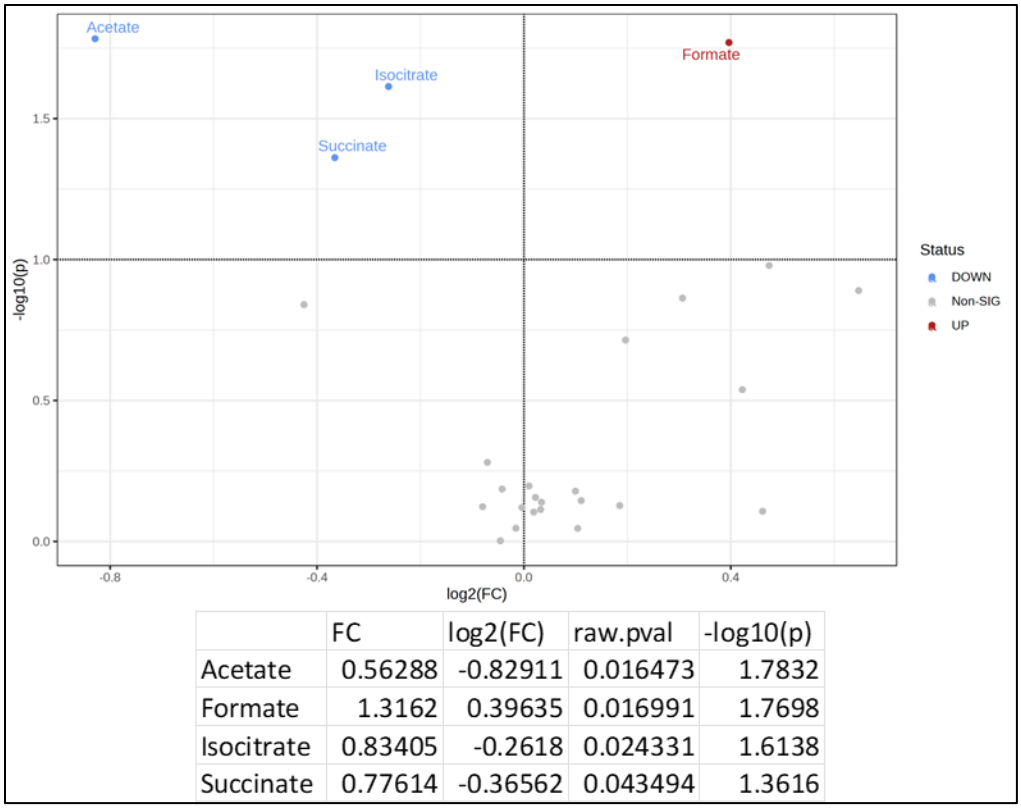
### 5.2.1 Urine Comparison A) ME/CFS Overall vs. Control Group

#### 5.2.1.1 Univariate Analysis




| | FC | log2(FC) | p | -log10(p) | q |
|---|---|---|---|---|---|
| Gluconic acid | 1.9889 | 0.99198 | 0.0001039 | 3.9834 | 0.015274 |
| L-Cystathionine | 2.2961 | 1.1992 | 0.0014027 | 2.853 | NS |
| Adipic acid | 0.5749 | -0.79862 | 0.0018528 | 2.7322 | NS |
| 4-Hydroxyphenylpyruvic acid | 1.6531 | 0.72515 | 0.013875 | 1.8578 | NS |
| 3-Phenylbutyric acid | 1.5091 | 0.5937 | 0.015968 | 1.7968 | NS |
| p-Hydroxymandelic acid | 1.5305 | 0.61397 | 0.030263 | 1.5191 | NS |

**Figure 5.2.1** Volcano plot of LCMS Urine Part 2 ME/CFS overall vs Control groups using test outcomes from the raw p-values. Metabolites with significantly higher (blue) or lower (red) concentrations in the ME/CFS cohort are highlighted (FC threshold 1.5 and uncorrected p-values ≤ 0.05) with non-significant metabolites represented in grey. The q-values of significant FDR-adjusted metabolites are shown (NS = q-value ≥ 0.05).




| | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|
| Acetate | 0.56288 | -0.82911 | 0.016473 | 1.7832 |
| Formate | 1.3162 | 0.39635 | 0.016991 | 1.7698 |
| Isocitrate | 0.83405 | -0.2618 | 0.024331 | 1.6138 |
| Succinate | 0.77614 | -0.36562 | 0.043494 | 1.3616 |

**Figure 5.2.2** Volcano plot of NMR Urine Part 2 ME/CFS overall vs Control groups. Metabolites with significantly higher (blue) or lower (red) concentrations in the ME/CFS cohort are highlighted (FC threshold 1.0 and uncorrected p-values ≤ 0.10) with non-significant metabolites represented in grey.

### 5.2.1.2 Multivariate Analysis (PCA)

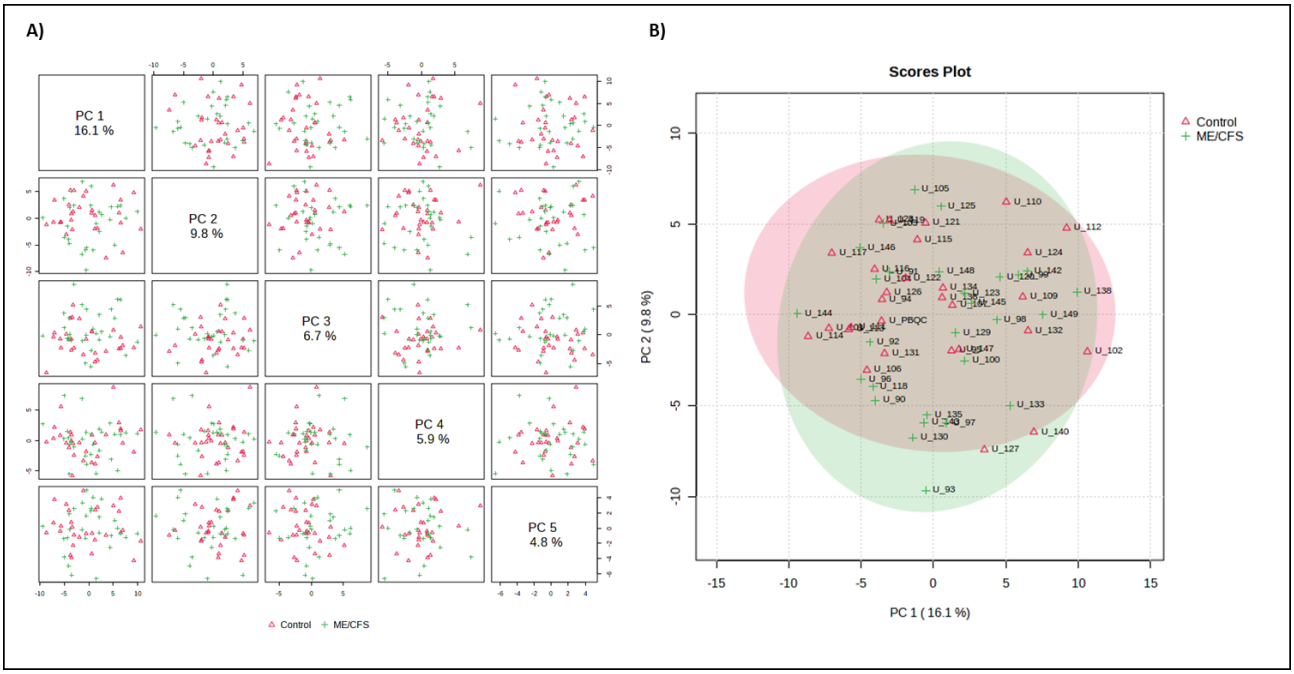



**Figure 5.2.3** PCA plots A) overview B) PC1 and PC2 only of LCMS Urine Part 2 ME/CFS overall vs. Control groups

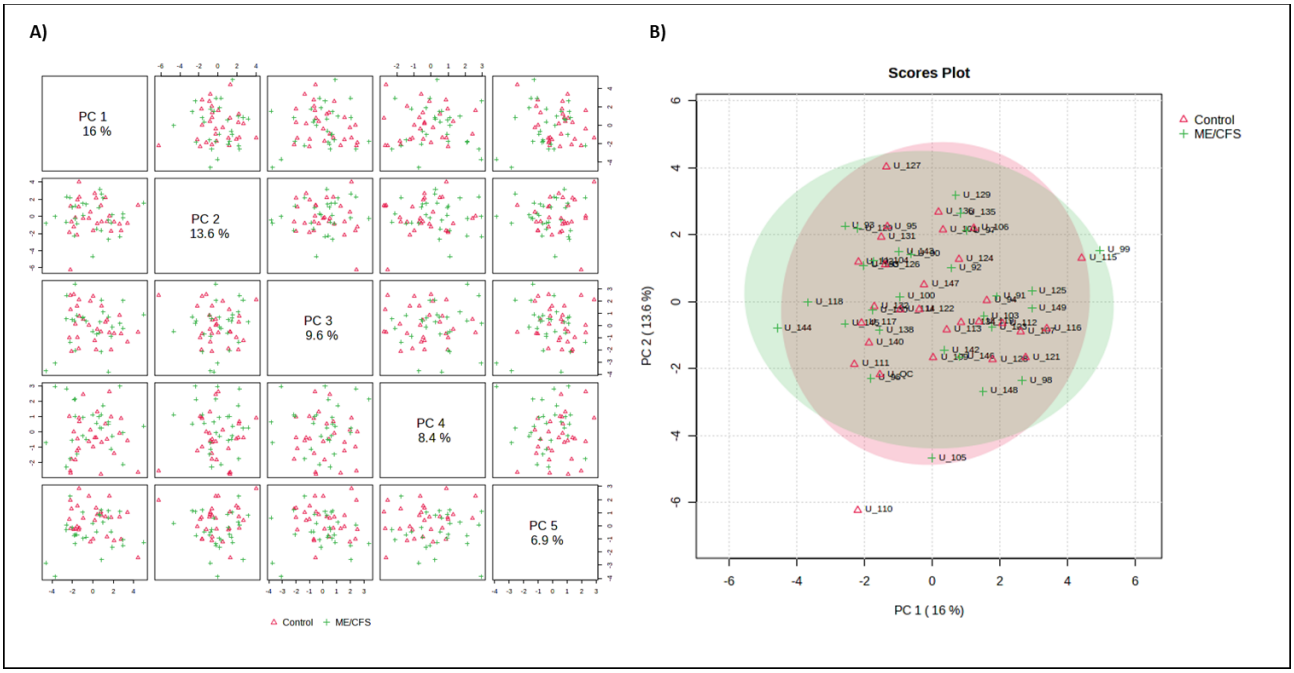



**Figure 5.2.4** PCA plots A) overview B) PC1 and PC2 only of NMR Urine Part 2 ME/CFS overall vs. Control groups

### 5.2.1.3 Multivariate Analysis (PLS-DA)

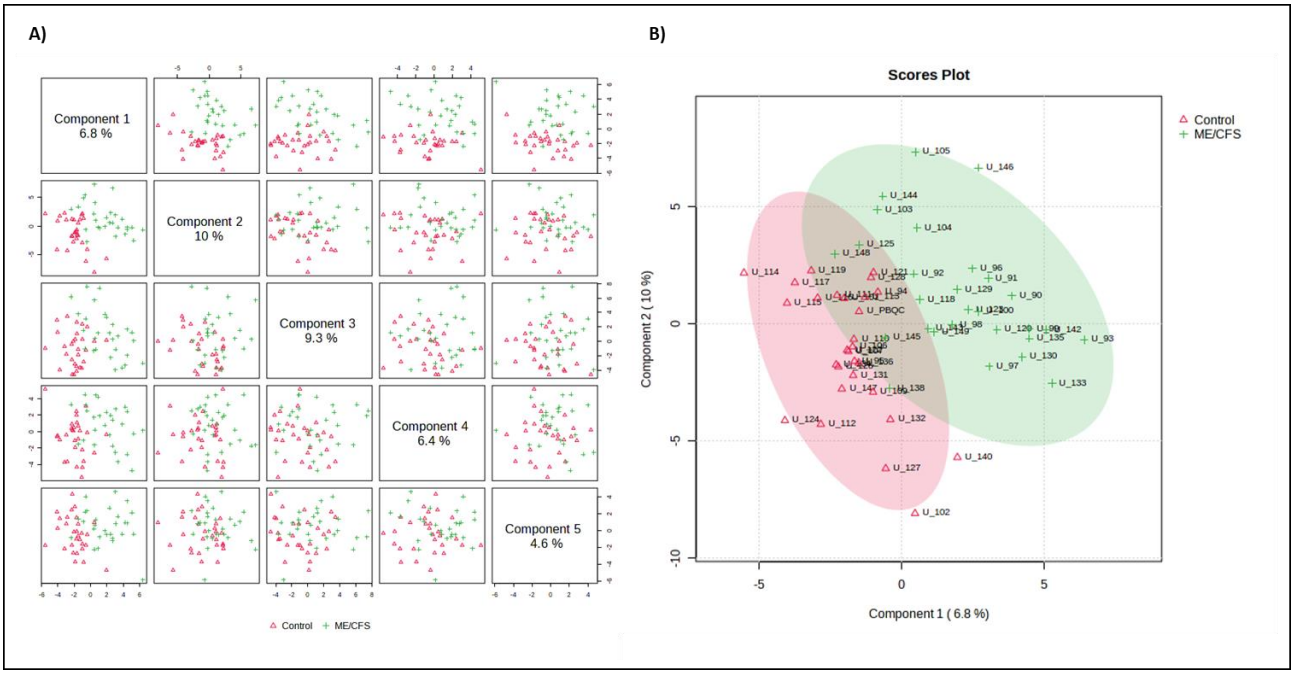



**Figure 5.2.5** PLSDA A) overview B) components 1 and 2 only of LCMS Urine Part 2 ME/CFS overall vs. Control groups

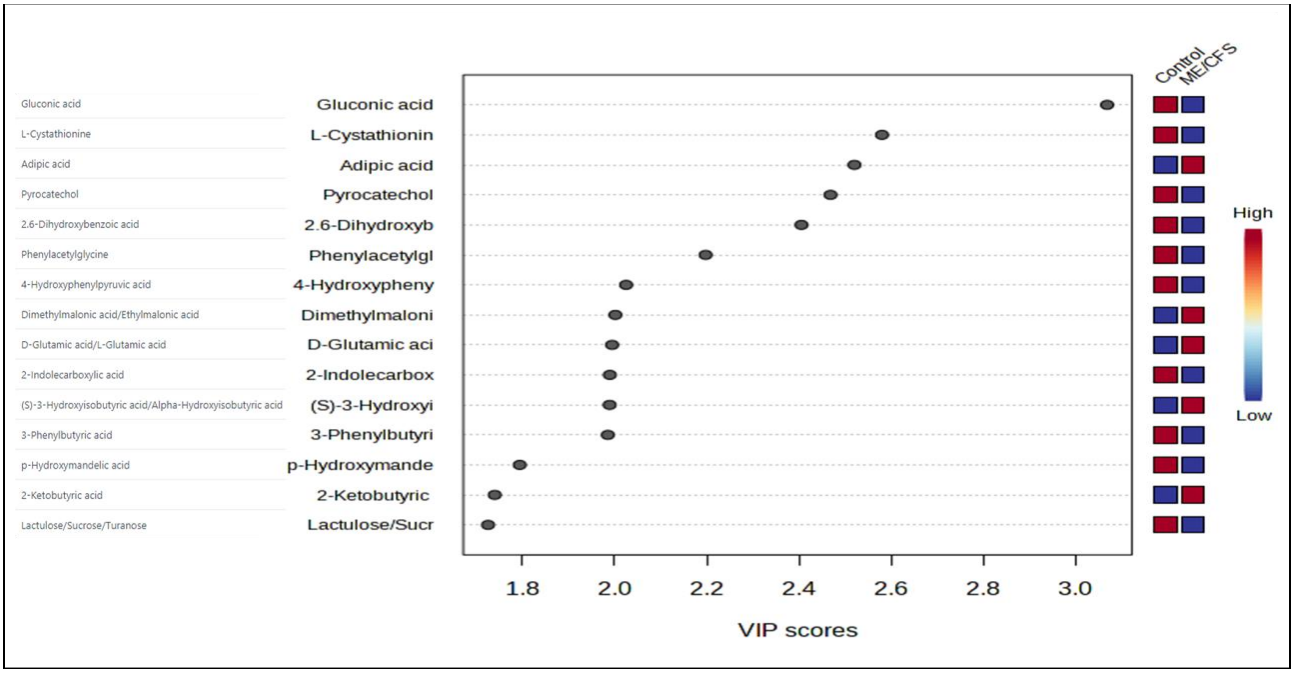



**Figure 5.2.6** VIP scores of corresponding PLSDA for LCMS Urine Part 2 ME/CFS overall vs. Control groups

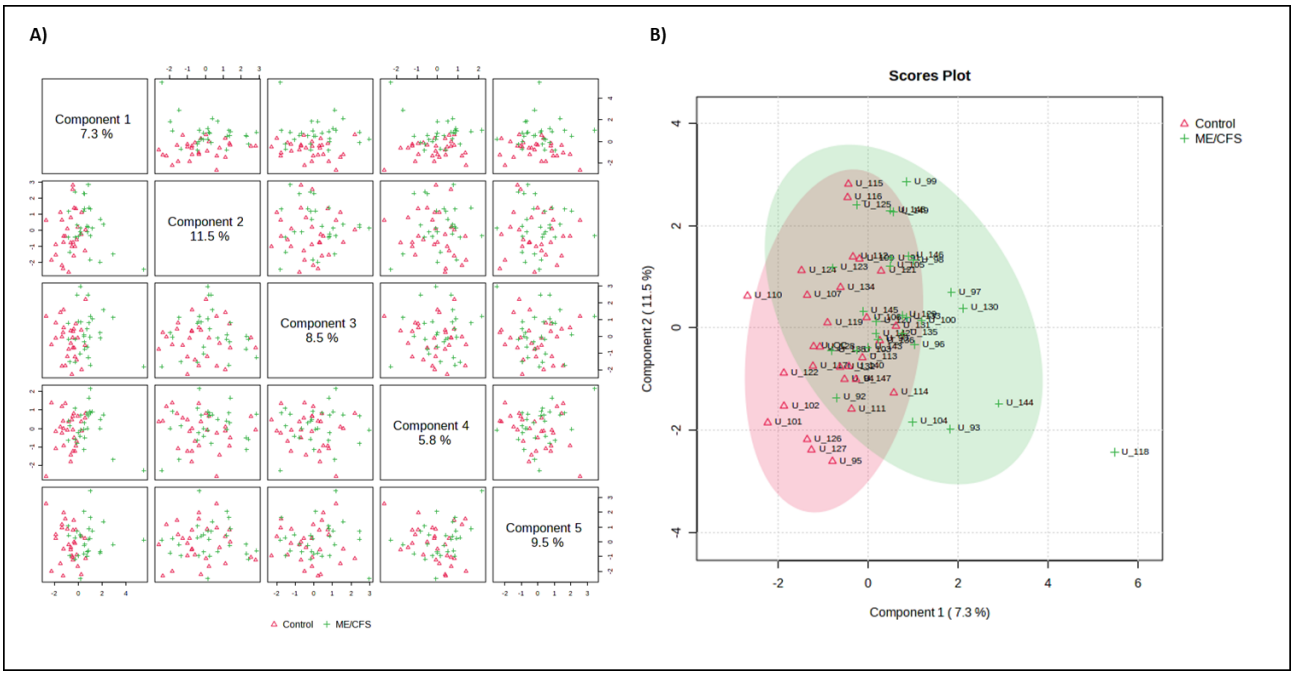



**Figure 5.2.7** PLSDA A) overview B) components 1 and 2 only of NMR Urine Part 2 ME/CFS overall vs. Control groups

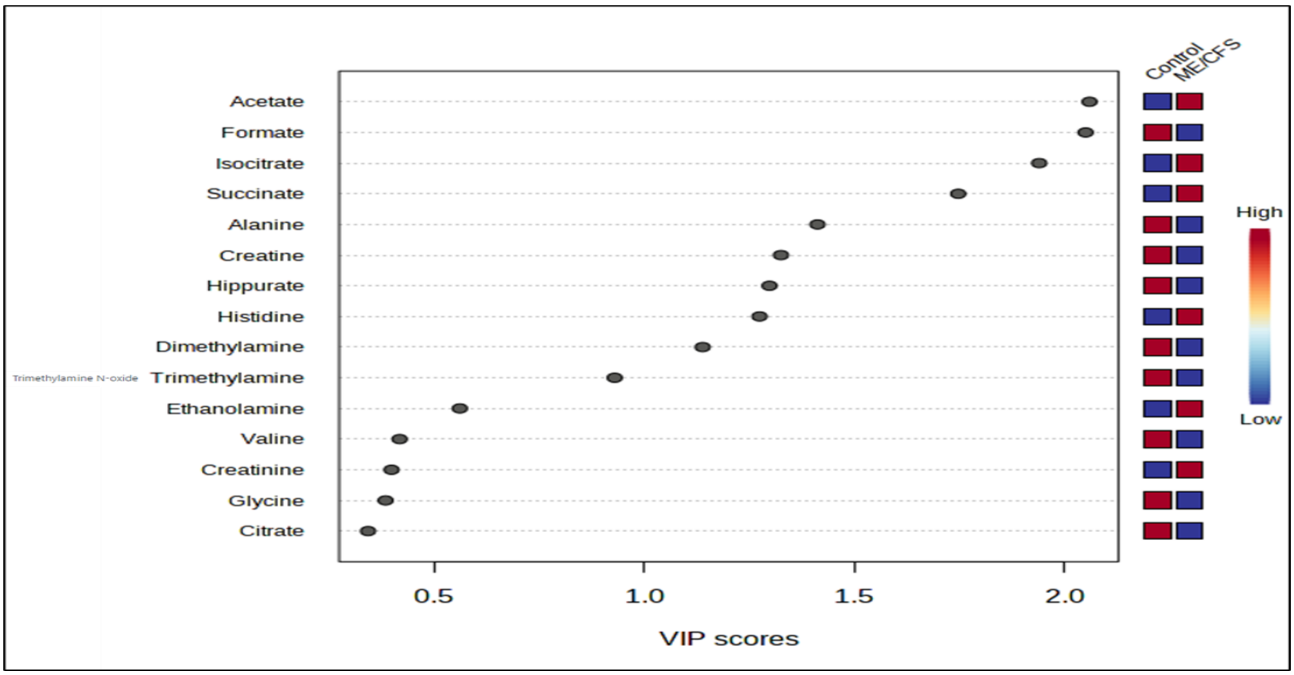
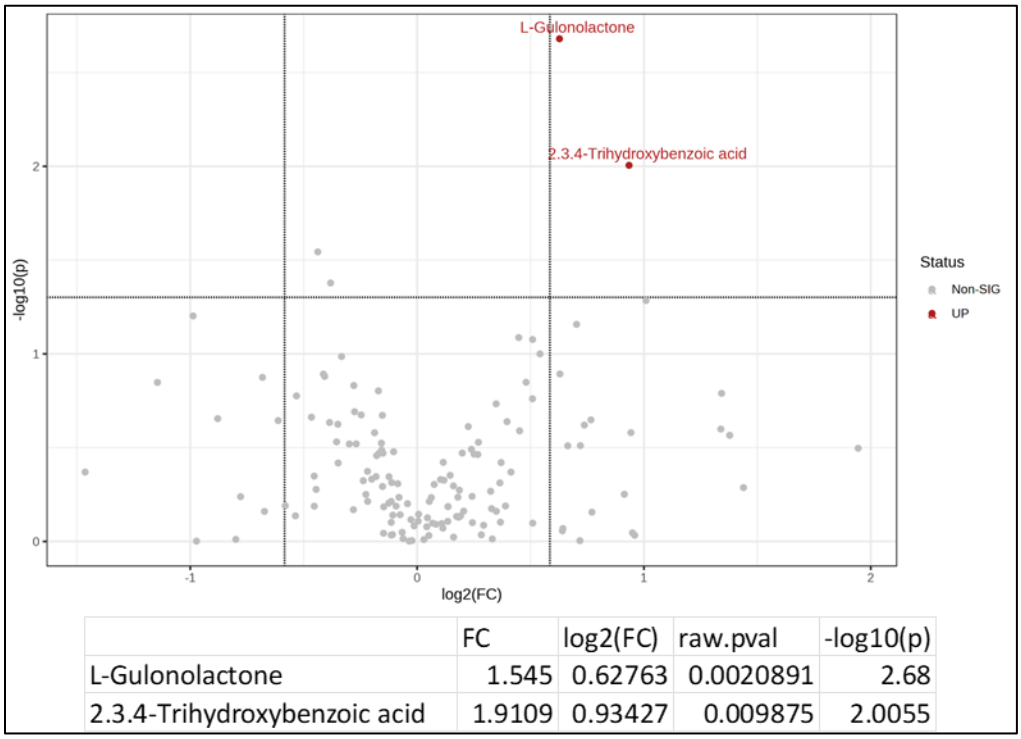



**Figure 5.2.8** VIP scores of corresponding PLSDA for NMR Urine Part 2 ME/CFS overall vs. Control groups

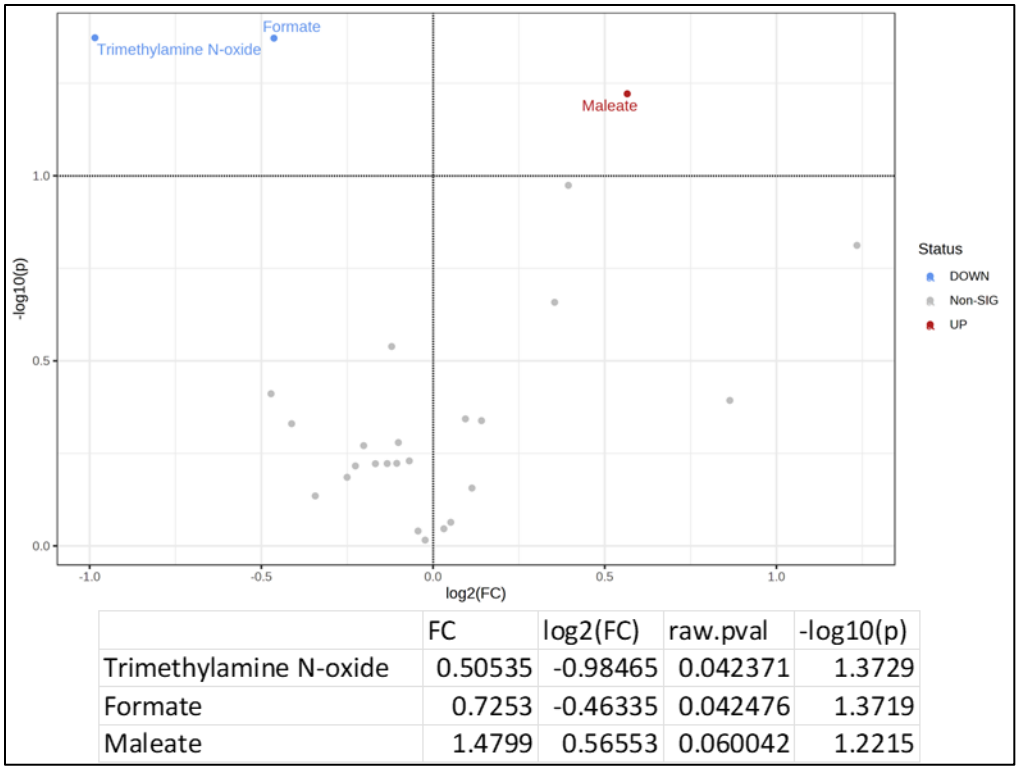
### 5.2.2 Urine Comparison B) ME/CFS with FM vs. ME/CFS without FM

#### 5.2.2.1 Univariate Analysis




| | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|
| L-Gulonolactone | 1.545 | 0.62763 | 0.0020891 | 2.68 |
| 2.3.4-Trihydroxybenzoic acid | 1.9109 | 0.93427 | 0.009875 | 2.0055 |

**Figure 5.2.9** Volcano plot of LCMS Urine Part 2 ME/CFS with FM versus ME/CFS without FM. Metabolites with significantly higher (red) or lower (blue) concentrations in the ME/CFS with FM cohort are highlighted (FC threshold 1.5 and uncorrected p-values ≤ 0.05) with non-significant metabolites represented in grey.




| | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|
| Trimethylamine N-oxide | 0.50535 | -0.98465 | 0.042371 | 1.3729 |
| Formate | 0.7253 | -0.46335 | 0.042476 | 1.3719 |
| Maleate | 1.4799 | 0.56553 | 0.060042 | 1.2215 |

**Figure 5.2.10** Volcano plot of NMR Urine Part 2 ME/CFS with FM versus ME/CFS without FM. Metabolites with significantly higher (red) or lower (blue) concentrations in the ME/CFS with FM cohort are highlighted (FC threshold 1.0 and uncorrected p-values ≤ 0.10) with non-significant metabolites represented in grey.

### 5.2.2.2 Multivariate Analysis (PCA)

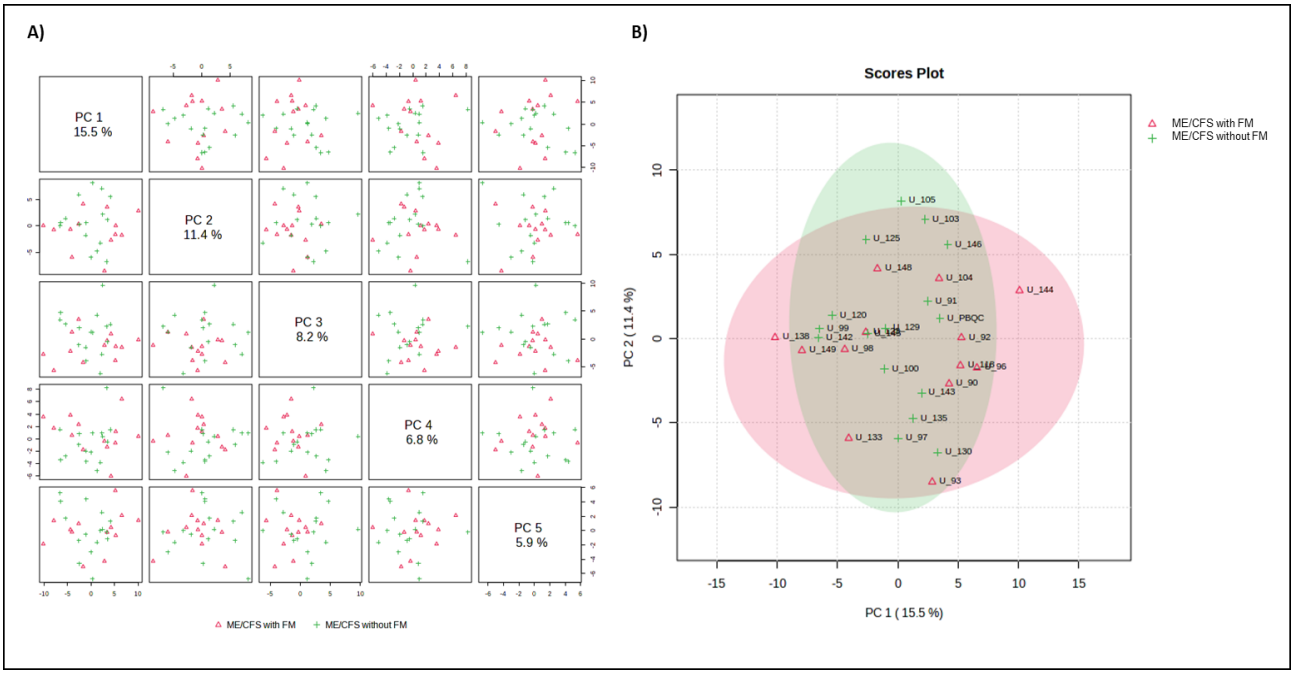



**Figure 5.2.11** PCA plots A) overview B) PC1 and PC2 only of LCMS Urine Part 2 ME/CFS with FM versus ME/CFS without FM

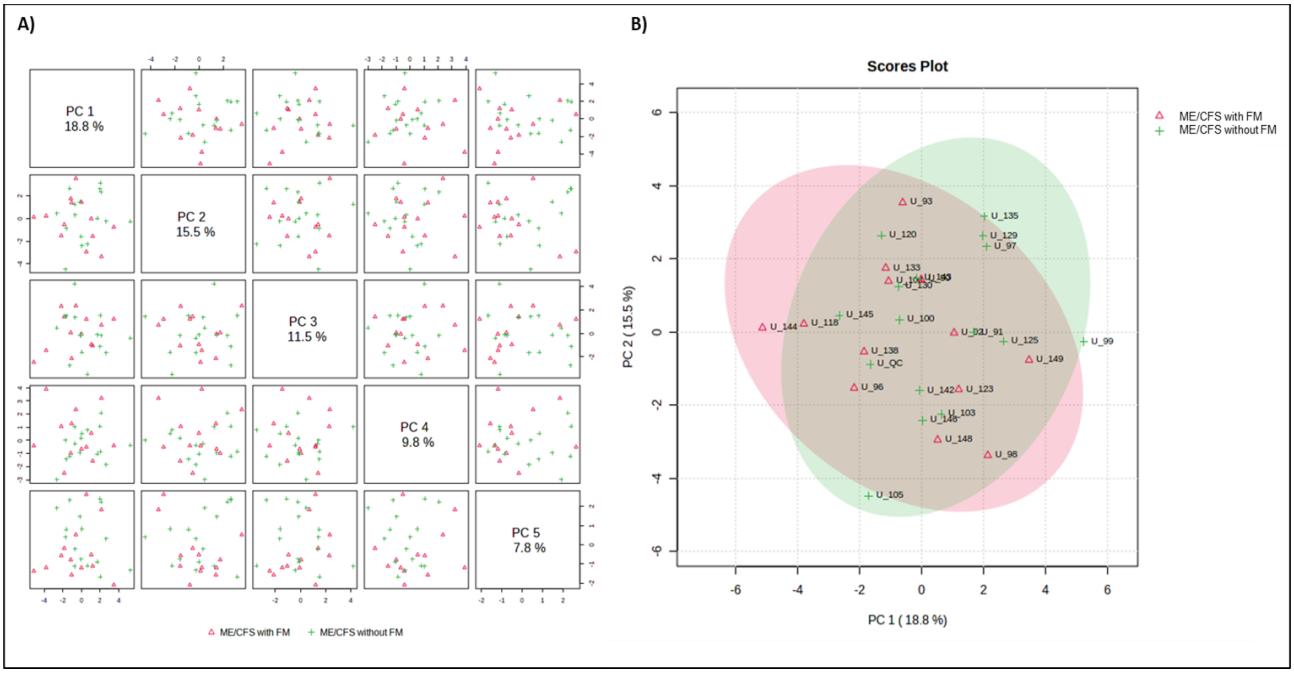



**Figure 5.2.12** PCA plots A) overview B) PC1 and PC2 only of NMR Urine Part 2 ME/CFS with FM versus ME/CFS without FM

### 5.2.2.3 Multivariate Analysis (PLS-DA)

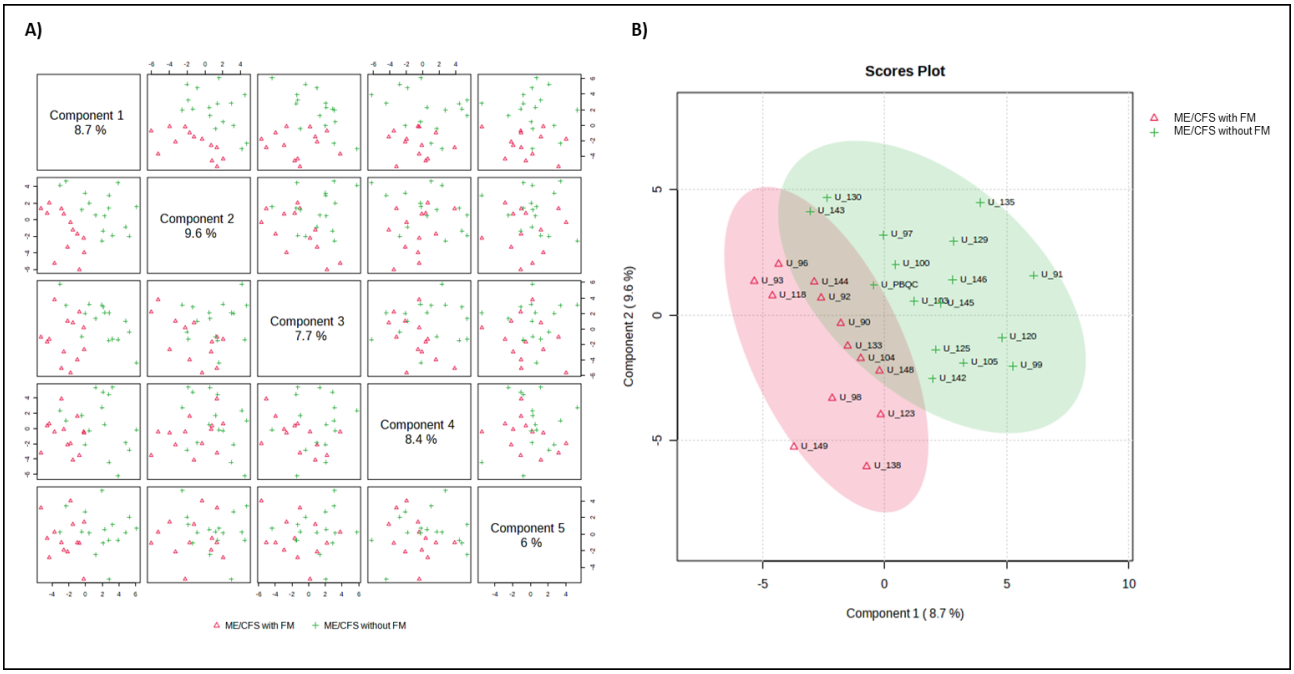



**Figure 5.2.13** PLSDA A) overview B) components 1 and 2 only of LCMS Urine Part 2 ME/CFS with FM versus ME/CFS without FM

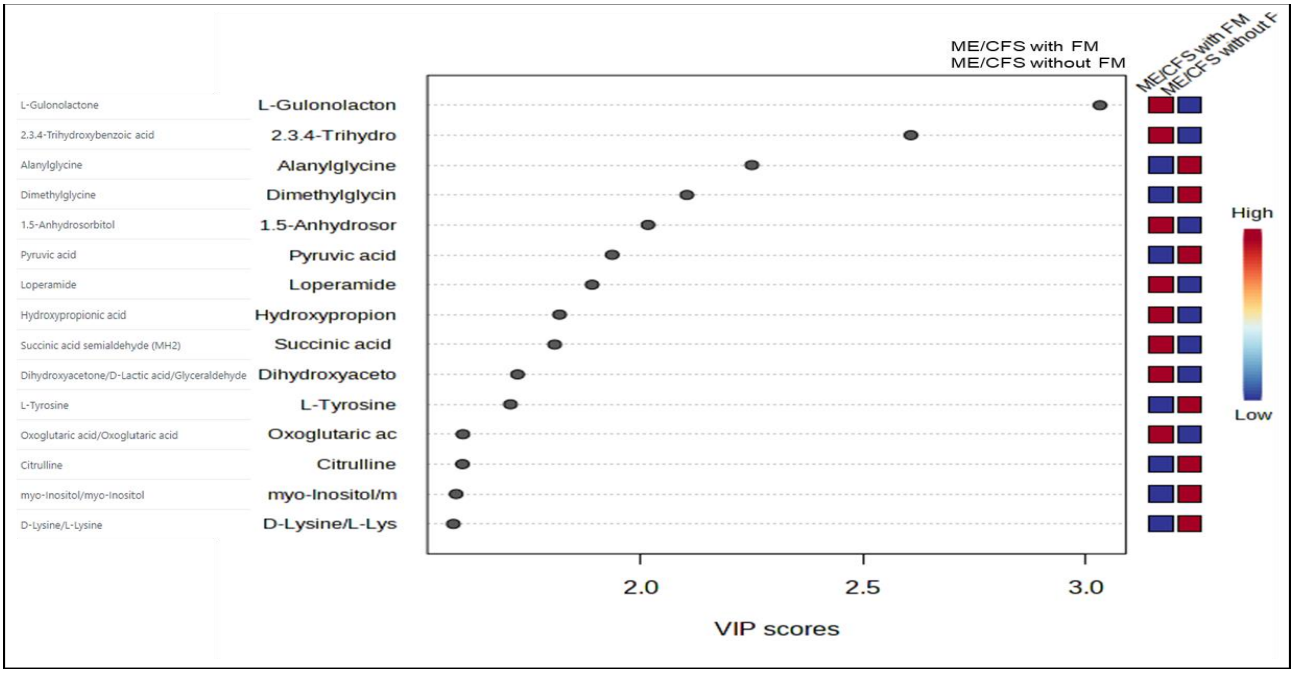



**Figure 5.2.14** VIP scores of corresponding PLSDA for LCMS Urine Part 2 ME/CFS with FM versus ME/CFS without FM

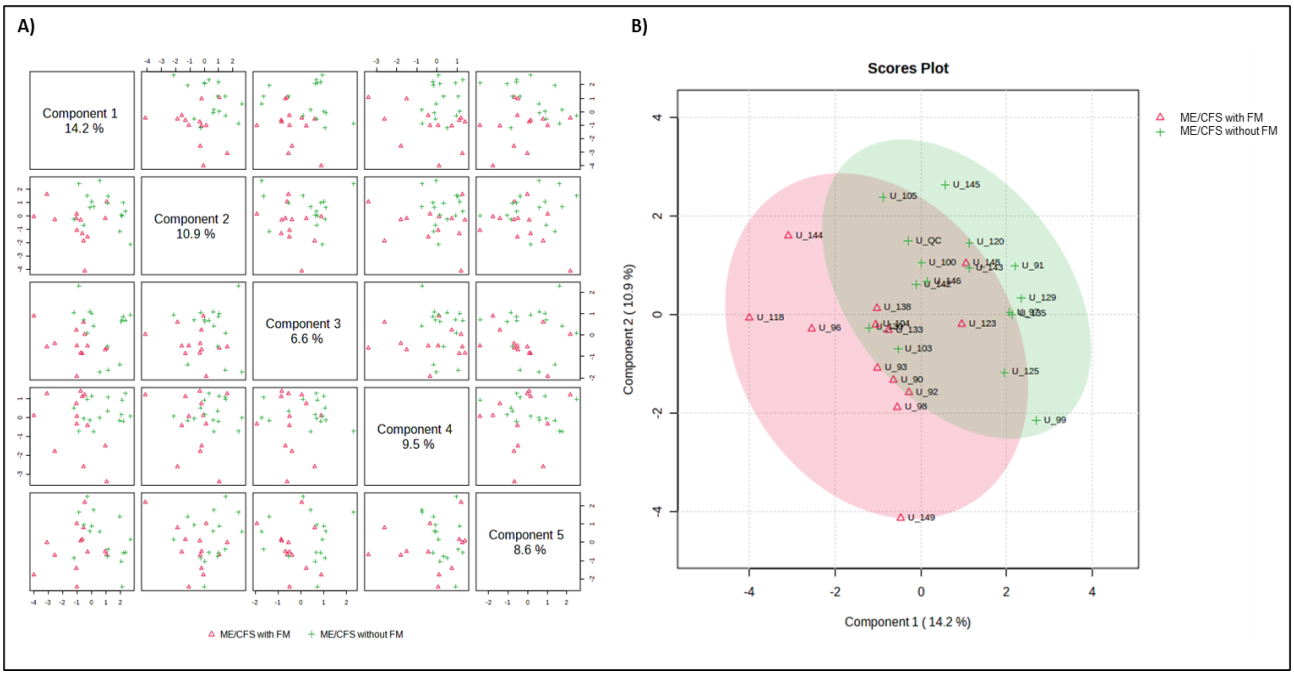



**Figure 5.2.15** PLSDA A) overview B) components 1 and 2 only of NMR Urine Part 2 ME/CFS with FM versus ME/CFS without FM

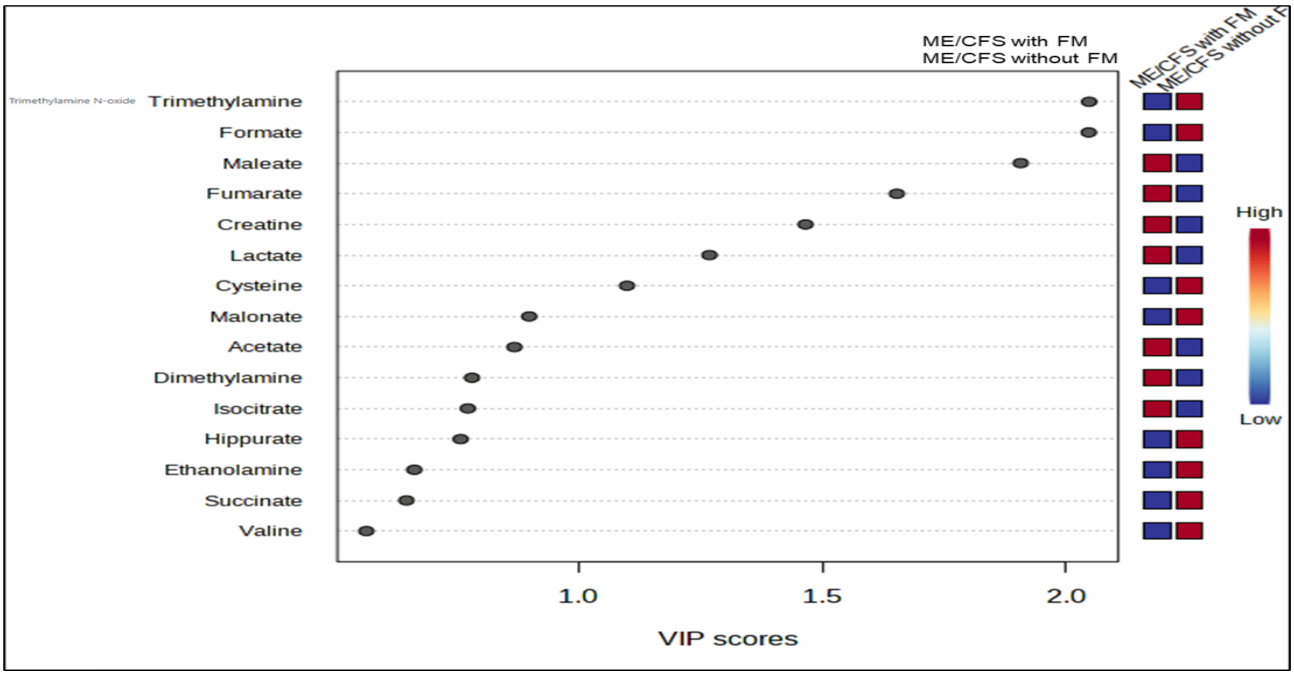
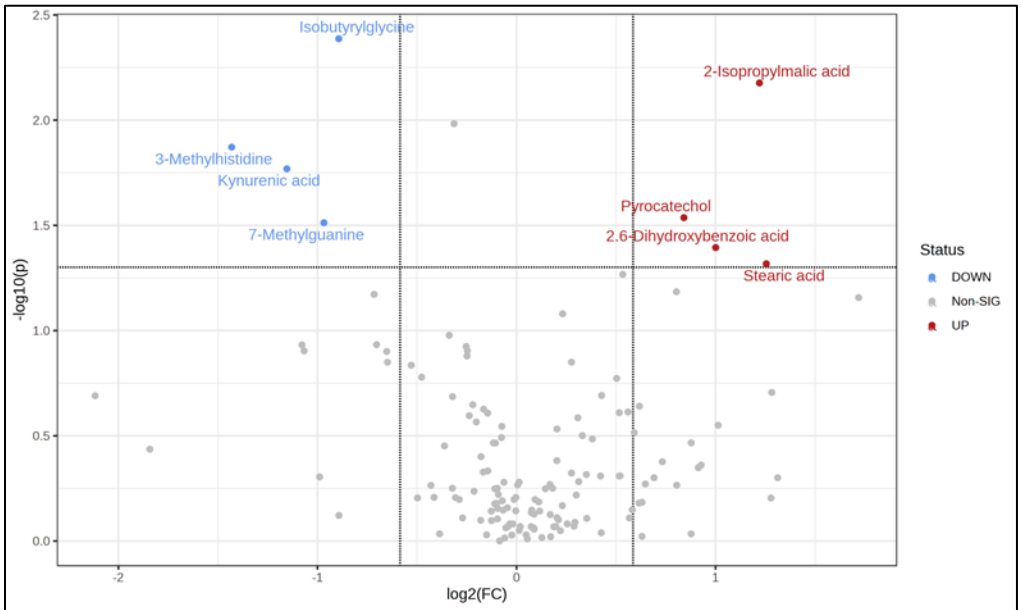



**Figure 5.2.16** VIP scores of corresponding PLSDA for NMR Urine Part 2 ME/CFS with FM versus ME/CFS without FM

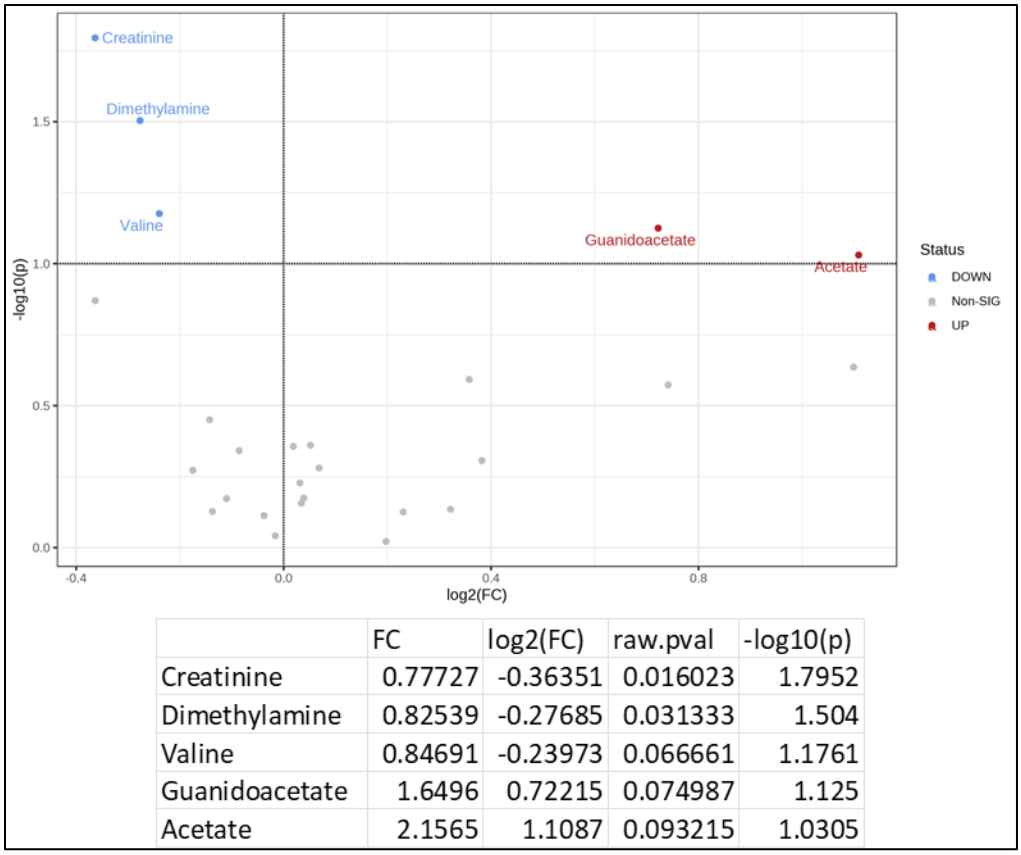
### 5.2.3 Urine Comparison C) ME/CFS with IBS vs. ME/CFS without IBS

#### 5.2.3.1 Univariate Analysis




| | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|
| Isobutyrylglycine | 0.53852 | -0.89293 | 0.0041049 | 2.3867 |
| 2-Isopropylmalic acid | 2.33 | 1.2204 | 0.0066571 | 2.1767 |
| 3-Methylhistidine | 0.37088 | -1.431 | 0.013437 | 1.8717 |
| Kynurenic acid | 0.44941 | -1.1539 | 0.017037 | 1.7686 |
| Pyrocatechol | 1.7913 | 0.841 | 0.029069 | 1.5366 |
| 7-Methylguanine | 0.51136 | -0.9676 | 0.030719 | 1.5126 |
| 2.6-Dihydroxybenzoic acid | 2.0009 | 1.0006 | 0.04032 | 1.3945 |
| Stearic acid | 2.3867 | 1.255 | 0.048133 | 1.3176 |

**Figure 5.2.17** Volcano plot of LCMS Urine Part 2 ME/CFS with IBS versus ME/CFS without IBS. Metabolites with significantly higher (red) or lower (blue) concentrations in the ME/CFS with IBS cohort are highlighted (FC threshold 1.5 and uncorrected p-values ≤ 0.05) with non-significant metabolites represented in grey.




| | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|
| Creatinine | 0.77727 | -0.36351 | 0.016023 | 1.7952 |
| Dimethylamine | 0.82539 | -0.27685 | 0.031333 | 1.504 |
| Valine | 0.84691 | -0.23973 | 0.066661 | 1.1761 |
| Guanidoacetate | 1.6496 | 0.72215 | 0.074987 | 1.125 |
| Acetate | 2.1565 | 1.1087 | 0.093215 | 1.0305 |

**Figure 5.2.18** Volcano plot of NMR Urine Part 2 ME/CFS with IBS versus ME/CFS without IBS. Metabolites with significantly higher (red) or lower (blue) concentrations in the ME/CFS with IBS cohort are highlighted (FC threshold 1.0 and uncorrected p-values ≤ 0.10) with non-significant metabolites represented in grey.

### 5.2.3.2 Multivariate Analysis (PCA)

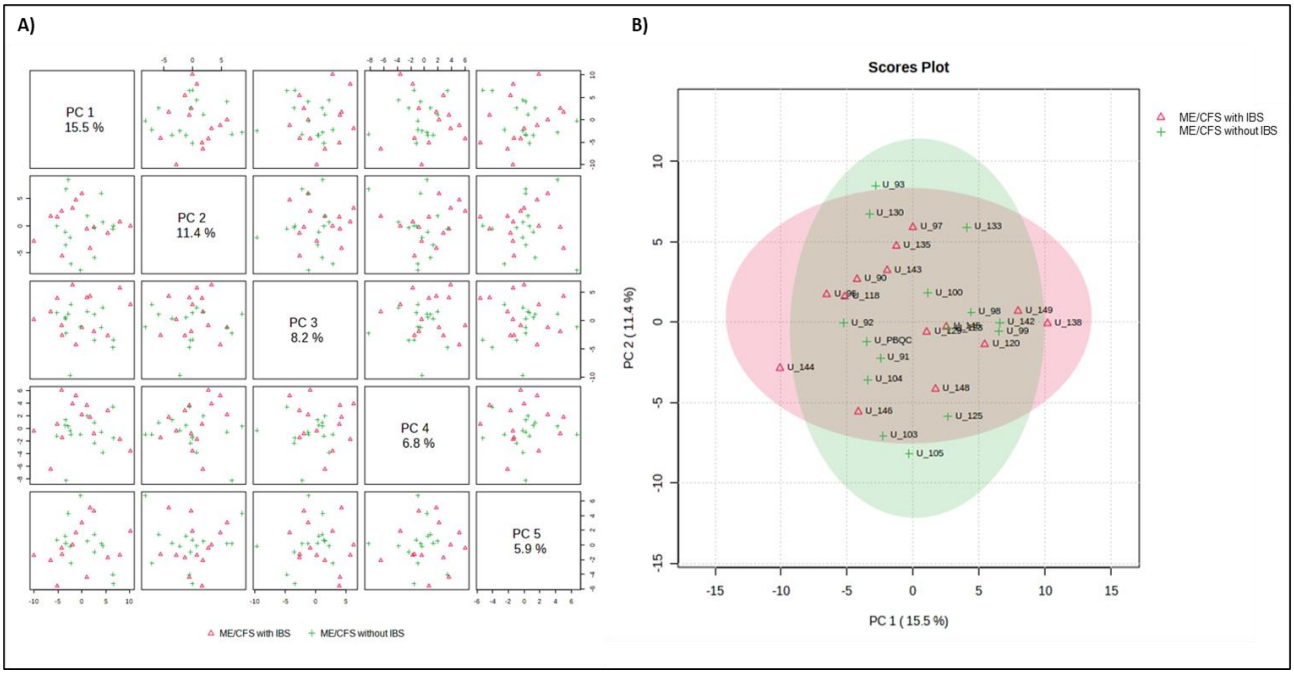



**Figure 5.2.19** PCA plots A) overview B) PC1 and PC2 only of LCMS Urine Part 2 ME/CFS with IBS versus ME/CFS without IBS

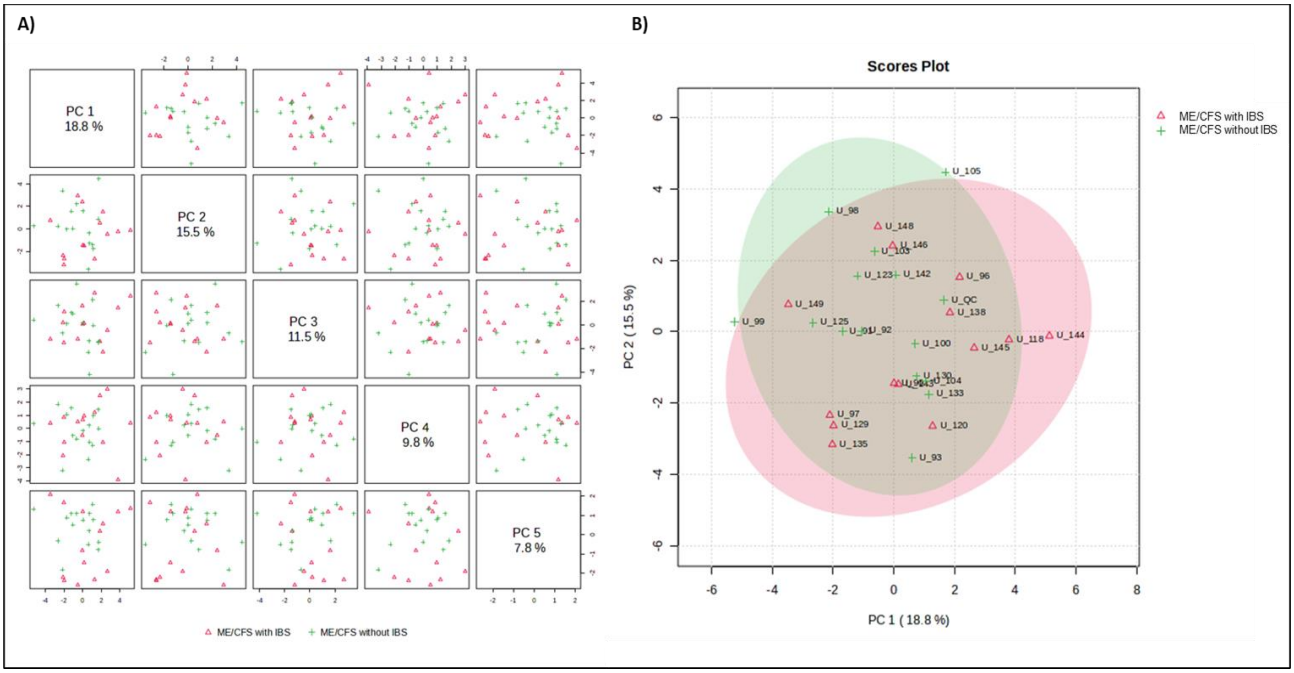



**Figure 5.2.20** PCA plots A) overview B) PC1 and PC2 only of NMR Urine Part 2 ME/CFS with IBS versus ME/CFS without IBS

### 5.2.3.3 Multivariate Analysis (PLS-DA)

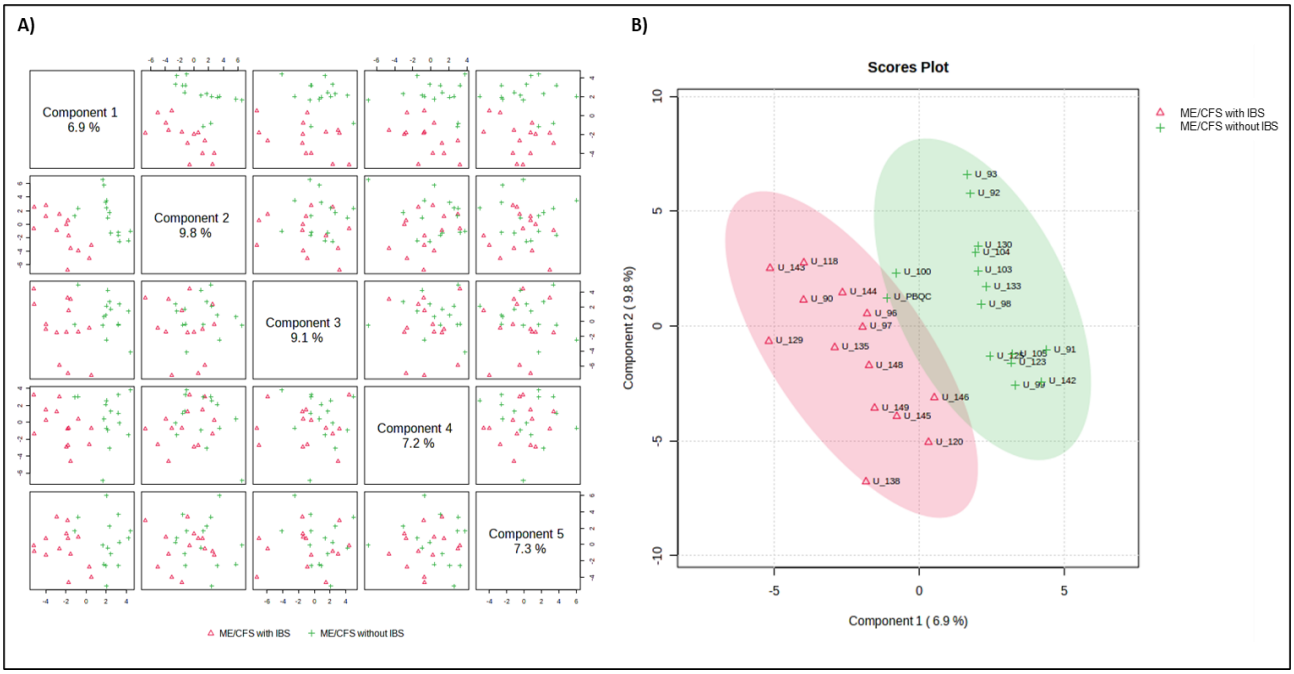



**Figure 5.2.21** PLSDA A) overview B) components 1 and 2 only of LCMS Urine Part 2 ME/CFS with IBS versus ME/CFS without IBS

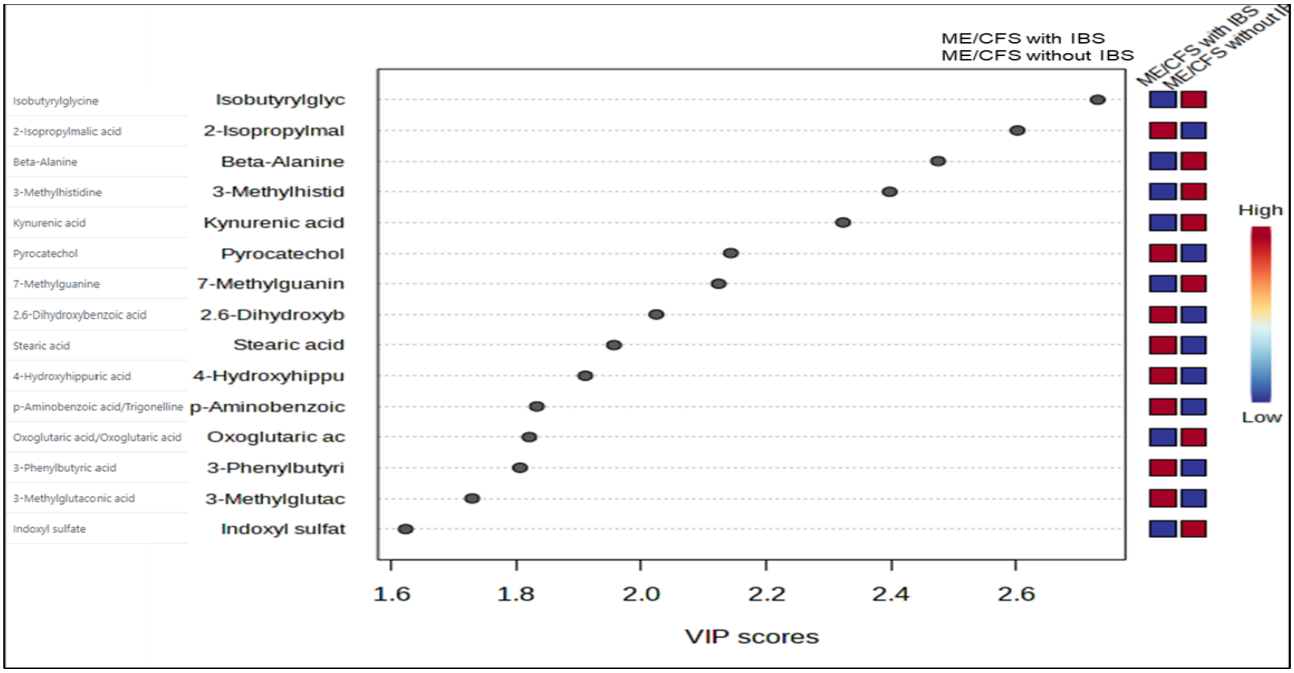



**Figure 5.2.22** VIP scores of corresponding PLSDA for LCMS Urine Part 2 ME/CFS with IBS versus ME/CFS without IBS

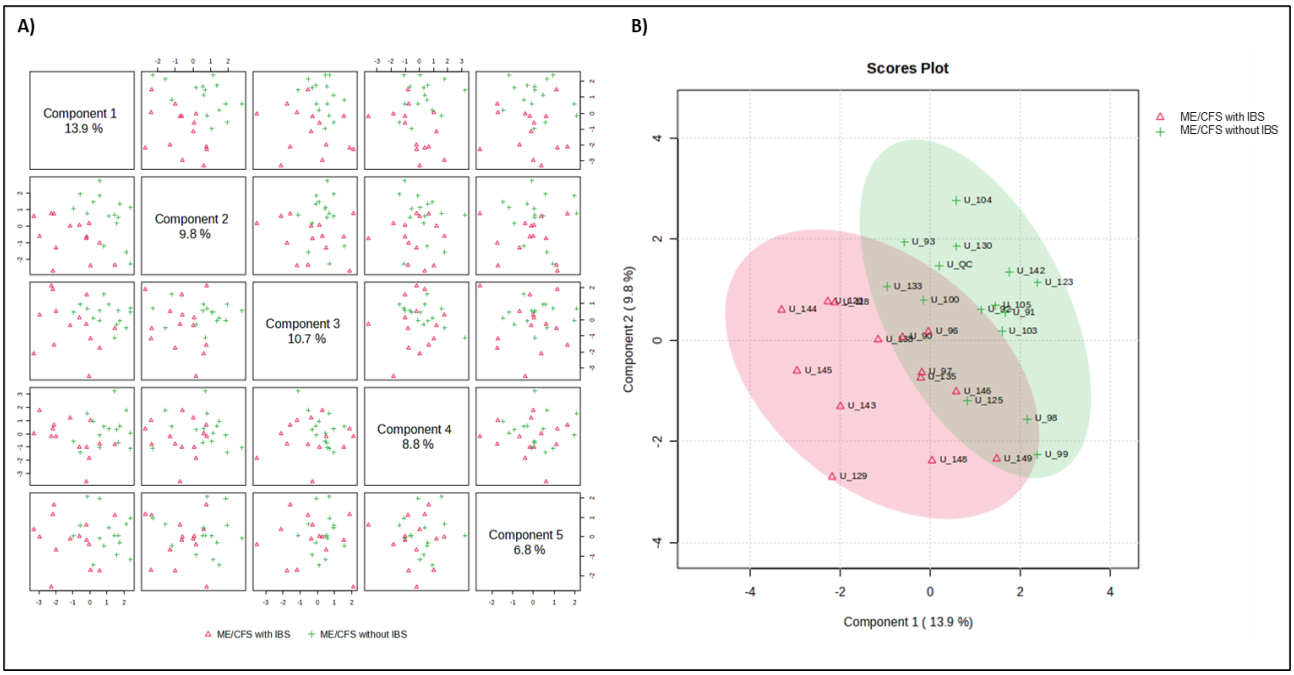



**Figure 5.2.23** PLSDA A) overview B) components 1 and 2 only of NMR Urine Part 2 ME/CFS with IBS versus ME/CFS without IBS

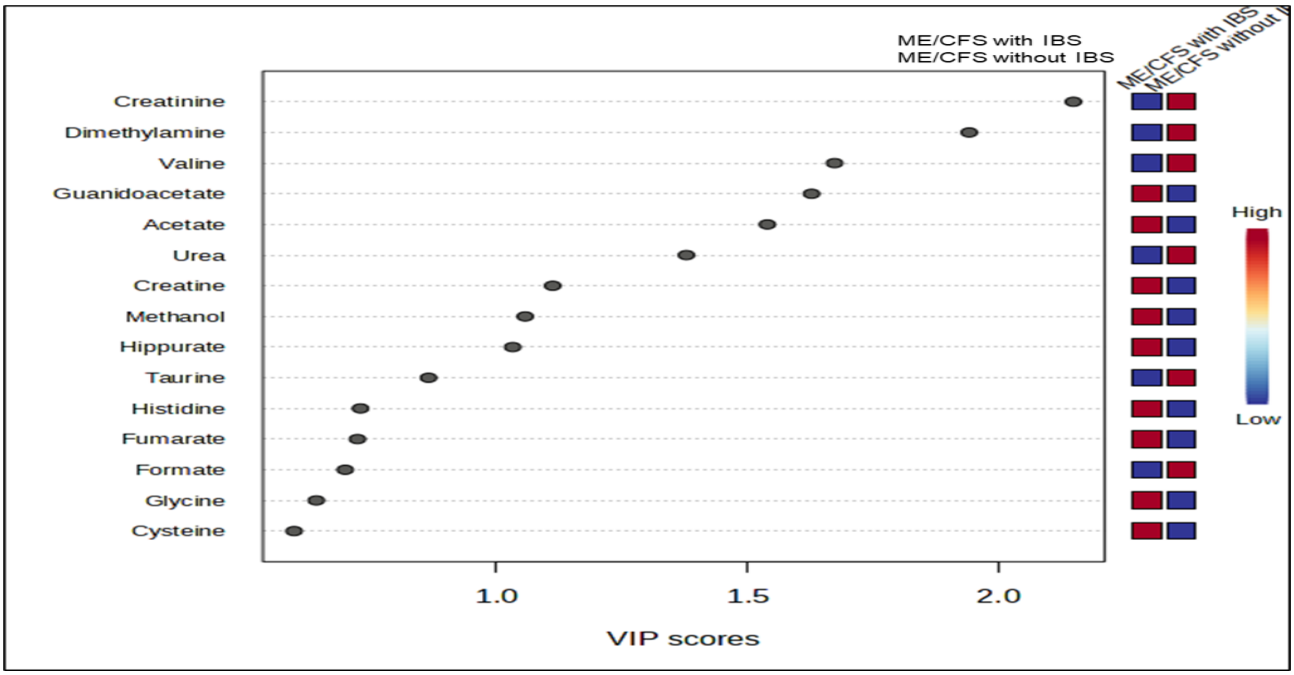
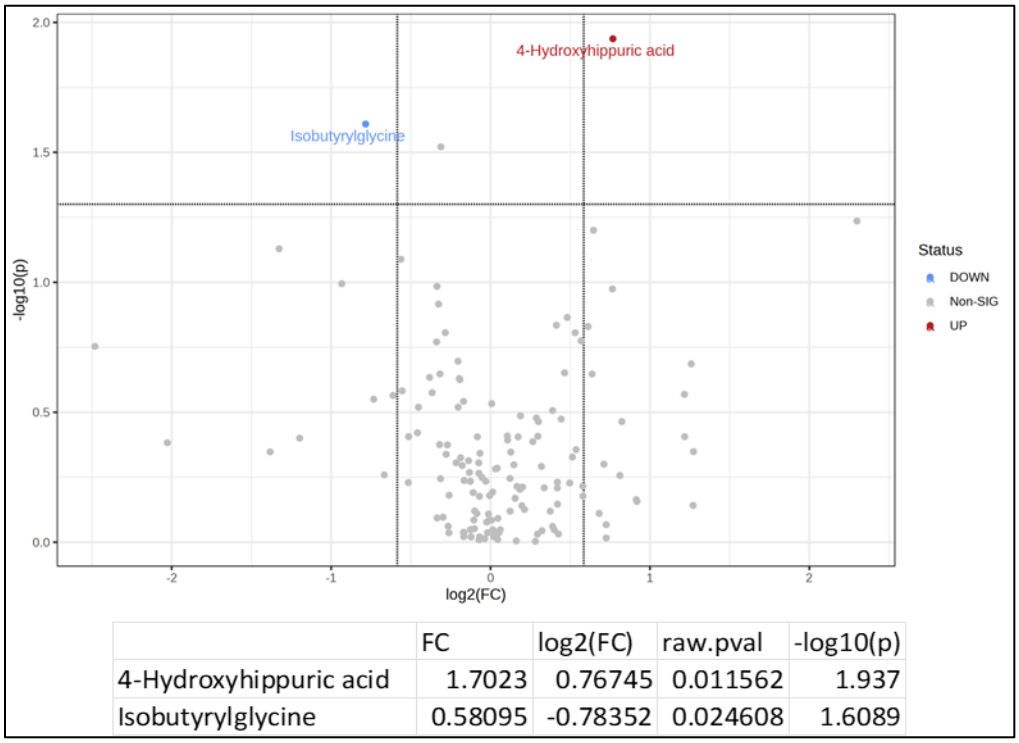



**Figure 5.2.24** VIP scores of corresponding PLSDA for NMR Urine Part 2 ME/CFS with IBS versus ME/CFS without IBS

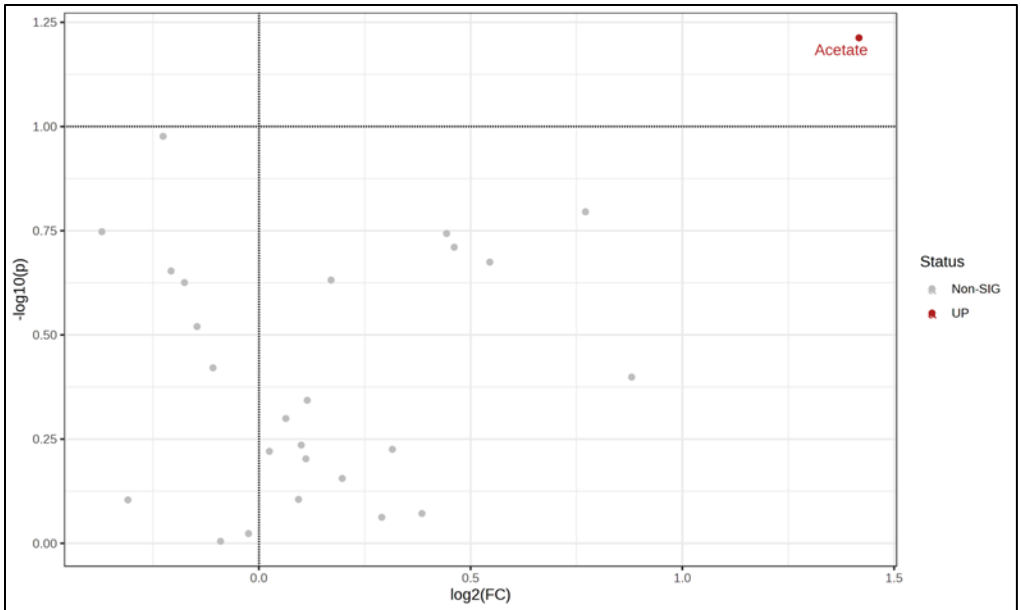
### 5.2.4 Urine Comparison D) Age-matched ME/CFS with IBS vs. ME/CFS without IBS

#### 5.2.4.1 Univariate Analysis




| | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|
| 4-Hydroxyhippuric acid | 1.7023 | 0.76745 | 0.011562 | 1.937 |
| Isobutyrylglycine | 0.58095 | -0.78352 | 0.024608 | 1.6089 |

**Figure 5.2.25** Volcano plot of LCMS Urine Part 2 age-matched ME/CFS with IBS versus ME/CFS without IBS. Metabolites with significantly higher (red) or lower (blue) concentrations in the age-matched ME/CFS with IBS cohort are highlighted (FC threshold 1.5 and uncorrected p-values ≤ 0.05) with non-significant metabolites represented in grey.




| | FC | log2(FC) | raw.pval | -log10(p) |
|---|---|---|---|---|
| Acetate | 2.6704 | 1.417 | 0.061268 | 1.2128 |

**Figure 5.2.26** Volcano plot of NMR Urine Part 2 age-matched ME/CFS with IBS versus ME/CFS without IBS. Metabolites with significantly higher (red) or lower (blue) concentrations in the age-matched ME/CFS with IBS cohort are highlighted (FC threshold 1.0 and uncorrected p-values ≤ 0.10) with non-significant metabolites represented in grey.

### 5.2.4.2 Multivariate Analysis (PCA)

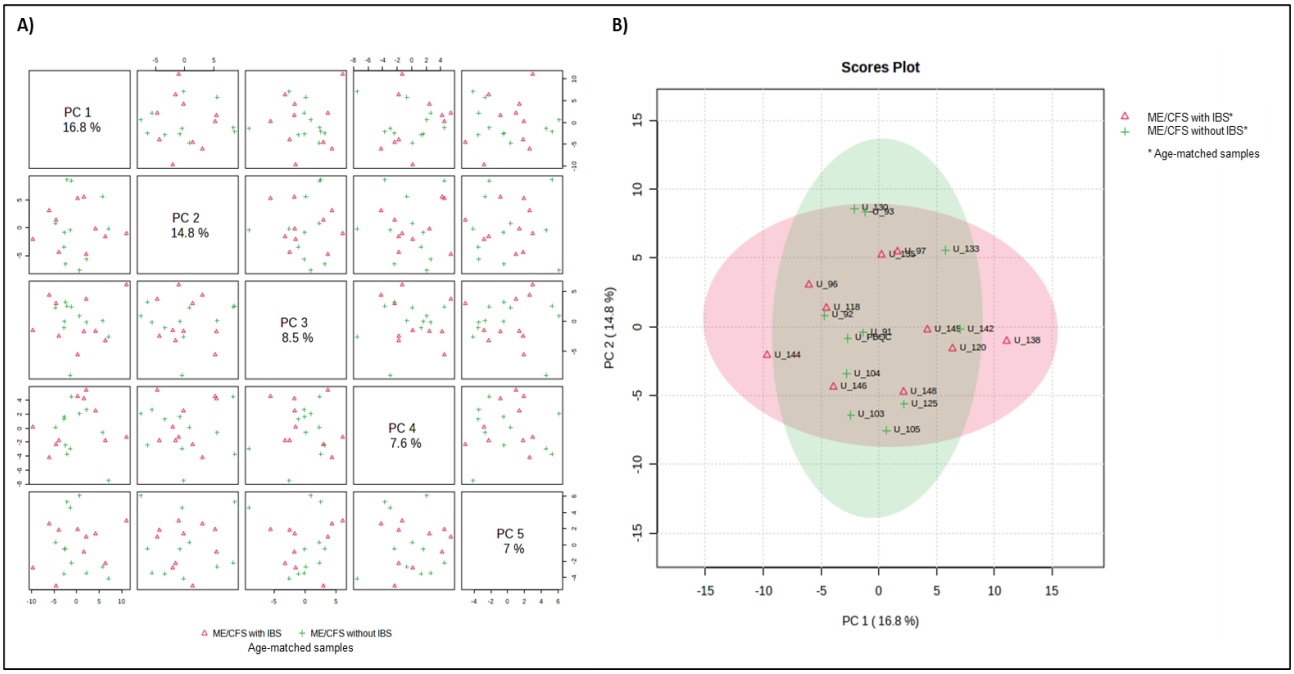



**Figure 5.2.27** PCA plots A) overview B) PC1 and PC2 only of LCMS Urine Part 2 age-matched ME/CFS with IBS versus ME/CFS without IBS

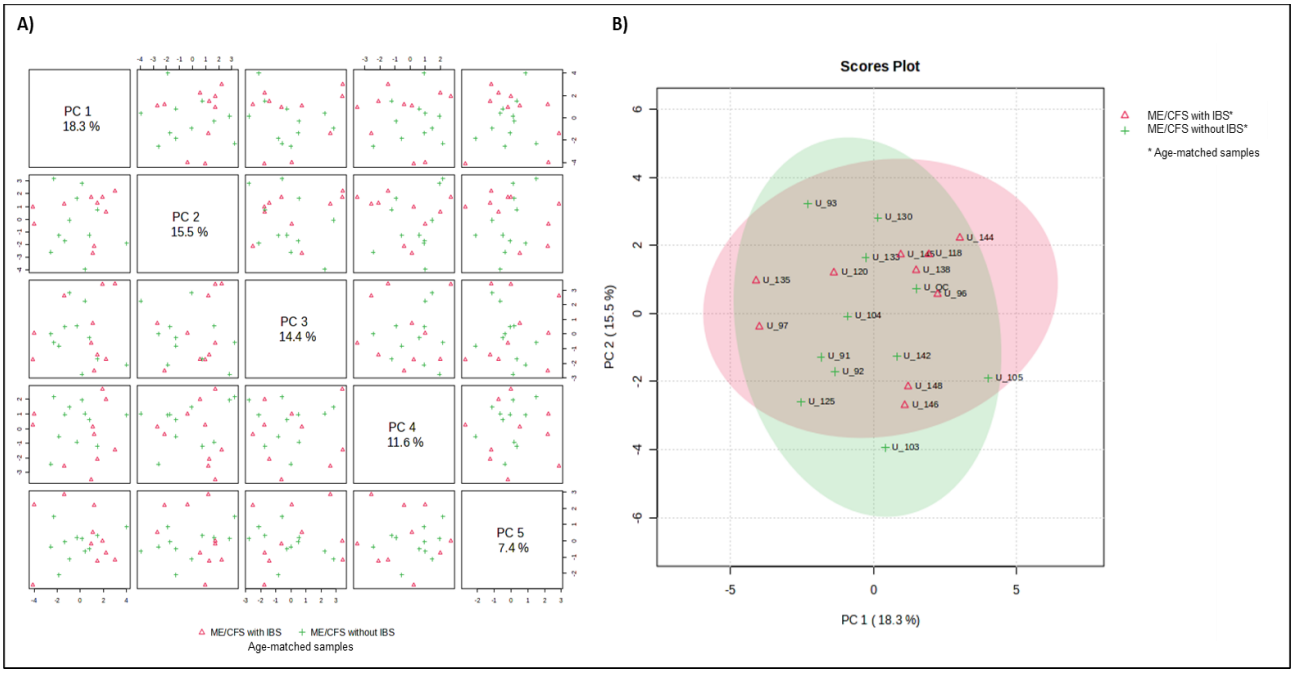



**Figure 5.2.28** PCA plots A) overview B) PC1 and PC2 only of NMR Urine Part 2 age-matched ME/CFS with IBS versus ME/CFS without IBS

### 5.2.4.3 Multivariate Analysis (PLS-DA)

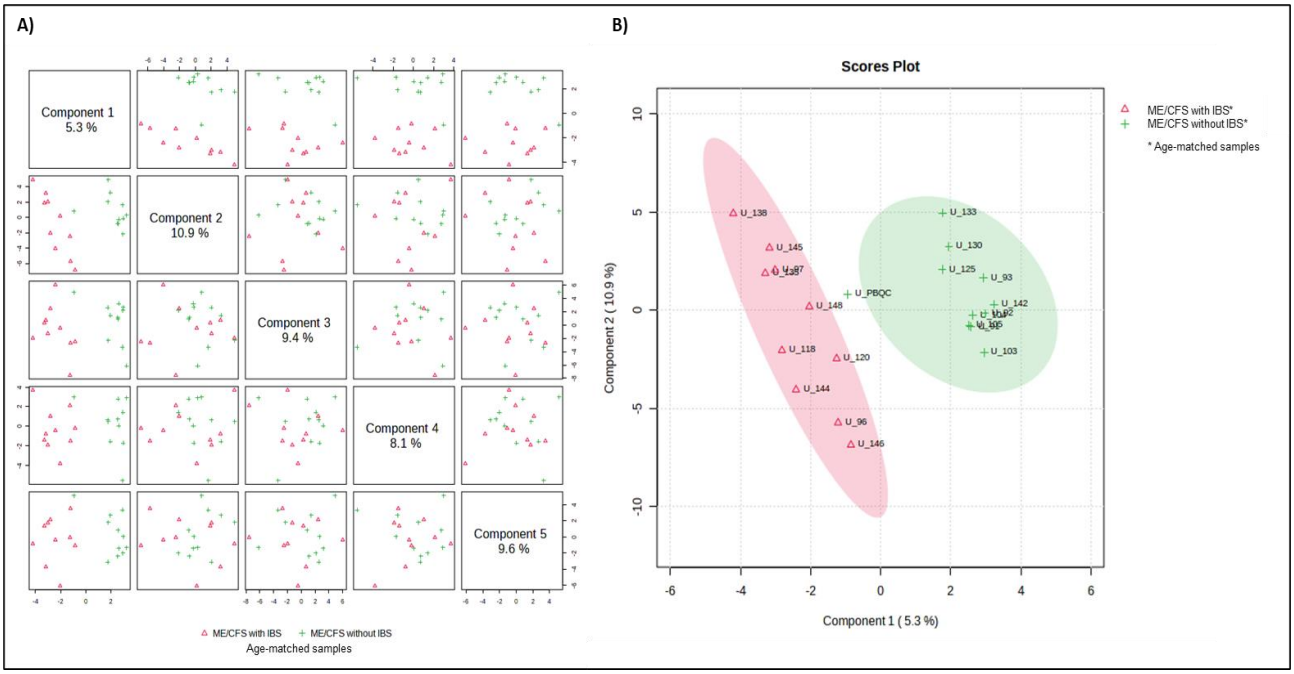



**Figure 5.2.29** PLSDA A) overview B) components 1 and 2 only of LCMS Urine Part 2 age-matched ME/CFS with IBS versus ME/CFS without IBS

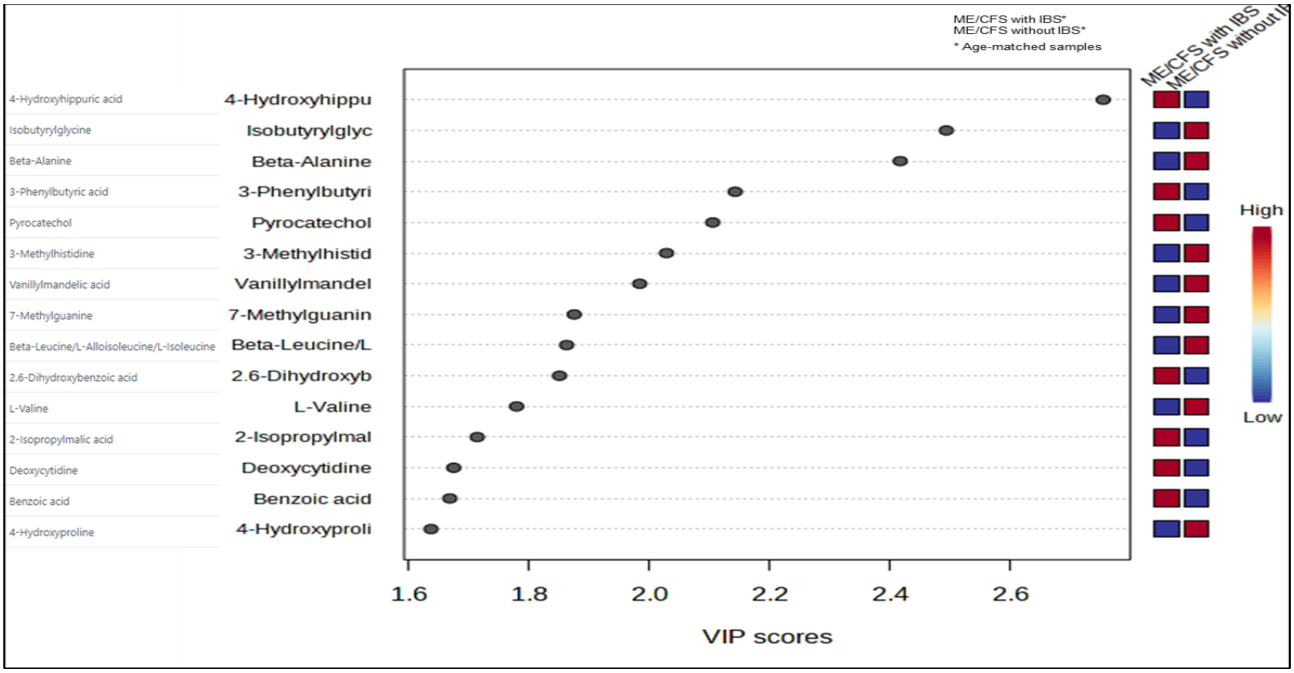



**Figure 5.2.30** VIP scores of corresponding PLSDA for LCMS Urine Part 2 age-matched ME/CFS with IBS versus ME/CFS without IBS

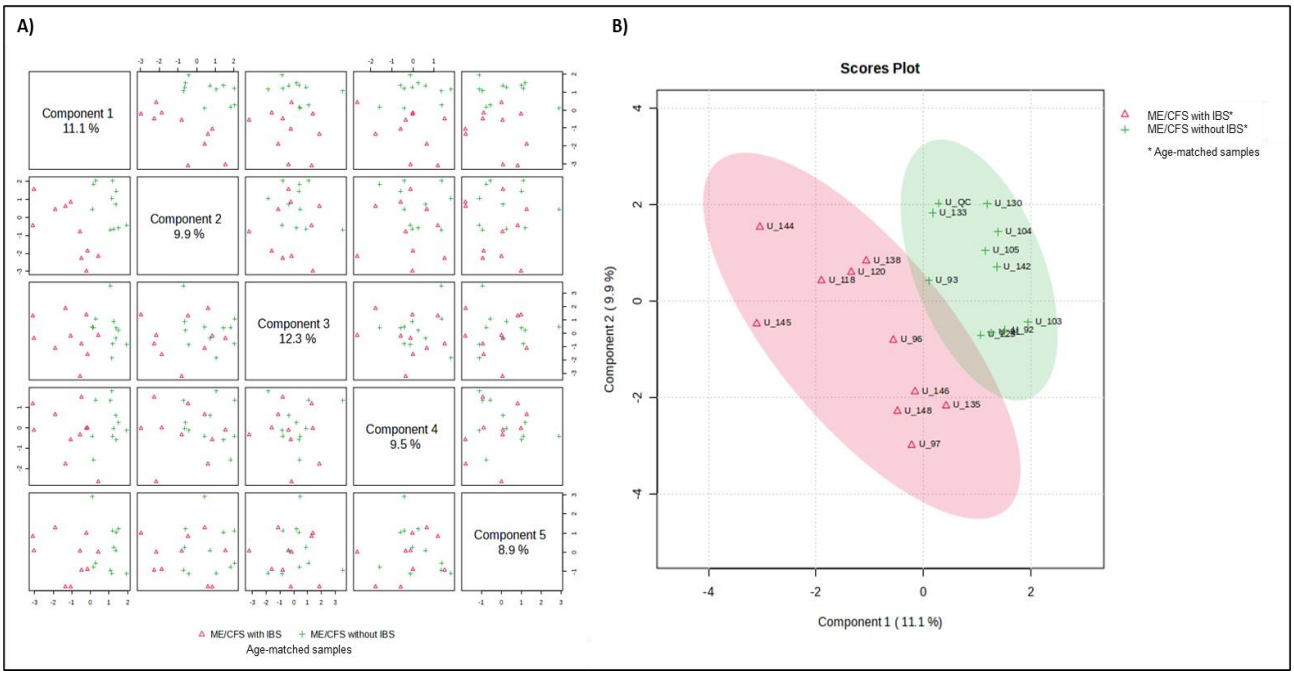



**Figure 5.2.31** PLSDA A) overview B) components 1 and 2 only of NMR Urine Part 2 age-matched ME/CFS with IBS versus ME/CFS without IBS

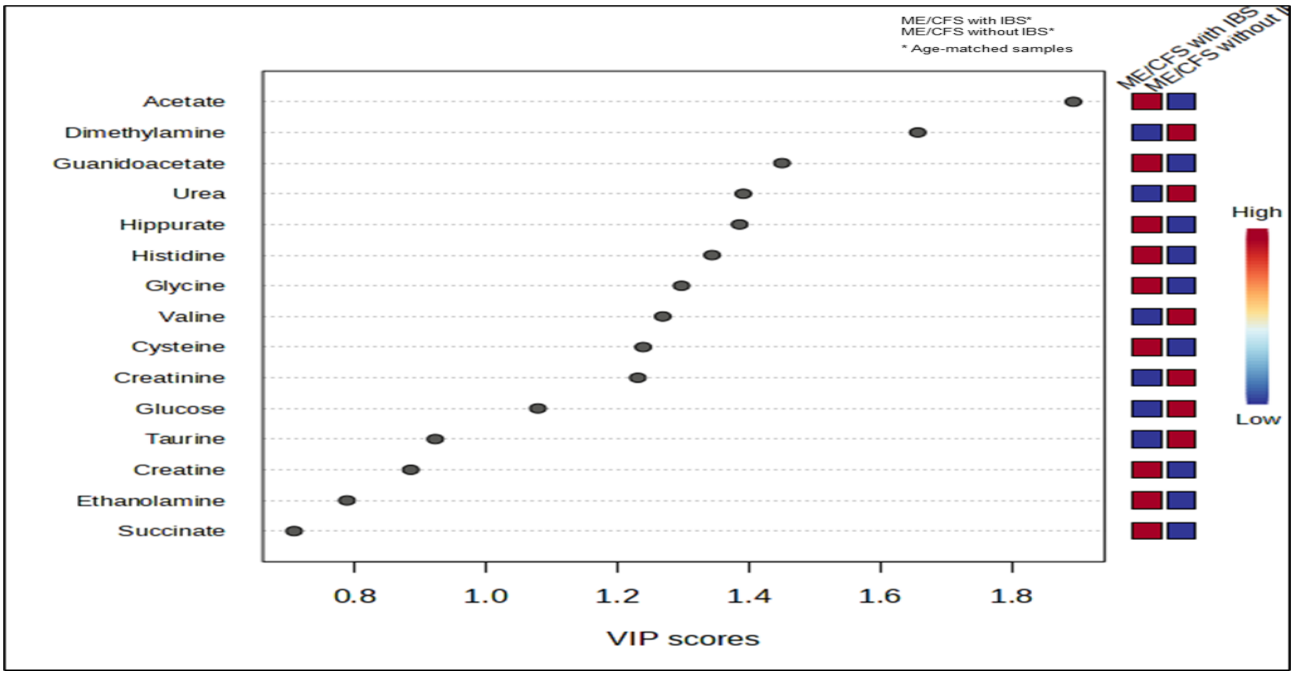



**Figure 5.2.32** VIP scores of corresponding PLSDA for NMR Urine Part 2 age-matched ME/CFS with IBS versus ME/CFS without IBS

## 5.3 Plasma and Urine Metabolomics Discussion

### 5.3.1 Variations in Plasma Metabolome

There were plasma metabolite features that were highlighted across several comparisons which showed inconsistent trends in their relative concentration depending on the comorbidity grouping. There were 18 metabolites, for example, betaine and succinate, that were elevated in both the Control group from Comparison A and in groups from Comparison C-D which are ME/CFS participants overall that have been defined according to their FM and IBS metadata. Glutamate showed a contrasting trend in the presence or absence of FM and IBS comorbidities where it was elevated in ME/CFS + FM (Comparison B) and ME/CFS - IBS** (Comparison D). Alpha-N-Phenylacetyl-L-glutamine and Alpha-Tocopherol showed a notable variation in trend where it was elevated in the ME/CFS overall group (Comparison A) and ME/CFS - FM (Comparison B). All these incongruent or interesting trends and patterns indicated that plasma metabolite characteristics may be different depending on the FM or IBS comorbidity and how participants are grouped.

### 5.3.2 Variations in Urine Metabolome

There were metabolite features that were highlighted across several comparisons which showed some inconsistent trends in their relative concentration depending on the comorbidity grouping. There were 10 metabolites, for example, formate and glycine, that were elevated in both the Control group from Comparison A and in groups from Comparison C-D which are ME/CFS participants overall that have been assigned according to their FM and IBS metadata. Cysteine and oxoglutaric acid showed a contrasting trend in the presence or absence of FM and IBS comorbidities where it was elevated in its relative concentration, i.e. if the metabolite was elevated in ME/CFS - FM (Comparison B), it was elevated in ME/CFS + IBS and/or IBS** (Comparison C and D), and vice versa. Further, succinate, ethanolamine, creatinine, and isocitrate showed a notable variation in trend where these metabolites were elevated in the ME/CFS overall group (Comparison A) but when looking at Comparison C-D with the ME/CFS participants, it depended on whether it was +/- FM, IBS, or IBS**. All these incongruent or interesting trends and patterns indicated that urine metabolite characteristics may be different depending on the FM or IBS comorbidity and how participants are grouped.

### 5.3.3 Metabolites in Plasma and Urine

Like Part One, the two metabolomic panels for Part Two with plasma and urine outcomes have been considered separately in this thesis. The following significantly differential metabolites appeared in both specimen types (Appendix 11): 1.5-Anhydrosorbitol, 3-Methylhistidine, 4-Hydroxyhippuric acid, acetate, beta-alanine, creatine, alanine, formate, gluconic acid, glutamate, glycine, histidine, lactate, malonate, methanol. myo-inositol, succinate, sucrose, and trigonelline. Of these metabolites across the two sample types, only 1.5-Anhydrosorbitol, beta-alanine, gluconic acid, and myo-inositol were highlighted as significant but in different comparative groups across the two sample types. Although these metabolites were highlighted in different comparative groups, the urine, and plasma sample types provide a different insight into the relative concentration trend of their respective grouping. It is thought that up to 46% of the variance in the host's circulating plasma metabolites can be explained by the gut microbiome (Puig-Castellví et al., 2023).

3-Methylhistidine, 4-Hydroxyhippuric acid, creatine, histidine, methanol, sucrose, and trigonelline appeared in the same comparative groups between the sample types with a consistent outcome in the trend of their relative concentration. However, acetate, formate, glycine, malonate, and succinate showed an opposite trend in their relative concentration although measured and significantly different in both sample types. These metabolites did not mirror each other in the relative concentration patterns of urine and plasma. For example, acetate assayed by NMR for Comparison A was elevated in the plasma of the Control group; however, it was elevated in the urine of the ME/CFS group. The discussion from Section 4.3.4 is also relevant here. A further look at these metabolites without the "noise" of the other metabolites is needed, and Comparison B-D requires some evaluation with a non-ME/CFS cohort involved. The different and varied trends found when considering the urine and plasma metabolome results alone, and metabolites across both biospecimen types indicated that altered metabolic patterns associated with ME/CFS are subtle yet complex and detailed.

However, some initial commentary can be provided regarding acetate, as it appeared across Comparison A-D in the urine panel, and in Part One with faecal and the other urine sample. Acetate or acetic acid is the most abundant short chain fatty acid in the gut, produced and excreted in large amounts by certain bacteria (HMDB0000042). Acetate and the related metabolism of acetyl-CoA confers numerous metabolic functions, including energy production,

lipid synthesis, and protein acetylation (Bose et al., 2019). It is one of the metabolites that NMR did measure, but not by LCMS. Lower urine acetate levels in ME/CFS patients have been observed compared to non-ME/CFS patients (Armstrong et al., 2015; McGregor et al., 2016, 2019); however, this study shows increased urine acetate in ME/CFS patients in Part One and Two. Intriguingly, in a study with lupus nephritis/systemic lupus erythematosus, urine acetate was higher in the urine of these patients compare to a control group, and it showed a trend towards decreasing after treatment with cyclophosphamide (Ganguly et al., 2020). These discrepancies could be attributed to the heterogeneity of study design, approaches, and participants in the respective studies and requires further investigation. However, faecal metabolomic outcomes on acetate between this study and Guo et al. (2023) showed similar outcomes whereby acetate was lower in ME/CFS. Blood acetate also showed similarities with previous ME/CFS studies where decreased levels were observed in the respective ME/CFS cohorts (Armstrong et al., 2015; McGregor et al., 2016, 2019). Differences by design aside, the variations across sample types are likely underpinned by different aspects of metabolic dysregulation associated with ME/CFS. However, like gut microbes, metabolites and their changes in concentration cannot be considered in isolation, but rather groups or “communities”. Further work would benefit from looking at the ratios of the common short chain fatty acids that present across all sample types with gut microbiome data. Granted that the Part Two samples were not collected at the same time point, looking at this data in this way may reveal a further understanding of these metabolites in ME/CFS heterogeneity.

### 5.3.4 Metabolites in Part One and Two

It is acknowledged that Pat Two of the study is not as extensive as Part One. This thesis did not address the shifts in urine metabolites from Part One and Part Two although samples for LCMS being assayed simultaneously in the same run and NMR results are comparable would allow for this work in the future. Granted that there are different participant cohorts included in Part One and Two, an immediate observation when looking between Table 4.2.1 and Table 5.2.1 shows differences in metabolite features and highlighted groups across Comparison A to D. Further investigation of sampling across time (longitudinal) could further understanding of the different biospecimen types, ME/CFS heterogeneity and subgroupings. With the data that is available, the selection of Part One and Two participants could be used to look at these two time points more closely.

# 6 CHAPTER SIX: General Discussion and Conclusion

## 6.1 Contributions

### 6.1.1 ME/CFS, Comorbidities and Heterogeneity

This thesis sought to investigate the bearing of FM and IBS comorbidity in ME/CFS with the outcomes from the gut microbiome and host metabolome. Often comorbidities are deemed potential confounders of a study. While this may be true and relevant commentary in the context of that specific study or topic of discussion, heterogeneity is inherently a feature of ME/CFS. Without the many dimensions and facets of the disease, it would not be what we know as "ME/CFS". In the attempt to understand and produce a "unifying" concept of an objective diagnostic panel for ME/CFS, the differences from the comorbidities must be considered. Comparison A, which looked at ME/CFS versus Control, only provided one lens to the possible differences in microbes and metabolites. However, Comparison B-D, which looked at ME/CFS +/- FM, ME/CFS +/- IBS, and an age-matched ME/CFS +/- IBS**, showed differences within the overall ME/CFS cohort. While there was the suggestion that IBS comorbidity could be an associated distinguishable subgroup, FM is not without variations in both gut microbiome and host metabolome measures. It does provide some indication that different comorbidities may have a different impact in the context of ME/CFS. Certainly, the variations in patterns from Comparison B-D underscores and sheds some light on the many challenges that ME/CFS faces from its definition, diagnosis, research, and clinical context. What is intriguing from this study is that the comparative groups, Comparison B-D, from the ME/CFS cohort were made up of the same participants that were assigned according to their FM or IBS status. It could be argued that this is an outcome of deliberate design; however, the various patterns in the ME/CFS cohort cannot deny the heterogeneity that warrants consideration and further investigation. Given this, the findings from this thesis supports the idea that future research investigations need to go beyond the traditional disease versus non-disease study designs, and one-medium, one-platform route. While this study was limited to the snapshots of Part One and Two of the study, and could not establish causation, prognosis, or any definitive pathophysiological mechanisms, it does support the notion that a multi-disciplinary and objective framework measured longitudinally is required for the multifaced issues in ME/CFS.

### 6.1.2 Complementary Methodologies and Different Biospecimen Types

The use of independent, but complementary methods to profile the gut microbiome from a variety of biospecimen types broadened data coverage and insights. No one method or approach can do everything. As standalone datasets both within and between the gut microbiome and metabolomics work, the outcomes are not all that versatile in explaining what might be going on in the disease. While the FM and IBS comorbidities in ME/CFS may be more clearly understood and "seen" with the functional predictions, and metabolomics work, the gut microbiome taxonomic data is still valuable in providing a context of dysbiosis. While the workload requirements were more onerous, the study shows the benefit and supports the approach and utility of considering a variety of platforms and approaches in statistics/data analysis and visualisation. Of course, numerous other metabolites and microorganisms exist that constitute the "entire" human metabolome and microbiome that were not measured in this study or are not currently known and remain inaccessible. The complete landscape of microbe-metabolite interactions in the gut is still largely unmapped (Muller et al., 2022). These "known unknown" features that were not covered by this thesis could potentially be assayed or obtained by other workflows available. There is the possibility for further work with what is currently available from this project and known information. For example, this study has a MS-based plasma lipidomic panel that was not presented in this thesis, MiMeDB can be re-queried, integrated meta-analyses with databases could be explored, existing raw spectra/FASTQ sequencing files and their annotations could be re-visited especially for low abundant features and remaining sample material (suitability of the stored samples, and ethics application allowing) could be used for panels with other molecules/markers, absolute quantitation, non-targeted profiling, and non-polar metabolites. However, there is the consideration for the "unknown unknown" features that are currently uncharacterised and not in the sphere of knowledge. Sometimes referred to as "dark matter", addressing these features is another avenue in the world of omics science, albeit with its unique own challenges, for biological discovery and understanding. There are multiple levels to the unknowns and limitations of research; they must be duly acknowledged to avoid mis- and over-interpretation of the data and its context. This is crucial for finding biologically meaningful and relevant relationships in an environment where multiple sources and types of data are being generated, shared, and integrated in a multi-omics fashion.

## 6.2 Future Directions

### 6.2.1 Study Challenges and Limitations

This study is not without limitations. The foremost challenge was securing a recruitment site and having enough participants. Reviews and adjustments of recruitment processes were constantly made throughout to ensure project progress and viability. This involved ethical approval amendments and changes to the supervisory and research team. Other limitations of the study reflected the following issues encountered:

- The inherent heterogenous and multifaceted nature of ME/CFS, and other debilitating, complex “syndrome” conditions.
- The common difficulties and hurdles that many studies face with not only obtaining participant numbers but also ensuring full completion of all study requirements.
- The logistics and practicalities of conducting a small-scale study with minimal personnel and resources with the sample collection and study requirements.
- Building the foundational two-way streets and bridges between clinical and research needs and the management of all the different stakeholders involved in a study.

The recruitment period did not meet the initial target sample size calculated where ME/CFS, FM, and Control (three) cohorts were sought from three different clinical practices and medical doctors that had a special interest in these complex conditions (see Section 2.1). These calculations were made during the very early stages of project planning and with a different research and supervisory team. Due to irreconcilable circumstances with two of the recruitment sites and clinicians who were initially interested in being involved with the project, the decision was made to focus on engaging participants from one site and clinician at the now-closed CFS Discovery Clinic in Melbourne. Instead of three cohorts, two cohorts, the ME/CFS overall and the Control group, were established. Further recruitment constraints were met when the announcement of Dr Don Lewis’s retirement was being brought forward much earlier than anticipated, and that CFS Discovery was permanently closing without any firm handover plans. Unfortunately, medical doctors and practices experienced, interested, and prepared to work in this area of medicine are few and far between. This event created a unique situation and selection bias whereby patients were both eager to support the research study but also understandably hesitant to join as the task of finding alternative medical care options became an overriding priority. To obtain adequate participation, non-ME/CFS relatives or household

members of ME/CFS patients were permitted to join the study as part of the control group. This concession was made recognising that there is a hypothesised inheritable (genetic) and contagious/transmissible (genetically unrelated but considered a close contact) causal link or component involved in ME/CFS (Underhill, 2015). This study was not geared to establish, prove, or disprove any causal or prognostic links in ME/CFS; however, investigations of the genetic risk and transmissibility of the disease among contacts may benefit future ME/CFS (and long COVID) studies.

Organising this study not only brought to light differences in opinions regarding ME/CFS as a disease state among the research team, which included scientists and clinicians but also underscored troublesome gaps in bridging the divide between the needs of research and clinical practice. Future projects would benefit from being aware of and mitigating these issues (Beckett et al., 2011). Unsurprisingly, all three clinicians generally regarded ME/CFS similarly as a complex biological disease; however, their approach to diagnosis, usage of governing clinical criteria, management, and consideration of comorbidities differed slightly. Had the participants come from different recruitment sites and clinicians, it would have been interesting to compare the microbiome and metabolomic outcomes. It would have also been possible to address the usage of different diagnostic approaches and criteria in ME/CFS and FM as discussed previously in the literature (Abbi & Natelson, 2013; Lim & Son, 2020; Natelson, 2019). It also became obvious early on that different stakeholders held diverse opinions on how the study would be designed and conducted. While everyone had the common interest of generally wanting to do better for ME/CFS, there was a mismatch of priorities and expectations. For example, while the assays used in this study could help contribute to the research of ME/CFS, they could not be used immediately for a routine patient diagnostic and management scenario. Other barriers included the extra time and resource constraints for the clinicians and their practices to undertake project-related tasks such as blood sample collection and participant referrals. The practicalities of research conduct are often cited as barriers for clinicians from committing fully to research-based activities, and unfortunately, the logistics and budget did not allow for the provision of extra assistance (Beckett et al., 2011). Further, there was at times an assumed power differential between research team members which although unnecessary and counterproductive, impacted the collaborative aspects of the project. Moving forward it is important to acknowledge that conflicts are part and parcel of any project and a balance between research and clinical needs, translation, and aspirations should be maintained. Everyone in a multidisciplinary project, especially for one as complicated and urgent as

ME/CFS, has a valuable and essential role, and these dynamic relationships must remain professional, productive, and engaged with open discussions, supported, and resourced activity. Aside from the small cohort numbers and limited recruitment avenues, this study had other shortcomings that are more reflective of the multifaceted complexity of ME/CFS. This study does not sufficiently capture all facets of heterogeneity that underlie all the various subgroups that possibly exist within ME/CFS. Overall, this study and future research undertakings would benefit from more participants and a more diverse cohort of ME/CFS cases in a longitudinal study design. Irrespective of the number of recruitment sites and clinician approaches to ME/CFS, the issue/s would remain with characterising the participants included in the study and the applicability of study findings to the wider ME/CFS population. If not for one issue, there is certainly something else that makes it difficult to produce a directly comparable, unified, and consistently reproducible understanding of the disease pathophysiology and mechanisms at play. In the first instance, participants were diagnosed according to the CCC criteria, and male and female participants were combined and presented in analyses throughout this thesis. There is clear and growing evidence of sex-related disparities in ME/CFS concerning prevalence, clinical phenotypes, and aetiological triggers before symptom onset (Thomas et al., 2022). Most of this study was represented by female participants; therefore, it is conceivable that findings are biased by or reflective of female patterns and biology. This study did not recruit enough male participants to look at data outcomes with an even split between the sexes; however, comparisons of the female-only with the male and female cohort outcomes could reveal any plausible metabolic and microbiome sex-related differences in ME/CFS. Future studies would benefit from a more closely sex-matched participant cohort where gender and sex biology are conscientiously considered in study designs (Thomas et al., 2022).

In another aspect, the findings from this study may not be generalisable to the wider population of ME/CFS. Using select diagnostic criteria may mean that findings do not apply as closely to individuals diagnosed with other case definitions. Although the age component of the comparative groups in this thesis was matched, analyses cannot determine whether microbiome and metabolomic outcomes are affected or influenced by age. That is, the relationship between age, ageing, life expectancy, and ME/CFS could not be evaluated in this study. Age-dependent changes in the microbiome and metabolome irrespective of ME/CFS, IBS or GI-related health issues, and disease state have been revealed extensively in the literature. It has been hypothesised that ME/CFS is associated with accelerated ageing and older age predicts a poorer prognosis (Kidd et al., 2016; Rajeevan et al., 2018). Likewise, an association between

the different ages, courses of disease onset, progression/deterioration, and duration of ME/CFS cases since diagnosis among participants was not considered in this thesis; however, questionnaire responses could potentially allow for these subsequent analyses to be conducted. Some studies have shown variations in data outcomes and cohorts depending on the age and timeframe that their participants had since been diagnosed with ME/CFS (Kidd et al., 2016; Xiong et al., 2023). Given that ME/CFS has a higher prevalence in females, it is plausible to consider the role of sex chromosomes and steroids at different stages of life and physical maturation in the development and moderation of the disease (Thomas et al., 2022). Future pathophysiological studies with sex, age, and disease duration considered would provide needed understanding of any prior, immediate, shorter- and longer-term considerations and implications of ME/CFS. Regardless of the sex and age limitations of this study, participants were primarily limited to those who could manage to complete the required tasks. Generally, this meant that the most severely affected individuals, those with cognitive or concentration issues, and/or those in the thick of a crash phase where any form of activity and communication was not possible were not represented. Geographically, participants were limited to those who resided in Australia. Part One of the study was a major positive drawcard for participants, including those who were primarily bedbound or housebound, to be able to complete tasks at home before returning their collection kit to the post office either by themselves or through their carer. For Part Two of the study, participants represented were limited to those who were well enough, accessibly close location-wise to attend the pathology collection centre and those comfortable with blood draws. Provided there is adequate funding and a suitably trained research team, there are several home-based, more convenient, and less invasive testing options becoming more readily available for both microbiome and metabolomic assay purposes which may better facilitate future participant involvement and research outcomes.

This thesis only looked at the FM and IBS comorbidities in ME/CFS in a binary fashion, that is ME/CFS overall versus Control (Comparison A) ME/CFS +/- FM (Comparison B) or ME/CFS +/- IBS (Comparison C, and D with the age-matched samples). More involved multivariate comparisons of the FM and IBS comorbidities and groupings were not considered. Further analyses of more than two groups are feasible with the study data collected and would involve comparing the ME/CFS with or without FM and IBS cohorts in the same analyses. Therefore, “Comparison E” would look at the metabolomic and microbiome outcomes of the ME/CFS + FM, ME/CFS + IBS, ME/CFS - FM, and ME/CFS - IBS cohorts. Additionally, this study does not have matched cohorts (IBS only or FM only without ME/CFS) with these conditions. However, beyond

these two comorbidities which already provide challenging diagnostic and management quandaries in ME/CFS, there are several other comorbidities. Table 2.3.1 and Table 2.3.2 summarise some of the other comorbidities that exist with ME/CFS and the non-ME/CFS (Control) cohort from Part One and Two participants. The workflow used in this thesis could also be applied to other comorbidities represented in these data tables. However, doing so is not only a time-consuming and onerous pursuit but perhaps one that does not truly represent the full disease situation (how long is the piece of string?) or the wider ME/CFS population. For example, someone in the ME/CFS with IBS group could also be ME/CFS with FM, ME/CFS without POTS, ME/CFS with TMJ, ME/CFS with Endometriosis (females only), and/or ME/CFS without Hypothyroidism. It was also noted that the Control group although ME/CFS-free at the time of participation was not completely without some comorbidities; however, it was out of the scope of this project to evaluate the risk and likelihood of developing ME/CFS. While stratifying the comorbidities provides a “layer by layer” insight, a concern is that the realm of comorbidities in ME/CFS is limitless. This leads to the conundrum of inter- and intra-heterogeneity where not only are there multiple comorbidities but not every person with ME/CFS has the same ones, experience, or history. Hypotheses from subgroups based on age and sex can be made relatively easily before evaluating data outcomes. Subgrouping based on the diagnostic absence or presence of comorbidities be it FM, IBS, or another condition, runs the risk of an artificial, paradoxical, and ‘subgroup within subgroup” effect and problematic invalid findings and observations because of HARKing which stands for Hypothesising After the Results are Known (Kerr et al., 1998). On top of the attempt to resolve exactly what is happening in ME/CFS amongst the comorbidities, symptomology adds another component to the “confounding factors”.

Analyses presented in this thesis did not take into consideration the symptoms of participants although questionnaire responses make this data available for further study investigations. Regardless of the comorbidities and their involvement, subgroups, or subtypes of ME/CFS potentially exist based on symptoms (Jonsjö et al., 2017; Vaes et al., 2023). While symptoms may be similar in name and presentation “at/above the surface”, what is happening “beneath the surface” may suggest otherwise. Further clarification of symptoms with gut microbiome, metabolomic, and other objective measures in ME/CFS could provide better insight into the disease burden and tailored management strategies without the overreliance on case definitions and differentials. It may also help with reducing the confusion with other chronic conditions with similar clinical challenges and presentations. The study collected questionnaire data that

captured information on participants' frequency and severity of symptoms. Microbiome and metabolome data could also be evaluated according to symptom score responses. However, the study did not capture any data on within-day and between-day variations in symptoms. Fluctuations, wax, and wanes are commonly described in ME/CFS both in severity and hierarchy; the variations presented among patients and within an individual patient cannot be captured by a single time point. Any future studies that would attempt to address these issues would require a well-powered longitudinal study design with real-time or continually monitored measurements. Given that there is an element of trust that participants have been diagnosed appropriately and accordingly for all their conditions, recall, and self-reported bias associated with questionnaire records, the integration of more objective measures from variables such as metabolites, microbes, and other clinical measurements, is important. With this being said, one's personal view and experience with their health should never be discounted or overlooked. Longitudinal research allows an individual to be their own control or baseline entity and distinguish between age and cohort effects. It also offers the opportunity to reduce the impact of bias, validate findings, improve statistical power, and dynamically understand the disease over a period in detail (M. Wang et al., 2017). Certainly, this would be a substantial undertaking highlighting the ever-important need for funding, approaches to deal with selective attrition and participant retention in a study, collaboration, data harmonisation, and consistent usage of suitable tools, technology, and platforms.

### 6.2.2 Long COVID and Global Health

Long COVID encompasses the ongoing or persistent health problems beyond the “resolution” of COVID-19 caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. Long COVID is also referred to as long-haul COVID, post-COVID conditions, post-COVID-19 syndrome, or post-acute sequelae of COVID-19 (PASC/PACS). More than 200 symptoms have been identified with impacts on multiple organ systems and they can develop regardless of the initial COVID-19 disease severity (Castanares-Zapatero et al., 2022; Davis et al., 2023). The underlying pathophysiology of long COVID is poorly understood with no definitive biomarkers or proven effective therapies (Davis et al., 2023; Kenny et al., 2023). Since the start of the COVID-19 (COVID) pandemic, discussions of long COVID and its comparisons to ME/CFS and post-infectious syndromes have been made with an eerie sense of Deja vu. In a bittersweet way, COVID has brought much-needed attention to ME/CFS (The BMJ, 2021; Yong, 2022). In terms of establishing disease aetiology and pathogenesis, perhaps one thing that long COVID does have in its favour, compared to ME/CFS, is the more obvious association and link to the acute infection (Komaroff & Lipkin, 2023). The extensive similarities between ME/CFS and long COVID suggest that there is some overlap in disease mechanisms and pathophysiology (Davis et al., 2023; Jason et al., 2023; Kenny et al., 2023; Komaroff & Lipkin, 2023).

The manifestations and adverse events that are observed in long COVID are not particularly new concepts; however, no other virus outbreak has affected so many people on the scale and in the time frame as SARS-CoV-2 (Davis et al., 2023; López-Hernández et al., 2023). At least 65 million individuals around the world have long COVID, based on a conservative estimated incidence of 10% of infected people and more than 651 million documented COVID-19 cases worldwide; the number is likely much higher due to many undocumented cases and widespread lack of awareness and knowledge of viral-onset conditions (Davis et al., 2023). The health, economic, societal, and personal burden due to COVID-19 is already far-reaching and overwhelming (Cutler & Summers, 2020; Mirin et al., 2022). Like ME/CFS, the aftermath of SARS-CoV-2 infection has presented significant challenges to patients, families, governing bodies, workplaces, healthcare providers, and researchers alike. Recruitment and sample collection for this study were completed in 2019 before the start of the pandemic; however, long COVID, disease heterogeneity, the gut microbiome, and host metabolome are now of particular interest and relevance to ME/CFS moving forward.

It has been estimated that 13-45% of people with persistent, debilitating symptoms following acute COVID-19 meet the National Academy of Medicine case definition for ME/CFS (Komaroff & Lipkin, 2023). For many of its symptoms long COVID is not like ME/CFS; it is ME/CFS (Friedman et al., 2021). While many long COVID patients will satisfy one or more of the case definitions of ME/CFS, it must be recognised that long COVID for many, contains symptoms that are other than ME/CFS (Friedman et al., 2021). While there is an overlap and similarity between the two syndromes, they cannot be considered synonymous at this stage (Friedman et al., 2021). The difficult question remains as to just how similar (or different) long COVID and ME/CFS are, and on what basis. Currently, these conditions could conceivably represent just two examples in a broader disease continuum, or be two comorbidities, albeit similar, that frequently appear together (Davis et al., 2023; Komaroff & Lipkin, 2023). Only time will tell if long COVID cases will recover or improve, to what extent, and if other comorbidities or health complications are at increased risk of developing in the longer-term (Ballouz et al., 2023; Komaroff & Lipkin, 2023; Legler et al., 2023). Given the variability of clinical presentation and severity of the disease, recovery from COVID-19 is likely to be heterogeneous, in terms of both time to recovery and completeness of recovery (E. Holmes et al., 2021).

There is also uncertainty and speculation that those already with pre-existing ME/CFS could have even worse exacerbations and poorer outcomes with long COVID and recovery from an acute COVID (re)infection as they have been experiencing the adverse multi-system symptoms, effects, and deconditioning for a longer time (Legler et al., 2023; Mclaughlin et al., 2023). More than three years on from the declaration of the pandemic, COVID-19 is here to stay; different variants continue to emerge, leading to newer infections and reinfections (Boufidou et al., 2023; Guedes et al., 2023; Willyard, 2023). The now combined challenges of ME/CFS and long COVID “coexisting” face the unknown of potentially worsened disease vulnerabilities, burden, severity, and prognosis. Indeed, if not for long COVID per se and the direct biological, and disease aspects of COVID, the pandemic and other global issues such as AI, smart technology, climate change, geopolitics, economic volatility, workplace environments, and altered personal livelihoods, have irrevocably brought (fortunate and unfortunate) changes to health, wellbeing, society, culture, media, human interaction, and global citizenship. Vaccinations for COVID-19 decrease the risk of severe illness; however, it is unclear whether vaccine administration may impact the prevalence of long COVID (Notarte et al., 2022). There is some debate over the details of vaccinations such as the number of doses, intervals between boosters, type of

vaccine, the variable host responses, and the relationship between vaccination and pre-existing long COVID symptoms which are described in detail elsewhere (Ayoubkhani et al., 2022; Brannock et al., 2023; Feikin et al., 2022; Notarte et al., 2022; Strain et al., 2022). Overall, although current evidence is inconclusive, available data suggest that COVID-19 vaccines are important factors for further immunological protection against potential reinfections from subsequent variants of COVID (Notarte et al., 2022; Strain et al., 2022). Again, all of this highlights the likely possibility of various heterogenous and subgroup trajectories which makes the exact definition and complete understanding of the long COVID and ME/CFS conditions convoluted and difficult undertakings in research and clinical practice. The biomedical research agenda moving forward needs to build on the existing knowledge from ME/CFS and other viral-onset conditions, including, but by no means limited to, changes in the gut microbiome and host metabolic phenotypes (Davis et al., 2023; Jason et al., 2023; Kenny et al., 2023; Komaroff & Lipkin, 2023). Given the complexity, scale, and burden of long COVID and ME/CFS, future research efforts require, more than ever, coordinated, international, and interdisciplinary approaches that perform clinical trials in parallel with mechanistic studies (Davis et al., 2023).

There are likely several intertwined mechanisms that may contribute to the development and persistence of long COVID. The general takeaway message from the numerous publications on long COVID irrespective of ME/CFS is that it is a prolonged, heterogenous, and multi-systemic issue affecting multiple organs with unclear disease mechanisms and pathophysiology (Alexander et al., 2023; Altmann et al., 2023; Bruzzone et al., 2023; Castanares-Zapatero et al., 2022; Davis et al., 2023; Espín et al., 2023; Guo, Yi, et al., 2023; F. He et al., 2021; E. Holmes et al., 2021; C. B. Jackson et al., 2021; Jason et al., 2021, 2023; Kenny et al., 2023; Komaroff & Lipkin, 2023; Kovarik et al., 2023; Legler et al., 2023; Q. Liu et al., 2022; López-Hernández et al., 2023; Mendelson et al., 2020; Morello et al., 2023; Nagata et al., 2023; Nguyen et al., 2023; Österdahl et al., 2023; Páez-Franco et al., 2022; Patel et al., 2023; A. Subramanian et al., 2022; Sudre et al., 2021; W. Tate et al., 2022; J. Xu et al., 2021; Yeoh et al., 2021; Zhan et al., 2023; D. Zhang et al., 2023). The role and importance of comorbidities and subgroups in the development, presentation, and trajectory of long COVID have been considered in studies so far and it is recognised that future research needs to address them (Alexander et al., 2023; Davis et al., 2023; Legler et al., 2023; Morello et al., 2023; A. Subramanian et al., 2022). ME/CFS is not the only condition to have been brought into the long COVID biomedical discussion; irritable bowel syndrome, fibromyalgia, and postural orthostatic tachycardia (POTS), amongst other conditions, have also been raised which adds another layer of involvedness to the already big

problem at hand (Alexander et al., 2023; Chadda et al., 2022; Chan & Grover, 2022; Clauw & Calabrese, 2023; Davis et al., 2023; Mallick et al., 2023). Not surprisingly, several hypotheses for long COVID pathogenesis have been proposed including perturbed metabolism, immune dysregulation, persisting reservoirs of SARS-CoV-2 in tissues, latent virus reactivation, autoimmunity, microbiome and microbial translocation, dysautonomia, endotheliopathy and dysfunctional signalling in the brainstem and/or vagus nerve (Davis et al., 2023; Kenny et al., 2023; Komaroff & Lipkin, 2023).

A growing number of studies with long COVID so far have highlighted alterations and disturbances in the gut and its microbiome (gut dysbiosis) and metabolomic profiles (see above for the references to some of these studies). These findings are some preliminary indications that there are shared phenotypic, pathophysiological, and potentially, mechanistic characteristics with ME/CFS. Viral persistence within the gastrointestinal (GI) tract may impact the gut microbiota and contribute to dysbiosis (Meringer & Mehandru, 2022). Long COVID GI symptoms include loss of appetite, nausea, weight loss, abdominal pain, heartburn, dysphagia, constipation, altered bowel motility, and irritable bowel syndrome (IBS) (Davis et al., 2023; Meringer & Mehandru, 2022). The pathophysiology and frequency of GI symptoms and long COVID are still not clearly defined or understood (Meringer & Mehandru, 2022). However, a survey of COVID-19 survivors from the Columbia University Irving Medical Centre reported that 220 of 749 (29%) patients had GI symptoms self-perceived to be related to COVID-19, and for 83 (11%) of the patients, a GI symptom was the most bothersome current symptom (Blackett et al., 2022). This survey also reported that women were more likely than men to report post-COVID-19 GI symptoms or to meet the criteria for IBS (Blackett et al., 2022). The review by Komaroff and Lipkin (2023) listed two long COVID gut microbiome studies that were comparable to ME/CFS and a very loose extent, this study (Q. Liu et al., 2022; Yeoh et al., 2021). Yeoh et al. (2021) and Q. Liu et al. (2022) used faecal shotgun metagenomic sequencing and different data analytical approaches so a direct comparison to this study was not possible.

Yeoh et al. (2021) found that gut microbiome composition during hospitalisation was significantly altered in patients with acute COVID-19 compared to uninfected matched controls irrespective of whether patients had received medication. Overall, they found several gut commensals with known immunomodulatory potential such as Faecalibacterium prausnitzii and Eubacterium rectale to be underrepresented in infected patients and low in faecal samples collected up to 30 days after resolution of infection. There was only one species feature that

was the same between Yeoh et al (2021) and this study. Collinsella aerofaciens using differential abundance analysis testing was shown to share the same trend; it was underrepresented in COVID-19 samples and ME/CFS study participants (Figure 3.4.13). In a different study and snapshot of time, Q. Liu et al. (2022) presented outcomes of patients at 6 months who had persistent and lingering symptoms following a confirmed diagnosis of acute COVID-19 and a matched non-COVID-19 control group that were recruited before the pandemic. This study is more in line with the timeframes of ME/CFS diagnostic protocols and its observations provide evidence of gut microbiome compositional alterations and GI-related health issues in patients with long-term complications from COVID-19. At 6 months, Q. Liu et al. (2022) observed that patients with PASC showed a significantly lower level of Collinsella aerofaciens, Faecalibacterium prausnitzii, Blautia obeum, and a higher level of Ruminococcus gnavus and Bacteroides vulgatus than non-COVID-19 controls ($p < 0.05$ LefSe <2); Figure 6.2.1. Some microbial features were identified in Figure 6.2.1 that also appeared in this study with a similar trend. Higher levels of R. gnavus (Figure 3.4.1), B. vulgatus (Figure 3.4.14), and Clostridium innocuum (Figure 3.4.13) were identified in the ME/CFS groups and lower levels of C. aerofaciens (Figure 3.4.13). Interestingly, B. thetaiotaomicron (Figure 3.4.13, Figure 3.4.15, and Figure 3.4.16) was both high and low in ME/CFS groups. This brief comparison requires further investigation and consideration of the GI system, gut microbiome, long COVID, and ME/CFS where microbiome-based profiling has utility as part of a prevention, risk mitigation, and management tool. Further, these studies provide a strong impetus for future studies where the gut microbiome is part of an integrated multi-omics approach to researching both long COVID and ME/CFS as a joint effort.

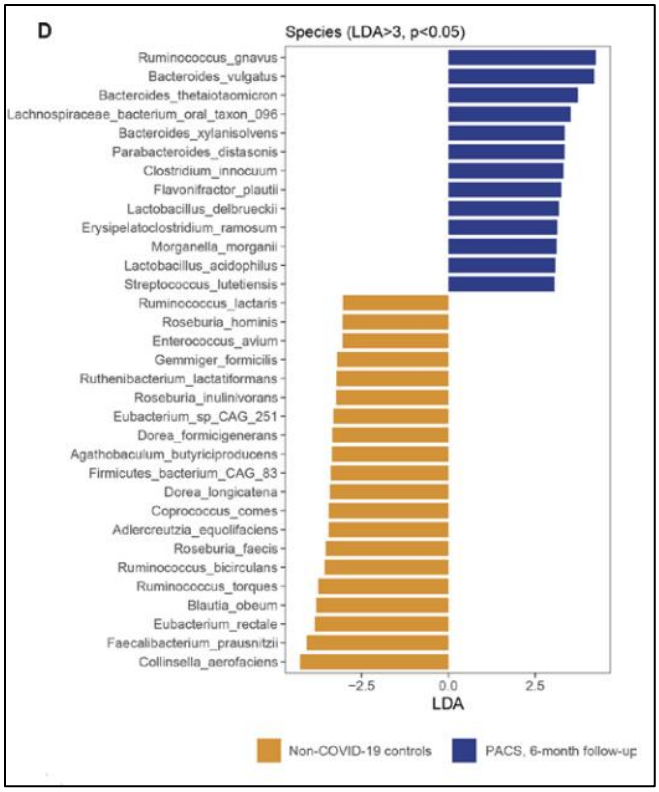



**Figure 6.2.1** Snippet from Figure 3 in Liu et al (2022)'s publication showing the linear discriminant analysis effect size in the gut microbiome of patients with PASC at 6 months.

As with any infectious disease, COVID-19 immediately triggers the immunological response, which is highly variable among patients and depends on personal characteristics and different involved risk factors (Bruzzone et al., 2023; C. B. Jackson et al., 2021). The interplay between infection, host response/immunological system, and the host, largely regulates the disease progression and is ultimately responsible for the severity and end outcomes of the disease (Bruzzone et al., 2023; E. Holmes et al., 2021; Miyazato et al., 2022). It is evident from the highly variable phenotypic responses and the systemic characteristics of the disease that these investigations require techniques such as the ones used in precision medicine (Bruzzone et al., 2023; Cen et al., 2023). Of the "omic" technologies aimed at genes, mRNA, proteins, and metabolites in a specific biological sample, metabolomics offers a particularly advantageous option for long COVID investigations (research, diagnostic, prognostic, monitoring, and treatments) because it is more sensitive to any phenotypic alteration (Bruzzone et al., 2023; Nicholson, 2021; Wishart, 2019). Metabolic information may complement and partially explain the phenotypic differences among long COVID-19 patients, and indeed assist with deciphering

the heterogeneity and overlapping conundrum owing to comorbidities and ME/CFS (Komaroff & Lipkin, 2023; López-Hernández et al., 2023; Wishart, 2019).

So far, profound metabolic dysregulation has been produced by SARS-CoV-2 infection that can be adequately characterised by metabolomics and lipidomics (Bruzzone et al., 2023). Similar metabolomic evidence from ME/CFS is emerging in people with long COVID with findings including reduced or disturbances in energy metabolism from fatty acids, amino acids, glucose via the TCA cycle, glycolysis, oxygen, and nucleotides central to energy metabolism, redox imbalance, changed metabolic state, and kynurenine pathway abnormalities (Bruzzone et al., 2023; F. He et al., 2021; Komaroff & Lipkin, 2023; Kovarik et al., 2023; López-Hernández et al., 2023). Given that the gut microbiome is responsible for the regulation of metabolism, the development of gastrointestinal mucosa, and an integral part of host immunity and responses, its connection to host metabolism plays an important part in discussions revolving around the management of ME/CFS and long COVID. However, the different techniques and approaches used in research impede proper comparison between studies and translation to human clinical application, particularly when considering the quantification of the changes (microbes and metabolites) associated with disease (Bruzzone et al., 2023; Galazzo et al., 2020). The findings in the gut microbiome and host metabolomic research in ME/CFS and long COVID make it obvious that future efforts would benefit from collaboration, larger study numbers, multi-omic and disciplinary longitudinal study designs, the prior knowledge, and several decades worth of experience established by ME/CFS, and the increased interest that is now held in post-infectious conditions because of COVID-19. For many reasons, it would be a poor use of skills, expertise, and resources, ignorant and short-sighted to consider one condition without the other. ME/CFS patients have suffered for far too long; long COVID patients should not experience a similar fate (Friedman et al., 2021).

### 6.2.3 Omics, Knowledge Application, and Translation

The gut microbiome, host metabolome, and its relationship are increasingly being recognised with further attributes discovered for its influence, association, and role in many health-related conditions including ME/CFS and long-COVID. A variety of tools, techniques, and technologies are becoming more readily accessible, available, and user-friendly in the growing field of omics and precision medicine for research and clinical practice. Metabolomics and metataxonomic analyses are only two approaches that can be used to investigate the gut microbiome, host metabolism, and indeed the broader aspects of biology, disease mechanisms, and pathophysiology. As discoveries and each avenue become more advanced, detailed, and involved, there are more things to explore and consider. Each dimension of a research problem and study requires advanced skills and training be it for wet laboratory procedures, sample collection, clinical practice, research/science communication, or statistics and data analysis. No single application can capture the entire complexity of most human diseases calling for integrated or multi-omic frameworks and approaches to provide a deeper, more comprehensive view of biology and disease (Karczewski & Snyder, 2018). Further, it is unlikely that a single biomarker, and therefore its underlying mechanism and pathology, can accurately and precisely identify all individuals with, or a predisposition for, ME/CFS. Other applications for gut microbiome analytical approaches include metagenomics and metatranscriptomics (Ojala et al., 2023). The wider reach of the omics field includes areas such as epigenomics, fluxomics, host genomics, genomics, glycomics, lipidomics, proteomics, and transcriptomics, which have also been utilised in numerous biomedical discoveries and developments to date. Every omics area has the potential to be integrated or combined with another to aid in data analysis, visualisation, and interpretation to determine the mechanism of a biological process (Krassowski et al., 2020). For ME/CFS, other than overcoming the challenges as discussed earlier with recruitment, further investigation of comorbidities and heterogeneity in the disease using these omic platforms is warranted.

Current omic-based approaches in ME/CFS are largely implemented to seek evidence, potential biomarkers, and the understanding of hypothesis-driven biological mechanisms that may underpin the disease. Research findings are yet to be developed and validated for board-approved, routine clinical use including the prevention, management, and treatment of ME/CFS. The potential of integrated omic models with longitudinal insight in translational medicine is rapidly being unveiled, adopted, and published; however, the shift of knowledge from the “bench

to bedside" is not without challenges. This comprises data analytical, accuracy and validation, interpretation, sampling, actionability, therapeutic, and monitoring considerations (Karczewski & Snyder, 2018; Krassowski et al., 2020). Of course, the questions and topics of life, reality, health, and disease at a philosophical level come into play. The "what is healthy?" can mean so much and take a variety of definitions. The computational needs and field also spark discussions over data management, security, ethics, privacy, insurance, society, and other issues regarding the usage of such data and information (Krassowski et al., 2020). The translation and route of research findings to human application is a detailed process and it needs to be done with responsibility. It is a new era for medicine, drug discovery, biomedical, and scientific research. The advent of machine learning, algorithms, and artificial intelligence in the omics field has facilitated rapid discoveries in health and disease and is becoming increasingly used in healthcare settings for diagnosis, prognostication, and outcome predictions (Pammi et al., 2022). With this comes the development and prospect of several models, tools, and platforms pitched toward improving the efficiency and tailoring of individual care. No doubt, these technologies with a precision-based and personalised focus have also formed the fundamental basis for numerous startup or new-age ventures in biological sample collection (for example, less sample required without the need for cold chain, better stability before reaching laboratory), high-throughput platforms, diagnostics, wearable monitoring devices, digital medical assistance, diet, nutrition, health promotion, wellness, therapeutics, drugs, supplements, and the like (M. Subramanian et al., 2020). There are several positive opportunities for mitigating and improving patient outcomes and experiences and identifying potentially at-risk individuals in ME/CFS. Certainly, much still needs to be done before any omic-based application can be applied specifically to a wider ME/CFS clinical or community/primary care setting. With the overwhelming abundance of consumer-based options, marketing hype, and accessible information in this space, it is critical that sound ME/CFS research continues with an acute awareness and caution of this burgeoning field and industry. ME/CFS individuals are already at their most vulnerable, they do not need to be led further astray or presented with a misleading narrative concerning their health.

## 6.3 Final Remarks

The overarching finding of this thesis is that the gut microbiome and host metabolome outcomes from ME/CFS can be viewed and potentially interpreted differently depending on FM or IBS comorbidity. While I did not look at any other comorbidities, the findings from this thesis indicate that they need to be given due consideration in ME/CFS. Moreover, these variations and research findings can also be influenced by the specific analytical approach or methods that were applied, and indeed by the biospecimen, metadata, and data-input types that were used to make these measurements and observations. These elements are all important in the pursuit of understanding the various complexities that present in ME/CFS. Throughout the journey of completing this study and thesis, and comprehending the extent of ME/CFS, I have come to appreciate and embrace that the gut microbiome and host metabolome are perhaps only some of the few pieces needed to solve the puzzle of this very intricate and heterogeneous disease. Further, it has highlighted and reaffirmed the fundamental importance of the individual and their experiences with ME/CFS which future research needs to be open and attuned to. Engaging all stakeholders in a dialogue about ME/CFS stands as a cornerstone for enhancing the understanding, outlook, and prospects of this disease. My contribution in the grand scheme of all things ME/CFS, and possibly now long COVID, seems modest, but they are not insignificant or in vain. It sheds light on the necessity of an objective framework and a pragmatic approach with the use of different but complementary omic-based technologies and biological analytical techniques and approaches for a more comprehensive and holistic command of the disease. These applications and fields are continually progressing, and it is encouraging for ME/CFS and many chronic diseases. As most things go, worthwhile and meaningful research takes time, effort, skill, expertise, determination, collaboration, and resources; ME/CFS especially demands and requires the whole gamut. My ultimate dream for ME/CFS biomedical research speaks to the larger aspirations within the entire ME/CFS community which is for the availability of a gold-standard, efficient, accessible, and reliable diagnostic and curative therapeutic protocol. In the meantime, I want to encourage others whether you are a health care provider, researcher, or lay person to be at least aware and respectful of the agonising biomedical reality of ME/CFS; ME/CFS is not “in the head” of a lazy, malingering person. I wish for continued productive research outcomes and discourse that allow for more individuals to receive appropriate and dignified care and support. It is crucial for individuals and everyone around them, from clinicians, allied health care workers, other service providers, friends, and family members, that we are all informed and empowered with the best available evidence and tools.

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# Appendices

Materials listed here in the Appendices can be viewed electronically the file "AmberJaaKwee_Thesis_2023_Appendix_Files" which accompanies this thesis.

**Appendix 1** Questionnaire Form

Questionnaire-1-Self-Report Questionnaire.pdf
Questionnaire-2-Symptom Questionnaire.pdf

**Appendix 2** Participant Cohort Metadata

ParticipantStudyMetadata_83_Part1.csv
ParticipantStudyMetadata_56_Part2.csv

**Appendix 3** Denaturing and Dilution Calculations for Illumina MiSeq Sample Loading

Denature DNA Pooled Library

Dilute pooled library from 3.5nM to 2nM (5 µL) with 10 mM Tris pH 8.5

$2\ nM \times 5\ \mu L = 3.5\ nM \times V_2$

$V_2 = 2.86\ \mu L$

2.86 µL of 3.5 nM pooled library + 2.14 µL of 10 mM Tris pH 8.5 = 5 µL of 2 nM pooled library

Following steps in the Illumina protocol for denaturing a 2 nM library (Illumina, 2013, 2019), 1 mL of a 10 pM denatured pooled library was prepared.

Denature PhiX Control Library

Dilute PhiX 10 nM stock to 2nM (5 µL) with 10 mM Tris pH 8.5

$2\ nM \times 5\ \mu L = 10\ nM \times V_2$

$V_2 = 1\ \mu L$

1 µL of PhiX 10 nM stock + 4 µL of 10 mM Tris pH 8.5 = 5 µL of 2 nM PhiX

Following steps in the Illumina protocol for denaturing a PhiX library (Illumina, 2013, 2019), 1 mL of a 10 pM denatured PhiX library was prepared.

Combined Denatured Pooled Library and PhiX Control Library

The pooled library with indexed amplicon samples was spiked with 10% PhiX control

432 µL of 10 pM pooled library + 48 µL of 10 pM PhiX library + 120 hybridization buffer (HT1) = 600 µL of 8 pM combined library for loading on to the MiSeq for sequencing

**Appendix 4** Script for QIIME2 processing of 16S rRNA amplicon sequences

Gut Microbiome/16SrRNA/QIIME2_AmberJaaKwee_Thesis_Appendix
The file can be viewed either in Notepad, R Studio, Visual Studio Code or similar

**Appendix 5** R Scripts for Gut Microbiome Data Analyses and Visualisation

Gut Microbiome/16SrRNA
Gut Microbiome/CultureMaldiTof

**Appendix 6** 16SrRNA Phyloseq Object

Gut Microbiome/16SrRNA/OriginalPhyloseqObjectAJK

**Appendix 7** Culture MALDI-TOF Phyloseq Object

Gut Microbiome/CultureMaldiTof/All-Species-Culture-Clean-AJK

**Appendix 8** LCMS and NMR Faecal and Urine Metabolome (Part One) Raw Data Matrices

Metabolomics/PartOneMetabolomicsRawDataMatrices

**Appendix 9** LCMS and NMR Faecal and Urine Metabolome (Part One) Results

Metabolomics/PartOneMetabolomicsResults

**Appendix 10** LCMS and NMR Blood Plasma and Urine Metabolome (Part Two) Raw Data Matrices

Metabolomics/PartTwoMetabolomicsRawDataMatrices

**Appendix 11** LCMS and NMR Blood Plasma and Urine Metabolome (Part Two) Results

Metabolomics/PartTwoMetabolomicsResults