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The Neurodevelopmental Impact of Stress and Inflammation During Pregnancy: Influence of the Maternal Microbiome and Gut Health

Thesis presented by

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for the degree of

Doctor of Philosophy

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Declaration

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

Author contributions

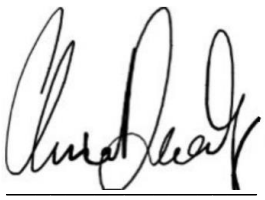
All the work conducted in this thesis was performed independently by the author with the following exceptions.

Chapter 2: Lars Wilmes carried out Z-score analysis.

Chapter 3: Jamie FitzGerald carried out bioinformatics.

Chapter 4: Jamie FitzGerald carried out bioinformatics.

Signed



Clara Deady

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Publications and presentations

Peer-reviewed publications

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Collins JM*, Keane JM*, **Deady C**, Khashan AS, McCarthy FP, O'Keeffe GW, Clarke G, Cryan JF, Caputi V, O'Mahony SM. Prenatal stress impacts foetal neurodevelopment: Temporal windows of gestational vulnerability. Neurosci Biobehav Rev. 2024 Sep;164:105793. doi: 10.1016/j.neubiorev.2024.105793. Epub 2024 Jul 5. PMID: 38971516.

Collins JM, Caputi V, Manurung S, Gross G, Fitzgerald P, Golubeva AV, Popov J, **Deady C**, Dinan TG, Cryan JF, O'Mahony SM. Supplementation with milk fat globule membrane from early life reduces maternal separation-induced visceral pain independent of enteric nervous system or intestinal permeability changes in the rat. Neuropharmacology. 2022 Jun 1;210:109026. doi: 10.1016/j.neuropharm.2022.109026. Epub 2022 Mar 10. PMID: 35283136.

Conference posters

Deady C, Mazzocchi M, Kara N, O'Mahony A, Shanahan R, Iesanu MI, McCarthy FP, McCarthy C, O'Keeffe GW, O'Mahony SM. Maternal Immune Activation and Microbiome Disruption leads to Differential Neurobehavioural Profiles in the Offspring. 2nd FinnBrain International Congress, Turku, Finland, 8th-9th June 2023.

Deady C, Mazzocchi M, Kara N, O'Mahony A, Shanahan R, Iesanu MI, McCarthy FP, McCarthy C, O'Keeffe GW, O'Mahony SM. Maternal Immune Activation and Microbiome Disruption leads to Differential Neurobehavioural Profiles in the Offspring. 36th ECNP Congress, Barcelona, Spain, 7th-10th 2023.

Deady C, Wilmes L, FP McCarthy FP, Clarke G, Keane J, McCarthy C, O'Keeffe GW, O'Mahony SM. Exposure to serum from biologically stressed pregnant women decreases neurite length in SH-SY5Y cells through a tumour necrosis factor-α-dependent mechanism. 37th ECNP Congress, Milan, Italy, 21st-24th September 2024.

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Manuscripts in preparation/submitted

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Deady C*,* Fitzgerald J, Crispie F, Cotter PD, McCarthy FP, McCarthy C, O'Keeffe GW, O'Mahony SM. Probiotic exposure in the prenatal period has differential effects on offspring behaviour in a healthy pregnancy. *To be submitted to Microbiome*.

Abbreviations list

5-HT – 5-hydroxytryptamine

ABX – Antibiotics

ADHD – Attention-deficit hyperactivity disorder

AI – Artificial intelligence

APC – Antigen presenting cell

ASD – Autism spectrum disorder

ASV – Amplicon sequence variants

BBB – Blood-brain barrier

BDNF – Brain-derived neurotrophic factor

BSA – Bovine serum albumen

CFU – Colony forming units

CRP – C-reactive protein

C-section – Caesarean-section

CXCL – Chemokine (C-X-C motif) ligand

DAPI - 4'-6-Diamidino-2-phenylindole

DMEM – Dulbecco's modified Eagle's medium

DSM-V – Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition

E – Embryonic day

FFAR – Free fatty acid receptor

FOS – Fructooligosaccharides

FMT – Faecal microbial transplant

GF – Germ-free

GFP – Green fluorescent protein

GOS – Galactooligosaccharides

HDAC – Histone deacetylase

HMO – Human milk oligosaccharides

HPA – Hypothalamic-pituitary-adrenal

HSD2 – Hydroxysteroid dehydrogenase type 2

IBS – Irritable bowel syndrome

iFABP – Intestinal fatty-acid binding protein

IFN – Interferon

IKK – IκB kinases

IL – Interleukin

IUGR – Intrauterine growth restriction

LBP – Lipopolysaccharide binding protein

LDH – Lactate dehydrogenase

LPS – Lipopolysaccharide

MEME – Minimum essential medium eagle

MFGM – Milk fat globule membrane

MHC – Major histocompatibility complex

MIA – Maternal immune activation

NDD – Neurodevelopmental disorders

NF-κB – Nuclear factor kappa-light-chain-enhancer of activated B cells

NSAID – Nonsteroidal anti-inflammatory drugs

OCD – Obsessive-compulsive disorder

P – Postnatal day

PBS – Phosphate buffered saline

PBS-T – Triton-X phosphate buffered saline

PCR – Polymerase chain reaction

PFA – Paraformaldehyde

Poly (I:C) – Polyinosinic:polycytidylic acid

RUPP – Reduced uterine placental perfusion

SCOPE – Screening for pregnancy endpoints

sCD14 – Soluble CD14

SCFA – Short-chain fatty acid

SEM – Standard error of the mean

siRNA – Small interfering RNA

TBS – Tris-buffered saline

TLR – Toll-like receptor

TNF – Tumour necrosis factor

TRAF – Tumour necrosis factor receptor associated factor

TRIF – Toll-IL-1 receptor domain-containing adapter-inducing interferon

TXTBS – Triton-X tris buffered saline

Abstract

Maternal immune disruption profoundly impacts developmental origins of disease. Infections, pregnancy complications, or adverse lifestyle factors disrupt the maternal-fetal immune balance, thereby compromising optimal neurodevelopment of the fetus. The health of the developing fetus is also determined by the maternal microbiome, which has been previously shown to shape and modulate long-term offspring outcomes. The unique bidirectional relationship between the maternal microbiome and immune system allows for potential manipulation of the maternal immune system through microbial modulation, and in turn, can influence neurodevelopment. This thesis explores how inflammatory stimuli and microbiome modulation influence brain development, offering novel insights into how these exposures can exert lasting effects on long-term offspring health.

First, the bioactive potential of maternal serum was examined from the previously completed Screening for Pregnancy Endpoints study. Identification of a biological "high-stress" group was based on inflammatory markers, measures of gut permeability, and tryptophan metabolism. Exposure to serum from "high-stress" pregnant women reduced neurite length in partially differentiated SH-SY5Y cells. This potentially harmful effect was due in part to phosphorylation of NF-κB p65 through a TNFα-dependent mechanism, highlighting the influential role of maternal inflammation.

Next, the dual-hit effect of prenatal exposure to lipopolysaccharide, acting as an inflammatory stimulus, and a cocktail of antibiotics, to disrupt the maternal microbiome, was investigated. There was a minor effect of a double-hit of inflammation exposure and microbial disruption on embryonic anthropometry and placental growth. Primarily, inflammation exposure led to significant behavioural deficits. The reported increase in anxiety-like behaviours is likely associated with a reduced expression of 5-HT1a receptors in the prefrontal cortex.

Finally, the potential of prenatal *Lacticaseibacillus rhamnosus* exposure in modifying neurodevelopment processes and behavioural outcomes via

modulation of the maternal microbiome in a healthy pregnancy was explored. Here, a significant improvement in sociability and repetitive-restrictive behaviours was observed. However, probiotic exposure was also found to increase anxiety-like behaviours, likely associated with an increase in active microglia in the amygdala and a reduction of butyrate-producing bacteria. This effect may be credited to an immunostimulatory response in the dams.

Overall, the results of this thesis provide novel insights into the harmful effects of maternal inflammation and a dysbiotic maternal microbiome during the development of the fetal brain and highlights the pressing need for appropriate microbiome-targeted therapeutics. These findings suggest a need for early identification of “at-risk” women in clinic, and careful consideration of probiotic consumption in expectant mothers.

Chapter 1

General introduction

1 Pregnancy and neurodevelopment

Pregnancy is a time of rapid and dynamic change that allows the body to support the development and growth of the fetus *in utero*. Across the 40 weeks of gestation, drastic changes occur in the human female body to make this possible. Pregnancy is a fine-tuned process that is meticulously governed by hormonal and physiological cues. A healthy pregnancy supports the development of the fetal nervous system which is optimal for later life.

1.1 Neurodevelopment from neural tube formation to postnatal life

The development of the nervous system begins in early gestation (**Figure 1**). During gastrulation which commences at week 3, the neural tissue forms from the ectoderm in a process termed neurulation to form the neural plate (Moon and Xiong, 2022). The neural plate, which is the earliest precursor of the central nervous system, folds in on itself at gestational week 3 to form the neural tube (Moon and Xiong, 2022; O'Rahilly and Müller, 1994). This continuous tube extends from head to tail in all chordates and contains the cerebrospinal fluid (Bueno et al., 2020; Moon and Xiong, 2022). This process is perhaps the most sensitive and detrimental for the health of the embryo. Failure to close the neural tube often results in embryonic death, particularly if the unclosed section is substantial (Moon and Xiong, 2022; Nikolopoulou et al., 2017). However, not all disruptions to neural tube closure are incompatible with life. Partial closure results in neural tube defects which can lead to complications in later life, including meningocele and myelomeningocele, both forms of spina bifida (Moon and Xiong, 2022; Nikolopoulou et al., 2017).

It is during this period that the nervous system begins to take shape into what we see in the human nervous system. Brain vesicles begin to expand to form the prosencephalon, mesencephalon, and rhombencephalon (Stiles and Jernigan, 2010). These sections continue to divide until the major features of the brain are formed (Stiles and Jernigan, 2010). This however is only a primitive map, and neural patterning is required for each brain area to form and eventually function

as required (Stiles and Jernigan, 2010). Neural patterning is heavily aided by various signalling cues which allow for correct neurogenesis and neuronal migration (Stiles and Jernigan, 2010). The neocortex, which will develop from the prosencephalon, consists of 6 layers with a carefully crafted cytoarchitecture (Cooper, 2008). Within this, each cortical layer contains different neuronal phenotypes (Stiles and Jernigan, 2010). Following migration, the immature neurons integrate into the neuronal network through axonal and dendritic growth (Brown et al., 2001; Stiles and Jernigan, 2010). The emerging axons are guided into the correct location through facilitatory or inhibitory signalling cues until it has reached its target (Brown et al., 2001; Stiles and Jernigan, 2010). This then forms a synapse with the neighbouring cell, allowing for electrochemical messages transferring information to be passed between the two cells (Stiles and Jernigan, 2010).

In this stage of brain development, however, the number of neuronal cells and synaptic connections formed is more than is required for later functioning. It is estimated that approximately 50% of the newly formed neurons are eliminated via nonpathological cell death via apoptosis and pruning (Stiles and Jernigan, 2010). The majority of neuronal cell death occurs in the prenatal period (Stiles and Jernigan, 2010). Programmed cell death of the glial population is normally reserved for the postnatal period (Stiles and Jernigan, 2010).

While further development is expected in the postnatal period; it is predominately fine tuning of the offspring's brain. Coupled with continued cellular pruning, differentiation and maturation of the neurons and glia continue during early life. The neurogenesis process continues, yet this is only seen in subventricular zone of the cortex and in the dentate gyrus of the hippocampus. This is thought to continue into adult life but only contributes to a small fraction of the overall population of neurons (Stiles and Jernigan, 2010). This is not the case for glial progenitor cells; which start populating the brain prenatally and this persists into adulthood (Cayre et al., 2009; Stiles and Jernigan, 2010). Glial progenitor cells develop in the subventricular zone and migrate to numerous regions within the brain including the hippocampus and the cortex. These glial

progenitor cells, primarily oligodendrocyte precursors, differentiate once reaching their required destination. Oligodendrocytes wrap tightly around the axonal sheath, acting as insulation that will propagate the electrical messages at a rapid speed (Stiles and Jernigan, 2010). The timing of these major neurodevelopmental processes is detailed in **Figure 1**. The success of each of these processes is critical for long-term neurodevelopmental outcomes in the offspring. Each of these developmental processes are sensitive to internal signalling cues, but also external influences such as maternal health, diet, stress, and environment. These external influences may be beneficial or detrimental in a case dependent manner.

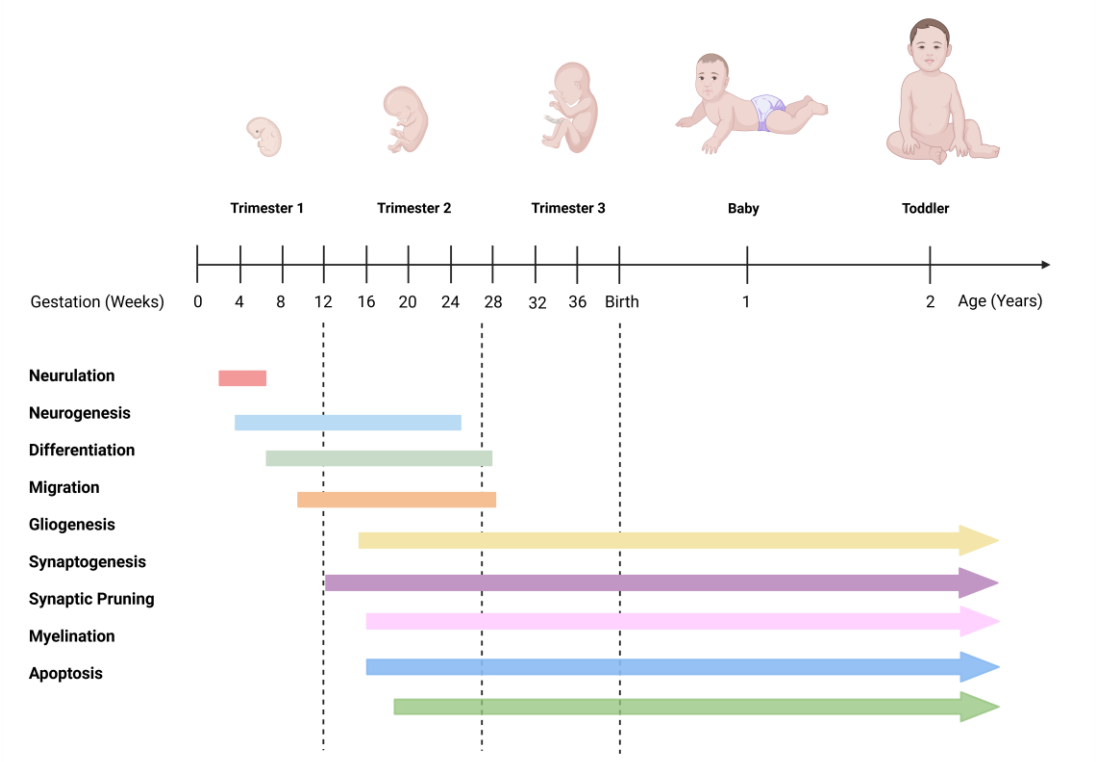


Figure 1: Major neurodevelopmental processes. *The timings of important developmental processes in the central nervous system across the three trimesters of pregnancy and the first two years of postnatal life.*

1.2 The importance of the placenta

The placenta acts as the interface between the mother and the developing fetus. The placenta is essential for fetal life and optimal fetal neurodevelopment. The placenta develops alongside the fetus *in utero*, forming from the trophectoderm layer of the blastocyst, approximately 5 days after fertilisation occurs (Covarrubias et al., 2023). Shortly after, the trophectoderm attaches to the endometrium of the uterus, eventually beginning to branch and divide to form villi (Benirschke et al., 2012; Covarrubias et al., 2023). By the end of the first trimester, the main structure of the placenta is fully formed but continues to expand its overall surface area (Covarrubias et al., 2023).

The placenta acts as both a functional and physical connection between the mother and the fetus, while also providing a protective barrier against harmful substances and serves numerous vital functions (Gude et al., 2004). It acts as a means for respiratory gas exchange, facilitating oxygen delivery to the fetus and the removal of carbon dioxide (Gude et al., 2004). The placental membrane is highly permeable, allowing for the diffusion of gas across numerous blood vessels (Gude et al., 2004). Nutrients, amino acids, and glucose are easily transported and metabolised within the placenta, providing the fetus with sufficient energy sources for growth (Gude et al., 2004). As the placenta is devoid of nerves, direct communication between the mother and the fetus relies on various blood-borne signals such as endocrine, autocrine, and paracrine cues (Gude et al., 2004). Mediators of particular importance to the health of the fetus and pregnancy include oestrogen and progesterone, cytokines, and various growth factors (Gude et al., 2004). The normal, yet critical, development and function of the placenta is necessary to facilitate the expected trajectory of the fetal brain. Pregnancy complications associated with placental dysfunction, such as pre-eclampsia, placenta previa, and placenta accreta, have all been linked to deleterious neurodevelopmental outcomes in the exposed offspring in later life (Huma et al., 2025; Liu et al., 2022; Redline, 2005; Sahu and Shrivastava, 2024).

1.3 Neurodevelopmental disorders

Neurodevelopmental disorders (NDDs) are defined in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-V) as "a group of conditions with onset in the developmental period, inducing deficits that produce impairments of functioning" (Morris-Rosendahl and Crocq, 2020). In the DSM-V, NDDs are subdivided into 6 major categories, these are detailed below in **Table 1**. Typically originating in very early childhood, NDDs can affect various parameters such as cognition, social behaviours, motor functioning, and behavioural alterations. These disorders persist into adulthood and dependent upon severity, can influence quality of life and prove to be a significant challenge. For most cases of NDDs, except those that are due to genetic mutations such as Fragile X syndrome, there is no direct cause (Hagerman et al., 2017). These disorders are often multifactorial, with both environmental exposure and genetic susceptibility contributing to their pathogenesis (van Loo and Martens, 2007).

Some of the most prevalent and extensively researched NDDs include autism spectrum disorder (ASD), attention-deficit hyperactivity disorder (ADHD), and schizophrenia. ASD encapsulates various conditions including autism, Asperger's syndrome, and childhood disintegrative disorder (Lord et al., 2018). The term spectrum covers the wide variety of symptoms associated with the various conditions but at its core, ASD typically presents with defects in social communication and interaction, in addition to restricted and repetitive behaviours (Lord et al., 2018). Social difficulties include lack of eye contact, inability to start or maintain a conversation, delayed or regressive speech, and difficulty in displaying emotions (Lord et al., 2018). For the behavioural patterns, repetitive motions commonly referred to as "stimming", excessive or restricted body language, lack in physical coordination, and specific fixations are just some of the symptoms (Lord et al., 2018). ADHD is often more severe in childhood and as the child advances into adulthood, the number of symptoms typically decreases. While often associated with difficulties relating to attention and impulsivity, those with ADHD often struggle with restlessness, mood swings, disorganisation, and frequent frustration (Leffa et al., 2022). Schizophrenia is the

only one of the 3 that involves some aspect of psychosis. Symptoms can be divided into what are termed positive and negative. Positive symptoms include visual or auditory hallucinations, delusions, disorganised thoughts and behaviour (Tandon et al., 2013). Negative symptoms encompass anhedonia and avolition, alongside an overall flat affect (Tandon et al., 2013).

This is not an exhaustive list of NDDs. Other disorders include intellectual disability, cerebral palsy, dyspraxia, tic disorders, Rett syndrome, Down syndrome, and dyslexia. Despite their importance and increasing prevalence, for the remainder of this thesis, ASD, ADHD, and schizophrenia will remain our focus of NDDs.

Table 1: Neurodevelopmental disorders as per the DSM-V definition, along with neurodevelopmental-adjacent disorders.

Neurodevelopmental Disorder Category	Definition	Examples	Brain regions implicated	Reviews
ASD	Deficient reciprocal social communication and the presence of restricted, repetitive, and inflexible patterns of behaviour	Category encompasses Autistic Disorder, Asperger's Syndrome, Childhood Disintegrative Disorder, and Pervasive Developmental Disorder Not Otherwise Specified	Amygdala, Insula, Orbitofrontal Cortex, Basal Ganglia, Caudate Nucleus, Hippocampus, Inferior Frontal Gyrus, Anterior Cingulate Cortex	(Ha et al., 2015)
ADHD	Persistent pattern of inattention and/or hyperactivity-impulsivity	Primarily Inattentive Type, Primarily Hyperactive-Impulsive Type, Combined Presentation	Prefrontal Cortex, Frontal Lobe, Medial Temporal Lobe, Corpus Callosum, Basal Ganglia, Anterior Cingulate Cortex	(Firouzabadi et al., 2022)
Motor Disorders	Coordination issues, stereotypic movements, or tics	Developmental Coordination Disorder, Stereotypic Movement Disorder, Tic Disorders	Basal Ganglia, Cerebellum, Sensorimotor Cortex, Thalamus, Parietal Lobe	(Subara-Zukic et al., 2022)
Intellectual Disabilities	Deficits in intellectual and adaptive functioning, that affects the ability to learn, retain, or process information	-	Dorsal Medial Prefrontal Cortex, Inferior Frontal Gyrus, Middle Frontal Gyrus, Cuneus, Anterior Cingulate Cortex	(Ma et al., 2021)
Communication Disorders	Language, speech sound, or pragmatic communication impairments	Language Disorder, Speech Sound Disorder, Social Communication Disorder, Childhood-Onset Fluency Disorder	Broca's Area, Planum Temporale, Inferior Frontal Gyrus, Basal Ganglia, Caudate Nucleus, Prefrontal Cortex	(Abbott and Love, 2023)
Specific Learning Disorder	Deficits in any area of information processing	Dyslexia, Dyscalculia, Dysgraphia	Inferior Frontal Gyrus, Tempero-Parietal Cortex, Occipito-Temporal Cortex, Cerebellum, Intraparietal Sulcus, Prefrontal Cortex, Precuneus, Posterior Cingulate	(Costa et al., 2022; Hernández-Vásquez et al., 2023; Üstün et al., 2021)
Other Neurodevelopmental Disorders	All other conditions that do not fit into the main categories but still involve neurological impairments from a developmental origin	Schizophrenia, Obsessive Compulsive Disorder, Rett Syndrome, Cerebral Palsy, Down Syndrome	Varies dependent upon neurodevelopmental disorder	(Hamadelseed et al., 2023; Hazari et al., 2019; Keshavan et al., 2020)

Abbreviations: ASD: Autism spectrum disorder; ADHD: Attention deficit hyperactivity disorder.

1.4 Mechanistic insights into neurodevelopmental disorders and critical periods

There are numerous mechanisms associated with certain NDDs, yet most NDDs are multifactorial, and a definitive cause cannot be easily elucidated (van Loo and Martens, 2007). Genetic NDDs stem from chromosomal abnormalities such as aneuploidies and duplications, or deletions in copy number variants or single gene mutations (Hagerman et al., 2017; Klein and Haydar, 2022; van Loo and Martens, 2007; Wu and Li, 2022). Genetic mutations are often associated with Rett syndrome or Fragile X syndrome, both rare developmental disorders that are linked to serious neurological complications (Hagerman et al., 2017; Vidal et al., 2019). These mutations are the sole cause of the NDDs, this is not seen in many other NDDs, yet there is strong genetic heritability (van Loo and Martens, 2007). The estimated genetic heritability of ASD is approximately 90%. However, not all people with ASD will give birth to children that will develop ASD. This indicates a role for environmental factors, indicating that they can modulate the neurodevelopmental outcome (Freitag, 2007; van Loo and Martens, 2007). A significant proportion of affected genes are related to neuronal connectivity, including SHANK3 and NRXN1 (Exposito-Alonso and Rico, 2022). While various NDDs have a diverse variety of symptoms, there is a substantial overlap between genetic factors across the different disorders (Exposito-Alonso and Rico, 2022).

Disrupted neurodevelopmental processes may or may not occur following genetic manipulations. Neuronal proliferation, migration, and differentiation are of utmost importance to overall brain health and disruptions to these processes contribute to various NDDs (Hu et al., 2014; Parenti et al., 2020). Other cellular mechanisms include synapse formation and incorrect synaptic pruning (Exposito-Alonso and Rico, 2022). Dysfunctional synaptic transmission has been linked to disruptions in numerous brain circuits, directly influencing cognition and behaviour (Exposito-Alonso and Rico, 2022). An impaired synapse has the potential to disrupt the expected excitatory/inhibitory balance which is seen in NDDs such as ASD (Exposito-Alonso and Rico, 2022). Connectivity defects are prevalent in many of the higher functioning cortical areas of the brain, and this

has been confirmed by functional magnetic resonance imaging (Exposito-Alonso and Rico, 2022; Hull et al., 2017). While there are no unique hallmarks in the ASD brain, recent findings suggest local overconnectivity and long-distance disconnection (Exposito-Alonso and Rico, 2022). This aberrant connectivity may reduce levels of the inhibitory neurotransmitter gamma-aminobutyric acid, reaffirming deficiencies in synaptic transmission (Exposito-Alonso and Rico, 2022).

However, in this thesis, our focus will be investigating environmental contributors to NDDs. Prenatal exposures have been linked to a wide variety of NDDs, but realistically, these only become influential in a fetus with specific genetic susceptibility (van Loo and Martens, 2007). Suboptimal neurodevelopmental outcomes can be traced to maternal infection and stress, environmental disasters, prenatal toxin exposure, along with dietary and lifestyle choices (Brown, 2006; Ünsel-Bolat et al., 2024; Urbonaite et al., 2022; Van Den Bergh et al., 2020; Williams and Ross, 2007). The risk of exposure to pollutants or harmful circulating factors in the maternal blood can be particularly disruptive to the developing fetal brain (Grandjean and Landrigan, 2014; Womack et al., 2024). While each of the abovementioned pathogenic mediators have their own specific mechanism of action and can influence the fetal brain differently, the overarching theme is often the same, whereby excessive levels of the insult can lead to increased inflammation and oxidative damage, epigenetic changes, and ultimately disrupt normal development (De Asis-Cruz et al., 2022; Scott et al., 2020). An overview of major contributors that increase the risk of NDDs is detailed in **Figure 2**.

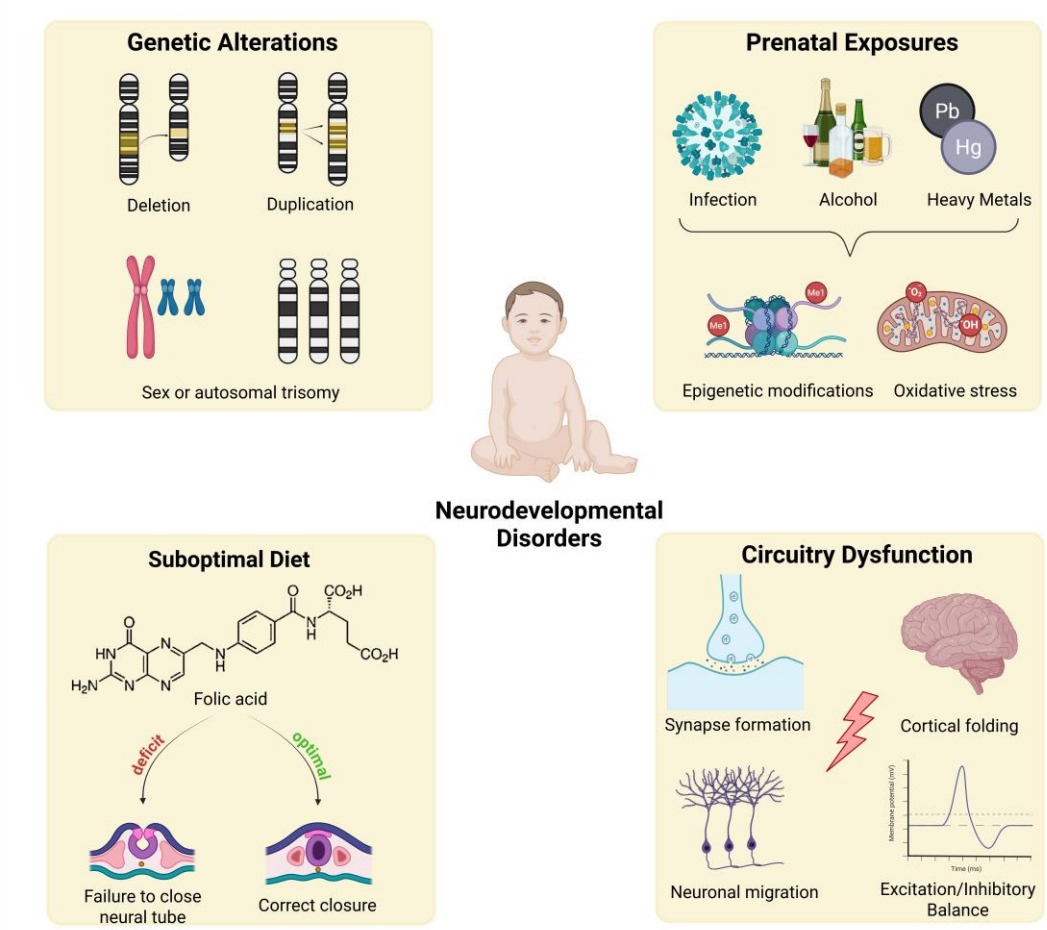


Figure 2: Contributors to neurodevelopmental disorders. *Possible mechanisms in which various genetic alterations, harmful prenatal exposures, a suboptimal maternal diet, and disruptions to certain neurodevelopmental trajectories and processes can increase the risk of neurodevelopmental disorders in the affected offspring.*

Regardless of the pathogenic mediators involved, the key to these prenatal disruptions, is the timing. The most important neurodevelopmental processes occur very early in pregnancy during the formation of the neural tube (Moon and Xiong, 2022). During this critical period, suboptimal alterations typically result in life-threatening or severe neural tube defects such as spina bifida or encephalocele (Kancherla, 2023). As the development of the nervous system continues, disruptions can affect developmental outcome and can be severe yet typically are not life-threatening and don't influence major functioning (Doi et al., 2022). As development progresses, the expected NDDs including ASD or ADHD, are more associated with connectivity issues and disruptions within minor brain circuits (Doi et al., 2022). Reducing the risk of exposure to harmful substances or

stressors during pregnancy is critical in protecting development of the fetal nervous system, yet this may not always possible.

2 Maternal immune system and inflammation: impacts on neurodevelopment

Maternal inflammation is a key physiological activity during pregnancy to maintain health of the developing fetus yet must be tightly regulated as high levels of maternal inflammation may disrupt critical neurodevelopment processes.

2.1 Maternal immune system during pregnancy

During pregnancy, the maternal immune system is one of the most important biological systems that protects the mother and the fetus from harmful pathogens but also aids in supporting the pregnancy itself. The maternal immune system undergoes dramatic changes to accommodate the allogenic fetus, starting at conception. Implantation of the embryo is marked by a period of increased inflammation (Fuhler, 2020). This allows the blastocyst to adhere to the surface of the endometrium, facilitating the tissue remodelling, while also stimulating angiogenesis, all of which are required for optimal invasion and placental formation (Dekel et al., 2014; Mor et al., 2011).

Following this process in early gestation, the immune system switches to an anti-inflammatory state, to ensure fetal tolerance (Fuhler, 2020). Paternal DNA contributes to half of the fetus, along with the fetal proportion of the placenta. Both contain antigens that are recognised as foreign by the maternal immune system (Fuhler, 2020). Typically, $CD4^+$ T-helper cells and $CD8^+$ cytotoxic T-cells identify these allogenic cells, stimulating clonal expansion of T-cell to target these foreign antigens (Fuhler, 2020; Ingulli, 2010). T-cell expansion is accompanied by various antigen presenting cells (APCs) such as macrophages and dendritic cells which begin phagocytosis (Fuhler, 2020). Ultimately, if this was to occur unopposed, it would lead to irreparable tissue damage to the fetus

(Fuhler, 2020). However, uterine natural killer and macrophages, which make up approximately 70% of the immune cells in the decidua and differ from systemic cytotoxic natural killer cells due to different surface receptors, ensure fetal protection (Faas and de Vos, 2017; Fuhler, 2020; Sentman et al., 2004). Furthermore, the major histocompatibility complex (MHC) expressed on fetal cells are non-traditional and are specific to the fetus (Fuhler, 2020). They lack the important MHC-I and MHC-II molecules which are used to determine allergenicity (Fuhler, 2020). Finally, maternal T-cell populations show pattern recognition towards fetal cells. Maternal APCs first process the fetal tissue before the fetal antigens are presented to the maternal T-cells (Fuhler, 2020). This is also accompanied by reduced APCs efficacy in the decidua (Fuhler, 2020). This restricted immune response in the primary state across the whole of gestation, functions to allow the semi-allogenic fetus to grow.

Yet, the maternal immune response during pregnancy is not global immunosuppression, this is primarily restricted to the decidua (Fuhler, 2020). Changes in both the maternal innate and adaptive immune system are also evident (Abu-Raya et al., 2020). The innate immune system typically enhances defence through upregulation of the complement system and neutrophils (Abu-Raya et al., 2020). Monocytes exist in a more intermediate phenotype, displaying both phagocytic and inflammatory properties (Abu-Raya et al., 2020). This is thought to maintain balance between inflammation and immunotolerance (Abu-Raya et al., 2020). Changes in the adaptive immune system include an increase in Th2 polarisation, which is initiated early in pregnancy to preserve an anti-inflammatory environment (Abu-Raya et al., 2020). Changes to suppressor T cells, T helper cells, and cytotoxic T cell populations all vary across the course of gestation and are dependent upon what is required by the body at each timepoint (Abu-Raya et al., 2020). B cells typically lose function as the pregnancy progresses, this is considered a protective function typically, maintaining fetal tolerance but may also increase the risk of maternal infection (Abu-Raya et al., 2020).

Finally, at the end of gestation, the anti-inflammatory environment shifts back towards a pro-inflammatory state (Fuhler, 2020; Mor et al., 2011). Inflammation is required to aid in parturition and to break down fetal tolerance. The increase of pro-inflammatory cytokines and chemokines trigger labour by stimulating uterine contractions and the breakdown of cervical and fetal membrane tissues (Romero et al., 2006). In the postpartum period, increased inflammation continues to aid in tissue repair and healing, but typically resides at 6 weeks and continues on for approximately 12 weeks unless there are complications (Dye et al., 2022; Groer et al., 2015). The cycle of maternal inflammation is carefully orchestrated and fine-tuned. Disruptions to this delicate equilibrium can result in implantation failure, miscarriage, pre-term birth, or neurodevelopmental disorders (Brown, 2006; Christiansen et al., 2006; Lin et al., 2024; Romero et al., 2006).

2.2 Causes of maternal inflammation

Maternal inflammation can stem from numerous origins, but predominately through prenatal infection. While the process of modified immunosuppression is necessary for fetal tolerance, it brings its challenges (Abu-Raya et al., 2020). Enhanced maternal immune cell surveillance and activity can prove to be detrimental for the fetus, but suppression of the immune response may leave the mother in a vulnerable position against infection (Fuhler, 2020; Han et al., 2021). There is increased susceptibility to certain infections during the gestational period, including listeriosis, malaria, influenza, and herpes simplex virus (Abu-Raya et al., 2020; Sappenfield et al., 2013). Once detected in the maternal system, the immune system sparks an inflammatory response by triggering a cascade of cytokines to mitigate the infection (Raj et al., 2014). This is commonly referred to as maternal immune activation (MIA). To combat this, vaccinations are recommended during pregnancy to reduce the chance of infection (Abu-Raya et al., 2020).

While maternal infection often leads to acute MIA, there are also numerous chronic conditions that can increase basal levels of inflammation. Maternal

conditions such as asthma, autoimmune diseases such as arthritis or lupus, major depressive disorder, or long-term stress have all been shown to induce inflammation in the mother (Han et al., 2021). Similarly, metabolic disorders like obesity or type 2 diabetes mellitus, or consumption of a high-fat, high-sugar diet can disturb the basal inflammatory state (Han et al., 2021). Several pregnancy complications are also associated with MIA, including pre-eclampsia and gestational diabetes mellitus (Han et al., 2021).

The duration of a maternal infection is significantly shorter in comparison to some life-long health issues or pregnancy complications. However, there is an acute yet intense immune reaction, the concentration of cytokines and chemokines induced by prenatal infection are significantly greater than most cases of chronic inflammation. These cases are typically low-grade levels of inflammation and unless there is a flare-up of symptoms, disruption of the maternal immune system is somewhat limited. However, as these conditions extend across the whole gestational period, the overall fetal exposure may be greater than a short-term infection. The contrasting durations and intensity of MIA may play a role in long-term neurodevelopmental outcomes (Bucknor et al., 2022).

2.3 Maternal immune activation due to infection: mechanisms and consequences

There is a strong link between MIA and NDDs in the exposed offspring. Perhaps one of the most well-studied cases is MIA due to maternal infection during the gestational period. The relationship between maternal infection and NDDs has been established through numerous global health emergencies, which saw a subsequent spike in the number of offspring presenting with suboptimal neurodevelopment. One key example was the 1964 rubella epidemic that led to an increase in the number of schizophrenia cases years later (Brown et al., 2001). Similarly, the Zika virus epidemic of 2016 was associated with increased incidence of microencephaly, and most recently, the SARS-CoV-2 global pandemic beginning in 2020 has been linked to various neurodevelopmental

sequelae (Antoniou et al., 2020; Duan et al., 2025; Dubey et al., 2022). The number of self-reported infections during pregnancy indicates that over 60% of women have at least one infection during pregnancy (Collier et al., 2009). The World Health Organisation (WHO) reported that 7% of all pregnancies suffer an infection that requires hospitalisation, highlighting the heavy burden that infectious diseases have on fetal neurodevelopment (Bonet et al., 2020).

There are two primary mechanisms by which maternal infection can disrupt the developing fetal brain. These mechanisms include vertical transmission, which occurs when the placenta fails to act as a physical barrier separating the mother from the fetus, allowing harmful substances to reach the fetus (Gude et al., 2004). Certain pathogens such as Zika virus, *Listeria monocytogenes*, rubella, and *Treponema pallidum* can circumvent this barrier (Arora et al., 2017; Megli and Coyne, 2022). This can occur through direct infection of extravillous trophoblasts causing damage to the syncytiotrophoblast layer of the placenta, or via transmission of infection from maternal immune cells to fetal cells in the decidua (Arora et al., 2017; Megli and Coyne, 2022). Pathogens can then directly infect the fetus, drastically increasing the risk on congenital abnormalities, NDDs, pre-term birth, and stillbirth (Megli and Coyne, 2022; Yates and Mulkey, 2024).

Vertical transmission is not possible in a proportion of maternal infections, such as typical influenza strains and SARS-CoV-2 as they fail to initiate significant viremia in the mother (Kanmaz et al., 2011; Lucot-Royer et al., 2024). Yet the developing fetus can be equally affected through indirect mechanisms that are typically due to environmental changes within the maternal body (Megli and Coyne, 2022). These changes can be severe and subsequently influence neurodevelopment (Yu et al., 2021). Following infection, there is a sharp increase in the production of pro-inflammatory cytokines to combat the infection. Cytokines such as interleukin (IL)-2 and IL-6 have been shown in preclinical animal models to cross the placental barrier, allowing the increased inflammation to directly interact with the fetal brain and disrupt numerous crucial mechanisms such as neuronal migration and differentiation, or disrupt

normal microglial function (McEwan et al., 2023; Ozaki et al., 2020; Vasistha et al., 2020; Woods et al., 2023). Separately, high levels of MIA can directly cause placental dysfunction, resulting in a hypoxic, growth restricted *in utero* environment, further impacting fetal growth (Baschat, 2014; Megli and Coyne, 2022; Yu et al., 2021).

This mechanism has been elucidated through an extensive number of MIA rodent models, revealing severe behavioural alterations alongside disruption to the normal neuroanatomy and neurochemistry of the exposed offspring's brain (Gzieło et al., 2023; Kalish et al., 2021; Ventura et al., 2020). The detrimental effect that MIA has on neurodevelopment is also seen in clinical studies that reveal similar findings. Cognitive and behavioural changes, along with an elevated risk of ASD and schizophrenia have all been linked to MIA (Hall et al., 2023). However, the timing, intensity, and duration of the maternal infection all play a role in determining the severity of the fetal neurodevelopmental outcomes (Bucknor et al., 2022; Doi et al., 2022).

2.4 The fetal immune system and its future role in neurodevelopment

The fetal immune system is a crucial system that, along with the maternal immune system, works to protect the developing fetus from potential harms. In addition to pathogens, the fetal immune system must protect against non-inherited maternal antigens which could contribute to graft versus host disease. Beginning to form around the fourth week of gestation, the fetal immune system originates from hematopoietic precursors in the liver (Gonzalez and Gonzalez, 2020). Throughout the course of gestation, various immune cells become detectable, including natural killer cells at 6 weeks, T and B lymphocytes at 8 weeks, and dendritic cells at 12 weeks. As the pregnancy continues, these immature immune cells develop further and gain cytotoxic activity. Both the composition and the function of the fetal immune system is dramatically different when compared to that of an adult. The innate immune system is the primary arm involved *in utero*, functioning to identify pathogen-associated

molecular patterns and initiating the appropriate response. In comparison, the adaptive immune system has rather limited capacity (Gonzalez and Gonzalez, 2020). There is substantial transfer of maternal immunity to the fetal compartment across gestation. Antibodies, inflammatory mediators, and cells capable of microchimerism are some of the components that can be transported to the fetus. This is heavily aided by the placenta, allowing easy passage of IgG antibodies. At the time of birth however, while functional, the immune system is still not mature enough to fully protect the now neonate. Due to this, the offspring relies on the transfer of passive immunity from the mother. This often comes in the form of maternal antibodies through the breastmilk (Gonzalez and Gonzalez, 2020).

In relation to future neurodevelopment, MIA can significantly alter fetal immune signalling. This can primarily occur through microglial activation, and this has been identified as a main driver in the associated increased risk of NDDs. Microglia originate from hematopoietic precursors in the yolk sac and populate the fetal brain before any other glial cell (Mastenbroek et al., 2024). Here, they act as regulators of neurogenesis, particularly in the ventricular and subventricular zones of the cortex. Due to their phagocytosing capabilities, microglia possess the ability to prune the available neural progenitor cell pool, allowing them to directly influence neuronal populations. The function of microglia goes beyond regulation of neurogenesis, as they also play a substantial role in guiding axons and interneurons to their correct place, particularly dopaminergic cells (Mastenbroek et al., 2024). Postnatally, these microglia continue to support brain development through the pruning of apoptotic neurons and oligodendrocyte precursors, along with weak or excessive synapses. Due to this, these cells play a substantial role in refining the brain circuits of the developing brain. However, this activity is heavily reliant on cytokine and chemokine signalling. In cases of MIA, this signalling becomes aberrant, and the normal microglial-neuronal crosstalk becomes disrupted. Exposure to excess inflammation can lead to activated microglia which can secrete both pro-inflammatory cytokines and reactive oxygen species. In turn, this can directly influence synaptic pruning in the brain, along with dysregulation of neural and

glial circuits. This is accompanied by changes in microglia receptor expression and altered transcriptome profiles. These changes have been revealed through numerous *in vivo* studies which then show links to unfavourable behavioural outcomes (Mastenbroek et al., 2024).

3 The microbiome

The microbiome plays a vital role in maintaining homeostasis in the body. Recent insights reveal the microbiomes' role in health and disease and is a key determinant of optimal neurodevelopment in the prenatal and postnatal period.

3.1 Overview of the microbiome

The microbiome can be defined as the collective term for the trillions of microorganisms that live both in and on us (Ursell et al., 2012). Consisting of bacteria, viruses, yeasts, protozoa, archaea, and parasites, these microbial cells have been found to outnumber human cells at a ratio of 1.3:1 (Cryan et al., 2019; Sender et al., 2016). Estimations suggest over 10 million of our genes are microbial in nature, surpassing over 99% of human genes (Cryan et al., 2019). While this number is startlingly high, it is rather unsurprising considering how eukaryotic cells evolved alongside microbes for millions of years (Suzuki et al., 2022). Research has shown that a niche microbiome can be identified in almost all parts of the human body, yet they are not all of equal significance in relation to human health (Cho and Blaser, 2012; Cryan et al., 2019). While a healthy urogenital, skin, and oral microbiome play a role in wellbeing, perhaps none is more intertwined with overall health than that of the gut (Cryan et al., 2019). The gut microbiome has been implicated in numerous diseases, from neurodevelopment to neurodegeneration, mental health disorders, cardiovascular health, metabolic health, skin health, as well as pain-associated conditions (Cryan et al., 2019; Gilbert et al., 2018; Knight et al., 2017; O'Riordan et al., 2025a). In-depth overviews of microbiome function and development will be discussed in the following sections.

3.2 The virome and phagosome: beyond bacteria

In most studies examining the microbiome, it is often the bacterial population, or the bacteriome that is studied or highlighted. Studies of the viral and phage microbiomes are limited in comparison to bacteria, yet this does not reflect the importance of their role. The bacteriome and virome in the gut have a similar count ratio, with phage making up the largest proportion of the virome (Cryan et al., 2019; Sutton and Hill, 2019). The human virome can have both a symbiotic and a pathogenic role in human health (Guerin and Hill, 2020; Shkoporov et al., 2022). Characterising the virome has proven to be challenging, as they lack conserved gene regions, making identification difficult (Sutton and Hill, 2019). Despite this, some important information regarding the virome during pregnancy and early life exists. The maternal virome has been reported to remain stable across pregnancy, in comparison to the changing bacterial populations, while the infant's virome appears to be less stable, and easily influenced via maternal transmission, feeding mode, and birth mode (Garmaeva et al., 2024).

Disruption to the maternal virome, seen during Zika infection, leads to significant impacts on offspring neurodevelopment and has been reviewed in detail elsewhere (Recaioglu and Kolk, 2023). An increase in Anellovirus positivity and copy number in maternal plasma has been recently linked to pre-term birth (Stout et al., 2023). Similarly, a unique gut virome signature has been revealed in women with pre-eclampsia (Lv et al., 2024). For the remainder of this thesis, when the microbiome is mentioned, it is referring to the bacterial population unless otherwise stated.

3.3 The microbiota-gut-brain axis

The bidirectional communication between the brain and the gastrointestinal tract has been described as the gut-brain axis. More recently, this has been re-framed as the microbiota-gut-brain axis to include the vast population of microbes that reside in the gut and their functional impact (Cryan et al., 2019). This axis has a monumental role in human health and in regulating homeostasis (Cryan et al., 2019; He et al., 2024; O'Riordan et al., 2025a). The enteric nervous

system, endocrine system, and immune system are all required for the microbiota-gut-brain axis to function optimally (Cryan et al., 2019; O'Riordan et al., 2025b; Yuan et al., 2023). Numerous communication pathways exist within the microbiota-gut-brain axis. Neural communication exists through the enteric nervous system and the vagus nerve. The vagus nerve acts as the fastest and most direct route between the gut and brain and can transmit information from the gastrointestinal tract through afferent fibres, and feedback through efferent fibres (Cryan et al., 2019). These long-reaching fibres synapse on the solitary tract nucleus in the medulla, which can further relay information to areas that influence emotion and memory (Cryan et al., 2019; Longo et al., 2023). The importance of the vagus nerve has been shown through numerous preclinical vagotomy studies, where severing this nerve influences behavioural outcomes (Cryan et al., 2019).

The immune cells that are present in the gut, along with both beneficial and harmful bacteria, can modulate neuroinflammation, which has been linked to numerous disease-states (Cryan et al., 2019). The endocrine system produces hormones and neuropeptides that influence brain signalling, similar to microbial metabolites such as neurotransmitters and short-chain fatty acids (SCFAs) (Cryan et al., 2019). SCFAs are produced from polysaccharides following bacterial fermentation as they are non-digestible and non-absorbable (O'Riordan et al., 2022). SCFAs have been shown repeatedly to influence host health through numerous physiological processes, with the most studied being acetate, propionate, and butyrate. SCFA can influence both brain and behaviour through both direct and indirect mechanisms. Gut-derived SCFAs can be taken up by the host through passive diffusion or protein transporters where they can be utilised by colonocytes as an energy source. Here, SCFAs, primarily butyrate, can be used to synthesis both glucose and lipids, which can then act as an energy source for the brain (O'Riordan et al., 2022). Germ-free (GF) rodents, which lack the conventional microbiota and in turn all of the beneficial metabolites, including SCFAs, have been shown to have disrupted mitochondrial respiration in colonocytes. In humans, it is estimated that 10% of daily energy contribution can be tied to SCFAs (Donohoe et al., 2011).

SCFAs can influence the host through the binding to receptors. These are G-protein coupled receptors, such as GPR41 or GPR43, or more recently termed free fatty acid receptor (FFAR) 2 and FFAR3 (O'Riordan et al., 2022). These receptors are present across the enteric nervous system, along with both the peripheral and central nervous system, ensuring that SCFAs have a long-list of targets. Through these receptors, SCFAs are able to modulate the function and motility of the gastrointestinal system, which further regulate intestinal permeability by altering tight junction expression. This influence on permeability also extends to the blood-brain barrier (BBB) (O'Riordan et al., 2022). Further receptor-mediated functions include signalling through the hypothalamic-pituitary-adrenal (HPA) axis which allows for production of numerous enteroendocrine and neuropeptide signalling. Some of these include neuropeptide Y which functions in appetite-regulation, the hormonal peptide YY and glucagon-like peptide 1 where once again both play a known role in eating and reward-like behaviour through signalling of the vagus nerve (O'Riordan et al., 2022).

The role of SCFAs in neuroinflammation has been consolidated in numerous rodent studies. A primary function behind this is through histone deacetylase (HDAC) inhibition. HDACs are a group of enzymes that remove an acetyl group from histones, allowing for chromatin remodelling and changes in gene expression. Through this inhibition via SCFAs, immunotolerance and a push towards an anti-inflammatory phenotype is favoured. Production of pro-inflammatory cytokines from neutrophiles is prevented by the major SCFAs, while also inhibiting activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (O'Riordan et al., 2022). There is a wealth of information regarding bacterial metabolites, such as serotonin and various catecholamines, along with SCFAs such as butyrate, propionate, and acetate, and their importance in maintaining both brain and gut health (Cryan et al., 2019). The mechanism in which SCFAs can influence the host are detailed in **Figure 3**.

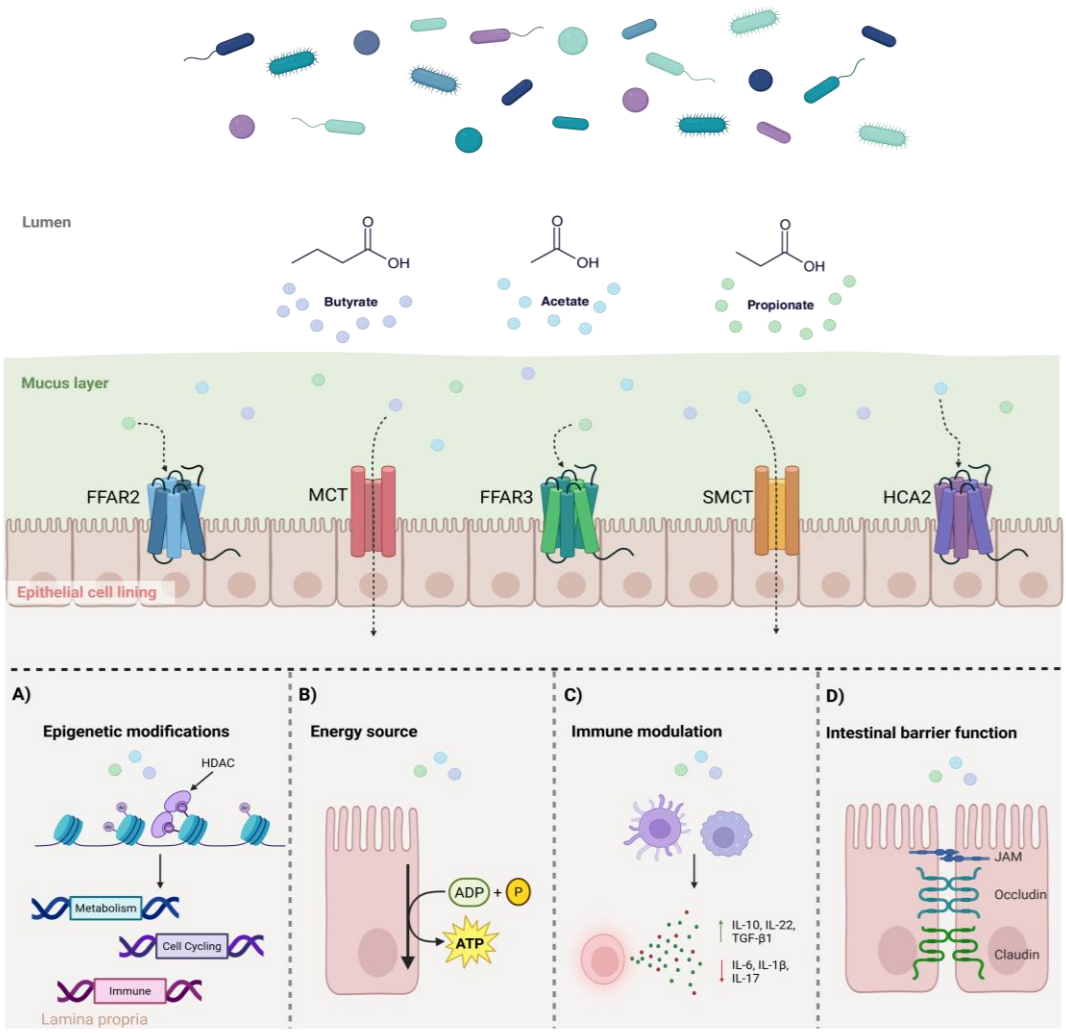


Figure 3: Mechanisms underlying functional impact of short-chain fatty acids. *Butyrate, acetate, and propionate, 3 of the major short-chain fatty acids, and the main receptors, both G-protein coupled receptors and transport channels. Short-chain fatty acids work through A) epigenetic modification through chromatin remodelling by histone deacetylases, and B) act as an energy source for colonocytes. In turn, these processes can lead to C) immunomodulation through a reduction in pro-inflammatory cytokines, and D) improved intestinal barrier integrity through increased expression of tight junction proteins. Through these 4 mechanisms, short-chain fatty acids can influence the health of the host. Abbreviations: FFAR: Free fatty acid receptors, MCT: Monocarboxylate transporter, SMCT: Sodium monocarboxylate transporter HCA: Hydroxycarboxylic acid, HDAC: Histone deacetylases, IL: Interleukin, JAM: Junction adhesion molecules.*

As mentioned, serotonin is a vital product made through the gut microbiome. Serotonin production is reliant upon the availability of the amino acid tryptophan, its precursor, from the diet. Approximately 90% of the body's serotonin is produced from enterochromaffin cells, and functions to facilitate communication between the brain and the gut (Akram et al., 2024). In the

gastrointestinal tract, serotonin influences peristaltic contractions and motility of the gut, enhance gut secretions, while also having an integral role in sensory perception of both fullness and pain (O'Mahony et al., 2015). In the central nervous system, serotonin functions separately, primarily involved in mood and stress regulation, along with regulation of sleep and appetite (Akram et al., 2024; O'Mahony et al., 2015). The serotonin that is produced in the gastrointestinal tract does not directly influence the brain. However, indirect communication is possible through modulation of the gut-brain axis via endocrine, immune, and neural signalling. Due to this, serotonin is both a potential means in which gut-brain axis signalling and in turn host health can become dysregulated but also can act as a potential therapeutic target (Xu and Lu, 2025).

Studies have established that the microbiota-gut-brain axis also plays a functional role in impacting BBB and intestinal barrier integrity, regulating neurotransmitter synthesis, and modulating inflammatory responses (Cryan et al., 2019). For this thesis, however, the primary interest lies in the behavioural changes that are impacted because of disruption in neurodevelopment. For decades, preclinical studies have highlighted the importance of a healthy gut microbiome, where dysbiosis adversely alters rodent behaviour (Hao et al., 2024; Liu et al., 2022). Similar evidence is now being reported in clinical studies (Nuzum et al., 2024; Xiong et al., 2023). However, in clinical studies it is challenging to determine the role of an abnormal gut profile in NDDs, and how significant an effect that the microbiome might have in contributing to these behavioural alterations.

3.4 The maternal microbiome during pregnancy

Pregnancy prompts the microbiome to adapt in numerous ways to aid in the support and growth of the fetus. The maternal microbiome is notably different from that of a healthy, non-pregnant counterpart. The typical changes seen in the maternal microbiome are discussed in the following sections.

3.4.1 Gut microbiome

During pregnancy, significant changes occur in the female body to allow for the rapid growth of the fetus, as well as to support and nourish the mother. The maternal gut microbiome is one of these systems that adapts and contributes substantially to healthy neurodevelopment (Kimmel et al., 2024; Orchanian and Hsiao, 2025; Pronovost et al., 2023; Vuong et al., 2020). Throughout pregnancy, the composition of the gut microbiome is influenced by the endocrine system, the immune system, and the metabolic system. During pregnancy, oestrogen and progesterone levels rise significantly. Both reproductive hormones contribute significantly to reshaping the maternal microbiome (Qi et al., 2021). This includes the promotion of beneficial genera that may influence energy harvesting such as *Akkermansia* and *Bifidobacterium* and modulate inflammation (Amato et al., 2024). Studies in Phayre's leaf monkeys noted a reduced microbial diversity that was negatively correlated with progesterone concentration (Mallott et al., 2020). Perhaps the most obvious work highlighting the role of female reproductive hormones on the microbiome comes from studies examining those with reduced or altered oestrogen function (Baker et al., 2017).

The microbiome of a healthy, pregnant woman has been compared to that of someone suffering from metabolic syndrome (Nuriel-Ohayon et al., 2016). This includes symptoms such as insulin resistance, weight gain, glucose intolerance, and continuous inflammation (Nuriel-Ohayon et al., 2016). This is coupled with a reduction in α-diversity and increased β-diversity in the microbiome (Koren et al., 2012). The similarities between pregnancy and metabolic syndrome have been shown via faecal microbial transfer (FMT) from women in the third trimester to mice which resulted in insulin resistance, weight gain, and a pro-inflammatory phenotype (Koren et al., 2012). Some of the same microbial signatures can also be seen, with reduced levels of SCFA-producing *Faecalibacterium* as is evident in the metabolic syndrome (Haro et al., 2016). In general, butyrate, propionate, and acetate are the most dominant SFCA metabolites evident in pregnancy (Hayward et al., 2020; Nilsen et al., 2020; Ziętek et al., 2021).

Other notable changes include an increased level of Firmicutes, *Bifidobacterium*, and the mucin-digesting *Akkermansia*, which are strongly associated with the growing need for energy extraction and storage (Gorczyca et al., 2022; Ionescu et al., 2022). Most of the alterations are seen in the later stages of pregnancy (Neuman and Koren, 2017). It is important to note that the overall composition of the maternal gut microbiome is also strongly influenced by multiple external factors, such as preconception lifestyle and diet, in addition to medications such as antibiotics (Gohir et al., 2015; Khan et al., 2016). All these changes together would be classified as dysbiosis in a non-pregnant individual but are essential during pregnancy to supporting the fetus development.

The maternal gut microbiome also appears to influence placental growth and development. This has been shown in preclinical animal models with a depleted microbiome, where placental growth was stunted and significantly reduced (Pronovost et al., 2023). This finding coincided with reduced volume and density at the site of maternal-fetal exchange, termed the placental labyrinth, which subsequently reduced fetal-placental vasculature (Pronovost et al., 2023). Several further animal studies have demonstrated the positive effects that SCFA supplementation has on the developing placenta, including increased placental size, improved spiral arteries, and reduction in inflammatory markers (Jin et al., 2022; Pronovost et al., 2023). This highlights the potential need for appropriate levels of SCFAs, such as butyrate and propionate, to be maintained across the gestational period.

3.4.2 Vaginal microbiome

While other microbiomes are less studied, emerging evidence has identified the importance of the vaginal and oral microbiomes on both maternal and fetal health. The vaginal microbial population drastically differs in composition and function in comparison to the gut microbiome. While the vaginal microbiome can be modified by the menstrual cycle, contraceptive use, and sexual activity, the conventional microbial population is heavily dominated by *Lactobacillus* (Saraf et al., 2021). *L. crispatus*, *L. iners*, and *L. gasseri* are some of the primary species

in a healthy vaginal microbiome, but there are approximately 20 different *Lactobacillus* species present (Saraf et al., 2021). While *Lactobacillus* predominance is not necessary for a healthy vaginal microbiome, high levels of lactic acid are required to prevent pathogenic opportunists such as human immunodeficiency virus or *Trichomonas vaginalis* (Saraf et al., 2021). As the lactic acid maintains an acidic pH of 3.5-4.5, along with production of other antimicrobial compounds such as hydrogen peroxide and bacteriocins, the vaginal microbiome provides excellent environmental protection (Saraf et al., 2021).

In relation to pregnancy, there is a dearth of information regarding changes in the vaginal microbiome. However, studies have shown certain changes within the composition of the vaginal microbiome when compared to healthy, non-pregnant women. The vaginal microbiome becomes more stable during the early stages of the first trimester and is known to become less diverse and rich in bacterial species and is consolidated by *Lactobacillus* species predominance (Aagaard et al., 2013; Romero et al., 2014). Infections can result in a decrease in *Lactobacillus*, leading to a reduction in the concentration of lactic acid which increases the pH environment (Diop et al., 2019; Saraf et al., 2021). This decrease of lactic acid and more neutral pH reduces protection and increases susceptibility to further infection (Diop et al., 2019; Saraf et al., 2021). Intrauterine infections, which have been linked to vertical transfer of vaginal infections, have been linked to several conditions such as placenta previa and cervical insufficiency, along with pre-term birth (Goldenberg and Thompson, 2003; Gonçalves et al., 2002; Park et al., 2009; Romero et al., 1992; Thorp et al., 1989).

3.4.3 Oral microbiome

The microbiome of the oral cavity plays an important role in our overall general health. Containing over 200 bacterial species, alterations can be linked with various disease-states (Paster et al., 2006; Willis and Gabaldón, 2020). In relation to oral disease, a strong association has been found between

periodontitis and an increased concentration of Spirochaetes and Bacteroidetes (Costalonga and Herzberg, 2014). Oral dysbiosis has even been linked to certain cancers, with an increase in *Capnocytophaga gingivalis* and *Prevotella melaninogenica* evident in oral cancer, and *Tannerella forsythia* and *Porphyromonas gingivalis* in oesophageal cancer (Mager et al., 2005; Peters et al., 2017). Interestingly, links between disruptions in the oral microbiome and cystic fibrosis, Alzheimer's disease, cardiovascular disease, pancreatic and colorectal cancer have been reported (Dominy et al., 2019; Fan et al., 2018; Kato et al., 2016; Teles and Wang, 2011; Whiley et al., 2015).

The oral microbiome also plays a role in maintaining a healthy pregnancy. Numerous studies have suggested the presence of a bidirectional communication pathway between the oral microbiome and the pregnancy itself (Saadaoui et al., 2021). When compared to a non-pregnant woman, there is a significant shift in bacterial populations in the oral microbiome. *Candida, Aggregatibacter actinomycetemcomitans*, *Streptococcus, Porphyromonas gingivalis,* and *Staphylococcus* are increased during pregnancy, along with the overall viable microbial load (Fujiwara et al., 2017). Periodontal diseases have been linked to unfavourable pregnancy outcomes including pre-term birth, low birth weight, and miscarriage (Farrell et al., 2006; Moore et al., 2004).

3.4.4 Placental microbiome

While the gut microbiome appears to play a significant role in supporting placental function, recent research has increasingly turned its attention to the possibility of a distinct placental microbiome. However, this topic remains highly controversial. Findings across studies vary dramatically, some provide compelling evidence for the presence of microbes within the placenta, while others report complete sterility (Aagaard et al., 2014; Kennedy et al., 2023; Pelzer et al., 2017; Perez-Muñoz et al., 2017). Assuming such a placental microbiome does exist, its origin remains unclear. One prevailing theory proposes that microbes may translocate from the gut microbiome. This idea is supported by studies in pregnant mice, which demonstrated bacterial migration from the gut

to the mammary glands and certain lymph nodes (Perez et al., 2007). Building on these findings, it has been hypothesized that microbes might reach the placenta via hematogenous transfer, passing through epithelial gaps in the gut lumen and eventually colonizing placental tissue (Walker et al., 2017).

However, a recent publication may have settled this long-standing debate. Kennedy et al. argue convincingly that previous reports of microbial presence in the placenta were likely the result of DNA contamination during clinical sample collection, ultimately reinforcing the view that the placenta is sterile (Kennedy et al., 2023).

3.5 Microbiome influences on neurodevelopment

The offspring's microbiome is malleable and plastic. It is easily influenced by numerous exposures across life. These have been linked to various neurodevelopmental outcomes in later life, and both are discussed in detail in the following sections. The various ways in which the maternal microbiome can influence neurodevelopment are delineated in **Figure 4**.

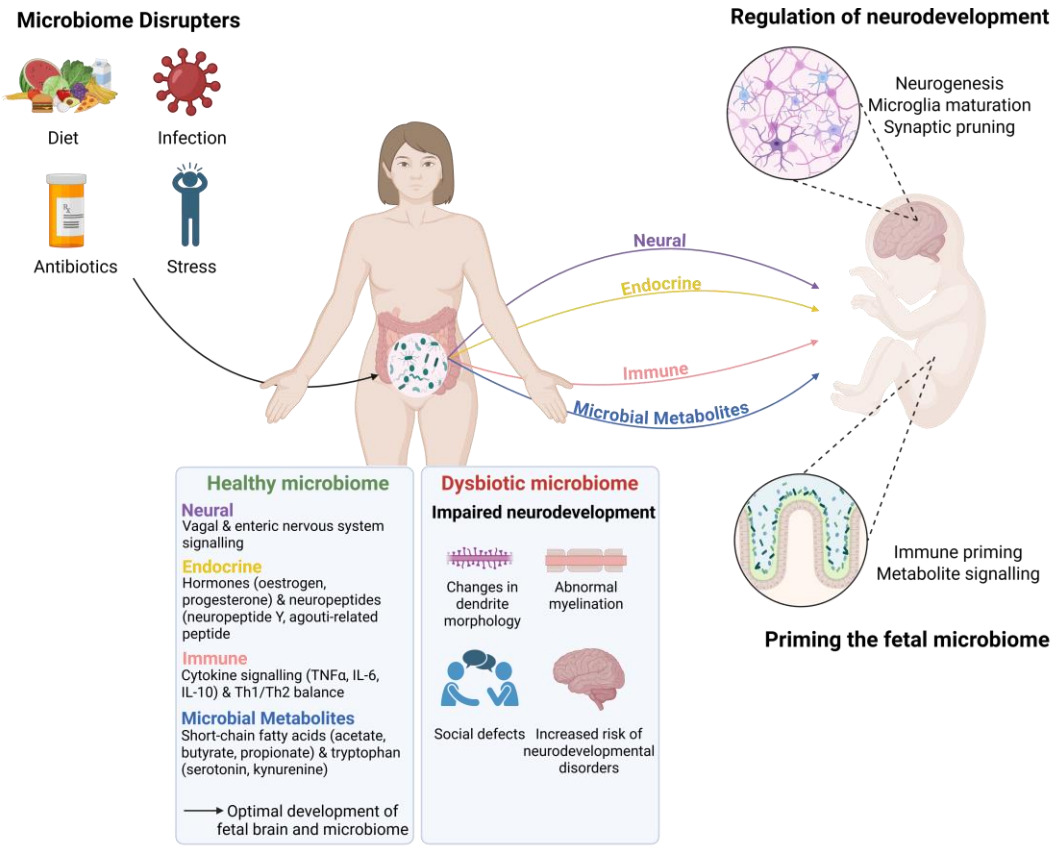


Figure 4: Schematic showing the role of the microbiota-gut-brain axis in pregnancy. *Four of the major routes of communication between the gastrointestinal tract and the brain are detailed, each of which are important for fetal development. Key processes in how the maternal microbiome regulates neurodevelopment and prepares the fetal microbiome are listed, along with suboptimal neurodevelopmental outcomes in cases of a dysbiotic microbiome. Potential disruptors of the maternal microbiome are also listed.*

3.5.1 Prenatal microbial influences

3.5.1.1 The impact of maternal antibiotic use during pregnancy

A Dutch study noted that approximately 20% of women are prescribed an antibiotic during pregnancy, with the prescription rate comparable across Europe (de Jonge et al., 2014). In human observational studies, prenatal antibiotic exposure significantly increases the risk of developing ADHD and ASD (Njotto et al., 2023; Straughen et al., 2023; Tao et al., 2022). Regarding the increased odds of developing ADHD, studies report varying levels of antibiotic exposure are required for neurodevelopmental consequences to occur, with one study reporting an association after one course and another study requiring three courses (Njotto et al., 2023; Straughen et al., 2023). Human maternal antifungal exposure has also been associated with an increased risk of developing ADHD;

however, this has only been noted in males, indicating that there may be a sex difference of prenatal exposures, which aligns with the current literature on sex differences in the prevalence of ADHD (Straughen et al., 2023). Antibiotics are often used in preclinical research to examine how the maternal microbiome influences fetal brain development.

3.5.1.2 The impact of prenatal psychological stressors on the microbiome and offspring brain

Maternal prenatal stress is an umbrella term encompassing psychological distress, physical stressors, and exposure to major life events. Psychological stress has been associated with altered fetal neurodevelopmental trajectories. One proposed mechanistic pathway involves stress-induced dysbiosis of the maternal gut microbiome, which may influence neurodevelopment through immune, endocrine, and metabolic signalling pathways. Although accumulating evidence supports the interplay between maternal stress, microbiome alterations, and fetal brain development, the causal mechanisms remain incompletely understood and warrant further investigation (Gur et al., 2017). In animal models, acute restraint stress is often used to model maternal psychological stress, through restricting the dam's movement for approximately 2-hours a day for multiple gestational days.

This stress paradigm has been shown to lead to increased anxiety and alterations in the levels of brain-derived neurotrophic factor (BDNF) in the placenta and the amygdala in adulthood, likely due to elevated IL-1β (Gur et al., 2017). The authors reported that the affected offspring's microbiome was disrupted compared to controls, with an decreased relative abundance of Firmicutes which are important for SCFA production, Bacteroidetes which play a function in nutrient absorption, and Rikenellaceae which has been previously linked to fear and temperament changes in children (Christian et al., 2015; Gur et al., 2017). Disrupted social behaviour is often reported following the restraint stress paradigm, accompanied by a profile of gut-brain axis alterations including altered microbial composition, neuroinflammation, and tryptophan

processing/metabolism (Galley et al., 2021; Gur et al., 2019). The alterations seen in the offspring's microbiome may be in part due to high levels of proinflammatory cytokines that are amplified in cases of psychological stress (Graham et al., 2022).

Shifts in infant microbial composition following chronic levels of self-reported maternal psychological prenatal stress are also seen in humans (Aatsinki et al., 2020). This includes an increased abundance in many possible inflammatory genera from the Proteobacteria group, such as *Haemophilus* and *Campylobacter*, as well as *Finegoldia* and *Veillonella* from the Firmicutes group (Aatsinki et al., 2020). The same study found a negative association between *Lactobacillus* and hair cortisol concentration, which measures the early and mid-gestation timepoint (Aatsinki et al., 2020). While there is no doubt that prenatal stress impacts cognition and motor development, the association with the maternal microbiome requires further elaboration (Van Den Bergh et al., 2020).

3.5.2 Postnatal microbial influences

3.5.2.1 How pre-term birth impacts long-term neurodevelopment

Pre-term birth, defined as delivery before 37 weeks' gestation, affects approximately 15 million infants annually (WHO, 2023). Following premature delivery, there is a high risk of a disability diagnosis in the affected offspring, this risk correlates with the degree of prematurity (Bourke et al., 2019). Major brain development occurs in the final trimester, and delivery during this critical window may lead to brain injury and other neurodevelopmental consequences (Matthews et al., 2018; Seki et al., 2021). The gut microbiome of these infants has previously been shown to have unique microbial profiles (Aguilar-Lopez et al., 2021). Using 16S sequencing, recent work has shown overgrowth of *Klebsiella* in the gastrointestinal tract of these premature infants, an increased abundance of *Bifidobacterium* and *Finegoldia* species were also noted. Interestingly, these changes were seen in those with severe brain injuries, while those without were reported to have elevated levels of *Streptococcus* and *Enterococcus* (Seki et al.,

2021). Despite the many microbial changes, *Klebsiella pneumoniae* was found to be significantly associated with brain injury, with the authors noting that *Klebsiella* overgrowth is a key mediator in the dysregulation of the infant's microbiome-immune-brain axis, perhaps aggravating the brain injury (Clarke et al., 2021; Seki et al., 2021).

3.5.2.2 The influence of caesarean-section on neurodevelopment

The newborn's microbiome is heavily influenced by the mode of delivery. During vaginal delivery, the infant is colonised with bacteria present in the mother's vaginal canal, including *Lactobacillus* which is beneficial and predominant in healthy women, and certain faecal bacteria, such as *Bacteroides* and *Escherichia* (Zhou et al., 2023). In contrast, Caesarean-section (C-section) delivery results in colonisation of *Staphylococcus* and *Klebsiella* (Zhou et al., 2023). Despite C-sections being a vital and often lifesaving healthcare intervention, they may be overutilised in high-to-middle income countries, and they may be associated with neurodevelopmental risks (Betrán et al., 2016). However, there is a significant overlap between C-section deliveries and pre-term births, potentially acting as a cofounding variable and complicating the effects on the offspring (Thanh et al., 2019). Research has examined the long-term effect of C-section on cognition and identified an increased risk of intellectual disability, ADHD, and ASD (Tianyang Zhang et al., 2019; Zhang et al., 2021).

Additionally, evidence shows, at least for ASD, that this association may be familial, confounded by genetic and/or environmental factors (Curran et al., 2015). Deficits in gross and fine motor skills, along with language development have been noted by 5 months old (Zheng et al., 2022). Problem-solving has also been found to be delayed at 3 years following C-section delivery (Sznajder et al., 2022). Interestingly, the risk of certain disorders, such as ASD, seems to be greater if the C-section is elective (Zhang et al., 2021). It is possible that the underlying reasons for the C-section and its consequential effects on altered microbial colonisation, may play a role in the neurodevelopmental deficits.

Preclinical research also has identified a role for the gut microbiome in the enduring behavioural effects induced by a C-section delivery (Cabré et al., 2022). Given the gut microbiome alterations following C-section delivery do not appear to endure, there remains an urgent need to demarcate the time windows when interventions should be applied. Disruption to metabolite production and overall functional capacity of certain microbial species may occur in early life that may have lasting consequences. The behavioural alterations that are seen in the human population have also been shown in preclinical studies and provide insight into the mechanisms underpinning these changes. C-section has been shown to negatively affect behaviour across the lifespan of mice, with increased levels of anxiety and altered sociability evident in adulthood (Morais et al., 2020). Interestingly, these neurobehavioural alterations were partially rescued following co-housing with mice born conventionally, allowing a natural transfer of the microbiome. Similar positive changes were also noted following administration of *Bifidobacterium breve*, highlighting the potential impact that a probiotic intervention may have on these known C-section deficits. It has also been noted that this model results in increased body weight (Martinez et al., 2017). Interestingly, prenatal and peripartum exposure to antibiotics interface with C-section delivery to modify microbiome profiles in human infants, a feature which needs to be integrated into a more translational animal model (Wong et al., 2020).

Research has attempted to ameliorate the effects of a C-section birth on microbial colonisation, by examining the impact of 'seeding' the neonate with maternal vaginal secretions. Seeding aims to recolonise the neonate's gut to match a vaginally born infant, which will have the microbes of the maternal gut and vagina, as opposed to the microbes of the maternal skin (Mueller et al., 2020). Recolonisation may alleviate the negative developmental outcomes that can be associated with C-section delivery (Zhou et al., 2023). While not widely used, preliminary results have found that vaginal seeding of C-section exposed infants leads to neonate intestinal recolonisation which partially matches a vaginally born infant (Dominguez-Bello et al., 2016; Zhou et al., 2023). Recolonisation also helped to regulate levels of important metabolites and

metabolic functions, including an increase in carbohydrate, amino acid, and energy metabolism and increased levels of L-lactic acid, homovanillic acid, and N-acetyl-D-glucosamine, which may potentially lead to better outcomes in later life (Dominguez-Bello et al., 2016; Zhou et al., 2023).

Individuals that received the vaginal microbial transplant had improved neurodevelopmental outcomes in comparison to those who received the saline control (Zhou et al., 2023). Separate research has shown that maternal FMT displays promising results in normalising gut bacterial composition in C-section compared to vaginal births in mice (Korpela et al., 2020). Conflicting research indicates no positive effect of seeding (Wilson et al., 2021). While findings suggest the possibility of intervention at this early stage, to prevent development of certain disorders, there is currently limited evidence supporting this technique given that most studies are too small and exploratory in nature to form an actionable evidence base at present. Others have highlighted the potential dangers of seeding the infant with a suboptimal vaginal microbiome and/or one containing harmful pathogens, including *Streptococcus agalactiae*, and various sexually transmitted infections, such as *Neisseria gonorrhoeae* or *Chlamydia trachomatis,* with screening of donor samples an important consideration in future guidelines (Cunnington et al., 2016).

3.5.2.3 The difference between feeding methods

The chosen method of infant feeding can help shape the overall health of the infant in later life; including an impact on various NDDs. Breastmilk is recognised as the “gold-standard” for infant nutrition, and this is in part due to its unique composition of micro- and macronutrients, healthy fats, and hormones necessary for brain health (Deoni et al., 2018; WHO, 2003). Infant formula, usually derived from cows or soy milk, still acts as a sound nutritional alternative but lacks complex compounds. Numerous infant formula companies now add additional supplements including prebiotics such as galactooligosaccharides (GOS) or human milk oligosaccharides (HMO). These additions not only improve the overall quality of the formula but have also been shown to modulate the

infants gut microbiome in a similar fashion to breastfeeding (Borewicz and Brück, 2024). Furthermore, when compared to normal formula, supplemented formula fed infants were found to have reduced proinflammatory cytokines and a similar immune profile to breastfed infants (Goehring et al., 2016). Vitamin D supplementation is common in formula; however, breastfed infants typically require additional supplementation as it is not present in adequate doses (Ziegler et al., 2014). Despite the improvements in formula composition, breastmilk is easier to digest and produces fewer gastrointestinal symptoms in comparison to formula (Huërou-Luron et al., 2010). Breastmilk composition changes substantially over time, and this is a potential drawback of infant formula. The components of colostrum, which is the first type of milk, is high in protein and factors to support the infants' immune system and is drastically different to mature milk which is richer in carbohydrates and fats (Ballard and Morrow, 2013). Breastmilk evolves alongside the developing infant, providing what is needed at each timepoint, and this in part may contribute to its favourable neurodevelopmental outcomes.

3.5.2.4 The neurodevelopmental benefits of breastmilk

Breastfeeding has been recommended by the WHO for a minimum of 6 months but should ideally remain part of the diet until the infant reaches 2-years (Kielbratowska et al., 2015; Taut et al., 2016; WHO, 2003). Through breastfeeding, the infant receives nutrients including essential proteins (lactoferrins and caseins), prebiotics, polyunsaturated fats, and immunoglobulins (Mosca and Giannì, 2017). These nutritional compounds positively influence the developing microbiome of the infant and can increase the abundance of beneficial microbes, including *Bifidobacterium*, and are also linked to a decreased abundance of other bacteria including *Clostridium* and *Bacteroides* species (Savage et al., 2018). Interestingly, infants fed breastmilk exclusively have a less diverse and more immature microbial composition than those receiving infant formula (Davis et al., 2022). However, in this context, low diversity is perhaps more beneficial for the infant and recent evidence supports the idea that

breastfeeding supports an appropriately paced assembly of the gut microbiome to control the acquisition of microbial species and functions (Shenhav et al., 2024). Feeding type is known to alter immune and metabolic gene expression and this ultimately enhances the transcription of genes that are associated with immune and metabolic processes (Milani et al., 2017). The microbial diversity in the gut of breastfed infants is reduced, and this is seen as a beneficial in very early life (O'Sullivan et al., 2015). Breastmilk encourages the survival and promotes growth of fewer bacterial species; however these genera, such as *Bifidobacterium* are commensal, beneficial bacteria that aid in HMO metabolism (O'Sullivan et al., 2015). In comparison, the microbiome of formula fed infants is more adult-like with a greater diversity and an increased presence of potentially pathogenic or inflammatory species (Milani et al., 2017; O'Sullivan et al., 2015). Breastfeeding for a recommended period is strongly associated with optimal neurodevelopment, including a reduced risk of ADHD, with a similar protective effect found with ASD and schizophrenia (Al-Farsi et al., 2012; Bar et al., 2016; Sørensen et al., 2005; Tseng et al., 2019a, 2019b).

Breastfeeding is also associated with greater IQ scores and enhanced cognition (Brahm and Valdés, 2017; Mortensen et al., 2002). Magnetic resonance imaging studies show white matter microstructural development is increased in areas associated with language, vision, and higher order thinking and cognition following longer periods of breastfeeding, or when comparing breastfed to formula-fed infants (Ottolini et al., 2020). The role that the gut microbiome has in the neurodevelopmental benefits of breastmilk is still to be fully elucidated, but one potential component, milk fat globule membrane (MFGM), has shown potential in improving certain neurodevelopmental outcomes following maternal separation in rats (Collins et al., 2022; O'Mahony et al., 2020). This includes spatial learning and memory, along with reducing visceral sensitivity stemming from the early life stress (Collins et al., 2022; O'Mahony et al., 2020). In the same study, a polydextrose and GOS prebiotic-mix also displayed potential in improving learning and memory along with helping the HPA axis return to baseline levels following restraint stress, with the combination of prebiotics and MFGM proving to have some greater effects (O'Mahony et al., 2020).

3.5.2.5 The impact of antibiotic use in the postnatal life

Antibiotics are often given prophylactically to infants in the neonatal intensive care unit and overuse is common, with earlier exposure during the first year of life a greater risk factor for subsequent difficulties (Slykerman et al., 2023; Stocker et al., 2023; Tripathi et al., 2012). Antibiotic exposure in early life is known to negatively impact the infant's gut microbiome, which is of lower diversity and more unstable, and thus more susceptible to being influenced by external factors compared to that of an adult (Gibson et al., 2015). Early antibiotic use can delay the maturation of the infant's microbiome and reduces the diversity of species present, negatively impacting the microbiota-gut-brain axis, and influencing brain function and behaviour (Cryan et al., 2019; Morowitz et al., 2022; Russell et al., 2021; Sherman et al., 2015).

Infants who received a course of antibiotics within their first 6 months have been found to have a higher risk of developing behavioural and emotional problems, as well as having deficits in language development (Slykerman et al., 2019). The result from this study strongly supports the accumulating evidence that the microbiome is involved in neurodevelopment. Interestingly, the suboptimal neurodevelopmental outcomes were not as severe in infants that received a course of antibiotics aged 6-12 months. These children had improved cognitive outcomes when compared to those that received antibiotics in the first 6 months of life (Slykerman et al., 2019). Infants receiving their first course of antibiotics aged 12-24 months were shown to have enhanced cognition in comparison to both previous timepoints (Slykerman et al., 2019). This suggests that a critical microbiota-gut-brain axis window may potentially exist. However, it is important to note that this does not mean that antibiotic exposure in later life will have no neurodevelopmental consequences. Research previously performed by the same group noted that antibiotic exposure within the first 12-months of life showed a negative impact on overall intelligence and reading ability (Slykerman et al., 2017). Additionally, higher levels of ADHD-like behaviour and depressive-like symptoms were noted in the periadolescent period, with an increased risk of ADHD being found if the exposure to antibiotics was within the first two years

(Njotto et al., 2023; Slykerman et al., 2017). However, the risk of children developing ASD is conflicting, with some studies reporting an increase in ASD risk following antibiotic usage in early life, whereas others found no association (Hamad et al., 2018; Njotto et al., 2023; Wimberley et al., 2018). In contrast to other findings, it has also been reported that oral vancomycin treatment can transiently alleviate ASD symptoms (Sandler et al., 2000). Despite this, it is important to note the small sample size of this study (10 participants), and the improvement significantly waned at the follow-up visit after the initial treatment. Animal studies also confirmed the results found from these observational human studies, demonstrating brain and behavioural changes following early life antibiotic administration (Kayyal et al., 2020; Lynch et al., 2023; Otten et al., 2022). Recent work has aimed to identify a specific temporal window in which microbial depletion is most critical (Lynch et al., 2023). For example, a more targeted depletion of the gut microbiome following broad-spectrum antibiotic treatment has unveiled varying microglia morphologies and gene expression in rodents, dependent upon time of treatment. This work highlights differential effects on cellular morphology and genetic expression dependent upon timing of the exposure, and this may be the key in relation to future neurodevelopmental outcomes. While this study only notes very subtle behavioural alterations, it provides insight into potential mechanisms.

3.5.2.6 The impact of postnatal stressors on the microbiome and offspring brain

The relationship between postnatal stressors, such as maternal separation, and neurodevelopment has also been explored, albeit mostly in animal studies. Maternal separation has long been used as a model of early life/postnatal stress, adverse exposures, and importantly, gut-brain axis dysfunction (O'Mahony et al., 2011). The isolation of the pups from postnatal day 2 until 12 has not only been shown to disrupt the serotonergic system, but to also alter microglia, and lead to a chronic state of inflammation via polyunsaturated fatty acids (Clarke et al., 2009; Nicolas et al., 2022; O'Mahony et al., 2008). Maternal separation has also

been shown to alter behaviour in rodents such as increased anxiety levels and reduced learning and memory (Collins et al., 2022; De Palma et al., 2015). Since the initial observation that this is also associated with microbiome disruption, it has subsequently been demonstrated that different aspects of the emergent phenotype are microbiome dependent (anxiety-, depression-like behaviour) and independent (HPA axis dysfunction) (De Palma et al., 2015; O'Mahony et al., 2009). A recent study noted that a subset of offspring exposed to maternal separation in early life showed a resilient phenotype to pain and depressive-like behaviours in later life (Wilmes et al., 2024). The resilient offspring were found to have a unique microbial profile, with metabolites involved in thiamine and pyruvate metabolism significantly altered (Wilmes et al., 2024). This concept of a resilient microbiome provides a unique opportunity for future intervention studies to build on these promising results.

Other postnatal stress models, such as the limited bedding and nesting model also disrupt normal maternal caregiving behaviours, which acts as a stressor in the young offspring. *Coprococcus* was found to be decreased in the late juvenile period with a reduction of *Rikenella* and the Rikenellaceae RC9 gut group in adulthood (Reemst et al., 2022). Parental social isolation has also been linked to a disrupted microbiome (Siddi et al., 2024). In this study, juvenile rats were housed individually until adulthood, the pups born to this generation, the F1 generation, displayed a disrupted gut microbiome, with an increase in Clostridales, Rhodospirillales, and Bacteriodales, accompanied with a near deficit of *Limosilactobacillus reuteri* (Siddi et al., 2024). While this study did not sample the microbiome from the generation receiving the postnatal stress, disruption to the microbiome was sufficient to negatively influence the following generation, inferring the severity of the microbial disruption.

In relation to human studies, there has been little work examining the effect that this specific stressor may have on neurodevelopmental outcomes. What is known however, is that separation of mother and child negatively impacts breastfeeding, which has been shown to impact long-term neurodevelopmental outcomes (Cimino and Cerniglia, 2023; Conti et al., 2021).

3.6 Microbiome and specific neurodevelopmental disorders

The association between the gut microbiome and neurodevelopment has gained increasing support, particularly through studies demonstrating distinct microbial compositions in individuals with NDDs compared to neurotypical controls. Selected NDDs and their corresponding microbiome profiles will be examined in the following sections.

3.6.1 Autism spectrum disorder

People on the ASD spectrum often suffer from gastrointestinal symptoms, which may be due to disruption of the microbiota-gut-brain axis (Alamoudi et al., 2022; Saurman et al., 2020). Disruption in the gut microbiome could relate to the relative bacterial abundance differences in 27 genera, including *Akkermansia*, *Bifidobacterium*, *Clostridium*, and *Ruminoccocus*, when comparing ASD to controls (Alamoudi et al., 2022; Sivamaruthi et al., 2020). Building on this, a recent study identified 10 bacterial strains that could be used to identify those as risk of ASD. Some of these strains include *Klebsiella oxytoca*, *Bifidobacterium longum*, *Streptococcus thermophilus*, and *Clostridium ramosum* (Ye et al., 2021). Unfortunately, this study was only performed on males. Recent analysis has identified a unique signal for individuals with ASD that has been driven by 591 microbes (Morton et al., 2023). This, coupled with 138 microbial encoding genes linked to ASD through shotgun sequencing, may prove to be a beneficial aid in diagnosis (Morton et al., 2023). While some of the results may be conflicting, a strong understanding of the microbial differences between those with ASD and neurotypical controls is emerging.

Gut microbiome differences are also observed in animal models of ASD, including greater relative abundance of Firmicutes and Clostridia, along with lower abundance of Bacteroidetes species (Alamoudi et al., 2022; Sauer et al., 2019). A marked reduction in the relative abundance of *Bifidobacterium* and *Blautia* were also shown in the BTBR mouse model of ASD. The authors note that this shift was linked to alterations in important bile acids and tryptophan

metabolism, both known for their role in regulating the gastrointestinal tract (Golubeva et al., 2017).

Of note, common ASD symptoms, particularly repetitive behaviours and sensory preferences, are linked to certain food choices, and typically a restrictive diet leads to a less diverse microbial composition (Yap et al., 2021). While this suggests that diet drives the microbiome alterations that have been reported, we should not discount the ongoing role of the gut microbiome in ASD symptom expression, or that it might represent a tractable target in this context.

In experimental research, FMTs from donors with ASD to mice transferred the donor's microbiome, led to ASD-like phenotype in the mice, with decreased communication and locomotion, and increased repetitive behaviours (Sharon et al., 2019). The shift in microbiome composition correlated with reduced social and repetitive behaviours, and *Eisenbergiella tayi* (02b40_Lachnospiraceae) correlated with increased repetition and social defects (Sharon et al., 2019). Overall, this study shows the potential causal role that the gut microbiome has in the pathophysiology of ASD, or at least of specific symptom profiles, and indicates that targeting the gut microbiome may be an effective therapeutic option for ASD.

Yet, other work has deduced potential mechanisms, such as vagus nerve activation in the modulation of certain ASD symptoms (Sgritta et al., 2019). Previous work has noted that administration of specific bacteria can activate vagus nerve signalling via intrinsic primary afferent neurons (Perez-Burgos et al., 2014). This has been seen following *Limosilactobacillus reuteri* administration in the *Shank3B*-/- mouse model, noting a negative impact on social interaction following vagotomy, indicating an indirect mechanism for beneficial microbes in altering symptom severity (Sgritta et al., 2019). Probiotic interventions, using species such as *Bifidobacterium longum*, *Lacticaseibacillus rhamnosus*, and *Lactobacillus acidophilus*, helped restore the microbiome and reduced the severity of gastrointestinal and autistic symptoms in children with ASD (Shaaban et al., 2018; Sivamaruthi et al., 2020). A 12-strain probiotic mix, given to young children with an ASD diagnosis for 3 months, drastically improved behavioural

symptoms, including stereotypical behaviour, hyperactivity, and social withdrawal (Khanna et al., 2025). Improvements were also seen with gastrointestinal issues, including constipation and diarrhoea (Khanna et al., 2025).

FMT interventions have also been tested, which involve antibiotic consumption, fasting, bowel cleansing, and a particular refinement of FMT from neurotypical donors along with stomach acid suppressants. This resulted in reductions in gastrointestinal symptoms, enhanced microbial richness and plasma metabolites, and alleviation of autistic symptoms such as social skills, speech, and irritability in children with ASD. Unfortunately, there was no placebo included in these trials (Kang et al., 2020, 2019, 2017). These positive changes occurred after the initial microbiome transfer therapy and persisted for 2-years following cessation of the transfer, with some symptoms improving further after the end of the study (Kang et al., 2019). Across the timeframe of the study, an increase in *Prevotella* was noted, along with significant decreases in the abundance of certain microbes that had low uncertainty (Morton et al., 2023). Abundance of *Anaerobutyricum* and *Butyricimonas* species, which are established SCFA producers, remained stable across the study which may be potentially crucial in modulating symptoms (Morton et al., 2023). While not yet deduced, it is possible that SCFAs may improve ASD symptoms by reducing glial inflammation, improving BBB integrity, reduce permeability, and overall neurological support (Silva et al., 2020). The beneficial effects of probiotics and microbial transplants highlight the intricate relationship between the gut microbiome and ASD, as well as the potential for it to act as a therapeutic target.

3.6.2 Attention deficit hyperactivity disorder

The gut microbiome has also been implemented as a mediating factor influencing ADHD. A significant amount of our understanding comes from human studies. When compared to those without ADHD, studies have found significant alterations in the composition of the gut microbiome, yet findings are not consistent (Cickovski et al., 2023a; Pinto et al., 2022; Stiernborg et al., 2023;

Wang et al., 2023). Differences between individuals with ADHD and controls include an elevation in abundance of *Odoribacter splanchnicus*, *Bifidobacterium* and Bacteroidaceae species, and lower abundance of *Faecalibacterium prausnitzii* (Gkougka et al., 2022a; Sukmajaya et al., 2021; Wang et al., 2023). Increased Actinobacteria abundance is also associated with reduced ADHD symptom severity (Wang et al., 2023). Given that the prior studies indicate a relationship between gut microbiome and ADHD, studies have since investigated the therapeutic use of probiotics in ADHD. One study found that *Lacticaseibacillus rhamnosus GG* administration, when given during pregnancy and early life, can significantly reduce the overall risk of ADHD and improve outcomes such as social and emotional functioning in children (Kumperscak et al., 2020). Beneficial impacts on behaviour in children with ADHD were also observed with *Bifidobacterium bifidum* consumption, and a multi-strain probiotic, which alleviated symptoms of inattention and impulsivity (Pinto et al., 2022).

However, no effect was seen with synbiotic administration of a *Lactobacillus* based probiotic and 3 different fermentable fibres on ADHD symptoms (Skott et al., 2020). The synbiotic seemed to have a greater effect on those with elevated levels of soluble vascular cell adhesion molecule-1 before the beginning of the trial, with these adults displaying improved emotional regulation. Apart from probiotic interventions, additional research has investigated the effect of FMT on ADHD, where a transplant from a neurotypical donor led to increased abundance of the anti-inflammatory and beneficial *Faecalibacterium prausnitzii*, reduced abundance of *Bifidobacterium longum*, and a reduction in ADHD symptoms (Hooi et al., 2022;Wang et al., 2022). While this result is promising, this outcome was in a single participant. To generate more conclusive evidence, extensive work is warranted to fully understand if targeting the microbiome via probiotics or FMT is a useful therapeutic intervention in ADHD. Furthermore, the use of animal models has been greatly underutilised in studying this topic. Limited models do exist and are validated, this includes the spontaneously hypertensive model and neonatal 6-Hydroxydopamine model (Kantak, 2022). Enriching the diet with omega 3 polyunsaturated fatty acids was found to ameliorate

symptoms of hyperactivity and impulsivity in males in a spontaneously hypertensive ADHD rat model, particularly through decreasing impulsive behaviours and improving attention (Dervola et al., 2012).

3.6.3 Schizophrenia

Schizophrenia is a mental health disorder also categorised as a NDD, although emerging later in life compared to ASD and ADHD, typically in the early 20s, is more convincingly associated with the potential modulatory role of the gut microbiome (McGuinness et al., 2022; Murray et al., 2023; Nikolova et al., 2021). Studies examining both males and females have noted that individuals with schizophrenia display a different gut microbial composition to those without schizophrenia, including a lower abundance of *Clostridium*, *Faecalibacterium* and *Ruminococcus* species, with greater abundance of *Lactobacillus*, *Collinsella*, and *Anaerococcus* (Li et al., 2020; Nguyen et al., 2019). At the genus level, individuals with schizophrenia had greater α- and β-diversity, and had a greater abundance of anaerobic bacteria, along with various species that typically reside in the oral microbiome (Zhu et al., 2020b). Taken together, these marked changes may indicate microbial dysbiosis. While limited animal work has been performed linking the microbiome and schizophrenia, one study demonstrated significant behavioural changes in mice that had received FMT from donors with schizophrenia (Zhu et al., 2020a). These changes include impaired learning and memory and psychomotor activity and highlights the severe disruption of the donor's microbiome and provides insight into how the microbial alterations seen in the gut may be affecting the gut-brain axis and behaviour.

While probiotic treatments have improved certain gastrointestinal symptoms, potentially due to their immunomodulatory effects, no definitive change in schizophrenia symptom profiles were evident (Dickerson et al., 2014; Tomasik et al., 2015). Of note, distinct microbial profiles are associated with different symptoms of schizophrenia, such as positive symptoms that include hallucinations and delusions, or negative symptoms such as anhedonia,

avolition, and issues with interpersonal relationships (Marder and Umbricht, 2023; Wang et al., 2023). Positive symptom phenotypes are positively correlated with *Lactobacillus*, while negative symptoms are positively correlated with Lachnospiraceae and Ruminococcaceae species abundance (Nocera and Nasrallah, 2022). The severity of symptoms can also be correlated with numerous bacterial species in the gut, and furthermore, the microbial profile is known to diversify following the first episode of psychosis (Li et al., 2020; Schwarz et al., 2018). Given that the literature highlights differential microbial profiles between patients based on their symptomology, future research should consider different phenotypes and trajectories of schizophrenia when assessing and implementing gut-based therapeutics. The potential for modulating the gut microbiome is not as clear with schizophrenia as it is for ASD and ADHD, yet the hope remains that careful microbial targeting, and regular psychotropic medication, may improve the outcomes and lives of those with schizophrenia.

3.7 Interplay between inflammation and the microbiome

The relationship between the gut microbiome and inflammation is complex and bidirectional (O'Riordan et al., 2025b). The gut has immunoregulatory effects, dampening the concentration of pro-inflammatory cytokines such as tumour necrosis factor (TNF)α and IL-6 and stimulating the production of anti-inflammatory cytokines such as IL-10 (Kim et al., 2025; Schirmer et al., 2016). Bacterial derived metabolites play a huge role in helping to maintain the immune system, through preservation of the gastrointestinal epithelial barrier and their interaction with immune cells resident in the gut (Kim et al., 2025). SCFAs, for instance, can maintain intestinal homeostasis and prevent inflammatory-based diseases by regulating gene transcription and increasing immunoglobulin production, while also acting as an energy source for the epithelial cells (Kim et al., 2025; O'Riordan et al., 2025b; Schirmer et al., 2016). Furthermore, the gut microbiome is required to promote the development and the maturation of the immune system, through mucin secretion, development of mucosal-associated

lymphoid tissue, and promotion of immune cell maturation (Lécuyer et al., 2014; Zhao et al., 2023).

In contrast, the microbiome may also drive the immune system towards a more activated and harmful state (O'Riordan et al., 2025b). Dysbiosis of the gut microbiome can negatively affect overall immune function. One of the most direct pathways responsible is through intestinal barrier damage, which when compromised, increases permeability of gap and tight junctions resulting in harmful pathogens invasion and intestinal damage, with the subsequent production of pro-inflammatory cytokines (Zhao et al., 2023). This disturbance of the gut microbiome can alter the production of SCFAs, mucin, and amino acid synthesis (Zhao et al., 2023). GF mice, which are a rodent model that lack a microbiome, have highlighted the importance of a healthy, balanced microbiome for the proper functioning of the immune system. Studies have revealed that GF mice have a reduced level of activation of the immune system in response to pathogen challenge, with reduced antibody production (Delgado-Ocaña and Cuesta, 2024).

SCFAs have been repeatedly shown to play an integral role in regulation of the immune system. These dietary fibre-derived molecules have the capability to influence both the differentiation and the function of numerous immune cells through HDAC inhibition. HDAC inhibition via SCFAs modulates the activity of B cells, macrophages, dendritic cells, and T cells (Kim, 2018) leading to a shift in the production of pro- or anti-inflammatory cytokines. Separately, tryptophan-derived metabolites such as indoles are known to further modulate T-cell activity, suppressing the activity of inflammatory Th17 cells and promoting the activity of regulatory T-cells (Gasaly et al., 2021). Leucine, isoleucine, and valine, all produced by the gut microbiome have been shown to support the immune system, particularly T cell maintenance. Other amino-acid related compounds, such as spermine and putrescine, have similar properties in relation to T cell regulation (Kim, 2018). Conversion of primary bile acids to secondary bile acids by the gut microbiome further contributes to immune system regulation via T-cell differentiation and macrophage cytokine production (Kim, 2018). Most of the

work surrounding this has been performed in rodent models. Building on this, numerous models of neurodevelopment, such as ASD and schizophrenia, support a combined role of the microbiome and inflammation in their pathogenesis (O'Riordan et al., 2025b).

Taken together, all of these microbial metabolites play a significant role in maintaining the immune balance, suppressing and regulating excess inflammation. However, with the microbiome and the immune system being so interconnected, it is easy to disrupt the homeostasis of both entities. Despite this, it also allows for a potential therapeutic approach in the form of an anti-inflammatory diet or a probiotic which will be discussed in more detail in the following sections.

3.8 Modulating the microbiome for neurodevelopment and brain health

The microbiome can be shaped by numerous external influences, and this provides opportunities for therapeutic intervention. While this section is not exhaustive, some of the key determinants will be discussed in the following sections.

3.8.1 The role of diet in shaping the microbiome

An individual's diet is of utmost importance to the composition of their gut microbiome, and in turn their overall health (Armet et al., 2022; Cryan et al., 2019). In fact, research has shown that changes in gut microbiome composition can occur within 24 hours of changing an individual's diet (David et al., 2014). This reflects the dynamic interaction between diet and the gut microbiome. The complex relationship between the gut microbiome, diet, and health outcomes has been consolidated through examining the Western diet. This diet is usually adopted by first-world countries and consists of high-fat, sugar, and salt, accompanied by a reduction in the number of plant and fibre intake (Malesza et al., 2021). The Western diet has been shown to induce inflammation and alter immune responses, reduce SCFA-producing bacterial species in the gut, and

increase the number of mucin-utilising microbes that are forced to obtain energy from the mucosal lining of the gut (Agus et al., 2016; Cabral et al., 2020; Li et al., 2025). Increases in Proteobacteria and *Bacteroides* species, and a reduction in Firmicutes is also linked to consumption of a Western diet (Agus et al., 2016; Cabral et al., 2020). The Western diet is also associated with numerous metabolic disorders and cancers, with the microbiome very much intertwined in the pathophysiology (Clemente-Suárez et al., 2023).

In comparison, the Mediterranean diet, which consists of high volumes of fruits and vegetables, nuts and legumes, healthy fats and fibre, has been shown to reduce the incidence of cancers, psychiatric conditions, and neurodegenerative diseases (Cryan et al., 2019). The Mediterranean diet is just one example of an anti-inflammatory diet whereby the focus is on whole, nutrient-rich foods that reduce systemic inflammation through the consumption of healthy fats, vitamins, antioxidants, dietary fibre, and polyphenols (Yu et al., 2024). The anti-inflammatory effects of a Mediterranean diet have been shown to have a restorative capacity on the gut microbiome, promoting an anti-inflammatory environment, stabilising diversity, and increasing SCFA production (García-Montero et al., 2021; Li et al., 2025). These findings offer the promising potential for the development of dietary interventions aimed at modulating the gut microbiome to enhance overall health outcomes. In terms of neurodevelopment, there is a wealth of existing research highlighting the importance of diet in the preconception and gestational period. Evidence has shown that elevated homocysteine levels is associated with poor psychomotor development, while folic acid, which aids in closure of the neural tube, is beneficial to verbal and cognitive development (Murphy et al., 2016; Murray et al., 2018; Roigé-Castellví et al., 2019; T. Suzuki et al., 2022).

Additionally, excessive consumption of highly processed foods is associated with lower infant developmental scores (de Lauzon-Guillain et al., 2022). Animal models have also demonstrated the potential for nutrient deficiencies, such as polyunsaturated fatty acids, in amplifying the effects of stressors such as maternal immune activation on locomotion and memory in affected offspring

(Leyrolle et al., 2021). However, when studies examine the effects of consumption of dietary micro- and macronutrients during pregnancy on neurodevelopment, the results are mixed, and this evidence was captured in a systematic review where limited associations were reported (Veena et al., 2016). However, examination of the maternal microbiome is missing from some of these studies in this systematic review, which is a likely mediator between maternal diet and infant neurodevelopment consequences.

Recent evidence has also evaluated links between the overall state of the maternal microbiome and the long-term health of the offspring (Daliry and Pereira, 2021). In humans, consumption of an overall anti-inflammatory diet, like the Mediterranean diet as previously mentioned, in the preconception period appears to lower the risk of impairments in numerous neurodevelopmental parameters, such as motor skills, communication, and problem-solving skills (Kyozuka et al., 2022). Diets rich in fat and sugar have also been linked to poor cognitive development, while also disrupting the gut microbiome of the offspring in these rodent models of obesity (Bhagavata Srinivasan et al., 2018; Sanguinetti et al., 2019). In humans, pre-pregnancy obesity was associated with alterations in the relative abundance of *Lactobacillus* and *Bifidobacterium* genera, which was subsequently associated with an increased risk of developing NDDs (Adane et al., 2016; Liu et al., 2021). Despite growing interest, the role of the microbiome in preconception diet has yet to be fully elucidated as most observational dietary studies do not yet routinely examine the maternal microbiome. However, future evidence from studies using animal models may give insight and direction towards a better understanding of the underlying mechanisms.

3.8.2 Prebiotics for brain and gut health

Prebiotics are defined as a “substrate that is selectively utilised by host microorganisms conferring a health benefit” (Gibson et al., 2017). Notable prebiotics include inulin, fructan, polyphenols, and digestive fibers (Cohen Kadosh et al., 2021; Cryan et al., 2019). Despite a long list of candidates, GOS and fructooligosaccharides (FOS) are the most researched and well-established.

Prebiotic administration in preclinical models has yielded promising results in improving both brain and gut health. Ingestion of oligosaccharides has been shown to increase hippocampal BDNF and N-methyl-D-aspartate receptor expression in adult rats (Sarkar et al., 2016). This has been linked to enhanced learning and memory in comparison to controls (Sarkar et al., 2016). Separately, they have also been shown to reduce stress-induced corticosterone release and improve behavioural outcomes in the open field test (Burokas et al., 2017). Inulin consumption has been found to improve overall gut health which has been shown through a rat model of distal colitis, while also increasing the abundance of *Lactobacillus* genera (Videla et al., 2001).

In terms of neurodevelopment, there is limited direct evidence that supports a beneficial role. Work in preclinical models is promising, with early life GOS administration increasing levels of BDNF in the hippocampus even after cessation of consumption and HMO was found to improve learning and memory up to one year after consumption (Oliveros et al., 2016; Williams et al., 2016). However, it has been challenging to replicate these results in clinic. Prebiotics were found to help reduce gut-associated symptoms in people with ASD but did not improve neurological symptoms of ASD or schizophrenia (Ansari et al., 2023). Building on this, prebiotics administration was unable to improve the deleterious neurodevelopmental outcomes associated with pre-term birth (Upadhyay et al., 2020). Despite this, there is still promise for prebiotics. High levels of GOS are found in human breastmilk, which may help contribute to the beneficial effects that breastfeeding has on neurodevelopment (Cryan et al., 2019). Infant formula is often supplemented with GOS, and it has been found to substantially increase the abundance of *Lactobacillus* and *Bifidobacterium* in the infant gut, almost mimicking that of breastfed infants (Cryan et al., 2019). The mechanism of action behind GOS is not fully apparent, and this is still a rather novel area of research in terms of neurodevelopment.

3.8.3 Probiotics for enhancing neurodevelopment and gut health

Probiotics have been previously defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Gibson

et al., 2017). Perhaps the most well-documented and familiar way to positively modulate the gut microbiome is the administration of probiotics, which has gained considerable interest as of late. These beneficial bacteria have been shown to exert their positive effects through numerous mechanisms. Probiotics possess the ability to immunomodulate the host through the production of IgA from B cells in gut-associated lymphoid tissue. Here, B cells undergo expansion and produce immunoglobulins in response to cytokines such as IL-6, in turn this also improves gut barrier defence. Furthermore, probiotics have the ability to interact with monocytes, lymphocytes, and macrophages in the gut via toll-like receptors (TLR). Through this, probiotics can upregulate the production of beneficial anti-inflammatory cytokines such as IL-10, while also restricting the production of pro-inflammatory cytokines like IL-6. This mechanism allows probiotics to regulate the host's immune state, limiting excessive inflammation and promoting immunotolerance. This is similar for chemokines. These inflammatory mediators play a greater role than just immune balance, in certain cases they can also promote healing and repair of the gastrointestinal mucosa. Probiotics can further influence the intestinal barrier by upregulating the secretion of mucus by goblet cells. This mucus acts as a protective barrier, both biochemically and physically. The expression of numerous tight junctions, including occludins and claudins, can also be modulated by probiotic consumption, ensuring that the epithelial barrier of the gastrointestinal tract maintains integrity and does not become leaky, allowing unwanted, foreign substances to pass through to the blood (Mazziotta et al., 2023). Finally, probiotic consumption allows for an increase in the number on Paneth cells in the gut, which in turn produce a number of antimicrobial peptides which protect against harmful, pathogenic bacteria that may be residing in the gut (Cazorla et al., 2018). Various probiotics can exert both immunoregulatory and immunostimulatory mechanisms through differential effects in terms of cytokine release (Mazziotta et al., 2023). While the exact mechanism behind whether a pro- or anti-inflammatory pathway is activated is still unclear, it is generally understood that probiotic administration has proven to exert numerous beneficial effects on the host (Cryan et al., 2019; Wieërs et al., 2020).

Probiotic administration has and continues to be examined as a potential way to improve both prenatal and postnatal neurodevelopmental outcomes. Results are inconsistent in clinical settings, often highly dependent upon duration and dosing (Eastwood et al., 2021; Godfrey et al., 2021; Korpela et al., 2018; Lin et al., 2023; Panchal et al., 2023; Slykerman et al., 2018). More promising evidence comes from preclinical studies where probiotics have been found to improve long-term neurobehavioural outcomes such as decreased anxiety and improved cognition, positively influence the offsprings' own microbiome, and protecting neurodevelopment in models of inflammation-based disease (Krishna et al., 2022; Niu et al., 2020; Radford-Smith et al., 2022; Zhou et al., 2022). The young, developing brain is incredibly plastic and normal developmental trajectory can be modified by various exposures and experiences, as discussed in depth above. Knowing this, preclinical perinatal probiotic administration shows great opportunity as a therapeutic target by reducing inflammation and improving the microbiota-gut-brain axis, however further clinical based studies are required to improve their translational benefits.

3.8.4 Postbiotics and their potential role

Postbiotics are defined as a "preparation of inanimate microorganisms and/or their components that confers a health benefit to the host" (Salminen et al., 2021). Postbiotics are a relatively new concept, but there is emerging evidence regarding the numerous mechanisms involved in their regulation of the microbiome. This includes immunomodulation, enhancing the epithelial barrier, and signalling via the nervous system (Salminen et al., 2021). The immunomodulatory and anti-inflammatory benefits from these compounds make them a potential therapeutic target. In infants, administration has been shown to improve the severity of gastrointestinal and allergic conditions (Guamán et al., 2024). However, in the context of neurodevelopment, this area is in its infancy requires further investigation. Prebiotic, probiotic, and postbiotic mechanisms of action are detailed below in **Figure 5**.

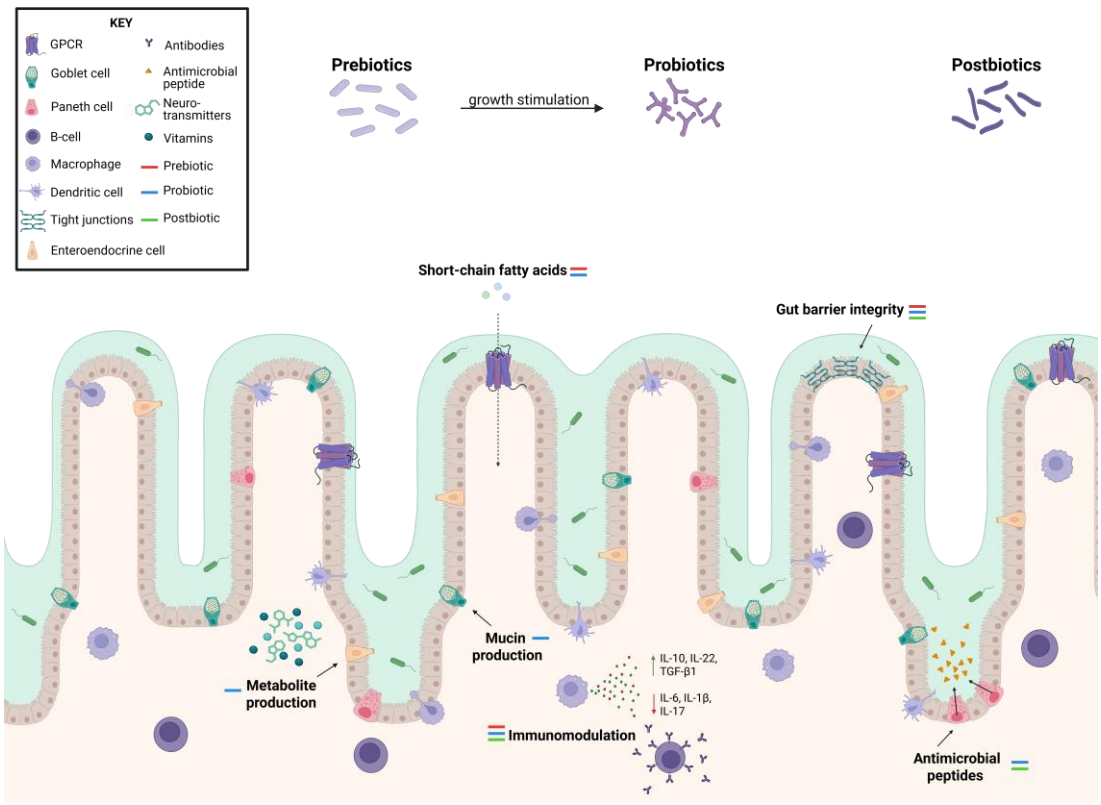


Figure 5: Schematic of different mechanisms of action for prebiotic, probiotics, and postbiotics. *Overview of how each microbiome-targeted intervention functions, denoted through a coloured line next to each mechanism. Mechanisms include production of short-chain fatty acids, metabolite production, mucin production, immunomodulation, maintaining hut barrier integrity, and finally, production of antimicrobial peptides. Prebiotic = red, probiotic = blue, postbiotic = green, as per the icon key. Abbreviations: GPCR: G-protein coupled receptor.*

4 Pre-eclampsia: A case of non-infectious maternal inflammation

Maternal inflammation is often thought to stem from infection. However, numerous pregnancies are implicated by cases of non-infectious inflammation. Pre-eclampsia is associated with maternal inflammation, its impact on neurodevelopment and the maternal microbiome will be discussed in the following sections.

4.1 Introduction to pre-eclampsia

Hypertensive disorders of pregnancy are an umbrella term for multiple disorders, including gestational hypertension, chronic hypertension, and pre-eclampsia,

which has the highest incidence rate, impacting 3-5% of global pregnancies (Mol et al., 2016). Pre-eclampsia is clinically diagnosed as *de novo* hypertension (≥140mmHg systolic and/or ≥90mmHg diastolic) after the 20th week of gestation in addition to one of the following: 1) proteinuria (≥300mg of protein in 24-hours); 2) uteroplacental dysfunction (fetal growth restriction); 3) maternal organ dysfunction, including neurological complications (such as hyperreflexia, changes in cognitive function, visual disturbances, or stroke), liver involvement (such as epigastric or right upper abdominal pain, increased transaminases levels), renal insufficiency (increased creatinine levels), and haematological issues (such as haemolysis or thrombocytopenia) (Brown et al., 2018; Tranquilli et al., 2014).

Despite the potential consequences of pre-eclampsia on the mother and the fetus, there is currently limited evidence regarding the molecular drivers of the pathophysiology. However, the predominant theory proposes defective placentation (Magley and Hinson, 2022). Ultimately, this leads to a reduced uteroplacental blood flow, resulting in placental ischemia and oxidative stress, which modifies the arteries from low-flow, high-resistance to high-flow, low-resistance vessels (Magley and Hinson, 2022). The continuous cycle of ischemia and reperfusion leads to the release of free radical species, pro-inflammatory cytokines, and antiangiogenic factors (Makris et al., 2007; Uzan et al., 2011). Endothelial dysfunction is also present as a result of these secretory mediators (Opichka et al., 2021). Women with pre-eclampsia tend to have altered inflammatory profiles, in particular increased levels of circulating pro-inflammatory cytokines, including TNFα, C-reactive protein (CRP), and IL-6 (Teran et al., 2001; Xiao et al., 2012). There is currently no cure for pre-eclampsia with the only effective treatment being delivery of the baby in addition to the clinical management of maternal hypertension with antihypertensives (Amaral et al., 2017).

4.2 Neurodevelopmental consequences of pre-eclampsia

The association between pre-eclampsia and NDDs has been identified primarily in epidemiological studies and in some preclinical models of disease. The strongest links exists with ASD, which is known to have a strong genetic component, however it is becoming increasingly evident that *in utero* experiences, such as pre-eclampsia, also have a role in its development (Lord et al., 2018). Studies have estimated that between 17% and 50% of ASD development is due to environmental contributions (Sandin et al., 2017, 2014). Numerous studies have noted that prenatal pre-eclampsia exposure increases the risks of ASD in the offspring even when controlling for confounding variables such as gestational age and birthweight (Dachew et al., 2018; Maher et al., 2018; Mann et al., 2010; Wade and Jenkins, 2016; Walker et al., 2015). Furthermore, it has been found that pre-eclampsia offspring who are often small for gestational age or growth restricted, have a 95% increased odds of developing ASD when compared to healthy babies (Maher et al., 2020b). The estimated increased risk of developing ASD from pre-eclampsia alone is approximately 32% (Dachew et al., 2018; Maher et al., 2018). The molecular mechanisms involved in this association are still unclear; however, *in vitro* studies have demonstrated that fetal neurons exposed to pre-eclampsia serum have altered cortical growth and branching, while no changes were evident upon exposure to placental explant cultures (Curran et al., 2018; Scott et al., 2018). This result would suggest that the increased risk is due to MIA, that is closely associated with pre-eclampsia (Jiang et al., 2018; Maher et al., 2019).

ADHD is characterised by impulsivity, high activity, and inattention (Gumusoglu et al., 2020). This too tends to be increased where mothers experience pre-eclampsia, with a 15% increased likelihood of developing ADHD (Maher et al., 2018). Looking at sibling-matched studies, it has been shown that these increased odds of developing ADHD is not due to familial environment or other genetic components, suggesting it is solely due to the *in utero* experience (Maher et al., 2020a). Likewise in studies combining small for gestational age babies with pre-eclampsia exposure, have demonstrated that the combination of these two

exposures leads to a 43% increased odds risk of developing ADHD (Maher et al., 2020a). MIA has been proposed as the potential mechanism (Böhm et al., 2019; Shelton et al., 2015; Wolford et al., 2017). Furthermore, sex-based differences are evident in the relationship between pre-eclampsia and ADHD. Studies have demonstrated that female offspring from a pre-eclampsia pregnancy appear to have a greater increased risk of ADHD, whereas in the normotensive pregnancy population, male offspring are most affected (Silva et al., 2014).

The overall risk of developing schizophrenia is also increased in association with pre-eclampsia, with studies suggesting an odds ratio of 1.3, this increases to 2.0 in cases of pre-term birth (Eide et al., 2013). Numerous studies have provided evidence linking pre-eclampsia with various cognitive issues. Infants tend to have a lower IQ when pre-eclampsia presents with intrauterine growth restriction (IUGR), in addition to mild cognitive impairments (Heikura et al., 2013; Many et al., 2003; Warshafsky et al., 2016). Other severe and debilitating conditions such as epilepsy and infantile spasms, cerebral palsy, and stroke at any age have also been associated with pre-eclampsia (Kajantie et al., 2009; Mann et al., 2011; Nahum Sacks et al., 2019; van Wassenaer et al., 2011).

4.3 Microbiome alterations in pre-eclampsia

Numerous studies have found drastic and significant shifts in the gut microbiome of women with pre-eclampsia in comparison to women with normotensive pregnancies. A 2021 study found significant decreases in *Varibaculum*, *Prevotella*, *Lactobacillus*, and *Porphyromonas* genera when compared to normotensive pregnancies, coupled with an increase in Bacteroidetes (Huang et al., 2021). In particular, *Prevotella*, which is known to aid in the protection against pathogens and produces SCFAs, was reduced by 50% in the microbiome of women with pre-eclampsia compared to healthy pregnant women (Huang et al., 2021; Kovatcheva-Datchary et al., 2015).

Studies have also found that women with pre-eclampsia tend to have lower levels of *Coprococcus*, which is a known producer of the important and beneficial SCFA, butyrate (Altemani et al., 2021). Interestingly, lower levels of

butyrate are also seen prior to the onset of symptoms of pre-eclampsia, and this is thought to be involved in increasing the risk of developing pre-eclampsia, highlighting the potential developmental role of the gut microbiome (Altemani et al., 2021). Furthermore, it has been shown that pre-eclampsia leads to an increased concentration of circulating lipopolysaccharide (LPS), which is known to be associated with inflammatory processes, in addition to increases in Gammaproteobacteria and Enterobacteriaceae (Kell and Kenny, 2016; Wang et al., 2019). The differences between the microbiome of normotensive and pre-eclamptic pregnancies have also been investigated in relation to gestational age, and while no differences were found during the second trimester, a significant shift was seen in the third trimester when compared to normotensive pregnancies (Wang et al., 2020). This finding showed an increase in Bacteroidetes and Proteobacteria, mirroring findings from other studies, and a decrease in Firmicutes, which is vital for energy harvesting (Wang et al., 2020).

Building on this, it has been found that certain gut microbiome deficits can be linked to the clinical symptoms of pre-eclampsia. *Akkermansia muciniphila*, a beneficial microbe that produces many metabolites from degrading intestinal mucus, such as SCFAs, has been associated with metabolic health (Zhang et al., 2019). Studies have found reduced *Akkermansia* in the intestines of women with pre-eclampsia, and this was negatively correlated with hypertension and proteinuria levels (Jin et al., 2022). These women displayed increased inflammation and significantly impaired barrier function, reduced faecal and placental levels of propionic and butyric acid in women with pre-eclampsia (Jin et al., 2022). Furthermore, they note the potential of incorporating the maternal gut profile as an additional diagnostic tool (Jin et al., 2022). Administration of *Akkermansia* and both butyrate and propionate to a preclinical rat model of pre-eclampsia ameliorated the clinical symptom of hypertension, improved gut barrier function, reduced placental inflammatory factors and improved spiral artery remodelling (Jin et al., 2022). Other FMT studies have also reported similar results of microbiome alterations, poor barrier function, and suggest a possible link between the gut-placental axis via *Fusobacterium* (Chen et al., 2020).

The disrupted microbiome seen in pre-eclampsia has opened up the possibility of microbiome-targeted therapeutic interventions. Probiotics have been suggested to benefit those women with pre-eclampsia by potentially positively modifying blood pressure, along with modifying both systemic and placental inflammation (Brantsæter et al., 2011). A summary of clinical findings is noted in **Figure 6**. A Norwegian study demonstrated that milk-based probiotics reduced the overall risk of developing pre-eclampsia, and its more severe phenotype (Brantsæter et al., 2011). This has been supported by the findings in other studies, where probiotic milk was found to reduce the risk of developing pre-eclampsia. Interestingly, this was only seen when the probiotic was taken in the later stages of gestation (Nordqvist et al., 2018). Furthermore, a genome-wide association study meta-analysis concluded that *Bifidobacterium* had a protective effect on the development of pre-eclampsia (Li et al., 2022). These effects may also extend to other genera, *Eubacterium*, Lachnospiraceae, and *Collinsella*, all known SCFA producers (Li et al., 2022). There is strong evidence that details the lack of SCFA-producing bacteria in the microbiome of women with pre-eclampsia. This is also true in pre-eclamptic animal models where probiotic supplementation has been shown to have a positive effect. Due to this, probiotic administration may potentially be a successful intervention in alleviating the maternal symptoms and lead to better outcomes for both the mother and baby.

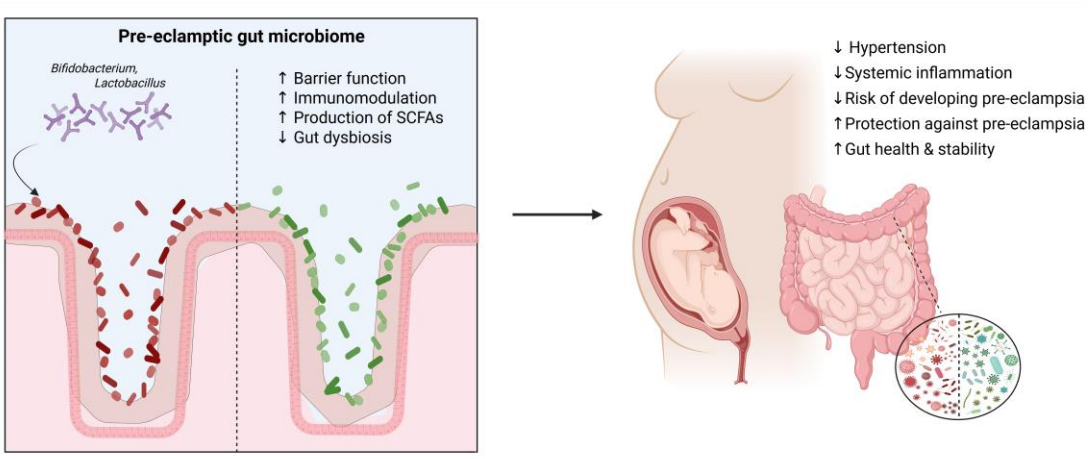


Figure 6: Clinical success of probiotics as a potential therapeutic intervention in pre-eclampsia. *Results from clinical studies revealing that administration of Lactobacillus and Bifidobacterium can improve the primary symptoms of pre-eclampsia and overall gut health, while also decreasing the overall risk of pre-eclampsia.*

5 Sex differences in Neurodevelopmental disorders

Biological sex can be described as a risk factor for certain NDDs. This is due in part to the influence of sex chromosomes, hormones, and the sexual dimorphism of the placenta, which behaves differently dependent upon the fetal sex. The mechanisms underpinning sex differences in the brain and the gut will be discussed in the following sections.

5.1 Sex-specific risk for neurodevelopmental disorders

NDDs tend to differ in prevalence and symptomatology across the two biological sexes, and this has led to what is often referred to as male bias. This is seen in three of the major neurodevelopmental disorders, ASD, ADHD, and schizophrenia.

5.1.1 Sex differences in autism spectrum disorder

ASD diagnosis ratio for male:female is currently 3:1 and is perhaps the most well-known example of this sex bias (Zablotsky et al., 2017). Typically, males present with more externalising behaviours, such as aggression and hyperactivity, along with higher motor and language scores (Breach and Lenz, 2023; Carter et al., 2007; May et al., 2016; Santos et al., 2022). Females display better executive

function and the ability to maintain reciprocal conversation but are also more likely to suffer with comorbid anxiety and depression (Breach and Lenz, 2023; May et al., 2014; Santos et al., 2022; Stacy et al., 2014). Differences are also seen in the neurobiological profile of males and females diagnosed with ASD. The male brain often displays cortico-cerebellum hypoconnectivity, whereas in the female brain, hyperconnectivity is present (Smith et al., 2019). The male cerebellum is often enlarged, whereas increased white matter volumes has been found in the female parietal and occipital lobe (Chen et al., 2017; Santos et al., 2022). This has been shown in numerous preclinical studies, mimicking the human evidence (Cho et al., 2017; Foley et al., 2015; Jung et al., 2018).

The high rate of diagnosis in males may be due to the more 'stereotypical' presentation of these disorders. Taking ASD as an example, females, as mentioned, often present symptoms in a different manner that may not be as clearly associated with NDDs (Santos et al., 2022). Females tend to have high rates of camouflaging behaviour, or 'masking' their symptoms that would be deemed as socially unacceptable, and this also may contribute to underdiagnosis (Lai et al., 2017). It is difficult to know if diagnostic inaccuracies are amplifying the diagnosis ratio and potentially inflating the sex gap. Regardless of whether this is true or not, there are marked differences between the male and female brain that may be contributing to these discrepancies. Examining why and how this sex differences occur is of utmost importance to understanding the individual risks and potential sex-based therapies going forward.

5.1.2 Sex differences in attention deficit hyperactivity disorder

In ADHD, the male:female diagnosis ratio is 2.3:1 in children, but the gap shrinks to 1.6:1 in the older population (Willcutt, 2012). Following a similar trend, males display an increased level of hyperactivity, aggression, and impulsivity, whereas females displayed decreased cognition and high levels of comorbidity with depression (Coxe et al., 2021; Gershon and Gershon, 2002; Loyer Carbonneau et al., 2021; Mphahlele et al., 2020; Rucklidge, 2008; Stibbe et al., 2020). Numerous neurobiological sex differences are associated with ADHD, including

electroencephalography differences in theta brain wave activity, with males having a global increase and females displaying a local increase, primarily in the frontal regions of the brain (Hermens et al., 2005). The hyperactivity and inattention symptoms typically associated with males have been correlated with decreased volumes of grey matter in the right cerebellum, right temporoparietal-opercular region, and the bilateral anterior and mid-cingulum (Biondo et al., 2022). However, in females that displayed these symptoms, increased volumes of grey matter were reported in both the cerebellar-fusiform region and temporoparietal-opercular region (Biondo et al., 2022). These discrepancies clearly highlight the importance that dimorphism in certain neural regions may have on behavioural outputs.

5.1.3 Sex differences in schizophrenia

Sex differences in schizophrenia are not as large as those reported for ASD or ADHD, with a male:female ratio of 1.4:1 (McGrath et al., 2008). Male diagnosis, often around the early 20s, is associated with negative symptoms including deficits in learning and memory, and social cognition, and a decreased prepulse inhibition (Kumari et al., 2004; Mu et al., 2020). Females tend to display fewer and less severe negative symptoms (Zorkina et al., 2021). In studying the neurobiological differences in the male schizophrenic brain, there is greater levels of axonal damage and a reduced brain volume coupled with enlarged ventricles (Lang et al., 2018; Mendrek and Mancini-Marïe, 2016).

5.2 Mechanisms underlying sex differences in neurodevelopmental disorders

5.2.1 Sex chromosomes

Being male increases the risk of a NDD diagnosis in later life. This male bias begins immediately at fertilisation with the determination of the sex chromosomes. There are two main reasons why the XY chromosomes are seen as unfavourable in this context, primarily due to hemizygosity. With only one copy

of the X chromosome from the mother, any potential harmful mutation is essentially unchallenged by what would be the second X chromosome in females (Lukin et al., 2024). It has been estimated that approximately 6% of NDDs can be linked to an X-linked coding variation (Martin et al., 2021). This in part may be due to the X chromosome containing approximately 20% of the genes associated with NDDs which are important for overall brain health, specifically cognition and neuronal plasticity (Brand et al., 2021).

The Y chromosome can also be linked to contributing to suboptimal development. Studies have shown that an additional Y chromosome, as in 47,XYY, doubles the risk of ASD and ADHD diagnosis (Ross et al., 2015). The rate of ASD diagnosis in this cohort is between 19-50%, but in the case of 47,XXY, while there are some notable learning disabilities, the ASD diagnosis rate is far lower at 5-10% (Ross et al., 2015). Females with a third X chromosome, which is seen in 47,XXX, do not have any additional protection against neurodevelopmental disorders. Those with 47,XXX have a significant increased risk of developing ASD and ADHD, along with defects in social cognition, language, and executive function (van Rijn, 2019). Studies examining sex chromosome trisomy reveal a unique role for the Y chromosome, but the mechanism involved are incompletely understood. Of course, having 47,XYY or 47,XXY syndrome is an extreme example, but it does highlight the role that the Y chromosome may have in combination with the disadvantageous single X chromosome.

5.2.2 Prenatal sex steroids

While oestrogen is neuroprotective in later life, there is no obvious protection in the prenatal period. In fact, exposure to high levels of prenatal androgens or oestrogen have both been linked to an increased ASD diagnosis rate in early life. While both hormones are crucial in fetal brain development, androgens for masculinisation of the brain and oestrogen contributing to neurogenesis, elevations of these sex steroids can be detrimental (Breach and Lenz, 2023; Denley et al., 2018).

In cases of polycystic ovarian syndrome in the mothers, the developing fetus is exposed to high levels of testosterone *in utero* (Cesta et al., 2020). Exposure can lead to an increased risk of ASD and ADHD diagnosis in later life, along with Tourette's chronic tic disorder (Cesta et al., 2020). Interestingly, females were found to be more impacted by this hormonal shift, highlighting the possibility of differential effects dependent upon sex (Cesta et al., 2020). Similar results are seen with congenital adrenal hyperplasia, characterised by increased testosterone exposure, leading to enhanced autistic traits (Knickmeyer et al., 2006). ASD has also been linked to elevated levels of oestradiol, oestriol, and oestrone in the amniotic fluid (Baron-Cohen et al., 2020). Due to the overall importance of oestrogen in brain development, this dysregulation may disrupt normal developmental trajectories and the endocrine system (Baron-Cohen et al., 2020). This has been demonstrated in mice with oestrogen disruption, which negatively influenced Purkinje cell growth and social behaviour (Hoffman et al., 2016). In a similar fashion the behavioural deficits seen in the mouse model of oestrogen disruption was only evident in males (Hoffman et al., 2016). Tight regulation of the prenatal sex steroids is of utmost importance due to the increased risk of ASD in the exposed offspring, and while not as obvious as other mechanisms, does influence differences between the sexes.

5.2.3 The sexually dimorphic placenta

A significant proportion of sex differences in NDDs can be directly linked to the placenta. The placenta is sexually dimorphic, and in turn grows and responds to stressors in a sex dependent manner. This leads to significant variation between male and female experiences *in utero*. The male placenta grows at a quicker rate, prioritising rapid fetal growth, while the female placenta remains more adaptable, and it is thought that this method of growth confers greater resilience to stressful *in utero* environments (Christians, 2022). Differences are also evident in immune, nutrient, and vascular gene expression between the two sexes (Gabory et al., 2012; Sood et al., 2006). In a high-fat mouse model, the female placenta gene expression regulating immune cell activity was predominant,

whereas the male placenta had greater nutrient and vascular gene expression (Gabory et al., 2012).

However, while these changes are of course important, the placental response to threats is the likely contributor to the sex-based differences in NDDs. Under immune threat of pre-eclampsia, the male placenta displays an exacerbated pro-inflammatory profile, coupled with high levels of apoptosis and increased NF-κB activation (Muralimanoharan et al., 2013). Females do not respond in this same way; there is no dramatic inflammatory response (Muralimanoharan et al., 2013). Improved modulation of immune factors within the female placenta may be a superior protective mechanism in comparison to the male placental response (Gabory et al., 2012; Sun et al., 2020). The elevated levels of pro-inflammatory cytokines in the male placenta may have a direct impact on the developing fetal brain by crossing the placental barrier and modulating neuronal growth (Dammann and Leviton, 1997; Woods et al., 2023).

Separately but perhaps of equal importance, there are differences between the expression of the 11β-hydroxysteroid dehydrogenase type 2 (HSD2) enzyme. 11β-HSD2 is a protective enzyme that is highly expressed in the syncytiotrophoblast layer of the placenta and acts as its own barrier to protect the fetus (Yang, 1997). Maternal glucocorticoids, such as cortisol, can damage the sensitive fetal brain, 11β-HSD2 is able to metabolise active cortisol to the inactive metabolite cortisone, which is not harmful (Sun et al., 1999). Studies have revealed that the male placenta has reduced expression of 11β-HSD2 when exposed to high levels of maternal glucocorticoids (Yu et al., 2022). Furthermore, at times of maternal stress, methylation of the HSD11B2 gene can downregulate expression (Peña et al., 2012). This may have a more pronounced impact in the male placentas, which would facilitate increased exposure to maternal glucocorticoids. Alterations in both the 11β-HSD2 enzyme and the exaggerated immune response seen in the male placenta may be contributing factors to the increased risk of NDDs in later life (Muralimanoharan et al., 2013; Peña et al., 2012; Yu et al., 2022). The male placenta seems to be primed to be more susceptible to stressful insults as it focuses on growth, whereas the female

placenta prioritises adaptation. These major sex differences are detailed below in **Figure 7**.

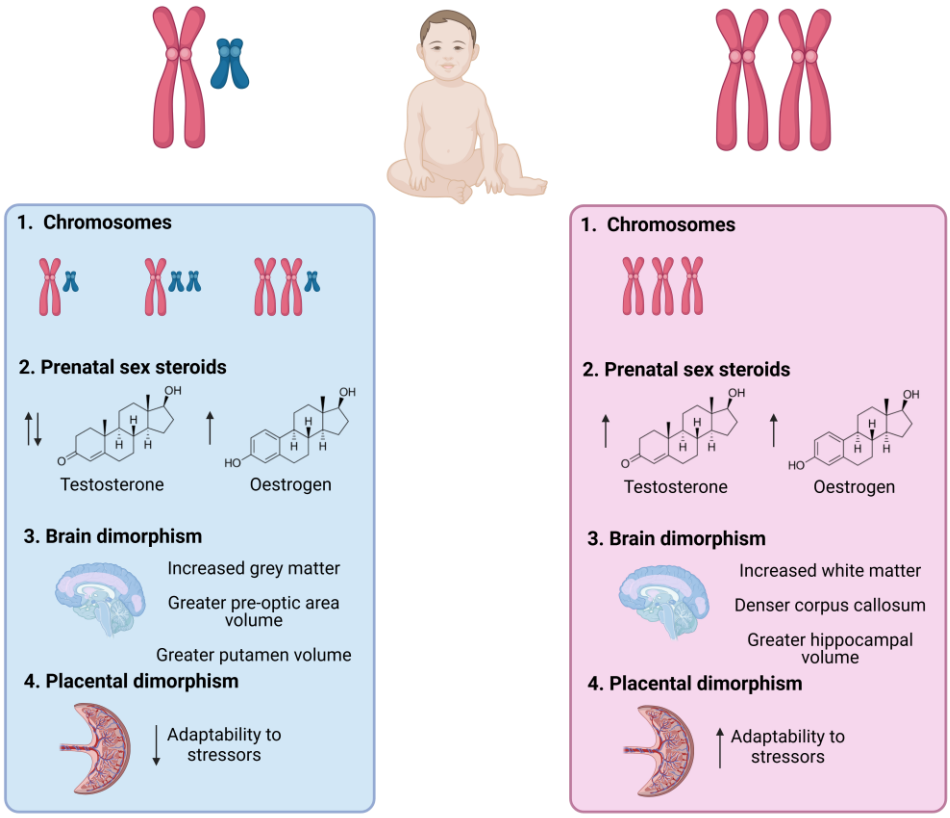


Figure 7: Significant cases of sexual dimorphism that can influence neurodevelopment. *Differences between male and female vulnerability leading to suboptimal neurodevelopmental. There are more opportunities for male sex trisomy in comparison to females. Exposure to elevated or decreased testosterone or increased oestrogen can negatively influence the male brain, whereas just elevated concentrations impact the female brain. Regional functional, structural, and connective differences seen between the sexes are seen in numerous neurodevelopmental disorders. Finally, sexual dimorphism of the placenta is responsible for differences in the fetus' capability to adapt to various stressors.*

5.2.4 Protective role of the female sex

Due to the abovementioned factors, which is not an exhaustive list, biological males appear more susceptible to NDDs, however females are not exempt from risk. Exposure to maternal inflammation or disruption of the gut microbiome can contribute to the development of ASD (Suprunowicz et al., 2024). The drastic difference in male:female diagnostic rates may in fact be due to non-typical

symptom presentation in females (Santos et al., 2022; Zablotsky et al., 2017). Despite this theory, the current statistics heavily favour enhanced male vulnerability and in part due to elevated prenatal sex steroid exposure, chromosomal abnormalities, and a less resilient placenta (Baron-Cohen et al., 2020; Hoffman et al., 2016; Martin et al., 2021; Muralimanoharan et al., 2013; Ross et al., 2015). From the beginning of puberty, females are protected further due to the sharp increase in oestrogen. Oestrogen is a potent antioxidant and anti-inflammatory mediator (Behl, 2002). This effect is typically seen decades later following menopause. The rapid decrease in oestrogen is often coupled with various neurological symptoms that can improve following hormone replacement therapy (Hogervorst et al., 2022). While *in utero* and in early prepubertal life, oestrogen is not protective, however, it reduces reactive oxygen species, promotes DNA repair, and reduces expression of pro-apoptotic genes (Bustamante-Barrientos et al., 2021; Crider and Pillai, 2017). Changes in oestrogen receptor expression and activity have previously been noted in ASD and schizophrenia, and there may be some potential therapeutic value in targeting these receptors (Crider and Pillai, 2017). Combining these factors provides females with a considerable advantage in comparison to their male counterparts. It is important however to continue research in this area to further explore why male offspring are particularly vulnerable and how their protection can be enhanced. However, it is important to note that the dimorphism of the brain and the placenta are not the only factors contributing to sex differences in neurodevelopment. Sex-specific variations in the microbiome have recently become of interest.

5.3 Sex differences in the microbiome

Perhaps not as familiar as sex dimorphism in the placenta and brain, sex differences in the gut microbiome are becoming more evident. The concept of sex-based differences is well-documented; however, the effect is quite modest, and evidence is limited. Female mice have higher levels of microbial species richness, as seen through α-diversity and abundance of certain genera such as

Akkermansia, *Veillonellaceae*, and *Enterobacteriaceae* (McGee and Huttenhower, 2021; Org et al., 2016; Yurkovetskiy et al., 2013). Numerous lifestyle and genetic factors can contribute to this dimorphism, as can the immune system (McGee and Huttenhower, 2021). However, hormonal influence continues to be the major contributor to sex differences. Before the onset of puberty, there is no evident sex differences in the gut microbiome (Ober et al., 2008). The differences between the male and female microbiome are only instigated by the increase and divergence of sex hormones in the adolescent period (Yurkovetskiy et al., 2013). Animal models have allowed for manipulation of the endocrine system to explore these effects. Testosterone mediates this divergence of the microbial community during puberty but the exact mechanism behind this is still unclear (Yurkovetskiy et al., 2013). Transfer of adult male caecal content into pubertal female mice led to masculinisation of the female's microbiome (Yurkovetskiy et al., 2013). These sex differences are maintained across adulthood in line with normal hormonal cycling (Ober et al., 2008). In later life we also see the importance of the sex hormones on the microbiome. Rat models of menopause, through oophorectomy, have shown that the reduction in oestrogen is associated with lower diversity, suggesting a protective role for oestrogen in maintaining a healthy, balanced gut microbiome (Moreno-Indias et al., 2016).

These sex differences seen in the gut microbiome are not just compositional however, they have the potential to be functional. Notably differences in glucose metabolism, carbohydrate metabolism, and energy harvesting can all be linked to gut microbiome sexual dimorphism (Gao et al., 2021; Wu et al., 2022). In terms of neurodevelopment, there is very limited evidence directly linking sex differences to NDDs, but inferences can be made. While there are no obvious sex differences in the microbiome before puberty, puberty itself occurs in a sex-dependent manner, heavily influenced by oestrogen and testosterone. Disruptions to the microbiome at this time may alter endocrine function, and in turn negatively influence the brain, which is still developing at this time. This area is one of great potential and further study could allow for a sex-based microbiome therapy.

6 Preclinical models in neurodevelopmental research

Neurodevelopmental research heavily relies on the use of preclinical models. Most of the clinical work in this field is observational and is unable to delve into mechanisms and in-depth neurological changes seen in the affected offspring. Here, some of the most relevant models will be discussed.

6.1 Models of immune activation

Preclinical models of MIA are the one of the most validated and widely used ways of studying neurodevelopmental outcomes. Two of the most renowned MIA models, LPS and polyinosinic:polycytidylic acid (Poly (I:C)) are both infection mimetics that trigger the maternal immune system (Bao et al., 2022).

LPS acts as a bacterial mimetic, derived from the cell wall of gram-negative bacteria (Bao et al., 2022). It binds to TLR4, activating both the MyD88-dependent pathway and Toll-IL-1 receptor domain-containing adapter-inducing interferon β (TRIF)-dependent pathway (Seeley and Ghosh, 2017). Subsequent activation of the activator protein 1, interferon regulatory factor 3, and NF-κB ensues (Seeley and Ghosh, 2017). This in turn influences the expression of numerous pro-inflammatory genes, including *Tnfα, Ifnα, Il6,* and *Il1β* (Hayden and Ghosh, 2012; Kawasaki and Kawai, 2014). This activation of the innate immune system has been linked to reduced placental and fetal birth weight, enhanced microglial activation, and alteration in numerous neurobehavioural outcomes in early life (Domínguez Rubio et al., 2017; Eloundou et al., 2019; Hsueh et al., 2018, 2017; Li et al., 2018).

In a similar fashion, Poly (I:C) also stimulates the maternal innate immune system as a viral mimetic (Bao et al., 2022). Activation is through binding to TLR3 which also activates the TRIF-dependent pathway (Bilbo et al., 2018). This in turn stimulates TNF receptor associated factor (TRAF) 3 and TRAF6 (Kawasaki and Kawai, 2014). TRAF3 recruits various IκB kinases (IKK) which allows for phosphorylation and dimer formation before translocation to the nucleus (Kawasaki and Kawai, 2014). Pro-inflammatory genes such as *Tnfα, Ifnα, Il6,* and *Il1β* are induced by NF-κB, activator protein-1, and interferon regulatory factor 3

(Bilbo et al., 2018; Kawasaki and Kawai, 2014). Poly (I:C) has also been shown to alter placental expression of immune-related genes (Barke et al., 2019). Furthermore, exposed offspring tend to have a dysregulated cytokine profile, with numerous pro-inflammatory cytokines present in the fetal brain, which in turn can disrupt neurotransmitter signalling and behavioural outcomes (Openshaw et al., 2019; X. Wang et al., 2019). In comparison to LPS models, Poly (I:C) more closely results in NDDs such as ASD and schizophrenia (Bao et al., 2022). The mechanism of both LPS and Poly (I:C) can be seen in **Figure 8.**

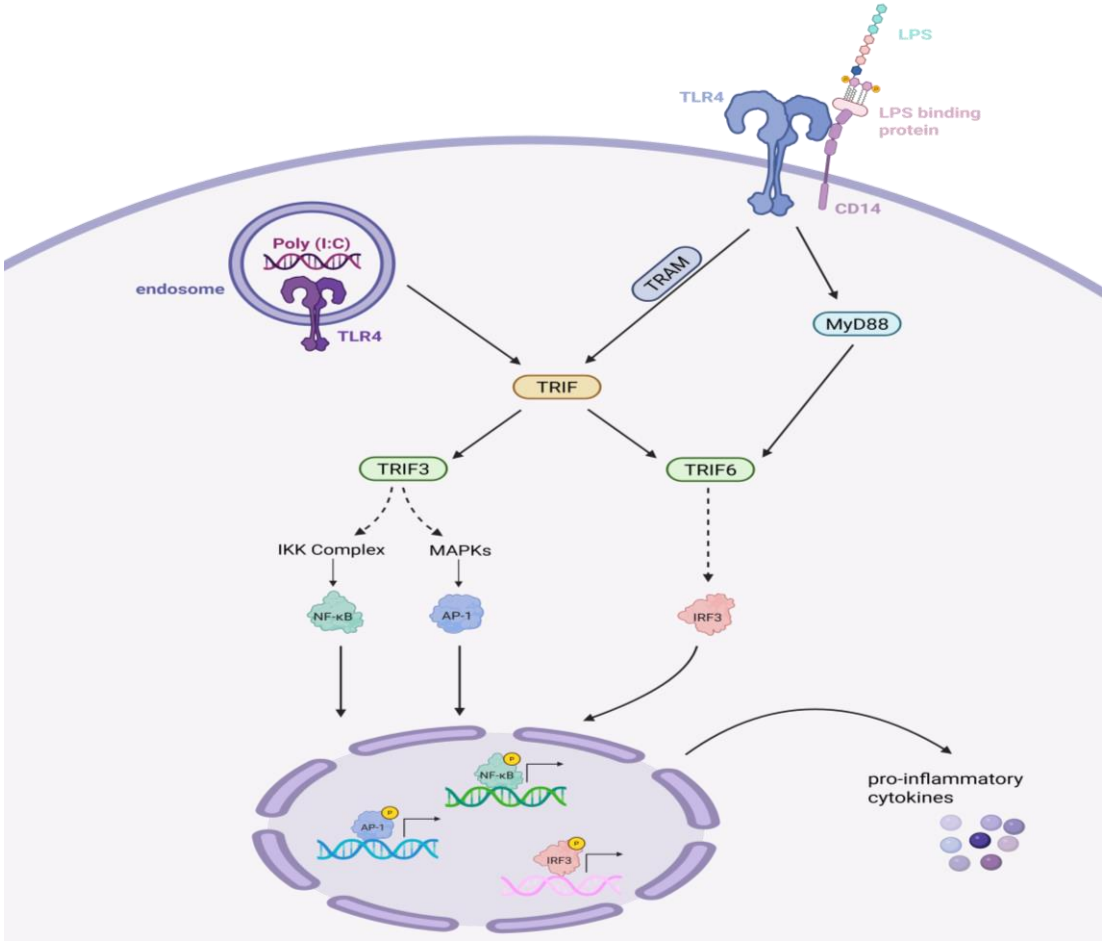


Figure 8: Cellular mechanism behind both MIA models of LPS and Poly (I:C) administration. *Graphic showing how administration of LPS or Poly (I:C) can induce inflammatory states in the cell. LPS binds to TLR4, activating both the MyD88-dependent and TRIF-dependent pathway. NF-κB, AP-1, and IRF3 are activated, influencing expression of inflammatory genes. Poly (I:C) binds to TLR3 and activates the TRIF-dependent pathway only which performs the same functions as previously described with LPS. Abbreviations: LPS: Lipopolysaccharide, Poly (I:C): polyinosinic:polycytidylic acid, TLR: Toll-like receptor, TRAM: TRIF related adaptor molecule, TRIF: Toll-IL-1 receptor domain-containing adapter-inducing interferon, IKK: IκB kinases, MAPKs:*

mitogen-activated protein kinase, NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells, AP: Activator protein, IRF: Interferon regulatory factor.

The LPS and the Poly (I:C) models are just two of the numerous models that can be used to examine the effect of MIA. Another notable model is the high-fat, high-sugar diet which can be examined both pre- and postnatally. As previously discussed, unfavourable diets such as the Western diet induce an inflammatory-like state. Examination of the effect that this diet may have on the offspring have revealed disruption to normal metabolic function, changes in anxiety-like behaviour, and alterations to enzymes involved in DNA methylation in the brain (Akhaphong et al., 2021; Hor et al., 2025; Mort et al., 2023). Other models include direct maternal injection of pro-inflammatory cytokines or administration of a live pathogen.

The overarching benefit of using these MIA models is the ability to administer the inflammatory trigger at various time points across gestation, which allows for in depth investigations of disruption to certain pathways or developmental process. However, results vary depending on dose, batch, timing, and duration of the administration (Bao et al., 2022). Both LPS and Poly (I:C) mechanisms and outcomes have been extensively reviewed by (Bao et al., 2022).

6.2 Microbiome-disrupted models

As the role of the maternal gut microbiome becomes more important in neurodevelopmental research, numerous models have been utilised to disrupt and deplete the microbiome. Initial studies using animal models often used high doses of antibiotics, sometimes as a cocktail, over longer durations that spanned critical time windows (Codagnone et al., 2019). Recently, there has been a shift in how these studies are performed, with more targeted microbial depletions at specific time points during gestation taking preference. Rodent studies have indicated that lower doses of a single antibiotic, in this case penicillin, during late pregnancy and early postnatal life, produces lasting effects in both sexes on gut microbiome, including increased cytokine expression in frontal cortex, modification of BBB integrity and altered behaviour in the offspring

(Leclercq et al., 2017). Prenatal exposure to low-dose penicillin during the last week of gestation has a sex-dependent effect on neurodevelopmental outcomes, with female mice becoming less anxious while males displayed abnormal social behaviours (Champagne-Jorgensen et al., 2020). In a similar fashion to models of MIA, antibiotic models allow for freedom with regard to the timing of the insult. Maternal microbiome dysbiosis at different times allow for careful examination of the effect of the disruption on different neurodevelopmental parameters. Furthermore, administration can be both acute and chronic.

Exposure to prenatal antibiotics has been shown to lead to persistent alterations in anxiety, sociability, and cognitive behaviours. Animal models also determined that the profile of behavioural effects in terms of anxiety, sociability, and cognitive behaviours due to a broad-spectrum antibiotic cocktail, were greater than in animals treated with just penicillin, except for greater deficits in social recognition (O'Connor et al., 2021). Mechanistic insights have also accrued that following exposure to an antibiotic cocktail mixture, offspring displayed altered tactile sensitivity which was rescued by concurrent administration of specific beneficial microbial metabolites (Vuong et al., 2020). To date there is limited evidence examining potential differences between absorbable and nonabsorbable antibiotics. Absorbable antibiotics, such as ampicillin, are easily absorbed into systemic circulation due to their chemical structure which allows for numerous off-target effects. Most animal models now use various combinations of nonabsorbable antibiotics such as vancomycin and neomycin, whose antimicrobial effects are confined more to the gut lumen due to poor absorption and lack systemic effects (Faas et al., 2023; González-Arancibia et al., 2022; Tochitani et al., 2016). The direct and indirect effects that absorbable antibiotics may have on neurodevelopment are yet to be fully elucidated, and it may prove that the different antibiotic drug classes along with different mechanisms of action (e.g. neurotoxic effects of metronidazole) may produce differential effects on both the offspring microbiome as well as neurodevelopmental parameters, depending on the doses used.

GF animals, while not as translational as antibiotic models, act as a complementary model to study the role of the maternal gut. In a similar fashion, offspring born to GF dams have also displayed deficits in tactile sensation via impaired thalamic axonogenesis (Vuong et al., 2020). The fetal brain from GF dams varies drastically in comparison to their colonised counterparts, where numerous genes involved in neural function are downregulated, while genes regulating neuronal and glial cell projection are increased (Husso et al., 2023). The abnormalities seen in the brain also extend to reductions in microglia numbers in the somatosensory cortex and the hippocampus with higher levels of hypothalamic cell death (Castillo-Ruiz et al., 2023). GF swine studies have revealed lower brain weight and reduced white matter volume in numerous regions with the latter connected to a lower level of oligodendrocyte proliferation (Ahmed et al., 2021). A reduction in both neural and glial stem cells have also been reported in GF zebra fish (Rea et al., 2022). Behaviourally, GF mice display increased anxiety-like behaviour and disruption in normal social behaviours (Clarke et al., 2013; Desbonnet et al., 2014).

These neurodevelopmental models of microbiome-disruption have confirmed the role that the maternal microbiome plays in modulating brain development. While as flexible as the models of MIA, they perhaps are less translatable and results are more varied, but crucial for advancing the preclinical knowledge, nonetheless.

6.3 Limitations and considerations in translational research

Pregnancy and fetal brain growth are two of the most controlled and measured physiological processes to occur in the human body. Due to the complexity of both, and the obvious numerous ethical considerations associated with pregnancy clinical trials, much of the mechanistic work relies on preclinical models, both *in vitro* and *in vivo*. While fundamental science has revolutionised this field and generated significant breakthroughs, it has limitations. Most of the significant results from preclinical work assume the same process or mechanism seen in a rodent model will match that of a developing human fetus.

While there can be an aspect of translatability in some *in vivo* studies, it is crucial to highlight the potential downfalls also.

Perhaps the most obvious discrepancy of preclinical *in vivo* work is simply the biological and physiological differences that are apparent between species (Choudhary and Ibdah, 2013; Denayer et al., 2014; Hartung, 2024). While mammals share similar biology, it is not an exact match, more so in the context of pregnancy due to differing gestational times and multifetal pregnancies. This makes rodent models of pregnancy exactly that, just a model. When inducing inflammation or microbial disruption for instance, the dose that is usually required to induce this is far greater than what would be seen in humans. For example, in the LPS model of MIA, studies may often administer 100 μg/kg to the pregnant dams which in turn successfully leads to neurobehavioural and neurocognitive alterations in the exposed offspring (Wischhof et al., 2015). However, this dose would supersede the concentrations evident in a bacterial infection, even in the most severe cases of sepsis and would most likely prove to be fatal (Opal and Glück, 2003).

Furthermore, there are many unique characteristics that shape the overall health of a pregnancy as well as developmental outcomes. Genetics, age, race, socioeconomic influences, obesity, and access to healthcare are just a small proportion of factors that influence how a fetus may respond to a prenatal challenge. Of course, *in vivo* preclinical models are simply unable to take account of these factors. Genetically similar and young animals are often included in these studies, and for consistency, are fed the same diet, have access to the same forms of enrichment, and are maintained in a temperature- and light-dark cycle-controlled environment. The lack of variability seen with *in vivo* studies acts as both an advantage and disadvantage for translatability.

Finally, in the context of drug discovery, recent reviews have estimated the predictive efficacy of animal models to match human outcomes ranges between 37-60%, which is relatively low (Hartung, 2024). Prediction of safety outcomes were found to be slightly higher at 70% (Hartung, 2024). These facts should be

considered when noting results of preclinical studies, but also highlights the drastic need for improved, more translatable, and accurate models of pregnancy.

7 Summary and conclusion

The development of the fetal brain is a sensitive and controlled process. One of the major determinants of postnatal health is the *in utero* experience, which is heavily influenced by maternal health. Maternal immune activation can exert deleterious effects on the offspring through upregulation of pro-inflammatory cytokines and negatively influencing the placenta. Separately, the maternal gut microbiome has been shown to shape the fetal brain through numerous potential mechanisms. Both prenatal insults have been shown separately to influence postnatal life.

Reviewing the literature, it is evident that both inflammation and the gut microbiome have a bidirectional, synergistic, and complex relationship. Despite this link, there has been limited examination of the cumulative effects of these mediators on neurodevelopmental outcomes in exposed offspring. Furthermore, with a surge in popularity of probiotic consumption to improve gut health, further investigations of potential beneficial effects on the plastic fetal brain are warranted.

8 Aims and objectives of this thesis

The overarching aim of this thesis is to investigate how maternal inflammation, and the maternal gut microbiome influence fetal neurodevelopmental trajectories and the offspring's gut microbiome. This work seeks to elucidate the neurobiological mechanisms by which maternal immune and microbial environments shape both the developing brain and the gut–brain axis of the offspring, with implications for understanding susceptibility to neurodevelopmental disorders.

8.1 Aim 1: *Can maternal serum-derived circulating factors modulate key processes in neuronal differentiation and development in vitro?*

Initially, serum from healthy pregnant participants (collected from the Cork cohort of the Screening for Pregnancy Endpoints study) were stratified based on previously analysed markers (Keane et al., 2021) into biologically "low-stress" and "high-stress" groups. To assess the neurodevelopmental impact of maternal systemic signals, serum from each group was applied to partially differentiated SH-SY5Y neuroblastoma cells, serving as an *in vitro* model of early neuronal development - ***Chapter 2.***

8.2 Aim 2: *Does a dual-hit exposure to maternal immune activation and gut dysbiosis synergistically impact the development of offspring microbiota-gut-brain axis?*

Building on evidence that maternal immune activation and prenatal maternal gut dysbiosis independently influence offspring outcomes, this study aimed to investigate the potential cumulative or interactive effects of these two exposures on the developing brain, behaviour, and gut microbiome of the offspring - ***Chapter 3.***

8.3 Aim 3: *Can maternal probiotic supplementation during healthy pregnancy modulate fetal neurodevelopmental trajectories and enhance offspring neurodevelopmental outcomes?*

Recognising the impact of a suboptimal maternal microbiome on development, we sought to determine the long-term developmental outcomes resulting from positive maternal microbial modulation using a beneficial bacterial species - ***Chapter 4.***

Chapter 2

Maternal Serum Demonstrates Bioactivity Capable of Attenuating Neurite Extension in SH-SY5Y Neuroblastoma Cells, an Effect Mediated Through a TNFα–Dependent Pathway

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Abstract

Prenatal stress is associated with deleterious neurodevelopmental consequences in affected offspring. Prenatal stress via exposure to high physiological levels of inflammation *in utero* may induce an inflammatory state in the fetal brain. However, inflammation is not only associated with disease-states but can also be seen in a healthy pregnancy. There is limited research examining the potential that exposure to biological mediators of stress may have on neurodevelopment. We aim to determine if certain circulating biological markers in maternal serum influence neurite growth in partially differentiated SH-SY5Y cells as a potential mechanism impacting neurodevelopment. Blood was collected at 20-weeks' gestation as part of the Screening for Pregnancy Endpoints (SCOPE) pregnancy cohort study. These factors, including pro-inflammatory cytokines, markers of tryptophan metabolism, and gut permeability that were previously analysed, were used to stratify women into low (n=10) and high (n=10) biological stress groups. Exposure to the serum categorised as “high-stress” significantly reduced neurite length in comparison to serum categorised as “low-stress”, with tumour necrosis factor-α playing a substantial role in mediating this reduction. The “high-stress” serum was subsequently found to increase the levels of phospho-Ser536-p65. Phosphorylation of Ser536 p65 has previously been shown to switch NF-κB from promoting neuronal growth, to inhibiting it. The reduction in neurite length seen following exposure to the “high-stress” serum was prevented when NF-κB p65 was knocked down. This study emphasises the potential negative impact that circulating factors may have on neuronal growth, and the mechanism behind it.

1 Introduction

In utero neurodevelopment is a complex, coordinated, and perhaps most importantly, time sensitive process. It has been well-characterised that prenatal stress can disrupt the optimal outcome of the developmental process, but the exact mechanisms remain unclear (Collins et al., 2024). In later life, this may manifest as behavioural and temperament difficulties in the offspring, deficits in motor skills, or cognitive impairment (Collins et al., 2024; O'Donnell et al., 2009; Van den Bergh et al., 2020). This has been shown in both preclinical work and in human studies (Aria et al., 2020; Bergh et al., 2005; Collins et al., 2024; Grace et al., 2016; Rahman et al., 2022; Vuong et al., 2020). Maternal prenatal stress further increases the risk of neurodevelopmental sequelae through its association with structural changes in the fetal brain such as altered white matter and decreased grey matter in key regions, along with disrupted connectivity (Wu et al., 2024).

Biological stress refers to the physiological response to a perceived threat that disrupts normal homeostasis. The response to this involves the interplay of psychological and physical changes, focused on coping with the perceived threat, responding to the threat, and finally restoring balance within the body. The response to stress is unique between individuals. Previous work in our group has identified a biological stress signature in maternal serum from healthy pregnant women at 20-weeks' gestation (Keane et al., 2021). Originally, the women were clustered dependent upon their high levels of self-reported stress, anxiety, and depression. These women were subsequently found to have elevated levels of defined circulating factors, including tumour necrosis factor-α (TNFα) and C-reactive protein (CRP), that could disrupt normal neurodevelopment.

Focusing on gut permeability and inflammatory cytokines, notably due to their bidirectional relationship, Keane et al., linked these with levels of self-reported measures of prenatal stress, depression, and anxiety, thus highlighting a biological stress signature. Of importance, this study linked elevated soluble CD14 (sCD14) and lipopolysaccharide binding protein (LBP) with levels of perceived social stress, and increased TNFα was associated with both stress and

depression. Furthermore, CRP was associated with increased anxiety. This signature may indicate an 'early warning marker' of active, biological prenatal stress.

Previously we have shown that circulating mediators in maternal serum from pre-eclamptic patients disrupted neuronal growth. In primary embryonic day (E) 18 cortical neurons, pre-eclamptic serum increased neurite number per soma, in addition to promoting both neurite branching and length (Curran et al., 2018). Building on this, we demonstrated using both neuronally differentiated SH-SY5Y cells and human neural progenitor cell line, ReNcell® VM, that pre-eclamptic maternal serum increased neurite length via an interleukin (IL)-6 dependent mechanism (Barron et al., 2023). These results indicate that maternal serum, from this pregnancy specific adverse outcome could alter typical neurite length due to alterations in the expression of certain inflammatory mediators. Yet, whether maternal serum from healthy pregnancies has such bioactivity is still to be elucidated (Keane et al., 2021).

Interestingly the biological signature of prenatal maternal stress from healthy pregnancies in our earlier study was characterised by elevated levels of TNFα, CRP, LBP, and sCD14 (Keane et al., 2021). Previous studies have shown that exposure to elevated levels of inflammation during pregnancy can directly impact the central nervous system, however the concentration levels are typically greater than in the original Keane et al. study and are often associated with disease-states (Brown et al., 2014; Hussain et al., 2022; Tomlinson et al., 2019). Increased TNFα has also been shown to cause gross tissue damage in human embryonic stem cell cerebral organoids (Benson et al., 2020). Increased apoptosis disrupted neuronal formation, in addition to the formation of scar-like structures were seen in the organoids following incubation with TNFα (Benson et al., 2020). Others have noted that the negative effects associated with TNFα are often concentration dependent, where low doses are required for normal cellular proliferation (You et al., 2021). However, at elevated concentrations, TNFα inhibits proliferation and induces unwanted apoptosis (You et al., 2021). This can vary dependent upon cell and tissue type, but the overall changes are relatively

consistent (You et al., 2021). This has further been supported via suppression of TNFα activity which improved neurite complexity in an *in vitro* model of inflammation model and in reducing developmental defects in an *in vivo* model of neonatal infection (Seleme et al., 2017; Yoon et al., 2020).

It is unclear as to whether altered intestinal permeability may also directly affect offspring neurodevelopment, but it has been hypothesised as a potential risk factor for prenatal conditions such as pre-eclampsia which has been shown to affect neurodevelopment (Gumusoglu et al., 2020; Koulouraki et al., 2023). In this study, our goal was to investigate if previously characterised serum had bioactive properties in our neuronal cell model and to understand the role of TNFα in this bioactivity. Using an *in vitro* model of neuronally differentiated SH-SY5Y cells, we aimed to investigate the effect of circulating factors in the maternal serum on neurite length, as a potential mechanism impacting neurodevelopment.

2 Materials and methods

2.1 Participant enrolment and serum collection

The Screening for Pregnancy Endpoints (SCOPE) study was an international multicentre prospective cohort study of nulliparous singleton pregnancies aimed to develop a screening test to predict adverse pregnancy outcomes (Kenny et al., 2014). The Cork participant cohort of the SCOPE study was recruited between November 2004 and January 2011 (n=1774). Ethical approval for the SCOPE study was obtained from all necessary local ethics committees (Cork ECM5 (10) 05/02/08)). Ethical approval for the use of the serum collected for this *in vitro* work was granted by Clinical Research Ethics Committee of the Cork Teaching Hospitals (protocol number: APC1004; approval number: APC-D-14). Informed consent was obtained from all women and participants could withdraw at any time. Exclusion criteria included women who were deemed to be at elevated risk for pre-eclampsia, having a small for gestational age infant, or preterm birth.

Previously, we selected a subset of the Cork cohort of the SCOPE study (n=209) which we used in this study. This subset consisted of 105 women with irritable bowel syndrome (IBS) and a randomly selected 104 women without IBS diagnosis (Keane et al., 2021). Blood samples were collected at 20-weeks' gestation, and serum was extracted and stored at -80˚C.

2.2 Stratification of samples

Keane et al. previously measured the levels of circulating tryptophan and its metabolite kynurenine, three measures of gut permeability; intestinal fatty-acid binding protein (iFABP), sCD14, LBP, and three measures of inflammation; CRP, IL-6, TNFα. To identify groups in our human whole population, a two-step cluster analysis was performed on the 20-week samples as described previously (Wilmes et al., 2024). Participants were not included in the cluster analysis if any data for the circulating markers was unavailable, leaving us with a new

population of (n=148). To ensure independence of input variables, initial step Z-scores were calculated on serum readouts using the following formula:

$$Z = \frac{(x_i - \bar{x})}{\bar{\sigma}}$$

In which x_i represents the test score of each individual, while $\bar{x}$ and σ represent the mean and standard deviation of the population, respectively. Subsequently, a combined Z-score for distinct pathological readouts was calculated by using the following formula:

$$ZCombined = \frac{\sum_1^i z_{test}}{Number\ of\ tests}$$

This combined Z-score was performed on parameters related to gut barrier integrity (LBP, CD14, iFABP), inflammation (CRP, TNFα, IL-6), and the Kynurenine/Tryptophan ratio. IBS diagnosis was not considered for producing the Z-score.

For the two-step cluster analysis, the individual values of $Z_{\text{Gut-barrier integrity}}$, $Z_{\text{Inflammation}}$ and the Kynurenine/Tryptophan ratio were used as continuous variables for each individual. For the identification of clusters, Log-likelihood was used as a distance measure for the pre-clustering step, while the Akaike information criterion was used as a cluster criterion to estimate the most appropriate number of clusters.

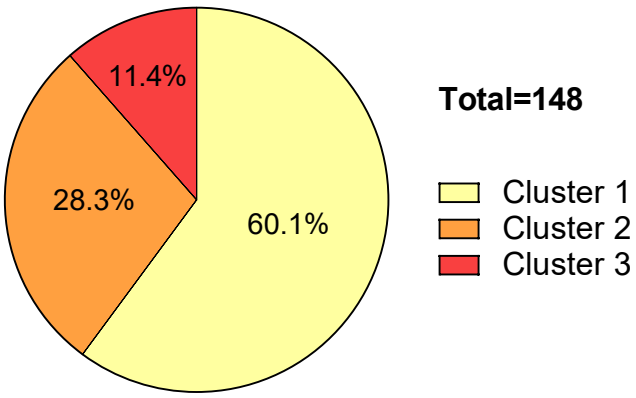


Figure 1: Stratification of SCOPE participants into clusters. *Cluster 1 = "low-stress", Cluster 2 = "medium-stress", Cluster 3 = "high-stress".*

Cluster 1 was identified as a group with low level of inflammation, a low kynurenine/tryptophan ratio, and high gut barrier integrity. Cluster 2 was identified as the middle group, with a mix of low and high values. Cluster 3 was identified as high inflammation, high kynurenine/tryptophan ration, and low gut barrier integrity (Fig. 1). To identify low-stress and high-stress samples, samples were selected based upon having Z-scores aligning with Cluster 1 or 3. The lowest overall samples were selected from Cluster 1 and deemed as "low-stress" (n=10), and the highest overall samples from Cluster 3 were defined as "high-stress" (n=10). The women were clustered based on biological stress alone, as the experience to psychological stress is unique to the individual. Following stratification of the women for our study, psychological stress was also evaluated and there was no correlation between the two. This ensures that any potential effect seen in this study is solely caused by biological markers.

2.3 Cell culture and treatments

Human neuroblastoma SH-SY5Y cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM)/Nutrient Mixture F-12 Ham's medium (Sigma Aldrich). Media was supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 2 mM L-glutamine (Sigma Aldrich). Cells were maintained in a T25 culture flask (Sarstedt) in incubator set at 37˚C and 5% CO_2. In all experiments, unless otherwise stated, cells were plated at a density of 12,500 cells/cm^2 once they reached 70-80% confluency in a 24-well plate. 10 µM retinoic acid (Sigma Aldrich) was added daily for the duration of the experiment to induce partial neuronal differentiation (Barron et al., 2024).

Unless stated otherwise, all treatments were administered 24 hours post plating and the experiment ended 72 hours after the first treatment. Final concentrations used include: 10 µM retinoic acid; 20 ng/mL of human TNFα recombinant protein (Fisher Scientific); 3% (v/v) maternal serum, 1 ng/mL of TNFα functional blocking antibody (Sarstedt), and 10 nmol of silencer small interfering RNA (siRNA) RELA (Thermo Fisher, Assay ID: s11914) (Barron et al., 2023). If n=10 for both "low-stress" and "high-stress" is not indicated, smaller sample sizes were selected at

random and based on sample availability. All experiments however had equal numbers of both “low-stress” and “high-stress”.

2.4 Neurite length measurements

To measure neurite length, live-cell imaging was performed using phase contrast at 20x magnification on an Olympus IX71 inverted microscope. Five non-overlapping images were taken for each well, and the length of five random neurites was measured per image using ImageJ software, with a total of 25 measurements taken per well. Neurite length was chosen as the main functional readout as previous work has shown that circulating factors in maternal serum alters neurite length (Barron et al., 2023).

2.5 Blocking TNFα activity

To ensure functionality of the TNFα blocking antibody, 1 ng/mL, 2 ng/mL, and 3 ng/mL were examined against 20 ng/mL of TNFα recombinant protein. The antibody was administered 24 hours after plating, and recombinant TNFα was administered 1 hour later. Functionality of the antibody was assessed by measuring its impact on neurite length versus recombinant TNFα treatment and the lowest functional dose was selected for further experiments.

2.6 Knockdown of NF-κB p65

To induce knockdown of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p65, 25,000 cells/cm^2 cells were transfected 24 hours post plating. Transfection complex was made in minimum essential medium eagle (MEME) (Sigma Aldrich) and contained 10 nmol siRNA RELA (si_p65) or siSCR negative control (Thermo Fisher), green fluorescent protein (GFP) using the TransIT-X2® Dynamic Delivery System (Medical Supply Co. Ltd). Transfection complex was left for 30 minutes prior to incubation of cells. Cells were also transfected with a separate GFP plasmid to aid visualisation of successful transfection and subsequently stained for NF-κB p65 to ensure no changes to basal neurite length by comparing with siRNA negative control. Transfection efficiency is approximately 30% and has been previously validated in the group.

2.7 Cytotoxicity assay

Cellular cytotoxic damage was measured based on extracellular release of lactate dehydrogenase (LDH) using the CyQUANT™ LDH Cytotoxicity Assay Kit (Invitrogen). Cell culture supernatant was collected 72 hours after treatment and stored at -20˚C until further use. At time of analysis, samples were thawed on ice and 50 µL of the cell media was added to 96-well plate, followed by the addition of 50 µL of the reaction mixture as per the manufacturer's instructions. Samples were incubated for 30 minutes in the dark at room temperature. 50 µL of the stop solution was added to end the reaction and absorbance was measured using the Multiskan FC Microplate Photometer (ThermoFisher Scientific, 51119000), at an excitation wavelength of 490 nm and an emission wavelength of 680 nm. To determine LDH release, the 680 nm absorbance value was subtracted from the 490 nm absorbance prior to calculation of the % cytotoxicity (Barron et al., 2023).

2.8 Immunocytochemistry

Cells were fixed for immunocytochemistry in 4% paraformaldehyde for 15 minutes prior to washing in 0.02% triton-X phosphate buffered saline (PBS-T). Non-specific binding was blocked by incubating cells in 5% bovine serum albumin (BSA) for 1 hour at room temperature. Cells were then incubated overnight at 4˚C in primary antibodies diluted in 1% BSA (primary antibodies used, all 1:500 dilution; β-III tubulin (R&D Systems, mouse anti- β-III tubulin, MAB1195); NF-κB p65 (Cell Signalling, mouse anti- NF-κB p65, 6956T); phospho- (Cell Signalling, rabbit anti-NF-κB p65 Ser536, 3033S)). Following overnight incubation cells were washed in PBS-T and incubated for 2 hours at room temperature in secondary antibody in 1% BSA (secondary antibodies used, all 1:500 dilution; goat anti-mouse Alexa fluor 488 secondary antibody (Invitrogen, A11001); goat anti-mouse Alexa fluor 594 secondary antibody (Invitrogen, A11005); goat anti-rabbit Alexa fluor 594 secondary antibody (Invitrogen, A11012). Following incubation, cells were washed in phosphate buffered saline (PBS) and imaged at 20x magnification on an Olympus IX71 inverted microscope. Four non-overlapping images were taken per well and mean fluorescence intensity was measured for four cells per image using ImageJ software, with a

total of 16 measurements taken per well. Antibodies were all previously validated by the company and positive staining confirmed with a negative control.

2.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism v8.3.0. Student's unpaired two-tailed t-test or two-way ANOVA was performed when required and further analysed using Fisher's LSD post-hoc test or Sidak's multiple comparisons test when appropriate. Results deemed significant when $p<0.05$. All data presented as mean ± standard error of the mean (SEM).

3 Results

3.1 Maternal characteristics for all participants in “low-stress” and “high-stress” groups

Clinical, socioeconomic, and demographic were recorded from each study participant. There were no significant differences between the maternal age, weight, number of cigarettes smoked, or units of alcohol consumed between the “low-stress” and the “high-stress” group. For ethnicity, marital status, years in education, employment status, and socioeconomic index, descriptive data was collected. For these variables, data was displayed as a percentage of that group.

Table 1: Demographic information

	"Low-stress" (n=10)	**"High-stress" (n=10)**
Ethnicity	Caucasian (100%)	Caucasian (100%)
Age (Years)	28.6 ± 1.002	29.3 ± 1.932
Weight (Kg)	68.4 ± 4.279	73.8 ± 2.920
Marital status	Married (100%) Single (0%)	Married (80%) Single (20%)
Years in education	<12 (10%) 12-13 (30%) >13 (60%)	<12 (0%) 12-13 (90%) >13 (10%)
Employment status	Full-time (80%) Part-time (20%) Student (0%) Unemployed (0%)	Full-time (80%) Part-time (0%) Student (10%) Unemployed (10%)
Socioeconomic index	<24 (20%) ≥24 (80%)	<24 (30%) ≥24 (70%)
Irritable bowel syndrome diagnosis	No (40%) Yes (60%)	No (70%) Yes (30%)
Cigarettes smoked (per day the week leading up to visit)	1 ± 1	1.2 ± 0.727
Alcohol exposure (units during first trimester)	6.072 ± 1.609	5.214 ± 1.408

Table 1: Demographic information for the "low-stress" and "high-stress" groups. *Mean ± SEM.*

3.2 Circulating factors in maternal serum differ between the "low-stress" and the "high-stress" groups

An unpaired students T-test revealed significant differences in various biological markers of interest between the biological "low-stress" and the "high-stress" groups. Of the inflammatory markers measured, only CRP (P=<0.0001) was significantly increased in the "high-stress" group, with no significant difference in IL-6 (P=0.1058) and TNFα (P=0.0894) between groups. Of the markers of gut health and permeability measured, there was no significant difference in iFABP (P=0.9899) between groups, but both sCD14 (P=0.0014) and LBP (P=0.0489) were significantly altered between the "low-stress" and "high-stress" groups. Finally, the concentration of L-Tryptophan was significantly decreased in the

"high-stress" group (P=0.0269). However, this reduction did not lead to any changes in the metabolite L-Kynurenine between the study groups (P=0.2322). However, the L-Kynurenine/L-Tryptophan ratio was significantly increased in the "high-stress" group (P=<0.0001) when compared to the "low-stress" group.

Table 2: List of markers as a defined biological stress signature

Biological marker	"Low-stress" (n=10)	"High-stress" (n=10)	P-value
CRP (µg/mL)	1.99 ± 0.51	10.37 ± 1.36	**<0.0001**
IL-6 (pg/mL)	0.22 ± 0.05	1.59 ± 0.80	0.1058
TNFα (pg/mL)	0.26 ± 0.13	0.67 ± 0.19	0.0894
iFABP (pg/mL)	5163.41 ± 554.21	5176.52 ± 851.66	0.9899
sCD14 (ng/mL)	736.34 ± 40.32	981.96 ± 51.19	**0.0014**
LBP (µg/mL)	11.57 ± 0.97	19.33 ± 3.55	**0.0489**
L-Tryptophan (pg/mL)	8065.48 ± 778.57	5710.7 ± 622.5	**0.0269**
L-Kynurenine (pg/mL)	184.65 ± 21.72	223.68 ± 22.90	0.2322
L-Kynurenine/Tryptophan ratio	0.02 ± 0.001	0.04 ± 0.002	**<0.0001**

Table 2: Biological markers used to profile the women into "low-stress" or "high-stress" categories. *iFABP: intestinal fatty-acid binding protein; sCD14: soluble CD14; LBP: lipopolysaccharide binding protein; CRP: C-reactive protein; IL-6: interleukin-6; TNFα: tumour necrosis factor-α. Unpaired students T-test. Mean ± SEM.*

3.3 "High-stress" serum reduces neurite length in differentiated SH-SY5Y cells compared to "low-stress"

Partially differentiated SH-SY5Y cells were treated with serum from either the biological "low-stress" and "high-stress" groups for 72 hours and neurite length was measured post-treatment. Exposure to "high-stress" serum significantly reduced neurite length when compared to the "low-stress" serum (P=0.0005) (Fig. 2A, 2B). This reduction in neurite length was not due to disruption in the cellular cytoskeleton measured by β-III tubulin fluorescence intensity (P=0.1880) (Fig. 2C, 2D) or due to a cytotoxic effect of maternal serum as measured using an LDH assay (P=0.5560) (Fig. 2E).

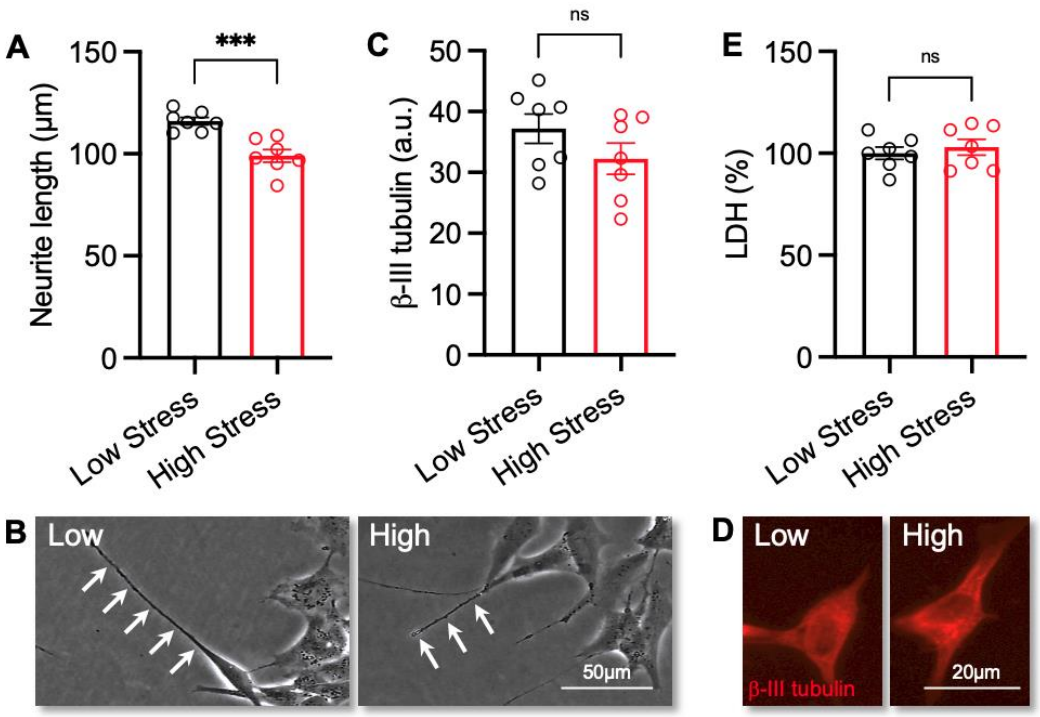


Figure 2: Exposure to maternal serum with a "high-stress" biological signature reduces neurite growth in differentiated SH-SY5Y cells. *Retinoic acid-differentiated SH-SY5Y cells were treated with 3% (v/v) serum with a "low-stress" (n=7) and "high-stress" (n=7) biological signature for 72h. Each serum sample was tested in triplicate on the same passage. (A) Graphs and (B) representative phase contrast micrographs of neurite growth in each experimental group. Neurites are indicated by white arrow heads. Scale bar = 50 µm. (C) Graphs and (D) representative photomicrographs of β-III tubulin (red) expression in each experimental group. Scale bar = 20 µm. (E) Graph of lactate dehydrogenase (LDH) activity expressed as a percentage of control in each experimental group. Data are presented as mean ± SEM and analysed using Student's unpaired t-test (*** $p < 0.001$; n.s. = not significant).*

3.4 Exposure to "high-stress" serum decreases neurite length in SH-SY5Y cells through a TNFα-dependent mechanism

Due to previous work in our lab, we have previously shown that TNFα can have a negative impact on neurite length in both differentiates SH-SY5Y cells along with primary neurons (Barron et al., unpublished). We validated the role of TNFα through a functional blocking antibody. This was first tested at various doses in competition with recombinant TNFα protein before testing this approach using serum as an inflammatory insult. There were significant changes in neurite length (Interaction (P=0.0228, F(3, 24)=3.819); Antibody effect (P=0.0422, F(3,24)=3.179); TNFα effect (P=0.0233, F(1, 24)=5.867)). Sidak's multiple comparisons test noted a significant difference between the control group and TNFα only (P=0.0035), indicating that the lowest dose of the functional blocking

antibody (1 ng/mL) was sufficient to block the effect of TNFα on neurite length (Fig. 3A).

Next, we investigated if blocking TNFα could equally negate the effect of both the "low-stress" and "high-stress" serum on neurite length. A two-way ANOVA revealed significant changes in neurite length (Interaction ($P=0.0003$, $F(1, 36)=16.07$); Antibody effect ($P=0.0006$, $F(1,36)=13.96$); Stress ($P=0.2314$, $F(1, 36)=1.475$)), with Fisher's LSD *post-hoc* test noting significant differences between Control: Low stress vs Control: High stress ($P=0.0007$), Control: High stress vs Antibody: Low stress ($P=0.0013$), and finally Control: High stress vs Antibody: High stress ($P=<0.0001$) (Fig. 3B, 3C). This result confirms that the higher circulating levels of TNFα in the "high-stress" group plays a role in neurite length reduction. Finally, there were no significant differences in β-III tubulin expression between any groups (Interaction ($P=0.1631$, $F(1, 36)=2.027$); Antibody ($P=0.0665$, $F(1, 36)=3.582$); Stress ($P=0.6120$, $F(1, 36)=0.2618$)) (Fig. 3D).

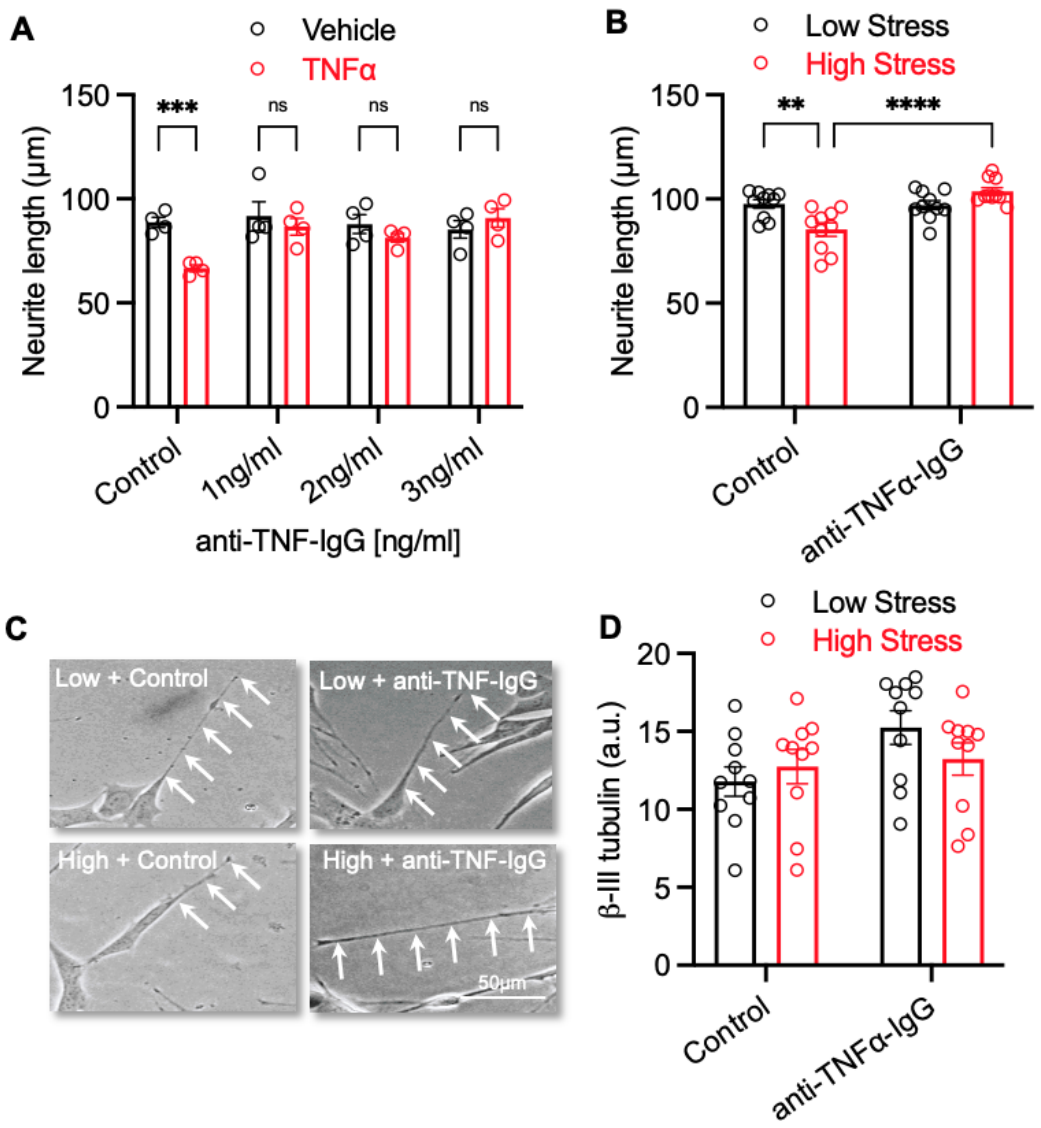


Figure 3: "High-stress" serum-induced deficits in neurite growth are mediated via a TNFα-dependent mechanism. *(A) Graph of SH-SY5Y cells treated with 20 ng/mL recombinant TNFα and cultured with or without increasing concentrations (1-3 ng/mL) of a TNFα function blocking antibody (anti-TNFα-IgG) for 72h. N=4 individual experiments. (B) Graph and (C) representative photomicrographs of SH-SY5Y cells treated with 3% (v/v) "low-stress" and "high-stress" SCOPE serum and cultured with or without 1 ng/mL anti-TNFα-IgG for 72h. Scale bar = 50 µm. Each serum sample was tested in triplicate on the same passage. (D) Graph of β-III tubulin expression from the experiment in B. N=10. Data are presented as mean ± SEM and analysed using a two-way ANOVA (** $p < 0.01$; *** $p < 0.001$; n.s. = not significant).*

3.5 Serum from "high-stress" serum increased phosphorylation of NF-κB p65 Ser536 in differentiated SH-SY5Y cells

To investigate the mechanism by which TNFα contributes to the reduction in neurite length, we examined possible changes in the status of the NF-κB p65 subunit due to its established roles in regulating neural growth (Gutierrez and

Davies, 2011). Differentiated SH-SY5Y cells were treated with “low-stress” or “high-stress” serum prior to immunocytochemical staining for both NF-κB p65 and phospho-NF-κB p65 Ser536. While there was no significant difference in total NF-κB p65 expression (P=0.3258) (Fig. 4A, 4C), incubated of cells with “high-stress” serum significantly increased phosphorylation of Ser536 residue in the NF-κB p65 subunit (P=0.0333) (Fig. 4B, 4C).

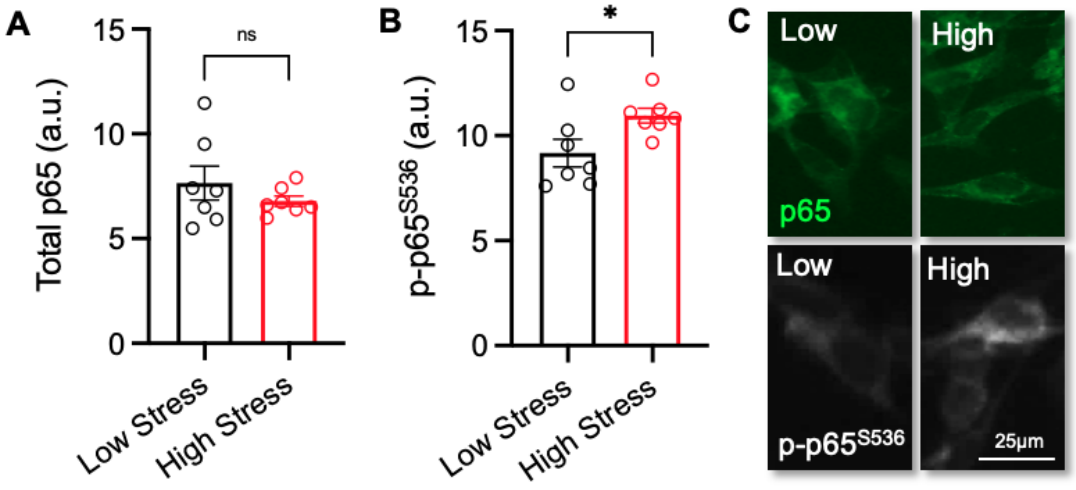


Figure 4: Exposure to serum with a “high-stress” biological signature increases phosphorylation of the Ser536 residue in the NF-κB p65 subunit in SH-SY5Y cells. *Retinoic acid-differentiated SH-SY5Y cells were treated with 3% (v/v) serum with a “low-stress” (n=7) and “high-stress” (n=7) biological signature for 72 h. Each serum sample was tested in triplicate on the same passage. (A-C) Graphs and representative photomicrographs of (A, C) total p65 (green) and (B, C) phospho-serine 536 p65 (p-p65S536) (greyscale). Scale bar = 25 µm. Data are presented as mean ± SEM and analysed using Student’s unpaired t-test (* $p < 0.05$; n.s. = not significant).*

3.6 NF-κB p65 knockdown prevents reduction in neurite length following exposure to “high-stress” serum

To further explore the role of NF-κB p65 in mediating the serum-induced effects on neurite growth, the NF-κB p65 subunit was knocked down via siRNA RELA transfection. Unpaired students T-test confirmed successful knockdown via quantification of fluorescence (P=0.0002) (Fig. 5A, 5B). Knock down of the NF-κB p65 subunit did not have any significant impact on basal neurite length (P=0.9413) (Fig. 5C). We then investigated if knockdown of NF-κB p65 subunit was sufficient to prevent TNFα-induced neurite length deficits. A two-way

ANOVA revealed significant differences between groups (Interaction (P=0.0027, F(1, 12)=14.13); RELA (P=0.0075, F(1, 12=10.29); TNFα (P=0.0144, F(1, 12)=8.16618)) (Fig. 5D, 5F). Fisher's LSD test showing significant differences between siSCR: No TNFα vs siSCR: TNFα (P=0.0005), siSCR: TNFα vs si_p65: No TNFα (P=0.0011), and finally between siSCR: TNFα vs si_p65: TNFα (P=0.0004) (Fig. 5D, 5F). No significant difference was found between si_p65: No TNFα vs si_p65: TNFα (P=0.5359), indicating that knockdown of NF-κB p65 was sufficient to protect against the TNFα-induced defects.

Finally, we investigated if RELA knockdown of NF-κB p65 would protect against the effects of the "high-stress" serum on neurite length. A two-way ANOVA revealed significant changes between the tested groups (Interaction (P=<0.0001, F(1, 36)=25.03); RELA (P=<0.0001, F(1, 36=19.28); Stress (P=<0.0001, F(1, 36)=23.64)) (Fig. 5E, 5G). Fisher's LSD *post-hoc* noted significant differences between siSCR: Low Stress vs siSCR: High Stress (P=<0.0001), siSCR: High Stress vs si_p65: Low Stress (P=<0.0001), and siSCR: High Stress vs si_p65: High Stress (P=<0.0001) (Fig. 5G). No significant difference was evident between si_p65: Low Stress vs si_p65: High Stress (P=0.9210), indicating that the reduction in neurite length previously seen following exposure to "high-stress" serum is mediated through the NF-κB p65 subunit.

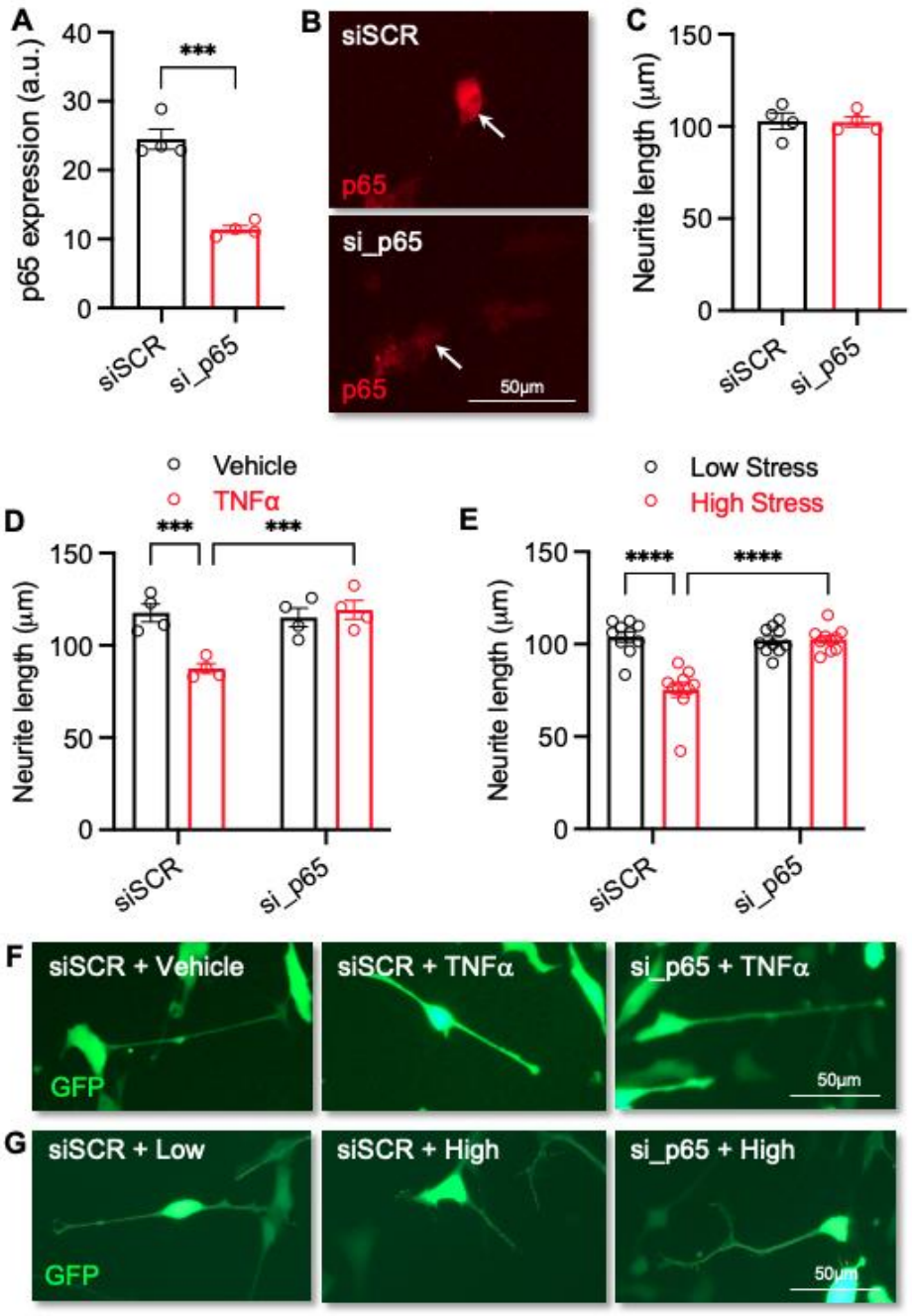


Figure 5: Knockdown of p65 prevents the detrimental effects of exposure to serum with a "high-stress" biological signature on neurite growth. *(A) Graph of p65 expression, (B) representative photomicrographs of p65 staining (red) and (C) graph of neurite length in retinoic acid-differentiated SH-SY5Y cells transfected with 10 nmol of a scrambled siRNA (siSCR) or an siRNA against the NF-kB subunit p65 (si_p65) and analysed at 72h post-transfection. (D) Graph of neurite length and (F) representative photomicrographs of SH-SY5Y cells transfected with 10 nmol of siSCR or si_p65 and cultured with or without 20 ng/mL TNFα for 72h. N=4 independent experiments. (E) Graph of neurite length and (G) representative photomicrographs of SH-SY5Y cells transfected with 10 nmol of siSCR or si_p65 and cultured in the presence of 3% (v/v) serum with a "low-stress" (N=10) and "high-stress" (N=10) biological signature for 72h. Each serum sample was tested in triplicate on the same passage. Scale bar = 50 µm. Data are presented as mean ± SEM and analysed using (A, C) a Student's unpaired t-test or (D, E) a two-way ANOVA with post-hoc Fisher's LSD test (*** $p < 0.001$; **** $p < 0.0001$; n.s. = not significant).*

4 Discussion

In this study, we used serum categorised as having a “low-stress” or “high-stress” biological phenotype based upon previously defined circulating factors in the blood and assessed their mechanistic role in altering processes important for neurodevelopmental using partially differentiated SH-SY5Y cells.

When compared to the “low-stress” group, exposure to “high-stress” serum significantly decreased neurite growth. This reduction did not appear to be due to cytotoxic damage as confirmed by the secretion of LDH, or alterations in β-III tubulin protein expression, one marker of the cellular cytoskeleton.

To examine the molecular mechanism involved in the reduction of normal neuronal growth, we used a functional blocking antibody to inhibit TNFα activity. Pretreatment of partially differentiated SH-SY5Y cells with this functional blocking antibody mitigated the negative effects of “high-stress” serum only on neurite growth. This effect was not due to any changes in β-III tubulin expression.

As TNFα was found to significantly contribute to the negative effect of “high-stress” serum on neuronal growth, we investigated the NF-κB signalling cascade, a key mediator of TNFα activity, in particular the p65 or RelA subunit of NF-κB. NF-κB consists of five structurally related proteins, including p65, p50, p52, RelB, and RelC (Gutierrez et al., 2008). TNFα is a known activator of the NF-κB pathway and has been shown to activate an IκB kinase (IKK)-β signalling cascade. This in turn leads to the formation of a p65/p50 heterodimer, which becomes phosphorylated at the Ser536 residue (Gutierrez et al., 2008). Here, we found that treatment of partially differentiated SH-SY5Y cells with “high-stress” serum did not alter the expression of total NF-κB, but increased phosphorylation of the Ser536 residue in the p65 subunit. Our findings of reduced neurite length coupled with increased phosphorylation of the Ser536 residue agree with previous studies (Gutierrez et al., 2008). Activation of this intracellular pathway following TNFα treatment has previously been shown to reduce neurite growth in sympathetic neurons, mimicking our results in partially differentiated SH-SY5Y cells (Gutierrez et al., 2008).

To confirm and further elucidate the role of NF-κB in mediating the serum-induced effects in neurite length, we used a siRNA approach to knockdown expression of the NF-κB p65 subunit. Transfected SH-SY5Y cell cells were then exposed to “low-stress” or “high-stress” serum for 72hr. Knockdown of NF-κB p65 subunit rescued the “high-stress” serum induced reduction in neurite length, confirming the specific molecular signalling mechanism involved.

NF-κB is a transcription factor which plays a central role in regulation of numerous genes, primarily in immunity and inflammation (Gavaldà et al., 2009), but its activation has also been shown to promote neurite growth in developing neurons (Gutierrez et al., 2008). Yet, its activation can also stunt neurite growth (Gutierrez et al., 2008). This somewhat paradoxical effect is dependent upon the mechanism of activation involved. Previous work has identified that activation via IKKβ inhibits neurite growth, and this kinase can be stimulated through TNFα (Sakurai et al., 2003). IKKβ, amongst other kinases, promotes phosphorylation of p65 Ser536 residue which is crucial to switching NF-κB from a promoting to an inhibitory role in neurons (Gutierrez et al., 2008). Furthermore, using S536A, a mutant protein that blocks Ser536 phosphorylation, abolished the inhibition of neural growth (Gutierrez et al., 2008). The formation of the phospho-Ser536-p65 complex is key in downregulating the genes that are necessary for promoting growth and upregulating genes that prevent growth (Gutierrez et al., 2008; Gutierrez and Davies, 2011), highlighting the importance of this post-translational modification in this mechanistic pathway. Other factors in the “high-stress” serum may also activate this pathway. Previous work has shown that CRP and IL-6 can also alter NF-κB gene expression, along with L-Tryptophan. Despite this, through blocking only TNFα, the neurite reduction was prevented, indicating a central role for TNFα in our experiment (Sun et al., 2019; Yue et al., 2016; Zhao et al., 2024).

Additionally, administration of brain-derived neurotrophic factor (BDNF) was found to stimulate dephosphorylation of p65 Ser536, showing that this mechanism is reversible (Gavaldà et al., 2009). However, this seems to be developmental stage-dependent, with BDNF decreasing phospho-Ser536-p65 in

embryonic neurons, but not in postnatal neurons (Gavaldà et al., 2009). This identifies the importance of this pathway in the prenatal period and reveals that after birth the phospho-Ser536-p65 pathway is not as significant to neuronal growth as different mechanisms take over (Gavaldà et al., 2009). In the prenatal period, there is a window of vulnerability where activation of phospho-Ser536-p65 cascade can alter neuronal growth, implying that high levels of TNFα in the maternal serum may be potentially harmful to the developing fetus. The importance of TNFα in having a substantial role in the "high-stress" serum-induced effects on neurite growth is not surprising given its role in systemic inflammation. It is highly plausible that the higher levels of TNFα seen in the serum are influencing other factors, such as IL-6, and potentially cause a cascade of downstream inflammatory factors that may amplify the effect. CRP was also found to be elevated in the "high-stress" serum and may be exacerbating the effects of TNFα. These cytokines have a bidirectional relationship and can sustain the pro-inflammatory environment (Palmqvist et al., 2008; Sproston and Ashworth, 2018; Tanabe et al., 2010). However, if this is the case, in this experiment TNFα was shown to be the primary instigator of the reductions in neurite length as no changes were observed when the activity was blocked.

Previous work in neuronally differentiated SH-SY5Y cells has also shown unique bioactive potential of maternal serum. While those at-risk of developing pre-eclampsia were excluded from the initial SCOPE study, previous work using serum from pre-eclamptic mothers also showed bioactive potential in the same model. Pre-eclampsia, a hypertensive condition occurring in the latter half of pregnancy, is characterised by hypertension along with either proteinuria, organ dysfunction, or uteroplacental dysfunction (Barron et al., 2023). This condition affects approximately 5% of all global pregnancies and has a strong association with an increased risk of neurodevelopmental disorders (Barron et al., 2023). We previously established that IL-6 was elevated in serum taken from pre-eclamptic women and upon exposure to cells, neurite length was increased. Unfortunately TNFα was not investigated here (Barron et al., 2023). However, the differential process in neural growth upon exposure to maternal circulating factors reveals

the varying mechanisms involved. Our “high-stress” serum only showed significant increases in the level of CRP, so it is difficult to deduce the role that IL-6 may be having here, if any. However, this still supports the overall concept of biological activity in the serum and highlights the importance of serum composition in affecting mechanisms important for neurodevelopmental outcomes.

However, while this study's results allow us to infer the potential role that specific factors may have on the developing brain, it is important to note a key limitation of this study. This work was performed in SH-SY5Y cells, taken from a neuroblastoma patient. The SH-SY5Y cell line is comprised of both neuroblast-like and epithelial-like cells. Following treatment with retinoic acid, neuroblast-like cells are partially differentiated into a more neuronal phenotype, but importantly these cells are not classified as fully neuronal. The use of SH-SY5Y cells in neurodevelopmental studies can be seen as an initial “screening tool” to probe mechanisms and in essence is the first step before progressing to more representative cellular models. Furthermore, our relatively small participant sample size and inability to examine potential confounders acts as a limitation of this study.

To further understand the effect that “high-stress” serum may have on neurodevelopment, it would be best to include more refined *in vitro* models of the fetal brain. Primary cells are an excellent model due to the mix of neurons and glia, which would allow us to examine complete neural networks. In this setting, it would be interesting to examine the effect that the TNFα may be having on other inflammatory markers and cell types such as primary or stem cells. For instance, does maternal serum increase the number of inflammatory-based cells in the culture or is there a synergistic of additive effect with the other pro-inflammatory cytokines IL-6 and CRP. Building on this, it would be of interest to examine the developmental mechanistic switch in phospho-Ser536-p65 activity in human stem cells as the previous work was performed in primary neurons isolated from animal models (Gavaldà et al., 2009). It would be interesting to also perform a similar experiment for the other circulating factors in the maternal

serum, particularly IL-6 and CRP. This would allow us to say with confidence if TNFα is the sole contributor to the changes seen in neurite length or is it just a primary instigator.

As noted previously, the women were stratified based on markers of biological stress, not psychological stress, although it was assessed as part of the SCOPE study. Importantly, all participants were generally healthy, bar some that were diagnosed with IBS. However, IBS diagnosis does not appear to be related to the observed changes since the percentage of participants with IBS in the “low-stress” group (60%) was higher than in the “high-stress” group (30%). Stress is usually associated with heightened cortisol, suggesting that this could be another mediator contributing to the observed phenotypes. To aid in our understanding of biological stress in this scenario, examination of cortisol levels would have been incredibly useful. High levels of cortisol could explain the elevated levels of inflammatory cytokines and gut permeability markers in the serum. Due to these women being classified as otherwise healthy, it is important to understand the potential risk that elevated levels of pro-inflammatory cytokines may be having in this context due to their known role in disrupting normal fetal neurodevelopment.

5 Conclusion

In summary, we stratified healthy pregnancies into biological “low-stress”, or “high-stress” phenotypes based on defined circulating factors identified in the maternal serum. The nine biological factors used to stratify the women signify inflammation, gut permeability, tryptophan metabolism, and are all crucial for maintaining optimal fetal health and additionally may play a role in neurodevelopment. When applied to partially differentiated SH-SY5Y cells, the biological “high-stress” group reduced neurite length, which was confirmed in part due to elevated levels of TNFα. The “high-stress” serum contributed to these deficits through increased phosphorylation of NF-κB p65 Ser536, which when knocked down, mitigated the reduction in neurite length evident upon exposure to maternal serum. These findings offer valuable insights into how exposure to “high stress” during pregnancy can impact processes important for neurodevelopment.

Chapter 3

Maternal Immune Activation and Antibiotics affect Offspring Neurodevelopment, Behaviour, and Microbiome

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Abstract

In utero exposure to an increased level of maternal inflammation or a disrupted maternal gut microbiome during pregnancy have been linked to several neurodevelopmental disorders in the offspring. Despite the strong links between these two adverse events, few studies looked at the interaction between the maternal gut microbiome and maternal immune activation (MIA) on the neurodevelopmental outcomes in the offspring. Here, we aim to determine if the maternal gut microbiome disruption exacerbated the impact of systemic inflammation on brain development, offspring behaviour, and long-term microbiome changes. A low dose of intraperitoneal lipopolysaccharide (LPS) was administered to pregnant Sprague Dawley rats from gestational day 12 to 18. Concurrently, an antibiotic cocktail (ampicillin, neomycin, vancomycin) was given in the drinking water to disturb the maternal microbiome. Embryos at gestational day 18 were found to have a reduced body size and weight, along with reduced placental weight following exposure to LPS, with some effects also seen with antibiotic exposure. Offspring exposed to LPS *in utero* were found to have increased anxiety-like behaviours and repetitive behaviours. No behavioural changes were noted from antibiotic exposure. The expression of 5-HT1a receptors in the prefrontal cortex was found to be reduced following LPS exposure. The offspring microbiome varied between the groups, with prenatal antibiotic exposure playing a role in reducing α-diversity and species richness in the periadolescent period. This study highlights the impact of prenatal exposures on different aspects of neurodevelopment. However, additional research is warranted to explore the role of the maternal immune system and microbiome on the offspring development, while also testing the potential therapeutic agents such as probiotics.

1 Introduction

Prenatal stressors during critical windows of fetal development have deleterious effects on the anatomy and physiology of numerous systems, particularly the nervous system (Minakova and Warner, 2018; Van Den Bergh et al., 2020; Yeramilli et al., 2023). The outcome of this *in utero* exposure may be associated with increased risks of developing neurodevelopmental disorders, such as autism spectrum disorder, attention deficit hyperactivity disorder, and schizophrenia, amongst other neuropsychiatric conditions (Beversdorf et al., 2018; Jallow et al., 2024; Lipner et al., 2019). The activation of the maternal immune system due to an infection during pregnancy is one particular prenatal stressor that has been associated with unfavourable neurodevelopmental outcomes (Vitor-Vieira et al., 2024; Xuan and Hampson, 2014; Zuckerman and Weiner, 2005). This connection has been consolidated by examining numerous global crises such as the rubella epidemic of 1964, the influenza pandemic of 1918, and more recently, the SARS-CoV-2 pandemic of 2020, all of which have been associated with an increased risk of a range of neurodevelopmental disorders (Brown et al., 2001; Brown, 2006; Brown et al., 2000; Dubey et al., 2022; Kępińska et al., 2020; Shook et al., 2022). The evidence from these diverse events suggests that it is not a specific set of pathogens that lead to these neurodevelopmental disorders, but rather that they may be linked via excessive and aberrant maternal immune activation. This has been shown through numerous rodent models which illicit similar neurobehavioural outcomes despite immune stimulation with different mimetics (Solek et al., 2018).

Administration of the viral RNA polyinosinic:polycytidylic acid or the bacterial cell wall endotoxin lipopolysaccharide (LPS) illicit strong immune responses in rodents (Bao et al., 2022; Bucknor et al., 2022). These models have been shown to result in altered levels of fundamental neurotransmitters such as serotonin and dopamine, disruption in the neuroanatomical architecture and increased microglia activation. These changes may result in severe behavioural changes and lead to the development of anxiety-like and autistic-like phenotypes (Aria et al., 2020; Estes and McAllister, 2016; Murray et al., 2019). Through studying the

cellular and functional changes, numerous drivers have been postulated. These include abnormal microglia activation affecting numerous cellular functions such as pruning and apoptosis, excessive generation of pro-inflammatory cytokines, or the hypermethylation of certain synaptic promoters (Labouesse et al., 2015; Solek et al., 2018).

Building upon this, the maternal gut microbiome has recently been identified as a key player in the development of the fetus (Sharon et al., 2016; Warner, 2019). The gut microbiome can be influenced directly by poor diet, medication use, and stress (Cryan et al., 2019). The gut-brain axis is defined as a bidirectional pathway of communication between the brain and the gut, with one being able to modify the other, positively or negatively, through vagus nerve activity, alterations in neurotransmitters, activation of the immune system, and certain bacterial metabolites (Cryan et al., 2019). Through this pathway, it is possible that prenatal stressors, such as maternal immune activation may disrupt the maternal gut subsequently causing neurodevelopmental complications and potentially having a cumulative effect.

Most of this work has been performed in animal models, examining the effect of microbial depletion or germ-free conditions on neurodevelopment. Germ-free rodents, which simply lack a gut microbiome, have been shown to display altered anxiety and social behaviours, reductions in choroid plexus integrity, and disrupted myelination (Forssberg, 2019; Hoban et al., 2016; Knox et al., 2023). Alterations in numerous transcriptional pathways that are involved in neuronal plasticity and neurotransmission have also been reported (Stilling et al., 2015). Through microbial depletion studies, a less drastic and more translatable model, it has been shown that suboptimal support from the maternal microbiome can lead to severe fetal neurodevelopmental complications that range from distorted tactile sensitivity to altered sociability levels in the offspring (Hassib et al., 2023; Vuong et al., 2020).

Antibiotic use during pregnancy is both common and often necessary to fight infections (Bookstaver et al., 2015; Nakitanda et al., 2024). While most antibiotics are accepted as safe, other classes such as streptomycin or

tetracycline may induce teratogenic effects (Bookstaver et al., 2015; Norwitz and Greenberg, 2009). Additionally, frequent antibiotic use can disrupt the microbiome through reduction in the beneficial microbial species, diversity, and the promotion of overgrowth of unfavourable species, which is postulated to lead to neurodevelopmental changes in animal models (Becattini et al., 2016; Ferrer et al., 2017).

In this study we combined an LPS model of maternal immune activation (MIA) and maternal gut dysbiosis to mimic a maternal infection that would require antibiotic treatment. We aimed to investigate the behaviour of the offspring following their exposure to the combination of prenatal stressors, with a specific focus on anxiety and autistic-like phenotypes, while also examining the impact on relevant brain areas and the preadolescent microbiome.

2 Materials and methods

2.1 Animals and housing

Male and female Sprague Dawley rats were purchased from Envigo, United Kingdom aged approximately 7 weeks old and housed in the Biological Services Unit, Western Gateway Building, University College Cork. All rats had water and standard chow *ad libitum*. Rats were housed in RC2F type cages (1575 cm^2) with sawdust bedding and cardboard tubes. All cages were held in a controlled environment; air temperature set to 21°C ± 1°C and a 12 hour light/dark cycle with light between 7am-7pm. Rats were allowed to habituate for 10 days before they were moved to a smaller breeding cage (960 cm^2). Embryonic day (E) 0 was defined upon identification of the vaginal plug. All procedures were conducted with approval from the Animal Experimentation Ethics Committee at University College Cork and the Health Products Regulatory Authority, in accordance with the recommendations of the European Directive 2010/63/EU.

2.2 Study design

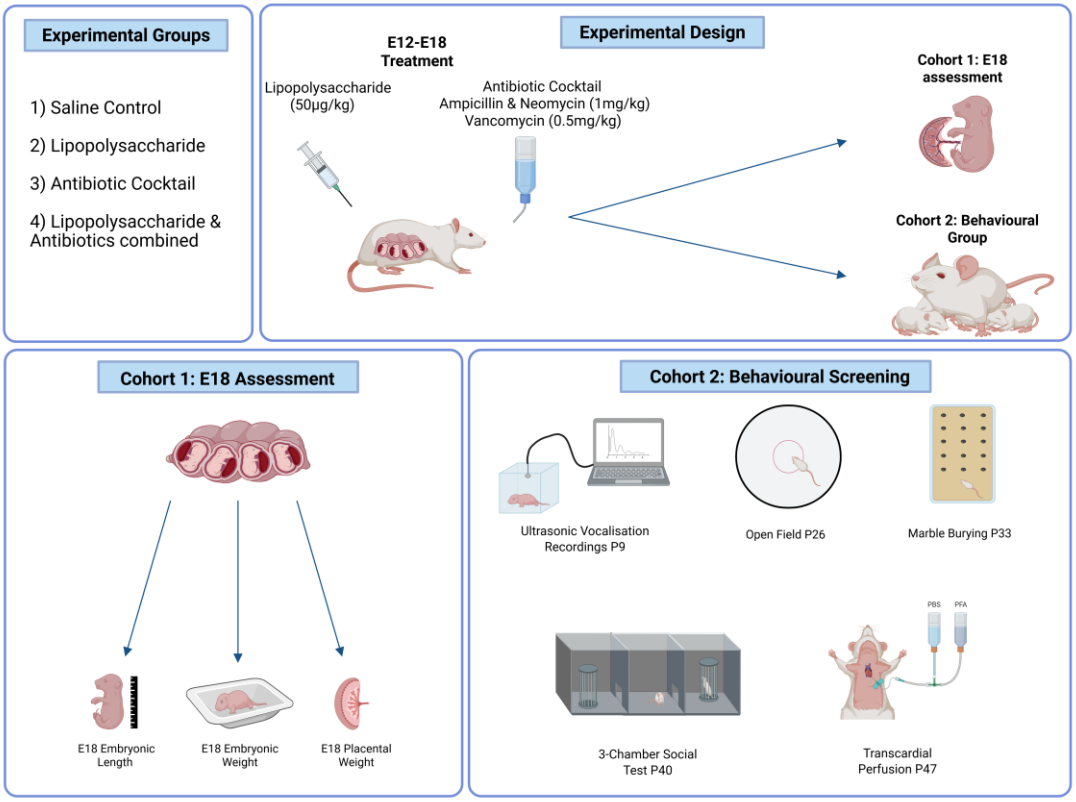


Figure 1: Study design detailing experimental groups, maternal treatments, and the fate of cohort 1 and 2. *Abbreviations: E: Embryonic day; P: Postnatal day.*

The dams were assigned to either cohort 1 or cohort 2. Both cohorts received identical treatment as discussed in section 2.3. However, rats from cohort 1 were culled via decapitation on E18 and the embryos were then assessed, as described in section 2.4. For cohort 1, there were 2 dams in the antibiotic (ABX) group, 3 dams in both the saline and the combined group (LPS/ABX), and 4 for the lipopolysaccharide (LPS) group. For cohort 2, each group had 6 dams, except for saline which had 5. On E18, the dams from cohort 2 were housed individually in small cages (960 cm^2) and allowed to give birth. Day of birth was assigned as postnatal day (P) 0. Pups stayed with their mothers until P21 when they were weaned and housed 2 males or 2 females per small cages (960 cm^2) and were used for all behavioural and postmortem examination (Fig. 1). This produced a total of 20 offspring for the saline group, 23 offspring for the LPS/ABX group due

to a single female pup born to one dam, and 24 offspring for both the LPS and the ABX groups. Dams were culled on P21.

2.3 Maternal immune activation and gut dysbiosis model

On E12, pregnant females were randomly assigned to one of 4 groups: saline, ABX, LPS, LPS/ABX. Dams were separated into cages based on their treatment group, with at least two dams in each cage. Those in the saline group received 0.5 mL of saline intraperitoneally with a 25G needle from E12-18, alternating the injection site from right to left, every other day. Those in the ABX group received 1 mg/mL of neomycin (Neomycin sulfate 1405-10-3, Discovery Fine Chemicals) and 1 mg/mL of ampicillin (Ampicillin Sodium Salt 69-52-3, Discovery Fine Chemicals) and 0.5 mg/mL of vancomycin (Vancomycin hydrochloride 1404-93-9, Discovery Fine Chemicals) dissolved in drinking water. The ABX water was changed every 3 days and was given on E12-18. Rats were weighed daily each morning. Dams received 50 µg/kg of LPS (Lipopolysaccharides from Escherichia coli O26:B6, Merck) dissolved in 0.5 mL saline intraperitoneally using a 25G needle from E12 to 18, alternating the injection site from right to left, every other day. Those in the LPS/ABX group received both LPS and antibiotics from E12 to18. Dosing for both LPS and ABX group was based on previous work in the lab which produced notable behavioural and/or functional deficits (Radford et al., unpublished).

2.4 Assessment of the embryos

Cohort 1 of the treated dams was taken on E18 for morphological assessment of the embryos. Dams were anesthetized using isoflurane (Vetflurane® 1000 mg/g), pedal, tail, and palpebral reflexes checked, and decapitated. Embryos were removed via laparotomy and placed into phosphate buffer saline (PBS). The weight and crown to rump length of each embryo was recorded, along with its placental weight. The sex of the embryo was determined by the anogenital distance.

2.5 Ultrasonic Vocalisation Recordings

On P9, the pups underwent ultrasonic recordings to measure their affective state. Pups were removed from their home cage one by one. Pups were placed in a small grey box which was placed under a styrofoam box that was used to insulate the calls and block out external noise. The microphone was threaded through the styrofoam box and hung approximately 10 cm above the pup. The pups were recorded for 3 minutes before being returned to their home cage and gently rolled in the bedding before being placed in the nest once again. Ultrasonic vocalisations were recorded and analysed using UltraVox XT v3.2.

2.6 Open Field Test

On P26, the open field test was performed to measure anxiety-like behaviour and locomotion. The pups were allowed to habituate to a separate room for 45 minutes before the beginning of the test. The circular open-field test (90 cm diameter) was situated in a dimmed room with the arena being brightly lit with lamps (1000 lux) to induce anxiety. The rats were allowed to explore the arena for 20 minutes. The rat was then removed and returned to its home cage, and the arena was wiped down with 70% ethanol to remove any olfactory cues before the next rat entered. The number of immobile episodes (a stationary period for >5 seconds) and time spent in the centre of the arena was scored using ANY-Maze v7.16.

2.7 Marble Burying

On P33, the pups underwent marble burying to measure autistic-like and anxiety-like behaviours. The rats were allowed to habituate to a separate room for 30 minutes. Following the habituation period, rats were placed into small cages (960 cm^2) with black marbles (15.9 mm diameter) arranged in a 4 x 5 arrangement on top of 5 cm of tightly packed sawdust bedding. The rats were allowed explore for 30 minutes before being removed from the test arena and returned to their home cage. The number of buried marbles (2/3 of the marble underneath the bedding) were then counted.

2.8 3-Chamber Social Test

On P40, the 3-chamber social test was performed to measure sociability and social novelty. Rats were allowed to habituate in a dimmed room for 45 minutes before beginning the test. The rectangular arena (57 cm x 99 cm) was divided into 3 separate chambers, connected by a small open doorway so the rat could explore all 3 chambers. The two outer chambers contained a small container to hold the non-test rats. In the first 10 minutes, the experimental rat was allowed to explore the whole arena. This is known as the habituation phase. The next 10 minutes, a novel, age- and sex-matched conspecific rat was included in one of the containers, with the other container empty. The time the experimental animal spent with a conspecific rat was recorded and defined as sociability. In the last 10 minutes, another novel, age- and sex- matched conspecific rat was included in the remaining container in the other chamber. The rat used in the sociability phase was left in its container. The experimental animal was allowed to interact with both conspecifics and the time spent with the new rat was measured and compared to the time spent with the now 'familiar' rat and defined as social novelty. Following the test, all rats were returned to their home cage, and the arena was wiped down with 70% ethanol to remove any olfactory cues. Sociability and social novelty were scored using BORIS v7.13.9.

2.9 Transcardial Perfusions

On P47, the rats were euthanised by intraperitoneal injection with a 25G needle with 0.8 ml/kg of 200 mg sodium pentobarbital (Euthatol). The palpebral, tail, and pedal reflexes were checked before beginning perfusion. The animal was secured to the dissection board, and the thoracic cavity was opened. With the heart still beating, a 15G cannula was inserted into the left ventricle. An incision was made in the right ventricle to allow blood to drain. Cold 10 mM PBS was pumped through the body using a Watson Marlow 101 U/R peristaltic pump until the perfusate ran clear. Following this, the pump was switched to 4% paraformaldehyde (PFA). Following the blanching of the liver and feet, stiffness of the neck, and fixation tremors, the rat was decapitated and the whole brain extracted.

2.10 Tissue Preparation

Following the brain removal, it was immersed in 4% PFA and stored at 4˚C overnight. The following day, the brain tissue was transferred to 30% sucrose and kept at 4˚C until fully submerged, then snap-frozen in liquid nitrogen, for long-term storage at -80˚C. 30 µm sections were cut using a freezing stage cryostat (Leica CM1950) and sections were kept in cryoprotectant (10 mM PBS with 30% ethylene glycol, 25% glycerol, 20% water) until mounting on Superfrost™ Plus Adhesion Microscope Slides and kept at -20˚C until used for immunohistochemistry.

2.11 5-HT1a Receptor Staining

The amygdala and prefrontal cortex were stained for 5-hydroxytryptamine (5-HT)1a receptor expression. Sections were removed from the freezer and brought to room temperature. Sections were washed 3 times, 10 minutes each using triton-X tris-buffered saline (TXTBS). Non-specific binding was then blocked using 10% goat serum in tris-buffered saline (TBS) for 1 hour. Following this, primary antibody (anti-5-HT1a receptor, Merck AB15350) (1:1000 dilution) and 1% goat serum was diluted in TBS. 200 µL was added to each slide and allowed to incubate overnight at 4˚C. Sections were washed 3 times, 10 minutes each with TXTBS. The secondary antibody (Alexa Flour 488 conjugated secondary antibody, Invitrogen) (1:500 dilution) was diluted in TBS with 1% goat serum and 200 µL was added to each slide. Following a 2 hour incubation at room temperature in the dark, slides were washed 3 times, 10 minutes each with TXTBS. Nuclei were stained with DAPI (4'-6-Diamidino-2-phenylindole, Sigma) (1:3000 dilution) in TBS for 5 minutes in the dark at room temperature. Finally, slides were washed 3 times, 10 minutes each with TBS before leaving to dry and cover-slipping with DAKO Fluorescence Mounting Medium. Slides were stored in the dark at 4˚C until imaged on the Olympus BX53 Upright Microscope. Intensity of receptor expression was measured using ImageJ software.

2.12 DNA Extraction

Faecal pellets were collected on P40, and DNA was extracted using QIAamp DNA Micro Kit (Qiagen) as per the manufacturer's instructions Quantification of DNA was assessed using ND-1000 Spectrophotometer (NanoDrop). Extracted DNA was stored at -80˚C until sent for sequencing.

2.13 DNA Sequencing

DNA was standardised to 5 ng/µL and the 16S library was prepared using a fivefold miniaturized version of the Illumina 16S metagenomics library preparation protocol on the Echo 525 (Labcyte). The initial polymerase chain reaction (PCR) was carried out using 0.5 µL DNA (at 5 ng/µL), 1 µL of both the forward and reverse primers (Illumina 16S metagenomics library preparation protocol) at 1 µM, and 2.5 µL 2 x Kapa Hifi Hotstart Ready mix. DNA and reagents were added to 96-well plates using the Echo 525 (Labcyte) and spun briefly to mix. The PCR conditions followed the Illumina 16S metagenomics protocol. Following this, the plates were spun, and 15 µL molecular grade water was added to each sample. Samples then underwent Ampure (Beckman Coulter) purification on the Beckman i7 (Beckman Coulter), the second PCR was set up again using the Echo 525 to transfer the liquid volumes to fresh plates. The reactions were again miniaturised once again to have a final volume of 5 µL 2x Kapa Hifi Hotstart Ready mix, 1 µL purified PCR product, 1 µL N7 index primer (Illumina), 1 µL S5 index primer (Illumina), and 2 µL molecular grade water. The plate was then spun, and index PCR performed as detailed in the Illumina 16S metagenomics protocol. No template controls were included in the initial PCRs and carried through all steps of library preparation. Samples were quantified using a Qubit High Sensitivity Assay and pooled equimolarly. An aliquot of the pool was then cleaned using a 0.8 X ratio of Ampure (Beckman Coulter) beads. The final pool was measured using Qubit and assessed on an Agilent high sensitivity chip (Agilent). The pool was diluted to 1 nM and mixed with 1 nM PhiX (Illumina) at 40% (v/v). The mixture was then loaded onto a P1 600 cycle cartridge and sequenced on the NextSeq 2000 following Illumina guidelines.

2.14 Bioinformatics

Illumina sequence data was retrieved via the *basespace* CLI (Illumina, USA). Sequence quality was evaluated using *FastQC* and *MultiQC* (Ewels et al., 2016) (Babraham Bioinformatics - FastQC A Quality Control tool for High Throughput Sequence Data), and synthetic sequences were removed using *Trimmomatic* (Bolger et al., 2014) (sliding filter window 6:15; minlength 299). *FIGARO* (FIGARO: An efficient and objective tool for optimizing microbiome rRNA gene trimming parameters | bioRxiv) was used to find optimal *dada2::filterAndTrim* parameters for each sample, which were then averaged to a single trimming value across the study (3' trim: 270,214; maxEE: 1,2; 5' trim: 0,0). Further *DADA2* (Callahan et al., 2016) parameters were set to determine and quantify unique amplicon sequence variants (ASVs) across the study: *learnErrors*: randomise = TRUE, nbases = $1x10^9$; *mergePairs*: minimum overlap of 25bp, maximum allowed mismatch of 0bp; *AssignTaxa*: RBD used the "consensus" method. Other parameters remained at default values. *DADA2* (assignSpecies) and DECIPHER (Wright, 2016) were used to generate taxonomic assignments from the Silva 138 release (Quast et al., 2013). *Decontam* (Davis et al., 2018) was used to detect contaminant ASVs based on prevalence between normal and control samples. Bimeras, contaminants, ASVs of unknown phylum, and ASVs outside of the expected length (397 – 428bp) were checked before being removed.

2.15 Statistics

As behavioural readouts were the primary outcome of this study, all power calculations were determined based on the marble burying test. Using GPower 3.1, to reach statistical significance, a total of 6 dams per group were required, with 12 males and 12 females per group to allow us to see sex differences within the group. Power calculations were approved by a biostatistician, Department of Mathematics, University College Cork.

For figures 2-7, statistical analysis was performed using GraphPad Prism v8.3.0. All analysis were carried out using a 2-way ANOVA, with Fisher's LSD *post hoc* test used where appropriate. Results were deemed significant when $p<0.05$. All data presented as mean ± standard error of the mean (SEM). For figures 2-6, 2

data points for both males and females were averaged to give 1 data point per sex per litter. For figure 7, 1 male and 1 female were used per litter.

For microbiome analysis, for visualisation purposes only, ASVs below 1% abundance and/or prevalence in ≥9 samples were combined to “other”. Remaining taxa were summed across different taxonomic levels (phylum, family, genus). Diversity analyses (α, β) were carried out using vegan (“R - Vegan Tutorial - Multivariate Analysis of Ecological Communities by Oksanen | PDF | Principal Component Analysis | Eigenvalues And Eigenvectors,” n.d.) to compute Shannon’s H, Inverse Simpson’s index, Chao1 richness, Bray-Curtis dissimilarity for PERMANOVA, the Hellinger transformation of abundances for constrained correspondence analysis, and significance of community structure (*vegan::betadisper, ::adonis2, ::anova*). For differential analysis, ASV count data was transformed using count-zero mutiplicative replacement of zeros with *zCompositions::cmultrepl* (Palarea-Albaladejo and Martín-Fernández, 2015) to allow a centre log-ratio transform.

3 Results

3.1 Inflammation and microbiome disruption during pregnancy reduces pup size and placental weight

Two-way ANOVA analysis of E18 body weight revealed significant interaction and LPS effects (Interaction Effect: P=0.0242, F(1, 20)=5.942; ABX Effect: P=0.0617, F(1, 20)=3.918; LPS Effect: P=0.0004, F(1, 20)=18.05). Fisher's LSD *post hoc* test noted numerous differences between *in utero* exposures. Body weight reduction was seen in LPS/ABX group compared to the saline control group (P=0.0002), as well as the LPS group (P=0.0026) and the ABX group (0.0003) (Fig. 2A). Overall crown-rump length did not show an interaction effect, but a two-way ANOVA noted a strong ABX and LPS effect (Interaction Effect: P=0.9171, F(1, 20)=0.0111; ABX Effect: P=0.0207, F(1, 20)=6.310; LPS Effect: P=0.0053, F(1, 20)=9.796). *Post hoc* testing revealed a decrease in crown-rump length in the LPS/ABX group in comparison to the ABX group (P=0.0477). This reduction in crown-rump length was also evident in the LPS group (P=0.0287) and the LPS/ABX group (P=0.0005) compared to the saline group (Fig. 2B). Two-way ANOVA also revealed an ABX effect on placental weight, but no interaction or LPS effect (Interaction Effect: P=0.0856, F(1, 20)=3.270; ABX Effect: P=0.0489, F(1, 20)=4.399; LPS Effect: P=0.2019, F(1, 20)=1.741). Fisher's LSD *post hoc* revealed a significant reduction in in placental weight in the ABX group (P=0.0243), the LPS group (P=0.0192), and in the LPS/ABX group (P=0.217) in comparison to the saline control group (Fig. 2C).

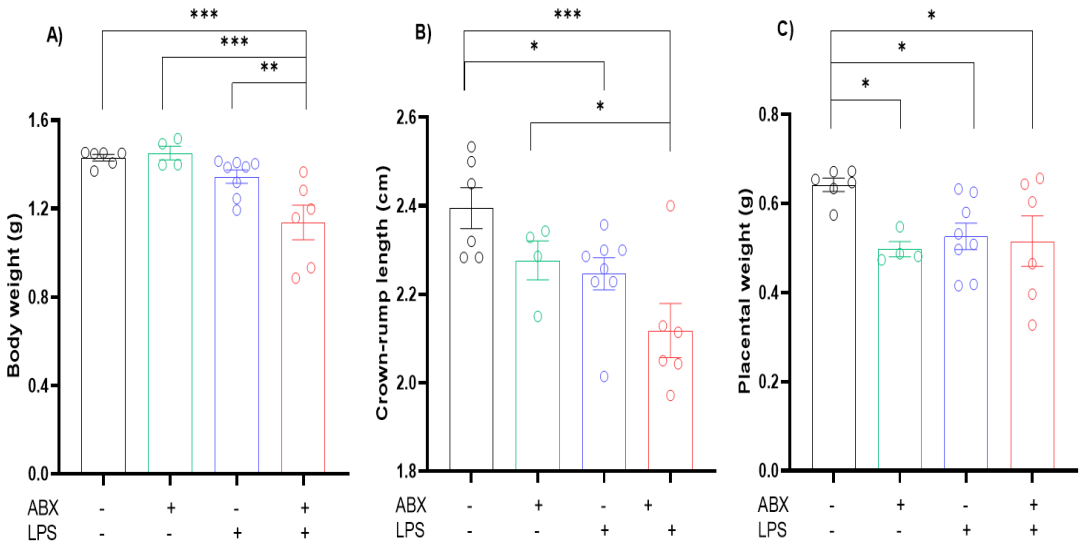


Figure 2: Significant morphological changes seen in E18 following inflammation and microbial disruption. *A) Pup weight on embryonic day 18. B) Pup crown-rump length on embryonic day 18. C) Placental weight on embryonic day 18. N=2-4 dams per group. 1 male and 1 female, 2 data points averaged to give 1 data point per sex per litter. *= P=<0.05, **=P=<0.01, ***=P=<0.001.*

3.2 Inflammation and maternal microbiome disruption during pregnancy does not alter the frequency or length of pup ultrasonic vocalisations

Two-way ANOVA revealed no significant changes seen in the number of calls (Interaction Effect: P=0.0629, F(1, 38)=3.672; ABX Effect: P=0.3648, F(1, 38)=0.8413; LPS Effect: P=0.4472, F(1, 38)= 0.5898) (Fig. 3A). Furthermore, no significant changes were seen in the total call duration across the 3-minute test period as seen with a two-way ANOVA (Interaction Effect: P=0.1390, F(1, 38)=2.284; ABX Effect: P=0.4062, F(1, 38)=0.7055; LPS Effect: P=0.9124, F(1, 38)= 0.0122) (Fig. 3B).

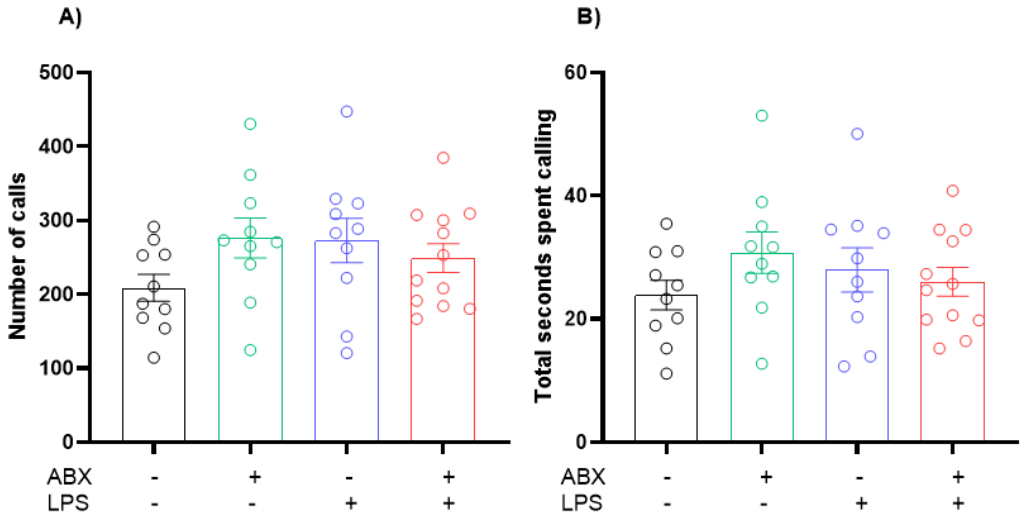


Figure 3: No significant changes seen in ultrasonic vocalisations following inflammation and microbial disruption. *A) Number of ultrasonic calls recorded within a 3-minute period. No significance found. B) Total time spent calling within a 3-minute period. No significance found. Recordings taken on postnatal day 9. N=5-6 dams per group. 1 male and 1 female, 2 data points averaged to give 1 data point per sex per litter.*

3.3 Rats show anxiety-like behaviour due to inflammation and maternal microbiome disruption *in utero*

Time spent in the centre of the arena, used as an indication of anxiety-like behaviour, was not statistically significant following a two-way ANOVA (Interaction Effect: P=0.1802, F(1, 42)=1.858; ABX Effect: P=0.3804, F(1, 42)=0.7857; LPS Effect: P=0.2754, F(1, 42)=1.221 (Fig. 4A). No significant differences were seen when examining the sexes either (Male: Interaction Effect: P=0.4660, F(1, 19)=0.5535; ABX Effect: P=0.1193, F(1, 19)=2.661; LPS Effect: P=0.0944, F(1, 19)=3.099) (Female: Interaction Effect: P=0.2426, F(1, 19)=1.455; ABX Effect: P=0.9905, F(1, 19)=0.0001; LPS Effect: P=0.8446, F(1, 19)=0.0394) (Fig. 4B).

Two-way ANOVA found a significant LPS effect in the number of immobile episodes, a measure of anxiety-like behaviour (Interaction Effect: P=0.1880, F(1, 42)=1.791; ABX Effect: P=0.7429, F(1, 42)=0.1091; LPS Effect: P=0.0084, F(1, 42)=7.638) (Fig. 4C). Fisher's LSD *post hoc* test revealed a significant increase in the number of immobile episodes in the LPS group in comparison to both the

saline group (P=0.007) and the ABX group (P=0.303). Finally, males displayed a significant LPS effect in number of immobile episodes following two-way ANOVA analysis (Interaction Effect: P=0.0814, F(1, 19)=3.386; ABX Effect: P=0.9334, F(1, 19)=0.0071; LPS Effect: P=0.0122, F(1, 19)=7.888) (Fig. 4D). Fisher's LSD *post hoc* test revealed a significant increase in the number of immobile episodes in the LPS group in comparison to the saline group (P=0.0046). No significant changes were seen in females (Interaction Effect: P=0.6666, F(1, 19)= 0.1915; ABX Effect: P=0.7486, F(1, 19)=0.1058; LPS Effect: P=0.1996, F(1, 19)=1.766) (Fig. 4D).

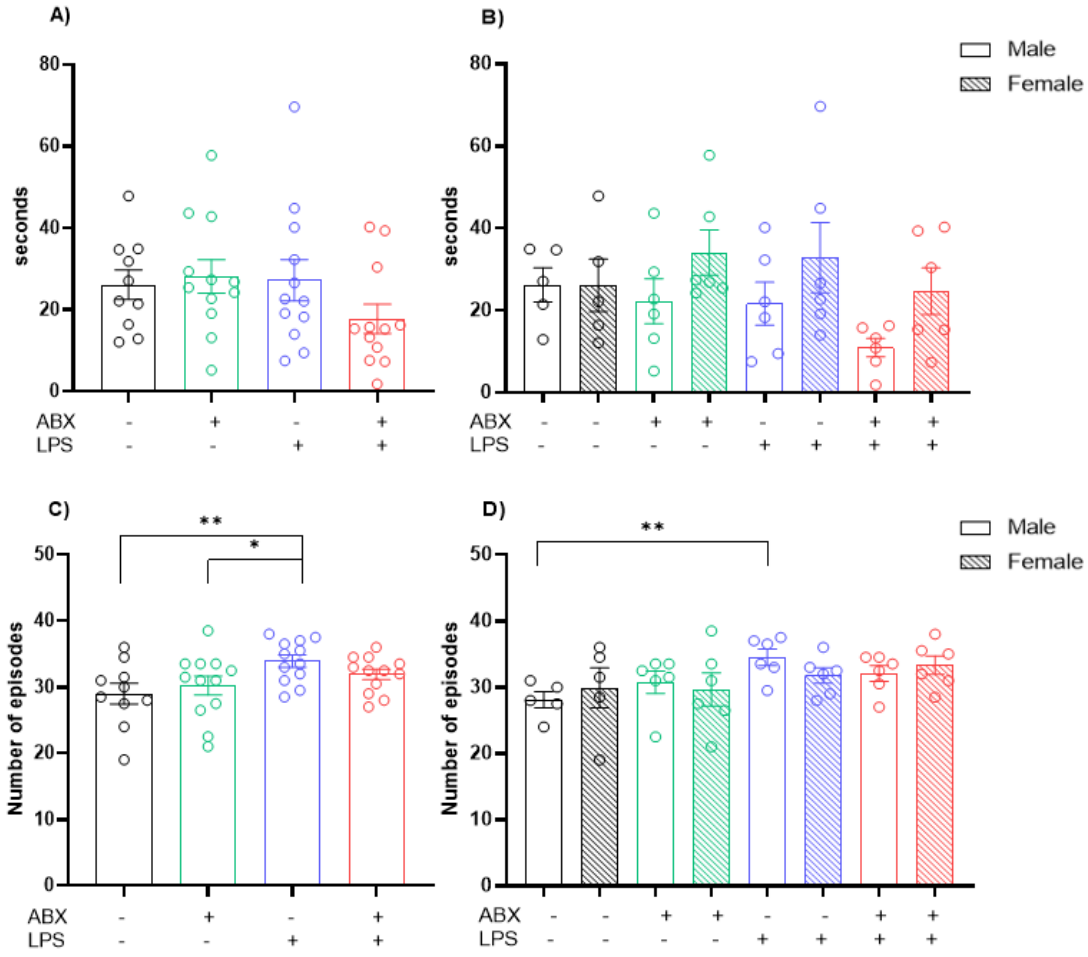


Figure 4: Increased anxiety following prenatal inflammation exposure. *A) Time spent in the centre within a 20-minute period. B) Sex differences in time spent in the centre within a 20-minute period. C) Number of immobile episodes (>5 stationary seconds) within a 20-minute period. D) Sex differences in the number of immobile episodes (>5 stationary seconds) within a 20-minute period. Behaviour performed on postnatal day 25. N=5-6 dams per group. 1 male and 1 female, 2 data points averaged to give 1 data point per sex per litter. *= P=<0.05, **=P=<0.01.*

3.4 Rats show stereotypical, repetitive behaviours due to inflammation *in utero*

Two-way ANOVA revealed statistically significant changes with an LPS effect in the number of marbles buried (Interaction Effect: P=0.8667, F(1, 42)=0.0285; ABX Effect: P=0.8302, F(1, 42)=0.0465; LPS Effect: P=0.0032, F(1, 42)=9.755) (Fig. 5A). Fisher's LSD *post hoc* test revealed a significant increase in the number of marbles buried in both the LPS group (P=0.0281) and combined LPS/ABX group (P=0.0261) compared to the saline control group. An increase in burying was also found between both the LPS group (P=0.0412) and the combined LPS/ABX group (P=0.0381) compared to the ABX group. Regarding the males, no main effect was seen with a two-way ANOVA (Interaction Effect: P=0.5251, F(1, 19)=0.4192; ABX Effect: P=0.7844, F(1, 19)=0.077; LPS Effect: P=0.0847, F(1, 19)=3.309) (Fig. 5B). Females displayed a strong LPS effect (Interaction Effect: P=0.6255, F(1, 19)=0.2461; ABX Effect: P=0.9943, F(1, 19)=5.169e-005; LPS Effect: P=0.0152, F(1, 19)=7.115) (Fig. 5B). Fisher's LSD *post hoc* test noted an increase in buried marbles in the LPS/ABX group (P=0.0271) in comparison to the ABX group.

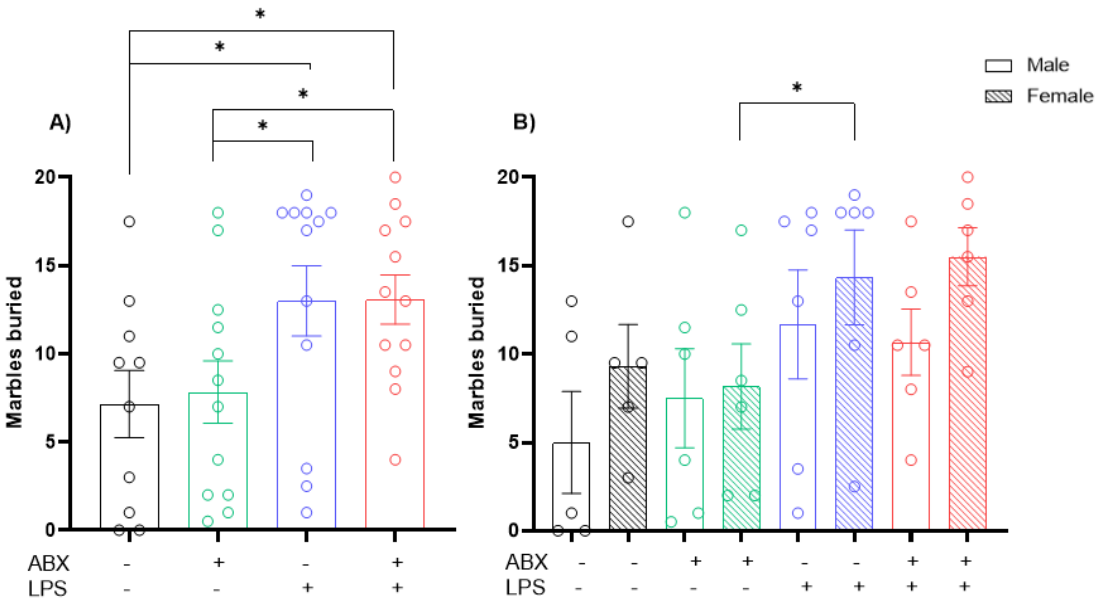


Figure 5: Increased repetitive behaviours following prenatal inflammation. *A) Number of marbles buried within a 30-minute period. B) Sex differences in number of marbles buried within*

*a 30-minute period. Behaviour performed on postnatal day 33. N=5-6 dams per group. 1 male and 1 female, 2 data points averaged to give 1 data point per sex per litter. *= P=<0.05.*

3.5 Rats do not show altered sociability due to inflammation or maternal microbiome disruption exposure *in utero*

Two-way ANOVA revealed no significant effect of *in utero* exposures on sociability (Interaction Effect: P=0.1427, F(1, 42)=2.231; ABX Effect: P=0.2235, F(1, 42)=1.527; LPS Effect: P=0.9814, F(1, 42)=0.0005) (Fig. 6A). No significant differences were seen within the sexes either (Male: Interaction Effect: P=0.3820, F(1, 19)=0.8009; ABX Effect: P=0.7015, F(1, 19)=0.1514; LPS Effect: P=0.5630, F(1, 19)=0.3465) (Female: Interaction Effect: P=0.2377, F(1, 19)=1.487; ABX Effect: P=0.1422, F(1, 19)=2.345; LPS Effect: P=0.3903, F(1, 19)=0.7728) (Fig. 6B). Regarding social novelty, a two-way ANOVA detected no main effects (Interaction Effect: P=0.4831, F(1, 42)=0.5007; ABX Effect: P=0.5745, F(1, 42)=0.3201; LPS Effect: P=0.0833, F(1, 42)=3.146) (Fig. 6C). When examining differences within the sexes, no significant alterations were observed (Male: Interaction Effect: P=0.3148, F(1, 19)=1.066; ABX Effect: P=0.3918, F(1, 19)=0.7680; LPS Effect: P=0.0903, F(1, 19)=3.186) (Female: Interaction Effect: P=0.9282, F(1, 19)=0.0083; ABX Effect: P=0.0696, F(1, 19)=3.698; LPS Effect: P=0.4844, F(1, 19)=0.5086) (Fig. 6D).

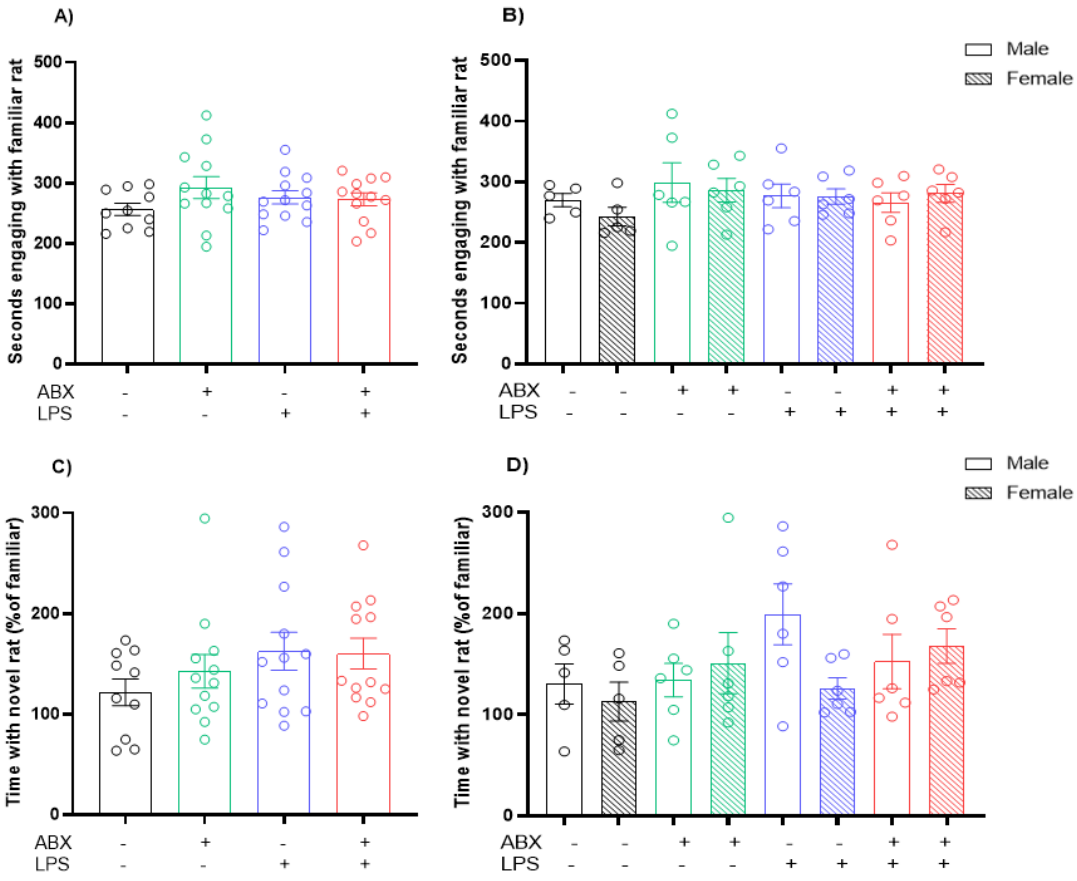


Figure 6: No changes to sociability following prenatal exposures. *A) Time spent with a novel, conspecific rat across a 10-minute period. B) Sex differences in time spent with a novel, conspecific rat across a 10-minute period. C) Time spent with a novel, conspecific rat across a 10-minute period expressed as a percentage of time spent with a familiar conspecific. D) Sex differences in time spent with a novel, conspecific rat across a 10-minute period expressed as a percentage of time spent with a familiar conspecific. Behaviour performed on postnatal day 40. N=5-6 dams per group. 1 male and 1 female, 2 data points averaged to give 1 data point per sex per litter.*

3.6 Inflammation reduces 5-HT1a receptor expression in the prefrontal cortex, but not in the amygdala

The intensity of 5-HT1a receptor expression in the amygdala was analysed via a two-way ANOVA and revealed no significant changes (Interaction Effect: P=0.0900, F(1, 41)=3.015; ABX Effect: P=0.2037, F(1, 41)=1.669; LPS Effect: P=0.6197, F(1, 41)=0.2501) (Fig. 7A). No significant changes were seen between males or females (Male: Interaction Effect: P=0.4224, F(1, 18)=0.6741; ABX Effect: P=0.5706, F(1, 18)=0.3338; LPS Effect: P=0.6868, F(1, 18)=0.1679) (Female: Interaction Effect: P=0.1362, F(1, 19)=2.421; ABX Effect: P=0.2385, F(1, 19)=1.481; LPS Effect: P=0.8091, F(1, 19)=0.06004) (Fig. 7B). The prefrontal

cortex revealed a significant reduction in 5-HT1a receptor expression with an LPS effect (Interaction Effect: P=0.4946, F(1, 39)=0.4754; ABX Effect: P=0.4190, F(1, 39)=0.6670; LPS Effect: P=0.0248, F(1, 39)=5.454) (Fig. 7D). Fisher's LSD *post hoc* test noted a reduction in receptor expression in the LPS/ABX group in comparison to the ABX group (P=0.0369) and the saline group (P=0.03). While no differences were seen between females, an LPS effect was found in males following two-way ANOVA analysis (Male: Interaction Effect: P=0.8994, F(1, 18)=0.0167; ABX Effect: P=0.9513, F(1, 18)=0.0038; LPS Effect: P=0.0269, F(1, 18)=5.808) (Female: Interaction Effect: P=0.3437, F(1, 16)=0.9522; ABX Effect: P=0.224, F(1, 16)=1.60; LPS Effect: P=0.2726, F(1, 16)=1.291) (Fig. 7E).

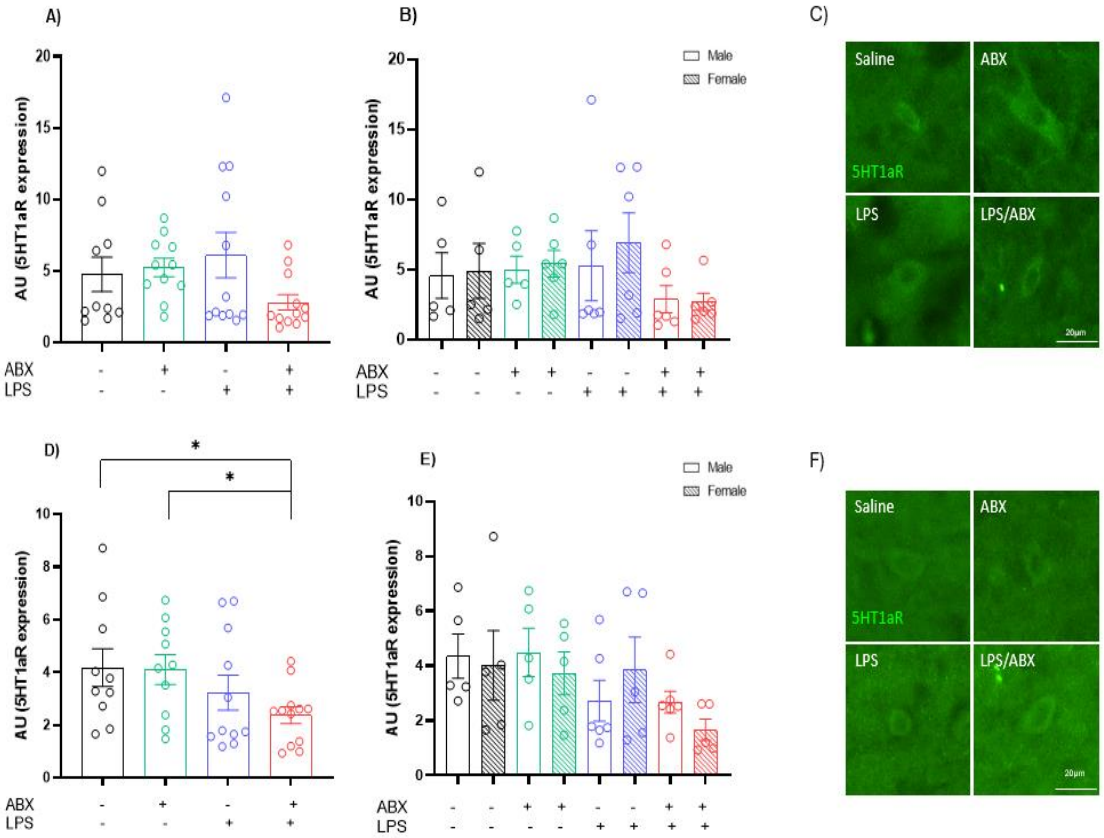


Figure 7: Reduction in 5-HT1a receptor expression in the prefrontal cortex following in utero exposure to inflammation. *A) 5-HT1aR expression in the amygdala. B) Sex differences in 5-HT1aR expression in the amygdala. 5-HT1aR expression. C) Representative photomicrographs on 5-HT1aR expression in the amygdala. D) 5-HT1aR expression in the prefrontal cortex. E) Sex differences in 5-HT1aR expression seen in the prefrontal cortex. Significant in males with two-way ANOVA only. F) Representative photomicrographs on 5-HT1aR staining in the prefrontal cortex. N=5-6 dams per group, 1 male and 1 female per litter. *= P=<0.05.*

3.7 Exposure to inflammation and maternal microbiome disruption *in utero* reduces diversity and alters the gut microbiome

High-throughput sequencing provided 16S rDNA profiles for 91 pup faecal samples (mean raw read-pairs per sample: 454K+/-129K, mean amplicon sequence variants (ASVs) per sample: 154K +/-38K merged read-pairs; mean unique ASVs per sample: 921 +/- 170). While microbiome composition was largely similar across groups and gender (Fig. 8A-C), disruption caused by ABX exposure *in utero* was still evident in pups at P40. Species richness (Chao1, Fig. 8E) was significantly lower in all ABX and LPS/ABX groups (t=-3.5-2.7, p=0.002-0.04), in addition to particularly low diversity in the male ABX group (Shannon's H; versus LPS: t=-2.5, p=0.02; versus female ABX: t=2.1, p=0.035; Fig. 8D). In addition to a significant effect on individual samples (α-diversity), treatment *in utero* also significantly changed microbial composition between treatment groups (β-diversity; PERMANOVA: F=3.1, p<0.001; Fig. 8F). In comparison, β-diversity was only weakly impacted by sex (PERMANOVA; F=1.3, p = 0.09).

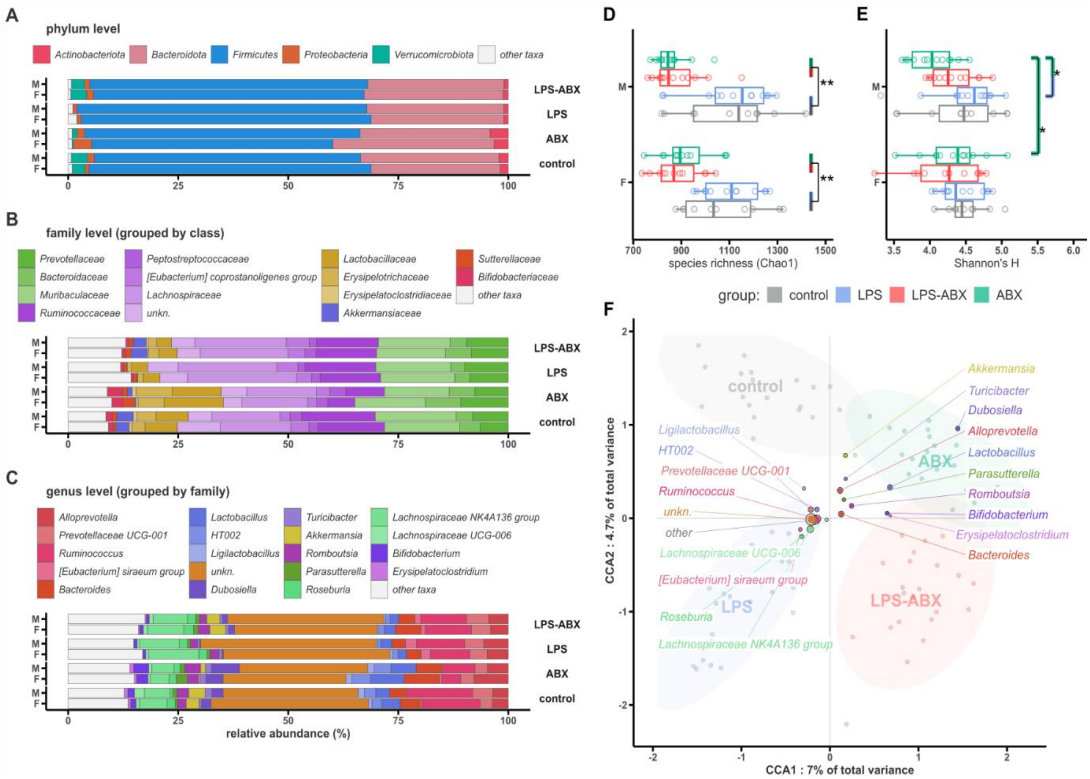


Figure 8: Microbiome disruption and inflammation lead to enduring changes in the microbiome of offspring. *A-C) Mean relative abundances of the pup microbiome at P40,*

*summarised at phylum, family and genus levels, grouped by group and sex. Taxa at family level share a colour if within the same class, while taxa at genus level share a colour if within the same family. Taxa not present above 1% in 9 or more samples are grouped to "other taxa". D) Microbiome disruption via ABX or LPS/ABX treatments significantly decreases species richness in the pup microbiome (Chao1 index) with respect to control or LPS groups, for both sexes. E) Disruption of the microbiome in the mother leads to significantly reduced α-diversity (Shannon's H) in the male ABX pups, compared to male LPS or female ABX. F) Pups exposed to different gestational conditions show significantly different microbiome composition at P40, as shown by the strong separation of samples and lack of overlap between different groups ($p < 0.001$). Group labels show the "average" position of each group (centroid), while coloured points show genera closest to the samples and groups where they were most abundant, with size of point indicating relative abundance. CCA: constrained correspondence analysis. N = 91 for all comparisons. *= P=<0.05, **=P=<0.01.*

Maternal exposure to ABX / LPS/ABX during gestation was also associated with significant decreases in specific ASVs; mainly in [homo/heterofermenters and aceto/acido/butyrogens] from the taxonomic classes Bacteroidia and Clostridia (Fig. 9). The relative abundance of a number of uncharacterised taxa (*Muribaculaceae, Lachnospiraceae, UBA1819/Ruminococcaceae*) was increased in ABX / LPS/ABX exposure groups. Exposure to both LPS and ABX *in utero* was associated with increases in ASVs from the *Eubacterium xylanophilum* clade. Notably, there were no significant differences between the control and LPS groups, nor between the ABX and LPS/ABX groups.

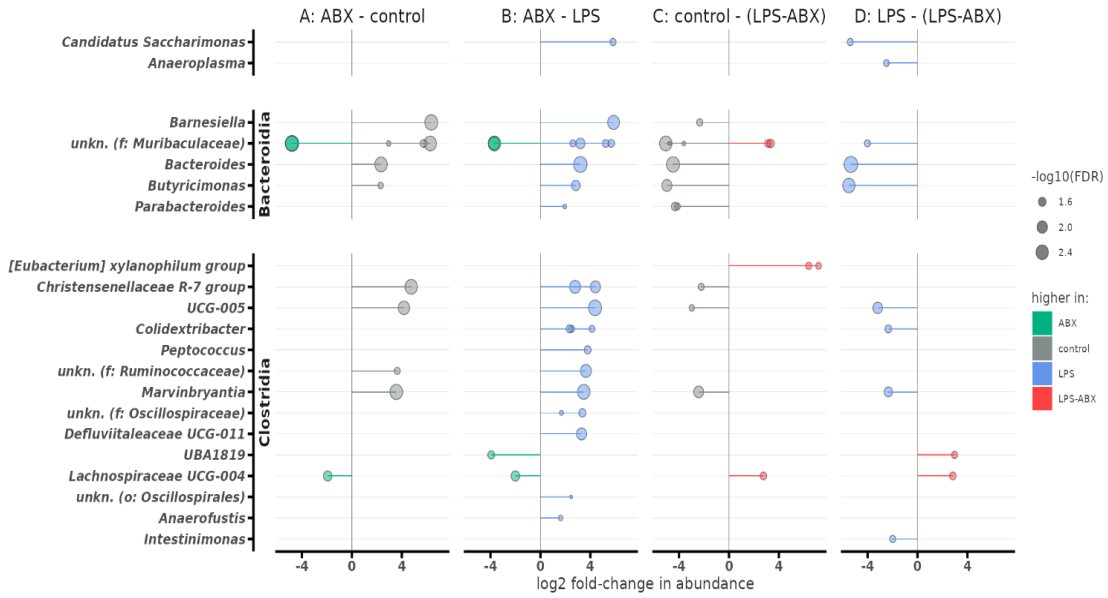


Figure 9: Many different populations of ASV are significantly affected by gestational microbiome disruption and inflammation at P40. *Taxa which are significantly different between groups, while controlling for the effects of sex and litter. All features are significantly different below a false discovery rate (FDR) correction of 0.05, with size of point showing the FDR value on*

a -log_{10} scale (larger points are more significantly different). X axis shows the log_2-fold change, i.e. how many doublings in abundance two groups differ by (L2FC 1 = $log_2(1)$ = 2 times more abundant, L2FC 3 = $log_2(3)$ = 8 times more abundant, etc.). In all cases, negative L2FC values indicate greater abundance in the left-hand side label. Abbreviations: f: family-level; o: order-level.

4 Discussion

In this study, we noted significant deleterious effects on offspring weight, size, and neurobehavioural outcomes following exposure to two different prenatal stressors. LPS administration, simulating maternal bacterial infection, produced the most pronounced deficits in behaviour and neurochemical changes within the brain, along with various morphological changes. These offspring were significantly more anxious (Chen et al., 2023; Depino, 2015; Lin et al., 2012) and had an increase in repetitive behaviours (Fortunato et al., 2017; Kirsten and Bernardi, 2017) which corresponds with the previous literature. These shifts in behaviour were associated with changes seen in 5-HT1a receptor expression in the prefrontal cortex, a key region in anxiety-related neural circuits. More discrete changes were noted following exposure to antibiotics *in utero*, where the main effect was evident in crown-rump length and placental weight. While cumulative effects were expected following exposure to both prenatal stressors, an interaction was only seen in the body weight of the embryos. This lack of a dual-hit effect of prenatal stressors on brain and behaviour is surprising. It was our expectation that the offspring exposed to both LPS/ABX would perform worse in the behavioural tests. Previous literature denotes the strong effect on neurodevelopmental sequalae that either stressor has on the offspring, hence a synergistic effect was hypothesised. Yet as the results show, this was not the case. It is difficult to state what may have blocked this effect, as this is the first time these exposures have been looked at in this context. One possible explanation for the lack of a dual hit is a potential anti-inflammatory effect of the antibiotics. If the antibiotics were able to suppress the inflammation that LPS would be expected to produce, the two insults would essentially be counterintuitive. While not the same as the work performed in this study, no cumulative effects were seen in a model of neonatal LPS exposure and early adulthood antibiotic exposure (Tejkalová et al., 2023). Potentially the negative modulation of the maternal microbiome is preventing the LPS from having full effect, if certain pro-inflammatory species were depleted by the antibiotics the LPS may not be able to produce the same level of inflammation. Separately, it is

likely that these two exposures work through separate mechanisms, MIA has been shown to influence neurodevelopment via the placenta (Woods et al., 2023), but antibiotic exposure may alter brain development through short-chain fatty acids for instance (Jiajia Jin et al., 2022; Vuong et al., 2020). By working through two separate pathways, it is possible that this study may not capture any cumulative effects.

Exposure to LPS alone or both LPS/ABX were required to reduce crown-rump length, LPS/ABX to decrease body weight, but exposure to either stressor was sufficient placental weight. This strongly indicates that LPS exposure results in IUGR in the fetuses that may be causing small for gestational age offspring. Previous work has shown that LPS exposure leads to IUGR (Liu et al., 2014) and in humans, both IUGR and being born small for gestational age have been linked to poorer neurodevelopmental outcomes (Sacchi et al., 2020). The growth restriction observed here may contribute to the behavioural changes seen later in life, but if this is the case, there is limited correlation with the severity of the outcomes. The placenta, which acts as the communicative interface between the mother and fetus for the duration of the pregnancy, is a vital organ necessary for sufficient oxygen and nutrient transport. Compromising the efficiency of the placenta is associated with numerous severe maternal complications, such as pre-eclampsia and placenta accreta spectrum, and may negatively influence fetal development (Cahill et al., 2018; Melchiorre et al., 2022). The reduction in placental weight seen in this study may be indicative of placental insufficiency and this in itself may lead to IUGR, fetal hypoxia, and pre-term birth (Audette and Kingdom, 2018; Gagnon, 2003). It remains challenging to uncouple if placental insufficiency or IUGR has a greater impact on the negative offspring effects.

Ultrasonic vocalisation recordings have long been used as an insight into communicative behaviour between the pup-mother dyad and can elicit maternal approach. These calls can be interpreted as the pups' affective state and can be informative about levels of postnatal stress. Various forms of prenatal stress have previously led to an increased number of calls emitted by the affected pups (Vitor-Vieira et al., 2024; Zimmerberg and Blaskey, 1998), but a decreased

number of calls has also been noted (Baharnoori et al., 2012). However, no significant effects were seen in this study, suggesting that pups exposed to prenatal stressors did not exhibit heightened stress levels compared to their control counterparts. This result is surprising, as vocalisation changes were expected in the offspring. It is hard to deduce why no significance was reported in this study but there are a few possible suggestions. It may have been a better approach to record vocalisations across multiple timepoints, to get a wider sampling range in case any alterations to vocalisations may emerge earlier or later. This has been done with several previous studies and not testing at various early life timepoints may have masked any effects (Hermans et al., 2024; Lai et al., 2014). Building upon this, LPS and antibiotic exposure has been shown to influence brain development, they may not affect areas involved in vocal behaviour. The periaqueductal grey and amygdala are key regions involved, and while we only examined 5-HT1aR expression in the amygdala, no changes were seen. It is very possible that the methods of prenatal stress do not evoke alterations in this behaviour.

The open field test revealed that offspring exposed to LPS showed greater anxiety-like behaviours, with an increased number of startled or freezing behaviours. In this, we found that the male offspring were driving the significant changes. While the increased number of freezing behaviours aligns with past literature, it is interesting that no changes were seen with the time spent in the centre of the arena, which would also be indicative of anxiety. This nuanced result may indicate that the anxiety seen from these offspring may not be baseline anxiety and may be more of fear avoidance. Both of these are regulated by partially distinct circuits, with overlap seen in the amygdala (Koutsikou et al., 2014; Yan et al., 2022), potentially explaining the differences seen here. The sex differences seen in this behaviour finding mimics what is normally found in the human population, where following prenatal stress, male offspring have 4- to 8-fold increased risk of developing various neurodevelopmental disorders (Bale, 2016). As previously mentioned, the increase in anxiety-like behaviours following prenatal stress is well-documented, though the precise mechanisms remain unclear. Potential contributors include disruptions of the serotonergic and

noradrenergic systems, microglia activation, and alterations in the neuroanatomy (Quagliato et al., 2021).

The marble burying test indicated high levels of repetitive and stereotypical behaviours following LPS exposure, paralleling characteristics of obsessive-compulsive disorder (OCD) in humans. While OCD, a subcategory of anxiety, is typically less associated with neurodevelopment, it still has developmental underpinnings (Geschwind, 2011). Although prenatal stress typically affects males to a greater extent, this behavioural analysis revealed that females were more engaged in the repetitive behaviours. Yet with that being said, females are more likely to develop OCD, which aligns with the findings in this study (Fawcett et al., 2020). Typically, males and females present OCD symptoms differently with tics and social phobias deemed more male-associated symptoms, and compulsion deemed more female associated (Mathes et al., 2019). The parameters of this behavioural test associates closer with the expected presentation of female OCD. But, because of this, it is entirely possible that the male offspring also may display OCD-like behaviours but simply another test would be required to uncover them.

Maternal immune activation via LPS administration has been previously linked to social behaviour deficits, with the exposed offspring typically showing reduced interaction with conspecifics when compared to their control counterparts (Dutra et al., 2023; Lee et al., 2021), interpreted as an autism-like phenotype However, this pattern was not replicated in the current study. In this study, no changes were seen in sociability or social novelty. Based on the current literature, our findings do not align with what has previously been shown. The discrepancies seen here could be stemmed back to various LPS dosing, as most studies administer one dose earlier or later than the first dose in this study (Dutra et al., 2023; Lee et al., 2021). Time of testing may also contribute, Dutra et al. noted opposing results on postnatal day 28 and 60, showing that social behaviour may change over time. Perhaps our timepoint of postnatal day 40 was too late, and any deficits caused by the LPS may have been compensated for. Not only is the lack of an LPS surprising, but direct antibiotic usage has previously be shown to

affect social behaviours in rodents (Hayer et al., 2023). However, in terms of the offspring exposed to antibiotics *in utero*, the results are not as clear. Studies report mixed findings of decreased sociability, but this finding has not been replicated in others (Hayer et al., 2023). Perhaps social behaviour is more difficult to characterise during this juvenile and adolescent period.

LPS exposure reduced the expression of 5-HT1a receptors in the prefrontal cortex, and this change was driven by affected male offspring. The 5-HT1a receptor is a well-documented inhibitory G-protein coupled serotonergic receptor that plays an important role in the presentation of various anxiety disorders. These heteroreceptors, often located on interneurons or pyramidal neurons, are implicated in the modulation of mood disorders (Akimova et al., 2009). Human imaging studies have revealed that these receptors are abundantly present in various regions of the brain, including the amygdala and prefrontal cortex (Garcia-Garcia et al., 2014). There was no significant difference in 5-HT1a receptor expression in the amygdala, which is surprising considering the role of the amygdala in anxiety. The decrease in 5-HT1a receptor expression seen in the prefrontal cortex was anticipated as the prefrontal cortex has long been involved in the neuronal signalling pathway associated with anxiety, with numerous projections to other important structures, such as the insula, locus coeruleus, and the stria terminalis (Bouras et al., 2023). Rodent studies have noted that 5-HT1a receptors have a role in both somatosensory development, but are also necessary in early postnatal life to mediate the emergence of normal and expected anxiety-like behaviours (Garcia-Garcia et al., 2014). Mice with non-functional 5-HT1a receptors have increased levels of anxiety and a critical developmental window of these receptors has been theorised (Garcia-Garcia et al., 2014). Depletion of these receptors, as seen here in this study, may be partly responsible for the increased anxiety-like behaviours seen in the male offspring, and the repetitive behaviours seen in the females. The serotonergic system begins formation between gestational day 11 – 12 (Lauder, 1990). Considering that one of the potential mechanisms behind maternal immune activation is disruption of serotonin neurotransmission (Quagliato et al., 2021), it is plausible that prenatal stress induced by LPS exposure may disrupt the correct formation

of the serotonergic system due to exposure coinciding with the beginning of this critical development window. The reduction in 5-HT1a receptor expression in the prefrontal cortex may explain why anxiety-like behaviours were seen males and not the females.

Differential behavioural profiles between the two sexes are well-documented, with numerous potential mechanisms proposed to account for this disparity. One major hypothesis involves the sexual dimorphism of the placenta. Studies have revealed that the male placenta responds and adapts to prenatal stressors differently in comparison to the female placenta (Bale, 2016; Pérez-Cerezales et al., 2018). There are a number of proposed molecular mechanisms underpinning these differences, including transcriptional sex differences in the placenta, glycogen availability affecting energy levels, and the ability to upregulate the protective enzyme 11β-hydroxysteroid dehydrogenase-2 when required, which converts cortisol to the inactive cortisone (O'Connell et al., 2013; Pérez-Cerezales et al., 2018; Wilcoxon et al., 2003). Furthermore, following early life stress, the levels of 17-β-hydroxysteroid dehydrogenase-3 were reduced in the male placenta. 17-β-hydroxysteroid dehydrogenase-3 converts androstenedione to testosterone, leading to reduced levels of testosterone in the brain, and this demasculinised phenotype has been shown to have behavioural implications (Bale, 2016). Despite the numerous and plausible hypotheses, the main conclusion is that the male fetus is not as well-equipped to deal with stressors in comparison to the female fetus. This typically leads the male to be impacted more than the female, with greater risk of neurodevelopmental disorders in later life.

The subsequent manipulation of the maternal microbiome following LPS or ABX administration was found to leave a long-lasting imprint on the microbial composition of the offspring. The β-diversity of the microbiome highlighted significant changes across all experimental groups, revealing that different groups are being driven by various bacteria with little-to-no overlap. Numerous populations of ASVs were also impacted by prenatal exposures, revealing variance between experimental groups in the abundance of different Clostridia

and Bacteroidia species. Differences were particularly evident when examining species richness across the experimental groups, with LPS/ABX and ABX alone showing a significant reduction in the overall number of species present when compared to LPS and the control groups. Reduced species richness has been previously linked to disrupted behaviour, mainly anxiety and depression, and is also associated with increased inflammation (Bear et al., 2023; Kumar et al., 2023; Lee et al., 2018. Yet, no behavioural alterations were found to be caused by *in utero* ABX exposure in this study, which suggests that the reduction in species was not substantial enough to impact behavioural changes. This is similar to the reduced levels of α-diversity seen in the ABX group when compared to LPS exposed offspring. Poor α-diversity is known to contribute to impaired gut and immune health, in addition to impacting behavioural outcomes (Lee et al., 2018; Li et al., 2023; Shevchenko et al., 2023; Q. Wang et al., 2022). Interestingly, the reduction in α-diversity was only seen in the male offspring. There are established differences in male and female microbiomes, so this is not unexpected (Shobeiri et al., 2022). Yet the reason why this outcome was evident could be related to many potential mechanisms, such as the impact of hormones or sex-related behavioural differences (d'Afflitto et al., 2022). It is surprising however that LPS exposure is insufficient to induce its own changes to the gut microbiome, as this has been previously established to cause the induce an inflammatory-like phenotype in the human microbiome previously (An et al., 2022; Candelli et al., 2021).

Despite the lack of effect upon exposure to antibiotics *in utero* on the offspring, maternal immune activation led to an increase in anxiety-like and repetitive-like behaviours and altered the serotonergic system in the prefrontal cortex. While this study focused on serotonergic signalling, we acknowledge that both MIA and antibiotic exposure likely impact immune pathways in both the brain and the gut of the offspring. It is possible that some of the behavioural outcomes seen in this study is in response to microglial activation or alterations in gut immune cells. Future studies should focus on neuroinflammation and possible ways to ameliorate the effects through positive modulation of the maternal microbiome. This could be achieved through administration of anti-inflammatory probiotics,

such as various *Lactobacillus* or *Bifidobacterium*. To date, there have been few studies investigating the therapeutic benefit of probiotics following maternal inflammation, but future studies are warranted to help reduce offspring exposure to inflammation *in utero* and improve their neurodevelopmental outcomes.

5 Conclusion

In conclusion, we examined the effect of MIA and maternal microbial dysbiosis on offspring neurodevelopment via *in utero* exposure to LPS and/or an antibiotic cocktail. While minimal changes were seen due to antibiotics, LPS exposure increased anxiety in males and repetitive-compulsive behaviour in females. These behavioural alterations are potentially mediated by the decreased expression of 5-HT1a receptors in the prefrontal cortex. This potentially disrupts the correct formation of the serotonergic system during a critical period in early life. This work provides further evidence of how the sensitive developing brain may respond to prenatal stress.

Chapter 4

Probiotic Exposure in the Prenatal Period has Differential Effects on Offspring Behaviour in a Healthy Pregnancy

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Abstract

Development of the fetal brain is a well-programmed process yet can be modulated by various factors throughout the course of gestation. The maternal microbiome is known to be a key regulator of neurodevelopment, and suboptimal maternal microbiome profiles have been linked to unfavourable outcomes in the offspring. Here, we aim to determine the potential effect of positive modulation of the maternal gut during pregnancy on neurobiological, behavioural, and microbial outcomes of the offspring. Pregnant Sprague Dawley rats had access to drinking water containing 1^9 CFU/mL *Lacticaseibacillus rhamnosus* from gestational day 11 until birth. Offspring underwent a battery of behavioural testing across the juvenile and periadolescent period. Postmortem immunohistochemical staining for 5HT1a receptors and Iba1 was performed, along with 16S sequencing of the offspring's microbiome. Exposure to the probiotic during this time significantly improved the emotional affect of the pups, increased sociability parameters, while also reducing repetitive behaviours. Interestingly, prenatal probiotic exposure was found to increase anxiety-like behaviours. This increase in anxiety was linked to an increase in the number of Iba1+ cells in the amygdala. This differential effect of *Lacticaseibacillus rhamnosus* on the offspring brain and behaviour may in part be related to an increased concentrations of inflammatory mediators including interleukin (IL)-1α and (C-X-C motif) ligand 1 (CXCL1) which were elevated in the dam's serum 21 days postpartum. Probiotic administration during pregnancy was found to be beneficial for the dam, increasing the number of short-chain fatty acid producing genera. However, analysis of the offspring's microbiome noted a reduction in butyrate producers. These novel findings reveal the nuanced effects of prenatal probiotic administration, highlighting the potential that *Lacticaseibacillus rhamnosus* has to affect developing brain regions in a differential manner. Future studies are essential to further understand these contrasting offspring effect and continue to work towards a microbiome-targeted anti-inflammatory supplement to improve neurodevelopmental outcomes.

1 Introduction

The maternal microbiome during pregnancy has recently become a focal point in the context of neurodevelopment. Both preclinical and clinical studies have emphasised the significance of an optimal, diverse gut microbiome during pregnancy (Koren et al., 2024; Pronovost et al., 2023; Sinha et al., 2023; Vuong et al., 2020). This has provided valuable insights into the connection between maternal gut health and long-term outcomes in the infant. Probiotics are defined as live microorganisms that, when taken in sufficient quantities can benefit the host and modify the host's microbiome (Cryan et al., 2019; Hill et al., 2014). Probiotics have been shown to work through several mechanisms such as modulating the immune system, producing short-chain fatty acids (SCFA) amongst other vitamins and metabolites, and strengthening the intestinal barrier (Cryan et al., 2019; Latif et al., 2023). In the context of pregnancy, probiotic use has been deemed safe, and it has been suggested that women of reproductive age are the group most likely to consume them (Ford et al., 2014; Sheyholislami and Connor, 2021).

The consumption of various probiotic species during pregnancy have proven to be beneficial in improving the health status of the vaginal microbiome, decreasing the risk of pre-term birth, reducing insulin resistance and the risk of gestational diabetes, all which can negatively influence neurodevelopment (Asemi et al., 2013; Luoto et al., 2010; Nordqvist et al., 2018; Seif El Dahan et al., 2022). Probiotic administration can modulate the developing brain and influence offspring neurodevelopmental outcomes. This concept has been examined through *in vivo* rodent models where probiotic administration direct to the offspring had reported beneficial effects. In a model of autism spectrum disorder (ASD) caused by maternal immune activation, postnatal administration of *Bacteroides fragilis* was shown to ameliorate unfavourable behaviours such as increased anxiety and altered communication, along with correcting impairments in gut permeability (Hsiao et al., 2013). Other studies include improved gut permeability and social behaviour following a prenatal lipopolysaccharide insult, and improvements in fear extinction and anxiety-like

behaviours in maternally separated offspring (Cowan et al., 2019; Peng et al., 2019; Wang et al., 2024). However, earlier administration of beneficial probiotics, during the prenatal period, may provide a more robust improvement in neurodevelopmental measures, particularly during periods of vulnerability in pregnancy. The prenatal brain is far more sensitive and remarkably more plastic than the postnatal brain, hence, earlier administration may potentially induce longer lasting modifications (Margolis and Gabard-Durnam, 2025). While postnatal administration can lead to positive neurodevelopmental changes, prenatal programming is already complete at this stage, hence the full potential of probiotics may be masked.

A probiotic intervention may prove to be useful during complications of pregnancy, especially those tied to high levels of inflammation. Probiotic use has reported beneficial effects in gestational diabetes and pre-term birth and may also reduce the risk of pre-eclampsia (Brantsæter et al., 2011). All the previously mentioned pregnancy complications are linked to deleterious neurodevelopmental outcomes in the offspring, including an increased risk of ASD, intellectual disabilities, and attention deficit hyperactivity disorder (Gumusoglu et al., 2020; Hee Chung et al., 2020; Ye et al., 2025). We hypothesise that a prenatal anti-inflammatory-based probiotic intervention may reduce the risk of unfavourable neurobiological and behavioural outcomes of the offspring born to a disease-model, but this must first be tested in a healthy pregnancy.

Numerous *Lactobacillus* species have demonstrated anti-inflammatory properties which have been shown in both *in vitro* and *in vivo* studies (Guo et al., 2023; Olimpio et al., 2022). *Lacticaseibacillus rhamnosus* has been shown to reduce pro-inflammatory cytokines such as interlekin-6 (IL-6), C-reactive protein, and tumour necrosis factor-α (TNFα), in addition to modulating immune cell phenotype (Chen et al., 2021; Y. Li et al., 2020). *L. rhamnosus* has shown great promise in improving inflammatory based conditions such as inflammatory bowel disease and post-injury inflammation (Guo et al., 2023; Olimpio et al., 2022). In the context of neurodevelopment however, *L. rhamnosus* treatment has been primarily used during the postnatal period, results showing improvements

in social and anxiety-like behaviours, improved gut health, and regulation of vasopressin genes in the hippocampus originally induced by a previous stressor (Kayyal et al., 2020; Wang et al., 2024). Yet there is limited research of their impact in the prenatal period, where the developing brain is more susceptible to different exposures.

In this study, we aimed to examine any potential effects that prenatal *L. rhamnosus* exposure may exert on neurodevelopmental-based behaviours in the early life period, in addition to examining effects on the offspring's brain and microbiome. Before using *L. rhamnosus* as an intervention, we initially wanted to examine maternal administration in a healthy rodent pregnancy to decipher any probiotic-induced changes on normal behaviours, neuroanatomy, and the microbiome prior to assessment in a disease-state model.

2 Materials and methods

2.1 Animals and Housing

Both male and female Sprague Dawley rats, aged approximately 7 weeks old, were purchased from Envigo, United Kingdom. The Sprague Dawley strain was selected due to their strong reproductive characteristics and neurodevelopmental phenotypes relevant to this study. The rats were homed and mated in the Biological Services Unit within Western Gateway Building, University College Cork. The offspring born to these rats were used in this study. Rats were allowed to habituate for 10 days before mating, housed in plastic cages (1575 cm^2) with sawdust bedding and cardboard tubes. For mating, rats were moved into a smaller cage (960 cm^2). Identification of a vaginal plug was denoted as embryonic day (E) 0. All cages were kept in a controlled environment. Rats had access to water and standard chow *ad libitum*. The room was both temperature (21°C ± 1°C) and humidity controlled, along with a strict 12-hour light cycle. All procedures performed within this study received approval from both the Animal Experimentation Ethics Committee at University College Cork and the Health Products Regulatory Authority, all in accordance with the recommendations of the European Directive 2010/63/EU.

2.2 Study design

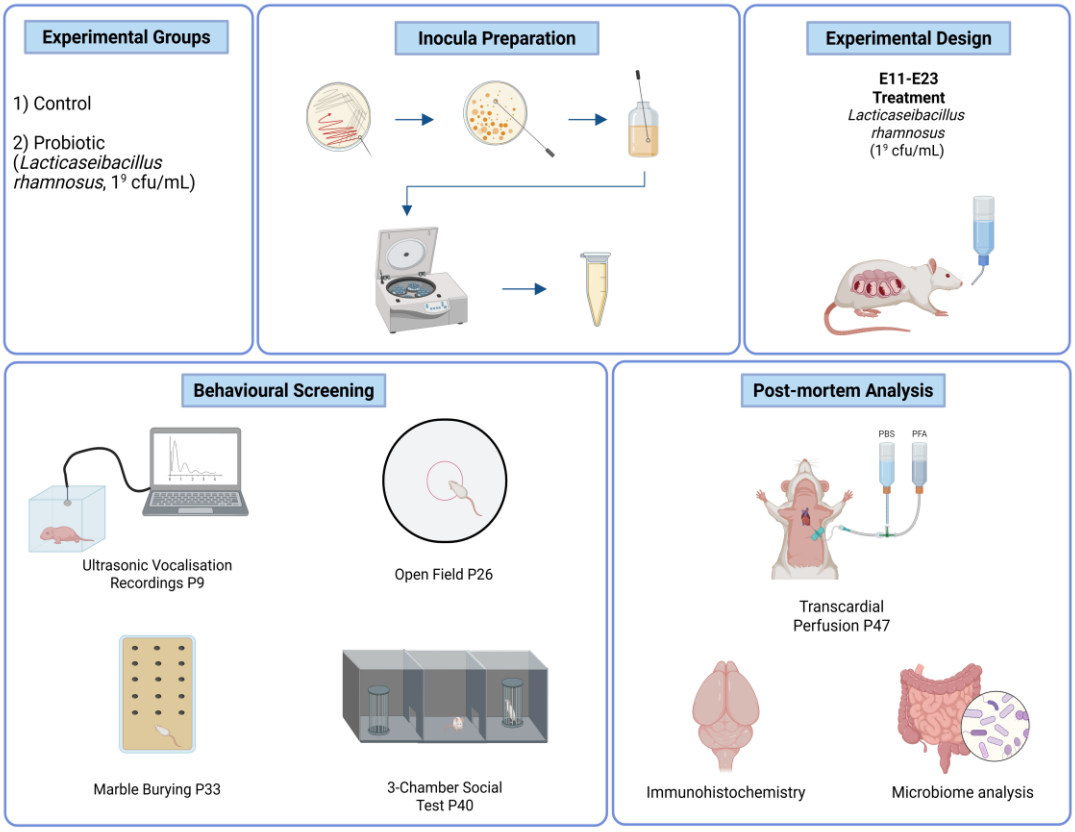


Figure 1: Study design detailing experimental groups, offspring behavioural tests, and fate of offspring. *Abbreviations: E: Embryonic day; P: Postnatal day.*

The pregnant dams were randomly assigned on E11 to one of two groups: probiotic or water control and were housed in groups of 2 or 3 with a total of 5 dams in each group. The probiotic-treated dams received *L. rhamnosus* in their drinking water from E11 until E23 or birth, whichever event occurred first. On E18, all the dams were housed individually in small cages (960 cm^2) where they remained for the duration of the pregnancy. The dams were provided with extra bedding at this time to aid in nest formation. All offspring remained with their mother until postnatal day (P) 21. At P21, the pups were weaned and housed in small cages (960 cm^2). 2 males and 2 females from each dam were used for all behavioural and postmortem examination (Fig. 1). The dams were culled on P21.

2.3 Preparation of *Lacticaseibacillus rhamnosus*

The *L. rhamnosus* inoculate was prepared using previously sequenced (Eurofins) glycerol stocks that were stored at -80˚C and cultured previously prepared agar plate (5 g agar, 0.25 g L-cysteine, and 27.5 g MRS powder) at 37˚C for 24 hours. One single colony was inoculated in 50 mL of liquid medium (27.5 g MRS powder and 0.25 g L-cysteine) incubated overnight in the anaerobic chamber. An OD600 reading was measured the following morning to confirm the bacteria were reaching the end of the exponential and beginning of the stationary phase which was confirmed using a growth curve (Fig. 2).

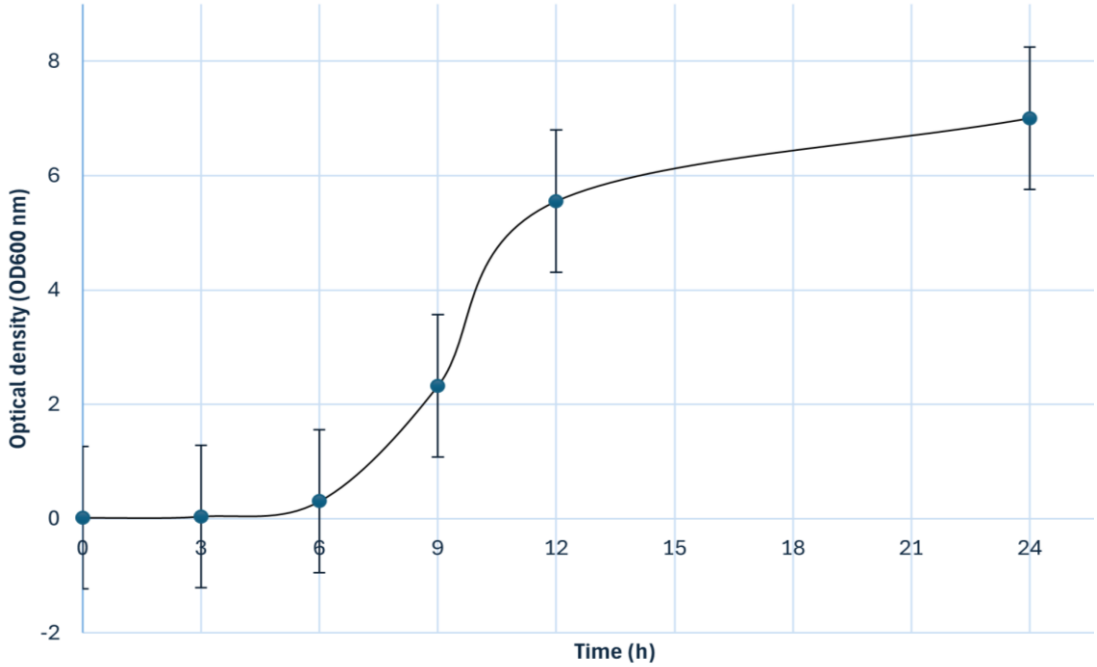


Figure 2: Growth curve of Lacticaseibacillus rhamnosus. *Bacteria grown in MRS broth at 37˚C for 24 hours, measured via optical density at 600 nm. Measurements taken at 3 hour intervals until hour 12, then again at hour 24 . Data presented as mean ± SEM. N=3 individual cultures.*

Following this, the liquid culture was spun at 4500 rpm for 10 minutes, the supernatant was removed and 20 mL of phosphate buffered saline (PBS) was added. The pellet was resuspended, and this process was repeated. 16 mL of PBS along with 4 mL of glycerol were then added to the falcon tube to resuspend the inoculate pellet prior to creating 1 mL stocks which were stored at -80˚C until further use. To determine the number of colony forming units (CFU), a serial

dilution was prepared using maximum recovery diluent by using 100 µL of the original stock. 100 µL of this was added to previously prepared agar plates using a L-shaped spreader and incubated for 48-hours before the number of colonies were counted in triplicate and the number of CFU/mL was deduced. Before administration to the pregnant dams, all batches of *L. rhamnosus* were sequenced (Eurofins) once again to confirm bacterial species.

2.4 Administration of probiotics

Each evening before the start of the dark phase (7pm), the pregnant dams received fresh water containing 1^9 CFU/mL of *Lacticaseibacillus rhamnosus* (probiotic). Each cage had access to two water bottles containing 75 mL each wrapped in tinfoil. The water levels were checked multiple times a day and replenished with water until the fresh probiotic was given each evening. Once the dams were separated into their own individual cages at E18, each dam had access to one water bottle containing 50 mL of water plus probiotic. Dams returned to water only on the day of birth or E23.

2.5 Ultrasonic vocalisation recordings

To measure the affective state of the exposed offspring, ultrasonic vocalisations emitted from the pups were recorded on P9. For this, 6 pups were randomly selected from each litter. Individually, each pup was placed into a small box and was covered with a larger styrofoam box to reduce as much external noise as possible. A microphone was then threaded through the box and was hung approximately 10 cm above the box containing the pup. Each pup was recorded for 3 minutes. Following the recording, the pup was returned to the home cage. UltraVox XT v3.2 was used to record vocalisations, and recordings were analysed using MATLAB R2024a.

2.6 Open field test

To measure anxiety-like behaviour, the open field test was performed on P26. Before the beginning of the test, the offspring were habituated in a dimly lit room for 45 minutes. The circular test arena measured 90 cm in diameter and was lit

using two lamps focused on the centre of the arena, measuring 1000 lux. The centred lights were used to create an anxiogenic area in the arena. Before each test subject, the whole arena was wiped down with 70% ethanol to limit the possibility of olfactory cues. The offspring were placed in the centre of the test arena and allowed to explore for 20 minutes before being returned to the home cage. The results of the open field test were analysed using ANY-Maze v7.16.

2.7 Marble burying

To measure stereotypical- and repetitive-like behaviours, marble burying was performed on P33. The pups were habituated to the test room for 30 minutes before the test commenced. 5cm of sawdust bedding was tightly packed into a small cage (960 cm^2) with black marbles (15.9 mm diameter) placed on top in a 4 x 5 arrangement. The offspring were introduced into the test arena and allowed to explore for 30 minutes. They were then carefully removed without disturbing the sawdust bedding and returned to their home cage. Buried marbles were defined as at least 2/3 marbles underneath the bedding.

2.8 3-Chamber social test

The final behavioural test was performed on P40, where the 3-chamber test was used to measure both sociability and social novelty. Offspring were allowed to habituate to the dimmed test room for 45 minutes. The rectangular test arena (57 cm x 99 cm) was divided into 3 chambers by two full-length walls which contained a small open passageway allowing the test subject to move freely between the 3 chambers. The two outer chambers, both had a small container to hold the non-test conspecifics. Before each test subject was placed into the arena, the whole apparatus was wiped down with 70% ethanol to minimise the possibility of any olfactory cues. The test is split into three 10-minute segments. The first segment was for habituation to the arena, where the test subject was allowed to explore the full arena. In the next segment, sociability, a novel, age-, and sex-matched conspecific was placed in one of the containers of the outer chamber, the opposing container remaining empty. The time that the test subject spent engaging with this conspecific was measured as a measure of sociability

readout. In the final segment, social novelty, another "new", age-, and sex-matched conspecific was introduced to the remaining container. The “old” conspecific is now deemed as ‘familiar’. The time that the test subject spent with both rats was measured and compared to assess social novelty. Following each test, the offspring and conspecifics were returned to their home cages. Both sociability and social novelty measurements were scored using BORIS v7.13.9.

2.9 Transcardial perfusions

The offspring were humanely culled on P47 via transcardial perfusion. The rats were first euthanised via intraperitoneal injection of 0.8 mL/kg of 200 mg sodium pentobarbital (Euthatol) with a 25G needle. Before commencement of the perfusion, the pedal, tail, and palpebral reflexes were examined. Following securement on the animal to the dissection board, the thoracic cavity was opened, and a 15G cannula was inserted into the left ventricle while the heart was still beating. Cold 10 mM phosphate buffer saline (PBS) was pumped through the cannula via a Watson Marlow 101 U/R peristaltic pump. An incision was made to the right ventricle, and PBS was allowed to flow until the perfusate ran clear and all blood was cleared from the body. 4% paraformaldehyde (PFA) was then pumped through the body to begin the fixation process. This was allowed to continue until the perfusion was complete, marked by fixation tremors, stiffness of the neck, along with blanching of both the liver and the feet. The head was swiftly decapitated and the whole brain was extracted for further fixation.

2.10 Tissue preparation

Post extraction, the whole brain was emersed in 4% PFA overnight and stored at 4˚C. After 24 hours, the brain was transferred to 30% sucrose and refrigerated until the brain sunk to the bottom of the tube. The brain tissue was snap-frozen using isopentane and liquid nitrogen and stored at -80˚C until sectioning. Sections were cut on a freezing stage cryostat (Leica CM1950) to a thickness of 30 µm. Sections were then kept in a cryoprotectant solution consisting of 10 mM PBS with 20% water, 25% glycerol, and 30% ethylene glycol. Required sections

were mounted onto Superfrost™ Plus Adhesion Microscope Slides and stored at -20˚C for further use.

2.11 5-HT1a receptor staining

Both the amygdala and the prefrontal cortex were stained for 5-hydroxytryptamine (5-HT)1a receptor expression. Sections were selected based on the Allen Institute Rat Brain Atlas. The mounted sections were removed from the freezer approximately 30 minutes prior to immunohistochemical staining. Sections were washed using triton-X tris-buffered saline (TXTBS) 3 times for 10 minutes to allow membrane permeabilisation and blocked for 1 hour at room temperature using a 10% goat serum solution in tris-buffered saline (TBS). After the blocking solution was removed, the primary antibody (rabbit anti-5-HT1a receptor, Merck, AB15350) (1:1000 dilution) was added to TBS along with 1% goat serum. 200 µL of primary antibody solution was added and incubated at 4˚C overnight. Following incubation sections were washed 3 times with TXTBS for 10 minutes each wash. The conjugated secondary antibody (Alexa Flour 488, Invitrogen) (1:500 dilution) and 1% goat serum was diluted in TBS. 200 µL of this solution was added to each slide and incubated for 2-hours at room temperature in the dark. Following this, the sections were washed a final 3 times with TXTBS for 10 minutes each. DAPI (4'-6-Diamidino-2-phenylindole, Sigma) (1:3000 dilution) nuclear stain was diluted in TBS and sections were incubated for 5 minutes in the dark. Sections were washed with TBS and left to dry in the dark to avoid any photobleaching before being cover-slipped with mounting media (DAKO Fluorescence Mounting Medium). Slides were stored at 4˚C in the dark until imaging on the Olympus BX43 Upright Microscope. A DAPI co-stain was used to identify nuclei for 5HT1aR staining intensity, however no additional co-stain was used to distinguish specific cell types. Intensity of the staining was measured in 20 cells across 4 images per brain region for each offspring using ImageJ software. Antibody was previously validated by the company and positive staining confirmed with a negative control.

2.12 Iba1 staining

To quantify the number of active microglia cells in the prefrontal cortex and the amygdala, the sections were stained for Iba1. Sections were selected based on the Allen Institute Rat Brain Atlas. Before commencing the immunohistochemical staining, mounted sections were allowed to come to room temperature, approximately for 30 minutes. Sections were washed 3 times, 10 minutes each wash with TXTBS to permeabilise the membrane. To minimise any non-specific binding, sections were blocked in 10% goat serum diluted in TBS. After 1 hour, the solution was removed and the primary antibody (rabbit anti-Iba1, FUJIFILM, Wako Pure Chemical Corporation 019-19741) (1:500) was diluted in TBS with 1% goat serum added. 200 µL of the primary antibody solution was applied to each slide and incubated overnight at 4°C. Following incubation, the sections were washed with TXTBS 3 times for 10 minutes. Subsequently, the conjugated secondary antibody (Alexa Fluor 594 conjugated secondary antibody, Invitrogen) (1:500) was diluted in TBS with 1% goat serum and 200 µL of the secondary antibody solution was added to each section and incubated at room temperature in the dark for 2 hours. Sections were then washed in TBS 3 times for 10 minutes each wash and allowed to dry in the dark before cover-slipping with mounting media (DAKO Fluorescence Mounting Medium). Sections were stored in the dark at 4°C until imaging using the Olympus BX43 Upright Microscope. The number of Iba1-positive cells were counted across 4 images per region for each offspring using ImageJ software. The morphology of the microglia was not taken into account in relation to overall cell count. Antibody was previously validated by the company and positive staining confirmed with a negative control.

2.13 Flow cytometry

Maternal serum was collected on P21 at time of cull and stored at -80˚C. The LEGENDplex™ Rat Inflammation Panel (13-plex) w/ VbP V02 (Biolegend, 741396) was used to measure inflammatory cytokines in the maternal serum as per the manufacturer's instructions. Samples were measured using flow cytometry (BD FACSCelesta) and cytokine levels quantified via the Biolegend software. For

analysis, any samples below the limit of detection, their value was halved and then used.

2.14 DNA extraction

Faecal pellets were collected from the offspring on P47 at time of cull. QIAamp Fast DNA Stool Micro Kit (Qiagen) was used to extract the DNA from the samples as per the manufacturer's instructions. Quantification and purity of DNA was measured using ND-1000 Spectrophotometer (NanoDrop).

2.15 DNA sequencing

Before commencing DNA sequencing, DNA concentration was standardised to 5 ng/µL. The required 16S library was then prepared using a five-fold miniaturised version of the Illumina 16S metagenomics library preparation protocol on the Echo 525 (Labcyte). The first polymerase chain reaction (PCR) included 0.5 µL DNA (at 5 ng/µL), 2.5 µL 2 x Kapa Hifi Hotstart Ready mix, and 1 µL of forward and reverse primers (Illumina 16S metagenomics library preparation protocol). Using the Echo 525, the DNA and all required reagents were introduced to a 96-well plate, spinning to mix. The PCR conditions followed the same Illumina 16S metagenomics protocol. 15 µL of molecular grade water was introduced into each well containing a sample. Following this, Ampure (Beckman Coulter) was added to all samples and purified on the Beckman i7 (Beckman Coulter). Samples were transferred to a fresh plate for the second PCR using the Echo 525 once again. The reactions were proportionally altered to have a final volume of 1 µL purified PCR product, 1 µL S5 index primer (Illumina), 1 µL N7 index primer (Illumina), 5 µL 2 x Kapa Hifi Hotstart Ready mix, and 2 µL molecular grade water. The plate was spun and PCR performed as per the Illumina 16S metagenomics protocol. In the initial PCRs, no template controls were included and carried through all steps of library preparation. Samples were then quantified using a Qubit High Sensitivity Assay and pooled equimolarly. To purify, an aliquot of the pooled samples underwent a 0.8 X ratio of Ampure (Beckman Coulter) beads, and the Qubit was used to measure the final pool. This was assessed on the Agilent high sensitivity chip (Agilent). Following this, the pool was diluted to a

concentration of 1 nM and mixed with 1 nM PhiX (Illumina) at 40% (v/v). Finally, the mixture was loaded onto a P1 600 cycle cartridge and sequenced on the NextSeq 2000 as per the Illumina guidelines.

2.16 Bioinformatics

Sequence data was obtained via basespace (Illumina, USA), with quality evaluated via *FastQC* (Babraham Bioinformatics) and *MultiQC* (Ewels et al., 2016), giving a median 338,376 (Q1: 245,703 Q3: 455,924) raw reads. Synthetic sequences were removed using *Trimmomatic* (Bolger et al., 2014; sliding filter window 6:15; minlength 299). The R library *DADA2* (Callahan et al., 2016) was used to preprocess reads (*filterAndTrim*: 3' trim: 279,219; 5' trim: 19,22; maxEE: 1,1) before identifying and quantifying unique amplicon sequence variants (ASV) across the study (*learnErrors*: randomise = TRUE, nbases = 1x10 10 ; *dada*: omega A & C: 1x10-80, self_consist=TRUE, pool=FALSE; *mergePairs*: minimum overlap of 30bp, maximum allowed mismatch of 0bp). Other parameters remained at default values. *Dada2::assignSpecies* and *DECIPHER::IdTaxa* (Wright, 2016) were used to generate taxonomic assignments from the *Silva* (Quast et al., 2013) database, release 138.2 (2024). The provenance of bimeras, ASVs of unknown phylum or domain, and ASVs outside of the expected length (397 – 428bp) was checked before removal. 16S rRNA phylogeny was estimated using *DECIPHER::TreeLine*. Final mean read count was a median 192,628 (Q1: 133,003 Q3: 244,185) per sample.

2.17 Statistics

For Figures 3-9, all statistical analysis was carried out using GraphPad Prism v8.3.0. Data analysed using unpaired student's t-test or 2-way ANOVA when appropriate, Fisher's LSD *post hoc* test used when required. In this study, all results were deemed significant when $p<0.05$. All data is presented as mean ± standard error of the mean (SEM).

For Figures 10-13, for visualisation purposes only, ASVs below 1.5% abundance and/or 6.5% prevalence were combined to "other". Remaining taxa were summed across different taxonomic levels (phylum, family, genus). Diversity

analyses (α, β) were carried out using *rtk* (Saary et al., 2017) to compute Shannon's H, Inverse Simpson's index, and Chao1 richness (*rtk*), abdiv (Bittinger, 2020) to compute Faith's phylogenetic distance (*faith_pd*), and vegan (*vegan*, 2012) to computer the Hellinger transformation of abundances for constrained correspondence analysis, significance of community structure (*betadisper, adonis2, cca*) and perform gradient analysis (*nmds, cca*). For differential analysis, ASV count data was transformed using count-zero multiplicative replacement of zeros (*zCompositions::cmultrepl*; Palarea-Albaladejo and Martín-Fernández, 2015) to allow a centre log-ratio (CLR) transform. Differences in α-diversity and ASV CLR values (n = 58) were incorporated into mixed-effect linear models (*lme4*; Bates et al., 2014) of experimental treatment and day with sex managed as a random effect (feature ~ treatment * day + (1|sex)). ASV differential abundance analysis used the Benjamini-Hockberg false-discovery rate (FDR) correction. Features differentially abundant across variables (*emmeans*, (Lenth et al., 2024) at an FDR < 0.05 were retained. Plots were assembled in R and RStudio, using the *vegan*, *ggplot2*, *patchwork*, and *vapoRwave* libraries (Oldach, 2024; "The Composer of Plots," n.d.; Wickham, 2009).

3 Results

3.1 *In utero Lacticaseibacillus rhamnosus* exposure improves male and female offspring affective state

For ultrasonic vocalisations assessment, 3 parameters were measured to give an insight into the offspring's affective state in early life; number of calls, duration spent calling, and average frequency of the calls. An unpaired student's t-test revealed a significant decrease in the number of calls from the probiotic group in comparison to the control group (P=0.0195) (Fig. 3A). A reduction was also seen in the total duration spent calling across the 3-minute test period in the probiotic group compared to the control group using an unpaired student's t-test (P=0.0106) (Fig. 3B). Both indicate that exposure to the probiotic during pregnancy positively influenced their affective state at this early stage of life. However, the average frequency of the offspring's calls did not differ between the two groups (Fig. 3C).

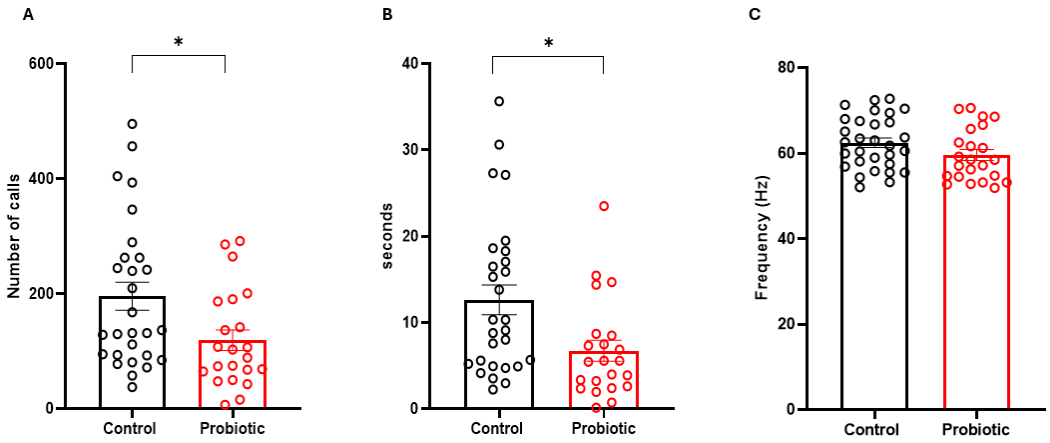


Figure 3: Probiotic exposure improves affective outcomes in males and females. *A) Number of USV calls, B) time in seconds spent calling, and C) the average frequency of the calls across the 3-minute test period. Tests were performed on postnatal day 9. 6 pups per litter, 4/5 litters per group. Data presented as mean ± SEM. *= P=<0.05.*

3.2 *In utero Lacticaseibacillus rhamnosus* exposure increases anxiety-like behaviour in female offspring

Time spent in the centre of the arena - both sexes compared: A two-way ANOVA revealed a significant difference in the time spent in the centre of the test arena, which can

be indicative of anxiety-like behaviours (Interaction Effect: P=0.0999, F(1, 34)=2.861; Probiotic Effect: P=0.0993, F(1, 34)=2.871; Sex Effect: P=0.0405, F(1, 34)= 4.536) (Fig. 4A). Further analysis with Fisher's LSD *post hoc* test revealed statistical differences between the control male group and the control female group, where males spent significantly less time in the centre in comparison to females (P=0.0089). The probiotic female group spent less time in the centre of the arena in comparison to control female group (P=0.0223).

Males alone: No significant difference was seen in the males only using an unpaired student's t-test (0.9980) (Fig. 4B).

Females alone: Unpaired student's t-test found significant differences between the time spent in the centre in control versus probiotic females (P=0.0499) (Fig. 4C).

Number of immobile episodes - both sexes compared: Examining the number of immobile episodes, which can be used as a measure of anxiety through freezing or startled-like behaviours, was significant following a two-way ANOVA (Interaction Effect: P=0.2510, F(1, 34)=1.364; Probiotic Effect: P=0.5667, F(1, 34)=0.3347; Sex Effect: P=0.0165, F(1, 34)= 6.357) (Fig. 4D). Using Fisher's LSD *post hoc* test, differences were found between the probiotic male group versus the probiotic female group (P=0.0134).

Males alone: Examining sexes individually through an unpaired student's t-test revealed no significant difference between control and probiotic males (P=0.0631) (Fig. 4E)

Females alone: No significant difference between control or probiotic treated females number for the immobile episodes using an unpaired student's t-test (P=0.6861) (Fig. 4F).

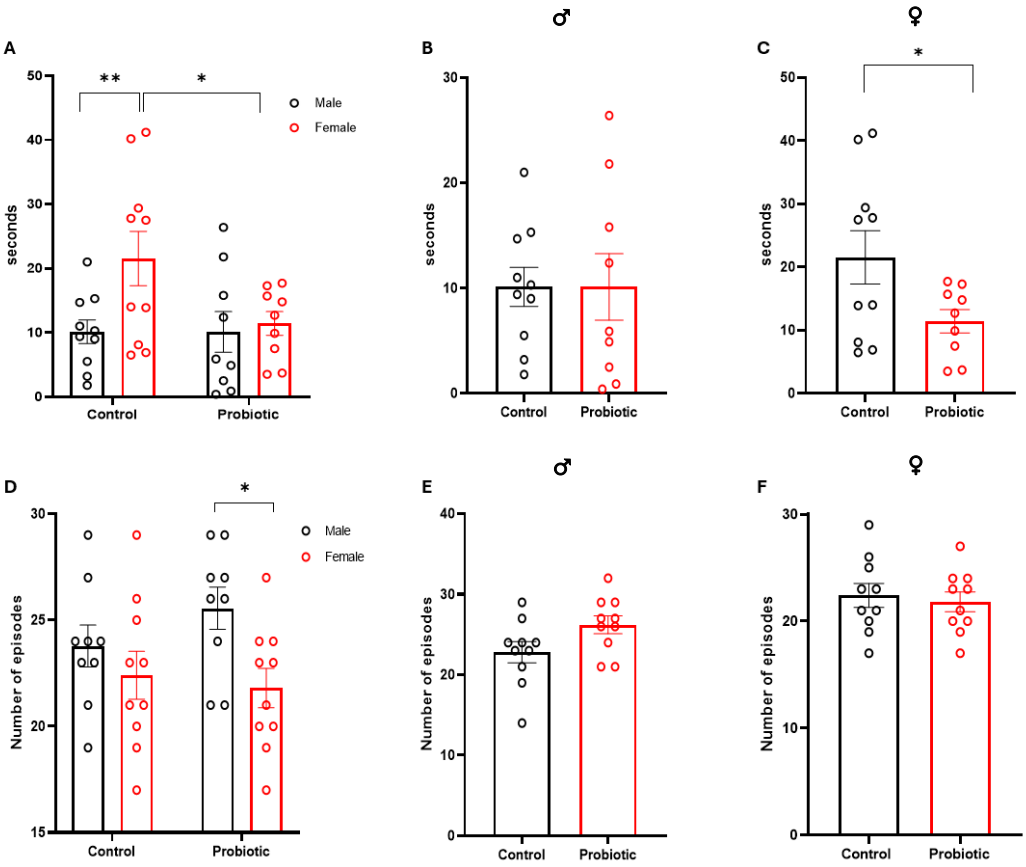


Figure 4: Probiotic exposure leads to increased anxiety levels in female offspring only. *A) time spent in the centre of the arena with both sexes compared, B) males alone, and C) females alone, D) number of immobile episodes with both sexes compared, E) males alone, and F) females alone across the 20-minute test period. Test were performed on postnatal day 25. 20 pups per group (10 males, 10 females) from 4/5 litters. Data presented as mean ± SEM. *= P=<0.05, **= P=<0.01.*

3.3 Female offspring show a reduction in repetitive-like behaviours after *in utero* exposure to *Lacticaseibacillus rhamnosus*

The marble burying test was used to examine potential repetitive- and stereotypical-like behaviours which may be associated with ASD or anxiety disorders such as obsessive-compulsive disorder. Using a two-way ANOVA, no significance was seen when comparing both sexes (Interaction Effect: P=0.1125, F(1, 30)=2.673; Probiotic Effect: P=0.0667, F(1, 30)=3.622; Sex Effect: P=0.4920, F(1, 30)= 0.4839) (Fig. 5A). Examining males only with an unpaired student's t-test, no significance was evident between the two groups (P=0.8674) (Fig. 5B). Yet, there was a significant difference in females alone using an unpaired student's t-test (P=0.0114) (Fig. 5C).

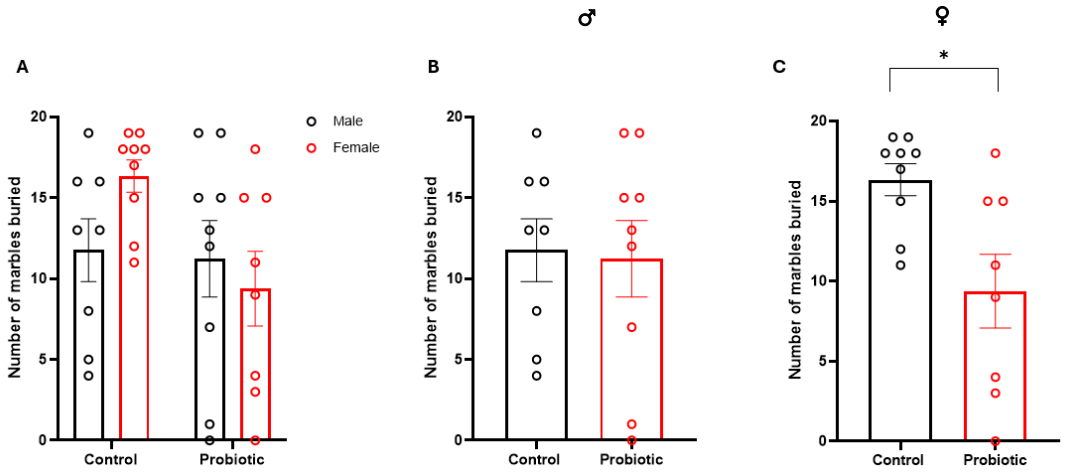


Figure 5: Reduction in repetitive-like behaviours in female offspring following probiotic exposure. *A) The number of marbles buried with both sexes compared, B) males alone, and C) females alone across the 30-minute test period. Tests were performed on postnatal day 33. 20 pups per group (10 males, 10 females) from 4/5 litters. Data presented as mean ± SEM. *= P=<0.05.*

3.4 *Lacticaseibacillus rhamnosus* exerts sex-specific improvements in social behaviours following *in utero* exposure

Time spent engaging with a novel conspecific - both sexes compared: To further explore other impacts that the probiotic may have on autism-like behaviours, the 3-chamber test was performed to examine social behaviour, through both sociability and social novelty. Examining sociability and comparing the two sexes, a two-way ANOVA revealed no significant differences (Interaction Effect: P=0.1760, F(1, 36)=1.905; Probiotic Effect: P=0.1346, F(1, 36)=2.343; Sex Effect: P=0.1833, F(1, 36)= 0.1833) (Fig. 6A).

Males alone: When examining the effect of males only, no significant changes were seen with an unpaired student's t-test (P=0.9276) (Fig. 6B).

Females alone: However, females exposed to the probiotic *in utero* spent significantly more time engaging with the novel conspecific in comparison to the control group, as seen with an unpaired student's t-test (P=0.0217) (Fig. 6C). This indicates that the probiotic increases sociability in the female offspring only.

Time spent engaging with a novel conspecific in comparison to a familiar conspecific - both sexes compared: The second parameter of the behavioural test, social novelty, was not found to be altered using a two-way ANOVA (Interaction Effect: P=0.3411, F(1, 30)=0.9358; Probiotic Effect: P=0.1084, F(1, 30)=2.738; Sex Effect: P=0.7366, F(1, 30)= 0.1152) (Fig. 6D).

Males alone: Conversely, when examining the individual sex effects of social novelty, it was the probiotic male group that engaged in a higher percentage of social novelty in comparison to the control group with an unpaired student's t-test (P=0.0386) (Fig. 6E).

Females alone: No changes were seen between control and probiotic females seen with an unpaired student's t-test (P=0.6557) (Fig. 6F). Unlike the other behavioural tests, the 3-chamber test reveals opposing sex effects in both parameters, indicating that the probiotic may have beneficial but differential effects in reducing an autism-like phenotype.

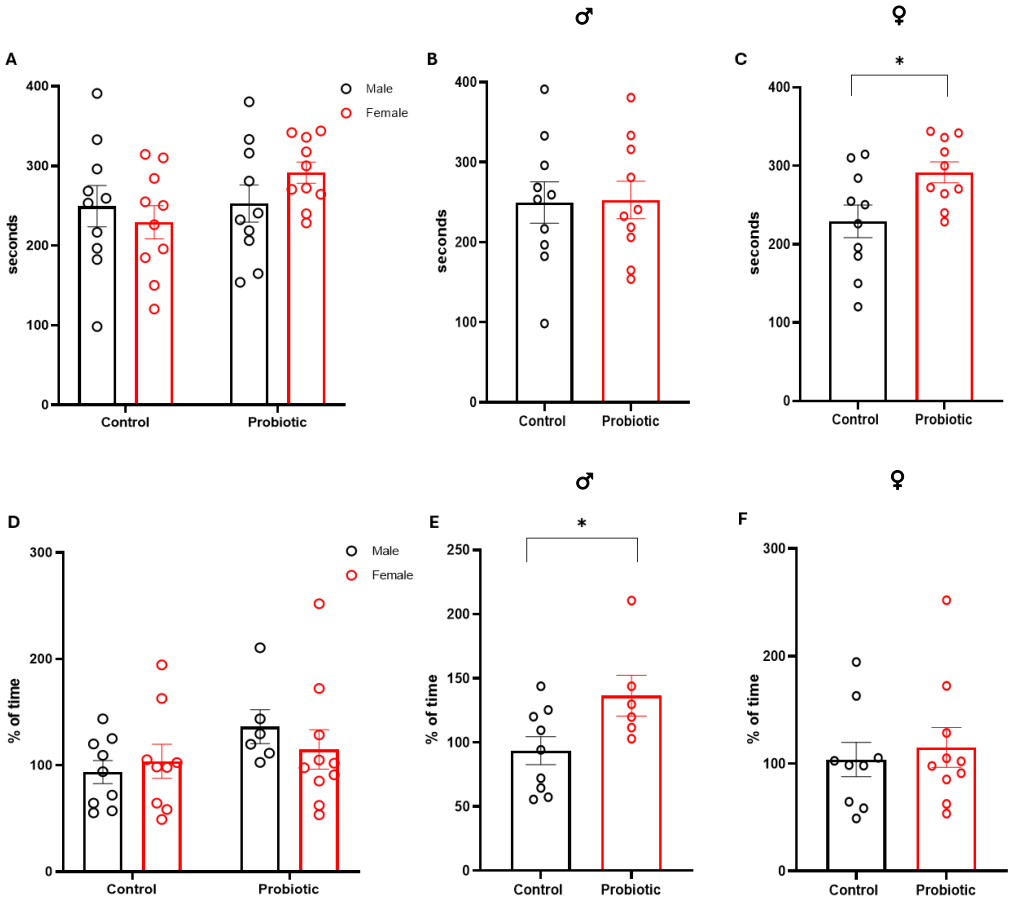


Figure 6: Improvements in different social behaviour parameters following probiotic exposure is sex dependent. *A) Time spent engaging with a novel conspecific with both sexes compared, B) males alone, and C) females alone across the 10-minute test period, along with D) time spent engaging with another novel conspecific expressed as a percentage of time spent with the familiar conspecific with both sexes compared, E) males alone, and F) females alone across the 10-minute test period. Tests were performed on postnatal day 40. 20 pups per group (10 males, 10 females) from 4/5 litters. Data presented as mean ± SEM. *= P=<0.05.*

3.5 5-HT1a receptor expression is not altered by *Lacticaseibacillus rhamnosus* exposure *in utero*

5-HT1a receptor expression in the prefrontal cortex - both sexes compared: To investigate a potential mechanism behind the behavioural changes evident, we examined the expression of 5-HT1a receptor in both the prefrontal cortex and the amygdala. A two-way ANOVA revealed no significant difference in receptor expression in the prefrontal cortex (Interaction Effect: P=0.6233, F(1, 28)=0.2467; Probiotic Effect: P=0.3129, F(1, 28)=1.056; Sex Effect: P=0.7523, F(1, 28)=0.1016) (Fig. 7A).

Males alone: Using an unpaired student's t-test, no significant changes were found in control versus probiotic male (P=0.2593) (Fig. 7C).

Females alone: No changes were found in control versus probiotic females using an unpaired student's t-test (P=0.7325) (Fig. 7D).

5-HT1a receptor expression in the amygdala - both sexes compared: There was also no significant difference in 5-HT1a expression in the amygdala. as noted with a two-way ANOVA (Interaction Effect: P=0.8487, F(1, 28)=0.03709; Probiotic Effect: P=0.6266, F(1, 28)=0.2420; Sex Effect: P=0.1434, F(1, 28)= 2.266) (Fig. 7E).

Males alone: No significant differences in 5-HT1a expression in control versus probiotic males (P=0.5691) (Fig. 7G).

Females alone: No changes seen in control versus probiotic females following an unpaired student's t-test(P=0.8560) (Fig. 7H).

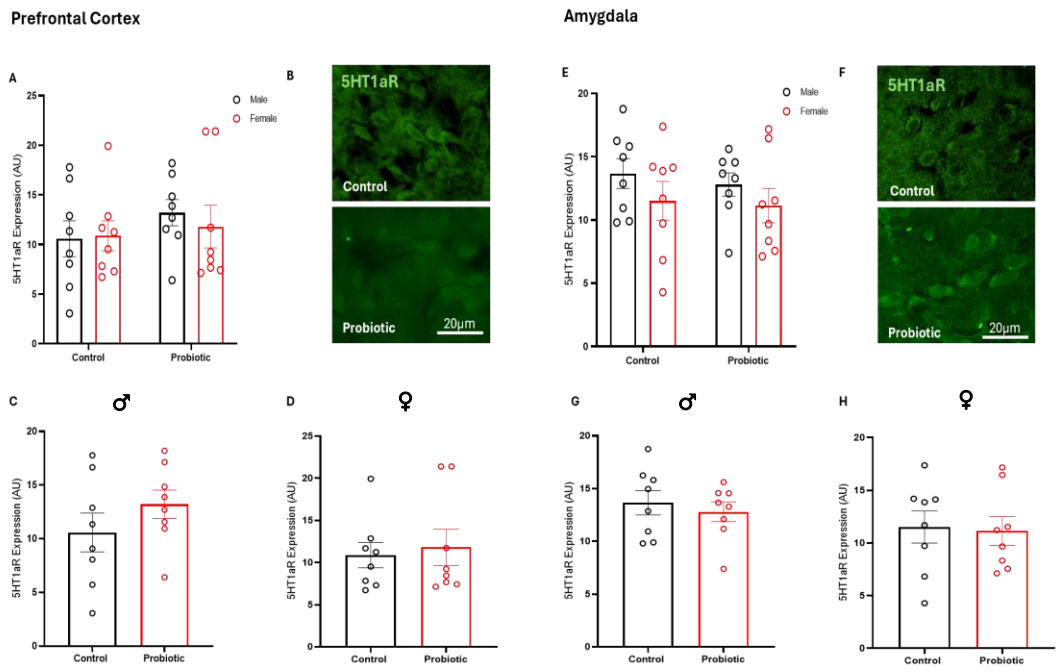


Figure 7: 5-HT1a receptor expression is not altered in the prefrontal cortex or the amygdala following probiotic exposure in utero. *A) 5-HT1aR expression in the prefrontal cortex for the sexes compared, B) representative photomicrographs, C) 5-HT1aR expression for males alone, and D) females alone. E) 5-HT1aR expression in the amygdala for the sexes compared, F) representative photomicrographs, G) 5-HT1aR expression for males alone, and H) females alone. Analysis performed on postnatal day 47. 16 pups per group (8 males, 8 females) from 4/5 litters. Data presented as mean ± SEM.*

3.6 *Lacticaseibacillus rhamnosus* exposure *in utero* increases the number of Iba1 positive cells in the female amygdala

Iba1+ cell count in the prefrontal cortex - both sexes compared: To examine if the *L. rhamnosus* was eliciting behavioural alterations through immune signalling in the brain, the number of Iba1 positive cells were counted as an indicator of activated microglia. Examining the number of cells of the prefrontal cortex, no significant differences were found between the groups (Interaction Effect: P=0.0551, F(1, 28)=4.005; Probiotic Effect: P=0.4657, F(1, 28)=0.5470; Sex Effect: P=0.0682, F(1, 28)=3.598) (Fig. 8A).

Males alone: When examining potential changes in males, no significant difference was found between control or probiotic using an unpaired student's t-test (P=0.3342) (Fig. 8C).

Females alone: No significant changes were seen with control versus probiotic females with an unpaired student's t-test (P=0.0992) (Fig. 8D).

Iba1+ cell count in the amygdala - both sexes compared: However, when using a two-way ANOVA to examine Iba1 positive cells in the amygdala, significant differences were evident following prenatal probiotic exposure (Interaction Effect: P=0.1018, F(1, 28)=2.861; Probiotic Effect: P=0.0244, F(1, 28)=5.665; Sex Effect: P=0.1311, F(1, 28)=2.419) (Fig. 8E). Using Fisher's LSD *post hoc* test, a significant increase in the number of Iba1 positive cells was seen in the probiotic female group when compared to the control female group (P=0.0076), and also the probiotic male group (P=0.0294).

Males alone: When examining males alone, no significance was found between the two groups (P=0.6101) (Fig. 8G) using an unpaired student's t-test.

Females alone: Finally, there was a significant increase in the number of Iba1 cells seen in the female probiotic group using an unpaired student's t-test in comparison to the female control group (P=0.0169) (Fig. 8H).

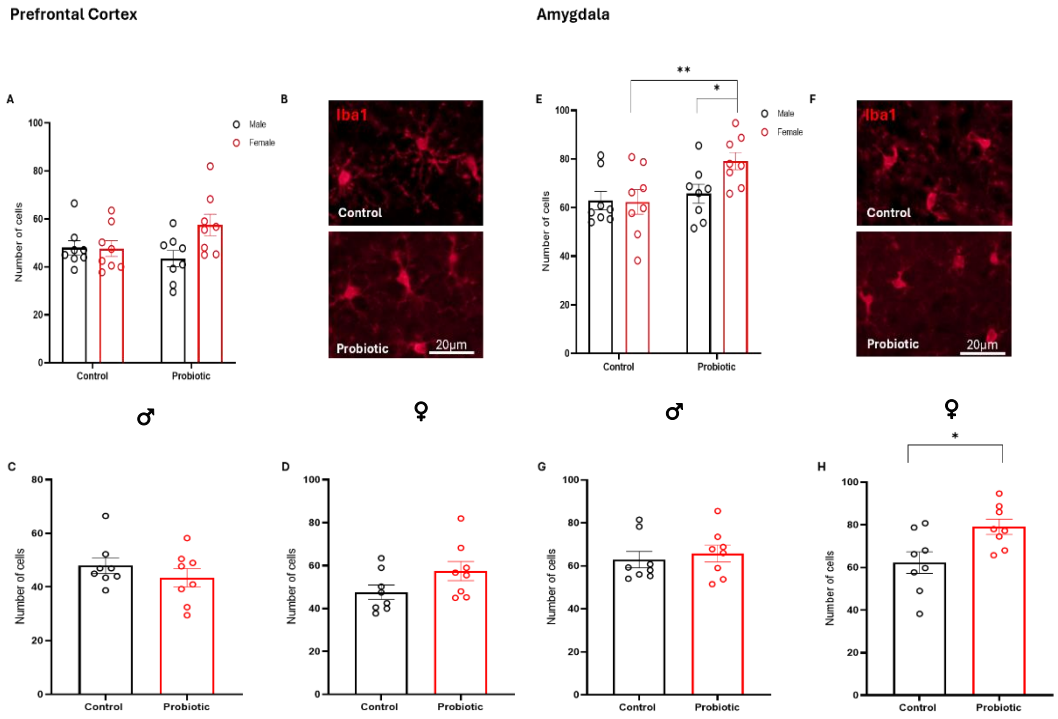


Figure 8: Number of Iba1 positive cells are increased in the amygdala in female offspring following probiotic exposure in utero. *A) Number of Iba1 positive cells in the prefrontal cortex for the sexes compared, B) representative photomicrographs, C) Number of Iba1 positive cells for males alone, and D) females alone. E) Number of Iba1 positive cells in the amygdala for the sexes compared, F) representative photomicrographs, G) Number of Iba1 positive cells for males alone, and H) females alone. Analysis performed on postnatal day 47. 16 pups per group (8 males, 8 females) from 4/5 litters. Data presented as mean ± SEM. *= P=<0.05, **= P=<0.01.*

3.7 Increase in circulating pro-inflammatory cytokines in postpartum dams following *Lacticaseibacillus rhamnosus* exposure during pregnancy

To examine if some of the changes seen in the offspring could be linked to increased levels of inflammation in the dams, 13 circulating cytokines were examined in the serum taken at P21 when the dams were euthanised. An unpaired student's t-test was used for cytokine analysis. For tumour necrosis factor-α (TNFα) (Fig. 9A) and IL-10 (Fig. 9B), as all dams were below the detection limit, it was not possible to compute a t-test and produce a p-value to perform statistical analysis. No significant changes were evident between the control and probiotic groups in circulating levels of interferon-γ (IFNγ) (P=0.8306) (Fig. 9C), monocyte chemoattractant protein-1 (MCP-1) (P=0.1027) (Fig. 9E), granulocyte-macrophage colony-stimulating factor (GM-CSF) (P=0.2924) (Fig. 9F), IL-18 (P=0.0797) (Fig. 9G), IL-12p70 (P=0.1836) (Fig. 9H), IL-1β (P=0.0586) (Fig. 9I), IL-17A (P=0.1752) (Fig. 9J), IL-33 (P=0.2437) (Fig. 9K), and IL-6 (P=0.2924) (Fig. 9M). A significant increase evident in circulating CXCL1 (P=0.0231) (Fig. 9D) and IL-1α (P=0.0149) (Fig. 9L) in the probiotic exposed dams compared to control dams.

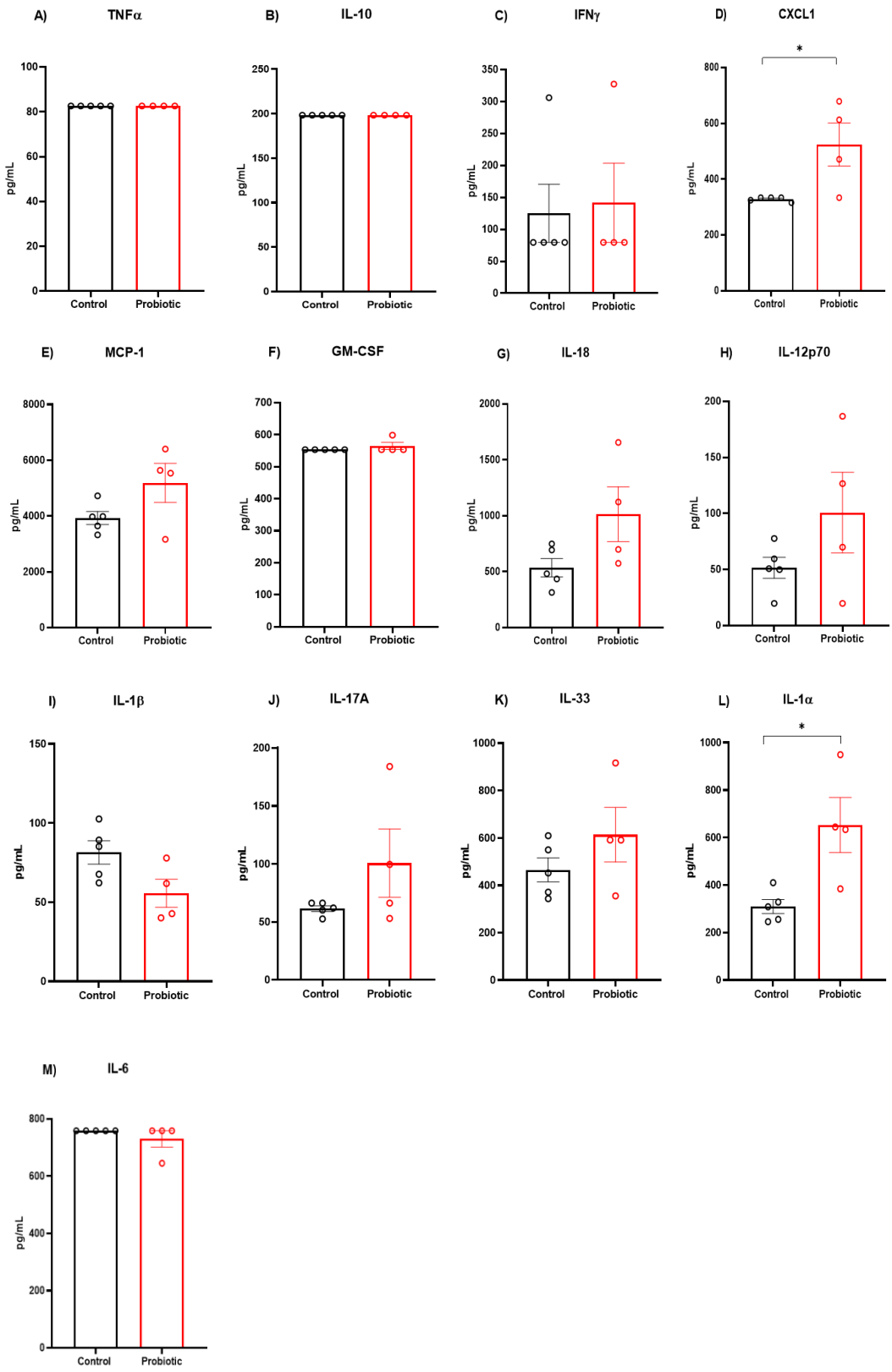


Figure 9*: *Increased circulating CXCL1 and IL-1α in postnatal dams after L. rhamnosus administration. *Concentration of A) TNFα, B) IL-10, C) IFNγ, D) CXCL1, E) MCP-1, F) GM-CSF, G) IL-18, H) IL-12p70, I) IL-1β, J) IL-17A, K) IL-33, L) IL-1α, and M) IL-6. Serum taken on postnatal day 21. 4/5 dams per group. Data presented as mean ± SEM. *= P=<0.05.*

3.8 *Lacticaseibacillus rhamnosus* exposure *in utero* alters is associated with alterations in the gut microbiome of the offspring

The gut microbial community of the pups was evaluated via 16S rRNA amplicon sequencing with reference to the control or probiotic treatment received by the dams (N = 20 offspring per group). Community composition was relatively similar in the offspring to that seen in the dams (Fig. 10A-C), (Fig. 12A-C). While no significant difference was observed in the α-diversity levels between the offspring from the control and probiotic group (Fig. 10D-F), there was a significant differentiation by treatment group in total community structure (β-diversity) between the two groups (constrained correspondence analysis; P=0.001), (Fig. 10G), indicating that some effect of the *L. rhamnosus* administration to the dams was transmitted to the pups, leading to a difference in the gut microbiome of the offspring.

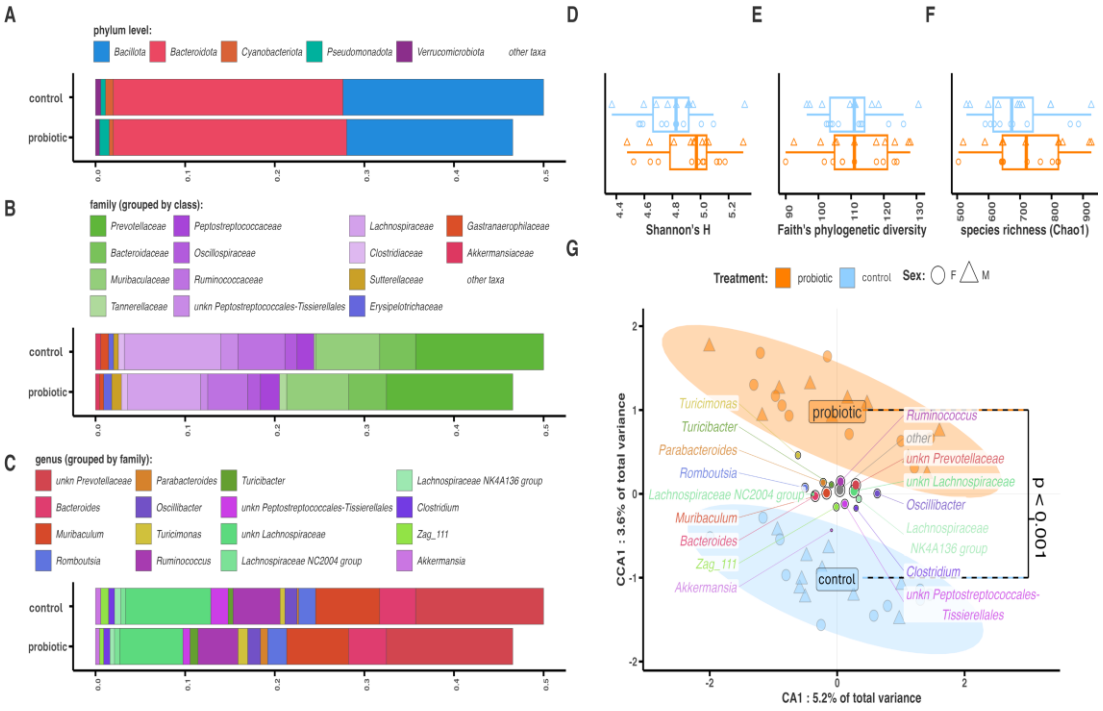


***Figure 10*: Prenatal probiotic exposure leads to enduring changes in the microbiome of offspring.** *A-C) Mean relative abundances of the pup microbiome at P47 at phylum, family, and genus level. Taxa at family level share a colour if within the same class, while taxa at genus level share a colour if within the same family. D-F) Probiotic exposure did not alter α-diversity as seen with Shannon's H, Faith's phylogenetic diversity, or Chao1 index. G) Offspring exposed to probiotic during gestation display significantly different microbiome composition at P47, as shown by the strong separation of samples and lack of overlap between different groups (p < 0.001). Group labels show the "average" position of each group (centroid), while coloured points show genera*

*closest to the samples and groups where they were most abundant, with size of point indicating relative abundance. CCA: constrained correspondence analysis. N = 40 for all comparisons. **=P=<0.01.*

Differential abundance testing was applied to determine what taxa were most strongly affected by prenatal *L. rhamnosus* exposure (Fig. 11). Although small increases were seen in uncharacterised Bacillota, pups in the probiotic group were more significantly associated (FDR < 0.05 – 0.001) with increased abundances of Bacteroidota, with large increases in ASVs assigned to *Bacteroides*, *Parabacteroides*, and *Muribaculum*. In addition to the enrichment of the Bacteroidota taxa, pups in the probiotic group were also associated with slightly lower abundances of butyrate producing ASVs from both Bacillota and Bacteroidota: *Butyribacter* and *Butyricimonas* respectively. No obvious changes were seen in *Lacticaseibacillus*.

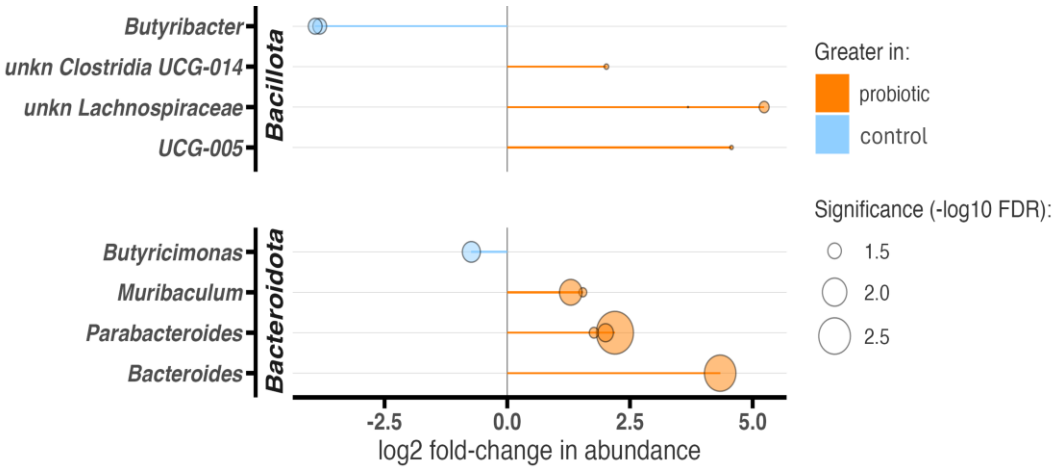


***Figure 11*: Many different populations of bacteria ASV are significantly affected by prenatal probiotic exposure.** *Taxa which are significantly different between the two groups P47. All features are significantly different below a false discovery rate (FDR) correction of 0.05, with size of point showing the FDR value on a -log_{10} scale (larger points are more significantly different). X axis shows the log_2-fold change, i.e. how many doublings in abundance two groups differ by (L2FC 1 = $log_2(1)$ = 2 times more abundant, L2FC 3 = $log_2(3)$ = 8 times more abundant, etc.). In all cases, negative L2FC values indicate greater abundance in the left-hand side label.*

3.9 Gestational *Lacticaseibacillus rhamnosus* administration shapes the postnatal maternal microbiome

16S rRNA amplicon sequencing of the dams at E18 (n=9) and P21 (n=9) demonstrated a strong change in gut microbiome composition between the two timepoints (Fig. 12A-C). Here, α-diversity measures such as number of species

(Chao1 index) and phylogenetic diversity (Faith's index) were both significantly increased at samples from the later timepoint (P21; P=<0.005, Fig. 12D-E), while comparisons of total community structure (β-diversity: Bray-Curtis & UNIFRAC) showed timepoint to have a strong and significant effect on composition (F=7.4, P=<0.001; Fig 12F). Notably, treatment of dams with probiotic did not show any significant difference in species richness or evenness (α-diversity), nor in community structure (β-diversity: Bray-Curtis & UNIFRAC), possibly due to a lack of effect at the community level, or limited sensitivity due to relatively small groups (4 probiotic dams, 5 control dams).

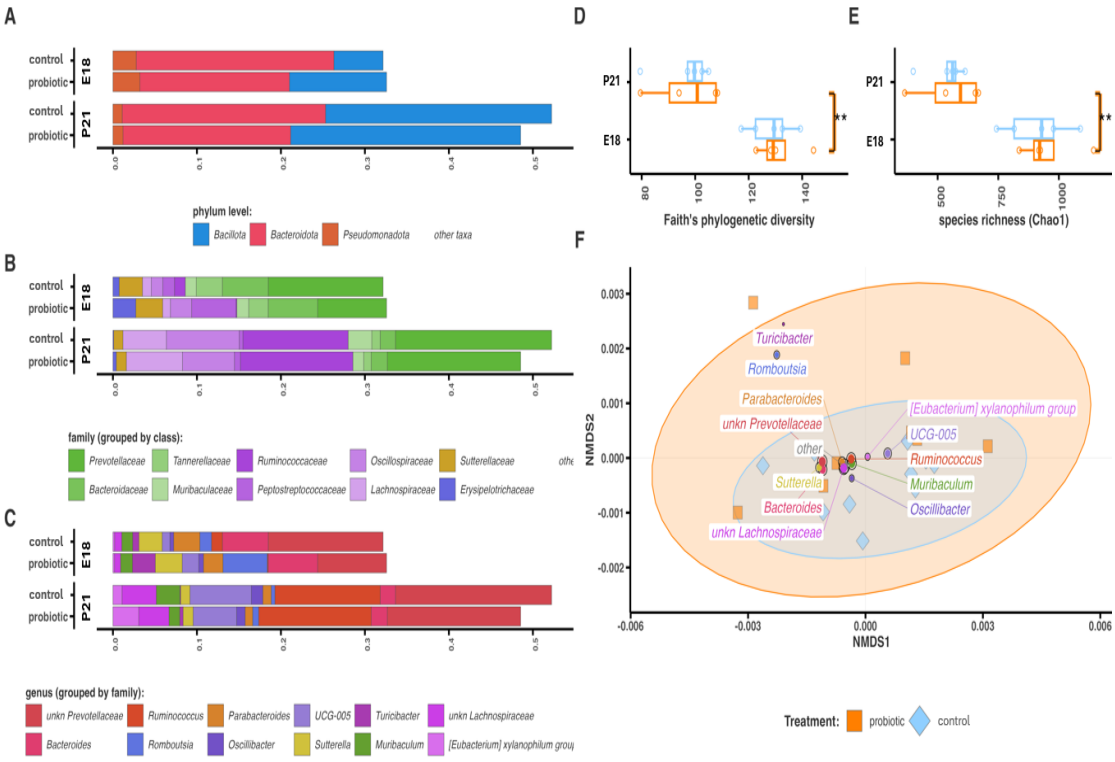


***Figure 12*: Probiotic administration during pregnancy leads to enduring changes in the microbiome of the dams.** *A-C) Mean relative abundances of the dam's microbiome at E18 and P21 at phylum, family, and genus level. Taxa at family level share a colour if within the same class, while taxa at genus level share a colour if within the same family. D) Probiotic significantly increases diversity of maternal microbiome at E18 in comparison to P21 (probiotic group only) as seen with Faith's phylogenetic diversity. E) Probiotic administration also increased species richness of maternal microbiome at E18 in comparison to P21 as seen with Chao1. F) No clear separation of maternal microbiome following probiotic administration. Group labels show the "average" position of each group (centroid), while coloured points show genera closest to the samples and groups where they were most abundant, with size of point indicating relative abundance. NMDS: non-metric multidimensional scaling. N = 9 for all comparisons at each timepoint. **=P=<0.01. E18 = embryonic day 18, P21 = postnatal day 21.*

However, changes associated with probiotic consumption were clearly evident in differential abundance testing (Fig. 13). At E18, probiotic consumption was linked to an increased relative abundance of Bacillota taxa, including clusters of uncharacterised Lachnospiraceae, Oscillospiraceae, and UCG-014 groups, as well as the genera *Colidextribacter*, *Actualibacter*, *Holdemania*, *Ruminococcus*, *Tyzzerella*, and *Lactocaseibacillus*. Notably, enrichment of Lachnospiraceae ASVs from the probiotic appeared to displace a number of alternate Lachnospiraceae taxa associated with controls. While the probiotic itself was not detected at P21, its effects persisted beyond treatment cessation. This was seen through the significant increase still evident in the same uncharacterised Lachnospiraceae, Oscillospiraceae, and UCG-014 ASVs. Additionally, significant increases are seen in *Blautia* and *Marvinbryantia* only in the probiotic group at P21. Finally, to explore whether microbiome development between E18 and P21 differed between the two groups, we assessed changes excluding those common to both treatments. While no unique changes were found in the control group, several additional Bacillota ASVs were uniquely associated with the probiotic group at P21 only.

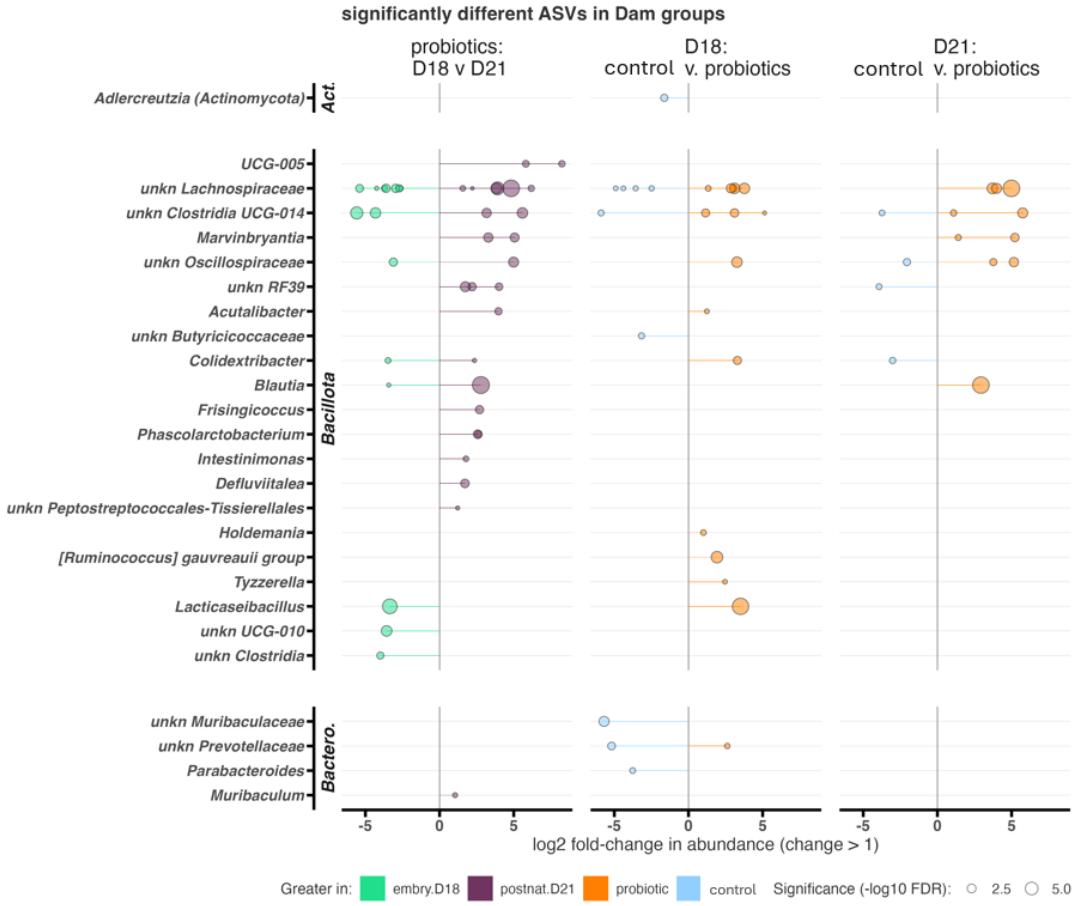


***Figure 13*: Many different populations of bacteria are significantly affected by maternal probiotic administration during pregnancy.** *Taxa which are significantly different between the control and probiotic-treated dams at E18 and P21. All features are significantly different below a false discovery rate (FDR) correction of 0.05, with size of point showing the FDR value on a -log_{10} scale (larger points are more significantly different). X axis shows the log_2-fold change, i.e. how many doublings in abundance two groups differ by (L2FC 1 = $log_2(1)$ = 2 times more abundant, L2FC 3 = $log_2(3)$ = 8 times more abundant, etc.). In all cases, negative L2FC values indicate greater abundance in the left-hand side label. E18 = embryonic day 18, P21 = postnatal day 21.*

4 Discussion

L. rhamnosus exposure *in utero* was found to have a beneficial impact on some of the key neurodevelopmental-based behavioural tests examined in this study. Improvements were seen in the pup's affective state through ultrasonic vocalisation testing, a reduction in repetitive-, stereotypical-behaviours as seen with the marble burying, and increased levels of sociability and social novelty in the 3-chamber test. These findings reveal that *L. rhamnosus* administration during pregnancy may promote a healthy environment for the fetus to develop in. Interestingly, despite *L. rhamnosus* initially being selected due to its anti-inflammatory properties, some paradoxical findings were found in the context of pregnancy (Chen et al., 2021; Li et al., 2020). An increase in anxiety-like behaviour was noted in the probiotic-exposed offspring, and this may be associated with the increase in the number of Iba1+ cells in the amygdala, an area that processes threat and fear-related stimuli. These activated microglia may be linked to the elevated circulating levels of inflammatory markers, IL-1α and CXCL1 in the maternal serum. Together the data suggests that *L. rhamnosus* may modulate fetal neurodevelopment in a differential manner, positively influencing various parameters such as a reduction in autism-like behaviours, while also inducing high levels of inflammation that may result in alternate behavioural consequences.

Ultrasonic vocalisation analysis has been extensively utilised to gain insights into the pup's affective state (Burgdorf et al., 2020). This sensory process is closely related to emotional processing and is important in early development (Burgdorf et al., 2020). In our study, the decrease in both the number of ultrasonic calls and the duration spent calling suggests a more regulated emotional circuitry. This may suggest that the pups are more resilient to stress, however, examination of the types of calls would be required to confirm this. The role of the microbiome in ultrasonic vocalisation production and modulation is not well understood; hence, it is difficult to underpin the mechanism behind these changes. Most microbiome-related ultrasonic vocalisations studies involve manipulation in the postnatal period and often do not examine behavioural changes in early life (Salia

et al., 2025; Septyaningtrias et al., 2020). Direct neomycin treatment of juvenile mice was found to increase the number of ultrasonic calls (Septyaningtrias et al., 2020). Despite this, there is very limited research examining ultrasonic vocalisations following prenatal maternal microbiome modulations. The results of our study, which showed that *L. rhamnosus* exposure reduced the pups' affective state, compliment the above-mentioned study where microbial disruption through antibiotic administration led to a more stressed, emotionally immature finding (Septyaningtrias et al., 2020). However, a previous study found an increase in the number of ultrasonic vocalisations following synbiotic administration directly to young juvenile mice (Nettleton et al., 2021). The increase seen was referred to as 'improved communication', yet as this is also seen with antibiotics directly, we propose that a reduction is a favourable outcome, perhaps a more refined and mature emotional processing, especially when coupled with some of the other positive behavioural alterations seen in this study.

In the marble burying test, female pups exposed to *L. rhamnosus* displayed a reduction in the number of marbles buried across the test-period. The test is used to infer the presence of autism-like behaviours, such as stereotypical and repetitive behaviours which are often seen with the developmental condition. Numerous studies have shown administration of various *Lactobacillus* and *Bifidobacterium* strains in adulthood for a prolonged period reduce these behaviours, even in disease-models (Engevik et al., 2020; Ghuge et al., 2023; Kantak et al., 2014). This also includes direct *L. rhamnosus* administration itself which was given in an obsessive-compulsive disorder model of adult mice (Kantak et al., 2014). The brain circuitry that is involved in marble burying is not as straightforward as some of the other tests in this study. Involving the prefrontal cortex, cortico-striatal pathway, along with the involvement of the dopaminergic system, it is possible that the *L. rhamnosus* is having a favourable impact on these sensitive structures perhaps through reducing inflammation or increasing the levels of SCFAs to promote brain health (Dalile et al., 2019).

Upon examination of social behaviour via the 3-chamber test, *L. rhamnosus* exposure *in utero* revealed marked but differential improvements in both test parameters with the two sexes. Females displayed increased time in the sociability arm of the test, whereas males showed improvement in the social novelty arm. The link between the microbiome and social behaviours has long been established (Sarkar et al., 2020; Wu et al., 2021). The microbiome is key in the development of the social brain, and positive modulation of the maternal gut is an unsurprising protective factor. Previous research has highlighted that prenatal stress during the final week of gestation leads to social complications in later life (Lee et al., 2007). At this point of gestation, the dams would have been administered *L. rhamnosus* for numerous days and administration would be concurrent with the development of the social circuitry in the fetal brain. Prenatal exposure to *Bifidobacterium dentium* has been found to produce similar results as to those in this study (Galley et al., 2024). The sole discrepancy between the two studies lies with the sex differences, however. Where our findings show increased female sociability and increased male social novelty, previous research noted converse results, such as increased male sociability and increased female social novelty but neither of these studies were of a similar design to ours (Galley et al., 2024; Gillette et al., 2022).

This improvement in social behaviours, in addition to the reduction in repetitive-like behaviours and improvements in the affective state strongly suggests that *L. rhamnosus* exposure during gestation positively modulates the developing fetus' gut-brain axis which leads to behavioural improvements in later life. Probiotic exposure *in utero* may be protective during certain windows of vulnerability, hence, explaining why numerous behavioural improvements are noted in this study (Cuinat et al., 2022). Particularly the combined results of the 3-chamber test and the marble burying indicate a reduction in autism-like behaviours, despite this not being a model of autism. There is strong evidence that the SCFAs and immune modulators produced directly from the probiotic, such as butyrate and propionate, can cross the placenta and directly influence neurodevelopment (Kimura et al., 2020). Further examination through metabolomics to measure SCFA quantities, or important markers such as brain-

derived neurotrophic factor would be useful here to help understand what is occurring mechanistically to improve behavioural phenotypes. There is also the potential for the probiotic to enhance the dams' milk which may continue to influence development in the very early postnatal life before the maternal gut returns to its normal composition. This enhancement may occur through altering the microbial composition of the milk, increasing the abundance of beneficial bacteria such as *Bifidobacterium* and *Lactobacillus* which can support the neonates developing gut. There is potential for the probiotic to modulate immune mediators also, such as a reduction of IL-6 and C-reactive protein as seen in humans, with associated neurodevelopmental benefits (Gonia et al., 2024).

Building on previous work that found an association between decreased 5-HT1a receptor expression in the prefrontal cortex and suboptimal behavioural outcomes, receptor expression was investigated here to examine if there was a correlation between favourable behavioural outcomes and enhanced receptor expression. These G-protein coupled serotonergic receptors play a vital role in mood disorders and ASD (Akimova et al., 2009; Kondaurova et al., 2023). Furthermore, the 5-HT1a receptor is known to be abundant in areas such as the hippocampus, raphe nuclei, prefrontal cortex, and amygdala, some of which are key brain areas in the circuitries behind our tested behaviours (Garcia-Garcia et al., 2014). Despite this, *L. rhamnosus* did not alter the expression of this receptor in either brain area examined. Yet this may be because this is not a disease-model and a healthy pregnancy, indicating that the advantageous alterations are caused by different neurochemical changes. Previous work has shown that *L. rhamnosus GG* increased 5-HT1a receptor expression in the hippocampus, but this was administered to adult male rats and not examined in any sort of developmental context (Işık et al., 2025). However, examining the hippocampus would not explain the changes seen in the ultrasonic vocalisations and the 3-chamber test as hippocampal involvement is very limited. It is still highly plausible that modulation of the offspring's serotoninergic system is behind these changes, either directly or indirectly. The serotoninergic system is comprised of numerous receptor subtypes, this includes 5-HT2a and 5-HT2c, which are known to play a similar or complimentary role to that of 5-HT1a in both

mood disorders and neurodevelopmental regulation (Alvarez et al., 2022; Gould et al., 2011; Ng et al., 2023). Beyond receptors, serotonin transporters have been shown to have a functional role in neurodevelopment. Studies on polymorphisms and knockouts have revealed that disruption of the serotonin transporter can be linked to ASD, increased anxiety-like behaviour, and reduced social behaviours (Kinast et al., 2013). Through regulation of synaptogenesis and dendrite development, the serotonergic system plays a substantial role in overall neurodevelopment that is complex and multifaceted (Kinast et al., 2013). It is plausible that there may be disruption to any of these parts that may in turn disrupt normal development.

Despite positive behavioural modifications, it is important to note that certain contrary findings were also found in our study. Probiotic exposure *in utero* decreased the amount of time that female offspring spent in the centre of the test arena, indicating increased anxiety, but potentially also reduced curiosity. Furthermore, no differences were found between the control and probiotic groups for distance or speed, indicating that the offspring were not just hyper-responsive or jumpy, but experiencing heightened arousal. Currently, the literature states that *L. rhamnosus* administration induces anxiolytic impacts. Previous studies have shown a reduction in anxiety-like behaviours in mice following *L. rhamnosus* administration, opposing the findings of this study. This was shown in both adult mice and maternally separated rat offspring (Bravo et al., 2011; McVey Neufeld et al., 2019; Schell et al., 2023). One study examining *L. rhamnosus* exposure in the prenatal period also showed anxiolytic effects (Zhou et al., 2022). However, the probiotic was administered from E18 until P5, much later in the gestational period than tested in the current study (Zhou et al., 2022). This finding, coupled with ours, strongly indicates that the timing of the administration is key. Here, the probiotic may have been given too early during gestation, when they fetal brain is too easily influenced by the maternal microbiome and immunomodulation. Later in gestation, such as E18, the fetal brain is more robust and less sensitive, and perhaps more likely to have a positive response to maternal probiotic supplementation.

Our findings reveal that these same female offspring exposed to prenatal *L. rhamnosus* had an increased number of Iba1+ cells in the amygdala, but not in the prefrontal cortex. Iba1+ is frequently used as a measure of activated microglia, which often are upregulated because of neuronal injury or inflammation (Jeong et al., 2013). The increased number of cells in the amygdala is indicative of fetal exposure to inflammation, an unwanted side effect of probiotic exposure *in utero*. The amygdala is often referred to as the brain's "fear centre", so it is not unexpected that neuroanatomical changes seen here manifest in a physical behavioural manner, as seen with the increased levels of anxiety. Of course, inflammation is not always detrimental and is required to some extent for certain critical processes. However, persistent and excessive inflammation can lead to deleterious consequences. Inflammation exposure *in utero* could alter microglia expression in the developing fetal brain (LaMonica Ostrem et al., 2024). Of note, administration of *L. rhamnosus* led to elevated circulating concentrations of IL-1α and CXCL1 in the dams on P21. Offspring exposure to these two pro-inflammatory cytokines during gestation may lead to neuroanatomical changes, altering normal behavioural outputs.

Interestingly, not all the major pro-inflammatory cytokines such as TNFα, IFNγ, and IL-6 were elevated in the maternal serum. The increased concentration of IL-1α and CXCL1, both markers of the innate immune system, indicates chronic, low-grade inflammation that still influences the neuroanatomy and behaviour of the exposed offspring. There is very limited information regarding rodent maternal cytokine levels in the postpartum period. Inflammatory mediators have been shown to be increased in the dam following administration of polyinosinic:polycytidylic acid (Lins et al., 2018). However, this is not a model of maternal immune activation, but rather a healthy pregnancy. Levels of pro-inflammatory cytokines and chemokines are expected to increase in later pregnancy to aid in parturition as is the case with human pregnancies (Fuhler, 2020; Mor et al., 2011). By P21 however, residual inflammation from birth should have resolved and both IL-1α and CXCL1 should be undetectable. Immunomodulation is proposed to be a key beneficial mechanism of probiotics, and we propose that this explains, in part, the unintended side effect (Mazziotta

et al., 2023). Probiotic strains, such as *L. rhamnosus*, bind to intestinal epithelial cells via toll-like receptors and this initiates immune stimulation (Mazziotta et al., 2023). Following this, there is a production of both anti-inflammatory antibodies such as IgA or T-reg cells, and pro-inflammatory cytokines such as IL-6 and IL-12 (Mazziotta et al., 2023). The *L. rhamnosus* treatment may be causing a dual immune response that may be contributing to the mixed behavioural and neuroanatomical effects in the exposed offspring. In addition to immunomodulation, other mechanisms could also be acting to produce these changes. These include neurohormone and neurotransmitter modulation, SCFA production, regulation of the hypothalamus-pituitary-adrenal axis, or influencing levels of BDNF in the offspring (Del Toro-Barbosa et al., 2020; Yong et al., 2020).

The examination of the offspring's microbiome may provide further explanation regarding the behavioural changes. Prenatal exposure to *L. rhamnosus* was found to leave an enduring imprint on the offspring's microbiome in the adolescent stage. Examining the β-diversity of the control and probiotic group notes significant differences between the groups, revealing a complete separation of composition with no overlap. While the probiotic group was found to have an increase in several *Bacteroides* and Firmicutes that are likely to produce beneficial SCFAs including propionate and acetate, a concern lies with the reduction of *Butyricimonas* and *Butyribacter*. Both genera are known to produce butyrate which is importantly linked to both anxiety and microglial activation. Previous work has revealed the immunomodulatory potential of butyrate, revealing its ability to suppress neuroinflammation both *in vitro* and *in vivo* (Caetano-Silva et al., 2023; Duan et al., 2021; Huuskonen et al., 2004). Separately, butyrate supplementation has been shown to reduce anxiety-like behaviours, with a reduction in the SCFA known to induce a more anxiogenic-like phenotype (Duan et al., 2021; Firoozi et al., 2025). Butyrate administration was shown to improve both anxiety-like behaviours and reduced microglia activation in the prefrontal cortex, both caused by high-fat diet-induced obesity (Duan et al., 2021). From this, the negative effect of the *L. rhamnosus* prenatal exposure may be in part due to a reduction in other key butyrate producers. Dependent upon the colonisation of the offspring's microbiome in the early postnatal period,

competition of certain species could have led to a reduction of beneficial butyrate producers. From this, the offspring's behaviour could be influenced by this coupled with the increased maternal inflammation.

The beneficial effects of the probiotic appear to have a greater impact on the maternal microbiome. While there is no clear separation of β-diversity in the microbial composition and the only significant changes in species richness and Faith's phylogenetic diversity a time effect, examination of differential bacterial abundances reveals a beneficial priming of the microbiome. *Lacticaseibacillus* was found to be increase at E18 as expected. Yet in comparison to E18, P21 probiotic-treated dams display an increase in numerous genera including *Blautia*, *Intestinimonas*, and *Phascolarctobacterium*. When compared to the microbiome of the control dams, those that received *L. rhamnosus* during pregnancy have a greater number of beneficial SCFA-producing bacteria with numerous favourable functions. This microbial composition could be described as an anti-inflammatory phenotype. This is the opposite as to what was seen in the offspring, which displayed a reduction in butyrate producers. While this may prove to be of benefit for overall maternal gut health, these dams did still display chronic low-grade levels on inflammation in the serum, however the effect was minimal and seen in a small group. This result suggests however that the positive enhancement of the maternal gut is separate from the increased levels of CXCL1 and IL-1α and are working through a different means.

The increase in SCFA-producing bacteria in the maternal microbiome may positively modulate offspring behaviour through transmission of beneficial metabolites through the milk. However, CXCL1 and IL-1α can also transfer to the offspring via this route and may be inducing inflammation in the pups that presents as increased anxiety. It is complicated to detangle the direct effect that the maternal microbiome has on the offspring in the postnatal period. Further examination of maternal milk metabolites, along with offspring faecal metabolites to examine the levels of butyrate, would be beneficial in furthering our understanding of the complicated mechanism of action of the *L. rhamnosus*. Future work should investigate how the harmful side effect occurred.

The sex differences found in the study are surprising when compared to the literature. Previous studies frequently report a male bias following exposure to a prenatal stressor, often accompanied with an increased risk of a neurodevelopmental disorder diagnosis in later life (Willcutt, 2012; Zablotsky et al., 2017). While probiotic administration is not classified as a prenatal stressor, if the prenatal probiotic induced an unfavourable state of inflammation, this could act as a stressor. Due to numerous biological factors and the sexual dimorphism of the placenta, male and female offspring can have drastically different *in utero* experiences. This results in the female sex being labelled as protective (Baron-Cohen et al., 2020; Hoffman et al., 2016; Martin et al., 2021; Muralimanoharan et al., 2013; Ross et al., 2015). However, while we do see positive behavioural alteration in both sexes, increased sociability and decreased repetitive-like behaviours in the females, in comparison to increased social novelty in the males, here, the female offspring also display disadvantageous behaviours. While exposure to a probiotic would not be considered a prenatal stressor, there is a certain overlap of process. Probiotics can be both immunoregulatory and immunostimulatory and they can modify the neuroendocrine system via hormones and neuropeptides. These metabolites and cytokines can cross the placenta and directly influence fetal neurodevelopment. Given the differential biological profiles between the two sexes, the female amygdala may be more vulnerable to the maternal levels of CXCL1 and IL-1α, which may lead to the increase in anxiety-like behaviour and microglial activity seen here.

While this study does provide evidence that *L. rhamnosus* during pregnancy may be beneficial for neurodevelopment, this research also highlights the potential negative side effects of an immunostimulatory probiotic in a healthy pregnancy. Perhaps prenatal probiotic administration is not optimal for this gestational timepoint, length of duration, or in a non-disease-model. Future work should examine the composition of both maternal and offspring metabolites as this would likely help in deducing the exact mechanism in which this probiotic works. *L. rhamnosus* does show great potential in improving neurodevelopmental outcomes but may be best utilised in a model of maternal immune activation,

such as an infection mimetic, pre-eclampsia, or high-fat diet-induced obesity for example.

5 Conclusion

In conclusion, the potential effect of *Lacticaseibacillus rhamnosus* exposure during the prenatal period on the offspring's brain, behaviour, and microbiome was examined. Maternal probiotic administration was found to improve the exposed offspring's affective state, increase sociability parameters, and decrease restrictive-repetitive behaviours. Despite these beneficial changes, female offspring displayed increased anxiety-like behaviour which was linked to increased microglial activation in the amygdala and potentially a decrease in butyrate-producing bacteria in the gut. This side effect was potentially mediated by an increase in CXCL1 and IL-1α in the maternal serum. This work demonstrates the primarily beneficial role of a prenatal probiotic on neurodevelopmental outcomes. However, this work also highlights the importance of testing probiotics in a healthy pregnancy before use in a disease-state model. Introduction of a potentially immunostimulatory probiotic into an already inflamed pregnancy could have deleterious consequences for the health of the developing fetus.

Chapter 5

General discussion

1 Overview and summary

This thesis demonstrates that elevated prenatal inflammation exerts a significant influence on long-term neurodevelopmental trajectories in exposed offspring. This effect was initially evident using serum from the previously completed Screening for Pregnancy Endpoints (SCOPE) study. Prior to this work, serum from healthy pregnant women was taken at gestational week 20. Originally the authors identified a biological stress signature that may have the potential to influence fetal development (Keane et al., 2021). In this thesis however, a subset of these women was identified as biologically "high-stress", classified according to markers of inflammation and gut permeability, along with tryptophan metabolism, all important for fetal health. This was used to identify a mechanism in which serum with bioactive potential can influence neuronal growth. Using an *in vivo* rat model of maternal immune activation (MIA), novel insights into the complex relationship between maternal inflammation and maternal microbiome disruption during pregnancy were examined and offspring outcomes characterised. Finally, the therapeutic potential for an anti-inflammatory based probiotic in improving neurodevelopmental outcomes in a healthy rat pregnancy were investigated.

In *Chapter 2*, a subset of women from the Cork cohort of the SCOPE study were stratified into 3 clusters; biological low-, medium- and high-stress. Using a proxy model of neuronal growth and development, the possible exposure effects of biological "high-stress" serum on neuronal morphology and outgrowth was investigated. For the first time, we demonstrated that exposure to serum from these biological "high-stress" pregnant women reduced neurite length in cells. This reduction was in part due to an increase in tumour necrosis factor (TNF)α. The elevated concentration of TNFα that was seen in the biological "high-stress" group increased phosphorylation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p65 subunit in SH-SY5Y cells. Neurite growth or arrest can be modulated by NF-κB activation. This study provides a mechanistic insight into the role of TNFα in altering neurite outgrowth which may in turn negatively influence neurodevelopmental outcomes in exposed offspring.

Building on these findings of how high inflammation may disrupt normal neuronal growth, in *Chapter 3*, it is reported for the first time the combined effect of MIA and maternal microbiome disruption on the developing offspring. Using a lipopolysaccharide (LPS) model of MIA in combination with an antibiotic cocktail, exposed embryos displayed a growth restricted phenotype. Significant developmental alterations following MIA exposure were reported. Juvenile male rats displayed an increase in anxiety, associated with a decrease in 5-hydroxytryptamine (5-HT)1a receptor expression in the prefrontal cortex. Periadolescent female offspring engaged in more repetitive-, restrictive-behaviours. Interestingly, there was no additive effect of MIA and microbiome dysbiosis on behavioural or neuroanatomical outcomes. A dual-hit effect was seen however on embryonic anthropometry and placental growth. Discrete changes in the offspring's microbiome following antibiotic prenatal exposure were also reported, yet this disruption didn't directly influence the brain or behaviour. Taken together, these findings suggest that exposure to inflammation poses a greater disruption to expected neurodevelopmental trajectories in comparison to a dysbiotic maternal microbiome. While both are suboptimal for a healthy pregnancy, these results support inflammation as a severe prenatal stressor that can lead to atypical development, despite both insults being administered at the same stage of gestation and for the same duration. Perhaps most importantly, this study reveals the differential risk that distinct prenatal exposures have on neurodevelopment.

Based on these findings, the need for an anti-inflammatory therapeutic agent is evident. Thus, in *Chapter 4*, prenatal exposure to *Lacticaseibacillus rhamnosus* was investigated in a healthy pregnancy to understand any effects of an anti-inflammatory species on different systems with regard to a safety perspective, before introduction into a disease-model. Differential effects of the probiotic on behavioural outcomes in the exposed offspring were reported. The probiotic positively influenced the affective state of the offspring, coupled with a reduction in repetitive-, restrictive-behaviours in females, in addition to a sex-specific improvement in sociability and social novelty. However, probiotic exposure in the prenatal period also induced some unexpected, potentially harmful

consequences including an increase in anxiety-like behaviours in juvenile female offspring, coupled with an increase in active microglia in the amygdala in the adolescent period, indicating potential vulnerability across transitional periods. This in part may be due to elevated levels of interleukin (IL)-1α and (C-X-C motif) ligand 1 (CXCL1) in maternal serum. Immunomodulation following probiotic administration is reported to be beneficial in cases of a healthy pregnancy, but this study urges caution regarding unwanted side effects.

Overall, the results obtained from *Chapters 2 – 4* of this thesis note the neurodevelopmental consequences linked to prenatal inflammation and maternal microbial modulation. This thesis supports the use of an anti-inflammatory microbiome-targeted therapy to reduce the inflammation-induced risk of neurodevelopmental sequalae, but highlights caution for unwanted immunomodulatory side effects. A summary of the investigations from this thesis are detailed in **Figure 1**.

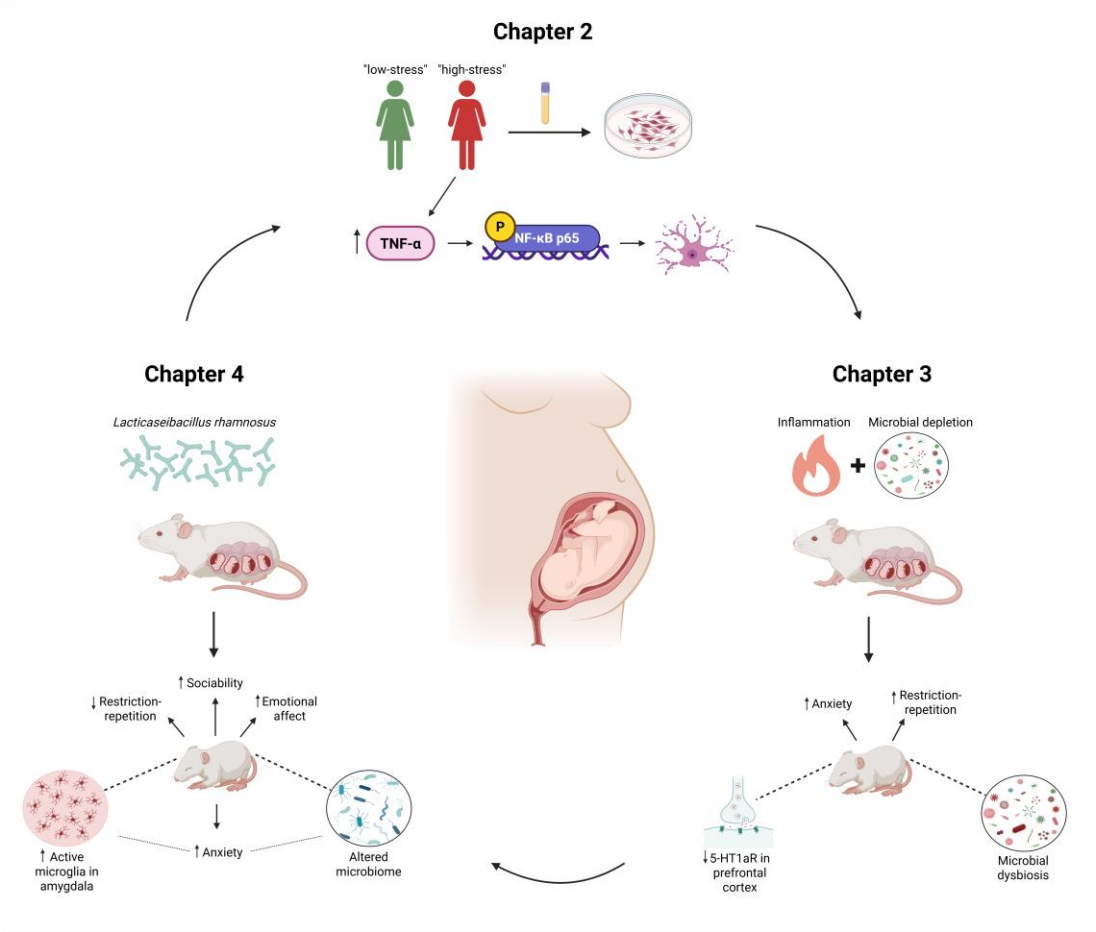


***Figure 1*: Overview of thesis findings.** *The major findings seen in each of the data chapters presented in this thesis summarised*.

2 Exploring translatability: bridging theory and practice

Translational research bridges the gap between basic scientific research and clinical practice. Examining and critiquing the translatability of the findings presented in this thesis allows us to understand the real-word value and adaptability of our research. Delving into the potential benefits of this work can significantly contribute to the field of neurodevelopmental research. However, this is not clear cut. Preclinical work is accompanied with numerous limitations and often the translatable aspect of science is called into question and justifiably so. Yet in recognising these hurdles, we open the door for appropriate evaluation and necessary reshaping of preclinical research. The field of neurodevelopmental research is evolving and rapidly expanding but is complicated by numerous logistical and ethical challenges that reduce certain exploratory or mechanistic impact. This forces most neurodevelopmental studies to be completed using *in vitro* or *in vivo* models. Despite the limitations of preclinical work, the work in this thesis has the possibility to inform future clinical decisions.

Although *Chapter 2* uses a proxy *in vitro* model of early brain development, the study's findings provide valuable insights that may translate to clinic. Previously in our lab, a biological stress signature was identified in the Cork cohort of the SCOPE study. Here, elevations in defined circulating pro-inflammatory factors were seen in a subset of these women. Yet, these women were classified as healthy pregnancies without complications with no evidence of systemic stress. A proportion of the women included in the study did have an irritable bowel syndrome (IBS) diagnosis. IBS is associated with low-grade systemic inflammation and symptoms can fluctuate during pregnancy, but there is a paucity of information surrounding this. However, there was no association with IBS and biological "high-stress" in this cohort. The observation of increased inflammation in this cohort highlights the important translational message that maternal inflammatory status cannot be inferred by clinical presentation alone. Even in the absence of disease, it is possible that subtle unexpected changes to

the maternal immune system may occur, potentially impacting fetal brain growth.

While the findings of this study reveal a potential mechanism by which elevated inflammatory factors may disrupt neurodevelopment, it highlights the need for a more in-depth health screening of expectant mothers. Inflammation is a critical process during conception, implantation, and parturition, and is an important regulator of these processes (Fuhler, 2020). However, throughout the remainder of the pregnancy, inflammation has the potential to be harmful to the developing brain and is linked to numerous neurodevelopmental disorders (NDD) (Antoniou et al., 2020; Brown et al., 2001; Dubey et al., 2022). While the magnitude of inflammation seen here in the biological "high-stress" group of women from the SCOPE study is unlikely to influence expected neurodevelopmental trajectories, the findings of unexpected inflammation in the maternal serum begs for increased vigilance. However, it is important to note that while TNFα and IL-6 levels seen in the "high-stress" group are normal for pregnancy, the concentration of C-reactive-protein (CRP) in these women are significantly elevated, bordering on levels that would be typical during an infection (Wirestam et al., 2021). Prenatal screening of various inflammatory mediators or markers of gut permeability may be of great benefit to protect both the fetal brain and the mother, with it being particularly relevant to women at an elevated risk of adverse pregnancy outcomes. Pregnancy conditions such as pre-eclampsia and gestational diabetes have strong links to a perturbed maternal immune system (Han et al., 2021). Furthermore, metabolic conditions or autoimmune diseases, which are increasingly common, exacerbate the risk of fetal inflammation exposure (Han et al., 2021). Lifestyle factors such as a Western-stye diet, physical inactivity, or smoking are known to intensify inflammation (Cabral et al., 2020; Celik and Yildiz, 2021; Kou et al., 2011; Li et al., 2025). The combined effect of these factors may increase the vulnerability of a fetus in an otherwise "low-risk" pregnancy. This vulnerability may be further increased due to genetic predisposition or an infection which is incredibly common during pregnancy (Collier et al., 2009).

Pre-emptive screening of expectant mothers across numerous gestational timepoints could allow for early identification of at-risk mothers. This could be simply performed during scheduled checkups. Taking a blood sample would allow for a quick inflammatory assessment using an enzyme-linked immunosorbent assay for example. CRP is often used to measure systemic inflammation, along with examination of different pro-inflammatory cytokines would also help to provide a clear picture as to who may be at-risk. From a clinical perspective, reducing the duration of inflammation exposure is of utmost importance in maintaining correct fetal neurodevelopmental trajectories. Multiple screening timepoints would allow elevated levels of inflammation to be rapidly identified; which in turn allow for further investigation to treat the underlying cause and measure the efficacy of the intervention. Reducing the overall magnitude and duration of inflammation exposure is of particular importance during critical periods of brain development.

Despite the suggestion of a prenatal anti-inflammatory intervention being a straightforward solution to the problem at hand, the number of appropriate interventions is greatly limited during pregnancy. Most pharmacological interventions, such as nonsteroidal anti-inflammatory drugs (NSAID), are contraindicated in pregnancy. While these drugs lower maternal systemic inflammation, most have teratogenic potential. NSAID use in the third trimester is associated with fetal kidney and cardiovascular damage (Black et al., 2019). Acetaminophen however, may be prescribed for a short period in the first and second trimester, if required, but this is not an appropriate long-term intervention to lower inflammation during pregnancy (Black et al., 2019). Due to the lack of safe and appropriate pharmacological interventions, alternative non-pharmacological anti-inflammatory approaches, such as dietary changes and healthy lifestyle modifications are recommended. Probiotics have shown great promise in preclinical findings but should be utilised with caution due to their immunomodulatory mechanisms. Clinical studies report improvement in pre-eclampsia symptoms following probiotic intervention, in part due to positive modulation of the maternal microbiome (Brantsæter et al., 2011; Nordqvist et al., 2018). Unfortunately, neither of these studies examine inflammatory

markers in the mothers but do hypothesise that the probiotics reduce pre-eclampsia risk through an inflammation-dependent mechanism. Furthermore, long-term neurodevelopmental outcomes were not examined in the offspring of either study, yet these findings are still promising.

Separately, these studies importantly add to a growing wealth of knowledge that probiotic usage can support maternal health in both the prenatal and postnatal period. Along with improving pre-eclampsia outcomes, probiotic administration in the prenatal period has improved insulin resistance and fasting blood sugar in cases of gestational diabetes, reduced the risk of pre-term birth, improved vaginal health, while also possibly ameliorating prenatal anxiety levels (Dibo et al., 2022; Seif El Dahan et al., 2022; Sun et al., 2024). *L. rhamnosus* HN001 administration during the prenatal and postnatal period was shown to reduce the severity of postpartum anxiety and depression (Slykerman et al., 2017). The benefit of improved maternal health in the peripartum period cannot be understated for supporting the fetus and infant's wellbeing.

Still, there is an urgent need for a clinical neuroprotective agent against fetal inflammation exposure. Melatonin and N-acetyl-L-cysteine have been shown to be protective in the fetal brain where high levels of maternal inflammation are induced in preclinical studies, with N-acetyl-L-cysteine showing promise in clinic in treating chorioamnionitis, however neurodevelopmental outcomes of the exposed offspring were not examined (Elsayed et al., 2021; Joseph et al., 2024; Malhotra et al., 2024).

Providing a safe and efficacious anti-inflammatory intervention without any teratogenic effects begins with preclinical work. Yet, how to bridge this gap between initial rodent studies and safety testing in a human fetus is a question without a straightforward answer and requires significant ethical considerations to protect both the mother and the developing fetus. Artificial intelligence (AI) may be a helpful tool. Data mining techniques through predictive machine learning may help in the rapid advancement of an anti-inflammatory intervention. Through screening of maternal health records, inflammatory markers, and long-term neurodevelopmental outcomes in the offspring, it may

give way to unique discrete patterns that may be missed by clinicians. This could provide vital safety information surrounding critical periods of brain development or potential contraindications. Examining a candidate therapeutic through AI could allow for rapid information of the predictive pharmacokinetics and pharmacodynamics of the intervention, while also highlighting safety concerns. This could allow for a unique personalised approach to treating or managing high levels of inflammation. Utilisation of AI and data mining may greatly improve the ability to fill the gap between preclinical work and clinical practice.

The findings of *Chapter 3* equally support enhanced maternal screening of inflammatory markers. Using a bacterial mimetic to stimulate MIA, chronic exposure to inflammation was harmful for offspring neurodevelopmental outcomes. However, the experimental design used in this thesis is not a direct reflection of a clinical setting (Opal and Glück, 2003). It would be very unlikely that maternal infection would reach the magnitude expected with our LPS model based off of previous research, or last throughout the course of the pregnancy (Fricke et al., 2018). Inflammation must be reduced, even in "low-risk" pregnancies that may be more vulnerable than expected. Antibiotics are given frequently in clinic to treat maternal infection, which reduces inflammation as a consequential effect (de Jonge et al., 2014). Here, limited alterations were seen following antibiotic exposure during the prenatal period, while LPS exposure was significantly more detrimental to the developing fetal brain.

However, the findings in this thesis do not infer that prenatal antibiotic exposure is without associated risks. The findings indicate that antibiotics may affect normal fetal growth, mimicking intrauterine growth restriction (IUGR) and stunted placental growth. However, it is important to note that the antibiotic cocktail used in this study, was composed of ampicillin, neomycin, and vancomycin and this combination would not be traditionally prescribed during pregnancy. Penicillin-based antibiotics, such as ampicillin, are commonly prescribed during pregnancy, with their use deemed safe by the Food and Drugs Administration and are labelled as a category B risk (Walling, 2006). Vancomycin is part of the glycopeptide class of antibiotics and are classified as category B,

generally considered safe during pregnancy (Nguyen et al., 2025). However, neomycin, as part of the aminoglycoside class of antibiotics is not recommended during pregnancy. Aminoglycosides are classified as category D and may be damaging to the fetus (Wang et al., 2025). The inclusion of neomycin in this study may explain the stunted growth pattern, and this is unlikely to be replicated in clinic due to its restricted use. As with all classes, antibiotic usage significantly disrupts the normal diversity of the gut microbiome which may further exacerbate any potential effects. Alterations in the composition of the maternal gut microbiome can lead to unfavourable changes in metabolite production, including the production of butyrate and propionate that are known to support fetal development. The importance of maternal metabolites in optimal fetal growth has been shown numerous times *in vivo* (Chen et al., 2022; Huang et al., 2022; Vuong et al., 2020).

While we did not observe indications of increased repetitive behaviour or effects on sociability, clinical findings have linked prenatal antibiotic exposure to an increased risk of autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD) in later life (Njotto et al., 2023; Straughen et al., 2023; Tao et al., 2022). In accordance with other studies, we observed a disruption of the exposed offspring's microbiome following prenatal antibiotic exposure was shown. However, it is difficult to pinpoint how this microbial disruption may have occurred. Ampicillin is the only antibiotic of the three used in this study that is absorbable and not restricted to the gastrointestinal tract. Due to this, ampicillin usage may disrupt the maternal vaginal microbiome, making it dysbiotic, and in turn this can disrupt normal seeding to the neonate's microbiome. Alternatively, the antibiotic-induced disruption to the maternal microbiome could compromise the quality of the milk, failing to support the neonatal microbiome during critical periods in early postnatal life. Despite this, it begs the question if children should undergo microbiome testing in cases where the mother received a course of antibiotics throughout the course of gestation to determine the need for an intervention, such as postnatal probiotics which may be less stimulatory for a less malleable brain.

Sampling the microbiome in early postnatal life may be beneficial to the offsprings future health. Based on the results seen in *Chapter 4*, a probiotic may not be an appropriate intervention unless there is justification for its use. The early postnatal microbiome should not be diverse, with a more immature composition deemed favourable to support *Bifidobacterium* and *Lactobacillus* (Hill et al., 2017; O'Sullivan et al., 2015; Savage et al., 2018; Shenhav et al., 2024). While the work presented in this thesis suggests caution when taking prenatal probiotics, more work is needed in this field to identify a suitable microbiome-targeted intervention. Prebiotics including human milk oligosaccharides (HMO) or various postbiotics may be a better route forward. However, if there is dysbiosis in the postnatal microbiome following suboptimal prenatal exposures, identifying this dysbiosis could allow for a personalised medicine approach, specifically targeting the disruption in the infant's microbiome rather that a generic probiotic that may in turn have negative neurodevelopmental effects. For maximum impact, the characterisation of the gut microbiome of neurotypical children should be used as a cautious comparison for those at-risk of neurodevelopmental disorders following prenatal exposure. For instance, one study noted that a *Bacteroidetes*-dominant gut microbiome was indicative of better motor, cognitive, and language scores in male toddlers (Tamana et al., 2021). Other longitudinal studies, such as INFANTmet, could also prove to be beneficial here (Hill et al., 2017).

Clinical studies have revealed great promise in probiotics improving maternal health and reducing unfavourable neurodevelopmental outcomes in the infant (Brantsæter et al., 2011; Godfrey et al., 2021; Korpela et al., 2018). Despite this, the results of *Chapter 4* urge caution when administering probiotics during pregnancy. While most of our findings proved to be beneficial for the offspring, the unwanted and unexpected increase in anxiety-like behaviour and inflammation in the offspring's amygdala is noteworthy, likely due to an increase in activated microglia. Of course, administration of a probiotic will modulate the gut microbiome and influence overall composition, but the effect seen will be unique to each host dependent upon lifestyle and diet, existing microbiome

composition, and other factors such as sex and genetics. However, it is possible that in an already healthy microbiome, introduction of a large quantity of a bacterial species may also introduce unwanted side effects. The gut microbiome does shift during pregnancy, typically associated with higher levels of inflammation, reduced α-diversity, and increased β-diversity (Koren et al., 2012; Nuriel-Ohayon et al., 2016). As previously mentioned, this phenotype mimics that of metabolic disorder (Nuriel-Ohayon et al., 2016). Yet in the case of pregnancy, this is the preferred and intended state that best supports fetal development. Introduction of a probiotic at such a sensitive time may not be the most efficient solution. The research presented in this thesis can inform future studies regarding the importance of careful species selection, along with dosing and timing of the administration to complement the maternal microbiome and support fetal neurodevelopment. It is important to highlight however the numerous limitations in relating these findings to clinic. Various yet complimentary reasons, such as biological and model differences, methodological heterogeneity, and safety regulation, all previously mentioned, prevent the results seen in this thesis from being taken at face value for human pregnancies. At human level there is very limited evidence of harm following probiotic exposure in pregnancy, with numerous studies finding a beneficial effect, or simply no effect on neurodevelopment. Yet in our findings, quite deleterious behavioural and anatomical effects were noted, this is unlikely to translate to human clinical studies with the same efficacy.

However, in saying that, based on the results of this thesis, it may be the best approach to only administer prenatal probiotics when there is a clear need, and the potential benefit to the fetus outweighs the potential risk. For this, maternal microbiome sampling via 16S rRNA sequencing or metabolomics would be a sound option during scheduled pregnancy checkups, allowing for direct identification of dysbiosis and allowing a personalised therapeutic approach. Of course, probiotics are not the sole method of a microbiome-targeted intervention. Dietary modifications may prove to be the best course of action, which would allow for a more natural holistic approach, one that is better studied and understood. Foods rich in protein, polyunsaturated fatty acids, and

micronutrients such as folic acid, iron, and choline are important strategies for supporting normal fetal neurodevelopment (Caffrey et al., 2021; Cortés-Albornoz et al., 2021; Ghazal and Naffaa, 2025; Miyake et al., 2023b). However, while this is recommended in clinic, major alterations to one's diet, especially during times of change can be difficult to adhere to. Expectant mothers may prefer adding a supplement on top of their normal dietary habits rather than significant dietary alterations, potentially increasing compliance and ease. Microbiome-targeted interventions should still be considered but in cases of healthy pregnancy, perhaps those with less immunomodulatory potential should be favoured over traditional probiotics. Dietary fibres and postbiotics may function in a more predictable, consistent manner and better support the maternal microbiome. While reduced levels of dietary fibre has been linked to increased neurodevelopmental complications in cohort studies, postbiotics are less well studied (Miyake et al., 2023a). Additional preclinical *in vivo* studies and clinical cohort studies are required to fully understand the influence of postbiotics on the developing fetus.

Overall, this thesis supports the need for regular maternal inflammatory and microbiome screening in clinic to help identify potential harmful exposures to the fetus, even in cases of "low-risk" pregnancies. This would allow clinicians to identify vulnerable, at-risk fetuses and consider if a microbiome-targeted intervention would be of benefit in supporting brain development. However, this thesis also suggests the need for caution when taking potential immunostimulatory products during pregnancy and perhaps only best consumed following a needs assessment by a clinician. Neonatal screening also may be beneficial to measure potential levels of dysbiosis following unwanted prenatal exposures.

3 Future directions

The results of this thesis advance the current knowledge regarding the influence of maternal inflammation and microbiome on normal offspring neurodevelopment. Nonetheless, there is a potential to build on the reported findings to further understand the complex interactions and mechanisms involved. In *Chapter 2*, the sole use of SH-SY5Y cells is a limitation of the project but is an important first screening tool to provide mechanistic insights. Future work should examine the effect that the biological "high-stress" serum has on a more representative model of fetal brain growth, with embryonic primaries or stem cells being potential cellular models. Despite this, perhaps the greatest potential stems from possible interactions between the circulating factors in the maternal serum.

TNFα, which was our primary interest, is known to interact with both IL-6 and CRP. The relationship between these three pro-inflammatory markers is bidirectional and often complex (Palmqvist et al., 2008; Sproston and Ashworth, 2018; Tanabe et al., 2010). Furthermore, inflammation as a whole is deeply intertwined with overall gut health and permeability and the two have a well-documented, often synergistic effect (Mishra et al., 2023; Shieh et al., 2020; Stephens and von der Weid, 2020). Regularly working as a positive feedback loop, inflammation has been shown to increase permeability of the intestinal barrier through alterations in both the structure and expression of the protective gap junctions (Al-Sadi et al., 2013b, 2013a; Ma et al., 2004). In turn, an increase in pro-inflammatory microbial components circulation, may present as low-grade chronic inflammation, or feed into the "leaky-gut hypothesis" (Fukui, 2016). Previous work has shown Caco-2 cells to have altered tight junction permeability following administration of TNFα (Al-Sadi et al., 2013b; Ma et al., 2004). This indicates that TNFα may be indirectly altering the markers of gut permeability that were used to profile the women in this study; soluble CD14 (sCD14), lipopolysaccharide binding protein (LBP), intestinal fatty acid binding protein (iFABP). Further examination of how these factors interact would provide valuable information as to what extent TNFα-driven inflammation directly

influences gut permeability and enhances microbial translocation into the bloodstream or if it is a downstream result. In a clinical setting, this could be utilised as a means to help identify a disrupted gut barrier.

In terms of tryptophan, the kynurenine pathway contributes to immune regulation and immunosuppression (Karahoda et al., 2020). Indoleamine 2,3-dioxygenase is one of the key enzymes required to convert tryptophan to kynurenine. It has been shown that mice that lack this enzyme produce a greatly exaggerated inflammatory response following an infectious or autoimmune-based inflammatory insult, (Sorgdrager et al., 2019). This deficiency was also found to have a protective-like role in a mouse mode of chronic gastric inflammation (El-Zaatari et al., 2018). Inflammation can be regulated via tryptophan metabolism along the kynurenine pathway, and the converse is also true. A recent study has highlighted the relationship between tryptophan and inflammation through examination of chronic inflammatory diseases. Here, it was evident that chronic inflammation depletes tryptophan in patient's serum. This depletion was shown to be inversely proportional to CRP levels (Harris et al., 2024). The kynurenine/tryptophan ratio, which was found to be significantly elevated in the biological "high-stress" group, can be used as a measure of kynurenine pathway activity and has also been tied to many chronic inflammatory conditions (Harris et al., 2024). Future work should continue to elucidate the relationship between tryptophan metabolism and inflammation during pregnancy. As with gut permeability markers, molecular manipulation of factors in the maternal serum may help to understand the relationship between TNFα-driven inflammation and tryptophan metabolism. If tryptophan is being metabolised into kynurenine at an accelerated rate in response to inflammation, there is a shift away from serotonin synthesis, tryptophan's other metabolite. There are potential implications of altered serotonin production on fetal brain development. Variations in normal tryptophan metabolite balance should be examined and potentially uncovering any harmful effects on fetal neurodevelopment.

The results of *Chapter 3* note the potential harm that prenatal inflammation exposure may have on normal developmental trajectories, revealing negative alterations to normal *in utero* growth, unfavourable behavioural changes, and disruption to normal serotonergic signalling. While it was noted that antibiotic exposure, or negative microbial modulation, did not negatively influence the offspring's brain or behaviour, positive modulation of the maternal microbiome could prove to be beneficial to combat MIA. An anti-inflammatory based probiotic should be investigated in this setting; most current work has examined this in the postnatal period and not the prenatal. Yet, there is potential for use of a prenatal intervention to limit damage to the fetal brain, rather than repairing existing damage. *Lacticaseibacillus rhamnosus*, used in *Chapter 4*, showed promise in supporting fetal neurodevelopment in a healthy pregnancy. However, increased anxiety-like behaviour and increased microglial activation in the offspring's amygdala were unexpected side effects. Firstly, further examination into the increased number of active microglia is warranted. Immunohistochemically co-staining alongside Iba1 would allow for identification of phenotype. Microglia can exist in two phases, pro-inflammatory or M1, or anti-inflammatory also called M2. Understanding this would allow further interpretation of the results seen in this chapter, notably, how is the offspring's immune environment modulated. The M1 state has previously been linked to an increased risk of neurodevelopmental disorders, and it is plausible that this might be the case here (Qin et al., 2023). Furthermore, examination of what are known as "dark microglia", identified through CD11b levels, could help provide insight into synaptic pruning, which has been implicates in numerous developmental conditions previously (Cornell et al., 2021). Due to the mild inflammatory-nature of the *L. rhamnosus* intervention, potentially this specific probiotic species would perform better in a disease-state model, rather than in a healthy pregnancy. The efficacy of *L. rhamnosus* to modulate the effect of maternal administration of LPS should also be examined. Not only would this provide information regarding its true anti-inflammatory capacity but provide more information on the associated negative side effect, allowing us to confirm that immunomodulation is not always beneficial unless there is a call for it.

Furthermore, *L. rhamnosus* should be examined in additional models of prenatal inflammation. The reduced uteroplacental perfusion pressure (RUPP) model is used to mimic human pre-eclampsia. Through surgical clamping of the uterine arteries and the lower abdominal aorta, the fetus is exposed to high levels of placental ischemia that imitates perfusion, while also leading to the key pre-eclamptic symptoms of hypertension and proteinuria (Li et al., 2012). Arguably, while the RUPP model has a number of limitations, it still may be a more translatable model of what is seen in clinic in comparison to LPS administration to mimic a bacterial infection. As previously mentioned, the dosing given in rodent models is far greater than what would ever be seen in clinic, and while these models do provide a vital understanding of certain mechanisms, there are perhaps better preclinical models to test probiotic efficacy (Opal and Glück, 2003). The RUPP model is not perfect. It fails to capture the perfusion-reperfusion seen in clinic, does not model early dysfunctional placentation, and clamping of major blood vessels can damage other maternal organs (Li et al., 2012; Morton et al., 2019). The RUPP model is typically used to investigate potential therapies for placental ischemia. However, the model still captures the inflammatory aspect of the condition well as a secondary feature and is closer to what is seen in clinic in comparison to infection mimetic models (Li et al., 2012) Testing *L. rhamnosus* in the RUPP model would provide valuable insights into the probiotics function and allow for a more accurate interpretation of how it may behave in clinic.

Despite some of the findings of *Chapter 4*, investigation into a microbiome-targeted supplement to support fetal neurodevelopment in a healthy pregnancy should not be abandoned. Postbiotics are an understudied concept but show exciting promise in positively modulating the microbiota-gut-brain axis. While performing similar actions to probiotics, including immunomodulation, supporting intestinal barrier integrity, and the production of antimicrobial peptides, postbiotics are potentially a safer and more reliable option during pregnancy as they are less likely to trigger unwanted immunostimulatory effects (Calvanese et al., 2025). Regardless of their promise, postbiotics have not been thoroughly examined in a neurodevelopmental context. As performed here,

prenatal postbiotic exposure should be examined in a healthy pregnancy to understand baseline changes to the offspring's brain, behaviour, and microbiome before introduction to a disease-model.

A greater understanding of how the maternal microbiome changes during pregnancy is needed. Many human observational studies produce conflicting findings and this is a rate-limiting step towards this concept progressing. Future work, despite the numerous associated challenges, should prioritise identifying a more definitive, predictable microbiome change in pregnant women. These studies should be longitudinal and follow-up with the offspring and potential NDD diagnosis. Production of a high-quality set of microbiome samples during pregnancy and long-term offspring outcomes is of utmost importance. Following confirmation of this, it could be utilised in gestational microbiome sampling that was previously suggested, to identify at-risk fetuses in a pregnancy that would otherwise be deemed "low-risk", with no obvious fear for the fetus' neurodevelopmental trajectories or overall health.

4 Overall conclusion

This thesis provides data that highlights the role of inflammation and the maternal microbiome in offspring neurodevelopment. Firstly, a potential mechanism by which circulating factors in the maternal serum may disrupt vital neurodevelopmental processes was identified. Further, the combined effects of prenatal inflammation and antibiotic exposure were characterised, revealing the lack of a dual-hit mechanism while demonstrating the harmful effects of MIA in neurodevelopment. The effects of prenatal inflammation were mediated in part by altered 5-hydroxytryptamine (5-HT)1a receptor expression leading to increased anxiety-like behaviours. Finally, while prenatal probiotic exposure can positively influence neurodevelopment, negative side effects potentially suggest that probiotics are best given in disease-states, and postbiotics may be a more appropriate supplement during a healthy pregnancy.

Initially, women were stratified based on tryptophan metabolism, gut permeability, and inflammatory mediators into biological "low-stress" and "high-stress" groups. Application of the "high-stress" serum led to phosphorylation of NF-κB p65 Ser536 via a TNFα-dependent mechanism, which in turn reduced neurite length in retinoic acid-differentiated SH-SY5Y cells.

Subsequently, the effect of LPS and antibiotic exposure in the mid-late gestational period was characterised. The exposure to MIA was found to increase repetitive like-behaviours in the female offspring, with an increase in anxiety-like behaviours through a reduction in 5-HT1a receptor expression in the prefrontal cortex of male offspring. Prenatal antibiotic exposure did not affect offspring behaviour or 5-HT1a expression. The concept of a dual-hit impact of LPS and antibiotics resulted in reduced placenta and fetal growth with a greater microbial dysbiosis due to antibiotic exposure.

Finally, given the deleterious effect of prenatal inflammation on fetal neurodevelopment, the potential beneficial effects of prenatal probiotic administration were investigated. *Lacticaseibacillus rhamnosus* enhanced pups' emotional maturity and affective state, improved social parameters, and reduced repetitive-like behaviours in a healthy pregnancy. However, female

offspring engaged in increased anxiety-like behaviours coupled with elevated Iba1+ cell counts in the amygdala. Together, these results note the differential effects that a probiotic may have in a healthy pregnancy.

The findings of this thesis report potential mechanisms in which harmful exposures during the critical periods can influence future offspring neurodevelopment. These prenatal exposures disrupt the normal behaviour, neuroanatomy, and microbiome of the young offspring. Increased immune activation during pregnancy is a growing clinical concern, work in this thesis provides valuable information towards a microbiome-targeted solution but also calls for future work to identify potential therapies with reduced immunostimulatory side effects.

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