

Metabolism in mood disorder across the gut-liver-brain axis



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A thesis submitted for the degree of Doctor of Philosophy

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August 2022

ii. Abstract

Background and Aims: Depression and nonalcoholic fatty liver disease (NAFLD) are common disorders that share a bidirectional relationship and continue to increase in prevalence. Both maternal depression and obesity during pregnancy increase the risk of neuropsychiatric disease in the offspring. Alterations to systemic immunity and gut microbiota composition are thought to contribute to these relationships, though the precise mechanisms are not known. In rodents, probiotic supplementation has been reported to have anti-inflammatory and antidepressant-like effects, yet it is not clear how modifying the microbiota of obese dams during the perinatal period may affect the behaviour, metabolism, and neuroplasticity gene expression of their offspring. Principally, this thesis explores the distinct effects of maternal perinatal probiotic intake and obesity (induced by chronic consumption of a high fat diet [HFD]) on the behaviour, brain-gut metabolism, and neuroplasticity gene expression of the offspring. Maternal diet-induced obesity was hypothesised to increase anxiety and depressive-like behaviour in the young offspring. Long-lasting behavioural changes were also expected in adult offspring. Additionally, I predicted that perinatal probiotic exposure would attenuate these behavioural changes and lead to changes in neuroplasticity gene expression alongside an increase in faecal short-chain fatty acids acetate, butyrate, and propionate. It was then determined whether a serum metabolomic profile of depressive symptoms could be detected in the context of metabolic dysfunction in humans, whereby I expected to observe an additive effect of depression on serum markers of dyslipidaemia and inflammation in NAFLD. **Methods:** Female CD-1 mice were fed either a HFD or control diet for 6 weeks prior to mating until three weeks postpartum (PND21). At the time of conception through to PND21, a multispecies probiotic supplement (or vehicle control) was administered through the drinking water. At the time of weaning, dams and half the juvenile offspring were assessed for anxiety and depressive-like behaviours, and the faecal, liver, blood, and brain metabolome were quantified using nuclear magnetic resonance (NMR) spectroscopy. In the offspring prefrontal cortex, mRNA expression of genes relating to neuroplasticity, astrocyte

metabolism, and inflammation were quantified. The remainder of the offspring were weaned onto regular chow and at 4-months age were profiled similarly to the juvenile offspring. In translational work, a human cohort with NAFLD where >90% of individuals were obese, serum NMR metabolite profiles were compared between patients with depressive symptoms in the last 12-months (n = 81), lifetime depression (n = 30) and no history of depressive symptoms (n = 107) using multivariate statistics. **Results:** Prolonged high-fat diet feeding reduced maternal gut SCFA abundance, increased markers of peripheral inflammation, and decreased the abundance of neuroactive metabolites in maternal milk during nursing. Both the juvenile and adult offspring of obese dams exhibited increased anxiety-like behaviour, which were prevented by perinatal probiotic exposure. Maternal probiotic treatment increased gut butyrate and brain lactate in the juvenile and adult offspring and increased the expression of prefrontal cortex *PFKFB3*, a marker of glycolytic metabolism in astrocytes. *PFKFB3* expression correlated with the increase in gut butyrate in the juvenile and adult offspring. The resilience of juvenile and adult offspring to anxiety-like behaviour was most prominently associated with increased brain lactate abundance. Individuals with NAFLD and recent depressive symptoms, but not lifetime depression, had a distinct serum metabolomic profile compared to those with NAFLD without depression. This signature was found to be independent of age, sex, medication, and disease severity. Serum triglycerides, VLDL, isoleucine, and the inflammatory biomarker of glycoprotein acetylation were key metabolites increased in patients with recent depressive symptoms. **Conclusions:** Taken together, the data in this thesis demonstrate a clear relationship between maternal diet-induced obesity and emotional dysfunction in the offspring. I also indicate that the gut microbiome may act as a key modifiable (and therefore treatable) feature of the relationship between maternal obesity and offspring brain function and behaviour. Lastly, I found that depressive symptoms in humans with liver disease, independent of demographic factors and liver disease severity, are associated with altered circulating metabolite concentrations such as increased serum isoleucine, triglycerides, and glycoprotein acetylation.

iii. Acknowledgements

I have been exceptionally fortunate to receive the intellectual, financial, and moral support from several key individuals and organisations throughout my DPhil. Firstly, I would like to thank my supervisor, Daniel Anthony. Since arriving as an exchange student from Brisbane, your support and guidance has been unwavering, and your generosity of spirit is something I aspire to. I count myself truly privileged to have learned so much from you during my DPhil and I look forward to future collaboration.

To my co-supervisors, Fay Probert, thank you for being there for every one of the 1,000+ metabolomic experiments in and out of this thesis, and for entrusting me with other exciting metabolomics projects. I am proud to call myself your (chronologically) #1 DPhil student. And Phil Burnet, working with you during my exchange inspired me to pursue this thesis, and I am very grateful for your support.

Jenna Hebert, Jay Roodselaar, and Abi Yates, thank you for your patience and teaching me how to work in the lab. I would also like to thank Daan Paget, who has not only become a dear friend to me but also aided with experiments on many an occasion. To my students, Trinity and Sabrina, and my other friends of the Anthony lab, I couldn't have hoped for a better community with which to learn with, and from.

I would like to thank Lincoln College, the Clarendon Fund, the Department of Pharmacology, and the Newton-Abraham electoral board for funding me and making this degree possible.

Mum and Dad, thank you for always being there for advice, or just to listen. I am very lucky to have great role models as parents. My brothers, thank you for being there if I needed a good chat or a good laugh. James and Keno especially, getting to hang out with you over the past few years has been one of the highlights of my degree. Lastly, to Micon, thank you for being with me on this journey through thick and thin. Your support has meant a great deal.

iv. Table of Contents

Metabolism in mood disorder across the gut-liver-brain axis.....	I
ii. Abstract.....	II
iii. Acknowledgements.....	IV
iv. Table of Contents	V
v. Abbreviations.....	XIII
vi. Gene name nomenclature.....	XVI
vii. List of Tables	XVII
viii. List of Figures	XVIII
ix. Conference presentations during candidature.....	XXI
x. Publications during candidature	XXII
1 INTRODUCTION.....	23
1.1. The gut microbiome: a recent history and current state of the field	23
1.2. The microbiota-gut-brain axis	25
1.2.1. The putative mechanisms of bug-to-brain communication.....	25
1.2.1.1. Vagus nerve.....	25
1.2.1.2. Immune system.....	28
1.2.1.3. Microbial metabolites and neurotransmitters	30
1.2.2. Perturbed microbiota-gut-brain axis metabolism in mood disorders	32
1.2.2.1. A critique of isolated microbiome analysis in mood disorders	32
1.2.2.2. Animal studies.....	33
1.2.2.3. Human studies	36
1.3. Maternal factors influencing offspring neurodevelopment and behaviour....	38

1.3.1. Spotlight on obesity.....	38
1.3.1.1. Clinical studies	39
1.3.2. Maternal care behaviour.....	41
1.3.3. The maternal microbiome.....	48
1.3.4. Maternal inflammation.....	53
1.3.5. Breast milk composition	58
1.3.6. Neurotransmitter Systems.....	61
1.3.7. Overview of maternal influences on the offspring and looking beyond the first filial generation	65
1.3.8. Novel antidepressant therapies are required	66
1.4. Modulation of the microbiota-gut-brain axis by prebiotics and probiotics	68
1.4.1. Prebiotics and probiotics modulate brain function and behaviour	68
1.4.1.1. Prebiotics modulate brain function and behaviour in animal models..	68
1.4.1.2. Probiotics modulate brain function and behaviour in animal models..	70
1.4.1.3. Prebiotics and probiotics in human studies.....	74
1.4.2. Prebiotics and probiotics during the perinatal period	78
1.5. Depression and metabolic disease: a bidirectional relationship	82
1.5.1. Obesity and depression.....	82
1.5.2. Depression and non-alcoholic fatty liver disease.....	84
1.6. Clinical (blood-based) applications for metabolomics in depression	87
1.7. Aims and hypotheses	89
2 Materials and Methods.....	91
2.1 Animals	91

2.2	Animal husbandry.....	93
2.3	Treatments.....	94
2.3.1	Probiotic administration.....	94
2.3.2	Diets.....	95
2.4	Behaviour.....	96
2.4.1	Open field test.....	96
2.4.2	Light-dark box	97
2.4.3	Forced swim test.....	98
2.4.4	Three-chamber social interaction test.....	99
2.4.5	Maternal care behaviour.....	100
2.4.5.1	Home cage observation	101
2.4.5.2	Pup retrieval test.....	101
2.5	Tissue collection.....	102
2.6	RNA isolation	103
2.7	DNA isolation	105
2.8	cDNA conversion and quantitative real-time polymerase chain reaction (qPCR)	106
2.9	Metabolomic Profiling	108
2.9.1	Sample preparation.....	108
2.9.1.1	Brain and liver	109
2.9.1.2	Plasma.....	109
2.9.1.3	Faecal.....	109
2.9.1.4	Milk	110

2.9.2	¹ H Nuclear Magnetic Resonance (NMR) spectroscopy	110
2.9.3	NMR data pre-processing	111
2.10	Human subjects.....	112
2.10.1	Clinical assessment and self-reported depression.....	112
2.10.2	Metabolomic profiling of human serum.....	112
2.11	Statistical analysis	114
2.11.1	Rodent parametric univariate analyses	114
2.11.2	Rodent nonparametric univariate analyses.....	115
2.11.3	Rodent multivariate analyses (PCA and PLS-DA)	115
2.11.4	Human univariate analyses	118
2.11.5	Human multivariate analyses (OPLS-DA)	119
3	Distinct effects of chronic high fat diet exposure and perinatal probiotic treatment on the maternal generation	121
3.1	Introduction	121
3.2	Experimental design.....	124
3.3	Results.....	125
3.3.1	Chronic high fat diet exposure induces maternal obesity.	125
3.3.2	Chronic high fat diet exposure alters aspects of maternal care behaviour during the first week of nursing.....	126
3.3.3	Chronic high fat diet exposure increased passive stress coping in dams.	128
3.3.4	Maternal obesity alters peripheral and central markers of innate immunity postpartum.	128

3.3.5	Maternal high fat diet and probiotic treatment differentially affect gut bacteria composition and modify the gut-brain axis metabolome.....	132
3.3.6	Maternal perinatal probiotic treatment increases the delivery of lactate and SCFAs to their offspring during nursing.	140
3.4	Discussion.....	143
4	Maternal probiotic treatment reverses anxiety-like behaviour and alters neuroplasticity gene expression in the offspring of obese dams	148
4.1	Introduction	148
4.2	Results.....	151
4.2.1	Juvenile but not adult body weight is altered by maternal diet and probiotic treatment.	151
4.2.2	Perinatal probiotic intake mitigates anxiety-like behaviours in the juvenile and adult offspring of obese dams.	153
4.2.2.1	Open field test.....	153
4.2.2.2	Light-dark box.	156
4.2.2.3	Forced swim test.....	157
4.2.2.4	Social interaction test.	159
4.2.3	Effects of maternal diet and probiotic treatment on peripheral and central markers of innate immunity in juvenile and adult offspring.	159
4.2.3.1	Liver gene expression.	159
4.2.3.2	Prefrontal cortex gene expression.....	162
4.2.4	Perinatal probiotic exposure alters glutamate receptor but not serotonin receptor mRNA expression in the prefrontal cortex of juvenile offspring.....	162

4.2.5 Perinatal probiotic treatment has long-lasting effects on neuroplasticity gene expression in the offspring prefrontal cortex.	163
4.2.6 Maternal probiotic intake alters expression of a genetic marker of astrocyte metabolic activity in the offspring prefrontal cortex.	166
4.3 Discussion.....	168
5 Effects of maternal obesity and probiotic treatment on the gut-liver-brain metabolome of the juvenile and adult offspring	174
5.1 Introduction	174
5.2 Results.....	176
5.2.1 Perinatal probiotic exposure increases faecal SCFA abundance in the juvenile and adult offspring.	178
5.2.2 Effects of maternal obesity and perinatal probiotic treatment on the offspring liver metabolome.	181
5.2.3 Maternal probiotic intake increases levels of circulating lactate in the juvenile offspring.	185
5.2.4 Maternal probiotic treatment exerts a long-lasting effect on brain energy metabolism in the offspring.	187
5.2.5 Brain lactate and faecal SCFAs correlate with prefrontal cortex gene expression	194
5.2.6 Elevated brain lactate accompanies the effect of early-life probiotic exposure on anxiety-like behaviour.....	197
5.3 Discussion.....	199
6 Investigating the serum metabolomic profile of recent depressive symptoms in individuals with pre-existing metabolic disease.....	207
6.1 Introduction	207

6.2	Results	209
6.2.1	Clinical indices and demographics overview	209
6.2.2	Clinical characteristics and severity of fibrosis do not identify recent or lifetime depressive symptoms in patients with NAFLD.	210
6.2.3	Multivariate analysis of the serum metabolome identifies a signature of recent depression in non-alcoholic fatty liver disease.....	215
6.2.4	The metabolomic signature of recent depressive symptoms is robust to demographic differences, including antidepressant medication usage.	217
6.2.5	Investigating depression-associated metabolites across the gut-brain axis in HFD-fed rodents and their offspring	220
6.2.5.1	Metabolite correlations in the F0 dams.....	221
6.2.5.2	Metabolite correlations in the F1 juvenile offspring.....	224
6.2.5.3	Metabolite correlations in the F1 adult offspring	224
6.2.6	The association between inflammatory gene expression and depressive-like behaviour in rodents	227
6.2.6.1	Gene expression correlations in the F0 dams	227
6.2.6.2	Gene expression correlations in the F1 juvenile offspring.....	228
6.2.6.3	Gene expression correlations in the F1 adult offspring	229
6.3	Discussion.....	231
7	Discussion	237
7.1	Summary of results	237
7.2	Relationship between brain metabolism, gene expression, and behaviour in the offspring	239

7.3 Relationship between faecal short-chain fatty acids and brain gene expression in the offspring.....	243
7.4 Investigating the relationship between the human and rodent blood metabolic profile to further understand the molecular biology of depression	246
7.5 General limitations and future directions.....	250
7.6 Translational perspectives and concluding remarks	254
References.....	258
Appendix A: Description of maternal care behaviours.....	280
Appendix B: Assessment of technical variation using tissue extraction method for NMR metabolomics.....	281
Appendix C NAFLD structured questionnaire	283
Appendix D PCA and PLS-DA matrices for models with >2 principal/predictive components	286
Appendix E Summary of PFC gene expression	293
Appendix F F1 (offspring) metabolome sex differences	295
Appendix G Summary of F1 adult metabolite changes that were identified as key metabolites in the juvenile offspring but not the adult offspring.....	296
Appendix H Rodent mRNA and metabolite correlations.....	297
Appendix I Maternal plasma isoleucine levels.....	301

v. Abbreviations

4EPS	4-ethylphenyl sulphate
5-HT	5-hydroxytryptamine (serotonin)
5-HIAA	5-hydroxyindoleacetic acid (serotonin metabolite)
ACTH	Adrenocorticotrophic hormone
ADHD	Attention deficit hyperactivity disorder
ALT	Alanine transaminase
ANOVA/AOV	Analysis of variance
ASD	Autism spectrum disorder
AST	Aspartate transaminase
AUC	Area under the curve
BBB	Blood brain barrier
BCAA	Branched chain amino acid
BD(A)I	Beck Depression (Anxiety) Inventory
BGOS	Bimuno® galacto-oligosaccharide
BMI	Body mass index
CCL2	Monocyte chemoattractant protein 1
CD	Crohn's Disease
CFU	Colony forming units
CI	Confidence interval
CMS	Chronic mild stress
CNS	Central nervous system
COSY	Correlation spectroscopy
CPMG	Carr-Purcell-Meiboom-Gill (NMR sequence)
CRH	Corticotropin releasing hormone
CRP	C-reactive protein
CSF	Cerebrospinal fluid
DASS	Depression anxiety stress scale
CSF-1R	Colony-stimulating factor-1 receptor
DDIT	DNA Damage Inducible Transcript 4
DHA	Docosahexaenoic acid
DOPAC	3,4-Dihydroxyphenylacetic acid (dopamine metabolite)
ELISA	Enzyme-linked immunosorbent assay
EPDS	Edinburgh Postnatal Depression Scale
EPM	Elevated plus maze
FA	Fatty acid
FDR	False discovery rate
FID	Free induction decay
FMT	Faecal microbiota transplant
FST	Forced swim test
GABA	Gamma-aminobutyric acid
GF	Germ Free
GFAP	Glial fibrillary acidic protein
eGFR	Estimated glomerular filtration rate
GGT	Gamma-glutamyl transferase
GI	Gastrointestinal
GR	Glucocorticoid receptor
GSSG	Oxidised glutathione
GWAS	Genome-wide association study

GWG	Gestational weight gain
HAD	Hospital anxiety and depression scale
HbA1c	Glycosylated haemoglobin
HDAC	Histone deacetylase
HDL	High-density lipoprotein
HFD	High fat diet
HIF-1	Hypoxia inducible factor 1
HMO	Human milk oligosaccharide
HPA	Hypothalamus pituitary adrenal (axis)
HSC	Haematopoietic stem cell
IBD	Inflammatory bowel disease
IBS	Inflammatory bowel syndrome
IDO	Indoleamine-2,3-dioxygenase 1
IFN	Interferon
IQR	Interquartile range
IU	International units
IVC	Individually ventilated cage
LAT1	Large amino acid transporter 1
LDB	Light-dark box
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LEPR	Leptin receptor
LPO	Lipoprotein
LPS	Lipopolysaccharide
LSM	Liver stiffness measurement
LTP	Long term potentiation
MBT	Marble burying test
MCB	Maternal care behaviour
MDD	Major depressive disorder
MIA	Maternal immune activation
MR	Mineralocorticoid receptor
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
MS	Mass spectrometry
MUC2	Mucin 2
NAA	<i>N</i> -Acetylaspartate
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NEGR1	Neuronal growth regulator 1
NHP	Nonhuman primate
NMDA(R)	N-methyl-D-aspartate (receptor)
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser effect spectroscopy
NS	Not significant
OFT	Open field test
OLFM4	Olfactomedin 4
(O)PLS-DA	(Orthogonal) partial least squares discriminant analysis
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase
PFC	Prefrontal cortex

PHQ9	Patient health questionnaire 9
PND	Postnatal day
PUFA	Polyunsaturated fatty acid
QIDS	Quick Inventory of Depressive Symptomatology
ROC	Receiver operator characteristic (curve)
SCFA	Short-chain fatty acid
SIT	Social interaction test
SPF	Specific pathogen free
SSRI	Selective serotonin reuptake inhibitor
STAI	State-trait anxiety inventory
TCA	Tricarboxylic acid (cycle)
TOCSY	Total correlation spectroscopy
TPH1/2	Tryptophan hydroxylase 1/2
TSP	3-(trimethylsilyl)propionic-2,2,3,3-d4 acid
TST	Tail suspension test
T2DM	Type 2 diabetes mellitus
VIP	Variable importance in projection
VLDL	Very low-density lipoprotein
VTA	Ventral tegmental area
WT	Wildtype

vi. Gene name nomenclature

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
TNF	Tumour necrosis factor
IL-1B	Interleukin 1 β
IL-6	Interleukin 6
TLR4	Toll-like receptor 4
SAA-2	Serum amyloid A2
BDNF	Brain-derived neurotrophic factor
5-HT_{1A}	Serotonin receptors
5-HT_{2A}	
5-HT₆	
GLUN1	NMDA receptor subunits
GLUN2A	
GLUN2B	
GLUN2C	
CREB1	cAMP responsive element binding protein 1
GSK3B	Glycogen synthase kinase-3 beta
PSD-95	Postsynaptic density protein 95
SYP	Synaptophysin
ATP1A2	Sodium/potassium-transporting ATPase subunit alpha-2
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
cFOS	FBJ murine osteosarcoma viral oncogene
ΔFOSB	FBJ murine osteosarcoma viral oncogene homolog B
ZIF-268	Zinc finger protein 268 / Early growth response protein 1
16S	16S ribosomal RNA gene
Lactobacillus	<i>Lactobacillus</i> spp.
Bifidobacterium	<i>Bifidobacterium</i> spp.

vii. List of Tables

Table 1.1 Summary of probiotic studies in rodent models on anxiety and depressive-like behaviours and brain metabolism	71
Table 1.2 Summary of probiotic studies on anxiety and/or depression in humans	76
Table 2.1 List of bacteria strains present in the Bio-Kult probiotic	94
Table 2.2 List of primers for qPCR	107
Table 3.1 Summary of F0 metabolite changes across each body site.....	138
Table 5.1 Summary of F1 juvenile metabolite changes across each body site.	191
Table 5.2 Summary of F1 adult metabolite changes across each body site.	192
Table 6.1 Demographic and clinical characteristics of the patient cohort according to self-reported depressive symptoms.	211
Table 6.2 Relationship between patient clinical and biochemical parameters and recent self-reported depressive symptoms.	213
Table 6.3 F0 Brain inflammatory gene expression vs. FST time immobile.....	228
Table 6.4 F0 Liver inflammatory gene expression vs. FST time immobile	228
Table 6.5 F1 juvenile brain inflammatory gene expression vs. FST time immobile	228
Table 6.6 F1 juvenile liver inflammatory gene expression vs. FST time immobile.....	229
Table 6.7 F1 adult offspring brain inflammatory gene expression vs. FST time immobile ...	229
Table 6.8 F1 adult offspring liver inflammatory gene expression vs. FST time immobile	230

viii. List of Figures

Figure 1.1 Summary of the putative mechanisms of communication between the gut microbiota and the brain.	26
Figure 1.2 Summary of the putative mechanisms by which maternal obesity is thought to influence the course of offspring neurodevelopment.....	66
Figure 1.3 Summary of the evidence from human and/or rodent studies that demonstrate overlapping pathophysiology between obesity and major depressive disorder.....	83
Figure 2.1 Timeline of behavioural experiments for the maternal (F0) and offspring (F1 juvenile and F1 adult) cohorts.	96
Figure 2.2 Overview of the biological samples collected for qPCR (Chapters 3-4) and ¹ H NMR metabolomics (Chapters 3,5).	102
Figure 2.3 Rodent metabolomic analysis workflow for principal component analysis.	116
Figure 2.4 Rodent metabolomics analysis workflow for partial least squares discriminatory analysis.	117
Figure 2.5 Overview of the analysis approach for the human serum metabolomics data....	120
Figure 3.1 Study design.....	124
Figure 3.2 Effect of maternal diet on body weight and litter size.	125
Figure 3.3 Maternal care behaviour.	127
Figure 3.4 Chronic high fat diet exposure increased passive stress coping in dams.	129
Figure 3.5 Chronic HFD feeding increases liver pro-inflammatory cytokine mRNA expression.	130
Figure 3.6 Prefrontal cortex toll-like receptor 4 and cytokine mRNA expression is altered by probiotic treatment and HFD, respectively.....	131
Figure 3.7 Relative abundance of maternal faecal <i>Lactobacillus</i> spp. and <i>Bifidobacterium</i> spp.	133
Figure 3.8 Unsupervised principal component analysis (PCA) of chronic HFD and perinatal probiotic treatment on the maternal (F0) faecal, liver, plasma, and brain metabolites.....	134
Figure 3.9 Maternal (F0) metabolome PLS-DA scores plots.....	135
Figure 3.10 Maternal (F0) metabolome PLS-DA loading plots.....	136
Figure 3.11 Metabolomic analysis of maternal milk at postnatal day 4.....	141
Figure 3.12 Summary of key metabolite changes in the dams from faeces, liver, blood, brain, and milk.....	142
Figure 3.13 Summary of top metabolites from partial least squares discriminant analyses (PLS-DA).....	146
Figure 4.1 Maternal HFD and probiotic intake increase offspring body weight in early life but not in adulthood.....	152
Figure 4.2 Maternal probiotic treatment reverses anxiety-like behaviour in the young and adult offspring in the open field test.....	154

Figure 4.3 Maternal perinatal probiotic treatment reduces anxiety-like behaviour in the juvenile offspring.....	155
Figure 4.4 Maternal probiotic intake reverses anxiety-like behaviour in the adult offspring of obese dams.....	156
Figure 4.5 Persistent effects of maternal obesity on depressive-like behaviour in young and adult offspring.....	158
Figure 4.6 F1 (PND21) juvenile liver and brain (PFC) pro-inflammatory gene expression..	160
Figure 4.7 F1 (PND112) adult liver and brain (PFC) pro-inflammatory gene expression. ...	161
Figure 4.8 Maternal probiotic treatment alters synaptic plasticity in juvenile offspring.	164
Figure 4.9 Maternal probiotic treatment has lasting effects on astrocytic metabolism and neuronal plasticity.....	167
Figure 5.1 Unsupervised principal component analysis of the offspring gut–brain metabolome reflects maternal metabotype influenced by diet and perinatal probiotic supplementation	177
Figure 5.2 Faecal PLS-DA scores plot with accompanying loadings plot for the juvenile and adult offspring.....	179
Figure 5.3 Liver PLS-DA scores plot with accompanying loadings plot for the juvenile and adult offspring.....	183
Figure 5.4 Plasma PLS-DA scores plot with accompanying loadings plot for the juvenile and adult offspring.....	186
Figure 5.5 Brain PLS-DA scores plot with accompanying loadings plot for the juvenile and adult offspring.....	188
Figure 5.6 Summary of key offspring metabolites in the juvenile offspring and the same metabolites shown in the adult offspring.....	193
Figure 5.7 Juvenile offspring: Correlation between key brain or faecal metabolites and the mRNA expression of genes that were significantly altered by maternal diet and/or probiotic treatment in the prefrontal cortex.	195
Figure 5.8 Adult offspring: Correlation between faecal or brain metabolites and the prefrontal cortex mRNA expression of genes that were identified as key metabolites in both the juvenile and adult offspring.	196
Figure 5.9 Key gut and brain metabolites altered by maternal probiotic treatment show correlations with a genetic marker of astrocytic metabolism and anxiety-like behaviour.	198
Figure 5.10 Comprehensive atlas of offspring gut-liver-brain after perinatal exposure to maternal obesity and maternal probiotic intake.....	200
Figure 6.1 Serum lipid biochemistry is altered by depression status in NAFLD.	212
Figure 6.2 Partial least squares discriminant analysis (PLS-DA) of clinical chemistry identifies recent, but not lifetime depressive symptoms in this cohort with non-alcoholic fatty liver disease....	214
Figure 6.3 Nuclear magnetic resonance spectroscopy (NMR) identifies a serum metabolic signature of recent depressive symptoms in NAFLD.	216
Figure 6.4 Univariate analysis and representative spectra of metabolite relative abundance derived from nuclear magnetic resonance (NMR) spectroscopy.	218

Figure 6.5 Metabolites most related to the effects of antidepressant (AD) treatment and age are unrelated to recent depressive symptoms.....	219
Figure 6.6 Sex differences in the serum metabolome are unrelated to recent depressive symptoms.....	222
Figure 6.7 Plasma branched chain amino acids (isoleucine and valine) are positively associated with passive stress coping in the forced swim test (FST) in maternal mice.	223
Figure 6.8 Plasma BCAAs are negatively associated with passive stress coping in the forced swim test (FST) in the juvenile offspring.....	225
Figure 6.9 Circulating metabolites do not significantly correlate with time spent immobile in the F1 adult offspring.	226
Figure 6.10 Summary of the significant Pearson correlations between inflammatory gene expression and depressive-like behaviour (total immobility time) in the FST.	227
Figure 7.1 Illustration of the relationship between faecal microbial metabolite profile and milk microbial metabolite profile through the correlation of butyrate levels in the female mouse dams....	243
Figure 7.2 Potential mechanisms describing how diet-induced metabolic syndrome (obesity/NAFLD) may lead to altered brain plasticity and low mood.	247
Figure 7.3 Summary of thesis findings by chapter.....	254

ix. Conference presentations during candidature

As presenting or co-presenting author

Beneficial Microbes Conference (virtual), 22-24 March 2021. Maternal probiotic intake during obesity ameliorates depressive-like behaviour and increases faecal short-chain fatty acids in offspring. Poster presentation.

Society of Biological Psychiatry (virtual), 29 April – 1 May 2021. Maternal probiotic intake in obese mice reduces anxiety-like behaviour in offspring and increases blood and brain lactate. Poster presentation.

The Harvard Chan Centre for the Microbiome in Public Health (HCMPH) Annual Symposium (virtual), 17 May 2021. Maternal probiotic intake in obese mice reduces anxiety-like behaviour in offspring and increases blood and brain lactate. Poster presentation.

European Association for the Study of the Liver (EASL) International Liver Conference (virtual), 23-26 June 2021. Serum nuclear magnetic resonance metabolomic signature can discriminate between immunoglobulin G4-related sclerosing cholangitis and primary sclerosing cholangitis. Poster presentation.

British Association for the Study of the Liver (BASL) Virtual Meeting, 21-24 September 2021. Serum nuclear magnetic resonance metabolomic signature can discriminate immunoglobulin G4-related sclerosing cholangitis and primary sclerosing cholangitis. Poster presentation.

European Crohn's and Colitis Organisation (ECCO) Congress (virtual), 16-19 February 2022. A serum metabolite panel predicts Crohn's disease relapse in patients discontinuing infliximab and continuing antimetabolite therapy: sub-analysis of the SPARE Trial. Oral and poster presentation.

European Crohn's and Colitis Organisation (ECCO) Congress (virtual), 16-19 February 2022. An inflammatory serum metabolomic signature predicts response to vedolizumab treatment in people with Crohn's Disease. Poster presentation.

x. Publications during candidature

Chan, S.Y., Probert, F., Radford-Smith, D.E., Hebert, J.C., Claridge, T.D.W., Anthony, D.C., et al. (2020). Post-inflammatory behavioural despair in male mice is associated with reduced cortical glutamate-glutamine ratios, and circulating lipid and energy metabolites. *Sci Rep* 10: 16857.

Healicon, R., Rooney, C.H.E., Ball, V., Shinozaki, A., Miller, J.J., Smart, S., Radford-Smith, D.E., et al. (2022). Assessing the effect of anesthetic gas mixtures on hyperpolarized ¹³C pyruvate metabolism in the rat brain. *Magn Reson Med* 88: 1324–1332.

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^ΘPublished material appears in Chapter 6 of this thesis

1 INTRODUCTION

1.1. The gut microbiome: a recent history and current state of the field

Comprehending the significance of very large (or very small) numbers is difficult, despite being inherent to descriptions of the biological and physical world. The human microbiome tends to be introduced with such massive orders of magnitude, for example the trillions of microbes known to inhabit the digestive tract. These quantifications, frankly estimations, might gloss over the fact that our interpretation of the microbiome remains limited by our own understanding and means of characterising it. Perhaps it is most insightful to perceive and interpret the human microbiome based on its function, rather than diversity and taxonomy, and relating this to our understanding of human physiology in health and disease. The human microbiome is mostly bacterial, but also incorporates commensal residing viruses, archaea, and fungi, the functional significance of which is much less understood. On average, there are 10 commensal bacteria for every nucleated cell in the human body (Sender et al., 2016a, 2016b), the vast majority of which are concentrated in the colon (large intestine). The modulation and impact of colonic 'gut' microbes on the brain and behaviour is the focus of this thesis.

Commensal gut microbes are essential to human health. Most prominently they have broad metabolic functions, are involved in the education and development of host immunity, and protection against the seeding of pathogenic enteric microbes (Shreiner et al., 2015). Through these intimate relations with host physiology, research on the gut microbiome has permeated all aspects of biomedical science. Initial characterisations of the human microbiome found it to be highly variable between individuals (Human Microbiome Project Consortium, 2012). Moreover, microbiota composition has not been found to reliably predict

healthy or disease-associated phenotypes (Human Microbiome Project Consortium, 2012, 2019a), despite a shift in microbiome composition compared to healthy controls being associated with obesity and other metabolic disorders, autoimmune disorders, and psychiatric disease. This is because associations do not distinguish meaningful differences from meaningless ones, and there is no distinction between cause and effect. This uncertainty remains a challenge of microbiome research in health and disease. This somewhat humbling revelation led to large scale, 'multi-omic' longitudinal studies to robustly characterise strain-specific microbiota profiles in healthy and disease states, with a focus on functional immune and metabolic outcomes (Lloyd-Price et al., 2017, 2019; Fettweis et al., 2019; Franzosa et al., 2019; Zhou et al., 2019). Along these lines, the fundamental aim of this thesis is to further understand the intersection between metabolic disease and mood disorders, with a focus on metabolism across the gut, liver, and brain. More broadly, current, and future investigations that aim to characterise microbial function and specific contribution to host physiology will be critical to converting research into clinical applications. More focus needs to be applied on achieving socioeconomic and ethnic diversity of humans participating in microbiome research, so that new findings can be applied to underrepresented populations with distinct microbiome signatures (Gaulke and Sharpton, 2018, 2019b). While the gut microbiome generally refers to the collective microbial genome, and the microbiota the actual organisms, the terms may be used interchangeably in this thesis.

1.2. The microbiota-gut-brain axis

One of the most exciting and rapidly evolving fields of neuroscience and biological psychiatry is the microbiota-gut-brain axis which explores the mutual influences and communications between the gut, its commensal microbes, and brain function. While much remains to be understood about how microbiota influence the brain and systemic physiology, significant progress in the last decade has provided some insight into the functional role microbiota play in brain health and disease (Cryan et al., 2019).

1.2.1. The putative mechanisms of bug-to-brain communication

Humans have never existed without a symbiotic relationship with microbes. By extension, the brain has never been without signals from the gut and its resident microbes. Seminal work in germ free mice established the important role that gut microbiota play to support the development of normal brain function and behaviour (Hegstrand and Hine, 1986; Sudo et al., 2004; Diaz Heijtz et al., 2011; Gareau et al., 2011; Neufeld et al., 2011; Clarke et al., 2013). Through this research, several bidirectional routes of communication have been identified (Figure 1.1). Principally, these include interaction with the nervous system, in particular the vagus nerve afferents, modulation of the host immune system, and the metabolism and production of small molecules including SCFAs and neuroactive compounds. Most likely these pathways operate in parallel.

1.2.1.1. Vagus nerve

The vagus nerve, the tenth cranial nerve, is the chief neural component of the brain-gut axis, and the primary parasympathetic nerve linking the brain with the viscera. Composed of 80% afferent (and 20% efferent) fibres, vagal afferent endings can respond to both distension of the gastrointestinal (GI) tract via mechanosensors as well as gut peptide hormones, neurotransmitters, and microbial metabolites through a diverse array of chemosensitive and other receptors (Forsythe et al., 2014).

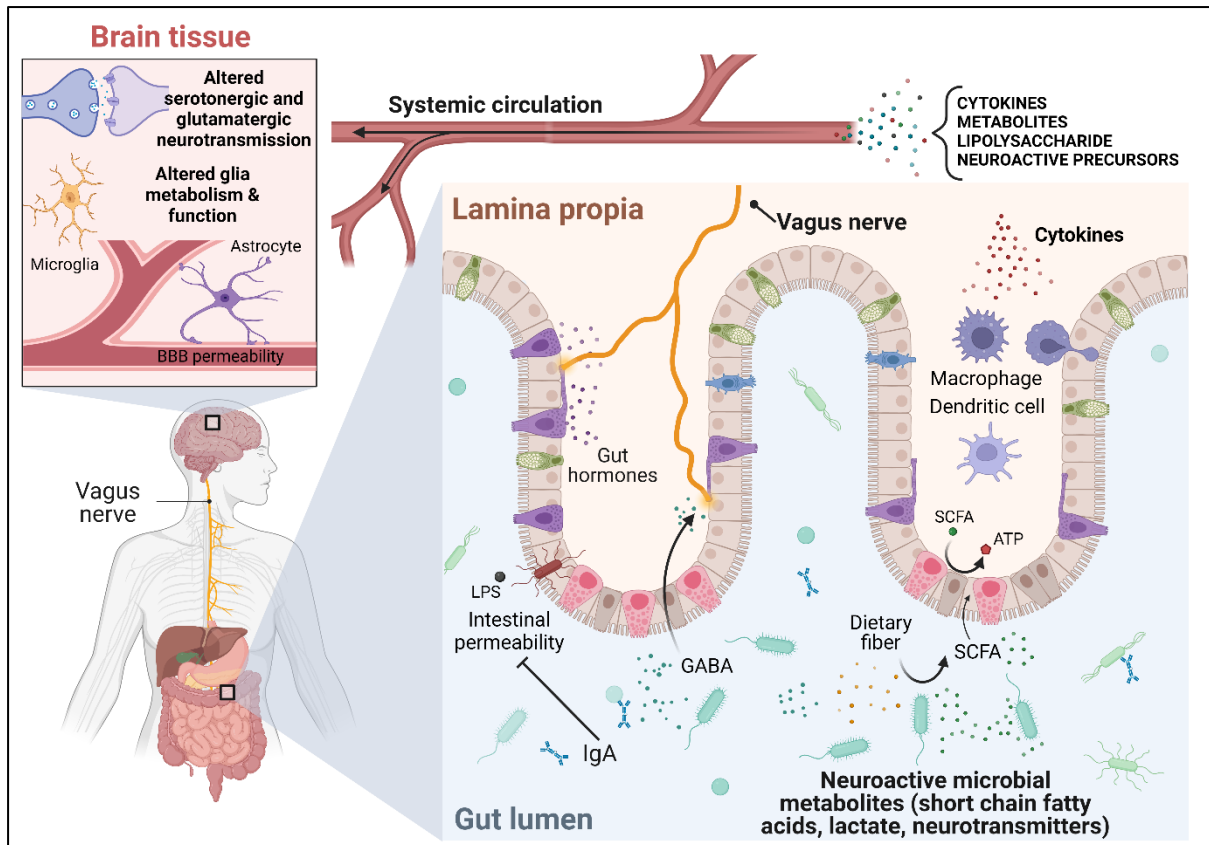


Figure 1.1 Summary of the putative mechanisms of communication between the gut microbiota and the brain.

Research utilising vagotomy has contributed most to our understanding of how the vagus nerve contributes to brain-gut communication, including signals from gut bacteria. In humans, vagotomy was a consequence of gastrectomy which was, historically, a treatment for duodenal ulcer. In a historical study of 118 patients following partial gastrectomy, a high incidence (43%) of psychiatric symptoms was observed (Sinclair-Gieben et al., 1962). Complete vagotomy has also been used for the treatment of morbid obesity (Kral, 1978), whereby the main cognitive symptom reported post-operatively was unexplained fatigue alongside altered hedonic perceptions of taste (Kral, 1983). Reciprocally, vagus nerve stimulation has been used as an adjunct treatment for severe depression (Aaronson et al., 2017).

In rodents, intraperitoneal injection of lipopolysaccharide (LPS) is used to model sickness behaviour associated with peripheral and central inflammatory responses. LPS is an integral structural component of the outer membrane of gram-negative bacteria and a potent

activator of the human immune system. Several studies noted that vagotomy reduced the effect of LPS on facets of sickness behaviour, including general activity two-hours post-injection (Layé et al., 1995), social behaviour (Bluthé et al., 1994), and food-motivated behaviour (Bret-Dibat et al., 1995). Levels of peripheral IL-1B, increased after LPS treatment, were unaffected by vagotomy (Bluthé et al., 1994; Layé et al., 1995). Cumulatively, these studies point to the contribution of the vagus nerve to changes in behaviour arising from peritoneal inflammation. Conversely, Van Dam et al. (2000) showed that vagotomy did not prevent an LPS-induced increase in brain IL-1B in rats. While sickness behaviour was not measured, the results indicate that the vagus nerve is not solely responsible for communicating gastrointestinal perturbations to the brain.

Studies conducted more recently have suggested that the vagus nerve may also mediate the effects of gut bacteria on brain function and behaviour. Bravo et al. (2011) showed that oral gavage treatment with *Lactobacillus rhamnosus* (JB-1) reduced anxiety and depressive-like behaviour in the elevated plus maze (EPM) and forced swim test (FST) in healthy mice, respectively. These changes were associated with altered messenger (m)RNA levels of $GABA_A$ and $GABA_B$ receptors in the brain. Vagotomy prevented the behavioural effects of *L. rhamnosus* (JB-1) administration as well as the associated changes in $GABA_A$ expression in the amygdala and hippocampus. This implicates the vagus nerve in some of the observed molecular and behavioural effects of the probiotic strain. Similarly, it was shown that anxiety-like behaviour associated with dextran sodium sulphate-induced colitis was mitigated by *Bifidobacterium longum* (NCC3001), only in animals with an intact vagus nerve (Bercik et al., 2011). Lastly, the bacterial species *Lactobacillus reuteri* was shown to act specifically via the vagus nerve in its ability to restore normal social behaviour in mouse models of autism spectrum disorder (ASD) when administered in drinking water (Sgritta et al., 2019). This was thought to be independent of other commensal microbes, as *L. reuteri* was also able to rescue aberrant social behaviour in germ-free (GF) mice, and supplementing ASD-like mice with *L. reuteri* did not change the overall gut microbiota composition of the animals. Despite the

rigorous nature of this study in elucidating the basic mechanism of action of *L. reuteri*, it remains unclear from all these studies how microbes interact with the vagus nerve. This is an important issue to resolve, as the latter study described here suggests that *L. reuteri* has the capacity to rescue social behaviour and restore oxytocin levels in the paraventricular nucleus of the hypothalamus as one strain among hundreds, at least. Understanding, and harnessing, this mechanism will be important in translating and applying microbiome-based therapeutics in the future via modulation of the vagus nerve.

1.2.1.2. Immune system

Inflammation is associated with depression in a bidirectional manner (Pitharouli et al., 2021). Chronic gut, liver, and brain inflammatory diseases such as inflammatory bowel disease (IBD), non-alcoholic fatty liver disease (NAFLD), and multiple sclerosis (MS) are associated with increased rates of depression (Boeschoten et al., 2017; Keefer and Kane, 2017; Labenz et al., 2020). The peripheral immune system, therefore, is a high plausible indirect route of communication between the collective gut microbiota, brain function, and behaviour.

Studies in GF mice established the essential role for commensal microbes in the maturation of the host innate and adaptive immune systems (Zheng et al., 2020) mediated by bacterial polysaccharide A (Mazmanian et al., 2005). GF mice have an underdeveloped immune system, including reduced number of adaptive immune cells and reduced development of secondary lymphoid structures (Powell et al., 2017). In the GI tract itself, converging mechanisms compartmentalise commensal microbes and prevent intrusion into sterile tissue and aberrant immune responses. A dense and insoluble luminal mucous barrier, largely formed of the mucin MUC2, is maintained by goblet cells in the epithelial lining of the large intestine. Within the mucous layer, dendritic cells sample microbial epitopes in a perpetual state of immunotolerance induced by MUC2-associated glycans (Shan et al., 2013; Zheng et al., 2020). In parallel, microbial production of butyrate induces the formation of regulatory T cells (Arpaia et al., 2013; Furusawa et al., 2013) which, together with the

tolerogenic dendritic cells, promote B cell secretory IgA production to further prevent pro-inflammatory innate immune activation in the gut (Macpherson and Uhr, 2004; Peterson et al., 2007).

Collectively, it has become apparent that the qualitative composition of the gut is crucial to maintaining systemic immune homeostasis. It is not just the presence or absence of microbes that affect host immunity, but rather the balance of an unfathomably large ecosystem. Dysbiosis of the microbiome by diet or other means therefore has the potential to disrupt immune homeostasis. Given that up to 80% of human immune cells reside in the GI tract (Faria and Weiner, 2005), it is conceivable that the downstream implications of disrupted immune homeostasis in the gut, such as increased circulating pro-inflammatory cytokines, induce or exacerbate chronic inflammatory diseases and psychiatric disorders (Powell et al., 2017).

In a maternal immune activation (MIA) model of ASD in mice, offspring showed increased intestinal permeability, gut microbiota dysbiosis, increased colonic inflammation by increased *IL-6* expression alongside reduced social behaviour (Hsiao et al., 2013). Treatment of the offspring with *Bacteroides fragilis* normalised levels of GI tract inflammation and reversed aberrant social behaviour, supporting an immune-mediated link between the microbiota, gut barrier integrity, and brain and behaviour (Hsiao et al., 2013). Distal to the gut, a complex microbiota has also been shown to be crucial for microglia maturation and ongoing function (Erny et al., 2015). Researchers found that microglia from GF mice, under homeostatic “steady-state” conditions, were immature and had a diminished immune response (Erny et al., 2015). Accordingly, microglia from GF mice failed to mount an appropriate immune response to intracranial injections of LPS or lymphocytic choriomeningitis virus, characterised by reduced mRNA expression levels of *TNF* and *IL-1B*. The precise coordinates of the intracranial injection was not reported, which limits the interpretability and reproducibility of this finding. Antibiotic depletion of the microbiota produced a similar immature

microglial phenotype in adult mice, suggesting an ongoing and highly plastic relationship between the gut microbiota and brain microglia (Erny et al., 2015).

The absence of microbiota has also been associated with altered microglia phenotype in aged mice (Mossad et al., 2022). Specific pathogen free (SPF), but not GF mice, showed an increase in intestinal permeability at ~2 years age compared to ~2 months age. This coincided with a detrimental accumulation of the microbial metabolite N 6-carboxymethyllysine in the aged microglia, which was not found in the brain of GF mice. GF mice showed reduced markers of brain oxidative stress and microglial dysfunction compared to SPF mice, which was attributed to the N 6-carboxymethyllysine. Thus, the commensal microbiota appears to play a role in normal microglia physiology and age-related pathology throughout the lifespan.

1.2.1.3. Microbial metabolites and neurotransmitters

Metabolites produced by microbiota are thought to be the key effector molecules that allow the microbiota to communicate and influence the function of distal organs, including the brain. Using untargeted metabolomics by mass spectrometry (MS) to compare the plasma of GF and SPF mice, Wikoff et al. showed that the gut microbiome affects the blood concentrations of many metabolites (Wikoff et al., 2009). Tryptophan, for example, was present at higher concentrations in GF mice, while serotonin was present at higher concentrations in SPF mice, which may suggest a role for the microbiota in the metabolism of dietary tryptophan to serotonin (Wikoff et al., 2009). Lai et al. (2021) performed untargeted metabolomics of the brain, blood, and faeces, allowing for an integrative view of the impact of the microbiome on metabolites across the gut-brain axis. In corroboration with Wikoff et al. (2009), Lai showed that SPF mice exhibit elevated levels of neurotransmitters in the gut compared to GF mice, including serotonin, GABA, glutamate, among others (Lai et al., 2021). Other, microbial-derived metabolites elevated in the serum of SPF mice compared to GF animals, such as indoxyl sulphate and indole-3-lactate, were also markedly elevated in the brain. In a different study by Chu et al. (2019), indoxyl sulphate was one of a panel of microbial metabolites required for normal fear extinction learning in mice, a behaviour that was

suggested to rely on microbiota-derived signals throughout the lifespan. Together, these studies substantiate the link between microbial metabolites, brain function, and altered behaviour.

Several studies have identified one or several metabolites that, when administered to GF/antibiotic-treated mice, partially or fully reproduce the effects of the presence of the gut microbiota on aspects of host immunity, brain function, and behaviour. Microbially-derived butyrate and propionate, for example, are potent histone deacetylase (HDAC) inhibitors *in vitro* and *in vivo*. In the colonic mucosa, these SCFAs have been shown to act via this mechanism to switch off dendritic cell pro-inflammatory cytokine expression and enhance *Foxp3* expression to drive the induction of regulatory T cells and immune tolerance (Arpaia et al., 2013). As previously discussed, ongoing signals from a complex gut microbiota are required for normal microglia function in the brain (Erny et al., 2015). By contrast, the microglia of GF mice lack these microbial signals, and display an immature phenotype including increased expression of survival and growth factors *CSF1R* and *DDIT4*, whereby expression is typically attenuated in mature microglia. 4-week supplementation with a mixture of SCFAs (acetate, propionate, and butyrate) in drinking water was shown to restore normal gene expression profiles in the GF mice, including reduced *DDIT4* expression, and reduce the number of *CSF1R*⁺ cells to levels exhibited by SPF mice (Erny et al., 2015). In a follow-up study, Erny et al. (2021) identified that the primary metabolite mediating microglial fitness in SPF mice was microbially-derived acetate. GF mice showed depleted levels of acetate in the brain and blood compared to SPF mice. Oral gavage of ¹³C-derived acetate in GF mice was shown to enter brain tissue after six hours, by which point the ¹³C label had been incorporated into tricarboxylic acid cycle metabolites.

Microbial metabolites can also facilitate the effects of the maternal gut microbiota on offspring brain development. Vuong et al. (2020) show that embryonic brain development markedly differs between GF and SPF mothers who are otherwise healthy. Specifically, axonal projections between the thalamus and cortex fail to connect properly, leading to defects in

sensorimotor behaviours. Metabolomic profiling of the maternal sera and offspring brain revealed reduced levels of certain microbial metabolites that, when supplemented to antibiotic-treated dams, restored normal axonogenesis and sensorimotor behaviour in the offspring (Vuong et al., 2020). In this way, microbial metabolites not only contribute to gut-brain communication across the lifespan, but also between mother and foetus during the perinatal period.

1.2.2. Perturbed microbiota-gut-brain axis metabolism in mood disorders

1.2.2.1. A critique of isolated microbiome analysis in mood disorders

Several studies in the last decade have found relationships between the gut microbiome and major depressive disorder (MDD) in humans (Cheung et al., 2019; Nikolova et al., 2021). Despite these significant findings, there is no consensus on which bacterial taxa, if any, are specific to depressive symptoms (Cheung et al., 2019). Interestingly, a meta-analysis of microbiome studies across a spectrum of psychiatric disorders found no real specificity between the relative abundance of different microbial taxa and a given disease, and no decrease in alpha-diversity (within-patient diversity or “richness”) (Nikolova et al., 2021). Rather, the most consistent observation were similar patterns of microbial changes between each disorder compared to the healthy control group, including a reduction in the abundance of butyrate-producing bacteria in patients with MDD, bipolar disorder, schizophrenia and anxiety (Nikolova et al., 2021). A high level of inter-study variation was observed. These discrepancies are likely due in part to methodological differences between studies, but also may be due to the substantial interindividual variation in gut microbiome composition (Human Microbiome Project Consortium, 2012). Given that functional metabolic processes are conserved across bacterial taxa (Human Microbiome Project Consortium, 2012), variable gut microbial taxonomy between depressed individuals is likely to exist, but may still give rise to highly convergent functional outcomes downstream. As a result, analysis of faecal and/or gut mucosal metabolites, or functional analysis of retrieved genomic data, may be more informative than describing the relative abundance of bacteria in the gut (Zierer et al., 2018).

This concept is supported by Visconti et al. (2019) investigating the inter-individual variation between the faecal microbiome and the faecal metabolome. It was found that the participants (>1,000 unrelated twins recruited from the UK twin registry) shared approximately double the number of metabolic pathways (82%) than microbial species (43%). Fundamentally, the study revealed that a variable microbial taxonomic signature does not necessarily lead to equivalent volatility in the downstream biochemistry. It is therefore more intuitive to explore the microbiome by using faecal metabolomic or annotated metagenomic methods that can report on function, as opposed to the classical 16S ribosomal RNA (rRNA) amplicon sequencing, which is relatively information poor.

1.2.2.2. Animal studies

Metabolites produced by the gut microbiota play a role in host pathways related to energy metabolism, immunity, and inflammation that connect the gut, liver, and brain (Nicholson et al., 2012). Direct evidence has been provided by studies that have demonstrated neurobehavioural changes associated with faecal microbiota transplantation (FMT) from humans with MDD into rodents. Zheng et al. (2016) showed that, in comparison to germ free (GF) mice colonised with microbes from healthy (control) individuals, GF mice receiving microbes from patients with MDD showed increased depressive-like behaviours after 2 weeks. This included a reduction in the time that the mice spent in the centre of an open field test (OFT) and increased time spent immobile in the FST. Depressive-like behaviour was linked to changes in carbohydrate and amino acid metabolism in faecal and serum samples, as well as in the hippocampus (Zheng et al., 2016). However, different metabolites within these pathways were either increased or decreased in mice receiving FMT from depressed individuals. Hippocampal glycine levels were reduced in mice who received the “depressed microbiota” compared to mice who received the “healthy microbiota”. Given that glycine is required to facilitate glutamatergic neurotransmission at N-methyl-D-aspartate receptors (NMDARs), this particular metabolic alteration may have contributed to the altered behaviours that were observed (Cummings and Popescu, 2015). For example, hypofunction of NMDARs by

alterations to the glycine binding site are thought to be implicated in the pathophysiology of schizophrenia in humans (Labrie and Roder, 2010). Kelly et al. (2016) investigated how FMT in mice from depressed patients mediated the neuroimmune and neuroendocrine pathophysiology of depressive-like behaviour. Depressed patients showed an increase in plasma pro-inflammatory cytokines, cortisol, and the kynurenine/tryptophan ratio. Following FMT, similar changes in plasma tryptophan metabolism were observed in recipient rats, but no changes in the level of pro-inflammatory cytokines or cortisol were observed. The rats also showed increased depressive-like behaviour. Both depressed individuals and the recipient rats showed reduced richness and diversity in the composition of the gut microbiome. From these studies, it can be concluded that microbe composition associated with depression can induce depressive-like behaviours in rodents, which may be mediated by host changes in energy metabolism and the levels of endogenous neuromodulatory metabolites. However, it remains uncertain from these studies whether microbiota that are associated with MDD in humans also directly contribute to inflammation. A more comprehensive assessment of host immunity post-FMT, for example the measurement of intestinal barrier integrity, circulating endotoxin levels, quantification of specific immune cell populations, and host response to immune challenges is warranted.

A recent systematic review evaluated 241 metabolomic studies in animal models of depression (Pu et al., 2021a). Individual metabolites were investigated by a simple vote-counting method. In the brain, levels of serotonin, dopamine, and GABA amongst others were significantly downregulated, whereas kynurenine and myo-inositol were consistently upregulated. In studies that specifically investigated the metabolite composition of the prefrontal cortex (PFC), only glutamate was significant, which was significantly downregulated. In the blood, the most consistently upregulated metabolite in animal models of depression was N-acetyl glycoprotein. These results will have been influenced by the underlying methodologies of each study, including the analysis technique (nuclear magnetic resonance [NMR] spectroscopy or MS) and how depressive-like behaviour was induced and

measure. Chronic mild stress (CMS) was the most common model of depression in the systematic review (Pu et al., 2020). This may have influenced the prevalence of inflammation-related metabolites present as CMS has been associated with the induction of peripheral inflammation (López-López et al., 2016; Pavlov et al., 2019). Additionally, MS was employed in the reviewed literature more often than NMR spectroscopy or magnetic resonance spectroscopy (MRS). While high inter-study variability was noted, trends towards decreased neurotransmitter levels in the brain, including reduced glutamate levels, and increased markers of inflammation in the brain and periphery were found to be the prominent features (Pu et al., 2021a).

It is worth noting that substantially less research has focused on anxiety-like behaviours in relation to the microbiota-gut-brain axis. In humans, however, anxiety and depressive symptoms are highly comorbid (Kaufman and Charney, 2000), and in a large proportion of animal models for depression, anxiety-like behaviour is often assayed as part of a battery of behavioural tests by virtue of the OFT or EPM (Zheng et al., 2016; Needham et al., 2022). In one study (Needham et al., 2022), anxiety-like behaviour was related to the gut-derived metabolite 4-ethylphenyl sulphate (4EPS). 4-ethyl phenol is derived from dietary tyrosine via gut microbiota metabolism before sulphation to 4EPS by the host. When gnotobiotic animals were colonised with single strains that either did or did not produce 4EPS, 4EPS was shown to accumulate in the brain after injection of probenecid, which prevents small molecule efflux across the blood brain barrier (BBB). It was therefore concluded that this gut-derived molecule was able to access the brain parenchyma. 4EPS was found to increase anxiety-like behaviour, but not cognition (Y-maze) or motor function (beam traversal, pole descent, wire hang tests), by disrupting oligodendrocyte maturation and myelination (Needham et al., 2022).

To conclude, there is substantial evidence for altered gut and brain metabolism in preclinical models of mood disorders – both in terms of the levels of endogenous neurotransmitters and metabolites that are exclusively microbially-derived. Much of this

evidence has accumulated recently in the last few years, and further research will be required to corroborate these findings in individuals with MDD and/or anxiety to assess the translational validity of candidate metabolites.

1.2.2.3. Human studies

Clinical studies have confirmed that there is perturbation of the microbiota-gut-brain axis, including alterations to individual metabolites, in patients with mood disorders. In two independent studies, the abundance of *Alistipes spp.* has been shown to be elevated in individuals with MDD compared to controls (Naseribafrouei et al., 2014; Jiang et al., 2015). Mechanistically, *Alistipes spp.* may be related to metabolic pathways associated with increased inflammation, as another study showed correlations with intensity of abdominal pain in irritable bowel syndrome (Saulnier et al., 2011). Through the use of gut microbiota sequencing in tandem with gut metabolomics analysis, Yang et al. (2020) related changes in gut microbiota composition between depressed and non-depressed individuals with changes to host metabolism. *Bacteroides spp.* were enriched in individuals with MDD and correlated with reduced faecal amino acid levels (leucine and proline) and increased lipid levels (2-indolecarboxylic acid and itaconic acid). Separately, the authors found that GABA and related metabolites were reduced in the gut in those with MDD compared to healthy controls, and the authors speculated that these findings may influence brain GABA levels in MDD. Li et al. (2022) elegantly demonstrated that a significant portion of the variation in serum GABA levels (up to 22%), which were reduced in individuals with depressed bipolar disorder compared to healthy controls, could be explained by whether or not the gut microbiota of a given individual contained genes essential for GABA synthesis. Moreover, GABA levels have shown to be increased in the blood of obese individuals after faecal microbiota transplant from lean individuals, which coincided with increased insulin sensitivity (Kootte et al., 2017). These studies provide further evidence that changes to the gut microbiota can affect the level of circulating neurotransmitters. Nevertheless, it remains unclear to what extent microbially-

produced neurotransmitters, for example GABA, directly contribute to central neurotransmitter levels (Strandwitz, 2018).

Two large ($n > 1,000$) cohorts with microbiome metagenomic data, alongside quality of life (QoL) or depression scores have shed further light on the possible links between gut microbial metabolism and MDD in humans (Valles-Colomer et al., 2019). In the first cohort, two metabolites were positively associated with QoL scores, including synthesis of 3,4-dihydroxyphenylacetic acid (DOPAC, a dopamine metabolite) and isovaleric acid (a SCFA). The correlation of QoL scores with DOPAC was reproduced in the second cohort. Butyrate-producing bacteria were positively associated with QoL in both cohorts (Valles-Colomer et al., 2019). Conversely, in a study of 113 children, chronic psychosocial stress showed a significant positive correlation with the faecal levels of several SCFAs, including butyrate and isovalerate (Michels et al., 2017). Overall, these correlational studies in humans support the preclinical evidence for a causal role of the gut microbiota and associated microbial metabolites in the pathogenesis of depressive symptoms. Many discrepancies remain in terms of the directionality of these metabolites, and how the composition of a “depressed” or “healthy” microbiome should be defined.

1.3. Maternal factors influencing offspring neurodevelopment and behaviour

1.3.1. Spotlight on obesity

Obesity is a common yet highly preventable metabolic disorder prevalent worldwide (Afshin et al., 2017). An audit led by the Royal College of Obstetricians and Gynaecologists in 2017 (NMPA Project Team, 2017) discovered that a quarter of pregnant women in the UK are obese, with a similar prevalence across continental Europe (Devlieger et al., 2016). Moreover, fewer than half of women in the UK have a pre-pregnancy body mass index (BMI) within the normal range (NMPA Project Team, 2017). Excessive gestational weight gain (GWG) is also a relatively common occurrence during pregnancy in the UK and other Western countries, including the USA and Australia (Gaillard, 2015). Entering pregnancy with an elevated BMI increases the likelihood of excessive GWG, as well as the risk of retaining weight postpartum (Campbell et al., 2016). Independent of pre-pregnancy BMI, 40% of women in Western countries gain excessive weight during pregnancy (Gaillard, 2015). It has also been shown that the majority of pregnant women gain excessive weight due to the consumption of a diet high in fat (Fung et al., 2001).

While the consequences of maternal obesity on offspring metabolism, obesity, and cardiovascular health have been widely reported (Leddy et al., 2008; Mulligan and Friedman, 2017), only more recently are thorough investigations being made into how maternal diet and obesity affect maternal and offspring behaviour, including the transmission of psychiatric disease risk through poor maternal health during gestation and the early life of the offspring. These potential mechanisms have been summarised in Figure 1.2. It is critical to have a mechanistic understanding of how the maternal uterine and lactational environment may mediate offspring neurodevelopmental morbidity, because pregnancy may represent an important window for targeted intervention to ameliorate foetal and offspring risk (Edlow, 2017).

Seminal studies in rodent (Bilbo and Tsang, 2010) and nonhuman primate (NHP) (Sullivan et al., 2010) offspring first observed the deleterious effects of maternal *peripartum* high fat diet feeding on offspring brain and behaviour. In rats, it was shown that expression of TLR4 and CD11b, a marker of macrophages and CNS-resident microglia, were significantly upregulated in the juvenile offspring of obese dams fed a diet high in saturated fat (Bilbo and Tsang, 2010). TLR4 activation is required for robust pro-inflammatory responses to bacterial endotoxin, but also mediated central inflammatory signalling and altered brain metabolism. In addition, the basal level of IL-1 β protein was significantly elevated in the hippocampus and periphery (liver) of both juvenile and adult offspring of HFD fed dams, compared to offspring of controls. These inflammatory changes in HFD-exposed offspring were associated with increased anxiety in the elevated plus maze. Moreover, Sullivan et al. (2010) linked changes in maternal HFD (mHFD) consumption during gestation with increased anxiety-like behaviour in juvenile female NHP offspring. This was also accompanied by perturbations to the serotonergic system, including decreased cerebrospinal fluid levels of 5-HT. These studies paved the way for future animal models investigating the underlying mechanisms behind diet-induced obesity during gestation and nursing and altered neurobiology and behaviour in offspring.

1.3.1.1. Clinical studies

Meanwhile, clinical studies, largely epidemiological in nature, have highlighted the translational relevance for this field of experimental research. Some of the first association studies in humans investigated the link between low birthweight – a complication of pre-term birth with significantly greater prevalence in maternal obesity (McDonald et al., 2010) – and behavioural problems in children. Initially, it was shown that very low birth weight contributed to education difficulties and hyperactive behaviour in children (McCormick et al., 1990; Kelly et al., 2001), independent of sociodemographic factors. Attentional problems were the focus of early studies in low-birthweight offspring (Breslau et al., 1988; McCormick et al., 1990; Ross et al., 1990; Klebanov et al., 1994; Pharoah et al., 1994; Chapieski and Evankovich, 1997;

Sykes et al., 1997; Stjernqvist and Svenningsen, 1999; Zubrick et al., 2000; Hille et al., 2001; Mick et al., 2002). Following this, Rodriguez et al. (2008) made the direct association between maternal obesity and attention deficit hyperactivity disorder (ADHD), independent of birthweight.

A 2010 study showed that in addition to inattentiveness, pre-pregnancy obesity and overweightness predicted difficulties with emotional intensity and regulation in children (Rodriguez, 2010). Studies over the subsequent decade affirmed the relationship between an elevated pre-pregnancy BMI and internalising and externalising behaviours (Robinson et al., 2013; Van Lieshout et al., 2013; Jo et al., 2015; Kong et al., 2018). Here, internalising behaviours correspond to affective disorders whereby problems of an individual are internalised, manifesting in symptoms of depression, social withdrawal (as opposed to antisocial behaviour), and anxiety. Other, related behavioural abnormalities, characterised by high levels of negative affect, include obsessive-compulsive disorder, dissociative disorders, and eating disorders. In externalising disorders, on the other hand, emotions and cognitions are manifested as maladaptive, antisocial behaviours towards the external environment. ADHD is a relatively common externalising disorder. Despite their opposing behavioural phenotypes, these categories often co-occur in a household or individual, and appear to have a shared aetiology (Cosgrove et al., 2011). Throughout childhood and adolescence, both internalising and externalising behavioural problems are more likely to occur after gestational and early-life exposure to maternal obesity (Van Lieshout et al., 2013). Analysis of the same pregnancy cohort followed up children prospectively between the ages of 5 and 17 and found a higher risk of affective disorder in those exposed to an elevated pre-pregnancy BMI (Robinson et al., 2013). Importantly, these and more recent studies with consistent findings adjust for potential causative and confounding factors – weight gain during pregnancy, birthweight, and the presence of maternal psychiatric disorders were common adjustments across these studies (Jo et al., 2015; Kong et al., 2018, 2020). More rigorous considerations were made in the most recent investigations, including maternal age, delivery method, country

of birth, socioeconomic status, marital status, smoking status, and the presence of a systemic inflammatory disease, for example Crohn's Disease (Kong et al., 2020). The continued clinical interest in this transgenerational relationship between obesity and behaviour, coupled with highly consistent findings in humans, highlights the need to uncover specific mechanisms of transmission, and accompanying therapeutics. While undoubtedly the relationship is complex with multiple contributing factors (Edlow, 2017), establishing key pathways involved will pave the way for more targeted prevention and treatment during gestation and early-life offspring.

1.3.2. Maternal care behaviour

It is generally accepted that the importance of the relationship between mother and infant transcends nutritional need. This was demonstrated in animals in early ethological studies (Harlow and Zimmermann, 1959; Harlow et al., 1965), which initially demonstrated that impact of contact and comfort on primate development independent on nutritional support. Infant primates raised in the absence of maternal care developed severe social deficits, while developing strong emotional attachment to inanimate objects that provided any source of comfort. While infants during the early postnatal period are typically hyporesponsive to stress compared to other life stages, maternal care (and neglect) remains a strong influence over neurodevelopment (Lupien et al., 2009).

Clinical studies have revealed important associations between childhood maltreatment and altered neurobiology and behaviour. Childhood neglect, in its various forms, is associated with increased risk of behavioural problems and psychiatric illness. Longitudinal studies are important for clinical studies of the effects of maternal care. A large longitudinal study in 2017 showed that poor maternal care provides context for a link between depression and elevated BMI in adult women (Sacks et al., 2017). This and other clinical studies indicate that female offspring may be more at risk of developing psychopathologies as a consequence of poor maternal care. Another study showed that female adolescents of mothers with MDD have a significantly greater likelihood of developing a psychiatric disorder (Gershon et al., 2011). Exposure to maternal neglect in childhood is also a risk factor for attempted suicide,

depression and anxiety, and other psychiatric disorders (Orso et al., 2019). A comprehensive systematic review of clinical studies on different forms of childhood maltreatment found neglect to have one of the highest associations with the development of depressive disorders in later life (Re et al., 2012). The environmental modulation of development during early life likely serves to allow the adaptation of subsequent generations to novel environments that may differ to those of previous generations. During the perinatal period the developing brain is particularly susceptible to environmental insult, including neglect. Prevailing theory suggests that exposure to early stress through lack of maternal (and paternal) care programs enhanced responsiveness to stress (Teicher et al., 2003; Pechtel and Pizzagalli, 2011). While this may be viewed as a neurodevelopmental adaptation to a perceived stressful environment, it is almost certainly detrimental in the present environment. Understanding the neurobiological sequelae of exposure to poor maternal care is therefore an important feature of both clinical and preclinical neuroscience and behavioural research.

The abnormal development of specific brain regions has been implicated in maternal neglect. Both the hippocampus and prefrontal cortex (PFC) undergo protracted postnatal development and neurogenesis prior to myelination and synaptic pruning, with the hippocampus continuing to develop during childhood and the PFC developing into the third decade of life (Davey et al., 2008; Abrahám et al., 2010). Both regions have a high density of glucocorticoid receptors (GRs) (Diorio et al., 1993; Patel et al., 2000). Physiologically, these GRs serve to maintain homeostasis by inhibiting further hypothalamic pituitary adrenal (HPA) axis activity during the stress response. They are also thought to be important to neuronal function and survival through the regulation of brain-derived neurotrophic factor (BDNF), a key protein involved in the regulation of brain synaptic plasticity (Anacker et al., 2011).

In rodents, maternal neglect has been shown to affect brain development and behaviour via overactivation of the HPA axis. Temporary intervals of maternal separation from pups activate the HPA axis, increasing the levels of circulating adrenocorticotrophic hormone (ACTH) and glucocorticoids (Levine and Wiener, 1988). Prolonged separation reduces

corticotropin releasing hormone (CRH) binding sites in the hypothalamus (Anisman et al., 1998), possibly due to negative feedback via the sustained elevation of circulating stress hormones. Lower levels of maternal care, specifically reduced licking and grooming of pups, correlates with reduced hippocampal GR mRNA levels, and increased CRH mRNA in the hypothalamus (Liu et al., 1997). Moreover, chronic corticosterone administration has been shown to hinder neurogenesis in the hippocampus in rodent models in a number of independent studies (Numakawa et al., 2017). Brain transcriptomic analysis has subsequently revealed that maternal care behaviour regulates the expression of >900 genes in the hippocampus (Weaver et al., 2006). Finally, it has been shown that low levels of maternal licking and grooming behaviour lead to persistent anxiety-like behaviours in the adult (3-month old) offspring (Champagne and Meaney, 2006; Weaver et al., 2006).

Poor maternal care has also been shown to increase anxiety behaviours in NHP offspring, which may be mediated by perturbations to tryptophan metabolism via increased glucocorticoid levels. Macaques exposed to maternal neglect displayed elevated anxiety-like behaviours (increased scratching behaviour) between 12 and 24 months of age, which correlated with reduced levels of 5-hydroxyindoleacetic acid (5-HIAA) in the cerebrospinal fluid (CSF) (Maestriperi et al., 2006). 5-HIAA is a major metabolite of serotonin and may indicate reduced brain serotonin synthesis. While glucocorticoids were not measured, other studies have shown social separation in early life to significantly increase plasma cortisol levels at 6 months age (Fahlke et al., 2000) and persistently elevate CRH in the CSF at 2 years age (Coplan et al., 1996). Glucocorticoids are known to induce the expression of tryptophan 2,3-dioxygenase, an enzyme that catalyses the first and rate-limiting step of tryptophan to kynurenine (Danesch et al., 1983; Nakamura et al., 1987). It is possible, therefore, that early life maternal separation increased circulating glucocorticoid levels, directing tryptophan metabolism towards kynurenine metabolism (in peripheral tissues or in the brain) and depleting brain tryptophan availability for serotonin synthesis. In parallel, early life stress has been shown to reduce the metabolism of kynurenine to kynurenic acid, a neuroprotective

agent, and increase metabolism to quinolinic acid, a neurotoxic agent (Coplan et al., 2021). Given that serotonin levels peak during the perinatal period (Hohmann et al., 1988), it is unsurprising that perturbations to signalling during this sensitive period could produce long-lasting behavioural changes reminiscent of affective disorders (Rebello et al., 2014). Recent evidence (Courtiol et al., 2018) indicates an immediate impact of serotonergic signalling in response to maternal presence, whereby PFC activity during the early postnatal period is reduced during maternal absence as opposed to maternal presence. This was shown to be mediated by 5-HT₂ receptors and is consistent with another study that demonstrated early life stress to increase the pre-mRNA editing (and reduce downstream activity) of the 5HT-2C receptor in adult mice (Bhansali et al., 2007).

The adverse effect of stress on neurodevelopment has been demonstrated retrospectively in humans by reduced hippocampal volume in adults with a history of childhood trauma (Bremner et al., 1997; Driessen et al., 2000). Women, but not men, who experienced low maternal care during early life have reduced hippocampal volume compared to matched control individuals (Buss et al., 2007). This points to the increased susceptibility of female offspring to poor maternal care. Similar findings have been identified in the PFC, whereby grey matter volume in the prefrontal cortex has been shown to be reduced in children exposed to early life stress in both longitudinal studies and when evaluated retrospectively (Hart and Rubia, 2012; Tyborowska et al., 2018; Heyn et al., 2019; Underwood et al., 2019). Total brain volume has also been shown to be reduced in children with a history of trauma, compared to typically developing children (Carrion et al., 2001; Hart and Rubia, 2012). Reduced hippocampal volume is also a risk factor for future psychiatric disease (Geuze et al., 2005). Other studies have been more equivocal – De Bellis et al. (1999) found no effect of post-traumatic stress disorder on hippocampal volume in children or adolescents compared to control individuals. Similarly, while Carrion et al. (2001) found that children (age 7-14) with a history of psychological trauma had a smaller total cerebral volume compared with control individuals, there was no difference in the relative volume of the hippocampus. In summary,

significant stress exposure prior to the complete development of the hippocampus and the PFC may lead to their precocious maturation at the expense of their full developmental potential (Teicher et al., 2003; Tyborowska et al., 2018).

Maternal depression has been demonstrated to be a modifying factor in the quality of maternal care behaviour, whereby depressive episodes are an obstacle to sensitive maternal care. Depressed mothers are less attentive and responsive to the needs of their offspring (Bernard-Bonnin, 2004). Substantial clinical evidence now exists to show that maternal depression affects the HPA axis of developing children. For example, children of mothers who exhibit depression show elevated morning cortisol levels at 6-10 years age (Lupien et al., 2000). In another study, elevated waking cortisol levels was identified in 13 year old children of mothers with postpartum depression (Halligan et al., 2004). In the same cohort, it was shown that the elevated cortisol levels in these children played a significant mediatory role in the development of depression at 16 years age (Halligan et al., 2007). Retrospective studies in humans have shown that both prenatal and postnatal maternal depression adversely affect cognitive and emotional development in offspring (Cogill et al., 1986; Caplan et al., 1989; Madigan et al., 2018). Furthermore, lower scores on parameters that measure parent-child relationships (i.e., reduced empathy and more emotional distance) significantly increase the risk of depression and anxiety in later life (Canetti et al., 1997; Meaney, 2001), increasing the risk of parental-offspring neglect and psychopathology between generations.

Preclinical studies investigating natural variations in maternal care (without human intervention i.e., maternal separation from pups) in rats show that natural variations in the extent of licking/grooming (LG) and arched-back nursing (ABN) of pups has significant influence on neurodevelopment and behaviour. Offspring of dams engaging in higher levels of LG show increased exploratory behaviour in an open field (Jensen Peña and Champagne, 2013). Another study showed that naturally occurring higher levels of LG and ABN increased the expression of NMDAR subunit and brain-derived neurotrophic factor (BDNF) mRNA, with increased hippocampal synaptogenesis and improved spatial memory (Liu et al., 1997).

Changes in offspring gene expression and behaviour may be mediated by epigenetic mechanisms (Meaney, 2001; Weaver et al., 2004; Champagne, 2008). Altered DNA methylation due to natural variation in maternal care that leads to changes in chromatin structure can result in the attenuation of hippocampal GR expression in pups exposed to less LG (Weaver et al., 2004). Interestingly, preclinical evidence also supports inheritance of maternal care behaviours, such that female mice exposed to lower levels of maternal care also exhibit poor maternal care to subsequent generations via epigenetic regulation of brain estrogen receptor alpha expression (Champagne, 2008). Association studies in humans have also been conducted, which show that an abusive or neglectful parent is far more likely to have a history devoid of adequate parental care (Dowdney et al., 1985).

Few studies have investigated how maternal diet or diet-induced obesity affects maternal care behaviours in rodents, or parenting style in clinical studies. A high fat, high protein diet in rats lead to increased ABN and reduced passive nursing (PN), as well as increased grooming behaviour (Bertino, 1982). This study is of limited validity as maternal care was observed from birth to weaning age (postnatal day 21), and only for 2 hours each day per animal. Maternal care exhibits natural variation throughout this period, and a more intensive observational study in the first week of life might have been more meaningful (Franks et al., 2011). Additionally, changes in maternal behaviour towards pups were not linked to any brain or behavioural changes in offspring. A more recent study, also using rats, looked at the effect of a high fat diet on (60% kcal from fat) in comparison to a control diet (10% kcal from fat) on maternal care behaviours from postnatal days 3 to 6 (Purcell et al., 2011). They found no differences in maternal care behaviour during the day (light period) over a 3-hour observation for each day. During the dark period, ABN and total nursing was increased in dams exposed to a palatable high fat diet, while time spent resting was reduced. No differences were observed between licking/grooming. This study also has several limitations; the rats were only started on the high fat or control diets after the second day of gestation, and straight after arriving to the facility. The effect of transport stress may confound results of stress due to diet.

Furthermore, weight gain in the mothers was not assessed, and it is unlikely that significant weight gain occurred during the feeding period. Short-term palatable high fat diet feeding may reduce anxious behaviour rather than induce depressive-like behaviours and deficits in maternal care, particularly in the absence of obesity (Xu et al., 2018). Moreover, evidence from another study suggests that an obese phenotype (significant weight gain) rather than high fat diet exposure alone is required to adversely impact offspring neurodevelopment and behaviour (Rincel et al., 2016). A third study (Baptissart et al., 2018) using inbred mice showed that the dams of neonate mice exposed to a high fat diet in utero were less interactive than neonates exposed to a control diet. Using a cross-fostering design, this was demonstrated to be independent of the mothers' diet, instead suggesting a role of offspring physiological changes that cue maternal interaction with pups. Importantly, females were fed the high fat diet (45% of kilocalories from fat) for six weeks prior to mating. This is an interesting concept, suggesting that even at this early stage, the relationship between mother and offspring is instigated bidirectionally (John, 2019). Another recent study of MCB in rats (Abuaish et al., 2018) used a high fat diet (60% of kilocalories from fat) that started 3 weeks prior to mating. MCB was assessed between the first and sixth postnatal day for 3 hours during the day and 3 hours during the night (at spaced intervals throughout the day and night). In this study, high fat dams were significantly heavier than their control counterparts. While this is perhaps one of the more comprehensive studies of the effect of maternal high fat diet feeding on MCB, no differences between control and high fat diet groups were found. Clearly, the preclinical evidence for an effect of high fat diet feeding on maternal care is mixed and needs clarification. The length of high fat diet feeding may also affect MCB. It may be that maternal care deteriorates over longer levels of pre-pregnancy high fat diet feeding.

Preliminary evidence in human studies similarly indicate that diet influences maternal care behaviour, although confounding variables are more difficult to control. A systematic review found that a healthy diet prior to and during pregnancy reduces the risk of perinatal anxiety and depression (Silva et al., 2019). Postpartum depression is also exacerbated by

poor diet (Okubo et al., 2011). On the other hand, a recent study conducted in the UK showed no evidence for overall diet being linked to postpartum depressive symptomology in humans, after adjusting for many potential confounders (Northstone et al., 2018). Thus, the evidence for diet playing a significant modifying role on maternal perinatal psychopathology is certainly mixed.

In summary, preclinical studies have begun to shed light on some of the key mechanisms mediating the effect of maternal neglect, a form of early life stress, on offspring neurodevelopment with consequences for behaviour. This evidence lends itself to the idea of foetal programming, whereby exposure to significant amounts of neglect or other stressors during the early postnatal window directs brain development along a specific pathway best adapted to a stressful environment. In a benign environment, these behaviours are maladaptive and likely increase the risk of internalising and externalising behavioural disorders in later life. When investigating the effect of maternal diet-induced obesity on maternal behaviours, the evidence is limited and mixed. Because of this, and the established effect of maternal care on offspring brain and behaviour, observed variations in maternal care should be reported in all studies investigating the gestational and early postnatal period, at least to eliminate possible confounding effects on results.

1.3.3. The maternal microbiome

The effect of maternal obesity on offspring brain and behaviour may be mediated, in part, by changes to the maternal and offspring gut microbiome. Placental inflammation due to high fat diet consumption has been demonstrated in non-human primates (Frias et al., 2011). Obesity status and the consumption of a high fat diet prior to and during pregnancy have been shown to affect the composition of the maternal and neonatal gut microbiome in humans (Collado et al., 2008; Santacruz et al., 2010; Chu et al., 2016) and rodents (Paul et al., 2016). Moreover, maternal gut dysbiosis induced by diet (Buffington et al., 2016) or antimicrobial treatment (Tochitani et al., 2016) has been shown to affect the offspring microbiome, as well as offspring brain and behaviour.

While the absence of a placental microbiome has been confirmed in humans (de Goffau et al., 2019), preclinical studies in mice have nonetheless revealed a profound effect of the maternal microbiome on prenatal development through the transfer of microbial metabolites from the dam to the offspring. Microbial metabolites contribute to foetal nourishment, but are also crucial for the origins of immunity (Macpherson et al., 2017) and neurodevelopment (Jašarević et al., 2015; Vuong et al., 2020). Maternal chronic mild stress during the first week of gestation has been shown to affect the maternal vaginal microbiome composition through a decrease in *Lactobacillus spp.*, markedly reducing the abundance of this taxa in offspring with associated changes to the plasma metabolome affecting energy balance and mitochondrial function (Jašarević et al., 2015). Brain amino acid levels were also reduced in early-life offspring, which likely have strong implications for neural development and behaviour (McDonald and Johnston, 1990). Indeed, the specific absence of certain or all microbes during early life has been shown to affect behaviour in later life. For example, Sudo et al. (2004) showed that GF mice exhibit downregulated NMDA receptor subunit NR2A in response to acute restraint stress, which was reversed by microbial reconstitution of Bifidobacteria (but not *Escherichia coli*) neonatally. Stress-induced changes in brain gene expression were not reversible by gut microbial reconstitution in adulthood. This seminal study established the importance of the commensal microbiota in normal conditioning of the HPA axis. An altered gut microbiota due to poor maternal diet likely also has a pervasive effect on metabolites reaching the foetus, which may prime offspring for disease. Germ-free mice colonised with the gut microbiota of offspring born to obese dams have increased intestinal permeability (determined by increased plasma fluorescein isothiocyanate dextran levels), hepatic inflammation (increased hepatic *TNF* mRNA and hepatic macrophage count), and susceptibility to non-alcoholic fatty liver disease (NAFLD), determined by histological examination of liver sections for steatosis and infiltration of inflammatory cells (Soderborg et al., 2018). Clearly, maternal microbial changes that occur because of perinatal obesity affect the composition of vertically transmitted metabolites to offspring during foetal and neonatal development.

One of the first pieces of evidence linking maternal high fat diet and offspring brain and behaviour via changes in the gut microbiota comes from a study by Buffington et al. (2016). In this study, female mice were fed a HFD (60% of kilocalories from fat) for 8 weeks prior to mating. This is a more appropriate time period to achieve a mouse model of diet-induced obesity (Aye et al., 2015). At parturition, litter sizes were significantly reduced compared to non-obese mothers who were maintained on a regular diet (13.4% kcal fat). Even though offspring mice were maintained on a 'regular' diet until adulthood, substantial differences in the gut microbiome were observed, mirroring some of the changes observed in the F0 maternal generation. These changes were replicated when offspring gut microbes were transplanted into germ free mice. Alongside changes to the microbiome, the offspring of dams fed a HFD exhibited social interaction deficits, and, with this, reduced oxytocin levels and potentiation of synaptic inputs to dopamine neurons in the ventral tegmental area (VTA) after the social interaction protocol. *Lactobacillus reuteri* was found to be the most reduced species in maternal HFD offspring compared to controls. Reconstitution of this species in maternal HFD offspring (4-weeks supplementation in drinking water post-weaning) reversed social and synaptic deficits, which was not the case for other depleted taxa. Heat-killed *L. reuteri* was unable to rescue social behaviour and had no effects on social interaction-induced long-term potentiation in the VTA of maternal HFD offspring. While this study provided some of the first compelling evidence for the role of specific microbes in neuropsychiatric disorders influenced by maternal nutrition, it contradicts other evidence that functional metabolite production likely plays more of a role in mediating changes to the gut-brain axis, rather than specific taxa (Cheung et al., 2019). With this in mind, microbial metabolite production was not reported as a result of maternal high fat diet, and functional metabolite changes as a direct result of *L. reuteri* administration were not described, despite this species having been demonstrated as effective in promoting GABA and histamine production *in vitro* (Mu et al., 2018). Maternal chronic variable stress exposure during the first week of gestation in mice has also been shown to reduce *Lactobacillus spp.* in the vaginal microbiome (early postpartum), and offspring gut (Jašarević et al., 2015). Offspring exposed to early prenatal stress also had

altered colon and plasma metabolite profiles, associated with energy and microbial metabolism. Finally, prenatal stress altered brain amino acid levels in offspring (postnatal day 2) in the hypothalamus and hippocampus. The effect of peripheral metabolic changes on the brain is particularly important during development as, relative to mature brain structures, certain brain regions are much more readily accessible to circulating factors (Jašarević et al., 2015). Offspring behavioural outcomes were not explored in this experiment. It is currently unknown whether maternal obesity significantly alters the normal composition of the vaginal microbiome, and this could be a mechanism by which maternal obesity leads to vertically transmitted changes in the offspring gut microbiome, with implications for behaviour as indicated by Buffington et al. (2016).

The effect of diet-induced microbial changes, but not the high-fat diet itself, on offspring behaviour has also been explored (Bruce-Keller et al., 2017). This allowed the direct effect of the microbiota to be assessed, rather than the whole array of changes induced by a maternal high fat diet, on offspring behaviour. Microbiota from male mice fed with a high-fat (60% kcal fat) diet for 3-months were transplanted into microbiota-depleted adult females. Obese-type microbiota did not induce weight gain or affect maternal care in dams, though female offspring were significantly heavier than control offspring at weaning and adulthood. In adult offspring, offspring males from dams with obese-type microbiota showed increased anxiety relative to controls, though in females no changes were found. All offspring showed microbiome changes that differed in accordance with maternal treatment. This study provided further evidence that maternal microbiota may program aspects of offspring behaviour.

The intrinsic ability of microbes associated with obesity to alter brain function and behaviour was also investigated by Bruce-Keller et al. (2015). Gut microbes from adult mice were depleted during a 2-week antibiotic protocol and recolonised with donor microbes from the caecal content of genetically identical mice fed a HFD (60% of kilocalories from fat) for 8 weeks. Compared to mice that received donor microbes from mice fed a control diet, microbes derived from a high fat diet increased anxiety-like behaviours in the OFT, EPM, and marble

burying test, in the absence of any weight gain in recipient mice. Increased plasma endotoxin and TLR4 expression and brain macrophage marker IBA1 and TLR4 expression were indicative of increased peripheral and central inflammation, respectively. Given that microbiota may partially mediate neuropsychiatric consequences of obesity, it can reciprocally be suggested that modulation of the microbiome in obese individuals, or individuals exposed to obesity with an altered microbiota composition, may be able to attenuate brain and behavioural deficits. The efficacy of such treatment may give rise to narrow therapeutic windows that correspond to important periods of neurodevelopment. For example, Buffington et al. (2016) found that microbes donated from conventionally housed mice reversed social deficits in germ free mice at 4 weeks old, but not at 8 weeks.

Diet-induced obesity also alters the innate immune response through changes in gut microbiota composition. While it is well known that commensal microbes interact with local immune cell populations in secondary lymphoid organs such as the gut (Round and Mazmanian, 2009), the mechanism by which microbiota influence haematopoiesis at primary lymphoid organs, for example the bone marrow, is less clear. Lee et al. (2019) demonstrated that a defined population of bone marrow resident macrophages are able to detect circulating cell-free bacterial DNA. The detection of this DNA drives the production of cytokines such as TNF and IL-1B which regulate the production of bone marrow haematopoietic stem cells (HSCs). While the complete absence of microbiota appears detrimental to primary immune cell development, modification to the microbiome by diet may also impact innate immune cell development. Luo et al. (2015) showed that a HFD altered the HSC niche in mice, with increased common myeloid as opposed to common lymphoid progenitors. Increased myeloid progenitors may underly more potent inflammatory responses to infection in obese individuals (Manicone et al., 2016) and increased peripheral and central *TLR4* expression (Strekalova et al., 2015). This effect was found to be mediated, at least in part, by changes to the gut microbiota, as depletion of gut microbes by oral antibiotics reversed changes to the HSC niche. Additionally, FMT from high fat diet mice to mice on a regular diet induced the same

effect as the direct ingestion of the high fat diet. By extension, high fat diet-induced changes to the microbiome could mediate depressive symptomology through immune dysfunction beginning in the HSC niche. Because innate immune memory appears to be transferable through the microbiota (Burgess et al., 2014), and the neonatal microbiota is significantly shaped by the mother (García-Mantrana et al., 2020), an adverse combination of microbes and immunomodulatory compounds during early life may impact the brain and periphery of developing offspring.

There remains a scarcity of studies that have investigated how obesity-induced changes to the maternal microbiome impact offspring neurodevelopment and behaviour. Most studies have investigated the link between obesity, the gut microbiota, and mood disorders in a single generation (Schachter et al., 2018). Some evidence points to the deficiency of *Bifidobacteria spp.* or *Lactobacillus spp.* in obese subjects mediating reduced beneficial metabolites (Schneeberger et al., 2015), though increased basal inflammation through increased circulating microbial endotoxin likely also plays a role (Gohir et al., 2019). There is a need to further investigate how manipulating the microbiota during the gestational and lactational period can protect against the physiological and neurological consequences of a maternal high fat diet on offspring. This will be explored in Chapters 3, 4, and 5 of this thesis.

1.3.4. Maternal inflammation

Perhaps the most striking example of the role of maternal inflammation programming offspring brain and behaviour comes from maternal infection during pregnancy and the increased risk of neuropsychiatric disorders in offspring. This has been investigated in preclinical models of maternal immune activation (MIA) and human epidemiological data. In humans, converging evidence from maternal autoimmune disorders, acute stress, and infection during pregnancy point to elevated maternal immune mediators in enhancing the risk of offspring psychopathology development. Moreover, viral infection during pregnancy can significantly alter normal neuronal development, as in the case of Zika virus infection (Mlakar et al., 2016). In rodents, congenital Zika virus infection impacted learning and memory in

adulthood, which presented with a reduced number of mature hippocampal neurons (Stanelle-Bertram et al., 2018). More generally, rodent MIA models demonstrate behaviours resembling autism spectrum disorders and schizophrenia, as well as anxiety-like behaviours (Estes and McAllister, 2016). The basal level of several neurotransmitters in different regions of the rodent brain have also been shown to be influenced by MIA and are thought to underly persistent behavioural changes (Winter et al., 2009). More recently, MIA has been associated with offspring depressive-like behaviour in preclinical models (Ronovsky et al., 2016) and in humans (Simanek and Meier, 2015). Mechanistic studies in rodents have attributed depressive-like behaviour to reduced neuron density and long-term potentiation (LTP) deficits in the hippocampus, reduced BDNF, and reduced serotonin in several brain regions including the PFC (Wang et al., 2009; Babri et al., 2014; Khan et al., 2014; Lin and Wang, 2014; Depino, 2015). Despite the apparent evidence of prenatal pro-inflammatory cytokines in programming offspring behaviour, it is important to note that maternal inflammation and foetal or neonatal exposure to maternal inflammatory mediators does not necessarily equate to a similar inflammatory response in the offspring (Estes and McAllister, 2016). It is largely unknown how elevated maternal cytokines alter brain development, though it is possible that they more directly affect synaptic plasticity and pruning, and neuronal function in offspring (Estes and McAllister, 2015). Given the broad range of behavioural outcomes in offspring associated with maternal immune activation, it seems unlikely that maternal inflammation is correlated with a specific disease. Rather, MIA may contribute to a wider spectrum of psychiatric illnesses, the penetrance of which may depend on other genetic and environmental factors. In humans, therefore, maternal infection and inflammation may act as a disease primer, increasing the risk of psychopathology manifesting later in life.

Obesity is an inflammatory condition. While different to an acute inflammatory episode, obesity is known to be associated with prolonged, chronic inflammation, and an increase in circulating pro-inflammatory cytokines including IL-6 (Das, 2001). Pregnancy is normally associated with immunological changes including enhanced pro-inflammatory responses to

LPS and viral challenges (Sureshchandra et al., 2019). Obesity during gestation exacerbates inflammatory changes associated with pregnancy which has implications for foetal development (Pendeloski et al., 2017). Maternal obesity during pregnancy in humans is associated with significant alterations to the placenta, including increased resident CD14+ and CD68+ macrophages, and an increase in the placental expression of several pro-inflammatory cytokines including IL-1 β , TNF, and IL-6 (Challier et al., 2008; Saben et al., 2013). Significant endocrine changes in the placenta also occur in response to maternal inflammation, mediated by IL-6 (Hsiao and Patterson, 2011). Increased maternal plasma concentrations of IL-6 were also observed in obese pregnant women compared to lean pregnant women (Stewart et al., 2007; Challier et al., 2008). Increased levels of endothelial intercellular adhesion molecule 1 and reduced levels of the anti-inflammatory cytokine IL-10 have also been observed early in pregnancy in obese pregnant women compared to lean pregnant women (Stewart et al., 2007). Increased serum IL-6 has been replicated in rodent models of maternal obesity, with a concomitant reduction in placental vascularisation (Kretschmer et al., 2020). Additionally, it has been shown that maternal IL-6 may transfer across the placenta and into foetal circulation (Zaretsky et al., 2004). Gestational weight gain, as opposed to pre-pregnancy obesity, may also be important. One study identified that every kilogram of gestational weight gain was associated with a 3% increase in serum C-reactive protein (CRP) and serum amyloid A (SAA) levels independent of pre-pregnancy BMI (Hrolfsdottir et al., 2016), though other inflammatory markers were unaffected. These studies clearly indicate that obesity during gestation may impact foetal development via excessive pro-inflammatory immune mediators.

The link between brain, behaviour, and immunity is evident in sickness behaviour. This is a robust evolutionary response, which arises upon the administration of a peripheral inflammatory stimulus such as endotoxin in both rodents (Bay-Richter et al., 2011) and humans (Lasselin et al., 2016). Early life infection may also result in persistent changes to brain and behaviour. For example, a longitudinal study in humans found an association between early life (<14 years of age) viral CNS infection and risk of schizophrenia

development in adulthood (Dalman et al., 2008). Other studies have discovered a similar association after a bacterial infection (Abraham et al., 2005; Blomström et al., 2014). Interestingly, the link between depression and immunity may be bidirectional, as depression at baseline has been shown to be prospectively associated with an increased risk of hospitalisation due to infection in the UK Biobank (Ronaldson et al., 2022). Strong epidemiological evidence has found that the risk of developing any common mental disorder was increased with childhood infection that required hospitalisation or other treatment (Köhler-Forsberg et al., 2019). While the trigger in this case may be caused by an infectious agent, peripheral inflammation is known to lead to *de novo* synthesis of cytokines in the brain (Yates et al., 2022). Therefore, adaptive CNS changes may occur via immune-mediated inflammatory pathways within the CNS. Chronic, stress-induced inflammation is thought to increase the risk of depression and other mood disorders via similar inflammatory mechanisms (Dantzer et al., 2008). Psychological stress, a key contributor to depression and other psychiatric conditions (Hammen, 2005), alters the neuroimmune system, in particular the induction of a pro-inflammatory state with cytokine signalling initiated by activated microglia (Anisman and Merali, 2003; Kiecolt-Glaser et al., 2003; Wohleb and Delpéch, 2017; Munshi et al., 2020). In humans, inflammatory biomarkers are associated with depression and depressive symptomology (Kowalska et al., 2020; Roman and Irwin, 2020), and attenuating this immune activation may be a prerequisite for antidepressant treatment efficacy (Liu et al., 2020a). Rodent models of early-life stress have connected the development of depressive-like behaviours with increased inflammatory tone that persists into adulthood (Hohmann et al., 2017). In this way, mood and depression may have an inflammatory origin that is conditioned early in development.

Microglia and astrocytes have been shown to play an important role in many aspects of neurodevelopment. For example, they participate in the genesis and pruning of synapses, regulation of apoptosis, and vascularisation of the CNS (Bolton and Bilbo, 2014; Cowan and Petri, 2018). The intimate relationship between microglia and neurons begins early in

development, whereby microglia regulate and refine neural circuits, and relay peripheral information to the brain, including signals from the microbiota (Schafer and Stevens, 2015; Li and Barres, 2018; Thion et al., 2018a, 2018b). Cytokines, produced by glia in the CNS, are thought to promote forebrain development in utero (Mousa et al., 1999), and based on preclinical and *in vitro* models, appear to have broad, important roles as growth factors and in supporting gliogenesis in the CNS (Mousa and Bakhiet, 2013). Of particular importance is IL-6, which both plays a key role in foetal brain development, but also in the association between maternal systemic inflammation and risk of psychiatric disorders in offspring (Bauer et al., 2007). Prenatal exposure to IL-6 was shown to cause deficits in spatial learning in adult offspring, with accelerated hippocampal neurodegeneration and increased GFAP and NMDA NR1 subunit mRNA expression (Samuelsson et al., 2006). Additionally, maternal serum IL-6 levels during pregnancy correlated with functional and structural changes to the amygdala as well as behavioural changes in 2-year-old human offspring (Graham et al., 2018). Clearly, the biology of IL-6 and other cytokines during early neurodevelopment is complex, contributing to normal development and pathogenesis in a dose-dependent manner, via both direct and indirect mechanisms.

As in systemic infection, maternal high fat diet and obesity can impact the developing brain via inflammatory mechanisms. Increased circulating IL-6, and other pro-inflammatory mediators, is associated with obesity during pregnancy in rodents (Sanders et al., 2014) and humans (Stewart et al., 2007; Challier et al., 2008; Curry et al., 2008; Catalano et al., 2009; Basu et al., 2011; Roberts et al., 2011; Friis et al., 2013; Christian and Porter, 2014; Sen et al., 2014; Holtan et al., 2015) though the extent of elevation compared to lean women likely fluctuates over the course of pregnancy and postpartum (Friis et al., 2013; Christian and Porter, 2014; Pendeloski et al., 2017). This obesity-induced activation of the maternal peripheral immune system has been shown in rodents to affect the offspring brain and behaviour in a manner resembling the consequences of maternal or early-life infection. One of the first demonstrations of this was by Bilbo and Tsang (2010). In offspring exposed to

maternal high-fat diet-induced obesity, increased hippocampal IL-1 β protein, but not gene expression, and microglial activation were found in adulthood compared to those born to dams fed a low-fat diet. Offspring of dams fed the HFD also showed increased anxiety-like behaviour in the EPM. The study by suggests that offspring microglia may be primed or sensitised to inflammatory responses in utero or early postnatal life due to maternal obesity, which persists into adulthood. Similar findings have been reported at weaning age, P21 (Cohen et al., 2016). Pro-inflammatory microglia that secrete more cytokines during this developmental period may contribute to the neuropathology of affective disorder associated with persistent central inflammation (Ratnayake et al., 2013).

Microglial activation has been discovered post-mortem in depressed victims of suicide. Increased *CCL2* expression was identified in the anterior cingulate cortex relative to non-psychiatric controls. An increase in the number of perivascular macrophages was also identified, suggesting further recruitment of circulating monocytes into the anterior cingulate cortex (Torres-Platas et al., 2014). Conversely, antidepressant treatment has been shown to limit microglial activation associated with depressive-like behaviours in mice (Kalkman and Feuerbach, 2016). Indeed, research on the relationship between microglial activation and mood disorders remains almost exclusively preclinical (Catale et al., 2020). Many animal studies have provided direct evidence for microglial activation in the foetal and adult brain after elevated maternal systemic inflammation, while other studies report synaptic and neurotransmitter deficits in the absence of microglial activation (Al-Haddad et al., 2019). Microglia therefore may play a role in the effect of maternal high fat diet on brain development and behaviour. Creating an inflammatory environment at such early periods during development, with or without direct changes to foetal microglia, appears to have significant, enduring consequences to brain and behaviour.

1.3.5. Breast milk composition

While evidence for the importance of human breast milk on developing offspring metabolism, immunity, and commensal gut microbe colonisation is considerable (Mulligan and

Friedman, 2017), the connection to psychopathology is only relatively recently being made (Di Benedetto et al., 2020). Breast milk is a source of live bacteria for the infant, possibly originating from the maternal gut (Rodríguez, 2014). As a result, changes to the maternal gut microbiome have direct consequences for the seeding of microbes in offspring through breast milk, and through this, may affect offspring neurodevelopment (Borre et al., 2014). In addition to essential macronutrients and micronutrients, Breast milk also contains prebiotic human milk oligosaccharides (HMO) which not only help proliferate but also shape optimal gut bacteria populations in offspring (De Leoz et al., 2015). This points to the overall importance of providing human breast milk as opposed to formula milk for the long-term metabolic health of developing offspring (Binns et al., 2016). Conversely, breast milk may also program adverse outcomes in offspring if the maternal diet is lacking certain micronutrients, or more likely in the United Kingdom, inducing maternal obesity. Maternal BMI influences overall breast milk composition (Moossavi et al., 2019). Mothers with a BMI > 25kg/m² had higher levels of insulin in the breast milk, and despite an overall reduction in fatty acid content, showed an increase in pro-inflammatory fatty acids like linoleic acid, an omega-6 fatty acid, and palmitic acid (Ellsworth et al., 2020). There was also a reduction in oleic acid. The microbiota composition of breast milk from obese mothers is less diverse (Cabrera-Rubio et al., 2012) and contains lower levels of *Bifidobacterium* than lean mothers (Collado et al., 2012). This is significant as *Bifidobacterium longum* subsp. *infantis* present in human milk is predicted to metabolise many HMOs present in human milk (Sela et al., 2008), from which SCFAs like acetate and butyrate are produced. Moreover, an elevated BMI has been suggested to modify the immunological composition of breast milk, with high levels of IL-6 (Collado et al., 2012), leptin (Andreas et al., 2014; Masuyama and Hiramatsu, 2014), and other pro-inflammatory mediators (Erlana and Fly, 2019) reported.

Clinical studies investigating the role of breastfeeding in the vertical transmission of psychopathology to the offspring face significant socioeconomic confounders. Initial studies on breastfeeding and its relationship with neurodevelopment focused on cognitive outcomes,

whereby breastfeeding was generally associated with predicting greater academic achievements compared to formula feeding (Bar et al., 2016; Horta et al., 2018). However, in these clinical studies, it is not possible to randomise mothers and their newborns to formula and breastfeeding groups. Additionally, because both obesity (Bann et al., 2017) and perinatal mental illness (English et al., 2018; Abel et al., 2019) are associated with socioeconomic inequality in the United Kingdom and globally, observational studies in humans have significant limitations (Kramer et al., 2011). When trying to observe the effect of maternal obesity and depression, independently or together against a control cohort on breast milk composition and the effect on childhood psychopathology development, obese/depressed mothers are more likely to switch to formula milk sooner (Taveras et al., 2003), have lower income, difficult social environments, reduced social support, and the young offspring are more likely to receive lower education standards. These factors independently increase the risk of adverse childhood neurodevelopment (Garratt et al., 2017).

Studies in mice have provided direct evidence for maternal obesity during lactation having the ability to program metabolic inflammation (Hafner et al., 2019) and precocious pubertal development (Wang et al., 2020) in the offspring. The latter finding was shown to be reversible with maternal microbial reconstitution during lactation, pointing to the role of altered vertical transmission of microbes and associated microbial metabolites during lactation as a mediator of adverse metabolic programming in offspring (Wang et al., 2020). Other bioactive molecules contained in breast milk, such as miRNAs, are differentially abundant in the milk of lean and obese dams (Chen et al., 2017). These miRNAs, contained in extracellular vesicles, have been shown to survive a simulation of gastric digestion *in vitro*, though their functions in milk remain largely unknown (Liao et al., 2017). Cross-fostering studies in rodents (Desai et al., 2014; Masuyama and Hiramatsu, 2014; Chen et al., 2017) have been important in confirming the independent and additive effects of lactational exposure to maternal obesity and high-fat diet.

Concerning the altered composition of breast milk in humans, few studies have related this to maternal psychopathology. Based on a recent review (Di Benedetto et al., 2020) of maternal psychopathology and altered breast milk composition, perinatal depression does not appear to significantly effect micronutrient content, though omega-3 polyunsaturated fatty acids (PUFAs) may be reduced in depression. Docosahexaenoic acid (DHA) is an omega-3 PUFA crucial for neurodevelopment (Innis, 2008; Colombo et al., 2011). Perinatally anxious and depressed mothers may also have increased secreted IgA in breast milk, which has been suggested to be a protective mechanism against other depressive-associated alterations in the mother that might otherwise be transmitted in the milk (Di Benedetto et al., 2020). Overall, there is a narrow scope of literature with a heterogeneous mix of human observational studies addressing how maternal breast milk components may contribute to offspring brain and behavioural development. Given that early-life offspring are dependent on breast milk for nutritional nourishment, immune mediators, while also being a critical source of commensal microbes, there is scant research on how different maternal nutritional and psychological states affect breast milk composition. Furthermore, there is preliminary evidence to suggest that maternal psychopathologies and obesity independently alter breast milk composition to the detriment of the offspring – likely by microbial and immune mechanisms. There remains a need for preclinical studies to investigate in more detail how depressive and anxious behaviours, in the presence of obesity, influence the bioactive content of milk throughout the nursing period. The metabolite composition of maternal milk is a subject of this thesis in Chapter 3. The metagenomic and transcriptomic content of milk in relation to maternal diet and behaviour are also lacking in current research.

1.3.6. Neurotransmitter Systems

The developing neurotransmitter systems of offspring exposed to maternal obesity have been suggested to be influenced by immune and microbial factors, which have been addressed in the previous sections. Moreover, the opportunity to ameliorate substantial changes to neurotransmission may exist only in infancy. Alterations to serotonin availability

during foetal development alters the normal formation of brain circuitry, resulting in enduring changes to adult anxiety-like behaviours (Ansorge et al., 2004; Bonnin et al., 2007). An early study investigating the role of commensal gut flora on serotonergic neurodevelopment found that recolonisation post-weaning was unable to normalise the increased levels of serotonin and its primary metabolite 5-HIAA in germ free mice (Clarke et al., 2013). While the complete lack of microbes in early life has been suggested to increase serotonin levels, maternal high-fat diet consumption may conversely suppress the offspring serotonergic system.

Maternal systemic inflammation during pregnancy may contribute to initial serotonin deficits in offspring. This has been suggested to occur by promoting placental kynurenine metabolism from tryptophan through upregulation of indoleamine-2,3-dioxygenase (*IDO1*) expression, a rate-limiting enzyme in the kynurenine metabolic pathway. This depletes foetal tryptophan availability in the foetus and limits central serotonin availability and production. Preclinically, this has been shown to occur in the murine placenta following prenatal infection and immune activation (Pfaff et al., 2008). Neonatal rabbits exposed to endotoxin in utero also show reduced tryptophan metabolism to serotonin in the cortex and paraventricular region as measured by α [^{11}C]methyl-L-tryptophan using positron emission tomography in vivo imaging (Kannan et al., 2011). Similarly, cortical and hippocampal serotonin levels as measured by enzyme-linked immunosorbent assay (ELISA) were reduced in the newborn rabbit brain exposed to prenatal endotoxin, compared to PBS control. Toll-like receptor 4 (TLR4) is critically involved in inflammatory responses to endotoxin (Beutler, 2000) and a gatekeeper for downstream induction of pro-inflammatory pathways, but during gestation can also respond to sterile pro-inflammatory signalling (Koga et al., 2014; Robertson et al., 2020). TLR4 activation initiates a host of cytokine responses and pro-inflammatory effector molecules via the nuclear translocation of nuclear factor kappa B (NFkB). Upregulation of maternal TLR4 is a risk for pre-term birth and foetal inflammatory response syndrome, characterised by systemic activation of the foetal immune system (Robertson et al., 2020). In humans, TLR4 expression was shown to be upregulated 9-10-fold in the placentas of obese women

compared to lean women. The expression of pro-inflammatory cytokines was also significantly greater (Yang et al., 2016). This may have implications for excessive pro-inflammatory signalling in the foetus, though more studies assessing the relationship between increased maternal/placental TLR4 expression and foetal inflammation, with outcomes assessing neurotransmission and behaviour, are required.

The placenta is an essential exogenous source of serotonin for the developing foetus. This was first shown by Bonnin et al. (2011), whereby maternal tryptophan is metabolised to serotonin in the placenta (shown in both mice and humans) before entering foetal circulation and allowing for normal forebrain development. Placental-derived serotonin is crucial for developing forebrain neurocircuitry, and its disruption by inflammation could be a mechanism by which prenatal maternal inflammation increases the risk of subsequent mental illness. Maternal immune activation in mice has been shown to alter placental serotonin production. Surprisingly, placental serotonin production from tryptophan increased with maternal inflammation (Goeden et al., 2016). Despite no increase in the expression of pro-inflammatory cytokines in the placenta or the foetal brain, placental *IDO1* expression was shown to increase 4-fold 24-hours after maternal immune activation with poly(I:C), and placental *tryptophan hydroxylase 1 (TPH1)* increased 2.5-fold. After another 24-hours, *TPH1* expression was significantly reduced compared to saline controls. Remarkably, this window of increased *TPH1* expression rapidly increased placental serotonin output, as well as foetal serotonin specifically in the forebrain (at gestational day 14). Kynurenine were also increased in the forebrain. This increase in foetal forebrain serotonin levels blunted serotonergic axon outgrowth (Goeden et al., 2016). While no behavioural experiments were conducted, the authors suggested that this phenomenon is maladaptive and may mediate, in part, the effect of maternal inflammation on increasing the risk of offspring anxiety and depressive-like behaviours in the offspring.

Preclinical studies have also investigated the effect of maternal diet-induced obesity on serotonergic perturbations in offspring, both at the juvenile age and in adulthood. Sullivan et al. (2010) showed that maternal high-fat diet consumption increased foetal tryptophan

hydroxylase 2 (*TPH2*) expression in the rostral dorsal raphe region of NHP macaques, as well as upregulation of the inhibitory 5-HT_{1A} receptor. *TPH2* is expressed in serotonergic neurons and is critical to the central production of serotonin. An increase in foetal serotonin here may have also caused the subsequent reduced serotonin levels in the cerebrospinal fluid (CSF) at juvenile age (4-months) reported in the study by Sullivan et al. (2010), with concomitant increased anxiety-like behaviour in female offspring. This study demonstrates that similar perturbations to serotonergic neurodevelopment may occur as a result of maternal metabolic inflammation, akin to maternal immune activation. A more recent study (Thompson et al., 2017) extended these findings into adult NHPs (11-months), with reduced serotonin levels, altered *TPH2* expression, and increased anxiety-like behaviours persisting despite being weaned onto a regular diet. These changes were exacerbated if the NHPs exposed to a high-fat maternal diet were also weaned onto the same diet. Overall, preclinical studies suggest that impaired development of the offspring serotonin system characterised by altered serotonergic activity may increase the risk of offspring behavioural problems with enduring effects.

Based on the postulated action of the antidepressant selective serotonin reuptake inhibitor (SSRI) agents, it is widely regarded that serotonergic neurotransmission plays a role in depression and anxiety in humans. In cohorts vulnerable to depression, reduced dietary tryptophan and subsequent serotonin deficits can induce temporary depressive symptoms (Smith et al., 1997). In terms of *TPH2* functionality in humans, polymorphisms in *TPH2* have also been associated with depression and anxiety (Del Colle et al., 2020). Perturbations to serotonin neurotransmission are associated with anxiety and depressive disorders in humans (Gross and Hen, 2004). Reduced serotonin neurotransmission may underly depressive behaviour, with an increase in neurotransmission thought to be a requirement for successful treatment with the selective serotonin reuptake inhibitor (SSRI) citalopram (Chaput et al., 1986). This may depend on reduced sensitivity over time to the inhibitory 5-HT_{1A} receptor, and hippocampal neurogenesis (Gross and Hen, 2004). Perhaps in some individuals in which

SSRIs are effective, this is correcting for serotonergic deficits that began in neurodevelopment. While maternal obesity has been associated with precocious puberty in females (Kubo et al., 2016), there is a lack of understanding as to how this also translates to altered brain function. Preclinical studies should further investigate the role of maternal pregravid obesity on brain development, and how this may translate to adversely affect the development of normal emotional behaviours in humans.

1.3.7. Overview of maternal influences on the offspring and looking beyond the first filial generation

In this section, factors associated with maternal pregravid obesity are described to coalesce and alter the normal course of neurodevelopment in offspring. TLR4 activation links maternal obesity with increased immune activation via both altered microbiota composition (endotoxin mediated inflammation), adipocyte proliferation (hypoxia and HIF-1 mediated inflammation), and by diet, whereby saturated fatty acids can also activate TLR4 (Korbecki and Bajdak-Rusinek, 2019; Tilg et al., 2020; Wu and Ballantyne). A state of chronic low-grade inflammation, exacerbated by normal immunological changes that occur during gestation, is likely to have an impact on the development of normal brain circuitry – in particular through alterations in the availability of serotonin during key neurodevelopmental windows. Sex differences may also occur, with females perhaps being more vulnerable to the effects of maternal obesity on developing depressive behaviour.

Given that depression and stress is also linked to excessive GWG (Paul et al., 2013), the effect of maternal obesity on offspring neurobiology and behaviour, coupled with adverse metabolic outcomes, may contribute to a perpetual cycle of poor maternal health across generations. The impact of maternal obesity of generation across subsequent generations has been explored in a recent study (Winther et al., 2019). F0 consumption of a diet consisting of 60% of kilocalories from fat for 14-weeks primed anxiety-like behaviours as assessed in the light-dark box (LDB) and elevated plus maze in female F2 (granddaughter) offspring. This was suggested to be due to heritable changes in HPA-axis functionality as assessed by gene

expression analysis, specifically the mineralocorticoid receptor (MR) in the hippocampus. Metabolic changes were also associated with F0 diet (Winther et al., 2019). This study emphasises the importance of understanding sexual dimorphism in intergenerational behavioural studies and highlights the need for pharmacological and/or lifestyle interventions to aid in reducing the intergenerational matrilineal transmission of vulnerability to mental illness.

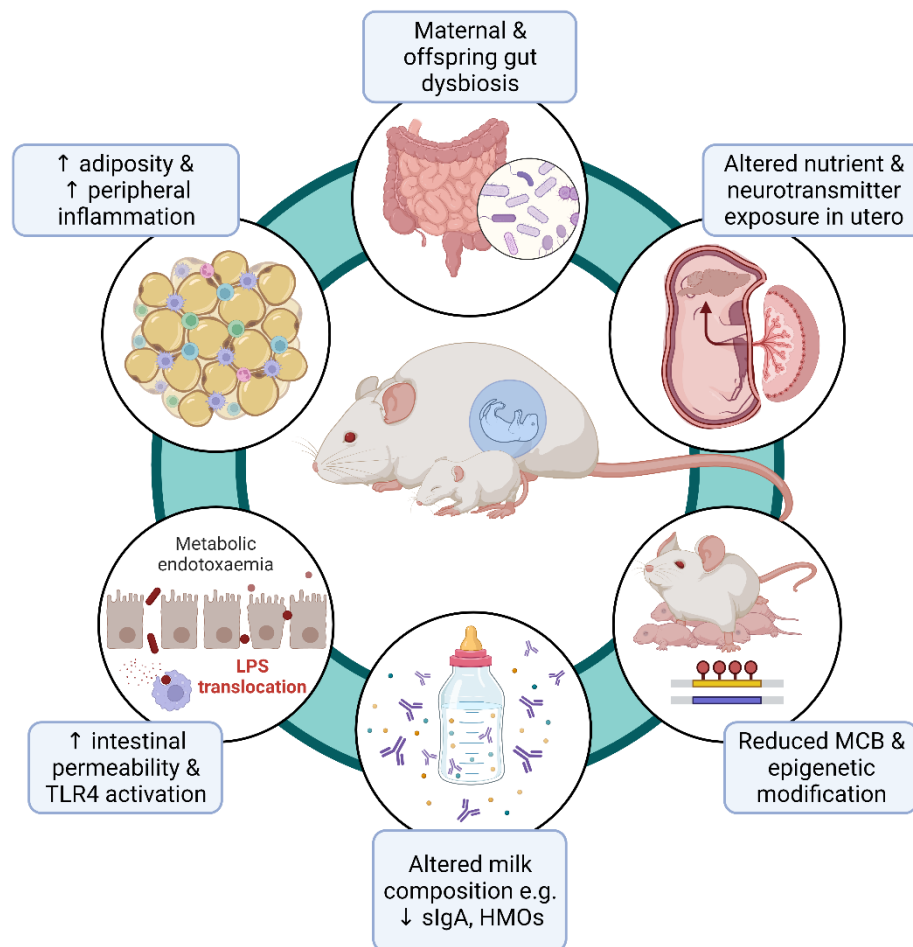


Figure 1.2 Summary of the putative mechanisms by which maternal obesity is thought to influence the course of offspring neurodevelopment.

1.3.8. Novel antidepressant therapies are required

Emerging data suggests that the average BMI increased during the UK 2020 lockdown period, amidst psychological stressors of isolation and possible financial strain. It is also pertinent to note that the association between obesity and psychopathology is greater in

women than men (Desai et al., 2009). Set against a background of broader economic and climate issues facing the world, which harbor psychological stress for current and future generations, there is a great need for cost-effective and safe methods of treating maternal obesity and comorbid psychological symptoms, with the parallel aim of mitigating the risk of mental illness in offspring. Conventional antidepressants, while very effective in some people, haven't reduced the prevalence of mood disorders in the last few decades, and this is despite a massive increase in availability and prescription of these medicines at the level of primary care, particularly in the UK (Jorm et al., 2017). NHS data has shown that the prescription of antidepressants is doubling every 10 years. In 1998 there were about 18 million prescriptions, 36 million in 2008 (Ilyas and Moncrieff, 2012), and the latest data from 2018 showed 71 million prescriptions being given out (Iacobucci, 2019). This increase can be partly attributed due to longer treatment regimens and an improvement in awareness, identification, and treatment of mental illness in the community over the last few decades. Over a similar period (1993-present), however, the prevalence of common mental disorders has remained comparatively stable (Baker, 2021), with between 15 and 20% of adults (and more women than men) experiencing a common mental disorder, for the most part anxiety or depression, in the last week at the time of polling. Because current antidepressant and anxiolytic agents are not necessarily reducing the prevalence of mental illness in society, and their use during pregnancy remains controversial (Mesches et al., 2020), adjunct therapies should continue to be investigated. Probiotics and prebiotics represent novel, safe (Allen et al., 2010) ways to mitigate risk of altered neurodevelopment and mental illness in offspring exposed to maternal diet-induced obesity. While they are unlikely to reverse an obese phenotype during pregnancy, they may promote resilience in the offspring by encouraging the production of molecules that support synaptic plasticity during key neurodevelopmental windows.

1.4. Modulation of the microbiota-gut-brain axis by prebiotics and probiotics

1.4.1. Prebiotics and probiotics modulate brain function and behaviour

Probiotics are defined as bacteria that elicit beneficial effects on host physiology when ingested (Sarkar et al., 2016). Prebiotics are substrates (typically fibre, carbohydrates, or saccharides indigestible by the host) that also confer a positive health effect when metabolised by commensal microbes, but do not include the microbes themselves (Gibson et al., 2017). Prebiotics and probiotics have become a mainstay of microbiota-gut-brain axis research, both as a tool to manipulate the host microbiota and as a potential therapeutic for mood disorder (Sarkar et al., 2016).

1.4.1.1. Prebiotics modulate brain function and behaviour in animal models

In rodents, galacto-oligosaccharide (GOS) prebiotics have been shown to elevate brain BDNF in the hippocampus. BDNF expression is thought to be important for ongoing neurogenesis in the adult hippocampus (Savignac et al., 2013), and its levels in human blood have been shown to be negatively correlated with depression severity (Qiu et al., 2022). BDNF protein was also shown to be elevated in the hippocampus of rats after five-week supplementation with the human milk oligosaccharide (HMO) 2'-fucosyllactose (Vázquez et al., 2015). In this case, associative learning behaviour was also assessed, and rats supplemented with the HMO tended to acquire the operant conditioning task in fewer sessions than the control group (Vázquez et al., 2015). Prebiotic supplementation has also been shown to modulate anxiety and depressive-like behaviour in rodents. Mice fed GOS for three weeks were shown to be less anxious than controls (drinking water only) in the LDB 24-hours after LPS administration (Savignac et al., 2016). The anxiolytic effect of the prebiotic was associated with an increase in gut *Bifidobacterium spp.* and an attenuation of IL-1B and 5-HT_{2A} receptor protein in the frontal cortex after LPS administration (Savignac et al., 2016).

Prebiotics have also been shown to mitigate the adverse behavioural effects of both early-life stress by maternal separation (O'Mahony et al., 2020) and chronic mild stress (Burokas et al., 2017) in rodents. This included reduced anxiety and depressive-like behaviour (Burokas et al., 2017) and an increased rate of spatial learning in the Morris water maze which was independent of early-life stress (O'Mahony et al., 2020). Lastly, postnatal supplementation of rats with GOS has been shown to reduce anxiety-like behaviour in the EPM at juvenile (3-week-old), adolescent (8-week-old) and adult (4-6-month-old) timepoints (Spitzer et al., 2021). Interestingly, this effect was only observed if rats were supplemented prior to weaning, and not when supplemented in adulthood, which suggests that the anxiolytic effects of GOS supplementation in unchallenged animals may depend on the modulation of brain function during a specific neurodevelopmental window. No overall effect of postnatal prebiotic supplementation was observed on the gut microbiome in any age group, though GOS supplementation was found to modify the relationship between branched-chain amino acid levels and age in the hippocampus (Spitzer et al., 2021). Overall, there is substantial preclinical evidence to support a neuromodulatory effect of prebiotics at both juvenile and adult timepoints, despite conflicting evidence in some cases. Mechanisms largely remain unclear due to inconsistent effects of prebiotics on the microbiome – some studies have reported a bifidogenic effect whereas others report no clear effects. A proportion of this variation may be due to inconsistencies in the age at which animals were treated, the length of treatment, the type of prebiotic, and the amount of prebiotic administered. Comparing the effects of prebiotics in GF and SPF animals would allow confirmation of prebiotics action by modification of the microbiome *in vivo*. This would be important, as GOSs have been shown to have microbiota-independent anti-inflammatory effects, possibly via TLR4-mediated IL-10 production (Lehmann et al., 2015). While the effects of prebiotics have not been assessed in GF animals, prebiotics have been shown to be effective in gnotobiotic animals colonised with just a single strain (Aoki et al., 2021).

1.4.1.2. Probiotics modulate brain function and behaviour in animal models

It is well-established that commensal bacteria contribute to host physiology, including brain function and behaviour (Cryan et al., 2019). The contributions of individual bacterial strains to developmental and homeostatic mechanisms involving the gut-brain axis in gnotobiotic models has been discussed in section 1.2. Here, studies involving probiotic supplementation as a potential therapeutic will be discussed (Table 1.1).

In BALB/c mice, which exhibit an innately anxious phenotype relative to other mouse strains (Belzung and Griebel, 2001), *Bifidobacterium longum* 1714 reduced anxiety-like behaviour in the OFT and reduced depressive-like behaviour in the FST, while *Bifidobacterium breve* 1205 reduced anxiety-like behaviour in the EPM (Savignac et al., 2014). Both probiotics were administered daily for three weeks prior to the start of behavioural testing, as 1×10^9 colony forming units (CFU)/mL by oral gavage. In each case, three-week treatment with escitalopram (daily oral gavage 20mg/kg) in the positive control group did not alter behaviour in each of these tests, relative to phosphate-buffered saline controls. It is possible that, as the BALB/c mice were healthy, the behavioural assays used may not have been sensitive enough to detect subtle changes in behaviour when the mice were unchallenged. Escitalopram treatment did reduce stereotypic behaviour in the marble burying test (MBT), as did both *Bifidobacterium* probiotics. While no insight was provided into how the probiotics elicited these behavioural changes, and why the behavioural changes appeared to be strain-dependent, a previous study in BALB/c mice using the probiotic *L. rhamnosus* (JB-1) showed that behavioural effects were dependent on the vagus nerve (Bravo et al., 2011).

Since these early studies that pioneered the field of probiotics as novel therapeutics for mood disorders, further efforts have been made to understand how certain strains exert their effects via the microbiota-gut-brain axis. In a brain *in vivo* MRS study, BALB/c mice were treated with *L. rhamnosus* (JB-1) daily for four-weeks, and brain imaging was performed weekly (Janik et al., 2016). A significant increase in brain glutamate + glutamine (Glx) levels

Table 1.1 Summary of probiotic studies in rodent models on anxiety and depressive-like behaviours and brain metabolism

Model organism (paradigm)	Probiotic (dose)	Duration of treatment	Effects	Reference
7-week-old male BALB/c mice (healthy)	<i>B. longum</i> 1714 or <i>B. breve</i> 1205 (1×10 ⁹ CFU, daily)	3 weeks prior to start of behavioural tests, 3 weeks during behavioural testing (6 weeks total)	↓ anxiety OFT, MBT and ↓ immobility in FST (<i>B. longum</i> 1714) ↓ anxiety EPM and MBT (<i>B. breve</i> 1205)	(Savignac et al., 2014)
6-week-old male BALB/c mice (vagotomy)	<i>L. rhamnosus</i> JB-1 (1×10 ⁹ CFU, daily)	4 weeks (behavioural assays started during final week of treatment)	↓ anxiety in EPM, OFT and decreased immobility in FST, altered hippocampal GABA _A receptor subunit expression, effects dependent on vagus nerve	(Bravo et al., 2011)
4-week-old male BALB/c mice (healthy MRI and MRS brain imaging)	<i>L. rhamnosus</i> JB-1 (1×10 ⁹ CFU, daily)	4 weeks (imaging performed throughout duration of treatment weeks 0,1,2,3,4, and 4 weeks post-treatment cessation)	↑ brain Glx at weeks 2,3,4,8 ↑ brain NAA at weeks 1,2,3,4 ↑ brain GABA at week 4 only No effect on hippocampal volume at any timepoint	(Janik et al., 2016)
Male Swiss albino mice 25-30g (28 days CMS)	<i>L. plantarum</i> MTCC 9510 (2×10 ¹⁰ CFU, daily)	4 weeks (duration of CMS paradigm)	CMS ↓ locomotion in the OFT, ↑ immobility in FST and TST, ↑ serum TNF, endotoxin, BBB and intestinal permeability, and ↑ brain NF-κB which were reversed by <i>L. plantarum</i> MTCC 9510 to non-stressed levels. <i>L. plantarum</i> MTCC 9510 without CMS had no significant effects on behaviour.	(Dhaliwal et al., 2018)
Male neonatal C57Bl/6 mice (maternal separation)	<i>B. pseudocatenulatum</i> CECT 7765 (1×10 ⁸ CFU, daily)	20 days (PND2 to PND21)	MS ↑ IFN-γ and IL-10 in small intestine and ↑ anxiety in EPM. <i>B. pseudocatenulatum</i> CECT 7765 ↓ intestinal IFN-γ and ↓ anxiety in EPM	(Moya-Pérez et al., 2017)
4-week-old male Sprague-Dawley rats (chronic HFD, 60% of kilocalories from fat)	<i>B. bifidum</i> W23, <i>B. lactis</i> W52, <i>L. acidophilus</i> W37, <i>L. brevis</i> W63, <i>L. casei</i> W56, <i>L. salivarius</i> W24, <i>Lc. Lactis</i> W19, <i>Lc. Lactis</i> W58 (3.75×10 ⁸ CFU/mL drinking water, daily)	5 weeks prior to start of behavioural tests, 10 weeks total.	Probiotic mixture ↓ immobility in FST, irrespective of diet. Chronic (10-16 week) HFD feeding ↑ plasma LPS and quinolinic acid, irrespective of probiotic treatment. Probiotic treatment ↑ plasma indole-propionic acid	Abildgaard et al. (2017)

was identified after two-weeks treatment. Brain Glx remained elevated for 4 weeks after cessation of probiotic treatment. In contrast, brain NAA and GABA were elevated after four weeks probiotic supplementation but returned to baseline levels immediately after treatment cessation. The study suggests that central levels of Glx levels may be of relevance to the enduring effects of *L. rhamnosus* (JB-1) on behaviour in mice, though the effects of probiotic supplementation on levels of glutamate and glutamine, individually, need to be resolved and quantified in future work.

Probiotics have also been shown to exert remedial effects in stressed rodents. In one study (Dhaliwal et al., 2018), male Swiss albino mice were subject to 28-days CMS or left undisturbed. Half of the CMS group and half of the control group received *Lactobacillus plantarum* MTCC 9510 by oral gavage during the same period. Chronic treatment with *L. plantarum* MTCC 9510 normalised locomotor activity in the OFT, which had been significantly reduced by CMS (Dhaliwal et al., 2018). Immobility in the FST and tail suspension test were also reduced by the CMS/probiotic compared to CMS/vehicle, but it was not discussed as to whether activity levels in the preceding OFT may have confounded these results. CMS increased serum TNF and brain NF- κ B, which were prevented by the probiotic. Increased peripheral inflammation may have occurred through increased intestinal permeability and increased circulating endotoxin levels after CMS, which were also attenuated by *L. plantarum* MTCC 9510 treatment (Dhaliwal et al., 2018). BBB integrity was also reduced by CMS and increased by the probiotic. Interestingly, the microbial metabolite butyrate has been shown to increase both gut barrier integrity (Fachi et al., 2019) and BBB integrity (Li et al., 2016a), possibly via increased HIF-1 expression (Fachi et al., 2019). In this study, caecal butyrate was significantly increased in CMS/probiotic mice relative to CMS/vehicle mice, though probiotic treatment alone did not significantly increase butyrate levels. Overall, this study suggests that chronic probiotic treatment can attenuate stress-induced inflammation and depressive-like behaviours in mice by increasing intestinal and brain barrier integrity.

In a maternal separation model of early-life stress, male offspring were treated with *Bifidobacterium pseudocatenulatum* CECT 7765 or vehicle between postnatal day (PND)2 and PND21 (Moya-Pérez et al., 2017). In the small intestine, maternal separation led to increased interferon gamma and interleukin (IL)-10, suggesting the induction of a Th1-type inflammatory response – interferon gamma being the archetypal Th1 cytokine (Berger, 2000). Increased IL-10 may represent the homeostatic response to attenuate the pro-inflammatory response to early-life stress. *B. pseudocatenulatum* CECT 7765 supplementation significantly reduced interferon gamma levels in the intestine, both after early-life stress and in non-stressed control mice. At PND41, approximately three weeks after cessation of probiotic treatment and early-life stress, mice showed increased anxiety-like behaviour in the EPM, which was attenuated by probiotic treatment (Moya-Pérez et al., 2017). This suggests that concurrent probiotic treatment is sufficient to prevent emotional dysfunction induced by early-life stress, though it was not tested whether probiotic treatment *after* early-life stress could also prevent early-life stress. On the whole, single-species probiotics have been shown to attenuate inflammation and anxiety-like behaviour in response to stress paradigms, though it remains unclear as to why a given probiotic strain is preferable over another.

The effects of multi-species probiotics have been less well investigated in rodents. Abildgaard et al. (2017) assessed the efficacy of eight bacterial strains (*Bifidobacterium* spp., *Lactobacillus* spp., *Lactococcus* spp. altogether) on depression-like behaviour after HFD-induced obesity in rats. Interestingly, probiotic intake was found to have a clear, independent effect on depressive-like behaviour in the FST. Total immobility time was significantly reduced in both lean and obese rats given the probiotic, whereas obesity with or without probiotic supplementation had no effect on depressive-like behaviour (Abildgaard et al., 2017). As expected, chronic HFD feeding elevated circulating levels of LPS, though in this case probiotics had no effect. Plasma metabolomics was performed by mass spectrometry. HFD-induced obesity led to many (209) metabolite changes compared to lean rats, including a highly significant increase in the tryptophan catabolite and NMDA agonist (Lugo-Huitrón et al.,

2013), quinolinic acid. Following FDR correction, probiotic treatment increased the levels of two tryptophan metabolites, indole-propionic acid and indole-acrylic acid, as well as acetylmethionine. While no significant diet x probiotic treatment interactions were found, indole-propionic acid has been shown to exert anti-inflammatory effects, attenuating astrocytic *CCL2* cytokine expression in a mouse model of experimental autoimmune encephalitis (Rothhammer et al., 2016). By exerting this central anti-inflammatory effect, indole-propionic acid may contribute to the antidepressant-like effects of the probiotic mixture.

In the above studies, only male rodents were used as test subjects. While the studies discussed here are not an exhaustive review of the current literature, the vast majority have reported the effects on males only. Further studies exploring the possible interaction between probiotic treatment and sex, in healthy subjects and in disease models, are urgently required to identify, validate, and translate the therapeutic effects of microbiota modulation in mood disorders.

1.4.1.3. Prebiotics and probiotics in human studies

The psychotropic effects of probiotics, and to a lesser extent prebiotics, have been assessed in humans, although the composition, timeframe, and nature of the human subjects varies greatly between studies (Table 1.2). In healthy subjects, a GOS prebiotic has been shown to significantly reduce the waking cortisol response after three weeks treatment (Schmidt et al., 2015). This effect was associated with reduced attentional vigilance to negative stimuli and increased vigilance to positive stimuli, which was significantly different from the placebo. Whilst this suggests some possibly anxiolytic effects of the prebiotic at a subconscious level, there were no significant changes in self-reported measures of anxiety or negative affect pre- and post-treatment. One possible reason for this was because the subjects were not clinically anxious or depressed at baseline. In fact, previous history of a psychiatric disorder was an exclusion criterion of the study (Schmidt et al., 2015). Another study stratified healthy individuals into high and low trait anxiety at recruitment (Johnstone et al., 2021) and found that four weeks GOS treatment reduced self-reported anxiety scores in

highly anxious participants, but not in individuals who had low levels of anxiety to begin with. This substantiates the notion that modifying the microbiota to improve mood may be most efficacious in people with mood disorders.

In people with irritable bowel syndrome (IBS), where anxiety is highly comorbid (Cho et al., 2011), GOS prebiotic treatment both increased levels of faecal *Bifidobacteria* and reduced anxiety scores using the Hospital Anxiety and Depression (HAD) scale at baseline and follow-up (Silk et al., 2009). Despite some evidence pointing to greater efficacy of prebiotics in people with greater symptom severity, the one study that has tested (GOS) prebiotic treatment in people with MDD found no significant effect on Beck Depression Inventory (BDI) score after an eight-week treatment regime (Kazemi et al., 2019). The study power was comparable with other studies (n = 36-38 per group). Anxiety scores were not measured. Notably, about 10% of individuals taking the prebiotic experienced mild adverse effects including gastrointestinal complaints. Overall, there is limited evidence for the efficacy of prebiotics for improving mood, which is supported by a recent meta-analysis (Liu et al., 2019). The evidence is especially sparse in people with clinically significant levels of anxiety and depression, and further studies are needed to determine whether prebiotics are effective in people with clinically significant anxiety disorders.

Probiotics have thus far been more extensively tested as a potential therapeutic for low mood and MDD. In healthy subjects, 30-day probiotic treatment with *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 reduces anxiety and depressive symptoms using the HAD scale (Messaoudi et al., 2011). Conversely, BDI and Beck Anxiety Inventory (BAI) scores were not significantly different between pre- and post-treatment. *Lactobacillus rhamnosus* (JB-1), which showed promising preclinical results in reducing basal levels of anxiety-like behaviour in healthy mice (Bravo et al., 2011), failed to improve mood or reduce anxiety in healthy volunteers after eight weeks treatment (Kelly et al., 2017).

Probiotics have also been trialled in individuals with subclinical levels of anxiety and depression. In individuals with mild to moderate levels of depression (Patient Health

Table 1.2 Summary of probiotic studies on anxiety and/or depression in humans

Paradigm	Probiotic (dose)	Duration of treatment	Effects	Reference
Adult males and females (healthy)	<i>L. helveticus</i> R0052 and <i>B. longum</i> R0175 (3×10 ⁹ CFU, daily)	30 days	↓ anxiety and depression HAD scores No effect on BDI or BAI scores	(Messaoudi et al., 2011)
Adult males (healthy)	<i>L. rhamnosus</i> JB-1 (1×10 ⁹ CFU, daily)	4 weeks	No differences between probiotic and placebo for BDI, BDAI, or serum cytokine levels	(Kelly et al., 2017)
Adult males and females (mild to moderate depression: PHQ9 score 5-19)	Bio-Kult probiotic – see list of strains in Table 2.1. (2×10 ⁹ CFU, daily)	4 weeks	No effect of probiotic on salivary cortisol or serum CRP. Probiotic ↓ PHQ9 scores	(Baião et al., 2022)
Adult males and females (moderate or severe depression: ≥11 QIDS-SR16 or ≥14 DASS-42)	<i>L. helveticus</i> R0052 and <i>B. longum</i> R0175 (3×10 ⁹ CFU, daily)	8 weeks	No effects of probiotic treatment on self-reported depression or anxiety scales, and no effect on serum CRP, IL-1B, IL-6, TNF, BDNF, or Vitamin D	(Romijn et al., 2017)
Adult males and females (MDD diagnosis, moderate-severe symptoms)	<i>L. acidophilus</i> , <i>L. casei</i> , and <i>B. bifidum</i> (6×10 ⁹ CFU, daily)	8 weeks	Probiotic ↓ BDI score compared to placebo. Probiotic ↓ serum CRP level compared to placebo	(Akkasheh et al., 2016)
Adult males and females (MDD diagnosis, mild-moderate symptoms)	<i>L. helveticus</i> R0052 and <i>B. longum</i> R0175 (1×10 ¹⁰ CFU, daily)	8 weeks	Probiotic ↓ BDI score compared to placebo. Probiotic ↓ serum kynurenine/tryptophan ratio compared to placebo	(Kazemi et al., 2019)
Adult pregnant women (at least mild anxiety or depression: ≥10 EPDS or ≥40 STAI)	<i>B. bifidum</i> W23, <i>B. lactis</i> W52, <i>L. acidophilus</i> W37, <i>L. brevis</i> W63, <i>L. casei</i> W56, <i>L. salivarius</i> W24, <i>Lc. Lactis</i> W19, <i>Lc. Lactis</i> W58 (5×10 ⁹ CFU, daily)	From 26-30 weeks gestation until delivery	No effect of probiotic on perinatal maternal depression (EPDS) or anxiety (STAI) scores.	(Browne et al., 2021)
Adult pregnant women (healthy)	<i>L. rhamnosus</i> HN001 (6×10 ⁹ CFU, daily)	From 14-16 weeks gestation until 6 months postpartum	Probiotic significantly decreased depression (EPDS) and anxiety (STAI) scores compared to placebo.	(Slykerman et al., 2017)
Adult pregnant women (obese: BMI≥30kg/m ²)	<i>L. rhamnosus</i> GG and <i>B. lactis</i> BB12 (6.5×10 ⁹ CFU, daily)	From 12-18 weeks gestation until 36 weeks gestation	No effect of probiotic on depression (EPDS) or anxiety (STAI-6) scores compared to placebo.	(Dawe et al., 2020)

Questionnaire [PHQ]9 score 5-19), four-week treatment with a multi-species probiotic (Bio-Kult Advanced®) significantly reduced PHQ9 scores, suggesting an antidepressant effect (Baião et al., 2022). In contrast with previous research in prebiotics (Schmidt et al., 2015), salivary cortisol levels were unaffected by probiotic treatment, and no changes in blood C-reactive protein (CRP) levels were identified (Baião et al., 2022). In individuals with moderate to severe depression, a probiotic mixture of *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 did not improve psychological outcomes after eight weeks treatment, including depression or anxiety (Romijn et al., 2017). The sample size was greater and the duration of treatment longer than a previously successful trial of the same probiotic mixture in healthy subjects (Messaoudi et al., 2011).

Lastly, probiotics have been trialled in individuals with a clinical diagnosis of MDD. In the two studies that have sought to assess the antidepressant efficacy of probiotics for clinically significant depression, both showed an improvement in BDI score at follow-up with the probiotics as compared to the placebo (Akkasheh et al., 2016; Kazemi et al., 2019). In both studies, both placebo and probiotic groups were receiving SSRI treatment – the use of the probiotic was therefore as an adjunct therapeutic. One study trialled *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 for eight weeks (Kazemi et al., 2019), and the other trialled *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium bifidum* for eight weeks (Akkasheh et al., 2016). In both cases, circulating inflammatory markers such as CRP and the kynurenine/tryptophan ratio were downregulated after probiotic supplementation compared to placebo.

Overall, probiotics have shown inconsistent effects in human trials, though greater symptom severity may lead to greater treatment efficacy. This conclusion is supported by a recent meta-analysis of probiotics for the treatment of depression (Liu et al., 2019). One possible reason for this could be an interaction with diet, which is often of poor quality in people with a severe psychiatric illness (Firth et al., 2018). On the one hand, beneficial microbial metabolites such as butyrate, propionate, and acetate may be lower in the gut of these

individuals, allowing for probiotics to exert a greater effect. Indeed, there is some evidence to support reduced colonic SCFA production in people with depression (Valles-Colomer et al., 2019; Liu et al., 2020b). On the other hand, the inverse may also be true, whereby a lack of dietary fibre provides a limited potential for probiotic proliferation in the colon after supplementation in individuals with MDD. Either way, future studies need to find a measurable biomarker, or panel of biomarkers, of treatment efficacy. This may include baseline and follow-up faecal metagenomic and metabolomic profiles alongside circulating inflammatory markers to determine the effect of the probiotic treatment on quantifiable aspects of gut-brain axis communication.

1.4.2. Prebiotics and probiotics during the perinatal period

Preclinical studies have investigated the effects of prebiotic and probiotic treatment during the perinatal period on offspring health outcomes, whereas clinical studies are lacking. There is currently no evidence for the consumption of prebiotics on maternal wellbeing outcomes – a recent systematic review did not identify any relevant studies using prebiotics (Desai et al., 2021). A feature of recent and ongoing clinical trials is the effect on pregnancy outcomes (Sohn and Underwood, 2017), and the prevention of autoimmune-related disease in infants with conflicting results of both beneficial and no effects reported (Wickens et al., 2013, 2018; Barthow et al., 2016; Baldassarre et al., 2018).

Maternal prebiotic intake has been shown to alter the behaviour of juvenile and adult offspring in mice (Hebert et al., 2021). Specifically, maternal GOS intake increased exploratory behaviour in young offspring compared to dams fed normal drinking water only. In adult offspring, anxiety-like behaviour was reduced, and social behaviour was increased by maternal GOS intake. Age-dependent changes in *NMDA* subunit receptor expression was noted alongside increased in offspring faecal butyrate and propionate (Hebert et al., 2021). Direct stimulation of early life gut microbes through neonatal prebiotic administration from PND3 to PND21 has also been shown to increase *NMDA* receptor subunit GluN2A, synaptophysin, and brain-derived neurotrophic factor protein levels in the hippocampus at P22

(Williams et al., 2016). These effects persisted from weaning into early adulthood, at 8-weeks of age, despite the cessation of prebiotic supplementation. While gut microbiota analysis was not performed in this study, the same galactooligosaccharide prebiotic has been shown to be Bifidogenic in adult rodents (Savignac et al., 2013).

A meta-analysis on the effect of probiotics on maternal metabolic health indicates that probiotic interventions improve glucose and lipid metabolism, including reducing insulin resistance (Han et al., 2019). Recently, probiotics have been investigated in clinical trials for their effect on maternal mental health during pregnancy and postpartum (Desai et al., 2021). Completed clinical studies have shown mixed results. Browne et al. (2021) aimed to assess the effect of prenatal probiotic (n = 20) or placebo (n = 20) consumption from mid- to late gestation until delivery on maternal depression and anxiety symptoms during pregnancy. Both Edinburgh Postnatal Depression Scale and State-Trait Anxiety Inventory scores decreased between the baseline and the eight-week follow-up in both probiotic and placebo groups. Thus, while there was a significant effect of time, there was no significant difference between intervention and control groups. No biomarkers of treatment response were used, and faecal microbiome sequencing was not performed. A study of 380 women who received either *Lactobacillus rhamnosus* HN001 (193) or placebo (187) from 14-16 weeks' gestation until six months postpartum were assessed for anxiety and depressive symptoms during pregnancy and postpartum (Slykerman et al., 2017). This study found a reduction in these symptoms in the probiotic group both during and after pregnancy than in those given a placebo. The large sample size and long duration of treatment of this study is a strength that should be replicated in future studies, particularly those that will assess offspring development. On the other hand, supplementation of *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* BB12, at a similar dose, did not affect depression and anxiety scores in an obese (BMI > 30.0kg/m²) cohort of 164 women (Dawe et al., 2020). Probiotics were taken from a similar timepoint, from mid-gestation until birth rather than post-weaning. Hence, this was an assessment of mental wellbeing during pregnancy only rather than postpartum.

The effects of maternal probiotic consumption on *offspring* health outcomes have also thus far only been investigated preclinically, and the scope of research here is limited. Mouse prenatal probiotic supplementation has been suggested to increase serum IgG and intestinal secretory IgA (Himaja et al., 2016) in the adult offspring, which may have immunoprotective roles (Di Benedetto et al., 2020). In line with this, antibody responses to hepatitis-B surface antigen were shown to be higher in offspring from BGOS-supplemented dams compared to controls (Himaja et al., 2016). Similarly, supplementation of rat dams with *Lactobacillus fermentum* CECT5716 during pregnancy and the first two weeks of lactation increased IgA in offspring pups at postnatal day 14 (Azagra-Boronat et al., 2020b). *L. fermentum* supplementation also had a positive effect on plasma fatty acid profile in both dams and offspring. The same experiment demonstrated that these effects may have been mediated by changes to the mammary milk composition, which showed increased polyunsaturated fatty acid content, reduced palmitic acid, and increased IgA in probiotic-supplemented dams compared to controls (Azagra-Boronat et al., 2020a). Despite these changes, *L. fermentum* CECT5716 was only detected in half of the supplemented dams (3/6) suggesting that presence of the probiotic in the milk was not a prerequisite for imparting positive immune effects on the offspring. Rather, changes to the intestinal microbiota, with subsequent systemic effects, may have mediated the beneficial effects on the rat milk composition (Azagra-Boronat et al., 2020a).

Overall, there is some evidence for a beneficial role of early life exposure, prenatal and postnatal, on maternal intake of prebiotics and/or probiotics to support normal brain and immune development. Further research in this area should be encouraged, particularly as to whether supplementation during pregnancy and lactation mitigates the consequences of maternal obesity on offspring neurobiology and behaviour. This is because there is some evidence that offspring microbial reconstitution has been suggested to reverse neurological and behavioural deficits induced by a maternal high-fat diet (Buffington et al., 2016), while other work suggests that microbial reconstitution post-weaning is not sufficient to rescue

neurological adaptations that arise during development (Clarke et al., 2013). Studies that include maternal diet are also needed because the type of diet consumed by the host, and the subsequent metabolic and inflammatory profiles associated with the diet, may significantly impact the efficacy of probiotic administration in terms of its anti-inflammatory properties and ability to modify the brain and behaviour (Ohland et al., 2013).

1.5. Depression and metabolic disease: a bidirectional relationship

1.5.1. Obesity and depression

Depression and obesity are both common, multifactorial disorders that impart severe global health and economic burdens (Malhi and Mann, 2018; Blüher, 2019). At an individual level, they can reduce the quality of life of those affected. Substantial epidemiological evidence exists for a bidirectional relationship between depression and obesity (Luppino et al., 2010; Jung et al., 2017; Pereira-Miranda et al., 2017). A meta-analysis of longitudinal studies found that obese individuals had a 55% increased risk of developing depression over time, while depressed persons were 58% more likely to become obese (Luppino et al., 2010). Reciprocally, weight loss has been shown to improve mood in people with depressive symptoms (Fuller et al., 2017). While antidepressant medication usage can cause short-term weight gain, medication status is not thought to mediate the relationship between obesity and depression (Gibson-Smith et al., 2016).

Obesity and depression share genetic risk factors. Genome wide association studies (GWAS) have identified overlap between the genetic risk for depression (Wray et al., 2018) and obesity (Locke et al., 2015) through single nucleotide polymorphisms near or in *OLFM4* and *NEGR1*. The association between *NEGR1*, obesity, and depression has been validated in independent human studies that cumulatively exceed 1.5 million individuals (Thorleifsson et al., 2009; Willer et al., 2009; Speliotes et al., 2010; Bradfield et al., 2012; Berndt et al., 2013; Levey et al., 2021). Transcriptomic analysis has mapped *NEGR1* expression in the CNS predominately to the hypothalamus in both humans (Levey et al., 2021) and rodents (Lee et al., 2012). *NEGR1*^{-/-} mice exhibit reduced brain volume, as well as reduced relative hippocampal volume (Singh et al., 2019). This is thought to be due to the fact that *NEGR1* is required for ongoing neurogenesis in the adult hippocampus in mice (Noh et al., 2019). *NEGR1*^{-/-} mice also demonstrate increased anxiety-like behaviour in the OFT and EPM, and increased depressive-like behaviour in the FST and tail suspension test compared to wildtype

controls (Noh et al., 2019). In the hypothalamus, *NEGR1*^{-/-} function may be related to the central regulation of feeding behaviours and energy balance (Boender et al., 2014).

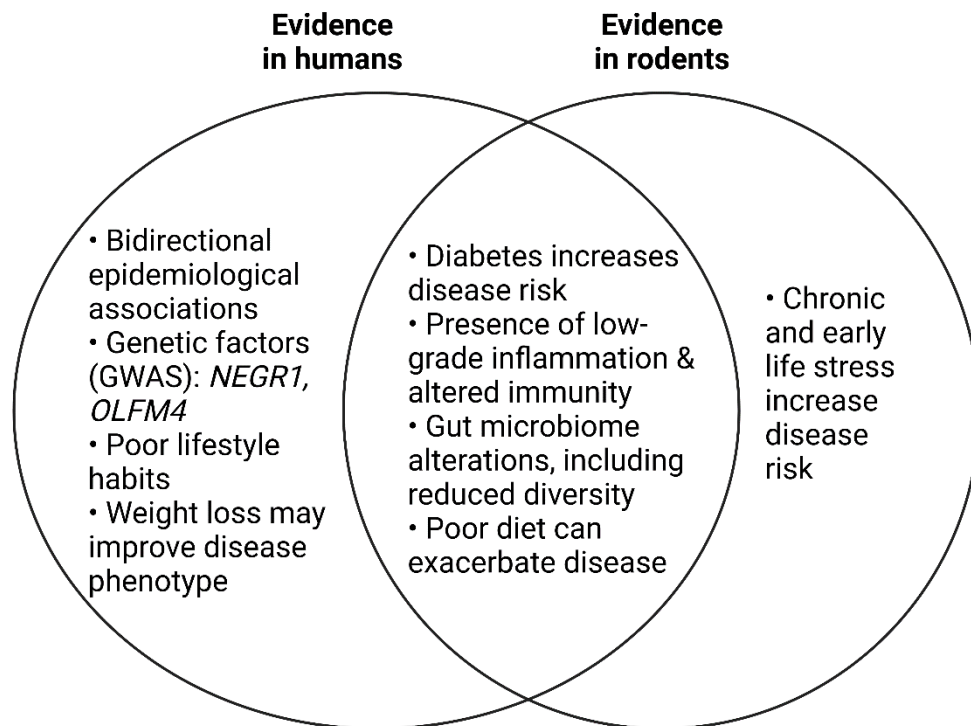


Figure 1.3 Summary of the evidence from human and/or rodent studies that demonstrate overlapping pathophysiology between obesity and major depressive disorder.

Chronic, low-grade inflammation is a hallmark of both obesity and depression (Milaneschi et al., 2019). In a large, case-control study of depression in the UK Biobank (Pitharouli et al., 2021), low-grade inflammation was identified in 21% of patients (defined as serum CRP > 3mg/L). In the same study, genetic risk for lifetime depression was found to be positively correlated with serum CRP. Interestingly, while this signature of inflammation in depression was found to be independent of BMI and other clinical and demographic features, the correlation with genetic risk was entirely dependent on BMI and smoking status. BMI was higher in individuals with depression. Thus, it was concluded that the genetic contribution to an inflammatory phenotype in depression is entirely dependent on these two factors (Pitharouli et al., 2021).

Limited preclinical evidence exists to support a role for the microbiota in the bidirectional relationship between obesity and depression. Transplanted gut microbiota from mice subject to HFD-induced obesity increased anxiety-like and stereotypic behaviour in recipient mice (Bruce-Keller et al., 2015). Recipient mice were first given broad-spectrum antibiotics to deplete the microbiome. Given that antibiotic treatment alone can alter behaviour (Vicentini et al., 2021), this may have confounded the results. Nevertheless, the obese-type gut microbiota were also found to increase intestinal permeability as well as peripheral and central inflammation without a significant change in body weight (Bruce-Keller et al., 2015).

Overall, there is substantial epidemiological evidence for a bidirectional relationship between obesity and depression, which may be mediated by chronic low-grade inflammation or other genetic factors associated with the regulation of food intake. Studies in humans are required to determine the putative role for obese-type gut microbiota in peripheral inflammation and depression.

1.5.2. Depression and non-alcoholic fatty liver disease

With an estimated worldwide prevalence of one in four individuals, non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disorder (Younossi et al., 2016). NAFLD has a close, bidirectional association with other obesity-related metabolic disorders. Metabolic syndrome, dyslipidaemia, and diabetes (T2DM) are established risk factors for NAFLD development and progression (European Association for the Study of the Liver (EASL) et al., 2016; Younossi et al., 2018). Alongside the presence of metabolic risk factors, NAFLD encompasses a progressive liver disease continuum, from noninflammatory steatosis (non-alcoholic fatty liver [NAFL]) to non-alcoholic steatohepatitis (NASH), an inflammatory subtype characterised by leukocyte infiltration, inflammation, and hepatocyte injury. Accumulating liver fibrosis (for example, from chronic NASH-related liver injury) may ultimately lead to advanced liver disease (cirrhosis) and associated liver-related complications. Due to the high prevalence of disease, the fraction of NAFLD patients that develop liver-related complications (<10%) (Ekstedt et al., 2015) make up a considerable proportion of individuals with primary liver

cancer (Wong et al., 2014) and those requiring liver transplantation (Wong et al., 2015). At an individual level, the rate of NAFLD progression, disease outcome, and response to treatment is variable, which may reflect a complex disease aetiology. At a community level, NAFLD contributes substantially to economic and health-care burdens (Allen et al., 2018). Identifying factors and disease biomarkers that influence the course of NAFLD is a crucial component of helping to prevent disease progression.

Emerging evidence points to a bidirectional association between NAFLD and depression; NAFLD has been found to increase the risk of anxiety and depression independently of other comorbid diseases (Labenz et al., 2020), and depressed individuals are more likely to have NAFLD than non-depressed individuals, particularly in the presence of obesity (Cho et al., 2021). Despite some epidemiological evidence, few studies have investigated clinicopathological and metabolic features of NAFLD patients with and without depression. Youssef et al. (2013) identified subclinical depression in over half of 567 patients with NAFLD, and clinical depression in 14%. Both subclinical and clinical depression were associated with more severe hepatocyte ballooning (reflecting hepatocellular injury) in liver biopsy sections. Conversely, Tomeno et al. (2015) identified a higher grade of steatosis alongside significantly elevated serum ferritin, C-reactive protein, aspartate transaminase (AST), and gamma-glutamyl transferase (GGT), though no differences in hepatocellular ballooning or fibrosis were found in NAFLD patients with depression ($n = 32$) compared to without ($n = 226$). Interestingly, less improvement in clinical chemistry parameters after a year of standard care was observed in patients comorbid with depression compared to NAFLD patients without depression. Similarly, individuals with stable depression (defined as full or partial remission) showed a greater improvement in alanine transaminase (ALT) and GGT levels after a year of standard care than patients with unstable depression (not in full or partial remission). This may indicate that NAFLD patients comorbid with depression do not respond to medical intervention as well as NAFLD patients without an affective disorder, and that recent depressive symptoms may be more pertinent to NAFLD than a lifetime history of depression.

Individuals with depression (and/or other psychiatric conditions) are more likely to lead an unhealthy lifestyle and less likely to adhere to a medical regimen, particularly those involving lifestyle adjustments (Penninx and Lange, 2018). Identifying and treating NAFLD patients with depression may improve disease treatment responses.

1.6. Clinical (blood-based) applications for metabolomics in depression

Metabolomics is an application and field of study that involves the simultaneous profiling of many metabolites (small molecular intermediates) in a biofluid or other medium (Beckonert et al., 2007). In blood, metabolite levels may reflect systemic changes in metabolism due to normal physiological processes, disease, pharmacological intervention, genotype, diet, and other environmental factors. The relative abundance of a constellation of metabolites may allow for the characterisation of a metabolomic signature of disease (Larkin et al., 2022; Radford-Smith et al., 2022) or even predict treatment response (Caspani et al., 2021) and disease progression (Probert et al., 2018).

Depression is underdiagnosed in primary care settings (Mitchell et al., 2009; Fekadu et al., 2022), while individuals with other chronic illnesses are at an increased risk of comorbid depression, which can affect prognosis (Evans et al., 2005). In these settings, a depression diagnosis often relies on self-report questionnaires with limited sensitivity and specificity (Wu et al., 2021). Depression is therefore a disorder that may benefit from new, complementary diagnostic approaches without placing additional strain on tertiary care centres.

Multivariate analysis of the blood metabolome has been shown to discriminate between individuals with depression and healthy controls (Zheng et al., 2012; Pan et al., 2018; Hung et al., 2021). In these cases, however, sample sizes were small and likely not reflective of the heterogeneity inherent to MDD and related subtypes. Larger studies have sought to identify metabolic changes associated with depression that may help to elucidate the biological mechanisms underpinning the disease. In a mass spectrometry metabolomic study, depression was associated with reduced serum levels of laurycarnitine (Zacharias et al., 2021), which may relate to altered lipid and energy metabolism in people with depression. Using NMR-based metabolomics, a pooled meta-analysis of >5,000 cases of depression and > 10,000 controls found significantly altered circulating lipid levels in people with depression,

including reduced HDL and increased VLDL and triglyceride (Bot et al., 2020). Another meta-analysis included both mass spectrometry and NMR techniques and identified tryptophan and kynurenine as being significantly reduced in depression compared to healthy controls (Pu et al., 2021b).

Whilst these large-scale studies have helped to progress understanding of depression pathophysiology on a biochemical level, the variation in the outcomes of each study emphasises the challenges of depression biomarker research. Despite intensive research into the pathophysiology of depression, breakthroughs in diagnosis and prognosis are impeded by the heterogeneity of people with the same diagnosis (Nandi et al., 2009). This heterogeneity encompasses different social, cultural, and physiological trajectories leading up to the point that the diagnosis was made. For example, some people may have developed depression where dietary factors and obesity may greatly contribute to their psychological symptoms. Others diagnosed with depression may have a normal weight and healthy diet, but have experienced psychological trauma, or are burdened with another medical disease like cancer, which contributes to their symptoms of depression. Further studies in this area need to consider comorbidities and other clinical and demographic features that may augment disease stratification. For example, depression associated with a specific disease or medication may have a clear biochemical fingerprint (Mukerji et al., 2021). Employing data-driven approaches to classify people with depression – as opposed to conventional methods – could identify patient subgroups which, despite overlapping clinical features at presentation, may differ in terms of prognosis and response to treatment (Beijers et al., 2019).

1.7. Aims and hypotheses

This introduction has summarised a) the contribution of the gut microbiota to brain function and behaviour via the microbiota-gut-brain axis, b) mechanisms by which maternal obesity may impact the neurodevelopment and behaviour of offspring, and c) the broader relationship between depression and metabolic dysfunction in humans and the application of metabolomics to depression. Previous studies have indicated effects of maternal HFD on offspring behaviour and brain function (Sullivan et al., 2014), which may be mediated by perturbations to the maternal and offspring microbiota (Buffington et al., 2016). In parallel, probiotics have emerged as a novel way to modify the microbiota-gut-brain axis in rodents (Bravo et al., 2011) and humans (Baião et al., 2022). Hitherto, it was unknown whether maternal probiotic intake may remedy the adverse effects of perinatal exposure to maternal obesity. Additionally, while the metabolomic profile of depressive symptoms has been characterised in several large studies (Bot et al., 2020; Pu et al., 2021b; Zacharias et al., 2021), it is unclear whether depression can be identified in a cohort with pre-existing metabolic dysfunction; some of the key metabolic pathways associated with depression relate to lipid and energy metabolism (Bot et al., 2020; Zacharias et al., 2021).

Principally, this thesis sought to explore the distinct effects of maternal perinatal probiotic intake and HFD-induced obesity on offspring behaviour, neuroplasticity gene expression, and gut-brain metabolism. It was then determined whether a serum metabolomic profile of depressive symptoms could be detected in the context of metabolic dysfunction in humans. Connections between the metabolic signature of depressive symptoms in NAFLD and depressive-like behaviours in the rodent offspring were investigated.

Specifically, this thesis aimed:

1. *To characterise the distinct effects of chronic high fat diet exposure and perinatal probiotic treatment on maternal behaviour and metabolite levels across the gut-brain axis.*

It was hypothesised that chronic HFD feeding would elevate markers of peripheral and central immune activation, reduce maternal care, increase passive stress coping behaviour in dams, and alter brain and peripheral metabolite levels. Conversely, it was expected that perinatal probiotic treatment would increase the abundance of faecal *Bifidobacterium spp.* and *Lactobacillus spp.* and attenuate the increase in brain and/or peripheral pro-inflammatory mediators, which is the topic of Chapter 3.

2. *To explore how maternal obesity affects affective behaviours and neuroplasticity gene expression in the offspring, and to see whether these changes could be reversed by maternal perinatal probiotic intake.*

Maternal diet-induced obesity was expected to increase anxiety and depressive-like behaviour in the young offspring. Long-lasting behavioural changes were also expected in adult offspring. Additionally, it was expected that perinatal probiotic exposure would attenuate these behavioural changes and lead to changes in neuroplasticity gene expression, which is investigated in Chapter 4.

3. *To investigate the metabolomic features associated with maternal obesity and probiotic intake in the offspring gut, liver, blood, and brain.*

We hypothesised that maternal probiotic intake would increase the abundance of gut acetate, propionate, and butyrate in the young offspring, though I did not expect this to last until adulthood (after offspring were weaned to regular chow for three months). I also expected maternal obesity to give rise to widespread changes in metabolism across the brain-gut axis, which is explored in Chapter 5.

4. *To profile the serum metabolomic signature of depressive symptoms in the context of pre-existing metabolic dysfunction.*

I expected to see an additive effect of depression on serum metabolite levels. Though patients already had dyslipidaemia in almost all cases, I expected depression to be associated with altered lipid and lipoprotein levels. Chapter 6 seeks to address this issue.

2

Materials and Methods

2.1 Animals

All licensed procedures were performed with UK Home Office approval, under licence P996B4A4E in accordance with the Animals (Scientific Procedures) Act (1986). All mice were housed in a pathogen-free facility under standard conditions (12-hour light-dark cycle 0700/1900hrs, $21 \pm 1^\circ\text{C}$, humidity $50 \pm 5\%$). Food and water, including specific dietary administrations, were provided *ad libitum*.

All mice were obtained from Charles River, Margate, UK, and all mice were housed in the same establishment for the study's duration thereafter. This is noteworthy, as a given animal facility exerts a fixed effect on the microbiota composition of housed animals (Parker et al., 2018).

The primary use of outbred mice for this thesis was chosen against the more common use of inbred strains in biomedical science (Festing, 2014). Usage of inbred strains is based primarily on the intuition that less genetic variability will result in reduced phenotypic variability and thus more statistical power when conducting experiments. It is understandable that this compels the use of common inbred strains, conveying scientific, financial, and ethical advantages. However, a recent and comprehensive systematic review (Tuttle et al., 2018) suggested that outbred mice showed no greater phenotypic variability than inbred mice across behavioural and other experiments. Excluding the specific requirements of inbred mice for precise genotypic regulation or standardisation for genetic, immunological, and disease models, outbred mice may be the way forward for biomedical research, given their cost-effectiveness. Moreover, the use of robust and diverse animals should be encouraged, so that any conclusions obtained from a set of experiments can be more generalised across animal conditions and populations. Background genetic variation may in fact buffer against inter-

laboratory effects that affect reproducibility and variability between isogenic stocks (Biggers and Claringbold, 1954; Phelan and Austad, 1994; Jensen et al., 2016).

2.2 Animal husbandry

Four-week-old male and female CD-1 IGS mice were purchased and housed four per cage (separated by sex) in individually ventilated cages (IVCs). Mice were habituated to the animal facility for one week before being randomly assigned to a treatment group. These mice comprised the parent (F0) generation. At the time of mating, cages were split into male and female pairs, whereby males were randomly assigned to a female. Mating pairs were checked at 0800hrs each day for the presence of vaginal plugs. The morning a vaginal plug was detected was designated as gestational day (E)0.5. F0 males were removed from the cage the following day.

F0 maternal mice were weighed twice a week prior to the start of gestation. The first filial (F1) generation were weighed every third day until weaning on postnatal day (PND)21. The F1 adult cohort were maintained in same-sex IVCs (5-6 per cage) and weighed once per week thereafter. Given that this housing arrangement was from juvenile age, no inter-male fighting was observed between male mice.

2.3 Treatments

2.3.1 Probiotic administration

Bio-Kult® is a multi-strain probiotic consisting of 14 live strains of bacteria (Table 2.1) and a minimum of 10 billion bacteria per gram. At the time of use it was the probiotic containing the most strains in a single product commercially available. At E0.5, female F0 mice were randomly assigned to receive either 4×10^7 CFU/mL, or sterile water as the vehicle control. This corresponded to intake of approximately 6.4 million live bacteria per gram body weight per day, based on the average daily water consumption of an adult mouse (0.12mL/g mouse/day).

Table 2.1 List of bacteria strains present in the Bio-Kult probiotic

<i>Bacillus subtilis</i> PXN® 21®
<i>Bifidobacterium bifidum</i> PXN® 23™
<i>Bifidobacterium breve</i> PXN® 25™
<i>Bifidobacterium infantis</i> PXN® 27™
<i>Bifidobacterium longum</i> PXN® 30™
<i>Lactobacillus acidophilus</i> PXN® 35™
<i>Lactobacillus delbrueckii</i> ssp. <i>bulgaricus</i> PXN® 39™
<i>Lactobacillus casei</i> PXN® 37™
<i>Lactobacillus plantarum</i> PXN® 47™
<i>Lactobacillus rhamnosus</i> PXN® 54™
<i>Lactobacillus helveticus</i> PXN® 45™
<i>Lactobacillus salivarius</i> PXN® 57™
<i>Lactococcus lactis</i> ssp. <i>lactis</i> PXN® 63™
<i>Streptococcus thermophilus</i> PXN® 66™

To prepare the probiotic, 800mg (8×10^9 colony forming units) Bio-Kult Advance® were added to 8mL sterile water and the solution thoroughly mixed. The solution was left for five minutes to allow separation, and the supernatant (~6mL) containing live bacteria was aspirated and dispensed into a sterile water bottle. Each water bottle was topped up to 200mL.

sterile water and mixed again. Water bottles were changed Monday, Wednesday, Friday, with fresh probiotic.

The dose and route of administration was chosen in line with previous research in probiotics (Dunne et al., 1999; Desbonnet et al., 2010) that demonstrate effects of the probiotics on gut microbiota composition, as well as oral administration through drinking water being the most ethically sound and minimally stressful method. Despite this, it should be noted that mice are consuming substantially more bacteria per kilogram of body weight than recommended for humans. Nevertheless, the dosage used is relatively low in comparison with other reports (Bravo et al., 2011).

2.3.2 Diets

Female F0 mice were randomly assigned to be maintained on either a high fat diet (HFD) consisting of 60% kilocalories from fat (research diet D12492), or a carbohydrate-matched control diet (CD) with 10% kilocalories from fat (research diet D12450J). Diets were purchased from Brogaarden, Denmark, and manufactured by Research Diets Inc, NJ, USA. This resulted in four conditions (CD/vehicle, HFD/vehicle, CD/probiotic, HFD/probiotic) with four dams per condition.

The HFD was chosen specifically for its ability to induce both behavioural changes (Winther et al., 2018) and perturbations to metabolism and gut microbiota ecology (Wankhade et al., 2018) in rodents. The specified control diet was chosen to minimise the variability between treatment and control groups, and to match the content of the high fat diet on all other macromolecules including sucrose.

F0 males were maintained on regular (RM1) chow prior to mating. Adult F1 offspring were maintained on RM1 chow post-weaning.

2.4 Behaviour

Established behavioural assays were used to assess differences in behaviour between treatment groups (Figure 2.1). In all cases, mice were allowed to habituate to the room for 30 minutes prior to behavioural testing. In each battery of behavioural tests, mice were assessed in the least stressful environment first, and the most stressful last. Typically, the open field test, light-dark box, and the forced swim test were performed consecutively, with about 90 minutes rest between tests for each animal. Each animal was returned to their home cage between tests. Male mice were always run before females, to avoid the scent of female mice in the apparatus that may influence their behaviour. Otherwise, the order of which animals were run was randomised. The behaviour was videotaped using a 1080p web camera (Huafu HI-Tech, China) and recorded on Apple QuickTime (Cupertino, CA). All behaviour was scored while blinded to treatment groups. Behavioural testing was completed with minimal noise disruption under controlled lighting conditions.

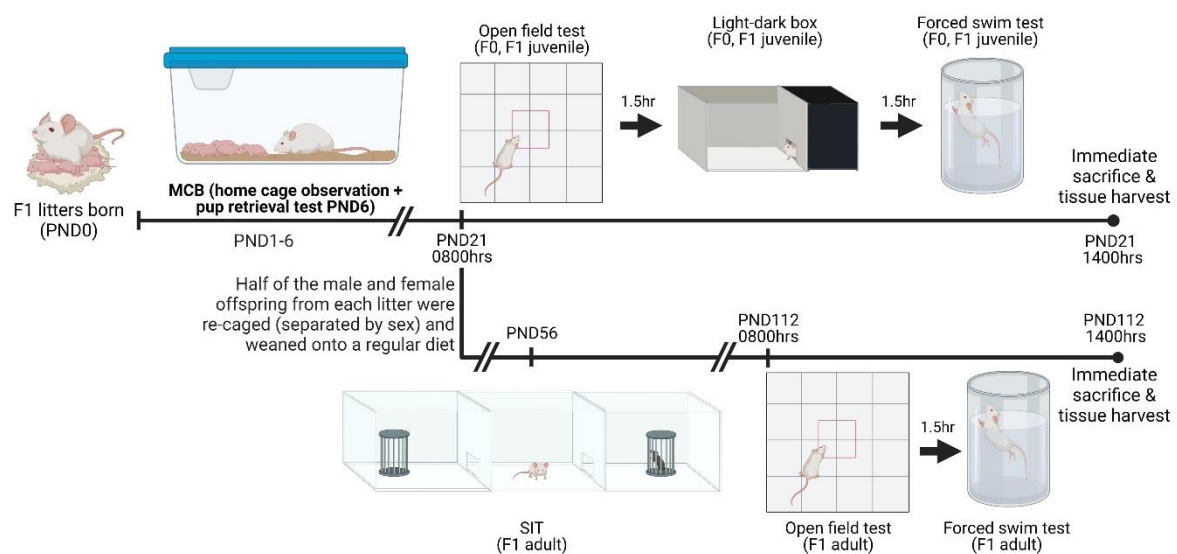


Figure 2.1 Timeline of behavioural experiments for the maternal (F0) and offspring (F1 juvenile and F1 adult) cohorts.

2.4.1 Open field test

The OFT is one of the most widely used tools in the assessment of animal behaviour. Given that rodents show innate aversions to novel, open environments, shorter assessments

of OFT activity (2-10 minutes) can sensitively detect differences in emotional 'anxiety-like' behaviour (Choleris et al., 2001). Longer or consecutive tests are more suited to assessing general activity after habituation to the novel environment. Experiments in this thesis utilised a 50x50x50cm³ (*lwxh*) open field constructed from painted wood, with a black floor and white walls. This apparatus contained four identical open fields, allowing for the simultaneous assessment of multiple animals. Lighting was fixed at 10 lux.

Introduction of mice to this environment was used to assess anxiety-like traits, primarily through quantifying the aversion of a pre-defined central zone (25x25cm²) – the most open area of the field. Aversion of open area and preference for the solid walls is known as thigmotaxis and is used as a proxy for anxiety-like behaviour. It is a commonly used parameter to screen for novel anxiolytic compounds or demonstrate anxiety-like behaviour in genetic models (Crawley, 1985; Bale et al., 2000; Prut and Belzung, 2003). Distance travelled was used to interpret activity levels displayed in a novel environment. Rearing behaviour was quantified as a measure of exploratory behaviour, which was expected to be reduced in more anxious animals. A rear was scored when the mouse lifted its forepaws and stood on its hind legs, either leaning onto the walls of the open field, or in the middle of the arena.

Here, mice were placed individually into the corner of the open field and were allowed to explore uninterrupted for five minutes. ANY-maze software (Stoelting Co., USA) was used to measure distance travelled (m), time spent in the central zone (s), and the number of entries into the central zone. The number of rears was scored manually. Each open field was cleaned with 70% ethanol between each animal tested.

2.4.2 Light-dark box

The innate preference of rodents for darker areas over lighter areas can be exploited as a measure of anxious behaviour in the light-dark box. The dissonance between a natural tendency to explore novel environments and neophobia, avoidance of the unfamiliar (particularly to more bright areas) is a mild stressor and can be used to quantify anxiety-like behaviour in a scenario that somewhat mimics a stressful environment frequently encountered

by wild mice. Increased tendency to explore the larger light compartment, which include total time spent, reduced latency to enter, increased rearing behaviour, and increased total number of entries, are interpreted as reduced anxiety-like behaviour.

Here, a small black covered compartment ($21 \times 16 \times 16 \text{ cm}^3$, $l \times w \times h$) was separated from the white, lighter (15 lux) open compartment ($46.5 \times 21 \times 21 \text{ cm}^3$, $l \times w \times h$) by a small opening ($3 \times 2.7 \text{ cm}^2$, $l \times w$). One face of this lighter compartment was transparent and faced the camera. Animals were placed gently in the dark enclosure and the lid was immediately put on. The test was run for five minutes. Time in the light compartment (s), latency to enter the light compartment with all 4 paws crossing over the threshold (s), number of crossings between compartments, and the number of rears was scored manually. The apparatus was cleaned with 70% ethanol between each animal.

2.4.3 Forced swim test

The FST is commonly used to assess depressive-like behaviour in rodents (Anyan and Amir, 2018). Antidepressants reduce immobility or 'floating' behaviour in the FST, which is the key parameter assessed (Cryan et al., 2001). In the FST, mice are placed gently into a tub of tepid water from which escape is not possible. Initially, the mice exhibit frantic escape behaviour typified by swimming around the container and climbing behaviour (peddling of the immersed paws against the side of the container). After a variable period, activity is interspersed with periods of (or continuous) immobility. This is described as a passive coping behaviour, whereby helplessness to the environment is learned (de Kloet and Molendijk, 2016). Because of this, behaviours of the forced swim test are also referred to as behavioural despair, or passive stress coping. In this thesis, these terms may be used alongside more general 'depressive-like behaviour' to describe outcomes of the FST. However, it is also noted that the FST has poor construct validity as an animal model of depression, and in recent years its use has become controversial (Reardon, 2019).

In this thesis, a rectangular box ($32 \times 17 \times 12 \text{ cm}^3$, $l \times w \times h$) was filled nine-centimetres deep with water (30°C). Light was set at 5 lux. To start the test, a mouse was placed facing

the centre of the apparatus. The first two minutes were not scored to avoid the effect of stress-induced hyper-locomotion (Strekalova et al., 2005). Time immobile (s) and the latency to first immobility (s) were scored for the last three minutes. Immobility behaviour was defined as doing the minimal amount of movement required to remain above water. Water was changed after every 2-3 mice. Mice were placed in a separate cage with paper towel for five minutes to allow for drying prior to being returned to their home cage.

2.4.4 Three-chamber social interaction test

Mice are social creatures by nature. They communicate to forage food, defend territory, nurse pups, and reproduce. While wild mice undoubtedly possess and require a more diverse and adaptable array of social behaviours, the natural tendency for laboratory mice to explore unfamiliar conspecifics over inanimate novel objects is rooted in their social instincts. Maternal peripheral immune activation, as well as various genetic defects, have been shown to reduce interaction time. Social dysfunction in humans characterised aspects of both internalising and externalising disorders, such as autism and schizophrenia. The three-chamber SIT has been used to screen mouse models of these human diseases using this paradigm (Nadler et al., 2004; Matveeva et al., 2019). In humans, anxiety and social deficits are related and may co-occur in autism. Social anxiety is an internalising behaviour associated with mood disorders (Rodebaugh et al., 2017).

The SIT used in this thesis was adopted from an established protocol (Yang et al., 2011). The apparatus consisted of a red transparent plexiglass box measuring 65x30x20 cm³ (*lwxh*). This was separated into three chambers of equal size by manually operated sliding doors. Room lighting was 0 lux. The SIT is divided into an initial habituation period followed by the testing phase. In the morning of testing (~0800-1100 hours), mice were individually placed in the central chamber with no access to the peripheral chambers. The doors were slid open after five minutes to then allow each mouse to explore the entire apparatus for a further five minutes. In the afternoon (~1300-1600 hours), each mouse was assessed individually during a ten-minute testing phase. Here, a novel C57Bl/6 mouse of the same sex was

enclosed in a small cage at the end of the right chamber. An empty cage was used as a novel object, placed in the corresponding position in the left chamber. The mouse to be tested was placed in the centre of the apparatus and the doors were immediately opened. The time spent in either the novel mouse or novel object chamber was recorded, and the social preference score calculated as time spent in the chamber with the novel mouse as a percentage of the total time exploring either of the peripheral chambers (novel mouse or novel object chambers). The apparatus, including each small cage, was cleaned with disinfectant and water (to remove odour) between each mouse tested.

2.4.5 Maternal care behaviour

Mice exhibit natural variations in postpartum maternal care (Champagne et al., 2007). Evaluating the amount and quality of maternal care behaviour (MCB) is critical when trying to comprehensively understand the influence of a maternal treatment or challenge, particularly if instigated during gestation or nursing, on offspring. This is particularly relevant if a chronic treatment, for example high fat diet, is expected to result in depressive-like behaviours during this period. Variations in MCB are known to affect the behaviour and gene expression in offspring (Jensen Peña and Champagne, 2013). Home cage observation is the primary method of assessing MCB as it is highly ethologically relevant. If performed appropriately discreet, it allows for the continuous non-disruptive assessment of maternal care. Cages can be videotaped, though live scoring is more accurate as the nest is often constructed away from the front of the cage. Similar maternal behaviours can also be difficult to discern.

Conversely, the pup retrieval test is used as a simple method of assessing maternal motivation toward her pups. This is a commonly used paradigm as it is quick and objective, quantifying the latency to retrieve pups dispersed from the nest (Carlier et al., 1982; Jensen Peña and Champagne, 2013). Because this test requires disruption to the nest, it should not overlap with home cage observation.

2.4.5.1 Home cage observation

Methods of MCB observation were adapted from a published protocol (Franks et al., 2011). Cages were not cleaned between P0 and P6 to avoid disrupting maternal behaviour. All home cage observations were carried out by the same researcher, which negated inter-researcher variability. Cages were scored live, for 4 hours daily between P1 and P6. Scoring was conducted in 3-minute intervals between 1000 and 1200 hours, and 1500 and 1700 hours. This resulted in 80 observations per day, and a total of 480 time-point observations per litter. A detailed description of scored home-cage maternal behaviours can be found in Appendix A. These included arched nursing, passive nursing, licking/grooming, self-grooming, nest building, eating or drinking (dam), climbing, or time spent passively away from the nest. The frequency of each behaviour during observation was calculated for each day and overall, from P1 to P6. Not all behaviours were mutually exclusive. Some could be conducted in parallel, for example, nursing and licking/grooming.

2.4.5.2 Pup retrieval test

After home observation of MCB was complete (1700 hours P6), the IVC was placed in an animal transfer station (biological safety cabinet) and the cage lid removed. The dam was briefly moved to a separate cage while three pups were displaced equidistant from the nest. Some disruption to the nest may have occurred to collect the pups if the nest was initially covered rather than open. The dam was then placed back on the nest, and the latency to retrieve each pup was recorded live.

2.5 Tissue collection

Following behavioural testing, animals were terminally anesthetized under isoflurane gas at 1400hrs (F0 and F1 juvenile offspring) or 1200hrs (F1 adult offspring), to minimize potential circadian variation in metabolic profiles (Figure 3.1). To collect plasma, blood was collected into lithium heparin-coated tubes via cardiac puncture with a heparinized 23G needle. Blood was left to stand for 30 minutes at room temperature, then centrifuged at 1300 x g for 10 minutes at 4°C. Plasma was stored at -80°C. Animals were transcardially perfused with cold saline (0.9% m/v) containing heparin (5000 USP/L) until the liver was clear of blood. Whole brain and liver portions were immediately dissected and snap frozen in isopentane. Faecal matter from the distal colon was collected, and all samples stored at -80°C until further use. A summary of the biological samples collected is shown in Figure 2.2.

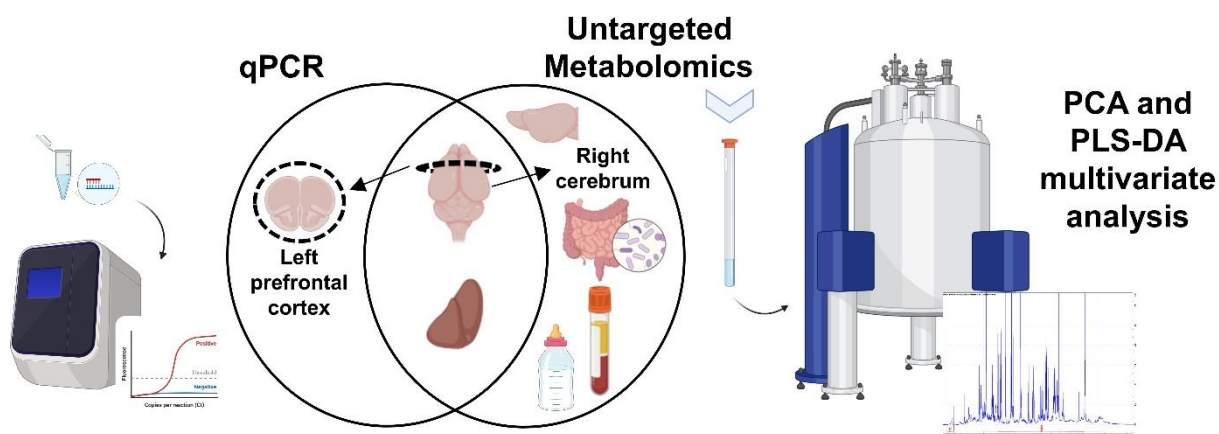


Figure 2.2 Overview of the biological samples collected for qPCR (Chapters 3-4) and ^1H NMR metabolomics (Chapters 3,5).

2.6 RNA isolation

Total RNA extraction was performed for the purpose of mRNA gene expression analysis using the QIAGEN QIAshredder and RNEasy kits as per the manufacturer's instructions. Frozen tissue weighing approximately 15mg (less for liver, more for brain) was dissected on a sterile Petri dish over dry ice. The frozen whole brain was cut into two hemispheres: the left hemisphere was thawed on ice and the prefrontal cortex (PFC) quickly dissected for RNA extraction; the right hemisphere was stored at -80°C for metabolomic analysis.

The dissected tissue was added to 300µL RLT lysis buffer containing 1% β -mercaptoethanol, homogenised for approximately 20 seconds using a pestle homogeniser (Kontes, UK), and then kept on ice. Another 300µL lysis buffer was added to the tissue lysate, which was then vortexed for 10 seconds. Half the volume (300µL) was transferred into a QIAshredder spin column. This was centrifuged for 3 minutes at full speed. All centrifugations were performed at room temperature (20°C). The remaining lysate was transferred to the spin column and centrifugation repeated. The QIAshredder column was discarded while the filtrate was mixed with an equal volume of ethanol (50% for liver, 70% for other samples). Half of this mix was transferred to an RNEasy spin column and centrifuged for 30 seconds at 10,000 rpm. The filtrate this time was discarded. This was repeated with the remaining sample. RW1 buffer was added to wash the column by centrifugation for 30 seconds at 10,000 rpm. This was followed by two washes with RPE buffer, the first for 30 seconds and the second for 2 minutes at the same speed. The spin column membrane was then dried by placing the column in a clean tube and centrifuging at full speed for a further minute. Finally, the RNA was eluted into a clean tube by adding nuclease free water (30µL for brain, 50µL for liver) to the membrane and centrifuging for one minute. The quantity and quality of RNA was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, UK). Extracted mRNA was deemed acceptable for downstream application in cDNA conversion and qPCR if the ratio of sample absorbance at 260nm and 280nm was ≥ 2 , and the ratio of sample absorbance at

260nm and 230nm was ± 1.8 -2.2. RNA not used immediately for downstream molecular biology was stored at -80°C .

2.7 DNA isolation

Faecal DNA isolation was performed for F0 maternal dams, using the QIAGEN QIAamp® Fast DNA stool mini kit as per the manufacturer's instructions. Approximately 200mg of stool was weighed into a sterile 2mL microcentrifuge tube over dry ice. The weighed sample was then placed on ice, and the remaining faecal matter was kept frozen for downstream metabolomic analysis. 1mL InhibitEX buffer was added to each sample. Samples were then homogenised with a pestle homogeniser before being centrifuged at room temperature for one minute at maximum speed. 15µL Proteinase K was then added to a new 1.5mL microcentrifuge tube, and 200µL supernatant from the faecal sample was added. 200µL lysis buffer (Buffer AL) was added, and the tube was vortexed for 15 seconds to form a homogeneous solution.

Samples were incubated at 70°C using a heat block for 10 minutes. 200µL absolute ethanol was added to the lysate, which was then mixed by vortexing. The solution (max 600µL) was added to the QIAamp spin column and centrifuged for one minute (room temperature, max speed). The collection tube containing the filtrate was discarded.

500µL wash buffer (Buffer AW1 and Buffer AW2) were consecutively added to the spin column, first centrifuging for one minute and then three minutes under the same conditions (room temperature, max speed). A final three-minute spin without wash buffer was also used to dry the column. A new collection was used and discarded after each spin.

Finally, DNA was eluted from the column using 200µL elution buffer (Buffer ATE), pipetted directly onto the column membrane. Care was taken not to touch the membrane with the pipette tip. The column was left for one minute at room temperature before being centrifuged for one minute to elute the DNA into a sterile microcentrifuge tube.

DNA concentration was measured using a NanoDrop 1000 spectrophotometer using the elution buffer as a blank.

2.8 cDNA conversion and quantitative real-time polymerase chain reaction (qPCR)

The high-capacity reverse transcription kit (Thermo Fisher Scientific, UK) was used to convert mRNA to cDNA. Thawed RNA was quantitated again using a NanoDrop prior to cDNA conversion. 1000ng RNA was used in combination with the reverse transcription master mix. Samples were centrifuged briefly before being added to a thermocycler (Bio-Rad, UK) for incubations at 25°C for 10 minutes, 37°C for 2 hours, and 85°C for 5 minutes. cDNA samples were stored at -20°C or kept on ice for downstream qPCR.

qPCR was performed using a LightCycler® 480 instrument (Roche Diagnostics, UK) with SYBR green master mix (Primerdesign, UK or Bio-Rad Laboratories Ltd, UK). 25ng cDNA was used per reaction. Primers were purchased from either Primerdesign, Integrated DNA Technologies (IA, United States), or Bio-Rad Laboratories Ltd and used at a working concentration of 300nM. *GAPDH* was used as the reference (housekeeping) gene for gene expression in the liver and brain, whilst *16S* was used as the reference gene for the relative quantification of bacteria in faeces. Primer sequences used are shown in Table 2.2. The generation of a melt curve was included after each run to confirm only a single PCR product was generated for a given reaction. Samples were run in duplicate, and $2^{-\Delta Ct}$ was calculated for each sample, where $\Delta Ct = (Ct_{\text{target gene}} - Ct_{\text{reference gene}})$. Fold change in gene expression was calculated by dividing the $2^{-\Delta Ct}$ of each treatment sample by the average $2^{-\Delta Ct}$ of the relevant control samples.

Table 2.2 List of primers for qPCR

Gene	Forward sequence (5' to 3')	Reverse sequence (5' to 3')	Reference
<i>GAPDH</i>	AACGACCCCTTCATTGAC	TCCACGACATACTCAGCAC	PrimerDesign
<i>TNF</i>	GCCTCCCTCTCATCAGTTCTAT	TTTGCTACGACGTGGGCTA	PrimerDesign
<i>IL-1B</i>	CAACCAACAAGTGATATTCTCCAT	GGGTGTGCCGTCTTTCATTA	PrimerDesign
<i>IL-6</i>	TCCATCCAGTTGCCCTCTTG	GGTCTGTTGGGAGTGGTATC	PrimerDesign
<i>TLR4</i>	CTGGCTAGGACTCTGATCATG	GCATTGGTAGGTAATATTAGGAACTA	(Strekalova et al., 2015)
<i>SAA-2</i>	TGGCTGGAAAGATGGAGACAA	AAAGCTCTCTCTTGCATCACTG	PrimerDesign
<i>BDNF</i>	GGATATTGCGAAGGGTTATTAGATT	GGAAGGTAATGTGTCTTGTGTTGAA	PrimerDesign
<i>5-HT_{1A}</i>	AACCAAGTTTGTGTCTCTCA	AGCACCTAAATAATTTTCTTCTCTGA	PrimerDesign
<i>5-HT_{2A}</i>	CAGGCAAGTCACAGGATAGC	TTAAGCAGAAAGAAAATCCACAG	(Hebert et al., 2021)
<i>5-HT₆</i>	CCTGACCCTCGGCATCCTG	GGTTCATGGTGCTATTACAGTATCC	PrimerDesign
<i>GLUN1</i>	CCAGACTAAAGATAGTGACAA	ACCATTGACTGTGAACTC	PrimerDesign
<i>GLUN2A</i>	GCTTTCCTTGAACCTTCAG	GGGGAGCTTTCCCTTTGGCTAAGTT	(Hebert et al., 2021)
<i>GLUN2B</i>	TTGGTGAGGTGGTCATGAAG	GGCTCTAAGAAGGCAGAAGGT	
<i>GLUN2C</i>	GGTTGCCATCACTGTCTTCA	CCACACGGACTTGCCAAT	PrimerDesign
<i>CREB1</i>	AGTGAAGTGAAGGCTTGTACCA	TGTGGCTGGGCTGAAC	(Iñiguez et al., 2021)
<i>GSK3B</i>	GACAAGCATTTAAGAACCAGAGA	ACCAGGTAAGGTAGACCTACATC	
<i>PSD-95</i>	TCTGTGCGAGAGGTAGCAGA	AAGCACTCCGTGAACCTCTG	(Zhao et al., 2021)
<i>SYP</i>	BioRad unique assay ID: qMmuCID0023269		BioRad
<i>ATP1A2</i>	BioRad unique assay ID: qMmuCID0024259		BioRad
<i>PFKFB3</i>	BioRad unique assay ID: qMmuCID0014746		BioRad
<i>cFOS</i>	AACCGCATGGAGTGTGTTGTTCC	TCAGACCACCTCGACAATGCATGA	(Iñiguez et al., 2021)
<i>ΔFOSB</i>	AGGCAGAGCTGGAGTCGGAGAT	GCCGAGGACTTGAACCTCACTCG	
<i>ZIF-268</i>	TCGGCTCCTTTCCTCACTCA	CTCATAGGGTTGTTGCTCGG	
<i>16S</i>	CGGCAACGAGCGCAACCC	CCATTGTAGCACGTGTGTAGCC	(Denman and McSweeney, 2006)
<i>Lactobacillus</i>	GGAAACAGRTGCTAATACCG	CACCGCTACACATGGAG	(Heilig et al., 2002)
<i>Bifidobacterium</i>	TCGCGTCYGGTGTGAAAG	CCACATCCAGCRTCCAC	(Rinttilä et al., 2004)

2.9 Metabolomic Profiling

Metabolomics enables the measurement of all small molecules present in a sample, under a set of circumstances. Proton nuclear magnetic resonance (^1H NMR) spectroscopy determines the chemical shift and relative abundance of ^1H within molecules. Because of this, deuterium (^2H) is used as an NMR buffer solvent to avoid interfering with ^1H signals arising from the sample. One-dimensional (1D) NMR is a common technique used to identify metabolites in this way. However, because the resonance peaks of some metabolite signals overlap and become indistinguishable from each other, 2D ^1H NMR may be used to identify all coupling networks between protons in a molecule, thus elucidating ambiguous metabolite signals. A combination of literature values, references to the human metabolome database (HMDB), and use of 2D correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY) were used to assign peaks in NMR spectra to a metabolite.

When it comes to metabolite extraction, there is no gold standard. Rather, the method for extracting metabolites when performing non-targeted metabolomics analysis depends on the category of metabolites to be analysed, and desired phases of extraction (e.g., aqueous, organic). Extraction methods should otherwise reflect the diversity and abundance of metabolites present in a biological sample, while being reproducible and straightforward. Generally, for non-targeted metabolomics it is desirable to capture a broad range of low molecular weight metabolites, while removing protein, nucleic acid, and any other compounds that may pollute the spectrum.

2.9.1 Sample preparation

Samples were prepared using an optimised approach based on previously published methods (Waters et al., 2001). For the metabolomic extraction and analysis of rodent brain tissue, methods developed in-house were shown to have minimal technical variation (Appendix B) following the approach of Parsons et al. (2009). Samples of one type from multiple groups were extracted altogether to minimize batch variation. Tissue and biofluid-specific extraction methods are described below.

2.9.1.1 Brain and liver

Between 100 and 125mg of fresh snap-frozen liver or brain (right cerebrum, excluding cerebellum and brainstem) was homogenised with a pestle and mortar on dry ice. Grinding dry tissue is more reproducible than homogenising wet tissue (Lin et al., 2007). Equipment was wiped using a dry paper towel to remove particulate matter between samples. Each ground sample was diluted 8-fold ($\mu\text{L}/\text{mg}$) in 50% acetonitrile (Sigma; v/v) in distilled water and further homogenised by vortex. Samples were centrifuged at $5,060 \times g$ for 5 minutes at 4°C . $750\mu\text{L}$ of supernatant was collected into a fresh tube, lyophilised, and stored at -80°C until the day of NMR analysis. Lyophilized brain and liver tissue samples were resuspended by vortex in $600\mu\text{L}$ of 75mM 'NMR buffer' (5:1 disodium phosphate [Na_2HPO_4] and monosodium phosphate [NaH_2PO_4] in 100% D_2O , pH = 7.4). Samples were centrifuged at $2,500 \times g$ for 5 minutes at 4°C to remove any particulate matter, before being transferred to a 5mm borosilicate NMR tube (Norell) using a glass pipette dropper.

2.9.1.2 Plasma

$100\mu\text{L}$ plasma was added to $500\mu\text{L}$ 75mM NMR buffer and mixed by vortex. This was transferred to a 5mm borosilicate NMR tube (Sigma) using a glass pipette dropper.

2.9.1.3 Faecal

Faecal metabolites were extracted as described previously (Hebert et al., 2021). ~30mg of starting material was used from juvenile mice, and ~75mg from F0 and F1 adult mice. Material was taken directly from the colon and multiple faecal pellets were collected and used for each mouse. 30mg faecal material was homogenised in $500\mu\text{L}$ NMR buffer, and 75mg in $750\mu\text{L}$ buffer, using a mechanical pestle homogeniser (DWK Life Sciences) and centrifuged at $16,100 \times g$ for 15 minutes at 4°C . The supernatant was collected into a clean Eppendorf and centrifuged again. $550\mu\text{L}$ was aspirated and added to $50\mu\text{L}$ 3-(trimethylsilyl)propionic-2,2,3,3- d_4 acid (TSP) in D_2O stock (0.05% by weight) to be used as an external reference. The final concentration of TSP in each sample was $5\mu\text{g}/\text{mL}$. Samples

were kept at -80°C until the day of NMR analysis, where the sample was added to a 5mm borosilicate NMR tube (Norell) using a glass pipette dropper.

2.9.1.4 Milk

At postnatal day (PND)4, pups from each litter were observed until the cessation of a nursing period. One pup from each litter was killed by cervical dislocation and the stomach rapidly dissected via visualisation of the 'milk spot', indicating a stomach full of recently ingested milk. The milk was transferred to a small tube, weighed, and diluted 8-fold ($\mu\text{L}/\text{mg}$) in 50% acetonitrile (Sigma; v/v) in distilled water. This mixture was homogenised using a mechanical pestle homogeniser, and centrifuged for 10 minutes at $13,000\times g$ at 4°C . 600 μL of supernatant was collected, and the remainder of supernatant discarded. The pellet was resuspended in the same volume of 50% acetonitrile, centrifuged, and another 600 μL of supernatant was collected. 1.2mL supernatant was lyophilised and stored at -80°C until the day of NMR analysis. Lyophilized milk samples were resuspended by vortex in 600 μL of 75mM NMR buffer. Samples were centrifuged at $2,500\times g$ for 5 minutes at 4°C prior to being transferred to a 5mm borosilicate NMR tube (Norell) using a glass pipette dropper.

2.9.2 ^1H Nuclear Magnetic Resonance (NMR) spectroscopy

The ^1H NMR spectra were acquired by using a 700 MHz Bruker AVII spectrometer operating at 16.4 Tesla equipped with a ^1H ($^{13}\text{C}/^{15}\text{N}$) TCI cryoprobe, as previously described (Chan et al., 2020). Sample temperature was stable at 310K. ^1H NMR spectra were acquired by using a one-dimensional nuclear Overhauser effect spectroscopy (NOESY) presaturation scheme for attenuation of the water resonance with a 2 second presaturation.

For brain samples, 32 data collections were performed. For liver, plasma, and faecal samples, an addition pulse sequence, the Wasted-II (formerly Painless-II) sequence (Aguilar et al., 2019), was applied with 32 repetitions of the pulse sequence (or 128 for juvenile faecal extracts), an acquisition time of 1.5s, a relaxation delay of 2s, and an inter-pulse delay of 287 μs . For milk and plasma, a spin-echo Carr-Purcell-Meiboom-Gill (CPMG) sequence was used under the same conditions as Wasted-II, but with a longer pulse interval of 400 μs . Milk

was run with 64 repetitions. These sequences were used for all future analyses of their respective samples. A fixed receiver gain was used for samples.

2.9.3 NMR data pre-processing

Resulting free induction decays (FIDs) were zero-filled by a factor of 2 and multiplied by an exponential function corresponding to 0.30 Hz line broadening prior to Fourier transformation. All spectra were manually phased, baseline corrected (using a 3rd degree polynomial), and chemical shifts referenced to the lactate-CH₃ doublet resonance at $\delta = 1.33$ ppm in Topspin 2.1 (Bruker, Germany), or in the case of faecal extracts to TSP at $\delta = 0$ ppm. Spectra were visually examined for errors in baseline correction, referencing, spectral distortion, or contamination and then exported to ACD/Labs Spectrus Processor Academic Edition 12.01 (Advanced Chemistry Development, Inc.). Baseline regions outside the spectrum region of interest were discounted from further analyses. All spectra of the same tissue were overlayed, and the region of each spectrum between 0.86–8.00 ppm, excluding the water region, was then binned by manually adding bins around each resonance signal, and the integral of each bin normalised to the sum of all integrals in the spectrum of each sample. Bins labelled with their corresponding treatment group and animal ID were exported to R (R Foundation for Statistical Computing; v3.3.1). Resonances were assigned by referring to the Human Metabolome Database (Wishart et al., 2018), reference to literature values (Govindaraju et al., 2000; Soininen et al., 2009; de Graaf et al., 2014; Cui et al., 2020), inspection of the 2D spectra, spiking of known compounds and 1D-TOCSY spectra.

2.10 Human subjects

The human study cohort comprised patients with non-alcoholic fatty liver disease (NAFLD) recruited from type 2 diabetes (T2DM) clinics and at-risk populations in primary care between October 2015 and August 2017, who had a fasting serum sample without freeze-thaw available for analysis ($n = 218$). The clinical characteristics of the source population have been previously described (Patel et al., 2018). Informed written consent was obtained from each patient and the protocol was approved by the Metro South Health and The University of Queensland Human Research Ethics Committees (HREC/15/QPAH/301; UQ2015001047).

2.10.1 Clinical assessment and self-reported depression

Clinical assessment included anthropometric measurements; routine hematologic, biochemical, and serologic tests; and liver ultrasound. Transient elastography was performed using FibroScan technology (Echosens, Paris, France) as previously described (Patel et al., 2018). Medical history was obtained during the initial consultation in the liver clinic using a structured questionnaire (Appendix C). Question items included self-reported sociodemographic characteristics, use of medications, previously diagnosed liver disease, and medical conditions including a clinical diagnosis of depression and whether they had depressive symptoms in the previous 12 months.

2.10.2 Metabolomic profiling of human serum

Fasted blood samples (minimum three hours) were collected on the morning of clinical assessment into BD Vacutainer tubes (BD 368968, NJ, USA), allowed to clot at room temperature for 3-4 hours, and centrifuged at $1,300 \times g$ for 10 minutes at room temperature. The supernatant (serum) was immediately frozen on dry ice and stored at -80°C prior to NMR sample preparation. Serum samples were thawed at room temperature and mixed by pipetting, before 150 μL of serum was added to 450 μL of 75 mM sodium phosphate buffer prepared in D_2O (pH 7.4). Samples were then transferred to a 5mm borosilicate glass NMR tube (Norell 502-7). All NMR samples were prepared on the day of analysis by the same individual. The time between thaw and NMR data acquisition did not exceed 9 hours. All NMR

experiments were acquired using a 700-MHz Bruker AVIII spectrometer as described in section 2.9.2 and published methods (Probert et al., 2018). Briefly, ^1H NMR spectra were acquired first using a 1D NOESY presaturation scheme (2 second presaturation). A spin-echo CPMG sequence with a pulse interval of 400 μs , 80 loops, an acquisition time of 1.5 seconds, 32 data collections, a relaxation delay of 2 seconds and a fixed receiver gain was used to suppress broad signals which occur from large molecular weight compounds in serum. This allowed us to quantify small molecular weight metabolites whilst providing some information on the relative abundance of different lipoprotein subclasses. ^1H COSY spectra were acquired on at least one sample in each group to aid in metabolite identification. For quality control, pooled plasma samples were spread throughout the run to monitor technical variation.

2.11 Statistical analysis

2.11.1 Rodent parametric univariate analyses

Statistical tests were performed in Prism 8 or 9 (GraphPad) and R (version 4.0.3). Initially, in the offspring, three-way analyses of variance (ANOVAs) were performed, to identify whether sex interacted with maternal diet and/or maternal perinatal probiotic intake, affecting the dependent (outcome) variable. If a statistically significant interaction with sex occurred, males and females were analysed separately with two two-way ANOVAs. Where no main effects of, or interactions with sex occurred, male and female data were consolidated into a single two-way ANOVA. In all cases, the number of male and female offspring within each group were similar and have been visualised in each figure. These processes served to simplify the interpretation of the main effects and their interaction. Prior to three-way or two-way ANOVA, Grubbs' test for outliers was performed on each group, and statistically significant outliers ($p < 0.05$) were removed from downstream analyses for behaviour, gene expression analysis, and metabolomics (where univariate analysis occurred). Normality of residuals was tested with the Shapiro-Wilk test ($p < 0.01$). Alternative tests were used for nonparametric data (see section 2.11.2). All univariate data were visualized as boxplots showing all data points, where the upper and lower bounds of the box indicate the interquartile range, the line within the box as the median, and the error bars denoting the full range of values. The significance threshold was set at $p < 0.05$ for behaviour and $q < 0.05$ for univariate metabolomic and Spearman rank correlation analyses adjusted for multiple testing using the false discovery rate method (Benjamini and Hochberg). For offspring gene expression data, significance was set at $p < 0.001$ using the Bonferroni method based on 50 independent tests. Mixed-effects models were used to assess the main effects of maternal diet and probiotic treatment on maternal and offspring weight over time. Tukey's correction for multiple comparisons were used to investigate post hoc comparisons when the interaction effect of a two-way ANOVA reached significance. If there was no significant interaction, only main effects were reported, and post hoc comparisons were not investigated.

2.11.2 Rodent nonparametric univariate analyses

Spearman's rank correlation test for heteroscedasticity was used to assess whether larger values of the outcome variable tended to have larger residuals, thus invalidating ANOVA. Significance was set at $p < 0.05$, the null hypothesis being homoscedasticity. The Shapiro-Wilk test for normally distributed data was also used, where $p < 0.01$. Tukey's correction for multiple comparisons was performed on normally distributed, homoscedastic data, to investigate group differences. Data that rejected the null hypothesis of a Gaussian distribution or heteroscedasticity were either log- or square root-transformed. This was most often the case for behavioural data. If these transformations still failed to satisfy the assumptions required to perform ANOVA, robust analogous ANOVAs using 20% trimmed means were run in R using the WRS2 package (Mair and Wilcox, 2020). These generated a Q statistic, analogous to the F statistic in 2-way ANOVA. This allowed the computation of nonparametric main effects and interactions.

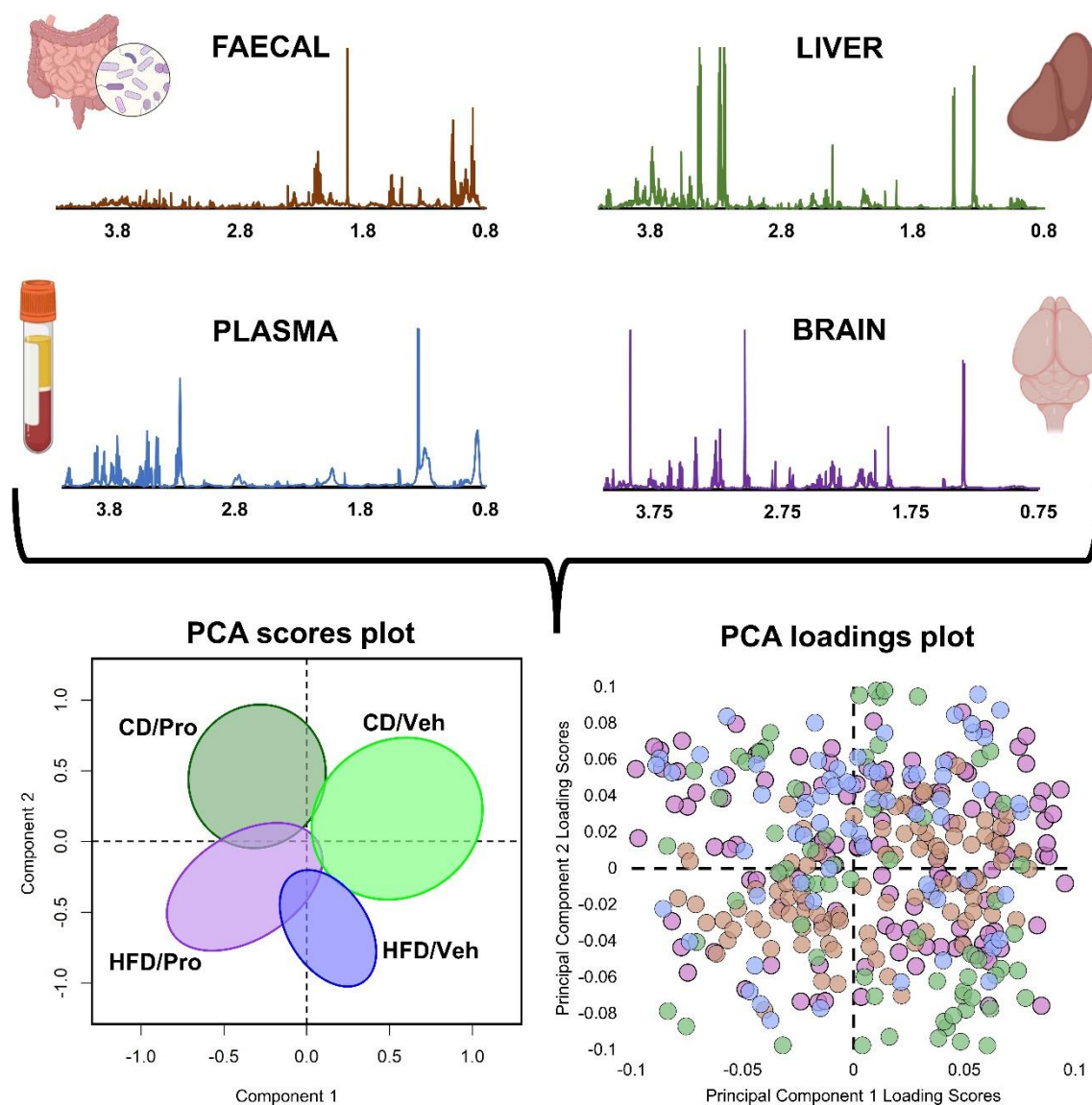
2.11.3 Rodent multivariate analyses (PCA and PLS-DA)

Metabolomic analysis was performed as described previously (Chan et al., 2020; Hebert et al., 2021) using in-house scripts and the ropls package (Thévenot et al., 2015). Principal component analysis (PCA) incorporated all metabolomic data from brain, liver, faeces, and plasma, and was used to initially visualise group differences in an unbiased manner (Figure 2.3). PCA was performed for individual tissues to inspect the data and identify outliers. Partial least squares discriminant analysis (PLS-DA) was then used to investigate group differences in the metabolome of each tissue/biofluid, and importantly, which variables (metabolites) contributed most to the variability between groups (e.g., maternal obesity vs lean, or maternal probiotic intake vs vehicle control). The PLS-DA analysis workflow is depicted in Figure 2.4.

RODENT METABOLOMICS ANALYSIS WORKFLOW

A Integrated principal component analysis (PCA)

- Visualise unsupervised variation between groups
- Investigate metabolites across the gut-brain axis that are driving unsupervised group separation



- Colours in the scores plot represent different experimental groups.
- Colours in the loadings plot represent metabolites from the different tissues/biofluids.

Figure 2.3 Rodent metabolomic analysis workflow for principal component analysis.

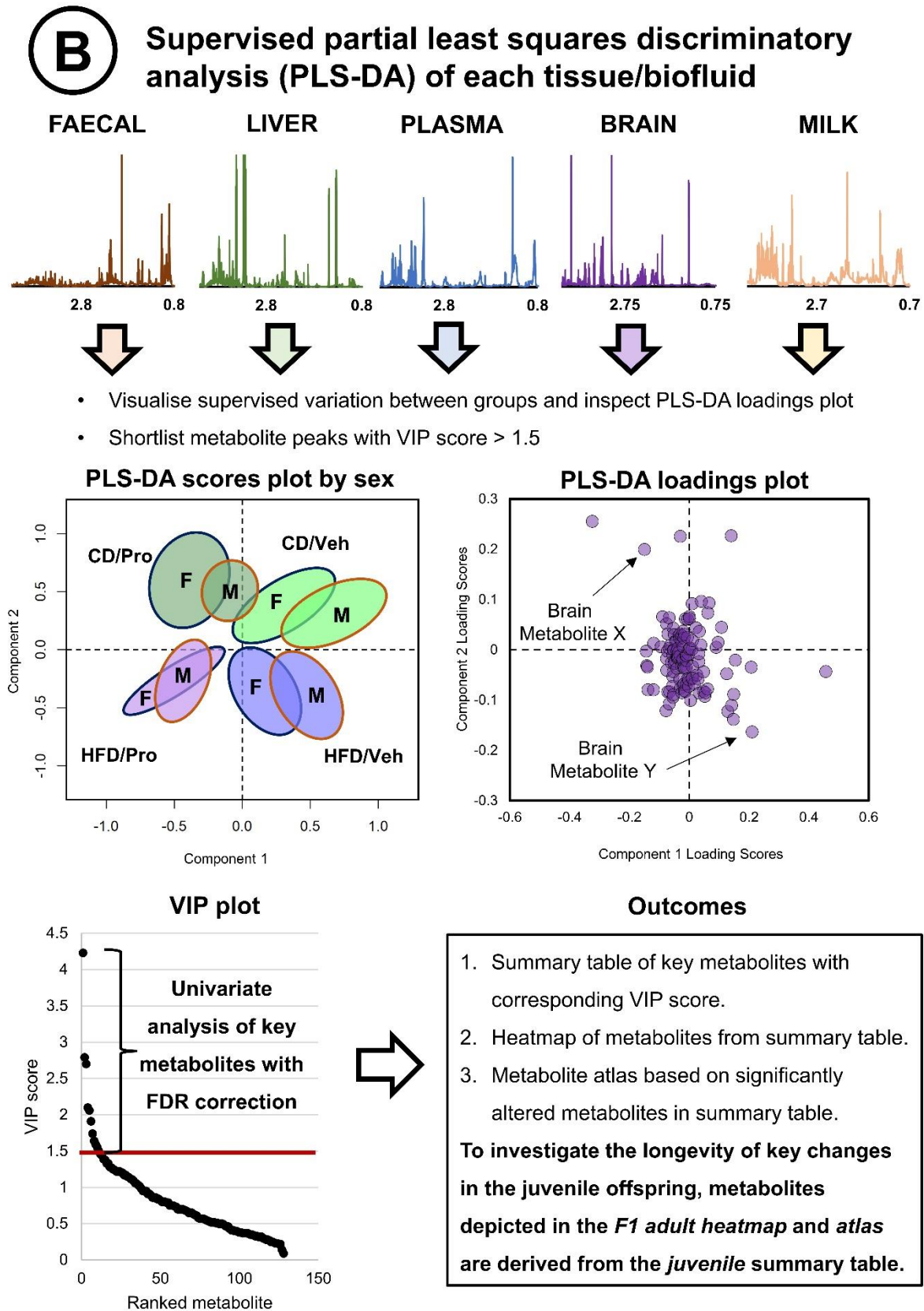


Figure 2.4 Rodent metabolomics analysis workflow for partial least squares discriminatory analysis. The Benjamini and Hochberg method of correction was applied to the univariate analysis of metabolites in each tissue/biofluid.

Metabolites from each PLS-DA with a variable importance in projection (VIP) score greater than 1.5 were considered important to the variability between groups and subject to univariate analysis. This is a common threshold used in the literature for metabolomics analysis using PLS (Chiu et al., 2016). Other metabolites were subject to univariate analysis independent of VIP score based on *a priori* interest. These metabolites included acetate, butyrate, and propionate, which are key products of microbial metabolism capable of altering host gene expression (Stilling et al., 2016). Additionally, metabolites with a VIP score > 1.5 in the *juvenile* offspring were interrogated in the *adult* offspring regardless of VIP score (Appendix H).

F0 and F1 juvenile metabolomic heatmaps were generated from the compilation of metabolites with a VIP score greater than 1.5 from each tissue/biofluid. These same metabolites are in the corresponding summary table in each Chapter. Sum-normalised spectral bins corresponding to each metabolite were z-scaled. Male and female metabolites were z-scaled separately in both juvenile and adult offspring, to remove sex as a confounder in the adult offspring. To allow for a direct comparison between juvenile and adult offspring, the F1 adult metabolomic heatmap was generated using the same list of metabolites as the F1 juvenile heatmap as described in Figure 2.4.

Tabulated descriptive statistics for the rodent metabolomics data, along with access to the raw metabolomics dataset, can be found at <https://doi.org/10.1073/pnas.2108581119> under [Data Availability](#).

2.11.4 Human univariate analyses

Significance level was set at $p < 0.05$. Individual biochemical parameters were analysed using one-way analysis of variance or the Chi-squared test for categorical data. Resonance peaks from the NMR spectrum corresponding to metabolite(s) were log-scaled and outliers were identified using Grubb's method of detection ($Q = 1\%$). Metabolites with a VIP score >1.5 were then analysed using the Brown-Forsythe ANOVA and p-values were adjusted for multiple comparisons using the Bonferroni method. Post hoc comparisons were

performed using Dunnett's test. Receiver operating characteristic (ROC) curves were built using the pROC package in R (Robin et al., 2011) with 95% confidence interval (CI).

2.11.5 Human multivariate analyses (OPLS-DA)

Multivariate PLS-DA and orthogonal (O)PLS-DA were performed on clinical and metabolomic data. The process is described in Figure 2.3, using a PLS-DA with 3 groups as an example. A ten-fold cross validation method with 100 iterations using a random sample of matched class sizes (the training set) was employed to build an ensemble of 1000 models. As part of this cross validation, an independent test set corresponding to 10% of the dataset was used to predict which class a sample belonged to, and thus the mean accuracy, sensitivity, and specificity over 1000 models was reported, $\pm$ standard error of the mean. Model performance metrics were also retrieved to assess goodness of fit, including the mean R^2X , R^2Y , and Q^2 .

(O)PLS-DA models were further validated against models built with randomly permuted data using the same external cross validation method, representing a null distribution. Models were tested against the null distribution using the two-sided Kolmogorov-Smirnov test. The most important metabolites found to be driving these group differences were identified using average VIP scores.

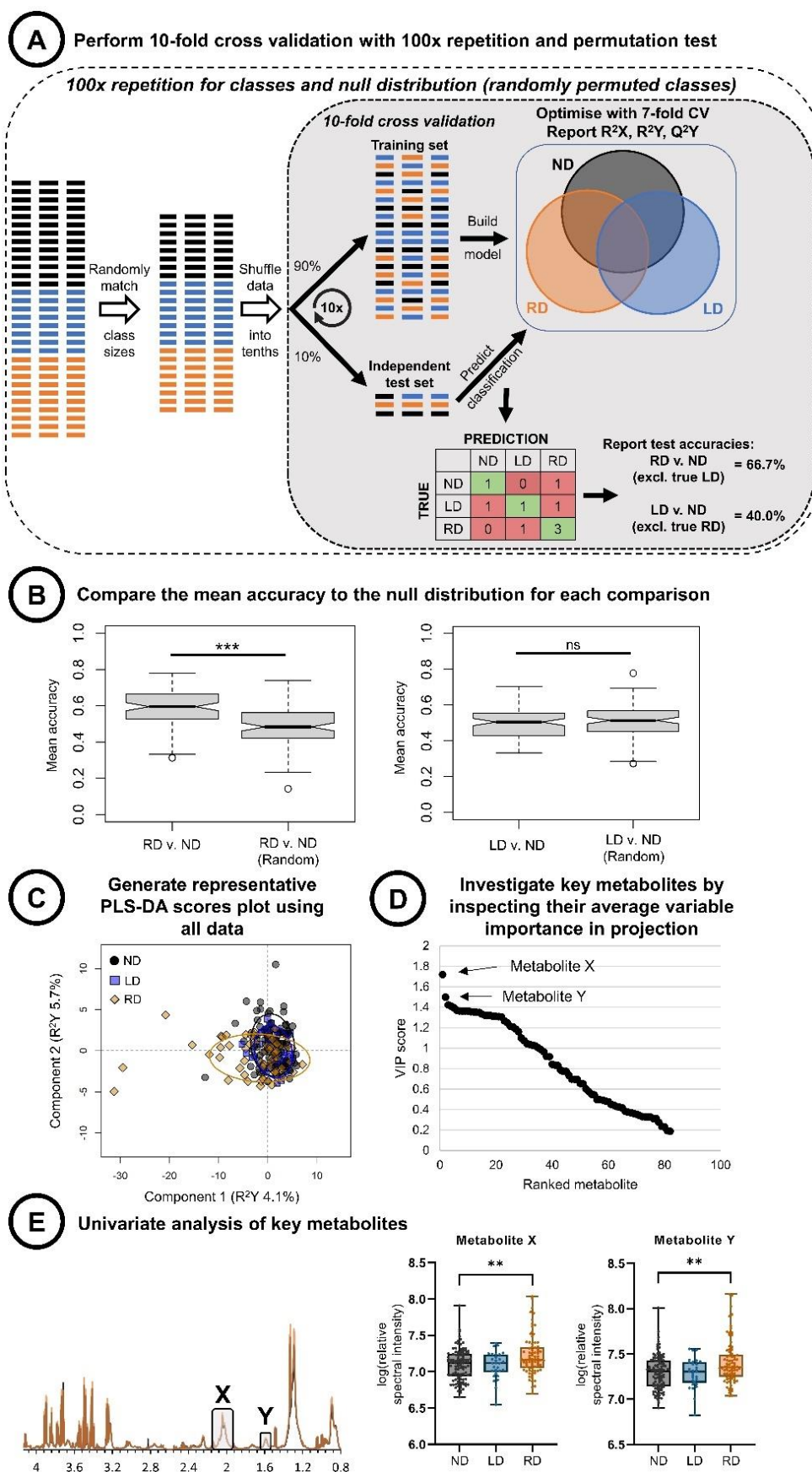


Figure 2.5 Overview of the analysis approach for the human serum metabolomics data.

3

Distinct effects of chronic high fat diet exposure and perinatal probiotic treatment on the maternal generation¹

3.1 Introduction

Obesity, depression, and anxiety are prevalent maternal complications during the perinatal period (Wisner et al., 2013; Fairbrother et al., 2015; Devlieger et al., 2016). Accumulating evidence points to a biological linkage between obesity and depression (Milaneschi et al., 2019). During the perinatal period in humans, gestational weight gain (GWG) and obesity are risk factors for maternal perinatal depression (Molyneaux et al., 2014, 2016; Catalano and Shankar, 2017; Zanardo et al., 2021), and depression may contribute to increased GWG (Hartley et al., 2015). Other reports have not linked weight gain during pregnancy and depressive symptoms in humans (Krause et al., 2009; Dachew et al., 2020), and it remains unclear what underlying mechanisms may drive the development of depression and anxiety that manifests during pregnancy.

This relationship is important because both perinatal obesity and mental illness in mothers has been suggested to impact the risk of both physical and mental disorders in the offspring (Cattane et al., 2021). This concept has been explored further in animal models (Edlow, 2017; Ortiz-Valladares et al., 2021), whereby altered behaviours in the offspring such as increased anxiety and depressive-like behaviour, and reduced social behaviour, have been attributed to maternal obesity. Most of these studies have focused on changes in the offspring, rather than investigating how adaptations to maternal care, immunity, or metabolism may instigate changes in the offspring (Sullivan et al., 2014).

¹ Results included in this Chapter have been published: <https://doi.org/10.1073/pnas.2108581119>

Dyar et al. (2018) explored the metabolomic changes induced by HFD (60% of kilocalories from fat) feeding for 10 weeks. The metabolome of multiple body sites including the medial PFC, liver, and serum were measured by MS. Over half of all metabolites detected in the liver were significantly altered in relative abundance by HFD, and 41% and 14% of metabolites were altered in the serum and medial PFC, respectively. Similarly, HFD feeding has been shown to alter brain energy metabolism (Hassan et al., 2019) and induce an overexpression of brain cholesterol (Tognini et al., 2020), glycerophospholipids, and ceramides (Sighinolfi et al., 2021).

Another possible mechanism of action is by perturbations to the maternal microbiome, which has been shown to be modified by obesity in humans (Kalbermatter et al., 2021). In rodents, maternal obesity disrupts the maternal and offspring microbiome, leading to altered offspring social behaviours. Modification of the juvenile *offspring* microbiome with a single-strain probiotic prevented aberrant social behaviour in the adult offspring (Buffington et al., 2016). In healthy, non-obese dams, the maternal microbiota support normal offspring neural (Vuong et al., 2020) and immune (Gomez de Agüero et al., 2016) development, prenatally through the transfer of microbial metabolites and immune factors to the offspring via the placenta (Vuong et al., 2020) and postnatally through the maternal milk (Gomez de Agüero et al., 2016). Thus, these studies have separately shown an adverse effect of maternal HFD on offspring social behaviour, which may be mediated by perturbations to the *offspring* microbiome, as well as the critical role of maternal microbes and metabolites on offspring development. Nevertheless, it remains unclear whether modification of the *maternal* microbiome can attenuate the adverse consequences of diet-induced obesity on aspects of maternal behaviour, inflammation, and energy metabolism. Therefore, the aim of this chapter was to characterise a model of maternal obesity induced by chronic HFD feeding and assess its impact on postpartum maternal care and affective behaviours, markers of inflammation, and perturbations to the metabolome across the gut-brain axis. In parallel, the effects of perinatal probiotic intake were assessed as a potential therapeutic. Here, it was expected that

chronic HFD consumption would be pro-depressant, anxiogenic, and pro-inflammatory. However, as the probiotic was only administered during gestation and nursing, by which time the dams were already obese after six weeks of HFD feeding, the probiotic was expected more to prevent adverse effects on *offspring* brain function and behaviour, rather than maternal behaviour.

3.2 Experimental design

An overview of the study design encompassing chapters 3-5 is summarised in Figure 3.1. Male and female mice were initially housed four per IVC, separated by sex. Each female cage was randomly assigned to receive a HFD (n = 8, 2 cages) or CD (n = 8, 2 cages), while males were maintained on regular chow. Female mice were fed the HFD for 6 weeks (between 5 and 11 weeks of age) before being arranged into mating pairs with the males in IVCs.

At the time of conception (E0.5), the probiotic supplement (or sterile water as the vehicle) was administered through the drinking water and the males were removed the following day. Maternal care behaviour was assessed between PND1 and PND6. Probiotic treatment continued until PND21. At this point, dams and approximately half the juvenile offspring (n = 13-15 per sex per group) were run through the OFT, LDB, and FST, and tissue was immediately harvested for qPCR (left prefrontal cortex, liver) and metabolomics (right cerebrum, liver, plasma, faecal matter). The remainder of the offspring were weaned onto regular chow and run through the SIT at 8 weeks of age. At PND112, adult offspring (n = 9-11 per sex per group) were run through the OFT and FST and tissue harvested for qPCR and metabolomics.

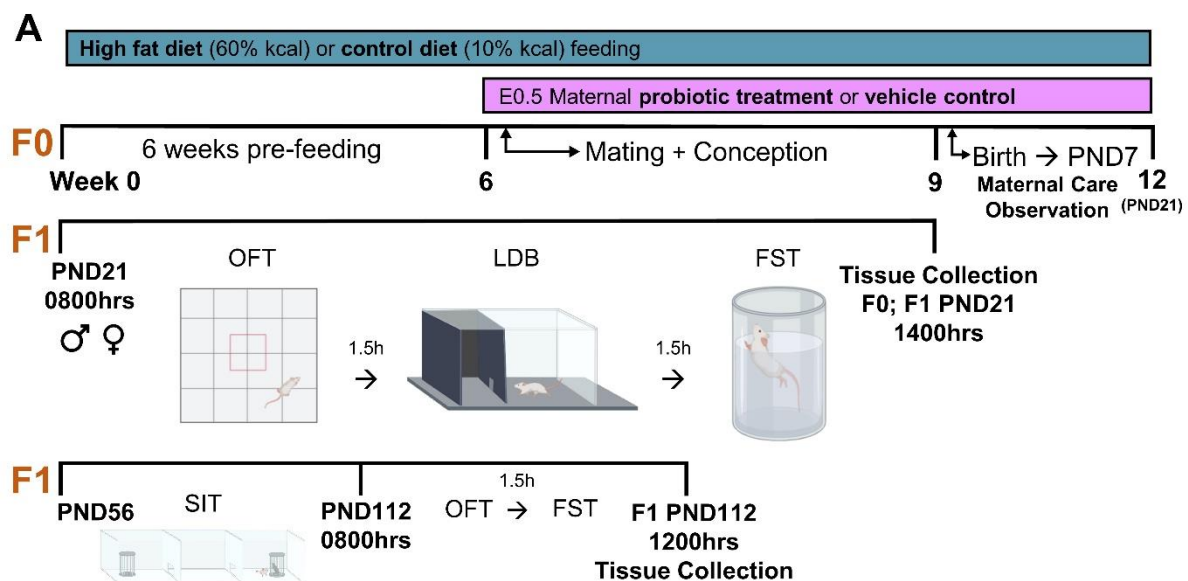


Figure 3.1 Study design.

3.3 Results

3.3.1 Chronic high fat diet exposure induces maternal obesity.

Maternal weight gain over time was analysed using a mixed effects model with fixed effects of time, high fat diet (HFD), and probiotic treatment grouping between 4 and 12 weeks of age (Figure 3.2a). Probiotic treatment did not begin until the beginning of gestation (E0.5). Sphericity of variances were not assumed. Significant main effects of both time ($F_{(2.13, 25.61)} = 82.98$, $p < 0.0001$) and diet ($F_{(1, 12)} = 9.62$, $p = 0.0092$) but not probiotic grouping ($F_{(1, 12)} = 1.24$, $p = 0.29$) were identified. This indicated that, independent of probiotic treatment grouping, HFD feeding significantly increased maternal weight. Time x probiotic ($F_{(15, 180)} = 2.82$, $p = 0.0006$) and time x diet ($F_{(15, 180)} = 11.09$, $p < 0.0001$) interactions were also identified.

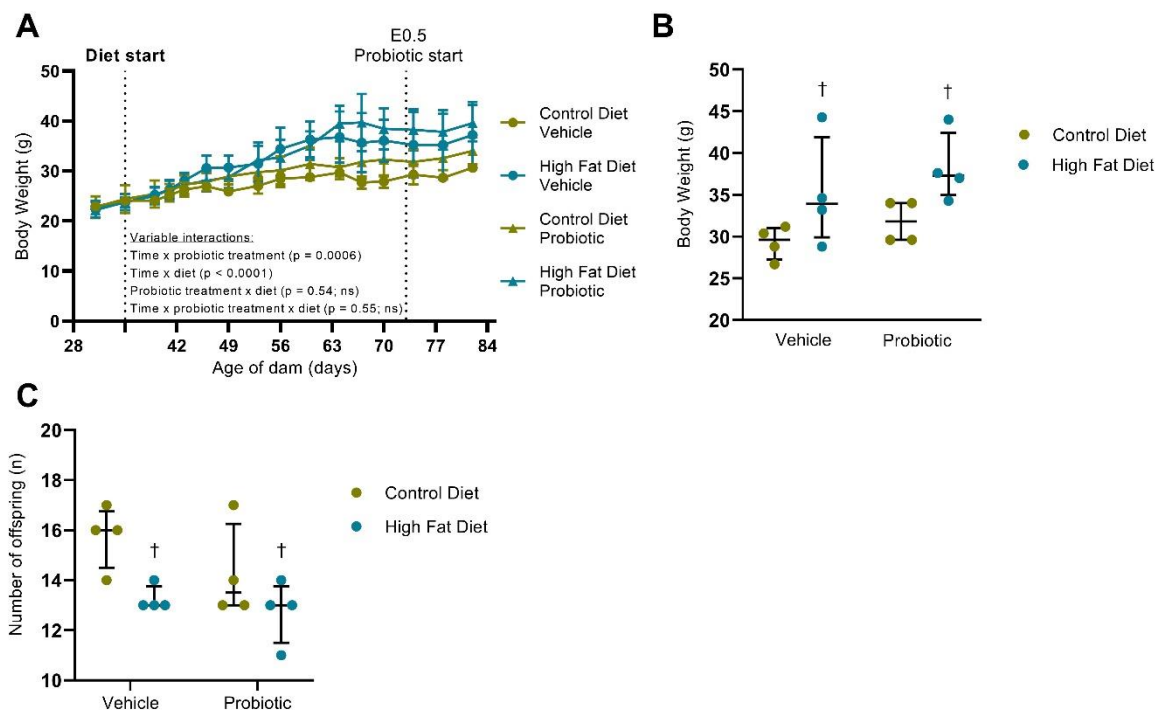


Figure 3.2 Effect of maternal diet on body weight and litter size. **(A)** Mixed-effects modelling of time, HFD, and probiotic treatment grouping on maternal body weight. Data presented as mean $\pm$ SD. **(B)** Weight difference at the time of mating (E0.5). **(C)** Effect of maternal diet on litter size. [†] $p < 0.05$, indicating main effect of diet. Data presented as median $\pm$ interquartile range showing all points. N = 4 per group.

At the time of mating, and after six weeks of dietary intervention, the female mice fed a HFD had developed an obese phenotype, defined as being >20% heavier than the control diet cohort. 2-way ANOVA confirmed this difference – at E0.5, maternal diet constituted the only weight difference between dams (Figure 3.2b; $F_{(1,12)} = 8.75$, $p = 0.012$). The size of each litter was also reduced in obese dams fed a HFD compared to lean dams fed a CD (Figure 3.2c) via a main effect independent of probiotic treatment ($F_{(1,12)} = 9.14$, $p = 0.011$). There was no significant interaction ($F_{(1,12)} = 0.57$, $p = 0.46$) and probiotic treatment had no effect ($F_{(1,12)} = 2.29$, $p = 0.16$).

3.3.2 Chronic high fat diet exposure alters aspects of maternal care behaviour during the first week of nursing.

MCB was assessed between PND1 and PND6, and the overall mean frequency of each behavioural trait during this period was used to assess differences in maternal care behaviour between groups. Few differences were observed between the groups. Two-way ANOVA revealed no main effects of gestational probiotic supplementation on any of the observed MCB traits (Figure 3.3). Arched nursing, passive nursing, and licking and grooming behaviours were also unchanged in frequency by maternal diet, with no significant interactions between diet and probiotic treatment (Figure 3.3a-c). Obese (HFD) dams spent more time passively away from the nest whilst not performing any other stereotypic behaviour compared to lean (CD) dams (main effect, $F_{(1,12)} = 11.94$, $p = 0.0048$), independent of probiotic treatment and with no interaction (Figure 3.3d). This seemed to be at the expense of the time spent building the nest, which, reciprocally, was significantly lower in obese dams ($F_{(1,12)} = 16.20$, $p = 0.0017$, Figure 3.3e). This main effect was also independent of probiotic treatment, and no interaction between diet and probiotic treatment was observed. Eating/drinking, climbing, and self-grooming behaviours were unaffected by diet or probiotic intake, with no significant interactions (Figure 3.3f-h). Lastly, a three-way mixed effects model for repeated measures was used to assess outcomes in the pup retrieval test of MCB. Pup collection (first, second, third collected repeated measures), diet, and probiotic treatment were included as fixed

effects. Only a fixed effect of pup collection was significant ($F_{(1.42, 15.6)} = 46.02$, $p < 0.0001$) but not diet ($F_{(1, 11)} = 1.24$, $p = 0.29$) or probiotic treatment ($F_{(1, 11)} = 0.003$, $p = 0.95$) and with no significant interactions (Figure 3.3i).

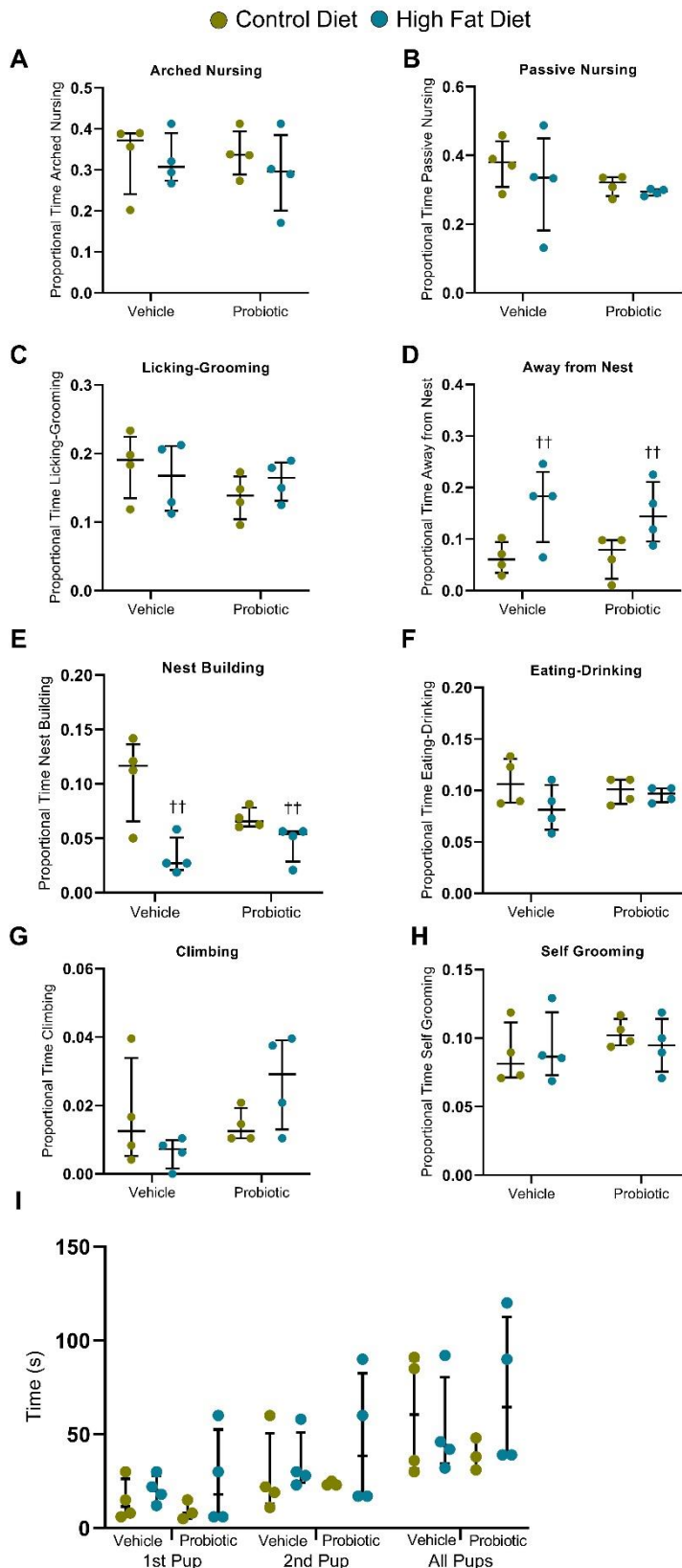


Figure 3.3 Maternal care behaviour.

(A) arched nursing, (B) passive nursing, (C) licking and grooming, (D) away from the nest whilst not performing any other stereotypic behaviour, (E) nest building, (F) eating/drinking, (G) climbing, (H) self-grooming, (I) time to retrieve pups after the period of maternal care observation. Data presented as median $\pm$ interquartile range showing all points. No significant interactions. $^{\dagger\dagger}p < 0.01$, indicating main effect of diet. $N = 4$ per group.

3.3.3 Chronic high fat diet exposure increased passive stress coping in dams.

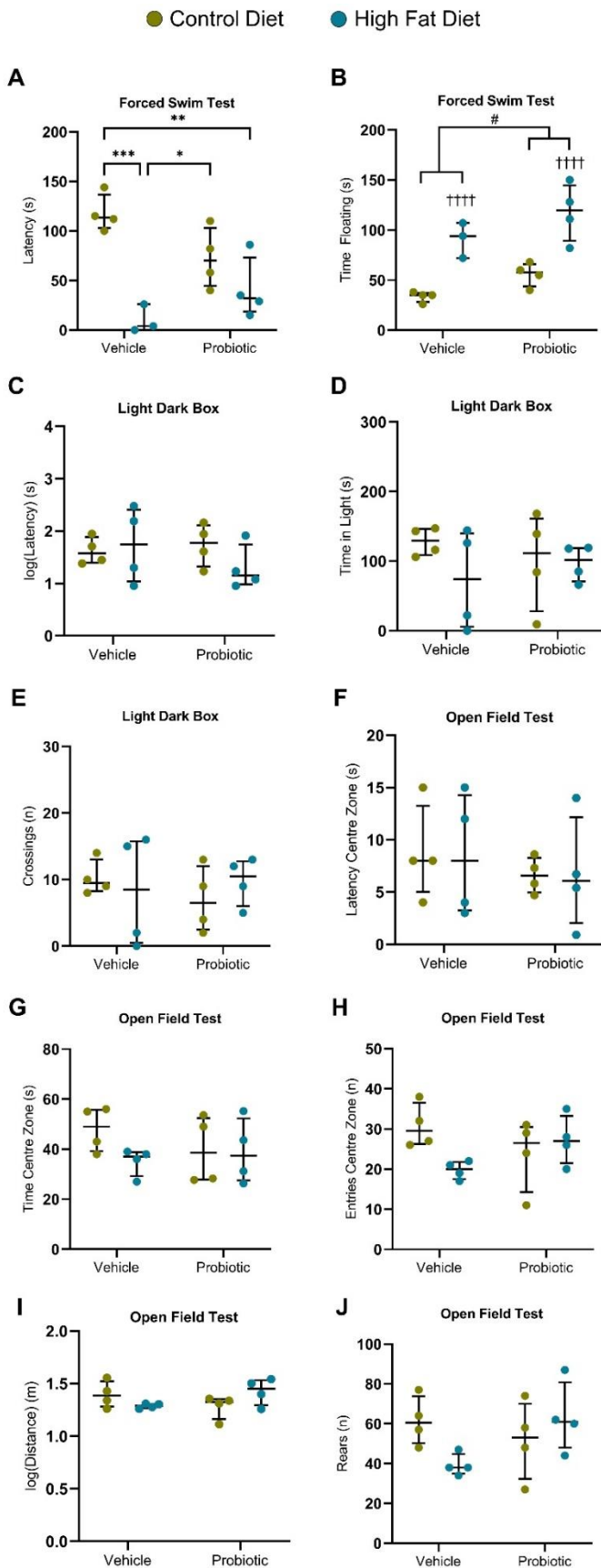
At the time of weaning (PND21), dams were assessed for postnatal anxiety- and depressive-like behaviour in the OFT, LDB, and FST. In the FST, there was a significant diet x probiotic treatment interaction for the latency to the first immobile period ($F_{(1, 11)} = 8.39$, $p = 0.015$, Figure 3.4a). A main effect of diet ($F_{(1, 11)} = 27.72$, $p = 0.0003$) was also identified. Post hoc Tukey tests revealed that CD/vehicle dams had increased latency to immobility compared to HFD/vehicle ($p = 0.0008$) and HFD/probiotic ($p = 0.0062$) dams, while CD/probiotic dams had an increased latency to immobility only relative to HFD/vehicle dams ($p = 0.035$). The time spent immobile was increased by HFD (main effect, $F_{(1, 11)} = 40.36$, $p < 0.0001$) and by perinatal probiotic intake (main effect, $F_{(1, 11)} = 6.786$, $p = 0.025$) with no significant diet x probiotic treatment interaction (Figure 3.4b). The mean increase in time spent immobile between vehicle and probiotic dams was 24.5 seconds (95% confidence interval [CI] 3.8 to 45.2 seconds), while the mean increase in time spent immobile between CD and HFD dams was 59.75 seconds (95% CI 39.05 to 80.45 seconds).

There were no differences in latency to enter light, time in light, or the number of crossings in the LDB between control and experimental maternal groups (Figure 3.4c-e).

Similarly, there were no differences between groups in the latency to enter the centre zone, total time spent in the centre zone, or number of entries to the centre zone of the OFT. Exploratory behaviour, estimated by the number of rearing events in the OFT, was also unaffected by maternal diet and probiotic treatment. Distance travelled for the duration of the OFT was unaltered by maternal diet and probiotic treatment (Figure 3.4f-j).

3.3.4 Maternal obesity alters peripheral and central markers of innate immunity postpartum.

The relative expression of mRNAs encoding inflammatory markers *TNF*, *IL-1B*, *IL-6*, *TLR4*, and *SAA-2* were investigated in the liver (Figure 3.5). Liver *TLR4* was investigated using the robust trimmed-means two-way ANOVA as homoscedasticity could not be assumed

**Figure 3.4** Chronic high fat diet exposure

increased passive stress coping in dams.

(A) Latency to immobility and **(B)** time spent immobile in the forced swim test, **(C)** latency, **(D)** time in light, and **(E)** crossings in the light-dark box, **(F)** Latency, **(G)** time, and **(H)** entries to the centre zone of the open field test, **(I)** distance travelled and **(J)** rearing in the open field test. Data presented as median $\pm$ interquartile range showing all points. †††p < 0.001, ††††p < 0.0001, indicating main effect of maternal diet. #p < 0.05, indicating main effect of maternal probiotic intake. **p < 0.01, ***p < 0.001 indicating significant post-hoc comparisons. N = 4 per group.

after square-root transformation of the data. Otherwise, expression levels were analysed using two-way ANOVA. The expression of *TNF* ($F_{(1, 12)} = 8.142$, $p = 0.015$) and *IL-6* ($F_{(1, 12)} = 17.55$, $p = 0.0013$) cytokines were reduced in the liver after probiotic intake as a main effect independent of diet with no significant interaction (Figures 3.5a and 3.5c, respectively). There was a main effect of maternal diet on liver *IL-1B* ($F_{(1, 12)} = 8.269$, $p = 0.014$) and *TLR4* ($Q_{(1, 12)} = 7.96$, $p = 0.022$) expression, which were elevated in obese dams relative to lean dams independent of probiotic treatment with no significant diet x probiotic treatment interaction (Figures 3.5b and 3.5d, respectively).

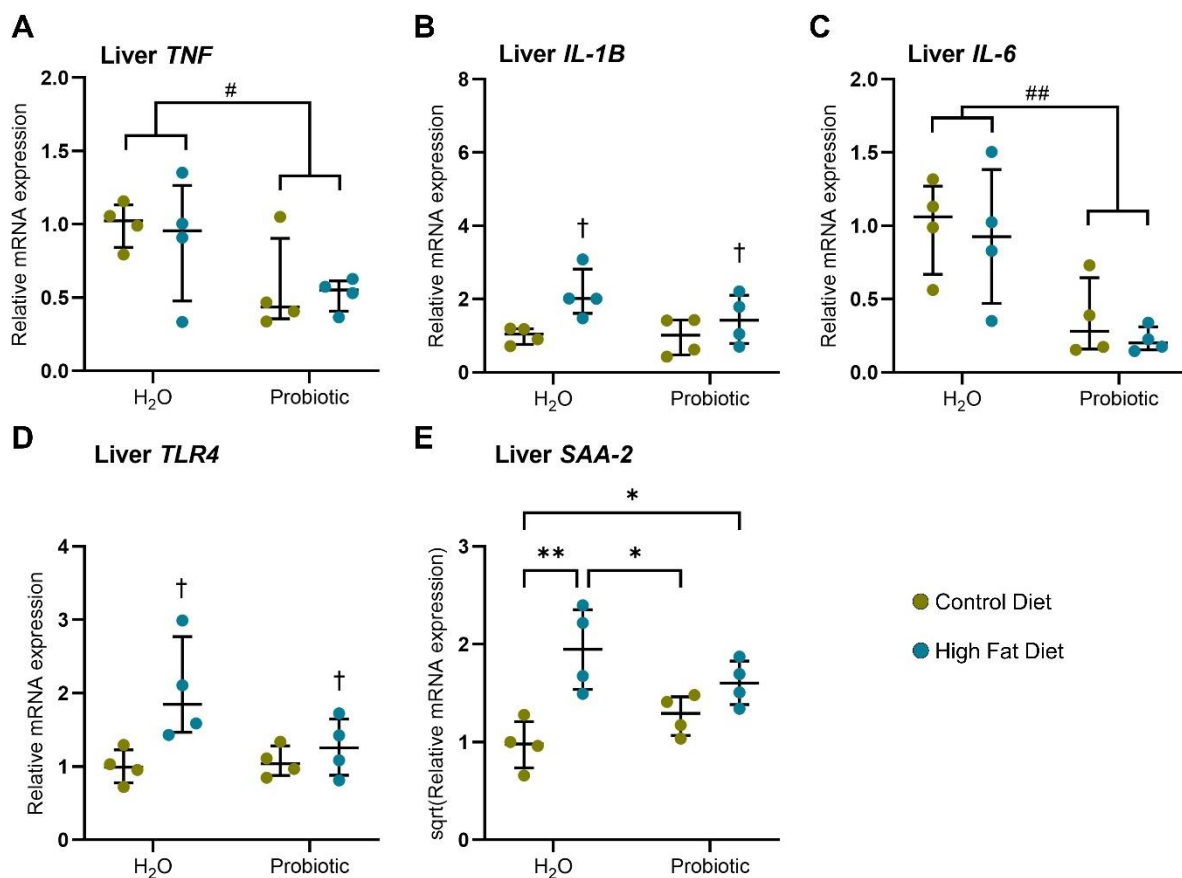


Figure 3.5 Chronic HFD feeding increases liver pro-inflammatory cytokine mRNA expression. **A)** Liver *TNF* expression was reduced by perinatal probiotic treatment, independent of diet, while **B)** *IL-1B* expression was increased by HFD feeding compared to the CD, independent of probiotic treatment. **C)** Liver *IL-6* was reduced by probiotic treatment relative to vehicle, **D)** liver *TLR4* and **E)** *SAA-2* were increased after chronic HFD feeding relative to CD. Data presented as median $\pm$ interquartile range showing all points, relative expression compared to vehicle/control diet. † $p < 0.05$, indicating main effect of maternal diet. # $p < 0.05$, ## $p < 0.01$, indicating main effect of maternal probiotic intake. * $p < 0.05$, ** $p < 0.01$, indicating significant Tukey post-hoc comparison compared to control group. $N = 4$ per group.

For the expression of liver SAA-2, a significant diet x probiotic interaction was identified ($F_{(1, 12)} = 4.76$, $p = 0.0497$). Tukey post-hoc testing revealed that HFD/vehicle dams had significantly greater expression of SAA-2 compared to CD/vehicle dams ($p = 0.0026$) and CD/probiotic dams ($p = 0.032$), while HFD/probiotic dams had significantly greater expression compared to CD/vehicle dams only ($p = 0.046$). There was also a significant main effect of maternal diet ($F_{(1, 12)} = 19.50$, $p = 0.0008$), whereby SAA-2 mRNA expression was increased in the liver of obese dams relative to lean dams regardless of probiotic treatment (Figure 3.5e).

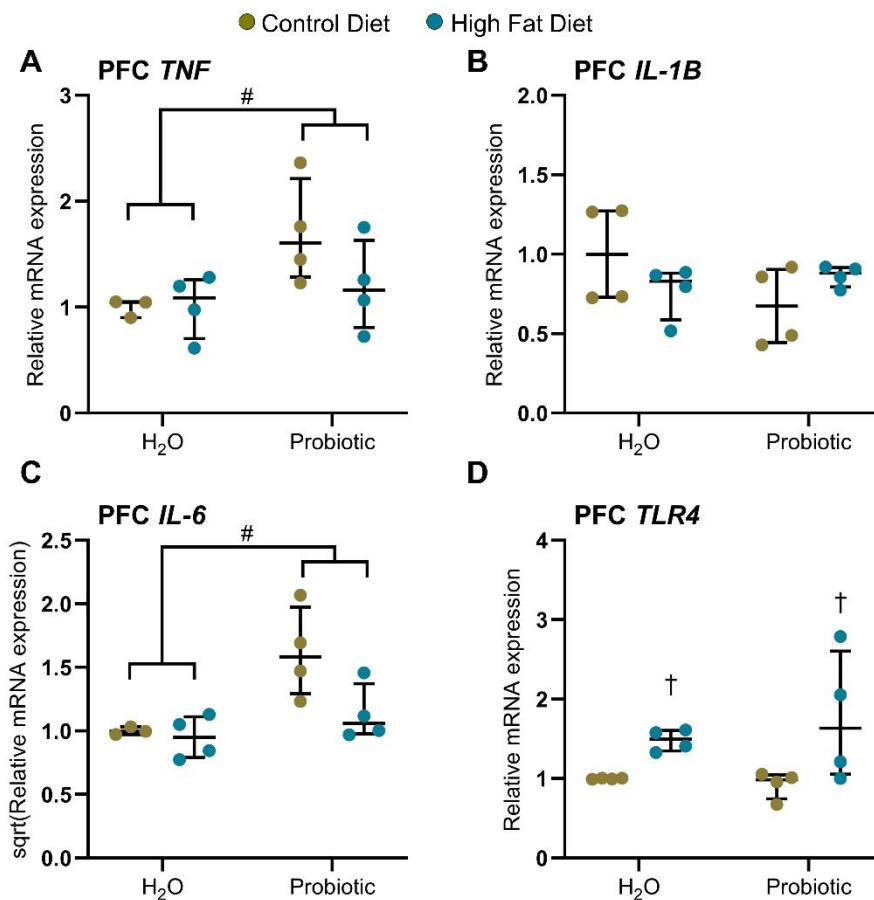


Figure 3.6 Prefrontal cortex toll-like receptor 4 and cytokine mRNA expression is altered by probiotic treatment and HFD, respectively. **A)** PFC *TNF* expression is increased in dams by perinatal probiotic treatment, **B)** *IL-1B* expression is unaffected, while **C)** *IL-6* expression is also increased by perinatal probiotic treatment. **D)** *TLR4* expression in the PFC is increased after chronic HFD feeding relative to the CD. Data presented as median $\pm$ interquartile range showing all points, relative expression compared to vehicle/control diet. $^{\dagger}p < 0.05$, indicating main effect of maternal diet. $^{\#}p < 0.05$, indicating main effect of maternal probiotic intake. $N = 4$ per group.

The relative expression of inflammatory markers *TNF*, *IL-1B*, *IL-6*, and *TLR4* were investigated in the prefrontal cortex (Figure 3.6). PFC *TNF* and *TLR4* were analysed using the non-parametric trimmed-means two-way ANOVA, whereas *IL-1B* and *IL-6* were analysed using two-way ANOVA. *TNF* and *IL-6* were increased (*TNF*: $Q_{(1, 11)} = 5.92$, $p = 0.035$, *IL-6*: $F_{(1, 11)} = 10.70$, $p = 0.0075$) in the PFC of probiotic-supplemented dams independent of diet, with no significant diet x probiotic interactions (Figures 3.6a and 3.6c, respectively). No significant interaction or main effects occurred for PFC *IL-1B* (Figure 3.6b). PFC *TLR4* ($Q_{(1, 11)} = 9.69$, $p = 0.024$) was elevated in obese dams independently of probiotic treatment with no significant diet x probiotic interaction (Figure 3.6d).

3.3.5 Maternal high fat diet and probiotic treatment differentially affect gut bacteria composition and modify the gut-brain axis metabolome.

The relative abundance of faecal *Lactobacillus spp.* and *Bifidobacterium spp.* were quantified in the F0 dams by qPCR (Figure 3.7). 2-way ANOVA revealed a main effect of probiotic treatment, which increased faecal *Lactobacillus spp.* levels in probiotic-treated dams at the time of pup weaning relative to vehicle (control) dams ($F_{(1, 10)} = 12.51$, $p = 0.0054$, Figure 3.7a). For *Lactobacillus spp.* levels there was no main effect of diet (HFD vs. CD) and no diet x treatment (probiotic, vehicle) interaction ($F_{(1, 10)} = 3.01$, $p = 0.11$). However, a significant diet x probiotic treatment interaction was observed for faecal *Bifidobacterium spp.* levels ($Q_{(1, 12)} = 9.58$, $p = 0.028$). Tukey post hoc tests revealed that CD/probiotic dams had significantly increased *Bifidobacterium spp.* levels relative to CD/vehicle ($p < 0.0001$), HFD/vehicle ($p < 0.0001$), and HFD/probiotic ($p = 0.0004$) dams (Figure 3.7b).

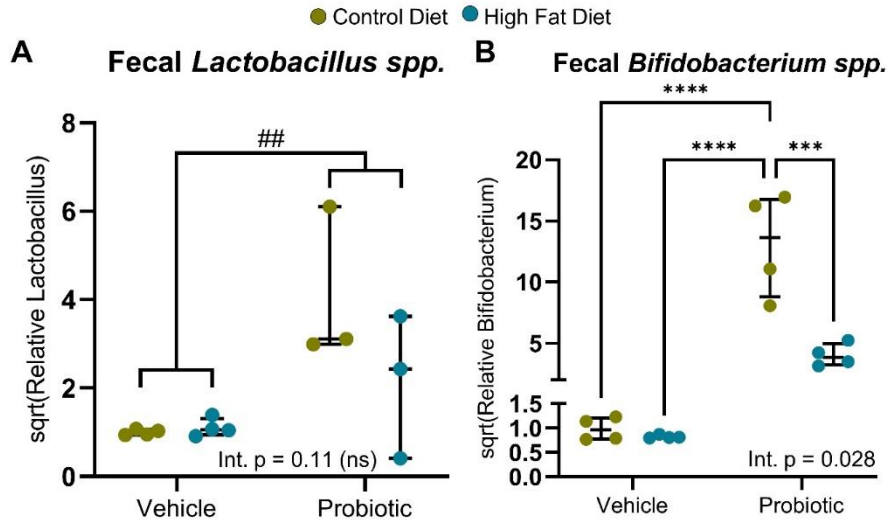


Figure 3.7 Relative abundance of maternal faecal **A)** *Lactobacillus* spp. and **B)** *Bifidobacterium* spp.

Unsupervised principal component analysis (PCA) of the integrated faecal, liver, brain, and blood metabolome revealed distinct metabolic profiles in dams with and without probiotic treatment, particularly in those that were not fed the HFD (Figure 3.8a). This variation occurred along the first principal component, where the R^2X was 0.28. Separation between CD/Veh and HFD/Veh dams was evident across principal component 3 (Figure 3.8b). The cumulative R^2X was 0.52, with three principal components. The corresponding PCA loadings plots (Figures 3.8c,d) were interrogated to understand which metabolites, and from which tissue or biofluid, contributed most to the group-specific variation in the integrated PCA. Faecal acetate, elevated by probiotic supplementation, contributes largely to the clustering of the CD/Probiotic dams. Maternal faecal, liver, and plasma metabolites contribute relatively equally to the clustering observed between the three other groups (CD/Vehicle, HFD/Vehicle, HFD/Probiotic), whereas the brain metabolome was relatively less important in overall metabolite variation in the dams.

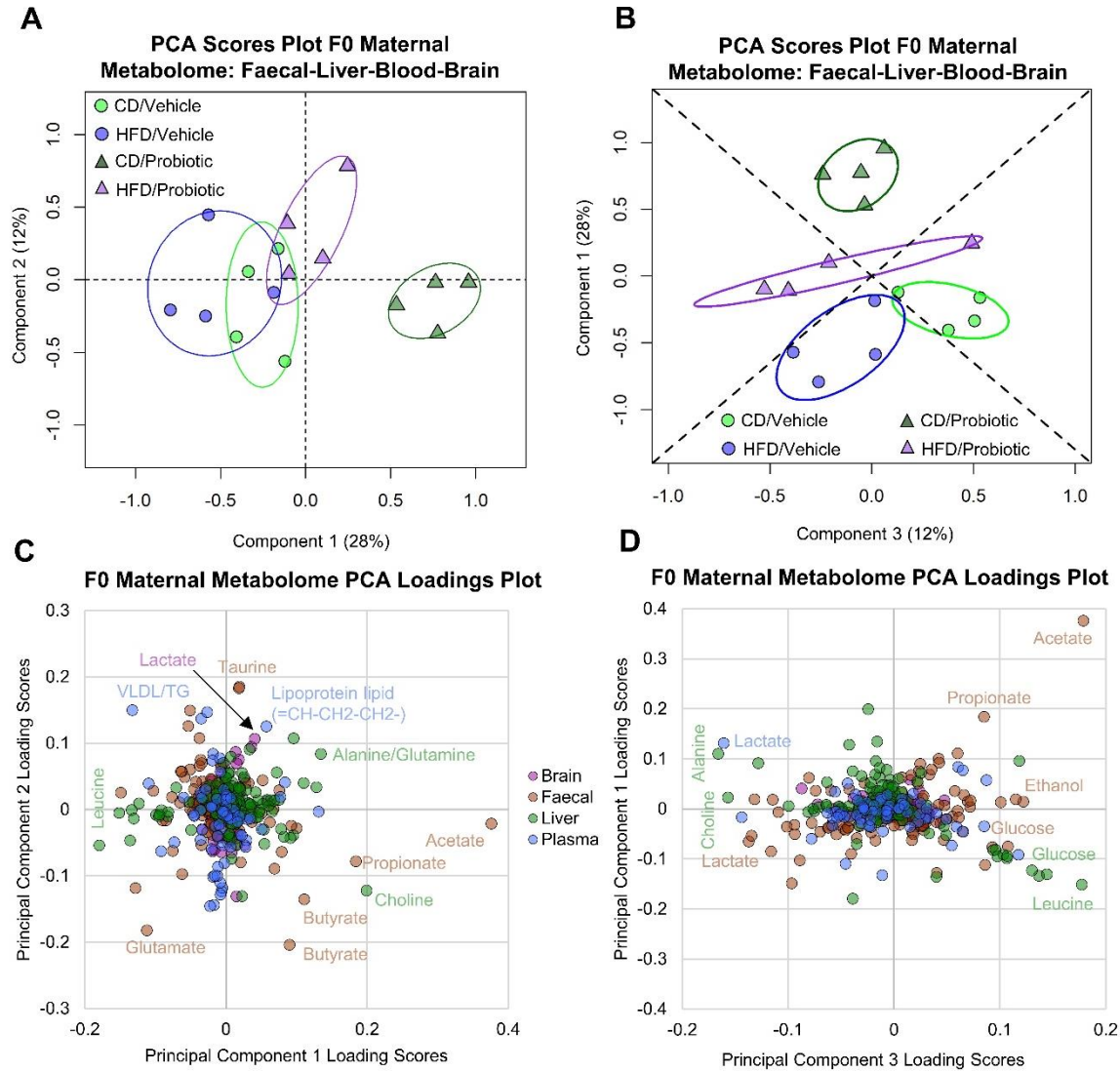


Figure 3.8 Unsupervised principal component analysis (PCA) of chronic HFD and perinatal probiotic treatment on the maternal (F0) faecal, liver, plasma, and brain metabolites. **A**) Untargeted PCA scores plot of the integrated faecal, liver, plasma, and brain metabolome PC1 vs PC2, **B**) Additional scores plot group separation across PC3, in particular between CD/Veh and HFD/Veh dams which was not evident in PC1 or PC2. **C**) Corresponding integrated PCA loadings plot between PC1 and PC2 and **D**) between PC1 and PC3. The R^2X of PC1-3 were 0.28, 0.12, and 0.12, respectively. $N = 4$ per group.

Supervised partial least squares discriminant analysis (PLS-DA) was used to investigate the metabolome of each individual tissue. PLS-DA confirmed the group differences observed by PCA and identified additional, tissue-specific variation in the metabolome between obese and lean dams with and without probiotic treatment (Figures 3.9 and 3.10). The top (ranked VIP) discriminatory metabolites identified by PLS-DA, for each tissue, are summarised in Table 3.1, and depicted in a heatmap with unsupervised hierarchical clustering of metabolites (Figure 3.12) and metabolite atlas (Figure 3.13).

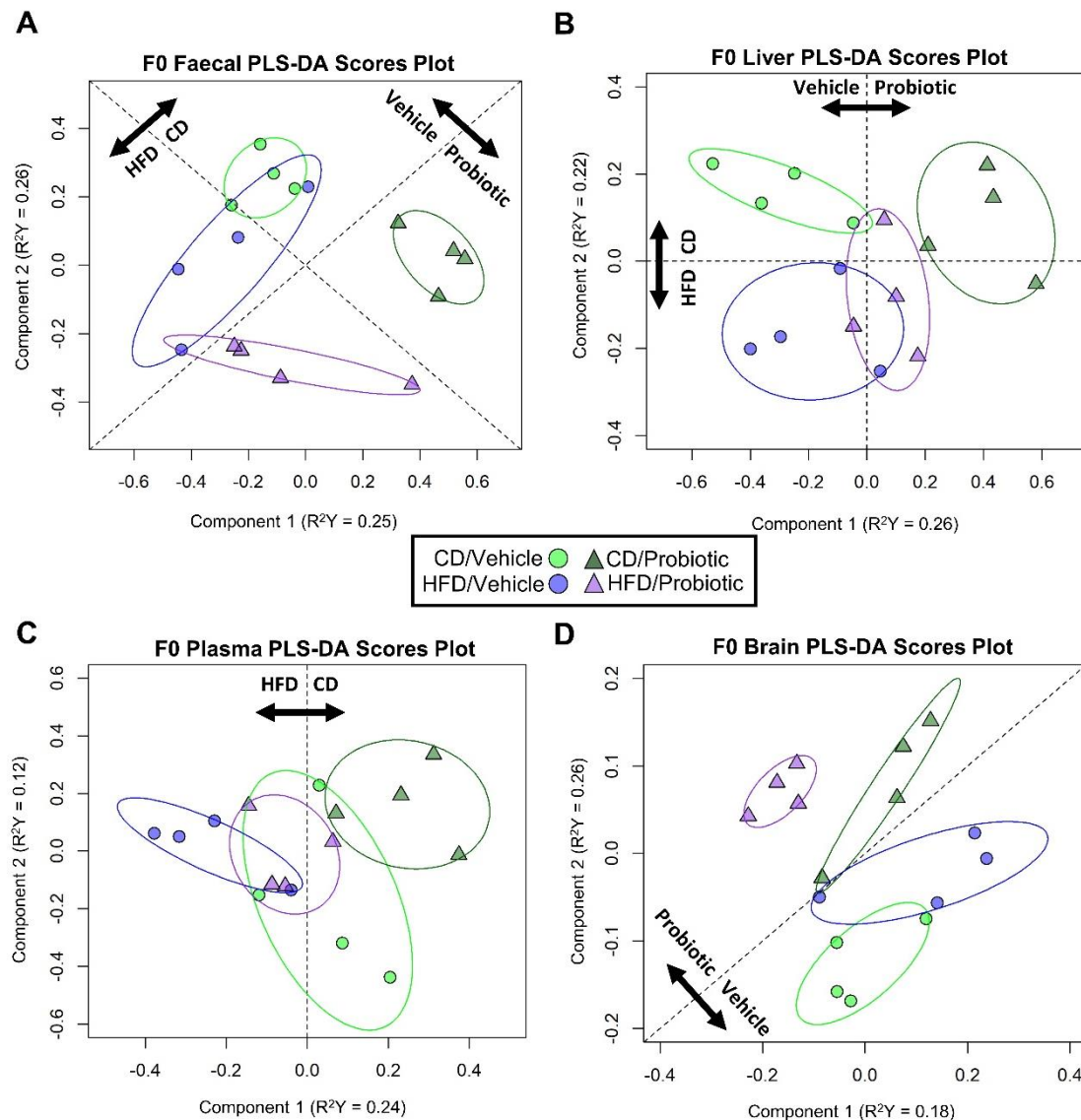


Figure 3.9 Maternal (F0) metabolome PLS-DA scores plots for **A)** faecal, **B)** liver, **C)** plasma, and **D)** brain compartments. N = 4 per group.

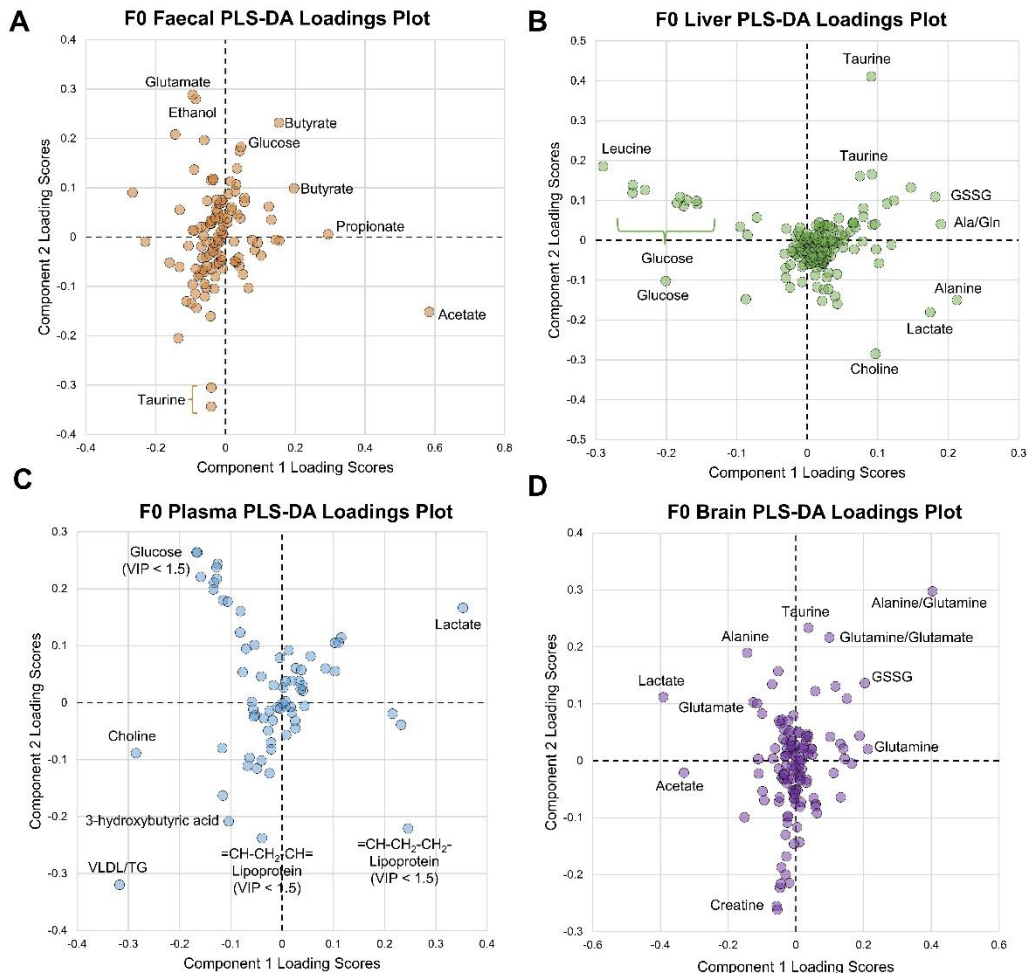


Figure 3.10 Maternal (F0) metabolome PLS-DA loading plots for **A)** faecal, **B)** liver, **C)** plasma, and **D)** brain compartments. N = 4 per group.

PLS-DA of the faecal metabolome revealed that group separation occurred orthogonally between components one and two (Figure 3.9a). The cumulative R^2X was 0.64, and the cumulative R^2Y was 0.71. The cumulative Q^2 was 0.33, with three components. Separation along the third predictive component is shown in Appendix D-1 between CD/Veh and HFD/Veh dams. Univariate two-way ANOVA revealed a significant interaction for faecal acetate ($F_{(1,11)} = 19.35$, $q = 0.0066$, Table 3.1). Post hoc Tukey tests revealed that CD/probiotic dams had significantly higher levels of faecal acetate relative to all other groups ($p = 0.0004$ compared to CD/vehicle and HFD/vehicle, $p = 0.0005$ compared to HFD/probiotic). Similarly, there was a significant diet x probiotic treatment interaction for faecal butyrate levels in dams ($F_{(1,12)} = 18.84$, $q = 0.05$). Post hoc Tukey tests revealed that CD/probiotic dams had significantly higher levels of faecal butyrate relative to all other dams ($p = 0.02$ compared to

CD/Veh and HFD/Veh, $p = 0.007$ compared to HFD/Pro dams). A significant interaction was identified for faecal propionate levels ($F_{(1, 12)} = 8.03$, $q = 0.032$) and post hoc Tukey tests showed that CD/probiotic dams also had significantly higher levels relative to HFD/probiotic dams ($p = 0.0048$), HFD/vehicle dams ($p = 0.0011$), and CD/vehicle dams ($p = 0.0019$). Lastly, there was a main effect of diet on faecal glucose levels ($F_{(1, 12)} = 45.90$, $q < 0.0001$) independent of probiotic treatment with no interaction. Levels were lower in dams fed a HFD relative to dams fed a CD.

In the liver, PLS-DA revealed differences in the metabolome of vehicle and probiotic-treated dams in the first component, and differences between HFD and CD dams in the second component (Figures 3.9b and 3.10b). The cumulative R^2X was 0.57, the cumulative R^2Y was 0.48, and the cumulative Q^2 was 0.26, with two components. A significant interaction was identified for liver choline ($F_{(1,12)} = 30.64$, $q = 0.0007$). Post hoc Tukey tests revealed that CD/probiotic dams had significantly higher levels of liver choline relative to all other groups ($p < 0.0001$ compared to all other groups). No other significant interactions were found for key liver metabolites. Liver leucine ($F_{(1, 12)} = 14.20$, $q = 0.0049$) and glucose ($F_{(1, 12)} = 13.95$, $q = 0.0049$) were reduced by probiotic treatment relative to vehicle as a main effect independent of diet, whereas alanine ($F_{(1, 12)} = 19.15$, $q = 0.016$) and GSSG ($F_{(1, 12)} = 13.14$, $q = 0.0049$) were increased by probiotic treatment relative to vehicle as a main effect independent of diet (Table 3.1).

Table 3.1 Summary of F0 metabolite changes across each body site.

F0	Metabolite	AOV main effect			AOV post-hoc analysis				VIP score	NMR ppm
		HFD v. CD	Probiotic v. Vehicle	Int.	CD/Veh	HFD/Veh	CD/Pro	HFD/Pro		
FAECAL	Acetate	-	-	**			↑***		3.34	1.92
	Ethanol	ns	ns	ns					2.53	1.18
	Glutamate	ns	ns	ns					2.33	2.37
	Taurine	ns	ns	ns					2.07	3.26
	Propionate	-	-	*			↑**		1.99	1.07
	Butyrate	-	-	*			↑*		1.95	1.56
	Glucose	↓****	ns	ns					1.68	4.65
LIVER	Choline	-	-	***			↑****		3.80	3.23
	Taurine	ns	ns	ns					3.80	3.42
	Leucine	ns	↓**	ns					2.91	3.72
	Glucose	ns	↓**	ns					2.40	4.65
	Alanine	ns	↑**	ns					2.17	1.48
	Lactate	ns	ns	ns					1.80	1.33
	GSSG	ns	↑**	ns					1.73	2.17
PLASMA	VLDL/TG	↑*	ns	ns					2.78	0.86
	Lactate	ns	↑*	ns					2.62	1.33
	Choline	↑****	↓*	ns					2.43	3.23
	3-hydroxybutyric acid	ns	ns	ns					1.52	1.20
BRAIN	Lactate	ns	ns	ns					3.42	1.33
	Creatine	ns	ns	ns					2.14	3.93
	Alanine	ns	↑**	ns					2.04	1.48
	Taurine	ns	ns	ns					1.94	3.42
	Glutamate	ns	ns	ns					1.81	2.36
	Acetate	ns	ns	ns					1.77	1.92
	GSSG	ns	ns	ns					1.63	3.75

*q < 0.05, **q < 0.01, ***q < 0.001, ****q < 0.0001 adjusted for false discovery using the Benjamini and Hochberg method. Int = interaction; VIP = variable importance in projection; AOV = analysis of variance, ns = not significant.

In plasma, PLS-DA revealed that group separation was evident across the first component only, between CD/probiotic and HFD/vehicle dams (Figures 3.9c and 3.10c). The cumulative R^2X was 0.64, the cumulative R^2Y was 0.36, and the cumulative Q^2 was 0.08, with two components. Two-way ANOVA of key plasma metabolites revealed no significant diet x probiotic treatment interactions. There was a main effect of perinatal probiotic treatment on increasing the relative abundance of plasma lactate ($F_{(1, 12)} = 10.87$, $q = 0.013$) relative to vehicle and independent of diet. Plasma choline was reduced by probiotic treatment relative to vehicle treatment as a main effect ($F_{(1, 12)} = 15.94$, $q = 0.0072$) and increased by HFD relative to CD as a main effect ($F_{(1, 12)} = 32.98$, $q < 0.0001$). Resonances corresponding to plasma very low-density lipoprotein (VLDL) and triglyceride were also increased in dams fed HFD relative to CD as a main effect ($F_{(1, 12)} = 7.88$, $q = 0.032$) independent of probiotic treatment (Table 3.1).

Lastly, the F0 brain (cerebrum) metabolome was investigated using PLS-DA and subsequent two-way ANOVA of key metabolites (Figures 3.9d and 3.10d, Table 3.1). Group separation between probiotic and vehicle treatment was evident between the first and second components. The cumulative R^2X was 0.49, the cumulative R^2Y was 0.44, and the cumulative Q^2 was 0.19, with two components. Two-way ANOVA revealed a significant main effect of probiotic treatment on brain alanine levels ($F_{(1, 12)} = 16.99$, $q = 0.0056$). Levels were increased in probiotic-fed dams relative to vehicle dams, independent of maternal diet and with no interaction. No other diet x probiotic interactions or main effects reached significance (Table 3.1).

3.3.6 Maternal perinatal probiotic treatment increases the delivery of lactate and SCFAs to their offspring during nursing.

The metabolite composition of maternal milk, collected on PND4, was analysed using PCA, PLS-DA, and two-way ANOVA as described in Figure 2.4. PCA revealed unsupervised group differences that were confirmed by PLS-DA (Figure 3.11a, b, c). Variation was apparent between vehicle and probiotic dams between components one and two, as well as orthogonal variation between CD and HFD-fed dams. For the PLS-DA, the cumulative R^2X was 0.79, the cumulative R^2Y was 0.37, and the cumulative Q^2 was 0.12, with two components. Following two-way ANOVA of the key metabolites identified by VIP score (Figure 3.11d), no significant diet x probiotic interactions occurred in any milk metabolites; thus, post hoc comparisons have not been described. Resonances corresponding to milk very low-density lipoprotein (VLDL) and triglyceride were increased in dams fed HFD relative to CD as a main effect ($F_{(1, 12)} = 6.77$, $q = 0.039$, Figure 3.9e) independent of probiotic treatment. Lactate ($F_{(1, 12)} = 9.70$, $q = 0.021$, Figure 3.9f) and butyrate/valerate ($F_{(1, 12)} = 9.65$, $q = 0.019$, Figure 3.9h) were increased by probiotic treatment as a main effect. The relative abundance of milk lactose was reduced by probiotic treatment relative to vehicle as main effect ($F_{(1, 12)} = 8.84$, $q = 0.017$, Figure 3.9g) and increased by HFD relative to CD as a main effect ($F_{(1, 12)} = 8.51$, $q = 0.031$). Butyrate was reduced by HFD relative to CD as a main effect ($F_{(1, 12)} = 9.92$, $q = 0.025$, (Figure 3.9i).

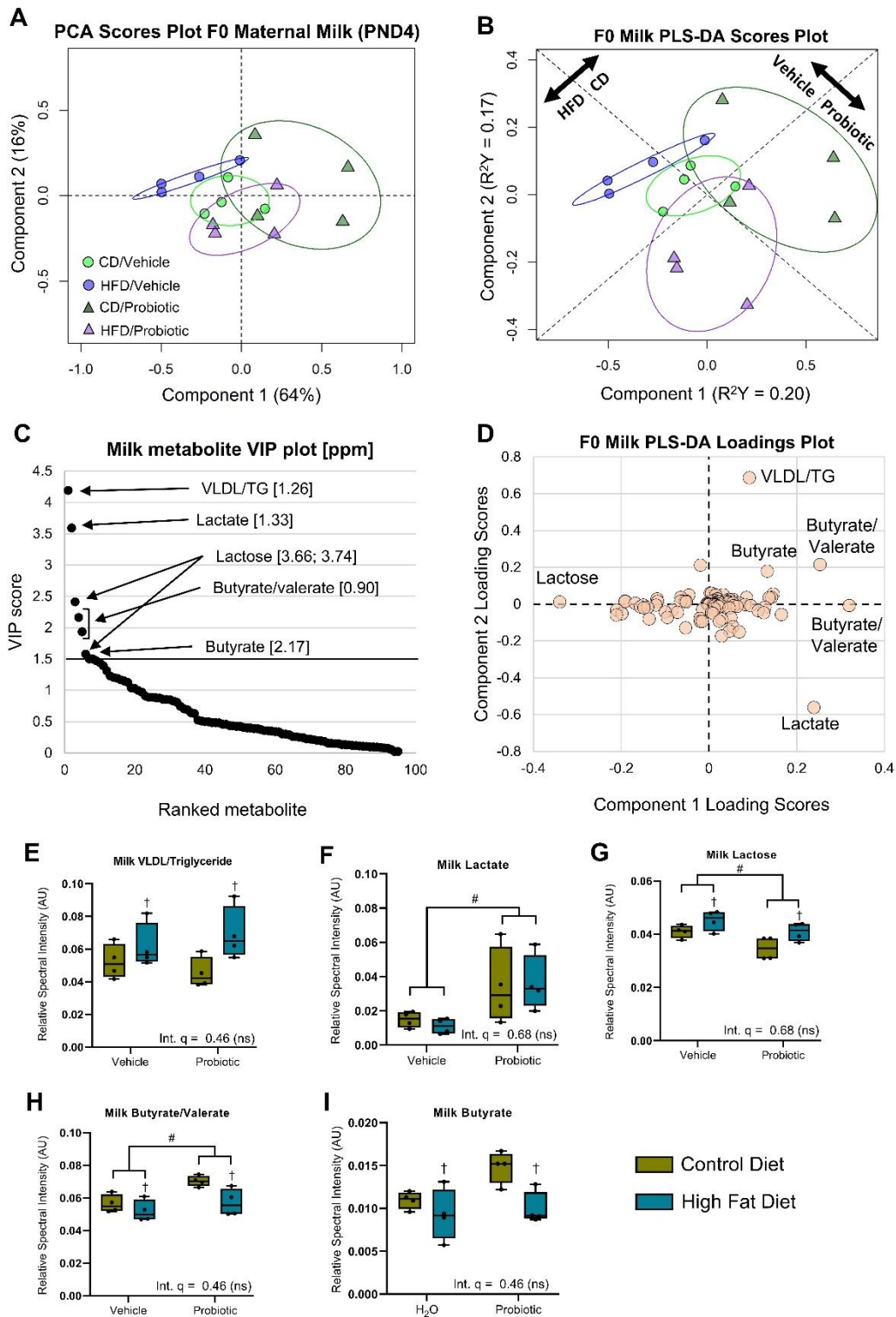


Figure 3.11 Metabolomic analysis of maternal milk at postnatal day 4. **A)** Unsupervised principal component analysis scores plot, **B)** supervised partial least squares discriminatory analysis scores plot, **C)** PLS-DA variable importance in projection plot, **D)** PLS-DA loadings plot, relative abundance of milk **E)** VLDL/triglyceride, **F)** lactate, **G)** lactose **H)** butyrate/valerate, and **I)** butyrate. Boxplots show min-max and all points. $^{\dagger}q < 0.05$, indicating main effect of maternal diet. $^{\#}q < 0.05$, indicating main effect of maternal probiotic intake. Int = interaction, ns = not significant. N = 4 per group.

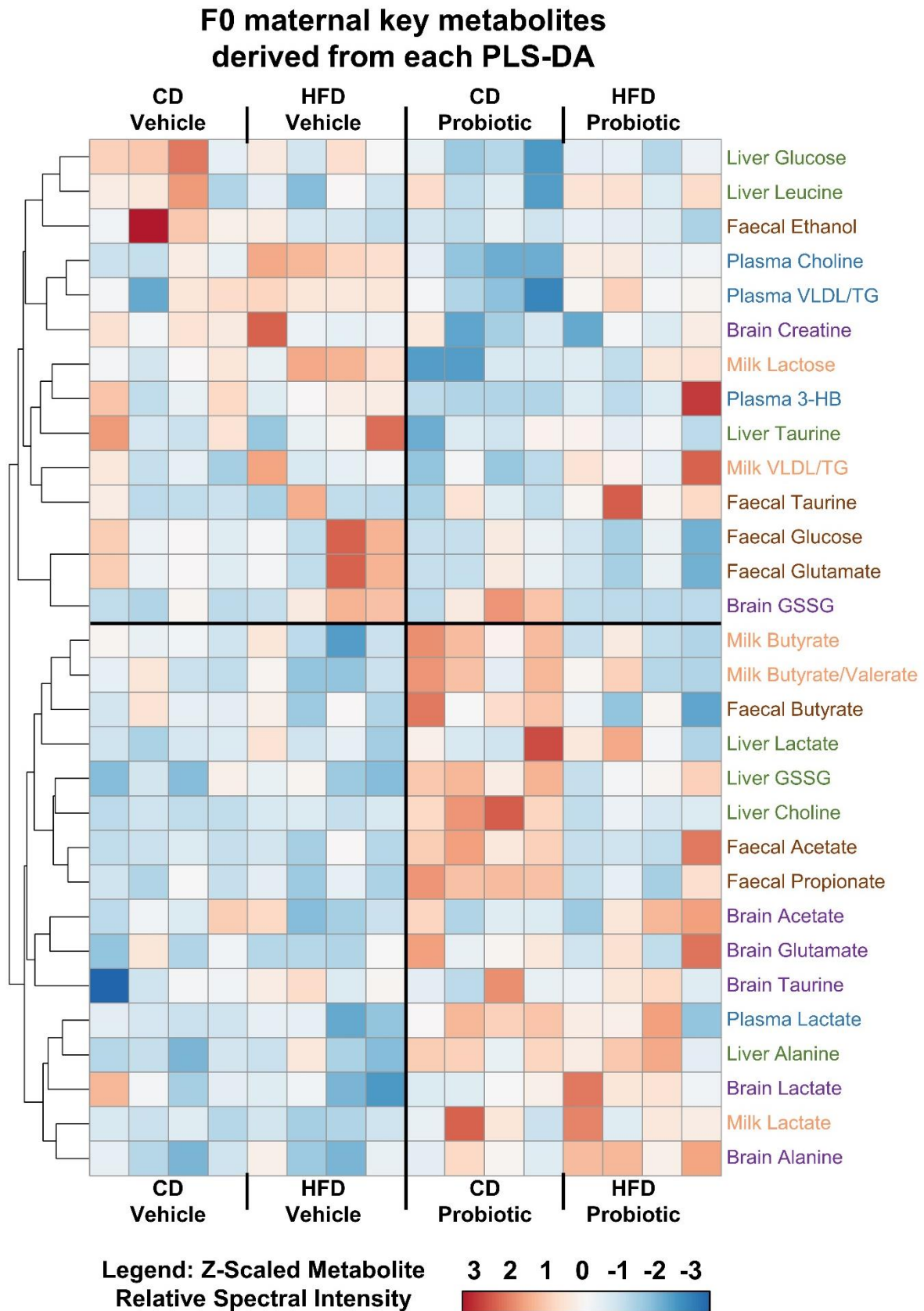


Figure 3.12 Summary of key metabolite changes in the dams from faeces, liver, blood, brain, and milk. Milk samples were collected 17 days prior to the other samples. 3-HB = 3-hydroxybutyric acid.

3.4 Discussion

Maternal obesity (Molyneaux et al., 2014) and excessive gestational weight gain (Zanardo et al., 2021) are risk factors for perinatal depression in humans. Despite this, few studies have investigated the effect of chronic high fat diet feeding on postpartum maternal behaviours. Rather, the vast majority focus exclusively on offspring outcomes associated with maternal interventions during the antenatal or early postnatal period. Given that maternal behaviour can affect the developmental trajectory of the offspring, including future anxiety behaviours (Weaver et al., 2006), it is important to understand the effects of chronic HFD feeding on both aspects of maternal care and postpartum emotional behaviours.

In this chapter, it was confirmed that six weeks HFD feeding induces an obese phenotype in female mice. Obese mice were less attentive to their offspring during the first postnatal week of life (as measured by time spent away from the nest), but the average time spent nursing, licking, and grooming was not different between obese and lean dams. As these latter attentive behaviours are thought to be the key actions that can influence offspring development (Champagne et al., 2003; Weaver et al., 2004), it is unlikely that the extra time spent away from the nest in obese dams would have adversely affected offspring development, as it appeared to only be at the expense of the average time spent nest building. While an adverse relationship between nest building and offspring developmental or behavioural outcomes has not been reported, nest-building is a highly motivated behaviour during pregnancy (Latham and Mason, 2004), and its reduction in favour of inactivity may be indicative of anhedonia. In parallel, the effects of maternal probiotic consumption during gestation and nursing on MCB were assessed here in both obese and lean dams, though no effects were found. This is consistent with our previous study of maternal prebiotic supplementation which found no effects of perinatal supplementation on maternal care (Hebert et al., 2021). To my knowledge, there have been no other reports of the effects of prebiotic or probiotic supplementation on maternal care.

The current study also found that maternal HFD-induced obesity, and to a lesser extent, maternal probiotic intake, led to increased passive stress coping behaviour in the FST. There were no effects of either diet or probiotic treatment on anxiety-like behaviour in the OFT or LDB. Many studies have assessed the effects of HFD feeding on depressive-like behaviour in the FST. A review of HFD feeding and the FST found that most studies have used male C57Bl/6 mice while the percentage fat, duration of feeding and percentage sucrose varied considerably (Lloyd and Reyes, 2020). Conversely, very few studies have investigated the effects of HFD-induced obesity on postpartum behaviour. In one study (Moazzam et al., 2021), female CD-1 mice were challenged with HFD for five weeks prior to mating, as well as through gestation and lactation (a similar paradigm to the present study design). In keeping with the results of this chapter, nest building was adversely affected by the HFD, and there was no significant effect on pup retrieval at PND5 (Moazzam et al., 2021). In terms of affective behaviour, maternal HFD feeding increased postpartum anxiety-like behaviour in the EPM at PND20, and reduced sucrose preference at pre-weaning (PND12-15) but not post-weaning (PND21-23). However, unlike the present investigation, no biochemical parameters were assessed to account for the relationship between the HFD feeding and altered postpartum behaviour.

In another study, HFD-induced antenatal obesity increased total immobility time in the tail suspension test post-weaning (after PND21), while intake of the dietary fibre, inulin, was protective (Liu et al., 2020c). Interestingly, high fibre diet also decreased immobility in lean dams fed a CD, suggesting that the effect may be independent of HFD. Serum LPS was increased in obese dams, but this was attenuated in obese dams fed the inulin fibre. Consistent with this, the current chapter has shown that liver *TLR4*, *IL-1B*, and *SAA-2* expression were upregulated after the HFD feeding compared to CD, which supports the peripheral pro-inflammatory effects of diet-induced obesity, potentially via reduced gut-barrier integrity and increased circulating endotoxin activating *TLR4* (Liu et al., 2020c). Moreover, HFD increased *TLR4* expression in the brain relative to dams fed a CD. Upregulated brain

TLR4 expression has been previously shown in a murine model of diet induced NAFLD (Strekalova et al., 2015) and may contribute to both pathological (Sartorius et al., 2012) and protective (Vasconcelos et al., 2021) mechanisms following peripheral inflammation.

While the study using inulin showed a protective effect on behaviour and pro-inflammatory gene expression induced by the HFD, it is important to note that the inulin was provided from the start of HFD feeding and prevented both an increase in weight gain during gestation and lactation as well as preventing increased energy intake (Liu et al., 2020c). Conversely, the probiotic supplementation in the present study was implemented after the establishment of maternal obesity, did not prevent an increase in weight gain, and was therefore not expected to mitigate its adverse effects on maternal behaviour and inflammatory markers. Nevertheless, probiotic treatment did affect the mRNA expression of pro-inflammatory cytokines in the liver and brain, reducing *TNF* and *IL-6* expression in the liver and increasing *TNF* and *IL-6* expression in the PFC. The peripheral changes in cytokine expression may be related to the effects of the probiotic on the gut, whereby *Bifidobacterium spp.* and *Lactobacillus spp.* were increased alongside elevated faecal SCFAs. A non-significant trend in decreased liver *TLR4* expression in HFD/probiotics dams compared to HFD/vehicle dams (Figure 3.5d) may indicate that the probiotic did improve gut barrier integrity in the obese animals, though the study is likely underpowered. Nonetheless, reduced intestinal permeability may account for the reduction in cytokine levels through improved immune tolerance. Further investigation through the quantification of occludin and claudin expression in the gut or measurement of plasma LPS levels would be required to confirm this. In the brain, the basal expression of cytokines are low (Gruol, 2015), and under physiological conditions *IL-6* and *TNF* are thought to regulate synaptic plasticity (Stellwagen and Malenka, 2006; Gruol, 2015). Therefore, it is unclear whether a two-fold increase in PFC cytokine expression equates to a pathological response to the probiotic consumption or an increase in synaptic plasticity. Histological interpretation of the glial phenotype (e.g., activated, or quiescent) may provide further insight.

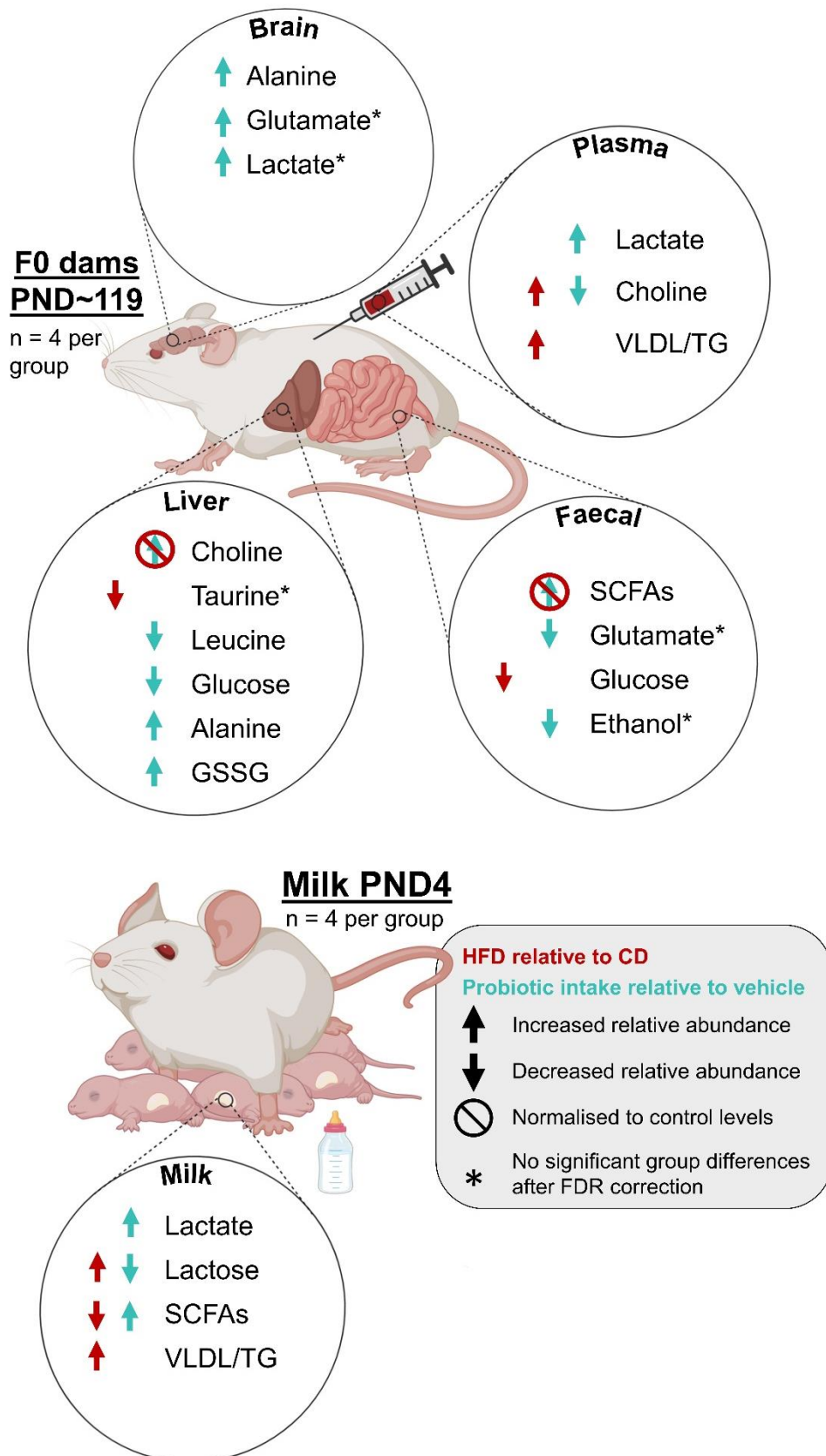


Figure 3.13 Summary of top metabolites from partial least squares discriminant analyses (PLS-DA), altered by HFD-induced obesity and/or perinatal probiotic intake, as listed in Table 3.1 (dams) and Figure 3.11 (milk).

Surprisingly, HFD feeding had little effect on brain and liver metabolite levels (Figure 3.13). One reason for this may be the profound changes in systemic energy metabolism that occur to support milk production during lactation. These changes, which include the mobilisation of fat stores and increased carbohydrate utilisation (Butte et al., 1999) may have partially masked changes to liver and brain metabolism associated with HFD intake. Another reason for this may be the small sample size. This is attributed to the experimental design, as only a small number of CD-1 mating pairs were required to produce a sufficient number of offspring (~50 per group including both juvenile and adult offspring). The sample size for the dams are also appropriate for the primary aim of this chapter, which were not to investigate subtle differences between each group of dams, but to characterise the most overt effects of maternal HFD-induced obesity on MCB and markers of inflammation. I also show that maternal probiotic supplementation alters the gut microbiota and metabolome, reduces peripheral cytokine levels, and increases lactate and SCFAs in the milk during nursing. Overall, the high fat diet and probiotic interventions had mostly independent effects on the maternal phenotype.

4

Maternal probiotic treatment reverses anxiety-like behaviour and alters neuroplasticity gene expression in the offspring of obese dams²

4.1 Introduction

Maternal obesity remains a significant issue in the United Kingdom and further afield (Devlieger et al., 2016; NMPA Project Team, 2017), with less than half of women in the UK having a healthy body mass index (BMI) between 18.5 and 25 (NMPA Project Team, 2017). Emerging data suggests that the average BMI increased during the UK 2020 lockdown period, amidst psychological stressors of isolation and possible financial strain. Consequently, there is a great need for cost-effective and safe methods of mitigating the risk of transferring mental and metabolic illnesses to offspring.

While the consequences of maternal obesity on offspring metabolism, obesity, and cardiovascular health have been widely reported (Leddy et al., 2008; Mulligan and Friedman, 2017), only more recently are thorough investigations being made into how maternal diet and obesity affect maternal and offspring behaviour, including the transmission of psychiatric disease risk through poor maternal health during gestation and the early life of the offspring. Sullivan et al. (2010) linked changes in maternal HFD consumption during gestation with increased anxiety-like behaviour in juvenile NHP offspring. This was also accompanied by perturbations to the serotonergic system, including decreased cerebrospinal fluid levels of 5-HT. In humans, both internalising and externalising behavioural problems are more likely to occur throughout childhood and adolescence after gestational and early-life exposure to maternal obesity (Van Lieshout et al., 2013).

² Results included in this Chapter have been published: <https://doi.org/10.1073/pnas.2108581119>

Obesity status and the consumption of a HFD prior to and during pregnancy have been shown to affect the composition of the maternal and neonatal gut microbiome in humans (Collado et al., 2008; Santacruz et al., 2010; Chu et al., 2016) and rodents (Paul et al., 2016). Moreover, maternal gut dysbiosis induced by diet (Buffington et al., 2016) or antibiotic treatment (Tochitani et al., 2016) has been shown to affect the offspring microbiome, as well as offspring brain and behaviour. Conversely, our laboratory has recently shown that maternal perinatal prebiotic intake reduces offspring anxiety-like behaviours, alters brain gene expression, and increases faecal SCFA levels (Hebert et al., 2021). Maternal prebiotic supplementation has also protected against increased peripheral inflammation in the offspring of obese dams (Paul et al., 2019).

The effects of maternal probiotic consumption on offspring health outcomes have thus far only been investigated through limited preclinical research. Mouse prenatal probiotic supplementation has been suggested to increase serum IgG and intestinal secretory IgA (Himaja et al., 2016) in adult offspring, which may have immunoprotective roles (Di Benedetto et al., 2020). Importantly, offspring probiotic treatment has been suggested to reverse neurological and behavioural deficits induced by a maternal high-fat diet (Buffington et al., 2016). However, it has also been suggested that microbial reconstitution post-weaning is not sufficient to rescue neurological adaptations that arise during development (Clarke et al., 2013). Currently, it is unknown how maternal probiotic intake affects offspring brain and behaviour, and whether it may remedy adverse effects of perinatal exposure to maternal obesity.

The preceding chapter demonstrated that perinatal probiotic supplementation altered the maternal faecal microbiome and metabolome and increased the delivery of lactate and SCFAs to the pups during their first week of life. Rodent (Vuong et al., 2020) and human (Valles-Colomer et al., 2019) studies have demonstrated neuromodulatory roles of gut and maternal-derived metabolites. Furthermore, preliminary research indicates a role for early life gut microbiota in the regulation of central astrocyte–neuron energy metabolism (Mărgineanu

et al., 2020). Lactate, which is produced in the brain by astrocytes, makes an important contribution to neuronal energy homeostasis (Barros, 2013; Magistretti and Allaman, 2018; Rabinowitz and Enerbäck, 2020) and brain signalling (Mosienko et al., 2015). Astrocyte dysfunction is associated with clinical depression and generates depressive-like phenotypes in rodents (Wang et al., 2017), although it remains untested whether interventions targeting the early life gut–brain axis can restore or enhance brain energy metabolism, alter markers of neuronal plasticity, or increase emotional resilience.

The aim of this chapter was to explore the effects of maternal obesity on anxiety and depressive-like behaviours and neuroplasticity gene expression in the offspring, and to see whether modifying the maternal microbiota with a multispecies probiotic could mitigate these changes. I expected that maternal obesity would induce negative affect in their offspring and that these effects are long-lasting. Markers of immune activation were quantified in the liver of dams and offspring, and the relative expression of inflammatory and plasticity-related genes were evaluated in the prefrontal cortex (PFC). The protracted structural and functional plasticity of the PFC throughout life (McEwen and Morrison, 2013) renders it a prime target to investigate the adverse consequences of maternal obesity on offspring brain and behaviour at juvenile and adult ages. Finally, I expected that maternal perinatal probiotic treatment would prevent increases in anxiety and depressive-like behaviour in the young and adult offspring, and that this would coincide with the increased expression of genes promoting neuronal survival and synaptic plasticity.

4.2 Results

4.2.1 Juvenile but not adult body weight is altered by maternal diet and probiotic treatment.

Offspring body weight was measured to determine whether the weight trajectory of the offspring was influenced by the weight of the dam, and whether this was further influenced by modification of the maternal microbiota by perinatal probiotic treatment (Figure 4.1). Change in body weight over time was analysed by three-way mixed effect modelling of time, maternal diet, and maternal probiotic treatment. In juveniles, sexes were mixed, and in adults, sexes were analysed separately. Analysis of body weight at set timepoints (PND21 and PND112) was performed using two-way ANOVA of males and females separately. Mixed effects analysis revealed that both maternal probiotic intake ($F_{(1, 217)} = 298.7$, $p < 0.0001$) and high-fat diet (HFD; $F_{(1, 217)} = 58.56$, $p < 0.0001$) independently increased juvenile body weight compared to controls (Figure 4.1a). There was also a significant main effect of time on increasing body weight ($F_{(3.652, 698.6)} = 2928$, $p < 0.0001$) and significant interactions: time x maternal probiotic treatment ($F_{(7, 1339)} = 55.73$, $p < 0.0001$); time x maternal diet ($F_{(7, 1339)} = 19.03$, $p < 0.0001$); probiotic x maternal diet ($F_{(1, 217)} = 19.71$, $p < 0.0001$) and time x probiotic x maternal diet ($F_{(7, 1339)} = 5.72$, $p < 0.0001$).

At PND21 (Figure 4.1b), significant maternal diet x probiotic interactions were present for males ($F_{(1, 107)} = 5.32$, $p = 0.023$) and females ($F_{(1, 88)} = 9.44$, $p = 0.0028$). Post hoc Tukey testing revealed significant pairwise differences between offspring of HFD/vehicle and CD/vehicle dams ($p < 0.0001$ for males and females). Furthermore, in each post hoc comparison, CD/probiotic and HFD/probiotic offspring were significantly heavier than CD/vehicle and HFD/vehicle offspring. There was no significant difference in body weight between CD/probiotic and HFD/probiotic offspring for males and females.

Apart from a main effect of time (females: $F_{(4.737, 161.0)} = 114.1$, $p < 0.0001$; males: $F_{(5.007, 194.5)} = 235.4$, $p < 0.0001$) body weight was not different between groups in adult female (Figure

4.1c) or male (Figure 4.1d) offspring with no significant interactions, also shown at PND112 (Figure 4.1e).

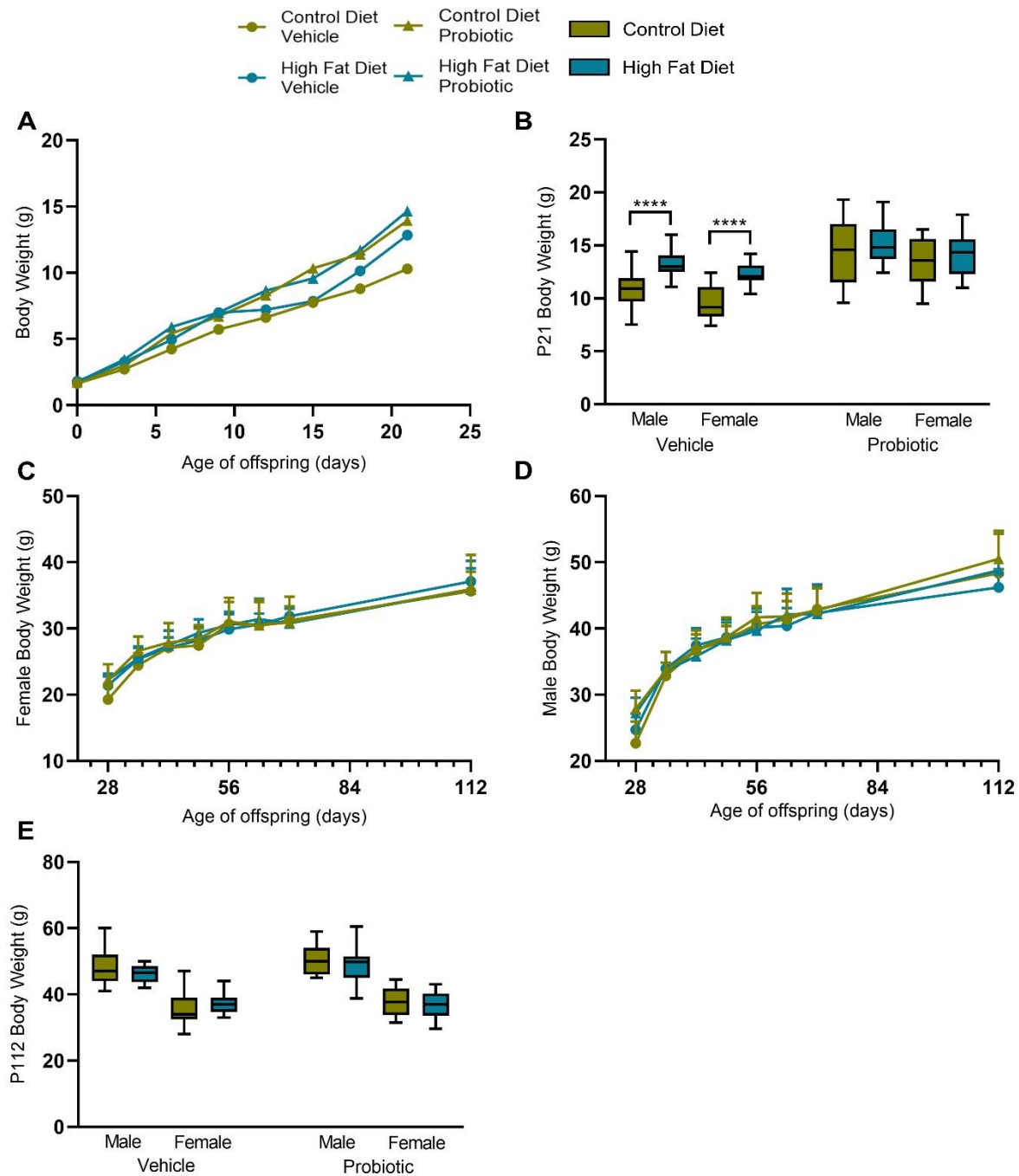


Figure 4.1 Maternal HFD and probiotic intake increase offspring body weight in early life but not in adulthood. **(A)** Juvenile offspring body weight between postnatal day (PND)0 and 21, **(B)** Juvenile offspring body weight at PND21. All probiotic juvenile offspring were also heavier than the vehicle offspring ($p < 0.05$). Body weight in adult **(C)** female and **(D)** male offspring, also shown at **(E)** PND112. Data presented as mean $\pm$ SD **(A,C,D)** or as boxplots indicating the median, interquartile range, minimum and maximum data points **(B,E)**. **** $p < 0.0001$, indicating significant Tukey post-hoc comparisons. $N = 55-62$ per group **(A)**; $22-33$ per group **(B)**; $9-16$ per group **(C-E)**.

4.2.2 Perinatal probiotic intake mitigates anxiety-like behaviours in the juvenile and adult offspring of obese dams.

In the following behavioural tests, where three-way ANOVA revealed that the outcomes were independent of sex, I only report the results of the subsequent consolidated two-way ANOVA. However, for completeness, the figures show how the F1 females and males are distributed.

4.2.2.1 Open field test.

In the OFT, two-way ANOVA revealed a significant maternal diet x probiotic treatment interaction on time spent in the centre zone for juvenile offspring ($F_{(1, 106)} = 9.23$, $p = 0.0030$, Figure 4.2a). Tukey post-hoc testing revealed that the HFD/vehicle offspring spent less time in the centre of the open field than CD/vehicle ($p = 0.0094$), CD/probiotic ($p = 0.0079$), and HFD/probiotic ($p = 0.0003$) offspring. There was also a main effect of maternal probiotic treatment ($F_{(1, 106)} = 9.23$, $p = 0.0030$). No interaction was observed for offspring rearing events, but there was a main effect of maternal probiotic treatment ($F_{(1, 107)} = 4.14$, $p = 0.044$), whereby perinatal probiotic exposure increased rearing events (Figure 4.2b) independent of maternal diet with no significant maternal diet x probiotic treatment interaction.

In the adult offspring, there was also a significant maternal diet x probiotic interaction for time in the centre zone of the OFT ($F_{(1, 75)} = 14.20$, $p = 0.0003$, Figure 4.2c). Post hoc testing showed that the reduced time in centre zone persisted in HFD/vehicle offspring relative to CD/vehicle ($p = 0.022$) and HFD/probiotic ($p = 0.0002$) offspring. A main effect also existed for maternal probiotic treatment, which increased time in the centre zone independent of maternal diet ($F_{(1, 75)} = 7.21$, $p = 0.0089$). There were no enduring effects on rearing behaviour in the adult offspring (Figure 4.2d).

Two-way ANOVA confirmed that, in both juvenile (Figure 4.3a) and adult (Figure 4.4a) offspring, overall locomotor activity was unaltered by maternal diet or probiotic treatment. In the juvenile offspring, latency to enter the centre zone was also unaffected by maternal diet or probiotic treatment (Figure 4.3b). There was a significant interaction between maternal diet

and probiotic intake on the total number of centre zone entries ($Q_{(1, 107)} = 4.87$, $p = 0.033$), though post hoc Tukey tests did not reveal any significant group differences (Figure 4.3c). In the adult offspring, centre zone entries were affected by a significant interaction ($F_{(1, 76)} = 21.20$, $p < 0.0001$, Figure 4.4b). Post hoc Tukey tests revealed that HFD/vehicle adult offspring entered

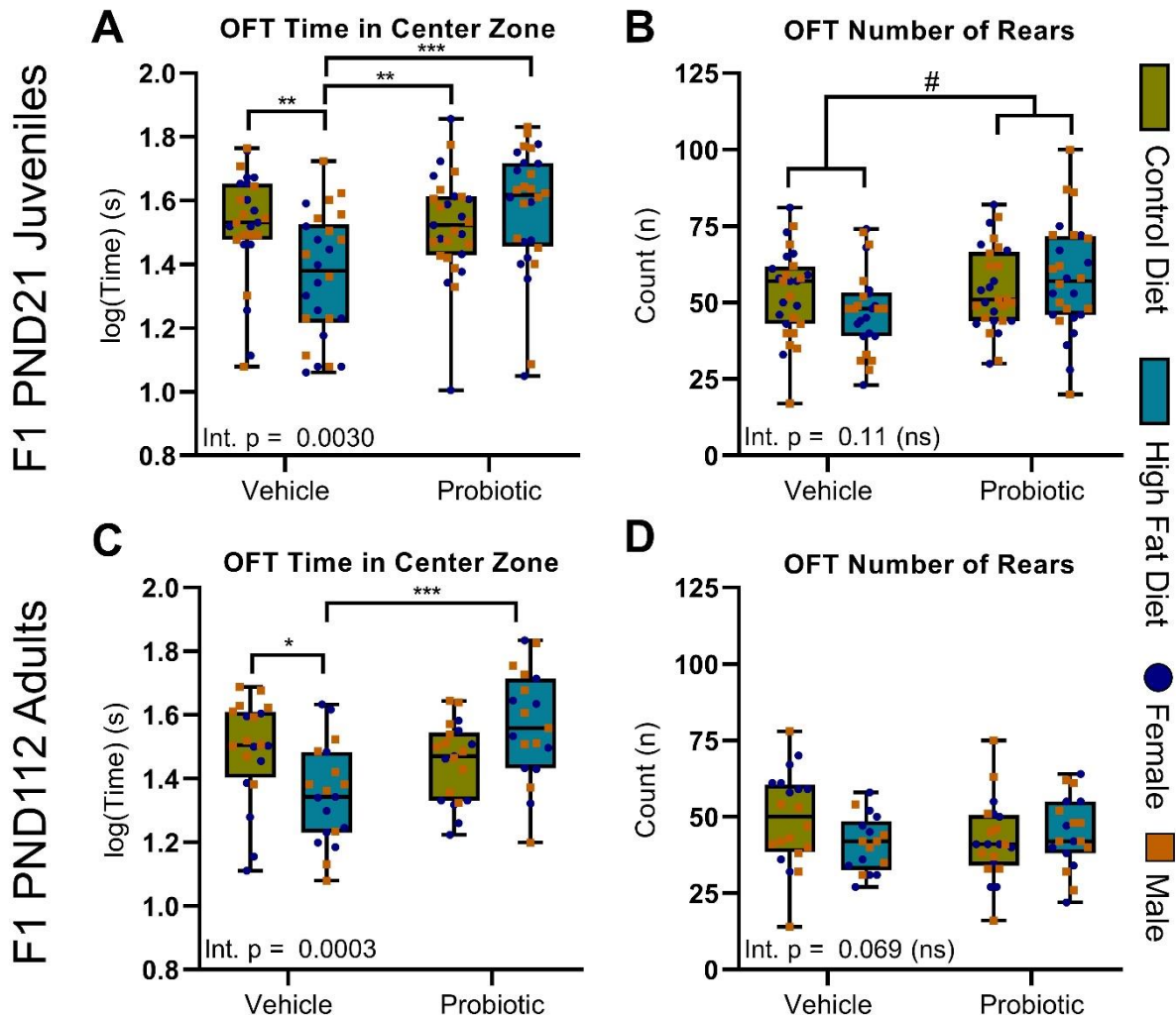


Figure 4.2 Maternal probiotic treatment reverses anxiety-like behaviour in the young and adult offspring in the open field test. Juvenile offspring (A) time in the centre zone and (B) rearing behaviour. Adult offspring (C) time in the centre zone and (D) rearing behaviour. Two-way ANOVA; data are presented as boxplots showing median, interquartile range, and min/max points. # $P < 0.05$ (indicating main effect of maternal probiotic treatment); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (indicating significant Tukey post hoc comparisons, computed only in the case of a significant diet $\times$ probiotic treatment interaction. Int., interaction; ns, not significant. $n = 26$ to 29 for juvenile offspring (13 to 15 male, 13 or 14 female) and 20 or 21 for adult offspring (10 or 11 male, 9 or 10 female) per group.

the centre zone significantly fewer times than both CD/vehicle offspring ($p = 0.014$) and HFD/probiotic offspring ($p < 0.0001$). CD/probiotic offspring had significantly fewer entries than HFD/probiotic offspring ($p = 0.0057$). Maternal diet increased latency to centre zone in the OFT in males (main effect, $F_{(1, 36)} = 4.33$, $p = 0.045$) independent of maternal probiotic treatment with no interaction (Figure 4.4c).

F1 PND21 Juveniles

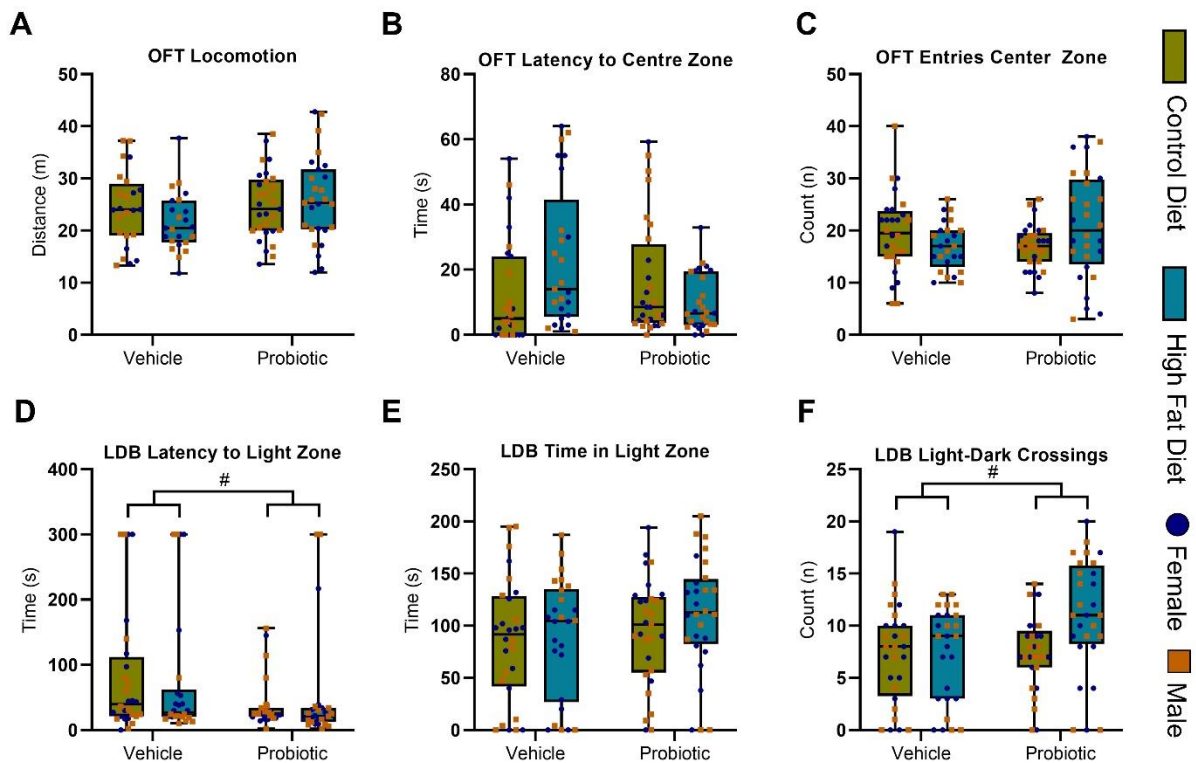


Figure 4.3 Maternal perinatal probiotic treatment reduces anxiety-like behaviour in the juvenile offspring. **(A)** Total distance travelled and **(B)** latency to enter the centre zone, and **(C)** centre zone entries in the open field test (OFT). **(D)** Latency to enter the light zone of the light-dark box (LDB), **(E)** total time spent in the light zone, and **(F)** LDB crossings # $p < 0.05$, indicating main effect of maternal probiotic intake. $N = 13-15$ per sex, per group.

F1 PND112 Adults

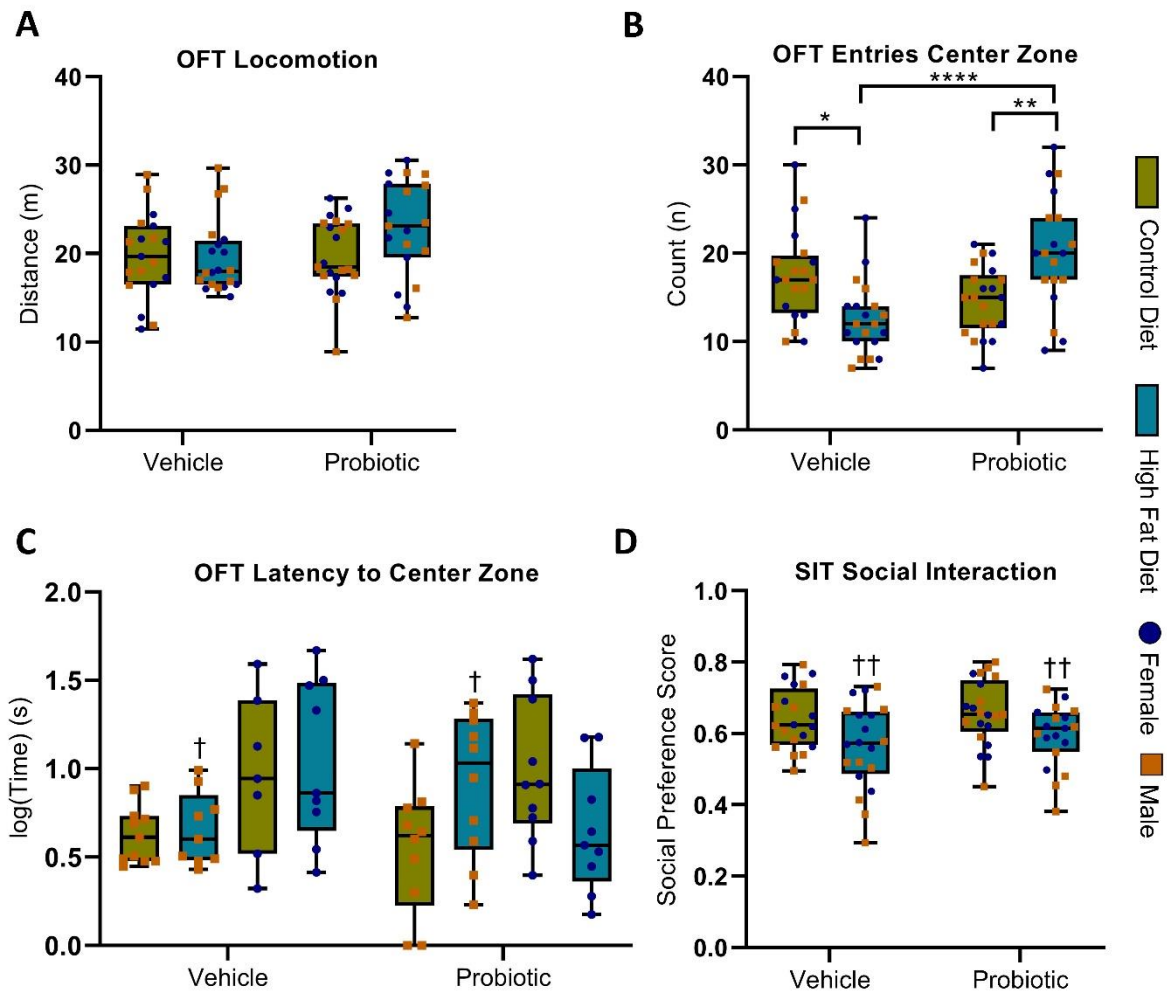


Figure 4.4 Maternal probiotic intake reverses anxiety-like behaviour in the adult offspring of obese dams. **(A)** Overall activity in the open field test (OLF), **(B)** centre zone entries, **(C)** latency to centre zone, **(D)** social preference score during social interaction test. Data presented as boxplots indicating the median, interquartile range, minimum and maximum points. $^{\dagger}p < 0.05$, indicating main effect of maternal diet. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, indicating significant Tukey post-hoc comparisons computed only in the case of a significant interaction. $N = 9-11$ per sex, per group.

4.2.2.2 Light-dark box.

There was no interaction between maternal diet and probiotic treatment on anxiety-like behaviour in the light-dark box in the juvenile offspring (Figures 4.3d-f). Perinatal probiotic exposure reduced latency to enter the light compartment (main effect, $Q_{(1, 107)} = 5.12$, $p = 0.031$, Figure 4.3d) and increased the number of light-dark crossing (main effect, $F_{(1, 107)} = 4.99$, $p = 0.028$, Figure 4.3f) in the juvenile offspring relative to vehicle offspring, independent

of maternal obesity and without a significant maternal x probiotic interaction. Behaviour in the light-dark box was not measured in the adult offspring.

4.2.2.3 Forced swim test.

In the juvenile offspring, maternal obesity increased passive stress coping in the juvenile offspring (Figures 4.5a,b). Time spent immobile was increased in the offspring of obese dams compared to the offspring of lean dams as a main effect ($F_{(1, 102)} = 5.75$, $p = 0.018$), while latency to the first immobile period was significantly lower in the offspring of obese dams relative to the offspring of lean dams, also as a main effect ($Q_{(1, 102)} = 4.54$, $p = 0.042$). In each case, no significant maternal diet x probiotic treatment interaction occurred, and there was no relationship between juvenile body weight and immobility time.

In the adult offspring, two-way ANOVA did not reveal an interaction for total time immobile (Figure 4.5c), but there were independent main effects for both maternal probiotic treatment ($Q_{(1, 75)} = 7.42$, $p = 0.010$) and maternal diet ($Q_{(1, 75)} = 4.12$, $p = 0.05$), whereby maternal HFD increased time immobile in adult offspring relative to CD adult offspring independent of probiotic, and maternal probiotic treatment reduced time immobile relative to offspring exposed to vehicle, independent of maternal diet. Regarding latency to immobility, a significant maternal diet x probiotic treatment interaction did occur ($F_{(1, 75)} = 5.06$, $p = 0.029$). Tukey post hoc tests indicated that HFD/vehicle offspring exhibited a significantly reduced latency to immobility compared to adult offspring from each other group ($p = 0.0087$ compared to CD/vehicle, $p = 0.0003$ compared to CD/probiotic, $p = 0.0006$ compared to HFD/probiotic, Figure 4.5d). Main effects of maternal diet ($F_{(1, 75)} = 5.75$, $p = 0.019$) and maternal probiotic treatment ($F_{(1, 75)} = 13.41$, $p = 0.0005$) were also identified. No relationship was identified between FST immobility and body weight in the adult offspring.

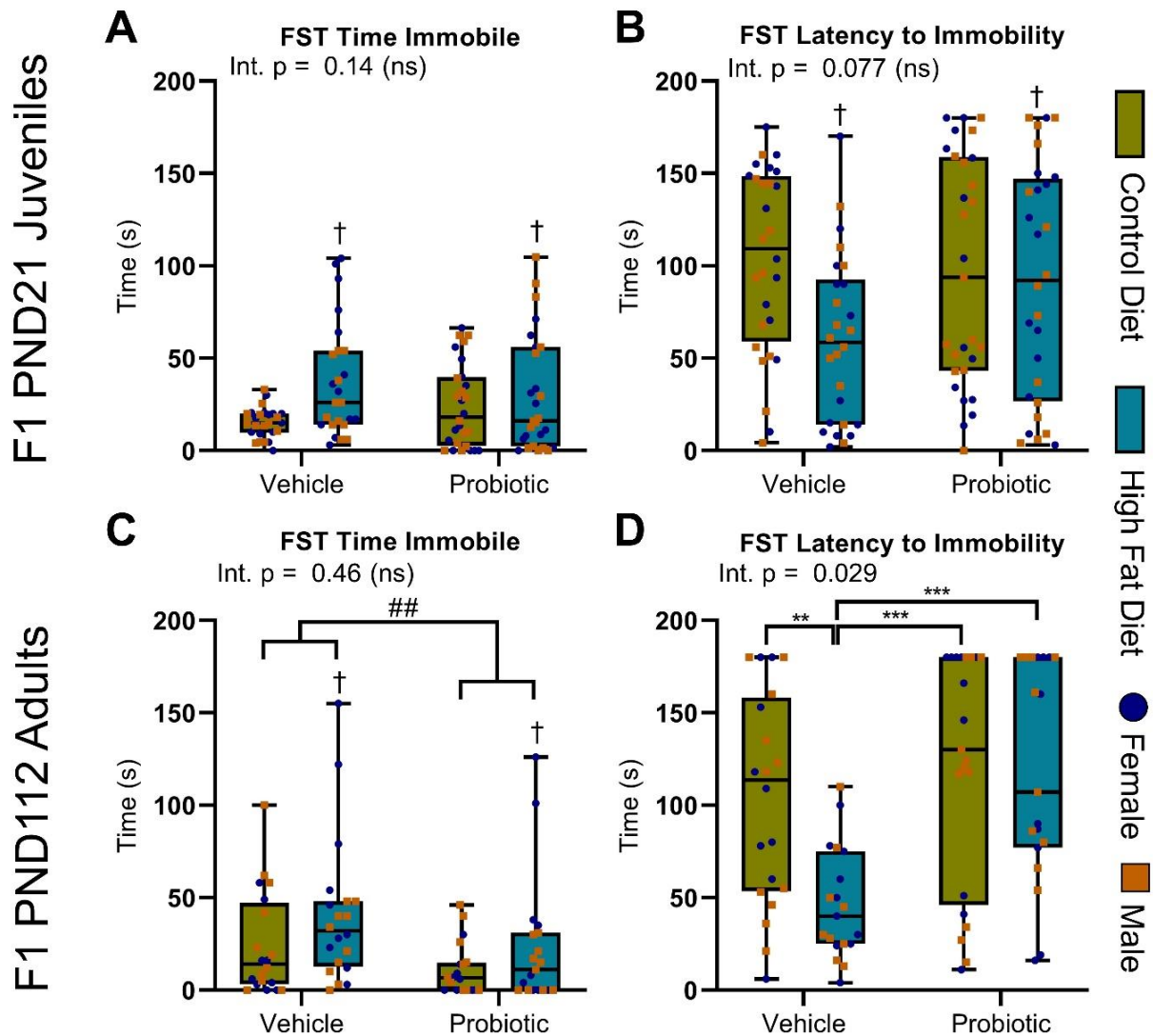


Figure 4.5 Persistent effects of maternal obesity on depressive-like behaviour in young and adult offspring. In the juvenile offspring, maternal obesity **(A)** increased immobility time and **(B)** reduced latency to immobility in the forced swim test (FST) relative to the offspring of lean dams. **(C)** Time spent immobile and **(D)** latency to immobility in the adult offspring. Two-way ANOVA; data are presented as boxplots showing median, interquartile range, and min/max points. $^{\dagger}P < 0.05$ (indicating main effect of maternal diet); $^{##}P < 0.01$ (indicating main effect of maternal probiotic treatment); $^{**}P < 0.01$; $^{***}P < 0.001$ (indicating significant post hoc comparison, computed only in the case of a significant diet $\times$ probiotic treatment interaction). Int., interaction; ns, not significant. $n = 26$ to 29 for juvenile offspring (13 to 15 male, 13 or 14 female) and 20 or 21 for adult offspring (10 or 11 male, 9 or 10 female) per group.

4.2.2.4 Social interaction test.

Behaviour in the social interaction test was assessed in the adult offspring at eight weeks of age. Two-way ANOVA revealed that maternal obesity reduced social interaction in the adult offspring at eight weeks of age relative to maternal control diet ($F_{(1, 76)} = 10.98$, $p = 0.0014$), independent of maternal probiotic treatment and with no significant interaction between maternal diet and probiotic treatment (Figure 4.4d).

4.2.3 Effects of maternal diet and probiotic treatment on peripheral and central markers of innate immunity in juvenile and adult offspring.

The relative expression of mRNAs encoding *TNF*, *IL-1B*, *IL-6*, and *TLR4* were quantified in the liver and prefrontal cortex of juvenile and adult offspring, to see whether early-life HFD exposure affected basal markers of inflammation. Significance level was set at $p = 0.0010$ prior to analysis using the Bonferroni method of correction.

4.2.3.1 Liver gene expression.

A significant maternal diet x probiotic interaction was identified for liver *IL-6* in the female juvenile offspring ($F_{(1, 18)} = 19.06$, $p = 0.0004$, Figure 4.6c). Post hoc Tukey tests revealed that the female juvenile offspring from HFD/vehicle dams had increased expression relative to the CD/vehicle ($p = 0.0002$), CD/probiotic ($p = 0.0002$) and HFD/probiotic ($p < 0.0001$) conditions. No significant interactions or main effects were observed in the male juvenile offspring. There was also a main effect of maternal probiotic supplementation on reducing *TLR4* expression in the liver of the juvenile offspring ($F_{(1, 44)} = 12.46$, $p = 0.0010$), relative to juvenile offspring from dams fed the vehicle, independent of maternal diet and with no sex differences and no significant maternal diet x probiotic interaction (Figure 4.6d). No significant main effects or significant interactions occurred for liver *TNF* or *IL-1B* in the male and female juvenile offspring (Figures 4.6a and 4.6b, respectively).

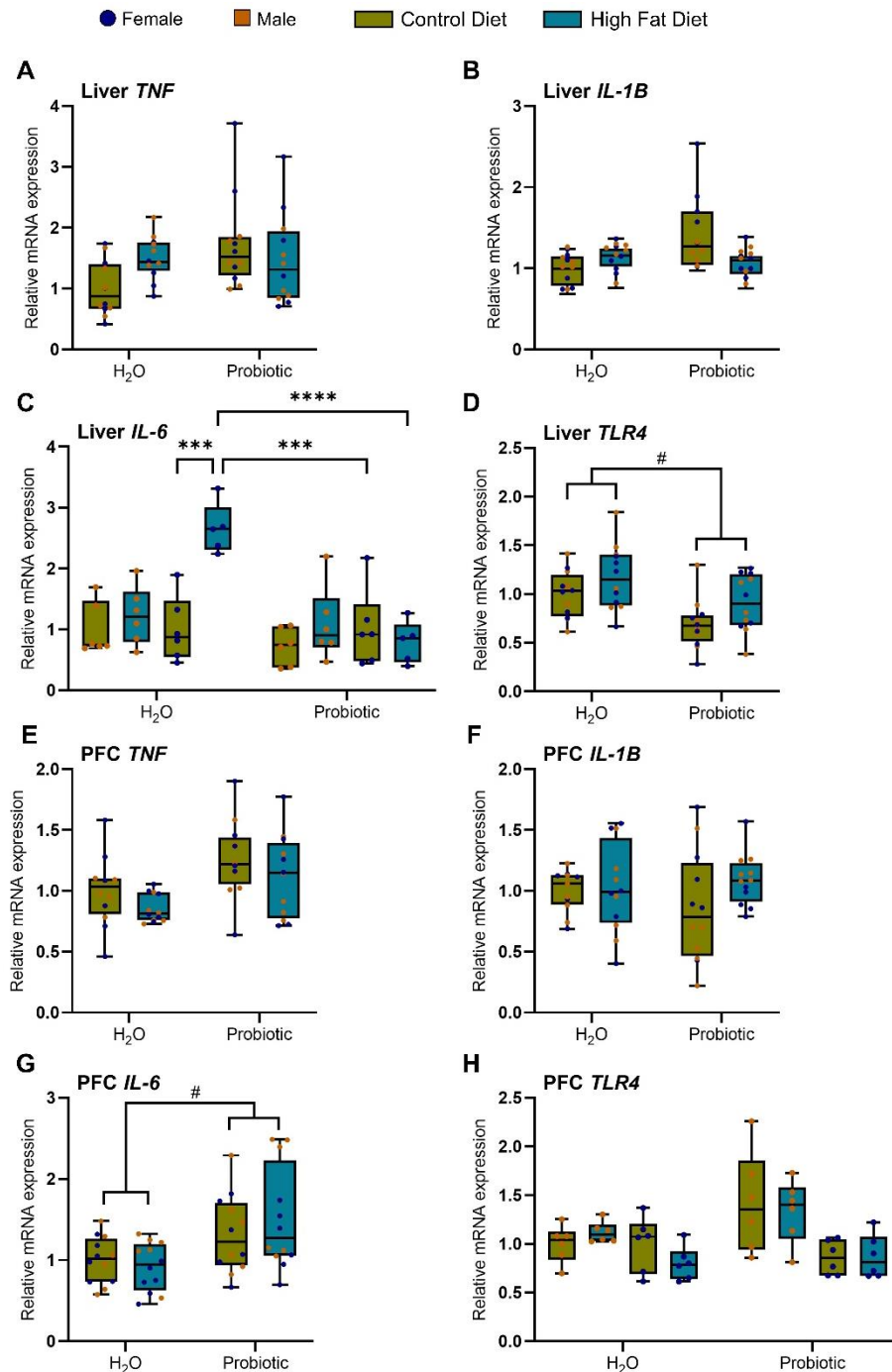


Figure 4.6 F1 (PND21) juvenile liver and brain (PFC) pro-inflammatory gene expression. Relative mRNA expression of liver **A)** *TNF*, **B)** *IL-1B*, **C)** *IL-6*, **D)** *TLR4*, and PFC **E)** *TNF*, **F)** *IL-1B*, **G)** *IL-6*, and **H)** *TLR4*. Data presented as boxplots $\pm$ interquartile range showing all points, relative expression compared to respective female or male vehicle/control diet offspring. N = 6 per group for 2-way ANOVA for each sex, or N = 12 per group if sex consolidated into a single 2-way ANOVA where no sex differences. #p < 0.001 indicating main effect of maternal probiotic intake. ****p < 0.0001, ***p < 0.001 indicating significant post-hoc comparison, performed only if a significant maternal diet x probiotic interaction was identified (interaction p < 0.001). Comparisons were adjusted for multiple testing using the Bonferroni method.

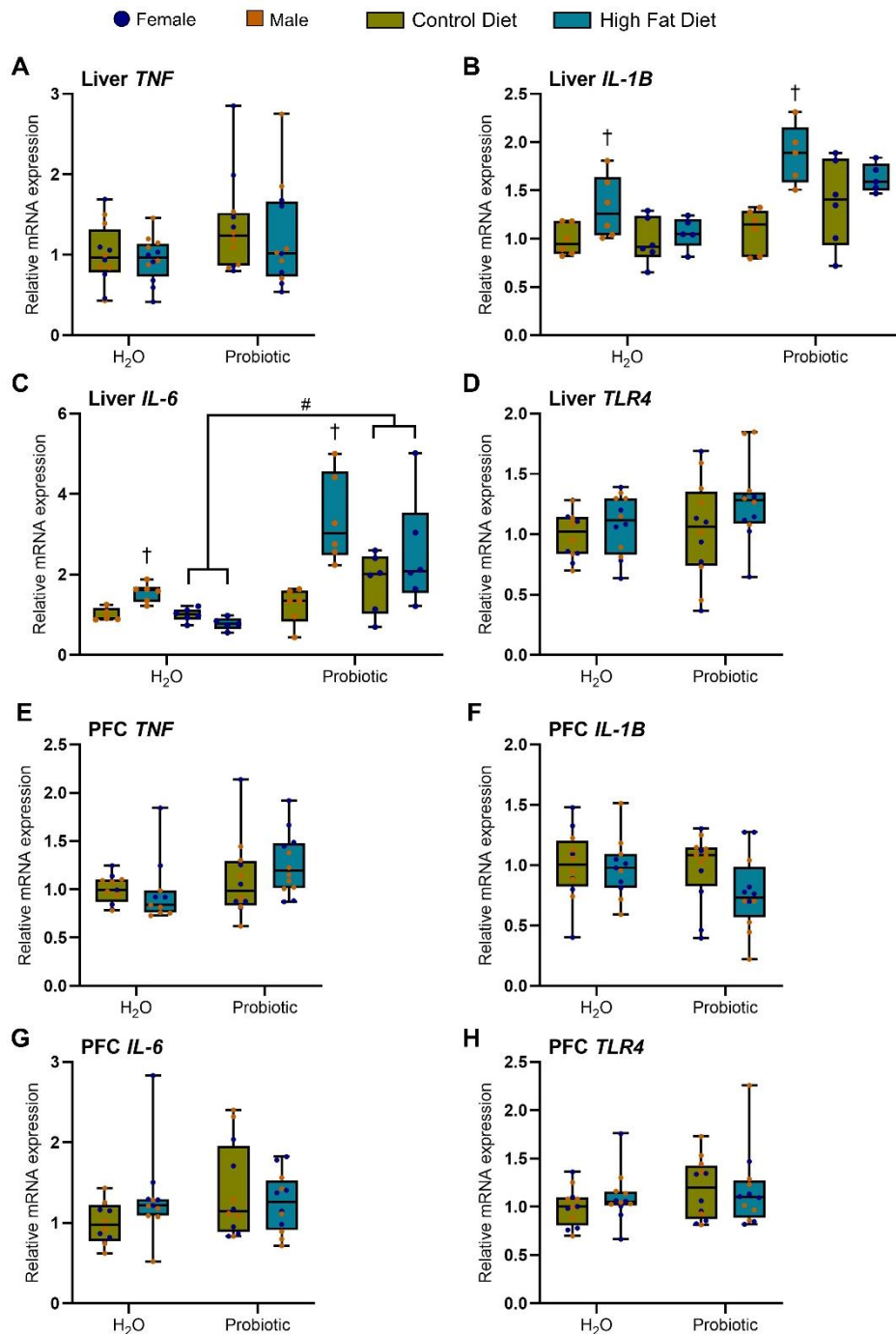


Figure 4.7 F1 (PND112) adult liver and brain (PFC) pro-inflammatory gene expression. Relative mRNA expression of liver **A) TNF**, **B) IL-1B**, **C) IL-6**, **D) TLR4**, and PFC **E) TNF**, **F) IL-1B**, **G) IL-6**, and **H) TLR4**. Data presented as boxplots $\pm$ interquartile range showing all points, relative expression compared to respective female or male vehicle/control diet offspring. N = 6 per group for 2-way ANOVA for each sex, or N = 12 per group if sex consolidated into a single 2-way ANOVA where no sex differences. $\dagger p < 0.001$, indicating main effect of maternal diet. $\#p < 0.001$ indicating main effect of maternal probiotic intake. Comparisons were adjusted for multiple testing using the Bonferroni method.

In the male adult offspring, there was a significant main effect of maternal diet on liver *IL-1B* ($F_{(1, 19)} = 26.48$, $p < 0.0001$) and *IL-6* ($F_{(1, 19)} = 33.83$, $p < 0.0001$) expression, whereby expression was increased in the offspring of obese dams relative to the offspring of lean dams, independent of probiotic and with no significant diet x probiotic interaction (Figures 4.7b and 4.7c, respectively). In the female adult offspring, there was a main effect of maternal probiotic intake on increasing liver *IL-6* expression ($F_{(1, 19)} = 19.64$, $p = 0.0003$) relative to the offspring of dams given vehicle, independent of maternal diet and with no significant diet x probiotic interaction (Figure 4.7c). No other significant main effects or interactions occurred.

4.2.3.2 Prefrontal cortex gene expression.

There was a significant main effect of maternal probiotic intake on brain *IL-6* expression, whereby mRNA levels were increased relative to the juvenile offspring of dams given vehicle ($F_{(1, 44)} = 12.49$, $p = 0.001$), independent of maternal diet (Figure 4.6g). No sex differences were observed, and there was no significant diet x probiotic interaction. No changes in the PFC mRNA levels of *TNF*, *IL-1B*, or *TLR4* were observed in the male and female juvenile offspring (Figures 4.6e,f,h).

There were no persistent changes in PFC cytokine expression in the adult offspring (Figures 4.7e-h).

4.2.4 Perinatal probiotic exposure alters glutamate receptor but not serotonin receptor mRNA expression in the prefrontal cortex of juvenile offspring.

Maternal perinatal probiotic treatment reduced expression of NMDA receptor subunits in the juvenile offspring, but not the adult offspring. Maternal probiotic intake had a main effect on reducing PFC expression of *GLUN2A* ($F_{(1, 44)} = 13.16$, $p = 0.0007$) relative to the offspring of dams given the vehicle, independent of maternal diet. No significant sex differences or interaction was observed (Figure 4.8a). *GLUN2B* expression was reduced in the juvenile females ($F_{(1, 19)} = 35.26$, $p < 0.0001$) of probiotic dams relative to the female offspring of dams given vehicle. No such effect was identified in the juvenile males ($F_{(1, 20)} = 4.216$, $p = 0.053$), and no significant interactions were identified after FDR correction (Figure 4.8b). In terms of

GLUN2C expression, two-way ANOVA revealed a significant maternal diet x probiotic interaction in females only ($F_{(1, 18)} = 15.28$, $p = 0.0010$, Figure 4.8c). Tukey post hoc tests showed that *GLUN2C* expression was increased in the female offspring of HFD/vehicle dams only (post hoc $p = 0.0003$ compared to offspring of CD/vehicle dams, $p = 0.0057$ compared to CD/probiotic, $p = 0.0025$ compared to HFD/probiotic). No significant effects were observed in the juvenile males for *GLUN2C* expression. *GLUN1* and *PSD-95*, constituent postsynaptic NMDA receptor components, were unaltered in the juvenile offspring (Appendix E-1).

In the adult offspring, there were no enduring effects of maternal probiotic treatment on the expression of the NMDA receptor subunits and on *PSD-95* expression in the PFC. No significant maternal diet x probiotic treatment interactions were identified, nor main effects of maternal diet, that survived Bonferroni's correction for multiple testing (Appendix E-2).

There were no effects of maternal probiotic treatment or maternal diet on the expression of serotonergic receptor subunits *5-HT1A*, *5-HT2A*, or *5-HT6* in the PFC in the juvenile or adult offspring (Appendix E).

4.2.5 Perinatal probiotic treatment has long-lasting effects on neuroplasticity gene expression in the offspring prefrontal cortex.

In the PFC of the juvenile offspring, two-way ANOVA revealed that there was a main effect of maternal probiotic intake on *CREB1* expression in the male offspring ($F_{(1, 20)} = 19.80$, $p = 0.0002$) but not the female offspring ($F_{(1, 20)} = 8.61$, $p = 0.0082$). In the males, *CREB1* expression was increased in the juvenile offspring of dams given the probiotic, relative to the offspring of dams given the vehicle control, independent of maternal diet (Appendix E-1). No significant interactions or main effects of maternal diet were identified. Expression of *SYP* was reduced in the male juvenile offspring of probiotic-supplemented dams compared to offspring of vehicle controls (main effect, $F_{(1, 20)} = 16.77$, $p = 0.0006$) but not females (no main effect, $F_{(1, 20)} = 1.00$, $p = 0.33$), with no significant interactions (Figure 4.8d). There was no main effect of maternal diet on *SYP* expression in males or females.

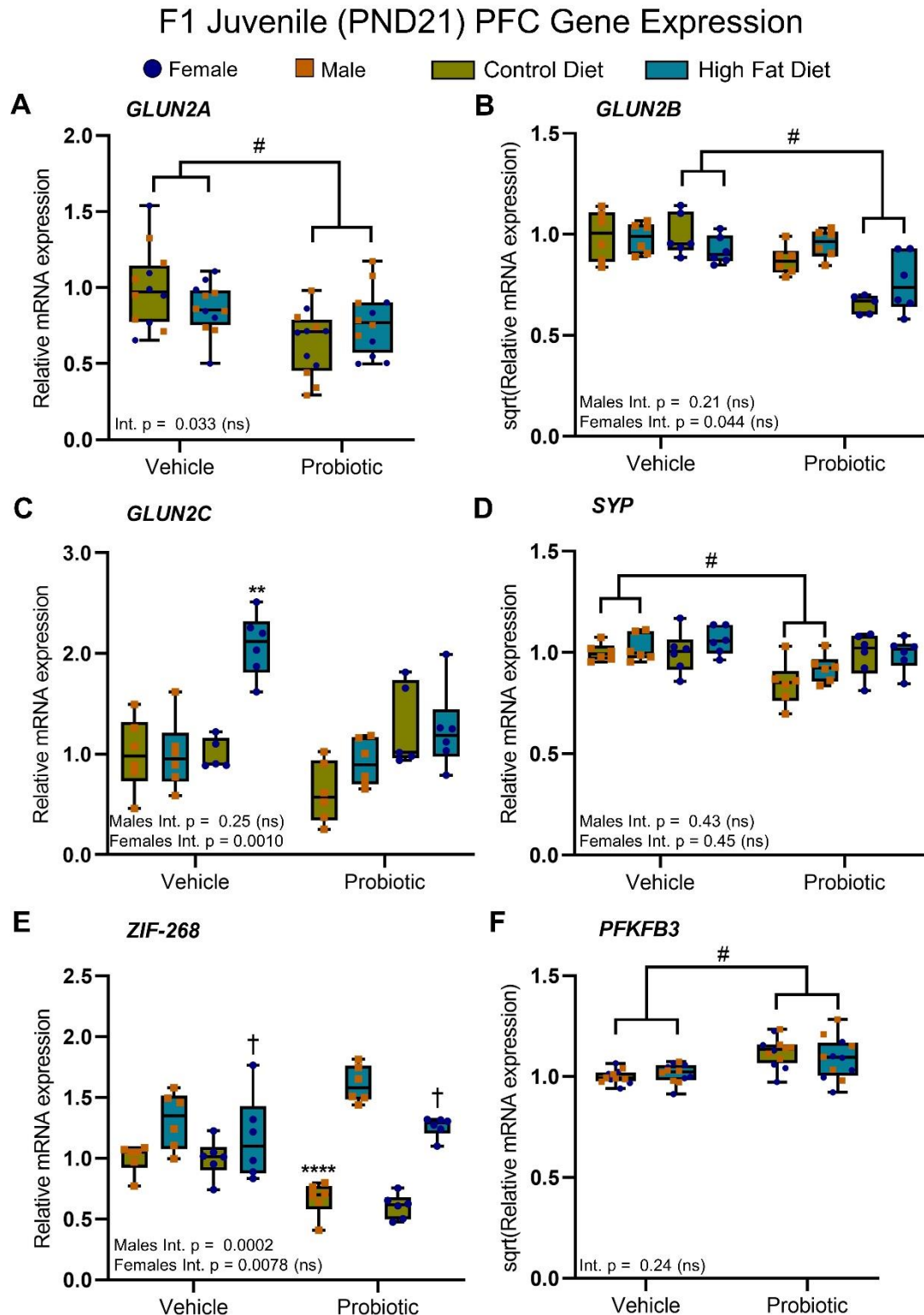


Figure 4.8 Maternal probiotic treatment alters synaptic plasticity in juvenile offspring. Juvenile PFC expression of (A) *GLUN2A*, (B) *GLUN2B*, (C) *GLUN2C*, (D) *SYP*, (E) *ZIF-268*, and (F) *PFKFB3*. Two-way ANOVA, data are presented as boxplots showing median, interquartile range, and min/max points. Int., interaction. † $P < 0.001$ (indicating main effect of maternal diet); # $P < 0.001$ (indicating main effect of maternal probiotic treatment); ** $P < 0.01$ compared to female offspring; **** $P < 0.0001$ compared to male offspring. ns, not significant. $N = 6$ per sex per group.

The expression of neuronal plasticity-related transcription factor *ZIF-268* showed an altered pattern of expression in the juvenile offspring of HFD-fed and CD-fed dams (Figure 4.8e). There was a main effect of maternal HFD, relative to maternal CD, on increasing expression in the female juvenile offspring ($F_{(1, 20)} = 24.99$, $p < 0.0001$) with no significant interaction ($F_{(1, 20)} = 8.73$, $p = 0.0078$). In males, there was a significant maternal diet x probiotic treatment interaction ($F_{(1, 20)} = 21.41$, $p = 0.0002$). Post hoc Tukey tests revealed significant differences, where male HFD/probiotic offspring had increased expression compared to both CD/probiotic and CD/vehicle male offspring ($p < 0.0001$) and HFD/vehicle male offspring had significantly greater expression compared to CD/probiotic male offspring ($p < 0.0001$). In the male juvenile offspring, there was also a main effect of maternal diet, whereby expression was significantly increased in offspring of obese dams relative to the offspring of lean dams ($F_{(1, 20)} = 85.24$, $p < 0.0001$, Figure 4.8e). There were no significant interactions or main effects for *BDNF*, *GSK3B*, *cFOS*, or $\Delta FOSB$ in the juvenile offspring ($p > 0.001$, Appendix E-1).

In the adult offspring, maternal probiotic treatment appeared to have enduring effects on neuroplasticity-related gene expression in the PFC (Figure 4.9). Two-way ANOVA revealed that the expression of *BDNF* ($F_{(1, 44)} = 70.53$, $p < 0.0001$, Figure 4.9a) and $\Delta FOSB$ ($F_{(1, 42)} = 33.60$, $p < 0.0001$, Figure 4.9e) were both increased by maternal probiotic intake as a main effect relative to the adult offspring of vehicle-fed dams, independent of maternal diet and with no significant interactions or sex differences. Conversely, *ZIF-268* expression was increased by maternal probiotic intake only in males (main effect relative to vehicle offspring, $F_{(1, 20)} = 34.99$, $p < 0.0001$) independent of maternal diet with no significant interaction (Figure 4.9c). In females, no main effects or interactions were observed for *ZIF-268* expression. This same pattern of expression was observed for *cFOS*, whereby there was a main effect of maternal probiotic intake on increasing expression only in males ($F_{(1, 20)} = 25.17$, $p < 0.0001$), independently of maternal diet, with no significant interaction (Figure 4.9d). In females, no main effects or interactions were observed for the expression of *ZIF-268* or *cFOS*. Lastly, there was a main effect of diet on *SYP* expression, with no sex differences (Figure 4.9b). Two-way

ANOVA revealed that expression was reduced in the offspring of HFD dams compared to adult offspring of CD dams ($F_{(1, 44)} = 12.86$, $p = 0.0008$), independent of maternal probiotic intake. No significant diet x probiotic interaction occurred. Neither *CREB1* nor *GSK3B* expression were altered in the PFC of the adult offspring (Appendix E-2).

4.2.6 Maternal probiotic intake alters expression of a genetic marker of astrocyte metabolic activity in the offspring prefrontal cortex.

Two-way ANOVA revealed a main effect of maternal probiotic intake on the expression of PFC *PFKFB3*, a marker of increased astrocytic metabolism through glycolysis. *PFKFB3* was increased in both the juvenile ($F_{(1, 44)} = 23.96$, $p < 0.0001$, Figure 4.8f) and adult ($Q_{(1, 44)} = 29.96$, $p = 0.001$, Figure 4.9f) offspring of dams treated with the probiotic compared to the offspring of vehicle controls, independent of maternal obesity and with no interaction or sex differences. *ATP1A2* expression was not altered in the juvenile or adult offspring (Appendix E).

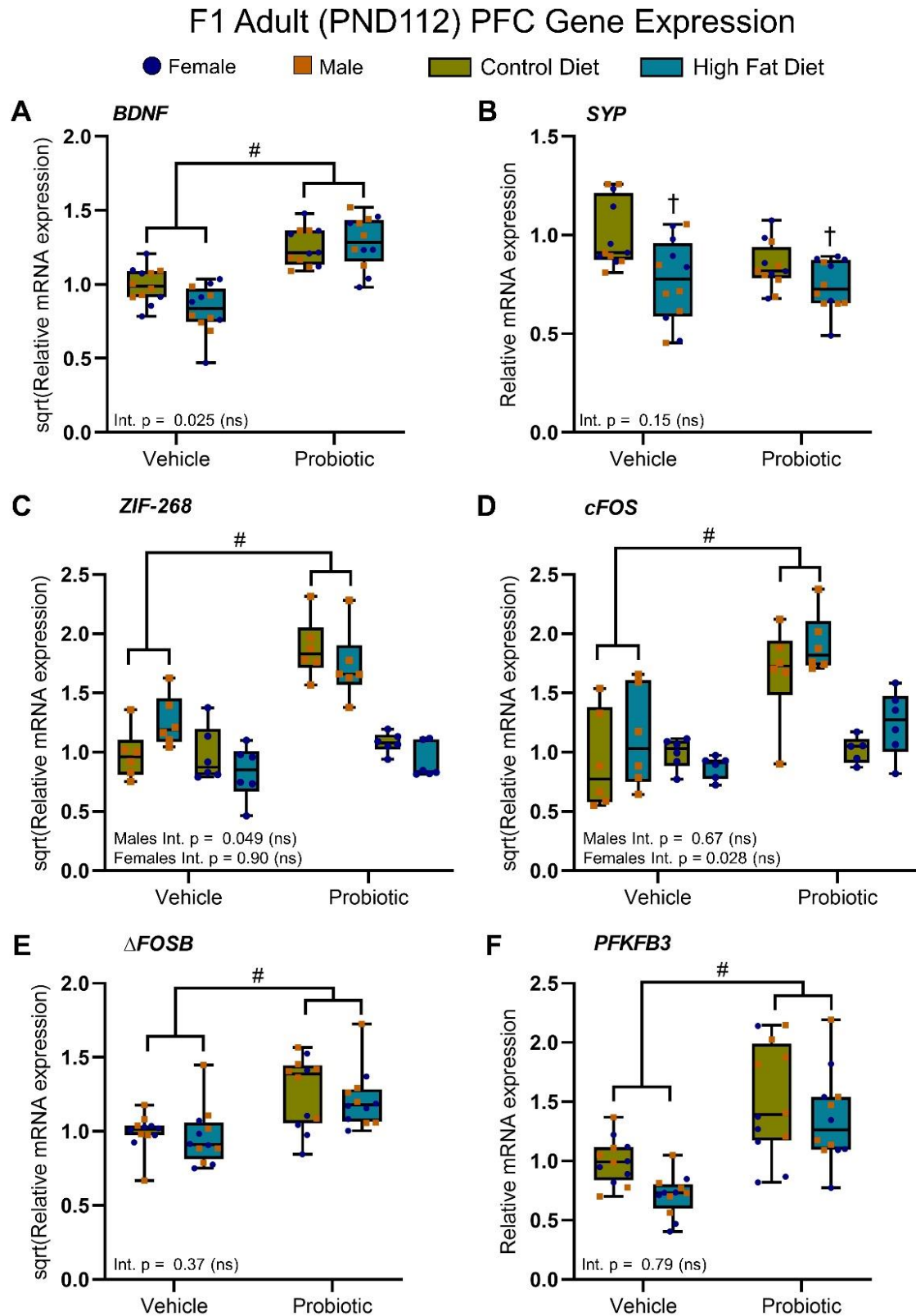


Figure 4.9 Maternal probiotic treatment has lasting effects on astrocytic metabolism and neuronal plasticity. Adult offspring PFC expression of (A) *BDNF*, (B) *SYP*, (C) *ZIF-268*, (D) *cFos*, (E) $\Delta FOSB$, and (F) *PFKFB3*. Data are presented as boxplots showing median, interquartile range, and min/max points. Int., interaction; ns, not significant. †P < 0.001 (indicating main effect of maternal diet); #P < 0.001 (indicating main effect of maternal probiotic treatment). N = 6 per sex per group.

4.3 Discussion

The impact of maternal diet-induced obesity on offspring behaviour has been previously described (Buffington et al., 2016; Winther et al., 2018). While microbial species have been found to be important in neuropsychiatric disorders influenced by maternal nutrition, the therapeutic effect of supplementing obese dams with a probiotic mixture during gestation and nursing has not been explored. Based on recent evidence demonstrating the potential anxiolytic effects of maternal prebiotic (Hebert et al., 2021) and probiotic (Niu et al., 2020) intake on young rodent offspring, I expected perinatal probiotic intake to reduce the adverse effect of maternal obesity on offspring behaviour. I also expected early HFD and probiotic exposure to differentially alter brain gene expression in young offspring.

The current chapter demonstrates that maternal obesity exerts long-term effects on behaviour and PFC plasticity gene expression. Maternal, perinatal multispecies probiotic supplementation ameliorated offspring anxiety-like behaviour associated with maternal obesity in both the young (PND21) and adult (PND112) offspring. Additionally, early life probiotic exposure reduced *GLUN2A/2B* mRNA, and elevated *PFKFB3* expression (a marker of increased astrocytic metabolism through glycolysis (Magistretti and Allaman, 2018)) in the PFC of young offspring relative to vehicle controls. *PFKFB3* was also elevated in the adult offspring exposed to perinatal probiotic supplementation relative to vehicle controls. *BDNF* and other plasticity-related genes were increased in adulthood after early life probiotic exposure, whereas *SYP* levels were reduced in the adult offspring of obese dams relative to the offspring of lean dams. Together, these data show that the intake of a multispecies probiotic supplement during gestation and nursing has a lasting influence on behaviour and neuroplasticity and can mitigate some of the behavioural symptoms of maternal obesity in the offspring.

The anxiolytic and antidepressant effects of perinatal probiotic exposure in this study are concordant with reports describing similar behavioural responses in rodent offspring: *Lactobacillus helveticus* NS8 intake in normal-weight dams from embryonic day (E) 13 to E22

during gestation has been shown to reduce offspring anxiety (Niu et al., 2020), and maternal perinatal prebiotic intake (which encourages the growth of endogenous *Bifidobacterium* spp.) reduced passive stress coping in the FST in adulthood (Hebert et al., 2021). Reduced social behaviour observed in the adult offspring of obese dams is associated with reduced *Bifidobacterium* spp. and *Lactobacillus* spp. (Buffington et al., 2016), but reconstitution with microbiota of control offspring through coprophagia reversed these deficits. These findings, in combination with our results, emphasize the important role of maternal microbiota and metabolites on early life neurodevelopment and behaviour (Vuong et al., 2020). Surprisingly, the juvenile offspring of obese dams exhibited increased anxiety-like behaviour (relative to the offspring of lean dams) in the OFT but not the LDB. In other studies that have assessed anxiety-like behaviour using both the OFT and the LDB, similar findings have been observed. Wada et al. (2018) found that high fat diet treatment in female adult C57Bl/6 mice reduced time in the centre zone of the OFT, but had no effect on anxiety-like behaviour in the subsequent LDB test. It is possible that the order of the tests contributed to the lack of an observed effect of diet in the LDB in both the study by Wada et al. and the present study. Given that all mice underwent testing in the OFT prior to the LDB, and that consecutive behavioural tests have been shown to increase overall anxiety levels in mice (Saré et al., 2021), the reduced signal-to-noise ratio of anxiety levels may have resulted in the greater variability observed in the LDB here.

It should also be noted that while maternal probiotic treatment attenuated depressive-like behaviour in the FST by increasing latency to float in the offspring of obese dams in the adult offspring, the effect was not significant in the juvenile offspring. Despite this, the ameliorative effect of maternal probiotic treatment on depressive-like behaviour in the juvenile offspring cannot be ruled out. In the adult offspring, the latency float in the offspring of obese dams was 44% of the control group without maternal probiotic treatment, but 98% of the control group with probiotic treatment. In the juvenile offspring, the latency to float was 58% and 94% respectively.

In this study, the expression of the *GLUN2A* and *GLUN2B* NMDAR subunits was reduced in the PFC of juvenile probiotic offspring. Our laboratory has previously found that maternal prebiotic supplementation reduced these subunits in the juvenile offspring hippocampus (Hebert et al., 2021), and others have shown that prebiotic supplementation reduced *GLUN2B* protein in the frontal cortex (Savignac et al., 2016) of adult mice. Consistent with the present data in juvenile mice, these reported changes in gene expression were associated with reduced anxiety-like behaviour. Thus, despite prebiotics and probiotics having distinct actions on the gut flora, the central mechanisms underlying their psychotropic effect may be similar. Antagonists of the NMDAR, including those selective to the *GLUN2B* subunit, have antidepressant activity in mice (Maeng et al., 2008) and humans (Berman et al., 2000; Zarate et al., 2006). It has been proposed that these antagonists preferentially block NMDAR stimulation of GABA inhibitory interneurons synapsing with glutamatergic pyramidal neurons, which ultimately enhances glutamate release from pyramidal neurons and PFC synaptic efficacy (Moghaddam et al., 1997; Homayoun and Moghaddam, 2007; Zanos and Gould, 2018). Alternatively, the antidepressant action of the NMDAR antagonist ketamine has been shown to be dependent on reduced presynaptic potentiation and increased postsynaptic potentiation of glutamatergic neurons (Lazarevic et al., 2021). I show that expression of postsynaptic density marker *PSD-95* was stable, while *SYP* expression, a presynaptic density marker, decreased in the male juvenile offspring of probiotic-fed dams alongside reductions in *GLUN2A* and *GLUN2B* expression relative to vehicle controls. These observations are largely consistent with the downstream effects of NMDAR antagonism in vivo (Duman et al., 2016).

In the adult offspring, gene-expression analysis revealed a long-term effect of maternal probiotic administration on synaptic plasticity. Increased *BDNF* and the immediate early genes *ZIF-268* and *cFOS* indicate an increase in the maintenance of BDNF-mediated synaptic plasticity in adult probiotic offspring (Bramham and Messaoudi, 2005). Indeed, both *ZIF-268* and *cFOS* expression are upregulated in hippocampal neuronal cell cultures within 20 minutes

of BDNF treatment, strongly suggesting a molecular role for these immediate early genes downstream of BDNF-induced synaptic plasticity (Alder et al., 2003). Because this mechanism is thought to underlie response to traditional antidepressant activity and mood resilience in adults (Bramham and Messaoudi, 2005; Castrén and Rantamäki, 2010), it may account for the long-term reduction in depressive-like behaviour in these offspring. The elevation of $\Delta FOSB$ in the adult offspring of probiotic dams may also contribute to the antidepressant-like behaviour. $\Delta FOSB$ is an important protein in reward-related neuronal plasticity (Nestler, 2008). Induced overexpression of $\Delta FOSB$ has been shown to counteract the behavioural effects of chronic social defeat stress in mice (Vialou et al., 2010; Donahue et al., 2014). However, because the phosphorylated form of the protein is stable *in vivo*, the protein expression of $\Delta FOSB$ should also be measured in future work to confirm its upregulation in the probiotic-exposed offspring (Ulery-Reynolds et al., 2009).

While the effects of maternal HFD on increasing aspects of anxiety and depressive-like behaviour in the offspring were in line with previous reports (Bilbo and Tsang, 2010; Giriko et al., 2013; Winther et al., 2018), the concomitant effects of maternal HFD on patterns of gene expression in the juvenile and adult offspring were inconclusive. Maternal HFD feeding increased liver *IL-1B* and *IL-6* expression in the male adult offspring, however this effect was not observed in the female adult offspring nor the juvenile offspring and is therefore unlikely to explain the effects of maternal diet on behaviour. Similarly, sex-specific effects of maternal HFD on PFC gene expression in the juvenile offspring did not mirror the behavioural changes, as the increase in anxiety and depressive-like behaviour was sex-independent. As maternal obesity is thought to have a wide range of effects on offspring development involving a combination of inflammatory, metabolic, and neurotransmitter pathways (Sullivan et al., 2014), the scope of targeted gene expression profiling here was insufficient to fully characterise the effects of maternal diet on brain function and its association with increased anxiety and depressive-like behaviour.

Indeed, the main limitation of the work described here is the unidimensional approach to assessing the effects of the probiotic on brain function and synaptic plasticity. Quantifying mRNA abundance is effective in discovering potential differences in the expression of target genes, and changes in mRNA expression are likely to precede congruent changes in protein expression. Nevertheless, it would be interesting to determine the consequences of these changes to brain function using an alternative method, for example protein expression or electrophysiological recordings of NMDAR activity in different brain regions. Moreover, quantifying mRNA does not take into account possible post-transcriptional inactivation or constitutive protein degradation. *PFKFB3*, for example, is transcribed and translated in neurons, yet remains largely absent due to continuous proteasomal degradation via the E3 ubiquitin ligase mechanism (Herrero-Mendez et al., 2009). Thus, to confirm that an upregulation of *PFKFB3* mRNA corresponds to an increase in the *glycolytic* metabolic rate of astrocytes, *PFKFB3* protein expression could be measured in brain tissue from mice administered with isotopically labelled metabolites (for example, ^{13}C labelled glucose) to quantify the rate of neuronal metabolism through glycolysis and the pentose phosphate pathway as opposed to the tricarboxylic acid (TCA) cycle. Conversely, Zhang et al. (2014) performed ex vivo transcriptomic analysis of neurons, astrocytes, and other purified brain cell types, and found that even at the mRNA level, *PFKFB3* was basally expressed at an order of magnitude higher in astrocytes than neurons. Moreover, the transcriptomic analysis suggested that an overexpression of *pyruvate dehydrogenase kinase 4* in astrocytes compared to neurons contributes to the very low level of pyruvate dehydrogenase (PDH) activity in astrocytes. Instead, the pyruvate generated from glycolysis is shunted towards lactate production via lactate dehydrogenase (LDH). Astrocytes express an LDH isoform which preferentially forms lactate from pyruvate (*LDHb*), allowing lactate to travel down a concentration gradient to be received by neurons, whilst neurons express an LDH isoform preferentially producing pyruvate (*LDHa*), allowing pyruvate to then be metabolised into Acetyl-CoA and through the TCA cycle (Zhang et al., 2014).

Finally, it would be important to understand more about NMDAR distribution, as opposed to purely quantifying expression levels. This is because the location of the receptor, specifically synaptic or extrasynaptic, is thought to drastically affect its function as either promoting neuronal survival (with concomitantly increased CREB and BDNF expression) or neuronal cell death (with concomitantly decreased CREB and BDNF expression), respectively (Hardingham and Bading, 2010).

In summary, maternal obesity increased anxiety- and depressive-like behaviour in young and adult offspring. Anxiety-like behaviour was prevented in the juvenile offspring of obese dams by maternal perinatal probiotic treatment, and both anxiety- and depressive-like behaviour were prevented in the adult offspring of obese dams that were exposed to perinatal probiotic treatment.

5

Effects of maternal obesity and probiotic treatment on the gut-liver-brain metabolome of the juvenile and adult offspring³

5.1 Introduction

Metabolic effects of chronic maternal HFD feeding are known to propagate to the *offspring*. For example, maternal obesity results in hepatic steatosis and impaired insulin sensitivity in the rodent offspring (Pereira et al., 2015; Paul et al., 2019; Savva et al., 2021) and dyslipidaemia in humans (Bucher et al., 2021). By contrast, the effects of maternal probiotic treatment on the offspring metabolome have hitherto not been explored. The aim of this chapter was to investigate whether maternal probiotic intake during the perinatal period would influence the gut, brain, liver, plasma, or brain metabolome in the young and adult offspring of obese dams.

While alterations in peripheral lipid metabolites are relatively well documented, few studies have investigated the brain metabolome of offspring exposed to maternal obesity (Zhu et al., 2018; Safi-Stibler et al., 2020). In one study, the effect of maternal diet (HFD vs control) was explored in male F1 six-month-old offspring (6 per group) in the liver, hypothalamus, and olfactory bulb by MS. While maternal diet had a strong effect on programming hepatic amino acid metabolism in the offspring, researchers found no lasting effects of maternal diet on brain metabolites in the two brain regions (Safi-Stibler et al., 2020). By contrast, Zhu et al. (2018) demonstrated that the offspring of obese dams can be discriminated with 100% accuracy from the offspring of lean dams based on brain PFC metabolites alone, also using MS techniques. Male F1 offspring (6-7-month-old, n = 8 per group) from either obese *LEPR^{db/+}* or lean WT

³ Results included in this Chapter have been published: <https://doi.org/10.1073/pnas.2108581119>

dams were compared, whereby the maternal mice heterozygous for leptin receptor deficiency displayed hyperphagia and 33% weight gain compared to WT mice prior to conception. Both saturated and unsaturated fatty acids were found to have accumulated in the PFC of the *LEPR^{db/+}* offspring compared to the WT offspring. However, because the genotype of the *offspring* also differed between the two groups in the same way as the F0 generation, it is unclear whether maternal obesity or offspring genotype was responsible for the observed increase in PFC lipid levels. Considering this conflicting evidence, it is important to determine how maternal obesity alters the offspring brain metabolome whilst considering possible effects of offspring sex and the longevity of such changes.

The gut microbiome is thought to modulate brain function and behaviour through gut-derived signalling molecules and the manipulation of host metabolism (Nicholson et al., 2012; Abildgaard et al., 2017, 2021; Vuong et al., 2020). Probiotic supplementation has been associated with changes in gut, blood, and brain metabolites thought to be derived from microbial metabolism. Furthermore, these metabolomic changes were associated with reduced anxiety and depressive-like behaviour in rodents (Abildgaard et al., 2017, 2021; Duan et al., 2021). While perturbations to the *maternal* microbiota induced by diet (Buffington et al., 2016) and antibiotic treatment (Vuong et al., 2020) have been shown to alter the *offspring* microbiota (Buffington et al., 2016), brain metabolome (Vuong et al., 2020), and behaviour, it is hitherto unclear as to whether the faecal metabolome, the functional readout of the gut microbiota, is also significantly affected by maternal diet or probiotic treatment. In the current study, I sought to investigate the metabolomic profile of the brain, gut, liver, and blood in the offspring using NMR-based techniques. I expected maternal obesity to give rise to long-lasting changes in offspring brain energy metabolism, whereas maternal probiotic supplementation was expected to increase faecal SCFA levels in the offspring.

5.2 Results

Juvenile and adult offspring brain, liver, plasma, and faecal samples were interrogated for metabolite composition using ^1H NMR. No sex differences were observed in the F1 juvenile offspring (Appendix F-1a, b) and group differences in metabolites were unrelated to sex differences in the F1 adults (Appendix F-1c, d).

Integrated PCA of all metabolites (relative abundance, z-scaled by sex) across the faecal, liver, blood, and brain identified unsupervised group differences in the juvenile offspring (Figure 5.1a and Appendix D-2). Maternal probiotic intake appeared to have a greater impact on the integrated metabolome than maternal obesity. In the PCA scores plot, the offspring of CD/probiotic dams, HFD/vehicle dams, and HFD/probiotic dams were clustered separately to each other, while CD/vehicle offspring showed some overlap between HFD offspring (of both vehicle and probiotic dams). The cumulative R^2X was 0.51, with six principal components. Consultation of the corresponding integrated PCA loadings plot (Figure 5.1b) revealed that, unlike in the dams, changes to the brain metabolome in the juvenile offspring were equal to or greater than the variation explained by the faecal, liver, or plasma metabolites. Specifically, brain metabolites appear to account for much of the variation between HFD/vehicle and HFD/probiotic offspring, while liver metabolites appear to explain much of the variation between CD/probiotic offspring and all other offspring (Figure 5.1b).

Similar clustering by group was observed in the integrated PCA (relative abundance, z-scaled by sex) of the adult offspring metabolome (Figure 5.1c and Appendix D-3). In the PCA scores plot, substantial unsupervised clustering by group was observed between the adult offspring of vehicle and probiotic dams. More variation between the offspring of HFD and CD dams was observed in the probiotic offspring than the offspring of vehicle-fed dams. The cumulative R^2X was 0.55 with six principal components. In the loadings plot (Figure 5.1d), plasma metabolites are important in the variation exhibited across the first principal component. Metabolite peaks corresponding to plasma glucose were decreased, whilst plasma resonances corresponding to αCH_2 , βCH_2 , VLDL/TG, and unsaturated fatty acyl

chains ($=\text{CH}-\text{CH}_2-\text{CH}_2$) were increased in the adult offspring of probiotic-fed dams relative to those fed the vehicle (Table 5.2).

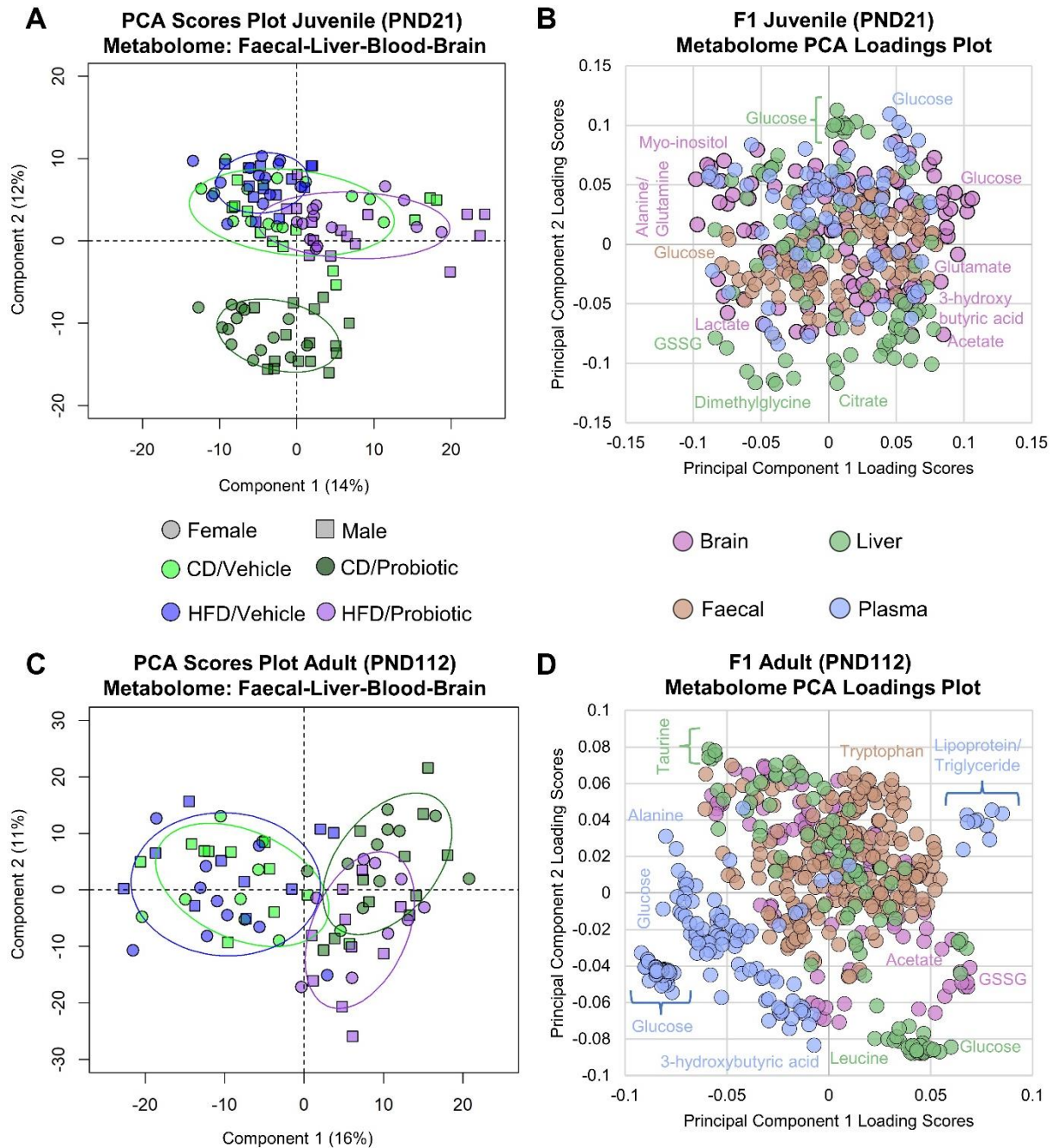


Figure 5.1 Unsupervised principal component analysis of the offspring gut–brain metabolome reflects maternal metabotype influenced by diet and perinatal probiotic supplementation. **(A)** PCA scores plot and **(B)** loadings plot of all juvenile offspring gut, liver, plasma, and brain metabolites at PND21. **(C)** PCA scores plot and **(D)** loadings plot of metabolites in the F1 adult offspring. N = 26 to 29 for juvenile offspring and 20 or 21 for adult offspring per group.

To better understand the discriminatory metabolites in each tissue individually, partial least squares discriminant analysis (PLS-DA) was used to investigate the key differences between male and female offspring of CD/vehicle, HFD/vehicle, CD/probiotic, and HFD/probiotic dams⁴ and the key metabolites identified by variable importance in projection (VIP) scores were investigated (Tables 5.1 and 5.2). In addition, the key metabolites across the brain-gut axis in the juvenile offspring were compared between the juvenile and adult offspring and depicted in the heatmap (Figure 5.6) and summary metabolite atlas (Figure 5.10).

5.2.1 Perinatal probiotic exposure increases faecal SCFA abundance in the juvenile and adult offspring.

PLS-DA of the juvenile faecal metabolome by sex, maternal diet, and maternal probiotic treatment revealed modest separation between CD/probiotic offspring and vehicle offspring in the first component, and clear separation by maternal diet was observed in both components one and two (Figure 5.2a). In the PLS-DA scores plot, greatest separation in the first two predictive components was observed between CD/probiotic and HFD/vehicle offspring, with no clear sex differences. However, modest separation between all CD offspring and all HFD offspring was also observed in component 3 (Appendix D-4). The cumulative R^2X was 0.49, the cumulative R^2Y was 0.22, and the cumulative Q^2 was 0.13, with three components. The PLS-DA loadings plot identified an overwhelming contribution of SCFA abundance – acetate, butyrate, and propionate, to faecal metabolite variation between offspring of probiotic and vehicle-treated dams (Figure, 5.2b).

Two-way ANOVA was used to investigate univariate statistical significance of each metabolite identified by PLS-DA in the juvenile offspring (Table 5.1). No significant interactions between maternal diet and maternal probiotic treatment were identified for the faecal metabolites and thus post hoc comparisons are not described (Table 5.1). Nevertheless,

⁴ Note that, in many cases, the R^2Y and Q^2Y values for the offspring PLS-DA models are small. A likely contributing factor is that offspring sex was included as a group when, in most cases, there was no clear difference in the metabolome between males and females especially in the juvenile offspring.

significant main effects of both maternal diet and probiotic treatment were identified. In terms of faecal SCFAs, acetate was not significantly increased by maternal probiotic treatment after FDR correction ($F_{(1, 103)} = 4.66$, $q = 0.096$), nor were its levels affected by maternal diet ($F_{(1, 103)} = 1.51$, $q = 0.22$). This was expected based on its position in the PCA loadings plot (Figure 5.2b) as while faecal acetate is increased in probiotic offspring relative to vehicle offspring (before FDR correction, $p < 0.05$) it also explains a large proportion of variation *within* each group from top left to bottom right of the PCA scores plot (Figure 5.2a).

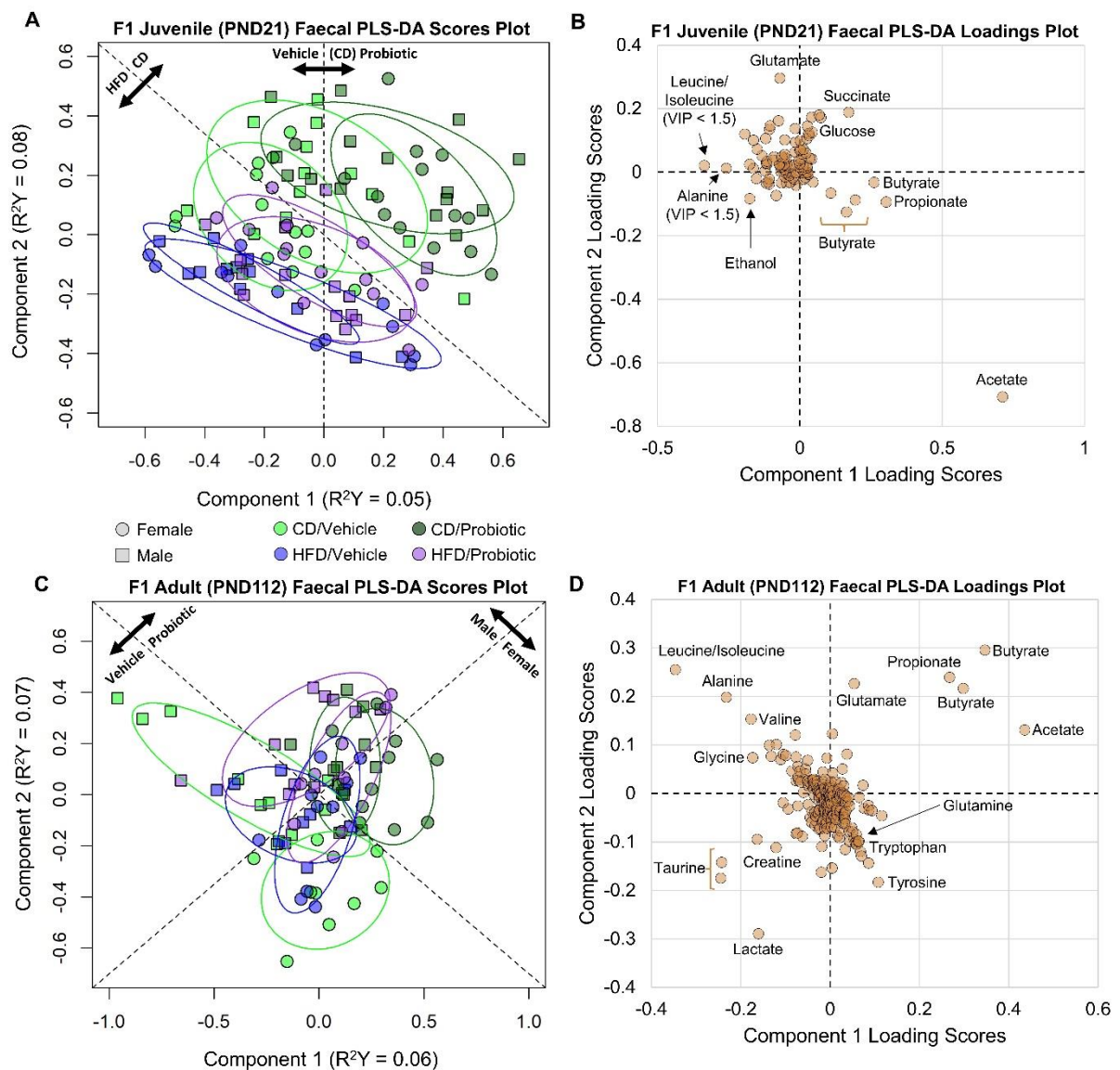


Figure 5.2 Faecal PLS-DA scores plot with accompanying loadings plot for the juvenile offspring (**A, B**) and adult offspring (**C, D**). N = 13-15 per group for juveniles and N = 9-11 per group for the adults.

Conversely, propionate and butyrate were increased as a main effect by maternal probiotic treatment relative to vehicle control offspring (propionate: $F_{(1, 102)} = 17.27$, $q < 0.0001$; butyrate: $F_{(1, 101)} = 13.83$, $q = 0.0007$, Figure 5.9c). Two-way ANOVA also revealed that faecal ethanol was reduced by maternal probiotic intake relative to vehicle ($F_{(1, 101)} = 27.87$, $q < 0.0001$). Faecal ethanol levels were also influenced by maternal diet, independent of maternal probiotic treatment (main effect, $F_{(1, 101)} = 19.22$, $q < 0.0001$). Ethanol levels were increased in the juvenile offspring of obese dams relative to lean dams. Maternal diet had a main effect on faecal succinate levels ($F_{(1, 102)} = 27.42$, $q < 0.0001$), faecal glucose levels ($F_{(1, 102)} = 301.6$, $q < 0.0001$), and faecal glutamate levels ($F_{(1, 103)} = 14.68$, $q = 0.0004$) which were reduced in the offspring of obese dams relative to lean dams independent of maternal probiotic treatment (Table 5.1).

PLS-DA of the adult faecal metabolome revealed little separation between the eight different groups (Figure 5.2c). Indeed, while the cumulative R^2X was 0.46, the R^2Y was only 0.13, and the Q^2 was 0.07, with two components. However, between the vehicle and probiotic adult offspring, some separation is driven by a relative increase in faecal butyrate, acetate, and propionate levels in the latter groups (Figure 5.2c,d). Orthogonally, male/female separation is visually apparent in the CD/vehicle adult offspring.

As in the juvenile offspring, two-way ANOVA did not reveal effects of maternal probiotic treatment ($F_{(1, 76)} = 3.10$, $q = 0.082$) or maternal diet ($F_{(1, 76)} = 0.016$, $q = 0.49$) on faecal acetate levels in the adult offspring. Faecal propionate ($F_{(1, 76)} = 6.57$, $q = 0.024$), butyrate ($F_{(1, 76)} = 8.71$, $q = 0.013$, Figure 5.9d) and glutamate levels, however, were increased in the adult offspring of probiotic dams relative to vehicle as a main effect, independent of maternal diet with no sex differences, while taurine and creatine levels were decreased (Table 5.2). Sex differences were observed in the levels of faecal leucine/isoleucine, lactate, alanine, valine, tyrosine, glycine, tryptophan, and glutamine, and thus males and females were analysed separately by two-way ANOVA. Statistically significant differences in metabolite levels between the adult offspring are summarised in Table 5.2. In males, faecal alanine and glycine

were reduced in CD/Pro only relative to controls. Tyrosine was reduced in HFD/Pro offspring only, and valine was reduced in both HFD/Veh and CD/Pro offspring relative to controls. Also in the males, lactate was reduced in the adult offspring of probiotic-treated dams relative to vehicle. In the females, leucine/isoleucine was increased in all probiotic offspring relative to vehicle. Tryptophan and glutamine were reduced in the offspring of obese dams relative to lean dams, while faecal valine levels were increased.

In addition, faecal ethanol, glucose, and succinate were profiled in the adult offspring (Appendix G), as they were significantly altered as key metabolites in the faeces of the juvenile offspring. In the adult males, there was a significant maternal diet x probiotic interaction for faecal ethanol, which was significantly increased in both HFD/Veh and CD/Pro offspring relative to controls. In both males and females, glucose was increased, and succinate was decreased in the offspring of probiotic-treated dams relative to vehicle (Appendix G).

5.2.2 Effects of maternal obesity and perinatal probiotic treatment on the offspring liver metabolome.

The PLS-DA scores plot of the juvenile liver metabolome revealed separation between HFD/vehicle and all probiotic offspring in the first component, and separation between CD/probiotic and all HFD offspring in the second component (Figure 5.3a). CD/vehicle offspring showed greater within-group variability in the first two components, but clustered together separately from the other juvenile offspring in components 3 and 4 (Appendix D-5). Male/female separation is observed in the CD/probiotic group only, in the first component. The cumulative R^2X was 0.88, the cumulative R^2Y was 0.44, and the cumulative Q^2 was 0.25, with eight components. Preliminary inspection of the PLS-DA loadings plot (Figure 5.3b) suggests that amino acid and glucose levels are the key changes influenced by maternal diet and probiotic treatment.

Two-way ANOVA uncovered a significant increase in liver lactate in offspring of probiotic-treated dams relative to vehicle (main effect, $F_{(1, 107)} = 15.44$, $q = 0.0003$, Table 5.1). A significant diet x probiotic interaction was identified for liver alanine ($F_{(1, 105)} = 7.42$, $q =$

0.023). Post hoc Tukey testing revealed that HFD/probiotic offspring had significantly increased liver alanine levels relative to control offspring ($p < 0.0001$). CD/probiotic offspring also had significantly increased alanine levels compared to HFD/vehicle offspring ($p = 0.0033$) and decreased alanine levels compared to HFD/probiotic offspring ($p = 0.050$). There was also a main effect of maternal probiotic treatment on increasing alanine levels relative to vehicle offspring independent of maternal diet ($F_{(1, 105)} = 35.45$, $q < 0.0001$). A significant diet x probiotic interaction was identified for liver glucose ($F_{(1, 106)} = 9.75$, $q = 0.0079$). HFD/vehicle offspring had increase glucose levels compared to CD/vehicle offspring ($p = 0.0042$) and compared to CD/probiotic and HFD/probiotic offspring (both $p < 0.0001$). Similarly, an interaction was identified for liver leucine ($F_{(1, 107)} = 9.00$, $q = 0.0079$) due to an increase in HFD/vehicle offspring compared to CD/vehicle ($p = 0.0060$), CD/probiotic ($p = 0.0002$) and HFD/probiotic ($p < 0.0001$). A significant interaction was identified for liver taurine ($F_{(1, 107)} = 20.36$, $q < 0.0001$), whereby levels in CD/probiotic juvenile offspring were relatively lower than CD/vehicle ($p < 0.0001$), HFD/vehicle ($p = 0.0018$) and HFD/probiotic ($p < 0.0001$). Liver oxidised glutathione (GSSG) was increased by maternal probiotic treatment (main effect, $F_{(1, 107)} = 39.87$, $q < 0.0001$) and reduced by maternal obesity ($F_{(1, 107)} = 6.00$, $q = 0.043$) without a significant interaction. Lastly, there was a main effect of maternal HFD intake on liver glutamine levels in the juvenile offspring ($F_{(1, 107)} = 10.60$, $q = 0.011$) whereby levels were increased in the offspring of obese dams compared to the offspring of lean dams (Table 5.1).

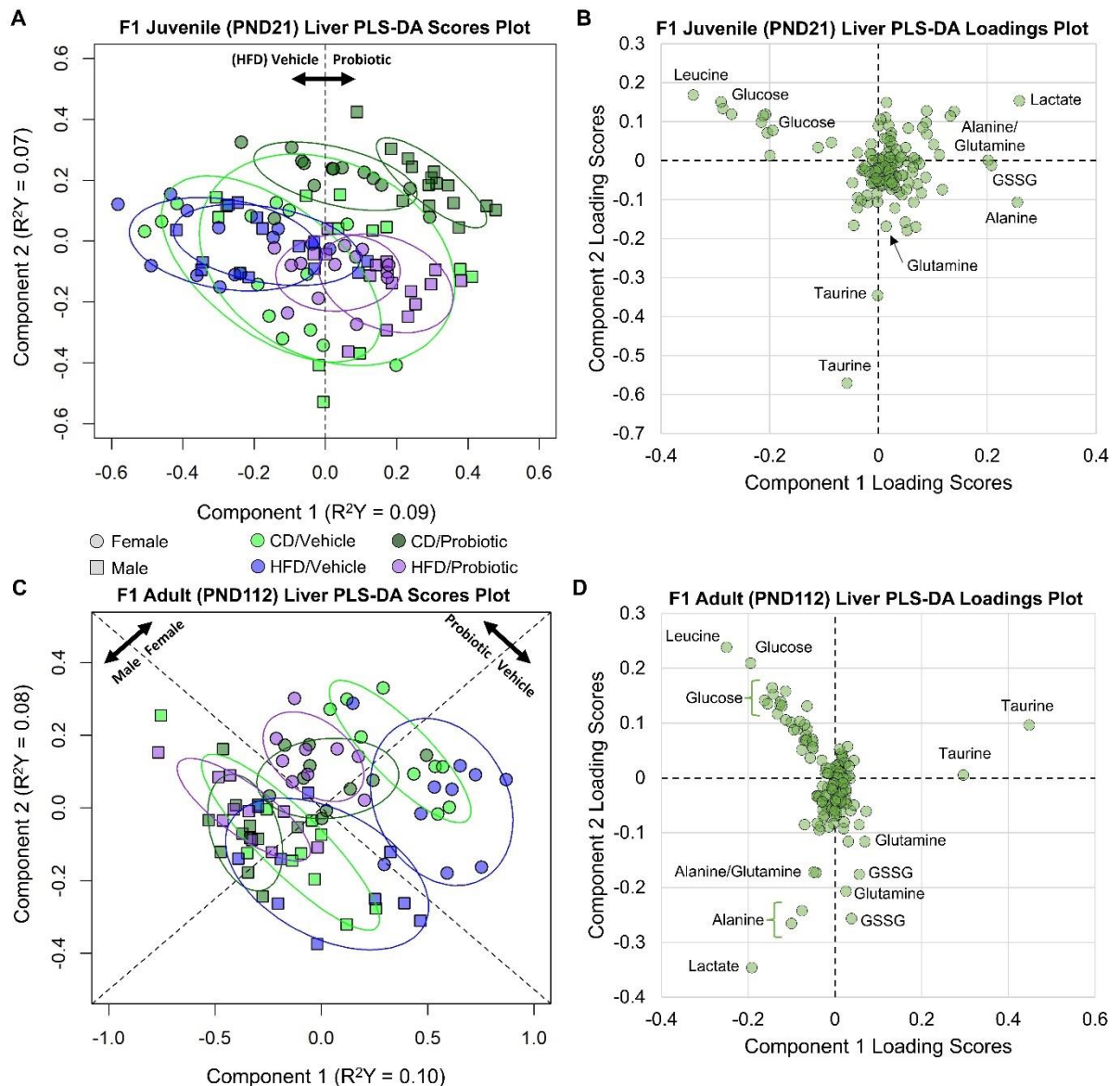


Figure 5.3 Liver PLS-DA scores plot with accompanying loadings plot for the juvenile offspring (**A, B**) and adult offspring (**C, D**). N = 13-15 per group for juveniles and N = 9-11 per group for the adults.

PLS-DA of the adult liver metabolome yielded a cumulative R^2X value of 0.65, a cumulative R^2Y value of 0.18, and a cumulative Q^2 value of 0.14, with two components (Figure 5.3c). Inspection of the PLS-DA scores plot showed some separation in the liver metabolome between adult offspring of probiotic and vehicle dams, independent of maternal diet. Orthogonal to this separation, a sex effect in the adult offspring is clear, independent of maternal factors. Interestingly, despite this separation by sex, the pattern of variation regarding

maternal probiotic treatment appears identical to the juvenile offspring (Figure 5.3c). As in the juvenile offspring, glucose and amino acids appear to contribute most to the variation in the PLS-DA model from inspection of the PLS-DA loadings plot (Figure 5.3d).

In line with the PLS-DA scores plot, three-way ANOVA of the adult liver metabolites identified sex differences in most metabolites. These were analysed separately by two-way ANOVA (Table 5.2). In the female adult offspring, liver lactate was increased by maternal HFD ($F_{(1, 34)} = 12.60$, $q = 0.0066$) relative to maternal CD. In the male adult offspring, there was a significant maternal diet x probiotic interaction in liver alanine levels ($F_{(1, 38)} = 12.69$, $q = 0.0060$). Tukey post hoc tests revealed that both HFD/vehicle ($p = 0.019$) and CD/probiotic ($p = 0.0004$) male offspring had significantly greater levels than CD/vehicle control male offspring. In the females, there was a main effect of maternal probiotic treatment ($F_{(1, 34)} = 17.74$, $q = 0.0010$), whereby levels were increased in the probiotic female offspring relative to vehicle female offspring. Glucose and leucine levels were increased in the adult male probiotic offspring relative to adult male vehicle offspring (glucose: $F_{(1, 38)} = 7.37$, $q = 0.018$; leucine: $F_{(1, 38)} = 6.71$, $q = 0.021$). In the adult female offspring, significant diet x probiotic interactions occurred for liver glucose ($F_{(1, 34)} = 8.55$, $q = 0.036$) and leucine ($F_{(1, 34)} = 7.66$, $q = 0.045$). Post hoc tests showed that, in both cases, levels were significantly lower in the female offspring of HFD/vehicle dams, than other groups (Table 5.2). In both males ($F_{(1, 38)} = 15.96$, $q = 0.0018$) and females ($F_{(1, 34)} = 41.51$, $q < 0.0001$), liver taurine was reduced by maternal probiotic treatment as a main effect relative to vehicle offspring, independent of maternal diet with no interaction. Lastly, liver glutamine was reduced in probiotic offspring relative to vehicle offspring as a main effect ($F_{(1, 76)} = 47.64$, $q < 0.0001$), independent of sex and maternal diet with no interaction.

5.2.3 Maternal probiotic intake increases levels of circulating lactate in the juvenile offspring.

In the PLS-DA scores plot of plasma by sex, maternal diet, and maternal probiotic treatment, little clustering is observed between groups (Figure 5.4a). Some separation between offspring of obese and lean dams may occur in component one. Orthogonally, in component two, there was some separation between probiotic and vehicle offspring, and HFD/probiotic cluster separately from all other offspring between components 3 and 4 (Appendix D-6). The cumulative R^2X was 0.81, the cumulative R^2Y was 0.30, and the cumulative Q^2 was 0.17, with five components. In the PLS-DA loadings plot (Figure 5.4b), glucose, lactate, as well as resonances corresponding to triglyceride and lipoprotein particles appear to be most altered by maternal diet and/or probiotic treatment.

Two-way ANOVA revealed distinct effects of maternal diet and probiotic treatment on plasma lactate levels (Table 5.1), which were increased in probiotic offspring relative to vehicle ($F_{(1, 107)} = 9.79$, $q = 0.0035$) and reduced in the offspring of obese dams relative to lean dams ($F_{(1, 107)} = 9.95$, $q = 0.0032$) with no interaction. Plasma VLDL/triglyceride levels were significantly increased in the juvenile offspring of obese dams relative to the offspring of lean dams (main effect, $F_{(1, 107)} = 33.43$, $q < 0.0001$). A significant diet x probiotic interaction was identified for plasma glucose ($F_{(1, 107)} = 11.92$, $q = 0.0040$). HFD/vehicle juvenile offspring had significantly increased glucose levels relative to CD/vehicle offspring ($p < 0.0001$) whereas CD/probiotic and HFD/probiotic offspring had significantly decreased levels ($p < 0.0001$ and $p = 0.0007$, respectively). A significant diet x probiotic interaction was identified for plasma valine ($F_{(1, 107)} = 8.22$, $q = 0.0050$). HFD/vehicle juvenile offspring had decreased levels relative to all other offspring ($p < 0.01$). Lastly, levels of plasma 3-hydroxybutyric acid were increased in the offspring of obese dams relative to lean dams (main effect, $F_{(1, 107)} = 19.22$, $q < 0.0001$).

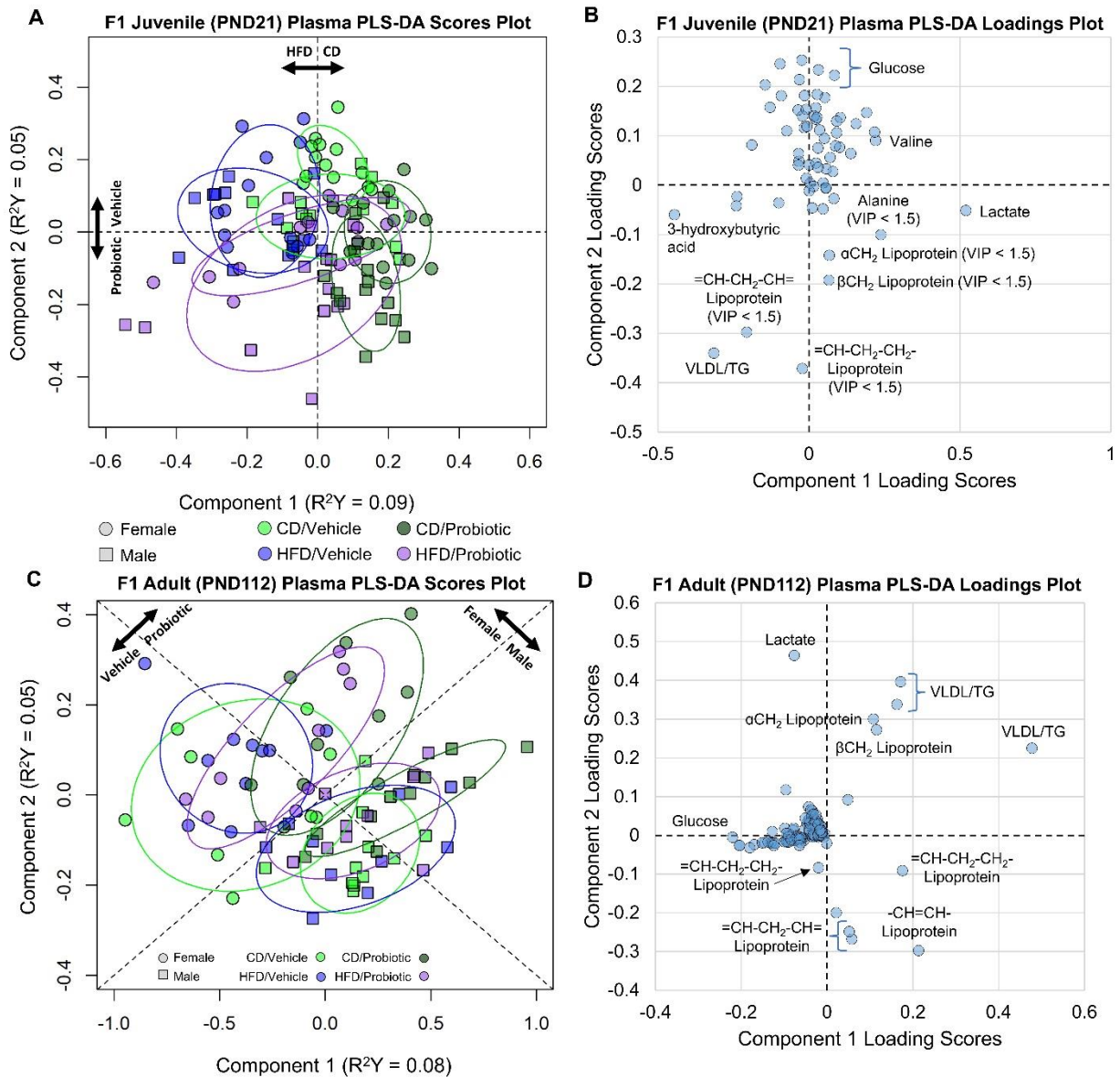


Figure 5.4 Plasma PLS-DA scores plot with accompanying loadings plot for the juvenile offspring (**A**, **B**) and adult offspring (**C**, **D**). N = 13-15 per group for juveniles and N = 9-11 per group for the adults.

As in the liver metabolome of the adult offspring, the PLS-DA scores plot of the F1 adult plasma metabolome reveals a clear effect of sex between components one and two, largely independent of maternal probiotic treatment or diet (Figure 5.4c). Orthogonal to this separation, some clustering is observed between adult offspring of probiotic and vehicle dams. The cumulative R^2X was 0.84, the cumulative R^2Y was 0.13, and the cumulative Q^2 was 0.09, with two components. The PLS-DA loadings plot (Figure 5.4d) suggests that clustering by sex

is due to different levels of plasma lactate, whereas variation by maternal probiotic treatment may be through altered levels of glucose and various lipoprotein particles.

No significant diet x probiotic interactions occurred in the adult plasma metabolome (Table 5.2). In females, plasma VLDL/triglyceride ($F_{(1, 34)} = 11.10$, $q = 0.013$) and α -CH-CH₂-CH₂ lipoprotein ($F_{(1, 34)} = 7.75$, $q = 0.027$) were elevated in the offspring of probiotic-supplemented dams relative to vehicle, while glucose levels were reduced ($F_{(1, 34)} = 12.05$, $q = 0.013$). α -CH₂ ($F_{(1, 76)} = 7.52$, $q = 0.027$) and β -CH₂ ($F_{(1, 76)} = 7.16$, $q = 0.027$) lipoprotein were increased in both male and female adult offspring of probiotic-treated dams relative to vehicle, independent of maternal diet.

In addition, 3-hydroxybutyric acid and valine were profiled in the adult offspring, as they were key metabolites altered in the juvenile offspring plasma (Appendix G). Levels of 3-hydroxybutyric acid were increased in the male adult probiotic offspring relative to vehicle ($F_{(1, 38)} = 14.36$, $q = 0.009$) whereas valine was increased in the same way only in the female offspring ($F_{(1, 34)} = 6.84$, $q = 0.034$).

5.2.4 Maternal probiotic treatment exerts a long-lasting effect on brain energy metabolism in the offspring.

In the juvenile offspring, the brain PLS-DA scores plot illustrates the variation between offspring of probiotic and vehicle dams across the second component (Figure 5.5a). Interrogation of the corresponding PLS-DA loadings plots shows that this variation can largely be attributed to changes in the relative abundance of brain lactate (Figure 5.5b, Table 5.1). Additional separation between CD/probiotic and HFD/probiotic offspring occurs in the first component. No clustering by sex is observed in any component, while separation between CD/vehicle and HFD/vehicle offspring can be observed in the third component (Appendix D-7). The cumulative R^2X was 0.67, the cumulative R^2Y was 0.32, and the cumulative Q^2 was 0.19, with five components.

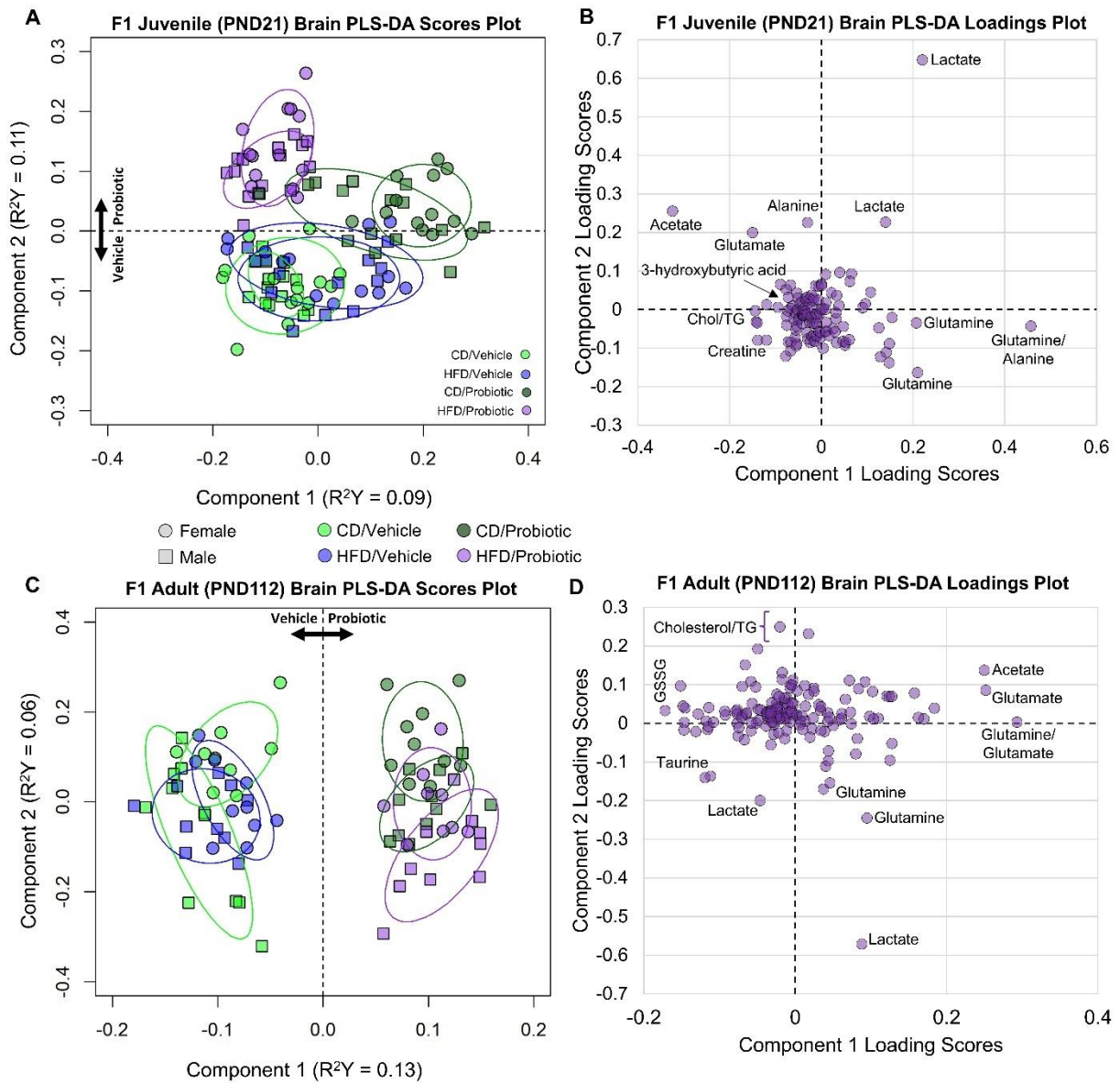


Figure 5.5 Brain PLS-DA scores plot with accompanying loadings plot for the juvenile offspring (**A, B**) and adult offspring (**C, D**). N = 13-15 per group for juveniles and N = 9-11 per group for the adults.

Two-way ANOVA revealed brain lactate to be significantly increased in the brain of juvenile offspring from probiotic-treated dams, relative to vehicle offspring ($F_{(1, 106)} = 108.2$, $q < 0.0001$, Figure 5.9a). This was independent of maternal diet and without a significant interaction. There was a significant diet x probiotic interaction for the juvenile offspring for brain creatine ($F_{(1, 106)} = 9.24$, $q = 0.012$, Table 5.1). Tukey post hoc testing revealed a significant reduction in brain creatine in HFD/vehicle offspring compared to CD/vehicle ($p < 0.0001$) and CD/probiotic offspring ($p = 0.0007$). HFD/probiotic offspring also had reduced brain creatine

compared to CD/vehicle offspring ($p < 0.0001$). Brain cholesterol/triglyceride levels were increased by maternal HFD relative to maternal CD (main effect, $F_{(1, 106)} = 30.64$, $q < 0.0001$) and decreased by maternal probiotic intake relative to vehicle (main effect, $F_{(1, 106)} = 9.18$, $q = 0.0041$). Brain alanine levels were increased by both maternal HFD (main effect, $F_{(1, 106)} = 39.65$, $q < 0.0001$) and maternal probiotic intake (main effect, $F_{(1, 106)} = 74.65$, $q < 0.0001$) relative to their respective controls, with no interaction. Brain glutamate was reduced by maternal HFD (main effect, $F_{(1, 105)} = 13.47$, $q = 0.0006$) but was increased in the juvenile offspring by maternal probiotic intake as a main effect relative to vehicle offspring ($F_{(1, 105)} = 16.45$, $q < 0.0001$) with no interaction. Brain glutamine levels were reduced by maternal HFD relative to maternal CD (main effect, $F_{(1, 106)} = 7.39$, $q = 0.010$). As in the plasma, brain 3-hydroxybutyric acid levels were increased in the juvenile offspring of obese dams relative to the offspring of lean dams ($F_{(1, 106)} = 42.08$, $q < 0.0001$), and levels in the brain were also reduced in probiotic offspring relative to vehicle offspring, without interaction ($F_{(1, 106)} = 10.61$, $q = 0.0024$). Lastly, there was a significant maternal diet $\times$ maternal probiotic treatment interaction in the juvenile offspring for brain acetate ($F_{(1, 105)} = 121.1$, $q < 0.0001$). Tukey post hoc testing revealed a significant reduction in brain acetate in HFD/vehicle and CD/probiotic offspring compared to CD/vehicle ($p = 0.0002$; $p < 0.0001$, respectively). HFD/probiotic offspring had increased brain acetate compared to all other groups ($p < 0.0001$).

Marked differences in the brain metabolome of adult offspring were observed between vehicle and probiotic groups in component 1 of the PLS-DA, independent of sex and maternal diet (Figure 5.5c). Some clustering, both by sex and by maternal diet, may be observed orthogonally in component 2. The cumulative R^2X was 0.44, the cumulative R^2Y was 0.19, and the cumulative Q^2 was 0.15, with two components. Key metabolites involved in the PLS-DA model are shown in the accompanying PLS-DA loadings plot (Figure 5.5d), including lactate, acetate, and glutamate.

Two-way ANOVA revealed a significant main effect increase in brain lactate in the female adult offspring of probiotic-fed dams relative to vehicle ($F_{(1, 34)} = 6.97$, $q = 0.030$, Figure

5.9b). Brain cholesterol/triglyceride levels ($F_{(1, 34)} = 16.66$, $q = 0.003$) and GSSG levels ($F_{(1, 34)} = 9.92$, $q = 0.017$) were reduced in the female adult offspring of obese dams relative to lean dams but there were no such changes in the adult male offspring. Similarly, brain taurine levels were increased in the female offspring of obese dams only, relative to the female offspring of lean dams ($F_{(1, 34)} = 9.56$, $q = 0.017$). In both males and females, brain acetate ($F_{(1, 76)} = 40.38$, $q < 0.0001$) and GSSG (M: $F_{(1, 76)} = 397.1$, $q < 0.0001$, F: $F_{(1, 76)} = 217.6$, $q < 0.0001$) were increased as a main effect by maternal probiotic treatment relative to the offspring of vehicle-treated dams, independent of maternal diet with no interaction.

In addition, brain glutamine ($F_{(1, 76)} = 10.49$, $q = 0.0046$) and alanine ($F_{(1, 76)} = 8.00$, $q = 0.016$) were increased as a main effect by maternal probiotic treatment relative to the offspring of vehicle-treated dams, independent of maternal diet with no interaction or sex differences (Appendix G). Brain 3-hydroxybutyric acid was decreased in the female adult offspring of obese dams relative to lean dams ($F_{(1, 34)} = 16.72$, $q = 0.0026$), and there were no enduring changes in brain creatine levels in the adult offspring.

Table 5.1 Summary of F1 juvenile metabolite changes across each body site.

F1 P21	Metabolite	AOV main effect			AOV post-hoc analysis				VIP score	NMR ppm
		mHFD v. mCD	Probiotic v. Vehicle	Int.	CD/Veh	HFD/Veh	CD/Pro	HFD/Pro		
FAECAL	Acetate	ns	ns	ns					4.09	1.92
	Glucose	↓****	ns	ns					1.91	4.65
	Succinate	↓****	ns	ns					1.89	2.41
	Ethanol	↑****	↓****	ns					1.88	3.66
	Glutamate	↓***	ns	ns					1.82	2.36
	*Butyrate	ns	↑***	ns					1.09	1.56
	*Propionate	ns	↑****	ns					1.33	1.07
LIVER	Lactate	ns	↑***	ns					3.62	1.33
	Taurine	-	-	****			↓****		3.20	3.27
	Alanine	-	-	**				↑****	2.92	1.48
	Glucose	-	-	**		↑**			2.18	3.56
	Leucine	-	-	**		↑**			1.97	3.72
	Glutamine	↑*	ns	ns					1.59	2.46
	GSSG	↓*	↑****	ns					1.53	2.18
PLASMA	Lactate	↓**	↑**	ns					2.33	1.33
	Glucose	-	-	**		↑****	↓****	↓****	1.88	3.68
	VLDL/TG	↑****	ns	ns					1.80	0.86
	3-hydroxybutyric acid	↑****	ns	ns					1.79	1.20
	Valine	-	-	**		↓**			1.76	0.99
BRAIN	Lactate	ns	↑****	ns					4.23	1.33
	Acetate	-	-	****		↓***	↓****	↑****	2.79	1.92
	Creatine	-	-	*		↓****		↓****	2.10	3.04
	Alanine	↑****	↑****	ns					1.74	1.48
	Glutamine	↓**	ns	ns					1.64	2.49
	Glutamate	↓***	↑****	ns					1.59	2.36
	Cholesterol/TG	↑****	↓**	ns					1.55	1.41
	3-hydroxybutyric acid	↑****	↓**	ns					1.50	1.20

*q < 0.05, **q < 0.01, ***q < 0.001, ****q < 0.0001 adjusted for false discovery using the Benjamini and Hochberg method. Int = interaction; VIP = variable importance in projection; AOV = analysis of variance, ns = not significant.

Table 5.2 Summary of F1 adult metabolite changes across each body site.

F1 PND112	Metabolite	AOV main effect			AOV post-hoc analysis				VIP score	NMR ppm
		mHFD v. mCD	Probiotic v. Vehicle	Int.	CD/ Veh	HFD/ Veh	CD/ Pro	HFD/ Pro		
FAECAL	Leucine/Isoleucine	ns	↑****	ns					4.22	0.95
	Lactate	-	↓****	*			↓*		3.25	1.33
	Alanine	-	-	*			↓*		3.18	1.48
	Taurine	ns	↓****	ns					3.00	3.27
	Creatine	ns	↓****	ns					2.72	3.04
	Valine	↑*	-	*		↓*	↓*		2.57	0.99
	Acetate	ns	ns	ns					2.41	1.92
	Butyrate	ns	↑*	ns					2.32	1.56
	Glutamate	ns	↑**	ns					2.30	2.36
	Tyrosine	-	-	*				↓*	2.19	6.88
	Propionate	ns	↑*	ns					2.04	2.16
	Glycine	-	-	*			↓**		1.81	3.56
	Tryptophan	↓****	ns	ns					1.64	7.25
	Glutamine	↓****	ns	ns					1.55	2.47
LIVER	Taurine	ns	↓*** ↓**	ns					4.51	3.27
	Lactate	↑*	ns	ns					3.81	1.33
	Alanine	-	↑***	*		↑*	↑***		2.44	1.48
	Leucine	-	↑*	*		↓*			2.41	3.72
	Glucose	-	↑*	*		↓*			1.94	4.65
	GSSG	ns	ns	ns					1.70	2.17
	Glutamine	ns	↓****	ns					1.57	3.79
PLASMA	VLDL/TG	ns	↑*	ns					4.01	1.27
	Lactate	ns	ns	ns					3.39	1.33
	-CH=CH- LPO	ns	ns	ns					2.92	5.30
	αCH ₂ LPO	ns	↑*	ns					2.22	2.24
	βCH ₂ LPO	ns	↑*	ns					1.99	1.59
	=CH-CH ₂ -CH= LPO	ns	ns	ns					1.91	2.76
	Glucose	ns	↓*	ns					1.79	3.85
	=CH-CH ₂ -CH ₂ LPO	ns	↑*	ns					1.71	2.00
BRAIN	Lactate	ns	↑*	ns					3.68	1.33
	Acetate	ns	↑****	ns					2.47	1.92
	GSSG	↓*	↑**** ↑****	ns					2.04	2.97
	Cholesterol/TG	↓**	ns	ns					1.87	0.95
	Taurine	↑*	ns	ns					1.80	3.27
	Glutamate	ns	ns	ns					1.58	3.75

*q < 0.05, **q < 0.01, ***q < 0.001, ****q < 0.0001 adjusted for false discovery using the Benjamini and Hochberg method. Int = interaction; VIP = variable importance in projection; AOV = analysis of variance, ns = not significant, LPO = lipoprotein. Blue and orange arrows represent significant changes in females and males only, respectively.

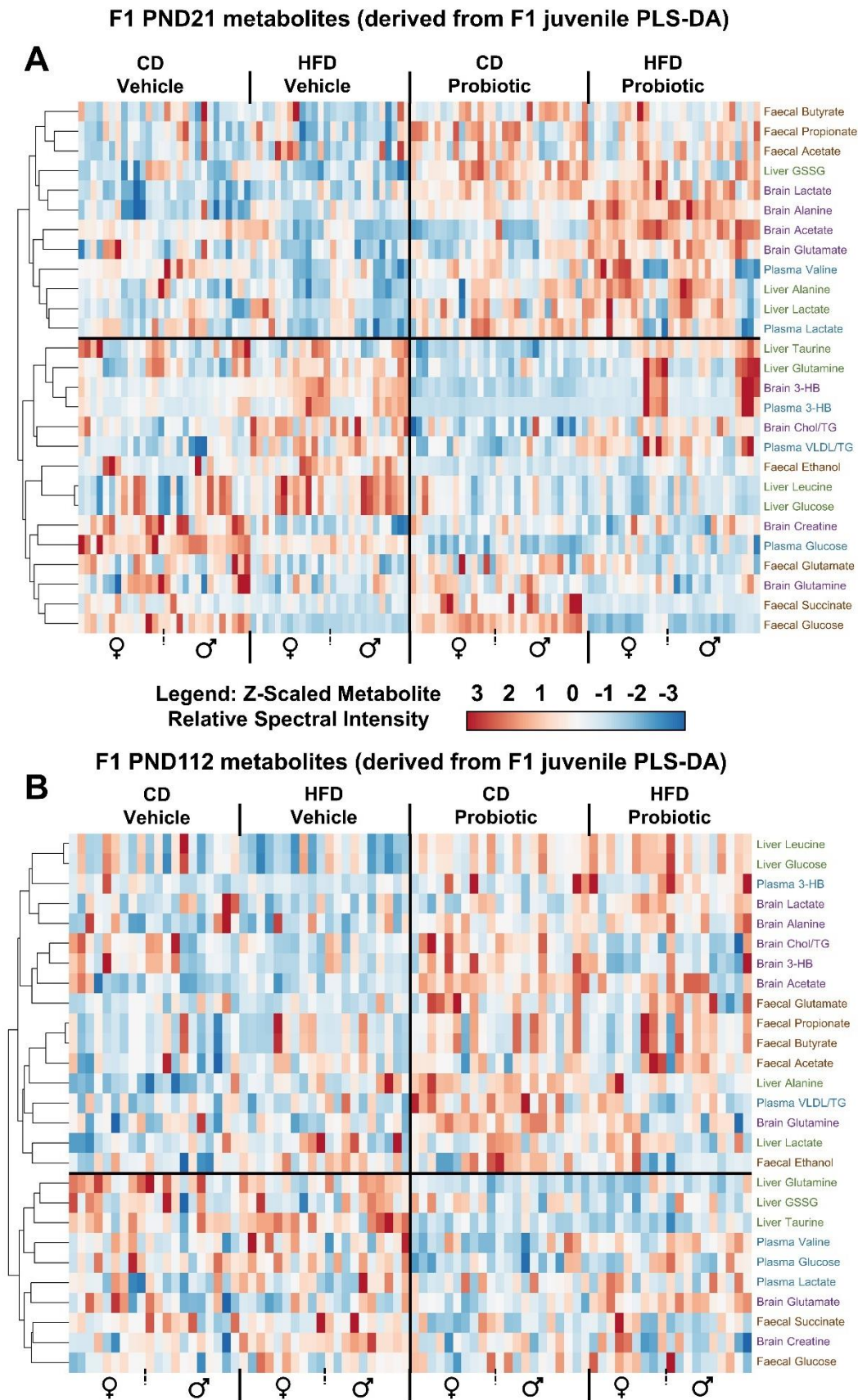


Figure 5.6 Summary of **A)** key offspring metabolites in the juvenile offspring, and **B)** the same metabolites shown in the adult offspring. N = 13-15 per sex per group in the juvenile offspring, and N = 9-11 per sex per group in the adult offspring.

5.2.5 Brain lactate and faecal SCFAs correlate with prefrontal cortex gene expression

We further investigated how key brain and faecal metabolite levels correlated with PFC gene expression in the juvenile (Figure 5.7) and adult (Figure 5.8) offspring. Correlations were performed between VIP brain metabolites based on the juvenile offspring, and genes whose mRNA expression was found to be significantly altered in the PFC of the juvenile offspring. In all cases, the animals used in each correlation correspond to the randomly selected samples used for gene expression analysis, which comprises six animals per sex, per condition (n = 48 per correlation). Tabulated correlation statistics can be found in Appendix H.

In the juvenile offspring, there was a significant negative correlation between brain lactate levels and expression of *GLUN2A*, *GLUN2B*, and *SYP*. A positive correlation existed between lactate and *PFKFB3* (Figure 5.9e) and lactate and *CREB1*. Increased alanine was associated with increased expression of *IL-6*, and glutamate was positively correlated with *ZIF-268* expression (Figure 5.7).

An increase in the faecal SCFAs acetate, propionate, and butyrate (Figure 5.9g) all significantly correlated with increased expression of *PFKFB3*, while acetate and propionate correlated positively with *CREB1* expression in the juvenile offspring. Propionate negatively correlated with *GLUN2A* and *GLUN2B* expression, while butyrate correlated negatively with *GLUN2C* and *SYP* expression (Figure 5.7).

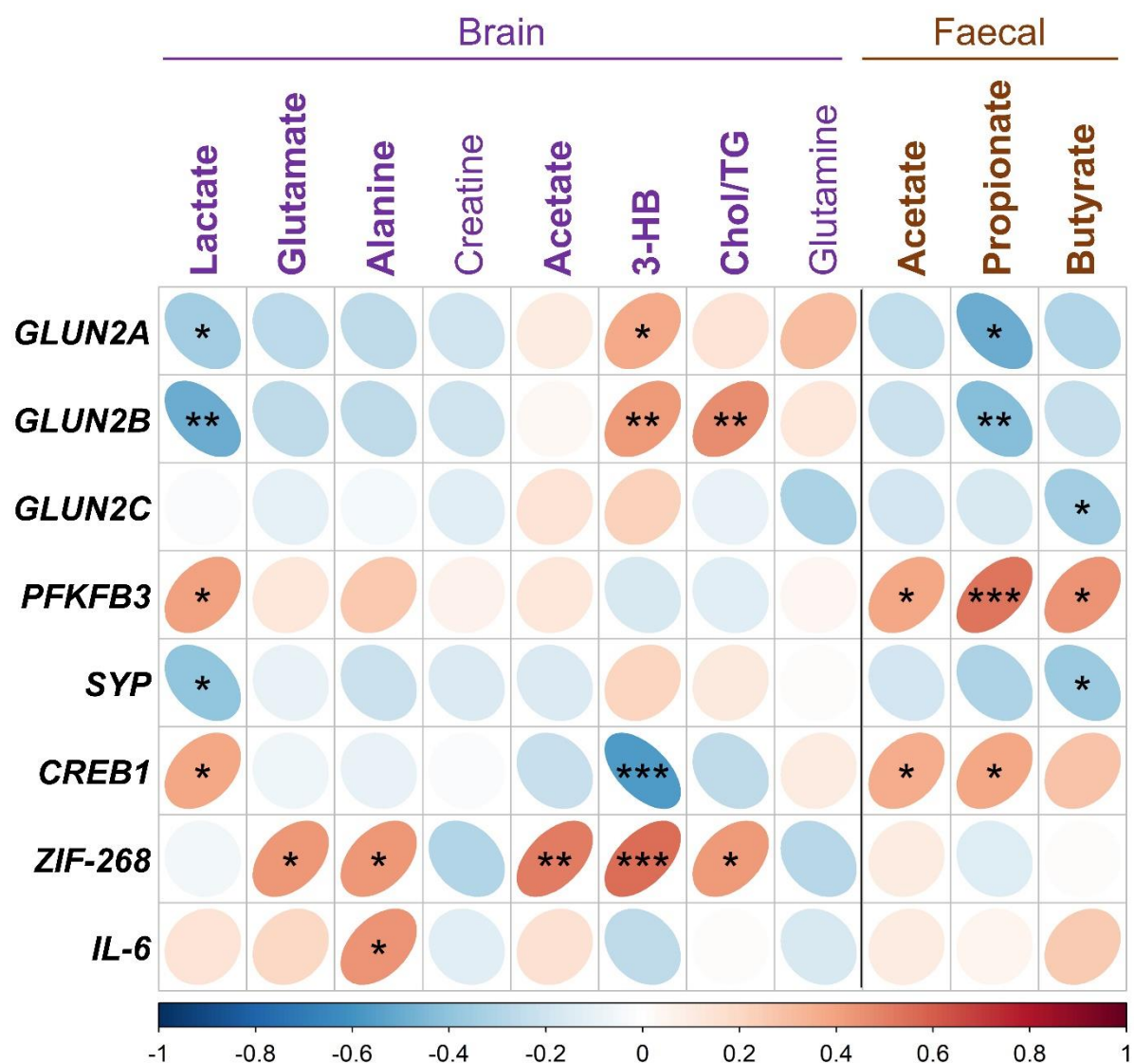


Figure 5.7 Juvenile offspring: Correlation between key brain or faecal metabolites and the mRNA expression of genes that were significantly altered by maternal diet and/or probiotic treatment in the prefrontal cortex.

For the same metabolite correlation analyses where PFC gene expression was also altered between groups in adult offspring, only faecal butyrate remained significantly correlated with PFC *PFKFB3* ($r = 0.40$, $q = 0.027$; Figures 5.8 and 5.9h). Brain lactate was not significant after correcting for the FDR ($r = 0.36$, $q = 0.057$, Figures 5.8 and 5.9f).

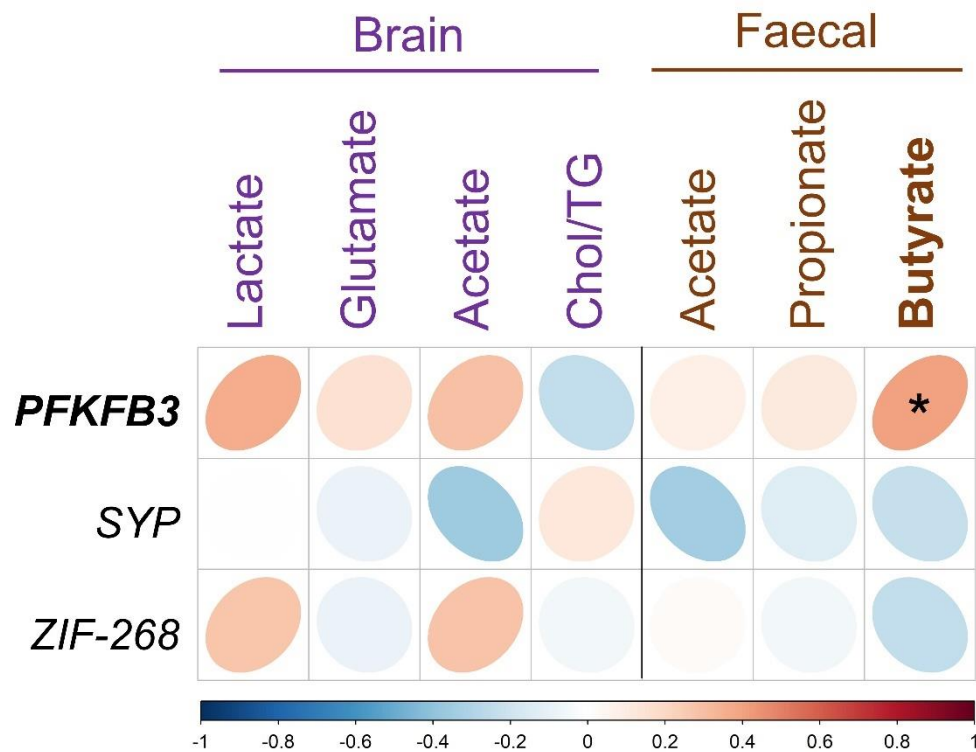


Figure 5.8 Adult offspring: Correlation between faecal or brain metabolites and the prefrontal cortex mRNA expression of genes that were identified as key metabolites in both the juvenile and adult offspring.

5.2.6 Elevated brain lactate accompanies the effect of early-life probiotic exposure on anxiety-like behaviour

Finally, I investigated how brain and faecal metabolites, in particular those that were discriminatory between offspring of lean, obese, and probiotic-supplemented dams, correlated with patterns of anxiety-like behaviour. In both juvenile (AUC 0.82) and adult (AUC 0.74) offspring, lactate was the most discriminatory brain metabolite between high (1st quartile) and low (4th quartile) anxiety-like behaviour (time in centre zone in the OFT), independent of experimental groupings (Figures 5.9i,j, respectively). Brain lactate levels correlated with reduced time in centre in the open field across all the juvenile ($r = 0.41$, $p < 0.0001$) and adult ($r = 0.33$, $p = 0.0034$) offspring (Figures k,l, respectively).

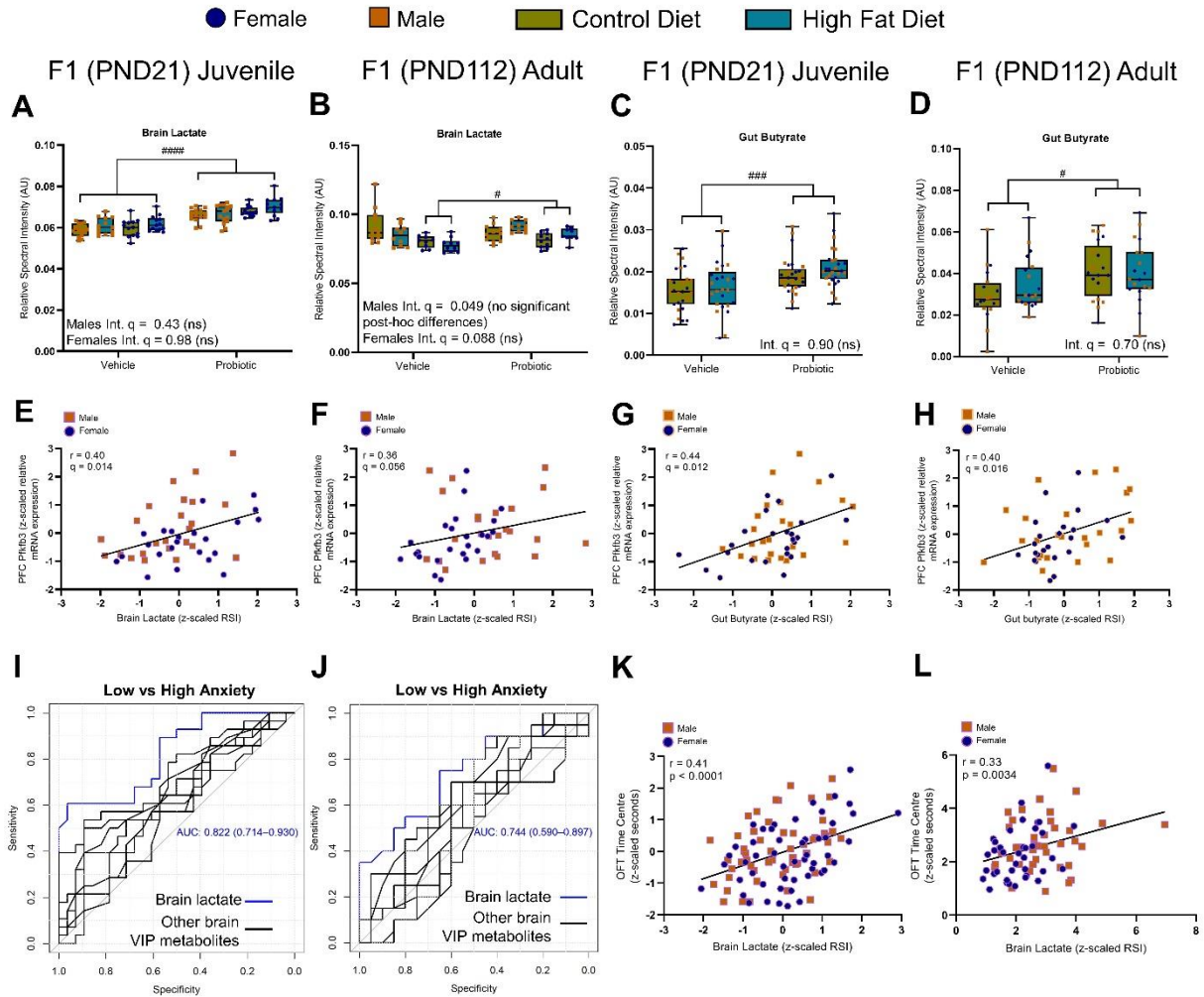


Figure 5.9 Key gut and brain metabolites altered by maternal probiotic treatment show correlations with a genetic marker of astrocytic metabolism and anxiety-like behaviour. Brain lactate levels in **A**) the juvenile offspring and **B**) the adult offspring. Gut butyrate levels in **C**) the juvenile offspring and **D**) the adult offspring. Correlation (Spearman's rank correlation coefficient) between brain lactate levels and PFC *PFKFB3* expression for **E**) the juvenile and **F**) the adult offspring. Correlations between gut butyrate and PFC *PFKFB3* expression for **G**) the juvenile and **H**) the adult offspring. Receiver operating characteristic curves (**I**, juvenile; **J**, adult) for brain lactate concentration compared to other discriminatory brain metabolites as classifiers for high and low anxiety-like behaviour in the OFT in juvenile ($n = 28$ low anxiety [10M, 18F], 28 high anxiety [15M, 13F]) and adult ($n = 20$ low anxiety [12M, 8F], 20 high anxiety [5M, 15F]) offspring, respectively. Correlation between brain lactate levels and OFT time in centre for **K**) the juvenile offspring and **L**) the adult offspring. (A–D) $n = 13$ to 15 juvenile offspring, $n = 9$ to 11 adult offspring per sex per group. Data are presented as boxplots showing median, interquartile range, and min/max points. AU, arbitrary units; Int., interaction; ns, not significant. # $P < 0.05$; ### $P < 0.001$; #### $P < 0.0001$ (indicating main effect of maternal probiotic treatment). Where sex differences occurred, separate two-way ANOVAs were performed.

5.3 Discussion

In the central dogma of biology, metabolomics is the biochemical component that contains information about the physiology of the organism that is most proximal to the phenotype. Variation in metabolite levels between experimental groups are the net product of changes to RNA and protein as well as the cellular regulatory mechanisms that govern their expression. Metabolic profiling, therefore, is a snapshot of the sum physiology of a given sample, and in more complex samples, such as gut tissue or faecal matter, also encompasses the interaction between host and microbial cells.

The aim of this chapter was to investigate, using untargeted ^1H NMR spectroscopy, how maternal HFD and probiotic intake differentially alter offspring metabolic states across the brain-gut axis. Given the clear effects of maternal probiotic intake on offspring brain gene expression, I expected to observe differentially abundant brain metabolites in the offspring of probiotic-fed dams compared to the offspring of dams fed the vehicle. In particular, the longstanding effect of increased brain *PFKFB3* expression in the probiotic offspring described in the previous chapter suggests that small yet detectable changes in brain energy metabolism in these offspring could be expected. Maternal obesity has been shown to affect fatty acid and energy metabolism in the PFC of male adult offspring (Zhu et al., 2018), but not in other brain regions such as the hypothalamus or olfactory bulb (Safi-Stibler et al., 2020). Moreover, the changes observed by Zhu et al. (2018) were confounded by genotype differences in the adult offspring. I expected to observe effects of maternal obesity on perturbed lipid and energy metabolism in the cerebrum of the juvenile offspring but expected these changes to be smaller in magnitude in the adult offspring who had been weaned to a regular diet for more than three months prior to profiling. Surprisingly, maternal probiotic intake appeared to exert the greatest influence on neurometabolism, particularly in the adult offspring, where the effect of maternal HFD was less clear in the PLS-DA scores plot.

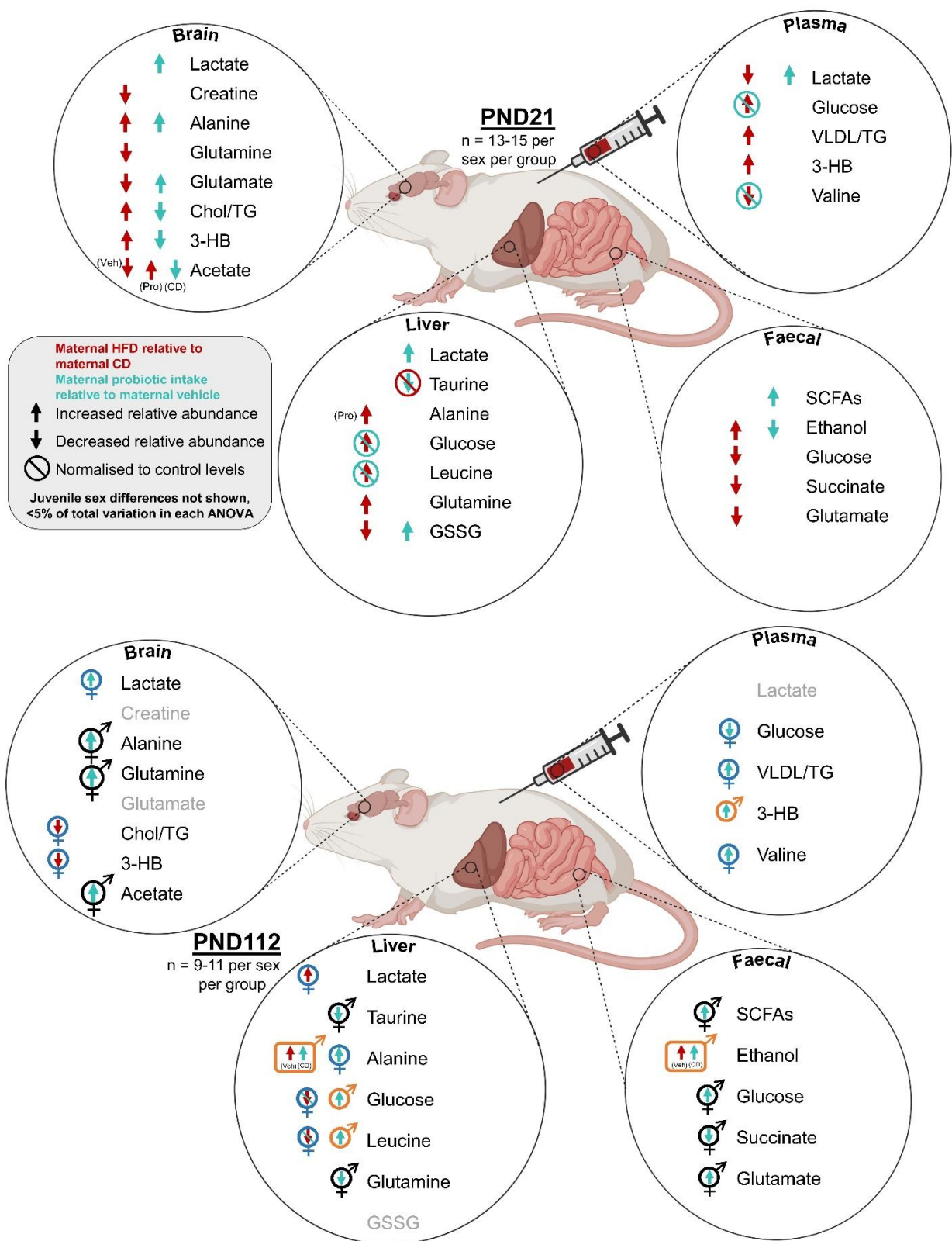


Figure 5.10 Comprehensive atlas of offspring gut-liver-brain after perinatal exposure to maternal obesity and maternal probiotic intake. Summary of top metabolites from partial least squares discriminant analyses (PLS-DA). Metabolite data is described in Tables 5.1 and 5.2, and Appendix G.

NMR-based metabolomic profiling revealed a significant increase in L-lactate in the brain, liver, and plasma of juvenile offspring after maternal probiotic intake compared to vehicle controls, with no overt sex differences (Table 5.1, Figure 5.10). While a direct link between maternal microbiota and increased brain lactate has not been reported, the importance of maternal microbiota and microbial metabolites in neurodevelopment during the perinatal period is recognized (Vuong et al., 2020). L-lactate has antidepressant-like properties when administered to adult mice (Carrard et al., 2018; Karnib et al., 2019), and the selective serotonin reuptake inhibitor fluoxetine has been shown to increase lactate release from astrocytes in vitro (Allaman et al., 2011). Here, the increase in cortical lactate in the juvenile offspring may be sourced from astrocytes via increased PFC *PFKFB3* expression stimulating the rate-limiting step of glycolysis, and the ensuing increase in pyruvate being preferentially metabolised into lactate. Alternatively, the increase in brain lactate could be from a microbial source. Increased milk lactate and *Lactobacillus spp.* were also associated with probiotic treatment in dams. Lactate, produced in the gut by both *Lactobacillus* and *Bifidobacterium spp.*, may enter systemic circulation via local sodium monocarboxylate transporters (Brooks, 2018). This may be one reason why lactate, unlike SCFAs, was not found to be increased in faeces as a result of probiotic supplementation but was instead found to be increased in the plasma and brain.

Top-ranked metabolites from each PLS-DA (Tables 5.1 and 5.2) were correlated with genes whose mRNA expression was found to be significantly altered in the PFC of the offspring. Here, the abundance of brain lactate correlated negatively with *GLUN2A/2B* expression and positively with *PFKFB3* and *CREB1* expression in the young offspring, and correlated with reduced anxiety-like behaviour in both the young and adult offspring. Lactate has been shown to directly modulate histone deacetylase (HDAC) activity in the mouse hippocampus, which was associated with reduced *GLUN2A* mRNA expression and resilience to chronic social defeat stress (Karnib et al., 2019). *PFKFB3* is a master regulator of lactate production through the glycolytic pathway in astrocytes (Bolaños, 2016) and may be regulated

in part by the microbiome (Margineanu et al., 2020). *ZIF-268* was positively correlated with the expression of a number of brain metabolites in the juvenile offspring including glutamate, alanine, acetate, 3-hydroxybutyric acid and cholesterol/TG levels. Given that *ZIF-268* is known to code for a transcription factor involved in neuronal plasticity, its expression likely lies upstream of many metabolic pathways (Knapska and Kaczmarek, 2004). Thus, this result is not surprising.

3-hydroxybutyric acid is thought to mediate neural stress responses, for example due to fasting, ketogenic diets, or exercise (Marosi et al., 2016). The increase in brain 3-hydroxybutyric acid in the juvenile offspring may be a direct result of increased fatty acid oxidation from the increased abundance of lipids present in the milk of obese dams (Schulz, 2013). This is supported by the fact that 3-hydroxybutyric acid was also significantly elevated in the plasma of the juvenile offspring of obese dams relative to the offspring of CD dams. A concomitant decrease in faecal glucose and succinate metabolites in HFD offspring compared to CD offspring suggests that the juvenile offspring exposure to maternal obesity induced a shift in gut microbiota ecology (De Vadder et al., 2016). Alongside *ZIF-268*, brain 3-hydroxybutyric acid correlated positively with PFC *GLUN2A/B* and negatively with *CREB1*. Further experiments are required to understand whether changes in the relative mRNA expression of these genes are adaptive responses to elevated brain 3-hydroxybutyric acid.

While not detected in brain tissue using NMR, the SCFAs butyrate and propionate are brain-permeable and, like lactate, capable of modulating gene transcription through HDAC modification in vivo (Arpaia et al., 2013; Karnib et al., 2019). Most notably, faecal butyrate correlated positively with PFC *PFKFB3* expression in both the juvenile and adult offspring. This observation provides evidence supporting the role of microbes and microbial metabolites, in particular, brain lactate and gut butyrate, in regulating mood behaviour and the expression of plasticity-related genes.

In juveniles, maternal obesity reduced brain and liver creatine levels, which were normalized after perinatal probiotic intake in the liver, but not in the brain. A systematic review

(MacDonald et al., 2019) collated data from studies employing in vivo brain ^1H magnetic resonance spectroscopy in MDD patients and found that creatine and glutamate were significantly downregulated in MDD. This is consistent with the brain metabolite changes I found in the juvenile offspring of obese dams. Low dietary creatine intake is a clinical risk factor for depression (Bakian et al., 2020), and creatine supplementation has shown promise as an adjunct supplement for MDD (Kondo et al., 2011). A promising theory for the pathophysiology of synaptic dysfunction in psychiatric disorders is that plastic changes are constrained by brain energy deficits (Harper et al., 2017). This has been supported by preclinical (Della et al., 2012) and clinical evidence (Song et al., 2021) implicating reduced creatine in depression and reduced creatine kinase flux in bipolar disorder, respectively. The results from our metabolomic study support accumulating evidence connecting maternal obesity during pregnancy to long-term changes in central metabolism and behaviour (Sullivan et al., 2014).

Faecal SCFA levels, specifically butyrate and propionate, were elevated in the juvenile offspring and, surprisingly, remained elevated in the adult offspring despite weaning to a regular diet without probiotic supplementation for >3 months. This suggests that early life exposure to the probiotic, possibly by maternal coprophagia or simply cohabitation, allows for long-term colonisation of the probiotic species that promote increased butyrate and propionate production. Few studies in rodents have assessed the longevity of maternal probiotic supplementation on the offspring faecal metabolome. Hsu et al. (2019) explored the effects of maternal CD, HFD, and HFD/probiotic (but not CD/probiotic) during gestation and nursing in male rat offspring at 16 weeks age (8 animals per group). In support of the results of the present study, a non-significant trend towards increased faecal butyrate levels were observed in the offspring of HFD/probiotic dams compared to CD dams (22% increase) and HFD dams (35% increase). In humans, conflicting evidence exists as to whether probiotic supplementation exerts a lasting effect on gut microbiota composition. Supplementation of adults with *Bifidobacterium infantis* 35624 for eight weeks increased *B. infantis* 35624 in

faeces compared to placebo, however levels of the probiotic declined to baseline levels at the follow-up timepoint, two weeks after ceasing the daily probiotic treatment (Charbonneau et al., 2013). In another study, probiotic *Bifidobacterium spp.* have been shown to colonise the adult human gut only transiently, for eight days (Bouhnik et al., 1992). Conversely, Maldonado-Gómez et al. (2016) found that supplementation with *Bifidobacterium longum AH1206*, in healthy individuals, remained detectable in stool for up to six months after discontinuation of the probiotic. Human studies investigating the effect of maternal perinatal probiotic supplementation on the offspring faecal microbiota have also exhibited conflicting results. One study investigated the effect of a *Lactobacillus*-containing probiotic supplement, between the last month of gestation and the first 3 months of lactation, on the longitudinal microbiota of the infants. 16S rRNA sequencing revealed that the infants had increased levels of faecal *Lactobacillus rhamnosus GG* (contained in the probiotic) relative to control infants at 3 months age, but not at 12 months (Dotterud et al., 2015). However, Schultz et al. (2004) found that in mothers supplemented with *Lactobacillus rhamnosus GG* during late pregnancy (but not lactation), the children had detectable faecal levels of *L. rhamnosus GG* until at least 12 months age. The infants of mothers who did not receive the probiotic had no detectable levels of *L. rhamnosus GG* at this timepoint.

There are two, somewhat opposing mechanisms to explain why probiotics persistently colonise the gut in some individuals but not others. On the one hand, colonisation success may depend on the pre-existing level of conspecific strains in the gut. Li et al. (2016) found that only probiotic strains of species already existing in the gut were able to be detected three months after probiotic treatment, the reason being that the species will already have the appropriate microenvironment with which to colonise successfully. On the other hand, Maldonado-Gómez et al. (2016) found that low levels of *B. longum* predicted *B. longum AH1206* engraftment success. Further metagenomic analysis suggested that colonisation success was related to the under-exploitation of certain carbohydrates produced in the gut, providing a window of opportunity for the probiotic strain to establish itself.

Perhaps more pertinent to this study is the fact that, while an indigenous “mature” microbiome is generally quite resistant to colonisation by probiotics, a microbe-deficient GF gut is readily colonised by probiotic species (Zmora et al., 2018). Accordingly, the neonatal gut may be much more readily colonisable with probiotic species, especially if these are obtained from the maternal milk or faecal matter. In humans, bacterial strains that colonise the infant gut in the first six months of life have been shown to persist at least for the next six years, even if they are not the dominant strain of a given species (Oki et al., 2018). Similarly, multi-strain probiotic administration to pre-term infants resulted in the increased abundance of probiotic *Bifidobacterium spp.* strains up to five months later (Yousuf et al., 2020). This evidence for the notion that early engraftment may be an important factor in the longevity of colonisation and may contribute to why both the juvenile and adult offspring of probiotic-treated dams showed increased SCFA abundance relative to controls.

One of the limitations of this study was the lack of accompanying microbiome sequencing in the offspring. While the limitations of microbiome profiling have been discussed in section 1.2.2.1 of this thesis, it would have been useful to confirm whether the probiotic strains administered perinatally to the F0 dams did in fact colonise the offspring gut at both juvenile and adult timepoints. Nevertheless, the persistent increase in SCFAs relative to the offspring of vehicle-treated dams is reassuring. The Bio-Kult probiotic mixture is known to produce lactate and acetate as the primary end-products of their metabolism (Tejero-Sariñena et al., 2012). Lactate is readily metabolised into either butyrate, propionate, or succinate by lactate-utilising bacteria in the gut (Brooks, 2018). A second limitation is that NMR spectroscopy does not provide full coverage the microbial metabolites produced in the gut. Indeed, many microbial metabolites are produced in too low a concentration ($< 1 \mu\text{M}$) to be detected by NMR. Combining both MS and NMR would provide the greatest metabolomic coverage and should be a priority in future work investigating the relationships between host and microbiota (Bhinderwala et al., 2018).

Fewer significant metabolites were identified in the adult offspring of obese dams compared to the adult offspring of lean dams. This may be due to the fact that all adult offspring had been fed a regular chow diet, and consequently the *metabolic* effects of maternal obesity were less apparent. Statistically, the reduced sample size per group in the adult offspring also reduced the power of the univariate metabolomic analysis, which also likely accounts for fewer significant metabolites identified. This is compounded by the fact that, in many cases, the concentration of metabolites in the liver, plasma, brain, and faeces differed systematically between male and female adult offspring. In such cases, male and females were analysed separately, further reducing the power of discovery relative to the juvenile offspring where the male and female offspring were analysed together. Lastly, I found that in each of the brain, liver, plasma, and faecal metabolic compartments, the effect of maternal HFD or maternal probiotic treatment differed in the male and female adult offspring relative to their respective control groups. For example, in the brain and plasma, the female adult offspring appeared to be more affected by maternal diet and maternal probiotic treatment, respectively, compared to the male adult offspring. These findings are important, as current preclinical research is skewed towards analysis of male offspring only (Hasebe et al., 2021), and the findings reported here, and by others (Ye et al., 2021), may be suggestive of an increased risk of metabolic perturbations due to maternal obesity in female offspring.

In summary, I discovered a reduced level of creatine and glutamate as being associated with behavioural dysfunction in the juvenile offspring of obese dams relative to the offspring of lean dams, pointing to disrupted brain energy metabolism in these offspring. Increased cortical lactate was identified as a potential source for the remedial effects of early life probiotic exposure, alongside a persistent increase in faecal SCFA levels.

6

Investigating the serum metabolomic profile of recent depressive symptoms in individuals with pre-existing metabolic disease⁵

6.1 Introduction

In Chapter 3, depressive-like behaviour in the FST was observed in a model of diet-induced obesity in postpartum dams. The depressive-like behaviour coincided with metabolic dysfunction, such as increased circulating lipid levels, and immune dysfunction, including increased hepatic expression of *TLR4*, *SAA-2*, and pro-inflammatory cytokines. Moreover, in Chapters 4 and 5, this depressive-like behaviour and metabolic dysfunction was found to propagate to the offspring. Obesity is also thought to intersect with the pathophysiology of depression in humans. Both longitudinal and cross-sectional studies have found that depression is associated with an increased risk of obesity (Milaneschi et al., 2019) and metabolic syndrome (Pan et al., 2012). This association is suggestive of an interactive relationship with overlapping pathophysiological mechanisms related to metabolic dysfunction and chronic, low-grade “metabolic” inflammation (Milaneschi et al., 2019). Here, in Chapter 6, the co-occurrence of major depressive disorder (MDD) with diet-induced metabolic dysfunction was investigated further in a human population with non-alcoholic fatty liver disease (NAFLD).

With an estimated worldwide prevalence of one in four individuals, non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disorder (Younossi et al., 2016) and has a close, bidirectional association with other obesity-related metabolic disorders (European Association for the Study of the Liver (EASL) et al., 2016). Depression, like NAFLD, is a

⁵ Results included in this Chapter have been published: [10.1371/journal.pone.0261555](https://doi.org/10.1371/journal.pone.0261555)

common and complex disorder, a global health and economic burden, and can substantially reduce the quality of life of those afflicted (Malhi and Mann, 2018). Emerging evidence points to a bidirectional association between NAFLD and MDD; NAFLD has been found to increase the risk of anxiety and depression independently of other comorbid diseases (Labenz et al., 2020), and depressed individuals are more likely to have NAFLD than non-depressed individuals independent of diabetes and obesity (Kim et al., 2019). While a blood metabolomic profile of depression in NAFLD has not been shown previously, NMR-based metabolomic studies of depression have found associations between both clinical and subclinical depressive symptoms and an altered circulating lipid profile (Bot et al., 2020). Consequently, I was interested in determining whether a fasting serum signature of recent, self-reported depressive symptoms could be identified in a heterogeneous NAFLD cohort with pre-existing metabolic disease using nuclear magnetic resonance (NMR)-based metabolomics. I expected depression to be associated with increased VLDL and triglyceride resonances as previously reported in individuals with depressive symptoms (Bot et al., 2020).

NMR metabolomic profiling was performed on 218 patients with NAFLD, who were stratified by depressive symptoms (recent, lifetime, or none), with a view to gaining further insight into the links between depression and chronic metabolic dysfunction. Here, serum NMR metabolite analysis provides a link between disturbed lipid metabolism, inflammation, and active mental health issues in NAFLD, irrespective of disease severity.

6.2 Results

6.2.1 Clinical indices and demographics overview

The clinical cohort comprised patients recruited from T2DM clinics at the Princess Alexandra Hospital in Brisbane, Australia, as well as at-risk individuals referred from primary care centres due to a history of T2DM, fatty liver, or metabolic syndrome (Patel et al., 2018). The recruitment period was between 2015 and 2017.

A diagnosis of NAFLD was determined by both including and excluding factors, as previously described (Patel et al., 2018). Liver ultrasound was performed to include those with fatty liver (hepatic steatosis), alongside the presence of established metabolic risk factors. A diagnosis of NAFLD was ruled out if patients had pre-existing alcohol-related liver disease, were currently consuming significant levels of alcohol (average ≥ 20 grams per day) or had other chronic liver diseases. Other exclusion criteria from the study included advanced chronic kidney disease (glomerular filtration rate < 15 mL/min), history of organ transplantation, and other suspected sources of fatty liver. For the present study, the only additional inclusion criterion was whether a serum aliquot with a single freeze-thaw was available for metabolomics analysis. Of the 252 patients in the clinical cohort, 218 (86.5%) had an available aliquot and were included in this study.

Overall, 45.0% of subjects were female, the mean age was 57.9 ± 12.4 years, and 78.0% were Caucasian. The mean BMI was 34.3 ± 7.5 kg/m². As expected, there was a high prevalence of T2DM (78.9%), dyslipidaemia (95.4%) and metabolic syndrome (82.6%). A reliable liver stiffness measurement (LSM), measured by transient elastography, was recorded for 197 patients (90.4%). The median LSM was 5.7 kPa, with a range from 2.8 to 47.2 kPa. A LSM ≥ 8.2 kPa, indicative of clinically significant fibrosis, was identified in almost a quarter of individuals (23.9%). 31 patients (15.7%) had an LSM ≥ 9.5 kPa, suggestive of advanced fibrosis, whilst 18 (9.1%) had an LSM ≥ 13 , indicative of cirrhosis.

Patient demographic and clinical indices, stratified by depressive symptoms, are described in Table 6.1. Of the 218 patients with NAFLD, 81 patients had recent self-reported depressive symptoms within the last 12-months at the time of blood sampling (RD = recent depressive symptoms). 30 patients had a history of depression (without recent depressive symptoms in the last 12-months; LD = lifetime depression), and 107 patients reported no history of depression (ND = no history of depression). Patients with NAFLD and recent depressive symptoms were younger ($p = 0.0007$ compared to lifetime depression; $p = 0.012$ compared to no depression), with a significantly higher fasting serum triglyceride level ($p = 0.022$ compared to lifetime depression; $p = 0.0015$ compared to no depression) and trend toward lower fasting serum high-density lipoprotein (HDL; $p = 0.099$ compared to no depression). As expected, there was a difference in antidepressant use between those with and without a history of depression ($p < 0.0001$). However, there were no significant differences in current use of medications for dyslipidaemia, presence of metabolic comorbidities, or liver disease severity (Table 6.1).

6.2.2 Clinical characteristics and severity of fibrosis do not identify recent or lifetime depressive symptoms in patients with NAFLD.

We next sought to determine whether clinical and biochemical variables could effectively stratify patients based on their depressive symptoms (Table 6.1). One-way ANOVA identified a significant difference in serum triglyceride level (mmol/L), which was elevated in those with recent depression (RD) compared to those with no history of depression (ND; $p = 0.001$) (Figure 6.1a). The serum triglyceride level of those with LD was identical to those with ND ($p = 0.96$). Similarly, regarding serum HDL level (mmol/L), a trending difference by one-way ANOVA ($p = 0.086$) appeared to be driven by reduced serum HDL in RD compared to ND ($p = 0.076$) whereas LD had very similar levels to ND ($p = 0.98$) (Figure 6.1b).

Table 6.1 Demographic and clinical characteristics of the patient cohort according to self-reported depressive symptoms.

	Depressive symptoms			p-value (one-way ANOVA or Pearson's chi- squared test)
	Recent depressive symptoms (<12months) N=81	Lifetime depression (no symptoms for >12months) N=30	No Depression N=107	
Sex (% female) †	46.9%	63.3%	38.3%	0.047*
Age (yrs)†	55 (45-61)	65 (55.75- 71.25)	61 (51.8-70)	0.0004***
BMI (kg/m²)†	33 (29.3-38.0)	30.3 (28.2- 37.8)	33.0 (29.1- 38.7)	0.22
eGFR (mL/min/1.73m²)†	90 (80-90)	87 (70-90)	88 (66-90)	0.10
Serum ALT (IU/mL)†	31 (22-51)	27 (19-39)	28 (20-47)	0.53
Serum AST (IU/mL)†	21 (15-28)	21 (16-29)	21 (15-32)	0.85
HBA1c (%)†	7.7 (6.6-9.0)	6.9 (6.1-8.4)	7.2 (5.9-8.8)	0.25
Serum Ferritin (µg/L)†	70 (38-141)	58 (25-146)	91 (48-159)	0.27
Serum Cholesterol (mmol/L)†	4.5 (3.6-5.5)	4.6 (3.5-5.8)	4.3 (3.5-5.1)	0.23
Serum Triglycerides (mmol/L)†	2.0 (1.6-2.9)	1.7 (1.2-2.0)	1.6 (1.0-2.3)	0.0009***
Serum HDL (mmol/L)†	1.0 (0.8-1.1)	1.2 (0.9-1.3)	1.1 (0.9-1.3)	0.086
Serum LDL (mmol/L)†	2.6 (1.7-3.2)	2.5 (1.8-3.8)	2.2 (1.7-3.0)	0.41
LSM kPa^{‡§}	5.6 (4.8-7.9)	4.8 (4.4-5.7)	6.1 (4.7-8.6)	0.10
LSM <8.2	65	26	59	0.20
LSM 8.2-9.5	3	1	1	
LSM 9.5-11.1	3	0	5	
LSM >13	12	0	6	
T2DM n (%)‡	72 (88.9)	23 (76.7)	86 (80.4)	0.19
Dyslipidaemia n (%)‡	77 (95.1)	29 (96.7)	102 (95.3)	0.94
Metabolic Syndrome n (%)‡	67 (82.7)	25 (83.3)	88 (82.2)	0.99
Current antidepressant use n (%)‡	37 (45.7)	12 (40.0)	0 (0.0)	<0.0001****
Current statin use n (%)‡	51 (63.0)	17 (56.7)	64 (59.8)	0.81
Current fibrate use n (%)‡	12 (14.8)	2 (6.7)	10 (9.3)	0.35

†Continuous data (median [IQR]) analysed using one-way analysis of variance; ‡Categorical data analysed using Pearson's chi-squared test. §LSM (liver stiffness measurement) presented for 197/218 patients with a reliable measurement. BMI, body mass index; eGFR, estimated glomerular filtration rate; ALT, alanine transaminase; AST, aspartate transaminase; HBA1c, glycated haemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein; T2DM, type 2 diabetes mellitus.

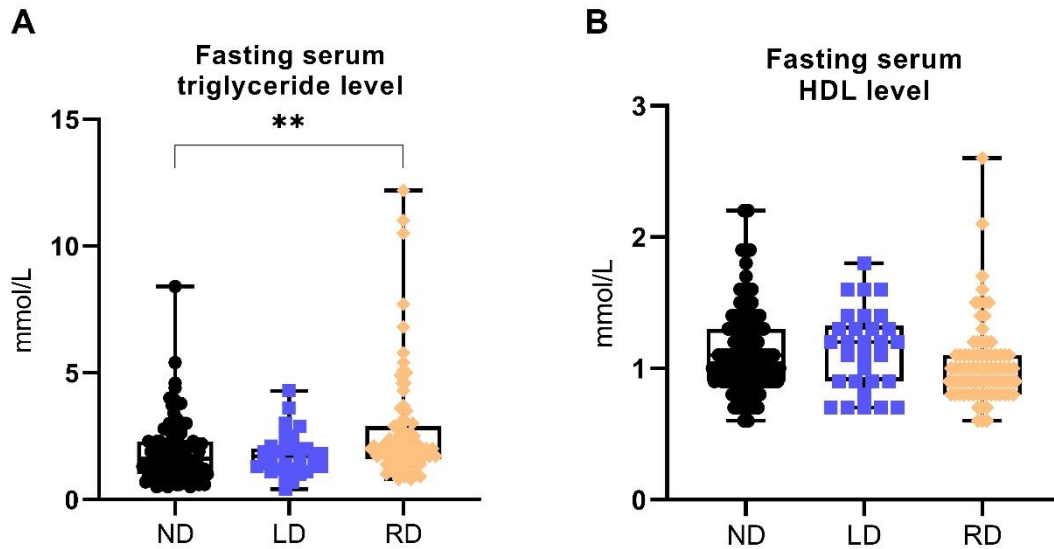


Figure 6.1 Serum lipid biochemistry is altered by depression status in NAFLD. The fasting serum level of **A)** triglycerides was significantly higher in those with RD compared to either those with LD or ND, while **B)** HDL level trended lower in those with RD compared to those with ND. * $P < 0.05$, ** $P < 0.01$. ND = no history of depression ($n = 107$), LD = lifetime history of depression but no recent depressive symptoms ($n = 30$), RD = recent depressive symptoms within the last 12 months ($n = 81$).

ROC curves were then built for clinical and biochemical parameters, to assess their discriminatory capacity regarding self-reported depressive symptoms (Table 6.2). Based on the observations in Table 6.1, which showed no significant differences between LD and ND groups, these groups were combined (LD/ND, $n = 137$) and compared to RD ($n = 81$) for the binary analysis. As expected, serum triglycerides were most discriminatory, with an AUC of 0.64 [95% CI 0.57-0.71, $p = 0.0006$]. Serum HDL was also discriminatory, with an AUC of 0.61 [95% CI 0.53-0.68, $p = 0.0071$], whereas other biochemical parameters were non-discriminatory (AUC < 0.60 and $p > 0.05$). Further, there was no difference between LSM (AUC 0.51 [95% CI 0.44-0.59]) and no significant difference in the number of individuals at each stage of fibrosis by LSM (Table 6.1, $p = 0.20$).

Table 6.2 Relationship between patient clinical and biochemical parameters and recent self-reported depressive symptoms.

	Depressive symptoms		
	Recent depressive symptoms (<12months) N=81	No depression or no symptoms for >12months N=137	ROC AUC Value (95% CI)
Serum Triglycerides (mmol/L)*	2.0 (1.6-2.9)	1.7 (1.2-2.0)	0.64 (0.57-0.71) p = 0.0006
Serum HDL (mmol/L)*	1.0 (0.8-1.1)	1.2 (0.9-1.3)	0.61 (0.53-0.68) p = 0.0071
HBA1c (%)*	7.7 (6.6-9.0)	7.2 (6.0-8.6)	0.58 (0.50-0.65) p = 0.056
eGFR (mL/min/1.73m ²)*	90 (80-90)	88 (69-90)	0.56 (0.49-0.63) p = 0.15
Serum Cholesterol (mmol/L)*	4.5 (3.6-5.5)	4.6 (3.5-5.8)	0.55 (0.47-0.63) p = 0.22
Serum LDL (mmol/L)*	2.6 (1.7-3.2)	2.5 (1.8-3.8)	0.54 (0.46-0.62) p = 0.30
Serum Ferritin (µg/L)*	70 (38-141)	91 (48-159)	0.54 (0.46-0.62) p = 0.30
Serum ALT (IU/mL)*	31 (22-51)	28 (20-45)	0.54 (0.45-0.61) p = 0.39
LSM kPa* [#]	5.6 (4.8-7.9)	5.8 (4.6-8.1)	0.51 (0.44-0.59) p = 0.80
Serum AST (IU/mL)*	21 (15-28)	21 (15-32)	0.51 (0.44-0.59) p = 0.69

*Continuous data (median [IQR]) analysed using a receiver operator characteristic (ROC) curve, reporting the area under the curve (AUC). [#]LSM (liver stiffness measurement) presented for 197/218 patients with a reliable measurement. eGFR, estimated glomerular filtration rate; ALT, alanine transaminase; AST, aspartate transaminase; HBA1c, glycated haemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

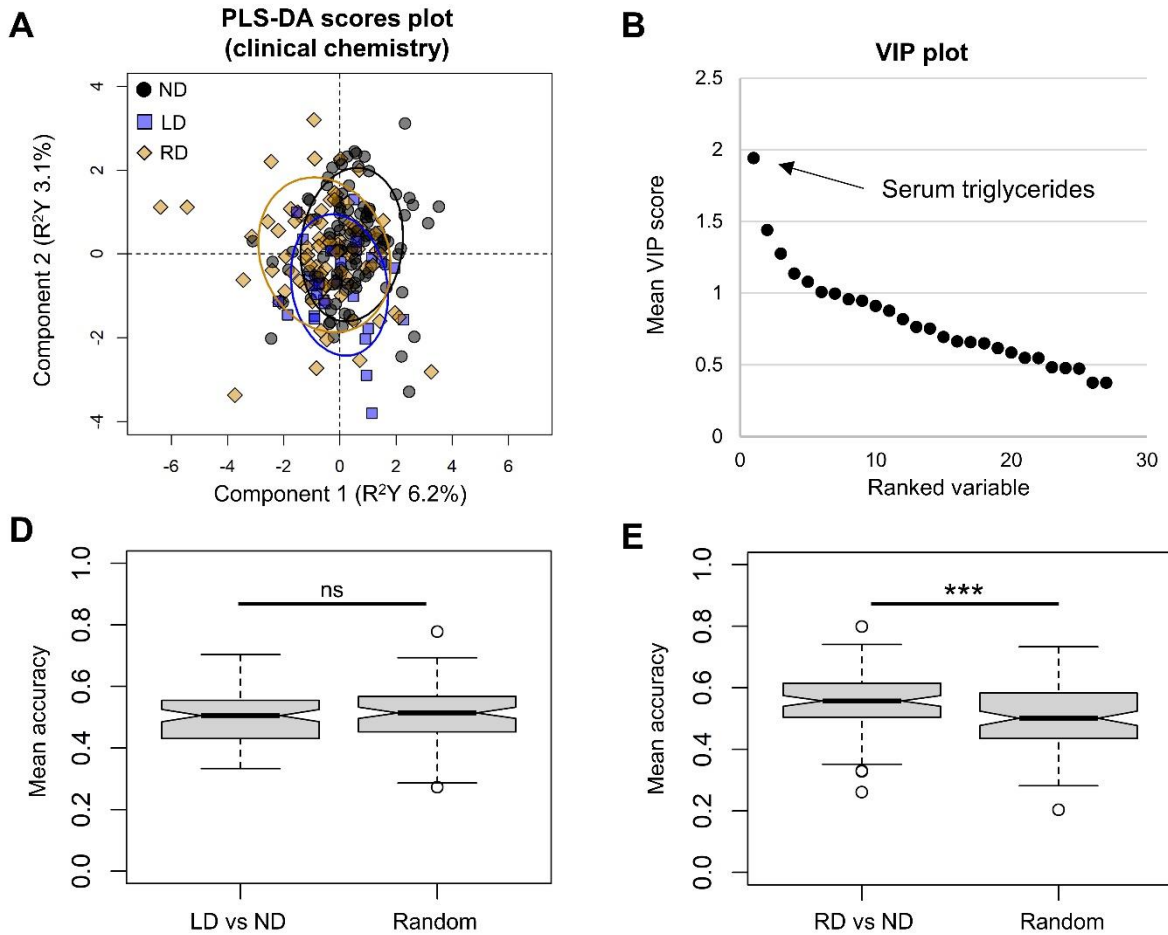


Figure 6.2 Partial least squares discriminant analysis (PLS-DA) of clinical chemistry identifies recent, but not lifetime depressive symptoms in this cohort with non-alcoholic fatty liver disease. **A)** PLS-DA scores plot, **B)** corresponding PLS-DA plot of variable importance in projection for each clinical variable, cross-validated accuracy of **C)** LD vs ND and **D)** RD vs ND. *** $P < 0.001$, ns = non-significant difference. ND = no history of depression ($n = 107$), LD = lifetime history of depression but no recent depressive symptoms ($n = 30$), RD = recent depressive symptoms within the last 12 months ($n = 81$).

PLS-DA of all clinical chemistry variables (Figure 6.2a) identified a significant difference between NAFLD patients with recent depression and no history of depression (accuracy 55.7%, $p < 0.0001$ compared to ND, Figure 6.2c), but not between NAFLD patients with a lifetime history of depression and no history of depression (accuracy $< 50.0\%$, $p = 1$ compared to ND, Figure 6.2d). Despite this significant difference, the multivariate model did not afford more information than the univariate analysis. Average VIP values (Figure 6.2b) confirm that serum triglyceride level contributed most to the model accuracy.

6.2.3 Multivariate analysis of the serum metabolome identifies a signature of recent depression in non-alcoholic fatty liver disease.

Multivariate PLS-DA of the serum metabolome was employed to determine whether a metabolomic signature of recent or lifetime depression could be identified in the context of NAFLD (Figure 6.3). A 10-fold cross-validation strategy with repetition using matched class sizes was employed to ensure that model accuracies > 50% were not due to overfitting. Models were compared to data with randomly permuted classes to determine whether they performed significantly better than the null distribution (random chance).

PLS-DA of the serum metabolome revealed that individuals with recent depression had a significantly different metabolomic signature than individuals with no history of depression (mean accuracy RD vs ND $60.0 \pm 7.8\%$, $p < 0.0001$, Figure 6.3a,c). Conversely, individuals with lifetime depression were indistinguishable from individuals with no history of depression based on their serum metabolic profile (mean accuracy LD vs ND $< 50\%$, $p = 1$, Figure 6.3a,d). The mean cumulative R^2X was 0.48 ± 0.04 for the model and 0.49 ± 0.03 for the null distribution ($p = 0.99$). The mean cumulative R^2Y was 0.22 ± 0.04 for the model and 0.18 ± 0.02 for the null distribution ($p < 0.0001$), while the mean cumulative Q^2 was -0.15 ± 0.08 for the model and -0.25 ± 0.08 for the null distribution ($p < 0.0001$). Metabolites were ranked by VIP from the PLS-DA (Figure 6.3b) and those with a score > 1.5 were subjected to univariate analysis using the Brown-Forsythe one-way ANOVA (Figure 6.4).

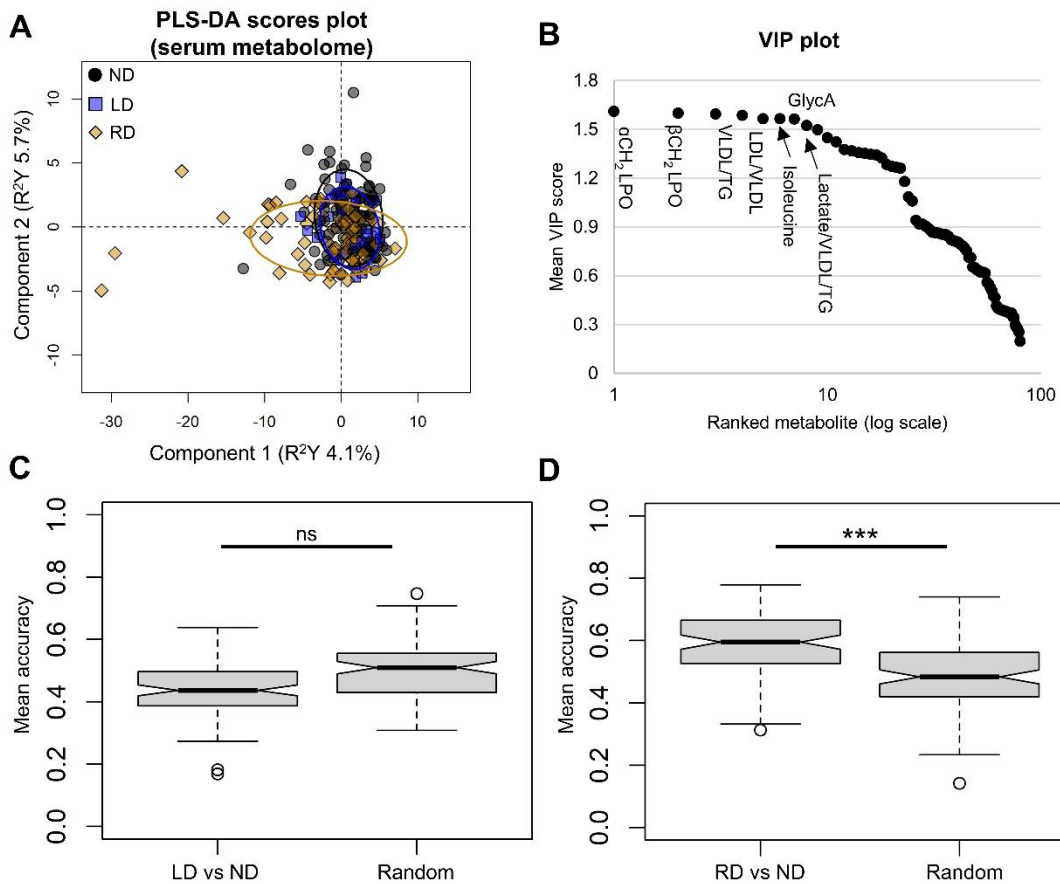


Figure 6.3 Nuclear magnetic resonance spectroscopy (NMR) identifies a serum metabolic signature of recent depressive symptoms in NAFLD. **A)** PLS-DA scores plot, **B)** corresponding PLS-DA VIP plot, **C)** mean cross-validated accuracy shows no difference in the serum metabolome between lifetime depression and no history of depression whilst **D)** there is a significant difference between recent depression and no history of depression. *** $p < 0.001$, ns = non-significant difference. ND = no history of depression ($n = 107$), LD = lifetime history of depression but no recent depressive symptoms ($n = 30$), RD = recent depressive symptoms within the last 12 months ($n = 81$), LPO = lipoprotein.

One-way ANOVA revealed that the level of α CH₂ group ($F_{(2, 161.5)} = 8.82$, $p = 0.0014$, Figure 6.4a), and β CH₂ group ($F_{(2, 161.8)} = 8.89$, $p = 0.0014$, Figure 6.4b) fatty acid (FA) chain resonances, corresponding to a mixture of lipoprotein subclasses, were significantly different between groups. Dunnett's post hoc comparisons test revealed that in all cases the FA abundance in those with RD, but not LD, was significantly greater than in those with ND ($p < 0.01$ in all cases). Methylene (-CH₂) group FA chains corresponding predominately to very low-density lipoprotein (VLDL), and triglycerides, were also significantly altered by depression status ($F_{(2, 161.5)} = 9.83$, $p < 0.0001$, Figure 6.4c), and post hoc testing revealed that this was

driven by a higher level in those with RD compared to those with ND ($p < 0.001$). Methyl group ($-\text{CH}_3$) resonances corresponding mainly to LDL and VLDL followed a similar pattern of abundance (Figure 6.4d). A significant difference among group means ($F_{(2, 170.1)} = 8.87$, $p = 0.0014$) was also driven by an increase in RD relative to ND ($p < 0.001$) while there was no difference between LD and ND. Serum isoleucine levels ($F_{(2, 145.9)} = 5.24$, $p = 0.045$) were higher in those with RD compared to ND ($p < 0.05$, Figure 6.4e). Glycoprotein acetyls ($F_{(2, 147.3)} = 6.68$, $p = 0.015$) (GlycA) was significantly altered according to depression status and found to be significantly increased in RD patients compared to ND patients ($p = 0.01$, Figure 6.4f). Lastly, the lactate peak within the VLDL/TG resonance also reached significance ($F_{(2, 165.2)} = 7.48$, $p = 0.0072$, Figure 6.4g) and was higher in the RD group compared to ND ($p < 0.01$) however given that the complementary lactate multiplet resonance at 4.09-4.13ppm was not significantly different between groups ($F_{(2, 115.5)} = 1.16$, $p = 0.32$, Figure 6.4h,i) this difference was most likely driven exclusively by the underlying lipoprotein resonances. In all cases, post hoc tests also included a comparison between LD and ND, though no significant difference was found for any of these metabolites ($p > 0.05$).

6.2.4 The metabolomic signature of recent depressive symptoms is robust to demographic differences, including antidepressant medication usage.

Sex, age, and current antidepressant usage were found to differ by depression status (Table 6.1) and thus were investigated to see whether they may also influence the serum metabolome. First, orthogonal (O)PLS-DA of the serum metabolome was performed between individuals with and without current antidepressant usage (Figure 6.5a). 10-fold cross-validation with repetition was applied. The mean model accuracy was $55.7 \pm 5.5\%$ ($p < 0.0001$ compared to the null distribution, Figure 6.5b). The mean cumulative R^2X , R^2Y , and Q^2 of the model were 0.54 ± 0.03 , 0.34 ± 0.04 , and -0.25 ± 0.16 , respectively.

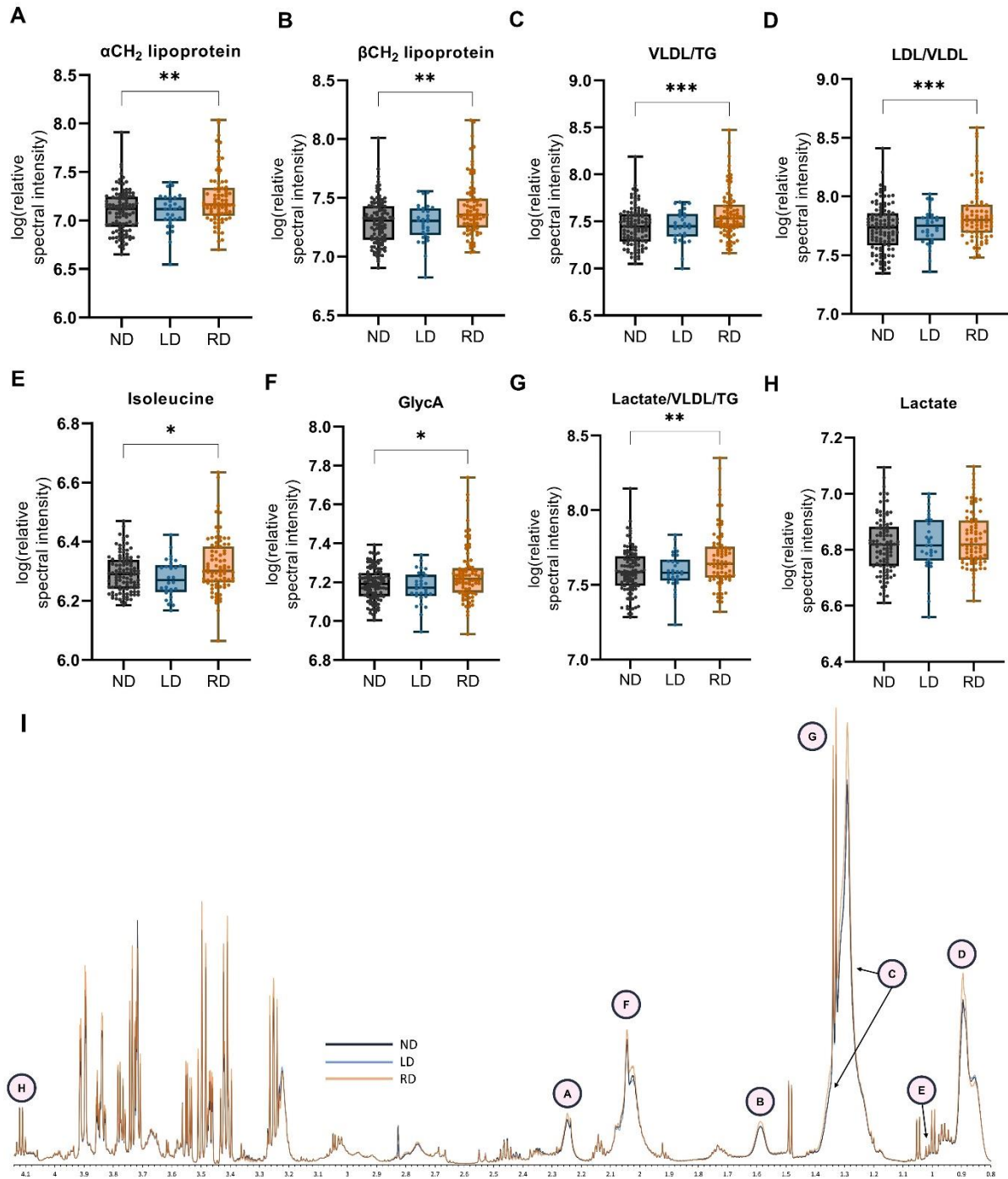


Figure 6.4 Univariate analysis and representative spectra of metabolite relative abundance derived from nuclear magnetic resonance (NMR) spectroscopy. Univariate boxplots of serum **A**) α CH₂ lipoprotein, **B**) β CH₂ lipoprotein, **C**) VLDL/TG, **D**) LDL/VLDL, **E**) isoleucine, **F**) glycoprotein acetylation, **G**) lactate/VLDL/TG, and **H**) lactate alone, with **I**) a representative NMR spectrum depicting the median magnitude of each peak corresponding to each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns = non-significant difference. ND = no history of depression ($n = 107$), LD = lifetime history of depression but no recent depressive symptoms ($n = 30$), RD = recent depressive symptoms within the last 12 months ($n = 81$). VLDL = very low density lipoproteins, TG = triglyceride, LDL = low density lipoproteins, GlycA = glycoprotein acetylation.

The NMR peaks contributing most to the model (VIP score ≥ 1.5) corresponded to β -glucose and glutamine (Figure 6.5c). Following identification of these metabolites, post hoc Welch's t-tests did not reveal a significant difference in metabolite levels between those currently using antidepressant medication, and those not (β -glucose $p = 0.11$, glutamine $p = 0.10$, Figures 6.5d,e, respectively).

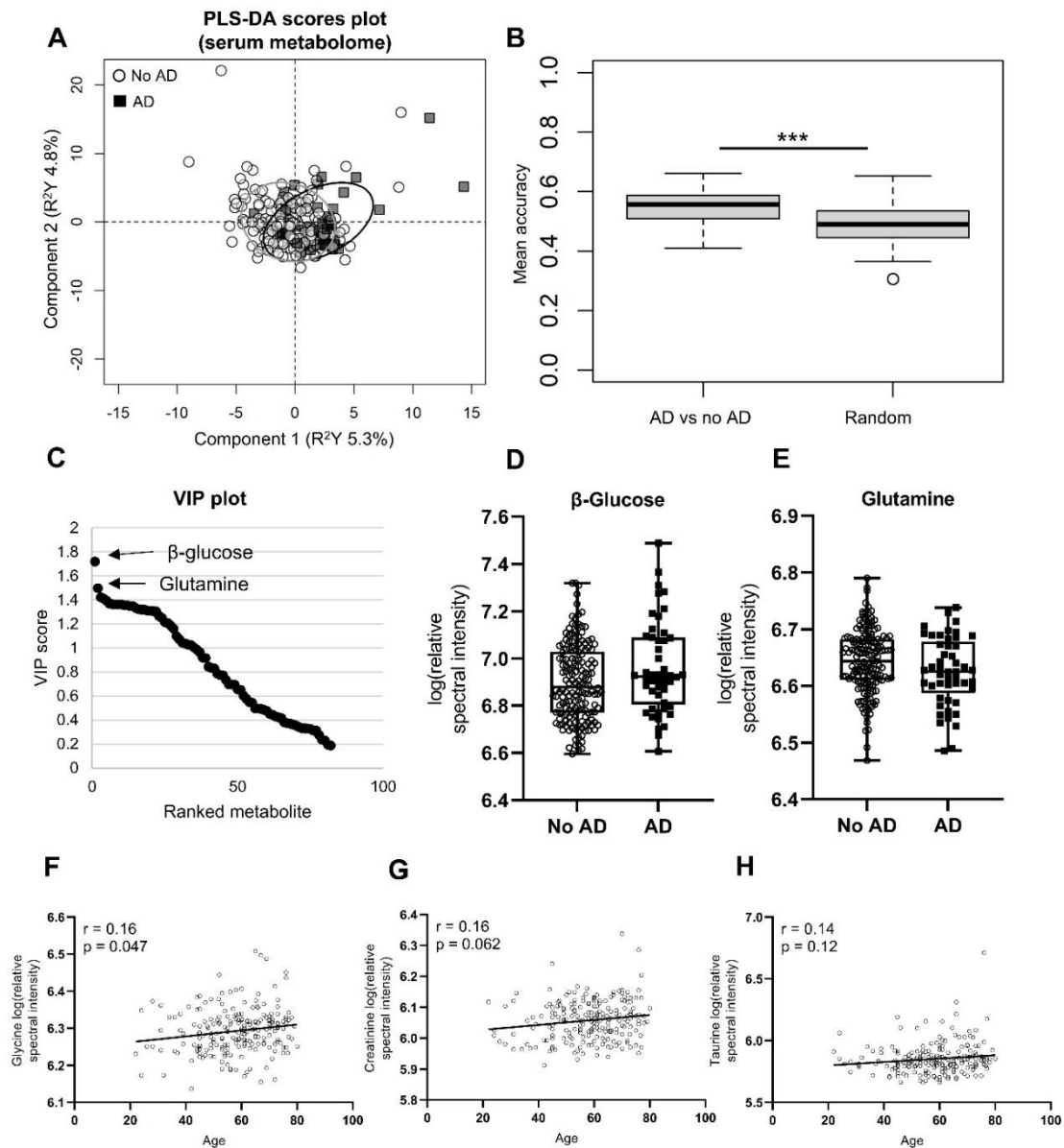


Figure 6.5 Metabolites most related to the effects of antidepressant (AD) treatment and age are unrelated to recent depressive symptoms. **A)** Orthogonal partial least squares discriminant analysis (OPLS-DA) of individuals with or without current AD treatment, **B)** mean accuracy of OPLS-DA with 10-fold cross validation and 100x repetition with class size matching, **C)** corresponding VIP plot, univariate representation of key metabolites **D)** serum glucose and **E)** glutamine, Pearson correlation between age and serum **F)** glycine, **G)** creatinine, and **H)** taurine. *** $P < 0.001$. $N = 49$ AD, $n = 166$ no AD.

Next, OPLS-DA of the serum metabolome was performed between males and females (Figure 6.6a). The mean model accuracy was $79.4 \pm 1.9\%$, ($p < 0.0001$ compared to the null distribution, Figure 6.6b). The mean cumulative R^2X , R^2Y , and Q^2 were 0.51 ± 0.02 , 0.51 ± 0.02 , and 0.34 ± 0.03 , respectively. The top contributory metabolites to the model (VIP score ≥ 1.5) were mobile choline, $-\text{CH}_3$ fatty acyl chain resonance (corresponding to HDL), creatinine, glycine, citrate, glucose, and valine (Figure 6.6c). Mobile choline, glycine, citrate, HDL, and glucose were higher in females compared to males (Welch's t-test, all comparisons $p < 0.0001$, Figures 6.6de,g,h,i), whereas creatinine (Welch's t-test, $p < 0.0001$) and valine (Welch's t-test, $p = 0.016$) were higher in males (Figures 6.6f,j).

Finally, significant Pearson correlations were found only between age and glycine ($r = 0.16$, $p = 0.016$, Figure 6.5f), taurine ($r = 0.14$, $p = 0.040$, Figure 6.5h), and creatine ($r = 0.16$, $p = 0.021$, Figure 6.5g), of which only glycine was significant after correcting for multiple testing ($p < 0.05$).

Overall, while sex does have a strong effect on the serum metabolome in this cohort, it was not the underlying cause of altered lipid and lipoprotein levels in those with recent depressive symptoms.

6.2.5 Investigating depression-associated metabolites across the gut-brain axis in HFD-fed rodents and their offspring

Metabolites act downstream of gene and protein expression and their relative quantification provides an insight into the biochemical basis of physiological and pathophysiological processes (Piazza et al., 2018; Wishart, 2019). While a number of circulating (serum) metabolites were found to be significantly associated with recent depressive symptoms in individuals with NAFLD, it remains unclear whether any of these circulating metabolites correlate with metabolic processes in the *brain*. The purpose of this section is to first investigate whether circulating (plasma) metabolites in mice correlate with passive stress coping behaviour in the forced swim test, and whether these metabolites are consistent with those identified in the human cohort. Secondly, I aim to understand how the

concentration of a circulating metabolite (associated with passive stress coping) correlates with metabolites in the brain, liver, and faeces. These further comparisons, particularly with the brain, afford an insight into how depression-associated circulating metabolomic biomarkers may be linked to underlying metabolic dysfunction in distal tissues.

Accordingly, the depressive-like behaviour (time immobile in the FST) in the F0 dams and the F1 offspring were each correlated, using Pearson's correlation coefficient, with the relative abundance of plasma metabolites in an untargeted fashion. Significant metabolites ($q = 0.10$) were identified following FDR correction (Benjamini and Hochberg) and related to the metabolites found in patients with NAFLD with or without recent depressive symptoms. It was also determined whether each metabolite was significantly correlated with metabolite concentrations in the brain, liver, and gut.

6.2.5.1 Metabolite correlations in the F0 dams

In the rodent F0 dams, both plasma isoleucine ($r = 0.73$, $p = 0.0021$, $q = 0.080$) and plasma valine ($r = 0.73$, $p = 0.0021$, $q = 0.080$) demonstrated a significant positive correlation with immobility in the FST (Figure 6.7a,b). In the brain, plasma isoleucine tended to be associated with elevated glutamine levels ($r = 0.49$, $p = 0.056$) and reduced glutamate levels ($r = -0.47$, $p = 0.063$). These were the top two metabolites as ranked by p-value, however due to the small overall sample size ($n = 16$) the correlations were not significant (Figure 6.7c). Similarly, plasma valine tended to be associated with brain glutamine levels ($r = 0.49$, $p = 0.053$) and brain taurine ($r = 0.48$, $p = 0.063$), but the correlations were not significant (Figure 6.7d). More robust correlations were identified between plasma isoleucine and liver metabolites (Figure 6.7e). Plasma isoleucine was significantly correlated with liver GABA ($r = 0.81$, $p = 0.00013$, $q = 0.016$), liver valine ($r = 0.78$, $p = 0.00036$, $q = 0.016$), liver glutamate ($r = 0.77$, $p = 0.00048$, $q = 0.016$), liver arginine ($r = 0.74$, $p = 0.0011$, $q = 0.029$), and isoleucine ($r = 0.66$, $p = 0.0059$, $q = 0.088$). Plasma isoleucine levels were also correlated with faecal methionine levels ($r = 0.73$, $p = 0.0012$, $q = 0.074$, Figure 6.7f) and non-significantly correlated

with faecal isoleucine ($r = 0.65$, $p = 0.0063$, $q = 0.18$). Plasma valine was not significantly correlated with any faecal metabolites after FDR correction ($q > 0.10$).

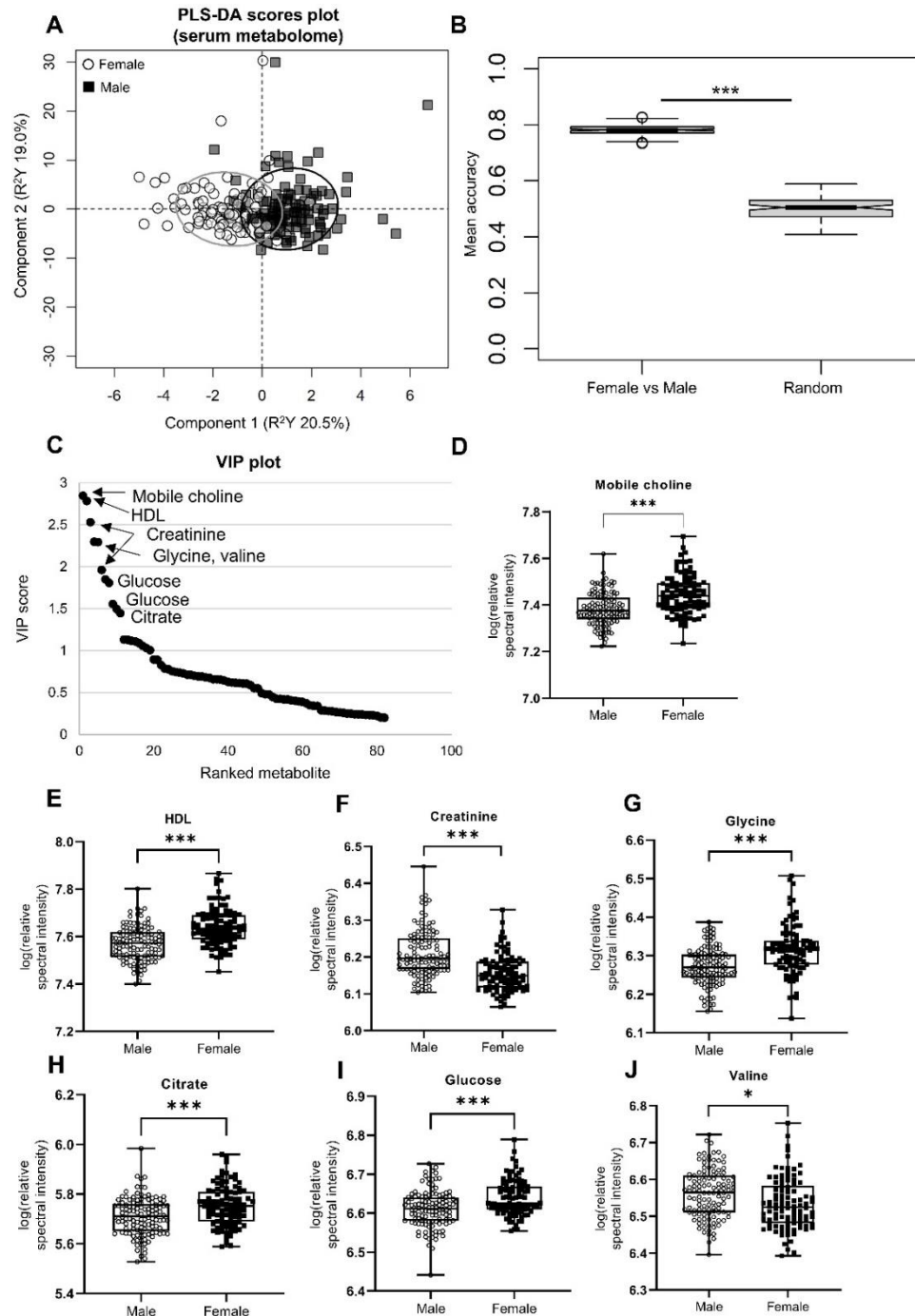


Figure 6.6 Sex differences in the serum metabolome are unrelated to recent depressive symptoms. **A)** Orthogonal partial least squares discriminant analysis (OPLS-DA) comparing the serum metabolome between males and females, **B)** mean accuracy of OPLS-DA with 10-fold cross validation and 100x repetition with class size matching, **C)** corresponding VIP plot, univariate representation of key metabolites **D)** serum mobile choline and **E)** HDL, **F)** creatinine, **G)** glycine, **H)** citrate, **I)** glucose, and **J)** valine. HDL = high density lipoprotein. N = 98 female, n = 120 male.

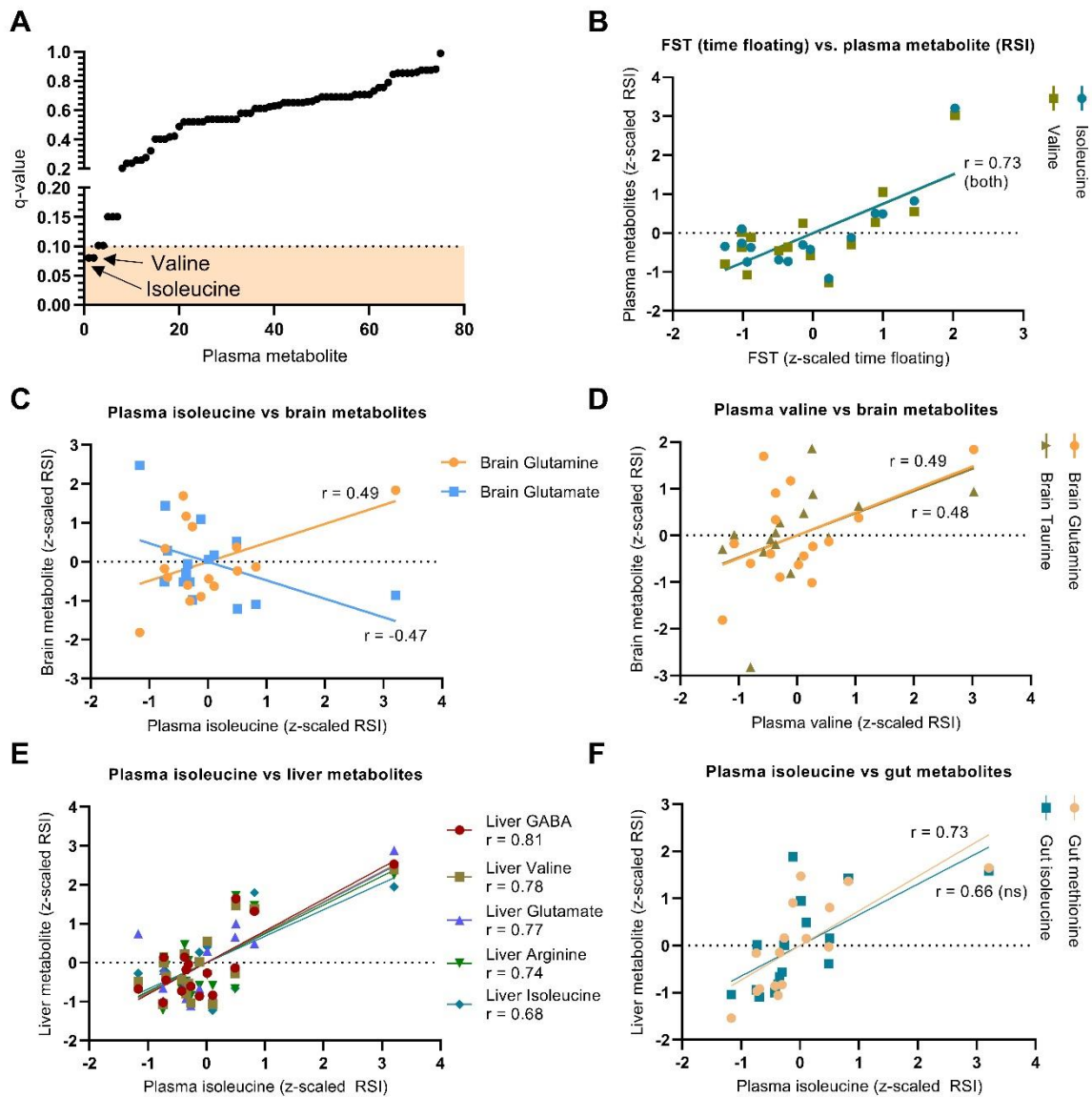


Figure 6.7 Plasma branched chain amino acids (isoleucine and valine) are positively associated with passive stress coping in the forced swim test (FST) in maternal mice. **A**) Plot of FDR-adjusted p-values (q-values) for all plasma metabolites correlated with time immobile in the FST, with the significance threshold for correlation, $q < 0.10$, indicated by the dotted line. Plot of **B**) the two significant metabolites plasma valine and isoleucine against time immobile in the FST, **C**) plasma isoleucine against the most highly correlated brain metabolites brain glutamine and glutamate, **D**) plasma valine against the most highly correlated brain metabolites brain glutamine and taurine, **E**) plasma isoleucine against liver GABA, valine, glutamate, arginine, and isoleucine, and **F**) plasma isoleucine against gut isoleucine and methionine. NS = non-significant correlation after FDR correction. $N = 16$ per group.

6.2.5.2 Metabolite correlations in the F1 juvenile offspring

In the F1 juvenile offspring, several circulating amino acids were associated with depressive-like behaviour in the FST (Figure 6.8a,b). Plasma leucine ($r = -0.37$, $p < 0.0001$, $q = 0.0042$), isoleucine ($r = -0.31$, $p = 0.00085$, $q = 0.030$), valine ($r = -0.30$, $p = 0.0014$, $q = 0.030$), arginine ($r = -0.26$, $p = 0.0051$, $q = 0.077$), glutamine ($r = -0.24$, $p = 0.010$, $q = 0.080$), lysine ($r = -0.24$, $p = 0.012$, $q = 0.080$), and phenylalanine ($r = -0.22$, $p = 0.019$, $q = 0.094$) were negatively correlated with immobility time in the FST, while glucose levels were positively correlated with immobility time ($r = 0.23$, $p = 0.016$, $q = 0.088$).

As serum isoleucine was identified as a metabolite associated with depressive symptoms in the human cohort, it was the focus of the gut-brain-axis correlation analysis in the F1 offspring.

In the F1 offspring brain (Figure 6.8c), plasma isoleucine was significantly negatively correlated with 3-hydroxybutyric acid ($r = -0.44$, $p < 0.0001$, $q = 0.00017$) and positively correlated with glycerophosphocholine ($r = 0.29$, $p = 0.0019$, $q = 0.063$). There was a non-significant positive correlation between plasma isoleucine and brain glutamate ($r = 0.20$, $p = 0.039$, $q = 0.29$). In the liver, 49 metabolite peaks were found to correlate with plasma isoleucine after FDR correction. The most important of these, as ranked by Pearson's r coefficient > 0.40 or < -0.40 , were similar to what was observed in the F0 dams, including GABA ($r = 0.65$, $p < 0.0001$, $q < 0.0001$), valine ($r = 0.63$, $p < 0.0001$, $q < 0.0001$), arginine ($r = 0.61$, $p < 0.0001$, $q < 0.0001$), isoleucine ($r = 0.45$, $p < 0.0001$, $q < 0.0001$), and glutamine ($r = -0.42$, $p < 0.0001$, $q < 0.0001$, Figure 6.8d). Faecal sarcosine correlated positively with plasma isoleucine levels ($r = 0.30$, $p = 0.0019$, $q = 0.055$, Figure 6.8e).

6.2.5.3 Metabolite correlations in the F1 adult offspring

Given that the adult rodent plasma metabolome was found to differ by sex (Figure 5.4c), correlation analysis was performed separately for the adult male and female rodents. In the adult female offspring, plasma alanine correlated positively with immobility time in the FST but did not reach significance after FDR correction ($r = 0.41$, $p = 0.011$, $q = 0.44$, Figure 6.9a,c).

In the adult male offspring, plasma 3-hydroxybutyric acid was the top metabolite (inversely) correlated with immobility time in the FST but was not significant ($r = -0.28$, $p = 0.071$, Figure 6.9b,d).

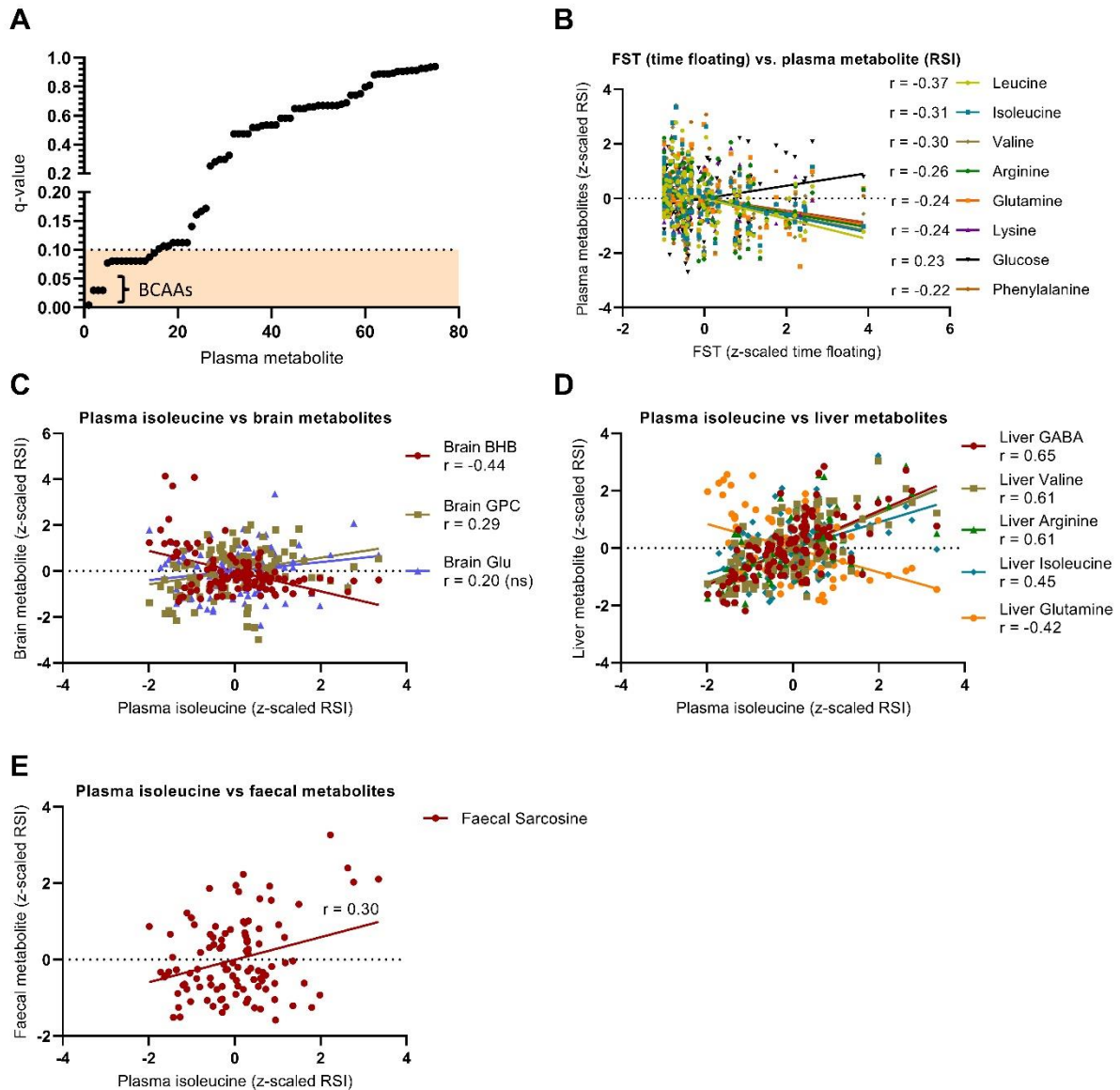


Figure 6.8 Plasma BCAAs are negatively associated with passive stress coping in the forced swim test (FST) in the juvenile offspring. **A**) Plot of FDR-adjusted p-values (q-values) for all plasma metabolites correlated with time immobile in the FST, with the significance threshold for correlation, $q < 0.10$, indicated by the dotted line. Plot of **B**) the significant circulating metabolites, including plasma isoleucine, against time immobile in the FST, **C**) plasma isoleucine against the most highly correlated brain metabolites BHB, GPC, and glutamate, **D**) plasma isoleucine against liver GABA, valine, arginine, isoleucine, and glutamine, and **E**) plasma isoleucine against gut sarcosine. NS = non-significant correlation after FDR correction. $N = 111$ per group. NS = non-significant correlation. BCAAs = branched chain amino acids, BHB = β -hydroxybutyric acid, GPC = glycerophosphocholine, Glu = glutamate, GABA = gamma-aminobutyric acid.

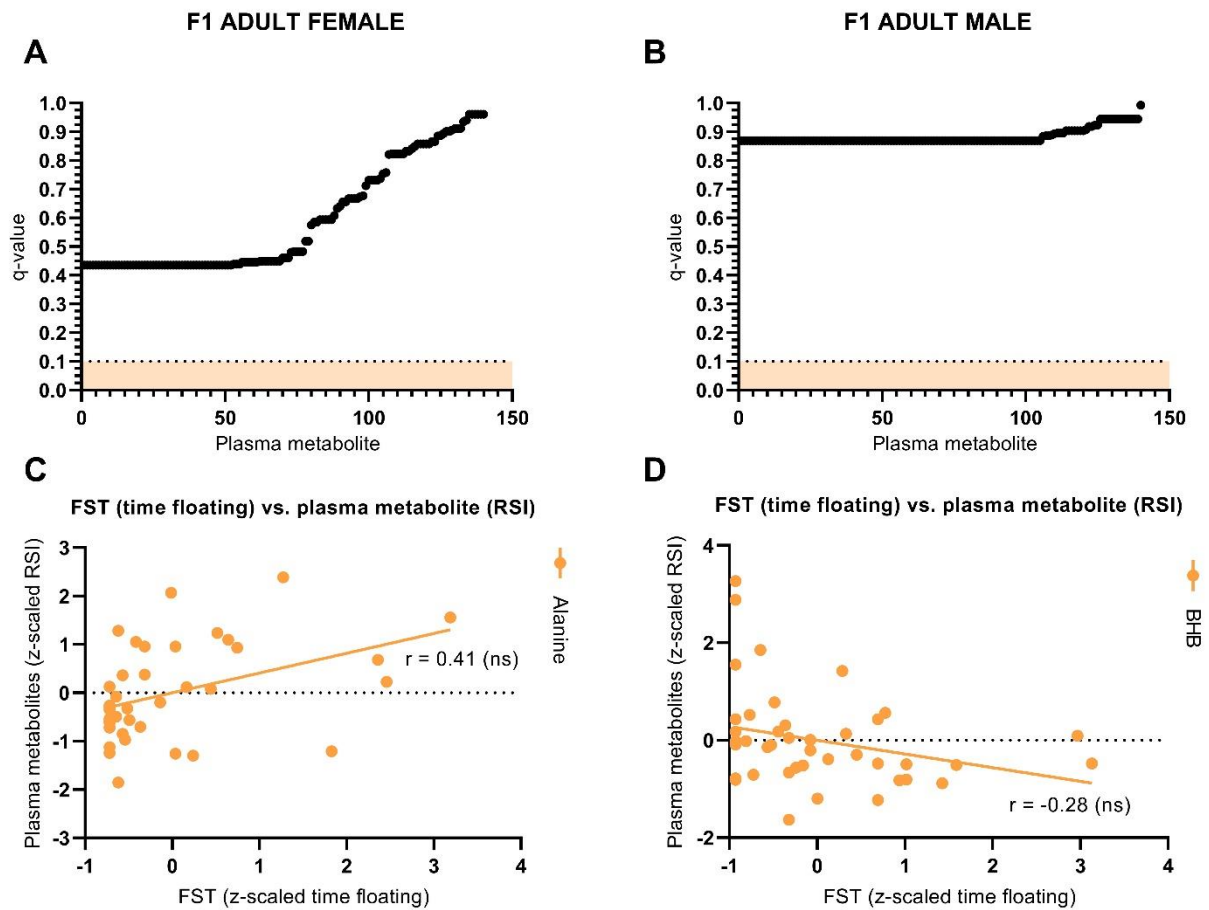


Figure 6.9 Circulating metabolites do not significantly correlate with time spent immobile in the F1 adult offspring. Plot of **A**) female and **B**) male FDR-adjusted p-values (q-values) for all plasma metabolites correlated with time immobile in the forced swim test (FST), with the significance threshold for correlation, $q < 0.10$, indicated by the dotted line. Plot of **C**) female and **D**) male immobility time against the most correlated plasma metabolite, alanine and BHB, respectively. NS = non-significant correlation, BHB = β -hydroxybutyric acid. N = 19-21 per group.

6.2.6 The association between inflammatory gene expression and depressive-like behaviour in rodents

As GlycA, an inflammatory marker, was found to be associated with recent depressive symptoms in patients with NAFLD, I sought to determine whether inflammatory gene expression in the prefrontal cortex or liver was correlated with passive stress coping depressive-like behaviour in the FST in rodents. Correlation was assessed using Pearson's correlation coefficient and corrected for the false discovery rate using the Benjamini and Hochberg method.

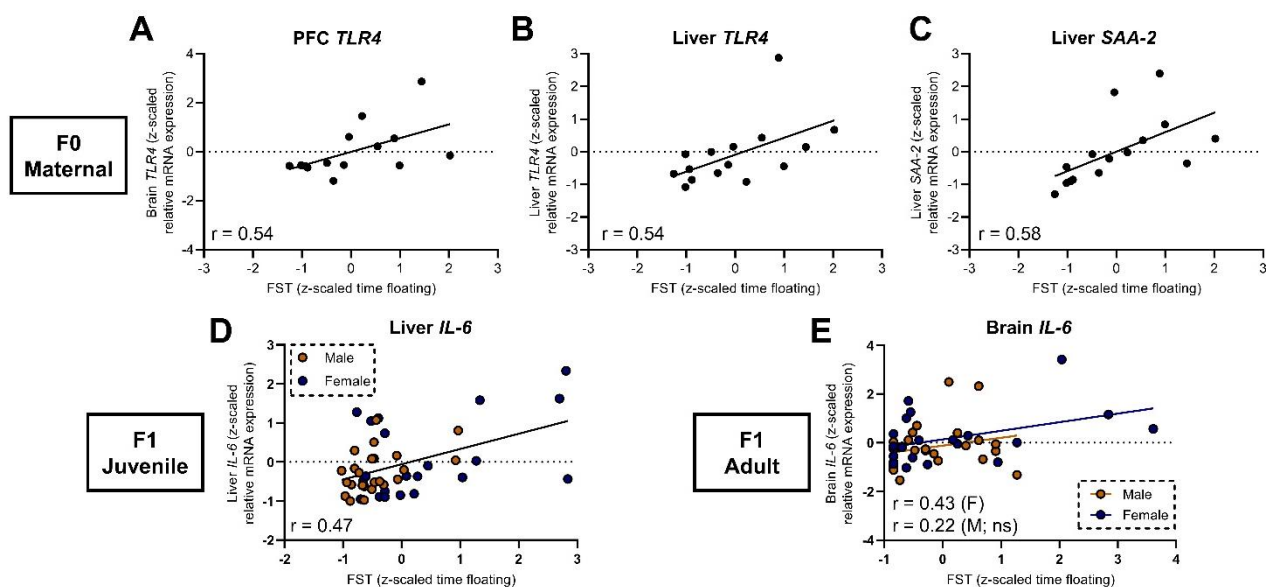


Figure 6.10 Summary of the significant Pearson correlations between inflammatory gene expression and depressive-like behaviour (total immobility time) in the FST. **A)** F0 PFC *TLR4* mRNA expression, **B)** F0 liver *TLR4* mRNA expression, **C)** F0 liver *SAA-2* mRNA expression, **D)** F1 juvenile liver *IL-6* mRNA expression, **E)** F1 adult brain *IL-6* mRNA expression.

6.2.6.1 Gene expression correlations in the F0 dams

PFC *TNF*, *IL-1B*, *IL-6*, and *TLR4* (Table 6.3), and liver *TNF*, *IL-1B*, *IL-6*, *TLR4*, and *SAA-2* (Table 6.4) were assessed for correlation with the extent of immobility time in the FST. PFC and liver *TLR4* gene expression were positively correlated with increased depressive-like behaviour (Figure 6.10a, b, respectively) in the dams. Liver *SAA-2* gene expression was also positively correlated with increased depressive-like behaviour (Figure 6.10c), though no correlations were significant after correcting for the false discovery rate.

Table 6.3 F0 Brain inflammatory gene expression vs. FST time immobile

Gene	Pearson R	p value (FDR q value)
<i>TNF</i>	-0.019	0.95 (0.95)
<i>IL-1B</i>	-0.081	0.77 (0.95)
<i>IL-6</i>	-0.17	0.56 (0.95)
<i>TLR4</i>	0.54	0.037 (0.15)

Table 6.4 F0 Liver inflammatory gene expression vs. FST time immobile

Gene	Pearson R	p value (FDR q value)
<i>TNF</i>	-0.29	0.30 (0.30)
<i>IL-1B</i>	0.45	0.091 (0.11)
<i>IL-6</i>	-0.48	0.067 (0.11)
<i>TLR4</i>	0.54	0.039 (0.097)
<i>SAA-2</i>	0.58	0.025 (0.097)

6.2.6.2 Gene expression correlations in the F1 juvenile offspring

No significant correlations were identified between brain inflammatory gene expression and depressive-like behaviour in the F1 juvenile offspring (Table 6.5), but there was a significant positive correlation with liver *IL-6* (Table 6.6 and Figure 6.10d).

Table 6.5 F1 juvenile brain inflammatory gene expression vs. FST time immobile

Gene	Pearson R	p value (FDR q value)
<i>TNF</i>	-0.029	0.85 (0.85)
<i>IL-1B</i>	0.20	0.18 (0.24)
<i>IL-6</i>	-0.25	0.087 (0.24)
<i>TLR4</i>	-0.21	0.15 (0.24)

No sex differences.

Table 6.6 F1 juvenile liver inflammatory gene expression vs. FST time immobile

Gene	Pearson R	p value (FDR q value)
<i>TNF</i>	0.16	0.27 (0.54)
<i>IL-1B</i>	-0.075	0.62 (0.67)
<i>IL-6</i>	0.47	0.0010 (0.004)
<i>TLR4</i>	0.065	0.67 (0.67)

No sex differences.

6.2.6.3 Gene expression correlations in the F1 adult offspring

PFC *IL-6* expression was significantly correlated with depressive-like behaviour in adult female offspring, but not adult male offspring (Table 6.7, Figure 6.10e). Significance was not maintained after correcting for the false discovery rate. No significant correlations existed between liver inflammatory gene expression and depressive-like behaviour in the adult female or male offspring (Table 6.8).

Table 6.7 F1 adult offspring brain inflammatory gene expression vs. FST time immobile

Gene	Sex	Pearson R	p value (FDR q value)
<i>TNF</i>	Female	0.32	0.14 (0.28)
	Male	0.12	0.58 (0.77)
<i>IL-1B</i>	Female	-0.079	0.72 (0.72)
	Male	0.17	0.42 (0.77)
<i>IL-6</i>	Female	0.43	0.039 (0.16)
	Male	0.22	0.29 (0.77)
<i>TLR4</i>	Female	0.18	0.40 (0.53)
	Male	0.057	0.79 (0.79)

Table 6.8 F1 adult offspring liver inflammatory gene expression vs. FST time immobile

Gene	Sex	Pearson R	p value (FDR q value)
<i>TNF</i>	Female	-0.098	0.65 (0.81)
	Male	-0.17	0.41 (0.55)
<i>IL-1B</i>	Female	-0.094	0.68 (0.81)
	Male	-0.26	0.23 (0.46)
<i>IL-6</i>	Female	-0.26	0.23 (0.81)
	Male	-0.28	0.20 (0.46)
<i>TLR4</i>	Female	-0.053	0.81 (0.81)
	Male	-0.010	0.97 (0.97)

6.3 Discussion

Despite growing evidence for the association between NAFLD and depression over the last decade (Labenz et al., 2020), the presence of depressive symptomology, particularly subclinical, is not typically assessed in the liver clinic. To gain further insight into the links between depression and chronic metabolic dysfunction, I performed serum NMR metabolomics to investigate metabolic perturbations associated with recent or lifetime depressive symptoms in patients with NAFLD. Here, I report a serum metabolic signature of recent (< 12 months) self-reported depressive symptoms, characterised by the increased relative abundance of serum triglycerides, low density lipoproteins, GlycA, and isoleucine.

A pooled analysis of NMR-based plasma metabolomics from 5,283 patients with depression and 10,145 control subjects identified similar biomarkers: higher levels of VLDL cholesterol, glycerides, diglycerides, triglycerides, total fatty acids, and the inflammatory marker GlycA were associated with depression, as were lower levels of HDL-cholesterol (Bot et al., 2020). Interestingly, many of these associations were strengthened when only current depression (3,265 patients) was included, defined as blood sampling within one month of diagnosis by clinical interview or questionnaire. A meta-analysis of depression in people with metabolic syndrome also identified a significant positive association between hypertriglyceridemia and depression, and a negative association between HDL levels and depression (Pan et al., 2012). The original studies pertaining to this meta-analysis used a mixture of self-reporting and structured interviews to classify depression. Associations between depression and metabolic perturbations were stronger in self-reported individuals rather than those diagnosed using structured clinical interviews, or by a clinician. I demonstrate that a similar serum metabolic signature of depression is detectable in a patient cohort with pre-existing metabolic dysfunction.

Alterations in the lipid spectrum identified in the present study may represent shared metabolic abnormalities linking depression to cardiometabolic diseases. In rodents, liver fatty acid synthesis may be increased by chronic stress due to changes in transcriptional activity of

key metabolic enzymes (Chuang et al., 2010). Chronic mild stress in rodents has been shown to significantly elevate plasma ALT and AST levels, triglycerides, and other lipid constituents compared to controls (Jia et al., 2016). Similarly, chronic stress in mice has been found to increase hepatic triglyceride and cholesterol levels whilst decreasing overall visceral fat mass (Liu et al., 2014). Increased lobular inflammation, hepatocyte injury, and serum pro-inflammatory cytokine levels were also noted in the stressed animals compared to controls. These preclinical studies reinforce the bidirectional association between NAFLD, cardiometabolic disease risk, and depression, which may be driven by alterations to peripheral inflammation and lipid metabolism.

Moreover, I found GlycA levels to be significantly increased in NAFLD patients with recent depression. This is consistent with other reports of an association between GlycA and depression symptom severity as well as depression persistence (Brunoni et al., 2020; Huckvale et al., 2020), and likely relates to the relationship between metabolic dysfunction, dyslipidaemia, depression, and the chronic, low-grade inflammation intrinsic to these conditions (Jung and Choi, 2014). By comparison, in the rodent dams fed either a CD or HFD, both peripheral (*TLR4*, *SAA2*) and central (*TLR4*) markers of inflammatory gene expression were correlated with depressive-like behaviour in the forced swim test. As the mRNA level of these genes were significantly elevated because of HFD intake (thesis section 3.3.4), the correlation provides further evidence that increased liver and brain inflammation may play a mediatory role in the association between diet-induced obesity and depressive-like behaviour. Indeed, in a UK Biobank epidemiological study of depression, participant BMI was found to account for a large proportion of the relationship between elevated circulating C-reactive protein levels and depression (Pitharouli et al., 2021). In the juvenile offspring, liver *IL-6* mRNA expression was significantly correlated with depressive-like behaviour, while in the adult offspring, brain *IL-6* showed a significant positive correlation with depressive-like behaviour. In the juvenile offspring, the observed correlation likely reflects the contribution of peripheral inflammation to the increase in immobility observed in the offspring of obese dams. In humans,

increased abundance of saturated fats in breast milk are associated with pro-inflammatory responses in infants (Kong et al., 2021). Therefore, the inflammation in these offspring may also be diet-derived, as the milk from HFD-fed dams contained elevated levels of triglyceride, and reduced levels of anti-inflammatory SCFAs (thesis section 3.3.6).

Lastly, I found serum isoleucine levels to be increased in people with NAFLD and recent depression, compared to patients with NAFLD without depression. As with serum lipid levels, serum isoleucine levels are already elevated in fasted patients with NAFLD compared to healthy controls (Kalhan et al., 2011; Gaggini et al., 2018). This may be due to increased protein turnover due to increased systemic inflammation, but may also arise through diet, whereby overnutrition and excessive BCAA consumption is thought to drive obesity and insulin resistance (Newgard et al., 2009; Solon-Biet et al., 2019; Yu et al., 2021). In addition to the metabolome-depression association analysis in humans, this chapter further explored the relationship between depression and metabolic disease by studying the effect of a high fat diet on mouse metabolism across the F0 and F1 generations. Mice exhibited a spectrum of immobility time in the FST, which was used to approximate depressive symptoms. Increased plasma isoleucine levels in the maternal mice showed a significant positive correlation with passive stress coping, which is consistent with the findings from the human study. Increased plasma isoleucine also coincided with increased glutamine levels and reduced glutamate levels in the brain. Our laboratory has previously identified a relationship between increased plasma isoleucine levels and post-inflammatory depressive-like behaviour induced by LPS administration (Chan et al., 2020). Interestingly, these findings were also associated with increased glutamine levels and reduced glutamate levels in the brain (Chan et al., 2020). Together, these findings support a relationship between circulating isoleucine levels and altered glutamate-glutamine levels centrally, though it is unclear whether this is related to the exhibition of altered depressive-like behaviours (see section 7.4 for an elaboration on this discussion).

In depression, BCAAs are implicated in the brain bioavailability of tryptophan, the precursor amino acid to serotonin. Both circulating tryptophan and isoleucine (as well as other BCAAs) compete for the same transporter for uptake into the brain, and so an increase in circulating BCAA levels is thought to competitively reduce brain tryptophan uptake, thereby reducing brain serotonin levels (Fernstrom, 2013; Kazemi et al., 2019). This concept is elaborated upon in section 7.4, with a mechanistic diagram of the relationship between blood BCAAs and brain tryptophan levels shown in Figure 7.2. Serum isoleucine has been shown to be elevated in people with MDD (Bot et al., 2020). Taken together, elevated blood isoleucine levels may contribute to the overlapping pathophysiology between depression and metabolic disease.

Few studies have investigated the effect of depression on NAFLD at a clinicopathological or biochemical level. Youssef et al. (2013) identified subclinical depressive symptoms (HAD score 1-7) in 53% of 567 patients with NAFLD, and clinically significant depressive symptoms (HAD score 8-21) in 14%. Both subclinical and clinical depression were associated with more severe hepatocyte ballooning (reflecting hepatocellular injury) in liver biopsy sections compared to those with no depressive symptoms (HAD score 0). In contrast, Tomeno et al. (2015) identified a higher grade of steatosis in those with depression (n = 32) compared to those without (n = 226), though no differences in hepatocellular ballooning or fibrosis were found. Interestingly, NAFLD patients with stable depression (defined as full or partial remission) also showed a greater improvement in ALT and GGT levels after a year of standard care than patients with unstable depression (not in full or partial remission). This may indicate that NAFLD patients comorbid with depression do not respond to medical intervention as well as NAFLD patients without an affective disorder, particularly in those with recent rather than a lifetime history of depressive symptoms.

Whilst depression has not been found to increase the likelihood of advanced fibrosis (Kim et al., 2019), managing comorbid depression remains an important facet of clinical care. Individuals with depression are more likely to lead an unhealthy lifestyle and less likely to

adhere to a medical regimen, particularly those involving lifestyle adjustments (Penninx and Lange, 2018). In patients with cardiovascular disease, the leading cause of premature death in NAFLD patients (European Association for the Study of the Liver (EASL) et al., 2016), persistent depressive symptoms (as opposed to none or remitting) predicted worse health-related quality of life after one year (Dekker et al., 2011). Therefore, identifying and treating NAFLD patients with recent depressive symptoms may improve quality of life relating to comorbid cardiovascular disease.

A key limitation of this study relates to the reporting of depression. Participants self-reported whether they had received a clinical diagnosis of depression, and whether they had symptoms in the previous 12 months. I used this data to stratify participants into groups with recent, lifetime, or no depressive symptoms. I did not have access to data from a validated depression scale or diagnostic interview, which may limit the diagnostic rigour of this study. However, single item self-report of depression has previously been shown to have reasonable correlation with validated interview schedules for depression (Zimmerman et al., 2006). Here, all patient interviews were performed by the same person using the same template, providing consistency. An advantage of our self-report measure is that it may more accurately reflect clinical practice, improving the clinical translatability of our findings to real world NAFLD clinics and sensitively capturing a metabolic signature of depressive symptomology in NAFLD, as was described previously in metabolic syndrome (Pan et al., 2012).

An additional limitation is the lack of detailed information regarding the dietary intake and physical activity of this NAFLD cohort, leading to uncertainty over the cause-and-effect relationships between depression, NAFLD, and lifestyle habits. Post-prandial inflammation can occur up to twelve hours after eating (Meessen et al., 2019). Despite highly variable lipid metabolism between individuals, post-prandial serum triglyceride metabolism has been shown to correlate strongly with GlycA levels within individuals, highlighting the important relationship between diet and inflammation (Mazidi et al., 2021). Here, I were unable to determine whether

pre-existing depression is driving eating behaviour and metabolic signals, or whether a poorer quality diet has led to inflammation, metabolic dysfunction, and subsequent depression.

Given that lifestyle interventions remain the key tool to halt or reverse liver disease progression in NAFLD, and that treating depression facilitates exercise and weight loss (Simon et al., 2010), it is important to identify and treat depression in liver clinics. Despite high rates of obesity and metabolic syndrome within the cohort, I identified a robust signature of recent depressive symptoms through altered serum lipid/lipoprotein composition in patients at varying stages of NAFLD. This result is concordant with previous, larger cohorts discriminating depressed from non-depressed individuals, and provides support for a link between disturbed lipid metabolism, liver disease, and active mental health issues. Further work in this area is encouraged to improve understanding of the aetiology of depressive symptoms in patients with metabolic dysfunction, with the aim of identifying novel therapies tailored to this patient population.

7

Discussion

7.1 Summary of results

Maternal obesity is a growing public health concern and is linked to an increased risk of neurodevelopmental and psychiatric disorders in humans (Devlieger et al., 2016; Kong et al., 2020). In parallel, research on the microbiota-gut-brain axis has increased exponentially over the past decade, exposing the far-reaching influence of the gut microbiome in health and disease (Cryan et al., 2019). The gut microbiome has become a target for novel therapeutics that aim to modify gut microbial metabolism for the benefit of host immunity, energy balance, and mental health. In this thesis, my overarching aim was to further understand the bidirectional relationship between depression and metabolic disease. In mice, I investigated the effects of maternal obesity on offspring behaviour, metabolism, and immune and brain plasticity gene expression. In parallel, I tested whether modification of the maternal microbiota with a multispecies probiotic could mitigate changes observed in the offspring of obese dams. In a human cohort with metabolic disease, I investigated whether depression, which was highly prevalent in the cohort, affected circulating metabolite concentrations and whether these metabolites were related to the obese rodent dams or their offspring.

The principal findings of this thesis, in chronological order, are as follows:

- HFD-induced maternal obesity increases time spent away from the litter during the first week of nursing, but not at the expense of licking or grooming the offspring.
- Perinatal probiotic treatment increases the level of lactate and SCFAs in the milk during the first week of nursing, irrespective of maternal diet.

- Supplementation with a multispecies probiotic modified the maternal microbiota during the perinatal period, which was sufficient to mitigate the anxiety-like behaviour observed in the juvenile and adult offspring of obese dams.
- Maternal probiotic treatment induces long-lasting changes to gene expression in the PFC of the offspring, including an increase in mRNAs encoding *PFKFB3*
- Maternal obesity and probiotic intake produce distinct, enduring effects on brain and peripheral energy metabolism in the offspring. Maternal probiotic intake was found to increase brain lactate in the juvenile offspring and faecal butyrate in the juvenile and adult offspring.
- Depressive symptoms in individuals with nonalcoholic fatty liver disease are also characterised by a distinct circulating metabolite profile, which includes increased levels of lipids and glycoprotein acetylation independently associated with metabolic disease.

Taken together, the data in this thesis demonstrate a clear relationship between maternal diet-induced obesity and emotional dysfunction in the offspring. This relationship was evident in the offspring of obese dams, which showed increased anxious and depressive-like behaviour accompanied by metabolic changes across the brain-liver-gut axis. By administering obese dams with a multispecies probiotic mixture and reversing the anxious phenotype in the juvenile and adult offspring, I also indicate that the gut microbiome may act as a key modifiable (and therefore treatable) feature of the relationship between maternal obesity and offspring brain function and behaviour. Lastly, I found that depressive symptoms in humans with liver disease, independent of demographic factors and liver disease severity, are associated with altered circulating metabolite concentrations such as increased serum isoleucine, triglycerides, and glycoprotein acetylation. In this Chapter, I will expand upon the observed relationships between (maternal) diet, key metabolites, and behaviour.

7.2 Relationship between brain metabolism, gene expression, and behaviour in the offspring

Changes in the metabolome are, most simply, the net contributions of both endogenous factors (DNA sequence, RNA expression, protein synthesis and modification, and a myriad of regulatory feedback loops) and exogenous factors (environment), including a key contribution from the diet (Bermingham et al., 2021). A bidirectional relationship exists between gene expression and metabolite levels locally, for example in specific brain regions (Shi et al., 2021), and is also thought to exist between compartments, for example, gut metabolites effecting brain gene expression (Hebert et al., 2021). It was therefore expected that correlations would exist in the offspring between the quantitative expression of target genes and metabolites.

In the juvenile offspring, brain metabolites were assessed for correlation with the expression of target genes that were significantly upregulated or downregulated by either maternal obesity or perinatal probiotic intake (*GLUN2A/B/C*, *PFKFB3*, *SYP*, *IL-6*, *CREB1*, and *ZIF-268*). Brain alanine was found to correlate significantly with PFC *IL-6* levels, while lactate correlated negatively with *GLUN2A*, *GLUN2B*, and *SYP* expression, and correlated positively with *PFKFB3* and *CREB1* expression.

When considering the association between brain alanine and prefrontal cortex *IL-6* expression observed in the juvenile offspring, it is important to consider the likely role of the cytokine and the cell type from which it may be produced. As a pleiotropic protein, *IL-6* performs a variety of functions depending on when and where it is expressed. A negative association may be observed between amino acids and *IL-6* expression peripherally during pathological inflammation, where marked amino acid catabolism is observed (McGaha et al., 2012). During exercise, *IL-6* is released from skeletal muscle to signal increased gluconeogenesis and muscle glucose uptake (Kistner et al., 2022). Peripheral alanine is depleted as it is used as the primary substrate for gluconeogenesis during long bouts of high-intensity exercise (Hennigar et al., 2017). In the brain, *IL-6* expression is predominately

astrocytic under normal physiological conditions, where it can exert neurotrophic and neuroprotective effects (Van Wagoner and Benveniste, 1999). Alanine contributes to glutamate-glutamine cycling between neurons and astrocytes by facilitating the transfer of the amino group back to astrocytes, but is not thought to play a major role in glutamate or other neurotransmitter biosynthesis through the metabolism of its carbon backbone (Bröer et al., 2007; Schousboe et al., 2014). In my thesis I have shown that the juvenile offspring of probiotic-treated dams exhibit an increase in both brain alanine and brain glutamate compared to vehicle controls. Moreover, probiotic offspring show increased PFC expression of the astrocyte metabolic marker *PFKFB3* and *IL-6*. These findings may reflect an increase in turnover of the glutamate-glutamine cycle, which relates increased astrocytic metabolic activity to increased glutamatergic neurotransmission through excitatory circuits (Tani et al., 2014). As the BBB prevents glutamate uptake from the periphery, my observation of increased brain glutamate is likely to be due to an increase in the synthesis of glutamate for glutamatergic neurotransmission (Zhou and Danbolt, 2014). Overall, it seems likely that the concerted increase in brain alanine and *IL-6* expression reflects an increase in astrocytic activity and not a direct cause-and-effect relationship between metabolite and cytokine expression. Further research is required to understand the dual roles of *IL-6* in both neuroinflammatory pathology and neuroprotection (Willis et al., 2020). Studies using isotopically labelled substrates could confirm whether maternal probiotic supplementation increases glycolytic metabolism through astrocytes, and whether this leads to an accumulation of alanine accompanied by increased *IL-6* expression.

Aside from alanine, brain lactate levels correlated with the expression of several genes involved in glutamatergic transmission and synaptic plasticity. Moreover, increased brain lactate was found to correlate with reduced anxiety-like behaviour in the OFT in both the juvenile and adult offspring more so than other brain energy metabolites. L-lactate has been demonstrated to exhibit antidepressant-like effects in adult mice (Carrard et al., 2018; Karnib et al., 2019). Similarly, fluoxetine may exert antidepressant effects in part by increasing lactate

release from astrocytes, which has been shown in vitro (Allaman et al., 2011). Functioning independently as both a signalling molecule (Magistretti and Allaman, 2018) and neuronal energy source (Pellerin and Magistretti, 1994; Bélanger et al., 2011), the pleiotropic properties of lactate may have contributed to the early life changes in NMDAR gene expression and behaviour. For example, lactate has been shown to increase histone deacetylase 2/3 (HDAC2/3) protein in the hippocampus of adult male C57Bl/6 mice (Karnib et al., 2019). Histone acetylases and deacetylases are enzymes that regulate the binding of histone proteins to DNA, and thus play a key role in transcriptional regulation. Deacetylases increase the affinity between histones and the DNA by removing an acetyl group from the histone tail and increasing its net positive charge. The result is a more tightly packed chromatin structure, reducing transcription. Accordingly, Karnib et al. (2019) found, alongside an increase in HDAC2/3 expression, reduced *GLUN2A* mRNA expression. This was accompanied by reduced anxiety-like behaviour in the Elevated Plus Maze after 10 days of chronic social defeat stress. Other target genes have been shown to be regulated by acute and chronic L-lactate administration. Carrard et al. (2018) demonstrated the antidepressant effect of L-lactate in adult male C57Bl/6 mice in the forced swim test, observing decreased immobility time compared to vehicle administration. This was accompanied by molecular changes downstream of lactate administration such as reduced GSK3 β and CREB protein. In my thesis, I have shown that the abundance of brain lactate, but not other brain metabolites, correlated highly with *GLUN2A/2B* expression changes across all young male and female offspring and correlated with reduced anxiety-like behaviour in both young and adult offspring. This supports the hypothesis that increased brain lactate promotes a resilient behavioural phenotype in mice.

One remaining question is how lactate levels may be regulated by the gut microbiome (or, indirectly, by maternal probiotic administration). One possible explanation is PFKFB3 (6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3). PFKFB3 is a master regulator of lactate production through the glycolytic pathway in astrocytes (Bolaños, 2016). Brain expression of *PFKFB3* has been shown to be regulated by the microbiome. Adult male

C57Bl/6 mice supplemented with prebiotics exhibit increased hippocampal *PFKFB3* expression (Margineanu et al., 2020). In my thesis, maternal probiotic intake was associated with a significant increase in *PFKFB3* expression relative to both the juvenile and adult offspring of dams given the vehicle.

In the adult offspring, *PFKFB3*, *SYP*, and *ZIF-268* expression, which remained significantly altered by maternal diet and/or probiotic treatment, was assessed for correlation with key brain metabolites. No significant correlations were found after correcting for the false discovery rate. Two factors may account for this. Firstly, the gene-metabolite correlations are limited by the fact that gene expression was measured in the PFC, whereas metabolite levels were generalised to the contralateral cerebrum. While this may be less of an issue in the juvenile offspring, where metabolite levels are highly correlated amongst all regions of the cerebrum at this early age, the transition to adulthood in mice is marked by more region-specific changes in metabolite levels (Ding et al., 2021). Consequently, the loss of certain gene-metabolite correlations in adulthood could be due to increased noise resulting from more distinct regional metabolite profiles within the F1 adult cerebrum. Secondly, *SYP* and *ZIF-268* did not follow the same pattern of expression in adult offspring compared to juvenile offspring, whereas *PFKFB3* expression was congruent with the juvenile offspring and tended to correlate with brain lactate levels in the same direction. Therefore, my thesis suggests that the direct association between *PFKFB3* expression and lactate levels alone may be long-lasting between the juvenile and adult offspring, but this should be retested using identical brain regions. This would be feasible as brain lactate levels are high and could be detected from a much smaller brain tissue sample than was used for untargeted metabolomics in this thesis.

7.3 Relationship between faecal short-chain fatty acids and brain gene expression in the offspring

In my thesis, I discovered that perinatal probiotic supplementation was sufficient to increase the levels of microbially-derived SCFAs in the milk of probiotic-fed dams during nursing, whereas maternal HFD feeding decreased SCFA levels. Breast milk serves as an important early source of live bacteria and milk oligosaccharide prebiotics for the development of the offspring gut microbiota (Martín et al., 2003; Li et al., 2021). The origin of milk microbiota remains unclear but may originate from the maternal gut (Rodríguez, 2014). In my work, this is supported by a similar pattern of variation between the maternal milk metabolome and maternal faecal metabolome despite being sampled at different timepoints (postnatal day 4 for milk and postnatal day 21 for faecal sample). As an example, butyrate levels were found to be higher in dams that received the probiotic in both the faeces and the milk. Similarly, levels were lower in dams that were fed a HFD in both faeces and milk, leading to a significant correlation between faecal butyrate and milk butyrate/valerate levels (Figure 7.1).

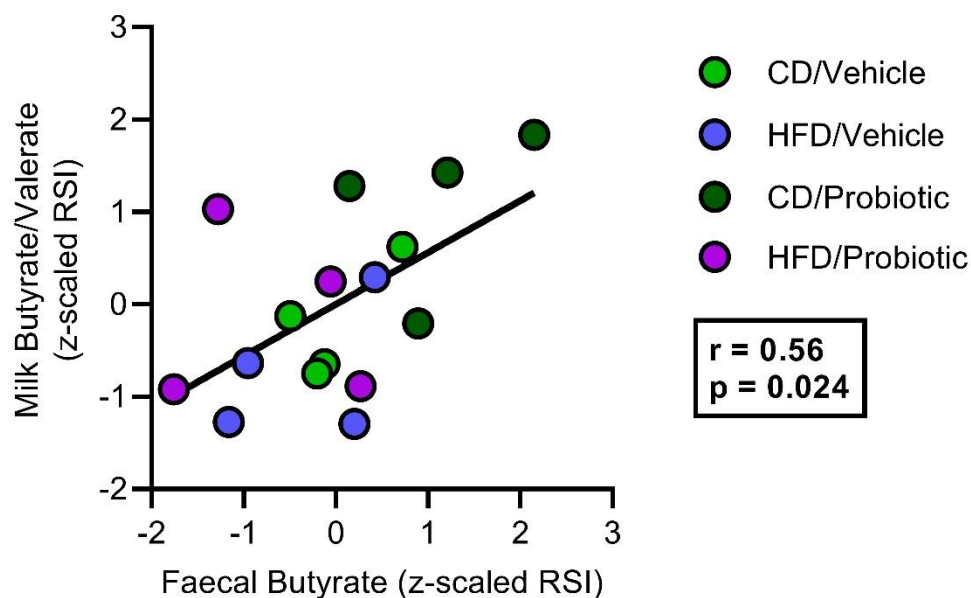


Figure 7.1 Illustration of the relationship between faecal microbial metabolite profile and milk microbial metabolite profile through the correlation of butyrate levels in the female mouse dams.

It is likely, therefore, that the increase in SCFA levels in the milk is due to the relative increase in faecal *Lactobacillus spp.* and *Bifidobacterium spp.* in the probiotic-treated dams. Conversely, I also showed that faecal *Bifidobacterium spp.* were reduced in dams fed the HFD, even if they had also been given the probiotic. This observation is supported by work in humans, which has shown that a diet high in fat (predominately soybean oil) reduces gut microbial diversity and total SCFA levels in a controlled isocaloric study relative to a low-fat diet (Wan et al., 2019). Because dietary fibre intake was matched between the HFD and control diet, it is possible that the reduced *Bifidobacteria* and butyrate levels observed in HFD-fed dams is due to the much lower carbohydrate level compared to the control diet. In humans, a low-carbohydrate diet is associated with reduced faecal butyrate and butyrate-producing bacteria levels (Duncan et al., 2007). While I did not quantitate the level of *Bifidobacterium spp.* in the milk of the dams, the observation of reduced faecal *Bifidobacterium spp.* may have mediated a decrease in milk levels of *Bifidobacterium spp.* and butyrate. Indeed, it has been shown in humans that the breast milk microbiota composition from obese mothers contains lower levels of *Bifidobacterium* than lean mothers (Collado et al., 2012), and that certain species of *Bifidobacterium* present in human milk are thought to metabolise milk oligosaccharides into SCFAs such as butyrate (Sela et al., 2008).

In the offspring, I was surprised to discover that the SCFA butyrate remained elevated in the faecal samples of the juvenile offspring of probiotic-treated at weaning age as well as the adult offspring from these same probiotic-treated dams at 4 months age. This may have contributed to the long-term changes in gene expression and behaviour exhibited by these offspring. While not detected in brain tissue using NMR, the SCFAs butyrate and propionate are brain-permeable and, like lactate, capable of modulating gene transcription through the modification of histone deacetylase activity (Arpaia et al., 2013; Karnib et al., 2019). In Chapter 5, I found that faecal butyrate was positively correlated with PFC *PFKFB3* expression in both the juvenile and adult offspring. Additionally, faecal propionate and butyrate levels correlated

negatively with NMDAR subunit expression in the PFC of juvenile offspring, while both metabolites were positively correlated with *CREB1* expression.

Unequivocally, gut butyrate levels can be manipulated in the offspring through maternal probiotic supplementation during the perinatal period. These effects are long-lasting and may relate to the altered prefrontal cortex gene expression in the offspring of dams treated with the probiotic. In future work I would like to confirm this hypothesis by investigating the direct contribution of individual SCFAs and lactate to long-term changes in depressive-like behaviour, plasticity gene expression, and brain energy metabolism.

7.4 Investigating the relationship between the human and rodent blood metabolic profile to further understand the molecular biology of depression

The mouse metabolite profiling in the F0 dams (Chapter 3) and F1 offspring (Chapter 5) provided an opportunity to draw comparisons between the rodent and human data in terms of the metabolomic correlates of depressive symptoms. Mice across all generations exhibited a spectrum of immobility time in the FST (Chapters 3,4), which was used to approximate depressive symptoms in comparison to the self-reported “recent depression” in those with pre-existing NAFLD (Chapter 6). The HFD used in this thesis has been used to model NAFLD in mice (Svegliati-Baroni et al., 2006; Flores-Costa et al., 2018). Metabolic disturbances such as steatosis (build-up of fat in the liver), obesity, and hepatic inflammation mirror the typical features observed in humans with NAFLD and minimal liver fibrosis. Moreover, NAFLD has been shown to independently increase the risk of anxiety and depression in humans (Labenz et al., 2020), which is also reflected in the high rate of recent and lifetime depressive symptoms in the NAFLD cohort described in this thesis. There, NAFLD is an appropriate condition by which to study the biological correlates of depression in relation to the results discovered in the mice.

In the F0 dams, plasma branched chain amino acids (BCAAs) isoleucine and valine showed a significant positive correlation with immobility time in the FST. Interestingly, those with NAFLD and recent depressive symptoms also showed increased serum isoleucine levels compared to those without a history of depression. Increased isoleucine has previously been related to depression in both humans (Bot et al., 2020) and rodents (Zemdegs et al., 2019; Chan et al., 2020). Circulating isoleucine levels can affect the brain bioavailability of tryptophan, the key substrate for serotonin synthesis. Tryptophan and isoleucine (as well as other large, neutral amino acids such as leucine and valine) compete for uptake by the transporter LAT1 into the brain. Therefore, increased circulating isoleucine levels are thought to competitively reduce brain tryptophan uptake, thereby reducing brain serotonin levels and

potentially altering behaviour (Fernstrom, 2013; Kazemi et al., 2019) (Figure 7.2). Altogether, elevated blood isoleucine levels may play a role in the shared pathophysiology between depression and metabolic disease.

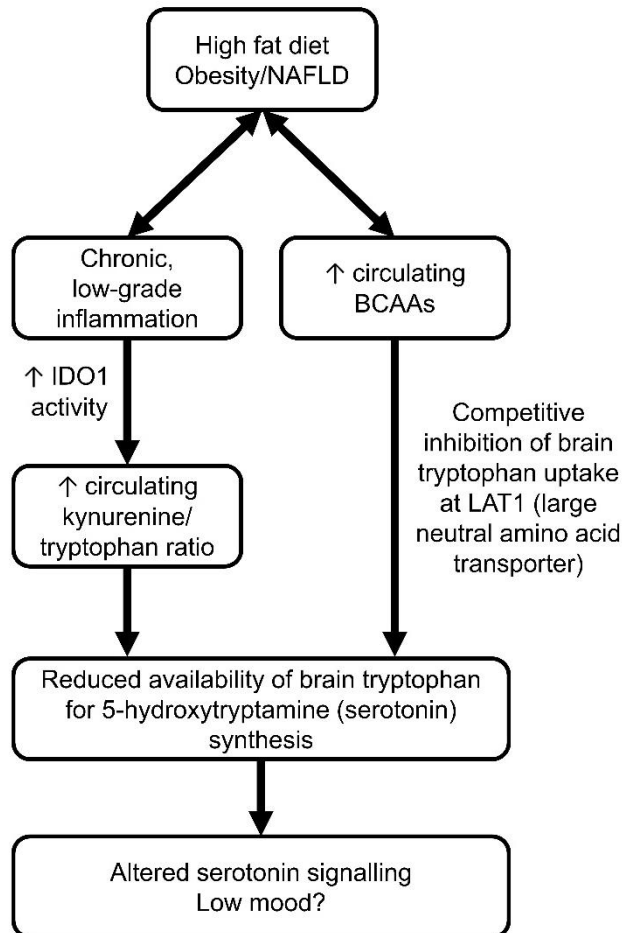


Figure 7.2 Potential mechanisms describing how diet-induced metabolic syndrome (obesity/NAFLD) may lead to altered brain plasticity and low mood. On the left side of the flowchart, chronic, low-grade inflammation is thought to upregulate peripheral indoleamine 2,3-dioxygenase (IDO1) activity, leading to increased kynurenine production from tryptophan and reduced brain tryptophan uptake for central serotonin synthesis. On the right, elevated circulating branched-chain amino acids may arise after prolonged high-fat diet feeding. BCAAs compete with tryptophan for the large neutral amino acid transporter (LAT1), reduce brain uptake of tryptophan and central serotonin synthesis. This may lead to altered neurotransmission and low mood.

To understand more about how circulating isoleucine may relate to altered brain metabolism, I related plasma isoleucine to the brain metabolome in the F0 dams. Plasma isoleucine correlated most with increased brain glutamine levels, and reduced brain glutamate. Our laboratory has previously shown that, in an LPS model of post-inflammatory depressive-like behaviour (as assessed by the FST), increased plasma isoleucine levels also coincided with increased glutamine levels and reduced glutamate levels in the brain (Chan et al., 2020). This may suggest that different models of depressive-like behaviour in rodents may converge on a similar mechanism involving altered circulating isoleucine and glutamate/glutamine ratios in the brain.

Increased isoleucine may arise due to an excessive level of dietary fat. Mice fed a HFD display elevated circulating BCAA levels alongside depressive-like behaviour and reduced extracellular hippocampal levels of serotonin (Zemdegs et al., 2019). In the same study, metformin reduced circulating BCAA levels and showed antidepressant-like effects in the HFD model of depression, suggesting that BCAA levels may indeed play a causal role in the pathophysiology of depression, especially in the context of metabolic dysfunction. Despite an apparent connection between diet-induced obesity and elevated circulating BCAA, it is unlikely to be the diet itself that directly contributes to elevated BCAA levels. In the study by Zemdeg et al. (2019), BCAA levels were matched between the HFD and CD, whereby increased energy consumption was derived exclusively from the increased lipid content. In the dams studied in Chapter 3, neither diet nor perinatal probiotic treatment had a significant effect on plasma isoleucine levels (Appendix I), suggesting that the association of isoleucine with passive stress coping may have also been independent of these factors. Rather than through diet, elevated fasting BCAAs are thought to be due to impaired catabolic pathways through branched chain keto acid and Acyl-CoA pathways (Siddik and Shin, 2019). To date, no study has tested whether enzymes responsible for BCAA catabolism are altered in expression or activity in individuals with depression, so it remains unclear whether this mechanism – which likely underlies the association with elevated BCAAs in obesity (Pietiläinen et al., 2008) – also may account for the observation of elevated isoleucine in individuals with depression.

In the F1 juvenile offspring, isoleucine was also a key plasma metabolite which correlated with FST immobility. In this case however, increased isoleucine was associated with *reduced* depressive-like behaviour. It is unclear why the relationship between plasma isoleucine levels and behaviour are reversed in the juvenile offspring. Decreased isoleucine in blood may be an allostatic response to the increased exposure to circulating BCAAs in utero. Moreover, in this thesis, maternal probiotic treatment had a marked effect on offspring central and peripheral metabolism, which may have disrupted the association between isoleucine and depressive-like behaviour as observed in the F0 dams. Indeed, perinatal

probiotic exposure appeared to increase the weight of the offspring at weaning age relative to the offspring of dams given the vehicle only. Conversely, in the juvenile offspring without probiotic exposure, maternal HFD treatment increased body weight at weaning age. In the F1 adult offspring, there were no significant correlations after FDR correction between plasma metabolite levels and immobility time in the FST.

Taken together, my thesis supports the notion of overlapping pathophysiology between depression and metabolic disease. Isoleucine may be important to this concept, because existing evidence suggests that its elevation may be a *consequence* of altered metabolism in obesity (Pietiläinen et al., 2008) and a contributory *cause* of depression by depleting brain bioavailability of tryptophan.

7.5 General limitations and future directions

In this thesis, maternal mice were treated with a probiotic containing *Bifidobacterium* spp. and *Lactobacillus* spp. (Table 2.1), both of which produce lactate as well as the SCFAs propionate, butyrate, and acetate (Ríos-Covián et al., 2016). I determined the sum therapeutic effect of these maternally ingested strains on the offspring metabolome, with the aim of identifying beneficial metabolites involved in resilience to anxiety-like behaviour induced by maternal obesity. While the rationale of employing any one strain as a potential antidepressant is limited, I did not resolve the precise source of the microbially derived metabolites or the contribution of individual strains and species to ameliorating the adverse effects of maternal obesity in the offspring. Several experiments could be designed to address these outstanding questions.

Firstly, it would be interesting to characterise more completely the effects of maternal obesity and maternal probiotic intake on the offspring gut environment. While NMR spectroscopy is relatively inexpensive and sample preparation is straightforward, it is insensitive and will only detect the most abundant microbial metabolites present in a sample. Combining the existing ^1H NMR data with mass spectrometry and either 16S rRNA amplicon sequencing, or metagenomic analysis, could be used in an experimental setting to identify a wider scope of alterations to the gut microbiota and microbial metabolites. In this study, such analyses were partly constrained by the limited amount of faecal material obtained from the juvenile offspring. Future experiments may require either pooling faecal material from the juvenile offspring at the time of culling, or serially collecting faecal pellets in the day(s) preceding the experimental endpoint. In a clinical setting, however, the high throughput, reproducible, and cost-efficient nature of NMR spectroscopy makes it an ideal tool for translational research between animals and humans. For example, NMR has been employed to profile the blood metabolomic profile of >100,000 individuals in the UK Biobank cohort (Julkunen et al., 2021). Moreover, multi-omics analyses that incorporate mass spectrometry or genomics generate many features, which increases the risk of false positive outcomes.

In parallel, it would be useful to confirm whether the effects of maternal obesity on the offspring are dependent on perturbations to the microbiome. By performing a faecal microbiota transplantation (FMT) from either HFD or CD offspring to juvenile GF mice and performing subsequent microbiome sequencing, Buffington et al. (2016) have shown that aberrant social behaviour due to maternal HFD is mediated by changes to the microbiome. Furthermore, FMT at 4-weeks age, but not 8-weeks age, was effective in reversing aberrant social behaviour, suggesting the presence of an early neurodevelopmental window during which microbiota modification could be beneficial. Similarly, FMT from obese maternal mice to GF maternal mice has also been shown to induce behavioural changes in male, 7-week-old offspring, such as increased anxiety-like behaviour in the OFT compared to FMT from lean maternal mice to GF maternal mice (Bruce-Keller et al., 2017). This latter approach would circumvent the issue that GF mice appear to show reduced anxiety-like behaviour compared to SPF controls (Luczynski et al., 2016), which might lead to the observation that FMT from either mHFD or maternal CD offspring would increase anxiety-like behaviour relative to the GF mice without any microbiota transplantation. By accompanying this maternal FMT approach with both gut metabolome and microbiota profiling in the dams and their offspring, it would be possible to confirm – with a high degree of certainty – the role of maternal HFD-induced dysbiosis in offspring emotional dysfunction in mice.

Equally, the same experiment could be conducted with maternal mice that had undergone probiotic supplementation. Because maternal perinatal multispecies probiotic supplementation was shown to have an independent anxiolytic effect on the juvenile and adult offspring, it would be expected that the maternal microbiota from probiotic-treated dams, when transplanted to GF dams, would have an equivalent effect on the offspring neurochemistry and behaviour. To understand the effect of the probiotic on the offspring brain and behaviour in more detail, one could then determine what microbial species or metabolites were most differentially abundant in the gut of the offspring. For example, because faecal butyrate and brain lactate were significantly elevated in the juvenile offspring and correlated with plasticity

gene expression in the juvenile PFC, it would be interesting to see if maternal supplementation with butyrate or lactate during the same perinatal window could recapitulate the effects of the probiotic in the offspring. In designing these experiments, the palatability of butyrate and lactate may need to be considered in a pilot study. Butyrate has been shown to be palatable in drinking water at 100mM, which was sufficient to significantly increase butyrate levels detected in the faeces of recipient mice, but water intake decreased in mice treated with 200mM butyrate in the drinking water (Smith et al., 2013). No literature currently exists to document the palatability of lactate in drinking water. It is unlikely that administration of sodium lactate by this method would be panicogenic, which may occur after acute lactate infusion and a very rapid rise in blood lactate concentration (Pitts and McClure, 1967).

Another limitation of the rodent work is that metabolite changes in the brain were not quantified for specific regions within the cerebrum, which would be useful to draw associations more accurately between the gene expression analysis in the PFC and downstream changes in metabolite levels. Additionally, while mRNA is useful for getting an indication of expression levels for several target genes, it does not always correlate with the expression of protein. Therefore, it would be important to confirm the changes identified, for example changes in the expression of NMDAR subunits, with protein-based assays (Western blot or enzyme-linked immunosorbent assay).

The candidate genes used in this study were based on a number of observations including the effects of maternal prebiotic supplementation on offspring brain function (Hebert et al., 2021), as well as the effects of lactate treatment on synaptic plasticity (Carrard et al., 2018) and early indications that the microbiota could modify markers of astrocytic metabolism (Margineanu et al., 2020). Due to the wide-ranging effect of both maternal obesity and probiotic supplementation on the offspring faecal, liver, and brain metabolome, an untargeted transcriptomic analysis of the PFC, and other brain regions, may be warranted. On the one hand, this would put the magnitude of gene expression changes observed in this study into the context of an untargeted approach. On the other hand, screening the transcriptome in an

unbiased fashion comes with its own set of limitations. A large change in gene expression under a given set of conditions does not necessarily equate to importance. Small changes in the expression of several genes may functionally converge to alter a phenotype more than a large change in the expression of a single gene with a different function. In practical terms, RNA sequencing is costly, and sample size per group may need to be reduced. Along with the reduced sensitivity and specificity in comparison to targeted qPCR, this may result in loss of signal (Evans, 2015). Conversely, due to the large number of genes screened with this approach and increased sample to sample variability compared with qPCR, false discoveries are also likely (Schurch et al., 2016).

In terms of the rodent metabolomics work, whereas ^1H NMR spectroscopy provides a snapshot of the relative abundance of metabolites in each sample, isotopically labelled metabolites (e.g., ^{13}C labelled glucose or acetate) can be used to estimate the rate of metabolism within a tissue. Depending on the positioning of the labelling at each carbon atom, the kinetics of different metabolic pathways can be inferred. As *PFKFB3* expression was significantly increased in the juvenile and adult offspring of dams receiving probiotic treatment compared to vehicle, it would be interesting to estimate the cerebral metabolic rate of astrocytes and neurons *ex vivo* using isotopically labelled acetate and glucose infusions, respectively followed by NMR spectroscopy (Mishra et al., 2018).

Lastly, when exploring the serum metabolomic profile of individuals with NAFLD and depression, several lipid resonances were identified as being significantly increased in those with recent depressive symptoms compared to those without a history of depressive symptoms. While this result answered the chief hypothesis of whether a distinct metabolic profile could be identified within the context of NAFLD and corroborated the key findings of an independent, larger metabolomics study in depression (Bot et al., 2020), due to the extraction method used the data on lipid-phase metabolites is limited to the broad lipid and lipoprotein peaks described in section 6.2.3. In the future, I aim to perform MS-based lipidomics analysis of the human sera to understand the circulating lipid profile of this cohort with more granularity.

7.6 Translational perspectives and concluding remarks

In summary, maternal obesity increased anxiety- and depressive-like behaviour in young and adult offspring. Anxiety-like behaviour was prevented in the juvenile offspring of obese dams by maternal perinatal probiotic treatment, and both anxiety- and depressive-like behaviour were prevented in the adult offspring of obese dams that were exposed to perinatal probiotic treatment. Furthermore, I identified increased cortical lactate and faecal butyrate in the offspring as potential sources for the increased resilience demonstrated by the offspring exposed to the probiotic during early life (Figure 7.3). Translationally, in humans with NAFLD, I identified a signature of altered lipid and amino acid metabolism in those with recent depressive symptoms compared to those without a history of depression (Figure 7.3). I aim to replicate these findings in a larger prospective cohort where the prevalence of depression has been assessed by a structured diagnostic interview.

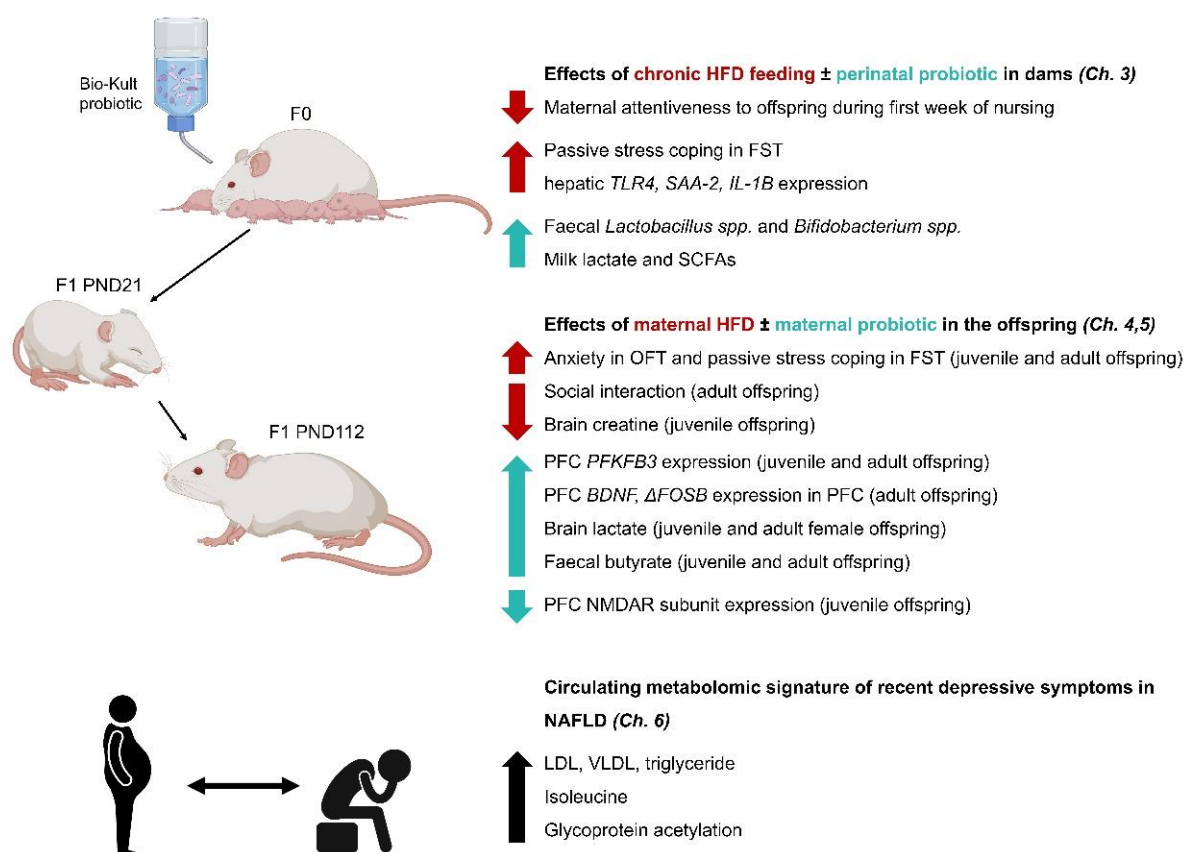


Figure 7.3 Summary of thesis findings by chapter.

In humans, lifestyle modifications are key to reducing the risk of maternal complications during pregnancy and supporting healthy offspring development. Current UK guidelines emphasise the importance of preventative strategies, whereby a healthy body mass index is achieved prior to conception (NICE, 2010; Denison et al., 2019). Nevertheless, the prevalence of maternal obesity in the UK doubled between 1989 and 2007 from 7.6% to 15.6% (Heslehurst et al., 2010), and may now be as high as 25% (Devlieger et al., 2016). As pharmacotherapy, including weight-loss therapy, during pregnancy raises safety concerns, optimising maternal nutrition during pregnancy and breastfeeding is a crucial aspect of pregnancy care (Denison et al., 2019). An established example of this is the relationship between maternal obesity, increased risk of neural tube defects in the offspring, and folic acid supplementation. Obese women of childbearing age are known to have reduced serum folate levels, even when adjusting for folate intake through diet or supplements (Mojtabai, 2004; Tinker et al., 2012; Knight et al., 2015). The underlying mechanisms for this may relate to differences in gut microbiota composition, chronic inflammation, insulin resistance and epigenetic factors between obese and lean women (van der Windt et al., 2021). As a result, guidelines for folate supplementation from one month preconception through the first trimester of pregnancy has changed from a “one-dose fits all approach” to a higher dosage (5mg daily as opposed to 0.4mg daily) in obese women.

I believe that this tailored approach to maternal nutrition could, in the future, be extended to target the gut (and milk) microbiota to reduce the risk of disease in the offspring, including mood disorders. Preliminary clinical evidence exists to support this idea. A study of >20,000 mother-child pairs revealed that children of mothers who followed an “unhealthy diet” high in processed foods during pregnancy were more likely to demonstrate externalising behavioural problems at 5 years of age compared to the children of mothers who followed a healthy diet (Jacka et al., 2013). This finding was independent of social deprivation, but it was unclear if it was independent of maternal BMI. More recently, it has been demonstrated in 213 mother-infant pairs that maternal prenatal gut microbiota composition was predictive of

internalising behaviour in children at 2 years age (Dawson et al., 2021). This study is the first to report a longitudinal association between maternal microbiota and offspring behaviour in humans, which previously had been reported in animal studies. Surprisingly, this effect was not mediated by infant gut microbiota. Maternal microbiota diversity was not predictive of child microbiota diversity at 6 months age, and there was no relationship between child gut microbiota composition at 6 months age and behaviour at 2 years age. Mediation analysis suggested that, while there was no overall association between maternal diet quality and offspring behaviour, maternal diet modified maternal gut microbiota diversity which likely indirectly influenced offspring behaviour outcomes (Dawson et al., 2021). Genus-level analysis of maternal microbiota revealed that levels of butyrate-producing bacteria (*Lachnospiraceae* and *Ruminococcaceae*) were lower in mothers with children who exhibited internalising behavioural problems. No direct association between maternal serum or faecal acetate, propionate, or butyrate levels and offspring behaviour was found, but samples acquired for SCFA analysis were taken at a different point in gestation (28 weeks) to the faecal microbiota sampling (36 weeks). In summary, this recent prospective human study supports current preclinical evidence that the maternal microbiota is associated with offspring development and behaviour. Continuing to assess child and adolescent behaviour in the future in this longitudinal fashion will be important in assessing the longevity of this association. Future studies in this area should aim to perform metagenomic analysis of the gut microbiome to retrieve information about collective microbiota function as opposed to taxonomy only. As microbiota metabolism is thought to be more conserved between individuals than taxa (Visconti et al., 2019), this approach may lead to more precise findings and shed more light on the metabolic changes occurring during offspring development in utero.

Microbiota modification by probiotic treatment or other methods may also aid directly in preventing excessive gestational weight gain and improving the maternal metabolic response to a dietary intervention. Turnbaugh et al. (2006) introduced the concept of an “obese-type” gut microbiome, the metabolic potential of which may account for why some

people find it difficult to lose weight. By extension, gut microbes associated with obesity could be targets for therapeutic intervention. In another study, faecal metagenomic analysis distinguished individuals who were either susceptible or resistant to a dietary weight-loss intervention (Diener et al., 2021). Differentially expressed microbial genes included an increase in starch-degrading amylases in those resistant to weight loss, which was independent of baseline BMI. Taken together, gut microbiota have the potential to modify energy harvest in humans. While there is some evidence to suggest that probiotic treatment can help with weight loss in humans (Álvarez-Arraño and Martín-Peláez, 2021), it is unclear whether probiotics are more or less effective than increasing daily fibre intake, which has also been shown to result in clinically-significant weight loss in humans (Ma et al., 2015).

Overall, dietary changes as recommended by a dietitian or other health professional are likely to be an appropriate first-line measure for women seeking to improve their metabolic health prior to, during, and after pregnancy. However, based on the results in this thesis and in line with current literature, direct modification of the maternal microbiota may also be appropriate to mitigate the risk of metabolic and/or mood disorders in the offspring. For example, in cases of low adherence to lifestyle interventions, antibiotic usage, or where dietary changes are not feasible during pregnancy, probiotic treatment may be warranted. Clinical research in this area should be encouraged to assess the efficacy of such an intervention. Alternatively, in the case that metagenomic or metabolomic analysis of maternal blood or faeces reveals a mediatory role of specific microbial metabolites in promoting normal offspring neurodevelopment [as has been demonstrated in rodents (Vuong et al., 2020)], future longitudinal studies are now warranted, to determine whether neuroactive metabolites in isolation can exert beneficial effects on offspring behaviour when administered during the perinatal period.

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Appendix A: Description of maternal care behaviours

Behaviour	Description
Nursing (passive)	Pups are suckling from dam, but dam has low arch in back or is lying on side.
Nursing (arched/active)	Pups are suckling from dam, with the dam having a high arch in her back.
Licking/grooming	Dam is licking pups.
Self-grooming	Dam is licking herself.
Nest-building	Dam is actively retrieving pieces of bedding to build the nest or shredding material to build the nest.
Eating/drinking	Dam is eating or drinking for herself.
Climbing	Dam is climbing around the bars of her cage.
Away from the nest	Dam is off the nest and is passive; not engaging in other behaviours.

Appendix B: Assessment of technical variation using tissue extraction method for NMR metabolomics

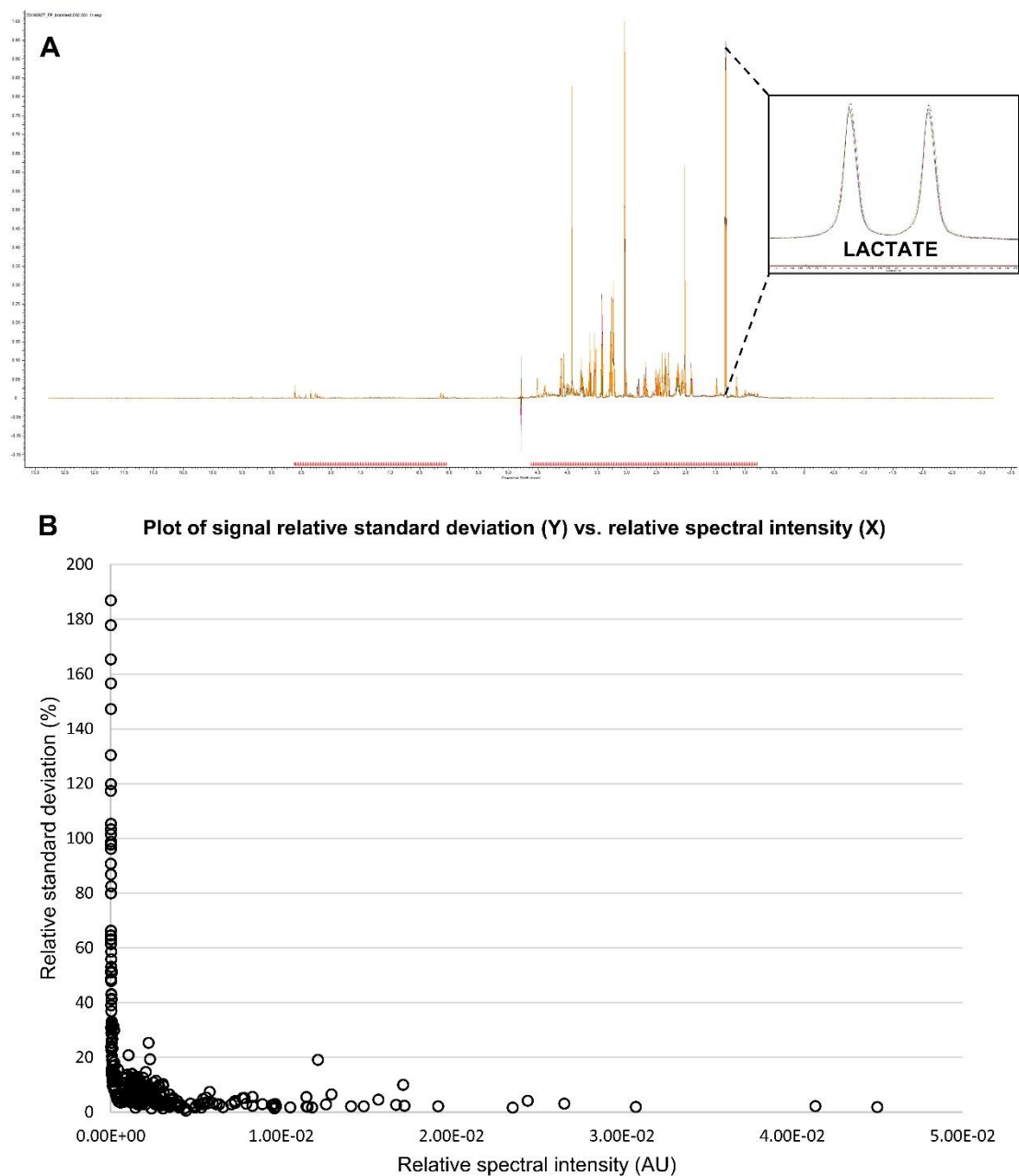


Figure B-1 Brain tissue extraction using all bins (including noise regions). **A)** Depiction of overlaid brain NMR spectra, including an insert to show the lactate doublet at 1.33ppm with very minimal technical variation in intensity. The red underline shows which regions of the spectra were included in the analysis. **B)** Plot of signal intensity against signal relative standard deviation (RSD) for each bin, indicating that only the smallest (noise) bins have an RSD >20%. N = 8 technical replicates.

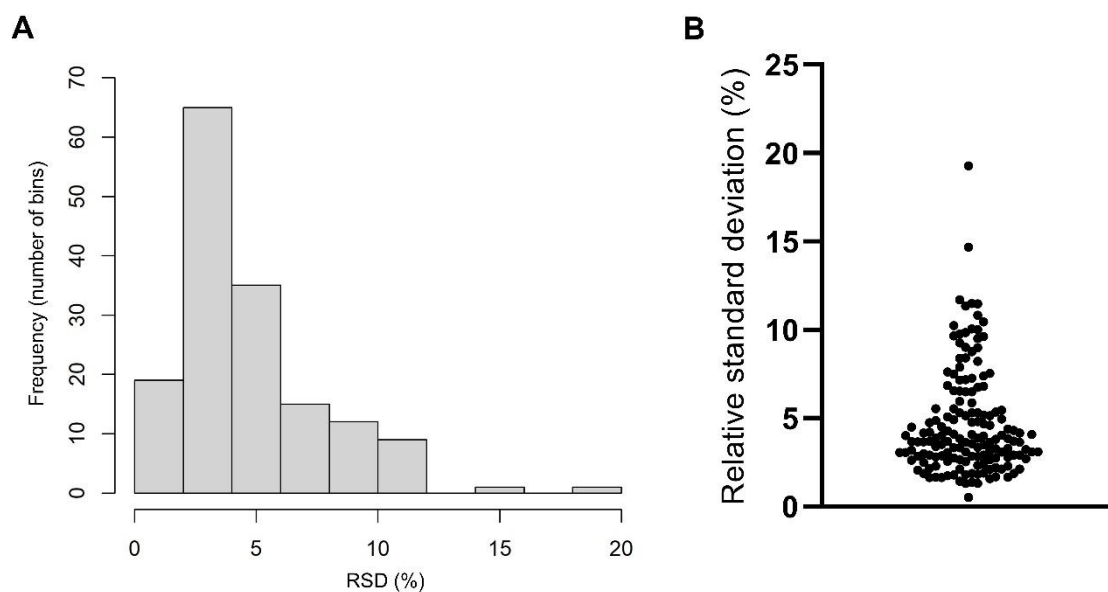


Figure B-2 Brain tissue extraction excluding noise regions. **A)** Histogram plotting the number of bins by relative standard deviation (RSD). All bins containing peaks fell within the pre-specified acceptable threshold of 0-20% RSD. **B)** Boxplot of RSD, indicating that most bins had an RSD<5%, and almost all bins had an RSD<10%. N = 8 technical replicates.

Appendix C NAFLD structured questionnaire

 Queensland Government Princess Alexandra Hospital NAFLD Assessment Visit 1	(Affix identification label here) URN: Family name: Given name(s): Address: Date of birth: Sex: ☐ M ☐ F
NAFLD Study ID	
Date Visit 1	/ /
General History	Referred: ☐ IPC ☐ PAH Diabetes ☐ Other: Previous Scores: NAFLD Fibrosis Score: _____ Fib 4: Risk Group: ☐ Low ☐ Indeterminate ☐ High Date of NAFLD Diagnosis: ____ / ____ / ____ ☐ Allergies:
Social	Country of Origin: Race: ☐ Caucasian Interpreter Required Yes ☐ No ☐ ☐ African Language: ☐ Asian ☐ ATSI ☐ Pacific Islander ☐ Other: Partner: Dependants: Social Supports (describe): Living Arrangements: ☐ Alone ☐ Partner ☐ Share accommodation ☐ with Dependents ☐ Unknown Pets: Hobbies/interests: Occupation: Hours per week: Centrelink benefits: No. of full years completed at school: Tertiary study: ☐ Nil ☐ Certificate ☐ Diploma ☐ Degree ☐ Post Graduate Qualification
Vitals	BP: Height: Pulse: Weight: Respirations: BMI: Temperature: Girth:
Weight History	Heaviest Weight: Weight in 2010: Lightest Adult Weight: Weight in 2000:
Metabolic Risks	☐ Obesity ☐ T2 Diabetes ☐ HTN ☐ Dyslipidaemia

Treatment for NAFLD so far:	☐ Nil ☐ Dietician (Community) ☐ Dietician (Hospital) ☐ Medication _____ ☐ Complementary Practitioner ☐ OTC / Herbal ☐ Other:					
Med. Diagnoses	☐ IHD ☐ Thyroid Disease ☐ COPD ☐ Psoriasis ☐ CKD ☐ Asthma ☐ OSA ☐ Malignancy ☐ Other:					
Drug & Alcohol	Current (last 3 mths):		Past:			
	Alcohol		Alcohol			
	Tobacco ____cigs /day x ____yrs		Tobacco ____cigs /day x ____yrs			
	Recreational Drugs		Recreational Drugs			
	Analgesia		Analgesia			
	Other:		Other:			
	Audit Questionnaire Completed: Yes ☐ No ☐					
	Audit Questionnaire Result:					
Mental Health	☐ Depression - on medication Yes ☐ No ☐ ☐ Anxiety - on medication Yes ☐ No ☐ ☐ Other: Problems in last 12 months? Yes ☐ No ☐ Admitted to Hospital? Yes ☐ No ☐ Previous / current major stressors: Previous / current coping strategies: Effectiveness of strategies: Psychiatrist: Yes ☐ No ☐ Name: Psychologist / Counsellor: Yes ☐ No ☐ Name:					
Medications (Including OTC / Herbal)	Medication Name	Dose	Route	Frequency	Start Date	End Date
Imaging (most recent)	☐ Abdominal Ultrasound – Date: ____ / ____ / ____ Where? ☐ Abdominal CT Scan – Date: ____ / ____ / ____ Where? ☐ Abdominal MRI – Date: ____ / ____ / ____ Where? ☐ Other:					
Imaging Result	☐ Hepatomegaly (size): ☐ Splenomegaly (size): ☐ Varices ☐ Nodular / irregular liver surface					

	Description:
Physical Examination	Digestive / Liver:
	Cardiovascular:
	Respiratory:
	Endocrine:
	Muscular:
	Skeletal:
	Lymph nodes:
	Nervous system:
	Malignancy (Type and Date):

Screening Bloods (Fasting)	Creat.		Free T4		HIV	
	eGFR		TSH		HBsAg	
	Protein		Ceruloplasmin		HBsAb	
	Albumin		AFP		HBcAb	
	Bilirubin		α1AT		HCV Ab	
	Bili (conj)		α1AT Genotype		HCV RNA	
	ALP		Transferrin Sat.		ANA	
	GGT		Ferritin		SMA	
	ALT		Hb		AMA	
	AST		Plt		Cholesterol	
	CK		WBC		Triglyceride	
	Glucose		MCV		HDL	
	HBA1C		INR		LDL	
	C-Peptide		PT			
	Insulin					
	Research Bloods: Yes ☐ No ☐ Serum Tubes _____ EDTA Tubes _____					
	Research Bloods collected by: ☐ Path QLD ☐ Study staff					
Post Visit 1 Referrals	Ultrasound: Yes ☐ No ☐					
	Fibroscan: Yes ☐ No ☐					
	Dietician: Yes ☐ No ☐					
	Psych: Yes ☐ No ☐					
	ECG: Yes ☐ No ☐					
Patient Education attended: Yes ☐ No ☐						
Fatty Liver Disease Flyer Provided: Yes ☐ No ☐						
Follow up appointment made:						
Letter to GP: Yes ☐ No ☐						
Letter to Endocrine: Yes ☐ No ☐						

Appendix D PCA and PLS-DA matrices for models with >2 principal/predictive components

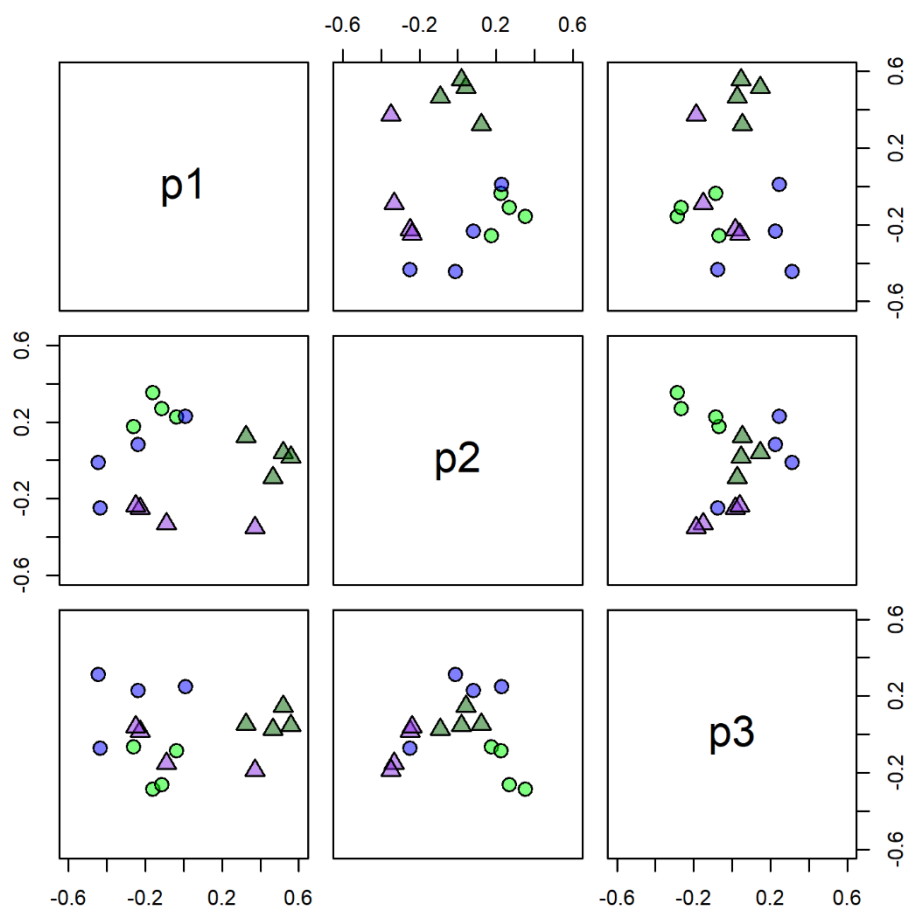


Figure D-1 Additional scores plot of the partial least squares discriminatory analysis (PLS-DA) of chronic HFD and perinatal probiotic treatment on the F0 (maternal) faecal metabolome. The third predictive component depicts increased CD/Veh and HFD/Veh group separation. The R^2Y of PC3 was 0.21 and the Q^2 was 0.070. $N = 4$ per group.

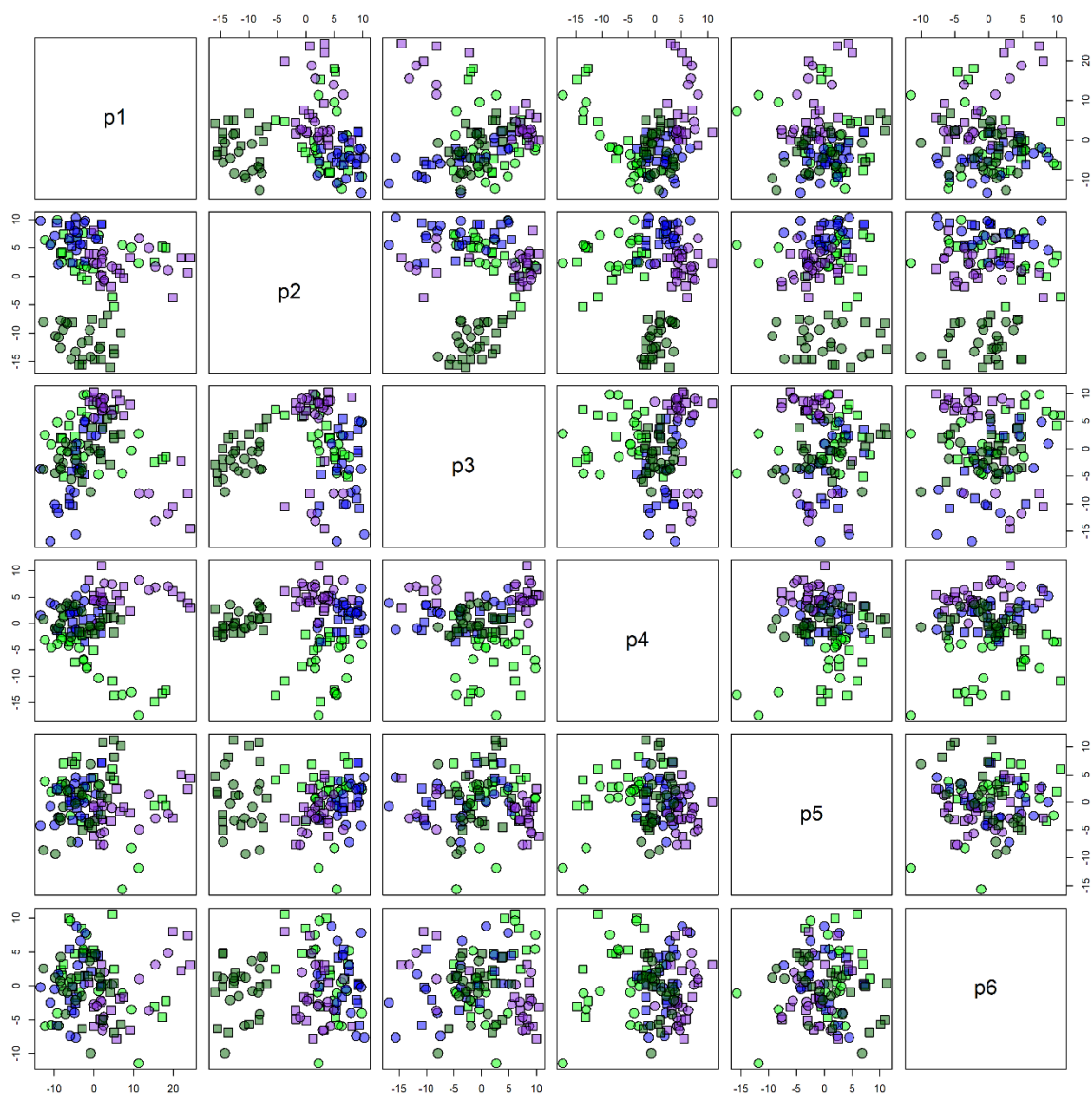


Figure D-2 Matrix of all principal components from the unsupervised principal component analysis (PCA) of the juvenile offspring faecal, liver, plasma, and brain metabolome. The PCA scores plot shows that CD/Veh male and female juvenile offspring separate from all other offspring in PC4. The R^2X of PC1-6 were 0.14, 0.12, 0.090, 0.065, 0.047, and 0.045, respectively. N = 13-15 per sex per group.

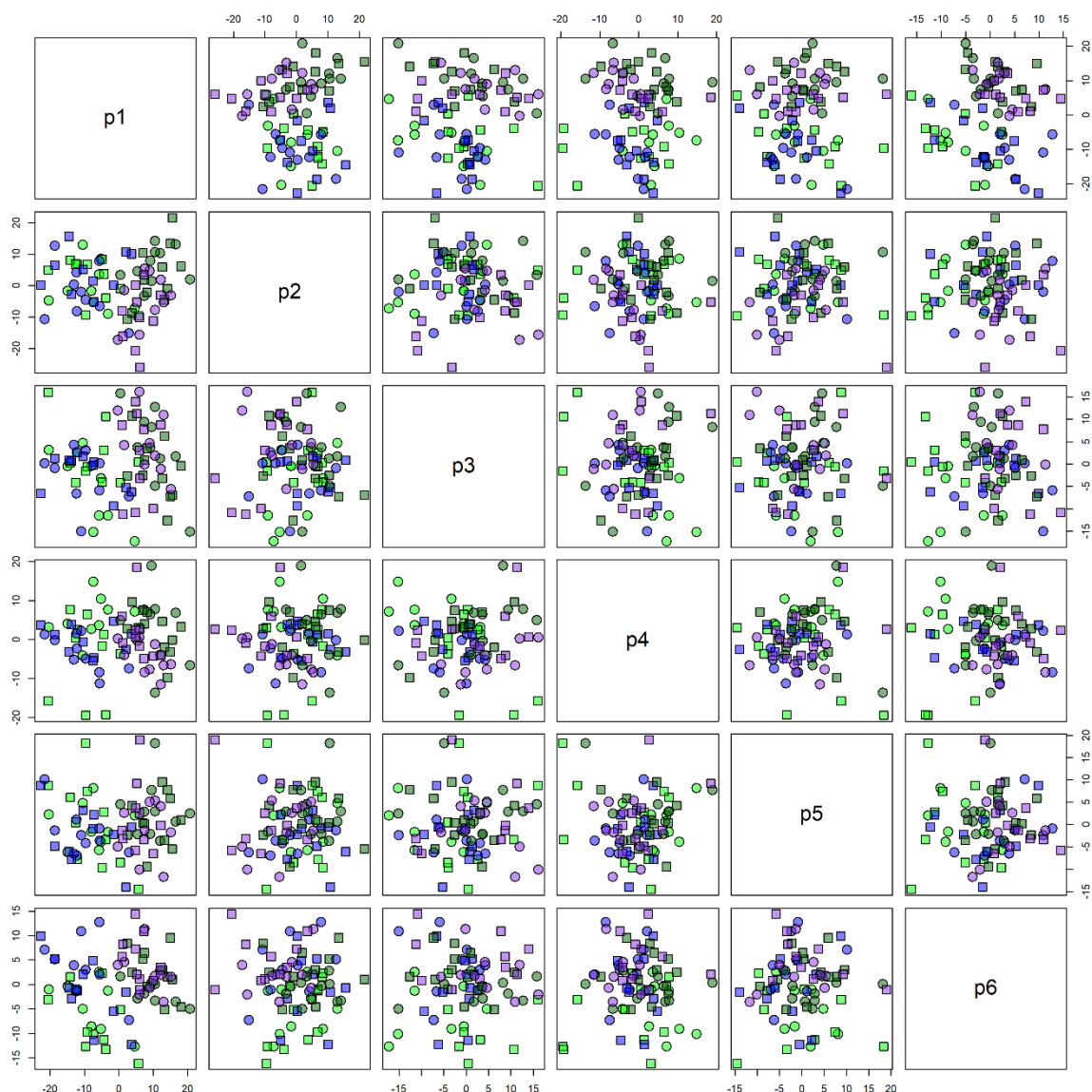


Figure D-3 Matrix of all principal components from the unsupervised principal component analysis (PCA) of the adult offspring faecal, liver, plasma, and brain metabolome. The PCA scores plot shows that while no unsupervised separation between CD/Veh and HFD/Veh adult offspring was evident in the first two principal components, modest separation between these groups can be observed in PC6. The R^2X of PC1-6 were 0.16, 0.11, 0.081, 0.071, 0.062, and 0.057, respectively. N = 9-11 per sex per group.

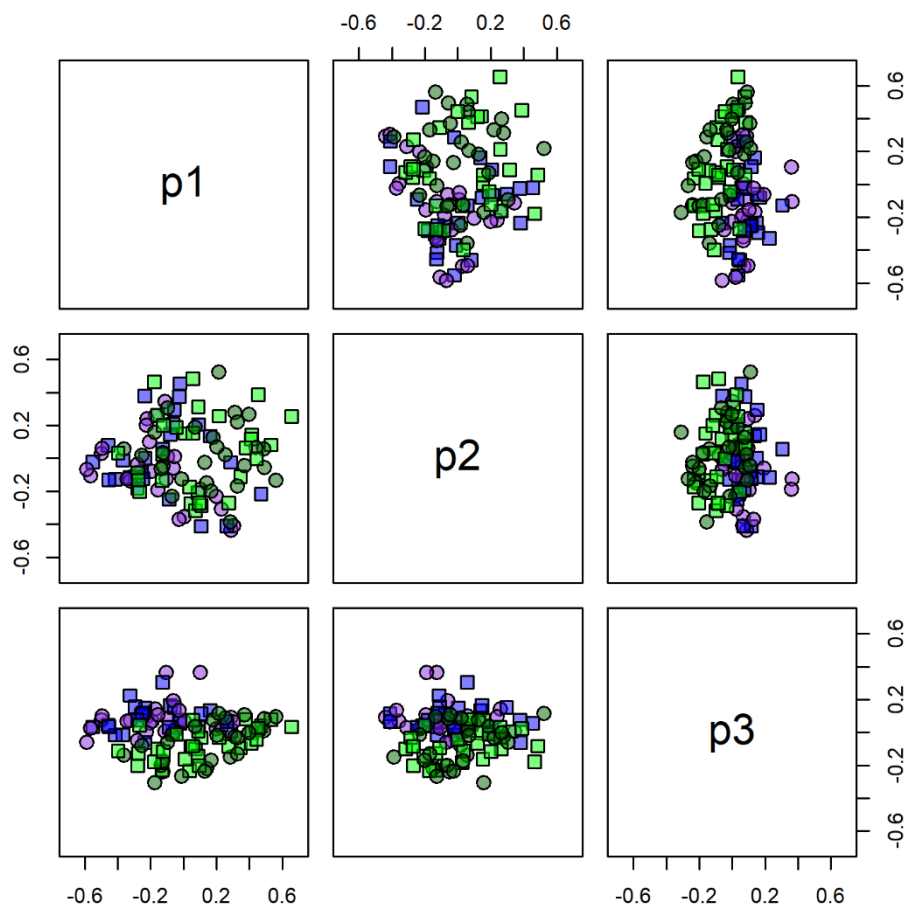


Figure D-4 Matrix of all predictive components from the partial least squares discriminatory analysis (PLS-DA) of the juvenile offspring faecal metabolome. The scores plot shows additional, modest separation between offspring of obese and lean dams in the third predictive component (PC3). The R^2Y of PC3 was 0.088 and the Q^2 was 0.048. $N = 13-15$ per sex per group.

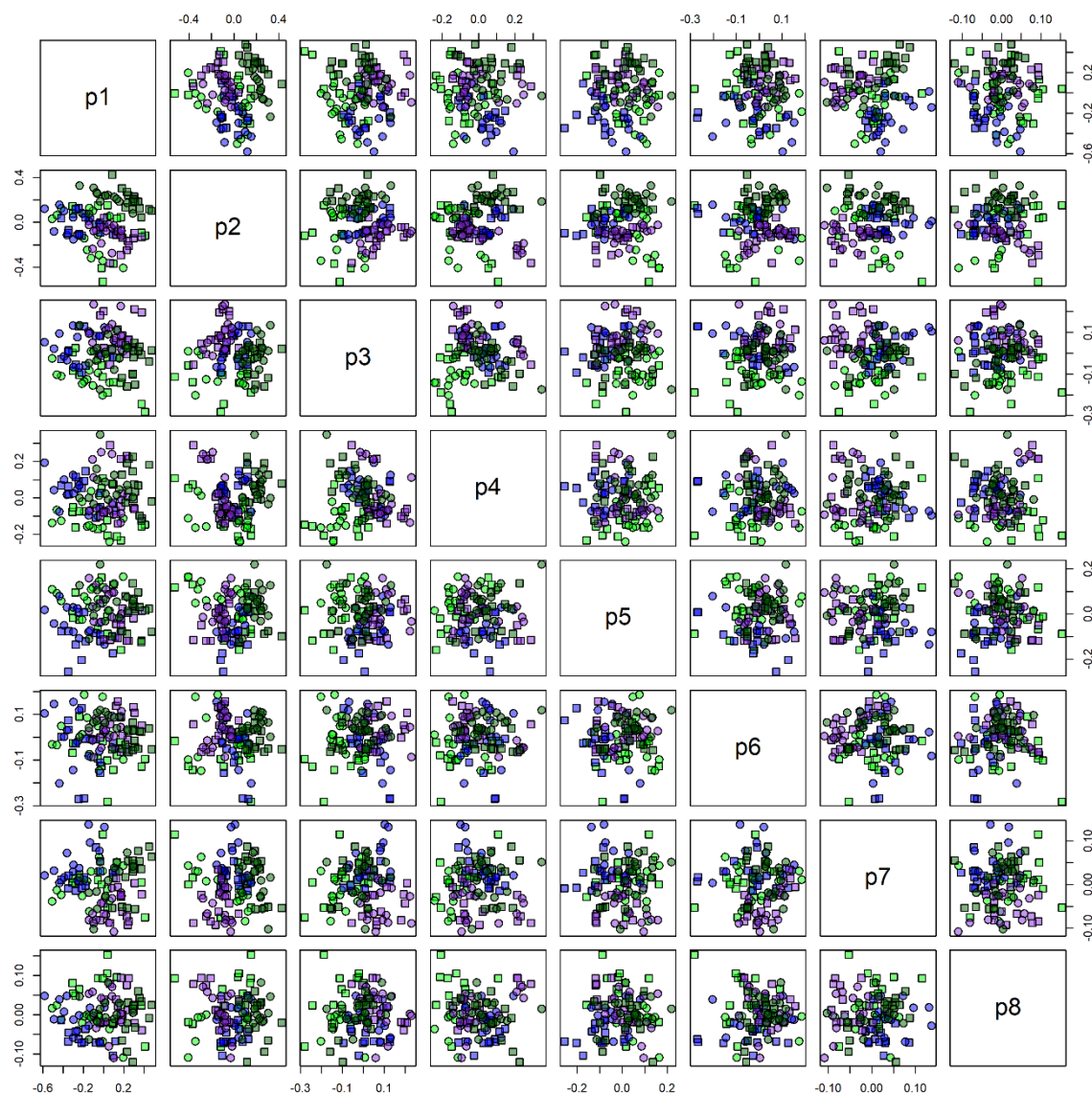


Figure D-5 Matrix of all predictive components from the partial least squares discriminatory analysis (PLS-DA) of the juvenile offspring liver metabolome. Whereas CD/Veh offspring show predominant within-group variation across the first two predictive components, they can be seen to cluster together separately from the other juvenile offspring in the in the third and fourth predictive components (PC3 and PC4). The R^2Y of PC1-8 were 0.090, 0.065, 0.053, 0.043, 0.044, 0.046, 0.053, and 0.048, respectively. The Q^2 of PC1-8 were 0.071, 0.049, 0.017, 0.026, 0.026, 0.041, 0.021, and 0.026, respectively. N = 13-15 per sex per group.

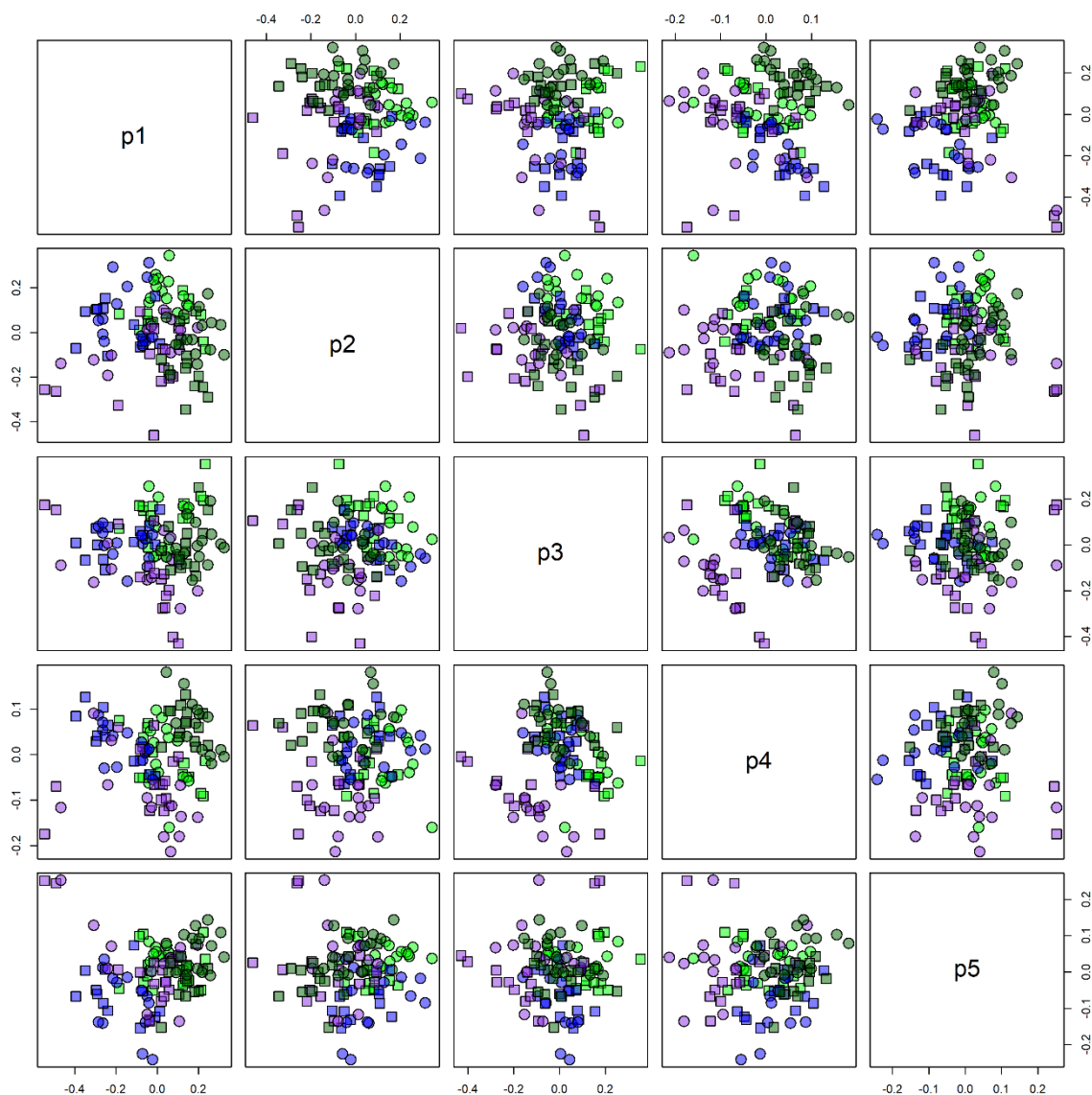


Figure D-6 Matrix of all predictive components from the partial least squares discriminatory analysis (PLS-DA) of the juvenile offspring plasma metabolome. The R^2Y of PC1-5 were 0.088, 0.050, 0.075, 0.063, and 0.030, respectively. The Q^2 of PC1-5 were 0.049, 0.034, 0.054, 0.031, and 0.018, respectively. $N = 13-15$ per sex per group.

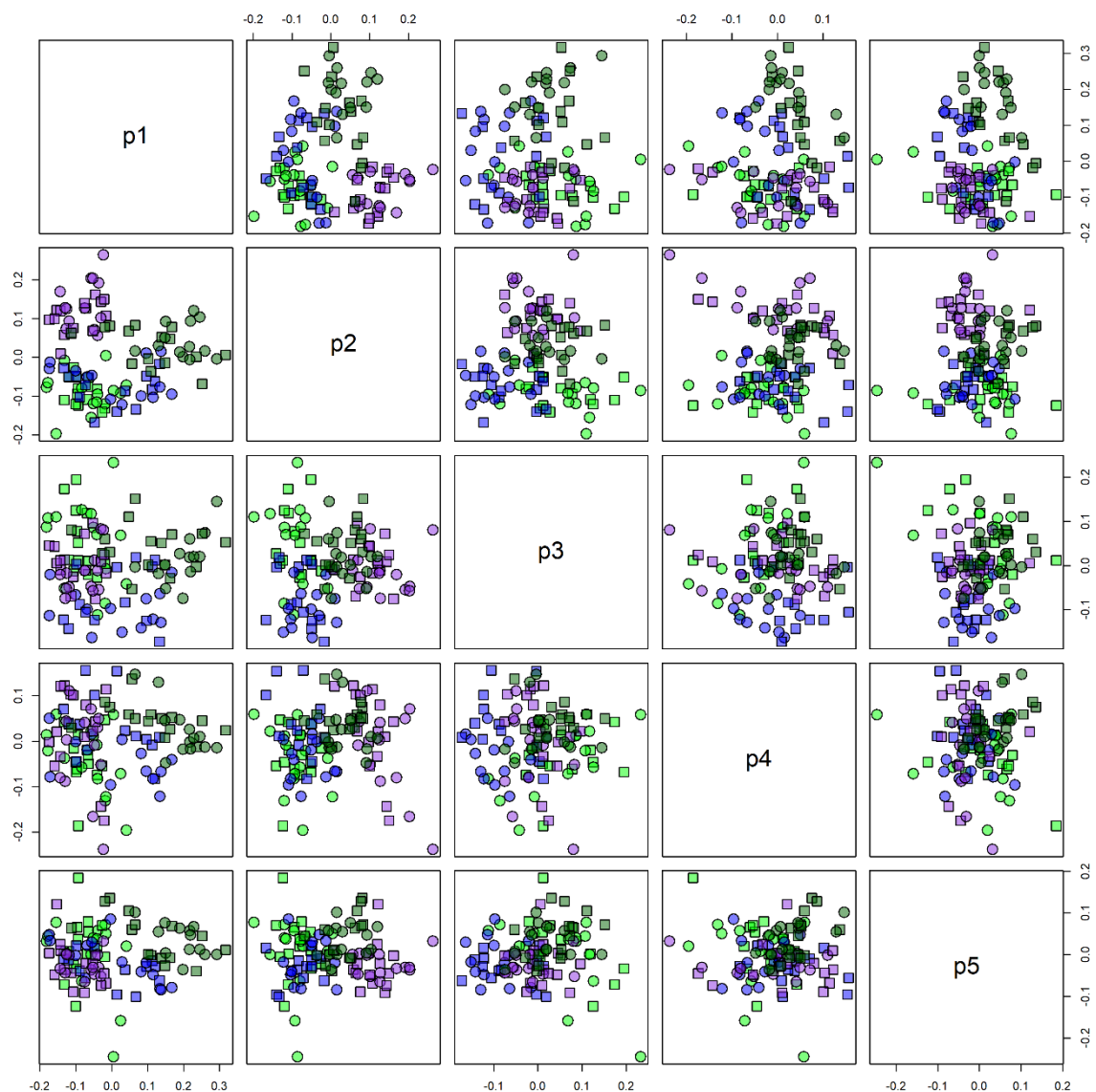


Figure D-7 Matrix of all predictive components from the partial least squares discriminatory analysis (PLS-DA) of the juvenile offspring brain metabolome. Separation between CD/Veh and HFD/Veh offspring, which is not clear in the first two predictive components, can be observed in the third predictive component (PC3). The R^2Y of PC1-5 were 0.088, 0.11, 0.060, 0.030, and 0.027, respectively. The Q^2 of PC1-5 were 0.059, 0.083, 0.040, 0.011, and 0.0069, respectively. N = 13-15 per sex per group.

Appendix E Summary of PFC gene expression

Table E-1 Summary of prefrontal cortex gene expression in the juvenile offspring

	FEMALE JUVENILE OFFSPRING				MALE JUVENILE OFFSPRING			
	Vehicle		Probiotic		Vehicle		Probiotic	
	Control Diet	High Fat Diet	Control Diet	High Fat Diet	Control Diet	High Fat Diet	Control Diet	High Fat Diet
<i>GLUN1</i>	1.00±0.65	1.42±0.47	0.85±0.09	0.95±0.33	1.00±0.09	1.00±0.30	0.55±0.29	1.00±0.55
<i>GLUN2A</i>	1.00±0.31	0.88±0.22	0.67±0.13[#]	0.66±0.17[#]	1.00±0.23	0.85±0.10	0.60±0.28[#]	0.90±0.19[#]
<i>GLUN2B</i>	1.00±0.21	0.86±0.13	0.43±0.06[#]	0.60±0.23[#]	1.00±0.25	0.97±0.15	0.76±0.13	0.91±0.13
<i>GLUN2C</i>	1.00±0.15	2.09±0.30**	1.28±0.42	1.24±0.41	1.00±0.36	0.99±0.35	0.62±0.30	0.92±0.23
<i>PSD-95</i>	1.00±0.08	1.14±0.07	0.98±0.12	0.95±0.07	1.00±0.11	1.03±0.09	0.90±0.11	0.96±0.09
<i>5-HT1A</i>	1.00±0.36	1.60±0.51	0.88±0.25	1.30±0.61	1.00±0.41	0.93±0.53	0.75±0.21	0.86±0.48
<i>5-HT2A</i>	1.00±0.25	1.15±0.32	0.85±0.21	0.92±0.21	1.00±0.44	0.64±0.24	0.55±0.15	0.66±0.33
<i>5-HT6</i>	1.00±0.27	1.27±0.26	0.68±0.17	0.84±0.07	1.00±0.48	0.80±0.26	0.87±0.43	1.02±0.46
<i>BDNF</i>	1.00±0.19	1.23±0.40	1.19±0.54	1.16±0.28	1.00±0.40	0.99±0.62	0.95±0.14	1.16±0.50
<i>CREB1</i>	1.00±0.15	1.03±0.26	1.56±0.37	1.05±0.06	1.00±0.20	0.98±0.08	1.33±0.10[#]	1.10±0.06[#]
<i>SYP</i>	1.00±0.10	1.06±0.07	0.99±0.11	0.99±0.08	1.00±0.04	1.02±0.07	0.85±0.11[#]	0.92±0.07[#]
<i>GSK3B</i>	1.00±0.15	0.86±0.27	1.28±0.27	0.84±0.14	1.00±0.18	0.86±0.08	1.28±0.12	1.02±0.20
<i>ZIF-268</i>	1.00±0.16	1.17±0.35[†]	0.60±0.10	1.26±0.08[†]	1.00±0.12	1.31±0.23****	0.67±0.14	1.61±0.15****
<i>cFOS</i>	1.00±0.49	0.88±0.31	0.67±0.11	1.05±0.49	1.00±0.47	1.47±0.56	1.03±0.25	1.50±0.44
<i>ΔFOSB</i>	1.00±0.08	0.82±0.22	1.38±0.63	1.36±0.29	1.00±0.32	1.09±0.54	1.94±0.42	1.65±0.69
<i>ATP1A2</i>	1.00±0.20	1.06±0.12	0.97±0.19	0.82±0.09	1.00±0.12	1.08±0.08	1.02±0.13	0.86±0.10
<i>PFKFB3</i>	1.00±0.09	1.03±0.12	1.21±0.20[#]	1.11±0.18[#]	1.00±0.05	1.04±0.08	1.32±0.12[#]	1.28±0.25[#]

Notes: Data presented as mean±SD, relative expression compared to respective female or male vehicle/Control Diet offspring. N = 6 per group for 2-way ANOVA for each sex, or N = 12 per group if sex consolidated into a single 2-way ANOVA where no sex differences. [†]p < 0.001, indicating main effect of maternal diet. [#]p < 0.001 indicating main effect of maternal probiotic intake. *p < 0.001 indicating significant post-hoc comparison if a significant diet x probiotic interaction was identified. Significance was adjusted for multiple testing using the Bonferroni method.

Table E-2 Summary of prefrontal cortex gene expression in the adult offspring

	FEMALE ADULT OFFSPRING				MALE ADULT OFFSPRING			
	Vehicle		Probiotic		Vehicle		Probiotic	
	Control Diet	High Fat Diet	Control Diet	High Fat Diet	Control Diet	High Fat Diet	Control Diet	High Fat Diet
<i>GLUN1</i>	1.00±0.10	0.91±0.10	0.95±0.06	0.95±0.10	1.00±0.11	1.05±0.15	1.24±0.10	1.06±0.10
<i>GLUN2A</i>	1.00±0.05	0.80±0.06	0.80±0.20	0.86±0.14	1.00±0.15	0.93±0.16	1.13±0.13	0.97±0.15
<i>GLUN2B</i>	1.00±0.19	0.88±0.10	1.01±0.09	0.90±0.14	1.00±0.20	1.07±0.22	1.22±0.16	1.06±0.15
<i>GLUN2C</i>	1.00±0.15	1.06±0.06	0.98±0.07	0.89±0.08	1.00±0.10	1.07±0.08	1.16±0.19	1.10±0.07
<i>PSD-95</i>	1.00±0.20	1.01±0.34	1.06±0.55	0.83±0.35	1.00±0.25	1.06±0.10	1.15±0.56	0.78±0.23
<i>5-HT1A</i>	1.00±0.23	0.78±0.09	0.76±0.11	0.63±0.11	1.00±0.22	0.93±0.20	0.85±0.36	0.78±0.18
<i>5-HT2A</i>	1.00±0.24	0.96±0.12	0.93±0.21	0.91±0.11	1.00±0.15	0.98±0.13	1.18±0.15	1.16±0.10
<i>5-HT6</i>	1.00±0.25	0.82±0.09	1.00±0.16	0.93±0.17	1.00±0.20	1.14±0.16	1.40±0.18	1.33±0.27
<i>BDNF</i>	1.00±0.32	0.75±0.31	1.67±0.34[#]	1.54±0.47[#]	1.00±0.15	0.68±0.19	1.49±0.30[#]	1.83±0.38[#]
<i>CREB1</i>	1.00±0.11	0.77±0.09	0.89±0.13	1.03±0.20	1.00±0.20	0.53±0.12	0.82±0.21	0.83±0.22
<i>SYP</i>	1.00±0.15	0.80±0.23[†]	0.87±0.14	0.78±0.16[†]	1.00±0.20	0.73±0.21[†]	0.82±0.09	0.71±0.08[†]
<i>GSK3B</i>	1.00±0.13	0.88±0.09	0.80±0.12	0.84±0.13	1.00±0.24	0.71±0.13	0.76±0.27	0.69±0.10
<i>ZIF-268</i>	1.00±0.50	0.73±0.36	1.17±0.18	0.87±0.29	1.00±0.45	1.62±0.58	3.58±0.99[#]	3.07±1.13[#]
<i>cFOS</i>	1.00±0.24	0.77±0.16	1.05±0.23	1.61±0.67	1.00±0.86	1.41±1.01	2.94±1.22[#]	3.72±1.03[#]
<i>ΔFOSB</i>	1.00±0.28	0.70±0.22	1.55±0.13[#]	1.42±0.37[#]	1.00±0.25	0.91±0.24	1.75±0.78[#]	2.01±0.71[#]
<i>ATP1A2</i>	1.00±0.26	0.89±0.25	0.91±0.12	0.94±0.21	1.00±0.20	0.78±0.18	0.91±0.27	0.70±0.18
<i>PFKFB3</i>	1.00±0.15	0.65±0.17	1.27±0.48[#]	1.28±0.37[#]	1.00±0.24	0.77±0.16	1.75±0.37[#]	1.44±0.42[#]

Notes: Data presented as mean±SD, relative expression compared to respective female or male vehicle/Control Diet offspring. N = 6 per group for 2-way ANOVA for each sex, or N = 12 per group if sex consolidated into a single 2-way ANOVA where no sex differences. [†]p < 0.001, indicating main effect of maternal diet. [#]p < 0.001 indicating main effect of maternal probiotic intake. *p < 0.001 indicating significant post-hoc comparison. Significance was adjusted for multiple testing using the Bonferroni method.

Appendix F F1 (offspring) metabolome sex differences

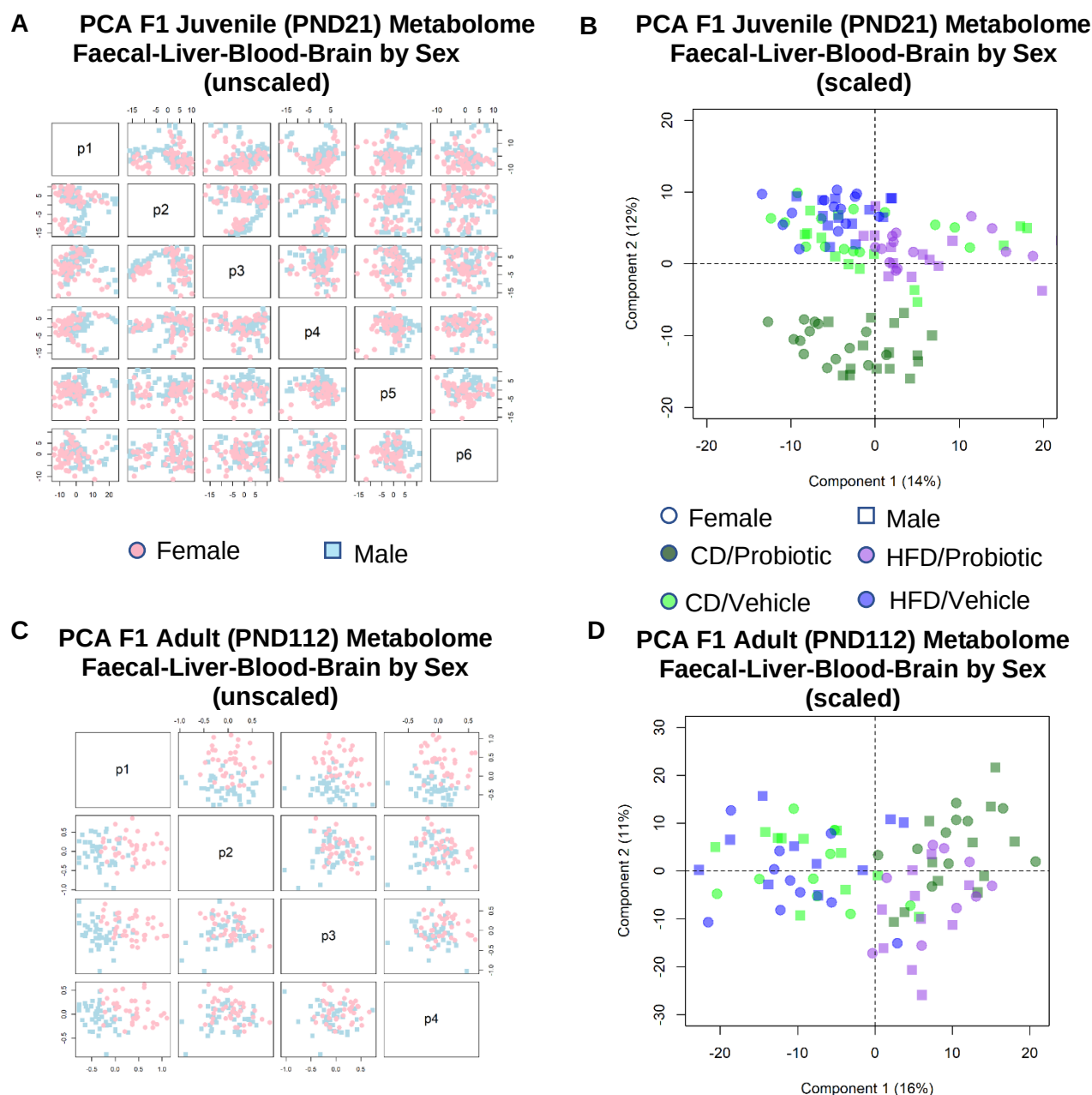


Figure F-1 Integrated principal component analysis (PCA) of the offspring faecal, liver, blood, and brain metabolome. **A)** PCA at PND21 ($n=111$) where male and female offspring are not independently z-scaled. Some clustering is observed in PC5, explaining $<5\%$ of total variance. Overall, very minimal clustering by sex is evident. Cumulative $R^2X = 0.51$. **B)** In the same mice where male and female offspring are independently z-scaled, the effect of each treatment (maternal obesity, maternal probiotic intake) affects males and females in the same way compared to the controls. $R^2X = 0.51$ with 6 predictive components. **C)** PCA at PND112 ($n=80$) where male and female offspring are not independently z-scaled. Strong sex-effects are observed in PC1, explaining 24% of total variance. Cumulative $R^2X = 0.57$. **D)** In the same mice where male and female adult offspring are independently z-scaled, the effect of each treatment (maternal obesity, maternal probiotic intake) affects males and females in the same way compared to the controls. $R^2X = 0.55$ with 6 predictive components.

Appendix G Summary of F1 adult metabolite changes that were identified as key metabolites in the juvenile offspring but not the adult offspring.

		AOV main effect			AOV post-hoc analysis					
F1 PND112	Metabolite	mHFD v. mCD	Probiotic v. Vehicle	Int.	CD/ Veh	HFD/ Veh	CD/ Pro	HFD/ Pro	VIP score	NMR ppm
FAECAL	Ethanol	-	-	**		↑*	↑**		0.80	1.19
	Glucose	ns	↑*	ns					1.00	3.84
	Succinate	ns	↓*	ns					1.35	2.41
PLASMA	3-hydroxybutyric acid	ns	↑**	ns					1.08	1.20
	Valine	ns	↑*	ns					0.61	1.00
BRAIN	Creatine	ns	ns	ns					0.86	3.04
	Alanine	ns	↑*	ns					0.81	1.48
	Glutamine	ns	↑**	ns					1.35	2.46
	3-hydroxybutyric acid	↓**	ns	ns					0.80	1.20

*q < 0.05, **q < 0.01, ***q < 0.001, ****q < 0.0001 adjusted for false discovery using the Benjamini and Hochberg method. Int = interaction; VIP = variable importance in projection; AOV = analysis of variance, ns = not significant. Blue and orange arrows represent significant changes in females and males only, respectively.

Appendix H Rodent mRNA and metabolite correlations

Table H-1 F1 (PND21) juvenile Spearman correlation analysis between prefrontal cortex gene expression and brain metabolites altered by maternal probiotic intake or obesity.

Brain Metabolite								
Brain (PFC) Gene	Lactate	Glutamate	Alanine	Creatine	Acetate	3-hydroxybutyric acid	Cholesterol/TG	Glutamine
GLUN2A	r = -0.35 q = 0.030	r = -0.27 q = 0.22	r = -0.26 q = 0.13	r = -0.20 q = 0.47	r = 0.10 q = 0.59	r = 0.37 q = 0.019	r = 0.14 q = 0.61	r = 0.30 q = 0.15
GLUN2B	r = -0.50 q = 0.0032	r = -0.26 q = 0.22	r = -0.26 q = 0.13	r = -0.21 q = 0.47	r = 0.035 q = 0.82	r = 0.42 q = 0.0083	r = 0.46 q = 0.0096	r = 0.13 q = 0.62
<i>GLUN2C</i>	r = -0.023 q = 0.88	r = -0.11 q = 0.51	r = -0.042 q = 0.78	r = -0.13 q = 0.55	r = 0.14 q = 0.54	r = 0.22 q = 0.18	r = 0.091 q = 0.63	r = -0.32 q = 0.15
PFKFB3	r = 0.40 q = 0.014	r = 0.13 q = 0.51	r = 0.26 q = 0.13	r = 0.068 q = 0.74	r = 0.12 q = 0.57	r = -0.17 q = 0.25	r = -0.13 q = 0.61	r = 0.046 q = 0.86
SYP	r = -0.40 q = 0.014	r = -0.10 q = 0.51	r = -0.22 q = 0.18	r = -0.16 q = 0.54	r = -0.16 q = 0.54	r = 0.21 q = 0.18	r = 0.11 q = 0.61	r = 0.01 q = 0.99
CREB1	r = 0.38 q = 0.032	r = -0.068 q = 0.51	r = -0.096 q = 0.59	-0.022 q = 0.77	r = -0.23 q = 0.48	r = -0.57 q = 0.0001	r = -0.26 q = 0.22	r = 0.10 q = 0.66
ZIF-268	r = -0.054 q = 0.88	r = 0.43 q = 0.020	r = 0.43 q = 0.012	-0.30 q = 0.41	r = 0.51 q = 0.0024	r = 0.55 q = 0.0001	r = 0.42 q = 0.013	r = -0.28 q = 0.16
IL-6	r = 0.14 q = 0.45	r = 0.20 q = 0.36	r = 0.44 q = 0.012	r = -0.13 q = 0.55	r = 0.16 q = 0.54	r = -0.26 q = 0.13	r = 0.017 q = 0.91	r = -0.17 q = 0.50

N = 48 per correlation analysis (24 female, 24 male).

Table H-2 F1 (PND21) juvenile Spearman correlation analysis between prefrontal cortex gene expression and faecal metabolites altered by maternal probiotic intake

	Faecal Metabolite		
Brain (PFC) Gene	Acetate	Propionate	Butyrate
<i>GLUN2A</i>	$r = -0.25$ $q = 0.23$	$r = -0.35$ $q = 0.028$	$r = -0.30$ $q = 0.064$
<i>GLUN2B</i>	$r = -0.22$ $q = 0.27$	$r = -0.43$ $q = 0.0056$	$r = -0.24$ $q = 0.12$
<i>GLUN2C</i>	$r = -0.19$ $q = 0.28$	$r = -0.17$ $q = 0.35$	$r = -0.34$ $q = 0.043$
<i>PFKFB3</i>	$r = 0.38$ $q = 0.034$	$r = 0.53$ $q = 0.0008$	$r = 0.44$ $q = 0.012$
<i>SYP</i>	$r = -0.19$ $q = 0.28$	$r = -0.31$ $q = 0.053$	$r = -0.37$ $q = 0.028$
<i>CREB1</i>	0.36 $q = 0.046$	0.38 $q = 0.019$	$r = 0.28$ $q = 0.10$
<i>ZIF-268</i>	0.10 $q = 0.49$	$r = -0.14$ $q = 0.46$	$r = 0.010$ $q = 0.97$
<i>IL-6</i>	$r = 0.10$ $q = 0.49$	$r = 0.056$ $q = 0.70$	$r = 0.25$ $q = 0.12$

N = 48 per correlation analysis (24 female, 24 male).

Table H-3 F1 (PND112) adult Spearman correlation analysis between prefrontal cortex gene expression and brain metabolites

	Brain Metabolite			
Brain (PFC) Gene	Lactate	Glutamate	Acetate	Cholesterol/TG
<i>PFKFB3</i>	$r = 0.36$ $q = 0.056$	$r = 0.16$ $q = 0.30$	$r = 0.29$ $q = 0.10$	-0.25 $q = 0.25$
<i>SYP</i>	$r = -0.01$ $q = 0.63$	$r = -0.086$ $q = 0.45$	$r = -0.36$ $q = 0.056$	$r = 0.12$ $q = 0.60$
<i>ZIF-268</i>	$r = 0.27$ $q = 0.15$	$r = -0.086$ $q = 0.45$	$r = 0.28$ $q = 0.10$	$r = -0.055$ $q = 0.71$

N = 48 per correlation analysis (24 female, 24 male).

Table H-4 F1 (PND112) adult Spearman correlation analysis between prefrontal cortex gene expression and faecal metabolites

	Faecal Metabolite		
Brain (PFC) Gene	Acetate	Propionate	Butyrate
<i>PFKFB3</i>	$r = 0.089$ $q = 0.83$	$r = 0.11$ $q = 0.71$	$r = 0.40$ $q = 0.016$
<i>SYP</i>	$r = -0.34$ $q = 0.060$	$r = -0.14$ $q = 0.71$	$r = -0.24$ $q = 0.13$
<i>ZIF-268</i>	$r = 0.027$ $q = 0.83$	$r = -0.054$ $q = 0.72$	$r = -0.25$ $q = 0.13$

N = 48 per correlation analysis (24 female, 24 male).

Appendix I Maternal plasma isoleucine levels

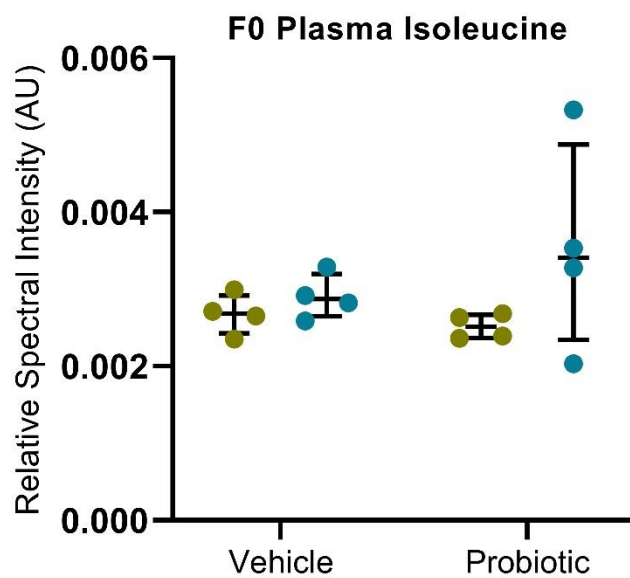


Figure I-1 Maternal (F0) plasma isoleucine levels are not significantly altered by high fat diet or perinatal probiotic treatment. However, it was noted that chronic HFD feeding numerically increased plasma isoleucine levels ($F_{(1, 12)} = 3.11$, $p = 0.10$). $N = 4$ per group.