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National University of Ireland, Cork

Department of Anatomy & Neuroscience

Head of Dept. John F. Cryan

Deciphering the Role of Microbially-Derived Metabolites on the Microbiota-Gut-Brain Axis

Thesis presented by

Simon Spichak

ORCID: 0000-0003-1226-5527

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Supervisor(s): Prof. John F. Cryan, Prof. Timothy G. Dinan

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Table of Contents

Declaration 4

Author Contributions 5

Acknowledgments 6

Publications 7

Abstract 8

Chapter 1: Introduction 9

The Microbiota 10

Modes of Communication between the Microbiota and the Brain 13

Major Microbial-Derived Metabolites 14

Early Life Factors: Delivery Mode, Breastfeeding, Antibiotics 17

Host Genetics and the Microbiota 18

Drugs and the Microbiota 19

Diet 21

Maternal Stress 25

Maternal Immune Activation 26

Postnatal Stress and the Microbiota 28

Sex-Specific Programming of Psychiatric Disorders Later in Life 29

Importance of the Microbiota: Observations from the Germ-Free Model 30

Generation of the GF Rodent 31

Physiological and Anatomical Differences in the GF Rodent 31

Microbiota-Gut Brain Axis Metabolite-Mediated Modulation of Glia 33

Astrocytes 33

Microglia 35

Oligodendrocytes 37

Sequencing the Gut Microbiota 39

Overall goal and Specific Aims 42

Aims Addressed in Chapter 2 42

Aims Addressed in Chapter 3 43

Chapter 2: Mining Microbes for Mental Health: Determining the Role of Microbial Metabolic Pathways in Human Brain Health and Disease 44

Graphical Abstract: 45

Abstract 46

Highlights 47

Introduction 48

2.0 Methods 50

3.0 Results and Discussion 55

4.0 Conclusion 95

5.0 Future Directions 99

Chapter 3: Microbially-Derived Short-Chain Fatty Acids Impact Astrocyte Gene Expression in a Sex-Specific Manner 187

Introduction 189

Experimental Procedures 191

Analysis 194

Results 195

Discussion 200

Competing Interests 203

Funding 203

Chapter 4: Discussion 204

4.1 Overview of Findings in Chapter 2 204

4.2 Overview of Findings in Chapter 3 212

4.3 Limitations of Human Data 213

4.4 Glia and the Microbiome: Where to Next? 214

4.5 SCFAs and the Microbiome: A Therapeutic Target Emerges 214

4.6 Tryptophan Metabolism and Microbiota: Diet to Neuroimmunity 215

4.7 Bile Acids and the Brain: More than a Gut Feeling? 216

4.8 Future Directions 216

References 219

Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

Simon Spichak

Author Contributions

My colleagues Thomaz F.S. Bastiaanssen, Dr. Kirsten Berding, Dr. Klara Vlckova, Prof. Gerard Clarke helped me with understanding the methods, techniques and limitations across human studies used for Chapter 2. Data from all studies were then synthesized and reanalyzed by me. Overall guidance was provided by my supervisors Profs. John F. Cryan and Timothy G. Dinan.

I set up the protocol for primary astrocyte culture with valuable input, training and support from Dr. Martin G. Codagnone. Dr. Anna V. Golubeva, Dr. Gerard M. Moloney and Dr. Jillian M. Brown helped optimize crucial steps of the cell culture process. Later support with culturing primary astrocytes was received from my colleagues Dr. Jillian M. Brown and Dr. Francisco Donoso. Subsequent RNA isolation, cDNA synthesis and PCR was performed by Dr. Francisco Donoso, Dr. Gerard M. Moloney, Dr. Jillian M. Brown and Dr. Eoin Gunnigle. Profs. John F. Cryan and Timothy G. Dinan provided valuable inputs into experimental design.

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Publications

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Boehme, M. *, Guzzetta K.E. *, Bastiaanssen, T.F.S. *, van de Wuow, M., Gual-Grau, A., Moloney, G.M., **Spichak, S.,** Olvarria-Ramirez, L., Fitzgerald, P., Morillas, E., Ritz, N., Jaggar, M., Cowan, C.S.M., Crispie, F., Donoso, F., Halitzki, E., Neto M.C., Sicchetti, M., Golubeva, A.V., Fitzgerald, R.S., Claesson, M. J., Cotter, P.D., O'Leary, O.F., Dinan, T.G., Cryan, J.F. 2020. Microbiota from Young Mice Selectively Counteracts the Effects of Aging Across the Microbiome-Gut-Immune-Brain Axis. *Submitted to Nature Aging.*

In Preparation

Spichak, S., Donoso, F., Moloney, G.M., Gunnigle, E., Brown, J.M., Codagnone, M.G., Golubeva, A.V., Dinan, T.G., Cryan, J.F. Sex-Specific Impact of SCFAs on Mouse Primary Cortical Astrocyte Gene Expression. *For submission to Neuronal Signaling*

Abstract

The trillions of microbial organisms residing in the gut, microbiota, are now recognized as major modulators of physiology and health, quickly becoming one of the most exciting emerging areas in neuroscience. Preclinical and clinical research alike suggests that the metabolites produced by these gut microbes modulate brain, behavior and disease. Short-chain fatty acids, tryptophan metabolites and bile acids are promising targets for new microbiome-based therapies. But, little is known about their mechanisms. To this end, the second chapter of the thesis collates 278 studies relating to the human microbiota-gut brain axis, identifying trends and technical/bioinformatics limitations. These studies across different disorders of the brain as well as healthy human behavioral functions. Then a 35 of these studies was reanalyzed with an up-to-date bioinformatics pipeline. New tools, mainly the gut-brain modules provide a predictive framework for identifying whether these gut microbial metabolic pathways are dysregulated in brain diseases and disorders. We uncovered evidence of disease-related alterations in microbial metabolic pathways in Alzheimer's Disease, schizophrenia, anxiety and depression. Previous human studies suggest that astrocyte immunity and metabolism is affected by short-chain fatty acids. Thus we grew primary male and female mouse astrocyte cultures, treating them with acetate, butyrate and propionate. Butyrate treatment (0 – 25μM) increased gene expression of Bdnf and Pgc1-α expression, implicating histone-deacetylase inhibitor pathways only in female cells. Acetate (0 – 1500 μM) positively correlated with Ahr and Gfap expression in males, suggesting an immune modulatory role. These findings show a novel sex-dependent impact of acetate and butyrate, but not propionate on astrocyte gene expression. These studies increase understanding of microbial metabolites and how they might impact the brain. It also provides guidance to improve and direct future investigations aimed at identifying the mechanisms of other metabolites.

Chapter 1: Introduction

> *"I then most always saw, with great wonder, that in the said matter there were many very little living animalcules, very prettily a-moving."* — Antonie van Leeuwenhoek.

In the 17th century, Leeuwenhoek first described live bacterial cells, isolated from his own mouth (Leidy, 1853). Since then, advances in germ-theory lead to a better understanding of the microbial world existing around us. It was even more recently that humans began exploiting the metabolic properties of these microbes for medicinal purposes. For almost a century, humans have exploited microbial cells for improving our own health. Since the discovery of penicillin almost a century ago (Fleming, 1946b), we have discovered many other microbial properties that confer humans with medical and functional benefits. Studies throughout the 1950s and 1960s emphasized the importance of these symbiotic microbes through germ-free animal models (Spichak et al., 2019).

In recent years, research into these microbial cells has re-emerged, cementing their status as important mediators of brain health and function (Cryan et al., 2019). Much of these studies have focused on the gut microbes, often measured from faecal samples or caecal biopsies. It is now recognized that human cells are outnumbered by bacterial cells (Sender et al., 2016b, Sender et al., 2016a), further supporting the importance of these microbes in physiology (Cryan et al., 2019). Important recent work even associates the metabolic pathways capable of generating neuroactive molecules with depression and quality of life scores (Valles-Colomer et al., 2019). The emergence of improved bioinformatics tools (Callahan et al., 2016, Callahan et al., 2017, McIver et al., 2018) allows scientists to dissect large swathes of 16S and shotgun sequencing. These insights are combined with neurophysiological measures across *in vitro, in vivo* and human models of diseases and disorders. Now it's becoming evident that the microbiota-gut brain axis influences host physiology via bidirectional signaling through the vagal nerve, inflammatory molecules, endocrine molecules, and

microbial metabolites (de Weerth, 2017, Erny et al., 2015, Bravo et al., 2011, König et al., 2016, Blanke et al., 2021, Kong et al., 2020, Marcondes Avila et al., 2020, Wang et al., 2020c, Chen et al., 2019a, Cryan et al., 2019, Gheorghe et al., 2019, Clarke et al., 2019). See **Figure 1** for an overview of the microbiota-gut-brain axis.

Additionally, many data sets are available for download from online repositories. Insight collected from open data informs our mechanistic experimental designs, providing important value to the field beyond the initial publication it was used for.

The Microbiota

The mammalian gastrointestinal (GI) tract is home to trillions of microbial organisms that colonize the gut during the birthing process (Benson et al., 2010, Gaulke et al., 2018). The collection of microorganisms serves important core functions including the modification of host, xenobiotic and dietary-derived molecules into bioactive metabolites that can impact host health and disease (Clarke et al., 2019, Sharon et al., 2014, Spanogiannopoulos et al., 2016, Morris et al., 2017, Sun et al., 2017). Many different factors influence microbiome composition and microbiota-gut-brain axis function (**see Figure 2**).

As a microcosm of the complexity within this field, there are different monikers for these microbes depending on genetic, ecological and host paradigms (Berg et al., 2020). Within this thesis however, the collective individuals within the gut ecosystem will be referred to as the microbiota while the collection of genetic information contained within the community will be referred to as the microbiome (Lederberg and McCray, 2001).

Collectively, there are millions more genes contained within the microbiome (>40 million) than within the human genome (~22 000) microbial cells in the GI tract are defined the gut microbiota (Tierney et al., 2019). We are only beginning to understand the functionality, diversity and interactions within these many members. New methods even allow researchers

to track the transmission of bacterial strains from mother to child (Segata, 2018, Yassour et al., 2018). For the Human Microbiome Project, it was estimated that 500 to 1000 unique species of microbes reside in our guts (Human Microbiome Jumpstart Reference Strains et al., 2010, Human Microbiome Project, 2012). As our bioinformatics tools improve, we may reorganize different genera. This was the case with the *Lactobacillus* genera was recently reclassified as 23 related genera (Zheng et al., 2020a).

Although the individuals within the gut microbiota be long to different kingdoms of life including archaebacteria, bacteria, viruses, protists and fungi, a majority of research has focused on the bacterial components of the gut microbiota (Cryan et al., 2019). In recent years, both the viral phage components and the fungal components of the gut microbiota have been recognized for their potential importance in physiology, brain health and function in both preclinical models and humans (Chin et al., 2020, Jiang et al., 2020, Zhang et al., 2019b, Seth et al., 2019).

It's also clear across converging evidence in the mammalian kingdom that microbiota differences associate with many different brain disorders. In this thesis, my chapters aim to provide insight specifically into neuropsychiatric, neurodevelopmental and neurodegenerative disorders. Differences in microbiome composition/diversity and production of microbial-derived metabolites such as short-chain fatty acids are seen across multiple human studies of depression (Valles-Colomer et al., 2019, Bharwani et al., 2020, Dinan and Cryan, 2019, Jiang et al., 2015, Lai et al., 2019, Liskiewicz et al., 2019, Zheng et al., 2020b), anxiety-related disorders (Jiang et al., 2018a, Slykerman et al., 2017, Stevens et al., 2018), anorexia nervosa (Borgo et al., 2017, Dominique et al., 2019, Hata et al., 2019, Kleiman et al., 2017b, Kleiman et al., 2015), autism (Ahmed et al., 2020, Carissimi et al., 2019, Grimaldi et al., 2018, Iovene et al., 2017, Kang et al., 2017, Kang et al., 2018a, Kong et al., 2019, Kong et al., 2020, Li et al., 2019d, Liu et al., 2019d, Luna et al., 2017), attention-deficit hyperactivity disorder (Aarts

et al., 2017, Jiang et al., 2018b, Prehn-Kristensen et al., 2018, Stevens et al., 2019, Wan et al., 2020, Wang et al., 2020b), schizophrenia (Schwarz et al., 2018, Severance et al., 2017, Shen et al., 2018, Xu et al., 2019, Yuan et al., 2019, Zhang et al., 2020d, Zheng et al., 2019, Zhu et al., 2019, Zhu et al., 2020), Alzheimer's disease (Haran et al., 2019, Liu et al., 2019b, MahmoudianDehkordi et al., 2019, Ticinesi et al., 2018, Vogt et al., 2017, Zhuang et al., 2018) and Parkinson's disease (Barichella et al., 2019, Bedarf et al., 2017, Heintz-Buschart et al., 2018, Heinzel et al., 2020, Hill-Burns et al., 2017, Keshavarzian et al., 2015, Li et al., 2019b, Li et al., 2017, Lin et al., 2018, Lin et al., 2019, Petrov et al., 2017, Unger et al., 2016). These studies are more closely analysed in Chapter 2.

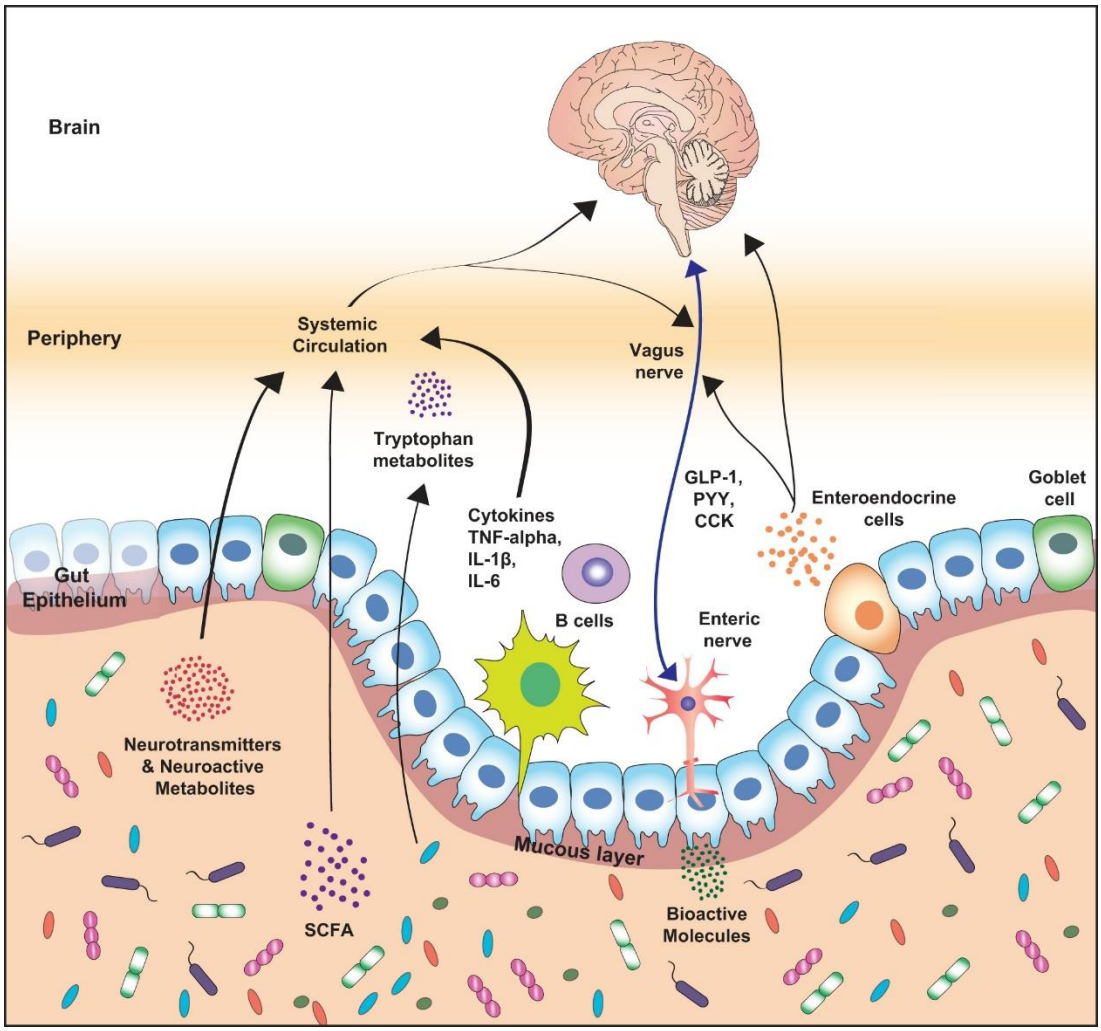

Figure 1. The various communication pathways involved in the microbiota-gut-brain axis. Individual microbes generate neurotransmitters and neuroactive metabolites that enter the peripheral circulation. Dietary fibres are fermented by colonic bacteria, producing a by-product of short-chain fatty acids (SCFAs) which signal with the gut epithelium, the enteric nervous

system, enteric immune cells and also reach peripheral circulation. By breaking down dietary tryptophan, bacteria can generate tryptophan metabolites which also impact the local gut environment or reach peripheral circulation. These microbes play an important role in regulating tryptophan metabolites within the host. In the periphery, these molecules may reach the brain impacting it directly. They may impact the brain indirectly through peripheral interactions with immune cells and other cells. Many other enteric immune cells release pro- or anti-inflammatory cytokines into peripheral circulation, impacting neuroimmunity. Bidirectional signalling through the vagus nerve is also a major carrier of information between gut microbes and the brain. At the enteric nerve interface, bioactive molecules produced by microbes may initiate signalling with vagal afferents. This figure is adapted from (Cryan et al., 2019).

Modes of Communication between the Microbiota and the Brain

Several mechanistic papers describe the connectivity between the microbiota, the enteric nervous system and the brain. Experiments involving microbiota perturbation and vagotomy in preclinical models show the importance of microbial-vagal signalling in neurogenesis (O'Leary et al., 2018, Cawthon and de La Serre, 2018, McVey Neufeld et al., 2019a, Zhang et al., 2020b, Lee et al., 2020b, Marcondes Avila et al., 2020). Intestinal microbiota interactions with the enteric or peripheral immune system, brain and microbiome are well-characterized in both preclinical models and humans (Xu et al., 2020, Sadler et al., 2020, Kong et al., 2020, Barra et al., 2020, Fulling et al., 2020, Dworsky-Fried et al., 2020, van de Wouw et al., 2020, Boehme et al., 2020, Gururajan et al., 2019, Murray et al., 2019, Xu et al., 2019). The gut microbiota also interacts with the cells of the enteric nervous system (Uhlig et al., 2020, Onyszkiewicz et al., 2019, Hyland and Cryan, 2016, McVey Neufeld et al., 2015) and the neuroendocrine system/hypothalamic-pituitary adrenal axis (Donoso et al., 2020, O'Mahony et al., 2020, Tian et al., 2019, Vodička et al., 2018, Golubeva et al., 2015, Perez-Burgos et al., 2013, Gur et al., 2015, Sudo et al., 2004). Additionally, microbial-derived metabolites including quorum signalling and other bacterial proteins (Uhlig et al., 2020, Breton et al., 2016, Dominique et al., 2019, Tennoune et al., 2014), various neurotransmitters (Strandwitz, 2018, Strandwitz et al., 2019, Yunes et al., 2016), tryptophan-associated metabolites (Golubeva et al., 2017, Israelyan et al., 2019, Jaglin et al., 2018, Lai et al., 2019, Reigstad et al., 2015, Rothhammer et al., 2016, Zhu et al., 2020), bile-acid metabolism (Enright et al., 2017, Golubeva et al., 2017) and short-chain fatty acids (Dalile et al., 2019, de Theije et al., 2014, Liu et al., 2019d, Matt et al., 2018, Priyadarshini et al., 2014, Provensi et al., 2019,

Reigstad et al., 2015, Sadler et al., 2020, Unger et al., 2016, van de Wouw et al., 2018, Zhao et al., 2018).

Major Microbial-Derived Metabolites

Dietary fibres that cannot be digested through host metabolism are fermented by colonic bacteria, forming short-chain fatty acids (SCFAs) (Stilling et al., 2016, Gill et al., 2020). While SCFAs enter peripheral circulation, it is unclear exactly how much enters the brain (Dalile et al., 2019). Nonetheless, recent studies underlie their importance in microbiota-gut-brain axis communication. One study found that SCFA supplementation attenuates the cortisol stress response in a psychosocial stress setting in a dose-dependent manner (Dalile et al., 2020). Another study suggests that acetate reaches the hypothalamus, impacting satiety signalling in the host (Frost et al., 2014). Across many preclinical studies, SCFAs modulate immunity, brain health and behaviour (Chen et al., 2019b, Erny et al., 2015, Matt et al., 2018, Hoffman et al., 2019, Lee et al., 2020a, Wang et al., 2020e, van de Wouw et al., 2018).

Tryptophan metabolites are produced from breaking down dietary proteins, playing important roles locally in the gut and throughout the host (Gheorghe et al., 2019, Kennedy et al., 2017, O'Mahony et al., 2015). Tryptophan metabolism is perturbed in psychiatric disorders like depression (Lai et al., 2019, Maes et al., 2011a, Rudzki et al., 2019, Maes et al., 2011b). This is further explored in animal models where perturbations in this metabolic pathway are associated with social impairments and anxiety-like or depressive-like behaviours (Golubeva et al., 2017, Clarke et al., 2013, Desbonnet et al., 2015, Hiroi et al., 2016, Jaglin et al., 2018, Tian et al., 2020).

Bile-acids form another intriguing class of metabolites, which undergo biotransformation by the gut bacteria and are also altered in some human brain disorders (Kiriyama and Nochi, 2019, MahmoudianDehkordi et al., 2019). Emerging preclinical studies suggest that bile-acid modulating bacteria are important for regulating brain health and behaviour (Golubeva et al.,

2017, Hoffman et al., 2019, Jena et al., 2018, McMillin et al., 2015, Nizamutdinov et al., 2017, Yanguas-Casas et al., 2017, Quinn et al., 2020).

These metabolites are discussed in more detail in Chapter 2. See Figure 2 for an overview of these metabolites within the microbiota-gut-brain axis.

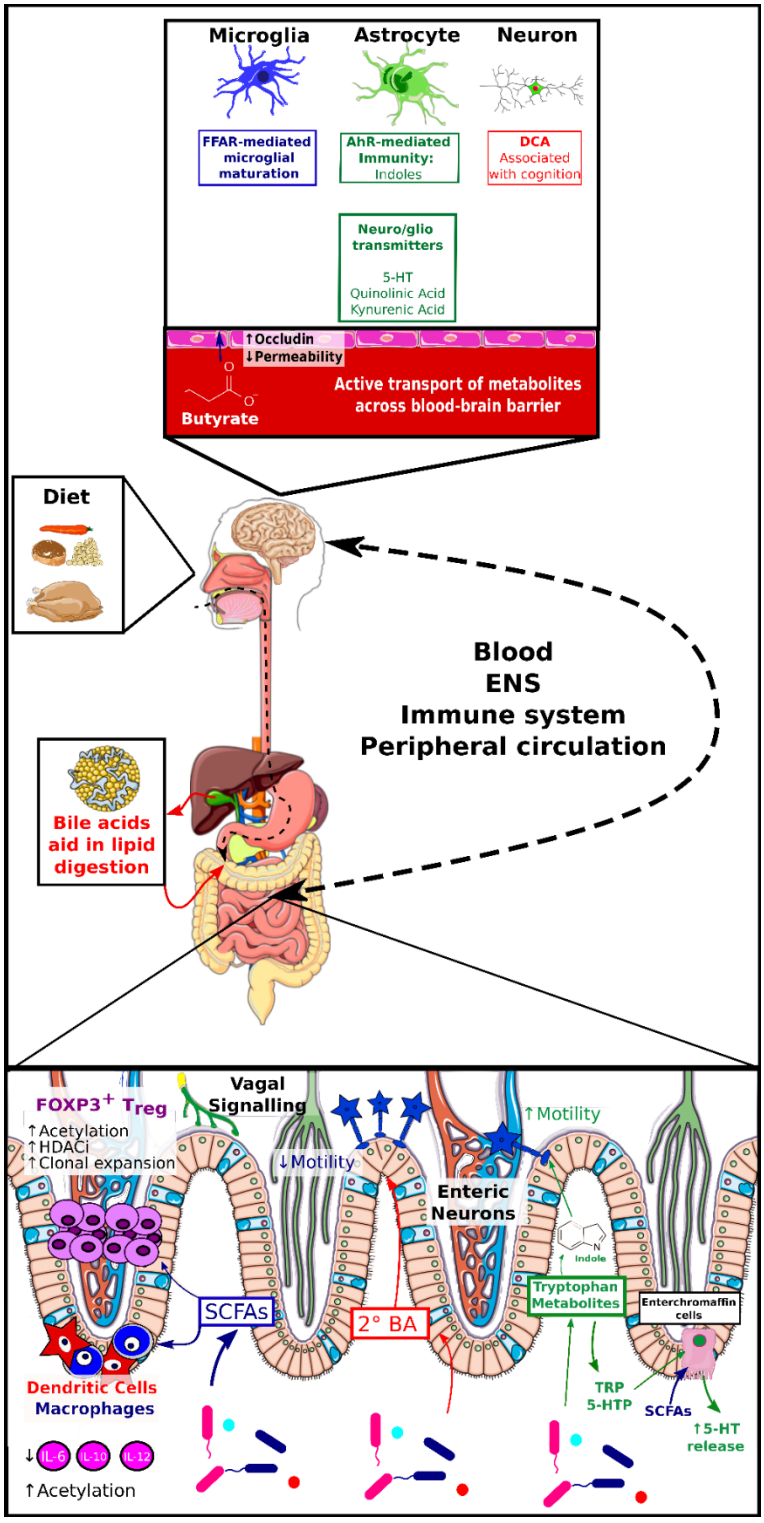

Figure 2. Potential pathways for microbiota-gut-brain axis communication. While it's is unclear exactly how microbial-derived metabolites impact the brain, there are several potential pathways. Non-digestible fibres are broken down into SCFAs which act as histone deacetylase inhibitors on FOXP3⁺ T_{Reg} cells in the gut, leading to clonal expansion. SCFAs many also influence the enteric dendritic and marcrophage cell population by increasing acetylation at specific gene targets impacting cytokine expression. SCFAs may also affect enterochromaffin cells in the gut, stimulating the release of serotonin into the lumen. Travelling through the blood, the SCFA butyrate may increase occludin expression at the blood-brain barrier as well as decrease its permeability to different molecules. If present in a sufficient concentration, SCFAs may impact microglial maturation through free-fatty acid receptor-mediated mechanisms. Bile acids used to aid in lipid digestion are deconjugated and biotransformed into secondary bile acids. These act on myenteric neurons to inhibit gut motility. In the

brain, there is evidence that the secondary bile acid, deoxycholic acid (DCA) is associated with cognition. Tryptophan derived from dietary protein sources impacts both the enteric and central nervous system environments. Briefly, bacteria may generate indole molecules which can act on myenteric neurons to increase gut motility. Tryptophan (TRP) or 5-Hydroxytryptophan (5-HTP) are also generated from dietary protein sources. TRP and 5-HTP can be converted into 5-HT in enterochromaffin cells. In the brain, indoles impact immunity through activation of the Aryl-Hydrocarbon receptor in astrocytes. Alternatively, TRP or 5-HTP can be transported across the blood-brain barrier and converted into the neurotransmitters 5-HT, quinolinic acid or kynurenic acid. It is unclear what role the vagal nerve pathway plays in mediating microbial-derived metabolite signalling.

Early Life Factors: Delivery Mode, Breastfeeding, Antibiotics

There are now a large number of studies showing a marked effect of mode of delivery on the gut microbiome (Jakobsson et al., 2014, Dominguez-Bello et al., 2010, Mueller et al., 2015, Biasucci et al., 2008, Bäckhed et al., 2015, Madan et al., 2016, Biasucci et al., 2010, Azad et al., 2013b, Salminen et al., 2004, Brumbaugh et al., 2016, Dogra et al., 2015, Grześkowiak et al., 2015, Martin et al., 2016, Hill et al., 2017, Tun et al., 2018, Reyman et al., 2019, Shao et al., 2019, Begum et al., 2019), although some studies have found less of an influence than others (Chu et al., 2017). Infants born via Caesarean-section (C-section) had a gut microbiota more similar to the maternal skin microbiota than the vagina (Jakobsson et al., 2014, Dominguez-Bello et al., 2010, Mueller et al., 2015, Biasucci et al., 2008), delayed *Bacteroides* colonization (Mueller et al., 2015, Biasucci et al., 2008), delayed *Lactobacillus* colonization (Dominguez-Bello et al., 2010), lower circulating chemokines (Jakobsson et al., 2014) and a higher risk of vertical obesity transmission from their mother (Tun et al., 2018). Thus, the immune changes during the birthing process are key determinants of the early microbiota and later neurodevelopment (Gur and Bailey, 2016)

Similar to humans, C-section mice initially had a significantly different gut microbiota from their mother (Hansen et al., 2014). In addition, they had significant differences in the abundance of bacteria in the orders Clostrodiales and Bacteroidales as well as lower systemic interleukin-10 expression (Hansen et al., 2014). (Morais et al., 2020) showed altered *Bifidobacterium spp.* abundance in C-section born mice as well as social, cognitive and anxiety deficits throughout life. These alterations were rescued by co-housing with vaginally-

born mice or via *B. breve* administration, further strengthening the connection between C-section, *Bifidobacterium spp.* and the brain (Morais et al., 2020).

Although the restoration of the microbiota in Caesarean born infants using a vaginal swab has been piloted, the long term consequences of such an intervention have yet to be studied (Dominguez-Bello et al., 2016). Alterations in the microbiota induced by C-section may play a functional role in predisposing such infants to a greater relative risk of allergy, asthma, obesity and Type 1 Diabetes (Bager et al., 2008, Tun et al., 2018, Cardwell et al., 2008, Stokholm et al., 2020). The relative contribution of such disturbances to brain health is less clear although epidemiology and animal studies are beginning to unlock some clear links (O'Neill et al., 2016, Fond et al., 2016, Moya-Perez et al., 2017, Martinez et al., 2017, Curran et al., 2017, Calatayud et al., 2019, Butler et al., 2020).

In addition to delivery mode, breastfeeding and early antibiotic use also shape the early microbiome and may impact later host behavior (Azad et al., 2014, Azad et al., 2013b, Hill et al., 2017, Slykerman et al., 2019). While it remains somewhat controversial, there is potential for mode of delivery, antibiotic use and breastfeeding in early life, to shape neurodevelopmental and neuropsychiatric trajectories.

Host Genetics and the Microbiota

There is some conflicting evidence regarding the role of host genetics in human and mouse gut microbiota colonization and composition (Goodrich et al., 2017, Rothschild et al., 2018, Chung et al., 2012, Zmora et al., 2018). Recently, a study using mice found that differences in host background amongst mouse strains, as well as gut microbiota composition impacted fructose metabolism (Ahn et al., 2020). Baseline difference in *Akkermansia*, shown to be a putative mediator of fructose metabolism, may be related to the differences in host genetics between strains (Ahn et al., 2020). Another study identified deleterious genetic variants in families with a genetic predisposition of Irritable Bowel Disease (Park et al., 2020). They

found 22 disease-associated microbial taxa that associated with 17 deleterious host genetic variants (Park et al., 2020). Interestingly, there is evidence of the ability of certain microbiota traits to predict phenotypic traits across large populations (Rothschild et al., 2020).

Drugs and the Microbiota

Over a quarter of pharmaceutical drugs have been shown to impact the composition of the microbiota (Le Bastard et al., 2018, Degroote et al., 2016, de Theije et al., 2014, Davey et al., 2013, Cussotto et al., 2018b, Panee et al., 2018, Flowers et al., 2019, Clarke et al., 2019, Zimmermann et al., 2019, Ma et al., 2020, Vidal-Martinez et al., 2020, Vich Vila et al., 2020), which has been implicated in both the efficacy and side effects of these drugs (Maier et al., 2018). Researchers hope that screening individuals for specific microbiota characteristics would help predict pharmaceutical treatment outcomes.

Different classes of antidepressant drugs including tricyclic antidepressants (Csiszar and Molnar, 1992) and selective serotonin inhibitors (Munoz-Bellido et al., 2000), as well as ketamine (Yang et al., 2017) which may be used in the future as a new treatment, impact the growth of bacteria. It is unclear if their bacteriocidal/bacteriostatic actions impact their efficacy. Prenatal exposure to selective serotonin reuptake inhibitors induces anxiety-like and depressive-like behavior in adulthood (Homberg et al., 2010) in rodents, though the implications of these results for humans are still being discussed (Gur et al., 2013). In addition, evidence suggests that these drugs may impact birth weight and motor development in humans, as well as in animal models (Hutchison et al., 2018); however potential mechanisms are unknown.

Antidepressants, including fluoxetine, are known to alter the microbiota and behavior in rodents (Yu et al., 2019, Yang et al., 2017, Sun et al., 2019b, Ramsteijn et al., 2020, Cussotto et al., 2019). In humans, antidepressants have been shown to transfer through breast milk and some can reach a clinically significant concentration in the infant's serum (Sachs and Drugs,

2013), though their effects on the infant have not yet been established (Glover and Clinton, 2016). While there are many other patient factors to consider, antidepressants do alter the microbiota of patients themselves (Zhang et al., 2019a, Valles-Colomer et al., 2019, Bharwani et al., 2020). (Bharwani et al., 2020) shows promise that the microbiota can even distinguish antidepressant-responders from non-responders.

Antipsychotics have been shown to impact the microbiota in rats and adolescent children, leading to weight gain (Davey et al., 2013, Morgan et al., 2014, Bahr et al., 2015). There is also evidence that microbiota profiles are influenced by antipsychotics in adult humans (Ma et al., 2020, Gorbovskaya et al., 2019, Flowers et al., 2019). It's unclear if there are similarities in the metabolic changes in adults and adolescents prescribed these medications.

Several recent human studies show the impact of nicotine, marijuana, alcohol and other recreational drugs on the human microbiome (Panee et al., 2018, Bjorkhaug et al., 2020, Seo et al., 2020, Hefner et al., 2019, Bajaj et al., 2019, Stadlbauer et al., 2019, Barengolts et al., 2018, Fulcher et al., 2018). Considering the different chemical and orexigenic properties of these drugs, its unsurprising that they impact the gut microbiota in distinct ways. In rodents, ethanol (Yan et al., 2011, Mutlu et al., 2009, Peterson et al., 2017, Frost et al., 2019, Jadhav et al., 2018) and cocaine exposure (Kiraly et al., 2016) have shown effects on the gut microbiota composition.

Exposure to antibiotics within the first three years of life in humans decreased microbiome stability and diversity while it transiently increased transcription of antibiotic resistant genes (Yassour et al., 2016) and increased adiposity in males during childhood (Azad et al., 2014). Moreover, neonatal exposure in rodents to antibiotics have been shown to alter the microbiota, brain inflammation and behavior (Leclercq et al., 2017), contribute to obesity

(Cho et al., 2012), increase visceral pain receptors and sensitivity (O'Mahony et al., 2014) and even alter the editing of a serotonin receptor 2C isoform (van de Wouw et al., 2019).

Diet

Unhealthy diet in humans leads to obesity and poor cardiovascular health but recent research has also shown its impact on neurocognitive development in both humans and rodents (Monk et al., 2013). Common rodent models of unhealthy diet include high-fat diet (36-60 % kcal from fat), Western diet (high fat and high sugar) as well as diets focusing on specific types of fats. Some of these studies have been criticized because improper controls are often selected and may introduce confounds that complicate the interpretation of admittedly intriguing results (Almeida-Suhett et al., 2017, Pellizzon and Ricci, 2018, Morrison et al., 2020).

Recent research indicates that fiber plays a more important role than fat in determining microbiome composition in these dietary paradigms (Morrison et al., 2020). In addition, both prenatal and adolescent exposure to a high fat diet changed the gut metabolome and microbiota composition in mouse, rat and macaques (Gohir et al., 2015, Oberbach et al., 2017, Buffington et al., 2016, Ma et al., 2014). Unhealthy prenatal diets lead to sex-specific differences in gene expression (Edlow et al., 2016, Graf et al., 2016), social deficits (Buffington et al., 2016, Graf et al., 2016), altered hypothalamic stress response (Grissom et al., 2017) and inflammation (Grissom et al., 2017, Du et al., 2012) in the offspring (see Table 2). This suggests that a greater emphasis should be placed on nutrition during pregnancy, though it is unclear if these changes in maternal microbiota directly impact the stress response in human infants.

Several nutrients may play a positive role in neurodevelopment and microbiota maturation. A high-fat diet supplemented with omega-3 polyunsaturated fatty acids increased the diversity of microbiota and enriched *Bifidobacterium* at a species level (Patterson et al., 2014). Omega-3 intake during pregnancy regulated the hypothalamic-pituitary-adrenal (HPA) axis activity,

shifted the maternal stress-induced gut microbiota composition to be more similar to an unstressed composition and conferred resilience to stress later in life; in contrast, a deficit in omega-3 polyunsaturated fatty acids affected the metabolome, impaired communication and social behavior, worsened immune function, while increasing depressive-like behavior (Robertson et al., 2017a, Weiser et al., 2015, Robertson et al., 2017b, Pusceddu et al., 2015, Leyrolle et al., 2020).

Prebiotics promote the growth of beneficial bacteria and include indigestible fibers that are fermented by colonic bacteria to produce short-chain fatty acids and provide a health benefit, though their effects on neurodevelopment have not been well studied (Gibson et al., 2017). Administration of the prebiotics galactooligosaccharide and inulin in mice reduced immune activation and intestinal permeability in offspring through gut microbiota modulation (Bouchaud et al., 2016). The prenatal administration of caprine milk oligosaccharide in mice has been shown to increase *Bifidobacteria* and butyric acid in the offspring colon (Thum et al., 2016). Interestingly, the addition of inulin to a mouse maternal high-fat diet abrogated the negative metabolic effects of the high-fat diet on offspring (Zou et al., 2018).

Probiotics are beneficial strains of bacteria that confer a health benefit to the host. Administration during pregnancy in humans can reduce the risk of atopy but not other immunity-related diseases like asthma (Elazab et al., 2013, Azad et al., 2013a), however, many current supplements lack proof of effectiveness (Reid and Kirjaivanen, 2005). More recent work shows promise for prenatal administration of *L. reuteri* LR92 DSM 2686 in the last four weeks of pregnancy to reduce the incidence and severity of colic, consistent with evidence from previous trials (Pourmirzaiee et al., 2020, Chau et al., 2015). It is unclear if prenatal probiotic intervention may also reduce the incidence of mental health and brain disorders later in the offspring.

An unhealthy diet commencing in early postnatal life also alters the microbiota composition (Turnbaugh et al., 2008) and results in different behavioral and inflammatory phenotypes across rodents and humans. High-fat diet after weaning and during adolescence altered the HPA-axis in female rats and impaired hippocampal memory and increased hippocampal lipopolysaccharide-induced cytokine response in the males (Boukouvalas et al., 2008, Boitard et al., 2014). Meanwhile high-fat diet bingeing during adolescence in mice increased anxiety and cocaine self-administration in adulthood (Blanco-Gandía et al., 2017). Meanwhile cafeteria diet during adolescence alters systemic inflammation in rats, and neuroinflammation in male mice (Fulling et al., 2020, Nicolas et al., 2020).

Studies in humans indicate the presence of acute effects in response to daily dietary intake (Johnson et al., 2019). Meanwhile, another study unveiled individual level differences in dietary responses to the exact same meal, attributed to host genetics and microbiome (Berry et al., 2020).

In rodents, *Lactobacillus* administration had similar effects to inulin, reducing anxiety through the HPA axis (Barrera-Bugueño et al., 2017). Interestingly, when combined with inulin, it did not affect the corticosterone levels and increased 5-HT_{1A} receptor mRNA in the hippocampus, a receptor associated with anxiety and depression (Barrera-Bugueño et al., 2017). Notably, *Lactobacillus rhamnosus* and *L. helveticus* administration in stressed infant rats (postnatal day 2 to 14) had a protective effect on fear conditioning memory and relapse after extinction in early life (Cowan et al., 2016). Some strains, such as *Bifidobacteria longum* and *Bifidobacteria breve*, can mediate anti-anxiety and anti-depressive behaviors in preclinical rodent models (Savignac et al., 2014). In mice, *Bifidobacterium breve* combined with prebiotics in early life exerted protection from the negative metabolic effects of a

Western diet (Mischke et al., 2018), showing promise for co-administration of prebiotics and probiotics.

A combination of *Lactobacillus rhamnosus* GG on its own or along with polydextrose and galactooligosaccharide protected maternally-separated (MS) male rats from MS-induced anxiety, while also rescuing hippocampal mRNA gene expression (McVey Neufeld et al., 2019b). Administration of the prebiotic inulin in young mice reduced the immunological changes associated with ageing while also reducing the infiltration of Ly-6^{hi} monocytes into the brain (Boehme et al., 2019). Taken together, these studies suggest targeting the gastrointestinal microbiota with prebiotics and probiotics is a promising strategy for attenuating the immune and behavioral effects of stress and ageing.

In humans, probiotics may reduce the risk of depression (Huang et al., 2016) and autism (Pärtty et al., 2015, Gilbert et al., 2013). While many studies have been conducted, their results thus far are confounded by differences in strains used and a lack of metadata. Thus far, various strains of *Lactobacillus* and *Bifidobacterium* were tested in student-stress conditions providing evidence that these probiotics may modulate stress or sleep (Nishida et al., 2017, Nishida et al., 2019, Kato-Kataoka et al., 2016, Rao et al., 2009, Takada et al., 2017, Takada et al., 2016, Tanida et al., 2016, Moloney et al., 2020). *Lactobacillus gasseri* CP2305 administration exerted protective effects against chronic stress on the microbiota, while also improving self-reported measures of anxiety and sleep disturbances (Nishida et al., 2019). While some probiotic strains characterized in rodents translated some neurophysiological effects in humans (Allen et al., 2016) many other strains do not (Kelly et al., 2017).

These findings further generate interest for psychobiotic research, looking to identify live bacterial strains that confer mental health benefits (Bambury et al., 2018, Butler et al., 2019, Dinan and Cryan, 2019). Nonetheless, there have been few randomized clinical trials

conducted in depressed or anxious populations, and even fewer studies finding strong benefits for these psychobiotics (Smith et al., 2019a). Even fewer studies have looked at the impact of administering prebiotics as an adjuvant treatment for depression or anxiety (Smith et al., 2019a). Another meta-analysis of randomized clinical trials did not find strong support for the administration of fermented food, prebiotics or probiotics for enhancing cognitive outcomes (Marx et al., 2020b). Looking across seven studies, a meta-analysis reported significant mood improvements for those taking pre or probiotics, noting that they may especially benefit individuals with irritable bowel syndrome co-morbidity (Noonan et al., 2020).

Maternal Stress

Maternal stress is modulated by the HPA axis and has been shown to impact this axis in the offspring. In offspring, maternal stress has increased serum levels of corticosterone, increased anxiety, social impairment and altered the resilience of different strains of rodents (Lee et al., 2016, Rana et al., 2015, Bale, 2015, Hiroi et al., 2016, Golubeva et al., 2015, Egerton et al., 2020, Donoso et al., 2020, Strzelewicz et al., 2019, Rincel et al., 2019). Maternal stress can also alter the gut and vaginal microbiota during pregnancy, decreasing diversity of maternal gut microbiota as well as dysregulating glucose metabolism in mice (Jašarević et al., 2017). (Golubeva et al., 2017) reported that specific gut microbes (*Bifidobacterium* and *Blautia*) in a strain of mice with social deficits, BTBR $T^{+}Itpr3^{tf}/J$, were associated with bile acid and tryptophan metabolism. Reductions in bile acid and alterations in tryptophan metabolism also associated with gastrointestinal dysfunction and social deficits in these mice (Golubeva et al., 2017). Varied prenatal stress interrupted normal pregnancy-related compositional changes in the mouse vaginal microbiota and also altered the protein content in the vaginal mucosa, which may have contributed to altered abundance of Firmicutes and Bacteroidetes in their offspring's gut microbiota (Jasarevic et al., 2015). After pregnant mice experienced restraint

stress specifically, female offspring showed more anxiety-like behaviors as well as decreases in brain-derived neurotrophic factor in the placenta concurrent with later decreases of its expression in the adult amygdala (Gur et al., 2017). Further investigation found MS-induced microbiome alterations (decreases in Bacteroides and Parabacteroides) persisting into adulthood, resulting in a decrease in sociability and serotonin metabolism.

In humans, ongoing maternal stress (inclusive of prenatal and postnatal stress) has been associated with altered microbiota mental health problems in adult offspring (Betts et al., 2015), as well as with influencing the development of the offspring microbiota over the first 110 days after birth (Zijlmans et al., 2015). Stress-induced alterations within the microbiome could contribute to neurodevelopment and impact future offspring behavior (Gur et al., 2015, Codagnone et al., 2019a). However, the mechanism by which the negative impact of maternal stress is transmitted to the offspring's microbiota is unknown, though IgA mediated immunity could be implicated (Kang et al., 2018b). Interestingly, exposure to intimate partner violence in pregnant women altered the infant microbiota, increasing *Weisella* and *Citrobacter* abundance (Naude et al., 2020). It's likely that these stressors also hold long-term consequences on brain development in humans, as they do in animals. It's unclear however, whether these microbiota alterations induced by the stress also play an important function.

Maternal Immune Activation

The maternal immune activation model is based around the controversial idea that maternal infections during pregnancy can impact psychiatric outcomes in the children (Estes and McAllister, 2016). A rodent model of maternal immune activation commonly administers viral mimetic poly(I:C) or bacterial lipopolysaccharide to produce psychiatric endophenotypes in offspring. Specifically, the viral mimetic poly(I:C) administered at E12.5 in mice altered the gut microbiota composition and increased gut leakiness by decreasing claudin expression, while also elevating intestinal cytokine levels, including interleukin-6 in

offspring (Hsiao et al., 2013). This model also elevated the bacterial production of, 4-ethylphenylsulfate, which induced anxiety-like symptoms in wild-type mice (Hsiao et al., 2013). However, there is heterogeneity in the time of administration of the viral mimetic poly(I:C) which can lead to the development of different biomarkers or behaviors common to different disorders including schizophrenia (Juckel et al., 2011, Li et al., 2009) or autism-spectrum disorder-like behaviors in mice (Malkova et al., 2012), and inconsistent depression-like endophenotypes in rats and mice (Ronovsky et al., 2016).

Though there exists no current research of its impact on the microbiota, the bacterial mimetic lipopolysaccharide can also induce behavioral phenotypes for anxiety, depression, or autism spectrum disorder in offspring (Depino, 2015, Oskvig et al., 2012); impairments in hippocampal development and neurogenesis (Escobar et al., 2011, Romero et al., 2007); and increased postnatal inflammation (Oskvig et al., 2012). In mice, lipopolysaccharide-induced maternal inflammation caused placental damage and fetal intestinal injuries that persisted in adulthood (Fricke et al., 2018).

Intriguingly, researchers discovered that this phenotype requires the influence of the gut microbiota in initiating the immune cascade (Kim et al., 2017). Segmented filamentous or human-microbiome commensal bacteria were required to initiate the intestinal found that segmented filamentous bacteria or human-microbiome commensals were required to initiate the T_H17 response (Kim et al., 2017). This cascade initiates a neuroinflammatory response which is required for developing MIA-induced behavioural abnormalities (Kim et al., 2017).

While there are few studies in humans, maternal immune activation could potentially impact the offspring, associated with brain connectivity, function and even retrospectively with schizophrenia (Guma et al., 2019).

Postnatal Stress and the Microbiota

Early postnatal stress impacts the HPA-axis and contributes to the programming of brain health in later life (Heim and Nemeroff, 2001). Different types of early postnatal stress (social isolation, maternal separation) alter the gut microbiota composition and metabolism in rats (Farshim et al., 2016, Doherty et al., 2018, O'Mahony et al., 2009, Vodička et al., 2018, van de Wouw et al., 2018) and their inflammatory profiles (Doherty et al., 2018, O'Mahony et al., 2009). Social isolation also impaired memory and learning in rats (Doherty et al., 2018). Finally, germ free mice were more vulnerable to restraint stress – resulting in higher adrenocorticotropic hormone and corticosterone in plasma (Sudo et al., 2004, Clarke et al., 2013), a reduction in glucocorticoid receptor mRNA and an increased stress response (Sudo et al., 2004). Remarkably, these effects were rescued with microbiota transplantation during adolescence but not adulthood (Sudo et al., 2004).

While few studies focused on the microbiota and stress in the early postnatal period, it's clear that many stress-related psychiatric disorders have marked alterations in the gut microbiome and metabolome (Cryan et al., 2019, Bastiaanssen et al., 2020, Bastiaanssen et al., 2019).

Figure 3. Factors affecting the microbiota-gut-brain axis. Many dietary, pharmacological, stress and infection-related factors influence the microbiota-gut-brain axis. These disruptions impact microbiota composition, the hypothalamic-pituitary-adrenal axis and microglial inflammation. Adapted from (Codagnone et al., 2019b).

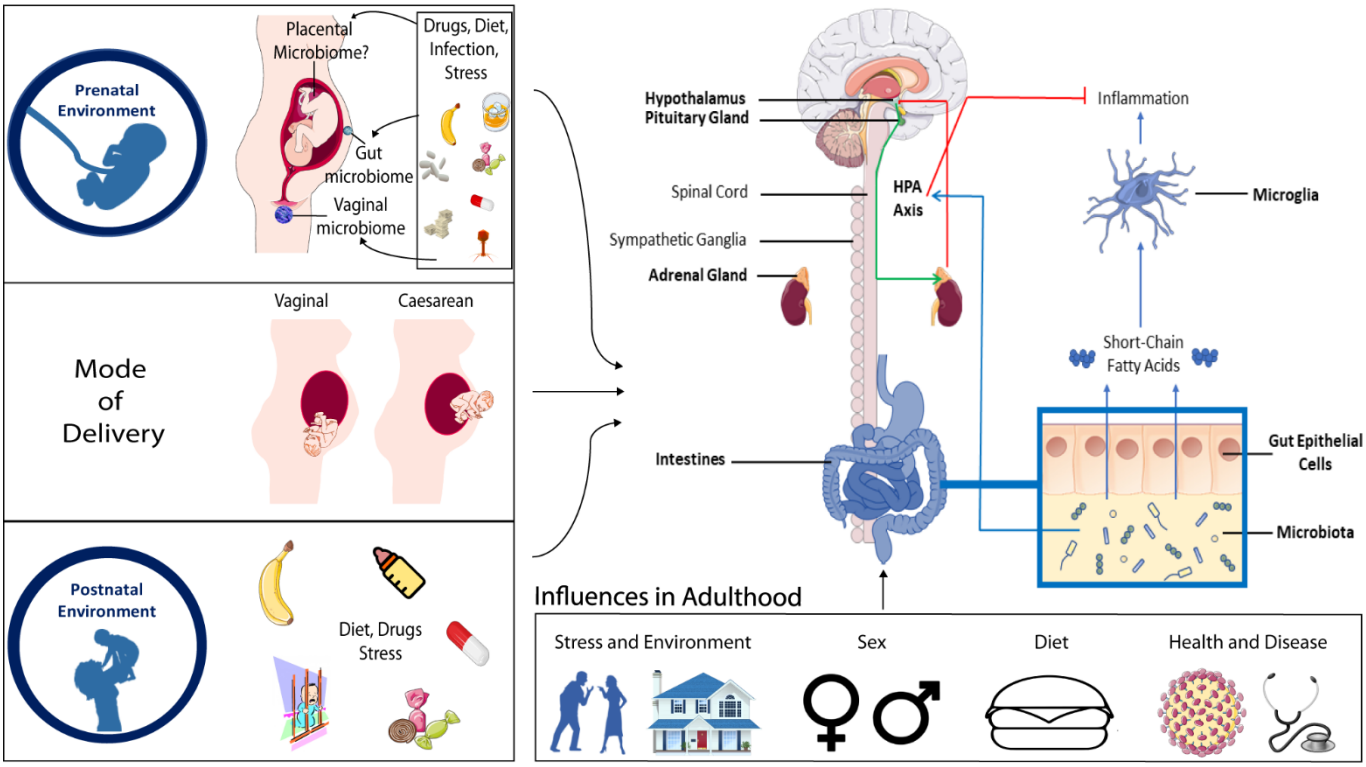

Figure 3 Factors affecting the microbiota-gut-brain axis. Many dietary, pharmacological, stress and infection-related factors influence the microbiota-gut-brain axis. These disruptions impact microbiota composition, the hypothalamic-pituitary-adrenal axis and microglial inflammation. Adapted from Codagnone & Spichak et al., (2019).

Sex-Specific Programming of Psychiatric Disorders Later in Life

Many psychiatric disorders differ among the sexes in terms of prevalence or onset – including autism (Yeargin-Allsopp et al., 2003), mood disorders (Merikangas et al., 2010), anxiety disorders (Merikangas et al., 2010) and schizophrenia (Aleman et al., 2003). The underlying biological basis of these sex differences is still unknown. Since microbiota-related alterations have shown sex-specific effects after exposure to prenatal or postnatal environmental stimuli (Steegenga et al., 2017, Bahr et al., 2015, de Theije et al., 2014, Gur et al., 2017), they may play a role in the sex-specific programming of health later in life (Jaggar et al., 2020). See **Figure 3** for the overlap between sex hormone levels, microbiota and brain dimorphism over time.

The gut microbiota and its metabolites influence the development of the microglia, and in its absence, development is disrupted in a sex-specific manner (Erny et al., 2015, Desbonnet et al., 2014, Thion et al., 2018). Abnormal microglial phenotypes were rescued with short-chain fatty acids, produced by certain members of the microbiota (Erny et al., 2015). The microglia are involved in synaptic pruning and maturation during neurodevelopment (see review by Salter (Salter and Beggs, 2014)).

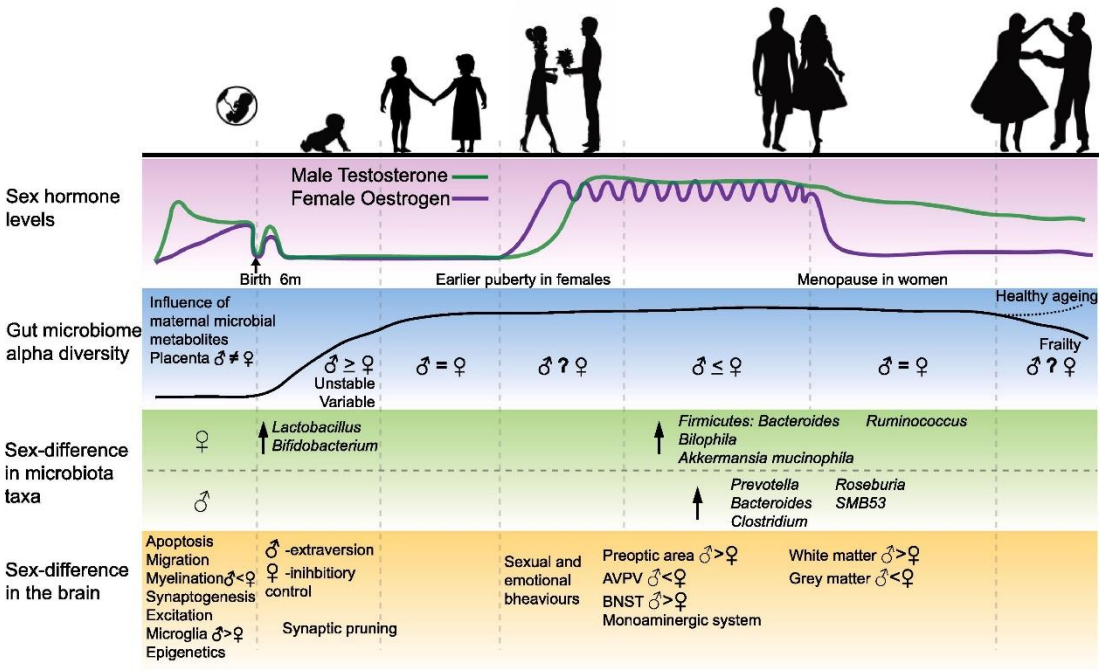

Figure 4 Variations between sex hormones, microbiota and brain dimorphism over the lifespan. Early sex differences in synaptic pruning correspond with sex differences in the gut microbiota of infants. Later in adulthood, females show increased alpha-diversity differences in gut microbiota and vast differences across neuropsychiatric disease and disorders. It is suggested that changes in microbiota may correspond to microbial androgen metabolism as well as brain function and disease. (Jaggar et al., 2020).

Importance of the Microbiota: Observations from the Germ-Free Model

The idea that microbes are essential for proper host development and function is not new. In 1885, Louis Pasteur posited the necessity of microbes for the existence of life (Pasteur, 1885). The first GF animal was the guinea pig, which followed only 10 years after Pasteur's publication (Nuttall and Thierfelder, 1895, Williams, 2014). Only decades later was the importance of this model truly recognized (Gordon and Pesti, 1971) and the concept of GF became more popular in the media. Interestingly, David Vetter, known as "the boy in the bubble" was also born in the year Gordon and Pesti published their seminal article. Vetter

suffered from a disorder known as severe compromised immune deficiency and had to live in a sterile environment.

Over the last few decades, several vertebrate models (rodent, bird, fish, pig) and invertebrate models (*Drospohila*) have provided insights on the impact and necessity of the gut microbiota for gut, immune and brain development (Rawls et al., 2004, Bates et al., 2006, Gordon et al., 1966, Savage et al., 1981, Erny et al., 2015, Hoban et al., 2016); anxiety, depression mood and social behavior (Hoban et al., 2017, Kraimi et al., 2018, Davis et al., 2016b, Neufeld et al., 2011, Desbonnet et al., 2014, Buffington et al., 2016); locomotion and feeding (Leitao-Goncalves et al., 2017, Davis et al., 2016a, Diaz Heijtz et al., 2011, Schretter et al., 2018); learning, memory and transcriptional/molecular changes (Clarke et al., 2013, Hoban et al., 2018, Stilling et al., 2018, Rawls et al., 2004, Gareau et al., 2011). The scope of this review will primarily include the GF rodent model and its contributions to our understanding of host microbiome-neuroimmune interactions.

Generation of the GF Rodent

The first GF rodent models involved the aseptic Caesarean section of pups from the mother, who were then hand-reared in a sterile isolator or cross-fostered by a germ-free mother. In the late 1950s (Gustafsson, 1959, Reyniers and Sacksteder, 1958), a technique for generating successive generations of GF rodents was developed, and similar techniques and equipment are still most frequently used today (Williams, 2014). Alternatively, embryos at the 2-cell stage can be implanted into pseudopregnant GF mothers, thus ensuring a sterile vaginal birth (Inzunza et al., 2005, Okamoto and Matsumoto, 1999).

Physiological and Anatomical Differences in the GF Rodent

GF animals show marked alterations in their gross anatomy, indicating the essential role that microbes have in host development. When compared to conventionally raised counterparts, GF rodents have alterations in bone health (Sjogren et al., 2012, Quach et al., 2018),

decreased weight of spleen, submandibular lymph nodes, liver as well as significantly larger colon (Reveley et al., 1983, Jeppsson et al., 1979, Gordon et al., 1966), liver metabolism (Li et al., 2018, Dempsey et al., 2018, Kindt et al., 2018) white blood cell count and distribution (Gordon et al., 1966), and even eye lipids (Oresic et al., 2009), in addition to various reported alterations in intestinal and brain measures (see (Spichak et al., 2019) for a thorough review). Their immune system is also profoundly immature (see **Fig. 5**).

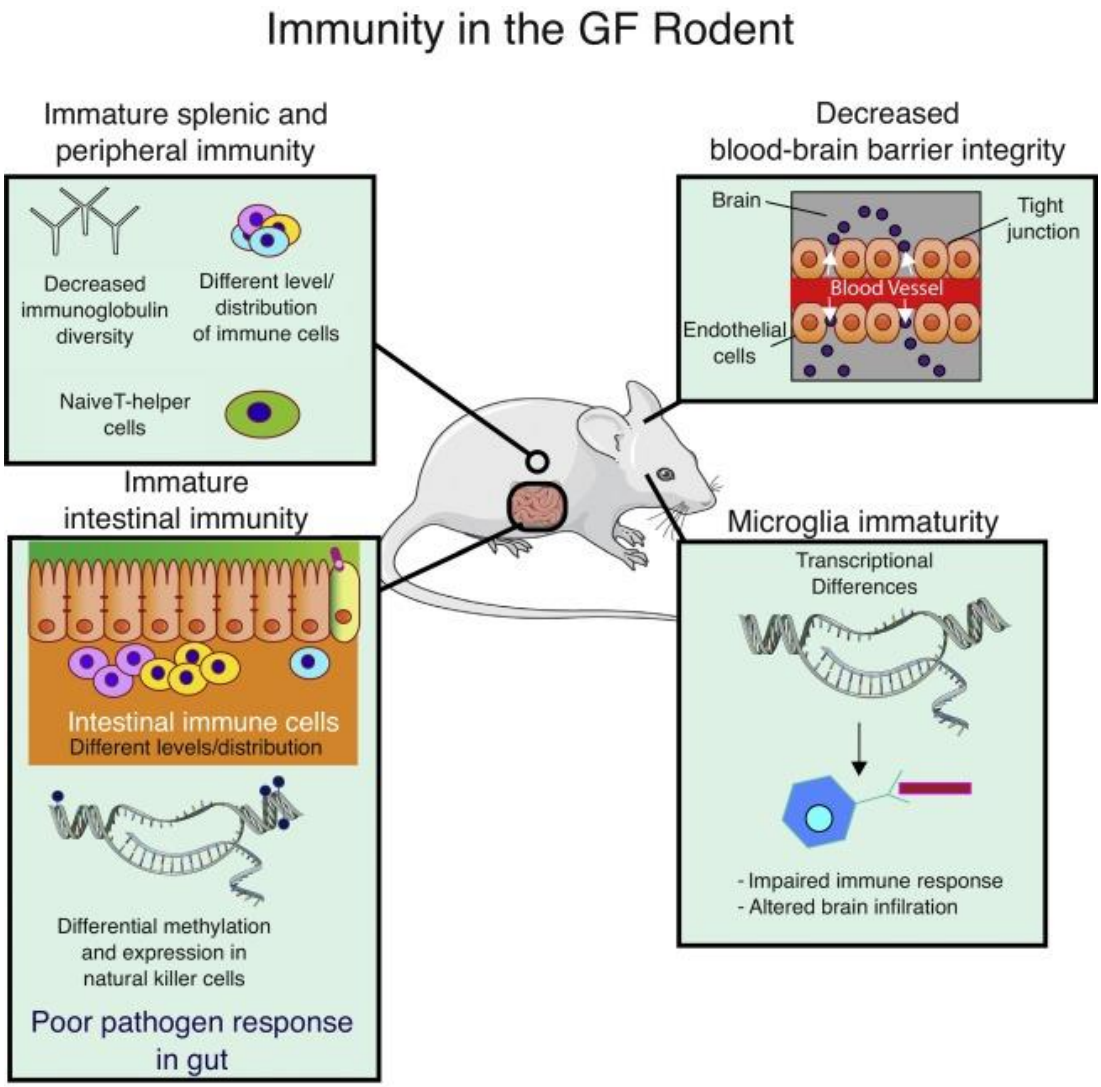

Figure 5. Profound immaturity in peripheral and CNS immunity in the GF rodent. Immune cell immaturity, transcription and distribution of these cells is altered. The blood-brain barrier is more permeable while microglia in the brain are immature. Adapted from (Spichak et al., 2019).

The behaviour, brain and immunity of the GF rodent is also markedly affected by the lack of a microbiota (see **Fig. 6**).

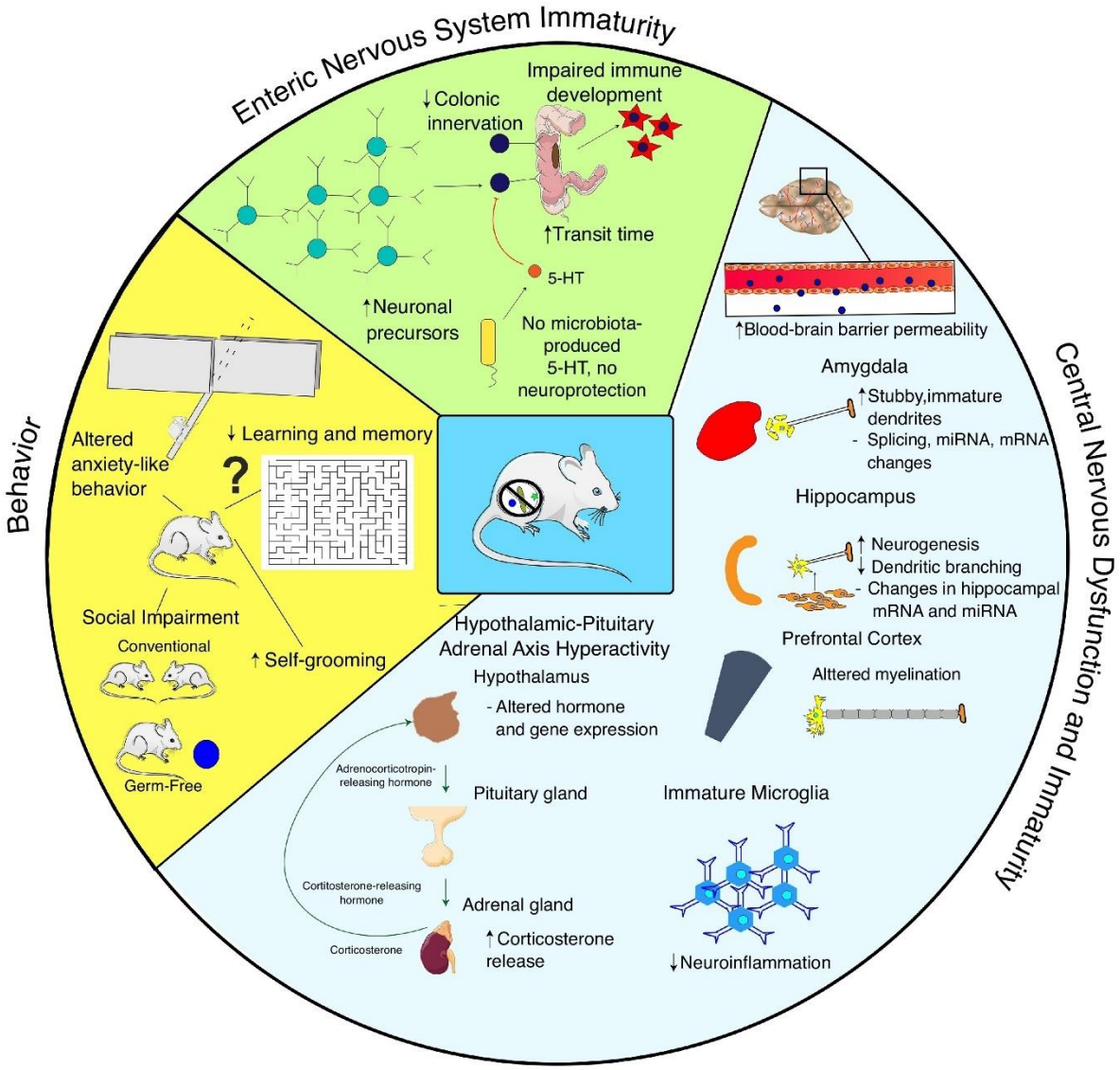

Figure 6 Phenotype of the GF rodent. This figure summarizes the most common findings across species and strain of GF rodent. GF rodents typically weigh less, have a significantly larger cecum and more cecal contents, possess altered liver metabolism, reduced gut motility, differences in bone density as well as striking changes to the structure of the gut. These GF rodents have learning and memory impairments, social impairments, altered anxiety-like behavior as well as an increase in self-grooming. Gut and peripheral immunity is severely compromised from the GF status of these rodents. As such, the lymphocyte immunoglobulin repertoire is attenuated, there is altered methylation in natural killer cells, and these rodents also possess resistance to some autoimmune diseases. In addition, their blood-brain barrier possesses increased permeability while the microglia in the brain have an immature phenotype. This microglia phenotype confers resistance against neurodegenerative disease. Finally, these GF rodents have many transcriptional changes in the amygdala and hippocampus contributing to an immature neuronal morphology, prefrontal cortex hypermyelination, and a hyperactive hypothalamic-pituitary adrenal axis.

Microbiota-Gut Brain Axis Metabolite-Mediated Modulation of Glia

Astrocytes

In the 1850s, Rudolf Virchow coined the term *neuroglia* referring to the glue-like substance that holds neurons in place (Parpura and Verkhratsky, 2012, Barres, 2008). Further research found different types of neuroglia, with much of it focused on describing the supporting cells of the brain, called the astrocytes (Barres, 2008). While widely recognized as modulators of

brain metabolism and immunity, recent work suggests these cells are genetically heterogenous and may even be involved in cognition, memory and behaviour (Khakh and Sofroniew, 2015, Khakh and Deneen, 2019, Alberini et al., 2018, Cao et al., 2013, Martin-Fernandez et al., 2017). It's also evident that astrocytes are involved in synaptic pruning during neurodevelopment (Chung et al., 2015). Until very recently, astrocytes were used as proxy-measurements of CNS inflammation during microbiota-gut-brain axis studies. However, striking evidence suggests that they directly interact with microbial metabolites to regulate aspects of the brain's immune response (Rothhammer et al., 2016).

Dietary-derived tryptophan-indole metabolites were shown to reduce inflammation in the experimental autoimmune encephalitis model of multiple sclerosis (Rothhammer et al., 2016). Indole metabolites activated the aryl-hydrocarbon receptor in astrocytes, leading to a downstream cascade activating the Interferon-I transcriptional pathway that reduced inflammation (Rothhammer et al., 2016). The indole metabolite indoxyl-3-sulfate is not produced in germ-free mice and is indeed shown to cross the blood-brain barrier to activate the aryl hydrocarbon receptor in astrocytes (Rothhammer et al., 2016). Humans with multiple sclerosis also showed a reduction in circulating indole metabolites that could act as agonists of the aryl-hydrocarbon receptor (Rothhammer et al., 2016). In adult mice, this pathway attenuates inflammation after stroke and reduces neurogenesis, perhaps preserving the neurogenic niche for more favourable conditions (Chen et al., 2019c)

Another study found sex differences in responses to a high-fat, high sucrose diet in both the microbiome and astrocytes (Daly et al., 2020). Interestingly, while female mice showed an increase in the $GFAP^{+}$ hypothalamic expression in the high fat, high sucrose condition compared to controls, this difference was absent in males (Daly et al., 2020). This suggested a reduction in astrogliosis only in females (Daly et al., 2020).

Even fewer studies focused on these interactions in humans. In autistic children, the astrocyte inflammation mark S100β is found increased in the bloodstream (Tomova et al., 2019). S100β is often used as a marker for brain inflammation or injury (Michetti et al., 2019), and in study negatively correlated with the abundance of gut *Bilophila* and *Carboxydothermus* (Tomova et al., 2019). Another study indicates that the gut-derived SCFA acetate reaches astrocytes in the hypothalamus, modulating satiety and appetite (Frost et al., 2014).

Microglia

Microglia, the brain's resident macrophages, mediate both immunity and synaptic pruning in the brain (Tremblay et al., 2011). Due to their importance in disease and neurodevelopment, impairments may contribute to different neurodevelopmental and neuropsychiatric disorders. In accordance with observations of peripheral immunity, microglia in GF animals are immature and cannot properly react to harmful stimuli (Erny et al., 2015, Castillo-Ruiz et al., 2018). In addition to global transcriptional differences with conventional mice (Erny et al., 2015), the microglia of GF rodents even have a different pattern of regional infiltration into the brain during development (Castillo-Ruiz et al., 2018). Further, altered cytokine and chemokine pathways in GF animals prevent the development of appropriate innate immune responses (Erny et al., 2015), which could impact synaptic pruning.

Another study found temporal and sex-dependent differences in microglia gene expression and function. RNA sequencing of microglia revealed differentially expressed genes during embryonic development, with more differences in between GF males and their conventional counterparts, than in GF females and their conventional controls (Thion et al., 2018). Interestingly, the magnitude of these differences is reversed in adulthood, with more differentially expressed genes between GF and conventional females, than between the males (Thion et al., 2018). In addition, while conventional mice had an increase in differentially accessible regions in their genetic material as the embryo matured, but not in the GF mice

(Thion et al., 2018). These results were shown to be translatable to humans, as there were transcriptomic similarities between microglia in both organisms during mid-gestation (Thion et al., 2018)).

Using an antibiotic depletion model, evidence for the necessity of the microbiota for proper microglia development has been strengthened (Erny et al., 2015, Thion et al., 2018). Impairments in the GF model were rescued with the introduction of a complex microbiota or through short-chain fatty acid-mediated activation of the Free Fatty Acid Receptor 2 (Erny et al., 2015). Short-chain fatty acids can be produced by the human and rodent gut microbiota through the fermentation of indigestible fibres (Stilling et al., 2016). The immature GF microglia also show impaired responses to ischemia (Singh et al., 2018) and bacterial lipopolysaccharide (Campos et al., 2016).

In mouse models of Alzheimer's disease, the microbiota is often targeted to influence microglial inflammation. One study supplemented the diet of a transgenic model of Alzheimer's disease, with a prebiotic soybean finding that it reduced microglial inflammation in the brain, attenuated the cognitive decline of the mice, reduced fecal lipopolysaccharide and increased the populations of *Lactobacilli* and *Bifidobacteria* in the gut (Lee et al., 2018). In other rodent models of Alzheimer's disease, antibiotic administration reduced microglial-related inflammation only in males (Mezo et al., 2020, Dodiya et al., 2019), mediated by changes in the levels of the 'M0' homeostatic microglial state (Dodiya et al., 2019). Additionally, administration of the butyrate producing probiotic *Clostridium butyricum* attenuated microglial inflammation and cognitive impairment in this transgenic model (Sun et al., 2020). Using the Amyloid-β induced BV2 microglial cell line, (Sun et al., 2020) found that butyrate administration reduced the expression of CD11b, COX-2 and reduced the phosphorylation of the transcription factor NF-κβ p65. There is a plethora of evidence

showing the functional involvement of short-chain fatty acids in the microglial pathogenesis across different mouse models of Alzheimer's disease.

These anti-inflammatory mechanisms may act in other disease states. The gut microbiota was necessary for activation of the neuroprotective microglial TLR4 response against viral-induced neurologic signalling (Brown et al., 2019). Other work indicates that microglia derived TGFα and VEGF-B mediated the astrocytic aryl-hydrocarbon receptor response in experimental autoimmune encephalitis; it was found that microglial activation was also dependent on dietary tryptophan metabolites (Rothhammer et al., 2018).

Interestingly the microbiota and microglia may also mediate behaviour. The tetracycline antibiotic minocycline inhibited microglial activation, attenuating anxiety and depressive-like behaviours in a high-anxiety breed of rats (Schmidtner et al., 2019). Minocycline treatment also reduced the caecal abundances of butyrate-producing families of bacteria Lachnospiraceae and Clostridiales Family XIII (Schmidtner et al., 2019). The abundance of these families, as well as the amount of 3-OH-butyrate in the serum were positively correlated with reductions in anxiety behaviours (Schmidtner et al., 2019).

Oligodendrocytes

The myelinating cells of the CNS, oligodendrocytes, also require a gut microbiota for proper maturation and function. Converging lines of evidence implicate the microbiota and its metabolites in oligodendrocyte function and gene expression.

Sex specific differences in myelination have been found in the prefrontal cortex GF mice (Gacias et al., 2016, Hoban et al., 2016, Radulescu et al., 2019, Lu et al., 2018). One study which used diffusion tensor imaging, showed that male C57BL/6 mice had hypomyelination in the corpus callosum, at 4 weeks of age, and internal capsule at 12 weeks of age, while females did not (Lu et al., 2018). In addition, differences in myelination between males and

females were significant in each investigated region (Lu et al., 2018). In contrast, Swiss Webster GF males displayed an upregulation in myelination genes in the prefrontal cortex, suggestive of hypermyelination – which was absent in females (Hoban et al., 2016). This was verified with electron microscopy and Western blot (Hoban et al., 2016).

One study administered a probiotic consisting of *Lactobacillus acidophilus* and *Bifidobacterium infantis* to pregnant C57BL/6 dams during pregnancy and weaning (Lu et al., 2020). Compared to controls, offspring from these dams showed increased protein expression of neuroglia-2, a marker of oligodendrocyte progenitor cells (Lu et al., 2020). In a model of maternal stress in rats, coadministration of a prebiotic with the milk-fat globule membrane prebiotic attenuated the negative impact of maternal stress-mediated increases on the expression of myelin-associated glycoprotein in the prefrontal cortex (O'Mahony et al., 2020).

The role of microbiota-mediated myelination was investigated in a genetic FVB mouse model of Huntington's disease. A reduction in myelin-related proteins was measured in the prefrontal cortex of these mice, suggesting hypomyelination (Radulescu et al., 2019). Much like the immature microglial phenotype, profound oligodendrocyte immaturity in the prefrontal cortex was also observed and measured using immunohistochemistry in the GF mice (Radulescu et al., 2019). In the corpus callosum of GF Huntington's mice, immunohistochemistry and electron microscopy uncovered hypermyelination (Radulescu et al., 2019). Interestingly, GF mice with genetically-induced Huntington's disease also had an increase in the proportion of small diameter axons as well as a decreased proportion of medium diameter axons (Radulescu et al., 2019). However, the results of this study were not stratified by sex.

A model of cuprizone-induced demyelination found that administration of the SCFA butyrate attenuated demyelination and enhanced remyelination, via its direct impact on oligodendrocytes (Chen et al., 2019b). Butyrate exerted its effects on remyelination by modulating the differentiation of immature oligodendrocytes (Chen et al., 2019b).

Research of multiple sclerosis in humans and corresponding rodent models finds that the microbiota mediates the autoantigen peripheral immune response during disease progression (Berer et al., 2017, Berer et al., 2011, Zeraati et al., 2019, McMurran et al., 2019, Miller et al., 2015) but little impact on subsequent remyelination (McMurran et al., 2019). Indeed, modulating the gut microbiota through diet in rodents modified disease severity in experimental autoimmune encephalitis (Libbey et al., 2018, Escribano et al., 2017). Microbial taxa reduced during the pathogenesis of multiple sclerosis produce SCFAs; accordingly, administration of SCFAs also attenuated the severity of disease in a rodent model of experimental autoimmune encephalitis indirectly via T-cell mediated effects (Mizuno et al., 2017a). Faecal microbiota transplants from healthy naïve mice, reduced the severity of experimental autoimmune encephalitis (Li et al., 2020a).

It’s clear that there is both direct and indirect influences of the gut microbiota and its metabolites on oligodendrocytes maturation, myelination and function.

Sequencing the Gut Microbiota

Reductions in the cost of RNA-sequencing as well as the innovations and improvements across these platforms enables the study of the gut microbiota (Claesson et al., 2017). Commonly, faecal samples are collected and stores at -80° C until the RNA is extracted and cDNA is synthesized (Bastiaanssen et al., 2019, Chen et al., 2020a, Cardona et al., 2012). This genetic material is amplified using the PCR reaction, either targeting multiple microbial

genes for whole-genome shotgun sequencing or only the hypervariable 16S rRNA gene for 16S sequencing (Bastiaanssen et al., 2019, Fouhy et al., 2016, Clooney et al., 2016). While 16S sequencing is less cost-intensive, it does not provide as much strain-level resolution as whole-genome sequencing (Bastiaanssen et al., 2019, Fouhy et al., 2016, Clooney et al., 2016). Next-generation sequencing platforms then provide readouts of RNA sequences (Bastiaanssen et al., 2019). These readouts are pre-processed and assigned to microbial taxa, functional pathways or potential neuroactive modules (Bastiaanssen et al., 2019, Valles-Colomer et al., 2019, Iwai et al., 2016, McIver et al., 2018). A recent study identified the importance of neuroactive gut-brain modules in mood disorders (Valles-Colomer et al., 2019). These gut-brain modules are inferred from microbiome composition and are validated on a large human dataset (Valles-Colomer et al., 2019). This tool is important for further investigations of the microbiome, adding on more functional input and insight.

The data analysis approach must account for the compositional nature of the microbiota to reduce spurious findings (Gloor et al., 2017, Quinn et al., 2018). Most microbiome studies measure the α-diversity of samples to determine the amount, evenness and distribution of difference species within each sample (Bastiaanssen et al., 2019). β-diversity metrics quantify the differences between two different groups, often displayed using Principal Component Analysis (or Principal Coordinate Analysis, when compositional methods are not used) (Gloor et al., 2017). Identifying differentially-abundant taxa is a common method for explaining the potential relationship between certain elements of the microbiota and host physiology (Gloor et al., 2017).

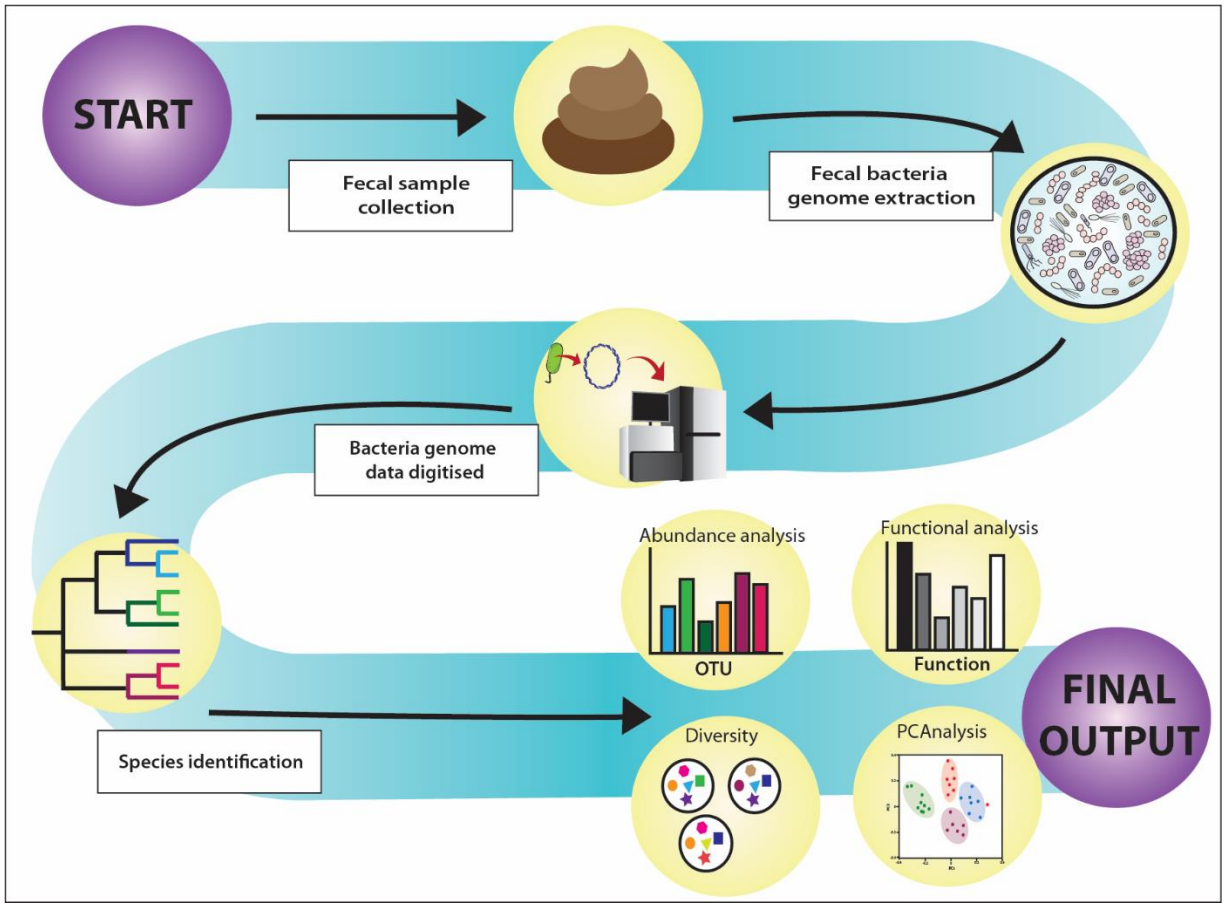

Figure 7. Pipeline of microbiota analysis, from faecal sample to final output. After fecal samples are collected, microbial genetic material is extracted. Microbial genes are amplified through PCR and the microbial genes are sequenced. Following this step, the microbiome information is digitized. Various bioinformatics tools are then used to identify the individual taxa within the sample. After generating a table containing the amount of different microbes found in each sample, bioinformatics analysis can determine whether one group expresses more of a specific taxa. Additionally, the amount of different bacteria and their distribution is compared between groups to assess different types of alpha diversity. The counts table can also be used to predict functional characteristics of the microbiota. Finally, a principal component analysis allows for the visualization of all samples, to determine whether groups cluster away from each other. This provides a measure of beta-diversity. Adapted from (Cryan et al., 2019).

Overall goal and Specific Aims

Given the importance of both the microbiota metabolites and glia in health and disease, my overall goal is to use two approaches to increase our knowledge of microbial metabolites and in particular SCFAs in brain health and disease

Aims Addressed in Chapter 2

While many studies analyse the microbiota in association with disorders or other aspects of the brain, they often find different results. Additionally, identifying differentially expressed microbes does not provide any functional information. A few studies also measure fecal short-chain fatty acids but other key metabolites, notably bile acids and tryptophan metabolites are rarely assessed. Since the development and validation of bioinformatics tools to identify neuroactive microbial pathways, these studies can be reanalyzed (Valles-Colomer et al., 2019). This chapter looks for differences or similarities in gut-brain module abundance across >200 human microbiome studies can be assessed. To our knowledge, this is the first such attempt at comparing and reconciling differences within these studies. With the addition of updated bioinformatics pipelines and taxonomic classifiers, a consistent pipeline is applied to analyse the data.

1. Although there is many studies on the microbiome in psychiatric disorders there is a lack of knowledge on the functionality of such changes in disease. Thus one of the major goals of this thesis is to synthesize, summarize and reanalysis of all existing human-microbiome-brain studies to probe for evidence of predicted microbial-metabolites associating with brain function, behaviour or disease in humans.
2. Use a standardized bioinformatics pipeline to determine if common microbiota-gut brain axis trends persist across existing human studies.

Aims Addressed in Chapter 3

A plethora of supporting studies suggest that SCFAs, especially butyrate, impact the glial components of the brain. While much of the focus has been on microglia, it is important to assess the specific roles of astrocytes within a homeostatic setting. (Frost et al., 2014) found that acetate impacts the glutamate-glutamine cycle in hypothalamic astrocytes, regulating appetite. SCFAs are also involved in the Krebs' cycle and thus may play a regulatory role in the brain. However, studies have not assessed whether there are sex-specific differences in cortical astrocyte function at physiologically-relevant levels of SCFAs. Thus the effects of SCFAs on gene expression of astrocytes may underpin some of the changes seen at a population level.

3. Determine whether physiologically relevant concentrations of microbially-derived SCFAs alter gene expression *in vitro* using primary cortical murine astrocyte cultures.
4. Determine if these effects *in vitro* are dependent upon sex.

Chapter 2: Mining Microbes for Mental Health: Determining the Role of Microbial Metabolic Pathways in Human Brain Health and Disease

Simon Spichak[1,2], Thomaz F.S. Bastiaanssen[1,2], Kirsten Berding[1,2], Klara Vlckova[1,2],

Gerard Clarke[2,3], Timothy G. Dinan[2,3], John F. Cryan[1,2*]

1. Department of Anatomy and Neuroscience, University College Cork, Cork, Ireland

2. APC Microbiome Institute, University College Cork, Cork, Ireland

3. Department of Psychiatry and Neurobehavioural Science, University College Cork, Cork, Ireland

** Corresponding author*

Prof. John F. Cryan

Email: j.cryan@ucc.ie

Tel: +353 21 4205426

Address: Room 3.86 Western Gateway Building, University College Cork, Cork, Ireland
Co-Author Postal Address: Room 5.35, Biosciences Institute, University College Cork, Cork, Ireland

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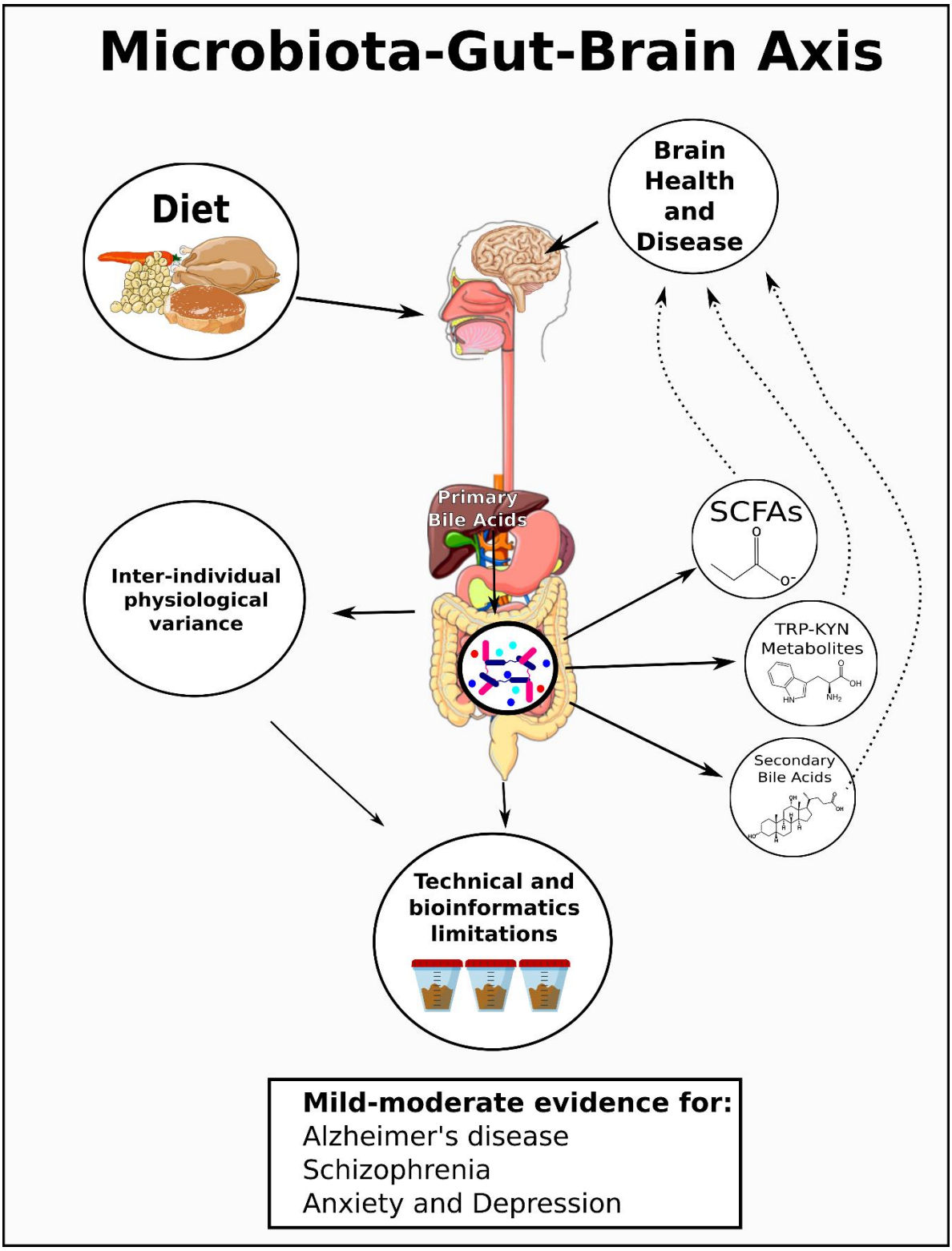
Graphical Abstract:



Abstract

There is increasing knowledge regarding the role of the microbiome in modulating brain and behaviour. Indeed, the actions of microbial metabolites are key for appropriate gut-brain communication in humans. Among these metabolites short-chain fatty acids, tryptophan, and bile acid metabolites/pathways show strong preclinical evidence for involvement in various aspects of brain function and behaviour. With the identification of neuroactive gut-brain modules, new predictive tools can potentially be applied to existing datasets.

We identified 278 studies relating to the human microbiota-gut-brain axis which included sequencing data. This spanned across psychiatric and neurological disorders with a small number also focused on normal behavioural development. With a consistent bioinformatics pipeline, thirty-five of these datasets were reanalyzed from publicly available raw sequencing files and the remainder summarized and collated. Among the reanalyzed studies, we uncovered evidence of disease-related alterations in microbial metabolic pathways in Alzheimer's Disease, schizophrenia, anxiety and depression. Amongst studies that could not be reanalyzed, many sequencing and technical limitations hindered the discovery of specific biomarkers of microbes or metabolites conserved across studies. Future studies are warranted to confirm our findings. We also propose guidelines for future human microbiome analysis to increase reproducibility and consistency within the field.

Keywords: Microbiota; brain; enteric-nervous system; short-chain fatty acids; bile acid; tryptophan; indole; psychiatry; neurodegenerative disease; diet

Highlights

- Microbially-derived metabolites are implicated in human brain health and disease
- Evidence is lacking for consistent microbial changes across studies
- Evidence suggests metabolites are involved in schizophrenia, anxiety/depression and Alzheimer's disease
- Technical sequencing and bioinformatics limitations hinder cross-study comparison
- Improved methods and data reporting are key to find robust associations

Introduction

1.1 Role of Metabolites in the Microbiota-Gut-Brain Axis

Since the serendipitous discovery of the antibacterial properties of penicillin in 1928, microbial metabolites have been harnessed for their various antimicrobial properties and are emerging as mediators of mammalian health and behaviour (Fleming, 1946b, Fleming, 1946a, O'Mahony et al., 2015, Blacher et al., 2017, Levy et al., 2017, McCarville et al., 2020). The mammalian gastrointestinal tract is colonized at birth by a diverse collection of microorganisms, collectively called the microbiota (Codagnone et al., 2019a, Theis et al., 2019). One of the core functions of the gut microbiota is the modification of host, xenobiotic and dietary-derived molecules into bioactive metabolites that can impact host health and disease (Clarke et al., 2019, Sharon et al., 2014, Spanogiannopoulos et al., 2016, Morris et al., 2017, Sun et al., 2017). The ecological community coexisting within a shared space is defined as the microbiome (Lederberg and McCray, 2001). One of the most surprising findings over the past decades is the cornucopia of genes within the microbiome that enable the production and modification of neuroactive metabolites which may modify gut-brain axis function (Zimmermann et al., 2019, Strandwitz et al., 2019, Lyte, 2014, Clarke et al., 2014, Tennoune et al., 2014, Lee et al., 2015). Most studies in this field characterize the predominant bacterial and archaeal components of the gut microbiota.

Microbial metabolites communicate through dynamic bi-directional pathways within the microbiota-gut-brain axis to mediate host brain immunity and physiology (Spichak et al., 2019, Erny et al., 2017, Pott and Hornef, 2012, Blacher et al., 2017, Levy et al., 2017, McCarville et al., 2020). They exert effects directly after being transported across the blood-brain barrier or indirectly through immune, neuroendocrine or vagal mechanisms (Alenghat, 2015, McCarville et al., 2020, Roager and Licht, 2018, Stilling et al., 2016, Fulling et al., 2019). Advances in sequencing technologies over the past decade enable the relatively rapid

and comprehensive illumination of the composition of the microbiome in the gut (Song et al., 2018, Bailey et al., 2019, Shakya et al., 2019). For the most part, sequencing of faecal samples is used as a surrogate of the gut microbiota composition of individuals. However, since most studies differ in methods, developing a consensus from this data is difficult (Pollock et al., 2018). Nonetheless, these studies are invaluable for assessing the role of the bacterial metabolites within the human host's central nervous system (CNS).

Three of the most-studied metabolic pathways within the gut microbiota are the short chain fatty acids (SCFAs), tryptophan metabolism and bile acid metabolism and will form the focus of our paper.

1.2 Aims and Scope

Knowledge on the role of the main microbial metabolic pathways influencing brain and behaviour is emerging. It is important to collate all the currently available datasets in order to identify gaps and point to novel areas of discovery. Thus, the aim of this paper is to assess metabolic signatures of different human brain health and disease states. All publicly available datasets will be reanalyzed and all existing data from the remaining studies will be collated. This study focuses on SCFAs, tryptophan pathway metabolites and bile acids. To the best of our knowledge, this is the first extensive analysis of the MGBA involving all publicly available data to determine whether any clear microbial composition and metabolic signatures emerge for psychological and psychiatric diseases. Briefly this will involve the following steps:

1. An extensive literature review (PubMed) on all studies involving sequencing the faecal microbiota in humans to compare with a functional or clinical brain measure, or disease status. Significant findings at the genera level related to the metabolites involved in the scope of this study will be recorded, along with significance and effect size, if available. Differentially abundant microbes known to be involved in metabolic

pathways (tryptophan, SCFA and bile acid) will be identified from the existing literature (Molinero et al., 2019, Roager and Licht, 2018, Valles-Colomer et al., 2019, O'Mahony et al., 2015).

2. A reanalysis of all publicly available datasets with a common, updated pipeline to identify differentially abundant microbes and gut-brain modules (GBMs). Recently the concept of GBMs has emerged, providing an additional predictive index for bacterial 16S rRNA gene sequencing studies (Valles-Colomer et al., 2019). Briefly, the authors performed an extensive literature review to inform the assembly of pathways with neuroactive potential in bacteria. Existing databases don't curate all these pathways or predict their ability to bypass the blood-brain barrier (Valles-Colomer et al., 2019). After construction and validation from genomes of human-associated microbes, GBMs were validated on a large cohort of human 16S rRNA gene sequencing data (Valles-Colomer et al., 2019). This revealed novel insight into the gut metabolic signatures of depression (Valles-Colomer et al., 2019). To fulfil a GBM, the microbe must possess each enzyme within the pathway (Valles-Colomer et al., 2019). Though this method does not directly measure the abundance of these metabolites, it provides stringent associations validated on large independent cohorts. In addition to changes in microbial composition and GBMs, effect sizes and 95% confidence intervals will also be reported.
3. Assess if common disease signatures exist across studies. If there is a specific host-microbe-metabolite interaction within a disease, we would expect a common unique signature of differentially-abundant taxa and GBMs across all studies of that disease.

2.0 Methods

2.1 Study Selection

PubMed database searches were conducted by searching for disease or health-related terms along with 'microbiome'. These terms were: obesity brain, anorexia, ADHD, ASD, PANDAS, schizophrenia, Alzheimer's Disease, amyotrophic lateral sclerosis, neurovascular, ischemia, temperament, personality trait, multiple sclerosis, IBS anxiety depression, fibromyalgia, migraine, stress AND human, posttraumatic, anxiety OR depression human faecal, alcohol-dependence, bipolar disorder, epilepsy, opioid use, smoking human faecal, human drug addiction faecal, sleep human faecal, human 'psychological stress', Rett syndrome. An example of one search would be: microbiome AND obesity brain. This search yielded a total of 3552 results on June 10th, 2020. The abstracts were manually searched and any studies not involving humans, the colonic microbiota or any brain or behaviour-related measures were excluded leaving 249 studies. 35 of these datasets were reanalyzed. 39 more studies published after June 10th 2020 were also included.

In the studies where the raw microbiome data was not reanalyzed the sequencing strategy, relevant results relating to differential abundance of microbes involved in neuroactive pathways and limitations were summarized.

2.2 Downloading datasets

Raw sequencing files (.fastq or .fastq.gz format) were downloaded from the European Nucletotide Archive or the Sequence Research Archive by generating a bash script to download the dataset (https://sra-explorer.info). For data deposited on the China National GeneBank Database Sequence Archive or Qiita sequencing files were downloaded by writing bash scripts to download each individual dataset (Gonzalez et al., 2018). Some data was also downloaded from the Metagenomic Rapid Annotations using Subsystems Technology (MG-RAST) using scripts from https://github.com/MG-RAST/MG-RAST-Tools (Meyer et al., 2008, Wilke et al., 2015). Two studies were excluded from reanalysis because one could not

be demultiplexed and another was sequenced using the SOLiD platform and could not be processed through the same pipeline.

2.3 Generating counts tables for 16S rRNA gene sequencing platforms

Raw sequencing files for each dataset were processed through the DADA2 pipeline (Callahan et al., 2016). Briefly, files were first filtered and trimmed to 200 base pairs (where possible with the following settings: trimLeft = 37, truncLen=237, maxEE=2, truncQ=2, maxN=0, rm.phix=TRUE) (Callahan et al., 2016). Next, sequence quality reports were generated using FastQC, using a threshold score of 28 (Andrews, 2010). If necessary, samples were filtered again and trimmed. Forward and reverse error rates (settings: nbases=1e8) were generated for each dataset, followed by merging of individual files into a sequence table and the removal of *de novo* bimeras (Callahan et al., 2016). The SILVA v132 training set was input into the RDP classifier in DADA2 to assign taxonomy to the sequence table (Glockner et al., 2017, Pruesse et al., 2019, Quast et al., 2013, Yilmaz et al., 2013, Wang et al., 2007).

Scripts used for bioinformatics analysis are found here: https://github.com/simon-sp/Mining-Metabolites.

2.4 Bioinformatics Analysis: Differentially Abundant Microbes

R version 3.6.3 was used in R Studio v1.2.5 for Ubuntu 18.04. Any amplicon-sequence variants (ASVs) with fewer than 2 raw counts were filtered out, and data was transformed using the centred-log-ratio (CLR) in ALDEx2 with 1000 Monte-Carlo sampling permutations (Fernandes et al., 2014, Quinn et al., 2018). Three studies were excluded where the majority of ASVs were filtered out, leaving a counts table with only 2-10 microbial taxa.

An overall PCA was generated by principal component analysis for visualisation and quality check purposes using the ggplot2 package (Wickham, 2016).

A list of differentially abundant microbes was generated with the Tjazi pairwise_DA_wrapper by incorporating the Wilcoxon Rank-Sum test for comparing the abundance of each individual microbe across groups, followed by a Benjamini-Hochberg post-hoc test (Bastiaanssen, 2019, Pounds and Cheng, 2004) . Microbes were reported if they had a $P_{adj} < 0.1$ and an effect size > 0.65 to increase the robustness of these findings. The 95% confidence intervals are also reported.

2.5 Bioinformatics Analysis: Differentially Abundant Gut Brain Modules

Raw sequencing data was transformed to be input into Piphillin for predicted functional analysis of the sequencing data (Iwai et al., 2016). The output of Piphillin produced a counts table of the Kyoto Encyclopdia of Genes and Genomes (KEGG) orthologs, which could then be used to assess the abundance of GBMs via omixerRpm, using the *GBM_v1.0* dataset (Kanehisa et al., 2019, Kanehisa and Goto, 2000, Kanehisa, 2019, Valles-Colomer et al., 2019). Differential abundance of GBMs was determined using the Tjazi pairwise_DA-wrapper. GBMs were reported if they had a $P_{adj} < 0.1$ and an effect size > 0.4 to increase the robustness of these findings. The 95% confidence intervals are also reported.

2.6 Generating Counts Tables for WGS Shotgun Analysis

First, adapter sequences were trimmed using bbduk (ktrim=r, mink=6, hdist=1, qtrim=rl, trimq=20, minlength=70, tpe, tbo, rcomp = T) followed by decontamination using bbmap (-Xmx16g, minid=0.95, qtrim=rl, trimq=10, untrim) against the masked human genome (Hg38) and merging using bbmerge (bbmerge-auto.sh, -Xmx24g, rem, k = 62, extend2=50, ecct) (Bushnell, 2020, Bushnell et al., 2017). The fastq.gz files were then processed through ‘biobakery_workflows wmgx’ run within a separate Miniconda environment (Python v2.7) with the following parameters: --bypass-strain-profiling --bypass-quality-control using the UniRef default databases for MetaPhlAn2 and HUMAnN2 (Truong et al., 2015, McIver et

al., 2018, Franzosa et al., 2018). The rest of bioinformatics analysis of the count tables for genes and gene pathways is described in Section 2.3 with two differences. Piphillin is not used because HUMAnN2 provides counts tables of gene pathways/proteins as outputs and thus do not need to be inferred. Counts tables for bacterial genes as well as gene pathways/proteins were first run through the guess_counts function within the Tjazi R library, before CLR transformation (Bastiaanssen, 2019). Two whole genome shotgun (WGS) studies were excluded from re-analysis because the publicly available dataset did not contain all sequenced samples or the fastq.gz files were not labelled.

3.0 Results and Discussion

3.1 Short-Chain Fatty Acids (SCFAs) in Brain Health and Disease

3.1.1 Biochemistry and Function

SCFAs are molecules consisting of a 1-6 carbon chain with a carboxylic acid group (Dalile et al., 2019). Colonic bacterial fermentation of non-digestible, non-absorbable fibres (inulin, cellulose, wheat bran and resistant starches) produces SCFAs as a by-product (Cummings, 1981). The following genera commonly found in the gut are known to produce SCFAs: *Akkermansia, Bifidobacterium, Lactobacillus, Lactocaseibacillus, Ligilactobacillus, Ruminococcus, Ruminoclustridium, Blautia, Bacteroides, Roseburia, Prevotella, Eubacterium, Fusicatenibacter, Faecalibacterium, Enterococcus, Clostridium* and *Coprococcus* (Takada et al., 2013, Dalile et al., 2019, Joseph et al., 2017, Valles-Colomer et al., 2019, Basson et al., 2016, Zheng et al., 2020a). It is unclear how these genera impact the absorption of SCFAs in the colon (Ruppin et al., 1980) nor how GI absorption may differ between individuals independent of these microbes (Dalile et al., 2019). Other factors that may impact differences in SCFA circulating concentrations include host genetics, dietary intake and colonic absorption of SCFAs (Dalile et al., 2019).

SCFA production involves overlap with pyruvate metabolism and other molecules involved in the Krebs Cycle (see **Figure 1**). The most abundant SCFAs in humans are acetate, butyrate and propionate (Dalile et al., 2019). They differ in their aliphatic tail length and the position of their carboxylic acid group (Dalile et al., 2019). These minor differences affect affinity and specificity to G-protein coupled receptors (GPCRs; (FFAR1, FFAR2, FFAR3, GPR109A, GPR164 and OR51E2)) (Dalile et al., 2019). SCFAs also act as histone deacetylase inhibitors in enteric neurons, enterochromaffin cells, and microglial cells (Stilling et al., 2016, Erny et al., 2015, Dalile et al., 2019, Woo and Alenghat, 2017, Yang et al., 2019). Through these

mechanisms, SCFAs impact host physiology by driving the expansion of $FOXP3^{+}$ T_{reg} cells (Woo and Alenghat, 2017), or mediating the release of IL-6, IL-10 and IL-12, dendritic cells and macrophages, in turn driving T cell maturation (Woo and Alenghat, 2017).

Many SCFA-sensing GPCRs are located on enteric immune and neuronal cells (Nohr et al., 2013, De Vadder et al., 2014). In the millimolar concentration range butyrate depolarizes enteric neurons (Neunlist et al., 1999), and reduces monocyte activation and mast cell degranulation (Digby et al., 2012, Diakos et al., 2006). Though a growing body of pre-clinical evidence suggests SCFAs are neuroactive metabolites influencing the brain and behaviour (Liu et al., 2020b, Sadler et al., 2020, van de Wouw et al., 2018, Lee et al., 2020a), few clinical studies thus far have reported on these effects in humans. Some promising evidence shows that SCFA production correlates with health outcomes in humans. For example, increasing dietary-fibre intake from an average of 12.12g daily to 37.10g over the course of 84 days modulated clinically-relevant host outcomes in Type 2 Diabetes, including reducing the levels of haemoglobin A1C (Zhao et al., 2018).

3.1.2 Potential of SCFAs to Cross the Blood-Brain Barrier

To reach the brain, SCFAs must cross the intestinal epithelium through passive diffusion or *via* monocarboxylate transporters (MCT1, SMCT1) (Bergersen, 2015, Chiry et al., 2006), before passing through the hepatic circulation without being completely depleted by hepatic enzymes (Stilling et al., 2016). In recently-deceased or fasting individuals, researchers found SCFAs in peripheral circulation were depleted to ~20% after passing through hepatic circulation (Stilling et al., 2016, Cummings and Macfarlane, 1997, Peters et al., 1992, Hamer et al., 2008).

SCFAs are transported across the blood-brain barrier by MCT1 or SMCT1, but it is unclear if they reach a relevant physiological concentration in the brain (Bergersen, 2015, Chiry et al.,

2006). One human study used PET *in vivo* imaging to microbially-produced acetate from the colon reached the hypothalamus to regulate satiety signalling (Frost et al., 2014). In addition, butyrate is involved in mediating the integrity and permeability of the blood-brain barrier by increasing occludin expression in preclinical models (Braniste et al., 2014, Li et al., 2016, Sun et al., 2016a, Sun et al., 2016b). Meanwhile, *in vitro* studies show that propionate can act on GPCR receptors at 1µM to promote neuroprotective pathways (Hoyles et al., 2018). The human metabolomic database assessed concentrations of SCFAs in the cerebrospinal fluid, finding ranges of 0-171µM for acetate, 0-6µM for propionate and 0-2.8µM for butyrate (Wishart et al., 2018). An older study performed gas chromatography on human brains, finding higher SCFA concentrations than the metabolomics study found in the cerebrospinal fluid (Bachmann et al., 1979). These studies are not conclusive but suggest that SCFAs do enter the brain. It is unknown if these SCFA levels induce effects on circumventricular organs such as the hypothalamus.

3.2 Tryptophan Pathway Metabolites

3.2.1 Biochemistry and Function of Bacterially-Produced Indoles

The gut microbiota can generate and modify neurotransmitters as well as their precursors, including serotonin and tryptophan (see Gheorghe et al. (2019), (Lee et al., 2015) for review). The potential for gastrointestinal microbes to metabolize tryptophan and its various metabolites was first characterized in the 1970s (Allison et al., 1974, Whitt and Demoss, 1975). In the decades since, metabolites exclusively produced by microbial enzymes yet communicate with the host, called indoles were functionally characterized (Lee et al., 2015, O'Mahony et al., 2015). While indoles are commonly produced by pathogenic strains of bacteria to improve their survival, they are also present in a symbiotic ecosystem (Lee et al., 2015). While the neurotransmitter serotonin is produced from the dietary-derived essential

amino acid tryptophan (Reigstad et al., 2015), indoles are produced by the breakdown of tryptophan using the bacterial enzyme tryptophanase (Lee et al., 2015).

Many of the bacterial strains capable of expressing tryptophanase are also involved in the other tryptophan metabolic pathways described below. These genera include *Bacteroides, Butyrivibrio, Clostridium, Enterococcus, Escherichia, Eubacterium, Haemophilus, Fusobacterium, Peptostreptococcus, Bifidobacterium, Parabacteroides, Megamonas, Anaerostipes, Ruminococcus* (Roager and Licht, 2018, Valles-Colomer et al., 2019, O'Mahony et al., 2015).

Indoles are present in the high nanomolar to low millimolar range in the colon (Bansal et al., 2010, Karlin et al., 1985). These metabolites, produced by the enzyme tryptophanase, signal with human intestinal epithelial cells in the millimolar concentration range, increasing tight junction resistance and mucin production (Bansal et al., 2010, Karlin et al., 1985). Indole metabolites also regulate enteric neuronal signalling and motility in the myenteric plexus through the aryl-hydrocarbon receptor (Obata et al., 2020). In the brain, indoles act on this same receptor in the central nervous system (CNS) astrocytes to regulate inflammation and immunity (Rothhammer et al., 2016, Rothhammer et al., 2018).

3.2.2 Biochemistry and Function of Serotonin

Microbial tryptophan metabolism regulates bioavailability of precursors required for host serotonin (5-HT) production (Kennedy et al., 2017, Yano et al., 2015). To produce 5-HT, tryptophan hydroxylase converts tryptophan into 5-hydroxytryptophan, which then requires an enzymatic decarboxylation reaction to form 5-HT (Gheorghe et al., 2019, Kennedy et al., 2017). 5-HT is important for gastrointestinal motility, absorption and secretion tract (Kennedy et al., 2017). ~95% of 5-HT is produced by the enterochromaffin cells in the gut and secreted into the lumen in response to different stimuli (Kuo et al., 2002, Gershon and

Tack, 2007). The enterochromaffin cells can uptake tryptophan or 5-hydroxytryptophan and generate serotonin via tryptophan hydroxylase (Kuo et al., 2002, Gershon and Tack, 2007). In disorders such as ulcerative colitis or irritable bowel syndrome, tryptophan hydroxylase 1 mRNA, serotonin transporter mRNA and serotonin transporter expression were markedly reduced (Coates et al., 2004). There is also cross-talk with SCFAs which modulate the expression of serotonin production within enterochromaffin cells by promoting tryptophan hydroxylase 1 gene expression (Reigstad et al., 2015).

The serotonergic system within the brain is involved in regulating cognition, mood and behaviour, and is dysfunctional in depression, anxiety and other neuropsychiatric disorders (Jacobs and Azmitia, 1992, Gheorghe et al., 2019).

3.2.3 Biochemistry and Function of Other Tryptophan Catabolites

Tryptophan is degraded in the colon and throughout the rest of the body by the ubiquitously expressed indoleamine-2,3-dioxygenase (IDO1) or tryptophan-2,3-dioxygenase in the liver (TDO2) (Seifert, 1993, Ruddick et al., 2006). The expression of these enzymes is increased past homeostatic levels by stress-released cytokines and elevated levels of glucocorticoids, toll-like-receptor activation or aryl hydrocarbon receptor activation (Morris et al., 2017, Maes et al., 2011b, Schrocksnadel et al., 2006, Kennedy et al., 2017). This increases the presence of downstream catabolites including quinolinic acid and kynurenic acid which act within the CNS or the enteric nervous system (ENS) (Morris et al., 2017, Maes et al., 2011b, Schrocksnadel et al., 2006, Kennedy et al., 2017).

Kynurenic acid is a GPR35 agonist in the gastrointestinal tract and in mononuclear immune cells in the ENS (Wang et al., 2006) and provides neuroprotection in the CNS as an antagonist of the N-methyl-D-aspartate (NMDA) receptor and the α-7-nicotinic receptor (Foster et al., 1984, Hilmas et al., 2001). Quinolinic acid on the other hand exerts agonistic

excitotoxic activity in the CNS through activation of the NMDA receptor (Foster et al., 1984).

Interestingly, in a recent a double-blind randomized placebo-controlled trial in humans it was found that probiotic supplementation with *L. plantarum* 299v altered kynurenine metabolites and improved cognition measures in individuals with major depressive disorder. However, the microbiota compositional changes were not characterized in this trial (Rudzki et al., 2019). It could be hypothesized that the changes introduced into the gut microbial ecosystem sufficiently altered the expression of hepatic TDO2, indirectly influencing peripheral tryptophan metabolism.

3.2.4 Transport into the Brain

Tryptophan is absorbed in the small intestine and transported into peripheral circulation and can be catabolized by IDO1 throughout the body or TDO2 in the liver (Seifert, 1993, Kennedy et al., 2017). Remaining tryptophan is transported across the blood-brain barrier via the large neutral amino acid transporter, where it can be converted to 5-HT or kynurenine catabolites (Ruddick et al., 2006). Kynurenic acid and quinolinic acid cannot cross the blood-brain barrier but other catabolites such as indoles and kynurenine have been detected in the brain (Morris et al., 2017, Maes et al., 2011b, Schrocksnadel et al., 2006, Kennedy et al., 2017, Gheorghe et al., 2019). Serotonin transporters in the brain can mediate the reuptake of excess 5-HT at the synaptic cleft, and is a common target of pharmaceutical interventions for depression and anxiety (Schwarcz et al., 2012).

3.3.1 Bile Acids and the Brain

Bile acids are molecules synthesized from cholesterol in the liver, characterized by amphipathic steroidal functional groups (Mertens et al., 2017, Kiriyama and Nochi, 2019). They play crucial roles facilitating the digestion and absorption of dietary lipids and fat-

soluble vitamins (Mertens et al., 2017, Kiriyama and Nochi, 2019, Enright et al., 2018). Most bile acids are generated through the hydroxylation reaction by CYP7A1, while the rest are synthesized via the alternative pathway involving the liver enzymes CYP271 and CYPB1 (Enright et al., 2018). In the mouse, the expression of these three enzymes is mediated by the host microbiota (Sayin et al., 2013). Shortly after they are generated in the liver, the bile acids are conjugated with taurine or glycine before being transported for storage in the gall bladder (Dawson and Karpen, 2015, Long et al., 2017). Once released to aid in the digestion and absorption of lipids, they travel through the gastrointestinal tract and can be deconjugated and bio transformed by gut microbes where they can be absorbed into peripheral circulation (Enright et al., 2017). These bile acids are also involved in cellular signalling, particularly as ligands for nuclear receptors and various transmembrane surface receptors (Mertens et al., 2017, Kiriyama and Nochi, 2019).

Bile salt hydrolases, enzymes produced by members of the mammalian gut microbiota, deconjugate bile acids (Long et al., 2017) (Fig. 1). Currently, these genera are known to produce this enzyme: *Bacteroides*, *Clostridium* cluster VIA, *Lactobacillus*, *Bifidobacterium*, *Eubacterium* (Molinero et al., 2019). In the gut, the primary bile acid deoxycholic acid, can inhibit colonic motility through the GpBAR1 (TGR5) receptor on enteric neurons (Sun et al., 2004a, Sun et al., 2004b, Poole et al., 2010). Disruptions and alterations in the gut microbiota contribute to bile acid dysregulation in the BTBR mouse model of autism-like behaviour (Golubeva et al., 2017). It is unclear how these gut microbial and bile acid changes relate to the behaviour in this model.

Conjugated and unconjugated bile acids, as well as taurine or glycine alone are potential neuroactive ligands in humans (Mertens et al., 2017, MahmoudianDehkordi et al., 2019). Taurine is thought to be neuroprotective as it functions as an agonist of glycine, $GABA_A$ and $GABA_B$ receptors in the brain (Albrecht and Schousboe, 2005, Boldyrev et al., 1999, Choe et

al., 2012, Hilgier et al., 2005, El Idrissi and Trenkner, 1999, Beetsch and Olson, 1998). It is unknown how much taurine is transported into the brain and if it is sufficient for signalling (Albrecht and Schousboe, 2005). Recently (Sharon et al., 2019a) showed that offspring of mice colonized with a human autism faecal microbiota produced less taurine than offspring of controls colonized with a neurotypical faecal microbiota. These mice were impaired in their social behaviours, suggesting a gut-brain connection is involved in these behaviours (Sharon et al., 2019a). Indeed, when they supplemented BTBR mice with taurine, characterized as socially-impaired, researchers could rescue these deficits (Sharon et al., 2019b)

Recently, a large multicentre metabolomics study of 1464 total participants found that the bacterially produced deoxycholic acid, as well as its glycine and taurine conjugated forms were increased in the serum metabolome of individuals with Alzheimer's Disease (MahmoudianDehkordi et al., 2019), suggesting increased 7α-dehydroxylation of cholic acid by the gut microbiota, as these metabolites cannot be produced by the host. Importantly, deoxycholic acid was also associated with cognitive decline, providing human evidence of a link between microbial bile acid metabolism and mental health (MahmoudianDehkordi et al., 2019).

3.4 Sequencing and Software

3.4.1 Sample Preparation and Sequencing Technology

There is great heterogeneity in sequencing preparation, sequencing strategy and downstream bioinformatics analysis despite multiple studies identifying a clear need and multiple efforts for standardization of these protocols (Fouhy et al., 2016, Clooney et al., 2016, Pollock et al., 2018, Aigrain et al., 2016, McLaren et al., 2019, Hogue et al., 2019, Santiago et al., 2014, Cardona et al., 2012).

Even before a sample is sequenced many factors influence the microbial community within it. Many studies reported a bias in different DNA extraction protocols biasing towards gram-positive or gram-negative bacteria (Watson et al., 2019), delivery-conditions and speed of the faecal sample and library preparation (Yeoh et al., 2019), fractional subsampling of faecal material (Yeoh et al., 2019), and storage (Panek et al., 2018, Chen et al., 2020a, Neuberger-Castillo et al., 2020, Carruthers et al., 2019).

Early microbiome studies used real time quantitative PCR (RT-qPCR) based techniques to amplify bacterial specific sequences from stool samples for species and genera-level identification. Other techniques hybridized fluorescent primers to these sequences for quantification or used terminal-restriction fragment length polymorphism analysis. These preliminary methods did not produce high-throughput, high coverage outputs and only describe the abundance of a few specific genera. There are indeed considerations in terms of bacterial load that could not have been addressed in these studies, making it difficult to draw robust conclusions about overall abundance without a clear picture of the entire microbiome (Vandeputte et al., 2017). With the decline in cost of sequencing, most high-throughput microarray-based technologies were replaced with next-generation sequencing, also known as high-throughput sequencing (NGS). NGS emerged as a method that provided untargeted information about the community as well as more reads and coverage (Bonk et al., 2018).

One method of sequencing the faecal microbiota involves the amplification of the hypervariable regions of bacterial 16S rRNA gene, found within the DNA of all bacteria. However, there is no universal consensus for selecting a hypervariable region to amplify despite substantial evidence showing its impact on the abundances of different detected taxa within a sample (Clooney et al., 2016, Kumar et al., 2011). The metagenomic GC content also biases the amplification process resulting in a decreased abundance of microbial taxa with higher GC content (Laursen et al., 2017). In addition, there is no consensus for

determining when single-end sequencing preparation is adequate and when paired-end sequencing methods must be used. While single-end reads often provide more coverage, paired-end reads provide more phylogenetic resolution (Werner et al., 2012, Chen et al., 2018).

When using a 16S rRNA gene based sequencing platform, there is great variation between different technological platforms such as 454 Roche Pyrosequencing, Illumina HiSeq and Illumina MiSeq (Clooney et al., 2016, Fouhy et al., 2016, Degnan and Ochman, 2012). Newer Illumina-based platforms improve coverage while reducing costs, predominantly replacing the use of 454 Roche Pyrosequencing (Degnan and Ochman, 2012). While 16S rRNA gene-base sequencing methods can accurately-identify taxa with genus-level resolution, WGS is required for quality species, strain and substrain identification in faecal samples. In addition, they identify previously uncultured bacteria and their genes. Since WGS amplifies all metagenomic information within a sample, it provides a more accurate view of the community composition and diversity while also providing functional information; however preferably amplified fragments however lead to overestimation in abundance of certain microbes (Clooney et al., 2016, Ranjan et al., 2016, Tessler et al., 2017). The currently most commonly-used platforms involve the use of Illumina sequencers however studies have not compared different WGS methods with each other.

3.4.2 Taxonomic Databases and Classifiers

Differences in taxonomic classification databases and taxonomic assignment likely contributed to inconsistent classification of microbial sequences across studies. In addition, researchers which conducted studies >3 years ago did not have access to more extensive taxonomic databases (Glockner et al., 2017, Pruesse et al., 2019, Quast et al., 2013, Yilmaz et al., 2013). Many existing studies have used the Greengenes database for assigning

microbial taxonomy, but this database is problematic because it has not been curated/updated since 2013 and thus cannot identify novel sequences (DeSantis et al., 2006). Greengenes has a significant overrepresentation of certain taxa; for example, at the species level ~15% of all sequences are assigned to *Faecalibacterium prausnitzii* (Allard et al., 2015). This is in contrast to other databases such as SILVA, which do not have a single species level assignment allocated to even 5% of all sequences within the database (Allard et al., 2015). This means that studies which used Greengenes to assign taxonomy were also a lot more likely to find an enrichment in *Faecalibacterium prausnitzii* and an underrepresentation of other taxa. In studies using untransformed relative abundance metrics, a non-specific assignment of *Faecalibacteirum prausnitzii* would affect the relative abundance of other identified genera.

One reason that different databases would assign a different classification to the same sequence is the size of the database (Balvociute and Huson, 2017). Having a larger taxonomic database can improve the specificity of these classifications since there will be more sequences with similarity to the read (Balvociute and Huson, 2017). Since taxonomic classes above the genus-level are very diverse, these differences were not reported in this analysis because they do not provide adequate resolution to infer the production of bacterial metabolites. Even bacterial members within the same family can differ in their enzymatic and metabolic capabilities.

In addition, the use of amplicon sequencing variants (ASVs) rather than operational taxonomic units (OTUs) provide more replicable and meaningful identification of taxa across studies (Callahan et al., 2017). However, many past studies have used and many still use OTUs, hindering comparison across datasets. Often, studies may even identify some OTUs belonging to one microbial genus increased in one group while also finding other OTUs

belonging to the same genus reduced in that same group. This confounds interpretation and replicability.

The gut microbiota functions as an ecological community with keystone species and genera necessary for its function. Identifying individual ASVs that are altered in a disease could help identify these keystone members. Thus, if an important keystone genus is disrupted, the metabolic output of the community is altered which may impact host health (Chng et al., 2020, Banerjee et al., 2018, Berg et al., 2020, Fisher and Mehta, 2014).

3.4.3 Compositional Data Analysis

Widely used relative abundance and general logarithmic transformations are inappropriate for microbiome data. Microbiome data is, by definition, compositional and thus using relative abundance, or rarefaction during processing is inappropriate and would skew study results (Gloor et al., 2017). In addition, issues within correlational analysis of compositional data have long been noted and are another challenge when analysing microbiome data (Gloor et al., 2017, Lovell et al., 2015, Friedman and Alm, 2012, Kurtz et al., 2015, Pearson, 1897). There is a known bias for spurious and negative correlations within microbiome datasets (Gloor et al., 2017). Additionally, we found many studies where rarefaction is used when processing reads. This involves subsampling of each sample's read counts to a common sequencing depth but results in a loss of information and precision (McMurdie and Holmes, 2014). Finally, it is also possible to mathematically model the bias within metagenomic experiments (McLaren et al., 2019). This would allow for reference calibration to correct these biases, but only if the data has already been compositionally transformed (McLaren et al., 2019).

3.4.4 Use of Outdated Tools and Software

Additionally, we also found that bioinformatics tools are often used after they are deprecated; a few studies described in Table 1 used Quantitative Insights into Microbial Ecology (QIIME) Version 1 past the date that it was still supported by its developers, while many studies did not specify the version used.

3.5 Healthy Humans

3.5.1 Infant Temperament and Behaviour

3.5.1.1 Studies Where Raw Microbiome Data Was Not Reanalyzed

The only WGS study found multiple associations between *Bifidobacterium, Clostridium* and *Bacteroides* species associated with brain connectivity and temperament. However, four 16S sequencing studies did not find any genus-level associations between infant temperament and microbiota composition (Carlson et al., 2018, Gao et al., 2019, Christian et al., 2015, Rosin et al., 2020). Two studies showed positive associations of increased *Bifidobacterium* abundance in infants with positive behaviours (soothability and emotional regulation) (Wang et al., 2020d, Aatsinki et al., 2019). Though Loughman et al. (2020) did not find associations with *Bifidobacterium, Prevotella* abundance was associated with behavioural problems. These studies rely on correlational analysis but since the microbiota is compositional by nature, these datasets are prone towards spurious correlations (see 3.4.3) (Gloor et al., 2017). Though *Bifidobacterium* and *Prevotella* participate in tryptophan and SCFA metabolic pathways (Valles-Colomer et al., 2019), it is still unclear whether these specific pathways are implicated in these behaviours. See Table 1 for more detail.

3.5.2 Adult Personality and Behaviour

3.5.2.1 Studies Where Raw Microbiome Data Was Not Reanalyzed

Many descriptive studies have associated individual genera of bacteria with personality traits. In healthy participants, Taylor et al. (2019) found a negative correlation of *Blautia* abundance with anxiety. Tillisch et al. (2017) did not assess anxiety but found a negative correlation of *Prevotella* abundance with negative affect. Interestingly, Kim et al. (2018) found associations between increased *Roseburia* abundance and conscientiousness while Johnson (2020) instead found *Oscillispira* associated positively with sociability. There were no consistent findings at the genus-level within these studies, resultant from limitations described in Table 1. Without additional metadata and strain level resolution, it is difficult to associate personality traits with microbial genera. While many studies identified associations with bacteria involved in SCFA and tryptophan metabolic pathways, the current state of the evidence for robust microbial associations with personality traits is weak.

3.5.2 Sleep Characteristics and Quality

3.5.2.1 Studies Where Raw Microbiome Data Was Reanalyzed

Liu et al. (2019a) collected faecal samples from ten individuals who reported insomnia and another ten who served as healthy controls. Though no GBMs were differentially abundant, *Alloprevotella* abundance was significantly reduced in individuals with insomnia ($p_{adj} < 0.1$, effect = -1.16; 95% CI: [-14.97; 0.17]). No other microbes or GBMs relating to SCFA, tryptophan or bile acid pathways were differentially abundant within this dataset.

3.5.2.2 Studies Where Raw Microbiome Data Was Not Reanalyzed

Few studies focused on associating sleep-quality and microbiota composition (see Table 1). Among these, two showed no genera-level associations between microbes and sleep (Liu et al., 2020c, Anderson et al., 2017). One 16S sequencing study found disruptions in across different sleep stages in individuals with a *Prevotella* enterotype (Ko et al., 2019). Smith et al. (2019) collected extensive metadata, allowing for the correlation of specific microbes to

sleep parameters. While *Holdemania* and *Corynebacterium* abundance negatively associated with number of awakenings, *Coprococcus* and *Neisseria* were associated with increased awakenings (Smith et al., 2019b). *Blautia* also negatively associated with sleep efficiency and total sleep time (Smith et al., 2019b). Though these findings are interesting, participants used faecal swabs to collect microbiota samples (Smith et al., 2019).

An additional three studies compared the gut microbiome of individuals with sleep disorders such as insomnia and narcolepsy type 1 to controls (Lecomte et al., 2020, Li et al., 2020c, Valentini et al., 2020). These compelling pilot studies point to possible associations of different microbial genera and healthy sleep.

While the interactions between circadian rhythm, sleep and the microbiome are compelling and gaining more traction (Godinho-Silva et al., 2019, Govindarajan et al., 2016, Li and Cui, 2018, Weger et al., 2018, Teichman et al., 2020), more human studies are necessary to investigate this interaction.

3.5.3 Ageing and Cognition

3.5.3.1 Studies Where Raw Microbiome Data Was Not Reanalyzed

No common genus-level differences associated with healthy cognitive ageing across existing studies (see Table 1). However, these studies all compared different subsets of unhealthy cognitive aging. One study compared healthy ageing to mild-cognitive impairment, another with Cirrhotic individuals, another with dementia and finally one was a 12 week crossover-double blind trial (Nagpal et al., 2019, Kim et al., 2020, Bajaj et al., 2016, Saji et al., 2019). While compelling large-cohort studies associated microbial populations with frailty and diet in aging (Ghosh et al., 2020, Meehan et al., 2015, O'Toole and Jeffery, 2018, Ticinesi et al., 2017, Verdi et al., 2018) they do not overtly focus on the cognitive aspects of ageing *per se*.

3.6 Neurodevelopmental Disorders

3.6.1 Attention-Deficit Hyperactivity Disorder

3.6.1.1 Studies Where Raw Microbiome Data Was Reanalyzed

One 16S sequencing study was reanalyzed, involving 19 individuals with Attention-Deficit Hyperactivity Disorder (ADHD) and 77 control participants, including a wide age-range for their participants (Aarts et al., 2017). Upon reanalysis, no significant differences within the microbial composition or GBM abundance were found (Aarts et al., 2017) (see Table 2).

The study is notable because 28 of these participants underwent a further fMRI analysis and found associations between microbial compositions with responses to reward anticipation (Aarts et al., 2017). Since fMRI data was not provided, this aspect of the study was not reanalyzed.

3.6.1.2 Studies Where Raw Microbiome Data Was Not Reanalyzed

Wan et al. (2020) used a WGS strategy to identify a reduction in KEGG Orthologs for dopaminergic pathways in individuals with ADHD. Consistent with Jiang et al. (2018b), ADHD individuals showed a reduction in the abundance of *Faecalibacterium* (Wan et al., 2020). In fact, *Faecalibacterium* abundance negatively associated with the total Conners Parent Rating Scales score, which assesses children's behavioural difficulties, as well as the hyperactivity index (Jiang et al., 2018b). No other common differences in microbial genera between ADHD and controls were reported across a set of five other studies (Stevens et al., 2019, Prehn-Kristensen et al., 2018, Szopinska-Tokov et al., 2020, Pärtty et al., 2015, Wang et al., 2020b). However, one of these studies used a compositional approach for their data analysis (Szopinska-Tokov et al., 2020). They found an increased relative abundance of *Ruminoclostridium 9* and *Ruminococcus 2* in ADHD individuals, and were able to correlate *Ruminococcus 2* with inattention scores ($B = 1.525$, $p = 0.001$) (Szopinska-Tokov et al.,

2020). Nonetheless, there is weak evidence for specific SCFA or tryptophan associated microbial pathway alterations in ADHD (see Table 2).

3.6.2 Autism Spectrum Disorder (ASD)

3.6.2.1 Studies Where Raw Microbiome Data Was Reanalyzed

Across seven reanalyzed studies (see Table 2), only two showed a robust effect of Autism Spectrum Disorder (ASD) on microbiota composition (Averina et al., 2020, Son et al., 2015, Pulikkan et al., 2018, Kang et al., 2019, Kong et al., 2019, Liu et al., 2019d, Strati et al., 2017). In the data collected by (Pulikkan et al., 2018), *Roseburia* abundance was increased in ASD ($p_{adj} < 0.001$, effect = 0.9; 95% CI: [-1.9, 10.38]). When stratifying individuals with ASD by the median Autism Treatment Evaluation Checklist score, those below the median of 62 showed a reduction in *Ruminoclostridium 9* ($p_{adj} < 0.1$, effect = -0.78; 95% CI: [-7.28, 1.80]) (Kong et al., 2019). However, none of these studies found any differentially abundant GBM pathways. Interestingly, in the dataset collected by Son et al. (2015), twins discordant for ASD showed no overall differences in microbiota composition. This may indicate that environmental factors such as diet as well as other factors are largely responsible for microbiota changes in these studies.

3.6.2.2 Studies Where Raw Microbiome Data Was Not Reanalyzed

Over 30 other studies assessed differences between the ASD microbiota and controls, or differences within ASD subgroups (see Table 2). Across the WGS studies, only one found changes in GBM abundance (Wang et al., 2019a). They reported decreased gut glutamate/glutamine metabolism in ASD individuals (Wang et al., 2019a).

Bacteroides abundance was increased in ASD groups amongst four datasets (Zhai et al., 2019b, Zurita et al., 2019, Coretti et al., 2018, Ahmed et al., 2020) and reduced in four (Dan et al., 2020, Niu et al., 2019, Ding et al., 2020, Zhang et al., 2020c). Similarly the relative

abundance of *Bifidobacterium* was increased in two datasets (Dan et al., 2020, Plaza-Diaz et al., 2019), and decreased in three others (Niu et al., 2019, Wang et al., 2020b, Zhang et al., 2020c). Another compelling argument from the use of ASVs over OTUs is identifying whether a specific genus is increased or decreased. For example, in Zurita et al, (2019), one *Ruminoccocus* OTU is increased in ASD while another is reduced. Until recently, the important *Lactobacillus* genera encompassed many distinct strains; with updated nomenclature it might be possible to differentiate amongst the genera and find other potential signatures (Zheng et al., 2020a). Overall, there is great heterogeneity in the methods, reporting and results.

Though they did not find correlations between ASD symptoms and faecal SCFAs, Berding and Donovan (2019) found that the SCFAs correlated strongly with diet. A separate study reported increased valerate and decreased butyrate in ASD faecal samples (Liu et al., 2019d).

As many as 90% of individuals with ASD display picky and repetitive eating behaviours, which can further impact their nutrient intake and microbiota (Kral et al., 2013). It's unclear whether SCFA dysregulation is a result of the microbial production or dysregulation in gut absorption. Along with host genetics, this is an important consideration for future studies.

3.6.3 Schizophrenia

3.6.3.1 Studies Where Raw Microbiome Data Was Reanalyzed

Four studies were reanalyzed (see Table 2), but we found differentially abundant genera in only two of these studies (Xu et al., 2020, Shen et al., 2018, Flowers et al., 2019, Nguyen et al., 2019). In these two studies, individuals with schizophrenia had higher abundances of the acetate-producing *Fusicatenibacter* ($p_{adj} < 0.001$, effect: 0.67; 95% CI:[-1.48; 7.56]; $p_{adj} < 0.001$ and effect =1.06; 95% CI: [-1.05; 7.60]) (Shen et al., 2018, Xu et al., 2020). In the samples collected by Xu et al. 2020, individuals with schizophrenia also showed an increase

in the following GBMs: Butyrate synthesis II ($p_{adj} < 0.001$, effect: 0.61; 95% CI:[-1.83; 5.41]), Kynurenine synthesis ($p_{adj} < 0.001$, effect: 0.68; 95% CI:[-2.10; 6.12]), and Inositol degradation ($p_{adj} < 0.001$, effect: 0.83; 95% CI:[-1.58; 6.96]). In addition, *Lactobacillus* abundance was reduced ($p_{adj} < 0.001$, effect: - 1.28; 95% CI:[-12.85; 0.11]) (Xu et al., 2020).

Perhaps *Fusicatenibacter* was only differentially abundant in the Chinese cohorts, compared to the North American cohorts due to dietary and environmental differences.

3.6.2.2 Studies Where Raw Microbiome Data Was Not Reanalyzed

Across three WGS studies, various *Lactobacillus* OTUs are increased in schizophrenia compared to controls (Zhu et al., 2020, Xu et al., 2020, Schwarz et al., 2018), however some OTUs were also reduced in one of the studies (Zhu et al., 2020). In two studies, *Bifidobacterium adolescentis* was increased in patients, while *Clostridium perfingens* was increased in one dataset (Xu et al., 2020) but reduced in the other (Zhu et al., 2020). Interestingly, this contrasts with the reduction found when reanalyzing the 16S dataset from (Xu et al., 2020). This discrepancy is resultant from the different sequencing and bioinformatics pipelines used. The majority of 16S sequencing studies assessed different subpopulations of schizophrenia and thus are difficult to compare with each other. Combined with reanalyzed results, there is evidence supporting *Lactobacillus* and *Bifidobacterium* dysregulation in schizophrenia, as well as potential changes in tryptophan and SCFA-related GBMs (see Table 2).

3.6.4 Pediatric Acute-onset Neuropsychiatric Syndrome and Pediatric Autoimmune Neuropsychiatric Disorder Associated with Streptococcal Infection

3.6.4.1 Studies Where Raw Microbiome Data Was Reanalyzed

One 16S sequencing study was reanalyzed but no relevant bacterial genera or differences in GBMs were found (Quagliariello et al., 2018).

3.6.5 Rett's Syndrome

3.6.5.1 Studies Where Raw Microbiome Data Was Reanalyzed

A small descriptive study was reanalyzed (Borghi and Vignoli, 2019) but no genus-level differences were found between Rett's Syndrome and age-matched controls. However, both faecal isobutyrate and isovalerate were increased in Rett's syndrome (see Table 2).

3.6.5.2 Studies Where Raw Microbiome Data Was Not Reanalyzed

Strati et al. (2016) found an increased abundance in faecal isobutyrate, isovalerate and propionate (see Table 2). However, after controlling for constipation and disease severity, no bacteria were differentially abundant within the disease group. There may be host-genotype microbiota associations involved in influencing SCFA production or absorption.

3.7 Epilepsy

3.7.1 Studies Where Raw Microbiome Data Was Reanalyzed

In the dataset from Lindefeldt et al. (2019), twelve children with epilepsy provided two faecal samples, one before commencing the ketogenic diet and three months afterwards (see Table 3). While no age-matched controls were included within the study, the children's parents served as a healthy control (Lindefeldt et al., 2019). The dataset was reanalyzed through the WGS pipeline described in **Section 2.6**. We found that the ketogenic diet increased abundance in L-Tryptophan biosynthesis pathways ($p_{adj} < 0.1$, effect = 0.9; 95% CI: [-1.07; 10.67]) and S-Adenosyl Methionine biosynthesis ($p_{adj} < 0.1$, effect = 0.63; 95% CI: [-1.89; 8.86]) (Lindefeldt et al., 2019). Though the study's authors found a reduction in relative abundance of *Bifidobacterium* their data was not treated compositionally (see 3.4.3 for limitations of non-compositional data approaches) (Lindefeldt et al., 2019).

Another reanalyzed study looked at individuals co-morbid with cerebral palsy and epilepsy (Huang et al., 2019a). While over 20 differentially abundant bacteria had an absolute effect size >0.65, there were no differences across GBM abundance (Huang et al., 2019a). From a bioinformatics perspective, this indicates that large genus-level differences do not always change the overall abundance of GBMs.

3.7.2 Studies Where Raw Microbiome Data Was Not Reanalyzed

Four 16S studies used different types of cohorts and comparisons (see Table 3). (Xie et al., 2017) assessed microbiome differences between epileptic infants and healthy controls. Two studies compared individuals with drug-responsive epilepsy, to drug-resistant epilepsy and controls from the same family (Peng et al., 2018, Zhang et al., 2018b). Peng et al. (2018) looked at the efficacy of dietary intervention while Safak et al. (2020) focused on idiopathic focal epilepsy. Another study compared same-family controls to epileptic individuals finding many genera-level differences (Liu et al., 2020a). As a result, we cannot make any definitive conclusions about GBM-related bacteria or signatures within epilepsy and epilepsy-responses to dietary or pharmacologic treatment. Interactions between diet, epilepsy, epileptic medication and the microbiome remain unclear.

3.8 Neurodegenerative Disease

3.8.1 Alzheimer's Disease

3.8.1.1 Studies Where Raw Microbiome Data Was Reanalyzed

Most of the raw sequences from one 16S study could not be aligned to ASVs (Liu et al., 2019b). In the other 16S sequencing study (Li et al., 2019a), a reduction was detected in the SCFA-producing *Ruminoclostridium 5* ($p_{adj} < 0.01$, effect = -0.67; 95% CI: [-8.52, 1.59] while the following SCFA-specific GBMs were upregulated when comparing Alzheimer's

Disease (AD) to healthy controls: isovaleric acid synthesis II ($p_{adj} < 0.1$, effect = 0.42; 95% CI: [-2.11; 5.62]), butyrate synthesis I ($p_{adj} < 0.1$, effect = 0.44; 95% CI: [-2.33; 6.25]), butyrate synthesis II ($p_{adj} < 0.1$, effect = 0.52; 95% CI: [-3.60; 4.79]), and acetate synthesis III ($p_{adj} < 0.1$, effect = 0.50; 95% CI: [-2.04; 5.92]) (Li et al., 2019a).

Though no differentially abundant microbes were identified when comparing individuals with mild cognitive impairment (MCI) to healthy controls, several SCFA and tryptophan related GBMs were increased: isovaleric acid synthesis II ($p_{adj} < 0.01$, effect = 0.43; 95% CI: [-2.48; 5.98]), butyrate synthesis I ($p_{adj} < 0.1$, effect = 0.44; 95% CI: [-2.33; 6.25]), acetate synthesis I ($p_{adj} < 0.01$, effect = 0.58; 95% CI: [-1.93; 5.86]), acetate synthesis II ($p_{adj} < 0.1$, effect = 0.47; 95% CI: [-1.81; 5.33]), acetate synthesis III ($p_{adj} < 0.01$, effect = 0.64; 95% CI: [-1.52; 6.26]), tryptophan synthesis ($p_{adj} < 0.1$, effect = 0.48; 95% CI: [-1.94; 6.71]), quinolinic acid synthesis ($p_{adj} < 0.01$, effect = 0.49; 95% CI: [-1.93; 6.43]), quinolinic acid degradation ($p_{adj} < 0.01$, effect = 0.56; 95% CI: [-1.93; 6.43]) (Li et al., 2019a).

Additionally, several other GBMs were differentially abundant in the MCI and AD groups compared to the controls, indicating an increase in overall pathways promoting excitatory neuronal signalling (Li et al., 2019a) (see Table 4).

3.8.1.2 Studies Where Raw Microbiome Data Was Not Reanalyzed

Bacteroides is found differentially abundant across the two 16S and one WGS study comparing AD to controls. However, it is found to be increased in two of these studies – one of which involves WGS (Haran et al., 2019, Vogt et al., 2017), and decreased in the third study (Zhuang et al., 2018). Additionally, *Alistipes* abundance was increased in the AD individuals in two of these studies (Haran et al., 2019, Vogt et al., 2017). Though findings from one reanalyzed dataset are strong (Li et al., 2019a), additional measures of metadata are

needed to disentangle GBM differences in AD or mild-cognitive impairment from sex, diet and age.

3.8.2 Multiple Systems Atrophy

3.8.2.1 Studies Where Raw Microbiome Data Was Not Reanalyzed

Three studies analyzing the gut microbial composition of individuals with Multiple Systems Atrophy (MSA) have been conducted (see Table 4), with two of these studies finding genus-level differences in bacterial abundance (Du et al., 2019, Engen et al., 2017, Tan et al., 2018). However, none of the genera are found differentially abundant across these two studies (Tan et al., 2018, Du et al., 2019). Interestingly, Tan et al. (2018) also found a reduction in faecal acetate, propionate and butyrate in their disease cohort. This data together suggests that MSA may alter the production or absorption of SCFAs, though it is unclear if individual microbial genera are involved.

3.8.3 Amyotrophic Lateral Sclerosis

3.8.3.1 Studies Where Raw Microbiome Data Was Not Reanalyzed

There were no consistent findings across three studies (Zhai et al., 2019a, Brenner et al., 2018, Mazzini et al., 2018, Zeng et al., 2020, Nicholson et al., 2020, Ngo et al., 2020) (see Table 4). (Blacher et al., 2019) did not find any significant microbes using a WGS approach but found an overall reduction in tryptophan metabolism-related genes in ALS compared with controls. Two other WGS studies did however find differentially abundant microbes, involved in SCFA and tryptophan metabolism (2020, Zeng et al., 2020). Indeed, there were alterations in serum tryptophan and nicotinamide metabolites suggesting the serum metabolome may have been altered by the gut microbiota (Blacher et al., 2019).

3.8.4 Parkinson’s Disease

3.8.4.1 Studies Where Raw Microbiome Data Was Reanalyzed

Surprisingly, across six studies (one WGS, five 16S) only 1 ASV was found differentially abundant (Bedarf et al., 2017, Heintz-Buschart et al., 2018, Aho et al., 2019, Pietrucci et al., 2019, Qian et al., 2018, Weis et al., 2019) (see Table 4). When stratifying 16S sequencing data from Weis et al. (2019) by gastrointestinal symptoms and L-DOPA dosage, there was one differentially abundant genus. Parkinson's disease (PD) individuals taking a L-DOPA dose of <300mg/day had a lower abundance of *Lactobacillus* than controls (effect = 0.83; 95% CI: [-2.10, 8.07]) (Weis et al., 2019). No GBMs related to SCFAs, tryptophan or bile-acid modifying bacteria were identified.

3.8.4.2 Studies Where Raw Microbiome Data Was Not Reanalyzed

Hill-Burns et al. (2017) found significant differences in bacterial abundance between PD and controls after controlling for covariates. They reported an increased abundance of *Bifidobacterium*, *Lactobacillus*, *Akkermansia* and *Roseburia* (Hill-Burns et al., 2017). Ren et al. (2020) used a generalized linear model to control for sex, age, body mass index and education and did not find any changes in these four genera in their cohort. Instead, they reported a reduction in *Ruminococcus* and *Blautia* in their PD group which had not experienced MCI (Ren et al., 2020). However, three other studies did find an increased abundance of *Lactobacillus* in PD (Petrov et al., 2017, Barichella et al., 2019, Cirstea et al., 2020). Additionally, three other 16S studies reported a reduction in *Roseburia* compared to their control cohorts (Barichella et al., 2019, Keshavarzian et al., 2015, Cristea et al., 2020). Four other 16S studies also found an increased abundance of *Akkermansia* (Keshavarzian et al., 2015, Vidal-Martinez et al., 2020, Li et al., 2019b, Zhang et al., 2020a) and *Bifidobacterium* (Petrov et al., 2017, Cirstea et al., 2020, Barichella et al., 2019, Tan et al., 2020). Interestingly, Lin et al. (2019) found *Akkermansia* was increased in the tremor PD

subtype when accounting for age, sex and diet. Finally, Unger et al. (2016) reported reductions in faecal acetate, butyrate and propionate. However, many of these studies used Greengenes, rarefaction and relative abundance. It is nonetheless fascinating that differences in microbial abundance at the genus-level were found in almost every PD study, especially those comparing different subtypes. It is unclear if the effect sizes in these studies are robust when data is analyzed in a compositional manner (see Table 4). If effect sizes are small, there is a higher probability that the effect sizes will not replicate in other studies. This may either obscure real differences in the PD microbiota or provide false positives.

3.9 Addiction and Substance Use

3.9.1 Alcohol

3.9.1.1 Studies Where Raw Microbiome Data Was Reanalyzed

Stadlbauer et al. (2019) collected faecal samples from participants before an acute 2mL alcohol binge, and one day afterwards in 15 healthy participants. We did not find any significant effects on the microbiota composition or GBMs in this dataset (Stadlbauer et al., 2019). The alcohol binge was likely too mild to exert any robust effects.

Another dataset focused on the long-term effects of alcohol-dependence on the gut microbiota (Bjorkhaug et al., 2019). Bacteria involved in SCFA and tryptophan metabolism were altered in the alcohol-dependent cohort (Bjorkhaug et al., 2019). Specifically, *Ruminococcus 2* abundance was increased ($p_{adj} < 0.1$, effect = 0.72, 95% CI: [-2.91; 6.75]) and a reduction in *Ruminoclostridium 9* ($p_{adj} < 0.001$, effect = - 0.99, 95% CI: [-7.99; 1.00]) (Bjorkhaug et al., 2019). Though SCFA-related GBMs were not altered, the tryptophan degradation module was reduced in alcohol-dependent subjects ($p_{adj} < 0.1$, effect = - 0.46, 95% CI: [-5.78; 2.47]) (Bjorkhaug et al., 2019). In addition, other GBMs suggested increased

GABA synthesis as well as a reduction in g-hydroxybutyrate and dopamine degradation (Bjorkhaug et al., 2019).

3.9.1.2 Studies Where Raw Microbiome Data Was Not Reanalyzed

Due to differences in cohorts, it is challenging to draw conclusions from other alcohol-related studies (see Table 5). Briefly, a WGS investigation using the SOLiD sequencing platform compared the microbiota of individuals with alcoholic dependence syndrome, alcoholic liver cirrhosis and control (Dubinkina et al., 2017). Dubinkina et al. (2017) reported an increased abundance of *Lactobacillus salivarius* in alcohol-dependent subjects. These results are not easily reconciled, with findings from Leclercq et al. 2014, where a three-week detoxification increased *Lactobacillus spp.* in alcohol-dependent subjects. Two other studies involving alcohol-dependence and alcohol overconsumption did not find any changes in *Lactobacillus* abundance (Tsuruya et al., 2016, Bjorkhaug et al., 2020). Meanwhile, a study of the microbiota and drinking habits of 212 twin pairs only found a reduction in *Roseburia* abundance associated with alcohol consumption, after correcting for heritability (Seo et al., 2020). Interestingly, one study found that *Haemophilia* abundance was associated with drinking only (Lin et al., 2020) while other genera associated with both drinking and smoking (*Bacteroides, Phascolarctobacteirum, Ruminococcus UCG-002, Ruminococcus UCG-003, Ruminoclostridium-9)*. Many of these genera are associated with SCFA/tryptophan metabolism.

Future studies must account for factors such as diet, heritability and drinking frequency to demystify the effects of alcohol on gut microbiota composition and metabolism.

3.9.2 Smoking and Tobacco Use

3.9.2.1 Studies Where Raw Microbiome Data Was Reanalyzed

Stewart et al. (2018) collected faecal samples from tobacco smokers, electronic cigarette users and controls (see Table 5). Though we did not uncover genus-level differences in microbial abundance, we found an increase in the tryptophan degradation module ($p_{adj} < 0.1$, effect = 0.84,, 95% CI: [-0.97; 8.52]) and the propionate synthesis III module ($p_{adj} < 0.1$, effect = - 0.80, 95% CI: [-9.86; 1.45])(Stewart et al., 2018).

3.9.2.2 Studies Where Raw Microbiome Data Was Not Reanalyzed

Among other studies assessing the microbiota composition of smokers, only a qPCR study identified microbial changes (Ishaq et al., 2017). Combined with results in 3.5.1.1, tobacco smoking may alter the overall metabolism of the gut microbiota.

3.9.3 Addiction and Recreational Drug Use

3.9.3.1 Studies Where Raw Microbiome Data Was Reanalyzed

There were no differentially abundant microbial or GBM-related associations within the (Barengolts et al., 2018) dataset of men characterized with a high-disease burden and opioid use.

3.9.3.2 Studies Where Raw Microbiome Data Was Not Reanalyzed

While Fulcher et al. (2018) reported specific changes in microbial abundance with many recreational drugs, Xu et al. (2017) did not find any differences between users and non-users when controlling for age and sex (see Table 5). Panee et al. (2018) recently found that *Prevotella* abundance in marijuana users was positively associated with cognitive functions. More studies investigating recreational drug use, controlling for age, sex and type of drug, must be conducted to deconvolute any potential changes.

3.10 Multiple Sclerosis and Demyelinating Diseases

3.10.1 Studies Where Raw Microbiome Data Was Reanalyzed

Across two 16S sequencing datasets, no differences in microbial abundance or GBMs related to SCFA, tryptophan or bile acid metabolism were identified (Miyake et al., 2015, Jangi et al., 2016) (see Table 6). When comparing individuals with neuromyelitis optica spectrum disorder (NMOSD) to control samples in the (Gong et al., 2019) dataset, there was a reduced abundance of *Streptococcus* in diseased individuals ($p_{adj} < 0.001$, effect = - 0.74, 95% CI: [- 6.40; 1.53]). The researchers also reported an overall reduction of faecal SCFAs and associations between acetate, butyrate and disease severity (Gong et al., 2019).

3.10.2 Studies Where Raw Microbiome Data Was Not Reanalyzed

There were no consistent effects across Multiple Sclerosis (MS) studies. Using WGS Ventura et al. (2019) found *Clostridium* increased across individuals with MS with Caucasian, Hispanic and African American ethnicities. Interestingly, (2017) compared faecal samples from 34 discordant twin pairs and did not find-any genus-level compositional changes when accounting for heritability. Other recent studies found a few dysregulated genera but did not take ethnicity into account (Ling et al., 2020b, Kishikawa et al., 2020).

A recent investigation by Reynders et al. (2020) found associations between multiple bacterial genera and clinical subtypes of MS. Another study also found differences in SCFA-producing genera between different subtypes of multiple sclerosis and controls (Saresella et al., 2020). Zeng et al. (2019) compared microbial and faecal SCFA abundance between MS, NMOSD and controls finding a reduction in acetate, butyrate and propionate when comparing either MS or NMOSD to controls. Interestingly, they also reported that faecal acetate and propionate are reduced in NMOSD individuals compared to those with MS (Zeng et al., 2019).

Together, these results suggest that measurements of faecal SCFAs and stratification by clinical subtype are crucial for uncovering any potential robust changes in bacterial abundance or GBMs.

3.11 Pain-Related Disorders

3.11.1 Fibromyalgia

3.11.1.1 Studies Where Raw Microbiome Data Was Reanalyzed

In the 16S dataset collected by Minerbi et al. (2019), only one bacterial genus was associated with the disease state (see Table 7). The abundance of *Sutterella* was increased in fibromylagia compared to controls living in the same address as the patient (p_{adj} <0.1, effect = 0.66; 95% CI: [-0.43; 0.92]) (Minerbi et al., 2019). However, no differences were found when comparing to overall controls in both this 16S dataset as well as in the samples from (Clos-Garcia et al., 2019).

3.11.1.2 Studies Where Raw Microbiome Data Was Not Reanalyzed

One study found several SCFA-associated bacteria differentially abundant between individuals with fibromyalgia and unrelated controls, corresponding to changes in serum SCFA concentrations (Minerbi et al., 2019). Compared to the 16S data produced from this cohort (discussed in **3.7.1.1**), WGS provides species level resolution and identifies many more differentially abundant microbes (Minerbi et al., 2019).

3.11.2 Irritable-Bowel Syndromes

3.11.2.1 Studies Where Raw Microbiome Data Was Not Reanalyzed

Ten 16S sequencing studies to date, investigated the associations between psychological well-being, IBS and the microbiota (see Table 7). While one study found *Bacteroides* abundance

positively associated with perceived stress (Peter et al., 2018b), while Jeffery et al. (2012) reported that it was reduced in Irritable-Bowel Syndromes (IBS) individuals compared with controls. Since no controls were included in the study by (Peter et al., 2018b), these results are not necessarily contradictory. Since many of these studies involved different probiotic, prebiotic and faecal microbiota transplant interventions and a lack of controls, the results of these studies could not be compared. Several studies do report changes in SCFA and tryptophan associated bacteria, with Labus et al. (2019) finding that *Clostridium* XIVa and *Coprococcus* associated with differences in brain connectivity between IBS and controls.

While the overall changes in microbiota composition are unclear, there is some evidence that manipulating its composition may improve various psychological aspects of IBS pathology.

3.11.3 Other Pain-Related Disorders

3.11.3.1 Studies Where Raw Microbiome Data Was Not Reanalyzed

A recent WGS study (see Table 7) reported the increased abundance of the kynurenine synthesis GBM and a reduction in quinolinic acid degradation in elderly women with migraines compared to healthy age-matched control (Chen et al., 2019). In addition, *Faecalibacterium prausnitzii* and *Bifidobacterium adolescentis* were reduced in the women who experienced migraines (Chen et al., 2019). However, this was the only microbiota study assessing migraines to date. Another conducted on a cohort with *myalgic encephalomyelitis/chronic fatigue* syndrome found negative correlations between *Faecalibacterium* and total sleep awakening (Kitami et al., 2020). Meanwhile, a study of chronic widespread pain patients found a decrease in *Coprococcus comes* abundance (Freidin et al., 2020)

3.12 Eating Disorders

3.12.1 Obesity

3.12.1.1 Studies Where Raw Microbiome Data Was Not Reanalyzed

Across studies of obesity where psychological or other brain measures were recorded, no genus-level associations were reported (see Table 8). However, one study used a commercially available dysbiosis test (GA-Map Dysbiosis) to compare the morbidly obese microbiomes to controls (Farup and Valeur, 2018). *Bacteroides*, *Prevotella* and faecal SCFAs were negatively associated with the WHO-5 Wellbeing Index Score within the obese group (Farup and Valeur, 2018). In addition, other SCFA and tryptophan modulating microbes, *Faecalibacterium prausnitzii* and *Dorea* were positively associated with this measure (Farup and Valeur, 2018). Recent studies associate microbiome, brain connectivity and structure as well as food craving (Dong et al., 2020b, Dong et al., 2020a). Another study finds alterations in aromatic amino acid metabolism in obesity impairing short-term memory (Arnoriaga-Rodríguez et al., 2020). There is insufficient evidence to conclude microbial-derived metabolites associate with psychological measures in obesity.

3.12.2 Anorexia Nervosa

3.12.2.1 Studies Where Raw Microbiome Data Was Reanalyzed

Using the raw dataset from (Borgo et al., 2017), we were unable to find any significant differences in microbial abundance or GBMs between anorexic individuals and controls. Another dataset (see Table 8) with a higher sample size however, found increased abundance in isovaleric acid synthesis I ($p_{adj} < 0.1$; effect = 0.44, 95% CI: [-2.80, 5.07]), quinolinic acid synthesis ($p_{adj} < 0.1$; effect = 0.48, 95% CI: [-2.13, 5.35]), and quinolinic acid degradation ($p_{adj} < 0.01$; effect = 0.42, 95% CI: [-2.33, 4.80]) (Mack et al., 2016). After gaining weight and subsequent release from the hospital, individuals with anorexia had a reduction in butyrate synthesis II compared to controls ($p_{adj} < 0.01$; effect = -0.43, 95% CI: [-4.88, 2.55])

(Mack et al., 2016). Importantly, ClpB was also elevated at baseline admission, compared to controls ($p_{adj} < 0.1$; effect = 0.43, 95% CI: [-2.30, 4.98]) (Mack et al., 2016). This *Escherichia coli* produced protein is an alpha-melanocortinin stimulating hormone mimetic, known to reduce appetite in mice (Tennoune et al., 2014).

3.12.2.2 Studies Where Raw Microbiome Data Was Not Reanalyzed

There were no consistent findings across microbial genera in six other studies (Morkl et al., 2017, Morita et al., 2015, Kleiman et al., 2015, Armougom et al., 2009, Schulz et al., 2020, Monteleone et al., 2020) (see Table 8). With the release of new tools interrogating GBM abundance may be important for identifying potential changes within the anorexic microbiota, especially ClpB production. All evidence considered, there is strong evidence for the involvement of microbially-derived ClpB but it's unclear if the microbes involved in its production also impact SCFA, tryptophan and bile-acid metabolism.

3.13 Neurovascular Disease

3.13.1 Studies Where Raw Microbiome Data Was Not Reanalyzed

Many preclinical studies identified butyrate as a potential neuroprotective agent for ischemia (Akhoundzadeh et al., 2018, Lee et al., 2020a, Sadler et al., 2020, Singh et al., 2018, Sun et al., 2016a). There are fewer studies assessing changes in the gut microbial composition responses to stroke in humans. One study compared the gut microbiota of infants who received hypothermia treatment for hypoxic ischemic encephalopathy (Watkins et al., 2017). Indeed compared to control infants, those undergoing treatment for ischemia showed a reduction of *Bacteroides* abundance (Watkins et al., 2017). Wang et al. (2018) assessed gut microbial composition in individuals after cerebral infarction but did not find any genus-level abundance changes compared to controls. The butyrate and tryptophan metabolism associated bacterial genera *Bacteroides, Parabacteroides, Akkermansia, Prevotella* and

Faecalibacterium were reduced after cerebral infarction when compared to controls (Ji et al., 2017).

Studies where participants were stratified by type of stroke and stroke severity uncovered more compositional differences that may impact SCFA, bile acid and tryptophan metabolism. Liu et al. (2020a) found many such genera which were altered when comparing participants who suffered post-stroke cognitive impairment with controls. Another study compared individuals post-stroke with no cognitive impairment along with those co-morbid with depression and cognitive impairment, finding few differences (Ling et al., 2020a). Another study stratified individuals with ischemic stroke by severity and found *Enterobacter* was reduced in severe ischemic stroke compared to mild stroke. Across two different studies comparing ischemic stroke to controls, *Akkermansia* was differentially abundant (Ji et al., 2017, Li et al., 2019c). However, in one study it was more abundant in the ischemic stroke (Li et al., 2019c) while it was reduced in the other study, though it only two individuals in the ischemic stroke cohort (Ji et al., 2017).

Polster at al. (2020) found robust differences and correlations within a large sample (N = 122) of individuals with cavernous angioma using a combination of 16S and WGS techniques. Compared with controls from the human microbiome project, individuals in the disease group showed an increased abundance of *Bacteroides thetaomicron* and *Odoribacter sphlancus* along with a reduction in *Bifidobacterium adolescentis* and *Faecalibacterium prausnitzii* (Polster et al., 2020). They found evidence that these changes in abundance promoted gut inflammation and increased lipopolysaccharide (LPS) synthesis pathways (Polster, 2020). Indeed, this robust methodology even identified differentially abundant species by cavernous angioma subtype and severity (Polster et al., 2020).

Exciting findings from (Polster et al., 2020) warrant more investigation into gut-brain communication after other neurovascular insults such as stroke. See Table 9 for more detail.

3.14 Stress and Psychiatric Disorders

3.14.1 Stress

3.14.1.1 Studies Where Raw Microbiome Data Was Not Reanalyzed

The effect of stress on the human microbiota is difficult to study, often involving associating lifetime stress metrics with microbiota composition (see Table 10). One 16S sequencing study identified that pregnant women that experienced more than 2 adverse childhood events had an increased abundance of *Prevotella* (Hantsoo et al., 2019). Another study of 75 women with pregnancy-related anxiety was unable to find genus-level associations between maternal anxiety and the infant meconium (Hu et al., 2019a). Interestingly, Naude et al. (2020) found infants born to mothers exposed to intimate partner violence has an increased abundance of *Citrobacter* and *Weisella*. (Carson et al., 2018) showed that *Fusobacterium* abundance was increased with stress in participants that identified as Black but not amongst other demographics, indicating host-mediated contributions to the microbiome stress responses. Even though there are clear links between stress and the microbiome (see for reviews (Cussotto et al., 2018a, Dinan and Cryan, 2012, Foster and McVey Neufeld, 2013, Liu, 2017) for extensive reviews of the subject), these studies indicate how much metadata is needed to properly stratify participants and identify some of these changes.

Another strategy focused on providing probiotic interventions, later comparing microbiome and stress metrics between individuals receiving controls or a placebo. Of three such randomized control trials, two found genus-level associations with psychological stress measures (Nishida et al., 2017, Nishida et al., 2019, Soldi et al., 2019). One study administered *Lactobacillus gasseri* CP2305 which reduced the magnitude of *Bifidobacterial* reduction after the stressor and increased faecal valerate concentrations (Nishida et al., 2019). Another study using the same probiotic intervention found different magnitudes of changes in

the abundances of *Corynebacterium* in addition to improved sleep quality and a reduction in stress symptoms in female participants (Nishida et al., 2017).

3.14.2 Posttraumatic Stress Disorder

3.14.2.1 Studies Where Raw Microbiome Data Was Not Reanalyzed

Two studies have assessed the impact of post-traumatic stress disorder on the gut microbiota. (Hemmings et al., 2017) did not find any differentially abundant genera when using a trauma-exposed comparison group (does not meet threshold for posttraumatic stress disorder). Bajaj et al. (2019) used a conventional control cohort, finding differences even after accounting for hepatic encephalopathy. In individuals without hepatic encephalopthay, the posttraumatic stress disorder individuals showed an increased abundance of *Streptococcus* and a reduction in *Acidaminococcus*, *Ruminococcus, Roseburia, Anaerostipes, Clostridium XIVA*a and *Pseudoflavonicbacter* compared to controls (Bajaj et al., 2019). In the subset of individuals with hepatic encephalopathy, posttraumatic stress disorder individuals only showed a reduction in *Subdoligranulum* (Bajaj et al., 2019).

3.14.3 Bipolar Disorder

3.14.3.1 Studies Where Raw Microbiome Data Was Not Reanalyzed

There were no consistent microbial changes observed across eleven 16S and WGS studies of bipolar disorder (see Table 10). In the two WGS studies, the SCFAs and tryptophan associated genera *Streptococcus, Clostridium, Oscillibacter* and *Bifidobacterum* were increased in bipolar individuals compared with controls (Rong et al., 2019, Lai et al., 2021). Due to differences in methodology and data analysis, other studies did not see these same differences in abundance. Evans et al. (2017) only reported reductions in *Facealibacterium* in bipolar individuals but did associate its abundance with sleep and depressive symptoms.

Contrary to these findings, Painold et al. (2019) reported increased *Faecalibacterium* abundance in bipolar disorder. Two other studies noted increased abundance of *Bacteroides* in bipolar disorder but their other findings differed (Hu et al., 2019b, Zheng et al., 2020b). Interestingly some of the studies did not find any genus-level differences in microbial composition (Coello et al., 2019, Vinberg et al., 2019, McIntyre et al., 2019). Coello et al. (2019) found that all the differences in abundance that they observed were explained by sex effects, heritability and smoking.

3.14.4 Depression and Anxiety

3.14.4.1 Studies Where Raw Microbiome Data Was Not Reanalyzed

Multiple studies have investigated the explanatory power of the gut microbiota in anxiety and depression (see Table 10). Only three 16S or WGS sequencing studies did not find any significant differences in major depressive disorder (MDD) compared to controls at the genus-level (Paulsen et al., 2017, Bharwani et al., 2020, Naseribafrouei et al., 2014). (Jiang et al., 2020) compared the gut microbiota of individuals currently undergoing a depressive episode to controls, finding increased abundance of *Akkermansia, Veillonella, Ruminococcus gnavus* and reductions in *Fusicatenibacter, Sutterella, Dialister*. A previous study (Jiang et al., 2015), reported a reduction in *Dialister* during active MDD. Other studies did not find any similarities with Jiang et al. (2020), thus it is unclear if the gut microbiota changes throughout depression or if these differences are a result of different methodologies.

Few other similarities in gut microbial signatures were reported across other studies. Two studies did report an increased abundance of the SCFA and tryptophan metabolism-associated microbe *Collinsella* in their respective depressed cohorts (Stevens et al., 2018, Zheng et al., 2016). Three studies also reported an increased abundance of *Blautia* in MDD (Huang et al., 2018, Jiang et al., 2015, Yang et al., 2020). When predicting clinical outcomes

from baseline microbiome data, researchers did not find that the microbiota in MDD predicted clinical response (Liśkiewicz et al., 2021). Nonetheless, they did find *Paraprevotella* strongly correlated with the Hamilton Depression Rating Scale-24 Item metric (Liśkiewicz et al., 2021)

Madan et al. (2020) compared rates of remission in psychiatric inpatients and aimed to identify microbial genera that predicted readmission or remission from severe depression or anxiety. *Coprococcus catus* was associated with moderate anxiety at admission and was reduced in individuals that had lower rates of remission from anxiety or depression (Madan et al., 2020). Interestingly, the *Coprococcus* genera was found increased in the depressed cohort by Huang et al. (2018). Other studies did report however, a reduction in *Coprococcus* abundance in depression (Valles-Colomer et al., 2019, Liu et al., 2016).

Valles-Colomer et al. (2019) used compositional data methods as well as large cohorts in their study, where they found *Coprococcus* as well as *Faecalibacterium* associated with a higher quality of life and that *Dialister* and *Coprococcus* were depleted in depression. Interestingly, the *Dialister* finding is consistent with other studies (Jiang et al., 2015, Jiang et al., 2020). Indeed, other studies also found negative correlations between *Facalibacterium* and anxiety or depression (Jiang et al., 2015, Jiang et al., 2018a, Stevens et al., 2018). The findings involving *Dialister* and *Faecalibacterium* are striking because they persisted despite non-compositional data analysis in other studies. A recent systematic-review also found a reduction in short-chain fatty acid producing bacteria such as *Faecalibacterium* across studies of anxiety and depression (Simpson et al., 2020).

3.15 Limitations of Existing Studies

There are many challenges that prevent researchers from drawing causal conclusions from their datasets beyond the technical and bioinformatics limitations discussed in Section 3.4,

especially in observational human studies (Ma et al., 2019c, Lynch et al., 2020, Koh and Bäckhed, 2020, Walter et al., 2020, Ma, 2020). See Table 11 for a description of these common limitations and available tools to address them. In addition to those limitations, even functional analysis of WGS data does not provide direct information about the proteomics or metabolomics within the gut community.

3.15.1 Sources of Inter-Individual Variance

One of the greatest challenges with human microbiota studies is making inferences about the composition of the colonic microbiota from faeces. There are known differences between the faecal and caecal microbiota composition in humans along with spatial variation across the gastrointestinal tract (Gevers et al., 2014, Lavelle et al., 2015). Finding healthy volunteers willing to provide one or multiple biopsies for a microbiota study is challenging. In addition, it's difficult to determine whether certain microbes are overrepresented in the faeces compared to others. The overall microbial load, though seldom measured, is an important determinant of microbiota composition (Vandeputte et al., 2017). It is also recognized that microbiota composition changes day-to-day in response to diet, circadian rhythms and sex hormones among other confounds (Jaggar et al., 2020, Johnson et al., 2019, Nobs et al., 2019, Markle et al., 2013).

In addition to long-term dietary patterns (Wu et al., 2011), food alters the microbiota on a smaller timescale as well. (Johnson et al., 2019) assessed day-to-day variations within microbiota composition by collecting detailed daily food diaries and daily faecal samples for seventeen days. While the microbiota composition was correlated with food preferences, it was not associated with individual nutrients (Johnson et al., 2019). Subject had different responses to the same types of foods, which could affect the microbiome for up to two days after consumption (Johnson et al., 2019). Meanwhile (Berry et al., 2020) reported that even

twins had different metabolic responses. Interestingly, the microbial composition of individuals explained more variation in postprandial lipemia than meal macronutrients (Berry et al., 2020). Metabolic disease and obesity is common amongst sufferers of anxiety and depression (Rajan and Menon, 2017) while food pickiness is common in ASD (Kral et al., 2013). In addition, microbiota correlates with both the diet and other peripheral health measures in elderly individuals (Claesson et al., 2012). Many neuropsychiatric disorders involve alterations in food preference (Greenwood et al., 2005, Yau and Potenza, 2013, Folley and Park, 2010). To detangle interindividual differences in dietary responses from microbial-brain-disease associations, multi-timepoint sampling and dietary records must be incorporated. Other considerations when collecting dietary-related data or integrating dietary interventions include study design, control selection, measuring subject compliance, diet-measurement error, participant bias and method of collecting dietary information (Swann et al., 2020, Willett, 2012).

Another large confound in many of these studies is the medication that individuals may take for their disease or disorder, as well as recreational alcohol and drug use (Maier et al., 2018, Fulcher et al., 2018, Cussotto et al., 2018b, Vich Vila et al., 2020, Forslund et al., 2015, Vieira-Silva et al., 2020, Barengolts et al., 2018, Peterfreund et al., 2012, Zhernakova et al., 2016, Falony et al., 2016, Panee et al., 2018, Bjorkhaug et al., 2019, Dubinkina et al., 2017, Seo et al., 2020, Tsuruya et al., 2016, Coello et al., 2019, Ishaq et al., 2017, Stewart et al., 2018).

In addition to diet, and drugs, sex hormones plays an important component in many of these neuropsychiatric disorders, the microbiota is also able to participate in 17-β-estradiol degradation (Valles-Colomer et al., 2019), and potentially other pathways (Fuhrman et al., 2014, Shin et al., 2019). (Shin et al., 2019) reported that the faecal abundance of multiple bacterial genera was associated with serum levels of testosterone and oestrogen in humans.

There are also limitations in diagnosing and subtyping different types of diseases and disorders. There are a wide spectrum of symptoms and conditions associated with the disorders mentioned within the study. The heterogenous nature of many disorders and conditions such as ASD, anxiety, depression and stress serve as large confounders (Feczko et al., 2019). Much of the metadata does not detail specific symptoms or subtypes of a diagnosis or a disorder. Having this information would allow for a higher resolution analysis of gut-brain interactions.

Even when accounting for host-genotype effects with larger cohorts, accounting for sex, body mass index and genotype, it is difficult to interpret microbiome-host associations without identifying the driving influence in such an interaction (Hughes et al., 2020). A preprint by (Rothschild et al., 2020) suggests that large cohort studies may require thousands of participants on order to reach 20% explanatory power for a certain host-trait with specific microbiota-associated metrics (Shannon diversity, relative microbial abundance). The collection of metadata is important to allow for a better comparison between studies and to identify differentially abundant microbes arising from confounding variables.

3.15.2 Reporting of Effect Sizes and Confidence Intervals

The magnitude of the effect size is also important to consider, as the microbiome is a dynamic system, and effect size measurements prove more informative than p-values alone though they are seldom reported (Sullivan and Feinn, 2012). In addition, tools involving linear discriminant analysis for identifying differentially expressed microbes and their effect sizes do not consider the compositional nature of microbiome data. Unfortunately, most studies did not report effect sizes.

4.0 Conclusion

Though the evidence for the involvement of individual microbial genera or GBMs related to SCFA, tryptophan or bile acid metabolism within humans is weak, we found several salient findings and features within these datasets. GBMs allow us to search metagenomic data for specific neuroactive metabolic pathways leading to mechanistic insights. Within a very short time after their release, several human (Butler et al., 2020; Chen et al., 2019; Tomizawa et al., 2019; Zhu et al., 2020) and preclinical studies have taken advantage of their descriptive properties (O'Connor et al., 2020; Van de Wuow et al. 2020).

Many studies involving healthy humans are currently investigating associations between temperament, cognition and personality across the lifespan. While these studies may continue to find various associations, without proper compositional data analysis these associations are likely spurious and biased towards negative correlations (Gloor et al., 2017). Even with compositional data methods, finding explanatory genera or ASVs may require thousands of participants to power the study (Hughes et al., 2020).

Neurodevelopmental disorders accounted for many of the human-microbiome-brain studies. While a WGS study suggests reductions in the KO abundance of dopamine pathways in ADHD (Wang et al., 2020b), it is hindered by a lack of compositional analysis. Other studies suggested correlations between *Faecalibacterium*, *Ruminococcus* and *Ruminoclostridium 9* with symptoms of ADHD (Jiang et al., 2018b, Szopinska-Tokov et al., 2020). These microbial genera may alter SCFA or tryptophan related pathways but must be further validated through metabolomic methods. Across dozens of ASD studies, very few consistencies were found across these studies. When reanalyzing raw microbiome data, very few differentially regulated microbes or GBMs reached the significance and effect size thresholds. In the dataset from Son et al. (2015), there were no differences in microbial composition within a sample of twins discordant for ASD. However, some of these studies

suggested the important interplay between diet and faecal SCFAs within ASD (Berding and Donovan, 2019, Liu et al., 2019d, Wang et al., 2020e). Meanwhile there is moderate level of evidence that *Lactobacillus* and *Bifidobacteria* are dysregulated amongst multiple schizophrenia studies, as well as dysregulation within SCFA and tryptophan-related GBMs (Zhu et al., 2020, Xu et al., 2020, Schwarz et al., 2018, Shen et al., 2018). While there are difficulties in determining strong associations because of the diversity and new nomenclature of *Lactobacillus* genera (Zheng et al., 2020a), we found evidence of broad dysregulation across most existing schizophrenia studies.

In one longitudinal study assessing the impact of ketogenic diet on the microbiota of young epileptic children, we found increased abundance of the Tryptophan Biosynthesis and S-Adenosyl Methionine Biosynthesis GBMs (Lindefeldt et al., 2019). This would imply different mechanisms for the efficacy ketogenic diet on epilepsy than seen in mice (Olson et al., 2018). In studies assessing the overall microbial differences found in epilepsy, we found that most studies assessed different cohorts making them difficult to compare with each other.

These results are not consistent with the findings in mice by Olson et al. (2018). In mice, the gut microbiota is required for mediating the anti-epileptic effects of the ketogenic diet; specifically, *Akkermansia* and *Parabacteroides* were implicated as mediators (Olson et al., 2018). In healthy adult humans, there is evidence that the ketogenic diet alters the gut microbiota and intestinal immunity, but more studies are needed to determine the mechanisms of anti-epileptic effects in humans (Ang et al., 2020).

Across neurodegenerative disorders, there is evidence of changes in SCFA and tryptophan-related GBM abundance in AD and MCI (Li et al., 2019a). However, most of this evidence is emergent from one reanalyzed study. Though the microbiota is an intriguing target for amytotrophic lateral sclerosis and multiple systems atrophy, we did not find enough studies

investigating this link to warrant a consensus. Preclinical evidence suggests PD pathogenesis can be initiated through α-Synuclein overexpression in the myenteric plexus, reaching the brain through the vagus nerve (O'Donovan et al., 2019, Holmqvist et al., 2014, Ulusoy et al., 2013, Uemura et al., 2018, Manfredsson et al., 2018). However, when reanalyzing raw data and accounting for recorded metadata we did not find evidence of consistent gut microbiota alterations. While PD is progressive and features many different subtypes, it may be necessary to stratify participants by medication and subtype. Nonetheless, this was a somewhat surprising finding.

A lack of dietary metadata may have hindered cross-comparison across alcohol-dependence studies. Though various genera involved in SCFA and tryptophan metabolism were identified across many of these datasets (Bjorkhaug et al., 2019, Seo et al., 2020, Dubinkina et al., 2017, Leclercq et al., 2014). Even with a small sample size consisting of 10 tobacco smokers and 10 controls, we found an increased abundance of the tryptophan degradation module and a reduction of the propionate synthesis III (Stewart et al., 2018). In addition, studies investigating the impact of recreational drug-use also reported differences in tryptophan and SCFA-associated genera (Fulcher et al., 2018, Panee et al., 2018). This must be taken into consideration when collecting metadata, as some of the strong microbiota-related changes between two groups may be explained by alcohol and drug use.

Reductions in faecal SCFA concentrations were reported in two studies investigating demyelinating diseases (Gong et al., 2019, Zeng et al., 2019). It is unclear if this is a result of subtle microbiota changes or gastrointestinal physiology within the disease group.

There are too few fibromyalgia and migraine microbiome-related studies to make definitive conclusions. However, one fibromyalgia study found altered microbial species associated with SCFA and tryptophan metabolism, as well as changes in serum levels of SCFAs

(Minerbi et al., 2019). Similarly the sole migraine-microbiota study reported an increased abundance of the kynurenine synthesis GBM (Chen et al., 2019). While few taxa were consistently associated with psychological metrics within IBS, interventions involving faecal matter transplantation of material high in *Bifidobacterium* (Mizuno et al., 2017b) or probiotic *Bifidobacterium* strains (Ma et al., 2019b, Pinto-Sanchez et al., 2017a) may improve the psychological dimensions of this disease.

Across studies of obesity involving 16S and WGS methods, we did not find differentially abundant microbes and microbial metabolic pathways consistently associated with psychological aspects of obesity. When reanalyzing studies of anorexia, we found an increased abundance in isovaleric acid synthesis I, quinolinic acid synthesis, quinolinic acid degradation when comparing anorexia to control individuals (Mack et al., 2016). While the ClpB GBM, produced by *Escherichia coli* (Tennoune et al., 2014), was elevated at admission compared to controls, after weight gain it was ameliorated (Mack et al., 2016). It is unclear whether microbial-host pathways involving ClpB and hunger also interact with SCFA and tryptophan metabolism.

Due to the heterogeneity of stroke and vascular disease conditions, it is difficult to make substantial comparisons between studies. However, (Polster et al., 2020) report convincing evidence for the involvement of specific microbial genera/species and a neurovascular condition in humans. However, rather these taxa were linked to LPS biosynthesis rather than SCFA production (Polster et al., 2020).

Several studies suggest lasting microbial changes in response to prenatal or postnatal stress (Naude et al., 2019, Hantsoo et al., 2019, Carlson et al., 2018) though these do not provide evidence for the involvement of SCFA, tryptophan or bile-acid modifying bacteria. Similar to stress, there are very few studies assessing the impact of posttraumatic stress disorder on the

microbiota. Though multiple studies have assessed the microbiota composition in bipolar disorder, there were no consistent signatures across studies. In fact, one study found sex effect, heritability and smoking explained all observed changes in the gut microbiota between bipolar disorder and controls (Coello et al., 2019). Meanwhile, across studies of anxiety and depression there is moderate evidence for *Dialister* and *Faecalibacterium* reductions in depression and anxiety (Valles-Colomer et al., 2019, Jiang et al., 2015, Jiang et al., 2020, Jiang et al., 2018a, Stevens et al., 2018). It is unclear if the metabolic pathways that these microbes contribute to, mainly SCFA and tryptophan-related pathways, impact the host phenotype.

5.0 Future Directions

In part due to the many limitations of existing 16S and WGS studies as well as their collected metadata, we did not find many consistent changes in the gut microbiota or their associated metabolic pathways. Despite the limitations outlined in **Section 3.15**, there is still potential for rigorous, well-designed human studies to uncover the potential roles of these metabolites. **Figure 2** briefly outlines the potential pathways and known interactions of SCFAs, tryptophan metabolism and deconjugated bile acids in brain function and health. Although none of these pathways have been directly linked to changes in the gut microbiota, we are hopeful that consensus guidelines for sequencing and downstream analysis of the human microbiota will contribute to uncover these changes. It is also difficult to compare studies within a human disease without the multiple publicly available datasets, detailed dietary information and medical information, effect sizes, confidence intervals or detailed bioinformatics procedures. The widespread use of relative abundance as opposed to methods incorporating the compositional nature of the microbiota (i.e. using the CLR transformation) is problematic within the microbiome field (Gloor et al., 2017, Gloor et al., 2016, Fernandes et al., 2014, Fernandes et al., 2013). Reporting effect sizes along with 95% confidence

intervals when finding differentially abundant microbes or metabolites would increase the interpretability of these results. For example, if a differentially abundant microbe is increased in one group, but its effect size has a very small lower bound (i.e. a large negative value), this is indicative of a spurious finding.

We provide some guidelines for scientists analyzing their microbiome data when building their pipeline and selecting their methodology (see Box 1).

In conjunction with metabolomic and proteomic studies, consistent well-designed bioinformatics pipeline can identify the involvement of microbially-associated SCFA, tryptophan and bile acid metabolites. There are still important questions that must be addressed or considered when designing these studies (see Box 2).

While we may standardize protocols and adapt to new sequencing platforms in the future, some researchers suggest the development of microbiome standards to better quantify microbial abundance within a sample (Ji et al., 2019, Venkataraman et al., 2018, Tourlousse et al., 2018, Vandeputte et al., 2017, Stämmler et al., 2016, Tkacz et al., 2018). In addition, a biobank of standardized references could be shared as controls across multiple studies (see Box 3).

This analysis provides a novel approach for understanding the mechanisms behind metabolite-mediated communication within the MGBA and reiterates many technical and bioinformatics considerations that must be acknowledged when interpreting results. Despite that, we found novel links between gut microbial metabolic pathways in schizophrenia, AD, and anxiety/depression.

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Conflicts of Interest

J.F.C, GC and T.G.D have research support from Cremo, Pharmavite, Dupont and Nutricia. These authors have spoken at meetings sponsored by food and pharmaceutical companies. All other authors report no potential conflicts of interest.

Figure Captions

Graphical Abstract. Dietary fibres, proteins and fats that are ingested by the host contain components which are metabolized by the host microbiota. Short-chain fatty acids (SCFAs) are produced from the fermentation of fibres and tryptophan-kynurenine (TRP-KYN) metabolites from dietary proteins. Primary bile acids derived from liver metabolism of aid in lipid digestion but can be deconjugated and bio-transformed into secondary bile acids. Though it's is unclear how these metabolites impact brain health and disease, we can look towards existing studies of faecal microbiota composition in humans for evidence. Inter-individual physiological variance as well as many technical and bioinformatics limitations hindered the replication of results across studies. However, by reanalyzing 35 studies with a consistent pipeline and comparing these results to other existing studies we found mild-to-moderate evidence of the involvement of these metabolic pathways in Alzheimer's disease, schizophrenia and anxiety/depression.

Figure 1. Microbial metabolic pathways. A summary of pathways used by different microbes to generate SCFA, tryptophan-kynurenine or bile acid related metabolites. Due to the amount of different microbial genera known to generate these metabolites, only a subset of these microbes is referred to in this figure. Many different genera use multiple metabolic pathways; it is yet unclear if all human enzymes in the kynurenine/tryptophan pathway are found in the microbiome. *5-HTP: 5-hydroxytryptophan; 5-HT: serotonin; AADC: aromatic amino acid decarboxylase; IDO1: indoleamine-2,3-dioxygenase; TDO1: tryptophan-2,3-dioxygenase; TH: tryptophan hydroxylase; K3H: kynurenine-3-hydroxylase; KAT: kynurenine amino-transferase; BSH: bile salt hydroxylase*.

Figure 2. Potential pathways for microbiota-gut-brain axis communication. While it's is unclear exactly how microbial-derived metabolites impact the brain, there are several potential pathways. Non-digestible fibres are broken down into SCFAs which act as histone

deacetylase inhibitors on FOXP3^{+} T_{Reg} cells in the gut, leading to clonal expansion. SCFAs many also influence the enteric dendritic and marcrophage cell population by increasing acetylation at specific gene targets. This leads to a decreased release of interleukin-6, interleukin-10 and interleukin-12. SCFAs may also affect enterochromaffin cells in the gut, stimulating the release of serotonin into the lumen. Travelling through the blood, the SCFA butyrate may increase occludin expression at the blood-brain barrier as well as decrease its permeability to different molecules. If present in a sufficient concentration, SCFAs may impact microglial maturation through free-fatty acid receptor-mediated mechanisms. Bile acids used to aid in lipid digestion are deconjugated and biotransformed into secondary bile acids. These act on myenteric neurons to inhibit gut motility. In the brain, there is evidence that the secondary bile acid, deoxycholic acid (DCA) is associated with cognition. Tryptophan derived from dietary protein sources impacts both the enteric and central nervous system environments. Briefly, bacteria may generate indole molecules which can act on myenteric neurons to increase gut motility. Tryptophan (TRP) or 5-Hydroxytryptophan (5-HTP) are also generated from dietary protein sources. TRP and 5-HTP can be converted into 5-HT in enterochromaffin cells. In the brain, indoles impact immunity through activation of the Aryl-Hydrocarbon receptor in astrocytes. Alternatively, TRP or 5-HTP can be transported across the blood-brain barrier and converted into the neurotransmitters 5-HT, quinolinic acid or kynurenic acid. It is unclear what role the vagal nerve pathway plays in mediating microbial-derived metabolite signalling.

Boxes

Box 1: Guidelines for metadata and bioinformatics analysis of human-microbiome-brain studies

1. If at all possible, ensure that extensive metadata is collected, including dietary intake, medication, supplement use, etc.
2. Make sure all software is up-to-date.
3. Use SILVA or other curated taxonomy databases instead of Greengenes.
4. Use compositional data method to transform the counts tables (ex. CLR).
5. Use compositional analysis methods to check for significance and employ a strict effect size cut-off.
6. Use compositional alternatives to standard correlational statistics.
7. Report effect sizes and confidence intervals along with p-values and p-adjusted values.
8. When possible, provide open access to datasets, scripts and pipelines to reproduce the results.

Box 2: Questions crucial for understanding the interactions between microbial metabolic pathways and the brain

1. How ubiquitous is the expression of any specific GBM across the same genera/species?
2. How explanatory are GBMs compared to metabolomic and proteomic faecal analysis?
3. Can we accurately develop a GBM framework for bile-acid metabolism?

4. How do we address causality when many microbes possess enzymes for multiple gut-brain modules?
5. How do we design studies to avoid the pitfalls of interindividual variation within the microbiome, metabolism and disease subtype/severity?

Box 3: Methods to improve cross-study replicability and provide more accurate microbial quantification

1. **Decomposition of Variance Using Replicate Sampling:** A combination of using technical replicates and spike-in controls to estimate absolute abundance.
2. **Spike-In:** Adding a known amount of synthetic 16S rRNA sequences to samples for estimation of absolute abundance.
3. **Reference Materials and Biobank:** Collecting and storing faecal samples from different cohorts of healthy individuals. This material would provide controls for multiple studies. It would allow for accurate quantification of variability between populations, labs and pipelines.

Figure 1.

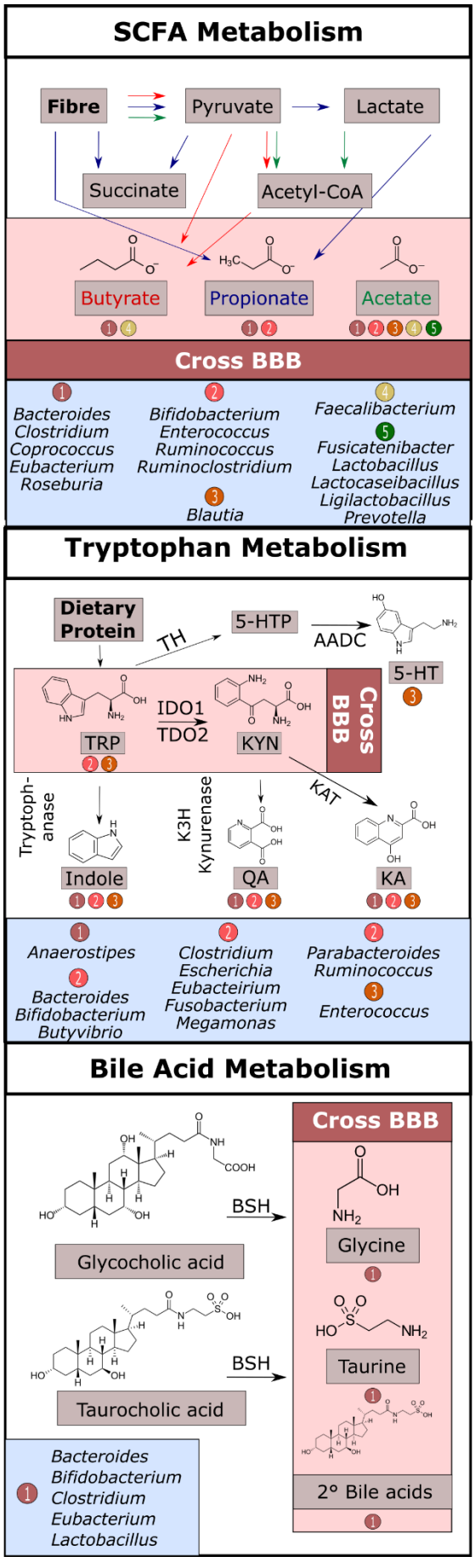
SCFA Metabolism
Fibre
Pyruvate
Lactate
Succinate
Acetyl-CoA
Butyrate
Propionate
Acetate
Cross BBB
Bacteroides
Clostridium
Coprococcus
Eubacterium
Roseburia
Bifidobacterium
Enterococcus
Ruminococcus
Ruminoclostridium
Blautia
Faecalibacterium
Fusicatenibacter
Lactobacillus
Lactocaseibacillus
Ligilactobacillus
Prevotella
Tryptophan Metabolism
Dietary Protein
TH
5-HTP
AADC
5-HT
IDO1
TDO2
TRP
KYN
Cross BBB
Tryptoph-anase
Indole
K3H Kynurenase
QA
KAT
KA
Anaerostipes
Bacteroides
Bifidobacterium
Butyvibrio
Clostridium
Escherichia
Eubacteirium
Fusobacterium
Megamonas
Parabacteroides
Ruminococcus
Enterococcus
Bile Acid Metabolism
BSH
Glycocholic acid
Taurocholic acid
Cross BBB
Glycine
Taurine
2° Bile acids
Bacteroides
Bifidobacterium
Clostridium
Eubacterium
Lactobacillus

Figure 2.

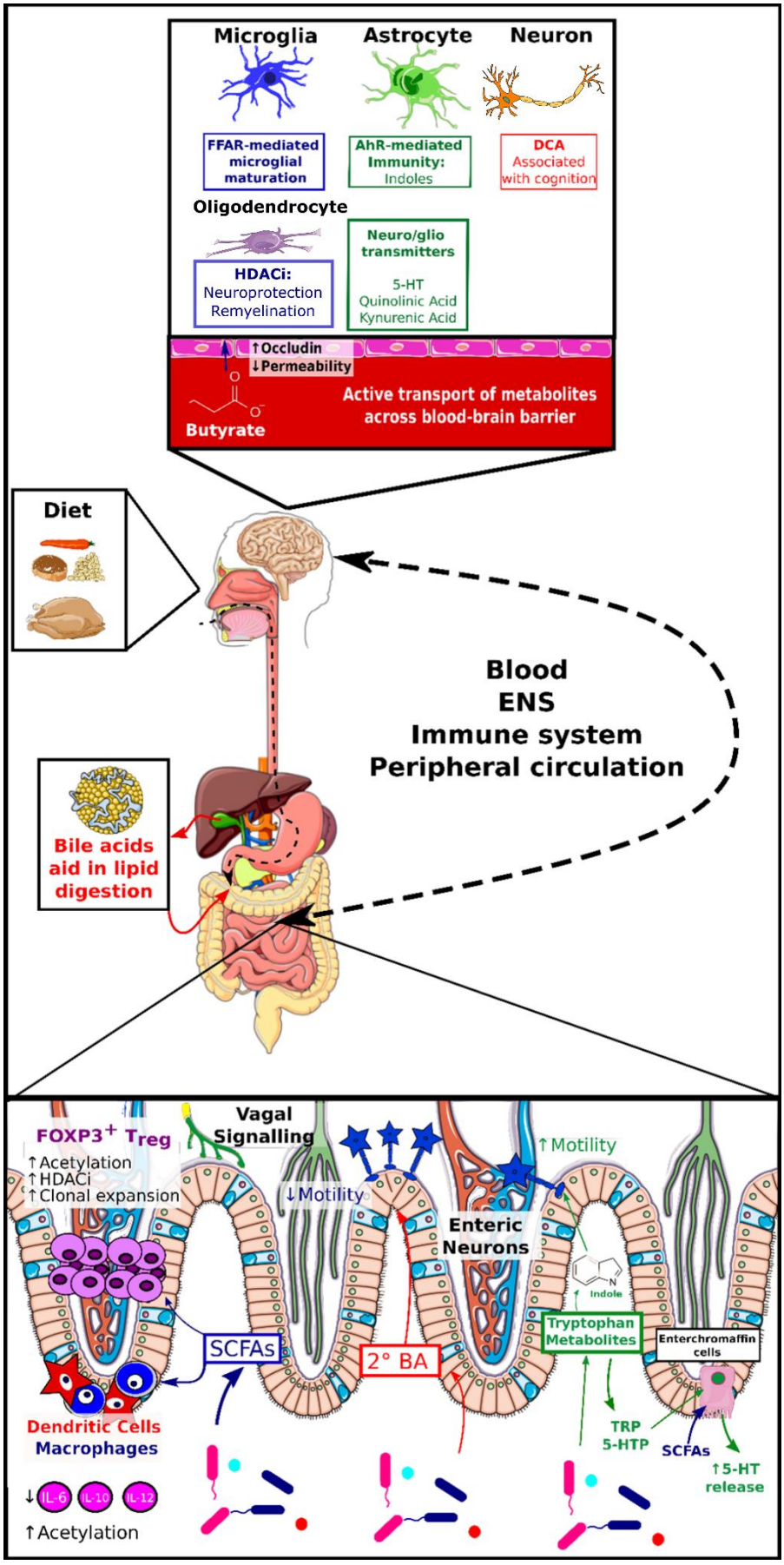
Microglia
Astrocyte
Neuron
FFAR-mediated microglial maturation
AhR-mediated Immunity: Indoles
DCA Associated with cognition
Oligodendrocyte
HDACi: Neuroprotection Remyelination
Neuro/glio transmitters
5-HT
Quinolinic Acid
Kynurenic Acid
↑Occludin
↓Permeability
Butyrate
Active transport of metabolites across blood-brain barrier
Diet
Blood
ENS
Immune system
Peripheral circulation
Bile acids aid in lipid digestion
FOXP3+ Treg
↑Acetylation
↑HDACi
↑Clonal expansion
Vagal Signalling
↓Motility
↑Motility
Enteric Neurons
Indole
Tryptophan Metabolites
Enterchromaffin cells
SCFAs
2° BA
Dendritic Cells
Macrophages
TRP
5-HTP
SCFAs
↑5-HT release
↓IL-6
IL-10
IL-12
↑Acetylation

Table 1. Microbiome-brain studies of healthy human cohorts.

Legend: LFC: log-2 fold change; LC: log change; RA: relative abundance; DASS-42: Depression Anxiety Stress Scales; ADHD: Attention-Deficit Hyperactivity Disorder; HE: hepatic encephalopathy; FOS: Fructo-oligosaccharide; HADS-A: Hamilton Anxiety and Depression Scales – Anxiety Subscale; HADS-A: Hamilton Anxiety and Depression Scales – Depression Subscale; *: $p_{adj} < 0.1$; **: $p_{adj} < 0.01$; ***: $p_{adj} < 0.001$; Reanalyzed studies are highlighted; 95% CI reported between square brackets [lower 95% CI; upper 95% CI]

Cohort Details	Sequencing (reanalysed)	Groups and Sample Size	SCFA/Tryptophan-Modifying Bacteria	BA-Modifying Bacteria	Other Metabolites/ GBMs	Specific Limitations	Ref
Infant Temperament and Stress	WGS (no)	N = 63 Infants	**Negative Emotionality (High vs Low Scoring Group)** ↑ *Bifidobacterium pseudocatenulatum* (LFC: 4.085) ↓ *Streptococcus vestibularis* (LFC – 3.12) **Regulation/Orienting (High vs Low Scoring Group)** ↑ *Bifidobacterium pseudocatenulatum* (LFC: 4.085) ↑ *Bifidobacterium catenulatum* (LFC: 4.177) **Functional Connectivity: Left Default Network (High vs Low Scoring Group)** ↓ *Clostridium perfingens* (LFC: 3.559) **Functional Connectivity: Left Fronto-Parietal Network (High vs Low Scoring Group)** ↑ *Enterococcus fragilis* (LFC: 3.765) ↑ *Collinsella* (LFC: 3.665) ↑ *Clostridium disporicum* (LFC: 3.415) ↑ *Prevotella copri*(LFC: 3.415)	**Negative Emotionality (High vs Low Scoring Group)** ↑ *Bifidobacterium pseudocatenulatum* (LFC: 4.085) **Regulation/Orienting (High vs Low Scoring Group)** ↑ *Bifidobacterium pseudocatenulatum* (LFC: 4.085) ↑ *Bifidobacterium catenulatum* (LFC: 4.177) **Functional Connectivity: Left Default Network (High vs Low Scoring Group)** ↓ *Clostridium perfingens* (LFC: 3.559) **Functional Connectivity: Left**	None		(Kelsey et al., 2021)

			↑ *Clostridium perfingens* (LFC: 3.415) ↑ *Clostridium tertium* (LFC: 3.367) ↑ *Clostridium* (LFC: 3.167) ↑ *Bacteroides caccae* (LFC: 3.164) ↓ *Streptococcus salivarius* (LFC : 3.397) ↓ *Enterococcus* (LFC = 3.042) **Functional Connectivity: Homologous Interhemispheric Network** ↑ *Eschericia coli (*LFC: 4.357) ↓ *Bifidobacteirum dentae (*LFC: 4.012)	**Fronto-Parietal Network (High vs Low Scoring Group)** ↑ *Clostridium disporicum* (LFC: 3.415) ↑ *Clostridium perfingens* (LFC: 3.415) ↑ *Clostridium tertium* (LFC: 3.367) ↑ *Clostridium* (LFC: 3.167) ↑ *Bacteroides caccae* (LFC: 3.164) **Functional Connectivity: Homologous Interhemispheric Network** ↓ *Bifidobacteirum dentae (*LFC: 4.012)			
	16S (no)	N = 34 Infants	None	None	None		(Rosin et al., 2020)
	16S (no)	N = 89 Infants	None	None	None	Genera level findings not reported; clustered microbiomes and associated these	(Carlson et al., 2018)

						clusters with infant cognition	
	16S (no)	N = 201 Infants	↓ *Prevotella**** associated with a decrease in behavioural problems	None	None	Potential bias in parent reported measures; DeSeq2 is inappropriate for microbiome analysis	(Loughman et al., 2020)
	16S (no)	N = 51 Infants (42 taking probiotics)	*Bifidobacterium*** associated with soothability				(Wang et al., 2020b)
	16S (no)	N = 301 Infants	↑ *Bifidobacterium* and *Streptococcus* associated with postive emotional regulation	↑ *Bifidobacterium* with positive emotional regulation		Greengenes, QIIME 1.9	(Aatsinki et al., 2019)
	16S (no)	N = 39	*None*	None	None	No genus-level associations with functional connectivity	(Gao et al., 2019)
	16S via 454 Pyrosequencing (no)	N = 77 Infants	None	None	None		(Christian et al., 2015)
Adult Emotion and Personality	16S (no)	N = 91 Healthy ♀ Focus on psychiatric measures	None	None	None		(Kleiman et al., 2017a)
	16S (no)	N = 15 Probiotics N = 15 Placebo	**<u>Probiotics vs Placebo</u>** ↑ *Bacteroides* sp.; associated with response accuracy neutral stimuli scores*, general depression scale* **<u>Probiotics vs Placebo</u>** ↑ *Bacteroides* sp.; associated with response accuracy neutral stimuli scores*, general depression scale*		None	Rarefaction	(Bagga et al., 2018)

	16S (no)	N = 135 Healthy Individuals	**<u>When accounting for fibre intake</u>** In males, DASS-42 anxiety scores negatively correlated with *Blautia* abundance	None	None	Greengenes, rarefaction	(Taylor et al., 2019)
	16S (no)	N = 672	↑ *Roseburia*** in high Conscientiousness group	None	Valine, leucine, isoleucine degradation pathways enriched in high neuroticism group***	Greengenes	(Kim et al., 2018)
	16S (no)	N = 655	**<u>Sociability (combination of extraversion, social skill and communication) as microbiome a predictor</u>** + *Oscillospira****	None	None	Sample collection in buffer to stabilize at room temperature	(Johnson, 2020)
	16S via 454 Pyrosequencing (no)	N = 40 Healthy Women (N = 33 in *Prevotella* cluster, N = 7 in *Bacteroides* cluster)	High *Prevotella* abundance associated with negative affect after negative valence picture block	None	None	Rarefaction	(Tillisch et al., 2017)
	16S gene array (no)	N = 60	No genus-levelenus-level associations reported	None	None		(Kim and Park, 2017)
	FISH (no)	N = 40 Focus on self-judgment and empathy measures	**<u>Negative Associations:</u>** *Lactobacillus*: cognitive depression* affective empathy** **<u>Positive Associations:</u>**		None		(Heym et al., 2019)

			Lactobacillus: self-judgment*** over identification*				
Sleep	16S (yes)	N = 10 Insomnia N = 10 Control	**None**	**None**	**<u>Insomnia vs Control</u>** ↓ *Alloprevotella* (effect = -1.16 [-14.97; 0.17]) *		(Liu et al., 2019a)
	16S (no)	N = 113 Focus on sleep	Disruptions in sleep across stages in *Prevotella* enterotype	None	None		(Ko et al., 2019)
	16S (no)	N = 22	None	None	None		(Liu et al., 2020c)
	16S (no)	N = 8 Control N = 7 Obstructive Sleep Apnoea	**<u>Apnea-Hypopnea Index Correlations</u>** *Eubacterium** (rho = 0.785) **<u>Wake After Sleep Onset</u>** *Escherichia*** (rho = 0.915) *Klebsiella** (rho = 0.768) **<u>Arousals Index</u>** *Clostridium** (rho = 0.852) *Ruminococcus** (rho = 0.738) *Oscillospira** (rho = 0.842)	None	None		(Valentini et al., 2020)
	16S (no)	N = 20 Acute Insomnia Disorder (AID) N = 38 Chronic Insomnia Disorder (CID) N = 38 Control	**<u>AID vs Control</u>** ↑ *Bacteroides** ↓ *Lachnospira** **<u>CID vs Control</u>** ↑ *Blautia**** ↓ *Faecalibacterium**** ↓ *Prevotella*** ↓ *Roseburia***	**<u>CID vs Control</u>** ↑ *Bacteroides**			(Li et al., 2020c)

	16S (no)	N = 37	None	None	None	Genus-level changes not reported	(Anderson et al., 2017)
	16S (no)	N = 35 Narcolepsy Type 1 (NT1) N = 41 Control	**NT1 vs Control** ↓ *Bacteroides**** ↑ *Flavonifactor***	**NT1 vs Control** ↓ *Bacteroides****			(Lecomte et al., 2020)
	16S (no)	N = 24	**Negative Associations** Sleep efficiency and total sleep time: *Blautia* Number of awakenings: *Holdemania, Corynebacterium,* **Positive Associations** Number of awakenings: *Coprococcus, Neisseria*	None	None	Faecal swab; no post-hoc testing for correlation coefficients	(Smith et al., 2019b)
Healthy Aging and Cognition	16S (no)	N = 11 MCI with Risk of AD N = 6 Aged-Control Randomized double-crossover intervention Ketogenic Mediterranean diet vs American heart association diet	**MCI vs Control at Baseline** ↑ *Phascolarctobacterium* ↓ *Dialister* ↑ *Bifidobacterium* after Mediterranean Ketogenic Diet in MCI but not in Controls	↑ *Bifidobacterium* after Mediterranean Ketogenic Diet in MCI but not in Controls		QIIME 1.9.1, Greengenes, rarefaction, Unbalanced groups	(Nagpal et al., 2019)
	16S (no)	N = 26 PBO N = 27 Probiotic 12 weeks *Bifidobacterium bifidum* BGN4 and *Bifidobacterium*	**Probiotic vs PBO** ↓ *Eubacterium* **In probiotic group** *Eubacterium* negatively correlated with serum BDNF	None	None		(Kim et al., 2020)

		longum BORI (1 × 109 CFU/d) 2 week washout					
	16S via 454 Pyrosequencing (no)	N = 37 Aged N = 39 Aged with Cirrhosis	**<u>Amnesia vs No Unimpaired</u>** ↑ *Paraprevotella* ↓ *Faecalibacterium* ↓ *Coprobacillus*	None	None		(Bajaj et al., 2016)
	T-RFLP (no)	N = 34 Dementia N = 94 Controls	**<u>Dementia vs Control</u>** ↓ *Bacteroides*		None		(Saji et al., 2019)

Table 2. Microbiome-brain studies involving neurodevelopmental disorders.

Legend: LFC: log-2 fold change; LC: log change; RA: relative abundance; DASS-42: Depression Anxiety Stress Scales; ADHD: Attention-Deficit Hyperactivity Disorder; HE: hepatic encephalopathy; FOS: Fructo-oligosaccharide; HADS-A: Hamilton Anxiety and Depression Scales – Anxiety Subscale; HADS-A: Hamilton Anxiety and Depression Scales – Depression Subscale; RTT: Rett's Syndrome; *: $p_{adj} < 0.1$; **: $p_{adj} < 0.01$; ***: $p_{adj} < 0.001$; Reanalyzed studies are highlighted; 95% CI reported between square brackets [lower 95% CI; upper 95% CI]

Cohort Details	Sequencing (reanalysed)	Groups and Sample Size	SCFA/Tryptophan-Modifying Bacteria	BA-Modifying Bacteria	Other Metabolites/ GBMs	Specific Limitations	Ref
ADHD	16S (yes)	N = 19 ADHD N = 77 Control	None	None	None	Wide age range	(Aarts et al., 2017)
	WGS (no)	N = 17 ADHD N = 17 HC	**<u>ADHD vs Control</u>** ↓ *Faecalibacterium** ↓ *Ruminococcus gnavus**	**<u>ADHD vs Control</u>** ↑ *Bacteroides caccae**	**<u>ADHD vs Control:</u>**		(Wan et al., 2020)

			↑ *Bacteroides caccae** ↑ *Odoribacter** ↑ *Enterococcus**		↓ in KO terms for dopamine pathways**		
	16S (no)	N = 10 ADHD + nutrient intervention N = 7 ADHD (placebo)	**ADHD Associations with Symptomology** ↑ *Bifidobacterium* associated with lower ADHD-IV-RS score (t = −2.3, df = 15, *p* = 0.04); possibly due to 3 outliers		None	Pilot study on intervention so no comparisons with controls	(Stevens et al., 2019)
	16S (no)	N = 14 ADHD N = 17 Control	**ADHD vs Control** ↓ *Prevotella* ↓ *Parabacteroides* ↑ *Neisseria*	None	None	Incomplete methods section, males only	(Prehn-Kristensen et al., 2018)
	16S (no)	N = 42 ADHD N = 15 Subthreshold ADHD N = 50 HC	**ADHD vs Control** ↑ *Ruminoclostridium 9*, Ruminococcus 2** **ADHD Medicated vs ADHD Unmedicated** *Ruminococcus 2* (B = 1.525, P = 0.001) associated with inattention score	None	None		(Szopinska-Tokov et al., 2020)
	16S (no)	N = 30 ADHD N = 30 Control	**ADHD vs Control (Genus-levelenus-level)** ↓ *Lactobacillus* ↑ *Fusobacterium* **ADHD vs Control (Species-Level)** ↑ *Bacteroides uniformis* ↑ *Bacteroides ovatus* ↑ *Sutterella stercoricanis* ↓ *Bacteroides coprocola*	**ADHD vs Control (Genus-levelenus-level)** ↓ *Lactobacillus* **ADHD vs Control (Species-Level)** ↑ *Bacteroides uniformis* ↑ *Bacteroides ovatus* ↓ *Bacteroides coprocola*	None	Rarefaction	(Wang et al., 2020b)

	16S via 454 Pyrosequencing (no)	N = 51 ADHD N = 32 Controls	**<u>ADHD vs Control</u>** ↓ *Faecalibacterium* ↓ *Dialister* ↓ *Faecalibacterium*; associated with total CPRS Score (Pearson Correlation: $p < 0.001$, $R^2 = -0.564$) and hyperactivity index score (Pearson Correlation: $p < 0.037$, $R^2 = -0.294$)	None		Rarefaction	(Jiang et al., 2018b)
	qPCR (no)	N = 35 Placebo N = 40 Probiotic	↓ *Bifidobacterium spp.* at 6 weeks of life in children that developed ASD/ADHD		None		(Pärtty et al., 2015)
ASD	WGS (yes, from species counts table)	N = 36 ASD N = 21 Control	None	None	None		(Averina et al., 2020)
	16S (yes)	N = 51 ASD N = 40 Control	None	None	None		(Son et al., 2015)
	16S (yes)	N = 20 ASD N = 20 Control	**<u>ASD vs Control</u>** ↑ *Roseburia**** (effect = 0.9 [-1.9; 10.38]	None	None		(Pulikkan et al., 2018)
	16S (yes)	N = 20 ASD N = 19 Control	None	None	None		(Kang et al., 2019)
	16S (yes)	N = 20 ASD N = 19 Control	**<u>ASD with ATEC score below median (62) vs ASD with score above median</u>** ↓ *Ruminoclostridium 9* (*effect = -0.78 [-7.28, 1.80])	None	None		(Kong et al., 2019)

	16S (yes)	N = 20 ASD Faecal samples taken before and after Vitamin A supplementation	None	None	None		(Liu et al., 2017)
	16S via 454 Pyrosequencing (yes – unable to identify bacterial sequences)	N = 40 ASD N = 40 Controls	None	None	None		(Strati et al., 2017)
	WGS (no)	N = 43 ASD (19 with GI symptoms, 24 without) N = 31 Controls	None	None	None	Focus on immune epitopes	(Wang et al., 2019b)
	WGS (no)	N = 39 ASD N = 40 Control	**ASD vs Control** ↑ *Veillonella parvula* ↑ *Butyrivibrio* unclassified ↑ *Streptococcus pasteurianus* ↑ *Lactobacillus rhamnosus* ↑ *Megasphera micronuciformis* ↑ *Lachnospiraceae bacterium 6163FAA* ↑ *Haemophilus haemolyticus* ↓ *Bifidobacterium longum* ↓ *Prevotella copri* ↓ *Bacteroides stercoris* ↓ *Dorea* unclassified ↓ *Lachnospiraceae bacterium 1456FAA* ↓ Eubacterium limosum	**ASD vs Control** ↑ *Lactobacillus rhamnosus* ↓ *Bifidobacterium longum* ↓ *Bacteroides stercoris*	None		(Zhang et al., 2020c)
	WGS (no)	N = 30 ASD N = 14 Controls	None	None	None		(Carisimi et

							al., 2019)
	WGS (no)	N = 166 Infants aged 6 weeks N = 158 Infants aged 1 year N = 129 Infants aged 2 years N = 140 Infants aged 3 years Assessing ASD-related social behaviors with Social Responsiveness Scale (SRS-2) T-scores	**<u>At One Year</u>** *Blautia producta* + association with SRS-2 **<u>At Two Years</u>** *Coprococcus* + association with SRS-2 *Ruminococcus gnavus* + association with SRS-2 *Bifidobacterium* + association with SRS-2 *Sutterella* + association with SRS-2 **<u>At Three Years</u>** *Bytyricoccus pulliacaerum* – association with SRS-2	**<u>At Two Years</u>** *Bifidobacterium* + association with SRS-2	None		(Laue et al., 2020)
	WGS (no – ASD and controls not specified in metadata)	N = 92 ASD N = 42 Control	**<u>ASD vs Control</u>** ↑ *Eggerthella lenta** ↑ *Eggerthella lenta* DSM2243* ↑ *Clostridium botulinum* A3 and Ba4* ↓*Bacteroides vulgaris***	**<u>ASD vs Control</u>** ↓*Bacteroides vulgaris***	**<u>ASD vs Control</u>** ↓ Glutamate/Glutamine metabolism		(Wang et al., 2019a)
	16S and WGS (no)	N = 143 ASD N = 143 Controls WGS: N = 30 ASD with Constipation (C-ASD) N = 30 Non-Constipated ASD (NC-ASD) N = 30 Controls	**<u>ASD vs Control</u>** ↑ *Dialister* ↑ *Escherichia-Shigella* ↑ *Bifidobacterium* ↓ *Prevotella 9* ↓ *Megamonas* ↓ *Ruminococcus 2* **<u>C-ASD vs NC-ASD</u>** ↑ *Alistipes*** ↑ *Anaerotruncus*** ↑ *Ruminoclostridium 6*** ↑ *Ruminococcus 2***	**<u>ASD vs Control</u>** ↑ *Bifidobacterium* **<u>C-ASD vs NC-ASD</u>** ↓ *Bacteroides***	None	QIIME 1.9, No post-hoc correction, rarefaction	(Dan et al., 2020)

			↑ *Subdolingranlum** ↑ *Coprococcus 1** ↑ *Blautia** ↑ *Roseburia** ↑ *Butyricoccus** ↑ *Ruminococcus 1** ↑ *Coprobacter** ↓ *Veillonella*** ↓ *Collinsella*** ↓ *Megasphera*** ↓ *Bacteroides***				
	16S (no)	N = 77 ASD N = 50 Control	**ASD vs Control** ↓ *Bacteroides** ↓ *Faecalibacterium** Negative association between *Faecalibacterium* and ASD severity Multiple other genera associated with ASD severity	**ASD vs Control** ↓ *Bacteroides**	None		(Ding et al., 2020)
	16S (no)	N = 60 ASD + Sleep disorder (ASD-S) N = 60 ASD without Sleep disorder	**ASD-S vs ASD** ↓ *Faecalibacterium* (also correlated to 3-hydroxybutyric acid abundance in feces)	None	None	Rarefaction	(Hua et al., 2020)
	16S (no)	N = 78 ASD N = 58 Controls	**ASD vs Controls** ↑ *Bacillus*** ↑ *Bacteroides*** ↑ *Bilophila*** ↑ *Parabacteroides*** ↑ *Sutterella***	**ASD vs Controls** ↑ *Bacteroides***	None	Qiime v1.9.1 (outdated since Jan 1, 2018)	(Zhai et al., 2019b)
	16S (no)	N = 30 ASD N = 20 Control	**ASD vs Controls** ↑ *Megamonas* ↓ *Eubacterium* ↑ Faecal valerate	**ASD vs Controls** ↓ *Eubacterium*	None		(Liu et al., 2019d)

			↓ Faecal butyrate				
	16S (no)	N = 21 ASD N = 23 Control	**ASD vs Control** ↓ *Faecalibacterium**** ↓ *Heamophilus****	None	None		(Kang et al., 2018a)
	16S (no)	N = 9 ASD N = 6 Control	None	None	None	Greengenes	(Sun et al., 2019a)
	16S (no)	N = 37 ASD + Probiotic (4 weeks) N = 77 ASD (no Probiotic) N = 40 Control Faecal samples not analysed after intervention	**ASD vs Controls (Baseline)** ↓ *Bacteroides**** ↓ *Bifidobacterium**** ↓ *Ruminococcus***** ↓ *Lachnospira**** ↓ *Roseburia**** ↓ *Blautia****	**ASD vs Controls (Baseline)** ↓ *Bacteroides**** ↓ *Bifidobacterium****	None		(Niu et al., 2019)
	16S (no)	N = 25 ASD N = 35 Control	**ASD vs Controls** ↓ *Lactobacillus* ↓ *Ruminococcus* ↑ *Bacteroides* ↑ *Akkermansia* ↑ *Coproccous* ↑ *Ruminococcus* (different OTU assigned to the same genera)	**ASD vs Controls** ↓ *Lactobacillus* ↑ *Bacteroides*	None	Greengenes	(Zurita et al., 2019)
	16S (no)	N = 24 ASD N = 24 Control FOS+Probiotics Interverntion	**ASD vs Controls at Baseline** ↓ *Bifidobacterium* ↓ *Veillonella* ↓ *Acidaminococcus* ↓ *Enterococcus* ↑ *Odoribacter* ↑ *Oscillispira* ↑ *Ruminococcus* **Day 80 vs Baseline ASD**	**ASD vs Controls at Baseline** ↓ *Bifidobacterium* **Day 80 vs Baseline ASD** ↑ *Bifidobacteriun longum* to control levels	↑ L-histidine and L-histamine over course of intervention	Rarefaction	(Wang et al., 2020e)

			↓ Acetate, butyrate, propionate; increases over 80 days of intervention ↑ *B. longum* to control levels ↓ *Clostridium* Most short term measures were not sustained after the end of the study				
	16S (no)	N = 63 ASD N = 27 Control	**<u>ASD vs Control</u>** ↑ *Aenerococcus* ↑ *Burkholderia,* ↑ *Desulfovibrio* ↑ *Oxalobacter* ↓ *Bilophila*			Lack of clarity in methods section, no post-hoc	(Tomova et al., 2019)
	16S (no)	N = 46 ASD N = 16 Control	None	None	None	Lack of clarity in methods section, no post-hoc	(Tomova et al., 2020)
	16S (no)	N = 76 ASD N = 47 Control	**<u>ASD vs Control</u>** Focus on co-abundance groups finding correlations with co-abundant *Bacteroides* ASVs and various ASD behaviours	None	None		(Chen et al., 2020b)
	16S (no)	N = 14 Unrestricted Diet ASD (split into PBO and B-GOS) N = 12 exclusion diet (split into PBO and B-GOS)	None	None	None	Rarefaction, reporting on genus-levelenus-level differences within groups is unclear	(Grimaldi et al., 2018)
	16S (no)	N = 48 ASD 30 with no mental regression (ANMR) 18 with mental regression (AMR)	**<u>ASD vs Controls</u>** ↑ *Bacillus* ↑ *Bifidobacterium* ↑ *Butyrivibrio* ↑ *Enterococcus* ↑ *Prevotella*	**<u>ASD vs Controls</u>** ↑ *Bifidobacterium*	None		(Plaza-Diaz et al., 2019)

		N = 57 Controls	↑ *Clostridium boltae* ↑ *Clostridium difficile* **AMR vs ANMR** ↑ *Enterococcus*				
	16S (no – unable to demultiplex)	N = 59 ASD N = 30 Control	**ASD vs Control** ↑ *Clostridium* ↑ *Pseudomonas* ↑ *Streptococcus* ↓ *Prevotella*	None	**None**	Greengenes, Qiime v1.9.1 (outdated since Jan 1, 2018), rarefaction	(Li et al., 2019d)
	16S (no)	N = 11 ASD N = 14 Control	**ASD vs Control** ↑ Faecal butyrate ↓ *Streptococcus*** ↓ *Coprocccus*** ↓ *Blautia*** ↓ *Eggerthella*** ↓ *Corynebacteirum*** ↑*Parabacteroides** ↑ *Bacteroides*** ↑ *Faecalibacterium prausnitzii*** ↑ *Roseburia*** ↑ *Ruminococcus***	**ASD vs Control** ↑ *Bacteroides***		Greengenes, QIIME 1.9, rarefaction	(Coretti et al., 2018)
	16S (no)	N = 45 ASD N = 45 Control	**ASD vs Control** ↓ *Flavonifractor***	None	None		(Ma et al., 2019a)
	16S (no)	N = 26 ASD N = 32 Control	Faecal butyrate associated with diet quality within ASD No butyrate producing bacteria reported to correlate with butyrate	None	None	Greengenes	(Berding and Donovan,

							2019, Berding and Donovan, 2018)
	16S (no)	N = 26 ASD N = 32 Control	**ASD + Temporally Unstable Microbiome vs ASD + Temporally Stable Microbiome** ↓ *Turcibacter** ↓ *Dorea** ↓ *Phascolarctobacterium**	None	None	Greengenes	(Berding and Donovan, 2019)
	16S (no)	N = 6 ASD N = 6 Control	**ASD vs Control** ↓ *Blautia* ↓ *Faecalibacterium*	None	None		(Inoue et al., 2016)
	16S (no)	N = 6 ASD Probiotic then PBO N = 4 ASD PBO then Probiotic VISBIOME crossover pilot trial	None	None	None		(Arnold et al., 2019)
	16S (no)	N = 35 ASD N = 6 Control	**ASD vs Control** ↓ *Streptococcus** ↓ *Vaillonella** ↓ *Escherichia**	None	None		(Zhang et al., 2018a)
	16S (no)	N = 21 ASD+GI problems (ASD^{GI}) N = 29 ASD^{noGI} N = 34 $Control^{noGI}$ N = 7 $Control^{GI}$	None	None	None		(Luna et al., 2017)

	16S via 454 Pyrosequencing (no)	N = 51 ASD N = 53 Neurotypical Siblings Controlled for GI Dysfunction	None	None	None		(Gondalia et al., 2012)
	16S via 454 Pyrosequencing (no)	N = 10 ASD N = 10 Other Neurodevelopmental Disorder (OND) N = 10 Control	Bacteria assessed at species-level; impossible to do reliably with 16S	None	None	Rarefaction, no post-hoc testing	(De Angelis et al., 2013)
	16S via 454 Pyrosequencing (no)	N =23 ASD without GI dysfunction N = 28 ASD with GI dysfunction N = 53 neurotypical siblings	No significant microbiome differences found	None	None		(Gondalia et al., 2012)
	qPCR (no)	N = 41 ASD N = 45 Non-ASD Siblings N = 45 Control	**ASD vs Control** ↑ *Bacteroides** ↑ *Ruminococcus*** ↓ *Prevotella** **Non-ASD Siblings vs Control** ↑ *Bacteroides*** ↑ *Ruminococcus***	**ASD vs Control** ↑ *Bacteroides** **Non-ASD Siblings vs Control** ↑ *Bacteroides***			(Ahmed et al., 2020)
	qPCR (no)	N = 30 ASD Received probiotics (*L. acidophilus, L. rhamnosus, B. longum)*	**After 3 Mo. Probiotics vs Baseline** ↑ *Lactobacillus**** ↑ *Bifidobacterium****	None	None	No control/PBO	(Shaaban et al., 2018)

	qPCR (no)	N = 30 ASD N = 30 Control	**ASD vs Control** ↑ *Clostridium difficile**** ↑ *C. paraputrificum** ↑ *C. clostridioforme**** ↑ *C. bolteae**** ↑ *C. clostridioforme****	None	None		(Kandeel et al., 2020)
	qPCR (no)	N = 23 ASD N = 22 Non-ASD Siblings N = 9 Control	**ASD vs Control** ↑ *Sutterella spp.** **Non-ASD Siblings vs Control** ↑ *Sutterella spp.**	None	None		(Wang et al., 2013)
	qPCR (no)	N = 10 ASD N = 10 Control Siblings N = 9 Unrelated Controls	*Desulfvibrio* correlated to autism intensity with ADI RRB **ASD vs Unrelated Control Baseline** ↑ *Lactobacillus spp.**** (no difference with siblings) **ASD After Probiotic vs Before Probiotic** ↓ *Bifidobacterium spp.**** ↓ *Desulfvibrio spp.****	**ASD vs Unrelated Control Baseline** ↑ *Lactobacillus spp.**** (no difference with siblings) **ASD After Probiotic vs Before Probiotic** ↓ *Bifidobacterium spp.****	None		(Tomova et al., 2015)
Schizophrenia	16S (yes)	N = 64 SZ N= 53 Control	**SZ vs Control** ↑ *Fusicatenibacter**** (effect = 0.67[-1.48; 7.56])	None	None		(Shen et al., 2018)
	16S (yes)	N = 40 SZ N = 40 Control	**SZ vs Control** ↓ *Lactobacillus**** (effect = -1.28 [-12.85; 0.11]) ↑*Fusicatenibacter**** (effect =1.06 [-1.05; 7.60]) ↑ *Ruminococcus 1****(effect = 0.80 [-2.23; 7.33] Butyrate Synthesis II*** (effect = 0.61 [-1.83; 5.41]) ↑ Kynurenine synthesis*** (effect = 0.68 [-2.10; 6.12]), Inositol Degradation***	**SZ vs Control** ↓ *Lactobacillus**** (effect = -1.28 [-12.85; 0.11])	**SZ vs Control:** ↓ Histamine Synthesis* (effect = -0.48 [-5.41; 1.89])		(Xu et al., 2019)

			(effect = 0.83 [-1.58; 6.96]),				
	16S (yes)	N = 21 taking atypical antipsychotics N = 16 taking Lithium or Lamotragine	None	None	None		(Flowers et al., 2019)
	16S (yes)	N = 25 SZ N = 25 Controls	None	None	None	Faecal swabs used	(Nguyen et al., 2019)
	WGS (no)	N = 84 SZ N = 84 Control	**SZ vs Control:** ↑ *Bifidobacterium adolescentis**** ↑ *Clostridium perfringens**** ↑ *Lactobacillus gasseri****	**SZ vs Control:** ↑ *Bifidobacterium adolescentis**** ↑ *Lactobacillus gasseri****	None		(Xu et al., 2019)
	WGS (no)	N = 90 drug-naïve SZ N = 81 Control	**SZ vs Control** ↑ *Eubacterium siraeum** ↑*Bacteroides plebius** ↑ *Bifidobacterium adolescentis** ↑ *Bifidobacteriun bifidum** ↑ *Bifidobacteriun dentium** ↑ *Bifidobacteriun longum** ↑ *Clostridium bolteae** ↑ *Clostridium ramosum** ↑ *Clostridium symbiosum** ↑ *Enterococcus faecium** ↑ *Lactobacillus crispatus**, ↑ *Limosilactobacillus fermentum** ↓ *Clostridium perfogens** ↓ *Bacteroides intestinales** ↓ *Bacteroides finegoldii** ↓ *Lactococcus lactis**	**SZ vs Control** ↑ *Eubacterium siraeum** ↑*Bacteroides plebius** ↑ *Bifidobacterium adolescentis** ↑ *Bifidobacteriun bifidum** ↑ *Bifidobacteriun dentium** ↑ *Bifidobacteriun longum** ↑ *Enterococcus faecium** ↑ *Lactobacillus crispatus**, ↑ *Limosilactobacillus fermentum**	None	Metadata is unlabelled	(Zhu et al., 2020)

			↓ *Lactobacillus acidophilus** ↓ *Lactobacillus johnsonii**	↓ *Bacteroides intestinales** ↓ *Bacteroides finegoldii** ↓ *Lactobacillus acidophilus** ↓ *Lactobacillus johnsonii*			
	WGS (no)	N = 28 First Episode Psychosis (FEP) N = 16 Matched Controls	**FEP vs Controls** ↑ *Lactobacillus*	**FEP vs Controls** ↑ *Lactobacillus*	None		(Schwarz et al., 2018)
	16S (no)	N = 40 Drug Naive SZ (DSZ) N= 85 Treated SZ (TSZ) N = 69 Control	**TSZ vs DSZ** ↑ *Escherichia* (LFC = 1.65)*** ↑ *Fusobacterium* (LFC = 2.43)** ↑ *Megasphaera* (LFC = 5.76)*** ↑ *Enterococcus* (LFC = 3.69)*** ↑ *Lactobacillus* (LFC = 5.02)*** ↑ *Streptococcus* (LFC = 2.67)*** ↑ *Shigellia* (LFC = 1.18)** ↑ *Veillonella* (LFC = 2.81)*** ↑ *Clostridium* (LFC = 1.26)** ↑ *Enterobacter* (LFC = 1.93)** ↑ *Ruminococcus* (LFC = 0.95)*** ↑ *Sutterella* (LFC = 1.06) **DSZ vs Control** ↑ *Escherichia* (LFC = 1.86)*** ↓ *Fusobacterium* (LFC = -2.99)*** ↓ *Megasphaera* (LFC = -4.60)*** **TSZ vs Control** ↓ *Bacteroides* (LFC = -0.73)** ↑ *Enterococcus* (LFC = 2.82)*** ↑ *Lactobacillus* (LFC = 3,74)*** ↑ *Parabacteroides* (LFC = -0.76)** ↑ *Shigella* (LFC = 1.66)*** ↑ *Streptococcus* (LFC = 1.29)	**TSZ vs Control** ↑ *Enterococcus* (LFC = 3.69)*** ↑ *Lactobacillus* (LFC = 5.02)***5.02)*** **TSZ vs Control** ↓ *Bacteroides* (LFC = -0.73)** ↑ *Enterococcus* (LFC = 2.82)*** ↑ *Lactobacillus* (LFC = 3,74)***74)***	None	Greengenes, rarefaction	(Ma et al., 2020)

			↓ *Turcibacter* (LFC = -2.04)*** ↑ *Veilonella* (LFC = 2.31)*** ↑ *Clostridium* (LFC = 1.67)**				
	16S (no)	N = 82 SZ N = 80 Control	**SZ vs Control** ↑ *Collinsella* ↑ *Prevotella* ↑ *Lactobacillus* ↑ *Eubacterium* ↑ *Corynebacterium* (negatively assocaited with negative SZ symptoms) ↑ *Succinovibrio*(correlated to severity of SZ symptoms) ↓ *Anaerostipes* ↓ *Faecalibacterium* ↓ *Aldercreutzia* ↓ *Butyricimonas*	**SZ vs Control** ↑ *Lactobacillus*	None		(Li et al., 2020 b)
	16S (no)	N = 48 SZ N = 48 Control	**SZ vs Control** No genera-level differences identified	None	None		(Nguyen et al., 2021)
	16S (no)	N = 26 SZ with no history of violence N = 16 SZ with violent behaviours (SZV)	**SZV vs SZ** ↓ *Delftia* ↓ *Allobaculum*	None	None		(Chen et al., 2021)
	16S (no)	N = 29 SZ in remission N = 29 SZ in disease onset Used controls from Human Microbiome project	**Remission vs Acute SZ** ↑ *Clostridium sensu stricto*	None	None		(Pan et al., 2020)
	16S (no)	N = 81 High Risk of Psychosis N = 69 Control	**Ultra High Risk vs High Risk** ↑ *Lactobacillus* ↑ *Prevotella*	None	None	Rarefaction	(He et al., 2018)

		N = 19 Ultra High Risk of Psychosis					
	16S (no)	N = 30 patients *B. breve A1* probiotic given daily for 4 weeks, washout for 4 weeks $1*10^{11}$ CFU daily	**<u>Responders vs Non-Responders (4 weeks vs Baseline)</u>** ↑ *Parabacteroides** Improved HADS and PANSS	None	None	Used QIIME 1.8, Greengenes, Pilot study	(Okubo et al., 2019)
	16S (no)	N = 20 Sampled before olanzapine and after 7 day washout 6 weeks later	None	None	None	Greengenes, Did not report differences between 6 weeks and baseline; generated heirarchical cluster for stratification	(Pelka-Wysiecka et al., 2019)
	16S (no)	N = 16 Controls N = 10 First Episode Drug Naive Schizophrenia	**<u>Schizophrenia vs Control</u>** ↓ *Faecalibacterium* ↓ *Fusicatenobacter* ↓ *Coprococcus 1* ↓ *Coprococcus 2* ↓ *Butyricoccus* ↑ *Actinomyces* ↑ *Eggerthella* ↑ *Anaerotruncus* ↑ *Flavonifactor* ↑ *Holdemania* ↑ *Eisenbergiella* ↑ *Prevotella* ↑ *Ruminoccocus gnavus* ↑ *Ruminoclostridium 5* ↑ *Dorea* ↑ *Hungatella* ↑ *Bilophila* ↑ *Oscillibacter*	None	None		(Zhang et al., 2019b)

			↑ *Prevotella* ↑ *Blautia*				
	16S (no)	N = 63 Schizophrenia N = 69 Controls	None	None	None	Genus-levelenus-level differences not reporter	(Zheng et al., 2019)
	T-RFLP (no)	N = 16 Schizophrenia Inpatients Sampled before and after intervention (6 months) Prebiotic: 3g/day 4G-β-D-galactosylsucrose	**Post- vs Pre- Prebiotic Intake** ↑ *Bifidobacteirum*** ↓ *Clostridium XIVa**	**Post- vs Pre-Prebiotic Intake** ↑ *Bifidobacteirum***			(Nagamine et al., 2018)
	qPCR (no)	N = 41 SZ N = 41 Control	**SZ vs Control** ↑ *Clostridium coccoides**** ↓ *Bifidobacterium spp.**** ↓ *Escherichia coli**** ↓ *Lactobacillus spp.**** Ameliorated after 24 weeks of risperidone	**SZ vs Control** ↓ *Bifidobacterium spp.**** ↓ *Lactobacillus spp.****	None	No control/PBO	(Yuan et al., 2018)
PANS/PANDAS	16S (yes)	N = 30 with PANS/PANDAS N = 70 Controls	None	None	None		(Quagliariello et al., 2018)
Rett's Syndrome	16S (yes)	N = 8 RTT N = 10 Control	**RTT vs Control** ↑ Faecal iso-butyrate** ↑ Faecal iso-valerate**	None	None		(Borghi et al., 2017)

	16S via 454 Pyrosequencing (yes)	N = 50 RTT N = 29 Control	**RTT vs Control** ↑ Faecal propionate* ↑ Faecal iso-butyrate*** ↑ Faecal iso-valerate** No differences even when accounting for constipation and severity	None	None		(Strati et al., 2016)

Table 3. Microbiome-brain studies involving epilepsy.

Legend: LFC: log-2 fold change; LC: log change; RA: relative abundance; DASS-42: Depression Anxiety Stress Scales; ADHD: Attention-Deficit Hyperactivity Disorder; HE: hepatic encephalopathy; FOS: Fructo-oligosaccharide; HADS-A: Hamilton Anxiety and Depression Scales – Anxiety Subscale; HADS-A: Hamilton Anxiety and Depression Scales – Depression Subscale; RTT: Rett's Syndrome; *: $p_{adj} < 0.1$; **: $p_{adj} < 0.01$; ***: $p_{adj} < 0.001$; Reanalyzed studies are highlighted; 95% CI reported between square brackets [lower 95% CI; upper 95% CI]

Cohort Details	Sequencing (reanalysed)	Groups and Sample Size	SCFA/Tryptophan-Modifying Bacteria	BA-Modifying Bacteria	Other Metabolites/ GBMs	Specific Limitations	Ref
Epilepsy	WGS	N = 12 Patients with epilepsy – before and after ketogenic diet	**After diet vs Before** ↑ L-tryptophan biosynthesis* (effect = 0.9 [-1.07; 10.67])	None	**After diet vs Before** ↑ SAM Biosynthesis* (effect = 0.63 [-1.89, 8.86] ↓ L-tyrosine biosynthesis* (effect = -0.60 [-12.29; 0.96]	Low sample size, no age-matched control	**(Lindefeldt et al., 2019)**
	16S (yes)	N = 25 Cerebral Palsy with Epilepsy (CPE) N = 21 Control	**CPE vs Control** ↓ *Acidaminococcus* (effect = -1.14 [-9.60; 1.17])*** ↓ *Akkermansia* (effect = -1.52 [-11.32; 0.40])*** ↓ *Alistipes* (effect = -1.33 [-10.13; 0.48])	**CPE vs Control** ↓ *Bacteroides* (effect = -0.72 [-7.75; 2.23])*** ↓ *Bifidobacterium* (effect = -2.97 [-24.09; 0.35]) ***	None		**(Huang et al., 2019a)**

			↓ *Bacteroides* (effect = -0.72 [-7.75; 2.23])*** ↓ *Bifidobacterium* (effect = -2.97 [-24.09; 0.35]) *** ↓ *Blautia* (effect = -1.83 [-14.30; 0.41]) ↓ *Catenibacterium* (effect = -1.67 [-11.67; 0.60]) *** ↓ *Clostridium sensu stricto 1* (effect = -0.96 [-11.22, 0.81]) *** ↓ *Collinsella* (effect = -1.88 [-12.63; 0.41]) *** ↓ *Desulfovibrio* (effect = -1.40 [-11.91; 0.53]) *** ↓ *Enterococcus* (effect = -1.56 [-13.23; 0.46]) *** ↓ *Escherichia/Shigella* (effect = -1.73 [-14.91; 0.43]) *** ↓ *Eubacterium* (effect = 1-.09 [-10.91; 0.61]) *** ↓ *Faecalibacterium* (effect = -1.70 [-12.12; 0.37])*** ↓ *Flavonifractor* (effect = -0.98 [-9.93; 1.26])*** ↓ *Gemella* (effect = -0.97 [-9.03; 1.85])*** ↓ *Haemophilus* (effect = -0.95 [-8.94; 1.39])*** ↓ *Klebsiella* (effect = -1.27 (-10.67; 0.93])*** ↓ *Lactobacillus* (effect = -0.70 [-8.59; 2.31])** ↓ *Methanobrevibacter* (effect = -0.89 [-8.59; 0.78])*** ↓ *Neisseria* (effect = -0.70 [-8.07; 2.44])** ↓ *Oscillibacter* (effect = -1.10 [-10.04; 1.18])***	↓ *Eubacterium* (effect = 1-.09 [-10.91; 0.61]) *** ↓ *Lactobacillus* (effect = -0.70 [-8.59; 2.31])**			

			↓ *Parabacteroides* (effect = -1.95 [-13.85; 0.39])*** ↓ *Prevotella_2* (effect = -0.67 [-8.09; 2.23])** ↓ *Prevotella*_9 (effect = -1.08 [-9.34; 0.90])*** ↓ *Ruminiclostridium_5* (effect = -1.06 [-9.16; 1.20])*** ↓ *Ruminiclostridium_9* (effect = -0.79 [-9.80; 1.25])*** ↓ *Streptococcus* (effect = -2.20 [-19.31; 0.38])*** ↓ *Sutterella* (effect = -0.79 [-12.31; 0.56])*** ↓ *Veillonella* (effect = -1.32 [-10.60; 0.45])***				
	16S (no)	N = 30 Idiopathic Focal Epilepsy N = 10 Control	None	None	**None**		(Safak et al., 2020)
	16S (no)	N = 55 Epilepsy N = 46 Control For validation cohort: N = 13 Epilepsy N = 10 Control	**Epilepsy vs Control** ↓ *Stutterella* ↓ *Klebsiella* ↓ *Lachnospiraceae* NK4A613 ↓ *Escherichia shigella* ↓ *Lachnoclostridium* ↑ *Prevotella* ↑ *Blautia* ↑ *Bifidobacterium* ↑ *Ruminococcaceae* UCG 014 ↑ *Ruminococcus gnavus* ↑ *Megamonas* ↑ *Akkermansia* ↑ *Eubacteirum hallili* **Drug-Resistant vs Responsive Epilepsy** ↑ *Blautia* ↑ *Bifidobacteirum*	**Epilepsy vs Control** ↑ *Bifidobacterium* ↑ *Eubacteirum hallili* **Drug-Resistant vs Responsive Epilepsy** ↑ *Bifidobacterium*			(Gong et al., 2020)

			↑ *Dialister* ↑ *Anaerostipes* ↑ *Subdoligranulum*				
	16S (no)	N = 20 Samples collected from children with refractory epilepsy before and 6 mo. after diet	**<u>After Diet vs Before</u>** ↓ *Faecalibacterium* ↓ *Leucabacter* ↓ *Actinobacter* ↓ *Coprobacter* ↓ *Lachnospiracea incertae sedis* ↑ *Bacteroides* **<u>Non-Responders vs Responders</u>** ↑ *Alistipes* ↑ *Clostridium i* ↑ *Oscillibacter* ↑ *Gordonibacter* ↑ *Lachnospiracea incertae sedis* ↑ *Helicobacter* ↑ *Blautia* ↑ *Dorea* ↑ *Ruminococcus2* ↑ *Fusicatenibacter* ↑ *Eggerthella* ↑ *Anaerotruncus* ↑ *Streptococcus*	**<u>After Diet vs Before</u>** ↑ *Bacteroides*	None	Unclear if comparisons were done in a paired manner; no age-matched controls	(Zhang et al., 2018b)

	16S (no)	N = 42 drug-responsive N = 49 drug-resistant N = 65 controls (from same families as patients)	**Drug-Resistant vs Responsive** ↑ *Bacteroides* ↑ *Barnesiell* ↓ Roseburia ↓ *Phoscolarctobacterium* *a*↓ Methanobrevibacter ↓ *Fusobacterium* ↓ *Coprococcus* ↓ *Neisseria* ↓ *Akkermansia* ↓ *Gemmiger* ↓ *Ruminoccoccus2* ↓ *Paraprevotella* ↓ *Coprobacillus* ↓ *Delftia* ↓ *Saccharibacteria incertae sedis* ↓ *Dorea* ↓ *Holdemania* ↓ *Atopobium* ↓ *Clostridium* XVIII	**Drug-Resistant vs Responsive** ↑ *Bacteroides*	None	No comparisons reported for controls; statistical methods unclear	(Peng et al., 2018)
	16S (no)	N = 30 healthy infants N = 14 epileptic infants	Difficult to interpret	None		Greengenes, No pair-wise group comparisons	(Xie et al., 2017)

Table 4. Microbiome-brain studies involving neurodegenerative disorders.

Legend: LFC: log-2 fold change; LC: log change; RA: relative abundance; DASS-42: Depression Anxiety Stress Scales; ADHD: Attention-Deficit Hyperactivity Disorder; HE: hepatic encephalopathy; FOS: Fructo-oligosaccharide; HADS-A: Hamilton Anxiety and Depression Scales – Anxiety Subscale; HADS-A: Hamilton Anxiety and Depression Scales – Depression Subscale; RTT: Rett's Syndrome; AD: Alzheimer's Disease, PD: Parkinson's Disease; MCI: mild-cognitive impairment; MSA: multiple-systems atrophy; ALS: amyotrophic lateral sclerosis*: $p_{adj} < 0.1$; **: $p_{adj} < 0.01$; ***: $p_{adj} < 0.001$; Reanalyzed studies are highlighted; 95% CI reported between square brackets [lower 95% CI; upper 95% CI]

Cohort Details	Sequencing (reanalysed)	Groups and Sample Size	SCFA/Tryptophan-Modifying Bacteria	BA-Modifying Bacteria	Other Metabolites/ GBMs	Specific Limitations	Ref

AD and MCI	16S (yes – extreme proportion of unmapped reads)	N = 33 AD N = 32 Mild Cognitive Impairment (MCI), N = 32 Controls	None	None	None	Too many unmapped reads	(Liu et al., 2019b)
	16S (yes)	N = 30 AD N = 30 MCI N = 30 Controls	**<u>AD vs Control</u>** ↓ *Ruminoclostridium 5*** (effect = -0.67[-8.52; 1.59]) ↑ Isovaleric-Acid Synthesis II* (effect = 0.42 [-2.11, 5.62]) ↑ Acetate Synthesis III* (effect = 0.50 [-2.04, 5.92]) ↑ Butyrate Synthesis I* (effect = 0.44 [-2.33, 6.25]) ↑ Butyrate Synthesis II* (effect = 0.52 [-3.60, 4.79]) **<u>MCI vs Control</u>** ↑ Isovaleric-Acid Synthesis II** (effect = 0.43 [-2.48; 5.98]) ↑ Acetate Synthesis I** (effect = 0.58 [-1.93; 5.86]) ↑ Acetate Synthesis II* (effect = 0.47 [-1.81; 5.33]) ↑ Acetate Synthesis III** (effect = 0.64 [-1.52; 6.26]) ↑ Tryptophan synthesis* (effect = 0.48 [-1.94, 6.71] ↑Quinolinic Acid Synthesis** (effect = 0.49 [-2.02; 6.43]) ↑Quinolinic Acid Degradation ** (effect = 0.56 [-1.93; 6.43])	None	**<u>MCI vs Control</u>** ↑ Glutamate synthesis I** (effect = 0.50 [-1.94; 5.90]) ↑ Glutamate synthesis II** (effect = 0.71 [-1.4; 6.25]) ↑ Histamine degradation* (effect = 0.46 [-2.36, 5.46]) ↑ p-Cresol Synthesis** effect = 0.64 [-1.61; 7.26]) ↑ClpB** (effect = 0.55 [-1.99; 6.30]) ↑17-Beta-Estradiol Degradation** (effect = 0.65 [-1.46; 6.58]) ↑ SAM Synthesis* (effect = 0.58 [-2.01; 5.92]) ↓ Glutamate Degradation II** (effect = -0.50 [-7.02, 2.42])		(Li et al., 2019a)

					↓ Vitamin K2 Pathway Synthesis II** (effect = -0.42 [-1.52; 6.3]) **<u>AD vs Control</u>** ↑ Glutamate Synthesis II* (effect = 0.45 [-2.03; 5.63]) ↑ Histamine Degradation* (effect = 0.46 [-2.51; 5.64]) ↑ p-Cresol Synthesis* (effect = 0.54 [-2.26, 5.82]) ↓ Vitamin K2 Pathway Synthesis II* (effect = -0.44 [-5.73, 2.83]) ↓ Glutamate Degradation II* (effect = -0.50 [-7.02, 2.42]) ↓ GABA Synthesis III* (effect = -0.48 [-5.90, 1.57]) ↓ Vitamin K2 Pathway Synthesis I (effect = -0.46 [-5.79, 2.03])		

	WGS (no)	N = 24 AD N = 33 Other Dementia N = 51 Controls	**AD vs Control** ↑ *Bacteroides** ↑ *Alistipes**** ↑ *Odoribacter**** **AD vs Other Dementia** ↑ *Odoribacter** ↓ *Eubacterium**** ↓ *Roseburia**	**AD vs Control** ↑ *Bacteroides**	None		(Haran et al., 2019)
	16S (no)	N = 43 AD N = 43 age and gender-matched controls	**AD vs Control** ↑ *Subdoligranulum*** ↓ *Bacteroides***	**AD vs Control** ↓ *Bacteroides***	None		(Zhuang et al., 2018)
	16S (no)	N = 25 AD N = 25 Control	**AD vs Control** ↑ *Blautia** ↑ *Bacteroides**** ↑ *Alistipes** ↑ *Phascolarctobacterium** ↓ *Bifidobacterium** ↓ *Dialister**** ↓ *Clostridium** ↓ *Turcibacter****	**AD vs Control** ↑ *Bacteroides**** ↓ *Bifidobacterium**	None	Greengenes	(Vogt et al., 2017)
	qPCR (no)	N = 40 Cognitively Impaired with Amyloidosis (AMY+) N = Cognitively Impaired No Amyloidosis (AMY-) N = 10 Control (Age and sex-matched)	**AMY+ vs AMY-** ↑ *Escherichia/Shigella**** ↓ *Eubacterium rectale**** **AMY+ vs Control** ↑ *Escherichia/Shigella**** ↓ *Bacteroides fragilis** ↓ *Eubacterium rectale**** **AMY- vs Control** ↑ *Escherichia/Shigella*** ↓ *Eubacterium rectale***	**AMY+ vs AMY-** ↓ *Eubacterium rectale**** **AMY+ vs Control** ↓ *Bacteroides fragilis** ↓ *Eubacterium rectale**** **AMY- vs Control** ↓ *Eubacterium rectale***	None		(Cattaneo et al., 2017)

	qPCR (no)	N = 20 AD Outpatients Prospective trial of probiotic treatment (28 days)	**AD after Probiotic vs Baseline** ↑ *Faecalibacterium prausnitzii***	None	None	Faeces stored at -18C	(Leblhuber et al., 2018)
MSA	16S (no)	N = 40 MSA N = 40 Control (spouses)	***MSA vs Control*** ↑ *Lactobacillus* ↑ *Gordonibacter* ↑ *Phascolarctobacterium* ↓ *Haemophilus*	***MSA vs Control*** ↑ *Lactobacillus*	None	Rarefaction	(Du et al., 2019)
	16S (no)	N = 6 MSA N = 11 Control	None	None	None		(Engen et al., 2017)
	16S (no)	N = 17 MSA N = 17 Control	***MSA vs Control*** ↑ *Bacteroides*** ↓ *Prevotella clara** ↓ *Paraprevotella**** ↓ Faecal acetate, propionate and butyrate	***MSA vs Control*** ↑ *Bacteroides***	None		(Tan et al., 2018)
ALS	WGS (no)	N = 37 ALS N = 29 age and BMI-matched controls	**ALS vs Control** ↓ Tryptophan metabolism genes				(Blacher et al., 2019)
	WGS and 16S (no)	16S N = 20 ALS N = 20 Control WGS N = 10 ALS N = 10 Control	**ALS vs Control** ↑ *Enterococcus columbae*	None	None		(Zeng et al., 2020)
	WGS & 16S (no)	N = 66 ALS N = 12 Neurodegenerative Control (ND)	**ALS vs Control** ↑ *Prevotella copri*	**ALS vs Control** ↑ *Bacteroides clarus*	None		(Nicholson et al., 2020)

		N = 61 Healthy Control	↑ *Phascolarctobacterium succinatutens* ↑ *Bacteroides clarus* ↑ *Dorea* ↑ *Escherichia* ↓ *Aldercreutzia equolifaciens* ↓ *Lachnospiraceae bacterium* 5 1 63FAA ↓ *Coprobacter fastidious* ↓ *Ruminococcus lactaris* ↓ *Eubacterium eligens* ↓ *Ruminococcus* sp 5 1 39BFAA ↓ *Bifidobacterium longum* ↓ *Roseburia intestinalis* ↓ *Eubacterium rectale* Decrease in butyrate producers **ALS vs ND** ↑ *Ruminoccous gnavus* ↑ *Veillonella parvula* ↓ *Lachnospiraceae* bacterium 3 1 57FAA CT1 ↓ *Lachnospiraceae* bacterium 1 1 57FAA ↓ *Lachnospiraceae* bacterium 5 1 63FAA ↓ *Parasutterella excrementihominis* ↓ *Roseburia hominis* ↓ *Burkholderiales* bacterium 1 1 47 ↓ *Oscillibacter*	↓ *Bifidobacterium longum*			
	16S (no)	N = 8 ALS N = 8 Control	None	None	None	No differential abundance testing	(Zhai et al., 2019a)
	16S (no)	N = 49 Motor Neuron Disease N = 51 Control	None	None	None		(Ngo et al., 2020)

	16S via 454 Pyrosequencing (no)	N = 25 ALS N = 32 Control	No differences in known microbes	None	None		(Brenner et al., 2018)
	qPCR (no)	N = 50 ALS N = 50 Control	**<u>ALS vs Control</u>** ↑ *Enterobacter* ↑ *Escherichia coli* ↓ *Clostridium*	None	None		(Mazzini et al., 2018)
Parkinson's Disease	WGS (yes)	N = 31 PD N = 28 Controls	None	None	None		(Bedarf et al., 2017)
	16S (yes – extreme proportion of unmapped reads)	N = 76 PD N = 21 idiopathic rapid eye movement sleep behaviour disorder N =78 Controls	None	None	None	Extreme proportion of unmapped reads	(Heintz-Buschart et al., 2018)
	16S (yes)	N = 64 PD N = 64 Controls	None	None	**<u>PD vs Control at Follow-up</u>** ↑ p-Cresol **synthesis (effect = 0.45 [-2.03; 5.18])		(Aho et al., 2019)
	16S (yes)	N = 80 PD N = 72 Controls	None	None	None		(Pietrucci et al., 2019)
	16S (yes)	N = 34 PD N = 25 Control	**<u>PD with Low L-DOPA (<300mg/day) dose vs Control</u>** ↓ *Lactobacillus* (effect = 0.83 [-2.10; 8.07]		None		(Weis et al., 2019)
	16S (yes, large proportion of	N = 45 PD N = 45 Controls (spouses)	None	None	None		(Qian et al., 2018)

	sequences could not be classified)						
	WGS (no)	N = 40 PD N = 40 Controls (spouses)	**PD vs Control** ↑ *Alistipes* ↑ *Holdemania* ↑ *Streptococcus* ↑ *Gordonibacter* ↑ *Lactobacillus* ↑ *Enterobacter* *Streptococcus salivarius* negatively correlated to L-DOPA dose equivalency *Enterobacter cloacae* positively correlated with unified Parkinson's Disease rating scale	**PD vs Control** ↑ *Lactobacillus*	None		(Qian et al., 2020)
	16S (no)	N = 197 PD N = 130 Controls	**PD vs Control (significant via Kruskal-Wallis and ANCOM after adjusting for covariates and COMT/AC)** ↑ *Bifidobacterium**** (Abundance: 0.0089 vs 0.0076) ↑ *Lactobacillus**** (Abundance: 0.0017 vs 0.0004) ↑ *Akkermansia**** (Abundance: 0.0476 vs 0.0185) ↓ *Roseburia* (OTU1)* (Abundance: 0.0073 vs 0.0125)	**PD vs Control (significant via Kruskal-Wallis and ANCOM after adjusting for covariates and COMT/AC)** ↑ *Bifidobacterium**** (Abundance: 0.0089 vs 0.0076) ↑ *Lactobacillus**** (Abundance: 0.0017 vs 0.0004)	None	Greengenes, Rarefaction, No direct comparison between individuals taking COMT/AC to those that aren't	(Hill-Burns et al., 2017)

	16S (no)	N = 13 PD-MCI N = 13 PD with no MCI (PD-NC) N = 13 Control Spouses	GLMs incorporated sex, age, bMI, education **<u>PD-MCI vs Control</u>** ↓ *Alistipes**** ↓ Odoribacter*** ↓ *Barnesiella**** ↓ *Butyricomonasi**** **<u>PD-MCI vs PD-NC</u>** ↓ *Alistipes**** ↓ Odoribacter*** ↓ *Barnesiella**** ↓ *Butyricomonasi**** ↑ *Ruminococcus**** ↑ *Blautia**** **<u>PD-NC vs Control</u>** ↓ *Ruminococcus**** ↓ *Blautia****				(Ren et al., 2020)
	16S (no)	N = 666 Aged individuals	Motor deficits indicating subthreshold parkinsonism associated with ↓ *Odoribacter*			Associative study looking to identify prodromal markers for Parkinsonism	(Heinzel et al., 2020)
	16S (no)	N = 80 PD N = 77 Controls	**<u>PD vs Control after accounting for age, sex, diet</u>** ↑ *Parabcteroides* ↓ *Prevotella* (reduced by 46.6%) **<u>PD Tremor-Subtype vs PD Non-Tremor Subtype accounting for age, sex, diet</u>**			Greengenes	(Lin et al., 2019)

			↑ *Clostridium* ↑ *Akkermansia* ↓ *Propionibacterium* ↓ *Sutterella* ↓ *Desulfvibrioo* **Positive Correlations:** *Bacteroides* abundance and TNFα				
	16S (no)	N = 89 PD N = 66 Control	**<u>PD vs Control</u>** ↑ *Lactobacillus**** ↑ *Bifidobacterium** ↓ *Faecalibacterium** ↓ *Prevotella** ↓ *Dorea****	**<u>PD vs Control</u>** ↑ *Lactobacillus**** ↑ *Bifidobacterium**			(Petrov et al., 2017)
	16S (no)	N = 29 PD N = 29 Controls	None	None	None	No genus information reported	(Hopfner et al., 2017)
	16S (no)	N = 104 PD N = 96 Control	**<u>PD vs Control</u>** ↑ *Bacteroides fragilis* ↑ *Lactobacillus acidophilus* ↑ *Megasphaera* ↑ *Veillonella* ↑ *Coriobacteria* ↑ *Akkermansia muciniphilia* ↑ *Bifidobacterium bifidum BGN4* ↑ *Bacteroides fragilis NCTC 9343* ↑ *Clostridium saccharolyticum WM1* Reduction in all fecal acetate, butyrate and propionate in low cognitive scoring patients	**<u>PD vs Control</u>** ↑ *Bacteroides fragilis* ↑ *Lactobacillus acidophilus* ↑ *Bifidobacterium bifidum BGN4* ↑ *Bacteroides fragilis NCTC 9343* ↓ Bile acid degradation pathways			(Tan et al., 2020)
	16S (no)	N = 25 PD Sequenced at baseline, 1 year, 2 year and 3 year follow-up	↓ *Roseburia* linked to development of non-motor, severity of mnesic-attention disorders ↓ *Roseburia* and *Faecalibacterium* at baseline linked to faster cognitive decline			Greengenes, collection at -20C	(Cilia et al., 2020)

			↑ *Oscillospira* at baseline linked to faster cognitive decline Results not significant after post-hoc correction				
	16S (no)	N = 63 PD N = 63 Healthy spouses (HS) N = 74 Control	**<u>PD vs Control</u>** ↑ *Oscillospira**** ↑ *Akkermansia**** ↓ *Fusobacterium*** **<u>PD vs HS</u>** ↑ *Oscillospira**** ↑ *Akkermansia**** ↓ *Fusobacterium*** **<u>Genera positively associated with disease stage and duration:</u>** *Parabacteroides, Akkermansia, Coprococcus, Bilophila, Collinsella, Methanobrevibacter, Eggerthella, Adlercreutzia*	None	None		(Zhang et al., 2020a)
	16S (no)	N = 64 PD N = 51 Control	**<u>PD vs Control</u>** ↑ *Veillonella**** (mean difference = 1.556) ↓ *Blautia** (mean difference = -0.596) ↓ *Butyrivibrio*** (mean difference = -0.951) ↓ *Coprococcus** (mean difference = -0.873)	None	None	Greengenes	(Vascellari et al., 2020)
	16S (no)	N = 24 PD N = 14 Controls	**<u>PD vs Controls</u>** ↑ *Enterococcus*** ↑ *Escherichia-Shigella** ↑ *Streptococcus*** ↑ *Proteus** ↓ *Blautia** ↓ *Faecalibacterium** ↓ *Ruminococcus**	**<u>PD vs Controls</u>** ↑ *Escherichia-Shigella***, ↑ *Enterococcus***	None	Rarefaction, 80% confidence level for SILVA alignment	(Li et al., 2017)

	16S (no)	N = 75 PD N = 45 Controls	None	None	None	Greengenes, rarefaction	(Lin et al., 2018)
	16S (no)	N = 10 PD N = 10 Controls	**PD vs Controls** ↑ *Akkermansia*,* ↑ *Parasutterella* * ↑ *Subdoligranulum** ↑ *Butyricimonas** ↓ *Clostridium** ↓ *Collinsella** ↓ *Bacteroides**	**PD vs Controls** ↓ *Bacteroides**	None	QIIME v1.8 (outdated since Jan 1, 2018), Greengenes	(Li et al., 2019b)
	16S (no)	N = 193 PD (de novo – 39, early – 57, mid-stage – 53, advanced – 44) N = 113 Controls	**PD vs Control** ↑ *Bifidobacterium** ↓ *Roseburia** ↓ *Ruminococcus*** **Mid-Stage and Advanced PD vs Control** ↑ *Lactobacillus****	**PD vs Control** ↑ *Bifidobacterium** **Mid-Stage and Advanced PD vs Control** ↑ *Lactobacillus****	**PD vs Control** ↑ *Bifidobacterium**: GABA pathway **Mid-Stage and Advanced PD vs Control** ↑ *Lactobacillus****: GABA pathway	Greengenes	(Barichella et al., 2019)
	16S (no)	N = 197 PD N = 103 Controls	**PD vs Control** ↑ *Bifidobacterium*** (4.02 fold change)* ↓ *Roseburia*** (0.71 fold change)* ↓ *Faecalibacterium prasunitzii**** (0.75 fold change)*	**PD vs Control** ↑ *Bifidobacterium**** (4.02 fold change)*	**PD vs Control** ↑ p-Cresol synthesis	Greengenes	(Cirstea et al., 2020)
	16S (no)	N = 9 PD N = 13 Controls	**PD vs Control** ↑ *Akkermansia**	None	None	Qiime 1.8	(Vidal-Martinez et al., 2020)
	16S (no)	N = 34 PD N = 31 Controls	**PD vs Control** ↑ *Bacteroides**	**PD vs Control** ↑ *Bacteroides**	None	Rarefaction, compared	(Keshavarzi

			↑ *Oscillospira** ↑ *Akkermansia** ↓ *Blautia** ↓ *Coprococcus** ↓ *Dorea** ↓ *Roseburia**			raw # of sequences compared raw # of sequences	an et al., 2015)
	16S (no)	N = 54 PD N = 34 Controls Enema and nutrition intervention	None	None	None	No genus information reported	(Hegelmaier et al., 2020)
	16S via 454 Pyrosequencing (no)	N = 74 PD N = 75 Controls	**PD (IBS+) vs PD (IBS-)** ↓ *Bacteroides* (LFC = -4.929) * ↓ *Prevotella* (LFC = -5.675) ***	**PD (IBS+) vs PD (IBS-)** ↓ *Bacteroides* (LFC = -4.929) *	None		(Mertsalmi et al., 2017)
	16S via 454 Pyrosequencing (no)	N = 72 PD N = 72 Controls	None	None	None	No genus information reported	(Scheperjans et al., 2015)
	qPCR (no)	N = 45 PD N = 35 Controls	**PD vs Control** ↑ *Lactobacillus* ↓ *Clostridium coccoides*** ↓ *Clostridium leptum** ↓ *Bacteroides fragilis**	**PD vs Control** ↑ *Lactobacillus* ↓ *Bacteroides fragilis**	None		(Hasegawa et al., 2015)
	qPCR (no)	N = 28 PD N = 17 Stable N=11 Deteriorated	**Follow-up vs Baseline (All PD)** ↓ *Bifidobacterium* ↓ *Clostridium leptum* subgroup ↓ *Bacteroides fragilis* group ↓ *Atopobium* cluster ↓ *Enterococcus* ↓ *L. gasseri* subgroup	**Follow-up vs Baseline (All PD)** ↓ *Bifidobacterium* ↓ *Bacteroides fragilis* group ↓ *Lactobacillus gasseri* subgroup	None		(Minato et al., 2017)

			↓ *Lactobacillus reuteri* subgroup ↓ *Prevotella* **Follow-up vs Baseline (Stable)** ↓ *Bifidobacterium* ↓ *Clostridium leptum* subgroup ↓ *Bacteroides fragilis* group ↓ *Atopobium* cluster ↓ *Enterococcus* ↓ *Lactobacillus. gasseri* subgroup ↓ *Lactobacillus reuteri* subgroup **Follow-up vs Baseline (Deteriorated)** ↓ *Lactobacillus gasseri* subgroup	↓ *Lactobacillus reuteri* subgroup **Follow-up vs Baseline (Stable)** ↓ *Bifidobacterium* ↓ *Bacteroides fragilis* group ↓ *Lactobacillus gasseri* subgroup ↓ *Lactobacillus reuteri* subgroup **Follow-up vs Baseline (Deteriorated)** ↓ *Lactobacillus gasseri* subgroup			
	qPCR (no)	N = 19 PD with COMT inhibitor N = 14 PD without COMT inhibitor	**COMT inhibitor (Entacapone) vs No COMT Inhibitor** ↓ *Faecalibacterium prausnitzii* **COMT inhibitor (Entacapone) vs Other COMT Inhibitors** ↓ *Faecalibacterium prausnitzii*	None	None		(Grun et al., 2020)
	16S qPCR (no)	N = 34 PD N = 34 Control	**PD vs Age-Matched Control** ↑ *Bifidobacterium**** ↓ *Faecalibacterium prausnitzii* ↓ *Lactobacilli/Enterococci**** ↓ Acetate** ↓ Butyrate** ↓ Propionate**	**PD vs Age-Matched Control** ↑ *Bifidobacterium**** ↓ *Lactobacilli/Enterococci****	None		(Unger et al., 2016)

Table 5. Microbiome-brain studies involving alcohol, nicotine and recreational drug use/addiction.

Legend: LFC: log-2 fold change; LC: log change; RA: relative abundance; DASS-42: Depression Anxiety Stress Scales; ADHD: Attention-Deficit Hyperactivity Disorder; HE: hepatic encephalopathy; FOS: Fructo-oligosaccharide; HADS-A: Hamilton Anxiety and Depression Scales – Anxiety Subscale; HADS-A: Hamilton Anxiety and Depression Scales – Depression Subscale; RTT: Rett's Syndrome; AD: Alzheimer's Disease, PD: Parkinson's Disease; MCI: mild-cognitive impairment; MSA: multiple-systems atrophy; ALS: amyotrophic lateral sclerosis*: $p_{adj} < 0.1$; **: $p_{adj} < 0.01$; ***: $p_{adj} < 0.001$; Reanalyzed studies are highlighted; 95% CI reported between square brackets [lower 95% CI; upper 95% CI]

Cohort Details	Sequencing (reanalysed)	Groups and Sample Size	SCFA/Tryptophan-Modifying Bacteria	BA-Modifying Bacteria	Other Metabolites/ GBMs	Specific Limitations	Ref
Alcohol Use and Dependence	16S (yes)	N = 15 Healthy participants, compared before and after acute binge	None	None	None	Binge is only 2mL of vodka	(Stadlbauer et al., 2019)
	16S (yes)	N = 15 Alcohol-Dependent N = 15 Control	**Alcohol-Dependent vs Control** ↑ *Ruminoccocus 2** (effect = 0.72 [-2.91; 6.75]) ↓ *Ruminoclostridium 9**** (effect = -0.99 [-7.99; 1.00]) ↓ Tryptophan degradation* (effect = -0.46 [-5.78; 2.47])	None	**Alcohol-Dependent vs Control** ↑ GABA synthesis III* (effect = 0.52 [-1.99; 5.66]) ↓ g-Hydroxybutyric acid (GHB) degradation** (effect = -0.77 [-6.99; 1.27]) ↓ Dopamine degradation* (effect = -0.56 [-7.71; 1.95])		(Bjorkhaug et al., 2019)
	Shotgun (no - SOLiD platform)	N = 72 Alcohol dependence syndrome (ADS)	***ADS vs Control*** ↑ *Lactococcus* ↑ *Lactobacillus salivarius* ↑ *Lactococcus lactis* subsp. *Cremoris* ↓ *Prevotella*	***ADS vs Control*** ↑ *Lactobacillus salivarius* **ALC vs Control**	None		(Dubinkina et al., 2017)

		N = 27 Alcoholic liver cirrhosis (ALC) N = 60 Controls	**<u>ALC vs Control</u>** ↑ *Bifidobacterium* (*B. longum*, *dentium*, and *breve*) ↑ *Streptococcus* (*S. thermophilus* and *mutans*) ↑ *Lactobacillus* species (*L. salivarius*, *antri*, and *crispatus*) ↓ *Coprococcus*	↑ *Bifidobacterium* (*B. longum*, *dentium*, and *breve*) ↑ *Lactobacillus* species (*L. salivarius*, *antri*, and *crispatus*)			
	16S (no)	N = 14 Non-Smoking, Non-Drinking N = 31 Smoking only N = 28 Drinking only N = 43 Smoking and drinking	**<u>Associations with Smoking and Drinking</u>** *Bacteroides*** *Phascolarctobacterium** *Ruminococcus UCG-002*** *Ruminococcus UCG-003*** *Ruminoclostridium 9**** **<u>Associations with Drinking Only</u>** *Haemophilus**	**<u>Associations with Smoking and Drinking</u>** *Bacteroides***	None		(Lin et al., 2020)
	16S (no)	N = 212 twins pairs	**<u>AUDIT score III (high alcohol consumption) vs Medium and Low Alcohol Consumption Groups</u>** ↑ *Prevotella copri** ↑ *Megamonas**** (4 OTUS) ↓ *Blautia obeum** ↓ *Roseburia** *Roseburia* survived correction for heritability	None	None	Greengenes	(Seo et al., 2020)
	16S via 454 Pyrosequencing & qPCR (no)	N = 13 Alcohol Dependent (6 with high intestinal permeability (IP), 7 without) N = 15 Controls	**<u>High IP vs Low IP</u>** ↓ *Ruminococcus* ↓ *Faecalibacterium* ↓ *Clostridium* ↓ *Bifidobacterium* spp. *Bifidobacterium* spp. and *Blautia* negatively correlated to IP **<u>After 3 Weeks of Detoxification</u>**	**<u>High IP vs Low IP</u>** ↓ *Bifidobacterium* **<u>After 3 Weeks of Detoxification</u>** ↑ *Bifidobacteria* spp. ↑ *Lactobacillus* spp	None		(Leclercq et al., 2014)

			↑ *Bifidobacteria* spp., ↑ *Lactobacillus* spp.				
	16S via 454 Pyrosequencings (no)	N = 16 Alcohol Dependent N = 48 Control	**Alcoholic vs Control** ↑ *Streptococcus* ↓ *Bacteroides* ↓ *Eubacterium* ↓ *Anaerostipes* **Alcoholic+Smoker vs Control Non-Smoker** ↑ *Streptococcus* ↓ *Bacteroides* ↓ *Eubacterium* ↓ *Anaerostipes* ↓ *Ruminococcus* **Alcohol+Non-Smoker vs Non-Smoker Control** ↓ *Bifidobacterium* ↓ *Anaerostipes* **Control Smoker vs Control Non-Smoker** ↓ *Faecalibacterium*	**Alcoholic vs Control** ↓ *Bacteroides* ↓ *Eubacterium* **Alcoholic+Smoker vs Control Non-Smoker** ↓ *Bacteroides* ↓ *Eubacterium* **Alcohol+Non-Smoker vs Non-Smoker Control** ↓ *Bifidobacterium*	None		(Tsuruya et al., 2016)
	16S rRNA for *Proteobacteria* and *Faecalibacterium* (no)	N = 28 Alcohol Overconsumption N = 25 Control	None	None	None	No associations found	(Bjorkhaug et al., 2020)
Opioids	16S (yes)	N = 99 High-disease burden/opioid use men	None	None	None		(Barengolts et al., 2018)
Nictoine/Tobacco/Smoking	16S (yes)	N = 10 Electronic Cigarette N = 10 Tobacco N = 10 Control	**Tobbacco Smoker vs Non-Smoker** ↑ Tryptophan Degradation* (effect = 0.84 [-0.97; 8.52]) ↑ Propionate Synthesis III* (effect = 0.94 [-0.90; 7.52])	None	**Tobbacco Smoker vs Non-Smoker**		(Stewart et al., 2018)

			↓ Propionate Synthesis II* (effect = -0.80 [9.86; 1.45])		↓17-beta-Estradiol degradation* (effect = - 0.74 [9.90; 1.19])		
	Shotgun (no)	N = 21 Smokers with Crohn's Disease N = 21 Smoker's without Crohn's Disease	None	None	None	No non-smoking controls	(Opstelten et al., 2016)
	454 Pyrosequencing (no)	N = 5 Continuing Smokers N = 5 Non-Smokers N = 10 Undergoing smoking cessation	None	None	None	Lack of genus-levelenus-level resolution	(Biedermann et al., 2013)
	qPCR (no)	N = 14 Smokers N = 6 Non-Smokers	**<u>Smokers vs Non-Smokers</u>** ↓ *Bifidobacterium**				(Ishaq et al., 2017)
	Fluorescence in-situ hybridization (no)	N = 101 with Crohn's (29 smokers) N = 58 Controls (8 smokers)	None	None	None	Lack of genera level resolution	(Benjamin et al., 2011)
Recreational Drug Use	16S (no)	N = 37 at two timepoints (HIV+ cohort)	↓ *Ruminococcus2* with methamphetamines, prescription drug use ↑ *Ruminoccus2* with synthetic drugs, 'poppers' use	None	None	Rarefaction	(Fulcher et al., 2018)

	16S (no)	N = 48 Users N = 45 Controls	No significance after controlling for sex and age				(Xu et al., 2017)
	16S (no)	N = 20 Marijuana users N = 19 Controls	*Prevotella* abundance associated positively with cognitive function in users				(Pane et al., 2018)

Table 6. Microbiome-brain studies involving demyelinating disease.

Legend: LFC: log-2 fold change; LC: log change; RA: relative abundance; DASS-42: Depression Anxiety Stress Scales; ADHD: Attention-Deficit Hyperactivity Disorder; HE: hepatic encephalopathy; FOS: Fructo-oligosaccharide; HADS-A: Hamilton Anxiety and Depression Scales – Anxiety Subscale; HADS-A: Hamilton Anxiety and Depression Scales – Depression Subscale; RTT: Rett's Syndrome; AD: Alzheimer's Disease, PD: Parkinson's Disease; MCI: mild-cognitive impairment; MSA: multiple-systems atrophy; ALS: amyotrophic lateral sclerosis; NMOSD: neuromyelitis optica spectrum disorder*: $p_{adj} < 0.1$; **: $p_{adj} < 0.01$; ***: $p_{adj} < 0.001$; Reanalyzed studies are highlighted; 95% CI reported between square brackets [lower 95% CI; upper 95% CI]

Cohort Details	Sequencing (reanalysed)	Groups and Sample Size	SCFA/Tryptophan-Modifying Bacteria	BA-Modifying Bacteria	Other Metabolites/ GBMs	Specific Limitations	Ref
Multiple Sclerosis and Other Demyelinating Conditions	16S (yes)	N = 60 MS N = 43 Control	*None*	None	None		(Jangi et al., 2016)
	16S (yes)	N = 84 NMOSD N = 54 Control	**NMOSD vs Control** ↑ *Streptococcus* (effect = -0.74 [-6.40; 1.63])*** ↓ Faecal SCFAs Acetate and butyrate negatively associated with severity				(Gong et al., 2019)
	16S via 454 Pyrosecuencing (yes)	N = 40 Controls N = 40 MS	None	None	**MS vs Control** ↓ GABA Degradation* (effect = -0.61 [-2.24, 8.46]) ↑ p-Cresol Synthesis*		(Miyake et al., 2015)

					(effect = -0.54 [-2.07, 8.10])		
	WGS (no)	N = 26 MS N = 77 Control	**<u>MS vs Control</u>** ↑ *Sutterella* sp.** (effect = 2.73) ↓ *Gemella morbillorum*** (effect = -0.95)	None	None		(Kishikawa et al., 2020)
	WGS and 16S (no)	N = 34 Discordant twin pairs	None	None	None		(Berer et al., 2017)
	WGS and 16S (no)	<u>Caucasion</u> N = 15 MS N = 15 Control <u>Hispanic</u> N = 16 MS N = 15 Control <u>African American</u> N = 14 MS N = 14 Control	**<u>Caucasian: MS vs Control</u>** ↑ *Akkermansia* ↑ *Clostridium* **<u>Hispanic: MS vs Control</u>** ↑ *Blautia* ↑ *Clostridium* ↑ *Dorea* ↑ *Holdemania* ↓ *Dialister* ↓ *Prevotella* **<u>African American: MS vs Control</u>** ↑ *Clostridium*				(Ventura et al., 2019)
	16S (no)	N = 22 MS N = 33 Control	**<u>MS vs Control</u>** ↑ *Blautia* ↑ *Flavonifractor* ↓ *Faecalibacterium* ↓ *Roseburia* ↓ *Haemophilus* ↓ *Bilophila* ↓ *Dorea* ↓ *Butyricicoccus* ↓ *Gemella* ↓ *Clostridium* XIVb			Greengenes	(Ling et al., 2020b)

	16S (no)	N = 26 Relapse-Remitting MS (RRMS) N = 12 Secondary Progressive MS (SPMS) N = 38 Control	**MS vs Control** ↑ *Akkermansia* ↑ *Collinsella* ↑ *Eubacterium* ↓ *Parabacteroides* ↓ *Roseburia* ↓ *Coprococcus* ↓ *Blautia* **SPMS vs Control** ↑ *Akkermansia* ↑ *Collinsella* ↓ *Roseburia* ↓ *Coprococcus* ↓ *Blautia* ↓ *Dorea* **RRMS vs Control** ↑ *Streptococcus* ↓ *Roseburia* ↓ *Coprococcus* ↓ *Blautia* ↓ *Lachnospira* ↓ *Ruminococcus* ↓ *Parabacteroides*	**MS vs Control** Increased serum intestinal-fatty acid binding protein correlated with *Parabacteroides*			(Saresella et al., 2020)
	16S (no)	N = 98 MS N = 120 Control	**MS vs Control** ↓ *Alistipes* (Effect = -0.18)* ↓ *Anaerotruncus* (Effect = -0.16)* ↓ *Butyricoccus* (Effect = -0.24)** ↓ *Clostridium* cluster IV (Effect = -0.35)*** ↓ *Gemmiger* (Effect = -0.30)*** ↓ *Lactobacillus* cluster IV (Effect = -0.18)* ↓ *Methanobrevibacter* (Effect = -0.20)* ↓ *Olsonella* (Effect = -0.19)* ↓ *Parabacteroides* (Effect = -0.15)* ↓ *Roseburia* (Effect = -0.17)* ↓ *Ruminococcus* (Effect = -0.17)*	**MS vs Control** ↓ *Lactobacillus* cluster IV (Effect = -0.18)*			(Reynders et al., 2020)

			↓ *Sporobacter* (Effect = -0.39)*** Many differences within clinical subtypes : *Butyricoccus, Clostridium* cluster IV and XCIII, *Gemmiger, Methanobrevibacter, Parabacteroides, Sporobacter*				
	16S (no)	N = 17 paediatric MS	None	None	None	Greengenes, no genus-levelenus-level associations reported	(Tremlett et al., 2016a)
	16S (no)	N = 18 Pediatric MS N = 17 Control	**MS vs Control** ↑ *Bilophila**** (FC = 3 [2.9; 3.2]) ↑ *Bifidobacterium**** (FC = 4.2 [3.9; 4.5]) ↑ *Desulfovibrio****(FC = 5.1 [4.7; 5.7]) ↑ *Prevotella copri****(FC = 5 [4.4; 5.6])	None	None		(Tremlett et al., 2016c)
	16S (no)	N = 15 Pediatric Relapse-Remitting MS N = 9 Control	None	None	None	No genus-levelenus-level changes reported	(Tremlett et al., 2016b)
	16S (no)	N = 15 Primary Progressive MS N = 15 Control	**MS vs Control** ↑ *Gemmiger*	None	None		(Kozhieva et al., 2019)
	16S (no)	N = 17 Pediatric MS	None	None	None	No genus-levelenus-level differences reported	(Nourbakhsh et al., 2018)

	16S (no)	N = 9 relapsing-remitting MS N = 13 Controls Given VSL-3 probioticcs	VSL3 Administration associated with ↑ *Lactobacillus, Streptococcus, Bifidobacterium*			Greengenes	(Tankou et al., 2018)
	16S (no)	N = 8 MS no fasting N = 8 MS with fasting Intermittent fasting (IF) pilot	None	None	None		(Cignarella et al., 2018)
	16S (no)	N = 34 relapsing-remitting MS N = 33 Neuromyelitis optica spectrumdisorder (NMOSD) N = 34 Control	**<u>MS vs Control</u>** ↑ *Streptococcus* ↓ *Faecalibacterium* ↓ *Prevotella 9* ↓ Faecal acetate*** ↓ Faecal butyrate* ↓ Faecal propionate*** **<u>NMOSD vs MS</u>** ↑ *Prevotella 9* ↓ Faecal acetate*** ↓ Faecal butyrate*** **<u>NMOSD vs Control</u>** ↓ Faecal acetate*** ↓ Faecal propionate*** ↓ Faecal butyrate***	None	None		(Zeng et al., 2019)

	16S (no)	N = 27 MS treated with dimethyl fumarate N = 9 MS treated with other therapy 12 week treatment	None	None	None	No genus-levelenus-level differences reported	(Storm-Larsen et al., 2019)
	16S (no)	N = 10 MS on high-vegetable/low protein diet (HV/LP) N = 10 MS on Western Diet (WD) Faecal samples collected at baseline and after 12 months	None	None	None	Greengenes, genus-levelenus-level differences not reporter	(Saresella et al., 2017)
	16S via Pyrosequencing (no)	N = 13 MS N = 13 neuro-Behçet Disease (NBD) N = 14 Control	**<u>MS vs Control</u>** ↑ *Coproccocus**** (LFC = 9.3) ↑ *Ruminococcus 2*** (LFC = 11.79) ↑ *Butyricoccus*** (LFC = 8.41) ↑ *Clostridium* XVIII** (LFC = 12.07) ↑ *Dorea** (LFC = 3.60) ↑ *Escherichia/Shigella** (LFC = 5.85) ↑ *Parabacteroides** (LFC = 7.05) ↑ *Gemmiger** (LFC = 4.43) ↓ *Succinivibrio** (LFC = 0.03) ↓ *Prevotella** (LFC = 0.12)	None	None		(Oezguen et al., 2019)

			<u>NBD vs HC</u> ↑ *Parabacteroides*** (LFC = 11.4) ↓ *Vampirovibrio** (LFC = 0.03) **<u>NBD vs MS</u>** ↑ *Butyricomonasi*** (LFC = 32.29) ↓ *Erysipelotichaceae incertae sedis** (LFC = 0.09)				
	Phylochip (no)	N = 8 Controls N = 7 MS (2 untreated) Measured change in RA after Vitamin D supplementation	**<u>MS Untreated vs Control</u>** ↑ *Akkermansia* ↑ *Faecalibacterium* ↑ *Coproccus*	None	None	Exploratory study	(Cantarel et al., 2015)
	Phylochip (no)	N = 16 NMOSD N = 16 MS N = 16 Control	**<u>NMOSD vs Control</u>** ↑ *Clostridium perfingensr**** ↑ *Coprococcus**** ↑ *Corynebacterium**** ↑ *Ruminoococcus**** ↑ *Trepenomaoe**** ↑ *Bacteroides**** ↑ *Blautia producta**** ↓ *Prevotella****	**<u>NMOSD vs Control</u>** ↑ *Bacteroides****	None		(Cree et al., 2016)
	FISH (no)	N = 25 MS (10 on ketogenic diet for 6 months) N = 14 Control	None	None	None		(Swidsinski et al., 2017)

Table 7. Microbiome-brain studies involving pain-related disorders.

Legend: LFC: log-2 fold change; LC: log change; RA: relative abundance; DASS-42: Depression Anxiety Stress Scales; ADHD: Attention-Deficit Hyperactivity Disorder; HE: hepatic encephalopathy; FOS: Fructo-oligosaccharide; HADS-A: Hamilton Anxiety and Depression Scales – Anxiety Subscale; HADS-A: Hamilton Anxiety and Depression Scales – Depression Subscale; RTT: Rett's Syndrome; AD: Alzheimer's Disease, PD: Parkinson's Disease; MCI: mild-cognitive impairment; MSA: multiple-

systems atrophy; ALS: amyotrophic lateral sclerosis; NMOSD: neuromyelitis optica spectrum disorder*: $p_{adj} < 0.1$; **: $p_{adj} < 0.01$; ***: $p_{adj} < 0.001$; Reanalyzed studies are highlighted; 95% CI reported between square brackets [lower 95% CI; upper 95% CI]

Cohort Details	Sequencing (reanalysed)	Groups and Sample Size	SCFA/Tryptophan-Modifying Bacteria	BA-Modifying Bacteria	Other Metabolites/ GBMs	Specific Limitations	Ref
Fibromyalgia	16S (yes)	N = 77 Fibromyalgia N = 79 Total Controls N = 11 first-degree relatives (controls) N = 20 household members of participating patients (controls) N = 48 unrelated controls	**Fibromyalgia vs Same Household Address as Patient** ↑*Sutterella* (effect = 0.66 [-0.43; 0.92])* **Fibromyalgia vs Unrelated Control** ↑ Serum butyrate ↓ Serum propionate ↓ Serum isobutyrate				(Minerbi et al., 2019)
	16S (yes)	N = 105 Fibromyalgia N = 54 Controls	None	None	None		(Clos-Garcia et al., 2019)

	WGS (no)	N = 77 Fibromyalgia N = 79 Total Controls N = 11 first-degree relatives (controls) N = 20 household members of participating patients (controls) N = 48 unrelated controls	**<u>Fibromyalgia vs Unrelated Control</u>** ↑ *Parabacteroides merdae* ↑ *Clostridium scindens* ↑ *Blautia hydrogentrophica* ↑ *Eisenbergella massiliensis* ↑ *Hungatella hathewayi* ↑ *Alistipes oderdonkii* ↑ *Blautia massilensis* ↑ *Butyricoccus desmolans* ↑ *Flavonifractor plautii* ↓ *Faecalibacterium prausnitzii* ↓ *Blautia faecis* ↓ *Haemophilus parainfluenzae* ↓ *Prevotella copri* ↓ *Bacteroides uniformis* ↑ Serum butyrate ↓ Serum propionate ↓ Serum isobutyrate				(Minerbi et al., 2019)
Irritable Bowel Syndrome (IBS)	16S (no)	N = 48 with IBS	↑ *Bacteroides* with higher perceived stress None		None	No control group, rarefaction	(Peter et al., 2018a)
	16S (no)	N = 38 IBS; Samples taken before and after gut-directed hypnotherapy	None	None	None	No hypnotherapy control, rarefaction	(Peter et al., 2018b)
	16S (no)	N = 11 Abdominal pain after flood disaster; received *B. infantis* M-63 N = 20 Control	**<u>Probiotic vs Control</u>** Improved anxiety score, mental component of QoL			Greengenes; genus-levelenus-level differences not reported	(Ma et al., 2019b)

	16S (no)	N = 211 Flood Survivors (80 with abdominal pain, 131 without) Subset of 72 consented to faecal samples	**<u>Abdominal Pain vs No Pain</u>** ↑ *Staphylococcus* ↑ *Megamonas* ↑ *Fusobacterium* **<u>IBS vs No IBS</u>** ↑ *Paraprevotella* No genus-levelenus-level differences for anxiety found				(Yusof et al., 2017)
	16S (no)	N = 10 IBS, sampled at 0, 4, 12 weeks after FMT	**<u>Donor for Responder vs Non-Responder</u>** ↑ *Bifidobacterium* No community change detected in responders or non-responders Reduction in HAM-A anxiety after 12 weeks in responders			Prospective trial	(Mizuno et al., 2017b)
	16S (no)	N = 37 IBS N = 20 age and sex matched controls	**<u>IBS vs Control</u>** ↑ *Bifidobacterium adolescentis**** ↑ *Dialister**** ↑ *Papilibacter**** ↑ *Dorea**** ↑ *Blautia**** ↑ *Sporobacter**** ↑ *Escherichia**** ↓ *Odoribacter**** ↓ *Alistipes**** ↓ *Bacteroides**** No genera level associations with anxiety or depression	**<u>IBS vs Control</u>** ↑ *Bifidobacterium adolescentis**** ↓ *Bacteroides****			(Jeffery et al., 2012)
	16S (no)	N = 17 IBS, sampled at 0, 1, 2, 4 weeks after FMT	**<u>Baseline: HAM-D >=8 vs HAM-D <8</u>** ↓ *Eubacterium* **<u>Week 4 vs Baseline HAM-D >=8</u>** ↑ *Eubacterium* **<u>HAM-D Responders vs Non-Responders :</u>** ↑ *Streptococcus*	**<u>Baseline: HAM-D >=8 vs HAM-D <8</u>** ↓ *Eubacterium* **<u>Week 4 vs Baseline HAM-D >=8</u>** ↑ *Eubacterium*	None	Prospective pilot	(Kurokawa et al., 2018)

	16S (no)	N = 44 IBS with moderate anxiety and/or depression N = 22 PBO N = 22 *B. longum NCC3001*	Improvement in HAD-D subscale for *B. longum* group	None	None		(Pinto-Sanchez et al., 2017b)
	16S (no)	N = 30 with refractory IBS; sequenced stool before FMT and 1 mo. after	**<u>1 Month vs Baseline in Responders</u>** ↑ *Methanobrevibacter* ↑ *Akkermansia*		↑ Quality of life 1 mo. and 3 mo. after FMT but not after 6 mo.	Prospective pilot	(Huang et al., 2019b)
	16S via 454 Pyrosequencing (no)	N = 65 IBS N = 21 Control	*Clostridium* XIVa, *Coprococcus* associated with differences in connectivity of cortical and subcortical networks between IBS and Control	None	None		(Labus et al., 2019)
	16S array (no)	N = 13 post-infectious IBS N = 19 general IBS N = 16 Control	No genus-level information	None	None		(Sundin et al., 2015)
	Fluorescence In-Situ Hybridization (no)	N = 44 IBS Receiving trans-GOS suppplementation or PBO (0g, 3.5g, 7g daily)	**<u>PBO vs trans-GOS 3.5g</u>** ↑ *Bifidobacterium spp.** ↑ *E. rectale/C. coccoides**** **<u>PBO vs trans-GOS 7g</u>** ↑ *Bifidobacterium spp.**** ↓ *Clostridium perfingensr** ↓ *Bacteroides/Prevotella**** ↓ HADS-A Score* ↑ QOL Score*	**<u>PBO vs trans-GOS 3.5g</u>** ↑ *Bifidobacterium spp.** ↑ *E. rectale/C. coccoides**** **<u>PBO vs trans-GOS 7g</u>** ↑ *Bifidobacterium spp.**** ↓ *Bacteroides/Prevotella****	None		(Silk et al., 2009)

	Primers from GA-map Dysbiosis Test (no)	N = 16 IBS, Sampled at 0, 1, 3, 12, 20/28 weeks after FMT	**<u>Responders vs Non-Responders</u>** ↑ *Bacteroides**** before FMT ↑ *Megasphera/Dialister** at week 1, 12, 20/28	None	No strong association with HADS-A or HADS-D (only significant at Week 3 vs baseline but becomes insignificant by week 20/28)	Prospective pilot	(Mazzawi et al., 2018)
	qPCR (no)	N = 40 IBS receiving short-chain FOS (scFOS) N = 37 PBO 4 week trial	**<u>scFOS vs PBO</u>** ↓ HAD-D score **<u>scFOS at D28 vs Baseline</u>** ↑ *Bifidobacterium** **<u>PBO at D28 vs Baseline</u>** ↑ *Roseburia/Eubacterium rectale*				(Azpiroz et al., 2017)
Other Pain Disorders	Shotgun (no)	N = 54 older women with migraines N = 54 Controls	**<u>Migraine vs Control</u>** ↓ *Faecalibacterium prausnitzii*** ↓ *Bifidobacteirum adolescentis** ↑ Kynurenine synthesis* GBMs ↓ Quinolinic Acid Degradation*	**<u>Migraine vs Control</u>** ↓ *B. adolescentis**	**<u>Migraine vs Control</u>** ↑ GABA Synthesis III* ↓ SAM Synthesis* ↓ Glutamate Degradation*		(Chen et al., 2019)
	16S (no)	N = 48 Myalgic encephalomyelitis /chronic fatigue syndrome (ME/CFS) N = 48 Control	**<u>ME-CFS vs Control</u>** ↑ *Blautia** ↑ *Coprobacillus*** ↑ *Eggerthella*** ↓ *Faecalibacteirum** ↓ *Lachnospira* ↓ *Collinsella* Negative correlation between *Faecalinacterium* and total sleep awakenings	None	None		(Kitami et al., 2020)

	16S (no)	N = 113 Chronic Widespread Pain (CWP) N = 1623 Control	**CWP vs Control** ↓ *Coprococcus comes* ***	None	None		(Freidin et al., 2020)

Table 8. Microbiome-brain studies involving eating-related disorders.

Legend: LFC: log-2 fold change; LC: log change; RA: relative abundance; DASS-42: Depression Anxiety Stress Scales; ADHD: Attention-Deficit Hyperactivity Disorder; HE: hepatic encephalopathy; FOS: Fructo-oligosaccharide; HADS-A: Hamilton Anxiety and Depression Scales – Anxiety Subscale; HADS-A: Hamilton Anxiety and Depression Scales – Depression Subscale; RTT: Rett's Syndrome; AD: Alzheimer's Disease, PD: Parkinson's Disease; MCI: mild-cognitive impairment; MSA: multiple-systems atrophy; ALS: amyotrophic lateral sclerosis; NMOSD: neuromyelitis optica spectrum disorder*: $p_{adj} < 0.1$; **: $p_{adj} < 0.01$; ***: $p_{adj} < 0.001$; Reanalyzed studies are highlighted; 95% CI reported between square brackets [lower 95% CI; upper 95% CI]

Cohort Details	Sequencing (reanalysed)	Groups and Sample Size	SCFA/Tryptophan-Modifying Bacteria	BA-Modifying Bacteria	Other Metabolites/ GBMs	Specific Limitations	Ref
Obesity	WGS (no)	N = 14 Obese N = 13 Non-obese	None	None	None		(Blasco et al., 2017)
	WGS (no)	N = 35 Obese N = 35 Non-obese	No genus-level differences or associations identified	None	None		(Palomo-Buitrago et al., 2019)
	WGS (no)	N = 65 Obese N = 51 Control	Bacterial genera positively associated with memory: *Bacteroides, Citrobacter, Enterobacter, Salmonella, Klebsiella* Specifically associated with verbal learning: *Ruminococcus* CAG353,	Bacterial genera positively associated with memory: *Bacteroides*			(Arnoriaga-Rodríguez et al., 2020)

			Roseburia CAG357, *Veillonella magna* Negatively associated with memory scores: *Eubacterium, Clostridium,* Proteobacteria *Roseburia* and *Bacteroidetes* associated with volume in left hippocampus Altered tryptophan metabolism in obesity associated with reductions in short term and working memory, as well as volume of frontal interior orbital right gyrus and left hippocampus				
	16S (no)	N = 86 Women with no food addiction (FA) N = 19 Women with FA	OTUs enriched in FA: *Megamonas* OTUs depleted in FA: *Bacteroides, Akkermansia, Eubacterium biforme.* Reduction is associated with decreased plasma indolepropionate in the brain reward system	OTUs depleted in FA: *Bacteroides*			(Dong et al., 2020b)
	16S (no)	N = 8 Obese with bariatric surgery	None	None	None	Greengenes	(Sanmiguel et al., 2017)
	16S (no)	N = 18 Obese undergoing bariatric surgery	Precueneus-Putamen connectivity and food addiction symptoms negatively associated with *Bacteroides*, *Ruminococcus, Holdemanella*	Precueneus-Putamen connectivity and food addiction symptoms negatively associated with *Bacteroides*	None		(Dong et al., 2020a)
	16S (no)	N =57 obese, N = 54 control	None	None	None		(Kreutzer et al., 2017)
	16S via 454	N = 20 Obese N = 19 Non-obese	None	None	None	No genera level	(Fernandez-Real

	Pyrosequencing (no)					differences reported	et al., 2015)
	GA-Map Dysbiosis Test	N = 102 Morbid Obesity N = 15 Control	**<u>Associations in Obese Group: WHO-5 Wellbeing Index</u>** Negatively associated with *Bacteroides spp.* and *Prevotella* Negatively associated with faecal acetate, butyrate and propionate Positively associated with *Faecalibacterium prausnitzii*, *Dorea* spp. **<u>Associations in Obese Group: Hopkin Symptom Checklist 10</u>** Negatively associated with *Faecalibacterium prausnitzii* Positively associated with *Bacteroides stercoris*	None	None		(Farup and Valeur, 2018)
Anorexia	16S (yes)	N = 15 Anorexia N = 15 Controls	None	None	None	None	(Borgo et al., 2017)
	16S (yes)	N = 55 Anorexia baseline (AN-1) N = 44 Anorexia after weight gain (AN-2) N = 55 Control	**<u>AN-1 vs Control</u>** ↑ Isovaleric acid synthesis I (effect = 0.44 [-2.80, 5.07])* ↑ Quinolinic acid synthesis (effect = 0.48[-2.13; 5.35])* ↑ Quinolinic acid degradation (effect = 0.42 [-2.33; 4.80])** **<u>AN-2 vs Control</u>** ↓ Butyrate Synthesis II (effect = -0.43 [-4.88; 2.55])**	None	**<u>AN-1 vs Control</u>** ↑ p-Cresol synthesis (effect = 0.49 [-2.37; 5.20])** ↑ S-Adenosylmethionine (SAM) synthesis (effect = 0.40 [-2.12; 5.05])* ↑ Glutamate synthesis II (effect = 0.46		(Mack et al., 2016)

					[-2.32; 5.03])** ↑ ClpB (ATP-dependent chaperone protein) (effect = 0.43 [-2.30; 4.98])* **<u>AN-2 vs Control</u>** ↓ Inositol degradation (effect = -0.43 [-5.09; 2.32]) *		
	16S (no)	N = 18 Anorexia N = 20 Athletes N = 26 Normal Weight N = 22 Overweight N = 20 Obese All women	None	None	None	Storage at -20C	(Mork l et al., 2017)

	16S (no)	N = 21 Anorexia at enrollment N = 16 Anorexia at discharge N = 29 Healthy women	**<u>Anorexia at Admission vs Control</u>** ↑ *Weissella** ↑ *Coprococcus** ↓ *Parabacteroides** **<u>Anorexia at Discharge vs Control</u>** ↑ *Collinsella** ↑ *Actinobacteria** ↑ *Parabacteroides**	None	None		(Monteleone et al., 2020)
	16S (no)	N = 19 Anorexia N = 20 Healthy Control	**<u>Anorexia at Admission vs Control</u>** ↑ *Anaerostipes** **<u>Anorexia at Discharge vs Control</u>** ↑ *Unclassified Lachnospiraceae*** ↑ *Fusicatenibacter** **<u>Anorexia at Admission vs Discharge</u>** ↓ *Bacteroides** ↑ *Unclassified Ruminococcaceae** ↑ *Unclassified Lachnospiraceae*** ↑ *Faecalibacterium** ↑ *Fusicatenibacter**	None	None		(Schulz et al., 2020)
	16S via 454 Pyrosequencing (no)	N = 16 at timepoint 1 (admission to hospital) N = 10 at timepoint 2 (discharge after nourishment)	↑ *Ruminococcus** after nourishment	None	None	Greengenes	(Kleiman et al., 2015)

	qPCR (no)	N = 25 Anorexia (11 binge eating, 14 restrictive) N = 21 Control	**<u>Anorexia vs Control</u>** ↓ Total bacteria*** ↓ *Clostridium coccoides**** ↓ *Clostridium leptum**** ↓ *Bacteroides fragilis**** ↓ *Streptococcus****	**<u>Anorexia vs Control</u>** ↓ *Bacteroides fragilis****	None		(Morita et al., 2015)
	qPCR (no)	N = 20 Obese N = 9 Anorexia N = 20 Control	**<u>Obese vs Control</u>** ↑ *Lactobacillus** **<u>Obese vs Anorexia</u>** ↑ *Lactobacillus** **<u>Anorexia vs Control</u>** ↑ *Methanobrevibacter smithii**	**<u>Obese vs Control</u>** ↑ *Lactobacillus** **<u>Obese vs Anorexia</u>** ↑ *Lactobacillus**	None		(Armougom et al., 2009)

Table 9. Microbiome-brain studies involving neurovascular disease.

Legend: LFC: log-2 fold change; LC: log change; RA: relative abundance; DASS-42: Depression Anxiety Stress Scales; ADHD: Attention-Deficit Hyperactivity Disorder; HE: hepatic encephalopathy; FOS: Fructo-oligosaccharide; HADS-A: Hamilton Anxiety and Depression Scales – Anxiety Subscale; HADS-A: Hamilton Anxiety and Depression Scales – Depression Subscale; RTT: Rett's Syndrome; AD: Alzheimer's Disease, PD: Parkinson's Disease; MCI: mild-cognitive impairment; MSA: multiple-systems atrophy; ALS: amyotrophic lateral sclerosis; NMOSD: neuromyelitis optica spectrum disorder*: $p_{adj} < 0.1$; **: $p_{adj} < 0.01$; ***: $p_{adj} < 0.001$; Reanalyzed studies are highlighted; 95% CI reported between square brackets [lower 95% CI; upper 95% CI]

Cohort Details	Sequencing (reanalysed)	Groups and Sample Size	SCFA/Tryptophan-Modifying Bacteria	BA-Modifying Bacteria	Other Metabolites/ GBMs	Specific Limitations	Ref
Neurovascular Disease	16S and WGS (no)	N = 122 Neruovascular Cavernous Angioma (CA) Controls from Human Microbiome Project	CA vs Control ↑ *Bacteroides thetaomicron**** ↑ *Odoribacter sphlancus**** ↓ *Bifidobacterium adolescentis**** ↓ *Faecalibacterium prausntzii**** Aggressive vs Non-Aggressive CA ↑ *Bifidobacterium adolescentis**** ↓ *Bacteroides eggerthii** CA with Symptomatic Hemmorhage vs CA No Hemmorhage ↑ *Faecalibacterium prausnitzii* ↑ *Oscillobacter*	CA vs Control ↑ *Bacteroides thetaomicron**** ↓ *Bifidobacterium adolescentis**** Aggressive vs Non-Aggressive CA ↑ *B. adolescentis**** ↓ *Bacteroides eggerthii**	None		(Polster et al., 2020)
	16S (no)	N = 8 Cerebral Infarction (CI) N = 2 Ischemic Stroke (IS) N = 10 Controls	**CI vs Control** ↓ *Bacteroides* ↓ *Parabacteroides* ↓ *Akkermansia* ↓ *Prevotella* ↓ *Faecalibacterium* **IS vs Control** ↑ *Escherichia*	**CI vs Control** ↓ *Bacteroides* **IS vs Control** ↓ *Bacteroides* **CI vs IS** ↑ *Bacteroides*	None	N = 2 for Ischemic Stroke	(Ji et al., 2017)

			↑ *Dialister* ↑ *Bifidobacterium* ↓ *Bacteroides* ↓ *Megamonas* ↓ *Parabacteroides* ↓ *Akkermansia* ↓ *Prevotella* ↓ *Faecalibacterium* ↓ *Ruminococcus* **CI vs IS** ↑ *Escherichia* ↑ *Bacteroides* ↑ *Megamonas* ↑ *Prevotella* ↑ *Ruminococcus* ↓ *Parabacteroides* ↓ *Akkermansia* ↓ *Faecalibacterium* ↓ *Dialister* ↓ *Bifidobacterium*	↓ *Bifidobacterium*			
	16S (no)	N = 30 Ischemic Stroke N = 30 Control	**Ischemic Stroke vs Control** ↑ *Odoribacter* ↑ *Akkermansia* ↑ *Victivallis* ↓ *Anaerostipes* ↓ *Ruminoclostridium 5* **Severe vs Mild Stroke** ↓ *Enterobacter*	None	None	Rarefaction	(Li et al., 2019c)
	16S (no)	N = 30 Post-Stroke Cognitive Impairment (PSCI) N = 35 non-PSCI	**PSCI vs non-PSCI** ↑ *Fusobacterium* ↑ *Bacteroides* ↑ *Colstridium* XIVa ↑ *Gemella* ↑ *Flavonifractor* ↓ *Prevotella* ↓ *Gemminger* ↓ *Alistipes*	**PSCI vs non-PSCI** ↑ *Bacteroides*	None		(Liu et al., 2020a)

			↓ *Ruminococcus* ↓ *Akkermansia* ↓ *Coprococcus* ↓ *Barnesiella* ↓ *Clostridium* IV ↓ *Odoribacter* ↓ *Methanobrevibacter* ↓ *Oxolobacter* ↓ *Hydrogenanaerobacterium*				
	16S (no)	N = 41 Post-Stroke Cognitive Impairment (PSCI) and Depression N = 25 non-PSCI	**PSCI vs non PSCI** ↓ *Fusicatenibacter* ↑ *Veilonella*	None	None		(Ling et al., 2020a)
	16S (no)	N = 10 Cerebral Infarction patients N = 10 Control	None	None	None	Not properly filtered, chloroplasts included in results	(Wang et al., 2018b)
	16S (no)	N = 10 infants with hypoxic ischemic encephalopathy treated with hypothermia N = 9 Control	**Hypoxic Ischemic Encephalopathy vs Control** ↓ *Bacteroides*** None		None		(Watkins et al., 2017)

Table 10. Microbiome-brain studies involving stress-related and psychiatric disorders.

Legend: LFC: log-2 fold change; LC: log change; RA: relative abundance; DASS-42: Depression Anxiety Stress Scales; ADHD: Attention-Deficit Hyperactivity Disorder; HE: hepatic encephalopathy; FOS: Fructo-oligosaccharide; HADS-A: Hamilton Anxiety and Depression Scales – Anxiety Subscale; HADS-A: Hamilton Anxiety and Depression Scales – Depression Subscale; RTT: Rett's Syndrome; AD: Alzheimer's Disease, PD: Parkinson's Disease; MCI: mild-cognitive impairment; MSA: multiple-systems atrophy; ALS: amyotrophic lateral sclerosis; NMOSD: neuromyelitis optica spectrum disorder*: $p_{adj} < 0.1$; **: $p_{adj} < 0.01$; ***: $p_{adj} < 0.001$; Reanalyzed studies are highlighted; 95% CI reported between square brackets [lower 95% CI; upper 95% CI]

Cohort Details	Sequencing (reanalysed)	Groups and Sample Size	SCFA/Tryptophan-Modifying Bacteria	BA-Modifying Bacteria	Other Metabolites/ GBMs	Specific Limitations	Ref
Stress	16S (no)	N = 50 Healthy subject in double-blind PBO RCT	None	None	None		(Soldi et al., 2019)
	16S (no)	N = 47 Black ♀ N = 33 white ♀	↑ *Fusobacterium** with stress in Black participants, but not in white participants	None	None	Greengenes	(Carson et al., 2018)
	16S (no)	N = 25 Low Adverse Childhood Events (ACE) (<2)	**<u>High ACE vs Low ACE Score</u>** ↑ *Prevotella****	None	None		(Hantsoo et al., 2019)

		N = 23 High ACE (>= 2) All participants pregnant at time of study					
	16S (no)	N = 75 Pregnancy-related anxiety associated to meconium of newborn	None	None	None	QIIME 1.9, Greengenes, No genus-level associations of identified microbes reported	(Hu et al., 2019a)
	16S (no)	N = 84 mothers (psychological stress collected) Infant faecal samples collected at birth, 4-12 weeks and 20-28 weeks	**<u>Mothers with Exposure to Intimate Partner Violence vs Control</u>** ↑ *Weisella**** at 4-12 weeks ↑ *Citrobacter*** at all timepoints	None	None	QIIME 1.7	(Naude et al., 2020)
	16S (no)	N = 31 Probiotic (*Lactobacillus gasseri* CP2305) N = 29 Placebo	**<u>Probiotic vs Placebo after stressor</u>** Smaller decrease in *Bifidobacteria* after stressor Increased faecal Valeric acid with probiotic	None	None		(Nishida et al., 2019)
	16S via 454 Pyrosequencing (no)	N = 16 PBO N = 16 Probiotic *L. gasseri* CP2305 Probiotic administration	**<u>Probiotic vs PBO after Stressor</u>** Differences in *Corynebacterium* Improved sleep quality Reduced stress symptoms in females	None	None	No post-hoc Identification of species-level differences with 16S	(Nishida et al., 2017)
	HITChip (no)	N = 28 high prenatal stress	None	None	None	Results difficult to interpret;	(Zijlmans et

		N = 28 low prenatal stress Measured composition at 5 points in first 110 days				table of p-values or statistics not provided; unclear if post-hoc used	al., 2015)
Post-Traumatic Stress Disorder (PTSD)	16S (no)	N = 29 PTSD N = 64 Controls	**PTSD without Hepatic Encephalopathy (HE) vs Control (no HE)** ↑ *Streptococcus* ↑ *Acidaminococcus* ↓ *Ruminococcus* ↓ *Roseburia* ↓ *Anaerostipes* ↓ *Colstridium* XIVAa ↓ *Pseudoflavonibacter* **PTSD with HE vs Control with HE** ↓ *Subdoligranulum*	None	None		(Bajaj et al., 2019)
	16S (no)	N = 18 PTSD N = 12 Trauma-exposed controls	None	None	None	Greengenes	(Hemmings et al., 2017)
Bipolar Disorder (BD)	WGS (no)	N = 31 BD N = 31 MDD N = 31 Control	**BD vs Control** ↑ *Streptococcus* ↑ *Clostridium* ↑ *Oscillibacter* ↑ *Bifidobacterium* ↑ *Bacteroides* **MDD vs Control** ↑ *Streptococcus* ↑ *Clostridium* ↑ *Oscillibacter* ↑ *Bifidobacterium*	**BD vs Control** ↑ *Bifidobacterium* ↑ *Bacteroides* **MDD vs Control** ↑ *Bifidobacterium* *Bifidobacterium* species and strains differed between BD and Control			(Rong et al., 2019)

			Various *Prevotella* and *Bifidobacterium* species and strains differed between BD and Control				
	WGS (no)	N = 25 BD N = 28 Controls	**BD vs Control** ↑ *Escherichia* ↑ *Bifidobacterium* ↑ *Lachnoclostridium* ↑ *Megasphera* ↑ *Clostridium* ↑ *Oscillibacter* ↑ *Acidaminococcus* ↑ *Streptococcus* ↓ *Bacteroides* Dysregulation in tryptophan metabolism pathway in BD	**BD vs Control** ↑ *Bifidobacterium* ↓ *Bacteroides*			(Lai et al., 2021)
	16S (no)	N = 115 BD N = 64 Control	**BD vs Control** ↓ *Faecalibacterium* **Associations** *Faecalibacterium* associated with higher PCS, lower PSQI and PHQ9 *Anaerostipes* associated with increased PCS	None	None		(Evans et al., 2017)
	16S (no)	N = 23 BD N = 23 Control	None	None	None	Greengenes	(McIntyre et al., 2019)
	16S (no)	N = 217 BD N = 165 MDD N = 217 Control	**BD vs Control (From top 5 LDA)** ↑ *Ruminococcus gnavus* (2 OTUs) ↑ *Clostridium sensu stricto* ↑ *Bacteroides* ↑ *Pseudomonas* (2 OTUs) ↓ *Prevotella 9* (2 OTUs) ↓ *Bacteroides* ↓ *Ruminococcus 2* ↓ *Klebsiella* **MDD vs Control (From top 5 LDA)**	**BD vs Control (From top 5 LDA)** ↑ *Bacteroides* (OTU) ↓ *Bacteroides* (OTU) **MDD vs Control (From top 5 LDA)** ↑ *Bacteroides* ↓ *Bacteroides* (5 OTUs)	None		(Zheng et al., 2020b)

			↑ *Citrobacter* ↑ *Fusobacterium* ↑ *Ruminococcus gnavus* ↑ *Bacteroides* ↑ *Ruminococcus 2* ↓ *Bacteroides* (5 OTUs) **BD vs MDD (From top 5 LDA)** ↑ *Blautia* ↑ *Bacteroides* ↑ *Lachnoclostridium* ↑ *Dialister* ↓ *Eubacterium rectale* ↓ *Eubacterium hallii* ↓ *Eggerthella* ↓ *Blautia* ↓ *Bacteroides*	**BD vs MDD (From top 5 LDA)** ↑ *Bacteroides* ↓ *Eubacterium rectale* ↓ *Eubacterium hallii* ↓ *Bacteroides*			
	16S (no)	N = 32 BD	None	None	None		(Benge sser et al., 2019)
	16S (no)	N = 113 BD N = 113 Control (37 unaffected relatives)	None	None	None	*Note all differences were explained by sex, family and smoking	(Coello et al., 2019)
	16S (no)	N = 52 BD (N = 12 BD-1 N = 38 BD-II) N = 20 After treatment with quietiapene N = 45 Control	**BD vs Controls** ↑ *Parabacteroides* ↑ *Bacteroides* ↓ *Roseburia* ↓ *Faecalibacterium* ↓ *Coprococcus* **BD-I (Baseline) vs BD-II (Baseline)** ↑ *Streptococcus* ↑ *Bacillus* ↑ *Veillonella* ↓ *Ruminococcus*	**BD vs Controls** ↑ *Bacteroides* **BD (treated) vs BD (untreated)** ↑ *Lactobacillus*			(Hu et al., 2019b)

			<u>BD (treated) vs BD (untreated)</u> ↑ *Klebsiella* ↑ *Lactobacillus* ↑ *Collinsella* ↑ *Paraprevotella* ↑ *Veillonella* ↓ *Alistipes*				
	16S (no)	N = 32 Bipolar disorder (BD) N = 10 Control	**<u>BD vs Controls</u>** ↑ *Faecalabacterium** **<u>Within BD</u>** ↑ *Lactobacillus*** associated with high IL-6 ↑ *Prevotella** with low LDL cholesterol ↑ *Roseburia*** with less depressive symptoms ↑ *Lactobacillus*** associated with high serum tryptophan ↑ *Prevotella** with low LDL cholesterol ↑ *Roseburia*** with less depressive symptoms	None	None	Greengenes	(Painold et al., 2019)
	16S (no)	N = 117 BD 49 treated with atypical antipsychotics (AAP) 68 non-AAP	**<u>AAP vs Non-AAP</u>** ↓ *Akkermansia* ↓ *Sutterella*	None	None		(Flowers et al., 2017)
	16S (no)	N = 128 Monozygotic twins discordant for BD	None	None	None		(Vinberg et al., 2019)
	qPCR (no)	N = 36 BD (before treatment) N = 27 Control	**<u>BD vs Control</u>** ↑ *Faecalibacterium prausnitzii** ↑ *Atopobium****	**<u>BD vs Control</u>** ↓ *Bifidobacteria**	None		(Lu et al., 2019)

			↑ *Enterobacter**** ↑ *Clostridium* cluster IV*** ↓ *Bifidobacteria**				
	qPCR (no)	N = 13 BD I N = 26 BD II N = 58 Control	None	None	None		(Aizawa et al., 2018)
Depression and Anxiety	16S and WGS (no)	N = 111 Psychiatric inpatients	*Coprococcus catus* and *Clostridium symbiosum* associated with moderate anxiety at admission ↓ *Coprococcus catus* associated with lower remission from anxiety and depression	None	None		(Madan et al., 2020)
	16S and WGS (no)	N = 1054	*Faecalibacterium*, *Coprococcus* associated with higher quality of life indicators *Dialister*, *Coprococcus* spp. depleted in depression	None	Synthesis of dopamine metabolite 3,4-dihydroxyphenylacetic acid positively correlated with quality of life		(Valles-Colomer et al., 2019)
	WGS (no)	N = 156 MDD N = 155 Control	**<u>MDD vs Control</u>** ↑ Multiple *Bacteroides* ASVs ↓ Multiple *Blautia* ASVs Many upregulated and downregulated ASVs assigned to *Eubacterium*	**<u>MDD vs Control</u>** ↑ Multiple *Bacteroides* ASVs	None		(Yang et al., 2020)
	16S (no)	N = 37 MDD N = 18 Control	None	None	None		(Naseribafrouei et al., 2014)
	16S (no)	N = 15 MDD, 11 Responders and 4 Non-Responders	None	None	None	Methods not clearly described	(Bharwani et al., 2020)

	16S (no)	N = 22 Depression/Anxiety N = 28 Control	**<u>Depression/Anxiety vs Control</u>** ↑ *Eubacterium* ↑ *Enterococcus* ↑ *Collinsella* ↓ *Faecalibacterium*	**<u>Depression/Anxiety vs Control</u>** ↑ *Eubacterium*	None		(Stevens et al., 2018)
	16S (no)	N = 23 MDD	Functional connectivity in lDLPFC inversely correlated with relative abundance of *Bacteroides*		None	Controls not in the scope of the study; focused on GABA	(Strandwitz et al., 2019)
	16S (no)	N = 24 Current depressive episode (CDE) N = 16 Control	**<u>CDE vs Control</u>** ↑ *Akkermansia* ↑ *Veillonella* ↑ *Ruminococcus gnavus* ↓ *Fusicatenibacter* ↓ *Sutterella* ↓ *Dialister*	None	None		(Jiang et al., 2020)
	16S (no)	N = 12 breast cancer survivors sampled at baseline and after 3mo Focus on psychosocial metrics	No significant microbiota differences after FDR adjustment	None	None		(Paulsen et al., 2017)
	16S (no)	N = 27 Control N = 27 MDD	**<u>MDD vs Control</u>** ↑ *Coprococcus** ↑ *Pseudomonas** ↑ *Blautia**			Greengenes	(Huang et al., 2018)
	16S (no)	N = 34 depressed + probiotic N = 37 + PBO N = 20 Non-depressed	No differences between groups *Ruminococcus gnavus* associated with DASS depression score* (Correlation = 0.37)	None	None	QIIME 1.9.1, Greengenes, rarefaction	(Chahwan et al., 2019)

	16S (no)	N = 17 MDD inpatients Samples collected at baseline and after 6wks treatment with Escitalopram	None	None	None	No genus level differences reported, no control arm	(Liskiewicz et al., 2019)
	16S (no)	N = 16 Inpatients at admission and 6 weeks later	*Paraprevotella* positively associated with Hamilton Depression Scale-24 Item score* (r=0.8)	None	None	Greengenes, rarefaction	(Liśkiewicz et al., 2021)
	16S (no)	N = 40 MDD taking psychotropics at three different timepoints	No genera-level associations with specific psychotropic medication within the cohort	None	None	Greengenes	(Tomizawa et al., 2020)
	16S (no)	N = 10 MDD N = 10 Control	**<u>MDD vs Control</u>** ↑ *Prevotella** (D1, D10 and D29) ↑ *Streptococcus** (D1, D10) ↑ *Clostridium XI** *(*D29)	None	None		(Lin et al., 2017)
	16S via 454 Pyrosequencing (no)	N = 58 MDD N = 63 Control	**<u>MDD vs Control</u>** ↑ *Collinsella* (RA = 4.2% vs 1.7%)* ↑ *Olsenella* (RA = 0.003% vs 0%)* ↑ *Blautia* (2 OTUs)** ↑ *Anaerostipes* (RA = 1.491% vs 0.303%)*** ↓ *Alistipes* (RA = 0.249% vs 0.761%)*	None	None		(Zheng et al., 2016)
	16S via 454 Pyrosequencing (no)	N = 38 Co-morbid anxiety and depression N = 8 Anxiety N = 14 Depression N = 10 Control	↑ *Bacteroides* in anhedonia	None	None		(Mason et al., 2020)

	16S via 454 Pyrosequencing (no)	N = 29 MDD responded N = 17 MDD active N = 30 Control	**Active MDD vs Control** ↑ *Blautia* ↑ *Oscillibacter* ↑ *Roseburia* ↓ *Bacteroides* ↓ *Dialister* ↓ *Faecalibacterium* ↓ *Prevotella* ↓ *Ruminococcus* **Responded MDD vs Control** ↑ *Bacteroides* ↑ *Roseburia* ↓ *Oscillibacter* ↓ *Prevotella* ↓ *Ruminococcus* ↓ *Faecalibacterium* Negative correlation between *Faecalibacterium* and depressive symptoms	**Active MDD vs Control** ↓ *Bacteroides* **Responded MDD vs Control** ↑ *Bacteroides*	**Responded MDD vs Control** ↓ *Escherichia/Shigella (ClpB)*		(Jiang et al., 2015)
	16S via 454 Pyrosequencing (no)	N = 40 Generalized Anxiety Disorder (GAD) N = 36 Controls N = 12 anti-depressant naïve patients N = 22 Control	**GAD vs Control** ↓ *Faecalibacterium** ↓ *Eubacterium rectale** ↓ *Sutterella** ↓ *Butyricoccus* ↑ *Bacteroides** ↑ *Ruminococcus gnavus** ↑ *Fusobacterium** **Treatment Naïve vs Control** ↑ *Lactobacillus** ↑ *Ruminococcus gnavus** ↑ *Fusobacterium** ↑ *Escherichia-Shigella** ↑ *Bacteroides** ↓ *Faecalibacterium** ↓ *Eubacteirum recetale* ↓ *Roseburia* ↓ *Subdoligranulum*	**GAD vs Control** ↑ *Bacteroides** **Treatment Naïve vs Control** ↑ *Lactobacillus** ↑ *Bacteroides** ↓ *Eubacteirum recetale*			(Jiang et al., 2018a)

	16S via 454 Pyrosequencing (no)	N = 40 with diarrhea-predominant IBS (IBS-D) N = 15 with Depression, N = 25 with comorbid patients (CM) N=20 Controls	**All Depression vs Control** ↑ *Bacteroides**** ↑ *Prevotella**** ↓ *Coprococcus****	**All Depression vs Control** ↑ *Bacteroides****	None		(Liu et al., 2016)
	16S via 454 Pyrosequencing (no)	N = 15 (co-morbid depression and diarrhoea predominant IBS) Treatments: Bifico probiotic (n = 8), duloxetine (n=6)	**Post vs Pre Bifico** ↓ *Bifidobacterium* **Post vs Pre Duloxetine** ↑ *Faecalibacterium*	**Post vs Pre Bifico** ↓ *Bifidobacterium*	**Post vs Pre Bifico** ↓ *Bifidobacterium* **Post vs Pre Duloxetine** ↑ *Escherichia/Shigella (*ClpB)		(Zhang et al., 2019a)
	16S (qPCR)	N = 43 MDD N = 57 Control	**MDD vs Control** ↓ *Bifidobacterium* ↓ *Lactobacillus*		None		(Aizawa et al., 2016)
	RFLP (no)	N = 56 OI N = 9 Control	**OI vs Control** ↑ *Clostridium* subcluster XIVa* **OI Depressed vs OI Non-Depressed** ↓ *Bifidobacterium*	**OI Depressed vs OI Non-Depressed** ↓ *Bifidobacterium*	None		(Ishii et al., 2019)

Table 11. Common limitations of human microbiome studies

Problem	Description	Solutions
Assigning taxonomy to sequences	Many studies use operational taxonomic units which are less precise and more prone to error.	ASVs are a more precise alternative which can be implemented through DADA2
Non-compositional data analysis	Counts data must be properly transformed to account for its relational data structure • Normalization with rarefaction or DESeq2 • Measuring distance between groups using Bray-Curtis, UniFrac, Jenson-Shannon; often used with Principal Co-ordinate Analysis • Pearson or Spearman Correlations (compositional data is prone to spurious correlation) • Differential abundance with LEfSe, DESeq, metagenomSeq	• Compositional normalization with ALDEx2 (i.e. CLR, IQR, ALR) • Measure distance between groups with the Aitchison metric in conjunction with Principal Component Analysis • SparCC, SpiecEasi, Φ for Correlations • Differential abundance with ALDEx2
Metadata collection	Often confounding variables are not measured or included in studies. There are several confounds that must be accounted for during analysis.	Participant data: • Food-frequency questionnaire • Alcohol-use • Smoking status • Prescription and recreational drug-use • Symptom frequency and severity
Bioinformatics Analysis	While adjusted p-values are often reported, studies seldom mention effect sizes or confidence intervals.	Report effect sizes and 95% confidence intervals. Sparse microbiome datasets are prone to uncertainty. If the confidence interval does not overlap with 0, then there is more certainty in the direction of the effect. Use gut-brain module analysis to provide more insight into your data. Deposit your data and code publicly if possible.
Updating tools for data analysis	Occasionally, studies use databases or tools that are no longer updated or supported i.e. QIIME version 1 or Greengenes database from 2013	Ensure that your bioinformatics tools and packages are regularly updated

Chapter 3: Microbially-Derived Short-Chain Fatty Acids Impact Astrocyte Gene Expression in a Sex-Specific Manner

Simon Spichak[1,2#], Francisco Donoso[2,3#], Gerard M. Moloney[1,2], Eoin Gunnigle[1,2], Jillian M. Brown[1], Martin Codagnone[1,2], Anna V. Golubeva[1,2], Timothy G. Dinan[1,2], John F. Cryan[1,2]*

1. Department of Anatomy and Neuroscience, University College Cork, Cork, Ireland

2. APC Microbiome Institute, University College Cork, Cork, Ireland

3. Department of Psychiatry and Neurobehavioural Science, University College Cork, Cork,

Ireland

* Corresponding author

[#] Authors contributed equally

Prof. John F. Cryan

Email: j.cryan@ucc.ie

Tel: +353 21 4205426

Address: Room 3.86 Western Gateway Building, University College Cork, Cork, Ireland

Co-Author Postal Address: Room 5.35, Biosciences Institute, University College Cork,

Cork, Ireland

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Abstract

Recent investigations in neuroscience implicate the role of microbial-derived metabolites in brain health and disease. Short-chain fatty acids are neuroactive metabolites produced by the gut microbiota. Short-chain fatty acid metabolites like acetate, propionate and butyrate have pleiotropic effects within the immune and enteric systems. They are crucial for the maturation of the brain's innate immune cells, the microglia and modulate other glial cells through the aryl-hydrocarbon receptor. *In vivo* these metabolites show neuroprotective and antidepressant properties. In humans, they even modulate the acute stress response and satiety. Together, these findings present a potential role for SCFA-astrocyte interactions. Our novel investigation tested the impact of physiologically relevant doses of SCFAs on male and female primary cortical astrocytes. We find that butyrate (0 – 25μM) positively correlates with *Bdnf* and *Pgc1-α* expression, implicating histone-deacetylase inhibitor pathways. Intriguingly, this effect is only seen in females. We also find that acetate (0 – 1500 μM) dosage positively correlates with *Ahr* and *Gfap* expression in males only, suggesting immune modulatory pathways. These findings show a novel sex-dependent impact of acetate and butyrate, but not propionate on astrocyte gene expression.

Keywords:

Short-chain fatty acid, Astrocyte, Microbiome, Neuro-immunity, Glia

Introduction

The microbiota-gut-brain axis emerged in recent years as a contributor or mediator of many neurophysiological and behavioural processes. The trillions of microorganisms within the mammalian gut, collectively called the microbiota, communicate with the brain through neuroendocrine, immune or vagal signalling (Cryan et al., 2019). In addition, they generate a cornucopia of neuroactive metabolites, absorbed into peripheral circulation, however it is still unclear if these metabolites act on brain cells directly. Recent work brought these metabolites into the spotlight, showing they were associated with host quality of life and depression in humans (Valles-Colomer et al., 2019).

Among the most promising candidates for modulating brain function are the short-chain fatty acids (SCFAs). SCFAs are fermented by-products, produced by specific colonic bacterial genera from dietary fibre. They consist of an aliphatic chain of carbon molecules with a carboxylic acid group. The most common types of SCFAs produced within the gut are acetate, butyrate and propionate, typically found in a 60:20:20 molar ratio (Dalile et al., 2019). SCFAs are also substrates easily used by colonocytes and other cell types to generate energy within the Krebs cycle.

SCFAs exert their action by acting as histone-deacetylase inhibitor (HDACi) intracellularly or by binding to G-coupled protein receptors (Dalile et al., 2019). In rodents, SCFA administration has shown to be neuroprotective against stroke (Sadler et al., 2020, Lee et al., 2020a), stress (van de Wouw et al., 2018), oligodendrocyte function (Chen et al., 2019b), and depressive-like behaviour (Yamawaki et al., 2018). These results are confounded by the broad influence of SCFAs on peripheral immune cells involved in both innate and adaptive immunity (Correa-Oliveira et al., 2016). However, SCFAs modulate blood-brain barrier permeability *in vitro* (Hoyles

et al., 2018) and are shown to cross the blood-brain barrier in rodent and primate studies (Dalile et al., 2019).

In humans, ^{13}C radiolabelled carbohydrate substrates are converted to acetate within the gut, reaching the hypothalamus through peripheral circulation leading to appetite suppression (Frost et al., 2014). Additionally, researchers observed elevations in the glutamate-glutamine cycle substrates within the hypothalamus (Frost et al., 2014). Another study provided healthy men with different doses of dietary-fibre that is converted into SCFAs or a placebo (Dalile et al., 2020). In a psychosocial stress paradigm, colon-derived SCFAs reduced the cortisol response with peripheral SCFA levels covarying with cortisol levels (Dalile et al., 2020). Interestingly, it did not alter the subjective response to the acute stressor, fear learning or extinction (Dalile et al., 2020).

The combination of preclinical and clinical data suggests that SCFAs exert both immune and metabolic effects within the central nervous system. Astrocytes within the brain are responsible for both metabolic regulation (Belanger et al., 2011) within the brain as well as neuroimmune functioning (Jensen et al., 2013). Recent work also implicates the metabolic functioning of cortical and subcortical astrocytes in learning and memory (Alberini et al., 2018). Additionally, dietary-derived tryptophan metabolites influence astrocytic immunity through the intracellular activation of the aryl-hydrocarbon receptor pathway (Rothhammer et al., 2016). To address whether SCFAs interact with cortical astrocytes, we used an enriched primary astrocyte culture.

Previous cell culture studies characterized the effects of supraphysiological levels on neuroimmune inflammation. While this provides insight into potential

neuroprotective functions, which could be induced by increasing the dietary-fibre intake, the question of what role SCFAs play in the homeostatic environment remain (Chen et al., 2007, Suh et al., 2010, Soliman et al., 2012, Singh et al., 2014, Wang et al., 2018a). These studies did not focus on identifying sex or region-specific effects as they were out of the scope of those respective studies. However, it's clear that sex may play a role in determining the physiological effects of SCFAs (Jaggar et al., 2020). Additionally, we aim to characterize the impact on primary astrocyte cultures, which have been seldom used in such studies. We also explored the potential of SCFAs to impact glutamate/glutamine metabolism, the aryl-hydrocarbon receptor pathway as well as genes related to HDACi.

Experimental Procedures

Animals

Experiments are authorised under a Euthanasia Only Project (2019-009). Experiments were conducted in accordance with guidelines established by University College Cork's Animal Welfare Body.

Seeding Primary Glial Culture

Litters of C57BL/6 mice were sexed and euthanized at postnatal day 1-3 (PND1-3). The mouse brains were removed from the skulls and the cortices were dissected out. The meninges and hippocampus were also removed. Cortices were pooled by sex into collection tubes, filled with DMEM-F12 (Gibco: 11320033), stored on ice. Pooled brains were washed with PBS 10mM, then incubated with 1.5mL of Trypsin/EDTA (0.25%) (Thermofisher: 25200072) at 37°C, 5% CO_2 for 20 minutes. Trypsin/EDTA (0.25%) was inactivated with 9mL of DMEM/F12 with 10% heat inactivated Fetal Bovine Serum (Thermofisher: 16140071). This mixture was

mechanically dissociated, transferred to a 15mL Falcon Tube and centrifuged at 200g, 21°C for 10 minutes. Supernatant was removed and resuspended in media. Media was composed of DMEM (Gibco: 16219961) with 10% heat inactivated Fetal Bovine Serum, 1% Penicillin/Streptomycin (Gibco: 15070063) and 2mM L-Glutamine (Thermofisher: 25030149) Cells were then seeded into T75 flasks with filtered caps, coated overnight with Poly-D-Lysine (Sigma-Aldrich: P6407-5MG). Half of the cell media was removed every 3-4 days and replenished with new media. Cells were grown at 37°C, 5% CO_2.

Primary Astrocyte Enrichment

After 14 days, cells were placed on an orbital shaker for 24 hours at 150rpm, 37°C, 5% CO_2. This process removed contaminating microglia from the culture. Media was aspirated, cells were washed with PBS 10mM and media was replenished. Cells were than shaken for 24 hours at 230rpm, 37°C, 5% CO_2 to remove oligodendrocyte precursor cells. Cells were washed with PBS 10mM and left for 1-2 days. Afterwards, astrocytes were dissociated using 3.0 mL Trypsin/EDTA (0.25%) before being seeded at a density of ~150 000 cells into a 6-well cell culture plate. Each well contained 1.5mL of media and 150 000 cells.

Primary Astrocyte Purity

To confirm enrichment, cells from three different litters were grown of coverslips and fixed with 4% PFA (Sigma-Aldrich: 158127) in triplicate. Briefly cells were first blocked for an hour with a solution of PBS 10mM with 0.1% Triton-X (Sigma-Aldrich X100) and 5% Donkey Serum (Sigma-Aldrich D9663). The primary antibodies, Rat anti-GFAP (1:250) (Thermofisher:13-0300) and Rabbit-anti IBA1 (1:1000) (Wako: 019-19741). was added in a PBS/0.1% Triton-X/2% Donkey

Serum solution and left at 4°C for 12 hours. After three washes, a secondary antibody, anti-Rat AlexaFluor 488 (Thermofisher: A-21028) and anti-Rabbit AlexaFluor 594 (Thermofisher: A-11012) were both added in a 1:500 dilution of PBS/0.1% Triton-X/2% Donkey Serum for two hours in a humid chamber. Cells were washed three times and DAPI (Thermofisher: D1306) was added at a concentration of 1:1000 in a PBS solution with 2% Donkey Serum. Finally, cells were mounted onto slides and GFAP/DAPI double positive cells were counted as astrocytes while DAPI positive cells or IBA1/DAPI double positive cells were counted as non-astrocytes. We found our enriched culture was >95% pure. In addition, T75 flasks were viewed under a microscope to ensure no microglia (small rounded cells adherent to the surface layer of attached cells) were present before further experiments.

Cell Culture Treatments

Once the cells adhered (after ~5 days), 150mL of media was removed, and new media with different concentrations of the short-chain fatty acids acetate (Sigma-Aldrich: S7545), propionate (Sigma-Aldrich: P1880) or butyrate (Sigma-Aldrich, 303410) diluted in culture media were added. Physiologically relevant concentrations were estimated based on the study by (Guardia-Escote et al., 2019). For acetate we tested concentrations at 0µM, 150 µM, 750 µM and 1500µM. For propionate we tested concentrations at 0µM, 3.5 µM, 17.5 µM and 35µM. For butyrate we tested concentrations at 0µM, 2.5 µM, 12.5 µM and 25µM.

Quantitative PCR

24 hours after treatment, cells were lysed and isolated according to the TRIzol protocol (Thermofisher: 15596026). RNA concentrations were then measured using

a Nanodrop-1000 (Thermofisher) followed by the generation of cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermofisher: 4368814). Using the primers listed in Table 1 (Eurofins) along with the SYBR™ Green PCR Master Mix kit (Thermofisher: 4309155), we conducted a quantitative PCR.

Table 1. PCR Primers

Pathway	Gene	Sequence Forward (5' -> 3')	Sequence Reverse (5' -> 3')	Reference
Astrocyte Marker	GFAP	GCTCCAAGATGAAACCA ACC	TTCAACCTTTCTCTCCAA ATCC	(Zeisel et al., 2018, Ramos-Garcia et al., 2020)
Aryl-Hydrocarbon Receptor Pathway	Aryl-hydrocarbon receptor	ACGGATGAAGAAGGACG AG	AAGGAGGACACAGATAG ATGG	(Ramos-Garcia et al., 2020, Rothhammer et al., 2016)
	S100β	TCTGTCTACACTCCTGTT ACTC	TCTCCATCACTTTGTCCA CC	(Tomova et al., 2019)
	IL-22	TGACGACCAGAACATCC AG	TAGAAGGCAGGAAGGAG CAG	(Monteleone et al., 2011)
	IFNAR1	TCTCAAAAACACATTCTC CCTC	CCATCCTTCTCCATGCTT ATC	(Rothhammer et al., 2016)
	CYP1B1	AAGGAAGGGGAGTGCG ATAG	AATAGATGGGGGAGATA GGAGG	(Rothhammer et al., 2016)
Glutamate-Glutamine Cycle	GLUL	TCTCTACACACCAACCC TTTC	ACCAACCTTCAACTCCT CAC	(Schousboe et al., 2014)
	GAD67	TGTGAGCCAAAGAGAAA AGATG	TGAGGGGGGAAAGAGA AGAG	(Schousboe et al., 2014)
	GLUD1	CTTCTTTACCACCTCTTC ACC	ACCTAAAAGCAAACCAC CTAAC	(Schousboe et al., 2014)
HDAC Inhibition Pathway	GDNF	TTCAACTCTTTTTCCCCC TTC	TTCCCCTATGTTCTCCTG TC	(Bourassa et al., 2016)
	BDNF	TTCCCCTATGTTCTCCTG TC	TACCATTCCCCACCTCC ATC	(Bourassa et al., 2016)
	NGF1	AGCAAAGCCAAGCAAAC C	CAAAACCCAACCAAACA AACC	(Bourassa et al., 2016)
	SP1	ATGCTGCTCAACTCTCC TC	GCTATTCTCTCCTTCTCC ACC	(Bourassa et al., 2016)
	PGC1-α	AACTCCTCCCACAACTC CTC	GCCGTTTAGTCTTCCTT TCC	(Bourassa et al., 2016)

Analysis

Each sample was analysed in duplicate for both target gene and reference gene (β-actin), and the relative mRNA expressions were calculated using the $2^{-\Delta\Delta Ct}$ method Linear regression was used to test for SCFA-dose responses (Livak and Schmittgen,

2001). Results in bar plots are presented as mean ± SEM. For presentation in heat maps, the means for gene expression were scaled. For linear regression, the SCFA dose was treated as a continuous independent variable while gene expression was treated as a continuous dependent variable. Graphs were made using ggplot2 in R 4.0.0. Linear regression was performed using the lm function in R 4.0.0. The code used for analysis and plotting is available here.

Results

Butyrate

Low levels of butyrate (2.5 - 25μM) did not significantly impact gene expression in males (see **Fig 1**). However, in females *Bdnf* expression positively associated with butyrate dose (df = 10, residual standard error = 0.9446, R^2= 0.4419, Adjusted R^2= 0.3861, F-Statistic = 7.918, p = 0.01835). Similarly, the expression of *Pgc1-α* was positively associated with butyrate dose in female astrocytes (df = 10, residual standard error = 0.9229, R^2= 0.3447, Adjusted R^2= 0.2792, F-Statistic = 5.26, p = 0.04475) but not in males (see **Table 2-3**).

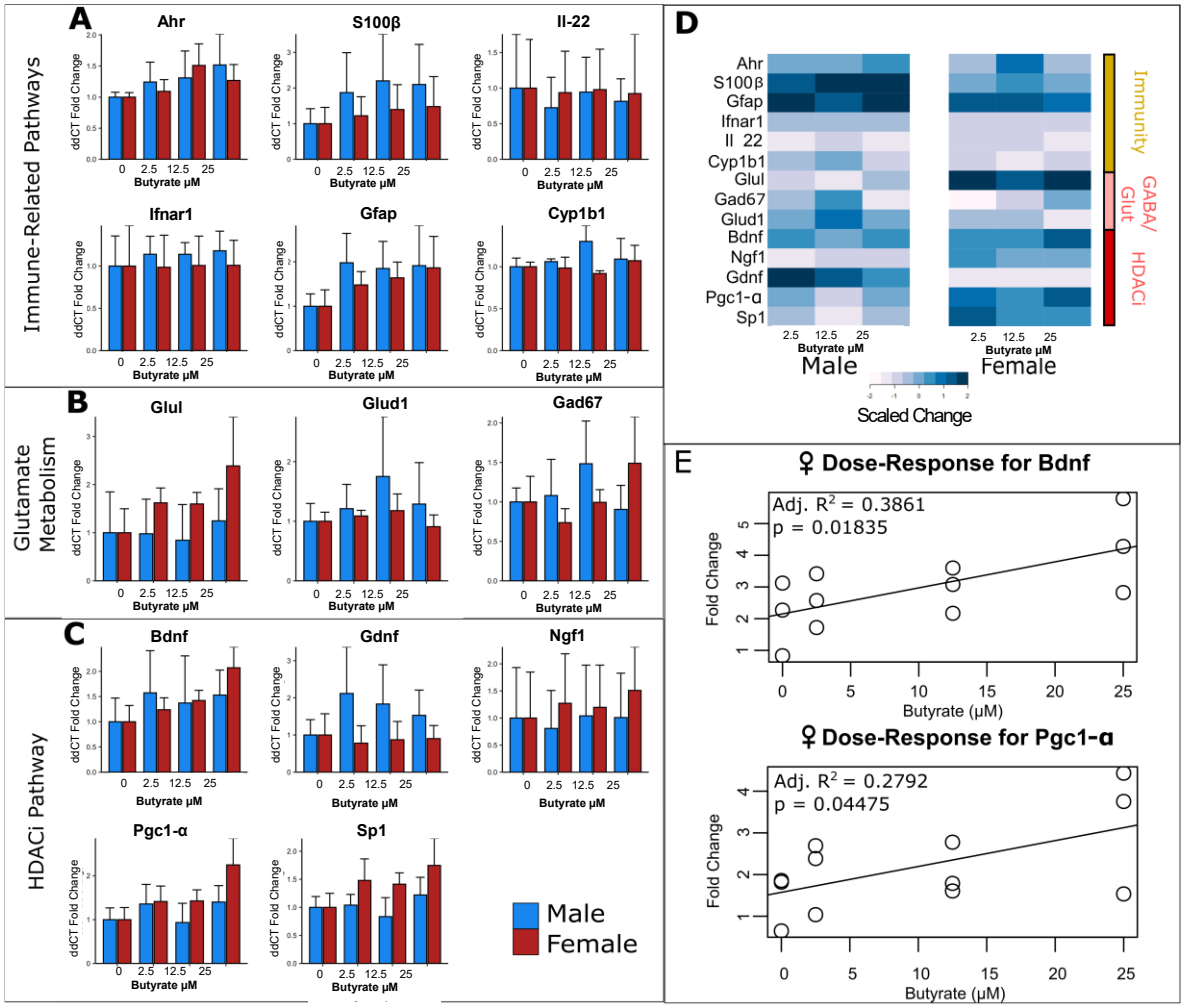

Fig 1. Impact of butyrate on cortical astrocyte gene expression. A-C. The impact of different concentrations of butyrate on immune-related, glutamine/GABA metabolism and histone deacetylase inhibitory pathways in male and female cortical astrocytes (N = 3 litters). Raw data was analyzed using the $2^{-\Delta\Delta CT}$ method. Plots show the means within each group normalized to the vehicle (0μM) with bars representing the standard error of the mean. D. A heatmap visualizing mean changes in gene expression across different genes. E. Using linear regression, there is a significant association between butyrate dose and *Bdnf* expression in female cortical astrocytes (df = 10, residual standard error = 0.9446, R^2= 0.4419, Adjusted R^2= 0.3861, F-Statistic = 7.918, p = 0.01835) but not in males (df = 10, residual standard error = 1.386, R^2= 0.01261, Adjusted R^2= -0.08612, F-Statistic = 0.1278, p = 0.7282). There is a significant association between butyrate dose and *Pgc1-α* in female cortical astrocytes (df = 10, residual standard error = 0.9229, R^2= 0.3447, Adjusted R^2= 0.2792, F-Statistic = 5.26, p = 0.04475) but not in males (df = 10, residual standard error = 0.6803, R^2= 0.1897, Adjusted R^2= -0.07913, F-Statistic = 0.1934, p = 0.6995).

Table 2. Linear regression results in male cultures treated with butyrate.

	Ahr		S100ß		Gfap		Ifnar		Il_22		Cyp1b1		Glul		Bdnf	
Predictors	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*
Dose	0.02 (-0.02 – 0.05)	0.328	0.03 (-0.08 – 0.14)	0.510	0.02 (-0.05 – 0.09)	0.503	0.01 (-0.02 – 0.03)	0.657	-0.00 (-0.06 – 0.05)	0.929	0.00 (-0.01 – 0.02)	0.612	0.01 (-0.07 – 0.08)	0.798	0.01 (-0.06 – 0.09)	0.728
Observations	12		12		12		12		12		12		12		12	
R^2 / R^2 adjusted	0.096 / 0.005		0.045 / -0.051		0.046 / -0.049		0.021 / -0.077		0.001 / -0.099		0.027 / -0.071		0.007 / -0.092		0.013 / -0.086	

	Ngf1		Gdnf		Gad67		Glud1		Pgc1-a		Sp1	
Predictors	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*
Dose	0.00 (-0.08 – 0.09)	0.914	0.00 (-0.09 – 0.10)	0.924	-0.00 (-0.05 – 0.04)	0.915	0.01 (-0.06 – 0.08)	0.709	0.01 (-0.03 – 0.05)	0.669	0.01 (-0.02 – 0.04)	0.611
Observations	12		12		12		12		12		12	
R^2 / R^2 adjusted	0.001 / -0.099		0.001 / -0.099		0.001 / -0.099		0.015 / -0.084		0.019 / -0.079		0.027 / -0.070	

Table 3. Linear regression results in female cultures treated with butyrate.

	Ahr		S100ß		Gfap		Ifnar		Il_22		Cyp1b1		Glul		Bdnf	
Predictors	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*
Dose	0.01 (-0.01 – 0.04)	0.342	0.02 (-0.05 – 0.08)	0.582	0.03 (-0.02 – 0.08)	0.219	0.00 (-0.04 – 0.04)	0.971	-0.00 (-0.07 – 0.07)	0.953	0.00 (-0.01 – 0.01)	0.653	0.05 (-0.02 – 0.11)	0.135	0.04 (0.01 – 0.07)	**0.018**
Observations	12		12		12		12		12		12		12		12	
R^2 / R^2 adjusted	0.091 / -0.000		0.031 / -0.066		0.147 / 0.062		0.000 / -0.100		0.000 / -0.100		0.021 / -0.077		0.209 / 0.130		0.442 / 0.386	

	Ngf1		Gdnf		Gad67		Glud1		Pgc1-a		Sp1	
Predictors	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*
Dose	0.02 (-0.07 – 0.10)	0.689	-0.00 (-0.05 – 0.05)	0.989	0.02 (-0.01 – 0.06)	0.183	-0.00 (-0.02 – 0.02)	0.668	0.04 (0.00 – 0.08)	**0.045**	0.02 (-0.01 – 0.06)	0.207
Observations	12		12		12		12		12		12	
R^2 / R^2 adjusted	0.017 / -0.082		0.000 / -0.100		0.170 / 0.087		0.019 / -0.079		0.345 / 0.279		0.154 / 0.069	

Acetate

Low levels of acetate (2.5 - 25µM) did not significantly impact gene expression in females (see **Fig 2**). However, in males *Ahr* expression positively associated with acetate dose (df = 10, residual standard error = 0.5271, R^2= 0.5457, Adjusted R^2= 0.5003, F-Statistic = 12.01, p = 0.00606). The expression of *Gfap* was positively associated with acetate dose in male astrocytes (df = 10, residual standard error = 0.8891, R^2= 0.4414, Adjusted R^2= 0.3855, F-Statistic = 7.902, p = 0.01844) but not in females (see **Table 4-5**).

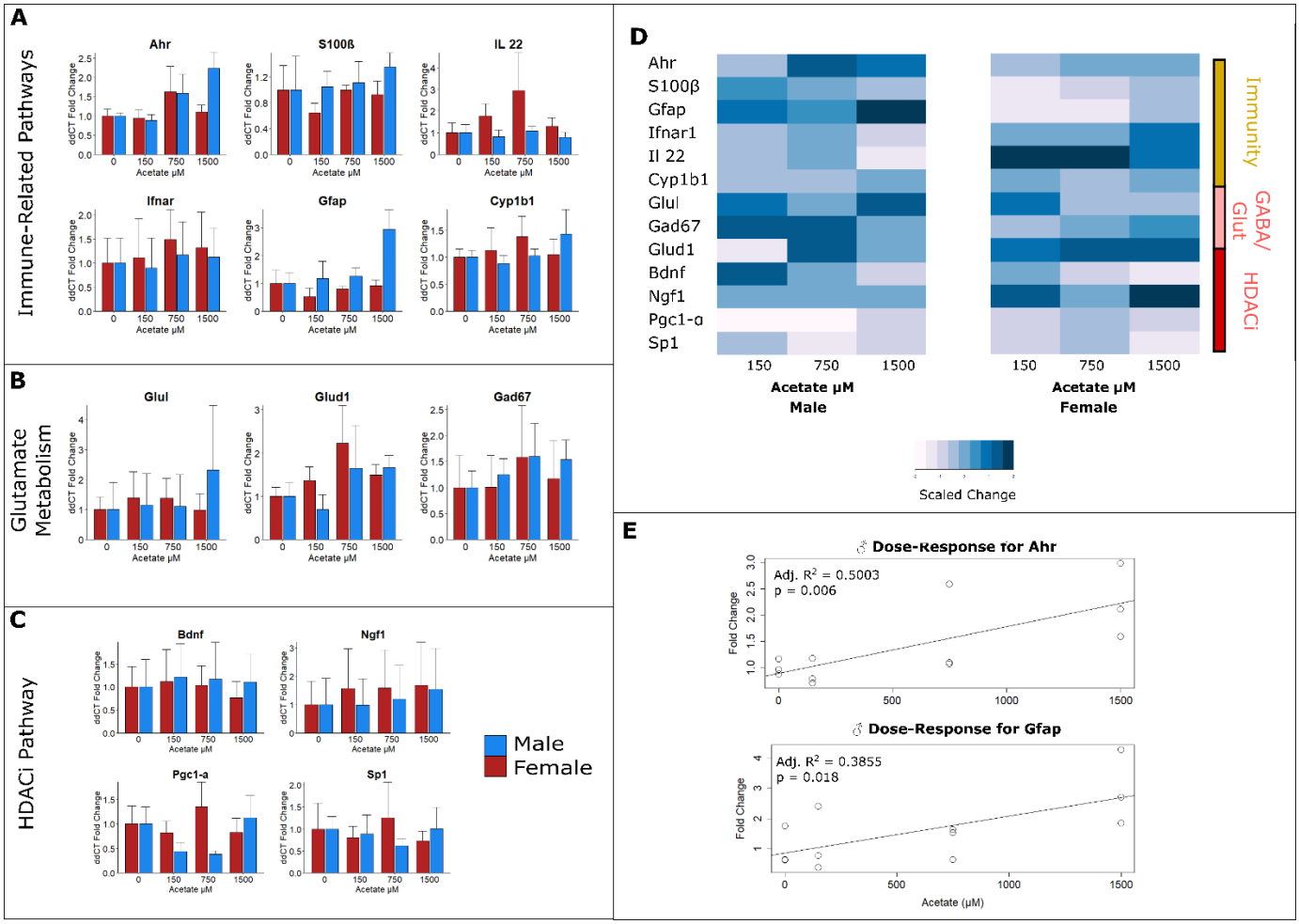

Fig 2. Impact of acetate on cortical astrocyte gene expression. A-C. The impact of different concentrations of butyrate on immune-related, glutamine/GABA metabolism and histone deacetylase inhibitory pathways in male and female cortical astrocytes (N = 3 litters). Raw data was analyzed using the $2^{-\Delta\Delta CT}$ method. Plots show the means within each group normalized to the vehicle (0µM) with bars representing the standard error of the mean. D. A heatmap visualizing mean changes in gene expression across different genes. E. Using linear regression, there is a significant association between acetate dose and *Ahr* expression in male cortical astrocytes (df = 10, residual standard error = 0.5271, R^2= 0.5457, Adjusted R^2= 0.5003, F-Statistic = 12.01, p = 0.00606) but not in females (df = 10, residual standard error = 0.6358, R^2= 0.0243, Adjusted R^2= -0.07327, F-Statistic = 0.2491, p = 0.6285). There is a significant association between butyrate dose and *Gfap* in male cortical astrocytes (df = 10, residual standard error = 0.8891, R^2= 0.4414, Adjusted R^2= 0.3855, F-Statistic = 7.902, p = 0.01844) but not in females (df = 10, residual standard error = 0.5173, R^2= 0.008557, Adjusted R^2= -0.09059, F-Statistic = 0.0863, p = 0.7749).

Table 4. Linear regression results in male cultures treated with acetate.

	Ahr		**S100ß**		**Gfap**		**Ifnar**		**Il_22**		**Cyp1b1**		**Glul**	
Predictors	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*
Dose	0.00 (0.00 – 0.00)	**0.006**	0.00 (-0.00 – 0.00)	0.413	0.00 (0.00 – 0.00)	**0.018**	0.00 (-0.00 – 0.00)	0.786	-0.00 (-0.00 – 0.00)	0.787	0.00 (-0.00 – 0.00)	0.141	0.00 (-0.00 – 0.00)	0.462
Observations	12		12		12		12		12		12		12	
R^2 / R^2 adjusted	0.546 / 0.500		0.068 / -0.025		0.441 / 0.386		0.008 / -0.092		0.008 / -0.092		0.204 / 0.124		0.055 / -0.039	

	Bdnf		**Ngf1**		**Gdnf**		**Gad67**		**Glud1**		**Pgc1-a**		**Sp1**	
Predictors	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*
Dose	0.00 (-0.00 – 0.00)	0.971	0.00 (-0.00 – 0.00)	0.670	3.62 (-3.47 – 10.71)	0.282	0.00 (-0.00 – 0.00)	0.342	0.00 (-0.00 – 0.00)	0.206	0.00 (-0.00 – 0.00)	0.505	-0.00 (-0.00 – 0.00)	0.994
Observations	12		12		12		12		12		12		12	
R^2 / R^2 adjusted	0.000 / -0.100		0.019 / -0.079		0.115 / 0.026		0.090 / -0.001		0.154 / 0.070		0.046 / -0.050		0.000 / -0.100	

Table 5. Linear regression results in female cultures treated with acetate

	Ahr		S100ß		Gfap		Ifnar		Il_22		Cyp1b1		Glul	
Predictors	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*
Dose	0.10 (-0.26 – 0.46)	0.554	0.01 (-0.21 – 0.24)	0.902	0.00 (-0.30 – 0.30)	0.993	0.13 (-0.48 – 0.74)	0.633	0.21 (-0.76 – 1.17)	0.640	0.04 (-0.26 – 0.34)	0.766	-0.00 (-0.59 – 0.58)	0.985
Observations	12		12		12		12		12		12		12	
R^2 / R^2 adjusted	0.036 / -0.060		0.002 / -0.098		0.000 / -0.100		0.024 / -0.074		0.023 / -0.075		0.009 / -0.090		0.000 / -0.100	

	Bdnf		Ngf1		Gdnf		Gad67		Glud1		Pgc1-a		Sp1	
Predictors	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*
Dose	-0.08 (-0.53 – 0.37)	0.699	0.21 (-0.96 – 1.37)	0.702	-0.18 (-0.43 – 0.07)	0.140	0.11 (-0.57 – 0.79)	0.728	0.23 (-0.26 – 0.73)	0.317	0.00 (-0.35 – 0.35)	0.999	-0.04 (-0.52 – 0.45)	0.875
Observations	12		12		9		12		12		12		12	
R^2 / R^2 adjusted	0.016 / -0.083		0.015 / -0.083		0.284 / 0.182		0.013 / -0.086		0.100 / 0.010		0.000 / -0.100		0.003 / -0.097	

Propionate

Propionate treatment (3.5 – 35µM) did not impact gene expression significantly (see Fig. 3 and Table 6-7).

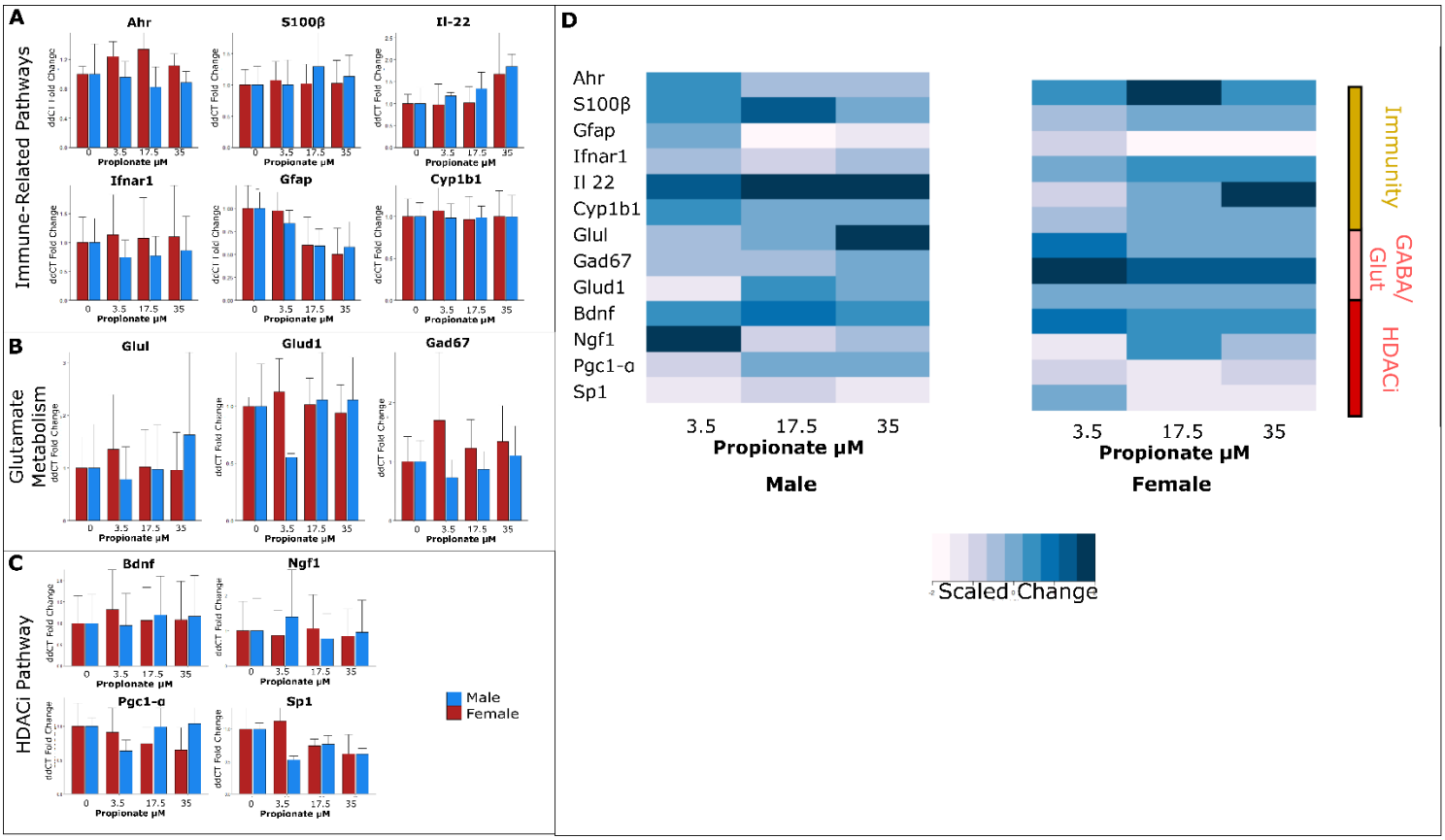

Fig 3. Impact of acetate on cortical astrocyte gene expression. A-C. The impact of different concentrations of butyrate on immune-related, glutamine/GABA metabolism and histone deacetylase inhibitory pathways in male and female cortical astrocytes (N = 3 litters). Raw data was analyzed using the $2^{-\Delta\Delta CT}$ method. Plots show the means within each group normalized to the vehicle (0µM) with bars representing the standard error of the mean. D. A heatmap visualizing mean changes in gene expression across different genes.

Table 6. Linear regression results in male cultures treated with propionate.

	Ahr		**S100ß**		**Gfap**		**Ifnar**		**Il_22**		**Cyp1b1**		**Glul**	
Predictors	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*
Dose	-0.00 (-0.02 – 0.02)	0.716	0.01 (-0.02 – 0.03)	0.706	-0.01 (-0.03 – 0.00)	0.141	-0.00 (-0.03 – 0.03)	0.928	0.02 (0.00 – 0.04)	**0.045**	0.00 (-0.01 – 0.01)	0.990	0.02 (-0.05 – 0.10)	0.562
Observations	12		12		12		12		12		12		12	
	Bdnf		**Ngf1**		**Gdnf**		**Gad67**		**Glud1**		**Pgc1-a**		**Sp1**	
Predictors	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*
Dose	0.01 (-0.05 – 0.07)	0.824	-0.01 (-0.08 – 0.07)	0.822	-0.18 (-0.43 – 0.07)	0.140	0.01 (-0.02 – 0.03)	0.642	0.01 (-0.02 – 0.03)	0.509	0.01 (-0.01 – 0.03)	0.520	-0.01 (-0.02 – 0.01)	0.311
Observations	12		12		9		12		12		12		12	
R^2 / R^2 adjusted	0.005 / -0.094		0.005 / -0.094		0.284 / 0.182		0.022 / -0.075		0.045 / -0.051		0.043 / -0.053		0.102 / 0.012	

Table 7. Linear regression results in female cultures treated with propionate.

	Ahr		**S100ß**		**Gfap**		**Ifnar**		**Il_22**		**Cyp1b1**		**Glul**	
Predictors	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*
Dose	0.10 (-0.26 – 0.46)	0.554	0.01 (-0.21 – 0.24)	0.902	0.00 (-0.30 – 0.30)	0.993	0.13 (-0.48 – 0.74)	0.633	0.21 (-0.76 – 1.17)	0.640	0.04 (-0.26 – 0.34)	0.766	-0.00 (-0.59 – 0.58)	0.985
Observations	12		12		12		12		12		12		12	
R^2 / R^2 adjusted	0.036 / -0.060		0.002 / -0.098		0.000 / -0.100		0.024 / -0.074		0.023 / -0.075		0.009 / -0.090		0.000 / -0.100	
	Bdnf		**Ngf1**		**Gdnf**		**Gad67**		**Glud1**		**Pgc1-a**		**Sp1**	
Predictors	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*	*Estimates*	*p*
Dose	-0.08 (-0.53 – 0.37)	0.699	0.21 (-0.96 – 1.37)	0.702	-0.18 (-0.43 – 0.07)	0.140	0.11 (-0.57 – 0.79)	0.728	0.23 (-0.26 – 0.73)	0.317	0.00 (-0.35 – 0.35)	0.999	-0.04 (-0.52 – 0.45)	0.875
Observations	12		12		9		12		12		12		12	
R^2 / R^2 adjusted	0.016 / -0.083		0.015 / -0.083		0.284 / 0.182		0.013 / -0.086		0.100 / 0.010		0.000 / -0.100		0.003 / -0.097	

Discussion

To our knowledge, this is the first *in vitro* study which assessed a physiologically relevant gradient of SCFAs in cortical astrocytes. Most *in vitro* studies of SCFA function pre-treated neuroimmune cells to induce inflammation before the addition of SCFAs and did not assess sex-differences (Yang et al., 2019, Yamawaki et al., 2018, Wang et al., 2018a, Singh et al., 2014, Chen et al., 2007, Hoyles et al., 2018). Thus, we used primary enriched male and female cortical astrocyte culture to investigate the potential impacts of butyrate, acetate and propionate on gene expression. We investigated the impact of SCFAs in aryl-hydrocarbon

receptor/immune signalling, glutamate/glutamine metabolism and histone-deacetylase inhibitor (HDACi) pathways.

When treating cells with butyrate, we did not see any impact on immune-related or glutamate-glutamine metabolism genes. However, in females we found that butyrate concentrations positively correlated with *Bdnf* and *Pgc1-α* expression. These genes may be activated downstream of HDACi activity. Intriguingly, these effects were only found in female cortical astrocytes.

Surprisingly, acetate treatment did not alter glutamate-glutamine related gene expression in the astrocytes. A previous human study found ^{13}C radiolabelled carbohydrate substrates are converted to acetate within the gut, reaching the hypothalamus through peripheral circulation leading to appetite suppression as well as changes to hypothalamic glutamate-glutamine metabolism (Frost et al., 2014). Acetate treatment did not impact any HDACi pathway-related genes but did alter immune gene expression in male cortical astrocytes. In male, but not female cortical astrocytes, acetate treatment positively correlated with *Ahr* and *Gfap* expression. Previous studies identified dietary-derived tryptophan metabolites as mediators of astrocytic immunity through the intracellular activation of the aryl-hydrocarbon receptor pathway (Rothhammer et al., 2016). Additional studies suggested that this receptor is also activated by SCFAs (Marinelli et al., 2019, Jin et al., 2017). These results suggest that acetate may also play a role in this astrocytic immune pathway. Meanwhile, propionate did not induce any dose-dependent or sex-specific responses in astrocyte gene expression.

There are practical limitations within the study. Prior to this investigation, there was a lack of insight into which pathways would be impacted by small concentrations of

SCFAs. There may be other pathways with larger undetected effects. Additionally, our study did not investigate the supernatants or metabolic responses within cell culture to these stimuli. Nonetheless, it is valuable insight for future mechanistic studies.

These results add to a growing collection of studies suggesting the importance of SCFAs for neuroprotection and behavior. In rodents, SCFA administration has shown to be neuroprotective against stroke (Sadler et al., 2020, Lee et al., 2020a), stress (van de Wouw et al., 2018), oligodendrocyte function (Chen et al., 2019b), and depressive-like behaviour (Yamawaki et al., 2018). SCFAs modulate blood-brain barrier permeability *in vitro* (Hoyles et al., 2018) and are shown to cross the blood-brain barrier in rodent and primate studies (Dalile et al., 2019). Now, we present evidence that mammalian astrocytes may mediate some of these effects in a sex-specific manner.

Competing Interests

J.F.C and T.G.D have research support from Cremo, Pharmavite, Dupont and Nutricia. These authors have spoken at meetings sponsored by food and pharmaceutical companies. All other authors report no potential conflicts of interests.

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Data Availability

Code used for analysis and plotting, as well as ddCT data is uploaded here.

CRediT Author Contribution

Simon Spichak: Investigation, Methodology, Writing - review & editing, Writing - original draft. **Francisco V. Donoso:** Investigation, Methodology, Writing - review & editing. **Gerard M. Moloney:** Investigation, Methodology, Writing - review & editing. **Eoin Gunnigle:** Investigation, Writing - review & editing. **Jillian M. Brown:** Methodology. **Martin Codagnone:** Methodology. **Anna V. Golubeva:** Methodology. **Timothy G. Dinan:** Conceptualization, Funding acquisition, Writing - review & editing. **John F. Cryan:** Conceptualization, Funding acquisition, Writing - review & editing.

Chapter 4: Discussion

4.1 Overview of Findings in Chapter 2

In Chapter 2, I reanalyzed 249 studies involving some measures of human brain health, psychiatric disorders, mood/behaviour and which sequenced the gut microbiome of participants. Where data was publicly accessible, studies were reanalyzing used a consistent pipeline which included assessing the abundance of neuroactive gut-brain modules.

Very few datasets involving healthy humans had publicly accessible data. Across six studies of infant temperament and microbiome, (Carlson et al., 2018, Gao et al., 2019, Christian et al., 2015, Wang et al., 2020d, Aatsinki et al., 2019, Loughman et al., 2020), only two found the same genus-level association of *Bifidoacterium* with positive behaviors (Wang et al., 2020d, Aatsinki et al., 2019). Four studies looked to correlate the abundance of gut microbes with aspects of personality and emotion (Tillisch et al., 2017, Taylor et al., 2019, Kim et al., 2018, Johnson, 2020). While each study found specific genus level associations with personality, emotion or anxiety, these were not consistent across studies. Next, the (Liu et al., 2019a) dataset was reanalysed finding that *Alloprevotella* abundance was significantly reduced in individuals with insomnia, compared to controls. However, there were no differences in gut-brain module abundance. Across four other studies that were not reanalyzed, two did not find any genera-level associations with sleep metrics (Liu et al., 2020c, Anderson et al., 2017). A 16S sequencing study associated sleep disturbance with the *Prevotella* enterotype (Ko et al., 2019). Another intriguing study found associations between multiple aspects of sleep quality with bacterial genera (Smith et al., 2019b). However, this study may be limited by its use of fecal swabs for sample collection. Five studies of healthy ageing and cognition all compared different subsets of unhealthy cognitive aging thus, no genus-level differences were found in common amongst them (Nagpal et al., 2019, Kim et al., 2020, Bajaj et al., 2016, Saji et al., 2019).

Many studies assessed the microbiome composition of people with neurodevelopmental disorders. Several of these datasets were reanalyzed. In attention-deficit hyperactivity disorder (ADHD), one reanalyzed study did not find any differences in microbial composition or GBMs (Aarts et al., 2017). Wan et al. (2020) used a WGS approach, finding a reduction in dopaminergic pathways in the ADHD microbiome, as well as a reduction in *Faecalibacterium*. Jiang et al. (2018b) also observed this reduction, correlating it to scores assessing behavioural difficulties and hyperactivity. Five other studies did not report similar findings (Stevens et al., 2019, Prehn-Kristensen et al., 2018, Szopinska-Tokov et al., 2020, Pärtty et al., 2015, Wang et al., 2020b).

Seven studies assessing the microbiome of children with autism spectrum disorders (Averina et al., 2020, Son et al., 2015, Pulikkan et al., 2018, Kang et al., 2019, Kong et al., 2019, Liu et al., 2019d, Strati et al., 2017). Only two studies showed significant differences in microbial abundance but none showed any changes in gut-brain modules. Thirty other studies were summarized and compared (see **Table 1**), finding very few consistent microbiome changes across the studies. One of the four studies which used a WGS approach reported a reduction in glutamate/glutamine metabolism in ASD (Wang et al., 2019a). A study by (Liu et al., 2019d). reported increased valerate and decreased butyrate in ASD faecal samples. Berding and Donovan (2019) reported that fecal SCFAs correlated strongly with diet. These results suggest that dietary preferences play a strong role in shaping the ASD microbiome.

Four studies assessing the schizophrenia microbiome were reanalyzed (Xu et al., 2020, Shen et al., 2018, Flowers et al., 2019, Nguyen et al., 2019). Across two of these studies, the schizophrenia microbiome was enriched with the acetate-producing

Fusicatenibacter (Shen et al., 2018, Xu et al., 2020). When looking for gut-brain modules, one of the datasets also found an increased abundance of butyrate synthesis, kynurenine synthesis and inositol degradation in the schizophrenia microbiome (Xu et al., 2020). Additionally, this dataset showed a reduction in *Lactobacillus* (Xu et al., 2020). However, across three WGS studies which were not reanalyzed (Zhu et al., 2020, Xu et al., 2020, Schwarz et al., 2018), the reported findings were not consistent.

A few studies analysed the microbiome in other neurodevelopmental disorders but were too few in number to draw any conclusions or comparisons (Borghi and Vignoli, 2019, Strati et al., 2016, Quagliariello et al., 2018).

One WGS epilepsy study was reanalyzed finding that the ketogenic diet increased the L-Tryptophan biosynthesis pathway in children with epilepsy (Lindefeldt et al., 2019). Another reanalyzed study compared children with cerebral palsy co-morbid with epilepsy to controls, finding over 20 differentially abundant microbes but no changes in gut-brain module abundance (Huang et al., 2019a). There were four 16S studies that could not be reanalyzed, however they each used different subsets of patients for comparisons (Zhang et al., 2018b, Peng et al., 2018, Xie et al., 2017, Safak et al., 2020).

Analyzing a dataset comparing Alzheimer's disease, mild-cognitive impairment and controls, we found multiple changes across microbial composition and gut-brain modules (Li et al., 2019a). In individuals with Alzheimer's disease, the microbiome showed a reduction in *Ruminoclostridium-5* and an enrichment in gut-brain modules involved in isovaleric acid synthesis, butyrate synthesis and acetate synthesis. Intriguingly, more gut-brain modules were found dysregulated when comparing the mild-cognitive impairment group to controls. This includes enrichment in isovaleric acid synthesis, butyrate synthesis,

acetate synthesis, tryptophan synthesis, quinolinic acid synthesis, quinolinic acid degradation. Three other studies (two 16S and one WGS) could not be reanalyzed; *Bacteroides* abundance was found increased in two of these studies (Haran et al., 2019, Vogt et al., 2017), but decreased in the third (Zhuang et al., 2018). Two of these studies also found *Alistipes* enrichment in individuals with AD (Haran et al., 2019, Vogt et al., 2017). However, none of these studies inferred the abundance of gut-brain modules nor did they measure fecal SCFAs. These findings provide moderate evidence for an increased SCFA production in Alzheimer's disease and mild cognitive impairment. This warrants further studies with more extensive metadata collection to untangle these interactions.

Three studies analysed the gut microbiome of individuals with Multiple Systems Atrophy, however the studies did not show consistent differences in the abundance of bacterial genera (Du et al., 2019, Engen et al., 2017, Tan et al., 2018). None of these studies were publicly accessible and thus were not reanalyzed. (Tan et al., 2018) found reductions in fecal SCFAs, suggesting they may be dysregulated in this disorder.

Three studies assessed the microbiome in Amyotrophic Lateral Sclerosis, however they all reported different results (Zhai et al., 2019a, Brenner et al., 2018, Mazzini et al., 2018). Using a WGS approach, (Blacher et al., 2019) found a reduction in tryptophan metabolism-related genes in ALS corresponding to alterations in serum tryptophan and nicotinamide metabolites.

Although Parkinson's Disease is among the most studied disorders in the context of the microbiota-gut-brain axis, our reanalysis did not find many differences in publicly accessible datasets (Bedarf et al., 2017, Heintz-Buschart et al., 2018, Aho et al., 2019, Pietrucci et al., 2019, Qian et al., 2018, Weis et al., 2019). However, after stratifying individuals by L-DOPA dose, we found one differentially abundant ASV of *Lactobacillus*

which was reduced compared to controls (Weis et al., 2019). Across ten studies that could not be reanalyzed, all of them found significant microbial community changes in Parkinson's disease even after accounting for covariates (Petrov et al., 2017, Barichella et al., 2019, Cirstea et al., 2020, Hill-Burns et al., 2017) (Keshavarzian et al., 2015, Vidal-Martinez et al., 2020, Li et al., 2019b, Unger et al., 2016, Ren et al., 2020, Lin et al., 2019). Perhaps information about pharmacological treatments, diet and lifestyle are required to uncover more consistent microbiome differences.

Two studies involving alcohol were reanalyzed (Stadlbauer et al., 2019, Bjorkhaug et al., 2019). In one study, the alcohol dependent cohort had an increased abundance of *Ruminococcus 2* and a reduction in *Ruminoclostridium 9*, as well as reduction in the tryptophan degradation gut-brain module (Bjorkhaug et al., 2019). Other studies that could not be reanalyzed all used different sequencing methods, cohorts and comparisons making it difficult to assess similarities across these studies (Dubinkina et al., 2017, Seo et al., 2020, Tsuruya et al., 2016).

One study involving smokers found that tobacco but not electronic cigarette users had a reduction in tryptophan degradation and propionate synthesis (Stewart et al., 2018). No other smoking studies which used next-generations sequencing were found. One datasets of recreational drug-use was reanalysed but no differentially abundant microbes or gut-brain modules were found (Barengolts et al., 2018). An additional three studies could not be reanalyzed. Among them, Fulcher et al. (2018) reported specific changes in microbial abundance associated with recreational drugs. (Xu et al., 2017) controlled for age and sex, finding no microbial changes associated with drug use. Finally, (Panee et al., 2018) reported a positive association between *Prevotella* abundance in recreational marijuana users and cognition.

Across two multiple sclerosis datasets, we did not find any differences in gut-brain modules or microbial abundance (Miyake et al., 2015, Jangi et al., 2016).. However,

when reanalyzing a dataset comparing individuals with neuromyelitis optica spectrum disorder to controls, we found reductions in *Streptococcus* in the disease group (Gong et al., 2019). The researchers also reported an overall reduction in fecal SCFAs also associating acetate and butyrate with disease severity (Gong et al., 2019). Across four studies that could not be reanalyzed, no differences were consistently reported (Ventura et al., 2019, Berer et al., 2017, Reynders et al., 2020, Zeng et al., 2019). (Zeng et al., 2019) reported reductions in fecal SCFAs when comparing either the multiple sclerosis or the neuromyelitis optic spectrum disorder to controls. The involvement of SCFAs in multiple sclerosis warrants further investigation.

While few studies looked specifically at the associations between pain-related disorders, the brain and the microbiome, there is nonetheless some evidence that warrants further investigations. There is some evidence from a WGS study that the gut microbiome influences fibromyalgia and serum SCFAs (Minerbi et al., 2019). Meanwhile, across several different irritable-bowel syndrome studies different microbes are associated with pain (Peter et al., 2018b, Peter et al., 2018a, Labus et al., 2019, Jeffery et al., 2012). These studies provide some evidence that the microbiome may be involved with the psychological aspects of irritable bowel syndrome. Meanwhile, a recent study found enrichment of the kynurening synthesis and quinolinic acid degradation gut-brain modules in elderly women with migraines (Chen et al., 2019).

Two anorexia nervosa datasets were reanalyzed (Borgo et al., 2017, Mack et al., 2016). We found significant differences only in the latter dataset which had a larger sample size. We saw enrichment in isovaleric acid synthesis, quinolinic acid synthesis, quinolinic acid degradation pathways in patients with anorexia (Mack et al., 2016). Notably, the alpha-melanocortinin stimulating hormone mimetic, known

to reduce appetite in mice (Tennoune et al., 2014) is restored to control level after weight gain and renourishment (Mack et al., 2016). While these changes were not reported in four other studies (Morkl et al., 2017, Morita et al., 2015, Kleiman et al., 2015, Armougom et al., 2009), authors did not measure the abundance of gut-brain modules. Future studies focusing on SCFAs and ClpB in anorexia nervosa are needed to better understand its role in satiety signalling.

While butyrate has been characterized as a neuroprotective agent for ischemia in preclinical studies (Akhoundzadeh et al., 2018, Lee et al., 2020a, Sadler et al., 2020, Singh et al., 2018, Sun et al., 2016a), few human studies have been conducted. None of these datasets were publicly accessible. (Wang et al., 2018b) did not find any changes in the gut microbiota after a cerebral infarction. In contrast, another study found that bacteria involved in butyrate and tryptophan metabolism (*Bacteroides, Parabacteroides, Akkermansia, Prevotella* and *Faecalibacterium*) were reduced following the cerebral infarction (Ji et al., 2017). When stratifying patients by type of stroke and severity, other researchers uncovered more microbial perturbations. For example, Liu et al. (2020a) found many genera associated with individuals showing post-stroke cognitive impairment. Two other studies reported conflicting differences in the abundance of *Akkermansia* in patients with ischemic stroke, with one study reporting an increased abundance while another reporting a reduction (Ji et al., 2017, Li et al., 2019c). (Polster et al., 2020) found robust differences and correlations within a large sample (N = 122) of cavernous angioma using 16S and WGS techniques. The abundance of several species was enriched in the cavernous angioma group including *Bacteroides thetaomicron* and *Odoribacter sphlancus* while the abundance of other species was reduced *Bifidobacterium adolescentis* and *Faecalibacterium prausnitzii* (Polster et al., 2020). They even reported differentially abundant species by cavernous angioma subtype and severity (Polster et al., 2020). However, they did not assess differentially abundant gut-brain modules. Nonetheless, similar large scale studies are crucial for understanding whether a certain microbiome composition is protective against stroke or neurovascular disease.

None of the studies involving stress or psychiatric disorders had publicly accessible datasets. Thus, none of this data could be reanalyzed. Across three studies of different types of stressors, the microbiome appears to play a role. One study found that adverse childhood events increased the abundance of *Prevotella* during pregnancy (Hantsoo et al., 2019). Other studies found that maternal stress influenced infant microbial composition (Hu et al., 2019a, Naude et al., 2020, Carson et al., 2018). These results suggest that stress should be closely monitored during pregnancy and that early-life insults may influence the microbiome composition in later life.

Two further studies analysed the impact of post-traumatic stress disorder on the gut microbial composition (Hemmings et al., 2017, Bajaj et al., 2019). While (Bajaj et al., 2019) reported many differences in microbial abundance in veterans suffering from this disorder, more studies are needed to confirm these findings in a broader population. Across ten studies of bipolar disorder, no consistent microbial changes were reported across studies (Rong et al., 2019, Vinberg et al., 2019, Schwarz et al., 2018, Painold et al., 2019, McIntyre et al., 2019, Evans et al., 2017, Coello et al., 2019, Hu et al., 2019b, Zheng et al., 2020b).

A total of 18 studies of depression and anxiety were reanalyzed and summarized. Across multiple studies of depression, *Faecalibacterium* shows a positive impact on quality of life/anxiety/depression (Jiang et al., 2015, Jiang et al., 2018a, Stevens et al., 2018, Valles-Colomer et al., 2019). Similarly, *Dialister* is depleted in depression across several other studies (Jiang et al., 2015, Jiang et al., 2020, Valles-Colomer et al., 2019). Indeed, other studies also found negative correlations between *Faecalibacterium* and anxiety or depression (Jiang et al., 2015, Jiang et al., 2018a, Stevens et al., 2018). These findings are especially interesting since they replicated in non-compositionally analysed data sets.

Together this data provides strong evidence that SCFA and tryptophan metabolism may be altered in Alzheimer's disease, depression, anxiety and schizophrenia.

4.2 Overview of Findings in Chapter 3

In Chapter 2, SCFAs were identified as important neuroactive metabolites. Despite these findings, the mechanisms behind these effects are unclear. Human studies suggested that SCFAs reach the brain and impact glutamate metabolism, stress, and satiety (Frost et al., 2014, Dalile et al., 2020). Preclinical and *in vitro* studies identified several potential pathways by which they impact neuroimmune cells: histone-deacetylase inhibition, glutamate-glutamine metabolism and aryl-hydrocarbon receptor/immune pathways (Erny et al., 2015, Yang et al., 2019, Yamawaki et al., 2018, Wang et al., 2018a, Singh et al., 2014, Chen et al., 2019b, Marinelli et al., 2019, Jin et al., 2017). These experiments often pre-treated cells to induce inflammation. Then supraphysiological levels of SCFAs, mainly butyrate, to observe any of its effects.

Recent investigations identified the important roles of astrocytes in brain and behavior, as well as in mediating the impact of microbial metabolites (Rothhammer et al., 2016, De Luca et al., 2020, Tomova et al., 2019, Khakh and Deneen, 2019, Alberini et al., 2018, Jensen et al., 2013, Cao et al., 2013). To address the potential role of SCFAs on naïve astrocytes, we generated enriched primary cultures and treated the cells with low, physiological doses of SCFAs. Addressing sex-differences across brain disorders and the microbiota-gut brain axis (Jaggar et al., 2020), we tested three different concentrations of butyrate, acetate and propionate on male and female murine cortical astrocytes separately. Using qPCR, the expression of a panel of genes was assessed after treatment.

When treated with butyrate, genes implicated in neuroimmune and glutamate metabolic pathways were not affected in males or females. However, in females but not in males, two genes (*Bdnf* and *Pgc1-* involved in the histone deacetylase inhibition pathway increased in a dose-dependent manner in response to butyrate. Meanwhile, only the neuroimmune pathway was altered by acetate treatment. We noticed a dose-dependent increase of *Ahr* and *Gfap* expression only in males in response to acetate. Finally, we did not find any dose-dependent or sex-specific effects of propionate on astrocyte gene expression.

4.3 Limitations of Human Data

Despite advances in sequencing and bioinformatics, it is still difficult to replicate findings across multiple studies. This is in part due to confounders emerging from the collection of metadata (medications, food-frequency questionnaires, symptoms/pathology), sequencing methods and the nature of the microbiome itself. When attempting to reanalyse studies with an up-to date bioinformatic pipeline, we demonstrated the wide confidence intervals of many differentially expressed microbes. Further, we found similar statistical uncertainties when inferring metabolites with neuroactive potential.

However, it remains clear even with these limitations that there are striking similarities across many microbiome cohorts involving schizophrenia, anxiety, depression and Alzheimer's disease. While we can infer metabolism from 16S or WGS sequencing, it does not provide more exact information about the metabolites, proteins, lipids and signals produced by gut microbes. While we used inferred neuroactive gut-brain modules, this database will likely be expanded in future studies as some neuroactive pathways, like bile metabolism, remain unannotated (Valles-Colomer et al., 2019). It will be important to further identify the metabolites

involved in these altered pathways to confirm these findings. Perhaps it might help us better understand the pathophysiology of these diseases or their symptoms.

4.4 Glia and the Microbiome: Where to Next?

As more researchers focus in on the gut microbiome, the glial cells of the brain have become an intriguing target. In the last decade, the importance of astrocytes and microglia in synaptic pruning and neurodevelopment has started to become unravelled (Paolicelli et al., 2011, Bilimoria and Stevens, 2015, Tremblay et al., 2011, Stephan et al., 2012, Sheridan and Murphy, 2013). Recent research looks to expand the role of microglia, oligodendrocytes and astrocytes beyond neurodevelopment and neuroinflammation including neuronal activity through negative feedback (Badimon et al., 2020), motor function and social interaction (Kana et al., 2019) and memory (De Luca et al., 2020, Drulis-Fajdasz et al., 2018, Alberini et al., 2018) amongst other functions. More microbiome studies are beginning to recognize the importance of glia within the brain (van der Lugt et al., 2018, Tse, 2017, Thion et al., 2018, Singh et al., 2018, Schmidtner et al., 2019, Sampson et al., 2016, Sadler et al., 2020, Rothhammer et al., 2016, Radulescu et al., 2019, Minter et al., 2016, McMurran et al., 2019, Matt et al., 2018, Leyrolle et al., 2019). Combined with new insights from single-cell RNA-sequencing technology (Zeisel et al., 2018, Van Hove et al., 2019, Sankowski et al., 2019, Prinz et al., 2019, Masuda et al., 2019), we may soon discover how specific microbial metabolites influence the brain, behavior and development.

4.5 SCFAs and the Microbiome: A Therapeutic Target Emerges

Several studies are already beginning to unravel the impacts of SCFAs on the brain and behaviour. SCFA supplementation attenuates the cortisol response in adult men after an acute psychosocial stressor in a dose-dependent manner (Dalile et al., 2020).

Another human study finds that acetate is able to reach the brain to influence satiety and glutamate/glutamine cycling in the hypothalamus (Frost et al., 2014). Recent advances in our understanding of prebiotic fibres pave the way for new personalized interventions to boost the levels of circulating SCFAs as an adjuvant for stress and eating disorders (Gill et al., 2020, Dalile et al., 2019, Fatahi et al., 2020, Deehan et al., 2020, Berding and Donovan, 2018). While one systematic review did not find that prebiotic intake impacted anxiety or depression measures (Liu et al., 2019c) while another found it associated with a lower odds-ratio for depression (Fatahi et al., 2020). Future studies are poised to identify specific effects of SCFAs on our cortisol response as well as determine whether they alleviate anxiety or depression. Since different fibres impact the microbiome in different ways (Gill et al., 2020, Deehan et al., 2020), this must be considered when suggesting appropriate prebiotic interventions.

4.6 Tryptophan Metabolism and Microbiota: Diet to Neuroimmunity

Tryptophan and indole metabolites are a source of intrigue, as more and more research focused on neuroinflammation and psychiatry suggest they play key roles. Dietary tryptophan metabolites were found to active the astrocyte aryl-hydrocarbon receptor (Rothhammer et al., 2018, Rothhammer et al., 2016). A systematic review of tryptophan/kynurenine metabolites suggests that perturbation of this delicate balance may contribute to schizophrenia, however more large-scale studies are needed (Pedraz-Petrozzi et al., 2020). Another meta-analysis finds that metabolism shifts towards the kynurenine pathway, away from serotonin production across many psychiatric disorders (Marx et al., 2020a). In schizophrenia, kynurenine metabolism then shifts towards the production of kynurenic acid while in mood disorders it shifts towards the production of excitotoxic quinolinic acid (Marx et al., 2020a). Future

studies may look to use diet as a means of influencing tryptophan or indole metabolism.

4.7 Bile Acids and the Brain: More than a Gut Feeling?

Several studies suggest that bile acids play intriguing roles beyond digestion, signalling through the microbiota-gut-brain axis. Several studies of neuronal and glial cells have identified the presence of bile acid receptors (Cani et al., 2013, Dempsey et al., 2018, Keitel et al., 2010, Mertens et al., 2017, Poole et al., 2010, Silva et al., 2012, Yanguas-Casás et al., 2017). Bile acid metabolism by the microbiota is implicated in social behaviour, gastrointestinal distress, neuroinflammation, depressive-like behaviors and the HPA axis across preclinical and *in vitro* models (Choudhuri et al., 2003, Golubeva et al., 2017, Hoffman et al., 2019, Jena et al., 2018, Klaassen and Aleksunes, 2010, McMillin et al., 2015, Mertens et al., 2017, Nizamutdinov et al., 2017, Yanguas-Casás et al., 2017, Yanguas-Casas et al., 2017, Wang et al., 2020a). A recent study found alterations in bile acid profiles in individuals with Alzheimer's and mild-cognitive impairments (MahmoudianDehkordi et al., 2019). A double-blind randomized clinical trial found that supplementation with the bile acid taurursodiol combined with sodium phenylbutyrate slowed motor decline in individuals with amyotrophic lateral sclerosis while another study found it increased survival (Paganoni et al., 2020a, Paganoni et al., 2020b). It is clear that bile acids may play important neuroprotective and anti-inflammatory roles in brain health, however more studies are needed to assess their effects across different brain disorders.

4.8 Future Directions

Many current human microbiome studies now focus on identifying gut-microbial metabolites derived from aromatic amino acids in the faeces and serum, to

understand their role in brain health (Arnoriaga-Rodríguez et al., 2020, Molinaro et al., 2020, Bar et al., 2020). There remain a multitude of unidentified microbial by-products that subtly impact our metabolism, serum metabolites and brain-signalling. Future bioinformatics studies will focus on identifying the range of metabolites and functional molecules produced by different genera or species of bacteria. In addition, different bacterial proteins might also contribute to cross-kingdom signalling. No example is as striking as ClpB, a bacterial protein was a mimetic of alpha-Melanacortonin Stimulating Hormone (Breton et al., 2016, Dominique et al., 2019, Tennoune et al., 2014). Moreover, quorum signalling molecules released by *Staphylococcus aureus* act as excitatory or inhibitory modulators of enteric nerves (Uhlig et al., 2020). Screening the supernatant proteins and secreted metabolites from a host of potential gut-bacteria could help identify new neuroactive molecules.

Additionally, open metabolomic, proteomic and lipidomic datasets could also be reanalysed. This could potentially identify new candidate signalling molecules altered due to gut microbiome disruptions.

However, we still don't know which metabolites may cross the blood-brain barrier and in what amount. Additionally, we do not understand the flux of these metabolites over the course of the day. Studies are only able to provide a snapshot of one moment in time. Studying the levels of these metabolites in the faeces and serum in conjunction with functional brain-imaging is the next step in understanding the impact of microbial metabolism on the brain. These studies would lead into the development of postbiotics or biologics mimicking beneficial microbial signals. Candidates could then be tested in preclinical disease models and later translated into humans. Microbial-derived metabolites will be key for understanding the mechanisms underlying the microbiota-gut-brain axis.

The next step would involve determining their potential mechanism of action. *In vitro* high-throughput studies could identify potentially neuroactive metabolites in *Caenorhabditis elegans* or *Danio rerio*. Alternatively, primary cultures or co-cultures of neurons and glia may be used to identify which cell types can respond to these metabolites. Metabolites with potential impacts on early neurodevelopment or neurobehavioral pathways would continue into *in vivo* rodent studies. Different doses are assessed on their microbiome modulatory properties as well as their impact on the brain. Once validated within a rodent model, these metabolites may be regulated either through dietary interventions or through oral supplementation. Potentially, some of these metabolites could even be delivered at supraphysiological levels to modulate brain-health in the coming decades.

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