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Bugs, brains and babies: The role of gut microbiota in preterm brain development

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A thesis submitted for the degree of
Doctor of Philosophy

2022

Abstract

The gut microbiota is increasingly recognised as a modulator of brain and behaviour. However, to date there is limited knowledge of the relationships between the early-life gut microbiota and brain structure in preterm infants, who are at risk for both altered brain development and gut bacterial dysbiosis.

This thesis aimed: to develop a brain imaging phenotype capturing diffuse white matter injury, a prevailing feature of the encephalopathy of prematurity (EoP) along with deep grey matter volume loss; to review the evidence that gut microbiota diversity and community composition associate with human brain development in infancy and childhood; to characterise and understand the drivers of preterm early-life gut microbiota diversity and community composition; and to investigate associations between the preterm gut microbiota and features of the EoP at term-equivalent age.

In the first study, using brain diffusion MRI data of 79 term and 141 preterm infants recruited to the Theirworld Edinburgh Birth Cohort (TEBC), I applied data reduction to diffusion MRI metrics across white matter tracts to derive single- and multi-metric latent general factors capturing diffuse white matter disease. I found that the general factors are influenced by preterm birth, suggesting they provide useful measures of global white matter microstructure to investigate upstream determinants of brain dysconnectivity among preterm children. I used these metrics in subsequent microbiota-brain analyses.

In the second study, I identified twenty studies that suggested the microbiome-gut-brain axis may operate across three domains in infancy and early childhood: general neurocognitive development, socio-emotional behaviours, and brain structure and function. However, there was substantial variation in the bacteria-brain/behaviour relationships reported, and I identified sources of clinical and methodological heterogeneity in the studies. I showed that there is very little understanding about microbiota-brain relationships in preterm infants in the extant literature.

In the third study, in 147 infants recruited to TEBC, I found that the majority of meconium samples were characterised by high relative abundances of *Staphylococcus* or *Streptococcus*, which minimally differed between term and preterm neonates. The microbiota community

of preterm infants by the time of discharge from hospital had high relative abundances of *Bifidobacterium* or *Enterobacteriaceae*; some samples also had high abundances of *Escherichia/Shigella, Klebsiella*, or *Enterococcus*. The preterm infant meconium sample composition was strongly influenced by mode of delivery, while the main drivers of gut microbiota composition prior to discharge from the hospital were the degree of prematurity, sex, and antibiotic exposure.

Finally, I took a whole-brain approach to investigate the associations between gut microbiota diversity and community composition prior to hospital discharge and brain imaging features of the EoP at term-equivalent age in 79 preterm neonates recruited to TEBC. Using dimensionality reduction, I identified four main axes of variance in the microbiota community composition data, driven by the abundances of *Bifidobacterium, Escherichia/Shigella-Enterobacteriaceae*, *Klebsiella*, or *Enterococcus.* I found that microbiota richness and the main axes of variance in community composition correlated with brain microstructure, particularly in the deep grey and white matter. This study provided the first evidence that the gut microbiota associates with common neuroimaging features of preterm brain dysmaturation.

This thesis provides evidence that general factors of diffusion MRI are useful for capturing global white matter changes associated with preterm birth; that the gut microbiota plays a role in a range of neurodevelopmental domains; and that the degree of prematurity is one of the main drivers of the preterm infant gut microbiota, which in turn may contribute to deep grey and white matter dysmaturation at term-equivalent age.

Lay summary

Around 11% of babies globally are born too early (before 37 weeks of gestation). Preterm infants are vulnerable to a range of conditions that can affect life-long health. Brain is one of the key organs affected by preterm birth and this can lead to problems with learning, motor skills and behaviour. Babies who are born too early are also exposed to microbes – bacteria, viruses and fungi – much earlier than their term-born peers. At birth, trillions of microorganisms colonise the gastrointestinal tract – termed as the gut microbiota – and these are important for many aspects of human health at all stages of life as they influence the immune system, metabolism and hormones. In recent years, researchers have additionally found that the gut microbiota associates with brain health, but less is known about this relationship in early life and in the context of brain adversities following preterm birth. This doctoral thesis presents four studies that investigate the impact of preterm birth on the development of the brain and the gut microbiota, and the relationship between these two systems.

In the first study, I investigated whether there are efficient ways to summarise the large and complicated set of diffusion MRI features. Diffusion MRI is a brain imaging technique used to study the microstructure of brain white matter bundles – the connections between different brain regions. I used statistical methods to calculate general factors which summarised the extent to which the diffusion MRI features are similar across the bundles. I found that if a baby had a 'weakness' in one of the connections, they were more likely to have weakness in others too. These general factors were associated with preterm birth, meaning that I could tell preterm babies apart from term-born peers just by observing general factors. These findings suggest that whole-brain estimates of microstructure are an efficient tool to study atypical brain development and can be used to understand causes and long-term consequences of the alterations in white matter bundles. The gut microbiota may be one of these causes as I investigated in the fourth study.

In the second study, I examined previously published literature that had reported associations between gut microbiota and brain or behaviour development in infants and children from birth up to five years of life. I found that gut microbiota correlates with brain development across many domains such as language, motor skills, temperament, social

behaviour as well as brain structure and function measured using neuroimaging methods. Despite these numerous findings, it was difficult to identify consistent bacterial signature associated with improved or impaired brain development. This could have resulted from the wide range of different methods used by different researchers. Furthermore, there was very little understanding about microbiota-brain relationships in preterm infants.

The third study characterised the microbial community composition of preterm infants shortly after birth and just before they were discharged from the neonatal intensive care unit. After birth, the microbiota community had a low diversity and had high levels of *Staphylococcus* bacteria – this composition was not too different from that observed in babies born at term. Birth mode – whether baby was born vaginally or via Caesarean section – strongly affected this composition. Before going home from the hospital, preterm baby gut microbiota had diversified and was dominated by different bacteria in different babies: *Enterobacteriaceae, Bifidobacterium, Escherichia/Shigella, Klebsiella*, or *Enterococcus.* The predominant bacterium in breastfed term infants is usually *Bifidobacterium*. The main influencers of this composition were the degree of prematurity (how young the baby was when he/she was born), antibiotics and sex. Infants who were born earlier and had been exposed to antibiotics had higher levels of *Klebsiella* which is a common pathogenic bacterium.

In the final study, I investigated whether this gut microbiota composition prior to hospital discharge associates with brain imaging findings. I found that microbiota richness and community composition correlate with the microstructural features measured using diffusion MRI. The effects were observed both in the white matter bundles, but also in the deep grey matter – brain structures in the centre of the brain that are important body's information relay stations and are involved in learning, memory and emotions. The bacteria most strongly correlated were *Escherichia/Shigella*, *Enterobacteriaceae, Klebsiella*, and *Veillonella* – these are commonly found in the preterm infant gut and have been found associated with developmental outcomes by other studies too.

This thesis shows that the gut microbiota is involved in brain development both in typical development and also following preterm birth. As the microbiota can be modified, it is an exciting avenue to promote brain health in preterm infants, but more research is needed to fully understand the linking mechanisms and design effective interventions.

Declaration of originality

I declare that this thesis is my own composition and that it has not been submitted for any other degree or professional qualification at this university or any other institution. Parts of the work comprising this thesis have been previously published as jointly-authored publications. All contributions and collaborations have been explicitly acknowledged in the text. I confirm that appropriate credit has been given within this thesis where reference has been made to the work of others.

Kadi Vaher

December 2022

Publications arising from this doctoral work

Publications presented as results chapters in this thesis

CRediT authorship contribution has been included within the respective results chapters.

Vaher, K., Galdi, P., Blesa Cabez, M., Sullivan, G., Stoye, D. Q., Quigley, A. J., Thrippleton, M. J., Bogaert, D., Bastin, M. E., Cox, S. R., & Boardman, J. P. (2022). General factors of white matter microstructure from DTI and NODDI in the developing brain. NeuroImage, 254, 119169. https://doi.org/10.1016/J.NEUROIMAGE.2022.119169

Vaher, K., Bogaert, D., Richardson, H., & Boardman, J. P. (2022). Microbiome-gut-brain axis in brain development, cognition and behavior during infancy and early childhood. Developmental Review, 66, 101038. https://doi.org/10.1016/J.DR.2022.101038

Other publications and preprints

Blesa Cábez, M.*, **Vaher, K.***, York, E. N., Galdi, P., Sullivan, G., Stoye, D. Q., Hall, J., Corrigan, A. E., Quigley, A. J., Waldman, A. D., Bastin, M. E., Thrippleton, M. J., & Boardman, J. P. (2023). Characterisation of the neonatal brain using myelin-sensitive magnetisation transfer imaging. MedRxiv. https://doi.org/10.1101/2023.02.01.23285326. *co-first authors

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Galdi, P., Blesa Cabez, M., Farrugia, C., **Vaher, K.**, Williams, L. Z., Sullivan, G., Stoye, D. Q., Quigley, A. J., Makropoulos, A., Thrippleton, M. J., Bastin, M. E., Richardson, H., Whalley, H., Edwards, A. D., Bajada, C. J., Robinson, E. C., & Boardman, J. P. (2022). Feature similarity gradients detect alterations in the neonatal cortex associated with preterm birth. BioRxiv, 2022.09.15.508133. https://doi.org/10.1101/2022.09.15.508133

Publications arising from rotation projects during the first year of the PhD programme

Bøstrand, S. M. K.*, **Vaher, K.***, Nooij, L. de, Harris, M. A., Cole, J. H., Cox, S. R., Marioni, R. E., McCartney, D. L., Walker, R. M., McIntosh, A. M., Evans, K. L., Whalley, H. C., Wootton, R. E., & Clarke, T.-K. (2021). Associations between alcohol use and accelerated biological ageing. Addiction Biology, e13100. https://doi.org/10.1111/ADB.13100. *co-first authors

Walker, R. M., **Vaher, K.**, Bermingham, M. L., Morris, S. W., Bretherick, A. D., Zeng, Y., Rawlik, K., Amador, C., Campbell, A., Haley, C. S., Hayward, C., Porteous, D. J., McIntosh, A. M., Marioni, R. E., & Evans, K. L. (2021). Identification of epigenome-wide DNA methylation differences between carriers of APOE ε4 and APOE ε2 alleles. Genome Medicine, 13, 1. https://doi.org/10.1186/s13073-020-00808-4

Walker, R. M., Bermingham, M. L., **Vaher, K.**, Morris, S. W., Clarke, T., Bretherick, A. D., Zeng, Y., Amador, C., Rawlik, K., Pandya, K., Hayward, C., Campbell, A., Porteous, D. J., McIntosh, A. M., Marioni, R. E., & Evans, K. L. (2020). Epigenome-wide analyses identify DNA methylation signatures of dementia risk. Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring, 12. https://doi.org/10.1002/dad2.12078

Presentations

Alterations in MRI biomarkers of myelination associated with preterm birth.

Organisation for Human Brain Mapping Annual Meeting, Glasgow, June 2022 (poster)

Paediatric Academic Societies Annual Meeting, Denver, April 2022 (poster)

General factors of white matter microstructure in the neonatal brain.

Neonatal Society Summer Meeting, London, June 2021 (oral talk)

International Society for Magnetic Resonance in Medicine Annual Meeting, May 2021 (virtual poster; conference abstract received the Summa Cum Laude merit award which recognises abstracts that score in the top 5% within major subject review category)

Acknowledgements

I am extremely grateful for my supervisors Professors James Boardman and Debby Bogaert for their guidance and support throughout my PhD journey. Their collective wisdom and expertise have been invaluable. It has been a privilege to work with them and be part of the friendly and collaborative teams they have set up. I would also like to thank my thesis committee chair Prof Andrew McIntosh for his insight and advice.

I thank the families participating in the Theirworld Edinburgh Birth Cohort for making the work presented in this thesis possible, and Theirworld for funding the cohort study. I also thank Wellcome for funding my PhD studies as part of the Translational Neuroscience PhD programme at the University of Edinburgh. I would like to thank the directors and coordinators of the PhD programme for a truly enjoyable and well-organised learning experience.

Throughout my studies, I have been fortunate to work alongside many talented scientists whom I am lucky to call my friends. I thank Dr Manuel Blesa for providing support in MRI data processing and for always having time for a coffee break. Dr Paola Galdi deserves a special thank you for her tireless help with all things related to statistics and code, and for the kindest words that kept me grounded during difficult times. I thank Dr Gemma Sullivan for her countless advice on all clinical topics, but also life in general. I am grateful for Gill Black, Dr Amy Corrigan and the radiographers in the Edinburgh Imaging Facilities for their superpowers in acquiring neonatal MRIs. I thank Paula Lusarreta Parga for her help with sample processing and all chats about life (but mostly sharing the frustration about the terrible public transport in Edinburgh). I am grateful for Dr Wouter de Steenhuijsen Piters for sharing his expertise in bioinformatics and data analysis, and for always approaching problems with good humour. I thank Justyna Binkowska for her support with data processing and for always having smiles and words of encouragement to share. I would like to thank all other past and present members of Boardman and Bogaert groups, both in Edinburgh and Utrecht, for their thoughts, scientific discussions and friendship. I could not have done it without you.

A very special thank you to the best thesis-writing buddy Niamh McSweeney. You (and the batch brew from the Union Brew Lab) really kept me going the past few months.

I would also like to thank my family for a lifetime of encouragement, love and support from near and from far, even without understanding the need to embark on yet another degree. Finally, I would like to express gratitude to my partner Karl-Mattias. You have seen me at my best and worst, and never lost faith in me.

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Abbreviations

3T	3 tesla
ADHD	attention deficit-hyperactivity disorder
AF	arcuate fasciculus
AIC	Akaike Information Criterion
ANOVA	analysis of variance
ASD	autism spectrum disorder
ASQ	Ages and Stages Questionnaire
ASV	amplicon sequence variant
ATR	anterior thalamic radiation
BDNF	brain derived neurotrophic factor
BIC	Bayesian Information Criterion
BPD	bronchopulmonary dysplasia
BSID	Bayley Scales of Infant Development
CB	cerebellum
CBCL	Child Behaviour Checklist
CC	corpus callosum
CCG	cingulum cingulate gyrus
CFI	comparative fit index
cGM	cortical grey matter
CRediT	Contributor Roles Taxonomy
C-section	Caesarean section
CSF	cerebrospinal fluid
CST	corticospinal tract
dGM	deep grey matter
dHCP	developing Human Connectome
dMRI	diffusion MRI
DTI	diffusion tensor imaging
ECBQ	Early Childhood Behaviour Questionnaire
EoP	encephalopathy of prematurity

FA	fractional anisotropy
FDR	false discovery rate
fNIRS	functional near-infrared spectroscopy
GA	gestational age
GABA	gamma-aminobutyric acid
GDI	Gesell Development Inventory
g-factor	general factor
IBQ	Infant Behaviour Questionnaire
IFOF	inferior fronto-occipital fasciculus
ILF	inferior longitudinal fasciculus
ISO	isotropic volume fraction
MaAsLin2	Microbiome Multivariable Associations with Linear Models
mPFC	medial prefrontal cortex
MRI	magnetic resonance imaging
MSE	Mullen Scales of Early Learning
NDI	neurite density index
NEC	necrotising enterocolitis
NMDS	non-metric dimensionality scaling
NNNS	NICU Network Neurobehavioural Scale
NNU	neonatal (intensive care) unit
NODDI	neurite orientation dispersion and density imaging
ODI	orientation dispersion index
PC	principal component
PCA	principal component analysis
PCo	principal coordinate
PCoA	principal coordinates analysis
PCR	polymerase chain reaction
PERMANOVA	permutational analysis of variance
PPROM	preterm premature rupture of membranes
qPCR	quantitative polymerase chain reaction
RCT	randomised controlled trial

RD	radial diffusivity
rRNA	ribosomal ribonucleic acid
saBIC	sample size adjusted Bayesian Information Criterion
SD	standard deviation
SRS	Social Responsiveness Scale
TEA	term-equivalent age
TEBC	Theirworld Edinburgh Birth Cohort
TLI	Tucker-Lewis index
TSS	total-sum scaling
UNC	uncinate fasciculus
WM	white matter

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Chapter 1. General introduction

The perinatal period is a critical time in central nervous system development during which preterm birth and neonatal clinical complications impose a risk of injury and dysmaturation to the developing brain, leading to increased prevalence of neurocognitive impairments among individuals born preterm. Maturation of key neurodevelopmental processes in preterm infants overlaps with the acquisition and progression of the gut microbiota, which itself is influenced by a range of perinatal factors. Increasingly, the gut microbiota is recognised to contribute to all aspects of human health, including brain development. However, it is unknown whether the gut microbiota impacts brain development after preterm birth.

This chapter provides the background and rationale for an integrative investigation of the gut microbiota and preterm brain dysmaturation. I first describe the epidemiology and outcomes of preterm birth, emphasising the neurodevelopmental sequalae of prematurity. Then, I provide an overview of the manifestation of preterm birth on brain macro- and microstructure as characterised using neuroimaging. Following, I describe the development of gut microbiota in early life and how it is affected by perinatal factors. Last, I discuss the evidence for microbiota modulation of brain and behaviour, focusing on the role of the microbiota in neurodevelopment. I end this chapter by outlining the scope and aims of this doctoral thesis.

1.1 Preterm birth

1.1.1 Epidemiology of preterm birth

Preterm birth, defined as birth before 37 weeks of gestation, is the leading cause of neonatal death (Liu et al., 2012). In 2014, the global preterm birth rate was 10.6% of all live births, equating to an estimated 14.84 million births (Chawanpaiboon et al., 2019). Preterm birth rate varies regionally; in 2020/2021 preterm birth rate in Scotland was 6.4% (Public Health Scotland, 2021). Preterm birth can be classified as extremely (birth < 28 completed weeks), very (28-32 weeks) and moderately preterm (32-37 weeks) (Blencowe et al., 2012), with around 15.5% of preterm deliveries occurring before 32 completed weeks of gestation (Chawanpaiboon et al., 2019). See Figure 1-1 for a graphical summary.

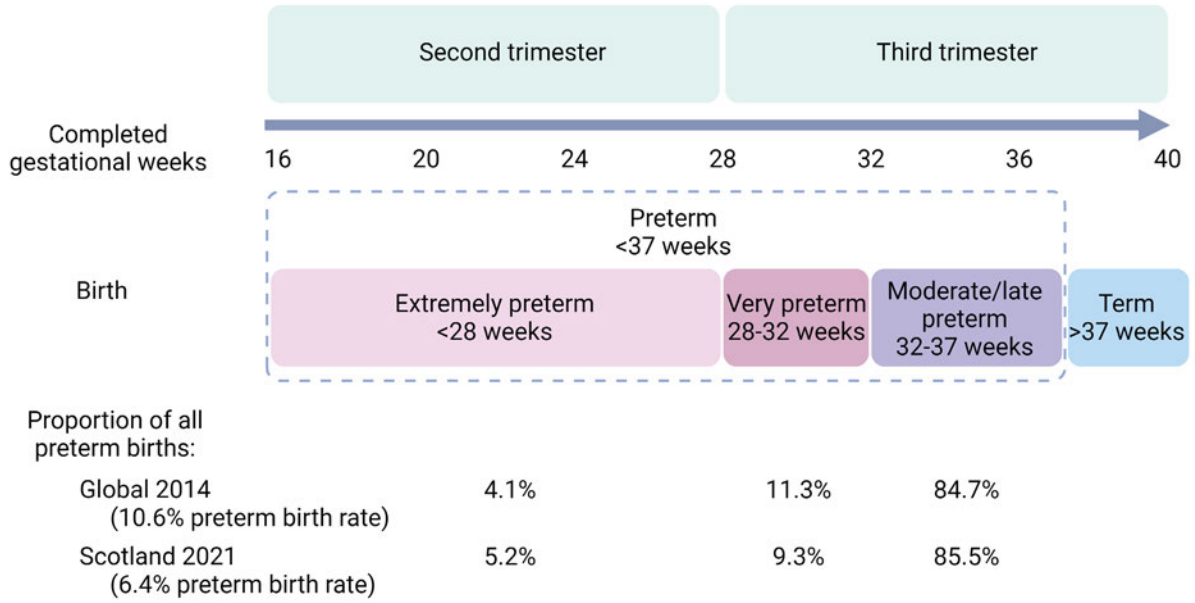


Figure 1-1. Overview of definitions and prevalence of preterm birth.
Definitions based on Blencowe et al. (2012); statistics of global preterm birth prevalence taken from Chawanpaiboon et al. (2019), and Scottish statistics are based on the most recent Births in Scotland report by Public Health Scotland (2021; Table 7.1). Figure created using Biorender.com.

Preterm birth may be provider-initiated or spontaneous. Medically-indicated preterm birth includes maternal/obstetric and foetal conditions such as pre-eclampsia, placental abruption, uterine rupture, intrauterine growth restriction, and foetal distress (Blencowe et al., 2013; Goldenberg et al., 2008). Spontaneous preterm birth, however, is a multi-factorial process and the causes remain incompletely understood, with around two-thirds of preterm births occurring without an evident risk factor (Ferrero et al., 2016). Previous preterm birth, maternal demographics such as age and body mass index, chronic disease, infection, poor nutrition, smoking, low socioeconomic status as well as psychosocial and genetic influences are some of the important risk factors for preterm birth (Blencowe et al., 2013; Ferrero et al., 2016; Goldenberg et al., 2008; Rocha et al., 2022).

1.1.1 Short- and long-term consequences of preterm birth

Despite the increase in the survival of extremely preterm infants due to advances in perinatal care (Myrhaug et al., 2019), many experience major short- and long-term health complications. Neonatal complications of preterm birth include sepsis, necrotising enterocolitis (NEC), respiratory problems such as bronchopulmonary dysplasia, and severe brain injury such as intraventricular haemorrhage (Platt, 2014; Stoll et al., 2015; Vogel et al., 2018). These conditions contribute to neonatal mortality but also have consequences on

lifelong health. For example, preterm infants are more likely to be re-hospitalised mainly due to respiratory complaints in infancy (Hannan et al., 2020), and are at an increased risk for developing asthma (Been et al., 2014), metabolic and cardiovascular disease (Markopoulou et al., 2019), and neurodevelopmental impairments (Johnson and Marlow, 2017; Wolke et al., 2019).

Neurodevelopmental deficits particularly contribute to the sequelae of preterm birth. Moderate to severe developmental disabilities, which include motor, sensory and cognitive impairments, affect 10-36% of children born very and extremely preterm, with greater rate of impairment as gestational age (GA) at birth decreases (Draper et al., 2020; Moore et al., 2012; Myrhaug et al., 2019; Pierrat et al., 2021; Serenius et al., 2013).

Cerebral palsy is the primary severe motor disorder following preterm birth, affecting 5-12% of very preterm infants (Pascal et al., 2018; Pierrat et al., 2021; Reid et al., 2016; Serenius et al., 2013; Spittle et al., 2018). Children born preterm are also at risk for developing milder motor difficulties which include deficits in coordination, balance, and gross and fine motor control, that affect around 20-26% of those born very and extremely preterm (Pascal et al., 2018; Spittle et al., 2018).

Cognitive impairments affect around 17% of very preterm infants (Pascal et al., 2018). These include an average of 12-13 points lower IQ scores (Kerr-Wilson et al., 2011; Twilhaar et al., 2018) and 0.4-0.7 standard deviations lower scores on tests of executive functioning such as working memory and processing speed (Allotey et al., 2018; Brydges et al., 2018) in preterm children and adolescents compared to their full-term peers. These deficits have important consequences on the academic performance in children born preterm (McBryde et al., 2020).

Preterm birth also has influences on mental health across the life course. Around 20% of preterm children exhibit a behavioural phenotype characterised by increased attentional and emotional problems, and difficulties with social interaction (Burnett et al., 2019; Johnson and Marlow, 2011). Additionally, the risk for neurodevelopmental and psychiatric disorders is increased. For example, compared to term-born peers, very and extremely preterm born children are three times more likely to be diagnosed with attention-deficit hyperactivity disorder (ADHD) (Allotey et al., 2018), around two to four times more likely to

be diagnosed with autism spectrum disorder (ASD) (Crump et al., 2021), and two times more likely to be hospitalised with any psychiatric disorder in adolescence and adulthood (Lindström et al., 2009).

Taken together, preterm birth increases risk for a range of adverse outcomes that persist into adulthood. Better understanding of the neurobiology and factors contributing to impaired neurodevelopment will help to identify therapeutic targets to reduce the global health burden associated with prematurity.

1.2 Preterm brain injury/dysmaturation

1.2.1 A brief overview of cellular vulnerability

The preterm period, i.e. the second and third trimesters of gestation, is a time of rapid development of many key complex cellular processes. Pre-oligodendrocytes constitute the majority of oligodendrocyte lineage until 28 weeks of gestation (Back et al., 2001). Thereafter, the pre-oligodendrocytes differentiate into postmitotic immature oligodendrocytes, which start ensheathment of white matter axons in preparation for myelination (Kinney and Volpe, 2018). This encapsulation is critical for axonal differentiation and function, which are similarly in a rapid phase of development during the preterm period. Between 20-24 weeks of gestation, thalamic afferent axons synapse on subplate neurons – the layer of cells beneath the pial surface that acts as a transient site of synaptic contact, providing axonal guidance for both ascending afferents and cortical projections – before entering the cerebral cortex between 24-32 weeks (Kinney and Volpe, 2017; Volpe, 2019). The commissural and cortico-cortico afferents follow a similar path, but in a delayed fashion. Axonal input, i.e. neuronal activity, first from the subplate neurons and then from afferent fibres from the thalamus and other cortical regions, is the driving force underlying dendritic development and synaptogenesis in the cerebral cortex (Chen and Ghosh, 2005; Volpe, 2019), resulting in increase of cortical surface area and development of sulci and gyri (Kinney and Volpe, 2017). The third trimester is also the time of gamma-aminobutyric acid (GABA)-ergic neuron migration through the white matter to the cortex (Xu et al., 2011). Microglia, the resident macrophages in the brain, heavily populate the white matter during preterm period; they have an important role for oligodendrocyte differentiation, synaptogenesis and synaptic pruning (Hammond et al., 2018). The preterm period is also a crucial time in astrocyte formation, which have multiple complex nutritive and supportive

roles in the brain (Reemst et al., 2016). In the cerebellum, the most rapid processes during the preterm period are proliferation and inward radial migration of the external granule cells (ten Donkelaar et al., 2003).

These key developmental processes are vulnerable to perturbations associated with environmental exposures following preterm birth. Preterm infants are at risk for hypoxic-ischaemic insults and inflammation, which via excitotoxic and free-radical-mediated processes can cause injury to the pre-oligodendrocytes, thalamic and subplate neurons, and the developing axons (Volpe, 2019, 2009). As part of the neuroinflammatory processes, microglia and astrocytes can further become destructive to other cells by secreting pro-inflammatory cytokines and enhancing excitotoxicity (Volpe, 2019). The pre-oligodendrocytes are particularly susceptible to these insults (Back and Volpe, 2018; Volpe et al., 2011). Although this cell population is replenished following primary injury, the subsequent differentiation into myelin-producing oligodendrocytes fails (Billiards et al., 2008; Volpe, 2019). This causes hypomyelination and a spectrum of white matter injury in the preterm brain, ranging from over cystic periventricular leukomalacia (PVL) affecting <3% of preterm infants (Van Haastert et al., 2011) to a more common diffuse white matter injury. These processes in turn lead to dysmaturation of the axons and the developing grey matter regions (Volpe, 2019). Injury to the proliferating external granule cells in the cerebellum alongside with impaired connections with the cerebrum contribute to cerebellar growth failure in preterm infants (Volpe, 2009).

In sum, preterm brain injury consists of a combination of dysmaturation of the developing white matter, cortical and deep grey matter, and cerebellum, commonly termed as encephalopathy of prematurity (EoP) (Volpe, 2009). Magnetic resonance imaging (MRI) has been valuable in characterising the manifestations of EoP which are summarised in the next section.

1.2.2 Magnetic resonance imaging of the preterm brain

MRI provides a safe, non-invasive technique for *in vivo* assessment of brain structure and function across the life-course. The small size and incomplete maturation of the neonatal brain presents specific challenges for MRI acquisition and processing. However, technical advancements in acquisition protocols, post-acquisition processing pipelines and the use of neonatal head coils have led to improved spatial resolution, signal-to-noise ratio, improved

tissue contrast, and motion correction (Batalle et al., 2018; Dubois et al., 2021). Major focal parenchymal pathologies of preterm birth such as cystic PVL, periventricular haemorrhagic infarction and cerebellar haemorrhage are readily visible on conventional structural MRI, but even in the absence of such injury, MRI studies of the preterm brain during the neonatal period have revealed both macro- and microstructural dysmaturation related to preterm birth. In the following sections, I summarise the structural and diffusion MRI phenotype of the preterm brain; principal findings are also outlined in Table 1-1.

Table 1-1. Principal MRI findings associated with preterm birth at term-equivalent age.
See text for details and references.

Imaging modality	MRI phenotype of EoP
Volumetric structural MRI	Reduced brain volume: white matter, cortical grey matter, deep grey matter Increased cerebrospinal fluid volume
Morphometric structural MRI	Reduced cortical surface area and gyrification Increased cortical thickness
Diffusion MRI	Reduced fractional anisotropy and neurite density, and increased diffusivity in the white matter Increased diffusivity and reduced neurite density in cortical grey matter

1.2.2.1 Structural MRI

MRI utilises the electromagnetic properties of hydrogen protons by measuring radio-frequency signals emitted by the protons after the application of electromagnetic field. Both the density of protons as well as the local tissue microenvironment influence the resulting contrast from each voxel. T_1 (longitudinal) and T_2 (transverse) relaxation properties form the basis of most structural MRI protocols. With T_1-weighting, which relates to the time for the hydrogen protons to return to equilibrium after radio-frequency field application (Lerch et al., 2017), fatty tissue, such as the white matter in the brain, appears bright whilst water appears dark (Paus et al., 2001). In contrast, T_2-weighting, which relates to the process by which spins are taken out of alignment with each other after the application the radio-frequency pulse (Lerch et al., 2017), causes fatty tissue to appear dark whilst water appears bright (Paus et al., 2001). In the neonatal brain, however, the water and fat content are different compared to the adult, leading to reversal of the normal contrasts between white and grey matter (Dubois et al., 2014; Paus et al., 2001).

Structural MRI is most often used in combination with tissue segmentation and anatomical parcellation which allows quantification of brain tissue volumes (total brain tissue, grey matter, white matter, cerebrospinal fluid (CSF)) as well as the volumes of specific brain regions of interest. During the last trimesters of pregnancy up until late childhood and adolescence, there is rapid growth in total and regional brain volumes (Bethlehem et al., 2022; Kersbergen et al., 2016; Makropoulos et al., 2016), but brain growth trajectories appear slower in preterm infants (Bouyssi-Kobar et al., 2016; Gui et al., 2019). At term-equivalent age (TEA; ~37-44 weeks of gestation), both global and regional brain tissue volumes are reduced in preterm infants compared to term controls, with a dose-dependent effect of prematurity (Alexander et al., 2019; Inder et al., 2005; Makropoulos et al., 2016; Padilla et al., 2015; Thompson et al., 2019). Yet, not all studies show reduced whole brain tissue volumes in preterm infants at group level (Alexander et al., 2019; Boardman et al., 2007; Thompson et al., 2019), suggesting that smaller brain is not an inevitable association of prematurity but rather driven by specific groups, e.g. those born at very low GA, with prolonged requirement for supplemental oxygen or intrauterine growth restriction (Boardman et al., 2007). In general, studies suggest reduction in the volumes of white matter (Alexander et al., 2019; Makropoulos et al., 2016), deep grey matter structures including the thalamus and basal ganglia (Ball et al., 2012; Boardman et al., 2006; Loh et al., 2017; Padilla et al., 2015; Srinivasan et al., 2007), and cortical grey matter (Alexander et al., 2019; Ball et al., 2012; Inder et al., 2005; Makropoulos et al., 2016; Padilla et al., 2015; Thompson et al., 2019) in the preterm compared to full-term infant. Cerebellum particularly has a rapid growth rate during the preterm period (Gui et al., 2019; Kersbergen et al., 2016) and some studies report smaller cerebellar volumes in preterm infants, however, the findings appear more mixed (Peterson et al., 2003; Shah et al., 2006; Srinivasan et al., 2006; Thompson et al., 2019). These reductions in tissue volumes are accompanied by increase in the CSF volume (Alexander et al., 2019; Gui et al., 2019; Makropoulos et al., 2016; Padilla et al., 2015; Thompson et al., 2019). In contrast, abnormal brain growth in preterm neonates may also be reflected by increases in the volume of some brain regions such as primary visual, motor and somatosensory cortices (Alexander et al., 2019; Thompson et al., 2019). This may be related to increased sensory input and unrestricted motor output in preterm infants during the neonatal period (Alexander et al., 2019).

Structural MRI can also be combined with surface-based processing to quantify measures related to the complexity and folding of the cerebral cortex, such as cortical thickness, surface area, sulcal depth and gyrification. The volumetric growth in early life is accompanied by increase in cortical surface area, thickness and curvature (Bethlehem et al., 2022; Dimitrova et al., 2021b; Gilmore et al., 2018; Makropoulos et al., 2016), representing increasing complexity of the cortical neurons. Preterm birth has been associated with reduced cortical surface area (Makropoulos et al., 2016), increased thickness (Dimitrova et al., 2021b) and reduced folding/gyrification at TEA (Shimony et al., 2016), which together with reduced cerebral cortex volume suggest altered cortical development in infants born preterm.

1.2.2.2 Diffusion MRI

Diffusion MRI (dMRI) probes tissue microstructure by measuring random diffusion of water molecules due to thermal energy. With this technique, by applying a range of different magnetic gradient directions, we can establish the preferential direction of water diffusion in a given voxel (Le Bihan, 2003). In an environment without restrictions such as the CSF, the displacement of water molecules is equal in all directions, i.e. isotropic, and water molecules will change direction due to their collision with other molecules. In a highly structured tissue such as the brain, water diffusion is restricted by axons, neuronal cell bodies, and other cells and macromolecules, making the diffusion directionally dependent, i.e. anisotropic (Pecheva et al., 2018; Figure 1-2A). This in turn enables inference about water content, axonal density, myelination and other aspects of brain microstructure.

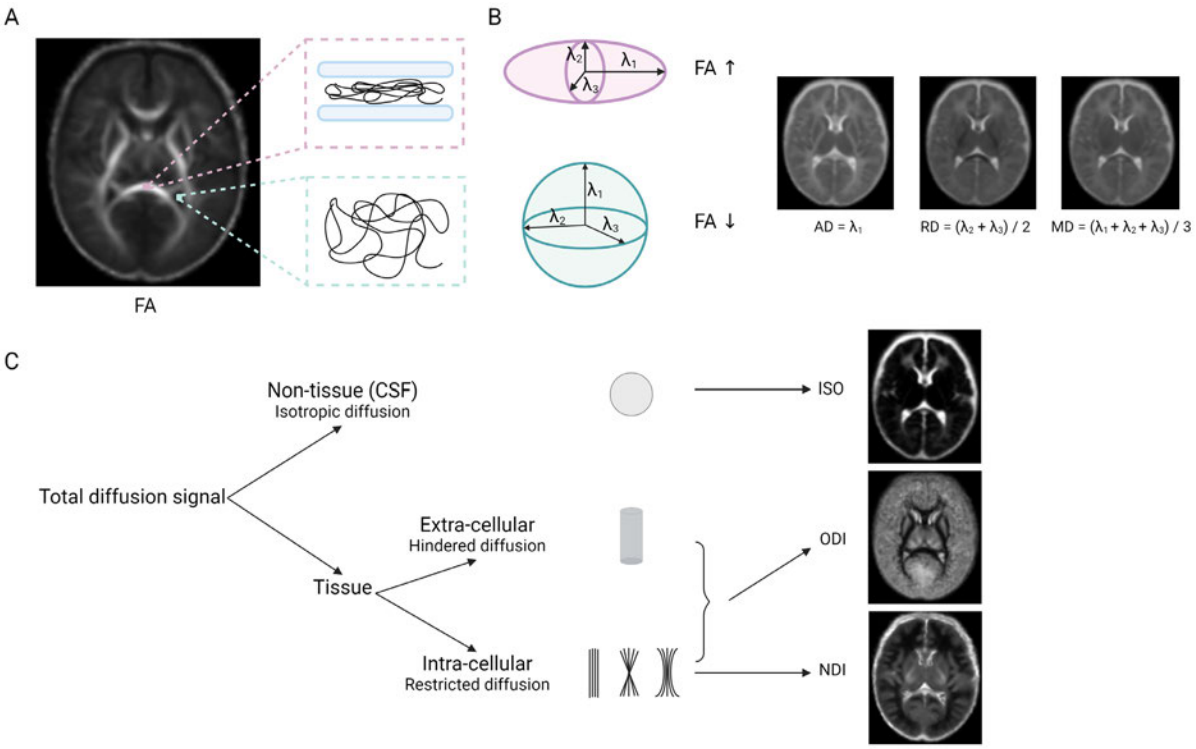


Figure 1-2. Diffusion MRI.
(A) Isotropic (green) and anisotropic (pink) diffusion in the brain. (B) Diffusion tensor ellipsoids with three principal axes of diffusion representing the voxels with anisotropic and isotropic diffusion alongside with representative brain maps of axial (AD), radial (RD) and mean (MD) diffusivities; representative fractional anisotropy (A) map has been provided in (A). (C) Neurite orientation dispersion and density imaging biophysical model alongside with representative brain maps of isotropic volume fraction (ISO), orientation dispersion index (ODI) and neurite density index (NDI). Brain maps were created by averaging the images of 20 preterm infants scanned at term-equivalent age. Adapted from (Pecheva et al., 2018; Rae et al., 2017; Tariq et al., 2016)*. Figure created using Biorender.com.*

The diffusion tensor imaging (DTI) model can be used to describe water diffusion in a 3D matrix (Basser et al., 1994), visualised as an ellipsoid (Figure 1-2B). DTI measures the magnitude of diffusion along the three perpendicular axes and a number of metrics can be derived. Axial diffusivity (AD) represents water diffusivity along the main fibre orientation; radial diffusivity (RD) is calculated as the average of the two axes perpendicular to the main axis, describing diffusivity across the fibres; mean diffusivity (MD) is the average of previous two, describing the magnitude of overall diffusivity within a voxel; and fractional anisotropy (FA) quantifies the directionality of water diffusion (Pierpaoli and Basser, 1996). FA takes values between 0 and 1, where 0 represents isotropic diffusion such as in free water, and 1 represents diffusion only in one direction.

Whilst DTI remains widely used to model brain microstructure, the metrics are not specific to biologically interpretable tissue properties and can be difficult to interpret in the

presence of multiple or crossing fibre populations (Tournier et al., 2011). To overcome these limitations, a number of biophysical models have been proposed, such as the neurite orientation dispersion and density imaging (NODDI; Zhang et al., 2012). NODDI model divides the diffusion signal into three compartments (Figure 1-2). The isotropic volume fraction (ISO) represents the non-tissue compartment where water diffuses freely, such as the CSF. The intra-neurite compartment represents highly restricted diffusion perpendicularly but unhindered diffusion along neurites, i.e. axons and dendrites. The extra-neurite compartment represents hindered/anisotropic diffusion within neuronal cell bodies. In addition to ISO, which captures water/CSF content within a voxel, NODDI provides metrics of neurite density index (NDI) and orientation dispersion index (ODI) to describe the neurite density and geometrical organisation of neurites, e.g. crossing fibres and fanning, respectively.

In the white matter, metrics derived from DTI and NODDI can be compared across individuals at every voxel alignment using techniques such as tract-based spatial statistics (TBSS (Smith et al., 2006)), or in combination with tractography-based white matter tract segmentation for tract-based analysis (e.g. Grotheer et al., 2022; Wakana et al., 2007). Applying these methods, studies have demonstrated that across the white matter FA and NDI increase, diffusivity metrics decrease and ODI remains relatively stable with increasing age at scan during the neonatal period, reflecting increasing axonal density/packing and myelination/axon ensheathment (Hüppi et al., 1998; Kersbergen et al., 2014; Kimpton et al., 2021; Partridge et al., 2004). At TEA, preterm infants have lower FA and NDI and increased MD and RD in much of the white matter, with a dose-dependent effect of prematurity (Anblagan et al., 2015; Anjari et al., 2007; Ball et al., 2010; Barnett et al., 2018; Batalle et al., 2017; Dibble et al., 2021; Thompson et al., 2019). These changes are interpreted as decreased white matter integrity associated with lower neurite density, (pre-)myelination and higher water content in the preterm brain.

dMRI has also been used to characterise the microstructural properties of the developing grey matter. Imaging studies during the preterm period have demonstrated that both FA and MD decrease, whilst ODI increases in the cortex until 38 weeks of gestation, reflecting increased dendritic arborisation (Ball et al., 2013b; Batalle et al., 2019; Eaton-Rosen et al., 2015). Interestingly, NDI appears to reduce in some cortical regions until 38 weeks of

gestation; thereafter, NDI increases while FA and ODI remain relatively stable, reflecting rapid increase in neuronal and organelle density (Batalle et al., 2019). In contrast, in term born infants, there appears to be continuous decrease in FA and MD, and increase in NDI and ODI in many parts of the cortex between 37-44 weeks of gestation (Dimitrova et al., 2021b). At TEA, preterm infants have higher diffusivity and lower NDI in many parts of the cortex (Bouyssi-Kobar et al., 2018; Dimitrova et al., 2021), while ODI and FA appear less affected at group level (Bouyssi-Kobar et al., 2018; Dimitrova et al., 2021b).

The deep grey matter, composed of structures including the thalamus and basal ganglia which relay and modulate information to and from the cortex, also undergoes microstructural changes during the preterm and early post-term period. Similarly to the white matter, FA increases and diffusivity decreases with age in the deep grey matter structures (Eaton-Rosen et al., 2015; Melbourne et al., 2016; Poh et al., 2015; Qiu et al., 2013). In addition, NDI increases during this time while ODI remains constant (Eaton-Rosen et al., 2015; Melbourne et al., 2016). These findings together suggest increasing neurite density and myelination in the deep grey matter structures. As described above, reduced deep grey matter volume is a common hallmark of preterm brain dysmaturation, but less is known about the impact of prematurity on the deep grey matter microstructural measures at TEA. Interestingly, reduced thalamic volume at TEA associates with increased thalamic diffusivity, suggesting larger extracellular space and reduced cellular density in the thalamus; thus, this microstructural change may underly the volumetric observations (Ball et al., 2012).

1.2.2.3 Predictive value of neonatal neuroimaging for long-term outcomes

Brain imaging of the preterm infants at TEA has demonstrated correlations between the MRI phenotype of EoP and later neurodevelopmental outcomes (Anderson et al., 2015). Both global and regional, such as the cerebellar and hippocampal, brain tissue volumes at TEA associate with cognitive and motor outcomes in childhood (Gui et al., 2019; Keunen et al., 2016; Thompson et al., 2008; Van Kooij et al., 2012a). The deep grey matter structure volumes have especially shown predictive value for motor outcomes (Kline et al., 2020b; Loh et al., 2017). Morphometric characteristics of the preterm neonate cortex, mainly cortical surface area and curvature, also correlate with outcomes (Kapellou et al., 2006; Kline et al., 2020a, 2020b). Furthermore, white matter microstructure at TEA, particularly lower FA

values in several white matter regions, such as the corpus callosum, fornix, cingulum, the corticospinal tract and inferior longitudinal fasciculus correlate with poorer cognitive, language and motor outcomes in toddlers born preterm (Barnett et al., 2018; Counsell et al., 2008; Dubner et al., 2020), and predict working memory at 5 years of life (Ullman et al., 2015). A common neonatal image phenotype of preterm birth comprised of diffuse white matter injury and tissue loss in the deep grey matter structures associates with lower developmental quotient at two years of life (Boardman et al., 2010).

The MRI signatures of reduced brain volumes, altered cortical structure and reduced white matter integrity persist into childhood and adulthood (Hadaya and Nosarti, 2020; Li et al., 2015; Loh et al., 2020; Meng et al., 2016; Nagy et al., 2011, 2009; Peterson et al., 2000; Young et al., 2019), suggesting long-term effects of preterm birth on brain structure. These characteristics also continue to have long-lasting correlations with cognitive measures (Dodson et al., 2018; Dubner et al., 2019; Hadaya and Nosarti, 2020; Nosarti et al., 2008; Papini et al., 2020).

Collectively, studies demonstrate that the MRI phenotype of EoP contributes to the neural basis of subsequent adverse neurodevelopmental outcome and that these measures may provide potential biomarkers for identifying those most at risk for neurodevelopmental adversity.

1.2.2.4 Diffuse white matter injury as a global phenomenon

Increasingly, it is recognised that the variance in water diffusion parameters is shared across the different white matter tracts across the life course (Cox et al., 2016; Girault et al., 2018; Penke et al., 2010), motivating the search for measures of global white matter integrity to capture diffuse white matter injury following preterm birth. For example, histogram-based peak width skeletonised metrics, which describe the variance in dMRI metrics within the white matter skeleton (Baykara et al., 2016), have demonstrated predictive value for differentiating between term and preterm infants at TEA (Blesa et al., 2020) and for language outcome two years later (Valavani et al., 2021). In addition, dimensionality reduction approaches, i.e. those based on principal component or factor analysis to calculate general factors, may be particularly useful in describing global white matter microstructure. The general factors of DTI metrics have shown utility in predicting cognitive abilities both in adults and children (Alloza et al., 2016; Lee et al., 2017; Penke et al., 2010),

and capturing diffuse white matter injury in preterm neonates (Telford et al., 2017). However, prior to this doctoral work, it was not known whether the NODDI metrics similarly share variance across neonatal white matter tracts.

Furthermore, the dMRI metrics themselves are highly correlated, suggesting they share overlapping information (Chamberland et al., 2019; De Santis et al., 2014; Geeraert et al., 2020). Recently, dimensionality reduction was applied to multimodal MRI which allowed to derive biologically-interpretable general factors that associated with age in late childhood and adolescence (Chamberland et al., 2019; Geeraert et al., 2020). Prior to this thesis, it was unknown whether such shared variance between the different DTI and NODDI metrics already exists in the neonatal brain and whether this approach may be useful in characterising preterm brain dysmaturation.

1.3 Gut microbiota in early life

The human body hosts a vast population of microorganisms including bacteria, archaea, viruses and fungi – collectively referred to as the microbiota. Bacteria are the highest in biomass, with an estimated ratio of 1.3 bacterial cells for every 1 human cell (Sender et al., 2016). To date, bacteria remain the most studied aspect of the microbiota and hereafter microbiota refers mainly to bacteria. The gastrointestinal tract particularly contains a high density and variety of microbes. Early life gut microbiota is increasingly recognised to play an important role in health (Sarkar et al., 2021). Gut microbiota has vital contributions to metabolism (Fan and Pedersen, 2020), immune maturation/response (D. Zheng et al., 2020), and hormonal regulation (Neuman et al., 2015). Imbalances in the early life gut microbiota community composition, referred to as dysbiosis, correlate with disease such as asthma (Stokholm et al., 2018), recurrent infections (Reyman et al., 2019), and childhood obesity (Korpela et al., 2017b). In the following sections, I summarise the development of the gut microbiota in infancy and how it is affected by perinatal covariates, focussing on the neonatal period in preterm infants.

1.3.1 Developing gut microbiota

In contrast to the long-standing hypothesis that the womb is sterile, some studies have suggested that infants acquire microorganisms *in utero* by demonstrating the detection of bacterial sequences in the placenta, amniotic fluid as well as the foetal meconium (Aagaard

et al., 2014; Collado et al., 2016; Rackaityte et al., 2020). More recent work has challenged these findings and emphasised contamination, lack of controls and batch effects as accounting for the effects, supporting the sterile womb hypothesis (Blaser et al., 2021; de Goffau et al., 2021; Lim et al., 2018; H. C. Wang et al., 2022). Regardless, it is widely accepted that the infant microbiome is rapidly populated at birth.

The infant gut microbiota develops rapidly during the first 2-5 years of life when it starts to resemble adult microbiota (Roswall et al., 2021; Yatsunenko et al., 2012). At birth, the neonate body is seeded by a homogeneous microbiome derived from maternal and environmental microbes and by 6 weeks of life, the infant microbiota structure has diversified with specific bacterial community compositions at different body sites (Chu et al., 2017). The infant gut is initially colonised by a simple community with low species complexity and high abundance of facultative anaerobes such as *Escherichia, Staphylococcus* and *Enterobacteriaceae* (Bäckhed et al., 2015; Bokulich et al., 2016; Chu et al., 2017; Ferretti et al., 2018; Koenig et al., 2011; Reyman et al., 2019; Yassour et al., 2018). This is followed by a rapid expansion of obligate anaerobes in the first weeks and months of life, when the gut microbiota is particularly dominated by *Bifidobacterium*, but the abundances of *Bacteroides* and *Clostridium* also increase (Bäckhed et al., 2015; Bokulich et al., 2016; Ferretti et al., 2018; Koenig et al., 2011; Reyman et al., 2019). As the infant matures and solid foods are introduced, the gut microbiota diversifies and starts to resemble adult-like microbiota around 3-4 years of age with continuously high abundances of *Bacteroides*, increasing levels of *Faecalibacterium, Prevotella* and *Ruminococcus,* and gradual decrease of *Bifidobacterium* (Bäckhed et al., 2015; Bokulich et al., 2016; Roswall et al., 2021; Stewart et al., 2018). For illustration of the dynamic microbiota changes in infancy and early childhood, see bottom panel of Figure 4-1. The gut microbiota continues to develop throughout childhood and adolescence (Derrien et al., 2019).

1.3.2 Gut microbiota development in preterm infants

Similarly to brain development, the very preterm infant gastrointestinal tract maturation is still ongoing at the time of birth. The preterm infant gut intestinal epithelium has increased permeability and decreased mucus production, leading to reduced epithelial barrier integrity (Healy et al., 2022; Henderickx et al., 2019). The gut epithelium is an important interaction point between the host and the microbes; this interaction impacts on the

development of the gut and immune system as well as affects nutrient digestion (Henderickx et al., 2019). The immature intestine of the preterm infant may also influence the development of beneficial symbiotic interaction between the host and the microbiota, leading to gut bacterial dysbiosis, which in turn can influence health. Indeed, understanding the development of preterm infant microbiota and how it contributes to long-term health outcomes has been recognised as a research priority (Groer et al., 2014).

Microbial succession in the new-born gut is significantly affected by GA, i.e. physical maturity. Longitudinal profiling of the preterm infant gut during the preterm period has revealed a patterned progression of the microbiota (Korpela et al., 2017a; La Rosa et al., 2014). In the earliest phase, the microbiota, similarly to term-born infants, is dominated by facultative anaerobes (*Bacilli*, such as *Staphylococcus, Enterococcus*). This stage is followed by the expansion of *Gammaproteobacteria (Enterobacteriaceae)*, and then by *Clostridia* or *Bifidobacterium*. The rate of this progression is affected by GA at birth so that the most premature infants have slowest progression of gut microbiota communities (Grier et al., 2017). Interestingly, shorter gestation associates with delayed acquisition of *Bifidobacterium*-rich microbiota even among normal range of gestational duration (Dogra et al., 2015).

Whilst the developmental pattern of gut microbiota in preterm infants over the neonatal period has extensively been described (e.g. see Beck et al., 2022; Rao et al., 2021), fewer studies have directly compared the differences in preterm gut microbiota with that of a healthy term baby. Comparison of term and preterm infants born at different GA-s has revealed a positive correlation between GA at birth and bacterial diversity during the first week of life (Chernikova et al., 2018; Dahl et al., 2018). Lower bacterial diversity in preterm infants may persist into early childhood (Fouhy et al., 2019; Toubon et al., 2022). Compositionally, in the first months of postnatal life, the preterm infant gut harbours lower levels of *Bifidobacterium* and *Bacteroides*, whilst members of *Proteobacteria* and potentially pathogenic taxa such as *Klebsiella* and *Enterococcus* are present in higher abundance (Arboleya et al., 2015, 2012; Chernikova et al., 2018; Dahl et al., 2018; Hill et al., 2017; Figure 1-3). There is considerable inter-individual variation in the microbiota composition in very and extremely preterm infants, which is initially dominated by *Staphylococcus*, and then transitions into communities dominated by *Klebsiella, Escherichia,* or *Enterococcus*,

whilst many infants are still able to transition into a *Bifidobacterium*-dominated gut microbiota (Beck et al., 2022; Rao et al., 2021; Figure 1-3).

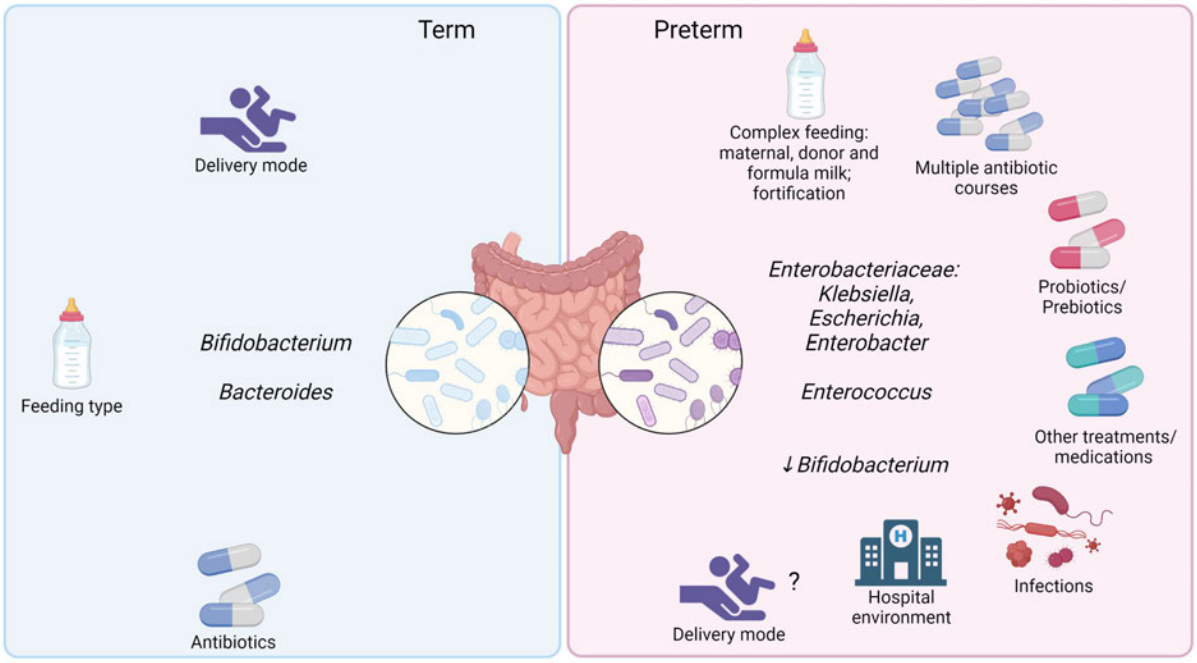


Figure 1-3. Gut bacterial communities and main factors affecting the assembly in term and preterm infants. *Figure created using Biorender.com.*

1.3.3 Factors affecting gut microbiota development in preterm neonates

The early life gut microbiota is dynamic and susceptible to a range of environmental influences. Compared to their term-born peers, preterm infants are exposed to a range of adverse medical, dietary and environmental influences that can impact on the microbiota development (Henderickx et al., 2019; Figure 1-3).

Detection of microbial metabolites in the foetal intestine suggests that foetal development is affected by microbes already before birth (Li et al., 2020). During pregnancy, the bacterial community structure of the maternal vaginal microbiota shifts and is typically dominated by one or two species of *Lactobacillus* (DiGiulio et al., 2015; Romero et al., 2014). Dysbiosis of the vaginal microbiota, particularly increased diversity and depletion of *Lactobacillus* species, associates with maternal inflammatory response, preterm prelabour rupture of foetal membranes (PPROM), and subsequent preterm birth as well as increased risk for neonatal sepsis (Brown et al., 2019, 2018; Chan et al., 2022; Huang et al., 2022). Vaginal dysbiosis may thus influence the early colonisation of the preterm neonate via mother-infant vertical transmission (S. Wang et al., 2020). Furthermore, PPROM and

chorioamnionitis, which precede 25-40% of preterm births (Amuel et al., 1998; Goldenberg et al., 2008), may directly expose the foetus to pathobionts (Chernikova et al., 2016). This body of research thus suggests that preterm infants may be exposed to adverse microbial influences already *in utero* and from the first moments of postnatal life. Interestingly, increased bacterial signal in the meconium of very preterm infants has been reported (Ardissone et al., 2014), suggesting early exposure to microbes.

Antibiotic exposure directly impacts the gut bacteria. Women with PPROM and at risk for preterm labour are routinely prescribed wide-spectrum antibiotics, which associate with prolonged pregnancy and reductions in chorioamnionitis and neonatal morbidities including infection, need for oxygen therapy and abnormal cerebral ultrasound (Kenyon et al., 2013). Erythromycin is a recommended antibiotic for PPROM (NICE, 2015), however, it may also exacerbate vaginal dysbiosis (Brown et al., 2018). Co-amoxiclav prescription, on the other hand, associates with increased incidence of NEC (Kenyon et al., 2013), suggesting adverse outcomes following perinatal manipulation of the microbiota. Nevertheless, assessment of the direct impact of prenatal antibiotics on the preterm infant microbiota is complicated as the majority receive postnatal antibiotics within first 24 hours of life for the treatment of (suspected) early-onset sepsis (Flannery et al., 2018).

Studies in both term and preterm infants suggest that early postnatal antibiotic exposure results in a large shift in microbiota community composition immediately following treatment, followed by a gradual recovery (Korpela et al., 2017a; Reyman et al., 2022). Evidence converges on reduced bacterial diversity and abundances of *Bifidobacterium* and *Bacteroides*, and increase of *Enterobacteriaceae*, including *Klebsiella* and *Enterobacter*, in association with neonatal antibiotic treatment in term and preterm infants (Arboleya et al., 2015; Bokulich et al., 2016; Chernikova et al., 2018; Fjalstad et al., 2018; Fouhy et al., 2012; Gasparrini et al., 2019; Greenwood et al., 2014; Reyman et al., 2022; Van Daele et al., 2022). In addition, exposure to antibiotics correlates with increased antimicrobial resistance genes (Fjalstad et al., 2018; Gibson et al., 2016; Reyman et al., 2022). Interestingly, however, a recent randomised control trial in preterm infants found no significant effects of antibiotic treatment on gut microbiota diversity or community composition (Russell et al., 2021). Some studies suggest that length of antibiotic treatment may further impact microbiota development. For example, long (> 5 days) compared to short (< 4 days) antibiotic

treatment associated with prolonged reduction of *Bifidobacterium* (Zwittink et al., 2018), decreased diversity and increased abundance of *Enterobacter* in preterm neonates (Greenwood et al., 2014). However, this effect is negligible compared to the initiation of antibiotics in the first place (Reyman et al., 2022). Preterm infants are often exposed to multiple courses of antibiotics during their neonatal unit stay, which may each exacerbate the dysbiosis.

Delivery mode is one of the most important factors influencing the initial gut microbiota colonisation in term-born infants in infancy and during the first year of life (Bäckhed et al., 2015; Reyman et al., 2019; Shao et al., 2019). Whilst vaginally delivered infant microbiota resembles their mothers' vaginal and rectal microbiota, Caesarean section (C-section) born infants are initially colonised with bacteria derived from skin and hospital surfaces (Bäckhed et al., 2015; Dominguez-Bello et al., 2010; Mitchell et al., 2020; Shao et al., 2019). These studies have demonstrated that C-section delivery associates with reduced abundances of *Bacteroides*, *Bifidobacterium* and *Escherichia*, and increased abundances of potentially pathogenic bacteria such as *Enterococcus* and *Klebsiella*. The differences gradually dissipate with age over the first year of life (Bäckhed et al., 2015; Reyman et al., 2019; Shao et al., 2019), while some suggest that the effect of delivery mode on gut microbiota may be visible for up to 4 years of life (Fouhy et al., 2019). The rate of C-section delivery is higher in preterm birth (Delnord et al., 2014). However, in contrast with term-born infants, increasing evidence suggests that delivery mode has minimal influences on gut microbiota diversity and composition in this population (Arboleya et al., 2015; Beck et al., 2022; Chernikova et al., 2018; Grier et al., 2017; Korpela et al., 2017a; Rao et al., 2021; Stewart et al., 2017a).

In term-born infants, compared to delivery mode, breastfeeding has a smaller effect on gut microbiota composition directly after birth, but it becomes the most significant driver from three months of life (Bäckhed et al., 2015; Shao et al., 2019; Stewart et al., 2018). Breastfed compared to formula-fed infants have increased abundances of *Bifidobacterium* and *Lactobacillus*, and decreased abundances of *Clostridium, Escherichia* and *Enterobacteriaceae* (Bäckhed et al., 2015; Bokulich et al., 2016; Reyman et al., 2019; Shao et al., 2019; Stewart et al., 2018). Cessation of breastfeeding has been demonstrated to have profound effects in shifting the microbiota towards a more adult-like composition around 1 year of life (Bäckhed et al., 2015; Stewart et al., 2018).

Whilst most studies in preterm infants report statistically significant differences in microbiota diversity and community composition between neonates receiving different milk types, there is less consistency in the reported effects (Aguilar-Lopez et al., 2021a). This may be due to the variety of feeding strategies the infants are exposed to during neonatal unit care to meet the nutritional requirements (Aguilar-Lopez et al., 2021a; Henderickx et al., 2019). Mother's own breast milk is the preferred source of nutrition for preterm infants, however, when the volumes are insufficient, pasteurised donor human milk is often recommended to avoid NEC (Quigley et al., 2018). Some studies have demonstrated differences between the microbiota of mother versus donor milk fed preterm infants. For example, infants fed mother's own breast milk have higher abundances of *Bifidobacterium* and lower abundances of *Pasteurellaceae* compared to infants fed donor milk (Kumbhare et al., 2022; Parra-Llorca et al., 2018; Piñeiro-Ramos et al., 2021). Yet, in comparisons with formula-fed infants, mother and donor milk groups are often combined due to small sample sizes (e.g. Aguilar-Lopez et al., 2021b; Gibson et al., 2016). Furthermore, the nutritional content of breast milk differs between mothers of term and preterm infants (Gidrewicz and Fenton, 2014) and milk is often fortified with macro- and micronutrients to achieve adequate growth (Brown et al., 2020; Örs, 2013). Several studies have demonstrated significant effects of fortification as well as the type of fortification (human- or bovine-milk-based) on preterm infant gut microbiota (Aguilar-Lopez et al., 2021b; Asbury et al., 2022). However, a recent study suggested that the source of human milk (mother or donor) is more important that the type of fortifier (Kumbhare et al., 2022). In sum, studying the effects of milk types on preterm gut microbiota development is difficult due to exposure to a range of different feeds. Randomised controlled trials (RCT) are needed to determine the best milk (donor vs formula) to use when maternal supply does not meet the nutritional needs of the baby, and embedded mechanistic multi-omic studies within such trials are likely to be informative (e.g. Young et al., 2022).

Some neonatal units across the world include probiotic administration as part of the standard care for infants born preterm because of a possible class-effect of multi-strain probiotics for reducing late-onset sepsis and NEC, ascertained by systematic review of clinically and statistically heterogeneous studies, though certainty of evidence is low (Poindexter et al., 2021; Sharif et al., 2020). Strains of *Bifidobacterium* and *Lactobacillus* are

the main constitutes in probiotics. Where administered, probiotics are the main drivers of gut microbiota composition in very and extremely preterm infants (Beck et al., 2022; Samara et al., 2022). *Bifidobacterium* strains have particularly shown to have prolonged persistence in the gut (Beck et al., 2022; van Best et al., 2020). Probiotic exposure in preterm infants associates with increased stability of the microbiota, accelerated maturation of the gut microbiota to a *Bifidobacterium*-dominated profile, and reduced levels of potentially pathogenic bacteria such as *Klebsiella* (Samara et al., 2022; van Best et al., 2020).

Very and extremely preterm infants are initially cared for in neonatal intensive care units. Neonatal units harbour a diverse microbiota comprised of human skin, oral and gut bacterial taxa (Bokulich et al., 2013; Brooks et al., 2018, 2014). Neonatal units may therefore act as a reservoir for bacterial colonisation in preterm neonates. This has been demonstrated by the overlap of specific bacterial strains, such as *Staphylococcus, Enterococcus,* and *Klebsiella,* between preterm infants and hospital surfaces (Brooks et al., 2018, 2014), the high number of shared strains of *Staphylococcus, Enterococcus*, and *Clostridium* between hospitalised preterm infants born at similar times (Olm et al., 2021), as well as the detection of probiotic species in the stools of infants not administered probiotics or before deliberate administration in neonatal units where probiotics are in use (‘unit cross-contamination’) (Beck et al., 2022; Costeloe et al., 2016b; Hickey et al., 2014; Kitajima et al., 1997). Moreover, a recent study showed that hospital site along with postnatal age was the main driver of gut microbiota as opposed to the prebiotic lactoferrin in a RCT (Young et al., 2022).

1.3.4 Gut microbiota in association with neonatal morbidities in preterm infants

Gut microbial dysbiosis in very and extremely preterm infants may increase the risk of infections and inflammatory processes (Groer et al., 2014). Indeed, gut microbiota associates with life-threatening conditions among very preterm infants including NEC and sepsis (Masi and Stewart, 2019). NEC affects around 7% of very preterm infants and has a mortality rate of 15-30% (Alsaied et al., 2020; Lin and Stoll, 2006). Although the pathogenesis of NEC remains unclear, inflammation in response to aberrant bacterial colonisation, rather than an infection from a specific causative pathogen, is hypothesised as an integral part in the disease (Claud and Walker, 2001; Masi and Stewart, 2019; Neu and Pammi, 2018). There is some converging evidence that increased abundance of *Proteobacteria*, especially *Gammaproteobacteria*, and decreased abundance of anaerobic

bacteria, especially those belonging to the phylum *Firmicutes* or *Bacteroidetes*, associate with NEC (Lindberg et al., 2020; Pammi et al., 2017; Warner et al., 2016). Others have suggested no specific microbial signature associated with NEC, but that higher abundance of *Bifidobacterium* may protect from the disease (Stewart et al., 2016). Late-onset neonatal sepsis, which affects 20-35% of extremely preterm neonates (Greenberg et al., 2017; Stoll et al., 2002), has also been associated with relocation of microorganisms from the gut to the bloodstream. This has been demonstrated by the dominance of the causative bacteria, including species of *Staphylococcus, Enterococcus, Streptococcus, Enterobacteriaceae* and *Escherichia,* in the gut prior to or at the time of diagnosis (El Manouni El Hassani et al., 2021; Shaw et al., 2015; Stewart et al., 2017b; Taft et al., 2015). Reductions in alpha diversity and in the levels of obligate anaerobes and *Bifidobacterium* also associate with sepsis diagnosis (Mai et al., 2013; Shaw et al., 2015; Stewart et al., 2017b).

To summarise, the gut microbiota of preterm infants follows a patterned developmental trajectory driven mainly by physical maturity. Challenges associated with preterm birth such as exposure to neonatal unit environment, antibiotics and different feeding strategies further influence the assembly of the microbiota community, but the effects vary between studies. Gut dysbiosis may in turn contribute to neonatal inflammatory and infectious conditions such as NEC and sepsis, which influence the mortality and long-term morbidities among preterm infants, although greater mechanistic understanding is required.

1.4 Microbiota-gut-brain axis – evidence of microbiota modulation of brain development

Increasing evidence from preclinical and human observational studies suggests a role for the gut microbiome in modulating brain and behaviour (for comprehensive reviews see Cryan et al., 2019; Spichak et al., 2021). Pathways of communication between the microbiota and the brain include neuronal signalling via the vagal nerve, modulation of immune system and inflammation, release of microbial metabolites and neurotransmitters, and modulation of the intestinal and blood-brain barrier function (Cowan et al., 2019; Cryan et al., 2019).

Infancy is a period of rapid development of both the brain and the gut microbiota. The recognition of the parallel development of these two system has led to the hypothesis of ‘nested sensitive periods’ when brain development interacts with gut microbiota

development to shape cognition and behaviour (Borre et al., 2014; Callaghan, 2020; Cowan et al., 2019). Perturbations of the gut microbiota in early life can therefore impact neurodevelopment and lead to adverse outcomes. In preterm infants, several critical neurodevelopmental processes discussed in section 1.2.1, which occur *in utero* for term infants under limited influence of bacteria, can be shaped by early exposure to environmental microbes and colonisation of the microbiota. Indeed, the gut microbiota may contribute to the pathogenesis of preterm brain injury/dysmaturation as suggested in recent review papers (He et al., 2022; Lu and Claud, 2018). In the following sections, I summarise the research on the role of gut microbiota in brain development.

1.4.1 Human observational studies

Evidence for correlations between early life microbiota diversity or community composition and brain development in human observational studies can in general be drawn from three types of research. First, case-control studies have identified altered microbiota profiles in individuals with neurodevelopmental disorders. Second, important drivers of the microbiota composition in infancy, such as delivery mode, breastfeeding and antibiotics, associate with cognitive outcomes and brain structure as assessed using MRI. The third line of evidence involves direct correlational analyses between neurodevelopmental outcomes and early life gut microbiota assessed using metagenomic sequencing.

1.4.1.1 Microbiota alterations in neurodevelopmental disorders

Increasing interest for the microbiota dysbiosis in neurodevelopmental disorders is exemplified by the large number of (systematic) reviews and meta-analyses published on this topic in the past 3-5 years. Given the gastrointestinal symptom comorbidities in children with ASD, gut bacteria compositional alterations have extensively been studied. Although most studies agree on overall differences in the microbiota community composition between neurotypical and autistic children, there is a large discrepancy in the specific bacterial differences reported and it is difficult to identify a clear 'microbiota signature of ASD' (Andreo-Martínez et al., 2022; Bundgaard-Nielsen et al., 2020; Iglesias-Vázquez et al., 2020; Soltysova et al., 2022; Xu et al., 2019). This inconsistency may be explained by the wide age range of children included in individual studies as well as in synthesis, and variation in the methods for measuring the microbiota (e.g. culture, polymerise chain reaction and sequencing methods have all been used), among other methodological

heterogeneities. Furthermore, it remains to be determined whether the microbiota alterations observed may be caused by differences in diet between autistic and neurotypical children, although a recent study suggested that diet had negligible effect on children's microbiota compared to chronological age and ASD diagnosis (Wan et al., 2022). Similarly, inconsistent findings have been reported in studies investigating the gut microbiota composition differences in children and adolescents with ADHD (Bundgaard-Nielsen et al., 2020; Gkougka et al., 2022; N. Wang et al., 2022). In sum, children with neurodevelopmental disorders have altered microbiota compositions, but exact bacterial signatures that are consistently altered across studies have not been identified.

1.4.1.2 Indirect evidence of microbiota association with brain development

Breast milk has recognised long-lasting beneficial effects for infant development. In particular, breastfeeding associates with improved performance in intelligence and general cognitive ability in childhood and adolescence even after controlling for maternal IQ and other confounding factors (Horta et al., 2015; Lopez et al., 2021). This effect appears to be mediated through brain structure. For example, breastfeeding in infancy associates with larger grey matter volumes in the cortex and subcortical regions in childhood (Luby et al., 2016; Núñez et al., 2022) and improved white matter myelination throughout infancy and early childhood (Deoni et al., 2018, 2013). Preterm infants may particularly benefit from increased exposure to human milk. At TEA, increased breast milk intake during the neonatal period in preterm infants associates with improved white matter microstructure indicated by increased FA (Blesa et al., 2019) and decreased MD (Ottolini et al., 2020) and improved brain growth indicated by the increase in volumes of deep grey matter (Belfort et al., 2016), amygdala, hippocampus and cerebellum (Ottolini et al., 2020), although the results with regional brain volumes at TEA remain inconclusive (Blesa et al., 2019). Research from our group recently demonstrated that increased breast milk intake in preterm neonates associates with a cortical imaging phenotype that more closely resembled that of term-born infants, including reduction in cortical grey matter volume, thickness and RD, and increase in FA (Sullivan et al., 2022). Collectively, these studies suggest that increased breast milk intake correlates with improvements in the MRI phenotype of EoP.

Intriguingly, C-section delivery has been associated with increased risk for neurodevelopmental disorders (Zhang et al., 2019) and poorer motor, social and cognitive

development (Khalaf et al., 2015; Polidano et al., 2017). Furthermore, C-section birth correlates with lower white matter integrity as indicated by decreased FA and myelin water fraction in full-term C-section-delivered infants at TEA and during infancy, with effects decreasing to non-observable at 3 years of life (Deoni et al., 2019). C-section birth is not generally considered a risk factor for neurocognitive impairment and these results may be confounded by other maternal and perinatal factors, including the obstetric indication for operative delivery. Indeed, a recent population-based study demonstrated that adjustment for the indication of C-section or familial genetic and environmental factors dramatically attenuates the association between C-section birth and increased risk for neurodevelopmental disorder, suggesting an absence of a causal relationship (T. Zhang et al., 2021).

Some studies further demonstrate that antibiotic exposure in infancy associates with neurodevelopmental outcomes. For instance, antibiotic exposure during the first year of life correlated with increased behavioural difficulties and cognition in childhood (Slykerman et al., 2019, 2017), and perinatal antibiotic exposure in preterm infants associated with increased attentional problems in childhood (Firestein et al., 2019). Whilst these associations survive adjustment for a number of covariates such as breastfeeding, delivery mode, maternal education and socioeconomic status, it remains to be determined whether these results are similarly confounded by indication for treatment or unmeasured familial factors.

Given the impact of these early life factors on the microbiota composition as described in section 1.3.3, it could be hypothesised that the microbiome is, at least partially, mediating the relationships observed with cognition and brain structure. However, to the best of my knowledge, studies to date have not yet specifically tested this hypothesis.

1.4.1.3 Neonatal inflammatory morbidities and neurodevelopment

Neonatal infection, including the diagnosis of sepsis or NEC, among very preterm infants associates with increased risk for adverse neurodevelopmental outcomes at follow-up, such as cerebral palsy and low cognitive scores (Schlapbach et al., 2011; Schulzke et al., 2007; Stoll et al., 2004). Risk for impaired neurodevelopmental outcomes is higher even without a positive blood culture (Mukhopadhyay et al., 2021). Neuroimaging studies have demonstrated that NEC and sepsis associate with white matter abnormalities visible on

conventional MRI (Glass et al., 2008; Lee et al., 2014; Shah et al., 2008) and increased water diffusivity in the white matter assessed using dMRI (Barnett et al., 2018; Lee et al., 2014). Although the link between infection and impaired neurodevelopment is likely multifactorial, inflammatory processes and cytotoxic mediators have been proposed as the key mechanisms (Strunk et al., 2014; Volpe, 2008). Indeed, recent research from our group has shown that interleukin-8, a pro-inflammatory cytokine, and a DNA methylation marker of inflammation correlate with MRI features of EoP including smaller deep grey matter volume and white matter dysmaturation (Conole et al., 2022; Sullivan et al., 2020). Thus, it could be hypothesised that gut dysbiosis in preterm infants contributes to the inflammatory cascade that can cause injury to the developing brain.

1.4.1.4 Direct evidence of microbiota associations with neurodevelopment

The past 6-7 years has seen the emergence of research into the direct associations of gut microbiota diversity and community composition with early cognitive and behavioural development. These studies of microbiota-gut-brain axis in infancy and early childhood are reviewed in detail in Chapter 4 as part of one of the aims of this thesis. Collectively, research has revealed that the gut microbiota associates with neurodevelopment across multiple domains, including general cognitive, language, motor, social and emotional development. Synthesis of these studies also revealed that the exact bacteria-behaviour relationships are complex and vary greatly between the studies, with potentially domain- and age-specific effects. Furthermore, clinical and methodological heterogeneity between studies is large, which hinders direct comparisons between studies. Nevertheless, given the increasing number of positive association studies published on this topic to date (34 studies of microbiota-brain/behaviour relationships in under 5-year-olds published since 2015, 14 of these published since June 2021 after submission of the review manuscript), a role for the microbiota in cognitive and behavioural development is probable, though mechanistic studies to elucidate these relationships are warranted. This effort can be accelerated using quantitative neuroimaging.

Neuroimaging provides a powerful *in vivo* assessment of brain structure. Although the field of 'radiomicrobiomics', i.e. the effort of combining sequencing-based microbiota data with quantitative neuroimaging features (De Santis et al., 2019), is still in emerging, incorporation of advanced imaging methods has demonstrated correlations between gut microbiota

composition with brain structure, function and metabolism across the life course as well as in health and disease (reviewed in P. Liu et al., 2019; Montoro et al., 2022). The use of neuroimaging may particularly be valuable in infancy to parse complex behavioural traits, which only become observable later in childhood, onto an intermediate phenotype. Neuroimaging may also reveal specific neural networks and microstructural features involved in the microbiota-behaviour relationships.

To date, only a handful of small studies (sample sizes < 65) in early childhood have investigated the microbiota-gut-brain axis using neuroimaging. I discuss these also in more detail in Chapter 4. These studies have applied structural (Carlson et al., 2021, 2018) and functional MRI (Gao et al., 2019) as well as functional near-infrared spectroscopy (Kelsey et al., 2021); the latter is used to estimate cortical hemodynamic activity that occurs in response to neural activity. Gut microbiota has been shown to associate with the volume and function of brain regions involved in emotion regulation (amygdala, medial prefrontal cortex, anterior cingulate and hippocampus), sensory-motor processing (precentral gyrus, supplemental motor area and insula), parietal regions important for language, sensory and emotional processing (angular gyrus and inferior parietal lobule) as well as deep grey matter regions (thalamus, caudate). Whilst these studies provide preliminary evidence that gut microbiota diversity and compositional features potentially correlate with brain structure and function, the results remain inconclusive, which could be due to methodological heterogeneity including age at microbiota sampling and scan, and approaches for deciding on the neuroimaging and microbiota features of interest. Furthermore, these studies do not rule out correlations with other brain networks, cortical morphometry, or microstructural properties derived from diffusion MRI.

The studies discussed this far in this section were conducted in typically developing term-born infants and children. Given the higher prevalence of neurodevelopmental impairments in preterm infants, investigations into the microbiota-brain relationships in this population may be beneficial to advance the understanding of the role of potentially modifiable factors, in this case the microbiota, in risk or resilience for adverse cognitive outcomes. Few groups have investigated direct associations between gut microbiota and cognitive development in preterm infants. The largest study (n = 372 with cognitive testing) conducted by Rozé and colleagues (2020) identified that very preterm neonates with gut microbiota compositions

characterised by higher relative abundances of *Enterococcus* or *Staphylococcus* had an increased risk for adverse outcomes defined as death or neonatal impairment at 2 years of life, whilst infants with higher abundances of *Escherichia, Enterobacter* or *Clostridium* were less at risk. Beghetti et al. (2021), on the other hand, studied a sample of 27 very preterm infants and demonstrated that *Bifidobacteriaceae* levels were less and *Enterococcaceae* levels more abundant at 1 month of life in infants who subsequently had neurodevelopmental impairments. A recent study of 24 preterm infants by Sarkar and colleagues (2022) showed that measures of neonatal microbiota richness positively associated with 2-year neurodevelopmental outcomes. They also observed associations with abundances of specific bacterial taxa, including *Enterobacteriaceae, Bifidobacterium, Klebsiella* and *Escherichia,* and different domains of neurodevelopment, yet, directions of effects were not specified. Two studies analysed data from a sample of 38 preterm neonates and by applying complex mathematical modelling were able to demonstrate how the correlations between microbiota composition and neurobehaviour at the time of discharge dynamically changed over the course of hospitalisation (Liu et al., 2022; Sun et al., 2020), potentially reflecting the rapid changes in microbiota composition over the neonatal period. In these two studies, taxa from the families of *Clostridiales* (e.g. *Veillonella*)*, Lactobacillales* (e.g. *Enterococcus*) and *Enterobacteriales* (e.g. *Escherichia/Shigella*) were identified as significant predictors for behavioural outcome.

Only one study in preterm infants has connected gut microbiota with neuroimaging findings. Seki et al. (2021) detected *Klebsiella*-dominated gut microbiota in extremely preterm neonates with severe brain injuries at TEA assessed using structural MRI, including periventricular haemorrhagic infarctions, intraventricular haemorrhages, cerebellar haemorrhages, and PVL. Multi-omics data integration using canonical correlation analysis revealed that *Klebsiella* abundance in the gut associated with a pro-inflammatory immunological profile including increased levels of γδT-cells and decreased levels of neuroprotective agents such as brain-derived neurotrophic factor (BDNF), suggesting that dysbiosis in preterm neonates may contribute to increased inflammation leading to brain injury. Interestingly, all infants in the study received probiotics (*Bifidobacterium bifidum* and *Lactobacillus acidophilus*) from first day of life until 34 weeks of gestation, suggesting that probiotic administration does not prevent *Klebsiella* overgrowth or brain injury.

Together, these studies suggest that gut microbiota composition in preterm neonates associates with behavioural and cognitive outcomes at discharge from neonatal unit as well as at 2 years of life. Gut microbiota dysbiosis also associates with severe brain injury. However, a consensus of a bacterial signature for adverse outcomes is difficult to identify due to varying directions of effects between studies. Furthermore, it remains unexplored to what extent gut microbiota may be associated with a more common and subtle preterm neuroimaging phenotype that includes reduced volumes and diffuse white matter dysmaturation.

1.4.2 Preclinical evidence

Whilst causal links between gut bacteria and brain or behavioural development have not been identified in humans, rodents provide good models to study the potential causative impact of gut microbiota on neurodevelopment. Germ-free mice are commonly used to study the impact of complete loss of microbiota on the central nervous system development. These loss-of-microbiota models also enable to assess the effect of specific microbial entities on the brain. For example, particular bacterial strains can be studied in isolation. This model can be expanded to research humanisation of the gut microbiota, which involves faecal microbiota transplantation from specific human conditions.

Studies in germ-free mice have emphasised the importance of bacterial exposure in early life for brain and behaviour development. For instance, germ-free mice demonstrate a lack of normal preference to novel social situations (Desbonnet et al., 2014; Stilling et al., 2018), impaired learning and memory (Chu et al., 2019; Hoban et al., 2018; Luk et al., 2018) as well as reduced anxious behaviours (Clarke et al., 2013; Diaz Heijtz et al., 2011). Importantly, several studies suggest a sensitive window in which recolonising germ-free mice with 'normal' gut bacteria can reverse the behavioural alterations. Microbiota introduction in early life, i.e. at birth or pre-weaning, as opposed to recolonisation in adulthood has been shown to rescue the observed behavioural phenotypes, including anxiety, social behaviours as well as fear responses (Buffington et al., 2016; Chu et al., 2019; Clarke et al., 2013; Diaz Heijtz et al., 2011). Interestingly, colonisation of germ-free mice as neonates with a consortium of human 'infant type' *Bifidobacterium* (*B. longum* subsp. *infantis, B. breve*, *B. bifidum*, and *B. dentium*) was sufficient to improve object recognition memory similarly to colonisation with conventional microbiota (Luk et al., 2018).

The behavioural phenotypes in microbiota-depleted animals are accompanied by morphological, cellular and molecular changes in the brain. Cellular and dendritic morphology is affected by microbiota as shown by extensive neuronal hypertrophy and dendritic arborization in the amygdala, but shorter, less complex neurons in the hippocampus of germ-free mice (Luczynski et al., 2016). Furthermore, increased density of immature dendritic spines were observed in the anterior cingulate cortex of germ-free mice, suggestive of a deficit in synaptic pruning (Luczynski et al., 2017). Germ-free mice also have increased adult hippocampal neurogenesis (Ogbonnaya et al., 2015). In contrast, neonatal antibiotic cocktail exposure led to decreased adult hippocampal neurogenesis (Keogh et al., 2021). Several lines of evidence suggest that gut microbiota depletion influences the microglia, including changes to microglia cell density and reduced maturity in terms of gene expression and morphology (Castillo-Ruiz et al., 2018; Chu et al., 2019; Erny et al., 2015; Matcovitch-Natan et al., 2016; Thion et al., 2018). Germ-free status and antibiotic exposure in neonatal mice also associate with increased myelination and expression of myelin-related genes in the prefrontal cortex (Hoban et al., 2016; Keogh et al., 2021). Microbiota alterations further affect the expression of various signalling molecules and neurotransmitters including BDNF and serotonin (Clarke et al., 2013; Desbonnet et al., 2015; Diaz Heijtz et al., 2011; Neufeld et al., 2011; Sudo et al., 2004) as well as levels of synaptic proteins such as postsynaptic density protein-95 and synaptophysin (Diaz Heijtz et al., 2011). Neuroimaging of the germ-free mouse brain post-weaning has shown decreased relative grey matter volume in olfactory bulbs, neocortex and cerebellum, increased relative volume of thalamus, decreased FA in the white matter as well as decreased myelin imaging marker (macromolecular proton fraction) throughout the brain compared to mice harbouring commensal microbes (Lu et al., 2018b). These studies collectively suggest that gut microbiota affects normal development of the brain.

Faecal transplantation studies have demonstrated the potential causative role of microbiota in human neurodevelopment. For example, microbiota transplantation from autistic individuals induced autism-like behavioural phenotypes in mice such as repetitive behaviour, and decreased locomotion, social behaviour and ultrasonic vocalisation compared to transplantation with microbiota from non-autistic individuals (Sharon et al., 2019). In addition, mice transplanted with a mixture of microbiota from three young adults

with ADHD had reduced white matter integrity marked by decreased FA and increased MD and RD both in white (internal capsule, fornix, corpus callosum) and grey matter regions (hippocampus), indicative of reduced myelination compared to mice transplanted with microbiota from age-matched typically-developing controls (Tengeler et al., 2020). Although the exact bacterial signature of ADHD and ASD remains inconclusive as discussed in section 1.4.1.1, these studies provide evidence that the gut dysbiosis observed in individuals with neurodevelopmental disorders may, at least partially, be causally linked with brain and behaviour phenotypes.

One group demonstrated that mice colonised with microbiota from a preterm infant with good postnatal growth rate had significantly more weight gain postnatally compared to mice colonised with microbes from an infant with poor postnatal growth (Lu et al., 2015). Colonisation with the poor-growth infant microbiota also led to increased intestinal and systemic inflammatory markers in mice. In a follow-up study they further showed that mice colonised with the poor-growth infant microbiota had decreased levels of markers of neuronal differentiation, oligodendrocyte development and myelination, and increased neuroinflammation (Lu et al., 2018a). Furthermore, transplantation with faecal microbiota from very preterm infants influenced associative learning and memory in a GA-dependent manner (Lu et al., 2022). These studies together suggest that preterm infant gut microbiota may be partially causative for phenotypic outcomes and contribute to inflammation and delayed brain development.

Together, this body of research in preclinical models provides evidence for causal role of the gut microbiota in brain and behavioural development.

1.5 Rationale and aims of this doctoral thesis

Preterm birth is closely associated with reduced white matter integrity, which is increasingly considered a whole brain phenomenon. However, previous research leaves uncertainty whether dimensionality reduction applied to diffusion MRI data is effective to capture diffuse/generalised white matter dysmaturation following preterm birth.

Research in preclinical models provides evidence that the gut microbiome is involved in neural development and behavioural regulation. Therefore, there is increasing interest in studying microbiome relationships with brain structure/function and behaviour in human

paediatric populations. To date, mounting evidence supports an association between the gut microbiota, brain, and cognition in early life but specific microbiome-brain/behaviour relationships appear inconsistent between studies, and preterm infants, who have a high burden of neurocognitive impairment, are not well-represented in the literature.

The neonatal period of the preterm infants presents unique developmental challenges associated with immaturity of the brain, gastrointestinal organs and the immune system, that are all shaped by early exposure to extrauterine life, including atypical exposure to microbes, both in terms of type and timing. Although preterm birth is closely associated with both brain and gut microbiota development, currently there is lack of understanding of whether and to what extent is there an interplay between early-life microbiota development and preterm brain dysmaturation. In particular, no studies to date have investigated associations between neonatal gut microbiota and MRI features of the EoP.

Gut microbiota is a potentially modifiable early life factor that could be utilised to promote brain health following preterm birth. Current evidence, however, does not demonstrate significant improvements in neurodevelopment after neonatal probiotic administration (Jacobs et al., 2017; Slykerman et al., 2018; Upadhyay et al., 2018). Greater understanding of the microbiota-gut-brain axis in preterm infants is therefore required to inform novel, targeted interventions in this population.

The aims of this PhD study are as follows:

1. To develop a whole-brain imaging marker capturing diffuse white matter injury in the preterm brain;
2. To review the evidence that gut microbiome diversity and community composition associate with human brain development in infancy and childhood;
3. To characterise early-life gut microbiota diversity and community composition in the first meconium of preterm and term control infants and at the end of neonatal unit care for the preterm group, and investigate their associations with neonatal clinical covariates;
4. To investigate associations between the diversity and community composition of the gut microbiota and brain structural integrity at term-equivalent age in infants born preterm.

Chapter 2 describes the main empirical methodologies used in the PhD study, detailing the study cohort, brain imaging acquisition, sample collection, and laboratory procedures.

I answer the first aim in Chapter 3 by developing a framework for deriving single- and multi-metric g-factors for DTI and NODDI metrics and quantifying their predictive utilities for GA at birth. The sample in this chapter consisted of term and preterm neonates of the Theirworld Edinburgh Birth Cohort (TEBC; Boardman et al., 2020) who had multimodal brain MRI obtained until January 2021.

I explore the second aim in Chapter 4 by carrying out a narrative review of the literature published up until June 2021 where associations between features of the gut microbiome derived from bacterial DNA sequencing and brain development, indexed by quantitative neuroimaging and/or cognitive/behavioural outcomes, were investigated. This review summarises gut microbiota-brain/behaviour associations in typically developing children from birth to 5 years.

I explore aims three and four in Chapters 5 and 6 using data from the TEBC. In Chapter 5, exploring the third aim, the participants were term and preterm neonates from whom faecal samples were collected. In Chapter 6, exploring the fourth aim, the sample consisted of study participants who had microbiome data matched with multimodal brain MRI.

Finally, in Chapter 7, I review the contribution of the main findings of this thesis and discuss avenues for future research in understanding the role of the gut microbiota in brain development.

Chapter 2. Materials and methods

2.1 Study design and participants

The participants were very and extremely preterm infants (GA at birth < 33 weeks) and term-born controls of the Theirworld Edinburgh Birth Cohort (TEBC), which is a single-centre (Simpson Centre for Reproductive Health, Royal Infirmary of Edinburgh, UK) longitudinal study designed to investigate the effects of preterm birth on brain structure and long-term outcome (Boardman et al., 2020). The study includes detailed biosampling, brain imaging and neurodevelopmental assessments from birth to 5 years of age (Figure 2-1).

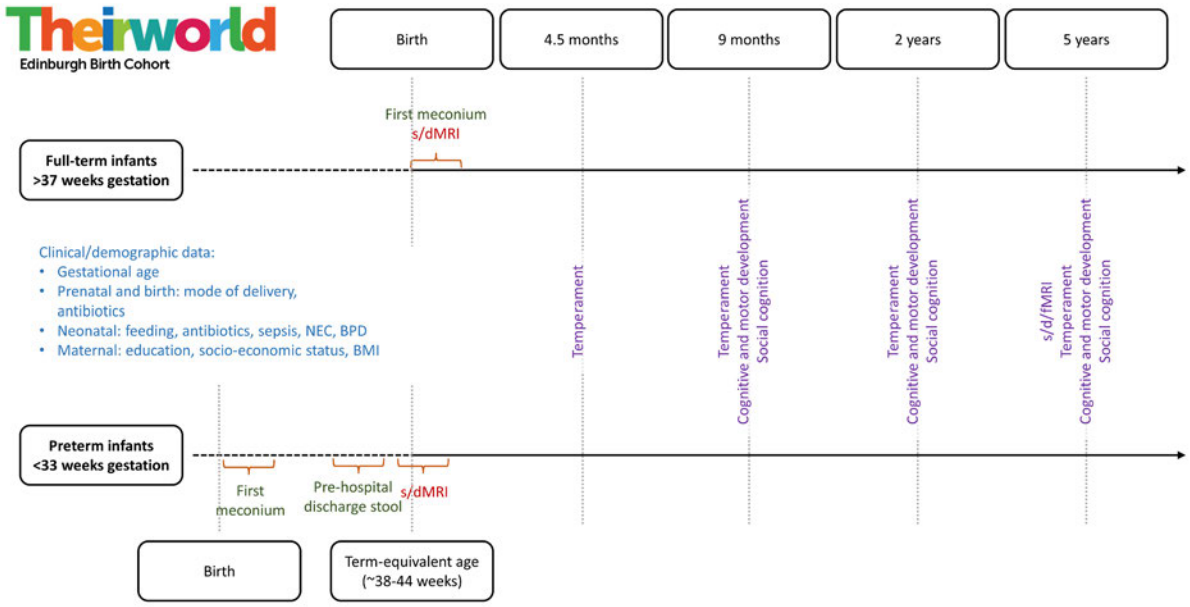


Figure 2-1. Overview of the Theirworld Edinburgh Birth Cohort study design.

The recruitment was conducted between November 2016 and September 2021. Recruitment and sampling was paused between March and June 2020 due to the COVID-19 pandemic.

Cohort exclusion criteria were death during neonatal period, major congenital malformations, chromosomal abnormalities, congenital infection, overt parenchymal lesions (cystic periventricular leukomalacia, haemorrhagic parenchymal infarction), post-haemorrhagic ventricular dilatation, or contra-indications to MRI. Term-born infants who required admission to the neonatal unit (NNU) were also excluded.

Ethical approval has been obtained from the National Research Ethics Service, South East Scotland Research Ethics Committee (11/55/0061, 13/SS/0143 and 16/SS/0154). Informed consent was obtained from a person with parental responsibility for each participant. The study was conducted according to the principles of the Declaration of Helsinki.

Details of study participant clinical and demographic characteristics are presented in each of the empirical results chapters.

2.1.1 Clinical data collection

Clinical data was collected by the study research fellows (Dr Gemma Sullivan, Dr David Q Stoye) and research nurses/midwives (Gillian Lamb, Gill Black, Dr Amy Corrigan) from antenatal and neonatal electronic patient records as part of the routine TEBC study data collection. Additional demographic data was obtained from parents using questionnaires during the study visits.

All infants were cared for in the Neonatal unit of the Simpson Centre for Reproductive Health, Royal Infirmary of Edinburgh, with standardised feeding, and antibacterial and antifungal guidelines. Preterm infants admitted to the NNU in the Simpson Centre for Reproductive Health are not routinely administered any pro- or prebiotic supplements.

2.1.1.1 Definition of neonatal morbidities and variables

Incidence of neonatal sepsis (early or late onset) was defined as detection of a bacterial pathogen from blood culture, or physician decision to treat with antibiotics for ≥ 5 days in the context of growth of coagulase negative *Staphylococcus* from blood or a negative culture but raised inflammatory markers in blood. Necrotising enterocolitis (NEC) was defined as stages II or III according to the modified Bell's staging for NEC which required medical treatment for ≥ 7 days or surgical treatment, respectively (Bell et al., 1978). Bronchopulmonary dysplasia (BPD) was defined as the requirement for supplemental oxygen or respiratory support at 36 weeks gestational age. Retinopathy of prematurity (ROP) was defined as requiring treatment with laser therapy or anti-VEGF. Birthweight z-scores were calculated according to International Fetal and Newborn Growth Consortium for the 21st Century (INTERGROWTH-21st) standards for preterm infants (Villar et al., 2016).

2.1.1.2 Neonatal antibiotic treatment

For this PhD study, I collected data on daily antibiotic exposure during NNU stay for the preterm neonates from neonatal electronic medical records. Three variables were composed to assess antibiotic exposure on the gut microbiota community composition: (i) exposure to antibiotics during the first three days of life, (ii) exposure to antibiotics at any other time during the NNU stay, and (iii) the total number of days of antibiotic treatment during NNU stay. As the length of NNU stay differs between infants and is dependent on GA at birth, the total number of antibiotic treatment days was divided by the number of days in NNU to create a variable reflecting the proportion (%) of antibiotic exposure days during NNU stay.

The antibiotic treatment in the Simpson Centre for Reproductive Health, Royal Infirmary of Edinburgh for all preterm infants with suspected and confirmed neonatal sepsis conformed to the following principles: babies up to 72 h of age were commenced on benzylpenicillin and gentamicin, babies > 72 h of age were commenced on piperacillin/tazobactam and vancomycin. To reduce unnecessary exposure to antibiotics, treatment was stopped in 48 h in the face of negative blood cultures and a low index of suspicion about infection. Some infants in the cohort were also exposed to azithromycin, cefotaxime, co-amoxiclav, flucloxacillin, linezolid, meropenem and metronidazole.

2.1.1.3 Neonatal nutrition data

Daily nutritional intake for preterm infants was collected from birth until discharge from the NNU using electronic patient records. Each day was categorised as consisting of exclusive maternal breast milk feeds, exclusive formula milk feeds, exclusive donor expressed milk feeds, or any combination of these feeding types. Data was available as the sum of each feeding type over the entire duration of NNU stay.

For consistency with our previous work demonstrating the influence of exclusive breast milk feeding on preterm brain development (Blesa et al., 2019; Sullivan et al., 2022), breast milk exposure was defined as the proportion (%) of inpatient days infants received exclusive breast milk feeds, which included both maternal and/or donor expressed breast milk. Infants were categorised into two groups based on breast milk exposure: high breast milk exposure was defined as exclusive breast milk feeds for ≥ 75% of inpatient days and low breast milk exposure was defined as exclusive breast milk feeds for < 75% of inpatient days.

2.2 Brain magnetic resonance imaging

2.2.1 Procedure

Infants were scanned at the Edinburgh Imaging Facility, Royal Infirmary of Edinburgh, University of Edinburgh, UK using a Siemens MAGNETOM Prisma 3 T MRI clinical scanner (Siemens Healthcare Erlangen, Germany) and a 16-channel phased-array paediatric head coil. Infants were scanned at TEA. Infants were fed and wrapped and allowed to sleep naturally in the scanner with monitoring of pulse oximetry, electrocardiography, and temperature. Flexible earplugs and neonatal earmuffs (MiniMuffs, Natus) were used for acoustic protection. All scans were supervised by a doctor or nurse trained in neonatal resuscitation. Each acquisition was inspected contemporaneously for motion artefact and repeated if there had been movement but the baby was still sleeping; diffusion MRI acquisitions were repeated if signal loss was seen in 3 or more volumes.

I assisted the clinical research fellows and research nurses/midwives with the infant scanning.

2.2.2 Imaging acquisition protocol

All MRI acquisition protocols are detailed in the study protocol paper (Boardman et al., 2020).

The following acquisitions were used in this PhD study: a 3D T_1-weighted (T_1w) magnetization- prepared rapid acquisition with gradient echo (MPRAGE) structural volume scan (repetition time (TR) = 1970 ms, echo time (TE) = 4.69 ms, inversion time = 1100 ms, flip angle = 9°, acquisition plane = sagittal, voxel size = 1 mm isotropic, FOV= 160 mm, acquisition time = 3:09 min), a T_2-weighted (T_2w) sampling perfection with application-optimised contrasts by using flip angle evolution (SPACE) structural scan (TR = 3200 ms, TE = 409 ms, acquisition plane = sagittal, voxel size = 1 mm isotropic, FOV = 128 mm, acquisition time = 2:13 min), and a multishell axial diffusion MRI (dMRI).

dMRI images were acquired in two separate acquisitions to reduce the time needed to re-acquire any data lost to motion artefacts: the first acquisition consisted of 8 baseline volumes (b = 0 s/mm^2 [b0]) and 64 volumes with b = 750 s/mm^2; the second consisted of 8 b0, 3 volumes with b = 200 s/mm^2, 6 volumes with b = 500 s/mm^2 and 64 volumes with b = 2500 s/mm^2 (acquisition time = 4:29 + 5:01 min). An optimal angular coverage for the

sampling scheme was applied (Caruyer et al., 2013). In addition, an acquisition of 3 b0 volumes with an inverse phase encoding direction was performed (acquisition time = 0:28 min). All dMRI images were acquired using single-shot spin-echo echo planar imaging (EPI) with 2-fold simultaneous multislice and 2-fold in-plane parallel imaging acceleration and 2 mm isotropic voxels; all three diffusion acquisitions had the same parameters (TR/TE 3400/78.0 ms).

2.2.3 Structural image reporting

Structural images were reported by a paediatric radiologist with experience in neonatal MRI (Dr. Alan J. Quigley) using an established system (Woodward et al., 2006). Images with evidence of post-haemorrhagic ventricular dilatation, cystic periventricular leukomalacia or central nervous system malformation were excluded from subsequent analysis.

2.2.4 MRI data pre-processing

Raw structural and diffusion images were visually inspected before pre-processing and low-quality images with major motion artefacts were discarded. I performed visual quality control of the images prior to pre-processing pipeline together with a postdoctoral research fellow (Dr Manuel Blesa Cabez).

dMRI processing was performed as follows: for each subject the two dMRI acquisitions were first concatenated and then denoised using a Marchenko-Pastur-PCA-based algorithm with MRtrix3's command *dwidenoise* (Tournier et al., 2019; Veraart et al., 2016); the eddy current, head movement and EPI geometric distortions were corrected using outlier replacement and slice-to-volume registration (Andersson et al., 2017, 2016, 2003; Andersson and Sotiropoulos, 2016) using *eddy* implemented in FMRIB Software Library (FSL) (Smith et al., 2004); bias field inhomogeneity correction was performed by calculating the bias field of the mean b0 volume and applying the correction to all the volumes (Tustison et al., 2010) using MRtrix3's *dwibiascorrect*.

The T_2w images were processed using the minimal processing pipeline of the developing human connectome project (dHCP) to obtain the bias field corrected T_2w and the brain mask (Makropoulos et al., 2018, 2014). Finally, the mean b0 EPI volume of each subject was co-registered to their structural T_2w volume using boundary-based registration using

FMRIB's Linear Image Registration Tool (FLIRT) (Greve and Fischl, 2009; Jenkinson et al., 2002; Jenkinson and Smith, 2001).

From the diffusion images I calculated the diffusion tensor imaging (DTI; fractional anisotropy (FA), mean, axial and radial diffusivities (MD, AD and RD)) and neurite orientation dispersion and density imaging (NODDI; neurite density index (NDI), isotropic volume fraction (ISO) and orientation dispersion index (ODI)) maps. The DTI model was fitted in each voxel using the weighted least-squares method *dtifit* as implemented in FSL using only the b = 750 s/mm^2 shell. NODDI maps were calculated using all shells and the recommended values of the parallel intrinsic diffusivity for neonatal white (1.45 µm^2/ms) or grey (1.25 µm^2/ms) matter (Guerrero et al., 2019; Zhang et al., 2012) using the original NODDI MATLAB toolbox (http://mig.cs.ucl.ac.uk/index.php?n=Tutorial.NODDImatlab).

2.2.4.1 Tract segmentation and derivation of general factors

Details of white matter tract segmentation and derivation of general factors capturing global white matter dysmaturation are provided in Chapter 3.

2.3 Gut microbiota

2.3.1 Faecal sample collection and processing

Faecal material was collected from dirty diapers by parents, NNU staff or research team. The samples were initially stored at -20°C as soon as possible after sample collection and then transferred to -80°C until further analyses. Faecal material was collected from the first bowel movement (meconium) from the term and preterm infants and a second faecal sample was collected from preterm infants prior to discharge from the NNU (referred to as pre-discharge sample going forward), which was generally around TEA. When preterm infants were transferred to another NNU prior discharge, efforts were made to collect the second sample prior to transfer from the Simpson Centre for Reproductive Health. A total of 143 meconium and 107 pre-discharge samples were collected during the study period.

2.3.2 DNA extraction

The bacterial DNA from faecal samples was extracted using an optimised protocol in the Bogaert group for DNA isolation of low biomass samples as previously described (Biesbroek et al., 2012; Reyman et al., 2019) involving phenol/bead beating in combination with the Agowa Mag Mini DNA Isolation Kit (LGC genomics, Germany). Samples were thawed on ice

for as little time as possible to obtain one 10µl inoculation loop of raw faeces (see below section 2.3.2.1 for additional information on meconium samples) which was added to a 2 ml screwcap tubes containing a mixture of 150 µl lysis buffer (Agowa Mag Mini DNA Isolation Kit, LGC genomics, Germany), 0.1 mm zirconium beads (BioSpec products, USA) in 650 µl lysis buffer, and 500 µl of phenol saturated with Tris-HCl (pH 8.0; BioSpec products, USA). Then, the samples were mechanically disrupted twice for 2 minutes at 2100 oscillations/minute using a bead beater (BioSpec products, USA). The samples were then centrifuged for 10 minutes at 5000 rpm at room temperature. Then, the aqueous phase was added to 1300 µl of binding buffer (Agowa Mag Mini DNA Isolation Kit, LGC genomics, Germany) with 10 µl magnetic beads (LGC genomics, Germany) in a sterile 1.5 ml Eppendorf tube and incubated for 30 minutes at room temperature on a thermos shaker (Hettich lab technologies, USA) to allow DNA binding. Then, the magnetic beads were washed twice with wash buffer 1 (Agowa Mag Mini DNA Isolation Kit, LGC genomics, Germany), once with wash buffer 2 (Agowa Mag Mini DNA Isolation Kit, LGC genomics, Germany), and the extracted DNA was eluted in a final volume of 50 µl.

To avoid potential cross-contamination from high-abundant samples to low-abundant samples, DNA from meconium and pre-discharge faecal samples was extracted on separate days. Samples were extracted in batches of 20 or 21. Each extraction was accompanied by two negative controls (200 µl of lysis buffer) and 1-2 positive controls (ZymoBIOMICS Microbial Community Standard [Zymo Research, USA] and/or a convenience saliva sample).

I assisted the research technician (Paula Lusarreta Parga) in the DNA extraction process and protocol optimisations.

2.3.2.1 DNA extraction protocol optimisation for meconium samples

Pilot extractions indicated that the DNA yield from the meconium samples was very low. Thus, optimisations were made for sample input with 2, 3 and 5 inoculation loops. With increasing sample inputs, interference with the extraction protocol was observed: in some samples, there was little separation of the aqueous phase following the first centrifugation and there was clumping of the magnetic beads, resulting in even lower DNA yields. The optimal sample input for the meconium samples that caused the least interference was determined to be two inoculation loops. However, additional modifications were made to the protocol: (i) increasing the lysis buffer volume to 200 µl; (ii) addition of six 2 mm glass

beads (Scientific Laboratory Supplies, UK) for more efficient mechanical sample disruption; (iii) to improve water phase separation, the initial centrifugation was increased to 15 minutes and an additional centrifugation for 5 minutes was performed for some samples to improve separation of the aqueous phase; however, when there was little separation of the aqueous layer, more volume from the other layers was included in the next extraction steps; (iv) the washing steps were performed with 400 µl of the buffers; (v) the DNA was eluted in a final volume of 35 µl to increase final DNA concentration. For some samples, extractions were conducted with multiple sample input amounts; for those, the highest concentration DNA sample was selected for sequencing.

In an additional optimisation experiment, the samples were pre-treated with proteinase K prior to the extraction protocol. However, this did not increase the DNA yield, thus, the above protocol, optimised for use in the group with low-biomass samples, was used for this study.

2.3.3 DNA yield quantification

The amount of extracted bacterial DNA was determined by quantitative polymerase chain reaction (qPCR) as described previously (Biesbroek et al., 2012; Bogaert et al., 2011) using primers specific for the bacterial 16S rRNA gene (forward: 5'-CGAAAGCGTGGGGAGCAAA-3', reverse: 5'-GTTCGTACTCCCCAGGCGG-3', TAMRA probe: 6FAM-ATTAGATACCCTGGTAGTCCA-MGB; Life Technologies, USA). The PCR mixture consisted of 12.5 µl of Taqman master mix (Applied Biosystems, USA), 1 µl of each primer (10 µM), 1 µl of the 5 µM 16S probe, 6.5 µl molecular grade water (miliQ, Life Technologies, USA), and 3 µl of the extracted DNA as template. Amplifications were performed using a Step One plus real-time PCR system (Applied Biosystems, USA) under the following conditions: 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. Standards for the qPCR comprised of extracted DNA from four bacteria (*Streptococcus pneumoniae, Staphylococcus aureus, Haemophilus influenzae* and *Moraxella catarrhalis*) that were equimolarly pooled to a final concentration of 1 ng/µl. The standard curve included five serial dilutions ranging from 1 ng/µl to 0.00001 ng/µl. A non-template control and extraction controls were included in each qPCR run.

2.3.4 Library preparation and 16S rRNA gene sequencing

Samples that yielded DNA concentration of > 0.18 pg/µl were considered for 16S ribosomal RNA (rRNA) gene sequencing. For the sequencing of the V4 hypervariable region of the 16S rRNA gene, amplicon libraries were generated by PCR using barcoded primers. This sequencing method utilises the conserved and hypervariable regions within the bacterial 16S rRNA gene. The conserved regions serve as primer binding sites to amplify the (fragments of) 16S rRNA gene and the hypervariable regions that contain species-specific information are used to differentiate between different bacteria and identify the composition of the microbiota. See Figure 2-2 for a graphical overview of amplicon-based sequencing.

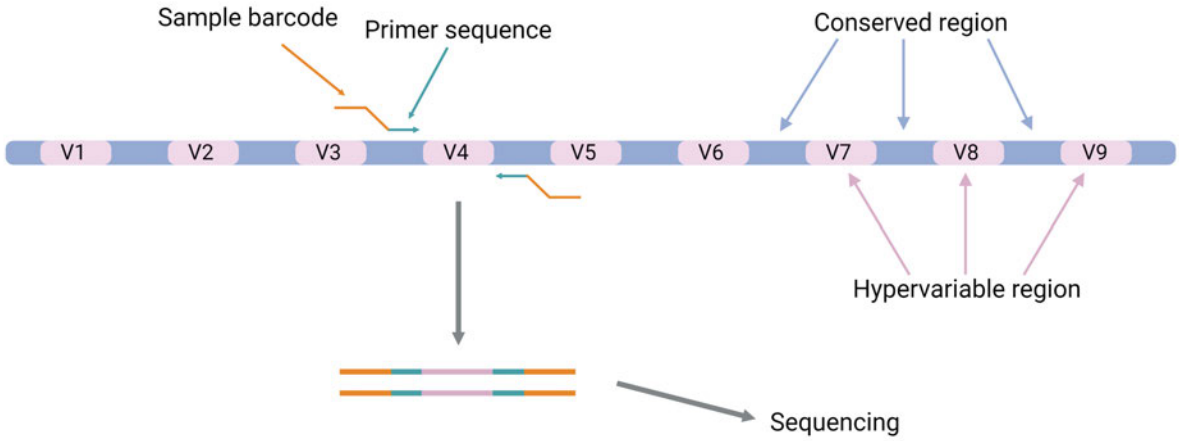


Figure 2-2. Graphical representation of the sequencing of the V4 hypervariable region of the 16S rRNA gene. *Figure created using Biorender.com.*

The PCR mix for each reaction consisted of 5 µl 5x Phusion High-Fidelity buffer (Life Technologies, USA), 0.5 µl Phusion® Hot Start II High-Fidelity DNA Polymerase (Life Technologies, USA), 2.5 µl of 2mM dNTPs mix (Merck Life Sciences, UK), 7 µl Nuclease free water, 2.5 µl of 5 µM 16S rRNA barcoded V4 forward primer (5 µM), 2.5 µl of 16S V4 barcoded reverse primer (5 µM), and 5µl of DNA as template. The V4 region of the 16S rRNA gene sequence primers used for amplification were 515F (5'-GTGCCAGCAGCCGCGGTAA-3') and 806R (5'-GGACTACCAGG-GTATCTAAT-3' (Caporaso et al., 2011). Amplification was performed on 96-well PCR plate using the Bio-Rad T100 PCR machine (Bio-Rad Laboratories, UK) under the following cycle conditions: 95°C for 2 minutes; 30 cycles of 98°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds; and a final extension of 72°C for 5 minutes.

Two mock DNA communities and a PCR non-template control were included in each MiSeq PCR run. The first mock community consisted of equimolarly pooled bacterial DNA from eleven species (*Bacteroides fragilis*, *Haemophilus influenzae*, *S. pneumoniae*, *Streptococcus pyogenes*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *haemolytic Streptococcus group A*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Moraxella catarrhalis*); the second mock community was the ZymoBIOMICS Microbial Community DNA Standard (Zymo Research, USA) at an estimated concentration of 20 pg/ul.

The amplified DNA concentration was quantified using Quant-iT™ PicoGreen® dsDNA Assay Kit according to manufacturer's instructions (Life Technologies, USA) and visualised on gel electrophoresis to ensure successful amplification. Samples with a visible band on gel electrophoresis were included in the amplicon pool along with DNA isolation negative and positive controls, and MiSeq PCR DNA positive and negative controls.

16S rRNA gene sequencing was performed on the Illumina MiSeq platform (Illumina, Eindhoven, the Netherlands) by Edinburgh Genomics (University of Edinburgh, UK) on a total of 191 samples (including for 6 samples which were excluded from further analyses due to neonatal death or congenital abnormalities, but yielded sufficient amount of DNA for library preparation), 23 negative and 18 positive controls in one run.

2.3.5 Bioinformatic data processing

2.3.5.1 16S rRNA gene sequencing data processing

16S rRNA gene sequencing data processing was performed in R (version 4.2.1) (R Core Team, 2022) as previously described (de Steenhuijsen Piters et al., 2022). Paired-end raw reads were filtered and trimmed (maxEE = 2; truncLen = 200/150 bp), merged, denoised, chimera filtered and binned into amplicon sequence variants (ASVs) using the DADA2 (version 1.16.0) in R (Callahan et al., 2016). Taxonomy was assigned using the DADA2 implementation of the naïve Bayesian classifier using the Silva v138.2 reference database. ASVs not assigned to the kingdom Bacteria or assigned to the family Mitochondria or class Chloroplast were removed. ASVs were named using taxonomic annotations combined with a rank number based on the relative abundance of each ASV in the dataset.

2.3.5.2 Sequencing data quality control and identifying potential contaminants

The resulting bacterial community profiles of the DNA extraction and MiSeq PCR negative and positive control samples were inspected.

Contaminant taxa were identified using the *isContaminant* function within the *decontam* package in R (Davis et al., 2018). DNA extraction blanks were used as negative controls and values from 16S qPCR (see section 2.3.3) were used for the measure of DNA concentrations. I used the "combined" method, which combines the probabilities from "frequency" (identifies contaminants by frequency that varies inversely with sample DNA concentration) and "prevalence" (identifies contaminants by increased prevalence in negative controls) algorithms with Fisher's method. To ensure the accuracy of the method, these contaminant ASVs were manually carefully inspected by plotting the 16S qPCR DNA concentration data against the relative abundance.

Second, in order to exclude ultra-rare taxa from the final dataset, I filtered the ASV table by removing ASVs that were identified at a relative abundance of < 0.1% and present in less than two samples (Subramanian et al., 2014).

Thereafter, the remaining list of ASVs were cross-matched to those taxa identified as contaminants by Salter et al (2014). These ASVs were manually inspected by plotting the 16S qPCR DNA concentration data against the relative abundance per isolation. Contaminant species were defined as ASVs abundant only in the lowest density samples in each isolation and/or only in isolation blanks. These ASVs were then additionally removed from the raw ASV list and the filtering of the ultra-rare taxa was repeated.

After excluding contaminating and ultra-rare taxa, the number of remaining reads per sample was investigated. Samples that had a final read count of less than 5000 were excluded from the final dataset. I additionally excluded duplicate samples (n=4) from the final dataset.

2.3.6 Derivation of microbiota characteristics

Bacterial diversity and community composition in the samples can be characterised on different levels and using a number of different indices. The main levels of microbiota characterisation are visualised in Figure 2-3 and explained below.

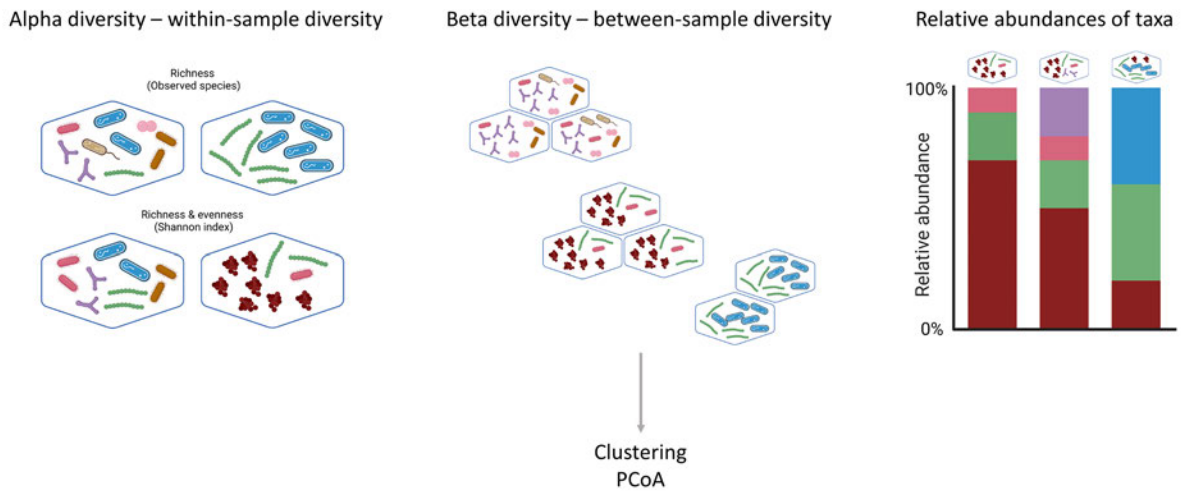


Figure 2-3. Different levels of microbiota characterisation.
Figure created using Biorender.com.

2.3.6.1 Alpha diversity

Measures of within-sample microbiota diversity (alpha diversity) were calculated using the *estimate_richness* function within *phyloseq* package in R (McMurdie and Holmes, 2013). Two indices of alpha diversity were estimated: Shannon index which is a measure of both microbiota richness and evenness, and observed species which is a measure of microbiota richness. Alpha diversity indices were calculated based on the ASV table after removal of contaminant taxa but before filtering of the ultra-rare taxa. This full ASV table was rarefied to the minimum sequencing depth (10200 reads) using *rarefy_even_depth* function within *phyloseq* package to ensure equal sequencing depth for each sample for estimating diversity indices.

2.3.6.2 Beta diversity

Beta diversity (between-sample diversity) was calculated as the Bray-Curtis dissimilarity (Bray and Curtis, 1957) matrix based on the total-sum-scaled (TSS-normalised; i.e. relative abundances) ASV table after filtering of the ultra-rare taxa using *vegdist* function within the *vegan* package (Oksanen et al., 2019). Beta diversity is commonly presented in a dimensionality reduction plot on which if two samples are further apart, they have higher beta diversity. Beta diversity is used as a measure of overall bacterial compositional differences between samples and is the basis of clustering or principal coordinates analyses.

2.3.6.3 Clustering

Hierarchical clustering was used to stratify infants and samples into groups based on gut microbiota composition. Clustering was performed on all samples based on the Bray-Curtis dissimilarity matrix calculated on relative abundance profiles (TSS-normalised ASV table). Hierarchical clustering of the samples was performed with the function *hclust* using average linkage as the method. Optimal number of clusters were identified based on Calinski-Harabasz and Silhouette width indices and the minimum cluster size was set to 3 samples.

2.3.6.4 Differential abundance testing

Microbiome Multivariate Association with Linear Models (MaAsLin2; Mallick et al., 2021) was used to identify bacterial ASV-level biomarkers associated with factors of interest. MaAsLin2 was chosen over other differential abundance testing tools due to its ability to accommodate a wide range of study designs, data types, and adjustments for covariates. In addition, MaAsLin2 has been shown to preserve statistical power, while having an adequate control over false positives (Mallick et al., 2021; Nearing et al., 2022). I applied TSS normalisation (i.e. calculation of relative abundances) on the ultra-rare-taxa-filtered ASV table prior to MaAsLin2 modelling. The exact parameters for MaAsLin2 modelling are provided in results chapters.

2.4 Statistical data analysis

Details of statistical data analysis are presented in each of the results chapters.

2.5 Code availability

2.5.1 Chapter 3

The segmented tracts in the ENA50 template space are available here: https://git.ecdf.ed.ac.uk/jbrl/ena. The code for tract propagation and average calculation, as well as scripts for data analysis are available here: https://git.ecdf.ed.ac.uk/jbrl/neonatal-gfactors.

2.5.2 Chapters 5 and 6

Scripts for data analysis are available here: https://github.com/kvaher/s1878972_thesis.

2.6 Supplementary materials

Supplementary materials accompanying this thesis are available in the OneDrive folder.

Chapter 3. General factors of white matter microstructure from DTI and NODDI in the developing brain

3.1 Chapter introduction

In this chapter, dimensionality reduction is applied to diffusion MRI data to derive whole-brain markers of generalised white matter dysmaturation. Previous work has suggested that the dysconnectivity and reduced white matter integrity associated with preterm birth are substantially whole brain phenomena. Furthermore, metrics derived from diffusion tensor imaging (DTI) and neurite orientation dispersion and density imaging (NODDI) share variance across tracts. These observations together motivate the search for efficient whole-brain estimates of white matter microstructure that capture (dys)maturational processes in early life. In this chapter, I apply principal component analysis and structural equation modelling to the neonatal brain MRI data from Theirworld Edinburgh Birth Cohort (TEBC) to: (1) determine general (g-) factors for DTI and NODDI metrics and evaluate whether a single factor captures substantial variance across major tracts; and (2) investigate the shared variance across dMRI metrics by deriving a multi-metric g-factor from DTI and NODDI, and quantify its predictive utility for GA at birth beyond uni-modal models. I hypothesised that g-factors associate with GA at birth and that they provide an efficient method for classifying generalised dysmaturation of white matter microstructure which is a core component of EoP. As such, they would be useful for investigating early life factors associated with adverse neurodevelopment. In Chapter 6, I apply the derived measures to study the correlations between gut microbiota community composition and brain development in preterm infants.

This chapter appears as a publication in *NeuroImage*:

Vaher, K., Galdi, P., Blesa Cabez, M., Sullivan, G., Stoye, D. Q., Quigley, A. J., Thrippleton, M. J., Bogaert, D., Bastin, M. E., Cox, S. R., & Boardman, J. P. (2022). General factors of white matter microstructure from DTI and NODDI in the developing brain. NeuroImage, 254, 119169. https://doi.org/10.1016/J.NEUROIMAGE.2022.119169

As first author, I designed the experiments, designed and conducted the white matter tract segmentation, conducted statistical analyses, and drafted and wrote the manuscript; I also contributed to MRI data acquisition. Dr Paola Galdi assisted with the statistical data analyses

(prediction modelling); Dr Manuel Blesa Cabez implemented the tract segmentation pipeline for the TEBC cohort. The CrediT authorship contribution statement is provided at the end of this chapter.

3.2 Abstract

Preterm birth is closely associated with diffuse white matter dysmaturation inferred from diffusion MRI and neurocognitive impairment in childhood. Diffusion tensor imaging (DTI) and neurite orientation dispersion and density imaging (NODDI) are distinct dMRI modalities, yet metrics derived from these two methods share variance across tracts. This raises the hypothesis that dimensionality reduction approaches may provide efficient whole-brain estimates of white matter microstructure that capture (dys)maturational processes. To investigate the optimal model for accurate classification of generalised white matter dysmaturation in preterm infants we assessed variation in DTI and NODDI metrics across 16 major white matter tracts using principal component analysis and structural equation modelling, in 79 term and 141 preterm infants at term equivalent age. We used logistic regression models to evaluate performances of single-metric and multimodality general factor frameworks for efficient classification of preterm infants based on variation in white matter microstructure. Single-metric general factors from DTI and NODDI capture substantial shared variance (41.8-72.5%) across 16 white matter tracts, and two multimodality factors captured 93.9% of variance shared between DTI and NODDI metrics themselves. General factors associate with preterm birth and a single model that includes all seven DTI and NODDI metrics provides the most accurate prediction of microstructural variations associated with preterm birth. This suggests that despite global covariance of dMRI metrics in neonates, each metric represents information about specific (and additive) aspects of the underlying microstructure that differ in preterm compared to term subjects.

3.3 Introduction

Diffusion tensor imaging (DTI) and neurite orientation dispersion and density imaging (NODDI) enable inference about the microstructural properties (such as water content, axonal density and myelination) of developing white matter from diffusion magnetic resonance imaging (dMRI) (Counsell et al., 2019; Tariq et al., 2016; Zhang et al., 2012). Neonatal dMRI has been valuable in assessing the impact of preterm birth on the developing brain; it reveals a preterm brain phenotype at term-equivalent age, which includes lower fractional anisotropy (FA) and neurite density index (NDI) and increased mean diffusivity (MD) throughout the white matter compared to term-born controls, with a dose-dependent effect of prematurity (Alexandrou et al., 2014; Anjari et al., 2007; Barnett

et al., 2018; Batalle et al., 2017; Blesa et al., 2020; Boardman and Counsell, 2019; Hüppi et al., 1998; Partridge et al., 2004; Pogribna et al., 2013). Importantly, the dysconnectivity and reduced white matter integrity associated with preterm birth is substantially a whole brain phenomenon (Girault et al., 2019; Telford et al., 2017). This motivates the search for efficient whole-brain estimates that would capture maturational processes in early life.

Studies have demonstrated that dMRI measures of white matter tracts across the brain are correlated (e.g. an individual with high FA in one tract is likely to have high FA across other tracts) and that this relationship is exists across the life course (Cox et al., 2016; Girault et al., 2019; Lee et al., 2017; Mishra et al., 2013; Telford et al., 2017; Wahl et al., 2010). This property has allowed the derivation of general factors (g-factors) of white matter microstructure (e.g. gFA), which associate with general cognitive functioning (Alloza et al., 2016; Cox et al., 2019; Penke et al., 2010) and age (Cox et al., 2016). Similar diffusion properties have been observed in early life and these predict cognitive abilities (Lee et al., 2017). Our group has previously reported that in neonates DTI-metric-based g-factors explain around 50% of variance in eight white matter tracts and associate with gestational age (GA) at birth (Telford et al., 2017).

The different dMRI metrics themselves as well as the derived g-factors are correlated (Chamberland et al., 2019; Cox et al., 2016; De Santis et al., 2014; Girault et al., 2019; Penke et al., 2010), suggesting that dMRI measures share overlapping information which can cause partial redundancies in data analysis. Recently, a dimensionality reduction framework based on multimodal principal component analysis (PCA) was proposed (Chamberland et al., 2019; Geeraert et al., 2020). Using this framework the authors identified a small number of microstructurally informative and biologically-interpretable components/factors which captured 80% of variance in dMRI and myelin-sensitive imaging metrics across the white matter tracts, which associated with age in a sample of typically developing 8-18-year-old children (Chamberland et al., 2019; Geeraert et al., 2020).

In this work, using a neonatal dataset and a neonatal white matter tract atlas based on established protocols (Pecheva et al., 2017; Wakana et al., 2007), we aimed to: (1) determine g-factors for DTI and NODDI metrics and evaluate whether a single factor captures substantial variance across major tracts; and (2) investigate the shared variance across dMRI metrics by deriving a multimodal g-factor from DTI and NODDI, and quantify its

predictive utility for GA at birth beyond uni-modal models. We hypothesised that g-factors would associate with GA at birth and that they would provide an efficient method for classifying generalised variation in white matter microstructure associated with preterm birth.

3.4 Materials and methods

3.4.1 Participants

The participants were preterm (with GA at birth < 33 weeks) and term born infants of the Theirworld Edinburgh Birth Cohort (TEBC) which is a longitudinal study designed to investigate the effects of preterm birth on brain structure and long term outcome (Boardman et al., 2020). The cohort exclusion criteria were major congenital malformations, chromosomal abnormalities, congenital infection, overt parenchymal lesions (cystic periventricular leukomalacia, haemorrhagic parenchymal infarction) or post-haemorrhagic ventricular dilatation. Ethical approval has been obtained from the National Research Ethics Service, South East Scotland Research Ethics Committee (11/55/0061, 13/SS/0143 and 16/SS/0154). Informed consent was obtained from a person with parental responsibility for each participant. The study was conducted according to the principles of the Declaration of Helsinki. The current study group contained 141 preterm and 79 term infants.

3.4.2 Data acquisition

Infants were scanned at the Edinburgh Imaging Facility: Royal Infirmary of Edinburgh, University of Edinburgh, UK using a Siemens MAGNETOM Prisma 3 T MRI clinical scanner (Siemens Healthcare Erlangen, Germany). A 16-channel phased-array paediatric head coil was used to acquire 3D T2-weighted SPACE images (T2w) (voxel size = 1mm isotropic, TE = 409 ms and TR = 3200 ms; acquisition time = 2:13 min) and axial dMRI data. Diffusion MRI images were acquired in two separate acquisitions to reduce the time needed to re-acquire any data lost to motion artifacts: the first acquisition consisted of 8 baseline volumes (b = 0 s/mm^2 [b0]) and 64 volumes with b = 750 s/mm^2; the second consisted of 8 b0, 3 volumes with b = 200 s/mm^2, 6 volumes with b = 500 s/mm^2 and 64 volumes with b = 2500 s/mm^2 (acquisition time = 4:29 + 5:01 min). An optimal angular coverage for the sampling scheme was applied (Caruyer et al., 2013). In addition, an acquisition of 3 b0 volumes with an inverse phase encoding direction was performed (acquisition time = 0:28 min). All dMRI images were acquired using single-shot spin-echo echo planar imaging (EPI) with 2-fold

simultaneous multislice and 2-fold in-plane parallel imaging acceleration and 2 mm isotropic voxels; all three diffusion acquisitions had the same parameters (TR/TE 3400/78.0 ms). Infants were fed and wrapped and allowed to sleep naturally in the scanner. Pulse oximetry, electrocardiography and temperature were monitored. Flexible earplugs and neonatal earmuffs (MiniMuffs, Natus) were used for acoustic protection. All scans were supervised by a doctor or nurse trained in neonatal resuscitation. Each acquisition was inspected contemporaneously for motion artefact and repeated if there had been movement but the baby was still sleeping; dMRI acquisitions were repeated if signal loss was seen in 3 or more volumes.

3.4.3 Data pre-processing

Prior to defining the current study group, raw structural and diffusion images were visually inspected before pre-processing and low-quality images were discarded. Fifteen out of 235 (6.38%; 7 preterm and 8 term infants) of acquisitions did not yield usable datasets across T2w or diffusion modalities due to motion or wakefulness during one or more sequences. Diffusion MRI processing was performed as follows: for each subject the two dMRI acquisitions were first concatenated and then denoised using a Marchenko-Pastur-PCA-based algorithm with Mrtrix3's command *dwidenoise* (Tournier et al., 2019; Veraart et al., 2016); the eddy current, head movement and EPI geometric distortions were corrected using outlier replacement and slice-to-volume registration (Andersson et al., 2017, 2016, 2003; Andersson and Sotiropoulos, 2016) using *eddy* implemented in FMRIB Software Library (FSL) (Smith et al., 2004); bias field inhomogeneity correction was performed by calculating the bias field of the mean b0 volume and applying the correction to all the volumes (Tustison et al., 2010) using Mrtrix3's *dwibiascorrect*. The T2w images were processed using the minimal processing pipeline of the developing human connectome project (dHCP) to obtain the bias field corrected T2w and the brain mask (Makropoulos et al., 2018, 2014). Finally, the mean b0 EPI volume of each subject was co-registered to their structural T2w volume using boundary-based registration using FMRIB's Linear Image Registration Tool (FLIRT) (Greve and Fischl, 2009; Jenkinson et al., 2002; Jenkinson and Smith, 2001).

NODDI and DTI maps were calculated in the dMRI processed images to obtain: fractional anisotropy (FA), mean, axial and radial diffusivities (MD, AD and RD), neurite density index

(NDI), isotropic volume fraction (ISO) and orientation dispersion index (ODI). DTI model was fitted in each voxel using the weighted least-squares method DTIFIT as implemented in FSL using only the b = 750 s/mm^2 shell. NODDI metrics were calculated using all shells and the recommended values for neonatal white matter of the parallel intrinsic diffusivity (1.45 μm^2/ms) (Guerrero et al., 2019; Zhang et al., 2012) using the original NODDI MATLAB toolbox (http://mig.cs.ucl.ac.uk/index.php?n=Tutorial.NODDImatlab). Representative maps of DTI and NODDI metrics for a preterm infant in the TEBC are provided in Supplementary Figure 3-1.

3.4.4 Tract segmentation

Whole brain tractography was performed in the ENA50 neonatal template space (Blesa et al., 2020, 2016) using SingleTensorFT tool within DTI-TK (Zhang et al., 2007, 2006) which generated white matter tractography from the ENA50 atlas tensor volume. Segmentation of white matter tracts was performed within the ENA50 atlas. Regions of interest (ROIs) used to delineate the tracts were drawn manually on the FA image, using the protocols outlined in Wakana et al. (2007) and Pecheva et al. (2017). Placement of ROIs is described in Supplementary Table 3-1 and these were drawn using the Paintbrush mode in ITK-SNAP (Yushkevich et al., 2006) (http://www.itksnap.org/). The ROIs were used to filter whole brain tractography either to select or to exclude tracts crossing the ROIs using TractTool within DTI-TK. The resulting tract images were binarized and manually refined. The white matter tracts delineated are shown in Figure 3-1.

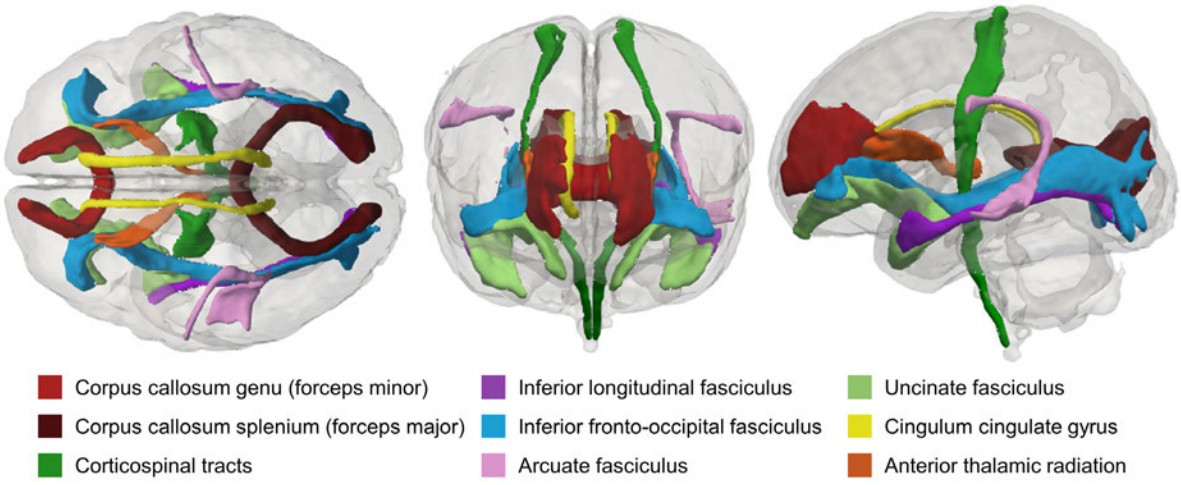


Figure 3-1. Visual representation of the generated white matter tracts in the ENA50 neonatal atlas space. *Shown in superior (left), anterior (centre) and lateral (right) views.*

3.4.5 Tract segmentation in subjects' native space and extraction of tract-averaged dMRI metrics

T2w processed images were registered to the ENA50 T2w structural template using rigid, affine and symmetric normalization (SyN) implemented in Advanced Normalization Tools (ANTs) (Avants et al., 2008). The resulting transformation was concatenated with the previously computed transformation from B0 to T2w and used to bring the tract ROIs defined in the ENA50 space to each subject's native space in a single step.

The average multi-tissue response function was calculated across the full population using the function *dwi2response* implemented in Mrtrix3 (Dhollander et al., 2019, 2016; Smith et al., 2022), with a FA threshold of 0.1. Then, using the function *dwi2fod* in Mrtrix3 the multi-tissue fibre orientation distribution (FOD) was calculated (Jeurissen et al., 2014) with the average response function using a spherical harmonic order (L_{max}) of 8. Only two (white matter and cerebrospinal fluid) response functions were used. Finally, a joint bias field correction and multi-tissue informed log-domain intensity normalisation on the FODs images was performed using the function *mtnormalise* in Mrtrix3 (Raffelt et al., 2017).

The tracts in native space were created using the iFOD2 algorithm using the command *tckgen* in Mrtrix3 (Tournier et al., 2010). The propagated tract ROIs were dilated and the original tract ROIs were used as seed images for the tractography, while the dilated tract ROIs were used as masks to constrain the tracts. The length of the fibres was set with a minimum length of 20 mm and a maximum of 250 mm. Finally, for each tract, a track density image (TDI) map (number of tracts per voxel) was created and normalised between 0 and 1 (Calamante et al., 2010) using the Mrtrix3's command *tckmap*. For each tract, the TDI map was multiplied by each of the DTI and NODDI maps, summed and divided by the average of the TDI map to calculate the weighted tract-averages for each of the DTI and NODDI metrics. We calculated TDI-weighted tract averages to better capture the core of the tracts and reduce bias arising from partial volume effects as highlighted by Parker et al. (2021).

3.4.6 Statistical analysis

All statistical analyses were performed in R (version 4.0.5) (R Core Team, 2022).

3.4.6.1 Effect of preterm birth on tract-averaged dMRI metrics

The tract-averaged dMRI parameters were adjusted for GA at scan by fitting a linear model of each scaled (z-transformed) metric on GA at scan and retaining the residuals. The distributions of the residualised dMRI metrics in each tract were assessed for normality using the Shapiro–Wilk test. Student's t-test or Mann–Whitney U-test as a non-parametric alternative was used to compare the tract-averaged values between term and preterm groups; Spearman's rho was used to investigate correlations between tract-averaged values and GA at birth. Reported p-values were adjusted for the false discovery rate (FDR) using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995).

3.4.6.2 Single-metric g-factors

The average Pearson's correlation coefficient for the inter- and intra-hemispheric associations between the tracts was calculated by first transforming the Pearson's r values to Fisher's Z, taking the average, and then back-transforming the value to Pearson's correlation coefficient (Corey et al., 1998).

One PCA was conducted for each of the seven DTI (FA, MD, AD, RD) and NODDI (NDI, ODI, ISO) parameters across the 16 tracts to quantify the proportion of shared variance between them. Thus, in each analysis, each subject was described by 16 features, computed as the tract-averaged values of a given metric across each tract. The first unrotated principal component (PC) scores were extracted as the single-metric g-factors. The g-factors were adjusted for GA at scan by fitting a linear model of each g-factor on GA at scan and retaining the residuals. We chose to adjust the g-factor scores for GA at scan in regression analyses rather than dMRI metrics prior to PCA to ensure that the covariances among the tracts are not 'washed out' by age when defining the g-factor. We report regression coefficients for linear models fitting a linear each of the residualised g-factors and GA at birth. All values were scaled (z-transformed) before fitting the models, thus, the regression coefficients are in the units of standard deviations. Reported p-values were adjusted for the FDR using the Benjamini-Hochberg procedure.

Structural equation modelling was used to investigate the extent that differences in GA at birth explain the shared variance across tracts (a common pathway model where GA has associations with only the latent g-factor), and the extent that GA at birth conveys unique information about individual tracts that is not conveyed via shared variance. First, we

evaluated the similarities between the g-factors obtained using PCA with the measurement model (confirmatory factor analysis [CFA] within the structural equation model) that was conducted for each metric using the R package *lavaan* (Rosseel, 2012). We used full information maximum likelihood estimation. Model fit was assessed according to standard fit indices: χ^2 test, root mean square error of approximation (RMSEA), comparative fit index (CFI), Tucker-Lewis index (TLI), and standardised root mean square residual (SRMR). Residual covariance paths (paths linking specific tracts to one another to account for the specific similarities between related tracts beyond their shared covariance across all tracts) were added between each of the bilateral tracts, the genu and splenium of the corpus callosum, as well as anatomically overlapping tracts (Dice Coefficient > 0.1 based on the dilated tract masks in template: ILF and IFOF in the same hemisphere, IFOF and UNC in the same hemisphere, ILF and UNC in the same hemisphere, and splenium of the corpus callosum and bilateral IFOF). Pearson's correlation coefficients were calculated for the g-factors derived using PCA and CFA.

Thereafter, we tested three models where 1) GA has associations with only the latent g-factor – a common pathway model; 2) GA has associations with each of the individual tracts separately and not with the latent factor – an independent pathways model; and 3) GA is associated with the latent factor and also with some specific factors – a common + independent pathways model (Cox et al., 2016; Tucker-Drob, 2013). To estimate the common + independent pathways model, we first included a path from GA to the latent g-factor, and then, in an iterative fashion, used modification indices (with a minimum value of 10) to include any additional paths from GA to specific tracts that substantially improved model fit. All models were adjusted for GA at scan at g-factor level. See Supplementary Figure 3-2 for graphical representation of the structural equation models. We used the χ^2 difference test (*aov* function within *lavaan*) and model fit indices (Akaike Information Criterion [AIC], Bayesian Information Criterion [BIC], and sample size adjusted Bayesian Information Criterion [saBIC]) to examine the fit differences between the models.

3.4.6.3 Multimodal g-factor

A multimodal PCA was conducted by pooling all tracts and metrics using a modification of an established framework (Chamberland et al., 2019; Geeraert et al., 2020). In summary, all metrics were analysed together in a single PCA, so that each observation was an individual

tract described by the 7 dMRI metrics, for a total of $n \times t$ observations, where n is the number of subjects and t is the number of tracts. The first and second PC were extracted as the multimodal g-factors which were averaged across the 16 tracts for each participant. To study the effect of GA at birth on the multimodal g-factors, we first adjusted the g-factors for GA at scan by fitting a linear model of each g-factor on GA at scan and retaining the residuals; then, linear regression models were fitted for each of the residualised multimodal g-factors and GA at birth. All values were scaled (z-transformed) before fitting the models, thus, the regression coefficients are in the units of standard deviations. Reported p-values were adjusted for the FDR using the Benjamini-Hochberg procedure.

3.4.6.4 Prediction modelling

We used the single- and multimodal g-factors as predictors in logistic regression models to discriminate between preterm and full-term infants. We measured classification accuracy, sensitivity and specificity using a 10-repeated 10-fold cross-validation scheme. In each of 10 repetitions data were randomly split in 10-folds of which 9-folds were used as training set to compute the PCs, adjust these for GA at scan, and train the prediction of preterm vs term subjects. The g-factors in the test set were computed and adjusted for GA at scan using the models retained from the training set. Then, the generalisation ability of the logistic regression model to predict term vs preterm group trained on the training set was assessed in the test set. Folds were stratified to preserve the proportion of term and preterm subjects of the whole sample. Accuracy was computed as the percentage of correctly classified subjects across folds and repetitions. We estimated the empirical distribution of chance by repeating the prediction analysis 1000 times after randomly assigning each subject to either the preterm or term group; permutation p-values were calculated by counting how many times the null models obtained an accuracy equal or greater than the original model. Sensitivity was calculated as the proportion of correctly identified preterm infants out of all cases classified as preterm. Specificity was calculated as the proportion of correctly identified term infants out of all those classified as term .

3.4.7 Data and code availability

Reasonable requests for original image and anonymised data will be considered through the BRAINS governance process (www.brainsimagebank.ac.uk) (Job et al., 2017). The segmented tracts in the ENA50 template space are available here: https://git.ecdf.ed.ac.uk/jbrl/ena.

The code for tract propagation and average calculation, as well as scripts for the data analysis in this paper are available here: https://git.ecdf.ed.ac.uk/jbrl/neonatal-gfactors.

3.5 Results

3.5.1 Study sample

The study group consisted of 220 neonates: 141 participants were preterm and 79 were term-born controls. Demographic details for participant characteristics are provided in Table 3-1. Among the preterm infants, 30 (21.3%) had bronchopulmonary dysplasia (defined as need for supplementary oxygen ≥36 weeks GA), 7 (5%) developed necrotising enterocolitis requiring medical or surgical treatment, and 27 (19.1%) had an episode of postnatal sepsis defined as either blood culture positivity with a pathogenic organism, or physician decision to treat for ≥5 days in the context of growth of coagulase negative staphylococcus from blood or a negative culture.

Table 3-1. Neonatal participant characteristics.
The last column reports the p-values of the group differences computed with t-test for continuous variables and Fisher's exact test for categorical variables. GA = Gestational age, M = male, F = female.

	Term (n=79)	**Preterm (n=141)**	**term vs preterm**
GA at birth (weeks)	39.65 (36.42 – 42.14)	29.48 (23.42 – 32.94)	n/a
Birth weight (grams)	3482 (2410 – 4560)	1334 (500 – 2510)	n/a
Birth weight z-score	0.48 (-2.30 – 2.57)	-0.02 (-3.13 – 2.14)	$p < 0.001$
GA at scan (weeks)	42.07 (38.28 – 46.14)	40.78 (36.56 – 45.84)	$p < 0.001$
M:F ratio	43:36	83:58	$p = 0.571$

3.5.2 Associations between preterm birth and tract-averaged dMRI metrics

Figure 3-2 and Supplementary Table 3-2 show tract-averaged dMRI parameter values for each of the 16 tracts for the term and preterm neonates. After adjusting for GA at MRI, in the majority of tracts FA was lower and MD, RD, AD and ISO were higher in preterm infants compared to term-born controls. However, ATR, CCG and CST showed only minimal or no differences in the DTI metrics between the two groups. There were groupwise differences in tract-averaged NDI and ODI values in a minority of the tracts (Figure 3-2).

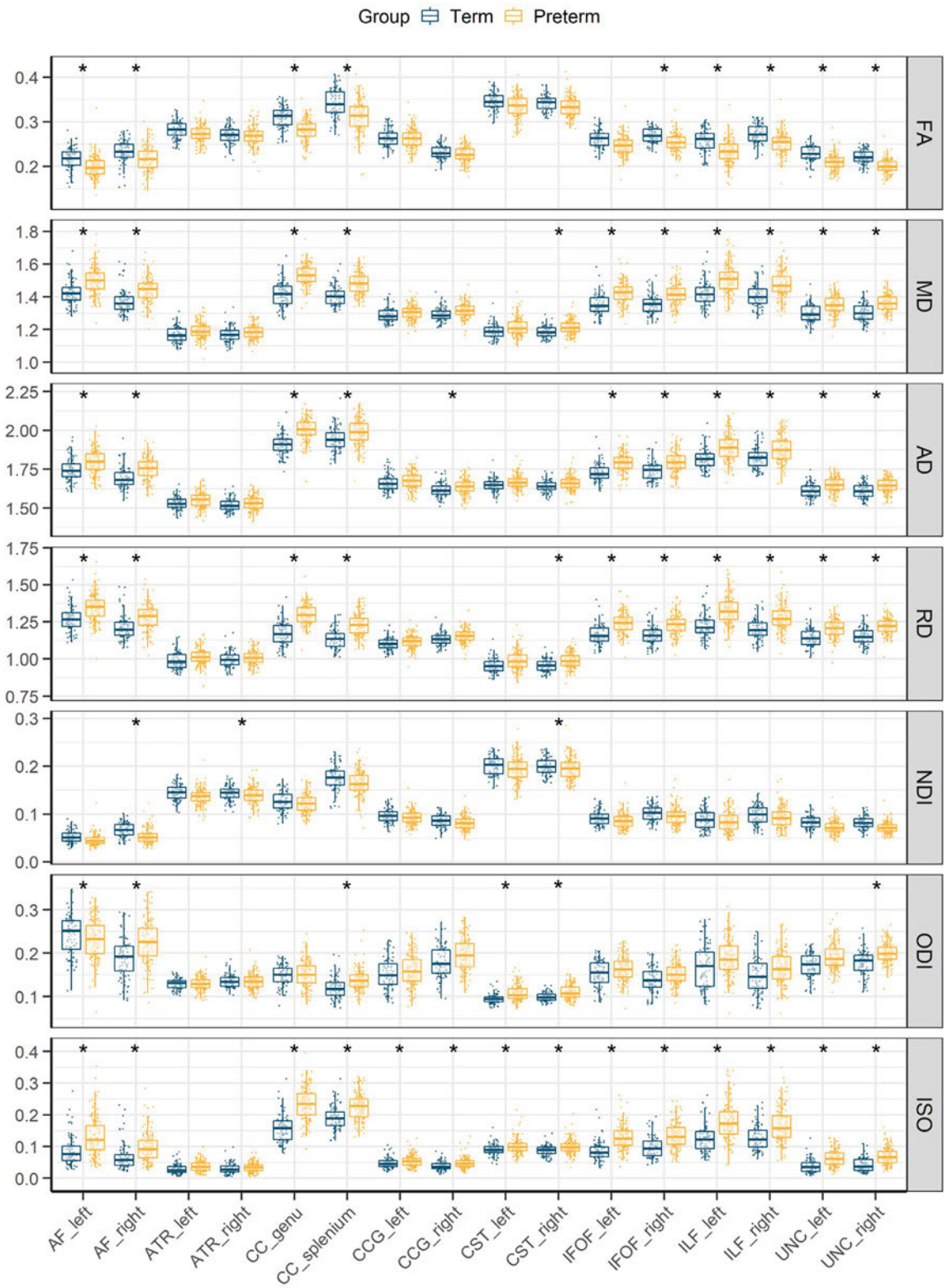


Figure 3-2. Tract-averaged diffusion characteristics of brain white matter tracts.
Asterisks (*) indicate statistically significant (FDR-corrected $p < 0.05$) differences in tract-averaged values between term and preterm infants after adjusting for age at scan. MD, AD and RD are in the units of $\times 10^{-3}$ mm^2 s^{-1}; other metrics are unitless. FA = fractional anisotropy, MD = mean diffusivity, AD = axial diffusivity, RD = radial diffusivity, NDI = neurite density index, ODI = orientation dispersion index, ISO = isotropic volume fraction, CC genu = corpus callosum genu/forceps minor, CC splenium = corpus callosum splenium/forceps major, CST = corticospinal tract, IFOF = inferior fronto-occipital fasciculus, ILF = inferior longitudinal fasciculus, AF = arcuate fasciculus, UNC = uncinate fasciculus, CCG = cingulum cingulate gyrus, ATR = anterior thalamic radiation.

3.5.3 Single-metric general factors of white matter microstructure

For all DTI and NODDI metrics, with the exception of ODI, metrics across tracts correlate positively (Figure 3-3). The mean (±SD) of the correlations was 0.601 (±0.294) for FA, 0.713 (±0.217) for MD, 0.573 (±0.199) for AD, 0.721 (±0.234) for RD, 0.628 (±0.250) for NDI, 0.351 (±0.287) for ODI, and 0.584 (±0.221) for ISO.

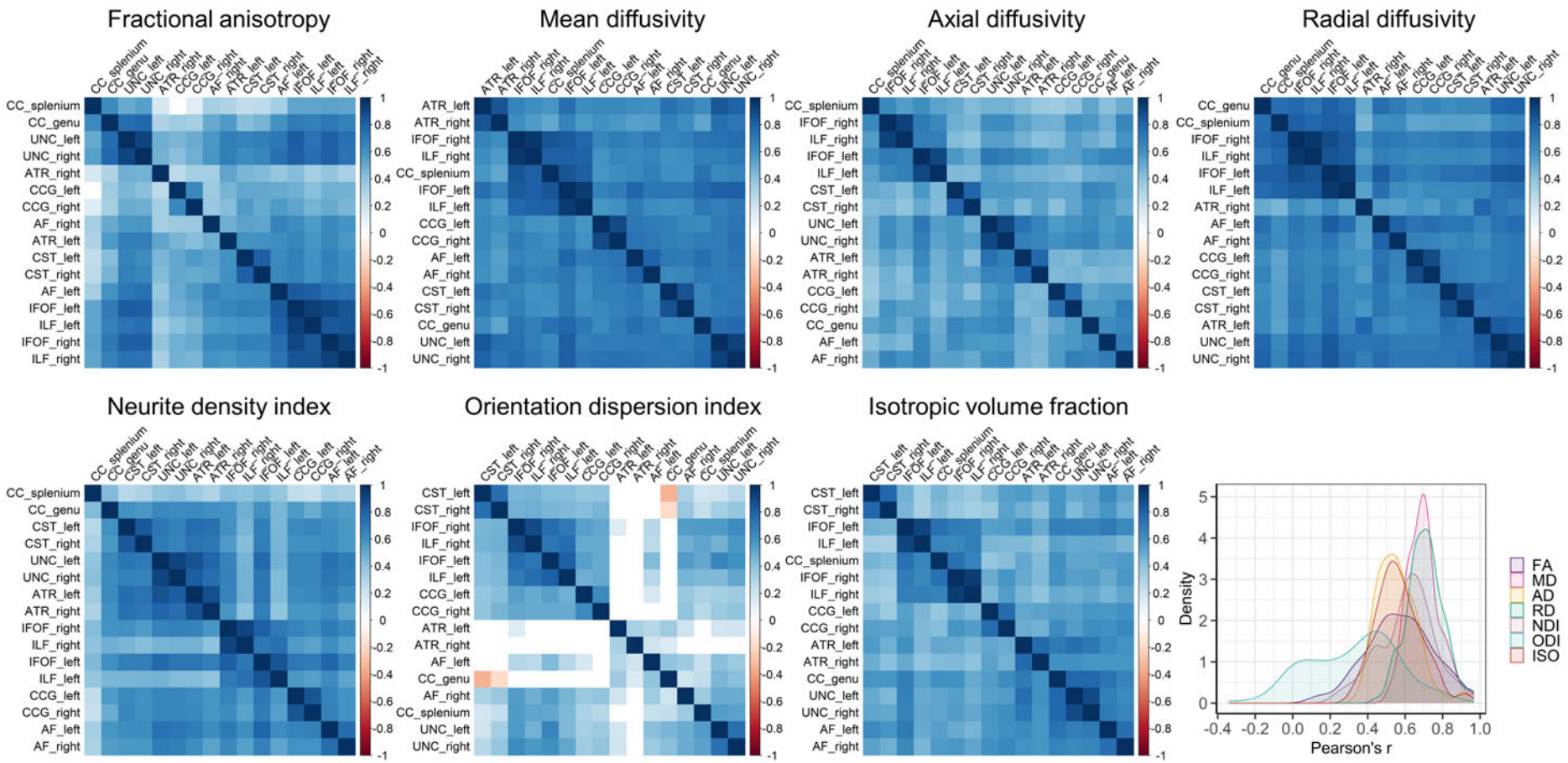


Figure 3-3. Heatmaps of inter- and intra-hemispheric associations (Pearson's r) for tract-averaged DTI (top row) and NODDI (bottom row) metrics.
In each case, the heatmaps are arranged by grouping highly correlated tracts around the diagonal. Blank squares represent correlations that were not nominally statistically significant (p>0.05). The plot on the bottom right represents the density of the correlation magnitudes. CC genu = corpus callosum genu/forceps minor, CC splenium = corpus callosum splenium/forceps major, CST = corticospinal tract, IFOF = inferior fronto-occipital fasciculus, ILF = inferior longitudinal fasciculus, AF = arcuate fasciculus, UNC = uncinate fasciculus, CCG = cingulum cingulate gyrus, ATR = anterior thalamic radiation.

We conducted separate PCAs for each of the 7 DTI and NODDI metrics on 16 white matter tracts to derive single-metric g-factors. For each metric, the scree plot provided evidence for a strong single factor capturing common variance across the tracts indicated by the comparatively large eigenvalue (Figure 3-4). This was less clear for ODI, which had a weaker first component and stronger second component compared to other dMRI metrics. The first PC is the g-factor for each of the white matter diffusion measures and this explained 61.3% variance in FA, 71.9% in MD, 59.9% in AD, 72.6% in RD, 63.9% in NDI, 41.8% in ODI, and 59.8% in ISO across the tracts. The tract loadings for the single-metric g-factors are presented in Table 3-2.

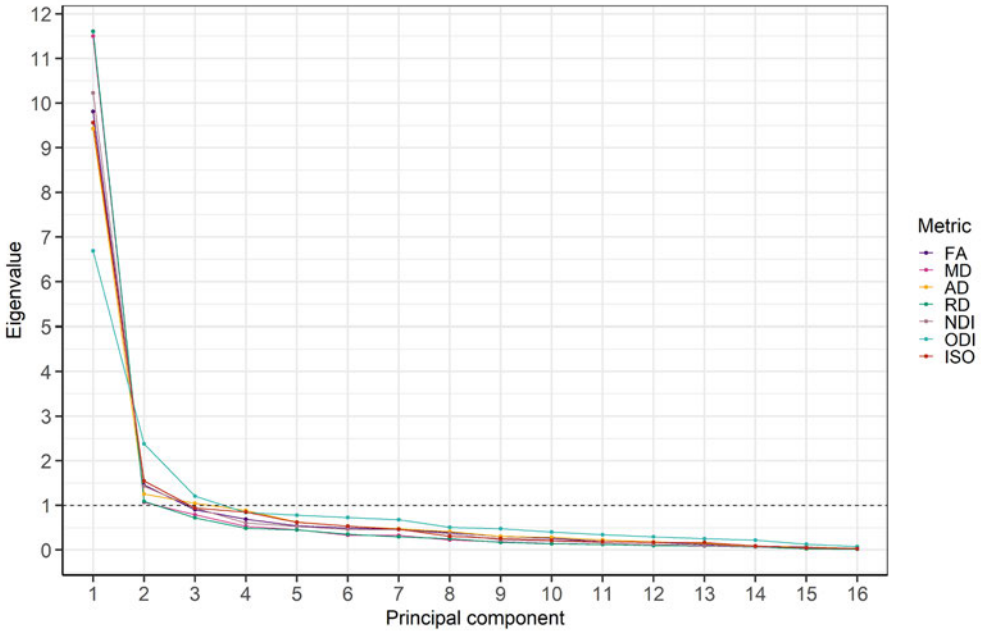


Figure 3-4. Scree plot for the principal component analysis.
Showing the eigenvalue against the number of components for each white matter tract dMRI metric. FA = fractional anisotropy, MD = mean diffusivity, AD = axial diffusivity, RD = radial diffusivity, NDI = neurite density index, ODI = orientation dispersion index, ISO = isotropic volume fraction.

Table 3-2. Tract loadings (correlation between the manifest variable and extracted component score) and explained variance for the first unrotated principal component (g-factor) for the seven dMRI metrics.

Tract	FA	MD	AD	RD	NDI	ODI	ISO
AF left	0.860	0.846	0.754	0.868	0.852	0.414	0.833
AF right	0.704	0.825	0.797	0.813	0.781	0.681	0.807
ATR left	0.758	0.834	0.748	0.839	0.871	0.192	0.794
ATR right	0.518	0.761	0.715	0.727	0.811	0.063	0.718
CC genu	0.772	0.846	0.782	0.853	0.740	0.184	0.860
CC splenium	0.539	0.832	0.668	0.785	0.467	0.637	0.746
CCG left	0.589	0.813	0.703	0.812	0.808	0.646	0.713
CCG right	0.618	0.834	0.758	0.824	0.797	0.645	0.760
CST left	0.779	0.832	0.760	0.833	0.818	0.544	0.695
CST right	0.784	0.825	0.699	0.835	0.800	0.616	0.649
IFOF left	0.936	0.933	0.871	0.945	0.913	0.882	0.856
IFOF right	0.928	0.890	0.810	0.911	0.853	0.883	0.778
ILF left	0.880	0.842	0.746	0.868	0.705	0.808	0.715
ILF right	0.877	0.844	0.750	0.868	0.718	0.853	0.729
UNC left	0.899	0.891	0.842	0.905	0.887	0.733	0.830
UNC right	0.899	0.905	0.848	0.916	0.866	0.765	0.846
Variance explained (%)	**61.341**	**71.899**	**58.938**	**72.562**	**63.917**	**41.785**	**59.775**

CC genu = corpus callosum genu/forceps minor, CC splenium = corpus callosum splenium/forceps major, CST = corticospinal tract, IFOF = inferior fronto-occipital fasciculus, ILF = inferior longitudinal fasciculus, AF = arcuate fasciculus, UNC = uncinate fasciculus, CCG = cingulum cingulate gyrus, ATR = anterior thalamic radiation, FA = fractional anisotropy, MD = mean diffusivity, AD = axial diffusivity, RD = radial diffusivity, NDI = neurite density index, ODI = orientation dispersion index, ISO = isotropic volume fraction.

After adjustment for GA at scan, there were significant associations between GA at birth and general factors of FA, MD, AD, RD and ISO (Figure 3-5). The strongest relationship was seen between GA at birth and gISO (GA at birth explained 11.06% of variance in gISO). Interestingly, GA at birth did not significantly associate with the g-factors of biophysical measures of white matter microstructure (NDI and ODI), mirroring the single-tract results described above.

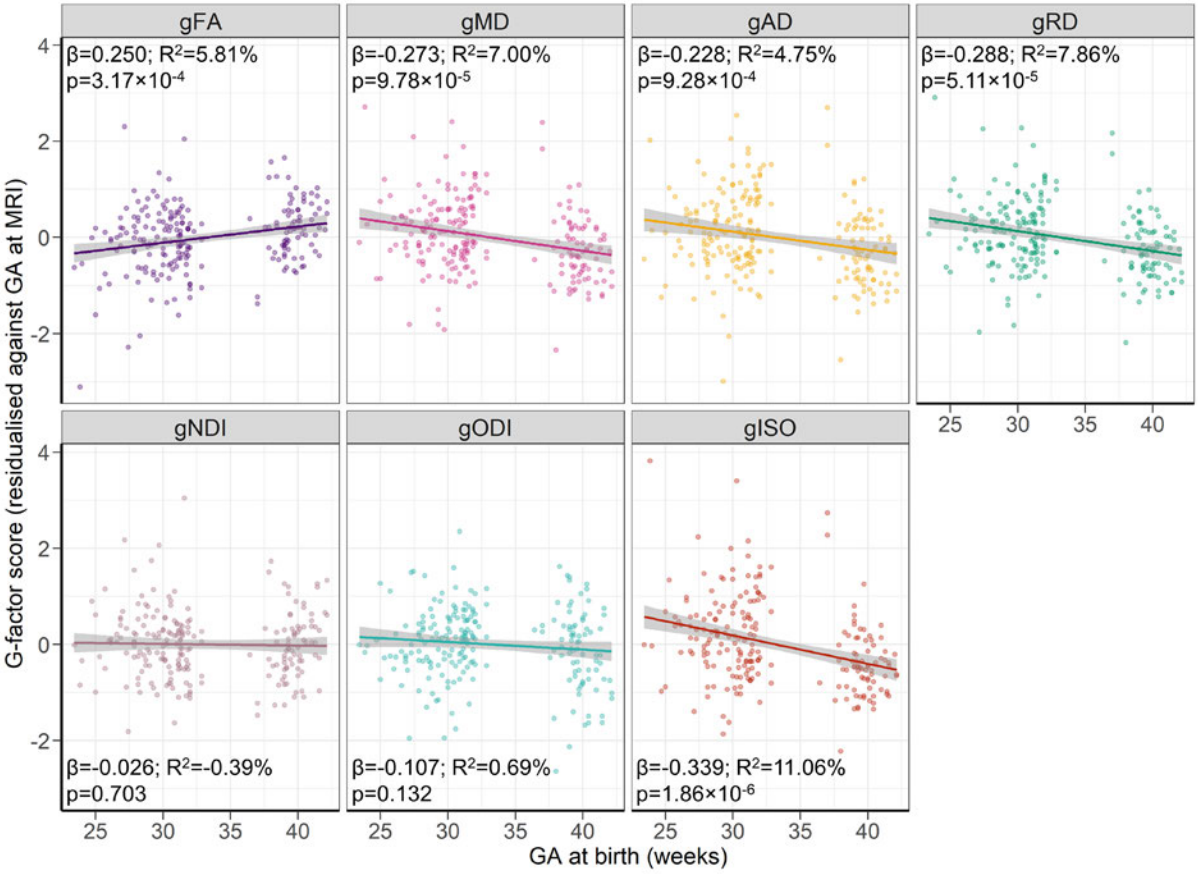


Figure 3-5. Associations between GA at birth and the g-factors of the seven dMRI metrics.
Regression lines and 95% confidence intervals (shaded) are shown for linear regression models between GA at birth and the g-factor scores, adjusted for GA at scan. The β coefficients are in standardised units so represent a standard deviation change in the residualised g-factor scores per standard deviation increase in GA at birth; variance explained in the model is shown in adjusted R2. Reported p-values are adjusted for the false discovery rate (FDR) using the Benjamini-Hochberg procedure. FA = fractional anisotropy, MD = mean diffusivity, AD = axial diffusivity, RD = radial diffusivity, NDI = neurite density index, ODI = orientation dispersion index, ISO = isotropic volume fraction.

To investigate the extent to which shared variance across all tracts explains differences in GA at birth, or whether specific tracts carry further information beyond generalised covariance, we used structural equation modelling. We observed that the measurement model (CFA) for each DTI and NODDI metric is highly collinear with the PCA results indicated by the similarities between the factor loadings (Supplementary Table 3-3; see Supplementary Table 3-4 for fit indices; all CFI > 0.93 (except ODI, CFI = 0.891)) and high positive correlations between the g-factors derived using PCA and CFA (all r > 0.98; Supplementary Table 3-5).

The structural equation modelling results showed that for the general factors of FA, MD, AD, RD and ISO there was evidence that GA at birth significantly associated with the g-factor (common model). The independent pathway model (where GA at birth associates with the

tract-specific values) fit significantly better than the model that only included the common pathway of GA at birth associations (Table 3-3; for factor loadings and regression coefficients see Supplementary Table 3-6), although it included the highest number of paths. We inspected the modification indices of the common pathway model to determine whether there are incremental, unique tract-specific effects of GA at birth which are not conveyed by the effect of GA at birth on the shared variance. The modification indices did not indicate additional tract-specific paths associated with GA at birth for any metric, thus, we were unable to construct models with both common and independent pathways and this suggests that the common model provides sufficient refinement of the properties of white matter microstructure that are affected by GA at birth.

Table 3-3. Model fit indices for each of the structural equation models linking GA at birth with the g-factors or individual white matter tracts.
P-values refer to the difference (χ2 difference test) between the common and the independent pathway models. For full parameter estimates in these models see Supplementary Table 3-6.

Metric	Model	χ2	df	χ^2 diff	p	AIC	BIC	saBIC
FA	Common	489.530	118	-	-	-449688	-449518	-449676.324
	Independent	313.520	103	176.010	$<2.2 \times 10^{-16}$	-449834	-449613	-449818.963
MD	Common	691.410	118	-	-	-450877	-450707	-450865.709
	Independent	491.110	103	200.290	$<2.2 \times 10^{-16}$	-451047	-450827	-451032.635
AD	Common	528.500	118	-	-	-449345	-449175	-449333.485
	Independent	421.360	103	107.140	5.70×10^{-16}	-449422	-449201	-449407.254
RD	Common	664.165	118	-	-	-450988	-450818	-450976.446
	Independent	441.838	103	222.330	$<2.2 \times 10^{-16}$	-451180	-450959	-451165.403
NDI	Common	455.697	118	-	-	-450056	-449887	-450045.040
	Independent	356.985	103	98.711	2.29×10^{-14}	-450125	-449904	-450110.382
ODI	Common	438.010	118	-	-	-448008	-447839	-447996.961
	Independent	363.457	103	74.553	6.82×10^{-10}	-448053	-447832	-448038.145
ISO	Common	543.819	118	-	-	-449594	-449424	-449582.735
	Independent	420.362	103	123.460	$<2.2 \times 10^{-16}$	-449687	-449467	-449672.822

FA = fractional anisotropy, MD = mean diffusivity, AD = axial diffusivity, RD = radial diffusivity, NDI = neurite density index, ODI = orientation dispersion index, ISO = isotropic volume fraction, AIC = Akaike Information Criterion, BIC = Bayesian Information Criterion, CFI = comparative fit index, TLI = Tucker-Lewis index, saBIC = sample size adjusted Bayesian Information Criterion.

3.5.4 Multimodal general factors of white matter microstructure

Next, we studied the shared variance of DTI and NODDI metrics across white matter tracts. The correlation matrices in Figure 3-6 show that the metrics form two clusters of positively correlated metrics: the first cluster represents positive correlations between FA and NDI, and the second cluster of positive correlations is formed of MD, RD, AD and ISO, while ODI appears to be a weaker member of the second cluster. These two clusters are negatively

correlated with each other. However, there is also variability in between-metric correlations between the different tracts. Nevertheless, the correlation matrix in the middle panel of Figure 3-6 highlights the similarity between the microstructural measures, which is consistent with them representing shared information about tract microstructure.

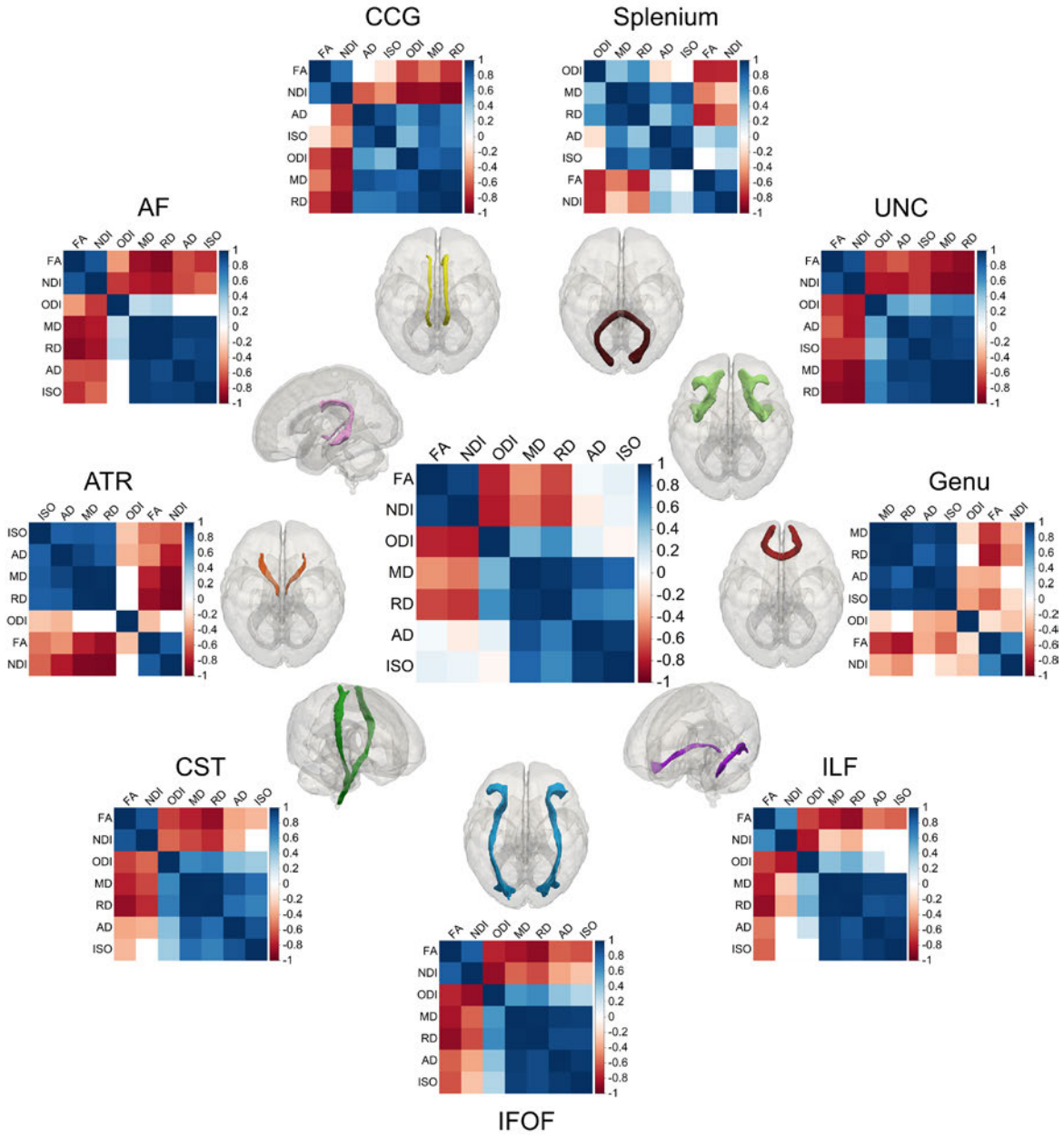


Figure 3-6. Correlation matrices of the seven diffusion measures.
The middle image represents the average of all white matter tracts. Matrices are re-organised using hierarchical clustering, grouping measures that have similar correlations together. Note that for bilateral tracts, the left and right values were averaged prior to performing the correlation. Genu = corpus callosum genu/forceps minor, splenium = corpus callosum splenium/forceps major, CST = corticospinal tract, IFOF = inferior fronto-occipital fasciculus, ILF = inferior longitudinal fasciculus, AF = arcuate fasciculus, UNC = uncinate fasciculus, CCG = cingulum cingulate gyrus, ATR = anterior thalamic radiation, FA = fractional anisotropy, MD = mean diffusivity, AD = axial diffusivity, RD = radial diffusivity, NDI = neurite density index, ODI = orientation dispersion index, ISO = isotropic volume fraction.

A PCA including all seven DTI and NODDI metrics revealed that 93.9% of the variability in dMRI metrics across white matter tracts is accounted by the first two PCs (Figure 3-7). The first PC (proportion of variance explained 60.0%, λ = 4.20) is mostly composed of RD and MD (both contributing negatively, 23.4% and 21.4%, respectively), and the second PC which captures 33.9% of variance in the data (λ = 2.38) is mostly driven by ISO (26.8%), AD (21.3%) and FA (18.9%). The loadings and contributions of the dMRI metrics to the first two PCs are presented in Table 3-4. RD and MD appear to be solely loading onto the PC1 (together contribute < 5% to the PC2), while the other dMRI metrics have more similar contributions to PC1 and PC2. The variability of between-tract correlations of dMRI metrics as mentioned above is also reflected in the clustering of tracts on the PC axes (Figure 3-8).

Table 3-4. dMRI metric loadings to the multimodal principal components.

Metric	**PC1**		**PC2**	
	loading	**contribution**	**loading**	**contribution**
FA	0.686	11.207	0.670	18.893
MD	-0.949	21.412	0.313	4.119
AD	-0.682	11.068	0.712	21.320
RD	-0.992	23.438	0.057	0.138
NDI	0.750	13.402	0.608	15.565
ODI	-0.693	11.412	-0.559	13.163
ISO	-0.582	8.062	0.798	26.803

FA = fractional anisotropy, MD = mean diffusivity, AD = axial diffusivity, RD = radial diffusivity, NDI = neurite density index, ODI = orientation dispersion index, ISO = isotropic volume fraction.

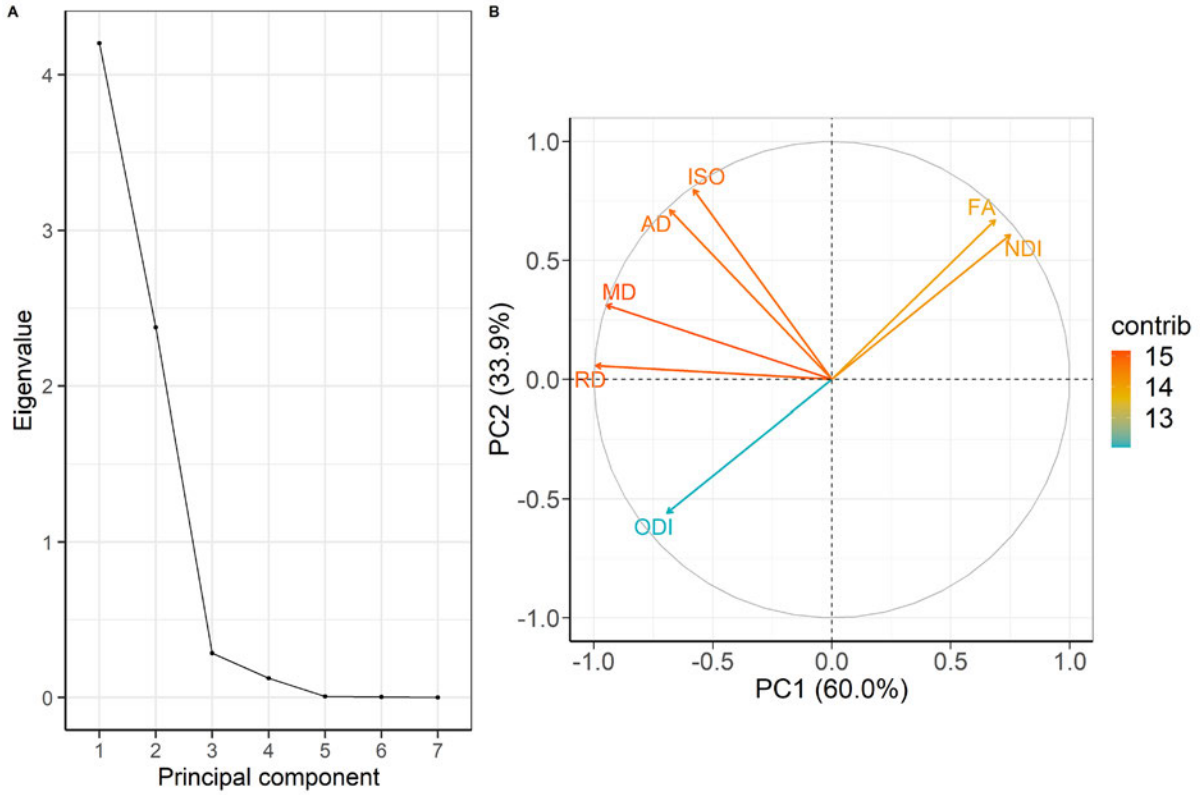


Figure 3-7. Multimodal PCA.
(A) Scree plot of the eigenvalues, (B) PCA variable contribution plot; the colours represent the contribution of the dMRI metric to the components. FA = fractional anisotropy, MD = mean diffusivity, AD = axial diffusivity, RD = radial diffusivity, NDI = neurite density index, ODI = orientation dispersion index, ISO = isotropic volume fraction.

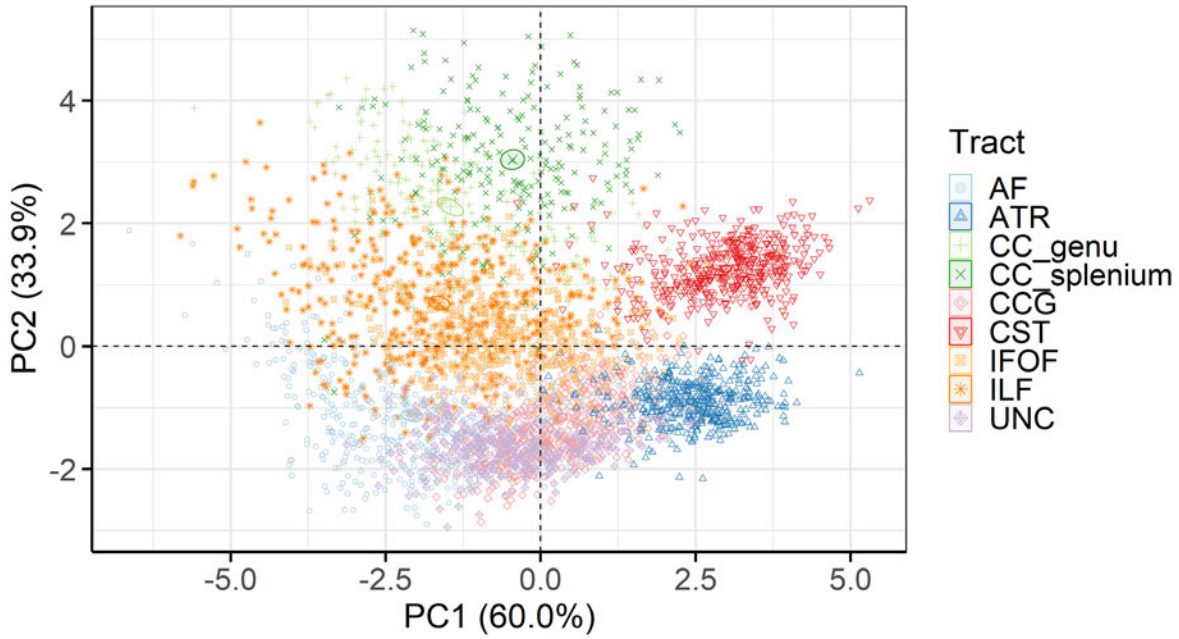


Figure 3-8. Visualisation of individual tract coordinates on the multimodal principal component axes.
CC genu = corpus callosum genu/forceps minor, CC splenium = corpus callosum splenium/forceps major, CST = corticospinal tract, IFOF = inferior fronto-occipital fasciculus, ILF = inferior longitudinal fasciculus, AF = arcuate fasciculus, UNC = uncinate fasciculus, CCG = cingulum cingulate gyrus, ATR = anterior thalamic radiation.

GA at birth was significantly associated with both multimodal g-factors (Figure 3-9): the first multimodal g-factor that has high negative contributions from RD and MD was positively, and the second multimodal g-factor with high positive contributions from ISO and AD was negatively associated with GA at birth. It is possible that individual tracts may contribute to varying degrees to the relationship with age (Supplementary Table 3-2).

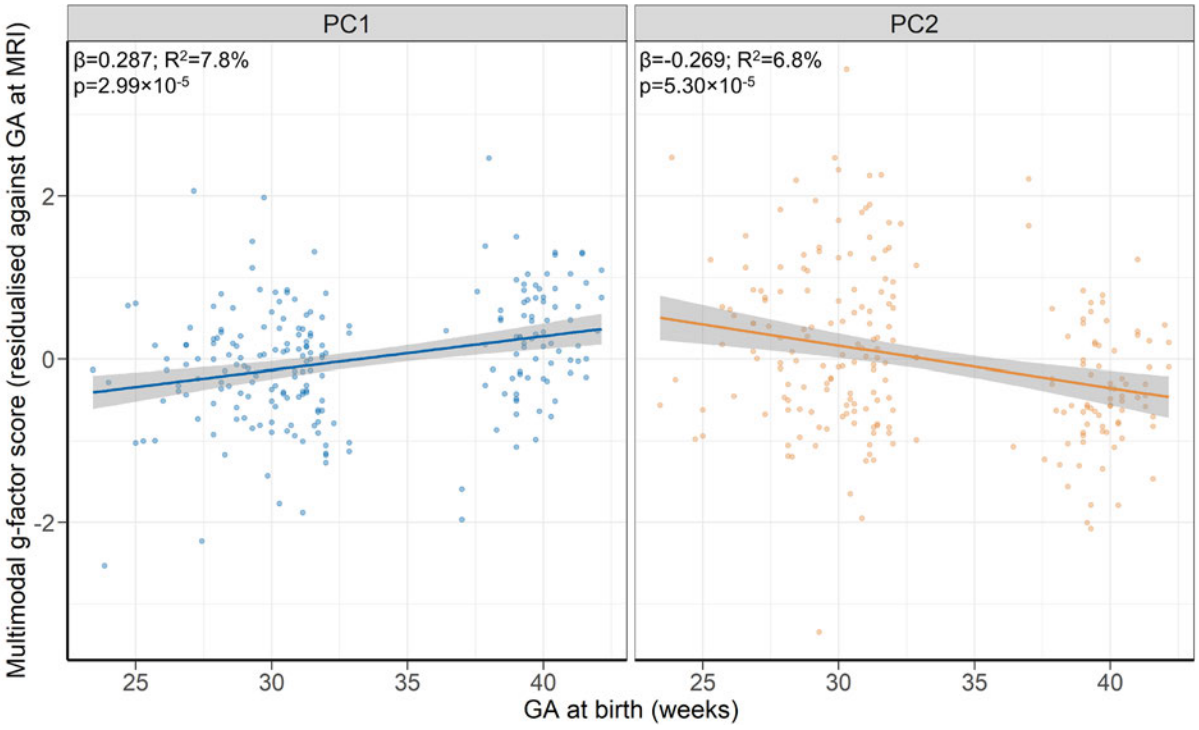


Figure 3-9. Associations between GA at birth and the multimodal g-factors.
The extracted multimodal principal components were averaged across the 16 tracts for each participant which resulted in a single estimate for the multimodal g-factors for each subject. Regression lines and 95% confidence intervals (shaded) are shown for linear regression models between GA at birth and the g-factor scores, adjusted for GA at scan. The β coefficients are in standardised units so represent a standard deviation change in the residualised g-factor scores per standard deviation increase in GA at birth; variance explained in the model is shown in adjusted R2. Reported p-values are adjusted for the false discovery rate (FDR) using the Benjamini-Hochberg procedure.

3.5.5 Utility of g-factors to classify infants based on gestational age

Given the high shared variance within and between the dMRI metrics across major white matter tracts and the significant associations between GA at birth and the derived g-factors, we asked whether g-factors are able to classify infants based on GA at birth (preterm vs term classification) (Table 3-5). Overall, the prediction accuracy for the single metric and multimodal g-factors only marginally exceeded chance (random classification accuracy in the current sample is 64.1% due to the imbalance of term and preterm infants). The highest prediction accuracy (75.2%) was achieved when incorporating all single metric g-factors in

one model, however, it has to be noted that this is the least parsimonious model with seven predictors compared to one and two in the other models.

Table 3-5. Prediction model results based on 10-repeated 10-fold cross validated logistic regression models using the single-metric g-factors and multimodal g-factors.
Reported values are mean and standard deviations computed across cross-validation folds and repetitions. Permutation p-values are computed over 1000 random permutations of the group variable.

	Accuracy	Sensitivity	Specificity	Permutation p-value
gFA	64.9±5.0	0.133±0.110	0.938±0.074	0.005
gMD	67.3±6.4	0.249±0.144	0.911±0.087	0
gRD	67.6±7.1	0.268±0.165	0.905±0.091	0
gAD	65.5±6.6	0.186±0.122	0.918±0.077	0
gNDI	64.1±1.0	0±0	1±0	0.210
gODI	64.0±3.2	0.034±0.056	0.979±0.043	0.703
gISO	67.7±8.0	0.364±0.154	0.852±0.093	0
All DTI	65.5±7.6	0.343±0.181	0.830±0.010	0.002
All NODDI	71.3±8.3	0.512±0.148	0.825±0.083	0
All single-metric	75.2±8.4	0.589±0.169	0.843±0.093	0
Multimodal PC1	67.9±6.9	0.273±0.153	0.906±0.090	0
Multimodal PC2	64.3±7.8	0.206±0.131	0.889±0.082	0.010
Multimodal PC1 and PC2	67.5±8.5	0.358±0.169	0.854±0.092	0

FA = fractional anisotropy, MD = mean diffusivity, AD = axial diffusivity, RD = radial diffusivity, NDI = neurite density index, ODI = orientation dispersion index, ISO = isotropic volume fraction, DTI = diffusion tensor imaging, NODDI= neurite orientation dispersion and density imaging.

3.6 Discussion

In this study, utilising the substantial shared variance within and between DTI and NODDI metrics across 16 major white matter tracts, we derive single- and multimetric g-factors which covary with GA at birth. Using structural equation modelling, we show that whilst the shared variance among tracts carries much of the white matter microstructural information about GA-based differences, there is modest additional unique information at the level of individual pathways that enhances term/preterm differentiation, though larger samples are required to reliably estimate the precise magnitudes and loci of the most informative white matter pathways. We demonstrate that combining single-metric g-factors from DTI and NODDI together in one prediction model offered the most efficient method for characterising variation in white matter microstructure associated with preterm birth, suggesting each metric carries additive information. These results add to the body of literature suggesting generalised dysmaturation of the white matter in the preterm neonates.

Variance in measures derived from dMRI is shared among white matter tracts in both neonates and adults. Previous studies suggest strong between-tract correlations of dMRI metrics in newborns, which show a decreasing trend from birth to 2 years of age (Girault et al., 2018; Lee et al., 2017). The between-tract correlations are weaker in adults compared to infants (Cox et al., 2016; Penke et al., 2010), and increase again at later ages (Cox et al., 2016). These results are supported by our current findings as the average correlation coefficients of the DTI metrics among white matter tracts in the current neonatal sample are of similar magnitude compared to previously reported in healthy term-born infants (Lee et al., 2017). Previous studies have utilised the shared white matter properties across tracts and have suggested that generalised measures to capture global white matter microstructure can be derived, which are useful for investigating global phenomena (Cox et al., 2016; Lee et al., 2017; Penke et al., 2010; Telford et al., 2017). Here, we report that the g-factors capture 58.9-72.6% of variance in DTI metrics, thus replicating our previous results in an independent, larger sample of neonates and different tract segmentation protocol (Telford et al., 2017). We additionally expand on the previous work and report that similarly to DTI metrics, in neonates there is substantial shared variance of NODDI metrics across white matter tracts (41.8-62.9%) as was previously reported in adult population (Cox et al., 2016). We observed the largest variance captured by a single g-factor for RD, while there was least evidence for a single latent factor for ODI, indicated by the comparably smaller eigenvalue for the first component. The weaker cross-tract correlations seen for ODI compared to other dMRI measures were previously also reported in the UK Biobank dataset (Cox et al., 2016) and together these results suggest that this measure of white matter microstructure may be capturing tract-specific rather than global effects. Age-related changes in between-tract correlations as discussed above are also reflected in the variance explained by the single-metric g-factors for each of the dMRI metrics, which is highest in the new-borns as reported in the current paper as well as by Lee et al. (2017) compared to early childhood (Lee et al., 2017) and adulthood (Cox et al., 2016; Penke et al., 2010). However, differences in the tract segmentation protocols, included tracts as well as models/approaches used for deriving the g-factors have to be taken into account when making these comparisons.

The correlations between different DTI and NODDI metrics themselves indicate that they share overlapping information in the brain (Chamberland et al., 2019; De Santis et al., 2014), but less is known about the covariance of dMRI measures in early development when water diffusion properties are different. By examining the covariance of dMRI metrics averaged over 16 white matter tracts, we observed that there are two clusters of positively correlated metrics: the first cluster includes measures of microstructural complexity and anisotropy of FA and NDI while the second cluster includes measures related to water diffusivity (MD, RD, AD and ISO); the metrics in these two clusters are in turn negatively correlated with one another. The highest positive correlations are between the pairs of FA-NDI (microstructural complexity and anisotropy), RD-MD (hindrance and degree of diffusivity) and AD-ISO (free/diffuse water). Importantly, the dMRI metric covariance structures vary slightly between tracts, confirming the tract-specific variability highlighted in the PCA plot. For example, the splenium of the corpus callosum appears to have weaker between-metric correlations overall although high correlations between MD-RD, FA-NDI and AD-ISO are still present. Interestingly, ODI, on average, appears to have weaker correlations with other dMRI metrics, which may further suggest between-tract variability of this measure of fibre orientation. Indeed, in the uncinate, inferior fronto-occipital fasciculi, cingulum cingulate gyri and corticospinal tracts, ODI is a part of the second cluster of positively correlated metrics, while it has very low correlations with other metrics in the inferior longitudinal fasciculi and the anterior thalamic radiation and is negatively associated with all other dMRI metrics in the genu of the corpus callosum. AD and ISO correlations with NDI, FA and ODI appear considerably weaker on average, possibly also due to variations in the dMRI metric covariance structure between tracts. Together, our results suggest that similarly to what is observed in adults and children (Chamberland et al., 2019; De Santis et al., 2014; Geeraert et al., 2020), the interdependence of dMRI measures is already present at birth.

Interestingly, however, the correlation patterns between the dMRI metrics vary in neonates compared to older children and adults. For example, whilst we observed positive correlations between AD and RD in neonates, these two measures of water diffusivity are negatively correlated in older children and adults (Chamberland et al., 2019; De Santis et al., 2019). Furthermore, these previous studies reported strong positive correlations between FA and AD, while in neonates, we observe a negative correlation between these metrics.

These differences could reflect maturation of the tracts with age. Yet, some other patterns appear similar across the life course such as the positive correlations between FA and NDI and their negative association with MD, as well as the overall weak correlation between ODI and other measures of white matter microstructure (Chamberland et al., 2019; Cox et al., 2016; De Santis et al., 2014; Geeraert et al., 2020).

We found that a considerable proportion of variance is shared across the dMRI metrics in neonates, which confirms previous observations in children and adolescents (Chamberland et al., 2019; Geeraert et al., 2020). We used this shared variance to derive multimodal g-factors of white matter microstructure using PCA as a data reduction technique. We found that two PCs explained a substantial amount (almost 94%) of the covariance among the seven DTI and NODDI metrics across 16 white matter tracts. In older children and adolescents, Chamberland et al. (2019) reported that 80% of variance in diffusion metrics was captured by two PCs and Geeraert et al. (2020) reported that three PCs were needed to capture 80% of variance diffusion and myelin-related metrics. Similarly in adults, three PCs have been shown to capture 80% of variance in white matter diffusion, myelin and structural metrics (De Santis et al., 2014). Thus, it appears that the diffusion properties of white matter tracts may be shared to a higher extent in neonates compared to older children and adults, which could reflect refinement of tract diffusion properties in development.

The interpretation of PCs derived from multiple input parameters requires consideration of the contribution and direction of individual metrics, and given that the PCs are orthogonal, they represent two uncorrelated aspects of the covariance between diffusion metrics. Here, multimodal PC1, which accounts for the largest proportion of variance in the data (60%), consists of measures sensitive to magnitude of diffusivity (RD and MD). In contrast, multimodal PC2, which accounts for 34% of variance, consists of measures of free water (ISO) as well as diffusion anisotropy (AD, FA). Therefore, as has been reported in children and adults, the main axes of covariance consist of dMRI metrics that share similarities in their sensitivity to different tissue properties (diffusivity and anisotropy) (Chamberland et al., 2019; De Santis et al., 2014; Geeraert et al., 2020); although the exact contributions of different parameters vary, which could be due to different white matter measures used in the studies. Including parameters that are more sensitive to myelin such as those from

magnetisation transfer imaging could further help to disentangle the microstructural properties of white matter in the developing brain.

We were then interested in testing whether the derived g-factors can be used as global measures to characterise atypical white matter development associated with low GA. After adjusting for age at scan, we report that gFA was positively and gMD, gAD and gRD negatively associated with GA at birth. Thus, we replicate previous results in a larger independent cohort and across more tracts (Telford et al., 2017). In addition, here we report significant negative association between GA at birth and gISO, which had the strongest correlation with GA at birth among the DTI and NODDI g-factors. Thus, those infants born preterm exhibit less coherent, but a greater magnitude of water diffusion across the major white matter tracts in the brain compared to term-born controls. Interestingly, despite the substantial variance reported in NDI and ODI across white matter tracts, gNDI and gODI are not significantly associated with GA at birth. This could indicate that these two metrics capture more specific aspects of tract composition, which may be less meaningful at a global level.

These results together suggest generally lower white matter integrity and higher water diffusivity in infants born preterm compared to term, and are in line with findings obtained using other analysis approaches such as tract-based spatial statistics (Barnett et al., 2018; Thompson et al., 2019) or tract-specific analyses (Pecheva et al., 2017). We used structural equation modelling to test whether the common variance shared among all tracts is sufficient to explain differences between infants born at varying GA. We found that the tract-specific (independent pathways) model is significantly better than the common model, suggesting there is incrementally valid, low level information for GA at birth contained in the unique tract-specific microstructural properties. However, it has to be noted that this model included the highest number of paths, and the residual variance that cannot be accounted for by the common factor constitutes both tract-specific aspects of microstructure and measurement error. We could not reliably detect any additional tract-specific pathways to be substantially more informative for GA at birth compared to the general/common factor, suggesting that the most parsimonious model, in which GA at birth affects the global/shared variance of the tracts, offers valuable distillation of the between-person differences in white matter microstructure that are pertinent for GA variability.

The prediction modelling results revealed that the single-metric g-factors (except for gNDI and gODI) achieved preterm vs term classification accuracy significantly higher than chance, but the classification accuracy was relatively low. It could be hypothesised that preterm birth has a diffuse effect on white matter microstructure, which is better captured by methods that do not rely on anatomically constrained regions (e.g. peak width of skeletonised metrics (Baykara et al., 2016; Blesa et al., 2020)). Nevertheless, the g-factors could carry information beyond the simple dichotomy of term vs preterm birth and could be useful for investigating other environmental or genetic/epigenetic exposures that are hypothesised to affect global white development (Boardman et al., 2014; Boardman and Counsell, 2019; Krishnan et al., 2017; Wheater et al., 2022), or for predicting neurocognitive outcomes as previously reported in adults and children (Cox et al., 2019, 2016; Lee et al., 2017; Penke et al., 2010).

We also report that the multimodal g-factors associate with GA at birth, which, given the correlations of the dMRI metrics with the multimodal g-factors, give a similar interpretation of the effect of GA at birth on dMRI metrics. However, despite the significant association, the preterm vs term classification accuracy achieved using the multimodal g-factors was, similarly to single-metric g-factors, relatively low. Interestingly, however, we achieved the greatest classification accuracy when combining all single metric g-factors together in one prediction model. These results may imply that despite global covariance of dMRI metrics in neonates, each one carries information on specific (and additive) aspects of the underlying microstructure that differ in preterm compared to term subjects. It is important to acknowledge that the model combining all single metric g-factors is by far the least parsimonious model tested, and increasing the number of predictors could artificially inflate the estimation of prediction accuracy. However, the combined single g-factor prediction model is by far the most successful one and we have used cross-validation with the aim to minimise bias and militate against the artificial inflation.

3.7 Conclusion

In this work, we extracted tract-averaged DTI and NODDI metrics from 16 major white matter tracts in 220 neonates of wide-ranging GA at birth. We then applied PCA as a data reduction technique to derive single- and multimodal general factors of white matter microstructure. These g-factors explained substantial variance within and between DTI and

NODDI metrics across white matter tracts and associated with GA at birth. Combining single-metric g-factors together in one prediction model achieved discriminating power between term and preterm infants. This framework for a principled approach for dMRI data reduction may be useful for investigating the upstream determinants and neurocognitive consequences of diseases characterised by atypical white matter development.

3.8 CrediT authorship contribution statement

Kadi Vaher: Conceptualization, Methodology, Formal analysis, Investigation, Data Curation, Writing Original Draft, Visualization, Funding acquisition; **Paola Galdi**: Conceptualization, Methodology, Formal analysis, Writing - Original Draft; **Manuel Blesa Cabez**: Methodology, Software, Writing - Original Draft; **Gemma Sullivan**: Investigation, Resources, Writing - Review & Editing; **David Q Stoye**: Investigation, Resources, Writing - Review & Editing; **Alan J Quigley**: Investigation, Resources; **Michael J Thrippleton**: Methodology, Software, Resources, Writing - Review & Editing; **Debby Bogaert**: Conceptualization, Supervision, Writing - Review & Editing; **Mark E Bastin**: Methodology, Software, Validation, Writing - Review & Editing; **Simon R Cox**: Conceptualization, Methodology, Writing - Review & Editing; **James P Boardman**: Conceptualization, Methodology, Writing - Original Draft, Supervision, Funding acquisition.

3.9 Chapter conclusion

This study demonstrated that a single g-factor captured substantial variance of DTI and NODDI metrics across 16 white matter tracts and two multi-metric factors captured over 90% of variance shared between DTI and NODDI metrics. The single- and multi-metric g-factors significantly correlated with GA at birth, however, prediction accuracy achieved for term vs preterm classification task only marginally exceeded chance with individual g-factors. The highest accuracy was achieved when all single-metric g-factors were used as predictors, and given the more straightforward interpretation in comparison to multi-metric g-factors, this motivated the use of single-metric g-factors in subsequent analyses integrating MRI and gut microbiota data in Chapter 6. Furthermore, single-metric g-factors have shown utility for functional outcomes as they associate with cognitive abilities both in adults and children born at term (Alloza et al., 2016; Lee et al., 2017; Penke et al., 2010), while multi-metric g-factors did not correlate with reading abilities in children (Geeraert et al., 2020). The extent to which these findings of neurocognitive outcomes can be translated to preterm population remains to be determined in future studies, although social cognition at 7 months was not associated with neonatal single-metric g-factors in preterm infants (Telford et al., 2017). Single-metric g-factors in preterm infants were recently shown to associate with a DNA methylation measure of inflammation (Conole et al., 2022), suggesting they are useful in studying early life factors associated with adverse outcomes in children born preterm. The microbiota may be one of those early life factors.

Chapter 4. Microbiome-gut-brain axis in brain development, cognition and behaviour during infancy and early childhood

4.1 Chapter introduction

In the previous chapter, I investigated whether whole brain measures may be utilised to capture white matter dysmaturation following preterm birth. These measures may be useful to study covariates related to brain development in preterm infants, such as the neonatal gut microbiota.

Indeed, the gut microbiota is increasingly recognised as the modulator of brain and behaviour. The first five years of life see the most rapid development of both the brain and the microbiota; however, the relationship between these two systems during the critical phase in development is not well understood. In this chapter, I carry out a narrative review to examine the evidence that gut microbiota diversity and community composition are associated with human brain development, indexed by neurodevelopmental assessments and/or neuroimaging, in typically developing pre-school children.

This chapter appears as a publication in *Developmental Review*:

Vaher, K., Bogaert, D., Richardson, H., & Boardman, J. P. (2022). Microbiome-gut-brain axis in brain development, cognition and behavior during infancy and early childhood. Developmental Review, 66, 101038. https://doi.org/10.1016/J.DR.2022.101038

As first author, I designed the search protocol, carried out the search and subsequent data extraction and synthesis, and drafted and wrote the manuscript. The CrediT authorship contribution statement is provided at the end of this chapter.

4.2 Abstract

The gut microbiota is increasingly recognised as a modulator of brain and behaviour but its role in early childhood, when the microbiome and the brain are both undergoing rapid development, is poorly understood. Preclinical work suggests there are critical windows during early life when bacterial signals are required for normal neurobehavioural development, whereas gut microbial dysfunction has been observed in patients with certain neurodevelopmental disorders. Here, we review the evidence that gut bacterial diversity and community composition affect brain structure/function and behaviour in typically developing pre-school children. Following narrative synthesis, we report that twenty studies suggest the microbiome-gut-brain axis may operate across three domains in infancy and early childhood: general neurocognitive development, socio-emotional behaviours, and brain structure and function inferred from neuroimaging. However, there is substantial variation in the bacteria-brain/behaviour relationships reported. We identified sources of clinical and methodological heterogeneity in the studies, including participant characteristics, small sample sizes, variations in DNA extraction and sequencing, and statistical analysis approaches. We propose that harmonisation of sample collection and data processing pipelines, longitudinal assessments, and mechanistic insights from whole metagenome analyses could improve understanding of the role of gut microbiome in brain development during early development. This will also promote comparability between studies and increase study power by allowing for meta-analyses. Greater knowledge of the role of gut microbiome in brain development may ultimately offer new avenues for promoting brain health in early life.

4.3 Introduction

Human life has never existed without microbes. The human body hosts an array of microorganisms, with the majority and most diverse set of microbes, including bacteria, viruses and fungi, present in the gastrointestinal tract. These, especially bacteria, play an important part in a range of physiological functions within the body, including host metabolism and immunity. The past few decades have seen an explosion of research into the bacterial influence on the brain via the microbiome-gut-brain axis (Cryan et al., 2019).

During the first few years of postnatal life the brain undergoes substantial changes, including increases in tissue volume and cortical complexity, organization of white matter

fibres, and myelination, with different regions and networks maturing at different rates (Gilmore et al., 2018; Figure 4-1). These changes underpin the acquisition of language, motor and social skills. In parallel to these intense periods in brain and cognitive development, the gut microbiome, which is rapidly populated at birth, similarly follows a dynamic developmental trajectory (Bäckhed et al., 2015; Reyman et al., 2019; Roswall et al., 2021; Yatsunenko et al., 2012; Figure 4-1). This has led to the notion of nested sensitive periods when brain development interacts with peripheral system development, in this case the gut microbiome, to shape the emergence of complex behaviours including learning and memory (Callaghan, 2020). It has been suggested that signals from the microbiome are required for certain aspects of brain development (Cowan et al., 2019; Stilling et al., 2014) and recent data from preclinical models support the theory that bacterial signals during critical windows in early life are required for typical neurobehavioural development (Buffington et al., 2016; Chu et al., 2019; Clarke et al., 2013; Diaz Heijtz et al., 2011; Sudo et al., 2004). Conversely, disruption of the microbiome development during these periods could impact concurrently developing cognitive functions (Callaghan, 2020; Cowan et al., 2019).

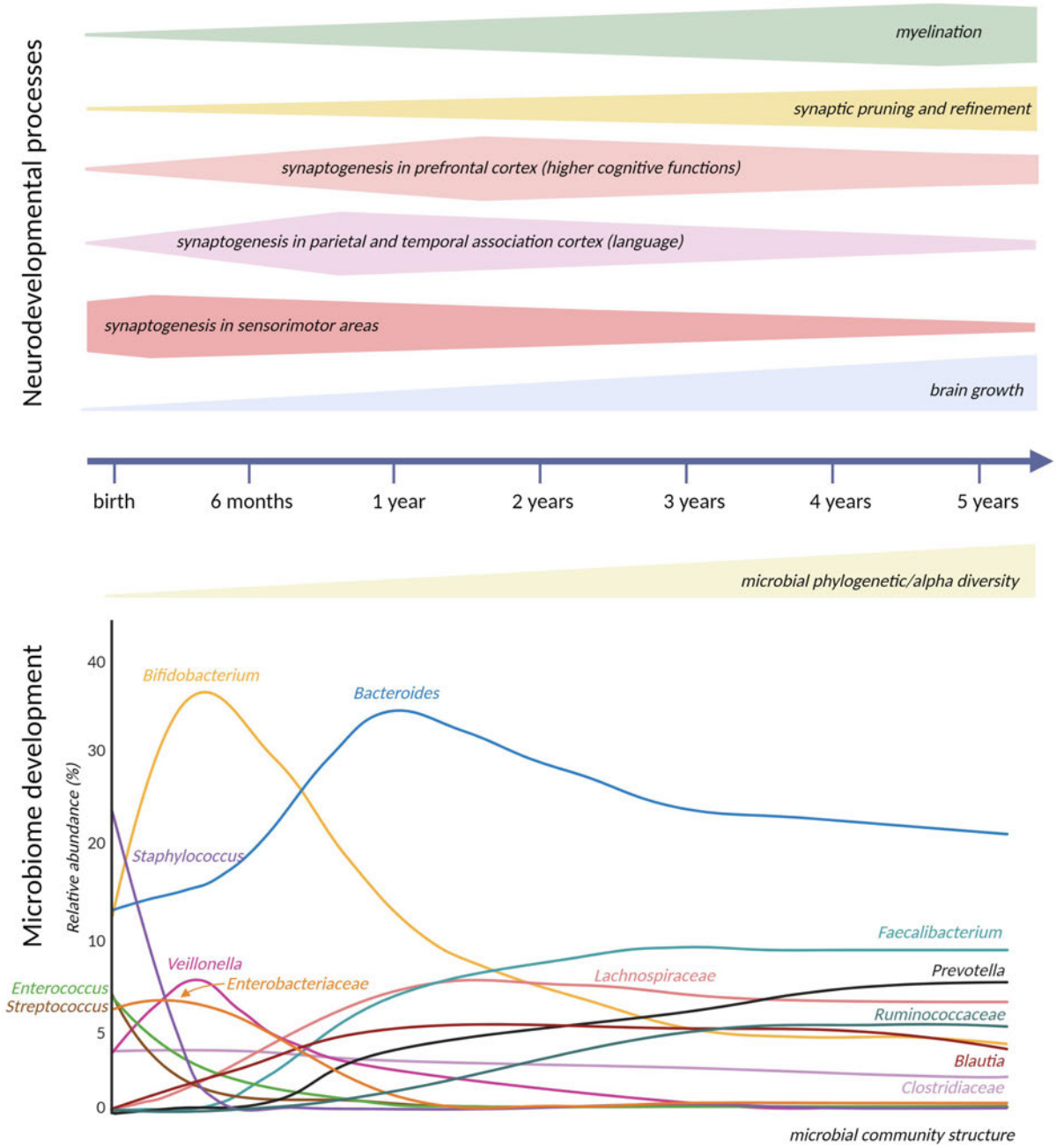


Figure 4-1. Development of the brain and microbiome during the first five years of life.
The top panel illustrates the time course of key neurodevelopmental processes that underpin the acquisition of language, motor and social skills, and that could serve as neurobiological mechanisms linking gut microbiome with neurodevelopmental outcomes; adapted from (Casey et al., 2005; Semple et al., 2013; Shonkoff and Phillips, 2000)*; the height of the diamonds signifies the intensity/peak of the process. The bottom panel illustrates the developmental trajectories of the gut microbiome alpha diversity and community composition. Colonization pattern based on data presented in (Roswall et al., 2021), focusing on the most abundant taxa across the first five years of life. Created with BioRender.com.*

Clinical investigation provides indirect evidence for a putative role of the gut microbiome in human brain development. First, disrupted gut microbiome profiles are reported in individuals with autism spectrum disorder and attention deficit-hyperactivity disorder (Jurek et al., 2020). Second, important drivers of the microbiome development in early life (e.g.

breastfeeding, delivery via *Caesarean*-section (C-section)) are associated with cognition as well as aspects of brain development as assessed by magnetic resonance imaging (MRI) (Anderson et al., 1999; Bauer et al., 2019; Belfort et al., 2016; Blesa et al., 2019; Deoni et al., 2019, 2018, 2013; Kar et al., 2021; Luby et al., 2016; Ottolini et al., 2020; Polidano et al., 2017). However, explorations into direct associations between gut microbial composition and neurocognitive and behavioural measures in early childhood have only emerged in the last 5-6 years. In this review, we evaluate the evidence that gut bacterial diversity and composition may affect human brain development. Here, we specifically examine features of the gut microbiome derived from bacterial DNA sequencing that are associated with brain development, indexed by quantitative neuroimaging, and/or neurobehavioural outcomes in typically developing children from infancy to 5 years.

4.4 Material and methods

We searched PubMed, Web of Science and Embase to identify studies that integrated bacterial DNA sequencing and quantitative neuroimaging or neurobehavioural assessments until June 2021. The search strategy used combined keywords describing gut microbiome with terms targeting: i) neuroimaging and brain features; or ii) child neurocognitive and behavioural outcomes (see Supplementary material for search details). Studies were included if they reported data from human participants between birth and 5 years without a diagnosis of a neurodevelopmental disorder at the time of recruitment, and if they directly investigated relationships between gut microbiome features derived from bacterial DNA sequencing (metagenomics or 16S-based) and quantitative neuroimaging or neurobehavioural assessments. We considered publications using any neuroimaging modality, and neurobehaviour could include general cognitive ability, motor development, social cognition and development, attention, language, communication, emotion recognition or temperament derived from direct observation or parent/teacher report. Single case studies, case-control studies solely focusing on microbiota differences between healthy/neurotypical and children with neurodevelopmental disorders, non-English language studies, and abstracts were excluded. Results are synthesised in tabular format for microbiota associations with measures of i) neurocognitive development, ii) socio-emotional behaviours/temperament, and iii) neuroimaging.

4.4.1 Risk of bias assessment

In the absence of a validated quality assessment tool for studies linking microbiome data with neurobehavioural and neuroimaging data, we summarised potential sources of bias arising from methodological heterogeneity between studies based on the STORMS guidelines (Mirzayi et al., 2021). We extracted data on the following study characteristics: age of participants at microbiome and neurodevelopmental assessments, participant exclusion criteria, bacterial DNA extraction method, sequencing methodology, microbiota features of interest, use of validated neurodevelopmental outcome assessments, neuroimaging modality and use of region-of-interest or whole-brain analyses, statistical models linking gut microbiota features with neurodevelopmental outcomes, adjustment for covariates, and correction for multiple comparisons (Supplementary Table 4-1).

4.5 Results and discussion

Our search yielded a total of 20 publications that associated features of gut bacterial composition with neurodevelopment indexed by neuroimaging and/or behavioural assessments. Due to the emerging nature of the field, all studies were published after 2015. We provide a synthesis of the studies structured around three domains: general neurocognitive development, socio-emotional behaviour and temperament, and brain structure and function inferred from neuroimaging. An overview of the methodologies in the included studies is presented in Table 4-1. We also refer the reader to Table 4-2 for a brief explanation of the terminology commonly used for gut microbiome measurement and characterisation.

Table 4-1. Overview of studies.
Note that some studies included multiple measures of neurodevelopment: of the 7 studies that assessed general neurocognitive development, one also reported microbiome correlations with socio-emotional behaviours and one reported microbiome correlations with neuroimaging; of the 13 studies that assessed socio-emotional behaviours, two also reported microbiome correlations with neuroimaging.

Neurodevelopmental domain; number of studies	**Assessment/measure used for microbiome-brain/behaviour associations**	**Gut microbiome sequencing method**
General neurocognitive development n=7	1 Mullen Scales of Early Learning (MSEL) 1 Gesell Development Inventory (GDI) 2 Ages and Stages Questionnaire (ASQ) 3 Bayley Scales of Infant Development (BSID)	7 16S rRNA gene sequencing
Socio-emotional behaviours n=13	5 Infant Behaviour Questionnaire (IBQ-R) 3 Child Behaviour Checklist (CBCL) 1 Social Responsiveness Scale (SRS-2) 1 Early Childhood Behaviour Questionnaire (ECBQ) 1 NICU Network Neurobehavioural Scale (NNNS) 1 Perceived Stress Scale for Children (PSS-C) 1 Emotional attention (using eye tracking) 1 Observational fear behaviour (Mask Task and Strange Situation)	2 metagenomics 12 16S rRNA gene sequencing
Structural and functional neuroimaging features n=4	2 structural MRI 1 functional MRI 1 functional NIRS	1 metagenomics 3 16S rRNA gene sequencing

Table 4-2. Glossary of terminology used for measuring and characterising the gut microbiome.

Term	Explanation
Sequencing methods	Gut microbiome is most commonly sequenced using one of two methods: • 16S ribosomal RNA (rRNA) amplicon-based sequencing utilises the conserved and hypervariable regions within the bacterial 16S rRNA gene. The conserved regions serve as primer binding sites to amplify the (fragments of) 16S rRNA gene and the hypervariable regions that contain species-specific information are used to differentiate between different bacteria and identify the composition of the microbiota (what bacteria are there). • Metagenomic shotgun sequencing involves sequencing the entire DNA as opposed to the 16S rRNA marker gene. This method enables both classification of bacteria as well as identification of their functional potential (what they are doing).
Alpha diversity	The diversity of bacteria within a sample. Alpha diversity can be calculated using different measures that differentially take into account the richness (i.e. number of different bacterial groups; higher number = higher diversity) and/or evenness (i.e. distribution of abundances of the groups; more similar distributions = higher diversity). Some measures additionally take into account the phylogenetic relatedness of the community members (closely related bacteria = less diversity). Common measures of alpha diversity include Shannon index, Simpson index, Chao1, Faith's Phylogenetic Diversity.
Beta diversity	The diversity of bacteria between samples. Similar to alpha diversity, beta diversity can be calculated using different measures that take into account the presence/absence of species, abundance of species and/or value placed on rare species. Beta-diversity is calculated as a similarity/dissimilarity matrix and commonly presented in a principal component analysis (or similar reduced dimensionality) plot: if two samples are further apart on this plot, they have high beta diversity. High beta diversity between samples generally means that the community composition is highly different between these samples. Beta diversity is commonly used as a marker of overall bacteria compositional differences between study participants. Common measures of beta diversity calculation include Bray-Curtis dissimilarity, UniFrac distances, Aitchison distance.

Bacterial composition	In most instances, researchers are interested in which bacteria correlate with the outcomes of interest. This question is most commonly answered using one of the following approaches:
	• Individual taxa level analyses where relative abundances of specific bacterial taxa are associated with variables of interest. This analysis can be conducted on different hierarchical levels in the taxonomic classification (Phylum->Class->Order->Family->Genus->Species->Strain).
	• Dimensionality reduction analyses where:
	  ○ the samples from study participants are clustered into groups based on the similarities in community composition; these clusters can then in turn be described by the dominant and/or discriminative bacteria
	  ○ the abundances of bacteria are represented as factors or co-abundance groups where each can be described by the increasing/decreasing abundance of specific taxa; study samples/participants in turn receive a score along these factors/co-abundance groups which are assessed for correlations with outcomes of interest

4.5.1 Gut microbiome associations with general neurocognitive development in early childhood

We identified seven studies which investigated associations between microbiota composition and general neurocognitive development in typically developing children (Table 4-3). In all seven studies, the gut microbiota was assessed using 16S rRNA amplicon sequencing. The studies used different measures to assess neurocognitive development,

including direct observation with the Mullen Scales of Early Learning (MSEL), Gesell Development Inventory (GDI), and the Bayley Scales of Infant Development (BSID); and parent/caregiver report using the Ages and Stages Questionnaire (ASQ). Two of the studies investigated the potential longitudinal effects of the gut microbiota composition in the first six months after birth on neurodevelopmental outcomes at age 2 to 3 years (Rozé et al., 2020; Sordillo et al., 2019), three studies investigated cross-sectional associations between gut microbiota and neurodevelopment in 18-month-old (Acuña et al., 2021) or 3-year-old children (Rothenberg et al., 2021; W. Zhang et al., 2021), and two studies used a combination of longitudinal and cross-sectional approaches where either cognitive outcomes (Carlson et al., 2018) or gut microbiota (Kort et al., 2021) were assessed at multiple timepoints. Six of these studies included children born within a normal range of gestation and one studied preterm infants (Rozé et al., 2020). We discuss the results below, starting from the earliest timepoints at which microbiome sampling was conducted.

The two longitudinal studies showed that gut microbiome composition in early infancy associates with neurocognitive outcomes later in childhood. Specifically, by applying data reduction to bacterial composition data from young infants (3-6 months of life), Sordillo et al. identified four bacterial co-abundance groups and found that the factor representing increased abundance of *Bacteroides* and decreased abundance of *Escherichia/Shigella* and *Bifidobacterium* negatively correlated with fine motor skills, while the factor representing increased abundance of different *Lachnospiraceae* and *Clostridiales* taxa and decreased abundance *Bacteroides* negatively correlated with communication as well as personal and social skills at 3 years of life (Sordillo et al., 2019). Their taxa-level analyses suggested similar results on the order- and family-level, although the genera-level associations were often different. In particular, similar to the associations with the co-abundance groups, different genera in *Clostridiales* order (e.g. *Ruminococcus, Oscillospira, Acidaminococcus*) were less abundant in children with typically developing communication and social/personal skills compared to those with potential atypical or delayed development. However, the results for motor development were different from the co-abundance group results, showing *Streptococcus* (*Lactobacillales* order) and *Klebsiella* (*Enterobacteriales* order) to have the strongest positive and negative associations with motor scores, respectively, highlighting the effect of methodological variability on results. These latter findings are interesting as

increased abundance of *Streptococcus* and *Klebsiella* in early infancy has also been associated with C-section birth (Reyman et al., 2019; Shao et al., 2019). The second longitudinal study was carried out in very and extremely preterm infants: Rozé and colleagues identified 5 clusters of preterm infants defined by their bacterial composition in the neonatal period, of which the *Enterococcus*- or *Staphylococcus*-dominated clusters were at higher risk for non-optimal 2-year neurodevelopmental outcome (defined as death or developmental delay (ASQ score <185)) compared to *Escherichia/Shigella-, Enterobacter*- or *Clostridium*-dominated clusters (Rozé et al., 2020). These cluster-level results were also in part paralleled by taxa-level analyses as a *Staphylococcus* species (*Staphylococcus caprae*) negatively and *Escherichia coli* positively correlated with ASQ scores (Rozé et al., 2020).

These results together suggest a potential role of increased abundance of *Escherichia/Shigella* and *Enterobacter* in early infancy for optimal neurocognitive development later in childhood, while the relationships with other bacteria (e.g. with members of *Clostridiales* order (*Ruminococcus, Clostridium*)) in this time period differ between the two studies. The differences could, at least in part, be explained by differences in study populations, timing of stool sampling, or other design features. Thus, further studies are required to determine the generalizability of these observations across populations.

Interestingly, the clusters characterised as having high abundance of *Enterococcus* or *Staphylococcus* mostly included infants born at an earlier gestational week (Rozé et al., 2020). This suggests that the composition of extremely preterm infants' gut microbiota might be more predictive of adverse neurodevelopmental outcomes (as compared to very preterm infants), and that the disrupted gut microbiota composition in extremely preterm neonates might have a larger effect on later neurodevelopmental outcomes compared to infants born at a later gestational week (Rozé et al., 2020).

Moving on from early infancy, Carlson et al. investigated whether gut bacterial composition in 1-year-old children correlates with neurocognitive development at the same time and/or at 2 years of life (Carlson et al., 2018). They identified three clusters of infants defined by their bacterial composition at 1 year of life, and found that the MSEL Early Learning Composite as well as expressive and receptive language subscale scores at 2 years of life were highest in the *Bacteroides*- and lowest in the *Faecalibacterium*-dominated clusters, but

they found no differences between the clusters in cross-sectional, 1-year MSEL scores. These results are consistent with the findings in Sordillo et al. (2019), where the co-abundance group characterised by decreased abundance of *Bacteroides* showed negative correlations with communication and social development scores, suggesting a positive effect of increased *Bacteroides* during the first year of life on language/communication development later in childhood. *Bacteroides* is one of the most abundant bacterial taxa in the human gut, reaching its highest abundance around 1 year of life and then stabilizing to adult levels around 3-5 years of life (Figure 4-1; Roswall et al., 2021). Increased abundance of *Bacteroides* in early infancy has also been associated with vaginal delivery (Bäckhed et al., 2015; Reyman et al., 2019; Shao et al., 2019) as well as with the cessation of breastfeeding around the first year of life (Bäckhed et al., 2015). Taken together, these results suggest that gut microbiome composition during the first year of life may be associated with neurocognitive outcomes at 2 and 3 years of life although the specific bacterial composition underlying these relationships varies between studies, possibly due to the different ages and populations studied.

In a cross-sectional study of 18-month-old children, Acuña et al. (2021) found that gut microbiota community structure was associated only with fine motor skills as assessed by BSID (3rd edition). By clustering infants into two community types, they observed that infants with higher fine motor scores were more likely to have *Firmicutes*-dominant (high abundance of *Lachnospiraceae, Streptococcus* and *Blautia*) compared to *Bacteroides*-dominant community type. Acuña et al. additionally demonstrated positive associations of fine motor scores with the abundance of *Bifidobacterium, Corprococcus, Enterococcus, Lactobacillus, Holdemanella, Roseburia* and *Veillonella,* and negative associations with the abundance of *Parabacteroides*. These results are partly consistent with the results by Sordillo et al. (2019), suggesting that increased abundance of *Bacteroides* in infancy and toddlerhood has negative effects on motor development, while *Bifidobacterium* and *Streptococcus* abundances may have beneficial effects on motor development in early childhood (Sordillo et al., 2019). These observations raise the possibility that some bacteria (e.g. *Bacteroides*) could have differential effects according to cognitive domain (e.g. language vs motor) and over time.

Gut microbiota composition during the second year of life may also be predictive of cognitive outcomes at 3 years. To understand how gut microbiota affects language development in 3-year-old children, Kort and colleagues used prediction modelling and identified that the best predictive model for 3-year language scores included language scores at 2 years, abundance of *Coprococcus* (from *Lachnospiraceae* family) at 2 years, and *Bifidobacterium* at 3 years of life, which all positively correlated with 3-year language scores (Kort et al., 2021). In individual taxa-level analyses, improved language at 3 years correlated with increased abundance of species in the genera of *Coprococcus, Bifidobacterium, Faecalibacterium, Roseburia, Bacteroides* and *Clostridium*, and decreased abundance of *Parabacteroides* and *Escherichia/Shigella*, among others, at 2 years of life. Importantly, these results provide further evidence that increased abundance of *Bacteroides* in the first two years of life positively correlates with language development, as reported previously (Carlson et al., 2018; Sordillo et al., 2019).

The oldest developmental timepoint at which relationships between gut microbiome profiles and cognitive outcomes have been studied is at the age of 3 years. Rothenberg et al. (2021) took a similar approach to Sordillo et al. (2019) in microbiome data reduction and found that the factor representing increased abundance of *Faecalibacterium*, *Clostridium* cluster *XIVa*, *Gemmiger*, *Phascolarctobacterium*, *Alistipes*, *Oscillibacter*, and *Sutterella*, and decreased abundance of *Blautia*, *Anaerostipes*, *Clostridium* cluster *XVIII*, and *Streptococcus* positively associated with both mental and psychomotor development assessed by the BSID (2^{nd} edition) in 3-year-old children. These results were partly consistent with taxa-level analyses as *Faecalibacterium* and *Gemminger* positively associated with psychomotor development, and *Faecalibacterium* and *Clostridium XlVb* positively associated with mental development. These results are also partly consistent with those observed by Kort et al. (2021) who reported that increased abundance of *Faecalibacterium* associates with improved cognitive (language) development. However, these results are in contrast to those observed in the study by Carlson et al. (2018), where high abundance of *Faecalibacterium* pointed towards lower cognitive and language scores, potentially suggesting that increasing abundance of *Faecalibacterium* after the first year of life has a positive effect on cognitive development while higher abundance of *Faecalibacterium* before that time may be unfavourable. Indeed, *Faecalibacterium* abundance in the gut starts to increase after 6

months of life, reaching adult levels around 3 years (Figure 4-1; Roswall et al., 2021), allowing the speculation that exposure to a higher abundance of *Faecalibacterium* too early could be unfavourable for cognitive outcomes.

Interestingly, taxa-level analyses in Rothenberg et al. (2021) additionally identified *Lachnospiraceae* abundance as negatively associated with cognitive development, which is in line with the findings in the study by Sordillo et al. (2019) where the co-abundance factor represented by increased abundance of *Lachnospiraceae* species negatively associated with communication and social skills. However, these results conflict those observed by Kort et al., who reported that members of *Lachnospiraceae* family (*Coprococcus, Roseburia*) at 2 years of life positively associated with language development at 3 years (Kort et al., 2021). The results regarding *Streptococcus* demonstrated by Rothenberg et al. (2021) also differ to those reported by Sordillo et al. (2019) and Acuña et al. (2021), who showed that higher abundance of *Streptococcus* associated with improved motor development, suggesting that early colonization with *Streptococcus* correlates with optimal neurocognitive development, but later in development increased abundance of *Streptococcus* might have a negative effect on neurodevelopment. *Streptococcus* abundance is relatively high in newborns, but decreases rapidly over the first few months of life (Figure 4-1; Roswall et al., 2021), suggesting that higher abundance of *Streptococcus* later in childhood is suboptimal.

A small study of 38 3-year-old children explored the associations between gut microbiome composition and neurodevelopment focused on different bacterial taxonomy levels and identified members of the *Firmicutes* phylum (e.g. *Ruminococcus*, *Hungatella*) to be positively and members of *Bacteroidetes* phylum (e.g. *Porphyromonas, Butyricimonas*) to be negatively associated with different subscales, particularly the motor scale, in the Gesell Developmental Inventory (W. Zhang et al., 2021). These results thus partly parallel those reported by Acuña et al. (2021) and suggest that members of the *Firmicutes* phylum may be positively associated with motor development, however, the specific genera- and species-level associations vary.

Four studies additionally evaluated associations between bacterial alpha diversity (within-sample diversity) and neurocognitive outcomes. Carlson et al. (2018) studied microbiota diversity in 1-year-old children and reported a negative association between different measures of alpha diversity and MSEL Early Learning Composite as well as expressive

language and visual reception subscale scores at 2 years of life while, similarly to cluster analyses, they did not observe any cross-correlational associations with 1-year cognitive scores. Relationships between bacterial alpha diversity and neurodevelopment were tested in three other studies (Acuña et al., 2021; Rothenberg et al., 2021; Sordillo et al., 2019), and no significant associations were found between 3-year neurocognitive scores as assessed by the parent-reported ASQ (Sordillo et al., 2019) or observational BSID at 18 months (Acuña et al., 2021) or 3 years (Rothenberg et al., 2021) and 5-month, 18-month or 3-year microbial diversity, respectively. However, the interpretation of these results is complicated as different alpha diversity metrics differentially take into account the richness and evenness (see Table 4-2) of microbial community composition.

4.5.2 Gut microbiome associations with socio-emotional behaviours

Thirteen studies have investigated associations between gut microbiome and socio-emotional development, including temperament, behavioural dysregulation, social behaviour, and stress (Table 4-4). Eleven of these studies have assessed the microbiota composition using 16S rRNA gene sequencing (Aatsinki et al., 2020, 2019b; Carlson et al., 2021; Christian et al., 2015; Fox et al., 2021; Loughman et al., 2020a, 2020b; Sobko et al., 2020; Sun et al., 2020; Y. Wang et al., 2020; W. Zhang et al., 2021), one study used whole metagenome sequencing (Kelsey et al., 2021), and one study used both approaches (Laue et al., 2020). The majority of these studies used questionnaires to characterise behavioural development (Infant and Early Childhood Behaviour Questionnaire, Child Behaviour Checklist (CBCL), Social Responsiveness Scale, Perceived Stress Scale for Children); and three have used direct observational behavioural measures (NICU Network Neurobehavioural Scale, emotional attention paradigm using eye-tracking, and laboratory fear paradigms (Mask Task and Strange Situation)). The results are discussed below, arranged based on the domain of socio-emotional behaviour studied: temperament, fear, behavioural dysfunction, and other traits (social development and stress).

4.5.2.1 Temperament

Infant temperament, defined as stable traits of reactivity and regulation (Rothbart and Gartstein, 2008), predicts social and attention behaviours in childhood (Abulizi et al., 2017) as well as personality and psychopathology in adulthood (Tang et al., 2020). Five studies have investigated correlations between gut microbiome composition and temperament

using the Infant or Early Childhood Behaviour Questionnaires (IBQ or ECBQ). Three of these studies used cross-sectional approaches whereby gut microbiome and temperament were measured at the same age, ranging from 1 month of life to 1- and 2-year-old children (Christian et al., 2015; Kelsey et al., 2021; Y. Wang et al., 2020), one study aimed to characterise the predictive value of gut microbiota at early infancy (2.5 months) to temperament in later infancy (6 months) (Aatsinki et al., 2019b), and one study investigated the relationship between gut microbiota at multiple timepoints during the first year of life and temperament in 1-year-old children (Fox et al., 2021).

During the first month of life, Kelsey et al. (2021) demonstrated that different *Bifidobacterium* species were more abundant in infants with higher levels of regulation/orienting and negative emotionality, while *Streptococcus* and *Schaalia* were more abundant in infants with lower levels of negative emotionality. The abundance of *Bifidobacterium* species in early infancy (2.5 months of life) may also predict later temperament traits: Aatsinki and colleagues (2019) used cluster analysis and showed that a cluster of infants characterised by high abundance of *Bifidobacterium* and *Enterobacteriaceae* had the highest levels of orienting/regulation compared to the clusters characterised by high abundance of *Bacteroides* or *Veillonella*. These cluster-based results are consistent with bacterial-taxa-level analyses as unidentified *Bifidobacterium* species positively correlated with regulation/orienting, surgency, and fear reactivity. Aatsinki et al. found several other bacterial taxa associated with temperament traits, including negative correlations between species in *Clostridiaceae* family or *Bacteroides* and negative emotionality, and between and *Streptococcus* and fear reactivity. These results lend further support to the notion that *Streptococcus*, *Bacteroides* as well as members of the families *Enterobacteriaceae* (e.g. *Enterobacter*) and *Clostridiaceae* (e.g. *Clostridium*) may play a role in neurocognitive development as discussed in the section 4.5.1 Gut microbiome associations with general neurocognitive development in early childhood.

Taking a longitudinal approach in exploring microbiota associations with temperament during the first year of life, Fox et al. identified early (1-3 weeks of life) and late infancy (12 months of life) as sensitive periods during which gut microbial community composition associated with temperament traits in 1-year-old children (Fox et al., 2021). Specifically, at 1-3 weeks of life, the abundance of *Bifidobacterium, Lachnospiraceae* and *Collinsella*

positively and the abundance of *Klebsiella* negatively associated with surgency/extraversion at 1 year of life, while the abundance of *Megamonas, Acidaminococcus* and *Ruminococcus* at 1 year of life positively and the abundance of *Lactobacillus* negatively associated with negative affectivity at the same age. In contrast, microbiota composition at 2 or 6 months of life was not associated with temperament traits in 1-year-old children (Fox et al., 2021), suggesting that gut microbiota composition during periods of early colonization and at 1 year of life, around cessation of breastfeeding, may exert effects on emotional/temperament development.

In line with this, Wang et al. additionally identified specific bacterial associations with temperament traits in 1-year-old children: the abundance of *Bifidobacterium* positively and the abundance of *Hungatella* negatively associated with different regulation/orienting subscales (Y. Wang et al., 2020). These results thus parallel the results in early infancy and suggest that the abundance of *Bifidobacterium* during the first few months of life (Aatsinki et al., 2019b; Fox et al., 2021; Kelsey et al., 2021) as well as at 1 year of life (Y. Wang et al., 2020) correlates with temperament traits (especially regulation/orienting and surgency/extraversion) during the first year of life. In contrast, *Bifidobacterium* levels did not correlate with temperament traits in 2-year-old children (Christian et al., 2015), suggesting that there may be temporal effects of specific bacterial taxa on temperament, in line with the temporal succession of gut microbiome (Figure 4-1); however, larger studies using longitudinal assessments of both microbiome and temperament are needed to test this hypothesis.

There may also be sex-specific associations between gut microbiota composition and temperament traits. For example, in 2-year-old children, several bacteria (*Ruminococacceae, Parabacteroides, Dialister, Rikenellaceae*) abundances correlated with surgency/extraversion only in boys (Christian et al., 2015), and in early infancy, the reported effects of *Bifidobacterium* on regulation and surgency (Aatsinki et al., 2019b) were not identified in girls in sub-group analyses. Conversely, in both studies gut microbiome composition in girls appears to be particularly correlated with fear reactivity, although different species-level associations were identified (Aatsinki et al., 2019b; Christian et al., 2015). Sex-specific effects of microbiota disruption on brain and behaviour in other contexts have been reviewed in detail elsewhere (for example see (Jaggar et al., 2020)).

In terms of the relationships between bacterial diversity and temperament, the studies report discrepant findings. While alpha diversity has been positively associated with surgency/extraversion in 2-year-old children (Christian et al., 2015), alpha diversity at 2 months negatively correlated with negative affectivity in 6-month-old infants (Aatsinki et al., 2019b) as well as in 1-year-old infants (Fox et al., 2021), although the latter result did not reach statistical significance. Conversely, there were no significant associations observed between alpha diversity and temperament traits during the first month of life (Kelsey et al., 2021). It could thus be hypothesised that, similarly to bacterial compositional effects, there may be temporal effects of alpha diversity on temperament though additional research is needed. Additionally, alpha diversity of the gut microbiota increases greatly over the first few years of life, especially around 4-6 months of life when solid foods are introduced to the diet (Reyman et al., 2019; Roswall et al., 2021), making comparisons of alpha diversity correlations with temperament traits at different ages difficult.

4.5.2.2 Fear-related behaviour

Whilst IBQ and ECBQ include fear reactivity as one of the subscales for negative affectivity trait, experimental approaches to study fear behaviour may reveal more specific information about development of fear processing during infancy. Indeed, preclinical studies suggest that microbiome is involved in fear- and anxiety-related behaviours (Diaz Heijtz et al., 2011; Hoban et al., 2018). Aatsinki et al. (2020) reported that increased attentional bias to fearful faces assessed by an emotional faces eye-tracking task correlated with decreased abundance of *Bifidobacterium, Lactobacillus, Prevotella* and *Haemophilus* and increased abundance of *Clostridium* at 2.5 months of life. These results seem in contrast to those observed by the same group previously (Aatsinki et al., 2019b) where abundance of *Bifidobacterium* positively and members of *Clostridiaceae* family negatively associated with parent-reported fear reactivity in early infancy, however, it is unclear to what extent negative emotionality/fear reactivity in temperament scales relates to attentional bias to fearful faces.

Moreover, in a small (n=14-19) pilot study, Carlson et al. (2021) reported that gut microbiota composition in 1-year-old children is associated with fear reactions in a non-social, but not social, fear paradigm. Specifically, they showed positive correlations between members of *Clostridiales* order, *Sutterella, Dialister* and *Erysipelotrichaceae* family and fear

reactions. Interestingly, they did not observe significant associations between gut microbiota and parent-reported fear behaviour (IBQ questionnaire), further suggesting that comprehensive assessment of fear processing is needed to fully understand the effects of gut microbiota on neurobehavioural and emotional development. Nevertheless, given the exploratory nature and small sample of this study, the results need to be replicated in a future study.

4.5.2.3 Behavioural problems

The CBCL is a parent reported questionnaire assessing a broad range of emotional and behavioural problems, and has been used in three studies investigating the relationships between gut microbiome and child development (Loughman et al., 2020b, 2020a; W. Zhang et al., 2021).

Loughman and colleagues used two cohorts in Australia to describe the relations between gut microbiome composition during various points during the first year of life (at 1, less than three, 6, and 12 months) and 2-year CBCL scores (Loughman et al., 2020b, 2020a). Gut microbiome composition at 1 month after birth did not significantly associate with behavioural dysfunction at 2 years (Loughman et al., 2020a), while several bacteria at around 2 months of life in a separate study were more or less abundant in children with behavioural problems at 2 years of life compared to those without behavioural problems (see Table 4-4) (Loughman et al., 2020b). For example, several *Streptococcus* and *Bacteroides* species were less abundant in children with behavioural problems (i.e. increased abundance of these bacteria associated with fewer behaviour problems). This is in line with the studies discussed in the section on Temperament as the increased abundance of these genera were also associated with decreased negative emotionality and fear reactivity in infancy (Aatsinki et al., 2019b; Kelsey et al., 2021) (see section 4.5.2.1 Temperament). Interestingly, bacteria in *Bifidobacterium* genera had both positive and negative associations with 2-year problem behaviour, suggesting that species-level associations may be important.

The discrepancies between the findings in two studies investigating relationships between gut microbiome in early infancy and 2-year behaviour problems (Loughman et al., 2020b, 2020a) could be due to different characteristics of the study populations: the study reporting associations between bacterial genera and problem behaviour was carried out in

infants with colic who had a higher baseline prevalence of behavioural problems (Loughman et al., 2020b). This is relevant because infantile colic has also been associated with aberrant intestinal microbiome (Dubois and Gregory, 2016), which raises the possibility that the potential to detect microbiome-behaviour associations is higher in children with divergent gut microbiome and/or behaviour compared to the general population.

Although there was no association between the gut microbiota at 6 months and problem behaviour at 2 years after adjustment for covariates, at 12 months the abundance of *Prevotella* was significantly lower in children with behavioural problems at 2 years (Loughman et al., 2020a). Interestingly, reduced abundance of *Prevotella* has also been observed in patients with autism spectrum disorder (F. Liu et al., 2019).

A small preliminary study exploring the associations between gut microbiome composition and problem behaviour in 38 3-year-old children cross-sectionally identified the phyla *Bacteroidetes* and *Actinobacteria* to be positively, and *Proteobacteria* and *Verrumicrobia* (e.g. *Akkermansia*) to be negatively correlated with different CBCL subscales (W. Zhang et al., 2021). This suggests that different bacteria may be important for behavioural development after the first year of life compared to those reported in early infancy (Loughman et al., 2020a) though additional research is needed to test this hypothesis.

Regarding bacterial alpha diversity, increased alpha diversity in early infancy positively associated with problem behaviour at 2 years in infants with colic (Loughman et al., 2020b). However, in typical development, alpha diversity during the first year of life does not appear to be statistically significantly associated with behavioural problems at 2 years of life, though the direction of effect was similar at all timepoints to that observed in infants with colic (Loughman et al., 2020a). These results further suggest population-specific effects of gut microbiota on behavioural development and highlight the possibility that the effects of microbiome on behavioural development may be greater (and more detectable) in vulnerable populations as also suggested above.

4.5.2.4 Other behavioural traits

Three studies have directly assessed the association between gut microbiome composition and other behavioural traits: social impairment (Laue et al., 2020), stress-related behaviour in neonatal unit (Sun et al., 2020), and perceived stress (Sobko et al., 2020).

Using a prospective, longitudinal assessment of gut microbiome composition via both 16S rRNA gene and shotgun metagenomic sequencing, Laue et al. (2020) demonstrated that increased abundance of several species within the *Lachnospiraceae* family (e.g. *Ruminococcus gnavus* and *torques, Blautia producta*) at 1 and 2 years associated with worse social behaviours in 3-year-old children assessed using the Social Responsiveness Scale (SRS); the associations between 3-year social behaviour and gut microbiome at 6 weeks and 3 years were less pronounced. Importantly, these results are in line with two other studies discussed above which reported a negative association between the abundance of *Lachnospiraceae* in early infancy and 3-year communication and personal/social skills (Sordillo et al., 2019), as well as a negative association between abundance of *Lachnospiraceae* and cognitive development at 3 years (Rothenberg et al., 2021). The longitudinal design of the study by Laue and colleagues additionally highlights the possibility of temporal effects of microbiome on behaviour and suggests that the timing of sampling and assessments is crucial in identifying potential correlations between the microbiome and behaviour. The use of metagenomic data by Laue et al. (2020) additionally enabled inferences about functional pathways, revealing that pathways involved in the urea cycle (L-ornithine de novo biosynthesis) and vitamin B6 biosynthesis (superpathway of pyridoxal 5'-phosphate biosynthesis and salvage) in 6-week and 1-year-old infants are positively associated with improved social behaviours. Bacterial alpha diversity, on the other hand, was not associated with SRS scores, further suggesting unknown effects of gut bacterial alpha diversity on neurodevelopment, as discussed in previous sections.

Two studies have investigated aspects of stress in relation to gut microbiome composition. Sun et al. (2020) used longitudinal sampling of gut microbiome in preterm infants during their stay in the neonatal intensive care unit and associated the composition of the gut microbiome to the stress score of the NICU Network Neurobehavioural Scale (NNNS) at term-equivalent age. The study identified four genera which had dynamic associations with the stress score during the neonatal period. The direction of effect for these genera-stress associations changed over the course of the first month of life, further implying that the specific relationships between microbiome and behaviour detected is age- or time-dependent. The strongest effects for all of these genera were observed around the latest timepoints assessed (i.e. at 4 weeks of life) when the abundance of *Enterococcus*, *Shigella,*

and an unknown bacteria in *Enterobacteriales* order negatively, while genus *Veillonella* positively correlated with stress scores. Interestingly, these results have some consistency with those observed in (Rozé et al., 2020) which reported that the clusters dominated by *Escherichia/Shigella* or *Enterobacter* (*Enterobacteriales* order) during the first postnatal month in preterm infants were less at risk for non-optimal neurodevelopmental outcomes at 2 years of life, and the abundance of species in *Veillonella* genera in 2.5-month-old infants have been both negatively and positively associated with 6-month temperament traits of regulation/orienting, surgency and fear reactivity (Aatsinki et al., 2019b). These results together suggest a potential role for these bacteria (*Enterococcus, Enterobacteriales (Enterobacter), Veillonella*) during the first few months of life on outcomes both in the neonatal period as well as later in infancy and childhood.

The second study looking at the relationships between gut microbiome and stress in childhood primarily focused on investigating the effects of an outdoor nature-related intervention on gut microbiome composition and perceived stress in 2-5-year-old children, however, their secondary analyses also included direct assessment of gut microbiome profiles and perceived stress scores (Sobko et al., 2020). In this study, bacterial alpha diversity negatively associated with perceived stress levels, and the diversity within the *Bacteroidetes* phyla negatively associated with stress scores.

4.5.3 Microbiome associations with structural and functional neuroimaging features in childhood

We identified four studies reporting associations between gut microbiota profiles and brain imaging features in early childhood (Table 4-5). In three studies, the gut microbiota was assessed using 16S rRNA gene-based amplicon sequencing (Carlson et al., 2021, 2018; Gao et al., 2019) and one study used metagenome sequencing (Kelsey et al., 2021). Brain structural and functional properties have been assessed in these studies using structural MRI (T1- and T2-weighted) (Carlson et al., 2021, 2018), functional MRI (Gao et al., 2019), and functional Near-Infrared Spectroscopy (fNIRS) (Kelsey et al., 2021). Although these studies to date are limited by small sample sizes (maximum sample size is 63 for participants with both measures), they suggest potential relationships between the gut microbiome and brain structure and function.

All four studies used alpha diversity as a microbiome feature of interest. As an exploratory analysis, Carlson et al. demonstrated that in a sample of 27 children with structural T1-weighted MRI scans, alpha diversity of the gut microbial taxa at 1 year of life positively correlated with the volume of left precentral gyrus, right angular gyrus and left amygdala at 2 years of life; while alpha diversity was uncorrelated with global measures of brain volume such as total grey and white matter or ventricular volume at 1 year of life (Carlson et al., 2018). However, as Carlson et al. observed negative correlations between alpha diversity and cognition (i.e. in opposite direction to those observed with regional volumes; see section 4.5.1 Gut microbiome associations with general neurocognitive development in early childhood), it is difficult to interpret the gut-brain structure findings. A more recent pilot study from the same group identified positive correlations between gut microbiota and fear responses (see section 4.5.2.2 Fear-related behaviour), but did not observe a correlation between bacterial alpha diversity at 1 month or at 1 year of life and volume of structures involved in fear processing (amygdala, hippocampus or medial prefrontal cortex (mPFC)) at 1 month or at 1 year (though note that n=13-14 for these analyses) (Carlson et al., 2021). These results together may potentially suggest that bacterial alpha diversity correlations with brain structure become apparent later in infancy/toddlerhood – but larger, longitudinal studies are needed to test this hypothesis.

Gut bacterial alpha diversity has also been a marker of interest in studies investigating associations between gut microbiota and brain function. Using resting state functional MRI Gao and colleagues demonstrated that gut bacterial alpha diversity in 39 1-year-old infants negatively correlated with functional connectivity between the left amygdala and thalamus as well as anterior cingulate cortex and insula at the same age, and positively correlated with functional connectivity between supplemental motor area and the inferior parietal lobule (Gao et al., 2019). Interestingly, the correlations of gut bacterial alpha diversity with the development of the connectivity between parietal and frontal brain areas may already be apparent earlier in infancy. Using fNIRS, Kelsey et al. reported that gut bacterial diversity positively correlated with connectivity within the fronto-parietal network in new-born infants (Kelsey et al., 2021). They also found taxa diversity to be linked with increased connectivity of the homologous-interhemispheric network. Making use of shotgun metagenomic data, which enables insight into functional properties of the microbiome,

Kelsey et al. (2021) additionally demonstrated that an increase in the diversity of virulence factors correlated with increased connectivity of the homologous-interhemispheric network, suggesting that the taxa diversity association with functional connectivity may be driven by increases in virulence factors.

These findings together raise the possibility that bacterial alpha diversity in early life affects the structure and function of the amygdala, which plays a central role in fear and emotion regulation, frontal precentral areas involved in motor processing (e.g. precentral gyrus and supplemental motor area), and parietal areas involved in language, sensory and emotional processing (e.g. angular gyrus and inferior parietal lobule). However, current literature does not rule out the effects of microbial diversity on other brain networks or on global/generalised properties. In addition, the correlations of microbial alpha diversity with brain structure and function may be age-dependent as the effects of alpha diversity on brain functional connectivity appear to be present already in early infancy while the effects on brain structure become evident after the first year of life.

Whilst all studies investigating the relationships between gut microbiome and brain development indexed by neuroimaging use bacterial alpha diversity as a feature of interest, the associations with bacterial community composition and specific bacterial taxa or their functionalities are less explored. Carlson and colleagues used a cluster analysis and reported that infants with *Bacteroides*-dominated microbial communities had the largest and infants with *Ruminococcaceae*-dominated microbial communities had the smallest volume of superior occipital gyrus at 1 year of life, and, conversely, infants with *Bacteroides*-dominated microbial communities had the smallest and infants with *Ruminococcaceae*-dominated microbial communities the largest volume of caudate nuclei at 2 years of life (Carlson et al., 2018). These results thus mirror the correlations between the microbiome community clusters and MSEL scores which were also only observed at 2 years (see section 4.5.1 Gut microbiome associations with general neurocognitive development in early childhood). As with alpha diversity, there were no effects of microbiota clusters on global measures of brain volume, which implies that microbiota-brain interactions may affect multiple discrete neural systems rather than global brain growth. Another study by the same group also found suggestive correlations between gut microbiota composition and the volume of fear- and emotion-related brain structures at 1 month and 1 year (amygdala,

hippocampus and mPFC) (Carlson et al., 2021). Namely, at 1 month, the relative abundance of *Streptococcus* associated with smaller hippocampal, amygdala and mPFC volumes, decreased abundance of *Staphylococcus* associated with larger amygdala and mPFC volumes, and increased abundance of *Bacteroides* associated with larger amygdala volume. In contrast, increased abundance of *Bacteroides* at 1 month associated with smaller mPFC volume at 1 year, whilst larger amygdala volume at 1 year was associated with increased abundance of *Lachnospiraceae* and decreased abundance of *Enterobacteriaceae* at 1 month of life. Therefore, the abundance of specific bacterial species may influence regional brain volumes throughout infancy, however, the specific bacteria-brain region relationships need to be replicated and expanded upon in larger studies.

Kelsey et al. (2021) additionally identified several bacterial taxa associations with brain functional connectivity (as measured with fNIRS), including positive associations of *Clostridium, Enterococcus* and *Bacteroides* species with the fronto-parietal network connectivity, and negative associations of *Bifidobacterium* species with homologous-interhemispheric network connectivity (i.e. connectivity between bilateral channels on an fNIRS cap plausibly covering frontal, temporal, and parietal regions). However, it remains to be determined in future longitudinal studies whether the relationships reported by Kelsey et al. replicate and persist beyond the new-born period.

The brain areas associated with bacterial diversity or composition in childhood have also been of interest in adult studies. For example, gut microbiota profiles have been associated with structural connectivity in limbic circuitry involving areas of anterior cingulate cortex and amygdala (Tillisch et al., 2017), and functional connectivity of the insula (Curtis et al., 2019) and the amygdala (L. J. Zheng et al., 2020). Furthermore, preclinical studies have also shown neurobiological alterations in the brains of animals with disrupted gut microbiota, including volumetric and morphometric changes in the amygdala, hippocampus, and anterior cingulate cortex (Luczynski et al., 2017, 2016; Ogbonnaya et al., 2015), coupled with changes in myelination (Hoban et al., 2016; Lu et al., 2018a) and expression of signalling molecules and neurotransmitters (Clarke et al., 2013; Desbonnet et al., 2015; Diaz Heijtz et al., 2011; Neufeld et al., 2011; Sudo et al., 2004). These studies can guide the hypotheses of future research with paediatric populations.

Importantly, incorporating brain imaging measures to the studies of microbiome-gut-brain axis may reveal the neurobiological pathways of gut microbiome influence on behaviour. For example, Kelsey and colleagues suggest that bacterial taxa and virulence factor diversity associations with behavioural temperament (see above section 4.5.2.1 Temperament) were mediated via homologous-interhemispheric connectivity (Kelsey et al., 2021). In addition, in the study by Gao et al., the connectivity between supplemental motor area and parietal cortex, which positively correlated with alpha diversity, also negatively correlated with Mullen Early Learning Composite scores at 2 years; however, they did not formally test for the possible mediation effect (Gao et al., 2019). These initial findings highlight the potential for this kind of research to reveal mechanistic pathways by which brain development links gut microbiome with behavioural and cognitive outcomes.

4.5.4 Summary of microbiome features associated with neurodevelopment

All 20 studies report some significant associations between microbiome features (clusters, taxa abundance, alpha diversity) and neurodevelopmental measures. However, differences in the direction and magnitude of bacteria-brain/behaviour relationships between studies are substantial. Figure 4-2 summarises several lines of convergence and divergence of the gut bacterial influence on brain and behaviour.

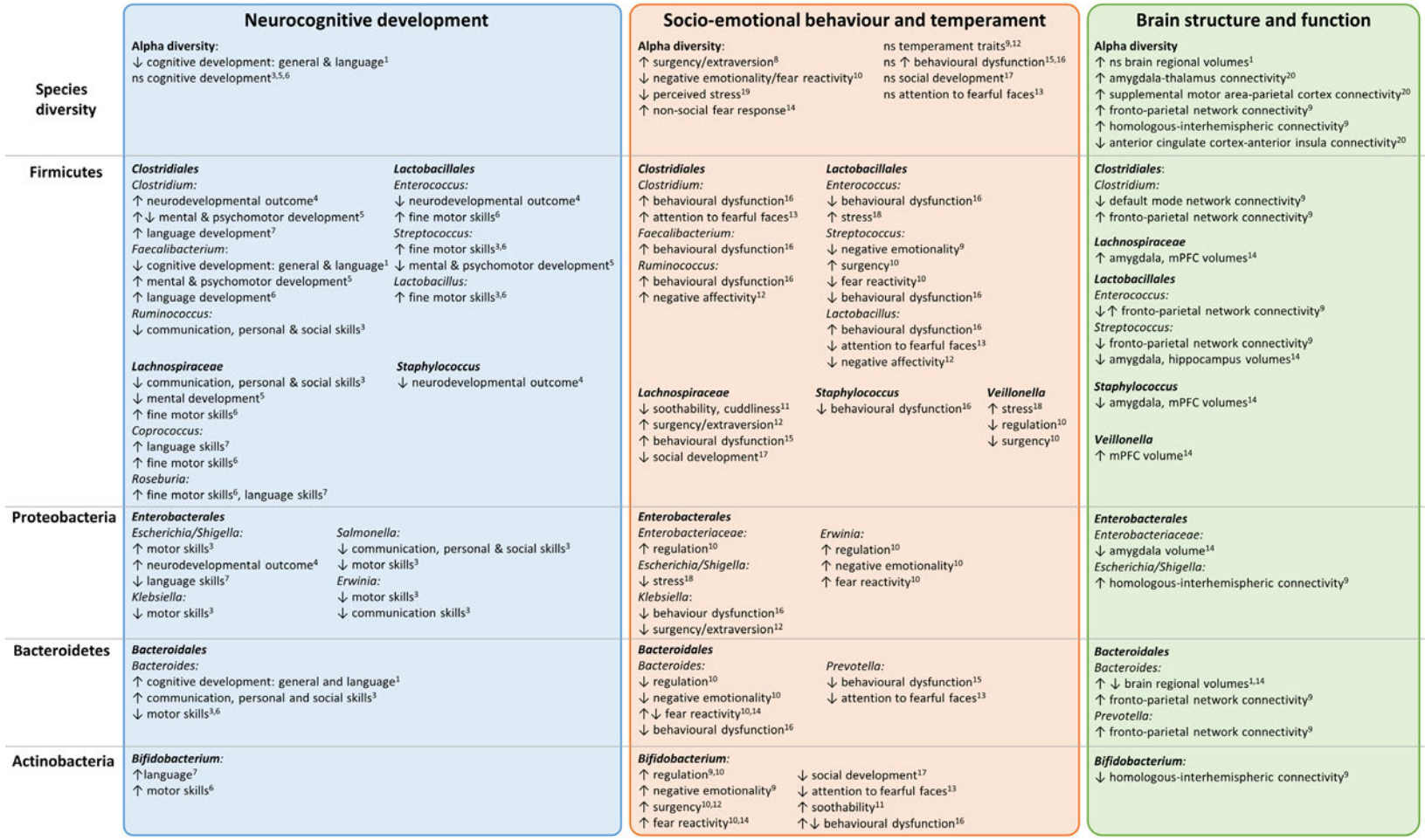


Figure 4-2. Bacterial features associated with aspects of neurodevelopment.

↑, ↓, and "ns" indicate whether the bacterial feature was positively, negatively, and/or not significantly associated with cognitive, behavioural or neuroimaging measures. The bacterial families and genera presented in this figure are those that were identified as correlated with neurodevelopment in at least two studies and/or with at least two different neurodevelopmental domains. The superscripts are study references: [1]Carlson et al., 2018, [2]Zhang et al., 2021, [3]Sordillo et al., 2019, [4]Rozé et al., 2020, [5]Rothenberg et al., 2021, [6]Acuña et al., 2021, [7]Kort et al., 2021, [8]Christian et al., 2015, [9]Kelsey et al., 2021, [10]Aatsinki et al., 2019, [11]Wang et al., 2020, [12]Fox et al., 2021, [13]Aatsinki et al., 2020, [14]Carlson et al., 2021, [15]Loughman, Ponsonby, et al., 2020, [16]Loughman, Quinn, et al., 2020, [17]Laue et al., 2020, [18]Sun et al., 2020, [19]Sobko et al., 2020, [20]Gao et al., 2019.

For example, although increased gut bacterial alpha diversity is generally considered a proxy for a healthy/stable microbiome (Shade, 2016) and is associated with improved health outcomes (e.g. reduced alpha diversity has been observed in patients with attention deficit-hyperactivity disorder and diabetes; Kostic et al., 2015; Prehn-Kristensen et al., 2018), it remains unclear from the published literature whether alpha diversity is associated with neurodevelopmental measures. Whilst neuroimaging studies suggested that alpha diversity in early life plays a role in the structure and function of several brain regions (including the amygdala, frontal precentral areas, and parietal regions), the associations with behavioural measures vary, with several studies reporting no significant associations between alpha diversity and features of cognitive or socio-emotional development. Importantly, recent systematic reviews have also reported inconsistent relationships between alpha diversity and psychiatric and developmental disorders such as depression, anxiety, autism spectrum disorder, schizophrenia and bipolar disorder (Ho et al., 2020; Nguyen et al., 2018; Sanada et al., 2020; Simpson et al., 2021).

Regarding bacterial taxa, we identified that the abundance of several bacteria is associated with multiple aspects of neurodevelopment. For instance, the abundance of *Bacteroides* appears to be positively associated with language and behavioural development, whilst it has negative effects on motor development. In early infancy, the abundance of *Enterococcus* and *Staphylococcus* seems to associate with poorer neurocognition and the abundance of *Escherichia/Shigella* and *Enterobacter* with improved neurocognition; in contrast, the abundance of *Enterococcus* and *Escherichia/Shigella* during the second year of life correlates with better and worse neurocognitive outcomes, respectively, suggesting potential age-dependent effects of specific bacteria on cognitive development. In parallel, the abundance of *Escherichia/Shigella*, *Streptococcus, Staphylococcus, Enterococcus*, and *Bacteroides* during the first month of life correlates with brain cortical connectivity and/or the volumes of fear- and emotion-related brain structures (amygdala, hippocampus and mPFC). On the other hand, the bacteria in *Clostridiales* order (e.g. *Clostridium, Faecalibacterium*) and *Lachnospiraceae* family (e.g. *Coprococcus, Roseburia*) do not have a clear direction of effect on neurodevelopmental measures, with studies reporting both positive and negative effects of these bacteria on language, motor, and socio-emotional development. Lastly, abundances of *Bifidobacterium, Lactobacillus, Bacteroides* and *Veillonella* have been

correlated with temperament traits and other measures of socio-emotional behavioural development, however, the directions of effect vary substantially between studies and behavioural measures. These inconsistencies could be explained by the vast methodological heterogeneity between studies, as discussed in the next section.

4.5.5 Methodological and statistical considerations, and avenues for future research

Whilst this review of 20 studies suggests that variations in gut microbiota composition during the first few years of life may be associated with neurocognitive and socio-emotional development and features of brain structure and function in early childhood, quantitative synthesis of microbiota-brain/behaviour associations was not possible due to clinical and methodological heterogeneity across studies. Comparison of sequencing-based human microbiome studies is especially complex due to the multitude of systemic biases introduced in various stages of research (Nearing et al., 2021). We extracted data on study characteristics to collate sources of potential bias arising from clinical and methodological heterogeneity (Supplementary Table 4-1), and we discuss these items in turn below, focusing on potential solutions for future studies.

4.5.5.1 Sample size

One of the limiting factors in existing studies is the sample size. Although almost half of the studies were only able to combine microbiome and brain/behaviour data from around 15-90 participants, several others were considerably larger and enabled associations between microbiome and neurodevelopmental data from >200 individuals. The small sample sizes are especially observed in the four studies that combined metagenomics data with neuroimaging, underlining the preliminary nature of these results and the need for replication in future studies. Small sample size is a particularly limiting factor in microbiota studies due to the high dimensionality of the data, i.e. much larger number of variables (bacterial taxa) than the number of cases/samples, which together with the compositional nature of the data impose challenges in applying classical statistical methods (Johnstone and Titterington, 2009; Tsilimigras and Fodor, 2016). Similarly, neuroimaging studies are often limited by small sample sizes due to the high cost. Neuroimaging data is often extremely noisy and variable, which coupled with a lot of analytic freedom may lead to challenges in downstream analyses and variability in results, especially when the sample size is insufficient and the hypotheses require examining individual differences in neural measures.

We propose that future studies use rigorous power calculations to inform sample sizes in order to limit both Type I and Type II errors and, where possible, constrain hypotheses and analysis approaches based on prior research. In addition, harmonisation of sampling protocols and processing pipelines in consortia, and other data-sharing initiatives, could enable scale-up of studies, increase study power and provide essential validation sets. Harmonisation of sampling and data processing could additionally promote comparability between studies and increase study power by allowing for meta- and mega-analyses.

4.5.5.2 Participant characteristics

Gut microbiome composition, cognition, behaviour and brain structure-function in the studies included in this review has been analysed at various timepoints from birth until three years of life. This provides a source of methodological heterogeneity that limits the generalizability of results across studies. It remains to be determined whether reported differences in bacteria-brain/behaviour relationships are dependent on the temporal development of the gut microbiota and/or the neurodevelopmental measures analysed, or on the differential effects of different bacteria on the brain at different neurodevelopmental timepoints in early childhood. Both the brain and gut microbiome undergo extensive changes in the first five years of life (Figure 4-1), thus, associations between the bacterial profiles and neurodevelopmental outcomes may depend on the timing of sampling and assessments. Therefore, the interacting temporal dynamics of these two systems via the microbiome-gut-brain axis and the potentially predictive value of gut microbiota profiles at different timepoints during infancy on neurocognitive outcomes later in childhood need to be explored in future studies with longitudinal sampling.

In addition to participant age at sampling and assessments, another source of heterogeneity in relation to participant characteristics is the inclusion or exclusion of participants based on specific clinical/demographic criteria. In light of the focus on typical development, studies included in this review excluded participants with neurodevelopmental delays, major illnesses, foetal ultrasound abnormalities, or visual/hearing impairments. Some studies additionally excluded participants based on specific criteria that could affect microbiome composition such as antibiotic use prior to study visits, C-section delivery, or formula feeding. Finally, several studies excluded participants based on low gestational age at birth while a few others focused exclusively on preterm infants. We chose not to exclude these

studies because preterm birth is not a neurodevelopmental disorder *per se* and many survivors have typical neurodevelopment. The use of different exclusion criteria impacts the ability to make direct comparisons of the results across studies and participant heterogeneities may need to be taken into account when drawing comparisons between studies.

4.5.5.3 DNA extraction and sequencing

DNA extraction has been suggested to be one of the steps that is at most risk of bias in microbiome research (Brooks et al., 2015; McLaren et al., 2019). Specifically, extraction efficiencies vary both between bacteria as well as extraction kits, resulting in higher or lower yields of DNA and biasing the detection of specific bacteria (Nearing et al., 2021). Moreover, systematic bias due to differences in DNA extraction protocols is increased in low biomass samples (Davis et al., 2019), such as those collected from young infants. The studies discussed in the current review have used a range of different DNA extraction kits and protocols, however, some studies have not specified the kit/protocol. This heterogeneity could thus partly underly the varying results between studies.

Nineteen studies discussed in this review used sequencing based on the amplification of hypervariable regions of the bacterial 16S rRNA gene and only two studies applied whole metagenome sequencing, which, in addition to genus-level identification of bacteria achieved by 16S rRNA gene-based sequencing, can more accurately identify bacterial species and strains, as well as the functional pathways (Kelsey et al., 2021; Laue et al., 2020). The higher cost and need for a larger amount of input DNA for shotgun metagenomic sequencing contribute to the continued prevalence of 16S rRNA gene sequencing. In addition, 16S rRNA gene sequencing could have higher sensitivity compared to metagenomics in low-microbial biomass samples (Hillmann et al., 2018; Pereira-Marques et al., 2019). Nevertheless, more widespread use of whole metagenome sequencing would support greater inference about the functional features, including metabolic pathways through which the gut microbiome influences brain development. Alternative methods have been introduced, however, to also infer functional information from 16S rRNA gene-based sequence data (e.g. PICRUSt (Langille et al., 2013)). In particular, the gut-brain modules developed by Valles-Colomer and colleagues enables assembly of pathways with

neuroactive potential in bacteria and could be used in future studies for functional insights inferred from 16S rRNA gene sequencing (Valles-Colomer et al., 2019).

Another source of variation in the sequencing methodologies is the use of a range of different hypervariable regions for 16S rRNA gene-based sequencing. This could explain variations of results between the different studies because the choice of 16S rRNA gene region can significantly alter the estimation of taxonomic composition and diversity (Clooney et al., 2016; Rintala et al., 2017; Yang et al., 2016).

4.5.5.4 Microbiota features of interest and statistical approaches

The application of different statistical analyses to describe bacteria-brain/behaviour relationships also varies between the studies. In particular, the choice of microbiome feature of interest differs between studies.

Several reports discussed above implemented different data reduction techniques to the microbiome data prior to associations with neurodevelopmental outcomes. These include clustering based on distance metrics applied to relative genus abundances (Aatsinki et al., 2019b; Acuña et al., 2021; Carlson et al., 2018; Rozé et al., 2020) or factor representations/co-abundance groupings of bacteria based on the correlations of the abundance of bacteria in the samples (Rothenberg et al., 2021; Sordillo et al., 2019). Whilst these methods are effective in reducing the number of multiple comparisons when associating the microbiota composition data with variables of interest (e.g. neurodevelopmental assessment scores), the comparisons across studies are complicated as the initial data reduction step (clustering or co-abundance grouping) is highly variable between studies. Additionally, even if the algorithms for initial data reduction are similar, the resulting microbiome groups or factors will be dependent on the samples present. For example, depending on the age of the children at the time of sample collection, the microbiome groups or factors are different due to the temporal nature of microbiome succession in the gut in early childhood (Bäckhed et al., 2015; Roswall et al., 2021; Stewart et al., 2018). Other participant characteristics (e.g. prematurity, infantile colic, exclusion of C-section-born children) might additionally affect the microbiome composition and thus the results of data reduction.

Clustering of the human gut microbiome samples initially led to the idea of enterotypes reflecting distinct compositions of microbial communities, however, it is debatable whether these enterotypes exist or how they should be defined (García-Jiménez and Wilkinson, 2019; Goodrich et al., 2014). Yet, the idea of combing existing large-scale microbiome datasets to define age-dependent bacterial clusters/factor compositions that could be applied in future studies is attractive as a means of standardisation to facilitate comparisons between studies. Another interesting approach for data reduction of microbiome datasets is based on calculating microbiota age (Stewart et al., 2018; Subramanian et al., 2014), which captures the relative maturity of microbiota composition. These and similar approaches for data reduction of multidimensional microbiome data could be of particular utility in studies investigating the relationship between gut microbiome and multidimensional neuroimaging data (Jenkinson et al., 2012).

Analyses relating taxa-level bacterial data with neurodevelopmental outcomes offer additional, valuable insights as these are, in theory, easier to compare between different studies. However, reporting of the findings differs across studies, with descriptions of per-feature analyses on different taxonomical levels (operational taxonomic units/species, genus, family, order, phyla) and inconsistencies in reporting of effect sizes and adjustments for multiple comparisons. Moreover, there are a variety of per-feature association methods that have been applied to study brain/behaviour associations with bacterial abundances (e.g. DESeq2, MaAsLin, voom, LefSE, standard correlation measures such as Pearson's or Spearman rank correlation, linear regression, Student's t-test or Wilcoxon signed rank test), which vary in options for normalisation, data transformation, models, and associated statistical inferences (Mallick et al., 2021, 2017; Nearing et al., 2022). Each of these methods have their limitations in terms of power, sensitivity, and rates of false positive and negative findings (Mallick et al., 2021; Nearing et al., 2022). Indeed, a recent study which compared 14 differential abundance testing methods demonstrated that the tools identified different sets of bacteria to be associated with features of interest, and that the tools have varying sensitivities to different aspects of the data (e.g. sample size, sequencing depth) and data pre-processing (e.g. transformation, normalisation, rarefaction, prevalence filtering) (Nearing et al., 2022). These differences could underly some of the between-study variations in the bacteria-neurodevelopment relationships observed. In addition,

metagenomic data has specific characteristics (noisy, non-normally distributed, sparse, zero-inflated) which call for special caution in using standard epidemiological multivariate statistical models. Importantly, microbiome data is compositional by definition and appropriate compositional data analytic methods in every step of the analyses (including transformation, normalisation, distance calculations as well as correlation with variables of interest) are crucial to avoid spurious correlations (Gloor et al., 2017; Spichak et al., 2021; Tsilimigras and Fodor, 2016).

Nevertheless, irrespective of the method used (clusters, factor compositions, taxa-level analyses), biologically relevant microbiome-brain/behaviour relationships could be expected to emerge from different analysis methods. In other words, the use of different analysis methodologies across studies could be seen as a strength if they produce comparable findings, demonstrating that these microbiome-brain/behaviour relationships are robust and observable across methodologies. The implementation of multiverse analyses (Steegen et al., 2016) or consensus approaches (as suggested by Nearing et al. (2022)) could thus be valuable to help ensure results are robust to choices in statistical analysis pipelines.

Moreover, the studies discussed aimed to identify individual bacteria that correlate with brain properties or behavioural traits. However, the abundances of different bacterial species are often intertwined and groups of different bacteria are co-occurring, suggesting that use of methods that enable the identification of bacterial networks or consortia which associate with brain or behaviour features may be important in future studies. Furthermore, trajectories and/or stability of microbiota composition during early life rather than composition at any specific timepoint may be important for neurocognitive outcomes, suggesting that longitudinal modelling of the microbiota profiles with neurocognitive outcome data could reveal novel associations within the microbiota-gut-brain axis in childhood.

In addition to identifying specific bacteria that correlate with brain and behaviour, most studies have used within-sample microbial diversity (alpha diversity) as a measure of interest and these results are often discussed as separate or independent from the microbial community results. There are multiple indices that can be calculated as measures of alpha diversity (e.g. Chao, Shannon and Simpson indices, number of observed species, Faith's Phylogenetic diversity), which differently take into account the richness (number of

taxonomic groups) and/or evenness (distribution of microbial abundances) of the microbial communities (see Table 4-2). Thus, depending on the measure used, alpha diversity may provide limited insights into the microbial community structure (Shade, 2016). In addition, alpha diversity of the gut microbiota increases greatly over the first few years of life, especially around 4-6 months of life when solid foods are introduced to the diet (Reyman et al., 2019; Roswall et al., 2021), making it difficult to compare microbial alpha diversity relationships with brain and behaviour across different timepoints in early childhood. Alpha diversity measures may especially lack resolution in early infancy when the gut is heavily dominated by *Bifidobacteria* (Reyman et al., 2019). We propose that future studies discuss alpha diversity associations with brain and behaviour in the context of the underlying microbial community structure in the samples.

4.5.5.5 Neurodevelopmental outcome assessments

To date, four studies have investigated the associations between childhood gut microbiota and brain development using neuroimaging. These studies took varying approaches for defining the outcome measures of interest and include both exploratory (e.g. volume of 90 grey matter regions) and hypothesis-driven (e.g. volume and connectivity of fear- and emotion-related brain structures, such as the amygdala and hippocampus) targets. Of note, current literature lacks explorations between gut microbiome and features of brain microstructure inferred from diffusion-weighted or magnetization transfer imaging, which enable inferences about the myelin content in the brain (Lazari and Lipp, 2021; Mohammadi and Callaghan, 2021). These investigations would be important as preclinical studies suggest changes in myelination (Hoban et al., 2016; Lu et al., 2018a) and neuronal morphology (Luczynski et al., 2016) in rodents with disrupted gut microbiota. Measures of brain microstructure have previously been associated with diet-induced changes in the microbiome in rats (Ong et al., 2018; Tengeler et al., 2020) as well as with gut microbiome in adults (Fernandez-Real et al., 2015; Tillisch et al., 2017). Moreover, diffusion MRI in infancy and early childhood has a strong predictive value for neurocognitive outcomes (for example see (Ball et al., 2015; Barnett et al., 2018; Counsell et al., 2008; Duerden et al., 2015; Girault et al., 2019; Ouyang et al., 2020; Van Kooij et al., 2012b)) and several factors that are established as important drivers of the microbiome development (e.g. breastfeeding, C-section delivery) are also associated with alterations in brain microstructure in infancy and

childhood (Bauer et al., 2019; Blesa et al., 2019; Deoni et al., 2019, 2018, 2013; Kar et al., 2021). However, it remains unexplored to what extent these associations may be mediated through the gut microbiome. In addition, magnetic resonance spectroscopy may provide another important avenue for exploration in microbiome-gut-brain axis studies as it can provide unique information about the metabolic and neurobiological substrates of the brain (Stovell et al., 2017), and has been applied in the study of the effects of gut microbiome alterations on the brain in preclinical (Janik et al., 2016; Menneson et al., 2019) as well as adult human studies (Ahluwalia et al., 2016; He et al., 2018).

Studies which have associated gut microbiota features with neurocognitive and behavioural outcomes have mostly relied on validated and standardised parent questionnaires (e.g. IBQ, ECBQ, ASQ, CBCL) or directly observed assessments (e.g. BSID, MSEL), spanning from one month to three years of life, which can be considered a major strength in these studies due to the standardised nature and wide applicability of these scales across populations. In addition, two other studies used experimental methods for the assessment of emotional attention (Aatsinki et al., 2020) and fear reactivity (Carlson et al., 2021), which revealed more nuanced bacteria-behaviour relationships not captured by broad-scale assessments.

In the light of the studies reporting microbiome differences in individuals with neurodevelopmental disorders (especially autism spectrum disorder and attention deficit-hyperactivity disorder (Jurek et al., 2020)), future studies could additionally benefit from incorporating early behavioural measures linked to those disorders. These may include experimental assessments of early social and attentional development, for example eye-tracking tasks measuring social cognition or attention maintenance/switching, or standardised tools such as the Autism Diagnostic Observation Schedule. The assessment of these behavioural measures in future studies would enable testing whether early life microbiome measures are predictive of later clinical diagnoses, and may also reveal novel mechanisms within the microbiome-gut-brain axis involved in the pathogenesis of certain neurodevelopmental disorders.

In addition, future studies looking into the relationships between the gut microbiome and early executive functions (e.g. using Behaviour Rating Inventory of Executive Function or Early Executive Functions Questionnaire) may reveal information about the specificity vs generalizability of microbiome-behaviour associations. In other words, these studies could

help to understand whether the microbiome-behaviour relationships are specific to certain domains of cognition (e.g. social cognition, language development) or whether these are reflective of a more domain-general impact (e.g. on executive functions). Understanding the specific/selective vs general impact of microbiome on behaviour may additionally help to reveal the neural circuits/pathways linking microbiome development with neurodevelopment.

Furthermore, as suggested by Kelsey and colleagues, incorporating brain imaging measures to the studies of microbiome-gut-brain axis may reveal important mechanistic neurobiological pathways whereby the gut microbiome may influence brain and behaviour that are difficult to detect when exclusively studying measures of overt behaviours (Kelsey et al., 2021). For example, by looking into the relationships between gut microbiome and brain structure/function inferred from neuroimaging, future studies may be able to clarify the timeline of the impact of the microbiome on child cognition and behaviour as neuroimaging studies could reveal early neural signatures that would be detectable before certain behaviours become observable/testable in early childhood. In addition, this research could help elucidate whether the microbiome has a general or specific impact on neurodevelopment by testing whether the correlations between microbiome composition and brain structure/function are observed across the brain or only in particular brain regions. Thus, future studies integrating all three data types (bacterial sequencing, multimodal neuroimaging as well as standardised and experimental behavioural assessments) will be valuable to understand the links between the gut microbiome and neural circuits involved in cognition and behaviour.

4.5.5.6 Covariates

Choice of covariates differs between the studies. Several studies took a combination of theoretical and data-driven approaches to identify the covariates to be included in the analyses, and include variables that have been previously associated with the gut microbiome or the neurodevelopmental outcome measure, and/or that associate with the gut microbiome and/or the neurodevelopmental outcome in univariate analyses in the study data. The most commonly used covariates included factors of the child (e.g. age at assessment/faecal sample collection, sex, gestational age at birth, birthweight, exposure to antibiotics and probiotics, breastfeeding status or length, mode of delivery), parents (e.g.

ethnicity, education level, age, income/socioeconomic status, maternal psychiatric history, BMI) as well as specific variables directly related to microbiota (e.g. storage conditions) or outcomes (e.g. head circumference, cognitive scores at an earlier age). Nevertheless, there were some studies that did not adjust for any potential confounders (Christian et al., 2015; Kort et al., 2021; Sobko et al., 2020; W. Zhang et al., 2021).

Covariate adjustment is necessary to identify the effects of gut microbiota on brain development and behaviour that are independent from other neurodevelopmentally important factors (e.g. birthweight, feeding, gestational age) and collection of such metadata relating to participant exposures and history enables measuring the effects of these exposures on the brain mediated by the gut microbiota. Furthermore, standardisation of metadata collection in future studies would facilitate comparisons between the studies.

4.5.5.7 Adjustment for multiple comparisons

As discussed above, the studies included in the current review have analysed a variety of microbiota and neurodevelopmental features of interest, leading to a multiple statistical tests correlating microbiome and brain/behaviour measures within each study. Thus, adjustment for multiple comparisons is important to avoid false positive results. Indeed, the majority of the included studies performed some correction for false positivity rate, but seven studies did not describe adjustment for multiple testing, raising the possibility of type 1 error.

4.6 Conclusion

This review synthesised findings from 20 studies which investigated relationships between gut microbiome profiles and neurodevelopmental outcomes in typically developing children from birth to five years of age. The studies used a range of outcome assessments including structural and functional neuroimaging, standardised neurocognitive assessments, and parent-reported temperament and behavioural dysfunction questionnaires; gut microbiome data were analysed using 16S rRNA gene or whole metagenome sequencing. Most studies report significant associations of microbiota clusters, abundance of specific taxa or alpha diversity with neurodevelopmental outcomes, however, differences in the nature of bacteria-brain/behaviour relationships between studies are substantial.

We identified several sources of methodological heterogeneity, including variations in gut microbiota sequencing, features of interest, statistical analysis decisions, and adjustment for confounding factors and multiple comparisons which could, at least partly, explain the differences observed in the results. We propose that standardisation of sampling and data processing pipelines could improve study power and enable validation of the current results in terms of the direction and magnitude of the effects. Adoption of reporting guidelines such as STORMS (Mirzayi et al., 2021) and pre-registration of analyses could enhance reliability and facilitate meta-analyses in future work. Importantly, however, apparent conflicting findings in the studies need to be considered within the substantial temporal development of gut microbiota, brain and behaviour during pre-school age. We propose that future large studies with longitudinal microbiota sampling and neurodevelopmental assessments across early years of life are required to identify the interacting temporal dynamics of the microbiome and the brain via the microbiome-gut-brain axis in childhood.

4.7 CRediT authorship contribution statement

Kadi Vaher: Conceptualization, Methodology, Investigation, Writing - Original Draft, Writing - Review & Editing, Funding acquisition; **Debby Bogaert:** Conceptualization, Writing - Review & Editing, Supervision; **Hilary Richardson:** Writing - Review & Editing; **James P Boardman:** Conceptualization, Methodology, Writing - Original Draft, Writing - Review & Editing, Supervision, Funding acquisition.

4.8 Chapter conclusion

In this chapter, I provided a comprehensive overview of gut microbiota-brain/behaviour relationships. Associations were reported across neurodevelopmental domains including general cognition, motor and language development as well as temperament and social behaviour. A handful of studies further integrated measures from structural and functional MRI or fNIRS. However, the exact bacteria-brain relationships reported varied greatly between studies and methodological heterogeneity was substantial. This work indicates that developing standardised practices in this field and longitudinal assessments may be required for elucidating the bacterial signals related to improved neurodevelopmental outcomes. Integrating advanced neuroimaging methods will further enhance understanding of the brain processes and networks involved in microbiota-gut-brain axis signalling.

A majority of the included studies in this review chapter were conducted in term infants. Preterm infants may particularly be vulnerable to gut microbiota dysbiosis due to exposure to extrauterine life during a period of rapid development of neuronal processes. A few more studies in preterm infants were published after the completion of this chapter; these have been summarised in Chapter 1 section 1.4.1.4. Taxa from the families *Bifidobacteriaceae, Enterobacteriaceae*, *Clostridiales* and *Enterococcacceae* are implicated, although directions of effect vary between studies.

There is increasing interest in the microbiota-brain relationships in infancy and childhood exemplified by the additional 14 studies published after the completion of the review. Whilst it remains out of scope of the current thesis to discuss these in detail, for completeness, main results of the additional studies are collated in Table 4-6. Collectively, there is growing evidence that gut microbiota associates with neurodevelopment in human paediatric populations.

Table 4-3. Summary of studies investigating associations between gut microbiome and general neurocognitive development.

Authors **Title** **Country** **Study name if applicable**	**Sample characteristics: Sample size, sex, and age at samples and assessments**	**Neurodevelopmental assessment**	**Gut microbiome: Sequencing method and platform Features of interest**	**Main findings**
Carlson et al., 2018 Infant Gut Microbiome Associated With Cognitive Development USA UNC Early Brain Development Study	• Microbiome analysis: n=89 (49 male, 40 female) at 1 year • Cognitive assessment: n=86 at 1 year, n=69 at 2 years	Mullen Scales of Early Learning: used the Early Learning Composite (ELC) as well as five subscales (gross motor, fine motor, visual reception, expressive language and receptive language skills)	• 16S rRNA gene sequencing, V1-V2 hypervariable region, Illumina MiSeq • Alpha diversity (Chao1, observed species, Shannon Index, Faith's Phylogenetic diversity) • Clustering: three groups of infants based on clustering analysis with Jensen-Shannon distance metric applied to relative genus abundance with Partitioning Around Medoids clustering algorithm ○ C1 (n=53): high *Faecalibacterium* ○ C2 (n=19): high *Bacteroides* ○ C3 (n=17): high *Ruminococcaceae*	Microbiota cluster status at 1 year of life associates with Mullen ELC score, and receptive and expressive language subscales at 2 years of life: highest in C2 and lowest in C1[a] Alpha diversity is negatively associated with Mullen ELC scores at 2 years of life, and with expressive language and visual reception subscales: alpha diversity accounts for 5-23% of variance in Mullen scores[b]
Zhang et al., 2021 Preliminary evidence for an influence of exposure to polycyclic aromatic hydrocarbons on the composition of the gut microbiota and neurodevelopment in three-year-old healthy children China	• n=38 (20 male, 18 female) at 3 years	Gesell Development Inventory (GDI): five behavioural domains (Adaptive; Gross motor; Fine motor; Language; and Personal social behaviours)	• 16S rRNA gene sequencing, V4-V5 hypervariable region, Illumina MiSeq • Relative abundance of bacterial taxa on phylum, class, order, family, genus and species level (main results on phylum level)	Associations between bacteria phyla and GDI scores (unadjusted analyses): • *Firmicutes*~Gross motor (rho=0.327) • *Bacteroidetes*~Gross motor (rho=−0.416) • *Fusobacteria*~Adaptive behaviour (rho=−0.334) With bacterial genera[c]: • Fine motor: ↓ *Porphyromonas*, ↓ *Butyricimonas* • Language: ↓ *Actinomyces*, ↓ *Faecalitalea*, ↓ *Megamonas*, ↑ *Sellimonas*, ↑ *Bifidobacterium* • Adaptive behaviour: ↑ *Ruminococcus*, ↑ *Ruminiclostridium*, ↑ *Anaerotruncus*, ↓ *Fusobacterium*, ↓ *Pseudomonas*, ↓ *Erysipelatoclostridium* • Gross motor: ↓ *Megasphera*, ↓ *Paraclostridium*, ↓ *Gemella*, ↑ *Hungatella*, ↑ *Mobiluncus* • Personal/social: ↓ *Paraclostridium*, ↓ *Lactococcus*, ↓ *Aeromonas*, ↓ *Granulicatella*, ↓ *Myroides*, ↑ *Parabacteroides*

Sordillo et al., 2019
 	
Association of the Infant Gut Microbiome With Early Childhood Neurodevelopmental Outcomes: An Ancillary Study to the VDAART Randomised Clinical Trial
 	
USA
	• n=309 (170 male, 139 female)
	• Microbiome analysis: at mean [range] of 5 months [3-6]
	• Cognitive assessment: at mean±SD of 3.0±0.07 years
		Ages and Stages Questionnaire (ASQ), reported by primary caregiver: domains of gross motor skills, fine motor skills, problem-solving ability, communication, and personal and social skills.	• 16S rRNA gene sequencing, V3-V5 hypervariable region, Roche pyrosequencing
			• Co-abundance groupings (factor representation) based on principal factor analysis applied to correlation matrix of Spearman rank correlations for the top 25 taxa; four resulting factors:
			  ○ F1: positive loadings for *Lachnospiraceae Dorea, L. Coprococcus* and an unclassified *Lachnospiraceae,* and unclassified *Clostridiales* taxa, negative for *Bacteroides*
			  ○ F2: positive loadings for *Klebsiella*, *Enterobacter* and unclassified *Enterobacteriaceae*
			  ○ F3: positive loadings for *Bacteroides*, negative for *Escherichia/Shigella*, *Bifidobacterium*
			  ○ F4: positive loadings for *Veillonella* and *Clostridium*
			• alpha diversity: Shannon index
			• Differential abundance of bacterial taxa
				Bacterial co-abundance scores associate with ASQ scores[d]:
				• F3 negatively associates with fine motor skills (β=-2.42)
				• F1 negatively associates with communication skills (β=-1.12) and personal and social skills (β=-1.44)
				
				Alpha diversity is not significantly associated with ASQ scores.
				
				Relative abundance of specific bacterial genera associate with ASQ scores[e]:
				• Communication scores negatively correlated with 3 genera in *Clostridiales* order (*Acidaminococcus* (β=-0.014) *Ruminococcus* (β=-0.07), and *Christensenella* (β=-0.003)), 2 genera in *Lactobacillales* order (an unclassified in *Enterococcaceae* family (β=-0.01) and an unclassified (β=-0.007)), 2 genera in *Enterobacteriales* order (*Salmonella* (β=-0.003), and *Erwinia* (β=-0.007)), and positively with *Chryseobacterium* in *Flavobacteriales* order (β=0.003)
				• Personal/social development scores negatively correlated with 3 genera from *Clostridiales* order (*Oribacterium* (β=0.002), *Oscillospira* (β=-0.04), and *Ruminococcus* (β=-0.07)), 2 genera in *Lactobacillales* order (*Weissella* (β=-0.003) and an unclassified in *Enterococcaceae* family (β=-0.01)), and *Salmonella* from *Enterobacteriales* order (β=-0.002)
				• Fine motor scores positively correlated with 2 genera in *Lactobacillales* order (*Lactobacillus* (β=0.003) and *Streptococcus* (β=0.03)), *Atopobium* in *Coriobacteriales* order (β=0.004), and *Actinomyces* in *Actinomycetales* order (β=0.006), and negatively associated with 3 genera in *Enterobacteriales* order (*Klebsiella* (β=-0.03), *Salmonella* (β=-0.003), and *Erwinia* (β=-0.005)), 2 genera in *Clostridiales* order (*Megasphaera* (β=-0.004) and *Phascolarctobacterium* (β=-0.01))

Rozé et al., 2020 Assessment of Neonatal Intensive Care Unit Practices and Preterm Newborn Gut Microbiota and 2-Year Neurodevelopmental Outcomes France EPIFLORE cohort	• Microbiome analysis: n=577 (300 male, 277 female) at median age [IQR] of 23 [22-26] days (DNA amplified and analysed for 484 infants) • Cognitive assessment: n=372 at 2 years corrected age (between 22-26 months) • Condition: preterm infants with gestational age at birth 24-31 weeks	Ages and Stages Questionnaire (ASQ), reported by primary caregiver: domains of gross motor skills, fine motor skills, problem-solving ability, communication, and personal and social skills.	• 16S rRNA gene sequencing, V3-V4 hypervariable region, Illumina MiSeq • Clusters based on taxonomic composition at genus level - relative abundance profiles clustered by the partitioning around medoids algorithm; supplemental cluster 6 defined by lack of DNA amplification (low bacterial load); random forest was used to identify bacterial genera driving the clusters: ○ C1 (n=240): *Enterobacter aerogenes* - C-section, lower risk of belonging to this cluster if breast milk in first week ○ C2 (n=68): *Clostridium sensu stricto* ○ C3 (n=61): *Escherichia/Shigella* - associated with higher GA (a more mature microbiota) ○ C4 (n=63): *Enterococcus* - lower GA ○ C5 (n=52): *Staphylococcus* - lower GA, C-section ○ C6 (n=93): low bacterial load - lower GA, C-section, lower risk of belonging to this cluster if breast milk in first week, increased risk if late-onset infection • Differential abundance of bacterial taxa	Cluster status associates with nonoptimal 2-year outcome - belonging to clusters 4, 5, or 6 significantly associates with nonoptimal 2-year outcome[f]: • C1 OR=2.79 • C2 OR=2.95 • C3 OR=1 (reference) • C4 OR=7.19 • C5 OR=5.06 • C6 OR=6.49 Specific bacterial taxa correlate with ASQ scores[g]: • *Staphylococcus caprae* r=−0.15, p=0.14 • *Escherichia coli* r=0.12, p=0.03
Rothenberg et al., 2021 Neurodevelopment correlates with gut microbiota in a cross-sectional analysis of children at 3 years of age in rural China China	• n=46 (28 male, 18 female) at 3 years (range 36.0–37.9 months)	Bayley Scales of Infant Development (BSID-II): Mental Developmental Index (MDI) and the Psychomotor Developmental Index (PDI)	• 16S rRNA gene sequencing, V3-V4 hypervariable region, Illumina MiSeq • Co-abundance groupings (factor representation) based on principal factor analysis applied to correlation matrix of Spearman rank correlations for the top 25 taxa; three resulting factors: ○ F1: positive loadings for *Faecalibacterium*, *Clostridium* cluster *XIVa*, *Gemmiger*, *Phascolarctobacterium*, *Alistipes*, *Oscillibacter*, and *Sutterella*, and	Bacterial co-abundance scores associate with BSID-II scores – F1 is positively associated with both MDI (β=3.9; explains 12% of variance in the model) and PDI (β=8.6; explains 24% of variance in the model)[h] Alpha diversity does not correlate with MDI or PDI scores. Some specific bacterial genera abundances were associated with MDI or PDI scores, but these were not significant after correcting for multiple comparisons[i]: • MDI:

			negative for *Blautia*, *Anaerostipes*, *Clostridium* cluster *XVIII*, and *Streptococcus* ○ F2: positive loadings for *Blautia*, *Roseburia*, *Ruminococcus*, *Collinsella*, *Cellulosibacter*, *Coprococcus*, and negative for *Bacteroides* and *Parabacteroides* ○ F3: positive loadings for *Faecalibacterium*, *Roseburia*, *Lachnospiraceae incertae sedis*, *Fusicatenibacter* and *Butyricicoccus*, and negative for *Bifidobacterium*, *Ruminococcus*, and *Megamonas* • Alpha diversity: number of observed OTUs, Shannon's diversity index, Faith's Phylogenetic Diversity, and Pielou's measure of evenness • Differential abundances of bacterial genera	○ Positive associations with: *Butyricimonas* (β=0.0016), *Flavonifractor* (β=0.0016), *Faecalibacterium* (β=0.0058), *Clostridium XlVb* (β=0.0011) ○ Negative associations with: *Lachnospiraceae incertae sedis* (β=-0.0037), *Granulicatella* (β=-0.00041), *Abiotrophia* (β=-0.00021) • PDI: ○ Positive associations with: *Faecalibacterium* (β=0.0054), *Gemmiger* (β=0.0043), *Butyricimonas* (β=0.00099)
Acuña et al., 2021 Infant Gut Microbiota Associated with Fine Motor Skills Spain PREOBE observational study cohort	• n=71 (26 female, 45 male) at 18 months	Bayley Scales of Infant Development 3rd edition (BSID-III): dichotomised (< or > median) scores for cognitive, receptive language, expressive language, composite language, gross motor and fine motor	• 16S rRNA gene sequencing, V1-V2 hypervariable region, Illumina MiSeq • Alpha diversity: number of taxa, Faith's phylogenetic diversity, Shannon index • Beta-diversity: weighted and unweighted UniFrac distances • Two enterotypes/community types based on Dirichlet multinomial mixture clustering - an unsupervised clustering method that uses Laplace approximation to identify groups of community assemblies genus level: ○ Group 1 (n=55): *Firmicutes*-dominant – most abundant bacteria are *Lachnospiraceae, Streptococcus* and *Blautia,* specific contribution from *Fusicatenibacter* and *Anaerostipes* ○ Group 2 (n=16): *Bacteroides*-dominant – specific contribution	Alpha diversity does not correlate with Bayley-III results. Beta diversity: weighted but not unweighted UniFrac distances associates with fine motor skills (R^2=0.04)[j] Community types: infants with above-median fine motor skills belong to the *Firmicutes*-dominant enterotype (odds ratio=0.27)[k] Bacterial genera[l]: • Above-median fine motor infants have higher abundance of *Bifidobacterium* (LFC≈2.1)*, Collinsella* (LFC≈1.9)*, Coprococcus* (LFC≈1.2)*, Enterococcus* (LFC≈9.9)*, Fusobacterium* (LFC≈7.3)*, Holdemanella* (LFC≈5.8)*, Lactobacillus* (LFC≈1.7)*, Propionibacterium* (LFC≈2.3)*, Roseburia* (LFC≈1.6)*, Veillonella* (LFC≈2.05) and an unassigned genus within *Veillonellaceae* (LFC≈4.5)

			from *Clostridium XIVa* and *Parabacteroides* • Differential abundance of bacterial genera	• Below-median fine motor infants have higher abundance of *Parabacteroides* (LFC≈1.8) and *Turicibacter* (LFC≈3.1)
Kort et al., 2021 Model Selection Reveals the Butyrate-Producing Gut Bacterium Coprococcus eutactus as Predictor for Language Development in 3-Year-Old Rural Ugandan Children Uganda	• n=139 at 24 and 36 months	Bayley Scales of Infant Development 3rd edition (BSID-III): language scores – raw (prediction model) and dichotomised (score ≥ or < 100; n=61 in below average group and n=78 in above average group; group comparisons) Score at 36 months were used as outcomes.	• 16S rRNA gene sequencing, V4 hypervariable region, Illumina MiSeq • Differential abundance of bacterial taxa	The model that best predicted[m] language scores at 36 months includes the three predictors which are all positively associated with language scores at 36 months (the standardised estimates from a linear regression model including the three predictors is given in brackets; adjusted R^2=0.31): • Language scores at 24 months (β=0.44) • *Coprococcus eutactus* at 24 months (β=1929) • *Bifidobacterium longum* at 36 months (β=417) Bacterial species at 24 months more abundant in[n]: • Language non-impaired group at 36 months (BSID-III ≥ 100): *Coprococcus eutactus, Bifidobacterium catenulatum, Bifidobacterium adolescentis, Faecalibacterium prausnitzi, Holdemanella biformis, Roseburia hominis, Eubacterium eligens, Campylobacter troglodytis, Prevotella copri, Intestinibacter bartlettii, Terrisporobacter petrolearius, Bacteroides xylanolyticus, Clostridium disporicum, Catenibacterium mitsuokai, Campylobacter troglodytis* • Language impaired group at 36 months (BSID-III < 100): *Granulicatella elegans, Parabacteroides, Bifidobacterium longum, Escherichia/Shigella, Campylobacter coli*

[a] covariates: caesarean section, paternal ethnicity, currently breastfeeding, sex, maternal education, paternal age, paternal ethnicity, twin status, and income

[b] covariates: older siblings, paternal ethnicity, sex, maternal education, paternal age, twin status, and income

[c] bacterial genera reported here based on visual inspection of the heat map for correlations of the highest magnitude with each GDI subscale; analyses unadjusted, significance level not reported; ↓ and ↑ indicate whether the bacterial genera is negatively or positively associated with the domain, respectively

[d] β-values are unstandardised (units refer to unit of factor score and unit of ASQ scores (not SDs)); covariates: age at ASQ assessment, vitamin D treatment group, clinical site, mode of delivery, sex, antibiotics in first days of life, gestational at birth, maternal age, marital status, educational level, family income, race/ethnicity, breastfeeding in first 6 months

[e] analysed using multivariate associations with linear models (MaAsLin) on dichotomised ASQ scores at the threshold for typical development; nominally significant ($p<0.05$) associations are reported; β-values are the MaAsLin coefficients; covariates: gestational age, race, gender, breast feeding in first 6 months of life, c-section, age at ASQ, maternal education, maternal marital status, maternal age, low income (<$30,000), antibiotics in the first days of life, treatment group and clinical site

[f] Nonoptimal 2-year outcome defined as death or developmental delay (ASQ score <185), available for n=394 (22 deaths (5.6%)); mixed effects logistic regression, odds ratios (OR) are reported; covariates: gestational age, maternal age, country of birth of the mother, mother level of education, birth weight Z-score, caesarean delivery, and individual therapeutics (surfactant, ductus arteriosus treatment before in the first 10 days of life, late neonatal infection, volume of enteral nutrition at day 7, gastrointestinal transit considered as regular, practice of skin-to-skin contact during the first week of life)

[g] adjustment for covariates not specified

[h] β-values are multiplied by the interquartile range of the factor scores and the units refer to number of points increase in MDI/PDI scores per IQR increase of factor score; covariates: mother's age, whether the mother completed high school, breastfeeding duration, child sex, child's age, child fish consumption in the previous 24 hours, child's weight z-score, attendance of preschool, C-section birth, and illness in the previous 12 months (upper respiratory illness, lower respiratory illness, diarrhoea, vomiting, or fever); all three factors were added together in one model as predictive variables alongside with the covariates.

[i] analysed using MaAsLin; β-values are the MaAsLin coefficients; MDI and PDI scores were first residualised against the covariates as in [h]

[j] Analysed using PERMANOVA; non-adjusted analysis

[k] Analysed using Fisher's exact test; covariates: maternal pregestational BMI and type of breast milk up to 3 months of life

[l] Analysed DESeq2 using non-normalised raw count tables; covariates: maternal pregestational BMI and type of breast milk up to 3 months of life; LFC=$\log_2$ fold change (estimated from Fig. 3)

[m] Best predictors selected using Mixed Integer Optimisation; predictors were selected from a total of 1170 potential predictors: one parameter indicating whether or not the mother of the child was included in the education intervention group, six anthropometric and cognitive parameters at 24 months, and 542 gut microbiota composition related parameters at 24 months and 621 parameters at 36 months; out of 60 best models including 2-4 parameters, language score at 24 months was included in 52 models, *Coprococcus eutactus* at 36 months in 42 models, and *Bifidobacterium* at 24 months in 19 models

[n] Analysed using two-tailed Mann-Whitney U-test for bacterial relative abundances; unadjusted analyses

Table 4-4. Summary of studies investigating associations between gut microbiome and socio-emotional behaviours.

Authors **Title** **Country** **Study name if applicable**	**Sample characteristics: Sample size, sex, and age at samples and assessments**	**Behavioural assessment**	**Gut microbiome:** **Sequencing method and platform** **Features of interest**	**Main findings**
Temperament				

Christian et al., 2015
Gut microbiome composition is associated with temperament during early childhood
USA
	• n=77 (41 male, 36 female) at a mean of 23.14 months (range 18-27 months)	Early Childhood Behaviour Questionnaire (ECBQ). 18 dimensions of temperament that load onto three composite scales: Negative Affectivity, Surgency/Extraversion, and Effortful Control	• 16S rRNA gene sequencing (bacterial tag-encoded FLX-Amplicon pyrosequencing), V1-V3 hypervariable region, Roche 454 FLX Titanium system
			• Alpha diversity: phylogenetic diversity, Shannon index
			• Beta diversity: weighted and unweighted UniFrac distances
			• Differential abundances of bacterial taxa
				Alpha diversity[a]:
				• Surgency/extraversion scale: associates positively with phylogenetic diversity in both boys and girls (r=0.414 and r=0.375, respectively)
				  ○ Subscales of sociability and high-intensity pleasure associate with phylogenetic diversity only in boys (r=0.55 and r=0.35, respectively
				• Effortful control scale: associates negatively with Shannon index in girls only (r=-0.38)
				
				Beta-diversity[b]:
				• In boys: unweighted UniFrac associates with Surgency/Extraversion scale as well as the subscales of sociability, high-intensity pleasure and activity level
				• In girls: unweighted UniFrac associates with fear subscale under Negative Emotionality
				
				Some specific bacterial genera associate with temperament dimensions[c]:
				• In boys:
				  ○ Sociability associates with an unidentified genus in *Ruminococcaceae* family (rho=0.37) and *Parabacteroides* (rho=0.44)
				  ○ High-intensity pleasure associates with *Dialister* (rho=0.37) and an unidentified genus in *Rikenellaceae* family (rho=0.43)
				  ○ Activity level associates with *Dialister* (rho=0.48) and an unidentified genus in *Rikenellaceae* family (rho=0.35)

				• In girls: Fear associates with an unidentified genus in *Rikenellaceae* family (rho=0.37)
Kelsey et al., 2021 Gut microbiota composition is associated with newborn functional brain connectivity and behavioural temperament USA	• n=63 (37 male, 26 female) for microbiome and temperament questionnaire; mean age [range]: 25 days [9-56]	Infant Behaviour Questionnaire Revised Short Form (IBQ-R): three general dimensions of Negative Emotionality (subscales of fear, distress to limitations, falling reactivity, sadness), Regulation/orienting (subscales of low intensity pleasure, cuddliness, duration of orienting, soothability), and Surgency/positive emotionality (subscales of activity level, smiling/laughing, high intensity pleasure, perceptual sensitivity, approach, vocal reactivity)	• shotgun metagenomics, Illumina NovaSeq 6000 • alpha diversity (Shannon index and Chao1 for both taxa and functional terms) • Relative abundance of taxa and functional terms	There were no significant direct associations between taxa or functional term diversity and temperament traits. Mediation analyses: taxa and virulence factor alpha diversity measures indirectly associated with temperament via homologous-interhemispheric connectivity[d]: • increased taxa alpha diversity and negative emotionality: Chao1 β=0.09, Shannon index β=0.12 • virulence factor alpha diversity and negative emotionality: β = 0.13 • virulence factor alpha diversity and regulation/orienting: β=-0.19 Relative abundance of bacteria associate with temperament traits[e]: • Negative emotionality: ↑*Bifidobacterium pseudocatenulatum* (LFC=4.085), ↓*Streptococcus vestibularis* (LFC=3.120), ↓*Schaalia radingae* (LFC=3.385) • Regulation/orienting: ↑*Bifidobacterium catenulatum* (LFC=4.177), ↑*Bifidobacterium pseudocatenulatum* (LFC=4.047)
Aatsinki et al., 2019 * Gut microbiota composition is associated with temperament traits in infants Finland FinnBrain Birth Cohort Study	• n=391 (159 male, 142 female) • gut microbiome: at 2.5 months (mean±SD of 65.2±13.4days days) • behaviour: at 6 months	Infant Behaviour Questionnaire Revised Short Form (IBQ-R SF): three main dimensions of negative emotionality (subscales: distress to limitations, fear, sadness and reversed scale of falling reactivity),	• 16S rRNA gene sequencing, V4 hypervariable region, Illumina MiSeq • Alpha diversity: Shannon and Chao1 indices • Clustering: three clusters based on Bray-Curtis dissimilarity using partitioning around medoids algorithm; clusters named based on the most discriminating bacteria: ○ *Veillonella dispar* (n=84) ○ *Bacteroides* (n=101)	Microbiota clusters associate with temperament dimensions: • *Bifidobacterium/Enterobacteriaceae*-cluster has highest and *Bacteroides*-cluster lowest scores of regulation and subscales of high intensity pleasure, cuddliness and duration of orienting • *Bacteroides*-cluster negatively associates with regulation (β=-0.18) compared to *Bifidobacterium/Enterobacteriaceae*-cluster[f] • *Veillonella dispar*-cluster negatively associates with regulation (β=-0.22) and cuddliness (β=-0.29)

	regulation/orienting (subscales: perceptual sensitivity, low intensity pleasure, cuddliness, duration of orienting and soothability) and surgency/positive emotionality (subscales: activity level, smiling and laughter, high intensity pleasure, approach, vocal reactivity)	○ *Bifidobacterium/Enterobacteriaceae* (n=116) • Abundance of bacterial taxa	compared to *Bifidobacterium/Enterobacteriaceae*-cluster[f] Alpha diversity (Shannon index) associates negatively with negative emotionality (β=-0.17) and fear reactivity (β=-0.27)[g] Specific bacterial genera associate with temperament traits[h]: • Surgency associates with *Atopobium* (LFC=−1.4), *Bifidobacterium* (LFC=1.2) and *Streptococcus*[i] (LFC=0.6) • Regulation associates with *Erwinia*[j] (LFC=1.0) • Negative emotionality associates with *Erwinia* (LFC=0.6), *Rothia* (LFC =0.8) and *Serratia* (LFC=1.3) • Fear reactivity associates with *Erwinia* (LFC=0.3), *Rothia* (LFC=0.6) and *Serratia* (LFC=0.6), *Peptinophilus* (LFC=1.0), and *Atopobium* (LFC=1.0) Several OTUs also associate with temperament traits[k]: • Surgency: *Veillonella dispar* (LFC=-1.3), unidentified *Bifidobacterium* (LFC=1.1) • Regulation: *Veillonella dispar* (LFC=-1.4), unidentified *Bifidobacterium* (LFC=1.4) and an unidentified *Veillonella* (LFC=2.1) • Negative emotionality: three *Clostridiaceae* family species (LFC between -2.3 and -2.6), unidentified *Bacteroides* (LFC=-1.6) • Fear reactivity: three *Clostridiaceae* family species (LFC between -1.4 and -1.5), unidentified *Bacteroides* (LFC=-0.8), two *Streptococcus* species (LFC=-0.7 and -0.6), *Veillonella dispar* (LFC=-0.6), two *Haemophilus parainfluenzae* OTUs (LFC 0.8 and 0.1), unidentified *Bifidobacterium* (LFC=1.1), unidentified *Veillonella* (LFC=1.2), unidentified *Peptoniphilus* (LFC=1.5), unidentified *Bacteroides* (LFC=1.9), unidentified *Parabacteroides* (LFC=2.0), *Bacteroides fragilis* (LFC=2.6)

Wang et al., 2020 Association between Gut Microbiota and Infant's Temperament in the First Year of Life in a Chinese Birth Cohort China	• n=51 (20 male, 31 female) at 1 year of life (mean±SD of 12.3±0.25 months)	Infant Behaviour Questionnaire-Revised (IBQ-R): 14 dimensions of temperament that are subdivided into three composite scales: extraversion, negative affectivity, and orienting/regulation	• 16S rRNA gene sequencing, V3-V4 hypervariable region, Illumina MiSeq • Abundance of bacterial taxa	Bacterial genera associate with temperament traits[l]: • Soothability is associated with abundance of *Bifidobacterium* (β=0.13) • Cuddliness is associated with abundance of *Hungatella* (β=−2.78) In unadjusted Spearman correlation analyses, *Akkermansi*a (positively with fear, duration of orienting, sadness, vocal reactivity, surgency and negative emotionality), *Hungatella* (negatively with smiling and laughter, cuddliness), *Blautia* (negatively with soothability), *Lachnospiraceae* (negatively with soothability, cuddliness, approach), *Faecalibacterium* (positively with vocal reactivity and surgency) and *Bifidobacterium* (positively with soothability) associate with different subscales of the temperament questionnaire.
Fox et al., 2021 Development of the infant gut microbiome predicts temperament across the first year of life US	• Total sample n=67 (32 female, 35 male) • Microbiome analysis at: 1-3 weeks (n=23), 2 months (n=25), 6 months (n=16) and 12 months (n=27) • Behaviour: at 12 months	Infant Behaviour Questionnaire – Revised (IBQ-R): 3 broad dimensions (negative affectivity, surgency/extraversion, orienting/regulation)	• 16S rRNA gene sequencing, V3-V4 region, Illumina MiSeq • Alpha diversity: Chao1 and Shannon indices • Beta diversity: robust Aitchison distances • Differential abundance of bacterial genera	Alpha diversity: suggestive negative association between microbiota Shannon index at 2 months and negative affectivity at 12 months (β=−0.57, p=0.06)[m]. Beta diversity[n]: • At 1-3 weeks associated with surgency/extraversion (R^2=0.276, p=0.012) • At 12 months suggestively associated with negative affectivity (R^2=0.101, p=0.094) Bacterial genera associated with temperament traits[o]: At 1-3 weeks: • Surgency/extraversion: *Bifidobacterium* (LFC≈5.3), *Lachnospiraceae* (LFC≈6.5), *Collinsella* (LFC≈6.9) positively associated; *Klebsiella* (LFC≈-5.75) negatively associated At 12 months: • Negative affectivity: *Megamonas* (LFC≈11.0), *Acidaminococcus* (LFC≈9.25) and *Ruminococcus* (LFC≈9.3) are positively associated; *Lactobacillus* (LFC≈-10.25) negatively associated
Fear-related behaviour				

Aatsinki et al., 2020 * Infant Fecal Microbiota Composition and Attention to Emotional Faces Finland FinnBrain Birth Cohort Study	• n=122 (65 male, 57 female) • microbiome analysis: at 2.5 months (mean±SD of 69±14 days) • behavioural analysis: at 8 months (±2 weeks)	Eye-tracking task for emotional attention: calculated measures of face and fear bias	• 16S rRNA gene sequencing, V4 hypervariable region, Illumina MiSeq • Alpha diversity: Shannon and Chao1 indices • Beta diversity: Bray-Curtis dissimilarity • Abundances of bacterial taxa	Alpha and beta diversity measures do not associated with face or fear bias. Specific bacterial taxa associate with fear bias[p]: • Negatively associated: *Lactobacillus* (LFC=-6.5), *Bifidobacterium* (LFC=-5.3), *Prevotella* (LFC=-5.7) and *Haemophilus* (LFC=-6.8) • Positively associated: *Clostridium* (LFC=5.4)
Carlson et al., 2021 Infant gut microbiome composition is associated with non-social fear behaviour in a pilot study USA	• Total sample: n=34 (23 male, 11 female); all participants were healthy full-term, had vaginal delivery, exclusive breastfeeding until 1 month, of life, and no antibiotic exposure later than two weeks before delivery until 1 month of life • Microbiome analysis: n=32 at 1 month; n=21 at 1 year • Behavioural assessments at 1 year (median [range] = 384 days [333–491])	• Mask Task of the Laboratory Temperament Assessment Battery: non-social fear expression; fear in response to four masks was coded for facial fear, vocal distress, bodily fear, escape behaviour, and startle response • Strange Situation: social wariness toward a stranger during the assessment • Infant Behaviour Questionnaire - Revised (IBQ-R): composite fear score	• 16S rRNA gene sequencing, V1-V2 hypervariable region plus *Bifidobacterium*-specific primers in a 4:1 Universal to *Bifidobacterium*, Illumina MiSeq • Alpha diversity principal components: 4 measures of alpha diversity (Shannon Diversity, Observed Species, Faith's Phylogenetic Diversity, and Chao1) were calculated and principal component analysis of these four alpha diversity measures (PC1 and PC2) were used as the predictors; at 1 month PC1 has positive loadings for all alpha diversity measures, PC2 has strong negative loading for Shannon Index and smaller positive loadings for other alpha diversity measures • Beta diversity: principal coordinates analysis of Weighted UniFrac was conducted at both timepoints and PC1 and PC2 were used as predictors; at 1 month PC1 has strong positive associations with *Bifidobacterium* and *Streptococcus* and strong negative associations with *Bacteroides,* PC2 at 1 month has strong positive associations with *Veillonella* and negative associations with an unnamed genus of *Enterobacteriaceae* and *Bifidobacterium*;	1-month alpha diversity PC2 positively associated with non-social fear response in the mask task (β=0.47, r^2=0.12; n=19)[q] 1-year Weighted UniFrac PC1 negatively associated with non-social fear response in the mask task (β=-1.15, r^2=0.26; n=14)[r] Nominally significant associations between bacterial genera at 1 year and non-social fear: • unnamed genus in *Clostridiales* order ~ vocal distress (β=30.82, r^2=0.23), ~ startle (β=11.67, r^2=0.38) • *Sutterella* ~ startle (β=8.06, r^2=0.41) • *Dialister* ~ escape behaviour (β=12.41, r^2=0.28) • members of the Clostridiales order that could not be confidently assigned to a family or genus ~ escape behaviour (β=15.44, r^2=0.19) • unnamed genus of Erysipelotrichaceae ~ escape behaviour (β=17.87, r^2=0.2) Social fear (Strange Situation) and parent-reported fear (IBQ-R fear index) are not associated with microbiota measures.

			at 1 year, PC1 has strong positive correlations with *Bacteroides* and strong negative correlations with *Veillonella, Dialister,* an unnamed genus of *Clostridiales, Bifidobacterium, and Lactobacillus,* PC2 has strong positive associations with *Bacteroides* and *Dialister* and strong negative associations with *Bifidobacterium* Relative abundance of bacterial genera	
Problem behaviour				
Loughman, Ponsonby, et al., 2020 Gut microbiota composition during infancy and subsequent behavioural outcomes Australia Barwon Infant Study	• n=201 (106 male, 95 female) for microbiome at 12 months and behavioural assessment at 2 years • n=182 for microbiome at 1 month • n=190 for microbiome at 6 months	Child Behaviour Checklist (CBCL): Internalizing, Externalizing and Total Problem subscales	• 16S rRNA gene sequencing, V4 hypervariable region, Illumina MiSeq • Alpha diversity: Shannon, Simpson, and Chao1 indices, and number of Observed species • Beta diversity: weighted and unweighted UniFrac distances • Abundances of bacterial taxa	Alpha diversity (Shannon index) at 1, 6 and 12 months positively, but not significantly associates with problem behaviour[s] (OR=2.6, OR=1.69 and OR=2.42, respectively). Beta diversity (unweighted UniFrac) at 12 months associates with problem behaviour (R^2=0.0092)[t], however, this may reflect differential multivariate dispersion. Abundance of bacteria at different timepoints associate with problem behaviour[u]: • At 6 months: *Sutterella* (LFC=-0.37) • At 12 months: *Prevotella* (LFC=-1.46), unidentified *Lachnospiraceae* (LFC=2.09) Association between low *Prevotella* and problem behaviours persist following adjustments for technical (storage) and other covariates (gestational age, mode of birth, antibiotic use during labour, breastfeeding at four weeks, number siblings, household pet ownership), but was attenuated for the unidentified *Lachnospiraceae*.
Loughman, Quinn, et al., 2020 Infant microbiota in colic: predictive associations with problem crying and	• n=118 (63 male, 55 female) for microbiome and behavioural outcomes	Child Behaviour Checklist (CBCL): Internalizing, Externalizing, and Total Problems subscales	• 16S rRNA gene sequencing, V3-V4 hypervariable region, Illumina MiSeq • Alpha diversity: Chao1 and Shannon-Weaver indices • Beta diversity: weighted and unweighted UniFrac distances	Alpha diversity in early infancy associates with increased odds for behaviour problems at 2 years of life (Shannon–Weaver index, OR=3.47)[v] Beta diversity does not significantly associate with problem behaviour.

subsequent child behaviour Australia Baby Biotics study	• Microbiome analysis: at mean±SD of 7.4±2.7 weeks • Behavioural outcome: at 2 years • Condition: infants with colic (crying and/or fussing)		• Abundances of bacterial taxa	54 bacterial OTUs associate with 2-year problem behaviour[w]: • Less abundant in problem behaviour case group: 3 *Streptococcus* species, 2 S*taphylococcus* species, 1 *Parabacteroides* species, 1 *Klebsiella* species, 1 *Enterococcus* species, 7 *Bifidobacterium* species, 6 *Bacteroides* species and 1 *Actinomyces* species • More abundant in problem behaviour case group: 2 *Ruminococcus* species, 1 *Parabacteroides* species, 1 *Lactobacillus* species, 1 *Faecalibacterium* species, 1 *Eggerthella* species, 3 *Collinsella* species, 5 *Clostridium* species, 6 *Bifidobacterium* species and 1 *Anaerostipes* species
Zhang et al., 2021 Preliminary evidence for an influence of exposure to polycyclic aromatic hydrocarbons on the composition of the gut microbiota and neurodevelopment in three-year-old healthy children	• Microbiome analysis: n=38 (20 male, 18 female) at 3 years • Behavioural analysis: n=25 at the same time	Child Behaviour Checklist with six core syndromes: Anxious/depressed, Withdrawn, Sleep problems, Somatic complaints, Aggressive behaviour, and Destructive behaviour; in addition, there are two broadband syndromes: Internalizing and Externalizing	Same as in Table 4-3	Associations between bacteria phyla and CBCL scores (unadjusted analyses): • *Bacteroidetes*~Withdrawn syndromes (rho=0.551 and Somatic complaints (rho=0.413) • *Actinobacteria*~Destructive behaviour (rho=0.589) and Externalizing behaviour (rho=0.471) • Proteobacteria~Withdrawn syndromes (rho=−0.435) • Verrucomicrobia~Anxious/depressed (rho=−0.476), Aggressive behaviour (rho=−0.542), Destructive behaviour (rho=−0.552), Internalizing behaviour (rho=−0.471), Externalizing behaviours (rho=−0.598), and total CBCL scores (rho=−0.410) With bacterial genera[x]: • *Actinobacillus* positively associated with all CBCL subscales • *Lactococcus* positively associated with all CBCL subscales • *Romboutsia* positively associated with all CBCL subscales • *Akkermansia* negatively associated with all CBCL subscales
Other behavioural traits				

Laue et al., 2020 Prospective associations of the infant gut microbiome and microbial function with social behaviours related to autism at age 3 years New Hampshire Birth Cohort Study	• Total cohort with behaviour analysis: at a mean±SD of 3.1±0.2 years, n=386 (199 male, 187 female) • Overlapping samples (any microbiome and behaviour data): n=273 • Microbiota analysis with 16S: at 6 weeks (n=166), at 1 year (n=158), a 2 years (n=129) and at 3 years (n=140) • Microbiota analysis with shotgun metagenomics: at 6 weeks (n=101) and at 1 year (n=103)	Social Responsiveness scale 2nd edition (SRS-2)	• 16S rRNA gene sequencing, V4-V5 hypervariable region, Illumina MiSeq • alpha diversity: Shannon, Simpson indices and count of detected ASVs • beta diversity: generalised UniFrac distances • abundance of bacterial taxa • shotgun metagenomics in a subset with Illumina NextSeq • features from shotgun metagenomics: alpha diversity, taxon and functional pathway relative abundances	Alpha diversity does not significantly associate with SRS-2 scores. Beta-diversity at 1 year of life associated with SRS-2 scores[y]. Bacterial taxa associate with SRS-2 scores[z]: • at 6 weeks: ○ from shotgun metagenomic sequence data: *Flavonifactor plautii* (*Ruminococcaceae* family; β=0.002) ○ from 16S: no significant associations • at 1 year: ○ from shotgun metagenomic sequence data: *Adlercreutzia equolifaciens* (*Coriobacteriaceae* family; β=0.002); *Ruminococcus torques* (*Lachnospiraceae* family; β=0.01); unidentified *Lachnospiraceae* (β=0.001); *Eubacterium dolichum* (*Erysipelotrichaceae* family; β=0.0003) ○ from 16S: *Blautia producta* (*Lachnospiraceae* family; β=0.34); unidentified *Lachnospiraceae* (β=0.01) • at 2 years: ○ from 16S: unidentified *Coprococcus* (*Lachnospiraceae* family; β=0.07); *Ruminococcus gnavus* (*Lachnospiraceae* family; β=0.17); unidentified *Bifidobacterium* (*Bifidobacteriaceae* family; β=0.06) • at 3 years: ○ from 16S: *Butyricicoccus pullicaecorum* (*Ruminococcaceae* family; β=0.01) Bacterial functional pathways[aa]: Pathways involved in urea cycle or vitamin B6 biosynthesis associate with SRS-2 scores. For example, increased abundance of L-ornithine de novo biosynthesis and the superpathway of pyridoxal 5'-phosphate biosynthesis and salvage pathways at 6 weeks and 1 year are negatively associated with SRS-2 scores at 3 years. At 1 year, the

				three most significant pathways are the superpathway of L-aspartate and l-asparagine synthesis, O-antigen building blocks biosynthesis—*Escherichia coli*, and pentose phosphate pathway (all negatively associated with SRS-2 scores).
Sun et al., 2020 Log-contrast regression with functional compositional predictors: linking preterm infants' gut microbiome trajectories to neurobehavioural outcome USA	• n=34 (17 male, 17 female) • Microbiome data collected between 5-28 postnatal days (each infant provided at least 5 samples) • Behavioural assessment at 36-38 weeks of postmenstrual age • Preterm infants	NICU Network Neurobehavioural Scale (NNNS); used the NSTRESS (Stress/abstinence scale) score with data divided into equal thirds (upper, mid and lower third); higher score indicates more stressful behavioural performance	• 16S rRNA gene sequencing, V4 hypervariable region, Illumina MiSeq • Abundance of bacterial taxa over time	Four known bacterial genera associate with NSTRESS scores during the neonatal period[bb]: • *Veillonella* (*Clostridiales* order): effect switches from negative to positive during the postnatal days from 5-28 • *Enterococcus (Lactobacillales* order): associated with higher stress scores during early postnatal days • *Shigella* (*Enterobacteriales* order): effect switches from positive to negative during the postnatal days from 5-28 • unclassified genus from *Enterobacteriales* order: increasing negative effect during the postnatal days 5-28 The strongest effect estimates are observed at around 26 days of postnatal life: *Enterococcus*, the unidentified *Enterobacteriales* genera and *Shigella* negatively, while genus *Veillonella* positively associate with NSTRESS scores.
Sobko et al., 2020 Impact of outdoor nature-related activities on gut microbiota, fecal serotonin, and perceived stress in preschool children: the Play&Grow randomised controlled trial Hong Kong	• n=45 (23 male, 22 female) • n=27 in intervention group (outdoor-play intervention program); mean±SD of 35.8±12.21 months • n=18 in control group; mean±SD of 35.4±8.51 months	Perceived Stress Scale for Children (PSS-C); anger score analysed separately	• 16S rRNA gene sequencing, V3-V4 hypervariable region, Illumina MiSeq • Abundance of bacterial taxa • Alpha diversity: Chao1 index • Alpha diversity of bacterial phyla: Shannon and Simpson indices for each phyla separately • Estimated functional pathways (PICRUST)	Microbial richness (Chao1) associates with PSS-C score: rho=-0.27 Correlations with alpha diversity for phyla and PSS-C scores: • *Bacteroidetes* (Shannon index: rho=-0.26 and Simpson index: rho=-0.24) • *Proteobacteria* Shannon index: rho=-0.23 (but p=0.07) PSS-C score associates with estimated pathways ($p<0.1$)[cc]: • Negatively with: betalain biosynthesis, indole alkaloid biosynthesis

	• Positively with: Isoquinoline alkaloid biosynthesis, C5-branched dibasic acid metabolism, propanoate metabolism, glyoxylate and dicarboxylate metabolism, pyruvate metabolism, glycolysis/gluconeogenesis, N-glycan biosynthesis, fatty acid metabolism, lipid biosynthesis proteins, nicotinate and nicotinamide metabolism, phosphonate and phosphinate metabolism, D-glutamine and D-glutamate metabolism, beta-alanine metabolism, prenyltransferases

* Overlapping cohorts

[a] Analysed in boys and girls separately using Pearson's correlation; unadjusted analyses

[b] Analysed in boys and girls separately using PERMANOVA; unadjusted analyses

[c] Analysed in boys and girls separately using Spearman rank correlation; unadjusted analyses

[d] Analysed using ordinary least squares path analysis; covariate adjustment not specified

[e] Analysed using LefSE, covariate adjustment not specified; ↓ and ↑ indicate whether a bacteria is enriched in low or high temperament trait groups, respectively (groupings based on median split); LFC: log-2 fold change

[f] Analysed using linear regression; covariates: infant sex and mode of delivery

[g] Analysed using linear regression; covariates: gestational age, infant age, sex, mode of delivery, breastfeeding and antibiotics intake

[h] Analysed using DESeq2; covariates: infant age, sex and mode of delivery; LFC=$\log_2$ fold change; FDR-corrected q-value<0.25 was considered statistically significant

[i] Association remains significant if additionally adjusted for gestational age, antibiotic treatments and breastfeeding status at 2.5 months of age

[j] Association remains significant if additionally adjusted for gestational age, antibiotic treatments and breastfeeding status at 2.5 months of age

[k] Analysed using DESeq2; covariates: infant sex, mode of delivery, gestational age, infant age during sampling, antibiotic treatments and breastfeeding status at 2.5 months of age; FDR-corrected q-value<0.25 was considered statistically significant

[l] Analysed using linear regression; covariates: delivery mode, feeding type, and probiotic consumption

[m] Analysed using multivariate linear regression; covariates: infant sex and breastfeeding duration

[n] Analysed using PERMANOVA; covariates: infant sex and breastfeeding duration

[o] Analysed using multivariate negative binomial mixed models in DESeq2; covariates: infant sex and duration of breastfeeding; LFC=$\log_2$ fold change; FDR-corrected p-value<0.1 was considered statistically significant

[p] Analysed using DESeq2; LFC=$\log_2$ fold change; covariates: mode of delivery, breastfeeding, infant age at faecal sampling, maternal depressive symptoms at the end of pregnancy, and infant sex

[q] Analysed using two-level mixed effects structure accounting for the within-subject correlations among the fear outcomes and within-subject but between-mask correlations of the outcome

[r] Analysed the same way as in [q]

[s] Problem behaviour case group defined as 1 SD above the mean of reference population (T≥60; n=22 of problematic behaviour case group in this study (~11%)); unadjusted analyses

[t] Analysed using PERMANOVA

[u] Analysed using voom on normalised abundance of taxa: using relative log expression with pseudo-counts, scaled the counts for each sample by the median across OTUs of the OTU count divided by the geometric mean of that OTU's count across all samples; unadjusted analyses, corrected for multiple comparisons; LFC=log fold change, relative to non-case group (negative values indicate lower expression in the behaviour case group as described in [s]

[v] Behaviour problem group was defined as a score of T≥60 (1 SD above the population normed mean) on one or more of the three CBCL subscales (n=20 of problematic behaviour case group in this study (17%)); covariates: sex, probiotic randomisation group, child age at baseline and maternal mental illness (at child age 2 years)

[w] Analysed using DESeq2; groups defined and same covariates as in [v]; results are FDR corrected

[x] bacterial genera reported here based on visual inspection of the heat map for correlations of the highest magnitude with CBCL scores; analyses unadjusted, effect sizes and significance level not reported

[y] Analysed using PERMANOVA; effect sizes not given; covariates: child age at follow-up, maternal education, maternal marital status, parity, maternal and paternal age at delivery, and child sex; results not significant if additionally adjusted for maternal smoking during pregnancy, early postnatal exclusive breastfeeding, delivery mode, perinatal antibiotic exposure, and continuous gestational age at delivery

[z] Analysed using MaAsLin; β-values are unstandardised (refer to % change of relative abundance per point increase in SRS scores); FDR-corrected q-value<0.25 was considered statistically significant; covariates: child age at follow-up, maternal education, maternal marital status, parity, maternal and paternal age at delivery, and child sex

[aa] Analysed as in [z]

[bb] Analysed using sparse log-contrast regression with functional compositional predictors; covariates: sex, delivery type, premature rupture of membranes, score of SNAPPE-II (acute physiology-perinatal extension), birthweight, % of feeding with mother's breast milk

[cc] Analysed using Spearman correlation; unadjusted analyses

Table 4-5. Summary of studies investigating associations between gut microbiome and structural and functional neuroimaging features

Study details: Authors Title Country Study name if applicable	**Sample characteristics: Sample size, sex, and age at samples and assessments**	**Neuroimaging: Modality Features of interest**	**Gut microbiome: Sequencing method and platform Features of interest**	**Main findings**
Carlson et al., 2018 * Infant Gut Microbiome Associated With Cognitive Development USA UNC Early Brain Development Study	• Microbiome analysis: n=89 (49 male, 40 female) at 1 year • Brain scans: n=46 at 1 year (median age 12.8 months), n=27 at 2 years (median age 25.1 months)	• Structural MRI, T1 • Volume of total intracranial volume, total gray matter, total white matter, total cerebrospinal fluid, lateral ventricle volume, and 90 gray matter ROIs segmented using ITK-SNAP and neonatal Automated Anatomical Labeling atlas template	Same as in Table 4-3	Alpha diversity at 1 year associates positively with the volumes of left precentral gyrus (Chao1), left amygdala (observed species), and right angular gyrus (Chao1) at 2 years[a] Microbiota clusters at 1 year associate with the volumes of right superior occipital gyrus at 1 year of life (largest in C2 and smallest in C3)[b], and with the volumes of left and right caudate nucleus at 2 years of life (smallest in C2 and largest in C3)[c]
Gao et al., 2019 * Gut microbiome and brain functional connectivity in infants-a preliminary study focusing on the amygdala USA UNC Early Brain Development Study	• n=39 (16 male, 23 female) for microbiota and brain scan; mean±SD age at scan: 13.5±1.3 months	• resting state functional MRI • functional connectivity of the amygdala using seed-based approach to generate voxel-wise temporal correlation measures for left and right amygdala • functional connectivity of other brain regions using nine seeds based on adult resting-state networks	• 16S rRNA gene sequencing, V1-V2 hypervariable region, Illumina MiSeq • Alpha diversity: Chao1, observed species, Shannon Index, Faith's Phylogenetic diversity	Alpha diversity associates with functional connectivity between[d]: • the left amygdala and thalamus (negative association; correlation coefficients between -0.33 and -0.55) • the anterior cingulate cortex and right anterior insula (negative association; correlation coefficients between -0.31 and -0.54) • the supplemental motor area and left parietal cortex (positive association; correlation coefficients between 0.29 and 0.41)

Kelsey et al., 2021
Gut microbiota composition is associated with newborn functional brain connectivity and behavioural temperament
USA
	• n=63 (37 male, 26 female) for microbiome and brain scan; mean age [range]: 25 days [9-56]	• resting state functional NIRS
		• three functional connectivity networks of interest based on signal correlations in regions of interest:
		  ○ fronto-parietal network
		  ○ default mode network
			Same as in Table 4-4	Taxa-level alpha diversity associates positively with:
				• Left fronto-parietal network: Chao1 β=0.71, Shannon β=0.14 in unadjusted models; Chao1 β=0.46, Shannon β=0.18 in adjusted modelse
				• Homologous-interhemispheric connectivity: Chao1 β=0.16, Shannon β=0.06 (unadjusted models)

		○ homologous-interhemispheric network		Virulence factor (a functional term) diversity (Chao1) associates positively with homologous-interhemispheric connectivity: β=0.22 in unadjusted model; β=0.23 in adjusted model[f] Relative abundance of bacteria associates with functional connectivity[g]: • Left default mode network: ↓ Clostridium perfingens (LFC= 3.559) • Left fronto-parietal network: ↑Enterococcus faecalis (LFC=3.765), ↑ Collinsella (LFC =3.665), ↑ Clostridium disporicum (LFC=3.548), ↑ Prevotella copri (LFC=3.523), ↑ Clostridium perfingens (LFC=3.415), ↑ Clostridium tertium (LFC=3.367), ↑ Robinsoniella peoriensis (LFC=3.265), ↑ Clostridium (LFC=3.167), ↑ Bacteroides caccae (LFC=3.164); ↓ Streptococcus salivarius (LFC=3.397), ↓ Enterococcus (LFC=3.042) • Homologous-interhemispheric network: ↑ Escherichia coli (LFC=4.357), ↓ Bifidobacterium dentium (LFC=4.012)
Carlson et al., 2021 Infant gut microbiome composition is associated with non-social fear behaviour in a pilot study USA	• Total sample: n=34 (23 male, 11 female); all participants were healthy full-term, had vaginal delivery, exclusive breastfeeding until 1 month, of life, and no antibiotic exposure later than two weeks before delivery	• Structural MRI, T1 and T2 • Volumes of hippocampus, amygdala (summed bilaterally) and medial prefrontal cortex (mPFC; the sum of bilateral cingulate gyrus anterior, straight gyrus rectus, superior frontal gyrus, medial orbital gyrus, subgenual frontal cortex, and pre- subgenual frontal cortex) parcellated using AutoSeg, NeoSeg, ITK-SNAP and the Gousias paediatric template	Same as in Table 4-4	Nominally significant associations: • 1-month Weighted Unifrac PC1 was positively associated with 1-year mPFC volume (β=22701, r2=0.34, p=0.046, n=14) • 1-year Weighted Unifrac PC1 was negatively associated with 1-year amygdala volume (β=-201.3 r2=0.29, p=0.034, n=13) Nominally significant associations between bacterial genera at 1 month and brain volumes at 1 month[h]: • *Streptococcus* ~ amygdala (β=-355, r^2=0.36; survives FDR correction), ~ hippocampus (β=-257, r^2=0.26), ~ mPFC (β=-7612, r^2=0.24) • *Bacteroides* ~ amygdala (β=233, r^2=0.23)

	until 1 month of life • Microbiome analysis: n=32 at 1 month; n=21 at 1 year • Brain scans: n=29 at 1 month (median [range] = 30 days [15–59]; n=16 at 1 year (median [range] = 384 days [333–491])	• *Staphylococcus* ~ amygdala (β=-8456, r^2=0.15), ~ mPFC (β=-239046, r^2=0.18) • *Lachnospiraceae* ~ mPFC (β=16622, r^2=0.21) Nominally significant associations between bacterial genera at 1 month and brain volumes at 1 year[i]: • *Bacteroides* ~ mPFC (β=-18833, r^2=0.36) • *Lachnospiraceae* ~ amygdala (β=616, r^2=0.33) • *Veillonella* ~ mPFC (β=52921, r^2=0.54) • *Enterobacteriaceae* ~ amygdala (β=-758, r^2=0.31)

*overlapping cohorts

[a] covariates: older siblings, paternal ethnicity, and total intracranial volume

[b] covariates: caesarean section, paternal ethnicity, currently breastfeeding, total intracranial volume

[c] covariates: caesarean section, paternal ethnicity, currently breastfeeding, total intracranial volume

[d] For each voxel ANCOVA was used to test for microbiome effects; covariates: older sibling, paternal ethnicity, birth weight, postnatal age at scan, sex, twin status, maternal/paternal education, residual frame-wise displacement; effect determined as significant if voxel-wise p<0.001 and cluster size threshold of 7 face-connected voxels. Post hoc cluster-level responses were generated using the average functional connectivity per subject for each cluster detected and then calculated Pearson correlation with alpha diversity.

[e] covariates: antibiotics, delivery method, breastfeeding, infant age, birthweight and weight at study visit, gestational age, income, sex, head circumference at birth

[f] covariates: antibiotics, delivery method, breastfeeding, infant age, infant weigh at study, gestational age, income, sex, maternal depression, head circumference at birth

[g] analysed using LefSE; covariate adjustment not specified; ↓ and ↑ indicate whether a bacteria is enriched in low connectivity or high connectivity groups, respectively (groupings based on median split); LFC: log-2 fold change

[h] Analysed using linear regression; n=27, covariates: age at 1 month scan, sex; mPFC=medial prefrontal cortex

[i] Analysed using linear regression; n=14, covariates: age at 1 year scan, sex

Table 4-6. Summary of additional studies investigating associations between gut microbiome and brain and/or behavioural development.

Authors **Title** **Country** **Study name if applicable**	**Sample characteristics: Sample size, sex, and age at samples and assessments**	**Neurodevelopmental assessment or neuroimaging**	**Gut microbiome: Sequencing method and platform Features of interest**	**Main findings**
Wei et al., 2022 Associations of maternal prenatal emotional symptoms with neurodevelopment of children and the neonatal meconium microbiota: A prospective cohort study China Shanghai Maternal-Child Pairs Cohort (Shanghai MCPC)	• Microbiome analysis: n=410 within 24h of birth (meconium), 228 boys, 182 girls • Behaviour: n=287 at 24 months • Recruitment targeted to balance women with emotional symptoms with those without emotional symptoms	Ages and Stages Questionnaire, Third Edition (ASQ-3) (developmental progress in 5 domains: communication, gross motor, fine motor, problem solving, and personal-social skills) Strengths and Difficulties Questionnaire (SDQ) (5 subscales: 4 subscales for difficulties (emotional, conduct, hyperactivity, and peer relationship) and 1 for strengths (prosocial behaviour))	• 16S rRNA gene sequencing, V3-V4 hypervariable region, Illumina MiSeq PE300 platform • Alpha diversity: microbial richness (Chao1 index and observed species) and evenness (Shannon diversity and Simpson diversity indices) • Beta diversity: Bray-Curtis, weighted UniFrac, unweighted UniFrac distances • Relative abundance of taxa	Alpha diversity: • negatively correlated with prosocial behaviour (from SDQ; Shannon, observed species and Chap1): effect size range ~-0.1 to -0.2[a] • No significant associations between any of the other SDQ or ASQ subscales Taxa (only tested those families and genera that were significantly different with ALDEx2 between babies whose mothers had emotional symptoms vs those without emotional symptoms):[b] • *Burkholderia*: negatively associated with fine motor scores (SDQ, effect size ~-0.1) • *Ralstonia*: negatively associated with fine motor scores (SDQ, effect size ~-0.4) • *Lactobacillus*: negative association with prosocial behaviour (effect size ~-0.6), positive with total difficulties (effect size ~0.11) and hyperactivity (effect size ~0.05) • *Burkholderiaceae*: no significant relationships with behaviour Mediation: meconium richness and *Lactobacillus* abundance mediate relationships between maternal emotional symptoms and prosocial behaviour.
Streit et al., 2021 Microbiome profiles are associated with cognitive functioning in 45-month-old children Germany	Final microbiota analysis sample: n=323 at 45 months (mean±SD 45.02±1.00 months, 53.2% female	Wechsler Preschool and Primary Scale of Intelligence - Third Edition (WPPSI-III): full-scale IQ, verbal IQ, performance IQ,	• 16S rRNA gene sequencing, V3-V4 hypervariable region, Illumina MiSeq • Metagenomic shotgun sequencing for a subset of 39 samples using Illumina HiSeq 4000	Variance explained by overall microbiome composition[c]: • Full-scale IQ: 13.3% • Verbal IQ: 15.8% • General language composite: 10.3% After adjusting for covariates[d] the associations were reduced, only verbal IQ remained significant

Pre-, Peri-, and Postnatal Stress: Epigenetic Impact on Depression; POSEIDON		general language composite	• Alpha diversity: observed species, Shannon's and Faith's phylogenetic diversity, and Pileou's Evenness • Relative abundance of genera and metabolic pathways	Alpha diversity[e]: negatively associated with cognition; after adjustment for covariates[f], only association between Faith's index and full-scale and verbal IQ and general language composite remained significant Taxa[g]: • Unidentified genus within *Enterobacteriaceae* negatively correlates with lower full-scale IQ (ß=-0.20; the only one significant after adjusting for multiple testing), verbal IQ (ß=-0.19), performance IQ (ß=-0.14) and language composite (ß=-0.21); full IQ association remains significant after adjusting for maternal age, shipment and maternal cigarette smoking during pregnancy • *Eubacterium* of *Erysipelotrichaceae* family negatively correlates with verbal IQ (ß=-0.20) • From metagenomics: ○ *Enterobacter cloacae* associated with full IQ, *Enterobacter cloacae* and *Enterobacter asburiae* were associated with the WPPSI-III PIQ subscale ○ Pathways: nominally significant negative association between full IQ and the predicted level of norspermidine after adjusting for smoking during pregnancy, present cigarette smoking, maternal age, and shipment (ß=-0.47)
Seki et al., 2021 Aberrant gut-microbiota-immune-brain axis development in premature neonates with brain damage Austria	• n=60, all extremely preterm infants born <28 weeks of gestation; mean GA at birth 25.15 weeks • Microbiome analysis: at day 3, 7 and 14; then biweekly sampling until discharge from hospital • aEEG: same timepoints as stool samples	• aEEG for monitoring neurophysiology/ cerebral activity: analysed by visual assessment of Burdjalov total maturational scores • NIRS for cerebral oxygenation: % of fraction of time spent with an oxygen supply	• 16S rRNA gene sequencing, V4 hypervariable region, Illumina MiSeq • Clusters: hierarchical clustering using Ward's method; identified 3 main and 8 sub-clusters that differed in the abundance of *Veillonella, Klebsiella, Escherichia-Shigella*, and *Bifidobacterium* • Abundance of genera	*Klebsiella* 1.7x more abundant at 4 weeks and less abundant shortly after delivery in infants with brain injury[h] With brain injury: ↑*Bifidobacterium* at 6 weeks, ↑*Finegoldia* shortly before discharge Without brain injury: ↑*Enterococcus, Escherichia-Shigella*, and *Streptococcus* at 3 days post-delivery; ↑*Enterococcus* 2 weeks post-delivery Clusters: in cluster C (*Klebsiella*-dominant) no healthy infant samples were observed -> Klebsiella domination in the gut associates with neurological pathologies

	• MRI: at term-equivalent age; 38 with no or mild brain injury; 15 with severe brain injury	deviating beyond 10% from the mean of total measurements • MRI: Kidokoro scores to assess presence of brain damage		Strongest indicators for white matter injury were *Klebsiella* levels, specific T-cell populations and production of cytokines and chemokines

Tamana et al., 2021
Bacteroides-dominant gut microbiome of late infancy is associated with enhanced neurodevelopment
Canada
CHILD (Canadian Healthy Infant Longitudinal Development) Cohort Study
	• n=405 (199 girls)
	• Microbiome analysis: at 4 (mean±SD 4.2±1.24 months) and 12.5 months (mean±SD 4.2±1.29 months)
	• Behaviour: at 1 and 2 years
		Bayley Scale of Infant Development Third Edition (BSID-III): cognitive, receptive and expressive communication, gross and fine motor scales	• 16S rRNA gene sequencing, V4 hypervariable region, Illumina MiSeq
			• Clusters: partitioning around medoids; applies LEfSE to identify unique taxa that differentiated the clusters
			• At 4 months: Proteobacteria-, Firmicutes- and Bacteroides-dominant clusters
			• At 12 months: *Proteobacteria*- (C1), *Firmicutes*- (C2) and *Bacteroides*-dominant (C3) clusters
			  ○ lowest richness and diversity in C1, highest in C2
			  ○ C3 enriched with sphingolipid metabolism and glycosphingolipid biosynthesis, metabolism of folate, biotin, pyruvate, vitamin B6, lipoic acid and fatty acid biosynthesis were enriched in this cluster
			• Bacterial taxa
				4 month clusters did not associate with Bayley scores.
				
				12 month clusters[i]:
				• no association between 12 month clusters and 1 year cognition
				• compared to C1, children in C3 and C2 had higher cognitive scores (4.8 and 5.3 points, respectively), language scores (4.2 and 4.1 points, respectively) and motor scores (3.1 and 3 points, respectively)
				• compared to C1, children in C3 and C2 had larger change in cognitive (3.3 and 3.8 points, respectively) and language (5.8 and 2.8 points, respectively) performance from 1 to 2 years of life; no difference for motor scores
				• in sex-stratified analyses, significant associations between clusters and cognitive development were only observed in males
				
				Taxa[j]:
				• different species of *Bacteroides* positively associated with increased cognitive and language development
				• *Prevotella* positively associates with motor development; also *Actinomyces* and unidentified genus from *Methylophilaceae*

Van De Wouw et al., 2022 Associations Between the Gut Microbiota and Internalizing Behaviours in Preschool Children	n=248, 3-5 years of age (mean=4.37 years, range 3.05-5.00), 130 male (52.4%)	Child Behaviour Checklist (CBCL)/1.5–5; analysed as continuous variables as well as dichotomised (clinically "at-risk" groups for internalizing and externalizing problems	• 16S rRNA gene sequencing, V3-V4 hypervariable region, Illumina MiSeq • Alpha diversity: observed Operational Taxonomic Units, phylogenetic diversity, Shannon diversity • Taxa abundance	Alpha diversity[k]: • Negative correlation between Shannon and internalising behaviours (Spearman rho = −0.134) • Negative correlation between Shannon and somatic complaints (Spearman rho = −0.144) Bacterial taxa[l]: no significant correlations after FDR correction. Strongest nominally significant correlations

Canada Alberta Pregnancy Outcomes and Nutrition (APrON) cohort		were defined as a T-score ≥60 and for clinical scales (e.g., somatic complaints) a T-score ≥65)		with internalising symptoms (and subscales) were with *Collinsella* and *Veillonella* positively, and *Eubacterium ruminantium* negatively

Laue et al., 2021
Sex-specific relationships of the infant microbiome and early-childhood behavioural outcomes
US
New Hampshire Birth Cohort Study (NHBCS)
	n=260, 144 boys
	
	Microbiome at 6 weeks, 1 year and 2 years (same as in (Laue et al., 2020))
	
	Behaviour at 3.2±0.3 years (mean±SD)
		Behavioural Assessment System for Children, second edition (BASC-2): internalizing behaviours (Anxiety and Depression), behaviours related to ADHD (Hyperactivity and Attention Problems),46 and behaviours that are related to autism (Social Skills and Developmental Social Disorders); four BASC-2 composite scales (Internalizing Problems, Externalizing Problems, Behavioural Symptoms Index, and Adaptive Skills) to capture broader phenotypes	• 16S rRNA gene sequencing, V4-V5 hypervariable region, Illumina MiSeq
			• Shotgun sequencing for a subset of 6-week and 1-year samples using NextSeq platform
			• Alpha diversity: Shannon index, Simpson index, count of ASVs
			• Beta diversity: generalised UniFrax
			• Bacterial taxa abundances
			• Metabolic pathways
				Alpha diversity[m]:
				• At 6 weeks: Correlates negatively with depression scores in boys (β =−1.81 points/std increase Shannon diversity); also with anxiety and internalising problems scales
				• At 1 year: no significant associations
				• At 2 years:
				  ○ In boys: positive associations with Developmental Social Disorders, Social Skills, and Adaptive Skills Composite scales
				  ○ In girls: negative associations with the same traits
				
				Beta diversity[n]:
				• No significant relationships between overall bacterial community structure and BASC-2 in fully adjusted models
				• In unadjusted models: in boys 6-week beta diversity associates with anxiety
				
				Bacterial taxa main results[o]:
				Associations in boys:
				• Adaptive skills: positive associations with *Bifidobacterium, Bacteroides, Streptococcus*; negative associations with *Klebsiella, Clostridium, and Haemophilus* at 6 weeks
				In full population:
				• Hyperactivity: associates negatively with *Faecalitalea* at 1 year, and *Blautia* at 2 years
				From metagenomics: no strong associations
				
				Metabolic pathways:

				• At 6 weeks: L-proline biosynthesis and methylphosphonate degradation positively associate with hyperactivity; superpathway of geranylgeranyl- diphosphate biosynthesis and (methylerythritol phosphate pathway associate positively with Externalizing Problems; NAD salvage pathway negatively associates with Internalizing Problems scores; catechol degradation associates negatively with Attention Problems • At 1 year: superpathway of L-aspartate and L-asparagine biosynthesis associate with better depression, behavioural symptoms, externalising problems and adaptive skills scores, superpathway of purine de novo biosynthesis II associates with better Attention Problems, Developmental Social Disorders, and Social Skills scores, and de novo pyrimidine biosynthesis associates with better Developmental Social Disorders scores, pathway of pyruvate fermentation, associates with worse Externalizing Problems and Behavioural Symptoms Index scores, plastidic and non-plastidic phosphatidylglycerol biosynthesis associated with better Behavioural Symptoms Index scores; bile acid dihydroxylation associated with worse Developmental Social Disorders and Adaptive Skills Composite scores; mevalonate pathway was associated with better Social Skills scores.
Guzzardi et al., 2022 Maternal pre-pregnancy overweight and neonatal gut bacterial colonization are associated with cognitive development and gut microbiota	• n=90 (53.3% male, 46.7% female) • Microbiome analysis: at first pass meconium (n=79) and at 3, 6, 12, and 36 months (n = 40, 47, 37, 21) • Behaviour: at the age of 6, 12, 18, 24, 36 and 60	Griffiths Mental Developmental Scales (GMDS) at 6-24 months Extended revised version (GMDS-ER) at 36–60 months	• 16S rRNA gene sequencing, V3-V4 hypervariable region, Illumina MiSeq • Alpha diversity: (PD whole tree, Chao1, Observed Species and Shannon • Beta diversity: generalised UniFrax • Clusters: Hierarchical clustering of samples was performed using UPGMA with weighted UniFrac as a distance measure	Meconium composition at genus level correlated significantly with practical reasoning scores at 60 months[p]: higher *Bifidobacterium* and *Veillonella* abundances were important determinants of higher practical reasoning scores; lower *Faecalibacterium, Ruminococcus, Dialister, Blautia, Roseburia, Coprococcus, Collinsella* (among others) were associated with lower Practical reasoning scores.

composition in pre-school-age offspring Italy Pisa birth Cohort (PISAC)	months (n = 24, 26, 26, 23, 27, 56)	GMDS includes six subscales: locomotor, personal-social, hearing and language, eye and hand coordination, performance and practical reasoning domains.	• Bacterial taxa abundances	Meconium composition also associated with cognition scores at 60 months: higher *Bifidobacterium* abundance was one of the main positive determinants of cognitive scores, while lower levels of *Ruminococcus, Collinsella, Blautia, Dialister* (among others) associated with lower cognitive scores.
Schoch et al., 2022 From Alpha Diversity to Zzz: Interactions among sleep, the brain, and gut microbiota in the first year of life Switzerland	• n=162 (46.3% female, 53.7% male) • Microbiota and behavioural development investigated at 3, 6 and 12 months • Neurodevelopment: additionally at 24 months • Sleep neurophysiology in a subset (n=33) at 6 months	Neurodevelopment: Ages and Stages Questionnaire: Collective Score for overall development by assembling the five key domains Communication, Gross Motor, Fine Motor, Problem Solving, and Personal Social Sleep neurophysiology: measured by EEG using 124 electordes	• 16S rRNA gene sequencing, V3 hypervariable region, Illumina MiSeq • Alpha diversity: Observed, Shannon Index, and Chao1 • Bacterial maturation index: used random forest to predict chronological age from bacterial composition and calculated difference between predicted and actual age • Enterotypes: classified samples into enterotypes A (*Bifidobacterium*) and B (*Bacteroides*) based on weighted UniFrac and Jensen-Shannon distance matrix	Sleep physiology (EEG)[q]: • At 6 months: ○ Enterotypes: *Bifidobacterium*-enterotype had reduced slow wave activity compared to *Bacteroides*-enterotype ○ Alpha diversity: not significantly associated • 6 month EEG power in theta frequency correlated negatively with alpha diversity at 12 months • 3 month alpha diversity or enterotype did not correlate with EEG features at 6 months Neurodevelopment[r]: • Concurrent analyses: alpha diversity positively associated with gross motor scores at 3 months; and with collective scores and gross motor scores at 12 months • Predictive analyses: enterotypes at 6 months associated with gross motor scores at 12 months - *Bifidobacterium*-enterotype had higher gross motor scores at 12 months
Beghetti et al., 2021 Early-life gut microbiota and neurodevelopment in preterm infants: any role for Bifidobacterium? Italy	• n=27 (55.6% female, 44.4% female), all very low birthweight and/or born with gestational age <32 weeks • Microbiome analysis at 1, 4, 7 and 30 days of life • Behaviour: at the age of 24 months corrected age	Revised Griffiths Mental Development Scale (GMDS-R): General Development Quotient (GQ) was calculated and normal development was defined as ≥ 88.7; in total 6 participants had neurodevelopmental impairment	• 16S rRNA gene sequencing, V3–V4 hypervariable region, Illumina MiSeq • Alpha diversity: inverse Simpson index • Beta diversity: estimated by weighted and unweighted UniFrac distances • Bacterial taxa relative abundances	Alpha diversity: no significant differences were found between infants with neurodevelopmental impairment vs normal development[s]. Beta diversity: infants with neurodevelopmental impairment had significantly different microbiota compositions[t] Taxa level: • *Bifidobacteriaecae* levels were higher in infants with neurodevelopmental impairments at earliest

				timepoints (day 1 and 4), at day 30 *Bifidobacteriaceae* levels tended to be lower in infants with neurodevelopmental impairment[u] • *Enterococcacceae* levels tended to be higher in infants with neurodevelopmental impairment at days 7 and 30[v] • at day 30 *Bifidobacterium* abundance was positively correlated with the General Development Quotient[w]
Xie et al., 2022 Relationship between the gut microbiota and temperament in children 1–2 years old in Chinese birth cohort China	• n=51 at 1 year (39.2% male) and n=41 at 2 years (39.0% male; n=37 with both timepoints) • Microbiota and behaviour both at 1 and 2 years	Infant Behaviour Questionnaire-revised (IBQ-R) at 1 year of age and the Early Childhood Behaviour Questionnaire (ECBQ) at 2 years of age: three domains and all subscales	• 16S rRNA gene sequencing, V3–V4 hypervariable region, Illumina MiSeq • Alpha diversity: Shannon and Simpson indices • Beta diversity: unweighted UniFrac distance • Relative abundance of bacterial genera	Alpha diversity was not significantly associated with temperament[x] Bacterial genera: • In non-adjusted analyses[y]: Eight genera were associated with 3 main dimensions (14 subscales) of IBQ-R; 16 genera were associated with 3 main dimensions (18 subscales) of ECB. ○ Strongest associations at 1 year: *Akkermansia*~positive with vocal reactivity and duration of orienting; *Bifidobacterium*~positive with soothability; *Faecalibacterium*~positive with vocal reactivity ○ Strongest associations at 2 years: *Blautia*~positive with sociability, *Faecalibacterium*~negative with high-intensity ○ pleasure, *Streptococcus*~negative with reversed soothability, *Bifidobacterium*~positive with perceptual sensitivity • in adjusted analyses[z]: ○ at 2 years *Lachnospiraceae* ~ positively with activity level and with attention shifts; *Anaerostipes* ~ positively with activity level; *Faecalibacterium* ~ negatively with high intensity pleasure and surgency; *Bifidobacterium* ~ negatively with perceptual sensitivity ○ do not report adjusted analyses for 1 year
Liu et al., 2022[aa] Multivariate log-contrast regression	• n=38 • microbiota: samples collected daily during the	NICU Network Neurobehavioural Scale (NNNS)	• 16S rRNA gene sequencing, V4 hypervariable region, Illumina MiSeq	At the three stages, the identified taxa associated with NNNS are different.

with sub-compositional predictors: Testing the association between preterm infants' gut microbiome and neurobehavioural outcomes US	first month of postnatal age; on average 11.4 samples per infant • behaviour: at discharge from neonatal unit around 36-38 weeks of gestation		• Abundances of taxa at 3 stages: 0-10 days, 11-2 days and 21-30 days; 62 genera were grouped into 11 predictor sets (based on sub-compositional predictor analysis)	• Overall: *Lactobacillales* and *Clostridiales* associate with NNNS • At stage 1: *Clostridiales* associates with NNNS • At stage 2: *Clostridiales* and *Burkholderiales* associate with NNNS • At stage 3: *Actinomycetales* and *Clostridiales* associate with NNNS With this modelling approach[bb], they did not identify directions of effects between bacterial taxa and specific NNNS subscales.
Delgadillo et al., 2022[cc] Associations Between Gut Microbes and Social Behaviour in Healthy 2-Year- Old Children USA	• n=77 (41 boys, 36 girls) • 18-27 months of age, mean age=23.14 months	Early Childhood Behaviour Questionnaire (ECBQ); focus on Effortful Control (EC) and subscales of attentional focusing, attentional shifting, cuddliness, inhibitory control, low intensity pleasure	• 16S rRNA gene pyrosequencing, V1-V3 hypervariable region, Roche 454 FLX Titanium system • 9 bacterial genera selected for analysis (linking gut bacteria with ASD): *Bacteroides, Prevotella, Ruminococcaceae, Akkermansia, Escherichia/Shigella, Alistepes, Parabacteroides, Lachnospiraceae, and Dialister.* • Alpha diversity: Shannon index • Beta diversity: Bray-Curtis dissimilarity on phylum and genus levels	Alpha diversity: no significant correlations with EC Beta diversity: no significant differences in community structure between high and low EC groups on phylum or genus level Bacterial taxa (unadjusted correlation analyses): • EC positively correlated with *Akkermansia* (r=0.25) and *Dialister* (r=0.24), and negatively with *Alistepes* (r=-0.25). • Cuddliness: positively associated with *Akkermansia* (r=0.26) and *Dialister* (r=0.23) • Attentional focusing negatively associated with *Alistepes* (r=-0.27) Bacterial taxa (adjusted models):[dd] • Cuddliness positively associated with *Akkermansia* (R^2=0.116, β=0.022); but not with *Dialister* (R^2=0.053, β=0.036) • Attentional focusing negatively associated with *Alistepes* (R^2=0.061, β=-0.011)
Chen et al., 2022 Characteristics of gut microbiota of term small gestational age infants within 1 week and their relationship with	• n=162 (41 small for gestational age (46.3% male); 121 appropriate for gestational age (62% male)); term-infants • faecal samples collected at days 1, 3, 5 and 7	Ages and Stages Questionnaire-3 (ASQ-3)	• whole genome sequencing, Illumina NovaSeq 6000 platform • alpha diversity: Chao1, abundance-based coverage estimator (ACE), Observed species, Simpson, and Shannon indices • beta diversity: Analysis of similarities (Anoism) • differentially abundant taxa	Correlations between microbiota and neurodevelopmental outcome only in the small for gestational age group Bacterial taxa[ee]: • Motor scores: negative correlated with *Bacteroides* day 1 (r=-0.41)

neurodevelopment at 6 months
Peijing, China
	• behavioural follow-up at 6 months (n=38 in the small for gestational age group)			• Communication scores: positively correlated with *Bacteroides* (r=0.88), negatively correlated with *Clostridium* (r=-0.74) at day 7
				
				Dichotomised into two groups (7 infants had poor communication)[ff]:
				• Alpha diversity: day 3 microbiota richness (Chao1, ACE, and Observed species indices) was higher in the poor communication group
				• Beta diversity: no significant differences in overall community composition
				• Differentially abundant taxa:
				  ○ Day 1: poor communication group had lower *Enterobacteriaceae, Streptococcaceae* and *Streptococcus*
				  ○ Day 5: poor communication score group had decreased relative abundances of *Staphylococcaceae* and *Staphylococcus*, and increased *Enterococcus*
				  ○ Day 7: poor communication score group had decreased abundances of *Bacteroides*, and increased *Corynebacterium*

Sarkar et al., 2022
Relationships of the very low birth weight infant microbiome with neurodevelopment at 2 and 4 years of age
USA
	• n=20 with follow-up data at 2 years; n=24 with follow-up data at 4 years; overlap n=17
	• sample 54% female
	• microbiota sampling weekly for 6 weeks during NNU stay and at 2 and 4 years
	• all very low birthweight (mean birthweight 1047.4 g)
		Battelle Development Inventory-2 Screening Test (BDI-2 ST)	• 16S rRNA gene sequencing, V4 hypervariable region, Illumina MiSeq
			• Alpha diversity: Shannon, Chao, Simpson indices; calculated for early (weeks 2-3) and late (4-6) faecal samples, and for the 2 and 4 year samples
			• Abundances of ASVs
			• Microbiota networks in infants who were vs who were not referred to neurodevelopmental clinic
				Alpha diversity[gg]:
				• Early NICU alpha diversity not correlated with 2-year neurodevelopment
				• Late NICU alpha diversity positively correlated with 2-year neurodevelopment:
				  ○ Chao index: adaptive (r=0.44) and personal-social scales (r=0.47)
				  ○ Simpson index: cognitive (r=0.47) and personal-social (r=0.54)
				• 2-year alpha diversity not associated with concurrent neurodevelopment
				• 4-year alpha diversity positively associated with concurrent neurodevelopment:
				  ○ Chao index: personal-social scales (r=0.42)
				  ○ Shannon index: personal-social (r=0.44)
				  ○ Simpson index: cognitive (r=0.47)

Taxa abundances[hh]:

- At 2 years:
  - Adaptability: 2 ASVs of *Enterobacteriaceae*
  - Cognition: 2 ASVs of *Enterobacteriaceae, Klebsiella*
  - Communication: *Enterococcus, Escherichia, Enterobacteriaceae, Bifidobacterium*
  - Motor: *Enterobacteriaceae, Klebsiella*
  - Personal-social: *Enterobacteriaceae, Proteus*
- At 4 years:
  - Adaptability: *Enterobacteriaceae, Klebsiella, Escherichia, Bifidobacterium, Proteus*
  - Cognition: *2 ASVs of Enterobacteriaceae, Enterococcus, Escherichia*
  - Communication: none
  - Motor: *Enterobacteriaceae*
  - Personal-social: *Enterobacteriaceae, Enterococcus*

Network analysis[ii]:

- At 2 years:
  - Non-referrals: *Bifidobacterium* forms strong network with other communities
  - Referrals: *Escherichia, Citrobacter, Enterobacteriaceae* and *Finegoldia* are higher in relative abundance and constitute a strong network
- At 4 years:
  - Non-referrals: *Finegoldia* and *Enterococcus* are higher in abundance and form a strong network
  - Referrals: *Escherichia* and *Clostridium* are more abundant and form connections with other bacteria
- The networks were not statistically different

[a] Analysed using multiple linear regression; covariates: maternal age, pre-pregnancy BMI, weight gain during preg- nancy, SES, passive smoking during early pregnancy, pregnancy complications, maternal inflammatory disease, postnatal depressive symptoms, district, child's sex, gestational age at birth, delivery mode, breastfeeding and complementary feeding at 6 months of age; not adjusted for multiple comparisons
[b] Analysed the same way as in a
[c] Analysed using REML within OSCA based on the abundance of 82 genera
[d] smoking during pregnancy, current smoking, breastfeeding, maternal education, multilingual upbringing, and shipment
[e] Analysed using linear regression; do not specify the covariates in the additional analysis
[f] Shannon: antibiotics at 6 months, gestational age; Pielou: antibiotics at 6 months; Faith's index: C-section birth; observed OTUs: gestational age
[g] Analysed using linear regression
[h] Analysed using Wilcoxon test and edgeR differential abundance test
[i] Covariates: birth mode, sex, maternal ethnicity, older sibling, breastfeeding status at 6 months, family income, maternal overweight, and age at sampling
[j] Analysed using generalised linear model adjusting for birth mode, gender, maternal race, birth order (siblings), breastfeeding status at 6 months, direct antibiotic exposure (0-12) months, family income, maternal pregnancy fruit intake (5-a-day method sum of servings of fruit, not including juices, plus servings of juice per day), and age at microbiota sampling
[k] Analysed using Spearman correlation, unadjusted, corrected for multiple comparisons using FDR, significance level set to p>0.15
[l] Analysed using Spearman correlation, no significant correlations after FDR corrections
[m] Analysed using linear regression; adjusted for gestational age, child sex, child age at follow-up, maternal education, parity, and maternal and paternal age, delivery mode, exclusively early-life breastfeeding, breastfeeding duration, and maternal smoking during pregnancy
[n] Analysed using PERMANOVA, same covariates as above
[o] Analysed using Maaslin2, adjusted for gestational age, maternal education, parity, delivery mode, maternal and paternal age at delivery, maternal smoking during pregnancy, early life exclusive breastfeeding, duration of any breastfeeding, and child age at follow-up
[p] Analysed using multivariate redundancy analysis and principal component analysis using Canoco; covariates: mode of delivery and sex
[q] For cross-sectional (concurrent) analyses and analyses using month 3 microbiota data: analysed using linear models with microbiota features as the predictors and EEG as the outcome; covariates: age, sex and breastfeeding; for longitudinal (predictive) analyses using 12 month microbiota data: analysed using linear models with microbiota features as the outcomes and EEG as the predictor; covariates: age, sex and breastfeeding
[r] Analysed using separate models for each behavioural domain score as the outcome and all three microbiota features as predictors; full information maximum likelihood; covariates: age, sex and breastfeeding, and a latent intercept for each construct across assessment time point; models were constructed both cross-sectionally (concurrent sample and behavioural score) and longitudinally (predictive: predicting later behavioural scores from microbiota data)
[s] Analysed using Wilcoxon test; no covariates specified
[t] Analysed using PERMANOVA; did not specify at which timepoint significant differences were observed; no covariates specified
[u] Analysed using Wilcoxon test; no covariates specified
[v] Analysed as above
[w] Analysed using Kendall rank correlation test; no covariates specified
[x] Analysed using Spearman correlation
[y] Analysed using Spearman correlation
[z] Analysed using multiple linear regression, covariates: breastfeeding, antibiotics, and probiotics consumptions

[aa] The sample in this study overlaps with that of (Sun et al., 2020)
[bb] Analysed using multivariate log-contrast regression with sub-compositional predictors
[cc] Overlapping cohort with that of (Christian et al., 2015)
[dd] Linear regression analyses, covariates: sex, delivery mode, diet, body composition, breastfeeding duration
[ee] Analysed using Pearson or Spearman correlation; unadjusted for covariates; multiple comparison correction not specified
[ff] Analysed using t-test or Mann-Whitney U-test for two group comparisons; unadjusted for covariates; multiple comparison correction not specified
[gg] Analysed using Pearson correlation analyses; unadjusted for covariates and multiple comparisons
[hh] Analysed using stepwise regression; covariates: gestational age at birth, days on oxygen, birthweight; multiple comparison adjustment not specified; effect sizes and directions not specified
[ii] Analysed using Network Construction and Comparison for Microbiome Data

Chapter 5. Microbiota profiles and drivers in preterm neonates

5.1 Introduction

Preterm infants are at risk for major short- and long-term health complications including necrotising enterocolitis (NEC), sepsis, recurrent infections, asthma/wheeze, as well as cognitive impairments (Been et al., 2014; Johnson and Marlow, 2017; Morgan et al., 2022; Platt, 2014). Many of these morbidities are associated with disturbances in the early life gut microbiota, which plays an essential role in immune function and metabolism, as well as can impact on the nervous and endocrine system (Sarkar et al., 2021).

The gut microbiota develops during the first 2-5 years of life when it starts to resemble adult microbiota (Roswall et al., 2021; Yatsunenko et al., 2012). Overall, in healthy vaginally delivered infants, during the first few weeks of life, the most abundant bacteria in the gut are facultative anaerobes such as *Escherichia* and *Staphylococcus,* followed by the rapid expansion of obligate anaerobes such as *Bifidobacterium*, *Bacteroides* and *Clostridium* (Bokulich et al., 2016; Nagpal et al., 2017b; Reyman et al., 2019).

Compared to infants born at term, preterm neonates are exposed to microorganisms and become colonised at an earlier maturation state of the gastrointestinal organs and the immune system (Healy et al., 2022; Henderickx et al., 2019). Previous work has demonstrated that although the general pattern of microbiota development is similar in term and preterm infants during their first months of life (Grier et al., 2017; Korpela et al., 2017a; La Rosa et al., 2014), the preterm infant gut has been characterised as dysbiotic, with lower bacterial diversity and abundances of *Bifidobacterium*, and higher levels of opportunistic pathogens such as *Klebsiella, Enterobacter, Enterococcus* and *Staphylococcus* (Healy et al., 2022). Importantly, differences in the microbiota composition between children born term and preterm have been detected up to four years of life (Fouhy et al., 2019).

The infant microbiota is dynamic and susceptible to a range of early environmental influences. In term infants, the main factors influencing gut microbiota composition in infancy are mode of delivery, breastfeeding and antibiotics (Bäckhed et al., 2015; Reyman et al., 2022, 2019; Roswall et al., 2021; Shao et al., 2019; Stewart et al., 2018). In preterm

infants, the most frequently studied variables include age, mode of delivery, antibiotic treatment and human milk consumption; however, there are substantial discrepancies between studies in the direction and size of the effects of these factors on the microbial diversity and individual bacterial taxa (Aguilar-Lopez et al., 2021a). These discrepancies could be resulting from the heterogeneous population and highlights the need to build up more knowledge on the early life determinants of gut microbiota in preterm infants.

Preterm infants are exposed to a multitude of medical, dietary and environmental adversities that can impact on the microbiota development (Henderickx et al., 2019). Very and extremely preterm infants, defined as born < 32 or < 28 completed weeks of gestation, respectively, are initially cared for in neonatal intensive care units (NNU), often for extended periods depending of the gestational age (GA) at birth. NNUs are considered a major source of microorganisms (Brooks et al., 2014), which are associated with nosocomial infections (Gastmeier et al., 2007; Parvez and Jarvis, 1999). In addition, antibiotics, established drivers of the microbiota development both in term and preterm infants (Bokulich et al., 2016; Fouhy et al., 2012; Gasparrini et al., 2019; Greenwood et al., 2014; Reyman et al., 2022), are the most commonly prescribed medications in NNUs (Clark et al., 2006) and around 80-90% of preterm infants are administered antibiotics during their first days of life (Flannery et al., 2018). Furthermore, compared to their term-born peers, preterm infants experience a range of different feeding practices including exposure to fortifiers and pasteurised donor milk which can influence gut microbiota development (Aguilar-Lopez et al., 2021b; Asbury et al., 2022; Gregory et al., 2016; Kumbhare et al., 2022; Piñeiro-Ramos et al., 2021). Infants in some previous studies have also been treated with probiotics, which confounds efforts to understand the general drivers of microbiota development in this population.

The gut microbiota is a potentially modifiable system, for example by using pre- and probiotic supplementation, making it attractive to study in the context of factors related to adverse outcomes in preterm infants. There is an urgent need to understand the drivers of gut dysbiosis in preterm infants because this will help develop rational treatment strategies of the many different supplements available, inform the design of efficacy and safety studies, as well as risk stratification for intervention trials.

5.2 Chapter aims

In this study, I aimed to:

a) Characterise the microbiota diversity and community composition in the meconium of term and preterm infants after birth, and in preterm neonatal faecal samples prior to discharge from the NNU;
b) Determine the perinatal drivers of gut microbial diversity and community composition in preterm infants.

5.3 Methods

The analyses presented in this chapter use a sample of term and preterm participants from the Theirworld Edinburgh Birth Cohort (TEBC; Boardman et al., 2020) who had meconium and/or pre-hospital discharge faecal samples collected. Details of study design, participant recruitment, and methods for faecal sample processing and microbiota sequencing are described in Chapter 2. In brief, microbiota profiles were generated by sequencing of the hypervariable V4 region of the 16S rRNA gene using Illumina MiSeq platform. Sequencing data were processed using the DADA2 pipeline (Callahan et al., 2016) to obtain the taxa abundance (amplicon sequence variant (ASV)) table. Participant clinical and demographic information was collected from antenatal and neonatal electronic patient records and parent questionnaires.

5.3.1 Statistical analysis

5.3.1.1 Study group characteristics

The study group baseline demographic and clinical characteristic tables were constructed using the package *tableone* in R (Yoshida et al., 2020). Normal distribution of continuous variables was assessed using Shapiro-Wilk's test and visual inspection of histogram and quantile-quantile plots using the package *MVN* in R (Korkmaz et al., 2014). Two-sample t-tests were used to compare the means of normally distributed continuous variables between term and preterm infants; Wilcoxon rank-sum tests were applied to compare differences in non-normally distributed continuous variables; Fischer's exact test was used to test for significant differences in categorical variables. P-values reported are not corrected for multiple comparisons.

5.3.1.2 Beta diversity

To assess differences in overall bacterial community composition, permutational analysis of variance (PERMANOVA) as implemented by the function *adonis2* in R package *vegan* (Oksanen et al., 2019) was used with the Bray-Curtis dissimilarity matrix as the outcome and perinatal features of interest as the predictors. To identify the effects of sample types and timepoints (term meconium, and preterm meconium and pre-discharge) on bacterial community composition, separate PERMANOVAs were performed for all pairwise comparisons. To assess the effects of perinatal features, univariable analyses were conducted with one perinatal feature at a time, separately for the meconium and pre-discharge samples; p-values were adjusted for multiple comparisons across the models using Benjamini-Hochberg method. Results with adjusted p-value < 0.1 were considered as statistically significant and followed up using differential abundance testing (see section 5.3.1.5).

5.3.1.3 Clusters

Clustering based on the Bray-Curtis dissimilarity matrix was performed using hierarchical clustering with average linkage. Clustering was conducted for descriptive purposes to identify and characterise main groups of samples and infants.

5.3.1.4 Alpha diversity

To assess differences in alpha diversity measures between the sample types and timepoints, linear mixed effect models were used as implemented in the R package *lmerTest* (Kuznetsova et al., 2017, function *lmer*) with alpha diversity index as the outcome, sample type (term meconium, preterm meconium and preterm pre-discharge) as the predictor, and participant ID fitted as a random effect to adjust for repeated measures. The assumption of normal distribution of the residuals was assessed by visual inspection of the model residuals. For observed species, this assumption was violated and therefore the values were $\log_{10}$-transformed, and in all analyses with observed species going forward the $\log_{10}$-transformed values were used. Post-hoc analyses were conducted using the package *emmeans* (Lenth, 2022). P-values were adjusted for multiple comparisons using Benjamini-Hochberg method (Benjamini and Hochberg, 1995) for the main effects across the models for the two alpha diversity indices, and separately for the three pairwise comparisons within the models for each alpha diversity index.

To assess the effects of different perinatal factors on alpha diversity indices, separate analyses were conducted for the preterm infant meconium and pre-discharge samples. Normal distribution of variables was assessed using Shapiro-Wilk's test and visual inspection of histogram plots using the package *MVN* in R (Korkmaz et al., 2014). Spearman correlation analyses were conducted to test for the associations between continuous perinatal variables and alpha diversity indices, and t-tests (pre-discharge samples) and Wilcoxon rank-sum tests (meconium samples) were applied to test for the differences in alpha diversity indices between groups of infants based on categorical perinatal variables. P-values were adjusted for multiple comparisons using Benjamini-Hochberg method separately for analyses involving meconium and pre-discharge samples. Significant (adjusted p-value < 0.1) effects in univariable models were followed up in a multivariable linear regression model to adjust for the potential confounding effects between the perinatal variables.

5.3.1.5 Differential abundance testing

Microbiome Multivariable Associations with Linear Models (MaAsLin2; Mallick et al., 2021) was used to test for the relationships between variables of interest and abundances of individual bacterial taxa. Total-sum-scaling (TSS-normalisation) was applied prior to running MaAsLin2 models. In all analyses, I tested for the effects on ASVs that were present with at least 1% of abundance (min_abundance = 0.01) in at least 5% of samples (min_prevalence = 0.05); the normalisation method within MaAsLin2 was set to "none"; all other arguments were used as by default. As by default in the method, I considered Benjamini-Hochberg method-adjusted p-values < 0.25 as statistically significant.

I tested for statistically significant differences in taxa relative abundances between preterm meconium and pre-discharge samples, and between term and preterm meconium samples. The model testing for differentially abundant ASVs between term and preterm meconium was adjusted for postnatal age at sample collection given that the preterm infant meconium samples were collected, on average, at a later postnatal day compared to term infant meconium.

Then, to understand the effects of perinatal factors on the abundances of bacterial taxa, I conducted separate analyses for preterm meconium and pre-discharge samples, focussing on the perinatal factors that were significantly (adjusted p-value < 0.1) associated with overall microbiota community composition as determined by PERMANOVA analyses.

Baseline analyses using meconium samples were adjusted for postnatal age at sample. Main analyses using pre-discharge samples concerned testing the effect of the degree of prematurity (GA group; very vs extremely preterm) on the taxa relative abundances, adjusting for GA at sample collection. Here, I chose to adjust for GA at sample collection instead of postnatal age due to the expectedly high collinearity between degree of prematurity and postnatal age at sample. Baseline models for other perinatal drivers were adjusted for GA group and GA at sample collection. In fully adjusted models, all clinical variables were tested simultaneously in one model.

5.3.1.6 BLAST

The BLAST tool within the National Center for Biotechnology Information (NCBI; https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify the bacterial species from the 16S sequence where appropriate.

5.4 Results

5.4.1 Sample characteristics

A total of 250 faecal samples from 206 cohort participants were collected during the study period. Seven infants were excluded from analyses due to neonatal death or congenital abnormalities. The mean postnatal and postmenstrual ages at sample collection are depicted in Table 5-1. 44 preterm infants had both meconium and pre-discharge samples obtained during the study period.

Table 5-1. Postnatal and postmenstrual ages at the time of sample collection.

	Full-term infants	**Preterm infants**	
	Meconium	Meconium	Pre-hospital discharge sample
Number of samples obtained	52	85	106
Postnatal age in days at sample collection (median [range])	1 [1, 13]	5 [1, 13]*	46.5 [9, 151]
Postmenstrual age in weeks at sample collection (mean (SD))	39.71 [37.29, 42.14]	29.57 [24.43, 33.57]	36.14 [29.43, 46.14]

**significantly different between term and preterm subjects (Wilcoxon rank-sum tests, unadjusted p < 0.001)*

While most full-term infants passed their first meconium within the first 2 days, preterm infants passed the first meconium, on average, 4 days later, as expected. The postnatal age for obtaining the pre-discharge stool sample is reflective of the time the infant spent in the NNU and the postmenstrual/gestational age at sample collection is reflective of GA at birth

(meconium) and GA at which infants were discharged from the NNU (pre-discharge sample). The pre-discharge samples were collected, on average, ~9 days before the infants were discharged from the NNU.

A flowchart detailing the exclusion of study subjects and samples is detailed in Figure 5-1. Clinical and demographic characteristics of the final study group (those with pre-processed 16S rRNA gene sequencing data; see sections 5.4.2 and 5.4.3 below) are provided in Table 5-2. In the final dataset, 26 preterm participants had matching meconium and pre-discharge faecal samples.

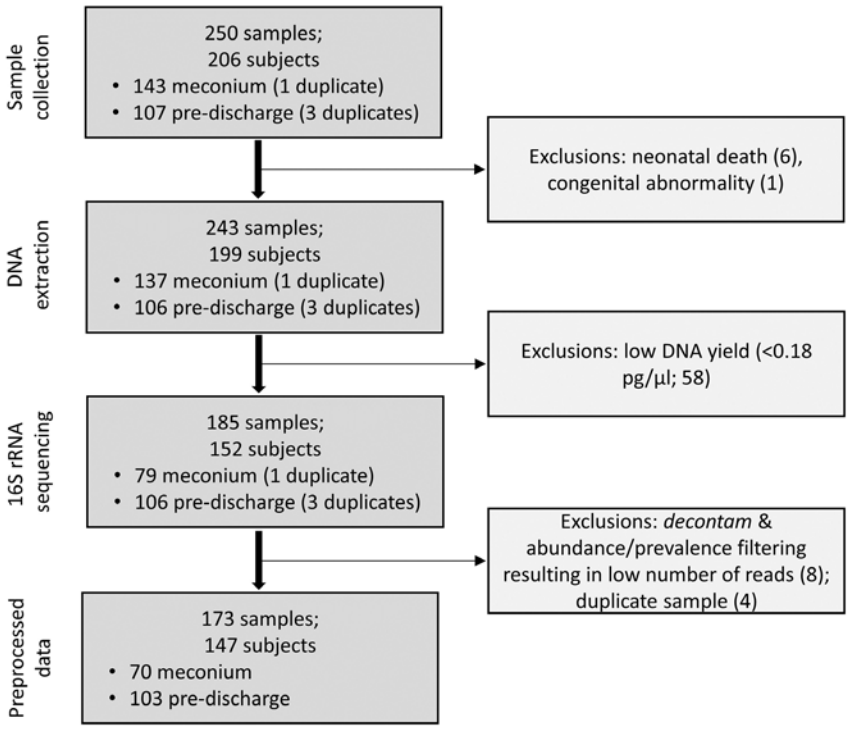


Figure 5-1. Flowchart detailing the inclusion and exclusion of samples following DNA extraction and sequencing data quality control.

Table 5-2. Baseline characteristics of the final study group.

Variable*	level	Full-term	Preterm	$p^{\$}$
Sample size		12	135	
GA at birth, weeks (median [range])		40.00 [37.71, 42.00]	29.14 [22.14, 32.86]	<0.001
Sex (%)	Male	3 (25.0)	71 (52.6)	0.078
	Female	9 (75.0)	64 (47.4)	
Birthweight, g (mean (SD))		3518 (486)	1255 (430)	<0.001
Birthweight z-score (median [range])		0.843 [-0.818, 2.331]	0.212 [-3.023, 2.141]	0.011
Mode of delivery (%)	C-section	1 (8.3)	94 (69.6)	<0.001
	Vaginal delivery	11 (91.7)	41 (30.4)	

Antenatal steroids given (%)	No	/	7 (5.2)	<0.001
	Yes		128 (94.8)	
Magnesium sulphate given (%)	No	/	31 (23.0)	<0.001
	Yes		104 (77.0)	
Labour antibiotics (%)	No	11 (91.7)	46 (34.1)	<0.001
	Yes	1 (8.3)	89 (65.9)	
Membrane rupture duration, hours (median [range])		10 [0, 63]	0 [0, 1043]	0.132
Days in NNU (median [range])		/	61 [6, 167]	
Bronchopulmonary dysplasia (%)	No	/	94 (69.6)	
	Yes		41 (30.4)	
Necrotising enterocolitis (%)	No	/	128 (94.8)	
	Yes		7 (5.2)	
Sepsis (%)	No	/	94 (69.6)	
	Yes		41 (30.4)	
Retinopathy of prematurity (%)	No	/	122 (90.4)	
	Yes		9 (6.7)	
	NA		4 (3.0)	
Antibiotics <72h of life (%)	No	/	29 (21.5)	
	Yes		106 (78.5)	
Antibiotics >72h of life (%)	No	/	58 (43.0)	
	Yes		77 (57.0)	
Total days of antibiotics during NNU stay (median [range])		/	4 [0, 49]	
% of days receiving antibiotics during NNU stay (median [range])		/	9.4 [0, 84.3]	
GA at discharge (median [range])		/	37.57 [30.57, 48.43]	
% of days receiving exclusive breast milk during NNU stay (median [range])		/	68.6 [0, 100]	
Breast milk exposure (%)	<75% inpatient days	/	73 (54.1)	
	≥75% inpatient days		62 (45.9)	
% of days receiving exclusive formula during NNU stay (median [range])		/	0 [0, 89.5]	
% of days receiving mixed feeds during NNU stay (median [range])		/	9.8 [0, 96.2]	
Feeding at discharge (%)	Breastfeeding	12 (100.0)	51 (37.8)	<0.001
	Formula feeding	0 (0.0)	43 (31.9)	
	Mixed feeding	0 (0.0)	41 (30.4)	
Maternal age, years (mean (SD))		33 (3)	32 (6)	0.401
Maternal BMI (median [range])		24.7 [20.9, 33.2]	26.7 [16.4, 46.6]	0.502
SIMD rank (%)	1	0 (0.0)	31 (23.0)	0.075
	2	1 (8.3)	29 (21.5)	

3	4 (33.3)	21 (15.6)
4	1 (8.3)	17 (12.6)
5	6 (50.0)	37 (27.4)

**Categorical variables are shown in absolute numbers with percentages (%); continuous, normally distributed variables as means with standard deviations (SD); continuous, non-normally distributed variables as medians with ranges. $^{\$}$Two-sample t-tests were used to compare the means of normally distributed continuous variables between term and preterm infants; Wilcoxon rank-sum tests were applied to compare medians of non-normally distributed continuous variables; Fischer's exact test was used to test for significant differences in categorical variables.*

5.4.2 Low bacterial DNA yield in meconium samples

58 out of 137 meconium samples (42.3%) did not yield sufficient amount of DNA for library preparation and sequencing. By comparing the participant and sample characteristics of those meconium samples that yielded sufficient amount of DNA for 16S rRNA gene sequencing with those that did not (Table 5-3), I observed that meconium samples with low bacterial DNA concentration were more likely from term-born infants or infants born at a later GA, and collected at an earlier postnatal day. This is consistent with the notion that the meconium of healthy term-born infants, on average, contains very low bacterial biomass.

Table 5-3. Characteristics of participants from whom meconium samples were collected.

Variable*	**level**	**Not included in MiSeq sequencing**	**Included in MiSeq sequencing**	***p*$^{\$}$**
Sample size		58	79	
Preterm (%)	No	36 (62.1)	16 (20.3)	<0.001
	Yes	22 (37.9)	63 (79.7)	
GA at birth, weeks (median [range])		39.00 [26.86, 42.14]	29.71 [23.86, 42.00]	<0.001
Postnatal age at sample collection, days (median [range])		2 [1, 13]	5 [1, 13]	<0.001
GA at sample collection, weeks (mean (SD))		36.13 (4.85)	31.51 (4.69)	<0.001
Sex (%)	Male	38 (65.5)	36 (45.6)	0.025
	Female	20 (34.5)	43 (54.4)	
Birthweight, g (mean (SD))		3230 [720, 4580]	1300 [562, 4420]	<0.001
Birthweight z-score (median [range])		0.513 [-1.588, 2.571]	0.355 [-3.023, 2.457]	0.178
Mode of delivery (%)	C-section	40 (69.0)	43 (54.4)	0.111
	Vaginal delivery	18 (31.0)	36 (45.6)	
Antenatal steroids given (%)	No	34 (58.6)	18 (22.8)	<0.001
	Yes	24 (41.4)	61 (77.2)	
Magnesium sulphate given (%)	No	38 (65.5)	31 (39.2)	0.003
	Yes	20 (34.5)	48 (60.8)	
Labour antibiotics (%)	No	34 (58.6)	30 (38.0)	0.024
	Yes	24 (41.4)	49 (62.0)	
Antibiotics <72h of life (%)	No	2 (3.4)	13 (16.5)	<0.001
	Yes	20 (34.5)	50 (63.3)	

	NA	36 (62.1)	16 (20.3)	
Antibiotics >72h of life (%)	No	13 (22.4)	21 (26.6)	<0.001
	Yes	9 (15.5)	42 (53.2)	
	NA	36 (62.1)	16 (20.3)	
Days in NNU (median [range])		41 [6, 99]	66 [13, 167]	0.056
Bronchopulmonary dysplasia (%)	No	18 (31.0)	45 (57.0)	<0.001
	Yes	4 (6.9)	18 (22.8)	
	NA	36 (62.1)	16 (20.3)	
Necrotising enterocolitis (%)	No	21 (36.2)	59 (74.7)	<0.001
	Yes	1 (1.7)	4 (5.1)	
	NA	36 (62.1)	16 (20.3)	
Sepsis (%)	No	16 (27.6)	37 (46.8)	<0.001
	Yes	6 (10.3)	26 (32.9)	
	NA	36 (62.1)	16 (20.3)	
Retinopathy of prematurity (%)	No	21 (36.2)	54 (68.4)	<0.001
	Yes	0 (0.0)	5 (6.3)	
	NA	37 (63.8)	20 (25.3)	
GA at discharge (median [range])		36.14 [31.14, 42.29]	37.57 [30.57, 48.43]	0.232
Maternal age, years (mean (SD))		33 (5)	31 (5)	0.004
Maternal BMI (median [range])		25.9 [18.1, 47.0]	26.0 [16.4, 43.2]	0.804
SIMD rank (%)	1	12 (20.7)	15 (19.0)	0.131
	2	8 (13.8)	21 (26.6)	
	3	10 (17.2)	10 (12.7)	
	4	17 (29.3)	12 (15.2)	
	5	11 (19.0)	21 (26.6)	

**Categorical variables are shown in absolute numbers with percentages (%); continuous, normally distributed variables as means with standard deviations (SD); continuous, non-normally distributed variables as medians with ranges. $Two-sample t-tests were used to compare the means of normally distributed continuous variables; Wilcoxon rank-sum tests were applied to compare medians of non-normally distributed continuous variables; Fischer's exact test was used to test for significant differences in categorical variables.*

5.4.3 Quality control

After filtering out the ASVs belonging to the phylogenetic groups 'Mitochondria', 'Chloroplast', 'Archae' or 'Eukaryota', a mean of 35196 raw reads (range 6452 – 109470) were generated per each biological sample. This was comparable between the biological samples and positive controls, while isolation and MiSeq PCR blanks had, as expected, significantly fewer reads (Figure 5-2).

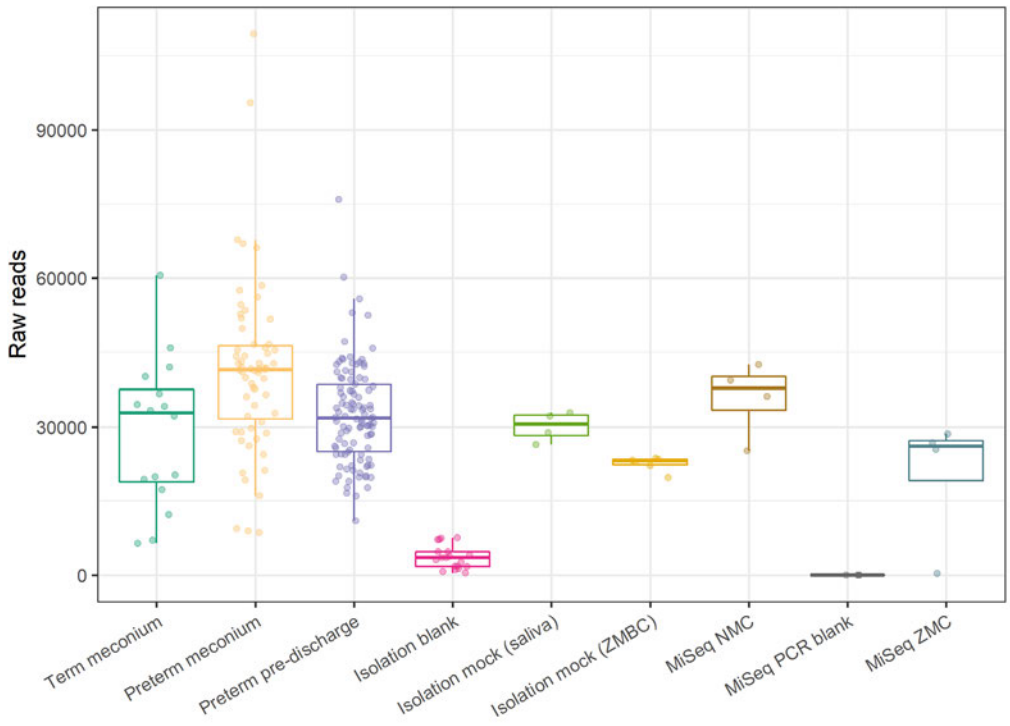


Figure 5-2. Number of raw reads produced during the 16S rRNA gene sequencing per each sample and control.

Low biomass samples, such as new-born infant faeces, are at risk for biases due to possible bacterial DNA contamination from processing reagents and environment (Davis et al., 2019; Nearing et al., 2021). To minimise the risk of contamination, I included negative (DNA isolation and MiSeq PCR blanks) and positive (mock communities) controls at the different processing stages alongside with samples. The resulting profiles of the positive and negative controls were inspected to ensure successful sequencing/taxa identification and to assess contamination, respectively (Figure 5-3). The profiles of the negative controls (Figure 5-3A) indicate fairly consistent profiles across the DNA isolation batches, with slight differences between the blanks from extraction including meconium and pre-discharge faecal samples, which could be due to higher cross-contamination from samples to negative controls during DNA extraction form pre-discharge samples. In negative controls, I identified high abundance of commonly identified contaminant taxa such as *Pseudomonas, Curvibacter, Schlegelella*, and *Comamonadaceae.* The profiles of the positive controls also indicate consistent profiles across DNA extraction batches and MiSeq qPCR plates (Figure 5-3B).

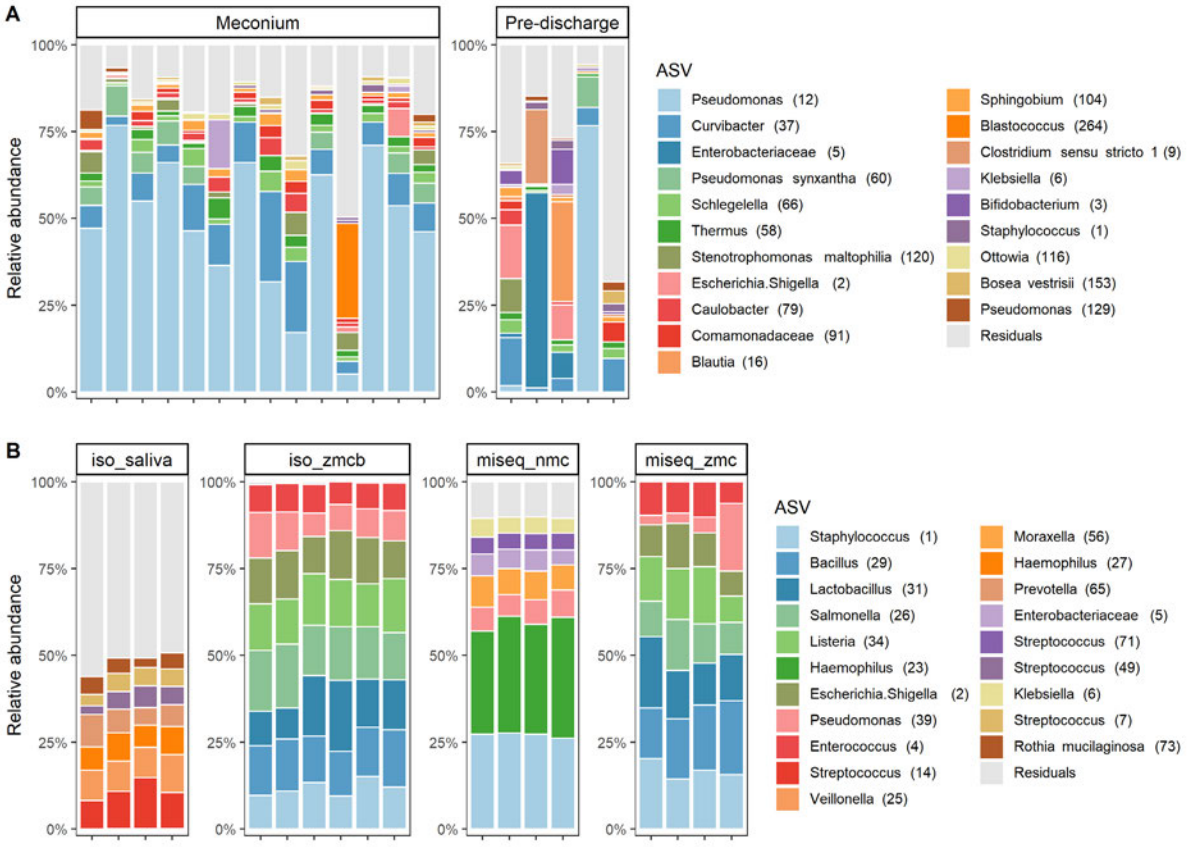


Figure 5-3. Relative abundances of top 20 bacterial ASVs identified in the control samples.
(A) DNA isolation negative controls, stratified by sample type alongside which the negative control was included. (B) Positive controls. Iso_saliva = a convenience saliva sample included as positive control in DNA isolations; iso_zmbc = ZymoBIOMICS Microbial Community Standard included as positive control in DNA isolations; miseq_nmc = a mock community consisting of equimolarly pooled bacteria (see Chapter 2 Materials and methods); miseq_zmc = ZymoBIOMICS Microbial Community DNA Standard included as a positive control during MiSeq PCR.

Next, the samples and identified taxa were filtered with a four step approach. First, potential contaminating ASVs were identified and removed from the data using the *decontam* R package (Davis et al., 2018). I applied the "combined" method which identified 72 contaminating taxa. These were manually inspected by plotting relative abundances against DNA concentration in the samples to ensure that the method did not identify common faecal taxa as contaminants (Supplementary Figure 5-1). From most negative controls, the *decontam* method removed > 75% of reads (Figure 5-4A). Second, by cross-matching the remaining list of ASVs to those taxa identified as contaminants by Salter et al. (2014) and plotting the DNA concentration against the relative abundance per DNA isolation batch, I additionally identified 27 contaminant ASVs (see examples in Supplementary Figure 5-2). Third, ASVs that were identified at a relative abundance of less than 0.1% and present in less than two samples were removed from the dataset (following Subramanian et al.

(2014)). This step removed 850 ultra-rare ASVs, resulting in 174 ASVs in the final dataset. Finally, samples that had a final read count of less than 5000 (n = 8) were additionally removed from the dataset; these 8 samples also had > 70% of the reads removed during the decontamination process, suggesting the remaining reads may not reliably represent the community composition (Figure 5-4B). In the final dataset, samples had a mean of 35416 reads (range 7534 – 109456). This demonstrates that, on average, the decontamination steps had a minimal effect on the read count of the samples included in the subsequent analyses, removing a mean of 3.75% of the raw reads from final included samples.

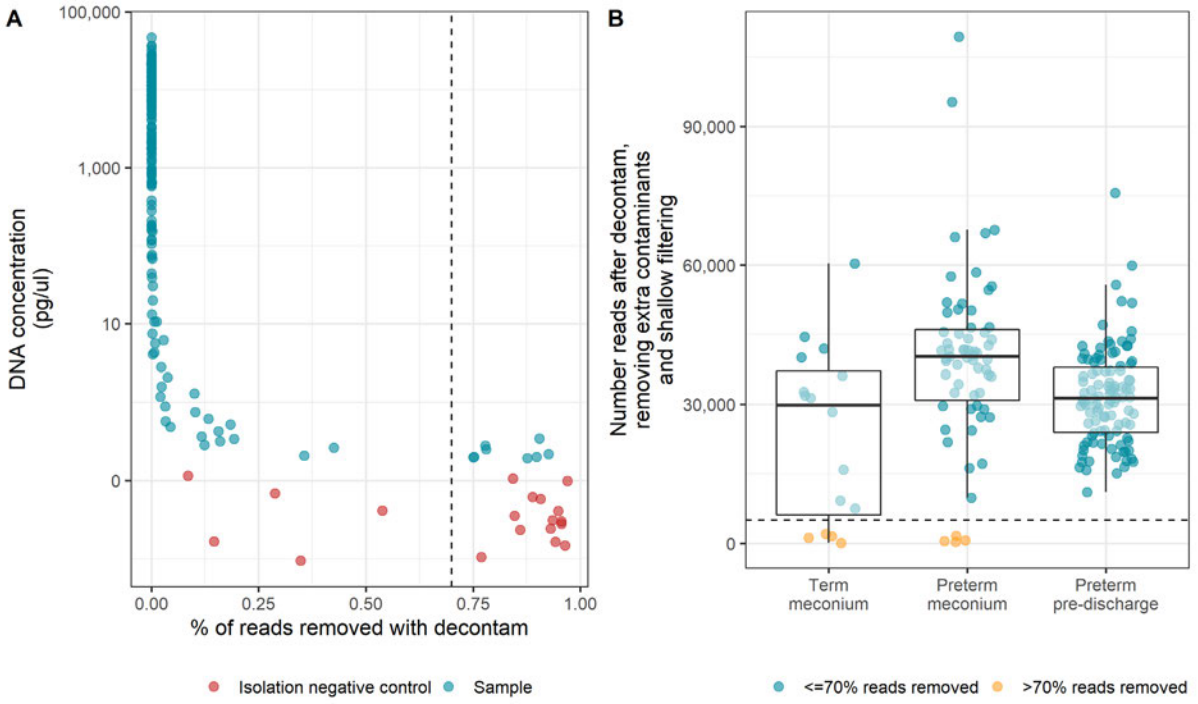


Figure 5-4. Sequencing reads following decontamination steps.
(A) Percentage of reads removed from samples and negative isolation controls using the "combined" method in decontam package over DNA concentration; dashed line indicates 70%. (B) Number of reads remaining in the three sample types following decontam and removal of additional contaminating taxa (cross-matched to contaminant list identified by (Salter et al., 2014), and shallow filtering (at a relative abundance of less than 0.1% and present in less than two samples); dashed line indicates 5000 reads.

5.4.4 First-pass meconium and neonatal faecal samples have distinct microbiota profiles

Bray-Curtis dissimilarity matrix was calculated on the TSS-normalised ASV table to identify differences in the overall community composition between the samples. Non-metric Multidimensional Scaling (nMDS) plot of the three sample types collected in the study (term meconium, and preterm meconium and pre-discharge) showed that the meconium and neonatal faecal samples have distinct microbiota community compositions (Figure 5-5). PERMANOVA revealed a significant effect of timepoint (meconium vs pre-discharge) on the

microbiota community composition in preterm infants (PERMANOVA, R^2 = 14.59%, p < 0.001) and also between term meconium and preterm pre-discharge (PERMANOVA, R^2 = 4.69%, p < 0.001). Furthermore, there was little separation of the term and preterm infant meconium samples (PERMANOVA, R^2 = 2.75%, p = 0.082), suggesting that overall the community compositions of meconium samples in term and preterm infants are similar. However, this analysis could be underpowered due to the small number of term infant meconium samples (n = 12) with available sequencing data.

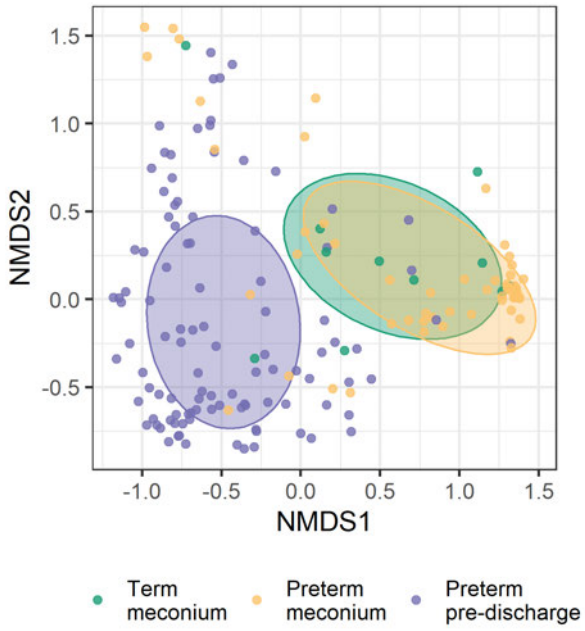


Figure 5-5. Non-metric multidimensional scaling plot based on Bray–Curtis dissimilarity between samples. *Data points and ellipses are coloured by sample type.*

To further characterise the microbiota composition and understand which bacterial taxa are driving most of the variance in the data, I used hierarchical clustering, which identified 11 clusters; 7 of the clusters contained more than three samples. The clusters contained 4-49 samples, whilst 7 samples did not cluster together with other samples (Figure 5-6). A majority of meconium samples were characterised by a community composition with high relative abundance of *Staphylococcus* (cluster 2, n = 49), whilst some meconium samples had very high abundances of *Streptococcus* (cluster 7, n = 4). Interestingly, based on NCBI BLAST tool this *Streptococcus* ASV had a 100% match to *Streptococcus agalactiae*, which is a group B *Streptococcus* associated with early-onset sepsis. The two preterm meconium samples not clustered together with other samples had high relative abundance of *Fusobacterium nucleatum.* In pre-discharge samples, I observed community compositions

with high relative abundances of *Bifidobacterium* (cluster 4, n = 41) or *Enterobacteriaceae* (cluster 3; n = 23). The three pre-discharge samples not clustering together with other samples had high relative abundances of taxa from the genera *Veillonella* or *Klebsiella*. I also observed three clusters that were not exclusively characteristic to meconium or pre-discharge samples; these were characterised by high abundances of *Escherichia/Shigella* (cluster 1, n = 31), *Klebsiella* (cluster 5; n = 11), or *Enterococcus* (cluster 6; n = 7).

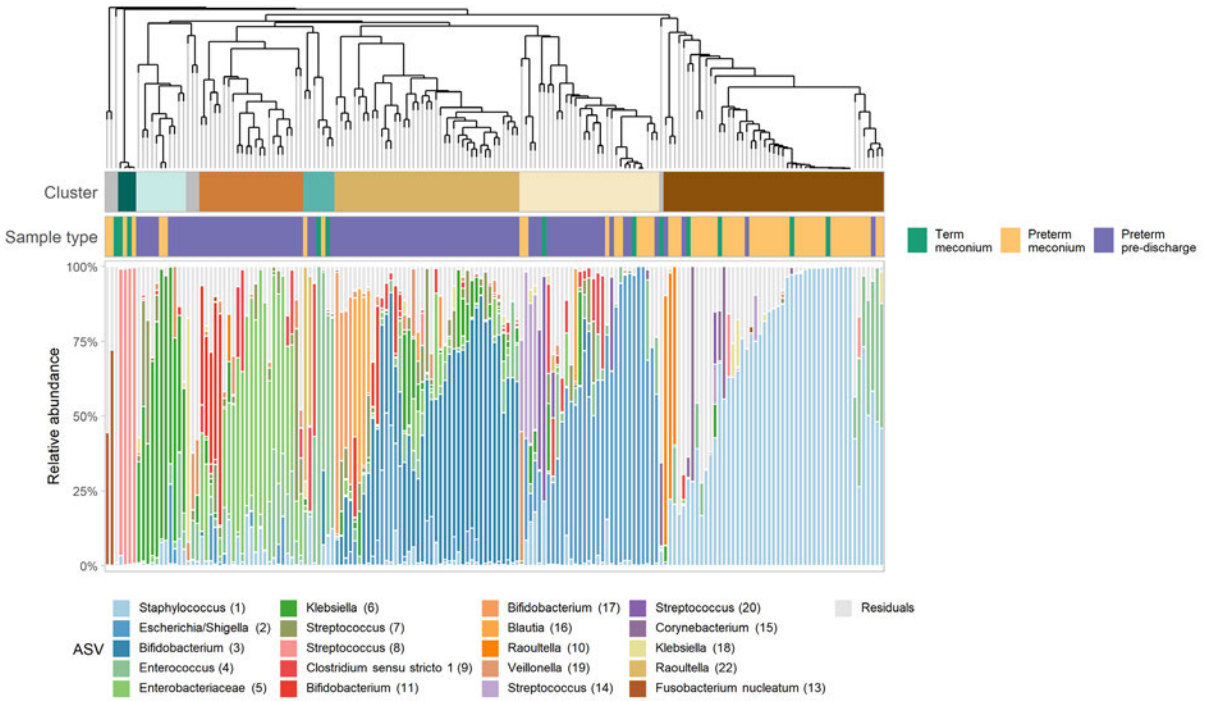


Figure 5-6. Relative abundances of the top 20 ASVs identified in the whole dataset.
Samples are ordered by clusters identified with hierarchical clustering with average linkage. Grey cluster bar indicates no cluster membership for a given sample.

Using MaAsLin2, I identified which bacterial taxa are differentially abundant between the three sample types. Although PERMANOVA revealed minimal differences between term and preterm meconium samples, I identified preterm meconium samples to have higher abundances of *Escherichia/Shigella* ($p = 0.057$, $q = 0.189$) and *Streptococcus* ($p = 0.100$, $q = 0.223$), while term meconium samples had higher abundance of *Corynebacterium* ($p = 0.021$, $q = 0.131$) (Figure 5-7A). In preterm infants, pre-discharge samples showed higher abundances of *Bifidobacterium* ($p = 2.13 \times 10^{-12}$, $q = 8.54 \times 10^{-12}$), *Clostridium sensu stricto* ($p = 2.05 \times 10^{-12}$, $q = 8.54 \times 10^{-12}$), *Streptococcus* ($p = 6.44 \times 10^{-14}$, $q = 1.03 \times 10^{-12}$; note that this is a different ASV to that characteristic of cluster 7), *Enterobacteriaceae* ($p = 3.08 \times 10^{-12}$, $q = 9.85 \times 10^{-12}$), *Enterococcus* ($p = 4.48 \times 10^{-9}$, $q = 1.19 \times 10^{-8}$), *Klebsiella* ($p = 9.90 \times 10^{-6}$, $q =$

1.98×10^{-5}), *Escherichia/Shigella* ($p = 7.08 \times 10^{-5}$, $q = 1.13 \times 10^{-4}$), different *Veillonella* ASVs (q range $6.16 \times 10^{-6} – 8.23 \times 10^{-4}$), and *Finegoldia* ($p = 1.25 \times 10^{-4}$, $q = 1.81 \times 10^{-4}$), whilst meconium samples had higher abundance of *Staphylococcus* ASVs (q range $1.35 \times 10^{-3} – 1.56 \times 10^{-12}$) (Figure 5-7B). These results parallel the distribution of the meconium and pre-discharge samples in the clusters.

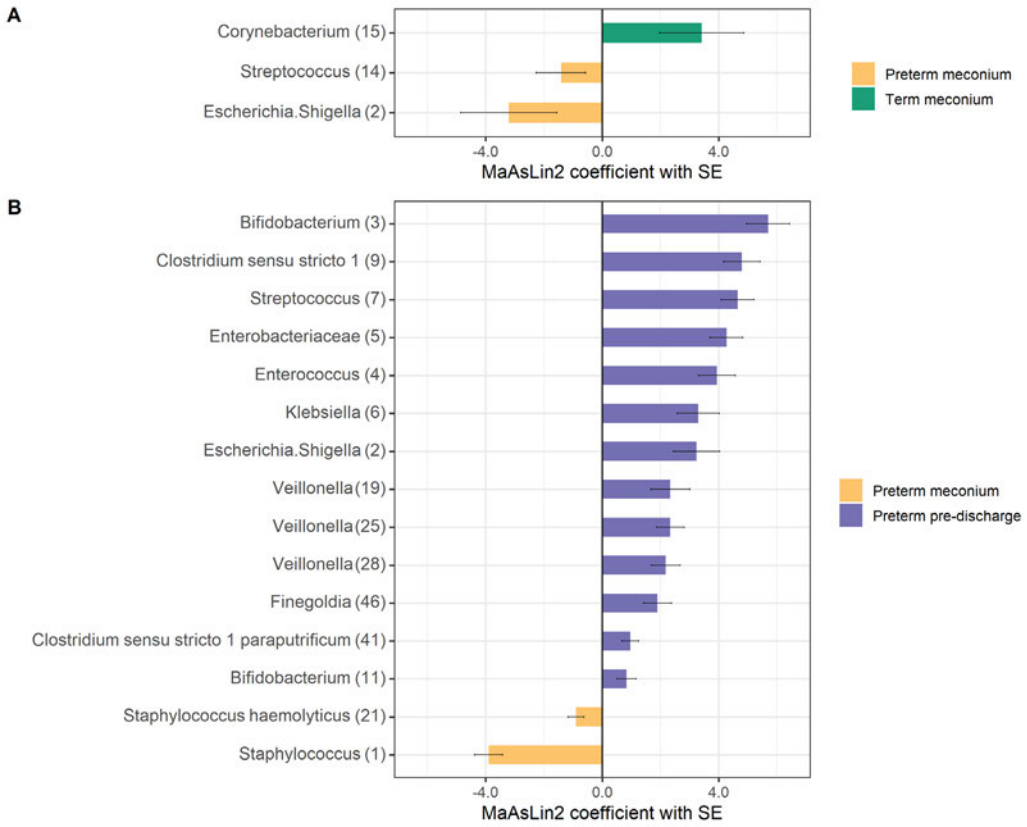


Figure 5-7. Differentially abundant taxa between sample types.
(A) Preterm versus term meconium samples (adjusted for postnatal age at sample collection); (B) preterm meconium versus pre-discharge samples (unadjusted). Analyses were conducted using MaAsLin2, testing differences in ASVs present with at least 1% of abundance in at least 5% of samples; results with FDR-corrected p-value < 0.25 are shown.

Alpha diversity also differed between the three sample types. Linear mixed effect models revealed a statistically significant effect of sample type/timepoint on Shannon index ($F = 47.244$, $p = 2.48 \times 10^{-15}$; Figure 5-8A): Shannon index was the highest in preterm pre-discharge samples ($p = 6.38 \times 10^{-5}$ and $p = 4.00 \times 10^{-14}$ for the comparisons with term meconium and preterm meconium samples, respectively) while there were no statistically significant differences between the term and preterm meconium samples ($p = 0.571$). There was also a statistically significant effect of sample type on the observed species ($\log_{10}$-transformed; $F = 22.876$, $p = 2.06 \times 10^{-9}$; Figure 5-8B), but interestingly, term infants had

significantly higher observed species in the meconium samples compared to preterm infants ($p = 7.75 \times 10^{-5}$), and pre-discharge samples had significantly higher observed species compared to preterm meconium ($p = 6.88 \times 10^{-9}$).

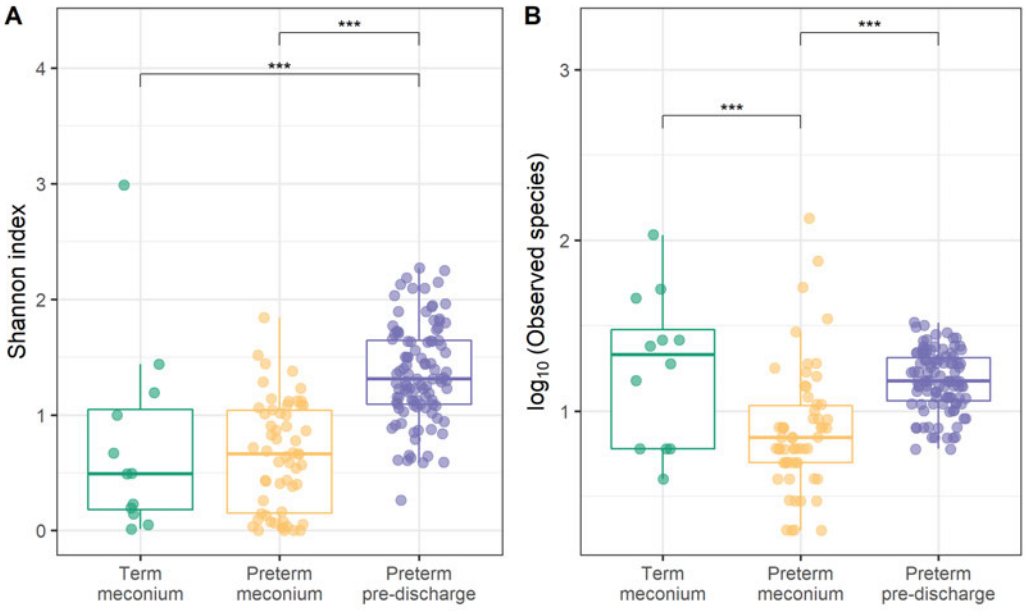


Figure 5-8. Microbiota alpha diversity in the sample types.
*Measured by (A) Shannon index and (B) observed species. *** indicates p < 0.001 in pairwise comparisons using emmeans following linear mixed effects model.*

5.4.5 Associations between preterm microbiota profiles and perinatal factors

Next, I investigated which perinatal factors shape the microbiota community composition and diversity. Due to the low number of term meconium samples with sufficient sequencing data, these analyses were performed only in preterm infants. In addition, these analyses were performed separately for the meconium and pre-discharge samples due to the highly different microbiota profiles in these sample types as well as to understand the temporal effects of perinatal factors on microbiota composition. GA at birth was dichotomised in these analyses to group the infants into extremely (GA at birth < 28 completed weeks) and very (GA at birth < 32 completed weeks) preterm.

5.4.5.1 Effects of perinatal factors on overall microbiota composition

Univariable PERMANOVA analyses (Table 5-4) revealed that bacterial community composition in preterm meconium strongly associates with postnatal age at sample collection, mode of delivery, and birthweight z-score. For pre-discharge samples, I observed the strongest effects for GA group (very vs extremely preterm), followed by postnatal age and GA at sample collection, antibiotic exposure, and sex. Interestingly, the effect of mode

of delivery was specific to meconium, suggesting recovery of the microbiota of C-section-born preterm infants over the neonatal period.

Table 5-4. Univariable PERMANOVAs investigating the effects of perinatal factors on bacterial community composition (beta-diversity) in preterm infant meconium and pre-discharge samples.
P-values were adjusted for FDR using the Benjamini-Hochberg method. Analyses were conducted separately for preterm meconium and pre-discharge samples. Sepsis, necrotising enterocolitis and bronchopulmonary dysplasia refer to diagnosis of these at any time during admission. NNU = neonatal unit.

	Meconium samples			Pre-discharge samples		
	R2 (%)	**p-value**	**Adjusted p-value**	**R2 (%)**	**p-value**	**Adjusted p-value**
Very vs extremely preterm	1.978	0.276	0.414	2.922	0.004	0.035
Postnatal age at sample collection	7.456	0.002	0.009	2.717	0.006	0.035
GA at sample collection	0.852	0.885	0.885	2.405	0.007	0.035
Sex	2.322	0.201	0.361	2.354	0.019	0.057
Mode of delivery	10.519	0.001	0.009	0.564	0.838	0.838
Labour antibiotics	1.855	0.362	0.465	1.395	0.164	0.273
Antibiotics <72h of life	1.331	0.615	0.692	1.192	0.288	0.392
Antibiotics >72h of life				2.500	0.017	0.057
% days of antibiotic exposure during NNU				1.290	0.193	0.289
Birthweight	3.545	0.059	0.133	0.993	0.384	0.443
Birthweight z-score	5.801	0.008	0.024	1.051	0.340	0.425
Sepsis				1.645	0.082	0.176
Necrotising enterocolitis				1.414	0.158	0.273
Bronchopulmonary dysplasia				0.832	0.554	0.594
High vs low breast milk exposure (≥/<75% of NNU days)				1.836	0.052	0.130

5.4.5.2 Differentially abundant taxa in association with perinatal factors

To understand how different perinatal factors are associated with the abundances of specific bacterial taxa, I conducted differential abundance analyses using MaAsLin2, focussing on the variables that had significant associations with microbiota beta diversity (adjusted p-value < 0.1). As age at sampling had a significant effect on both meconium and pre-discharge sample compositions, models were adjusted for postnatal age or GA at sampling, respectively.

The meconium of vaginally born preterm infants contained higher abundance of *Escherichia/Shigella* (p = 0.048, q = 0.172), among others, whilst *Staphylococcus* was more abundant in the gut of C-section born infants (p = 0.002, q = 0.023; Figure 5-9A). Birthweight z-score negatively associated with the abundance of *Staphylococcus* (p = 0.018, q = 0.217;

Figure 5-9C). In the full multivariable model, where the effects of mode of delivery, birthweight z-score and postnatal age at sample collection were tested simultaneously, C-section born infants had higher relative abundance of *Staphylococcus* (p = 0.007, q = 0.156; Figure 5-9B), whilst no significant effects remained for birthweight z-score, suggesting the results for birthweight z-score observed in the baseline model could be contributed to mode of delivery.

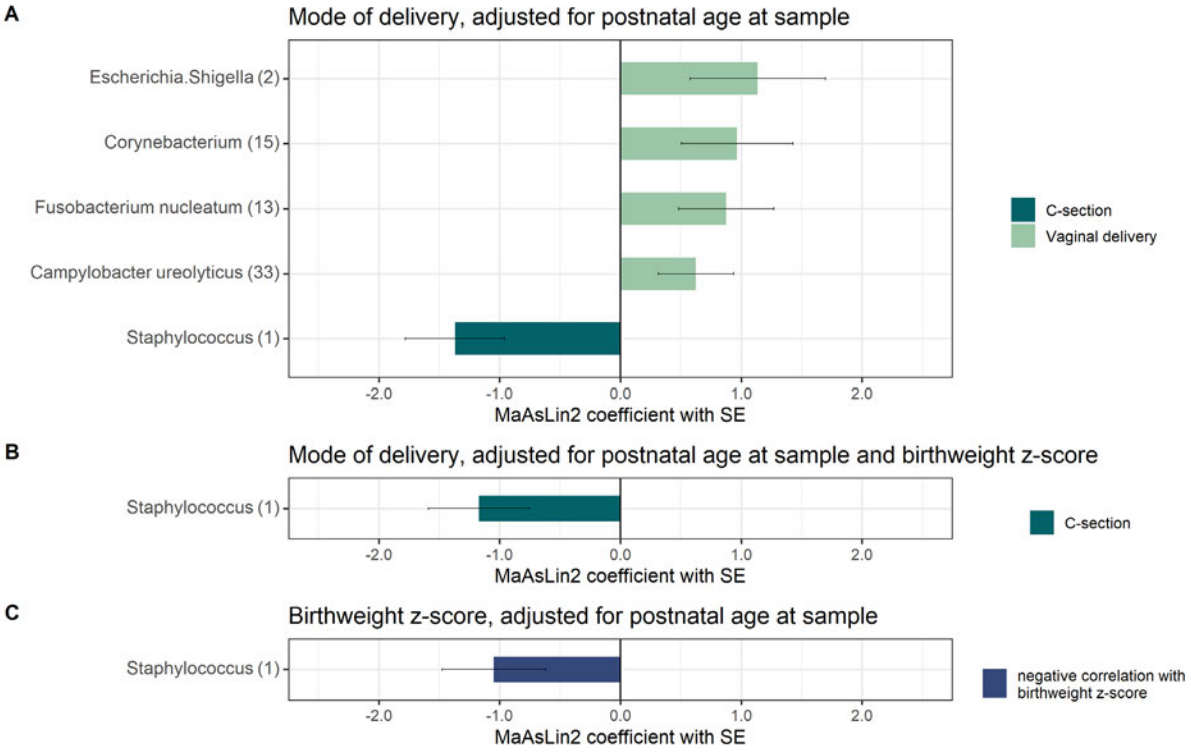


Figure 5-9. Differentially abundant ASVs in relation to perinatal clinical factors in preterm meconium samples.
Analyses were conducted using MaAsLin2, testing differences in ASVs present with at least 1% of abundance in at least 5% of samples; results with FDR-corrected p-value < 0.25 are shown. (A) Effect of mode of delivery in baseline model (adjusted for postnatal age at sampling); (B) Effect of mode of delivery in full model (adjusted for postnatal age at sampling and birthweight z-score); (C) Effect of birthweight z-score in baseline model.

By the time of hospital discharge, the faecal samples of extremely compared to very preterm infant gut had higher relative abundances of *Veillonella* ASVs (q range 0.008 – 0.078) and *Klebsiella* (p = 0.024, q = 0.160), and lower levels of *Staphylococcus* (p = 0.004, q = 0.037; Figure 5-10A, top panel). Next, I investigated the effects of other significant drivers of the microbiota community composition: sex and exposure to antibiotics. In baseline models (adjusted for GA group and GA at sample collection; Figure 5-10A), male infants had higher relative abundances of *Veillonella* (p = 0.034, q = 0.216), *Bifidobacterium* (p = 0.023, q = 0.171), and *Streptococcus* (p = 0.0007, q = 0.011); and antibiotic-exposed infants had

higher relative abundance of *Klebsiella* (p = 0.014, q = 0.110). In the fully adjusted model (Figure 5-10B), where the effects of GA group, GA at sample collection, sex, and antibiotic exposure were tested simultaneously, the main results paralleled those in the baseline model, although the relationships between GA group and *Klebsiella*, and sex and *Veillonella* were no longer statistically significant after adjusting for multiple comparisons.

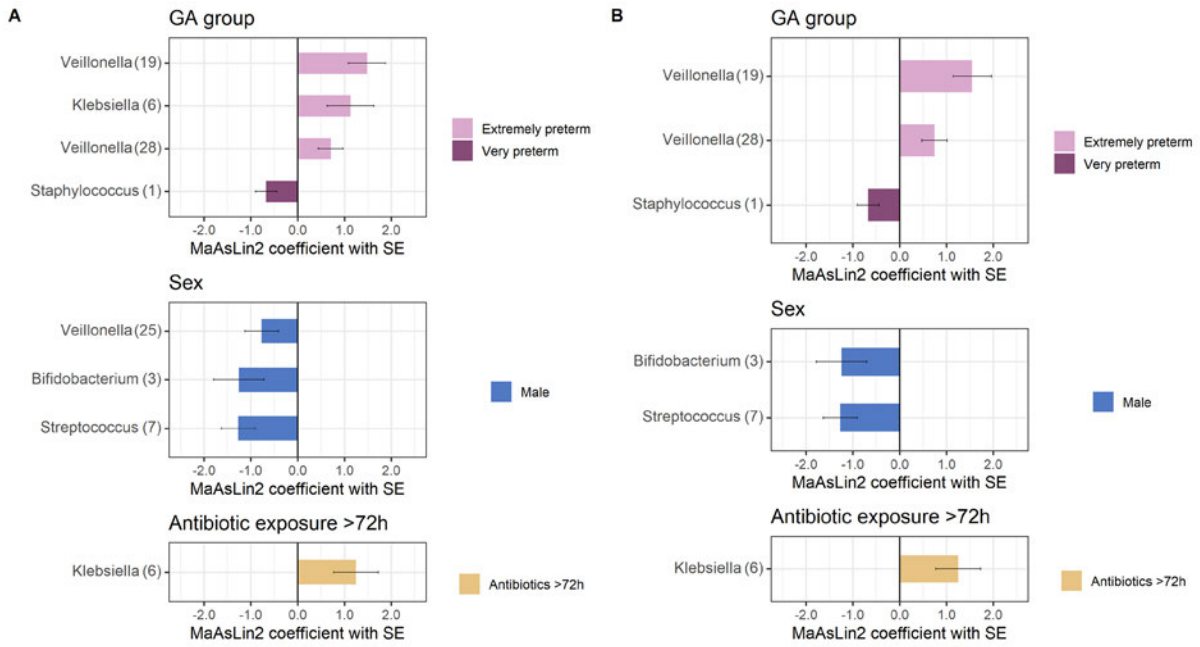


Figure 5-10. Differentially abundant ASVs in relation to perinatal clinical factors in preterm pre-discharge samples.
Analyses were conducted using MaAsLin2, testing differences in ASVs present with at least 1% of abundance in at least 5% of samples; results with FDR-corrected p-value < 0.25 are shown. (A) Baseline model, adjusted for GA group and GA at sample collection; (B) Fully adjusted model, testing all variables simultaneously, adjusting for GA at sample collection.

Given the putative role of gut microbial dysbiosis in the development of NEC and sepsis (Olm et al., 2019; Pammi et al., 2017; Stewart et al., 2017b, 2016), I re-ran the fully adjusted MaAsLin2 model excluding infants who were diagnosed with sepsis or NEC during their NNU stay (n = 26). These results, in most parts, were similar to these observed in the baseline models in the full sample (Figure 5-11).

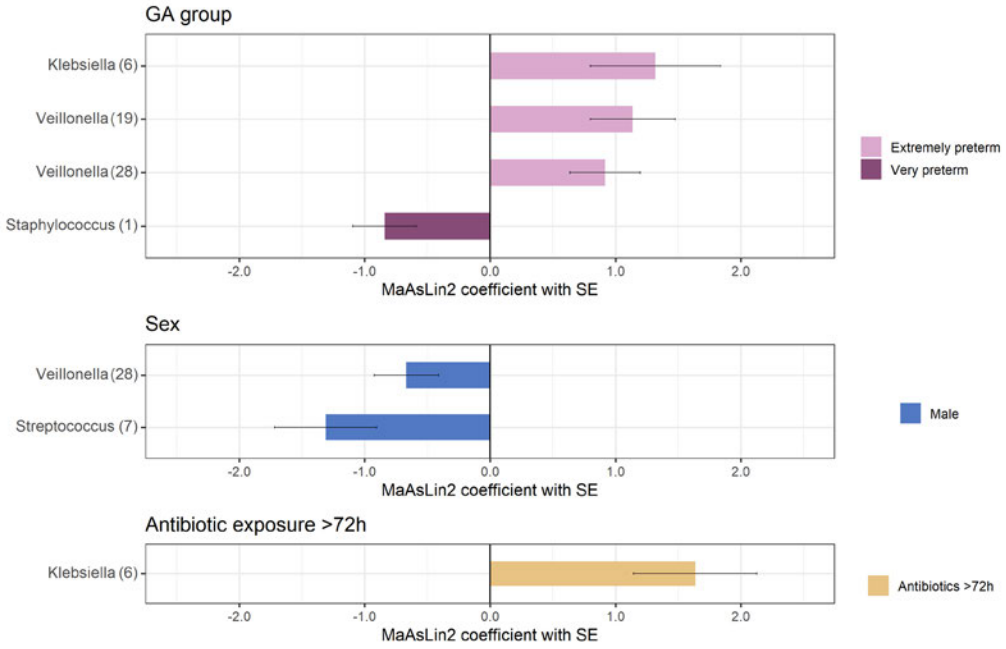


Figure 5-11. Differentially abundant ASVs in relation to perinatal clinical factors in the pre-discharge samples of infants not diagnosed with sepsis or NEC during their hospital stay.
Analyses were conducted using MaAsLin2, testing differences in ASVs present with at least 1% of abundance in at least 5% of samples; results with FDR-corrected p-value < 0.25 are shown. All variables were tested simultaneously, additionally adjusting for GA at sample collection.

5.4.5.3 Associations between perinatal factors and bacterial alpha diversity

With regard to bacterial alpha (within-sample) diversity, overall, different perinatal variables had minimal associations in univariable models (Table 5-5 and Table 5-6). Although postnatal age and mode of delivery had large effects on meconium microbiota composition, interestingly, none of the perinatal variables tested had significant effects on microbiota diversity indices in meconium samples.

Table 5-5. Effects of perinatal factors on alpha diversity indices in preterm meconium samples.
P-values were adjusted for FDR using the Benjamini-Hochberg method.

	Shannon index			log10 Observed species		
	statistic	**p-value**	**Adjusted p-value**	**statistic**	**p-value**	**Adjusted p-value**
GA group	W = 451.5	0.546	0.900	W = 339	0.250	0.645
Postnatal age at sample collection	rho = 0.005	0.969	0.988	rho = -0.123	0.356	0.712
GA at sample collection	rho = -0.052	0.699	0.968	rho = -0.219	0.098	0.645
Sex	W = 351	0.286	0.645	W = 343	0.232	0.645
Mode of delivery	W = 320	0.271	0.645	W = 299	0.148	0.645
Labour antibiotics	W = 312.5	0.866	0.988	W = 261.5	0.281	0.645
Antibiotics <72h of life	W = 299	0.911	0.988	W = 266	0.626	0.940
Birthweight	rho = 0.017	0.898	0.988	rho = -0.002	0.988	0.988
Birthweight z-score	rho = 0.08	0.550	0.900	rho = 0.192	0.150	0.645

In pre-discharge samples, observed species was higher in extremely compared to very preterm infants, as well as in infants diagnosed with NEC, although the number of infants who developed NEC was very small. In addition, observed species positively associated with postnatal age and GA at sample collection as well as birthweight z-score. Although the direction of effect for the age variables was similar for the Shannon index, the effect sizes were smaller and the tests did not reach statistical significance. This suggests that prematurity and age have stronger effects on microbiota richness than evenness. I also observed that male infants and those diagnosed with sepsis had higher observed species, however, these effects were not statistically significant after adjusting for multiple comparisons.

Table 5-6. Effects of perinatal factors on alpha diversity indices in preterm pre-discharge samples.
P-values were adjusted for FDR using the Benjamini-Hochberg method. Sepsis, necrotising enterocolitis and bronchopulmonary dysplasia refer to diagnosis of these at any time during admission. NNU = neonatal unit.

	Shannon index			log10 Observed species		
	statistic	**p-value**	**Adjusted p-value**	**statistic**	**p-value**	**Adjusted p-value**
GA group	t = -1.088	0.280	0.365	t = -3.547	0.001	0.006
Postnatal age at sample collection	rho = 0.16	0.106	0.290	rho = 0.416	0.000	0.000
GA at sample collection	rho = 0.122	0.218	0.321	rho = 0.399	0.000	0.000
Sex	t = 1.668	0.099	0.290	t = 2.282	0.025	0.125
Mode of delivery	t = 0.863	0.391	0.475	t = -0.854	0.396	0.475
Labour antibiotics	t = 0.388	0.699	0.699	t = 1.109	0.270	0.365
Antibiotics <72h of life	t = -1.269	0.215	0.321	t = -1.961	0.060	0.225
Antibiotics >72h of life	t = -0.723	0.471	0.524	t = -1.507	0.135	0.292
% days of antibiotic exposure during NNU	rho = 0.121	0.225	0.321	rho = 0.14	0.160	0.292
Birthweight	rho = -0.04	0.689	0.699	rho = -0.146	0.140	0.292
Birthweight z-score	rho = 0.139	0.162	0.292	rho = 0.254	0.010	0.057
Sepsis	t = -1.58	0.121	0.292	t = -2.116	0.039	0.169
Necrotising enterocolitis	t = -1.903	0.190	0.316	t = -6.89	0.006	0.046
Bronchopulmonary dysplasia	t = -0.508	0.613	0.657	t = -1.841	0.071	0.236
High vs low breast milk exposure (≥/<75% of NNU days)	t = 0.825	0.412	0.475	t = 1.396	0.166	0.292

To test the independence of the factors associated with observed species in pre-discharge samples, I performed a multivariable model testing the effects of GA group and birthweight z-score simultaneously, adjusting for GA at sample collection. Here, I excluded the infants

with NEC due to the small number (n = 3). This model confirmed the positive association between birthweight z-score and observed species, but the effect for the degree of prematurity was attenuated (Table 5-7).

Table 5-7. Results of the multivariable model testing associations between perinatal factors and bacterial richness (Observed species) in the pre-discharge samples.

Predictor	β	SE	95% CI	p-value
GA group	0.325	0.204	-0.080 – 0.731	0.115
Birthweight z-score	0.259	0.090	0.080 – 0.438	**0.005**
GA at sample collection	0.309	0.099	0.113 – 0.506	**0.002**

5.5 Discussion

This study investigated the microbiota diversity and community composition of meconium and faecal samples prior to discharge from NNU in very and extremely preterm infants. The data showed that (1) preterm meconium and pre-discharge faecal samples differ in bacterial diversity and community compositions; (2) term infant meconium samples have higher bacterial richness compared to preterm meconium, but the differences in overall bacterial community composition are minimal; (3) the main drivers of meconium community composition in preterm infants are postnatal age and mode of delivery; and (4) by the time of hospital discharge, preterm infant gut microbiota composition has been shaped by the degree of prematurity, sex, and antibiotic exposure.

5.5.1 Development of the microbiota over the neonatal period

The data support previous studies showing a dynamic development of the gut microbiota over the neonatal period in preterm infants, which includes increase in bacterial alpha diversity (richness as well as evenness) and shift in community composition from the first days of life to the discharge from hospital. Initially, the majority of preterm infants had community compositions characterised by high relative abundances of *Staphylococcus*, which decreases in abundance by the time of discharge from the NNU. By that time, the majority of preterm infants had gut microbiota profiles characterised by high relative abundances of either *Bifidobacterium* or *Enterobacteriaceae*, while some infants retained community compositions high in *Escherichia/Shigella* or *Klebsiella.* Pre-discharge samples additionally had higher relative abundances of *Clostridium*, *Enterococcus,* and *Veillonella*

compared to meconium. These results are in agreement with previous studies which also demonstrate dominance of *Staphylococcus* in the preterm infant gut in the first week of life (Beck et al., 2022; Grier et al., 2017; Klopp et al., 2022; Korpela et al., 2017a; La Rosa et al., 2014; Moles et al., 2013). Thereafter, the bacterial communities transition to states dominated by *Bifidobacterium* or *Enterobacteriaceae,* including *Klebsiella* (Beck et al., 2022; Grier et al., 2017; Hui et al., 2021; Klopp et al., 2022; Korpela et al., 2017a; La Rosa et al., 2014; Moles et al., 2013; Rao et al., 2021), while others also describe high abundances of *Enterococcus*, *Escherichia,* and *Veillonella* preterm infant faeces (Hui et al., 2021; Moles et al., 2013; Rao et al., 2021).

5.5.2 Microbiota in association with gestational age at birth

Previous studies have reported significant effects of prematurity and GA at birth on the bacterial composition of meconium samples (Ardissone et al., 2014; Klopp et al., 2022; Morais et al., 2020). Preterm status explained around 3% of variance in the overall community composition of meconium samples. Although this effect size was similar to those observed for the strongest perinatal drivers of the preterm pre-discharge samples, it was not statistically significant. This could be explained by low power as only 12 term infant samples had sequencing data for analyses. There were some differences at the ASV level, especially increase in the abundances of *Escherichia/Shigella* and *Streptococcus* and decrease in *Corynebacterium* in the preterm compared to term infant meconium. Indeed, *Escherichia/Shigella* and *Streptococcus* have previously been reported as common taxa in preterm meconium samples (Klopp et al., 2022). The increased abundance of *Corynebacterium* in term infant meconium is intriguing, as this taxa is a common skin commensal and associated with C-section delivery (Dominguez-Bello et al., 2010), and only one term infant in the current cohort was born via C-section. In contrast, I did not observe a significant difference between the bacterial community composition of meconium obtained from very or extremely preterm infants, which could potentially be due to the overshadowing effect of factors like delivery mode and postnatal age on meconium bacterial composition. In addition, I identified two preterm meconium samples with high relative abundances of *Fusobacterium nucleatum*. This observation is interesting as this bacterium has previously been linked to adverse pregnancy outcomes such as preterm birth (Vander Haar et al., 2018).

Term and preterm infant meconium also differed in bacterial richness as term infant meconium had significantly higher Observer species. This observation may potentially be attributed to the higher postnatal age at preterm infant meconium sample collection in line with findings that there is a decrease in bacterial alpha diversity in the first days of life due to environmental filtering before a gradual increase (Beck et al., 2022; Enav et al., 2022). Indeed, delayed passage of meconium in preterm infants is expected based on previous literature and is reflective of gestation-dependent feeding practice and maturation of bowel processes (Bekkali et al., 2008). Some studies have also suggested a prolonged reduction in gut bacterial richness in preterm compared to term infants (Fouhy et al., 2019; Gibson et al., 2016), whilst others report no differences in bacterial diversity between term and preterm neonates (Chernikova et al., 2018; Tauchi et al., 2019).

Although the degree of prematurity did not have an effect on meconium sample composition in preterm infants, there were significant differences by the time of discharge from the hospital, potentially suggesting accumulating effect of neonatal exposures. The abundance of *Klebsiella*, a known pathogen associated with nosocomial infections such as NEC (Olm et al., 2019; Pammi et al., 2017), positively associated with the degree of prematurity. Microbiota profile dominated by *Klebsiella* in association with lower GA at birth has also been previously reported in a longitudinal study of preterm gut microbiota development (Beck et al., 2022), suggesting that higher degree of prematurity at birth may be associated with increase in pathogenic bacteria that persist until discharge from the neonatal care. *Veillonella*, Gram-negative commensal obligate anaerobic bacteria and a common resident oral bacteria, was also significantly more abundant in extremely compared to very preterm infant gut. Interestingly, the gut microbiota of pregnant women who delivered preterm compared to those with normal pregnancies was enriched for common oral bacteria and the abundance of oral bacteria correlated with GA at birth (Yin et al., 2021), suggesting a potential source of the *Veillonella* in the preterm infant gut. In the context of preterm infant care, it could be hypothesised that prolonged feeding via nasogastric tubes could potentially contribute to increased oral-to-gut translocation of bacteria. In addition, nasogastric tubes as part of the NNU equipment may influence the exchange of bacteria between the environment and the infant (Brooks et al., 2014; Petersen et al., 2016). Infants born at a younger GA spend a longer time in neonatal intensive care

and thus have extended exposure to environmental bacteria in the hospital. Indeed, a previous study additionally found increased *Veillonella* in association with increased NNU stress (D'Agata et al., 2019). Nevertheless, many studies suggest a potential non-pathogenic, or protective, role of this bacteria as higher *Veillonella* abundance has been found in preterm infants without NEC (Lindberg et al., 2020; Ward et al., 2016; Zhou et al., 2015) and in association with appropriate postnatal growth (Younge et al., 2019). *Veillonella* has also been reported as one of the signature taxa in 4-month-old term infant microbiota, interpreted as reduced oxygen concentration and utilisation of lactic acid (Bäckhed et al., 2015). Furthermore, the abundance of *Staphylococcus*, a facultative anaerobe which was the dominant bacteria in meconium samples, negatively associated with degree of prematurity. This might potentially suggest a "younger-looking" microbiota at the time of discharge of infants born at an older GA.

5.5.3 Microbiota in association with perinatal factors

In addition to prematurity, I determined the perinatal clinical factors associated with microbiota development in preterm infants. Interestingly, the effect of the clinical factors varied between meconium and faecal samples, suggesting temporal effects of perinatal variables in shaping the microbiota.

Mode of delivery is considered one of the main drivers of microbiota composition over the first year of life. In the current study, I found that, in preterm infants, mode of delivery had the strongest effect on the bacterial composition in meconium samples, but had lost its effect by the time of discharge from the hospital. This is partly in line with previous research demonstrating the "recovery" of gut microbiota profiles over the first few months of life in full-term C-section born infants (Reyman et al., 2019; Shao et al., 2019), whilst other studies suggest that the differences in the gut microbial species between vaginally and C-section delivered children remain visible for up to 4 years of life (Fouhy et al., 2019). It could further be hypothesised that the potent exposures related to preterm birth that accumulate over hospital admission overshadow mode of delivery effect in later samples.

C-section-delivered infants particularly had a higher abundance of *Staphylococcus*. This is in line with previous studies demonstrating higher abundance of *Staphylococcus* in C-section born infants in the first days of life (Dominguez-Bello et al., 2010; Reyman et al., 2019) and suggest that infants delivered by C-section initially acquire a bacterial composition similar to

that normally found on skin surfaces (Dominguez-Bello et al., 2010). In contrast, Korpela et al (2017) reported higher levels of *Staphylococcus* in vaginally delivered preterm infants over the course of NNU stay. I additionally observed some evidence for increased *Escherichia/Shigella* in the meconium of vaginally delivered infants, although it was not significant after additional adjustment for birthweight z-score. Increased *Escherichia* abundance vaginally delivered infants has also been observed previously, for example, in the meconium and first week of life of full-term infants (Reyman et al., 2019; Wong et al., 2020) and in very preterm infants (Stewart et al., 2017a). The finding of increased *Corynebacterium* in vaginally delivered infant meconium in the baseline model is interesting as *Corynebacterium* is a typical skin-associated bacteria and previously reported to associate with C-section delivery in term infant meconium (Dominguez-Bello et al., 2010). However, these discrepancies could highlight the differential effect of delivery mode on microbiota composition between full- and preterm infants, which could arise from exposure to intensive care immediately following birth. Indeed, although delivery mode is an established driver of the early life gut microbiota in full term infants, there is mixed evidence to what extent it has an impact on the microbiota in preterm infants (Aguilar-Lopez et al., 2021a), with some studies reporting no or weak effects (e.g. Beck et al., 2022; Stewart et al., 2017). In the current study, the sampling of the first meconium may have allowed the detection of the effect related to delivery mode in very and extremely preterm infants.

Although I identified a significant effect of delivery mode on preterm meconium microbiota composition, the results suggest no differences in microbiota alpha diversity between vaginally and C-section-delivered infants. This is in line with several other studies reporting no differences in alpha diversity indices between delivery modes among preterm infants (e.g. Korpela et al., 2017; Mshvildadze et al., 2010; Stewart et al., 2017; Wandro et al., 2018).

I further identified a negative association between birthweight z-score and *Staphylococcus* in the preterm meconium. Extreme negative deviations of birthweight expected from GA at birth are related to intrauterine growth restriction which associates with neonatal morbidities (Malhotra et al., 2019). A few studies have investigated the impact of intrauterine growth restriction (or being born small for gestational age) on the gut microbiota (Chen et al., 2022; Hiltunen et al., 2021), but the effect remains poorly

understood. In the current study, however, the effect of birthweight z-score was lost when additionally adjusting for delivery mode, suggesting that birthweight z-score was acting as a proxy for mode of delivery. Indeed, among infants with meconium samples in the current study, those born via C-section had significantly lower birthweight as well as birthweight z-score.

Interestingly, however, birthweight z-score also associated with increased bacterial richness prior to discharge from the hospital independently from age at sampling and degree of prematurity, though no significant association with overall bacterial composition was observed. This result is partly in line with the work by Chen et al (2022) showing reduced microbiota diversity in full-term infants born small for gestational age during the first week of life. Birthweight z-score could be related to a range of factors and morbidities, such as prolonged hospitalisation and poor growth postnatally (Malhotra et al., 2019; Sharma et al., 2016), which can further shape the gut microbiota.

Increasingly, studies have investigated the independent role of sex on gut microbiota (Valeri and Endres, 2021), however, only a few studies have done so in infancy. In the current study, sex explained ~2% of variance in microbiota beta-diversity. I found that male infants prior to discharge from the hospital had higher abundances of *Streptococcus, Bifidobacterium* and *Veillonella*. Higher levels of *Bifidobacterium* in full-term male infants have also been reported before (Nagpal et al., 2017a), though the results regarding *Streptococcus* are in conflict with those by Cong et al. (2016) who reported increased *Lactobacillales* in female preterm infants over the first month of life. Nevertheless, the impact of infant sex on gut microbiota development remains poorly understood, with other studies reporting no significant effects (e.g. Beck et al., 2022).

Male infants are at an increased risk for mortality and major morbidities related to preterm birth such as bronchopulmonary dysplasia, severe brain injury, retinopathy of prematurity, and NEC, although the differences in mortality between males and females are getting smaller (Garfinkle et al., 2020). Thus, the sex effects on microbiota could be confounded by other perinatal factors. In the current sample of preterm infants, there were no statistically significant differences in perinatal clinical factors or neonatal morbidities between males and females, except a higher birthweight and birthweight z-score in male infants. These factors, however, did not have significant effects on the microbiota community composition

in pre-discharge samples after adjusting for confounding. In addition, sex effects on bacterial taxa were observed even after adjusting for GA at birth, GA at sampling, and antibiotic exposure, suggesting an independent role of sex on microbiota development. Therefore, it could be speculated that the independent sex-related differences in microbiota profiles may potentially play a role in the differential susceptibility of male and female preterm infants to neonatal morbidities. However, this hypothesis remains to be tested in future studies.

Human milk consumption is a known driver of the preterm infant gut microbiota composition, particularly related to increased abundance of *Bifidobacterium*, although the specific effects vary greatly between studies (Aguilar-Lopez et al., 2021a). Surprisingly, in the current study, I found no evidence that the level of exclusive breast milk exposure has an effect on the preterm gut microbiota composition or diversity. This discrepancy with previous studies may be attributed to the different classification of feeding type between studies. Here, I defined breast milk exposure as the proportion of inpatient days infants received exclusive breast milk feeds, which included both maternal and/or donor expressed breast milk. It has been reported that donor and maternal breast milk have different effects on preterm gut microbiota composition (Gregory et al., 2016; Piñeiro-Ramos et al., 2021). Thus, combining the two milk types may have biased the results. Nevertheless, preterm infants, including the babies in the current cohort, are exposed to a variety of feeding strategies during their hospital stay, including simultaneous exposure to maternal, donor and formula milk. This limits the opportunities to study the impact of the different feeding types separately. For example, in the current study, only 7 infants with pre-discharge samples received exclusively mother's own breast milk, only 2 infants never received any mother's breast milk, and no infants received 100% exclusive formula or donor milk feeds during their hospital stay. Much larger samples may be needed to tease out the specific effects of maternal, donor and formula milk in the heterogeneous preterm population with complex nutritional needs.

I found significantly higher abundance of *Klebsiella* in those infants who were exposed to antibiotics after three days of life. Based on NCBI BLAST tool, this 16S rRNA gene sequence had 100% overlap with *Klebsiella pneumoniae* which is considered to belong to the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella species, Acinetobacter baumannii,*

Pseudomonas aeruginosa, and *Enterobacter* species) pathogen family (Boucher et al., 2009) and associates with nosocomial infections including the development of NEC (Olm et al., 2019; Pammi et al., 2017). These results are in line with previous studies as outgrowth of *Klebsiella* following antibiotic exposure has been reported in full-term (Reyman et al., 2022) as well as preterm infants without NEC (Pammi et al., 2017). *Klebsiella* abundance has also been associated with increased antibiotic resistance genes in antibiotic-exposed preterm infants (Gibson et al., 2016). Due to the small number of infants diagnosed with NEC in the TEBC cohort, I was unable to investigate the unique bacterial signature associated with NEC. Importantly, however, antibiotic exposure was also associated with increased *Klebsiella* abundance in infants not diagnosed with NEC or sepsis during their hospital stay, suggesting an independent effect of antibiotic exposure and that it is not solely a proxy for a major illness.

Studies in full- and preterm neonates have shown temporary effects of antibiotic exposure on the developing gut microbiota, with large shifts in community composition immediately after antibiotic exposure, followed by a gradual recovery (Korpela et al., 2017a; Reyman et al., 2022). Here, the temporal effects of antibiotic exposure could not be studied due to the single faecal sample collected per infant; the length of time between the end of antibiotic treatment and faecal sample collection was also unknown. Thus, it is possible that some more temporary/subtle effects of antibiotic exposure were not captured in the current study. However, the results suggest that some effects of antibiotic exposure during NNU stay are still evident just before discharge from the hospital.

Interestingly, it appears that exposure to antibiotics rather than length of exposure plays a role in shaping the intestinal microbiota of preterm infants as I observed no effect of the duration of antibiotic exposure on the overall microbiota composition. Although there is some evidence in previous studies that the effect of antibiotic exposure on the microbiota varies with the duration of treatment, this effect is negligible compared to the initiation of antibiotics (Greenwood et al., 2014; Reyman et al., 2022). In the current study, I did not detect any effect for the duration of treatment, however, this could be related to low variance in the data with the mean of antibiotic treatment duration of 3 days, and 85% of study infants were on antibiotic treatment less than 20% of inpatient days. Furthermore, antibiotic exposure for the treatment of early onset sepsis (< 3 days of life) had no effect on

microbiota composition, although the effect of new-born antibiotic treatment on gut microbiota has been reported by others before (Arboleya et al., 2016a; Chernikova et al., 2018). This could also be related to low variance in the data as almost 80% of the preterm neonates were exposed to antibiotics in the first three days of life for treatment of suspected or confirmed early onset sepsis.

5.5.4 Strengths and limitations

The preterm participants of this observational study were cared for in a single NNU in Scotland, ensuring a similar environmental bacterial background for all infants. Furthermore, preterm infants were all cared for under the same standardised feeding and antibiotic treatment regimes, with the same access to donor milk and no exposures to probiotics.

Another strength of the current study is the detailed quality control of the 16S rRNA gene sequencing data. Low biomass samples, such as those collected from neonates, are at an increased risk for biases due to possible bacterial DNA contamination from processing reagents and environment (Davis et al., 2019; Nearing et al., 2021). I included positive and negative control samples at each of the sample processing steps, which enabled to investigate the background and stability of profiles between processing batches. I detected very low contamination levels in the negative controls indexed by the extremely low bacterial yield and number of sequencing reads. Furthermore, the inclusion of negative controls enabled the identification of contaminant taxa in the dataset and their removal from subsequent analyses. In addition, all samples in the current study were sequenced during the same MiSeq sequencing run, which minimises biases due to differences in sequencing depths that could arise between different runs.

This study also has several limitations. First, DNA extraction did not yield sufficient amount for the sequencing of ~40% of meconium samples. Although various protocol adjustments were tested, I was not able to test and optimise different bacterial DNA extraction kits or protocols in order to ascertain if the DNA yield could have been improved. The protocol used in the current study has been optimised for low dense bacterial communities such as those in the respiratory tract (Biesbroek et al., 2012), but it is possible that some reactions in the protocol were inhibited due to the consistency of and/or additional material present in meconium samples. Indeed, it has been reported previously that meconium samples

contain PCR inhibitors and the choice of DNA extraction kit can impact on the ability to extract and detect bacterial DNA (Stinson et al., 2018). Nevertheless, low bacterial DNA in meconium samples has also been reported previously (Ardissone et al., 2014; Klopp et al., 2022), with higher detection of bacterial DNA in the meconium of preterm infants (Ardissone et al., 2014). This is also consistent with the results in the current study which showed that the extremely low-dense samples were more common among meconium samples of term infants and those collected at an earlier postnatal age.

Second, although the study aimed to characterise the preterm gut bacterial composition at the time of discharge from hospital (i.e. at a similar GA for all infants) to characterise the accumulating effects of neonatal exposures, for practical reasons, some infants had the pre-discharge sample collected considerably earlier (in 13% of cases the sample was collected > 3 weeks before discharge, with a maximum of 8 weeks before discharge). This resulted in a range of different GAs at sample collection, which was also positively associated with postnatal age at sample collection. In addition, due to the varying GA at birth, the pre-discharge samples were collected at a wide range of postnatal ages as the time spent in NNU is strongly affected by GA at birth. Therefore, due to the high collinearities between prematurity and ages at sample collection, it was difficult to disentangle the effect of GA at birth from those associated with postnatal age at sample collection, and the effect of postnatal age at sample collection from GA at sample collection. Indeed, it remains debated whether chronological age (i.e. time post-birth) or postconceptional age (i.e. physical maturity) is more important for gut microbiota development in preterm infants (Korpela et al., 2017a; La Rosa et al., 2014; Shen et al., 2021). Large studies with matched data with regard to both gestational and postnatal age at sample collection are required to disentangle these effects.

The third limitation of the study relates to the level of detail available for some of the perinatal exposures. Antibiotic and feeding data spanned the entire NNU stay, thus, due to the varying time between sample collection and hospital discharge, it may not have been fully representative of all the exposures prior to the pre-discharge sample collection for all infants. I aimed to overcome this limitation by using dichotomised variables or using continuous exposure data as proportion of the days spent in the NNU. Nevertheless, bias due to the difference between the timing of data and sample collection cannot not be ruled

out, and it may have prevented to find evidence for more temporal effects. In addition, only a small number of infants had matching meconium and pre-discharge samples collected and sequenced, thus I was unable to follow the trajectories of gut microbiota development in individual infants, which could vary depending on prematurity or early life exposures.

Another limitation relates to the use of 16S rRNA gene sequencing, limiting the understanding of the microbiome functional properties in association with perinatal clinical factors. Thus, the application of whole metagenome shotgun sequencing in the future will allow inference about the changes in functional metabolic capacity. However, intriguingly, a recent longitudinal study found weak evidence for early life factors, except probiotic exposure, shaping microbiota functional properties (Beck et al., 2022).

5.6 Conclusion

The neonatal period is a phase with rapid development of the gut microbiota, which plays a key role in health outcomes. Preterm infants are particularly at risk for gut dysbiosis-associated morbidities. Here, I demonstrate a substantial shift in microbiota diversity and community composition from preterm birth until hospital discharge. Mode of delivery was the strongest driver of the early colonisation, whilst the degree of prematurity, sex, and antibiotic exposure during neonatal care associated with microbiota composition prior to discharge from the hospital. This study contributes to the understanding of the drivers of microbiota development following very preterm birth.

Chapter 6. Neonatal microbiota and brain dysmaturation in preterm infants

6.1 Introduction

Accumulating evidence suggests a role for the microbiota in neurodevelopment, spanning the domains of general neurocognition, language, motor, social, and emotional development (see Chapter 4 and Vaher, Bogaert, et al. (2022) for a detailed review). Preterm infants are at an increased risk for adverse neurodevelopmental outcomes (Johnson and Marlow, 2017; Morgan et al., 2022). They comprise a unique population for whom critical neurodevelopmental processes, such as neurogenesis and neuronal migration, microglia migration and morphogenesis, myelination, and synaptogenesis, can be influenced by extrauterine life, including early colonisation of the immature gastrointestinal system by microorganisms. Preterm infant gut microbiome has previously been characterised by low species diversity (Chernikova et al., 2018; Moles et al., 2013), delayed maturation (La Rosa et al., 2014), and dominance of potentially pathogenic bacteria such as *Klebsiella* and *Escherichia* and low levels of *Bifidobacterium* (Arboleya et al., 2012; Arboleya, Sánchez, et al., 2016; Barrett et al., 2013; Rao et al., 2021; Stewart et al., 2016, 2017; see also Chapter 5). Gut dysbiosis in preterm infants associates with increased risk for major morbidities including necrotising enterocolitis and sepsis (Olm et al., 2019; Pammi et al., 2017; Stewart et al., 2017b, 2016). However, to date, there is poor understanding of the relationship between early-life gut microbiota and brain development following preterm birth.

Brain injury/dysmaturation in preterm infants is multifactorial and includes developmental disturbances affecting the developing white matter, cortical and deep grey matter, brain stem and cerebellum, commonly termed as encephalopathy of prematurity (EoP) (Volpe, 2019). In the absence of focal brain injury, neonatal neuroimaging has revealed a preterm brain phenotype at term-equivalent age (TEA), which includes reduced volume (Alexander et al., 2019; Makropoulos et al., 2016; Thompson et al., 2019), dysmaturation of cortical and deep grey matter structures (Ball et al., 2013b, 2012; Boardman et al., 2006; Dimitrova et al., 2021b; Makropoulos et al., 2016; W. Wang et al., 2022), and reduced white matter integrity (Barnett et al., 2018; Blesa et al., 2020; Telford et al., 2017; Thompson et al., 2019).

These features are predictive of later neurodevelopmental outcomes (e.g. Barnett et al., 2018; Boardman et al., 2010; Counsell et al., 2008; Gui et al., 2019; Keunen et al., 2016; Loh et al., 2017; Ouyang et al., 2020; Van Kooij et al., 2012), making them attractive to study as intermediate phenotypes for early identification of infants most at risk for impaired neurodevelopment.

Experimental manipulation of the gut microbiota in animal models has demonstrated causal links between preterm infant gut microbiota and neurodevelopment. For example, mice transplanted with gut microbiota from a preterm infant with poor postnatal growth demonstrated decreased markers of neuronal differentiation, oligodendrocyte development and myelination, and increased levels of neuroinflammation compared to mice transplanted with microbiota from an infant with adequate postnatal growth (Lu et al., 2018a). Furthermore, colonisation of mice with preterm infant microbiota influenced associative learning and memory (Lu et al., 2022).

To my knowledge, only a few previous studies have investigated gut microbiota in correlation with preterm infant neurodevelopment. These studies suggest a dynamic association between the neonatal microbiota composition and neurobehaviour at the time of discharge from the neonatal intensive care unit (Liu et al., 2022; Sun et al., 2020). Three studies additionally suggest that the neonatal gut microbiota composition associates with 2-year neurodevelopmental outcomes (Beghetti et al., 2021; Rozé et al., 2020; Sarkar et al., 2022). Although most of these studies have been small ($n < 40$; except the study by Rozé et al. (2020) where 372 infants with cognitive testing were included), they motivate research on the role of microbiota in preterm brain injury/dysmaturation.

Increasingly, neuroimaging methods are incorporated in the study of microbiota-gut-brain axis (P. Liu et al., 2019; Montoro et al., 2022), but only a few have done so in infancy and childhood. These include investigation of brain regional volumes (Carlson et al., 2021, 2018), functional connectivity (Gao et al., 2019; Kelsey et al., 2021), and electrical activity (Schoch et al., 2022). The only study linking preterm neonatal gut microbiota with brain MRI features at TEA found *Klebsiella*-dominated gut microbiota communities, alongside a pro-inflammatory immunological profile, to associate with severe brain injury (periventricular haemorrhagic infarction, intraventricular haemorrhage, cerebellar haemorrhage, or

periventricular leukomalacia) (Seki et al., 2021). However, no studies have investigated the role of gut microbiota in brain MRI features of EoP in the absence of major focal lesions.

6.1.1 Chapter aims

In this study, I aimed to test the hypothesis that the preterm infant neonatal gut microbiota diversity and community composition associate with brain MRI features of EoP at TEA.

6.2 Methods

The analyses presented in this chapter use a sample of preterm participants from the Theirworld Edinburgh Birth Cohort (TEBC; Boardman et al., 2020) who had matching data of multimodal brain MRI acquired at TEA and 16S rRNA gene sequencing from pre-hospital discharge faecal samples. Details of study design, participant recruitment, and methods brain MRI acquisition and pre-processing as well as faecal sample processing and 16S rRNA gene sequencing are described in Chapter 2. In brief, structural T_2-weighted and diffusion MRI were acquired at 3T at TEA; faecal samples were collected prior to discharge from neonatal unit, and microbiota profiles were generated by sequencing of the hypervariable V4 region of the 16S rRNA gene using Illumina MiSeq platform. Participant clinical and demographic information was collected from antenatal and neonatal electronic patient records and parent questionnaires.

6.2.1 Brain imaging features of interest

6.2.1.1 Volumetric features

The volumes were calculated from the tissue parcellation obtained from the dHCP pipeline using Draw-EM (Makropoulos et al., 2018, 2016). The volumes of interest were: total tissue (the sum of the volumes of cortical grey matter, white matter, deep grey matter, cerebellum, brainstem, hippocampi and amygdalae), cortical grey matter, deep grey matter, white matter, cerebellum, and the ventricles (Figure 6-1 top panel). For cortical grey matter, deep grey matter, white matter and the cerebellum both raw and relative (i.e. normalised to total tissue volume) volumes were considered in the analyses to quantify absolute growth of the tissues as well as that relative to total brain growth, respectively.

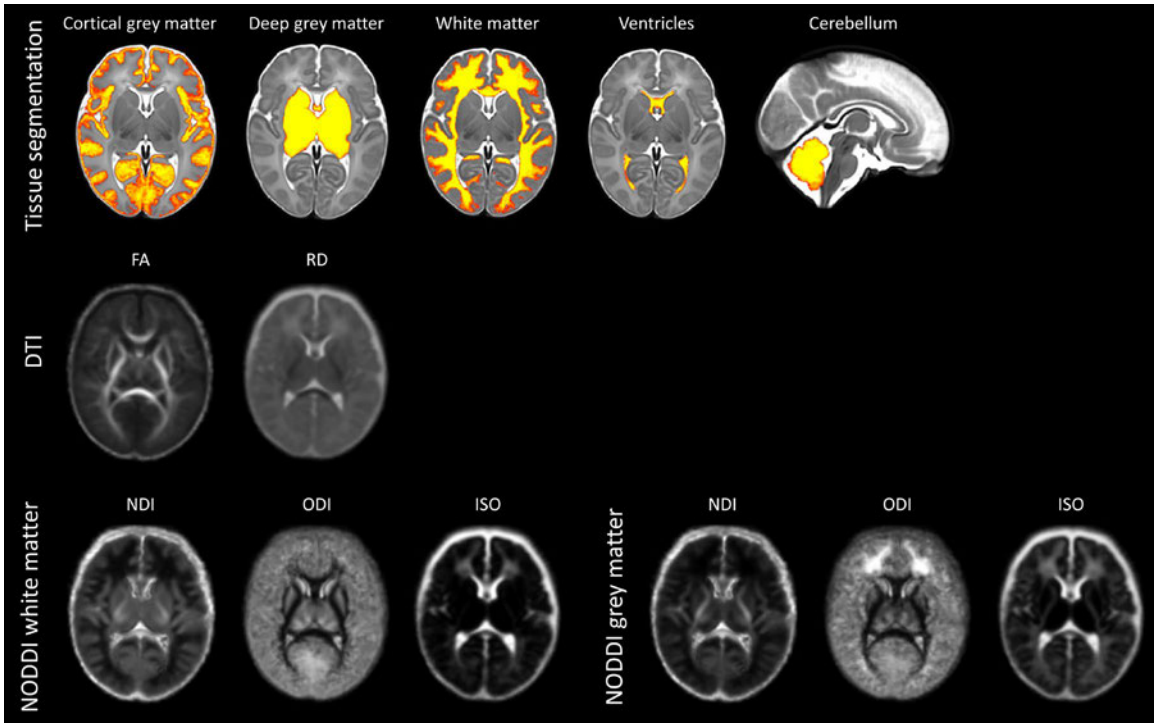


Figure 6-1. Representative brain maps.
Top panel: segmentation of the brain tissues of interest, overlaid on the Developing Human Connectome Project 40-week T2w template; middle panel: diffusion tensor imaging maps; bottom panel: neurite orientation dispersion and density imaging maps using the parallel diffusivity values for neonatal white matter (left) and grey matter (right). Maps are averaged over 20 random participants in this study.

6.2.1.2 Microstructural features

Representative DTI and NODDI maps of the study participants are provided in Figure 6-1 (middle and bottom panel).

6.2.1.2.1 Grey matter microstructure

For cortical and deep grey matter and the cerebellum, the mean DTI (fractional anisotropy (FA), radial diffusivity (RD)) and NODDI (neurite density index (NDI), orientation dispersion index (ODI), isotropic volume fraction (ISO)) metrics were calculated. The NODDI maps in these tissues were calculated using the recommended value for the parallel intrinsic diffusivity of neonatal grey matter (1.25 $\mu m^2/m$) (Guerrero et al., 2019).

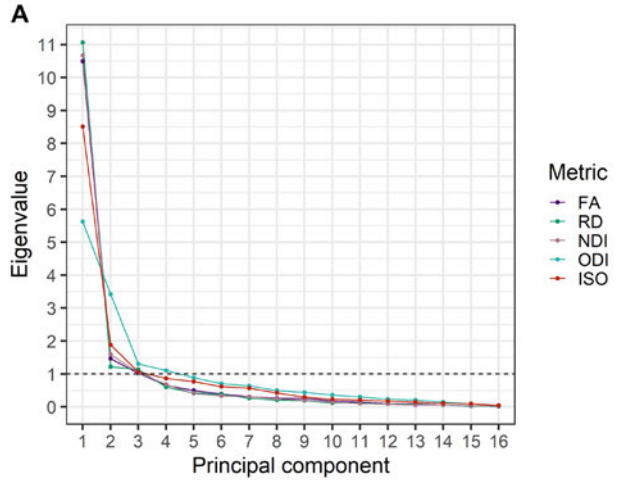
For the cortex, mean gyrification index, thickness, sulcal depth, curvature and surface area as measures of cortical complexity were also calculated (Makropoulos et al., 2018).

6.2.1.2.2 White matter microstructure

To capture global white matter dysmaturation, I segmented the major white matter tracts and derived general factors (g-factors) for each of the DTI (FA, RD) and NODDI (NDI, ODI, ISO) metrics for the current study group as described in detail in Chapter 3 and (Vaher et al.,

2022b). The only difference with Chapter 3 was in the image registration to ENA50 template (Blesa et al., 2020, 2016) space which previously was conducted by registering the T_2w processed image to ENA50 T_2w structural template and then combining it with the transformation obtained from the diffusion-to-structural registration. Here instead, for tract segmentation in subject native space, subject FA maps were registered to the ENA50 FA template using rigid, affine and symmetric normalization (SyN) implemented in Advanced Normalization Tools (ANTs) (Avants et al., 2008). Then, the tract-density-image-weighted averages for FA, RD, NDI, ODI and ISO were calculated for each of the 16 white matter tracts as described in Chapter 3. For white matter tracts, the NODDI metrics were calculated using the recommended values of the parallel intrinsic diffusivity for neonatal white matter (1.45 $\mu m^2/ms$) (Guerrero et al., 2019; Zhang et al., 2012) using the original NODDI MATLAB toolbox (http://mig.cs.ucl.ac.uk/index.php?n=Tutorial.NODDImatlab). Thereafter, separate principal component analyses (PCA) were conducted for each of the metrics across the 16 white matter tracts and the first PC score was extracted as the single-metric g-factor. In Chapter 3, g-factors were derived for a sample of both preterm and term neonates whilst here the sample consisted exclusively of preterm infants. Therefore, the tract loadings, eigenvalues and variance explained by the g-factors of the current preterm sample are provided in Figure 6-2. Similarly to what was previously reported (Chapter 3; Vaher et al., 2022), the g-factors captured substantial variance in diffusion MRI metrics across the tracts.

A



B

Tract	FA	RD	NDI	ODI	ISO
AF left	0.873	0.813	0.882	0.470	0.770
AF right	0.740	0.744	0.843	0.617	0.687
ATR left	0.770	0.827	0.841	0.238	0.772
ATR right	0.790	0.822	0.846	0.119	0.731
CC genu	0.714	0.807	0.699	0.324	0.830
CC splenium	0.474	0.664	0.455	0.622	0.657
CCG left	0.785	0.813	0.799	0.566	0.620
CCG right	0.663	0.779	0.759	0.389	0.590
CST left	0.854	0.884	0.893	0.361	0.744
CST right	0.854	0.890	0.866	0.429	0.691
IFOF left	0.914	0.926	0.926	0.841	0.855
IFOF right	0.922	0.884	0.856	0.856	0.689
ILF left	0.850	0.843	0.795	0.787	0.739
ILF right	0.877	0.843	0.762	0.790	0.670
UNC left	0.865	0.853	0.872	0.700	0.793
UNC right	0.892	0.876	0.860	0.705	0.774
Variance explained (%)	**65.589**	**69.149**	**66.699**	**35.134**	**53.172**

Figure 6-2. Single metric general factors in the current study group.
(A) Scree plot for the principal component analysis, showing the eigenvalue against the number of components for each white matter tract diffusion MRI metric. (B) Tract loadings (correlation between the manifest variable and extracted component score) and explained variance for the first unrotated principal component (g-factor) for the diffusion MRI metrics. FA = fractional anisotropy, RD = radial diffusivity, NDI = neurite density index, ODI = orientation dispersion index, ISO = isotropic volume fraction.

6.2.1.2.3 Justification of the choice of diffusion MRI metrics

FA is the most commonly used measure to describe microstructural complexity and anisotropy. I chose to omit mean and axial diffusivity measures from the current analyses due to the very high correlations between mean diffusivity and RD, and axial diffusivity and ISO as demonstrated in previous work (Chapter 3; Vaher et al., 2022). NDI and ODI were included in the current work to capture more specific aspects of the microstructural organisation. Although NDI has very high correlations with FA in the neonatal white matter, it may reveal additional properties in the grey matter given the differing correlations of FA and NDI with age at scan and in association with prematurity in the cortex (Batalle et al., 2019; Bouyssi-Kobar et al., 2018; Dimitrova et al., 2021b).

6.2.2 Microbiota features of interest

6.2.2.1 Alpha diversity

Shannon index and observed species were calculated as measures of microbiota alpha diversity as described in Chapter 2. Previous work has shown associations between microbiota diversity and brain regional volumes (Carlson et al., 2018) and functional connectivity (Gao et al., 2019; Kelsey et al., 2021) in paediatric samples.

6.2.2.2 Community composition

I sought to reduce the multidimensionality of microbiota composition data into a meaningful set of variables capturing the variation in the microbiota compositional data. First, I calculated Bray-Curtis dissimilarity matrix based on the total-sum-scaled (TSS) amplicon sequence variant (ASV) table including samples and ASVs in those infants that had both microbiota and structural/diffusion MRI as described in Chapter 2. Then, principal coordinates analysis (PCoA) was performed on the dissimilarity matrix using the function *pcoa* within the *ape* package in R (Paradis and Schliep, 2019). Cailliez transformation was applied to correct for negative eigenvalues (Cailliez, 1983). The scree plot of the eigenvalues alongside with proportion of variance explained were inspected to determine the optimum number of coordinates to extract. To determine the bacterial drivers of the main axes of variance (i.e. the extracted principal coordinates (PCo-s)), Spearman correlation coefficients were calculated between the PCo scores and the relative abundances of the ASVs. Similar approaches for microbiota community data reduction have been applied previously and these have demonstrated associations with volumes of fear- and emotion-related brain

structures (Carlson et al., 2021) as well as neurocognitive development (Rothenberg et al., 2021; Sordillo et al., 2019).

6.2.2.3 Individual bacterial taxa

In order to identify associations between brain imaging features and additional microbes that were not the main biomarkers driving the microbiota community composition data (i.e. less abundant taxa), individual taxa-level analyses were conducted using Microbiome Multivariable Associations with Linear Models (MaAsLin2; Mallick et al., 2021; see below section 6.2.3.6).

6.2.3 Statistical analyses

All statistical analyses were performed in R (version 4.2.1) (R Core Team, 2022).

6.2.3.1 Study group characteristics

The study group baseline demographic and clinical characteristic tables were constructed using the package *tableone* in R (Yoshida et al., 2020). Normal distribution of continuous variables was assessed using Shapiro-Wilk's test and visual inspection of histogram and quantile-quantile plots using the package *MVN* in R (Korkmaz et al., 2014). Values are presented as mean and standard deviation (SD) or median and range for normally or non-normally distributed continuous features, respectively, and counts with percentages of total study group for categorical variables.

6.2.3.2 Association of brain imaging features with GA at scan and the degree of prematurity

For contextualisation of the global volumetric and microstructural neuroimaging features during the neonatal period in the current study group, I performed separate linear regression models for each neuroimaging feature as the outcome and GA at birth and at scan as the predictors. All values were scaled (z-transformed) before fitting the models, thus, the regression coefficients are in the units of standard deviations. Here, p-values were not corrected for multiple comparisons.

6.2.3.3 Association of microbiota features with the degree of prematurity

Normal distribution of microbiota features (PCo-s and alpha diversity indices) was assessed using Shapiro-Wilk's test and visual inspection of histogram and quantile-quantile plots using the package *MVN* in R (Korkmaz et al., 2014). I calculated Spearman rank correlations between each of the microbiota features. I also compared the values between extremely

(GA < 28 completed weeks) and very (GA < 32 completed weeks) preterm infants using two-sample t-tests for normally distributed features or Wilcoxon rank-sum tests for non-normally distributed features. These analyses were performed for the contextualisation of the microbiota features.

6.2.3.4 Baseline models

First, the microbiota features were adjusted for GA at sample collection by fitting a linear model of each feature on GA at sample collection and retaining the residuals. I chose to adjust for sampling age in this manner rather than using it as a covariate in the model with the brain MRI feature as the outcome to avoid spurious correlations between GA at microbiota sampling and brain MRI features. Then, linear regression model was performed for each residualised microbiota feature as the predictor, each MRI feature as the outcome, and GA at birth and at scan added as covariates. All values were scaled (z-transformed) before fitting the models, thus, the regression coefficients are presented in the units of standard deviations. Regression diagnostic plots were visually inspected to ensure approximate conformation to assumptions. P-values were adjusted for the FDR across all models using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995) separately for volumetric and microstructural MRI features. Due to the exploratory nature of the study and given the correlated nature of the neuroimaging measures, results with adjusted p-values < 0.25 were considered as noteworthy and investigated further in the following analyses (sections 6.2.3.5 and 6.2.3.6). FDR correction of the p-values provides a balance between type I and type II errors (Storey and Tibshirani, 2003), though it needs to be acknowledged that this was an arbitrary threshold for determining statistical significance. However, this is the default in MaAsLin2 (Mallick et al., 2021) and there is precedence of using this cut off in previous microbiota-brain/behaviour studies (Aatsinki et al., 2019b, 2019a; Kelsey et al., 2021).

To quantify the variance in each brain imaging metric accounted for by the microbiota PCo-s or diversity indices, the incremental R^2 was calculated as the difference between the multiple R^2 of each model with that from the null model including only the covariates (GA at birth and at scan) as predictors. I used analysis of variance (ANOVA) to test whether the baseline model with the microbiota feature as the predictor fit the data significantly better compared to the null model.

6.2.3.5 Covariate identification and adjustment

The clinical variables that were significantly (FDR-adjusted p-value < 0.1) associated with microbiota alpha- or beta-diversity in the pre-discharge samples in Chapter 5 (sex, birthweight z-score, antibiotics >72h of life, and necrotising enterocolitis (NEC)) were tested for associations with brain MRI features. Normal distribution of brain MRI features was assessed using Shapiro-Wilk's test and visual inspection of histogram and quantile-quantile plots using the package *MVN* in R (Korkmaz et al., 2014). Two-sample t-tests were used to compare the means of normally distributed continuous variables between the groups of the categorical covariates; Wilcoxon rank-sum tests were applied to compare differences in non-normally distributed MRI features; Spearman correlation analysis was used to test for significant associations between birthweight z-score and MRI features. Variables that were nominally significantly ($p < 0.05$) associated with at least one of the brain MRI features, were added as a covariates in the fully adjusted model. These variables were: birthweight z-score, sex and NEC. Only three infants in the study were diagnosed with NEC, thus, instead of adding this variable as a covariate, a sensitivity analysis was performed excluding infants with NEC.

6.2.3.6 Baseline taxa-level analyses

Taxa-level analyses were conducted using MaAsLin2 for those brain MRI features that were significantly (adjusted p-value < 0.25) associated with any of the microbiota feature in the baseline models. To achieve alignment of the MaAsLin2 models with the initial baseline regression model, I first adjusted the brain MRI features for GA at birth and at scan by fitting a linear model of each feature on GA at birth and at MRI, and retaining the residuals. These residuals were then used independently as the predictors in the MaAsLin2 models with the TSS-normalised ASV table as the outcome. Due to the relatively small sample size in comparison with the number of bacterial taxa, in all analyses, I tested for effects on ASVs that were present with at least 1% of abundance (min_abundance = 0.01) in at least 10% of samples (min_prevalence = 0.1); the normalisation method within MaAsLin2 was set to "none"; all other arguments were used as by default. As by default in the method, I considered Benjamini-Hochberg method-adjusted p-values < 0.25 as statistically significant.

6.2.3.7 Deep grey matter regional analysis

Main analyses revealed significant associations between microbiota features (PCo2 and observed species) and deep grey matter microstructure (FA, NDI, ODI). To aid interpretation of these results, baseline models were repeated for these microbiota and microstructure features in the specific regions (thalamus, caudate, subthalamic and lentiform nuclei; Figure 6-3).

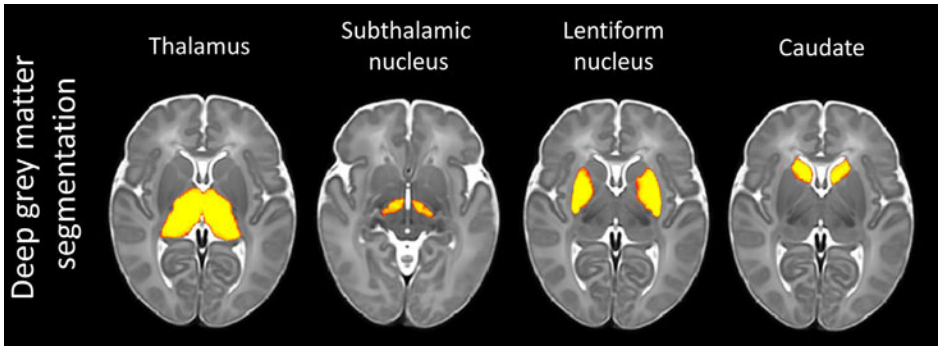


Figure 6-3. Segmentation of deep grey matter structures.
Overlaid on the Developing Human Connectome Project 40-week T2w template.

6.3 Results

6.3.1 Participant characteristics

Of the 103 preterm infants with faecal microbiota data obtained prior to discharge from the neonatal unit, 79 had matching multimodal MRI data. The clinical and demographic characteristics of the study group are provided in Table 6-1**.** In this sample of preterm infants, 24 (30.4%) had bronchopulmonary dysplasia, 3 (3.8%) developed NEC, 5 (6.3%) developed retinopathy of prematurity, and 19 (24.1%) had one or more episodes of postnatal sepsis. On average, the MRI scans were acquired ~4 weeks after the faecal sample collection.

Table 6-1. Participant characteristics.

Variable*	level	Preterm infants, n=79
GA at birth, weeks (median [range])		29.86 [22.14, 32.86]
Sex (%)	Male	43 (54.4)
	Female	36 (45.6)
Birthweight, g (mean (SD))		1311 (448)
Birthweight z-score (median [range])		0.153 [-2.520, 2.141]
GA at MRI, weeks (mean (SD))		40.56 (1.76)
Postnatal age at faecal sample collection, days (median [range])		45 [9, 151]
GA at faecal sample collection, weeks (median [range])		35.86 [32.43, 46.14]

Weeks between faecal sample collection and MRI (mean (SD))		3.81 (2.65)
Mode of delivery (%)	C-section	58 (73.4)
	Vaginal delivery	21 (26.6)
Antenatal steroids given (%)	No	5 (6.3)
	Yes	74 (93.7)
Magnesium sulphate given (%)	No	25 (31.6)
	Yes	54 (68.4)
Labour antibiotics (%)	No	31 (39.2)
	Yes	48 (60.8)
Membrane rupture duration, hours (median [range])		0 [0, 934]
Days in NNU (median [range])		60 [17, 167]
Bronchopulmonary dysplasia (%)	No	55 (69.6)
	Yes	24 (30.4)
Necrotising enterocolitis (%)	No	76 (96.2)
	Yes	3 (3.8)
Sepsis (%)	No	60 (75.9)
	Yes	19 (24.1)
Retinopathy of prematurity (%)	No	73 (92.4)
	Yes	5 (6.3)
	NA	1 (1.3)
Antibiotics <72h of life (%)	No	17 (21.5)
	Yes	62 (78.5)
Antibiotics >72h of life (%)	No	41 (51.9)
	Yes	38 (48.1)
Total days of antibiotics during NNU stay (median [range])		3 [0, 47]
% of days receiving antibiotics during NNU stay (median [range])		9.1 [0, 70.8]
GA at discharge (median [range])		37.43 [34.43, 48.43]
% of days receiving exclusive breast milk during NNU stay (median [range])		66.3 [0, 100]
% of days receiving exclusive formula during NNU stay (median [range])		0 [0, 89.5]
% of days receiving mixed feeds during NNU stay (median [range])		9.2 [0, 96.2]
Feeding at discharge (%)	Breastfeeding	32 (40.5)
	Formula feeding	18 (22.8)
	Mixed feeding	29 (36.7)
Maternal age, years (mean (SD))		32.89 (5.43)
Maternal BMI (median [range])		26.5 [16.4, 46.6]
SIMD rank (%)	1	17 (21.5)
	2	11 (13.9)
	3	13 (16.5)
	4	10 (12.7)
	5	28 (35.4)

**Categorical variables are shown in absolute numbers with percentages (%); continuous, normally distributed variables as means with standard deviations (SD); continuous, non-normally distributed variables as medians with ranges.*

The final sample sizes for linking microbiota features with brain MRI are the following: volumetric and cortical structural complexity analysis n = 76, white matter microstructure analysis n = 74, and cortical and deep grey matter and cerebellar microstructural diffusion analysis n = 74.

6.3.2 Global volumetric and microstructural neuroimaging features in association with GA at MRI and the degree of prematurity

For contextualisation of the neuroimaging features during the neonatal period, I tested the association of each of the MRI feature with GA at MRI and at birth (Supplementary Table 6-1).

Expectedly, GA at MRI had significant associations with most of the global MRI features. Absolute tissue volumes positively correlated with GA at MRI; the relative volumes of cortical grey matter and cerebellum also followed this trend while white matter and deep grey matter relative volumes negatively associated with age at scan. Cortical microstructural indices including thickness, sulcal depth, gyrification index and surface area increased while measures of diffusivity, RD and ISO, decreased with advancing age at scan. Deep grey matter FA and NDI increased whilst RD, ODI and ISO decreased with increasing age at scan. The directions of effect were the same with global white matter microstructure. Similar trends can also be seen in the cerebellum, although ODI effect is in the opposite direction.

GA at birth also significantly associated with several global neuroimaging features, albeit with significantly smaller effect sizes compared to GA at MRI. Increasing GA at birth was positively correlated with the absolute volumes of total brain tissue, white matter and cortical grey matter, and negatively with ventricular volume. Interestingly, however, total deep grey matter volume was not significantly associated with the degree of prematurity. The relative volume of white matter positively and the relative volume of cerebellum negatively correlated with GA at birth. Associations between cortical microstructure and GA at birth largely followed similar trends as with GA at scan, showing positive correlations with sulcal depth, gyrification index and surface area; although the relationships with diffusion MRI measures varied showing positive correlation with cortical ODI and a weak evidence for a negative correlation with cortical NDI. In the deep grey matter, ODI was positively and ISO and RD negatively correlated with GA at birth. In the cerebellum, both FA and NDI negatively correlated with GA at birth. White matter diffusion MRI features were not

significantly associated with GA at birth, suggesting that the association observed in the sample of both term and preterm infants (Chapter 3; Vaher et al., 2022) was driven by the differences between term and preterm groups, which cannot be explained solely by GA at birth.

6.3.3 Main axes of variance in the microbiota data

PCoA of the Bray-Curtis dissimilarity matrix revealed a 4-coordinate solution based on a scree plot (Figure 6-4A) which together explained 40.9% of variance in the microbiota community composition data. To understand the bacterial taxa driving the main axes of variance, I calculated Spearman correlation coefficients between the relative abundance of each ASV and each principal coordinate (Figure 6-4B). This revealed that PCo1 had strong negative correlations with *Bifidobacterium* and *Cutibacterium*; PCo2 had strong negative correlation with *Escherichia/Shigella* and positive with an unidentified ASV in *Enterobacteriaceae* family*;* PCo3 had a strong negative correlation with *Klebsiella*; and PCo4 had a strong negative correlation with *Enterococcus*. These correlations remained after residualisation against GA at sample collection.

For contextualisation of the microbiota features, I investigated the associations of the features with one another (Figure 6-5A) and in association with the degree of prematurity (Figure 6-5B). The PCo-s were orthogonal and showed very weak correlations with one another with Spearman rank correlation, suggesting that each of them captures an independent aspect of the variance in beta diversity. The two alpha diversity indices were moderately correlated with one another. Observed species additionally had a moderate correlation with PCo1. After adjustment for GA at sample collection, PCo3 was significantly lower in extremely compared to very preterm infants (Figure 6-5B; W = 987, p-value = 0.010), thus paralleling the results in Chapter 5 where I found increased abundance of *Klebsiella* in the extremely preterm infant samples.

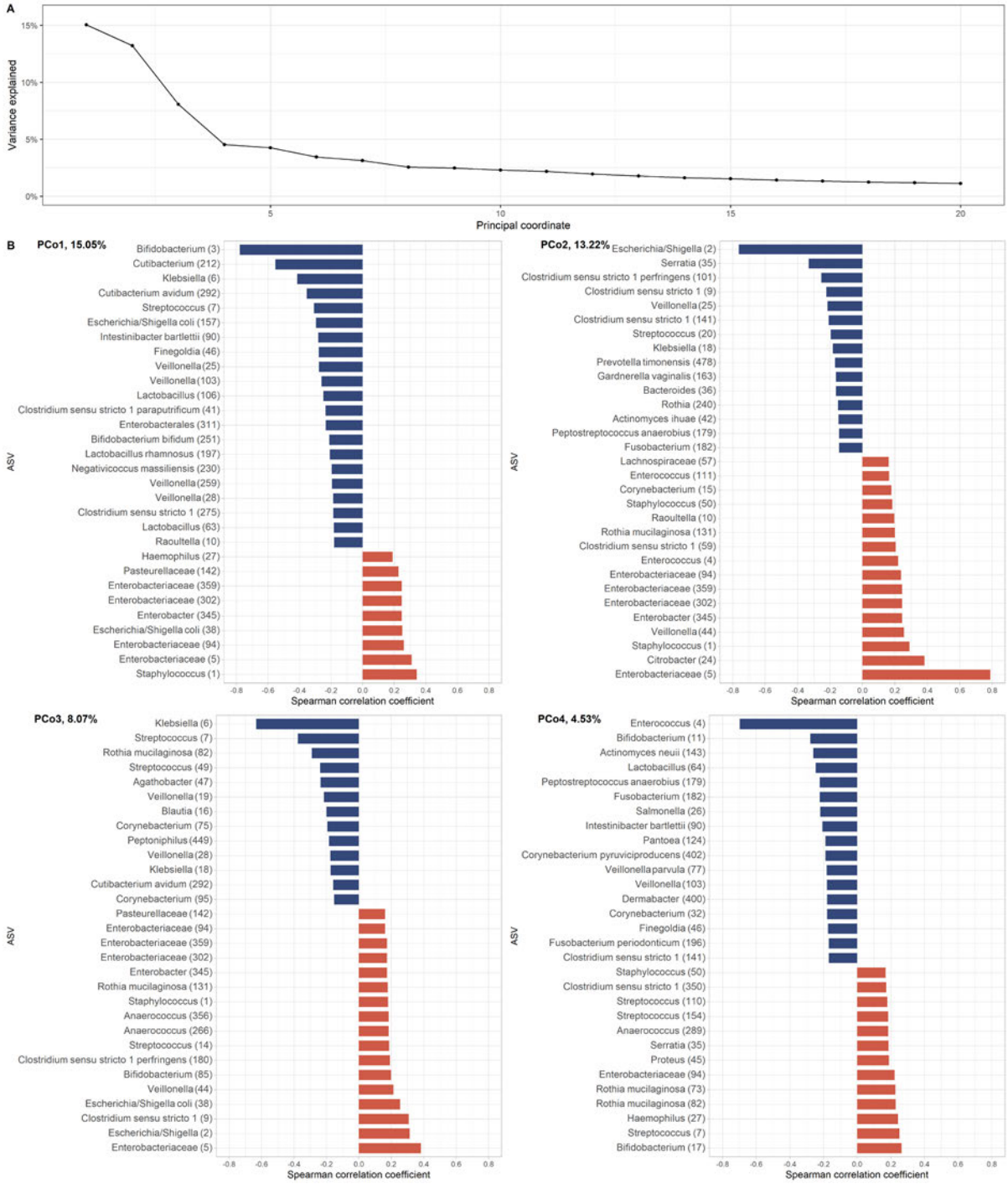


Figure 6-4. Dimensionality reduction of the microbiota community composition data.
(A) Scree plot of the variance explained by the first 20 principal coordinates calculated on the Bray-Curtis dissimilarity matrix. (B) Bacterial taxa correlations with the first four orthogonal principal coordinates (PCo), showing the top 30 strongest correlations for each PCo. The % refers to the variance explained by each of the PCos.

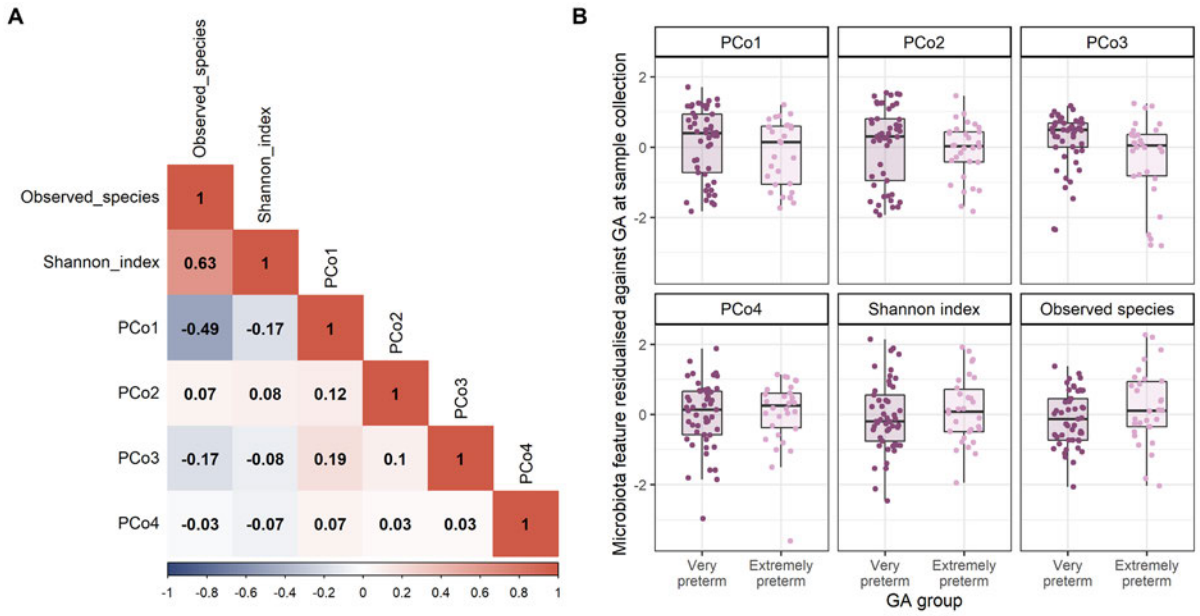


Figure 6-5. Microbiota features.
(A) Spearman rank correlations between the different microbiota PCo-s and alpha diversity indices. (B) Microbiota community and diversity features in association with the degree of prematurity; the only statistically significant difference is for PCo3 where extremely preterm infants have lower values (analysed using Wilcoxon rank-sum test, see text). GA = gestational age, PCo = principal coordinate.

6.3.4 Gut microbiota associations with brain volumes

Brain volumetric measures were associated with the PCo1 of the microbial community composition, which negatively correlated with total brain tissue and white matter volume, and positively with relative deep grey matter volume (Figure 6-6). The incremental R^2 upon adding the PCo1 to a null model was 2.6% for total brain tissue, 5.4% for white matter, and 8.3% for deep grey matter relative volume (Supplementary Table 6-2). There was also a nominally significant association between relative cortical volume and PCo2, which captured an additional 2.6% of variance in the relative cortical volume measure (Supplementary Table 6-2). However, none of these associations remained significant after adjustment for multiple comparisons.

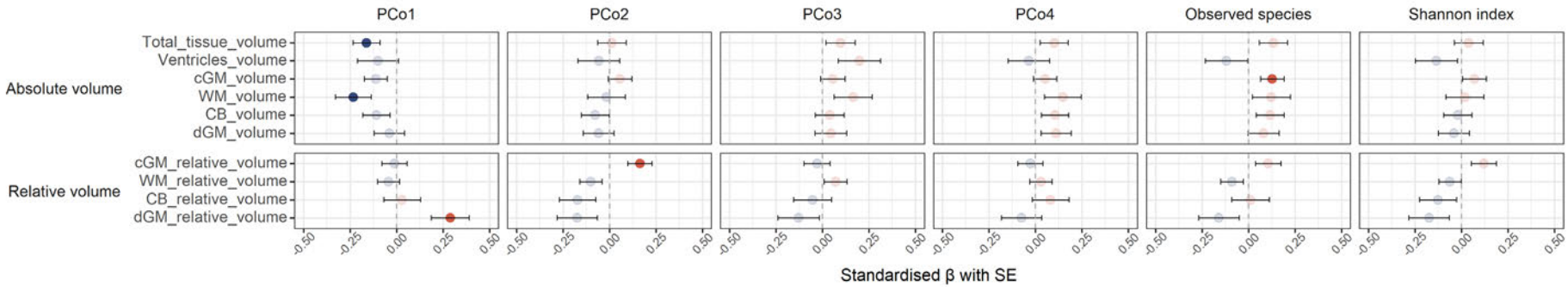


Figure 6-6. Microbiota associations with brain volumetric measures.
Models are adjusted for gestational age at birth and at scan. Full colour points indicate nominal p-value < 0.05. cGM = cortical grey matter; WM = white matter; dGM = deep grey matter, CB = cerebellum.

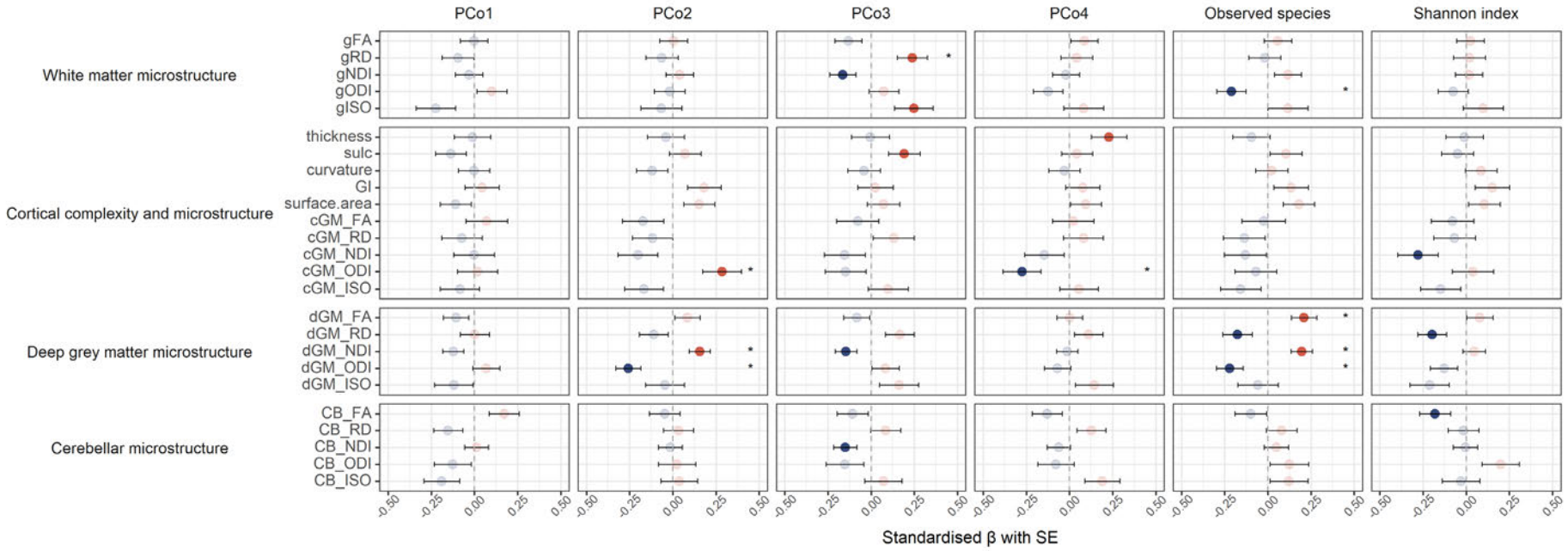


Figure 6-7. Microbiota associations with brain microstructural measures.

Models are adjusted for gestational age at birth and at scan. Full colour points indicate nominal p-value < 0.05; asterisks (*) indicate FDR-adjusted p-value < 0.25. FA = fractional anisotropy; RD = radial diffusivity; NDI = neurite density index; ODI = orientation dispersion index; ISO = isotropic volume fraction; cGM = cortical grey matter; dGM = deep grey matter, CB = cerebellum; sulc = sulcal depth; GI = gyrification index, g = general factor.

6.3.5 Gut microbiota associations with brain microstructure

Brain microstructural features also showed associations with microbiota community composition (Figure 6-7, Supplementary Table 6-3). PCo2 mainly associated with deep grey matter microstructure (incremental R2 was 6.4% for ODI and 2.3% for NDI). PCo3 associated with measures of global white matter microstructure (gRD, gNDI and gISO; incremental R^2 was 5.6%, 2.7% and 6.0% respectively). PCo4 mostly associated with cortical grey matter complexity (thickness; incremental R^2 5.2%) and microstructure (ODI; incremental R^2 7.5%).

With regard to bacterial alpha diversity, microbiota richness (observed species) associated with deep grey matter microstructure, showing positive associations with FA and NDI (incremental R^2 3.9% and 3.4%, respectively), and negative associations with ODI and RD (incremental R^2 4.3% and 2.7%, respectively). The correlations with Shannon index overall followed similar trends, but were weaker. Observed species further negatively associated with ODI in the white matter.

The relationships between PCo2 and deep grey matter NDI/ODI, PCo3 and gRD, PCo4 and cortical grey matter ODI, and observed species and deep grey matter FA, NDI and ODI as well as gODI survived the threshold of multiple comparison testing and were investigated further in sensitivity analyses. The associations, except for the correlation between observed species and gODI, remained statistically significant when adjusting for birthweight z-score and sex (Figure 6-8A, Supplementary Table 6-4A) and when excluding infants with NEC (Figure 6-8B, Supplementary Table 6-4B).

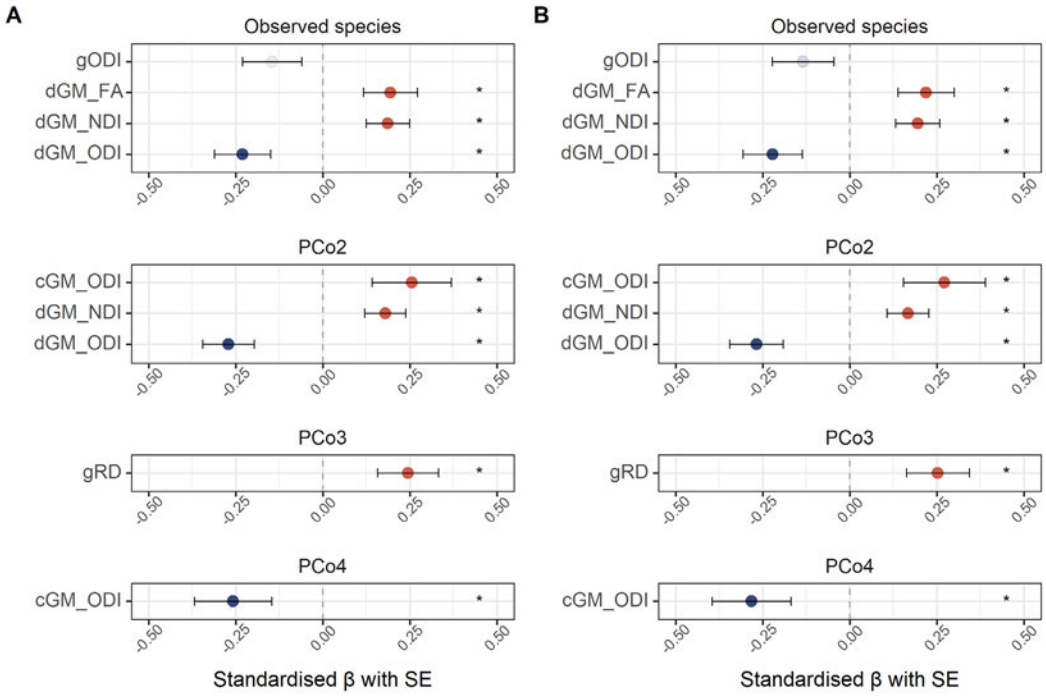


Figure 6-8. Microbiota associations with brain MRI features in sensitivity analyses.
(A) Model with birthweight z-score and sex as additional covariates to GA at birth and at scan; (B) Model excluding infants with NEC (n = 3), adjusted for GA at scan and at birth, birthweight z-score and sex. Full colour points indicate nominal p-value < 0.05; asterisks (*) indicate FDR-adjusted p-value < 0.05. FA = fractional anisotropy; NDI = neurite density index; ODI = orientation dispersion index; cGM = cortical grey matter; dGM = deep grey matter, g = general factor.

6.3.6 Multivariate analyses of gut microbiota taxa and brain structure

MaAsLin2 results investigating associations between individual bacterial taxa and brain microstructural features were partially in line with the results obtained using the microbial community PCo-s, but also revealed novel relationships not captured with the PCo-s (Figure 6-9). *Bifidobacterium*, the strongest negative driver of the PCo1, was not significantly associated with any of the brain microstructural measures tested. This was not surprising as volumetric features, which showed nominally significant correlations with the mainly *Bifidobacterium*-driven PCo1, were not included here. *Escherichia/Shigella*, the strongest negative driver of PCo2, showed significant associations with deep grey matter microstructure: negative associations with FA (p = 0.061, q = 0.204) and NDI (p = 0.0007, q = 0.004), and positive associations with ODI (p = 0.014, q = 0.061). These results correspond with the PCo analyses as PCo2 had the strongest negative association with deep grey matter ODI, and a smaller positive association with deep grey matter NDI. *Enterobacteriaceae*, the strongest positive driver of PCo2, negatively associated with deep grey matter ODI (p = 0.004, q = 0.025), thus corresponding to the PCo analyses as PCo2 negatively correlated with

deep grey matter ODI. *Enterococcus*, the strongest negative driver of PCo4, was not associated with any of the features tested, although PCo analyses suggested a potentially positive association between *Enterococcus* and deep grey matter ODI. *Klebsiella*, the strongest negative driver of PCo3, was interestingly positively associated with deep grey matter NDI (p = 0.021, q = 0.089) and negatively with deep grey matter ODI (p = 0.056, q = 0.162), although PCo analyses indicated a stronger association between PCo3 and gRD. *Klebsiella* was ranked at the top of the list of associations with gRD (p = 0.063, q = 0.328), however, none of the taxa-level associations reached statistical significance.

These analyses also revealed novel relationships between brain MRI features and bacterial taxa beyond the main drivers of the community composition variance. In particular, the data showed positive correlations between deep grey matter FA/NDI and different *Veillonella* ASVs (q range 0.018 – 0.209), *Streptococcus* (q range 0.204 – 0.209), and *Finegoldia* (q range 0.204 – 0.209), and negative correlations between deep grey matter ODI and *Veillonella* ASVs (q range 0.083 – 0.115). Additionally, there were positive associations between *Staphylococcus* and ODI both in the cortex (p = 0.034, q = 0.149) and white matter (p = 0.032, q = 0.138).

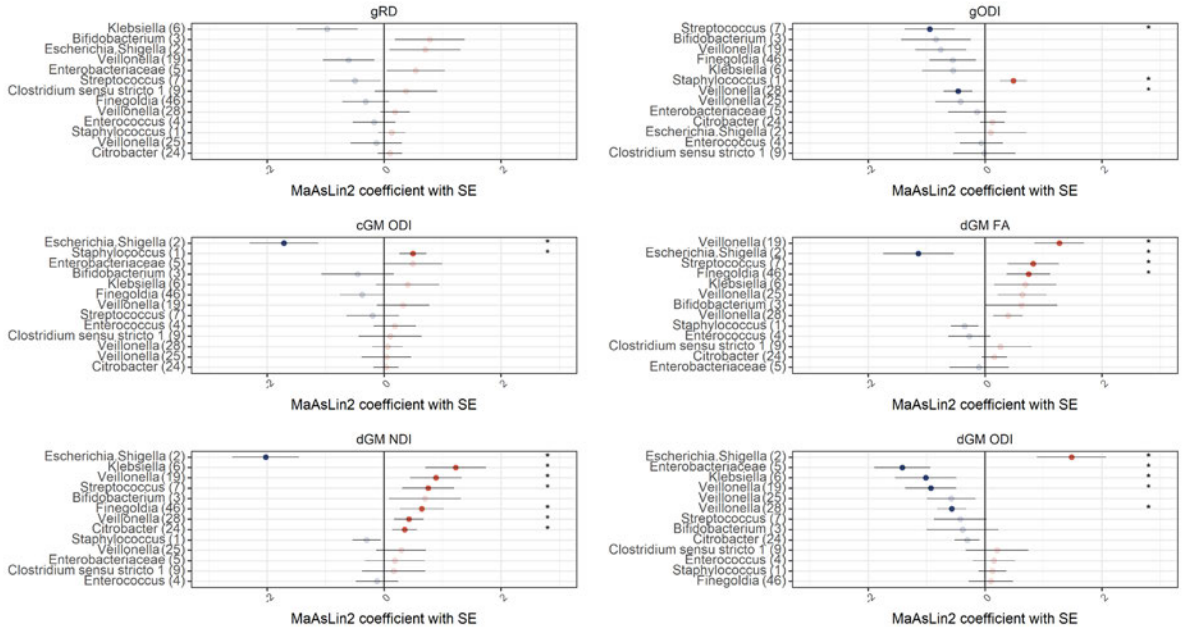


Figure 6-9. Taxa-level analyses correlating brain microstructural features with the relative abundances of ASVs.

Analyses were conducted using MaAsLin2, testing differences in ASVs present with at least 1% of abundance in at least 10% of samples. Full colour points and asterisks (*) indicate FDR-adjusted p-value < 0.25. FA = fractional anisotropy; NDI = neurite density index; ODI = orientation dispersion index; cGM = cortical grey matter; dGM = deep grey matter, g = general factor.

6.3.7 Regional analysis in the deep grey matter structures

The deep grey matter consists of distinct brain structures (thalamus, caudate, lentiform and subthalamic nuclei), but the MR signal can be affected by partial volume effects from adjacent structures including cerebrospinal fluid and white matter. Therefore, to further understand which specific structures are driving the association between the gut microbiota features and deep grey matter microstructure, I conducted region-wise analysis, focussing on the main features of interest showing significant associations with deep grey matter microstructure (PCo2 and observed species) and adjusting for GA at birth and at MRI.

These regional analyses (Figure 6-10, Supplementary Table 6-5) revealed that the positive association of PCo2 with deep grey matter NDI and negative association with deep grey matter ODI were mainly driven by the values in the thalamus and lentiform nucleus. The positive association between observed species and deep grey matter NDI was driven by the values in most regions with the exception of caudate; the positive association between observed species and deep grey matter FA was driven by the values in the subthalamic nucleus; and the negative association between observed species and deep grey matter ODI was driven by the values in the lentiform nucleus.

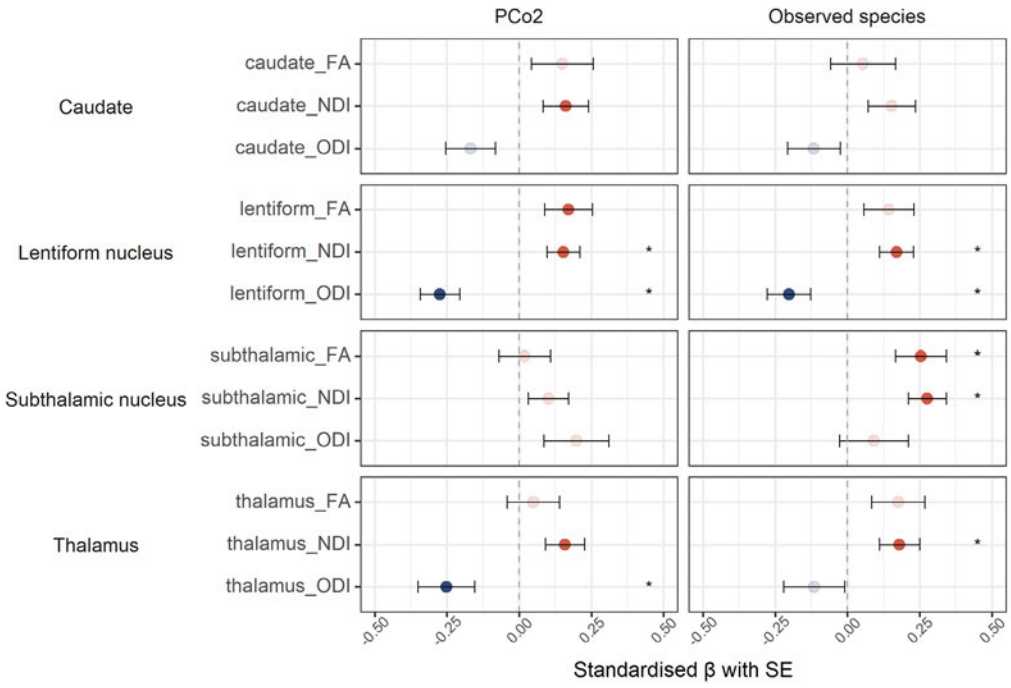


Figure 6-10. Microbiota associations with deep grey matter microstructural measures.
Models are adjusted for gestational age at birth and at MRI. Full colour points indicate nominal p-value < 0.05; asterisks (*) indicate FDR-adjusted p-value < 0.05. FA = fractional anisotropy; NDI = neurite density index; ODI = orientation dispersion index.

6.4 Discussion

This study integrated data from multimodal MRI and 16S rRNA gene sequencing in a sample of 79 preterm infants to characterise the associations of the gut microbiota community composition and diversity with brain structure. The data showed that the preterm gut microbiota composition and diversity particularly associate with neuroimaging markers of microstructure in the white and deep grey matter. As the microbiota is modifiable, these data motivate further research into the potential of modulating gut microbiota to promote brain health following preterm birth.

6.4.1 Dimensionality reduction of microbiota community composition data

I used principal coordinates analyses and observed that a considerable proportion of variance in microbiota community composition prior to hospital discharge in preterm infants could be explained by the first four latent variables. These captured mostly the relative abundance of the most abundant taxa: *Bifidobacterium*, *Escherichia/Shigella*, *Enterobacteriaceae, Klebsiella,* and *Enterococcus.* These taxa were also observed as the drivers of microbiota clusters in Chapter 5 of this thesis. The principal coordinates were minimally affected by the degree of prematurity at birth as only PCo3 values (negatively driven by the abundance of *Klebsiella*) were slightly lower in extremely compared to very preterm infants. In addition, the data in Chapter 5 showed that these top abundant taxa were minimally affected by the clinical variables tested. The exceptions were the abundance of *Klebsiella*, which was significantly more abundant in extremely compared to very preterm neonates and in infants exposed to antibiotics, and *Bifidobacterium* that was slightly more abundant in male infants, although the latter effect did not replicate in sensitivity analyses. These result might suggest that the core microbiota is personalised and less affected by clinical covariates as has been reported before (Wandro et al., 2018).

Similar approaches in microbiota data reduction have been used before in studies of microbiota-gut-brain axis in early childhood (Carlson et al., 2021; Rothenberg et al., 2021; Sordillo et al., 2019). In all these studies, the resulting principal axes of variance in microbiota data differ, suggesting population- or study-dependent effects on microbiota data reduction as might be expected.

6.4.2 Complex relationships between neonatal microbiota and brain structural development

The derived microbiota beta diversity PCo-s and microbiota richness significantly associated with several features of brain development, primarily with measures of tissue microstructure in white and deep grey matter. Concurrent diffuse white matter injury and dysmaturation of deep grey matter structures is a common preterm neonatal image phenotype which negatively associates with subsequent neurodevelopmental outcomes (Boardman et al., 2010). The variance explained by the different microbiota features over and beyond the effects of GA at scan and at birth was modest, ranging between 2.3-8.3%, though it remains to be investigated in future studies to what extent these effect sizes can be considered biologically/functionally meaningful. I further investigated the microbiota associations with brain structure at individual taxa-level, which provided additional support for some of the PCo correlations, but also revealed novel relationships. Interestingly, the data showed that different features of the microbiota composition associated with different neuroimaging phenotypes, suggesting specific bacteria-brain structure relationships.

Reduced brain volume is a commonly found feature of EoP. To my knowledge, only one study has investigated the associations between gut microbiota and global brain tissue volumes in early childhood, which suggested no significant relationships between microbiota composition and global tissue volumes (Carlson et al., 2018). However, possible associations between microbiota composition and the volumes of some specific brain regions have been shown (Carlson et al., 2021, 2018). Specifically, one study demonstrated that higher abundance of *Bifidobacterium* in infancy may be positively correlated with medial prefrontal cortex volume (Carlson et al., 2021). This is interesting in the context of the current study as the microbiota beta diversity PCo1 was nominally negatively associated with total brain tissue and white matter volume and positively with deep grey matter relative volume. Negative values on this PCo were mainly related to increased abundance of *Bifidobacterium*, thus, these results could potentially be reflecting increasing brain white matter size in association with increasing abundance of *Bifidobacterium*. The interpretation of the relative regional brain tissue volumes is more complex as these are calculated as the proportion of total brain tissue. In the current study, I found that the relative volume of deep grey matter negatively associates with GA at MRI; that is, the proportion of deep grey

matter of the entire brain tissue gets smaller with advancing age, probably due to increasing proportion of the cortical GM. Therefore, the positive association between PCo1 and deep grey matter relative volume inversely follows the trend with GA at scan and further suggests that increasing abundance of *Bifidobacterium* may positively correlate with improved brain growth in the neonatal period. However, replication in an independent cohort will be needed to validate these findings.

Bifidobacterium is the predominant bacterium in vaginally delivered breastfed infants during the first year of life and it is often considered a positive influence on general health in infancy (Arboleya et al., 2016b; Sarkar and Mandal, 2016; Saturio et al., 2021). It is sometimes used as a probiotic to promote gut health although clinical efficacy is uncertain (Costeloe et al., 2016a; Sharif et al., 2020). Previous studies have also identified correlations between *Bifidobacterium* levels in infancy or childhood and neurodevelopment. For example, positive correlations have been shown with motor development (Acuña et al., 2021; Sordillo et al., 2019), language (Kort et al., 2021; W. Zhang et al., 2021), positive temperament traits such as regulation, soothability and surgency (Aatsinki et al., 2019b; Fox et al., 2021; Kelsey et al., 2021; Y. Wang et al., 2020), and improved neurocognitive outcomes in preterm infants (Beghetti et al., 2021). Animal studies have further suggested a causal role of *Bifidobacterium* in behaviour and brain function. For example, colonisation with *Bifidobacterium* rescued impaired recognition memory, anxiolytic behaviour, motor impairments and social deficits in germ-free mice (Luk et al., 2018), as well as depression-like behaviours and reduced noradrenaline levels in a rat maternal separation model of depression (Desbonnet et al., 2010). However, whilst *Bifidobacterium* strains are commonly used probiotics and shift the microbiota community composition (Beck et al., 2022), to date, the evidence for improving effects of probiotics on neurodevelopment in early childhood is poor (Jacobs et al., 2017; Slykerman et al., 2018; Upadhyay et al., 2018).

White matter is considered one of the predominant site of injury/dysmaturation in the preterm brain (Volpe, 2019). Increasingly, the dysmaturation of white matter is recognised as a whole-brain phenomenon (Blesa et al., 2020; Girault et al., 2019; Telford et al., 2017), motivating the use of global measures to capture alterations in white matter integrity in the developing brain. In this study, using g-factors as measures of global white matter microstructure, I observed that the microbiota beta diversity PCo3 was positively associated

with the g-factor of RD. RD, a measure derived from diffusion MRI calculated as the diffusion perpendicular of the main axis, is the main contributor related to increasing FA and decreasing MD in early development and is thought to relate to the rapid myelination during this period (Colby et al., 2013), although this interpretation is challenging in the context of pathology and crossing fibres (Wheeler-Kingshott and Cercignani, 2009; Winklewski et al., 2018). G-factor of RD negatively associates with GA at MRI and is higher in the preterm compared to term-born infants (see Chapter 3), suggesting decreasing water diffusivity with increasing age and maturity at birth.

Intriguingly, negative values on PCo3 mainly indicated greater relative abundance of *Klebsiella*, a known common pathogen that is hospital-acquired and often observed at high abundance in preterm infants (e.g. Beck et al., 2022; Rao et al., 2021; Stewart et al., 2017; also see Chapter 5 where I observed higher *Klebsiella* in extremely compared to very preterm infants) and in association with the development of NEC (Olm et al., 2019; Pammi et al., 2017). Paradoxically, then, these results suggest decreasing gRD with increasing relative abundance of *Klebsiella.* Although not statistically significant after multiple testing, PCo3 was also positively correlated with the g-factor of ISO and negatively with the g-factor of NDI. Thus, these results together provide evidence for the interpretation of increased water content and diffusivity and decreased neurite density in the preterm white matter in association with decreased abundance of *Klebsiella*. The negative association between the abundance of *Klebsiella* and gRD was not significant in individual taxa-level analyses, despite being the top-ranked ASV, suggesting that the relationship between gut microbiota and white matter microstructure is likely to be related to a more complex microbiota community than simply an increase in the abundance of a single pathogenic taxa. Indeed, whilst *Klebsiella* was the strongest driver of PCo3, other taxa also contribute to the variance related to this dimension of community composition.

Interestingly, a previous study suggested increased *Klebsiella* abundance around 1-2 months of postnatal life in a small sample of preterm infants with severe parenchymal brain injury (Seki et al., 2021). The results in the current chapter are therefore in contrast with these findings as I found increased *Klebsiella* abundance to associate with markers of improved white matter integrity. Previous studies in healthy term infants have shown increased *Klebsiella* abundance in infancy to negatively associate with fine motor scores (Sordillo et

al., 2019) and surgency (Fox et al., 2021), suggesting a negative *Klebsiella*-neurodevelopment relationship. The discrepancies with previous studies could, at least to some extent, be attributed to study population differences. For example, in contrast to the study by Seki and colleagues (2021), the sample here consisted of preterm infants with no major parenchymal brain injury and with no exposure to probiotics, and the studies by Sordillo et al. and Fox et al. investigated term-born infants. It could further be hypothesised that preterm infants with *Klebsiella*-dominated microbiota in the current study may experience accelerated white matter development in response to increased sensory stimulation in the neonatal period, potentially reflecting compensatory mechanisms.

Deep grey matter microstructure was another feature associated with microbiota composition and diversity, suggesting that microbiota associates with cellular and dendritic morphology as previously demonstrated in preclinical models of microbiota disruptions (e.g. Chu et al., 2019; Luczynski et al., 2016, 2017; Ogbonnaya et al., 2015). Reductions in deep grey matter volume, particularly in the thalamus and lentiform nucleus, are commonly observed in preterm infants (Ball et al., 2012; Boardman et al., 2006; Loh et al., 2017; Padilla et al., 2015; Srinivasan et al., 2007); reduced thalamic volume associates with reduced FA and increased MD and RD in the thalamus as well as in white matter (Ball et al., 2012; Boardman et al., 2006). Here, I observed that beta diversity PCo2, which was mainly driven by reduced abundances of *Escherichia/Shigella* and increased abundances of an unidentified taxa in the *Enterobacteriaceae* family, positively associated with NDI and negatively with ODI in the deep grey matter. These results were partially supported by the MaAsLin2 models which showed positive correlations between the abundance of *Escherichia/Shigella* and deep grey matter ODI, whilst deep grey matter FA and NDI negatively associated with *Escherichia/Shigella* abundance. The abundance of the unidentified *Enterobacteriaceae*, in contrast, was significantly associated only with reduced values of deep grey matter ODI, suggesting that the association of PCo2 and deep grey matter FA and NDI was mainly driven by the relative abundance of *Escherichia/Shigella.*

Regional analysis revealed that the associations between beta diversity PCo2 and deep grey matter microstructure were mainly driven by the diffusion MRI measures in the thalamus and lentiform nucleus. Previous work assessing microstructural features in the thalamus as a representative deep grey matter structure has demonstrated that both FA and NDI increase

during the preterm period from birth up to TEA; this has been interpreted to reflect increasing myelination and axonal volume (Eaton-Rosen et al., 2015; Melbourne et al., 2016). The results described here support these findings as I also observed positive correlations of deep grey matter FA and NDI with GA at MRI. In contrast, ODI has previously been shown to remain constant over this period in the thalamus (Eaton-Rosen et al., 2015), whilst here the data showed a negative association between deep grey matter ODI and GA at MRI. This discrepancy could potentially be attributed to the NODDI parameter settings as here I used the parallel diffusivity values optimised to neonatal grey matter, which strongly affect the derived ODI values (Guerrero et al., 2019). The exact cellular basis for the ODI values in the deep grey matter remain unknown; in the white matter, higher ODI (i.e. more dispersed diffusion) is interpreted as increased fibre crossing and fanning (Kunz et al., 2014), whilst in the cortical grey matter it is thought to reflect more elaborated dendrites and axons (Eaton-Rosen et al., 2015). Further complicating the interpretation of deep grey matter ODI is the small positive association with GA at birth in the current study. Interestingly, however, the beta diversity PCo2 correlations with deep grey matter NDI and ODI follow those observed with GA at MRI, suggesting that PCo2 and microbiota richness associate with microstructural markers that relate to more mature deep grey matter microstructure.

Escherichia/Shigella has been associated with brain/behaviour in previous studies of microbiota-gut-brain axis in infancy and childhood. For example, increased abundance of *Escherichia* has been related to improved motor skills (Sordillo et al., 2019), general neurodevelopment in preterm infants (Rozé et al., 2020) as well as decreased stress scores at the time of neonatal unit discharge (Sun et al., 2020). The abundance of this bacterium has also been positively associated with the functional connectivity of the homologous-interhemispheric network in infancy (Kelsey et al., 2021), and found in decreased abundance in preterm infants with severe brain injury in the first days of life (Seki et al., 2021). Age at scan positively correlated with NDI and negatively with ODI in the deep grey matter, suggesting that higher NDI and lower ODI are markers of improved deep grey matter microstructure. Thus, the current findings that *Escherichia/Shigella* negatively associates with NDI and positively with ODI appear in contrast with these previous studies, which could potentially highlight the differences in the populations, timepoints, and

neurodevelopmental features studied. However, future research is needed to understand the relationship between deep grey matter NODDI and functional outcomes for the interpretation of these findings.

Cortical grey matter microstructure associated with microbiota beta diversity PCo-s. PCo2, which had negative contribution from *Escherichia/Shigella* and positive from *Enterobacteriaceae,* positively correlated with cortical ODI. Moreover, PCo4, which had a strong negative contribution from *Enterococcus,* negatively correlated with cortical ODI. In the current study group, cortical ODI did not change with increasing age at MRI, which is similar to the plateau effect of ODI increase after 38 weeks of GA reported previously (Batalle et al., 2019). The results also showed a positive association between cortical ODI and GA at birth, suggesting lower values in infants born more preterm; similarly, lower GA at birth has previously been associated with higher proportion of extremely negative cortical ODI values at TEA (Dimitrova et al., 2021b). Thus, higher ODI values could be interpreted as improved cortical microstructure reflecting increased neurite orientation complexity/dendritic arborisation in the cortex. Therefore, it is interesting that PCo2 associated negatively with deep GM ODI (lower values mean more mature development), but positively with cortical ODI (higher values mean more mature development). These results were paralleled in the taxa-level analyses as *Escherichia/Shigella* negatively associated with cortical ODI, further suggesting a negative association of this bacteria with brain microstructural development as observed with deep grey matter. With regard to PCo4, in taxa-level analyses I found no evidence that the abundance of *Enterococcus* significantly associated with cortical ODI, potentially suggesting a more complex relationship between microbiota community composition and cortical microstructure. Furthermore, preterm brain dysmaturation, in terms of growth and microstructure, is heterogeneous in the cortex and consists of both accelerated and decelerated maturation depending on the region (Alexander et al., 2019; Dimitrova et al., 2021b; Galdi et al., 2020), suggesting that cortical phenotype in the preterm brain is not as simple as delayed maturation. This could also potentially lead to unexpected and complex patterns of associations.

Taxa-level analyses revealed relationships between brain imaging features and the abundance of several bacteria that were not captured as the main drivers of the first four beta diversity PCo-s. First, different *Veillonella* ASVs, Gram-negative commensal obligate

anaerobic bacteria, significantly correlated with deep grey matter diffusion measures: positively with FA and NDI, and negatively with ODI, thus following the associations of these deep grey matter features inversely with the abundance of *Escherichia/Shigella*. Further, increased abundance of *Veillonella* negatively associated with g-factor of ODI, suggesting improved microstructural coherence in the white matter. Interestingly, in Chapter 5 of this thesis, I found that *Veillonella* ASVs were significantly more abundant in extremely compared to very preterm infant gut prior to discharge from the neonatal unit, suggesting that prematurity-related bacteria may play a role in brain microstructural development. Here, however, I found a potentially positive role of *Veillonella* in preterm infant brain microstructure development indicated by increased FA and NDI in the deep grey matter and reduced gODI in the white matter. A positive association between *Veillonella* and preterm infant health has also been demonstrated in previous studies where decreased *Veillonella* abundance was found in infants with NEC (Lindberg et al., 2020; Ward et al., 2016; Zhou et al., 2015) and poor postnatal growth (Younge et al., 2019).

Previous studies of microbiota-gut-brain axis in infants have found associations between the abundance of *Veillonella* and behaviour, but the literature is complex for determining the direction of effect. For example, a small study in preterm infants demonstrated that *Veillonella* abundance at the time of hospital discharge positively associated with stress scores (Sun et al., 2020), suggesting poorer neurobehavioural performance. In term-born infants, studies have found higher *Veillonella* abundance in association with reduced regulation temperament trait (Aatsinki et al., 2019b) as well as increased fear response and amygdala volume (Carlson et al., 2021), while other studies suggest positive associations between *Veillonella* abundance and fine motor scores (Acuña et al., 2021) or practical reasoning skills (Guzzardi et al., 2022). It could be speculated that *Veillonella* plays different roles in brain function and behaviour at different developmental phases and it remains to be established to what extent these relationships may be mediated by brain microstructure around the time of birth.

Second, the data showed significant associations between increased *Streptococcus* abundance and increased ODI in the white matter, as well as increased FA and NDI in the deep grey matter. *Streptococcus* is a common commensal genus colonising the gut in the first few months of life. Increased abundance of *Streptococcus* in infancy has previously

been associated with improved motor development (Acuña et al., 2021; Sordillo et al., 2019), decreased negative emotionality (Kelsey et al., 2021), as well as decreased fear reactivity and increased surgency (Aatsinki et al., 2019b), whilst it has also been shown to negatively associate with the volumes of amygdala and medial prefrontal cortex (Carlson et al., 2021) and functional connectivity in the fronto-parietal network (Kelsey et al., 2021). Third, increased abundance of *Staphylococcus* associated with reduced white matter microstructural coherence as indicated by the positive association with gODI, whilst it also associated positively with ODI in the cortex which reflects improved cortical microstructure, though the effect sizes were smaller compared with the top-ranked ASVs for these microstructural features (*Streptococcus* and *Escherichia/Shigella*, respectively). Interestingly, a previous study in preterm infants suggested increased *Staphylococcus* in association with poorer neurodevelopmental outcomes (Rozé et al., 2020); in term infants, *Staphylococcus* abundance has been associated with smaller amygdala and medial prefrontal cortex volumes (Carlson et al., 2021). Indeed, as also found in Chapter 5 of this thesis, the abundance of *Staphylococcus* in the gut is high at the beginning of life whereafter it is gradually replaced by more niche-specific microbes (Beck et al., 2022; Roswall et al., 2021), suggesting that temporal effects may be important. Lastly, *Finegoldia* abundance correlated with improved markers of deep grey matter microstructure as indicated by increased levels of FA and NDI. In contrast, Seki et al (2021) observed increased *Finegoldia* abundance in preterm infants with severe brain injury shortly before discharge from NNU.

Interestingly, the data showed that microbiota richness indicated by the observed species index positively correlated with markers of improved brain structural development. This includes positive associations with FA and NDI and negative associations with RD and ODI in the deep grey matter, mainly due to values in the thalamus, lentiform and subthalamic nucleus, as well as a negative correlation with ODI in the white matter. These results together reflect increasing microstructural coherence in correlation with increased bacterial richness, potentially reflecting a parallel maturation of the two systems. Although increased alpha diversity is generally viewed as a marker of healthy microbiota, the interpretation is often difficult (Shade, 2016), especially in early life when the gut microbiota is dominated by a few taxa. I sought to overcome this limitation by correlating the microbiota beta diversity PCo-s and alpha diversity indices which revealed a moderate positive correlation between

observed species and PCo1. Therefore, it was interesting that the associations between observed species and deep grey matter microstructure paralleled those observed with PCo2 although these two microbiota measures were not correlated.

Previous work has reported some evidence for positive correlations between alpha diversity and brain functional connectivity between fronto-parietal cortical regions (Gao et al., 2019; Kelsey et al., 2021), but negative associations with connectivity in regions involved in emotional processing (amygdala-thalamus, and anterior cingulate-insula) (Gao et al., 2019). Alpha diversity was also associated with regional brain volumes (Carlson et al., 2021, 2018), but the results remain contradictory, potentially suggesting age-related differences. The correlations between alpha diversity and behavioural outcomes similarly remain unclear as many studies report no significant relationships (see Chapter 4 and (Vaher et al., 2022a)) including a recent study in preterm infants (Beghetti et al., 2021). Thus, it remains to be investigated in future studies with longitudinal follow up to what extent the current neonatal findings relating microbiota richness with brain microstructure translate into behavioural outcomes.

6.4.3 Strengths and limitations

This is the first time microbiota community composition and diversity were examined in an infant cohort in relation to brain volumetric, morphometric and diffusion MRI features associated with preterm birth. This study included a sample of preterm infants without major parenchymal lesions, thus, it is representative of the majority of infants born very and extremely preterm. The sample size (n = 74 for microbiota-neuroimaging associations) is larger than that of previous work examining gut microbiota association with brain structure and function in infancy and childhood, with the prior work conducted in sample sizes of less than 65 (Carlson et al., 2021, 2018; Gao et al., 2019; Kelsey et al., 2021; Schoch et al., 2022; Seki et al., 2021). There is a scarcity of metagenomics data alongside with multi-modal neuroimaging (De Santis et al., 2019; P. Liu et al., 2019; Montoro et al., 2022), particularly in the neonatal population, making this a valuable contribution to the microbiota-neuroimaging field. In addition, most previous studies have not evaluated infants born at < 32 weeks gestation, yet, the prevalence of adverse neurocognitive outcomes is especially high in this population.

Although the sample size was larger than previous studies incorporating microbiota data with neuroimaging in infancy and childhood, it is still one of the major limitations of the current study given the high inter-individual variation both in brain microstructural (Dimitrova et al., 2020) as well as in the microbiota development in preterm infants (Magne et al., 2006). The latter was also illustrated in the current study by the relatively low proportion of variance explained by the first PCo. Small sample size and high heterogeneity coupled with multidimensional nature of both microbiota sequencing and neuroimaging datasets and a lot of analytic freedom are the main limitations of microbiota-neuroimaging studies, leading to reduced power and variability in the results.

Here, the choice of neuroimaging measures was based on established measures targeting whole brain dysmaturation in preterm infants, including brain tissue volumes to characterise brain growth and microstructural development targeting both water content and cellular complexity. While this comprehensive characterisation could be considered a strength of the current study, it may have introduced type I error given the small sample size in comparison with the neuroimaging features studied. Furthermore, this whole brain approach may have hindered the detection of more specific brain structures associated with microbiota community composition as was revealed for the deep grey matter structures. Future studies with larger sample sizes are needed to investigate whether the global effects observed in association with white matter and cortical microstructure are driven by specific tracts or cortical regions, respectively. In addition, the cellular features underlying diffusion MRI metrics in the neonatal deep grey matter structures remain to be studied in conjunction with post-mortem tissue pathology.

With regard to microbiota features, I used established composite measures of microbiota diversity and community composition to overcome the multidimensionality of the dataset. The compositional data, consisting of potentially hundreds or thousands of bacterial taxa, is especially prone to the risk of type I error due to the number of correlations that could be tested. Therefore, here, I took two complementary approaches to study the correlations between microbiota community composition and brain structure. First, similarly to previous studies of microbiota-gut-brain axis in infancy (Carlson et al., 2021; Rothenberg et al., 2021; Sordillo et al., 2019), dimensionality reduction was applied to construct latent variables that captured the main variance related to bacterial composition; these principal coordinates

were used in the main analyses testing for the association between microbiota composition and brain structural features. Next, these analyses were followed up with individual taxa-level correlations using MaAsLin2 models applying a stringent ASV filtering threshold. It could be hypothesised that meaningful microbiota-brain relationships should arise irrespectively of the method used, though it is important to note that the MaAsLin2 models reversed the predictors and outcomes compared to the PCo-based analyses. Variable residualisation against covariates was used to maximise alignment of these two analysis approaches and it was reassuring that for the majority of brain features, the top-ranked taxa were also those most strongly associated with the significant microbiota PCo. Nevertheless, the data also showed some novel relationships from bacteria that were not the main drivers of the microbiota community data variance, suggesting that similar multiverse or consensus analyses may be beneficial in identifying microbiota-brain relationships.

Another limitation of the current study relates to the wide age range of microbiota sampling. Although the analyses were adjusted for gestational age at sample collection, the wide sampling age range could have had profound effects on the microbiota community compositions and thereby cause noise in the subsequent microbiota-brain relationships. In addition, due to the small sample size, age-stratified analyses could not be performed which would have allowed to investigate whether there are any specific developmental windows during the neonatal hospital admission where the microbiota-brain correlations are the strongest. This is particularly important to study in early infancy when both the microbiota and the brain are undergoing rapid changes. Future studies with longitudinal microbiota sampling will be needed to test this hypothesis.

This study was observational in design, thus, any suggestion of causal influence of the microbiota on brain structure is circumstantial. The relationships observed may instead be reflecting different disease burdens which may be causally linked to the development of both gut microbiota and brain structure. Future studies in animal models are encouraged to investigate whether the microbiota associations with neuroimaging features of EoP can be recapitulated. In line with this hypothesis, it has recently been shown that mice transplanted with microbiota from individuals with attention-deficit hyperactivity disorder have reduced structural integrity both in the white and grey matter measured using diffusion MRI (Tengeler et al., 2020).

Finally, although the neuroimaging features of EoP included here have demonstrated predictive value for neurocognitive outcomes following preterm birth, it remains to be investigated in future work with longitudinal follow up to what extent the microbiota-brain correlations observed in the current study translate to functional outcomes in the infants.

6.5 Conclusion

Preterm infants are at risk for both suboptimal brain and microbiota development – two systems which are increasingly acknowledged to have a bidirectional communication. In this study, using a sample of very and extremely preterm infants, I found that the gut microbiota richness and community composition are associated with common neuroimaging features of preterm brain dysmaturation. Particularly, microstructural development in the deep grey matter correlated with microbiota richness and community compositions driven by the abundances of *Escherichia/Schigella* and *Enterobacteriaceae*, whilst white matter microstructure associated with community compositions driven by the abundances of *Klebsiella*. This study contributes to the understanding of the neural substrates potentially underlying microbiota-behaviour relationships in preterm infants.

Chapter 7. General discussion

7.1 Summary of findings

In this thesis I integrated data from multimodal brain MRI and amplicon-based metagenomic sequencing of the gut microbiota with the primary aim to identify gut bacterial signature associated with brain dysmaturation following preterm birth.

Previous work has demonstrated that white matter dysmaturation in the preterm brain is substantially a whole-brain phenomenon. However, less was known whether single- or multimodal MRI data dimensionality reduction can effectively capture the generalised reduced white matter integrity associated with preterm birth. Work presented in Chapter 3 thus contributes to the development of global neuroimaging markers in the neonatal brain.

A substantial body of research has shown that the gut microbiota plays a role in brain development and behaviour. Chapter 4 offers a detailed review to the extent that the gut microbiota associates with cognition, behaviour and brain structure and function in infancy and early childhood.

Gut microbiota diversity and community composition are affected by a range of perinatal factors, however, previous research leaves uncertainty about the effects in preterm infants. Chapter 5 contributes to the field by characterising gut microbiota community composition in a new cohort of very and extremely preterm infants and furthers the knowledge of the drivers shaping the preterm infant gut microbiota during neonatal unit care.

Finally, whilst preterm birth influences the development of both the gut microbiota and brain structure, the association between these two processes in the vulnerable preterm population is poorly understood. The work presented in Chapter 6 addresses this important gap in the field.

7.1.1 General factors of white matter microstructure from DTI and NODDI in the developing brain

In Chapter 3, I derived global neuroimaging estimates of white matter integrity and investigated if these may be useful to capture preterm brain dysmaturation at TEA. I applied principal component analysis to capture the shared variance of DTI and NODDI metrics

across tracts, as well as across the metrics themselves. This method allowed derivation of single- and multi-metric general (g) factors in the neonatal brain. I found that single-metric g-factors captured substantial shared variance (41.8-72.5%) across 16 white matter tracts, and two multi-metric factors captured 93.9% of variance shared between DTI and NODDI metrics themselves. These results were in line with previous research in adult and paediatric populations and introduced for the first time the use of NODDI measures in the g-factor framework in neonates. Regression analyses revealed that the g-factors, apart from those derived using NDI or ODI, significantly associated with preterm birth and suggested increased diffusivity and water content in the white matter of the preterm compared to term-born infants, in line with previous literature. The strongest effects were observed for gISO and gRD: GA at birth explained 11.06% and 7.86% of variance in these metrics, respectively. Although the multi-metric g-factors also significantly associated with prematurity, the effect sizes and variance explained by GA at birth did not exceed the ones observed for gISO and gRD. I then applied logistic regression and cross-validation to determine if g-factors are able to classify infants based on term/preterm status. The results showed that, although statistically significant, the prediction accuracy of the single- and multi-metric g-factors only marginally exceeded chance. Here, as for the regression analyses, the prediction accuracy of the multi-metric g-factors was similar to that achieved using gRD or gISO. Last, I found that the highest prediction accuracy was achieved with the model incorporating all single metric g-factors, which I interpreted to reflect specific and additive aspects carried by each of the metrics with regard to the underlying microstructure. Therefore, I chose to use single-metric g-factors for investigating the associations with gut microbiota.

7.1.2 Microbiome-gut-brain axis in brain development, cognition and behaviour during infancy and early childhood

In Chapter 4, I reviewed the evidence that gut microbiota diversity and community composition associate with human brain development in infancy and childhood. I identified 20 studies that had integrated data from gut bacterial DNA sequencing and neurodevelopmental and/or -imaging assessments in typically developing children from birth up to 5 years of life. Gut microbiota to date has been linked with a range of neurodevelopmental domains, including general cognitive, motor and language

development, as well as temperament, social behaviour and fear responses. A handful of studies had further incorporated structural and functional MRI or functional near-infrared spectroscopy. The main finding of this chapter was that while features of the gut microbiota significantly associate with one or more neurodevelopmental outcomes in most studies, differences in the specific bacteria-brain/behaviour relationships between studies are substantial. This emerging field exhibits a great deal of methodological heterogeneity, which hindered quantitative synthesis or meta-analysis of the results. Sources of heterogeneity were related to variability in gut microbiota sequencing method, choice of microbiota and/or neurodevelopmental features of interest, approaches for statistical analyses as well as approaches for adjustment of confounding factors. Furthermore, the reviewed studies left uncertainty about the relationships between microbiota community composition and brain dysmaturation in very and extremely preterm infants; yet, neurocognitive adversities are particularly high in this population.

7.1.3 Microbiota profiles and drivers in preterm neonates

In Chapter 5, using 16S rRNA gene sequencing, I characterised the gut bacterial diversity and community composition of term and preterm infants shortly after birth, and in preterm infants prior to discharge from the neonatal unit. I also determined the perinatal drivers shaping the preterm infant microbiota during the hospital stay. The first meconium samples in the majority of both term and preterm neonates were dominated by *Staphylococcus*. By the time of hospital discharge, the gut microbiota of preterm infants had diversified, both in terms of richness and evenness, and there was high between-subject variability in the microbiota profiles. The majority of preterm infants prior to discharge had gut microbiota profiles characterised by high relative abundances of either *Bifidobacterium* or *Enterobacteriaceae*, while some infants retained community compositions high in *Escherichia/Shigella* or *Klebsiella*. *Clostridium*, *Enterococcus,* and *Veillonella* were also abundant. These results were in line with previous work characterising preterm neonatal microbiota development (e.g. Beck et al., 2022; Hui et al., 2021; Rao et al., 2021). The main drivers of the preterm infant microbiota compositions shortly after birth were mode of delivery and postnatal age. Degree of prematurity, sex and antibiotic exposure during neonatal unit stay affected microbiota compositions prior to hospital discharge. This investigation contributes to the field by promoting the understanding of the most influential

early-life factors associated with microbiota composition in a representative cohort of very and extremely preterm infants without exposure to probiotics.

7.1.4 Neonatal microbiota and brain dysmaturation in preterm infants

Finally, in Chapter 6, I tested the hypothesis that preterm infant gut microbiota associates with brain MRI features of EoP at TEA. Previous studies investigating the relationships between preterm neonatal microbiota and brain development were limited to the identification of neurocognitive delays 2-3 years after microbiota sampling (Beghetti et al., 2021; Sarkar et al., 2022), so the role of early childhood events must be considered, or detection of severe parenchymal injury (Seki et al., 2021), which does not explain the impairments faced by the majority of neonatal intensive care survivors. Through the use of structural and diffusion MRI in a cohort of representative very and preterm infants, I was able to study features related to a common preterm infant imaging phenotype including reduced tissue volumes and altered microstructure. First, I applied principal coordinates analysis to the microbiota beta diversity data; this method derived four latent factors explaining over 40% of variance in the microbiota community composition data. The factors captured the relative abundance of the top taxa in the current study: *Bifidobacterium*, *Escherichia/Shigella*, *Enterobacteriaceae, Klebsiella,* and *Enterococcus.* I found that several microbiota features were significantly associated with brain microstructural measures derived from diffusion MRI, particularly in the white and deep grey matter. The data showed that deep grey matter microstructure, especially NDI and ODI, correlated with microbiota richness as well as community compositions driven by the abundances of *Escherichia/Shigella* and *Enterobacteriaceae*. White matter microstructure, particularly RD, was mainly associated with the abundance of *Klebsiella*. Gut microbiota was minimally correlated with brain volumetric measures. The data of this chapter provides the first evidence that neonatal gut microbiota associates with common neuroimaging features of preterm brain dysmaturation.

7.2 Implications and future directions

7.2.1 Studying brain development with global neuroimaging measures

The use of global neuroimaging measures in this doctoral work was motivated by previous research suggesting generalised white matter dysconnectivity in the preterm brain (Blesa et al., 2020; Telford et al., 2017), the findings that diffusion MRI metrics in the brain are

correlated (De Santis et al., 2014), and previous work demonstrating associations between g-factors and cognition in adults and children born at term (Alloza et al., 2016; Lee et al., 2017; Penke et al., 2010). Furthermore, use of principled global MRI features aid data integration with other multidimensional datasets, such as that derived from sequencing of the microbiota. In this thesis, I applied global neuroimaging measures to capture variance associated first with GA at birth and then with gut microbiota diversity and community composition.

G-factors of white matter microstructure are relatively well-established in the neuroimaging field. Yet, the maturation rate measured by DTI and NODDI over the neonatal period varies between the different white matter tracts (Kersbergen et al., 2014; Kunz et al., 2014). This notion is also supported by the data in Chapter 3 showing varying DTI and NODDI values between the major tracts. Therefore, the vulnerability of the tracts to environmental as well as microbial exposures may also vary due to the ongoing (and different state of) cellular processes, e.g. pre-myelination evens or axonal density, which might not have been captured by using the generalised measures.

I also chose to study the effect of gut microbiota on whole cortex morphological and diffusion measures. There is precedence from previous works for studying cortical maturation using global morphological measures in association with preterm birth as well as with cognitive outcomes in paediatric populations (e.g. Girault et al., 2020; Kline et al., 2019). In recent work from our group, we demonstrated that breast milk intake, a known driver of the microbiota, associated with whole cortex volume, thickness, FA and RD (Sullivan et al., 2022), supporting the use of global MRI features in the study of early life determinants of cortical development. However, preterm brain dysmaturation is especially heterogeneous in the cortex and displays both accelerated and decelerated development depending on the region (Alexander et al., 2019; Dimitrova et al., 2021b). Furthermore, cortical morphometry in different regions may differentially contribute to the correlation with cognitive outcomes (e.g. Kline et al., 2020). Thus, it could be hypothesised that the gut microbiota exhibits region- or network-specific effects in the cortex, which may not be detectable using average measures.

All in all, the work in this thesis does not rule out more specific effects of the microbiota on different brain networks, regions and white matter tracts. Thalamocortical connectivity may

especially be interesting to study in the context of the microbiota-gut-brain axis given the findings in Chapter 6 showing significant associations between microbiota and deep grey matter microstructure, particularly in the thalamus and the lentiform nuclei. Thalamocortical connectivity is reduced following preterm birth (Ball et al., 2013a) and associates with cognitive outcomes in this population (Ball et al., 2015). In addition, a recent study suggested an association between gut microbiota alpha diversity with amygdala-thalamic connectivity in 1-year-old infants (Gao et al., 2019), further motivating future research of the impact of the gut microbiota on brain networks involving the thalamus.

7.2.2 Advanced neuroimaging modalities to study the preterm brain

This doctoral study contributed to the characterisation of neonatal brain microstructure using NODDI both in the white and grey matter. The advantage of NODDI is that it enables to disentangle neurite density and geometrical orientation, which both contribute to measures derived from the DTI model, such as FA (Zhang et al., 2012). Previous studies had demonstrated changes in NODDI metrics over the preterm neonatal period in the white and grey matter (Eaton-Rosen et al., 2015; Kimpton et al., 2021; Melbourne et al., 2016; Poh et al., 2015; Qiu et al., 2013). This thesis adds to this literature by describing the effect of prematurity on NODDI metrics in white matter tracts and deep grey matter. Furthermore, in Chapter 6, NODDI was sensitive to detecting correlations between gut microbiota composition and deep grey matter microstructure, which would have remained undetected with the use of only DTI. Future work incorporating histological analysis will be needed to clarify the cellular morphology correlates of diffusion MRI measures in the neonatal brain, particularly in the deep grey matter.

Preclinical work has suggested a role for the microbiota in myelination (Hoban et al., 2016; Keogh et al., 2021). Indeed, myelination is a key neural process affected by preterm birth due to the vulnerability of pre-oligodendrocytes to early life insults (Back and Volpe, 2018; Volpe et al., 2011). Therefore, the use of myelin-sensitive imaging acquisitions such as magnetisation transfer imaging (MTI) in the study of microbiota-brain relationships in preterm infants would be an exciting avenue for future research. This hypothesis is also supported by the finding that microbiota composition correlates with RD in the white matter, which has been suggested to be sensitive to myelin pathologies, though it lacks specificity (Lazari and Lipp, 2021; Mancini et al., 2020; Song et al., 2002). MTI was the latest

addition to the TEBC imaging protocol, however, as only a small subset of the cohort had matching faecal microbiota and MTI data, the current study was not powered to include this modality.

7.2.3 Normative modelling to study atypical development of the brain and gut microbiota in preterm infants

Preterm infants comprise a heterogeneous group with a diverse neurocognitive outcome spectrum, which could lead to reduced power in group-level analyses. Recently, approaches to study atypical brain development in preterm infants by measuring individual deviation from normative development have been proposed; these approaches may be clinically useful as the individual deviations correlate with perinatal factors and developmental outcomes (Dimitrova et al., 2021a, 2020). Indeed, brain charting, i.e. the efforts of providing reference standards of brain morphological development across the life course by integrating data across multiple studies, have gained traction in recent years (Bethlehem et al., 2022; Rutherford et al., 2022). As an alternative method, brain age, or the difference between actual age and that predicted from multimodal neuroimaging data, has been used to characterise brain maturation following preterm birth in neonates (Galdi et al., 2020; Taoudi-Benchekroun et al., 2022) as well as children (Kelly et al., 2022) and adults (Hedderich et al., 2021).

Similarly, the gut microbiota of preterm infants has high inter-individual variation, illustrated by the existence of multiple microbiota clusters prior to hospital discharge demonstrated in Chapter 5 of this thesis as well as in previous studies (e.g. Rao et al., 2021; Stewart et al., 2017). Combined with the rapid development of the microbiota in infancy, this suggests that characterisation of the microbiota at a single sampling point and its association with brain imaging markers is not enough to fully understand the interactions between these two systems in early life. Rather, longitudinal assessment to enable identification of group- and/or individual-level trajectories may be important to elucidate the associations between microbiota development and brain dysmaturation. Whilst some studies of microbiota-gut-brain axis in paediatric samples discussed in Chapter 4 of this thesis had included multiple microbiota sampling points, the analyses were still cross-sectional by design and did not taken into account longitudinal microbiota development in relation to outcomes. In the current study, although two microbiota sampling points were included, only a small

proportion of participants had matching samples due to transfers to other hospitals, withdrawal of consent or sample exclusion due to low DNA yield or following quality control; thus, longitudinal modelling was not included.

In the absence of longitudinal sampling in individual studies, comparison of the gut microbiota with normative charts would therefore be attractive. Indeed, to utilise microbiota as a biomarker for risk stratification for interventions, single timepoint assessment may still be needed. One method with this goal is calculation of microbiota age to understand to what extent early life factors, such as prematurity, nutrition or antibiotics, lead to atypical development or a dysmaturation phenotype of the gut microbiota compared to what could be expected based on age and other demographics in representative normative data. There is precedence for the use of microbiota age modelling in the literature and this has revealed that deviation between actual and microbiota-based prediction of chronological age associates with malnourishment (Subramanian et al., 2014) and breast milk intake (Stewart et al., 2018). It could be hypothesised that preterm infant microbiota looks younger compared to those born at term.

However, with regard to preterm infants, there is a question of what should be considered the respective normative population. Given the physical immaturity of the gastrointestinal organs and much earlier exposure to environmental microbes, it could be argued that preterm infants comprise a unique population with regard to their microbiota development. This is supported by the data of the patterned microbiota succession in preterm infants depending on gestational/postmenstrual age, i.e. physical maturity (Grier et al., 2017; Korpela et al., 2017a; La Rosa et al., 2014). Accordingly, the earlier microbiota phases do not occur in term infants as they are exposed to microbes at a later age and physical maturation state. Therefore, prediction of gestational age, as opposed to chronological age as previously done in microbiota age modelling, may be more appropriate for the preterm infant population. Future efforts for defining a reference/normative trajectory (or indeed multiple possible trajectories given the heterogeneous population and personal nature of the microbiota) of the gut microbiota in preterm infants over the neonatal period would be useful for detecting infants most at risk for microbiota dysbiosis-related morbidities. This effort is expected to be challenging due to the methodological heterogeneity of microbiota data curation.

Calculation of atypicality/dysmaturation indices of the gut microbiota as well as the brain would be another avenue for a principled approach for data dimensionality reduction for integrating multidimensional datasets. This would allow for a more individualised approach for data analysis and the arising knowledge may be useful for future design of personalised microbiota-targeted therapies.

7.2.4 Studying mechanistic and causal pathways

The current study investigated gut microbiota diversity and community composition at the taxonomic level. Future work will further incorporate data from whole metagenome shotgun sequencing to infer functional properties of the microbiota in association with perinatal covariates and brain structural development as well as determine species-level effects.

The microbiota may exert its influence on neural processes through a multitude of pathways including release of small molecules and modulation of the immune system. Therefore, incorporation of other 'omics approaches such as metabolomics, proteomics and host transcriptomics and epigenomics from different tissues is important to understand the host-microbe cross-talk and unravel the pathways linking preterm infant gut dysbiosis with brain dysmaturation. For instance, metabolomic profiles in blood, stool and urine associate with neonatal morbidities such as sepsis and necrotising enterocolitis (Renwick and Stewart, 2022) as well as ASD (Dan et al., 2020; Needham et al., 2021). Microbial metabolites associated with ASD have also been shown to induce autism-like behaviours in rodents (Bermudez-Martin et al., 2021; Meeking et al., 2020), suggesting a causal mechanistic pathway between metabolic products of gut bacteria and behaviour.

Inflammatory mediators may be attractive to study in the preterm population. The gut microbiota is important for early life immune system maturation (Gensollen et al., 2016; Gollwitzer and Marsland, 2015) and gut dysbiosis has been implicated in neonatal inflammatory morbidities such as sepsis and NEC (Masi and Stewart, 2019) – conditions which themselves are independent predictors of adverse brain and neurocognitive outcomes (Barnett et al., 2018; Lee et al., 2014; Stoll et al., 2004). Thus, it could be hypothesised that suboptimal microbiota development contributes to or reinforces the inflammatory cascade associated with early exposure to extrauterine life. For example,

excessive immune response triggered by lipopolysaccharide expressed by gram-negative bacteria has been proposed as one of the mechanisms inducing NEC (Hunter and De Plaen, 2014). Therefore, understanding of the microbiota signatures associated with systemic inflammation may further help to understand the pathways linking gut microbiota development with brain structure. The TEBC sampling schedule includes a panel of immune mediators in blood as well as salivary DNA methylation. The latter can be utilised to derive proxies of systemic inflammation which may be more stable compared to blood-based markers (Stevenson et al., 2020) and salivary DNA methylation compared to blood may be more representative of that in the brain (Braun et al., 2019; Smith et al., 2015). Previous work from our group demonstrated that interleukin-8 in blood and a DNA methylation proxy of C-reactive protein in saliva associated with white matter dysmaturation (Conole et al., 2022; Sullivan et al., 2020), however, the effect of inflammatory markers on deep grey matter microstructure, to the best of my knowledge, has not been studied. It would be of great interest in future work to investigate whether systemic inflammation is mediating some of the effects observed between microbiota and brain microstructure. Indeed, it was demonstrated in a recent study that pro-inflammatory immunological tone accompanied pathogenic *Klebsiella* overgrowth in infants with severe brain injury, suggesting that aberrant development of the gut-microbiota-immune-brain axis may contribute to brain injury following preterm birth (Seki et al., 2021). It remains to be investigated to what extent this microbiota-immune regulation replicates in the context of a more common preterm brain phenotype.

Causal links between the microbiota and brain dysmaturation in clinical datasets could further be studied by utilising genetic studies and application of methods such as Mendelian randomisation. Indeed, genome-wide association and targeted single nucleotide polymorphism studies have identified genetic variants associated with preterm birth (Zhang et al., 2017), gut microbiota (Kurilshikov et al., 2021), brain structure (Smith et al., 2021) as well as preterm brain injury (Boardman et al., 2014; Krishnan et al., 2017). In addition to enabling the study of causal inference (e.g. preterm birth on microbiota, microbiota on brain structure), incorporation of genetic data in future work in cohorts such as the TEBC may help to distinguish between brain dysmaturation arising from genetic variation from that arising as a result of early exposure to microbes.

In addition to including other biological data in clinical cohorts, mechanistic studies in preclinical models and/or *in vitro* cell/organoid cultures are important for effective design of microbiota-based interventions for optimal brain maturation. For example, preterm intestinal-derived organoids provide a unique opportunity for mechanistic investigation of host-microbe interactions (Stewart et al., 2020). This model was recently used to demonstrate that microbial metabolites impact the epithelial barrier function in a microbiota cluster-specific manner (Beck et al., 2022). Development of multi-organ culture systems may be an exciting avenue for further understanding of microbe-intestine-brain interactions. Given that patient-derived organoids retain the genetic and epigenetic susceptibility of the host, these approaches in the future may pave way for personalised medicine.

Understanding the mechanistic pathways linking gut microbial dysbiosis with brain dysmaturation in preterm infants will inform interventions targeted at promoting brain health following preterm birth. These studies would also help to understand the cause-effect relationships between microbiota and neuronal structure/function.

7.2.5 Modifying the microbiota to improve brain health

Given the accessibility and recognition of the role of the gut microbiota in many aspects of health, there is growing interest to modulate the gut microbiota to promote preterm infant outcomes by administering probiotics mainly containing strains of *Bifidobacterium* and *Lactobacillus*. Probiotics are thought to exert beneficial effects by promoting maturation of the preterm infant gut microbiota to a *Bifidobacterium*-dominated profile, i.e. to one more closely resembling that of the healthy term infant microbiota, and reducing levels of pathobionts and pro-inflammatory cytokines (Samara et al., 2022; van Best et al., 2020). Whilst probiotics are used to reduce mortality and prevent sepsis and NEC, the certainty of the evidence is low due to multitude of biases in trial designs (Sharif et al., 2020). Furthermore, larger trials and those performed in extremely preterm infants show no effects (Costeloe et al., 2016a; Sharif et al., 2020). Clinicians further have concerns about exposing immunologically susceptible infants (< 1000 g at birth) to non-pharmaceutical grade products, sparse mechanistic understanding, conflicting data on efficacy and safety, uncertainty about the optimal constitution of preparations (e.g. product, dosing, duration), and cross-contamination within nurseries (Poindexter et al., 2021). Probiotics have also

shown to associate with a small risk of probiotic-induced sepsis (e.g. Bertelli et al., 2015). These findings highlight the need to further study the mechanistic actions of probiotics and careful definitions of patient groups who would most benefit from microbiota-based interventions.

The accumulating evidence for gut bacteria influences on brain function has led to increased interest in manipulating the gut microbiota for brain health and a new classification of probiotics/psychotropics – psychobiotics – has been suggested (Dinan et al., 2013). Preterm infants may especially be the population to most benefit from microbiome-targeted therapies for protection against brain dysmaturation and subsequent neurodevelopmental impairments (Lu and Claud, 2018). Administration of *Bifidobacterium* strains to germ-free mice promotes memory and synaptic density (Luck et al., 2020; Luk et al., 2018) while *Lactobacillus* administration prevents antibiotic-induced effects on social behaviours and cytokine expression in the brain (Leclercq et al., 2017). In human adults, administration of probiotic cocktails consisting of various strains of *Bifidobacterium* and *Lactobacillus* associates with altered brain functional connectivity and reduced depression and anxiety scores (Bagga et al., 2018; Tillisch et al., 2013). The current evidence from RCT-s, however, does not implicate improved neurodevelopmental outcomes following probiotic treatment; furthermore, brain outcomes in trials have been cursory, if assessed at all (Jacobs et al., 2017; Sharif et al., 2020; Slykerman et al., 2018; Upadhyay et al., 2018). Strain-specific effects may be important to consider here, however, it could also be argued (and may well be more likely) that the relationship between gut microbiota and brain development is more complex than "one pathogen – impaired neurodevelopment" or "*Bifidobacterium* – improved neurodevelopment", which may have to be taken into account for designing microbiota-targeted interventions. This hypothesis is supported by the apparent lack of consistent bacterial signature or specific taxa associated with improved neurodevelopmental outcomes as discussed in Chapter 4.

The work in Chapter 6 further adds to this notion. For example, I found some limited evidence that increased *Bifidobacterium* abundance may be associated with improved brain growth, however, there were no significant relationships between *Bifidobacterium* and brain microstructure. Next, although *Klebsiella* is a common pathogen associated with neonatal morbidities, I found a paradoxically positive correlation between the principal

coordinate negatively driven by the abundance of *Klebsiella* and gRD, a marker of increased diffusivity and potentially decreased myelination in the white matter. In individual taxa-level analyses, relative abundance of *Klebsiella* further correlated positively with NDI in the deep grey matter, which could be considered a marker of improved microstructure. Furthermore, deep grey matter microstructure associated with the abundances of *Escherichia/Shigella*, *Enterobacteriaceae* and *Veillonella*. These findings therefore suggest that there may not be a single bacterial signature related to improved developmental processes across the brain, but it may be dependent on the ongoing maturational process of which stage and pace varies between brain regions/networks. Some suggestions for future research as outlined in the previous sections in this chapter may help to investigate this hypothesis.

7.3 Strengths and limitations

Several strengths and limitations of this PhD study have already been discussed in Chapters 4-6. In Chapter 4, I outlined the sources of methodological heterogeneity and general limitations in the field of investigating microbiota relationships with neurodevelopment in infancy and early childhood. Chapters 5-6 discussed strengths and limitations specific to methods and analyses therein.

This doctoral work was the first study to integrate information about gut microbiota derived from 16S rRNA gene sequencing and multimodal MRI characterising brain volumetric and microstructural maturation in a very and extremely preterm infants without major parenchymal brain injuries. I used data from a single-centre birth cohort representative of a typical preterm population in terms of antenatal and neonatal characteristics. The preterm infants in this cohort have approximately equal representation from different quintiles of the Scottish Index of Multiple Deprivation, suggesting that the sample is representative of different socioeconomic groups in Scotland, although the effects of socioeconomic status were not directly investigated or controlled for. Deep clinical phenotyping allowed me to characterise the sample in terms of nutritional and antibiotic exposure, yet, the effects of timing and type of antibiotics need to be clarified in future studies with longitudinal sampling. None of the infants in the cohort were exposed to probiotics, which enabled unconfounded investigation of common early life drivers of the preterm infant microbiota.

The imaging protocol applied in this cohort included multi-shell diffusion acquisitions with high b-values that enabled implementation of advanced biophysical modelling. Indeed, the use of multimodal neuroimaging at TEA to study the relationships with neonatal microbiota should be considered a major strength in the current study as it allowed to overcome a limitation of previous studies in preterm infants where there has been a significant time lag between microbiota sampling (e.g. during the first few months of life) and neurodevelopmental outcome assessments (e.g. at 2-3 years) (e.g. Beghetti et al., 2021; Rozé et al., 2020; Sarkar et al., 2022).

By conducting a narrative review, I identified 20 studies published between 2015-2020 and I provided a detailed overview of the microbiota correlations encompassing multiple neurodevelopmental and -imaging domains in infancy and early childhood. This is a relatively new, but fast-growing field, and standards and conventions to link microbiota with behavioural or brain imaging data have not yet been established. This resulted in a range of methodological heterogeneities between studies that hindered quantitative synthesis. I further discussed approaches for reducing this heterogeneity which would improve comparability between studies. Given the rapidly evolving field as illustrated by 14 additional studies published since the submission of the review, future studies could particularly benefit from re-analysis of pooled raw data, i.e. mega-analysis, using a consistent statistical approach to increase power and clarify the potentially age-specific microbial signatures associated with the different neurodevelopmental domains. A recent study made a step towards this goal, however, none of the re-analysed datasets included typically developing infants and pre-school children (Spichak et al., 2021).

A limitation of this observational cross-sectional study is that cause-effect relationships could not be studied. Instead, the resulting correlations could potentially result from a pleiotropic effect of other factors influencing both the gut microbiota and brain structure, accumulation of neonatal exposures on both systems, or reflect the parallel maturation stage of the brain and microbiota. Above, I discussed some options for future research to improve causal or mechanistic inference.

Another limitation of the work presented in this thesis is that the source of bacteria in the infant gut could not be identified due to the absence of maternal and hospital environment sampling. Furthermore, I did not study the potential effect of the global COVID-19 pandemic

on infant microbiota. Hospital regulations, for example regarding family visitation and the use of masks, significantly changed with the pandemic. Thus, it could be hypothesised that this affected the NNU environmental microbiota, which in turn could have affected neonatal colonisation. Studying the effects of potential intrauterine exposure to microbes, e.g. by investigating the correlations with histologic chorioamnionitis or PPROM, was additionally out of scope of this current thesis.

This thesis was limited to the characterisation of bacteria, though interactions between different kingdoms of microorganisms can affect colonisation in preterm infants (Rao et al., 2021).

In this thesis, I was unable to investigate the potential mediating effect of the gut microbiota in the associations between breast milk exposure and improved brain development. Much larger samples may be needed to investigate this hypothesis.

Inclusion of term control infants in future studies may help to understand whether the bacteria-brain relationships vary depending on prematurity. This would be beneficial to ascertain whether a more term-infant-like gut microbiota composition in preterm infants prior to hospital discharge associates with a brain phenotype that is more similar to a term-born infant.

Due to sample size limitations in the microbiota-MRI matching sample, I did not investigate sex-specific effects of the microbiota on neuroimaging features. Male sex is a recognised determinant of adverse neonatal morbidity and associates with increased risk for brain dysmaturation and neurodevelopmental outcomes (Barnett et al., 2018; Garfinkle et al., 2020; Linsell et al., 2018). In Chapter 5, the data suggested an independent effect of sex on microbiota profiles, which could be hypothesised to contribute to the differential risk of impairment associated with infant sex. Furthermore, several studies have identified sex-specific associations between the microbiota and behaviour in paediatric populations (Aatsinki et al., 2019b; Christian et al., 2015; Laue et al., 2021) as well as across the life-course as reviewed by Jaggar et al. (2020). Given the heightened risk for adverse neurodevelopmental outcomes in male preterm infants, future studies in larger cohorts are motivated to perform sex-disaggregated analyses in studying microbiota effects on preterm brain development.

Due to ongoing follow-up appointments in the TEBC cohort, developmental outcome measures were not included in the current thesis. Future work will incorporate standardised cognitive and behavioural measures to understand if the microbiota-brain relationships observed in neonates translate to functional outcomes.

7.4 Concluding remarks

This thesis provides four original research studies that generated the following main findings. First, DTI and NODDI metrics share variance across the brain white matter tracts which enables derivation of g-factors as global estimates of white matter microstructure associated with preterm birth; g-factors provide tractable metrics for investigating determinants of brain development. Second, gut microbiota associates with brain and behavioural development across multiple domains, but there is high variability in the specific effects. Third, gut microbiota in preterm infants has a profound shift in bacterial diversity and community composition from birth to hospital discharge, and the drivers of microbiota composition over the neonatal period include the degree of prematurity, sex and antibiotic exposure. Fourth, gut microbiota community composition and diversity prior to hospital discharge correlate with global measures of microstructure in the white and deep grey matter, which are key components of EoP. These results support the hypothesis that gut microbiota development associates with brain dysmaturation in the vulnerable population of very preterm infants at risk for neurocognitive impairments. Future work on mechanistic and causal pathways, regional specificity and implications in terms of functional/behavioural outcomes are warranted to guide the design of optimal microbiota-targeted interventions for brain health in infants born preterm.

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