

Mom's diet matters:

Effects of mouse maternal prebiotic intake on
the offspring brain and behaviour



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A thesis submitted in partial fulfilment of the requirements for the degree
of Doctor of Philosophy in the University of Oxford.

Michaelmas 2019

ii. Abstract

Background: Studies are increasingly pointing to the gut microbiome as an important modulator of brain development and function. The galacto-oligosaccharide prebiotic Bimuno® (BGOS) has been shown to alter behaviour and brain gene expression in rodents when administered orally during both early life and adulthood. BGOS also influences gut microbiome composition, production of short-chain fatty acids (SCFAs) through fermentation, and host immune function and metabolism. In addition, the maternal microbiome is important for offspring brain and behaviour development. Therefore, BGOS supplementation during pregnancy and lactation in mice may modulate the maternal gut microbiome and, in turn, influence offspring behaviour and brain gene expression.

Methods: CD1 dams were randomly assigned to receive either normal drinking water or drinking water supplemented with BGOS during gestation and suckling. Offspring were tested at weaning age or adulthood, and a cross-foster design was employed in a separate cohort to differentiate between effects of prenatal and postnatal maternal BGOS intake. Acute or early-life lipopolysaccharide was also administered to determine whether maternal BGOS mitigates the brain and behaviour effects of a pro-inflammatory challenge. Exploratory, anxiety-like, and passive stress-coping behaviours were assessed. In the brain, relative gene expression of glutamate and serotonin receptors, brain-derived neurotrophic factor, and pro-inflammatory markers was quantified in the prefrontal cortex and hippocampus. To explore mechanisms that may underlie changes in the offspring brain and behaviour, immune cell composition and function and host plasma metabolite profiles were also assessed in offspring. Finally, maternal and offspring faecal microbiome diversity, composition, and metabolites were analysed to explore potential

relationships between the maternal microbiome, the offspring gut microbiome, and the offspring brain, behaviour, immunity, and metabolism.

Results: Maternal BGOS supplementation during gestation and suckling increased exploratory behaviour and reduced anxiety and behavioural despair in young CD1 offspring at weaning age. In cross-fostered pups, both prenatal and postnatal maternal BGOS increased exploration, while postnatal BGOS additionally reduced anxious behaviour. Maternal BGOS also reduced expression of cortical and hippocampal glutamate and serotonin receptor genes in young offspring. In addition, immune cell composition and function and plasma metabolite profiles were altered. In adult offspring, perinatal BGOS induced long-lasting and sex-dependent changes in behaviour, brain gene expression, and plasma metabolites. Finally, while offspring faecal microbiome beta diversity, composition, and metabolite profiles were significantly different in young BGOS pups compared to controls, including increased levels of SCFAs, minimal microbial changes were detected in maternal faecal samples. Differentially abundant taxa, but not faecal metabolites, correlated with changes in offspring behaviour and gene expression.

Conclusion: Maternal BGOS produced long-lasting changes in behaviour and brain gene expression in young and adult offspring. These differences were accompanied by changes in plasma metabolite profiles and immune function. Although differences in beta diversity and abundances of specific taxa were detected in offspring faecal samples, BGOS did not substantially alter the maternal faecal bacteria. This suggests that maternal BGOS-induced changes in the offspring brain and behaviour are independent of changes in the maternal microbiome. Therefore, future studies will need to explore how maternal BGOS intake changes the offspring gut microbiome and whether there is a causal relationship between the offspring microbiome and brain and behaviour changes.

iii. Acknowledgements

It has been surreal to witness three years of pipetting, troubleshooting qPCR, bemoaning smeared Western blots, watching mice run around in boxes, and spending hours aligning figures come together into a completed thesis. Although it is only my name on the title page, it took an army to get here, led by my supervisors Professors Phil Burnet and Daniel Anthony. Thank you so much for your guidance and support. It is astonishing to think back to October of 2016 and contemplate how much I have learned from both of you. I could not have asked for better mentors during this process.

I have had many collaborators in the past three years, and I need to thank the following extremely busy postdocs who somehow found the time, energy, and patience to help me in the lab: Naveed Akbar, for hosting me in your lab and taking the time to show me step-by-step how to extract and stimulate bone marrow-derived macrophages; Fay Probert, for running my ^1H NMR samples, teaching me how to run the multivariate analysis, and showing me that I should have been using R all along; Nick Ilott, along with the Oxford Genomics Centre and the Oxford Centre for Microbiome Studies, for prepping, sequencing, and processing my DNA samples and guiding me on the statistical analysis. In addition, this work required many hands. I could not have done this ambitious project without Daniel Radford-Smith's help in running and scoring behavioural tests and helping with tissue collection. I am also grateful to Kary Chan and Pippa Gleave for helping with behavioural testing.

And finally, this DPhil would not have been possible without my Oxford family. The NET group (Kalina, Katerina, Amy, Shi Yu, Sonia, Rita, Dan) – you made the lab a joy to work in. To my parents – I wouldn't be here without you (literally). Thanks for putting up with me through 20 years of school, and I promise I'll get a real job now. To all my Oxford friends – there are too

many to list, but you made Oxford a home away from home. And finally, thank you, Tariq, for bouncing ideas around with me, listening to me complain, supporting and encouraging me, and being my writing buddy. You have been my rock.

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v. Abbreviations

ASD	autism spectrum disorder
ASV	amplicon sequence variant
BDNF	brain-derived neurotrophic factor
BGOS	Bimuno ® galacto-oligosaccharide
BMDM	bone marrow-derived macrophage
CNS	central nervous system
FMT	faecal microbiome transplant
FST	forced swim test
GABA	gamma-aminobutyric acid
GF	germ-free
GOS	galacto-oligosaccharide
HDAC	histone deacetylase
HPA	hypothalamic-pituitary-adrenal
I.P.	intraperitoneal
IFN- γ	interferon- γ
IL-10	interleukin-10
IL-1 β	interleukin-1 β
IL-6	interleukin-6
LDB	light-dark box
LPS	lipopolysaccharide
MIA	maternal immune activation
NMDAR	N-methyl-D-aspartate receptor
NMR	nuclear magnetic resonance
OFT	open field test
OPLS-DA	orthogonal partial-least squares discriminant analysis
PCA	principal component analysis
PCoA	principal coordinate analysis
PFC	prefrontal cortex
qPCR	quantitative polymerase chain reaction
rRNA	ribosomal RNA
S.C.	subcutaneous
SCFA	short-chain fatty acid
SIT	social interaction test
SPF	specific pathogen-free
TLR	toll-like receptor
TNF- α	tumour necrosis factor- α
Treg	regulatory T cell
VIP	variable importance in projection

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1. Introduction

1.1 Trillions of stowaways: the gut microbiome

The role of the gut microbiome in human health and disease represents one of the most rapidly growing domains of research in biomedical science. When the Human Genome Project was completed, geneticists were surprised to find that only ~20,000 protein coding genes were predicted by human DNA. The trillions of microorganisms that live inside and on the surface of the human body may help to account for some of the missing traits and diversity. The largest collection of bacteria is found in the gastrointestinal tract (Turnbaugh et al. 2007), which will be the focus of this thesis. These microbial symbionts are crucial for immune system development and function, nutrient sensing, and metabolism (Kau et al., 2011).

A diverse microbiome promotes health for the host, although it is still unclear what a “healthy” gut microbiome looks like because there is a large amount of variation in gut microbiome composition between healthy individuals (Huttenhower et al. 2012). A shift in microbiome composition compared to healthy controls is associated with disorders in the gut as well as metabolic disorders, allergy, asthma, and central nervous system (CNS) disorders. It is unknown, however, whether a shift in the gut bacteria community is a cause or symptom of these conditions, and this remains a challenge of microbiome research in both health and disease (Carding et al., 2015).

In addition to the composition of the microbial community, it is crucial that gut bacteria remain contained within the intestinal lumen. The mucosal epithelium maintains this compartmentalisation by providing a physical barrier sealed with tight junctions. Epithelial cells also discriminate between pathogenic and commensal bacteria and regulate the appropriate

immune response. While pathogenic bacteria induce a pro-inflammatory immune response, “good” bacteria, such as species in the *Lactobacillus* and *Bifidobacterium* genera, inhibit pro-inflammatory pathways (Artis 2008). Furthermore, stress, inflammation, or poor diet can increase permeability of the gut epithelium. This results in stimulation of local and systemic pro-inflammatory responses (Cani et al., 2009; Smith et al., 2010; Söderholm et al., 2002). Thus, gut microbiome composition and compartmentalisation are crucial for host health.

Germ-free (GF) mice, otherwise genetically identical to conventional mice, are physiologically distinct in a number of ways and highlight the importance of the gut microbiome in normal development. GF mice show deficits in immune system function, including gut-associated lymphoid tissue development, antibody production, T cell proliferation, and susceptibility to pathogens and infections (Round and Mazmanian 2009). Metabolically, GF mice fed a high-fat and high-sugar Western diet are resistant to obesity (Bäckhed et al., 2007). Observations of differences in behaviour and brain function (Sudo et al. 2004) stimulated investigations into the influence of the gut microbiome on the CNS.

1.2 I’ve got a gut feeling: the microbiome-gut-brain axis

The common adage, “I’ve got a gut feeling,” is indeed supported by the last decade of research. Neurobiologists have been increasingly interested in the “microbiota-gut-brain axis” – the connections between our brains and the bacteria that live symbiotically in our gastrointestinal tracts.

1.2.1 Growing up in a bubble: Evidence from germ-free mice

This domain of research was popularised by studies in GF mice. Remarkably, researchers reported altered brain function and behavioural patterns in these aseptic animals, although there is some disagreement in the literature about the direction of changes. Lower levels of locomotor

activity and increased anxiety-like behaviour have been reported in GF mice (Lu et al. 2018). Increased locomotor activity and decreased anxiety-like behaviour, however, have also been described, along with increased monoamine turnover in the brain (Heijtz et al. 2011). GF mice display altered central gene expression patterns, increased plasma corticosterone levels, and reduced myelination in the prefrontal cortex (Hoban et al., 2016; Neufeld et al., 2011). Interestingly, both increased and decreased (Sudo et al. 2004; Heijtz et al. 2011) brain-derived neurotrophic factor (BDNF) have been reported in the hippocampus of GF mice. Although it is clear that GF mice fundamentally differ from conventional mice in behaviour and central gene expression patterns, further studies are necessary to clarify why disparities in findings about the directions of change exist. In addition, certain mouse models of brain pathologies fail to develop in GF mice, including models of Parkinson's disease (Sampson et al. 2016) and Alzheimer's disease (Harach et al. 2017). This highlights that the gut microbiome and the presence or absence of particular microbes are not inherently "good" or "bad" for the body. These studies do show, however, that gut bacteria are necessary for normal physiology.

Colonisation of GF mice at different time points in the lifespan has provided further evidence for the role of bacteria in brain development. Faecal microbiome transplant (FMT) involves transferring gut microbes from one individual to another to colonise the gut. This involves homogenising faecal samples from the cecum, colon, or stool and administering to an animal via oral gavage. Colonising GF mice with single species of bacteria early postnatally reversed the exaggerated hypothalamic-pituitary-adrenal (HPA) axis response to restraint stress that is characteristic of these animals (Sudo et al. 2004). However, reconstituting GF mice with the gut microbes of conventional mice in adulthood did not revert anxiolytic behaviour to normal (Neufeld et al, 2011). This implies a critical period early in development for the influence of the gut

microbiome on the brain. The importance of the early-life gut microbiome will be key to the rationale and the hypotheses in this thesis.

Although establishing causation in microbiome-brain studies is challenging, “humanising” GF mice or mice treated with high levels of antibiotics to deplete the gut bacteria with FMTs from humans with psychiatric disorders has been useful in investigating the causal role of the gut microbiome in these conditions. Transplanting faecal samples from patients with depression to mice, for example, recapitulated depression-like symptoms in the animals. These humanised mice exhibited higher levels of pro-inflammatory cytokines and depression-like behaviour than mice transplanted with samples from healthy individuals (Kelly et al. 2016). In another study, FMTs from children with autism spectrum disorder (ASD) or typically developing (TD) children were transplanted into mice. These mice were mated, and the offspring were tested for behavioural and molecular changes. ASD mice displayed deficits in social and anxiety-like behaviour. Several pathways in the brain were found to be downregulated in ASD mice based on RNA-sequencing data. In addition, differential splice events were identified in ASD mice in genes that have previously been associated with autism in humans (Sharon et al. 2019). FMT is a useful tool for exploring the causal relationship between the gut microbiome and brain disorders. While it provides convincing evidence for a microbiome-brain link, understanding the reasons for the causal link requires exploring the structure and function of the microbiome itself.

1.2.2 Microbial composition and the brain

Studies of microbiota composition in mouse models and human cohorts have rapidly populated the literature. Two DNA sequencing techniques are used to characterise the gut microbial community: 16S sequencing, which involves amplifying and sequencing the bacteria-specific small subunit of the ribosomal RNA gene, and metagenomics, which requires shotgun

DNA sequencing and assembly of all the DNA in a sample. The latter has a higher resolution, which allows for differentiation between lower taxa, and provides information about the presence of bacterial genes, such as enzyme-coding genes. It also accounts for other members of the microbiome besides bacteria, including viruses, fungi, and archaea. Metagenomics, however, is more costly and computationally demanding. For these reasons, 16S sequencing remains the most common technique for gut microbiome characterisation in human and animal studies.

The diversity of the microbiome and specific taxa have been associated with neurological and psychiatric disorders in human studies. Differences in gut microbial communities based on faecal DNA have also been reported in autism spectrum disorder (De Angelis et al. 2013), multiple sclerosis (Jangi et al. 2016), and Alzheimer's disease (Vogt et al., 2017), among others. Reproducibility and standardisation, however, pose challenges in the design and interpretation of experiments. Depression, for example, has been associated with altered microbiome composition, and increases in *Bacteroides*, *Faecalibacterium*, and *Prevotella* and decreases in *Clostridium XIX*, *Oscillibacter*, and *Roseburia*, were described in a cohort with active depression (Jiang et al., 2015). In contrast, while another study found a marginal association between the order *Bacteroidales* and depression, no other differences were identified at lower taxonomic levels (Naseribafrouei et al. 2014). Cohort-control studies in the microbiome-gut-brain axis field are becoming increasingly popular. While the results are exciting, researchers need to be cautious about experimental design, statistical power, high levels of inter-individual variation, and the limitations of 16S sequencing.

The majority of studies follow this formula of identifying simple correlations between gut microbiome diversity or specific taxa and a phenotype. Some studies have begun to explore mechanisms using sequencing data. Valles-Colomer et al. (2019) used shotgun metagenomics to identify “gut-brain modules,” microbial pathways that produce compounds with the potential to

interact with the nervous system. Even in the case of metagenomics, however, identifying protein-coding genes that correlate with a phenotype only provides evidence for a potential functional role of the microbiome. More work is needed to unravel the neural, immune, and metabolic mechanisms of microbiome-brain communication in health and disease.

1.2.3 Putative mechanisms of microbiome-brain communication

Possible mechanisms that link gut bacteria to brain function and behaviour include modulation of vagus nerve activity, development and function of the immune system, and circulation of microbial metabolites. These pathways are summarised in **Figure 1.1**.

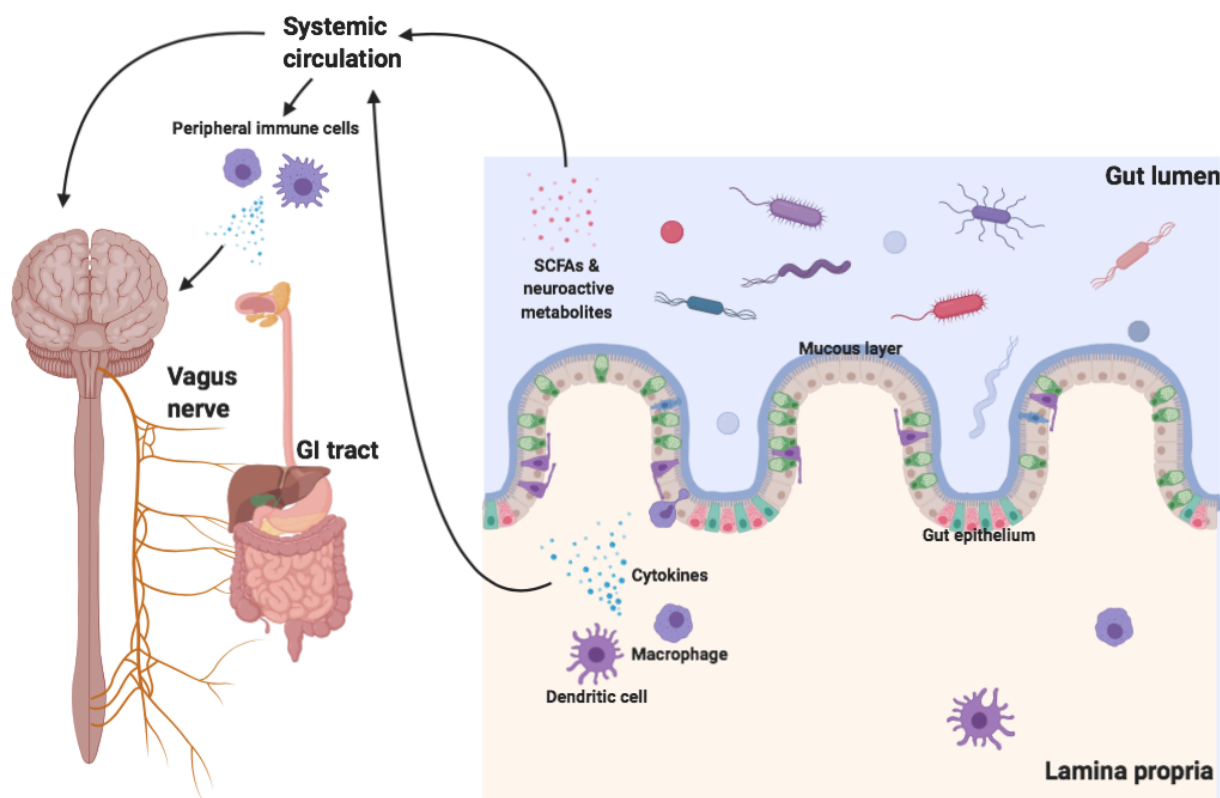


Figure 1.1. Schematic representation of vagal, metabolic, and immune microbiome-gut-brain pathways.

1.2.3.1 *Vagus nerve*

Gut microbes modulate activity of the vagus nerve, which connects the gut to the brainstem. Administration of the probiotic species *L. rhamnosus* reduced anxiety- and depression-like behaviour and stress-induced corticosterone release. It also altered central gamma-aminobutyric acid (GABA) receptor expression in BALB/c mice. However, a vagotomy prevented these changes, suggesting that vagus nerve activity was necessary for the neurobehavioural changes (Bravo et al. 2011). In addition, constitutive vagus nerve activity was shown to modulate hippocampal BDNF expression and neurogenesis in mice (O’Leary et al. 2018), supporting the hypothesis that regular vagus nerve stimulation is necessary for normal brain function. In contrast, vagotomy did not prevent the effects of antibiotics on anxiety-like behaviour in BALB/c mice (Bercik et al., 2011). Together, this evidence suggests that there are both vagus nerve-dependent and -independent mechanisms for microbiome-gut-brain communication.

1.2.3.2 *Peripheral immune system development and function*

The role of the microbiome in innate immune system function and, in turn, the influence of peripheral immunity on the brain are well-established. The immune system may therefore provide an indirect link between the gut microbiome and the brain. For example, commensal gut microbes produce short-chain fatty acids (SCFAs) that help to keep microbes compartmentalised in the intestinal lumen by reinforcing epithelial integrity (Peng et al., 2007; Peng et al., 2009; Suzuki, Yoshida, & Hara, 2008) and inhibit the growth of pathogenic bacteria by maintaining a low pH (Cherrington et al. 1991). Beyond the gut, microbes regulate myeloid cell proliferation and function (Deshmukh et al. 2014; Khosravi et al. 2015) and gene expression in immune cells through DNA methylation and histone acetylation (Alenghat et al., 2013; Chang et al., 2014; Ganai et al., 2012). Microbial metabolites, including SCFAs, are important mediators between the gut

microbiome and immune cell proliferation and function (Trompette et al. 2014; Arpaia et al. 2013; Chang et al. 2014; Krishnan et al. 2018). This evidence illustrates that gut bacteria and their metabolites are dynamic regulators of the immune system.

1.2.3.3 The gut microbiome, immune signalling, and the brain

On the other side of the microbiome-immune system-brain relationship, the brain's resident immune cells as well as peripheral immune cells and cytokines regulate normal brain function.

Microglia are the brain's resident macrophage cells (Salter and Stevens 2017), and increased microglia activation has been associated with human psychiatric disorders (Bayer et al., 1999; Torres-Platas et al., 2014) and stress-induced deficits in depression behaviour (Couch et al. 2013), anxiety, and social interaction (Lehmann et al., 2019). Microglia can also respond to peripheral inflammatory signals, such as tumour necrosis factor (TNF)- α , and recruit pro-inflammatory monocytes to the brain (D'Mello, Le, and Swain 2009) and promote anxiety-like behaviour in mice (McKim et al. 2018). There are also complex relationships between the cellular level of the innate immune system and brain function and behaviour. For example, the pro-inflammatory myeloid cells Ly6C^{hi} monocytes are necessary for hippocampal neurogenesis, an important process in cognition and mood regulation (Filiano et al. 2016), and behavioural and neuroinflammatory response to stress (Wohleb et al., 2013). Finally, levels of circulating cytokines, such as TNF α , IL-1 β , and IL-6, have also been associated with psychiatric and mood disorders (Costello et al., 2019; Köhler et al., 2017; Masi et al., 2015) and can induce depression and anxiety behaviour in animal models (Karson et al., 2013; Kaster et al., 2012; Munshi, Parrilli, & Rosenkranz, 2019; Swiergiel & Dunn, 2007) and in humans (Chiu et al., 2017; Renault et al., 1987). There is strong evidence that peripheral immune cell pro-inflammatory activity and

recruitment to the brain are associated with mood and behaviour dysregulation, although more causal evidence is needed.

There have been an increasing number of studies on a gut microbiome-immune system-brain relationship. The absence of a gut microbiota in GF mice, for example, resulted in abnormal microglia that demonstrated altered gene expression profiles, an immature phenotype, increased numbers, and a reduced immune response to an LPS challenge, and these irregularities were rescued with FMT from specific pathogen-free (SPF) mice (Erny et al. 2015). In addition, GF mice exhibited a blunted increase in depression-like behaviour after LPS compared to SPF mice (Campos et al. 2016). Probiotics, however, reduced microglia activation and monocyte infiltration into the brain in a mouse model of liver inflammation (D'mello et al. 2015). These studies show that the presence of a microbiome is necessary for both normal immune and brain function. Furthermore, encouraging the colonisation of certain bacteria can promote an anti-inflammatory response that is beneficial for the host.

Antibiotics, which deplete the gut microbiome in large and chronic doses, decreased hippocampal neurogenesis in mice and performance in the novel object recognition test, a measure of spatial and object recognition memory. Administration of probiotics reversed the deficits, and these improvements were dependent on the presence of Ly6C^{hi} monocytes. Interestingly, reconstituting the mice with a faecal transplant from conventional mice did not improve hippocampal and behavioural dysfunction (Möhle et al. 2016). It is unclear why probiotics but not re-colonisation rescued immune function and behaviour. It shows that the role of the immune system in the microbiome-gut-brain axis is not as simple as the presence or absence of a diverse set of microbes. More work is needed to clarify the relationships between a complex microbiota, specific species in the gut, immune function, and the brain.

Increased gut permeability may also link the gut microbiome to immune system activation and, in turn, brain and behavioural dysfunction (Kelly et al. 2015). For example, higher serum concentrations of enterobacteria-derived LPS and IgA and IgM antibodies against LPS were found in depressed subjects compared to controls. Given that enterobacteria reside in the gut, the authors suggested that LPS was translocated from the intestines to the blood (Maes, Kubera, & Leunis, 2008; Maes et al., 2012). Stress, which increases the likelihood of developing depression and other mental illness later in life (Hammen 2005), also increases intestinal permeability in humans (Vanuytsel et al. 2014) and animal models (Zareie et al. 2006; Moussaoui et al. 2014). This might explain the elevated peripheral inflammation in some individuals with depression (Köhler et al. 2017).

1.2.3.4 Microbial metabolites

Finally, circulating microbial metabolites allow the bacteria in the gut to communicate with the brain. The role of bacterial metabolites in immune function has already been discussed, and this provides one possible link between microbiome composition and brain function. Erny et al. (2015), for example, found that the microglia abnormalities in GF mice could be rescued with administration of SCFAs. In addition, gut microbiome-derived compounds may be involved in mood disorders and psychiatric disease. Chronic stress was found to shift faecal metabolic profiles in mice, including a reduction in the SCFA propionic acid, and this was associated with increased depression-like behaviour and altered neurotransmitter metabolism in mice. Intrarectal administration of propionate reversed these changes (Li et al., 2018). A mix of SCFAs also reduced anxiety and depression behaviour in mice (van de Wouw et al. 2018), while butyrate improved depression-like behaviour after LPS and increased histone acetylation in the hippocampus (Yamawaki et al. 2018). Few human studies have been conducted to support these conclusions.

Faecal SCFA concentration negatively correlated with depression severity in a moderate-size cohort of Polish women (Skonieczna-zydecka et al. 2018), but more studies are needed. SCFAs have also been found to be involved in appetite regulation (Frost et al. 2014; Byrne et al. 2016) and the progression of neuroinflammation and neurodegenerative disease (Sharma, Taliyan, and Singh 2015; Soliman et al. 2012; Ryu et al. 2005).

These behavioural and molecular results suggest that administration of microbial metabolites is neuroprotective. Supplementing conventional mice with these compounds via the drinking water or injections, however, may increase the circulating levels beyond what would be expected with a normal microbiome. Furthermore, some evidence suggests that microbial compounds can in fact promote behavioural deficits and the development of neurodegenerative disease. Administration of SCFAs promoted neurodegeneration and neuroinflammation in a mouse model of Parkinson's (Sampson et al. 2016). Administration of SCFAs has also been associated with ASD-like behaviours (Shultz et al. 2015), depression-like behaviour (Gundersen and Blendy 2009), anxious behaviour, and reduced social interaction in rodents (Shams et al., 2019). Furthermore, the maternal immune activation (MIA) mouse model of ASD, which involves administering the viral mimetic and TLR3 agonist Poly(I:C) to pregnant dams (Meyer 2014), is associated with elevated microbial compounds, including 4-ethylphenylsulfate (4EPS) and indolepyruvate, which have previously been associated with autism in humans. Administration of 4EPS induced autism-like behaviour (Hsiao et al., 2013). It is possible that a pathological increase in gut permeability can release abnormally high levels of metabolites into the bloodstream, resulting in autism or other disorders. There may be an ideal level of circulating bacteria-derived compounds, but the details are not understood. In addition, while most studies have focused on the direct effects of metabolites on the brain via, for example, epigenetic modifications, the possible

indirect effects on peripheral immunity should not be discounted. Finally, the functions of metabolites beyond the SCFAs, such as bile acids and tryptophan, need to be explored. Nevertheless, the evidence is growing that microbe-derived compounds can modulate brain function.

1.3 Dietary modulation of the microbiome

1.3.1 Probiotics and the microbiome-gut-brain axis

Probiotics are live strains of bacteria that exert a beneficial effect on the host (Hill et al. 2014). Microbiome-gut-brain axis research has primarily focused on the effects of *Bifidobacterium* and *Lactobacillus* strains (Wang et al., 2016). From the *Bifidobacterium* genus, *B. longum* and *B. breve* improved performance in a variety of memory tasks in mice (Savignac et al., 2015). *B. longum*, but not *Lactobacillus rhamnosus*, was also anxiolytic (Bercik et al., 2011), and reduced circulating cytokines in mice infected with a pathogen (Bercik et al., 2010). From the *Lactobacillus* genus, *L. rhamnosus* improved deficits in social interaction and anxious behaviour after social defeat and increased levels of splenic Treg cells (Bharwani et al., 2017). *L. reuteri* restored social interaction behaviour and LTP in the ventral tegmental area following interactions with a novel mouse in animal models of autism (Buffington et al. 2016; Dooling et al. 2018). Multispecies probiotics that combine these genera are also used. A mix of *L. helveticus* and *B. longum* reversed decreases in BDNF and increases in pro-inflammatory cytokines in the brain and serum after LPS administration (Mohammadi et al., 2018). While there is increasing evidence that probiotics can improve behavioural parameters in animal models, there is conflicting evidence as to whether these benefits translate to humans. Single- and multispecies probiotics have demonstrated efficacy in reducing depression in human cohorts (Pinto-Sanchez et al. 2017; A. A. Mohammadi et al. 2015; Tillisch et al. 2013). However, although *L. rhamnosus* reduced anxiety and depression behaviour

as well as stress-induced corticosterone release in mice (Bravo et al. 2011), this probiotic did not have any effects on cognitive or mood parameters in humans (Kelly et al. 2017). In addition, a probiotic-containing milk drink improved mood in subjects with low mood. However, the milk had a moderate detrimental effect on mood in the healthier participants, although the authors did not discuss this trend. Subjects taking the probiotic milk also recalled fewer words in a long-term memory test than controls (Benton, Williams, and Brown 2007). This discrepancy might be due to the publication bias towards positive results in preclinical studies. The mixed results with probiotics might also be due to the complex nature of the gut microbiome. Microbes are not isolated from one another – they interact and behave synergistically, inhibiting or promoting the abundance or metabolic activity of neighbouring bacteria (Carey et al. 2018; Worrich, Musat, and Harms 2019). It is difficult to predict what adding one microbe to the mix will do. For this reason, supplements that target multiple types of resident bacteria in the gut may have more widespread effects (Burnet 2012).

1.3.2 Prebiotics and the microbiome-gut-brain axis

Less work has been done on the effects of prebiotic supplementation on the brain and behaviour. Prebiotics are compounds that are not digested by the host but instead are metabolised by one or a few select strains of gut bacteria. These compounds promote the proliferation and metabolic activity of commensal (beneficial) microbes and improve host health (Gibson and Roberfroid 1995). Rather than targeting a specific strain, prebiotics promote the growth of a variety of bacteria that are beneficial to the host. Furthermore, prebiotics target bacteria that are indigenous to the gut, which may be more effective than adding new bacteria that are transient (Brigidi et al. 2003). If the taxa of interest are not present in the gut already, however, prebiotics are unlikely to have an effect. The targets of most prebiotics are *Bifidobacteria* and *Lactobacilli* (Macfarlane,

Steed, & Macfarlane, 2008; Vulevic et al., 2008; Vulevic et al., 2015), which are common probiotics associated with altered neurobehavioural patterns in animal and human studies. Other bacteria such as *Clostridia* and *Bacteroides*, however, can also metabolise prebiotics. Furthermore, fermentation of prebiotics can have ripple effects on the overall composition of the microbiome due to cross-feeding on metabolites between microbes (Macfarlane, Steed, and Macfarlane 2008). The prebiotic compound galacto-oligosaccharides (GOS), for example, shifted the composition of the mouse gut microbiome. Differentially abundant genera included *Bifidobacterium* as well as *Coprococcus*, *Oscillibacter*, and *Allobaculum*, among others (Cheng et al. 2018). Therefore, prebiotics can have extensive effects on the microbiome and on the host.

Oligosaccharides and inulin are the most common prebiotics in microbiome-gut-brain research, likely because they have a history of safe use commercially and are not sensitive to the harsh acidic environment of the gastrointestinal tract (Macfarlane, Steed, and Macfarlane 2008). GOS has been shown to increase the GluNR1 and GluNR2A subunits of the N-methyl-D-aspartate receptor (NMDAR) in adult mice. In addition, while it increased BDNF expression in the dentate gyrus of the hippocampus, it decreased BDNF in the CA3 region. These changes corresponded to an increase in faecal *Bifidobacterium* (Savignac et al., 2013). GOS also increased cortical neuronal firing in response to NMDA, suggesting functional differences in glutamatergic transmission (Gronier et al. 2017). Furthermore, a diet rich in GOS increased GluNR1 and BDNF and decreased GluNR2B expression in the prefrontal cortex and increased time spent in the central zone of an open field. These changes positively correlated with increases in *Lactobacillus* in the gut, although causation between gut microbes and the brain and behaviour was not demonstrated (Mika et al. 2018). Prebiotics can also have long-lasting effects on the brain. Neonatal GOS intake resulted in increased hippocampal BDNF and GluNR2A expression in adult mice (Williams et al. 2016). In

addition to oligosaccharides, the prebiotics 3' and 6'Sialyllactose also influence anxiety-like behaviour and hippocampal neurogenesis (Tarr et al. 2015). In one study, the prebiotics GOS and fructo-oligosaccharides (FOS) altered gut microbiome composition and increased gut SCFA levels in mice. Interestingly, in contrast to the most findings, this included a decrease in *Bifidobacterium*. GOS and FOS also reduced chronic stress-induced anxious and depressive behaviour and corticosterone release as well as reduced expression of the GluNR2A subunit of the NMDAR and increased BDNF (Burokas et al. 2017). While prebiotics putatively increase “good” bacteria, *Bifidobacterium* decreased in this study. It is possible that the high dose of prebiotics confounded the results. Anxiety-like behaviour, however, improved. Therefore, the exact relationships between relative abundances of specific taxa in the gut and the brain and behaviour are not clear. Furthermore, comprehensive analyses of global changes in the gut microbiome are lacking in many of these studies.

Neuroprotective effects have also been demonstrated with administration of oligosaccharides and other prebiotics. In a mouse model of amyotrophic lateral sclerosis (ALS), GOS delayed the onset of disease, increased lifespan, and reduced cell death and neuroinflammation. The proposed mechanism was encouraging the proliferation of microbes that promote absorption and synthesis of B vitamins (Song et al., 2013).

The effects of prebiotics in a “synbiotic” supplement, a combination of probiotics and prebiotics, have also been studied. This combination supports the colonisation and survival of the probiotics in the gut by giving the bacteria a competitive advantage over resident microbes. Combining prebiotics with probiotics also increases resulting production of SCFAs compared to probiotics alone (van Zanten et al. 2012). A mixture of the prebiotics GOS and polydextrose and the probiotic *L. rhamnosus*, for example, was more effective than either supplement alone in

restoring altered behaviours and central gene expression after maternal separation stress in rats (Neufeld et al., 2019).

Finally, prebiotics reverse the effects of “unhealthy” diets. Prebiotics influence central regulation of appetite (Arora et al., 2012; Cani et al., 2005) as well as microglia activation, hippocampal long-term potentiation, and cognitive function in obese insulin-resistant rats (Chunchai et al. 2018). Furthermore, the prebiotic oligofructose reduced food intake, improved insulin resistance, and decreased corticosterone levels (de Cossío et al. 2017). Interestingly, these results are similar to the findings after SCFA supplementation in animals. This evidence illustrates the wide-ranging effects of prebiotic supplementation on brain function, neuroinflammation, and behaviour.

1.3.3 Mechanisms of brain and behaviour modulation via probiotic and prebiotic consumption

The mechanisms that mediate probiotic- and prebiotic-induced changes in the brain and behaviour parallel the mechanisms underlying the microbiome-gut-brain axis. Administration of probiotics, for example, results in differential activation of the vagus nerve. The *SHANK3B*^{-/-} mouse model of autism had a distinct gut microbiome compared to controls, as well as deficits in social behaviour and social interaction-induced synaptic plasticity in the ventral tegmental area. The probiotic *L. reuteri* reversed these behavioural changes and shifted the gut microbiome composition to more closely resemble that of control mice. In addition, vagotomy prevented the probiotic-induced changes (Dooling et al. 2018), reiterating the importance of the vagus nerve as a possible mediator between probiotic supplementation and changes in the brain and behaviour (Bercik et al., 2011).

Probiotics modulate immune function, and this is due in part to their ability to improve integrity of the mucosal epithelial barrier (Rautava, Arvilommi, and Isolauri 2006; Resta-Lenert

and Barrett 2003; Hardy et al. 2013). They also compete for nutrients and, in turn, deplete levels of pathogenic bacteria (Maltby et al. 2013). In addition, gut immune cells sample the luminal contents, and the presence or absence of different microbes result in distinct downstream immune cascades in the gut and systemically. *Lactobacilli* and *Bifidobacteria*, for example, tend to promote anti-inflammatory cytokine profiles and proliferation of Treg cells (Hardy et al., 2013). The immunomodulatory properties of probiotics have consequences for the CNS. In the experimental autoimmune encephalomyelitis (EAE) mouse model of multiple sclerosis, species of *Lactobacillus* delayed onset and reduced the severity of MS symptoms. The probiotics also reduced pro-inflammatory cytokine secretion in the spleen and increased Treg cell proliferation in the periphery as well as infiltration in the cerebellum. (Lavasani et al. 2010). In addition, *L. rhamnosus* supplementation reversed social defeat-induced deficits in social behaviour, and this was associated with a reduction in dendritic cell activation in the spleen. Overall, *L. rhamnosus* did not cause any global changes in microbiome composition, but it did reverse social defeat-related changes in faecal metabolites. Whether the source of those metabolites was the probiotic, the host, or other microbes in the gut, however, was not clear (Bharwani et al. 2017), and the evidence that probiotics can alter microbiome composition inconsistent (McFarland 2014). More research is needed on the fate of probiotics in the gut, global effects on the gut microbial community (Derrien and van Hylckama Vlieg 2015), and the relevance to CNS function.

Prebiotics interact with the immune system by promoting proliferation of the anti-inflammatory microbes mentioned previously as well as by increasing metabolite production in select bacteria. *In vitro*, fermentation of GOS and other prebiotics in faecal cultures stimulated production of SCFAs, and supernatants from the faecal cultures containing the fermentation products reduced LPS-induced TNF- α secretion from peripheral blood mononuclear cells

(PBMCs) (Liu, Gibson, & Walton, 2016). *In vivo*, GOS reversed LPS-induced anxiety-like behaviour and abnormal gene expression in the brain. This included a reversal of elevated IL-1 β expression (Savignac et al., 2016). Other *in vitro* and *in vivo* studies in humans have described increased anti-inflammatory cytokine production, reduced pro-inflammatory markers in PBMCs, and increases in *Bifidobacterium* after oligosaccharide supplementation (Vulevic et al. 2015, 2013; Lecerf et al. 2012). Furthermore, prebiotics may also have direct effects on host immune function in the gut and systemically by interacting with TLR4 on immune cells (Eiwegger et al. 2010; Capitán-Cañadas et al. 2014; Bode et al. 2004). The anti-inflammatory phenotypes *in vitro* and *in vivo* suggest that prebiotic-induced changes in microbiome composition and metabolites, as well as the prebiotic compound itself, have functional consequences for the host immune system and the brain.

Finally, prebiotics modify gut microbial metabolic profiles. Prebiotics are fermented by *Bifidobacteria* and *Lactobacilli* to produce SCFAs *in vitro* (Hopkins & Macfarlane, 2003; Liu et al., 2016), the effects of which on the brain and behaviour as well as the immune system have already been discussed. *In vivo*, high-fibre foods positively correlate with levels of SCFAs (Cuervo et al., 2013) and SCFA-producing bacteria (De Filippo et al. 2010). In mice, GOS altered faecal metabolic profiles, including myo-inositol, oleic acid, and amino acids (Cheng et al. 2018). There are also associations between prebiotic feeding, microbial metabolites (predominately SCFAs), and the brain. In rodent studies GOS resulted in an increase in acetate in rats that was accompanied by increased cortical NMDAR subunit expression and reduced 5-HT_{2A} (Kao et al., 2018). The work by Burokas et al. (2017), showed oligosaccharide-induced changes in behaviour and central gene expression correlated with SCFA levels. In the anxious BALB/c mouse strain, short-chain and long-chain oligosaccharides resulted in increased SCFAs and reduced anxiety-like and

repetitive behaviours and deficits in sociability (Szklyany et al. 2019). These studies provide evidence for microbial metabolites as mediators between prebiotic intake and changes in the brain. Strong evidence beyond associations for causation, however, is still needed. The putative mechanisms of the effects of the BGOS prebiotic on the brain and behaviour are summarised in **Figure 1.2**.

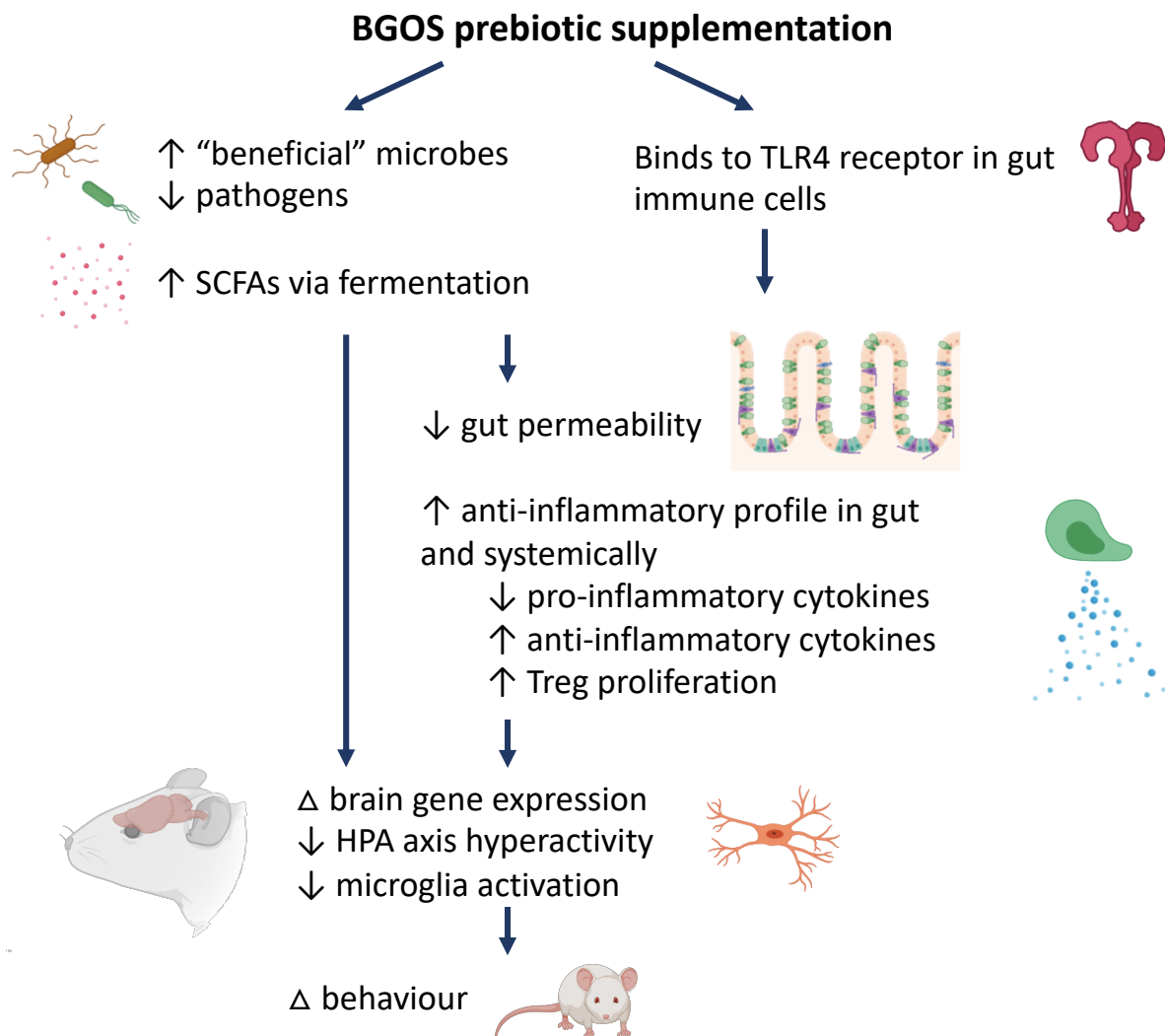


Figure 1.2. Putative mechanisms linking the prebiotic BGOS and the brain and behaviour in rodent models.

1.4 The maternal gut microbiome and offspring neurodevelopment

So far, this review has focused on the microbiome-gut-brain axis within individual human subjects or animal models. In individuals, probiotic or prebiotic supplements can modulate this axis. While adding probiotics to a gut microbial community can be beneficial for the host's health, prebiotics encourage the growth and metabolic activity of resident bacteria, shift the composition of the microbiome, and promote host health. Although there is growing evidence for a connection between the gut microbiome and brain health and disease, few studies have interrogated the relationship between the maternal microbiome and the offspring brain. Given the importance of the gut microbiome in early life for brain development, this is an important question.

Studies in the MIA mouse model of ASD suggest that offspring brain development is related to the composition of the maternal microbiome. Kim et al. (2017) found that offspring of mice from Taconic Biosciences (Tac mice) exhibited the typical MIA phenotype, while offspring from Jackson Laboratories dams (Jax mice) did not show the MIA characteristics. The authors reported that this was due to the segmented filamentous bacteria in the intestines of Tac mice. These bacteria promote differentiation of Th17 cells and interleukin (IL)-17a production in the gut, and the authors found that the Th17 cells were necessary for the MIA phenotype in offspring. After colonising Jax mice with a species of segmented filamentous bacteria, their offspring displayed the MIA phenotype, suggesting that the gut microbiome was causally related to the offspring brain and behavioural deficits. These findings were replicated in a related study (Lammert et al. 2018). These studies not only demonstrate the role of the maternal microbiome in the offspring brain and behaviour, but they also illustrate mouse supplier as a possible source of variation between experiments in different types of mice.

Other studies have supported the importance of the mother's gut microbiome beyond the MIA model. Antibiotic treatment in mothers has been shown to reduce social interaction behaviour and prepulse inhibition of the acoustic startle response reflex, a measure of sensorimotor gating that is deficient in schizophrenia (Degroote et al., 2016). Antibiotics during pregnancy also resulted in reduced bacterial load and diversity in maternal faecal samples. Pups from these dams exhibited reduced locomotor activity, rears, and time in the central zone of an open field. Cross-fostering antibiotics pups to control dams, however, prevented these behavioural changes. This result suggests that postnatal exposure to the depleted maternal microbiome caused these differences (Tochitani et al. 2016). This is in contrast to the Kim et al. (Kim et al. 2017), where cross-fostering MIA Tac pups to Jax mothers did not rescue the MIA phenotype, indicative of an effect *in utero*.

GF mice studies have provided additional evidence in support of the role of the maternal microbiome in *in utero* neurodevelopment. Increased blood-brain barrier permeability was found in GF embryonic mice as well as reduced expression of the tight junction protein occludin, suggesting that the maternal microbiome modulates brain development *in utero* (Braniste et al. 2014). Transcriptome analysis of microglia in male embryonic brains showed that microglia in GF mice had many differentially expressed genes, a result that corresponded with increased numbers of microglia in GF mice relative to SPF mice (Thion et al. 2018). These increases in microglia were also reported just after birth in GF pups along with altered patterns of apoptosis and cytokine levels. In this study, however, no differences were found in number of microglia in the embryonic brain (Castillo-Ruiz et al. 2018), which contrasts with Thion et al. (2018). Nevertheless, there is strong evidence for maternal microbiome-dependent changes in brain development.

1.4.1 Mechanisms of maternal microbiome influence on the offspring brain

The question remains as to how a perturbed or absent maternal microbiota produces changes in the offspring brain and behaviour. There is disagreement in the evidence as to whether the progeny effects are due to *in utero* or early postnatal factors. It is likely that the maternal microbiome plays a role during both periods: *in utero*, through modulation of the maternal and offspring immune systems and circulating microbial metabolites, or early postnatally, via direct exposure to the mother's gut and vaginal microbes or breastmilk. These mechanisms are summarised in **Figure 1.3**.

1.4.1.1 *The maternal and offspring immune systems*

Given that the maternal immune system is closely linked to offspring brain development, an immune-related mechanism may be one of the factors at play. There are extensive observations of offspring brain and behavioural deficits in the rodent MIA model, including changes in behaviour, plasticity and synaptogenesis, central cytokines, cortical lamination, and excitatory/inhibitory imbalances (Choi et al., 2016; Corradini et al., 2018; Garaya et al., 2012; Giovanoli et al., 2016; Smith et al., 2007). In mice, maternal exposure to LPS also results in increases in pro-inflammatory markers in mothers as well as increased corticosterone and anxiety-like behaviour in offspring (Babri, Doosti, and Salari 2014). In addition, maternal levels of the pro-inflammatory cytokine IL-6 also correspond to anxiety and deficits in social interaction and prepulse inhibition behaviour in offspring. Importantly, these abnormal behaviours are rescued with anti-IL-6 antibodies, suggesting that the pro-inflammatory cytokine is causally related to behavioural dysfunction in offspring (Smith et al., 2007). Studies in humans have also described correlations between maternal circulating levels of cytokines during pregnancy and psychiatric illness, cognitive function, and brain connectivity (Brown et al. 2004; Buka et al. 2001; Graham

et al. 2018; Rudolph et al. 2018). These studies demonstrate the importance of the maternal immune system for brain development in progeny. It may partially mediate the relationship between the maternal microbiome and the offspring brain and behaviour.

The maternal microbiome can also shape the offspring immune system, which can influence brain function in the pups. Differentiating between the influences of the maternal microbiota and the offspring microbiota on offspring immune development is a challenge. One study addressed this with a unique experimental design: GF dams were colonised during pregnancy with an *E. coli* strain that does not survive in the intestines, returning the dams to GF status during the suckling period. This resulted in an increase in numbers of immune cells in the gut in offspring. Small intestinal innate lymphoid cells and colon lamina propria intestinal mononuclear cells increased in pups from transiently colonised dams. The small intestines of pups from colonised dams also had gene expression profiles that were distinct from those of GF dams, including genes for antimicrobial peptides and metabolic processes. These changes were attributed to maternal antibodies that promote the transfer of microbe-derived compounds to neonates during pregnancy and suckling. The pups of colonised dams also had dampened inflammatory responses to immune challenges, such as injections of live pathogens or LPS (Gomez de Agüero et al. 2016). The maternal gut microbiome prepares the neonatal immune system for postnatal microbial colonisation, and it is necessary for proper offspring immune development.

1.4.1.2 Microbial metabolites and the offspring brain

Another possible mechanism by which maternal gut bacteria modulate offspring brain development is through production of microbial metabolites. The microbial metabolite 5-aminovaleric acid, for example, can enter the embryonic brain and act as a weak GABA receptor agonist. It has been proposed as a mediator between the maternal microbiome and the development

of ASD (Sharon et al. 2019). Other bacterial by-products with neuroactive properties may influence brain development.

The ability of SCFAs to act as epigenetic modifiers makes them attractive candidates as mediators of the maternal microbiome-offspring brain relationship. In keeping with its role as an HDAC inhibitor, maternal butyrate supplementation increased histone acetylation in adult female offspring in the promoters of genes involved in lipid synthesis in mice (Huang et al., 2017). Similarly, acetate supplementation during gestation in mice resulted in increased histone acetylation at the *Foxp3* promoter in offspring (Thorburn et al. 2015). More studies are needed to explore the influence of maternal microbial metabolites on the offspring epigenetic landscape, brain function, and other aspects of offspring health.

1.4.1.3 Offspring gut microbiome composition

The offspring gut microbiome closely resembles that of the community of bacteria in the maternal gut in rodents. This is due to co-housing, maternal care behaviour, and coprophagia (consumption of faeces) (Friswell et al. 2010; Jašarević et al. 2017; Ubeda et al. 2012) as well as ingestion of probiotic- and prebiotic-rich breastmilk (Shen et al. 2011). Similarly, in a human study, the maternal gut microbiome strongly influenced gut microbiome composition in infants. Of the 187 metagenomic operational taxonomic units (MetaOTUs) found in vaginally delivered infants, 135 were found in their mothers, suggesting vertical transmission of gut microbes from mother to neonate (Bäckhed et al. 2015). Therefore, alterations to the maternal microbiome may affect the offspring microbiome and, in turn, the offspring brain.

1.4.1.4 The vaginal microbiome

Although it will not be a focus of this thesis, the vaginal microbiome is one of the first direct exposures of neonates to a diverse community of bacteria (Mackie, Sghir, and Gaskins

1999). In addition to the gut microbiome, this microbial ecosystem has been shown to influence offspring neurodevelopment. Maternal stress in mice during pregnancy resulted in changes in vaginal microbiome composition, notably decreased *Lactobacillus*, that was mirrored by changes in the neonate gut at postnatal day (P)2. Stressed mothers had distinct vaginal proteomic profiles compared to controls, suggesting that the changes in microbial metagenome had functional consequences. Furthermore, these changes were associated with altered amino acid availability in the offspring brain (Jašarević et al., 2015). A causal link between the vaginal microbiome and the offspring brain was established in a later study. These experiments showed that gavaging caesarean section-delivered pups with vaginal fluid from stressed mothers induced altered stress hormone levels and gene expression in the brain. These inoculated C-section pups resembled the offspring that were vaginally delivered from stressed mothers. However, vaginal fluid from control pups only partially rescued the phenotypes of the pups from stressed dams (Jašarević et al. 2018), suggesting that there are other mechanisms at work. These results show that the vaginal microbiome has an impact on brain function in pups.

This section has summarised studies that interrogated the link between the maternal microbiome and brain function in pups. Most studies have reported associations between the presence/absence or the composition of a mother gut microbiome and behaviour, gene expression, or other measures of brain function in progeny. Few papers have provided evidence for causality or the mechanisms that underlie these associations. Studies examining the role of the maternal microbiome in other aspects of offspring health can help generate hypotheses about mechanisms. In addition, evidence of the influence of the gut microbiome on immune function and the brain in adult animals are also relevant. More studies, however, will be necessary to fully elucidate the mechanisms relating the maternal microbiome to the offspring brain.

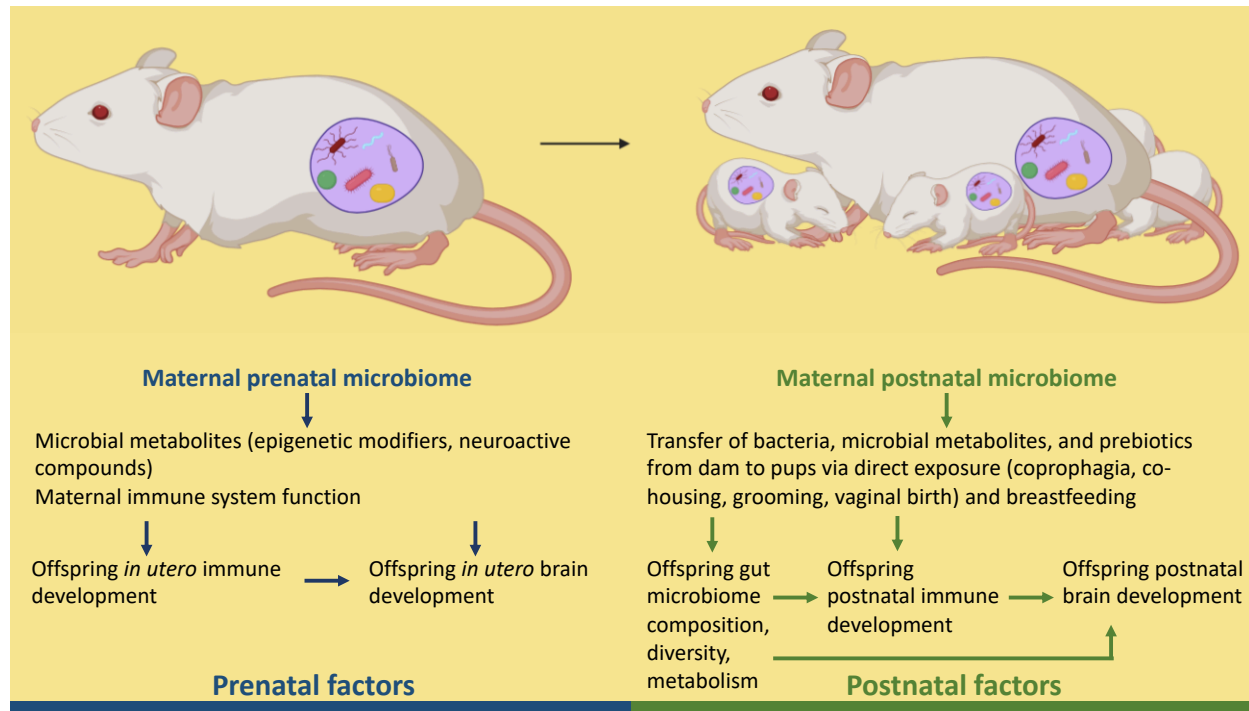


Figure 1.3. Potential links between the maternal prenatal and early postnatal microbiome and offspring neurodevelopment.

1.4.2 Dietary modulation of the maternal microbiome and pup health

1.4.2.1 Maternal probiotics and prebiotics and the offspring brain

Maternal supplementation with probiotics or prebiotics allows researchers to investigate the effects of manipulating the maternal microbiome on the offspring brain. To date, however, these studies are limited. In one set of experiments, inulin and oligosaccharide administration during pregnancy reduced anxiety-like behaviour and oxidative stress markers in the brain in rat foetal and P22 brains after exposure to the toxins rotenone or acrylamide. The maternal prebiotics also rescued depletion of *Bifidobacterium* and *Lactobacillus* (Krishna et al., 2015; Krishna & Muralidhara, 2015, 2018a, 2018b). In addition, there is evidence that probiotics can influence the brain in progeny. A probiotic mixture reversed F1 (offspring) behavioural changes and brain pro-

inflammatory cytokine and GABA levels following MIA (Wang et al., 2019). Similarly, maternal separation-induced increases in prelimbic and infralimbic phosphorylated mitogen-activated protein kinase were also reversed in rat pups by maternal probiotic intake (Cowan, Stylianakis, and Richardson 2019). More research is needed to fully understand how supplements that target the maternal gut microbial community have an impact on offspring brain development.

1.4.2.2 Maternal prebiotics and offspring gut microbiome, immune function and metabolism

Although there is limited evidence for dietary modification of maternal gut bacteria and the effects on the brain in pups, some studies have examined effects on the offspring gut, immune function, and metabolism. Caprine milk oligosaccharides, but not GOS, increased levels of caecal propionate and butyrate, gut microbiome diversity, and *Bifidobacterium* in mouse offspring (Thum et al., 2016). Perinatal intake of a GOS/inulin mixture increased colon size (Desbuard et al. 2012), while maternal FOS supplementation increased gut IFN- γ , IgA, and SCFA levels in pig litters (Le Bourgot et al. 2017).

In addition, several studies have examined the influence of maternal prebiotic intake on immune function in progeny. Mouse maternal FOS intake during pregnancy and lactation resulted in altered gut microbiome composition in offspring from P0 to P21 and at P98. Maternal FOS also reduced symptoms of skin inflammation in progeny in a mouse model of dermatitis (Fujiwara et al., 2010; Fujiwara et al., 2008). Some immune changes in progeny occur independently of modifications to offspring microbiome composition. In a mouse model of wheat allergy, the addition of a GOS/inulin mixture to the maternal diet reduced allergy symptoms and IgE, IL-4, and IL-5 and increased TGF β and IL-10. T cell numbers changed in pups as well, with reduced Th2 response and increased Th1 response and Treg cell activity (shifting the Th2/Th1 balance towards Th1 is desirable in the allergy response). In the offspring gut, maternal prebiotic increased

gut barrier integrity but had no effect on microbiome composition or SCFA levels, pointing to an offspring gut microbiome-independent effect of maternal prebiotic intake (Bouchaud et al. 2016). Maternal high-fibre diet resulted in increased levels of SCFAs in offspring until P22, but not at P30, and did not change offspring microbiome composition in mice. The maternal microbiome, however, did change, particularly in the *Bacteroidetes* and *Firmicutes* phyla. SCFAs in dams increased as well. This suggests that high fibre changed the maternal microbiome and, in turn, levels of SCFAs, which may transfer to pups *in utero* and or during lactation. Treg cells also increased in the thymus and spleen in offspring (Nakajima et al. 2017). This is in agreement with the conclusion in Bouchaud et al. (2016) that modifications to offspring immune function may occur independently of changes in the gut microbiome. Thorburn et al. (2015) reported that high-fibre diet during gestation improved symptoms in a mouse model of asthma in adult offspring. The high-fibre diet also altered the gut microbiome composition in dams to increase production of acetate. The authors concluded that fermentation of the fibre in dams resulted in elevated acetate, which increased histone acetylation at the *Foxp3* promoter to increase Treg cells. Caesarean transfer of foetuses at E18 to mothers given a control diet resulted in identical reductions in allergy symptoms. This result demonstrates that the effects were independent of microbial transfer from dam to pups.

Maternal prebiotic intake can also influence offspring metabolism and obesity risk. In rats, the prebiotic oligofructose reduced maternal weight, energy intake, and hyperglycaemia and altered the faecal microbiome and serum metabolic profiles during a high-fat diet. These changes were associated with reduced body weight and increased satiety hormone level in offspring (Paul et al., 2016). This was in agreement with a study showing that a high-prebiotic fibre diet during gestation and suckling increased levels of the glucagon-like peptide (GLP)-1, a hormone involved

in insulin secretion and satiety in rat offspring. Reduced plasma glucose was also reported in progeny. The microbiome was not investigated in this study, however, and no mechanism was proposed (Maurer and Reimer 2011). Nevertheless, these studies illustrate that maternal prebiotic intake can influence offspring metabolism and obesity risk, characteristics that are associated with brain function and behaviour (Bruce-Keller et al., 2015; Cournot et al., 2006; Elias et al., 2005; Gunstad et al., 2007; Sabia et al., 2009).

Human studies support the findings in animal models. A meta-analysis found that maternal probiotic treatment resulted in a modest but significant reduction in offspring risk of atopic dermatitis, an inflammatory skin condition (Pelucchi et al. 2012). Probiotic administration during pregnancy also reduced offspring incidence of allergic disease (Dotterud et al., 2010) but only resulted in mild changes in the infant microbiome – increased *L. rhamnosus* was detected in both maternal and offspring stool samples, while *L. acidophilus* and *B. animalis* were not found in infant faecal samples after maternal intake of these probiotics. Furthermore, none of these probiotics modified offspring gut microbiome composition or diversity (Dotterud et al. 2015). This was in agreement with a study showing that *L. rhamnosus* administration during pregnancy resulted in significantly higher levels of this species in the neonatal gut at six months, but not at twelve months old, and also reduced incidence of atopic eczema (Gueimonde et al., 2006). Similarly, FOS supplementation increased faecal *Bifidobacterium* in pregnant mothers but not in one-month old infants. There was no correlation between maternal and offspring levels of *Bifidobacteria* (Jinno et al. 2017).

1.4.2.3 Adverse effects?

Given that prebiotics and probiotics are, by definition, beneficial to the host, the assumption is that boosting the maternal microbiome with prebiotics or probiotics should benefit

progeny as well. There is evidence, however, of both beneficial and detrimental phenotypes (Barouei, Moussavi, and Hodgson 2012; Needell et al. 2017). Administration of a probiotic mixture to dams during gestation and suckling, for example, had mixed results on offspring stress hormone levels and immune function in rats. The maternal probiotic had similar effects as maternal separation stress in offspring – both resulted in increased levels of the stress hormones adrenocorticotrophic hormone and corticosterone in offspring and altered the gut microbiome in a similar manner. The probiotic did, however, increase plasma and faecal IgA, an antibody that helps to confine bacteria to the intestinal lumen (Barouei, Moussavi, and Hodgson 2012). In addition, administration of SCFAs (which increase in dams with probiotic and prebiotic supplementation) to rats prior to pregnancy actually reduced *Bifidobacterium*, along with *Clostridium* and *Bacteroides*, in offspring in a Kilham Rat Virus (KRV) model of type 1 diabetes. Paradoxically, maternal SCFA administration also reduced SCFA levels in Peyer's patches (follicles that make up part of the gut-associated lymphoid tissue) compared to controls. Furthermore, while SCFAs reduced the pro-inflammatory cytokine IFN- γ , they reduced Treg numbers compared to controls in Peyer's patches (Needell et al. 2017). Based on this evidence, supplements that modify the maternal microbiome modulate stress and immune responses in offspring, but offspring may exhibit a mix of detrimental and beneficial phenotypes in offspring.

1.4.2.4 Maternal diet, breastmilk, and offspring health

Maternal prebiotic and probiotic supplementation can also influence composition of the breastmilk, which plays an important role in development of the offspring immune system and gut microbial community (Ding and Schloss 2014; Belderbos et al. 2012; Rogier et al. 2014). FOS intake during pregnancy and lactation altered gene expression profiles in breastmilk and increased levels of IL-27, a cytokine involved in T cell differentiation and the allergic response (Kubota et

al. 2014). A synbiotic containing *Bifidobacteria* and *Lactobacilli* as well as FOS increased total antioxidant capacity, IgA, and TGF β 1 in breastmilk (Nikniaz et al., 2013a; Nikniaz et al., 2013b). Similarly, a mixed-species probiotic during pregnancy and lactation resulted in increased levels of the anti-inflammatory cytokines TGF β 1 and IL-10 in breastmilk and increased secretory IgA in new-born stool samples. The probiotic also, however, elevated IL-6 in breastmilk, and this increase tended to correlate with lower BMI at birth (Baldassarre et al. 2016). While the functional relevance of these changes in breastmilk composition for the infant is unknown, a mix of beneficial and detrimental characteristics was reported with maternal probiotic and prebiotic intake. Again, more studies are necessary to determine how these effects on cytokine profiles in the breastmilk affect offspring.

1.5 Hypotheses and aims of thesis

The latter half of this literature review has summarised the widespread effects of maternal prebiotic and probiotic supplementation on the gut microbiome, microbial and host metabolism, and immune function in progeny as well as maternal breastmilk composition. Given that these parameters can influence CNS function, it is possible that the offspring brain and behaviour will be affected by supplements that target the maternal microbiome. A prebiotic will be administered in this project because prebiotics may be more effective than probiotics in anti-inflammatory potential and shifting microbiome composition to promote host health (Liu et al., 2016). Apart from the few studies on the effects of maternal prebiotic feeding on oxidative stress in the brain (Krishna et al., 2015; Krishna & Muralidhara, 2015, 2018a, 2018b), however, no studies have investigated the influence of prebiotic feeding in mothers on the offspring brain and behaviour in animals or humans.

A commercial formulation of GOS called Bimuno®, or BGOS, promotes the growth of *Bifidobacteria* in the gut and is anti-inflammatory *in vivo* and *in vitro*. It has also been shown to alter behavioural patterns in rodent behavioural tests in adult mice. The multigenerational effects of maternal BGOS intake, however, have not been investigated. This thesis explores the influence of perinatal prebiotic intake on the offspring brain and behaviour (summarised in **Figure 1.4**).

1.5.1.1 Hypothesis 1: Prebiotic intake during pregnancy and suckling modifies the offspring brain and behaviour

Prebiotic supplementation during pregnancy and suckling may cause neurobehavioural changes in offspring at weaning.

Given the influence of BGOS and other formulations of the GOS prebiotic on behavioural patterns in adult animals, and the potential for multigenerational effects of prebiotic feeding, I hypothesised that administration of BGOS during pregnancy and lactation similarly affects offspring behaviour. I also hypothesised that both *in utero* and postnatal factors are responsible for any behavioural changes, given that both periods have been reported as important for offspring development in the literature.

In order to investigate changes in the brain in offspring, I compared gene expression using a candidate gene approach based on findings in adult studies. Given the anti-inflammatory effects of maternal prebiotic intake in progeny, maternal BGOS may similarly reduce deficits in behaviour and gene expression following an acute LPS injection. Furthermore, I did not expect maternal prebiotic intake to produce appreciable changes in healthy animals because previous studies have only investigated behavioural effects of prebiotics in animals challenged with LPS or chronic stress or in models of neurodegenerative disease (Song et al. 2013; Burokas et al. 2017; Helene M. Savignac et al. 2016). I expected the LPS challenge to reveal latent differences in these offspring

by producing different neurobehavioural responses to peripheral inflammation. This will be the topic of Chapter 3.

1.5.1.2 Hypothesis 2: Perinatal prebiotic intake alters peripheral immune function and host metabolism

This review has outlined the role of the immune system in brain function and the immunomodulatory effects of maternal prebiotic intake. Therefore, I hypothesised that perinatal BGOS feeding alters immune function in pups. Specifically, I expected to see a reduced cellular pro-inflammatory response, increased anti-inflammatory response, and altered counts of circulating immune cells.

In addition, I hypothesised that maternal prebiotic intake alters offspring metabolic profiles. Furthermore, a comprehensive analysis of plasma metabolic profiles in pups may generate hypotheses about the small molecules involved in changes in the brain, behaviour, and immunity for future studies. This will be explored in Chapter 4.

1.5.1.3 Hypothesis 3: Maternal prebiotic supplementation results in long-lasting changes in adult offspring

At about three weeks of age, mice are weaned, and after exposure to the diet and microbes in the animal facility environment, effects of maternal prebiotic intake may dissipate. On the other hand, some interventions or insults during the *in utero* and early postnatal periods can cause long-lasting changes that last into adulthood. Given this critical period of development, I hypothesised that the brain, behavioural, and peripheral changes in pups after maternal prebiotic feeding last until adulthood. This question will be answered in Chapter 5.

1.5.1.4 *Hypothesis 4: Maternal prebiotic feeding modifies maternal and offspring gut microbiome composition and metabolism*

Finally, the effects of maternal BGOS intake on maternal and offspring gut microbiome composition and metabolite profiles will be explored in Chapter 6. Microbial metabolites, especially SCFAs, are a common thread in the maternal microbiome literature. ¹H NMR metabolomics was used to explore global changes in faecal metabolite profiles in dams and offspring. Specifically, I hypothesised that maternal BGOS increases levels of faecal SCFAs in mothers and pups. Finally, I hypothesised that BGOS alters both maternal and offspring microbiome composition, including increased levels of *Bifidobacterium*.

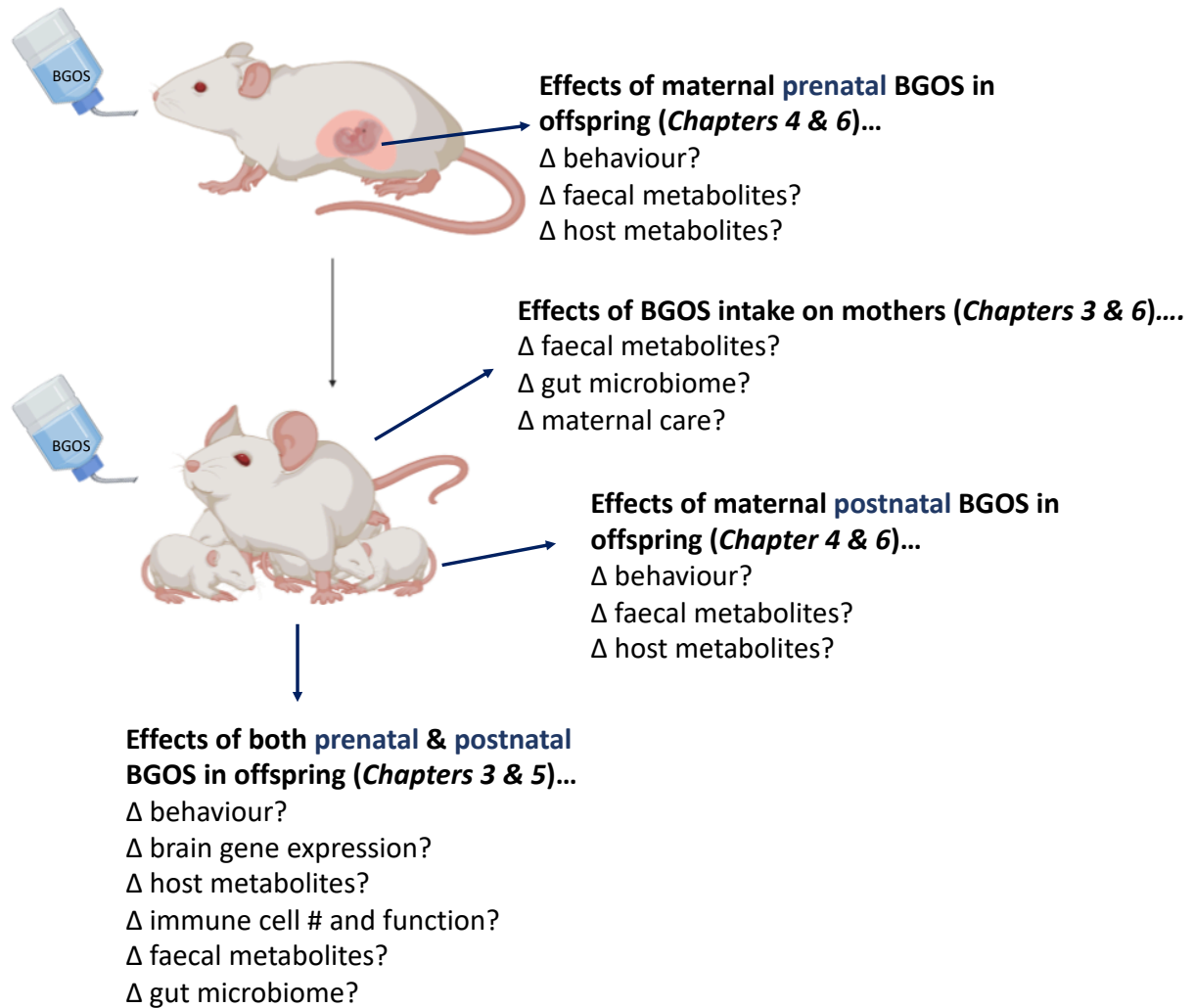


Figure 1.4. Summary of thesis questions.

2. Materials and Methods

2.1 Animals

All experiments were carried out with local ethical approval and a UK Home Office licence granted under the Animals (Scientific Procedures) Act (1986). Female (28-30 g) and male (30-32 g) CD1 mice (Charles River, UK) were housed under normal laboratory conditions (12-h light-dark cycle, lights on at 7 a.m., 21 ± 1 °C, humidity $50 \pm 5\%$).

2.2 Breeding

Male and female mice were housed in individually ventilated cages (IVCs). After habituating to the animal facility for at least one week, they were organised into mating pairs. Pairs were checked daily for vaginal plugs. The date a vaginal plug was found was noted as E0. Males were kept with females until E14 to minimise stress for the dam. At E14, males were removed from the cages. Pups were weighed at pre-specified time points.

2.3 Drugs

2.3.1 BGOS administration

When a vaginal plug was found, females were randomly assigned to receive either normal drinking water or water supplemented with 1.5 mg/mL BGOS (approximately 0.25 mg/g per day). This dose was chosen because it was shown to increase *Bifidobacterium* levels in female mice in a small pilot study (**Appendix A**). Although the dose is lower than the 0.5 mg/g BGOS dose that has been published previously (Savignac et al. 2013; Williams et al. 2016; Savignac et al. 2016; Gronier et al. 2017), it was shown in the pilot study to increase *Bifidobacterium* while the previously published dose did not. I also did not have a primary outcome in offspring on which to test multiple doses, and testing several doses on every outcome in this study would have required a prohibitive number of mice. Therefore, only one dose was possible. In adult rodents, neurobehavioural effects of BGOS have been detected after three weeks (Savignac et al. 2013; Williams et al. 2016; Savignac et al. 2016; Gronier et al. 2017), which aligns with the gestational period in the mouse. We continued BGOS supplementation during lactation as well to capture potential postnatal effects of maternal prebiotic feeding. The BGOS was weighed in a weigh boat and added to 200 mL water. The bottles were shaken briefly with one finger over the spout to prevent spillage. The weigh boat was then rinsed with 50 mL water to collect any remaining powder into the bottle. The bottles were rinsed out and refilled Monday, Wednesday, and Friday every week. Assignment to BGOS or water was performed using the “randomise range” function in Google Sheets.

2.3.2 LPS administration

LPS from *Escherichia coli* O111:B4 (Sigma-Aldrich) was dissolved the morning of injections in sterile 0.9% sodium chloride. For intraperitoneal (I.P.) injections in pups at weaning

age, LPS was administered in a single dose of 1 mg/kg. For subcutaneous (S.C.) injections for P9 pups, LPS was delivered at 3 mg/kg. Pups were randomly assigned to receive saline or LPS.

For the early-life LPS dose, P9 was chosen based on a study that reported deficits in anxiety-like and social behaviour in adulthood after LPS at this age, as well as increased microglia activation (Li et al. 2018). Unfortunately, I found that the 10 mg/kg dose used in this study was lethal, so I reduced the dose to 3 mg/kg. In addition, at P9, pups are in the middle of rapid increases in brain volume and neurogenesis, which makes it a vulnerable period for inflammatory insults to brain development (Semple et al. 2013).

2.4 Behaviour

Standard tests were used to assess changes in behavioural patterns in pups. Mice were transported to the behaviour room and habituated for 30 minutes. The open field test (OFT), light-dark box (LDB), and the forced swim test (FST) were performed in that order. While the decision to run the OFT before the LDB was arbitrary, the FST was performed last to avoid the influence of a substantial stressor on behaviour in the other tasks (Habr et al. 2014). These tests were chosen to determine whether changes in exploration, anxiety-like behaviour, passive stress coping, and social preference after BGOS/GOS supplementation were multigenerational (Savignac et al. 2016; Burokas et al. 2017). Each mouse had approximately two hours of rest between tests. Male mice were run before females to minimise the effects of female scent on male behaviour. All behavioural tests had to be run on the same day because mice were transported to the Department of Pharmacology and needed to be tested and culled the day they were moved. The animals were run in a random order. In addition, the observer was blinded to the treatment groups during behaviour scoring.

2.4.1 Open field test (OFT)

The OFT (**Figure 2.1A**) is used in most comprehensive assessments of animal behaviour. When the test is performed for a brief period of time (usually 2–10 minutes), it measures the degree of exploration of a novel environment more so than baseline locomotor activity. General activity can be quantified in a longer test once the mouse has habituated and the environment is no longer novel.

In the OFT, distance travelled is a measure of activity in the novel environment. Rearing behaviour can also be quantified as a measure of exploratory behaviour. A rear occurs when the mouse elevates its forepaws, keeping the hind paws on the floor, to assess the environment. In addition, mice tend to stay close to the walls, a phenomenon known as thigmotaxis. The time spent in the central zone of the open field is therefore a proxy for anxiety-like behaviour, and more time in the centre is interpreted as less anxiety. Anxiolytic drugs increase locomotor activity, time in centre, and rearing behaviour (Gould, 2009).

Throughout the course of the experiments, two open fields were used: a large open field (84x57x40cm³, *Lxwxh*) with black walls and floor, or a small open field (50x50x50cm³, *Lxwxh*) with a black floor and white walls. The small open fields were constructed by dividing a large square into four compartments. This apparatus was built and used in later experiments because the smaller size was determined to be more appropriate for a mouse. This also increased the efficiency of behavioural testing by allowing me to run four animals at a time. Both apparatuses were constructed from painted wood. Lighting was measured using the “Lux Light Meter Pro” app and adjusted to 10 lux. The centre was defined as one-third of the open field area.

The mouse was placed in one corner of the open field and recorded for five minutes. The following parameters were measured with ANY-maze software (Stoelting Co.): distance travelled

(cm), time in centre zone (s), and entries into the centre zone (s). Total number of rears was scored manually by an independent observer blinded to the treatment groups.

2.4.2 Light-dark box (LDB)

The OFT and the LDB (**Figure 2.1B**) are advantageous because they are ethological measures of innate behaviour and attempt to capture the mouse's reaction (fight or flight, freezing behaviour) to a situation that mimics its natural environment. In the LDB, the bright light and novel environment act as mild stressors for the mouse. This test measures the balance between the mouse's tendency to explore and neophobia (avoidance of the unfamiliar). Increases in exploration in the light compartment – time spent, latency to enter, entries to the light side, and rearing in the light – correspond to reduced anxiety-like behaviour (Gould, 2009).

In this set of experiments, mice were placed in a box with two compartments: a small black compartment with a lid ($21 \times 16 \times 16 \text{ cm}^3$, $L \times w \times h$) and a white, brighter (15 lux) open compartment ($46.5 \times 21 \times 21 \text{ cm}^3$, $L \times w \times h$) separated by a small opening ($3 \times 2.7 \text{ cm}^2$, $L \times w$). One transparent side faced the camera. To begin the test, the mouse was placed in the dark side, and the lid was immediately put on. The test was run for five minutes. Time in the light side (s), latency to enter the light (s), number of crossings between sides, and number of rears were scored manually.

2.4.3 Forced swim test (FST)

The FST (**Figure 2.1C**) was first described by Porsolt and colleagues as a novel screen for antidepressant activity (Porsolt, Pichon, and Jalfre 1977), and most current antidepressants do indeed reduce “depression-like” immobility behaviour in the FST. In this test, mice are placed into a tub of water. They swim around frantically in an attempt to escape in the first two minutes. Eventually, they exhibit passive floating behaviour. This immobility has been described as behavioural despair, or an unwillingness to exert effort to escape. The FST, however, has poor

construct validity as a model of depression. Floating could be considered as an adaptive, rather than maladaptive, strategy to coping with stress. Furthermore, although traditional antidepressants that target monoamines effectively reduce time floating, it is unclear how well the FST would perform as a screen for novel antidepressants (Gould, 2009). For this reason, immobility/floating behaviour will not be referred to as depression-like behaviour in this thesis. Instead, we will refer to floating as passive stress coping or behavioural despair.

A box (32x17x12 cm³, *Lxwxh*) was filled to 9 cm with water at 30 °C. Light was adjusted to 25 lux. A mouse was placed in a corner of the apparatus for a total of five minutes. Time floating (s) and latency to float (s) were scored in the last three minutes. Floating behaviour was defined as the minimal amount of movement necessary to stay afloat.

2.4.4 Three-chamber social interaction test (SIT)

Mice are social and curious animals, and they tend to approach unfamiliar conspecifics. This is likely rooted in their cooperative approaches to foraging, defending territories, and avoiding predators. Genetic modification or maternal immune activation can reduce interaction time. In addition, this behavioural deficit is used as a model of impaired social approach behaviour in humans with autism (Yang, Silverman, and Crawley 2016)

The social interaction test (SIT, **Figure 2.1D**) was adopted from a published protocol (Yang, Silverman, and Crawley 2016) and was performed in adult offspring only. The apparatus was a transparent red Plexiglas box (65x30x20 cm³, *Lxwxh*) divided into three equal-sized chambers separated by sliding doors. The lighting of the room was measured to be 2 lux. The test consisted of two parts: a habituation phase and a testing phase. In the morning, during the ten-minute habituation phase, mice were placed in the central chamber with the doors closed. The doors were opened after five minutes to allow the animal to explore the entire apparatus for the

remaining five minutes. In the afternoon, for the ten-minute testing phase, a novel C57Bl/6 mouse of the same sex was placed in a wired cup in one chamber, and an empty wired cup was placed in the other chamber, serving as a novel object. The mouse was placed in the centre of the apparatus, and the doors were immediately opened. The mice were tracked with ANY-maze software. The social preference score was calculated as the percentage of time spent in the chamber with the novel mouse compared to the total time exploring either the novel mouse chamber or the novel object chamber. The apparatus and wired cups were cleaned with Anistel, an animal facility disinfectant, in between tests. Water was also used to remove the smell of the disinfectant.

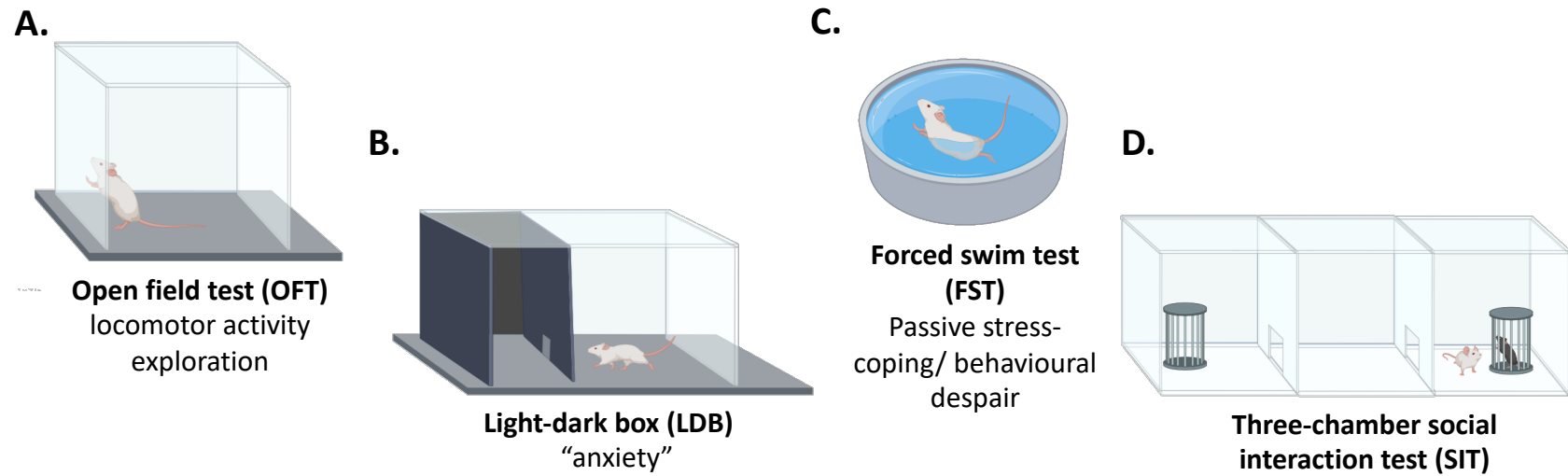


Figure 2.1 Schematics of mouse behavioural tests in pups. **A)** The OFT, **B)** LDB, **C)** FST, and **D)** SIT were performed in offspring.

2.4.5 Maternal care behaviour

Assessing maternal care behaviour is useful in understanding the influence of a maternal treatment or challenge on offspring. In this project, it was necessary to test the possibility that the prebiotic feeding made the dams more or less attentive to their pups, thus changing behaviour and brain gene expression in offspring. Home cage observation is the most useful measure of maternal care because it assesses the dam's behaviour in a natural environment without disrupting the nest. It can be challenging for the experimenter, however, to differentiate between similar behaviours (grooming and nursing, for example). Quantifying the latency to retrieve pups that are dispersed from the nest by the experimenter is another common technique. This test is advantageous because it is quicker and less subjective. In this case, however, the disruption to the nest is less ethologically relevant than the home cage observation test (Franks, Curley, and Champagne 2011). Given that these paradigms both have advantages and disadvantages, both tests were used to assess maternal care behaviour.

2.4.5.1 *Home cage observation*

An abbreviated version of a previously published protocol was used (Franks, Curley, and Champagne 2011). Cages were transported to the observation room and left to acclimate for 30 minutes. Maternal care behaviour was observed on P2 and P4 between 8:00 – 9:00 and 17:00 – 18:00. Behavioural observations were recorded every two minutes for a total of 120 observations per dam. The experimenter was blinded to treatment conditions. The following behaviours were scored: nursing, licking/grooming, nest building, and time absent from the nest.

2.4.5.2 *Pup retrieval test*

After the home cage observation was complete, the dam was moved to a separate cage. Three pups were removed from the nest, each placed in a different corner of the home cage. The dam was then returned to the home cage. The latency to retrieve the first pup and the time to retrieve all pups were recorded.

2.5 Tissue collection

After the last behavioural test, animals were anaesthetised with isoflurane gas, and blood was collected via cardiac puncture in either heparin-coated tubes or 0.5 M EDTA-coated tubes. Whole blood, used for full blood counts, was stored at 4 °C until ready to be analysed. When plasma was collected, blood was centrifuged for 10 minutes at 1300 g at 4 °C and then stored at -80 °C until further processing and analysis. The right atrium was pierced, and the circulatory system was then transcardially perfused with heparinized saline (0.9% sodium chloride) using a perfusion pump connected to a needle in the left ventricle to purge any residual blood. Brains were then removed and the prefrontal cortex (PFC) and hippocampus were dissected using a scalpel blade and forceps and immediately frozen on dry ice.

Spleens were weighed immediately after the dissection, and body weights from the day before were used to calculate spleen:body weight ratios.

To collect faecal samples, the intestines were removed, and faecal pellets were dissected from the colon.

Legs were removed for bone marrow collection and put on wet ice. We ensured that they contained intact femurs and tibias. The day after mice were culled, bone marrow was collected under sterile conditions for culture of bone marrow-derived macrophages.

Dissection tools were autoclaved and cleaned with 70% ethanol between dissections. Skin and muscle were dissected away to expose the tibia and femur from each leg. The bones were then flushed into a petri dish with 10 mL ice-cold Dulbecco's Phosphate-Buffered Saline (DPBS, Sigma-Aldrich) using a 10 mL syringe and 25G needle. The DPBS and bone marrow were filtered through a 70 μ m strainer into a 50 mL falcon tube, and the petri dish was washed with an additional 10 mL DPBS and added to the solution. The bone marrow was centrifuged for five minutes at 400 g. The supernatant was removed, and the bone marrow was reconstituted in 2 mL freezing solution: 10% dimethylsulfoxide (DMSO, Sigma-Aldrich) in foetal bovine serum (FBS, Sigma-Aldrich). The bone marrow was split into two cryovials and stored at -80 °C for a month, then moved to liquid nitrogen storage to prevent deterioration.

2.6 RNA extraction

PFC and hippocampi were homogenised in approximately 15 μ L/mg Tri Reagent (Sigma-Aldrich) in a Tissue Lyser (Qiagen) for two minutes at 40 Hz and centrifuged for five minutes at room temperature at maximum speed. The supernatant was collected and combined with one-fifth volume of chloroform, shaken for 30 seconds to mix, and incubated for five minutes at room temperature. The samples were then centrifuged for 10 minutes at 4 °C at maximum speed. The clear top layer was collected, and an equal volume of isopropanol was added and mixed by pipetting. After incubating for five minutes on ice, the samples were centrifuged for ten minutes at max speed at 4 °C. The supernatant was discarded, and the RNA pellet was purified with 500 μ L 70% ethanol. The samples were centrifuged again at max speed for one minute at 4 °C. The supernatant was discarded, and the white RNA pellet was left to dry until it turned transparent (about five minutes). The

RNA was then reconstituted in 20-30 μL nuclease-free water and the concentration was measured using the NanoDrop ND-1000 (Thermo Scientific).

2.7 DNA extraction

Faecal samples were homogenized in 10 $\mu\text{L}/\text{mg}$ 6M guanidine hydrochloride (Sigma-Aldrich) for four minutes at 40 Hz using the Tissue Lyser. Samples were centrifuged for five minutes at room temperature. The supernatant was removed, and 10% silicon dioxide (Sigma-Aldrich) in nuclease-free water was added to isolate the DNA. The pellet was washed with 100 mM NaCl in 70% ethanol, then re-suspended in nuclease-free H_2O and heated in a water bath at 55 $^{\circ}\text{C}$. The DNA solution was isolated, and DNA quality and concentration were determined using the NanoDrop.

2.8 Quantitative PCR (qPCR)

SYBR Green (Life Technologies) was used for detection of amplified DNA. qPCR reactions were run using the Applied Biosystems QuantStudio 6 Flex Real-Time PCR System (Thermo Scientific). A melt curve was generated after each run to confirm a single PCR product. Transferrin receptor (TFRC) was used as a housekeeping gene for brain regions, and beta-2-microglobulin (B2M) was used for BMDMs. Raw Ct values for reference genes were compared across groups to ensure that there were no effects of treatment on reference gene expression. All samples were run in triplicate, and triplicates labelled as outliers by the QuantStudio software were removed before analysis. $2^{-\Delta\text{Ct}}$ was calculated for each sample, where $\Delta\text{Ct} = (\text{Ct}_{\text{target gene}} - \text{Ct}_{\text{reference gene}})$. Fold change in gene expression was then calculated by dividing each group by the average $2^{-\Delta\text{Ct}}$ value for the control group. The primer sequences are shown in **Table 2.1**.

Table 2.1 qPCR primer sequences.

Gene or bacterial taxa	Forward sequence (5' to 3')	Reverse sequence (5' to 3')	Amplicon size (bp)	Reference
TNF- α	GCCTCCCTCTCATCAGTTCTAT	TTTGCTACGACGTGGGCTA	94	(Couch et al. 2015)
GluNR1	ATCATCCTGCTGGTCAGCGA	AGCAGAGCCGTACATTCTT	137	Verrall 2008*
GluNR2A	GCTTTCCTTGAACCCCTTCAG	GGGGAGCTTTCCCTTTGGCTAA GTT	142	Verrall 2008*
GluNR2B	TTGGTGAGGTGGTCATGAAG	GGCTCTAAGAAGGCAGAAGGT	166	Verrall 2008*
IL-6	CTGCAAGAGACTTCCATCCAGTT	GAAGTAGGGAAGGCCGTGG	70	(Atarashi et al. 2008)
BDNF	AGCTGAGCGTGTGTGACAGT	ACCCATGGGATTACACTTGG	161	(Musazzi et al. 2009)
5-HT2A	CAGGCAAGTCACAGGATAGC	TTAAGCAGAAAGAAAATCCCA CAG	93	(Couch et al. 2015)
YM1	GGATGGCTACACTGGAGAAA	AGAAGGGTCACTCAGGATAA	178	(Ji et al. 2013)
Fizz1	CCCTCCACTGTAAACGAAG	GTGGTCCAGTCAACGAGTAA	176	(Ji et al. 2013)
TFRC	TGGGTCTAAGTCTACAATGGCT	CCCTCATGACGAATCTGTTTG	114	Verrall 2008*
Beta actin	CGTTGACATCCGTAAAGACC	AACAGTCCGCCTAGAAGCAC	281	(Ji et al. 2013)
18S	GTAACCCGTTGAACCCATT	CCATCCAATCGGTAGTAGCG	151	(Abernathy et al., 2016)
16S	CGGCAACGAGCGCAACCC	CCATTGTAGCACGTGTGTAGCC	130	(Denman and McSweeney 2006)
<i>Bifidobacterium</i>	TCGCGTCYGGTGTGAAAG	CCACATCCAGCRTCCAC	243	(Rinttilä et al., 2004)
<i>Lactobacillus</i>	GGAAACAG(A/G)TGCTAATACCG	CACCGCTACACATGGAG	544	(Heilig et al. 2002)
<i>Clostridium</i> <i>coccoides</i> / <i>Eubacterium</i> <i>rectale cluster</i>	CGGTACCTGACTAAGAAGC	GTTCTCCTAATATCTACGC	236	(Rinttilä et al. 2004)
<i>Bacteriodes</i>	GAGAGGAAGGTCCCCCAC	CGCTACTTGGCTGGTTCAG	106	(Layton et al. 2006)
<i>Escherichia coli</i>	CATGCCGCGTGTATGAAGAA	CGGGTAACGTCAATGAGCAAA	96	(Penders et al. 2005)
<i>Faecalibacteriu</i> <i>m prausnitzii</i>	AGATGGCCTCGCGTCCG	CCGAAGACCTTCTCTCTCC	199	(Wang, Cao, & Cerniglia, 1996)
<i>Roseburia</i>	GCGGTRCGGCAAGTCTGA	CCTCCGACACTCTAGTMCGAC	81	(Ramirez-Farias et al. 2009)
<i>Methanobacteri</i> <i>aceae</i>	AGGAATTGGCGGGGAGCAC	TACCGTCGTCCACTCCTTCCTC	284	Fwd: (Stahl and Amann 1991) Rev: (Raskin et al., 1994)
<i>Oscillibacter</i>	GGCAATGGRCGCAAGYCTGACC	ATTCDHGAGGTACCGTCTTCTR CTC	105	(Morel et al. 2015)
<i>Coproccoccus</i>	CWRKGYAAGTCTGAAGTGAA	CCKRYACTCTAGCTRVAYAGT	74	(Morel et al. 2015)
<i>Allobaculum</i>	ACCTGCGGTGCATTAGYTGG	GCATYGCTCGTTCAGGCTTGC	169	(Morel et al. 2015)

*Primers were designed using the Primer 3 software (primer3.ut.ee, run by Dr Louise Verrall, DPhil Thesis 2008). PCR products spanned exon-exon boundaries such that any contaminating genomic DNA would be unlikely to amplify given its size or, if it were, would be distinguishable by size from mRNA PCR products. Specificity of primers for

each transcript was confirmed using the nucleotide-nucleotide alignment search (<http://ncbi.nlm.nih.gov/BLAST/>), and product sequencing (Department of Biochemistry, University of Oxford).

2.9 Bone marrow-derived macrophages (BMDM) culture and stimulation

Macrophages are critical effector cells of the innate immune system. They are tissue-resident phagocytic cells that are involved in homeostasis, clearance of debris, secretion of growth factors, pathogen recognition, and cytokine production. Macrophages are derived from hematopoietic stem cells in the bone marrow (Geissmann et al. 2010). They can be derived from bone marrow cells *in vitro* in the presence of macrophage colony-stimulating factor (M-CSF). They can then be simulated and tested for changes in gene and protein expression, morphology, and physiology (Weischenfeldt and Porse 2008).

L929-conditioned media (LCM) was used to provide M-CSF factors for the BMDMs. L929 cells, which secrete M-CSF, were plated in a T75cm² flask in media: RPMI (VWR International) with 10% FBS (Life Technologies) and 1x L-glutamine/penicillin/streptomycin (Sigma-Aldrich). Once the cells reached confluence, they were passaged 1:10 in T175 cm² flasks and left until the media turned yellow/orange (about seven days). The supernatants were collected and spun for 10 minutes at 1000 g to eliminate debris, aliquoted in 50 mL Falcon tubes, and stored at -20 °C.

Day 0 – 6: Frozen bone marrow was thawed in a 37° C water bath for five minutes. The cells were washed with Dulbecco's Modified Eagle's Medium (Sigma-Aldrich). Samples were centrifuged at 1000 g for ten minutes. The supernatant was discarded, and the cell pellet was re-suspended in culture media (10% FBS, 20% LCM, and 1x Pen-strep/glutamine). The cells were plated in two 10 cm uncoated petri dishes per mouse. Cells were fed with 3 mL media on Day 3 and Day 6.

Day 7: Although bone marrow can yield a number of different cell types, macrophages can easily be selected for because they rapidly adhere to surfaces. Media was discarded, and cells were dislodged using 0.25% trypsin (Sigma Aldrich). After neutralising cells with fresh media (without LCM), the BMDMs from each mouse were pooled, re-plated, and incubated at 37° C for 20 minutes. Media was aspirated and discarded, and fresh media was added, leaving only the adherent macrophages.

Day 8: In the morning, BMDMs were trypsinized and re-plated in 12-well plates. Cells from each mouse were split into three wells each, one well for each stimulation condition. In the evening, cells were stimulated to polarise into M1 (pro-inflammatory state characterised by release of IL-6, TNF- α , and other pro-inflammatory markers) or M2 (anti-inflammatory state characterised by YM1, Fizz1, and other anti-inflammatory markers) (Layoun, Samba, and Santos 2015). For the M1 condition, 10 ng/mL IFN γ and 100 ng/mL LPS were added. For M2, 10 ng/mL IL-4 was added.

Day 9: BMDMs were lysed using 300 μ L/well Lysis Buffer from the mirVana miRNA isolation kit (Thermo Fisher). RNA was then isolated according to kit instructions. Briefly, an equal volume of acid phenol-chloroform was mixed with cell lysates, centrifuged to separate out the aqueous RNA-containing phase, and filtered through a glass fibre filter to purify the RNA.

This protocol is summarised in **Figure 2.2**.

with mass spectrometry. Hydrogen protons have a quality known as “spin.” This spin has a direction, similar to the north pole of a magnetic field. When an external magnetic field is applied, hydrogen nuclei align with this field. When an exact frequency of energy is applied to the system, the protons flip to align in the opposite direction of the external magnetic field. This is the resonance frequency, and it is dependent on the proton’s chemical environment. Hydrogen nuclei that bond to an electronegative atom are less shielded from the external magnetic field, and it takes less energy for these protons to flip orientation. ^1H NMR measures the magnetic field at which protons are at resonance (**Figure 2.3A**). Different compounds have characteristic peaks on the NMR spectrum depending on the chemical structure. Deuterated solvent (D_2O) is used to avoid interfering with the ^1H proton signal in the compounds of interest.

^1H NMR can be used in an untargeted manner to assess the metabolic “fingerprint” of a sample or group of samples. It is a highly reproducible technique that is widely used for diagnostic purposes. One-dimensional (1D) ^1H NMR is the quickest and most basic technique for metabolomics analysis (**Figure 2.3B**). Metabolite signals, however, overlap with one another. To differentiate between overlapping metabolites, 2D ^1H NMR is useful (**Figure 2.3C**). In this case, correlation spectroscopy (COSY) and total correlated spectroscopy (TOCSY) analyse proton spins that are coupled to each other. In other words, the spins of protons in a chain in the same compound are correlated, and this information can be used to separate ambiguous signals (Bjerrum 2015). Approximately 90 plasma metabolites and 60 faecal metabolites (Lamichhane et al. 2015) can be detected with this technique.

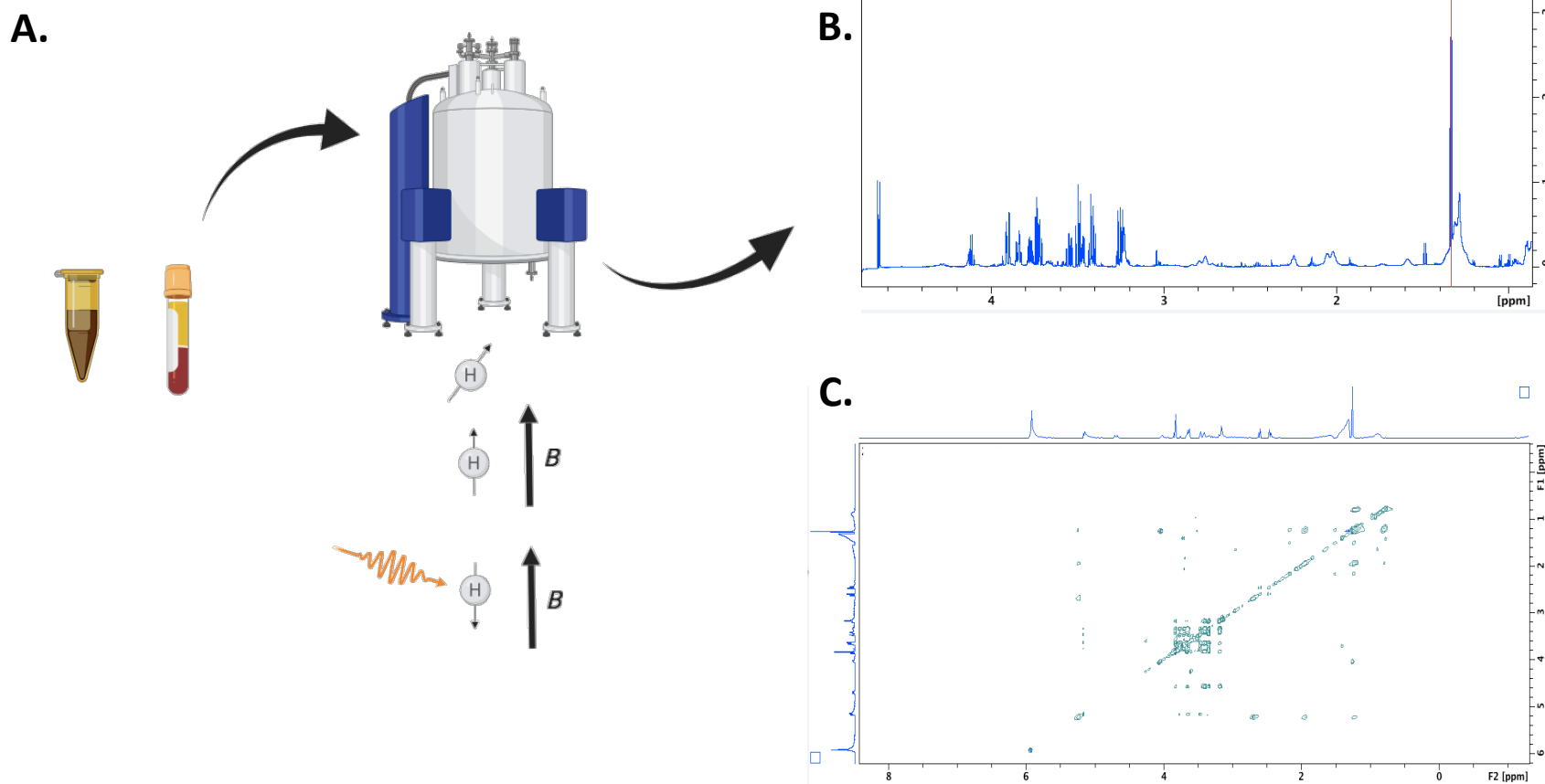


Figure 2.3 A) Procedure for characterisation of faecal and plasma metabolic profiles with ^1H NMR. B) Example of a 1D NMR spectrum. C) Example of a 2D NMR spectrum; ppm: parts per million.

2.11.1 Sample processing

Plasma was thawed at room temperature, vortexed, and centrifuged briefly at max speed; 150 μ L plasma was added to the 60 μ L 75 mM phosphate buffer (5:1 disodium phosphate [Na_2HPO_4] and monosodium phosphate [NaH_2PO_4] in 100% D_2O , pH = 7.4). The solution was transferred to 5 mm Borosilicate NMR tubes (Sigma) with a glass pipette dropper.

In addition, 60–70 mg of faecal sample was thawed at room temperature and diluted with 600 μ L buffer with 5 $\mu\text{g/mL}$ 3-(trimethylsilyl)propionic-2,2,3,3- d_4 acid (TSP) as a standard. Samples were homogenized in a Tissue Lyser at 40 Hz for four minutes and centrifuged at 16100 g for 15 minutes. The supernatant was transferred to a new Eppendorf tube, and the centrifuging process was repeated. All processing occurred at 4 $^\circ\text{C}$. Samples were then stored at -80°C . On the day of running the faecal extracts in the NMR, the samples were thawed at room temperature and briefly vortexed and centrifuged at max speed; 400 μ L sample was combined with 150 μ L buffer and transferred to an NMR tube.

Samples were acquired using a 700-MHz Bruker AVII NMR Spectrometer operating at 16.4 T equipped with a ^1H TCI cryoprobe. Temperature was maintained at 310 K. ^1H spectra were acquired using a one-dimensional Nuclear Overhauser Effect Spectroscopy (NOESY) pre-saturation to reduce the water signal with a 2 s pre-saturation. For plasma, a spin-echo Carr-Purcell-Meiboom-Gill (CPMG) sequence was used to suppress signals resulting from large molecular weight compounds such as proteins.

2.11.2 Spectra processing

Spectra were processed in Topspin 4.0.5 (Bruker). Spectra were phased and baseline-corrected using a third-degree polynomial. For plasma, the centre of the lactate

doublet was calibrated to 1.33 parts per million (ppm). For faecal extracts, TSP was calibrated to 0 ppm. The spectra were then exported to ACD/Labs Spectrus Processor Academic Edition 12.01 (Advanced Chemistry Development, Inc.). The water region was removed, as well as the noise regions up to 0.82 ppm and after 7.46 ppm. The spectra were divided into 0.02 ppm segments called “bins,” and the integral of each bin was calculated. For samples containing EDTA, this signal was removed as well. For each sample, the integrals were then normalised to the sum of all the integrals for the sample’s spectra. Finally, remaining noise regions were removed. The bins, labelled with classes (treatment groups) and animal IDs, were then exported to R for principal component analysis (PCA) and multivariate analysis. Metabolites were identified by referring to literature values and the human metabolome database (Wishart et al. 2009). COSY and TOCSY spectra were used to separate overlapping signals. The ^1H NMR process workflow is summarised in **Figure 2.4**.

2.12 Statistical analysis

2.12.1 Univariate analysis

Statistical tests were run in Prism (Graphpad) and R (R Foundation for Statistical Computing). Normality of the residuals was tested with the Shapiro-Wilk test, and homogeneity of variances was tested with Spearman’s rank correlation test for heteroscedasticity (alpha threshold for significance set at $p < .01$). Grubbs’ test for outliers was used before statistical analysis, and statistically significant outliers were excluded. Non-Gaussian or heteroscedastic data were square root- or log-transformed. Analysis of variance (ANOVA) was used to analyse main effects and interactions of maternal diet and LPS treatment and, in the case of interactions, pairwise group differences. In the first

instance, three-way ANOVAs were run to identify two-way or three-way interactions with sex. To simplify interpretation of the results, if a statistically significant interaction with sex was detected, the males and females were analysed separately with two two-way ANOVAs. When there were no interactions with sex, however, male and female data were consolidated into one two-way ANOVA to examine main effects and interactions. When a statistically significant interaction was detected, post-hoc tests with Bonferroni corrections for multiple comparisons were used to investigate group differences. In the case of pairwise comparisons, student's *t*-tests were used. Welch's correction was used for data with unequal variances. The threshold for significance for ANOVAs and pairwise tests was $p < .05$. Data were plotted in boxplots, where boxes comprise the median and interquartile range, and the error bars denote minimum and maximum values. Mean (*M*), standard deviation (*SD*), degrees of freedom (including numerator and denominator degrees of freedom for ANOVAs), and *F* and *t* scores were reported.

Performing ANOVAs on heteroscedastic or non-Gaussian data can yield misleading results. This is often the case with biological data, especially animal behavioural tests. When data failed to meet the assumptions of parametric tests even after square-root or log-transformations, robust alternatives were used. For the two-way and three-way ANOVAs, analogous ANOVAs based on 20% trimmed means were performed in R using the *WRS2* package (Mair and Wilcox 2019). A trimmed mean is computed by removing the top 20% most extreme values and taking the arithmetic mean of the remaining data. The resulting test statistic, *Q*, is analogous to the *F* statistic in parametric ANOVAs and is used to test null hypotheses of main effects and interactions (Wilcox 2012). For pairwise comparisons, the Kolmogorov-Smirnov test was used in Prism or R.

These tests are robust to outliers and do not require normally distributed residuals or equal variances.

To explore associations between dependent variables, correlation matrices were built using Pearson product-moment correlation coefficients and p -values using the *corrplot* package in R (Wei 2017). Because these initial analyses were exploratory, no corrections for multiple comparisons were applied. After identifying significant correlations, however, these relationships can be confirmed using proper power analysis in future studies.

2.12.2 Accounting for litter effects

The effects of maternal diet, LPS, and/or sex on outcomes in offspring are not independent because pups within the same litter may be more similar to each other than pups in different litters. In other words, my data has a hierarchical, or nested, design with maternal diet, LPS, and sex as fixed effects and litter as a random effect. If litter explains a large proportion of the variance, this would artificially inflate the statistical power, but the ANOVA described in the previous section cannot account for this. In order to confirm that the results remained the same after accounting for litter effects, a linear mixed-effects model was used to determine whether factors remained significant. The linear mixed-effects model fit by restricted maximum likelihood was built using the “lmer” function in the *lmerTest* package for each outcome (Kuznetsova, Brockhoff, and Christensen 2017), and p values were calculated using the “anova” function in R. Results are summarised in **Appendix C**.

3.1.1.1 Multivariate analysis (for ^1H NMR data)

Analysis of metabolomics data was performed as previously described (Jurynczyk et al. 2017). Sum-normalized bucket integrals were imported into R software. PCA plots of data that were Pareto-scaled (a scaling method that reduces effects of large signals without inflating noise) were built for initial visualization of the data and to identify outliers using the *pcaMethods* package (Stacklies et al., 2007). Orthogonal partial-least squares discriminant analysis (OPLS-DA) was then used to investigate differences in the metabolic profiles between groups using the *ropls* package in R (Thévenot et al., 2015). OPLS-DA is a supervised multivariate analysis method that attempts to generate models to explain differences between two classes. These models can also give information about which metabolites drive differences between groups.

OPLS-DA models were built by multiple iterations of randomly separating data into training and test sets. Training sets were used to build the models using a seven-fold internal cross-validation procedure. The models were then tested by forced classification of samples in the independent test sets in a ten-fold external cross-validation scheme to determine the accuracy, sensitivity, and specificity of the models. For sample sizes less than ten, eight-fold external cross-validation was used. In addition, goodness of fit was assessed with R^2X (a measure of how well the models explained variation in the observations) and R^2Y (how good the separation between groups was). Finally, predictive ability of the models was assessed with Q^2 . A total of 1000 models were built for each pairwise group comparison (800 models if eight-fold cross-validation was used for smaller group numbers). In addition, the same model-building and external cross-validation procedure was used to build models from data randomly sorted into classes – the average

accuracy of these models is always expected to be 50%. If the true models performed significantly better than random chance in categorizing the samples in the test sets ($p < .001$, Kolmogorov-Smirnov test), a representative OPLS-DA model was generated, and metabolites driving the group separations were identified using the average variable importance in projection (VIP) scores. In the graph of VIP scores vs. ranking, the inflection point was used to determine which variables were important in building the models.

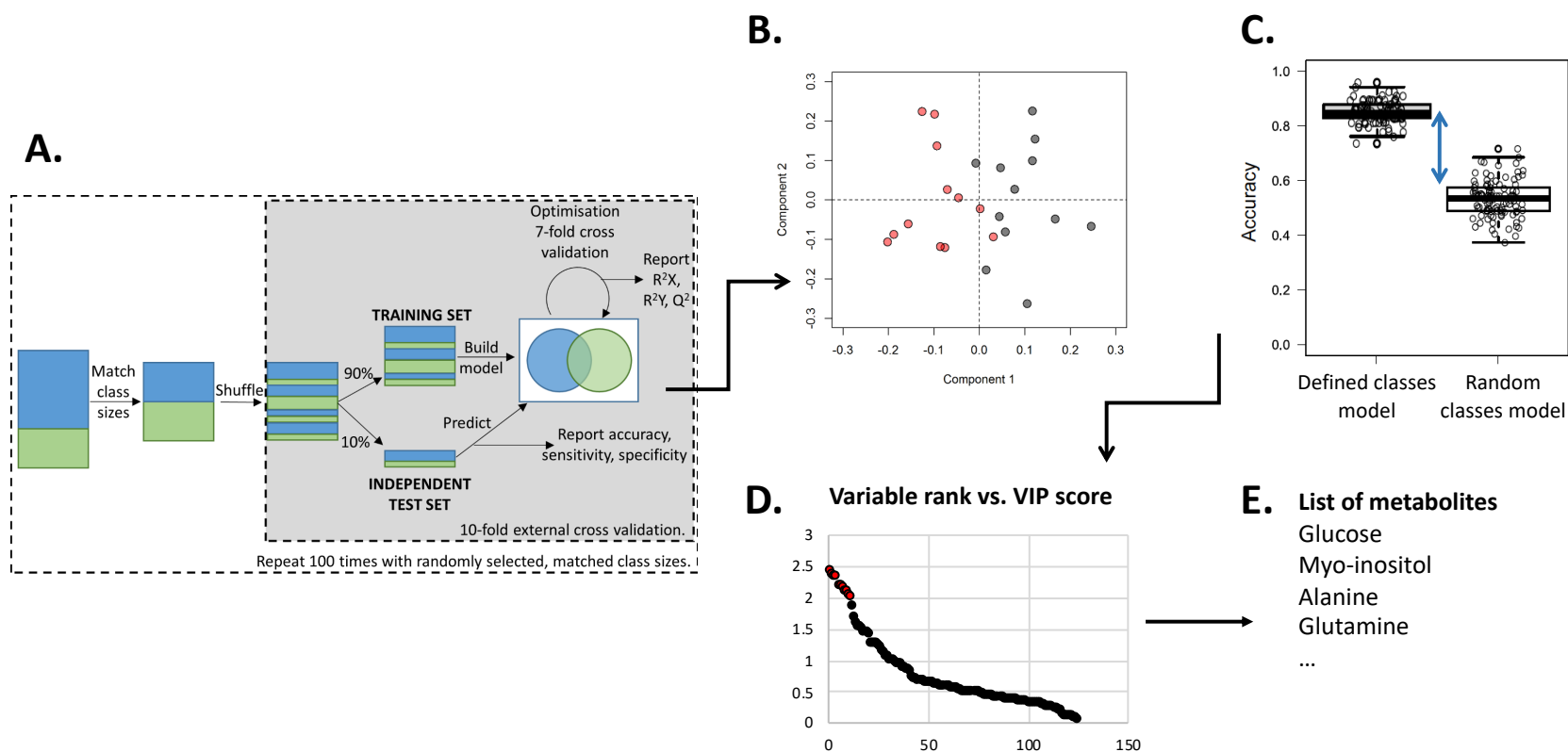


Figure 2.4 OPLS-DA analysis procedure. **A)** OPLS-DA procedure for building models and testing models with 10-fold external cross-validation. **B)** Example of one OPLS-DA model. **C)** Accuracy is compared to a model of random classes. **D)** Variables corresponding to top VIP are selected for metabolite identification. **E)** The last step is to use the high-scoring variables (spectral bins) to determine which metabolites that drive the separation between groups. *OPLS-DA figure (A) and accuracy plot (C) courtesy of Dr. Fay Probert.*

2.13 16S Sequencing

DNA sequencing is an accessible and informative tool for the comparison of gut microbiota between individuals and groups. Similar to ^1H NMR, amplicon-based DNA sequencing can give a microbial fingerprint of a community of bacteria. In this technique, the gene that encodes the 16S small subunit of ribosomal RNA is used to infer the phylogenetic structure of a community. This gene is highly conserved, specific to bacteria, rarely horizontally transferred, and contains variable regions that can be used as evolutionary clocks to differentiate between bacterial taxa in a sample. This allows for the comparison of the number of individual taxa within samples (alpha diversity). Taxonomic abundance profiles can also be compared between treatment and control groups or patient populations and healthy cohorts (beta diversity). The relative abundances of specific taxa can also be compared between groups. The 16S region is the only known marker that is present in all bacteria whose function is highly conserved, allowing random DNA mutations to be used as a measure of evolution. This makes it the most common tool for investigating microbiome diversity and relative abundances of taxa (Janda and Abbott 2007; Hugerth and Andersson 2017).

The 16S sequencing is computationally manageable and affordable because only a small fraction of the DNA is sequenced. There are, however, several limitations. Researchers must select one of the variable regions in the 16S rRNA gene to amplify and sequence because the entire gene is too long to sequence. This makes comparisons between studies using different variable regions challenging. In addition, PCR amplification errors make it difficult to differentiate between rare or closely-related taxa and sequencing artefacts. There is also poor resolution at the species level. Furthermore, it is impossible to infer the functional profile of a microbiome from 16S sequencing (Schloss, Gevers, and Westcott 2011). Shotgun sequencing of the metagenome is a more recent

technique for analysing the microbiome. It involves shearing all the DNA into small segments, sequencing those fragments, and reassembling. This method provides data on the composition of a microbial community as well as the presence of other DNA, such as protein-coding genes. This can provide information about the functional potential of the microbiome. Metagenomics, however, is more costly and computationally challenging than 16S sequencing. For these reasons, the former remains the dominant technique for comparing gut microbiota (Sharpton 2014).

2.13.1 16S rRNA amplicon library preparation and sequencing

Library preparation and sequencing were performed at the Oxford Genomics Centre (OGC). DNA samples were normalised to 12.4 ng in 2.5 µl volume. Primers targeting the V3-V4 region of the 16S rRNA gene were used for amplification (Klindworth et al. 2013). The full-length primer sequences, using standard International Union of Pure and Applied Chemistry (IUPAC) nucleotide nomenclature, were: 16S Amplicon PCR Forward Primer = 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and 16S Amplicon PCR Reverse Primer = 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC. Following a post-amplicon PCR clean-up, a second indexing PCR was applied using NexteraXT indices. Following the second PCR clean-up, libraries were then pooled and diluted to a 10nM final multiplex library ready for sequencing on Illumina MiSeq (V3 chemistry). An average of 99147 (range 505–152758) 300bp paired-end reads were generated.

2.13.2 Data processing

Quality of raw sequencing data was assessed using FastQC (v0.11.07). Following quality assessment, downstream processing was performed using *dada2* (Callahan et al. 2016) which was implemented in pipeline_dada2.py from the NGSSKit repository

(<https://github.com/nickilott/NGSKit>). Given the drop in quality at ~200bp, we decided to proceed with the analysis using the first read in the pair (V3 region). The `filterAndTrim` function in *dada2* was used to truncate the forward and reverse reads. Retained primer sequences were removed from forward reads (17bp) which resulted in final read lengths of 183bp. Error learning, de-replication, and sample inference were performed using *dada2* with default parameters. Taxonomy was assigned to amplicon sequence variants (ASVs) using the `assignTaxonomy` function in *dada2* and the NCBI RefSeq training data (https://zenodo.org/record/2541239/files/RefSeq-RDP16S_v2_May2018.fa.gz). ASV count tables were used for downstream analysis.

2.13.3 Alpha and beta diversity

The *phyloseq* package in R (McMurdie and Holmes 2013) was used for all statistical analysis of diversity and differentially abundant taxa. To investigate global changes in microbiome composition, the Chao1 measure of alpha diversity (number and distribution of ASVs in each sample) was compared between H₂O and BGOS animals in the F0 and F1 cohorts using absolute ASV counts. The Bray-Curtis measure of beta diversity (how different each sample is from every other sample based on relative ASV abundances) was used to explore global differences in microbiome composition between groups. Permutational multivariate analysis of variance (PERMANOVA) was used to determine whether microbiome composition was significantly different between groups.

2.13.4 Identification of differentially abundant taxa

To identify differentially abundant taxa between groups, differential gene expression analysis based on the negative binomial distribution (DESeq) was performed using the *deseq2* package in R (Love, Huber, and Anders 2014). Differentially abundant taxa were plotted in a heat

map and in boxplots. In offspring samples, because there are strong cage and litter effects in microbiome data (Ubeda et al. 2012), linear mixed-effects analysis with litter as a random effect was performed on log10-transformed relative abundances to determine which ASVs were robust to litter effects. Differentially abundant ASVs that were detected by both DEseq and mixed-effects analysis ($p < .05$) were considered to be significantly different in offspring.

2.14 Cohorts

Table 2.2. Summary of the animal cohorts used and outcomes measured in this thesis.

Cohort	Description	Groups: dams	Groups: pups (# males/# females)	Age at testing/tissue collection	Outcomes measured and associated chapters
1	Pups from control dams or BGOS-fed dams were injected with LPS or saline.	1) Control: n = 3 2) BGOS: n = 5	1) H2O/Saline: n = 21 (11/10) 2) H2O/LPS: n = 20 (10/10) 3) BGOS/Saline: n = 22 (11/11) 4) BGOS/LPS: n = 21 (10/11)	Between P22–27	Pups: Body weights, behaviour, gene expression (brain, BMDMs), select bacteria taxa and 16S sequencing (faecal DNA), spleen:body weight ratios, full blood counts Chapters 3, 4, and 6
2	Dams were given BGOS or water. At birth, half of the litters were cross-fostered to a dam of the opposite diet.	1) Control: n = 6 2) BGOS: n = 6	1) H2O: n = 32 (16/16) 2) H2O → BGOS: n = 32 (16/16) 3) BGOS → H2O: n = 32 (16/16) 4) BGOS: n = 32 (16/16)	Between P22–23	Pups: Behaviour, plasma and faecal metabolic profiles Chapters 3, 4, and 6
3	Dams were given BGOS or water. Pups were injected with LPS or saline at P9.	1) Control: n = 6 2) BGOS: n = 5	1) H2O/Saline: n = 20 (10/10) 2) H2O/LPS: n = 20 (10/10) 3) BGOS/Saline: n = 20 (10/10) 4) BGOS/LPS: n = 20 (10/10)	7–8 weeks	Dams: Maternal care behaviour Pups: Brain gene expression, plasma and faecal metabolic profiles Chapters 5 and 6

3. Perinatal prebiotic supplementation alters behaviour and central gene expression in young offspring

3.1 Introduction

The aim of this chapter is to establish whether maternal prebiotic intake influences changes in behaviour and central gene expression resulting from an acute lipopolysaccharide (LPS) challenge in offspring at weaning age.

3.1.1 The LPS model: peripheral inflammation and behavioural deficits

LPS is a component of the gram-negative bacterial cell wall. When administered to a mouse, it stimulates a peripheral immune response mediated by pro-inflammatory cytokines, including interleukin (IL)-6, tumour necrosis factor (TNF)- α , and IL-1 α and β . Immediately after LPS exposure, mice exhibit sickness behaviour, which includes reduced grooming and locomotor activity and social withdrawal (Dantzer and Kelley 2008). The first observation of LPS-induced sickness behaviour came from Miller (1964) when he observed that mice injected with the endotoxin exhibited reduced motivation to press a lever for food or a sweet drink. Later, Yirmiya et al. (1999) drew a parallel between the symptoms of sickness behaviour and clinical depression. He demonstrated that peripheral administration of cytokines reduced rats' motivation to drink a sucrose solution or approach a female mouse, behaviour that resembles anhedonia. These deficits were reversed with antidepressant treatment.

In rodents, to differentiate between short-term sickness behaviour and long-term “depression” and other behavioural deficits, a model is used in which animal are injected with 1 mg/kg LPS or less 24 hours before behavioural testing. In this model, anxiety, anhedonia, and passive stress coping can be detected in behavioural tests while locomotor activity returns to

baseline (Painsipp et al., 2011). In addition, microglia activation and pro-inflammatory cytokines are elevated in the brain 24 hours post-injection, particularly in the prefrontal cortex (PFC) and hippocampus (Hoogland et al., 2015; Wang et al., 2014).

A pilot study (**Appendix B**) showed that an acute I.P. injection of 1 mg/kg LPS increased expression of the cytokines TNF- α , IL-6, and IL-10 as well as cyclooxygenase-2 (COX-2) and toll-like receptor (TLR)4 in the prefrontal cortex 1.5 hours post-injection. Plasma TNF- α was also approximately 600-fold higher than baseline at this point. After 24 hours, peripheral TNF- α returned nearly to baseline levels. Elevated TNF- α mRNA, however, was still detected in both the PFC and cerebellum. There were no changes in IL-1 β or IL-6 after 24 hours. TNF- α was therefore selected as a marker of LPS-induced CNS inflammation for this study.

Given the anti-inflammatory effects of BGOS in adult rodents and humans (Liu, Gibson, & Walton, 2016; Vulevic et al., 2008; Vulevic et al., 2015), maternal BGOS may reduce the central and behavioural response to an endotoxin in pups. Prebiotics promote the proliferation of beneficial microbes with anti-inflammatory properties. Therefore, offspring may benefit from perinatal prebiotic feeding through exposure to maternal microbes or microbial metabolites.

3.1.2 Regions of interest: the PFC and hippocampus

Studies on the effects of prebiotic intake on the brain have predominately investigated the PFC and the hippocampus (**Figure 3.1**). The PFC has historically been defined as the region of the cerebral cortex that receives projections from the mediodorsal nucleus of the thalamus, although other thalamic nuclei project to the PFC in mice. In our study, the PFC encompasses the anterior cingulate, prelimbic, infralimbic, and orbital areas (Carlén 2017). The PFC is involved in learning, long-term memory storage, emotion and stress regulation, goal-directed behaviour, working memory, and other high-level cognitive functions (Miller, Freedman, & Wallis, 2002; Morgan,

Romanski, & LeDoux, 1993; Shaw & Wallis, 2008; Sullivan & Gratton, 2002). Abnormal PFC function is also implicated in a number of psychiatric disorders, including depression, PTSD, autism spectrum disorder, and schizophrenia (Amaral, Schumann, and Nordahl 2008; Murrough et al. 2016; Arnsten et al. 2015; Sullivan and Gratton 2002).

The hippocampus also has diverse functions in learning and memory and stress and emotion regulation. Hippocampal dysfunction, especially deficits in neurogenesis, is linked to psychiatric illnesses (Fanselow and Dong 2010). Stress or inflammation, which increase the likelihood of depression in humans and animal models, can reduce neurogenesis. In contrast, antidepressant medications increase neurogenesis (Bremner et al., 1995; Chen et al., 2001; Levita et al., 2014; Malberg et al., 2000; McKinnon et al., 2009; Schoenfeld & Cameron, 2015).

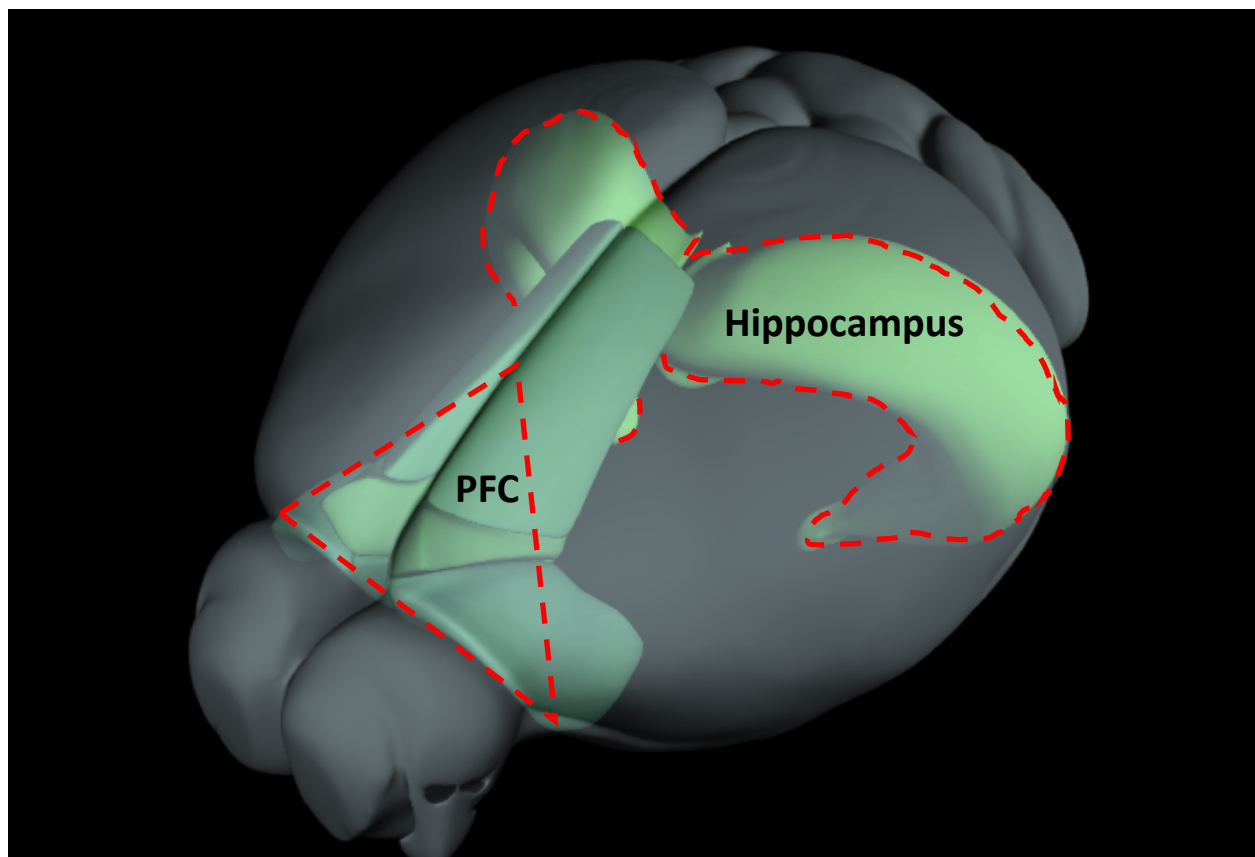


Figure 3.1. Overhead view of PFC and hippocampus. This image from the Allen Mouse Brain Connectivity Atlas illustrates the hippocampus (posterior) and the PFC (anterior) that were dissected from mouse brains for gene expression analysis (Oh et al. 2014). Dashed red lines indicate the approximate regions that were dissected.

3.1.3 Genes of interest: NMDAR subunits, 5-HT_{2A} receptor, BDNF

Genes that have previously been demonstrated to change with oligosaccharide intake (Burokas et al., 2017; Savignac et al., 2013, 2016) were selected for expression analysis. Altered genes include N-methyl-D-aspartate receptor (NMDAR) subunits, 5-hydroxytryptamine (5-HT)_{2A} receptors, and brain-derived neurotrophic factor (BDNF). Cortical and hippocampal NMDARs are glutamate-gated ionotropic receptors involved in excitatory neurotransmission and synaptic plasticity. These are heterotetrameric receptors that typically consist of a mixture of GluNR1 and GluNR2 subunits. The composition of the receptor determines its function, including the tendency to promote synaptic long-term potentiation or long-term depression. Hyperactivation of NMDARs is excitotoxic, while NMDAR hypofunction can lead to cognitive impairment (Paoletti, Bellone, and Zhou 2013).

5-HT_{2A} receptors are members of the 5-HT₂ subclass of serotonin receptors. These excitatory receptors are highly expressed in the PFC and hippocampus. 5-HT_{2A} signalling has been associated with anxiety and depression in animal models and human studies. The exact role of 5-HT_{2A} in mood, however, is not clear. 5-HT_{2A} knockout mice display reduced anxious behaviour (Weisstaub et al., 2006), while 5-HT_{2A} agonists are anxiolytic (Nic Dhonnchadha, Bourin, and Hascoët 2003). In humans, decreased hippocampal 5-HT_{2A} receptor binding has been reported in depressed patients in a positron emission tomography (PET) study (Mintun et al. 2004). Increased cortical 5-HT_{2A} binding, however, was found in a later PET study (Bhagwagar et al. 2006) while increased expression of the receptors was described in post-mortem brains of teen suicide victims (Pandey et al. 2002). Furthermore, 5-HT_{2A} agonists, such as psilocybin, have been shown to have antidepressant properties in human subjects (Griffiths et al. 2016; Carhart-Harris et al. 2018;

Kraehenmann et al. 2015). While 5-HT_{2A} is likely important in behaviour, the nature of the relationship needs to be explored further.

Finally, BDNF is necessary for neurogenesis, neuronal development and survival, and synaptic plasticity in both the PFC and hippocampus. It is involved in learning and memory as well as mood regulation. Reduced hippocampal and cortical BDNF is associated with psychiatric disorders, especially depression. Antidepressants, on the other hand, stimulate BDNF production. Boosting BDNF, which is associated with increased neurogenesis and survival, is believed to be integral to the therapeutic effects of antidepressants (Bredy et al., 2007; Castrén, 2004; Leal et al., 2015; Pandey et al., 2008; Sales et al., 2019; Yu & Chen, 2011).

LPS has extensive effects on gene expression in the brain. Savignac et al. (2016) reported that 0.75 mg/kg LPS I.P. increased GluNR2B and 5-HT_{2A} in the frontal cortex 24 hours post-injection. Similarly, Maher et al. (2014) found increased levels of whole-brain GluNR2B after a similar dose of LPS. LPS has also been shown to reduce brain BDNF mRNA and protein (Guo et al., 2014; Martin et al., 2013; Sriram et al., 2016).

3.1.4 Chapter aims

The primary aim in this chapter is to test whether maternal BGOS supplementation during gestation and suckling improves LPS-induced behavioural and gene expression abnormalities. Specifically, I expected LPS to reduce exploration and increase innate anxiety behaviour and passive stress-coping behaviour. In the brain, I expected LPS to increase expression of NMDAR subunits, TNF- α , and 5-HT_{2A} and decrease BDNF. I hypothesised that F0 BGOS reverses these effects. Given the associations between these genes and behaviour, I also explored significant correlations in a correlation matrix. Finally, I examined the differential contributions of the prenatal and postnatal periods. Given the importance of the *in utero* and early postnatal periods for

brain development, I hypothesised that maternal BGOS intake during both periods contribute to altered behavioural patterns in a cross-fostering design. I also tested whether the variation in behaviour is independent of changes in maternal care behaviour.

3.2 Experiment 3A design: Effects of maternal BGOS on offspring behaviour and brain gene expression

The design of experiment 3A is summarised in **Figure 3.2**. Mating pairs were arranged. At E1, dams were randomly assigned to receive either normal drinking water (H₂O) or water supplemented with prebiotic (BGOS). This feeding continued through gestation and suckling. Between P22–P27, pups were randomly assigned to receive an I.P. injection of either saline or 1 mg/kg LPS. Behavioural testing was performed 24 hours later. After the FST, mice were immediately culled, and the PFC and hippocampus were dissected from each brain for qPCR. For data that passed the normality and homogeneity of variances tests, a standard three-way or two-way ANOVA was used to examine the influence of F0 diet (H₂O vs. BGOS), acute treatment (saline vs. LPS), and sex (male vs. female) on behaviour and gene expression. For datasets that failed these tests ($p < .01$) even after eliminating outliers and/or log- or square root-transformations, a robust three-way or two-way ANOVA based on trimmed means was used. In the cases where there were no significant interactions with sex, data were consolidated for two-way ANOVAs to simplify interpretation of the results. Pearson correlations between gene expression and behaviour were evaluated to investigate potential relationships between gene expression and behaviour (N = 10–11 males and N = 10–11 females; N = 20–22 total).

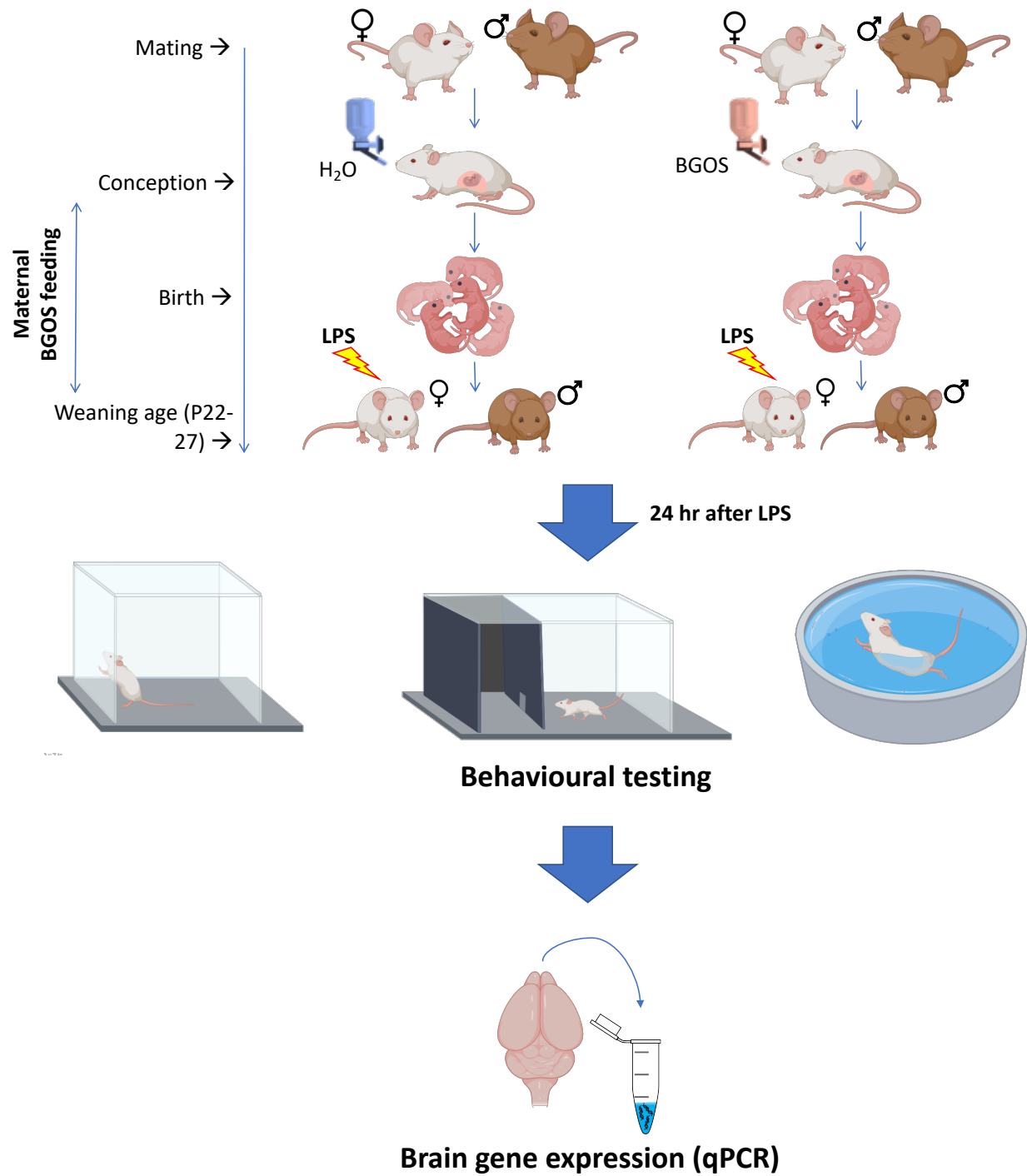


Figure 3.2. Diagram of Experiment 3A.

3.3 Experiment 3A Results

3.3.1 Maternal prebiotic intake increases exploratory behaviour in the OFT

Interactions and main effects of F0 diet and acute treatment were assessed for three outcomes in the OFT: number of rears, time spent in the centre (s), and distance travelled (m). Three-way ANOVAs were performed first to determine if there were any interactions with sex. No significant F0 diet x sex, acute treatment x sex, or F0 diet x acute treatment x sex effects were identified for any of the outcomes, so data were consolidated for two-way ANOVAs.

There was a main effect of F0 diet on rearing in the OFT, $F(1,80) = 21.05, p < .0001$. The number of rears in the BGOS pups ($M = 51.30, SD = 1.70$) was significantly greater than rears in pups from dams given water ($M = 39.01, SD = 0.41$, **Figure 3.3A**). The effect of acute treatment between saline-treated and LPS-treated pups was not significant, $F(1,80) = 0.12, p = .74$. There was also no significant F0 diet x acute treatment interaction, $F(1,80) = 0.31, p = .58$.

There were no effects of F0 diet, $F(1,80) = 0.33, p = .56$, or acute treatment, $F(1,80) = 0, p = .99$, or interaction effects between the two variables, $F(1,80) = 0.17, p = .68$, on time spent in the centre (**Figure 3.3B**). For distance travelled, there was a main effect of acute treatment, $F(1,80) = 5.22, p = .02$. LPS-treated ($M = 35.12, SD = 9.48$) pups covered less distance in the open field than saline-treated pups ($M = 39.77, SD = 8.94$). There was no main effect of F0 diet, $F(1,80) = 0.01, p = .91$, or interaction, $F(1,80) = 3.1, p = .08$ (**Figure 3.3C**).

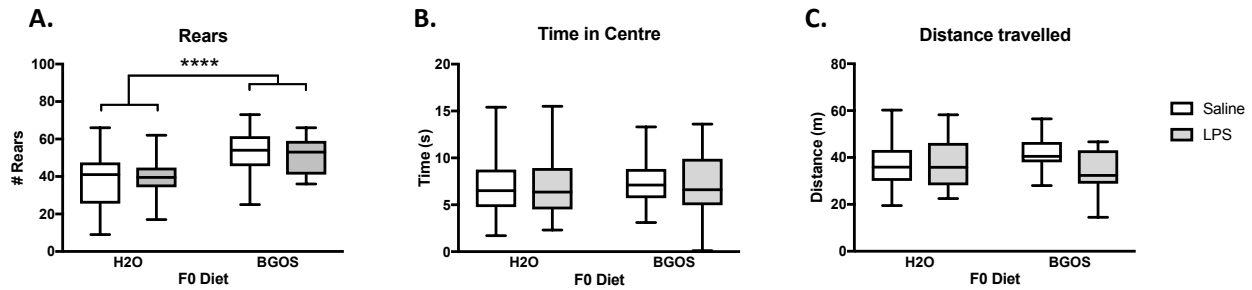


Figure 3.3. Maternal probiotic increases rearing in pups in the OFT. **A)** A significant main effect of maternal diet showed that maternal BGOS supplementation increased rearing in offspring. There was no effect of F0 BGOS on **B)** time in centre or **C)** distance travelled, but a significant main effect of acute treatment ($p < .05$) indicated that acute LPS reduced distance travelled. There were no two-way or three-way interactions between F0 diet or acute treatment and sex, so data from male and female pups were consolidated. Two-way ANOVA, *** $p < .001$, $N = 20-22$.

3.3.2 Maternal BGOS reduces anxiety-like behaviour in the LDB

Number of entries into the light side, number of rears, latency to enter the light (s), and total time spent in the light (s) were evaluated in offspring. Because latency to enter light and time in light data failed to meet the assumptions of homogeneity of variances and normality of residuals, the robust two-way ANOVA was used for these data. For all outcomes, with the exception of time in light, there were no interactions with sex.

There was a significant main effect of F0 diet on entries into the light, $F(1, 80) = 9.07$, $p < .01$. BGOS pups ($M = 11.08$, $SD = 0.72$) crossed over to the light side significantly more than H₂O pups ($M = 8.41$, $SD = 0.57$). There were no effects of acute treatment, $F(1, 80) = 1.06$, $p = .004$, or interaction, $F(1, 80) = 0.01$, $p = .91$ (**Figure 3.4A**). Similarly, F0 diet had a significant effect on rearing in the LDB, $F(1, 80) = 15.36$, $p < .001$. Offspring of BGOS-fed dams ($M = 24.88$, $SD = 1.44$) reared in the light significantly more than pups of dams given water ($M = 16.68$, $SD = 0.25$). Again, there were no effects of LPS, $F(1, 80) = 0.16$, $p = .69$, or interaction effects, $F(1, 80) = 0.33$, $p = .57$ (**Figure 3.4B**). According to the robust two-way ANOVA, there were no effects of

F0 diet, $Q = 2.27$, $p = .14$, acute treatment, $Q = 0.63$, $p = .43$, or interaction, $Q = 0.38$, $p = .54$ on latency to enter the light (**Figure 3.4C**).

Time in light was the only outcome where an F0 diet x acute treatment x sex interaction was detected, $Q = 5.55$, $p = .03$, with the robust three-way ANOVA. There was also an overall main effect of F0 diet, $Q = 6.39$, $p = .02$. The effects of sex, $Q = 0.21$, $p = .66$, acute treatment, $Q = 1.12$, $p = .30$, F0 diet x sex, $Q = 0$, $p = .93$, F0 diet x acute treatment, $Q = 0.29$, $p = .60$, and sex x acute treatment, $Q = 2.13$, $p = .16$, were not significant. Male and female data were then analysed separately with robust two-way ANOVAs. In males, there were no main effects of F0 diet, $Q = 3.45$, $p = .09$, or acute treatment, $Q = 3.70$, $p = .08$, and no interaction effect, $Q = 1.92$, $p = .20$ (**Figure 3.4D**). Similarly, in females, there were no main effects of F0 diet, $Q = 3.01$, $p = .11$, or acute treatment, $Q = .07$, $p = .80$, and no interaction effects, $Q = 3.67$, $p = .08$ (**Figure 3.4E**).

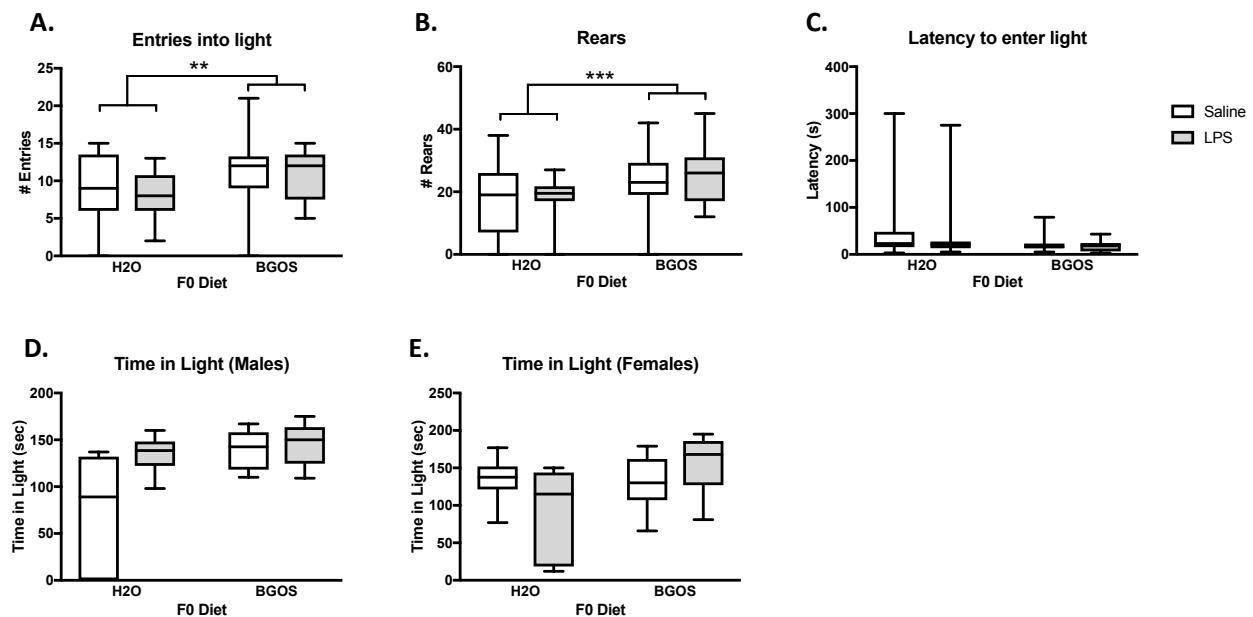


Figure 3.4 Maternal prebiotic intake increases explorations in the light side of the LDB. Significant main effects of maternal diet showed that F0 BGOS increased **A)** entries into the light and **B)** rearing in the light side. **C)** There was no effect of maternal BGOS on latency to enter the light. Because a sex x diet x treatment interaction was detected for time in light, data were plotted for **D)** males and **E)** females separately. No effect of time in light was detected.

Two-way ANOVA (parametric or based on trimmed means for nonparametric data), ** $p < .01$, *** $p < .001$. N = 10–11 males/females; N = 20–22 total.

3.3.3 F0 BGOS reduces floating behaviour in the FST in offspring

There were no interactions between F0 diet or acute treatment and sex. Latency to float (s) and time floating (s) data were square root-transformed to meet the criteria for the two-way ANOVA, and square root-transformed means and standard deviations are provided. There was a main effect of F0 diet on latency to float, $F(1, 80) = 7.16, p < .01$. The latency to float was longer in BGOS pups ($M = 7.39, SD = 0.83$) than in H₂O pups ($M = 5.35, SD = 0.05$). There was no effect of LPS, $F(1, 80) = 0.52, p = .47$, and no interaction, $F(1, 80) = 0.67, p = 0.42$ (**Figure 3.5A**).

Similarly, there was a main effect of F0 diet on total time floating, $F(1, 80) = 11.69, p < .001$. Maternal BGOS resulted in significantly less floating behaviour ($M = 5.39, SD = 1.40$) than pups from dams that received water ($M = 7.54, SD = 0.23$). There was no effect of LPS, $F(1, 80) = 3.34, p = .07$, and no interaction, $F(1, 80) = 1.75, p = .19$ (**Figure 3.5B**).

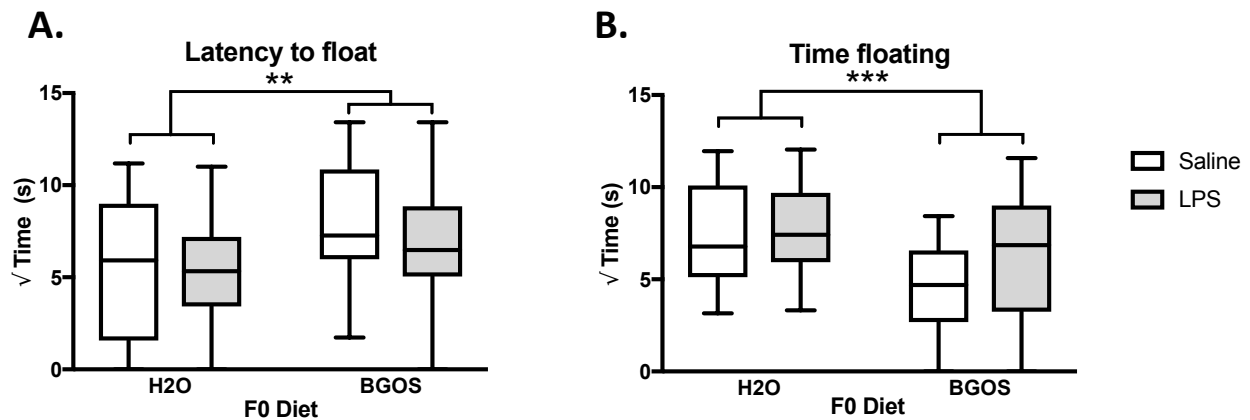


Figure 3.5. Maternal prebiotic reduces passive stress-coping behaviour in the FST. Significant main effects of maternal diet showed that BGOS pups demonstrated **A**) increased latency to the first bout of floating and **B**) reduced time floating. Two-way ANOVA, ** $p < .01$, *** $p < .001$. N = 10–11 males/females; N = 20–22 total.

3.3.4 Maternal prebiotic intake and acute LPS alter NMDAR subunit, BDNF, and 5-HT_{2A} expression in the offspring brain

3.3.4.1 Prefrontal cortex

There were no interactions between F0 diet or acute treatment and sex on gene expression. Maternal BGOS reduced expression of all three of the NMDAR subunits measures. Means and standard deviations of relative expression are given. There was a main effect of F0 diet on GluNR1 expression in the PFC, $F(1, 80), p < .01$. There was significantly less expression in BGOS pups ($M = 0.88, SD = 0.02$) than in H₂O pups ($M = 1.00, SD = 0.03$). There were no effects of acute treatment, $F(1, 80) = 0.60, p = .44$, or interaction, $F(1,80) = 0.06, p = .82$ (**Figure 3.6A**). Similarly, GluNR2A expression was significantly decreased in pups of BGOS-fed dams ($M = 0.83, SD = 0.013$) compared to offspring of dams that received water ($M = 0.99, SD = 0.014$), $F(1, 78) = 19.17, p < .0001$. There was no interaction, $F(1,78) = 0.28, p = .60$, or effect of LPS, $F(1, 78) = 0, p < .99$ (**Figure 3.6B**). Finally, GluNR2B expression was reduced in BGOS pups ($M = 0.83, SD = 0.018$) compared to H₂O pups ($M = 1.00, SD = 0.013$), $F(1, 80) = 12.35, p < .001$. There was no interaction, $F(1, 80) = 0, p = .95$, or effect of acute treatment, $F(1, 80) = 0.2230, p = .36$, on GluNR2B expression (**Figure 3.6C**).

Maternal BGOS reduced 5-HT_{2A} mRNA ($M = 0.92, SD = 0.16$) compared to pups from dams given drinking water ($M = 1.00, SD = 0.23$), $F(1, 80) = 6.39, p = .01$. The interaction, $F(1, 80) = 0.45, p = .50$, and main effect of acute treatment, $F(1, 80) = 0.73, p = .40$, were not significant (**Figure 3.6D**). There were no effects of F0 diet, $F(1, 78) = 2.42, p = .12$, acute LPS, $F(1, 78) = 3.24, p = .08$, or interaction effects, $F(1, 78) = 2.22, p = .73$, on BDNF expression (**Figure 3.6E**). According to the robust two-way ANOVA, there were no significant main effects of F0 diet, $Q =$

0.19, $p = .67$, treatment, $Q = 1.90$, $p = .18$, or interaction, $Q = 1.57$, $p = .22$, on TNF- α (**Figure 3.6F**).

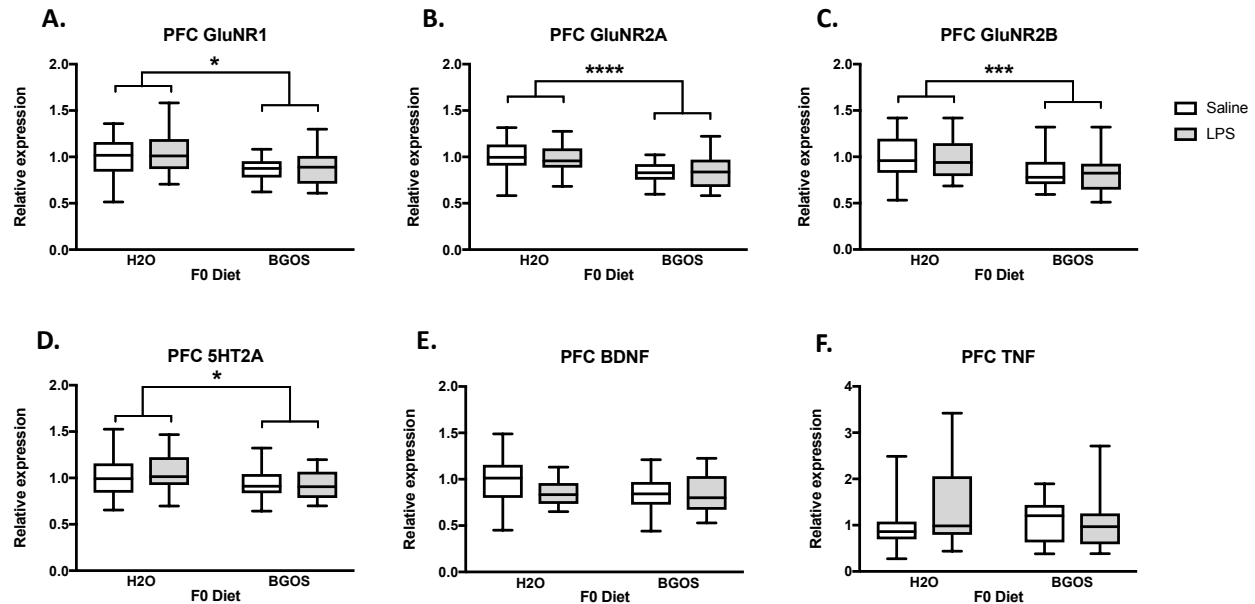


Figure 3.6. Maternal prebiotic intake altered expression of key genes in the PFC in pups. Significant main effects of maternal diet indicated that F0 BGOS during pregnancy and suckling reduced expression of the NMDAR subunits **A)** GluNR1, **B)** GluNR2A, and **C)** GluNR2B as well as **D)** 5-HT_{2A} in the PFC in offspring. There were no effects of maternal diet on **E)** BDNF or **F)** TNF- α . Two-way ANOVA (parametric or based on trimmed means for nonparametric data), * $p < .05$, *** $p < .001$, **** $p < .0001$. N = 10–11 males/females; N = 20–22 total.

3.3.4.2 Hippocampus

There were no interactions between F0 diet or acute treatment and sex on gene expression. In the hippocampus, both F0 BGOS and acute LPS reduced NMDAR subunits. There were significant main effects of F0 diet, $F(1, 76) = 15.85$, $p < .001$, and acute treatment, $F(1, 76) = 7.38$, $p < .01$, on GluNR1 mRNA. Expression was significantly lower in BGOS pups ($M = 0.75$, $SD = 0.05$) than H₂O pups ($M = 0.88$, $SD = 0.09$). Acute LPS also resulted in significantly less GluNR1 mRNA ($M = 0.76$, $SD = 0.15$) compared to saline ($M = 0.86$, $SD = 0.18$). The F0 diet x acute treatment interaction effect on GluNR1 was not significant, $F(1, 76) = 0.77$, $p = .38$ (**Figure 3.7A**).

GluNR2A was also reduced in BGOS pups ($M = 0.79$, $SD = 0.09$) compared to H₂O pups ($M = 0.95$, $SD = 0.08$) and LPS-treated ($M = 0.80$, $SD = 0.20$) compared to saline-treated pups ($M = 0.93$, $SD = 0.24$). Main effects of both F0 diet, $F(1, 76) = 10.89$, $p < .01$, and acute treatment, $F(1, 76) = 6.60$, $p = .01$, were significant. There was no interaction, $F(1, 76) = 0.04$, $p = .83$, (**Figure 3.7B**). Finally, there were significant main effects of F0 diet, $F(1, 77) = 12.91$, $p < .001$, and acute treatment, $F(1, 77) = 8.76$, $p < .01$, on GluNR2B mRNA. Expression was lower in BGOS pups ($M = 0.76$, $SD = 0.12$) than in H₂O pups ($M = 0.94$, $SD = 0.09$). LPS ($M = 0.77$, $SD = 0.22$) also resulted in lower GluNR2B mRNA levels than saline ($M = 0.92$, $SD = 0.25$). There was no F0 diet x acute treatment interaction, $F(1, 77) = 0.15$, $p = .70$ (**Figure 3.7C**).

F0 BGOS reduced 5-HT_{2A} in pups ($M = 0.78$, $SD = 0.003$) compared to H₂O pups ($M = 0.91$, $SD = 0.13$), $F(1,77) = 6.92$, $p = .01$. There was no effect of acute treatment, $F(1,77) = 2.78$, $p = .10$ on hippocampal 5-HT_{2A} expression. The interaction was also not significant, $F(1,77) = 3.07$, $p = .10$ (**Figure 3.7D**). There was a significant effect of acute treatment on BDNF expression, $F(1, 77) = 16.71$, $p < .001$, but no effect of F0 diet, $F(1, 77) = 1.03$, $p = .31$, and no interaction, $F(1, 77) = 0.13$, $p = .72$. Lower BDNF expression was found in pups after LPS ($M = 0.76$, $SD = 0.23$) compared to saline ($M = 0.98$, $SD = 0.25$, **Figure 3.7E**). Based on the robust two-way ANOVA, there were no effects of F0 diet, $Q = 0.11$, $p = .75$, or treatment, $Q = 1.89$, $p = .18$, or interaction effects, $Q = 0.80$, $p = .38$, on TNF- α expression (**Figure 3.7F**).

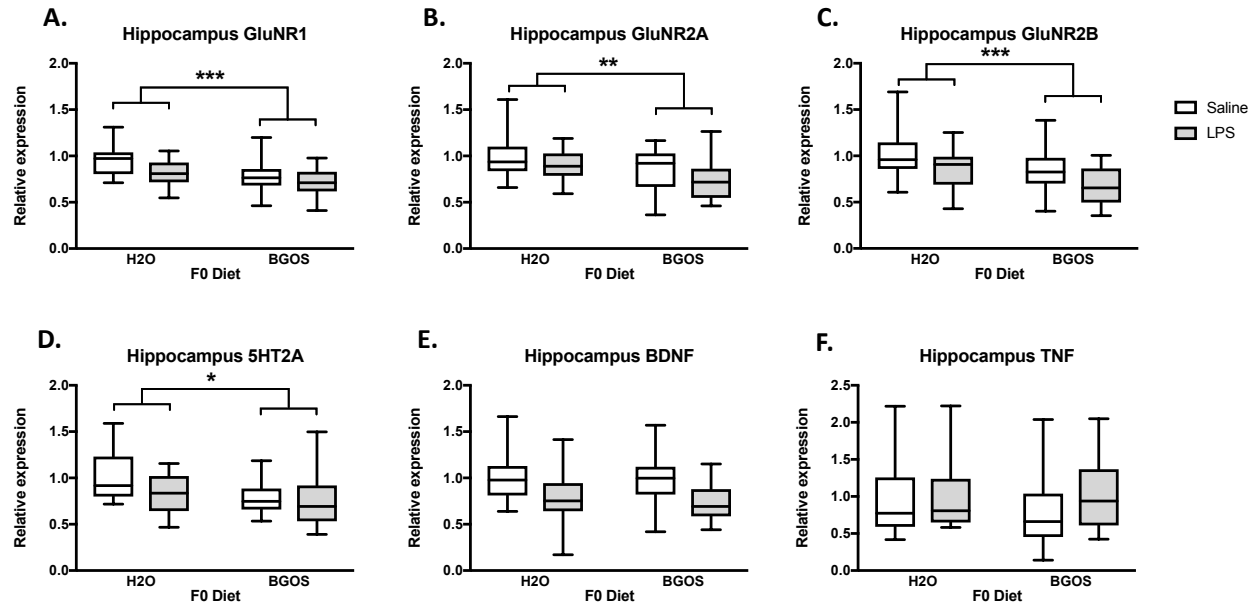


Figure 3.7. Maternal BGOS intake alters expression of key genes in the hippocampus in progeny. Significant main effects of F0 diet showed that maternal BGOS reduced expression of **A)** GluNR1. Both F0 BGOS and acute LPS reduced expression of **B)** GluNR2A and **C)** GluNR2B. **D)** F0 BGOS reduced 5-HT_{2A}, while significant main effects of acute treatment indicated that **E)** acute LPS reduced BDNF ($p < .001$). **F)** Neither maternal diet nor acute LPS had any effect on TNF- α in pups. Two-way ANOVA (parametric or based on trimmed means for nonparametric data), * $p < .05$, ** $p < .01$, *** $p < .001$. N = 10–11 males/females; N = 20–22 total.

3.3.5 Behaviour correlates with changes in cortical and hippocampal changes in gene expression

We performed exploratory analysis using Pearson correlations to determine if the changes in behaviour were associated with altered central gene expression (**Figure 3.8**). Rears in the OFT correlated with the most genes. There were moderate but significant negative correlations between number of rears and PFC GluNR2A ($r(82) = -0.22, p = .04$), PFC 5-HT_{2A} ($r(82) = -0.23, p = .03$), and hippocampal GluNR1 ($r(82) = -0.22, p = .04$). In addition, distance travelled in the OFT negatively correlated with hippocampal BDNF ($r(79) = -0.22, p = .04$). Finally, time spent in the light of the LDB negatively correlated with hippocampal GluNR2A ($r(77) = -0.24, p = .03$) and GluNR2B ($r(78) = -0.24, p = .04$).

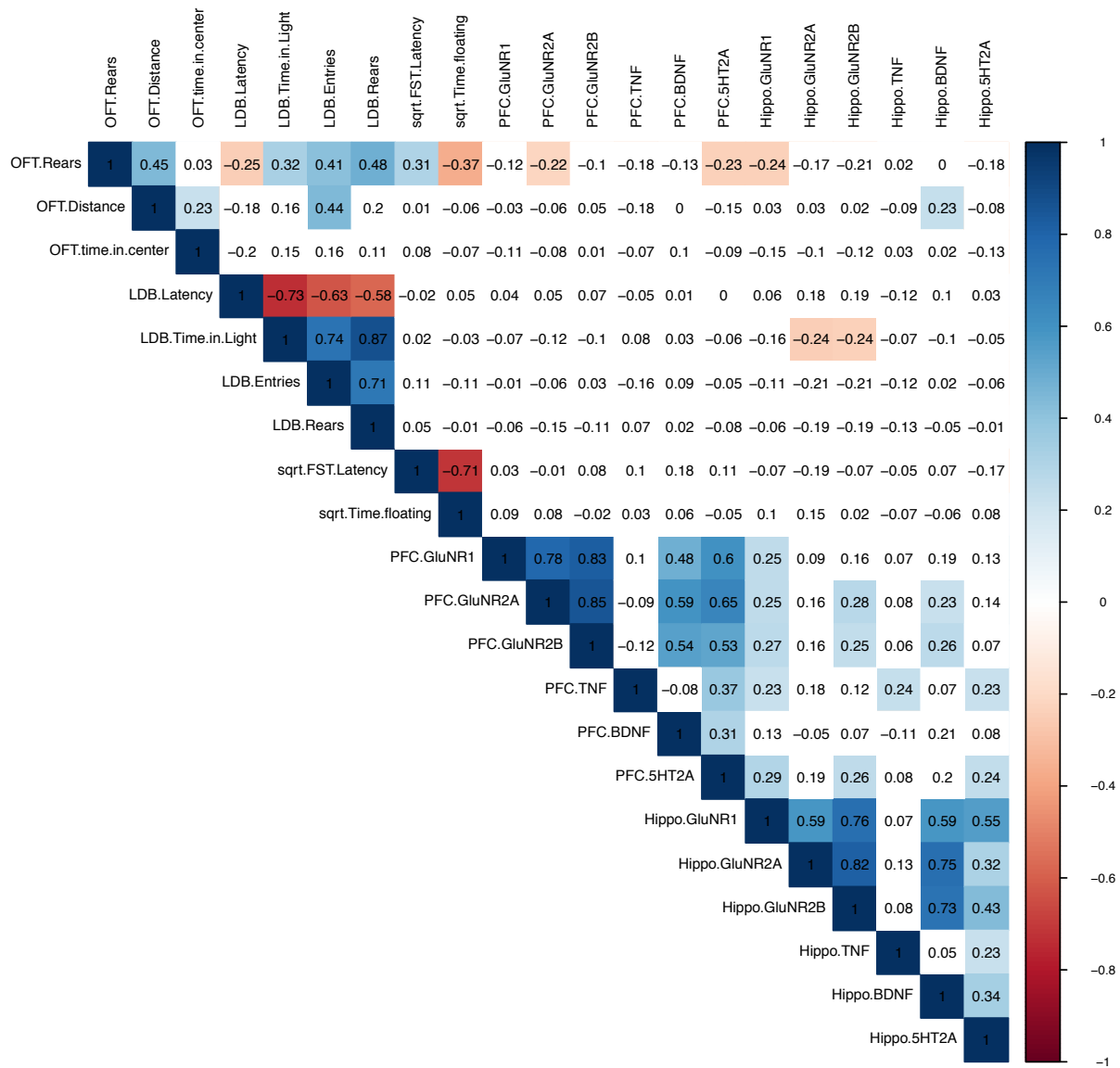


Figure 3.8. Behaviour correlates with some gene expression patterns. A correlation matrix of behavioural and central gene expression data is shown. Each square contains Pearson product-moment correlations. The coloured squares correspond to significant correlations, and the colour indicates the direction and magnitude of the correlation according to the scale on the right. N = 10–11 males/females; N = 20–22 total.

3.4 Experiment 3B design: Effects of prenatal and postnatal maternal BGOS on offspring behaviour

The design of experiment 3B is shown in **Figure 3.9**. After observing altered behavioural patterns in offspring of BGOS-fed mice, a cross-fostering design was employed to differentiate

between effects of prenatal and postnatal maternal BGOS intake on offspring behaviour. Mating pairs were arranged, and dams were randomly assigned to receive plain water or water supplemented with BGOS on E1. Feeding continued through gestation and pregnancy as in experiment 3A. Shortly after birth, however, half of the litters were cross-fostered to a dam of the opposite treatment. Entire litters were swapped. This resulted in four groups: pups that were born to and suckled by an H₂O dam (H₂O/H₂O); pups born to an H₂O dam and suckled by a BGOS dam (H₂O/BGOS); pups born to a BGOS dam and suckled by an H₂O dam (BGOS/H₂O); and pups born to and suckled by a BGOS dam (BGOS/BGOS). The OFT and LDB were then performed between P22–P23. Because no two- or three-way interactions with sex were identified, two-way ANOVAs were run with F0 prenatal diet and F0 postnatal diet as the independent variables (N = 15–16 males and N = 15–16 females; N = 31–32 total).

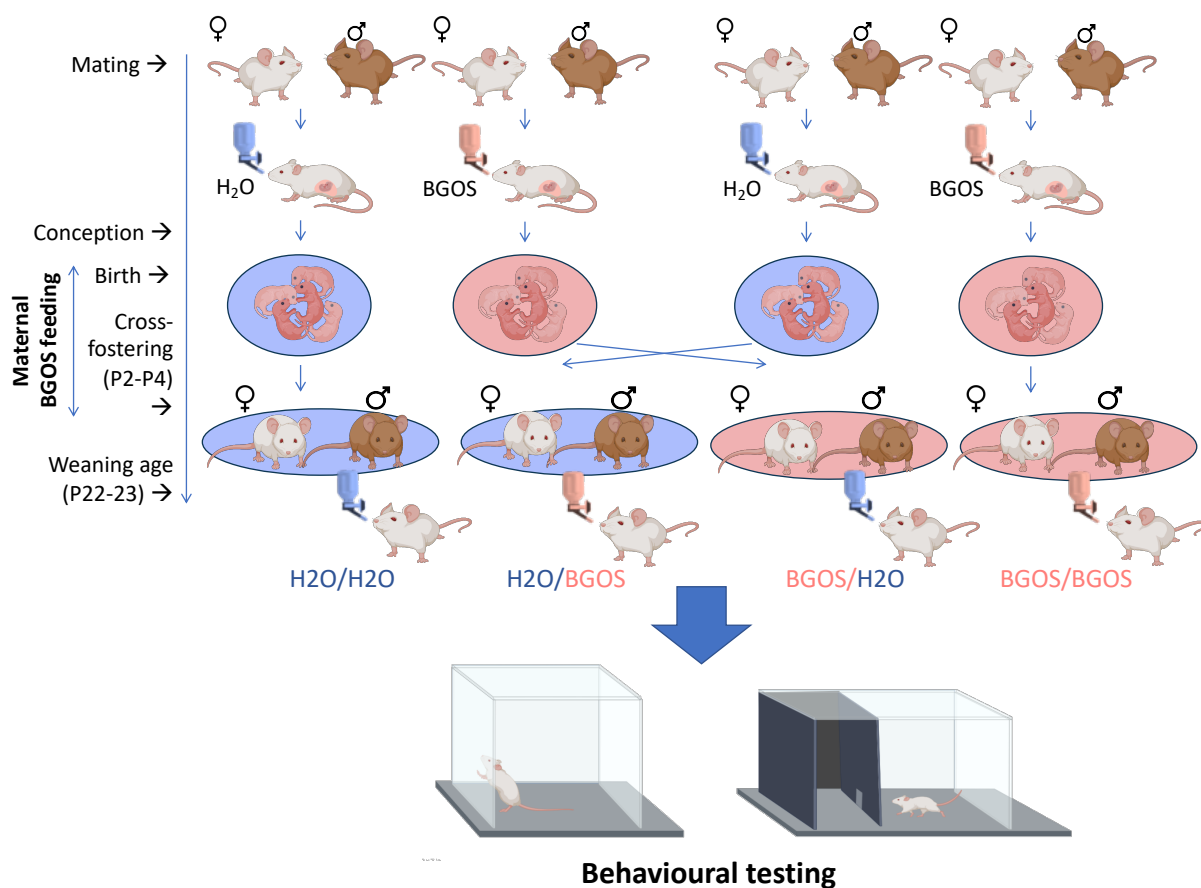


Figure 3.9. Diagram of experiment 3B.

3.5 Experiment 3B results

3.5.1 Both prenatal and postnatal maternal prebiotic increase exploratory behaviour in the OFT

In the OFT, there were significant main effects of both F0 prenatal diet, $F(1, 123) = 26.29$, $p < .0001$, and F0 postnatal diet, $F(1, 123) = 38.09$, $p < .0001$. Pups whose mothers received BGOS, regardless of whether they were cross-fostered (BGOS/H₂O and BGOS/BGOS, $M = 58.52$, $SD = 13.70$), reared more overall than pups whose mothers received plain water (H₂O/H₂O and H₂O/BGOS, $M = 48.66$, $SD = 11.06$). In addition, pups suckled by a dam given BGOS, regardless of their maternal diet *in utero* (H₂O/BGOS and BGOS/BGOS, $M = 59.64$, $SD = 7.53$), reared more than pups suckled by dams given water (BGOS/H₂O and BGOS/BGOS, $M = 47.64$, $SD = 6.56$).

There was no interaction effect, $F(1, 123) = 0.12, p = .72$ (**Figure 3.10A**). There were no effects of prenatal F0 diet, $F(1, 123) = 0.25, p = .62$, postnatal F0 diet, $F(1, 123) = 3.05, p = .08$, or interactions, $F(1, 123) = 0.03, p = .86$, on distance travelled (**Figure 3.10B**). Based on the robust two-way ANOVA, there were no effects of F0 postnatal diet, $Q = 1.15, p = .29$ or F0 prenatal diet, $Q = 0.88, p = .35$, or interaction effects, $Q = 3.68, p = .06$ (**Figure 3.10C**).

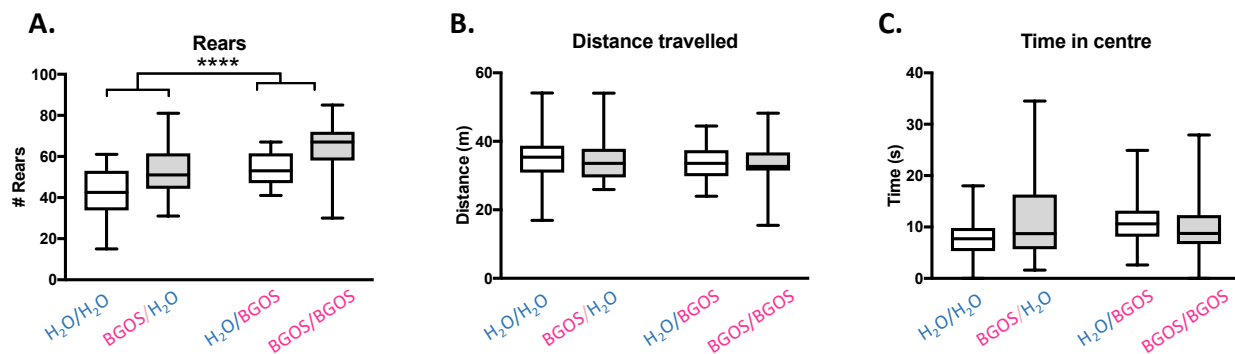


Figure 3.10. Both prenatal and postnatal maternal BGOS supplementation increased rearing in the OFT. A) Significant main effects of postnatal F0 diet showed pups that were weaned to a BGOS dam (H₂O/BGOS and BGOS/BGOS) exhibited increased rearing in the OFT compared to those weaned to an H₂O dam, regardless of their birth mother. Similarly, significant main effects of prenatal F0 diet showed that pups of BGOS dams (BGOS/H₂O and BGOS/BGOS) reared more than pups from H₂O dams ($p < .0001$). There were no differences in **B)** distance travelled or **C)** time in centre. Two-way ANOVA (parametric or based on trimmed means for nonparametric data), **** $p < .0001$. N = 15–16 males/females; N = 31–32 total.

3.5.2 Maternal postnatal, but not prenatal, BGOS intake reduces anxiety-like behaviour in the LDB

All LDB outcomes, apart from entries into the light side, failed the normality and/or heteroscedasticity tests. Based on the robust two-way ANOVA, there was a significant main effect of F0 postnatal diet on time in light, $Q = 9.18, p < .01$. There were no effects of prenatal diet, $Q = 0.52, p = .48$, or interaction effects, $Q = 3.07, p = .09$ (**Figure 3.11A**). Likewise, there was a main effect of F0 postnatal diet on rears in the light side, $Q = 8.99, p < .01$, but no effect of F0 prenatal diet, $Q = 0.32, p = .57$, or interaction effects, $Q = 3.55, p = .06$ (**Figure 3.11B**). There were no

effects of F0 postnatal diet, $F(1, 124) = 1.66, p = .20$, or F0 prenatal diet, $F(1, 124) = 0.18, p = .67$, or interaction effects, $F(1, 124) = 0.15, p = .70$, on entries into the light (**Figure 3.11C**). Latency to enter the light was also not affected by F0 postnatal diet, $Q = 2.83, p = .10$, F0 prenatal diet, $Q = 1.58, p = .22$, or an interaction, $Q = 3.63, p = .06$ (**Figure 3.11D**).

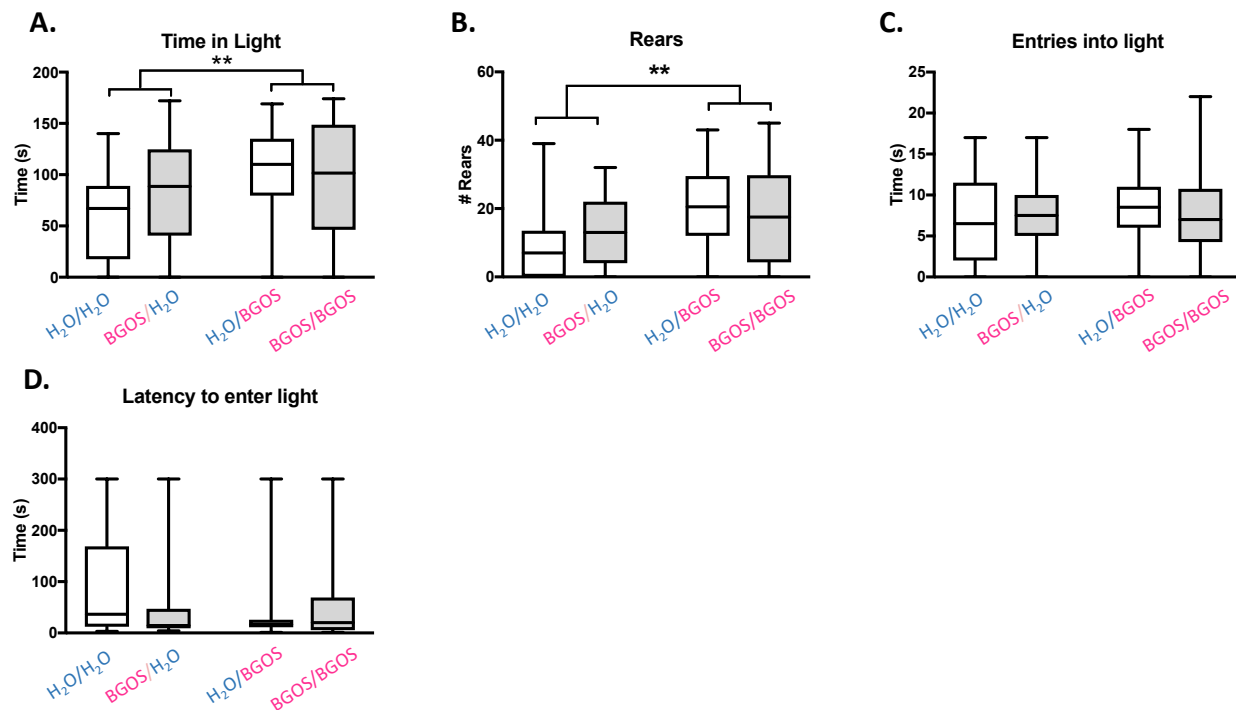


Figure 3.11. Maternal BGOS during suckling, but not *in utero*, reduces anxiety-like behaviour in the LDB. Significant main effects of postnatal F0 diet showed that early postnatal maternal BGOS increased **A**) time in light and **B**) rearing in the light. Neither prenatal nor postnatal maternal BGOS feeding changed **C**) entries or **D**) latency into the light. Two-way ANOVA (parametric or based on trimmed means for nonparametric data), ** $p < .01$. $N = 15$ – 16 males/females; $N = 31$ – 32 total.

3.6 Experiment 3C design: Effects of BGOS on maternal care behaviour

A final experiment was performed to assess whether BGOS alters maternal behaviour. Dams were randomly assigned to BGOS or plain water at E1 as in previous studies. At P2 and P4, home cage maternal care behaviour was scored. After the home cage observation period, the pup retrieval test was performed. Unpaired student's *t*-tests were performed to test the hypothesis that

BGOS supplementation has no effect on maternal care behaviour. To minimise the possibility of a Type II error (concluding BGOS does not influence maternal care when there is in reality a significant effect), robust methods and corrections for multiple comparisons were not used ($N = 5-6$).

3.7 Experiment 3C results

3.7.1 Prebiotic intake has no effect on maternal care behaviour

There were no effects of BGOS on frequency of maternal nursing, $t(9) = 1.22, p = .25$, nest building, $t(10) = 0.61, p = .56$, licking/grooming, $t(10) = 0.42, p = .68$, or being away from the nest, $t(9) = 1.4, p = .19$, in the home cage (**Figure 3.12A**). In the pup retrieval test, there were also no differences between BGOS and plain water on latency to retrieve the first pup, $t(10) = 1.41, p = .19$, or all pups, $t(10) = 2.09, p = .06$ (**Figure 3.12B**).

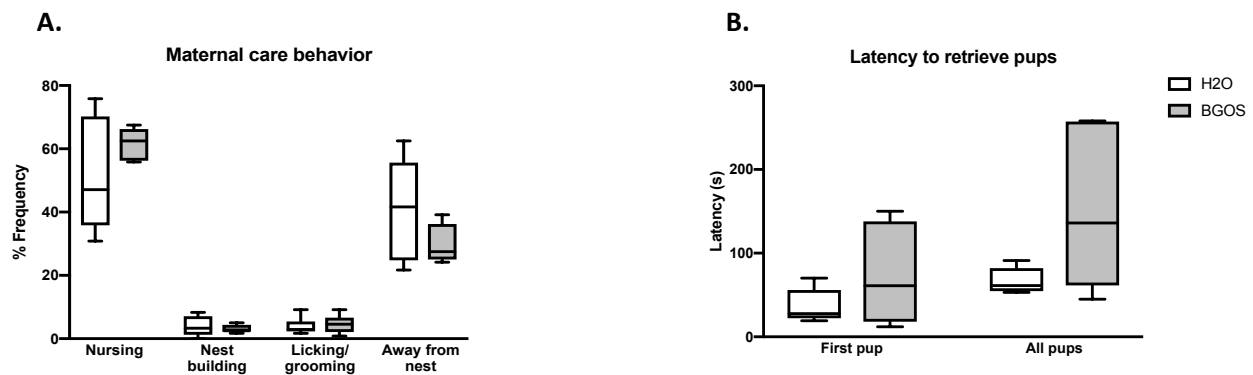


Figure 3.12. Maternal BGOS has no effect on maternal care behaviour. Prebiotic supplementation did not change **A)** frequency of maternal care behaviours or **B)** latency to retrieve pups. Student's unpaired t-test, $N = 5-6$.

3.8 Discussion

3.8.1 Summary of results

Although our group has previously reported the impact of BGOS on the brain and behaviour in adult rodents, the influence of BGOS intake during gestation and suckling on pups

has not been explored. I tested whether maternal prebiotic intake alters offspring behaviour and central gene expression. I also explored the relative contributions of prenatal and postnatal maternal BGOS supplementation on offspring behaviour. Finally, I confirmed whether BGOS influences maternal care behaviour. I show that my hypothesis was correct – maternal BGOS feeding increased exploration and reduced anxious and passive stress-coping behaviour. F0 BGOS also altered expression of NMDAR subunits, BDNF, and 5-HT_{2A} in the offspring brain. Surprisingly, acute LPS had minimal effects on offspring behaviour. Although LPS induced changes in gene expression in the hippocampus, it did not increase expression of the pro-inflammatory cytokine TNF- α . Finally, prenatal and postnatal BGOS had differential effects on offspring behaviour, and these effects were not mediated by changes in maternal care behaviour.

3.8.2 Maternal BGOS, but not acute LPS, alters offspring behaviour

In experiment 3A, we report that BGOS supplementation during pregnancy and suckling increased exploration in the OFT, reduced innate anxiety in the LDB, and reduced behavioural despair in the FST. To date, no studies have been published on the influence on prebiotic intake on offspring behaviour. One study has tested the effects of maternal probiotic administration on behaviour in a maternal immune activation mouse model of autism. Administration of a probiotic containing *Bifidobacterium* and *Lactobacillus* strains during pregnancy and suckling rescued abnormal social and anxiety-like behaviour in adult offspring and pro-inflammatory cytokines in foetal brains (Wang et al., 2019). The tendency towards increased exploration and reduced anxious behaviour agree with the findings in this chapter. The effects of maternal prebiotic feeding on adult offspring will be explored in Chapter 5.

There were minimal effects of acute LPS on behaviour 24 hours post-injection. Most papers have reported increased anxiety in the LDB and immobility in the FST the day after a single

I.P. injection of LPS at similar doses (Couch et al., 2015; O'Connor et al., 2009; Zhao et al., 2016). Some studies, however, have reported minimal or no changes in behaviour after 24 hours (Biesmans et al., 2013; Wang et al., 2011). The inconsistencies may be due to discrepancies in the potency of different batches of LPS. This might also be due to publication bias, given that researchers are unlikely to publish data on null effects of LPS. We did not expect to see large differences in offspring behaviour without a challenge to induce abnormal behaviour. Finding robust F0 BGOS-mediated changes in healthy mice, however, provides strong evidence that there are differences in brain development.

3.8.3 Maternal BGOS and LPS alter expression of NMDAR subunits, 5-HT_{2A}, and BDNF, and these changes modestly correlate with behaviour

In the PFC and hippocampus, maternal BGOS reduced expression of NMDAR subunits. All subunits decreased, suggesting that the total number of NMDARs was downregulated in BGOS pups. NMDAR antagonists, such as ketamine, reduce anxiety and behavioural despair in animal models (Engin, Treit, and Dickson 2009) and have antidepressant effects in humans (Murrough et al. 2013; McGirr et al. 2015). In addition, in our study, exploratory and anxiety-like behaviour negatively correlated with NMDAR subunits in both the PFC and hippocampus. A reduction in NMDAR in offspring following maternal prebiotic intake may cause reduced anxiety in offspring. Furthermore, GOS prebiotics have been shown to alter expression of NMDAR subunits in adult rodents. Although the decrease in NMDAR subunits here is in contrast to the increase in NMDAR subunits found in Savignac et al. (2013), it is consistent with other studies reporting reduced mRNA and protein after GOS intake (Burokas et al., 2017; Savignac et al., 2016). The results in this chapter show that the effects of GOS on NMDAR expression are multigenerational.

Maternal BGOS also reduced 5-HT_{2A} mRNA in the PFC and hippocampus. This is consistent with a study showing that BGOS reduced 5-HT_{2A} in mice treated with LPS 24 hours post injection (Savignac et al., 2016). Although there were no associations between anxious behaviour in the LDB or behavioural despair in the FST, there was a modest negative correlation between rears in the OFT and 5-HT_{2A} in our study. The downregulation of 5-HT_{2A} may be related to the increase in exploratory behaviour.

Surprisingly, acute LPS had no effect on TNF- α expression. This disagrees with our pilot study in which LPS increased PFC TNF- α 24 hours post injection in adult CD1 mice. This elevation is believed to contribute to the long-lasting changes in behaviour. Pro-inflammatory signals, for example, activate brain indoleamine-2,3-dioxygenase, which metabolises kynurenine to quinolinic acid. Quinolinic acid is an NMDAR agonist that is neurotoxic in large quantities that has been associated with depression symptoms and may link LPS to abnormal behaviour in animal models (Heisler and O'Connor 2015; Raison et al. 2010). Neither the expected elevation of central TNF nor the behavioural deficits, however, occurred here. In addition, based on previous studies, I had hypothesised that LPS would increase 5-HT_{2A} (Couch et al., 2015; Savignac et al., 2016). The absence of an effect of acute LPS on 5-HT_{2A} expression and behaviour, may be due to the lack of a pro-inflammatory response in the brain (Couch et al., 2013; Weisstaub et al., 2006).

Although there were no changes in TNF- α , acute LPS decreased expression of NMDAR subunits in the hippocampus. It is interesting that both maternal BGOS and LPS were associated with reduced hippocampal NMDAR expression. Because quinolinic acid, an NMDAR agonist, increases following LPS (A. K. Walker et al. 2013), there may be a compensatory decrease in NMDAR subunit expression. The relationships between inflammation, the gut microbiome and microbial metabolites, and central NMDAR should be investigated further. Acute LPS also

reduced hippocampal BDNF, which is consistent with previous studies (Guo et al., 2014; Li et al., 2017; Martin et al., 2013; Sriram et al., 2016; Zhao et al., 2017). There were no effects of maternal BGOS, however, on cortical or hippocampal BDNF and minimal correlations between BDNF expression and behaviour. This suggests that changes in BDNF were not related to the altered behavioural outcomes. In the hippocampus, quantifying BDNF expression in different subfields may reveal expression differences that are hidden when whole hippocampi are homogenised.

It should be noted that the FST was run before collecting tissue, and this may have influenced gene expression patterns. Acute forced swim stress has been shown to alter hippocampal and PFC gene expression, including expression of genes associated with glutamatergic synaptic transmission (Bohacek et al. 2015; Floriou-Servou et al. 2018; Yuen et al. 2011). Therefore, future studies that attempt to replicate gene expression changes would need to incorporate acute forced swim stress. Although this limits the generalisation of the gene expression findings to non-stressed mice, all pups in this study underwent the same stressor with the same amount of time between testing and tissue collection, which supports the internal validity of this experiments. In addition, other brain regions are involved in these behaviours beyond the PFC and hippocampus, including the amygdala, hypothalamus, pituitary, locus caeruleus, and bed nucleus of the stria terminalis (Walker, Miles, and Davis 2009; Bruchas et al. 2009; Tanaka et al. 2000; Gray 1993; Frenois et al. 2007), and should be explored in future studies.

A correlation matrix was used to explore associations between behaviour and gene expression. The significant correlations between OFT rears, OFT distance travelled, and LDB time in light with relative gene expression ranged from -0.25 to 0.25 , which are modest correlations. The p values were also close to the threshold of $.05$, and given the large number of comparisons, these correlations should be interpreted with caution. Although we were primarily interested in

correlations between behaviour and gene expression, the correlation matrix revealed interesting associations between behavioural outcomes. Outcomes within the same behavioural tests strongly correlated with each other. In the LDB, for example, the four outcomes, which are all measures of innate anxiety, correlated with each other. Although not all outcomes passed the threshold for a significant main effect of F0 diet in the two-way ANOVAs (entries into the light and rears), there were trends towards reduced anxiety in the parameters that were not significant (time in the light and latency to enter the light). The strong correlations among behaviours in the LDB reinforce the conclusion that maternal BGOS reduces innate anxiety. It also demonstrates the importance of measuring multiple parameters in each behavioural test.

Interestingly, rears in the OFT correlated with nearly every behavioural outcome. Maternal BGOS also had the strongest effect on rears in both experiment 3A and 3B based on *F* scores. This suggests that rearing in the OFT is an informative and reproducible behavioural measure.

Several mechanisms likely mediate the relationship between maternal prebiotic and central gene expression and behaviour in offspring. Microbe-derived compounds, transferred either from the mother or offspring gut microbiota, are good candidates. The short-chain fatty acids (SCFAs) propionate and butyrate, for example, negatively correlate with hippocampal GluNR2A and GluNR2B, respectively (Burokas et al. 2017). Administration of SCFAs produced similar behavioural phenotypes to those reported in this chapter (Yamawaki et al. 2018; van de Wouw et al. 2018). I will explore correlations between microbes, bacterial metabolites, central gene expression, and behaviour in Chapter 6.

Every behavioural and gene expression outcome was tested in a three-way ANOVA first for interactions with sex. Because we were primarily interested in F0 diet x LPS interactions and main effects, data were consolidated for two-way ANOVAs when there were no interactions with

sex. With the exception of time spent in the light side of the LDB, there were no other significant differences due to sex. More sex differences were detected in adult male and female offspring of prebiotic-fed dams, so the effect of gender might be more substantial in adulthood. This will be explored in Chapter 5.

3.8.4 Prenatal and postnatal maternal BGOS intake both influence offspring behaviour

Both prenatal and postnatal prebiotic feeding increased rearing in the OFT. These relationships were highly significant. Although prenatal BGOS did not significantly affect anxiety behaviour in the LDB, it is possible that its effect was too small to register at this level of statistical power. The mean time in light, rears, and entries were higher in the BGOS/H₂O group than the H₂O/H₂O group, although these differences did not reach statistical significance. In addition, the correlation matrix showed that rears in the OFT are associated with anxiety in the LDB. I conclude from these results that both prenatal and postnatal BGOS intake influence behaviour, although the effects of postnatal maternal prebiotic are more robust. Therefore, postnatal factors (exposure to the maternal gut microbiome and bacterial metabolites and breastmilk) are primarily responsible for variation in offspring behaviour.

Importantly, the findings in the cross-fostered cohort show that my initial behavioural findings were reproducible. In each study, only 3–4 litters were used per group, and this is one limitation of this study. Animals from the same litter are more similar to each other than pups from other dams. I could have improved this experimental design by using more litters and fewer animals per litter. Nevertheless, the replication of behavioural findings in the cross-fostering study suggests that my results are robust. In addition, results of the mixed-effects analysis that accounts for litter as a random effect can be found in **Appendix C**. Most of the behavioural findings remained significant or close to significant after accounting for litter effects.

3.8.5 BGOS does not affect maternal care behaviour

BGOS did not alter frequency of maternal behaviours in the home cage or performance in the pup retrieval test. Maternal care behaviour is an important factor in offspring behaviour and brain development. It is necessary to account for changes in maternal behaviour in multigenerational studies (Berger et al., 2018; Champagne et al., 2003; Meaney, 2001). This result suggests that the brain and behaviour changes in this study are not mediated by differences in maternal care.

3.8.6 Conclusion

BGOS intake during gestation and suckling increases exploration and reduces anxious and passive stress-coping behaviour in healthy offspring. Both *in utero* and postnatal factors contribute to the variation in behaviour. Maternal BGOS supplementation also produces changes in gene expression, including reduced NMDAR and 5-HT_{2A} expression in the PFC and hippocampus. These results establish that supplementing pregnant and suckling dams with prebiotic influences brain development and behaviour in progeny. The subsequent chapters in the thesis will focus on how maternal prebiotic intake changes peripheral immunity and metabolism, long-lasting effects in adult offspring, and the roles of the maternal and offspring gut microbiomes.

4. Peripheral metabolic and immune effects of maternal prebiotic intake in offspring

4.1 Introduction

The aim of this chapter is to explore whether maternal prebiotic intake influences changes in immune function and the plasma metabolome following an acute LPS challenge in offspring.

4.1.1 Prebiotic intake, the gut microbiome, and plasma metabolites

The gut microbiome is integral to host metabolism locally in the gut and systemically (Nicholson et al. 2012; Holmes et al. 2011). Metabolomics, analysis of the set of metabolites present in an organism, is a useful tool to study the influence of the microbiome and prebiotics on the host in an unbiased manner. Microbial compounds, including short-chain fatty acids (SCFAs), bile acids, branched chain amino acids, and tryptophan and tyrosine metabolites, can be detected in plasma and serum. They have diverse effects on host physiology in the gut and beyond (Pedersen et al. 2016; Golubeva et al. 2017; Swann et al. 2011; Kelly et al. 2016).

Gut bacteria also regulate body mass index (BMI) and energy metabolism, including blood glucose, lipoproteins, and triglycerides (Fu et al. 2015; Velagapudi et al. 2010; Vrieze et al. 2012). While the mechanisms underlying the interaction between the gut microbiome and energy metabolism are unclear, the relationship likely involves microbial compounds, such as bile acids and SCFAs, that bind to farnesoid X receptor (FXR) and free fatty acid receptor 2 (FFAR2) in the intestines and liver and modulate metabolic processes (Ge et al. 2008; Gao et al. 2009; Pars us et al. 2017). Gut bacteria are also involved in extracting energy from food (Turnbaugh et al. 2006; Murphy et al. 2010).

Beneficial effects of prebiotics on glucose and lipid homeostasis have generally been reported (Everard et al. 2011; Delzenne and Williams 2002), although detrimental effects of galacto-oligosaccharides (GOS) on glucose metabolism have also been described, including increased blood glucose levels in a mouse study (Cheng et al. 2018). There have been few studies, however, on the effects of maternal prebiotic supplementation on offspring blood metabolites. One study showed that a high-fibre diet during pregnancy and lactation altered levels of satiety hormones and expression of genes involved in glucose and lipid metabolism and reduced glucose levels in rats. In addition, supplementation of a *Lactobacillus* probiotic during breastfeeding in human subjects altered the serum metabolome in infants, including reduced levels of fatty acids and increased tryptophan (Chorell et al. 2013). The effects of maternal prebiotic intake on the plasma metabolome in offspring, however, are unknown.

4.1.2 The gut microbiome and peripheral immune function

The gut microbiome is an important regulator of macrophage function. Bone marrow-derived macrophages (BMDMs) from aged conventional mice, for example, but not aged germ-free mice, displayed deficits in antimicrobial killing capacity and an enhanced pro-inflammatory response to LPS. This macrophage dysfunction in aged mice was likely due to increased systemic inflammation resulting from microbial dysbiosis and increased intestinal permeability (Thevaranjan et al. 2017). Colonisation of the gut with segmented filamentous bacteria enhanced production of IL-23 in mouse bone marrow-derived dendritic cells in response to a parasite (Burgess et al. 2014). In addition, *in vitro* treatment with the probiotic *Bacillus amyloliquefaciens* enhanced macrophage M1 polarisation and phagocytic ability (Ji et al. 2013). These studies demonstrate the impact of gut microbes on immune cell function, from the progenitor stage in the bone marrow to the differentiated state. Therefore, maternal BGOS may influence BMDM M1 or

M2 polarisation by altering offspring microbiome composition and function. In this chapter, BMDMs were collected from pups to test the effects of maternal prebiotic intake and acute LPS on cellular pro-inflammatory and anti-inflammatory responses.

Exposure to an infection or other immune stimulus can “train” the innate immune system and alter subsequent immune responses. Bone marrow monocytes from post-septic mice, for example, secreted more pro-inflammatory cytokines in response to LPS *in vitro* than controls (Bomans et al. 2018). Similarly, a vaccine augmented TNF- α , IL-1 β , and IFN- γ in peripheral blood mononuclear cells collected from human subjects up to three months after the injection (Kleinnijhuis et al. 2012). Therefore, it is reasonable to expect *in vivo* LPS to augment expression of M1 markers and reduce M2 markers *in vitro* 24 hours post-injection, and if maternal BGOS is anti-inflammatory in offspring, it may reverse this effect.

In addition to immune cell function, gut microbiome composition has also been associated with white blood cell (WBC) counts, including the ratio of neutrophils to lymphocytes in healthy human subjects (Yoon et al. 2018) and WBC counts in bone marrow transplantation patients (Schluter et al. 2019). Acute LPS can also alter WBC levels and composition (Kolomaznik et al. 2014; Yates et al. 2011; Wrotek et al. 2016; Tavares et al. 2006). I expected the pro-inflammatory *in vivo* LPS challenge and maternal BGOS, a potentially anti-inflammatory treatment, to have opposing effects on offspring WBC counts and composition.

4.2 Chapter aims

The primary aim of this chapter is to determine whether maternal prebiotic (BGOS) feeding during pregnancy and suckling alters peripheral metabolite profiles and immune function of offspring. ^1H NMR was used to quantify metabolites in offspring plasma. I hypothesised that maternal prebiotic feeding during gestation, suckling, and both periods produces changes in

plasma metabolic profiles in offspring. Apart from providing additional evidence that these animals are biologically different, this approach was also used to identify specific differentially abundant metabolites and, if possible, generate hypotheses about their effects on behaviour for future studies. Finally, I tested whether the short-chain fatty acid (SCFA) acetate was higher in pups born to or suckled by prebiotic-fed dams. BGOS boosts acetate production when fermented and increases levels of acetate-producing microbes, namely *Bifidobacterium* (Grimaldi et al. 2017). Exposure to the maternal microbiome may also increase acetate levels in offspring.

Immunologically, based on previous studies, I expected acute LPS to change the composition of WBCs, increasing neutrophils and decreasing monocytes and lymphocytes (Tavares et al. 2006). In contrast, I hypothesised that maternal BGOS increases WBC counts in pups. In addition, I expected maternal prebiotic to promote an anti-inflammatory phenotype in BMDMs, decreasing M1 marker expression and increasing M2 marker expression, while I expected LPS to promote a pro-inflammatory phenotype. I also anticipated that maternal BGOS reverses LPS-induced increases in spleen/body weight ratios in pups. Finally, given the relationship between the gut microbiome and BMI, I hypothesised that maternal prebiotic produces changes in offspring body weight.

4.3 Experiment 4A design: Effects of maternal BGOS and acute LPS on offspring plasma metabolite profiles

Plasma was collected from pups in Chapter 3, experiment 3B after behavioural testing. A principal component analysis (PCA) plot was built from plasma ¹H NMR spectral bins to explore trends and identify outliers. Bins were then analysed with orthogonal partial least squares discriminant analysis (OPLS-DA). OPLS-DA models were used to determine which global metabolic profiles were significantly different in each pairwise comparison. For significant

models, metabolites corresponding to top variable importance in projection (VIP) scores were identified and plotted in a heat map. Plasma acetate was measured by evaluating the integral at 1.91–1.93 parts per million (ppm) in the spectra. The integral sums between groups were compared with a two-way ANOVA (prenatal diet x postnatal diet). Because of the small numbers of males and females, data from both sexes were combined for metabolite analysis (N = 6 males and N = 6 females; N = 12 total). The experimental design is summarised in **Figure 4.1**.

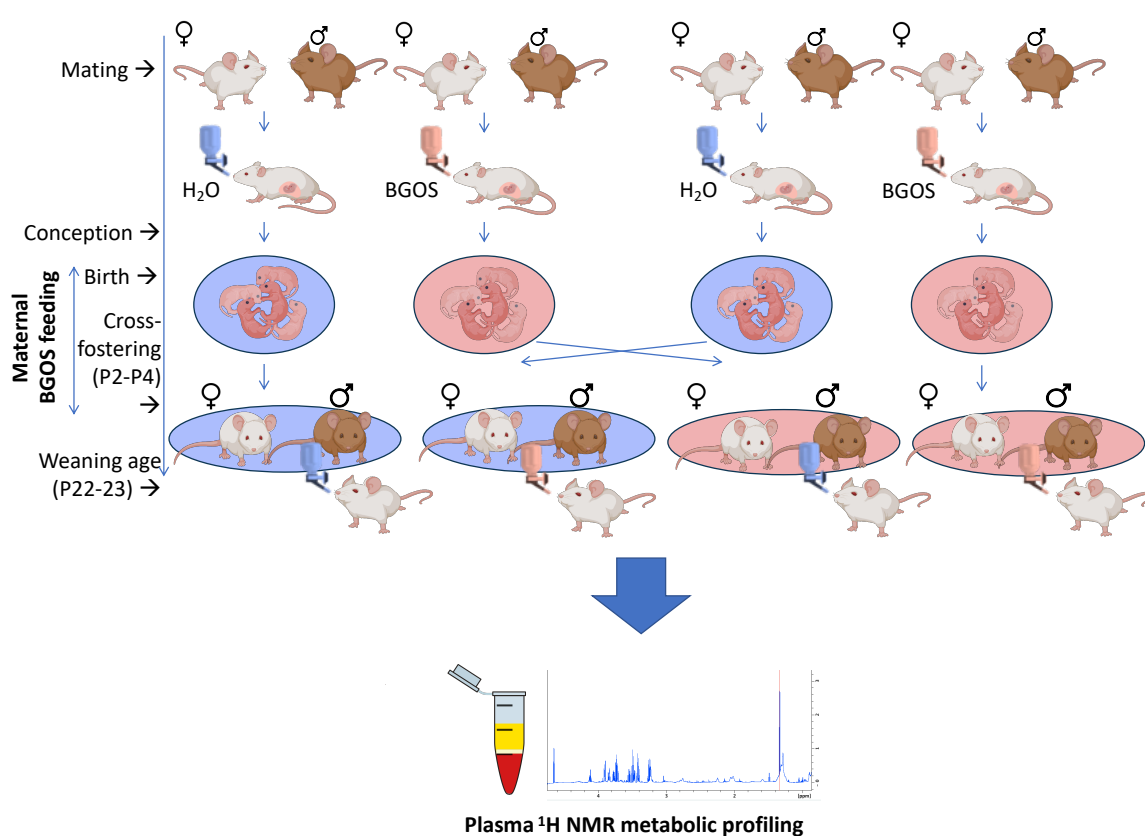


Figure 4.1. Diagram of experiment 4A.

4.4 Experiment 4A results

4.4.1 Maternal BGOS intake during gestation alone, suckling alone, and both gestation and suckling result in metabolite profiles that are distinct from control pups

Pareto-scaled data were visualised in a PCA plot to explore trends and identify outliers (**Figure 4.2**). No data points were removed. Although there was substantial overlap between the four groups in the plot of principal component (PC) 1 and PC2, H₂O/H₂O and BGOS/BGOS tended to cluster towards the left of PC1, while H₂O/BGOS and BGOS/H₂O pups tended to cluster towards the right.

OPLS-DA was performed to examine plasma metabolic differences in more detail. Four pairwise comparisons were run: H₂O/H₂O vs. BGOS/BGOS, H₂O/H₂O vs. BGOS/H₂O, H₂O/H₂O vs. H₂O/BGOS, and H₂O/BGOS vs. BGOS/H₂O. The ensemble of H₂O/H₂O vs. BGOS/BGOS OPLS-DA models performed significantly better than random chance in classifying data (**Figure 4.3A**). In addition, both cross-fostered groups (H₂O/BGOS, **Figure 4.3B** and BGOS/H₂O, **Figure 4.3C**) had significantly different metabolic profiles than controls. BGOS/H₂O and H₂O/BGOS pups' plasma profiles, however, were not significantly different from each other (**Figure 4.3D**). Average accuracies as well as R²X, R²Y, and Q² values are summarised in **Table 4.1**.

4.4.2 Glucose and lipids drive the separation between BGOS pups and controls

Spectral bins corresponding to top VIP scores (**Figure 4.4**) for each significant model were assigned to metabolites where possible. All high-ranking signals in the H₂O/H₂O vs. BGOS/BGOS model were in the glucose region (**Table 4.2**). In the H₂O/H₂O vs. H₂O/BGOS model (**Table 4.3**) and the H₂O/H₂O vs. BGOS/H₂O model (**Table 4.4**), glucose signals also ranked highly. In addition, lipid signals also received top VIP scores. These signals included hydrogen protons attached to (-CH₂-) and -CH₃ carbons in lipid chains and were located in regions associated with

chylomicron (Chyl)- and very low density lipoprotein (VLDL)-associated lipids (Soininen et al. 2009). Ambiguous or unknown signals are indicated in tables with multiple metabolite assignments and/or question marks.

To visualise the directions of change between groups, spectral bins that corresponded to top VIP scores in at least one of the three significant models were used to construct a heat map (**Figure 4.5**). BGOS/BGOS pups had lower glucose levels than controls. Cross-fostered pups (H₂O/BGOS and BGOS/H₂O) had higher glucose levels than controls and BGOS/BGOS animals. In addition, while there were no differences in plasma lipoprotein-associated lipids between BGOS/BGOS and H₂O/H₂O mice, cross-fostered pups had lower levels of plasma lipoprotein-associated lipids. Boxplots that show group differences in top spectral bins can be found in **Appendix D**.

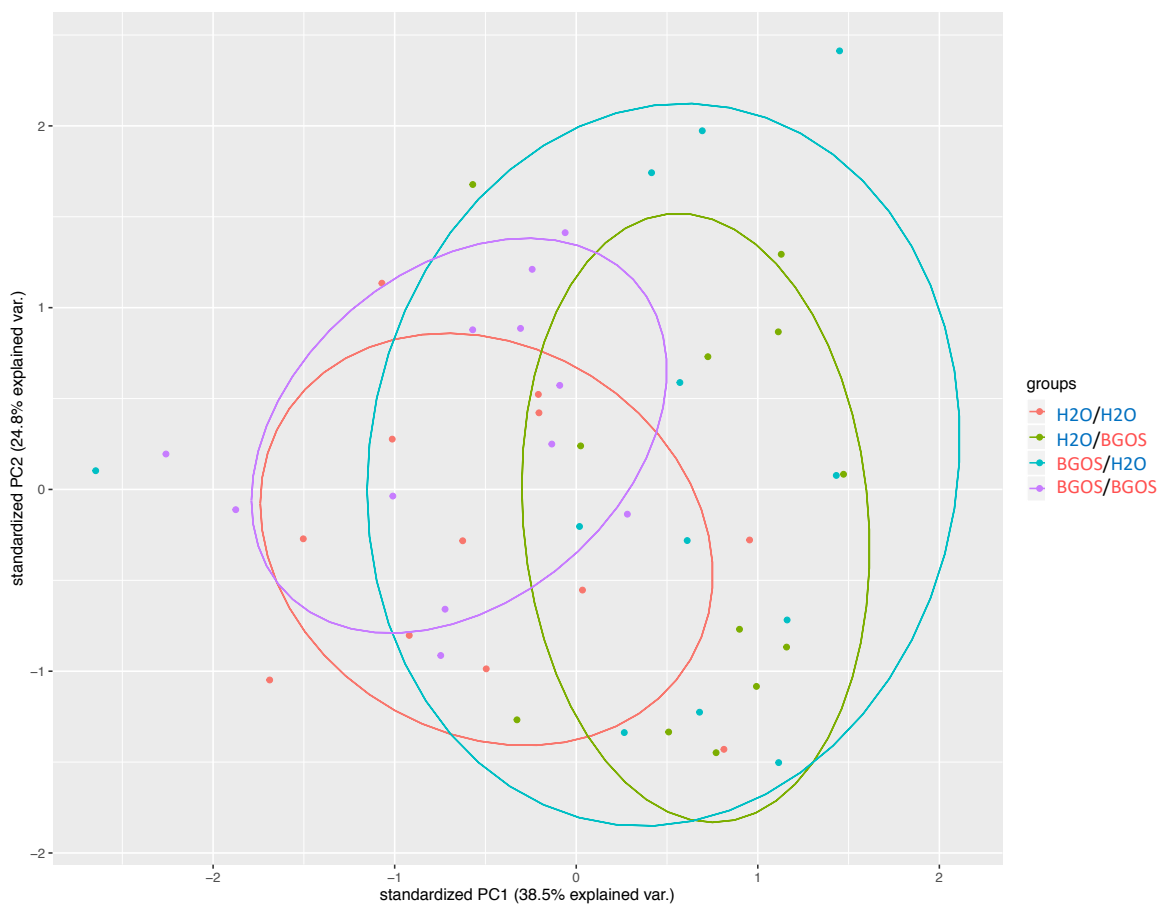


Figure 4.2. PCA plot of plasma ^1H NMR pareto-scaled spectral bins. N = 6 males/females; N = 12 total.

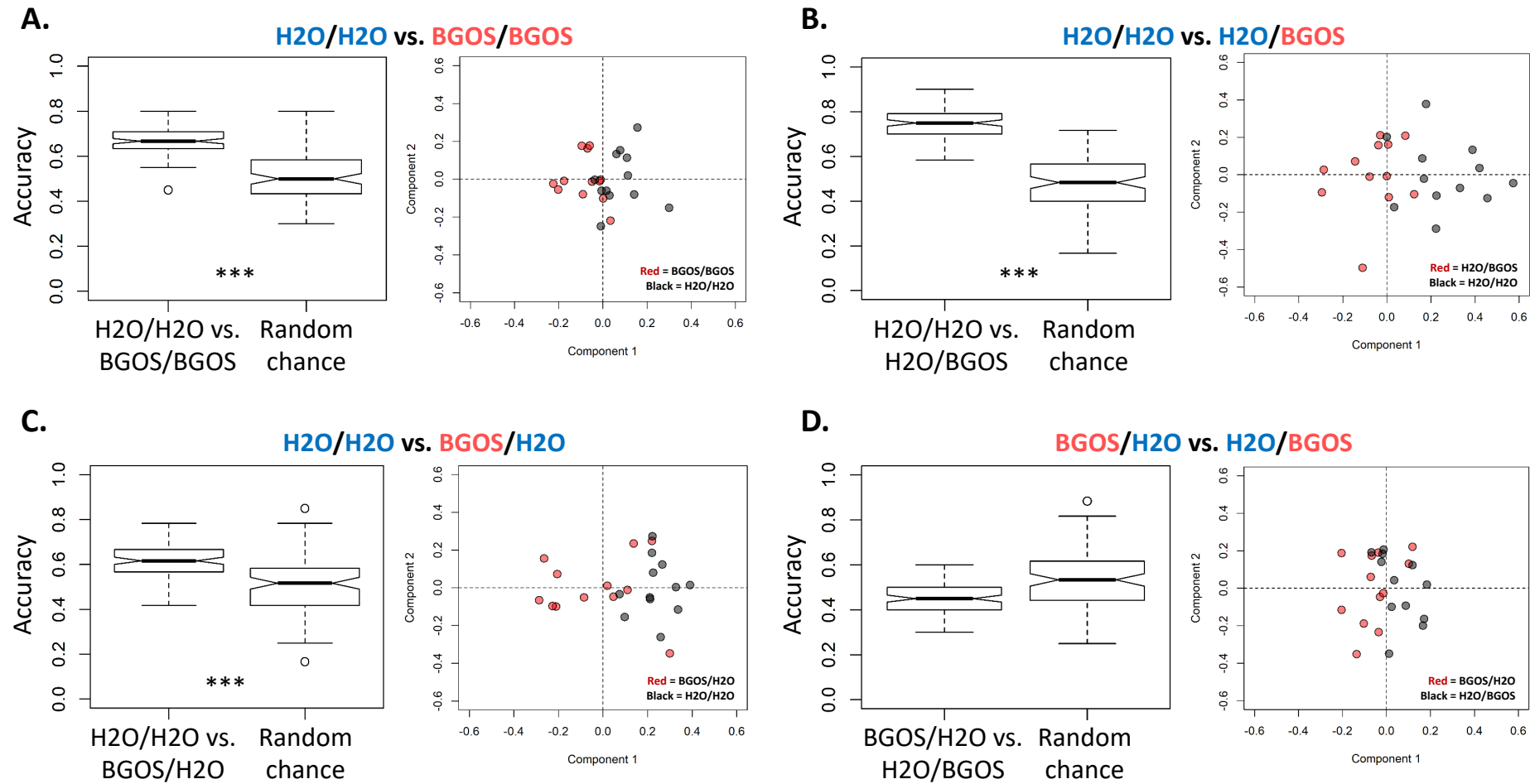
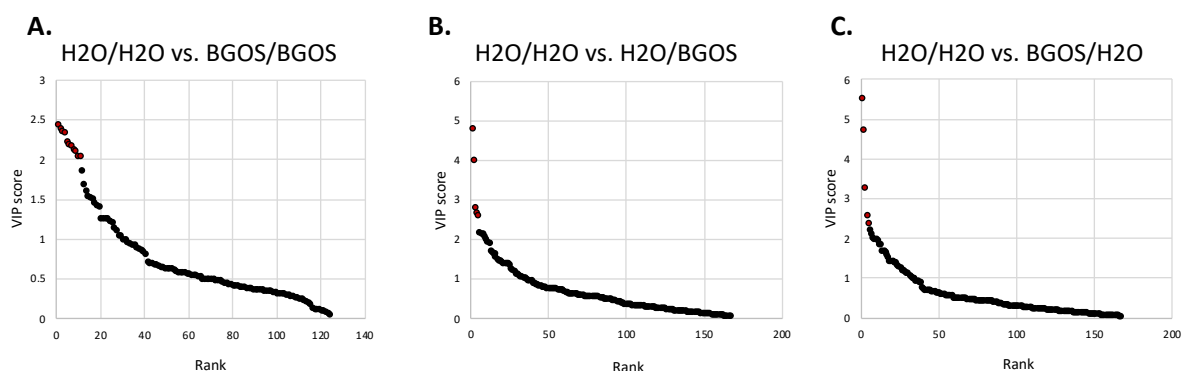


Figure 4.3. OPLS-DA results: average accuracies of 1000 models compared to random data classification (left) and representative OPLS-DA scores plots. The OPLS-DA models performed significantly better than chance in classifying A) H₂O/H₂O vs. BGOS/BGOS, B) H₂O/H₂O vs. H₂O/BGOS, and C) H₂O/H₂O vs. BGOS/H₂O. D) The ensemble of BGOS/H₂O vs. H₂O/BGOS models was not significant. Kolomogorov-Smirnov, *** $p < .001$. N = 6 males/females; N = 12 total.

Table 4.1. Summary of OPLS-DA models, average accuracies, R^2X , R^2Y , and Q^2 values.

Group A	Group B	Average accuracy of A vs. B models $\pm$ SD*	Significantly better performance than random chance?	Average $R^2X \pm$ SD*	Average $R^2Y \pm$ SD*	Average $Q^2 \pm$ SD*
H ₂ O/H ₂ O (n = 12)	BGOS/BGOS (n = 12)	68.6 $\pm$ 6%	Yes	0.70 $\pm$ 0.02	0.75 $\pm$ 0.01	-0.04 $\pm$ 0.10
H ₂ O/H ₂ O (n = 12)	H ₂ O/BGOS (n = 12)	74.4 $\pm$ 7%	Yes	0.77 $\pm$ 0.01	0.82 $\pm$ 0.01	0.27 $\pm$ 0.06
H ₂ O/H ₂ O (n = 12)	BGOS/H ₂ O (n = 12)	61.3 $\pm$ 7%	Yes	0.76 $\pm$ 0.77	0.74 $\pm$ 0.01	-0.34 $\pm$ 0.11
H ₂ O/BGOS (n = 12)	BGOS/H ₂ O (n = 12)	43.9 $\pm$ 6%	No	0.72 $\pm$ 0.02	0.58 $\pm$ 0.01	-0.81 $\pm$ 0.18

**Figure 4.4. Scatter plots of VIP scores vs. rank for each significant OPLS-DA model. Top VIP scores are in red.****Table 4.2. Top-ranking spectral bins, VIP scores, metabolite assignments for H₂O/H₂O vs. BGOS/BGOS model.**

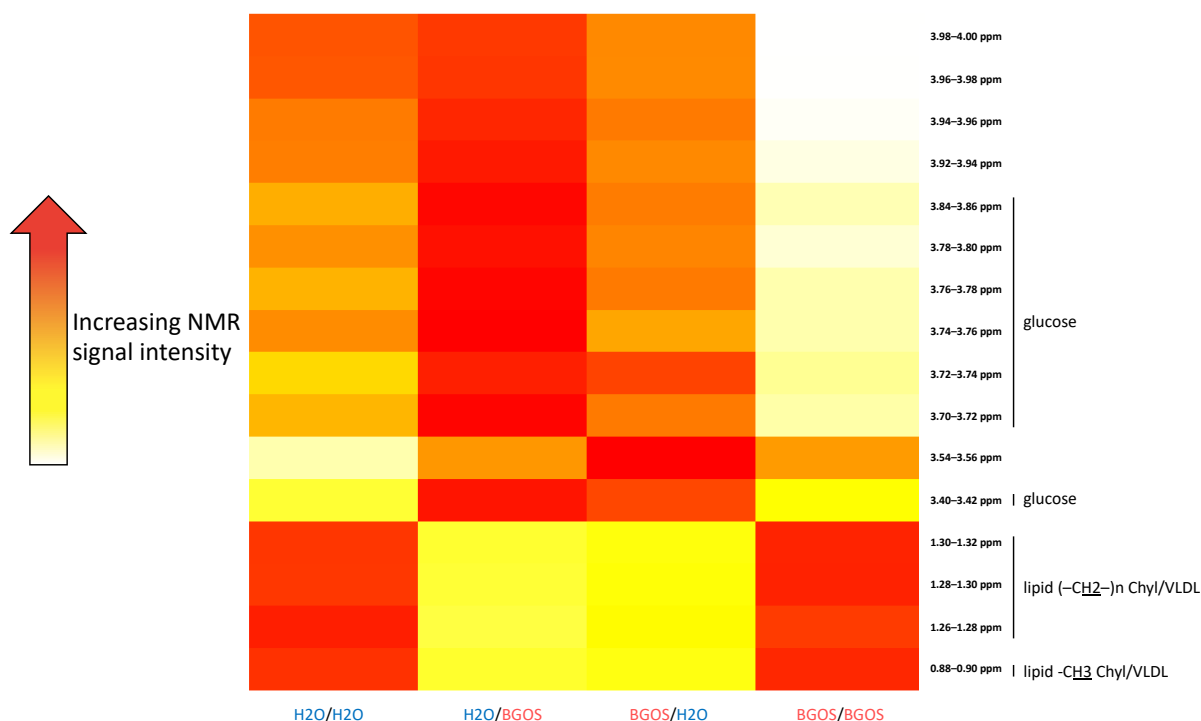
H ₂ O/H ₂ O vs. BGOS/BGOS		
Bin	VIP	Assignment
3.78–3.80 ppm	2.440603	glucose
3.74–3.76 ppm	2.379624	glucose
3.70–3.72 ppm	2.346387	glucose
3.92–3.94 ppm	2.341482	???
3.54–3.56 ppm	2.211992	glycine/glucose/???
3.72–3.74 ppm	2.189453	glucose
3.94–3.96 ppm	2.175821	???
3.96–3.98 ppm	2.123174	histidine/???
3.98–4.00 ppm	2.108703	histidine/???
3.76–3.78 ppm	2.04214	glucose
3.84–3.86 ppm	2.035347	glucose

Table 4.3. Top-ranking spectral bins, VIP scores, metabolite assignments for H₂O/H₂O vs. H₂O/BGOS model.

H2O/H2O vs. H2O/BGOS		
Bin	VIP	Assignment
1.28–1.30 ppm	4.812658	lipid (-CH ₂ -)n Chyl/VLDL
1.30–1.32 ppm	4.015521	lipid (-CH ₂ -)n Chyl/VLDL
0.88–0.90 ppm	2.800421	lipid -CH ₃ Chyl/VLDL
1.26–1.28 ppm	2.685543	lipid (-CH ₂ -)n Chyl/VLDL
3.40–3.42 ppm	2.589501	glucose

Table 4.4. Top-ranking spectral bins, VIP scores, metabolite assignments for H₂O/H₂O vs. BGOS/H₂O model.

H2O/H2O vs. BGOS/H2O		
Bin	VIP	Assignment
1.28–1.30 ppm	5.500132	lipid (-CH ₂ -)n Chyl/VLDL
1.30–1.32 ppm	4.718891	lipid (-CH ₂ -)n Chyl/VLDL
0.88–0.90 ppm	3.264758	lipid -CH ₃ Chyl/VLDL
3.72–3.74 ppm	2.584928	glucose
1.26–1.28 ppm	2.367069	lipid (-CH ₂ -)n Chyl/VLDL

**Figure 4.5.** Heat map comparing spectral bins and corresponding metabolites between groups. To visualise group differences in metabolite levels, spectral bins with top VIP scores in at least one of the significant OPLS-DA models were used to construct a heat map.

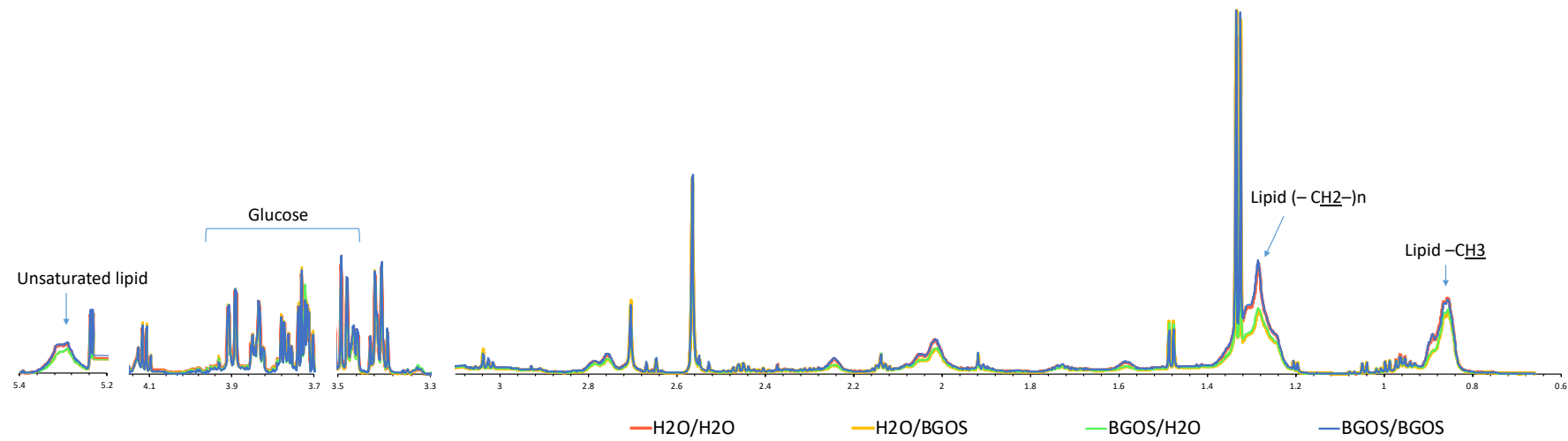


Figure 4.6. Average CPMG spectra values for each group. Major glucose and lipid regions are indicated.

4.4.3 Plasma acetate is elevated only in pups born to and suckled by BGOS-fed dams

There was a significant prenatal diet x postnatal diet interaction effect on plasma acetate levels in pups, $F(1, 44) = 6.46$, $p = .01$. There were no main effects of prenatal diet, $F(1, 44) = 2.05$, $p = .16$, or postnatal diet, $F(1, 44) = 2.69$, $p = .11$. Bonferroni post hoc tests revealed that BGOS/BGOS pups had significantly higher acetate levels than BGOS/H₂O, $p = .04$, and H₂O/BGOS pups, $p = .03$. The difference between H₂O/H₂O and BGOS/BGOS was not significant, $p = .21$ (Figure 4.7).

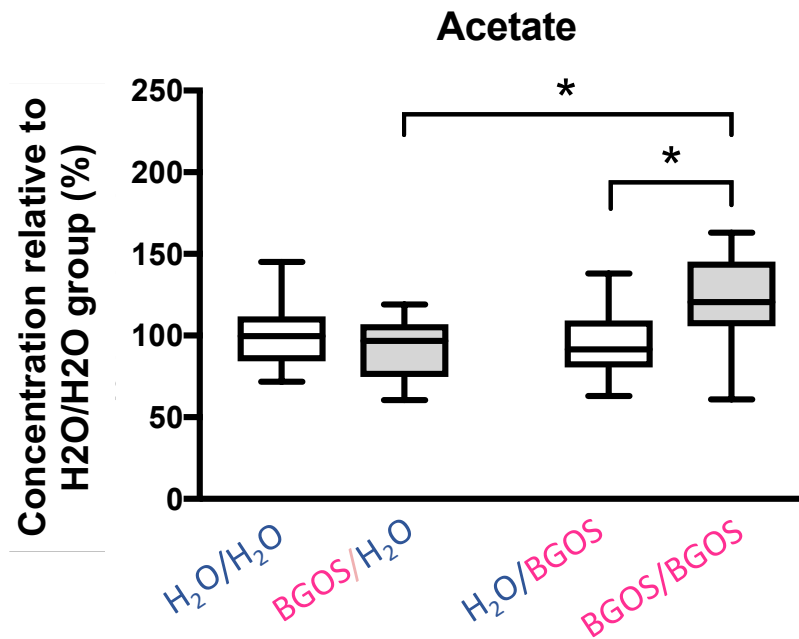


Figure 4.7. Maternal BGOS intake during gestation and suckling increases plasma acetate in pups. There was a significant prenatal diet x postnatal diet interaction effect on plasma acetate levels in pups. There were no main effects of prenatal diet or postnatal diet. Bonferroni post hoc tests revealed that BGOS/BGOS pups had significantly higher acetate levels than BGOS/H₂O and H₂O/BGOS pups. Two-way ANOVA, * $p < .05$. N = 6 males/females; N = 12 total.

4.5 Experiment 4B design: Effects of maternal BGOS and acute LPS on offspring immune cell function and composition

Pups from the Chapter 3, experiment 3A cohort were weighed at P7, P14, and P21. When pups were culled after behavioural testing, whole blood was collected for full blood counts, and spleens were harvested and immediately weighed to calculate spleen/body weight ratios.

For blood counts and spleen/body weight ratios, parametric or robust three-way ANOVAs were performed. F0 diet, acute treatment, and sex were the factors in a 2 x 2 x 2 design. If no significant interactions with sex were detected, male and female data were consolidated, and a two-way ANOVA was run to test for F0 diet x acute treatment interactions and main effects of F0 diet and acute treatment (N = 20–22). In the BMDM stimulation experiment, macrophages expressed IL-6 and YM1/Fizz1 (markers of M2 polarisation in macrophages that indicate an anti-inflammatory state) at very low levels unless polarised into M1 or M2 states, respectively. Therefore, to analyse gene expression, robust two-way ANOVAs were performed on IL-6 and TNF- α expression in the M1 condition (media with IFN- γ and LPS) only. For YM1 and Fizz, robust two-way ANOVAs were only performed on in the M2 condition (media with Il-4, N = 3–4 males/females; N = 6-8 total). Pre-LPS body weights were analysed with t-tests to explore effects of F0 BGOS at P7, P14, and P21 (N = 45–67 total), and *p* values were corrected for multiple comparisons using the Holm-Sidak method. Sex was not accounted for in body weight analysis. Also, a repeated measures ANOVA was not used to analyse body weights because the identities of pups were not kept track of between weighing days. Finally, correlations between blood counts, metabolites, brain gene expression and behaviour were explored using Pearson product-moment correlations.

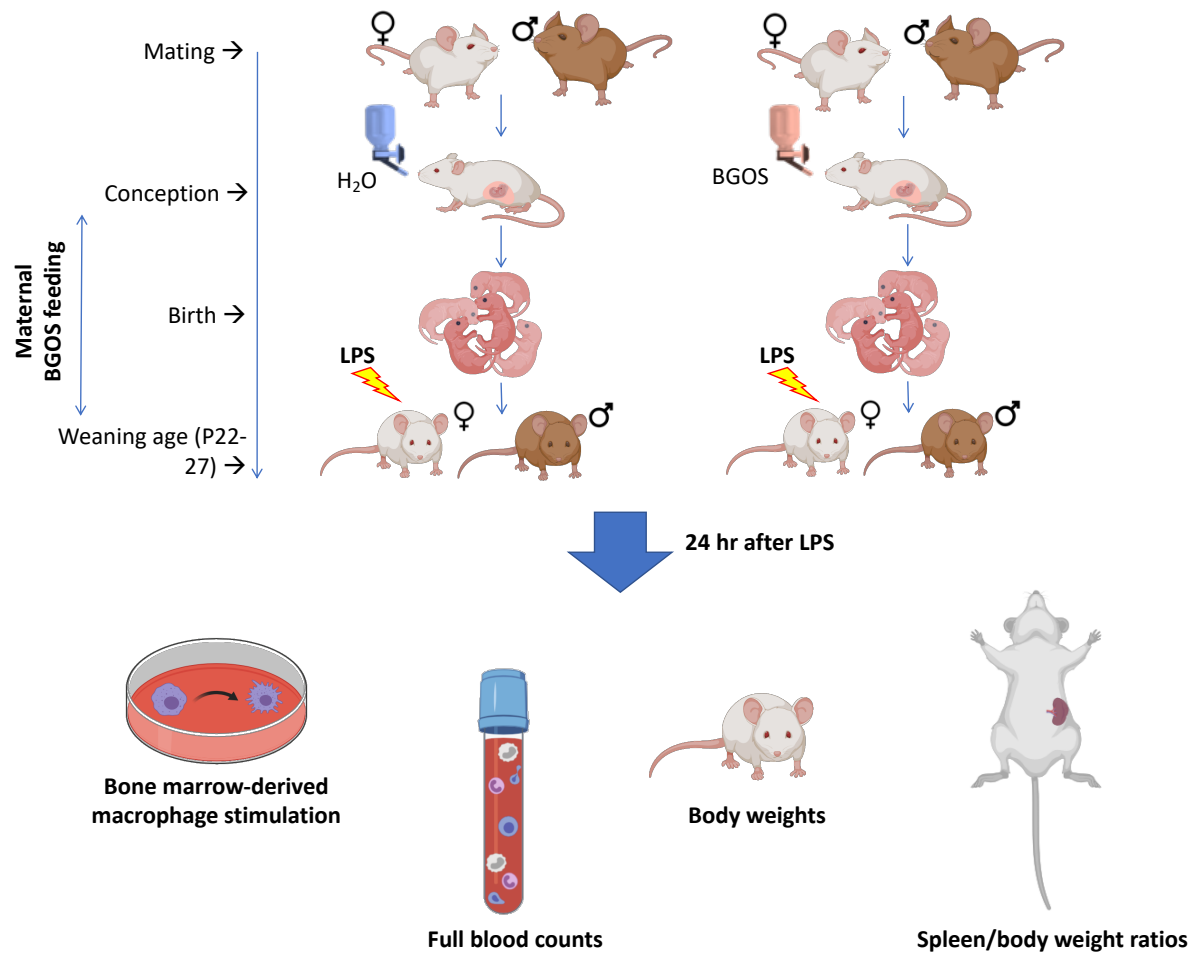


Figure 4.8. Diagram of experiment 4B.

4.6 Experiment 4B results

4.6.1 Perinatal prebiotic intake reduces the pro-inflammatory response in offspring macrophages *in vitro*

BMDMs successfully developed from non-differentiated bone marrow cells (**Figure 4.9A**) to adherent macrophages (**Figure 4.9B**). In the LPS- and IFN- γ -induced M1 state, maternal BGOS significantly reduced macrophage TNF- α expression, $Q = 6.38$, $p = .03$, but there was no effect of acute treatment, $Q = 0.42$, $p = .53$, and no interaction effect, $Q = 0.18$, $p = .68$ (**Figure 4.9C**). The

effect of F0 diet on IL-6 expression was not significant, $Q = 4.86, p = .06$, and there were no effects of treatment, $Q = 0.38, p = .56$, and no interaction effect, $Q = 0, p = .93$ (**Figure 4.9D**). In the IL-4-induced M2 state, there were no effects of F0 diet, $Q = 2.07, p = .21$, or acute treatment, $Q = 0.01, p = .92$, and no interaction effect, $Q = 0.03, p = .87$, on the M2 marker YM1 (**Figure 4.9E**). Similarly, there was no effect of F0 diet, $Q = 0.19, p = .68$, or acute treatment, $Q = 1.32, p = .28$, and no F0 diet x acute treatment interaction effect, $Q = 0.42, p = .54$, on Fizz1 expression (**Figure 4.9F**).

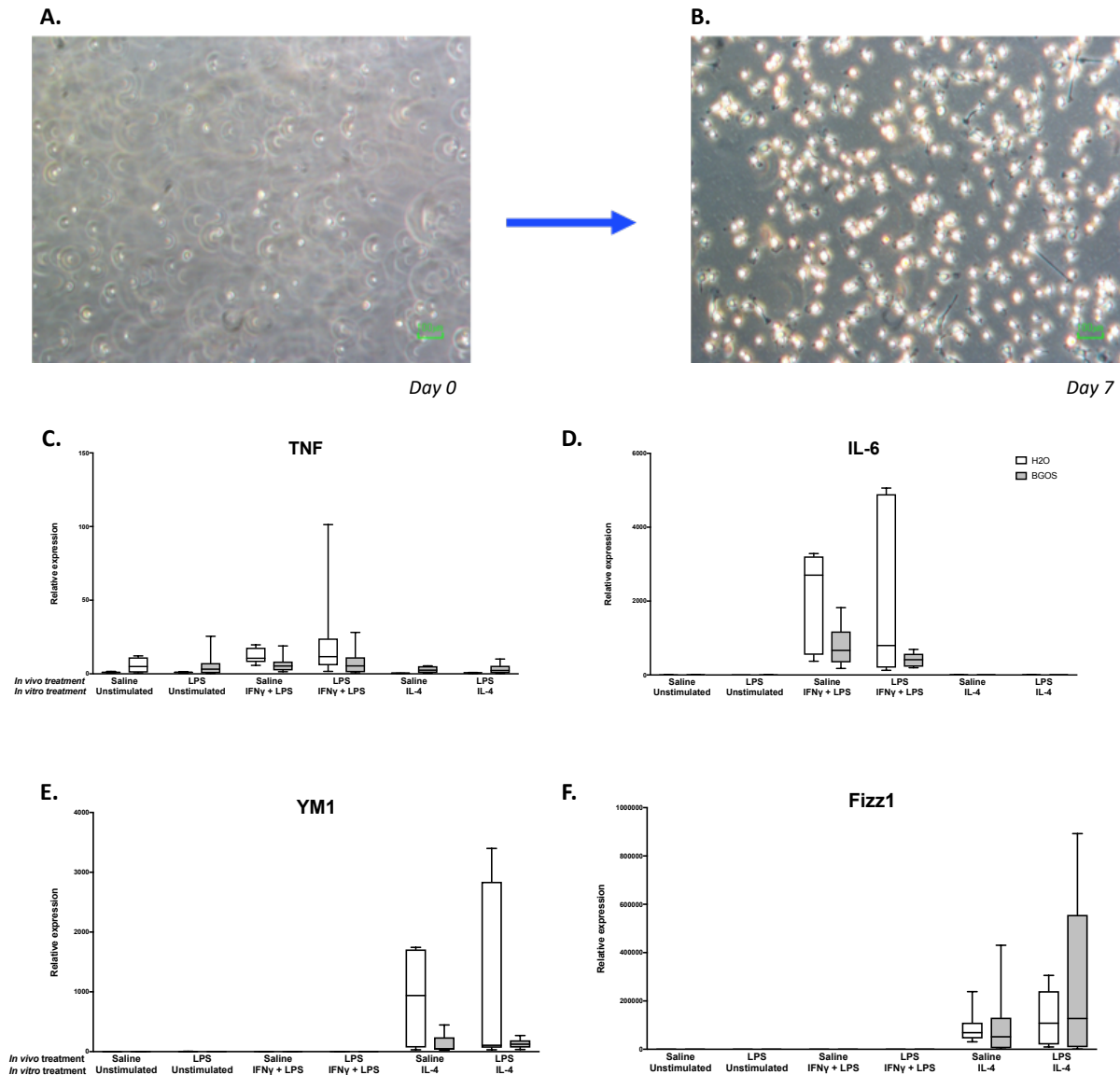


Figure 4.9. Maternal prebiotic intake reduces pro-inflammatory response in BMDMs. **A)** Floating non-differentiated bone marrow cells on Day 0. **B)** Adherent macrophages on Day 7. Projections can also be seen in the mature macrophages. Gene expression is plotted relative to the saline/unstimulated condition. **C)** In the M1 state (IFN- γ + LPS), a significant main effect of F0 diet showed that maternal BGOS significantly reduced TNF- α ($p = .03$). **D)** The main effect of F0 diet on IL-6 in BGOS pups did not reach significance ($p = .06$). In the M2 state (IL-4), there were no effects of F0 BGOS on **E)** YM1 or **F)** Fizz1 expression. Two-way ANOVA (based on trimmed means for nonparametric data), $N = 3-4$ males/females; $N = 6-8$ total.

4.6.2 Maternal prebiotic increases WBC counts in offspring, while LPS alters the composition of WBCs 24 hours post-injection

According to the robust three-way ANOVA, there was a significant F0 diet x sex interaction on total WBC counts, $Q = 4.83, p = .04$. There was also a significant main effect of F0 diet $Q = 6.55, p = .02$. There were no significant main effects of acute treatment, $Q = 0.98, p = .34$, or sex, $Q = 1.98, p = .18$. The F0 diet x acute treatment, $Q = 0.07, p = .79$, acute treatment x sex, $Q = 1.10, p = .31$, and F0 diet x acute treatment x sex interaction effects, $Q = 0.23, p = .64$, were also not significant. Data were then split into male and female WBC counts for two-way ANOVAs to determine the reason for the interaction. In males, there was a significant main effect of F0 diet on number of WBCs, $Q = 7.83, p = .02$. WBCs were elevated in male BGOS pups compared to H₂O pups (**Figure 4.10A**). There was no effect of acute treatment, $Q = 1.44, p = .26$, and no significant F0 diet x acute treatment interaction effect, $Q = 0.02, p = .90$. In females, however, there were no effects of F0 diet, $Q = 0.12, p = .74$, or acute treatment, $Q = 0, p = .96$, and no interaction effect, $Q = 0.50, p = .49$, on WBC counts (**Figure 4.10B**).

Lymphocytes, neutrophils, and monocytes were measured as percentages of WBCs. There was a significant main effect of acute treatment on the WBC lymphocyte fraction, $Q = 7.36, p = 0.01$, but no effect of F0 diet, $Q = 0.81, p = .37$, or interaction effect, $Q = 0.13, p = .72$. Pups treated with LPS had a significantly lower percent lymphocytes than saline-treated animals (**Figure 4.10C**). There was a significant main effect of acute treatment on the neutrophil fraction of WBCs, $F(1, 78) = 8.00, p < .01$. In contrast to percent lymphocytes, however, percent neutrophils was significantly higher in LPS-treated pups ($M = 24.40, SD = 6.91$) compared to saline-treated animals ($M = 20.41, SD = 6.05$). There was no effect of F0 diet, $F(1, 78) = 0.56, p = .46$, and no interaction effect, $F(1, 78) = 2.17, p = .15$ (**Figure 4.10D**). Finally, there were no effects of F0 diet, $Q = 0.43$,

$p = .52$, or acute treatment, $Q = 3.43$, $p = .07$, and no interaction effect, $Q = 0.20$, $p = .66$, on percent monocytes in pups (**Figure 4.10E**).

Absolute counts of blood lymphocytes, neutrophils, and monocytes were also calculated and compared between groups. There was a significant effect of F0 diet on lymphocyte counts, $Q = 5.07$, $p = .03$, but no effect of acute treatment, $Q = 0.62$, $p = .44$, or F0 diet x acute treatment interaction, $Q = 0.13$, $p = .73$. Similarly, there was a significant main effect of F0 diet on absolute numbers of neutrophils, $Q = 5.89$, $p = .02$. There was no effect of acute treatment, $Q = 1.50$, $p = .23$, and no interaction effect, $Q = 0.32$, $p = .58$. Maternal BGOS increased both lymphocyte (**Figure 4.10F**) and neutrophil (**Figure 4.10G**) counts in offspring.

There was a significant F0 diet x sex interaction on blood monocyte levels, $Q = 10.05$, $p = .004$. In addition, there was a main effect of F0 diet, $Q = 9.25$, $p = .005$. The acute treatment, $Q = 0.03$, $p = .86$, sex, $Q = 0.66$, $p = .42$, F0 diet x acute treatment, $Q = 2.08$, $p = .16$, acute treatment x sex, $Q = 0$, $p = .95$, and F0 diet x treatment x sex interactions, $Q = 7.28$, $p = .98$, were not significant. Male and female data were separated for two-way ANOVAs. In males, maternal BGOS significantly increased monocyte levels, $Q = 18.64$, $p < .01$, while there was no effect of acute treatment, $Q = 0.03$, $p = .87$, or F0 diet x acute treatment interaction, $Q = 0.97$, $p = .35$ (**Figure 4.10H**). In contrast, there were no effects of F0 diet, $Q = 0.01$, $p = .93$, or acute treatment, $Q = 0.01$, $p = .93$, and no interaction effects, $Q = 1.12$, $p = .30$, on absolute blood monocyte levels in female pups (**Figure 4.10I**).

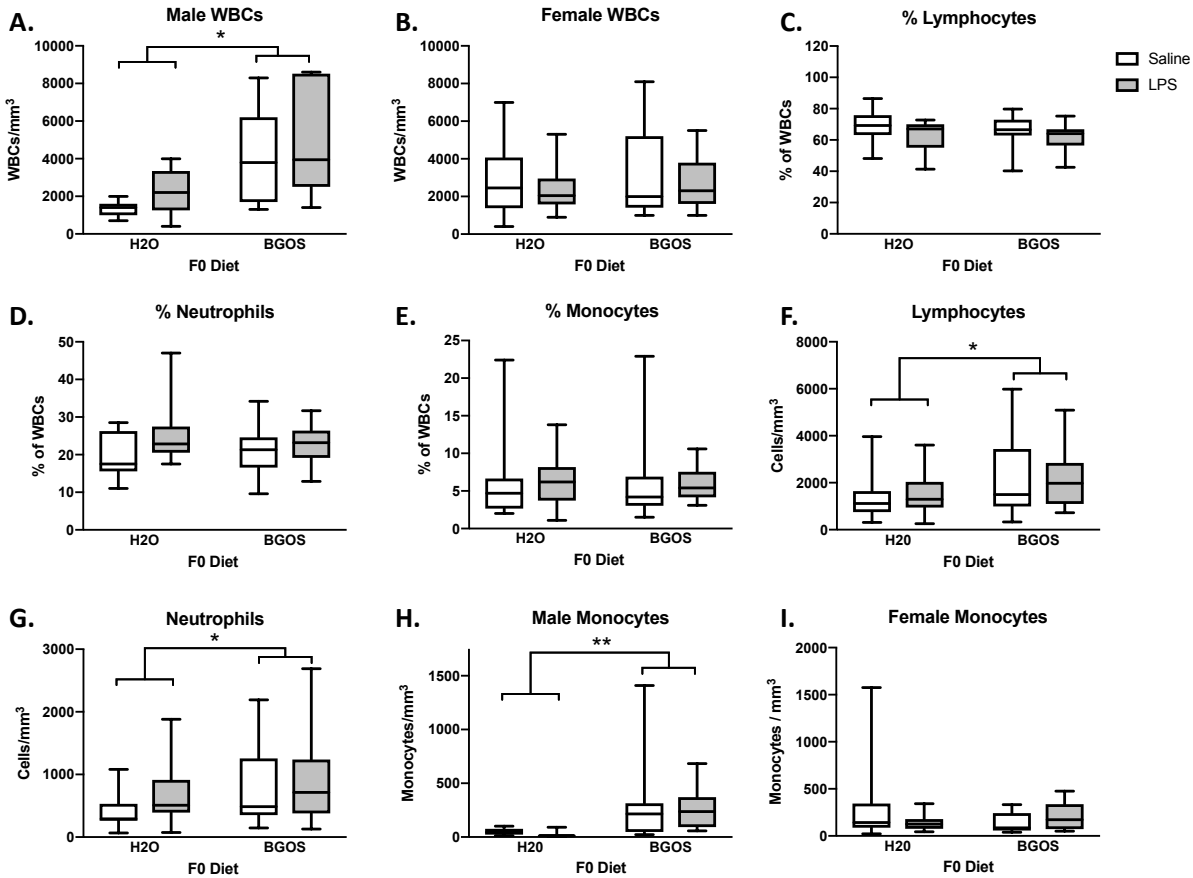


Figure 4.10. Acute LPS modifies WBC composition, while maternal prebiotic intake increases WBC counts in pups. A) A significant main effect of F0 diet showed that maternal prebiotic increased WBC counts in males but not B) females. C) Significant main effects of acute treatment showed that LPS reduced % lymphocytes ($p = .01$) but D) increased % neutrophils 24 hours post-injection ($p < .01$). E) Neither maternal prebiotic nor acute LPS affected % monocytes in pups. Significant main effects of F0 diet indicated that maternal prebiotic feeding increased both F) absolute numbers of lymphocytes and G) neutrophils. H) However, a significant main effect of maternal BGOS showed that monocyte counts were elevated in male BGOS pups, but I) there was no difference in female pups. Two-way ANOVA (parametric or based on trimmed means for nonparametric data), * $p < .05$, ** $p < .01$. $N = 10-11$ males/females; $N = 20-22$.

4.6.3 Maternal BGOS and acute LPS do not alter spleen/ body weight ratios

There was no significant effect of F0 diet, $F(1, 77) = 0.22$, $p = .64$, or acute treatment, $F(1, 77) = 0.40$, $p = .64$, on spleen/body weight ratios. The interaction effect also did not reach significance, $F(1, 77) = 3.93$, $p = .05$ (Figure 4.11).

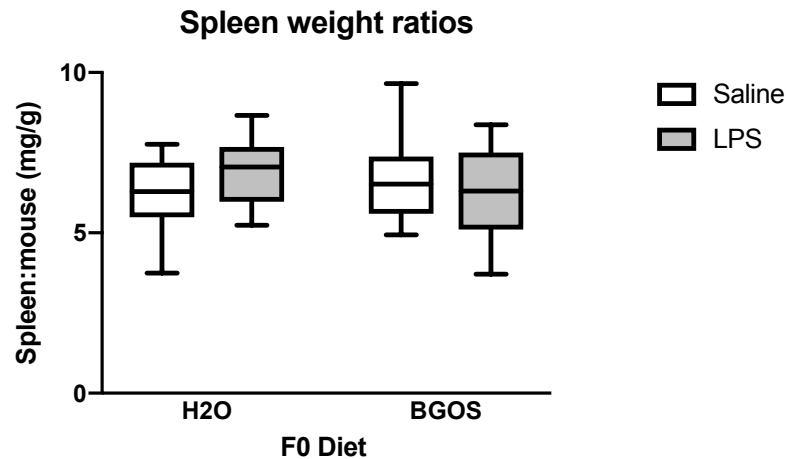


Figure 4.11. Maternal prebiotic and acute LPS do not significantly alter spleen/mouse body weight ratios. Two-way ANOVA. N = 10–11 males/females; N = 20–22.

4.6.4 Maternal BGOS reduces offspring body weight

F0 BGOS had significant effects on offspring body weight (g) at P7, $t(112) = 4.47$, $p < .001$, P14, $t(109) = 3.38$, $p < .01$, and P21, $t(108) = 5.05$, $p < .001$. Mean body weights at P7, P14, and P21, respectively, for BGOS pups were the following: $M = 4.98$, $SD = 0.56$; $M = 7.94$, $SD = 1.02$; $M = 13.98$, $SD = 1.51$. Mean body weights for H₂O pups were the following: $M = 4.48$, $SD = 0.62$; $M = 7.34$, $SD = 0.75$; $M = 12.39$, $SD = 1.79$.

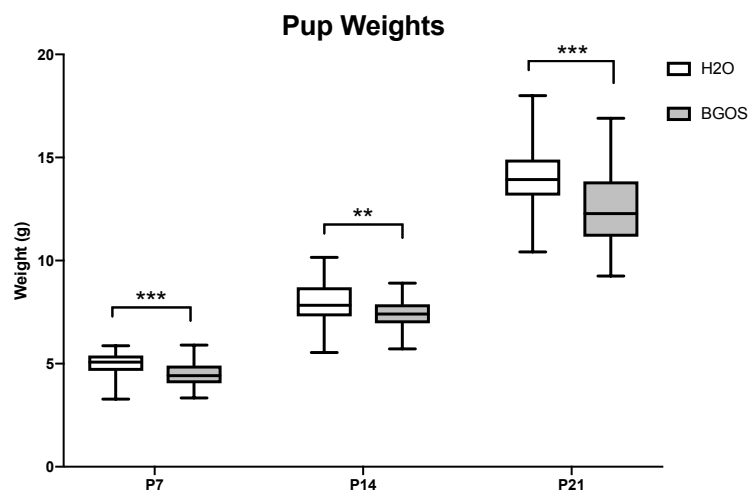


Figure 4.12. Maternal prebiotic reduces offspring body weight. Unpaired student's t-test, * $p < .05$, ** $p < .01$, *** $p < .001$. N = 45–67.

4.6.5 Blood counts correlate with behavioural outcomes in offspring

There were significant correlations between blood counts and behaviour in offspring. Number of WBCs positively correlated with time in the centre of the OFT, $r(81) = 0.23, p = .04$, and entries into the LDB $r(80) = 0.25, p = .02$, and negatively correlated with latency to enter the light in the LDB, $r(81) = -0.24, p = .03$. Percent lymphocytes negatively correlated with time in the centre of the OFT, $r(82) = -0.26, p = .02$, and positively correlated with latency to float in the FST, $r(82) = 0.25, p = .02$. Percent monocytes correlated with time in the centre of the OFT, $r(80) = 0.26, p = .02$, and negatively correlated with distance travelled in the OFT, $r(80) = -0.25, p = .02$, and latency to enter the light in the LDB, $r(79) = -0.31, p < .01$. Absolute monocyte counts positively correlated with time in centre in the OFT, $r(80) = 0.35, p < .01$, and negatively correlated with latency to enter the light in the LDB, $r(81) = -0.29, p < .01$. Numbers of monocytes also significantly correlated with BDNF expression in the PFC, $r(78) = 0.23, p = .04$. Finally, numbers of neutrophils significantly correlated with time in centre of the OFT, $r(81) = 0.27, p = .01$, and entries in the LDB, $r(80) = 0.25, p = .02$, and negatively correlated with latency to enter the light side of the LDB, $r(80) = -0.22, p = .04$, and latency to float in the FST, $r(81) = -0.24, p = .03$ (**Figure 4.13**).

Correlations between plasma acetate and glucose and behavioural outcomes were also explored. The only significant correlation was between plasma acetate and rears in the OFT, $r(45) = 0.29, p = .04$ (**Figure 4.14**).

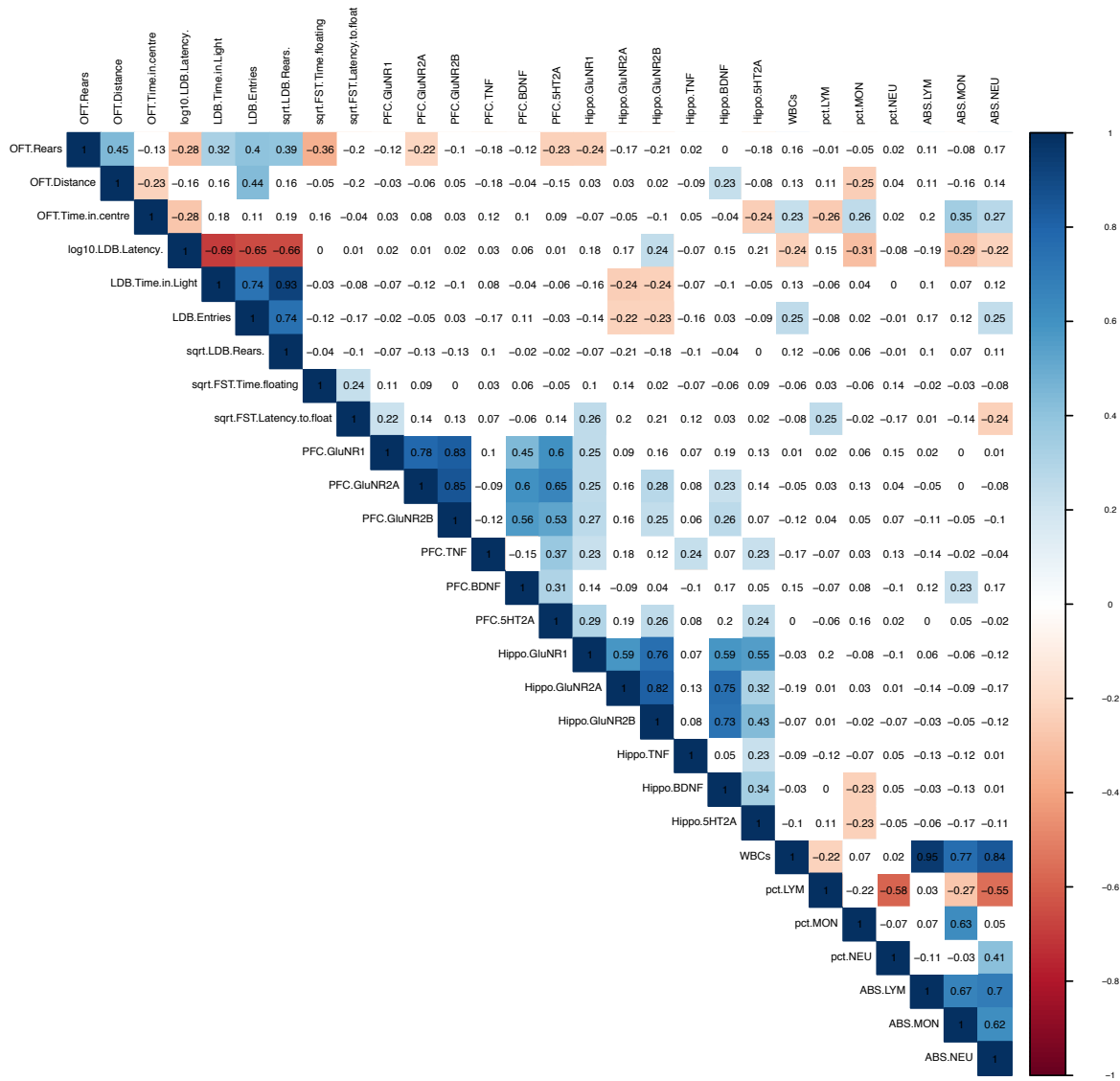


Figure 4.13. Pearson product-moment correlations between behaviour and gene expression and blood counts in offspring. N = 10–11 males/females; N = 20–22 total.

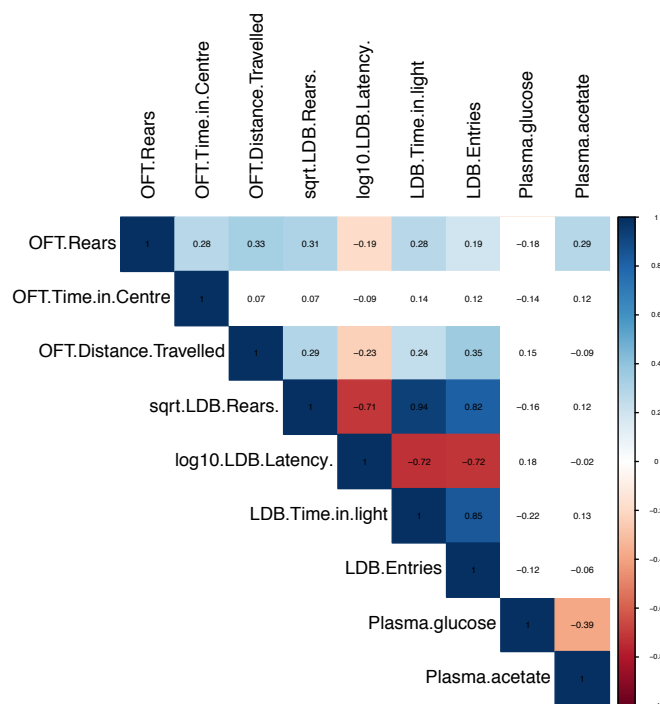


Figure 4.14. Pearson product-moment correlations between behaviour and plasma metabolites in offspring. N = 6 males/females; N = 12 total.

4.7 Discussion

4.7.1 Summary of results

The purpose of this chapter was to determine whether maternal prebiotic feeding influences offspring metabolism and immunity in offspring. I found that maternal BGOS reduces plasma glucose in pups. Perinatal prebiotic intake also blunts the pro-inflammatory response in offspring BMDMs *in vitro* and increases WBC counts, while acute LPS alters the composition of WBCs. Finally, maternal prebiotic intake reduces pup body weights.

4.7.2 Maternal BGOS reduces offspring plasma glucose levels and increases acetate

The top spectral bins in the H₂O/H₂O vs. BGOS/BGOS OPLS-DA model were in the glucose region, and I found that this was due to a drop in plasma glucose levels in BGOS pups.

This is consistent with a study in rats reporting reduced plasma glucose levels in offspring whose mothers were given a high-fibre diet, a change that likely involved gut bacteria (Maurer and Reimer 2011). In rodents, faecal microbiome transplant (FMT) from glucose intolerant mice to germ-free animals induced glucose intolerance (Suez et al. 2014). Similarly, in humans, FMT from healthy lean donors to recipients with metabolic syndrome resulted in increased insulin sensitivity (Vrieze et al. 2012). One possible mechanism by which gut bacteria regulate glucose metabolism is modulation of intestinal permeability and systemic inflammation, which are associated with insulin resistance and obesity. A “bad” high-fat diet, for example, increases endotoxemia and plasma glucose, while prebiotics have the opposite effect (Cani, Amar, et al., 2007; Cani, Neyrinck, et al., 2007). In addition, microbial metabolites, including SCFAs and bile acids, influence secretion of gut and pancreatic hormones that are involved in glucose homeostasis (Gao et al., 2009; Lin et al., 2012; Parséus et al., 2017). Furthermore, BGOS was effective in reducing insulin and total cholesterol in overweight adult human subjects (Vulevic et al. 2013). The differences in glucose levels in this study suggest that maternal BGOS also has metabolic benefits and should be investigated in the context of metabolic disorders.

Surprisingly, the cross-fostered pups (H₂O/BGOS and BGOS/H₂O) were metabolically more similar to each other than the offspring that were not cross-fostered (H₂O/H₂O or BGOS/BGOS). This was reflected in reduced glucose and elevated lipid levels in the cross-fostered animals compared to the pups that were suckled by their birth mothers. Of course, this may also indicate a limitation of the experimental design – the act of cross-fostering *per se* may have altered the plasma metabolites. For example, if dams do not nurse the cross-fostered pups with as much fatty breastmilk as they do their own offspring, the resulting reduction of plasma lipids may cause cross-fostered pup to eat more of the carbohydrate-rich chow to compensate for

less milk. This could explain the increase in plasma glucose levels. Furthermore, this finding that cross-fostered offspring have distinct metabolite profiles compared to those that remain with their birth mothers is consistent with a study showing that cross-fostered pups exhibit metabolic differences, including changes in food intake, triglycerides, cholesterol, and insulin (Matthews et al. 2011). Although the conclusions about the effects of BGOS intake compared to plain water during both gestation and suckling from this study are still valid, in future cross-fostering studies, all litters (both pups that are switched to dams of the opposite treatment group *and* those moved to a dam receiving the same treatment) should be swapped to avoid this potential confound.

Finally, we tested our *a priori* hypothesis that maternal BGOS increases plasma acetate in pups. Consistent with our hypothesis, BGOS/BGOS pups had the highest levels of plasma acetate. This supports the possibility that the effects of maternal prebiotic intake on offspring are bacteria-dependent. Prebiotics are fermented by gut microbes to produce SCFAs, including acetate, which have widespread effects. Metabolically, both elevated (Perry et al. 2016) and decreased acetate (Murphy et al. 2010) have been associated with impaired glucose homeostasis, suggesting that acetate may be related to the changes in plasma glucose in BGOS pups. Consistent with this idea, there was a significant negative correlation between plasma acetate and glucose. Finally, there was a significant correlation between plasma acetate and rears in the OFT. Acetate crosses the blood-brain barrier and can influence the brain and behaviour in rodents (Frost et al. 2014), suggesting that the maternal BGOS-induced increase in acetate may be causally related to the changes in exploratory behaviour.

4.7.3 Maternal prebiotic reduces the pro-inflammatory response in offspring BMDMs and increases WBC counts

Maternal BGOS reduced expression of TNF- α in offspring BMDMs after stimulation with LPS and IFN- γ . IL-6 expression was also reduced in the M1 state in BGOS pups, although this difference was just above the threshold for significance. This variation in gene expression was specific to the M1 markers, as there was no difference in the M2 markers YM1 and Fizz1. This finding is interesting in light of studies that have demonstrated an impact of the gut microbiome on BMDM function (Thevaranjan et al. 2017; Ji et al. 2013).

Because the macrophage progenitor cells were collected from the bone marrow and differentiated *in vitro*, F0 diet-mediated changes in pro-inflammatory response likely involve epigenetic modifications. Histone methylation in bone marrow progenitor cells, for example, programs macrophages towards a pro-inflammatory phenotype in a mouse model of type 2 diabetes (Gallagher et al. 2015). In my study, the altered programming in BGOS pups may have been due to increased acetate, which increases histone acetylation (Thorburn et al. 2015; Soliman et al. 2012).

Acute LPS, in contrast, had no effect on BMDM M1 or M2 polarisation. We had expected BMDMs from animals exposed to LPS *in vivo* to exhibit enhanced pro-inflammatory signalling in the M1 state due to trained innate immunity. The dose used in this study, however, was low (Lew et al. 2013). This dose was chosen because similar doses have been shown to produce behavioural changes without causing long-lasting peripheral inflammation or sickness (Frenois et al. 2007). It is possible, however, that a single low dose of LPS was not sufficient to produce measurable changes in BMDM function.

Maternal BGOS also increased the overall number of WBCs, but this effect was specific to male offspring. Perinatal prebiotic intake increased lymphocytes and neutrophils in all offspring, but only increased monocytes in males. This discrepancy may be due to the lower average number of WBCs in control H₂O/H₂O males compared to females.

LPS also altered the composition of WBCs at 24 hours post-injection. The fraction of neutrophils increased, while the lymphocyte percentage decreased. This agrees with our hypothesis and previous studies showing similar changes in WBC composition after LPS exposure. There were, however, no changes in percent monocytes, while previous studies report both increases and decreases in monocyte levels after LPS (Yates et al. 2011; Kolomaznik et al. 2014; Tavares et al. 2006). These results suggest that maternal prebiotic produces global changes in absolute WBC counts, while acute LPS selectively changes the relative percentages of WBCs.

Finally, the F0 diet x acute treatment interaction effect on spleen:body weight ratios just missed the threshold for statistical significance. An increase in spleen weight relative to body weight is associated with LPS-induced inflammation (Liverani et al. 2014). Thus, the trend towards reduced relative spleen weight data in BGOS pups is consistent with my hypothesis that maternal prebiotic has an anti-inflammatory effect in offspring.

There were modest correlations between blood cell counts and behaviour, especially time in the centre of the OFT and latency to enter the light in the LDB. Correlations between BMDM gene expression and the brain and behaviour outcomes were not evaluated due to the small group numbers and large variation in the data. As discussed in the previous chapter, there is a high risk of false positives when many comparisons are made. These significant associations, however, provide rationale for testing causal relationships between blood cell counts, other measures of immune function, and the brain and behaviour in offspring after maternal BGOS supplementation.

4.7.4 Perinatal BGOS reduces offspring body weight

BGOS pups had lower body weights than control animals, although it is unclear whether this change in body weight was due to a difference in food intake or metabolic rate. Gut bacteria are involved in body weight regulation, and gut microbiome composition is correlated with body weight. The *Firmicutes/Bacteroides* ratio, for example, is elevated in obese human subjects and rodents, and this ratio decreases with weight loss. In addition, gut bacteria more efficiently extract calories from food and contribute to weight gain in *ob/ob* mice, a leptin-deficiency model of obesity (Ley et al. 2006; Turnbaugh et al. 2006). Administration of SCFAs is also associated with lower body weight and plasma free fatty acid levels and normalises weight gain in animal models of obesity (Ge et al. 2008; H. V. Lin et al. 2012; Gao et al. 2009). Although the mice in the current study were healthy, it is possible that the impact of maternal prebiotic intake on offspring body weight was due to altered offspring microbiome composition and function, including production of SCFAs.

4.7.5 Conclusion

Maternal prebiotic intake alters metabolism and immune function in young offspring. Metabolically, BGOS pups exhibit lower glucose and higher acetate levels. These animals also had lower average body weights. In addition, perinatal prebiotic feeding produced changes in macrophage function *in vitro* and WBC counts. These findings were consistent with the original hypotheses. My proposed mechanism is that administration of prebiotic to the pregnant and suckling dams alters offspring metabolism and immune function through the transfer of immunomodulatory microbes or microbial compounds via exposure to the mothers' microbiome in the faeces or breastmilk.

5. Long-term effects of maternal prebiotic intake on adult offspring behaviour, brain gene expression and plasma metabolite profiles

5.1 Introduction

The aim of this chapter is to explore whether maternal prebiotic intake influences the effects of an early-life inflammatory challenge on brain gene expression, behaviour and plasma metabolite profiles in adult offspring.

5.1.1 Neurodevelopmental consequences of early-life inflammation

The early postnatal period is critical for brain development. A pro-inflammatory insult can disrupt this process and cause brain and behavioural dysfunction in adulthood. Neonatal lipopolysaccharide (LPS), for example, is associated with deficits in anxiety, social interaction, anhedonia, and passive stress-coping behaviour in adult rodents. These behavioural alterations are accompanied by changes in brain gene expression, such as cytokines, brain-derived neurotrophic factor (BDNF), and cortical N-methyl-D-aspartate (NMDAR) subunits (Blom et al., 2006; Custódio et al., 2018; Harré, Galic, Mouihate, Noorbakhsh, & Pittman, 2008; Li et al., 2018). Other long-term consequences of an early postnatal immune challenge for brain development include reduced neurogenesis (Wanga et al. 2013; Dinel et al. 2014), cognitive deficits (Spencer, Heida, and Pittman 2005) alterations in sleep that resemble autism spectrum disorder (Missig et al. 2018), and increased microglia activation (Lei et al. 2017; Sominsky et al. 2012; Claypoole, Zimmerberg, and Williamson 2017). Deficits in neurodevelopment and behaviour have also been described in human studies investigating associations between inflammatory markers in the first weeks and months of life and brain development and cognition later in life (Jiang et al., 2014; Leviton et al., 2016; O'Shea et al., 2014). Neonatal immune activation in animal models may help us understand

neurodevelopmental dysfunction that is associated with early-life inflammation (Jiang, Cowan, Moonah, & Petri, 2018; Rantakallio, Jones, Moring, & Von Wendt, 1997).

5.1.2 Maternal and offspring microbiota and long-term brain development

Evidence in animal models suggests that the neonatal gut microbiome is a critical factor in long-term brain development (Heijtz et al. 2011), and colonisation is largely influenced by maternal bacteria. At birth, the neonatal gut microbiome closely resembles the vaginal microbial community, and by weaning age, the structure looks more like the maternal gut microbiome. This pattern occurs in both humans and mice (Jašarević et al. 2017; Bäckhed et al. 2015). Furthermore, pre-, pro-, and antibiotics in early life can influence brain development. Administration of BGOS to neonatal rats resulted in elevated hippocampal BDNF, synaptophysin, and GluNR2A protein in adulthood (Williams et al. 2016), while antibiotic administration to mouse dams during late pregnancy and suckling produced deficits in anxiety and social behaviour in adult offspring (Leclercq et al. 2017). In addition, early postnatal administration of a *Bifidobacterium* probiotic rescued maternal separation-induced changes in neurotransmitter concentrations, behaviour, and corticosterone levels in adult mice (Moya-Pérez et al. 2017). These studies demonstrate that maternal and early postnatal microbiota mediate long-term brain development. Providing the maternal and offspring gut bacteria with BGOS may therefore mitigate the neurobehavioural effects of a neonatal LPS challenge.

5.2 Chapter aims

The aim of this chapter is to test whether consumption of BGOS during pregnancy and suckling influences behaviour, brain gene expression, and plasma metabolite profiles after an early-life exposure to LPS. Generally, I expected the pro-inflammatory LPS challenge and anti-inflammatory BGOS to have opposing effects in offspring. I specifically hypothesised that P9 LPS

increases anxiety and behavioural despair and produces deficits in exploratory and social behaviour, and that maternal prebiotic intake prevents these changes. In the brain, based on the results in young pups in chapter three, I anticipated that maternal BGOS reduces NMDAR subunits and 5-HT_{2A} expression in the PFC and hippocampus, and prevents increased expression of these genes in LPS-treated mice. Correlations between brain gene expression and behaviour would suggest that causal relationships between genes of interest and behaviour should be investigated in future studies in adult offspring following maternal BGOS. Metabolically, I expected maternal BGOS to normalise plasma metabolite profiles, including glucose and acetate, in LPS-treated mice. In saline-injected mice, I hypothesised that maternal prebiotic intake would reduce plasma glucose and increase acetate as it did in pups at weaning.

5.3 Experimental design

Five dams were randomly assigned to receive BGOS during gestation, and six dams received plain drinking water. At postnatal day (P)9, pups in each litter were randomly assigned to receive subcutaneous injections of either 3 mg/kg lipopolysaccharide (LPS) or saline. This age and route of administration were based on previous studies showing brain and behavioural deficits in adulthood (Li et al., 2018; Missig et al., 2018). P9 is roughly equivalent to 39–40 weeks of gestational age in a human (Semple et al. 2013), so this was used as a model of immune activation in a newborn. Because maternal BGOS was not expected to produce measurable changes in healthy adult offspring, the purpose of this challenge was to induce brain, behavioural, and metabolic deficits.

Neonatal body weights were recorded at P9 (before saline/LPS injections), P10, and P14 (N=31–38). Neonatal (P9–P14) body weights were analysed using a two-way ANOVA (F0 diet x P9 treatment) at each timepoint. It should be noted that given the large number of pups at this stage

(N=138 across all groups) and the challenge in keeping track of animals, a repeated measures design was not used. Sex was also not taken into account in neonates. Four litters from each diet were used for the remainder of the study. Adult body weights were recorded before the forced swim test (FST) at ~P56 (N = 10 males, N = 10 females, N = 20 total). Adult body weights (~P56) were analysed using a three-way ANOVA (F0 diet x P9 treatment x sex).

At ~P22, pups were weaned onto normal drinking water and housed with same-sex littermates (2–5/cage). At approximately six weeks of age, offspring were tested in the social interaction test (SIT). Social index was calculated as a percentage of the total exploration time (time in the chambers containing the novel mouse and the novel object) spent in the chamber with the novel mouse. At eight weeks old, the open field test (OFT), light-dark box (LDB), and FST were performed. Mice were culled immediately after the FST. Brain tissue (prefrontal cortex [PFC] and hippocampi) was collected for qPCR gene expression analysis, and plasma was collected for ¹H NMR. Genes for N-methyl-d-aspartate receptor (NMDAR) subunits, 5-hydroxytryptamine (5-HT)_{2A}, and brain-derived neurotrophic factor (BDNF) were analysed. Glucose and acetate were compared between groups using the spectral integral values from 3.76–3.78 ppm and 1.91–1.93 ppm, respectively.

For behaviour and gene expression as well as glucose and acetate NMR signals, parametric or robust three-way ANOVAs were used to explore main effects of and interactions between F0 diet (H₂O or BGOS), P9 treatment (saline or LPS), and sex (male or female). If no two- or three-way interactions with sex were identified, data were consolidated for analysis with a two-way ANOVA. Otherwise, male and female were analysed separately with two-way ANOVAs. A correlation matrix was used to explore associations between adult behaviour and brain gene

expression. Because this analysis was exploratory, correlations were not corrected for multiple comparisons.

A principal component analysis (PCA) was performed for initial visualisation of the NMR data. Orthogonal partial least squares discriminant analysis (OPLS-DA) was used to determine significantly different plasma metabolomes. Models for four pairwise comparisons were built: H₂O/Saline (Sal) vs. BGOS/Sal, H₂O/LPS vs. BGOS/LPS, H₂O/Saline vs. H₂O/LPS, BGOS/Sal vs. BGOS/LPS. Variable importance in projection (VIP) scores were used to identify metabolites with the most variation between groups. The experimental design is summarised in **Figure 5.1**.

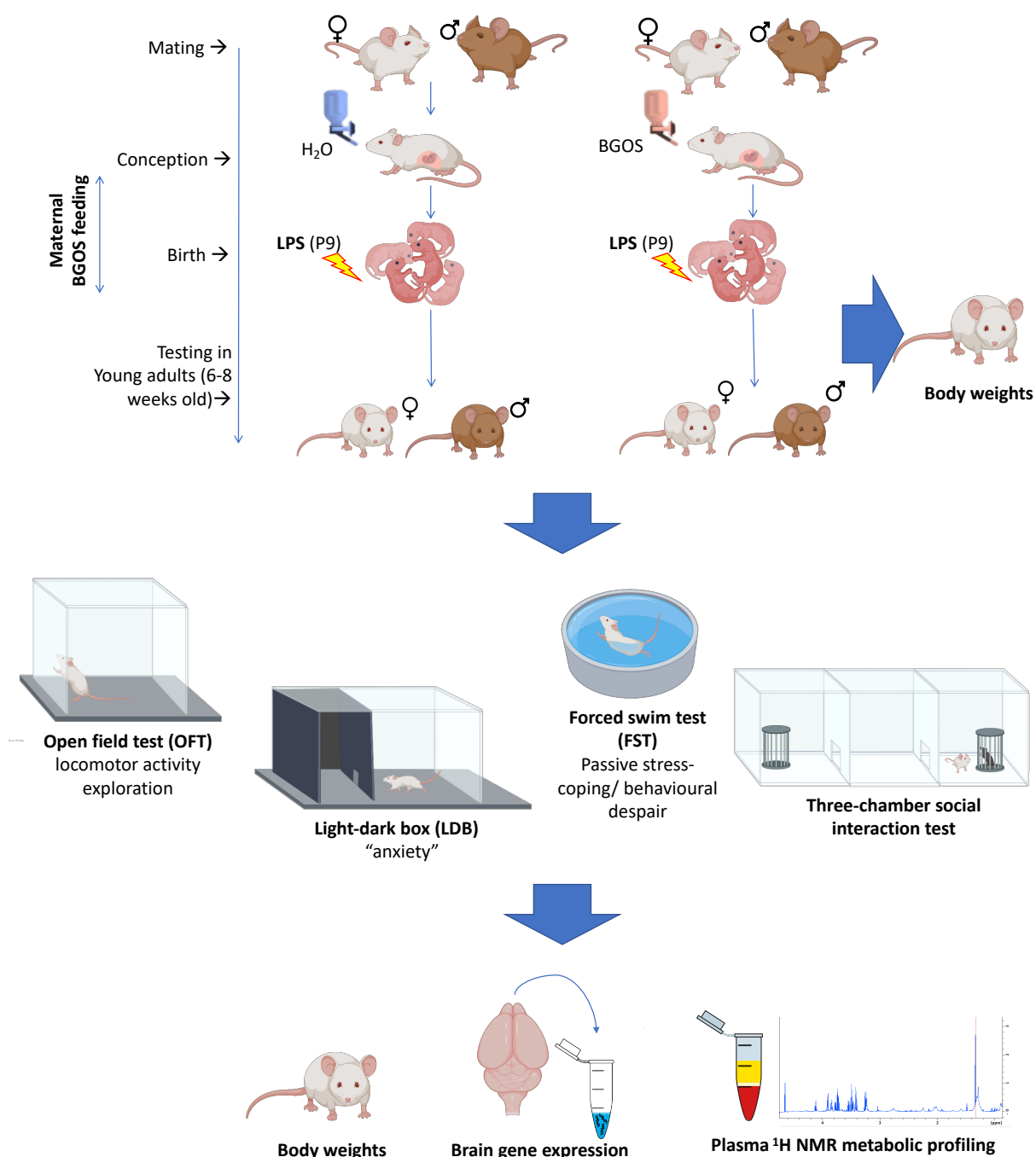


Figure 5.1. Diagram of experimental design.

5.4 Results

5.4.1 LPS reduces body weight in neonates

Neonatal offspring body weights were recorded at P9 (before saline/LPS injections), P10, and P14. At baseline before administration of LPS (age P9), there were no differences between groups – the main effects of F0 diet, $F(1, 135) = 3.22, p = .07$, and P9 treatment, $F(1, 135) = 0.12, p = .73$, and the F0 diet x P9 treatment interaction, $F(1, 135) = 0, p = .99$, were not significant (**Figure 5.2A**). At P10, however, there was a significant main effect of P9 treatment, $F(1, 135) = 20.89, p < .0001$. P10 LPS-treated pups ($M = 6.37, SD = 0.84$) weighed significantly less than saline-treated animals ($M = 7.00, SD = 0.77$). The effects of F0 diet, $F(1, 135) = 2.62, p = .11$, and the interaction between F0 diet and P9 treatment, $F(1, 135) = 0.42, p = .52$, were not significant at P10 (**Figure 5.2B**). Similarly, at P14, there was a significant main effect of P9 treatment on body weight, $F(1, 135) = 35.07, p < .0001$. LPS-treated animals ($M = 7.45, SD = 1.01$) weighed significantly less than saline-treated mice ($M = 8.44, SD = .93$) five days after LPS exposure. There were no significant effects of F0 diet, $F(1, 135) = 0.54, p = .47$, and no F0 diet x P9 treatment interaction, $F(1, 135) = 0.10, p = .75$ (**Figure 5.2C**).

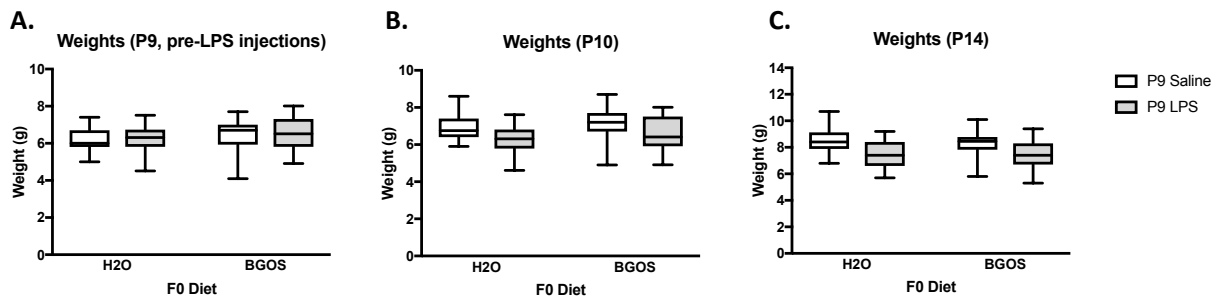


Figure 5.2. P9 LPS results in significant weight loss one and five days post injection. A) At baseline, there were no differences between groups. At both B) P10 and C) P14, significant main effects of P9 treatment showed that pups treated with subcutaneous LPS weighed significantly less than those treated with saline ($p < .0001$ for both P10 and P14). Two-way ANOVA. $N=31-38$ total/group.

In contrast to neonatal offspring, there was no effect of P9 treatment on body weight in adult animals at P56, $F(1, 72) = 0, p = .96$. There was, however a significant effect of F0 diet, $F(1, 72) = 10.86, p < .01$. BGOS offspring ($M = 33.32, SD = 5.85$) weighed significantly less than H₂O offspring ($M = 34.80, SD = 5.40$). The only other significant factor was sex, as expected, $F(1, 72) = 542.46, p < .0001$, given that male mice ($M = 39.28, SD = 2.01$) always weigh more than their female counterparts ($M = 28.85, SD = 2.17$). None of the interactions were significant, including F0 diet x P9 treatment, $F(1, 72) = 0.08, p = .78$, F0 diet x sex, $F(1, 72) = 0.90, p = .34$, P9 treatment x sex, $F(1, 72) = 0.90, p = .35$, and F0 diet x P9 treatment x sex, $F(1, 72) = 0.38, p = .54$ (**Figure 5.3**).

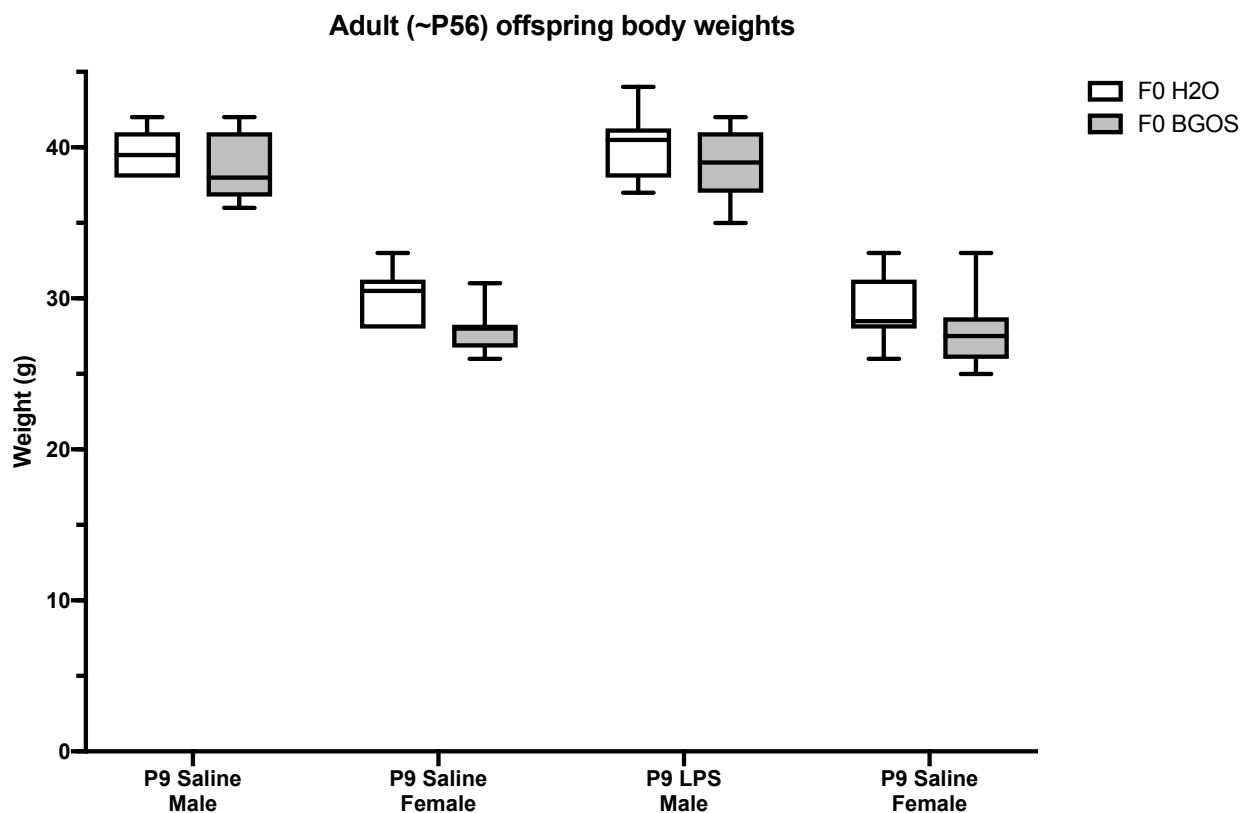


Figure 5.3. Maternal BGOS reduces average body weight in adult offspring. There was a main effect of F0 diet indicating that maternal prebiotic reduced body weights ($p < .01$). Three-way ANOVA. N = 10 males/females; N = 20 total.

5.4.2 Maternal BGOS reduces behavioural despair in the FST, while early-life LPS prevents this change

In the FST, there was a main effect of F0 diet, $F(1, 74) = 5.51, p = .02$, on time floating. BGOS pups ($M = 40.04, SD = 19.10$) spent significantly less time floating than H₂O pups ($M = 58.80, SD = 5.24$). There was no main effect of P9 treatment, $F(1, 74) = 1.50, p = .22$, but there was a significant interaction between the two factors, $F(1, 74) = 4.64, p = .03$. Bonferroni post-hoc tests showed that effect of F0 diet on time floating was dependent on P9 treatment – BGOS/Saline animals spent more time floating than H₂O/Saline animals, $p = .01$, but no other comparisons were significant (**Figure 5.4A**).

There was a main effect of P9 treatment on latency to float in the FST, $F(1, 74) = 5.14, p = .04$. Based on square-root transformed means, animals treated with early-life LPS ($M = 5.27, SD = 0.47$) exhibited a shorter latency to float than pups treated with saline ($M = 7.16, SD = 1.43$). There was no effect of F0 diet, $F(1, 74) = 0.53, p = .47$, and no interaction effect, $F(1, 74) = 2.10, p = .15$ (**Figure 5.4B**).

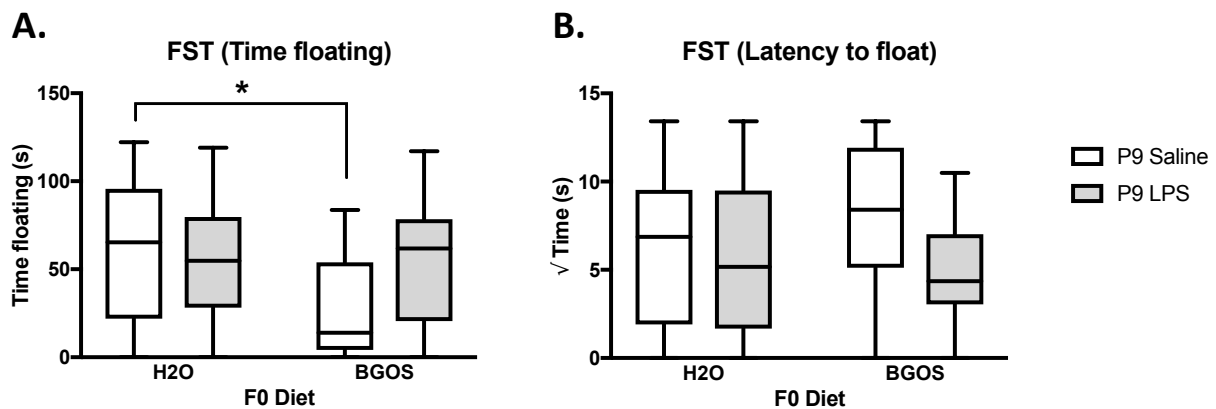


Figure 5.4. Maternal BGOS and early-life LPS have opposing effects on time floating in the FST. A) There was a significant F0 diet x P9 treatment effect on time floating. Post hoc tests showed that maternal BGOS reduced time floating in the FST, while P9 LPS increased floating. **B)** A main effect of P9 treatment indicated that P9 LPS significantly increased latency to float ($p = .04$). Two-way ANOVA, * $p < .05$. N = 10 males/females; N = 20 total/group.

5.4.3 Maternal BGOS has sex-dependent effects on anxiety-like behaviour in the LDB

In the LDB, there was a significant main effect of F0 diet on latency to enter the light side, $F(1, 74) = 6.14, p = .02$. Based on log-transformed means, BGOS pups exhibited a shorter latency to enter the light ($M = 1.07, SD = 0.01$) compared to H₂O pups ($M = 1.20, SD = 0.01$). There was no effect of P9 treatment, $F(1, 74) = 0.61, p = .44$, and no interaction effect, $F(1, 74) = 0.92, p = .34$ (**Figure 5.5A**). There was also a significant effect of P9 treatment on number of rears, $Q = 4.74, p = .04$, based on a robust two-way ANOVA. Offspring treated with early-life LPS reared less in the light side of the LDB than saline-treated pups. There was no effect of F0 diet, $Q = 0, p = .97$, and no interaction effect, $Q = 0.75, p = .49$, on number of rears in the LDB (**Figure 5.5B**). Entries into the light were not affected by F0 diet, $Q = 0.67, p = .42$, P9 treatment, $Q = 0.08, p = .78$, or F0 diet x P9 treatment interaction, $Q = 0.08, p = .79$ (**Figure 5.5C**). Finally, there was a significant F0 diet x sex interaction on time in light in the three-way ANOVA, $F(1, 70) = 13.06, p < .001$. There was also a significant effect of sex, $F(1, 70) = 7.90, p < .01$. No other effects were significant, including F0 diet, $F(1, 70) = 0.04, p = .85$, P9 treatment, $F(1, 70) = 1.26, p = .27$, F0 diet x P9 treatment, $F(1, 70) = 0.24, p = .62$, P9 treatment x sex, $F(1, 70) = 0, p = .98$, and F0 diet x P9 treatment x sex, $F(1, 70) = 1.93, p = .17$. Male and female data were then separated to further examine the F0 diet x sex interaction. In males, there was a significant effect of F0 diet on time in light, $F(1, 34) = 5.80, p = .02$. Male BGOS offspring ($M = 134.67, SD = 3.61$) spent significantly more time in light than male H₂O offspring ($M = 108.10, SD = 15.00$). There was no effect of P9 treatment, $F(1, 34) = 0.53, p = .47$, and no interaction effect, $F(1, 34) = 1.42, p = .24$. In females, there was also a significant effect of F0 diet on time in light, $F(1, 36) = 7.56, p < .01$. In contrast to males, female pups from BGOS-fed dams ($M = 129.05, SD = 9.83$) spent less time in the light

than female H₂O pups ($M = 153.00$, $SD = 0.99$). There was no effect of P9 treatment, $F(1, 36) = 0.77$, $p = .39$, and no interaction effect, $F(1, 36)$, $p = .48$, on time in light in female offspring.

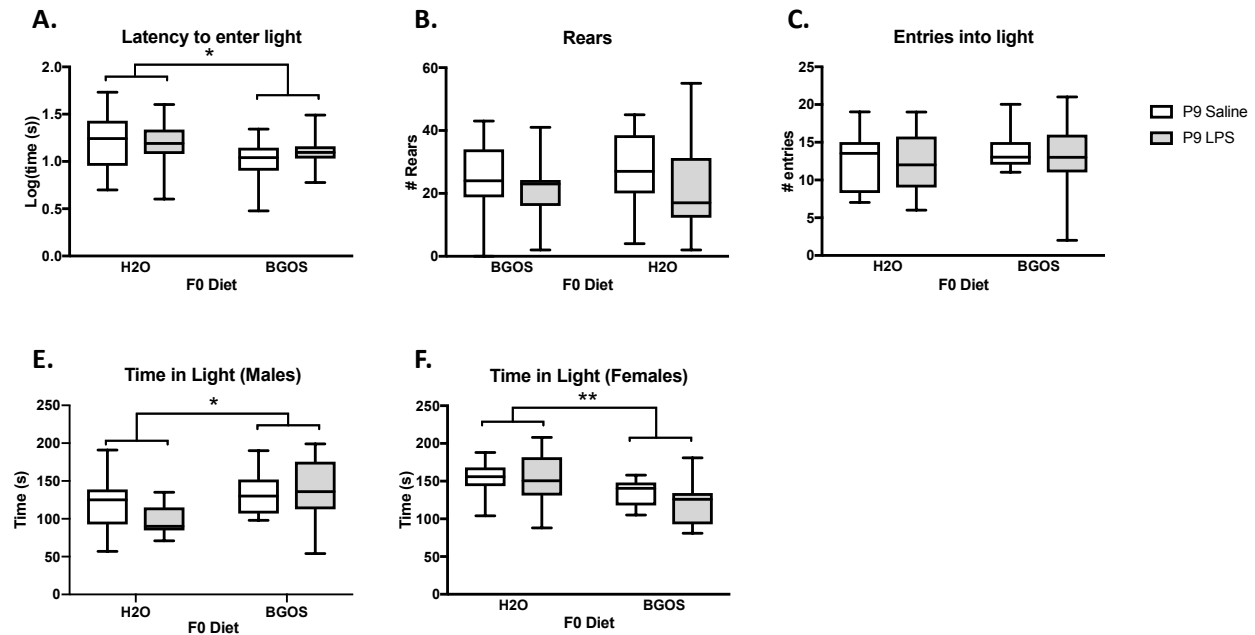


Figure 5.5. Maternal BGOS has long-lasting, sex-dependent effects on anxiety-like behaviour in the LDB. **A)** A significant main effect of F0 diet showed that F0 BGOS reduced latency to enter light in the LDB. **B)** A significant main effect of P9 treatment indicated that P9 LPS reduced rearing behaviour ($p = .04$), but **C)** neither maternal BGOS nor P9 LPS affected entries into the light. Perinatal BGOS had sex-specific effects on time in the light – **E)** in males, a significant main effect of F0 diet showed that maternal BGOS was associated with increased time in the light, but **F)** in females, a significant main effect of F0 diet showed that F0 BGOS resulted in reduced time in light. Two-way ANOVA, * $p < .05$, ** $p < .01$. $N = 9-10$ males/females; $N = 19-20$ total/group.

5.4.4 Maternal BGOS increases social preference in adult offspring

There was a significant main effect of F0 diet on social interaction behaviour, $F(1, 76) = 4.22$, $p = .04$, in the SIT. BGOS offspring spent significantly more time exploring the mouse chamber ($M = 62.85$, $SD = 2.67$) than H₂O pups ($M = 59.27$, $SD = 2.08$) relative to the novel object chamber. There was no effect of P9 treatment, $F(1, 76) = 3.70$, $p = .06$, and no interaction effect, $F(1, 76) = 0.06$, $p = .81$.

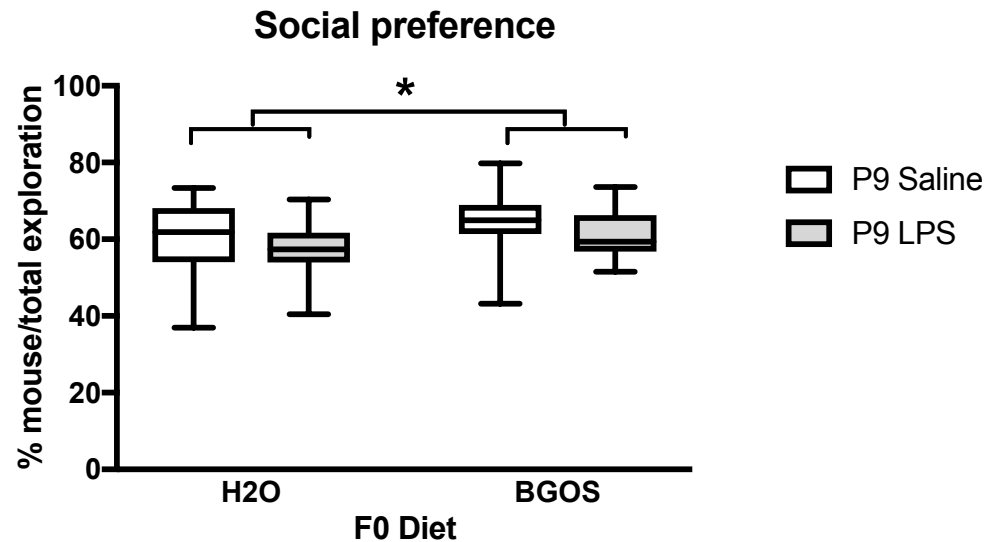


Figure 5.6. Maternal BGOS increases social preference in the SIT. There was a main effect of F0 diet on social preference, which indicated that maternal BGOS increased social preference. Two-way ANOVA, * $p < .05$. $N = 10$ males/females; $N = 20$ total/group.

5.4.5 Maternal BGOS reduces rearing behaviour in the OFT in adult offspring

F0 diet had a significant effect on rearing behaviour in the OFT, $F(1, 76) = 5.64, p = .02$. BGOS offspring ($M = 40.18, SD = 1.74$) reared significantly less than H2O pups ($M = 46.75, SD = 2.90$). P9 treatment did not have a significant effect on rears, $F(1, 76) = 1.40, p = .24$, and there was no interaction effect, $F(1, 76) = 0.09, p = .77$ (**Figure 5.7A**). No significant effects of F0 diet, $F(1, 76) = 1.66, p = .20$, P9 treatment, $F(1, 76) = 1.16, p = .28$, or F0 diet x P9 treatment interaction, $F(1, 76) = 0.88, p = .35$, on distance travelled were found (**Figure 5.7B**). Also, there were no significant main effects of F0 diet, $F(1, 76) = 0.10, p = .75$, or P9 treatment, $F(1, 76) = 0, p = .99$, and no interaction effect, $F(1, 76) = 0.46, p = .50$, on time in centre (**Figure 5.7C**).

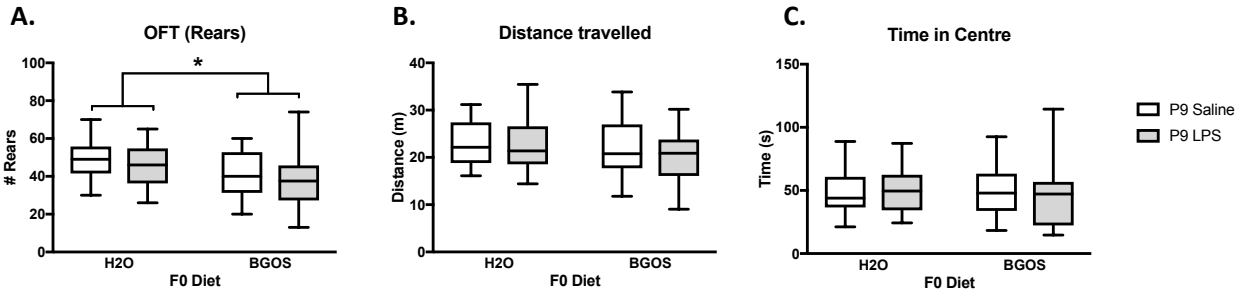


Figure 5.7. Maternal BGOS reduces rearing behaviour in the OFT in adult offspring. A) A significant main effect of F0 diet showed that maternal prebiotic intake resulted in reduced rearing in the OFT. There were no effects of either maternal prebiotic or P9 LPS on B) distance travelled or C) time in centre. Two-way ANOVA (parametric or based on trimmed means for nonparametric data), * $p < .05$. $N = 10$ males/females; $N = 20$ total/group.

5.4.6 Perinatal BGOS has sex-dependent effects on NMDA and 5-HT_{2A} expression in the PFC

In the three-way ANOVA of PFC GluNR1 expression, there was a significant F0 diet x P9 treatment x sex interaction, $F(1, 72) = 10.76$, $p < .01$. There was also a significant F0 diet x P9 interaction effect, $F(1, 72) = 7.00$, $p = .01$. There were no other significant effects or interactions, including F0 diet, $F(1, 72) = 0.25$, $p = .62$, P9 treatment, $F(1, 72) = 1.57$, $p = .21$, sex, $F(1, 72) = 0.15$, $p = .70$, F0 diet x sex, $F(1, 72) = 1.58$, $p = .21$, and P9 treatment x sex, $F(1, 72) = 0.03$, $p = .87$. Two-way ANOVAs for the separate male and female data were performed to further explore the significant three-way interaction. In males, there were no significant main effects of F0 diet, $F(1, 36) = 0.24$, $p = .63$, or P9 treatment, $F(1, 36) = 0.50$, $p = .48$, and no F0 diet x P9 treatment interaction, $F(1, 36) = 0.17$, $p = .68$ (**Figure 5.8A**). In female offspring, however, there was a significant F0 diet x P9 treatment interaction, $F(1, 36) = 21.28$, $p < .0001$. Bonferroni post-hoc tests showed that H₂O offspring treated with P9 saline had significantly lower GluNR1 expression than BGOS offspring treated with saline, $p < .001$. In addition, there was significantly lower GluNR1 expression in BGOS pups administered LPS than BGOS offspring injected with saline, $p < .01$. Neither F0 diet, $F(1, 36) = 1.88$, $p = .17$, nor P9 treatment, $F(1, 36) = 1.22$, $p = .28$, had significant main effects on PFC GluNR1 expression in females (**Figure 5.8B**).

There was a significant F0 diet x P9 treatment interaction effect on PFC GluNR2A, $F(1, 75) = 6.23, p = .01$. There were no main effects of F0 diet, $F(1, 75) = 0.07, p = .80$, or P9 treatment, $F(1, 75) = 0.21, p = .65$. No post-hoc comparisons were significant (**Figure 5.8C**). Similarly, there was a significant interaction on GluNR2B expression, $F(1, 76) = 6.39, p = .01$, and the main effects of F0 diet, $F(1, 76) = 0.02, p = .90$, and P9 treatment, $F(1, 76) = 2.41, p = .12$, were not significant. Bonferroni post-hoc tests revealed a significant difference between BGOS offspring treated with saline and BGOS offspring administered LPS, $p = .03$ (**Figure 5.8D**).

5-HT_{2A} expression was influenced by an F0 diet x P9 treatment x sex interaction in a robust three-way ANOVA test, $Q = 7.07, p = .01$. There was also a significant main effect of F0 diet, $Q = 16.48, p < .001$. The other effects and interactions were not significant, including P9 treatment, $Q = 1.14, p = .30$, sex, $Q = 1.58, p = .22$, sex x F0 diet, $Q = 0.04, p = .84$, sex x P9 treatment, $Q = 3.24, p = .08$, and F0 diet x P9 treatment, $Q = 0.26, p = .61$. In males, the two-way ANOVA showed a significant main effect of F0 diet, $Q = 9.58, p < .01$. There was no effect of P9 treatment, $Q = 0.35, p = .56$, and no interaction, $Q = 2.96, p = .10$. There was lower 5-HT_{2A} expression in male offspring of BGOS-fed dams compared to those from dams given plain water (**Figure 5.8E**). Similarly, there was a significant effect of F0 diet on 5-HT_{2A} expression in females, $Q = 7.42, p = .01$. As with males, expression of 5-HT_{2A} was lower in female BGOS offspring than H₂O offspring. There was no effect of P9 treatment, $Q = 3.36, p = .01$, and no interaction effect, $Q = 4.12, p = .06$ (**Figure 5.8F**).

There was no effect of F0 diet, $F(1, 76) = 1.04, p = .31$, P9 treatment, $F(1, 76) = 1.78, p = .19$, or F0 diet x P9 treatment interaction, $F(1, 76) = 2.91, p = .09$, on PFC BDNF expression (**Figure 5.8G**).

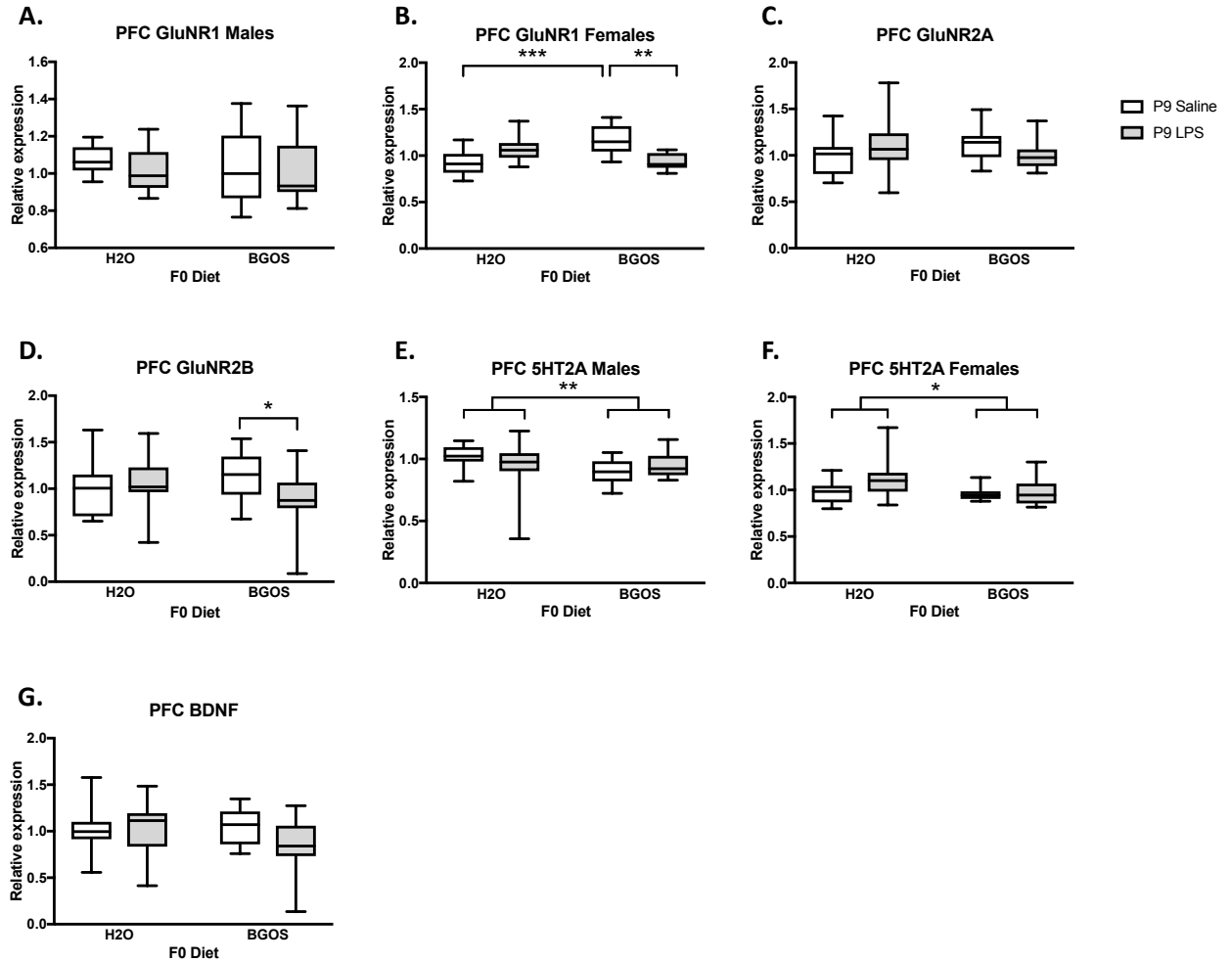


Figure 5.8. Perinatal BGOS produces long-lasting, sex-specific changes in PFC gene expression. **A)** There were no effects of maternal prebiotic intake or P9 LPS on GluNR1 expression in male offspring. **B)** In female offspring, however, there was an F0 diet x P9 treatment effect – post hoc tests showed that maternal BGOS increased GluNR1 expression, while P9 LPS prevented this change. There was a significant F0 BGOS x P9 LPS interaction on **C)** GluNR2A, but no post hoc test were significant. **D)** There was also a significant F0 BGOS x P9 LPS interaction on GluNR2B expression in the PFC. Post hoc tests showed that GluNR2B expression was lower in BGOS/LPS offspring than in BGOS/Saline offspring. Significant main effects of F0 diet showed that 5-HT_{2A} expression was downregulated in **E)** male and **F)** female BGOS offspring. **G)** There were no effects of maternal BGOS or early-life LPS on BDNF expression. Two-way ANOVA (parametric or nonparametric based on trimmed means), * $p < .05$, ** $p < .01$, *** $p < .001$. N = 9–10 males/females; N = 19–20 total/group.

5.4.7 Maternal prebiotic reduces NMDAR subunit expression in the hippocampus

There was a significant F0 diet x sex effect on hippocampal GluNR1 expression in the three-way ANOVA, $F(1, 72) = 5.18, p = .03$. The effects of F0 diet, $F(1, 72) = 3.49, p = .07$, P9 treatment, $F(1, 72) = 1.04, p = .31$, sex, $F(1, 72) = 0.02, p = .88$, F0 diet x P9 treatment, $F(1, 72)$

$= 2.15, p = .15$, P9 treatment x sex, $F(1, 72) = 0.04, p = .84$, and F0 diet x P9 treatment x sex, $F(1, 72) = 0.58, p = .45$, were not significant. Male and female data were separated for two-way ANOVAs. In males, there was a significant effect of F0 diet on GluNR1 expression, $F(1, 36) = 9.70, p < .01$. Male BGOS offspring ($M = 0.92, SD = 0.07$) had lower GluNR1 expression than H₂O offspring ($M = 1.04, SD = 0.02$). There was no effect of P9 treatment, $F(1, 36) = 0.85, p = .36$, or interaction between F0 diet and P9 treatment, $F(1, 36) = 2.80, p = .10$ (**Figure 5.9A**). In females, however, there was no effect of F0 diet, $F(1, 36) = 0.07, p = .79$, P9 treatment, $F(1, 36) = 0.30, p = .59$, or interaction, $F(1, 36) = 0.22, p = .64$ (**Figure 5.9B**). There was also no effect of F0 diet, $F(1, 76) = 0.33, p = .57$, or P9 treatment, $F(1, 76) = 0.27, p = .60$, and no interaction effect, $F(1, 76) = 0.22, p = .64$, on hippocampal GluNR2A expression (**Figure 5.9C**). There was a significant main effect of F0 diet on hippocampal GluNR2B expression, $F(1, 75) = 6.73, p = .01$. GluNR2B mRNA was significantly reduced in BGOS offspring ($M = 0.94, SD = 0.07$) compared to H₂O animals ($M = 1.025, SD = 0.01$). There was no effect of P9 treatment, $F(1, 75) = 2.43, p = .12$, and no F0 diet x P9 treatment interaction, $F(1, 75) = 1.77, p = .19$ (**Figure 5.9D**).

The effects of F0 diet, $F(1, 75) = 1.49, p = .22$, and P9 treatment, $F(1, 75) = 0.49, p = .49$, and the interaction effect, $F(1, 75) = 1.10, p = .30$, on 5-HT_{2A} expression were not significant (**Figure 5.9E**). Similarly, there were no effects of F0 diet, $F(1, 75) = 3.06, p = .08$, or P9 treatment, $F(1, 75) = 0.84, p = .36$, and no F0 diet x P9 treatment interaction, $F(1, 75) = 0.02, p = .89$, on hippocampal BDNF expression (**Figure 5.9F**).

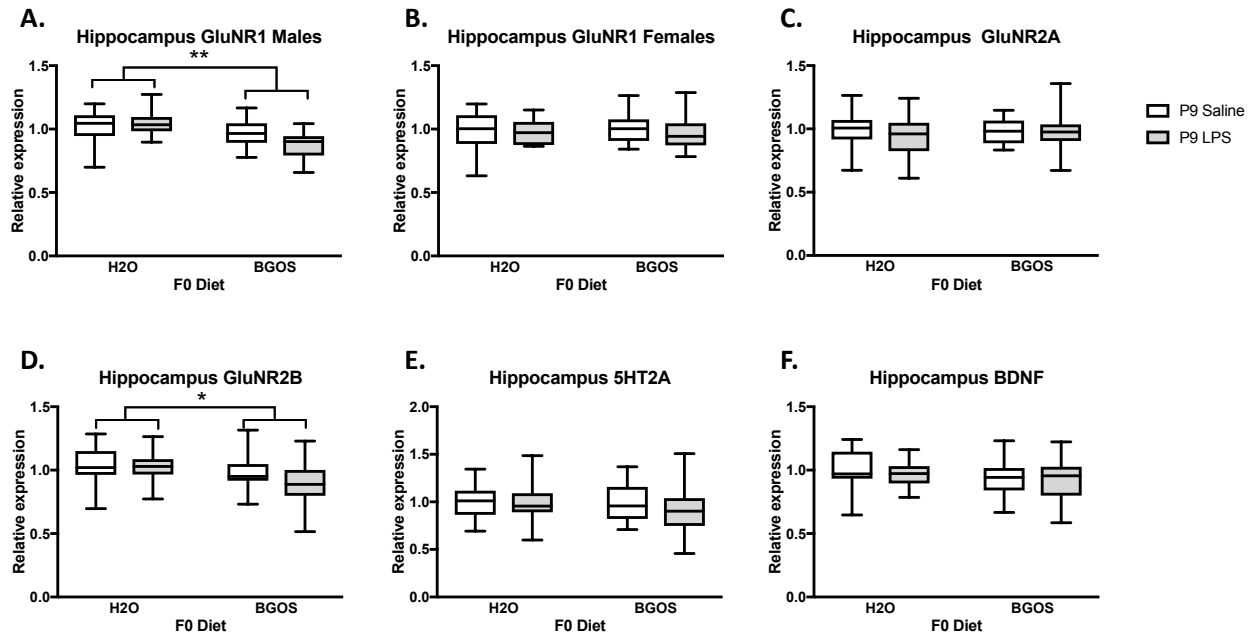


Figure 5.9. Maternal BGOS reduces hippocampal NMDAR subunits. A) Significant main effects of F0 diet showed that maternal BGOS reduced GluNR1 expression in male offspring but B) not in female offspring. C) There was no effect of F0 diet or P9 treatment on hippocampal GluNR2A, but D) significant main effects of P9 treatment showed that perinatal prebiotic intake reduced GluNR2B expression. E) There were no effects of F0 BGOS or P9 LPS on hippocampal 5-HT_{2A} F) or BDNF expression. Two-way ANOVA (parametric or nonparametric based on trimmed means), * $p < .05$, ** $p < .01$. N = 9–10 males/females; N = 19–20 total/group.

5.4.8 Anxiety-like behaviour in the LDB correlates with hippocampal NMDAR expression

Pearson correlations were used to explore associations between gene expression and behavioural outcomes in adult offspring (Figure 5.10). Time in light in the LDB correlated with the most genes in the brain. There were significant negative correlations between time in light and hippocampal gene expression, including GluNR1 ($r(77) = -0.32, p < .01$), GluNR2A ($r(77) = -0.30, p < .01$), GluNR2B ($r(77) = -0.45, p < .0001$), 5-HT_{2A} ($r(77) = -0.25, p = .03$), and BDNF ($r(77) = -0.30, p < .01$). Entries into the light and rears in the LDB also negatively correlated with brain gene expression. Entries correlated with reduced PFC mRNA, including expression of GluNR2A ($r(77) = -0.25, p = .03$), GluNR2B ($r(77) = -0.25, p = .03$), and BDNF ($r(77) = -0.25, p = .04$). Rears were associated with reduced hippocampal expression of GluNR2A ($r(77) =$

$-0.24, p = .04$), GluNR2B ($r(77) = -0.30, p < .01$), and BDNF ($r(77) = -0.25, p = .02$). Finally, distance travelled in the OFT negatively correlated with hippocampal GluNR2B expression ($r(77) = -0.24, p = .03$).

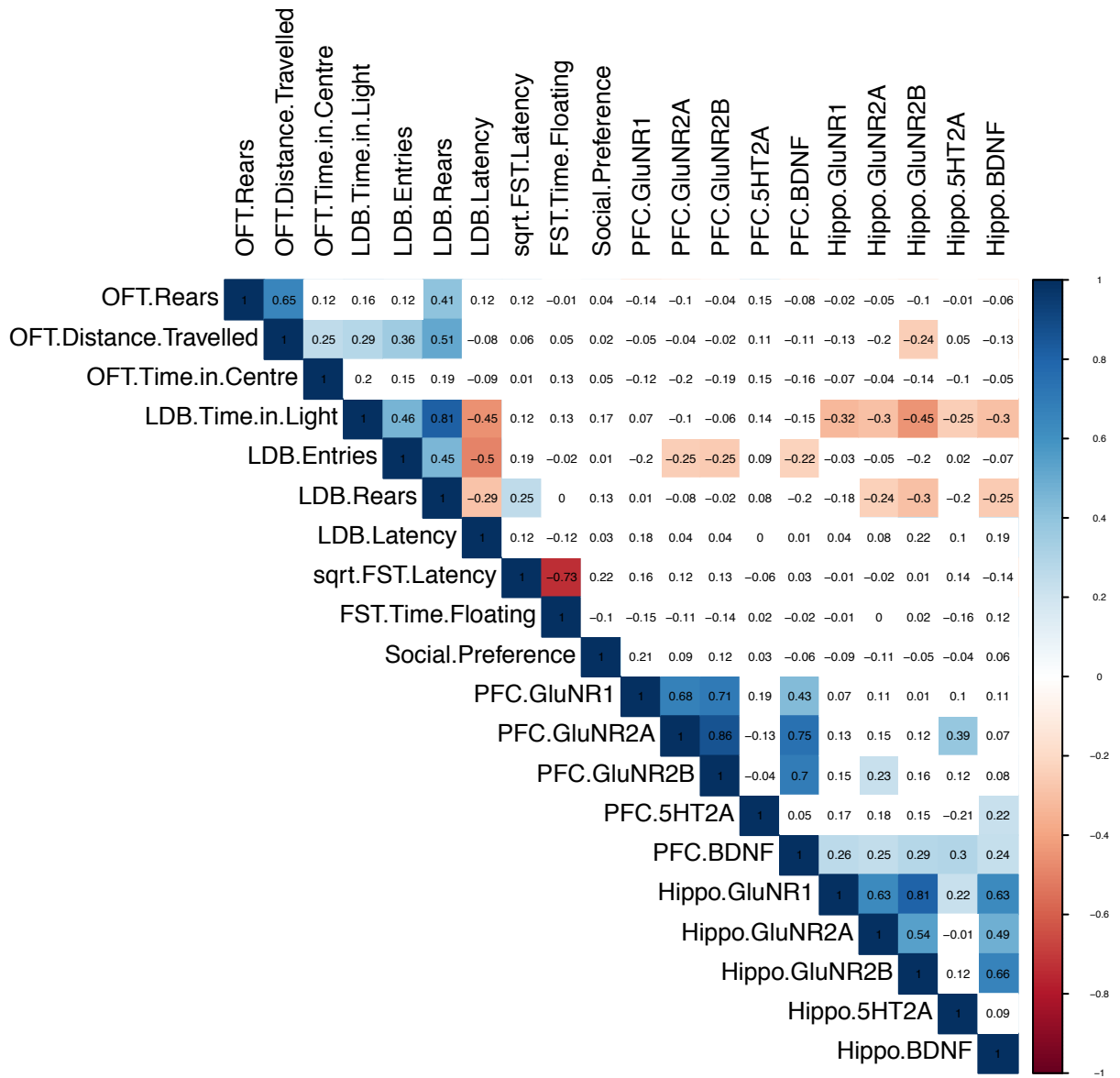


Figure 5.10. Correlation matrix showing associations between behavioural outcomes and gene expression. A correlation matrix of behavioural and central gene expression data is shown. Each square contains Pearson product-moment correlations. The coloured squares correspond to significant correlations, and the colour indicates the direction and magnitude of the correlation according to the scale on the right. N = 9–10 males/females, N = 20 total/group.

5.4.9 Maternal BGOS alters plasma lipid levels in adult offspring

^1H NMR spectral bins were Pareto-scaled and plotted in a PCA plot (**Figure 5.11**). No obvious group clustering was seen, and no data points were removed. According to OPLS-DA, however, the $\text{H}_2\text{O}/\text{Sal}$ vs. BGOS/Sal (**Figure 5.12A**) and $\text{H}_2\text{O}/\text{LPS}$ vs. BGOS/LPS (**Figure 5.12B**) models were significant, indicating that these groups had distinct plasma metabolite profiles. The $\text{H}_2\text{O}/\text{Sal}$ vs. $\text{H}_2\text{O}/\text{LPS}$ model (**Figure 5.12C**) and the BGOS/Sal vs. BGOS/LPS model (**Figure 5.12D**), however, were not significant. The results of the OPLS-DA analysis, including accuracies and R^2X , R^2Y , and Q^2 scores, are summarised in **Table 5.1**. Top VIP scores for the $\text{H}_2\text{O}/\text{Sal}$ vs. BGOS/Sal (**Figure 5.13A**) and $\text{H}_2\text{O}/\text{LPS}$ vs. BGOS/LPS models (**Figure 5.13B**) were identified, and metabolites were assigned to the top bins (summarised in **Table 5.2** and **Table 5.3**, respectively). All top-ranking bins fell in lipid regions. Lipoprotein-associated lipid signals in the 0.84–0.90 ppm and 1.26–1.36 ppm regions were roughly divided into lipid signals associated with chylomicron (Chyl)/very low-density lipoprotein (VLDL) and low-density/high-density lipoproteins (HDL/LDL) (Liu, Tang, Nicholson, & Lindon, 2002; Soininen et al., 2009). Similar bins were identified for the $\text{H}_2\text{O}/\text{Sal}$ vs. BGOS/Sal and $\text{H}_2\text{O}/\text{LPS}$ vs. BGOS/LPS models. Bin integral values were plotted in a heat map to compare values between groups (**Figure 5.14**). Signal intensity in peaks corresponding to Chyl/VLDL-associated lipids were lower in offspring of BGOS-fed dams (BGOS/Sal and BGOS/LPS) than H_2O offspring ($\text{H}_2\text{O}/\text{Sal}$ and $\text{H}_2\text{O}/\text{LPS}$). In contrast, signals corresponding to HDL/LDL were elevated in BGOS offspring compared to H_2O offspring.

Data were also analysed separately for males and females to confirm that there were not any sex-specific effects of maternal prebiotic on offspring plasma metabolite profiles. The separate

results for male and female offspring were similar to the results for all data combined. The full adult male and female OPLS-DA analysis, as well as boxplots comparing the integrals at each bin between groups, is found in **Appendix E**.

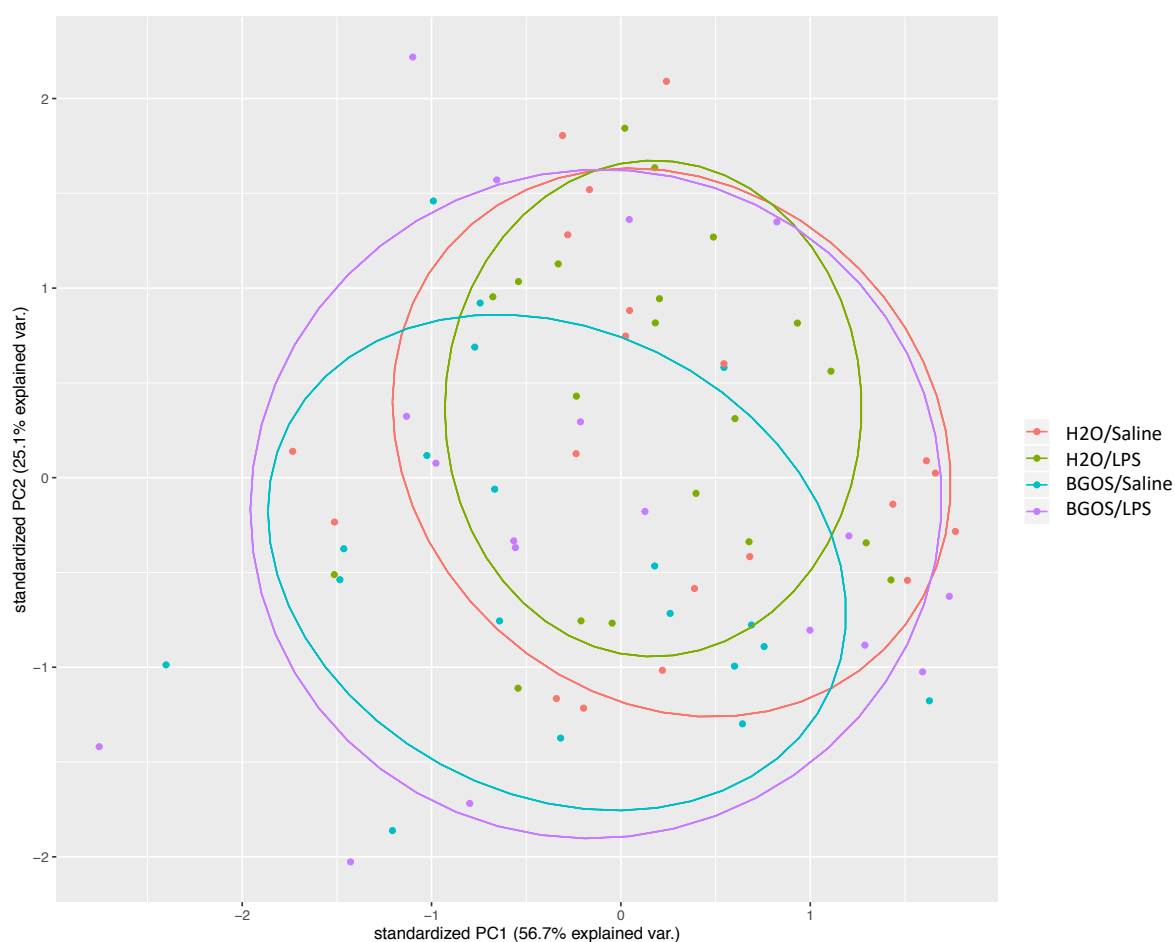


Figure 5.11. PCA plot of Pareto-scaled ¹H NMR spectral bins. PC1 and PC2 are plotted. N = 8–10 males/females; N = 18–20 total/group.

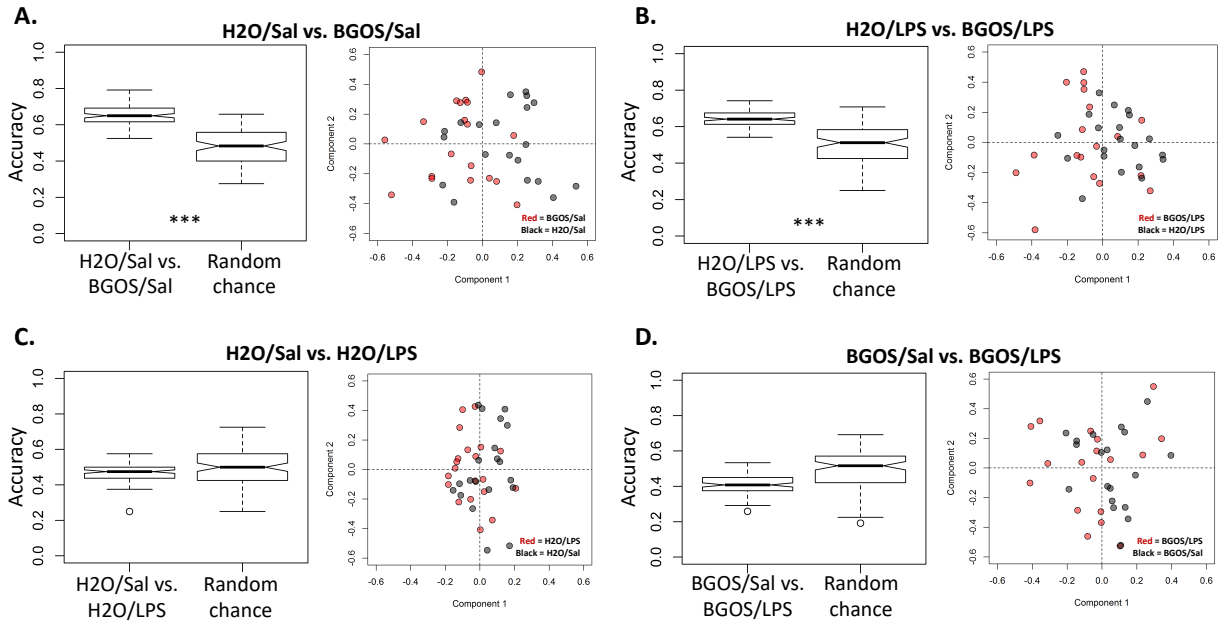


Figure 5.12. OPLS-DA model accuracies and representative scores plots. Accuracy of ensemble of 1000 OPLS-DA models (left) and representative scores plots (right) are shown for **A)** H₂O/Sal vs. BGOS/Sal, **B)** H₂O/LPS vs. BGOS/LPS **C)** H₂O/Sal vs. H₂O/LPS, and **D)** BGOS/Sal vs. BGOS/LPS. Kolmogorov-Smirnov, *** $p < .001$. $N = 8-10$ males/females; $N = 18-20$ total/group.

Table 5.1. Summary of OPLS-DA results.

Group A	Group B	Average accuracy of A vs. B models $\pm$ SD*	Significantly better performance than random chance?	Average $R^2 X \pm$ SD*	Average $R^2 Y \pm$ SD*	Average $Q^2 \pm$ SD*
H2O/Saline (n = 20)	BGOS/Saline (n = 19)	65.2 $\pm$ 5%	Yes	0.84 $\pm$ 0.01	0.44 $\pm$ 0.08	0.18 $\pm$ 0.05
H2O/LPS (n = 20)	BGOS/LPS (n = 18)	64.4 $\pm$ 5%	Yes	0.87 $\pm$ 0.01	0.60 $\pm$ 0.03	-0.17 $\pm$ 0.11
H2O/Saline (n = 20)	H2O/LPS (n = 20)	47.2 $\pm$ 5%	No	0.87	0.35 $\pm$ 0.01	-0.53 $\pm$ 0.10
BGOS/Saline (n = 19)	BGOS/LPS (n = 18)	41.1 $\pm$ 5%	No	0.89	0.35 $\pm$ 0.02	-0.63 $\pm$ 0.12

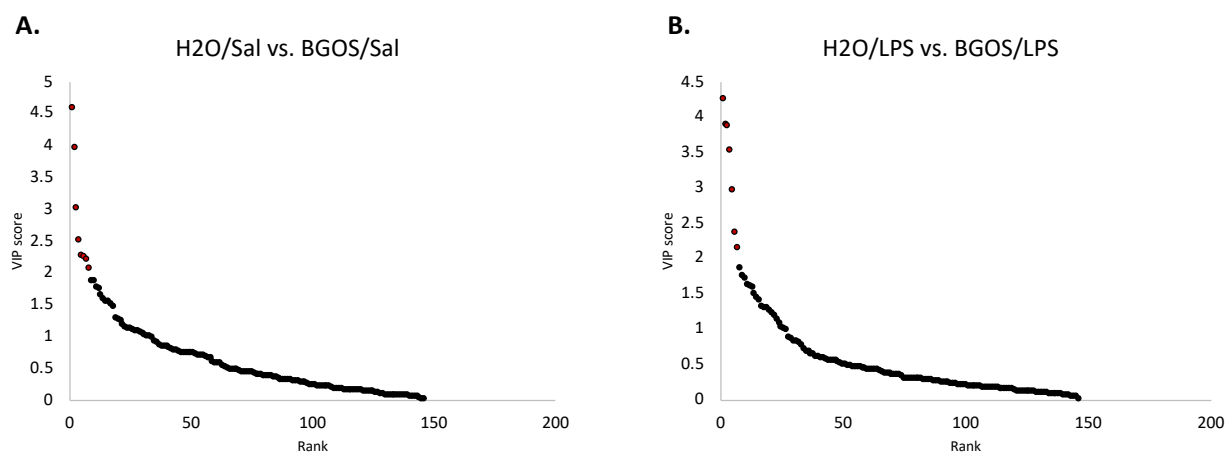


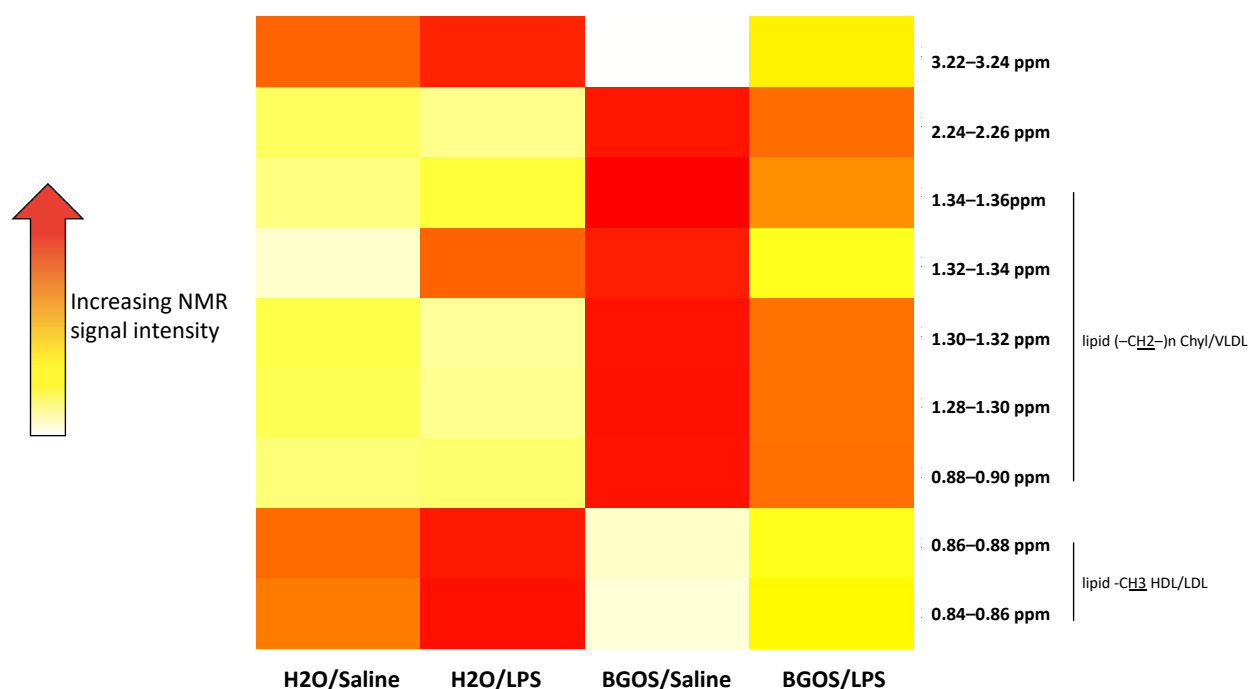
Figure 5.13. VIP scores and rank for spectral bins. Plots of all spectral bins and VIP assignments for **A)** H₂O/Sal vs. BGOS/Sal and **B)** H₂O/LPS vs. BGOS/LPS. Top bins are in red.

Table 5.2. Top bins and corresponding VIP scores and metabolites assignments for H₂O/Saline vs. BGOS/Saline.

H2O/Sal vs. BGOS/Sal		
Bin	VIP score	Assignment
1.28–1.30 ppm	4.5890309	lipid (-CH ₂ -)n Chyl/VLDL
1.30–1.32 ppm	3.97084306	lipid (-CH ₂ -)n Chyl/VLDL
3.22–3.24 ppm	3.02245966	lipid
0.88–0.90 ppm	2.5212338	lipid (-CH ₃) Chyl/VLDL
0.84–0.86 ppm	2.27297212	lipid (-CH ₃) HDL/LDL
1.32–1.34 ppm	2.2458549	lactate/lipid (-CH ₂ -)n
2.24–2.26 ppm	2.2096544	lipid
1.34–1.36 ppm	2.08051635	lipid (-CH ₂ -)n Chyl/VLDL

Table 5.3. Top bins and corresponding VIP scores and metabolites assignments for H₂O/LPS vs. BGOS/LPS.

H ₂ O/LPS vs. BGOS/LPS		
Bin	VIP score	Assignment
1.28–1.30 ppm	4.26767165	lipid (-CH ₂ -)n Chyl/VLDL
3.22–3.24 ppm	3.90385494	lipid
1.30–1.32 ppm	3.87448167	lipid (-CH ₂ -)n
0.84–0.86 ppm	3.5362072	lipid (-CH ₃) HDL/LDL
0.86–0.88 ppm	2.97303842	lipid (-CH ₃) HDL/LDL
0.88–0.90 ppm	2.36454717	lipid (-CH ₃) Chyl/VLDL
2.24–2.26 ppm	2.14964805	lipid

**Figure 5.14. Heat map comparing spectral bins and corresponding metabolites between groups.** To visualise group differences in metabolite levels, spectral bins with top VIP scores in at least one of the significant OPLS-DA models were used to construct a heat map.

5.4.10 Perinatal prebiotic has no effect on plasma acetate or glucose in adult offspring

Glucose and acetate peaks were compared between groups. There were no effects of F0 diet, $F(1, 73) = 2.93$, $p = .09$, P9 treatment $F(1, 73) = 0.03$, $p = .86$, or interaction, $F(1, 73) = 0.32$,

$p = .58$, on glucose levels (**Figure 5.15A**). There were also no effects of F0 diet, $F(1, 73) = 1.22$, $p = .27$, P9 treatment, $F(1, 73) = 0.16$, $p = .70$, or interaction, $F(1, 73) = 0.01$, $p = .91$, on acetate levels (**Figure 5.15B**).

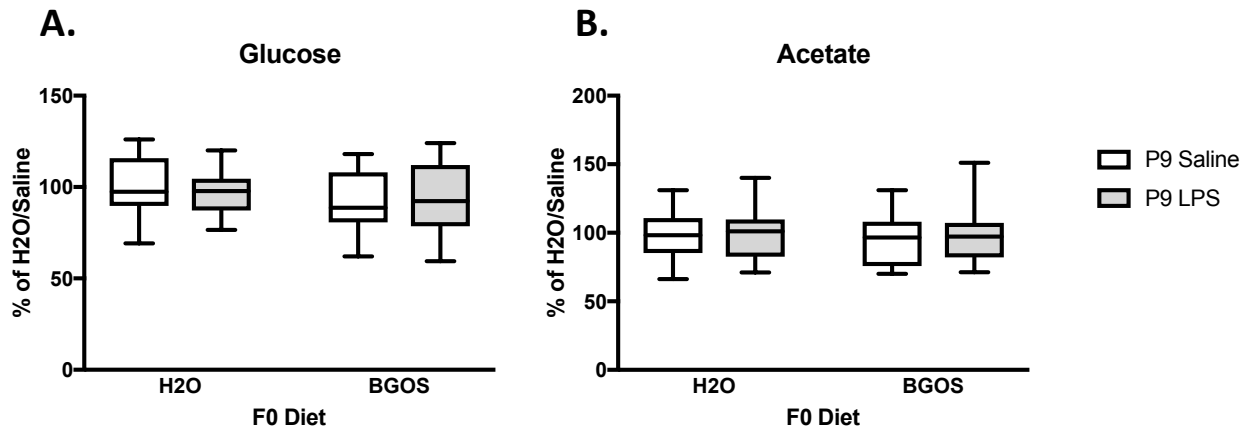


Figure 5.15. Maternal BGOS effects on offspring plasma glucose and acetate. Maternal BGOS does not alter plasma levels of **A**) glucose or **B**) acetate in adult offspring. Two-way ANOVA. $N = 8$ – 10 males/females; $N = 18$ – 20 total.

5.5 Discussion

5.5.1 Summary of results

The effects of maternal prebiotic intake and early-life inflammation in offspring last into adulthood. Body weights were reduced in BGOS adult offspring, which is consistent with the results in Chapter 3. Maternal prebiotic intake also had long-lasting effects on behaviour, some of which were sex-dependent. Behavioural despair in the FST in all offspring was reduced by maternal BGOS, while this change was prevented by early-life LPS. In the LDB, however, although maternal BGOS reduced latency to enter the light, its effects on time spent in the light were dependent on the sex of the offspring. Maternal BGOS increased social preference in adult offspring. In the OFT, BGOS offspring displayed significantly fewer rears than H₂O animals, which contrasts with the finding in young offspring. Perinatal BGOS feeding also had long-lasting

and sex-specific effects on NMDAR subunit and 5-HT_{2A} expression in the brain. Finally, maternal BGOS intake changed plasma metabolite profiles in adult offspring, and this difference was primarily driven by differences in lipid composition in adult offspring. Acetate and glucose, however, did not change.

5.5.2 LPS exposure causes weight loss in neonates, while maternal prebiotic intake reduces body weight in adult offspring

P9 LPS resulted in significant weight loss both one and five days after the injection. This reduced body weight was expected based on previous studies reporting weight loss after LPS due to diminished food-motivated behaviour and food intake (Bluthé et al. 1999; Bret Dibat et al. 1995; Y. H. Liu et al. 2015). Previous studies suggest that reduced maternal care towards the LPS-treated pups was not a likely cause of the weight loss (Spencer et al. 2006). This result provides evidence that the subcutaneous injection was effective in producing an inflammatory response and sickness behaviour. Additional tests, such as measuring peripheral cytokines in the hours and days after LPS exposure, would be helpful to confirm this. Body weight normalised in adulthood, indicating that development was mostly normal after the initial inflammatory challenge. It is also important to note that all litters consisted of co-housed LPS- and saline-treated pups. Co-housing and exposure to the dam's microbes were therefore insufficient to prevent the short-term weight loss after LPS. This might be because nine days of postnatal exposure to the maternal BGOS was insufficient to blunt the pro-inflammatory response. Long-term brain and behavioural effects of early-life LPS after the full three weeks of maternal BGOS feeding during suckling, however, were prevented by maternal BGOS intake.

There was no effect of F0 BGOS on neonatal body weights. In adulthood, however, BGOS offspring weighed significantly less than H₂O offspring. This is consistent with the results in

chapter four showing that young BGOS pups weighed less than H₂O offspring from P7 to P21. As discussed in the previous chapter, the gut microbiome is involved in body mass index (BMI) regulation and energy homeostasis. It is possible that maternal prebiotic-induced changes in the offspring gut microbiome are long-lasting and result in reduced body weight in adulthood.

5.5.3 Maternal BGOS has long-lasting sex-dependent effects on behaviour

Perinatal prebiotic intake has a variety of effects on behaviour in adult offspring. Some of these changes were consistent with our original hypotheses, while others were unexpected. In the FST, for example, maternal BGOS alone reduced floating time as anticipated. Importantly, this shows that the attenuating effects of maternal prebiotic on behavioural despair are long-lasting. Early-life LPS alone did not change behaviour in the FST, but it did prevent the BGOS-induced decrease in floating time. P9 LPS alone may have been insufficient to induce behavioural despair, but the effects were revealed when the early-life LPS was combined with maternal BGOS. Although latency to float data did not result in a significant interaction, it showed a similar pattern to the time floating data. Therefore, maternal prebiotic may produce behavioural changes in the FST through modification of the offspring immune system. This is consistent with the findings in Chapter 4 where BGOS pups had altered composition of white blood cells and a blunted pro-inflammatory phenotype in M1 bone marrow-derived macrophages. In addition, early-life immune activation is associated with both increased inflammation and behavioural deficits in adulthood (Custódio et al., 2018; Harré et al., 2008; Li et al., 2018; Missig et al., 2018). Therefore, P9 LPS may diminish the benefits of perinatal BGOS by reversing its anti-inflammatory effects.

The results of the LDB test were more complex. My hypothesis was supported by the observation that latency to enter the light was shorter in BGOS offspring compared to H₂O progeny, although there were no effects of BGOS or P9 LPS on entries into the light or number of

rears. Effects of maternal prebiotic on time in light, however, differed in male and female offspring. While the expected increase in time spent in the light by BGOS animals was observed in male offspring, female mice spent less time in the light side. Similarly, in the OFT, maternal prebiotic reduced exploratory behaviour. Based on the findings in Chapters 3 and 4, I had indeed expected rearing frequency to change. In young offspring, however, maternal BGOS intake increased rears, while the opposite was found in adult animals.

The increase in social interaction in the SIT in BGOS offspring was consistent with our original hypothesis. Galacto- and fructo-oligosaccharide (GOS and FOS) supplementation promoted social behaviour in chronically-stressed mice (Burokas et al. 2017) and a combination of BGOS and gluten-restriction improved social skills in children with autism spectrum disorder (Grimaldi et al. 2018). This is the first study, however, showing a transgenerational effect of prebiotic intake. Changes in the offspring microbiome after maternal prebiotic feeding may influence the development of social behaviour (Sherwin et al. 2019). In addition, although the effect of P9 LPS on social interaction was just above the threshold for significance, the trend towards reduced social behaviour was also in the direction that I expected based on previous studies (Li et al., 2018). These results indicate that maternal prebiotic intake and early-life inflammation have opposing effects on social behaviour in adult offspring that may reflect disparate effects of the two treatments on the neonatal immune system.

Overall, perinatal prebiotic feeding tended to produce similar changes in behaviour in adult offspring as in young pups in the FST and LDB. Some behaviours, such as time spent in light in female offspring and rearing in the OFT, changed in the opposite direction to those in young progeny. Nevertheless, the fact that the same behaviours differed in adult offspring as in pups at weaning supports the theory that the neural circuits underlying these behaviours are modified by

maternal prebiotic feeding. Furthermore, sex-dependent effects of maternal diet and dietary supplements on adult offspring behaviour and gene expression have been reported previously (McKee et al. 2018; Keleher et al. 2018; Ye et al. 2018; Surzenko et al. 2019). The sex differences in anxiety-like behaviour may be explained by an interaction between exposure to maternal BGOS in early life and estrogen and estrogen receptors, which are involved in anxiety-like behaviour (Eid et al. 2019). The increase in cortical GluNR1 may also leave females susceptible to anxiety-like behaviour (Victoriano et al. 2019). In summary, maternal BGOS feeding may induce long-lasting changes in the neonatal brain that alter these behaviours, although the exact phenotype is not consistent across age and sex.

5.5.4 Perinatal prebiotic intake has sex-specific effects on gene expression in the PFC and hippocampus in adult offspring

I had hypothesised that maternal prebiotic intake prevents increases in cortical and hippocampal NMDAR expression after early-life LPS exposure in adult offspring based on previous studies (Harré et al. 2008) and the gene expression results in Chapter 3. Maternal BGOS, however, tended to upregulate cortical NMDAR expression in offspring, while early-life LPS prevented this increase. This F0 diet x P9 treatment interaction was true for GluNR2A and GluNR2B expression in both sexes, but GluNR1 was only altered by F0 BGOS and P9 LPS in female offspring. PFC GluNR1 did not differ between groups in male offspring. Similar to some behavioural results, this result reflected a change in direction between weaning age and adulthood. In contrast, in the hippocampus, maternal prebiotic reduced GluNR2B expression in both sexes, but only reduced GluNR1 in males. While P9 LPS did not affect NMDAR expression in the hippocampus, maternal prebiotic did reduce expression as it did in young offspring in Chapter 3.

Overall, the effects of maternal BGOS on both brain NMDAR subunit expression and behaviour are sex- and age-dependent.

Cortical 5-HT_{2A} expression was downregulated in male and female BGOS offspring. Although I had hypothesised that maternal prebiotic intake would normalise upregulated 5-HT_{2A} in offspring treated with LPS as it did in adult CD1 mice (Savignac et al., 2016), this overall decrease in BGOS offspring is consistent with our findings in chapter three in pups at weaning. In contrast to expression in the PFC, maternal BGOS did not change 5-HT_{2A} mRNA in the hippocampus in adult offspring. This result indicates that although hippocampal 5-HT_{2A} was reduced in young offspring at weaning, this change was not long-lasting.

In summary, some of the same genes with altered expression levels in young BGOS pups were also differentially expressed in adulthood. In adult offspring, however, expression of cortical NMDAR subunits changed direction in adult BGOS offspring, while hippocampal NMDAR subunits and 5-HT_{2A} showed similar expression patterns as young offspring. Also, NMDAR subunit expression was sex-specific. Although further experiments are needed to determine the functional significance of these gene expression changes, these results indicate that the effects of perinatal BGOS intake on the brain are long-lasting. It is possible that a microbe-derived epigenetic modifier is taken up by the neonatal brain and induces enduring epigenetic changes. Changes in such metabolites in offspring will be investigated in the next chapter.

5.5.5 Hippocampal NMDAR subunit expression was associated with anxiety-like behaviour in the LDB

A correlation matrix was used to explore potential associations between gene expression and behaviour. There were strong negative correlations between NMDAR subunit expression and time spent in the light side of the LDB. Interestingly, young pups displayed the same negative

association at weaning age. Although false positives are likely in tests involving multiple comparisons, the *p* value for the correlation coefficient between GluNR2B expression and time in light in the adult offspring was low enough that it would still be significant even after applying a correction (e.g. Bonferroni). This, in addition to the replication of the correlation found in young pups, suggests that reduced hippocampal expression may be causally related to anxiety in the LDB and should be investigated in future studies using genetic knockout mice or pharmacological methods.

It is noteworthy that rearing behaviour in the OFT did not correlate with other behavioural outcomes, with the exception of rearing in LDB. This is in contrast to the associations between OFT rears and every other behavioural test in young offspring in Chapter 3. This suggests that rearing behaviour in weaning-age animals is more closely linked to anxiolytic behaviour than in adult animals and therefore should not be interpreted in the same manner in young and adult mice. This finding may also explain the discrepancy between rearing behaviour in young and adult offspring in this study.

5.5.6 Maternal BGOS produces long-term changes in the plasma metabolome

¹H NMR was used to identify overall changes in plasma metabolite profiles and specific differentially abundant metabolites. Maternal prebiotic intake produced significant differences in the plasma metabolic “fingerprint” of adult offspring. The metabolites that were associated with top VIP scores were in the lipid regions. Specifically, I found that perinatal BGOS reduced HDL/LDL-associated lipids and increased Chyl/VLDL-associated lipids. Differentiating lipoproteins beyond this level of specificity would require additional experiments. A reduced LDL/HDL ratio, however, is considered to be beneficial for cardiovascular and metabolic health (Ference et al. 2017; Klop, Elte, and Cabezas 2013). Therefore, maternal BGOS may have

metabolic benefits for adult offspring and should be explored in the context of metabolic disorder models. Furthermore, LDL and HDL levels are associated with anxiety and depression (Fang et al., 2013; Huang, Wu, Chiang, & Chen, 2003; Van Reedt Dortland et al., 2010) and may have consequences for the brain and behaviour in adult offspring.

Based on the ^1H NMR results in Chapter 3, I tested glucose and acetate signals using univariate analysis. Glucose and acetate, which were respectively reduced and elevated in young BGOS pups, did not differ in adult BGOS offspring compared to controls. Glucose homeostasis and microbial acetate production may normalise when pups are weaned to normal drinking water, suggesting that these changes are dependent on direct exposure to maternal BGOS. It should be noted that these adult animals underwent the FST approximately two hours before being culled, while the cohort of weaning-age pups that were tested for metabolic differences in Chapter 4 did not do the FST. This stressor may have masked differences in some metabolites. Nevertheless, acetate or other microbial factors may cause brain and immune changes in young pups (Frost et al., 2014; Smith et al., 2013; Soliman et al., 2012; van de Wouw et al., 2018) that endure after levels of these compounds or microbes have normalised.

5.5.7 Conclusion

Maternal prebiotic intake has sex- and age-dependent effects on behavioural despair, anxiety-like behaviour, social preference, and brain NMDAR and 5-HT_{2A} gene expression in progeny. It reduces passive stress-coping behaviour and increases sociability in all offspring, while it primarily has anxiolytic effects in male offspring. Maternal BGOS tends to increase NMDAR subunit expression in the PFC and decrease expression in the hippocampus. It also alters plasma lipid composition in adult offspring. The final chapter will aim to identify candidate microbial taxa and metabolites that mediate the changes in young and adult offspring.

6. Maternal prebiotic intake alters the gut microbiome in young offspring

6.1 Introduction

The aim of this chapter is to determine whether maternal BGOS intake changes gut microbiome metabolite production, composition, and diversity in dams, young offspring, and adult offspring.

Modulation of the gut microbiome by BGOS intake has already been demonstrated to increase absolute levels of *Bifidobacteria*, *Lactobacilli*, and the *Eubacterium rectale-Clostridium coccoides* group and decreases in the *Bacteroides-Prevotella* group *in vitro* and *in vivo*. In addition, fermentation of BGOS produces short-chain fatty acids (SCFAs) (Grimaldi, Swann, Vulevic, Gibson, & Costabile, 2016; Liu et al., 2016), and SCFAs and other microbe-derived metabolites are important for brain, immune, and metabolic health (Arpaia et al., 2013; De Vadder et al., 2014; Lin et al., 2012; Soliman et al., 2012; van de Wouw et al., 2018). The effects of maternal BGOS supplementation on the offspring gut microbiome, however, and the potential link between the offspring microbiome and brain are unknown. Offspring gut microbiome composition closely resembles that of the maternal gut (Bäckhed et al. 2015; Ferretti et al. 2018; Funkhouser and Bordenstein 2013). In rodents, this is due to transmission of microbes through co-housing and coprophagia (Friswell et al. 2010; Jašarević et al. 2017; Ubeda et al. 2012) as well as consumption of probiotic- and prebiotic-rich breastmilk (Shen et al. 2011). Therefore, exposure to an altered maternal microbiome may modify the offspring microbiome and, in turn, the offspring brain.

6.2 Chapter aims

The purpose of this chapter is to use metabolomics and genomic approaches to identify candidate compounds and microbes that may mediate the effects of maternal prebiotic intake on

the offspring brain, immunity and metabolism that were demonstrated in the previous chapters. In addition, given that BGOS increases levels of *Bifidobacterium* in rodents and humans, I hypothesised that the relative abundance of faecal *Bifidobacterium* is higher in both dams and young pups through direct contact with maternal microbes. Furthermore, because BGOS is fermented by gut microbes to produce short-chain fatty acids (SCFAs), I also hypothesised that BGOS increases levels of faecal acetate, butyrate, and propionate in dams, young pups, and adult offspring. I used ^1H nuclear magnetic resonance (NMR) to explore changes in SCFAs and other faecal metabolites as a result of prenatal and postnatal maternal prebiotic supplementation in the cross-fostered cohort. Finally, I hypothesised that differentially abundant microbial factors, including taxa and metabolites, correlate with brain gene expression and behaviour outcomes.

6.3 Experimental design

A schematic of the experimental design is shown in **Figure 6.1**. Although most studies that examine host-microbiome interactions focus on sequencing faecal DNA, both sequencing and metabolic profiling are important – analysing DNA with 16S sequencing addresses the question, “Who is there?”, while metabolomics answers, “What are they doing?”. For faecal metabolic profiling, a pilot study was performed to verify that the extraction technique produced reliable and reproducible results (**Appendix F**). Faecal extracts from the following animals were then collected for ^1H NMR: BGOS-fed and control dams (Chapter 4 and 5 cohorts, $N = 10\text{--}12/\text{group}$), pups at weaning age (Chapter 3/4 cross-fostered cohort, $N = 6\text{--}8$ males/females, $N = 12\text{--}14$ total/group), and adult offspring (Chapter 5, $N = 9\text{--}10$ males/females, $N = 19\text{--}20$ total/group). Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were performed on Pareto-scaled integrals of 0.02 parts per million (ppm) bins in Nuclear Overhauser effect spectroscopy (NOESY) spectra. A summary of the OPLS-DA models in this

chapter can be found in **Table 6.1**. Male and female mice were combined for all analysis in young pups because of the low group numbers of males and females alone. In adult offspring, the combined data for males and females are presented in this chapter, while the separate analyses for males and females can be found in **Appendix E**.

Metabolite identification was attempted for all spectral bins using the Human Metabolome Database (HMDB) and previous studies (Kostidis et al., 2017; Lamichhane et al., 2015; Lin et al., 2016) as well as two-dimensional Total Correlated Spectroscopy (TOCSY) spectra. Bins with ambiguous signals were either assigned to multiple metabolites or designated with “???” when no candidate metabolites could be assigned. Integral values of the following spectral regions were also calculated to compare faecal SCFA levels between groups: acetate (1.90–1.94 ppm), butyrate (1.54–1.58 ppm), and propionate (1.06–1.08 ppm). SCFA signals were analysed with unpaired t-tests for dams. For young offspring, a two-way ANOVA was used to determine effects of prenatal diet, postnatal diet, and prenatal x postnatal diet interactions. In adult offspring, a three-way ANOVA was initially used, but if no significant interactions with sex were found, data were consolidated, and two-way ANOVAs were performed to investigate effects of F0 diet, P9 treatment, and F0 diet x P9 treatment interactions.

16S ribosomal RNA (rRNA) sequencing was used to compare microbiome composition between groups, investigate relative abundances of amplicon sequence variants (ASVs), and identify differentially abundant taxa. Initially, I had used qPCR to compare absolute quantities of select taxa of interest based on previous studies (Morel et al. 2015). I decided, however, that 16S sequencing would be a better method for a comprehensive and unbiased look at global and specific changes in the maternal and offspring gut microbiomes. Results from qPCR can be found in **Appendix J**.

For 16S sequencing of the offspring gut microbiome, faecal DNA from the cohort in Chapter 3 was collected. Because lipopolysaccharide (LPS) had minimal effects on the brain and behaviour, only samples from the H₂O/Sal and BGOS/Sal groups were used (N = 10 males/females, N = 20 total/group). In addition, faecal DNA from BGOS-fed and control dams (Chapter 4 and 5 cohorts, N = 12/group) was collected. The bacteria-specific 16S rRNA V3-V4 region was amplified and sequenced. The forward V3 reads were processed, assessed for quality, and assigned to taxa in the *dada2* pipeline (Callahan et al. 2016). To investigate global changes in microbiome composition, alpha diversity (Chao1) was compared between H₂O and BGOS animals in the F0 and F1 cohorts using absolute ASV counts. Beta diversity (Bray-Curtis dissimilarity matrices) was used to explore global differences in microbiome composition between groups using relative ASV abundances. Microbiome composition was compared between the following groups with principal coordinate analysis (PCoA) plots and permutational multivariate analysis of variance (PERMANOVA): male and female offspring, F0 and F1, F0 H₂O and F0 BGOS, F1 H₂O and F1 BGOS, and litters. To identify differentially abundant taxa between groups, differential gene expression analysis based on the negative binomial distribution (DESeq) was used (Love, Huber, and Anders 2014). Differentially abundant taxa were plotted in a heat map and in boxplots. In addition to DESeq, because there are strong cage and litter effects in microbiome data (Ubeda et al. 2012), linear mixed-effects analysis with litter as a random effect was performed on log₁₀-transformed relative abundances to determine which ASVs were robust to litter effects. ASVs that were detected by both DESeq and mixed-effects analysis ($p < .05$) were considered to be significantly different between BGOS and H₂O pups.

Finally, correlation matrices using Pearson product-moment correlation coefficients were constructed to explore associations between behaviour, gene expression, faecal metabolites, and

log-transformed differentially abundant ASVs in young pups and adult offspring. Immune parameters were also included to investigate potential microbiome-immune-brain links. Because this analysis was exploratory, p values were not corrected for multiple comparisons.

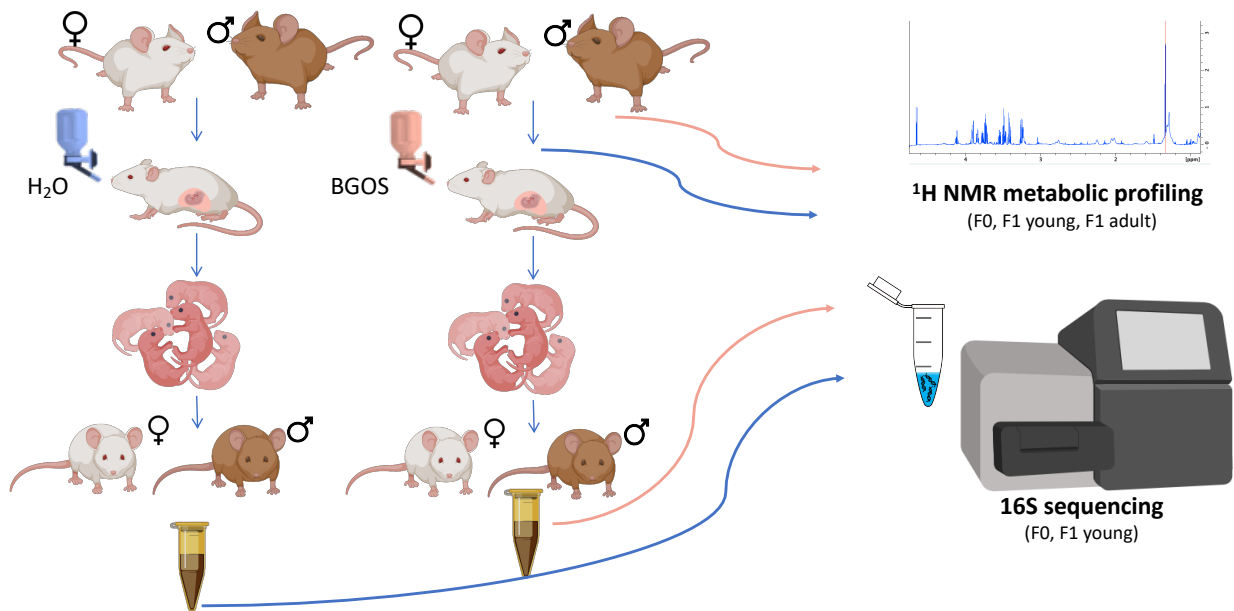


Figure 6.1 Diagram of experiment design. Faecal metabolite extracts and DNA were collected from dams, young offspring from the cross-fostering cohort, and adult offspring.

Table 6.1. Summary of OPLS-DA models and cohorts used for faecal ^1H NMR analysis

Group A	Group B	Cohort
H ₂ O	BGOS	Dams (<i>Chapter 4/5</i>)
H ₂ O/H ₂ O	BGOS/BGOS	Young pups (<i>Chapter 3/4</i>)
H ₂ O/H ₂ O	H ₂ O/BGOS	Young pups (<i>Chapter 3/4</i>)
H ₂ O/H ₂ O	BGOS/H ₂ O	Young pups (<i>Chapter 3/4</i>)
BGOS/H ₂ O	H ₂ O/BGOS	Young pups (<i>Chapter 3/4</i>)
H ₂ O/Sal	BGOS/Sal	Adult offspring (<i>Chapter 5</i>)
H ₂ O/LPS	BGOS/LPS	Adult offspring (<i>Chapter 5</i>)
H ₂ O/Sal	H ₂ O/LPS	Adult offspring (<i>Chapter 5</i>)
BGOS/Sal	BGOS/LPS	Adult offspring (<i>Chapter 5</i>)

6.4 Results

6.4.1 BGOS has no effects on maternal faecal metabolite profiles

Faecal extracts were collected from dams for ^1H NMR metabolic profiling. BGOS had no effect on faecal metabolite profiles (**Figure 6.2**). In addition, faecal SCFA levels were compared between groups based on the *a priori* hypothesis that fermentation of BGOS should increase faecal SCFA levels. There were no effects of BGOS intake, however, on faecal acetate, $t(19) = 1.86$, $p = .08$ (**Figure 6.3A**), butyrate, $t(20) = 1.57$, $p = .13$ (**Figure 6.3B**), or propionate, $t(20) = 1.09$, $p = .29$ (**Figure 6.3C**).

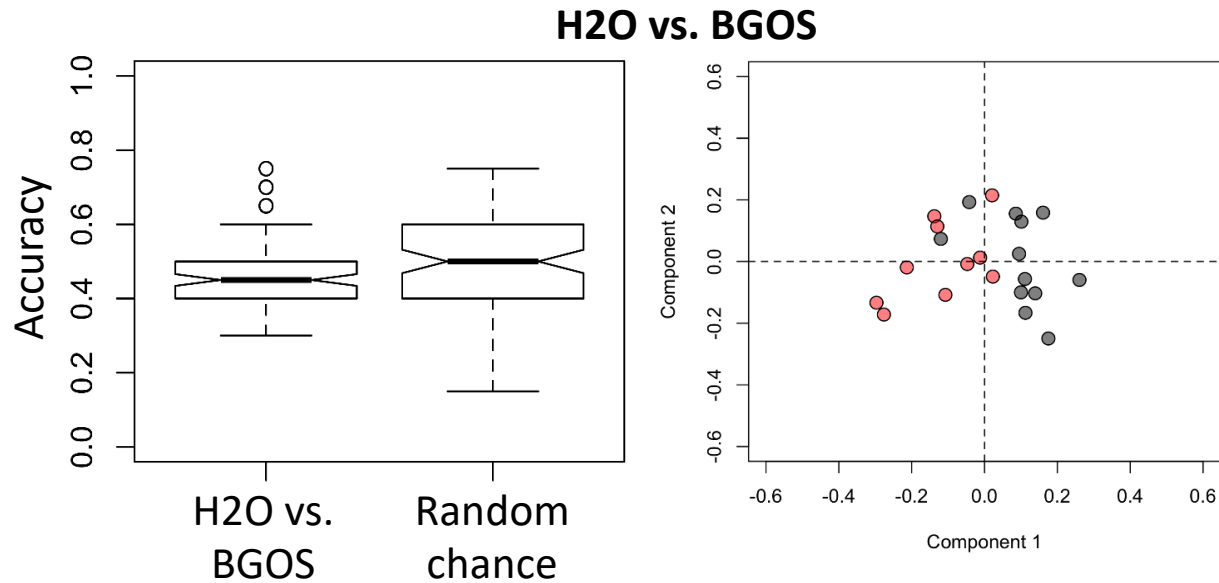


Figure 6.2. BGOS has no effect on maternal faecal metabolic profiles at offspring weaning (~3 weeks after birth). N = 10–12.

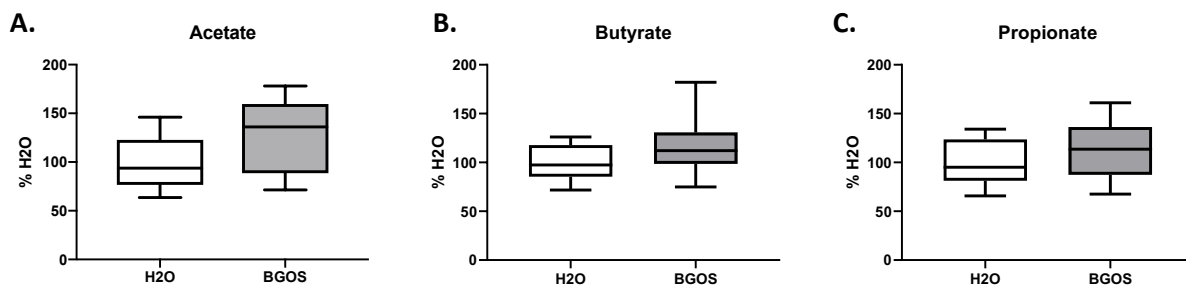


Figure 6.3. BGOS has no effect on maternal faecal SCFA levels at offspring weaning (~3 weeks postpartum). BGOS had no effect on maternal faecal levels of A) acetate, B) butyrate, or C) propionate. Unpaired t-test, N = 10–12.

6.4.2 Postnatal, but not prenatal, maternal prebiotic feeding alters offspring faecal metabolites, including butyrate, propionate, glucose, and leucine/isoleucine

Faecal extracts were collected from offspring at weaning age in the cross-fostered cohort from Chapter 4. A PCA plot was used for initial visualisation of the data, and there was no obvious clustering between groups (**Figure 6.4**). According to the OPLS-DA analysis, however, the

H₂O/H₂O vs. BGOS/BGOS (**Figure 6.5A**) and H₂O/H₂O vs. H₂O/BGOS (**Figure 6.5B**) models performed significantly better than chance, indicating an effect of postnatal maternal BGOS intake. The H₂O/H₂O vs. BGOS/H₂O model was not significant, suggesting that prenatal maternal BGOS feeding did not change faecal metabolite profiles (**Figure 6.5C**). BGOS/H₂O mice had significantly different faecal metabolite profiles, however, than H₂O/BGOS mice (**Figure 6.5D**). The detailed results of the OPLS-DA analysis, including R²X, R²Y, and Q² values, are summarized in **Table 6.2**.

Spectral bins with high variable importance in projection (VIP) scores from the following OPLS-DA models were identified: H₂O/H₂O vs. BGOS/BGOS (**Figure 6.6A**), H₂O/H₂O vs. H₂O/BGOS (**Figure 6.6B**), and BGOS/H₂O vs. H₂O/BGOS (**Figure 6.6C**). Metabolites corresponding to the top spectral bins in the H₂O/H₂O vs. BGOS/BGOS model are summarised in **Table 6.3**. Identifiable signals belonged to butyrate, glucose, and leucine/isoleucine (leucine and isoleucine peaks overlap and cannot be easily distinguished). These metabolites were also important in building the H₂O/H₂O vs. H₂O/BGOS (

Table 6.4) and the BGOS/H₂O vs. H₂O/BGOS models (**Table 6.5**). In addition to butyrate, leucine/isoleucine, and glucose signals, the succinate peak also differed in magnitude between BGOS/H₂O and H₂O/BGOS groups.

A heat map was used to visualise the direction of change for each signal (**Figure 6.7**). Faecal butyrate was elevated in pups that were suckled by a BGOS-fed dam (H₂O/BGOS and BGOS/BGOS groups), while glucose and leucine/isoleucine signals were reduced in these animals. Succinate levels were highest in BGOS/H₂O pups. Boxplots comparing integral values between groups can also be found in **Appendix G**.

Based on my *a priori* hypothesis that faecal SCFA levels would be elevated in offspring, I also performed two-way ANOVAs to determine the effects of prenatal and postnatal BGOS on acetate, butyrate, and propionate signals in offspring faecal extracts. Means are expressed as percentages based on integral values relative to the H₂O/H₂O group. There was a significant main effect of postnatal diet on butyrate, $F(1, 48) = 12.74, p < .001$. The average integral of the butyrate region in pups suckled by a BGOS-fed dam ($M = 120.12, SD = 7.13$) was significantly higher than in pups suckled by a control dam ($M = 97.57, SD = 3.34$). There was no effect of prenatal diet, $F(1, 48) = 0.18, p = .67$, or interaction, $F(1, 48) = 1.37, p = .25$ (**Figure 6.8A**). Similarly, there was a significant main effect of postnatal diet on propionate, $F(1, 47) = 16.84, p < .001$. The average integral of the propionate region in pups suckled by BGOS-fed dams ($M = 117.34, SD = 2.24$) was significantly higher than in pups suckled by a control dam ($M = 98.29, SD = 4.89$). There was no effect of prenatal diet, $F(1, 47) = 1.18, p = .29$, and no interaction, $F(1, 47) = 0.16, p = .67$ (**Figure 6.8B**). There were no significant effects of prenatal diet, $F(1, 48) = 0.31, p = .58$., postnatal diet, $F(1, 48) = 0.61, p = .44$, or prenatal x postnatal diet interaction, $F(1, 48) = 0.34, p = .56$, on faecal acetate (**Figure 6.8C**). Furthermore, the effects of postnatal maternal BGOS intake on faecal butyrate and propionate remained after accounting for random litter effects using linear mixed-effects analysis (see **Appendix C** for details).

Average NOESY spectra for faecal samples from the H₂O/H₂O and BGOS/BGOS groups are shown in **Figure 6.9**, and butyrate, propionate, and glucose signals are labelled. Higher intensity signals in the butyrate and propionate regions can be seen in the BGOS/BGOS group.

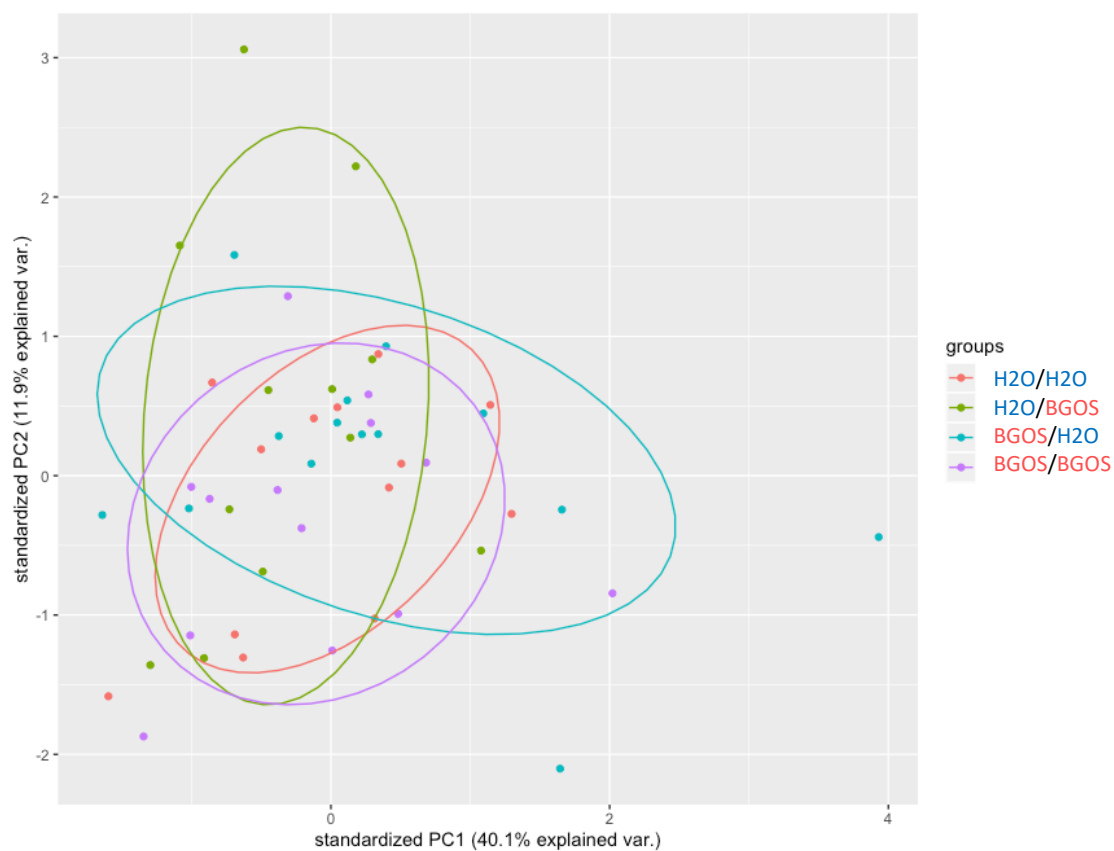


Figure 6.4. PCA plot of Pareto-scaled ^1H NMR spectral bins. PC1 and PC2 are plotted. $N = 6\text{--}8$ males/females; $N = 12\text{--}14$ total/group.

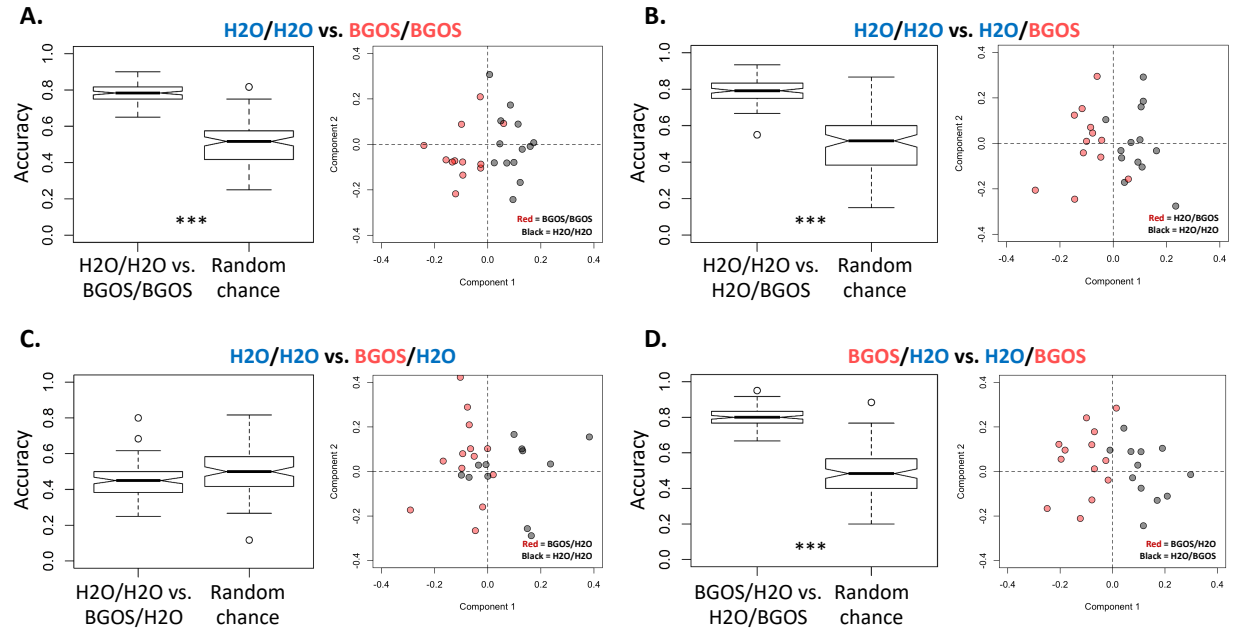


Figure 6.5. OPLS-DA model accuracies and representative scores plots. Accuracies of ensembles of 1000 OPLS-DA models (left) and representative scores plots (right) are shown for **A)** H₂O/H₂O vs. BGOS/BGOS, **B)** H₂O/H₂O vs. H₂O/BGOS, **C)** H₂O/H₂O vs. BGOS/H₂O, and **D)** BGOS/H₂O vs. H₂O/BGOS pups from the cross-fostered cohort. N = 6–8 males/females; N = 12–14 total/group.

Table 6.2. Summary of OPLS-DA results.

Group A	Group B	Average accuracy of A vs. B models $\pm$ SD*	Significantly better performance than random chance?	Average $R^2 X \pm$ SD*	Average $R^2 Y \pm$ SD*	Average $Q^2 \pm$ SD*
H ₂ O/H ₂ O (n = 13)	BGOS/BGOS (n = 13)	78.1 $\pm$ 6%	Yes	0.65 $\pm$ 0.05	0.88 $\pm$ 0.03	0.51 $\pm$ 0.05
H ₂ O/H ₂ O (n = 13)	H ₂ O/BGOS (n = 12)	79.3 $\pm$ 6%	Yes	0.69 $\pm$ 0.04	0.89 $\pm$ 0.03	0.42 $\pm$ 0.07
H ₂ O/H ₂ O (n = 13)	BGOS/H ₂ O (n = 14)	45.1 $\pm$ 9%	No	0.71 $\pm$ 0.03	0.78 $\pm$ 0.03	-0.66 $\pm$ 0.22
H ₂ O/BGOS (n = 12)	BGOS/H ₂ O (n = 14)	80.1 $\pm$ 6%	Yes	0.71 $\pm$ 0.03	0.89 $\pm$ 0.02	0.49 $\pm$ 0.09
H ₂ O (F0) (n = 12)	BGOS (F0) (n = 10)	47.1 $\pm$ 10%	No	0.58 $\pm$ 0.03	0.83 $\pm$ 0.03	-0.42 $\pm$ 0.30

*SD = standard deviation

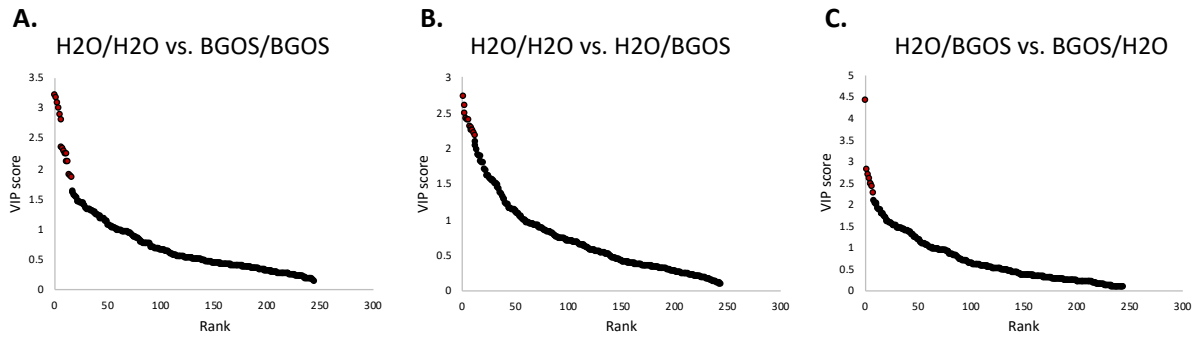


Figure 6.6. VIP scores and ranks for spectral bins. Plots of all spectral bins and VIP assignments for **A)** H₂O/H₂O vs. BGOS/BGOS, **B)** H₂O/H₂O vs. H₂O/BGOS, and **C)** BGOS/H₂O vs. H₂O/BGOS. Bins with top VIP scores are in red.

Table 6.3. Top bins and corresponding VIP scores and metabolite assignments for H₂O/H₂O vs. BGOS/BGOS model.

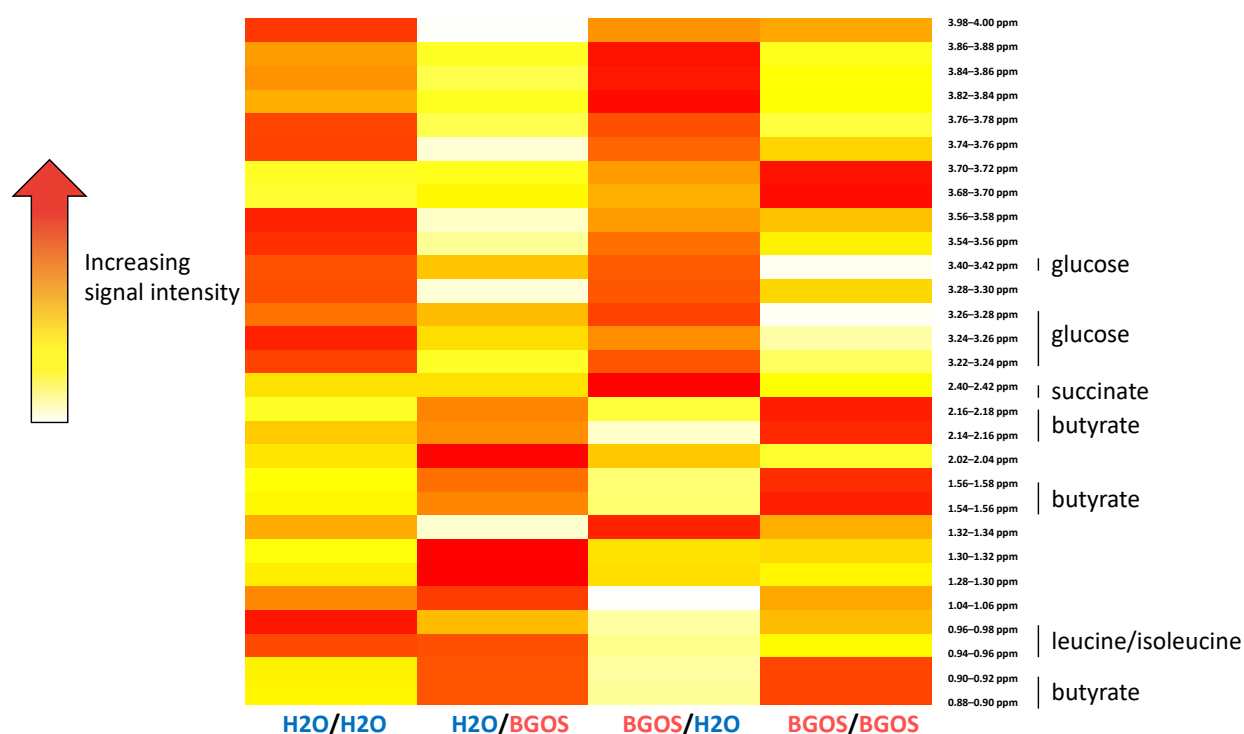
H₂O/H₂O vs. BGOS/BGOS		
Bin	VIP score	Assignment
2.16–2.18 ppm	3.21157841	butyrate
3.26–3.28 ppm	3.17381059	glucose
1.54–1.56 ppm	3.07797034	butyrate
1.56–1.58 ppm	3.00166582	butyrate
0.88–0.90 ppm	2.88096595	butyrate
3.24–3.26 ppm	2.80688128	glucose
3.40–3.42 ppm	2.35494593	glucose
0.94–0.96 ppm	2.32885349	leucine/isoleucine
3.68–3.70 ppm	2.29833894	???
3.22–3.24 ppm	2.24563612	glucose
0.96–0.98 ppm	2.24058751	leucine/isoleucine
0.90–0.92 ppm	2.11299258	butyrate
3.76–3.78 ppm	2.10888241	glucose/galactose/glycerol
2.14–2.16 ppm	1.908454	butyrate
3.70–3.72 ppm	1.88355012	glucose/galactose/fructose/lysine
3.54–3.56 ppm	1.84851504	glycine/fructose/glycerol

Table 6.4. Top bins and corresponding VIP scores and metabolite assignments for H₂O/H₂O vs. H₂O/BGOS model.

H₂O/H₂O vs. H₂O/BGOS		
Bin	VIP score	Assignment
3.98–4.00 ppm	2.73289969	???
1.30–1.32 ppm	2.59268906	valerate/isoleucine
0.88–0.90 ppm	2.48393342	butyrate
3.56–3.58 ppm	2.42097264	glycine/fructose/glycerol
3.54–3.56 ppm	2.39648913	glycine/glucose
0.96–0.98 ppm	2.3960421	leucine/isoleucine
1.28–1.30 ppm	2.30073614	???
3.76–3.78 ppm	2.28439191	glucose/galactose/glycerol
1.56–1.58 ppm	2.25814186	butyrate
3.28–3.30 ppm	2.25671265	???
3.74–3.76 ppm	2.2104495	glucose/galactose/glycerol
2.02–2.04 ppm	2.17847669	???

Table 6.5. Top bins and corresponding VIP scores and metabolite assignments for BGOS/H₂O vs. H₂O/BGOS model.

BGOS/H ₂ O vs. H ₂ O/BGOS		
Bin	VIP score	Assignment
2.40–2.42 ppm	4.41816582	succinate
1.32–1.34 ppm	2.80813192	lactate/valerate
1.04–1.06 ppm	2.68898092	propionate/valine
0.88–0.90 ppm	2.5890085	butyrate
3.86–3.88 ppm	2.46559143	glucose/galactose/fructose
3.84–3.86 ppm	2.46342846	glucose/galactose
0.94–0.96 ppm	2.42028156	leucine/isooleucine
3.82–3.84 ppm	2.27344436	glucose/galactose/fructose

**Figure 6.7.** Heat map comparing spectral bins and corresponding metabolites in young offspring. To visualise group differences in metabolite levels in pups from the cross-fostered cohort, spectral bins with top VIP scores in at least one of the significant OPLS-DA models were used to construct a heat map.

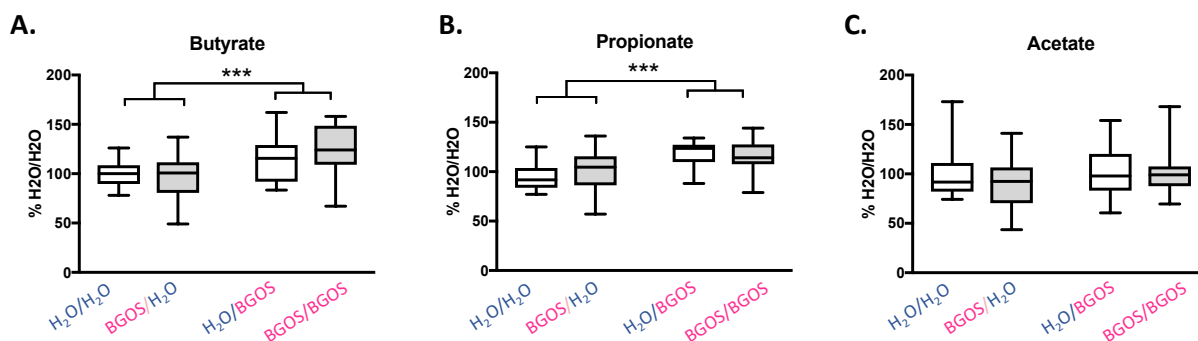


Figure 6.8. Postnatal maternal prebiotic intake increases butyrate and propionate. There were significant main effects of maternal BGOS supplementation on faecal levels of **A)** butyrate and **B)** propionate in young offspring. **C)** There were no effects on faecal acetate. Two-way ANOVA, *** $p < .001$, $N = 6-8$ males/females; $N = 12-14$ total/group.

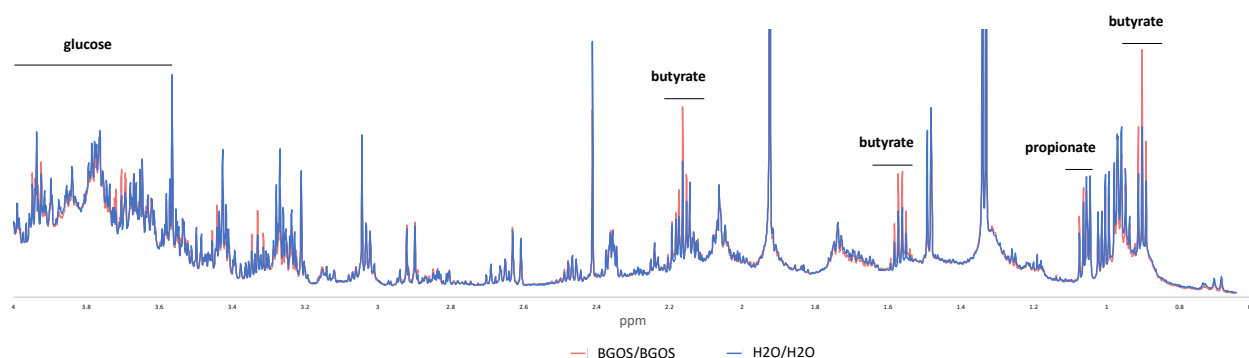


Figure 6.9. Average NOESY ¹H NMR spectra. Average spectra for BGOS/BGOS (pink) and H₂O/H₂O (blue) pups. Butyrate, propionate, and glucose regions are indicated.

6.4.3 P9 LPS alters the faecal metabolome in adult offspring, but maternal BGOS has no effect

Faecal extracts were collected from offspring at approximately P56 from the adult cohort used in Chapter 5. Data were plotted in a PCA plot for initial visualisation of the data, and there was no obvious clustering between groups (**Figure 6.10**). According to the OPLS-DA analysis, maternal BGOS had no effect on faecal metabolite profiles – there were no differences between the H₂O/Sal vs. BGOS/Sal groups (**Figure 6.11A**) or H₂O/LPS vs. BGOS/LPS groups (**Figure 6.11B**). The only significant model was H₂O/Sal vs. H₂O/LPS, indicating a significant effect of

early-life LPS on adult faecal metabolites (**Figure 6.11C**). The BGOS/Sal vs. BGOS/LPS model, however, was not significant (**Figure 6.11D**). The OPLS-DA results are summarised in **Table 6.6**.

Spectral bins with the highest VIP scores were identified (**Figure 6.12**), and metabolites were assigned to bins where possible. Glucose, succinate, and choline drove the separation between groups (**Table 6.7**), and these metabolites were lower in the H₂O/LPS group compared to controls (**Figure 6.13**). Boxplots showing differences between groups for each top-ranking bin can be found in **Appendix I**.

SCFA levels were also compared between groups using ANOVAs. Because no interactions with sex were found in the three-way ANOVAs, all data were consolidated for two-way ANOVAs. No significant effects were found. Acetate was not significantly affected by F0 diet, $F(1, 75) = 0.45, p = .50$, P9 treatment, $F(1, 75) = 0.09, p = .77$, or an F0 diet x P9 treatment interaction, $F(1, 75) = 0.05, p = .83$ (**Figure 6.14A**). There were also no significant effects of F0 diet $F(1, 75) = 2.96, p = .09$, P9 treatment, $F(1, 75) = 0.02, p = .89$, or F0 diet x P9 treatment interaction, $F(1, 75) = 0.29, p = .59$, on butyrate (**Figure 6.14B**). Finally, the main effects of F0 diet, $F(1, 75) = 0.95, p = .33$, and P9 treatment, $F(1, 75) = 0.20, p = .66$, and the interaction, $F(1, 75) = 0.14, p = .71$, on propionate were not significant (**Figure 6.14C**).

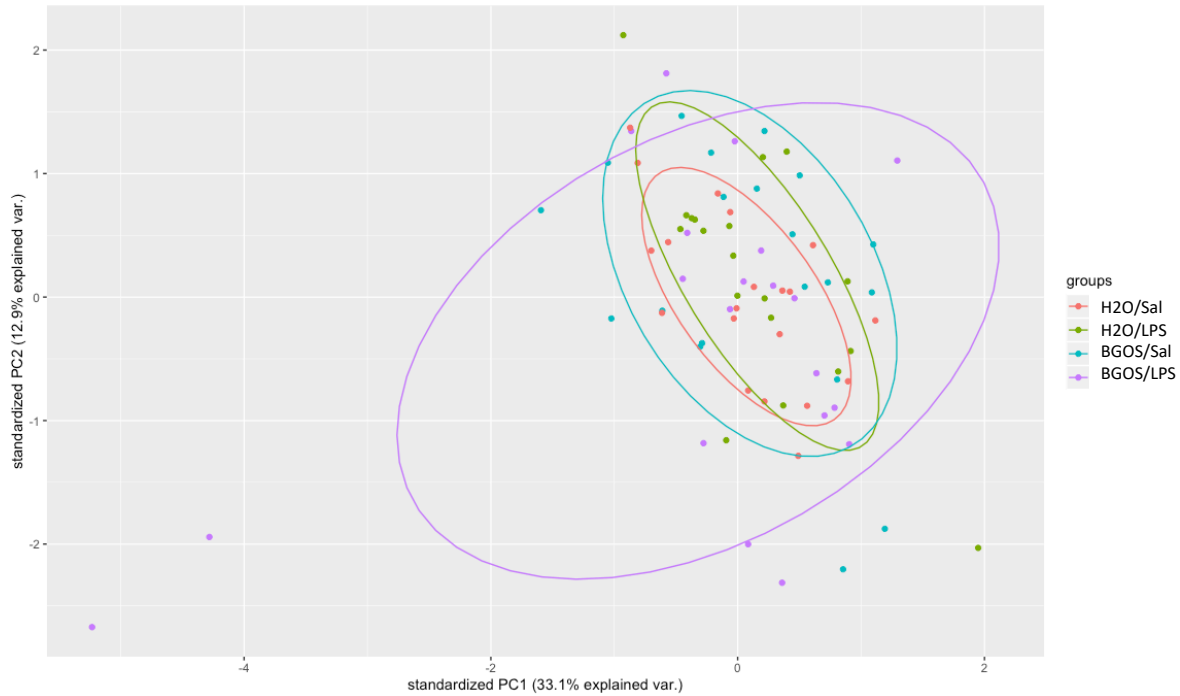


Figure 6.10. PCA plot of Pareto-scaled ^1H NMR spectral bins in adult faecal extract samples. PC1 and PC2 are plotted. $N = 9\text{--}10$ males/females; $N = 19\text{--}20$ total/group.

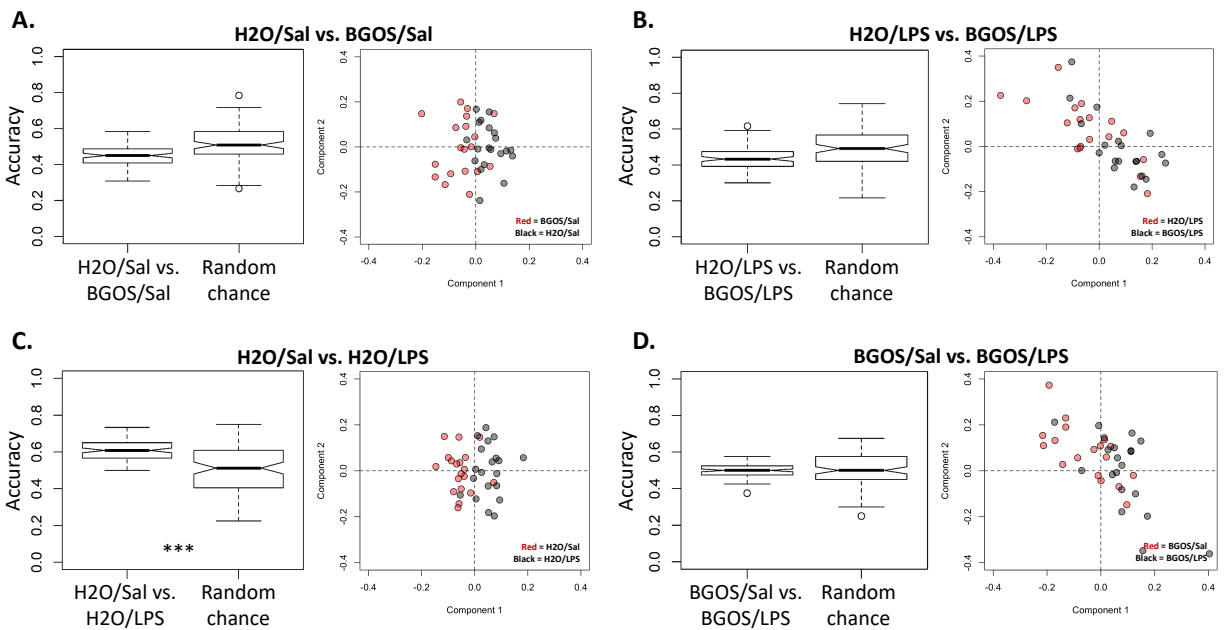


Figure 6.11. OPLS-DA model accuracies and representative scores plots for adult offspring. Accuracies of ensembles of 1000 OPLS-DA models (left) and representative scores plots (right) are shown for **A)** $\text{H}_2\text{O}/\text{Sal}$ vs. BGOS/Sal , **B)** $\text{H}_2\text{O}/\text{LPS}$ vs. BGOS/LPS **C)** $\text{H}_2\text{O}/\text{Sal}$ vs. $\text{H}_2\text{O}/\text{LPS}$, and **D)** BGOS/Sal vs. BGOS/LPS adult offspring faecal extracts. Kolmogorov-Smirnov, *** $p < .001$, $N = 9\text{--}10$ males/females; $N = 19\text{--}20$ total/group.

Table 6.6. Summary of OPLS-DA results for adult offspring faecal ^1H NMR.

Group A	Group B	Average accuracy of A vs. B models $\pm$ SD*	Significantly better performance than random chance?	Average $R^2 X \pm$ SD*	Average $R^2 Y \pm$ SD*	Average $Q^2 \pm$ SD*
H ₂ O/Saline (n = 20)	BGOS/Saline (n = 19)	45.1 $\pm$ 6%	No	0.48 $\pm$ 0.01	0.79 $\pm$ 0.01	-0.48 $\pm$ 0.13
H ₂ O/LPS (n = 19)	BGOS/LPS (n = 20)	43.5 $\pm$ 6%	No	0.70 $\pm$ 0.02	0.69 $\pm$ 0.03	-0.76 $\pm$ 0.21
H ₂ O/Saline (n = 20)	H ₂ O/LPS (n = 19)	61.1 $\pm$ 5%	Yes	0.54 $\pm$ 0.02	0.74 $\pm$ 0.01	-0.24 $\pm$ 0.11
BGOS/Saline (n = 19)	BGOS/LPS (n = 20)	50.6 $\pm$ 4%	No	0.61 $\pm$ 0.01	0.62 $\pm$ 0.03	-0.44 $\pm$ 0.15

*SD = standard deviation

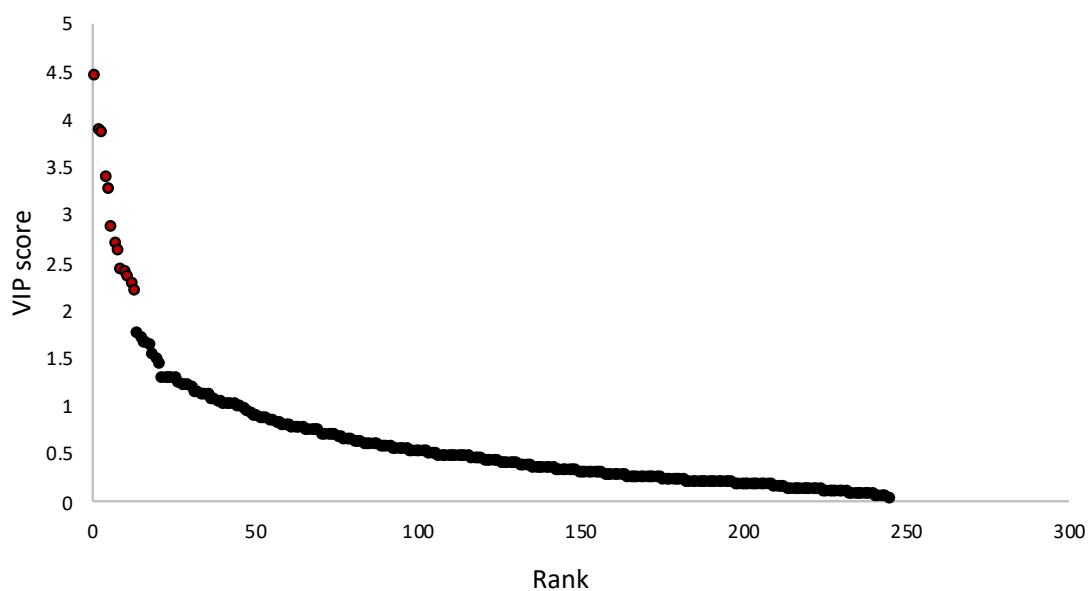
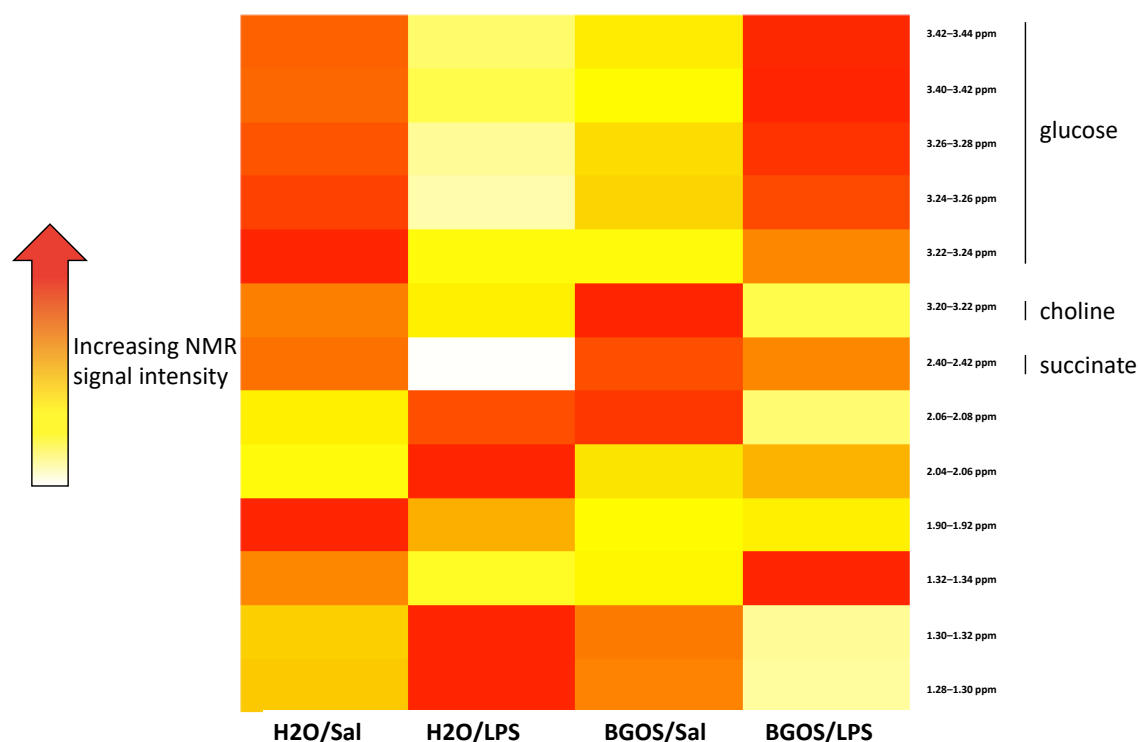
H₂O/Sal vs. H₂O/LPS**Figure 6.12. VIP scores and ranks for spectral bins.** Plots of all spectral bins and VIP assignments for the H₂O/Sal vs. H₂O/LPS OPLS-DA model.

Table 6.7. Top bins and corresponding VIP scores and metabolite assignments for H₂O/Sal vs. H₂O/LPS model.

H ₂ O/Sal vs. H ₂ O/LPS		
Bin	VIP score	Assignment
1.32–1.34 ppm	4.456508	lactate/valerate
3.26–3.28 ppm	3.893011	glucose
2.40–2.42 ppm	3.8609	succinate
2.04–2.06 ppm	3.407303	proline/glutamate/N-acetyl groups
3.42–3.44 ppm	3.279639	glucose
3.24–3.26 ppm	2.864693	glucose
3.40–3.42 ppm	2.708908	glucose
3.20–3.22 ppm	2.631511	choline
1.28–1.30 ppm	2.425702	???
1.30–1.32 ppm	2.409952	valerate/isoleucine
1.90–1.92 ppm	2.358686	acetate/arginine
2.06–2.08 ppm	2.294994	proline/glutamate/N-acetyl groups
3.22–3.24 ppm	2.211318	glucose
3.26–3.28 ppm	3.893011	glucose

**Figure 6.13. Heat map comparing spectral bins and corresponding metabolites between adult offspring groups.**

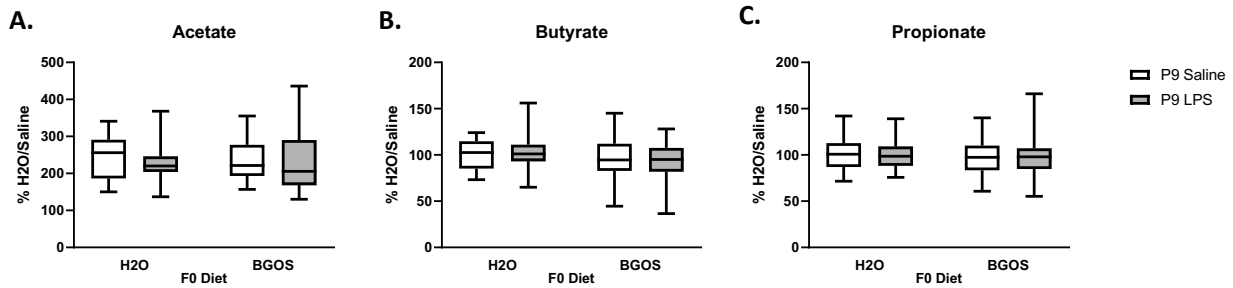


Figure 6.14. Maternal prebiotic intake has no effect on faecal SCFA levels in adult offspring. There were no significant effects of maternal BGOS supplementation on faecal levels of **A)** butyrate, **B)** propionate, or **C)** acetate in adult offspring. Two-way ANOVA, N = 9–10 males/females; N = 19–20 total/group.

6.4.4 16S sequencing quality

DNA was extracted from maternal and offspring faecal samples and diluted for sequencing. Average Phred scores (confidence in the assigned base pair [bp]) for each bp position were used to assess sequence quality in every sample. All samples had acceptable quality scores (Phred score > 25) up until read lengths of ~200–250 bp (**Figure 6.15**). This was expected given that sequence quality degrades with increasing read length. In addition, all sequence reads had acceptable average quality scores across all samples (**Figure 6.16**).

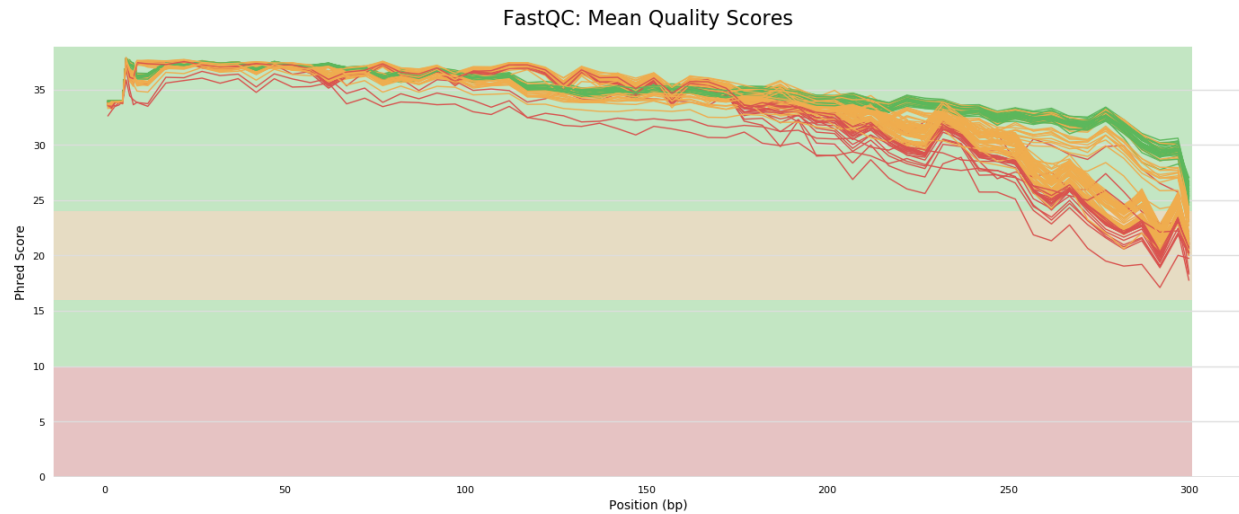


Figure 6.15. FastQC Quality Scores. Average Phred scores for each bp position. Each line is a different sample. All sequencing reads were of acceptable quality (average Phred score > 25) up until ~200–250 bp.

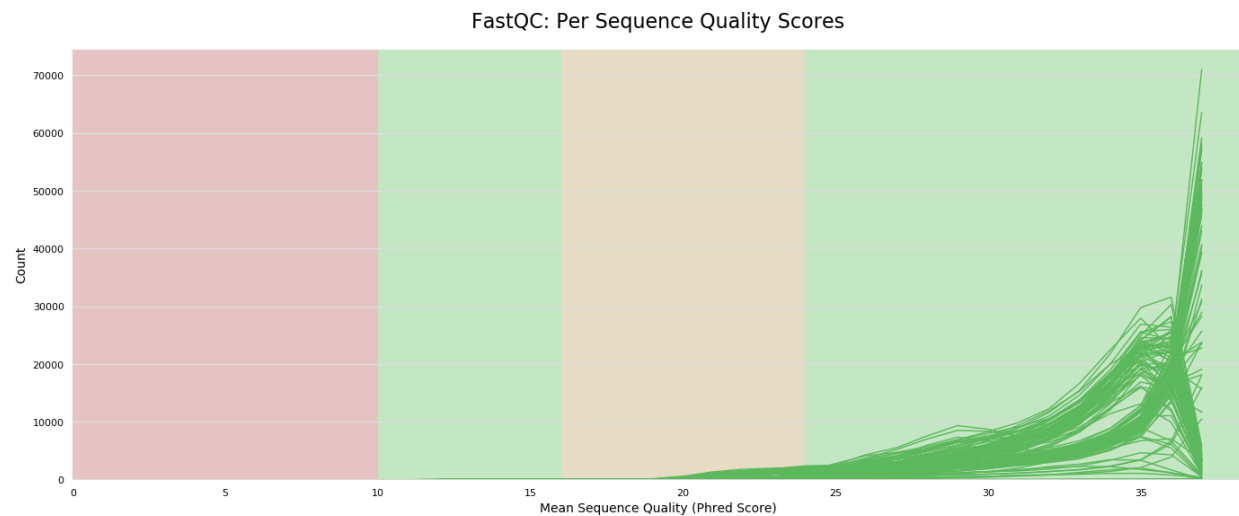


Figure 6.16. Number of reads and average sequence quality scores for each sample.

6.4.5 16S sequencing ASVs and taxonomic assignment

Samples had between 455 and 135,372 reads ($M = 86,335$, $SD = 29,895$). Two samples with very low read counts (< 600) were removed before analysis, and both samples came from the F0 BGOS group. The remaining samples had at least 7259 reads ($M = 89,103$, $SD = 25,957$).

Between ~100–350 ASVs were called per sample in the *dada2* pipeline, with the exception of the two samples with very low reads (**Figure 6.17A**). As expected, few ASVs were very prevalent, while most ASVs were rare (**Figure 6.17B**). In addition, most ASVs had low abundance, while few ASVs had high abundance and high prevalence (**Figure 6.17C**).

High-level taxonomic assignment was performed to confirm that the samples contained the expected phyla. As would be anticipated in the mouse gut, Bacteroidetes and Firmicutes made up the vast majority of the microbes present (**Figure 6.18**). In addition, most ASVs were able to be assigned at the phylum-level, and a smaller percentage could be assigned at the lower taxonomic levels, with very few assigned at the species level. This is expected for the resolution of 16S sequencing (**Figure 6.19**). ASVs that could not be assigned to a phylum were removed before analysis.

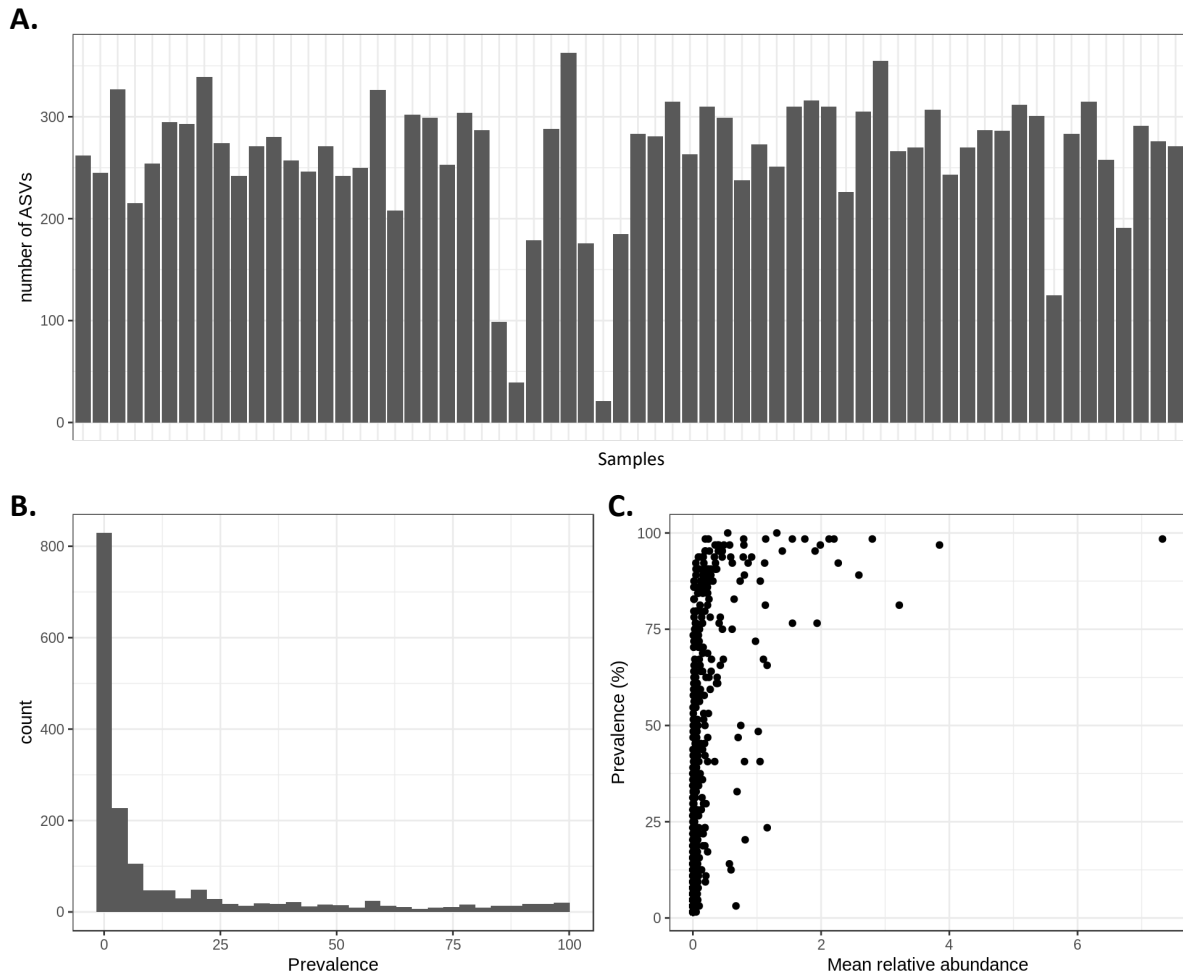


Figure 6.17. Number of ASVs called per sample and prevalence. **A)** Bar graph showing number of ASVs detected per sample. **B)** Histogram showing ASV prevalence (percentage of samples in which each ASV was found). **C)** Scatter plot showing mean abundance and prevalence of each ASV.

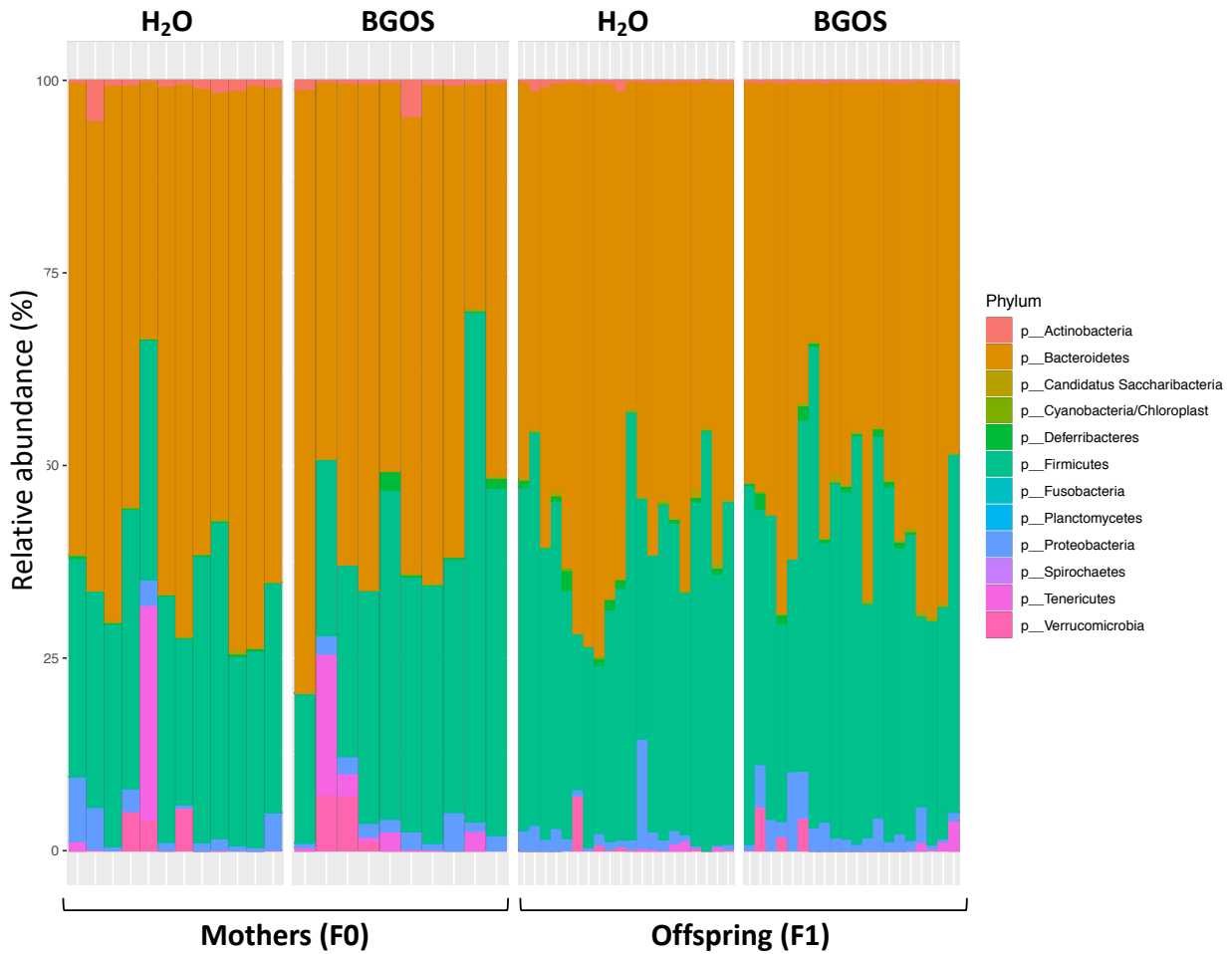


Figure 6.18. Taxonomic assignments for dams and offspring (relative abundances at the phylum level). Each multi-coloured bar represents faecal microbiome composition of one sample.

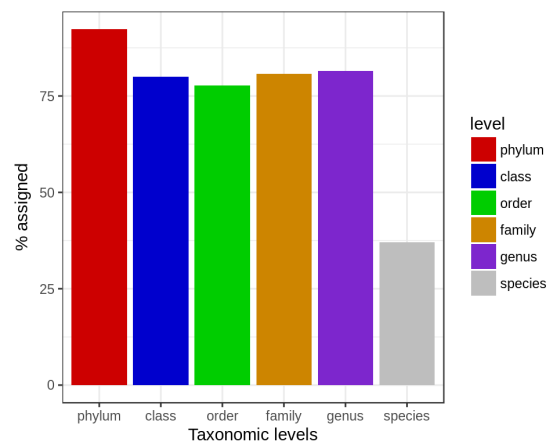


Figure 6.19. Percent ASVs assigned at each level. Nearly all ASVs could be assigned at the phyla level. Fewer ASVs could be assigned to lower taxonomic levels, and the resolution of 16S is poor at the species level.

6.4.6 Pups of BGOS-fed dams have significantly different microbiome composition than control offspring

A Welch's t-test was used to compare the Chao1 measure of alpha diversity (number and distribution of ASVs within samples) between BGOS-fed and control dams and their offspring. There was no effect of BGOS on alpha diversity in F0, $t(18.60) = -1.07$, $p = .30$ (**Figure 6.20A**). Similarly, there was no effect of maternal diet on alpha diversity in offspring, $t(24.10) = -1.50$, $p = .15$ (**Figure 6.20B**).

Bray-Curtis dissimilarity was used to quantify beta diversity in dams and offspring. PCoA plots were built to visualise whether groups clustered based on beta diversity, and PERMANOVA tests were used to determine whether there were significant differences in beta diversity between groups. Because there were no differences in beta diversity between male and female offspring, $F(1, 38) = 1.49$, $R^2 = 0.04$, $p = .12$, offspring of both sexes were combined for the remaining analysis. The clearest separation was between F0 dams and F1 offspring (**Figure 6.21A**). There was substantial overlap, however, among dams (**Figure 6.21B**). In agreement with the PCoA plots, the PERMANOVA showed that offspring had significantly different microbiomes than mothers, $F(1, 60) = 8.29$, $R^2 = 0.12$, $p < .001$, but there were no differences between H₂O and BGOS dams, $F(1, 20) = 1.00$, $R^2 = 0.05$, $p = .42$. In the PCoA plots of offspring alone, pups tended to cluster by maternal diet (**Figure 6.21C**) and litter (**Figure 6.21D**). Because of the nested design (litters nested within groups), R^2 values in these tests represent the maximum variance explained by maternal diet, $F(1, 38) = 3.80$, $R^2 = 0.09$, $p < .001$, or litter, $F(6, 33) = 5.59$, $R^2 = 0.50$, $p < .001$, rather than the actual variance explained.

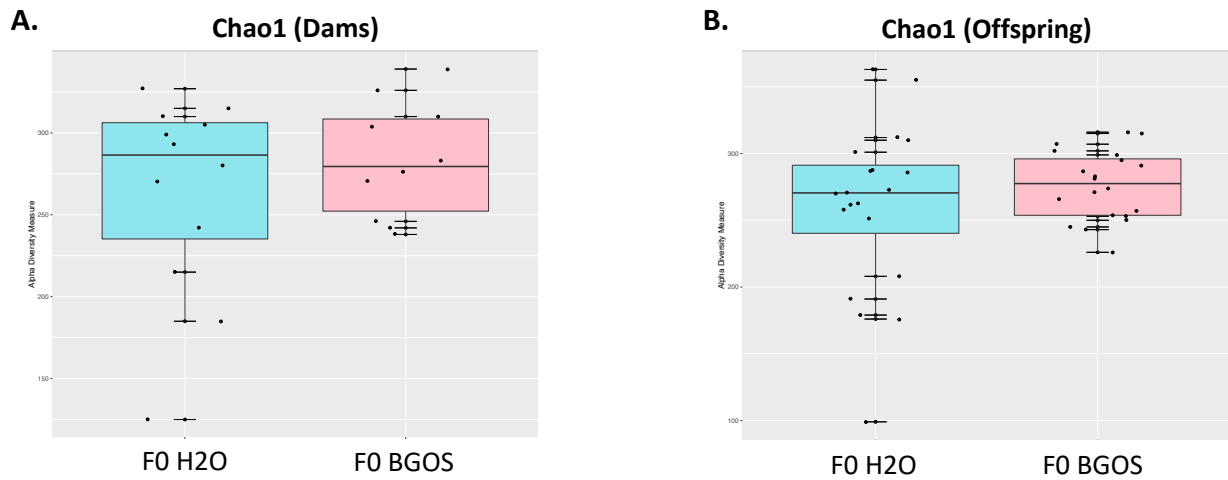


Figure 6.20. BGOS has no effect on alpha diversity in dams or offspring. Absolute numbers of ASVs per sample were used to calculate the Chao1 measure of alpha diversity in **A)** F0 and **B)** F1 animals. There were no significant differences in either cohort. Unpaired Welch's t-test, N = 10–12 dams/group; N = 10 male/female offspring, N = 20 total/group.

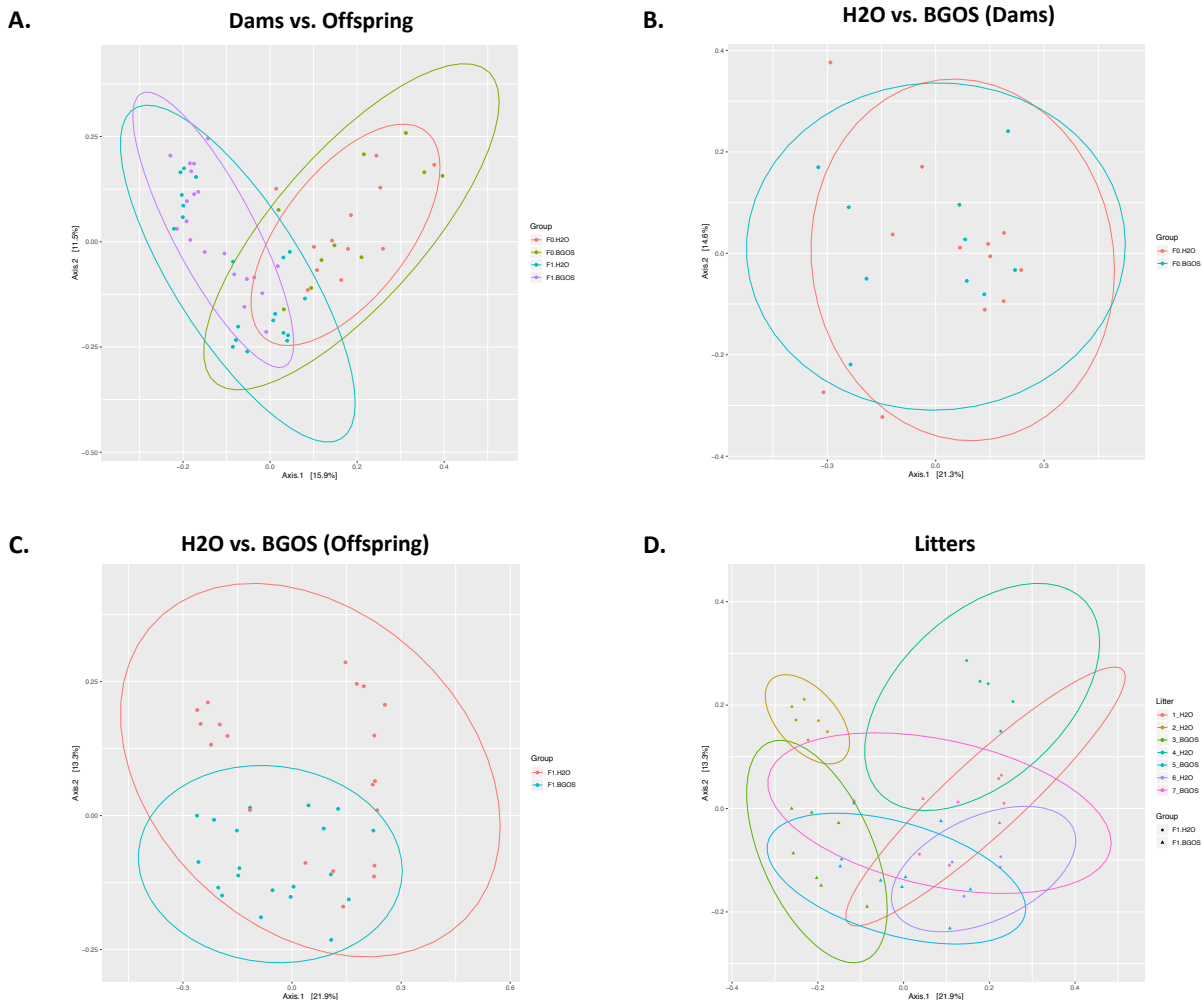


Figure 6.21 PCoA plots of beta diversity in dams and offspring. Relative ASV abundances were used to calculate beta diversity using Bray-Curtis dissimilarity matrices. **A)** There was a clear separation between mothers and pups but **B)** no separation between H₂O and BGOS dams. Offspring clustered by **C)** maternal diet and **D)** litter.

6.4.7 Relative abundances of taxa in the Bacteroidetes and Actinobacteria phylum differ between H₂O and BGOS mothers and pups

DEseq was used to identify differentially abundant taxa between H₂O and BGOS groups in the F0 and F1 cohorts. DEseq analysis resulted in 5 differentially abundant ASVs in dams and 20 ASVs in offspring. Relative abundances of all 25 ASVs labelled at the genus level were combined in a heat map to visualise difference between groups (**Figure 6.22**). For a more detailed look, each ASV was plotted in a boxplot and labelled with the lowest taxonomic level assigned by

dada2 for mothers (**Figure 6.23**) and offspring (**Figure 6.24**). It should be noted that each boxplot corresponds to one ASV and not the total relative abundance for the taxon in the graph title.

In dams, three ASVs associated with the *Porphyromonadaceae* family in the Bacteroidetes phylum, including one ASV corresponding to the *Barnesiella* genus, were more abundant in BGOS-fed dams than control dams (**Figure 6.23A–C**). *Moryella*, a genus in the *Lachnospiraceae* family of the *Clostridia* class, was also elevated in BGOS-fed dams (**Figure 6.23D**). Finally, an ASV that could only be assigned to the Firmicutes phylum was more abundant in BGOS mothers than controls (**Figure 6.23E**).

A number of taxa were differentially abundant in offspring as a result of maternal BGOS supplementation. The species *Bacteroides caecimuris* was less abundant in BGOS pups than H₂O pups (**Figure 6.24A**). An ASV associated with the *Barnesiella* genus (*Porphyromonadaceae* family) was also significantly reduced in BGOS pups (**Figure 6.24B**). Finally, *Bifidobacterium* was reduced in BGOS pups (**Figure 6.24C**). Other taxa (**Figure 6.24D–T**) were detected by DEseq as differentially abundant between groups but were not significant when analysed with linear mixed-effects analysis, indicating that the contributions of maternal prebiotic feeding could not be separated from random litter effects for these taxa.

Because differences in the ASVs assigned to *Bifidobacterium* and *Barnesiella* could not be assigned to specific species, the effects of maternal diet on total *Bifidobacterium* and *Barnesiella* were tested. Log-transformed relative abundances of ASVs from these genera were pooled and tested using linear mixed-effects analysis with maternal diet as a fixed effect and litter as a random effect. The effects of maternal diet on relative abundances of total *Bifidobacterium* just missed the threshold for significance, $F(1, 4.05) = 7.54, p = .05$ (**Figure 6.25A**). In addition, there was no effect of maternal diet on total *Barnesiella*, $F(1, 4.53) = 2.02, p = .22$ (**Figure 6.25B**).

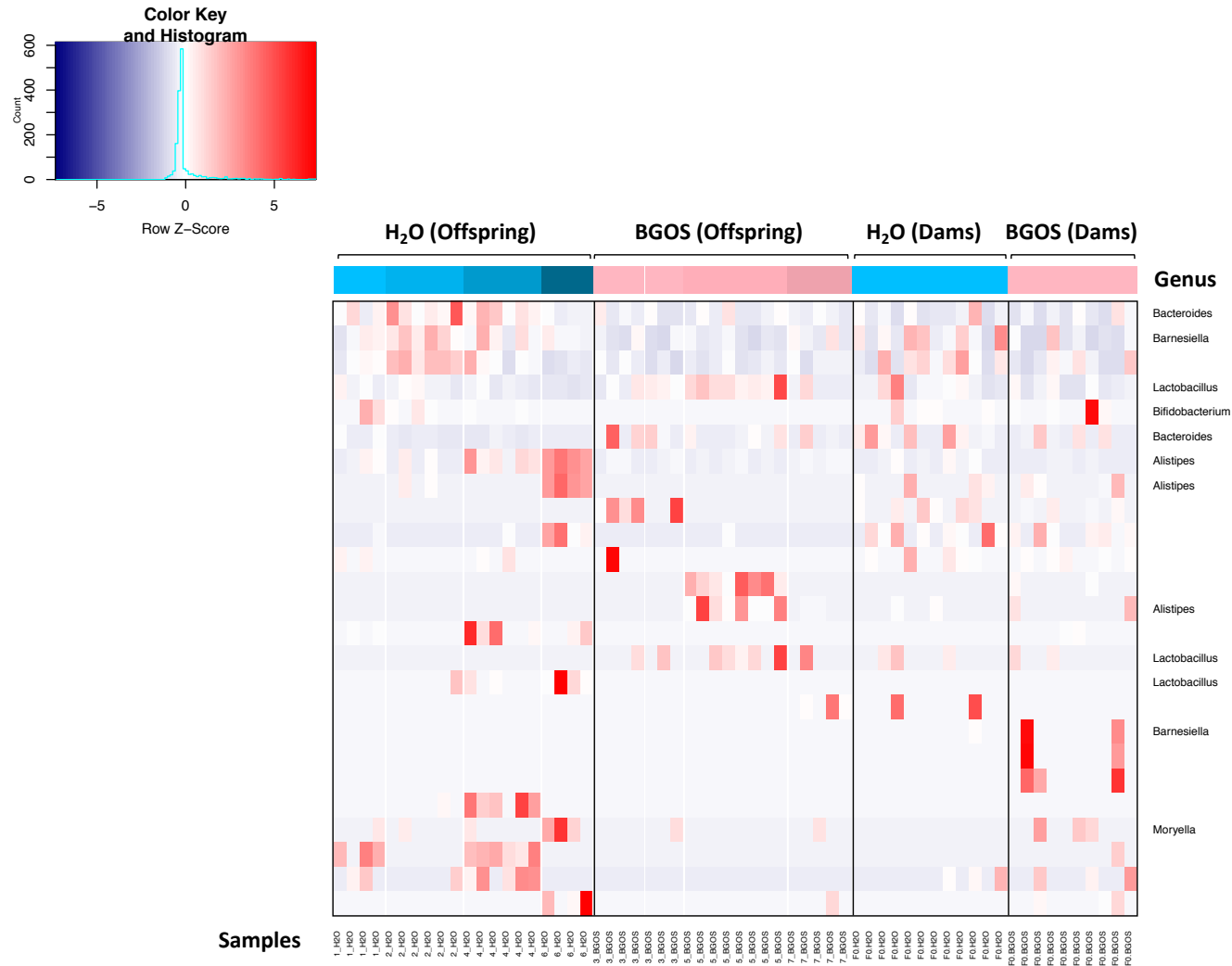


Figure 6.22. Heat map of differentially abundant taxa. Relative abundances of ASVs identified by DESeq as differentially abundant between H₂O (blue) and BGOS (pink) young offspring in F0 or F1 are shown. Genus is indicated where possible, and samples are on the x-axis. In addition, pups from different litters are denoted with different shades of blue or pink.

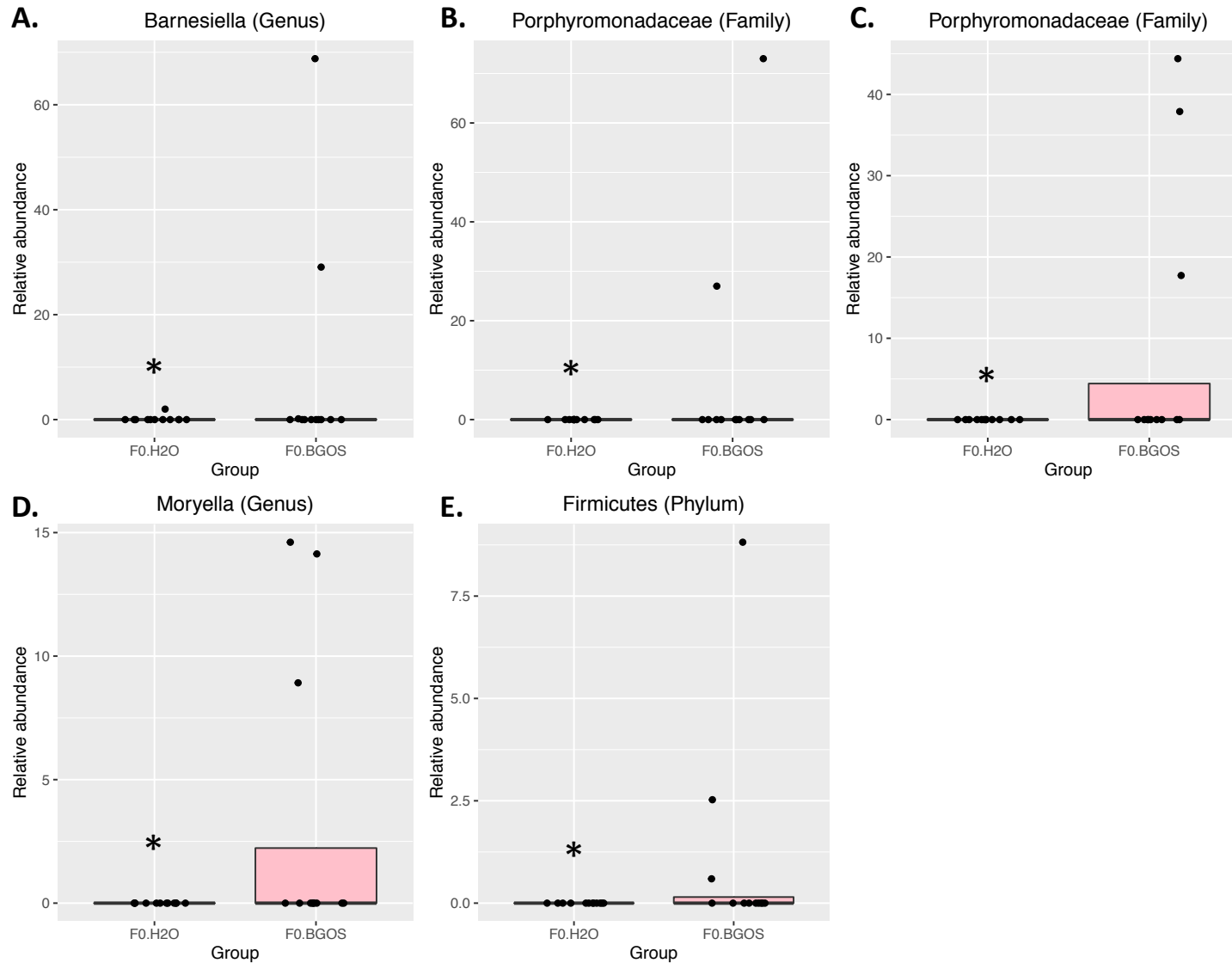
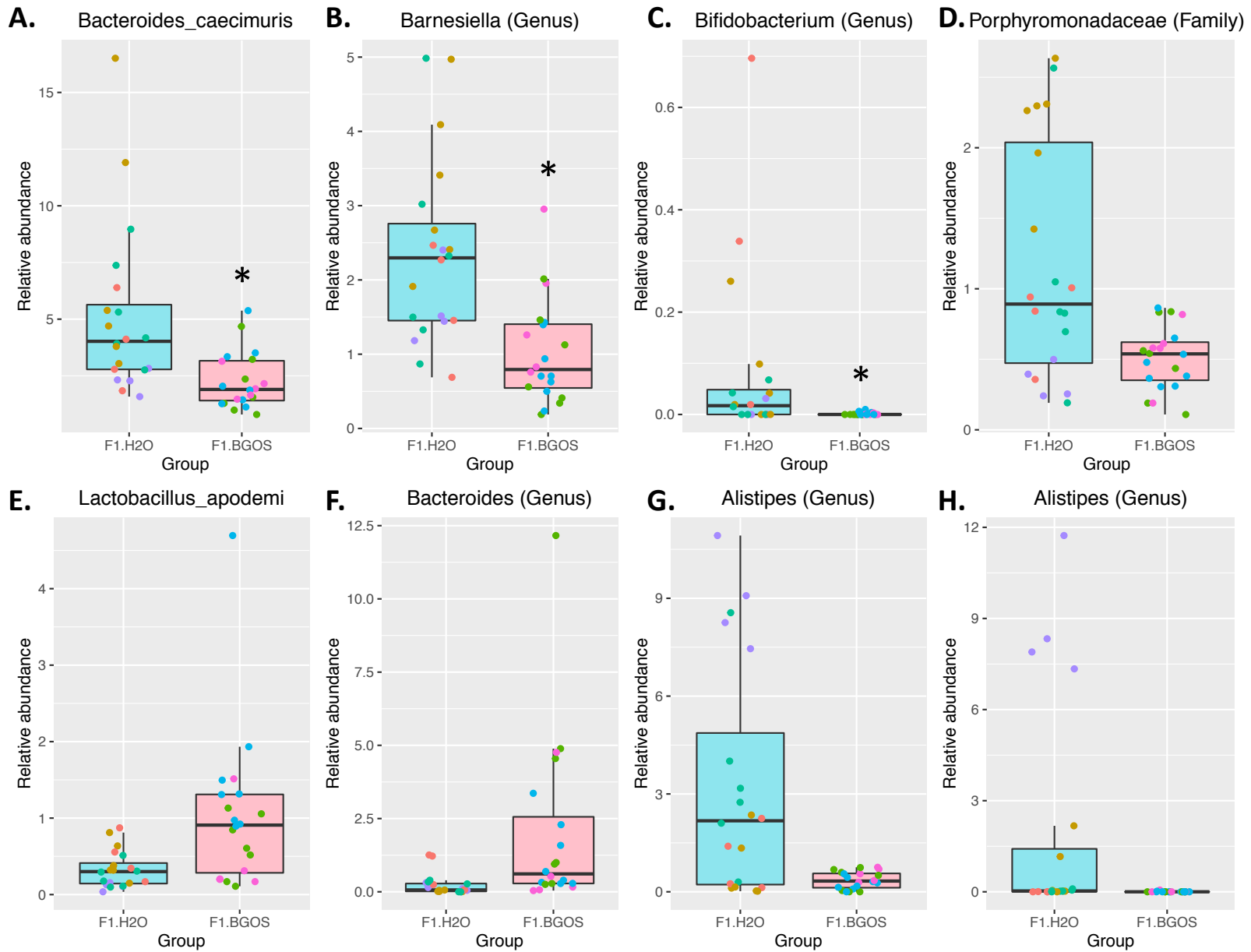
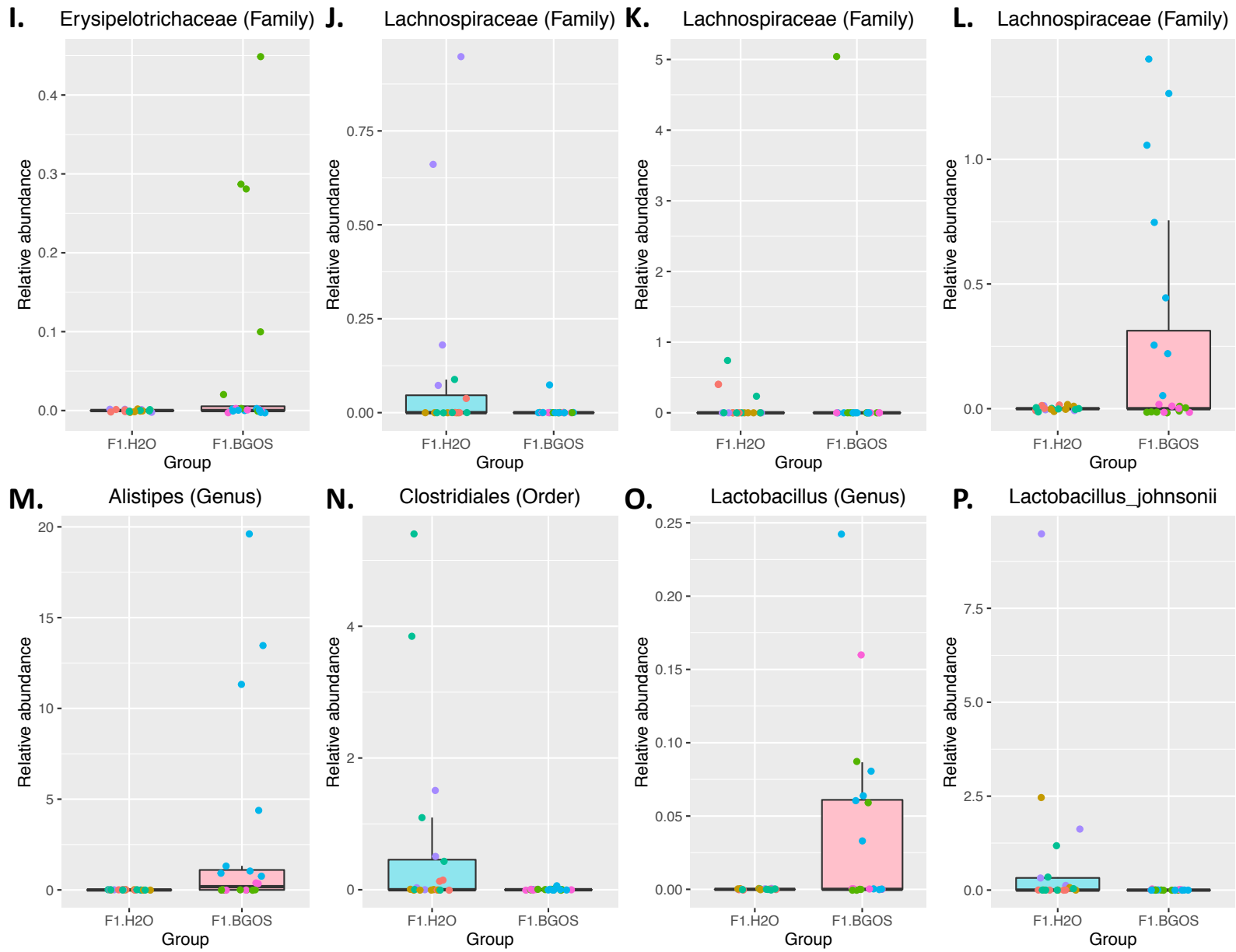


Figure 6.23. Relative abundances of taxa detected by DEseq in dams. Each boxplot represents one ASV, and the lowest taxonomic level assigned by *dada2* is indicated in the boxplot titles. DEseq, * $p < .05$, $N = 10-12$ /group.





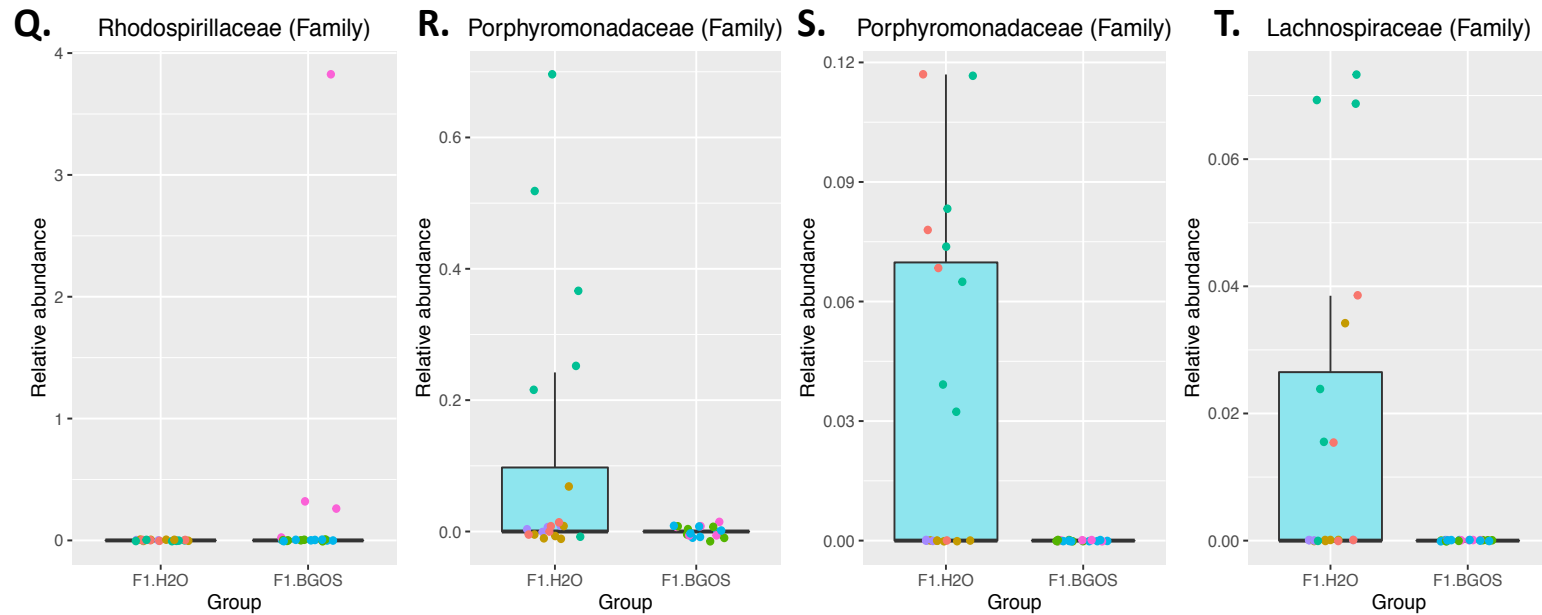


Figure 6.24. Relative abundances of taxa detected by DEseq and mixed-effects analysis in offspring. Each boxplot represents one ASV, and the lowest taxonomic level assigned by *dada2* is indicated in the boxplot titles. Data points are coloured by litter. DEseq and mixed-effects analysis, $*p < .05$, $N = 10$ males/females; $N = 20$ total/group.

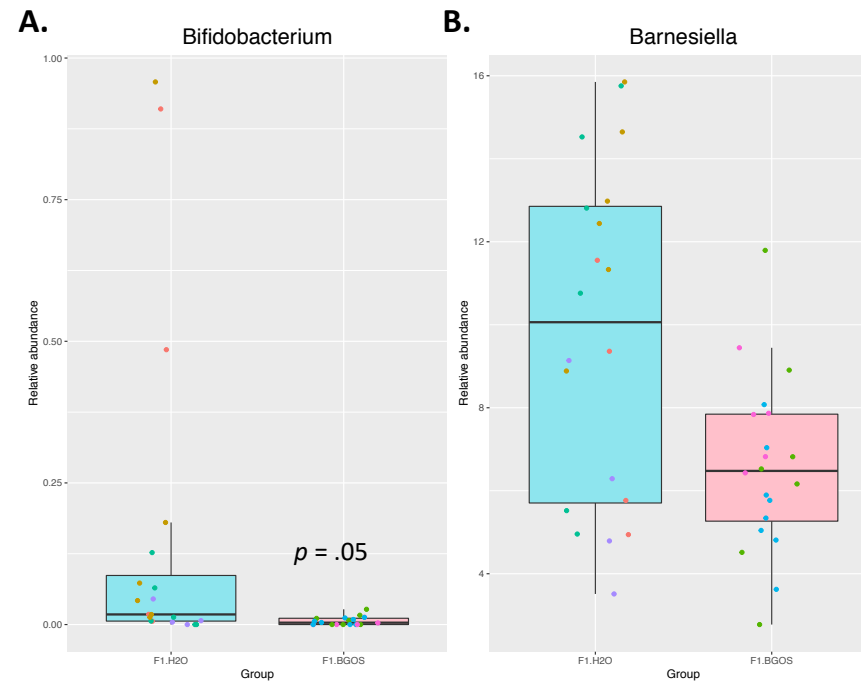


Figure 6.25. Relative abundances of total *Bifidobacterium* and *Barnesiella* in offspring. Because ASVs corresponding to unknown species in the *Bifidobacterium* and *Barnesiella* genera were differentially abundant between H₂O and BGOS pups, ASVs in these genera were pooled and compared between groups using linear mixed-effects analysis. There were no significant differences between groups, although the effect of F0 diet on *Bifidobacterium* was marginal.

6.4.8 Differentially abundant taxa, but not metabolites, correlate with behaviour and brain gene expression in offspring

Behaviour, brain gene expression, blood counts, and differentially abundant ASVs (log-transformed relative abundances) were included in a correlation matrix to investigate associations between microbes and brain changes in offspring (**Figure 6.26**). These data came from the offspring cohort treated with acute saline/LPS in Chapter 3. There were significant correlations between the species *Bacteroides caecimuris* and behaviour in the light-dark box (LDB), including latency to enter the light, $r(37) = 0.33$, $p = .04$, and entries into the light, $r(37) = -0.32$, $p = .04$. In the brain, relative abundance of *Bacteroides caecimuris* positively correlated with expression of the N-Methyl-D-aspartate receptor subunit GluNR2A in the prefrontal cortex (PFC), $r(38) = 0.38$, $p = .02$, as well as hippocampal tumour necrosis factor-alpha (TNF- α), $r(36) = 0.40$, $p = .01$, and the 5-hydroxytryptamine (5-HT)-2A receptor, $r(37) = 0.33$, $p = .04$. The *Barnesiella* genus, which could not be assigned to a specific species, correlated with the following outcomes: rears in the open field test (OFT), $r(38) = -0.42$, $p < .01$, distance travelled in the OFT, $r(38) = -0.35$, $p = .02$, latency to enter the light, $r(38) = -0.11$, $p = .02$, LDB entries into the light, $r(37) = -0.43$, $p < .01$, LDB rears in the light, $r(37) = -0.36$, $p = .02$, time floating in the forced swim test (FST), $r(38) = 0.32$, $p = .04$, PFC expression of GluNR2A, $r(38) = 0.38$, $p = .02$, and hippocampal expression of GluNR1, $r(36) = 0.49$, $p < .01$, GluNR2A, $r(37) = , p < .01$, GluNR2B, $r(37) = , p < .01$, and 5-HT2A, $r(37) = , p < .01$. Finally, the ASV in the *Bifidobacterium* genus was associated with time spent in the centre of the open field, $r(38) = -0.43$, $p < .01$.

Relationships between behaviour and faecal metabolites (selected from the metabolites corresponding to top VIP scores in the OPLS-DA analysis) were also explored using a correlation

matrix (**Figure 6.27**). These data came from the cross-foster cohort (Chapters 3 and 4). There were no correlations between faecal metabolites and behaviour.

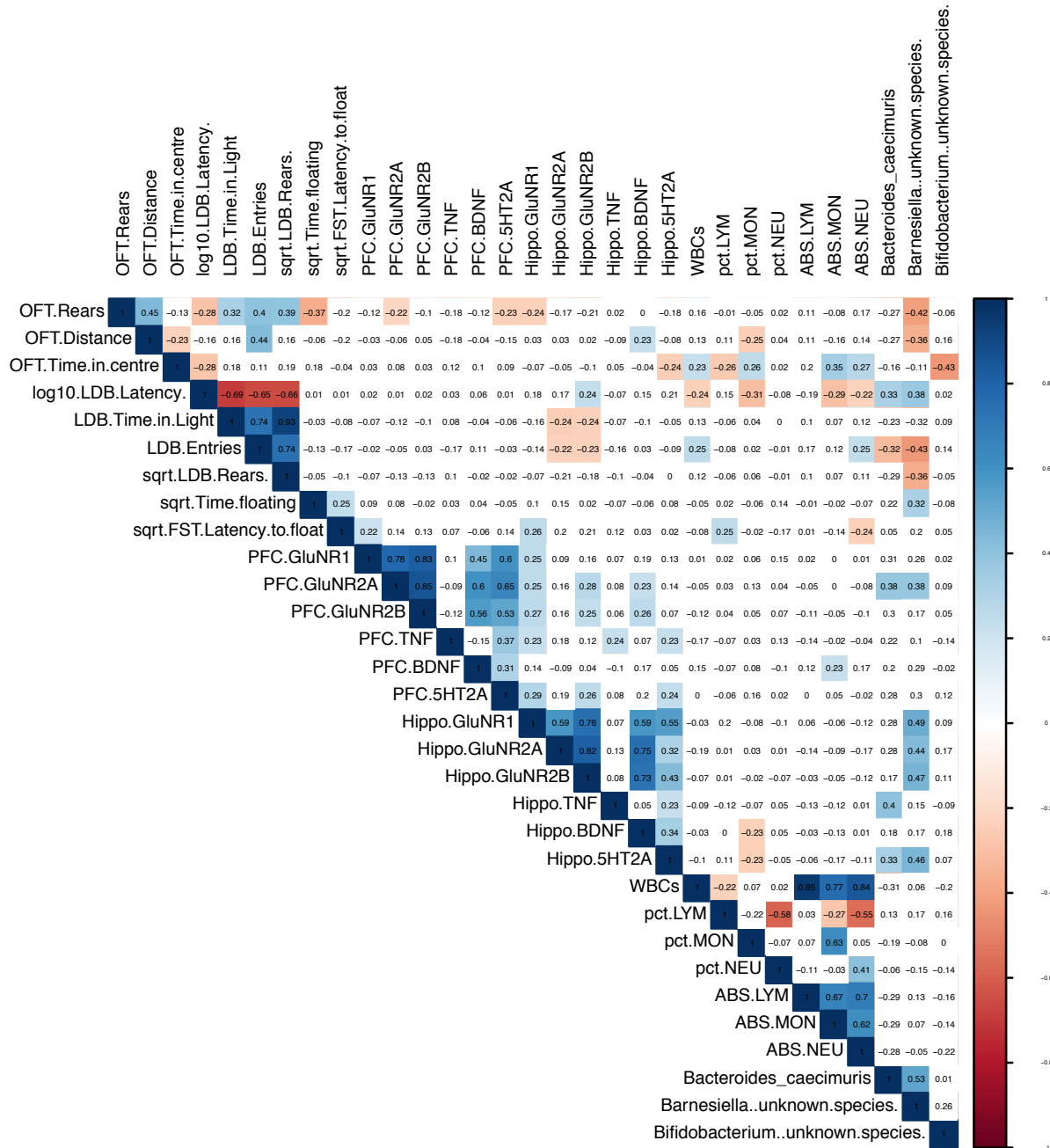


Figure 6.26. Correlation matrix comparing differentially abundant taxa with brain, behaviour, and immune parameters. Pearson correlations between outcomes in young H₂O and BGOS offspring. Coloured boxes indicate significant correlations ($p < .05$).

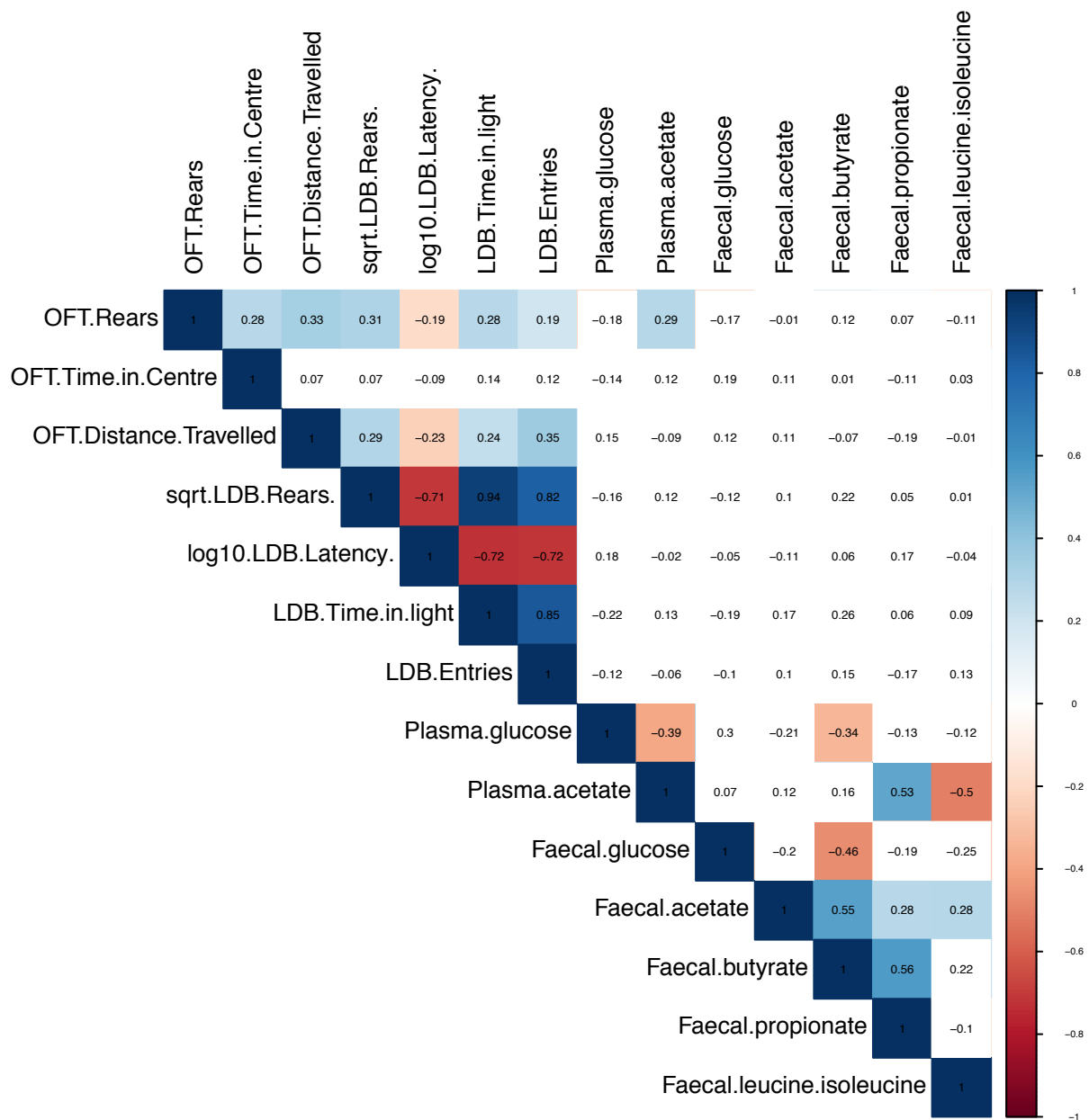


Figure 6.27. Pearson correlations between faecal metabolites and behaviour in the cross-foster cohort.

6.5 Discussion

6.5.1 Summary of results

The purpose of this chapter was to determine whether BGOS supplementation alters gut microbiome composition or metabolic profiles in suckling mothers, pups at weaning age, and adult offspring. In suckling dams, BGOS had no effect on metabolite profiles and produced mild changes in microbiome composition. In young offspring, however, postnatal BGOS resulted in global changes in faecal metabolite profiles in offspring that were driven by increases in butyrate and leucine/isoleucine signals and reductions in glucose. Propionate was also found to be elevated in BGOS pups, while acetate did not change. Microbiome composition in young offspring also differed in BGOS pups compared to controls, and taxa from the *Bacteroides*, *Barnesiella*, and *Bifidobacterium* genera were differentially abundant between groups. In adults, however, while early-life LPS produced mild changes in faecal metabolic profiles, adult BGOS offspring could not be distinguished from adult H₂O offspring based on faecal metabolite profiles. Finally, Pearson correlation analysis revealed significant correlations between microbes and behaviour and brain gene expression. The differences in microbial composition and metabolites in young, but not adult, offspring, and the significant correlations, suggest that maternal BGOS supplementation changes the offspring microbiome in early life, which may lead to changes in brain development. By adulthood – several weeks after weaning to normal drinking water – these microbial changes have normalised, but differences in the brain and behaviour can still be detected. Furthermore, because there were few measurable changes in maternal faecal samples, it is likely that effects of maternal BGOS are transferred through a different route, such as the breastmilk.

6.5.2 BGOS has no effect on maternal faecal metabolite profiles and minimal changes in microbiome composition

BGOS had no effects on faecal metabolite profiles in dams, and contrary to my hypothesis, there were no differences in faecal acetate, propionate, or butyrate levels. In addition, I could not detect global changes in the maternal gut microbiome based on Bray-Curtis dissimilarity matrices. While DEseq did detect five taxa that were differentially abundant between BGOS-fed dams and controls, these differences were driven by two or three outliers per group. It is possible that large microbiome shifts that occur in the perinatal period, including increases in *Actinobacteria*, mask BGOS-induced changes in the maternal gut microbiome (Koren et al. 2012).

6.5.3 Postnatal maternal prebiotic intake alters faecal metabolite profiles and SCFA levels in young, but not adult, offspring

Despite the lack of changes in the maternal microbiome, postnatal maternal BGOS produced significant changes in faecal metabolite profiles in young offspring. Although I had hypothesised that both prenatal and postnatal prebiotic maternal BGOS intake increase faecal SCFAs, butyrate and propionate only increased in pups suckled by BGOS-fed dams. While I had found that plasma acetate increased in BGOS pups in Chapter 4, faecal acetate levels did not change, and there was no correlation between faecal and plasma acetate levels. A small human study showed that faecal acetate levels reflect variation in rates of absorption of acetate from the gut rather than production of the metabolite but that this was not true for butyrate or propionate (Vogt & Wolever, 2003). This finding may explain the discrepancy here. In addition to the SCFAs, glucose and leucine/isoleucine signals were also reduced in pups suckled by BGOS-fed dams. Finally, the decrease in faecal glucose was consistent with the reduction in plasma glucose, but

there was no correlation between faecal and plasma glucose, suggesting that the reduced plasma glucose was not related to altered microbial glucose metabolism.

The robust effect of postnatal BGOS on faecal metabolite profiles suggests that if brain, behaviour, or other changes are mediated by the gut microbiome, these differences are likely caused by postnatal factors rather than *in utero* factors. This finding is also consistent with a study that used a cross-fostering design to show that the offspring gut microbiome is determined by the nursing dam rather than the birth mother (Daft et al. 2015). In this case, maternal BGOS may change faecal metabolite levels by transferring metabolites, fermentable compounds, or microbes to offspring via the breastmilk (Shen et al. 2011; Jost et al. 2014; Marcobal et al. 2010).

In adult offspring, there were significant differences between animals that received early-life LPS and saline in H₂O offspring but not in BGOS offspring. Although the OPLS-DA model comparing saline-treated pups to LPS-treated animals was weak (low accuracy and R² and Q² values), it is possible that early-life inflammation has long-lasting effects on the offspring microbiome and that this difference is prevented by maternal prebiotic intake. Few studies have investigated the influence of neonatal inflammation on microbiome function or composition later in life. One study tested the influence of neonatal exposure to LPS on microbiome composition in adolescence in hamsters but did not find any effects (Sylvia, Deyoe, and Demas 2018). The effects of early-life LPS on the adult microbiome should be investigated further in future studies.

Finally, although I had hypothesised that functional changes in the gut microbiome would last until adulthood, there were no maternal BGOS-induced changes in faecal metabolites in adults. This shows that while brain and behaviour changes can be detected in adult offspring, microbiome changes are not long-lasting. Although it will be necessary to perform 16S sequencing on faecal samples from adult offspring, I would predict that there are no differences in microbiome

composition in adults based on the metabolic data. Therefore, after offspring are separated from the mother and weaned to normal drinking water, microbiome composition and metabolic function likely normalise and are determined primarily by environmental and dietary factors. Nevertheless, changes in the gut microbiome in early life may produce changes in the brain and behaviour that last into adulthood (Clarke et al. 2013; Heijtz et al. 2011; Stilling et al. 2015).

6.5.4 Maternal BGOS supplementation alters microbiome composition in young offspring, and differentially abundant taxa correlate with some brain and behaviour metrics

Bray-Curtis dissimilarities were used to determine whether gut microbiota in BGOS pups were more similar to each other than to gut microbiota in H₂O pups. In addition, correlation analysis was used to investigate associations between differentially abundant taxa and behaviour. There were global changes in gut microbiome composition based on analysis of 16S rRNA ASVs in faecal DNA, which was expected based on studies showing differences in microbiome composition after consumption of galacto-oligosaccharide prebiotics (Cheng et al. 2018). After accounting for random litter effects, there were three significant ASVs that were differentially abundant between BGOS and H₂O pups: *Bacteroides caecimuris* and ASVs from the *Barnesiella* and *Bifidobacterium* genera were significantly reduced in BGOS pups.

Bacteroides caecimuris is a member of the gram-negative, obligate anaerobic *Bacteroides* genus. Although *Bacteroides caecimuris* has not been linked to brain gene expression or behaviour in previous studies, other *Bacteroides* species have emerged as important players in the gut microbiome-brain axis. *Bacteroides fragilis*, for example, prevented autism-like behaviour and gut permeability in offspring in the maternal immune activation mouse model (Hsiao, McBride, et al. 2013), and total *Bacteroides* negatively correlated with a resting-state functional magnetic resonance imaging (fMRI) signature of depression in human subjects (Strandwitz et al. 2019). In

contrast, increased *Bacteroides* has been associated with early-life adversity and reduced social behaviour in mice (Rincel et al. 2019). *Bacteroides caecimuris* may be an important member of the *Bacteroides* genus in the microbiome-gut-brain axis.

An ASV associated with *Barnesiella* significantly correlated with a number of behaviour and gene expression outcomes. The ASV could not be assigned to a specific species, but increased *Barnesiella* has been associated with autism spectrum disorder in humans (Liu et al., 2019). Although this study did not test autism-like behaviours, this supports the finding that this taxon is associated with increased anxiety and behavioural despair.

The decrease in a *Bifidobacterium* taxon was surprising, given that BGOS has previously been shown to increase *Bifidobacterium* (Grimaldi et al., 2016; Liu et al., 2016). When I consolidated all ASVs in the *Bifidobacterium* genus, there was also a trend towards reduced relative abundance of total *Bifidobacterium* in BGOS pups. In addition, my initial qPCR analysis also showed reduced copy numbers of *Bifidobacterium* 16S rRNA per gram of faeces in BGOS offspring (**Appendix J**). For the most part, *Bifidobacterium* did not correlate with brain gene expression or behaviour, so the decrease in *Bifidobacterium* in BGOS pups is likely independent of brain and behaviour changes. Furthermore, although *Bifidobacterium* strains are generally considered to impart benefits on the host, it is important to not place too much weight on specific taxa as “good” or “bad” (Cirstea, Radisavljevic, and Finlay 2018). Finally, partial sequencing of the 16S gene gives limited resolution, especially at the species level, and cannot give information about the effects of BGOS on relative abundances of specific species or strains of *Bifidobacterium* (J. S. Johnson et al. 2019).

There were no significant correlations between faecal metabolites and offspring behaviour. Although the increase in SCFAs may be beneficial for other aspects of offspring health, there is

likely another mediator between the altered gut microbiome and the changes in offspring behaviour. Alternative mechanisms that were discussed in the thesis introduction include other microbe-derived metabolites that cannot be detected with ^1H NMR, modulation of vagus nerve activity, or changes in immune function that were not investigated in this project.

It is interesting that the differentially abundant taxa significantly correlated with a number of brain and behaviour metrics. It should be noted that this correlation analysis was exploratory. There is a high risk of false positives with such a large number of tests, but I did not correct p values for multiple comparisons because I was more interested in developing hypotheses for future studies than definitively establishing correlations between microbial factors and specific brain and behavioural outcomes. Nevertheless, these correlations provide the rationale for testing causality in future experiments. This could be accomplished by performing a faecal transplant from suckling dams or neonates receiving normal drinking water or water supplemented with BGOS into germ-free or antibiotics-treated mice.

Finally, the lack of changes in the maternal microbiome, combined with the exclusively postnatal effect of BGOS supplementation on faecal metabolites, suggest that the effects of maternal BGOS on the offspring brain and behaviour occur during the suckling period. My hypothesised mechanism was that BGOS alters composition and function of the maternal gut microbiome and, in turn, the offspring gut microbiome via direct exposure to these microbes due to co-housing and coprophagia. These results, however, indicate that there are not substantial changes in the maternal gut microbiome. Therefore, it is likely that BGOS alters the offspring gut microbiome and brain through a different mechanism. BGOS may alter metabolites or microbes in the breastmilk and, as a result, produce changes in the offspring microbiome and brain. This hypothesis should be investigated in future studies.

6.5.5 Conclusion

Maternal prebiotic intake alters gut microbiome composition and metabolism in young offspring. Microbial taxa that are less abundant in pups of BGOS-fed dams correlate with brain gene expression and behaviour. This provides rationale for investigating a causal relationship between the offspring microbiome and brain and behaviour in future studies. In addition, there were minimal changes in the maternal gut microbiome after BGOS feeding and a strong postnatal effect of maternal BGOS on offspring faecal metabolite profiles. These results suggest that rather than direct exposure to maternal gut microbes, changes to breastmilk composition may provide a better explanation for the differences in brain gene expression, behaviour, immunity, and metabolism in BGOS pups compared to controls. This revised hypothesis should be considered in follow-up experiments.

7. Discussion

7.1 Summary of results

Studies on the microbiome-gut-brain axis have rapidly populated the literature, including research on the effects of prebiotic supplementation on the brain and behaviour in both animal and human studies. Studies on neurobehavioural effects of maternal diet on offspring, and the role of the maternal and offspring microbiota in offspring brain development, however, are lacking.

The primary aim of this thesis was to determine whether maternal intake of the prebiotic BGOS changes behaviour and brain gene expression in young offspring. This was explored in Chapter 3 by feeding pregnant and suckling CD1 females the BGOS prebiotic in drinking water from conception until weaning. Offspring underwent behavioural testing 24 hours after a lipopolysaccharide (LPS) challenge, and brain tissue was collected immediately after. I specifically expected LPS to reduce exploration and increase innate anxiety behaviour and passive stress-coping behaviour. In the brain, I had expected LPS to increase expression of N-methyl-D-aspartate receptor (NMDAR) subunits, tumour necrosis factor (TNF)- α , and 5-hydroxytryptamine (5-HT)_{2A} and decrease brain-derived neurotrophic factor (BDNF). I had hypothesised that F0 BGOS reverses these effects. While LPS administration had minimal effects, the brain and behaviour changes from baseline in offspring were consistent with my hypothesis: offspring from BGOS-fed dams were more exploratory, exhibited less anxiety-like and passive stress-coping behaviour, and had altered gene expression in the prefrontal cortex (PFC) and hippocampus. Other results not described in this thesis suggest that altered maternal BGOS intake can also alter brain gene expression in an inbred mouse strain. C57Bl/6 mice were given BGOS at a dose of 1.5 mg/mL, and offspring showed altered gene expression of NMDAR subunits and cytokines in the

frontal cortex and cerebellum (**Appendix K**). This result reinforces the findings in Chapter 3 by showing that the effects of maternal prebiotic intake on gene expression occur in different strains of mice.

The remaining experiments in Chapters 3–6 built on these results. The cross-fostering study confirmed my hypothesis that prenatal and postnatal BGOS influence behaviour in offspring, but, importantly, it showed that the effects of postnatal maternal BGOS are more robust. In addition, a test of maternal care ruled out differences in maternal behaviour as a cause of brain and behaviour changes in offspring. The findings from this thesis are summarised in **Figure 7.1**.

The aim of Chapter 4 was to explore whether maternal prebiotic intake influences changes in immune function and the plasma metabolome in offspring. The goal of these experiments was to identify specific immune and metabolic factors that characterise these animals and, if possible, generate hypotheses about their effects on the brain and behaviour for future studies. BGOS pups tended to have higher levels of white blood cells (WBCs), and their bone marrow-derived macrophages (BMDMs) had a blunted pro-inflammatory response *in vitro*. Metabolically, BGOS pups were characterised by reduced plasma glucose and increased acetate levels. Although correlation analyses showed that these factors were not correlated with gene expression or behavioural outcomes, these findings demonstrate that maternal BGOS has widespread effects on offspring health.

In Chapter 5, I tested the hypothesis that the brain, behaviour, and metabolic changes in young pups last until adulthood. The same genes and behaviours were indeed affected in adult offspring as in young pups, but the changes were sex-specific, and some outcomes (NMDAR expression in the PFC) changed direction. In addition, early-life LPS reversed some BGOS-induced changes, including behavioural despair in the forced swim test (FST) and NMDAR

expression in the PFC, suggesting that the pro-inflammatory effects of LPS and the anti-inflammatory effects of maternal BGOS cancel each other out. In addition, there were long-term changes in metabolite profiles that were driven by changes in lipoprotein-associated lipids. This chapter showed that maternal BGOS has long-lasting and sex-specific effects on the brain and behaviour and that it may be anti-inflammatory in offspring.

Finally, Chapter 6 explored the involvement of maternal and offspring microbiome composition and metabolite profiles in the changes identified in the previous chapters. Surprisingly, there were minimal changes in the maternal microbiome after BGOS supplementation, but this might be explained by large shifts in the microbiome during pregnancy that mask any changes due to prebiotic intake. Importantly, this suggests that the maternal gut microbiome is not involved in the neurobehavioural changes in offspring. Instead, rather than direct exposure to maternal gut microbes, changes to breastmilk composition may provide a better explanation for the differences in brain gene expression, behaviour, immunity, and metabolism in BGOS pups compared to controls. This is supported by the effect of postnatal, but not prenatal, maternal BGOS intake on offspring faecal metabolite profiles. Finally, correlations between microbial factors and brain gene expression and behaviour provide the rationale for exploring a causal relationship between the altered microbiome in BGOS pups and the brain in future studies.

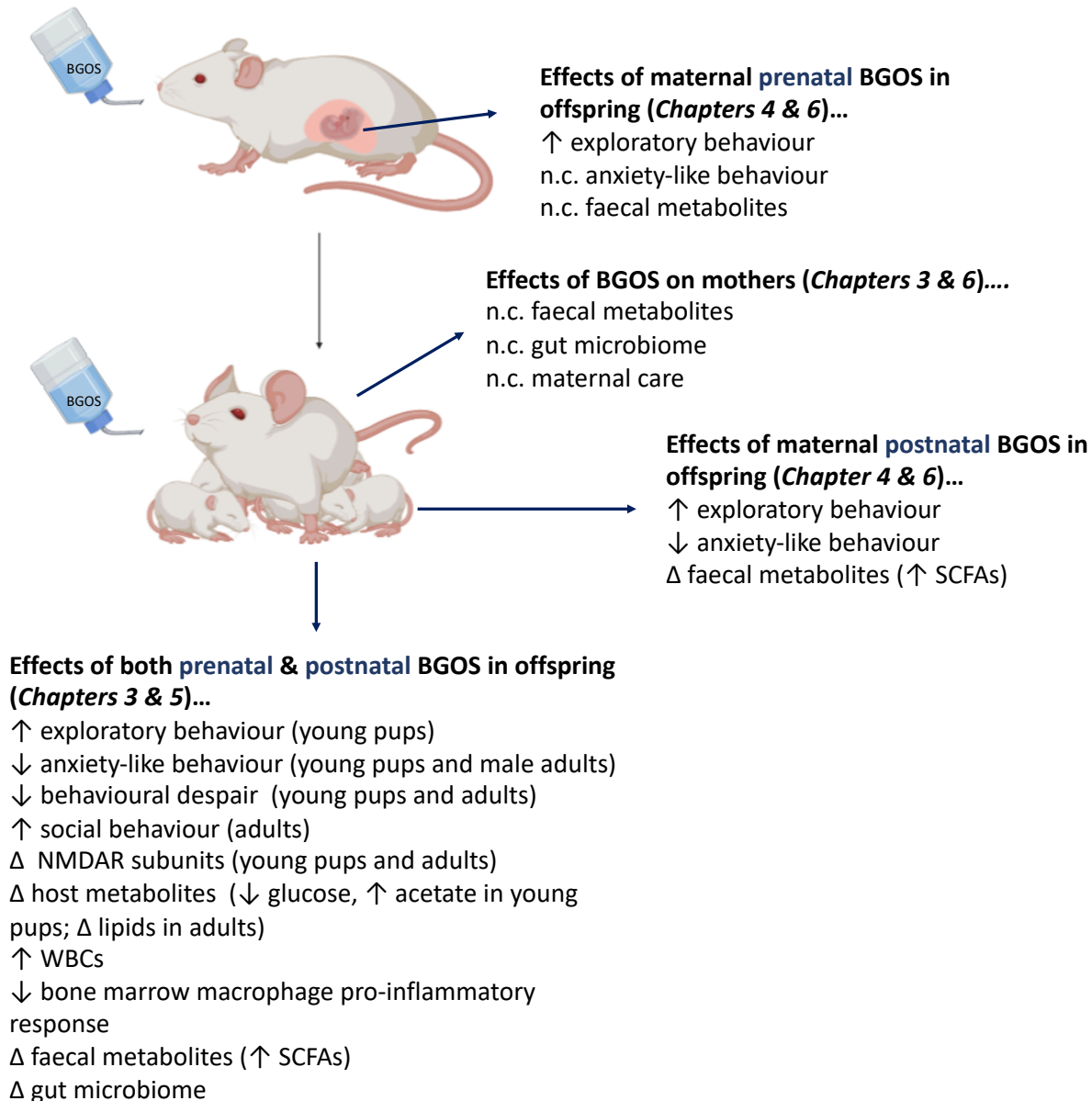


Figure 7.1. Summary of findings. No change = “n.c.”

7.2 Potential mechanisms

Changes in offspring brain gene expression may be related to the altered concentrations of microbial metabolites. Maternal prebiotic feeding resulted in higher levels of plasma acetate and faecal butyrate and propionate compared to pups from control dams. These elevated levels of

SCFAs may come from breastmilk. Breastmilk itself contains high levels of SCFAs (Z. Jiang et al. 2016), while compounds in breastmilk, such as human milk oligosaccharides, can also be fermented in the offspring gut to produce SCFAs (Xiao et al. 2018). BGOS supplementation in mothers may increase SCFA levels in offspring through one or both of these mechanisms. High-fibre diet, for example, increases oligosaccharide levels in maternal milk in rats (Hallam et al. 2014). The influence of prebiotic intake on breastmilk composition, however, should be investigated further in future studies. SCFAs can also cross the blood-brain barrier (Minamiyama et al. 2004; Frost et al. 2014). The most likely mechanism by which SCFAs modulate gene expression is by acting as histone deacetylase (HDAC) inhibitors. SCFAs have been shown to inhibit mRNA and protein expression of HDAC3 and HDAC5 *in vitro* (Zheng et al. 2020), and oral administration of butyrate increases acetylation at histone H3 in the brain in mice. In young offspring from B-GOS-fed dams, increased SCFAs may increase histone acetylation and, in turn, increase expression of genes/proteins that negatively regulate expression of NMDAR subunits and 5-HT_{2A}. Future studies will need to investigate brain HDAC activity in offspring following maternal prebiotic feeding. In addition, if SCFA transport to the brain could be prevented by experimentally blocking monocarboxylate transporters in endothelial cells, this could confirm whether transport of SCFAs into the brain is necessary for changes in gene expression in offspring.

Sex differences were common in this study, particularly in adult offspring. This is not surprising, considering that differences between males and females have been reported previously in studies exploring the effects of maternal diet (S. A. Johnson et al. 2017; Bilbo and Tsang 2010; Sasaki et al. 2013; Mckee et al. 2018; Keleher et al. 2018; Ye et al. 2018; Surzenko et al. 2019). Although the results of 16S sequencing in Chapter 6 showed that maternal BGOS-induced changes in offspring microbiome composition and metabolite profiles were the same in male and female

offspring, these microbial changes may have differential effects on the male and female brain. The gut microbiome has been shown to modulate behaviour and brain gene expression in a sex-dependent manner. For example, recolonisation of female, but not male, germ-free (GF) mice normalised hyperlocomotor activity and anxiolytic behaviour (Luk et al. 2018), while male, but not female, GF mice have reduced hippocampal BDNF and 5-HT (Clarke et al. 2013). Sex differences in anxiety-like behaviour may be explained by an interaction between the early-life microbiome and estrogen and estrogen receptors, which are involved in anxiety-like behaviour (Eid et al. 2019) and hippocampal serotonin concentrations (Imwalle, Gustafsson, and Rissman 2005) and NMDAR levels (Cyr et al. 2001), although the interaction between gut bacteria and estrogen has not yet been investigated. Future studies could explore the effects of maternal BGOS in ovariectomised offspring to confirm whether estrogen or estrogen receptors are involved in neurobehavioural changes following maternal BGOS supplementation. Finally, given that NMDAR antagonists have been shown to have anxiolytic properties (Victoriano et al. 2019), the increase in cortical GluNR1 may also leave females susceptible to anxiety-like behaviour.

7.3 Implications of relationship between maternal diet and offspring microbiome and brain

There is a dearth of research on the effects of maternal diet on offspring and the role of the offspring microbiome, but the results in this thesis suggest that this topic warrants further investigation. The early postnatal period is critical for brain development, and the offspring microbiome is emerging as an increasingly important player in early neurodevelopment and a link between maternal and offspring health. Maternal high-fat diet, for example, was associated with altered microbiome composition and deficits in social interaction behaviour and neural circuitry in adult mouse offspring. Normalising the offspring gut microbiome by co-housing high-fat diet

pups with control animals prevented these changes, indicating a causal role of the offspring microbiome in maternal diet-induced brain and behavioural dysfunction (Buffington et al. 2016). Similarly, in the maternal immune activation (MIA) mouse model of autism, deficits in intestinal permeability and behaviour were reversed with *Bacteroides fragilis* treatment, which may again point to a causal relationship between an altered offspring microbiome and brain (Hsiao et al., 2013). Finally, prenatal stress altered the offspring gut microbiome (Jašarević et al. 2017), corticosterone stress response, and hypothalamic gene expression. Inoculation with vaginal microbes from non-stressed dams partially rescued the gut microbiome and brain changes in offspring, suggesting a connection between the maternal vaginal microbiome, the offspring gut microbiome, and the offspring brain (Jašarević et al. 2018). In addition to high-fat diet, inflammation, and stress, this thesis adds maternal prebiotic intake as another modulator of the offspring microbiome and brain.

A number of studies that were discussed in the introduction demonstrated the influence of the maternal microbiome on offspring development. The effects of MIA on offspring, for example, are dependent on the bacteria present in the maternal gut (Kim et al. 2017; Lammert et al. 2018). The results in Chapter 6, however, suggest that changes in the maternal microbiome are not necessary for modulation of the offspring microbiome and brain. Of the mechanisms proposed in the introduction, the most likely explanation in this study is transfer of fermentable compounds, microbes, or metabolites to pups via the breastmilk. Prebiotic intake has been shown to modify breastmilk composition, including immunomodulatory compounds (Nikniaz, Mahdavi, et al. 2013; Nikniaz, Ostadrahimi, et al. 2013; Baldassarre et al. 2016; Kubota et al. 2014). In addition, the breastmilk is an important vehicle for transmission of maternal gut and skin microbes to offspring (Jost et al. 2014; Funkhouser and Bordenstein 2013; Ferretti et al. 2018). This might explain the

changes in brain gene expression and behaviour, immune function, and host and microbial metabolite profiles in pups in the absence of changes in the maternal microbiome.

Although much more work is needed, this set of experiments provides initial evidence that consuming a prebiotic during pregnancy and lactation can have beneficial effects on the brain in offspring. Prebiotics could provide a safe and economical supplement for pregnant women to promote brain health in children. Although a commercial formulation of galacto-oligosaccharides was used here, it is possible that simply consuming a diet high in fibre from fruit, vegetables, nuts, and legumes in the perinatal period may produce similar benefits for infants and children. The results in these experiments show that the effects of perinatal dietary supplements on offspring brain developments warrant further exploration in both animal models and human subjects.

7.4 Limitations and future directions

This was a largely descriptive and exploratory project that investigated many variables. Although experiments like this are important as a starting point, the changes identified in offspring need to be followed up with mechanistic studies. An obvious next step is to test the causal relationship between the offspring microbiome and the neurobehavioural outcomes in BGOS pups through faecal transplants or co-housing in germ-free or antibiotics-treated animals. GF or antibiotics-treated mice could be colonised with gut bacteria from pups of BGOS-fed dams to determine whether the changes in gut microbiome or metabolites cause the differences in brain gene expression or behaviour. The causal relationship between brain gene expression and behaviour could also be explored by administering an NMDAR or 5-HT_{2A} antagonist before behavioural testing to determine whether blocking these receptors normalises behavioural changes following maternal BGOS. Furthermore, I did not measure protein changes in the brain. This will be important to examine in future experiments using Western blotting or enzyme-linked

immunosorbent assay (ELISA) considering that mRNA levels do not always correlate with protein concentrations in tissue (Maier, Güell, and Serrano 2009). It will also be important to explore the functional consequences of the changes in gene expression and, potentially, protein levels, such as an electrophysiology study in pups of BGOS-fed dams to test NMDAR function.

In addition, maternal milk should be tested for composition, metabolite profiles, immunomodulatory compounds, oligosaccharides, and other constituents that could influence offspring brain development. If possible, maternal milk from BGOS-fed dams could be administered to naïve mouse pups to determine whether the neurobehavioural changes can be replicated.

The BGOS dose used in this study was chosen based on a small pilot study that was shown to increase faecal *Bifidobacterium* in C57Bl/6 female mice (**Appendix A**). Testing multiple doses on all the outcomes in these experiments would have required a prohibitive number of mice, and we did not have an *a priori* primary outcome for a more suitable pilot test. This is a key limitation of the study. In future studies, however, a dose-response could be possible using select outcomes that changed significantly after maternal BGOS, such as increased rearing in the OFT and faecal SCFAs and reduced NMDAR expression. A dose-response curve would also bolster the conclusions from this study.

Candidate genes were selected in the brain based on previous BGOS studies in adult rodents (Savignac et al., 2013, 2016; Williams et al., 2016). Now that changes in brain gene expression have been identified with qPCR, an unbiased microarray or RNA sequencing approach would be useful to identify more differentially expressed genes and get an unbiased picture. Similarly, although ^1H NMR is an affordable and easy tool, mass spectrometry would give a more comprehensive picture of the changes in host and microbial metabolites.

Changes in emotional behaviours, including exploratory, anxiety-like, passive stress-coping, and social behaviour, were found following maternal BGOS supplementation. The choice to examine affective behaviours was made based on previous studies that have described changes in these types of behavioural tests after prebiotic feeding in adult rodents. A larger repertoire of behaviours, however, should be explored in the future. In light of the changes in NDMAR subunit expression, it will be particularly important to investigate the effects of perinatal BGOS on cognitive behaviours, such as the Morris water maze or the Y-maze spatial recognition task. Similarly, a larger number of brain regions should be tested in the future. The PFC and hippocampus were a good start given previous studies on prebiotic feeding in adults rodents. Other regions of interest that are key in emotional behaviours include the hypothalamus, the pituitary, the amygdala, the bed nucleus of the stria terminalis (BNST), the nucleus accumbens, the ventral tegmental area, and specific regions of the PFC, such as the medial PFC.

The purpose of this project was to investigate whether maternal prebiotic intake produces changes in the offspring brain and behaviour, immunity, and metabolism as a proof of principle. LPS challenges were employed to help reveal these changes, but the effects of LPS administration were subtle. From a translational perspective, although the BGOS pups exhibited reduced anxiety and behavioural despair, and increased social interaction and exploratory behaviour, it is difficult to say whether these changes from already healthy offspring should be interpreted as “good” or “bad.” Therefore, maternal BGOS intake should be tested in well-validated disease model. In a separate study, maternal BGOS was tested for neuroprotective effects in mice deficient in *Oxr1*, a gene that codes for a protein that regulates neuronal response to oxidative stress. *Oxr1* knockout mice exhibit rapid neurodegeneration in the cerebellum and motor deficits (Oliver et al. 2011). The results of this study can be found in **Appendix L**. I did not have sufficient group numbers to

draw meaningful conclusions about effects of maternal BGOS in this model. Also, this was a severe disease model, and maternal BGOS should be tested in a milder model of neuroinflammatory or psychiatric disease. In addition, I explored immune function and host metabolism in offspring to explore candidates that mediate changes in the offspring microbiome and the brain. The immune and metabolic changes, however, were interesting on their own and should be explored further in rodent models of inflammatory or metabolic disorders.

Finally, prebiotic intake may be a promising supplement for pregnant and breastfeeding women and produce beneficial effects on mental health in children. Although further experiments in animals are necessary first, the effects of perinatal prebiotic intake on the gut microbiome, brain development, and emotional and cognitive well-being should be studied prospectively in human subjects.

7.5 Conclusion

Maternal prebiotic intake modulates brain gene expression and behaviour, immune function, and metabolism in offspring. BGOS also produces changes in offspring gut microbiome composition and metabolism in the absence of changes in the maternal gut microbiome. Future studies will be necessary to determine whether the distinct characteristics in BGOS offspring are caused by changes in offspring gut bacteria and, if so, what mechanisms mediate this relationship.

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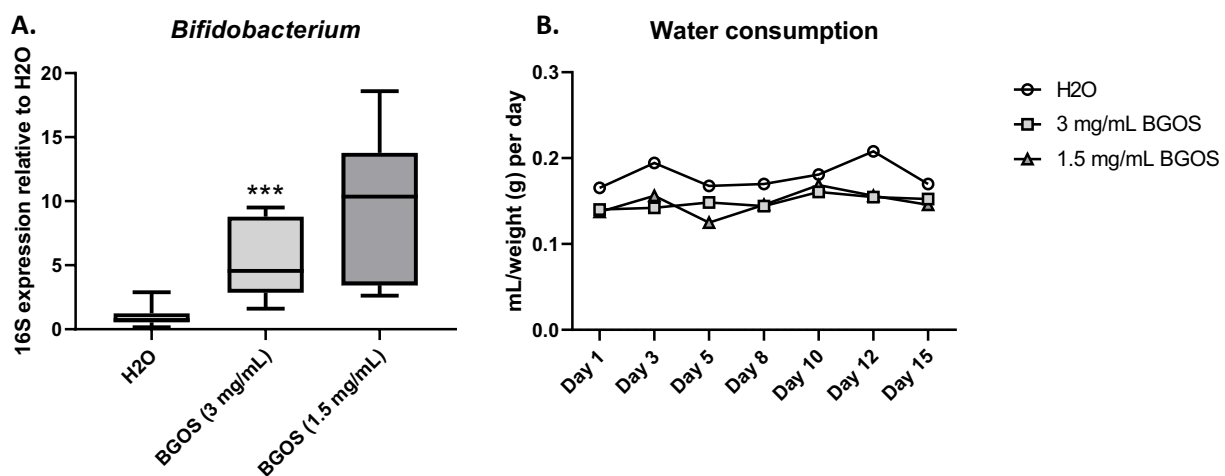
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Appendix A: BGOS dosing pilot study

A small study was performed to determine an appropriate dose of prebiotic for supplementation in the drinking water. The outcome of interest was *Bifidobacterium* levels relative to total bacteria in faecal samples. C57Bl/6 dams (N = 4/group) were administered either normal drinking water, 3 mg/mL BGOS, or 1.5 mg/mL BGOS. Faecal samples were collected, and DNA was extracted for qPCR. *Bifidobacterium* levels were analysed by normalising expression of *Bifidobacterium*-specific 16S rRNA to total bacteria (16S universal sequence).

According to the one-way ANOVA, there were significant differences in relative *Bifidobacterium* levels between groups, $F(2, 20) = 8.71$, $p < .01$. Post-hoc comparisons showed that the 1.5 mg/mL dose of BGOS resulted in a significant increase in *Bifidobacterium* relative to the control group, $p < .001$, while the 3 mg/mL dose was not significant relative to controls, $p = .06$ (**Supplementary Figure 1A**). In addition, volume of water consumed was monitored for the first two weeks of BGOS administration to confirm that the prebiotic did not influence water consumption (**Supplementary Figure 1B**).

There were several limitations to this study. Group numbers were low, and all animals in each group were housed in the same cage. In addition, these females were not pregnant/suckling. These aspects of the pilot study design may explain why I did not see a significant increase in *Bifidobacterium* in BGOS-fed dams. Nevertheless, this dose of BGOS did influence offspring development and gut microbiome composition.



Supplementary Figure 1. A) Expression of *Bifidobacterium* 16S relative to total bacteria and normalised to control group. B) Average mouse water consumption per gram per day.

Appendix B: Confirmation of neuroinflammatory response to 1 mg/kg LPS

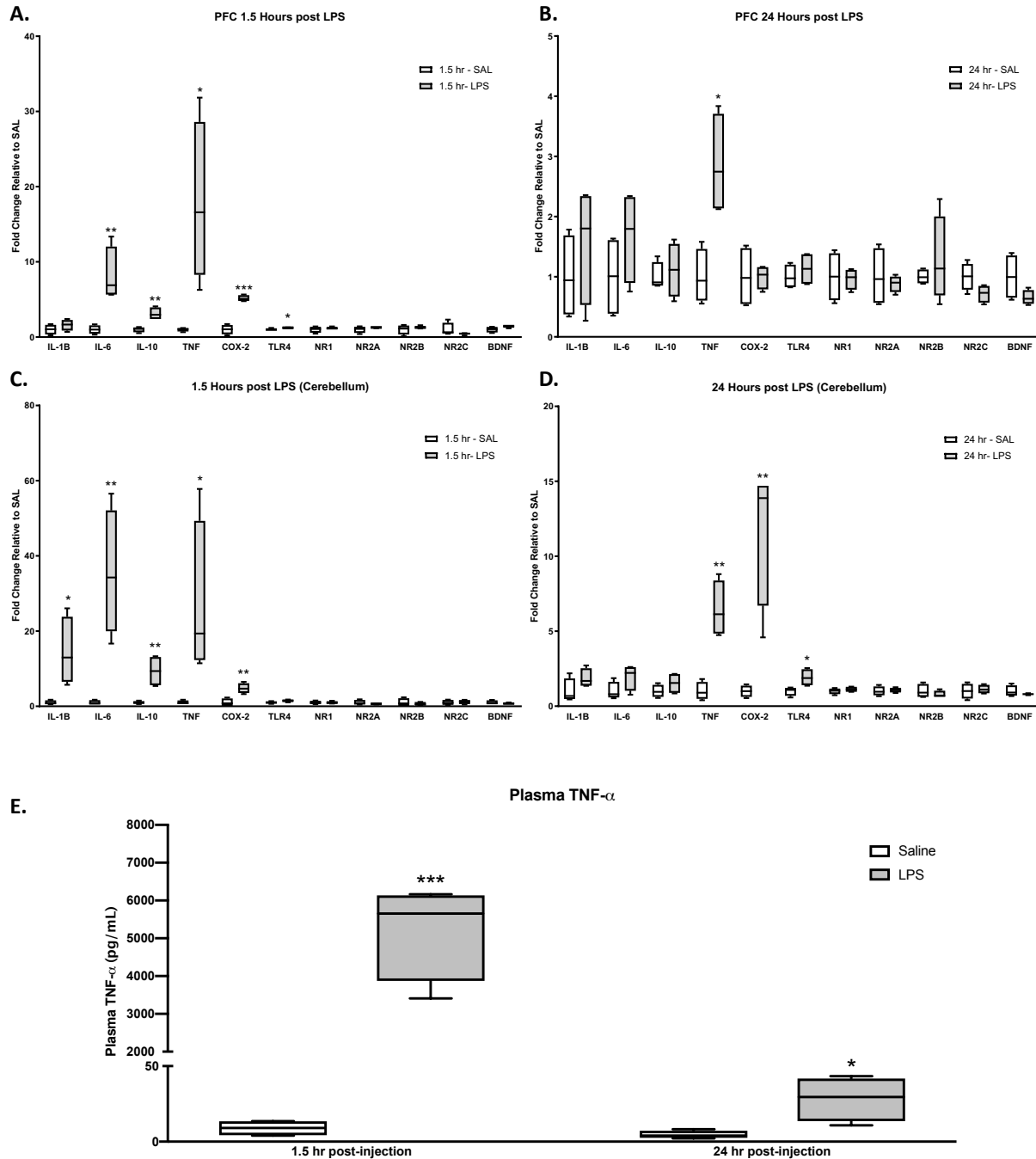
Eight-week old male CD1 mice (N = 4/group) were injected with 1 mg/kg LPS before tissue collection to confirm an inflammatory response to intraperitoneal LPS. Prefrontal cortex (PFC) and cerebellum (Cb) were collected either 1.5 or 24 hours after injections. The reason the cerebellum was collected was because I had started my DPhil with a mouse model (*Oxr1* ^{-/-}) that developed severe neurodegeneration in the cerebellum. I had originally planned to do additional experiments looking at maternal BGOS administration in wild-type C57Bl/6 mice to confirm that maternal BGOS influences gene expression in the cerebellum. T-tests were used to compare brain gene expression between groups.

At 1.5 hours post LPS, expression of the following genes was elevated compared to saline-treated animals in the PFC: COX-2, $t(6) = 11.02$, $p < .0001$, IL-10, $t(6) = 4.64$, $p < .01$, IL-6, $t(6) = 3.93$, $p < .01$, TNF- α , $t(6) = 3.15$, $p = .03$, and TLR4, $t(6) = 2.86$, $p = .03$ (**Supplementary Figure 2A**). At 24 hours, only TNF- α was significantly elevated in LPS-treated animals, $t(6) = 2.87$, $p < .01$ (**Supplementary Figure 2B**). For this reason, TNF was selected as the proinflammatory marker to investigate in CD1 pups.

In the cerebellum, expression of COX-2, $t(6) = 4.38$, $p < .01$, IL-6, $t(6) = 4.11$, $p < .01$, IL-10, $t(6) = 4.06$, $p < .01$, IL-1 β , $t(6) = 2.95$, $p = .03$, and TNF- α , $t(6) = 2.45$, $p = .04$, was elevated in LPS animals (**Supplementary Figure 2C**). At 24 hours, TNF- α , $t(6) = 5.54$, $p < .01$, COX-2, $t(6) = 4.43$, $p < .01$, and TLR4, $t(6) = 2.88$, $p = .03$, were significantly elevated compared to controls (**Supplementary Figure 2D**).

In plasma, TNF- α protein was significantly elevated in plasma after 1.5 hours, $t(6) = 8.19$, $p < .001$. It returned nearly to baseline after 24 hours, although the difference in plasma TNF- α

concentration between the saline and LPS groups remained significant, $t(6) = 3.18$, $p = .02$ (Supplementary Figure 2E).



Supplementary Figure 2. Central and peripheral effects of I.P. LPS. A–D) Central expression of inflammatory markers and NMDA receptors 1.5 and 24 hours after LPS injections and plasma levels. **E)** Plasma levels of TNF- α 1.5 and 24 hours after LPS.

Appendix C: Results of linear mixed-effects analysis

I worked under the assumption that data from offspring in the same litter were independent. About halfway through writing this thesis, it became clear that this assumption needed to be tested. Rather than re-doing the work done so far, I have summarised the results of the linear mixed effects analysis here, including main effects and interactions. Most of the outcomes that I had found to be significant remained significant or marginal after accounting for litter effects.

Supplementary Table 1. Mixed-effects analysis of effects of maternal BGOS, acute LPS, and sex on young offspring.

	F0 Diet	p < .05?	Treatment	p < .05?	Sex	p < .05?	F0 Diet x Treatment	p < .05?	F0 Diet x Sex	p < .05?	Treatment x Sex	p < .05?	F0 Diet x Treatment x Sex	p < .05?
OFT.Rears	0.05744759		0.61882907		0.06280966		0.77778369		0.93286703		0.06509878		0.26975584	
OFT.Distance	0.8925618		0.00135599	*	0.31303065		0.120206191		0.72588345		0.10304657		0.82349175	
OFT.Time.in.centre	0.6024735		0.9398687		0.06742439		0.0045031	*	0.34506005		0.16408612		0.12718047	
log10.LDB.Latency.	0.07342042		0.25976822		0.02615532	*	0.585813103	*	0.85614641		0.31024856		0.0082497	*
LDB.Time.in.Light	0.01474199	*	0.2473054		0.72788085		0.581346738	*	0.75556681		0.02160297	*	0.00083651	*
LDB.Entries	0.22015693		0.11357769		0.83046952		0.570554758	*	0.38478263		0.24462193		0.0063234	*
sqrt.LDB.Rears.	2.04E-05	*	0.9811867		0.68984376		0.840174502	*	0.78002371		0.00896632	*	0.00013686	*
sqrt.FST.Time.floating	0.1253541		0.05192441		0.65013263		0.178563204	*	0.881178		0.79927349		0.42620319	
sqrt.FST.Latency.to.float	0.0114898	*	0.81354499		0.16209175		0.269201138	*	0.36842359		0.88029407		0.9125727	
PFC.GluNR1	0.09307605		0.62230849		0.95505118		0.984725459	*	0.65108781		0.80582798		0.9799554	
PFC.GluNR2A	0.10230132		0.33065819		0.88768169		0.910102354	*	0.23851913		0.60621166		0.68761087	
PFC.GluNR2B	0.07869072		0.10013756		0.63939952		0.975346564	*	0.61049849		0.81193868		0.93737043	
PFC.TNF	0.79553961		0.06851636		0.01522281	*	0.076996296	*	0.10660508		0.08476015		0.12805912	
PFC.BDNF	0.40521218		0.28979856		0.01088193	*	0.47522617	*	0.35598399		0.852358		0.84187894	
PFC.5HT2A	0.18428553		0.69137708		0.04198388	*	0.124254268	*	0.19173831		0.25666693		0.21847476	
Hippo.GluNR1	0.01634282	*	0.00802937	*	0.97574098		0.398284506	*	0.20195455		0.5343321		0.24377729	
Hippo.GluNR2A	0.04404982	*	0.01395198	*	0.76746222		0.840196903	*	0.48724694		0.69431634		0.98267821	
Hippo.GluNR2B	0.03541474	*	0.00458282	*	0.09970957		0.669437139	*	0.24359955		0.76963944		0.83485783	
Hippo.TNF	0.70471839		0.35133695		0.03857174	*	0.31031178	*	0.0421505	*	0.65149329		0.63340259	
Hippo.BDNF	0.29781423		0.00013129	*	0.15199756		0.768689345	*	0.18309082		0.60221782		0.53256659	
Hippo.5HT2A	0.11965965		0.10990432		0.23297485		0.086346688	*	0.99805041		0.13298399		0.57139075	
WBCs	0.30689615		0.26295213		0.31591366		0.714985243	*	0.02059941	*	0.19638993		0.67882103	
pct.LYM	0.63839023		0.01758399	*	0.00956424	*	0.292799411	*	0.92298052		0.5999642		0.23473296	
pct.MON	0.64214874		0.07096144		0.04652507	*	0.420025071	*	0.96094066		0.22940639		0.29835261	
pct.NEU	0.67389918		0.00473965	*	0.08535975		0.146854449	*	0.13263403		0.77436354		0.49375987	
ABS.LYM	0.2823296		0.57041192		0.78350829		0.703761139	*	0.04836357	*	0.11029916		0.61749797	
ABS.MON	0.57674985		0.06677912		0.5833729		0.650311463	*	0.41954488		0.16497225		0.3939832	
ABS.NEU	0.32931507		0.01984371	*	0.00723309	*	0.39435441	*	0.30251426		0.17469165		0.98180106	

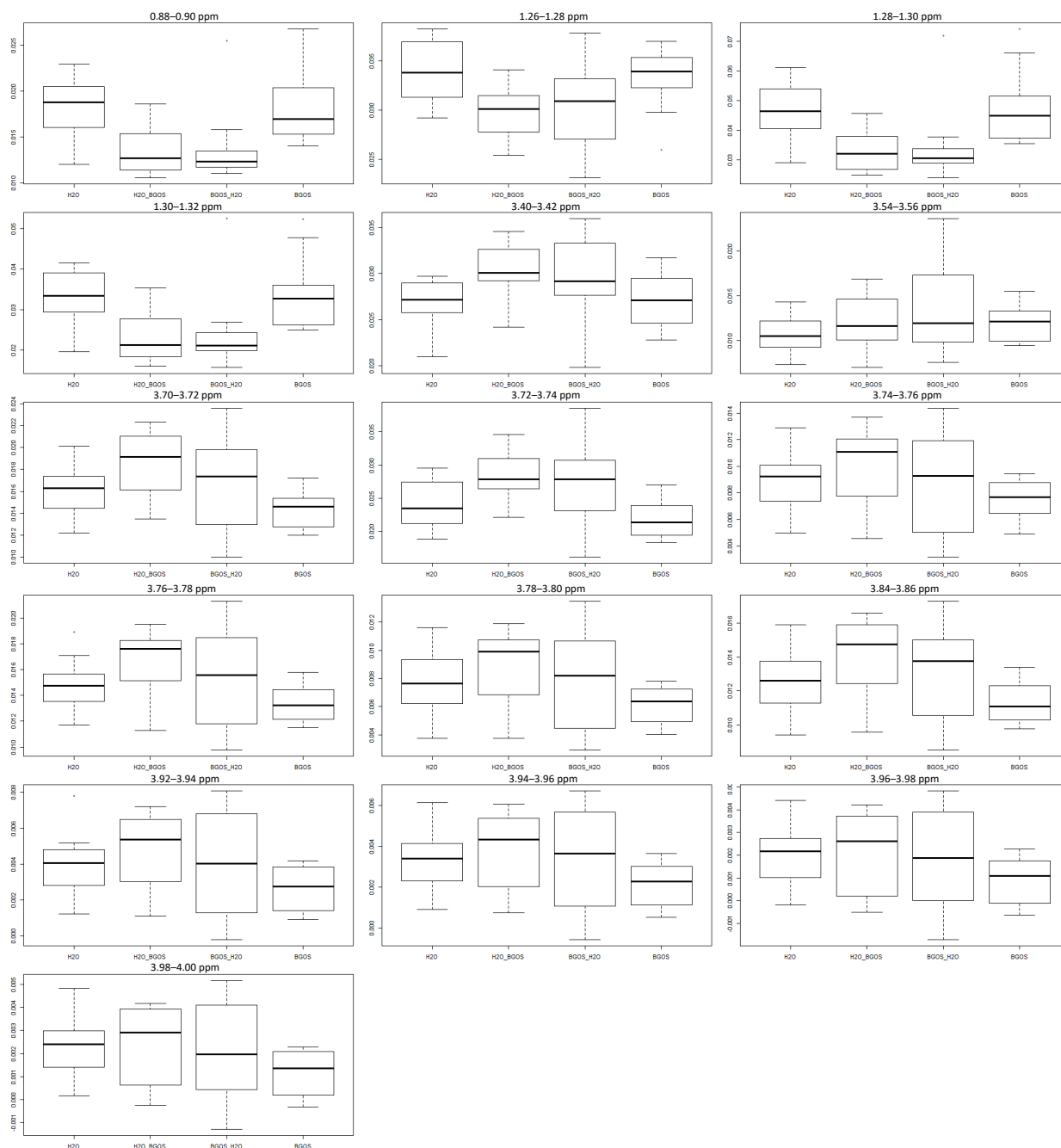
Supplementary Table 2. Mixed-effects analysis of effects of prenatal maternal BGOS, postnatal maternal BGOS and sex on young offspring.

	Prenatal Diet	p < .05?	Postnatal Diet	p < .05?	Sex	p < .05?	Prenatal x Postnatal	p < .05?	Prenatal x Sex	p < .05?	Postnatal x Sex	p < .05?	Prenatal x Postnatal x Sex	p < .05?
OFT.Rears	0.017544439	*	0.006968795	*	0.830109403		0.942159708		0.99700547		0.195401147		0.049266889	*
OFT.Time.in.Centre	0.130895092		0.90818424		0.352804758		0.379142586		0.4319855		0.320210268		0.239225698	
OFT.Distance.Travelled	0.803384453		0.351430021		0.831659215		0.984679683		0.56689455		0.646451014		0.225691107	
sqrt.LDB.Rears.	0.70906949		0.079917915		0.075238578		0.202613715		0.49435283		0.330486161		0.562105599	
log10.LDB.Latency.	0.619586958		0.18987887		0.564778119		0.28678172		0.52594919		0.879937099		0.179201965	
LDB.Time.in.light	0.777237753		0.063554062		0.310551013		0.261805007		0.19044749		0.215077114		0.301860206	
LDB.Entries	0.886758616		0.455393883		0.197378101		0.845888133		0.39222842		0.164279896		0.131947484	
Plasma.acetate	0.364182573		0.760421908		0.000149018	*	0.776133714		0.23247264		0.941981872		0.443545957	
Faecal.acetate	0.56239651		0.423607468		0.216593092		0.533962549		0.5718098		0.676526073		0.028765548	*
Faecal.butyrate	0.804361578		0.045328168	*	0.131126175		0.47985989		0.32670285		0.93965608		0.157140622	
Faecal.propionate	0.758434025		0.049774343	*	0.041240688	*	0.868760972		0.554175		0.502837311		0.22675955	

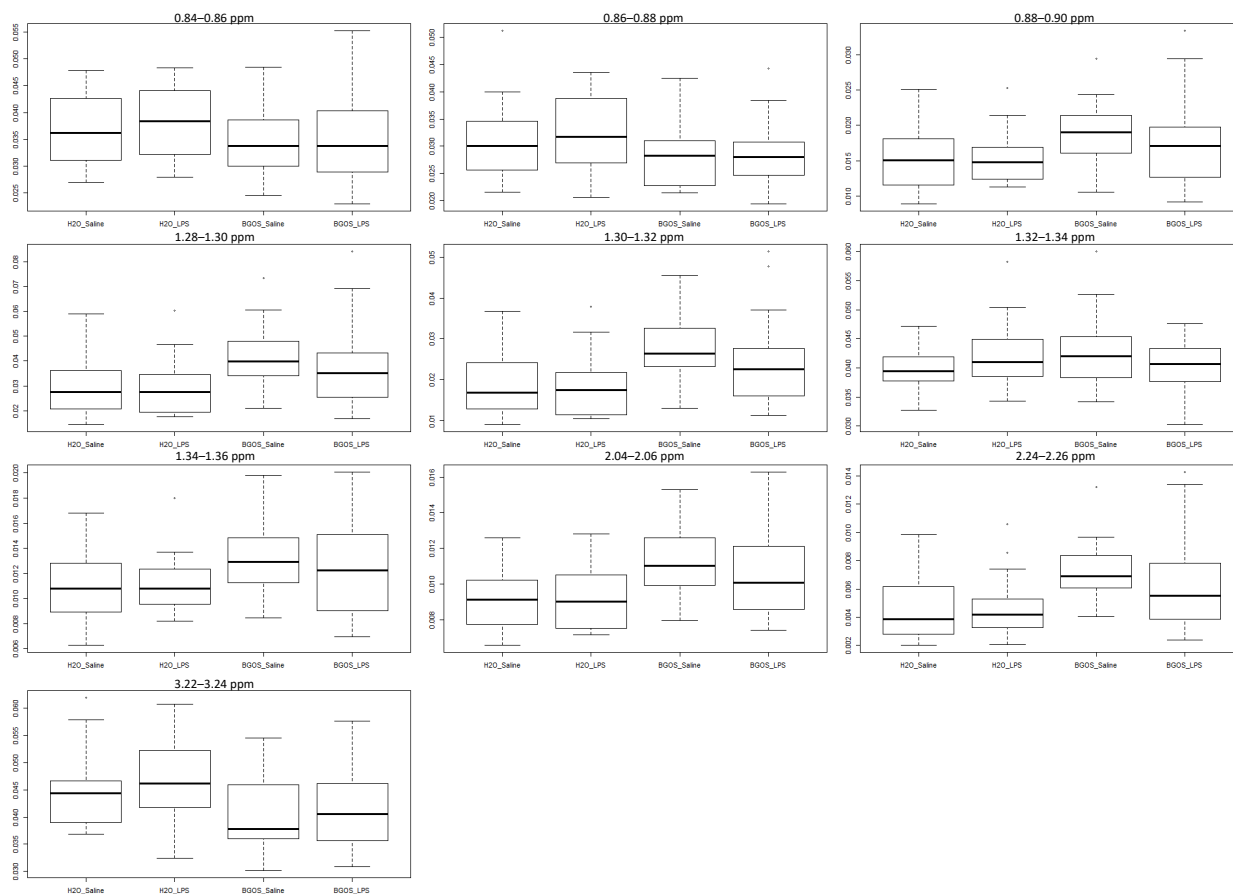
Supplementary Table 3. Mixed-effects analysis of effects of maternal BGOS, acute LPS, and sex in adult offspring.

	F0 Diet	p < .05?	P9 Treatment	p < .05?	Sex	p < .05?	F0 Diet x P9 Treatment	p < .05?	F0 Diet x Sex	p < .05?	P9 Treatment x Sex	p < .05?	F0 Diet x P9 Treatment x Sex	p < .05?
OFT.Rears	0.46933844		0.090801621		0.66746345		0.74752392		0.06040063		0.04725032	*	0.68427994	
OFT.Distance.Travelled	0.80958713		0.070119848		0.17941103		0.16572229		0.24782438		0.75743259		0.10286561	
OFT.Time.in.Centre	0.75036887		0.992103497		0.40778531		0.50526434		0.96800661		0.71823804		0.21390967	
LDB.Time.in.Light	0.97823494		0.19619104		0.00622501	*	0.56762891		0.00050941	*	0.94016288		0.11663416	
LDB.Entries	0.46987167		0.481191147		0.00404952	*	0.7560831		0.37792034		0.87638277		0.87268409	
LDB.Rears	0.89579389		0.070625914		0.00934001	*	0.49155247		0.04453262	*	0.62540381		0.02359019	*
log10.LDB.Latency.	0.39014508		0.730587054		0.07965911		0.62009434		0.11441986		0.5434729		0.68441793	
sqrt.FST.Latency	0.62167017		0.085745974		0.26790352		0.30081416		0.60514968		0.25573388		0.53666041	
FST.Time.Floating	0.12213281		0.21400903		0.86518356		0.03397598	*	0.17447524		0.15801779		0.88363911	
Social.Preference	0.03594941	*	0.019894841	*	0.08279771		0.63522605		0.70946683		0.15292741		0.5035259	
PFC.GluNR1	0.6164223		0.213724804		0.69828405		0.01003891	*	0.21242321		0.86823986		0.00160022	*
PFC.GluNR2A	0.8158352		0.635416982		0.23880042		0.01401153	*	0.26049148		0.56940481		0.29896915	
PFC.GluNR2B	0.93784868		0.087608327		0.35571998		0.00955816	*	0.1625691		0.42012965		0.02439964	*
PFC.5HT2A	0.10334726		0.2690628		0.09654398		0.89099966		0.70420376		0.17007806		0.04833684	*
PFC.BDNF	0.21133903		0.619131336		0.91858925		0.12504792		0.35865594		0.22591138		0.72390943	
Hippo.GluNR1	0.36596876		0.297104322		0.68730692		0.11163425		0.03321867	*	0.86665181		0.39907558	
Hippo.GluNR2A	0.70681571		0.577766046		0.87626084		0.66907148		0.69756209		0.72439849		0.96773515	
Hippo.GluNR2B	0.25219272		0.134150366		0.81263484		0.13460718		0.15067496		0.52651782		0.2055593	
Hippo.5HT2A	0.56612809		0.250245558		0.93976039		0.28387849		0.56738732		0.58168413		0.44210853	
Hippo.BDNF	0.4072499		0.350991811		0.36786689		0.88641334		0.15002728		0.93876139		0.08862922	

Appendix D: Boxplots of significant ^1H NMR plasma spectral bins in young offspring

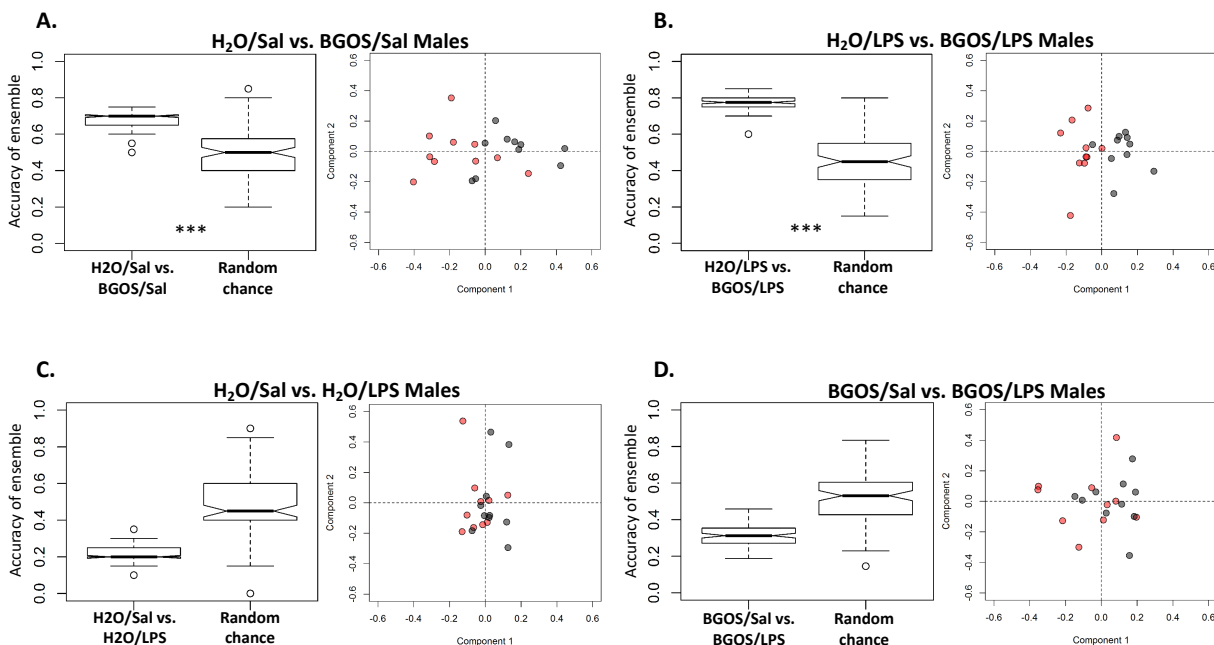


Supplementary Figure 3. Top spectral bins in young offspring plasma OPLS-DA models. Boxplots showing integral values of 0.02 ppm spectral bins corresponding to top VIP scores in young offspring plasma samples.

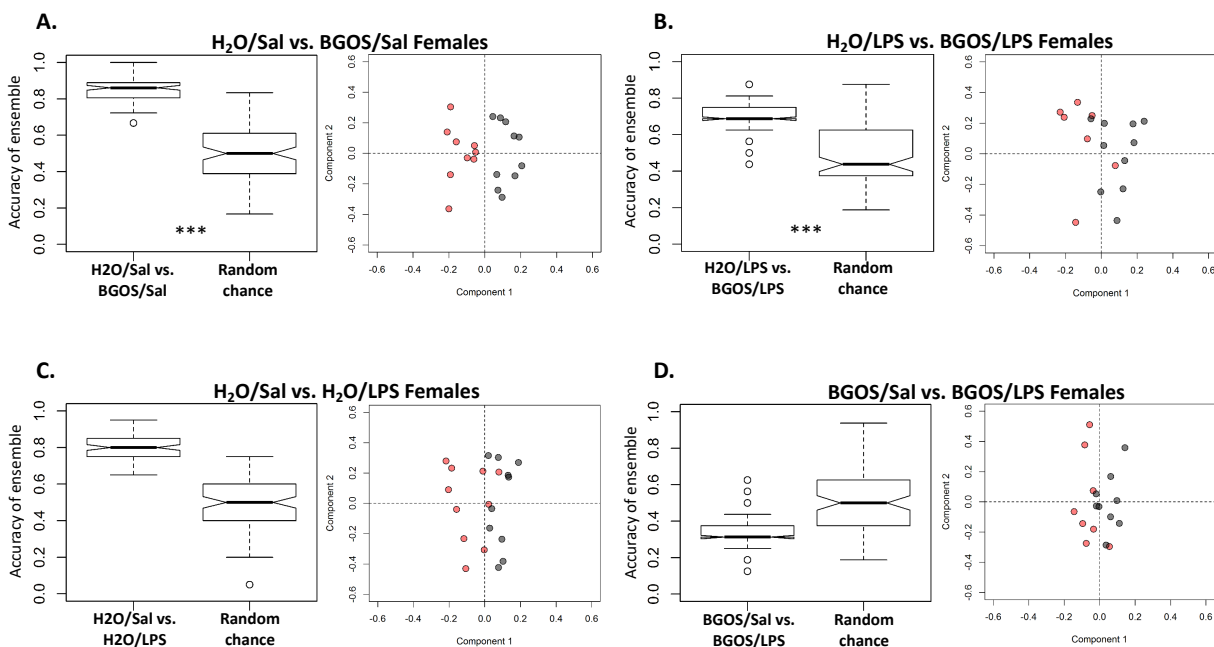
Appendix E: Adult plasma ^1H NMR boxplots and analysis separated by sex

Supplementary Figure 4. Top spectral bins in adult offspring plasma OPLS-DA models. Boxplots showing 0.02 ppm spectral bins corresponding to top VIP scores in adult offspring plasma samples.

In addition to the ^1H NMR analysis presented in Chapter 5, I have included the analyses for male and female adult offspring to demonstrate that the results are similar. The only difference is that in females, there is a significant effect of early-life LPS on plasma metabolite profiles.



Supplementary Figure 5. Maternal BGOS significantly alters plasma metabolites in young male offspring. OPLS-DA accuracies and scores plots for **A)** H₂O/Saline vs. BGOS/Saline, **B)** H₂O/LPS vs. BGOS/LPS, **C)** H₂O/Saline vs. H₂O/LPS, and **D)** BGOS/Saline vs. BGOS/LPS young male offspring.



Supplementary Figure 6. Maternal BGOS and early-life LPS significantly alter plasma metabolites in young female offspring. OPLS-DA accuracies and scores plots for **A)** H₂O/Saline vs. BGOS/Saline, **B)** H₂O/LPS vs. BGOS/LPS, **C)** H₂O/Saline vs. H₂O/LPS, and **D)** BGOS/Saline vs. BGOS/LPS young female offspring.

Supplementary Table 4. Bins corresponding to top VIP scores and metabolite assignments for H2O/Sal vs. BGOS/Sal males.

H2O/Sal vs. BGOS/Sal Males		
Bin	VIP score	Assignment
1.28–1.30 ppm	4.31166912	lipid (-CH ₂ -)n Chyl/VLDL
1.30–1.32 ppm	3.48906747	lipid (-CH ₂ -)n Chyl/VLDL
3.22–3.24 ppm	3.19597463	lipid
0.86–0.88 ppm	2.90190372	lipid (-CH ₃) HDL/LDL
0.84–0.86 ppm	2.88274269	lipid (-CH ₃) HDL/LDL

Supplementary Table 5. Bins corresponding to top VIP scores and metabolite assignments for H2O/LPS vs. BGOS/LPS males.

H2O/LPS vs. BGOS/LPS Males		
Bin	VIP score	Assignment
1.28–1.30 ppm	4.096673315	lipid (-CH ₂ -)n Chyl/VLDL
1.30–1.32 ppm	3.847767085	lipid (-CH ₂ -)n Chyl/VLDL
0.86–0.88 ppm	2.768888045	lipid (-CH ₃) HDL/LDL
3.22–3.24 ppm	2.405924263	lipid
1.28–1.30 ppm	4.096673315	lipid (-CH ₂ -)n Chyl/VLDL

Supplementary Table 6. Bins corresponding to top VIP scores and metabolite assignments for H2O/Sal vs. BGOS/Sal females.

H2O/Sal vs. BGOS/Sal Females		
Bin	VIP score	Assignment
1.28–1.30 ppm	3.492455379	lipid (-CH ₂ -)n Chyl/VLDL
3.22–3.24 ppm	3.4041808	lipid
1.30–1.32 ppm	3.216421333	lipid (-CH ₂ -)n Chyl/VLDL

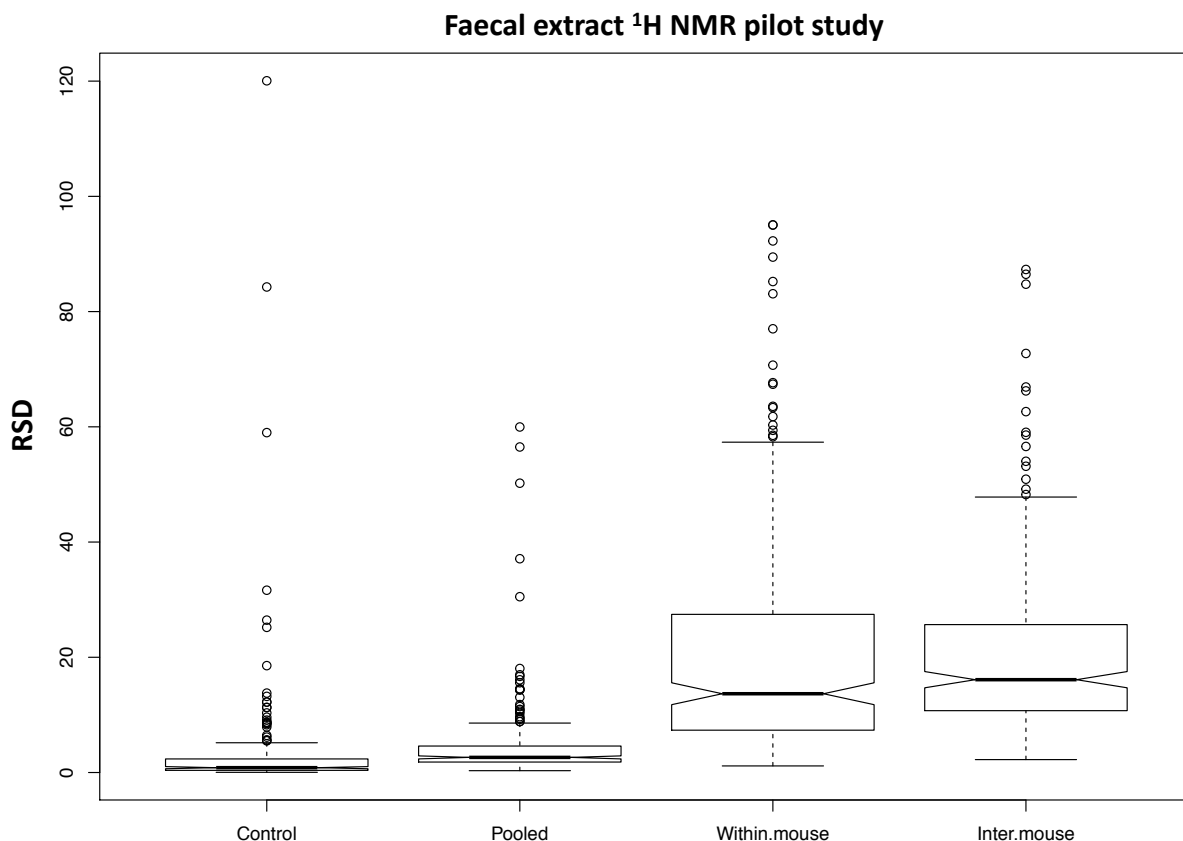
Supplementary Table 7. Bins corresponding to top VIP scores and metabolite assignments for H2O/LPS vs. BGOS/LPS females.

H2O/LPS vs. BGOS/LPS Females		
Bin	VIP score	Assignment
3.22–3.24 ppm	4.138112694	lipid
0.84–0.86 ppm	4.016324904	lipid (-CH ₃) HDL/LDL
0.86–0.88 ppm	3.358723499	lipid (-CH ₃) HDL/LDL
1.28–1.30 ppm	2.628489212	lipid (-CH ₂ -)n Chyl/VLDL
1.30–1.32 ppm	2.341536082	lipid (-CH ₂ -)n Chyl/VLDL

Supplementary Table 8. Bins corresponding to top VIP scores and metabolite assignments for H2O/Sal vs. H2O/LPS females.

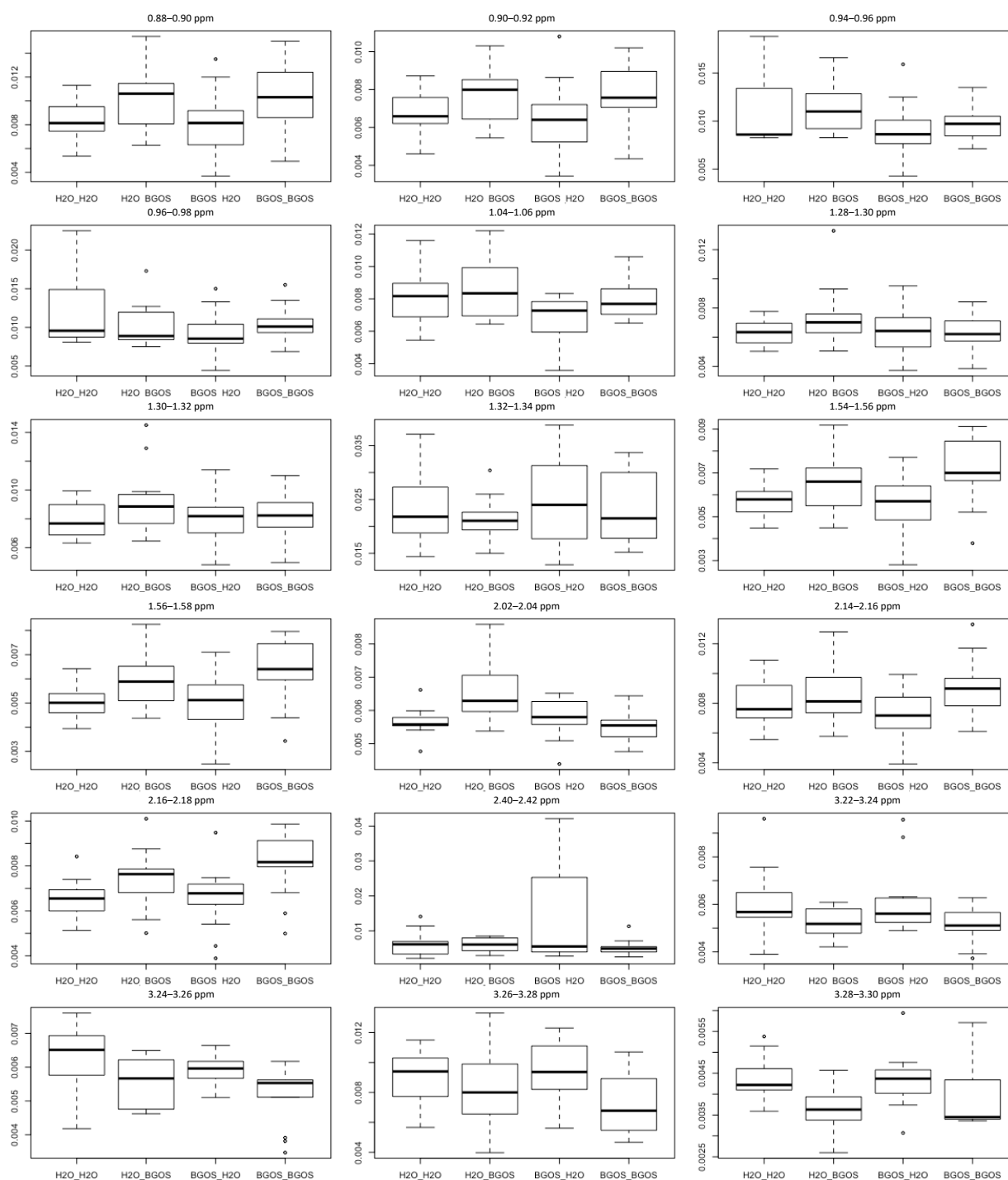
H2O/Sal vs. H2O/LPS Females		
Bin	VIP score	Assignment
1.32–1.34 ppm	4.60688965	lactate/lipid (-CH ₂ -)n
0.84–0.86 ppm	3.36516592	lipid (-CH ₃) HDL/LDL
0.86–0.88 ppm	2.93561385	lipid (-CH ₃) HDL/LDL

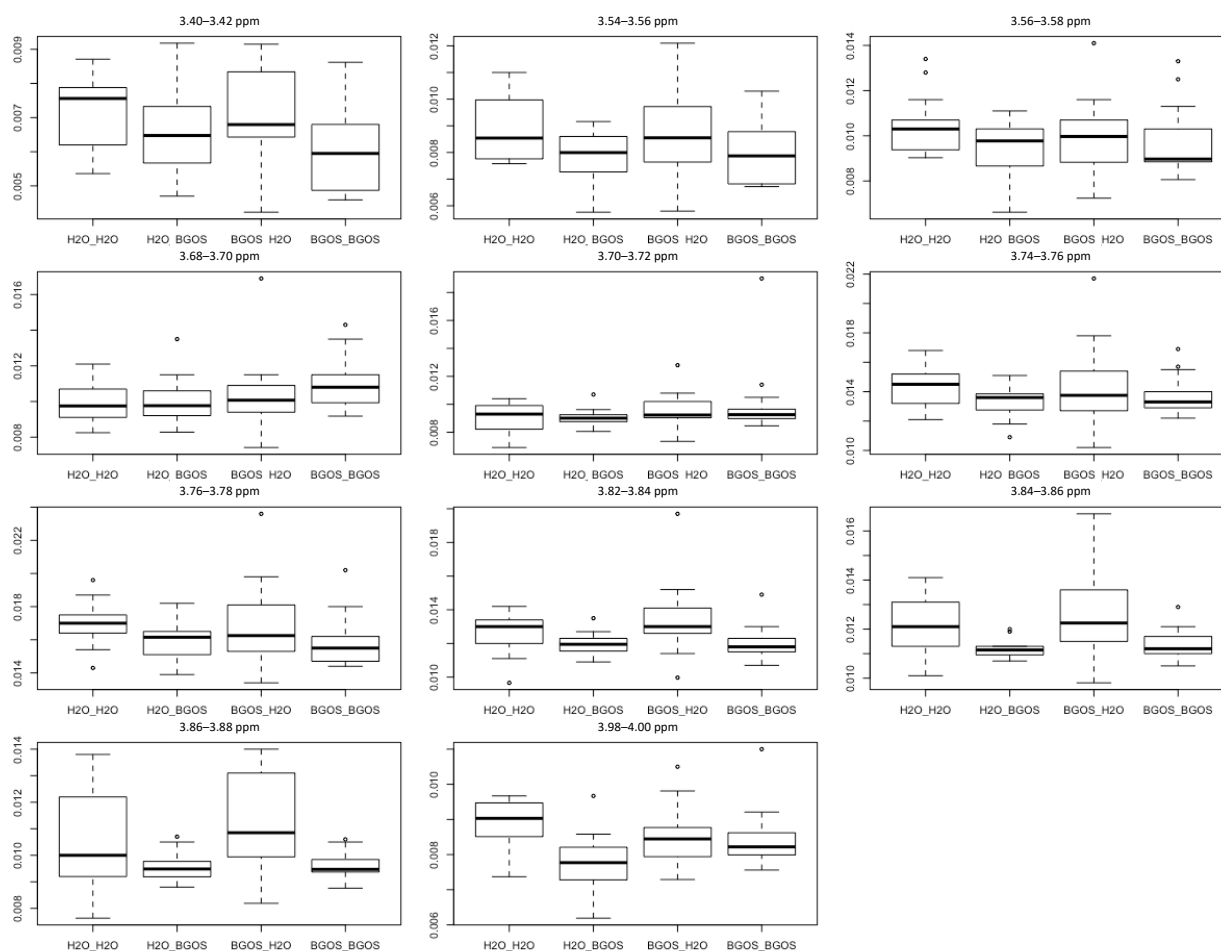
Appendix F: Pilot study for faecal metabolite extraction technique



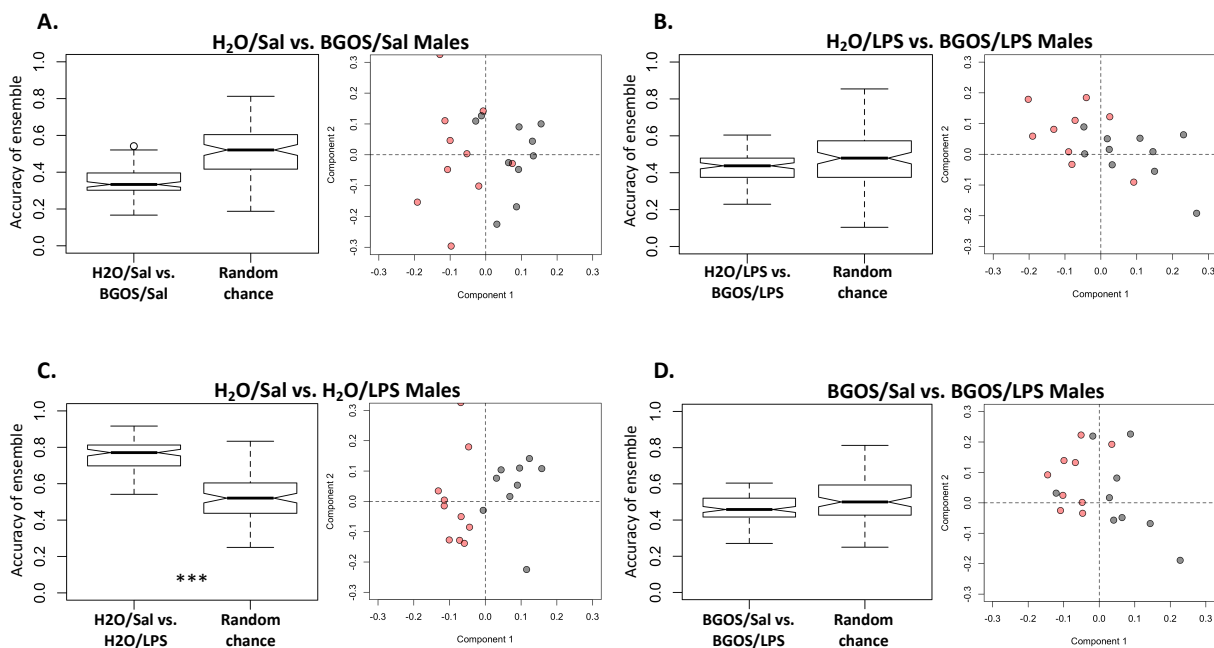
Supplementary Figure 7. Faecal metabolite extraction assessment. Faecal samples from CD1 mice were collected to assess the faecal metabolite extraction technique. One sample was run in the NMR machine three times to quantify inter-run variation (“Control”, N = 3 runs). Faecal samples from five mice were mixed, split into five samples, and extracted separately to quantify variation in the metabolite extraction technique (“Pooled”, N = 5 replicates). In addition, three separate faecal pellets from the same mouse were homogenised and extracted separately to quantify within-mouse variation (“Within.mouse”, N = 3 samples). Finally, five samples from six different mice were collected and extracted separately to measure inter-mouse variation (“Inter.mouse”, N = 6 mice). Relative standard deviation (RSD) was calculated for each bin within each group. As expected, Control and Pooled had very low average RSD. Within.mouse was surprisingly high, so I collected as many faecal pellets as possible for the samples in Chapter 6 to get a representative average for each mouse.

Appendix G: Boxplots of significant ^1H NMR faecal spectral bins in young offspring

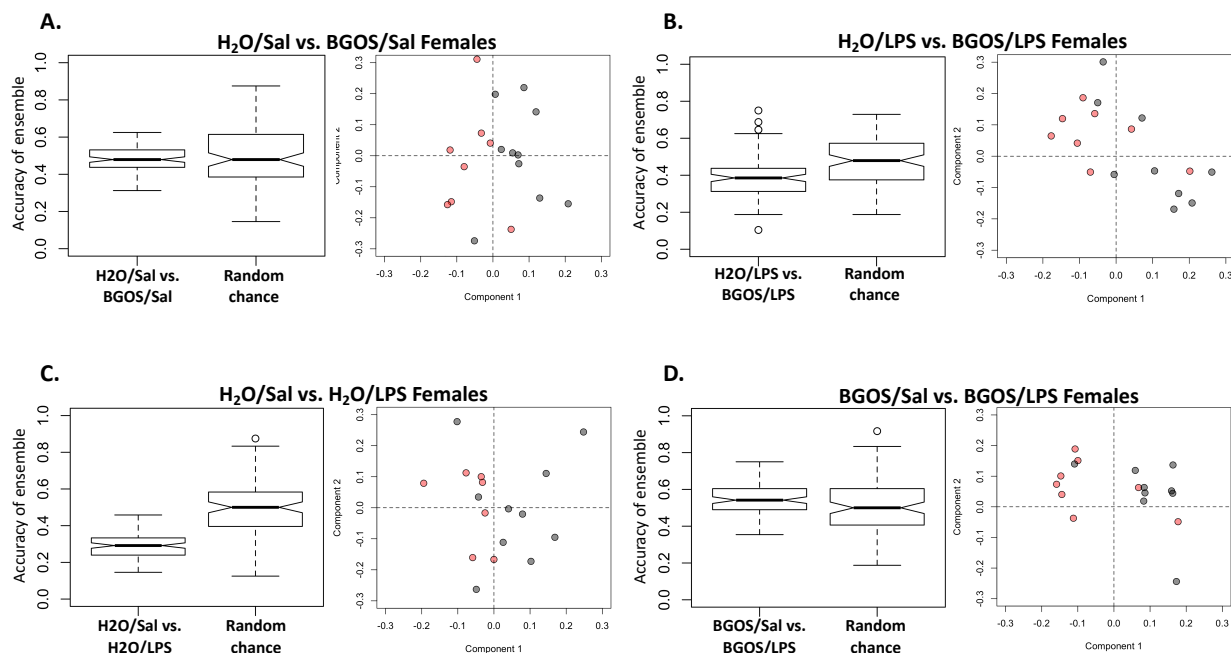




Supplementary Figure 8. Top spectral bins in young offspring faecal extract OPLS-DA models. Boxplots showing 0.02 ppm spectral bins corresponding to top VIP scores in young offspring faecal samples.

Appendix H: Adult faecal ^1H NMR analysis separated by sex

Supplementary Figure 9. Accuracy of OPLS-DA models and scores plots for adult offspring male faecal extracts. Differences in faecal metabolite profiles in LPS-treated adult offspring appear to be driven by males.

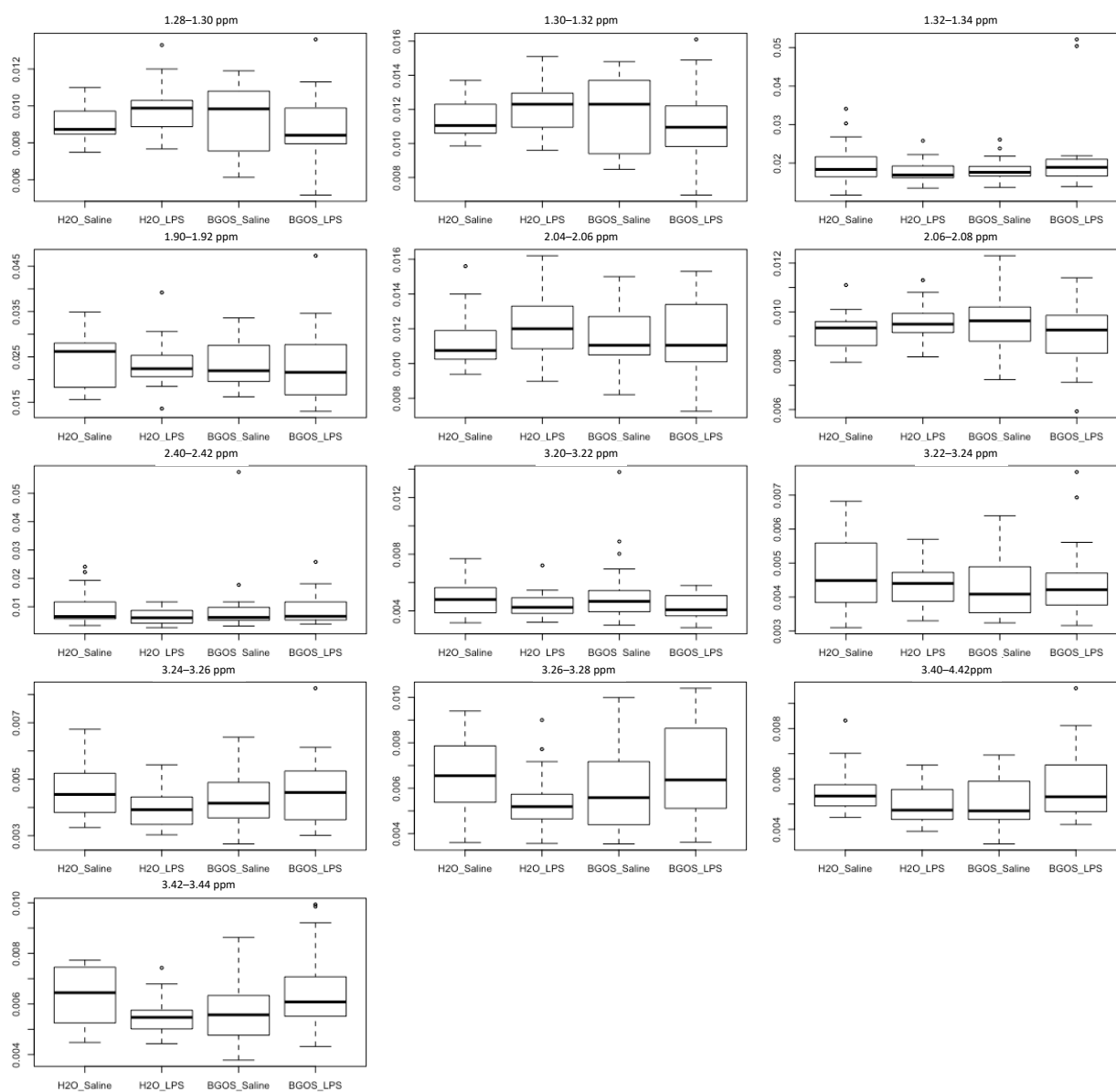


Supplementary Figure 10. Accuracy of OPLS-DA models and scores plots for adult offspring female faecal extracts.

Supplementary Table 9. Bins corresponding to top VIP scores and metabolite assignments for H2O/Sal vs. H2O/LPS male faecal extract samples.

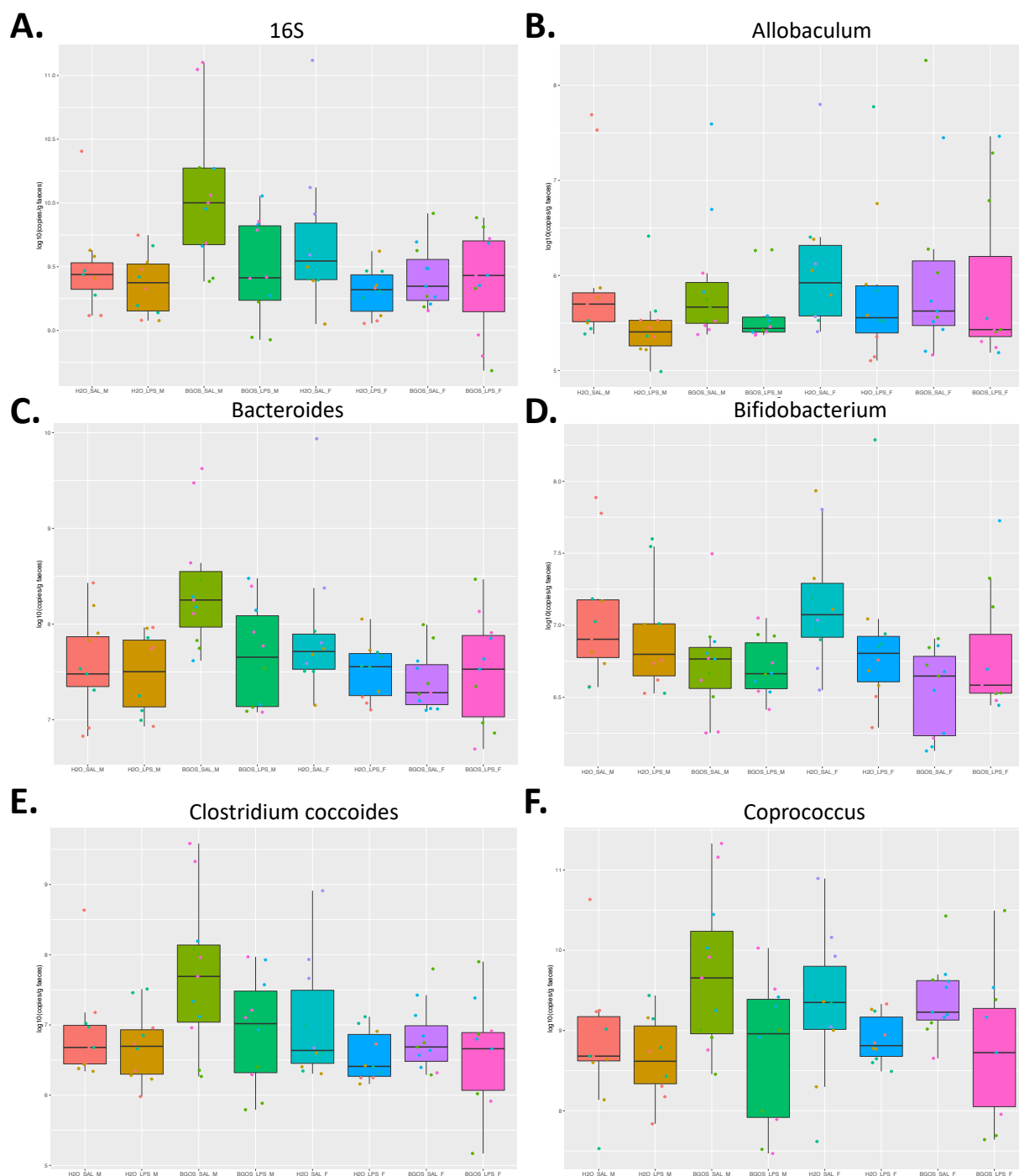
H2O/Sal vs. H2O/LPS Males		
Bin	VIP score	Assignment
1.90–1.92 ppm	4.80997196	acetate/arginine
2.40–2.42 ppm	3.43389217	succinate
2.04–2.06 ppm	3.37646853	proline/glutamate/N-acetyl groups

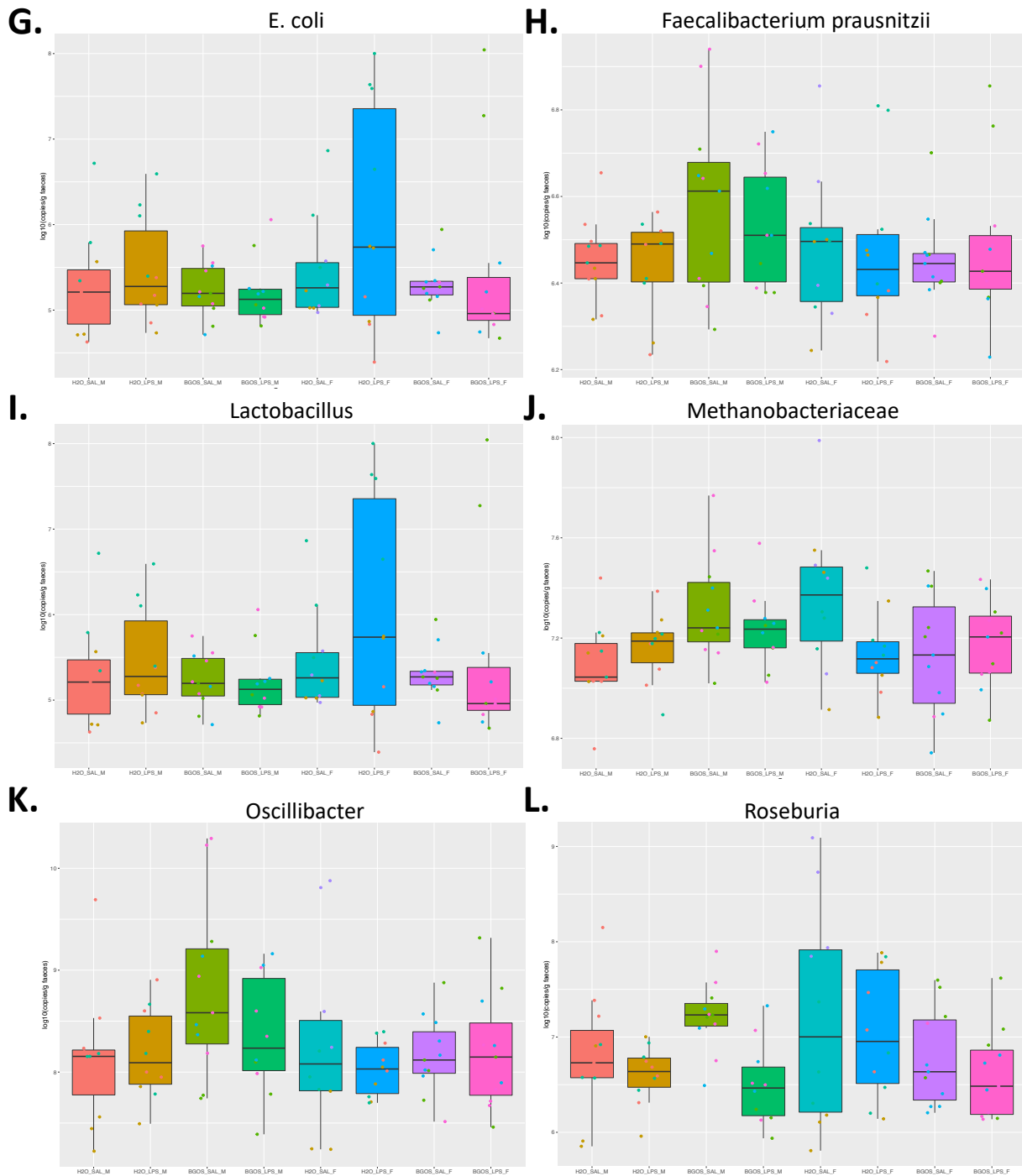
Appendix I: Boxplots of significant ^1H NMR faecal spectral bins in adult offspring



Supplementary Figure 11. Top spectral bins in young offspring faecal extract OPLS-DA models. Boxplots showing 0.02 ppm spectral bins corresponding to top VIP scores in young offspring faecal samples.

Appendix J: qPCR results for bacterial taxa of interest





Supplementary Figure 12. Boxplots showing qPCR analysis of bacterial taxa of interest in young offspring. Faecal DNA was collected for qPCR. Amplified DNA was used to construct standard curves for calculation of absolute 16S copy number per gram of faeces. Boxplots are separated by sex, and dots are coloured by litter.

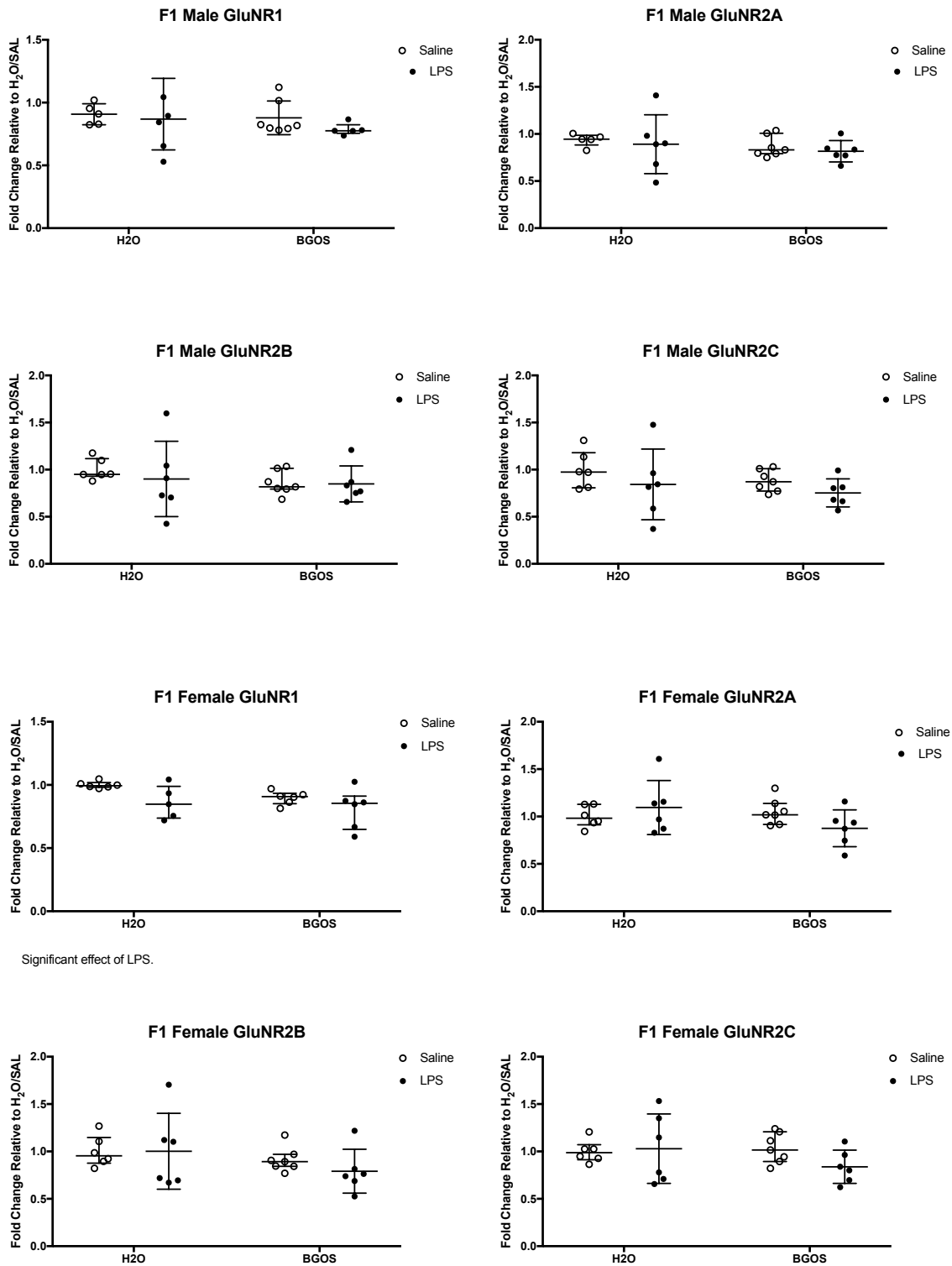
Supplementary Table 10. Mixed-effects analysis results for qPCR of select bacterial taxa in young offspring.

	F0.Diet	p < .05?	Treatment	p < .05?	F0.Diet x Treatment	p < .05?	F0.Diet x Sex	p < .05?	Treatment x Sex	p < .05?	F0.Diet x Treatment x Sex	p < .05?
16S	0.62267905		0.00206671	*	0.52948131		0.02117554	*	0.33695333		0.03191787	*
Allobaculum	0.97753361		0.08796856		0.61319312		0.80978012		0.42147359		0.88969268	
Bacteroides	0.5741984		0.02233914	*	0.66066101		0.00297727	*	0.28584271		0.0244779	*
Bifidobacterium	0.02597547	*	0.63808375		0.06582756		0.62999929		0.701874		0.23982386	
Clostridium coccoides	0.70987141		0.0126582	*	0.36072229		0.08780327		0.46608818		0.30446417	
Coprococcus	0.4630672		0.0010438	*	0.10334702		0.1342138		0.7564103		0.42062627	
E.coli	0.67262484		0.1471997		0.36901338		0.58004567		0.16829212		0.44728482	
Lactobacillus	0.45462678		0.00605649	*	0.56033081		0.18606138		0.59702082		0.73634263	
Methanobacteriaceae	0.61964288		0.26506492		0.51228629		0.00692696	*	0.35519491		0.02178081	*
Oscillibacter	0.77937212		0.44367345		0.37855625		0.18883795		0.37171491		0.27980395	
Faecalibacterium prausnitzii	0.16511414		0.82177477		0.95470929		0.08474139		0.55994435		0.62149477	
Roseburia	0.51993674		0.13402035		0.02127397	*	0.16887791		0.01748394	*	0.83291652	

Appendix K: Maternal BGOS intake alters brain gene expression in young C57Bl/6 offspring

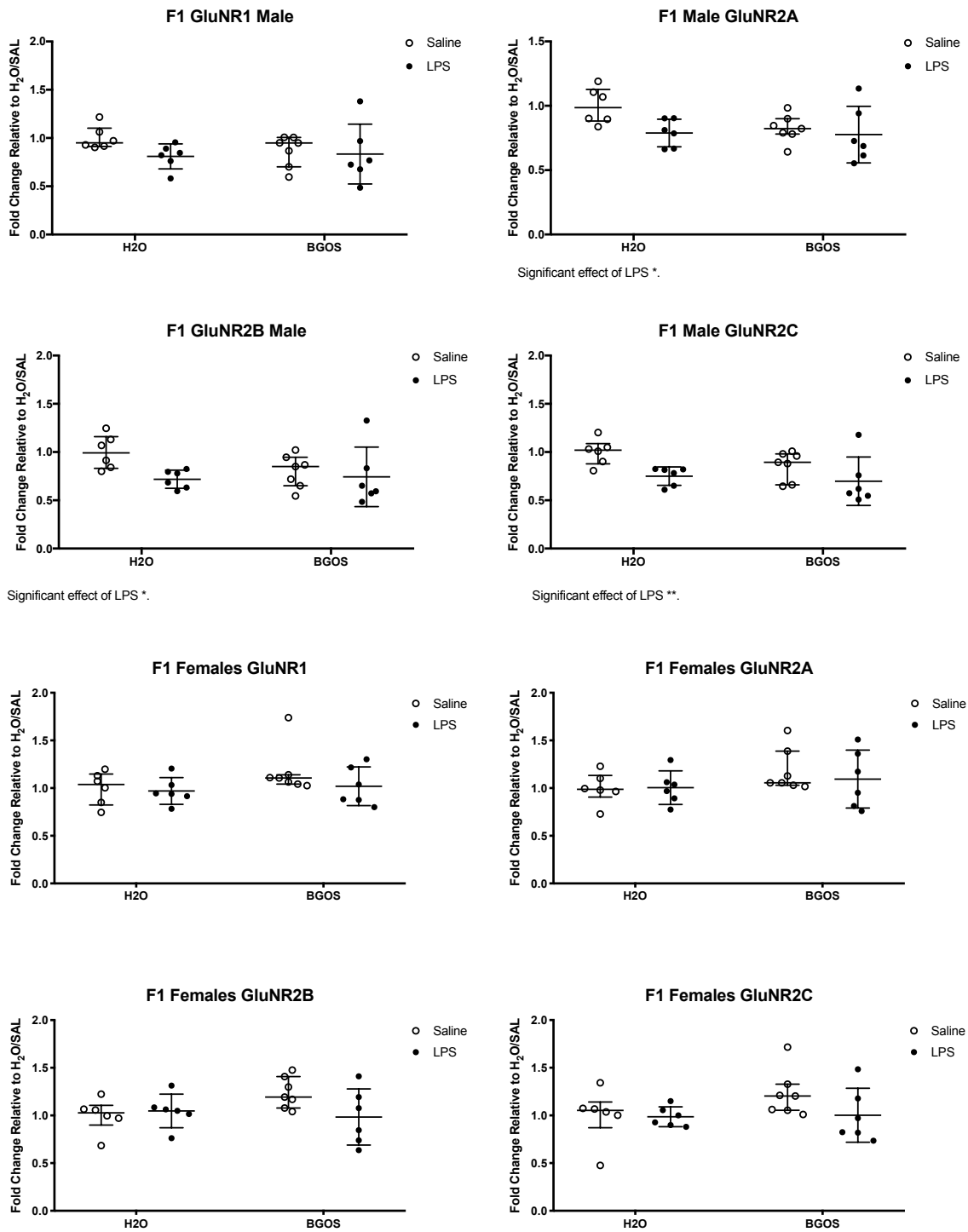
C57Bl/6 dams were supplemented with 1.5 mg/mL BGOS during gestation and weaning. Pups were injected with 0.83 mg/mL LPS at P21 and culled 24 hours later. Frontal cortex, hippocampus, cerebellum, and brainstem were collected for qPCR analysis of genes for NMDAR subunits and cytokines (IL-1 β and IL-6 – TNF- α was not measured because I did not have a good set of primers at the time). Brain gene expression data are summarised in **Supplementary Figures 13–18**. Maternal BGOS reduced expression of NMDAR subunits in the cerebellum and brainstem, while acute LPS reduced NMDAR expression in the brainstem and hippocampus. Faecal samples were also collected from offspring, and DNA was extracted for quantification of *Bifidobacterium* with qPCR (**Supplementary Figure 19**). Finally, plasma was collected for acetate quantification using an Acetate Assay Kit (colorimetric, Abcam, **Supplementary Figure 20**). Maternal BGOS had no effect on *Bifidobacterium* levels or plasma acetate concentrations in offspring.

Frontal Cortex



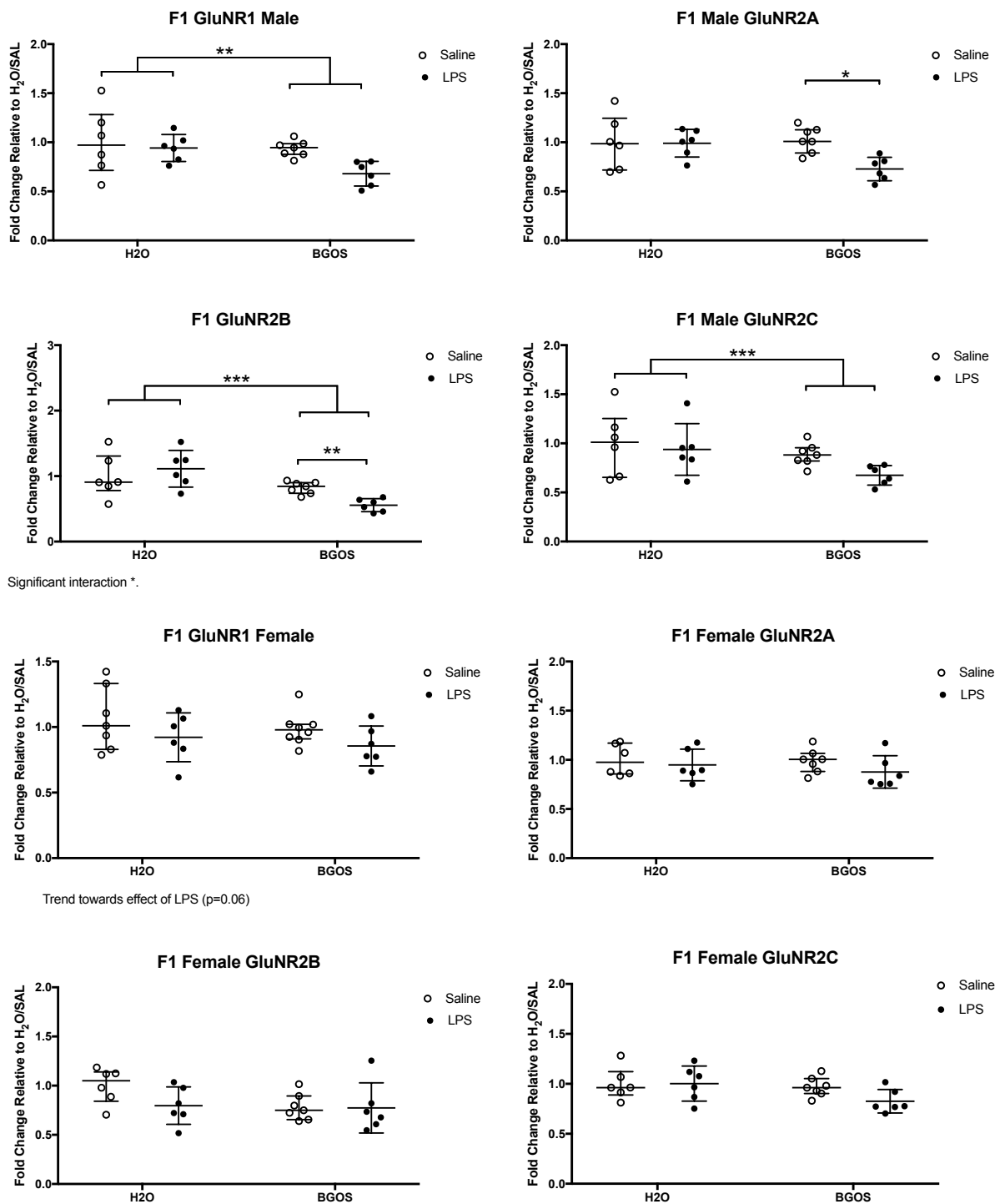
Supplementary Figure 13. Cortical NMDAR expression in male and female offspring following maternal BGOS and acute LPS.

Hippocampus



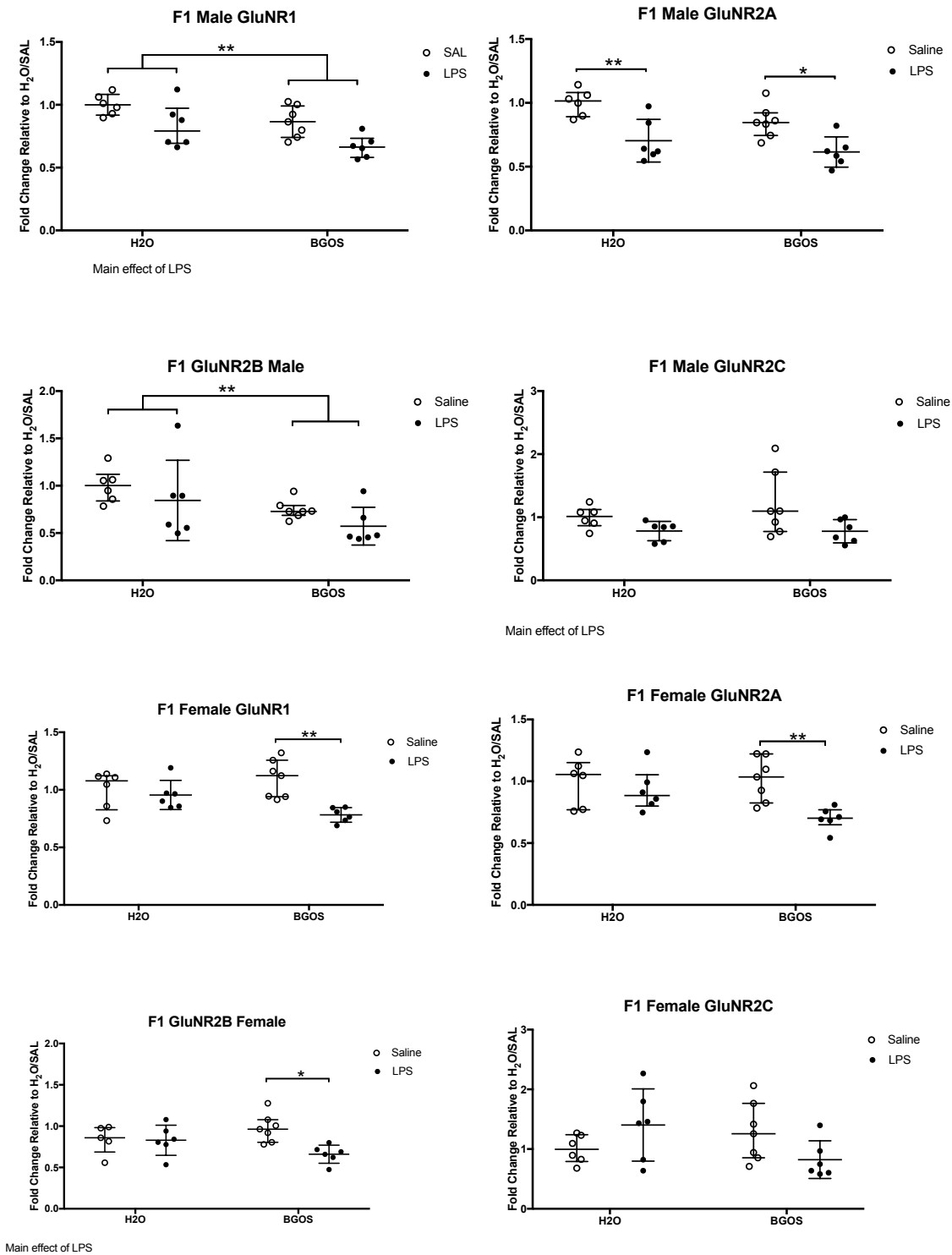
Supplementary Figure 14. Hippocampal NMDAR expression in male and female offspring following maternal BGOS and acute LPS.

Cerebellum



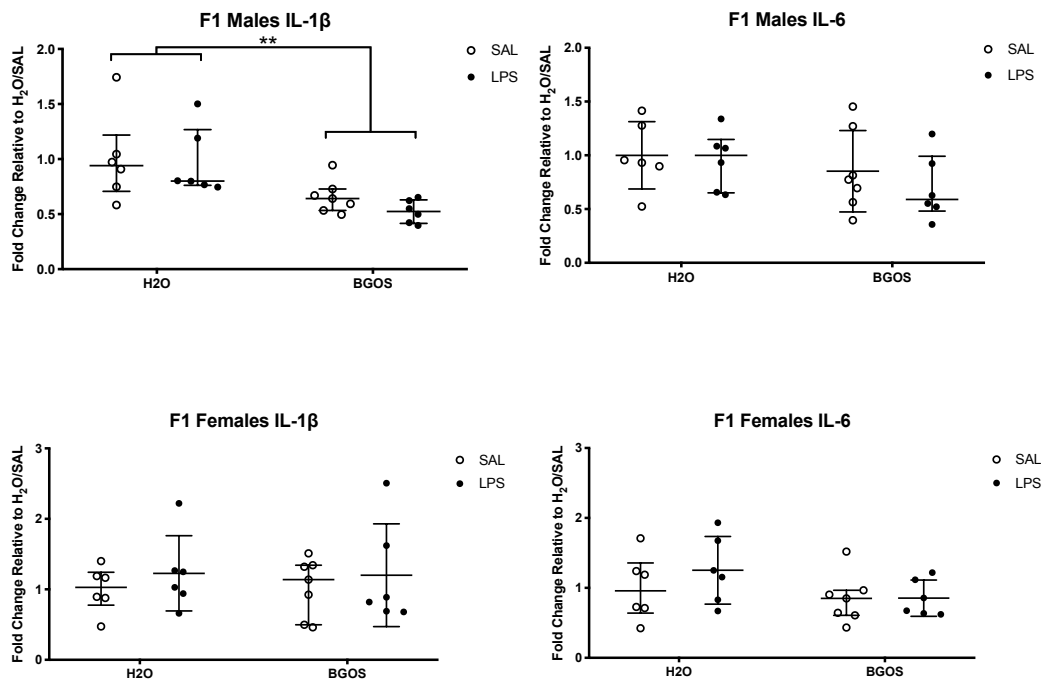
Supplementary Figure 15. Cerebellum NMDAR expression in male and female offspring following maternal BGOS and acute LPS.

Brainstem

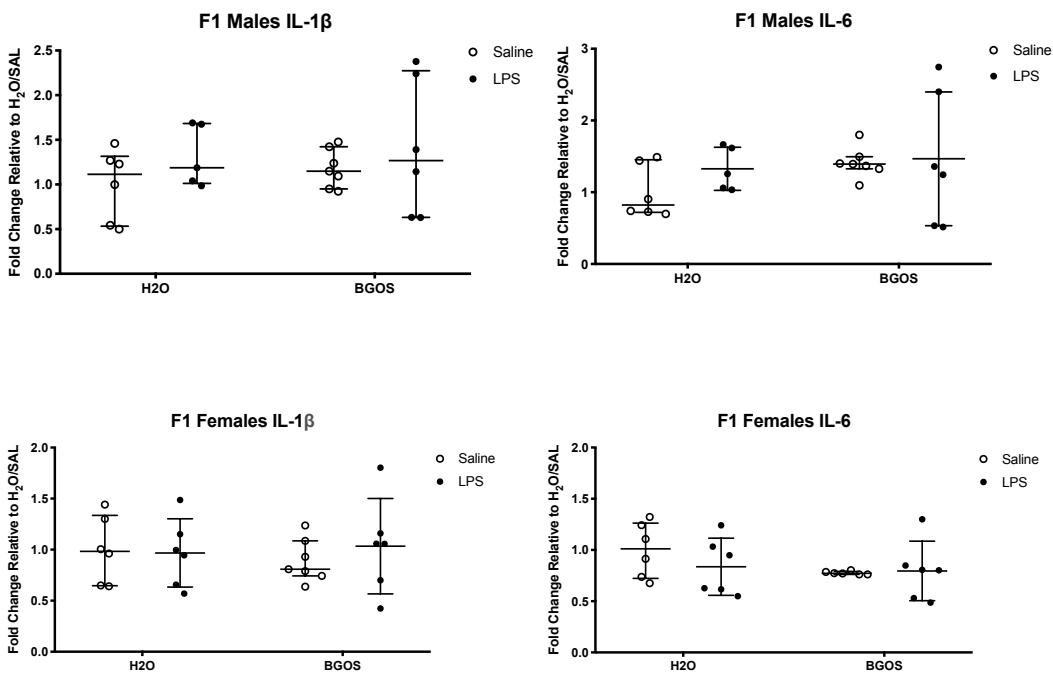


Supplementary Figure 16. Brainstem NMDAR expression in male and female offspring following maternal BGOS and acute LPS.

Frontal cortex

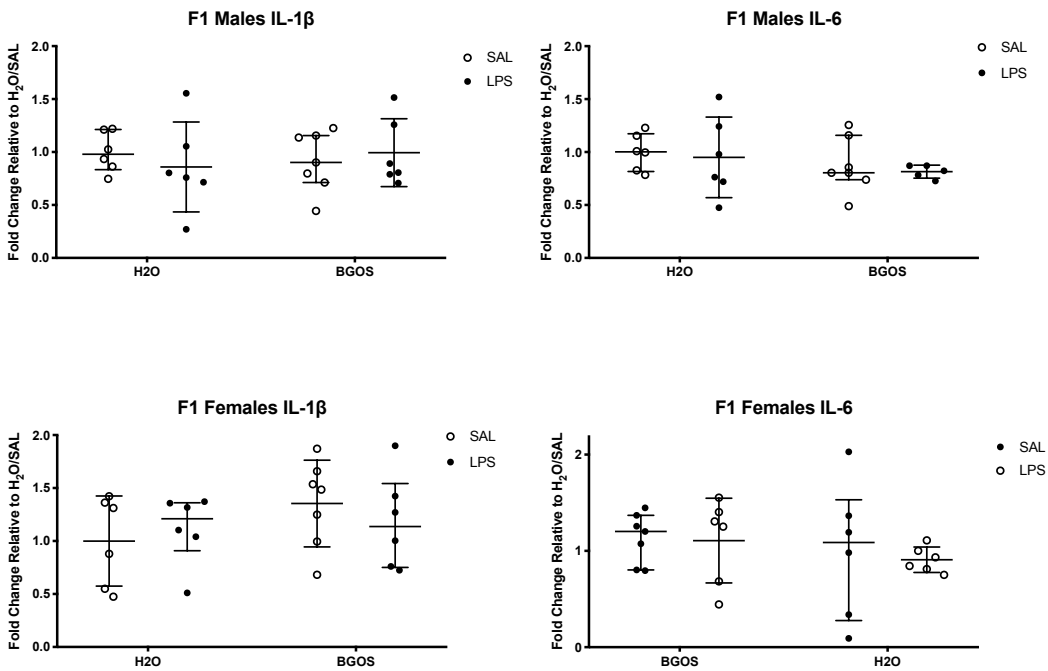


Hippocampus

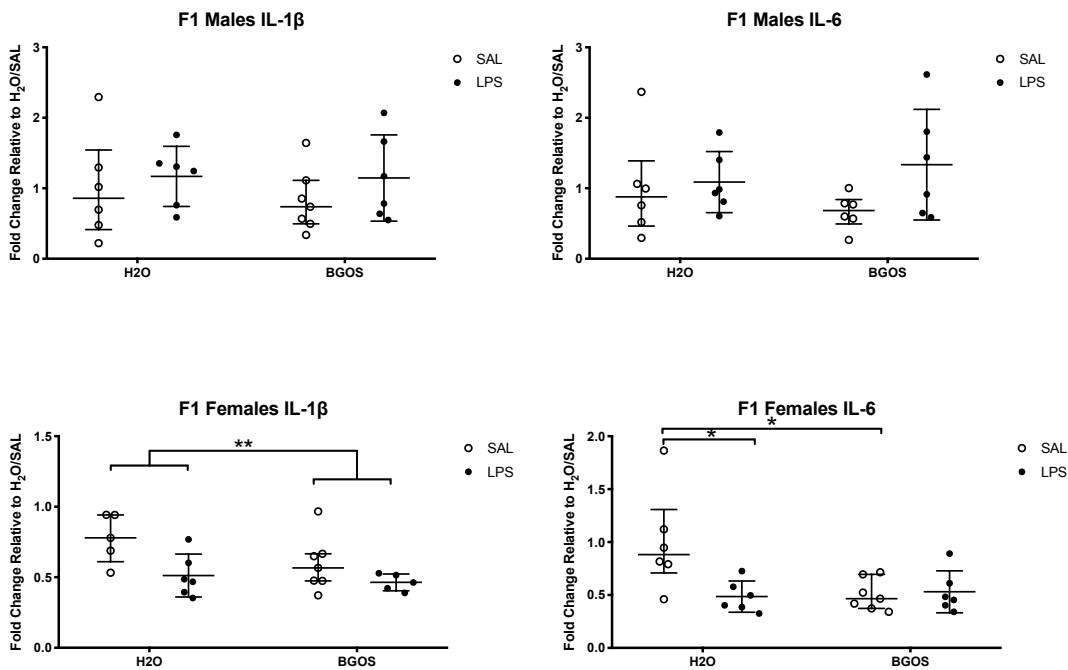


Supplementary Figure 17. Cortical and hippocampal cytokine expression in male and female offspring following maternal BGOS and acute LPS.

Cerebellum

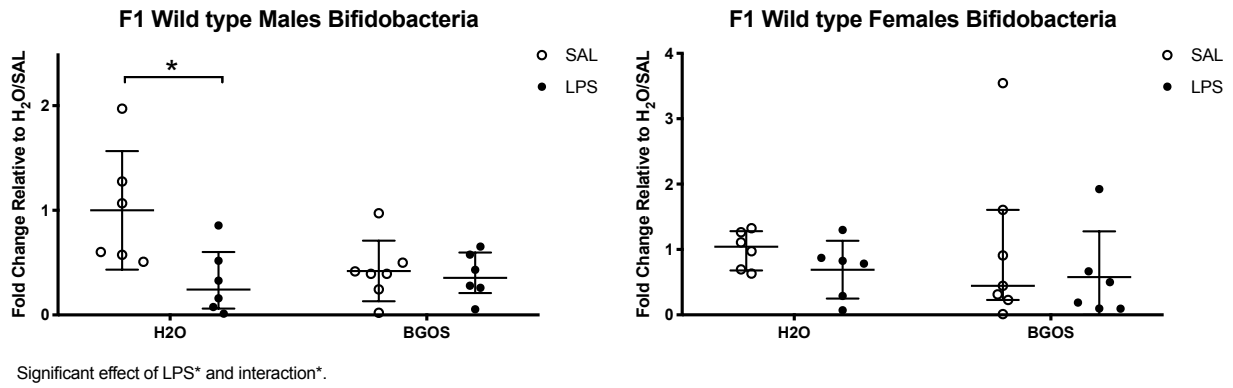


Brainstem

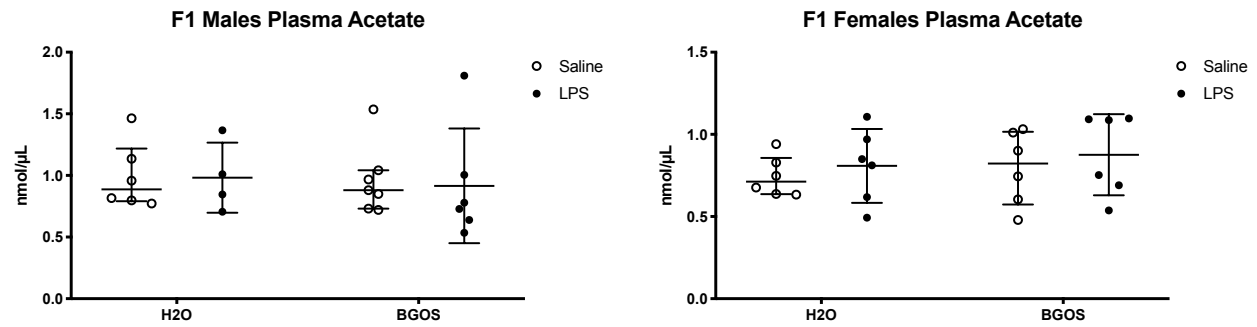


Significant effect of LPS * and interaction*.

Supplementary Figure 18. Cerebellum and brainstem cytokine expression in male and female offspring following maternal BGOS and acute LPS.

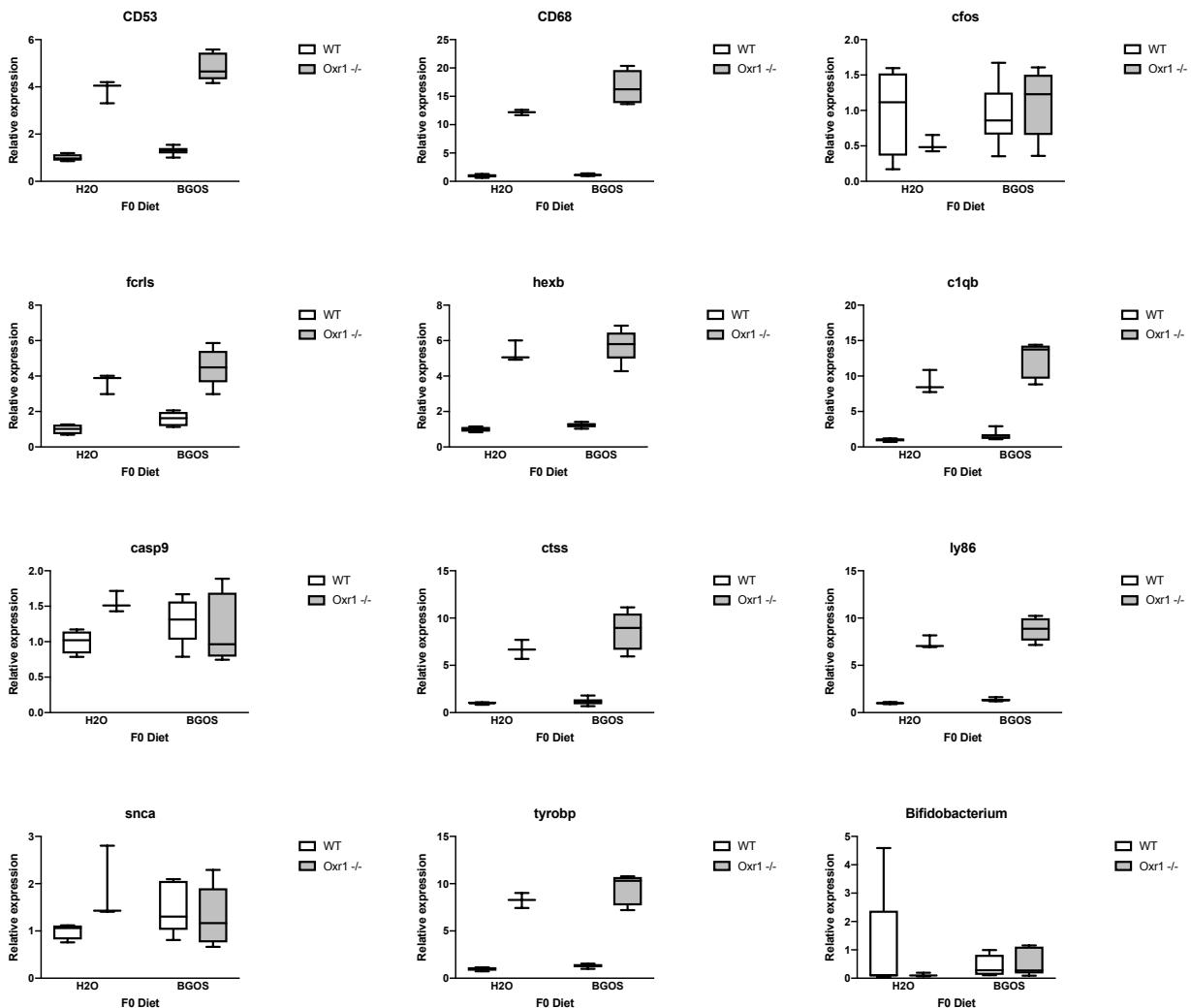


Supplementary Figure 19. Effects of maternal BGOS and acute LPS on faecal *Bifidobacterium* in C57Bl/6 offspring.



Supplementary Figure 20. Effects of maternal BGOS and acute LPS on plasma acetate in C57Bl/6 offspring.

Appendix L: Maternal BGOS in *Oxr1* ^{-/-} model of ataxia and neurodegeneration



Supplementary Figure 21. Effects of maternal prebiotic intake on cerebellum markers associated with *Oxr1* ^{-/-} neurodegeneration. Heterozygous male and female mice were bred to generate wild-type/heterozygous (grouped together as WT because hets do not have any measurable phenotype) and *Oxr1* ^{-/-} knockouts against a C57Bl/6 background. BGOS was added to drinking water at a dose of 3 mg/mL. Offspring were culled at P21, and cerebellums were collected for RNA extraction and qPCR of genes that had previously been identified as elevated in *Oxr1* ^{-/-} animals. It appears that maternal BGOS exacerbates *Oxr1* ^{-/-} pathology based on gene expression, but because of small group numbers (N = 3–8/group), it is difficult to draw meaningful conclusions.

Appendix M: Conferences attended during DPhil

- Hebert, J.C.,** Probert, F., Anthony, D.C., & Burnet, P.W.J. (2019, October). Mouse maternal prebiotic intake alters behaviour, brain gene expression, and blood and faecal metabolomes in offspring. Oral and poster presentation at Exploring Host-Microbiome Interactions in Health and Disease, Cambridge, U.K.
- Hebert, J.C.,** Radford-Smith, D.E., Probert, F., Akbar, N., Anthony, D.C., & Burnet, P.W.J. (2019, July). Effects of mouse maternal prebiotic intake on the offspring brain and behavior. Poster presentation at the Annual Broad-ISF Symposium, Jerusalem, Israel.
- Hebert, J.C.,** Radford-Smith, D.E., Probert, F., Anthony, D.C., & Burnet, P.W.J. (2019, April). Effects of maternal prebiotic intake on anxiety-like behavior and the blood metabolome in offspring. Poster presentation at British Neuroscience Association conference, Dublin, Ireland.
- Hebert, J.C.,** Radford-Smith, D.E., Akbar, N., Chan, K.W., Anthony, D.C., & Burnet, P.W.J. (2019, January). Perinatal prebiotic alters anxiety- and depression-like behavior, NMDA receptor expression, and macrophage function in offspring. Oral and poster presentation at Mind, Mood, Microbes, Amsterdam, Netherlands.