

# **The Role of Dietary Fibres in the Gut-Microbiota-Brain Axis**

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## Abstract

Dietary Fibre (DF) is increasingly recognised as a key regulator of the gut-microbiota-brain (GMB) axis; however substantial interindividual variability exists in responses to DF interventions. This thesis examined how DF type, biological sex, dietary context, and genetic risk influence gut microbiota composition, metabolic activity, and downstream mental and cognitive health outcomes. Using preclinical models, baseline sex differences in gut microbiota composition, and gut-metabolomic profiles were first characterised, revealing pronounced male-female divergence. Building on this foundation, the effects of isolated DF interventions were investigated using inulin and psyllium, selected for their contrasting fermentability. Inulin induced marked, sex-dependent alterations in gut microbiota composition and metabolic output, whereas psyllium elicited more modest, largely sex-independent effects. A combined DF intervention generated heterogeneous responses, highlighting interactive effects between fibre types. Together, these findings demonstrate that DFs are not functionally equivalent and that fermentability and host sex critically influence GMB communication. To translate these mechanistic insights into humans, the impact of DF within whole dietary patterns was assessed. In adults with mild to moderate anxiety and/or depression, short-term adherence to a high-fibre Mediterranean-style diet resulted in rapid shifts in gut microbiota composition and metabolite profiles, which were associated with improvements in mental health outcomes. These results highlight the sensitivity of the GMB axis to high-DF vs low-DF dietary patterns. Finally, habitual DF intake was examined in relation to cognitive performance in older adults with Subjective Cognitive Impairment (SCI) or Mild Cognitive Impairment (MCI), stratified by *APOE4* genotype. Higher DF intake was associated with better cognitive performance in non-*APOE4* carriers, whereas no clear associations were observed in *APOE4* carriers, underscoring the importance of genetic context in shaping dietary responsiveness. Collectively, this thesis advances the understanding of DFs as a biologically active dietary component and supports the development of personalised, DF-based strategies to support mental and cognitive health.

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## List of Abbreviations

|        |                                                                    |
|--------|--------------------------------------------------------------------|
| AD     | Alzheimer's Disease                                                |
| AMPs   | Anti-Microbial Peptides                                            |
| AOAC   | Association of Official Analytical Chemists (AOAC)                 |
| BBB    | Blood Brain Barrier                                                |
| BDNF   | Brain-derived Neurotrophic Factor                                  |
| CBF    | Cerebral Blood Flow                                                |
| CNS    | Central Nervous System                                             |
| DALYs  | Disability Adjusted Life Years                                     |
| DASH   | Dietary Approaches to Stop Hypertension                            |
| DF     | Dietary Fibre                                                      |
| DFs    | Dietary Fibres                                                     |
| DOPAC  | 3,4-Dihydroxyphenylacetic acid                                     |
| DSB    | Digit Span Backwards                                               |
| EFSA   | European Food Safety Agency                                        |
| ENS    | Enteric Nervous System                                             |
| F:B    | Firmicutes to Bacteroidetes ratio                                  |
| FDR    | False Discovery Rate                                               |
| FMT    | Faecal Microbiota Transplantation                                  |
| FSA    | Food Safety Agency                                                 |
| GABA   | Gamma Aminobutyric Acid                                            |
| GAD-7  | Generalised Anxiety Disorder-7                                     |
| GBD    | Global Disease Burden                                              |
| GDNF   | Glial cell-line Derived Neurotrophic Factor                        |
| GI     | Glycaemic Index                                                    |
| GIT    | Gastrointestinal Tract                                             |
| GLP-1  | Glucagon-like Peptide 1                                            |
| GMB    | Gut-Microbiota-Brain                                               |
| GPCRs  | G-protein Coupled Receptors                                        |
| HPA    | Hypothalamic-Pituitary-Adrenal                                     |
| IBD    | Inflammatory Bowel Disease                                         |
| IBS    | Irritable Bowel Syndrome                                           |
| ICD    | International Classification of Diseases                           |
| ISAPP  | International Scientific Association for Probiotics and Prebiotics |
| KO     | Knockout                                                           |
| LCMS   | Liquid Chromatography Mass Spectrometer                            |
| L-DOPA | L-3,4-dihydroxyphenylalanine                                       |
| MCI    | Mild Cognitive Impairment                                          |
| MCTs   | Monocarboxylate Transporters                                       |
| MDP    | Mediterranean Dietary Pattern                                      |
| MEDAS  | Mediterranean Diet Adherence Screener                              |
| MIND   | Mediterranean-DASH diet Intervention for Neurodegenerative Delay   |
| MUFA   | Monounsaturated Fatty Acids                                        |
| NCDs   | Non-Communicable Diseases                                          |
| NDNS   | National Diet and Nutrition Survey                                 |
| NGF    | Nerve Growth Factor                                                |
| NMR    | Nuclear Magnetic Resonance                                         |

|           |                                                  |
|-----------|--------------------------------------------------|
| NOR       | Novel Object Recognition                         |
| NSP       | Non-Starch Polysaccharides                       |
| OF        | Open Field                                       |
| OTUs      | Operational Taxonomic Units                      |
| PCA       | Principal Component Analysis                     |
| PERMANOVA | Permutational Analysis of Variance               |
| PHQ-9     | Patient Health Questionnaire-9                   |
| POMS      | Profile of Mood States                           |
| PPP       | Pentose-phosphate Pathway                        |
| PUFA      | Polyunsaturated Fatty Acids                      |
| PYY       | Peptide YY                                       |
| qRT-PCR   | Quantitative Real-Time Polymerase Chain Reaction |
| RCT       | Randomised Controlled Trial                      |
| ROs       | Resistant Oligosaccharides                       |
| RS        | Resistant Starch                                 |
| SACN      | Scientific Advisory Committee on Nutrition       |
| SART      | Sustained Attention to Response Test             |
| SCFAs     | Short-chain Fatty Acids                          |
| SFA       | Saturated Fatty Acids                            |
| SCI       | Subjective Cognitive Impairment                  |
| TCA       | Tricarboxylic acid                               |
| TDF       | Total Dietary Fibre                              |
| TMA       | Trail Making A                                   |
| TMD       | Total Mood Disturbance                           |
| WD        | Western Diet                                     |
| WHO       | World Health Organisation                        |
| 5-HT      | 5-Hydroxytryptamine                              |

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## **Chapter 1: General Introduction**

Thesis Marrium Liaquat

## 1.1 Mental Health Disorders are a Global Health Burden

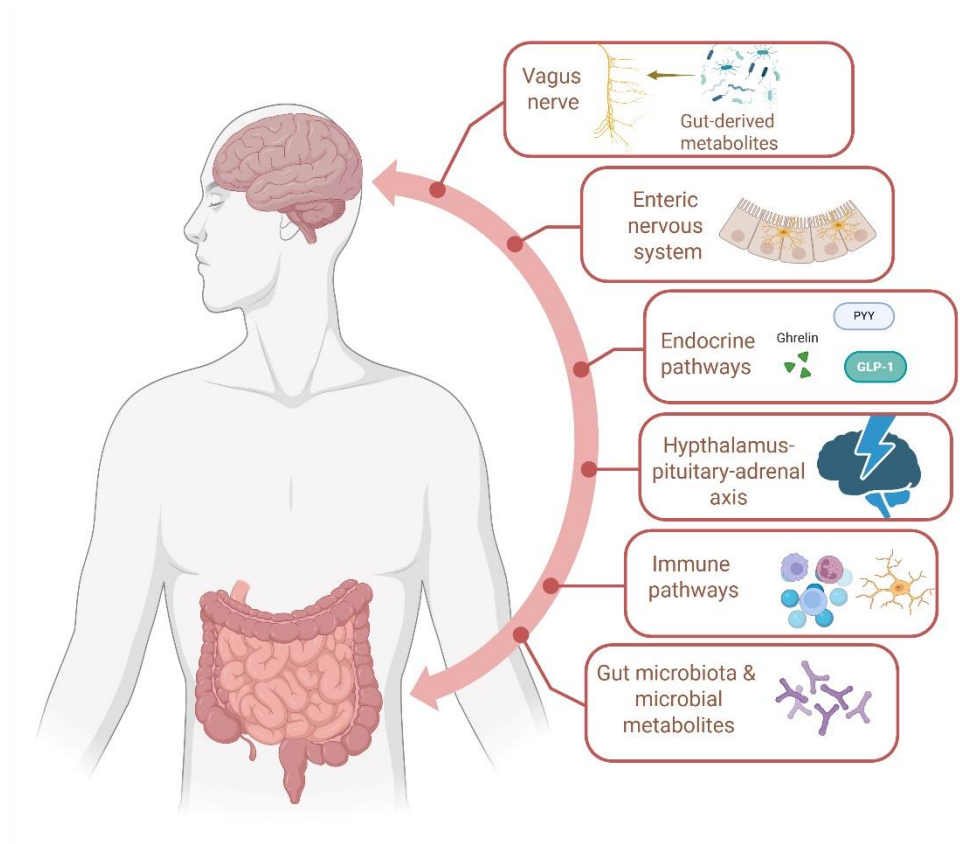
The World Health Organisation (WHO) defines mental health as “a state of well-being in which every individual realises his or her own potential, can cope with normal stresses of life, can work productively and fruitfully, and is able to make a contribution to his or her community” (WHO, 2013). The International Classification of Diseases, 11<sup>th</sup> Revision (ICD-11), categorises mental disorders to include conditions such as anxiety disorders, depression, bipolar disorder, post-traumatic stress disorder, schizophrenia, eating disorders, disruptive behaviour and dissocial disorders, and neurodevelopmental disorders (WHO, 2022). Among these, depression and anxiety disorders are the most common (Liubertiene et al., 2025).

Mental disorders account for a large burden of disability worldwide. It is estimated that 418 million disability-adjusted life years (DALYs) are attributable to mental disorders, representing 16% of global DALYs (Arias et al., 2022). According to the Global Disease Burden (GBD) study, mental disorders were the seventh leading cause of DALYs in 2019, which has worsened since 1990 when it was ranked as 13<sup>th</sup>. Over this period, the global DALYs attributable to mental disorders increased from 80.8 million to 125.3 million (Collaborators, 2022). In 2019, mental health conditions accounted for 7% of all ill health in the UK, with associated societal costs estimated at £117.9 billion, which is equivalent to approximately 5% of the UK GDP (Gross Domestic Product) (McDaid & Park, 2022). According to the 2023/2024 Survey of Mental Health and Wellbeing in England, common mental health conditions including depression and anxiety affect one in five adults (20.2%) (Liubertiene et al., 2025). The prevalence is higher among women (24.2%) than men (15.4%) (Liubertiene et al., 2025).

Addressing modifiable risk factors such as nutrition, housing, education, and economic insecurity can help prevent mental health disorders (Saxena et al., 2006). With a growing shift towards preventative healthcare, the field of “Nutritional Psychiatry” has emerged (Adan et al., 2019; Sarris et al., 2015), highlighting the link between diet and brain health, particularly for mood disorders such as anxiety and depression (Sanz et al., 2025; Xiong et al., 2023). Central to this diet-brain relationship is the gut microbiota, which plays a key role in systemic and neurological health (Berding, Vlckova, et al., 2021; Cryan et al., 2019; Schneider, O’Riordan, et al., 2024).

## 1.2 Gut-Brain Axis

A ‘gut feeling’ or the sensation of ‘butterflies’ in the stomach are common demonstrations for the complex physiological and molecular relationships between gut and brain function. The gut-brain axis is the bidirectional communication comprised of central, enteric and autonomic nervous systems and the hypothalamic-pituitary-adrenal (HPA) axis. The key pathways of interest in this communication include the *vagus* nerve, the immune and enteroendocrine systems, the neurotransmitters and the gut microbiota along with their associated metabolites (Chakrabarti et al., 2022), as outlined in Figure 1.1.



**Figure 1.1 | Gut-Brain axis involves different communication pathways.**

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1. The *vagus* nerve is the tenth cranial nerve extending from the brainstem through the neck and thorax to the abdomen and regulates internal organ functions such as digestion, heart rate and respiratory rate. It is also called the “wanderer nerve”, because of its long path through the human body (Breit et al., 2018; Rosas-Ballina et al., 2011). The efferent fibres of the *vagus* nerve send signals down from the brain to the gut, and

account for 10-20% of all vagal fibres, with the afferent fibres sending signals up from the intestinal walls to the brain, and accounting for 80-90% of all vagal fibres (Breit et al., 2018). This division of efferent and afferent nerve fibres highlight the prime importance of the gut environment in regulating the *vagus* nerve stimulation and as a modulator of brain responses.

2. The Enteric Nervous System (ENS), also known as intrinsic nervous system consists of a nerve plexus (~200-600 million neurons) embedded in the intestinal wall, extending across the whole gastrointestinal tract from the oesophagus to the anus (Furness et al., 2014). It has more neurons than the spine (Breit et al., 2018), which sense the dynamic intestinal microenvironment via the enteroendocrine and immune cells in the intestinal walls, which facilitate signalling from the gut lumen to the ENS (Geng et al., 2022).

3. The enteroendocrine system involves several gut hormones and peptides (such as peptide YY, glucagon-like peptide 1, ghrelin) released by the enteroendocrine cells (Zheng et al., 2023), into the blood circulation. These mediate the gut-brain communication in numerous ways, e.g. these hormones and peptides may cross the blood-brain barrier (BBB) or synergistically stimulate the *vagus* nerve (Geng et al., 2022).

4. The HPA axis is a major neuro-endocrine system which responds to stress by releasing corticotrophin-releasing hormone from the hypothalamus, which subsequently releases adrenocorticotrophic hormone from the pituitary thus facilitating cortisol release from the adrenal glands (Rusch et al., 2023). Cortisol is a stress hormone, which also affects gut microbiota by influencing gut transit time, intestinal permeability, and nutrient availability, which in turn impacts gut microbiota composition and diversity (Mayer, 2000). It contributes to the bidirectional gut-brain communication by influencing cognitive processes like learning and memory as well as normal neurodevelopment (plasticity) and function (Rusch et al., 2023).

5. The immune system plays a crucial role in gut-brain communication. Enterocytes which are present all along the intestinal tract express innate immune receptors, which distinguish between *harmful* and *harmless* signals in the gut, and release cytokines and chemokines. These molecules act as chemical messengers, facilitating the communication between the gut environment, the immune system and the central nervous system (CNS) (Zheng et al., 2023).

6. Neurotransmitters are a diverse class of endogenous chemical messengers that transmit information between neurons or between neurons and other cell types including immune cells, glial cells and gland cells. The gut serves as a production site for many major neurotransmitters (Teleanu et al., 2022), such as gamma-aminobutyric acid (GABA), 5-hydroxytryptamine (5-HT) also called serotonin, dopamine, norepinephrine and acetylcholine. These neurotransmitters play an important role in gut-brain communication (Lyte, 2014), and are involved in various functions such as learning, cognition, memory, attention and other brain functions (Loh et al., 2024). Most of these neurotransmitters cannot cross the BBB due to their low lipid solubility but can indirectly modulate brain activity via *vagus* nerve or local interaction with the ENS (Zheng et al., 2023). However, certain precursors (also produced in the gut) can cross the BBB, and neurotransmitter-metabolising enzymes present in the brain endothelial cells convert these precursors into active neurotransmitters within the brain (Lattera et al., 1999). For example, unlike dopamine, its precursor L-DOPA can cross the BBB and is subsequently converted into dopamine by L-DOPA decarboxylase and monoamine oxidase enzymes in the capillary endothelial cells (Hardebo & Owman, 1980). This study of microbe-derived neurochemicals is referred to as “microbial endocrinology” (Lyte, 2014).

7. The gut microbiota and its metabolites play a major role in Gut-Brain communication, influencing BBB and overall brain functions such as immunity and neuroplasticity. These effects have been linked to cognitive function and neurodegeneration (Boehme et al., 2023). The following section explores this role in greater detail.

### 1.3 Role of the Gut Microbiota in Gut-Brain Crosstalk: Gut-Microbiota-Brain (GMB) Axis

The term “microbiota” refers to the collection of microorganisms in an ecosystem, and “microbiome” refers to the collection of microorganisms along with their collective genetic material (Gibson et al., 2017). The “Gut microbiota” refers to the collection of microorganisms in the gastrointestinal tract (GIT) including bacteria, archaea, viruses and fungi (Valeri & Endres, 2021). Bacteria make up the majority of the gut microbiota community and are taxonomically classified into phyla, classes, orders, families, genera, species and strains. To date, 25 different phyla, and ~ 2,000 genera and 5,000 species

have been identified (Thomas & Segata, 2019). Among the 25 phyla, the most abundant ones include Firmicutes, Bacteroidetes, Actinobacteria, Cyanobacteria, Fusobacteria, Proteobacteria, and Verrucomicrobia. Out of these phyla, Firmicutes and Bacteroidetes constitute 70-90% of the total gut microbiota in healthy humans (Berding, Vlckova, et al., 2021).

The inter-individual variability in the gut microbiota, driven by factors such as age, sex, ethnicity, lifestyle, and diet (Rinninella et al., 2019), poses a major challenge in defining a universally healthy gut microbiota (Thomas & Segata, 2019). The relationship between the two dominant phyla, expressed as the Firmicutes:Bacteroidetes (F:B) ratio has gathered significant attention as a potential biomarker for health (Van Hul et al., 2024), and a disturbed ratio has been associated with various pathologies such as obesity and metabolic disorders (Houtman et al., 2022; Magne et al., 2020), and gastrointestinal issues such as IBS and IBD (Tsai et al., 2025). It is also suggested that resistance (ability to resist changes) and resilience (ability to return to the baseline state) (Bäckhed et al., 2012) of the gut microbiota or microbial richness (number of unique microbes) and diversity (amount of different microbes) could define a healthy microbiota community (Berding, Vlckova, et al., 2021).

However, the definition of a “healthy gut microbiota” remains debated. Rather than specific microbial species, the presence of key microbial functions, referred to as “functional core” may be more important, as similar metabolic roles can be performed by different microbes across individuals (Thomas & Segata, 2019). Large cohort studies show that while only about 45% of bacterial species are shared between individuals, approximately 82% of their metabolic pathways are conserved, highlighting greater functional similarity than compositional overlap (Van Hul et al., 2024; Visconti et al., 2019).

The gut microbiota has a symbiotic relationship with the host, which can be either beneficial, neutral or pathogenic. It is shaped and influenced by various factors (endogenous and exogenous) encountered throughout the life course including but not limited to birth (e.g. mother’s health and mode of delivery), diet, and antibiotic usage (Valeri & Endres, 2021). The gut microbiota is ranked as the fourth most important factor

in programming brain health in early life along with host genetics, and the pre- and post-natal environment (Codagnone et al., 2019). A vast number of association studies have explored the possible modulatory role of the gut-microbiota in learning, memory, anxiety, stress, neurodevelopment, and neurodegeneration (Chakrabarti et al., 2022). Alterations in gut microbiota composition have been linked to various neurological pathologies and diseases (Bonnechere et al., 2022). Meta-analyses of studies on populations with major depressive disorder (M. Chen et al., 2022; Nikolova et al., 2021; Sanada et al., 2020) and anxiety (Lai & Xiong, 2025) showed significant associations between gut microbiota and disease patterns. Another meta-analysis summarising associations between the abundance of specific gut microbiota and cognitive development (Putri et al., 2023), suggested a potential causal link, as antibiotic treatment was associated with reduced cognitive performance later in life among individuals without prior cognitive impairment (Ye et al., 2024). Furthermore, faecal microbiota transplantation (FMT) has been shown to significantly improve symptoms of depression and anxiety in patient with *Clostridioides difficile* infections (Koppenol et al., 2022). Research focus is now turning towards gut-microbiota-based therapeutic and nutritional strategies for improving brain health and preventing neurodegeneration (Chakrabarti et al., 2022).

### 1.3.1 Barriers to Gut-Microbiota-Brain Signalling

There are two primary barriers which regulate GMB signalling: (1) the intestinal barrier, and (2) the blood-brain barrier. Both are dynamic and can be influenced by factors such as the gut microbiota, inflammation, stress (Aburto & Cryan, 2024; Mayer et al., 2022), genotype (Montagne et al., 2020), and dietary patterns (Hargrave et al., 2016).

#### Intestinal Barrier

The intestinal barrier separates the gut microbial ecosystem from the gut-linked immune system. It comprises of two principal layers. The first consists of epithelial cells which are connected by tight junctions. The second is the mucus layer, which consists of glycans (complex sugar molecules) varying in thickness and composition (Mayer et al., 2022). The mucus layer is further categorised into the outer layer (facing the gut lumen) and the inner layer (attached to the epithelial cells). The outer layer is inhabited by commensal microbes and provides nutrients (e.g. glycans) in the absence of dietary

fibres. The inner layer is free from microbes and protects the epithelial cells from gut microbiota via physical separation, innate immune mechanisms (e.g. antimicrobial peptides (AMPs)), and adaptive immune mechanisms (such as secretory immunoglobulin A) (Mayer et al., 2022).

The major component of the mucus layer is MUC2 mucin (secreted by goblet cells), the only gel-forming mucin along with other components such as FCGBP, CLCA3, ZG16, AGR2, and immunoglobulins (Johansson et al., 2011). MUC2 of the inner firm mucus layer is insoluble, and that of the outer loose mucus layer is readily soluble. This change in solubility is probably due to proteolytic cleavages of MUC2 in the outer mucus layer, allowing the mucus to expand four times in volume, thus facilitating commensal bacteria to thrive (Johansson et al., 2011; Johansson et al., 2008). Bacteria bind to this mucus via adhesins (found at the tip of bacterial fimbriae). The O-glycan repertoire on MUC2 mucin has been observed to be variable in different species (Hansson, 2020). As the gut bacteria have symbiotic binding to the outer side of inner mucus layer, the inner side of this layer is devoid of bacteria, thus maintaining the integrity of the intestinal barrier. Studies have shown that feeding low-fibre Western-type diets, render the inner mucus layer more penetrable to bacteria, allowing them to come closer to the host epithelium (Desai et al., 2016; Schroeder et al., 2018), where the first line of intestinal barrier defence fails. The status of the mucus layer is balanced between replenishment by goblet cells and degradation by gut bacteria (Desai et al., 2016). When the goblet cells sense the increased level of bacterial products, they respond by releasing a mucus plume to wash away these bacteria. These lines of defence can be exhausted, which leads to stress responses and overt inflammatory reactions, once an overwhelming number of bacteria reach the immune cells of lamina propria (Hansson, 2020). A healthy balance of gut microbiota and their derived metabolites is crucial in maintaining the integrity of the intestinal structure by maintaining the tight junctions between epithelial cells and by reducing the gut-associated immune cells activation (Mayer et al., 2022; Rosell-Cardona et al., 2025).

### Blood-Brain Barrier

The blood brain barrier (BBB) is a physiological barrier between the circulatory system and the cerebrospinal fluid. It is composed of capillary endothelial cells, sealed by tight

junctions, astrocytes and pericytes. Paracellular diffusion of water-soluble substances from the blood to the brain is typically restricted by tight junction proteins. These proteins are dynamic structures and their expression changes under physiological and pathological conditions (Braniste et al., 2014). Like tight junction proteins in the intestinal barrier, tight junctions in the BBB are also influenced by the gut microbiota. The absence of gut microbes in germ-free mice is associated with decreased expression of tight junction proteins causing structural alterations in the BBB, leading to increased permeability compared to conventional mice (Braniste et al., 2014). The gut microbiota exhibits a causal relationship, as evidenced by a decrease in BBB permeability when germ-free mice receive faecal transplantation from conventional mice (Braniste et al., 2014; Parker et al., 2020).

In summary, the gut microbiota plays a key role in GMB communication by influencing the integrity of the intestinal and blood-brain-barriers, which is essential for healthy signalling along this axis.

#### 1.4 Role of Diet in Modulating the Gut-Microbiota-Brain Axis

Changes in dietary pattern or in the intakes of select diet-derived bioactives are increasingly recognised for their ability to modulate the GMB axis (Schneider, Balasubramanian, et al., 2024), and its effect on mood, cognitive function and the progression of neurodegenerative disorders (Schneider, O'Riordan, et al., 2024). Dietary changes can alter the composition of the gut microbiota within days (Bourdeau-Julien et al., 2023; David et al., 2014).

Among various dietary patterns, the Western and Mediterranean diets have been the most extensively studied for their distinct and contrasting effects on the gut microbial communities (Mafe & Busselberg, 2025; Schneider, O'Riordan, et al., 2024). The Western diet is characterised by foods high in refined carbohydrates (sugars), saturated fats and salt, and low in dietary fibres (DFs), complex carbohydrates and micronutrients (Kendig et al., 2021). This dietary pattern is associated with a reduced overall microbial diversity and alterations in the gut microbiota composition, including an increased F:B ratio, elevated abundance of Proteobacteria, and decreased levels of Bacteroidetes and Actinobacteria (Clerici et al., 2025). These microbial shifts contribute to gut dysbiosis,

which is associated with intestinal epithelial barrier dysfunction, diminished neuroplasticity and neurotransmitter production, and heightened systemic and neuroinflammation (Martinez Leo & Segura Campos, 2020).

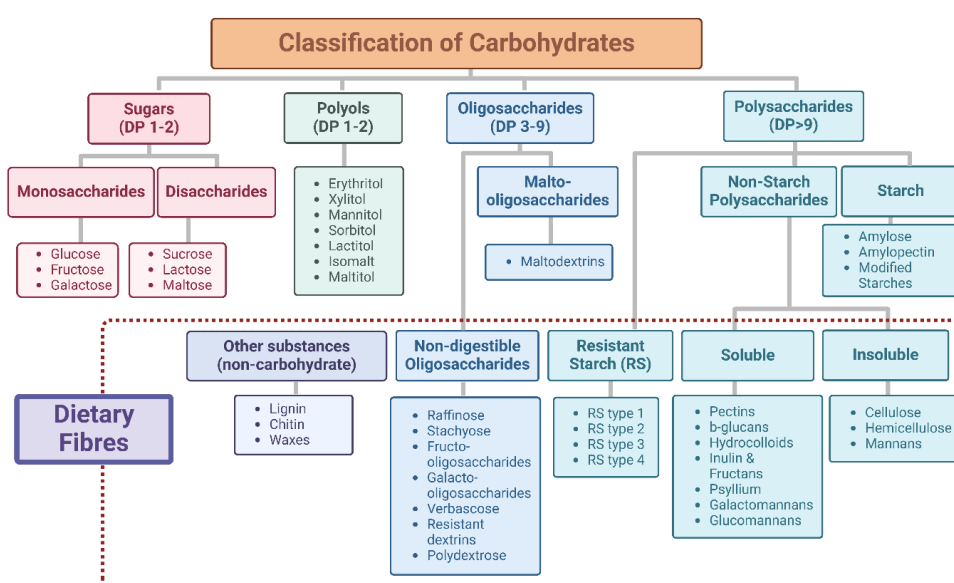
On the other hand, a Mediterranean diet characterised by high intake of fruits, vegetables, whole grains, legumes, fish, healthy fats, and limited consumption of confectionary along with red and processed meat and is consistently associated with better cognitive function and lower risk of neurodegenerative diseases (Mafe & Busselberg, 2025). Habitual consumption of Mediterranean diet is also associated with slower cognitive decline and better cognitive performance (Jennings et al., 2024; McEvoy et al., 2024; Picone et al., 2024; Shannon et al., 2019). Like a Mediterranean diet, other neuroprotective diets such as the Dietary Approaches to stop hypertension (DASH) and the Mediterranean-DASH diet Intervention for Neurodegenerative Delay (MIND diet) are also enriched in high fibre foods like whole grain cereals, fruits, vegetables, legumes and nuts. These dietary patterns have been involved in modifying various biological processes involved in cognitive decline like neuroinflammation, oxidative stress, hippocampal neurogenesis impairment, neurovascular dysfunction and gut-brain axis disruption (Cremonini et al., 2019).

Among the beneficial components of these diets, DFs plays a crucial role in modulating the gut microbiota, serving as a primary substrate for microbial fermentation (Cronin et al., 2021). These dietary patterns promote a more balanced and diverse gut microbiota composition (Garcia-Mantrana et al., 2018; Merra et al., 2020). High-level adherence to a Mediterranean-style diet is associated with higher levels of fibre-degrading bacterial genera such as *Lachnospira* and *Prevotella* (De Filippis et al., 2016). Another study linked high adherence to a Mediterranean diet with lower *Escherichia coli* levels, and a higher *Bifidobacteria/E.coli* ratio (Mitsou et al., 2017). Animal and human translational studies suggest that gut-microbiota related changes could be the main drivers of the beneficial effects of traditional Mediterranean diet (Khavandegar et al., 2024; Tosti et al., 2018).

## 1.5 Dietary Fibres as Gut Microbial substrate and their associated metabolites

### 1.5.1 Dietary Fibres

The European Food Safety Agency (EFSA) defines DFs as “non-digestible carbohydrates plus lignin”, which includes the main types of DF i.e., non-starch polysaccharides (NSP), resistant starch (RS), resistant oligosaccharides (ROs), and other substances associated with dietary fibre polysaccharides, especially lignins (EFSA Panel on Dietetic Products, 2010). The International Scientific Association for Probiotics and Prebiotics (ISAPP) defines DFs as non-digestible plant-derived carbohydrates comprising at least 3 units of individual sugars (ISAPP, 2019). Definitions of DFs from different authorities differ to some extent because of differences in the analytical methods used to determine DFs, as well as their ability to get digested and solubilise in the small intestine (Stephen et al., 2017). A detailed classification of carbohydrates and DFs is outlined in Figure 1.2.



**Figure 1.2 | Classification of Carbohydrates and Dietary Fibres**

Created with BioRender.com; DP: Degree of polymerisation

### 1.5.2 Dietary Fibre quantification methods

The analytical methods for DF quantification have evolved over years. Since DF is an important dietary component, also declared on food labels, it must be accurately quantified (Stephen et al., 2017).

The total dietary fibre (TDF) method 985.29 developed by the Association of Official Analytical Chemists (AOAC) has been the official method for DF quantification in

numerous countries (De Vries, 2000). In addition to TDF quantification, AOAC also developed methods to quantify specific types of fibre, e.g. insoluble DF (AOAC 991.42), soluble DF (AOAC 993.19), oligosaccharides (AOAC 2001.02), resistant starch (AOAC 2002.02) or  $\beta$ -glucans (AOAC 995.16)(McCleary et al., 2012). In the UK, the Englyst method (Englyst et al., 1992) for the quantification of non-starch polysaccharides (NSP) was accepted and used by the-present Food Standards Agency (FSA) for nutrition labelling purposes, until 1999. This method is still used in the McCance and Widdowson's Table of Food Composition. However, the latest version of McCane and Widdowson's: The Composition of Foods released in 2014 reports both NSP (using Englyst method) and DF values (using the AOAC method), proposing that DFs intake in the future might be described by both methods. The UK Scientific Advisory Committee on Nutrition (SACN) recommends the use of the recent AOAC method 2009.01, which is in line with most other countries (SACN, 2013).

### 1.5.3 Dietary recommendations for dietary fibres

The recommended daily intake of DF in the UK for different age groups is detailed in table 1.1, with an intake of 30g per day recommended for adults. Despite increasing awareness on health benefits of DFs, the mean DF intake in UK adults (16.4g/day) has remained far below the recommendation (30g/day) as per the National Diet and Nutrition Survey (NDNS) data from 2019 to 2023 (NDNS, 2025). According to this recent NDNS data, only 4% of the UK adult population met the DFs recommendation of 30g/day (NDNS, 2025).

**Table 1.1**|Recommendations by the Scientific Advisory Committee on Nutrition (SACN), UK

| Age group      | Recommended DF intake          |
|----------------|--------------------------------|
| < 2 years      | No quantitative recommendation |
| 2 to 5 years   | 15 g/day                       |
| 5 to 11 years  | 20 g/day                       |
| 11 to 16 years | 25 g/day                       |
| > 16 years     | 30g/day                        |

(SACN, 2013)

#### 1.5.4 Health Benefits of Dietary Fibres

A systematic review and meta-analysis of 64 prospective studies found that a higher DF intake was associated with a 23% reduction in all-cause mortality (HR:0.77; 95%CI (0.73,0.82)) (Ramezani et al., 2024). Additionally, meta-analyses of observational studies have linked higher DF consumption to lower odds of depression (Aslam et al., 2024; Yang et al., 2024), reduced anxiety (Aslam et al., 2024), and better cognitive function (Prokopidis et al., 2022).

The health effects of DFs have long been associated with GIT physiological functions such as anti-constipation effects, delayed gastric emptying and glucose absorption, and enhanced fat excretion (Perez-Jimenez, 2024). However, evidence from the last decade shows that health benefits of DFs go far beyond that, with the gut microbiota acting as an important mediator (Deehan et al., 2024; Perez-Jimenez, 2024; Pushpass et al., 2022). The gut microbiota feeds on DFs, which acts as a prebiotic. A prebiotic is defined by ISAPP as “a substrate that is selectively utilised by host microorganisms conferring a health benefit” (Gibson et al., 2017; Vinelli et al., 2022).

#### 1.6 Dietary fibres as Modulators of the Gut-Microbiota-Brain (GMB) Axis

Whilst several foods and dietary components are known to modulate the GMB axis, DFs and their capacity to act as substrates for microbial digestion have been identified as key modulators of the gut microbiota and its subsequent signalling to the brain (Berding, Carbia, et al., 2021; Berding, Vlckova, et al., 2021; Schneider, Balasubramanian, et al., 2024). It has been proposed that ~30% of the DFs from grain products, and 75-90% of DFs from fruits and vegetables is accessible for microbial fermentation. DFs can also shape the gut microbiota composition by increasing the faecal bulk and gut transit time (Schneider, Balasubramanian, et al., 2024).

DF interventions have been shown to reduce anxiety (Azpiroz et al., 2017; Hall et al., 2024; P. P. Jackson et al., 2023; Johnstone et al., 2021; Silk et al., 2009) and depression (Farhangi et al., 2018; Hall et al., 2024; P. P. Jackson et al., 2023) in humans and improve cognitive function (Abe et al., 2024; Berding, Long-Smith, et al., 2021), likely through beneficial shifts in the gut microbiota composition. A randomised placebo-controlled

trial in healthy adults with mild-to-moderate levels of anxiety and depression looked at the effect of individual DFs oligofructose and 2'fucosyllactose, alone and in combination. Both oligofructose and oligofructose/2'fucosyllactose led to significant changes in several bacterial taxa including *Bifidobacterium*, *Bacteroides*, *Roseburia*, and *Faecalibacterium prausnitzii*. These gut bacterial changes were accompanied by significant improvements in depression and anxiety, as well as cortisol awakening response (P.P. Jackson et al., 2023). A 12-week RCT in adults with metabolic syndrome found that a prebiotic blend of diverse DFs significantly reduced inflammation (high sensitivity C-reactive protein) and self-reported affective scores, alongside increased abundance of SCFA-producing bacteria *Bifidobacterium* and *Parabacteroides* (Hall et al., 2024). Table 1.2 summarises human studies on various DF interventions and their effects on GMB communication.

Despite substantial evidence on individual DF types, studies examining DFs (consumed as fibre rich foods, rather than supplements or extracts) within a whole diet remain limited. This thesis will explore the role of DFs as part of a whole Mediterranean diet, on the gut microbiota and brain health.

**Table 1.2 |** Dietary fibre interventions in humans and their impact on the microbiota and brain health outcomes

| References                 | Intervention                                              | Population                                                             | Study design                                                   | Study duration (Country)            | Gut microbiota changes                                                                                                             | Cognitive/ behavioral/ neurological changes                                                                |
|----------------------------|-----------------------------------------------------------|------------------------------------------------------------------------|----------------------------------------------------------------|-------------------------------------|------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------|
| (Azpiroz et al., 2017)     | scFOS (5g/day) vs placebo                                 | IBS patients with rectal hypersensitivity (n=79, M/F=19/60, 18-60 yrs) | Parallel, placebo-controlled, randomised, double-blind study   | 4 weeks (France, Spain)             | ↑Faecal <i>Bifidobacteria</i>                                                                                                      | ↓Anxiety score                                                                                             |
| (Johnstone & Kadosh, 2025) | GOS (5.5g/day) vs maltodextrin                            | Healthy children (n=40, M/F=15/25, 6-14 yrs)                           | Double-blind, randomised, placebo-controlled trial             | 28 days (UK)                        | Gut microbiota was not sequenced                                                                                                   | Positive trends were observed as following<br>↓ Trait anxiety levels<br>↓ Negative emotional responses     |
| (Johnstone et al., 2021)   | Biotis™ GOS (~5.5g GOS) vs maltodextrin                   | Healthy females (n=64, 18-25 yrs)                                      | Double-blind, placebo-controlled intervention                  | 4 weeks (UK)                        | ↑ <i>Bifidobacterium</i> ,                                                                                                         | ↓Anxiety,<br>↓Negative emotional bias and<br>↑positive bias in the dot-probe task                          |
| (Silk et al., 2009)        | Trans-GOS (3.5 g/day or 7 g/day) vs placebo               | IBS patients (n=44, M/F=16/28, 20-79 yrs)                              | Single-centre, parallel, cross-over, controlled clinical trial | 12 weeks (UK)                       | ↑ <i>Bifidobacterium</i> ,<br>↑ <i>Eubacterium rectale</i> ,<br>↑ <i>Clostridium coccoides</i><br>↓ <i>Clostridium perfringens</i> | Significant difference in stool consistency,<br>Improved flatulence & bloating,<br>Improved anxiety scores |
| (Mysonhimer et al., 2023)  | FOS and GOS 5g each/day vs control (Lactaid low-fat milk) | Healthy adults (n=24, M/F=10/14, 25-45 yrs)                            | Single-blinded, Randomised                                     | 4 weeks each intervention with >= 4 | ↑ <i>Bifidobacterium</i><br>↑ <i>Actinobacteriota</i>                                                                              | No differences in stress markers and mental health outcomes                                                |

|                                     |                                                                                                 |                                                                                                    | controlled,<br>crossover<br>trial                            | weeks<br>washout<br>period<br>(USA) |                                                                                                                                       |                                                                                                                                                                               |
|-------------------------------------|-------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------|--------------------------------------------------------------|-------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>(Schmidt et al., 2015)</b>       | FOS or Bimuno® (B-GOS) vs maltodextrin                                                          | Healthy volunteers (n=45, M/F = 22/23, 18-45 yrs)                                                  | Double-blind randomised, controlled trial                    | 3 weeks (UK)                        | Gut microbiota was not sequenced                                                                                                      | ↓ Cortisol awakening reactivity in B-GOS group                                                                                                                                |
| <b>(P. P. Jackson et al., 2023)</b> | Oligofructose (8g/day) and 2'fucosyllactose (2g/day) (alone and in combination) vs maltodextrin | Healthy adults with mild-to-moderate levels of anxiety and depression (n=92, M/F=30/62, 18-50 yrs) | Double-blind, Randomised, parallel, placebo-controlled trial | 5 weeks (UK)                        | ↑ <i>Bifidobacterium</i> , <i>Bacteroides</i> , <i>Roseburia</i> , <i>Faecalibacterium</i> in oligofructose and the combination group | Improvements in Beck Depression Inventory, State Trait Anxiety Inventory Y1 and Y2, Positive and Negative Affect Schedule Score and cortisol awakening response in all groups |
| <b>(Smith et al., 2015)</b>         | Oligofructose enriched inulin (5g) vs maltodextrin                                              | Healthy adults (n=47, M/F=19/28, 19-30 yrs)                                                        | Double-blind, crossover, placebo-controlled trial            | 4 hours (UK)                        | Gut microbiota was not sequenced                                                                                                      | ↑ Accuracy on recognition memory task<br>Improved recall performance                                                                                                          |
| <b>(Leyrolle et al., 2021)</b>      | Inulin (16g/day) vs maltodextrin                                                                | Obese adults (n=106, 18-65 yrs)                                                                    | Single-blinded, multicentric, placebo-controlled trial       | 3 months (Belgium)                  | ↑ <i>Coprococcus</i> levels at baseline were linked to better mood outcomes after inulin supplementation                              | Moderate beneficial effect on emotional competence and cognitive flexibility                                                                                                  |

|                                            |                                                                                    |                                                              |                                                                    |                   |                                                                                                      |                                                                                                                   |
|--------------------------------------------|------------------------------------------------------------------------------------|--------------------------------------------------------------|--------------------------------------------------------------------|-------------------|------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------|
| <b>(Vaghef-Mehrabani et al., 2023)</b>     | Inulin (10g/day) vs maltodextrin with a healthy calorie-restricted diet            | Obese women with Major Depressive Disorder (n=45, 20-50 yrs) | Double-blind, Randomised, controlled, clinical trial               | 8 weeks (Iran)    | Gut microbiota was not sequenced, although gut permeability was measured with no changes observed    | No effect observed on depressive symptoms                                                                         |
| <b>(Medawar et al., 2024)</b>              | Inulin (30g/day) vs Equicaloric placebo                                            | Over-weight young adults (n=59, M/F=40/19, 18-42 yrs)        | Randomised, controlled, crossover trial                            | 14 days (Germany) | ↑ SCFA-producing <i>Bifidobacteriaceae</i><br>Changes in >60 predicted functional signaling pathways | ↓ Brain activation towards high-caloric food stimuli                                                              |
| <b>(Abe et al., 2024)</b>                  | Partially hydrolysed guar gum (5g/day) vs maltodextrin                             | Healthy elderly individuals (n=61, M/F=31/30, >60 yrs)       | Double-blind, Randomised, placebo-controlled, parallel group trial | 12 weeks (Japan)  | Gut microbiota was not sequenced                                                                     | Improved visual memory scores<br>Improved vigour-activity scores<br>Decreased scores for confusion-bewilderment   |
| <b>(Berding, Long-Smith, et al., 2021)</b> | A DF supplement (12.5g Litesse® ultra (>90% polydextrose polymer)) vs maltodextrin | Healthy female participants (n=18, 18-40 yrs)                | Double-blind, Randomised, placebo-controlled crossover study       | 4 weeks (Ireland) | ↑ <i>Ruminiclostridium</i>                                                                           | Improved cognitive flexibility<br>Better performance in sustained attention                                       |
| <b>(Farhangi et al., 2018)</b>             | Nutriose®06 a resistant dextrin (10 g/day) vs maltodextrin                         | Women with T2DM (n=55, 30-65 yrs)                            | Randomised, triple-blind, placebo-controlled clinical trial        | 8 weeks (Iran)    | ↑ <i>Bifidobacterium</i> ,<br>↑ <i>Fusobacterium</i> ,<br>↓ <i>Clostridium perfringens</i>           | ↓Cortisol levels<br>↓inflammatory markers e.g. IFN $\gamma$ , IL12, IFN10/IL10 ratio, LPS<br>↑DASS and GHQ scores |

|                                |                                                                                                                                |                                                                      |                                                   |                 |                                                      |                                                                          |
|--------------------------------|--------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------|---------------------------------------------------|-----------------|------------------------------------------------------|--------------------------------------------------------------------------|
| <b>(Hall et al., 2024)</b>     | Prebiotic fibre blend (10g/day) and healthy eating advice vs healthy eating advice only                                        | Adults with metabolic syndrome (n=53, M/F=17/37, 18-75 yrs)          | Open-label, parallel, Randomised controlled trial | 12 weeks (UK)   | ↑ <i>Bifidobacterium</i><br>↑ <i>Parabacteroides</i> | ↓ Perceived stress, anxiety and depression                               |
| <b>(Lawton et al., 2013)</b>   | Wheat bran supplemented breakfast cereal containing 5.4g fibre (3.5 g from wheat bran) vs non-intervention baseline monitoring | Low dietary fibre consumers (<15g/day) (n=153, M/F=72/81, 18-50 yrs) | Single-Centre, multi-site, intervention trial     | 14 days (UK)    | Gut microbiota was not sequenced                     | Improved subjective perception of well-being                             |
| <b>(Sandberg et al., 2018)</b> | Whole grain (WG) rye bread (with resistant starch type 2) vs white wheat flour bread                                           | Healthy adults (n=38, M/F= 8/30, 52-70 yrs)                          | Randomised, cross-over, controlled trial          | 3 days (Sweden) | Gut microbiota was not sequenced                     | Improved mood ratings<br>↑ Insulin sensitivity<br>↓ Inflammatory markers |

CAD, Coronary artery disease; DASS, Depression, Anxiety and Stress scale; DF, Dietary Fibre; FOS, Fructo-oligosaccharide; GHQ, General Health Questionnaire; GOS, Galacto-oligosaccharide; IBS, Irritable Bowel Syndrome; scFOS, short-chain Fructo-oligosaccharides; T2DM, Type-2 diabetes mellitus.

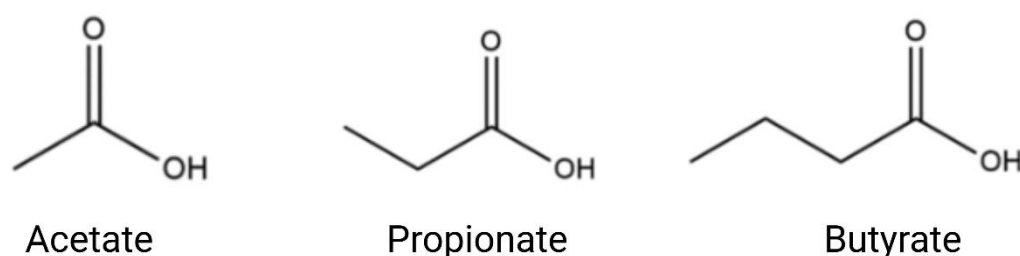
### 1.7 Short-chain fatty acids (SCFAs) derived from dietary fibres

SCFAs are saturated fatty acids with a chain length ranging from one to six carbon atoms. They are produced primarily in the colon as a result of bacterial fermentation of undigested DFs. Small quantities of SCFAs are also produced from amino-acid fermentation (Dalile et al., 2019), and dietary (poly)phenols can indirectly enhance overall SCFAs production (Rodriguez-Daza et al., 2021). Depending on the DF content of the diet, approximately 500–600 mmol of SCFAs are produced in the gut per day (Dalile et al., 2019). Acetate (acetic acid, C2), propionate (propionic acid, C3) and butyrate (butyric acid, C4) are the predominant SCFAs (Figure 1.3) in the human colon, found in a typical ratio of 60:20:20 (Mann et al., 2024). Butyrate is primarily absorbed by the gut epithelial cells, propionate by the liver, and acetate by the peripheral tissues (Mann et al., 2024).

SCFAs exert several effects locally to improve gut health such as the maintenance of intestinal barrier integrity and protecting from intestinal inflammation. They also affect distant organs, reaching the brain via the BBB, with abundant expression of monocarboxylate transporters (MCTs), in the brain endothelial cells (Dalile et al., 2019). The abundant MCTs expression on the brain endothelial cells facilitate SCFAs in crossing the BBB and reaching the brain (Silva et al., 2020; Vijay & Morris, 2014), where they exert neuroactive effects, despite their minimal uptake (Dalile et al., 2019). The circulating levels of SCFAs are crucial, and the highest levels are reported in the portal circulation, with acetate 3.8 mM, propionate 1.1 mM, and butyrate 0.29 mM (Mukhopadhyaya & Louis, 2025). These SCFAs concentrations lowers in hepatic circulation and the lowest levels have been reported in the peripheral circulation, where the mean concentrations are reported to be 70  $\mu\text{mol/l}$  for acetate, 5  $\mu\text{mol/l}$  for propionate, and 4  $\mu\text{mol/l}$  for butyrate (Cummings et al., 1987).

Studies on the physiological concentrations of SCFAs in the brain are scarce (Silva et al., 2020). The concentrations in the human brain have been reported as 17.0 pmol/mg of tissue for butyrate and 18.8 pmol/mg of tissue for propionate (Bachmann et al., 1979). The concentrations for acetate, propionate and butyrate in the cerebrospinal fluid (CSF)

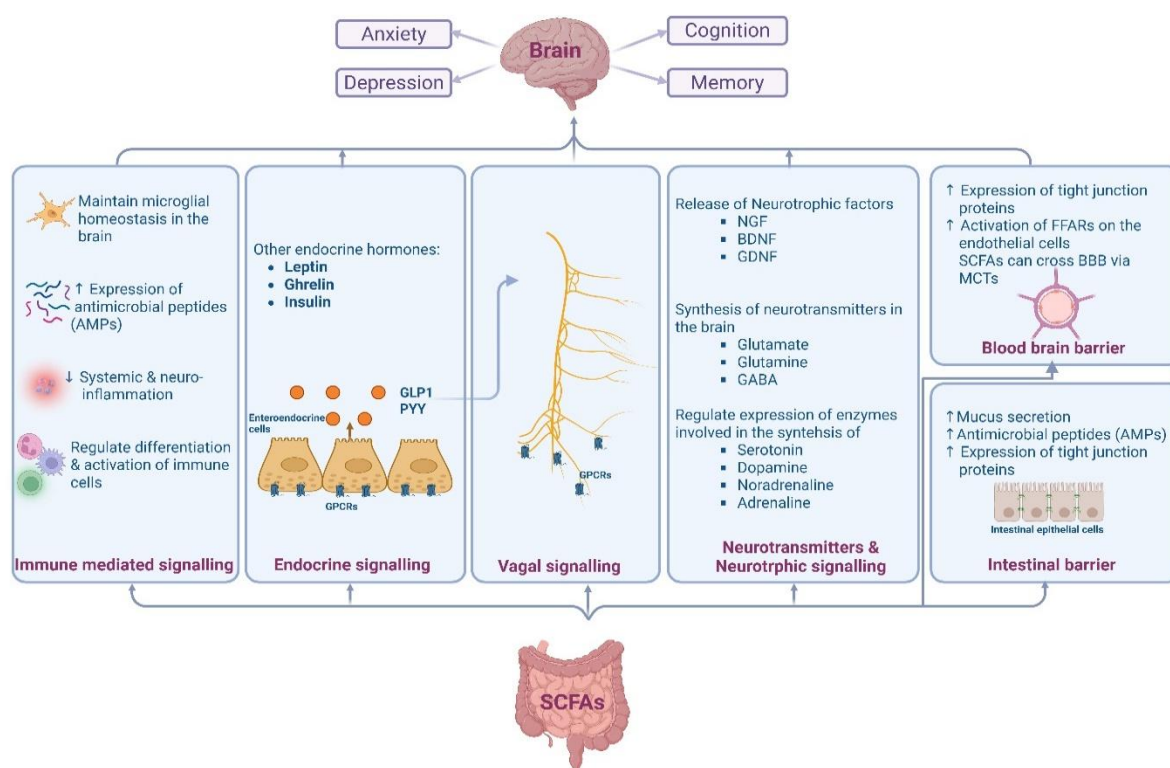
have been reported as 0-171  $\mu\text{M}$ , 0-6  $\mu\text{M}$  and 0-2.8  $\mu\text{M}$ , respectively (<http://www.hmdb.ca/>; O'Riordan et al., 2022).



**Figure 1.3 | Short-chain fatty acids (SCFAs) structures for acetate (C-2), propionate (C-3) and butyrate (C-4)**

### 1.8 Role of SCFAs in Gut-Microbiota-Brain Communication

SCFAs may directly or indirectly influence communication along the GMB axis because of their neuroactive properties and involvement in gut–brain signalling pathways through immune, endocrine, and other mechanisms (Stilling et al., 2016), also outlined in Figure 1.4.



**Figure 1.4 | SCFAs in Gut-Microbiota-Brain Communication**  
Created with BioRender.com

### 1.8.1 Immune-mediated SCFAs signalling

SCFAs regulate the GMB crosstalk by reducing neuroinflammation both directly and indirectly.

(1) SCFAs maintain a healthy gut environment by increasing the expression of AMPs on the gut epithelial cells, which modulate the production of immune mediators (e.g. IL-18) and maintain epithelial integrity, thus preventing microbial translocation into the circulation. SCFAs also regulate the differentiation, recruitment and activation of neutrophils, dendritic cells, macrophages, monocytes, and T cells (Correa-Oliveira et al., 2016). As SCFAs can reach the blood stream, they also modulate immune cell function in the systemic circulation potentially influencing brain and neuronal function (Dalile et al., 2019), by reducing systemic inflammation which is crucial in modulating neuroinflammation (Varatharaj & Galea, 2017).

(2) SCFAs directly modulate the immune pathways in the brain partly by maintaining microglial homeostasis. Microglia are brain macrophages-like cells and maintain tissue homeostasis by scavenging pathogens and dying cells. SCFAs can cross the BBB and control microglial structure and maturity (Erny et al., 2021; Erny et al., 2015). Acetate, fuels microglial energy metabolism, where it is converted into acetyl-CoA, ultimately regulating the metabolic pathways like the tricarboxylic acid (TCA) cycle (Erny et al., 2021).

### 1.8.2 Endocrine signalling

SCFAs influence the secretion of gut hormones such as glucagon-like peptide 1 (GLP-1) and peptide YY (PYY), which modulate the GMB communication (Dalile et al., 2019). These gut hormones are released from the enteroendocrine cells of the colon as result of G-protein coupled receptors activation by SCFAs (Larraufe et al., 2018; Psichas et al., 2015). These hormones influence brain circuits involved in food intake and appetite regulation either via the systemic circulation (Freeland & Wolever, 2010) or the *vagus* nerve (Sam et al., 2012).

SCFAs also influence other metabolic hormones including leptin, ghrelin, and insulin. However, the role of these hormones in brain function have been studied less extensively than GLP-1 and PYY (Dalile et al., 2019; O'Riordan et al., 2022).

### 1.8.3 Vagal signalling

SCFAs may directly activate vagal afferent fibres. Vagal sensory neurons actively express G-protein coupled receptors (GPCRs), FFAR3 and OLFR78, both of which have binding affinities for SCFAs. SCFAs influence the CNS via the *vagus* nerve (De Vadder et al., 2014; Goswami et al., 2018). The mediating role of the *vagus* nerve was confirmed when the anorectic effect of propionate was lost in the absence of *Ffar3* receptors from vagal neurons in vagal-FFAR3 Knockout (KO) mice (Cook et al., 2021).

### 1.8.4 Neurotransmitter changes

SCFAs also mediate the GMB communication through changes in neurotransmitters, and neurotrophic factors such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and glial cell-line derived neurotrophic factor (GDNF) (Dalile et al., 2019). These proteins regulate the growth, survival and differentiation of synapses and neurons, thus playing critical role in learning, memory and brain-related disorders (Morís & Vega, 2003). Butyrate injection positively affected mood-related behaviours in rats (Varela et al., 2015) and mice (Sun et al., 2016) alongside an increase in neurotrophic factors. It is also known to regulate the expression of BDNF, through epigenetic modulation, which is associated with increased neurogenesis and synaptic plasticity (Wei et al., 2015).

SCFAs regulate the expression of enzymes involved in the synthesis of neurotransmitters like serotonin, dopamine, noradrenaline and adrenaline (Silva et al., 2020). Acetate is known to significantly increase the levels of glutamate, glutamine and GABA in the hypothalamus, as confirmed after incorporating <sup>13</sup>C-labelled acetate (Frost et al., 2014). Serotonin - a neurotransmitter in the CNS and the periphery, may be affected by SCFAs. More than 90% of serotonin is produced in the gut by the enterochromaffin cells, and intraluminal administration of SCFAs into the rat proximal colon stimulated serotonin release from enterochromaffin cells (Fukumoto et al., 2003).

### 1.8.5 Blood brain barrier (BBB) changes

BBB integrity is crucial to brain development and homeostasis and SCFAs play an important role in maintaining a healthy BBB. The barrier integrity is measured through both functional permeability, and the expression of tight junction proteins which reduce paracellular permeability (O'Riordan et al., 2022). Propionate (Hoyles et al., 2018) and

butyrate (Braniste et al., 2014) treatment improved BBB integrity in human cerebro-microvascular endothelial cell line and germ-free mice, respectively.

Accumulating evidence indicates that SCFAs have therapeutic potential in mental and cognitive disorders through modulation of the GMB axis, as summarised in Table 1.3. Most studies focus on the three most abundant SCFAs acetate, propionate, and butyrate, due to their well-documented physiological benefits. Chronic stress induced depression-like behaviour in mice was associated with impaired hippocampal neurogenesis and disrupted BBB integrity, which coincided with reduced gut-derived SCFAs. Supplementation with SCFAs reversed these neurogenic deficits, repaired BBB damage and reduced microglial activation, underscoring their potential as dietary interventions for depression (Tang et al., 2022). Alongside lower concentrations of SCFAs, a reduced abundance of SCFA-producing bacteria has been associated with anxiety (Jiang et al., 2018; Xiong et al., 2023) and depression (Xiong et al., 2023; Yu et al., 2023). A mouse model of long-term DF deficiency showed impaired cognitive function which was significantly associated with gut microbiota dysbiosis ( $\downarrow$ Bacteroidetes and  $\uparrow$ Proteobacteria). Although a rapid change in gut microbiota differentiation was observed before cognitive impairment, which highlights a possible causal impact of the gut microbiota composition on cognitive outcomes. When these fibre deficient mice were treated with SCFAs, they showed significantly improved memory (increased object location memory, temporal order memory and the ability to build a nest) when compared with mice without SCFAs supplementation (Shi et al., 2021).

These findings highlight the contribution of DFs to cognitive and brain health and delineates the causal impact of SCFAs produced from DF fermentation.

**Table 1.3 | SCFAs and their physiological role in the Gut-Brain Axis**

| References            | Intervention                                                                                     | Species                         | Duration | Model                                                                  | Findings                                                                                                                                                                                                                                         |
|-----------------------|--------------------------------------------------------------------------------------------------|---------------------------------|----------|------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| (Tang et al., 2022)   | SCFAs                                                                                            | C57BL/6J mice                   | 8 weeks  | High-fructose diet and chronic stress induced depression               | SCFAs prevented depression-like behaviors by reducing BBB damage, suppressed microglial activation and neuroinflammation; and rescuing hippocampal neurogenesis decline                                                                          |
| (Lanza et al., 2022)  | Propionate and butyrate<br>Sodium propionate and sodium butyrate at doses of 10, 30 and 100mg/kg | CD1 mice (females)              | 4 hours  | Nitroglycerine (NTG) induced migraine                                  | ↓ markers of inflammation<br>↑ protective antioxidant enzymes<br>↓ reduced histological damage in whole brain and small intestine<br>Modulation of intestinal microbiota                                                                         |
| (Lai et al., 2022)    | SCFAs<br>(67.5mM acetate, 40mM butyrate, 25.9mM propionate)                                      | C57BL/6J mice (males)           | 29 days  | Methamphetamine (METH) induced neuro-inflammation and behavior change  | SCFAs changed METH-induced behaviours, ↓ <i>Parabacteroides</i> and ↑ <i>Lactobacillus</i>                                                                                                                                                       |
| (Lupori et al., 2022) | SCFAs<br>(25mM sodium propionate, 40mM sodium butyrate, 67.5mM sodium acetate)                   | C57BL/6J mice (male and female) | 5 weeks  | Environmentally enriched (EE) mice vs standard rearing (ST) mice       | SCFA administration in ST mice mimics EE induced microglial remodeling and ocular dominance plasticity                                                                                                                                           |
| (Shi et al., 2021)    | SCFAs<br>(67.5mM sodium acetate, 40mM sodium butyrate, 25.9mM sodium propionate)                 | C57BL/6J mice (males)           | 15 weeks | Fibre deficiency (FD) induced cognitive decline and gut dyshomeostasis | SCFA supplementation restored FD-diet induced synaptic protein loss in hippocampus, inhibited microglial activation and phagocytosis to the synapse and neuroinflammation. Improved colonic and systemic inflammation; and some memory functions |

|                              |                                                                                                             |                               |                                                             |                                        |                                                                                                                                                                            |
|------------------------------|-------------------------------------------------------------------------------------------------------------|-------------------------------|-------------------------------------------------------------|----------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>(Liu et al., 2021)</b>    | SCFAs<br>(Pre-treatment with acetate, propionate and butyrate at a ratio of 3:1:1 at 500 mg/kg body weight) | C57BL/6J mice (males)         | 7 days pre-treatment to cecal ligation and puncture surgery | Sepsis-associated encephalopathy (SAE) | SCFAs pretreatment significantly reduced neuronal degeneration and behavioral impairment, ↓ levels of IL-1 $\beta$ , IL-6 and ↑ expression of tight junction proteins      |
| <b>(Sadler et al., 2020)</b> | SCFAs (25.9mM sodium propionate, 40mM sodium butyrate, and 67.5mM sodium acetate vs salt matched control)   | C57BL/6J male and female mice | 4 weeks                                                     | Photothrombic stroke model             | Improved recovery and cortical reorganization after stroke<br>Modulation in poststroke synaptic plasticity<br>Modulation in microglial activity and immune cell regulation |
| <b>(Liu et al., 2025)</b>    | SCFAs (67.5mM sodium acetate, 25mM sodium propionate, 40mM sodium butyrate)                                 | Sprague-Dawley male rats      | 4 weeks                                                     | Perioperative neurocognitive disorder  | SCFAs reversed cognitive impairments by ↓neuronal damage and ↑synaptic plasticity<br>↑Histone acetylation and ↑BDNF                                                        |

#### ***Human Studies***

| <b>References</b>            | <b>Intervention</b>                                                                          | <b>Population</b>                  | <b>Study Design</b>                                             | <b>Duration</b> | <b>Model</b>                                              | <b>Findings</b>                                                                                            |
|------------------------------|----------------------------------------------------------------------------------------------|------------------------------------|-----------------------------------------------------------------|-----------------|-----------------------------------------------------------|------------------------------------------------------------------------------------------------------------|
| <b>(Dalile et al., 2020)</b> | SCFAs mixture (doses equivalent to fermentation of 10g or 20g arabinoxylan oligosaccharides) | Healthy men (n=66), Age (23.6±2.4) | Triple-blind, randomised, placebo-controlled intervention trial | 1 week          | Healthy men performing psychosocial stress and fear tasks | Both doses of SCFAs significantly attenuated cortisol response to psychosocial stress compared to placebo. |

|                              |                                                              |                                     |                                                                  |        |                                                               |                                                                                                                                                           |
|------------------------------|--------------------------------------------------------------|-------------------------------------|------------------------------------------------------------------|--------|---------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>(Dalile et al., 2024)</b> | Butyrate (colon delivery capsules delivering 5.28g butyrate) | Healthy men (n=71), Age (25.2±4.82) | Triple-blind, randomised, placebo-controlled, intervention trial | 1 week | Healthy men performing psychobiological stress and fear tasks | Significant modulation of fear memory at subjective level, but no changes at physiological levels<br>No changes were observed in response to acute stress |
|------------------------------|--------------------------------------------------------------|-------------------------------------|------------------------------------------------------------------|--------|---------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------|

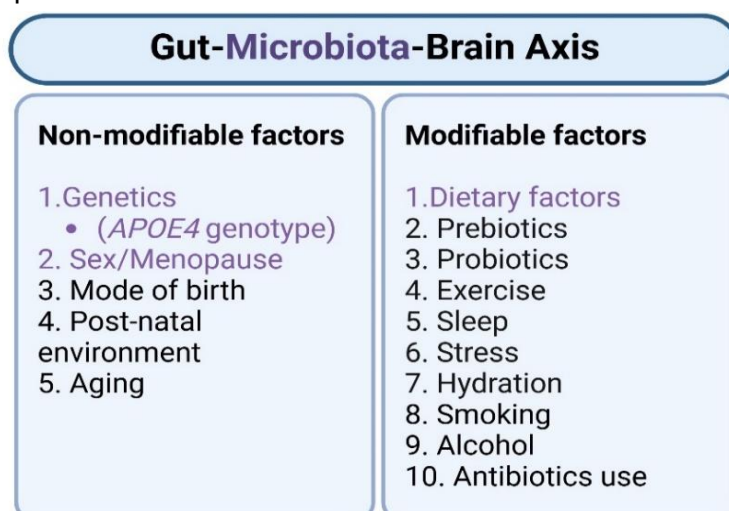
Age is presented as mean ± standard deviation (SD).

## 1.9 Factors affecting the Gut-Microbiota-Brain Communication

The “microbiota” component of the GMB axis is influenced by several modifiable and non-modifiable factors as outlined in Figure 1.5.

For the adult microbiome, mode of delivery and infant feeding are the primal non-modifiable factors shaping the gut microbiota composition. Infants delivered vaginally are exposed to their mother’s microbiota at birth as compared to those born via caesarean section (Wen & Duffy, 2017). Ethnicity, genetic makeup (Qin et al., 2022) and biological sex (Org et al., 2016) are additional non-modifiable factors influencing the gut microbiota composition. In addition, age-associated alterations in gut microbiota are also evident (Ghosh et al., 2022). In addition to diet (section 1.4) and in particular DFs (section 1.6), as discussed above, a variety of other factors including smoking, alcohol consumption, physical activity, stress, and sleep contribute to shaping the gut microbiota composition (Pedroza Matute & Iyavoo, 2023). Many of these factors are modifiable and offer potential avenues for promoting a healthier microbiota profile (Figure 1.5).

The primary focus of this PhD is to investigate DFs as a modifiable factor in promoting a healthy GMB axis. In parallel, we aim to explore how non-modifiable risk factors, specifically biological sex and *APOE4* genotype, may modulate the impact of DFs, given that both are recognised as independent contributors to cognitive health and gut microbiota composition.



**Figure 1.5 | Factors modulating the adult gut microbiota at individual level and mediating gut-microbiota-brain axis**

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### 1.10 Sexual dimorphism in Gut Microbiota composition and brain health

Biological sex is identified as an important modulator of gut microbial composition (Valeri & Endres, 2021). Sex mediated gut microbiota differentiation starts in early life. On the first day of life, male infants show higher *Bifidobacterium* levels as compared to female infants (Nagpal et al., 2017). Within the first 30 days, stool sample analysis reveals that male neonates have lower  $\alpha$ -diversity (observed number of bacterial species), lower abundance of Clostridiales, and higher abundance of Enterobacteriales compared to females (Cong et al., 2016).

Both dynamic changes (during the onset of puberty and menopause) and steady-state (during adulthood) levels of sex hormones play a role in shaping the gut microbiota (Valeri & Endres, 2021), which has been observed in several human studies (Ding & Schloss, 2014; Falony et al., 2016; Takagi et al., 2019; Zhernakova et al., 2016). In two large human cohorts, the Belgian Flemish Gut Flora Project (n=1,106) and the Dutch LifeLines-Deep study (n=1,135), sex (10<sup>th</sup> strongest effect size among 69 factors) was associated with overall microbiome community variation (Falony et al., 2016).

Sexual dimorphism in gut microbiota composition is likely due to the influence of sex hormones. A bidirectional relationship exists between these sex hormones and the gut microbiota: 1) sex hormones (at specific concentrations) appear to modulate the gut microbial composition (Koren et al., 2012; Org et al., 2016; Santos-Marcos et al., 2023; Yurkovetskiy et al., 2013), whilst 2) the gut microbiota regulates the levels of circulating levels of sex hormones (Baker et al., 2017; Peters et al., 2022).

#### 1.10.1 Sexual dimorphism in brain health

In recent decades, neuroscience has seen a marked increase in research on sex differences, with findings consistently supporting distinctions in brain structure and functional connectivity, which have implications for cognitive and emotional behaviour (Hodgetts & Hausmann, 2022).

These differences in the brain exist at both macro and micro levels. At the macro-structural level, the average male brain is larger and heavier than the average female brain, even after controlling for differences in body size (Ruigrok et al., 2014; Williams et al., 2021). These differences in brain structure are also region-specific. A meta-analysis

of 2,186 individual MRI scans showed that women have larger grey matter volumes in the thalamus, while men have larger grey matter volumes in the amygdala, hippocampus, and putamen (Ruigrok et al., 2014). At the microstructural level, differences exist in synaptic density, neuron number, and cell size (Hodgetts & Hausmann, 2022). Studies have shown that men possess a greater number of neurons in the frontal and temporal neocortex compared to women (Stark et al., 2007), whereas women typically exhibit higher neuronal density as compared to men (Witelson et al., 1995).

Sex plays a significant role in cognitive function, influencing hippocampal plasticity, and contributing to the development of various brain disorders (Yagi & Galea, 2019). Women show a greater prevalence of disease and more severe cognitive symptoms in Alzheimer's disease (Irvine et al., 2012; McPherson et al., 1999). On the other hand, men show greater cognitive impairment in Schizophrenia (Han et al., 2012) and Parkinson's disease (Cereda et al., 2016) as compared to women. These prominent sex differences in brain structure and function highlight the need for sex-specific approaches when developing treatment and prevention strategies for brain health, as tailored interventions may more effectively address the underlying mechanisms in each sex.

#### 1.10.2 Sex-specific responses to dietary patterns and dietary fibres

Biological sex is an important determinant of individual responses to diet and can influence the impact of diet on metabolic health and disease risks (Andrews et al., 2024). A Mediterranean diet enhances metabolic health and lowers the risk of type 2 diabetes. Bedard et al reported greater improvements in insulin sensitivity in men compared to women following this dietary pattern (Bedard et al., 2012). Another Mediterranean diet intervention for 12 weeks showed more pronounced metabolic changes in men as compared to women (Leblanc et al., 2014). In a 12-months RCT on the Mediterranean diet, men benefited from lowered systolic and pulse pressure, while women uniquely showed improvements in arterial stiffness (Jennings et al., 2019). Collectively the evidence suggests that men tend to experience more pronounced cardiometabolic improvements following a Mediterranean-style diet.

DF is an important component of a Mediterranean-style dietary pattern and exerts sex-specific health benefits as growing evidence suggests (Benítez-Páez et al., 2021; Fernandez et al., 1995; S. Kadyan, G. Park, P. Singh, et al., 2023; Tashiro et al., 2020;

Zhang et al., 2020). An observational study reported a significant association between daily DF intake and perceived health status and immune function in men, but not in women (Fernstranda et al., 2017). Psyllium supplementation led to 17% reduction in triglyceride levels in men, whereas women showed a 16% increase (Vega-López et al., 2002).

Inulin supplementation differentially modulated gut microbiome and metabolome, with distinct sex-specific mechanisms of action, in a humanised murine model of ageing (S. Kadyan, G. Park, B. Wang, et al., 2023). In chronic stress, combined administration of fructo-oligosaccharides and galacto-oligosaccharides attenuated anxiety-like behaviour in female mice only. This effect was linked to modulation of tight junction proteins in the distal colon, and hippocampus, and restoration of stress-induced goblet cell loss in the colon (Jiang et al., 2023). Despite the sex-specific behavioural effects, stress-induced BBB disruption, elevated pro-inflammatory cytokines, and gut microbiota alterations were mitigated in both male and female mice (Jiang et al., 2023).

Not just the quantity, but the quality of DFs is also crucial for sex-specific effects. In a randomised controlled, parallel study, participants consumed either a low-GI or high-GI Mediterranean diet, matched for carbohydrates (270 g/d) and fibre (35 g/d) content. In women, the high-GI diet significantly increased the plasma glucose concentrations from the first day, with a 37% rise after 12 weeks. In contrast, no significant changes were observed in men across both diets (Vitale et al., 2023). Current evidence suggests that DFs confer greater metabolic and physiological benefits in men, while effects in women are less consistent, except for one study showing reduced anxiety-like behaviour following DFs supplementation (Jiang et al., 2023). A meta-analysis across different populations observed that nutritional interventions were significantly more effective in alleviating symptoms of anxiety and depression in females (Firth et al., 2019).

These sex-specific responses underscore the need to further investigate underlying mechanisms and include both sexes in experimental models to better understand the role of dietary interventions, particularly DFs and inform personalised nutritional interventions. This thesis investigates the mechanisms underlying sexual dimorphism in response to dietary changes, particularly DF intake, and examines their role in anxiety, depression, and cognitive health.

### 1.11 *APOE4* genotype; modulator of Gut Microbiota and brain health

The *APOE* gene encodes a multifunctional protein, apolipoprotein E (APOE), primarily involved in lipid metabolism, inflammation and neurobiology. APOE serves as a ligand with its most well described function being the transport of lipids in the periphery and the brain. It has three different isoforms (APOE2, APOE3, APOE4), which differ in structure, stability and physiologic effect (Emrani et al., 2020; Huang & Mahley, 2014). *APOE3* is the most common allele and considered to be the normal isoform and accounts for 70-80% of the gene pool, whereas the *E2* and *E4* alleles account for 5-10% and 10-15%, respectively (Huang et al., 2004). APOE2 and APOE4 differ from APOE3 by single amino acid substitutions at position 112 or 158 (Huang & Mahley, 2014).

The *E4* allele (*APOE4*) is the strongest common genetic risk factor for Alzheimer's disease (AD) and also for earlier stages of cognitive decline like Mild Cognitive Impairment (MCI) (Krysicio et al., 2006). Evidence suggests that *APOE4* carrier status influences cognition even in pre-MCI states such as Subjective Cognitive Impairment (SCI) (Müller-Gerards et al., 2019). Systemic and central metabolic dysfunction associated with *APOE4* allele is evident often decades before any symptoms appear (Hoffman et al., 2019).

*APOE4* carriers present with a distinct gut-microbial diversity which potentially contributes to the pathological metabolic effects of *APOE4* genotype (Hoffman et al., 2019; Maldonado Weng et al., 2019; Parikh et al., 2020; Tran et al., 2019; Yanckello et al., 2022). At the family level, the abundance of Ruminococcaceae, a group of fermentative anaerobic bacteria involved in DFs degradation and SCFAs production, was found to be the lowest in *APOE4/APOE4* carriers, followed by *APOE3/APOE4*, *APOE3/APOE3* and *APOE2/APOE3* genotypes (Tran et al., 2019). This gut dysbiosis associated with *APOE4* genotype is known to exacerbate systemic inflammation and metabolic dysfunction (Hoffman et al., 2019; Parikh et al., 2020; Tran et al., 2019).

Early neurodegenerative changes in *APOE4* carriers (Reiman et al., 2005) and the distinct gut microbiota composition may also influence the efficacy of microbiota-targeted strategies such as DFs intervention. A supporting study showed that inulin enhanced systemic metabolism and improved gut microbiota balance and beneficial microbial metabolites such as SCFAs, tryptophan metabolites, bile acids etc in an *APOE4* mouse model, compared to the *APOE3* control group (Hoffman et al., 2019). The neurological

benefits of inulin were observed in both *APOE3* and *APOE4* variant mice, although the underlying mechanisms were not entirely the same for both genotypes (Yanckello et al., 2022). In *APOE3* (5xFAD) mice, inulin altered metabolic pathways related to tryptophan and tyrosine in the periphery, whereas in *APOE4* (5xFAD) mice, it led to changes in TCA cycle, Pentose-phosphate pathway (PPP) and bile acid pathways. Gut microbial composition and metabolites also responded differentially to inulin between genotypes (Yanckello et al., 2022).

In humans, while some studies support the role of DFs in a healthy gut-brain communication (Aslam et al., 2024; Berding, Carbia, et al., 2021), limited evidence suggests that *APOE4* carriers may be less responsive to DF interventions compared to non-*APOE4* individuals. For example, *APOE4* carriers showed no changes in postprandial fat metabolism (Wolever et al., 1997) or cholesterol-lowering response (Uusitupa et al., 1992) to  $\beta$ -glucan rich oat bran, unlike non-*APOE4* carriers. Contrarily, a study in older adults showed a protective effect of DFs against cognitive decline, only in subjects with *APOE4* genotype (Unión-Caballero et al., 2023). Overall, given the impact of *APOE4* carrier status on the gut microbiota, there is a real need to further investigate its impact on DFs-mediated GMB interactions and associated GMB responses, and to gain an understanding of underpinning mechanisms.

### 1.12 Literature Review Conclusions and Research Gaps

Human brain health spans along a continuum, from optimal brain function to neurocognitive and emotional dysfunctions to dementia and significant mental ill-health, including transient states in between. This spectrum highlights the need for preventive strategies during healthy or transitional phases to delay or prevent progression to advanced pathology (Collaborators, 2022; Schneider, O'Riordan, et al., 2024). Diet quality is a key contributor, and there is clear evidence that healthy dietary patterns promote better cognition and mental health, and that the gut microbiota plays an important role in this diet-brain crosstalk, which is referred to as GMB axis. The “microbiota” component of this axis is influenced by several modifiable and non-modifiable factors, as previously outlined in Figure 1.5.

Whilst various dietary factors can influence the gut microbiota, DFs and their capacity to act as substrates for microbial fermentation has been identified as a major regulator of

the gut microbiota, their associated metabolites and subsequent GMB signalling. Given the wide range of gut-derived metabolites with physiological activity and involvement in gut brain axis (Connell et al., 2022), this thesis focuses primarily on SCFAs as they are the most prominent metabolites produced from DFs and are emerging key mediators of cognitive function (Wang et al., 2021). Much is known about how individual DFs influence the GMB communication (as summarised in Table 1.1), but their role within broader dietary patterns such as a Western or Mediterranean diet remain largely unexplored and will be investigated as part of the MediMood trial.

Furthermore, *APOE4* genotype and female sex are recognised risk factors for cognitive decline and key modulators of gut microbiota composition. Understanding how these factors shape the impact of DFs on GMB communication is crucial for advancing personalised fibre-based approaches to support cognitive health.

### 1.13 Aims and Outline of the thesis

The overall aim of this thesis is to investigate the role of DFs in GMB communication, focusing on how non-modifiable risk factors for cognitive decline such as biological sex and *APOE4* genotype may influence this relationship. To achieve this, we aim to explore the role of biological sex in GMB communication in healthy rodents consuming a refined, western-style diet (Chapter 2). Furthermore, we explore the role of DFs with varying fermentability (both isolated and in combination) in GMB communication and how this differs in males and females (Chapter 3). We also delivered an RCT to investigate the impact of DFs as part of a whole Mediterranean diet on brain health in adults with mild to moderate depression and/or anxiety (Chapter 4). Lastly, we conducted a cross-sectional analysis to explore the role of *APOE4* genotype in shaping the effect of DFs on cognitive health in people with SCI or MCI (Chapter 5).

Together this thesis aims to highlight the important role of DFs in GMB communication and emphasise that biological sex and *APOE4* genotype are potential factors to be considered when evaluating DF-based strategies for supporting cognitive health and preventing cognitive decline.

### Hypotheses:

We hypothesise that:

- Sex-specific differences will be observed in the Gut-Microbiota-Brain axis responses to DF supplementation.
- A short-term high-fibre Mediterranean diet intake will improve mild to moderate depression and/or anxiety through gut microbiota and gut-derived metabolites changes.
- *APOE4* genotype will shape the role of DFs in cognitive health through gut microbiota changes.

## **Chapter 2: The Gut-Brain-Metabolic axis is modulated by sex in rodents consuming a low-fibre Western-type diet**

Thesis Marrium Liaquat

## 2.1 Introduction

A suboptimal diet is a major modifiable risk factor for non-communicable diseases (NCDs). According to the Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) 2021, dietary risk factors contributed to 7.2 million deaths and 178 million disability-adjusted life years (DALYs) worldwide (Sanz, Cryan et al. 2025). Of these, 81.2 million DALYs were linked to low intake of whole grains, fruits and vegetables, which typifies a nutrient-poor, energy-dense western diet (Collaborators 2024). A western diet is characterised by low intake of whole plant foods (wholegrains, fruits and vegetables), low dietary fibre, and high levels of refined carbohydrates (Clemente-Suarez, Beltran-Velasco et al. 2023). Chronic consumption of a western diet is associated with a decline in both physical and psychological health (Clemente-Suarez, Beltran-Velasco et al. 2023). Diet influences human health in part by modulating the gut microbiota, which plays a pivotal role in maintaining the function of multiple organ systems (O'Neill, Monteleone et al. 2016, Chen, Chen et al. 2019, Berding, Vlckova et al. 2021, Anand and Mande 2022, Jamar and Pisani 2023, Li, Yin and Shi 2024).

Gut dysbiosis is strongly associated with refined, western diets and is a key underlying mechanism in diet-related metabolic and cognitive disorders (Soto, Herzog et al. 2018). Indeed, the adoption of a refined, Western-style diet among U.S. immigrants is associated with a loss of beneficial gut microbes and the subsequent onset of dysbiosis, coinciding with higher rates of metabolic diseases (Vangay, Johnson et al. 2018). Furthermore, antibiotic treatment which affects the gut microbiome has been reported to alleviate anxiety and depressive behaviours induced by an obesogenic diet, suggesting a microbe-mediated effect (Soto, Herzog et al. 2018).

The prevalence and progression of metabolic diseases are sex-dependent (Maggi and Della Torre 2018, Varghese, Song and Singer 2021). This may in-part be related to how sex-differences influence response to specific dietary constituents and patterns, however, to date such interactions are poorly characterised (Pontifex, Vauzour and Muller 2024). Factors mediating these sex differences include sex hormones (Maggi and Della Torre 2018, Santos-Marcos, Mora-Ortiz et al. 2023), sex chromosome dosage (Link, Chen et al. 2015), body composition and nutrient metabolism (lipids and carbohydrates) (Ethun 2016), resting metabolic rate (Cunningham 1982), and the gut microbiota (Santos-

Marcos, Mora-Ortiz et al. 2023, Zhu, Qi et al. 2023). Of these factors, the gut microbiota is known to modulate the health effects of diet, primarily through its involvement in nutrient metabolism and in influencing the fate and function of dietary components (Sanz, Cryan et al. 2025). Sex-specific metabolic response to a refined western diet have been observed in animal models such as *Drosophila* (De Groef, Wilms et al. 2021) and mice (Zheng, Li et al. 2022). These differences may be attributed to the complexity of dietary components and the distinct ways in which male and female gut microbiota respond to them. Not only the metabolic responses, but many neurological conditions also exhibit clear sex differences in prevalence, severity, and treatment outcomes (Li and Singh 2014).

Given the widespread consumption of western style refined diets, known to adversely affect brain health (Connell, Blokker, et al., 2024), it is crucial to investigate how these dietary patterns differentially impact males and females. Moreover, the gut microbiota and its metabolites, key modulators of brain function, may play a pivotal role in mediating the sex-specific effects. Understanding these interactions is essential for developing targeted, sex-informed strategies to preserve brain health. In this study, we investigate sex differences across the Gut-brain-metabolic axis in animals consuming a purified rodent diet with characteristics similar to that of a refined/processed western-style diet (e.g. refined carbohydrates, low dietary fibre and phytochemical content). Additionally, we examine the role of the gut microbiota and its associated metabolites as potential mechanistic drivers of these sex differences and consequences for health.

## 2.2 Materials and Methods

### 2.2.1 Study Approval

All experimental procedures were conducted per the specifications of the United Kingdom Animal Scientific Procedures Act, 1986 (Amendment Regulations 2012), and were reviewed and approved by the Animal Welfare and Ethical Review Body (AWERB). The reporting of the study outcomes complies with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines (Percie du Sert et al., 2020).

### 2.2.2 Overview of the experimental procedure

Male (n=10) and female (n=10) C57BL/6J mice sourced from Charles River (Margate, UK) were maintained in individually ventilated cages (n=5 per cage), in a controlled environment (21 ± 2°C; 12-h light/dark cycle; light from 7:00 AM). The mice were fed *ad libitum* on a standard diet (RM3-P; Special Diet Services (SDS), Horley, UK) up to the age of 10 weeks, ensuring normal development and stabilisation of the gut microbiota (Laukens et al., 2016). After which, the mice were transferred to a western-style purified diet; low fat (LF) (D12450Ji, Research Diets, USA) for the remaining 10-week experimental period (see Supp Table S2.1 for detailed dietary composition). Upon completion of the dietary intervention and behavioural testing (see below for more details), 20-week-old animals were sedated with a mixture of isoflurane (1.5%) in nitrous oxide (70%) and oxygen (30%), and transcardially perfused with ice-cold saline containing 10 IU heparin (Sigma-Aldrich, UK). Blood samples were kept on ice for 30 min, centrifuged at 10,000 x g for 5 min at room temperature, followed by sera collection. Brains were rapidly removed, and brain regions were dissected, snap frozen and stored at -80°C until further analysis. The small intestine of each mouse was collected, stripped of mesenteric fats and the length was measured from stomach to caecum with the intestinal content collected. The remaining intestinal tissue was divided into three equal parts, approximately representing the duodenum (part 1), the jejunum (part 2) and the ileum (part 3). Each of these sections were then cut open using a midline incision and the intestinal mucosa was gently scrapped off with the blunt edge of a glass slide and snap-frozen in dry ice. Additionally, caecum and liver samples were collected, weighed, snap-frozen in dry ice, and stored at -80°C until further analyses.

### 2.2.3 Behavioural assessment

All behavioural tests were performed at the end of the experiment after 10-week intervention as described (Pontifex et al., 2022). Briefly, the Open Field (OF) task was performed as a measure of anxiety-like behaviour. The animals were individually placed in a square arena (50 cm x 50 cm x 50 cm) illuminated with dim lighting (100 lux) and were allowed to move freely for a 10-min period. Mice were tracked for the distance travelled, velocity and time spent in the centre/periphery of the maze using Ethovision software (Noldus Information Technology, Inc.).

The Novel Object Recognition (NOR) task was performed as a measure of recognition memory. All experimentation was performed in dim lighting (100 lux). On day 1 (habituation), animals were placed into an empty maze for 10 min. On day 2, animals were conditioned to a single object for a 10-min period. On day 3, animals were exposed to two identical objects for a 15-min period, and after an interval of 1 h, mice were placed back into the testing arena which now contains one familiar object and one novel object. Videos were analysed for a 5-min period, after which if an accumulative object exploration of 8 seconds failed to be reached, the analysis continued for the full 10-min or until 8 seconds was achieved. Those not achieving 8 seconds of object exploration were excluded from the analysis (Denninger et al., 2018). A discrimination index (DI) was calculated as  $DI = (TN - TF)/(TN + TF)$ , where TN is the time spent exploring the novel object, and TF is the time spent exploring the familiar object.

The Y maze spontaneous alternation test was performed as a measure of spatial working memory, as previously described (Pontifex et al., 2022). Each animal was observed for 7 min to record zone transitions and locomotor activity, using Ethovision software. Spontaneous alternation was calculated using the formula (number of alternations/maximum number of alternations x 100).

#### 2.2.4 Gut microbiota DNA extraction and 16S rRNA amplicon sequencing

Gut microbial DNA was isolated from approximately 50mg caecum content from n=5 randomly selected animals using the QIAamp PowerFecal Pro DNA kit (Qiagen, Manchester, UK) as per the manufacturer's instructions. DNA was quantified using a Nanodrop 2000 Spectrophotometer (Fisher Scientific, UK). Quality assessment was performed using agarose gel electrophoresis to detect DNA integrity, purity, fragment size and concentration. The 16S rRNA amplicon sequencing of the V3-V4 hypervariable region was performed with an Illumina NovaSeq 6000 PE250. Sequence analysis was performed using Uparse software (Uparse v7.0.1001) (Wang et al., 2007), using all the effective tags. Sequences with  $\geq 97\%$  similarity were assigned to the same operational taxonomic units (OTUs). A representative sequence for each OTU was screened for further annotation. For each representative sequence, Mothur software was performed against the SSUrRNA database of SILVA Database 138 (Quast et al., 2013). Alpha diversity was assessed using Chao1, Shannon Entropy, and Simpson indices, whilst beta diversity was assessed using

Aitchison's distance values, obtained after CLR (centred-log-ratio) transformation of Euclidean distance values. Statistical significance was determined by Kruskal-Wallis or Permutational Multivariate Analysis of Variance (PERMANOVA), combined with a false discovery rate (FDR) approach used to correct for multiple testing.

### 2.2.5 $^1\text{H}$ NMR Metabolomics

Metabolites were analysed and quantified by  $^1\text{H}$  NMR analysis in the caecum (~ 50 mg) and cortex (~ 30 mg) of the same n=5 animals per group utilised for the microbiome analysis. Frozen tissue content was thoroughly lysed and homogenised in a deuterated phosphate buffer (1.9 mM  $\text{K}_2\text{HPO}_4$ , 8.1 mM  $\text{NaH}_2\text{PO}_4$ , and 1 mM sodium 3-(trimethylsilyl)-propionate- $\text{d}_4$  in deuterated water (Goss Scientifics, Crewe, United Kingdom)) and diluted to a tissue-to-buffer ratio of 13 (e.g. 54 mg in 700  $\mu\text{L}$ ). After homogenising and centrifugation, 650  $\mu\text{L}$  of supernatant was transferred into a 5-mm NMR tube for spectral acquisition.

The cortex tissue (~ 20-40 mg) was lysed and extracted with ice-cold methanol/chloroform separation. The samples were incubated at  $-20^\circ\text{C}$  for 15 min, followed by centrifugation at  $17,000 \times g$  for 3 min. The aqueous phase (on top) was evaporated until dryness using a Savant™ SpeedVac™ High-Capacity Concentrator (Cat. SC210A-230) and reconstituted with 550  $\mu\text{L}$  of deuterated phosphate buffer and then transferred into a 5-mm NMR tube for spectral acquisition.

High resolution  $^1\text{H}$  NMR spectra were recorded on a 600-MHz Bruker Avance spectrometer fitted with a 5-mm TCI proton optimized triple resonance NMR inverse cryoprobe and a 24-slot autosampler (Bruker, Rheinstetten, Germany). Sample temperature was controlled at 300 K. Each spectrum consisted of 128 scans of 65,536 complex data points with a spectral width of 20 ppm (acquisition time 2.6 s). The noesypr1d presaturation sequence was used to suppress the residual water signal with low power selective irradiation at the water frequency during the recycle delay ( $D1 = 2$  s) and mixing time ( $D8 = 0.01$  s). A  $90^\circ$  pulse length of 11.4  $\mu\text{s}$  was set for all samples. Spectra were transformed with a 0.1-Hz line broadening and zero filling, manually phased, baseline corrected and referenced by setting the trimethylsilylpropanoic acid methyl signal to 0 ppm. Metabolites were identified using information found in the

literature or on the web (Human Metabolome Database, <http://www.hmdb.ca/>) and quantified using the software Chenomx® NMR Suite 8.6™.

### 2.2.6 RNA extraction and qRT-PCR

Total RNA was isolated from intestinal part-1 (duodenum) and brain cortex (n=6, randomly selected from each group) using the Qiazol reagent (Qiagen, UK). Two µg of total RNA was treated with DNase I (Invitrogen, UK) and used for complementary DNA (cDNA) synthesis using Invitrogen™ Oligo (dT) primers and M-MMLV reverse transcriptase enzyme. Quantitative real-time PCR (qRT-PCR) reactions were performed using SYBR green detection technology on the Applied Biosystems Quant Studio 5 Real-Time PCR system (Life Technologies). Results are expressed as log<sub>2</sub> transformed values of relative gene expression which has been scaled to the average across all samples per target gene and normalised to the geometric mean of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) reference gene.

### 2.2.7 Triglyceride Analysis

Serum and liver tissue triglyceride levels were measured using a triglyceride assay kit (Abcam, Ab65336) following manufacturer's protocol. Approximately 100mg tissue was washed in cold PBS and homogenised in 1 mL of 5% NP-40/ddH<sub>2</sub>O solution. Homogenates were heated to 80-100°C for 2-5 minutes until the NP-40 solution became cloudy and then cooled down to room temperature. Standards and samples were added to a 96-well plate with cholesterol esterase/lipase and incubated for 20 mins with constant agitation to convert triglycerides into glycerol and fatty acids. Reactions were terminated with assay reaction mix, and absorbance was measured at 570 nm using a microplate reader.

### 2.2.8 Statistical Analyses

Data analysis was performed in GraphPad Prism version 10 (GraphPad Software, CA, USA). All data are presented as mean ± standard deviation (SD), unless otherwise stated. Outliers were identified using ROUT method (q=1%), and data were checked for normality/equal variances using the Shapiro-Wilk test. Further analyses were performed using unpaired t-tests or Mann-Whitney U-test based on the normality of the data.

Statistical analysis of 16S rRNA amplicon sequencing data was performed on RStudio (version 4.4.1). Microbial abundance data was CLR (centred log-ratio) transformed after filtering the dataset excluding all genera and features with <10% prevalence, except for alpha diversity metrics where we are interested in rare taxa. Beta diversity was reported after Permutational Multivariate Analysis of Variance (PERMANOVA), and ordinations were built using Principal Component Analysis (PCA). Differential bacterial abundance analyses were performed using linear models built for each genus to test multiple transformations accounting for different data distribution. P values for all the contrasts were corrected for False Discovery Rate (FDR) using the Benjamini & Hochberg's procedure. Procrustes analysis (PA), investigating the congruence of the metabolomics and microbiome data, was conducted using M<sup>2</sup>IA (Ni et al., 2020).

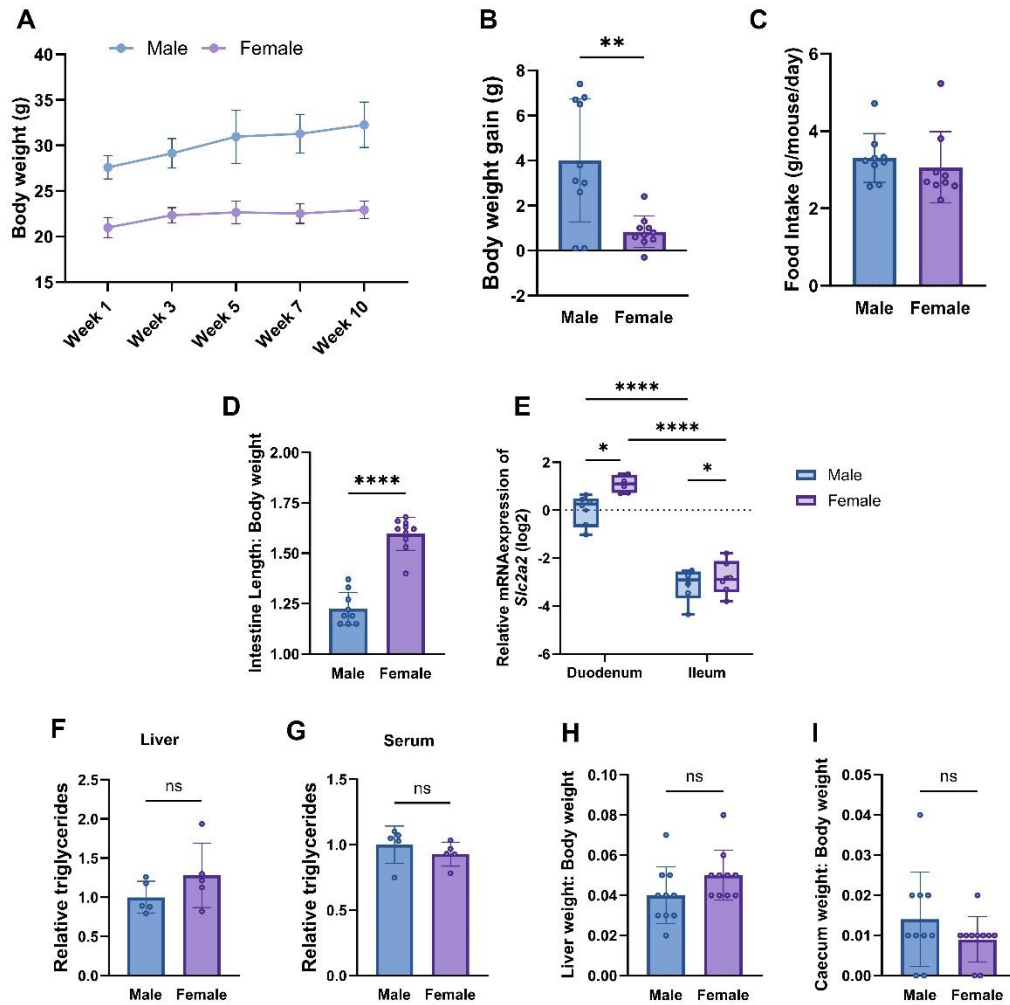
Gut-Brain Modules (GBMs) were identified based on PICRUSt2 predictions which are based on several gene family databases including Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs (KO), Enzyme Commission (EC) numbers and MetaCyc pathway abundances (Bastiaanssen et al., 2023). These PICRUSt2 outputs have been validated with various microbiome datasets (Gavin M. Douglas et al., 2020). GBM modules are annotated for function, pathway, structure and potential to cross the intestinal epithelium and the blood-brain barrier, and their abundances were derived from an orthologue abundance table using Omixer Reference Pathways Mapper (Valles-Colomer et al., 2019). GBM counts were CLR-transformed after removing the features with <10% prevalence, followed by fitting a linear regression model and FDR correction using Benjamini and Hochberg method. q-values of 0.1 and 0.2 were used as a cut-off for statistical significance for genera and GBMs, respectively. CLR transformed data was reported for the final figures as presented.

Statistical analysis of the metabolomics data was carried out using Metaboanalyst 6.0 (Pang et al., 2024). Data were normalised by sum, scaled by autoscaling and log<sub>2</sub> transformed. PCA was employed to illustrate the clustering of different metabolites across the groups. Volcano plot presents comparisons in the direction Male/Female, with an FDR threshold of 0.1. Heatmap shows Ward clustering on Euclidean distance measures.

## 2.3 Results

### 2.3.1 Distinct physiological differences exist between male and female animals consuming a refined diet

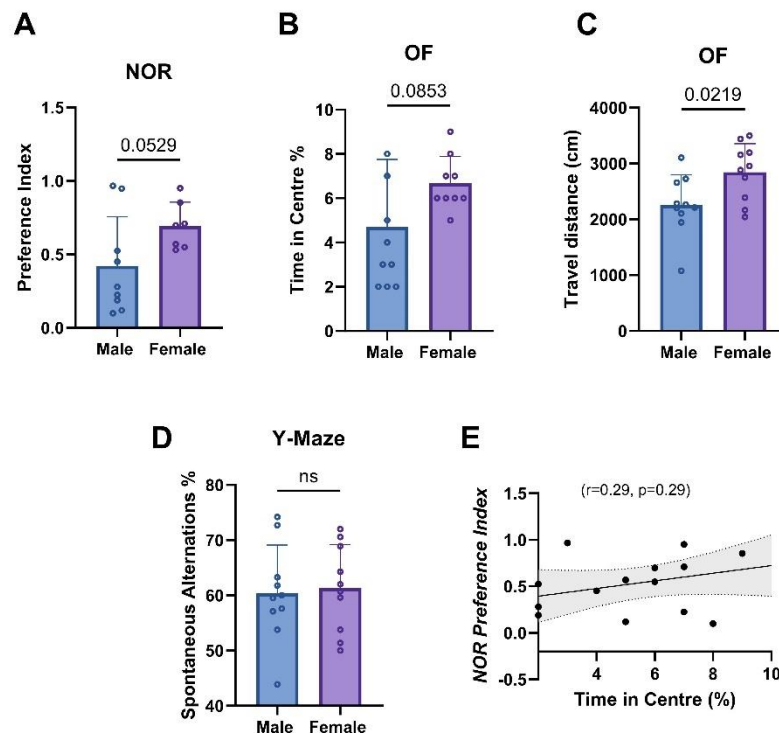
Body weight increased over the 10-week experiment (Fig. 2.1A), with males showing significantly greater weight gain than females ( $p < 0.01$ , Fig. 2.1B), despite similar food intake (Fig. 2.1C). Females exhibited a 30% longer intestinal length than males ( $p < 0.001$ , Fig. 2.1D), which was accompanied by greater glucose transporter *Slc2a2* expression ( $p < 0.05$ , Fig. 2.1E) in both the proximal and distal parts of the small intestine. Despite these sex-specific differences in intestinal morphology and gene expression, hepatic ( $p > 0.05$ , Fig. 2.1F) and serum ( $p > 0.05$ , Fig. 2.1G) triglyceride levels, as well as liver ( $p > 0.05$ , Fig. 2.1H) and caecum weights ( $p > 0.05$ , Fig. 2.1I), were similar between sexes.



**Figure 2.1 | Sex-specific physiological differences are evident in animals consuming a refined LF diet.** (A) Body weight trajectory across the 10-week study. (B) Body weight gain was ~4 times higher in males vs females, (C) despite no significant increases in food intake. (D) Females had 30% longer intestines compared to males, accompanied by (E) higher glucose transporter (*Slc2a2*) expression in both proximal and distal parts of the intestine. (F) Liver and (H) serum triglycerides, and (I) caecum and (F) liver weight showed no sex-specific differences. Data are presented as mean  $\pm$  S.D. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ .

### 2.3.2 Behavioural tests of cognition are not significantly different across males and females consuming a refined diet

Female mice performed nominally better on the NOR task than males ( $p=0.053$ , Fig. 2.2A), although this difference did not reach the conventional threshold for statistical significance. OF task (measure of anxiety) and Y maze (measure of spontaneous working memory) were not significantly different across sex ( $p=0.09$  and  $p>0.05$ , respectively Fig 2.2B and Fig 2.2D). However, males showed significantly less movement than females in the OF task ( $p=0.02$ , Fig 2.2C). NOR performance did not correlate with OF task performance ( $r=0.29$ ,  $p=0.29$ , Fig 2.2E) suggesting that the results of the NOR were independent of anxiety-related behaviours.

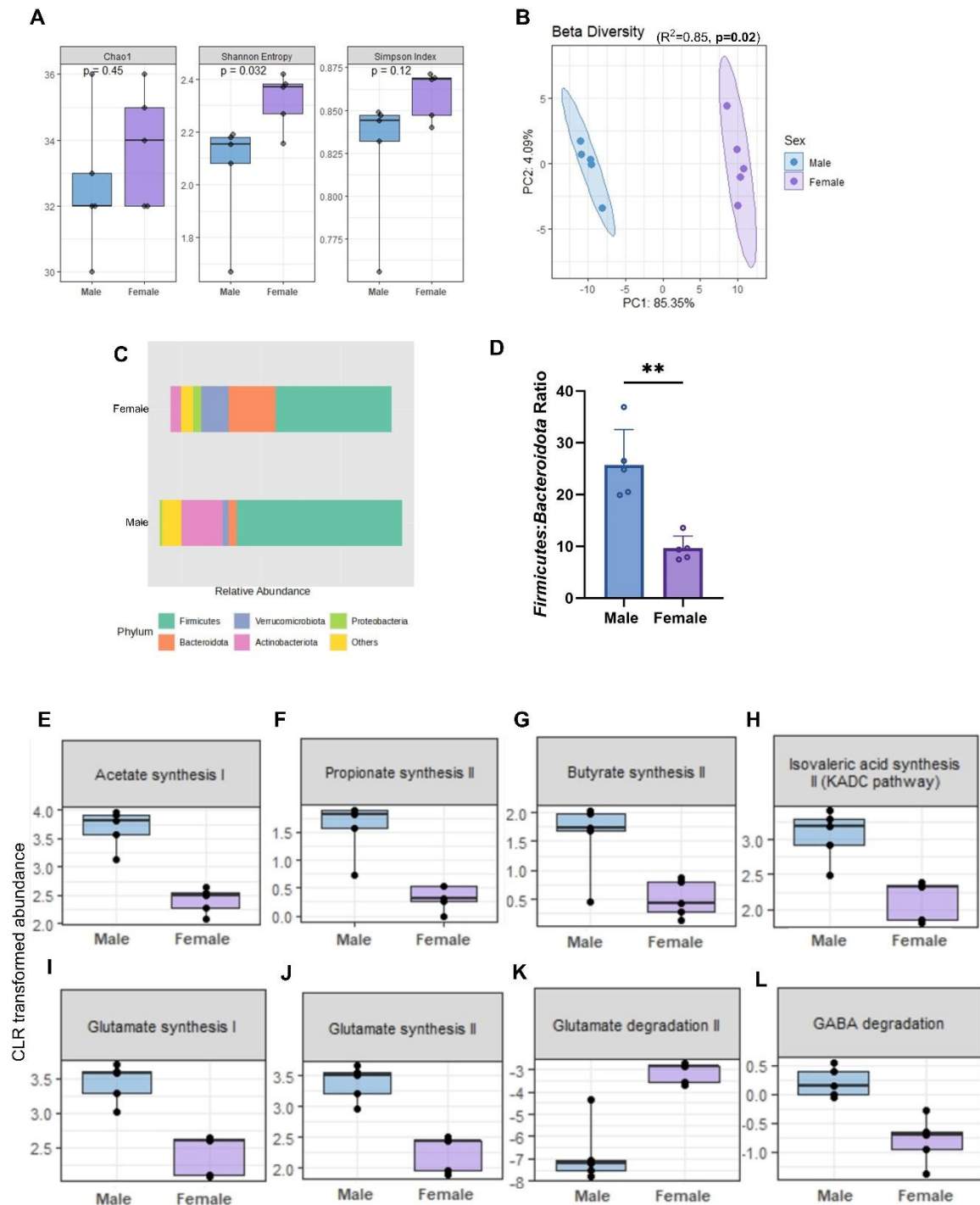


**Figure 2.2 | Subtle differences in anxiety and NOR performance were observed across sex.** (A) Recognition memory as assessed via NOR was nominally lower in males compared to females, whilst (B) Anxiety like behaviour determined via OF was nominally better in females, however neither of these measures reached statistical significance. (C) Females moved significantly more than males in the OF task. (D) Y-Maze performance was comparable across sexes. (E) No correlation was observed between NOR and OF task performance.

### 2.3.3 Male and female animals presented distinct gut microbial profiles despite consuming the same refined diet

Alpha diversity was measured using Chao1, Shannon and Simpson indices. Chao1 index, a measure of species richness was not significantly different between groups ( $p=0.45$ , Fig 2.3A), whilst Shannon index which accounts for both richness and evenness was higher in females ( $p=0.03$ , Fig 2.3A) than males. Simpson index did not show any significant difference ( $p=0.12$ , Fig 2.3A). Beta diversity as shown by Principal Component Analysis (PCA) based on OTUs revealed a distinct clustering of gut microbial communities as observed between males and females based on Euclidean distance (PERMANOVA  $R^2=0.85$ ,  $p=0.02$ , Fig 2.3B). At the phylum level, significant difference was observed in the relative abundance of all major phyla between groups (Fig 2.3C). The relative abundance of gut bacteria from the phylum Bacteroidota was approximately six times higher in females, whereas bacteria from the phylum Firmicutes were about 1.4 times more abundant in males (Fig 2.3C). Consequently, males exhibited a higher Firmicutes to Bacteroidota (F:B) ratio compared to females ( $p<0.001$ , Fig 2.3D). At the genus level, a number of bacteria showed sex-specific differences in response to LF diet (Table 2.1, Supp Fig S2.1).

The GBM predictions showed significant sex-specific differences in metabolic pathways involved in gut-brain communication (Supp Fig S2.2). Select modules are presented in Figure 2.3 (E-L) and a full list added as a supplementary table (Supp Fig S2.2). Predicted SCFAs synthesis for acetate (from Acetyl-CoA I), propionate (from propanoyl-CoA and acetate/lactate), butyrate (from crotonyl-CoA/butanoate and acetate/acetyl-CoA) and isovalerate (KADC pathway) were significantly higher in males ( $q<0.1$ , Fig 2.3E-H). Glutamate, an important metabolite in brain health, showed lower predicted synthesis and higher degradation in females as compared to males ( $q<0.1$ , Fig 2.3I-K). GABA, the chief inhibitory neurotransmitter, showed less predicted degradation in females than males ( $q<0.1$ , Fig 2.3L).



**Figure 2.3 | Distinct microbial compositions are observed between males and females consuming a refined LF diet** (A) Females have significantly higher  $\alpha$ -diversity compared to males and possess (B) distinct microbial communities as highlighted by  $\beta$ -diversity, (C) Relative abundance of the gut microbiota at phylum level and (D) the Firmicutes:Bacteroidota (F:B) ratio. Gut Brain Modules (GBMs) predicted higher synthesis of SCFAs including (E) acetate, (F) propionate, (G) butyrate, and (H) isovalerate in males ( $q < 0.1$ ). Females showed lower (I,J) predicted synthesis of glutamate, and (K) higher glutamate degradation, and (L) lower degradation of GABA ( $q < 0.1$ ).

**Table 2.1** | Differentially abundant bacterial genera between refined LF diet fed males and females

| <b>Bacterial Genera</b>            | <b>Males</b> | <b>Females</b> | <b>P val</b> | <b>FDR</b> |
|------------------------------------|--------------|----------------|--------------|------------|
| <i>Acetatifactor</i>               | 2.44 ± 0.22  | 3.44±0.30      | 0.001        | 0.002      |
| <i>Akkermansia</i>                 | 0.89 ± 1.91  | 4.85 ± 0.46    | 0.004        | 0.008      |
| <i>Alistipes</i>                   | 3.02 ± 0.11  | 2.45 ± 0.46    | 0.043        | 0.066      |
| <i>Anaerostipes</i>                | -3.07 ± 0.64 | 0.55 ± 0.18    | 0.000        | 0.000      |
| <i>Anaerotruncus</i>               | -0.61 ± 0.72 | 1.43 ± 0.41    | 0.001        | 0.003      |
| <i>Bacillus</i>                    | -3.18 ± 0.39 | -3.72 ± 0.12   | 0.030        | 0.047      |
| <i>Bacteroides</i>                 | -0.15 ± 0.61 | 1.65 ± 0.59    | 0.003        | 0.007      |
| <i>Bifidobacterium</i>             | 5.81 ± 0.49  | -3.41 ± 0.71   | 0.000        | 0.000      |
| <i>Bilophila</i>                   | -3.26 ± 0.45 | 2.18 ± 0.51    | 0.000        | 0.000      |
| <i>Butyrivicoccus</i>              | -1.77 ± 0.85 | -3.72 ± 0.12   | 0.002        | 0.005      |
| <i>Candidatus Soleaferrea</i>      | -2.03 ± 0.67 | -3.63 ± 0.21   | 0.002        | 0.005      |
| <i>Clostridium sensu stricto 1</i> | -3.18 ± 0.39 | 2.08 ± 0.48    | 0.000        | 0.000      |
| <i>Enterorhabdus</i>               | -0.44 ± 0.29 | 1.26 ± 0.48    | 0.000        | 0.001      |
| <i>Faecalibaculum</i>              | 5.56 ± 0.31  | 5.18 ± 0.12    | 0.055        | 0.082      |
| <i>Incertae Sedis</i>              | 1.56 ± 0.22  | 0.33 ± 0.43    | 0.001        | 0.003      |
| <i>Intestinimonas</i>              | 1.01 ± 0.56  | -3.72 ± 0.12   | 0.000        | 0.000      |
| <i>Jeotgalicoccus</i>              | -2.93 ± 0.59 | -3.72 ± 0.12   | 0.030        | 0.047      |
| <i>Lachnospiraceae A2</i>          | 2.34 ± 0.51  | 3.08 ± 0.16    | 0.025        | 0.043      |
| <i>Lachnospiraceae UCG-004</i>     | 1.54 ± 1.02  | -3.72 ± 0.12   | 0.000        | 0.000      |
| <i>Lachnospiraceae UCG-006</i>     | 1.38 ± 0.64  | -3.72 ± 0.12   | 0.000        | 0.000      |
| <i>Lactobacillus</i>               | 5.21 ± 0.39  | 3.00 ± 0.64    | 0.000        | 0.001      |
| <i>Lactococcus</i>                 | -2.90 ± 0.63 | -1.61 ± 0.62   | 0.020        | 0.036      |
| <i>Muribaculum</i>                 | 1.85 ± 0.37  | 0.90 ± 0.29    | 0.003        | 0.008      |
| <i>Oscillibacter</i>               | 1.51 ± 0.48  | 0.09 ± 0.59    | 0.006        | 0.011      |
| <i>Parabacteroides</i>             | -3.26 ± 0.45 | 3.57 ± 0.31    | 0.000        | 0.000      |
| <i>Parasutterella</i>              | -0.32 ± 0.52 | 1.49 ± 0.41    | 0.001        | 0.002      |
| <i>Romboutsia</i>                  | -3.26 ± 0.45 | 2.65 ± 1.01    | 0.000        | 0.000      |
| <i>Roseburia</i>                   | 2.99 ± 0.68  | 0.87 ± 0.28    | 0.000        | 0.001      |
| <i>Turicibacter</i>                | -3.26 ± 0.45 | -1.87 ± 0.90   | 0.025        | 0.043      |
| <i>Tuzzerella</i>                  | 0.06 ± 0.64  | -3.72 ± 0.12   | 0.000        | 0.000      |

CLR transformed abundance values are mentioned as Mean ± Standard Deviation.

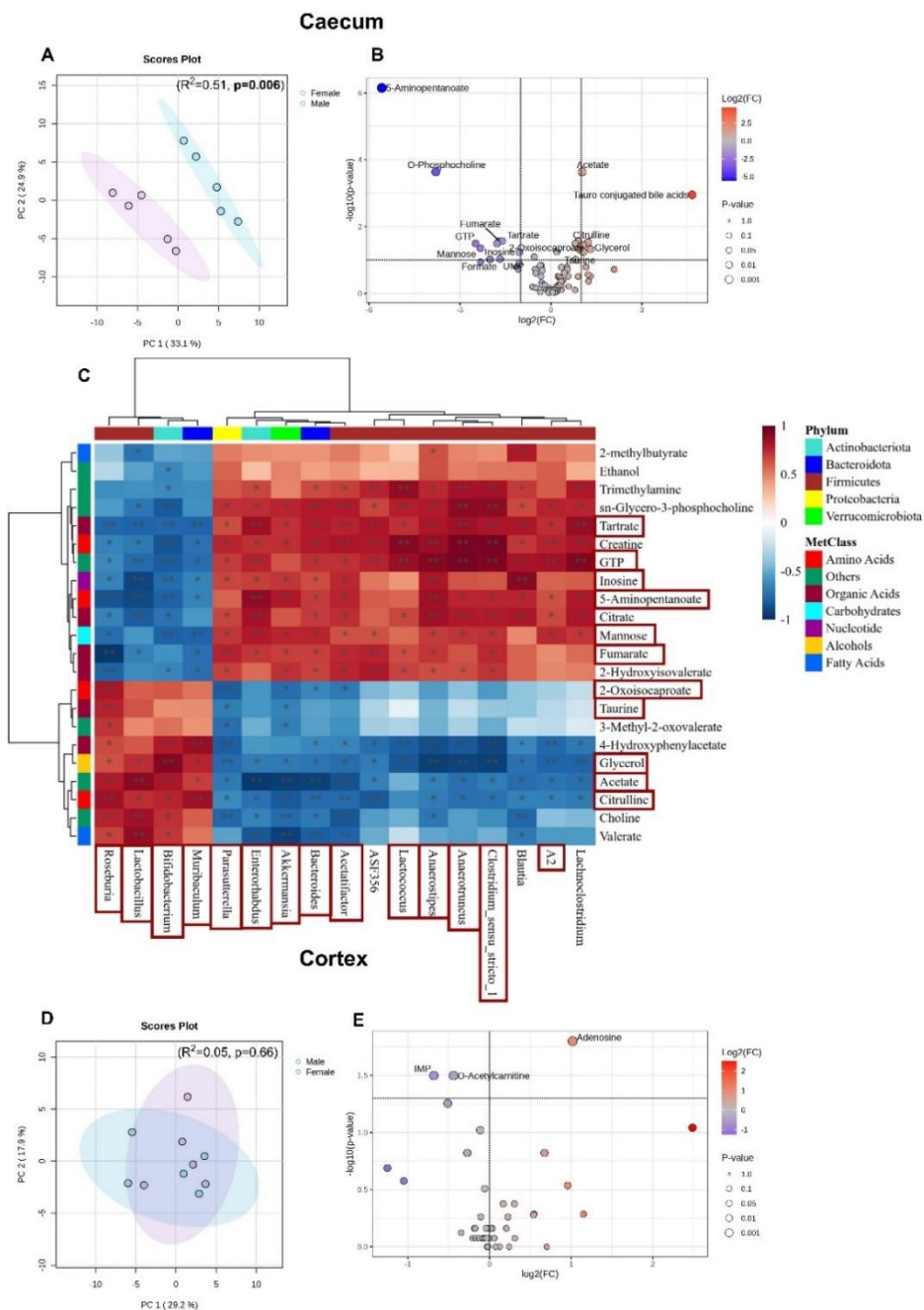
### 2.3.4 Caecal metabolomic differences between sexes mirrored distinct microbial profiles, whereas cortical metabolomic profiles did not

To investigate the metabolic implications of these sex-specific microbial profiles, metabolomic analyses of caecal content and brain (cortex) homogenates was conducted. There were profound caecal metabolomic differences across sex as showcased by the PCA plot, computed using Euclidean distance values ( $R^2=0.51$ ,  $p=0.006$ , Fig 2.4A). The extent of these differences can be seen through the volcano plot which highlights all 15 significantly distinct metabolites ( $FDR<0.1$ , Fig 2.4B). The metabolites 5-aminopentanoate, O-phosphocholine, fumarate, GTP, mannose, inosine, formate, UMP, tartrate and 2-oxoisocaproate were found at higher levels in the female caecal content ( $FDR<0.1$ , Fig 2.4B), whilst, acetate, tauro-conjugated bile acids, citrulline, taurine and glycerol were at lower levels ( $FDR <0.1$ , Fig 2.4B). A full heatmap is given in the supplementary data which shows interindividual variability/difference within each group (Supp Fig S2.3).

Procrustes analysis revealed a strong similarity between the gut microbiota and gut metabolome in males and females ( $R=0.67$ ,  $p=0.02$ , Supp Fig S2.4). Spearman correlation analysis highlighted a potential relationship between the statistically significant bacterial genera *Akkermansia*, *Parasutterella*, *Bacteroides*, *Enterorhabdus*, *Acetatifactor*, *Lactococcus*, *Anearostipes*, *Anaerotruncas*, *Clostridium sensu stricto 1*, and *Lachnospiraceae-A2* and a subset of the significantly altered metabolites, namely 5-aminopentanoate, inosine, GTP, tartrate, mannose, fumarate and 2-oxoisocaproate (Fig 2.4C). In correspondence to the GBMs, the gut levels of SCFAs were lower in females. The serum SCFA levels were lower in females (Supp Fig S2.5A-C), as well as glutamate levels (Supp Fig S2.5D).

In contrast to the pronounced sex differences observed in the caecal metabolome, the cortical metabolomic profile was broadly similar between males and females ( $R^2 = 0.05$ ,  $p = 0.66$ ; Fig. 2.4D), with only three metabolites differing significantly. IMP and O-acetylcarnitine were present at higher levels in female brains, whereas adenosine was lower ( $p<0.05$ ; Fig. 2.4E) as compared to males. The cortical metabolome showed no overall relationship with gut microbial composition. Among the metabolites altered by

sex, only *O*-acetylcarnitine demonstrated a cross-tissue association, correlating with the caecal metabolite taurine ( $r = -0.68$ ,  $p = 0.04$ , Supp Fig S2.6).



**Figure 2.4 | Distinct metabolomic profiles are observed between males and females consuming a refined LF diet** (A) PCA plot showing distinct separation between male and female caecum metabolites, and (B) Volcano plot depicting 15 significantly different caecal metabolites between groups with direction of comparison Male/Female (FDR  $q=0.1$ ), and (C) Correlation analysis between caecal metabolites and gut microbiota conducted using M<sup>2</sup>IA, significantly different metabolites and bacterial genera are highlighted in red rectangles. (D) PCA plot showing subtle separation between male and female cortex metabolites, and (E) Volcano plot depicting 3 significantly different cortex metabolites between groups with direction of comparison Male/Female ( $p<0.05$ ). \* $p<0.05$ , \*\* $p<0.01$

## 2.4 Discussion

Emerging research highlights sexual dimorphism in response to dietary patterns and components. Recognising and understanding these differences is vital for identifying sex-specific health and disease patterns (Pontifex, Vauzour, et al., 2024). In this study, we examine the physiological, metabolic and microbial differences as well as the broader gut-brain dynamics that exist across young healthy female and male mice maintained on a standard refined diet. Our findings reveal pronounced sex-specific physiological, microbial and metabolic differences in the periphery, which were somewhat more nuanced within the brain.

Physiological and metabolic differences between the sexes are believed to be partly driven by greater energy demands associated with reproduction (Hudry et al., 2016). Indeed, the longer small intestines exhibited by females offers a greater propensity for nutrient digestion and absorption (McKenney et al., 2023). However, additional factors such as microbiome-driven influences may also contribute to this difference. Female-enriched bacterial genera showed strong positive correlations with small intestine length, whereas male-enriched genera showed strong negative correlations (Supp Table S2.2). This greater propensity of females for nutrient digestion and absorption is further supported by increased expression of the intestinal glucose transporter (*Slc2a2*). *Slc2a2* principally mediates glucose transport from the lumen to the bloodstream, and its expression upregulates rapidly in response to elevated luminal glucose (Shen et al., 2024). Although not particularly in the context of intestinal expression, *Slc2a2* has been known to exert sex-specific functional differences (Pasula et al., 2024). Together, these factors likely contribute to the consistently higher postprandial glucose levels observed in females than males, despite similar or lower glucose intakes (Bartholomae et al., 2019; Basu et al., 2006; Sicree et al., 2008).

Despite these physiological differences, and similar food consumption (Fig 2.1B), females gained less body weight than males ( $p < 0.01$ , Fig 2.1B), consistent with findings from other studies showing greater protection against diet-induced weight gain (de Souza et al., 2022; Maric et al., 2022; Zhu et al., 2023). This lower weight gain in females may stem from interactions between sex hormones and the gut-microbiota, as these hormones are known to shape the gut microbiota composition (Liaquat, Minihiene, et al.,

2025). Zhu et al, showed that germ-free females are heavier than conventional females, whilst male weight is unaffected by microbial status (Zhu et al., 2023). Notably, males remained heavier than females in both conditions, indicating the involvement of additional factors. Some evidence further suggests that males have higher blood glucose levels than females due to androgen-driven gut microbiota changes (Gao et al., 2021). However, our findings reveal a sex-specific response to a refined diet, which may explain discrepancies with studies using standard diets. This underscores that sexual dimorphism depends strongly on dietary composition.

Females had a distinct gut microbiota composition, as shown by significantly higher  $\alpha$ -diversity and distinct  $\beta$ -diversity than males (Fig 2.3), also confirmed by literature (de la Cuesta-Zuluaga et al., 2019; Org et al., 2016; Valeri & Endres, 2021). The relative abundance of the phyla Firmicutes and Bacteroidota (F:B ratio) plays a key role in maintaining intestinal homeostasis and has been strongly implicated in the development of obesity (Braun et al., 2009; Eehalt et al., 2010). Males showed a significantly higher F:B ratio, which is also confirmed by other studies (Ali et al., 2024), and is frequently cited as a hallmark of obesity (Houtman et al., 2022).

The Firmicutes phylum includes both SCFA-producing bacteria (Zhu et al., 2023), and species that do not contribute to SCFA production (Houtman et al., 2022; Ley et al., 2006; Magne et al., 2020). SCFAs provide approximately 10% of the total dietary energy in humans (McNeil, 1984), and the bacterial variations within the Firmicutes phylum among SCFA producers and non-producers pose contradictory interpretation about F:B ratio and its association with weight gain, as it's not a true representative of proposed functional capacity for energy harvest (Houtman et al., 2022). Higher F:B ratio in males was accompanied with higher abundance of SCFA-producing bacterial genera within the Firmicutes phylum including *Intestinimonas*, *Butyricicoccus*, *Faecalibacterium*, and *Roseburia* (Table 2.1). These microbes may mediate the greater weight gain observed in males by extracting more energy from the diet in the form of SCFAs. Acetate, which was significantly higher in the male caecum, is linked to obesity and can also stimulate insulin secretion and increase food consumption through activation of the parasympathetic nervous system (Perry et al., 2016; Trent & Blaser, 2016).

In addition to compositional changes, functional differences in the metabolic capacity of microbiota are important in microbe-mediated weight gain (Martinez et al., 2016). The predicted capacity of the gut microbiota to synthesize SCFAs including acetate (from Acetyl-CoA I), propionate (from propanoyl-CoA and acetate/lactate), butyrate (from crotonyl-CoA/butanoate and acetate/acetyl-CoA), and isovalerate (KADC pathway), was significantly higher in males (Fig 2.3 E-H). Caecum metabolomic profile further supported this finding, as evidenced by higher caecal acetate, propionate and butyrate levels in males compared to females (Supp Fig S2.5 A-C). Among which only caecal acetate levels were significantly different ( $p < 0.05$ ), which also positively correlated with SCFA producers in the gut including *Muribaculum*, *Bifidobacterium*, *Lactobacillus*, and *Roseburia* (Fig 2.4C). SCFAs serve as energy source for the intestinal epithelial cells, and mouse models of obesity show elevated levels of SCFAs in the lumen (Schwartz et al., 2010). Though conflicting evidence exists regarding increased energy availability via SCFA production, given the positive impact of SCFAs on metabolism (De Vadder et al., 2014; Martinez et al., 2016). Whilst males exhibit a higher energy harvest from gut derived SCFAs, females exhibit higher levels of other energy metabolites in the gut, such as GTP, UMP, mannose, fumarate, formate, which supports our findings that females exhibit greater nutritional efficiency than males. Females were also suggestive of a healthier gastrointestinal environment and barrier function due to higher levels of O-phosphocholine (Braun et al., 2009; Eehalt et al., 2010) and lower levels of taurine-conjugated bile acids (Ali et al., 2024) as compared to males.

Similar to the gut, we also observed sex-specific differences in the cortex metabolomic profile. Females had significantly higher levels of IMP and O-Acetylcarnitine in the brain cortex, which are known to protect against neuropsychiatric disorders (Fisher et al., 2002) and age-related defects (Rosca et al., 2009), respectively. We observed predominantly higher glutamatergic neurotransmission in males and higher GABAergic neurotransmission in females. In males, there was higher predicted glutamate synthesis and lower degradation in the gut (Fig 2.3 I-K). Additionally, female gut microbiota predicted reduced GABA degradation (Fig 2.3L) and showed elevated gut levels of 5-aminopentanoate, a GABA analogue involved in GABA-mediated signalling (Dhaher et al., 2014). Females also had higher abundance of *Akkermansia* in the gut, which is known to

produce GABA, regardless of the presence of GABA precursors (Konstanti et al., 2024). GABA and GABA-producing bacteria are also associated with improved metabolic health (Marques et al., 2016; Tian et al., 2011). Furthermore, a strong correlation between brain O-acetylcarnitine and gut taurine suggests that gut microbial metabolites may influence brain energy metabolism. These cortical and gut-brain module differences were modest, likely because the animals were healthy and lacked any underlying pathology, so large or overt alterations were not expected. Nonetheless, we detected subtle metabolic differences that may become biologically important in the context of dietary interventions or disease.

This study reveals sex-specific metabolic differences likely influenced by distinct gut microbiota. Whilst males harvested more energy from gut derived SCFAs, females were more efficient in glucose uptake from the intestine and displayed significantly higher levels of several other energy-related metabolites in the gut, suggesting sex-specific strategies for meeting metabolic demands. they also had significantly higher levels of numerous other energy metabolites in the gut. These findings underscore the gut microbiota's pivotal role in mediating sex-specific dietary responses and support its potential as a target for personalised nutrition and disease prevention. Future research should explore the mechanistic links among diet, gut microbiota, and metabolic health across sexes to inform tailored dietary strategies.

## **Chapter 3: Comparative analysis of dietary fibres impact on sex-specific Gut-Microbiota-Brain communication**

Thesis Marrium Liaquat

### 3.1 Introduction

The bidirectional communication that exists between the gut and the brain (gut-microbiota-brain axis) has the potential to influence brain health (Chakrabarti et al., 2022), and therefore represents a promising therapeutic target. Although the gut microbiota is altered via several genetic, health and environmental factors (Cryan et al., 2019), diet is one of the most powerful and accessible means to modulate gut microbiota composition and function (Cryan et al., 2019; Schneider, O'Riordan, et al., 2024).

Changes in dietary patterns, particularly increased intake of DFs are increasingly recognised for their capacity to modulate the GMB axis (Berding, Carbia, et al., 2021; Berding, Vlckova, et al., 2021; Schneider, Balasubramanian, et al., 2024). Cross-sectional and prospective cohort studies consistently report positive associations between higher DF intake and better cognitive and mental health (La Torre et al., 2021; Liaquat, Le Gall, et al., 2025; Prokopidis et al., 2022; Yang et al., 2024). However, fibres differ markedly in origin, chemical structure, and physicochemical properties (solubility, viscosity, and fermentability), which shape both their effects on gut microbiota composition (Thomson et al., 2021) and the pathways through which they influence brain function (La Torre et al., 2021).

Inulin and psyllium are soluble dietary fibres that differ markedly in their fermentation profiles due to their distinct structures (Thomson et al., 2021). Inulin, a  $\beta$ -2,1-linked fructan, is highly fermentable in the proximal colon and acts as a prebiotic by selectively promoting bacteria such as *Lactobacillus* and *Bifidobacterium* (Pontifex et al., 2021). Psyllium, composed largely of arabinoxylans, forms a viscous gel that increases stool bulk and is only moderately and slowly fermentable throughout the colon. Because fermentability is central to fibre-microbiota interactions, these differences have important functional implications. Inulin primarily drives microbial growth, whereas psyllium's high water holding capacity influences digestion, nutrient absorption, and metabolic processes. Notably, combining these fibres can produce distinct effects (Pontifex et al., 2021).

Biological sex is a critical yet overlooked factor in dietary interventions (Pontifex, Vauzour, et al., 2024). As discussed in chapter 2, sex differences influence both gut microbiota

composition and functional capacity, which is also known to influence GMB axis (Hokanson et al., 2024). Although sex-specific responses to dietary interventions have been reviewed (Pontifex, Vauzour, et al., 2024) and DFs are known to modulate gut microbiota (Drew et al., 2018; Zochling et al., 2025), sex-specific effects of isolated DFs, particularly within the GMB axis remain unexplored.

Key gaps remain in understanding: (i) sex-specific physiological responses to distinct DFs, (ii) fibre-specific microbial and metabolomic signatures, and (iii) how gut microbial and metabolic shifts influence brain metabolomic profiles. To address these gaps, we investigated the effects of inulin, psyllium and their combination on physiology, gut microbiota composition, gut and brain metabolomes, and markers of BBB integrity. We hypothesised that fermentable and less-fermentable DFs would elicit distinct, sex-specific microbial and metabolic responses extending from the gut to the brain. Our findings provide new insight into how specific fibres modulate the microbiota-host axis in a sex-dependent manner and reveal mechanistic links that may inform personalised fibre-based strategies to optimise gut and brain health.

## 3.2 Materials and Methods

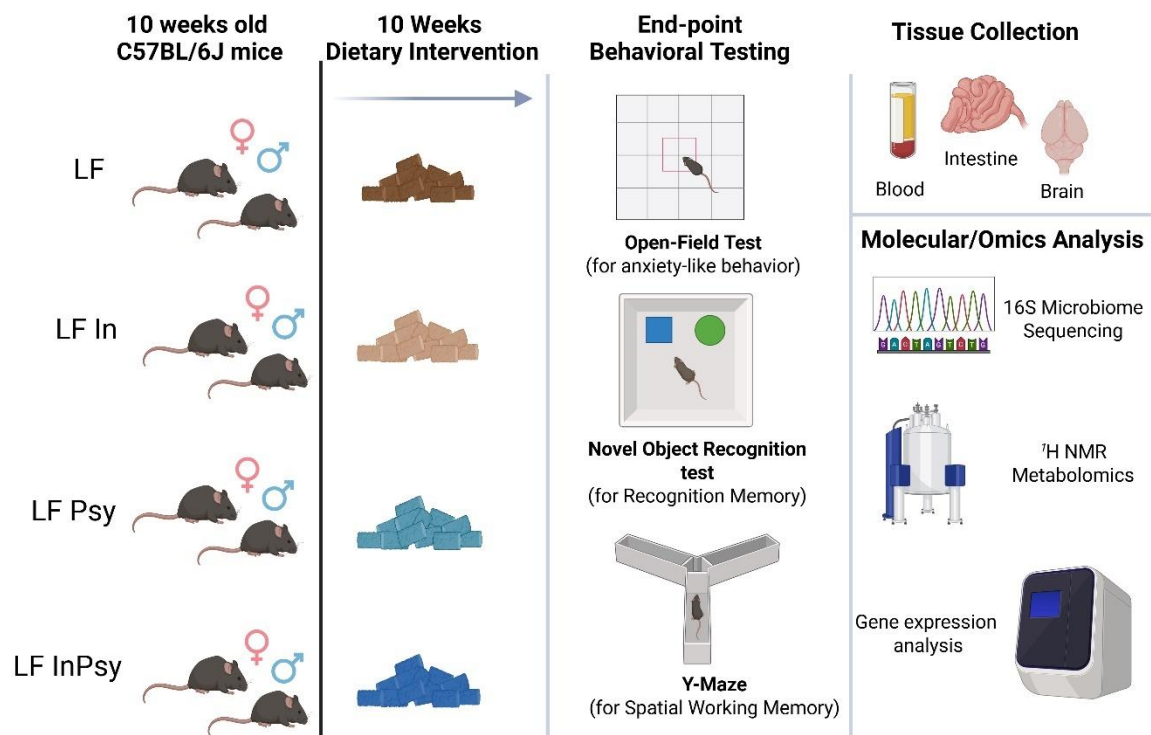
### 3.2.1 Study Approval

All experimental procedures were conducted per the specifications of the United Kingdom Animal Scientific Procedures Act, 1986 (Amendment Regulations 2012), and were reviewed and approved by the Animal Welfare and Ethical Review Body (AWERB). The reporting of the study outcomes complies with ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines (Percie du Sert et al., 2020).

### 3.2.2 Overview of the experimental procedure

Male (n=40) and female (n=40) C57BL/6J mice sourced from Charles River (Margate, UK) were maintained in individually ventilated cages (n=5 per cage), in a controlled environment (21 ± 2°C; 12-h light/dark cycle; light from 7:00 AM). The mice were fed *ad libitum* on a standard diet (RM3-P; Special Diet Services (SDS), Horley, UK) up to the age of 10 weeks, ensuring normal development and stabilisation of the gut microbiota (Laukens et al., 2016). After which, the mice were transferred to one of the four purified diets: low fat (LF, D12450J), LF with 35 g inulin/kg (LFI<sub>n</sub>, D23012401), LF with 35 g

psyllium/kg (LFPsy, D23012402) and LF with 17.5 g inulin and 17.5 g psyllium/kg (LFInPsy, D23012403) for the remaining 10-week experimental period (see Supp Table S3.1 for detailed dietary composition). Upon completion of the dietary intervention and behavioural testing (see below for more details), 20-week-old animals were sedated with a mixture of isoflurane (1.5%) in nitrous oxide (70%) and oxygen (30%), and transcardially perfused with ice-cold saline containing 10 IU heparin (Sigma-Aldrich, UK). Blood samples were kept on ice for 30 min, centrifuged at 10,000 x g for 5 min at room temperature, followed by sera collection. Brains were rapidly removed, and brain regions were dissected, snap frozen and stored at -80 °C until further analysis. The small intestine of each mouse was collected, stripped of mesenteric fats and the length was measured from stomach to caecum with the intestinal content collected. The remaining intestinal tissue was divided into three equal parts, approximately representing the duodenum (part 1), the jejunum (part 2) and the ileum (part 3). Each of these sections were then cut open using a midline incision and the intestinal mucosa was gently scrapped off with the blunt edge of a glass slide and snap frozen in dry ice. Additionally, caecum and liver samples were collected, weighed, snap-frozen in dry ice, and stored at -80 °C until further analyses. An overview of the study design is presented in Figure 3.1.



**Figure 3.1| Overview of the experimental design**

LF: Low fibre; LF In: Low fibre with inulin; LF Psy: Low fibre with psyllium; LF InPsy: Low fibre with inulin and psyllium. Created with BioRender.com.

### 3.2.3 Behavioural assessment

All behavioural tests were performed at the end of the experiment after 10-week intervention as described (Pontifex et al., 2022). Briefly, the Open Field (OF) task was performed as a measure of anxiety-like behaviour. The animals were individually placed in a square arena (50 cm x 50 cm x 50 cm) illuminated with dim lighting (100 lux) and were allowed to move freely for a 10-min period. Mice were tracked for the distance travelled, velocity and time spent in the centre/periphery of the maze using Ethovision software (Noldus Information Technology, Inc.).

The Novel Object Recognition (NOR) task was performed as a measure of recognition memory. All experimentation was performed in dim lighting (100 lux). On day 1 (habituation), animals were placed into an empty maze for 10 min. On day 2, animals were conditioned to a single object for a 10-min period. On day 3, animals were exposed to two identical objects for a 15-min period, and after an interval of 1 h, mice were placed back into the testing arena which now contains one familiar object and one novel object. Videos were analysed for a 5-min period, after which if an accumulative object exploration of 8 seconds failed to be reached, the analysis continued for the full 10-min or until 8 seconds was achieved. Those not achieving 8 seconds of object exploration were excluded from the analysis (Denninger et al., 2018). A discrimination index (DI) was calculated as  $DI = (TN - TF)/(TN + TF)$ , where TN is the time spent exploring the novel object, and TF is the time spent exploring the familiar object.

The Y maze spontaneous alternation test was performed as a measure of spatial working memory, as previously described (Pontifex et al., 2022). Each animal was observed for 7 min to record zone transitions and locomotor activity, using Ethovision software. Spontaneous alternation was calculated using the formula (number of alternations/maximum number of alternations x 100).

### 3.2.4 Gut microbiota DNA extraction and 16S rRNA amplicon sequencing

Gut microbial DNA was isolated from approximately 50mg caecum content from n=5 randomly selected animals using the QIAamp PowerFecal Pro DNA kit (Qiagen, Manchester, UK) as per the manufacturer's instructions. DNA was quantified using a Nanodrop 2000 Spectrophotometer (Fisher Scientific, UK). Quality assessment was

performed using agarose gel electrophoresis to detect DNA integrity, purity, fragment size and concentration. The 16S rRNA amplicon sequencing of the V3-V4 hypervariable region was performed with an Illumina NovaSeq 6000 PE250. Sequence analysis was performed using Uparse software (Uparse v7.0.1001) (Wang et al., 2007), using all the effective tags. Sequences with  $\geq 97\%$  similarity were assigned to the same operational taxonomic units (OTUs). A representative sequence for each OTU was screened for further annotation. For each representative sequence, Mothur software was performed against the SSUrRNA database of SILVA Database 138 (Quast et al., 2013). Alpha diversity was assessed using Chao1, Shannon Entropy, and Simpson indices, whilst beta diversity was assessed using Aitchison's distance values, obtained after CLR (centred-log-ratio) transformation of Euclidean distance values. Statistical significance was determined by Kruskal-Wallis or Permutational Multivariate Analysis of Variance (PERMANOVA), combined with a false discovery rate (FDR) approach used to correct for multiple testing.

### 3.2.5 $^1\text{H}$ NMR Metabolomics

Metabolites were analysed and quantified by  $^1\text{H}$  NMR analysis in the caecum (approximately 50 mg) and cortex (approximately 30 mg) of the same  $n=5$  animals per group utilised for the 16S microbiome analysis. Frozen tissue content was thoroughly lysed and homogenised in a deuterated phosphate buffer (1.9 mM  $\text{K}_2\text{HPO}_4$ , 8.1 mM  $\text{NaH}_2\text{PO}_4$ , and 1 mM sodium 3-(trimethylsilyl)-propionate- $\text{d}_4$  in deuterated water (Goss Scientifics, Crewe, United Kingdom)) and diluted to a tissue-to-buffer ratio of 13 (e.g. 54 mg in 700  $\mu\text{L}$ ). After homogenising and centrifugation, 650  $\mu\text{L}$  of supernatant was transferred into a 5-mm NMR tube for spectral acquisition.

The cortex tissue ( $\sim 20\text{-}40$  mg) was lysed and extracted with ice-cold methanol/chloroform separation. The samples were incubated at  $-20^\circ\text{C}$  for 15 min, followed by centrifugation at  $17,000 \times g$  for 3 min. The aqueous phase (on top) was evaporated until dryness using a Savant™ SpeedVac™ High-Capacity Concentrator (Cat. SC210A-230) and reconstituted with 550  $\mu\text{L}$  of deuterated phosphate buffer and then transferred into a 5-mm NMR tube for spectral acquisition.

High resolution  $^1\text{H}$  NMR spectra were recorded on a 600-MHz Bruker Avance spectrometer fitted with a 5-mm TCI proton optimized triple resonance NMR inverse

cryoprobe and a 24-slot autosampler (Bruker, Rheinstetten, Germany). Sample temperature was controlled at 300 K. Each spectrum consisted of 128 scans of 65,536 complex data points with a spectral width of 20 ppm (acquisition time 2.6 s). The noesypr1d presaturation sequence was used to suppress the residual water signal with low power selective irradiation at the water frequency during the recycle delay ( $D1 = 2$  s) and mixing time ( $D8 = 0.01$  s). A  $90^\circ$  pulse length of  $11.4 \mu\text{s}$  was set for all samples. Spectra were transformed with a 0.1-Hz line broadening and zero filling, manually phased, baseline corrected and referenced by setting the trimethylsilylpropanoic acid methyl signal to 0 ppm. Metabolites were identified using information found in the literature or on the web (Human Metabolome Database, <http://www.hmdb.ca/>) and quantified using the software Chenomx<sup>®</sup> NMR Suite 8.6<sup>™</sup>.

### 3.2.6 RNA extraction and qRT-PCR

Total RNA was isolated from intestinal part-1 (duodenum), and brain cortex ( $n=6$ , randomly selected from each group) using the Qiazol reagent (Qiagen, UK). Two  $\mu\text{g}$  of total RNA was treated with DNase I (Invitrogen, UK) and used for complementary DNA (cDNA) synthesis using Invitrogen<sup>™</sup> Oligo (dT) primers and M-MMLV reverse transcriptase enzyme. Quantitative real-time PCR (qRT-PCR) reactions were performed using SYBR green detection technology on the Applied Biosystems Quant Studio 5 Real-Time PCR system (Life Technologies). Results are expressed as  $\log_2$  transformed values of relative gene expression which has been scaled to the average across all samples per target gene and normalised to the geometric mean of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) reference gene.

### 3.2.7 Statistical Analyses

Data analysis was performed in GraphPad Prism version 10 (GraphPad Software, CA, USA) and R Studio (version 4.4.3). All data are presented as mean  $\pm$  standard deviation (SD), unless otherwise stated. Outliers were identified using ROUT method ( $q=1\%$ ), and data were checked for normality/equal variances using the Shapiro-Wilk test. Further analyses were performed using two-way ANOVA or mixed model analysis based on the normality of the data and to account for missing values.

Statistical analysis of 16S rRNA microbiome sequencing data was performed on RStudio version 4.4.3. Microbial abundance data was CLR (centred log-ratio) transformed after filtering the dataset excluding all genera and features with <10% prevalence, except for alpha diversity metrics where we are interested in rare taxa. Beta diversity was reported after Permutational Multivariate Analysis of Variance (PERMANOVA), and ordinations were built using Principal Component Analysis (PCA). Differential bacterial abundance analyses were performed using linear models built for each genus to test multiple transformations accounting for different data distribution. P values for all the contrasts were corrected for False Discovery Rate (FDR) using the Benjamini & Hochberg's procedure. Procrustes analysis (PA), investigating the congruence of the metabolomics and microbiome data, was conducted using M<sup>2</sup>IA (Ni et al., 2020).

Gut-Brain Modules (GBMs) were identified based on PICRUSt2 predictions which are based on several gene family databases including Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs (KO), Enzyme Commission (EC) numbers and MetaCyc pathway abundances (Bastiaanssen et al., 2023). These PICRUSt2 outputs have been validated with various microbiome datasets (Gavin M. Douglas et al., 2020). GBM modules are annotated for function, pathway, structure and potential to cross the intestinal epithelium and the blood-brain barrier, and their abundances were derived from an orthologue abundance table using Omixer Reference Pathways Mapper (Valles-Colomer et al., 2019). GBM counts were CLR-transformed after removing the features with <10% prevalence, followed by fitting a linear regression model and FDR correction using Benjamini and Hochberg method. q-values of 0.1 and 0.2 were used as a cut-off for statistical significance for genera and functional modules (GBMs), respectively. CLR transformed data was reported for the final figures as presented.

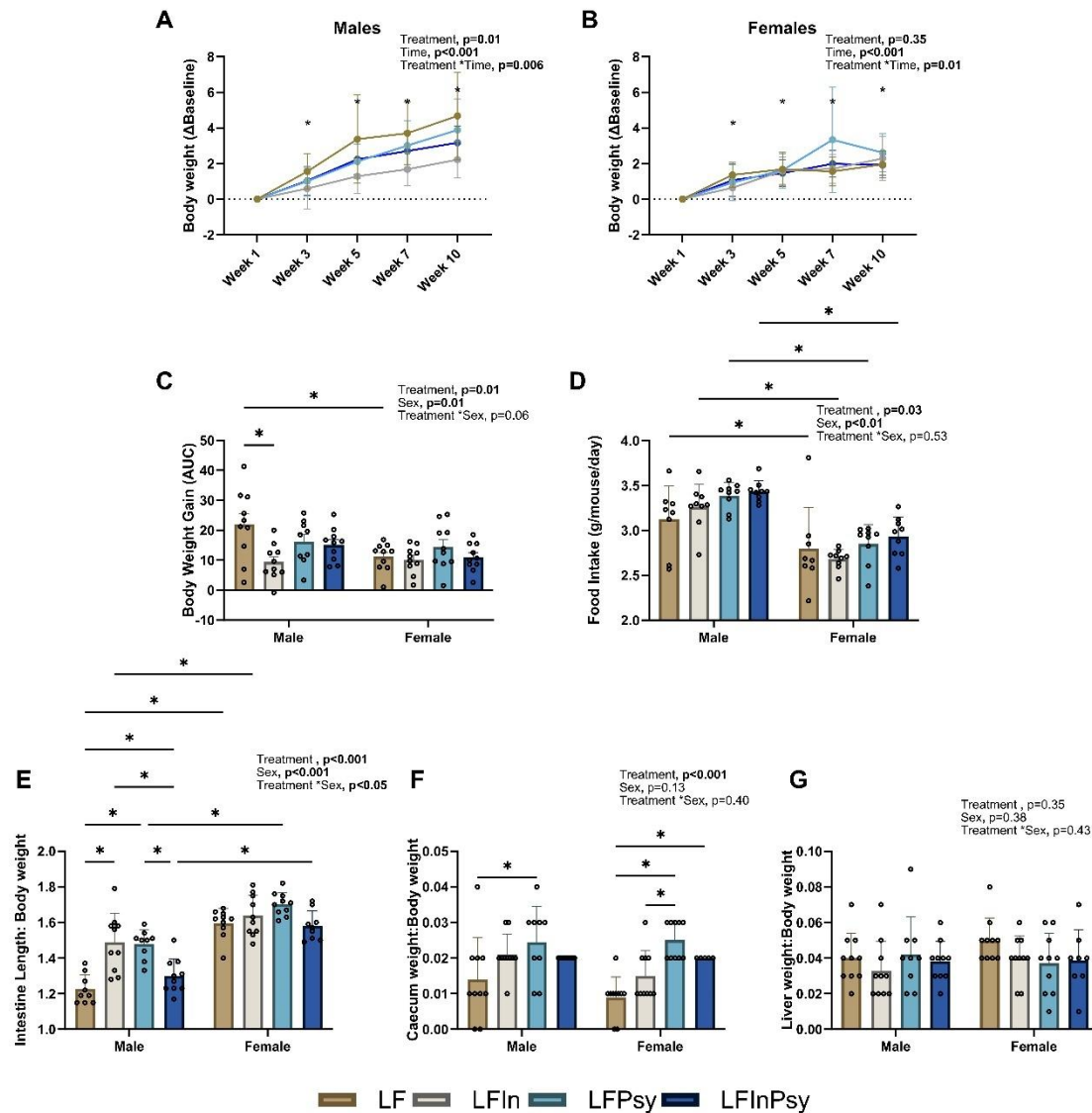
Statistical analysis of the metabolomics data was carried out using Metaboanalyst 6.0 (Pang et al., 2024). Data were normalised by sum, scaled by autoscaling and log2 transformed. PCA was employed to illustrate the clustering of different metabolites across the groups. Heatmap shows Ward clustering on Euclidean distance measures.

### 3.3 Results

#### 3.3.1 Dietary Fibres (DFs) exhibit sex-specific physiological changes in mice

Body weight trajectories over the 10-week period revealed a significant reduction in weight gain in males following DF exposure (Fig 3.2A), that was not present in females (Fig 3.2B). The reduction in weight gain observed in males following DF treatment was also apparent in the area under the curve (AUC) analysis; with AUC reduced by 57%, 26%, and 31% across the inulin, psyllium and combination diets, respectively, however statistical significance according to post hoc analysis was limited to only the inulin-treated group ( $p=0.0006$ ). The differences were very close to significant for psyllium ( $p=0.07$ ) and the combination ( $p=0.06$ ) groups. Marked sex difference in weight gain were also apparent from the AUC analysis with males gaining approximately twofold more weight than females (Fig. 3.2C), consistent with their higher daily food intake ( $p<0.05$ ; Fig. 3.2D).

Female intestines were 30% longer than males ( $p<0.0001$ , Fig 3.2E). Interestingly, DF intervention had no effect on intestinal length in females, whereas it increased the intestinal length in males, with ~21% increase observed in inulin and psyllium groups ( $p<0.0001$ ). However, the increase was relatively smaller ~6% in inulin-psyllium combination group ( $p=0.04$ , Fig 3.2E). Psyllium increased caecum weight ( $p<0.05$ ) in both males and females, while the combined inulin-psyllium intervention produced a significant increase only in females (Fig 3.2F). No change in liver weight was observed following DF supplementation in either sex (Fig 3.2G).



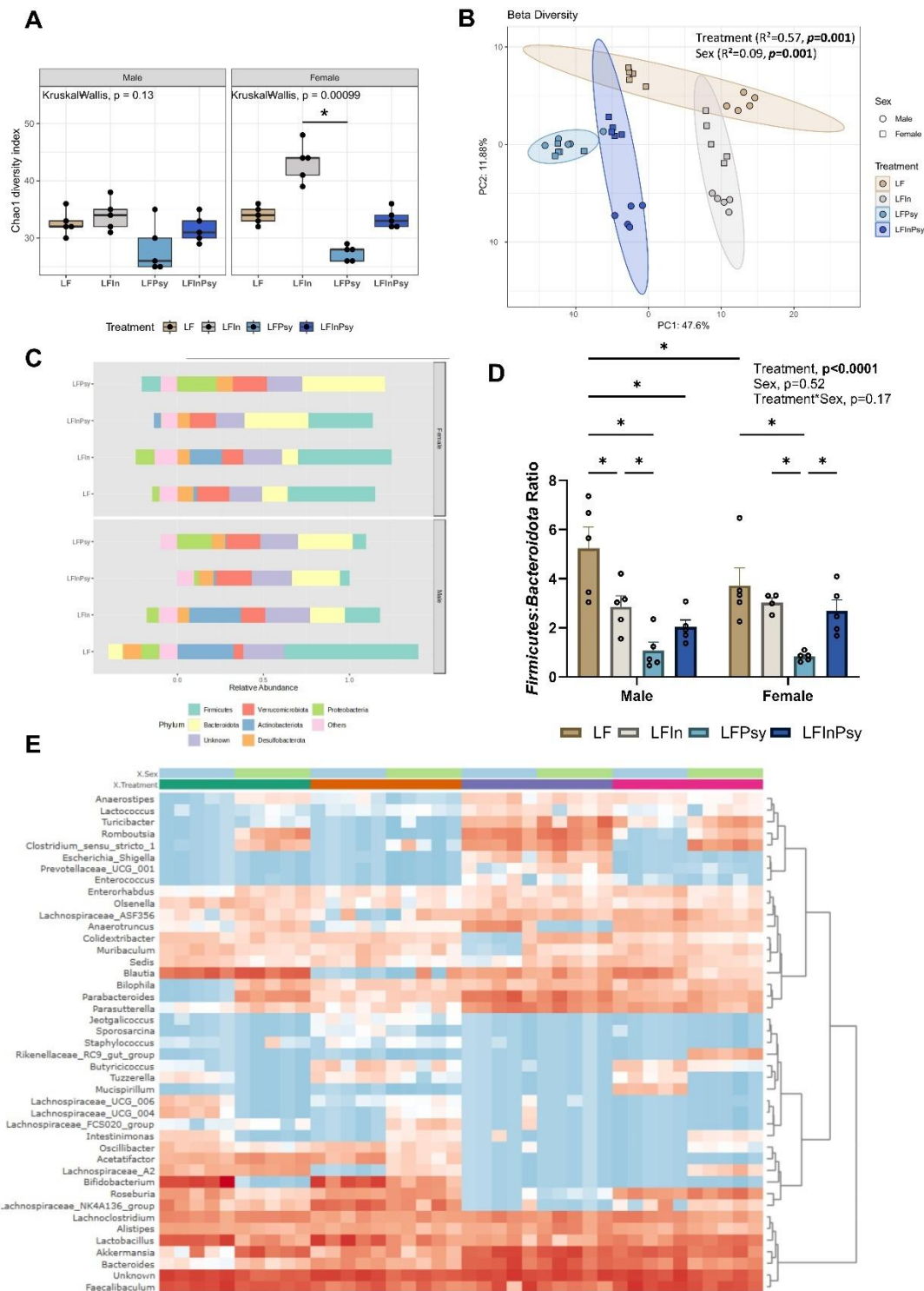
**Figure 3.2 | Sex-specific physiological responses to dietary fibre (DF) supplementation.** DFs significantly reduced body weight gain in (A) males and (B) females, plotted as change from the baseline. (C) Histogram showing area under the curve (AUC) for body weight changes over the 10-week intervention in males and females. (D) Females consumed significantly less food than males across all dietary groups. (E) Intestine length was unaffected by DFs in females but significantly increased in males. (F) Psyllium supplementation increased caecum weight irrespective of sex, while (G) liver weight remained unchanged across all groups.

### 3.3.2 Male and female animals possess distinct gut microbiota changes following dietary fibre supplementation

Alpha diversity, assessed using the Chao1 index (a measure of species richness), was significantly altered by dietary intervention in females ( $p < 0.05$ ; Fig. 3.3A), whereas no such effect was observed in males. Post hoc analysis revealed a ~58% increase in inulin treated females compared to those on the psyllium intervention. In contrast, both Shannon and Simpson diversity indices (measures of species richness and evenness, and species dominance, respectively) were not altered by dietary intervention in either sex ( $p > 0.05$ , Supp Fig S3.1). Beta diversity analysis using Euclidean distance values revealed distinct clustering of the gut microbial communities by DF treatment ( $R^2 = 0.57$ ,  $p = 0.001$ ) and sex ( $R^2 = 0.09$ ,  $p = 0.001$ , Fig 3.3B). Intriguingly this separation by sex was apparent for all DF interventions except for psyllium (Fig 3.3B). The relative abundance of gut bacteria at the phylum level was distinctly altered by DF interventions in both sexes (Fig 3.3C, Supp Table S3.2) with the Firmicutes:Bacteroidota (F:B) ratio significantly influenced by diet ( $p < 0.0001$ , Fig 3.3D). Post hoc analysis highlighted that all DF interventions (inulin, psyllium and combination) significantly reduced F:B ratio in males ( $p < 0.01$ ), whereas for females, this was limited to only psyllium ( $p = 0.001$ , Fig 3.3D).

Widespread dietary, sex and interaction effects were also apparent at the genus level as depicted in the heatmap (Fig 3.3E). In line with the beta diversity analysis, there were significant genus shifts in response to psyllium with no prominent sex differences, including an increase in *Bacteroides* ( $p < 0.01$ ), *Prevotellaceae-UCG-001* ( $p < 0.01$ ), *Parabacteroides* ( $p < 0.01$ ), *Bilophila* ( $p < 0.01$ ), *Turicibacter* ( $p < 0.01$ ), *Enterococcus* ( $p < 0.01$ ), *Lactococcus* ( $p < 0.01$ ), *Clostridium sensu stricto 1* ( $p < 0.01$ ), *Anaerostipes* ( $p < 0.01$ ), *Romboutsia* ( $p < 0.01$ ), *Parasutterella* ( $p < 0.01$ ), *Escherichia/Shigella* ( $p < 0.01$ ) and *Akkermansia* ( $p < 0.01$ ); and a decrease in *Bifidobacterium* ( $p < 0.01$ ), *Muribaculum* ( $p < 0.01$ ), *Faecalibaculum* ( $p < 0.01$ ), *Lactobacillus* ( $p = 0.01$ ), *Staphylococcus* ( $p = 0.03$ ), *Lachnospiraceae-A2* ( $p < 0.01$ ), *Acetatifactor* ( $p < 0.01$ ), *Lachnospiraceae FCS020 group* ( $p = 0.04$ ), *Lachnospiraceae NK4A136* ( $p < 0.01$ ), *Lachnospiraceae UCG-006* ( $p = 0.03$ ), *Roseburia* ( $p < 0.01$ ), *Tuzzerella* ( $p = 0.01$ ) and *Oscillibacter* ( $p < 0.01$ ) (Fig 3.3E, Supp Table S3.3).

Within the DF treatments, inulin showed clear sex-specific differential abundance for *Lachnospiraceae A2*, *Lachnospiraceae ASF356*, *Butyricicoccus*, *Corynebacterium*, *Erysipelatoclostridium*, *Lachnospiraceae FCS020 group*, *Olsenella*, *Sporosarcina*, *Tuzzerella*, and *Ruminococcaceae UBA1819* (Fig 3.3E). A detailed mapping of these genera is presented as boxplots in Supp Figure S3.2.



**Figure 3.3 | Dietary fibre (DF) induced gut microbiota remodelling in males and females.** (A) Inulin significantly increased chao1 alpha diversity index in females compared with psyllium. (B) Beta diversity revealed clear sex-specific clustering, except in psyllium groups, where sex differences were diminished. (C) Phylum-level relative abundance differed across dietary groups. (D) Psyllium reduced the Firmicutes:Bacteroidota ratio in both sexes, whereas inulin reduced this ratio only in males. (E) A detailed heatmap highlights distinct bacterial abundances patterns, demonstrating sex-dependent effects of inulin and largely sex-independent effects of psyllium.

### 3.3.3 Dietary fibres drives sex-specific shifts in caecal metabolomic profiles

Distinct clustering was observed in the gut metabolomics profile of both male and female mice following DF supplementation ( $F=9.68$ ,  $R^2=0.45$ ,  $p=0.001$ , Figure 3.4A), with the psyllium group showing the most distinct separation. Two-way ANOVA revealed that 49 metabolites were significantly altered by DFs treatment ( $p<0.05$ ), 12 metabolites exhibited significant sex differences ( $p<0.05$ ), and 13 were influenced by a significant DF x sex interaction (Fig. 3.4B; a complete list of significant metabolites is provided in Supplementary Table S3.4). Psyllium alone and in combination with inulin showed significantly lower levels of energy metabolites and energy derivatives including glycerol, 3-hydroxybutyrate, 2-oxoglutarate, alpha-ketoisovalerate, hypoxanthine, xanthine, creatine, uracil, and ribose as compared to LF and inulin supplemented groups; along with a higher abundance ( $p<0.05$ ) of amino acids (Fig 3.4C). Another cluster of metabolites predominantly including TCA organic acids such as succinate, fumarate and malate were observed to be highly abundant in psyllium fed group (Fig 3.4C). In contrast, a large metabolite cluster, composed of primary carbon metabolites specifically glucose, mannose, and fructose was enriched in the inulin-psyllium combination group (Fig 3.4C). Two-way ANOVA revealed a significant main effect of sex for glucose ( $p<0.01$ , Fig 3.4D), mannose ( $p<0.01$ , Fig 3.4E), and fructose ( $p<0.01$ , Fig 3.4F). Additionally, fructose exhibited a significant main effect of treatment ( $p<0.001$ , Fig 3.4F).

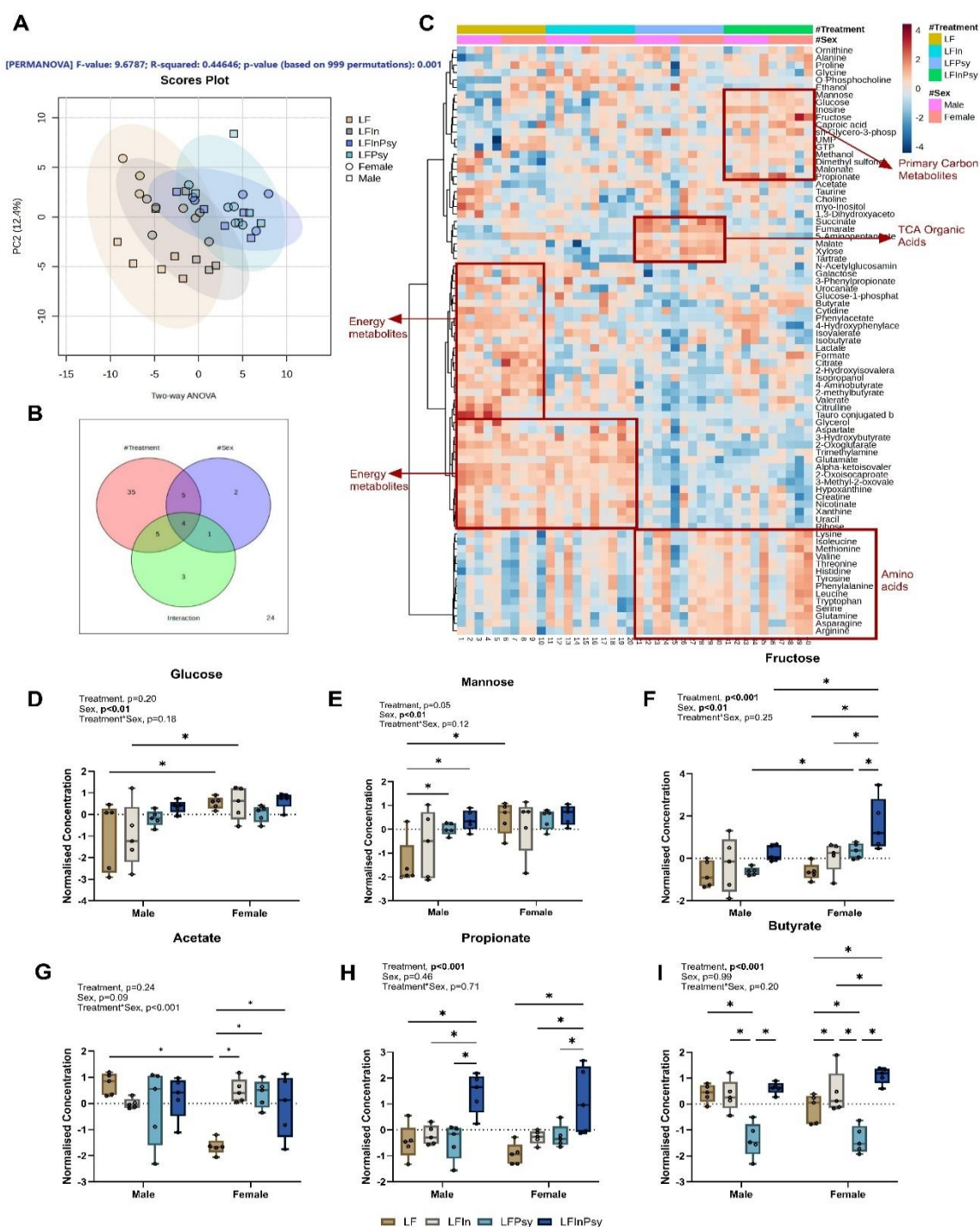
Procrustes analysis revealed a strong congruence between the gut microbiome and metabolome profiles in both males and females (Supp Fig S3.3, S3.4). In males, the strongest congruence existed for the inulin group ( $R=0.83$ ,  $p<0.001$ ), followed by psyllium ( $R=0.79$ ,  $p<0.01$ ) and inulin-psyllium combination ( $R=0.66$ ,  $p=0.03$ ) (Supp Fig S3.3). In females, inulin group showed the strongest congruence ( $R=0.79$ ,  $p<0.01$ ) followed by inulin-psyllium combination ( $R=0.75$ ,  $p<0.01$ ) and the psyllium group ( $R=0.67$ ,  $p=0.02$ ) (Supp Fig S3.4).

SCFAs are the primary microbial metabolites produced during DF fermentation. Although DF supplementation did not elicit a significant main effect of treatment ( $p=0.24$ ) or sex ( $p=0.09$ ) on acetate levels, there was a significant effect of treatment\*sex interaction ( $p<0.001$ , Fig 3.4G). In females, all DF interventions increased acetate concentrations, whereas no changes were observed in males ( $p<0.05$ , Fig 3.4G). Propionate levels were

significantly elevated by the combined inulin-psyllium treatment in both sexes, while neither fibre alone altered propionate concentrations ( $p < 0.05$ , Fig 3.4H).

Butyrate levels exhibited a significant main effect of treatment ( $p < 0.001$ , Fig 3.4I). Post hoc comparisons indicated that psyllium supplementation reduced butyrate levels in both sexes ( $p < 0.05$ , Figure 3.4I). Notably, the inulin-psyllium combination had no significant effect on butyrate levels in males, whereas it significantly increased butyrate concentrations in females ( $p < 0.05$ , Figure 3.4I). This increase in butyrate in females positively correlated with butyrate-producing bacteria in the inulin-psyllium group including *Butyricicoccus* ( $p < 0.01$ ), *Prevotellaceae-UCG-001* ( $p < 0.01$ ), *Intestinimonas* ( $p < 0.05$ ), *Escherichia/Shigella* ( $p < 0.01$ ), *Muribaculum* ( $p < 0.05$ ), *Bacteroides* ( $p < 0.05$ ), *Roseburia* ( $p < 0.01$ ), *Clostridium sensu stricto 1* ( $p < 0.05$ ), *Turicibacter* ( $p < 0.05$ ) and *Lactobacillus* ( $p < 0.01$ ); whilst negatively correlating with *Lachnospiraceae\_FCS020 group* ( $p < 0.05$ ), *Acetatifactor* ( $p < 0.01$ ), *Blautia* ( $p < 0.05$ ), *Colidextribacter* ( $p < 0.01$ ), *Anaerovorax* ( $p < 0.01$ ), *Lachnoclostridium* ( $p < 0.01$ ), *Lachnospiraceae A2* ( $p < 0.01$ ) and *Paludicola* ( $p < 0.01$ ) (Supp Fig S3.7).

Following the pronounced reduction in butyrate observed in psyllium-supplemented groups, we identified significant sex-specific associations between gut butyrate concentrations and microbial genera. In psyllium-fed females, butyrate levels were negatively correlated with *Colidextribacter* ( $p < 0.05$ ), whereas this correlation was positive in males ( $p < 0.05$ , Supp Fig S3.6). In females, butyrate was positively correlated with several taxa, including *Lachnospiraceae-NK4A136 group* ( $p < 0.05$ ), *Blautia* ( $p < 0.05$ ), *Olsenella* ( $p < 0.01$ ), *Oscillibacter* ( $p < 0.05$ ), *Acetatifactor* ( $p < 0.01$ ) and *Lachnospiraceae\_FCS020 group* ( $p < 0.01$ ). In contrast, psyllium-fed males exhibited a broader pattern of correlations across multiple genera, as shown in the correlation heatmap (Supp Fig S3.6).

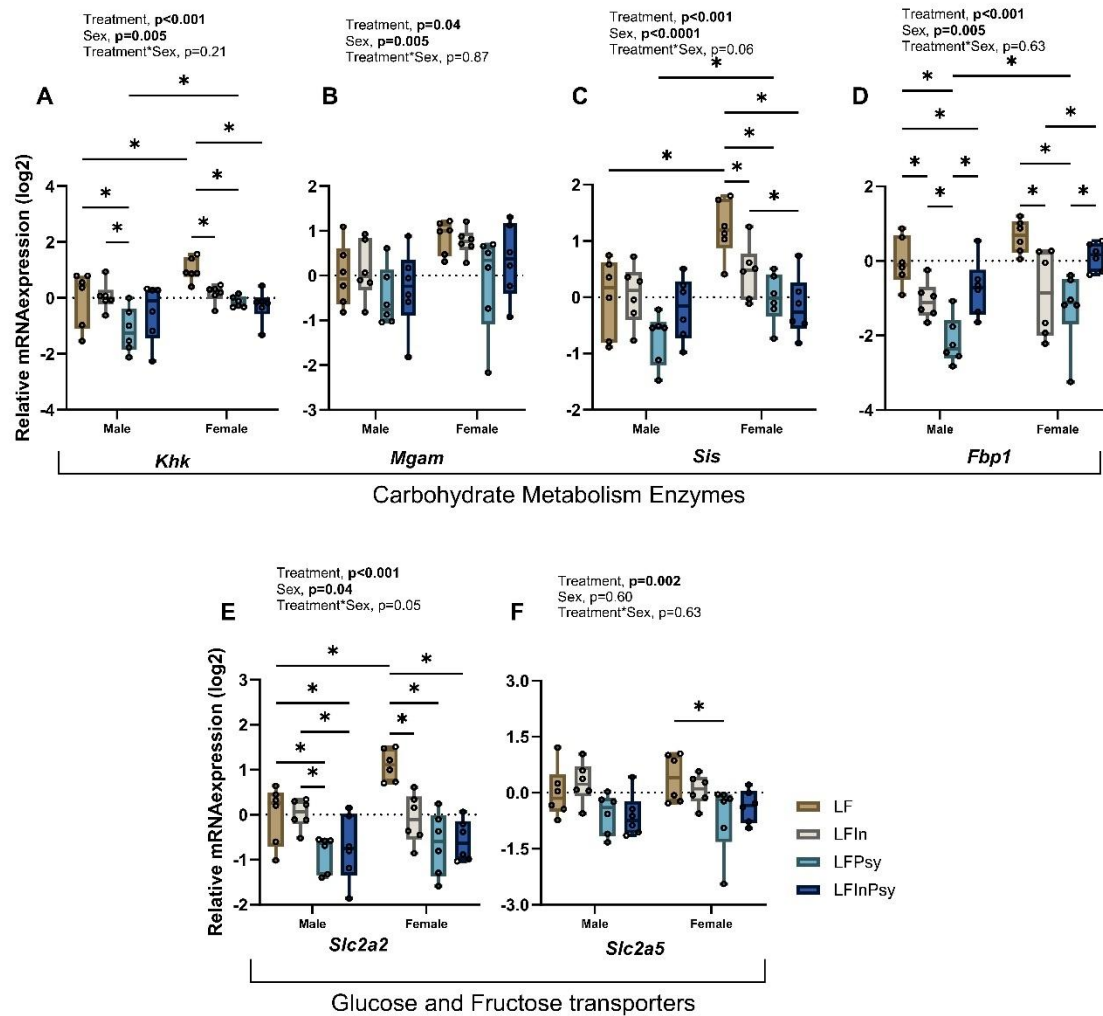


**Figure 3.4 | Dietary fibre (DF) induced alterations in gut metabolomic profiles in both males and females.** (A) PCA plot revealed distinct clustering of the gut metabolome across DF interventions. (B) Two-way ANOVA identified metabolites that differed significantly by treatment, sex and their interaction. (C) A detailed heatmap of significantly altered metabolites demonstrated group-wise clustering. (D) Glucose, (E) mannose and (F) fructose levels were altered in the gut. (G) Acetate levels increased significantly in females following DF supplementation, (H) propionate levels increased following combined inulin and psyllium supplementation, and (I) butyrate levels decreased significantly following psyllium supplementation in both sexes.

### 3.3.4 Intestinal gene expression reveals differential regulation of carbohydrate-metabolism enzymes and transporters

Following the differential abundance of glucose, mannose and fructose in the gut, we quantified the duodenal expression of genes involved in carbohydrate metabolism and uptake. We assessed how enzymes that process carbohydrates and sugars respond to different DFs in males and females. Two-way ANOVA revealed clear treatment and sex effects, indicating that both factors shape intestinal carbohydrate metabolism (Fig 3.5A-D). Psyllium was observed to be a strong influencer; it lowered the expression of *Ketohexokinase (Khk)*, (a fructose metabolising enzyme) in both males ( $p=0.01$ ) and females ( $p=0.01$ , Fig 3.5A). Psyllium also reduced starch degradation enzyme (*Mgam*) expression in both sexes, though not enough for the change to reach significance ( $P>0.05$ , Fig 3.5B). The enzyme *Sis*, responsible for breaking down sucrose and maltose, showed a striking pattern, females responded to all DFs with significantly reduced expression ( $p<0.01$ ), while the expression in males remained largely unchanged ( $p>0.05$ , Fig 3.5C). Finally, key gluconeogenesis enzyme (*Fbp1*) expression dropped sharply in both sexes when animals were given inulin ( $p<0.05$ ) or psyllium ( $p<0.05$ ). Interestingly, the combined effect of the two fibres was only significant in males ( $p=0.04$ , Fig 3.5D), highlighting a sex-specific metabolic response.

Additionally, DF supplementation showed significant main effects of treatment ( $p<0.001$ ) and sex ( $p=0.04$ ) on the expression of glucose transporter *Slc2a2*, with reductions observed in both males ( $p=0.01$ ) and females ( $p<0.0001$ ) following psyllium and inulin-psyllium treatments (Fig 3.5E). In contrast, inulin alone reduced *Slc2a2* expression only in females ( $p=0.0005$ , Figure 3.5E). Despite substantial differences in gut fructose concentrations (Fig 3.4F), the expression of the fructose transporter *Slc2a5* remained largely unchanged. The only significant effect was a reduction in *Slc2a5* expression in females following psyllium supplementation ( $p=0.04$ , Figure 3.5F). The expression levels of these enzymes and transporters did not exhibit any significant correlations with the corresponding gut sugar concentrations.



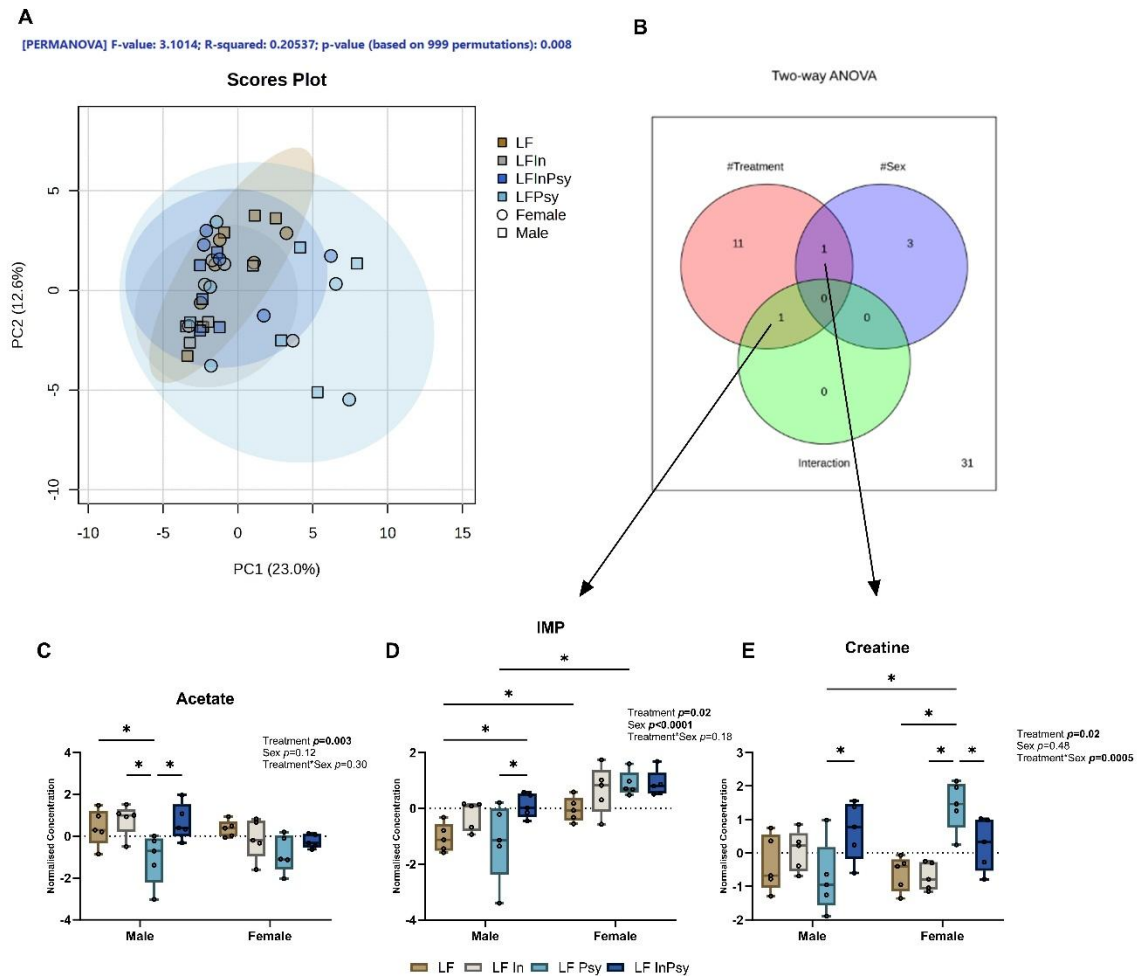
**Figure 3.5 | Dietary fibre (DF) dependent modulation of carbohydrate metabolism enzymes and associated transporters in males and females.** DF supplementation reduced the expression of carbohydrate metabolism enzymes including (A) Ketohexokinase (*Khk*), (B) maltase glucoamylase (*Mgam*), (C) sucrase isomaltase (*Sis*), and (D) fructose-1,6-bisphosphatase (*Fbp1*). (F) Glucose transporter *Slc2a2* and (G) fructose transporter *Slc2a5* expression also differed significantly in response to DF supplementation.

### 3.3.5 Dietary fibres (DFs) influence the cortical metabolomic profile

After 10 weeks of dietary intervention, behavioural tests of cognition were performed in 20-weeks old mice. There was a near significant effect of sex on time spent in the centre of the open field test as a measure of anxiety ( $p=0.07$ , Supp Fig S3.8A), but no significant effect of treatment ( $p=0.78$ , Supp Fig S3.8A), or treatment and sex interaction ( $p=0.80$ , Supp Fig S3.8A). In Y-Maze performance (Supp Fig S3.8B) and the novel object recognition (Supp Fig S3.8C), no significant effect of treatment ( $p>0.05$ ), sex ( $p>0.05$ ) or their interaction ( $p>0.05$ ) was observed.

Despite no difference in behaviour, metabolomic analysis of the cortex revealed distinct metabolic profiles according to DF intervention, as evidenced by clear clustering (PERMANOVA:  $F=3.10$ ,  $R^2=0.21$ ,  $p=0.008$ ; Fig. 3.6A). Subsequent two-way ANOVA identified 13 cortical metabolites that were significantly modulated by DF supplementation; including acetate ( $q=0.09$ ), choline ( $q=0.09$ ), creatine ( $q=0.09$ ), IMP ( $p=0.09$ ), isoleucine ( $q=0.09$ ), lactate ( $q=0.09$ ), N-acetylaspartate ( $q=0.09$ ), pantothenate ( $q=0.09$ ), succinate ( $q=0.09$ ), UDP-glucose ( $q=0.09$ ), uridine ( $q=0.09$ ), valine ( $q=0.09$ ) and citrate ( $q=0.09$ ). Four metabolites exhibited significant sex effects: including guanosine ( $q=0.09$ ), IMP ( $q<0.01$ ), inosine ( $q=0.09$ ) and serine ( $q=0.01$ ). Creatine showed a significant treatment\*sex interaction effect ( $p<0.01$ ), along with a significant treatment effect ( $p=0.02$ , Figure 3.6E). Detailed concentrations in each group and statistical analyses results are presented in Table 3.1 and plotted as heatmap (Supp Fig S3.9). Psyllium showed a distinct cortex metabolomic profile (Fig 3.6A), significantly dropping acetate levels in male cortex ( $p<0.05$ , Fig 3.6C), and significantly increasing creatine levels in female cortex ( $p<0.05$ , Fig 3.6E).

Procrustes analysis revealed stronger congruence between the gut microbiome and cortex metabolome profiles in males as compared to females, however the associations were not significant ( $p>0.05$ ). In males, R coefficients ranged from 0.36 to 0.49 between groups ( $p>0.05$ , Supp Fig S3.10), whereas, in females, R coefficients ranged from 0.07 to 0.29 ( $p>0.05$ , Supp Fig S3.11).



**Figure 3.6 | Dietary fibre (DF) induced alterations in cortical metabolomic profiles in males and females.** (A) PCA plot revealed distinct clustering of the cortical metabolome across DF interventions. (B) Two-way ANOVA identified metabolites that differed significantly by treatment, sex and their interaction. Two-way ANOVA analysis of select significant metabolites including (C) acetate, (D) IMP and (E) creatine.

**Table 3.1** | ANOVA results for Cortex Metabolomics Profile

| Treatment                | LF         | LFIn       | LFPsy     | LFInPsy    | Treatment<br>(adj.p) | Sex<br>(adj.p) | Interaction<br>(adj.p) |
|--------------------------|------------|------------|-----------|------------|----------------------|----------------|------------------------|
| <b>Acetate</b>           |            |            |           |            | <b>0.09</b>          | 0.47           | 0.69                   |
| Male                     | 0.54±0.33  | 0.69±0.27  | 0.21±0.25 | 0.84±0.16  |                      |                |                        |
| Female                   | 0.51±0.28  | 0.58±0.30  | 0.33±0.24 | 0.43±0.24  |                      |                |                        |
| <b>Choline</b>           |            |            |           |            | <b>0.09</b>          | 0.92           | 0.69                   |
| Male                     | 0.27±0.18  | 0.41±0.15  | 0.13±0.12 | 0.68±0.13  |                      |                |                        |
| Female                   | 0.24±0.10  | 0.45±0.21  | 0.34±0.25 | 0.35±0.22  |                      |                |                        |
| <b>Creatine</b>          |            |            |           |            | <b>0.09</b>          | 0.83           | <b>0.02</b>            |
| Male                     | 6.05±3.23  | 6.76±2.32  | 2.73±2.66 | 9.42±0.44  |                      |                |                        |
| Female                   | 5.76±3.05  | 6.70±2.96  | 5.40±3.52 | 6.28±3.75  |                      |                |                        |
| <b>Guanosine</b>         |            |            |           |            | 0.15                 | <b>0.09</b>    | 0.24                   |
| Male                     | 0.05±0.03  | 0.05±0.02  | 0.01±0.02 | 0.06±0.03  |                      |                |                        |
| Female                   | 0.01±0.02  | 0.05±0.03  | 0.04±0.03 | 0.00±0.01  |                      |                |                        |
| <b>IMP</b>               |            |            |           |            | <b>0.09</b>          | <b>0.00</b>    | 0.69                   |
| Male                     | 0.46±0.29  | 0.69±0.28  | 0.20±0.18 | 1.06±0.24  |                      |                |                        |
| Female                   | 0.61±0.27  | 0.98±0.42  | 0.77±0.48 | 0.93±0.50  |                      |                |                        |
| <b>Inosine</b>           |            |            |           |            | 0.51                 | <b>0.09</b>    | 0.69                   |
| Male                     | 0.30±0.21  | 0.40±0.09  | 0.20±0.16 | 0.46±0.12  |                      |                |                        |
| Female                   | 0.26±0.15  | 0.36±0.20  | 0.11±0.05 | 0.22±0.15  |                      |                |                        |
| <b>Isoleucine</b>        |            |            |           |            | <b>0.09</b>          | 0.93           | 0.76                   |
| Male                     | 0.04±0.02  | 0.04±0.02  | 0.02±0.02 | 0.05±0.01  |                      |                |                        |
| Female                   | 0.04±0.02  | 0.04±0.02  | 0.03±0.02 | 0.03±0.02  |                      |                |                        |
| <b>Lactate</b>           |            |            |           |            | <b>0.09</b>          | 0.54           | 0.69                   |
| Male                     | 10.00±5.26 | 8.33±2.99  | 4.73±4.90 | 13.12±1.25 |                      |                |                        |
| Female                   | 10.08±5.29 | 10.17±4.88 | 6.58±4.79 | 8.61±5.26  |                      |                |                        |
| <b>N-Acetylaspartate</b> |            |            |           |            | <b>0.09</b>          | 0.93           | 0.76                   |
| Male                     | 4.84±2.46  | 5.63±1.96  | 2.37±2.46 | 7.13±0.96  |                      |                |                        |
| Female                   | 4.94±2.50  | 5.67±2.51  | 4.09±2.84 | 5.29±3.19  |                      |                |                        |
| <b>Pantothenate</b>      |            |            |           |            | <b>0.09</b>          | 0.27           | 0.76                   |
| Male                     | 0.01±0.01  | 0.02±0.01  | 0.00±0.01 | 0.02±0.01  |                      |                |                        |
| Female                   | 0.02±0.02  | 0.03±0.02  | 0.02±0.02 | 0.02±0.01  |                      |                |                        |
| <b>Serine</b>            |            |            |           |            | 0.12                 | <b>0.01</b>    | 1.00                   |
| Male                     | 0.60±0.33  | 0.66±0.29  | 0.29±0.30 | 1.06±0.12  |                      |                |                        |
| Female                   | 0.69±0.38  | 0.87±0.38  | 0.59±0.39 | 0.88±0.54  |                      |                |                        |
| <b>Succinate</b>         |            |            |           |            | <b>0.09</b>          | 0.48           | 0.80                   |
| Male                     | 0.08±0.03  | 0.07±0.04  | 0.04±0.04 | 0.17±0.04  |                      |                |                        |
| Female                   | 0.08±0.04  | 0.10±0.05  | 0.09±0.07 | 0.12±0.08  |                      |                |                        |
| <b>UDP-glucose</b>       |            |            |           |            | <b>0.09</b>          | 0.94           | 0.69                   |
| Male                     | 0.11±0.07  | 0.17±0.06  | 0.02±0.04 | 0.15±0.04  |                      |                |                        |
| Female                   | 0.10±0.08  | 0.13±0.05  | 0.09±0.07 | 0.12±0.10  |                      |                |                        |
| <b>Uridine</b>           |            |            |           |            | <b>0.09</b>          | 0.83           | 0.75                   |
| Male                     | 0.06±0.04  | 0.10±0.03  | 0.03±0.06 | 0.12±0.02  |                      |                |                        |
| Female                   | 0.06±0.04  | 0.10±0.04  | 0.06±0.05 | 0.08±0.05  |                      |                |                        |
| <b>Valine</b>            |            |            |           |            | <b>0.09</b>          | 0.93           | 0.76                   |
| Male                     | 0.09±0.06  | 0.10±0.03  | 0.04±0.04 | 0.12±0.01  |                      |                |                        |
| Female                   | 0.07±0.04  | 0.09±0.04  | 0.07±0.04 | 0.08±0.04  |                      |                |                        |

|                |           |           |           |           |             |      |      |
|----------------|-----------|-----------|-----------|-----------|-------------|------|------|
| <b>Citrate</b> |           |           |           |           | <b>0.09</b> | 0.83 | 0.69 |
| <i>Male</i>    | 0.24±0.15 | 0.38±0.14 | 0.13±0.12 | 0.40±0.04 |             |      |      |
| <i>Female</i>  | 0.26±0.13 | 0.34±0.13 | 0.25±0.16 | 0.22±0.11 |             |      |      |

Data are presented as mean±SD

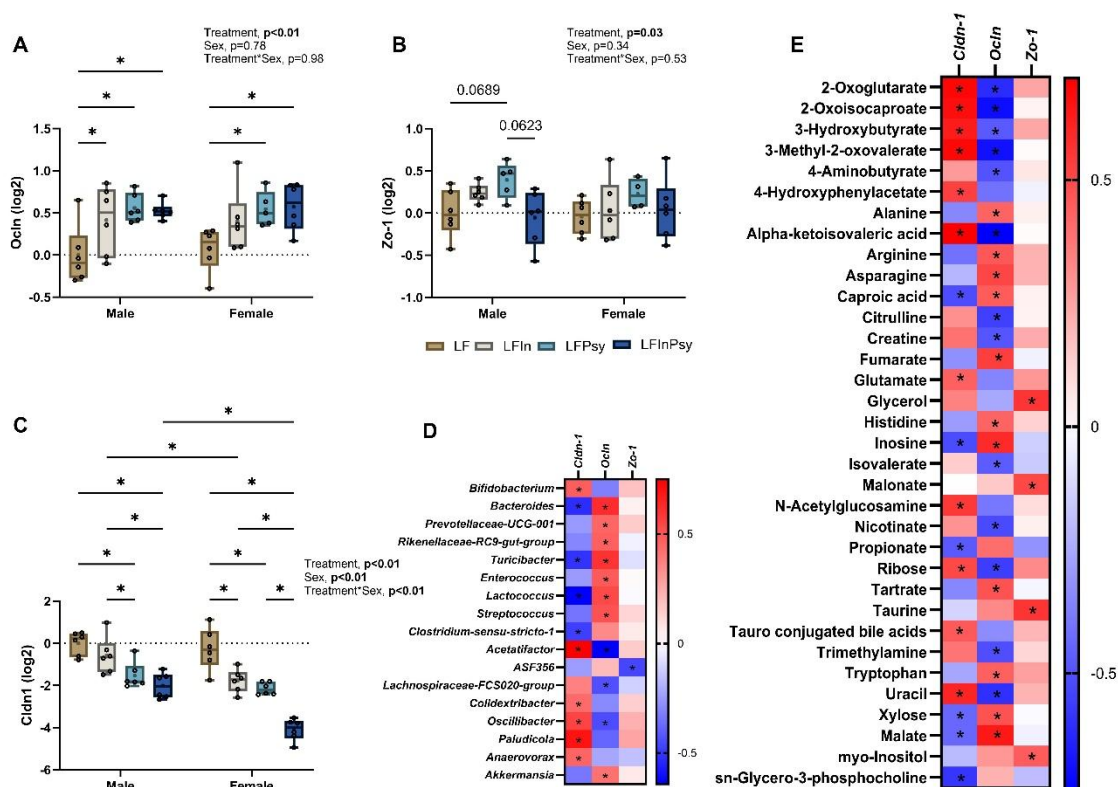
### 3.3.6 Dietary Fibres (DFs) influences sex-specific tight junctions gene expression in the brain cortex

Gut microbiota and their metabolites can alter BBB permeability. Following significant changes in gut microbiota and their metabolites, we measured tight junction gene expression in the brain cortex to determine whether microbiota-driven gut metabolic shifts translate into structural changes at the BBB, that could explain the observed brain metabolomic differences.

Two-way ANOVA analysis revealed a significant DF treatment effect on the expression of tight junction genes in the cortex including occludin (*Ocln*) ( $p < 0.01$ , Fig 3.7A), zonulin-1 (*Zo-1*) ( $p = 0.03$ , Fig 3.7B) and claudin-1 (*Cldn-1*) ( $p < 0.01$ , Fig 3.7C). Sex effect ( $p < 0.01$ ) and treatment \* sex interaction effect ( $p < 0.01$ ) was only significant for *Cldn1* expression (Fig 3.7C). Psyllium and inulin-psyllium combination increased *Ocln* expression in both males and females, whereas inulin only increased *Ocln* expression in males ( $p < 0.05$ , Figure 3.7A). Psyllium also increased the expression of *Zo-1* in males, but it didn't reach statistical significance ( $p = 0.07$ , Fig 3.7B).

On the other hand, *Cldn1* expression significantly declined with DF intervention in both sexes (Fig 3.7C), with the strongest effect observed in inulin-psyllium combination groups ( $p < 0.05$ , Fig 3.7C). This DF-linked reduced *Cldn1* expression suggests a better BBB integrity at the tight junctions (Sladojevic et al., 2019). *Cldn1* expression was negatively correlated with *Bacteroides* ( $R = -0.53$ ,  $p = 0.00$ ), *Turicibacter* ( $-0.51$ ,  $p = 0.01$ ), *Lactococcus* ( $R = -0.64$ ,  $p = 0.00$ ), and positively correlated with *Acetatifactor* ( $R = 0.75$ ,  $p = 0.00$ ), and *Oscillibacter* ( $R = 0.55$ ,  $p = 0.00$ ). On the contrary, *Ocln* expression was positively correlated with *Bacteroides* ( $R = 0.62$ ,  $p = 0.00$ ), *Turicibacter* ( $R = 0.60$ ,  $p = 0.00$ ), *Lactococcus* ( $R = 0.53$ ,  $p = 0.00$ ), and negatively correlated with *Acetatifactor* ( $R = -0.64$ ,  $p = 0.00$ ) and *Oscillibacter* ( $R = -0.45$ ,  $p = 0.03$ ). Detailed correlations are plotted in the heatmap (Fig 3.7D), with correlation coefficients reported in the Supp Table S3.5.

These tight junction gene expressions strongly correlated with gut metabolites (Figure 3.7E), with detailed correlations coefficients reported in Supp Table S3.6. Gut levels of propionate were negatively correlated with the *Cldn1* expression ( $R = -0.48$ ,  $p = 0.02$ , Fig 3.7E). No correlations were observed among the metabolites measured in the cortex and the tight junction genes.



**Figure 3.7 | Dietary fibre (DF) induced modulation of tight junction gene expression in the cerebral cortex of males and females.** (A) Psyllium supplementation increased occludin (*Occludin*) expression in both sexes, whereas inulin increased *Occludin* expression only in males. (B) Zonulin-1 (*Zo-1*) expression showed diet related trends but did not reach statistical significance. (C) Claudin-1 (*Cldn-1*) expression decreased significantly following DF supplementation in both sexes, psyllium and combination effects were consistent across sexes, while inulin reduced *Cldn-1* expression only in females. Cortical tight junction gene expression was strongly correlated with (D) gut microbiota composition and (E) gut metabolites.

### 3.4 Discussion

This study demonstrates that DFs differentially influence host physiology, gut microbial and metabolomic profiles, and ultimately the GMB axis. We provide evidence to suggest that the impact of DFs, particularly that elicited by inulin was modulated by sex. Intriguingly, the impact of psyllium was largely sex independent, with gut microbial and metabolic profiles shifting in a consistent manner across both sexes. These divergent microbial and metabolic responses were accompanied by corresponding alterations in brain tight junction gene expression, reflecting both microbiota-dependent and microbiota-independent influences of DFs on the gut–brain axis. Collectively, this work highlights that DFs are not equivalent and that this is particularly evident when considering their sex-specific impacts.

Females exhibited significantly longer small intestines than males, consistent with previous reports suggesting enhanced nutrient absorption to meet the increased energy demands associated with pregnancy and lactation. DF deficiency is known to markedly reduce the intestinal size over both short and long-term periods (Hunt et al., 2021). Notably, DF supplementation restored intestinal length in males. In contrast, females exhibited no change, suggesting that their inherently longer intestinal length (McKenney et al., 2023), which is also discussed in Chapter 2, is not dependent on DF status. Longer intestines are predictive of enhanced nutrient absorption. Despite consuming significantly more food than females across all DF groups (Figure 3.2D), males receiving inulin showed reduced weight gain (Figure 3.2E), which may reflect increased energy costs associated with the transit of bulkier fibre masses through the GIT, although quantitative evidence for this remains limited (Zochling et al., 2025). Inulin has been shown to reduce body weight and BMI in multiple RCTs and meta-analyses (Reimer et al., 2024), although these studies did not assess sex-specific effects. Its impact on weight is likely mediated through modulation of the gut microbiota and increased SCFA production (Alonso-Allende et al., 2024), and evidence indicates that inulin induces sex-specific shifts in gut microbial composition (S. Kadyan et al., 2023).

An enlarged caecum accompanies the consumption of DFs (Drew et al., 2018), and is the major intestinal site for microbial fermentation (Brown et al., 2018). Addition of psyllium

(an effective bulking agent) to the diet, forms gel-like masses in the GI tract, encapsulating nutrients and preventing absorption while increasing caecum size (Zochling et al., 2025). Whereas inulin possesses minimal water-holding capacity as compared to psyllium (Geng et al., 2024). This bulking effect of psyllium is physicochemical rather than hormonal or microbiota-driven, which explains sex-independent increase in caecum weight. The microbiota-dependent effects of psyllium observed in beta diversity clustering and in phylum and genus level abundance differences (Figure 3.3B-E) were also independent of sex, which may be explained by the minimal fermentability of psyllium, and the fact that psyllium effects are often functional rather than compositional such as affecting the colonic pH or mucosal hydration which leads to changes which are uniform across both sexes (Zochling et al., 2025). The F:B ratio which is frequently cited as a hallmark of obesity (Houtman et al., 2022) was higher in males compared to females, consistent with other studies (Ali et al., 2024). Psyllium supplementation significantly reduced the F:B ratio in both sexes, whereas inulin-induced reductions occurred only in males (Figure 3.3D). Psyllium reduced several butyrate-producing genera in both sexes, including *Butyricicoccus*, *Lachnospiraceae UCG-004*, *Lachnospiraceae UCG-006*, *Lachnospiraceae FCS020 group*, *Intestinimonas*, *Lachnospiraceae A2*, *Roseburia* and *Lachnospiraceae NK4A136 group* (Figure 3.3E), consistent with the significantly lower butyrate concentrations measured in the gut (Figure 3.4I). However, inulin-psyllium combination differentially affected key butyrate-producers, increasing the abundance of *Butyricicoccus* in males and *Intestinimonas* in females. This represents lower fermentability of psyllium in both males and females.

DFs, especially psyllium, reprogrammed the gut microbiota's metabolic signature, dampening sex-specific differences. In the psyllium groups, NMR-based metabolomics analysis of the caecal content confirmed higher concentrations of amino acids in the gut (Figure 3.4C). Psyllium increases viscosity of the intestinal content (Gibb et al., 2023), and slows transit time, which can lead to reduced microbial access to amino acids, thus less efficient utilisation and higher concentrations in the gut. Viscous fibres are known to slow gastric emptying and macronutrient absorption from the gut (La Torre et al., 2021). Increased psyllium-induced water retention can also cause the dilution effect and less protein fermentation. Correspondingly, psyllium and combination treatments reduced

energy derivatives (Figure 3.5C), likely due to altered transit time and slower energy metabolism from bulking effects. Sex specific differences also emerged in luminal glucose, mannose, and fructose (Fig 3.4D-F), likely reflecting slower carbohydrate digestion and absorption following DF supplementation.

These gut microbiota and gut metabolomic changes appear to extend to the brain and relate to BBB integrity. Gut-derived SCFAs can cross the BBB, with butyrate showing the highest permeability, followed by propionate and acetate (Choe, 2025). We detected only acetate in the brain tissue, and its levels were lowest in the psyllium-fed group in both sexes, although statistically significant only in males ( $p < 0.05$ ). Acetate in the brain is known to support astrocytic glucose uptake and glycolysis, contributing to its neuroprotective potential (Peng et al., 2025). Despite lower brain acetate, psyllium supplementation was associated with improved BBB integrity, reflected by increased *Ocln* and decreased *Cldn1* expression in both sexes. This suggests that psyllium's protective effects on the BBB may occur independently of direct SCFA transport, potentially via upstream gut microbiota-driven mechanisms. Gut propionate levels significantly increased in response to DF combination and showed a negative correlation with *Cldn1* expression in the brain, consistent with enhanced BBB integrity (Sladojevic et al., 2019).

A fibre-deprived diet has been linked to cognitive impairment, which is distinct from normal brain ageing (Shi et al., 2021), while several human and preclinical studies report beneficial cognitive effects of DF intake (Berding, Carbia, et al., 2021; He et al., 2025; Sun et al., 2022; Yang et al., 2024). However, the mechanistic basis remains poorly defined and likely varies with fibre fermentability. Notably, SCFA interventions (both in vitro and in vivo) have been shown to upregulate tight junction proteins and enhance BBB integrity in germ-free or disease models (Fock & Parnova, 2023), offering a plausible mechanistic link with DF-induced microbial fermentation.

Together, these findings demonstrate that DFs reshape the gut microbiota and metabolite profiles in a sex-dependent manner and extend their influence to the brain. Psyllium intervention, despite reducing the SCFA levels, improved the BBB tight junction integrity in both males and females. In contrast, inulin exerted sex-dependent effects, suggesting

that the physiological impact of individual fibre types on the gut-brain axis may differ markedly between sexes.

## **Chapter 4: Impact of a high-fibre Mediterranean vs. a low-fibre Western diet on the Gut-Microbiota-Brain axis in adults with mild to moderate depression or anxiety**

Thesis Marrium Liaquat

## 4.1 Introduction

Mental disorders account for a large burden of disability worldwide. Recent estimates suggest that approximately 418 million disability-adjusted life years (DALYs) are attributable to mental disorders, representing around 16% of global DALYs (Arias et al., 2022). According to the International Classification of Diseases, 11<sup>th</sup> Revision (ICD-11), mental disorders encompass conditions such as anxiety disorders, depression, bipolar disorder, post-traumatic stress disorder, schizophrenia, eating disorders, disruptive behaviour and dissocial disorders, and neurodevelopmental disorders (WHO, 2022). Among these, depression and anxiety disorders are the most prevalent (Liubertiene et al., 2025).

Lifestyle factors are well established determinants of mental health, with diet and nutrition representing key modifiable factors capable of preventing mental ill health. With a growing shift towards preventative healthcare, the field of “Nutritional Psychiatry” has emerged (Adan et al., 2019; Sarris et al., 2015), highlighting the interrelationship between diet and brain health, particularly in mood-related disorders such as anxiety and depression (Sanz et al., 2025; Xiong et al., 2023). A meta-analysis of 16 independent RCTs across 45,826 participants, found that dietary interventions (aimed at decreasing unhealthy food intake and improving nutrient intake) had a modest positive effect on depressive symptoms ( $g=0.275$ , 95% CI=0.10 to 0.45,  $p=0.002$ ) (Firth et al., 2019). Strong evidence from longitudinal studies linked the Mediterranean diet to reduced depression risk, showing a 33% lower incidence of depression in the highest vs lowest Mediterranean diet adherence groups (RR=0.67, 95% CI 0.55-0.82) (Lassale et al., 2019). Another cross-sectional analysis of population scale observational studies showed associations between higher Mediterranean diet scores and lower odds of depressive symptoms, with an adjusted OR of 0.87 (95% CI 0.84-0.91) (Nicolaou et al., 2020).

A Mediterranean diet characterised by high intake of fruits, vegetables, whole grains, legumes, healthy fats, and limited consumption of confectionary, and red and processed meat is consistently associated with better cognitive function and lower risk of neurodegenerative diseases (Mafe & Busselberg, 2025). The majority of this evidence comes from longitudinal or cross-sectional studies in the general population, and there

is limited evidence in people suffering from depression (Gianfredi et al., 2023), and a major need of gold-standard evidence from RCTs (Esgunoglu et al., 2021).

A central mechanism linking diet to brain is the gut microbiota, which plays a key role in systemic and neurological health (Berding, Vlckova, et al., 2021; Cryan et al., 2019; Schneider, O'Riordan, et al., 2024). Dietary changes can alter the composition of the gut microbiota within days (Bourdeau-Julien et al., 2023; David et al., 2014). Dietary compositions and patterns demonstrate a robust potential to modulate the GMB axis (Schneider, Balasubramanian, et al., 2024), and its effect on mood, cognitive function and the progression of neurodegenerative disorders is well reported (Schneider, O'Riordan, et al., 2024). Among various dietary patterns researched, the Mediterranean-style dietary patterns (MDP) and the Western style diets (WD) have been the most extensively studied for their distinct and contrasting effects on the gut microbial communities (Mafe & Busselberg, 2025; Schneider, O'Riordan, et al., 2024).

The Western diet is characterised by foods high in simple/refined carbohydrates (sugars), saturated fats and salt, and low in dietary fibres (DFs), complex carbohydrates and micronutrients (Kendig et al., 2021). Relative to a more healthy diet, this dietary pattern is associated with a reduced overall microbial diversity and alterations in the gut microbiota composition, including an increased F:B ratio, elevated abundance of Proteobacteria, and decreased levels of Bacteroidetes and Actinobacteria (Clerici et al., 2025). These microbial shifts contribute to gut dysbiosis, which in turn is associated with compromised intestinal epithelial barrier, reduced neuroplasticity and neurotransmitter synthesis, and heightened systemic and neuroinflammatory responses (Martinez Leo & Segura Campos, 2020). Conversely, a Mediterranean dietary pattern promotes a more balanced and diverse gut microbiota composition (Garcia-Mantrana et al., 2018; Merra et al., 2020). High-level MDP adherence characterised by high plant-based foods is associated with higher levels of fibre-degrading bacterial genera such as *Lachnospira* and *Prevotella* (De Filippis et al., 2016).

Whilst several foods and dietary components are known to modulate the GMB axis, DFs and their capacity to act as substrates for microbial digestion have been identified as a key modulator of gut microbiota and its subsequent impact on brain signalling (Berding, Carbia, et al., 2021; Berding, Vlckova, et al., 2021; Schneider, Balasubramanian, et al.,

2024). It has been proposed that ~30% of the DF from grain products, and 75-90% of DF from fruits and vegetables is accessible for microbial fermentation (Schneider, Balasubramanian, et al., 2024).

DF interventions have been shown to reduce anxiety (Azpiroz et al., 2017; Hall et al., 2024; P. P. Jackson et al., 2023; Johnstone et al., 2021; Silk et al., 2009) and depression (Farhangi et al., 2018; Hall et al., 2024; P. P. Jackson et al., 2023) in humans and improve cognitive function (Abe et al., 2024; Berding, Long-Smith, et al., 2021), likely through beneficial shifts in the gut microbiota composition and their metabolites produced including SCFAs. Much is known about how individual DFs influence the GMB communication (as summarised in chapter 1, section 1.6), but their role within broader dietary patterns such as MDP and WD remain largely unexplored and was investigated as part of the MediMood RCT.

The MediMood study was designed to address key gaps in the literature, as limited evidence exists regarding (i) the short-term effects of MDP and WD on anxiety and depression, (ii) the contribution of DFs within the whole dietary patterns to mental health and cognitive outcomes, and (iii) gut microbiota alterations associated with short-term MDP and WD interventions, and their link with mental health and cognition. We conducted this cross-over RCT, in which we investigated the impact of a high-fibre Mediterranean vs a low-fibre Western diet on GMB axis in adults with mild to moderate depression and/or anxiety, over a short period of 5 days.

## 4.2 Design and Methodology

### 4.2.1 Overview

MediMood is a single-centre cross-over RCT conducted at the Norwich Medical School, UEA and the NHS Clinical Research Facility (CRF) intervention centre, based at the Quadram Institute (QI), in Norwich, UK. The trial was registered on ClinicalTrials.gov (NCT05927376). The study locations included the CRF and UEA Wolfson and Wellcome Brain Imaging Centre (UWWBIC).

### 4.2.2 Ethical conduct of the study

The conduct, evaluation, and documentation of the study abide by Good Clinical Practice guidelines and the guiding principles of the Declaration of Helsinki. The study was approved by NHS London Queen Square Research Ethics Committee (Study ID 22/LO/0796). Informed consent was received from all participants by trained research staff.

#### 4.2.3 Recruitment and Screening

An online study platform (<https://app.mantal.co.uk/medimood>) was designed to screen participants, after an initial telephone screening. Participants were recruited from Norfolk, UK using advertisement posters/leaflets, invitation emails and social media. The participants were deemed to be eligible if they; i) had mild to moderate depression, scoring 5-14/27 on the Patient Health Questionnaire (PHQ-9) and/or anxiety, scoring 5-14/21 on the Generalised Anxiety Disorder (GAD-7) questionnaire, ii) were not already following a MDP (score of <8/14 on MEDAS scale), and iii) were able to undertake an MRI scan. Both PHQ-9 and GAD-7 are used in research and primary health care services, and are interpreted depending on the total score, which is categorised as follows; 0-4 none, 5-9 mild, 10-14 moderate, 15-19 moderately severe, 20-27 severe for PHQ-9, and similarly the scoring system of GAD-7 is 0-4 no to low risk, 5-9 mild, 10-14 moderate, 15+ severe. Participants with mild to moderate level of anxiety and/or depression were eligible to participate in the study.

Participants were ineligible if they had any dietary restrictions (e.g. vegan or vegetarian), allergies or intolerances to the study food provided. If they reported antibiotics use, their participation was postponed until 1 month after treatment to allow their gut microbiota composition to return to its habitual state. Participants were requested to keep their supplementation use and physical activity levels stable during their participation. Full inclusion and exclusion criteria are published in the protocol paper (Esgunoglu et al., 2024), and added as a supplementary table to this thesis (Supp Table S4.1).

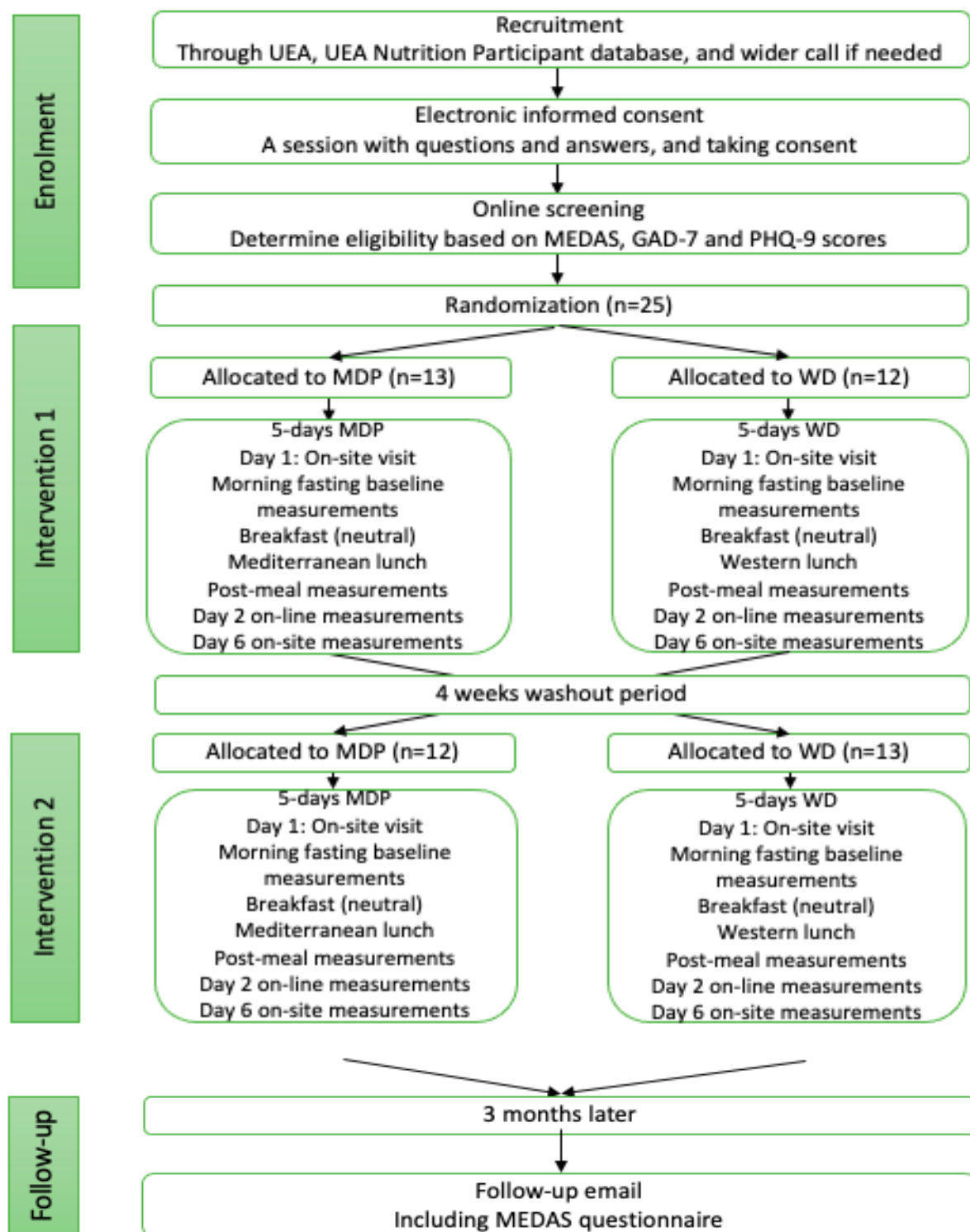
#### 4.2.4 Study Design

This was a cross-over RCT with two dietary intervention arms for 5 days each, i) high-fibre MDP and ii) low-fibre WD. The sample size was calculated based on data from a previous cross-over trial of the effect of MDP adherence in a young healthy adult group, and

detailed calculation is published as part of the protocol (Esgunoglu et al., 2024). Participants received both treatment and were randomly assigned to either the MDP or WD in arm 1 with a 23-days washout period in between (to reduce participant heterogeneity based on a 28-day menstrual cycle) as outlined in Figure 4.1. To capture habitual dietary intake, participants were asked to complete the European Prospective Investigation into Cancer and Nutrition study Food Frequency Questionnaire (EPIC FFQ) before their baseline visit. Participants were followed up 3-months after the end of intervention to assess their on-going adherence to a MDP.

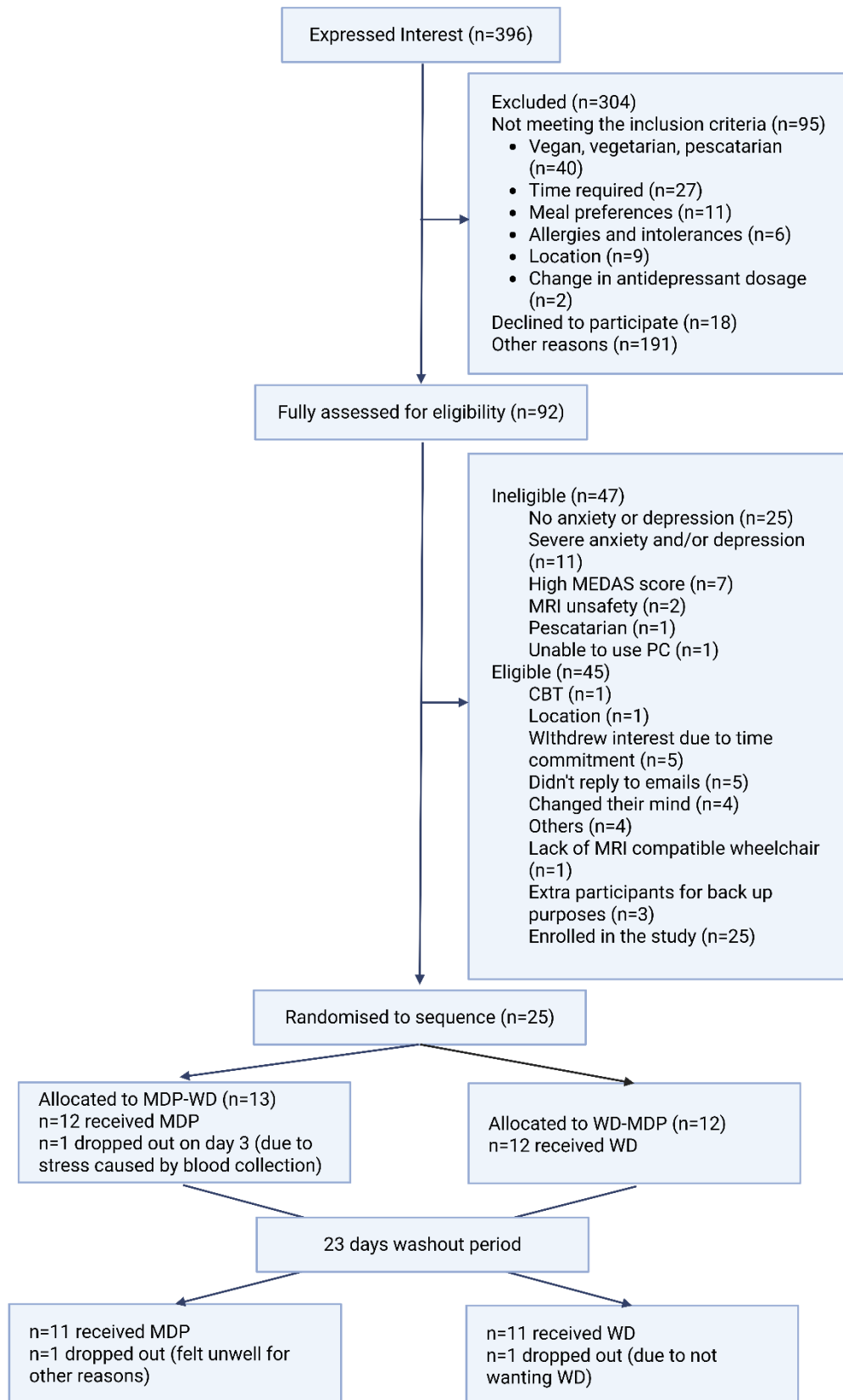
A week prior to the start of intervention, the participants received dietary booklets containing grocery checklist, detailed instructions on food preparation, and a food diary to assess compliance. They received a full 5-days grocery basket via supermarket delivery, a day before each intervention.

The detailed participant flow, following the CONSORT framework is illustrated in Figure 4.2.



**Figure 4.1 | MediMood Study flow diagram.**

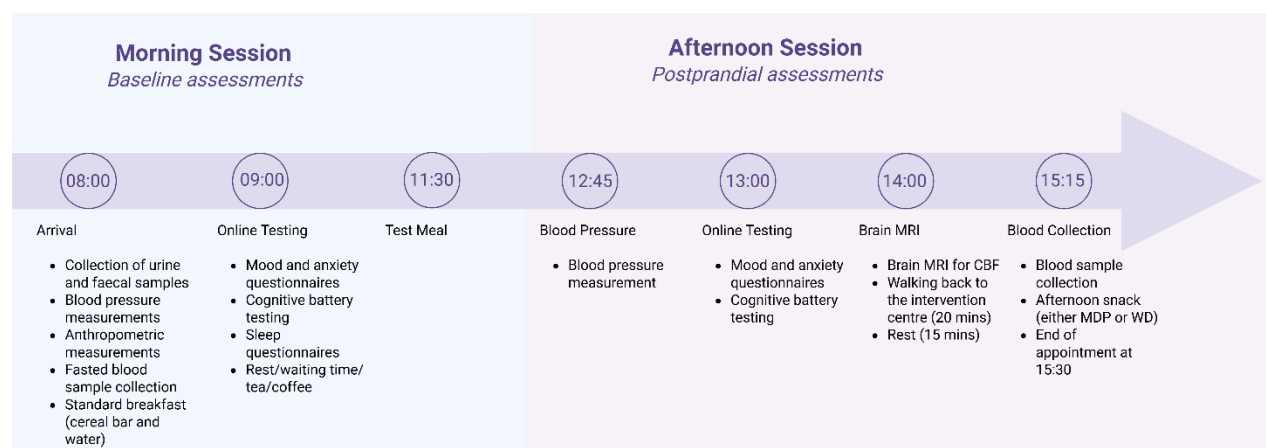
GAD-7, Generalised Anxiety Disorder; MDP, Mediterranean-style dietary pattern; MEDAS, Mediterranean Diet Adherence Screener; PHQ-9, Patient Health Questionnaire; WD, Western-style diet



**Figure 4.2 | Flow of participants through the trial according to the CONSORT guidelines**

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Participants were required to visit the study site, four times in total, 2 x day-1 visits, (baseline and postprandial day) for 8 hours and 2 x day-6 visits (end of treatment) for 2.5 hours). The sequence of events during the study visits is presented in Figure 4.3. Participants were asked to collect urine samples on the morning of their day-1 visits, and faecal samples within 24 hours before their study visits. On the study visits, these biological samples were collected, along with height, weight and blood pressure measurements. A 30ml fasted blood sample was collected, followed by a standard snack breakfast (a honey and oat cereal bar plus water). The first online testing session started at 09:00, assessing mood, anxiety and cognition. The test meals were served at 11:30 (detailed composition in the Supp table 4.2), and the participants were requested to aim to finish the meal in 30 minutes. At 12:45, a postprandial blood pressure measurement was taken, and the participants were administered online mood, anxiety and cognitive testing at 13:00, and they underwent an MRI scan at 14:00. Postprandial blood samples were collected at approximately 15:15, followed by an afternoon snack, with the day-1 visits concluding at 15:30. On day-6 visits, the morning part before the lunch meals was repeated. Detailed study design is also published as part of the protocol paper (Esgunoglu et al., 2024).



**Figure 4.3 | Sequence of events during the study visits**

On day-1 visits, participants underwent all these events. On day-6 visits, participants underwent the morning sessions only, until before the test meal. CBF, Cerebral blood flow; MDP, Mediterranean-style dietary pattern; WD, Western diet.

Created with BioRender.com

### 4.2.5 Dietary Composition

The 5-day diets in both intervention arms provided approximately 2000 kcal/day ( $\pm 10\%$ ; range 1800-2200 kcal/day). They were designed to represent opposing extremes on the Mediterranean Diet Adherence Screener (MEDAS) scale, where the MDP arm scored 13/14 (excluding optional wine consumption), while WD arm scored 0/14. For the MDP arm, the PREDIMED diet was used as a reference standard (Rodriguez-Rejon et al., 2014) and the WD was based on the extreme nutrient intakes (lowest or highest 2.5% depending on dietary component) of the UK population using the UK National Diet and Nutrition Survey (NDNS) data. Table 4.1 represents the nutrient composition of the full 5 days diet, and the nutrient composition of test lunch meals is presented in Supplementary table 4.2.

**Table 4.1 | Nutrient composition table for the 5 days test diets**

|                      |          | <b>Mediterranean Diet<br/>(mean <math>\pm</math> SD)</b> | <b>Western Diet<br/>(mean <math>\pm</math> SD)</b> |
|----------------------|----------|----------------------------------------------------------|----------------------------------------------------|
| <b>Energy</b>        | Kcal/day | 1878 $\pm$ 46                                            | 2027 $\pm$ 79                                      |
| <b>Carbohydrates</b> | g/day    | 154.2 $\pm$ 16.2                                         | 230.8 $\pm$ 24                                     |
|                      | %        | 32.8 $\pm$ 3.0                                           | 45.5 $\pm$ 4.6                                     |
| <b>Free Sugars</b>   | g/day    | 0.3 $\pm$ 0.6                                            | 35.7 $\pm$ 10.4                                    |
| <b>Fibre</b>         | g/day    | 34.8 $\pm$ 6.4                                           | 10.6 $\pm$ 2.9                                     |
| <b>Proteins</b>      | g/day    | 80.8 $\pm$ 19.1                                          | 64.6 $\pm$ 11.4                                    |
|                      | %        | 17.1 $\pm$ 4                                             | 12.7 $\pm$ 2.3                                     |
| <b>Total fat</b>     | g/day    | 105.0 $\pm$ 8.9                                          | 94.2 $\pm$ 11.2                                    |
|                      | %        | 50.3 $\pm$ 4.6                                           | 41.8 $\pm$ 4.6                                     |
| <b>SFA</b>           | g/day    | 16.1 $\pm$ 2.0                                           | 37.1 $\pm$ 3.9                                     |
|                      | %        | 7.2 $\pm$ 1.0                                            | 16.5 $\pm$ 1.3                                     |
| <b>MUFA</b>          | g/day    | 43.5 $\pm$ 6.1                                           | 5.0 $\pm$ 2.6                                      |
| <b>PUFA</b>          | g/day    | 16.9 $\pm$ 7.5                                           | 2.6 $\pm$ 3.0                                      |
| <b>Omega-3 PUFA</b>  | g/day    | 2.4 $\pm$ 1.5                                            | 0.7 $\pm$ 0.1                                      |
| <b>Omega-6 PUFA</b>  | g/day    | 9.3 $\pm$ 6.6                                            | 0.6 $\pm$ 0.7                                      |

%, contribution to total energy intake as percent; g, grams; kcal, kilocalories; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids, SFA, saturated fatty acids.

#### 4.2.6 Primary Outcomes

##### 4.2.6.1 Mood and Anxiety

The co-primary outcomes were the ‘contentment’ domain from Bond-Lader and ‘anxiety’ domain from the Profile of Mood States (POMS). Two instruments were employed for these mood measurements. The Bond-Lader Scale is a 16-item visual analogue scale and measures alertness, calmness and contentment (Bond & Lader, 1974). POMS is a 65-item questionnaire with each question having 0-4 scale answers (five points), and assesses anxiety, depression, anger, vigour, fatigue, confusion and an overall Total Mood Disturbance (TMD) score (McNair et al., 1971). Contentment from the Bond-Lader was selected as the primary mood indicator as identified in our group’s systematic review (Esgunoglu et al., 2021), with the anxiety domain from POMS being the primary anxiety endpoint.

#### 4.2.7 Secondary Outcomes

##### 4.2.7.1 Cognitive Assessment

Changes in cognition were assessed using a cognitive battery administered using the NeurOn online platform (<https://neuropsychology.online/>). The following tests were included in the cognitive battery:

1. Reaction Time Test for motor function
2. Digit Span Test for executive function
3. Trail Making Test A for executive function
4. Sustained Attention to Response Test (SART) for executive function and attention
5. Word Encoding for episodic memory
6. Word Recognition for episodic memory
7. Go No-Go for executive function and impulse control
8. Fragmented Letters Test for visuospatial function

In addition to these, a global cognition score was calculated. To do so, an inclusion criterion was defined, including only participants who achieved a full score on the Fragmented Letters task. One key main variable was selected from each task as follows:

1. Reaction Time: Mean value of reaction speed after false answers were excluded,
2. Digit Span Backwards: Number of correctly recorded digits,

3. Trail Making A: Total interval time,
4. SART: Number of wrong answers,
5. Word Recognition: Source memory of correct answers (%),
6. Go No-Go: Error rate (%)
7. Fragmented Letters: Total correct answers (%) was used as the eligibility criterion, hence not included in the global score

The variables chosen from Reaction Time, Trail Making A, SART and Go/No-Go were reversed, so higher numbers always reflected an improvement. All scores were converted into percentages (except the ones which were already in percentages) with the average of the % in the individual six tasks (1-6 as listed above) representing the global score.

#### 4.2.7.2 Gut Microbiota Profiling

The gut microbiota was profiled using 16S rRNA amplicon-based Sequencing of faecal samples (Liaquat, Le Gall, et al., 2025). Microbial DNA was extracted using the QIAamp PowerFaecal Pro DNA kit (Qiagen) following the manufacturer's instructions. Extracted DNA samples were quantified using NanoDrop (ND-1000 spectrophotometer). Agarose gel electrophoresis was used for quality assessment, which distinguished DNA integrity, purity, fragment size and concentration. The V3-V4 hypervariable region of the 16S rRNA gene was amplified with Illumina NovaSeq 6000 PE250 platform. Sequence analysis was performed using the Uparse software (Uparse v7.0.1001), using all effective tags. Sequences with  $\geq 97\%$  similarity were assigned to the same Operational Taxonomic Unit (OTU). A representative sequence for each OTU was screened for further annotation. Each representative sequence was aligned against the SSuRNA database 138 (Pontifex, Connell, et al., 2024). Alpha diversity was assessed using Chao1, Shannon, and Simpson diversity indices, while beta diversity was assessed using Aitchison distance (Liaquat, Le Gall, et al., 2025).

#### 4.2.7.3 $^1\text{H}$ NMR Untargeted Faecal Metabolomics

Faecal metabolites were analysed and quantified using  $^1\text{H}$  NMR metabolomics analysis. The preparation method has been reported previously (Liaquat, Le Gall, et al., 2025). Approximately 50mg of faecal sample was extracted with 800  $\mu\text{l}$  of NMR buffer. The samples were vortexed until completely homogenised. After mixing and centrifugation

(15,000 g for 5 mins), 700 µl of clean supernatant was transferred to a 5-mm NMR tube for spectral acquisition. NMR spectra were recorded on a 600-MHz Bruker Avance spectrometer fitted with a 5-mm TCI proton-optimised triple resonance NMR inverse cryoprobe and a 24-slot autosampler (Bruker, Coventry, England). Sample temperature was maintained at 300K (27 °C). Each spectrum consisted of 128 scans of 65,536 complex data points with a spectral width of 20 ppm (acquisition time 2.6 s).

The noesypr1d presaturation sequence was used to suppress the residual water signal with low power selective irradiation at the water frequency during the recycle delay ( $D1=2s$ ) and mixing time ( $D8=0.01s$ ). A 90° pulse length 11.4 µs was set for all samples. Spectra were transformed with a 0.1-Hz line broadening and zero filling, manually phased, baseline corrected and referenced by setting the TSP methyl signal to 0ppm. Metabolite identification and quantification was done using the software Chenomx® NMR Suite 8.6™.

#### 4.2.7.4 LCMS/MS Targeted Serum Metabolomics

Sera SCFAs were quantified via a targeted metabolomics approach using Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) combined with Multiple Reaction Monitoring (MRM) mode. Extraction and derivatisation of SCFAs was adapted from the literature (Dei Cas et al., 2020) and further modified as per our publication (Liaquat, Le Gall, et al., 2025).

The analytical system consisted of Waters Acquity UPLC equipped with Water Xevo TQ-S mass spectrometer that is controlled by MassLynx v4.2 software for data acquisition, analyses, and data management. Chromatographic separation was achieved on Kinetex® 2.6 µm XB-C18, 50 x 2.1mm column (Phenomenex, Torrance, CA, USA). Column temperature was maintained at 60 °C with 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B, with a constant flow rate of 0.5 mL/min. The elution gradient began at 90% A, which linearly decreased to 50% at 6 min. From 7 to 7.5 min, solvent B was 100%, which was maintained at 10% hereafter until 13 min.

#### 4.2.8 Statistical Analysis

Statistical analyses were performed using RStudio (version 4.4.3) and GraphPad Prism version 10 (GraphPad Software, CA, USA). All data are presented as mean  $\pm$  standard deviation (SD) unless otherwise stated. We excluded 3 participants from the analyses due to dropout during prior to completing both study arms; and an additional 2 participants were excluded from the microbiome and metabolome analysis due to missing faecal samples.

After identifying outliers (ROUT method,  $q=1\%$ ) and assessing normality of the data by Q-Q plots, two-way repeated measures ANOVA was performed for raw values from both diets (WD and MDP) at both timepoints (baseline and day-6). For comparisons between score changes, paired t-test was used. Metabolomics analysis was performed using MetaboAnalyst version 6.0 online software. Data was normalised by sum, followed by log transformation and auto-scaling. The normalised data was further analysed on GraphPad prism version 10. After identifying outliers (ROUT method,  $q=1\%$ ), a mixed-effect model was run (to account for missing values in the data).

Microbiome analyses were performed on CLR (centred log-ratio) transformed data after filtering the datasets excluding all genera and features with  $<10\%$  prevalence, except for alpha diversity metrics where we are interested in rare taxa. Alpha diversity was measured after adjusting for rarefaction curves, and a linear model approach was used to compare differences, followed by Tukey's multiple comparison based on the normality of data. Beta diversity was reported after pairwise Permutational Multivariate Analysis of Variance (PERMANOVA) testing on Aitchison distance values (Euclidean distance over CLR transformed values) to assess microbiota divergence between groups, allowing 1,000 permutations. Ordinations were built using Principal Component Analysis on Aitchison distance values, and the PCA plots present variance ( $R^2$ ) and p values for each plot.

Gut Brain Modules (GBMs) were identified based on PICRUSt2 predictions, which are based on several gene family databases including Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs (KO), Enzyme Commission (EC) numbers, and MetaCyc pathway abundances (Bastiaanssen et al., 2023). These PICRUSt2 outputs have been

validated with various human microbiome datasets (Gavin M. Douglas et al., 2020). GBM modules are annotated for function, pathway, structure, and potential to cross the intestinal epithelium and the BBB, and their abundances were derived from an orthologue abundance table using Omixer Reference Pathways Mapper (Valles-Colomer et al., 2019). GBM counts were CLR transformed after removing the features with <10% prevalence, followed by fitting a linear regression model and FDR correction using Benjamini and Hochberg method.

Differential bacterial abundance analyses were performed using linear models built for each genus to test multiple transformations accounting for different data distribution. p values for all the contrasts were corrected for False Discovery Rate (FDR) using Benjamini & Hochberg's procedure. q-values of 0.2 was used as a cutoff for statistical significance for genera and functional modules. CLR transformed data was reported for the final figures as presented.

## 4.3 Results

### 4.3.1 Recruitment and Sample characteristics

A total of 396 people expressed interest and were provided with the participant information sheet. Out of these, 304 people were excluded based on several reasons, and 92 were fully assessed for eligibility. Twenty-five participants were finally enrolled in the study, n=13 was randomised to MDP diet as their first arm, and n=12 was allocated to the WD arm. After a 23 days washout period, they were allocated to the other dietary pattern.

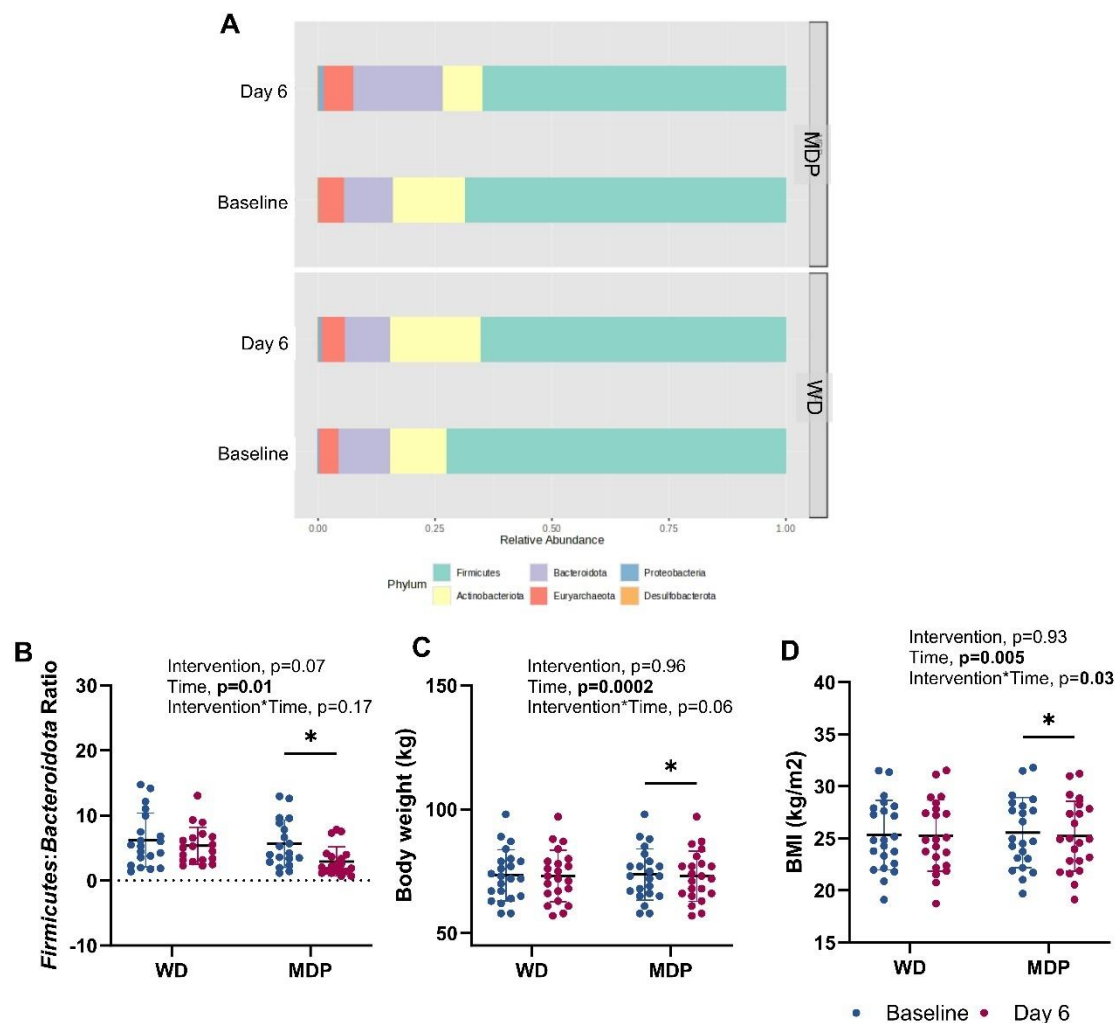
**Table 4.2 | Participant characteristics (n=25)**

| Characteristics          | Mean $\pm$ SD   | Number (n) | Percentage (%) |
|--------------------------|-----------------|------------|----------------|
| Age (years)              | 41.6 $\pm$ 20.7 | NA         | NA             |
| Sex                      |                 |            |                |
| Female                   | NA              | 16         | 64             |
| Male                     |                 | 9          | 36             |
| MEDAS score (0-14)       | 5.2 $\pm$ 1.3   | NA         | NA             |
| MEDAS adherence groups   |                 |            |                |
| Lowest scorers (0-4)     |                 | 7          | 28             |
| Lower scorers (5-7)      |                 | 18         | 72             |
| PHQ-9 score (0-27)       | 6.8 $\pm$ 2.9   | NA         | NA             |
| PHQ-9 groups             |                 |            |                |
| None (0-4)               |                 | 5          | 20             |
| Mild (5-9)               |                 | 15         | 60             |
| Moderate (10-14)         |                 | 5          | 20             |
| GAD-7 score (0-21)       | 6.7 $\pm$ 1.9   | NA         | NA             |
| GAD-7 groups             |                 |            |                |
| None (0-4)               |                 | 3          | 12             |
| Mild (5-9)               |                 | 20         | 80             |
| Moderate (10-14)         |                 | 2          | 8              |
| BMI (kg/m <sup>2</sup> ) | 25.3 $\pm$ 3.2  | NA         | NA             |

The values presented were taken from screening. SD, Standard deviation; MEDAS, Mediterranean Diet Adherence Screener; PHQ-9, Patient Health Questionnaire-9 which assesses depressive symptoms. GAD-7, Generalised Anxiety Disorder-7 which measures anxiety.

#### 4.3.2 High-fibre MDP led to significant changes in bacterial phyla, reduced F:B ratio and lower BMI in only 5 days

A prominent change was observed in the gut microbiota at the phylum level (Fig 4.4A). The ratio of two prominent bacterial phyla Firmicutes and Bacteroidetes (F:B ratio) was significantly affected by the MDP intervention ( $p < 0.05$ , Fig 4.4B); however, it was unaffected during the WD intervention ( $p > 0.05$ , Fig 4.4B). The F:B ratio is widely associated with weight gain and obesity (Magne et al., 2020), with the body weight in the MDP arm reduced from 74 kg to 73 kg ( $p < 0.05$ , Fig 4.4C) associated with a  $0.3 \text{ kg/m}^2$  decrease (1.3%) in BMI ( $p < 0.05$ , 4.4D).

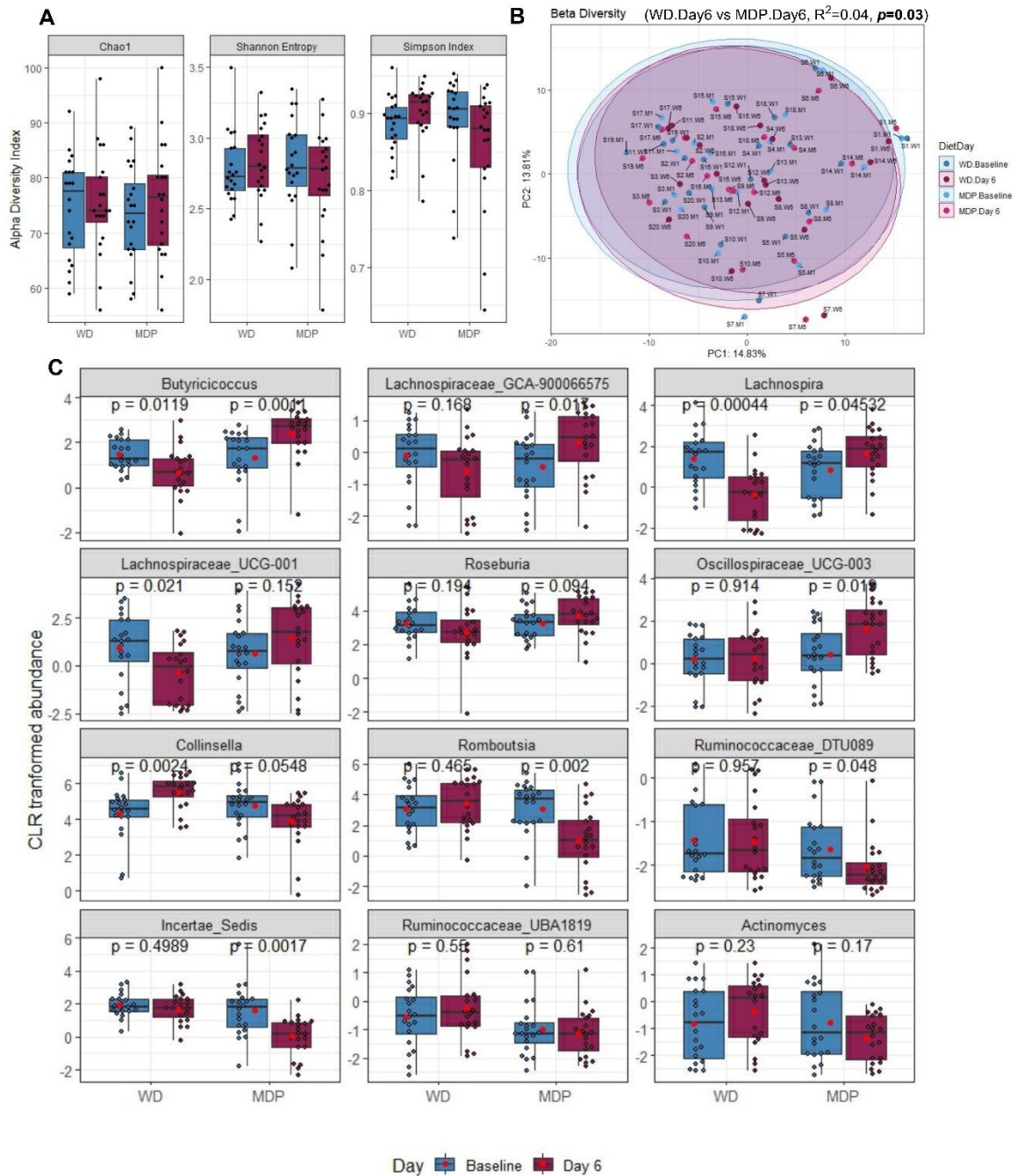


**Figure 4.4 | The relative abundance of gut microbiota at phylum level at baseline and after (day 6) 5-day intervention with a low-fibre Western diet (WD) or a high-fibre Mediterranean-style diet (MDP).** (A) Differences were observed in the relative abundance of the gut microbiota at phylum level. (B) Firmicutes to Bacteroidetes (F:B) ratio was unaffected during the WD intervention but significantly decreased following the MDP ( $p=0.007$ ). (C) Body weight ( $p=0.0001$ ) and (D) BMI ( $p=0.0005$ ) of the participants showed similar trend as the F:B ratio. Statistical analyses were performed using two-way repeated measures ANOVA, followed by FDR method to correct for multiple comparisons.  $*=p<0.05$ .

#### 4.3.3 High-fibre MDP and Low-fibre WD display marked shifts in intestinal microbiota

Neither intervention showed any significant differences in alpha diversity, as measured by Chao1, Shannon Entropy and Simpson indices ( $p>0.05$ , Fig 4.5A). Pairwise PERMANOVA analysis demonstrated significant beta diversity differences between MDP Day 6 and WD day 6 gut microbiota abundance ( $R^2=0.04$ ,  $p=0.03$ ,  $F=1.72$ , Fig 4.5B).

At the genus level, general linear model demonstrated significant differences in 12 genera among groups ( $FDR<0.2$ , Fig 4.5C). Of these bacteria, 4 bacterial genera were significantly altered following the WD intervention, including a decrease in *Butyricicoccus* ( $p=0.01$ ), *Lachnospira* ( $p<0.001$ ) and *Lachnospiraceae\_UCG-001* ( $p=0.02$ ), and an increase in *Collinsella* ( $p=0.002$ ). Eight genera were significantly altered following the MDP intervention; *Butyricicoccus* ( $p=0.001$ ), *Lachnospiraceae\_GCA-900066575* ( $p=0.02$ ), *Lachnospira* ( $p=0.05$ ) and *Oscillospiraceae\_UCG-003* ( $p=0.02$ ) significantly increased, whereas *Collinsella* ( $p=0.05$ ), *Romboutsia* ( $0.002$ ), *Ruminococcaceae\_DTU089* ( $p=0.05$ ) and *Incertae sedis* ( $p=0.002$ ) significantly decreased.



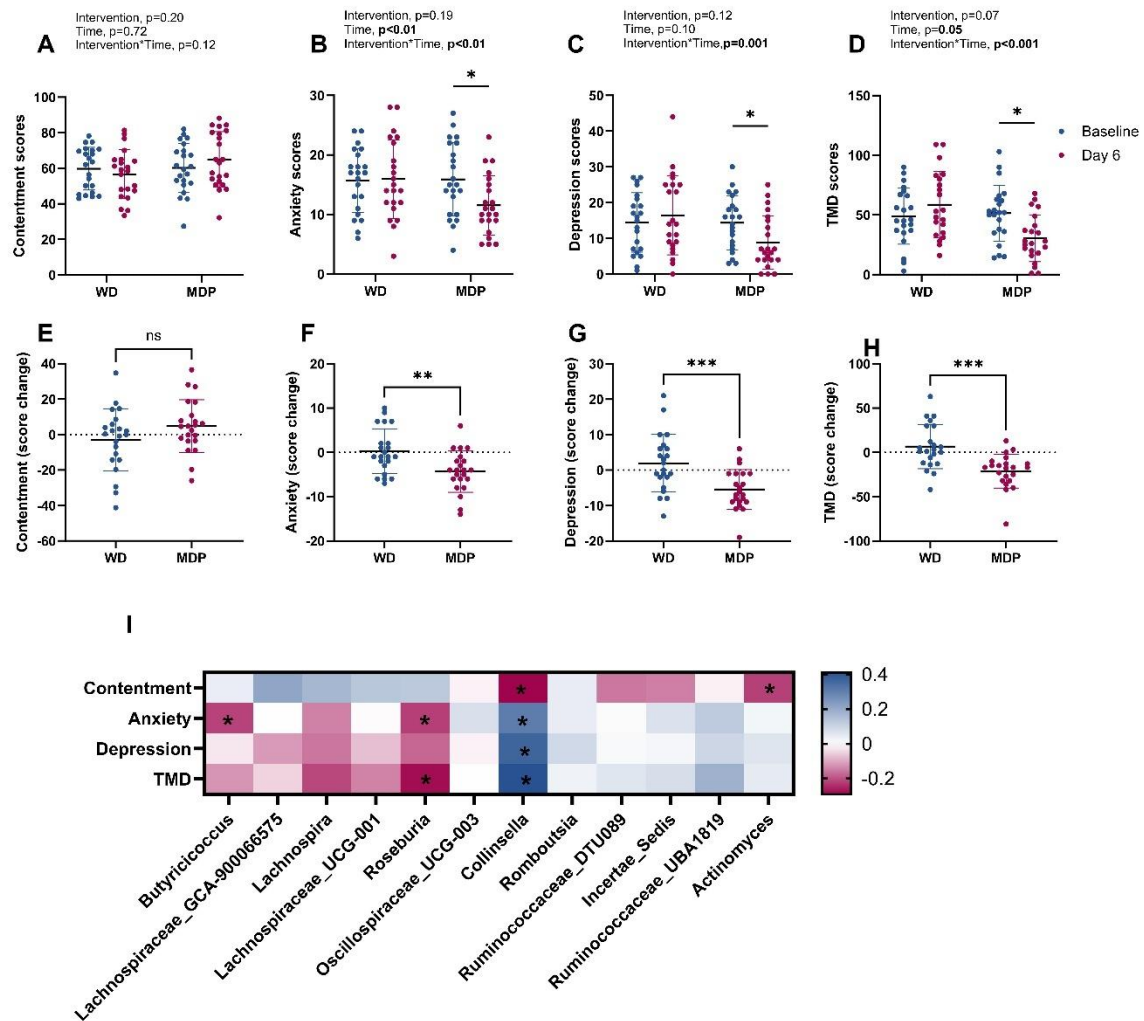
**Figure 4.5 | Microbiota diversity at baseline and following a 5-days intervention (day 6) with a low-fibre Western-style diet (WD) and a high-fibre Mediterranean-style dietary pattern (MDP).** (A) Alpha diversity is presented as boxplots for Chao1, Shannon and Simpson indices. (B) Beta diversity presented as PCA plot of Euclidean distance values. Pairwise PERMANOVA analysis showed a significant difference between WD.Day6 and MDP.Day6. Variance  $R^2$  and  $p$  values are plotted. (C) Abundance of gut bacterial genera was significantly altered by the short-term dietary interventions. General linear model was applied for all possible comparisons among 4 groups,  $FDR < 0.2$  was used as a cut off. Within diet comparison was performed using pairwise Kruskal-Wallis test and  $p$  values are reported on the plot. The red dots indicate mean values.

#### 4.3.4 High-fibre MDP over a period of 5 days significantly improved anxiety, depression and total mood disturbance

The mental health data has been presented at length and included in the PhD thesis of Latife Esgunoglu, who was awarded her PhD in March 2025. Here it is briefly summarised and introduced in order to correlate our select mental and cognitive health outcomes with microbiome and metabolomics data.

Significant intervention effects were observed for anxiety ( $p < 0.01$ , Fig 4.6B), depression ( $p < 0.01$ , Fig 4.6C), and TMD ( $p < 0.01$ , Fig 4.6D). After 5 days of MDP intervention, anxiety scores decreased by 27%, whereas WD led to a minimal change (Fig 4.6B, F). Contentment was unaffected by either intervention ( $p > 0.05$ , Fig 4.6A). Depression scores improved by 38% following MDP, while WD increased depression scores by 16% (Fig 4.6 C, G). Looking at the composite (of all 6 POMS domains) TMD scores improved by 28% following the MDP intervention relative to WD ( $p < 0.01$ , Fig 4.6D, H).

These mental health outcomes were also correlated with the gut microbiota changes (Fig 4.6I). Contentment scores were negatively correlated with *Collinsella* ( $r = -0.30$ ,  $p = 0.009$ , Fig 4.6I) and *Actinomyces* ( $r = -0.22$ ,  $p = 0.05$ , Fig 4.6I) abundance, both of which have reported detrimental effects on brain health (Gomez-Arango et al., 2018; Lin et al., 2024). Anxiety scores were negatively correlated with butyrate-producing bacterial genera *Butyricicoccus* ( $r = -0.22$ ,  $p = 0.05$ ) and *Roseburia* ( $r = -0.22$ ,  $p = 0.05$ ), and positively correlated with *Collinsella* ( $r = 0.31$ ,  $p = 0.006$ ). Depression scores were positively correlated with *Collinsella* ( $r = 0.36$ ,  $p = 0.009$ ). TMD scores were negatively correlated with butyrate-producers *Roseburia* ( $r = -0.28$ ,  $p = 0.01$ ), and positively correlated with *Collinsella* ( $r = 0.41$ ,  $p < 0.001$ ) abundance (Fig 4.6I).



**Figure 4.6 | Contentment, anxiety, depression and total mood disturbance (TMD) scores before (baseline) and after (Day 6) 5-days intervention with a low-fibre Western-style diet (WD) or a high-fibre Mediterranean-style dietary pattern (MDP).**

(A) No significant changes were observed in the contentment scores. (B) Anxiety, (C) Depression, and (D) TMD scores improved significantly after the MDP intervention. The score change (baseline to day 6) for (E) contentment was not significantly different between the two treatment groups. Although, the intergroup difference in change between groups was evident for (F) anxiety, (G) depression and (H) TMD. (I) Various significant correlations were observed between the genera and scores for contentment, anxiety, depression and TMD. Statistical analyses were performed using two-way repeated measures ANOVA for raw score values, and paired t-test was performed to compare score changes. Spearman correlation analysis was performed to identify significant correlations. \*= $p<0.05$ , \*\*\*= $p<0.0001$ , ns=non-significant.

#### 4.3.5 High-fibre MDP over a period of 5 days significantly improved executive function

Cognition was assessed using different cognitive tasks to assess cognitive domains including motor function (assessed by reaction time cognitive task), sustained attention (assessed by sustained attention response task), executive function (assessed by DSB task, trail making A task), episodic memory (recognition and source memory from the word recognition task) and impulse control (assessed from go no/go task). Variables from these cognitive tasks were used to calculate an overall cognitive score. The impact of both WD and MDP interventions on these cognitive tasks are presented in Table 4.3.

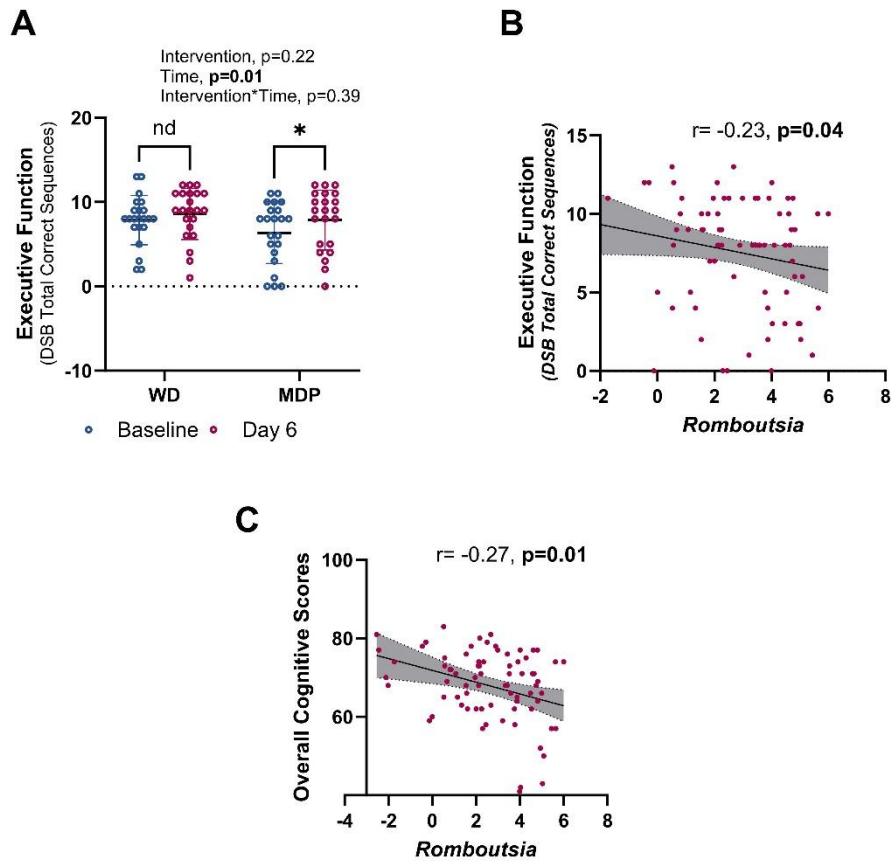
Executive function showed a significant time effect, as assessed by DSB total correct sequences ( $p=0.01$ ), DSB total correct digits ( $p=0.01$ ) and TMA task ( $p=0.04$ ). Although the post hoc analysis suggested temporal changes within the MDP group, the absence of a significant treatment\*time interaction indicates that these changes were not significantly different between treatments. Episodic memory, as assessed by the source memory variable from the word recognition task also showed a significant time effect ( $p=0.02$ ). A significant time effect was also observed for the overall cognitive score ( $p=0.03$ ). However, of all these cognitive domains, which showed a significant time effect, only executive function (determined by the DSB task total correct sequences) showed a significant within MDP group effect following the multiple comparison testing, with an increase in function ( $p<0.05$ , Fig 4.7A).

The executive function scores negatively correlated with *Romboutsia* abundance ( $r = -0.23$ ,  $p=0.04$ , Fig 4.7B). *Romboutsia* also negatively correlated with the overall cognitive score ( $r = -0.27$ ,  $p=0.01$ , Fig 4.7C). This bacterial genus is associated with lower levels of an enzyme involved in converting tryptophan to serotonin in the brain (Cooke et al., 2022). It is also associated with increased risk of Parkinson's disease dementia (Ji et al., 2024). Although, some conflicting evidence suggests the lower abundance of *Romboutsia* is linked to depression symptoms in Parkinson's disease patients (Xie et al., 2022).

**Table 4.3** | Impact of 5-days WD and MDP interventions on cognitive performance

|                                                        | N  | Baseline     | N  | Day 6        | P<br>Intervention | P<br>Time | P<br>Intervention*<br>Time |
|--------------------------------------------------------|----|--------------|----|--------------|-------------------|-----------|----------------------------|
| Motor Function (assessed by RT)                        |    |              |    |              |                   |           |                            |
| WD                                                     | 20 | 288 ± 20     | 20 | 309 ± 49     | 0.64              | 0.06      | 0.19                       |
| MDP                                                    | 19 | 290 ± 30     | 21 | 299 ± 38     |                   |           |                            |
| Sustained Attention (assessed by SART)                 |    |              |    |              |                   |           |                            |
| WD                                                     | 21 | 135 ± 39     | 22 | 133 ± 38     | 0.72              | 0.63      | 0.98                       |
| MDP                                                    | 21 | 140 ± 43     | 22 | 137 ± 47     |                   |           |                            |
| Executive Function (assessed by DSB correct sequences) |    |              |    |              |                   |           |                            |
| WD                                                     | 22 | 7.9 ± 2.9    | 22 | 8.6 ± 3.0    | 0.22              | 0.01      | 0.39                       |
| MDP                                                    | 22 | 6.4 ± 3.7    | 22 | 7.9 ± 3.5    |                   |           |                            |
| Executive Function (assessed by DSB correct digits)    |    |              |    |              |                   |           |                            |
| WD                                                     | 22 | 40.8 ± 19.9  | 22 | 47.4 ± 20.4  | 0.21              | 0.01      | 0.66                       |
| MDP                                                    | 22 | 32.2 ± 21.9  | 22 | 41.7 ± 23.0  |                   |           |                            |
| Executive Function (assessed by TMA)                   |    |              |    |              |                   |           |                            |
| WD                                                     | 21 | 23350 ± 7015 | 21 | 21855 ± 6888 | 0.86              | 0.04      | 0.57                       |
| MDP                                                    | 21 | 22698 ± 6174 | 21 | 21850 ± 5458 |                   |           |                            |
| Episodic Memory (WR, recognition memory)               |    |              |    |              |                   |           |                            |
| WD                                                     | 22 | 89.2 ± 8.1   | 21 | 92.1 ± 6.5   | 0.30              | 0.26      | 0.37                       |
| MDP                                                    | 22 | 92.1 ± 7.3   | 20 | 92.7 ± 4.5   |                   |           |                            |
| Episodic Memory (WR, source memory)                    |    |              |    |              |                   |           |                            |
| WD                                                     | 22 | 67.6 ± 24.3  | 22 | 73.4 ± 21.6  | 0.81              | 0.02      | 0.81                       |
| MDP                                                    | 22 | 68.5 ± 20.5  | 22 | 75.5 ± 20.8  |                   |           |                            |
| Impulse Control (GNG % of incorrect answers)           |    |              |    |              |                   |           |                            |
| WD                                                     | 22 | 3.5 ± 2.8    | 22 | 3.6 ± 2.8    | 0.88              | 0.36      | 0.68                       |
| MDP                                                    | 21 | 3.4 ± 2.4    | 22 | 3.9 ± 3.0    |                   |           |                            |
| Overall Cognitive Score                                |    |              |    |              |                   |           |                            |
| WD                                                     | 20 | 68.7 ± 7.5   | 22 | 69.6 ± 7.8   | 0.67              | 0.03      | 0.68                       |
| MDP                                                    | 20 | 67.7 ± 6.2   | 22 | 69.1 ± 8.8   |                   |           |                            |

DSB, Digit Span Backwards; GNG, Go No-Go; MDP, Mediterranean-style dietary pattern; RT, Reaction Time; SART, Sustained Attention to Response Task; TMA, Trail Making A; WD, Western-style diet; WR, Word Recognition

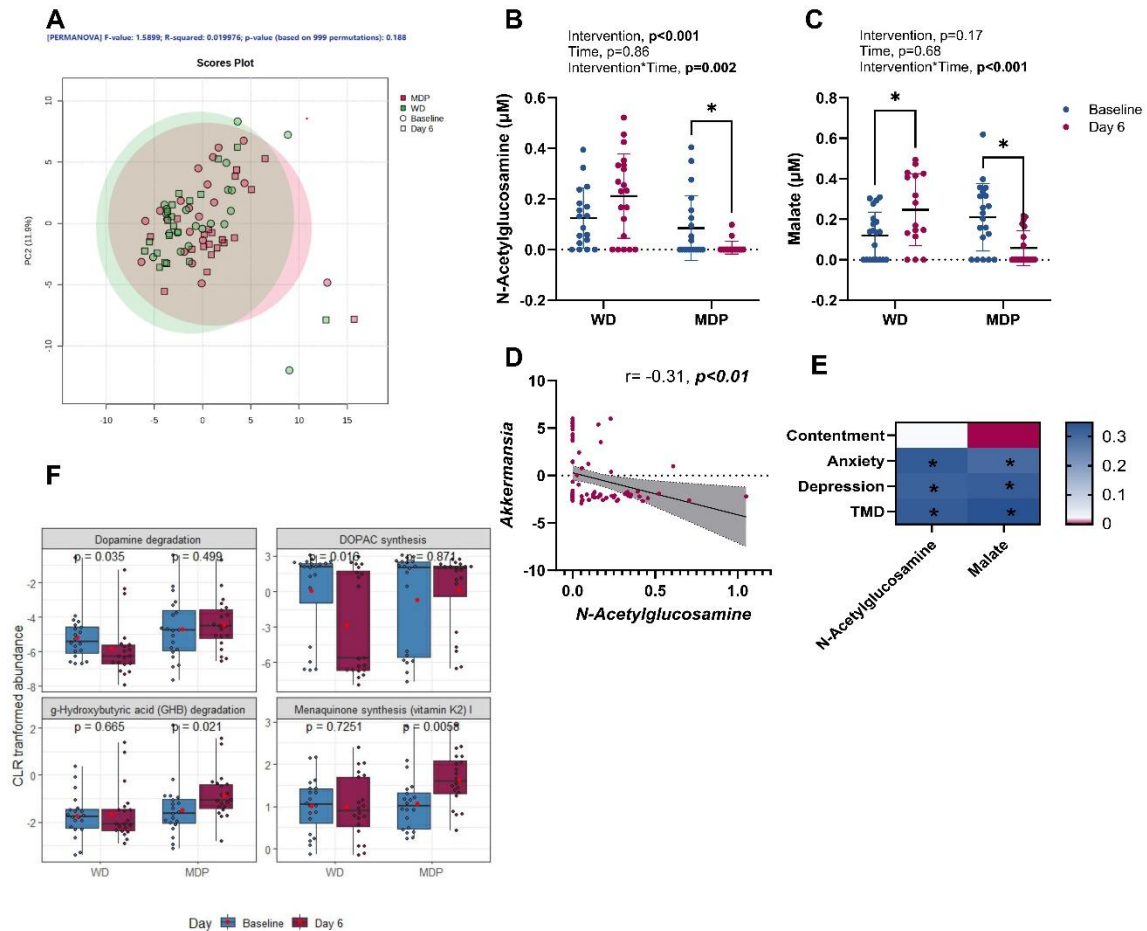


**Figure 4.7 | Executive function improved following 5 days Mediterranean-style dietary pattern (MDP) intervention.** (A) Executive function improved following 5-days MDP intervention, although there was no significant interaction effect and (B) negatively correlated with *Romboutsia* abundance in the gut. (C) *Romboutsia* also negatively correlated with the overall cognitive scores. Mixed effects model was used for statistical comparisons, after removing outliers. Spearman rank correlation analysis was performed for computing correlations.  $*=p<0.05$ .

#### 4.3.6 Diet-induced faecal metabolomic shift could influence mental health outcomes

Faecal metabolomics analysis did not demonstrate a significant difference between dietary intervention groups, as determined by PERMANOVA analysis ( $R^2=0.02$ ,  $p=0.2$ , Fig 4.8A). Two-way repeated measures ANOVA showed significant differences in N-acetylglucosamine (NAG) (Fig 4.8B) and malate (Fig 4.8C) which dropped after the MDP intervention. In contrast to the MDP, the WD intervention increased malate levels after 5-days ( $p<0.05$ , Fig 4.8C). Both of these metabolites positively correlated ( $p<0.05$ ) with anxiety, depression and TMD scores (Fig 4.8E).

*Akkermansia* contain glycoproteases, which can degrade the protein core of intestinal mucin (Luis & Hansson, 2023), and it was found to be negatively correlated ( $r=-0.31$ ,  $p<0.01$ ) with NAG (a mucin degradation product) ( $r= -0.31$ ,  $p<0.01$ , Fig 4.8D). Functional GBM predictions showed significant alterations in certain gut metabolites involved in gut-brain communication pathways. Predicted GBMs showed a significant decrease in dopamine degradation and resulting DOPAC (3,4-Dihydroxyphenylacetic acid) synthesis following the WD intervention (Fig 4.8F), and a significant increase in g-hydroxybutyric acid (GHB) degradation and vitamin K2 synthesis following MDP intervention (Fig 4.8F).

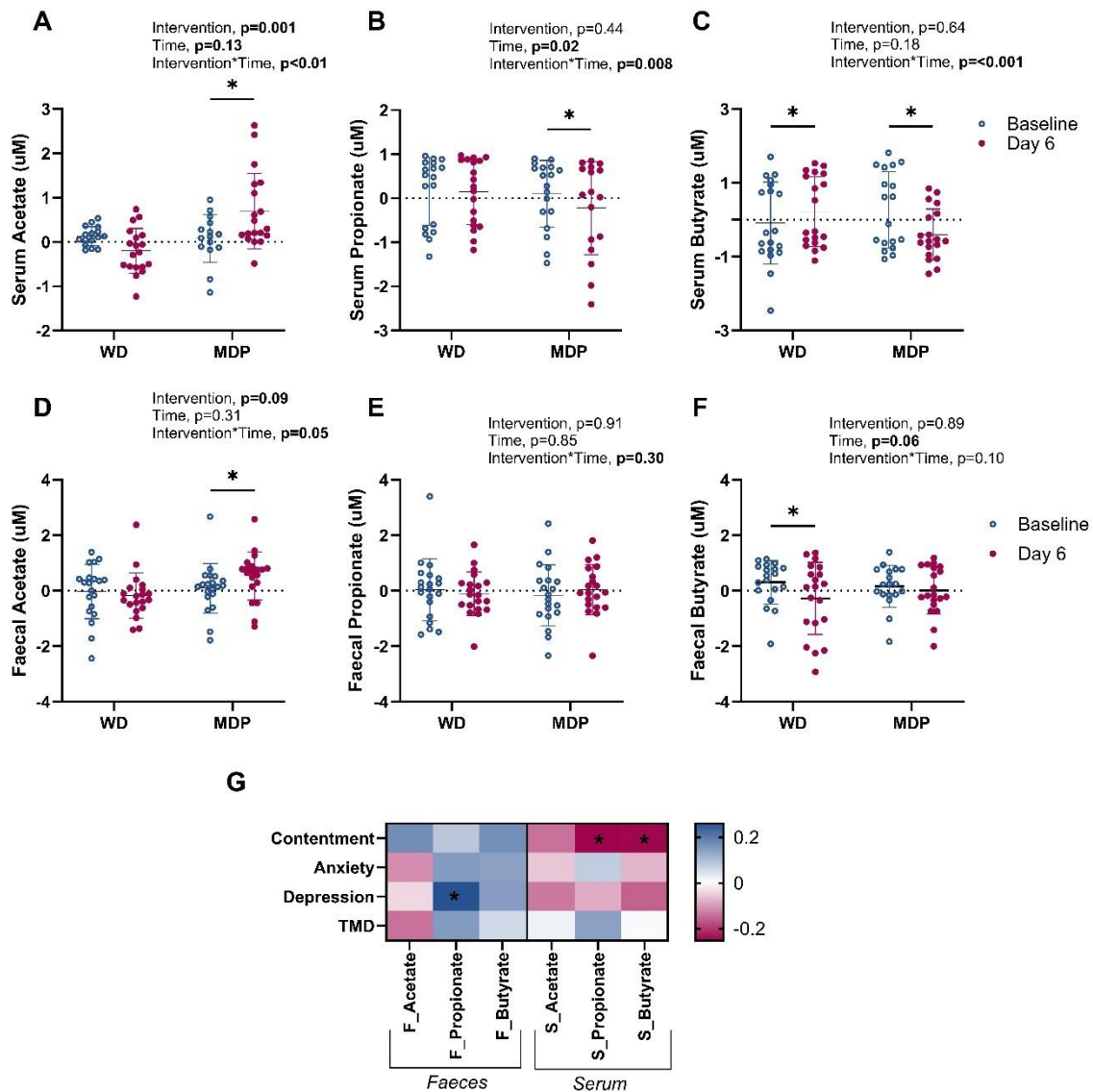


**Figure 4.8 | Select gut metabolites at baseline and following (day 6) a low-fibre Western-style diet (WD) and a high-fibre Mediterranean-style diet (MDP), and associations with mental health outcomes** (A) PCA plot demonstrated no significant metabolomic differences following a WD versus an MDP. However, further two-way repeated measures ANOVA showed (B) a significant drop in N-Acetylglucosamine (NAG) following the MDP intervention, and (C) a significant drop in malate concentrations following MDP and an increase following the WD intervention. (D) NAG levels were negatively correlated with *Akkermansia* abundance. (E) Both NAG and Malate levels positively correlated with anxiety, depression and total mood disturbances (TMD). (F) Predicted Gut-Brain Modules (GBMs) showed a significant increase in g-Hydroxybutyric acid degradation and vitamin K2 synthesis following the MDP intervention. The WD reduced dopamine degradation and associated DOPAC synthesis. Statistical analysis was performed using two-way repeated measures ANOVA for metabolites comparisons. Spearman rank correlation analysis was performed to calculate correlations. Linear regression model was fitted for GBM analysis followed by pairwise Kruskal Wallis test for within diet comparisons.  $*=p < 0.05$ .

#### 4.3.7 Faecal SCFAs and SCFA-producing bacteria increased during MDP and reduced during the WD intervention

SCFA levels were differentially influenced by short-term WD and MDP interventions. Acetate levels increased following the MDP intervention in both serum ( $p < 0.01$ , Fig 4.9A) and faecal samples ( $p = 0.03$ , Fig 4.9D). However, propionate showed an opposite trend, with serum levels decreasing following the MDP intervention ( $p < 0.01$ , Fig 4.9B), whilst no changes were observed in the faecal levels (Fig 4.9E). Serum butyrate levels were decreased following the MDP ( $p < 0.01$ , Fig 4.9C) but increased following the WD intervention ( $p = 0.05$ , Fig 4.9C). Whereas, a significant drop was observed in the faecal butyrate levels, following the WD intervention ( $p = 0.01$ , Fig 4.9F).

Serum concentrations of butyrate ( $r = -0.25$ ,  $p = 0.03$ ) and propionate ( $r = -0.25$ ,  $p = 0.03$ ) were negatively correlated with the contentment scores. Faecal propionate levels were positively correlated with depression scores ( $r = 0.26$ ,  $p = 0.02$ ) (Fig 4.9G).



**Figure 4.9 | Serum and faecal levels of SCFAs at baseline and following (day 6) a 5-days intervention with a low-fibre Western-style diet (WD) and a high-fibre Mediterranean-style diet (MDP) and associations with mental health outcomes**

Serum levels of (A) acetate significantly increased following the MDP intervention, whereas (B) serum propionate levels significantly dropped. (C) Serum butyrate levels showed a significant increase following the WD intervention and a significant reduction following the 5-days MDP intervention. Faecal levels of (D) acetate significantly increased after the MDP intervention. (E) No significant changes were observed in faecal propionate levels, whereas (F) butyrate levels significantly decreased following the WD intervention. (G) Significant correlations were observed between SCFAs levels and mental health outcomes. Statistical analysis was performed using two-way repeated measures ANOVA or mixed model analysis due to missing values. Data presented as mean and standard deviation. Spearman rank correlation analysis was done to calculate correlations.  $*=p<0.05$ .

#### 4.4 Discussion

Adherence to a Mediterranean-style dietary pattern is well established as a long-term strategy to support cognition and mental health (Knight et al., 2016; Shannon et al., 2019) (Esgunoglu et al., 2021; Shannon et al., 2019); as well as to reduce systemic inflammation (Meng et al., 2025). However, its short-term effects remain less well understood (Esgunoglu et al., 2021). Current evidence is largely from prospective cohort studies, with insufficient gold-standard RCT data. Furthermore, underlying mechanisms and the role of the gut microbiota in mediating the MDP-brain effects are not yet fully elucidated. In this study, we compared a high-fibre MDP and a low-fibre WD over a 5-days period, examining change in the gut microbiota composition and metabolite profiles. The MDP intervention led to favourable shifts in the gut microbiota, which were associated with improvements in mood, anxiety and executive function.

We found that both dietary interventions distinctly shaped the gut microbiota. These changes in response to short-term dietary interventions have been reported across studies with intervention period ranging from 48 hours to 7 days (Bourdeau-Julien et al., 2023; David et al., 2014; Forteza et al., 2022), however no study yet explored how these short-term mediterranean diet induced microbial changes linked to anxiety, depression and cognition. Overall, and consistent with other studies, no significant difference was observed in the alpha diversity metrics, (Barber et al., 2021), however, beta diversity differed significantly following the MDP and WD interventions, (Bourdeau-Julien et al., 2023; David et al., 2014). The MDP intervention significantly shifted the gut microbiota composition towards a reduced F:B ratio, a marker previously linked to psychiatric conditions (Faraj et al., 2021). A systematic review investigating the gut microbiota in individuals with depression and anxiety reported that, among the seven studies examining depression, three found an increased relative abundance of Firmicutes, while four observed a decreased abundance (Cao et al., 2025). Evidence remains inconsistent, as a meta-analysis of eight cohorts with depressive phenotypes reported lower Firmicutes, and higher Bacteroidota species (Liang et al., 2022), whilst another study found an elevated F:B ratio in depression (Lin et al., 2017). A lower F:B ratio has been associated with improved outcomes in depression, as evidenced by studies showing that antidepressants such as fluoxetine (Zhang et al., 2021) and escitalopram (Shen et al.,

2021) reduce the F:B ratio. Our findings align with these observations, as MDP significantly lowered the F:B ratio in parallel with improvements in depressive symptoms.

We observed a marked increase in the abundance of butyrate-producing bacterial species in response to the MDP, whereas the WD reduced these bacteria; a pattern consistent with previous studies investigating the gut microbiota responses to MDP and WD interventions (Barber et al., 2021). However, our study is the first one to associate these short-term MDP associated gut microbiota changes with mental health and cognition. There was a higher abundance of butyrate-producers following the MDP diet (Barber et al., 2021), among which *Butyricicoccus* and *Roseburia* abundance was negatively correlated with anxiety score, and *Roseburia* was also negatively correlated with TMD scores (Fig 4.6I). *Roseburia* is known to improve intestinal barrier function and inflammation (Kasahara et al., 2018). Low-DF diets are linked to higher abundance of *Collinsella* (Gomez-Arango et al., 2018), similar to what we observed in the WD intervention. The *Collinsella* abundance was positively correlated with anxiety, depression and TMD, and negatively correlated with contentment scores (Fig 4.6I).

Inflammation is a key mechanism in the pathogenesis of anxiety and depression (Meng et al., 2025), influenced by the gut microbiota along with their metabolites and lipopolysaccharides (LPS). Gut microbial metabolites and LPS can interact with macrophages and stimulate immune responses, leading to the release of proinflammatory factors influencing inflammatory markers and depressive symptoms (Cao et al., 2025; Liu et al., 2023). The excessive production of inflammatory cytokines such as IL-1a, IL-1b, TNF-a, and IL-6, in the periphery, could cross the BBB and act on receptors expressed by neurons and glial cells leading to neuroinflammation (Carlessi et al., 2021). SCFAs are integral microbial metabolites involved in gut-brain communication (Dalile et al., 2019). SCFAs and SCFA-producing bacteria are depleted in patients with anxiety and depression (Simpson et al., 2021), and their administration exert antidepressant effects by ameliorating depression-related neuroinflammation (Tang et al., 2022).

Both serum and faecal acetate levels increased following the MDP intervention, while propionate levels significantly dropped in the serum following the MDP intervention. Butyrate exhibited a divergent trend, serum levels declined following the MDP and

increased following the WD intervention, whereas WD led to a drop in faecal levels. These opposing responses highlight the complexity of SCFAs dynamics, suggesting that not all metabolites respond uniformly to short-term dietary shifts. The reduction in serum butyrate after the high fibre MDP may reflect increased local utilisation by the colonocytes, altered hepatic metabolism or increased conversion to acetyl-CoA and ultimately acetate, rather than reduced microbial production. These SCFAs may potentially be cross-feeding other gut microbiota and possibly influencing other mechanisms, as lower serum butyrate and propionate levels were associated with better contentment scores (Fig 4.9G). However, this contradicts with literature which reports lower serum butyrate levels in depression patients as compared to healthy controls, and higher baseline butyrate levels were associated with a greater likelihood of remission at 6 months follow-up (Schiweck et al., 2025).

Habitual consumption of plant-based diets promotes enrichment of fibre-degrading gut bacteria such as *Lachnospira* among the Firmicutes and is also associated with higher levels of SCFAs in the gut (De Filippis et al., 2016). Both *GCA-900066575* and *Lachnospiraceae* *UCG-001* bacteria from the Lachnospiraceae family, are potent producers of butyrate (Hu et al., 2024), which increased after MDP and decreased after the WD intervention, along with *Butyricicoccus* and *Oscillospiraceae\_UCG-003* (Fig 4.5C), which is linked to depression (Liang et al., 2022). The anxiolytic and antidepressant effect of SCFAs is also confirmed in literature (Cheng et al., 2024; Schiweck et al., 2025; van de Wouw et al., 2018). Functional module predictions showed higher GHB degradation following the MDP intervention. GHB is both a precursor and metabolite of GABA, which acts as a neurotransmitter in the CNS, and can cross the BBB (van Noorden et al., 2016). Lower levels of DOPAC (a dopamine metabolite), and reduced dopamine degradation following the WD diet (Fig 4.8F) predicted lower dopaminergic signalling. This suggests decreased dopamine metabolism via the monoamine oxidase (MAO) pathway (Graves et al., 2020) indicating lower dopamine turnover following the WD intervention, which could be one of the potential gut microbial mediators affecting mental health.

Executive function refers to a group of high-order cognitive abilities such as working memory, inhibitory control, cognitive flexibility, planning, reasoning and problem solving

(Cristofori et al., 2019). We observed a within MDP improvement in executive function following 5 days of intervention. Data from the EPIC-Norfolk study suggests higher mediterranean diet adherence to be associated with better global cognition, verbal episodic memory and simple processing speed (Shannon et al., 2019). In a recent study, participants showed faster processing speed and improved executive functioning on days following higher than usual adherence to the mediterranean diet (Parker & Hamm, 2025). One potential mechanism by which the MDP may enhance executive functioning is through modulation of the gut microbiota. In our findings, executive functioning was negatively correlated with the abundance of *Romboutsia*, a genus that is involved in bile acids metabolism (Connell, Le Gall, et al., 2024). A high quantity of dietary fibre is known to bind bile acids, preventing them from reabsorption (Zochling et al., 2025), potentially shifting the gut microbiota abundance towards lower *Romboutsia* levels and reduced tryptophan metabolism. *Romboutsia* was also negatively correlated with the overall cognitive scores (Fig 4.7C).

Regarding the faecal metabolomic profile, no significant differences were observed between the two dietary interventions (Fig 4.8A). Faecal levels of NAG and malate significantly dropped after the MDP intervention, and were positively correlated with anxiety, depression and TMD scores (Fig 4.8E). As an intermediate of tricarboxylic acid cycle, malate has previously been reported to show a negative association with functional brain connectivity in regions involved in emotional regulation (Coley et al., 2021). NAG is a key constituent of the intestinal mucin, which serves as a substrate for *Akkermansia* species. Chronic supplementation with *Akkermansia muciniphila* has been shown to restore intestinal barrier function and alleviate anxiety-related behaviours by promoting a healthier mucus layer (Coletto et al., 2023). In our study, we observed significantly lower faecal NAG levels following the MDP intervention. This reduction may reflect increased utilisation of NAG by elevated *Akkermansia* populations, or reduced mucin degradation, both of which are consistent with improved intestinal barrier integrity. A compromised intestinal barrier contributes to “leaky gut”, which weakens the containment of microbial products, allowing them to leak from the intestine into the circulation, which results in chronic low-grade inflammation which is observed in various psychological and neurological disorders (Rutsch et al., 2020).

Overall, our study is the first RCT to report that short-term high-fibre MDP intervention significantly improved anxiety and depression as well as executive functioning in adults with mild to moderate depression or anxiety. These effects were potentially driven by increased abundance of SCFA-producing bacteria and strengthened intestinal mucosal barrier integrity by reduced mucin degradation.

## **Chapter 5: *APOE4* genotype shapes the role of dietary fibre in cognitive health through gut microbiota changes**

Thesis Marrium Liaquat

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Title: *APOE4* genotype shapes the role of dietary fibre in cognitive health through gut microbiota changes

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## 5.1 Introduction

The *APOE4* genotype is the strongest genetic risk factor for Alzheimer's disease (AD) and also for earlier stages like Mild Cognitive Impairment (MCI) (Krysio et al., 2006). Evidence suggests that *APOE4* genotype influences cognition even in pre-MCI state such as Subjective Cognitive Impairment (SCI) (Müller-Gerards et al., 2019). Systemic and central metabolic dysfunction associated with this neuropathology is evident often decades before any symptoms appear (Hoffman et al., 2019). *APOE4* carriers also present with a distinct gut-microbial diversity which potentially contributes to the pathological metabolic effects of *APOE4* genotype (Hoffman et al., 2019; Maldonado Weng et al., 2019; Parikh et al., 2020; Tran et al., 2019; Yanckello et al., 2022).

The intestinal microbiome (Cheung et al., 2019; Kolobaric et al., 2024; Liu et al., 2019) plays a key role in various physiological changes that contribute to cognitive decline via disrupted gastrointestinal function, weakened gut barrier integrity, and alterations in intestinal neurotransmitters and immune markers (Bosco & Noti, 2021), which bi-directionally communicate with the brain (Connell et al., 2022; Dalile et al., 2019). Therefore, the gut-microbiota is emerging as a tractable target to affect cognitive functions thorough microbiome-gut-brain axis mainly via microbial metabolites (Chakrabarti et al., 2022; Saji et al., 2019).

Microbiome research has unveiled the role of dietary fibres (DFs) in modulating the host microbiota and associated metabolites such as the short-chain fatty acids (SCFAs) in human health and disease (Cronin et al., 2021). Different types of DFs offer various metabolic benefits to the host, largely determined by their physicochemical properties, such as solubility, viscosity and fermentability, which also serve as the basis for many DF

classification systems (Bijkerk et al., 2004; Cronin et al., 2021). A key aspect of this relationship is understanding how each specific type of DF shapes the composition of the host's microbiota (Cronin et al., 2021). Neuroprotective diets like the Mediterranean diet (MedDiet), Dietary Approaches to stop hypertension (DASH) and the Mediterranean-DASH diet Intervention for Neurodegenerative Delay (MIND diet) have in common a high consumption of DF and fibre-rich foods like whole grain cereals, fruits, vegetables, legumes, and nuts. These dietary patterns modify various biological processes involved in cognitive decline including neuroinflammation, oxidative stress, hippocampal neurogenesis impairment, neurovascular dysfunction and gut microbiota dysbiosis (Cremonini et al., 2019).

The biological mediators driving these physiological effects remain largely unknown; SCFAs are the microbial metabolites from bacterial fermentation of DF in the intestine and are often considered key candidate mediators. SCFAs might be directly or indirectly involved in communication along the Microbiome-Gut-Brain axis because of their direct neuroactive properties or involvement in gut-brain signaling pathways through immune and endocrine systems (Dalile et al., 2019). One such SCFA, butyrate, has been reported to influence the release of serotonin and gut hormones in the enteric nervous system which can stimulate the vagus nerve and trigger endocrine signaling impacting brain function (Stilling et al., 2016). SCFAs also exert several effects locally to improve gut health such as the maintenance of intestinal barrier integrity and protection from intestinal inflammation. SCFAs can also cross the blood-brain barrier (BBB) via monocarboxylate transporters (MCTs) abundantly expressed on endothelial cells (Dalile et al., 2019) and potentially centrally active. Reduced abundance of SCFA-producing bacteria is associated with higher odds of AD pathology (Verhaar et al., 2021), with similar gut microbiota changes also reported in cognitive impairment (Li et al., 2019; Liu et al., 2019; Pan et al., 2021).

There is substantial evidence that *APOE4* genotype and cognitive 'health' are associated with the gut microbiome composition and metabolism, and DF is a promising substrate for modulating gut-bacterial diversity and ultimately cognitive function. Therefore, gut microbiota targeted interventions, such as increasing DF intake, may be a promising approach to promote beneficial changes in the gut microbiome. However, currently the

role of DF and DF-derived microbial metabolites (SCFAs), and their associations with cognitive status and *APOE4* genotype are unknown, and the subject of the current analyses.

Therefore, in this study, we investigate the associations between *APOE4* genotype status, DF intake, and SCFA profiles in those with Subjective Cognitive Impairment (SCI) or Mild Cognitive Impairment (MCI), compared to healthy controls.

## 5.2 Materials and Methods

### 5.2.1 Subjects and Methods

The study participants were recruited for the CANN (Cognitive Ageing, Nutrition and Neurogenesis) and COMBAT (The impact of Cranberries On the Microbiome and Brain in healthy Ageing sTudy) trials registered in clinicaltrials.gov as NCT02525198 and NCT03679533, respectively. The CANN study recruited 259 participants, aged  $\geq 50$  years, with SCI or MCI based on criteria developed by the National Institute of Aging-Alzheimer's Association, with no indication of clinical dementia (Irvine et al., 2018). Briefly, the classification of MCI was based on established criteria, requiring (1) self-reported memory decline over 2–3 years, (2) preserved functional independence, indicated by a Functional Activities Questionnaire score of less than 6, (3) absence of dementia, defined as a Montreal Cognitive Assessment score of 18 or higher (Nasreddine et al., 2005), and (4) no significant depression, determined by a Geriatric Depression Scale-15 score of less than 10 (Yesavage & Sheikh, 2008). Additionally, MCI classification required cognitive impairment of at least one standard deviation below the age- and education-adjusted mean on at least one neuropsychological test assessing memory (California Verbal Learning Test-II (Woods et al., 2006)), language (Boston Naming Test (Roth & Helm-Estabrooks, 2018)), visuospatial function (Figure Copy task of the Repeatable Battery for the Assessment of Neuropsychological Status (Randolph et al., 2010)), attention (Digit Span task, Forward or Backward, from the Wechsler Adult Intelligence Scale (Wechsler, 2019)), or executive function (Trail Making Test, Part A or B (Reitan, 1955)). Participants meeting all criteria except for cognitive impairment were classified as SCI. Cognitively healthy adults were selected from the COMBAT study as a control group. The COMBAT study recruited 60 adults, aged 50-80 years, with no subjective memory complaints as

assessed by the Cognitive Change Index (CCI) questionnaire (Flanagan et al., 2022). All groups (HC, SCI, MCI) were matched for age, sex and BMI. The participants were grouped based on *APOE* genotype, into *APOE4* carriers (E3/E4 and E4/E4, with E2/E4 excluded) and non-*APOE4* carriers (E3/E3 and E2/E3). The participants were split (independent of their cognitive status) into tertiles based on their DF consumption, into low (<15.03g/day), medium (15.03-20.85g/day), and high (20.85-41.35g/day) consumers. Faecal samples were collected during the baseline visit using the collection vessels (NHS-approved Easy sampler collection kit). The storage container was placed in a cool dry location prior to returning to the research facility at the earliest opportunity (i.e. at the study visit) and subsequently stored at -80°C until analysis.

### 5.2.2 Chemicals & Reagents

All SCFA standards, namely acetic acid (C2:0), propionic acid (C3:0), butyric acid (C4:0), isobutyric acid (C4:0), 2-methylbutyric acid (C5:0), valeric acid (C5:0), isovaleric acid (C5:0), caproic acid (C6:0) and isocaproic acid (C6:0) were purchased from Merck (UK). Acetic acid d-4, propionic acid d-2 and 2-isobutoxyacetic acid were used as internal standards. For derivatisation, 3-nitrophenylhydrazine hydrochloride (3-NPH), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and pyridine were purchased from Merck (UK). All the solvents used in the experimental procedures were of HPLC grade.

### 5.2.3 LCMS\MS based quantification of serum SCFAs

Sera short-chain fatty acids (SCFAs) were quantified by targeted metabolomics using Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) combined with Multiple Reaction Monitoring (MRM) mode. Extraction and derivatisation of SCFAs was adapted from literature and further modified (Dei Cas et al., 2020). 40 µL serum was diluted with 500 µL ice-cold methanol and incubated on dry ice for 15 mins. The samples were centrifuged (14800 rpm, 5 mins) and supernatants were filtered using 0.45 µm PTFE filters. The filtered extracts were evaporated until dryness using a Savant™ SpeedVac™ High-Capacity Concentrator (Cat. SC210A-230) and reconstituted with 40 µL of methanol. 20 µL of reconstituted sample was mixed with 20 µL of internal standard mix (20ppm ac-d4, pro-d2, isobutoxyacetic acid). For derivatisation, 10 µL of 3-NPH (~50mM

3-NPH solution) and 10  $\mu$ L of EDC (~50mM EDC solution) made in 7% pyridine (v/v methanol) were added to the reconstituted extracts and incubated at 37°C for 30 min in a standard incubator (Model B 28, Binder, Tuttlingen, Germany). The derivatisation reaction was quenched by adding 20  $\mu$ L of 0.1% formic acid before being run on the LC-MS/MS system.

The analytical system consisted of Waters Acquity UPLC equipped with Water Xevo TQ-S mass spectrometer that is controlled by MassLynx v4.2 software for data acquisition, analyses and data management. Chromatographic separation was achieved on Kinetex® 2.6  $\mu$ m XB-C18, 50 × 2.1 mm column (Phenomenex, Torrance, CA, USA). Column temperature was maintained at 60 °C with 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B, with a constant flow rate of 0.5 mL/min. The elution gradient began at 90% A, which linearly decreased to 50% at 6 mins. From 7 to 7.5 mins, solvent B was 100%, which was maintained at 10% hereafter until 13 mins.

#### 5.2.4 $^1\text{H}$ NMR Faecal Metabolomic Analysis

Faecal metabolites were analysed and quantified using  $^1\text{H}$  NMR metabolomics analysis. The preparation method was similar to that reported previously (Tran et al., 2019). Briefly, 50 mg of human faecal sample was mixed with 800  $\mu$ L of NMR buffer [0.26 g  $\text{Na}_2\text{HPO}_4$ , 1.44 g  $\text{K}_2\text{HPO}_4$ , 17mg TSP (sodium 3-(trimethylsilyl)-propionate- $\text{d}_4$  (3-Trimethylsilyl) propionic-2,2,3,3- $\text{d}_4$  acid sodium salt) in 100 ml deuterated water]. The samples were vortexed until completely homogenised. After mixing and centrifugation (15,000 g for 5 mins), 550  $\mu$ L of the supernatant was transferred to a 5-mm NMR tube for spectral acquisition. NMR spectra were recorded on a 600-MHz Bruker Avance spectrometer fitted with a 5-mm TCI proton-optimised triple resonance NMR inverse cryoprobe and a 24-slot autosampler (Bruker, Coventry, England). Sample temperature was maintained at 300 K (27 °C). Each spectrum consisted of 128 scans of 65,536 complex data points with a spectral width of 20 ppm (acquisition time 2.6 s).

The noesypr1d presaturation sequence was used to suppress the residual water signal with low power selective irradiation at the water frequency during the recycle delay ( $\text{D1} = 4\text{s}$ ) and mixing time ( $\text{D8} = 0.01\text{s}$ ). A 90° pulse length of 8.1  $\mu$ s was set for all samples.

Spectra were transformed with a 0.3-Hz line broadening and zero filling, manually phased, baseline corrected and referenced by setting the TSP methyl signal to 0 ppm. Metabolite identification and quantification was done using the software Chenomx (V 8.6).

#### 5.2.5 Microbial DNA extraction and 16S rRNA amplicon sequencing

Microbial DNA was extracted from 150-250mg faecal samples using QIAamp PowerFaecal Pro DNA kit (Qiagen) following the manufacturer's instructions. Extracted DNA samples were quantified using NanoDrop (ND-1000 spectrophotometer). Agarose gel electrophoresis was used for quality assessment, which distinguished DNA integrity, purity, fragment size and concentration. The V3-V4 hypervariable region of the 16S rRNA gene was amplified with Illumina NovaSeq 6000 PE250 platform. Sequence analysis was performed using the Uparse software (Uparse v7.0.1001), using all the effective tags. Sequences with  $\geq 97\%$  similarity were assigned to the same Operational Taxonomic Unit (OTU). A representative sequence for each OTU was screened for further annotation. Each representative sequence was aligned against the SSuRNA database of SILVA database 138 (Pontifex, Connell, et al., 2024). Alpha diversity was assessed using Chao1, Shannon and Simpson diversity indices whilst beta diversity was assessed using Aitchison distance.

#### 5.2.6 Dietary Assessment

Baseline dietary assessment was conducted using the validated European Prospective Investigation into Cancer and Nutrition FFQ (Irvine et al., 2018) and Scottish Collaborative Group FFQ version 6.6 (Hollis et al., 2017) for the CANN and COMBAT cohorts, respectively. Nutrient and fibre intake was established from the FFQ data using the seventh edition of McCance and Widdowson food composition tables (Roe et al., 2015).

#### 5.2.7 Statistical Analyses

Statistical analyses were performed using RStudio version 4.4.0 and GraphPad Prism version 10 (GraphPad Software, CA, USA). All data are presented as mean  $\pm$  standard deviation (SD) unless otherwise mentioned. After identifying outliers (ROUT method,

q=1%) metabolomics data from both serum LCMS/MS and faecal NMR analyses were log transformed and analysed using Mann-Whitney tests or two-way ANOVA, followed by Benjamini and Hochberg method for FDR correction. Spearman correlations were performed to assess associations among metabolites, dietary intake, and bacterial abundance.

Microbiome analyses were performed on CLR (centred log-ratio) transformed data after filtering the datasets excluding all genera and features with <10% prevalence, except for alpha diversity metrics where we are interested in rare taxa. Alpha diversity was measured after adjusting for rarefaction curves and a linear model approach was used to compare differences in alpha diversity metrics within a group, while accounting for the effect of other variables.

Beta diversity was reported after pairwise Permutational Multivariate Analysis of Variance (PERMANOVA) testing on Aitchison distance values (Euclidean distance over CLR-transformed values) to assess microbiota divergence between groups, allowing 1,000 permutations. Ordinations were built using Principal Component Analysis on Aitchison distance values, and the PCA plots present variance ( $R^2$ ) and p values for each plot (Fig 5.4). Gut-Brain Modules (GBMs) and Gut-Metabolic Modules (GMMs) were identified based on PICRUSt2 predictions, which are based on several gene family databases including Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs (KO), Enzyme Commission (EC) numbers and MetaCyc pathway abundances (Bastiaanssen et al., 2023). These PICRUSt2 outputs have been validated with various human microbiome datasets (Gavin M. Douglas et al., 2020). GBM modules are annotated for function, pathway, structure and potential to cross the intestinal epithelium and the blood-brain barrier, and their abundances were derived from an orthologue abundance table using Omixer Reference Pathways Mapper (Valles-Colomer et al., 2019). The same was done for GMMs (Vieira-Silva et al., 2016). GBM and GMM counts were CLR-transformed after removing the features with <10% prevalence, followed by fitting a linear regression model and FDR correction using Benjamini and Hochberg method.

Differential bacterial abundance analyses were performed using linear models built for each genus to test multiple transformations accounting for different data distribution. *P* values for all the contrasts were corrected for False Discovery Rate (FDR) using Benjamini

& Hochberg's procedure. q-values of 0.1 and 0.2 were used as a cut-off for statistical significance for genera and functional modules, respectively. CLR transformed data was reported for the final figures as presented.

## 5.3 Results

### 5.3.1 Participant characteristics

A total of 170 participants were included in the analyses, comparing 73 SCI and 60 MCI participants with 37 older healthy adults as controls (Table 5.1). The mean  $\pm$  SD age and BMI of the participants was  $65.8 \pm 6$ y and  $25.7 \pm 3.7$  kg/m<sup>2</sup>. Overall, 56% were female and 44% were *APOE4* carriers. The participants in each DF tertile group were matched for age, sex and BMI between *APOE4* and non-*APOE4* carriers, except Low DF consumer group (where they were not matched for sex, due to limited number of participants) (Supp Table 2).

**Table 5.1** | Baseline characteristics of the participants

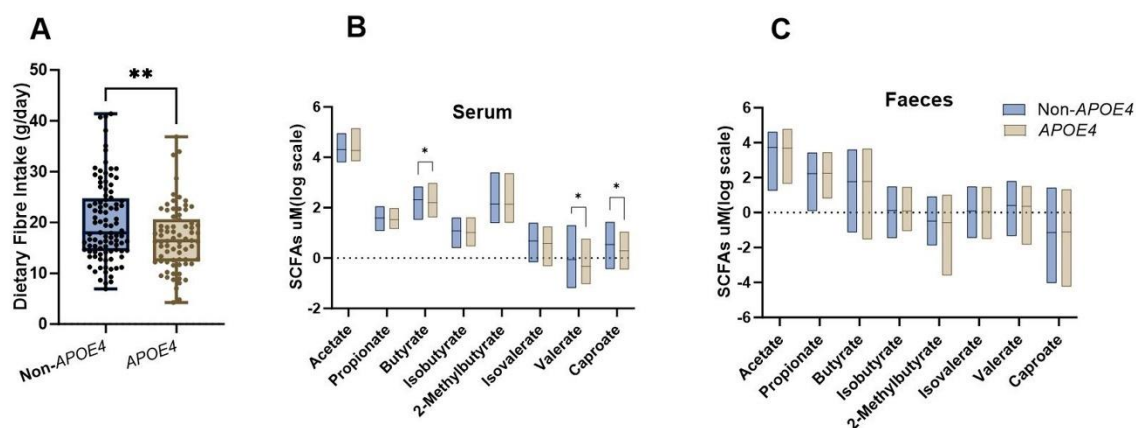
|                                   | Overall        | Low DF Consumers<br>(n=57)    |                              | Medium DF<br>Consumers (n=56) |                              | High DF Consumers<br>(n=57)   |                          |
|-----------------------------------|----------------|-------------------------------|------------------------------|-------------------------------|------------------------------|-------------------------------|--------------------------|
|                                   |                | Non- <i>APOE4</i><br>carriers | <i>APOE4</i><br>carrier<br>s | Non- <i>APOE4</i><br>carriers | <i>APOE4</i><br>carrier<br>s | Non- <i>APOE4</i><br>carriers | <i>APOE4</i><br>carriers |
| <b>n</b>                          |                | 29                            | 28                           | 28                            | 28                           | 39                            | 18                       |
| <b>HC</b>                         | 37             | 10                            | 3                            | 4                             | 2                            | 17                            | 1                        |
| <b>SCI</b>                        | 73             | 7                             | 14                           | 14                            | 19                           | 14                            | 5                        |
| <b>MCI</b>                        | 60             | 12                            | 11                           | 10                            | 7                            | 8                             | 12                       |
| <b>Age (y)</b>                    | 65.8<br>(6.0)  | 65.2<br>(6.7)                 | 64.8<br>(6.3)                | 66.4<br>(4.4)                 | 66.0<br>(6.4)                | 65.8<br>(6.1)                 | 66.6<br>(6.0)            |
| <b>Sex, M/F<br/>(%F)</b>          | 75/95<br>(56%) | 7/22<br>(76%)                 | 12/16<br>(57%)               | 15/13<br>(46%)                | 11/17<br>(61%)               | 20/19<br>(49%)                | 10/8<br>(44%)            |
| <b>BMI<br/>(kg/m<sup>2</sup>)</b> | 25.7<br>(3.7)  | 24.9<br>(3.0)                 | 28.0<br>(4.5)                | 25.1<br>(3.0)                 | 25.9<br>(4.6)                | 25.4<br>(2.8)                 | 25.2<br>(3.7)            |

Data are mean (SD) or as stated otherwise.

DF, dietary fibre; HC, healthy controls; SCI, subjective cognitive impairment; MCI, mild cognitive impairment

### 5.3.2 *APOE* genotype and cognitive status influence dietary fibre intake and circulating SCFAs

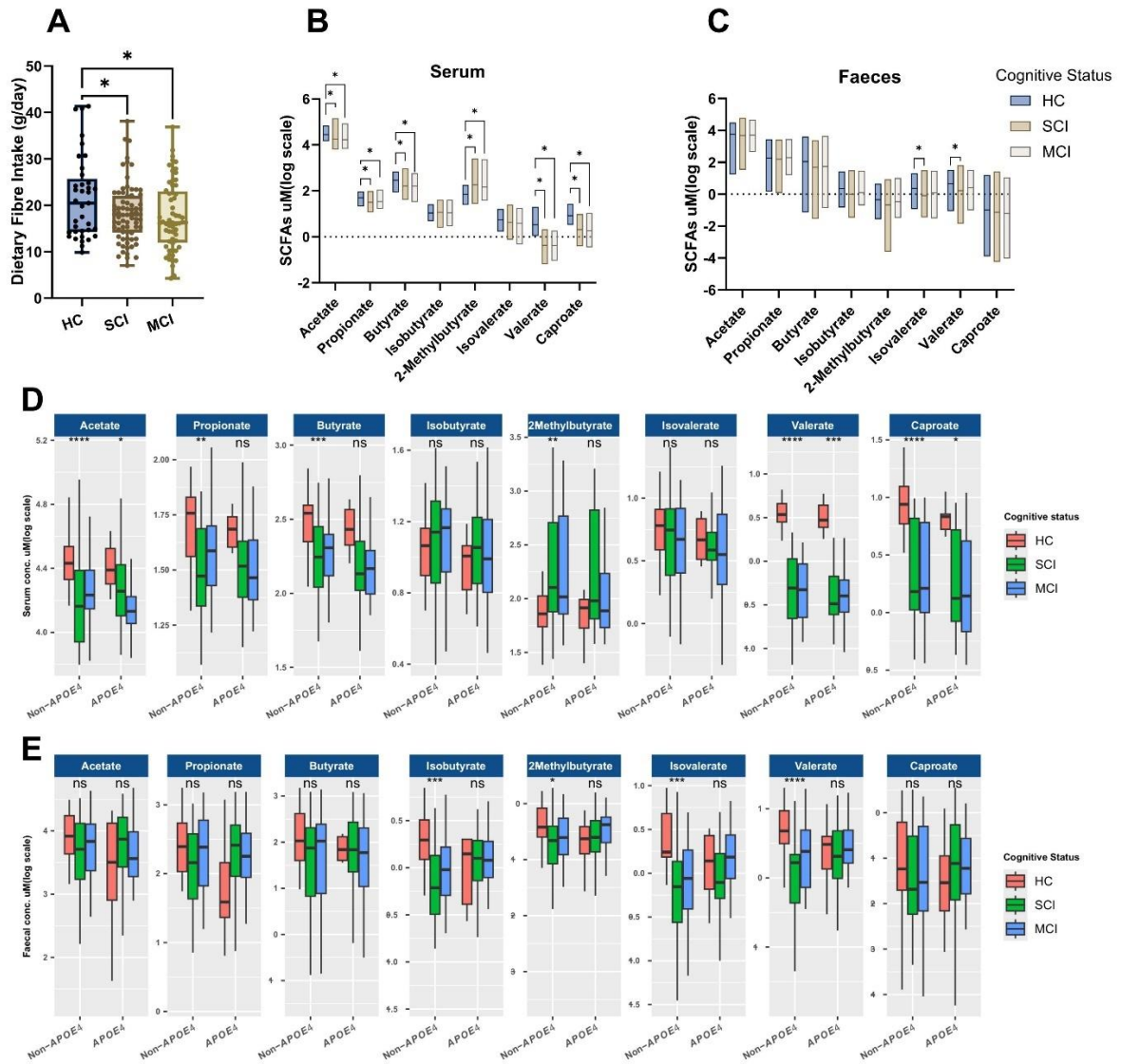
*APOE4* carriers consumed on average 16% less DF compared to non-*APOE4* carriers ( $F(1, 73) = 7.295$ ;  $p=0.009$ , Fig 5.1A), which corresponded with lower circulating SCFAs (Fig 5.1B). Specifically, serum levels of butyrate, valerate and caproate were significantly lower in *APOE4* carriers (Fig 5.1B). No differences were observed in faecal SCFAs concentrations between the genotypes (Fig 5.1C). DF consumption did not affect either serum (Supp Fig. 5.1A) or faecal SCFAs levels (Supp Fig. 5.1B) which confirmed that the observed variation in serum SCFAs between *APOE4* and non-*APOE4* carriers is independent of DF intake. Additionally, we were also interested to see whether the response to DF intake is *APOE4* genotype dependent. No significant associations between DF intake and either serum (Supp Fig 5.1C) or faecal (Supp Fig 5.1D) level of SCFAs were evident in either non-*APOE4* or *APOE4* carriers.



**Figure 5.1 | Association between *APOE4* genotype, dietary fibre (DF) intake and SCFAs concentrations.** A) DF intake (g/day) in non-*APOE4* and *APOE4* carriers. B) Serum and C) Faecal SCFAs concentrations ( $\mu\text{M}$  log scaled) in individuals with non-*APOE4* and *APOE4* genotype. Statistical comparisons were done by Mann-Whitney U tests and multiple comparisons were adjusted for false discovery rate (FDR). Data are represented as mean  $\pm$  standard deviation. \* =  $p<0.05$  and \*\* =  $p<0.01$ .

The cognitive status of individuals was also associated with DF consumption. Compared to SCI and MCI participants, healthy controls consumed 18% and 22% more DF, respectively ( $F(2, 167) = 3.568$ ;  $p=0.03$ ; Fig 5.2A). This was also mirrored by significantly lower serum levels of straight SCFAs ( $p<0.05$ ) in cognitively impaired individuals, including acetate, propionate, butyrate, valerate and caproate (Fig 5.2B). Contrarily, a branched form of butyrate, 2-Methylbutyrate was significantly higher ( $p<0.05$ ) in cognitively impaired individuals as compared to healthy controls (Fig 5.2B). Faecal levels of SCFAs were only significantly different for C-5 isovalerate and valerate ( $p<0.05$ ) between healthy and SCI individuals (Fig 5.2C). These differences in SCFAs were also independent of DF intake as confirmed for *APOE4* genotype.

To establish if *APOE4* genotype influences how cognitive status relates to SCFA levels, we split the cohort based on their *APOE4* genotype and observed that, non-*APOE4* carriers had more significant associations between serum SCFAs and cognitive status, as compared to *APOE4* carriers (Fig 5.2D). Cognitive status was also associated with faecal SCFAs differences in non-*APOE4* carriers only, for isobutyrate, 2-methylbutyrate, isovalerate and valerate (Fig 5.2E).

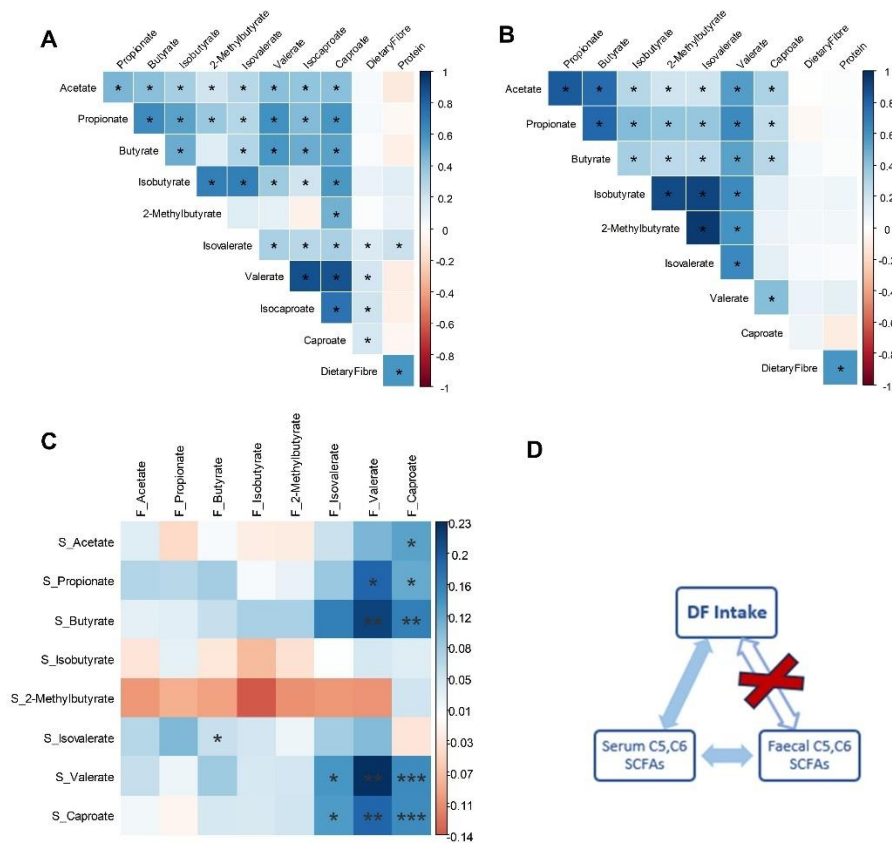


**Figure 5.2 | Association between cognitive status, *APOE4* genotype, dietary fibre (DF) intake and SCFAs concentrations.** A) DF intake (g/day) in healthy controls (HC), subjective cognitive impairment (SCI) and mild cognitive impairment (MCI) participants. B) Serum and C) Faecal SCFAs concentrations ( $\mu\text{M}$  log scaled) in HC, SCI, and MCI participants. D) Serum SCFAs concentrations ( $\mu\text{M}$  log scaled) in individuals with non-*APOE4* and *APOE4* genotype and split based on their cognitive status. E) Faecal SCFAs concentrations ( $\mu\text{M}$  log scaled) in individuals with non-*APOE4* and *APOE4* genotype and split based on their cognitive status. Statistical comparisons were done by Kruskal-Wallis test and significance levels are presented on the plots; ns = non-significant ( $p > 0.05$ ), \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .

### 5.3.3 Faecal SCFAs do not reflect circulating SCFA levels and dietary fibre intake

No significant difference in either serum or faecal levels of SCFAs based on DF intake was observed (Supp Fig S5.1 A, B). DF intake was not correlated ( $p>0.05$ ) with either serum (Fig 5.3A) or faecal (Fig 5.3B) SCFAs; except with serum isovaleric ( $R^2=0.16$ ,  $p<0.05$ ), valeric ( $R^2=0.19$ ,  $p<0.05$ ), isocaproic ( $R^2=0.20$ ,  $p<0.05$ ) and caproic ( $R^2=0.17$ ,  $p<0.05$ ) acids. Protein as the primary precursor of branched SCFAs, was positively correlated with serum isovaleric levels ( $R^2=0.21$ ,  $p<0.05$ , Fig 5.3A) only. No correlations were observed between protein or DF intake and faecal SCFAs.

Faecal C5-C6 SCFAs were correlated ( $p<0.05$ ) with serum C5-C6 SCFAs which is supported by their minimal utilization in the gut and liver. However, these faecal C5-C6 SCFAs are also positively correlated with serum C2-C4 (acetate, propionate and butyrate) SCFAs (Fig 5.3C), which is possibly due to metabolism of serum C5-C6 SCFAs to serum C2-C4 SCFAs, which represents an indirect association between serum C5, C6 SCFAs and DF intake (Fig 5.3D).



**Figure 5.3 | Serum and faecal SCFAs correlation with dietary fibre (DF) and protein intake.** A) Serum SCFAs correlation with DF and protein intake; B) Faecal SCFAs correlation with DF and protein intake. C) Correlation between serum and faecal SCFAs. Spearman correlations are plotted, and significant correlations are annotated as  $*$ = $p<0.05$ ,  $**$ = $p<0.01$ , and  $***$ = $p<0.001$ . D) Association between DF Intake and C5, C6 SCFAs, blue arrows indicate significant positive association and red cross (x) indicates non-significant association.

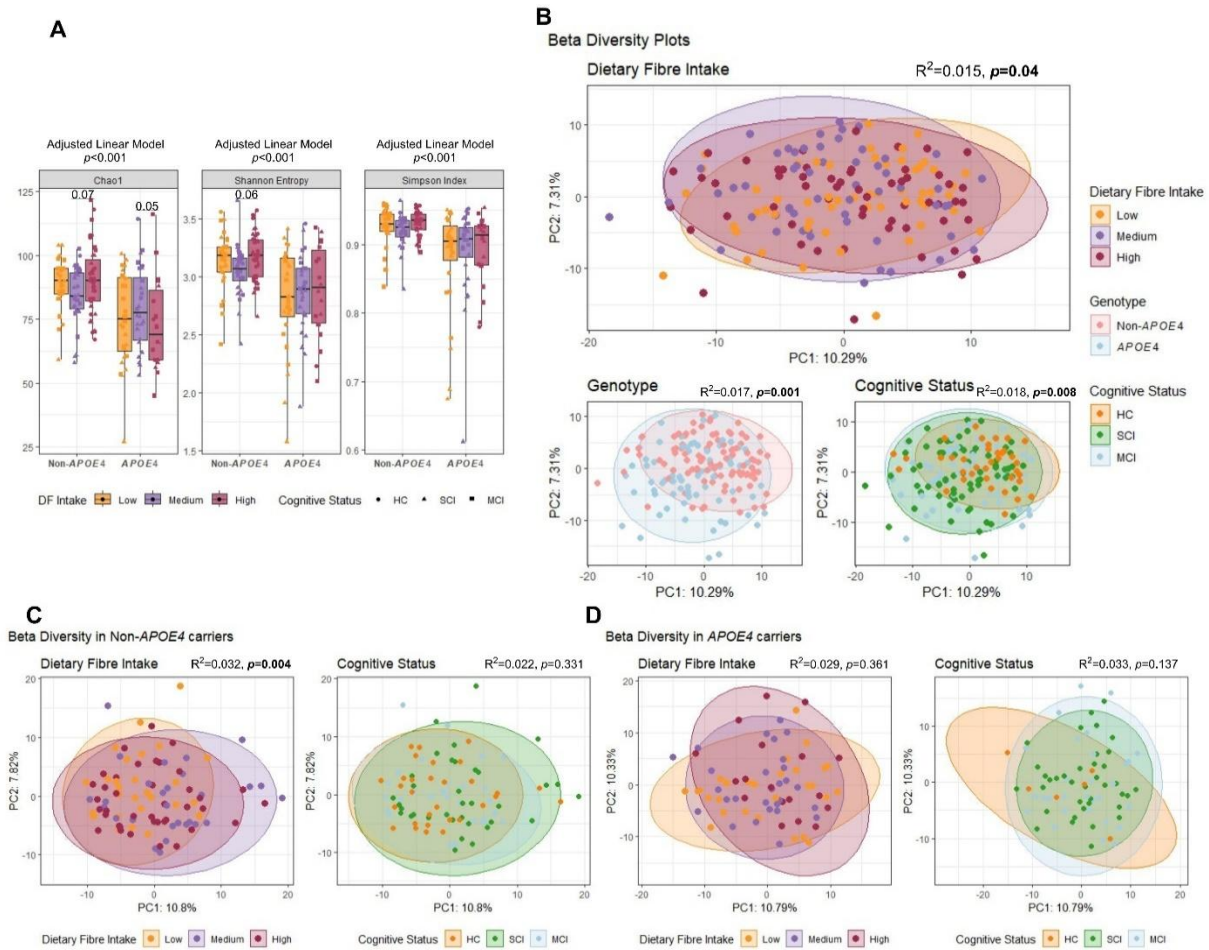
#### 5.3.4 Differential influence of *APOE4* genotype and DF intake on gut microbiota and circulating SCFAs

*APOE4* carriers had significantly lower alpha diversity, as measured by Chao1 ( $p<0.001$ ), Shannon Entropy ( $p<0.001$ ) and Simpson index ( $p<0.001$ ), compared to non-*APOE4* carriers (Fig 5.4A). PERMANOVA analysis demonstrated that beta diversity was also significantly influenced by *APOE4* genotype ( $R^2=0.017$ ;  $p=0.001$ , Fig 5.4B).

Non-*APOE4* carriers responded differently to DF intake as reflected by their  $\alpha$  and  $\beta$ -diversity indices. Although, DF intake influenced species richness (measured by Chao1 index) in both non-*APOE4* ( $p=0.07$ ) and *APOE4* ( $p=0.05$ ) genotypes, species evenness (measured by Shannon Entropy) was only influenced in non-*APOE4* carriers (albeit not significantly,  $p=0.06$ ) (Fig 5.4A). Participants with medium DF consumption exhibited higher  $\alpha$ -diversity (Chao1 and Shannon Entropy indices) among *APOE4* carriers and lower  $\alpha$ -diversity among non-*APOE4* carriers compared to low or high DF consumers (Fig 5.4A).

$\beta$ -diversity was also significantly influenced by DF intake ( $R^2=0.015$ ;  $p=0.04$ ; Fig 5.4B) and pairwise PERMANOVA identified a significant difference between medium and high DF consumers ( $R^2=0.013$ ;  $p=0.04$ ) only. However, splitting the cohort based on their *APOE* genotype showed that the influence of DF intake on  $\beta$ -diversity was solely within the non-*APOE4* carrier subgroup ( $R^2=0.032$ ;  $p=0.004$ ; Fig 5.4C) versus *APOE4* carriers ( $R^2=0.029$ ;  $p=0.361$ ; Fig 5.4D). Non-*APOE4* carriers had a significant difference between low vs medium DF consumers ( $R^2=0.03$ ;  $p=0.005$ ) and medium vs high DF consumers ( $R^2=0.03$ ;  $p=0.02$ ).

$\beta$ -diversity was also significantly influenced by cognitive status ( $R^2=0.018$ ;  $p=0.008$ ) (Fig 5.4B). PERMANOVA analysis highlighted that HC showed significant difference in  $\beta$ -diversity compared to cognitively impaired groups; HC vs SCI ( $R^2=0.02$ ;  $p=0.003$ ) and HC vs MCI ( $R^2=0.02$ ;  $p=0.03$ ). However, there was no difference between SCI and MCI. *APOE4* genotype had no influence on the association of cognitive status in changing  $\beta$ -diversity (Fig 5.4C, D).

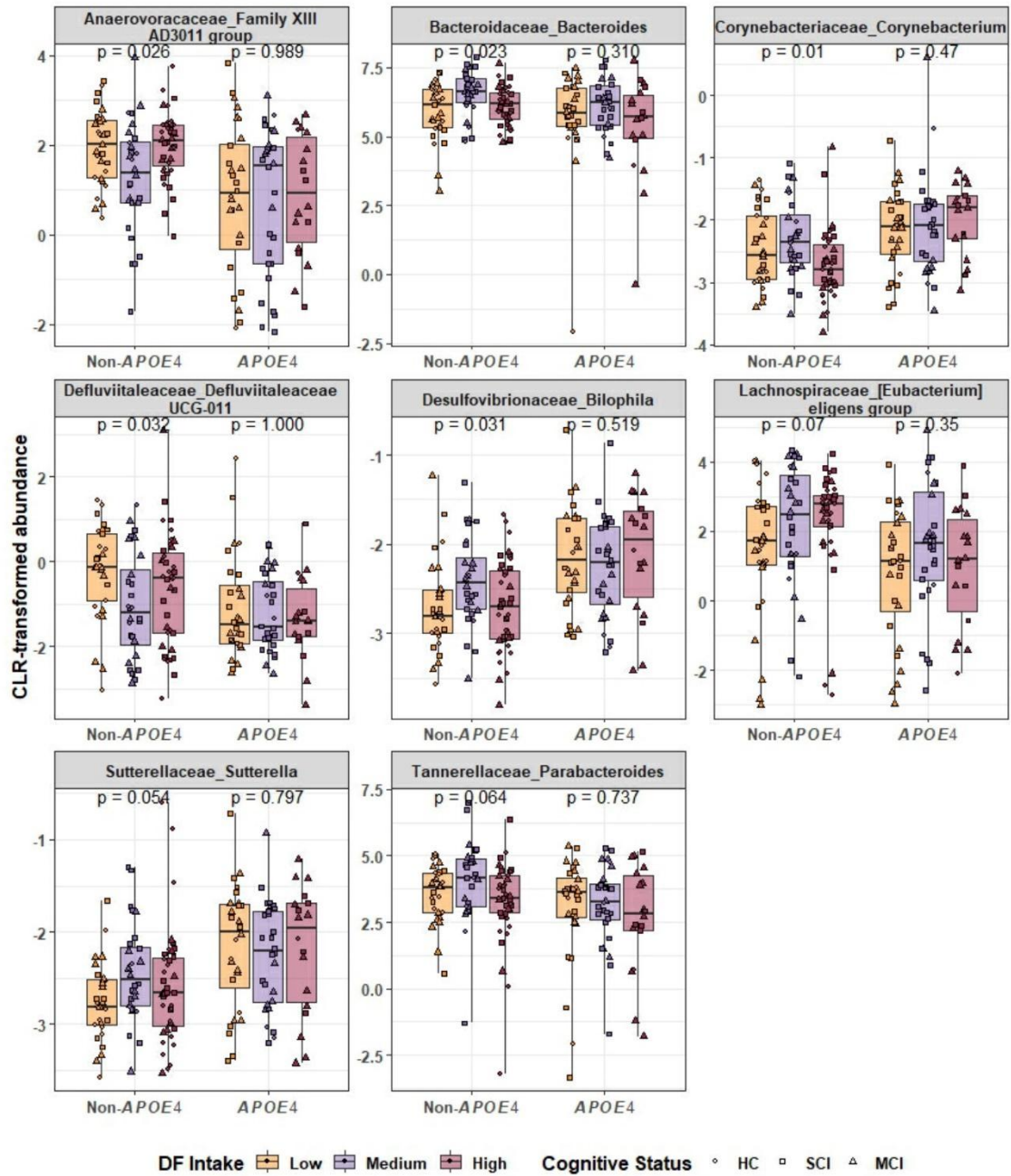


**Figure 5.4 | Alpha and beta diversity indices and associations between *APOE4* genotype, cognitive status, and dietary fibre (DF) intake.** A) Alpha diversity indices including Chao1, Shannon Entropy and Simpson Index, B) PCA plots presenting  $\beta$ -diversity changes based on dietary fibre (DF) intake, genotype, and cognitive status, C) PCA plots presenting  $\beta$ -diversity in non-*APOE4* carriers based on DF intake and cognitive status. D) PCA plots presenting  $\beta$ -diversity in *APOE4* carriers based on DF intake and cognitive status. Linear regression model was used for alpha diversity analyses between genotypes while adjusting for participants' cognitive status and DF intake; p values are plotted on top of the plots. Alpha diversity comparisons within each genotype were adjusted for participants' cognitive status and select p values reported on the plots. PERMANOVA analyses were performed on Aitchison distance values for  $\beta$ -diversity; variance ( $R^2$ ) and p values are plotted on each PCA plot.

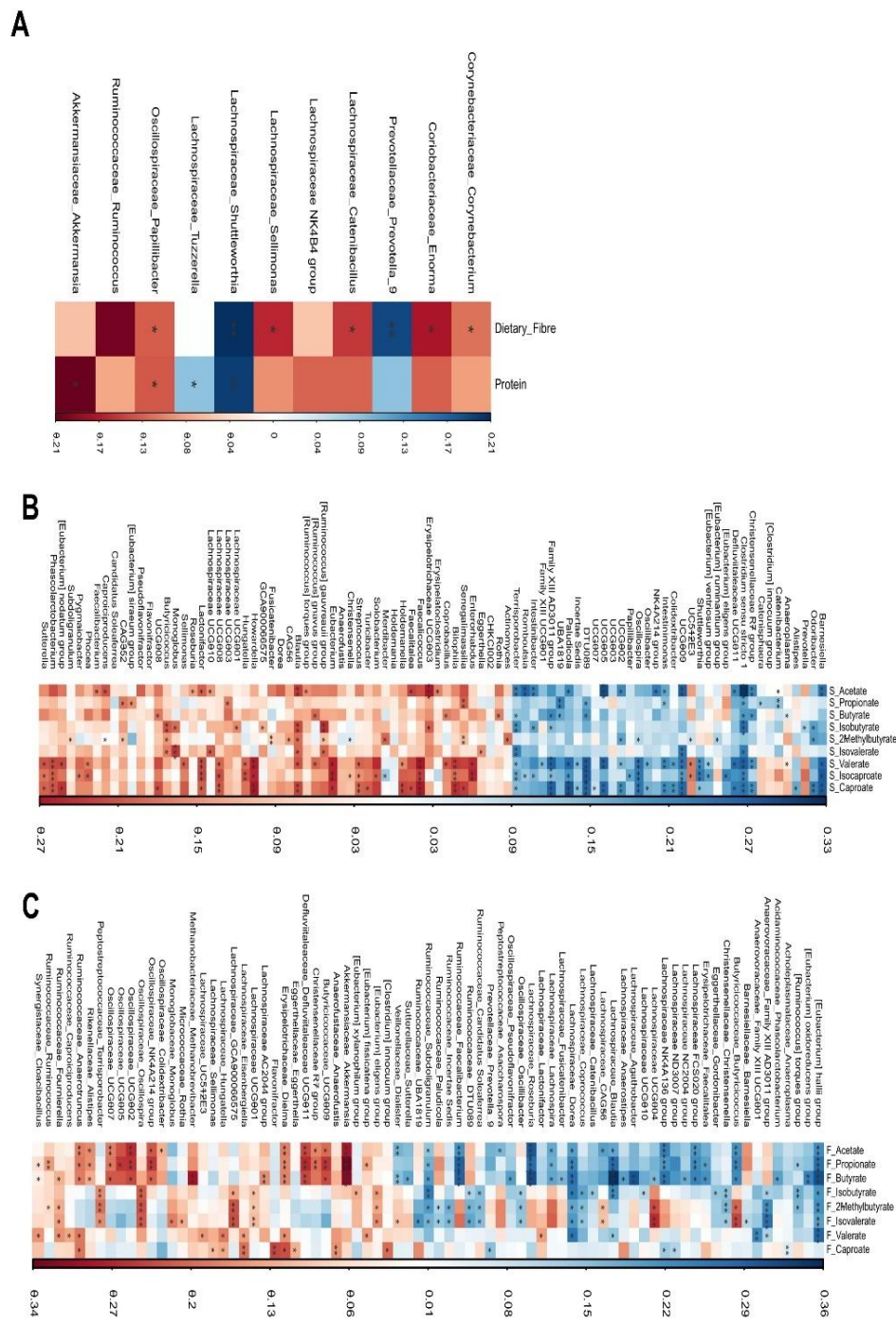
Various bacterial genera were differentially abundant between non-*APOE4* and *APOE4* carriers (Supp Table S5.1), which is consistent with the significant  $\beta$ -diversity differences between both genotypes. This differential bacterial abundance was affected by DF intake (Supp Fig S5.2), with significant changes observed within non-*APOE4* carriers only (Fig 5.5). *Bacteroides* and *Parabacteroides* were higher in non-*APOE4* carriers (FDR=0.04 and 0.03, respectively, Supp Table 5.1). *Bacteroides* genera is one of the most common DF degraders in the gut; and was significantly ( $p=0.02$ ) higher in higher DF tertiles in non-*APOE4* carriers only, although this increase was not linear (Fig 5.5). A comparable opposite trend was observed in *Parabacteroides* abundance ( $p=0.06$ ) in non-*APOE4* carriers only (Fig 5.5). In contrast, higher DF intakes were associated with a lower abundance of *Corynebacterium* ( $p=0.01$ ) and *Defluviitaleaceae UCG-011* ( $p=0.03$ ) in non-*APOE4* carriers, whereas no significant difference was observed in *APOE4* carriers. *Sutterella* and *Bilophila* levels were also lower in non-*APOE4* compared to those with the *APOE4* allele. In non-*APOE4* carriers, both these genera showed significantly higher but non-linear abundance with higher DF intake (Fig 5.5).

DF intake was negatively correlated with the presence of several bacterial genera ( $p<0.05$ ), including *Corynebacterium*, *Enorma*, *Catenibacillus*, *Sellimonas* and *Papillibacter* (Fig 5.6A). However, *Prevotella* and *Shuttleworthia* abundance increased ( $p<0.05$ ) with an increase in DF intake (Fig 5.6A). Significant correlations were also found between several bacterial genera and circulating (Fig 5.6B), and faecal SCFA levels (Fig 5.6C). Correlations in serum were more significant ( $p<0.01$ ) for C5-C6 SCFAs, however in faeces C2-C4 (acetate, propionate and butyrate) showed stronger correlations ( $p<0.01$ ) than other SCFAs.

Major SCFA-producing bacteria including *Butyricicoccus*, *Dorea*, *Blautia* and *Sutterella* were positively ( $p<0.05$ ) correlated with SCFAs (C2-C4) in the faeces but negatively correlated with them in the serum ( $p>0.05$ ); except for *Butyricicoccus* which showed a consistent negative correlation ( $p<0.05$ ) with branched SCFAs (C4, C5) in both serum and faeces (Fig 5.6B, C).



**Figure 5.5 | Association between dietary fibre (DF) intake and abundance of bacterial genera within non-APOE4 and APOE4 carriers.** Kruskal-Wallis test was performed to compare the effect of DF intake within each genotype, p values are reported within individual boxplots.



**Figure 5.6 | Spearman correlations among bacterial genera, SCFAs and their precursors dietary fibres (DF) and proteins.** A) Bacterial genera significantly correlating with DF and protein intake B) Bacterial genera correlations with serum SCFAs C) Bacterial genera correlations with faecal SCFAs. Significant correlations are presented as \*= $p < 0.05$ , \*\*= $p < 0.01$  and \*\*\*= $p < 0.001$ .

### 5.3.5 Role of *APOE4* genotype and dietary fibre intake in altering Gut-Brain and Gut-Metabolic modules

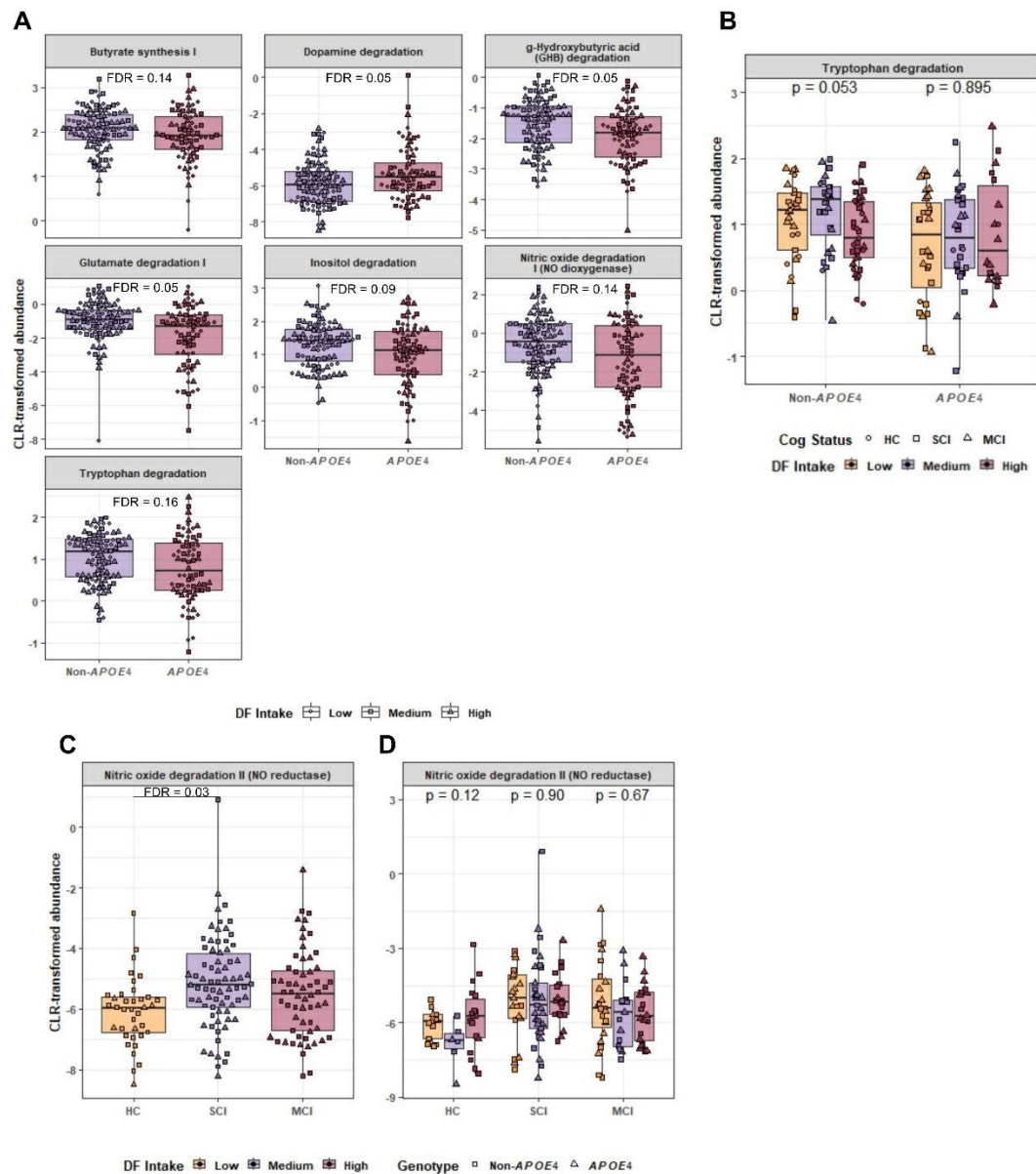
*APOE4* genotype was associated with significantly altered gut-brain modules (Fig 5.7A). *APOE4* carriers had significantly lower representation of butyrate synthesis I (FDR=0.14), and degradation of gamma-hydroxybutyric acid (GHB) (FDR=0.05), glutamate I (FDR=0.05), inositol (FDR=0.09), nitric oxide I (FDR=0.14) and tryptophan (FDR=0.13) as compared to non-*APOE4* carriers (Fig 5.7A). *APOE4* carriers showed a higher predicted degradation of dopamine as compared to non-*APOE4* carriers (FDR=0.05). These pathways were confirmed by significant KO enzyme differences in both *APOE4* and non-*APOE4* carriers (Supp Fig S5.3).

DF intake did not show any significant role in altering these pathways within either genotype, but a trend towards lower tryptophan degradation ( $p=0.05$ ) was observed with high DF intake in non-*APOE4* carriers only (Fig 5.7B). The cognitive status of the participants was significantly associated with nitric oxide degradation via NO reductase; and SCI participants reported the highest comparative degradation (Fig 5.7C), however this was not affected by DF intake (Fig 5.7D). Several Gut-Metabolic modules (GMMs) were also significantly influenced by *APOE4* genotype (Supp Fig S5.4) and DF intake (Supp Fig S5.5).

### 5.3.6 *APOE4* carriers show negative association between serum SCFAs and their predicted synthesis in the gut

Functional predictions from the PICRUSt2 output showed lower levels of butyrate synthesis in *APOE4* carriers (Fig 5.7A), confirmed by significantly lower predicted levels of essential enzymes involved in butyrate synthesis i.e., butyryl CoA dehydrogenase, phosphate butyryl transferase and butyrate kinase (Supp Fig 5.3). *APOE4* carriers showed a negative association between serum acetate, propionate and butyrate, and the predicted capacity of gut microbiota to produce these SCFAs (Supp Fig 5.6). In *APOE4* carriers, serum acetate concentration was negatively ( $p<0.05$ ) associated with the GBM acetate synthesis I (representing acetate synthesis from acetyl-CoA I), whereas non-*APOE4* carriers showed a slightly positive association (Supp Fig 5.6).

*APOE4* carriers also showed similar negative association for serum propionate ( $p=0.05$ ) and butyrate ( $p=0.04$ ) with propionate synthesis II (synthesis from acetate and lactate via propanoyl-CoA) and butyrate synthesis II (synthesis from acetate and butanoate via crotonyl-CoA and Acetyl-CoA), respectively, whereas non-*APOE4* carriers showed positive associations (Supp Fig 5.6). Linear regression analysis was performed to explore these relationships, and no significant associations were observed between faecal levels of SCFAs and their predicted synthesis by gut bacteria.



**Figure 5.7 | Differentially abundant Gut-Brain Modules (GBMs) and their association with dietary fibre (DF) intake.** A) Differentially abundant Gut-Brain Pathways between non-*APOE4* and *APOE4* carriers. Linear regression analyses were performed on centered log ratio (CLR) transformed data. p values are reported following Benjamini & Hochberg FDR correction. B) Tryptophan degradation in non-*APOE4* and *APOE4* carriers based on DF intake. Kruskal-Wallis tests were used to compare means based on DF intake within each genotype and p values are mentioned on the plot. C) Nitric oxide degradation in SCI (Subjective Cognitive Impairment) and MCI (Mild Cognitive Impairment) participants vs HC (Healthy Controls). Linear regression analysis was performed, and p values are reported following Benjamini & Hochberg FDR correction. D) Nitric oxide degradation in SCI and MCI participants vs HC based on DF intake. Kruskal-Wallis tests were used to compare means based on DF intake within each cognitive group. Non-*APOE4* and *APOE4* carriers are presented with different shapes in both plots.

## 5.4 Discussion

*APOE* genotype influences various physiological processes (Iacono & Feltis, 2019; Martinez-Martinez et al., 2020), including gut microbiota composition (Maldonado Weng et al., 2019; Tran et al., 2019) and microbe-associated-metabolites like SCFAs (Tran et al., 2019; Zajac et al., 2022). However, its interaction with dietary intake and metabolism, particularly DF (a primary substrate for gut microbiota), remains unclear. Our study is the first to suggest that DF intervention as a microbiota-targeted approach may help prevent cognitive impairment, but only in non-*APOE4* carriers.

*APOE4* carriers had lower DF intake, possibly due to *APOE*'s role in metabolism (Norwitz et al., 2021), which may influence dietary preferences resulting in *APOE4* carriers having an aversion for certain foods including those rich in DF. Alternatively, a differential gut microbiota could drive these dietary behaviour changes (Medawar et al., 2021). We observed differential gamma-hydroxybutyric acid (GHB) degradation between *APOE4* and non-*APOE4* carriers, which is known to influence dietary behaviours (Goodwin et al., 2005; Ottani et al., 2007). Further research is needed to understand the mechanisms behind reduced DF intake in *APOE4* carriers.

It is important to establish if the differences in DF consumption also drive variations in DF-derived metabolites like SCFAs. Previous research on DF intake and SCFA levels has been inconsistent, with some studies finding correlations while others do not. Vinelli and colleagues conducted a systematic review of 44 studies and found that 26 of these studies reported no significant differences in individual SCFA levels (Vinelli et al., 2022). SCFAs profile depends on the dose (Deehan et al., 2020; Deroover et al., 2017), and type and structure of DF consumed (Deehan et al., 2020; den Besten et al., 2013; Vinelli et al., 2022), and a lack of fermentable fibres may shift fermentation towards protein, producing branched-chain fatty acids (Koh et al., 2016). Other bioactives, like polyphenols also influence SCFAs production by shaping gut microbiota (Zhang et al., 2022). We focused mainly on DFs as primary precursors for SCFAs, and our analysis suggests that the differences in serum butyrate, valerate and caproate between *APOE4* and non-*APOE4* carriers are independent of DF intake, aligning with our previous findings on genotype-associated SCFAs variations (Tran et al., 2019).

Previous studies, including our own, have linked gut microbial diversity to the *APOE* genotype (Hoffman et al., 2019; Parikh et al., 2020; Tran et al., 2019; Zajac et al., 2022). Our findings further reveal distinct gut bacterial diversity between *APOE4* and non-*APOE4* carriers, potentially explaining the lower SCFA-producing bacteria and serum SCFA levels in *APOE4* carriers. The predicted SCFA-producing capacity of the gut microbiota was significantly associated with serum acetate, propionate and butyrate levels, but this relationship differed by *APOE4* genotype. In non-*APOE4* carriers, microbial SCFA synthesis aligned with serum SCFA levels, whereas *APOE4* carriers showed an opposite trend (Supp Fig 5.6). This suggests metabolic differences between *APOE4* and non-*APOE4* carriers, warranting further investigation into the underlying mechanisms.

Strong evidence supports the role of DF in shaping gut microbiota (Cronin et al., 2021; Deehan et al., 2020), yet its interaction with *APOE4* genotype remains unclear. Our findings show that DF intake significantly correlates with gut microbial diversity only in non-*APOE4* carriers, suggesting that metabolic disruptions linked to *APOE4* genotype (Carvalho-Wells et al., 2012; Dallongeville et al., 1992) may influence DF metabolism. Further analysis highlights significant bacterial differences in response to DF intake exclusively in non-*APOE4* carriers, reinforcing the idea that *APOE4* carriers are relatively unresponsive to DF-driven gut microbiota changes.

Higher DF intake in non-*APOE4* carriers is associated with decrease in *Corynebacterium*, a bacteria linked to conditions such as depression (Park et al., 2023), schizophrenia (Li et al., 2020), and autism spectrum disorders (Strati et al., 2017). DF intake also correlated with *Defluviitaleaceae* UCG-011 in non-*APOE4* carriers, which may help maintain cognitive function through the gut-brain axis (Cui et al., 2023). Additionally, *Bilophila*, *Eubacterium eligens*, *Sutterella*, *Bacteroides* and *Parabacteroides* were associated with DF intake in non-*APOE4* carriers. *Eubacterium eligens* group has been linked to improved cognitive function and increased SCFA production (Mukherjee et al., 2020), while *Parabacteroides*, known for acetate production helps prevent inflammation (Lei et al., 2021). The changes in bacterial abundances were not always linear, potentially due to differences in DF composition, which we could not account for due to limited data availability, as both cohorts used different FFQs which used AOAC fibre and NSP fibre for DF quantification in CANN and COMBAT cohorts, respectively.

DF-derived bacterial changes in non-*APOE4* carriers predict better cognitive health. We found that non-*APOE4* carriers are more responsive to changes in DF intake and subsequently SCFA levels in SCI and MCI compared to HC. Further research is needed to determine whether these differences stem from distinct microbiota between *APOE4* and non-*APOE4* carriers or unique metabolic mechanisms that shape microbiota composition. DF-derived SCFAs modulate the intestinal permeability (Cammann et al., 2023) which could be another potential contributor to gut microbiota linked cognitive decline in *APOE4* carriers. DF-derived SCFAs support microbial cross-feeding, influencing overall gut metabolism (Morrison & Preston, 2016). Nitric oxide (NO), a key microbial metabolite, is involved in neuronal signalling (Lundberg & Weitzberg, 2022), synaptic plasticity, memory function (Hardingham et al., 2013), and cerebral blood flow regulation (Carter et al., 2021). Elevated NO degradation in SCI group may contribute to cognitive decline, potentially preventable by enhancing NO production. Gut microbiota can mediate NO production via dissimilatory nitrate reduction to ammonia (DNRA) pathway (Vermeiren et al., 2009), and we observed a significant increase in dissimilatory nitrate reduction with higher DF intake (Supp Fig. S5.5A). While this was not associated with cognitive status or *APOE* genotype (Supp Fig. S5.5B), we propose that high DF intake might support NO production, potentially counteracting NO degradation in SCI through SCFA-driven cross-feeding.

Moreover, reduced straight SCFAs have been reported in the faeces of MCI individuals vs healthy controls; and has been suggested as early diagnostic biomarkers for MCI and potential targets for disease prevention (Gao et al., 2023). Most of the other studies also report lower faecal SCFA levels in neurocognitive disorders like anorexia nervosa, Parkinson's disease (PD), AD and depression (O'Riordan et al., 2022). However, faecal concentrations of SCFAs are neither a true representative of SCFA production nor circulating levels, as confirmed by Chen et al., in patients with PD who presented lower faecal but higher plasma concentrations of acetate (S. J. Chen et al., 2022). This low concordance between faecal and serum SCFAs has also been reported in other cohorts (TwinsUK and ZOE PREDICT) (Nogal et al., 2023). Moreover, we observed that individuals with differing cognitive statuses exhibited more pronounced differences in serum SCFA concentrations compared to faecal SCFA levels. Although most of the literature reports

faecal levels of SCFAs, we did not find any correlation between faecal SCFA and DF intake. Interestingly, serum C5 and C6 SCFAs showed significant positive correlation with DF intake, proposing that these SCFAs may not be entirely metabolised in the gut or the liver. Hence their elevated serum levels potentially reflecting higher DF consumption, higher production in the gut and ultimately faster absorption in the circulation. The majority of SCFAs produced in the gut are utilized locally as an energy substrate by gut epithelial cells with the remainder absorbed into the portal circulation, where most of the C2-C4 SCFAs are metabolized in the liver (Dalile et al., 2019). These results align with previous findings reported by Cummings and colleagues, which showed that the levels of acetate, propionate, and butyrate are approximately five times lower in peripheral venous blood (79  $\mu\text{mol/l}$ ) compared to portal blood (375  $\mu\text{mol/l}$ ) (Cummings et al., 1987), indicating significant utilization of these SCFAs in the liver. However, it proposes that SCFAs (C5-C6) tend to enter the systemic circulation (as indicated by the positive correlation between serum and fecal levels), where they are eventually metabolized into smaller SCFAs (C2-C4) which have the most physiological effects.

Among all SCFAs, butyrate has the greatest impact at physiological level, thus posing higher relevance for butyrate producing bacteria (Fusco et al., 2023). *Lachnospiraceae* and *Ruminococcaceae* families have bacteria mainly known for butyrate production (Fusco et al., 2023), but also produce other SCFAs like other genera including *Roseburia* (Nie et al., 2021), *Blautia* (Freitas et al., 2022), *E.hallii* (Engels et al., 2016; Udayappan et al., 2016), and *Faecalibacterium* (Martin et al., 2023). We observed these genera to be positively correlated with faecal SCFAs, but contrarily, *Roseburia*, *Blautia* and *Faecalibacterium* were negatively correlated with the serum levels of SCFAs. These conflicting associations with serum and faecal SCFA levels have also been reported by other studies (S. J. Chen et al., 2022; Wang et al., 2023), supporting that faecal SCFAs are not a true representation of physiologically circulating levels highlighting the need to focus more on systemic concentrations.

Complex gut-bacterial cross-feeding mechanisms may drive DF-induced microbiome shifts in non-*APOE4* carriers, potentially altering gut metabolism. For instance, we predicted significantly lower tryptophan degradation in *APOE4* carriers, which was further confirmed by a lower predicted abundance of tryptophanase enzyme in *APOE4*

carriers (Supp Fig 5.3). Tryptophan degradation is associated with the synthesis of serotonin and other metabolites involved in the modulation of neurotransmission and elimination of amyloid- $\beta$  peptides from the brain (S. J. Chen et al., 2022; Wang et al., 2023). Previous research has reported changes in tryptophan metabolism in non-*APOE4* mice in response to inulin supplementation, while no such alterations were noted in *APOE4* mice (Yanckello et al., 2022). It may therefore be speculated that the effect of DF on altered tryptophan metabolism may be independent of tryptophan content and rather dependent on DF-derived microbial cross-feeding of microbes involved in tryptophan metabolism (Huang et al., 2023), which are also shaped by the *APOE* genotype.

Our novel findings indicate that non-*APOE4* carriers tend to consume more DFs and are more responsive to DF related microbial changes, than *APOE4* carriers. DF-derived metabolites (i.e., SCFAs) and their associations with cognitive status were also found to be more strongly associated in non-*APOE4* carriers. Non-*APOE4* carriers show positive association with predicted SCFAs synthesis and their serum levels, as opposed to negative associations in *APOE4* carriers. These findings indicate that *APOE4* and non-*APOE4* carriers may follow different physiological pathways, necessitating further investigation to understand the genotype-dependent responses to DF intake and its metabolism.

Overall, we suggest that DF-induced changes in the microbiome and resulting serum SCFAs may be an effective strategy for preventing cognitive decline, but primarily in non-*APOE4* carriers.

However, some limitations should be stressed along with potential areas of future research endeavors. This is a single center study which may not represent the diversity of different populations and dietary patterns. In addition, a larger sample size would have greatly strengthened the validity of our results, however the level of significance and physiological trends observed offer a compelling reason for further research to consolidate these findings in a larger population. Also, our analysis includes total DF, it did not consider the individual types of dietary fibre, which may differentially affect the gut microbiota composition and subsequent metabolites. Lastly, controlled DF intervention studies could further clarify the causal or associative link among the *APOE4* genotype, gut microbiota, DF intake, related metabolites, and cognitive benefits.

## **Chapter 6: General Discussion**

Thesis Marrium Liaquat

This thesis aimed to investigate the role of DFs in the gut-microbiota-brain axis. Collectively, the findings demonstrate that DFs play an integral role in shaping the gut-microbiota-brain communication, and this crosstalk is influenced by non-modifiable factors like biological sex and *APOE4* genotype. This highlights the need for and the importance of personalised DF-based interventions to target the gut-microbiota-brain axis.

## 6.1 Main Findings

Across multiple experimental models, DF interventions consistently modulated the gut microbial composition and its metabolic output. To interpret these effects within the context of gut-microbiota-brain communication, it was essential to first establish baseline sex differences, as demonstrated in Chapter 2, which revealed clear male-female divergence in gut microbiota, gut metabolome and gut-microbiota-brain interactions. Building on this foundation, Chapter 3 examined how isolated DF interventions differentially shape these pathways. Inulin and psyllium were selected as complementary fibres due to their contrasting fermentability profiles, rapidly fermentable versus slowly fermentable, allowing a distinction between gut microbiota-dependent and -independent effects (e.g. stool bulking, changes in gut transit time etc). Inulin produced marked alteration in gut microbiota composition and metabolomic profiles in a distinctly sex-dependent manner, whereas psyllium induced significant but largely sex-independent changes. The combined inulin-psyllium intervention yielded mixed responses, highlighting potential interactions between fibre types. Collectively, these findings demonstrate that DFs are not functionally equivalent and that both DF fermentability and biological sex critically influence their downstream effects on gut-microbiota-brain communication. This underscores the importance of considering DF type and host sex when designing targeted DF-based interventions.

Beyond examining the effects of isolated DFs, it is essential to understand how DFs influence the gut-microbiota-brain axis when consumed as part of whole dietary patterns. Chapter 4 addressed this by investigating the impact of a high-fibre Mediterranean style diet compared with a low-fibre Western diet in adults experiencing mild to moderate level of anxiety and/or depression. Notably, adherence to the

Mediterranean diet for as little as five days was sufficient to induce beneficial shifts in the gut microbiota composition and metabolite profiles, which were associated with improvements in mental health outcomes. These findings highlight the rapid responsiveness of the gut-microbiota-brain axis to DF-rich dietary patterns in human populations.

Building on the investigation of DFs in individuals with mild to moderate mental health symptoms, Chapter 5 extended this work to cognitive health in older adults with SCI or MCI. Given the established role of *APOE4* genotype as a genetic risk factor for cognitive decline, this chapter specifically examined the DF consumption in relation to cognitive status stratified by *APOE4* carrier status. The findings demonstrate that habitual DF consumption is associated with cognitive performance in older adults, with these associations being more pronounced in non-*APOE4* carriers. In contrast, *APOE4* carriers showed no clear associations between DF intake and cognitive status. Together these results highlight the importance of considering *APOE4* genotype when targeting DF-based interventions which aim to modify the gut-microbiota-brain axis to support cognitive health.

## 6.2 Overall discussion of experimental chapters

There is growing body of literature evidence demonstrating that DFs are key modulators of the gut microbiota and associated metabolic output, with downstream implications for overall metabolic health and brain function (Dalile et al., 2019; Prokopidis et al., 2022; Shi et al., 2021; Yang et al., 2024). This thesis corroborates these observations across both animal and human models, while extending the existing knowledge by demonstrating that the influence of DFs on the gut-microbiota-brain axis is not uniform and is strongly shaped by biological sex, fibre fermentability, and host genotype.

Sex-specific responses to dietary interventions have been increasingly recognised recently (Pontifex, Vauzour, et al., 2024), but remain under-explored in gut-microbiota-brain research. Distinct sex-differences exist in microbiota composition (Hokanson et al., 2024; Ma & Li, 2019), and this thesis further explores the differences in microbiota derived metabolite profiles and gut-brain signalling pathways (Chapter 2). Importantly, this work advances the field by showing that fermentable DFs such as inulin amplify

these sex-specific responses, whereas slowly fermentable fibres such as psyllium diminishes any sex-specific effects (Chapter 3). This finding challenges the common assumption that the type of DF alone predicts DF efficacy and highlights the importance of host biological context in shaping dietary responses. Psyllium induced substantial microbiota and metabolomic changes despite limited fermentability partially contradicts the prevailing view that microbial fermentation is the primary mechanism through which DFs exerts systemic effects. Instead, these findings support the evidence that physical properties of fibres, such as bulking capacity, water-retention, and transit time may independently influence gut microbial ecology and host metabolism. The mixed responses observed with combined inulin-psyllium supplementation further suggest that fibre-fibre interactions may not be additive, underscoring the complexity of designing multi-fibre interventions.

In human studies, the rapid modulation of gut microbiota and metabolite profiles following adherence to high-fibre Mediterranean-style diet is consistent with prior evidence (Merra et al., 2020; Perrone & D'Angelo, 2025). However, the demonstration that these changes can occur within as little as five days and are significantly associated with mental health outcomes in individuals with mild to moderate depression and anxiety, is both striking and clinically relevant. This finding builds on the existing literature by highlighting the temporal sensitivity of the gut-microbiota-brain axis and suggests that dietary interventions may exert measurable neurobiological effects over clinically meaningful timeframes, identifying an effective fast-acting, accessible approach to improve mental health. In the context of cognitive ageing, the association between habitual DF consumption, and cognitive status in older adults aligns with epidemiological evidence linking fibre-rich diets to better cognitive outcomes (Prokopidis et al., 2022). Notably, the differential responses observed according to *APOE4* genotype represent a novel contribution (Chapter 5). The lack of association between DF consumption and cognitive status in *APOE4* carriers was unexpected and suggests that genetic risk may modulate responsiveness to microbiota-targeted dietary interventions. This finding challenges the assumption that dietary strategies confer uniform benefits across genetically diverse populations and reinforces the need for precision nutrition approaches.

Together, these findings contribute new insights into the heterogeneity of DF responses and highlight that DF influence the gut-microbiota-brain axis through multiple, context-dependent mechanisms. By integrating animal and human data, this thesis provides evidence that DF efficacy is shaped not only by fibre type and fermentability, but also by biological sex, and genetic background. These observations advance the field beyond one-size-fits-all dietary recommendations and underscores the importance of personalised strategies when leveraging DFs to support brain and mental health.

### 6.3 Strengths and Limitations

A key strength of this thesis is the integrative, multi-model approach used to investigate the role of DFs in gut-microbiota-brain communication. By combining controlled animal experiments with human randomised controlled trial and a cross-sectional observational study, this work bridges mechanistic insights with translational relevance. Another major strength is the explicit consideration of biological sex and genetic risk (*APOE4* genotype) in the GMB communication. This approach strengthens the case for personalised nutrition strategies.

The use of multi-omics methodologies, including gut microbiota profiling, metabolomics, gene expression analyses, and behavioural and cognitive assessments represents a further strength. Integrating microbial, metabolic and host outcomes enabled a systems-level understanding of how DFs exert their effects locally in the gut and peripherally in the serum and brain. This revealed mechanisms beyond simple changes in microbial composition.

Despite these strengths, there are limitations which should be acknowledged. First, results from animal models cannot be fully extrapolated to the human phenotype. While rodent studies enable controlled manipulation of the diet and mechanistic exploration, there are inter-species differences in metabolism and in the gut microbiota speciation and metabolism and they cannot fully replicate the complexity of human dietary behaviours, psychosocial factors, or long-term disease trajectories. Second, the causal inference in cross-sectional human studies is limited. While associations between DF intake, gut microbiota, metabolites, and brain-related outcomes were robust, residual confounding from lifestyle, and baseline diet cannot be fully excluded. Finally,

microbiome and metabolomics analyses were limited in resolution, relying primarily on taxonomic and predicted functional profiling rather than direct measures of microbial activity or metabolic flux. Future studies incorporating metagenomics, meta-transcriptomics, and longitudinal sampling would strengthen mechanistic interpretation.

Taken together, the strengths of this thesis lie in its novel, integrative, and personalised approach to understanding DF mediated gut-microbiota-brain interactions. While the limitations reflect common challenges in nutritional and microbiome research, they also highlight clear opportunities for future work, building on and being informed by the contribution of the findings presented.

## 6.4 Future Directions and Impact

The findings of this thesis highlight several important avenues for future research to address gaps and disparities.

### 6.4.1 Need for personalised dietary intervention trials

Future research should prioritise the development of personalised DF interventions through systemic stratification of participants by key biological determinants, including biological sex, genetic risk factors (such as *APOE4* genotype), and baseline gut microbiota composition. In particular, well-powered human RCTs with prospective recruitment based on *APOE* genotype and stratification by biological sex are warranted. Such designs would enable robust examination of the differential effects of DF types on mental health outcomes alongside cognitive performance.

Following the establishment of efficacy and mechanistic insight in metabolically and cognitively healthy populations, this approach should be extended to cohorts with mental health complaints and cognitive decline along different severity levels on the spectrum. This staged research strategy would allow evaluation of DF-based interventions across the health-disease continuum while minimising confounding by advanced pathology. Ultimately, these studies would facilitate the identification of biological responders and non-responders to specific DF types and combinations, thereby advancing precision nutrition approaches for mental and cognitive health.

#### 6.4.2 High resolution multi-omics approaches for strong mechanistic insights

Future studies should employ high-resolution, integrative multi-omics approaches to strengthen mechanistic understanding of DF-mediated effects on gut-microbiota-brain communication. The combined application of metagenomics, meta-transcriptomics and proteomics would enable direct characterisation of microbial functional capacity, active metabolic pathways and host-microbe signalling processes, moving beyond taxonomic associations in both animal and human models. Building on our findings demonstrating differential modulation of carbohydrate and energy metabolism pathways by distinct DF types (Chapter 3), these pathways warrant systemic investigation across a broader range of DF sources in both preclinical and human intervention studies. Such approaches would facilitate the identification of DF types most effective in targeting specific metabolic and signalling pathways relevant to gut-microbiota-brain interactions, thereby informing mechanism-driven selection of DF interventions.

#### 6.4.3 Whether dietary pattern changes are better than individual nutrient supplementation?

An important future research direction emerging from this work is the need to directly compare whole dietary pattern interventions with individual nutrient or DF supplementation strategies in the context of mental and cognitive health. Future RCTs should compare DF supplementation alone, dietary pattern modification (e.g. high-fibre whole food diets), and combined approaches. Addressing this question is critical for informing translational dietary recommendations, as dietary pattern interventions may better reflect habitual eating behaviours, whereas targeted supplementation may offer greater precision. Clarifying the relative and combined efficacy of these approaches will be essential for advancing evidence-based, scalable nutritional strategies for mental and cognitive health.

#### 6.4.4 How do DF interventions compare with first-line treatments for mental health conditions?

There is a rigorous need to evaluate DF interventions relative to established first-line treatments for mild to moderate mental health conditions, including psychological therapies. While the present findings indicate that DF-rich dietary interventions can

improve mental health outcomes via changes in gut-microbiota composition and metabolic pathways, its comparative clinical effectiveness remain unexplored. Future RCTs should therefore incorporate comparative designs to assess DF-based interventions against standard first-line care, as well as combined treatment arms to evaluate potential additive or synergistic effects. Such trials should prioritise early-stage populations with mild to moderate symptoms, where lifestyle-based interventions are most clinically appropriate. Establishing how DF interventions perform relative to, or in combination with, existing first-line treatments is essential for determining their potential role as standalone, adjunctive or preventative strategies within stepped-care models for mental health. This evidence will be critical for informing clinical guidelines and the integration of DF-based approaches into routine mental health care.

#### 6.4.5 Public health and policy implications of DF based strategies for better mental health

Given the high prevalence of mild to moderate mental health symptoms and the substantial economic and societal burden associated with these conditions, DF-based interventions represent a potentially scalable, low-risk approach to early intervention and prevention. Future studies should evaluate the effectiveness of DF-focused dietary strategies when embedded within public health frameworks, such as primary care, workplace wellbeing programmes, and community-based interventions. This includes assessing real-world feasibility and adherence, as well as the capacity of DF focused strategies to reduce demand on mental health services and prevent symptom escalation.

In parallel, health economic analyses are needed to determine the cost-effectiveness of DF-based mental health interventions relative to existing care pathways and to model their potential impact on productivity, healthcare utilisation, and long-term public expenditure. Generating such evidence will be essential for informing dietary guidelines, mental health prevention policies, and cross-sector strategies that integrate nutrition into mental health promotion.

#### 6.5 Overall Conclusion

This PhD project makes a significant contribution to the understanding of how DFs influence gut-microbiota-brain communication, demonstrating that fibre fermentability,

biological sex, and genetic risk critically shape host responses. By challenging the notion that all DFs exert uniform health benefits, this work supports a shift towards and informs precision nutrition strategies rather than one-size fits-all recommendations. Rapidly fermentable inulin and slowly fermentable psyllium exerted distinct, fibre-specific and sex-dependent effects, while these fibres combined produced non-additive responses, underscoring the complexity of DF interactions. At the dietary pattern level, short-term adherence to a high-fibre Mediterranean diet rapidly altered gut microbiota and metabolite profiles and was associated with improved mental health outcomes in adults with mild to moderate anxiety and/or depression symptoms. Extending these findings to cognitive ageing, habitual DF intake was associated with cognitive performance in older adults in an *APOE4*-dependent manner. Collectively, these findings support a shift towards personalised, mechanism-driven DF-based strategies for optimising mental and cognitive health.

The findings have important implications for nutritional guidance, mental health, and cognitive ageing, suggesting that targeted DF interventions may represent a low-cost, accessible, and scalable approach to support brain health. More broadly, this research strengthens the evidence base linking diet, the gut microbiome, and brain function, contributing knowledge to the fields of nutrition, neuroscience, microbiology, and public health.

## List of Communications

1. **Liaquat, M.**, Le Gall, G., Scholey, A., Pontifex, M. G., Bastiaanssen, T. F. S., Muller, M., Minihane, A.M., Vauzour, D. (2025). *APOE4* genotype shapes the role of dietary fibers in cognitive health through gut microbiota changes. *Gut Microbes*, 17(1). <https://doi.org/10.1080/19490976.2025.2526133>
2. **Liaquat, M.**, Minihane, A.M., Vauzour, D. & Pontifex, M.G. (2025). The gut microbiota in menopause: Is there a role for prebiotic and probiotic solutions? *Post Reproductive Health*, 31(2):105-114. 10.1177/20533691251340491
3. Esgunoglu, L., **Liaquat, M.**, Gillings, R., Lazar, A.S., Leddy, A., Brooks, J., Penny, W., Sami, S., Hornberger, M., Stevenson, E., Jennings, A. & Minihane, A.M. (2024). The acute effect of a Mediterranean-style dietary pattern (MDP) on mood, anxiety and cognition in UK adults with mild to moderate anxiety and depression: the MediMood randomised controlled trial protocol. *BMJ Open*, 14:e082935. <https://doi.org/10.1136/bmjopen-2023-082935>
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5. Connell, E., Gall, G.L., McArthur, S., Lang, L., Breeze, B., **Liaquat, M.**, Pontifex, M.G., Sami, S., Pourtau, L., Gaudout, D., Müller, M., & Vauzour, D. (2026). A novel Mediterranean diet-inspired supplement reduces hippocampal amyloid deposits and microglial activation through the modulation of the microbiota gut-brain axis in 5xFAD mice. *Gut Microbes*, 18(1). <https://doi.org/10.1080/19490976.2026.2614030>

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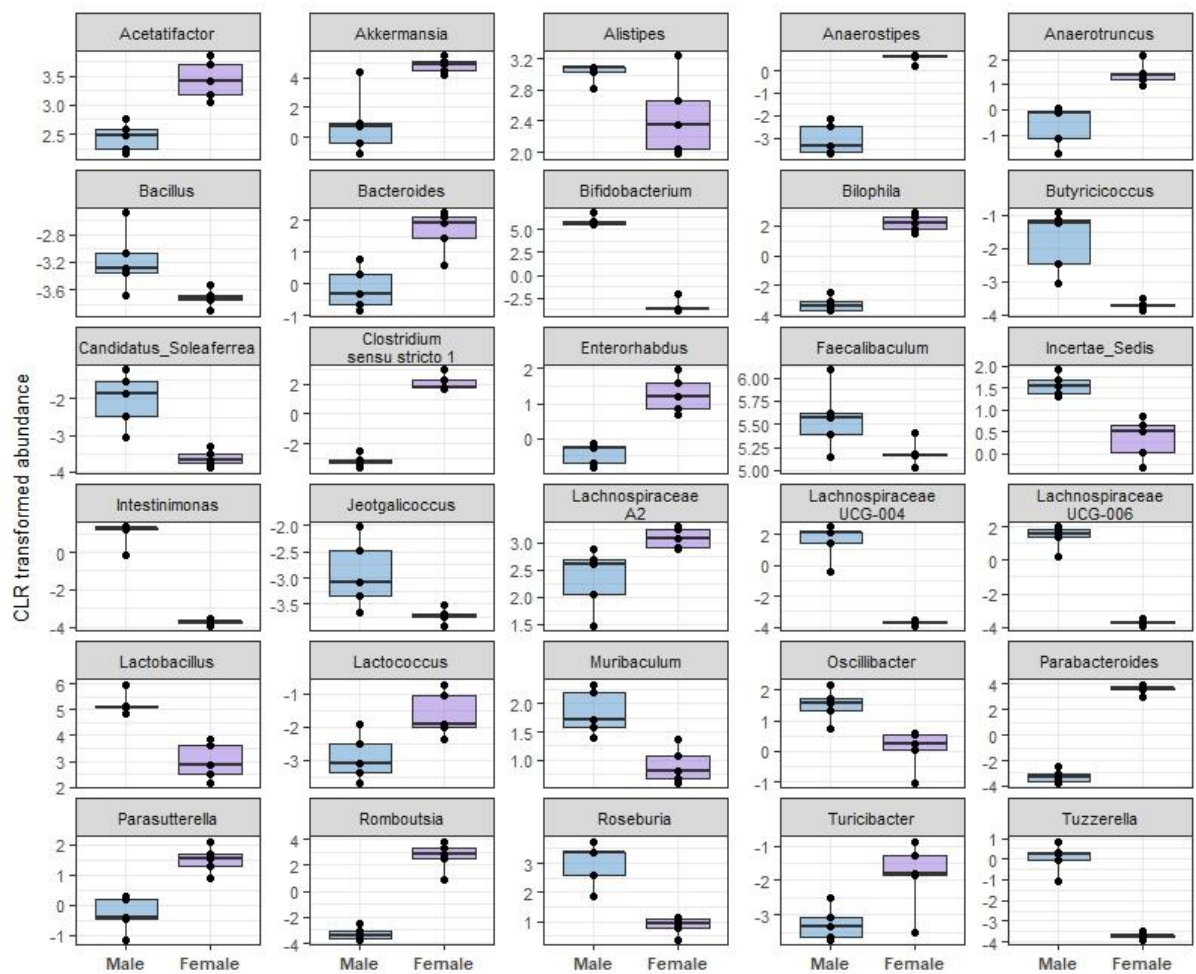
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## Appendices

**Supplementary Table S2.1 | Dietary Composition of the Low-fibre Western style purified diet**

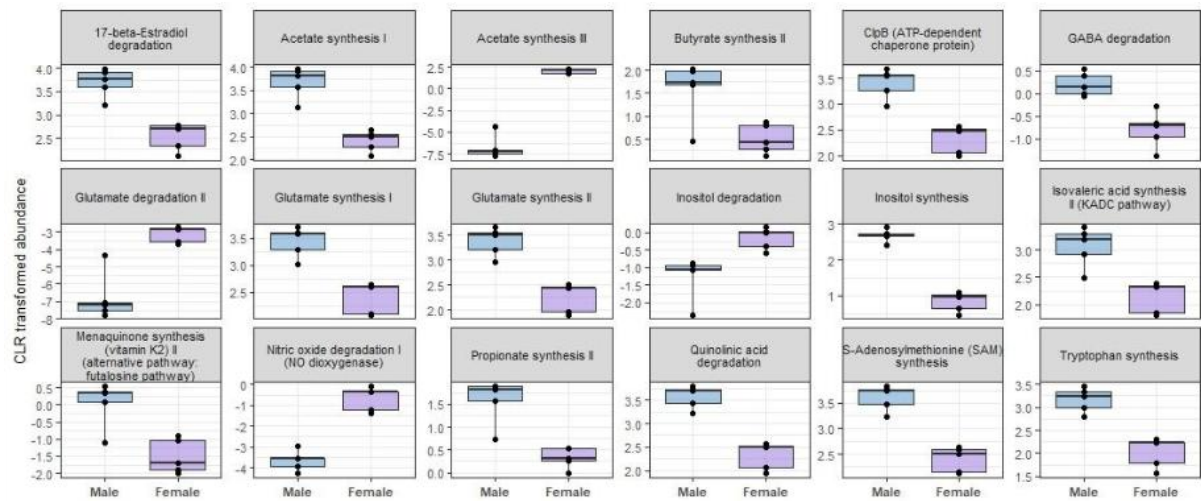
| <b>Product #</b>                           | <b>D12450J</b>         |              |
|--------------------------------------------|------------------------|--------------|
|                                            | <i>10 kcal% Fat</i>    |              |
|                                            | <i>50 gm Cellulose</i> |              |
|                                            | <b>gm%</b>             | <b>kcal%</b> |
| <b>Protein</b>                             | 19                     | 20           |
| <b>Carbohydrate</b>                        | 67                     | 70           |
| <b>Fat</b>                                 | 4                      | 10           |
| <b>Total</b>                               |                        | 100          |
| <b>Kcal/gm</b>                             | 3.85                   |              |
| <b>Ingredients</b>                         | <b>gm</b>              | <b>kcal</b>  |
| <b>Casein</b>                              | 200                    | 800          |
| <b>L-Cystine</b>                           | 3                      | 12           |
| <b>Corn Starch</b>                         | 506.2                  | 2024.8       |
| <b>Maltodextrin 10</b>                     | 125                    | 500          |
| <b>Sucrose</b>                             | 68.8                   | 275          |
| <b>Cellulose, BW200</b>                    | 50                     | 0            |
| <b>Lard</b>                                | 20                     | 180          |
| <b>Soybean Oil</b>                         | 25                     | 225          |
| <b>Mineral Mix S10026</b>                  | 10                     | 0            |
| <b>Dicalcium Phosphate</b>                 | 13                     | 0            |
| <b>Calcium Carbonate</b>                   | 5.5                    | 0            |
| <b>Potassium Citrate, 1 H<sub>2</sub>O</b> | 16.5                   | 0            |
| <b>Vitamin Mix V10001</b>                  | 10                     | 40           |
| <b>Choline Bitartrate</b>                  | 2                      | 0            |
| <b>Yellow Dye #5, FD&amp;C</b>             | 0.04                   | 0            |
| <b>Blue Dye #1, FD&amp;C</b>               | 0.01                   | 0            |
| <b>Total</b>                               | <b>1055.05</b>         | <b>4057</b>  |
| <b>gm</b>                                  |                        |              |
| <b>Total Fibre</b>                         | 50                     |              |
| <b>Insoluble Fibre</b>                     | 50                     |              |
| <b>Soluble Fibre</b>                       | 0                      |              |
| <b>gm%</b>                                 |                        |              |
| <b>Total Fibre</b>                         | 4.7                    |              |
| <b>Insoluble Fibre</b>                     | 4.7                    |              |
| <b>Soluble Fibre</b>                       | 0                      |              |



**Supplementary Figure S2.1** | Refined LF diet shows significant sex-specific abundance differences in the gut bacterial genera. FDR<0.1

**Supplementary Table S2.2** | Spearman Correlation between the intestine length and gut microbiota abundance

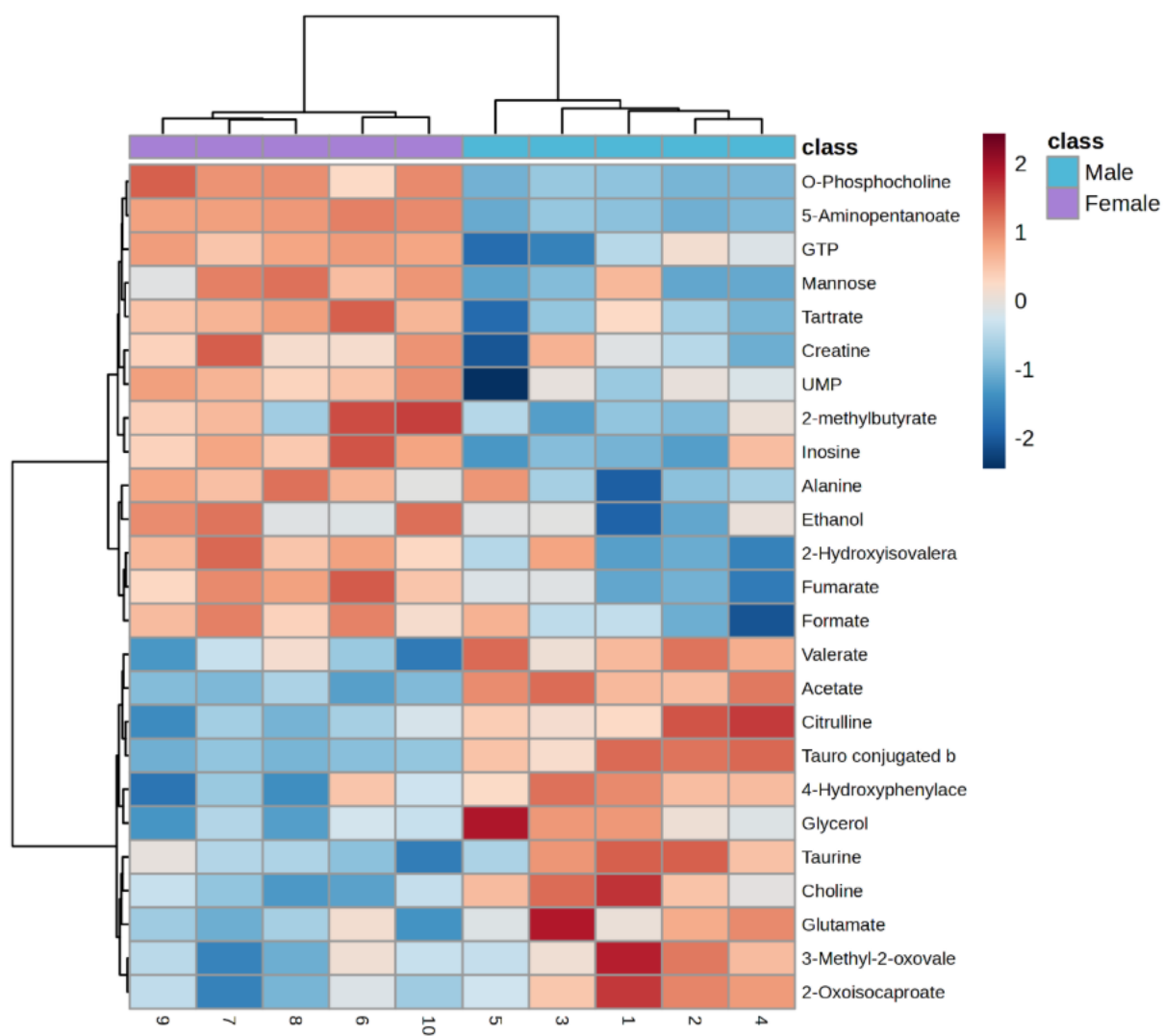
| Genus                              | R Coef. | P Value     | Genus                                | R Coef. | P Value     |
|------------------------------------|---------|-------------|--------------------------------------|---------|-------------|
| <i>Bifidobacterium</i>             | -0.84   | <b>0.01</b> | <i>Anaerostipes</i>                  | 0.97    | <b>0.00</b> |
| <i>Corynebacterium</i>             | -0.57   | 0.11        | <i>ASF356</i>                        | 0.39    | 0.29        |
| <i>Olsenella</i>                   | -0.32   | 0.40        | <i>Blautia</i>                       | 0.18    | 0.64        |
| <i>DNF00809</i>                    | 0.56    | 0.12        | <i>GCA-900066575</i>                 | -0.57   | 0.11        |
| <i>Enterorhabdus</i>               | 0.82    | <b>0.01</b> | <i>Lachnoclostridium</i>             | 0.67    | <b>0.05</b> |
| <i>Bacteroides</i>                 | 0.55    | 0.13        | <i>Lachnospiraceae-FCS020-group</i>  | 0.45    | 0.22        |
| <i>Muribaculum</i>                 | -0.74   | <b>0.03</b> | <i>Lachnospiraceae-NK4A136-group</i> | -0.61   | 0.09        |
| <i>Prevotellaceae-UCG-001</i>      | -0.57   | 0.11        | <i>Lachnospiraceae-UCG-004</i>       | -0.92   | <b>0.00</b> |
| <i>Alistipes</i>                   | -0.76   | <b>0.02</b> | <i>Lachnospiraceae-UCG-006</i>       | -0.92   | <b>0.00</b> |
| <i>Rikenellaceae-RC9-gut-group</i> | -0.57   | 0.11        | <i>Roseburia</i>                     | -0.65   | 0.07        |
| <i>Parabacteroides</i>             | 0.93    | <b>0.00</b> | <i>Tuzzerella</i>                    | -0.94   | <b>0.00</b> |
| <i>Mucispirillum</i>               | -0.57   | 0.11        | <i>Butyricicoccus</i>                | -0.92   | <b>0.00</b> |
| <i>Bilophila</i>                   | 0.89    | <b>0.00</b> | <i>Colidextribacter</i>              | -0.34   | 0.36        |
| <i>Sporosarcina</i>                | -0.57   | 0.11        | <i>Intestinimonas</i>                | -0.94   | <b>0.00</b> |
| <i>Erysipelatoclostridium</i>      | -0.57   | 0.11        | <i>Oscillibacter</i>                 | -0.60   | 0.10        |
| <i>Faecalibaculum</i>              | -0.34   | 0.37        | <i>Anaerotruncus</i>                 | 0.84    | <b>0.01</b> |
| <i>Turicibacter</i>                | 0.89    | <b>0.00</b> | <i>Candidatus-Soleaferrea</i>        | -0.92   | <b>0.00</b> |
| <i>Enterococcus</i>                | -0.37   | 0.33        | <i>Harryflintia</i>                  | -0.57   | 0.11        |
| <i>Lactobacillus</i>               | -0.70   | <b>0.04</b> | <i>Incertae-Sedis</i>                | -0.81   | <b>0.01</b> |
| <i>Lactococcus</i>                 | 0.56    | 0.12        | <i>Paludicola</i>                    | -0.01   | 0.99        |
| <i>Streptococcus</i>               | 0.07    | 0.87        | <i>UBA1819</i>                       | -0.57   | 0.11        |
| <i>Jeotgalicoccus</i>              | -0.80   | <b>0.01</b> | <i>Anaerovorax</i>                   | 0.24    | 0.52        |
| <i>Staphylococcus</i>              | 0.17    | 0.67        | <i>Romboutsia</i>                    | 0.99    | <b>0.00</b> |
| <i>Clostridium-sensu-stricto-1</i> | 0.94    | <b>0.00</b> | <i>Parasutterella</i>                | 0.76    | <b>0.02</b> |
| <i>Defluviitaleaceae-UCG-011</i>   | -0.57   | 0.11        | <i>Escherichia/Shigella</i>          | -0.57   | 0.11        |
| <i>A2</i>                          | 0.92    | <b>0.00</b> | <i>Akkermansia</i>                   | 0.62    | 0.08        |
| <i>Acetatifactor</i>               | 0.61    | 0.09        |                                      |         |             |



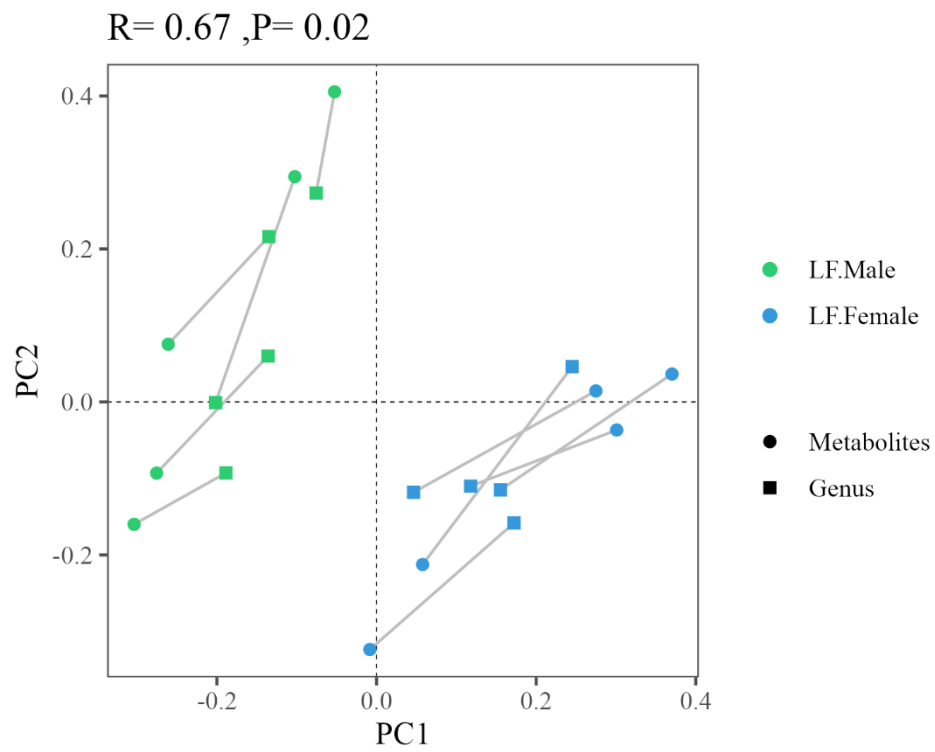
**Supplementary Figure S2.2** | Refined LF diet shows significant sex-specific differences in the gut brain modules (GBMs). FDR<0.1

**Supplementary Table S2.3** | General Linear Model Analysis of the Gut Brain Modules (GBMs)

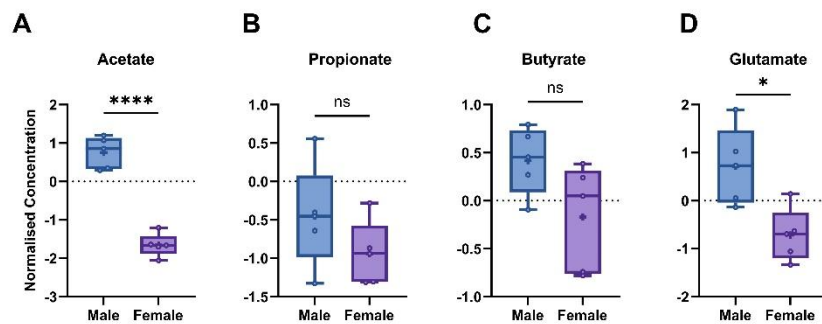
| GBMs                                                                          | SexFemale | SexFemale   |
|-------------------------------------------------------------------------------|-----------|-------------|
|                                                                               | Estimate  | Pr(> t ).BH |
| p-Cresol synthesis                                                            | -0.36     | 0.35        |
| Inositol synthesis                                                            | -1.83     | <b>0.00</b> |
| p-Cresol degradation                                                          | 0.39      | 0.79        |
| Inositol degradation                                                          | 1.10      | <b>0.01</b> |
| Quinolinic acid degradation                                                   | -1.27     | <b>0.00</b> |
| Propionate synthesis III                                                      | -0.29     | 0.40        |
| Isovaleric acid synthesis I (KADH pathway)                                    | 1.29      | 0.35        |
| Isovaleric acid synthesis II (KADC pathway)                                   | -0.91     | <b>0.00</b> |
| S-Adenosylmethionine (SAM) synthesis                                          | -1.21     | <b>0.00</b> |
| Glutamate degradation II                                                      | 3.66      | <b>0.00</b> |
| Butyrate synthesis I                                                          | -0.51     | 0.32        |
| 17-beta-Estradiol degradation                                                 | -1.16     | <b>0.00</b> |
| Butyrate synthesis II                                                         | -1.08     | <b>0.02</b> |
| Quinolinic acid synthesis                                                     | -0.51     | 0.11        |
| Propionate synthesis II                                                       | -1.25     | <b>0.00</b> |
| Glutamate degradation I                                                       | 0.00      | 1.00        |
| GABA degradation                                                              | -1.01     | <b>0.00</b> |
| Kynurenine synthesis                                                          | 0.31      | 0.87        |
| Nitric oxide synthesis II (nitrite reductase)                                 | 0.22      | 0.87        |
| Tryptophan synthesis                                                          | -1.15     | <b>0.00</b> |
| Nitric oxide degradation I (NO dioxygenase)                                   | 2.99      | <b>0.00</b> |
| Tryptophan degradation                                                        | 0.13      | 0.79        |
| Glutamate synthesis I                                                         | -1.03     | <b>0.00</b> |
| Glutamate synthesis II                                                        | -1.13     | <b>0.00</b> |
| ClpB (ATP-dependent chaperone protein)                                        | -1.08     | <b>0.00</b> |
| GABA synthesis III                                                            | -0.43     | 0.11        |
| Acetate synthesis III                                                         | 8.84      | <b>0.00</b> |
| Nitric oxide synthesis I (NO synthase)                                        | 0.45      | 0.79        |
| Acetate degradation                                                           | -0.46     | 0.19        |
| Menaquinone synthesis (vitamin K2) I                                          | 0.10      | 0.79        |
| Menaquinone synthesis (vitamin K2) II (alternative pathway: futasine pathway) | -1.55     | <b>0.01</b> |
| Acetate synthesis I                                                           | -1.27     | <b>0.00</b> |



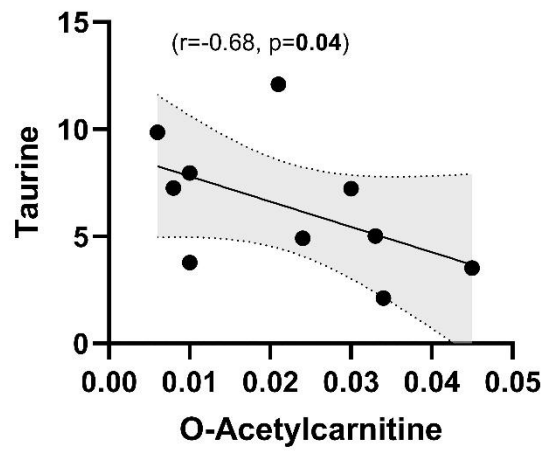
**Supplementary Figure S2.3** | Refined LF diet shows significant sex-specific differences in the caecum metabolomics profile.



**Supplementary Figure S2.4** | Procrustes analysis of the congruence of metabolite and microbiome profiles



**Supplementary Fig S2.5** | Caecum levels of SCFAs and glutamate were lower in females than males.

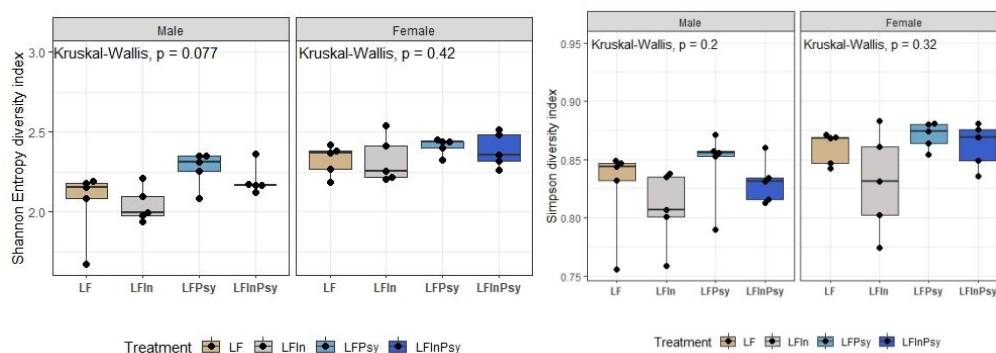


**Supplementary Fig S2.6 |** Caecum levels of taurine significantly correlates with O-Acetylcarnitine levels in the cortex

**Supplementary Table S3.1 | Dietary Composition of the intervention diets**

| <b>Product #</b>                         | <b>D12450J</b>         |              | <b>D23012401</b>     |              | <b>D23012402</b>      |              | <b>D23012403</b>                           |              |
|------------------------------------------|------------------------|--------------|----------------------|--------------|-----------------------|--------------|--------------------------------------------|--------------|
|                                          | <i>10 kcal% Fat</i>    |              | <i>Modified with</i> |              | <i>Modified with</i>  |              | <i>Modified with</i>                       |              |
|                                          | <i>50 gm Cellulose</i> |              | <i>35 gm Inulin</i>  |              | <i>35 gm Psyllium</i> |              | <i>17.5 gm Inulin<br/>17.5 gm Psyllium</i> |              |
|                                          | <b>gm%</b>             | <b>kcal%</b> | <b>gm%</b>           | <b>kcal%</b> | <b>gm%</b>            | <b>kcal%</b> | <b>gm%</b>                                 | <b>kcal%</b> |
| Protein                                  | 19                     | 20           | 19                   | 20           | 19                    | 20           | 19                                         | 20           |
| Carbohydrate                             | 67                     | 70           | 65                   | 70           | 65                    | 70           | 65                                         | 70           |
| Fat                                      | 4                      | 10           | 4                    | 10           | 4                     | 10           | 4                                          | 10           |
| Total                                    |                        | 100          |                      | 100          |                       | 100          |                                            | 100          |
| Kcal/gm                                  | 3.85                   |              | 3.77                 |              | 3.75                  |              | 3.76                                       |              |
| <b>Ingredients</b>                       | <b>gm</b>              | <b>kcal</b>  | <b>gm</b>            | <b>kcal</b>  | <b>gm</b>             | <b>kcal</b>  | <b>gm</b>                                  | <b>kcal</b>  |
| Casein                                   | 200                    | 800          | 200                  | 800          | 200                   | 800          | 200                                        | 800          |
| L-Cystine                                | 3                      | 12           | 3                    | 12           | 3                     | 12           | 3                                          | 12           |
| Corn Starch                              | 506.2                  | 2024.8       | 493                  | 1972         | 497.5                 | 1990         | 495.3                                      | 1981         |
| Maltodextrin 10                          | 125                    | 500          | 125                  | 500          | 125                   | 500          | 125                                        | 500          |
| Sucrose                                  | 68.8                   | 275          | 68.8                 | 275          | 68.8                  | 275          | 68.8                                       | 275          |
| Cellulose, BW200                         | 50                     | 0            | 50                   | 0            | 50                    | 0            | 50                                         | 0            |
| Inulin, Orafit HP                        | 0                      | 0            | 35                   | 53           | 0                     | 0            | 17.5                                       | 26           |
| Psyllium                                 | 0                      | 0            | 0                    | 0            | 35                    | 35           | 17.5                                       | 18           |
| Lard                                     | 20                     | 180          | 20                   | 180          | 20                    | 180          | 20                                         | 180          |
| Soybean Oil                              | 25                     | 225          | 25                   | 225          | 25                    | 225          | 25                                         | 225          |
| Mineral Mix<br>S10026                    | 10                     | 0            | 10                   | 0            | 10                    | 0            | 10                                         | 0            |
| Dicalcium<br>Phosphate                   | 13                     | 0            | 13                   | 0            | 13                    | 0            | 13                                         | 0            |
| Calcium<br>Carbonate                     | 5.5                    | 0            | 5.5                  | 0            | 5.5                   | 0            | 5.5                                        | 0            |
| Potassium Citrate,<br>1 H <sub>2</sub> O | 16.5                   | 0            | 16.5                 | 0            | 16.5                  | 0            | 16.5                                       | 0            |
| Vitamin Mix<br>V10001                    | 10                     | 40           | 10                   | 40           | 10                    | 40           | 10                                         | 40           |
| Choline Bitartrate                       | 2                      | 0            | 2                    | 0            | 2                     | 0            | 2                                          | 0            |
| Yellow Dye #5,<br>FD&C                   | 0.04                   | 0            | 0.025                | 0            | 0                     | 0            | 0                                          | 0            |
| Red Dye #40,<br>FD&C                     | 0                      | 0            | 0.025                | 0            | 0.05                  | 0            | 0.025                                      | 0            |
| Blue Dye #1,<br>FD&C                     | 0.01                   | 0            | 0                    | 0            | 0                     | 0            | 0.025                                      | 0            |
| <b>Total</b>                             | <b>1055.05</b>         | <b>4057</b>  | <b>1076.85</b>       | <b>4057</b>  | <b>1081.35</b>        | <b>4057</b>  | <b>1079.15</b>                             | <b>4057</b>  |
| <b>gm</b>                                |                        |              |                      |              |                       |              |                                            |              |
| Total Fibre                              | 50                     |              | 85                   |              | 85                    |              | 85                                         |              |
| Insoluble Fibre                          | 50                     |              | 50                   |              | 75                    |              | 62.5                                       |              |

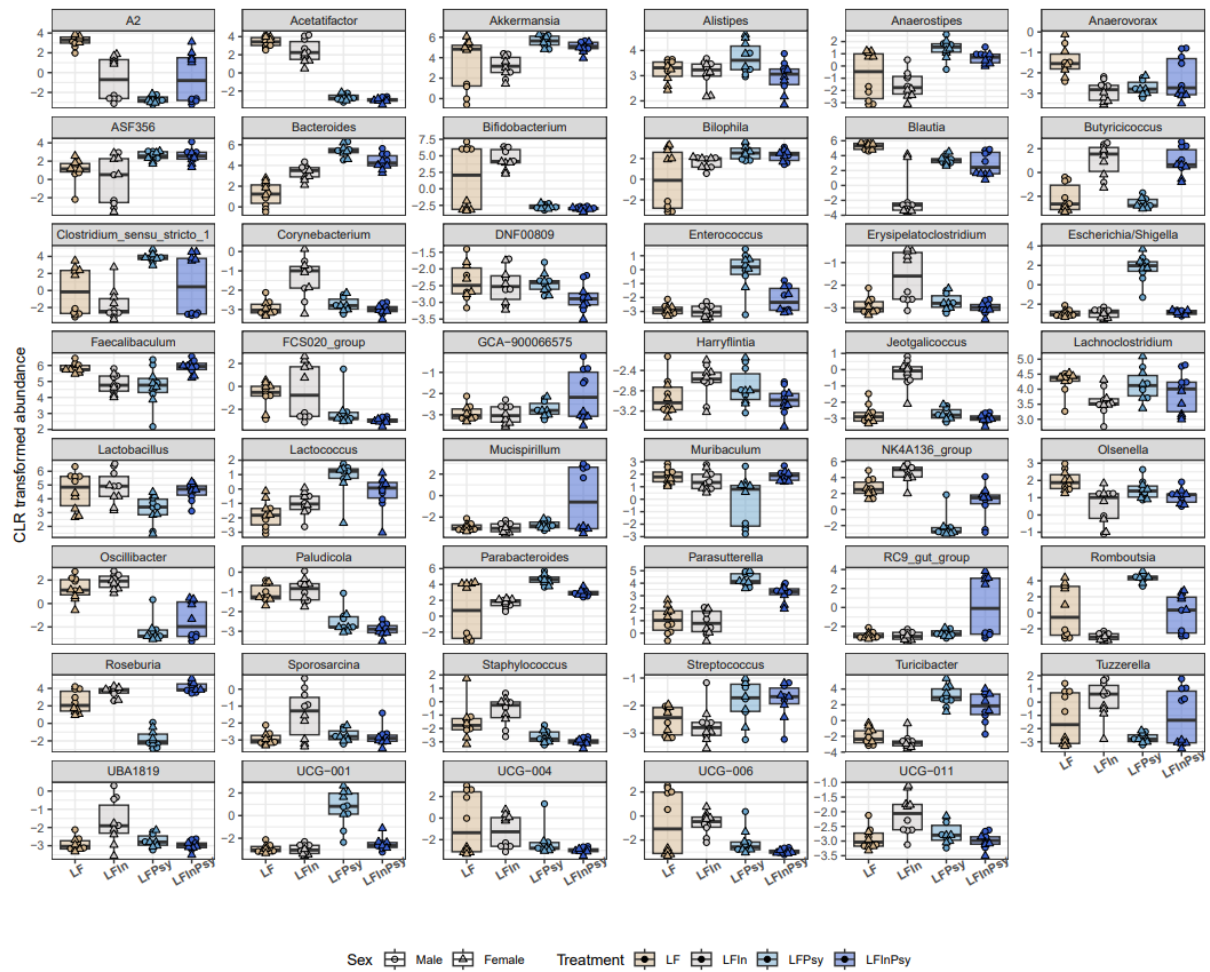
|                 |     |     |     |      |
|-----------------|-----|-----|-----|------|
| Soluble Fibre   | 0   | 35  | 7.7 | 21.4 |
| <b>gm%</b>      |     |     |     |      |
| Total Fibre     | 4.7 | 7.9 | 7.9 | 7.9  |
| Insoluble Fibre | 4.7 | 4.6 | 6.9 | 5.8  |
| Soluble Fibre   | 0   | 3.3 | 0.7 | 2.0  |



**Supplementary Figure S3.1 | Shannon and Simpson alpha diversity indices.** Kruskal Wallis test was used for statistical analyses, and significance p values are mentioned on the plots.

**Supplementary Table S3.2** | Relative Abundance of the Gut Microbiota at Phylum level

|                          | LF    |        | LF In |        | LF Psy |        | LF InPsy |        |
|--------------------------|-------|--------|-------|--------|--------|--------|----------|--------|
|                          | Male  | Female | Male  | Female | Male   | Female | Male     | Female |
| <b>Firmicutes</b>        | 0.78  | 0.51   | 0.20  | 0.54   | 0.08   | -0.11  | 0.05     | 0.37   |
| <b>Bacteroidota</b>      | -0.08 | 0.15   | 0.20  | 0.10   | 0.32   | 0.48   | 0.28     | 0.37   |
| <b>Actinobacteriota</b>  | 0.33  | 0.02   | 0.31  | 0.19   | 0.01   | 0.00   | 0.02     | -0.04  |
| <b>Proteobacteria</b>    | -0.10 | -0.04  | -0.07 | -0.11  | 0.20   | 0.22   | 0.03     | 0.00   |
| <b>Verrucomicrobiota</b> | 0.05  | 0.19   | 0.14  | 0.12   | 0.20   | 0.20   | 0.21     | 0.15   |
| <b>Desulfobacterota</b>  | -0.11 | 0.09   | 0.07  | 0.07   | 0.08   | 0.10   | 0.08     | 0.07   |
| <b>Others</b>            | -0.11 | -0.11  | -0.11 | -0.14  | -0.10  | -0.09  | 0.10     | -0.10  |
| <b>Unknown</b>           | 0.24  | 0.19   | 0.26  | 0.22   | 0.22   | 0.21   | 0.23     | 0.17   |



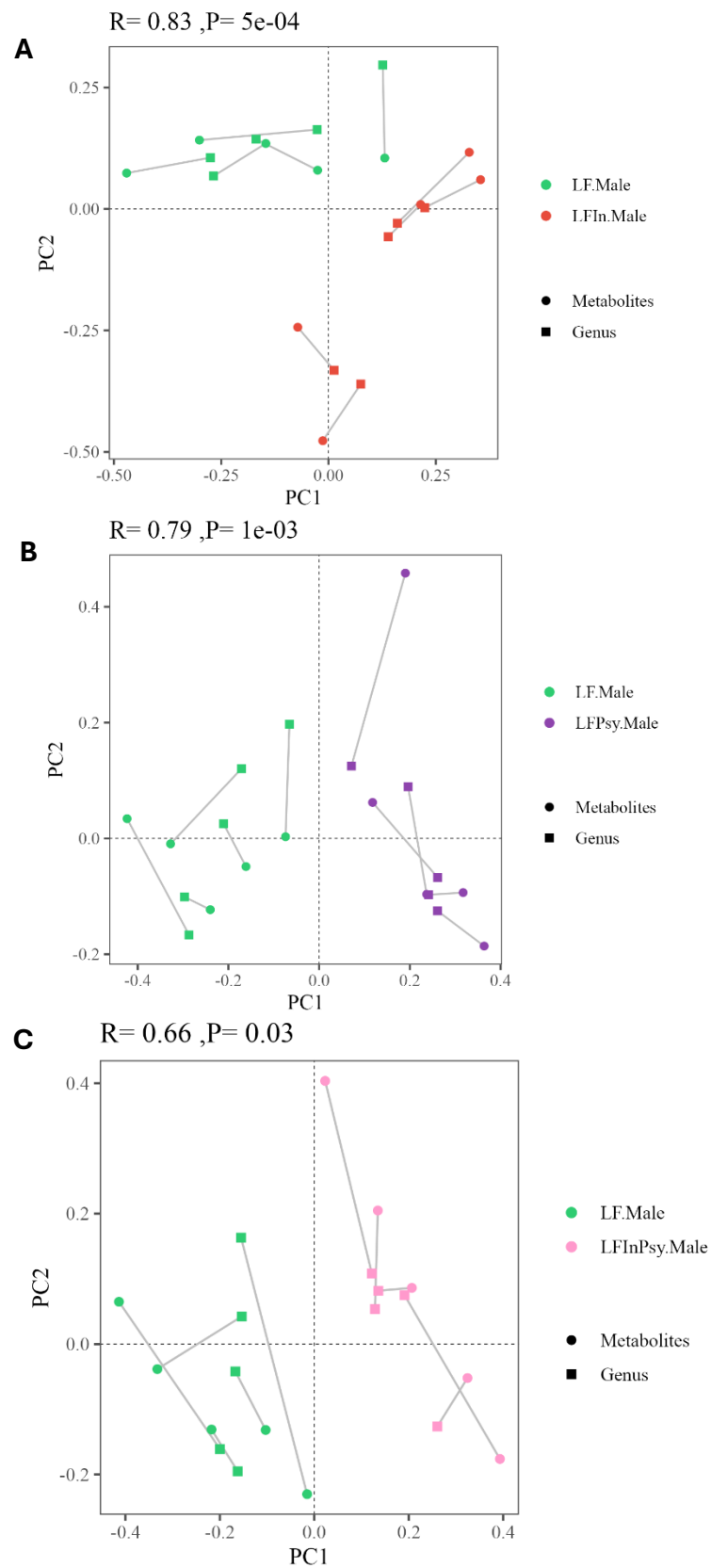
**Supplementary Figure S3.2** | Differentially abundant bacterial genera exhibiting significant treatment effect (FDR<0.1) and prominent sex-specific differences within treatments.

**Supplementary Table S3.3** | General Linear Model Analysis of the gut microbiota composition at genus level

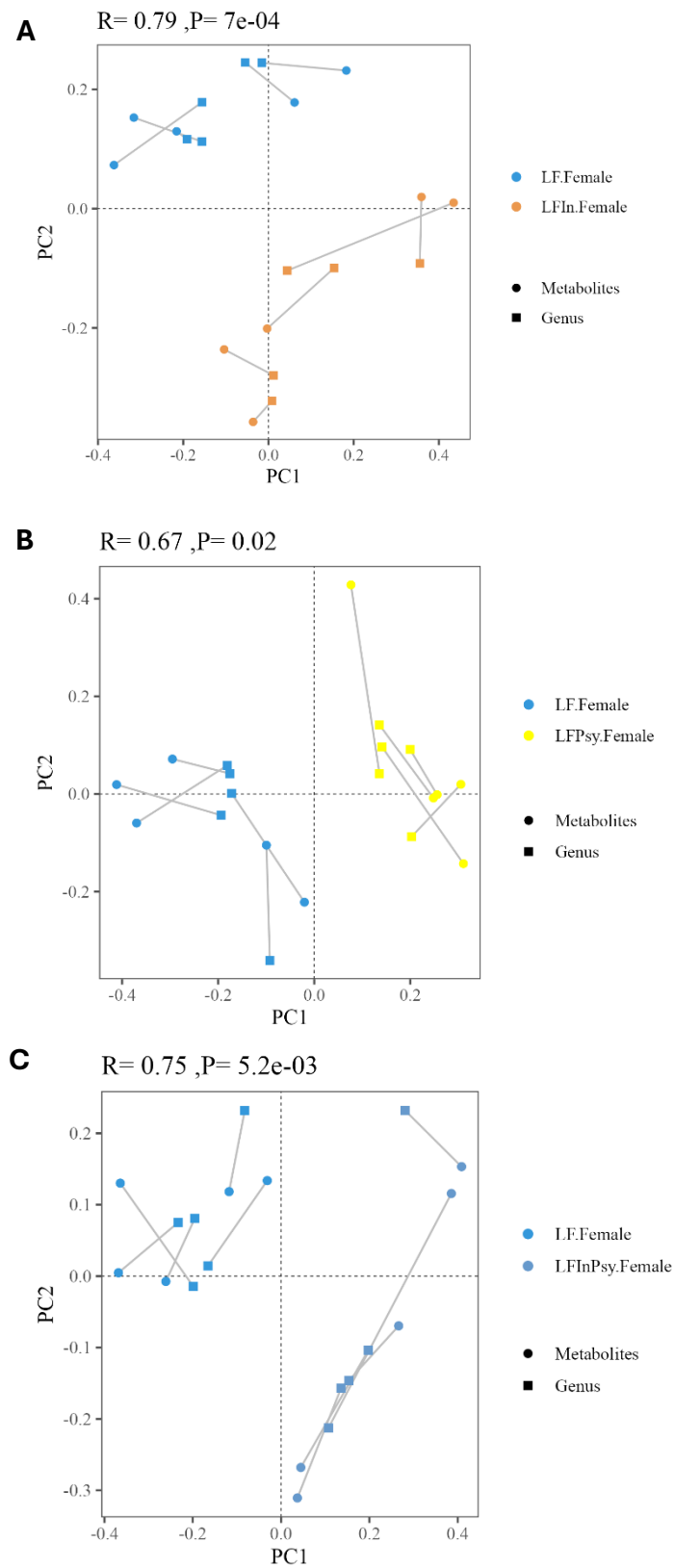
|                                    | Sex<br>Female | Treatment<br>LF In | Treatment<br>LF Psy | Treatment<br>LF InPsy | Sex<br>Female | Treatment<br>LF In | Treatment<br>LF Psy | Treatment<br>LF InPsy |
|------------------------------------|---------------|--------------------|---------------------|-----------------------|---------------|--------------------|---------------------|-----------------------|
|                                    | Estimate      | Estimate           | Estimate            | Estimate              | Pr(>  t ).BH  | Pr(>  t ).BH       | Pr(>  t ).BH        | Pr(>  t ).BH          |
| <i>Bifidobacterium</i>             | -2.96         | 2.86               | -4.43               | -4.69                 | <b>0.00</b>   | <b>0.01</b>        | <b>0.00</b>         | <b>0.00</b>           |
| <i>Corynebacterium</i>             | -0.15         | 1.56               | 0.19                | -0.07                 | 0.60          | <b>0.00</b>        | 0.55                | 0.88                  |
| <i>Olsenella</i>                   | -0.17         | -1.45              | -0.52               | -0.89                 | 0.60          | <b>0.00</b>        | 0.14                | <b>0.02</b>           |
| <i>DNF00809</i>                    | -0.09         | -0.12              | -0.05               | -0.49                 | 0.66          | 0.78               | 0.81                | 0.05                  |
| <i>Enterorhabdus</i>               | 0.06          | 0.05               | 0.51                | 0.64                  | 0.84          | 0.97               | 0.20                | 0.12                  |
| <i>Bacteroides</i>                 | -0.08         | 2.12               | 4.20                | 3.14                  | 0.84          | <b>0.00</b>        | <b>0.00</b>         | <b>0.00</b>           |
| <i>Muribaculum</i>                 | 0.18          | -0.34              | -2.01               | 0.04                  | 0.72          | 0.75               | <b>0.00</b>         | 0.94                  |
| <i>Prevotellaceae-UCG-001</i>      | 0.41          | -0.06              | 3.59                | 0.40                  | 0.26          | 0.97               | <b>0.00</b>         | 0.46                  |
| <i>Alistipes</i>                   | -0.25         | -0.11              | 0.49                | -0.27                 | 0.26          | 0.83               | 0.08                | 0.43                  |
| <i>Rikenellaceae-RC9-gut-group</i> | 1.25          | -0.06              | 0.19                | 3.06                  | <b>0.04</b>   | 0.97               | 0.81                | 0.00                  |
| <i>Parabacteroides</i>             | 1.89          | 1.01               | 3.98                | 2.29                  | <b>0.00</b>   | 0.32               | <b>0.00</b>         | <b>0.01</b>           |
| <i>Mucispirillum</i>               | -1.66         | -0.06              | 0.19                | 2.62                  | <b>0.00</b>   | 0.97               | 0.78                | <b>0.00</b>           |
| <i>Bilophila</i>                   | 1.61          | 1.70               | 2.62                | 2.35                  | <b>0.00</b>   | <b>0.02</b>        | <b>0.00</b>         | <b>0.00</b>           |
| <i>Sporosarcina</i>                | -0.82         | 1.46               | 0.19                | 0.11                  | <b>0.00</b>   | <b>0.00</b>        | 0.60                | 0.88                  |
| <i>Erysipelatoclostridium</i>      | 0.36          | 1.29               | 0.19                | -0.07                 | 0.17          | <b>0.00</b>        | 0.55                | 0.88                  |
| <i>Faecalibaculum</i>              | -0.36         | -0.99              | -1.15               | 0.06                  | 0.23          | <b>0.01</b>        | <b>0.00</b>         | 0.89                  |
| <i>Turicibacter</i>                | 1.24          | -0.65              | 5.14                | 3.81                  | <b>0.01</b>   | 0.39               | <b>0.00</b>         | <b>0.00</b>           |
| <i>Enterococcus</i>                | -0.24         | -0.11              | 2.81                | 0.73                  | 0.54          | 0.95               | <b>0.00</b>         | 0.12                  |
| <i>Lactobacillus</i>               | -0.84         | 0.27               | -1.37               | -0.05                 | <b>0.03</b>   | 0.78               | <b>0.01</b>         | 0.92                  |
| <i>Lactococcus</i>                 | 0.29          | 0.77               | 2.59                | 1.55                  | 0.54          | 0.22               | <b>0.00</b>         | <b>0.01</b>           |
| <i>Streptococcus</i>               | -0.05         | -0.18              | 0.69                | 0.74                  | 0.84          | 0.78               | <b>0.04</b>         | <b>0.03</b>           |
| <i>Jeotgalicoccus</i>              | -0.31         | 2.52               | 0.02                | -0.24                 | 0.18          | <b>0.00</b>        | 0.92                | 0.46                  |
| <i>Staphylococcus</i>              | 0.07          | 0.88               | -1.06               | -1.43                 | 0.84          | 0.09               | <b>0.03</b>         | <b>0.00</b>           |
| <i>Clostridium sensu stricto 1</i> | 3.53          | -1.57              | 3.96                | 0.69                  | <b>0.00</b>   | 0.10               | <b>0.00</b>         | 0.49                  |
| <i>Defluviitaleaceae UCG-011</i>   | 0.13          | 0.83               | 0.19                | -0.07                 | 0.54          | <b>0.00</b>        | 0.44                | 0.88                  |

|                                      |       |       |       |       |             |             |             |             |
|--------------------------------------|-------|-------|-------|-------|-------------|-------------|-------------|-------------|
| <i>Lachnospiraceae A2</i>            | 2.34  | -3.81 | -5.90 | -3.70 | <b>0.00</b> | <b>0.00</b> | <b>0.00</b> | <b>0.00</b> |
| <i>Acetatifactor</i>                 | -0.33 | -0.99 | -6.14 | -6.40 | 0.26        | <b>0.01</b> | <b>0.00</b> | <b>0.00</b> |
| <i>Anaerostipes</i>                  | 0.27  | -0.75 | 2.20  | 1.46  | 0.60        | 0.33        | <b>0.00</b> | <b>0.02</b> |
| <i>ASF356</i>                        | 0.48  | -1.06 | 1.52  | 1.54  | 0.51        | 0.26        | 0.06        | 0.06        |
| <i>Blautia</i>                       | -0.11 | -6.84 | -1.82 | -2.41 | 0.87        | <b>0.00</b> | 0.05        | <b>0.01</b> |
| <i>GCA-900066575</i>                 | -0.79 | -0.06 | 0.19  | 0.88  | <b>0.00</b> | 0.95        | 0.54        | <b>0.00</b> |
| <i>Lachnoclostridium</i>             | -0.12 | -0.67 | -0.09 | -0.37 | 0.60        | <b>0.02</b> | 0.75        | 0.19        |
| <i>Lachnospiraceae FCS020 group</i>  | 0.99  | 0.30  | -1.53 | -2.27 | <b>0.09</b> | 0.83        | <b>0.04</b> | <b>0.00</b> |
| <i>Lachnospiraceae NK4A136 group</i> | -0.49 | 1.90  | -4.90 | -1.74 | 0.49        | <b>0.02</b> | <b>0.00</b> | <b>0.03</b> |
| <i>Lachnospiraceae UCG-004</i>       | -0.87 | -0.72 | -1.76 | -2.47 | 0.23        | 0.57        | 0.05        | <b>0.01</b> |
| <i>Lachnospiraceae UCG-006</i>       | -1.04 | -0.01 | -1.60 | -2.35 | <b>0.07</b> | 0.99        | <b>0.03</b> | <b>0.00</b> |
| <i>Roseburia</i>                     | -0.16 | 1.21  | -4.17 | 1.69  | 0.69        | <b>0.02</b> | <b>0.00</b> | <b>0.00</b> |
| <i>Tuzzerella</i>                    | -2.40 | 1.50  | -1.47 | 0.12  | <b>0.00</b> | <b>0.01</b> | <b>0.01</b> | 0.88        |
| <i>Butyricicoccus</i>                | -1.19 | 3.24  | -0.40 | 3.07  | <b>0.00</b> | <b>0.00</b> | 0.43        | <b>0.00</b> |
| <i>Colidextribacter</i>              | 0.74  | -0.04 | -1.15 | -0.77 | 0.23        | 0.97        | 0.13        | 0.38        |
| <i>Intestinimonas</i>                | 0.45  | -0.11 | -1.52 | -0.21 | 0.60        | 0.97        | 0.14        | 0.88        |
| <i>Oscillibacter</i>                 | -0.09 | 0.59  | -3.64 | -2.74 | 0.84        | 0.41        | <b>0.00</b> | <b>0.00</b> |
| <i>Anaerotruncus</i>                 | -0.97 | -1.09 | -0.42 | 1.61  | 0.23        | 0.37        | 0.69        | 0.12        |
| <i>Candidatus Soleaferrea</i>        | -0.56 | -0.50 | -0.47 | -0.20 | <b>0.02</b> | 0.16        | 0.14        | 0.64        |
| <i>Harryflintia</i>                  | -0.13 | 0.31  | 0.19  | -0.07 | 0.37        | 0.10        | 0.27        | 0.85        |
| <i>Incertae Sedis</i>                | -0.42 | -0.37 | -0.35 | 0.44  | 0.29        | 0.61        | 0.51        | 0.46        |
| <i>Paludicola</i>                    | -0.24 | 0.16  | -1.43 | -1.83 | 0.23        | 0.72        | <b>0.00</b> | <b>0.00</b> |
| <i>UBA1819</i>                       | -0.58 | 1.21  | 0.19  | -0.07 | <b>0.02</b> | <b>0.00</b> | 0.55        | 0.88        |
| <i>Anaerovorax</i>                   | -0.61 | -1.46 | -1.29 | -0.82 | <b>0.02</b> | <b>0.00</b> | <b>0.00</b> | <b>0.02</b> |
| <i>Romboutsia</i>                    | 2.38  | -3.15 | 4.17  | -0.25 | <b>0.00</b> | <b>0.00</b> | <b>0.00</b> | 0.88        |
| <i>Parasutterella</i>                | 0.13  | -0.19 | 3.24  | 2.14  | 0.72        | 0.83        | <b>0.00</b> | <b>0.00</b> |
| <i>Escherichia/Shigella</i>          | 0.03  | -0.02 | 4.67  | 0.06  | 0.88        | 0.97        | <b>0.00</b> | 0.89        |
| <i>Akkermansia</i>                   | 0.79  | -0.21 | 2.27  | 1.73  | 0.18        | 0.92        | <b>0.00</b> | <b>0.02</b> |

The general linear model (GLM) analysis was done using LF dietary group and male sex as the reference groups for analyses



**Supplementary Figure S3.3 |** Procrustes Analysis (PA) shows strong congruence between gut microbiota and gut metabolite profiles in males.



**Supplementary Figure S3.4** | Procrustes Analysis (PA) shows strong congruence between gut microbiota and gut metabolite profiles in females.

**Supplementary Table S3.4** | Two-way ANOVA Results of the Caecum Metabolomics Profile

|                             | Treatment (adj.p) | Sex (adj.p)     | Interaction(adj.p) |
|-----------------------------|-------------------|-----------------|--------------------|
| Alpha-ketoisovaleric acid   | <b>4.11E-12</b>   | 0.34335         | <b>0.042538</b>    |
| 3-Methyl-2-oxovalerate      | <b>4.06E-11</b>   | 0.2607          | <b>0.0237</b>      |
| 2-Oxoglutarate              | <b>4.06E-11</b>   | 0.81035         | 0.67432            |
| Tauro conjugated bile acids | <b>1.26E-10</b>   | <b>0.000194</b> | 8.69E-10           |
| 2-Oxoisocaproate            | <b>3.71E-10</b>   | <b>0.028727</b> | 0.09875            |
| O-Phosphocholine            | <b>1.04E-09</b>   | <b>0.000194</b> | 6.95E-08           |
| Butyrate                    | <b>1.34E-08</b>   | 0.998           | 0.4031             |
| 5-Aminopentanoate           | <b>1.34E-08</b>   | <b>0.028727</b> | <b>2.30E-18</b>    |
| Trimethylamine              | <b>6.19E-08</b>   | <b>0.000764</b> | 0.30081            |
| Xylose                      | <b>1.82E-07</b>   | 0.50603         | 0.4031             |
| Tartrate                    | <b>4.06E-07</b>   | 0.87216         | 0.087778           |
| 3-Hydroxybutyrate           | <b>5.41E-06</b>   | 0.74467         | 0.78232            |
| Uracil                      | <b>6.64E-06</b>   | 0.93671         | 0.52882            |
| Fumarate                    | <b>6.64E-06</b>   | 0.29343         | 0.31073            |
| Propionate                  | <b>6.64E-06</b>   | 0.66647         | 0.78232            |
| 2-Hydroxyisovalerate        | <b>8.99E-06</b>   | 0.83996         | <b>0.054312</b>    |
| Citrulline                  | <b>9.02E-06</b>   | 0.74467         | 0.091474           |
| Glutamate                   | <b>4.70E-05</b>   | 0.51914         | 0.52882            |
| 4-Hydroxyphenylacetate      | <b>8.52E-05</b>   | <b>0.000342</b> | <b>0.011613</b>    |
| 4-Aminobutyrate             | <b>0.000115</b>   | 0.2607          | 0.78232            |
| Ribose                      | <b>0.00012</b>    | 0.87216         | 0.78232            |
| Histidine                   | <b>0.000138</b>   | 0.49431         | 0.78232            |
| Succinate                   | <b>0.000162</b>   | 0.93671         | 0.77089            |
| Malate                      | <b>0.000215</b>   | 0.48794         | 0.31073            |
| 2-methylbutyrate            | <b>0.000215</b>   | 0.54565         | 0.16458            |
| Inosine                     | <b>0.000343</b>   | 0.12837         | <b>0.022571</b>    |
| Valerate                    | <b>0.000465</b>   | 0.98041         | <b>0.052667</b>    |
| Phenylacetate               | <b>0.000759</b>   | 0.13476         | <b>0.0237</b>      |
| Formate                     | <b>0.000823</b>   | <b>0.028727</b> | 0.14364            |
| Fructose                    | <b>0.001311</b>   | <b>0.0395</b>   | 0.44767            |
| Citrate                     | <b>0.001425</b>   | 0.51914         | 0.33129            |
| Alanine                     | <b>0.00178</b>    | 0.22822         | 0.14364            |
| Isopropanol                 | <b>0.002085</b>   | 0.34526         | 0.5093             |
| Glycerol                    | <b>0.002324</b>   | 0.47879         | 0.24332            |
| Galactose                   | <b>0.00427</b>    | 0.34597         | 0.31073            |
| Nicotinate                  | <b>0.00427</b>    | 0.36603         | 0.41337            |
| Xanthine                    | <b>0.00427</b>    | 0.5512          | 0.75577            |
| Threonine                   | <b>0.010395</b>   | 0.74467         | 0.68605            |
| Caproic acid                | <b>0.022282</b>   | 0.51914         | 0.78232            |
| Arginine                    | <b>0.025049</b>   | 0.5925          | 0.93483            |
| Glutamine                   | <b>0.025049</b>   | 0.55128         | 0.67432            |
| Lactate                     | <b>0.026333</b>   | 0.72934         | 0.47057            |

|                            |                 |                 |                 |
|----------------------------|-----------------|-----------------|-----------------|
| <b>3-Phenylpropionate</b>  | <b>0.027558</b> | 0.62604         | 0.91927         |
| <b>Creatine</b>            | <b>0.032318</b> | 0.74467         | 0.88164         |
| <b>Choline</b>             | <b>0.033356</b> | 0.028727        | 0.41337         |
| <b>N-Acetylglucosamine</b> | <b>0.036065</b> | 0.87216         | 0.91927         |
| <b>Taurine</b>             | <b>0.03866</b>  | 0.61651         | <b>0.028727</b> |
| <b>Isovalerate</b>         | <b>0.0395</b>   | 0.55128         | 0.36627         |
| <b>Leucine</b>             | <b>0.043531</b> | 0.61651         | 0.75633         |
| <b>Mannose</b>             | 0.074691        | <b>0.028727</b> | 0.31073         |
| <b>Aspartate</b>           | 0.18858         | 0.87216         | <b>0.042538</b> |
| <b>GTP</b>                 | 0.21665         | <b>0.028727</b> | <b>0.006063</b> |
| <b>Glucose</b>             | 0.23932         | <b>0.028727</b> | 0.39061         |
| <b>Acetate</b>             | 0.27135         | 0.30882         | 0.011281        |
| <b>Urocanate</b>           | 0.40553         | 0.57317         | <b>0.0237</b>   |

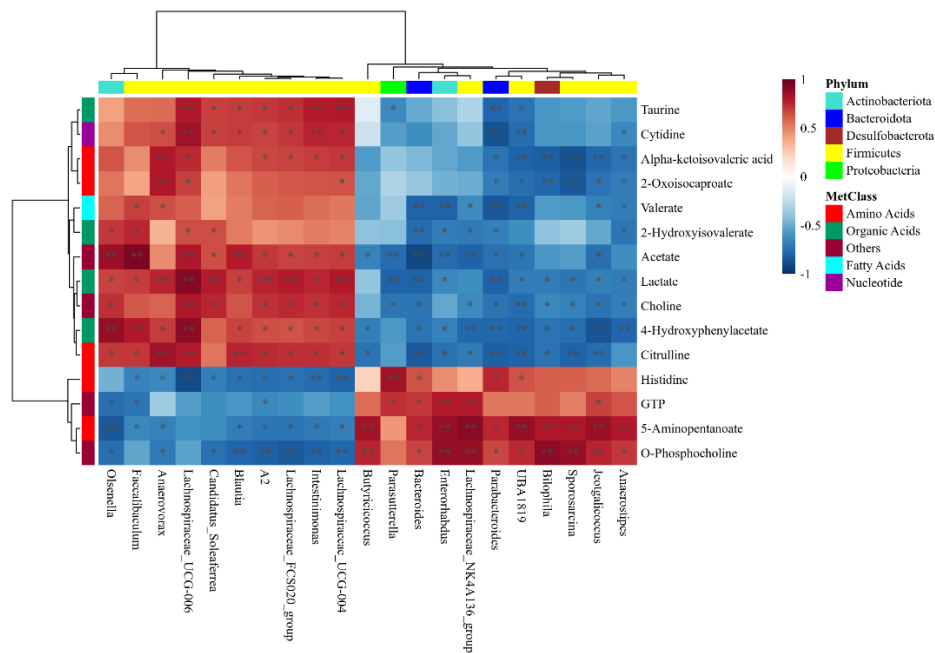
**Supplementary Table S3.5** | Spearman correlation between differentially abundant gut bacteria and tight junction gene expression in brain cortex

| Bacterial genera                    | <i>Cldn-1</i> |             | <i>Ocln</i> |             | <i>Zo-1</i> |             |
|-------------------------------------|---------------|-------------|-------------|-------------|-------------|-------------|
|                                     | R             | P           | R           | P           | R           | P           |
| <i>Bifidobacterium</i>              | 0.47          | <b>0.02</b> | -0.32       | 0.14        | 0.18        | 0.42        |
| <i>Bacteroides</i>                  | -0.53         | <b>0.00</b> | 0.62        | <b>0.00</b> | 0.04        | 0.86        |
| <i>Prevotellaceae-UCG-001</i>       | -0.26         | 0.23        | 0.45        | <b>0.03</b> | 0.15        | 0.51        |
| <i>Rikenellaceae-RC9-gut-group</i>  | -0.31         | 0.15        | 0.46        | <b>0.03</b> | -0.04       | 0.87        |
| <i>Turicibacter</i>                 | -0.51         | <b>0.01</b> | 0.60        | <b>0.00</b> | -0.08       | 0.71        |
| <i>Enterococcus</i>                 | -0.25         | 0.23        | 0.48        | <b>0.02</b> | 0.01        | 0.71        |
| <i>Lactococcus</i>                  | -0.64         | <b>0.00</b> | 0.53        | <b>0.00</b> | -0.01       | 0.96        |
| <i>Streptococcus</i>                | -0.35         | 0.09        | 0.51        | <b>0.01</b> | 0.12        | 0.59        |
| <i>Clostridium-sensu-stricto-1</i>  | -0.49         | <b>0.02</b> | 0.34        | <b>0.11</b> | 0.06        | 0.79        |
| <i>Acetatifactor</i>                | 0.75          | <b>0.00</b> | -0.64       | <b>0.00</b> | 0.15        | 0.51        |
| <i>ASF356</i>                       | -0.25         | 0.25        | 0.20        | 0.36        | -0.47       | <b>0.03</b> |
| <i>Lachnospiraceae-FCS020-group</i> | 0.37          | 0.08        | -0.44       | <b>0.04</b> | -0.12       | 0.61        |
| <i>Colidextribacter</i>             | 0.43          | <b>0.04</b> | -0.30       | 0.17        | 0.14        | 0.53        |
| <i>Oscillibacter</i>                | 0.55          | <b>0.00</b> | -0.45       | <b>0.03</b> | 0.23        | 0.31        |
| <i>Paludicola</i>                   | 0.69          | <b>0.00</b> | -0.39       | 0.07        | 0.26        | 0.23        |
| <i>Anaerovorax</i>                  | 0.45          | <b>0.03</b> | -0.22       | 0.31        | -0.16       | 0.48        |
| <i>Akkermansia</i>                  | -0.34         | 0.10        | 0.41        | 0.05        | 0.06        | 0.79        |

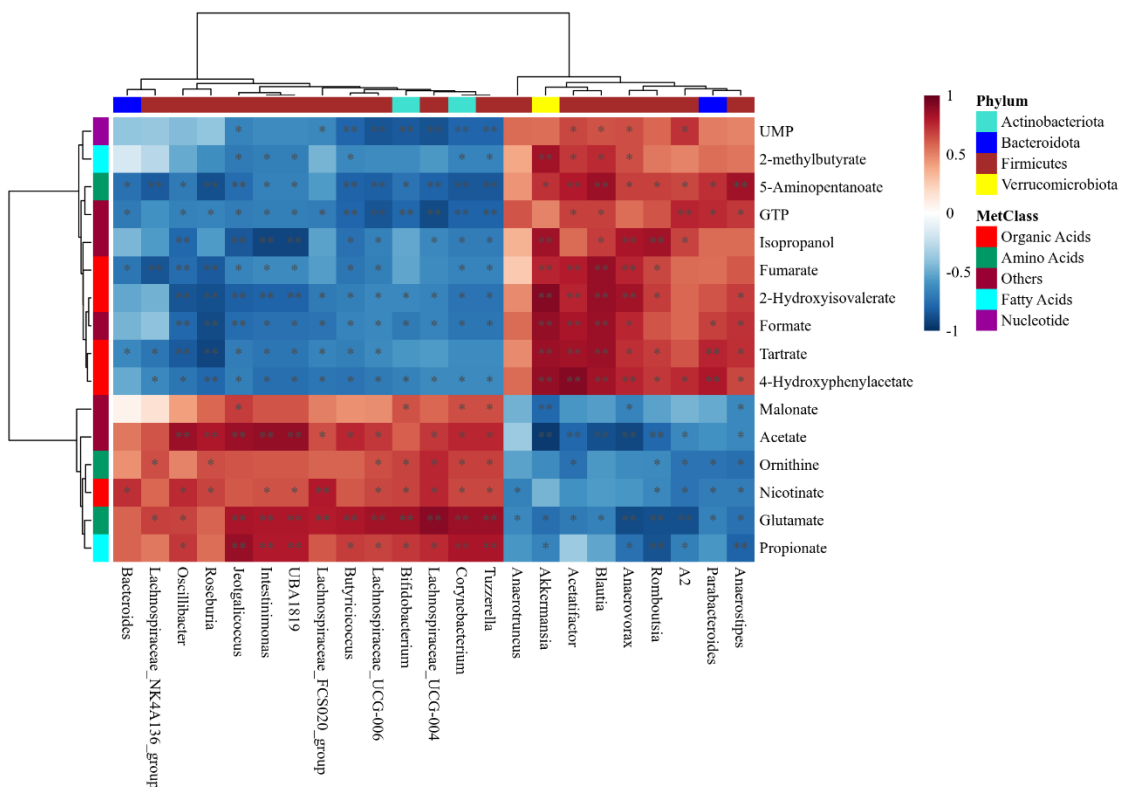
**Supplementary Table S3.6** | Spearman correlation between differentially abundant gut bacterial metabolites and tight junction gene expression in brain cortex

| Gut Metabolites             | <i>Cldn1</i> |             | <i>Ocln</i> |             | <i>Zo-1</i> |             |
|-----------------------------|--------------|-------------|-------------|-------------|-------------|-------------|
|                             | R            | P           | R           | P           | R           | P           |
| 2-Oxoglutarate              | 0.69         | <b>0.00</b> | -0.61       | <b>0.00</b> | 0.25        | 0.27        |
| 2-Oxoisocaproate            | 0.66         | <b>0.00</b> | -0.70       | <b>0.00</b> | 0.03        | 0.89        |
| 3-Hydroxybutyrate           | 0.63         | <b>0.00</b> | -0.48       | <b>0.02</b> | 0.24        | 0.28        |
| 3-Methyl-2-oxovalerate      | 0.68         | <b>0.00</b> | -0.69       | <b>0.00</b> | 0.01        | 0.96        |
| 4-Aminobutyrate             | 0.28         | 0.19        | -0.50       | <b>0.02</b> | 0.06        | 0.77        |
| 4-Hydroxyphenylacetate      | 0.53         | <b>0.00</b> | -0.41       | 0.05        | -0.04       | 0.87        |
| Alanine                     | -0.35        | 0.10        | 0.43        | <b>0.04</b> | 0.04        | 0.86        |
| Alpha-ketoisovaleric acid   | 0.71         | <b>0.00</b> | -0.74       | <b>0.00</b> | 0.01        | 0.96        |
| Arginine                    | -0.41        | <b>0.05</b> | 0.46        | <b>0.03</b> | 0.21        | 0.34        |
| Asparagine                  | -0.21        | 0.32        | 0.51        | <b>0.01</b> | 0.21        | 0.34        |
| Caproic acid                | -0.52        | <b>0.00</b> | 0.45        | <b>0.03</b> | 0.04        | 0.87        |
| Citrulline                  | 0.31         | 0.15        | -0.56       | <b>0.00</b> | 0.03        | 0.88        |
| Creatine                    | 0.39         | 0.06        | -0.49       | <b>0.02</b> | 0.23        | 0.30        |
| Fumarate                    | -0.32        | 0.13        | 0.53        | <b>0.00</b> | -0.04       | 0.86        |
| Glutamate                   | 0.44         | <b>0.03</b> | -0.35       | 0.10        | 0.29        | 0.19        |
| Glycerol                    | 0.34         | 0.10        | -0.25       | 0.24        | 0.56        | <b>0.00</b> |
| Histidine                   | -0.29        | 0.17        | 0.43        | <b>0.04</b> | 0.12        | 0.59        |
| Inosine                     | -0.52        | <b>0.00</b> | 0.59        | <b>0.00</b> | -0.14       | 0.52        |
| Isovalerate                 | 0.14         | 0.52        | -0.47       | <b>0.02</b> | -0.15       | 0.49        |
| Malonate                    | 0.00         | 0.99        | 0.15        | 0.50        | 0.51        | <b>0.02</b> |
| N-Acetylglucosamine         | 0.55         | <b>0.00</b> | -0.40       | 0.06        | 0.09        | 0.68        |
| Nicotinate                  | 0.30         | 0.16        | -0.52       | <b>0.01</b> | 0.04        | 0.86        |
| Propionate                  | -0.48        | <b>0.02</b> | 0.40        | 0.06        | -0.31       | 0.16        |
| Ribose                      | 0.50         | <b>0.01</b> | -0.56       | <b>0.00</b> | 0.32        | 0.14        |
| Tartrate                    | -0.35        | 0.10        | 0.48        | <b>0.02</b> | -0.03       | 0.90        |
| Taurine                     | -0.13        | 0.55        | 0.33        | 0.13        | 0.56        | <b>0.00</b> |
| Tauro conjugated bile acids | 0.46         | 0.03        | -0.33       | 0.12        | 0.20        | 0.37        |
| Trimethylamine              | 0.38         | 0.06        | -0.51       | <b>0.01</b> | 0.11        | 0.62        |
| Tryptophan                  | -0.26        | 0.22        | 0.44        | <b>0.04</b> | 0.26        | 0.24        |
| Uracil                      | 0.60         | <b>0.00</b> | -0.60       | <b>0.00</b> | 0.21        | 0.36        |
| Xylose                      | -0.44        | <b>0.03</b> | 0.48        | <b>0.02</b> | -0.02       | 0.94        |
| Malate                      | -0.44        | <b>0.03</b> | 0.64        | <b>0.00</b> | -0.04       | 0.85        |
| myo-Inositol                | -0.20        | 0.34        | 0.29        | 0.19        | 0.45        | <b>0.04</b> |
| sn-Glycero-3-phosphocholine | -0.58        | <b>0.00</b> | 0.21        | 0.33        | -0.19       | 0.39        |

### LF vs Inulin Males

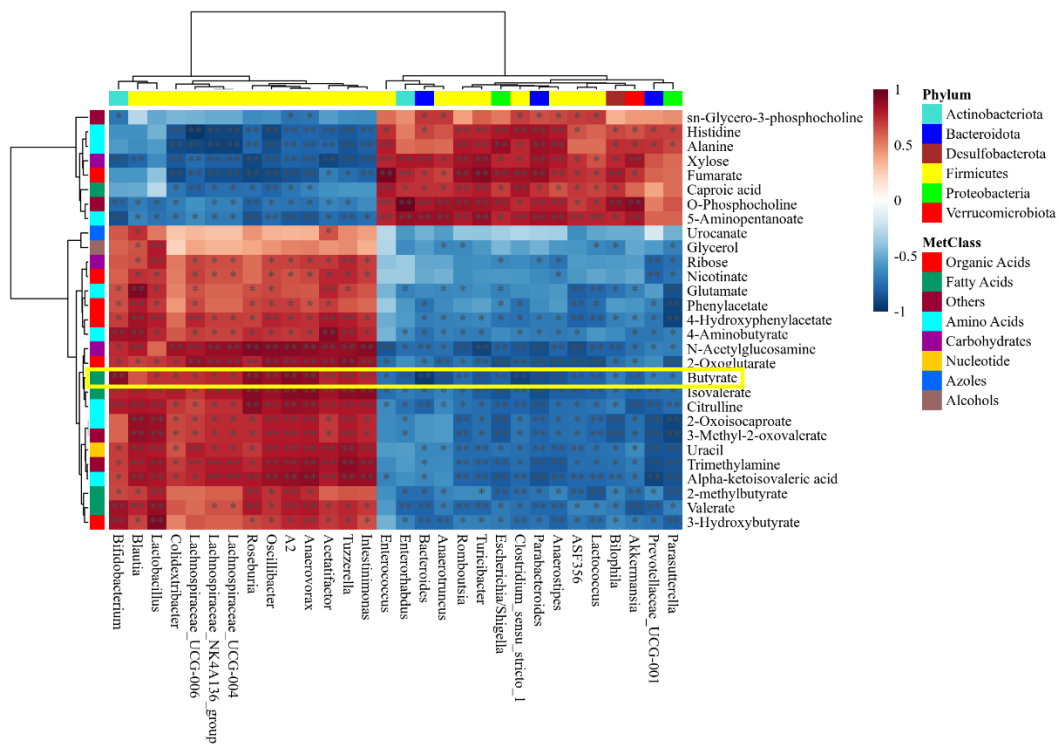


### LF vs Inulin Females

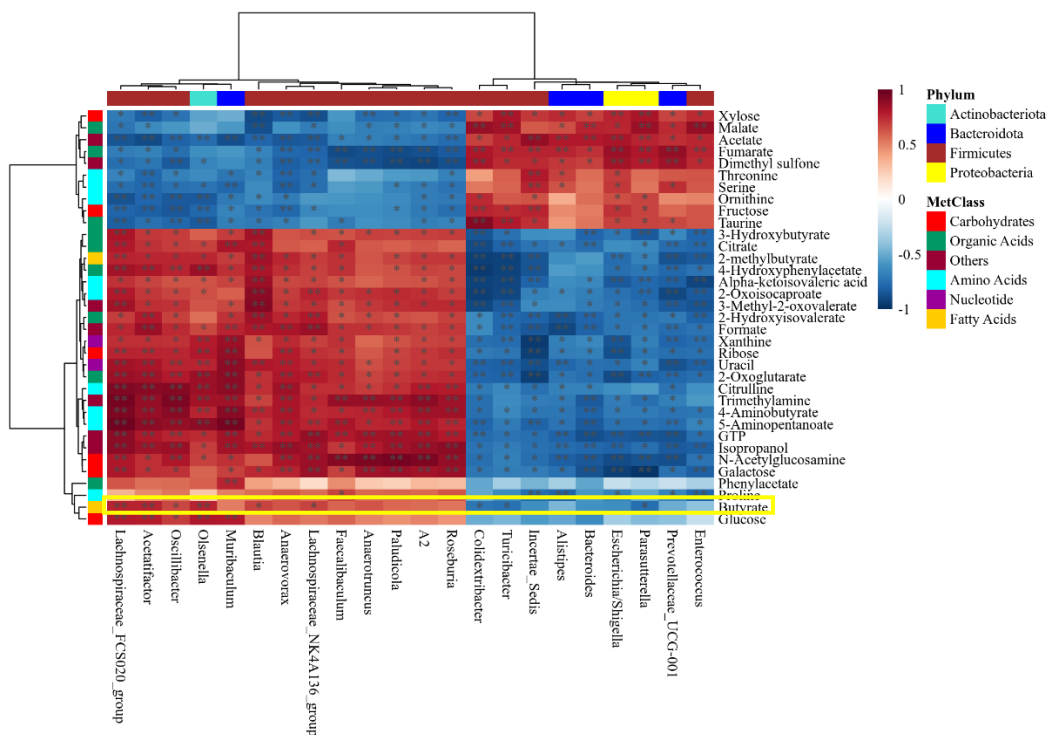


**Supplementary Figure S3.5** | Correlation analysis between caecal metabolites and gut microbiota conducted using M<sup>2</sup>IA, in males and females fed with inulin supplemented diet. Significant correlations are annotated by asterisks. \*=p<0.05 and \*\*=p<0.01.

## LF vs Psyllium Males

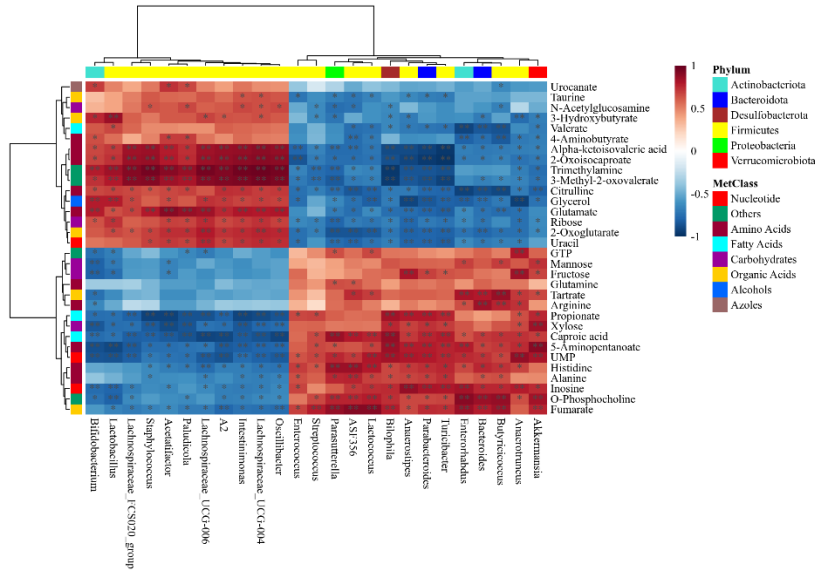


## LF vs Psyllium Females

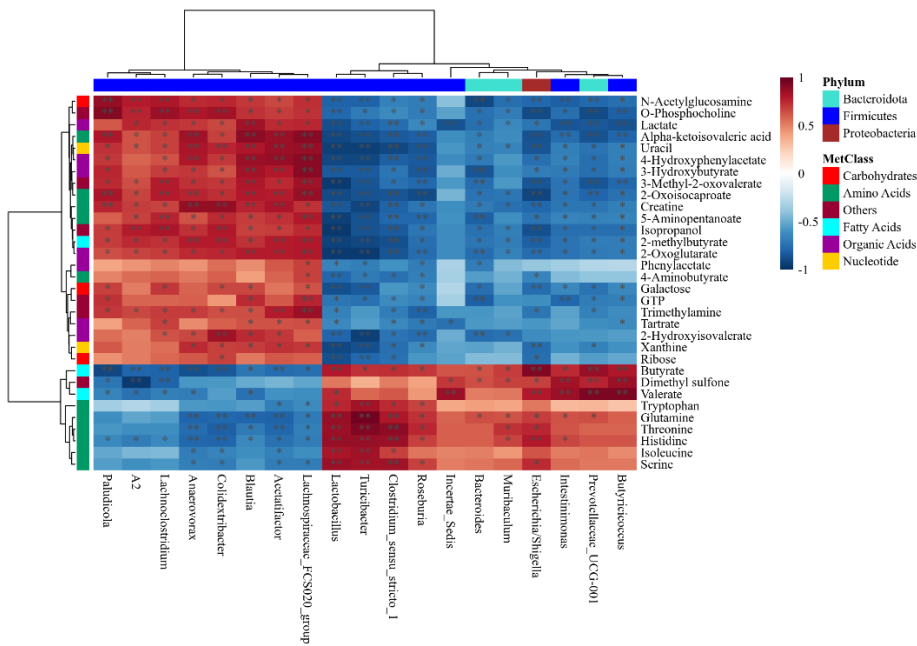


**Supplementary Figure S3.6 |** Correlation analysis between caecal metabolites and gut microbiota conducted using M<sup>2</sup>IA, in males and females fed with psyllium supplemented diet. Significant correlations are annotated by asterisks. \* =  $p < 0.05$  and \*\* =  $p < 0.01$ .

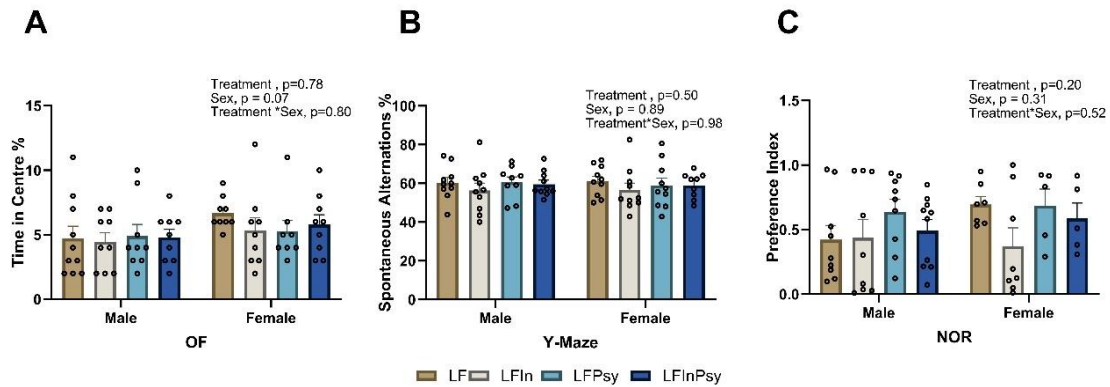
### LF vs Inulin-Psyllium Males



### LF vs Inulin-Psyllium Females

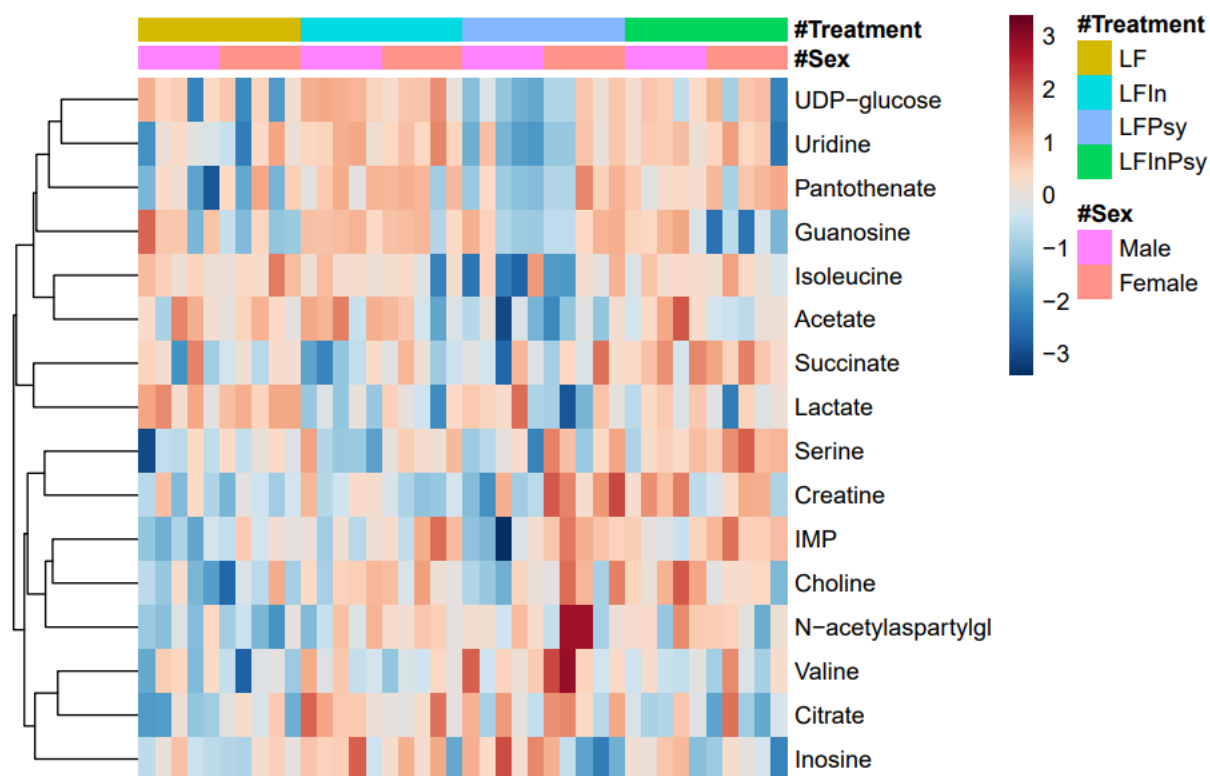


**Supplementary Figure S3.7** | Correlation analysis between caecal metabolites and gut microbiota conducted using M<sup>2</sup>IA, in males and females fed with inulin-psyllium combination diet. Significant correlations are annotated by asterisks. \*= $p < 0.05$  and \*\*= $p < 0.01$

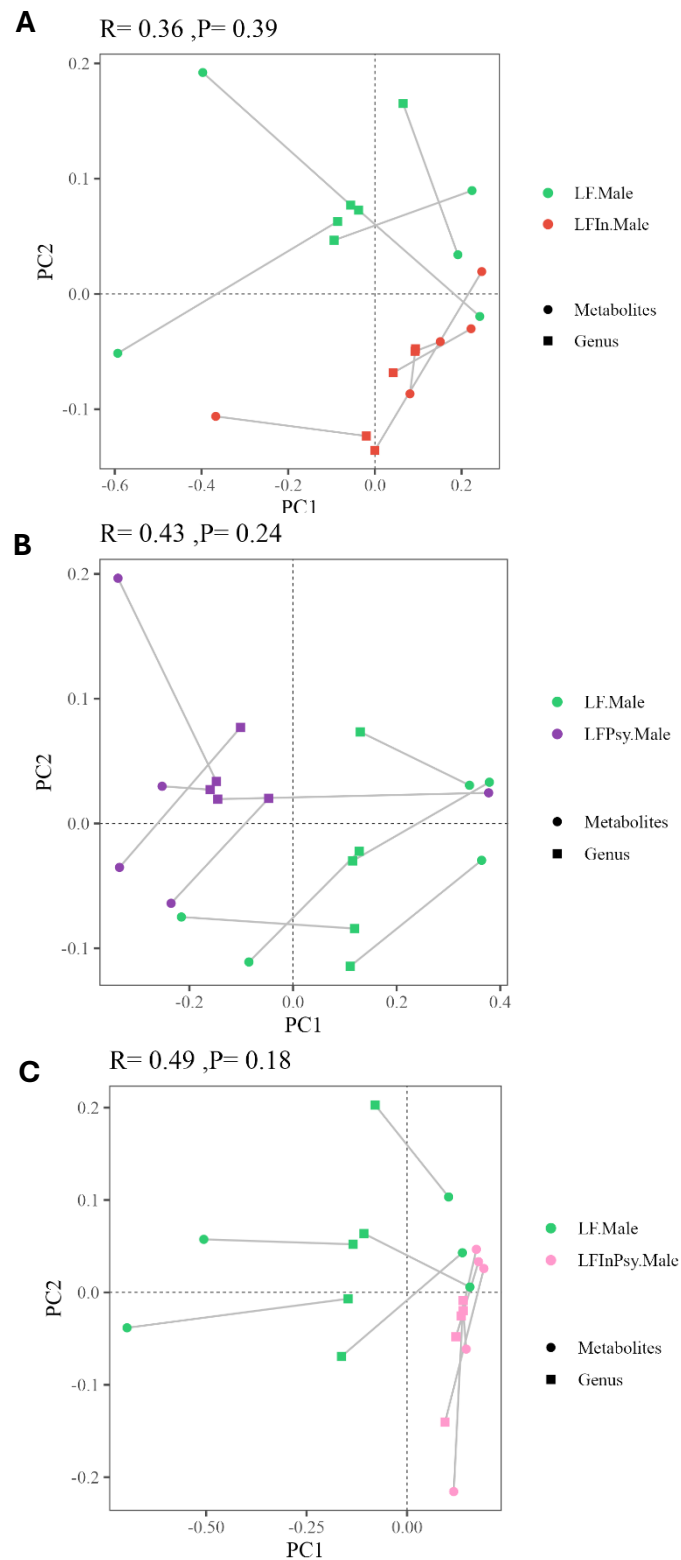


### Supplementary Figure S3.8 | Dietary fibre interventions did not alter behaviour in males and females.

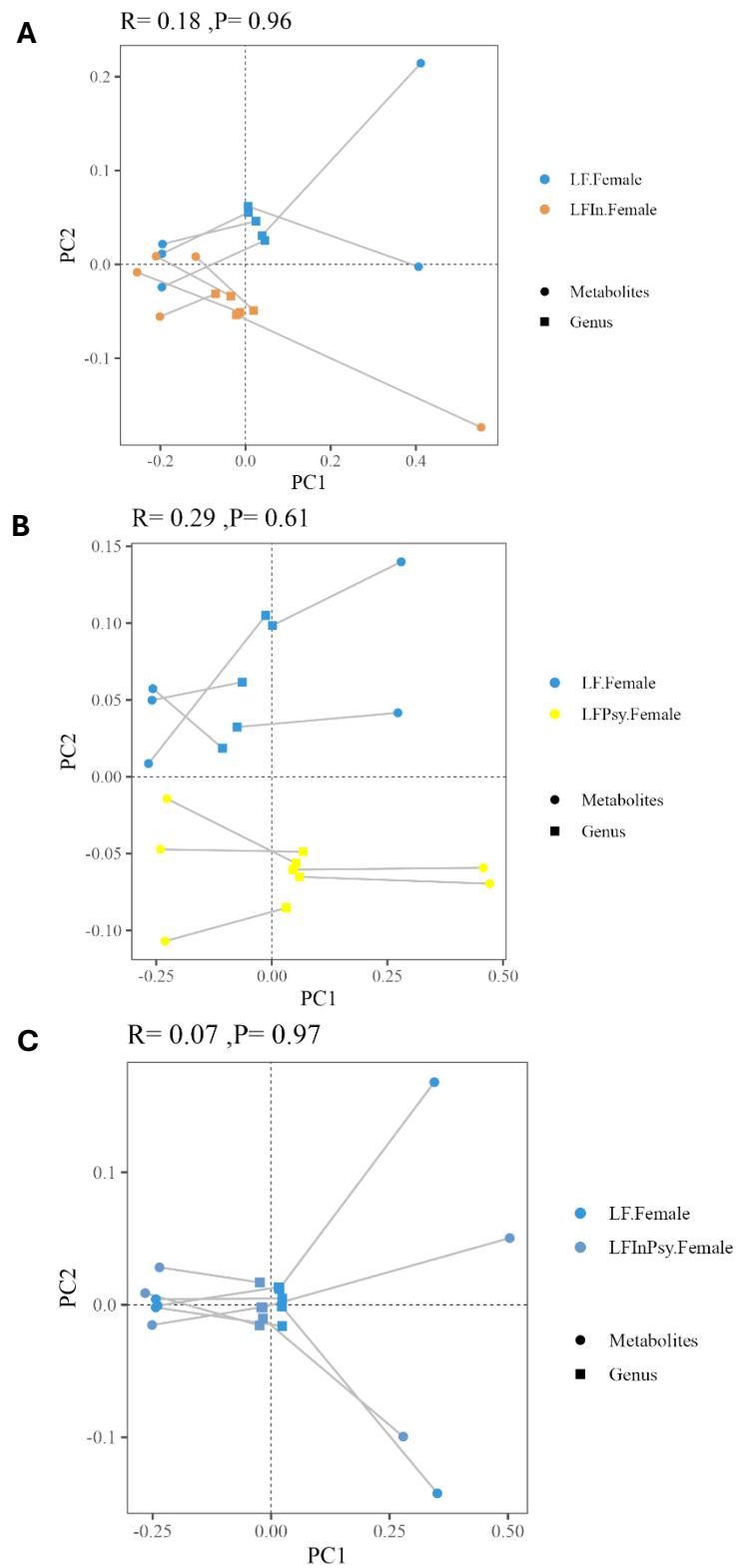
(A) Anxiety like behaviour determined via Open field (OF) task was unaffected by DF interventions in both males and females. Whereas (B) Y-Maze performance and (C) Recognition memory as assessed by novel object recognition task were comparable across DF supplementation in both sexes. Two-way ANOVA was used to calculate statistical significance, and p values are plotted on each graph.



**Supplementary Figure S3.9** | Heatmap showing differentially abundant metabolites in the brain cortex region.



**Supplementary Figure S3.10** | Procrustes Analysis (PA) shows congruence between gut microbiota and cortex metabolite profiles in males.



**Supplementary Figure S3.11** | Procrustes Analysis (PA) shows congruence between gut microbiota and cortex metabolite profiles in females.

**Supplementary Table S4.1** | The eligibility criteria

| <b>Inclusion criteria</b>                                                                                                                                                                                                                                  | <b>Exclusion criteria</b>                                                                                                       |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------|
| Males and females aged 18 or over                                                                                                                                                                                                                          | Vegan, vegetarian                                                                                                               |
| Mild to moderate level anxiety and/or depression (PHQ-9 and/or GAD-7 scores of 5-14)                                                                                                                                                                       | Allergic to any of the study components, for example, nuts and fish                                                             |
| Low MDP adherence (MEDAS score $\leq 7/14$ )                                                                                                                                                                                                               | On antianxiety and/or antidepressant medication which has changed in the last 3 months or likely to change in the next 3 months |
| Able to have an MRI scan                                                                                                                                                                                                                                   | Unwilling or unable to make changes to their diet for 10 days (2x5 days period)                                                 |
| Computer literate with internet access                                                                                                                                                                                                                     | Unable to attend the intervention centre                                                                                        |
| Fluent in written and spoken English                                                                                                                                                                                                                       | MEDAS score $> 7$                                                                                                               |
| Gave consent for the study team to contact their GP                                                                                                                                                                                                        | Not fluent in written and spoken English                                                                                        |
| Willing and able to comply with all study procedures including diet                                                                                                                                                                                        | MRI unsafety                                                                                                                    |
|                                                                                                                                                                                                                                                            | Not agreed for the study team to contact their GP                                                                               |
|                                                                                                                                                                                                                                                            | Not prepared to make changes to diet for 10 days (2x5 days period)                                                              |
| GAD-7, Generalised Anxiety Disorder (includes 7 items); GP, general practitioner; MDP, Mediterranean-style dietary pattern; MEDAS, Mediterranean Diet Adherence Screener tool (includes 14-items); PHQ-9, Patient Health Questionnaire (includes 9-items). |                                                                                                                                 |

**Supplementary Table S4.2** | Nutrient composition of the test lunch meals (day 1) taken from the product labels

|                      |      | <b>Mediterranean Diet</b> | <b>Western Diet</b> |
|----------------------|------|---------------------------|---------------------|
| <b>Energy</b>        | Kcal | 1013                      | 984                 |
| <b>Carbohydrates</b> | g    | 45                        | 123                 |
|                      | %    | 18.3                      | 50.3                |
| <b>Free Sugars</b>   | g    | 0                         | 83                  |
| <b>Fibre</b>         | g    | 10.6                      | 2.9                 |
| <b>Proteins</b>      | g    | 60                        | 37.5                |
|                      | %    | 24.3                      | 15.3                |
| <b>Total fat</b>     | g    | 63                        | 37.3                |
|                      | %    | 57.4                      | 34.4                |
| <b>SFA</b>           | g    | 8.8                       | 15.2                |
|                      | %    | 7.8                       | 14                  |

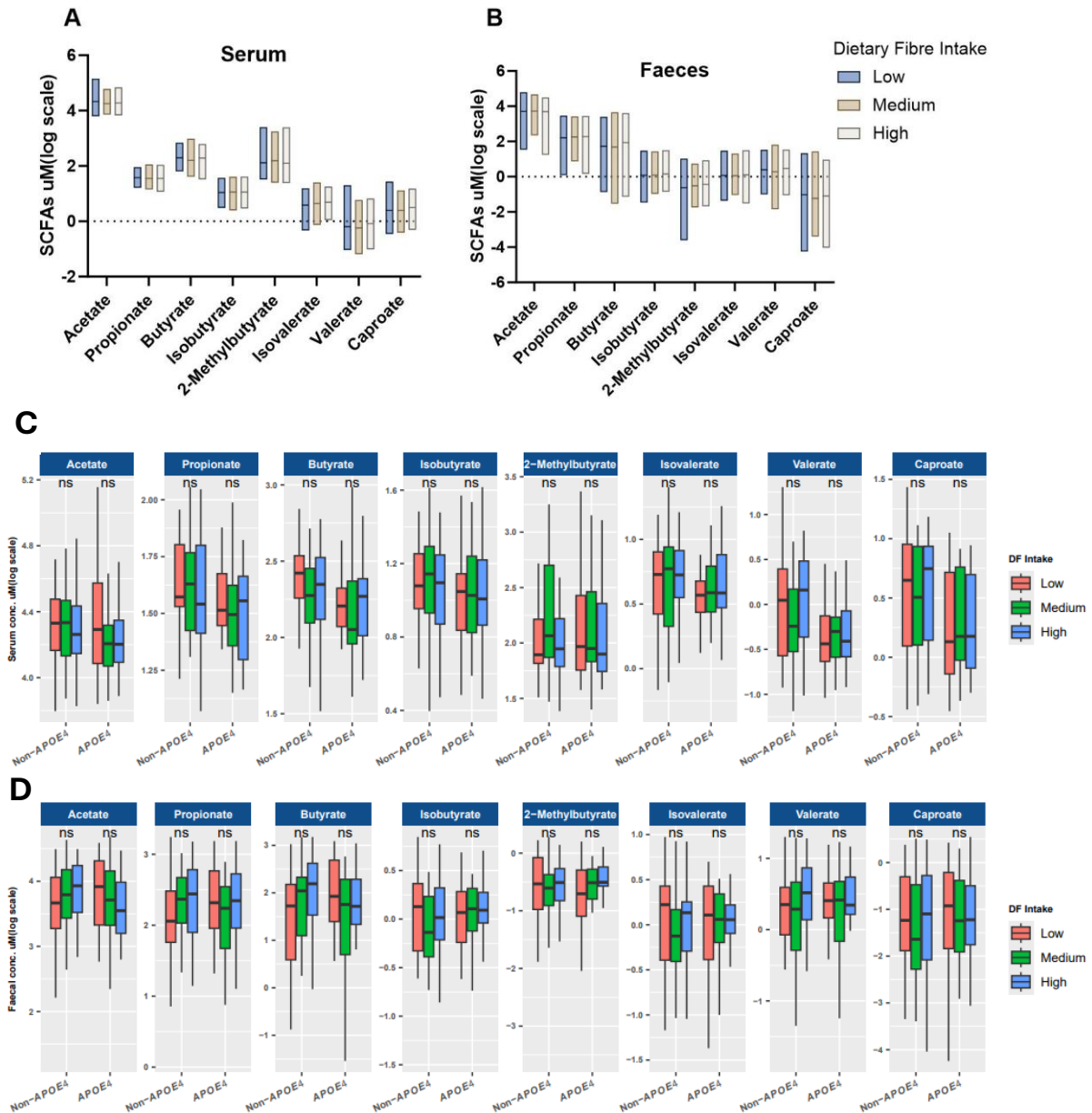
%, contribution to the daily energy intake as percent; g, grams; kcal, kilocalories; SFA, saturated fatty acids

**Supplementary Table S5.1** | Differentially abundant bacterial genera within non-*APOE4* and *APOE4* carriers

| Genus                              | Genotype          | Mean   | SD    | FDR * |
|------------------------------------|-------------------|--------|-------|-------|
| <i>Phascolarctobacterium</i>       | <i>APOE4</i>      | -2.138 | 0.629 | 0.03  |
|                                    | Non- <i>APOE4</i> | -2.466 | 0.528 |       |
| <i>Family XIII AD3011 group</i>    | <i>APOE4</i>      | 0.795  | 1.578 | 0.00  |
|                                    | Non- <i>APOE4</i> | 1.770  | 0.995 |       |
| <i>Family XIII UCG-001</i>         | <i>APOE4</i>      | -0.232 | 1.177 | 0.00  |
|                                    | Non- <i>APOE4</i> | 0.692  | 1.087 |       |
| <i>Mogibacterium</i>               | <i>APOE4</i>      | -1.753 | 1.350 | 0.02  |
|                                    | Non- <i>APOE4</i> | -2.399 | 1.116 |       |
| <i>Bacteroides</i>                 | <i>APOE4</i>      | 5.782  | 1.554 | 0.04  |
|                                    | Non- <i>APOE4</i> | 6.176  | 0.885 |       |
| <i>Butyricicoccus</i>              | <i>APOE4</i>      | 2.043  | 1.243 | 0.08  |
|                                    | Non- <i>APOE4</i> | 2.528  | 1.489 |       |
| <i>Butyricicoccaceae_UCG-008</i>   | <i>APOE4</i>      | -2.004 | 0.781 | 0.02  |
|                                    | Non- <i>APOE4</i> | -2.444 | 0.794 |       |
| <i>Clostridium sensu stricto 1</i> | <i>APOE4</i>      | 2.426  | 2.554 | 0.03  |
|                                    | Non- <i>APOE4</i> | 3.531  | 1.567 |       |
| <i>Corynebacterium</i>             | <i>APOE4</i>      | -2.088 | 0.702 | 0.02  |
|                                    | Non- <i>APOE4</i> | -2.504 | 0.620 |       |
| <i>Defluviitaleaceae UCG-011</i>   | <i>APOE4</i>      | -1.198 | 1.050 | 0.06  |
|                                    | Non- <i>APOE4</i> | -0.631 | 1.267 |       |
| <i>Bilophila</i>                   | <i>APOE4</i>      | -2.184 | 0.592 | 0.00  |
|                                    | Non- <i>APOE4</i> | -2.624 | 0.519 |       |
| <i>Eggerthellaceae_CHKCI002</i>    | <i>APOE4</i>      | -1.662 | 1.529 | 0.08  |
|                                    | Non- <i>APOE4</i> | -2.187 | 1.449 |       |
| <i>Gordonibacter</i>               | <i>APOE4</i>      | -1.082 | 1.429 | 0.10  |
|                                    | Non- <i>APOE4</i> | -1.542 | 1.485 |       |
| <i>Faecalicoccus</i>               | <i>APOE4</i>      | -2.084 | 0.959 | 0.08  |
|                                    | Non- <i>APOE4</i> | -2.560 | 0.933 |       |
| <i>Faecalitalea</i>                | <i>APOE4</i>      | -1.779 | 1.486 | 0.10  |
|                                    | Non- <i>APOE4</i> | -2.361 | 1.201 |       |
| <i>Solobacterium</i>               | <i>APOE4</i>      | -1.933 | 1.386 | 0.06  |
|                                    | Non- <i>APOE4</i> | -2.485 | 1.123 |       |
| <i>Turicibacter</i>                | <i>APOE4</i>      | -2.121 | 0.696 | 0.06  |
|                                    | Non- <i>APOE4</i> | -2.456 | 0.792 |       |
| <i>Eubacterium</i>                 | <i>APOE4</i>      | -2.028 | 0.920 | 0.04  |
|                                    | Non- <i>APOE4</i> | -2.486 | 0.840 |       |
| <i>[Eubacterium] eligens group</i> | <i>APOE4</i>      | 1.132  | 1.868 | 0.04  |
|                                    | Non- <i>APOE4</i> | 2.012  | 1.753 |       |
| <i>Anaerostipes</i>                | <i>APOE4</i>      | 3.558  | 1.638 | 0.10  |
|                                    | Non- <i>APOE4</i> | 4.123  | 1.260 |       |
| <i>Coproccoccus</i>                | <i>APOE4</i>      | 3.994  | 1.791 | 0.03  |

|                                              |           |        |       |      |
|----------------------------------------------|-----------|--------|-------|------|
|                                              | Non-APOE4 | 4.705  | 0.757 |      |
| <b><i>Lachnospiraceae_GCA-900066575</i></b>  | APOE4     | -0.680 | 1.229 | 0.08 |
|                                              | Non-APOE4 | -0.155 | 1.316 |      |
| <b><i>Lachnospiraceae_UCG-008</i></b>        | APOE4     | -2.066 | 0.676 | 0.10 |
|                                              | Non-APOE4 | -2.375 | 0.750 |      |
| <b><i>Odoribacter</i></b>                    | APOE4     | 0.452  | 1.622 | 0.05 |
|                                              | Non-APOE4 | 1.086  | 1.262 |      |
| <b><i>Colidextribacter</i></b>               | APOE4     | 0.652  | 1.674 | 0.02 |
|                                              | Non-APOE4 | 1.527  | 1.231 |      |
| <b><i>Oscillospiraceae_NK4A214 group</i></b> | APOE4     | 1.570  | 2.206 | 0.03 |
|                                              | Non-APOE4 | 2.613  | 1.918 |      |
| <b><i>Oscillospira</i></b>                   | APOE4     | -1.295 | 1.188 | 0.08 |
|                                              | Non-APOE4 | -0.729 | 1.467 |      |
| <b><i>Pseudoflavonifractor</i></b>           | APOE4     | -2.032 | 0.762 | 0.03 |
|                                              | Non-APOE4 | -2.470 | 0.856 |      |
| <b><i>Oscillospiraceae_UCG-005</i></b>       | APOE4     | 2.179  | 1.637 | 0.10 |
|                                              | Non-APOE4 | 2.839  | 1.409 |      |
| <b><i>Prevotella</i></b>                     | APOE4     | -1.823 | 1.441 | 0.02 |
|                                              | Non-APOE4 | -2.446 | 0.827 |      |
| <b><i>Prevotella_7</i></b>                   | APOE4     | -1.301 | 2.232 | 0.03 |
|                                              | Non-APOE4 | -2.192 | 1.689 |      |
| <b><i>Anaerotruncus</i></b>                  | APOE4     | -1.577 | 0.960 | 0.06 |
|                                              | Non-APOE4 | -2.029 | 1.059 |      |
| <b><i>Incertae Sedis</i></b>                 | APOE4     | 2.160  | 1.387 | 0.02 |
|                                              | Non-APOE4 | 2.807  | 0.826 |      |
| <b><i>Phoceia</i></b>                        | APOE4     | -1.930 | 0.649 | 0.03 |
|                                              | Non-APOE4 | -2.370 | 0.899 |      |
| <b><i>Streptococcus</i></b>                  | APOE4     | -2.091 | 0.848 | 0.03 |
|                                              | Non-APOE4 | -2.545 | 0.727 |      |
| <b><i>Sutterella</i></b>                     | APOE4     | -2.200 | 0.635 | 0.01 |
|                                              | Non-APOE4 | -2.599 | 0.551 |      |
| <b><i>Cloacibacillus</i></b>                 | APOE4     | -1.924 | 1.348 | 0.08 |
|                                              | Non-APOE4 | -2.377 | 1.023 |      |
| <b><i>Parabacteroides</i></b>                | APOE4     | 2.966  | 1.860 | 0.03 |
|                                              | Non-APOE4 | 3.592  | 1.459 |      |

Data is reported as mean and SD of the centered log ratio (CLR) transformed abundance of each bacterial genera. Linear regression model was used for each genera and *p* values for all the contrasts were corrected for false discovery rate (FDR) using Benjamini & Hochberg's procedure. q-value of 0.1 was used as a cut-off for statistical significance.



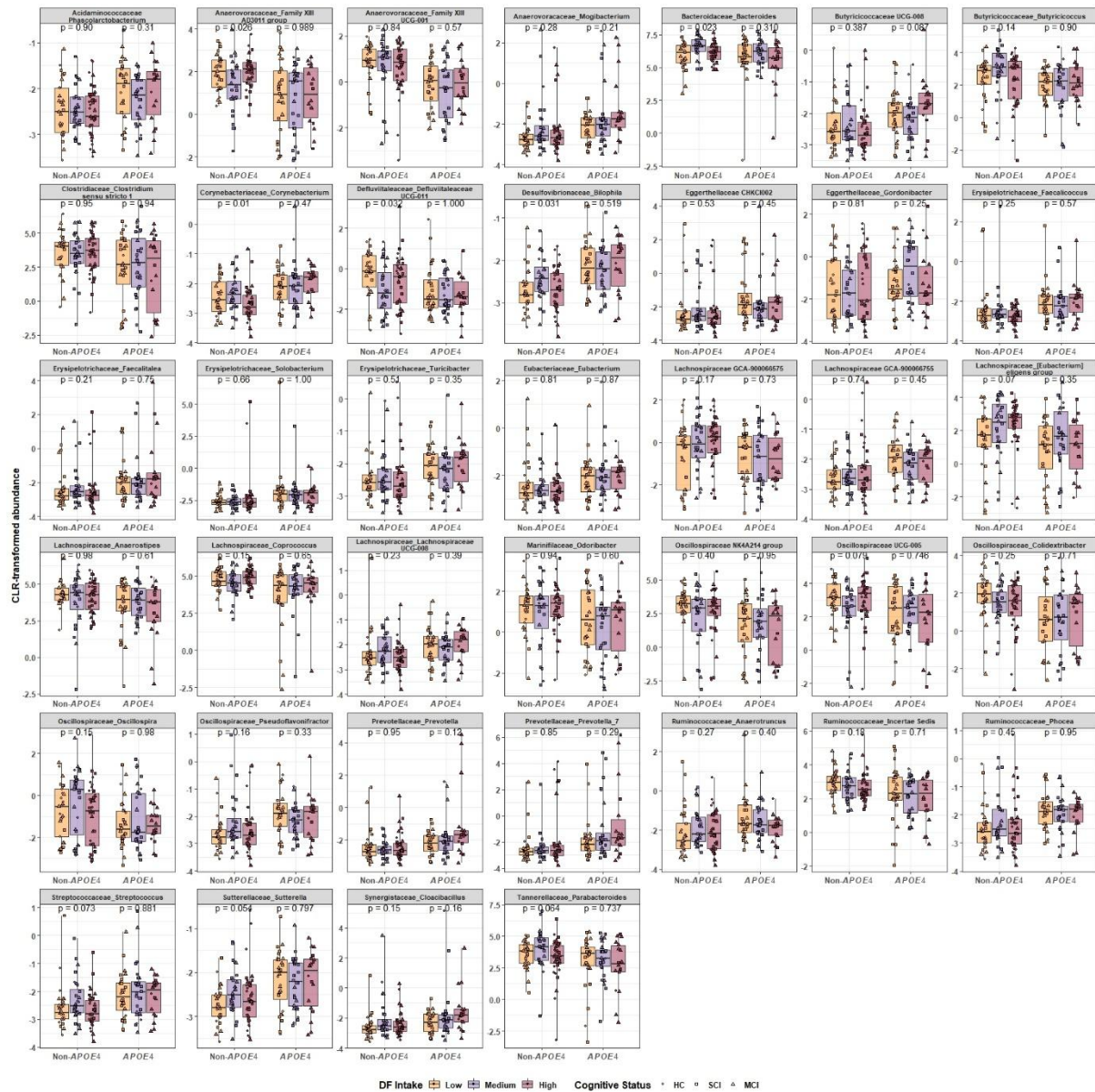
**Supplementary Figure S5.1** | Association between dietary fibre (DF) intake, *APOE4* genotype, and SCFAs concentrations. A) Serum and B) Faecal SCFAs concentrations ( $\mu\text{M}$  log scaled) in participants with low, medium, and high DF consumption. C) Serum SCFAs concentrations ( $\mu\text{M}$  log scaled) in people with non-*APOE4* and *APOE4* genotype and split based on their DF intake. D) Faecal SCFAs concentrations ( $\mu\text{M}$  log scaled) in people with non-*APOE4* and *APOE4* genotype and split based on their DF intake. Kruskal-Wallis test was performed for comparisons and significance levels are presented on the plots; ns = non-significant ( $p > 0.05$ ).

**Supplementary Table S5.2 | Age, Sex and BMI comparisons between non-APOE4 and APOE4 carriers in DF consumer groups**

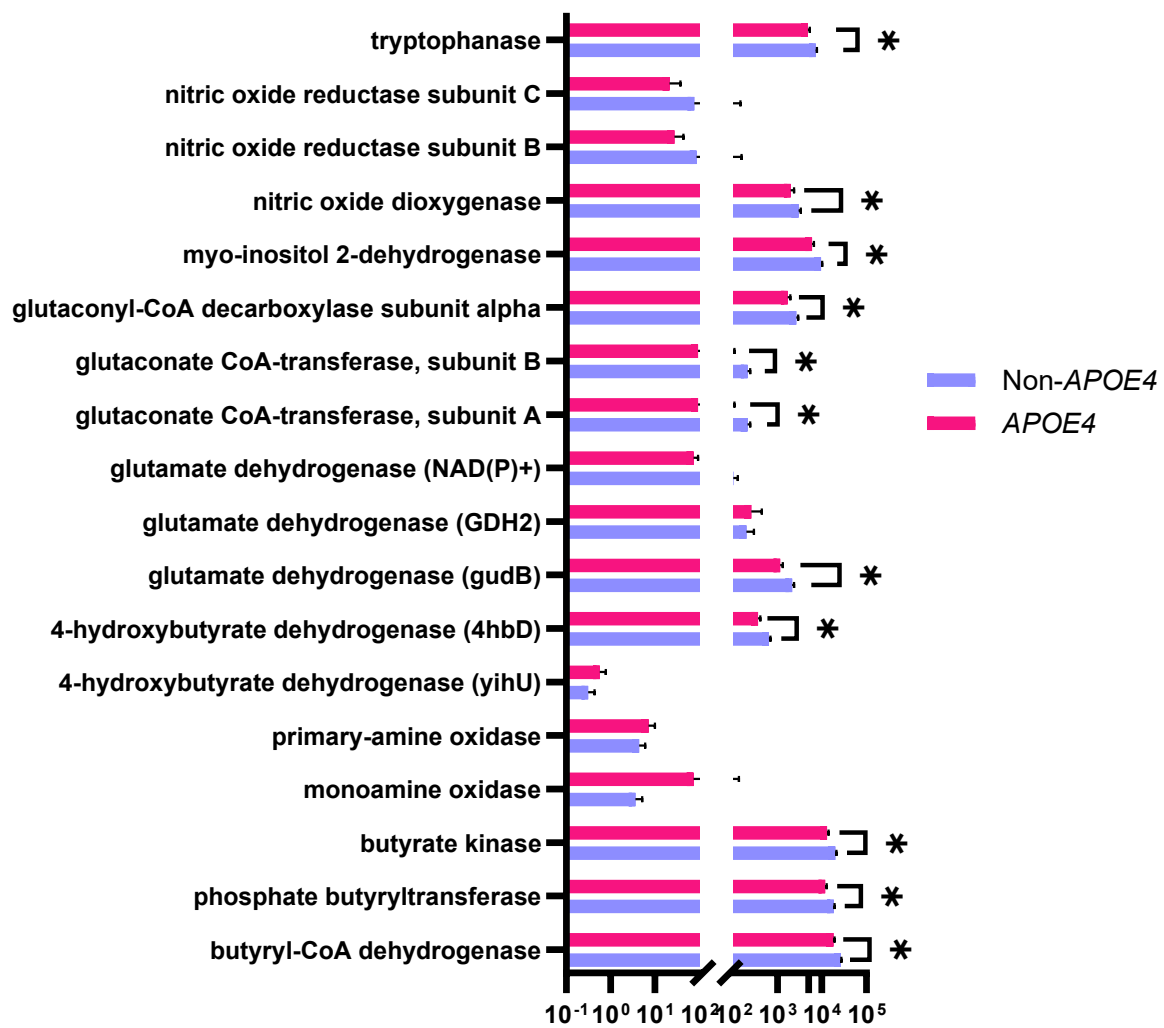
| Overall                       |                | Low DF Consumers<br>(n=57) |                   |           | Medium DF Consumers<br>(n=56) |                       |          | High DF Consumers<br>(n=57) |                   |          |
|-------------------------------|----------------|----------------------------|-------------------|-----------|-------------------------------|-----------------------|----------|-----------------------------|-------------------|----------|
|                               |                | Non-APOE4<br>carriers      | APOE4<br>carriers | P- values | Non-APOE4<br>carriers         | APOE4<br>carrier<br>s | P-values | Non-APOE4<br>carriers       | APOE4<br>carriers | P-values |
| <b>Age (y)</b>                | 65.8<br>(6.0)  | 65.2<br>(6.7)              | 64.8<br>(6.3)     | 0.73      | 66.4<br>(4.4)                 | 66.0<br>(6.4)         | 0.73     | 65.8<br>(6.1)               | 66.6<br>(6.0)     | 0.57     |
| <b>Sex, M/F<br/>(%F)</b>      | 75/95<br>(56%) | 7/22<br>(76%)              | 12/16<br>(57%)    | 0.03      | 15/13<br>(46%)                | 11/17<br>(61%)        | 0.08     | 20/19<br>(49%)              | 10/8<br>(44%)     | 0.67     |
| <b>BMI (kg/m<sup>2</sup>)</b> | 25.7<br>(3.7)  | 24.9<br>(3.0)              | 28.0<br>(4.5)     | 0.20      | 25.1<br>(3.0)                 | 25.9<br>(4.6)         | 0.29     | 25.4<br>(2.8)               | 25.2<br>(3.7)     | 0.85     |

Data are mean (SD) or as stated otherwise.

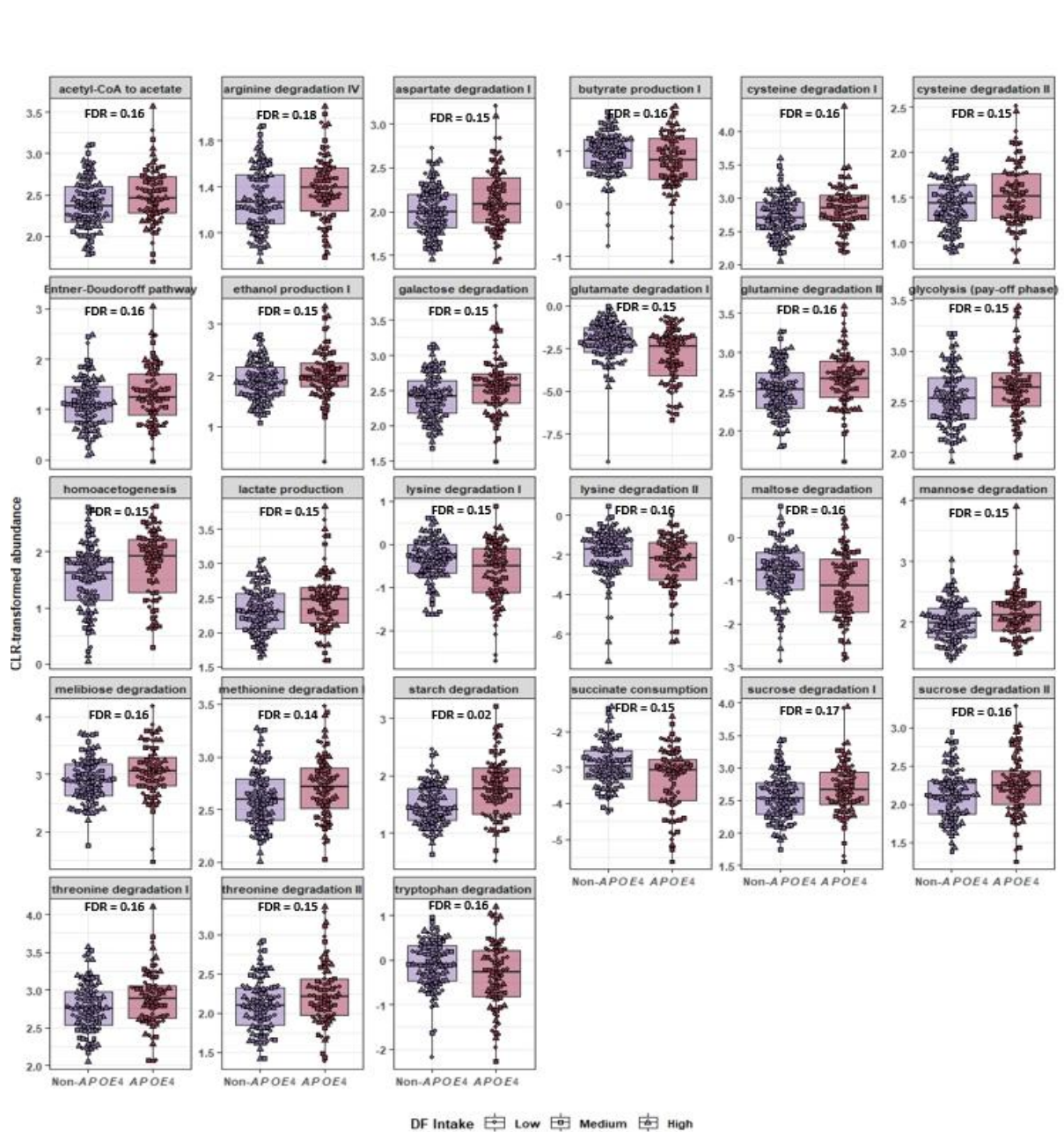
DF, dietary fibre; T-test was used to compare between-genotype differences for Age and BMI within each group, and Chi-square test was used for sex comparisons.



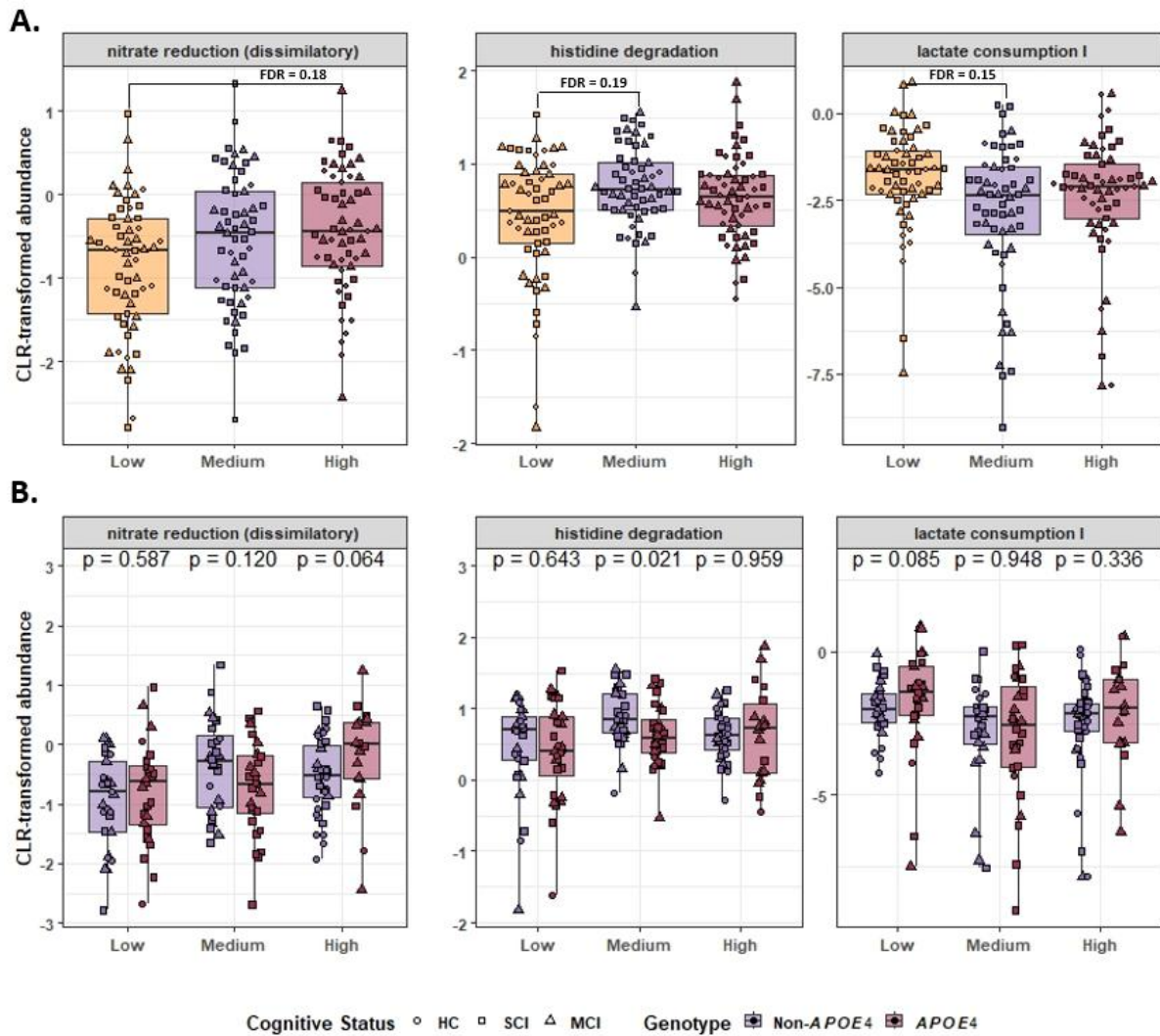
**Supplementary Figure S5.2** | Differentially abundant bacterial genera within non-APOE4 and APOE4 carriers split by dietary fibre (DF) intake. Kruskal-Wallis test was performed to compare the effect of DF intake within each genotype, p values are reported on individual boxplots.



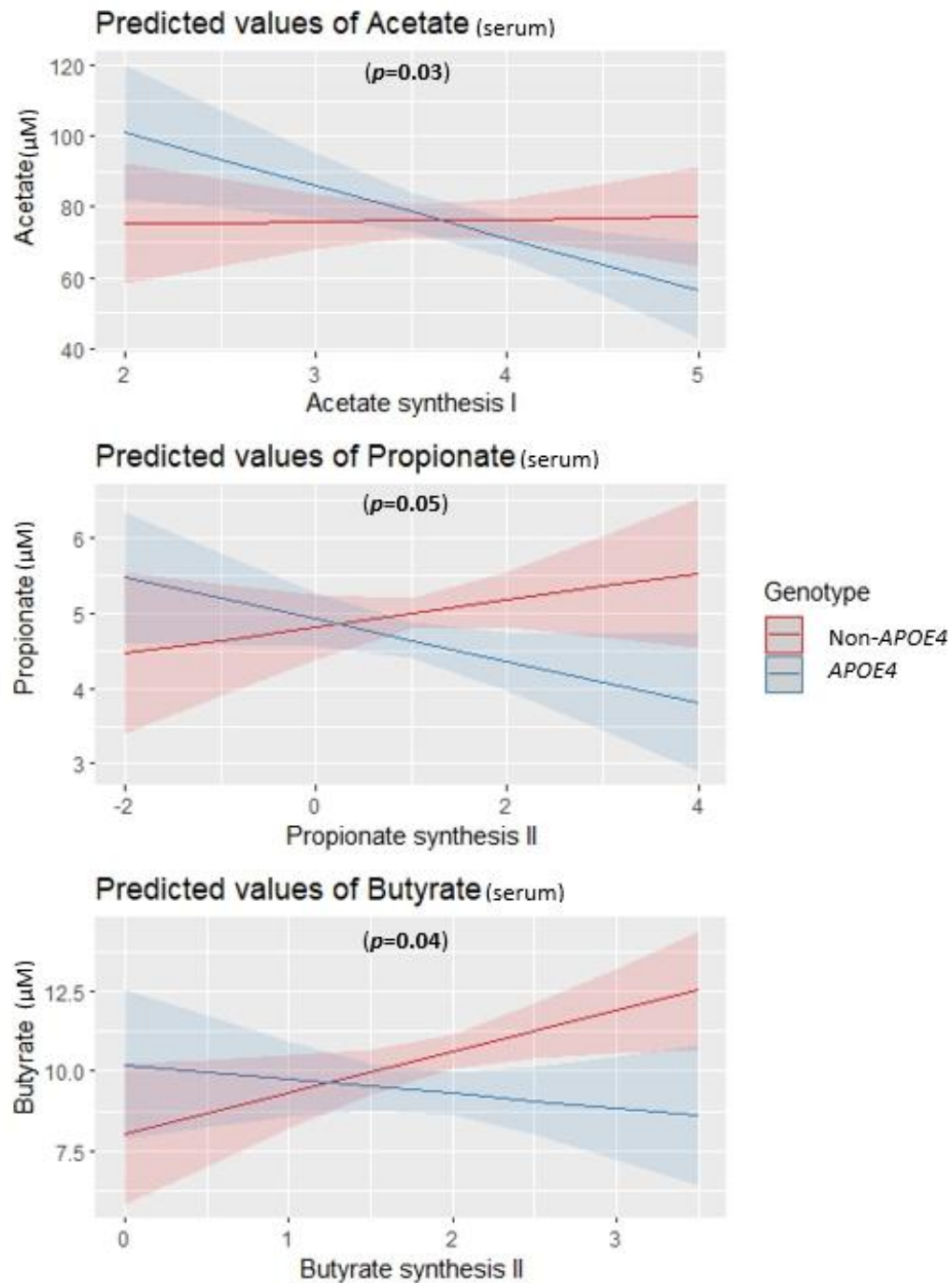
**Supplementary Figure S5.3** | PICRUSt2 predicted KEGG Orthologue (KO) enzymes abundance in non-APOE4 and APOE4 carriers. Data are presented as mean  $\pm$  SEM of enzyme abundance values (log 10 transformed). Multiple Mann-Whitney tests were used to compare groups followed by Benjamini and Hochberg FDR correction. \* = p<0.05.



**Supplementary Figure S5.4** | Differentially abundant Gut-Metabolic Modules (GMMs) based on *APOE4* genotype and the association with dietary fibre (DF) intake. Linear regression analysis was performed, and p values are reported following Benjamini & Hochberg FDR correction.



**Supplementary Figure S5.5 | Differentially abundant Gut-Metabolic Modules (GMMs) based on dietary fibre (DF) intake and the association with *APOE4* genotype.** A) Differentially abundant Gut-Metabolic modules based on DF intake. Linear regression analysis was performed, and p values are reported following Benjamini & Hochberg FDR correction. B) Role of *APOE4* genotype within each group. Kruskal-Wallis tests were used to compare means based on genotype within each DF intake group and p values are mentioned on the plots.



**Supplementary Figure S5.6** | Association between Gut-Brain Modules (predicting SCFAs syntheses) and serum SCFA levels. Linear regression analysis was performed between predicted SCFAs synthesis (x-axis) and their serum levels (y-axis), and significant interactions ( $p<0.05$ ) between GBM modules for SCFAs synthesis and *APOE4* genotype are plotted for respective SCFAs.