Longitudinal Relations Between Early Life Stress, Gut Microbiota, and Executive Functioning from Pregnancy to Childhood

Henrik Eckermann

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Longitudinal Relations Between Early Life Stress, Gut Microbiota, and Executive Functioning from Pregnancy to Childhood

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Contents

Chapter 1

General Introduction

In 2004, Sudo et al. documented aberrant activation of the hypothalamus–pituitary–adrenal (HPA) axis in mice bred and raised entirely devoid of microorganisms (germ-free mice) (Sudo et al., 2004). When subjected to stress-inducing physical restraint, these germ-free mice exhibited heightened endocrine responsiveness, as evidenced by increased levels of stress biomarkers such as plasma adrenocorticotropic hormone and corticosterone, compared to conventionally colonized mice. Furthermore, the germ-free mice exhibited diminished levels of hippocampal and cortical brain-derived neurotrophic factor (BDNF), a protein intimately linked with neuroplasticity, learning, and memory. Remarkably, the reintroduction of specific microbes to the germ-free mice mitigated hyperactivity of the HPA axis at six weeks of age, but less effectively so at 14 weeks of age (corresponds to human ages of approximately 11.5 and 22 years, respectively (Dutta & Sengupta, 2016)). The findings by Sudo et al. point at the existence of critical periods during which specific bacteria are required for normal neurodevelopment.

These findings sparked broader interest in the role of bacteria for neurodevelopment and many experimental animal studies have since followed. Collectively, they added evidence for a causal effect of bacteria on brain development and functioning as well as the presence of neurodevelopmental time windows (Borre, O'Keeffe, et al., 2014). However, a notable gap remains when translating these findings from animal models to human studies. This has prompted calls by leading researchers for the greater involvement of psychologists and behavioral scientists in bridging this divide (Sarkar et al., 2018).

In response to this call and the growing recognition of the critical role played by the gut microbiota in neurodevelopmental processes, the overarching goal of this thesis is to help bridge the translational gap between animal models and human studies. Our objective is to integrate insights garnered from three themes of experimental animal research with human research. These themes are the influence of maternal prenatal stress (Theme 1) and early-life stress in offspring (Theme 2) on offspring microbiota development. The third theme looks at the microbiota composition in infancy and early childhood in relation to learning and memory later in life (Theme 3). In the following sections of this introductory chapter, we briefly delve into the relation between stress and the gut microbiota, providing context for themes 1 and 2, before examining how the gut microbiota may modulate brain function, as addressed in Theme 3. The last sections of this chapter list the studies utilized for this thesis as well as its general outline.

Table 1.1: Content of this thesis.

Theme No	Theme Title	Chapter Title	Study Sample	Chapter No
1	Maternal stress and offspring microbiota development	Maternal Pre- and Postnatal Stress and Maternal and Infant Gut Microbiota Features	SMILEY	2
2	Early life stress and microbiota development	Does entry to center-based childcare affect gut microbial colonization in young infants?	BIBO	3
		Daily skin-to-skin contact alters microbiota development in healthy full-term infants	SKIPPY	4
3	Early microbiota development and learning and memory	Can gut microbiota throughout the first 10 years of life predict executive functioning in childhood?	BIBO	5

1.1 Stress and the gut microbiota

Stress denotes a condition wherein the typical physiological equilibrium of an organism is disrupted by a real or perceived challenge (Salomon, 2013). Acute stress triggers the activation of the HPA axis, leading to a release of corticotropin-releasing hormone (CRH) from the hypothalamus. This stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH), which in turn stimulates the adrenal glands to produce cortisol (or corticosterone in rodents) (Karin et al., 2020). Elevated cortisol levels negatively feedback on the secretion of CRH and ACTH. This response is evolutionarily preserved, priming the individual for defense or escape from the perceived threat. Upon cessation of the threat, normal physiological balance should be restored. However, prolonged activation of the stress response can cause the HPA axis to become dysregulated (Salomon, 2013). A dysregulation can manifest as abnormal secretion of cortisol and ACTH, impacting the metabolism, immunity, behavior as well as gut physiology and functioning (Karin et al., 2020; Leigh et al., 2023).

Both HPA-axis activation or dysregulation can influence gut physiology and functioning (Leigh et al., 2023), for instance by impacting the function of the intestinal barrier. The intestinal barrier refers to the complex system of epithelial cells, mucus layers, and immune cells that

regulate the selective permeability of the intestinal mucosa. Selective permeability is crucial for maintaining gut homeostasis and preventing the translocation of harmful pathogens and antigens into the bloodstream. Dysfunction of this barrier is associated with many chronic illnesses such as Alzheimer's disease, inflammatory bowel diseases, food allergy, irritable bowel syndrome, rheumatic diseases and other autoimmune conditions such as type-1 diabetis mellitus (Horowitz et al., 2023).

Studies have evidenced a relation between chronic stress and heightened permeability of the intestinal barrier in humans and animals (Li et al., 2013; Vanuytsel et al., 2014). In humans, stress-induced hyperpermeability affects both the small and large intestine, with mast cells suggested as potential mediators (Vanuytsel et al., 2014; Wallon et al., 2007). These findings are congruent with pharmacological investigations demonstrating that prolonged exposure to glucocorticoids or corticotrophin-releasing factor can disrupt gut barrier integrity (Meena et al., 2023; Shukla et al., 2021; Teitelbaum et al., 2008; Zheng et al., 2013). Chronic stress or administration of corticotrophin-releasing factor have been observed to impact the colonic gut barrier by perturbing tight junction proteins, augmenting paracellular permeability, elevating levels of proinflammatory cytokines (Machorro-Rojas et al., 2019; Nozu et al., 2017), increasing infiltration of mast cells (Lauffer et al., 2016) and mononuclear cells (Vicario et al., 2012), and upregulating toll-like receptor 4 expression (Yu et al., 2013). These changes, observed in both murine models and human subjects, are linked to local and systemic inflammation and alterations in microbiota composition (Karl et al., 2017).

The previous description of the effects of stress on intestinal barrier function merely serves as one example of how stress can influence gut physiology and function. Stress furthermore affects gut motility, visceral sensitivity, gastrointestinal hormone secretion, bile acid synthesis and secretion, and mucosal immune function as was recently extensively reviewed by Leigh and colleagues (Leigh et al., 2023). Unsurprisingly, by changing the living environment of intestinal bacteria, stress indirectly affects the gut microbiota composition. In the following, we will briefly elaborate on the effect of stress on gut bacteria and how these bacteria can also influence the mammalian body's response to stress.

In 1974, researchers found decreased levels of lactobacilli in stressed mice (Tannock & Savage, 1974). This finding has since been confirmed in rhesus monkeys and in rodent pups subjected to early life maternal separation stress (Bailey & Coe, 1999; De Palma et al., 2015; De Santa et al., 2024). Subsequent animal studies found that stress can affect the gut microbiata in various hosts, including cattle (Czech et al., 2022), horses (Glimstedt, 1959), pigs (Tang et al., 2022), non-human primates (Bailey & Coe, 1999; Bailey et al., 2004) and rodents (Desbonnet et al., 2010; Foster et al., 2017; S. M. O'Mahony et al., 2009, 2011) and across different stress models including acoustic stress, heat, water avoidance, overcrowding and maternal separation (Czech et al., 2022; De Santa et al., 2024; Delaroque et al., 2021; Lee et al., 2017; Z. Zhang et al., 2023). Collectively, these studies provide evidence that moderate to severe physical and psychological stressors can impact the gut microbiota composition in mammals.

Moreover, the rodent research underlying the first theme of this thesis (maternal stress and offspring microbiota development) indicates that stress even impacts the microbiota of offspring from stressed dams with potentially generational consequences (Brawner et al., 2020; Gur et al., 2019; Hantsoo et al., 2019; Jašarević et al., 2017, 2018; Yeramilli et al., 2023). Interestingly,

transferring the vaginal microbiota from stressed mother rats to non-stressed pups has been found to be sufficient to significantly change the pups' microbiota and modify their stress response later in life (Jašarević et al., 2018). This suggests that the effects of stress are mediated by transferring microbes from the dam to the offspring. As we elaborate in Chapter 2 as part of Theme 1, human studies are yet inconclusive about how maternal stress affects the infant microbiota. Our study analyzed three maternal stool samples obtained during and after pregnancy as well as four infant stool samples obtained at two, six and 12 weeks as well as eight months postpartum. In addition, we measured prenatal and postnatal maternal stress using diverse questionnaires and hair cortisol and cortisone to investigate the relations between maternal prenatal and postnatal stress and the maternal and infant microbiota. Hence, our study provides important evidence to shed light on the question whether and how prenatal (and postnatal) maternal stress is related to infant microbiota development in humans.

Lastly, it is important to highlight that microbes are not just influenced by stress. They may moderate or mediate the effects of stress on the mammalian body. For example, maternal separation is a commonly used stressor in rodents where the offspring is separated from the dam early in life. If separation occurs long enough, maternal stress leads to activation of the HPA axis in the offspring with subsequent alternations in microbiota composition, increased gut permeability and systemic inflammation, which results in an adult depressive and anxiety-like phenotype (Bailey & Coe, 1999; Desbonnet et al., 2010; Foster et al., 2017; S. M. O'Mahony et al., 2009, 2011). De Palma et al. reported that bacteria are required to induce the altered phenotype (De Palma et al., 2015). Comparing germ-free mice with specific pathogen free mice, they showed that stress leads to activation of the HPA-axis in both types of mice but the depressive and anxiety-like phenotype only occurs in mice that have gut bacteria. When colonizing germ-free mice that underwent maternal separation stress as well as a non-stressed control group of germ-free mice, they found that stressed mice have different gut microbiota composition and expressed the depressive and anxiety-like phenotype. Interestingly, the administration of specific bacteria can prevent this cascade by preventing increased gut permeability, systemic inflammation and thereby the depressive and anxiety-like phenotype (Abildgaard et al., 2017; Ait-Belgnaoui et al., 2014; Ait-Belgnaoui et al., 2012; Bravo et al., 2011; De Palma et al., 2015; Gareau et al., 2007; Lew et al., 2019; Messaoudi et al., 2011). Thus, animal studies suggest that bacteria mediate some of the negative effects of stress and that certain beneficial bacteria can protect the host from these negative effects.

Due to ethical considerations, demonstrating the effects of early life stress on the gut microbiota in humans poses challenges. Therefore, in Theme 2, we aim to explore if a naturally occurring stressor affects infant microbiota development in healthy human infants (Chapter 3). Specifically, we look at the effects of the entrance into center-based childcare at 10 weeks of age. Entering center-based childcare involves relatively early maternal separation and changes in environments and caregivers. As we summarize in Chapter 3, previous evidence found that the entrance can lead to the activation of the HPA-axis in young infants as evidenced by increased cortisol levels. Furthermore, in Chapter 4 we investigate whether a destressing intervention implemented during the first five postnatal weeks influences microbiota development. The first five weeks are crucial for both the microbiota and neurodevelopement (Borre, O'Keeffe, et al., 2014). Our studies were the first studies that investigated whether each of these early life factors was associated with alternations in gut microbiota composition and development.

1.2 The gut microbiota and the brain

Contemporary challenges persist among psychologists, behavioral scientists, and psychiatrists in effectively diagnosing, treating, and comprehensively understanding the pathogenesis of chronic conditions including bipolar disorder, major depressive disorder, schizophrenia, ADHD, and autism. Recent research exploring the interaction between intestinal microorganisms and the human body suggests a promising avenue for improving our understanding of the underlying mechanisms of these conditions and others (Alam et al., 2017; Dinan et al., 2014). For example, bacteria play a critical role in the metabolism of the amino acid tryptophan, with the most important genera being *Clostridium*, *Burkholderia*, *Streptomyces*, *Pseudomonas*, and *Bacillus* (Kaur et al., 2019). Tryptophan serves as the only precursor for serotonin, an important monoamine neurotransmitter involved in regulating central nervous system transmission and gastrointestinal functions (K. Gao et al., 2020). Additionally, tryptophan can be broken down into kynurenine, tryptamine, and indole, which play roles in influencing neuroendocrine activities and immune responses in the gut (K. Gao et al., 2020). Furthermore, serotonin synthesized in the gut can impact the permeability of the vagus nerve and the blood-brain-barrier (Alam et al., 2017), modulate gastrointestinal inflammation (De Vadder et al., 2018), secretion and peristalsis as well as other physiological functions (Strandwitz, 2018). Lastly, intestinal production of serotonin can influence central serotoninergic pathways by altering tryptamine and tryptophan supply.

The documented influence of gut bacteria on the tryptophan metabolism alone illustrates how this research can add explanatory potential to existing pathophysiologic models. However, it is only one of numerous established pathways that all add to this explanatory potential. Together these pathways are referred to as the microbiota-gut-brain-axis. Communication along this axis is bidirectional, meaning that the central nervous system also influences intestinal functioning and the gut microbiota. The pathways have been summarized extensively (Cryan et al., 2019) and describing all of them goes beyond the scope of this introduction. However, it is important to note that gut microbes can influence the brain via the immune system, the vagus nerve and the enteric nervous system as well as by producing metabolites such as branched chain amino acids and short-chain fatty acids (Cryan et al., 2019).

These insights are not only relevant for enhancing existing disease models but also for elucidating the development of fundamental cognitive functions related to learning and memory (Theme 3). Several factors are known to influence cognitive development including genetics and early caregiving and environment (Bouchard & McGue, 2003; Han et al., 2023). Microbiota-gut-brain-axis research has the potential to further improve our understanding. Animal studies have demonstrated that gut bacteria can influence cognitive functions related to learning and memory. (Alemohammad et al., 2022; Sarkar et al., 2018).

An initial study exploring the connection between bacteria and cognition revealed memory deficits and decreased levels of hippocampal BDNF in germ-free mice when contrasted with mice colonized under normal conditions (Gareau et al., 2011). Other studies found that mice and rats administered human milk oligosaccharides exhibited improved performance across various cognitive assessments, encompassing working memory, spatial learning and reinforcement-based learning when compared to rodents treated with a vehicle (Vázquez et al., 2015). Human

milk oligosaccharides are non-digestible compounds found in milk that promote the growth and activity of beneficial bacteria in the gut. These and similar compounds that can be found in food (e.g. fructo-oligosaccharides) are referred to as a prebiotic. Studies conducted in rats yielded similar results, demonstrating the positive effects of human milk oligosaccharides on novel object recognition, spatial memory, and long-term potentiation, which persisted even a year after initial exposure to the prebiotic (Oliveros et al., 2016).

Also, the administration of beneficial bacteria showed effects on cognitive functioning. For example, in a study by Wu et al., mice were given an *Akkermansia muciniphila* subtype with a high-fat diet for 10 months, leading to significantly enhanced spatial memory functioning (Wu et al., 2020). In line with these results, Yang et al. found that increased gut permeability and pro-inflammatory cytokines as well as impaired spatial learning and memory functioning induced by a high-fat diet were ameliorated by administration of *Akkermansia muciniphila* (Y. Yang et al., 2019). Also, other bacteria such as *Bifidobacterium* and *Lactobacillus* have been shown to cause improvement of depression, mood, and cognitive function and memory in animal models (Davari et al., 2013; Desbonnet et al., 2010; O'Hagan et al., 2017).

While evidence from animal models is rapidly accumulating, human studies remain limited (Alemohammad et al., 2022; Cooke et al., 2022), as summarized in Chapter 5. Specifically, no human study had yet explored the association between the gut microbiota from infancy to early childhood and childhood cognition. In Chapter 5, as part of Theme 3, our study was the first to investigate the relation between gut microbiota during the first 10 years of life and executive functioning at ages eight and 10 years.

1.3 Studies utilized in this thesis

1.3.1 SMILEY study

The SMILEY study (Study of MIcrobiota and Lifestyle in the Early Years) is a longitudinal investigation focusing on the lifestyle and well-being of pregnant women and their infants. Participants were recruited through the Baby & Child Research Center's (BRC) network of midwifery practices in the Nijmegen region in the Netherlands and through social media between December 2019 and April 2021. A total of 160 participants provided written informed consent. Data utilized for this thesis include measurements during pregnancy (at 18 and 32 weeks of gestation) and at four postnatal assessment moments (at two, six, and 12 weeks, and eight months postpartum).

1.3.2 BIBO study

The BIBO (Basal Influences on Baby Development) study is a prospective longitudinal cohort study focusing on the associations between prenatal and early-life environmental factors and the psychobiological development of children (see (Beijers et al., 2010, 2013)). A total of 193 healthy mother-offspring dyads were recruited in pregnancy. Data utilized for this thesis include multiple assessments spanning the first 10 postnatal years, including fecal samples collected at

ages one, three, and four months, six years, and 10 years, as well as information on relevant covariates obtained through questionnaires and a logbook.

1.3.3 SKIPPY study

The SKIPPY study represents the first preregistered and adequately powered randomized controlled trial aimed at assessing the efficacy of daily Skin-to-Skin Contact (SSC), in comparison to care-as-usual (CAU), across a broad spectrum of outcomes in healthy mothers and their full-term infants. The study was originally designed to partially replicate findings related to maternal postnatal depressive symptoms observed in a prior long-term SSC intervention involving healthy full-term infants (A. Bigelow et al., 2012). In the SKIPPY study, 127 mothers were randomly assigned to the treatment (SSC) and the control group (CAU). Mothers in treatment group were instructed to provide a specific SSC intervention in the first five postnatal weeks whereas mothers in the control group received no specific instructions. Infant fecal samples were obtained as a secondary outcome measure at two and five weeks as well as at one year postpartum. For the purposes of this dissertation, these samples and data spanning the first year after birth were utilized.

1.4 Thesis outline

In addition to this general introduction (Chapter 1), the thesis comprises four empirical studies organized into three themes, as outlined in Table 1.1. Chapter 2 investigates the association between prenatal and postnatal maternal stress and infant microbiota development using data from the SMILEY study. Chapter 3 examines the influence of early life stress (entrance to center-based childcare) on gut microbial colonization in healthy Dutch infants, drawing on data from the BIBO study. Chapter 4 explores the impact of a destressing intervention early in life on gut microbiota development in healthy Dutch infants, using data from the SKIPPY study. Finally, Chapter 5 investigates whether microbiota composition in the first 10 years of life predicts executive functioning measured at age eight and 10 using data from the BIBO study. Chapter 6 provides a summary and discussion of findings from all studies.

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Chapter 2

Maternal Pre- and Postnatal Stress and Maternal and Infant Gut Microbiota Features

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Eckermann, H. A., Lustermans, H., Parnanen, K. Lahti, L., & de Weerth, C. (2024). Maternal Pre- and Postnatal Stress and Maternal and Infant Gut Microbiota Features.

2.1 Abstract

Maternal stress can have short and long term adverse (mental) health effects for the mother and her child. Previous evidence suggests that the gut microbiota may be a potential mediator and moderator for the effects of stress via various pathways. This study explored the maternal microbiota trajectory during pregnancy as well as the association between pre- and postnatal maternal stress and features of the maternal and infant gut microbiota during and after pregnancy. In line with previous research, we hypothesized that maternal stress would be positively related to maternal and infant microbiota volatility and that infants of highly stressed mothers would show a relative increase in Proteobacteria and a relative decrease in *Bifidobacterium*. We collected maternal stool samples at 18 and 32 weeks of pregnancy and 8 months postpartum. Infant stools samples were obtained at 2, 6 and 12 weeks and 8 months postpartum. All samples were analyzed using shotgun metagenome sequencing. We also collected several measures of maternal stress (self-reported depression, anxiety, and stress, and hair cortisol and cortisone), most at the same time points as the microbiota samples. Our data indicated that the maternal microbiota does not undergo drastic changes from the second to the third trimester of pregnancy but that the postpartum microbiota differs significantly from the prenatal microbiota. Furthermore, we identified associations between several stress measures and the maternal and infant gut microbiota features at different time points including positive and negative associations with alpha diversity, beta diversity and individual microbial phyla and species relative abundances. Also, the maternal stress composite score, the perceived stress score and the log-ratio of hair cortisol and cortisone were all positively associated with infant microbiota volatility. Our study provides evidence that maternal prenatal and postnatal stress is related to both the maternal and the infant microbiota. Collectively, this and previous studies indicate that maternal stress does not uniformly associate with most gut microbial features. Instead, the associations are highly time point specific. Regarding infant microbiota volatility, we have consistently found a positive association between stress and infant microbiota volatility. This warrants future research investigating this link in more depth.

2.2 Introduction

A growing body of research indicates that an allostatic overload caused by acute or chronic stress during pregnancy and postpartum can raise the risk of short and long term adverse (mental) health outcomes for the mother and her child (Beydoun & Saftlas, 2008; Bussières et al., 2015; Egmose et al., 2022; Graignic-Philippe et al., 2014; Hsu & Wickrama, 2018; Van den Bergh et al., 2020; Walker et al., 2020). Animal studies showed that the gut microbiota may be a potential mediator and moderator for the effects of stress (Bailey et al., 2004; De Palma et al., 2015; Desbonnet et al., 2010; Golubeva et al., 2015; Jašarević et al., 2015, 2017; S. M. O'Mahony et al., 2009) via various pathways (Cryan et al., 2019; Kimmel et al., 2023). However, human studies investigating these associations remain scarce and limited. In this study, we explore the associations between pre- and postnatal maternal stress and features of the human maternal and infant gut microbiota during and after pregnancy.

The gut microbiota can influence host-health for instance via the gut brain axis (Cryan et

al., 2019). The maternal microbiota plays a special role. Prenatally, it informs the fetus (Kaisanlahti et al., 2023) and programs the functioning of the fetal intestine (Husso et al., 2023). Postnatally, it is the most important source of microbes for the initial colonization of the infant gut by microbes through vertical transmission (H. P. Browne et al., 2022; Valles-Colomer et al., 2022, 2023; Van Daele et al., 2019; S. Wang et al., 2020). Accordingly, variables that affect the maternal microbiota, such as prenatal stress, may also directly or indirectly affect the health of the offspring (Kimmel et al., 2023) and should be explored and defined. In the following, we briefly summarize the status of the literature on maternal microbiota studies in relation to pregnancy and then maternal and infant microbiota studies in relation to prenatal and postnatal stress.

Previous literature indicates that the maternal gut microbiota does not undergo significant changes during pregnancy (DiGiulio et al., 2015; H. Yang et al., 2020), although one study reported drastic changes (Koren et al., 2012). A recent study suggests that changes may depend on host factors such as pre-pregnancy BMI (pBMI) and parity (Kennedy et al., 2022); more specifically, the gut microbiota would change more in primiparous women with low pBMI, and less in multiparous women or women who have a high pBMI. Altogether, these studies leave remaining uncertainty as to whether the maternal gut microbiota changes because of pregnancy, whether it returns to its pre-pregnancy state afterwards and whether this depends on host characteristics. Addressing this uncertainty may help shed light for understanding the short- and long-term implications of pregnancy for maternal health.

Although a link between stress and the gut microbiota has been established in non-pregnant individuals (Leigh et al., 2023), prenatal stress has barely been explored as a factor that may potentially influence the maternal gut microbiota during pregnancy. To our knowledge, there are only two previous human studies that investigated associations between prenatal stress and the maternal gut microbiota during pregnancy (Hechler et al., 2019; Naudé et al., 2020). These studies were limited to one stool sample per mother in late pregnancy. Furthermore, they had relatively low sample sizes (N = 70; N = 84) and a limited taxonomic resolution at genus level (based on 16S rRNA sequencing), as well as measures of stress exclusively based on self-report. Nonetheless, these studies found that fecal specimens from mothers exposed to intimate partner violence had higher proportions of the family Lactobacillaceae and lower proportions of Peptostreptococcaceae at birth (Naudé et al., 2020) and that maternal general anxiety (State-Trait-Anxiety Inventory; STAI (Spielberger, 1989)) was related to overall microbiota composition, mainly driven by differences in *Parasutterella*, *Staphylococcus*, *Rothia*, *Oxalobacter* and several bacteria belonging to the class of Clostridia (Hechler et al., 2019).

More studies examined the associations between maternal prenatal stress and the infant gut microbiota (Dutton et al., 2023; Galley et al., 2023; Mepham et al., 2023; Weiss & Hamidi, 2023; Zijlmans et al., 2015). Collectively, they identified associations between prenatal stress and infant microbiota features including alpha- and beta diversity, as well as relative abundances of individual genera. Besides several unique findings, five out of 10 studies found a positive association between prenatal stress and Proteobacteria (one study found the opposite; reviewed in Mepham et al. (2023)). Also, four out of 10 studies found a negative association with *Bifidobacterium* (Dutton et al., 2023; Galley et al., 2023; Jahnke et al., 2021; Zijlmans et al., 2015), while one found the opposite (Weiss & Hamidi, 2023). Studies found both positive (Dutton et al., 2023; Rojas et al., 2023; Zijlmans et al., 2015) and negative (Galley et al., 2023; Jahnke

et al., 2021) associations with alpha diversity. Prenatal stress was defined heterogeneously between studies and time points of stress measurement and stool sampling varied considerably, although most studies included an infant stool sample taken at around two to three months of age. These differences impede reproducibility of findings. Moreover, Rojas et al. (2023) and Van den Bergh et al. (2020) report measurement- and trimester-specific associations between prenatal stress and the infant gut microbiota and other outcome measures, respectively. This implies that reproducibility of findings may have been further hampered by the possibility that the effects of prenatal stress may be trimester specific. Lastly, most of these studies (nine out of 10) were limited to a taxonomic resolution at the genus level (due to the use of 16S rRNA sequencing) and only two out of 10 studies collected more than one infant stool sample (Dutton et al., 2023; Zijlmans et al., 2015).

In sum, there is a lack of studies focusing on the association between prenatal stress and the maternal microbiota during pregnancy. Across mother and infant microbiota studies, differences between studies related to the stress measurements utilized and the time points of stress and microbiota sampling necessitate a larger number of studies and standardization of research methodology to disentangle the association between prenatal stress and the maternal and infant gut microbiota. Moreover, studies are yet to investigate the magnitude of intra-individual shifts, referred to as microbiota volatility (T. Bastiaanssen et al., 2021), even if repeated stool sampling was performed. Microbiota volatility has recently been positively linked to stress (T. Bastiaanssen et al., 2021). While it remains unclear whether stress causes a more volatile microbiota or vice versa, a recently published randomized controlled trial found that a destressing intervention reduced microbiota volatility in infants (Eckermann et al., 2024). More research is needed to determine whether volatility is a biomarker of stress, which factors influence it and how it relates to other health outcome measures. The present study on healthy low-risk mothers and their infants used shotgun metagenomic sequencing to analyze three stool samples of the mother, at 18 and 32 weeks of gestation and at eight months postpartum, and four infant stool samples in the first eight months of life. Maternal stress was measured at all these time points using different self-report questionnaires. Additionally, maternal stress was objectively assessed by means of two hair cortisol samples reflecting chronic stress pre- and postnatally.

Our data allow an in-depth exploration of the associations between pre- and postnatal stress and the maternal and infant gut microbiota by addressing the following research questions: 1) Can we observe changes in the microbiota from the second to the third trimester of pregnancy and does the microbiota at eight months postpartum differ from the microbiota during pregnancy? 2) How is maternal prenatal and postnatal stress related to the maternal gut microbiota during and after pregnancy? We explore how our results compare to previously published findings by investigating alpha diversity, beta diversity and phylum and species level abundances. We furthermore hypothesize that prenatal stress is positively related to maternal gut microbiota volatility based on previous findings (T. Bastiaanssen et al., 2021; Eckermann et al., 2024). 3) Does maternal pre- and postnatal stress associate with the infant gut microbiota development? Our results will add to the existing evidence and help to disentangle the association between prenatal stress and infant gut microbiota development. We hypothesize that prenatal maternal stress will be positively related to Proteobacteria and negatively related to Lactobacilli and Actinobacteria, specifically *Bifidobacterium* based on previous research. Furthermore, we hypothesize that infants of mothers who experience more stress pre- and postnatally have a more

volatile gut microbiota.

2.3 Methods

2.3.1 Participants

Participants are mother-infant dyads from the ongoing longitudinal SMILEY study (Study of MIcrobiota and Lifestyle in the Early Years), which investigates lifestyle and well-being of pregnant women in relation to infant development (Epstein et al., 2024; Lustermans et al., 2024). Pregnant women were recruited through the Baby & Child Research Center's network of midwifery practices in the Netherlands and via social media, between December 2019 and April 2021. Inclusion criteria were $\geq$ 18 years of age, mastery of the Dutch language, singleton pregnancy, and pre-pregnancy BMI $\leq$ 30, and exclusion criteria were severe obstetric complications, and (severe) mental or physical health issues (i.e. mental health problems that require psychiatric treatment and/or medication). Inclusion criteria for the infant were born at $\geq$ 37 weeks of pregnancy, birth weight of $\geq$ 2500 g, and a 5-min Apgar score of $\geq$ 7. The study was approved by the Ethics Committee of the Faculty of Social Sciences of the Radboud University under the blanket research line 'pregnancy-4years Developmental Psychobiology Lab' (SW2017-1303-497), including two amendments (ECSW-2019-051 and ECSW2020-021). In total, 160 participants enrolled in the study by providing written informed consent. The current study contains data from both measurements in pregnancy (18 and 32 weeks of gestation) and four measurements after birth (2, 6 and 12 weeks and 8 months postpartum). Supplementary Figure 2.6 shows a flow-chart of participant numbers at each assessment moment. Note that some participants were excluded after completing one or more measurement rounds. Table 2.1 shows demographic data for all infant-mother dyads that were included in the current study (i.e. that provided at least one maternal or infant stool sample).

Table 2.1: Descriptives of demographics, maternal stress variables and covariates (N=151)

	Mean (SD)/ N (%)	Median [Min, Max]	Missing
Demographics			
M. age (in years)	32.0 (3.60)	32.0 [22.4, 41.7]	0 (0.0 %)
M. education			
High	130 (86.1%)		0 (0.0 %)
Low/medium	21 (13.9%)		
Parity			
First born	74 (49.0%)		0 (0.0 %)
I. sex			
Boy	71 (47.0%)		9 (6.0%)
Girl	71 (47.0%)		
I. birthweight (in grams)	3540 (414)	3530 [2570, 4840]	9 (6.0%)
I. gestational age at birth (w)	40.1 (1.09)	40.0 [37.9, 42.7]	9 (6.0%)

I. age 2 w postpartum (d)	15.6 (7.06)	14.0 [10.0, 70.0]	34 (22.5%)
I. age 6 w postpartum (d)	46.3 (7.11)	45.0 [14.0, 84.0]	32 (21.2%)
I. age 12 w postpartum (d)	86.7 (10.8)	87.0 [14.0, 150]	17 (11.3%)
I. age 32 w postpartum (d)	248 (23.7)	242 [204, 320]	42 (27.8%)
M. gestational week 18 w gestation	19.5 (1.43)	18.7 [17.3, 24.6]	0 (0%)
M. gestational week 32 w gestation	31.3 (1.19)	31.3 [29.1, 35]	1 (0.7%)
Maternal Stress Variables			
EPDS 18 w gestation	4.52 (4.09)	3.00 [0, 21.0]	0 (0.0 %)
EPDS 32 w gestation	5.31 (4.38)	5.00 [0, 24.0]	1 (0.7%)
EPDS 32 w postpartum	4.64 (4.67)	4.00 [0, 29.0]	44 (29.1%)
STAI 18 w gestation	32.5 (8.86)	31.0 [20.0, 57.0]	0 (0.0 %)
STAI 32 w gestation	32.9 (8.80)	32.0 [20.0, 75.0]	1 (0.7%)
STAI 32 w postpartum	31.8 (9.50)	31.0 [20.0, 80.0]	44 (29.1%)
PSS-10 18 w gestation	11.0 (5.51)	11.0 [2.00, 28.0]	0 (0.0 %)
PSS-10 32 w gestation	11.9 (5.42)	11.0 [0, 27.0]	1 (0.7%)
PSS-10 32 w postpartum	11.0 (5.84)	10.0 [0, 33.0]	45 (29.8%)
PRAQR2-B 18 w gestation	5.91 (2.68)	5.00 [3.00, 15.0]	0 (0.0 %)
PRAQR2-B 32 w gestation	6.09 (2.51)	6.00 [3.00, 14.0]	1 (0.7%)
PRAQR2-H 18 w gestation	8.56 (3.07)	8.00 [4.00, 15.0]	0 (0.0 %)
PRAQR2-H 32 w gestation	7.95 (2.91)	8.00 [4.00, 19.0]	1 (0.7%)
PSAS-RSF-C 2 w postpartum	19.6 (4.28)	19.0 [12.0, 35.0]	10 (6.6%)
PSAS 6 w postpartum	80.7 (17.5)	78.0 [53.0, 157]	10 (6.6%)
PSAS-RSF-C 12 w postpartum	19.2 (4.55)	18.0 [11.0, 39.0]	12 (7.9%)
Cortisol (log) 6-15 w gestation	0.120 (1.11)	0.250 [-3.48, 4.02]	43 (28.5%)
Cortisol (log) 15-23 w gestation	0.628 (0.944)	0.645 [-2.39, 5.18]	40 (26.5%)
Cortisol (log) 23-32 w gestation	1.23 (0.630)	1.21 [-0.863, 4.12]	40 (26.5%)
Cortisol (log) 4-8 w postpartum	0.977 (0.971)	1.15 [-2.41, 2.72]	64 (42.4%)
Cortisone (log) 6-15 w gestation	0.918 (0.946)	1.01 [-2.03, 2.49]	40 (26.5%)
Cortisone (log) 15-23 w gestation	1.69 (0.836)	1.83 [-1.27, 3.02]	40 (26.5%)
Cortisone (log) 23-32 w gestation	2.64 (0.502)	2.65 [-0.232, 3.67]	40 (26.5%)
Cortisone (log) 4-8 w postpartum	2.04 (0.818)	2.16 [-0.412, 3.67]	60 (39.7%)
Log-ratio HCS/HCN 6-15 w gestation	-0.842 (1.06)	-0.892 [-5.59, 2.70]	43 (28.5%)
Log-ratio HCS/HCN 15-23 w gestation	-1.06 (0.739)	-1.16 [-2.76, 2.93]	40 (26.5%)
Log-ratio HCS/HCN 23-32 w gestation	-1.41 (0.510)	-1.39 [-4.20, 1.37]	40 (26.5%)
Log-ratio HCS/HCN 4-8 w postpartum	-1.14 (0.615)	-1.10 [-3.19, 0.171]	64 (42.4%)
Other Variables			
Pre-pregnancy BMI	23.0 (2.75)	22.5 [17.3, 30.4]	1 (0.7%)
Physical activity 18 w gestation	4.09 (2.03)	4.00 [0, 11.0]	0 (0.0 %)
Physical activity 32 w gestation	3.79 (2.06)	4.00 [0, 14.0]	1 (0.7%)
Dutch Healthy Diet index 18 w gestation	89.5 (16.7)	90.4 [47.3, 127]	0 (0.0 %)
Dutch Healthy Diet index 32 w gestation	87.3 (17.6)	90.1 [38.8, 122]	1 (0.7%)
Presence of pet(s)			
No	76 (50.3%)		0 (0.0 %)
Yes	75 (49.7%)		

M. antibiotics use sample M1		
No	130 (86.1%)	19 (12.6%)
Yes	2 (1.3%)	
M. antibiotics use sample M2		
No	144 (95.4%)	6 (4.0%)
Yes	1 (0.7%)	
M. antibiotics use sample M3		
No	95 (62.9%)	56 (37.1%)
Yes	0 (0.0%)	
Delivery mode		
vaginal	125 (82.8%)	9 (6.0%)
c-section	17 (11.3%)	
I. antibiotics use sample I1		
No	138 (91.4%)	10 (6.6%)
Yes	3 (2.0%)	
I. antibiotics use sample I2		
No	134 (88.7%)	16 (10.6%)
Yes	1 (0.7%)	
I. antibiotics use sample I3		
No	139 (92.1%)	10 (6.6%)
Yes	2 (1.3%)	
I. antibiotics use sample I4		
No	137 (90.7%)	14 (9.3%)
Yes	0 (0%)	
I. feeding sample I1		
Breastmilk	91 (60.3%)	10 (6.6%)
Formula	6 (4.0%)	
Mixed	44 (29.1%)	
I. feeding sample I2		
Breastmilk	103 (68.2%)	10 (6.6%)
Formula	13 (8.6%)	
Mixed	25 (16.6%)	
I. feeding sample I3		
Breastmilk	96 (63.6%)	10 (6.6%)
Formula	20 (13.2%)	
Mixed	25 (16.6%)	
I. feeding sample I4		
Breastmilk	28 (18.5%)	43 (28.5%)
Formula	27 (17.9%)	
Mixed	53 (35.1%)	

Note:
M. = Maternal. I. = Infant. w = weeks. d = days. HCS = Hair cortisol. HCN = Hair cortisone. Samples M1-M3 = Time point of maternal microbiota sampling in chronological order (18 and 32 weeks of gestation and 8 months postpartum). Samples I1 - I4 = Time point of infant microbiota sampling in chronological order (2, 6 and 12 weeks and 8 months). PRAQR2-B = Fear of giving birth. PRAQR2-H = fear of a handicapped child. Missing information on infant sex can be explained by the fact that some participants only completed the first two measurement rounds (see supplementary Figure 2.6).

2.3.2 Procedure

This study is based on multiple assessments that took place at 18 and 32 weeks of gestation and 2, 6 and 12 weeks and 8 months postpartum. At each wave, participants were asked to complete online questionnaires measuring maternal stress and potential covariates (e.g. demographics, maternal diet, antibiotic use). Also, at 18 and 32 weeks of gestation and 8 months postpartum, mothers collected a fecal sample of themselves. At 2, 6 and 12 weeks and 8 months postpartum, mothers collected a fecal sample of their infant. For each fecal sample, a paper questionnaire was completed by the mother to assess maternal and/or infant health and medication use. In addition, around 32 weeks of gestation and 8 weeks postpartum a researcher collected a maternal hair sample for hormone assessments during a laboratory visit.

2.3.3 Measures

2.3.3.1 Fecal samples

Mothers were instructed to collect a stool sample of themselves and their infant at different time points at home. After collection, they were instructed to store the sample immediately in their own freezer, at ± -20 °C. Participants brought the samples to the lab in a cool box with ice when visiting our lab or a researcher picked up the samples at home with a mobile ± -18 °C freezer. In the lab, the samples were first stored in a -20 °C freezer and within a few weeks switched to a -80 °C freezer, where they were kept until being sent to Baseclear (BaseClear BV, The Netherlands) for analysis (see supplementary methods for microbial DNA extraction and taxonomic profiling). The number of collected stool samples per time point are shown in supplementary Table 2.2. Among the infants, their mothers collected all four samples for 92 infants, three samples for 43 infants, two samples for eight infants, and one sample for one infant. Among the mothers, 97 provided all samples followed by 50 who provided two and four who provided only one sample.

2.3.3.2 Hair cortisol

Maternal hair cortisol and cortisone was used as a biomarker of physiological stress, capturing cortisol and cortisone secretion over longer periods of months. During the laboratory visit, a trained researcher cut a hair sample of around 100-150 hairs from the posterior vertex of the

Table 2.2: Number of stool samples per time point.

Origin	Stage	Week	n	Abbreviation
Mother	Prenatal	18	150	M1
Mother	Prenatal	32	147	M2
Mother	Postnatal	32	98	M3
Infant	Postnatal	2	139	I1
Infant	Postnatal	6	136	I2
Infant	Postnatal	12	139	I3
Infant	Postnatal	32	100	I4

Table 2.3: Number of hair cortisol samples, corresponding period of cortisol and cortisone production and abbreviations used throughout the manuscript for these samples.

Stage	Collection week (M ± SD)	Measured period (weeks)	n	Cortisol	Cortisone	Log-ratio
Prenatal	32.28 ± 1.52	6 - 15	108	HCS1	HCN1	HCR1
Prenatal	32.28 ± 1.52	15 - 23	111	HCS2	HCN2	HCR2
Prenatal	32.28 ± 1.52	23 - 32	111	HCS3	HCN3	HCR3
Postpartum	8.19 ± 1.17	4 - 8	87	HCS4	HCN4	HCR4

head of the mothers, as close as possible to the scalp. From the sample taken at 32 weeks pregnancy we analyzed three segments of two centimeters as measured from the scalp. From the sample taken at 8 weeks postpartum we used a 1 cm segment. On average hair grows one centimeter a month. Accordingly, the different segments reflect different periods of the hormones exposure prenatally and postnatally as shown in Table 2.3. Table 2.3 furthermore introduces abbreviations that represent these different periods for simplicity. The samples were packed individually in aluminum in an envelope and stored until all samples were collected. Thereafter, they were sent out for analysis to the Dresden LAB Service, Germany. The hormone concentrations of the samples were assayed using a column-switching Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS). Due to the COVID-19 measures that were being applied at the moment of data collection, several lab visits had to be cancelled. This resulted in a relatively high number of missing data for the hair samples (Table 2.1).

2.3.3.3 Stress questionnaires

2.3.3.3.1 EPDS The Edinburgh Postnatal Depression Scale (EPDS, McDonald's ω ranged between .88 - .94) (Cox et al., 1987)) was used to measure depressive symptoms over de last seven days. It consists of 10 items, reported on a four-point scale, translating to a score of zero to three points. Total scores can range from zero to 30, with a higher score indicating more depressive symptoms. A score of 14 and higher is considered "probable depression". An example item of the EPDS is: "I have been able to laugh and see the funny side of things" (reversed item).

2.3.3.3.2 STAI-state The State-Trait Anxiety Inventory (STAI, McDonald's ω ranged between .93-.96) was used to measure how anxious the participant was feeling at the moment. This subscale consists of 20 items, reported on 4-point scales ranging from one to four. Total scores can range from 20 to 80, with a higher score indicating more experienced anxiety. A score of 45 or higher is considered "high anxiety". An example item of the STAI-state is: "I feel calm" (reversed item).

2.3.3.3.3 PSS-10 The Perceived Stress Scale-10 (PSS-10, McDonald's ω ranged between .89-.93) was used to measure how stressful certain situations were perceived over the previous month. It consists of 10 items, reported on a five-point scale, with answers ranging from "never" (zero) to "often" (four). Total scores can range from zero to 40, with a higher score indicating more experienced stress. A score of 27 or higher is considered "high perceived stress". An example item of the PSS-10 is: "In the last month, how often have you felt confident about your ability to handle your personal problems?" (reversed item).

2.3.3.3.4 PRAQR2 Two subscales of the Pregnancy Related Anxiety Questionnaire-Revised (PRAQR2; McDonald's ω ranged between .80-.85) namely 'Fear of giving birth' (three items; PRAQR2-B) and 'Worries about bearing a handicapped child' (four items; PRAQR2-H), were used to measure pregnancy specific anxiety symptoms. The items were answered on a five-point Likert scale, with a range from one (definitely not true) to five (definitely true), reflecting how the participant felt during that pregnancy. Sum scores on the two scales ranged from three to 15 and four to 20, respectively. A higher score was an indication of more pregnancy-specific anxiety. An example item of the subscale 'Fear of giving birth' is "I am anxious about the delivery" and an example of the subscale 'Worries about bearing a handicapped child' is "I am afraid the baby will be mentally handicapped or will suffer from brain damage".

2.3.3.3.5 PSAS The Postpartum Specific Anxiety Scale (PSAS, McDonald's $\omega = .95$) was used to measure specific anxiety symptoms during the postpartum period. It consists of 51 items, reported on a four or five-point scale. The total score can range from 51 to 204, with a higher score indicating more postpartum anxiety. A score of 112 or higher is considered "a clinical level of anxiety". In addition, to reduce participant burden, the 12-item PSAS-Research Short Form – Crisis (RSF-C, McDonald's ω ranged from .81-.83) was used as well (Silverio et al., 2021). Total scores range from 12 to 48, and a score of 26 and higher is proposed to be the cut-off score for a clinical level of anxiety. An example item of both the PSAS and the PSAS-RSF-C is: "I have worried more about my relationship with my partner than before my baby was born". At two and 12 weeks postpartum, the PSAS-RSF-C was completed, while at six weeks postpartum the PSAS was completed.

2.3.3.3.6 Maternal stress composite We created a composite stress score (maternal stress; MS) from three highly correlated (supplementary figures 2.7-2.8) questionnaires (EPDS, PSS-10, STAI) to reduce the complexity of the analysis and reporting. However, to alleviate

the issue of reproducibility due to the use of different stress measurements, we also ran all analyses with the single stress measurements and reported if results differed meaningfully from the composite score. The PRAQR2 (prenatal) and the PSAS (postnatal) were left out of the MS variable because otherwise the composite score would represent a different measure pre- and postnatally.

2.3.3.4 Potential Covariates

All the following covariates were obtained via questionnaires during the measurement rounds as described in the Procedures section. Maternal age was calculated in years, based on the age at the measurement at 18 weeks of pregnancy. Maternal education included seven categories (one to seven), with categories ≥ 6 reflecting high education (degree of applied sciences or university) and categories ≤ 5 reflecting low/medium education. Maternal pre-pregnancy body mass index (pBMI) was based on the self-reported weight and length prior to the current pregnancy. A score for maternal diet quality was available at 18 weeks and 32 weeks of gestation. Specifically, a food frequency questionnaire (Looman et al., 2017; Siebelink et al., 2011; Streppel et al., 2013) was obtained and used to calculate the total score of the Dutch Healthy Diet index (Looman et al., 2017). A higher score reflected a higher total diet quality. A maternal activity score was available at 18 weeks and 32 weeks of gestation. This score was based on the total score of the Pregnancy Physical Activity Questionnaire (Chasan-Taber et al., 2004). A higher score indicated more physical activity. Antibiotic use was included as a dichotomous variable and reflected whether the mother or infant had used any antibiotics at the day or the week before taking the stool sample. Furthermore, parity, infant sex, gestational age, delivery mode and feeding mode (i.e. exclusively breastfed, formula fed or mixed) were available to include as covariate. Covariate selection is further explained in the Statistical analysis section.

2.3.4 Statistical analysis

The statistical analyses were performed in R (version 4.3.1) (R Core Team, 2022). All analyses were preregistered (https://doi.org/10.17605/OSF.IO/CKE84) and the code is openly available with a permanent DOI (https://doi.org/10.5281/zenodo.11611111). Missing covariates were imputed using predictive mean matching (m = 50) (Kleinke, 2017) using the *mice* (v3.16.0) package (van Buuren & Groothuis-Oudshoorn, 2011). Deviations of results from complete case analyses are reported. Several features of the gut microbiome were examined, including alpha diversity, beta diversity, species and phylum level relative abundances and volatility. For alpha diversity, we calculated Faith and Shannon diversity indices using the *mia* (v1.11.4) package (Ernst et al., 2022). For beta diversity, we used Aitchison distance (Euclidean distances of centered-log-ratio transformed abundances) and we report if results deviate if Bray-Curtis is used instead. Volatility was calculated as described by Bastiaanssen et al. (T. Bastiaanssen et al., 2021). For differential abundance analyses we used *MaAsLin2* (Mallick et al., 2020), which has performed well according to multilple benchmark studies (Nearing et al., 2022; Pelto et al., 2024). Results were corrected for multiple testing using the Benjamini–Hochberg procedure (Benjamini & Hochberg, 1995). All adjusted p-values can be found in the column q in the supplementary tables.

We used Bayesian robust linear models to investigate associations between the maternal stress variables and alpha diversity and volatility using the *brms* (v2.21.0) package (Bürkner, 2018) with default priors and a student t distribution for the response variable. In these Bayesian models we reject the null hypothesis if the 95% highest probability density interval (HDI) excludes zero for non-directional hypotheses or if more than 95% of the posterior distribution is larger or smaller than zero, respectively, for directional hypotheses (indicated by $P(\beta \geq 0)$ in text). For beta diversity analyses we applied PERMANOVA with the *adonis2* function from the *vegan* (v2.6.4) package (Oksanen et al., 2022). We accounted for non-independence by specifying random intercepts in models that included repeated samples of an individual and additionally performed analyses per time point. To determine the model structure, we created directed acyclic graphs (DAG) for the infant and mother samples separately (supplementary figures 2.9-2.10). We tested conditional independence and adjusted the DAG if necessary. DAGs graphically represent the knowledge and assumptions of the analyst. Some of the assumptions can be tested (testing conditional independence). Given these assumptions, the DAG dictates which covariates must be included to answer the research questions of interest (Cinelli et al., 2020). While some covariates need to be included to reduce bias (i.e. physical activity, parity and maternal age and education for the maternal samples and delivery mode, gestational age and parity for the infant samples), others can optionally be included (in this case pBMI for the maternal samples and maternal education, feeding mode, gestational age, and child sex for the infant samples). We used leave-one-out cross-validation (Vehtari et al., 2017) to decide whether such optional covariates should be included to improve model fit.

2.4 Results

2.4.1 1) Can we observe changes in the microbiota from the second to the third trimester and does the microbiota at 8 months postpartum differ from the microbiota during pregnancy?

2.4.1.1 Alpha diversity

We did not detect changes in the alpha diversity during pregnancy. However, we observed that Faith diversity decreased 8 months postpartum compared to at 18 (β = -1.851, 95% HDI = [-2.387; -1.337], $P(\beta \geq 0) = 0$) and 32 (β = -1.562, 95% HDI = [-2.073; -1.043], $P(\beta \geq 0) = 0$) weeks of gestation on average. A similar trend was observed when looking at the difference in Shannon diversity between 8 months postpartum and 18 (β = -0.081, 95% HDI = [-0.162; 0.008], $P(\beta \geq 0) = 0.03$) and 32 (β = -0.055, 95% HDI = [-0.14; 0.032], $P(\beta \geq 0) = 0.104$) weeks of gestation (Figure 2.1).

2.4.1.2 Beta diversity and differential abundance analysis

Similarly, we did not find evidence that beta diversity is different between 18 weeks and 32 weeks of gestation. Beta diversity at eight months postpartum differs significantly compared to

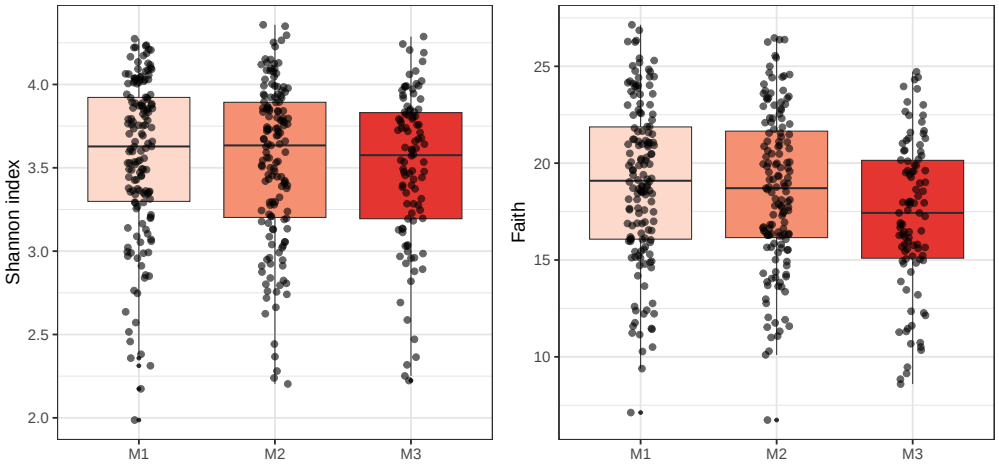

Figure 2.1: Shannon and Faith alpha diversity across time points for maternal microbiota samples. Samples were taken at 18 (M1) and 32 (M2) weeks of pregnancy and at 8 months postpartum (M3). Alpha diversity dropped significantly from M1 and M2 to M3 for both indices.

18 weeks ($p = 0.008$) and the 32 weeks ($p = 0.035$) of gestation. However, effect sizes were very small ($R^2 \leq .01$) and there is no visible separation from maternal samples within pregnancy and postpartum (supplementary Figure 2.11). Looking at individual species, we only found a small increase in *Bifidobacterium pseudocatenulatum* between 18 and 32 weeks of gestation (FDR = .039). We found many individual species to be differentially abundant (FDR $\leq$ 0.1) between 32 weeks of pregnancy and eight months postpartum (supplementary Table 2.4). Mainly we observed a decrease in *Akkermansia muciniphila* and *Anaerostipes hadrus* as well as an increase in *Fusicatenibacter saccharivorans* and an unclassified species of *Ruminococcaceae*. Lastly, in the beta diversity models, we observed an interaction between parity and pBMI, such that pBMI was only associated with microbiota composition in multiparous mothers.

2.4.2 2) How is maternal prenatal and postnatal stress related to the maternal gut microbiota during and after pregnancy?

2.4.2.1 Alpha diversity

Prenatally, our data indicated no or only a weak association between MS and the alpha diversity measures (supplementary Table 2.5). However, we found a negative association between the PRAQR2-B (fear of giving birth) with both the Shannon (β = -0.251, 95% HDI = [-0.388; -0.112], $P(\beta \geq 0) = 0$) and Faith index (β = -0.125, 95% HDI = [-0.239; -0.009], $P(\beta \geq 0) = 0.018$) at 18 weeks but not 32 weeks of pregnancy (Figure 2.2A).

Postnatally, we only detected an association between the maternal stress composite (MS) and alpha diversity when exploring non-linear associations by segmenting the predictor variable into low, medium and high stress. Specifically, we found that the difference in alpha diversity between the medium and high stress group varied closely around zero, indicating that these groups do not differ in alpha diversity. However, the low stress group had consistently lower alpha diversity than the medium and high stress groups. While the HDIs for most contrasts overlapped slightly with zero, the model indicated that Shannon index in low stress mothers is lower at 8 months postpartum (β = -0.487, 95% HDI = [-0.888; -0.065], $P(\beta \geq 0) = 0.01$) compared to the medium stress group (Figure 2.B). The effect size for the contrast with the high stress group is similar but the HDI is wider due to the smaller group sizes of low and high stress (β = -0.459, 95% HDI = [-1.05; 0.126], $P(\beta \geq 0) = 0.063$)

For the time point at 8 months postpartum we also investigated whether the PSAS was related to alpha diversity and found that the directions of the estimates were in line with the association reported for the prenatal PRAQR2-B (fear of giving birth) for Shannon (β = -0.097, 95% HDI = [-0.318; 0.123], $P(\beta \geq 0) = 0.188$) and Faith diversity (β = -0.155, 95% HDI = [-0.36; 0.047], $P(\beta \geq 0) = 0.068$). However, we could not reject the null hypothesis for the PSAS.

Looking at the hair cortisol and cortisone concentrations, our models indicate no, or a weak association between most hormone measures and alpha diversity (supplementary Table 2.5). However, the log-ratio of cortisol and cortisone (HCR2) was positively related to both alpha diversity measures at 18 weeks of gestation (β = 0.234, 95% HDI = [0.029; 0.436], $P(\beta \geq 0) =$ 0.987; Figure 2.2C; β = 0.192, 95% HDI = [0.024; 0.358], $P(\beta \geq 0) = 0.988$; Figure 2.2D).

2.4.2.2 Beta diversity and differential abundance analysis

We did not find evidence that any of the stress variables was associated with beta diversity. In addition, there were only a few associations between the stress variables and individual species, including *Blautia* for the PSS-10 and *Bacteroides cellulosilyticus* for hair cortisone (HCN3). Supplementary tables 2.6-2.7 list the coefficients of all the single species that were associated with any of the stress variables ($FDR \leq 0.1$) across all time points (supplementary Table 2.6) and per time point (supplementary Table 2.7).

2.4.2.3 Volatility

Lastly, MS was not associated with the maternal microbiota volatility between 18 and 32 weeks of pregnancy and 32 weeks of pregnancy and 8 months postpartum (β = -0.018, 95% HDI = [-0.128; 0.1], $P(\beta \geq 0) = 0.381$), also not for any of the separate questionnaires (including PRAQR2 and PSAS) or hair cortisol/cortisone. The microbiota volatility from 32 weeks of pregnancy to 8 months postpartum was significantly higher than the volatility between the second and the third trimester (β = 0.722, 95% HDI = [0.532; 0.905], $P(\beta \geq 0) = 1$) as would be expected due to the longer time interval between taking the samples.

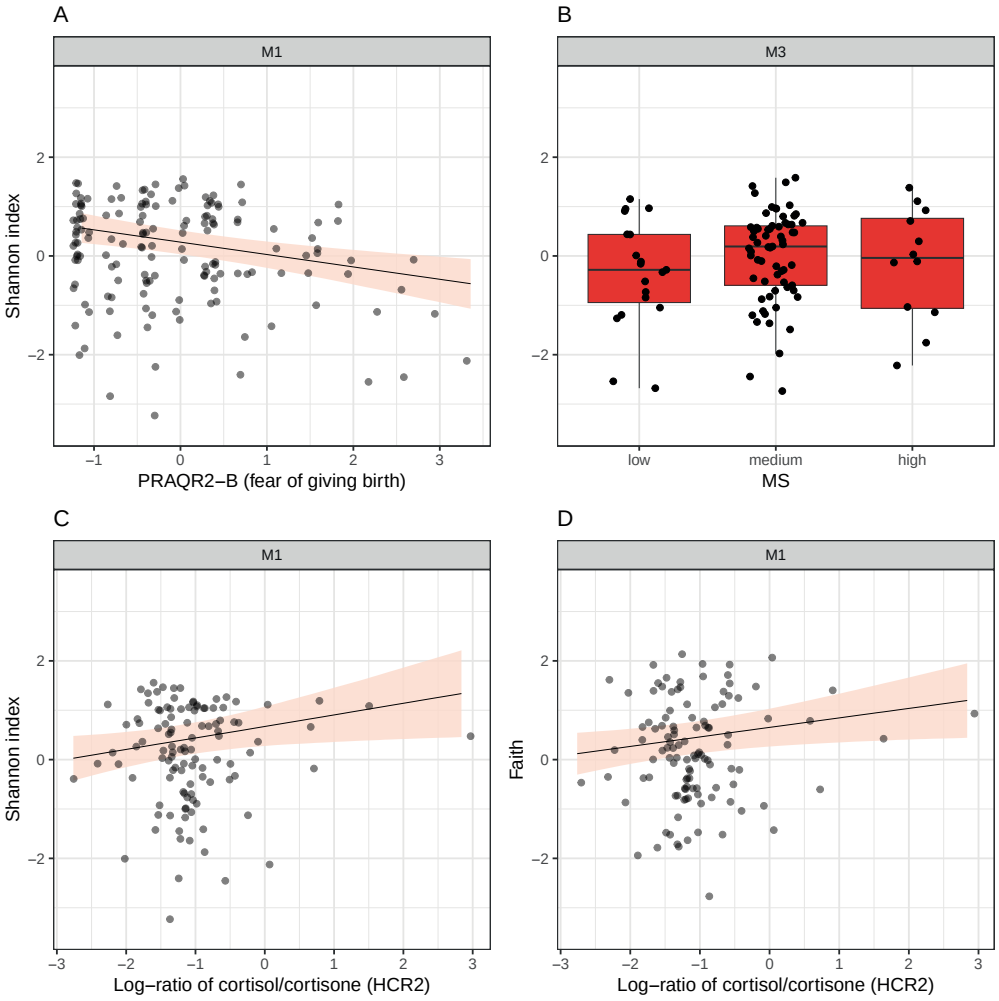

Figure 2.2: Shannon alpha diversity for maternal microbiota samples obtained at 18 weeks of pregnancy (M1) and at 8 months postpartum (M3) plotted with the PRAQR2-B (fear of giving birth; A) and the MS (i.e. composite score based on EPDS, STAI, PSS-10; B). For the plot with PRAQR2-B, we added random noise to avoid overplotting of the data points. The regression line and the colored shaded region illustrate our slope estimate including the 95% credible interval. We used the median for all covariates to generate the posterior distribution for the slope parameter.

2.4.3 3) Does maternal pre- and postnatal stress associate with the infant gut microbiota development?

2.4.3.1 Alpha diversity

While we did not find a significant association between the MS composite score measured at 18 weeks of pregnancy, the STAI was negatively associated with infant Shannon diversity at 2 weeks postpartum (β = -0.169, 95% HDI = [-0.331; -0.007], $P(\beta \geq 0) = 0.022$). Also, the PRAQR2-H (fear of a handicapped child) at 32 weeks of pregnancy was positively associated with Shannon diversity at 2 weeks postpartum (β = -0.184, 95% HDI = [-0.34; -0.03], $P(\beta \geq 0) = 0.011$), see also supplementary Table 2.8. In contrast, we found a positive association between MS (β = 0.054, 95% HDI = [0.001; 0.11], $P(\beta \geq 0) = 0.974$) and specifically the EPDS (β = 0.144, 95% HDI = [0.001; 0.281], $P(\beta \geq 0) = 0.978$) measured at 32 weeks of pregnancy and Faith diversity at 6 weeks postpartum and between PRAQR2-B (fear of giving birth) measured at 18 and 32 weeks of pregnancy and Shannon diversity at 12 weeks postpartum (β = 0.185, 95% HDI = [0.033; 0.339], $P(\beta \geq 0) = 0.99$).

Furthermore, we identified a negative association between hair cortisone (HCN1) and Faith diversity at eight months postpartum (β = -0.349, 95% HDI = [-0.612; -0.091], $P(\beta \geq 0)$ = 0.004), with similar trends seen also for hair cortisone (HCN2) as well as when using Shannon diversity (see supplementary Table 2.8). Also, the log-ratio of hair cortisol and cortisone (HCR2) was positively related to Shannon diversity at 8 months postpartum (β = 0.387, 95% HDI = [0.022; 0.749], $P(\beta \geq 0) = 0.982$). Figure 2.3 shows all associations where we rejected the null hypothesis.

Our data further indicated that there is no or only a weak association between postnatal maternal stress variables and alpha diversity at most time points in the infant samples (see supplementary Table 2.9 for the corresponding β coefficients of all time points and stress measures). However, we found negative associations between Shannon diversity and EPDS (β = -0.247, 95% HDI = [-0.458; -0.036], $P(\beta \geq 0) = 0.011$) and PSS-10 (β = -0.221, 95% HDI = [-0.429; -0.006], $P(\beta \geq 0) = 0.021$) at 8 months postpartum. In line with that, comparing the groups of low and high MS we found that at 8 months postpartum, infants of low stress mothers had higher Shannon alpha diversity than infants of high stress mothers (β = 0.961, 95% HDI = [0.143; 1.737], $P(\beta \geq 0) = 0.99$), see Figure 2.4.

2.4.3.2 Beta diversity and differential abundance analysis

The MS or its individual stress variables were not significantly associated with beta diversity. However, we observed that the PRAQR2-B (fear of giving birth) at 18 weeks of pregnancy was associated with beta diversity in infants sampled at 12 weeks (R^2 = .011, p = .023) and 8 months (R^2 = .015, p = .040) postpartum. Hair cortisol (HCS3) was associated with beta diversity at 2 (R^2 = .017, p = .006) and 12 weeks (R^2 = .015, p = .022) postpartum and hair cortisone (HCN3) was associated with beta diversity at 2 (R^2 = .016, p = .004) and 8 months (R^2 = .022, p = .025) postpartum. Neither the questionnaires, nor the hair cortisol and cortisone concentrations obtained postnatally were associated with beta diversity of the infant samples.

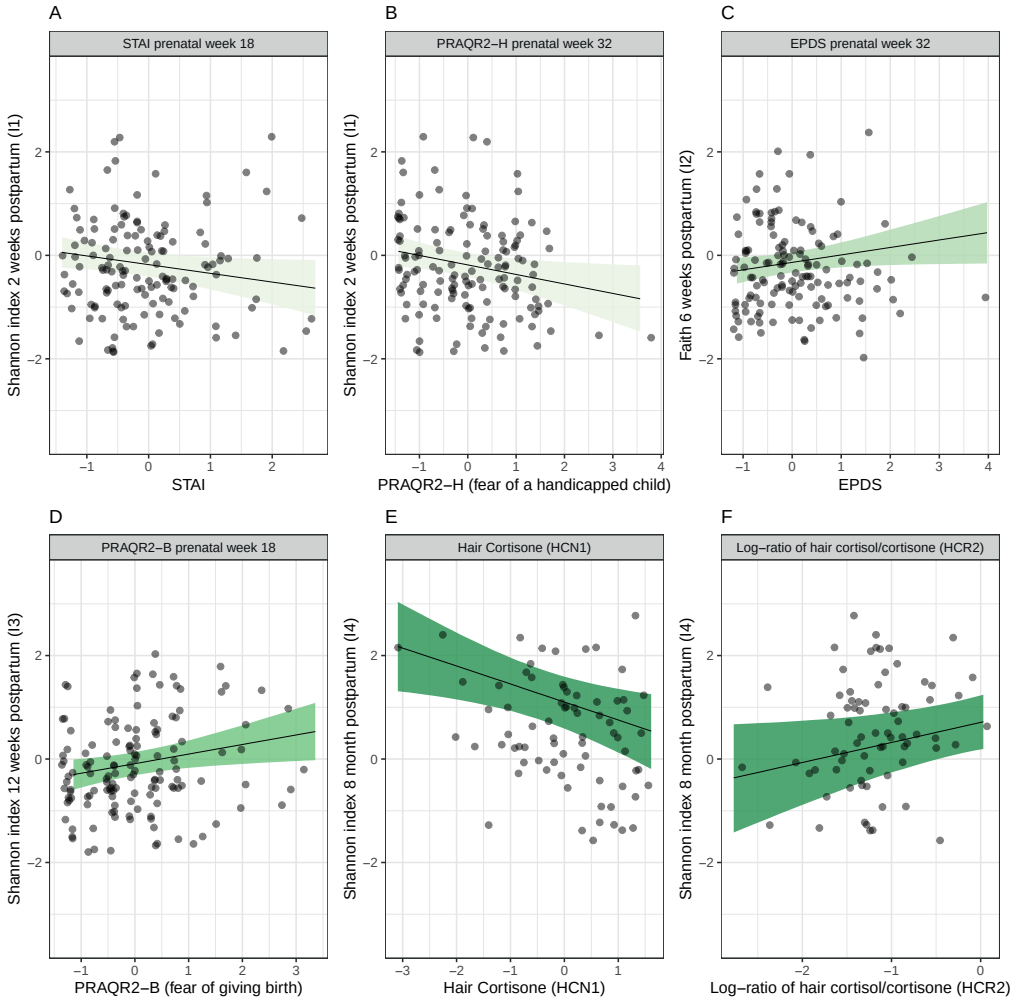

Figure 2.3: Alpha diversity at different time points (indicated in the y-axis label by shade coloring from light to dark green) plotted against different stress measures (indicated in the x-axis label). The time point of the maternal stress measurement is indicated in the box above the plot. The regression lines and the colored shaded regions illustrate our slope estimates including their 95% credible interval. We used the median for all covariates to generate the posterior distribution for the slope parameters. HCN1 = Hair cortisol reflecting maternal stress between week 6 and 15 of gestation, HCR2 = log-ratio of hair cortisol and cortisone reflecting stress between 15 and 23 weeks of gestation.

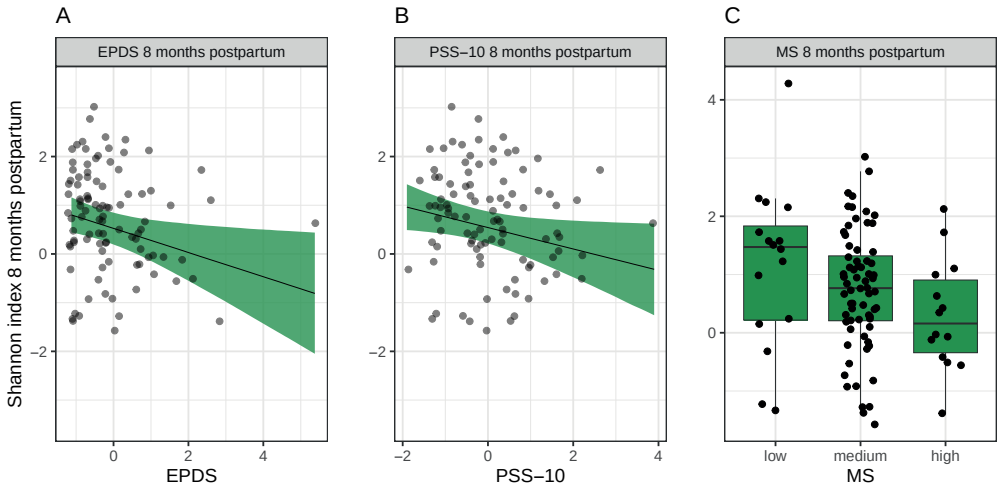

Figure 2.4: Shannon alpha diversity of infant microbiota samples collected at 8 months postpartum plotted against different stress measures (indicated in the x-axis label). The time point of the maternal stress measurement is indicated in the box above the plot. The regression lines and the colored shaded regions illustrate our slope estimates including their 95% credible interval. We used the median for all covariates to generate the posterior distribution for the slope parameters. MS = Maternal stress composite score.

Several bacterial species were associated with the postnatal stress variables (supplementary Tables 2.10-2.12 list all species and phyla related to any of the stress variables across time and at the separate time points (FDR ≤ 0.1)). Among other associations, we found positive associations between *Bacteroides fragilis* and hair cortisol (HCS3 and HCS2). This association was present across all samples and at several stool sampling time points individually. It became particularly strong when looking at the log-ratio of hair cortisol and cortisone (HCR3) and the infant sample at 6 weeks postpartum. Furthermore, we observed negative associations between *Flavonifractor plautii* and *Bacteroides ylanisolvens* and hair cortisol (HCS3) as well as *Bacteroides thetaiotaomicron* and hair cortisol (HCS2). We did not find evidence for a positive association between any of the stress variables and Proteobacteria or for a negative association with Lactobacilli or *Bifidobacterium*. Instead, the postnatal stress measures MS, EPDS and PSS-10 were positively associated with Firmicutes across all time points.

2.4.3.3 Volatility

We found a positive association between MS at two weeks postpartum and volatility between 2 and 6 weeks postpartum ($\beta = 0.178$, 95% HDI = [0.046; 0.311], $P(\beta \geq 0) = 0.996$), see Figure 2.5A. We found the same for all questionnaires individually. Also, the PSS-10 at 6 weeks postpartum was positively associated with volatility between 6 and 12 weeks postpartum, see Figure 2.5B. Lastly, we found that the log-ratio of hair cortisol and cortisone (HCR2) was positively related to volatility between 12 weeks and eight months postpartum ($\beta = 0.287$, 95% HDI = [-0.007; 0.573], $P(\beta \geq 0) = 0.974$), see Figure 2.5C.

2.5 Discussion

We investigated whether we could detect changes in the maternal microbiota from the second to the third trimester and whether the maternal microbiota at eight months postpartum differed from the prenatal microbiota. Furthermore, we examined how prenatal and postnatal maternal stress were related to the maternal and infant microbiota. We hypothesized that prenatal stress would be positively associated to maternal microbiota volatility and that pre- and postnatal maternal stress would be negatively related with the abundance of *Bifidobacterium* and positively associated with the abundance of *Proteobacteria* and with infant microbiota volatility in early life. In the following we will summarize and discuss the results.

2.5.1 1) Maternal microbiota during and after pregnancy

Our data supports previous findings indicating that the maternal microbiota does not undergo major changes between the second and the third trimester (DiGiulio et al., 2015; H. Yang et al., 2020). However, we did observe changes from pregnancy to eight months postpartum. Specifically, we detected a significant decrease in Faith alpha diversity at eight months postpartum as compared to the first and the second trimester. While we also observed that beta diversity was significantly different at eight months postpartum compared to the second trimester of

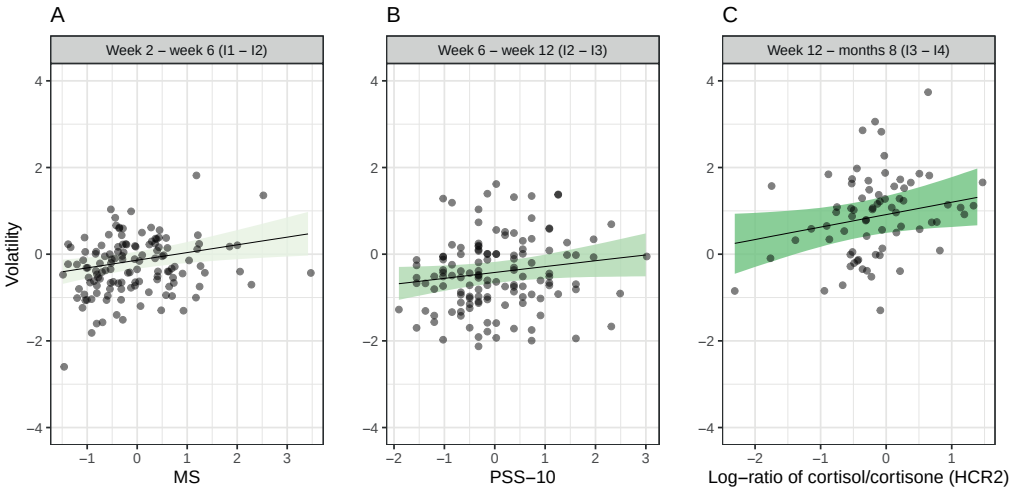

Figure 2.5: Volatility at different time point intervals (indicated by shade color from light green to dark green) of the infant stool samples and the postnatal maternal stress composite score (MS) (A), PSS-10 (B) and log-ratio of hair cortisol and cortisone (HCR2) (C). The time points used to calculate volatility are indicated in the box at the top of each plot. The regression line and the colored shaded region illustrate our slope estimate including the 95% credible interval. We used the median for all covariates to generate the posterior distribution for the slope parameter. HCR2 = log-ratio of hair cortisol and cortisone reflecting maternal stress between 15 and 23 weeks of gestation.

pregnancy, the effect size of this finding does not warrant interpreting it as a major change. Nevertheless, the decrease in Faith alpha diversity as well as observed decreases in, for example, *Akkermansia muciniphila*, *Anaerostipes hadrus*, *Streptococcus thermophilus* and *Streptococcus salivarius* as well as increases in, for example, *Fusicatenibacter saccharivorans*, *Ruminococcaceae* and *Clostridiales bacterium* indicate that the microbiota postpartum differs significantly from the prenatal microbiota. The observed changes could be due to physiological changes induced by shifting from the pregnant to the non-pregnant state and to the induction of breastfeeding but could also be due to lifestyle changes related to the postpartum period, which is characterized by alterations in sleep, activity, diet, and other behavioral changes. While we collected prenatal data on lifestyle factors such as diet, physical activity, and sleep, we did not collect these variables at eight months postpartum, limiting our analysis in that regard. To further study the potential changes induced by pregnancy or the shift into the non-pregnant state, it would be interesting to include more follow up samples as well as samples from before conception and to collect data about lifestyle changes associated with the pregnancy and postpartum period. Moreover, we observed that pre-pregnancy body mass index (pBMI) was associated with microbiota composition in multiparous but not primiparous women across time. This is in contrast with previous research that found an association between pBMI and changes in the gut microbiota throughout pregnancy only in primiparous women (Kennedy et al., 2022). However, our data and analyses are not comparable to those of the previous study as they collected samples also in the first trimester and included gestational weight gain in their statistical models. The potential interaction between parity and pBMI in relation to gut microbiota trajectories in the perinatal period warrants further research.

2.5.2 2) Maternal prenatal and postnatal stress and the maternal microbiota

We found limited evidence for an association between MS and alpha diversity. Namely, when comparing the low MS group with the medium or high MS group we found that only at eight months postpartum mothers reporting low levels of stress had slightly lower Shannon diversity than mothers reporting medium or high levels of stress. Interestingly, we did find that the PRAQR2-B (fear of giving birth) measured at 18 weeks pregnancy was negatively associated with alpha diversity at 18 weeks of pregnancy with both Shannon and Faith diversity. Similar results were observed at 32 weeks of pregnancy and therefore this association appeared to be more robust compared to the findings related to MS. The fact that we find different associations for MS and PRAQR2-B can be explained because of the limited overlap between those variables (supplementary figures 2.7-2.8) and the different time points the microbiota samples were obtained. Indeed, the PRAQR2-B (fear of giving birth) subscale assesses very specific prenatal anxieties whereas the MS assesses stress in general. Furthermore, we detected two associations between measures of maternal stress with individual microbial species. First, we found a positive association between the PSS-10 and *Blautia* throughout all time points. Second, we found a negative association between hair cortisone (HCN3) and *Bacteroides cellulosilyticus* at 32 weeks of pregnancy. Lastly, we could not reject our null hypothesis that maternal stress is not related to maternal microbiota volatility. Possible explanations include that there is indeed no association between maternal stress and microbiota volatility during pregnancy. Alternatively,

it is possible that the stress experienced in our sample of highly educated women, was not severe enough to induce significant changes in the more stable adult maternal microbiota. It is also possible that increased volatility could be observed at earlier stages of pregnancy such as from the first to the second trimester. Altogether these findings provide evidence that maternal stress, specifically fear of giving birth, is associated with the the maternal gut microbiota at different time points in the perinatal period.

2.5.3 3) Maternal prenatal and postnatal stress and the infant microbiota

Depending on the type of stress measurement and the time of maternal stress and infant microbiota sampling, we found contrasting associations between maternal stress and features of the infant gut microbiota. For example, we found negative associations between the STAI measured at 18 weeks of pregnancy as well as the PRAQR2-H (fear of a handicapped child) measured at 32 weeks of pregnancy and infant Shannon diversity at two weeks postpartum. Also, hair cortisone (HCN1) and MS at eight months postpartum were negatively related to alpha diversity at eight months postpartum. In line with this, the log-ratio of hair cortisol and cortisone (HCR2) was positively related to infant Shannon diversity at eight months postpartum. In contrast, the EPDS measured at 32 weeks and the PRAQR2-B (fear of giving birth) measured at 18 and 32 weeks of pregnancy were positively related to Shannon diversity at six and 12 weeks postpartum, respectively. While these findings provide strong evidence that prenatal maternal stress is related to infant microbiota alpha diversity, they also show that the association is not uniform across time or between different measures of stress. This is in line with Rojas et al. (Rojas et al., 2023) and van den Bergh et al. (Van den Bergh et al., 2020), who reported measurement- and trimester-specific associations between prenatal stress and the infant gut microbiota or other outcome measures, respectively. Our data indicate that, in addition, the potential effect of maternal stress also differs depending on time of measurement of the infant gut microbiota. We will touch upon a possible explanation when discussing our findings on volatility further below.

Next to the associations with alpha diversity, we found that the PRAQR2-B (fear of giving birth), hair cortisol (HCS3) and hair cortisone (HCN3) were related to infant beta diversity at different time points. We also identified many individual species related to our stress measurements across time and at the individual time points (supplementary Tables 2.10-2.12). Most notably, we found a positive association between infant *Bacteroides fragilis* and maternal hair cortisol in the second and third trimester (HCS2 and HCS3) that was present across time and at the individual time points. *Bacteroides fragilis* has been associated with reduced levels of aggressive behavior, emotional reactivity, externalizing behavior, sadness, and impulsivity as well as an increase in inhibitory control and lower reported incidents of family turmoil (Flannery et al., 2020). Next to that it was associated with lower reported incidents of family turmoil (Flannery et al., 2020). Also, animal studies found that *Bacteroides fragilis* has protective effects against pathogen-induced gut inflammation and an autism-spectrum-disorder phenotype induced by maternal immune activation (Hsiao et al., 2013). This means that our results are either in contrast with previous findings or, possibly, that the association between stress and *Bacteroides fragilis* is not linear, such that, e.g., while moderate levels of stress may be positively associated with it, more extreme levels of stress may lead to a relative decrease of this bacterium.

Furthermore, we found a negative association between *Flavonifractor plautii* at week six and maternal stress as measured by hair cortisol in the third trimester (HCS3). *Flavonifractor plautii* has been negatively associated with the development of asthma later in life (Stokholm et al., 2018a) and was able to strongly suppress Th2 immune responses in mice (Ogita et al., 2020). Furthermore, in line with our findings, *Flavonifractor plautii* has been previously reported to be negatively associated with a maternal stress composite score at six weeks postpartum (Dutton et al., 2023) (which was based on STAI, EPDS, PSS and scales assessing several types of trauma and PTSD). We also reported previously that *Flavonifractor* was increased in a group of infants that received a destressing skin-to-skin contact intervention in the first five weeks of their lives (Eckermann et al., 2024). Thus, our findings are in line with the literature and suggest that maternal physiological stress is related to a relative decrease in infant *Flavonifractor* at around six weeks postpartum. Lastly, in contrast to previous literature (Dutton et al., 2023; Galley et al., 2023; Jahnke et al., 2021; Mepham et al., 2023; Zijlmans et al., 2015), we did not find evidence for a positive association between prenatal stress and Proteobacteria or for a negative association with *Bifidobacteroium*.

Given the complexity of the microbial ecosystem and its diversity between different geographic regions and cultures, it may be challenging to find uniform associations between maternal stress with many of the microbiota features such as alpha diversity and relative abundances. However, in line with our hypothesis, we found that postnatal MS, PSS-10, and the log-ratio of hair cortisol and cortisone (HCR2) were associated with microbiota volatility between week two and six, week six and 12 and week 12 and eight months, respectively. With this study and previous studies, more research is accumulating that infant microbiota volatility is positively related to maternal stress. Namely, volatility has been reported to be increased in humans reporting higher levels of stress during exam periods, experimentally stressed mice, patients with inflammatory bowel disease and decreased in infants who received a destressing intervention in the first five weeks of their life (T. Bastiaanssen et al., 2021; Clooney et al., 2021; Eckermann et al., 2024). In addition, a similar metric indicated higher volatility (lower stability) over nine samples taken in the first 100 days of life in infants with colic (de Weerth et al., 2013). Furthermore, in line with our previous study of another group of Dutch infants (Eckermann et al., 2024), we found again that gestational age was negatively related to microbiota volatility, especially with volatility between week six and week 12 postpartum.

There are many potential mechanisms through which maternal stress could induce volatility in the infant gut microbiota. For example, maternal stress may induce physiological changes in the mother that ultimately lead to a different composition and quality of breastmilk or changes in breastfeeding frequency and overall duration. We included feeding mode into the volatility models and found that between two and six weeks postpartum, mixed feeding was negatively associated with volatility compared to exclusive breastfeeding, whereas exclusively formula fed infants tended to have highest volatility in that period. However, between week six and 12 as well as between week 12 and eight months postpartum, exclusively breastfed infants had significantly lower microbiota volatility than mixed or fomula fed infants. This could be explained by the fact that as the diversity in diet increases (from exclusively breastfeeding to the introduction of increasingly complex solid food), the microbiota changes drastically initially.

It will be interesting to see if future studies on maternal stress and the infant microbiota can consistently identify an association between stress and volatility. Then, the question remains

whether volatility is a direct consequence of stress or whether people with higher microbiota volatility are more susceptible to experiencing stress. A study that tracks a few individuals with daily high frequency sampling of stress (e.g., with ecological momentary sampling) and the gut microbiota may be able to provide evidence about the timeline of this association. But the question would remain whether volatility has any negative or positive effects on health by itself or whether what causes volatility simultaneously causes alternations in health.

2.5.4 Strengths and limitations

Strengths include the many microbiota sampling time points of both mothers during pregnancy and at eight months postpartum and infants in the first year of life. These allowed us to evaluate our research questions on microbiota volatility. Also, our microbiota samples, in conjunction with measurements of maternal stress via reported stress as well as objectively measured stress (hair cortisol/cortisone), provided data covering many time points sampled individually in previous studies researching the association between maternal stress and the maternal and infant gut microbiota. This, and analyzing questionnaires that measure perinatal specific and general stress, helped to shed light on the consistency and reproducibility of findings across studies. To analyze our microbiota samples, we used shotgun metagenomic sequencing, allowing us to work with higher taxonomic resolutions compared to previous studies. Limitations include that we did not measure potentially relevant covariates at eight months postpartum such as physical activity, sleep, and diet. This limited our analyses on factors contributing to the changes in the maternal microbiota from 32 weeks of pregnancy to eight months postpartum. Also, we studied a healthy, highly educated sample in a developed country. In other settings maternal stress levels can be substantially higher and more variable, potentially leading to different results than in our study. Lastly, including analyses on functional profiling of the gut microbiota was beyond the scope of this study, but is a logical next step in this area of research.

2.5.5 Conclusion

Our study provides evidence that maternal prenatal and postnatal stress is related to both the maternal and the infant microbiota. Collectively, our and previous studies indicate that, apart from a positive association with microbiota volatility in infants, maternal perinatal stress is not uniformly associated with most microbiota features and that the associations are highly time point specific (i.e. sensitive to when both the stress and the microbiota assessments are made). The consistent findings on volatility warrant future research investigating these associations further and in more depth.

2.6 Supplemental Material

2.6.1 Supplementary Methods

2.6.1.1 Microbial DNA extraction

DNA was extracted from feces using the ZymoBIOMICS DNA 96 Magbead kit (ZY-D4302, Zymo Research) and an automated extraction robot, the Kingfisher Flex Purification System. DNA concentration was assessed using a fluorometer (Infinite F200, Tecan) with the Quant-iT™ dsDNA Broad-Range Assay Kit (VXQ33130, Invitrogen). A liquid handler (Biomek i7 Automated Workstation) was used to perform the in-house optimized Nextera XT Library preparation (FC-131-1096, Illumina). In summary, 0.25 ng of genomic DNA was used as a template for the library preparation. A tagmentation step was carried out at 55 degrees Celsius for 5 minutes using a transposome to add Illumina adapters, creating fragments of approximately 300 bp. The tagmented DNA with the Illumina adapter was then employed as a template for PCR amplification and to add unique index primers. The PCR protocol included 72°C for 3 minutes, 95°C for 30 seconds and 12 cycles ofthe following: 95°C for 10 seconds, 55°C for 30 seconds, 72°C for 30 seconds, 72°C for 5 minutes and hold at 10°C.

The PCR products were purified using Agencourt© AMPure® XP (A63882 Becker Coulter). DNA was measured using the fluorometer (Infinite F200 Pro Tecan) and fluorometric analysis (Quant-iT™ dsDNA High Sensitivity, Invitrogen). The purified PCR products were equimolarly pooled, followed by sequencing on the Novaseq 6000 (Illumina) for a minimum of 1500 Mb per sample. FASTQ read sequence files were generated using bcl2fastq version 2.20 (Illumina). Initial quality assessment was based on data passing the Illumina Chastity filtering. Subsequently, reads containing PhiX control signal were removed using by aligning to the PhiX reference sequence (Bowtie2.2.6) and taking the unaligned reads. In addition, reads containing (partial) adapters were clipped (up to a minimum read length of 50bp) by using fastq-mcf from the ea-utils package v1.04.807. The second quality assessment was based on the remaining reads using the FASTQC quality control tool version 0.11.8.

2.6.1.2 Microbial taxonomic profiling

MetaPhlAn (Truong et al., 2015) v4.0 was used to conduct taxonomic profiling of the metagenomic samples. This tool uses a library of clade-specific markers to provide quantification at species level for microbial entities, including bacteria, archaea, viruses, and eukaryotes. The default settings were applied when running MetaPhlAn. HUMAnN3 (Abubucker et al., 2012) v3.0 was used to perform functional profiling. When provided with an input metagenome, HUMAnN3 generates a sample-specific reference database, which is constructed by concatenating and indexing the pangenomes of species identified by MetaPhlAn in the sample. Pangenomes refer to pre-clustered and pre-annotated catalogues of open reading frames that are found across isolated genomes from a particular species (K. Huang et al., 2014). Subsequently, using HUMAnN3, sample reads were mapped against this database to quantify the presence and abundance of genes in a species-stratified manner. Unmapped reads were further used in a translated search against UniRef90 (Suzek et al., 2015) to include abundances of gene

families that are taxonomically unclassified but functionally distinct. Lastly, HUMAnN3 reconstructed metabolic pathway abundance, to determine community-total, species-stratified, and unclassified gene family abundance. This reconstruction was based on the subset of gene families annotated to metabolic reactions, based on reaction and pathway definitions from MetaCyc (Caspi et al., 2016). Enzyme abundances at the level-4 Enzyme Commission (EC) categories were further computed by summing the abundances of individual gene families annotated to each EC number. The annotations were based on UniRef90-EC annotations obtained from UniProt (The UniProt Consortium et al., 2023). A phylogenetic tree was obtained using the chocophlan database *mpa_vJan21_CHOCOPhlAnSGB_202103* (obtained from https://github.com/biobakery/MetaPhlAn/blob/master/metaphlan/utils/mpa_vJan21_CHOCOPhlAnSGB_202103.nwk).

2.6.2 Supplementary Tables

Table 2.4: Bacterial species differentially abundant between maternal samples collected at 32 weeks of pregnancy and 8 months postpartum.

Feature	Coefficient	q
s__Actinomyces_oris	-0.002	0.022
s__Actinomyces_SGB17168	0.002	0.051
s__GGB3510_SGB4687	-0.002	0.062
s__Clostridia_bacterium_UC5_1_1D1	0.003	0.047
s__Clostridia_unclassified_SGB66170	-0.003	0.051
s__GGB3571_SGB4778	0.003	0.051
s__Rothia_mucilaginosa	-0.004	0.005
s__Faecalibacillus_faecis	-0.004	0.040
s__Clostridia_unclassified_SGB6276	-0.004	0.041
s__Clostridia_unclassified_SGB4121	0.005	0.001
s__Eubacterium_ventriosum	-0.005	0.071
s__Streptococcus_parasanguinis	-0.006	0.001
s__GGB9522_SGB14921	0.006	0.001
s__Lachnospiraceae_unclassified_SGB4894	-0.006	0.027
s__Romboutsia_timonensis	-0.006	0.062
s__Phocaeicola_massiliensis	-0.006	0.082
s__GGB3570_SGB4777	0.007	0.000
s__Methanobrevibacter_smithii	-0.007	0.061
s__Odoribacter_splanchnicus	-0.008	0.015
s__Parabacteroides_distasonis	-0.008	0.019
s__GGB9627_SGB15081	0.009	0.001
s__GGB9699_SGB15216	0.009	0.019
s__Turicibacter_sanguinis	-0.010	0.001
s__Clostridiales_bacterium_KLE1615	0.011	0.027
s__Agathobaculum_butyriciproducens	0.011	0.039
s__Barnesiella_intestinihominis	-0.011	0.057
s__Streptococcus_salivarius	-0.016	0.000
s__GGB9632_SGB15089	0.016	0.007
s__Lachnospiraceae_bacterium_WCA3_601_WT_6H	-0.016	0.025
s__Streptococcus_thermophilus	-0.018	0.080
s__Ruminococcaceae_unclassified_SGB15265	0.020	0.010
s__Fusicatenibacter_saccharivorans	0.030	0.011
s__Anaerostipes_hadrus	-0.031	0.005
s__Akkermansia_muciniphila	-0.042	0.039

Note:
The leading "s" indicates phylogenetic Species level.

Table 2.5: Posterior distribution of the beta coefficients corresponding to the different prenatal and postnatal stress measures per time point and alpha diversity index per maternal sample.

Stress Variable	Sample	Index	M	SD	95% HDI	P($\beta \geq 0$)
MS	M1	shannon	0.050	0.076	[-0.096;0.201]	0.739
MS	M2	shannon	-0.037	0.085	[-0.204;0.129]	0.328
MS	M3	shannon	0.053	0.092	[-0.125;0.237]	0.718
MS	M1	faith	0.016	0.059	[-0.099;0.133]	0.605
MS	M2	faith	0.032	0.061	[-0.087;0.15]	0.706
MS	M3	faith	0.043	0.066	[-0.082;0.179]	0.743
STAI	M1	shannon	0.070	0.073	[-0.075;0.211]	0.834
STAI	M2	shannon	-0.052	0.086	[-0.229;0.111]	0.270
STAI	M3	shannon	0.036	0.094	[-0.148;0.222]	0.646
STAI	M1	faith	0.033	0.057	[-0.078;0.144]	0.720
STAI	M2	faith	0.022	0.062	[-0.1;0.141]	0.634
STAI	M3	faith	0.031	0.069	[-0.101;0.166]	0.675
EPDS	M1	shannon	0.032	0.076	[-0.116;0.183]	0.671
EPDS	M2	shannon	-0.017	0.086	[-0.178;0.157]	0.423
EPDS	M3	shannon	0.049	0.094	[-0.13;0.237]	0.700
EPDS	M1	faith	0.023	0.060	[-0.098;0.137]	0.645
EPDS	M2	faith	0.042	0.064	[-0.083;0.166]	0.749
EPDS	M3	faith	0.042	0.070	[-0.086;0.187]	0.729
PSS	M1	shannon	0.016	0.073	[-0.128;0.159]	0.583
PSS	M2	shannon	-0.038	0.084	[-0.205;0.125]	0.330
PSS	M3	shannon	0.064	0.095	[-0.121;0.248]	0.753
PSS	M1	faith	-0.035	0.058	[-0.15;0.077]	0.273
PSS	M2	faith	0.018	0.062	[-0.098;0.143]	0.618
PSS	M3	faith	0.049	0.070	[-0.085;0.187]	0.762
PRAQR2-H	M1	shannon	0.015	0.071	[-0.121;0.158]	0.581
PRAQR2-H	M2	shannon	0.019	0.076	[-0.128;0.171]	0.603
PRAQR2-H	M1	faith	0.014	0.055	[-0.091;0.124]	0.597
PRAQR2-H	M2	faith	-0.012	0.052	[-0.114;0.087]	0.408
PRAQR2-B	M1	shannon	-0.251	0.071	[-0.388;-0.112]	0.000
PRAQR2-B	M2	shannon	0.210	0.073	[0.067;0.353]	0.998
PRAQR2-B	M1	faith	-0.125	0.059	[-0.239;-0.009]	0.018
PRAQR2-B	M2	faith	0.066	0.052	[-0.034;0.168]	0.900

HCS2	M1	shannon	-0.063	0.074	[-0.21;0.077]	0.196
HCS3	M2	shannon	0.002	0.107	[-0.207;0.211]	0.506
HCS2	M1	faith	0.070	0.056	[-0.044;0.177]	0.892
HCS3	M2	faith	0.068	0.082	[-0.094;0.23]	0.798
HCN2	M1	shannon	-0.120	0.082	[-0.267;0.054]	0.087
HCN3	M2	shannon	-0.168	0.177	[-0.498;0.122]	0.213
HCN2	M1	faith	-0.012	0.069	[-0.135;0.139]	0.434
HCN3	M2	faith	-0.039	0.086	[-0.213;0.137]	0.309
HCR2	M1	shannon	0.234	0.104	[0.029;0.436]	0.987
HCR3	M2	shannon	0.187	0.154	[-0.104;0.496]	0.892
HCR2	M1	faith	0.192	0.085	[0.024;0.358]	0.988
HCR3	M2	faith	0.097	0.127	[-0.156;0.348]	0.780
PSAS	M1	shannon	-0.097	0.112	[-0.318;0.123]	0.188
PSAS	M1	faith	-0.155	0.104	[-0.36;0.047]	0.068

Note:
M = median of the posterior distribution. HCS = hair cortisol, HCN = hair cortisone, HCR = log-ratio of hair cortisol and cortisone and the measured times include 23-32 (1), 15-23 (2) and 6-15 weeks of gestation as well as 4-8 weeks postpartum (4). M1 - M3 = Time point of maternal microbiota sampling in chronological order (18 and 32 weeks of gestation and 8 months postpartum). PRAQR2-B = Fear of giving birth. PRAQR2-H = Fear of a handicapped child. MS = maternal stress composite score.

Table 2.6: Bacterial species identified in the maternal microbiota samples associated with any of the maternal stress variables. Results are based on a multilevel model fit to all time points.

Stress Variable	Feature	Coefficient	q
PSS-10	s__Blautia_sp_MSK_20_85	0.009	0.078

Note:
The leading "s" indicates phylogenetic Species level.

Table 2.7: Bacterial species identified in the maternal microbiota samples associated with any of the maternal stress variables. Results are based on models fit per maternal microbiota sampling time point.

Stress Variable	Sample	Feature	Coefficient	q
HCN3	M2	s___Bacteroides_cellulosilyticus	-0.024	0.068

Note:
The leading "s" indicates phylogenetic Species level. HCN3 = Hair cortisone reflecting cortisone levels between 23 and 32 weeks of pregnancy.

Table 2.8: Posterior distribution of the beta coefficients corresponding to the different prenatal stress measures per time point and alpha diversity index per infant sample.

Time	Sample	Stress Variable	Index	M	SD	95% HDI	P($\beta \geq 0$)
18	I1	MS	shannon	-0.054	0.031	[-0.116;0.006]	0.041
18	I1	MS	faith	0.017	0.026	[-0.034;0.066]	0.746
18	I1	STAI	shannon	-0.169	0.083	[-0.331;-0.007]	0.022
18	I1	STAI	faith	0.042	0.069	[-0.1;0.171]	0.728
18	I1	EPDS	shannon	-0.131	0.088	[-0.299;0.046]	0.069
18	I1	EPDS	faith	0.037	0.074	[-0.107;0.181]	0.691
18	I1	PSS-10	shannon	-0.108	0.082	[-0.268;0.056]	0.093
18	I1	PSS-10	faith	0.050	0.068	[-0.084;0.181]	0.770
18	I1	PRAQR2-B	shannon	-0.023	0.082	[-0.187;0.135]	0.386
18	I1	PRAQR2-B	faith	0.036	0.066	[-0.093;0.167]	0.707
18	I1	PRAQR2-H	shannon	-0.121	0.079	[-0.272;0.038]	0.062
18	I1	PRAQR2-H	faith	0.029	0.063	[-0.093;0.154]	0.676
18	I2	MS	shannon	-0.038	0.029	[-0.094;0.018]	0.092
18	I2	MS	faith	0.027	0.027	[-0.024;0.079]	0.845
18	I2	STAI	shannon	-0.132	0.076	[-0.283;0.017]	0.041
18	I2	STAI	faith	0.079	0.071	[-0.058;0.217]	0.869
18	I2	EPDS	shannon	-0.096	0.083	[-0.258;0.065]	0.120
18	I2	EPDS	faith	0.053	0.078	[-0.096;0.213]	0.751
18	I2	PSS-10	shannon	-0.057	0.078	[-0.212;0.092]	0.229
18	I2	PSS-10	faith	0.068	0.071	[-0.069;0.209]	0.830
18	I2	PRAQR2-B	shannon	0.032	0.082	[-0.128;0.195]	0.652
18	I2	PRAQR2-B	faith	0.087	0.074	[-0.057;0.234]	0.881
18	I2	PRAQR2-H	shannon	-0.019	0.073	[-0.164;0.122]	0.398
18	I2	PRAQR2-H	faith	0.033	0.069	[-0.101;0.168]	0.689

18	I3	MS	shannon	0.016	0.030	[-0.044;0.075]	0.697
18	I3	MS	faith	0.019	0.028	[-0.037;0.072]	0.756
18	I3	STAI	shannon	0.054	0.080	[-0.097;0.213]	0.753
18	I3	STAI	faith	0.082	0.073	[-0.061;0.225]	0.868
18	I3	EPDS	shannon	0.058	0.087	[-0.112;0.229]	0.749
18	I3	EPDS	faith	0.065	0.080	[-0.092;0.222]	0.797
18	I3	PSS-10	shannon	0.010	0.082	[-0.155;0.168]	0.548
18	I3	PSS-10	faith	-0.001	0.074	[-0.144;0.145]	0.495
18	I3	PRAQR2-B	shannon	0.185	0.079	[0.033;0.339]	0.990
18	I3	PRAQR2-B	faith	0.100	0.071	[-0.044;0.231]	0.919
18	I3	PRAQR2-H	shannon	0.057	0.075	[-0.088;0.207]	0.778
18	I3	PRAQR2-H	faith	0.062	0.069	[-0.071;0.198]	0.819
18	I4	MS	shannon	-0.055	0.045	[-0.14;0.037]	0.112
18	I4	MS	faith	-0.002	0.047	[-0.096;0.09]	0.481
18	I4	STAI	shannon	-0.042	0.116	[-0.271;0.182]	0.358
18	I4	STAI	faith	0.081	0.118	[-0.157;0.305]	0.752
18	I4	EPDS	shannon	-0.165	0.132	[-0.422;0.097]	0.104
18	I4	EPDS	faith	-0.073	0.136	[-0.34;0.196]	0.291
18	I4	PSS-10	shannon	-0.218	0.125	[-0.463;0.031]	0.042
18	I4	PSS-10	faith	-0.046	0.129	[-0.299;0.206]	0.362
18	I4	PRAQR2-B	shannon	-0.125	0.109	[-0.34;0.085]	0.125
18	I4	PRAQR2-B	faith	-0.092	0.113	[-0.317;0.124]	0.204
18	I4	PRAQR2-H	shannon	0.038	0.117	[-0.196;0.263]	0.621
18	I4	PRAQR2-H	faith	0.163	0.113	[-0.058;0.386]	0.926
32	I1	MS	shannon	-0.040	0.033	[-0.105;0.022]	0.112
32	I1	MS	faith	0.025	0.026	[-0.025;0.076]	0.833
32	I1	STAI	shannon	-0.125	0.085	[-0.293;0.041]	0.074
32	I1	STAI	faith	0.031	0.068	[-0.106;0.162]	0.672
32	I1	EPDS	shannon	-0.109	0.086	[-0.273;0.068]	0.104
32	I1	EPDS	faith	0.045	0.069	[-0.088;0.18]	0.746
32	I1	PSS-10	shannon	-0.051	0.087	[-0.216;0.124]	0.277
32	I1	PSS-10	faith	0.103	0.069	[-0.033;0.236]	0.936
32	I1	PRAQR2-B	shannon	-0.021	0.090	[-0.194;0.156]	0.407
32	I1	PRAQR2-B	faith	0.056	0.071	[-0.081;0.199]	0.789
32	I1	PRAQR2-H	shannon	-0.184	0.079	[-0.34;-0.03]	0.011
32	I1	PRAQR2-H	faith	-0.046	0.066	[-0.176;0.082]	0.244
32	I2	MS	shannon	0.002	0.031	[-0.06;0.065]	0.527
32	I2	MS	faith	0.054	0.028	[0.001;0.11]	0.974
32	I2	STAI	shannon	-0.039	0.086	[-0.202;0.132]	0.326
32	I2	STAI	faith	0.123	0.076	[-0.024;0.272]	0.948
32	I2	EPDS	shannon	0.014	0.081	[-0.148;0.172]	0.565

32	I2	EPDS	faith	0.144	0.071	[0.001;0.281]	0.978
32	I2	PSS-10	shannon	0.037	0.080	[-0.124;0.189]	0.679
32	I2	PSS-10	faith	0.101	0.073	[-0.04;0.243]	0.916
32	I2	PRAQR2-B	shannon	0.044	0.086	[-0.124;0.209]	0.696
32	I2	PRAQR2-B	faith	0.098	0.078	[-0.056;0.248]	0.896
32	I2	PRAQR2-H	shannon	-0.122	0.076	[-0.272;0.024]	0.051
32	I2	PRAQR2-H	faith	-0.016	0.070	[-0.152;0.121]	0.411
32	I3	MS	shannon	0.042	0.031	[-0.018;0.102]	0.915
32	I3	MS	faith	0.023	0.028	[-0.032;0.079]	0.795
32	I3	STAI	shannon	0.143	0.081	[-0.019;0.3]	0.960
32	I3	STAI	faith	0.056	0.075	[-0.088;0.205]	0.776
32	I3	EPDS	shannon	0.124	0.084	[-0.036;0.296]	0.931
32	I3	EPDS	faith	0.085	0.078	[-0.07;0.233]	0.864
32	I3	PSS-10	shannon	0.032	0.083	[-0.133;0.191]	0.650
32	I3	PSS-10	faith	0.026	0.074	[-0.121;0.171]	0.632
32	I3	PRAQR2-B	shannon	0.172	0.087	[0.003;0.341]	0.977
32	I3	PRAQR2-B	faith	0.118	0.076	[-0.031;0.269]	0.940
32	I3	PRAQR2-H	shannon	0.032	0.078	[-0.123;0.183]	0.665
32	I3	PRAQR2-H	faith	0.026	0.071	[-0.111;0.162]	0.646
32	I4	MS	shannon	-0.050	0.043	[-0.132;0.037]	0.125
32	I4	MS	faith	-0.014	0.045	[-0.101;0.075]	0.381
32	I4	STAI	shannon	-0.018	0.117	[-0.248;0.207]	0.441
32	I4	STAI	faith	0.051	0.118	[-0.174;0.29]	0.673
32	I4	EPDS	shannon	-0.133	0.119	[-0.355;0.111]	0.132
32	I4	EPDS	faith	-0.032	0.123	[-0.276;0.209]	0.395
32	I4	PSS-10	shannon	-0.218	0.118	[-0.444;0.015]	0.032
32	I4	PSS-10	faith	-0.127	0.123	[-0.369;0.115]	0.150
32	I4	PRAQR2-B	shannon	-0.005	0.119	[-0.238;0.225]	0.483
32	I4	PRAQR2-B	faith	0.028	0.119	[-0.21;0.26]	0.597
32	I4	PRAQR2-H	shannon	-0.049	0.120	[-0.292;0.18]	0.337
32	I4	PRAQR2-H	faith	0.073	0.121	[-0.178;0.298]	0.720
HCN1	I1	HCN1	shannon	-0.037	0.099	[-0.23;0.159]	0.352
HCN1	I1	HCN1	faith	-0.041	0.075	[-0.19;0.102]	0.292
HCN1	I2	HCN1	shannon	-0.045	0.098	[-0.238;0.151]	0.321
HCN1	I2	HCN1	faith	0.034	0.082	[-0.128;0.193]	0.663
HCN1	I3	HCN1	shannon	-0.045	0.100	[-0.245;0.147]	0.328
HCN1	I3	HCN1	faith	-0.108	0.086	[-0.283;0.056]	0.102
HCN1	I4	HCN1	shannon	-0.255	0.142	[-0.53;0.03]	0.037
HCN1	I4	HCN1	faith	-0.349	0.134	[-0.612;-0.091]	0.004
HCN2	I1	HCN2	shannon	0.027	0.099	[-0.175;0.214]	0.606
HCN2	I1	HCN2	faith	0.020	0.075	[-0.132;0.163]	0.605

HCN2	I2	HCN2	shannon	0.002	0.097	[-0.187;0.195]	0.510
HCN2	I2	HCN2	faith	0.043	0.081	[-0.121;0.197]	0.702
HCN2	I3	HCN2	shannon	-0.011	0.099	[-0.203;0.184]	0.454
HCN2	I3	HCN2	faith	-0.079	0.088	[-0.25;0.093]	0.180
HCN2	I4	HCN2	shannon	-0.111	0.156	[-0.429;0.184]	0.235
HCN2	I4	HCN2	faith	-0.282	0.148	[-0.574;0.004]	0.027
HCN3	I1	HCN3	shannon	0.008	0.102	[-0.193;0.207]	0.533
HCN3	I1	HCN3	faith	-0.118	0.078	[-0.27;0.038]	0.068
HCN3	I2	HCN3	shannon	-0.152	0.096	[-0.343;0.035]	0.059
HCN3	I2	HCN3	faith	-0.067	0.087	[-0.24;0.1]	0.217
HCN3	I3	HCN3	shannon	0.024	0.100	[-0.169;0.222]	0.595
HCN3	I3	HCN3	faith	-0.086	0.090	[-0.256;0.093]	0.170
HCN3	I4	HCN3	shannon	-0.098	0.134	[-0.36;0.163]	0.233
HCN3	I4	HCN3	faith	-0.106	0.130	[-0.366;0.145]	0.192
HCN4	I1	HCN4	shannon	0.015	0.114	[-0.2;0.246]	0.556
HCN4	I1	HCN4	faith	-0.100	0.087	[-0.275;0.065]	0.123
HCN4	I2	HCN4	shannon	-0.153	0.103	[-0.349;0.05]	0.070
HCN4	I2	HCN4	faith	-0.049	0.094	[-0.231;0.139]	0.297
HCN4	I3	HCN4	shannon	0.068	0.100	[-0.127;0.261]	0.753
HCN4	I3	HCN4	faith	0.030	0.089	[-0.138;0.212]	0.635
HCN4	I4	HCN4	shannon	-0.117	0.148	[-0.395;0.189]	0.210
HCN4	I4	HCN4	faith	-0.103	0.138	[-0.376;0.163]	0.222
HCR1	I1	HCR1	shannon	0.067	0.101	[-0.133;0.266]	0.751
HCR1	I1	HCR1	faith	0.101	0.074	[-0.045;0.244]	0.914
HCR1	I2	HCR1	shannon	0.004	0.102	[-0.195;0.206]	0.514
HCR1	I2	HCR1	faith	0.059	0.083	[-0.106;0.219]	0.761
HCR1	I3	HCR1	shannon	-0.053	0.102	[-0.255;0.143]	0.300
HCR1	I3	HCR1	faith	0.043	0.089	[-0.133;0.215]	0.690
HCR1	I4	HCR1	shannon	0.209	0.165	[-0.113;0.539]	0.898
HCR1	I4	HCR1	faith	0.238	0.177	[-0.101;0.594]	0.919
HCR2	I1	HCR2	shannon	-0.110	0.093	[-0.286;0.078]	0.116
HCR2	I1	HCR2	faith	-0.018	0.069	[-0.152;0.119]	0.395
HCR2	I2	HCR2	shannon	-0.070	0.090	[-0.248;0.104]	0.218
HCR2	I2	HCR2	faith	0.011	0.084	[-0.156;0.171]	0.549
HCR2	I3	HCR2	shannon	-0.013	0.092	[-0.192;0.169]	0.443
HCR2	I3	HCR2	faith	0.085	0.082	[-0.075;0.244]	0.854
HCR2	I4	HCR2	shannon	0.387	0.184	[0.022;0.749]	0.982
HCR2	I4	HCR2	faith	0.256	0.176	[-0.102;0.592]	0.929
HCR3	I1	HCR3	shannon	0.056	0.096	[-0.138;0.238]	0.719
HCR3	I1	HCR3	faith	0.035	0.070	[-0.103;0.171]	0.698
HCR3	I2	HCR3	shannon	0.070	0.091	[-0.103;0.252]	0.785

HCR3	I2	HCR3	faith	0.046	0.079	[-0.11;0.203]	0.719
HCR3	I3	HCR3	shannon	0.008	0.092	[-0.173;0.188]	0.532
HCR3	I3	HCR3	faith	0.046	0.082	[-0.113;0.21]	0.715
HCR3	I4	HCR3	shannon	0.066	0.153	[-0.221;0.376]	0.673
HCR3	I4	HCR3	faith	0.041	0.150	[-0.245;0.347]	0.614
HCR4	I1	HCR4	shannon	0.150	0.112	[-0.072;0.369]	0.912
HCR4	I1	HCR4	faith	0.054	0.086	[-0.113;0.223]	0.736
HCR4	I2	HCR4	shannon	0.117	0.104	[-0.087;0.32]	0.870
HCR4	I2	HCR4	faith	0.146	0.088	[-0.024;0.319]	0.952
HCR4	I3	HCR4	shannon	0.121	0.101	[-0.084;0.312]	0.885
HCR4	I3	HCR4	faith	0.082	0.090	[-0.099;0.256]	0.820
HCR4	I4	HCR4	shannon	0.172	0.159	[-0.148;0.478]	0.866
HCR4	I4	HCR4	faith	0.124	0.150	[-0.166;0.418]	0.797
HCS1	I1	HCS1	shannon	0.028	0.098	[-0.157;0.23]	0.618
HCS1	I1	HCS1	faith	0.044	0.073	[-0.101;0.188]	0.729
HCS1	I2	HCS1	shannon	-0.019	0.097	[-0.217;0.165]	0.423
HCS1	I2	HCS1	faith	0.092	0.086	[-0.075;0.26]	0.850
HCS1	I3	HCS1	shannon	-0.060	0.095	[-0.249;0.12]	0.263
HCS1	I3	HCS1	faith	-0.030	0.085	[-0.202;0.133]	0.357
HCS1	I4	HCS1	shannon	-0.084	0.153	[-0.381;0.216]	0.292
HCS1	I4	HCS1	faith	-0.156	0.153	[-0.453;0.148]	0.156
HCS2	I1	HCS2	shannon	-0.067	0.093	[-0.247;0.118]	0.235
HCS2	I1	HCS2	faith	0.001	0.070	[-0.14;0.137]	0.504
HCS2	I2	HCS2	shannon	-0.050	0.089	[-0.224;0.126]	0.284
HCS2	I2	HCS2	faith	0.048	0.080	[-0.107;0.203]	0.725
HCS2	I3	HCS2	shannon	-0.020	0.094	[-0.212;0.158]	0.417
HCS2	I3	HCS2	faith	0.003	0.084	[-0.165;0.165]	0.516
HCS2	I4	HCS2	shannon	0.120	0.170	[-0.212;0.455]	0.756
HCS2	I4	HCS2	faith	-0.141	0.169	[-0.487;0.179]	0.195
HCS3	I1	HCS3	shannon	0.051	0.096	[-0.14;0.237]	0.706
HCS3	I1	HCS3	faith	-0.050	0.072	[-0.192;0.09]	0.243
HCS3	I2	HCS3	shannon	-0.047	0.092	[-0.225;0.14]	0.299
HCS3	I2	HCS3	faith	-0.011	0.083	[-0.167;0.154]	0.446
HCS3	I3	HCS3	shannon	0.024	0.095	[-0.165;0.204]	0.601
HCS3	I3	HCS3	faith	-0.026	0.086	[-0.193;0.146]	0.381
HCS3	I4	HCS3	shannon	-0.059	0.155	[-0.36;0.248]	0.348
HCS3	I4	HCS3	faith	-0.092	0.150	[-0.389;0.203]	0.264
HCS4	I1	HCS4	shannon	0.081	0.112	[-0.134;0.304]	0.769
HCS4	I1	HCS4	faith	-0.059	0.085	[-0.231;0.104]	0.243
HCS4	I2	HCS4	shannon	-0.059	0.101	[-0.265;0.131]	0.276
HCS4	I2	HCS4	faith	0.044	0.086	[-0.121;0.213]	0.697

HCS4	I3	HCS4	shannon	0.110	0.100	[-0.092;0.301]	0.865
HCS4	I3	HCS4	faith	0.046	0.089	[-0.125;0.225]	0.699
HCS4	I4	HCS4	shannon	-0.016	0.157	[-0.317;0.298]	0.460
HCS4	I4	HCS4	faith	-0.049	0.145	[-0.335;0.233]	0.369

Note:
M = median of the posterior distribution. p = proportion of the posterior distribution larger than zero. The column 'Time' indicates when the stress variable was measured during pregnancy or which period cortisol and cortisone were measured in, whereby HCS = hair cortisol, HCN = hair cortisone, HCR = log-ratio of hair cortisol and cortisone and the measured times include 23-32 (1), 15-23 (2) and 6-15 weeks of gestation as well as 4-8 weeks postpartum (4). I1 - I4 = Time point of infant microbiota sampling in chronological order (2, 6 and 12 weeks and 8 months). PRAQR2-B = Fear of giving birth. PRAQR2-H = Fear of a handicapped child. MS = maternal stress composite score.

Table 2.9: Posterior distribution of the beta coefficients corresponding to the different postnatal stress measures per time point of infant microbiota sampling and alpha diversity index.

Stress Variable	Sample	Index	M	SD	95% HDI	P($\beta \geq 0$)
MS	I1	shannon	-0.006	0.092	[-0.186;0.171]	0.474
MS	I1	faith	0.078	0.075	[-0.065;0.229]	0.850
STAI	I1	shannon	-0.061	0.094	[-0.237;0.132]	0.261
STAI	I1	faith	0.064	0.076	[-0.084;0.213]	0.801
EPDS	I1	shannon	0.011	0.090	[-0.169;0.186]	0.548
EPDS	I1	faith	0.078	0.079	[-0.082;0.225]	0.838
PSS-10	I1	shannon	0.026	0.085	[-0.136;0.2]	0.619
PSS-10	I1	faith	0.065	0.069	[-0.075;0.197]	0.828
PSAS	I1	shannon	-0.759	0.639	[-1.98;0.529]	0.116
PSAS	I1	faith	-0.313	0.516	[-1.318;0.704]	0.270
HCS4	I1	shannon	0.081	0.111	[-0.151;0.288]	0.771
HCS4	I1	faith	-0.058	0.084	[-0.228;0.103]	0.242
HCN4	I1	shannon	0.018	0.113	[-0.202;0.24]	0.562
HCN4	I1	faith	-0.100	0.087	[-0.275;0.066]	0.125
HCR4	I1	shannon	0.150	0.113	[-0.069;0.373]	0.911
HCR4	I1	faith	0.054	0.087	[-0.115;0.225]	0.734
MS	I2	shannon	-0.040	0.086	[-0.206;0.129]	0.320
MS	I2	faith	-0.011	0.077	[-0.162;0.14]	0.444
STAI	I2	shannon	-0.112	0.086	[-0.277;0.057]	0.092
STAI	I2	faith	-0.028	0.079	[-0.186;0.126]	0.352

EPDS	I2	shannon	-0.037	0.083	[-0.194;0.13]	0.324
EPDS	I2	faith	-0.072	0.074	[-0.218;0.074]	0.164
PSS-10	I2	shannon	0.031	0.077	[-0.122;0.181]	0.656
PSS-10	I2	faith	0.060	0.071	[-0.082;0.196]	0.808
PSAS	I2	shannon	-0.229	0.159	[-0.535;0.085]	0.076
PSAS	I2	faith	-0.047	0.144	[-0.324;0.237]	0.371
HCS4	I2	shannon	-0.058	0.101	[-0.258;0.137]	0.281
HCS4	I2	faith	0.046	0.087	[-0.122;0.219]	0.697
HCN4	I2	shannon	-0.154	0.103	[-0.356;0.048]	0.068
HCN4	I2	faith	-0.048	0.093	[-0.231;0.133]	0.302
HCR4	I2	shannon	0.116	0.104	[-0.092;0.316]	0.869
HCR4	I2	faith	0.146	0.087	[-0.025;0.317]	0.952
MS	I3	shannon	0.058	0.071	[-0.083;0.195]	0.797
MS	I3	faith	-0.015	0.065	[-0.138;0.115]	0.404
STAI	I3	shannon	0.050	0.069	[-0.085;0.186]	0.765
STAI	I3	faith	-0.004	0.065	[-0.132;0.119]	0.474
EPDS	I3	shannon	0.047	0.073	[-0.095;0.19]	0.744
EPDS	I3	faith	-0.028	0.067	[-0.158;0.105]	0.334
PSS-10	I3	shannon	0.069	0.076	[-0.079;0.221]	0.820
PSS-10	I3	faith	-0.014	0.069	[-0.147;0.123]	0.423
PSAS	I3	shannon	-0.167	0.573	[-1.272;0.95]	0.388
PSAS	I3	faith	0.022	0.526	[-1.034;1.028]	0.517
HCS4	I3	shannon	0.110	0.100	[-0.09;0.299]	0.864
HCS4	I3	faith	0.046	0.088	[-0.131;0.214]	0.703
HCN4	I3	shannon	0.070	0.099	[-0.137;0.253]	0.760
HCN4	I3	faith	0.032	0.090	[-0.149;0.204]	0.641
HCR4	I3	shannon	0.120	0.100	[-0.076;0.316]	0.882
HCR4	I3	faith	0.081	0.090	[-0.096;0.257]	0.822
MS	I4	shannon	-0.202	0.107	[-0.414;0.004]	0.028
MS	I4	faith	-0.124	0.107	[-0.334;0.087]	0.123
STAI	I4	shannon	-0.102	0.107	[-0.311;0.107]	0.170
STAI	I4	faith	-0.059	0.108	[-0.265;0.161]	0.296
EPDS	I4	shannon	-0.247	0.107	[-0.458;-0.036]	0.011
EPDS	I4	faith	-0.177	0.111	[-0.391;0.045]	0.054
PSS-10	I4	shannon	-0.221	0.109	[-0.429;-0.006]	0.021
PSS-10	I4	faith	-0.114	0.112	[-0.331;0.112]	0.152
PSAS	I4	shannon	-0.148	0.103	[-0.351;0.051]	0.076
PSAS	I4	faith	-0.121	0.104	[-0.327;0.082]	0.123
HCS4	I4	shannon	-0.014	0.158	[-0.319;0.304]	0.462
HCS4	I4	faith	-0.051	0.144	[-0.335;0.231]	0.359
HCN4	I4	shannon	-0.118	0.147	[-0.416;0.164]	0.205

HCN4	I4	faith	-0.102	0.138	[-0.368;0.177]	0.226
HCR4	I4	shannon	0.173	0.158	[-0.13;0.482]	0.865
HCR4	I4	faith	0.122	0.148	[-0.159;0.42]	0.796

Note:
The stress measurement took place at the time of microbiota sampling (except for the hair derived measures). M = median of the posterior distribution. p = proportion of the distribution larger than zero. HCS = hair cortisol, HCN = hair cortisone, HCR = log-ratio of hair cortisol and cortisone. The number behind the abbreviation indicates that the hair sample reflects the time period of 4-8 weeks postpartum. I1 - I4 = Time point of infant microbiota sampling in chronological order (2, 6 and 12 weeks and 8 months). MS = maternal stress composite score.

Table 2.10: Bacterial species identified in the infant microbiota samples associated with any of the maternal stress variables. Results are based on a multilevel model that was fit to all time points.

Stress Variable	Feature	Coefficient	q
EPDS	s__Clostridium_fessum	0.000	0.002
EPDS	s__GGB9627_SGB15081	0.000	0.041
EPDS	s__Blautia_SGB4805	0.000	0.061
EPDS	s__Intestinimonas_butyriciproducens	0.001	0.001
EPDS	s__Actinomyces_graevenitzii	0.001	0.034
EPDS	s__Gemmiger_SGB15299	-0.003	0.041
EPDS	s__Senegalimassilia_anaerobia	0.003	0.061
EPDS	s__Streptococcus_parasanguinis	0.005	0.047
EPDS	s__Ruminococcus_gnavus	0.019	0.027
HCN1	s__Blautia_glucerasea	0.000	0.095
HCN3	s__Blautia_sp_AF19_10LB	-0.002	0.000
HCN3	s__Flavonifractor_plautii	-0.020	0.014
HCR1	s__Alistipes_finegoldii	-0.002	0.010
HCR2	s__Bacteroides_fragilis	0.044	0.010
HCR3	s__Bacteroides_fragilis	0.068	0.003
HCS2	s__Bacteroides_fragilis	0.031	0.028
HCS3	s__Bacteroides_fragilis	0.049	0.024
HCS4	s__Streptococcus_parasanguinis	-0.009	0.083
MS	s__Clostridium_fessum	0.000	0.085
MS	s__Intestinimonas_butyriciproducens	0.001	0.002
MS	s__Actinomyces_graevenitzii	0.001	0.058
MS	s__Senegalimassilia_anaerobia	0.003	0.051

MS	s__Bacteroides_xylanisolvens	0.005	0.057
MS	s__Streptococcus_parasanguinis	0.005	0.058
PRAQR2-B	s__Phocaeicola_vulgatus	0.004	0.092
PRAQR2-H	s__Prevotella_copri_clade_A	0.001	0.052
PSS-10	s__Actinomyces_graevenitzii	0.001	0.048
PSS-10	s__Intestinimonas_butyriciproducens	0.001	0.089
PSS-10	s__Bacteroides_xylanisolvens	0.006	0.007
STAI	s__Intestinimonas_butyriciproducens	0.001	0.002
STAI	s__Senegalimassilia_anaerobia	0.003	0.031

Note:
The leading "s" indicated phylogenetic Species level. HCS = hair cortisol, HCN = hair cortisone, HCR = log-ratio of hair cortisol and cortisone. The number behind the abbreviation indicated the measured time interval including 23-32 (1), 15-23 (2) and 6-15 weeks of gestation as well as 4-8 weeks postpartum (4). PRAQR2-B = Fear of giving birth. PRAQR2-H = Fear of a handicapped child. MS = maternal stress composite score.

Table 2.11: Bacterial phyla identified in the infant microbiota samples associated with any of the maternal stress variables. Results are based on a multilevel model that was fit to all time points.

Stress Variable	Feature	Coefficient	q
EPDS	p__Firmicutes	0.032	0.032
MS	p__Firmicutes	0.029	0.081
PSS-10	p__Firmicutes	0.031	0.050

Note:
MS = maternal stress composite score.

Table 2.12: Bacterial species identified in the infant microbiota samples associated with any of the maternal stress variables. Results are based on models that were fit per time point as indicated by the "Sample" and "Time" columns.

Time	Sample	Stress Variable	Feature	Coefficient	q
32	I3	HCS3	s__Bacteroides_fragilis	0.056	0.062
32	I2	HCS3	s__Bacteroides_fragilis	0.096	0.041
32	I2	HCS2	s__Bacteroides_thetaiotaomicron	-0.034	0.074
32	I3	HCS2	s__Bacteroides_fragilis	0.037	0.075
32	I2	HCS2	s__Bacteroides_fragilis	0.068	0.013

32	I3	HCN3	s__Faecalicatena_contorta	-0.002	0.000
32	I2	HCN3	s__Odoribacter_splanchnicus	-0.007	0.094
32	I4	HCN3	s__Blautia_sp_AF19_10LB	-0.010	0.000
32	I2	HCN3	s__Bacteroides_xylanisolvens	-0.038	0.022
32	I2	HCN3	s__Flavonifractor_plautii	-0.039	0.022
32	I4	HCN1	s__Sutterella_wadsworthensis	-0.023	0.096
32	I3	EPDS	s__Intestinimonas_butyriciproducens	0.000	0.013
32	I3	EPDS	s__Roseburia_intestinalis	0.002	0.013
32	I3	EPDS	s__Actinomyces_graevenitzii	0.002	0.047
32	I4	EPDS	s__Parabacteroides_merdae	0.002	0.080
32	I3	MS	s__Intestinimonas_butyriciproducens	0.000	0.013
32	I3	MS	s__Roseburia_intestinalis	0.001	0.013
32	I3	MS	s__Actinomyces_graevenitzii	0.001	0.020
32	I4	MS	s__Parabacteroides_merdae	0.001	0.095
18	I3	PRAQR2-B	s__Intestinimonas_butyriciproducens	0.000	0.059
18	I3	PRAQR2-B	s__Clostridia_unclassified_SGB4121	0.001	0.059
18	I3	PRAQR2-B	s__Roseburia_intestinalis	0.001	0.059
32	I3	PRAQR2-B	s__Actinomyces_graevenitzii	0.002	0.067
18	I3	PRAQR2-B	s__Actinomyces_graevenitzii	0.003	0.000
32	I4	HCR3	s__Intestinibacter_bartlettii	-0.003	0.001
32	I4	HCR3	s__Cutibacterium_avidum	-0.006	0.000
32	I3	HCR3	s__Bacteroides_fragilis	0.069	0.044
32	I2	HCR3	s__Bacteroides_fragilis	0.138	0.004
32	I3	HCR2	s__Bacteroides_fragilis	0.051	0.023
32	I2	HCR2	s__Bacteroides_fragilis	0.093	0.005
32	I4	HCR1	s__Alistipes_finegoldii	-0.010	0.011
32	I3	STAI	s__Intestinimonas_butyriciproducens	0.000	0.003
32	I3	STAI	s__Roseburia_intestinalis	0.002	0.003
32	I4	STAI	s__Parabacteroides_merdae	0.002	0.038
32	I3	STAI	s__Actinomyces_graevenitzii	0.003	0.005

Note:
The column 'Time' indicates when the stress variable was measured during pregnancy. HCS = hair cortisol, HCN = hair cortisone, HCR = log-ratio of hair cortisol and cortisone. The number behind the abbreviation indicates the measured times including 23-32 (1), 15-23 (2) and 6-15 weeks of gestation. I1 - I4 = Time point of infant microbiota sampling in chronological order (2, 6 and 12 weeks and 8 months). PRAQR2-B = Fear of giving birth. MS = maternal stress composite score. The leading 's' indicates phylogenetic Species level.

2.6.3 Supplementary Figures

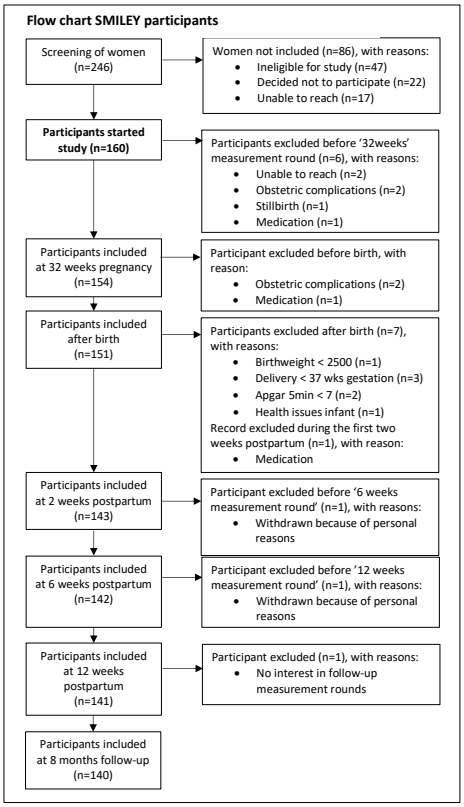

Figure 2.6: Flow chart SMILEY participants.

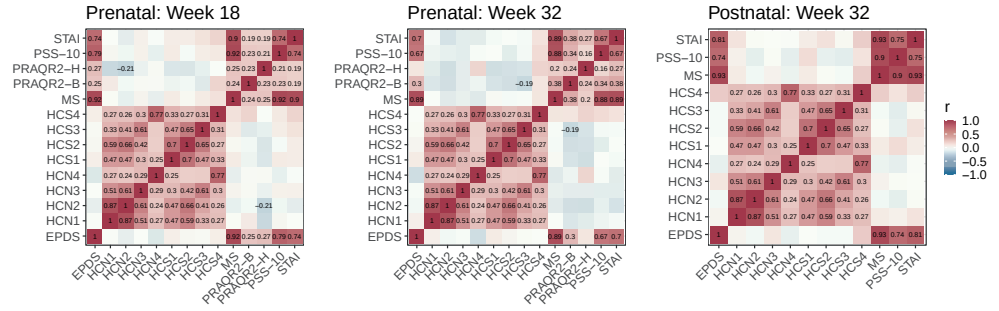

Figure 2.7: Pearson correlations between the stress variables at the time points of maternal microbiota sampling. If the corelation was significant, the coefficient estimate is shown in the tile, otherwise not. PRAQR2-H = fear of a handicapped child. PRAQR2-B = fear of giving birth. HCS = hair cortisol, HCN = hair cortisone, HCR = log-ratio of hair cortisol and cortisone. The measured times of hair cortisol and cortisone include 6-15 (1), 15-23 (2) and 23-32 (3) weeks of gestation as well as 4-8 weeks postpartum (4).

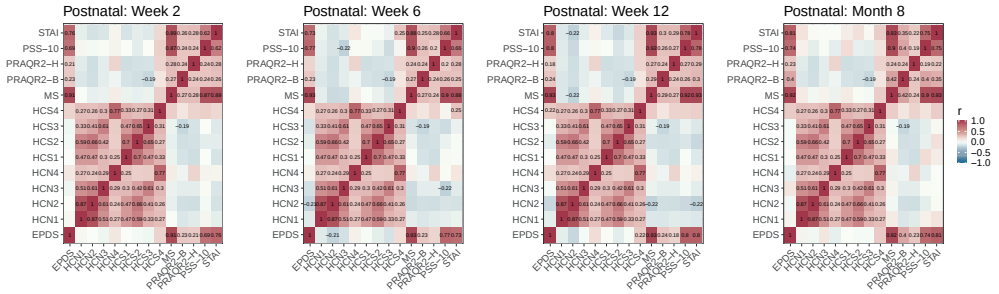

Figure 2.8: Pearson correlations between the stress variables at the time points of infant microbiota sampling. If the corelation was significant, the coefficient estimate is shown in the tile, otherwise not. PRAQR2-H = fear of a handicapped child. PRAQR2-B = fear of giving birth. HCS = hair cortisol, HCN = hair cortisone, HCR = log-ratio of hair cortisol and cortisone. The measured times of hair cortisol and cortisone include 6-15 (1), 15-23 (2) and 23-32 (3) weeks of gestation as well as 4-8 weeks postpartum (4).

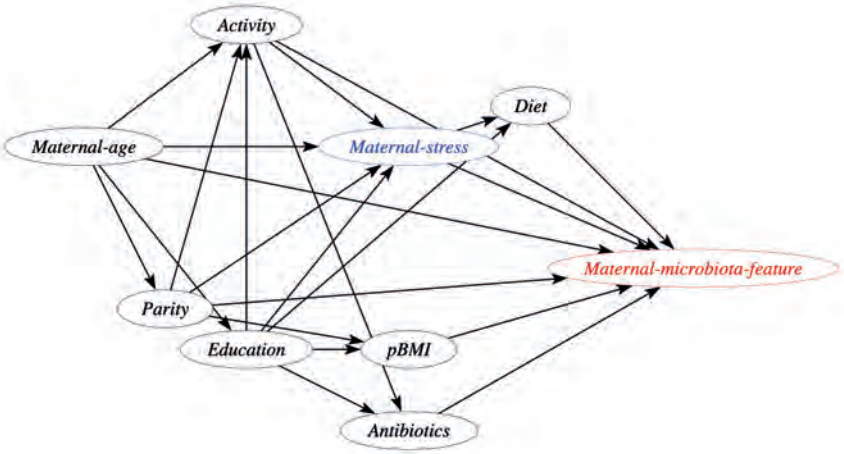

Figure 2.9: Directed acyclic graph depicting the assumptions underlying the analyses for the maternal microbiota samples. pBMI = pre-pregnancy BMI. Activity = Pregnancy Physical Activity Questionnaire. Diet = Dutch Healthy Diet index.

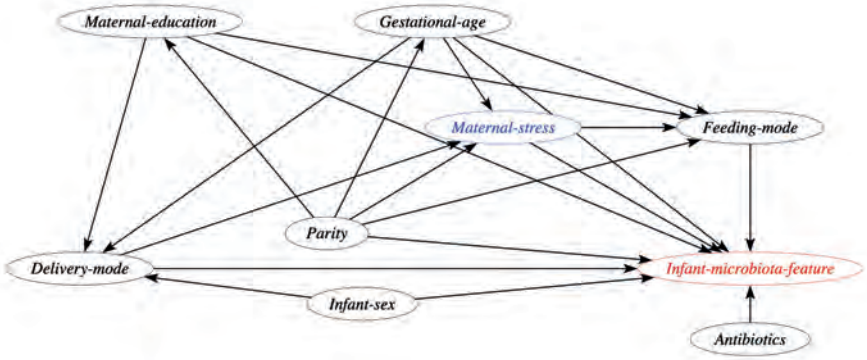

Figure 2.10: Directed acyclic graph depicting the assumptions underlying the analyses for the infant microbiota samples. Note that for analyses that use prenatal stress the direction of the arrow between gestational age and maternal stress reverses.

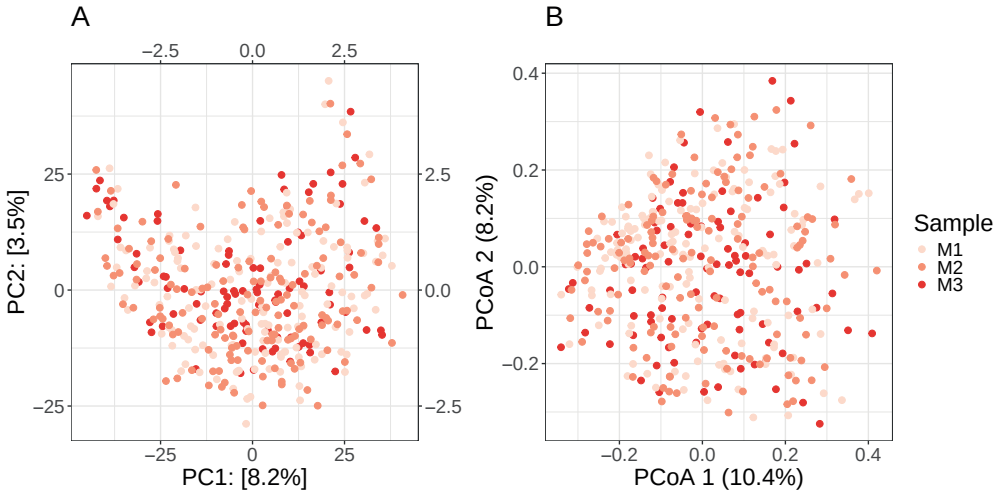

Figure 2.11: Visualization of beta diversity of all maternal samples using either Aitchison distance (A) or Bray-Curtis similarity (B). Samples were taken at 18 (M1) and 32 (M2) weeks of pregnancy as well as 8 months postpartum (M3).

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Chapter 3

Does Entry to Center-Based Childcare Affect Gut Microbial Colonization in Young Infants?

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3.1 Abstract

Entry to center-based childcare (CC) at three months of life can be an important challenge for infants as it includes major stressors such as long maternal separations and frequently changing caregivers. Stress and the new environment may in turn alter the composition of the gut microbiota with possible implications for future health outcomes. As part of an ongoing longitudinal study, we investigated whether CC, as compared to being cared for by the parents at home, alters the composition of the gut microbiota, while accounting for known covariates of the infant gut microbiota. Stool samples of infants who entered CC (n=49) and control infants (n=49) were obtained before and four weeks after CC entrance. Using Redundancy analysis, Random Forests and Bayesian linear models we found that infant gut microbiota was not affected in a uniform way by entry to CC. In line with the literature, breastfeeding, birth mode, age, and the presence of siblings were shown to significantly impact the microbial composition.

3.2 Introduction

The human gut microbiota refers to a complex and dynamic population of microorganisms that resides in the human gastrointestinal tract and has recently become object of much scientific endeavors. Intestinal bacteria as part of this ecosystem play central roles in human health and disease (Cryan et al., 2019; Marchesi et al., 2016). They are essential for nutrition, intestinal function, the education of the developing immune system and the protection of the host from invading microbial pathogens (Adlerberth & Wold, 2009; Gonzalez et al., 2011; Leslie et al., 2019). Via a bi-directional communication system, intestinal bacteria may even influence brain development and behavior (Cryan et al., 2019; de Weerth, 2017; Derrien et al., 2019; Mayer, 2011).

Multiple factors contribute to the development of the human gut microbiota in infancy and the microbial composition becomes relatively stable within the first 3–5 years of life, although some reports describe a longer development phase (Robertson et al., 2019; Rodríguez et al., 2015). Because intestinal bacteria influence the development of important host physiological systems within this early and critical developmental time period (Borre, Moloney, et al., 2014) it is crucial to understand the factors that influence the establishment of the early gut microbiota. In the present study we will concentrate on an early life factor that can potentially disrupt healthy gut microbial colonization and negatively affect microbial composition: entry into childcare at the age of 3 months. In the following paragraphs, we will outline how gut microbial colonization occurs and why entering childcare at that young age may disrupt this process.

Gut microbiota development is a highly dynamic and individual process. The current consensus is that the first major exposure to microbes happens during the birthing process and is highly dependent on mode of delivery (Dominguez-Bello et al., 2010; Dominguez-Bello et al., 2016; Korpela & De Vos, 2018). The first inoculation during natural childbirth clearly resembles the maternal fecal microbiota, with potential input from the vagina and other parts of the urogenital tract (Korpela & De Vos, 2018). In contrast, infants delivered through a Caesarean section (C-section) are colonized with common skin and environmental microbes (Dominguez-Bello et al.,

2010). Nevertheless, this difference in microbiota composition between children born vaginally or by C-section seems to gradually decrease, although some later life impact has been reported (Sevelsted et al., 2015; Tamburini et al., 2016).

The initial inoculum initiates a succession of events leading to the development of a child's own microbiome. In this dynamic process the microbial abundance increases over time, with large fluctuations in the microorganisms present and their relative abundance (Koenig et al., 2011). Diversity generally increases, aerobes are succeeded by facultative and then strict anaerobes and, roughly up until the introduction of the first solid foods, a well-constrained range of stereotypical bacteria emerge in the faeces. Exclusive breast-feeding generally selects for genera specialized in the utilization of complex human milk oligosaccharides, such as *Bifidobacterium* (Sela et al., 2008) and to a lesser extent *Bacteroides* spp, as they can compete for the same ecological niche (Marcobal et al., 2010). By studying both Western and non-Western populations it has been shown that differences exist with regards to community membership, but that the overall temporal dynamics are similar over populations, with aberrant development after C-section delivery, use of antibiotics or early termination of breast-feeding (Blanton et al., 2016; Korpela & De Vos, 2018; Planer et al., 2016; Smith et al., 2013; Subramanian et al., 2014).

Microbial colonization is characterized by large inter- and intra-individual variability, with large, abrupt, community shifts with interludes of relative stability of varying lengths of time (Favier et al., 2002; Koenig et al., 2011; Palmer et al., 2007; Trosvik et al., 2010). Sometimes these shifts occur together with life events that likely instigate considerable environmental pressure, such as antibiotics use, fever, and introduction of formula feeding (Palmer et al., 2007). Animal models that use maternal separation (MS) to induce early life stress, suggest that early life stress is another such environmental pressure that can induce shifts in the microbiota. MS provokes an adult depressive and anxiety-like phenotype, along with altered immune function, activation of the hypothalamic-pituitary-adrenal (HPA) axis and disruption of the offspring's microbiota (Bailey & Coe, 1999; Desbonnet et al., 2010; Foster et al., 2017; S. M. O'Mahony et al., 2009, 2011). De Palma et al. (2015) demonstrated the importance of the microbiota to induce these behavioral changes in animals. In germ free mice HPA axis regulation was altered by early life stress, but for the actual induction of the behavioral changes the microbiota was required. This indicated that MS-induced changes in host physiology led to intestinal dysbiosis, which in turn was needed for behavioral changes to occur (De Palma et al., 2015).

The entrance into center-based childcare (CC) typically starts at three months of life in the Netherlands and includes early life stressors such as long maternal separations, new and frequently changing caregivers and peers, and exposure as well as adaption to a new physical environment. Entering CC produces significant increases in cortisol levels as compared to being cared for in the home environment. Cortisol levels continue to increase until at least a month after entering (Ahnert et al., 2004; Albers et al., 2016; Watamura et al., 2010; Waynforth, 2007). CC has also been related to other symptoms and illnesses including diarrhea, respiratory illnesses, otitis media, and skin complaints (Beijers et al., 2011a). These findings indicate that entering CC at this young age can be an early life stressor for infants.

This study explores the effects of the entrance to CC on the developing microbiota of 3-month-old infants by comparing infants attending CC to infants being cared for at home. In line with the above-mentioned animal studies, we expected CC to be associated with changes in

microbial composition. Specifically, we tested for differences in the relative abundances at a genus-like level (see Methods) as well as differences in alpha diversity by combining univariate Bayesian approaches with multivariate methods including the Random Forests machine learning algorithm that has previously been shown to be useful in microbiome data analyses (Blanton et al., 2016). We included breastfeeding as a potential protective factor for microbial development, and accounted for known confounders, namely birth mode, antibiotics, age, and the presence of siblings (Stewart et al., 2018).

3.3 Methods

3.3.1 Participants

As part of a larger and ongoing longitudinal study (BIBO), 220 mothers were followed since the third trimester of pregnancy, to investigate the influences of early environmental and caregiving factors on child development. Uncomplicated singleton pregnancy, proficiency in the Dutch language, no drug use, and the absence of physical and mental health problems were criteria for initial inclusion. Eight of the 220 women were excluded due to preterm birth or for other medical reasons. In addition, 19 mothers discontinued participation in the study during the first three postpartum months because of personal circumstances. All remaining infants (N=193) were healthy and born at full term (37 weeks). Infants who had used antibiotics (n=4) were excluded from the current study. In the first four months of life, mothers collected 9 fecal samples from their infants. Two samples were available for use in this study: Ten weeks post-partum, before entrance to CC (PRE), and 4 weeks after the PRE sample (POST). After eliminating infants who did not provide stool samples for both time points, the final sample size consisted of 49 infants who entered CC (group CC) and 49 infants who were cared for at home (group HOME). Table 3.1 shows demographic variables for both groups. The age of the HOME group infants was slightly lower than that of the CC group, both for sample PRE ($p<0.001$) as for sample POST ($p<0.001$), using Welch's t-test. There were no significant differences between groups for any other of the shown variables. Within the CC group, infants varied in the number of half-days of childcare per week (Mdn=4, IQR=3-4). We tested in a separate analysis whether the number of half-days was associated with gut microbiota composition beyond just the grouping variable, but this effect did not modify the conclusions (Supplementary Table 3.4). This study and all its experimental protocols were approved by and carried out in accordance with the Ethical Committee of the Faculty of Social Sciences, Radboud University Nijmegen (ECG/AvdK/07.563). Informed consent was obtained from each mother.

Table 3.1: Descriptive statistics for demographic variables of infants and mothers included in the present study.

	CC (n = 49)	HOME (n = 49)	p-value
Sex			
Male	29	25	0.417
Female	20	24	
Age (days) PRE			
mean (sd)	87.8 (±) 16.0	76.7 (±) 6.3	<0.001
Min	58	68	
Max	123	90	
Age (days) POST			
mean (sd)	118.4 (±) 16.1	106.5 (±) 5.9	<0.001
Min	90	97	
Max	154	116	
Maternal Age (years)			
mean (sd)	32.9 (±) 3.0	32.2 (±) 3.6	0.306
Min	25.1	24.9	
Max	42.0	40.1	
Birthweight (grams)			
mean (sd)	3630.4 (±) 508.9	3636.0 (±) 438.4	0.955
Min	2708	2810	
Max	4600	4700	
Breastfeeding (Birth - PRE)			
mean (sd)	5.4 (±) 2.9	5.7 (±) 2.3	0.558
Min	0	0	
Max	11.4	8.9	
Breastfeeding (PRE - POST)			
mean (sd)	4.0 (±) 2.9	3.8 (±) 2.8	0.832
Min	0	0	
Max	8.5	8.2	
Formula-feeding (Birth - PRE)			
mean (sd)	1.5 (±) 2.4	1.4 (±) 2.1	0.558
Min	0	0	
Max	7.7	7.0	
Formula-feeding (PRE - POST)			
mean (sd)	1.8 (±) 2.4	2.0 (±) 2.3	0.832
Min	0	0	
Max	5.9	6.0	
Proportion breastfeeding (Birth - PRE)			
mean (sd)	0.8 (±) 0.4	0.8 (±) 0.3	0.558

Min	0	0	
Max	1	1	
Proportion breastfeeding (PRE - POST)			
mean (sd)	0.7 (±) 0.5	0.6 (±) 0.5	0.832
Min	0	0	
Max	1	1	
Siblings			
Yes	25	32	0.186
No	23	17	
C-Section			
Yes	6	3	0.294
No	42	45	

3.3.2 Microbiota covariates

The mothers received diaries towards the end of their pregnancy with instructions to take weekly notes about breastfeeding and formula-feeding from week 1 until week 27 after birth. For each week, the average number of breast- and/or formula-feedings per day were noted. To determine the effect of breastfeeding we defined two breastfeeding variables: the average number of feedings per day before and during the investigative period (i.e. birth to when the PRE-sample was obtained and PRE to when the POST-sample was obtained). The average number of breastfeedings included the feeding of expressed breastmilk through a bottle. We included the breastfeeding variables, age, siblings and C-section as covariates in all linear models. For 5 infants (4 in CC (8%), 1 in HOME (2%)) breastfeeding data was missing completely. For the univariate analyses the missing values were imputed using multiple imputation (see methods). For the multivariate analysis and visualization the original data was used.

3.3.3 Feces collection, DNA isolation and microbiota profiling

The parents were instructed to collect the fecal samples at home and to store them at -20 degrees Celsius. For transportation, samples were kept in coolers and then stored at -20 and later at -80 degrees Celsius before being processed at the Laboratory of Microbiology at Wageningen University. DNA isolation from fecal samples has been described elsewhere in detail (Salonen et al., 2010). In brief, DNA was isolated using a combination of column purification and Repeated-Bead-Beating. Purity and concentration of DNA were assessed with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). The analysis was then performed utilizing a previously benchmarked custom made, phylogenetic microarray, the Human Intestinal Tract Chip (HITChip) (Claesson et al., 2009; Rajilić-Stojanović et al., 2009). The HITChip contains a duplicated set of 3,631 probes, which target the V1 and V6 hypervariable regions of the 16 S rRNA gene of 1140 intestinal bacterial phylotypes. After extraction of DNA, the full-length 16S rRNA gene was amplified by PCR using primers T7prom-Bact-27-for and Uni-1492-rev41. This was followed by in vitro transcription and labelling of the resulting RNA

with Cy3/Cy5 before hybridization to the array. The signal intensity data from the microarray hybridizations were collected from the Agilent G2505C scanner (Agilent Technologies) using the Agilent Feature Extraction software, version 10.7.3.1 and pre-processed using an in-house MySQL database and custom R scripts. Each scanner channel from the array was spatially normalized separately using polynomial regression, followed by outlier detection and filtering in each set of probes with a χ^2 test. Each sample was hybridized at least twice to ensure reproducibility. Duplicate hybridizations with a Pearson correlation <0.95 were not considered for further analysis. Microbiota profiles were summarized to genus-like 16S rRNA gene sequence groups with a sequence similarity >90% referred to as species and relatives ('et rel.'). Measurements of probes that belong to the same phylotype were normalized with Robust Probabilistic Averaging (Lahti et al., 2011, 2013). Log10-transformed hybridization signals were used as a proxy for bacterial abundance.

3.3.4 Statistical analyses

3.3.4.1 Microbiota analysis

All analyses were performed in R version 3.5. Bacterial richness was calculated at the probe level by using an 80% quantile threshold for detection of each individual probe. Diversity within a sample is termed alpha diversity and was calculated using the Shannon metric. HITChip signals were transformed to relative abundance. To determine the dynamics of microbial groups we calculated their coefficient of variation (CoV). CoV is a standardized measure of dispersion defined as the ratio of the standard deviation (σ) to the mean (μ). For the redundancy analysis (RDA) and the Bayesian robust linear models we applied centered-log-ratio (clr) transformation (Aitchison, 1986). The clr-transformation of relative abundances allows for the application of statistical methods that have been developed for real random variables, such as RDA and the Bayesian models (Gloor et al., 2017). To determine the multivariate effects of CC and the number of half-days in CC on overall microbiota composition, we performed redundancy analysis (RDA) while accounting for the following variables that are known to influence microbiota composition: breastfeeding (average number of feedings during and before the investigative period), birth mode (C-section vs natural birth), age in days, and the presence of siblings using the function rda from the vegan package (Oksanen et al., 2020).

To determine the univariate effects of CC entry on alpha diversity and each bacterial group individually, we performed Bayesian hierarchical robust linear models as described by Krushke et al.(Kruschke, 2013). Bayesian data analysis provides several advantages over classical null hypothesis group comparison methods. These include richer information about parameter estimates, as the method provides complete distributional information about model parameters such as means and standard deviations, including credibility intervals of all possible combinations of these parameters. Furthermore, Bayesian data analysis delivers more precise information about the uncertainty when estimating group differences. The robust linear model presented by Krushke et al. in particular has advantages over standard linear models: The model is able to accurately estimate the mean (μ) and standard deviation (σ) when outliers are present as it utilizes the student t-distribution instead of the gaussian distribution. Furthermore, standard linear models assume homogeneity of variance between groups while this assumption is often

violated in the context of differential abundance testing (Kruschke, 2013). In addition, an environmental factor could lead to a change in the variance of a distribution rather than (only) a change in the mean. This possibility is not considered in standard models. Our model allows the standard deviationto vary between the groups by modeling it as a linear function of the grouping variables CC, time, siblings and birth mode. The model can be written as:

$$y_i \sim T(\nu, \mu, \sigma)$$

where

$$\mu = \beta_{0i[j]} + \beta_1 \times CC + \beta_2 \times time + \beta_3 \times CC \times time + \beta_4 \times age + \beta_5 \times bf + \beta_6 \times sib + \beta_7 \times csec + \beta_8 \times sib \times csec$$

and

$$\sigma = \beta_{\sigma 0} + \beta_{\sigma 1} \times CC + \beta_{\sigma 2} \times time + \beta_{\sigma 3} \times CC \times time + \beta_{\sigma 4} \times sib + \beta_{\sigma 5} \times csec + \beta_{\sigma 6} \times sib \times csec$$

Besides μ (mean) and σ (standard deviation (SD)), the model consists of the parameter ν, which represents the normality parameter (low values lead to long and heavy tails, whereas as ν increases, the distribution approaches the gaussian distribution). The j in $\beta_{0i[j]}$ indicates that each subject can deviate from the overall mean (often referred to as partial pooling or mixed effects modeling).

The goal is to infer differences in μ and σ of the assumed distribution of relative bacterial abundances between the subgroups (CC PRE, CC POST, HOME PRE and HOME POST), while accounting for the effects of the other environmental variables. This implies multiple comparisons of model parameters. In the Bayesian framework we can use a normal prior centered at 0 with a standard deviation of 1 for each comparison of interest across all the models so that the Bayesian 95% credible interval (CI) of the effect size is always more likely to include zero compared to the classical confidence interval, which makes Bayesian inference a more conservative approach (Gelman et al., 2012). The robust linear models were fitted using the package *brms*, which uses the probabilistic programming language Stan (Bürkner, 2017; Carpenter et al., 2017). Stan utilizes Hamilton Monte Carlo (HMC), a Markov chain Monte Carlo (MCMC) method, to estimate parameters. To ensure proper convergence of the chains, we investigated individual chains using Shinystan and screened diagnostic parameters (divergent transitions and rhat values) (Gabry & Veen, 2020). To visualize the posterior predictive intervals we utilized the tidybayes package (Kay, 2020). Brms allows multiple data sets as input, which enables the use of multiple imputation. We used predictive mean matching (PMM) as implemented in the mice package (van Buuren & Groothuis-Oudshoorn, 2011) to impute the missing data points within the breastfeeding variable. PMM is robust against misspecification of the imputation model while performing as well or better than common parametric imputation models under various missingness conditions with up to 20–30% missing values (Kleinke, 2017; Marshall et al., 2010). In contrast, the variable breastfeeding had 10% missing. The multiple imputation resulted in

10 new datasets that were used to fit the Bayesian models. We also explored the same models using listwise deletion.

Finally, to evaluate whether we could predict childcare entrance based on microbiota composition after one month, we used the Random Forests machine learning algorithm (RF) with relative abundance data. RF is a tree-based ensemble learning method that is well suited for classification based on microbial abundances (Knights et al., 2011). We performed repeated 10-fold cross-validation (10 × 10) to estimate the classification accuracy using the caret package (Kuhn, 2008).

3.4 Results

3.4.1 Infant microbiota composition and dynamics

Overall, the infant microbiota was dominated by only a few genera from four Phyla; Actinobacteria, Firmicutes (specifically members from the Class Bacilli), Bacteroidetes and Proteobacteria. At the genus level, these were *Bifidobacterium* spp. with a mean relative abundance of 51%, followed by facultative anaerobes from the Bacilli (bacteria related to *Streptococcus* (16.8%), *Enterococcus* (3.4%), *Lactobacillus plantarum* et rel (2.9%) and *Granulicatella* (1.0%)). The cumulative mean relative abundance of these groups was more than 75%. Additionally, the variation in relative abundance of these taxa at the population level was very high. For instance, the relative abundance of *Bifidobacterium* spp. ranged from completely dominating (89%) to almost absent (0.2%) (Fig. 3.1A,B). Apart from being the most dominant taxa at the population level these were also among the most variable within subjects as determined by their CoV (Fig. 3.1C). The fact that the most abundant taxa display the highest variability implies a highly variable microbiota.

3.4.2 Effects of childcare entry on infant microbiota

3.4.2.1 Redundancy analysis of the effects of childcare entry on infant gut microbiota composition

We determined whether childcare (CC) entry affected overall microbiota community composition using Redundancy Analysis (RDA). RDA is a direct gradient analysis technique which summarizes linear relationships between components of response variables (microbiota) explained by a set of explanatory variables (CC and covariates) by multiple linear regression of the multiple response variables on the multiple explanatory variables. To determine how the different environmental variables interact with and impact the microbiota, we calculated their simple effects (i.e. the effect of the environmental variable on the microbiota without any other covariates) as well as the conditional effects (the impact on the microbiota when the effect of the other variables are partialled out). This allowed us to determine the effect of each variable on its own, but also their combined effects.

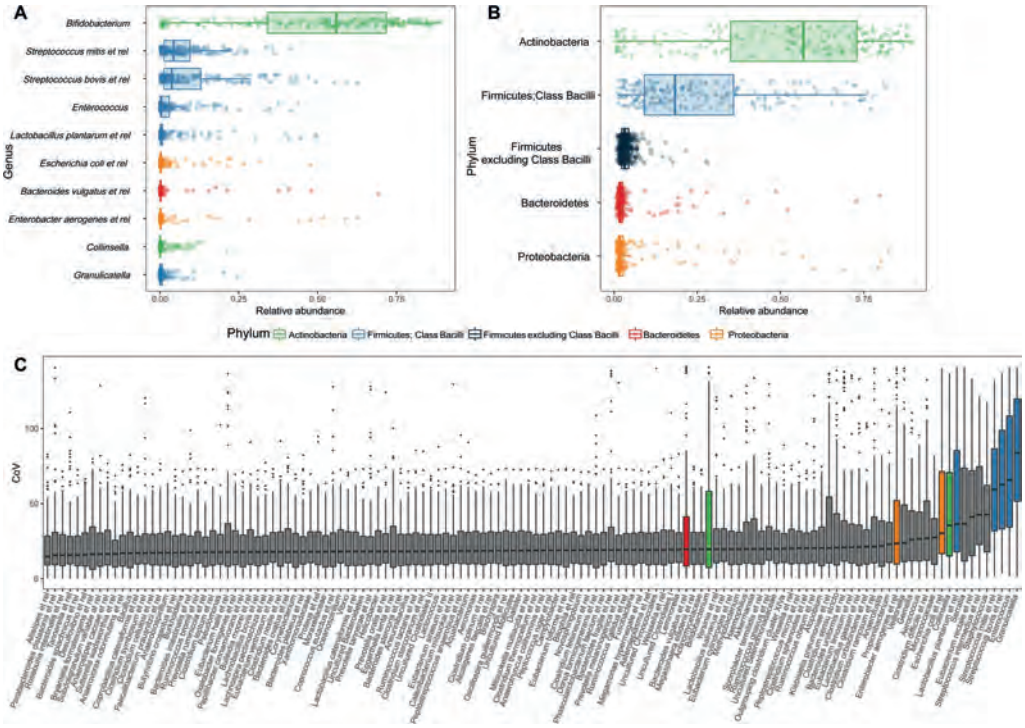

Figure 3.1: Infant microbiota composition and dynamics. (A,B) Relative abundance of major genus-like groups and their Phyla with mean abundance of >1% at the population level. (C) Coefficient of Variation (CoV) of all genus-like groups. The infant microbiota is highly variable as it is dominated by only a few taxa, which are also among the most variable. GREEN - Actinobacteria; BLUE - Firmicutes, Class Bacilli; BLACK - Firmicutes excluding Class Bacilli; RED - Bacteroidetes; ORANGE - Proteobacteria.

Table 3.2: RDA models output.

	Df	Variance	F	Pr (>F)	R2
Simple effects					
Time	1	0.317	1.4265	0.129	0.008
CC	1	0.274	1.2314	0.256	0.007
Age	1	0.436	1.9677	0.028*	0.011
Sibling	1	0.456	2.0602	0.019*	0.011
Birth-mode	1	0.559	2.5288	0.015**	0.014
Breastfeeding	2	1.605	3.7085	0.001***	0.032
CC × Time	3	0.781	1.172	0.226	0.019
Conditional effects					
Age	1	0.436	2.0652	0.018*	0.004
Sibling	1	0.598	2.8338	0.003**	0.010
Birth-mode	1	0.609	2.8843	0.002**	0.010
Breastfeeding	2	1.663	3.9364	0.001***	0.027

We did not find a significant effect of CC entry or the number of half-days in CC (Supplementary Table 3.1), compared to staying at home, on the microbiota. Neither in separation nor combined with other environmental variables. Nevertheless, birth mode, feeding mode, age, and siblings, were significantly correlated to the microbiota in concordance with literature (Stewart et al., 2018). The strongest effect was from breastfeeding, with decreasing effect sizes for birth mode, siblings and age. All simple and conditional effects and corresponding p-values and their respective effect sizes (R^2) are shown in Table 3.2. R^2 reflects the percentage of variation explained out of the total microbiota variation; i.e. a higher R^2 implies a stronger effect. All these findings are combined in a tri-plot visualizing the relation of the environmental variables with each other and their resulting effect on the microbiota (Fig. 3.2A). The relation between the variation explained by the environmental variables and their overlapping conditional effects is visualized in a Venn diagram (Fig. 3.2B).

3.4.2.2 Bayesian group comparisons of effects of entry on individual microbial groups and microbiota diversity

To gain more insight on the association of CC entry and other environmental variables with individual bacterial groups and microbiota diversity, we performed Bayesian hierarchical robust linear models. Bayesian approaches provide more detailed information about the uncertainty when estimating parameters such as group differences or slope parameters in linear models. The robust linear model as described by Kruschke et al. is particularly well suited to model distributions when outliers are present and to address common model violations in standard linear models such as heterogeneity of variance (Kruschke, 2013). In the following, for covariates, effect refers to the magnitude of the slopes whereas for the group comparisons effect refers to the difference in the means. To compute the difference in means between two groups (e.g. CC-PRE - CC-POST) the calculated posterior distributions of their means are subtracted. We make a

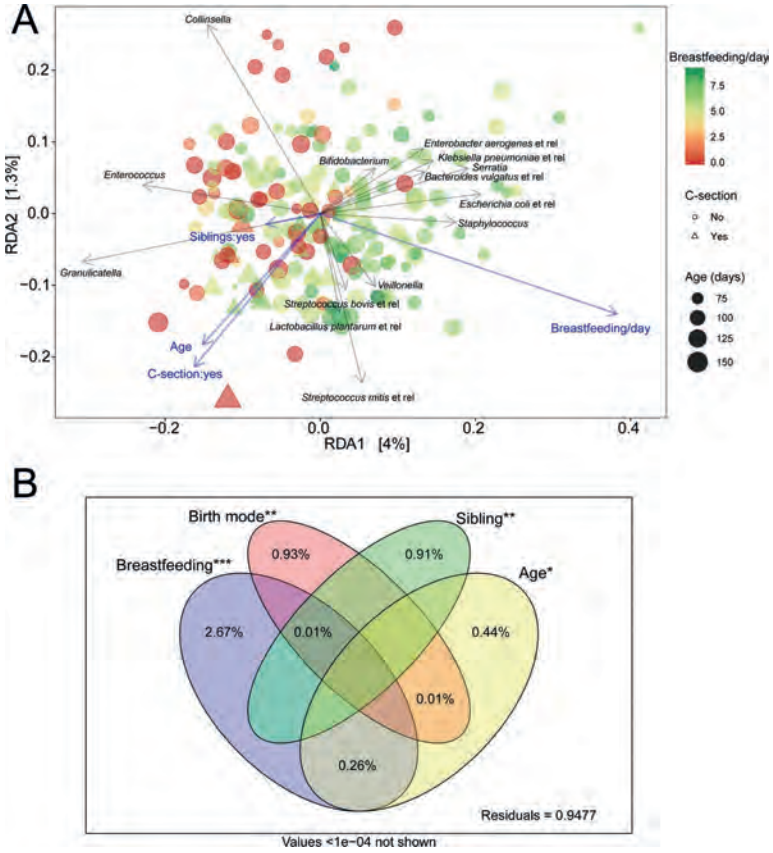

Figure 3.2: The impact of birth mode, breastfeeding, siblings and age on the infant gut microbiota. (A) Redundancy analysis (RDA) visualizing microbiota composition of all fecal samples (n = 196) colored by the number of breast-feedings and the size of the points scaled by age in days. Individuals born by C-section are represented as triangles. RDA displays and explains the variation explained in the microbiota, constrained by the predictor variables. Blue arrows depict the significant environmental variables and grey arrows the abundance of bacterial groups. Length of the arrows is a measure of fit. The longer the arrow the higher the association. (B) Venn diagram visualizing the partitioning of the variation explained by the significant predictors. P< 0.001, P < 0.01, P < 0.05.

statement with confidence about the effect size being larger than zero when 95% of the posterior distribution excludes zero. The use of listwise deletion instead of multiple imputation led to similar results.

Figure 3.3 shows the posterior distributions of interest whereby red coloring indicates that we can make a claim with confidence. Within the CC or HOME group (Fig. 3.3A), temporal effects were as follows. Within CC, bacteria related to *Streptococcus bovis* and *Staphylococcus* were lower after one month, whereas within HOME bacteria related to *Granulicatella* and *Aerococcus* were higher, while those related to *Enterobacter aerogenes* and *Oxalobacter formigenes* were lower. We did not detect differences in relative bacterial abundances between the two groups (Fig. 3.3B, right graph) before CC entry, while after one month the relative abundance of the Proteobacteria related to *Enterobacter aerogenes* and *Klebsiella pneumoniae* was lower in the HOME group, while that of *Streptococcus intermedius* was higher (Fig. 3.3B, left graph).

Figure 3.3C shows that infants born via C-section showed higher relative abundances of bacteria related to *Granulicatella*, *Aerococcus* and Micrococcaceae, but lower relative abundances of *Bifidobacterium*. Infants with siblings were found to have lower levels of *Staphylococcus* and also a lower SD of this taxon. A lower SD was also confirmed for *Enterococcus*, without differences in the mean. Finally, as can be seen in Fig. 3.3D, a higher number of daily breast-feedings was associated with more bacteria related to *Staphylococcus* and less to *Enterococcus*, *Collinsella*, *Eggerthella lenta* and *Oxalobacter formigenes*. A higher age was positively associated with bacteria related to *Streptococcus mitis*, *Streptococcus intermedius* and *Gemella*.

We used the same approach to compare microbiota diversity between groups. Table 3.3 shows the estimated difference in the means and standard deviations between the groups as well as the magnitude of the slopes for the covariates. Within the CC or HOME groups our model estimates that there is no temporal effect on diversity as well as between the two groups before CC entrance. However, when comparing HOME and CC one month after entrance, the average diversity was estimated to be lower in the CC group. Figure 3.4 shows the means of the Shannon diversity index per group with 95% CI (black point range) as well as the observed values (black points) and the posterior predictive interval (blue bar). To calculate the predictive interval, we used the median for the average number of breast-feedings and the median age at PRE and POST, respectively. The Bayesian predictive intervals illustrate the uncertainty of the predictions: the estimated distributions for the CC/HOME groups are very much overlapping despite the mean difference, whereas C-Section seems to have a stronger impact on diversity. Age and breastfeeding did not reach our predefined threshold to make a statement with confidence about the effect being > 0 and there was no difference in the estimated standard deviations between groups.

3.4.2.3 Random Forest analysis of non-linear relationships between childcare entry and microbiota composition

Finally, we used the random forests (RF) algorithm to determine if we could accurately classify whether an infant belonged to the HOME or CC group after one month. The latter would indicate a characteristic effect of CC on the infant gut microbiota. The benefit RF has over the linear models is its ability to detect non-linear associations. The prediction accuracy using

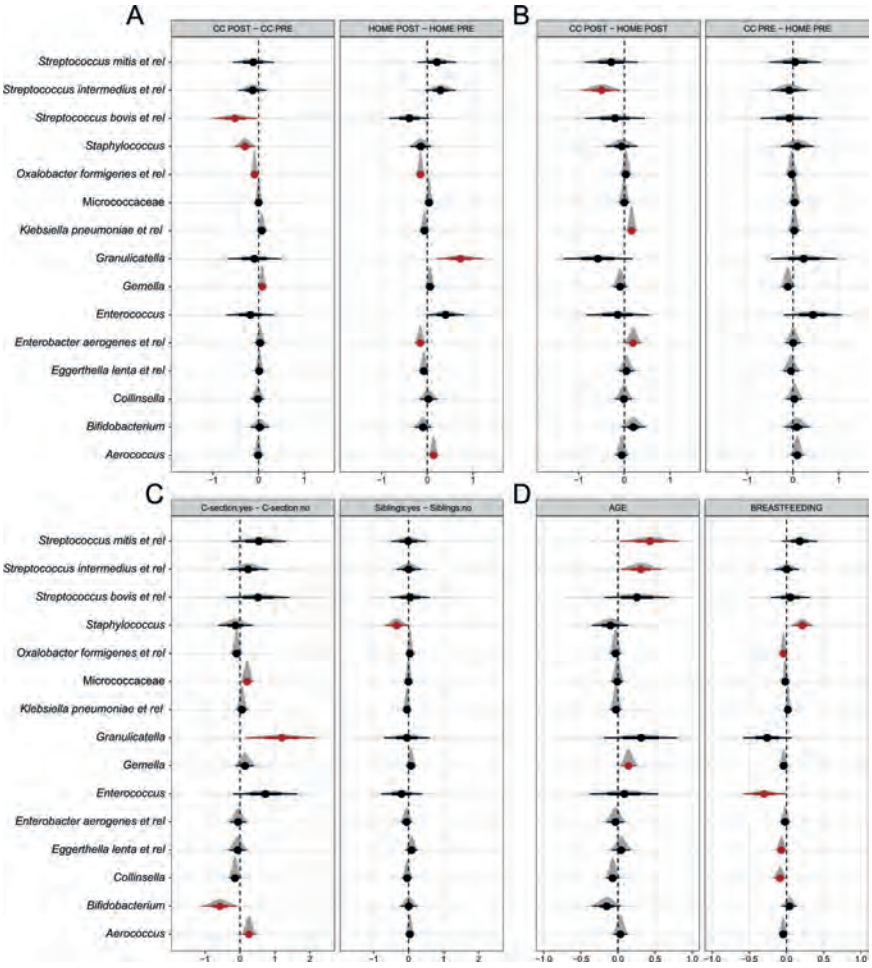

Figure 3.3: Bayesian hierarchical robust linear model posterior distributions for individual bacterial group differences. (A) within group effects, (B) between group effects and (C) C-section and siblings (D) age and breastfeeding. For the covariates (D) the x-axis refers to the magnitude of the slopes whereas for the group comparisons (A–C) it refers to the difference in the means. Taxa are shown in red when the probability that the absolute effect size is >0 exceeds 0.95, given our model and the data. 88

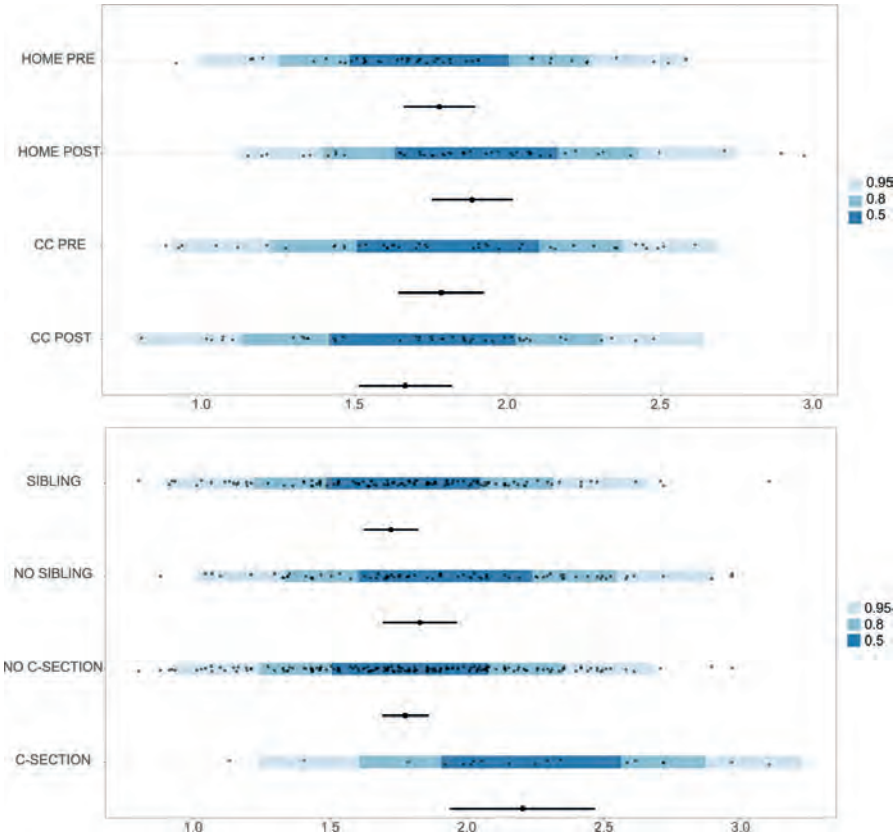

Figure 3.4: Bayesian hierarchical robust linear model group posteriors for microbiota alpha diversity. Means of Shannon diversity per group with 95% CI are shown with the black point range. The observed values are shown as black points within the blue bar. The Bayesian posterior predictive intervals are shown in blue. The predictions were made using the median for average number of breast-feedings and median age at PRE and POST.

Table 3.3: Estimated model parameters for microbiota diversity (Shannon).

Comparison	Median	95% CI
Difference in means		
CC PRE - HOME PRE	0.01	[-0.18, 0.19]
HOME POST – HOME PRE	0.11	[-0.01, 0.23]
CC POST – CC PRE	-0.12	[-0.26, 0.03]
CC POST – HOME POST	-0.22	[-0.41, -0.03]
Covariates		
C-section:yes – C-section:no	0.43	[0.15, 0.70]
Sibling:yes – Sibling:no	-0.11	[-0.27, 0.06]
Age	0.06	[-0.07, 0.20]
Breastfeeding	-0.06	[-0.13, 0.01]
Difference in SD		
CC PRE – HOME PRE	0.07	[-0.02, 0.17]
HOME POST – HOME PRE	0.01	[-0.09, 0.10]
CC POST – CC PRE	0.02	[-0.13, 0.19]
CC POST – HOME POST	0.08	[-0.04, 0.22]
C-section:yes – C-section:no	0.06	[-0.06, 0.24]
Sibling:yes – Sibling:no	-0.02	[-0.12, 0.07]

repeated cross validation was close to random classification with 53.5%, suggesting that CC did not produce a strong uniform shift in microbiota composition.

3.5 Discussion

This study examined the effect of entry into center-based childcare (CC) on gut microbial composition of 3-4-month-old infants by comparing the microbial composition between infants that entered CC at 3 months and infants that were cared for at home (HOME) at that age. For all infants, we assessed microbial composition at two time points: At 10 weeks of age (PRE), which was before CC entrance for the CC group, and 4 weeks later, or 4 weeks after entrance for the CC group (POST). We accounted for known covariates including age, presence of siblings (yes vs no), delivery mode (natural vs C-section) and the average number of breast-feedings per day in the period before measurement of the microbiota. We combined multivariate (Redundancy analysis and Random Forest algorithm) with Bayesian univariate statistical methods to test our hypothesis that CC entrance is associated with changes in microbial composition over time.

The microbiota of all the infants exhibited a low microbial diversity as it was dominated by only a few typical bacterial groups from the phylum Actinobacteria (*Bifidobacterium* spp and *Collinsella*), facultative anaerobes from the Firmicutes (such as *Streptococcus* spp, *Lactobacillus* spp and *Enterococcus* spp), Proteobacteria (*E.coli* and bacteria related to *Enterobacter aerogenes*) and Bacteroidetes (*Bacteroides* spp). These findings are in line with findings from previ-

ous studies in the US and Europe (Germany, Finland, Sweden and the Netherlands) (Borewicz et al., 2019; Stewart et al., 2018; Yatsunenko et al., 2012).

In contrast to breastfeeding, birth mode, age, and the presence of siblings, CC was not associated with gut microbiota composition according to Redundancy analysis (RDA). In line with that, we could not achieve higher accuracy than by chance using the Random Forest algorithm to classify CC vs HOME using the POST samples. Bayesian univariate analyses, that also take the individuality of the starting microbiota into account, did show a few taxa to be differently distributed between the two groups in the POST samples. In the HOME group the relative abundances of Proteobacteria related to *Enterobacter aerogenes* and *Klebsiella pneumoniae* were lower while that of *Streptococcus intermedius* was higher compared to the CC group at time POST. Except for bacteria related to *Streptococcus intermedius*, these bacterial groups were also heavily influenced by other environmental variables. Using the same Bayesian approach, we observed a lower Shannon alpha diversity in the CC group compared to the HOME group at time POST. All in all, our results show that entrance to CC does not result in a complete and homogeneous 'disruption' or 'dysbiosis' of the microbiota as reported for rodents subjected to early life stress.

There are several possible explanations for the lack of a general effect of the entrance to childcare on infants' gut microbiota. First, although CC entrance may be considered a major stressor in a young infant's life that is accompanied by substantial rises in stress hormones (Ahnert et al., 2004; Albers et al., 2016; Watamura et al., 2010; Waynforth, 2007), it might not be comparable in severity to maternal separations in rodents. E.g. in our CC sample infants were taken care of by surrogate caregivers, while in maternal separation paradigms with rodent pups, there is no surrogate caregiver during the separation (S. M. O'Mahony et al., 2011). Also, Dutch infants typically attend childcare for around 2 days a week (Beijers et al., 2013); this contrasts with practices elsewhere in the world and may not be enough exposure to produce major effects on the microbiota. Second, rodents lack the genotypical variation found in human populations and are kept and studied in controlled laboratory environments in which the individual variation in gut microbiota is small (Nguyen et al., 2015; Yates et al., 2016). Hence, in rodent models the effects of environmental pressures can be studied in complete isolation. This may lead to a much greater response to an individual stressor than it normally would when other (stronger) environmental drivers of microbiota composition are present. Finally, the infant gut microbiota at 3–4 months of age is still in an unstable, dynamic, highly individual developmental stage (Favier et al., 2002; Koenig et al., 2011; Palmer et al., 2007; Stewart et al., 2018; Trosvik et al., 2010). This was confirmed by a very large intra and inter-individual microbiota variability in our population. Microbiota at these young ages appears to show large fluctuations, probably resulting from a myriad of environmental influences. Additionally, infants of the present study went to different childcare centers, which exposed them to different built environments, caregivers and other infants with different microbiomes, thereby increasing the variety of environmental influences on the microbiota. Nevertheless, a generalizable stress-related effect across centers would most likely have been detectable, despite these (unknown) and potential center-specific confounding variables. Other factors than those we controlled for are e.g. fever, contact with animals, and/or genetics (Palmer et al., 2007; Stewart et al., 2018). The instability of the infant microbiota might also possibly be an intrinsic property of the dynamics of the gut microbial colonization and may obscure the effects of individual environmental factors that may each have only a modest

influence on gut microbiota composition. In other words, an environmental factor would need to be very strong to generate a universal disruption in the microbial composition that overrides the normally occurring fluctuations in infant gut microbiota in the first months of life.

The results of our study showed significant associations with known important environmental factors. Their effects were often partly overlapping and impacted similar bacterial groups (Fig. 3.2A,B), possibly indicating an accelerated colonization process in infants born by C-section. For instance, being older and being born by C-section were both positively associated with the abundance of the highly variable facultative anaerobes from the Bacilli and related to *S. bovis* and *S. mitis L. plantarum* and *Granulicatella* at the expense of *Bifidobacterium* and several Proteobacteria. Contrarily, a younger age and being born vaginally were both associated with higher relative abundances of *Bifidobacterium*, *Collinsella* and several Proteobacteria in line with previous research of (Dutch) infants (Penders et al., 2006; Yatsunenko et al., 2012). The latter pattern, except for *Collinsella*, was also associated with having no siblings. C-section delivery was associated with higher Shannon diversity. This increase in diversity may be the result of the lower proportions of the generally dominant *Bifidobacterium* in this group of infants, as has been previously reported (Jakobsson et al., 2014).

Breastfeeding showed the strongest association with infant gut microbiota composition. However, surprisingly, breastfeeding was only weakly related to *Bifidobacterium*, but was rather more associated with increases in mainly *Staphylococcus* and to a lesser extent, Proteobacteria (*Serratia*, *E. coli*, *Klebsiella pneumoniae* and *Enterobacter aerogenes* et rel) and *Bacteroides vulgatus* et rel. Breastfeeding was also strongly associated with a decrease in *Enterococcus* and *Collinsella*. In recent years in European countries, infant formulae have often been supplemented with prebiotics such as short chain galacto-oligosaccharides (scGOS) alone, or in a mixture with a chicory root derived inulin containing long chain fructo-oligosaccharides (lcFOS) (Authority, 2014). Prebiotics mimic the bifidogenic effect of oligosaccharides found in human milk thus preventing the difference in *Bifidobacterium* abundance that was previously associated with formula feeding (Borewicz et al., 2019). Furthermore, human milk itself has been found to contain bacteria, including Proteobacteria, as well as *Staphyloccocus*, which is generally associated with the skin (Ward et al., 2013). The latter has also been previously found to be depleted in formula-fed infants in concordance with our earlier findings (Borewicz et al., 2019). In total, the environmental variables explained a non-redundant ~5.9% of the microbiota composition. This is in the same order of magnitude as previously reported in other human studies (Falony et al., 2016). This means that in infants generally only a relatively small proportion of the gut microbial composition can be explained by the factors that are most commonly accounted for.

Despite the fact that we did not find an effect of CC entrance on the microbiota at the group level even after using different statistical approaches, it is not possible to conclude that there was no disrupting effect on the microbiota at the individual level. Individual infants did show large changes in the microbiota between the two assessments, but these changes were not uniform across individuals. Although temporal microbial dynamics at the population level, with regards to bacterial succession patterns, have been shown to be universal over different cultures and geography (Yatsunenko et al., 2012) it is known that this process is very variable between individuals (Favier et al., 2002; Koenig et al., 2011; Palmer et al., 2007; Trosvik et al., 2010). Future studies including larger populations and especially repeated measurements from before and after life changing events are necessary to determine whether the resulting bacterial shift

is typical or deviant for a specific individual. Subsequently, potential sub-groups of infants displaying specific signatures of dysbiosis can be determined, and their microbial signatures related to possible adverse health outcomes later in life. However, given the importance of gut microbial colonization for the development of the microbiota-gut-brain axis and the future health of the individual (Borre, Moloney, et al., 2014), it is also possible that the colonization process might be robust against perturbations such as entrance to CC, hence explaining the lack of microbial change at the group level.

This is the first human study that examined the effect of entrance to center-based childcare on microbiome development. The strengths are that it included a relatively large population of healthy infants with a natural variation of environmental variables and an ecologically valid stressor as opposed to typical rodent studies that generally include low number of individuals under strictly controlled laboratory conditions. Another strength is that we used a combination of different multivariate and Bayesian univariate statistical methods to analyze the data. However, there are also some weaknesses with regards to this study. Our population was ethnically and socio-economically uniform (Beijers et al., 2010). This can be seen as an advantage, but it precludes generalization to the larger population. Also, to conclude whether the microbiota is disrupted at an individual level, more sample time points than just PRE and POST would be needed. For example, it would be interesting to follow infants entering childcare for a longer period of time, as elevated infant cortisol has been observed throughout the first months in childcare (Albers et al., 2016). Larger and more stable differences in the gut microbiota of infants attending childcare and those being taken care of at home, may only appear after a longer period of time in childcare.

3.6 Conclusion

Entering center-based childcare has been shown to produce large increases in stress hormones in infants and can therefore be considered a significant stressor in early life. Childcare includes maternal separation and in animal models early life maternal separation has been found to lead to large shifts in gut microbial composition. However, in the present study infant gut microbiota was not impacted in a uniform way by entering childcare at the age of 3 months. Large shifts in gut microbiota were observed, but were idiosyncratic to individual infants and were also observed in infants not attending center-based childcare. In general, the infants' gut microbiota was found to be intrinsically very dynamic. Other environmental variables, namely breastfeeding, birth mode, age, and the presence of siblings, were shown to significantly impact the microbial composition, with effects that were largely overlapping and typically included the most abundant and variable taxa. Our results suggest that in infants the stress-inducing effects of childcare entry might not be as strong as the maternal separation paradigms of animal models. Alternatively, general effects may potentially only become visible after longer periods of childcare entry and when infants are older and their gut microbiota has become more stable.

3.7 Supplementary Tables

Table 3.4: RDA models output with Childcare attendance as half days instead of as a grouping factor.

Model Parameter	Sum of Squares	Mean Sum of Squares	F	Df	p	R2
Childcare	43.52	43.525	1.096	1.00	0.334	0.006
Time	61.44	61.441	1.548	1.00	0.082	0.008
Breastfeeding	204.80	204.797	5.158	1.00	0.001	0.027
Age	62.19	62.186	1.566	1.00	0.113	0.008
Childcare:Time	29.75	29.751	0.749	1.00	0.691	0.004
Residuals	7,305.03	39.701	-	184.00	-	0.948
Total	7,706.73	-	-	189.00	-	1.00

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Chapter 4

Daily Skin-to-Skin Contact Alters Microbiota Development in Healthy Full-Term Infants

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4.1 Abstract

The gut microbiota is vital for human body development and function. Its development in early life is influenced by various environmental factors. In this randomized controlled trial, the gut microbiota was obtained as a secondary outcome measure in a study on the effects of one hour of daily skin-to-skin contact (SSC) for five weeks in healthy full-term infants. Specifically, we studied the effects on alpha/beta diversity, volatility, microbiota maturation, and bacterial and gut-brain-axis-related functional abundances in microbiota assessed thrice in the first year. Pregnant Dutch women (n = 116) were randomly assigned to the SSC or care-as-usual groups. The SSC group participants engaged in one hour of daily SSC from birth to five weeks of age. Stool samples were collected at two, five, and 52 weeks and the V4 region was sequenced. We observed significant differences in the microbiota composition, bacterial abundances, and predicted functional pathways between the groups. The SSC group exhibited lower microbiota volatility during early infancy. Microbiota maturation was slower in the SSC group during the first year and our results suggested that breastfeeding duration may have partially mediated this relation. Our findings provide evidence that postpartum SSC may influence microbiota development. Replication is necessary to validate and generalize these results. Future studies should include direct stress measurements and extend microbiota sampling beyond the first year to investigate stress as a mechanism and research SSC's impact on long-term microbiota maturation trajectories.

4.2 Introduction

The human gastrointestinal tract is inhabited by a complex population of bacteria. These bacteria allow the digestion of dietary fibers, providing absorption of nutrients and energy(Cerqueira et al., 2020). They play an important role in intestinal integrity and immune functioning (Gensollen et al., 2016; Y.-J. Zhang et al., 2015). In addition, gut bacteria can influence the brain via the gut-brain axis, a bidirectional pathway between the gut and the brain (Cryan & Dinan, 2012; Cryan et al., 2019). The complex mechanisms underlying this bidirectional communication are still subject of study and have been thoroughly summarized elsewhere (Cryan et al., 2019). Briefly, the gut microbiota can influence human physical and mental development via the immune system, tryptophan metabolism, the vagus nerve and the entericnervous system. This communication involves microbial metabolites such as short-chain fatty acids, branched chain aminoacids and peptidoglycans. During the early stages of life, the gut microbiota and other co-evolving systems are particularly sensitive to environmental disturbances. Furthermore, the establishment of a healthy gut microbiota during early life is important for the functioning of other systems, such as the immune system(Claesson et al., 2012; Sekirov et al., 2010). Therefore, it is important to understand how the gut microbiota develops and how it is influenced during infancy. The present study investigated the potential effects of a randomized controlled trial (RCT) involving daily skin-to-skin contact (SSC) between mothers and their full-term infants on the developing gut microbiome.

The fetal gut is thought to be virtually sterile, although the sterility of the intra-uterine environment and meconium is still subject of debate (Milani et al., 2017). Starting from birth, the

mother is the infant's main source of microbial gut colonization via vaginal delivery, breastfeeding, and frequent close contact. This transmission of bacteria will remain detectable even at older ages (Valles-Colomer et al., 2023; Van Daele et al., 2019; L. Yang et al., 2021).Subsequently, other household members, close contacts, as well as pets, may become sources of bacteria (Hermes et al., 2020; Valles-Colomer et al., 2023). The infant's gut microbiota starts to increase in diversity and develops towards an adult-like microbiota as solid foods are introduced and breastfeeding is cessated(Bäckhed et al., 2015), although important changes are still seen into middle childhood (Ou et al., 2022). Finally, infant health, use of antibiotics, hygiene and infant genetics are important factors that contribute to the development of the gut microbiome (Korpela et al., 2017; Van Daele et al., 2019).

During SSC the naked infant, dressed only in a diaper, is placed on the bare chest of the mother (WHO, 2003). SSC can be considered de-stressing (Beijers et al., 2016; Cristóbal Cañadas et al., 2022; Ionio et al., 2021) and has been shown to be beneficial for both mother and infant. In pre-term infants, SSC immediately after birth is associated with improved health outcomes, as well as a reduced mortality rate for the infant, and improved caregiving behavior and lower postpartum depression for the mother (Bergman et al., 2004; Conde-Agudelo & Díaz-Rossello, 2016; de Alencar et al., 2007; Dombrowski et al., 2001; Feldman et al., 2002, 2014; Meder et al., 2021; Moore et al., 2016; Sinha et al., 2021; WHO Immediate KMC Study Group, 2021). After the first postnatal hours, daily SSC with pre-terms is associated with better physical outcomes and improved development of the brain and the cardiovascular system (Feldman & Eidelman, 2003; Feldman et al., 2014; Föhe et al., 2000). Interestingly, the effect of daily SSC on cognitive function in pre-terms was still seen in young adulthood (Charpak et al., 2017; Ropars et al., 2018). While the focus of these studies remains on pre-term infants, some studies have investigated full-term infants, although these studies are mostly limited to SSC in the first hours after birth. They have indicated several benefits of SSC right after birth, such as improved cardiovascular health, improved sleep, and weight gain (Ionio et al., 2021; Moore et al., 2016). Concerning the mother, SSC performed on full-term infants right after birth is associated with a decrease in anxiety and longer breastfeeding duration (Mehler et al., 2020; Moore et al., 2016; Walters et al., 2007). Prolonged, daily SSC is associated with a reduction in maternal depressive symptoms (A. Bigelow et al., 2012), anxiety and stress (A. E. Bigelow & Power, 2020). The SKIPPY study, of which this current study is part, was the first to perform an RCT to study an SSC intervention on both maternal and infant outcomes in a healthy, full-term sample (Cooijmans et al., 2017). The study showed that performing a daily hour of SSC during the first five prenatal weeks may reduce maternal anxiety and fatigue symptoms, increase infant sleep and reduce infant crying, and extend exclusive and continued breastfeeding durations (Cooijmans, Beijers, Brett, & de Weerth, 2022; Cooijmans, Beijers, Brett, & Weerth, 2022; Cooijmans, Beijers, & De Weerth, 2022). At three years of age, the children that had received the SSC intervention also showed fewer internalizing and externalizing behavioral problems (Rheinheimer, Beijers, Bruinhof, et al., 2022). However, the results did not indicate that this daily hour of SSC influenced mother-infant interaction quality and maternal depressive, stress, and pain symptoms, as was found in earlier intervention studies (Cooijmans, Beijers, Brett, & de Weerth, 2022; Rheinheimer, Beijers, Cooijmans, et al., 2022).

There are multiple pathways in which mother-infant SSC could potentially influence the infant gut microbiome. Firstly, as mentioned before, a daily hour of SSC was associated with fewer

maternal anxiety and fatigue symptoms (Cooijmans, Beijers, Brett, & de Weerth, 2022). Previous studies have indicated a link between maternal postnatal distress (including anxiety) and breast milk microbiota, which in turn could influence the gut microbiome of breastfed infants (P. D. Browne et al., 2019; Ziomkiewicz et al., 2021). Secondly, breastfeeding shapes the infant gut microbiome (van den Elsen et al., 2019). The extension of exclusive and continued breastfeeding duration caused by a daily hour of SSC could therefore influence the infant gut microbiome. Thirdly, SSC itself may also be de-stressing for the infant. Stress during the early developmental stages has been found to influence the gut microbiota and gut-brain axis in rodents (Bailey et al., 2011; S. O'Mahony et al., 2017). Therefore, a de-stressing practice such as SSC could potentially alter the colonization of the gut (de Weerth, 2017; Galley et al., 2014; Xu et al., 2020). Lastly, SSC could also provide an additional opportunity for the exchange of microbes between mother and child. As previous studies have shown, constant contact between microbial communities increases their similarity (Dill-McFarland et al., 2019; Drell et al., 2017) and the maternal skin microbiome has shown to provide a source of bacteria for the infant (H. P. Browne et al., 2022; Ferretti et al., 2018).

To the best of our knowledge, there are no previous studies on the effects of SSC on the infant gut microbiome. The current study investigates the effects of a five-week daily hour of SSC between mothers and full-term infants, compared to care-as-usual (CAU), on the infant gut microbiome. We hypothesized that the treatment and the control group differ in (1) alpha diversity, (2) beta diversity, (3) genus level abundances, (4) volatility, (5) microbiota age and (6) functional pathways related to gut-brain communication. While most hypotheses are non-directional, we hypothesized that SSC infants have less volatile microbiota, since stress has been shown to increase gut microbiota volatility (T. Bastiaanssen et al., 2021).

4.3 Methods

4.3.1 Study design

This RCT included two parallel groups: an intervention group and a passive control (Cooijmans et al., 2017). Ethics approval was granted by the ethics committee of the Social Science Faculty of Radboud University (ECSW2015-2311-358). This study was registered in the Netherlands Trial Register (NTR5697). It followed CONSORT guidelines and the protocol was published (Cooijmans et al., 2017).

4.3.2 Participants

Expectant mothers from the Nijmegen region were recruited between April 2016 and September 2017. Recruitment was performed with the help of a database of pregnant mothers who expressed interest in participating in scientific research, as well as via promotions at pregnancy clubs, baby fairs, and baby shops. Prenatal characteristics were examined during the last trimester of pregnancy, using an eligibility survey. Expectant women were eligible to participate when they were at least 18 years old, had good physical and mental health, had a singleton

pregnancy, were not using drugs during pregnancy, and had sufficient understanding of the Dutch language. After the birth of their child, mothers were excluded if their child was born before 37 weeks, had congenital anomalies, a birth weight less than 2500 g, and/or a 5-minutes Apgar score of < 7.

4.3.3 Procedure

4.3.3.1 Prenatal

Detailed study information was provided to eligible women during a home visit between weeks 34-36 of gestation. After informed consent was obtained, only mothers allocated to the SSC group were encouraged to engage in at least one uninterrupted daily hour of SSC, starting immediately after birth until including the fifth postnatal week (Dutch mothers are entitled to 10 to 12 weeks of paid leave after birth). Detailed written and oral instructions about the SSC protocol, optimal SSC position and safety were provided. CAU mothers received no additional information, and all mothers were encouraged to contact the principal investigator when experiencing any problems during the study by phone, text message or email. Besides SSC, both conditions underwent the same procedures.

4.3.3.2 Postnatal

All mothers reported daily information on SSC, holding (clothed physical contact), and no-contact in 15-min intervals every 2-3h in a logbook throughout the 5-week intervention period. All mothers were contacted weekly by telephone (postnatal day 5 and 13) or text-message/email (postnatal day 21 and 28) to remind mothers to complete the logbook, ask for questions/comments and, for SSC mothers, to discuss SSC obstacles. When their child was two weeks, five weeks and one year of age, parents were instructed to collect a fecal sample. The feces were collected directly from the first diaper that day, with the help of a provided plastic scoop. Parents were instructed to avoid any contact with surfaces or humans and put two or three scoops into a sterilized plastic tube. In addition to that, a stool questionnaire was completed, providing information on date of collection and infant health. After being temporarily stored in the home freezer (-20 degrees Celsius), the samples were collected during the home visits at 5 weeks and 1 year and then stored at -80 degrees Celsius. During the same home visits, questionnaires were obtained with information on covariates, such as gestational age, birth mode and feeding patterns.

4.3.4 DNA Extraction and Processing to Microbiota Features

The samples were transported to the Laboratory of Microbiology at Wageningen University and stored at -80°C until they were processed for DNA extraction as published previously (Ramiro-Garcia et al., 2018) and described in the following: DNA extraction was carried out with the Maxwell 16 TOTAL RNA system (Promega, Wisconsin, USA) in conjunction

with Stool Transport and Recovery Buffer (STAR; Roche Diagnostics Corporation, Indianapolis, IN). The V4 region of the 16S ribosomal RNA (rRNA) gene was amplified in duplication, generating amplicons of approximately 290bp. PCR reactions comprised 0.5µl Phusion Green Hot Start II High-Fidelity DNA polymerase (Thermo Scientific, US) at 2U/µl, 1µl of 10um barcoded primers 515F-n(5'-GTGYCAGCMGCCGCGGTAA-3') and 806R-n(5'-GGACTACNVGGGTWTCTAAT-3'), 10µl 5xPhusion Green HF Buffer (Thermo Scientific, US), 1µl of 10mM dNTPs mix (Promega Corporation, US), 36.5µl Nuclease-free water, and 1µl of 20ng/µl DNA template. PCR involved an initial denaturation period of 30s at 98°C, followed by 25 cycles of denaturation (98°C, 10s), annealing (50°C, 10s), and extension (72°C, 10s), concluding with a final elongation step (72°C, 7min). PCR products were validated through gel electrophoresis and purified using the HighPrep® PCR kit (MagBio Genomics, Alphen aan den Rijn, Netherlands). DNA concentration was assessed using a fluorometer (DS-11; DeNovix) with the Qubit® dsDNA BR Assay Kit (Life Technologies, Leusden, Netherlands). Barcoded samples belonging to the same library (200ng) were combined. Each library incorporated 69 unique barcode tags, with 2 specifically designed for artificial control communities representing human gut microbiota. The mixture underwent purification again to achieve a final volume of 40µl using the HighPrep® PCR kit. Sequencing was performed on the Illumina platform at Eurofins Genomics in Germany. Data was pre-processed from raw genetic sequences to amplicon sequence variants (ASV) tables using the NGTax2 pipeline (version 2.1.74) with the SILVA database (version 138.1). The sequence data, together with the metadata, were stored in a TreeSummerizedExperiment (TreeSE) container (R. Huang et al., 2021) for further analysis. To analyze whether the gut microbiota differs between the SSC and the CAU groups, the different features of the infant gut microbiome were evaluated. For alpha diversity, we calculated Shannon, inverse Simpson, Faith and Chao1 indices. For beta diversity, we calculated the Aitchison distance and Bray Curtis similarity. For volatility, we calculated the Aitchison distance sequentially between intra-individual samples (T. Bastiaanssen et al., 2021). The microbiota age was determined as described by Subramian et al. (Subramanian et al., 2014) and we controlled for age in the regression models. We used samples from other longitudinal studies (BIBO and BINGO) to train the Random Forest model. Functional pathways, specifically, the gut-brain-module (GBM) were calculated as described by Bastiaanssen et al. (T. F. S. Bastiaanssen et al., 2022).

4.3.5 Statistical analysis

In the following, we briefly describe the statistical analysis. For a more detailed description see the preregistration (https://doi.org/10.17605/OSF.IO/S45MU). All analyses were performed in R (version 4.2.1) (R Core Team, 2022) and the code has been published (https://doi.org/10.5281/zenodo.8121370). Missing covariates and outcome variables were imputed using predictive mean matching (m = 50) (Kleinke, 2017) with the package *mice* in R (van Buuren & Groothuis-Oudshoorn, 2011). Deviations of results from complete case analyses were to be reported. For each feature of the gut microbiota (alpha diversity, beta diversity, genus level abundances, volatility, microbiota age and GBM), we performed an intention to treat (ITT) and a per protocol (PP) analysis. We used Bayesian robust linear models to regress alpha diversity, volatility and microbiota age on SSC and covariates. These models were fit using the *brms*

package (Bürkner, 2018) with default priors and a student t distribution for the response variable. For differential abundance analysis (genus level and GBMs), we utilized ANCOMBC,(Lin & Peddada, 2020) LinDA,(Zhou et al., 2022) and MaAsLin2.(Mallick et al., 2021) The *adonis2* function from the *vegan* package was used for beta diversity analyses (Oksanen et al., 2022). We accounted for non-independence by specifying random intercepts in models that included repeated samples of an individual. The different alpha diversity indices were calculated with the R package miam (Ernst et al., 2022).

4.3.5.1 Covariates

In the ITT analyses, we can assume that randomization prevented confounding of the average causal effect estimate (supplementary Figure 4.16). Therefore, we only added covariates that may improve the precision of our estimate of interest in a data-driven manner (Cinelli et al., 2020). Possible mediators of the effect of SSC, such as breastfeeding, had to be excluded to estimate the total effect of SSC (Cinelli et al., 2020). Breastfeeding was only added to determine the magnitude of the direct effect of SSC on the microbiota once an effect of SSC was detected. If the direct effect is smaller than the total effect upon inclusion, while SSC is positively related to breastfeeding and breastfeeding is related to the microbiota, this would suggest a mediating role of breastfeeding under the assumed directed acyclic graph (supplementary Figure 4.16) (Cinelli et al., 2020). For the Bayesian models, we used leave-one-out cross-validation (Vehtari et al., 2017) to evaluate whether any of the following variables improved model fit per microbiome feature: C-section, birth weight, siblings (yes/no), sex, Apgar (at 5 minutes), gestational age at birth, and education level. To determine whether SSC had an effect across all samples and over time, we modeled SSC in interaction with age across the models that included the samples obtained at 2 and 5 weeks. When modeling the samples at 52 weeks, we omitted the interaction term. For the PP analyses, we did not use the data-driven approach because confounding of the total effect estimate is possible and we needed to make stronger assumptions (Hernán & Hernández-Díaz, 2012; Hernán et al., 2013). Measured variables that may influence whether an individual actually received the treatment (irrespective of assigned treatment), would need to be included to avoid confounding. Here, we included the following additional covariates in the models: birth weight, gestational age at birth, education level, C-section and sex. Sensitivity analyses were performed using complete case analyses as well as leaving out all covariates for the ITT analyses. Results did not differ meaningfully unless reported otherwise.

4.4 Results

4.4.1 Descriptive statistics

Participation in stool sample collection and eligibility resulted in 116 participants (Figure 4.1). For one stool sample, PCR amplification did not provide enough material for sequencing. While there was no loss to follow-up in this RCT, some mothers did not provide stool samples for all time points leading to a total of 315 analyzed samples: 105 at week two, 107 at week five and 103 at week 52. Across all time points, 11 phylum-level groups and 162 genus-level groups

were identified. The SSC group provided longer total SSC duration (intention-to-treat (ITT): 2067.67 ± 850.65 min; per-protocol (PP): 2905.90 ± 497.52 min) than the CAU group (ITT: 308.17 ± 442.41; PP: 308.17 ± 442.41) according to independent sample t-tests ($p < 0.001$). The average daily SSC duration between groups is furthermore depicted in Figure 4.2. An overview of the baseline characteristics of the infants and their mothers, including information on missing data, is presented in Table 4.1. In the following, we present for each preregistered feature of the microbiota the results of the ITT analysis and in case of meaningful deviation, the PP analysis. Relevant parameter estimates, such as β-coefficients of the Bayesian robust linear models, are mentioned in the text. To inspect all model coefficients, see supplementary Figures 4.8-4.13.

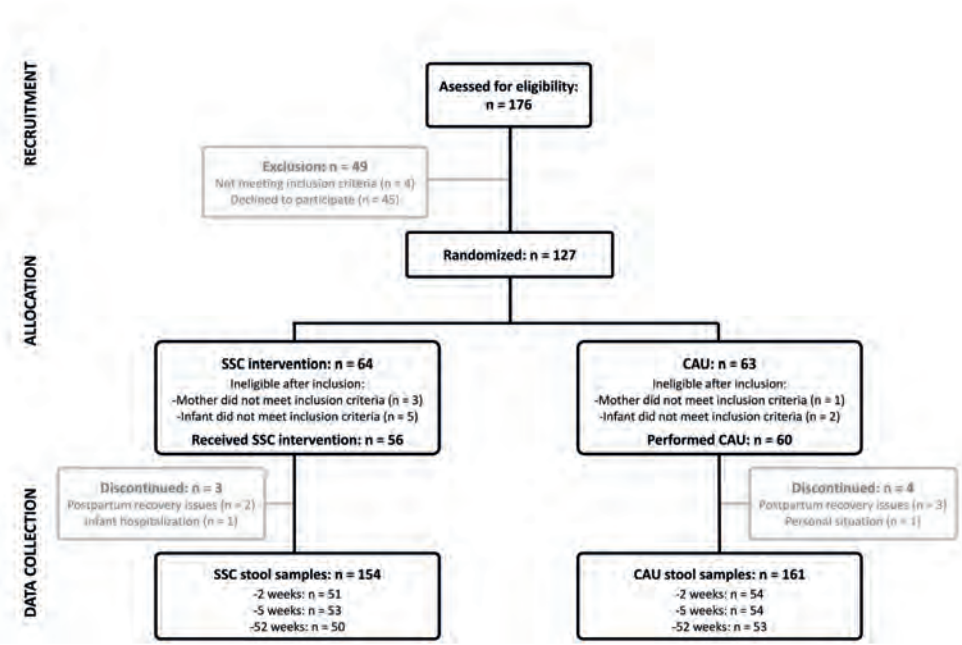

Figure 4.1: Participant flow diagram, including the number of participants at each of the trial stages. SSC = skin-to-skin contact. CAU = care-as-usual.

Table 4.1: Descriptive statistics and group comparisons for mother–infant dyads of the SKPPY study.

	CAU	SSC	Overall

	(N=60)	(N=56)	(N=116)
Sex			
Male	26 (43.3%)	33 (58.9%)	59 (50.9%)
Female	34 (56.7%)	23 (41.1%)	57 (49.1%)
C-section			
No	55 (91.7%)	51 (91.1%)	106 (91.4%)
Yes	3 (5.0%)	4 (7.1%)	7 (6.0%)
Missing	2 (3.3%)	1 (1.8%)	3 (2.6%)
Gestational age (weeks)			
Mean (SD)	40.0 (1.10)	40.1 (1.01)	40.0 (1.05)
Median [Min, Max]	40.2 [37.1, 42.1]	40.2 [36.6, 42.1]	40.2 [36.6, 42.1]
Birth weight (grams)			
Mean (SD)	3570 (386)	3650 (415)	3610 (401)
Median [Min, Max]	3520 [2880, 4620]	3670 [2740, 4850]	3610 [2740, 4850]
Siblings			
No	28 (46.7%)	27 (48.2%)	55 (47.4%)
Yes	32 (53.3%)	29 (51.8%)	61 (52.6%)
Exclusive breasfeeding duration (months)			
Mean (SD)	2.78 (1.47)	3.14 (1.86)	2.96 (1.68)
Median [Min, Max]	3.00 [0, 5.00]	3.00 [0, 9.00]	3.00 [0, 9.00]
Missing	9 (15.0%)	7 (12.5%)	16 (13.8%)
Age week 2 (days)			
Mean (SD)	14.9 (1.25)	14.1 (1.60)	14.5 (1.49)
Median [Min, Max]	14.0 [14.0, 19.0]	14.0 [8.00, 16.0]	14.0 [8.00, 19.0]
Missing	13 (21.7%)	8 (14.3%)	21 (18.1%)
Age week 5 (days)			
Mean (SD)	35.9 (2.33)	35.1 (1.88)	35.5 (2.13)
Median [Min, Max]	35.0 [28.0, 40.0]	35.0 [28.0, 39.0]	35.0 [28.0, 40.0]
Missing	13 (21.7%)	7 (12.5%)	20 (17.2%)
Age week 52 (days)			
Mean (SD)	371 (7.56)	370 (7.89)	371 (7.70)
Median [Min, Max]	369 [364, 399]	368 [362, 406]	368 [362, 406]
Missing	9 (15.0%)	9 (16.1%)	18 (15.5%)
Antibiotics week 2			
No	47 (78.3%)	43 (76.8%)	90 (77.6%)
Yes	1 (1.7%)	2 (3.6%)	3 (2.6%)
Missing	12 (20.0%)	11 (19.6%)	23 (19.8%)
Antibiotics week 5			
No	46 (76.7%)	42 (75.0%)	88 (75.9%)
Yes	2 (3.3%)	1 (1.8%)	3 (2.6%)
Missing	12 (20.0%)	13 (23.2%)	25 (21.6%)
Antibiotics week 52			

No	50 (83.3%)	46 (82.1%)	96 (82.8%)
Yes	1 (1.7%)	1 (1.8%)	2 (1.7%)
Missing	9 (15.0%)	9 (16.1%)	18 (15.5%)

4.4.2 Main Results

4.4.2.1 Alpha Diversity

Our results indicated, across different alpha diversity indeces, time points and ITT and PP analyses, that SSC had no effect on alpha diversity (Figure 4.3). Infants with siblings had lower Shannon alpha diversity scores (β = -0.31, 95% HDI [-0.52;-0.09], $P(\beta < 0) = 0.997$) in early infancy (2 and 5 week samples) but no longer in late infancy (1 year samples). Results regarding siblings were similar for Chao1 and inverse Simpson but the difference disappeared mostly when the Faith index was used.

4.4.2.2 Beta Diversity

Figure 4.4 shows results of a PCA of the centered-log-ratio transformed abundances (Aitchison distance). Samples obtained at one year (triangles) were clearly separated from samples obtained at 2 and 5 weeks. There seems to be no apparent separation of SSC and CAU samples considering all samples, also not when other principal components are inspected (not shown here). Within the 1-year samples (triangles), the SSC samples (grey) may occur more frequently closer to the early infancy samples than the CAU samples. Statistically, PERMANOVA identified that the microbiota composition of CAU and SSC samples differed significantly in the early infancy samples ($p = 0.016$) but not in the late infancy samples ($p = .087$). To derive the direct (rather than the total) effect of SSC under the assumed directed acyclic graph (supplementary Figure 4.16), we fitted new models including breastfeeding as a covariate. The effect remained significant, and the effect size unchanged, suggesting that SSC has an effect on microbiota composition independent of its effect on breastfeeding duration. In the PP analyses the effect size increased slightly, but the standard error increased as well due to the lower sample size, leading to a non-significant effect (p = .058). Note also that the result was sensitive to the choice of another distance metric. When using Bray Curtis similarity, the main effect was no longer significant (p = .377) while the interaction term suggested that SSC may have only had an effect on the microbiota at 5 weeks ($p = .069$). Using Bray Curtis similarity, SSC only showed a significant effect in the PP analyses if fitting a model to all samples ($p = .035$).

4.4.2.3 Genus Level Abundances

Because recent benchmark studies showed that differential abundance analysis results can depend heavily on the specific method that is applied (Cappellato et al., 2022; Nearing et al., 2022), we used several methods to evaluate the robustness of findings across methods. While we focus on the results of MaAsLin2 in this section, Figure 4.5 indicates whether a taxon was identified by one or more methods (denoted as M (MaAsLin2), L (LinDA) or A (ANCOMBC),

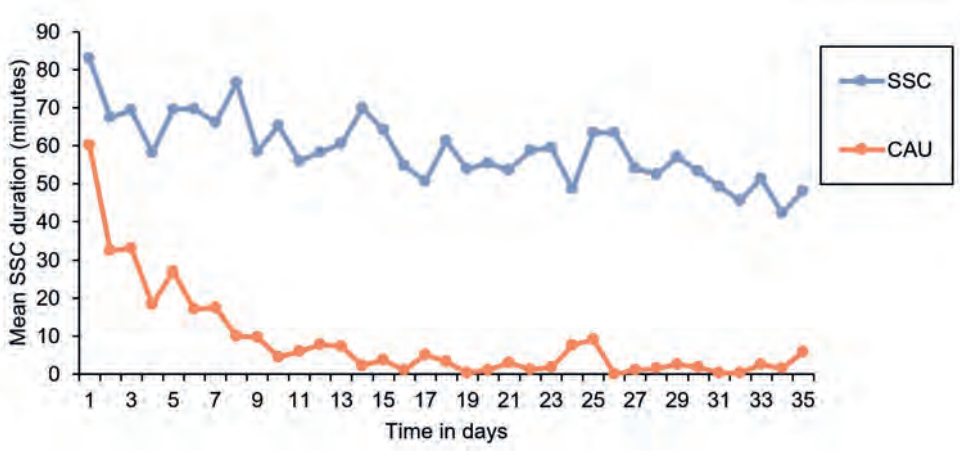

Figure 4.2: Mean daily skin-to-skin contact (SSC) duration in the SSC and care-as-usual (CAU) condition based on data derived from the intention-to-treat selection.

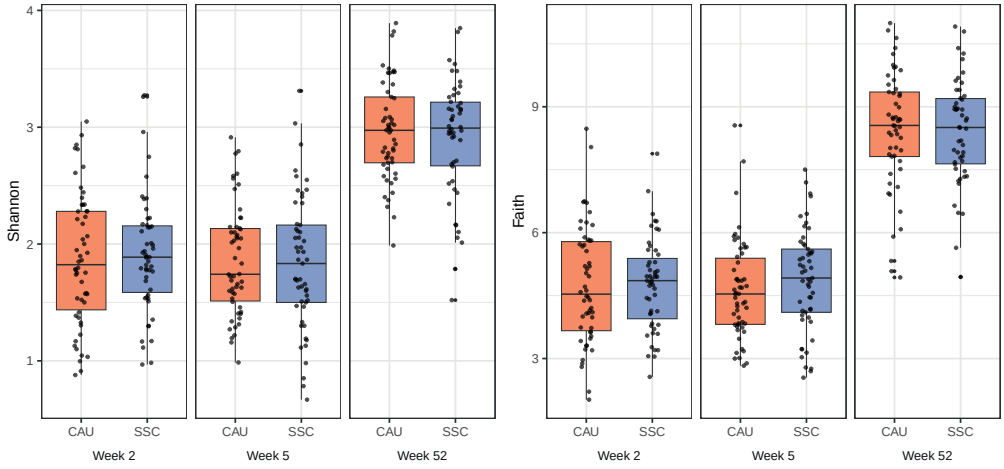

Figure 4.3: Alpha diversity indices between treatment (SSC) and control (CAU) group per sampling time point.

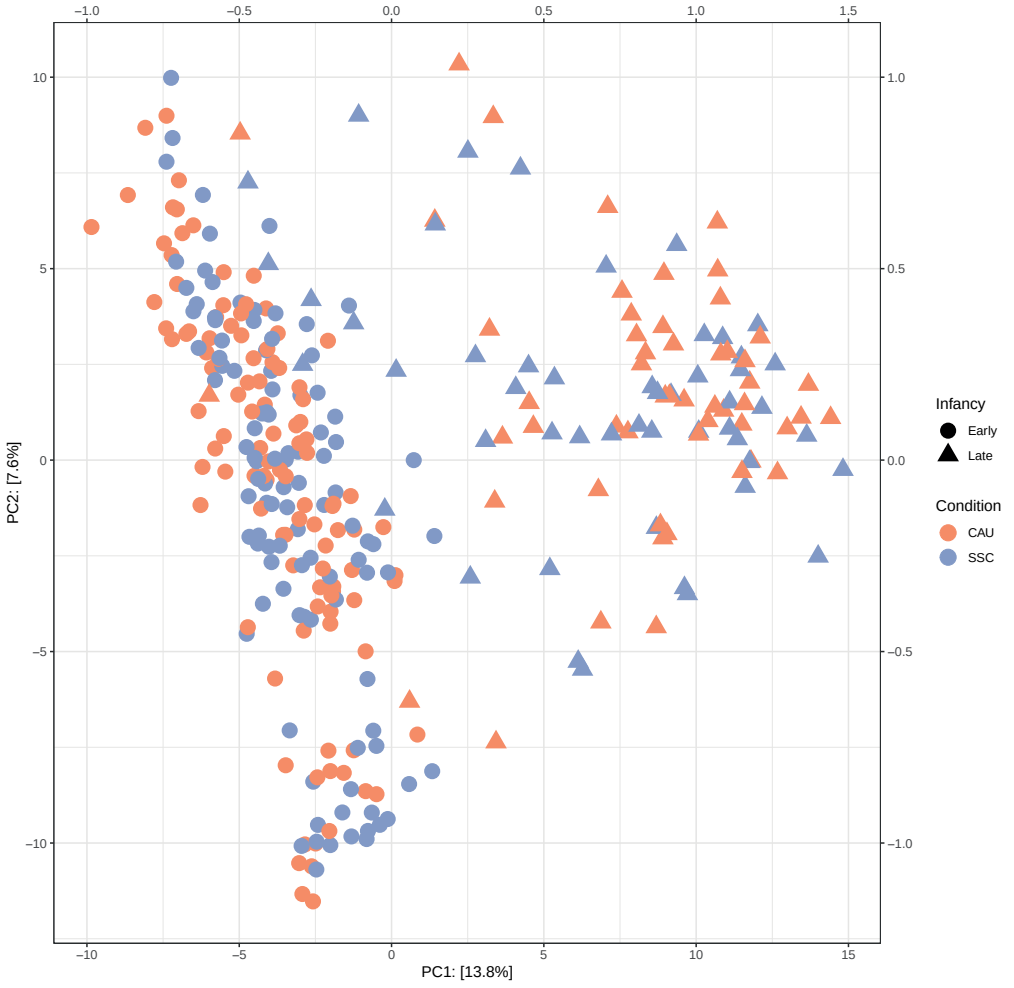

Figure 4.4: All samples plotted on the first 2 dimensions of a PCA of Euclidean distances of CLR transformed abundance values (Aitchison Distance).

respectively). MaAsLin2 detected lower relative abundance of *Faecalibacterium*, *Eubacterium hallii*, and *Rothia* and increases in *Flavonifractor*, *Lacticaseibacillus*, *Bacteroides* and *Megasphaera* in the SSC group compared to the CAU group. The top six genera in Figure 4.5 stand out as they were identified as a driver of differences between groups in at least two methods. From the heatmap we can further infer that some genera were only differentially abundant in either early or late infancy. Note that ANCOMBC as the most conservative method did not detect any genera as differentially abundant.

4.4.2.4 Microbiota Volatility

Microbiota volatility is defined as the intra-individual change in microbiota composition over time and was calculated as described in Bastiaanssen et al. (T. F. S. Bastiaanssen et al., 2022). We fitted Bayesian robust linear models to volatility scores in early infancy (2-5 weeks) and from early to late infancy (5-52 weeks) (Figure 4.6). Volatility was lower in the SSC group in early infancy than in the CAU group (β = -0.31, 95% HDI [-0.62;0], $P(\beta < 0) = 0.95$) rejecting the null hypothesis that SSC has no influence on microbiota volatility. This effect remained unchanged after including breastfeeding in the regression model, suggesting that SSC affects microbiota volatility in early infancy, independent of its effect on breastfeeding. Note that in the PP analyses for early infancy, the effect size increased slightly, whereas the highest density interval widened with smaller sample size (β = -0.4, 95% HDI [-0.88;0.09], $P(\beta < 0) = 0.92$). While average volatility was also lower in the SSC group when looking at the distances between the 5 and 52 weeks samples, the effect did not pass the decision criterion (β = -0.16, 95% HDI [-0.47;0.15], $P(\beta < 0) = 0.80$) to reject the null hypothesis. Breastfeeding (β = -0.13, 95% HDI [-0.22;-0.03], $P(\beta < 0) = 0.99$) and gestational age (β = -0.25, 95% HDI [-0.46;-0.04], $P(\beta < 0)$ = 0.99) were negatively related with volatility (breastfeeding only for volatility between 5 and 52 weeks).

4.4.2.5 Microbiota Age

Microbiota age was estimated as described in Subramanian et al. by training a Random Forest model using samples from two other Dutch longitudinal studies (BIBO, BINGO) in the age range of 7-561 days (Figure 4.7A) (Subramanian et al., 2014). The Random Forest model performance was comparable to that of Subramanian et al. (Subramanian et al., 2014) as 64% of variance in age could be explained by the microbiota. We evaluated model fit and significance by computing the correlation of predictions and actual ages using the SKIPPY samples (r = 0.862, $p < 0.001$). Besides illustrating the results of the differential abundance analysis, Figure 4.5 shows the 15 most important features of the RF model with non-zero abundance in the SKIPPY samples (denoted by R next to the taxon name in Figure 4.5). To predict microbiota age, *Faecalibacterium* was the most informative next to other genera that are known to either dominate early infancy due to breastfeeding (*Staphylococcus* and *Bifidobacterium*) or to only occur with the cessation of breastfeeding and introduction of solid food as dominance of *Bifidobacterium* disappears. Note that we have lower coverage of the ages around 1 year in BIBO and BINGO compared to the coverage of ages in early infancy (Figure 4.7A) resulting in higher uncertainty in the estimation of the microbiota age reference as compared to the time

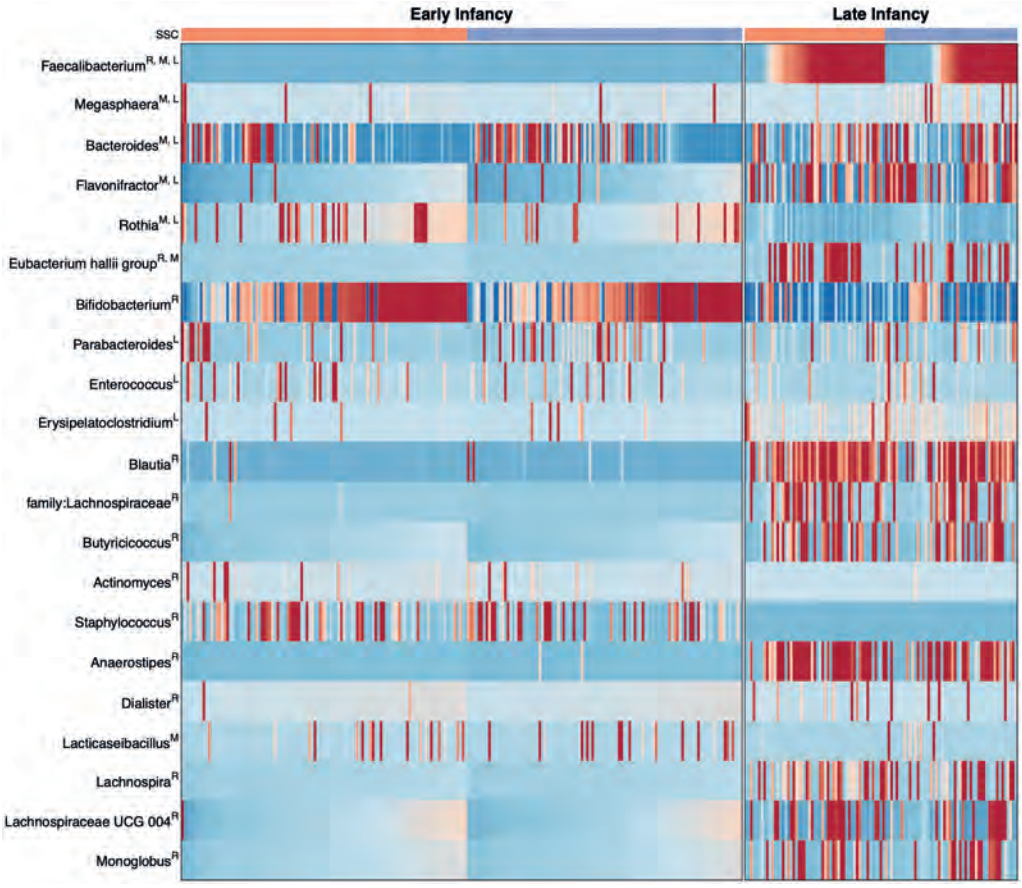

Figure 4.5: Heatmap of clr-transformed bacterial abundances that were either differential abundant between treatment (SSC) and control (CAU) or important for the microbiota age model. The relative abundance values have been scaled to zero mean and unit variance in order to highlight differences in the variation relative to the mean level within each taxonomic group. The color scale has been limited to the interval [-1, 1] (from dark blue to dark red). The letter behind each genus indicates whether it was important for the microbiota age model (R, see corresponding section) or identified by any of the differential abundance analysis methods (M = Maaslin2, L = LinDA).

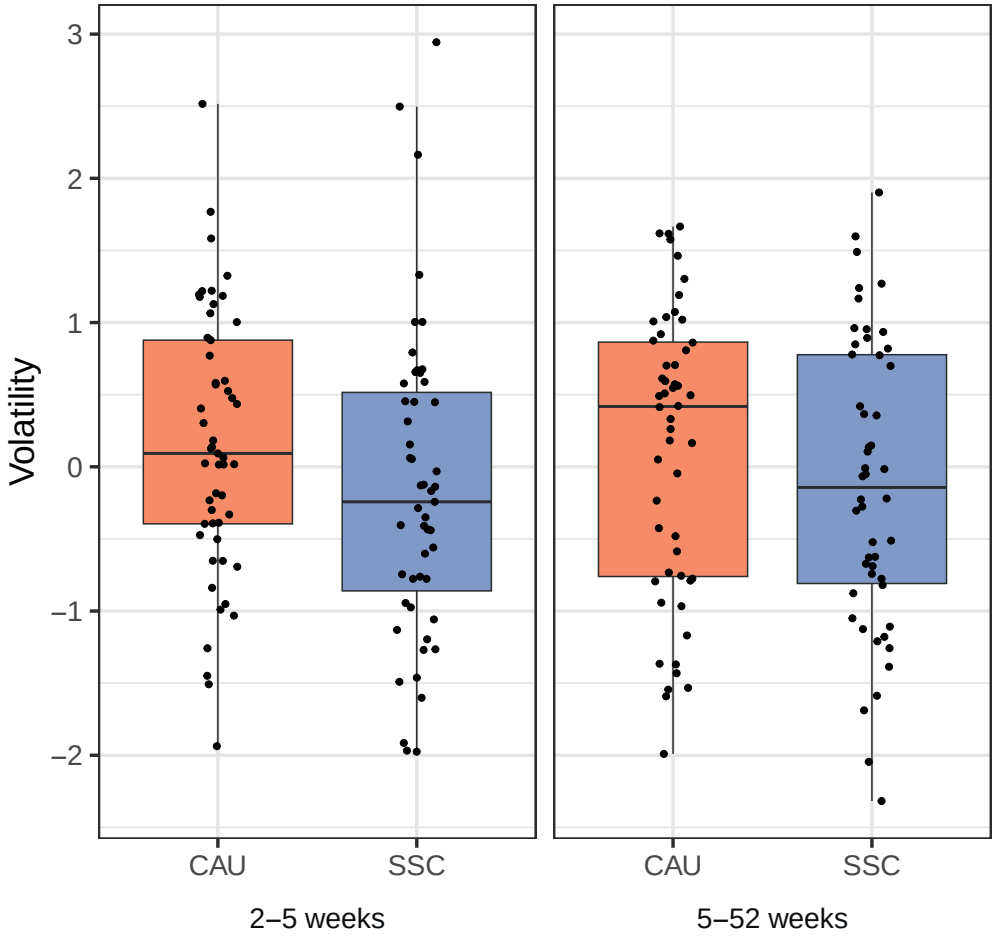

Figure 4.6: Volatility (intra-individual Aitchison Distance) between treatment (SSC) and control (CAU) group for the sample sequence from 2 to 5 weeks and 5 to 52 weeks.

period early in infancy. Nevertheless, we could compare the microbiota ages of the treatment and control groups in the SKIPPY cohort. Figure 4.7B depicts the microbiota age scores per time point between treatment groups in the SKIPPY samples. The samples obtained at one year show relatively low microbiota age, indicating that the SKIPPY cohort has lower microbiota ages than the BIBO and BINGO cohorts. The Bayesian robust regression models indicated that treatment was associated with lower microbiota age with an average decrease of 25.65 days (β = -25.65, 95% HDI [-50.37;-1.31], $P(\beta < 0) = 0.98$) at one year of age. After adding breastfeeding, the effect size shrank (β = -19.32, 95% HDI [-47.90;8.01], $P(\beta < 0) = 0.90$)) while breastfeeding was associated with lower microbiota age as expected (β = -7.27, 95% HDI [-13.94;-0.62], $P(\beta < 0) = 0.98$). These results indicate that SSC intervention influenced microbiota development with visible effects at 1 year of age. Breastfeeding duration may have partially mediated this effect. An exploratory dose-response analysis within only the treatment group indicated that there was no stronger effect on microbiota age when subjects provided more hours of SSC. Thus, infants in the treatment group were probably mostly above the minimal number of hours needed to produce the observed effect on microbiota age (supplementary Figure 4.14).

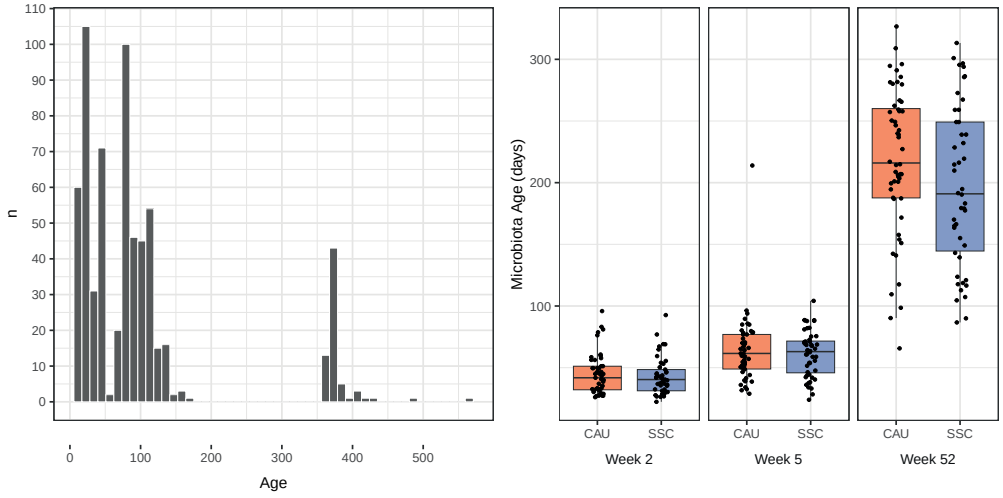

Figure 4.7: A. Age of sample collection for the samples used to train the Random Forest model. B. Predicted microbiota age for the samples analyzed in this study between treatment (SSC) and control (CAU) per time point.

4.4.2.6 Gut Brain Module Abundances

We estimated 44 metabolic pathways that are related to gut-brain communication (gut brain modules) (Valles-Colomer et al., 2019). These pathways include, for example, serotonin degradation or histamine production. Using MaAsLin2 ($FDR \leq 0.2$), we found that nitric oxide

degradation was lower (FDR = 0.166) while butyrate synthesis I (FDR = 0.169) and acetate synthesis III (FDR = 0.148) were higher in the SSC group compared to the CAU group (supplementary Figure 4.15).

4.5 Discussion

We hypothesized that a skin-to-skin intervention (SSC) applied in the first weeks of life in full-term infants would influence features of the gut microbiota via several potential mechanisms. These include physical transmission (Ferretti et al., 2018), prolonged breastfeeding (Cooijmans, Beijers, Brett, & Weerth, 2022) and by having a stress-reducing, stabilizing effect on the developing infant (Beijers et al., 2016). The microbiota features that were investigated included alpha-diversity, beta-diversity, genus level abundances, volatility, microbiota age and abundances of selected predicted functional pathways (gut brain modules; GBMs). The results provide evidence that a postpartum skin-to-skin intervention may influence gut microbiota development.

4.5.0.1 Lower Microbiota Volatility in the SSC group

Infants that received the SSC intervention significantly differed in their overal microbiota composition and had lower microbiota volatility in early infancy. Volatility has been found to be higher in stressed mice, in humans reporting higher experienced stress during exam periods, and in patients with inflammtory bowel disease (T. Bastiaanssen et al., 2021; Clooney et al., 2021). In addition, a related metric indicated higher volatility (lower stability) in infants with colic (de Weerth et al., 2013). We furthermore observed that longer gestational age was associated with lower volatility in both time windows, possibly indicating that infants who are further in their physical development for their chronological age have more stable gut microbiota.

4.5.0.2 Lower Microbiota Age in the SSC group

Infants in the SSC group had a lower microbiota age at one year of age compared with the control group. Our results further indicated that breastfeeding may have partially mediated this effect of SSC on microbiota age, which is in line with previous research showing that breastfeeding duration is negatively associated with microbiota maturation (Bäckhed et al., 2015; Depner et al., 2020). However, after controlling for months of exclusive breastfeeding or alternatively, age of weaning, SSC infants still had on average 19 or 15 days, respectively, lower microbiota age. In addition to breastfeeding, lower microbiota age has been associated with exposure to antibiotics, delivery via cesarean section (Bokulich et al., 2016), and asthma (Depner et al., 2020). We found that siblings and educational level were positively associated with microbiota age at one year. The former is in line with previous research showing that infants with older siblings show faster maturation of the microbiota (Baniel et al., 2022) and specifically earlier colonization with *Faecalibacterium prausnitzii* (Laursen et al., 2017). Colonization with *Faecalibacterium prausnitzii* is a characteristic of microbiota maturation (Roswall et al., 2021) and this taxon was the most important feature in our microbiota age model.

It is important to note that environmental factors appear to alter the dynamics of microbiota maturation rather than the speed of maturation. For example, formula-fed infants or infants born via cesarean section showed initially higher microbiota ages (in the first 6-12 months) before they started to show lower or similar microbiota ages compared to breastfed or vaginally born infants at later developmental stages (Bokulich et al., 2016). A reverse dynamic was observed for antibiotic exposure with initially lower and later temporarily higher microbiota age compared to inviduals not exposed to antibiotics (Bokulich et al., 2016). The diverse associations with microbiota age raise the question of which specific aspects of microbiota maturation at which specific ages may be beneficial or possibly unfavorable for a healthy development.

To investigate this further, it would be desirable for future research to utilize a common microbiota age model trained on large samples for the respective geographic region. Combined with sampling for a longer time span (e.g. the first 3 years), it may be possible to describe different trajectories of microbiota maturation that may be related to environmental variables, and in longitudinal follow-ups, to health outcomes in later life. Finally, while increasing alpha diversity is a characteristic of microbiota maturation, we did not observe differences in alpha diversity between the groups. Our observation that siblings were associated with lower alpha diversity and with higher microbiota age might seem contrary, but note that having one or more siblings was only negatively associated with alpha diversity in early infancy and not at 1 year of age in line with findings in another Dutch cohort (Hermes et al., 2020) as well as with those of other studies (Adlerberth et al., 2007; Azad et al., 2013; Christensen et al., 2022; Laursen et al., 2015).

4.5.0.3 Differential Abundance of Individual Genera between groups

Differential abundance analysis highlighted several genera (*Faecalibacterium*, *Megasphaera*, *Bacteroides*, *Flavonifractor*, *Rothia* and *Eubacterium hallii*) as well as predicted functional pathways (nitric oxide degradation, butyrate synthesis I and acetate synthesis III) as differentially abundant between the groups. *Faecalibacterium prausnitzii* is well known as a butyrate producer and because of that, and its negative relationship with inflammatory bowel diseases, it is generally considered a health promoting species (Laursen et al., 2017; Miquel et al., 2013). Laursen et al.(Laursen et al., 2017) found that the prevalence was between around 55% and 80% at 9-10 months of age and increased to almost 100% at 16 months of age in three separate infant populations. The abundance of *Faecalibacterium* has been found to further increase substantially after 1 year of age but not anymore from 3 to 5 years of age (Laursen et al., 2017; Roswall et al., 2021). In our study sample, the prevalence of *Faecalibacterium* at 12 months of age was 86.8% for the CAU and 68.0% for the SSC group. This finding, combined with the previously discussed findings of the microbiota age model, indicate a slower speed of microbiota maturation in the SSC group along a normal trajectory of microbiota development up until the first year of life. This is supported by the fact that while the microbiota of infants typically matures along similar trajectories, differences in speed between individuals have been documented (Roswall et al., 2021).

In contrast to *Faecalibacterium*, the abundance of *Bacteroides* was higher in the SSC group. Increased abundances at 1 year of age have previously been associated with enhanced neurodevelopment (Tamana et al., 2021). *Megasphaera* was more abundant in the SSC group. This

genus includes butyrate- and proprionate-producing strains (Duncan et al., 2007) and has been negatively associated with diarrheal cryptosporidiosis (Carey et al., 2021) and maternal stress (Dutton et al., 2023). However, the relationship with maternal stress was only present at 6 weeks and 3 months of age and reversed at 6 months of age. *Flavonifractor*, also higher in the SSC group, has been negatively associated with the development of asthma later in life (Stokholm et al., 2018b) and was able to strongly suppress Th2 immune responses in mice (Ogita et al., 2020). Furthermore, *Flavonifractor plautii* was negatively associated with maternal stress in samples obtained at 6 weeks, 3 months and 6 months (Dutton et al., 2023). *Rothia*, with a slightly lower relative abundance in the SSC group, has been reported to be increased in formula fed infants by two studies (one study found the opposite) (Z. Wang, 2019) and to be negatively associated with childhood asthma (Arrieta et al., 2015). Lastly, *Eubacterium hallii* (reclassified as *Anaerobutyricum soehngenii* (Shetty et al., 2018)) was lower in the SSC group. *Anaerobutyricum soehngenii* can produce butyrate by utilizing the byproducts of other early colonizers that metabolize human milk oligosaccharides (Chia, 2018; Dedon et al., 2023). It has been positively associated with infant colic (Pham et al., 2017) and is under investigation for its benefits related to glucose metabolism (Gilijamse et al., 2020).

4.5.0.4 Summary and Conclusion

In summary, we found evidence that SSC affected microbiota composition in early infancy (2 and 5 weeks) and development in early and late infancy as measured by volatility and microbiota age. Differential abundance and microbiota age analyses highlighted individual genera that differed between groups. We proposed three mechanisms of action through which SSC may affect the microbiota. First, the transmission route appeared least relevant for the observed effects. Skin bacteria, such as *Staphylococcus*, were not differentially abundant between groups. The groups did not yet differ in the number of exclusively breastfed infants at 5 weeks (80.2% vs 78.4%). Differences in breastfeeding were observed only at later ages. Thus, we can assume that physical contact, as occurring naturally through maternal caregiving and breastfeeding, was sufficient to transmit bacteria that were also transmitted through increased direct physical contact by the SSC intervention. Furthermore, previous research demonstrated that most skin bacteria obtained from the mother are mainly detectable shortly after delivery but not at later time points (Ferretti et al., 2018) as they do not colonize the gut. Second, we showed that breastfeeding is one of the mechanisms via which the SSC intervention altered the microbiota maturation long after the intervention at 1 year of age. Third, considering prior evidence (T. Bastiaanssen et al., 2021) and the lack of differences in breastfeeding in early infancy, we hypothesize that the effect of SSC on volatility in early infancy may be due to its de-stressing effect. Future longitudinal studies would need to measure stress in addition to the measures we collected in order to confirm the proposed mechanism of SSC influencing microbiota volatility by acting as a stress buffer.

This preregistered study is the first RCT assessing effects of SSC on the developing gut microbiota. Strengths include the randomized controlled design with blind recruitment and a low drop-out rate throughout the intervention phase. Also, the microbiota and relevant covariates were sampled at several time points and included a follow-up microbiota sample long after the intervention had finalized. This allowed us to estimate the effect of SSC on the microbiota

development and to test one possible mechanism. Furthermore, we investigated a broad set of features of the microbiota and took measures to ensure robustness of our results. Our analyses revealed that it is important to look beyond alpha diversity, beta diversity and differential abundance analysis, and include features that reflect microbiota development (volatility and microbiota age). Our study also has limitations. Since we did not measure all (time-varying) variables that influence protocol adherence, we may not exclude potential confounding bias in the per-protocol analysis estimates (Hernán & Hernández-Díaz, 2012; Hernán et al., 2013). Note however, this limitation is not relevant for our intention-to-treat analysis, which is regarded as the preferred analytic approach for RCTs. Finally, the generalizability of the study is limited given the relatively homogeneous sample with mainly families of highly educated mothers.

In conclusion, we provide evidence that a postpartum skin-to-skin intervention in full-term infants may influence gut microbiota composition and volatility in early infancy as well as microbiota age (for chronological age) in late infancy. The effects on microbiota age may have been partially mediated by SSC prolonging breastfeeding duration. It is highly desirable to replicate these findings to validate their robustness and establish their generalizability. Future studies would benefit by extending microbiota sampling beyond the first year of life in order to investigate the effect of SSC on microbiota maturation at later time points. Including variables that measure stress would allow for the investigation of whether the intervention has an effect on microbiota volatility by reducing stress.

4.6 Supplementary Figures

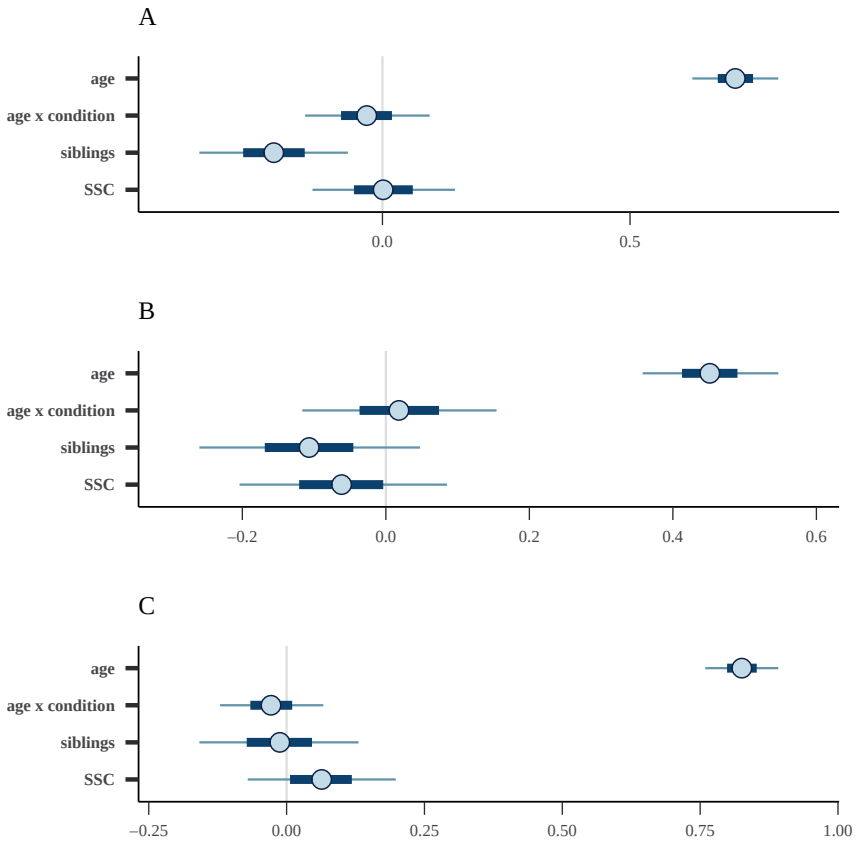

Figure 4.8: Posterior distributions (mean, 50% and 95% of the probability mass) of beta coefficients for the alpha diversity models (intention-to-treat analysis) using Shannon (A), Chao1 (B) or Faith (C) index.

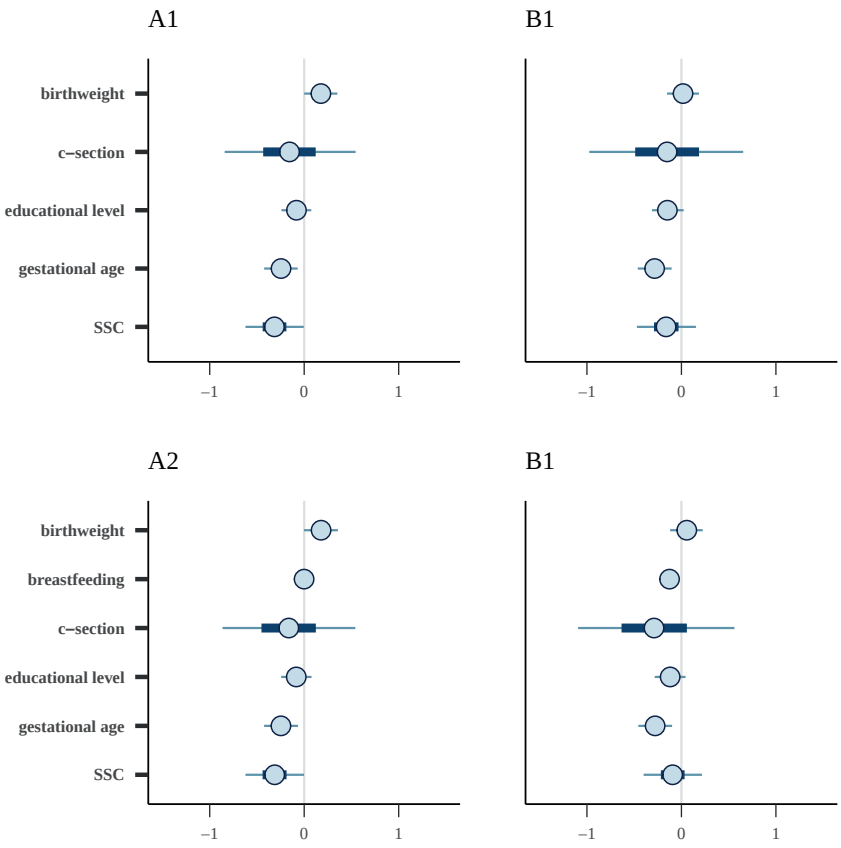

Figure 4.9: Posterior distributions (mean, 50% and 95% of the probability mass) of beta coefficients for the volatility models (intention-to-treat analysis) using volatility between 2 and 5 weeks (A) and 5 and 52 weeks (B) without (1) and with (2) breastfeeding.

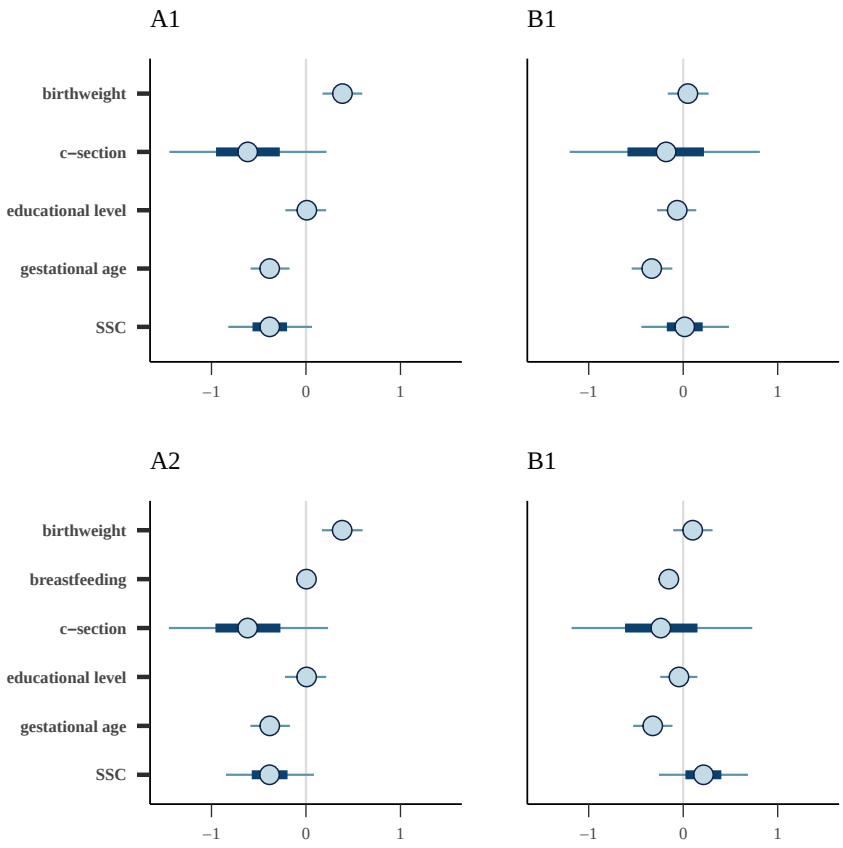

Figure 4.10: Posterior distributions (mean, 50% and 95% of the probability mass) of beta coefficients for the volatility models (per-protocol analysis) using volatility between 2 and 5 weeks (A) and 5 and 52 weeks (B) without (1) and with (2) breastfeeding.

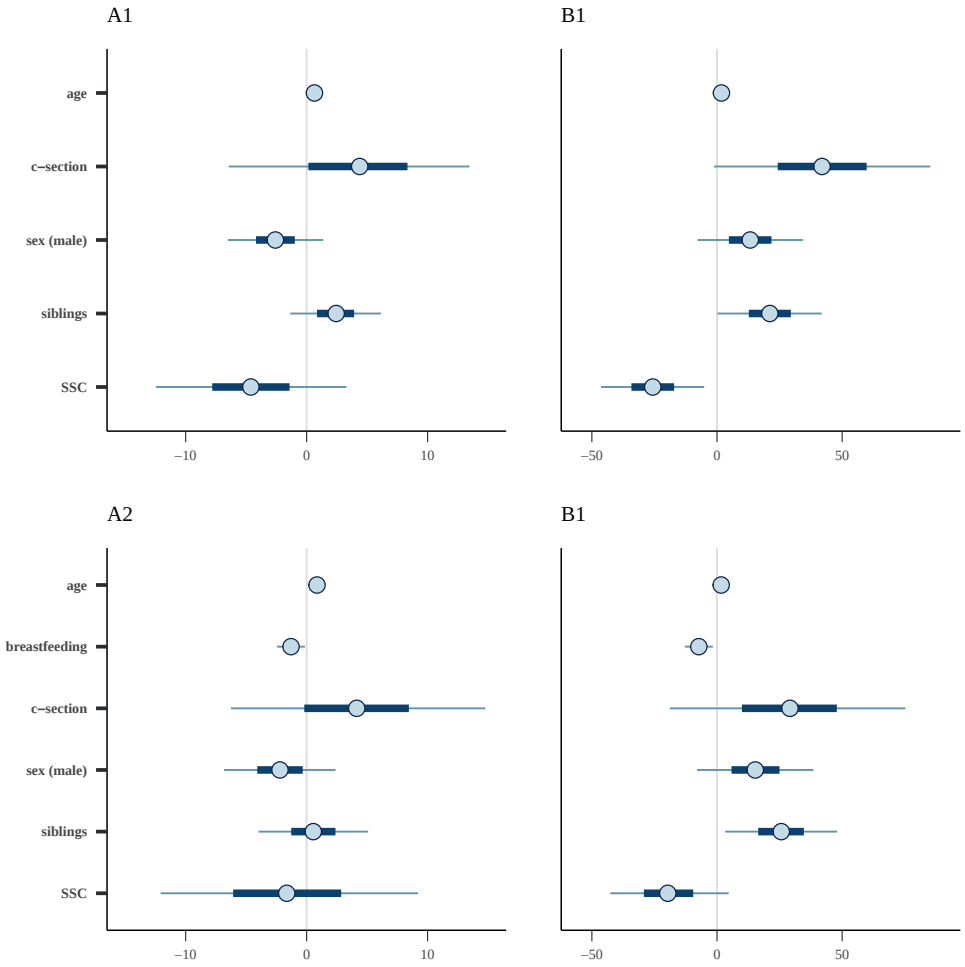

Figure 4.11: Posterior distributions (mean, 50% and 95% of the probability mass) of beta coefficients for the microbiota age models (intention-to-treat analysis) using microbiota age scores in early (A) and late infancy (B) without (1) and with (2) breastfeeding.

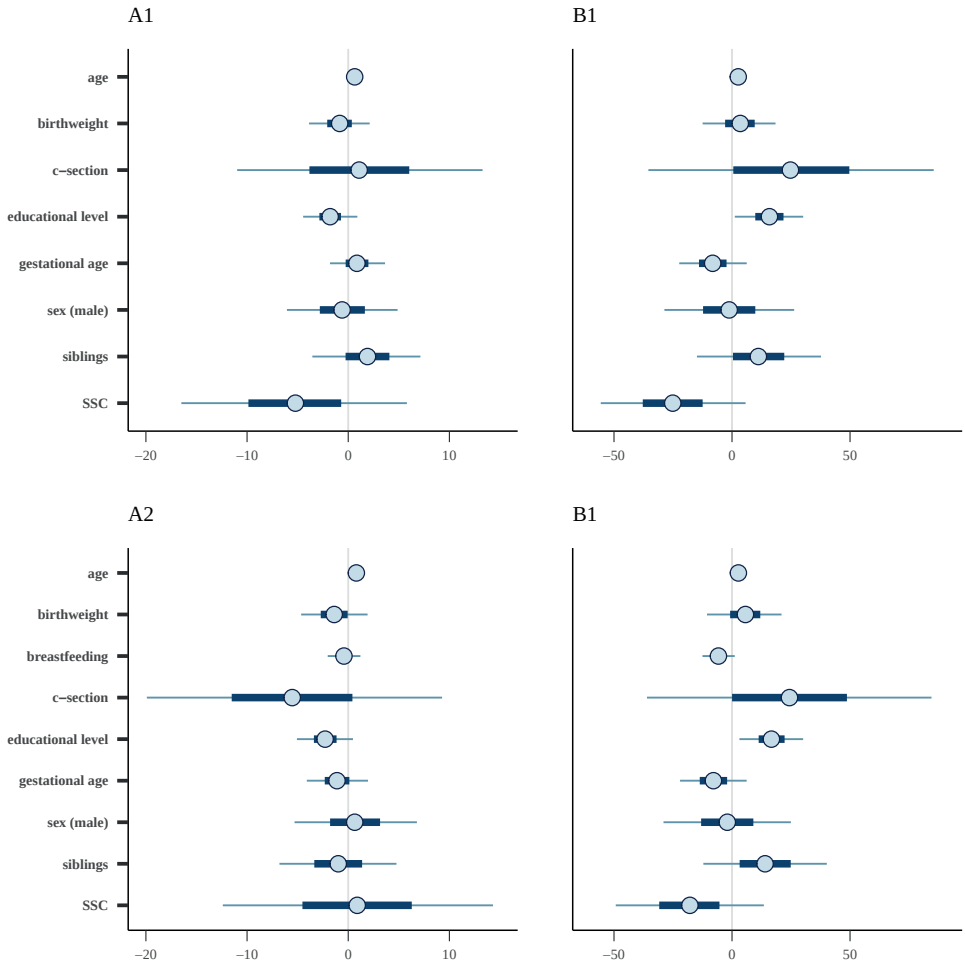

Figure 4.12: Posterior distributions (mean, 50% and 95% of the probability mass) of beta coefficients for the microbiota age models (per-protocol analysis) using microbiota age scores in early (A) and late infancy (B) without (1) and with (2) breastfeeding.

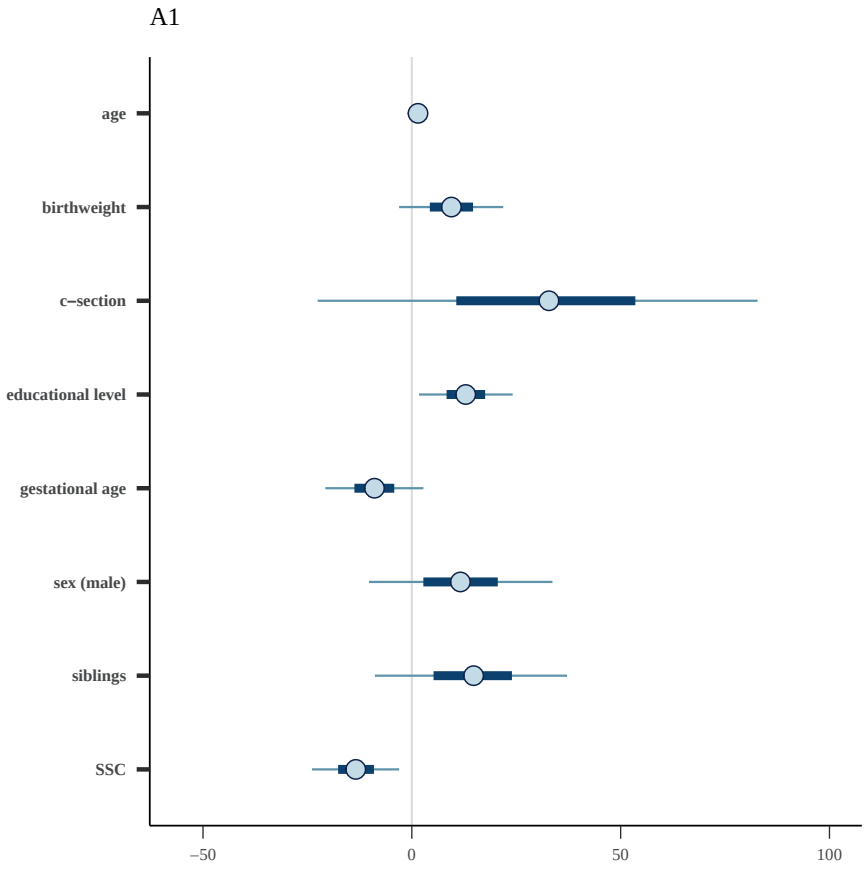

Figure 4.13: Posterior distributions (mean, 50% and 95% of the probability mass) of beta coefficients for the microbiota age models (dose-response analysis) using microbiota age scores in late infancy without (A1) and with (A2) breastfeeding.

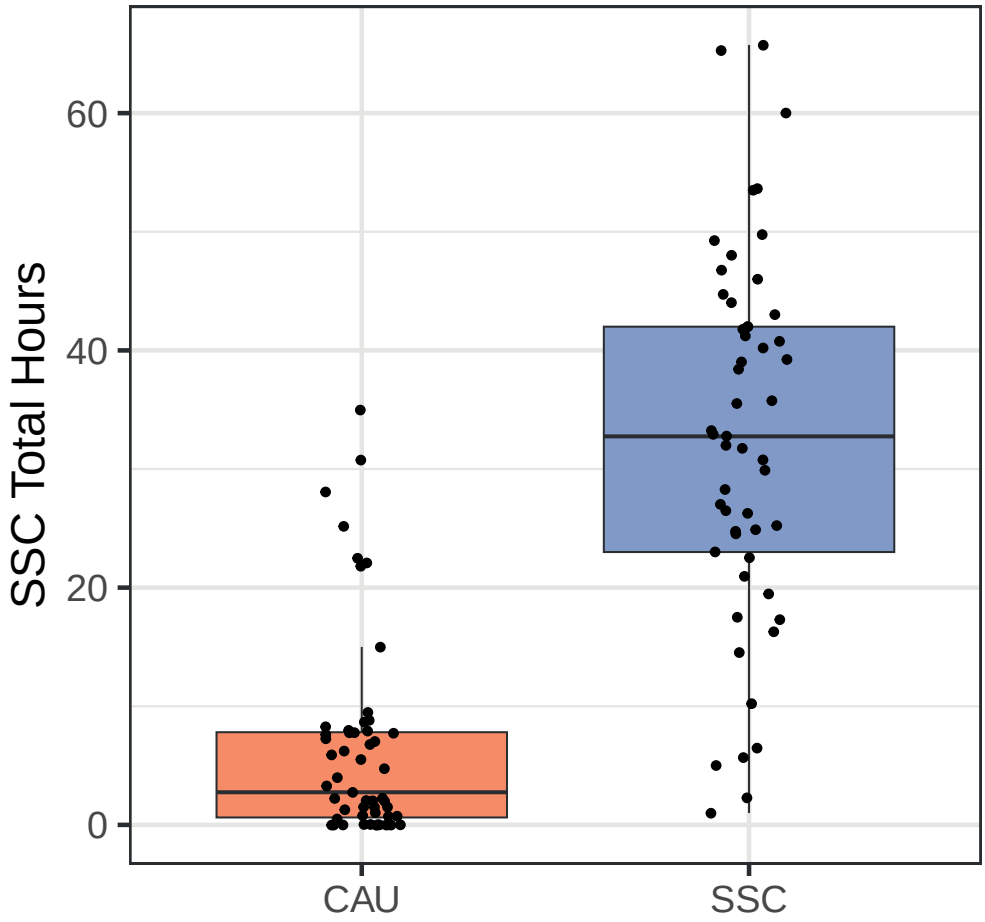

Figure 4.14: Boxplots of the number of provided skin-to-skin hours between groups.

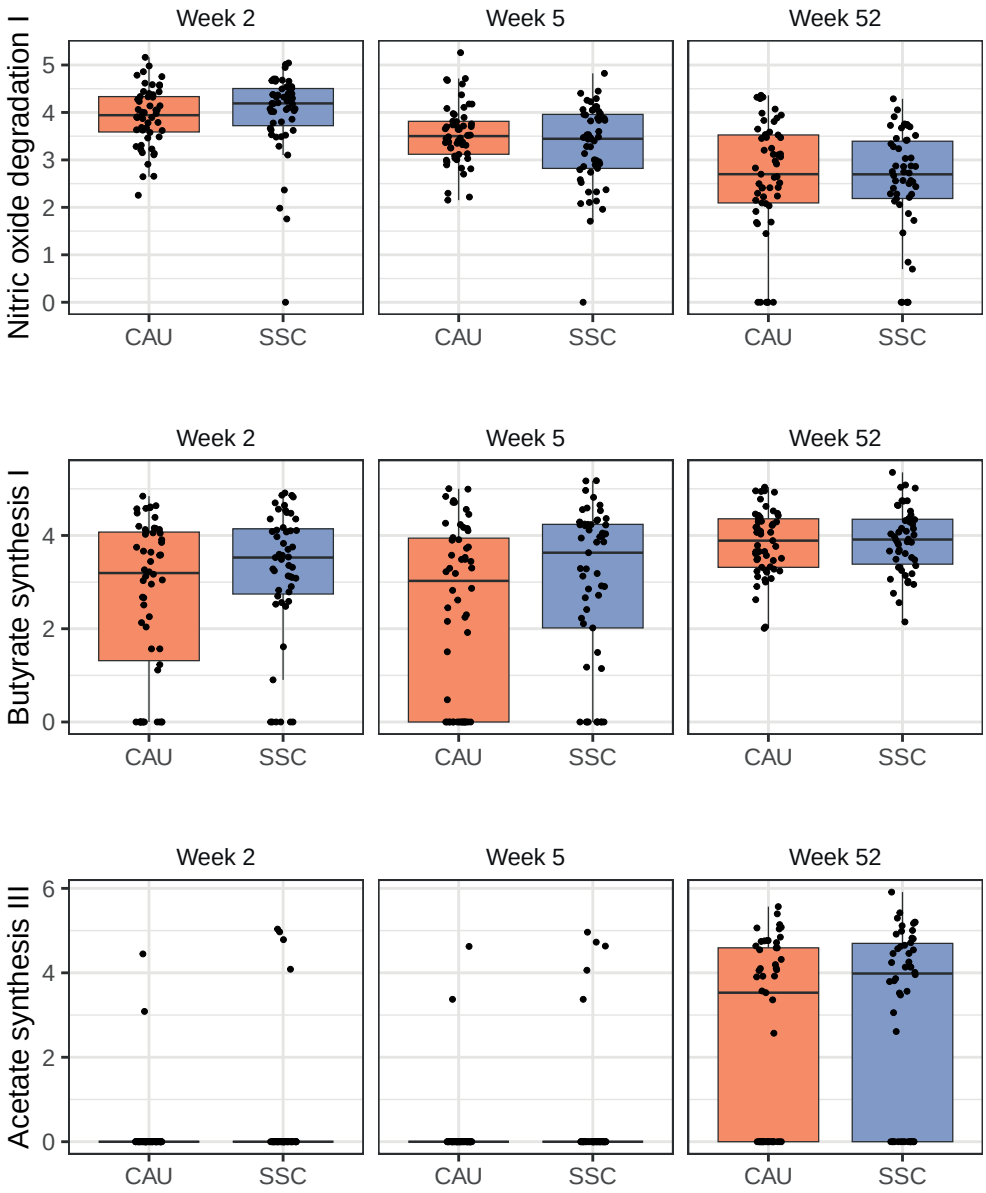

Figure 4.15: Boxplots of gut-brain-modules that were differentially abundant between SSC (blue) and CAU (red).

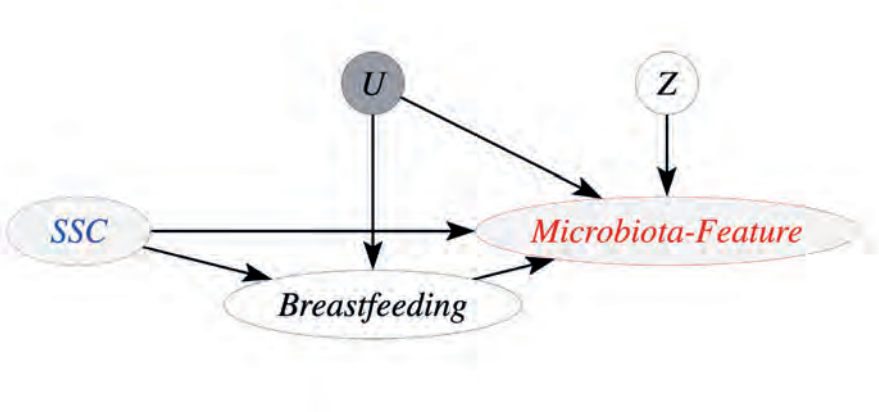

Figure 4.16: Directed acyclic graph depicting assumptions for the statistical analyses. U represents unmeasured variables. Z reflects all the measured variables that are known to influence the gut microbiota that are added in a data-driven approach in some of our models as they may improve precision of our estimate of interest (see covariate section in the methods and Cinelli et al. (2020). Note that we performed sensitivity analysis by leaving out any variables Z. Given the randomization of participants into skin-to-skin (SSC) or care-as-usual, there are no arrows pointing towards SSC and the total effect estimate of SSC on the microbiota is assumed to be unbiased in contrast to any other potential effects that may be confounded by U.

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Chapter 5

Can Gut Microbiota Throughout the First 10 Years of Life Predict Executive Functioning in Childhood?

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5.1 Abstract

Animal models suggest that the gut microbiota can influence cognitive development and functioning via various pathways. In line with that, a first human study found associations between infant fecal microbiota composition and cognition at two years of age. This longitudinal study investigated whether fecal microbiota composition in infancy and childhood is associated with executive functioning in childhood. We followed healthy individuals from birth to their 10th year of life. Executive functioning was assessed using the Digit Span working memory test at 10 years of age and the ecologically valid Behavior Rating Inventory for executive functioning at 8 and 10 years. Stool samples were collected at month 1, 3 and 4 as well as at 6 and 10 years. The V4 region of the 16S ribosomal RNA was analyzed to determine microbial composition at the genus level. Using established statistical techniques for microbiota analysis we did not find associations between fecal microbiota composition and executive functioning after accounting for breastfeeding, maternal education, child sex and age. Our study results are most compatible with the absence or only a weak relationship between infant and childhood fecal microbiota composition and executive functioning in childhood, in healthy community children.

5.2 Introduction

The gut-brain axis is a complex bidirectional network where the gut and the brain are connected via various pathways (Mayer et al., 2014). The gut microbiota, the ecosystem of microorganisms in the intestinal lumen, is a critical part of this network. It can generate endocrine-, neurocrine- and immune-related signals that can shape the development and functioning of the central nervous system (de Weerth, 2017; Mayer, 2011). In the present study we investigated whether infant and childhood fecal microbiota (FM) composition as a measure of the distal gut microbiota, can predict individual variation in executive functioning (EF) in childhood. Identifying and describing possible relationships between the (early) FM and EF is a necessary first step towards developing prevention, diagnostic, and intervention strategies targeting the microbial ecosystem in the gut. The following paragraphs will elucidate the importance of EF and why the FM is a relevant study variable for psychologists researching child cognitive development (Sarkar et al., 2018).

EF comprises cognitive functions central to goal-directed, efficient and adaptive behavior (Huizinga & Smidts, 2010). These include inhibition, shifting, self-monitoring, planning, attention, and working memory. Proper EF is crucial for every-day-life and academic achievement (Huizinga & Smidts, 2010; Huizinga et al., 2018). Disruption of EF has negative effects on health outcomes and is itself part of several psychiatric disorders, such as attention deficit hyperactivity disorder, bipolar disorder and schizophrenia (Testa & Pantelis, 2009). Thus, proper development of EF is crucial for the quality of life of the individual. EF emerges as the output of various neural networks during the first years of life (Goldstein & Naglieri, 2014) and continues to develop during childhood, adolescence and even adulthood (Best & Miller, 2010). These neural networks rely on the development of frontal and posterior cerebral cortex and subcortical regions (Goldstein & Naglieri, 2014). The plasticity of these networks is maximal early in life (Diamond, 2013). As extensively reviewed (Borre, O'Keeffe, et al., 2014; Varier

et al., 2020), the colonization of the intestines by microbes occurs simultaneously with and influences this pivotal period of brain development. E.g., experimental rodent studies revealed that the gut microbiota affects social behavior, cognitive performance and neurobiology in brain regions related to learning and memory (Ohland et al., 2013; Savignac et al., 2015; Vázquez et al., 2015; T. Wang et al., 2015) and that there exists a time window in early life for such effects to take place (Buffington et al., 2016; Sudo et al., 2004). Therefore, it is necessary to study the gut microbiota in infancy in relation to EF later in life to disentangle such early programming effects (Borre, O'Keeffe, et al., 2014). In sum, EF is important for the quality of life of the individual. It develops early in life and continues to develop throughout adulthood. The gut microbiota may influence both the early development and current functioning of the brain.

At present, there is a lack of human developmental studies that focus on the relationship between the FM and cognitive functioning. Studies that found associations focused on temperament (Aatsinki et al., 2019), attention to emotional faces (Aatsinki et al., 2020) or social behaviors related to autism (Laue et al., 2020). One study provided indirect support for a potential relationship between the early FM and later cognitive functioning as antibiotic treatment in the first 2 years of life was related to worse cognitive functioning at 11 years (Slykerman et al., 2019). Earlier, the first human developmental study (Carlson et al., 2018) examined whether FM around 1 year of age is related with cognitive functioning and global and regional brain volumes at 1 and 2 years of age in 89 typically developing infants. Cluster analysis of bacterial abundances identified 3 groups that significantly predicted cognition at 2 but not 1 year of age. Lower alpha diversity was related to higher cognitive functioning at 2 years of age. Furthermore, FM was weakly related to regional brain volumes at 1 (N = 46) and 2 (N = 27) years of age, as well as brain functional connectivity (N = 39) (Carlson et al., 2018; W. Gao et al., 2019). A limitation of this study was that there was only one measurement of the FM, while infants show rapid shifts in microbial composition and diversity. Hence, multiple sample time points are necessary to distinguish temporary shifts in composition from more stable characteristics ((Bäckhed et al., 2015; de Meij et al., 2016; de Muinck & Trosvik, 2018). The finding that higher alpha diversity was associated with lower cognitive scores (Carlson et al., 2018) might indicate that low alpha diversity at around 1 year of age is beneficial for infant cognitive development or that there are underlying environmental factors that cause lower alpha diversity and have a positive influence on cognitive development. For instance, previous research found that breastfed infants have a more stable FM composition that is dominated by the genus Bifidobacterium and is therefore less diverse (Stewart et al., 2018). Breastfeeding also has been positively associated with cognitive functioning in previous studies (Kim & Choi, 2020).

Based on the outlined preclinical studies and the first human study (Carlson et al., 2018; W. Gao et al., 2019), we hypothesized (1) that FM composition in infancy and in childhood is associated with childhood EF, (2) that there is a negative association between alpha diversity in infancy and childhood EF, and (Kruschke, 2013) that (infant) FM samples can be grouped into clusters of community similarity that are differentially associated with EF. In the context of hypothesis 2, we also investigated whether alpha diversity in childhood was related to childhood EF. Finally, we explored whether microbiota compositional change over time (volatility) is associated with EF in childhood. To investigate our hypotheses, we took the dynamic nature of the FM into account by analyzing stool samples obtained at 5 different time points, from here on referred to

as T1-T5. Three stool samples per child were collected in infancy at months 1, 3 and 4 (T1-T3). Two more samples were collected at 6 and 10 years of age (T4-T5). We furthermore collected questionnaire and test measurements of EF at 8 and 10 years of age and tested our hypotheses using diverse complementary statistical and machine learning approaches as described in our preregistration (https://aspredicted.org/uc98s.pdf). This study represents a further important step in translating findings from animal studies into human research. It is the first study that examined the relationship between FM and EF beyond the age of toddlerhood and spanning a period of 10 years.

5.3 Methods and Materials

5.3.1 Participants

Participants are children from the 193 healthy mother-infant dyads from the ongoing longitudinal BIBO study, that started in the third trimester of pregnancy and is ongoing (Beijers et al., 2011b). Mothers were recruited on a voluntary basis during late pregnancy as they responded to flyers that were spread among midwife practices in the cities of Nijmegen, Arnhem, and surrounding areas. Inclusion criteria were an uncomplicated singleton pregnancy, no drug use and no current physical or mental health problems. Furthermore, all infants were healthy, born at full term ($\geq$ 37 weeks), and with a 5-min APGAR score $\geq$ 7. Out of 220 women, 8 were excluded due to medical reasons such as preterm birth. Another 19 women discontinued the study within the first 3 postpartum months due to personal circumstances. When the children were 10 years old, 177 mother-child dyads were still participating. Table 5.1 shows demographic variables for the 156 study participants that had complete data for this study. Table 5.2 shows participant numbers at the different time points. All mothers gave written informed consent and the ethical Committee of the Faculty of Social Sciences, Radboud University Nijmegen approved the study (ECG/ AvdK/07.563, ECG300107, ECG13012012, SW2017-1303-497, SW2017-1303-498).

5.3.2 Procedure

Mothers completed demographic questionnaires in the third trimester of pregnancy (M = 37.4, SD = 1.4 weeks) as well as questionnaires about the delivery and the infant immediately after birth. Information about breastfeeding was obtained weekly through diaries (0-6 months) and through monthly health interviews (0-12 months). When the children were 8 and 10 years old, mothers filled in the BRIEF questionnaire. The Digit Span test scores were obtained during a home visit at 10 years.

Table 5.1: **Demographic characteristics of subjects**

Characteristic	N = 156
Antibiotics	
Birth - T1	1 (1.0%)
Birth - T2	2 (1.8%)
Birth - T3	2 (2.3%)
5 years - T4	22 (17%)
9 years - T5	6 (4.5%)
Child age at EF collection	
8 years	8.04 (8.02, 8.11)
10 years	10.15 (10.03, 10.23)
Child age at FM collection	
T1	28.00 (27.00, 28.00)
T2	82 (75, 89)
T3	112 (107, 119)
T4	6.06 (6.01, 6.15)
T5	10.11 (9.96, 10.21)
Other	
Alcohol during pregnancy	21 (15%)
Birthweight	3,618 (3,233, 3,931)
Child sex	
female	71 (46%)
male	85 (54%)
Delivery mode	
assisted vaginal	15 (9.9%)
cesarean section	8 (5.3%)
vaginal	128 (85%)
Firstborn	69 (44%)
Gestational length (days)	282 (275, 287)
Maternal education	
Secondary education	30 (19%)
College or University	126 (81%)
Smoking during pregnancy	2 (1.5%)

Note. Categorical variables show number of participants per category (%). Continuous variables show median and interquertile range. Missing values for any of the listed variables were omitted to calculate shown descriptive statistics. Abbreviations: EF, executive functioning; FM, fecal microbiota.

Table 5.2: Sample size per time point of stool sample collection and outcome variable.

Time	DS F	DS BW	DS LNS	BRIEF
T1	135	132	131	144
T2	125	123	122	131
T3	123	121	120	129
T4	139	137	136	144
T5	146	146	145	146

Note. DS F = Digit Span Forwards. DS BW = Digit Span Backwards. DS LNS = Digit Span Letter-number sequencing. BRIEF = Behavior Rating Inventory of Executive Functioning.

5.3.3 Measures

5.3.3.1 Fecal Samples

We instructed parents to collect fecal samples at 9 time points postpartum as well as one sample at 6 and 10 years of age. Due to financial constraints, we could use only 3 out of 9 samples in infancy. After collection and temporary storage at -20 ∘ C at home, samples were transported in coolers and later stored at -80 ∘ C. Next, they were processed at the Microbiology Laboratory at Wageningen University as described in a previously published protocol (Gu et al., 2018; Ramiro-Garcia et al., 2018) (supplement 1). 16S rRNA sequencing was carried out on a Illumina HiSeq2000 sequencing platform at Eurofins Genomics, Germany. We utilized the NG-Tax 2.0 pipeline (Poncheewin et al., 2020) to process to amplicon sequence variants. Only reads with matching barcodes were kept. Amplicon sequence variants were obtained by assigning reads to each sample based on distinguishable barcodes. The SILVA_132_SSU 16S rRNA gene reference database was used for taxonomic assignment (Quast et al., 2012).

5.3.3.2 Digit Span (Forwards, Backwards and Letter-number sequencing)

The Digit Span is part of the Wechsler Intelligence scale for children and measures working memory (Petrosko, 1975). For the Digit Span tests, a trained instructor reads out numbers and the child has to repeat these either in the given order (Digit Span forwards) or backwards (Digit Span backwards). The maximum score for each subtest is 14. For the Digit Span letter-number sequencing test, the instructor reads out a number of letters and numbers. The child has to memorize and give back the numbers in ascending order and then the letters in alphabetical order. The maximum score for Digit Span letter-number sequencing is 30 points. For all Digit Span tests each level of difficulty consists of 2 trials whereby each correct trial is worth 1 point. As soon as both trials are answered incorrectly the test is finished. Previous research suggests that the different Digit Span tests measure different aspects of working memory functioning (Gerton et al., 2004; Rosenthal et al., 2006; St Clair-Thompson & Allen, 2013). This was also reflected by low-moderate correlation strength among our Digit span sub-scores ($r < 0.41$). As

norms were only available for the total score at the time of our preregistration, we used the raw sub-scores and corrected for age and gender within the analysis.

5.3.3.3 Behavior Rating Inventory of Executive Function (BRIEF)

Designed to achieve high ecological validity (Gioia & Isquith, 2004), the BRIEF was frequently utilized in clinical practice and research settings to measure daily life EF in children (Huizinga & Smidts, 2010). The BRIEF consists of 75 items that measure 8 scales: Inhibition, Shift, Emotional Control, Initiate, Working Memory, Plan/Organize, Organization of Materials and Monitor. Based on these scales, the age and gender normed T-scores for the Metacognition- and Behavior Regulation Indices can be obtained. It is advised to use these sub-scales when the T-scores between them differ significantly (Huizinga & Smidts, 2010). That was not the case in our sample. Therefore, we calculated age- and gender normed T-scores for the total score of the BRIEF as a general indicator of EF. A higher BRIEF score indicates lower EF. Note that we used the mean of the 8 and 10 year scores for the Random Forest models. In the linear models that relate alpha diversity or volatility to EF, we applied a multilevel structure for the repeated measurement.

5.3.3.4 Confounding Variables

Figure 5.1 shows a directed acyclic graph (Williams et al., 2018) based on our literature review. Directed acyclic graphs graphically depict potentially confounding variables of the association between an exposure (here FM) and an outcome (EF). They furthermore provide a set of rules to identify variables that reduce or induce bias when being adjusted for in a statistical model (Cinelli et al., 2020). The rationale behind our assumed graph (Figure 5.1) is as follows: socio-economic status, age and sex can influence EF measurements (Cuevas et al., 2014; Grissom & Reyes, 2019) and FM (Bolnick et al., 2014; Bowyer et al., 2019; de Muinck & Trosvik, 2018). As we have no direct measurement of socio-economic status, maternal education serves as a proxy in our study and might itself also influence both FM (e.g. via diet) and EF of the child. Age of the child during the EF task was included as it is expected to increase the precision of the effect of interest (Cinelli et al., 2020). Age and child sex were left out for the models that included the BRIEF scores as these were normed by age and sex. Breastfeeding is a strong driver of the gut microbiota in infancy while there is contradictory research about a relationship with later EF (Belfort et al., 2016; Rochat et al., 2016). For the analyses that include the childhood FM, diet is a confounding factor that we cannot adjust for. We might partially mitigate a potential bias under the assumption that maternal education has an effect on the child's diet (Cinelli et al., 2020). However, confounding effects of diet in childhood cannot be entirely ruled out given our data. In infancy, variation in diet is reflected in the amount of received breastfeeding. We considered other variables not shown in the graph: Gestational age and birthweight are not expected to influence EF unless the infant was born preterm or has low birthweight (<2500g) (Houdt et al., 2019). To infer the total effect of the FM under the assumed directed acyclic graph model, all variables shown in the graph have to be included. However, several covariate structures were explored to also give room to other candidate directed acyclic graphs (e.g. a graph where breastfeeding is not related to EF). These did not lead to different conclusions.

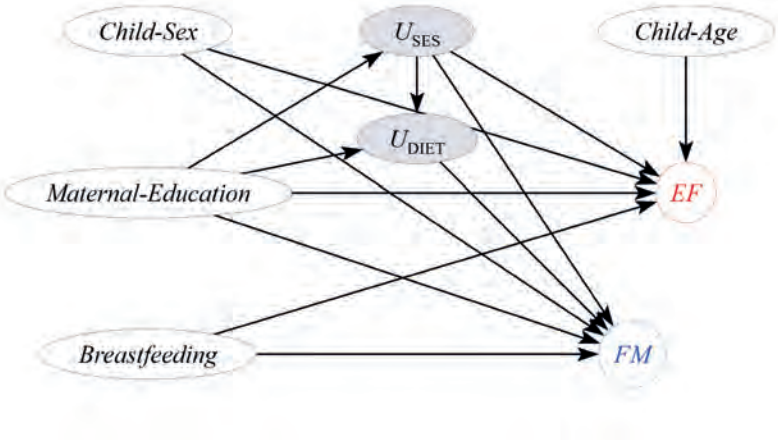

Figure 5.1: Directed acyclic graph based on literature review. Grey variables with a leading U reflect unmeasured variables, for example: SES, unobserved socioeconomic status; FM, fecal microbiota; EF, executive functioning. The graph applies to each time point of FM measurement and each EF measurement separately.

5.3.4 Statistical Analysis

We performed our analyses in R (R Core Team, 2020) version 4.0.2 and Stan (Carpenter et al., 2017) version 2.21.0. We used the packages microbiome (Shetty & Lahti, 2019) and phyloseq (McMurdie & Holmes, 2014) to process microbiome data in R. Per time point and outcome variable, data from all mother-infant dyads that provided fecal samples and the outcome variable were used. Among those subjects there was very little missingness in the covariates (education: 0.7% - 1.7%, age: 0.8% - 2.4%). We performed complete case analyses in these cases.

5.3.4.1 Code availability

The code corresponding to all statistical analyses is publicly available (doi: https://doi.org/10.5281/zenodo.5026029).

5.3.4.2 Random Forest Regression

The Random Forest algorithm (Breiman, 2001) is invariant to scaling of inputs, computationally efficient, appropriate for high dimensional data, able to predict non-linear relationships and thus well suited to analyze microbiome data (Belk et al., 2018; Louppe, 2014; Namkung, 2020). For each outcome and time point of microbiota determination, we fitted a Random Forest model

using the ranger package (Wright & Ziegler, 2017) with relative abundances. First, we tuned the hyperparameters mtry and sample.fraction using the package tuneRanger, which uses the mean squared error as out of bag error. Next, 10x 4-fold cross-validation was performed to estimate Pearson correlations of predicted values and leave-out values. To obtain a distribution of Pearson correlations under the null hypothesis, we performed the same procedure (incl. hyperparameter tuning) after permutation of the outcome variable (1000 permutations). We used the median Pearson correlation of the cross-validation procedure to obtain the p-value. Since we tested 20 Random Forest models for significance, we accounted for multiple testing using the Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995). We also explored the Random Forest algorithm for feature selection as described in Bommert et al. (2020). Briefly, Random Forest accuracy was evaluated by the set of top scoring taxa that were identified in a 4-fold cross-validation and then used to train a new model on the whole data set. This was performed for each outcome and time point separately.

5.3.4.3 Bayesian Linear Models

We fitted Bayesian robust Linear Models to regress EF on Shannon diversity and volatility, respectively. Shannon diversity is a commonly used measure of alpha diversity. Experiments with alternative alpha diversity indices (observed richness, Chao1, inverse Simpson) yielded similar results. For volatility, we calculated intra-subject Aitchison distance sequentially resulting in four volatility scores for each individual: T1-T2, T2-T3, T3-T4 and T4-T5. This allowed us to determine whether volatility in infancy, between infancy and childhood and in childhood is associated with EF. Covariates were included for both Shannon and volatility models as described. Maternal education was modeled using an ordinal regression approach as described by McElreath (2020) to respect the ordinal nature of this variable (Liddell & Kruschke, 2018). We used cmdstanr (Gabry et al., 202-) to fit the models. The cmdstanr package utilizes the probabilistic programming language Stan (Carpenter et al., 2017). Stan estimates parameters using the Hamilton Monte Carlo (HMC) method. All continuous predictors were standardized to ease interpretability and setting prior distributions for the parameters. Priors were set based on prior predictive simulations such that the parameter space was only mildly restricted and the same priors could be used across all models. A Gaussian prior with a mean of 0 and a standard deviation of 0.5 was used for all β coefficients across all models. Assigning a prior probability to the effect centered at 0 and constraining the model to not consider highly unrealistic slope sizes results in a more conservative model compared to the classical approach (Gelman & Tuerlinckx, 2000; Gelman et al., 2012). Note that changing prior distributions to more or less constrictive priors did not influence the results. Posterior predictive checks and residual plots were used to evaluate appropriateness of the model. Finally, we evaluated correct functioning of the HMC method by screening chain plots and diagnostic parameters such as divergent transitions and rhat4 values (Gabry et al., 2019).

5.3.4.4 Partitioning Around Medoids

We applied the Partitioning around Medoids clustering algorithm to the centered-log-ratio transformed genus level abundances (Aitchison distance) (Gloor et al., 2017) using the R package

cluster. For each time point we determined whether clustering is present based on Prediction Strength (Tibshirani & Walther, 2005) and the Silhouette Index utilizing functions from the packages cluster and fpc. Both measures are absolute measures of cluster strength that indicate support for clustering when they fall above a predefined threshold. The Calinski-Harabasz index is a relative measure of cluster strength that reflects which number of clusters is most likely (assuming that clusters are present). We considered clusters to be valid if either Prediction Strength is ≥ 0.9 or Silhoutte Index is ≥ 0.5 as recommended by Koren et al. (2013).

5.4 Results

Figure 5.2 shows the distributions for all outcome variables including their means, ranges, medians and interquartile ranges. In the following sections, we describe the results per analysis method.

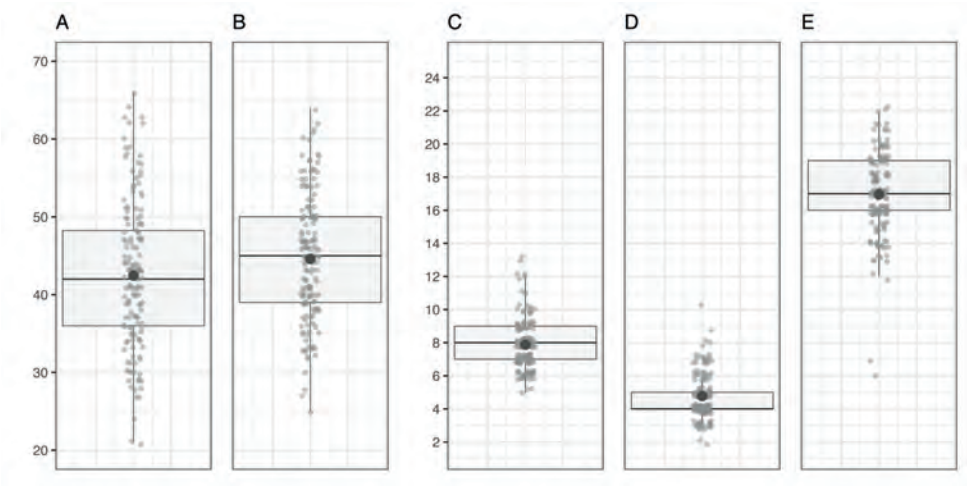

Figure 5.2: For each outcome, a boxplot is shown on the first layer. On a second layer, the corresponding single data points are shown in light grey as well as a larger black point depicting the mean. Small random noise is added to the single data points to avoid overplotting. (a) BRIEF (8 years), (b) BRIEF (10 years), (c) Digit Span forwards, (d) Digit Span backwards and (e) Digit Span letter-number sequencing.

5.4.1 Predicting EF from Genus Level Abundances - Random Forest Regression

Table 5.3 shows the median correlation between the Random Forest predictions and the leave-out values with the corresponding p-values and q-values for each model. If relative abundances

Table 5.3: Correlation between Random Forest prediction and real data.

Outcome	Median	Mean	SD	p	q
T1					
DS Forwards	-0.14	-0.14	0.14	0.94	0.85
DS Backwards	-0.07	-0.09	0.13	0.77	0.83
DS LNS	0.17	0.14	0.17	0.10	0.74
BRIEF	0.20	0.18	0.11	0.04	0.74
T2					
DS Forwards	0.03	0.00	0.15	0.46	0.79
DS Backwards	0.25	0.24	0.15	0.01	0.49
DS LNS	0.02	0.00	0.15	0.52	0.79
BRIEF	-0.13	-0.11	0.13	0.90	0.83
T3					
DS Forwards	-0.01	0.02	0.17	0.60	0.83
DS Backwards	-0.12	-0.10	0.19	0.87	0.83
DS LNS	0.03	0.04	0.14	0.48	0.79
BRIEF	-0.06	-0.06	0.11	0.74	0.83
T4					
DS Forwards	0.06	0.06	0.15	0.38	0.79
DS Backwards	-0.13	-0.10	0.14	0.88	0.83
DS LNS	0.09	0.09	0.12	0.25	0.75
BRIEF	0.05	0.04	0.14	0.44	0.79
T5					
DS Forwards	0.03	0.00	0.14	0.46	0.79
DS Backwards	0.14	0.12	0.12	0.13	0.74
DS LNS	0.05	0.05	0.11	0.39	0.79
BRIEF	0.17	0.17	0.13	0.08	0.74

Note. SD = standard deviation. DS = Digit Span.

at Genus level are associated with EF, we would expect a significant correlation between the Random Forest predictions and the known outcome. Only two models yielded significant results: Predicting the combined BRIEF scores and Digit Span Backwards from the samples obtained at T1 and T2, respectively. However, after applying the Benjamini-Hochberg procedure to correct for multiple testing they no longer remain significant. In general, the correlation coefficients vary closely around zero indicating that it was not possible to predict EF scores based on Genus level relative abundances at any time point. In a separate exploratory analysis, we used feature selection prior to fitting the final models to avoid potential overfitting to uninformative features. This approach did not change results meaningfully (see supplementary Table 5.4).

5.4.2 Bayesian Linear Models

5.4.2.1 Associations between Shannon Diversity and EF

Figures 5.3 and 5.4 summarize 20 linear models by showing the posterior distributions of the slope coefficients for alpha diversity and the covariates, respectively. Figure 5.3 corresponds to our hypothesis that alpha diversity is associated with EF. For every slope, we evaluate the proportion of the distribution that lies above or below zero. The higher this proportion, the more confident we can be that the relationship is positive or negative, respectively. We conclude that the association is positive or negative with confidence, if the 95% credible interval (CI) (grey area) excludes zero. Despite 2 sub-figures (Figure 5.3C T1 and Figure 5.3B T5) indicating otherwise, this was not the case for any slope parameter (see supplementary tables 5.5-5.24 for exact CIs). In addition to looking at the 95% CI, we can also evaluate consistency in the most likely direction of the association within infancy or childhood across our EF measures. If the most likely direction is consistent this could be interpreted as evidence for an association between alpha diversity and EF. For example, the relationship between alpha diversity and Digit Span Backwards (B) is consistently estimated to be most likely negative in infancy. However, we can observe consistency in the opposite direction across the three infant time points for Digit Span Forwards (C). Thus, the direction is not consistent across EF measures. In sum, we cannot observe evidence for an association between alpha diversity and EF using linear models. Among the covariates (Figure 5.4), the model is confident that maternal education is positively associated with Digit Span letter-number sequencing. Also, for Digit Span Backwards this relationship is likely ($P(\beta_{edu} > 0) = 0.95$). Sex and breastfeeding were not associated with EF scores although there is a tendency ($P(\beta_{male} > 0) = 0.97$) for boys to score higher on Digit Span letter-number sequencing. Note that these results do not differ depending on the alpha diversity index chosen (supplementary Figures 5.9 - 5.12).

5.4.2.2 Associations between Volatility and EF

Figure 5.5 summarizes our exploratory analyses (not preregistered) that investigate a potential association between microbiota volatility (supplementary Figure 5.13) and EF in childhood (see supplementary tables 5.25-5.40 for exact CIs). Each curve depicts a posterior distribution of a slope corresponding to the association between volatility at the given time point and EF in childhood. Applying the same criteria as outlined in the former section where we used Bayesian Linear models, we do not observe a relationship between microbiota volatility and EF. For time pair T2-T3 the direction of the association is most likely positive for three out of four EF measures (note that BRIEF must be interpreted inversely). This might indicate a positive relationship between volatility in that specific infant time window and EF in childhood that could potentially be identified with higher samples sizes. Similarly, for T4-T5 we observe that the majority of the posterior distribution indicates a positive slope across the three digit span measures.

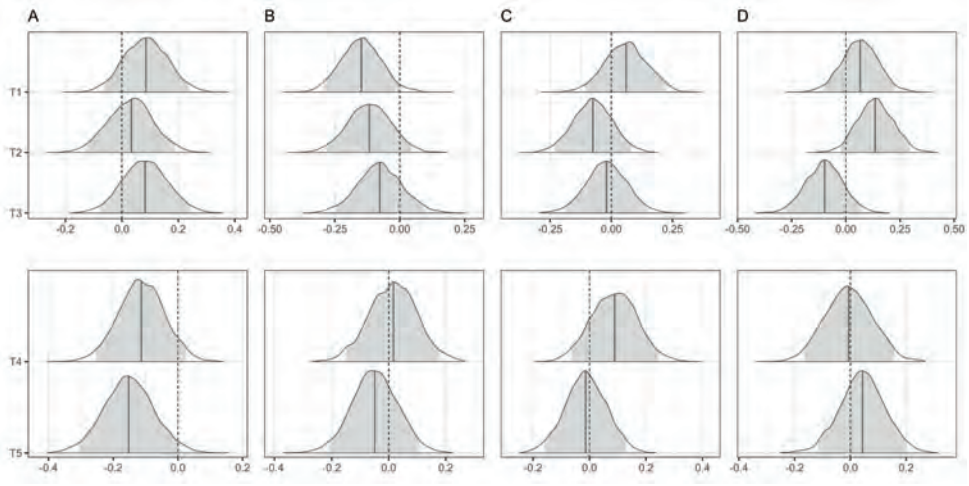

Figure 5.3: Posterior distributions (x-axis) of the beta coefficients of alpha diversity (Shannon) for each outcome (a–d) and time point (y-axis) when stool samples were obtained. The grey areas reflect the 95% credible intervals of the estimates. (a) Digit Span forwards, (b) Digit Span backwards, (c) Digit Span letter-number sequencing and (d) BRIEF.

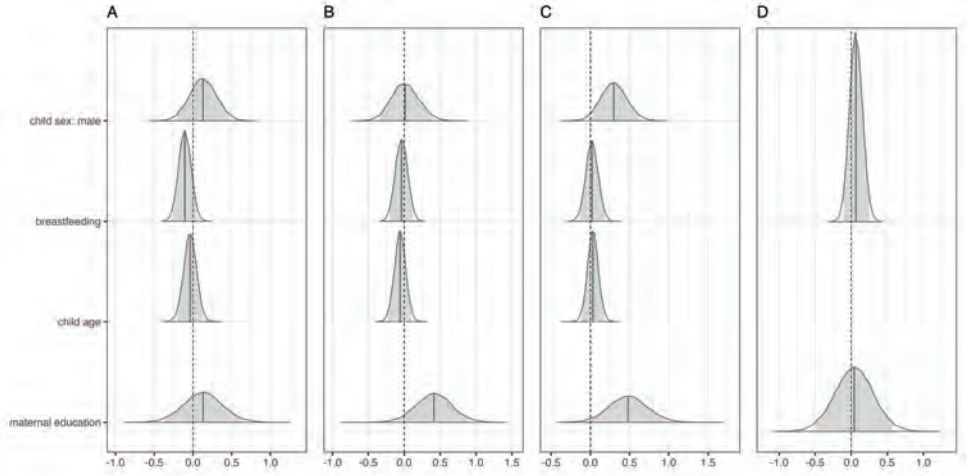

Figure 5.4: Posterior distributions of the beta coefficients of the covariates for each outcome averaged over all time points of microbiota sampling. The grey areas reflect the 95% credible intervals of the estimates. (a) Digit Span forwards, (b) Digit Span backwards, (c) Digit Span letter-number sequencing and (d) BRIEF.

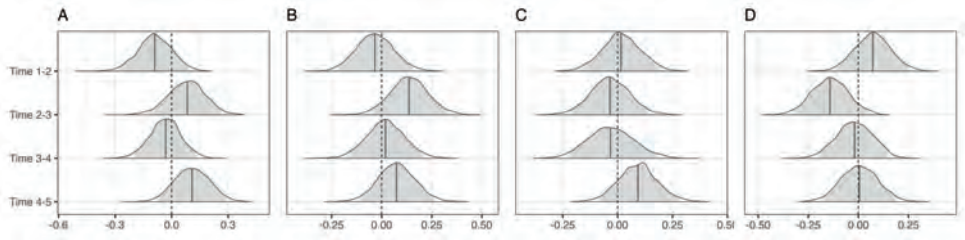

Figure 5.5: Posterior distributions (x-axis) of the beta coefficients of volatility for each outcome (a–d) and time point pair (y-axis) when stool samples were obtained. Grey areas reflect the 95% credible intervals of the estimates. (a) BRIEF, (b) Digit Span forwards, (c) Digit Span backwards and (d) Digit Span letter-number sequencing.

5.4.3 Identifying Clusters of FM composition - Partitioning Around Medoids

Neither Prediction Strength nor Silhouette Index indicated the presence of clustering at any of the five microbiota assessment moments (Figure 5.6). This was also the case when we used the cluster algorithm on all infant or childhood samples at once. Therefore, no follow-up analyses comparing EF between clusters were warranted. Note that we preregistered the k-means algorithm rather than Partitioning around Medoids. The k-means algorithm led to similar results. We presented Partitioning Around Medoids here as this makes our analyses more comparable to previous research (Carlson et al., 2018).

5.5 Discussion

This study examined the relationship between infant and childhood fecal microbiota (FM) composition and childhood executive functioning (EF). Our results did not reveal any consistent associations between FM composition and EF. The Random Forests algorithm, that is able to detect complex non-linear relationships, was unable to predict EF from Genus level relative abundances at any given time point. In line with that, Bayesian robust linear models found no association between alpha diversity or volatility and EF. Finally, we did not find that infant microbiota composition can be described by clusters based on the Partitioning Around Medoids method.

There are several potential explanations for the absence of statistical associations between FM composition and EF in our data. Considering that we explored the data using diverse complementary statistical approaches and also used 5 different time points of microbiota sampling that we could relate to our outcomes, the most likely explanation might be that there is no or only a weak relationship between the FM composition at the genus level and EF in childhood as assessed by our methods (parent questionnaires and digit span tests). That does not mean that gut microbes may not play a role in EF in humans in general. It is possible that effects of the microbiota on EF become apparent when looking at high-risk populations as opposed to

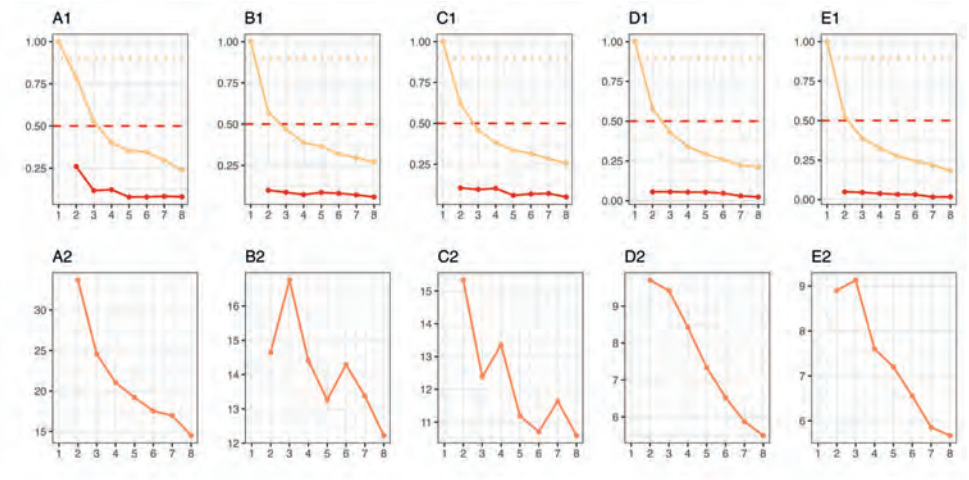

Figure 5.6: The y-axis shows absolute (a1–e1) and relative (a2–e2) measures of cluster strength for the stool samples obtained at T1 (a), T2 (b), T3 (c), T4 (d) and T5 (e). The x-axis shows the number of clusters the calculation is based on. Silhouette index and prediction strength are indicated in red and yellow, respectively, including their predefined thresholds. Neither of these measures exceeds the threshold. Therefore, the Calinski–Harabasz index (a2–e2) will not be interpreted.

a low-risk healthy population as in our study. Our hypotheses were mainly based on animal models that have identified associations between the gut microbiota and cognitive functioning (Sarkar et al., 2018). In these models, higher risk is induced by an intervention (e.g. stressors or antibiotics). At the same time, animal models have far less variation in the gut microbiota (Lagkouvardos et al., 2016). Therefore, animal studies may more easily reveal effects that in the human population are obscured by the large variability in environments and the more complex microbial ecosystems. Also, the natural gut microbial ecosystem in infancy is highly variable even within infants (de Muinck & Trosvik, 2018), as was also observed in our data throughout the first 10 years of life when looking at volatility (supplementary Figure 5.13) or Shannon alpha diversity (Figures 5.7 and 5.8). Correlation coefficients between alpha diversity values over all time points ranged between 0.22 and .29, indicating high intra-individual variability. To find an association between any individual variable that is highly variable over time might require very large sample sizes. And indeed, a large-scale study showed that effect sizes of FM-covariate associations are often surprisingly small, requiring very large sample sizes to be identified (Falony et al., 2016).

The absence of clustering in our data (determined with Partitioning around Medoids) as opposed to the data of e.g., Carlson et al. (2018) illustrates other important challenges in microbiome studies regarding cross-study comparisons, some of which have been discussed recently (Moreno-Indias et al., 2021). These challenges can include different choices regarding the sequenced region of the 16S gene or the pipeline used to process the sequencing data. For example, the V2 region has been shown to have higher resolution for lower-rank genera than the V4 region (Bukin et al., 2019). Other challenges arise because researchers choose different statistical methods or apply them differently. For instance, we applied thresholds to define the presence of clustering based on Koren et al. (2013). According to these thresholds, there would have been no clustering in the data of Carlson et al. (2018) either. As a final example, a Dirichlet Multinomial Mixture Model (Holmes et al., 2012), that we fitted as part of another project on the data of this study (Ou et al., 2022), can identify 3 clusters in infancy and 4 clusters in childhood (exploratory pairwise comparisons between these clusters can be found in the supplement). These examples illustrate that a lack of standardization of the many necessary analysis choices in microbiome studies makes a cross-study comparison difficult and in many cases impossible (Moreno-Indias et al., 2021).

Limitations of our study are that we could not take into account the functionality of the gut microbial ecosystem or look at species or even strain level. Relating (predicted) microbial neuroactive metabolites directly to EF was beyond the scope of this study but would be informative as the metabolites reflect an important pathway through which bacteria exert effects on the host. Also, it is possible that only single species or strains of a genus are associated with EF while the genus is not. Furthermore, given the high variability of the FM, partially caused by known uncontrolled variables such as diet (childhood), time of defecation, stool consistency and others, our sample size is too small to be confident that there is not a weak association between genus level FM and EF. Note, however, that the sample size is large compared to the earlier human studies (Carlson et al., 2018; W. Gao et al., 2019). Lastly, our study sample consisted of highly educated women with uncomplicated pregnancies and giving birth to healthy, full-term infants. This limits the generalizability of our findings. Strengths of the current study include using a longitudinal design over a long time span. This allowed us to determine FM at 3 distinct time

points in infancy and at 6 and 10 years of age in childhood. Repeated microbiota sampling makes our findings more robust compared to studies that analyzed a single sample. Furthermore, we used the Digit Span memory task in combination with the ecologically valid BRIEF questionnaire to measure different aspects of EF. The repeated measurement of the BRIEF resulted in a more robust estimation of daily life EF. Finally, we utilized different complimentary and sophisticated statistical methods to evaluate our hypotheses while accounting for important confounding variables.

5.6 Conclusions

In conclusion, we did not find a relationship between infant or childhood fecal microbiota composition and executive functioning in childhood. Future studies might benefit from a higher taxonomic resolution than the genus level, repeated assessments, and larger sample sizes, as well as the addition of the (predicted) functional assessment of the gut microbial ecosystem.

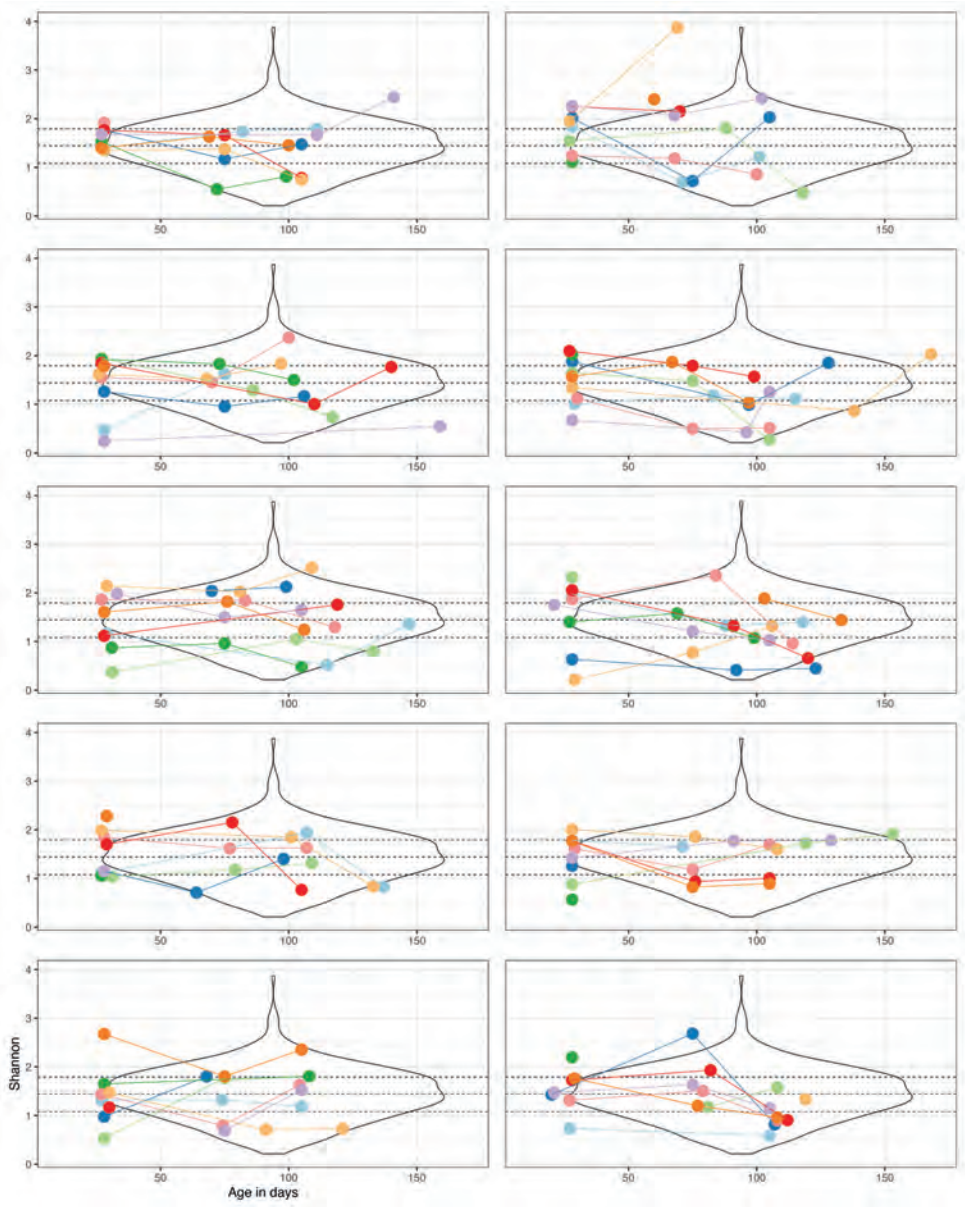

Figure 5.7: Alpha diversity (Shannon) for each sample in infancy. On each plot maximal eight infants (maximum eight colors) are shown to enable tracking the paths of the individuals without cluttering. The three dashed lines represent the 25%, 50% and 75% quantiles of all Shannon values in infancy. The violin plot in the background shows the corresponding whole distribution of Shannon diversity of our infant samples.

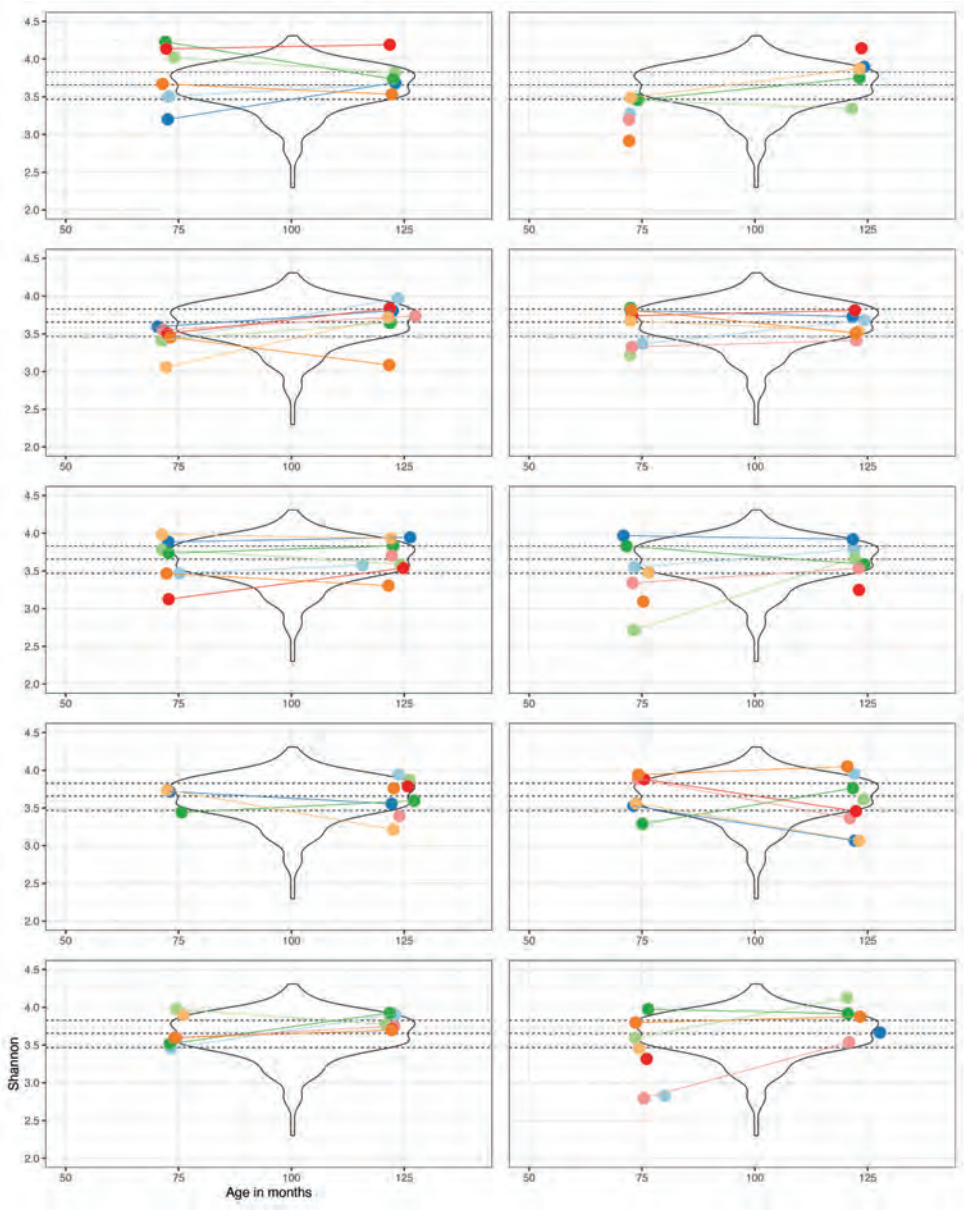

Figure 5.8: Alpha diversity (Shannon) for each sample in childhood. On each plot maximal eight children (maximum eight colors) are shown to enable tracking the paths of the individuals without cluttering. The three dashed lines represent the 25%, 50% and 75% quantiles of all Shannon values in childhood. The violin plot in the background shows the corresponding whole distribution of Shannon diversity of our childhood samples.

5.7 Supplementary Materials

5.7.1 Supplementary Methods

5.7.1.1 DNA Extraction

DNA extraction was performed via Maxwell 16 TOTAL RNA system (Promega, Wisconsin, USA) with Stool Transport and Recovery Buffer (STAR; Roche Diagnostics Corporation, Indianapolis, IN). We amplified the V4 region of the 16S ribosomal RNA (rRNA) gene in duplication, generating amplicons with length of around 290bp. Each PCR reaction consisted of 0.5µl 2U/µl Phusion Green Hot Start II High-Fidelity DNA polymerase (Thermo Scientific, US), 1µl 10um barcoded primers 515F-n(5'-GTGYCAGCMGCCGCGGTAA-3') and 806R-n(5'-GGACTACNVGGGTWTCTAAT-3') (Apprill et al., 2015), 10µl 5xPhusion Green HF Buffer (Thermo Scientific, US), 1µl 10mM dNTPs mix (Promega Corporation, US), 36.5µl Nuclease-free water and 1µl 20ng/µl DNA template. PCR was performed with 30s initial denaturation period at 98°C followed by 25 cycles of denaturation (98°C, 10s), annealing (50°C, 10s) and extension (72°C, 10s) and a final elongation (72°C, 7min). We verified PCR products by gel electrophoresis and purified them by the HighPrep® PCR kit (MagBio Genomics, Alphen aan den Rijn, Netherlands). DNA concentration was determined by fluorometer (DS-11; DeNovix) with Qubit® dsDNA BR Assay Kit (Life Technologies, Leusden, Netherlands). Barcoded sample belonging to the same library (200ng) was pooled together. Each library included 69 unique barcode tags, whereby 2 of them were specially designed for artificial control communities representative of human gut microbiota (Ramiro-Garcia et al., 2018). The mixture was again purified to a final volume of 40µl using HighPrep® PCR kit.

5.7.2 Supplementary Results

5.7.2.1 Alternative Alpha diversity Indices yield similar results

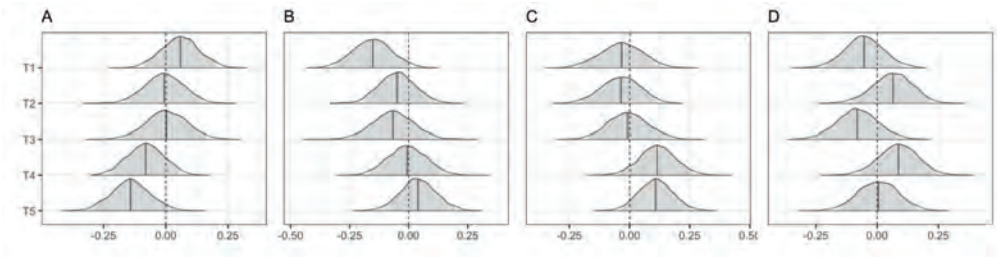

Figure 5.9: Posterior distributions (x-axis) of the beta coefficients of alpha diversity (richness) for each outcome (A-D) and time point (y-axis) when stool samples were obtained. The grey areas reflect the 95% credible intervals of the estimates. A = BRIEF. B = Digit Span Forwards. C = Digit Span Backwards. D = Digit Span Letter Number Sequencing.

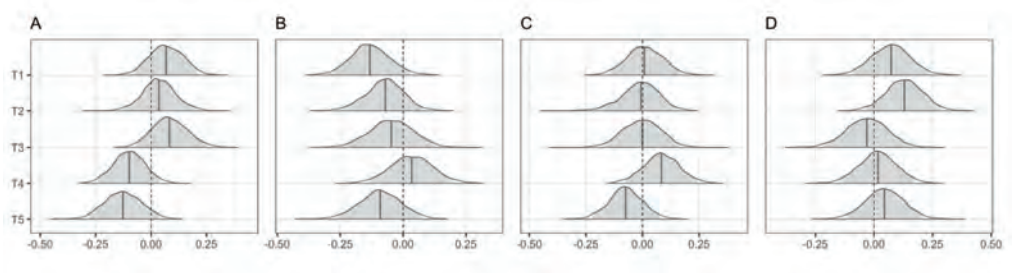

Figure 5.10: Posterior distributions (x-axis) of the beta coefficients of alpha diversity (inverse Simpson) for each outcome (A-D) and time point (y-axis) when stool samples were obtained. The grey areas reflect the 95% credible intervals of the estimates. A = BRIEF. B = Digit Span Forwards. C = Digit Span Backwards. D = Digit Span Letter Number Sequencing.

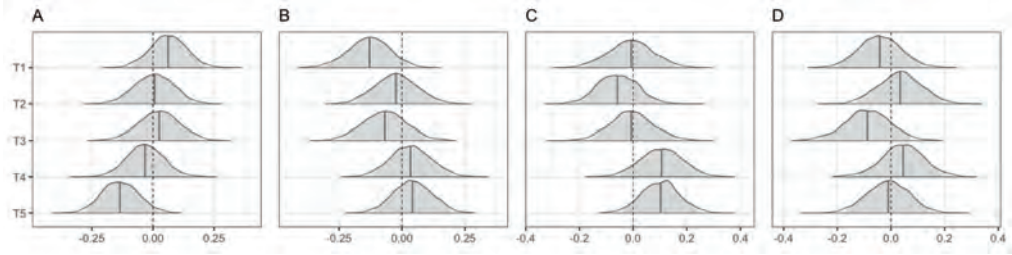

Figure 5.11: Posterior distributions (x-axis) of the beta coefficients of alpha diversity (Fisher) for each outcome (A-D) and time point (y-axis) when stool samples were obtained. The grey areas reflect the 95% credible intervals of the estimates. A = BRIEF. B = Digit Span Forwards. C = Digit Span Backwards. D = Digit Span Letter Number Sequencing.

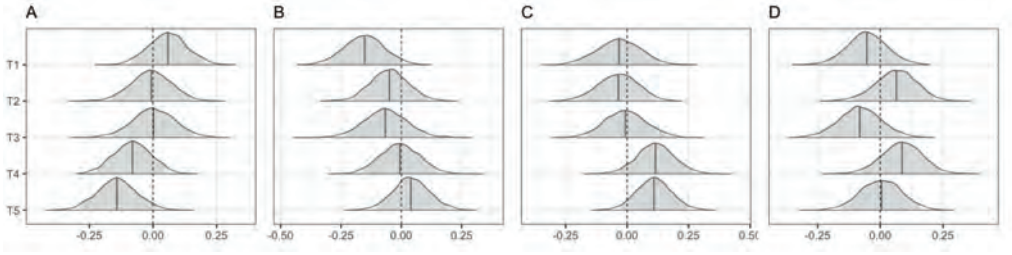

Figure 5.12: Posterior distributions (x-axis) of the beta coefficients of alpha diversity (Chao 1) for each outcome (A-D) and time point (y-axis) when stool samples were obtained. The grey areas reflect the 95% credible intervals of the estimates. A = BRIEF. B = Digit Span Forwards. C = Digit Span Backwards. D = Digit Span Letter Number Sequencing.

5.7.2.2 Microbiota Volatility

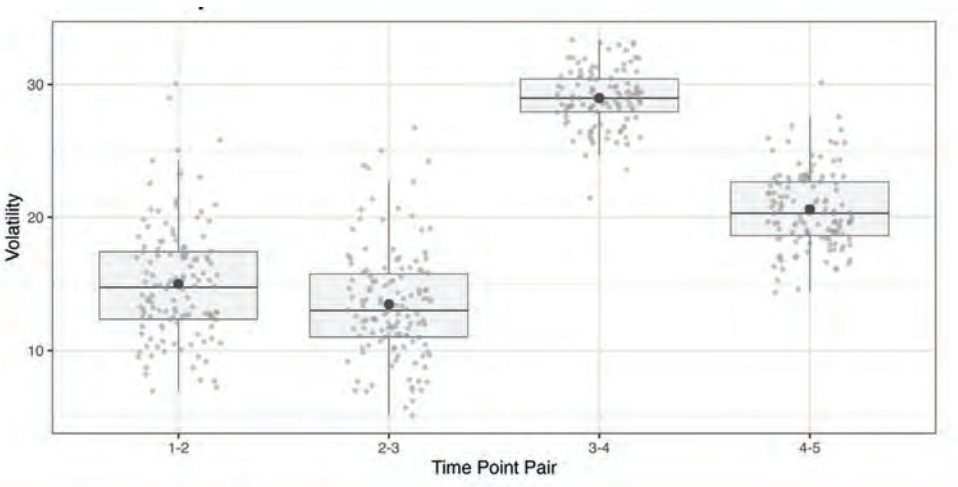

Figure 5.13: Distributions of microbiota volatility for each sequential time point pair (indicated on x-axis). Grey points show single data points plotted over boxplots. The larger black dot represents the mean.

5.7.2.3 Dirichlet Multinomial Mixture Clusters

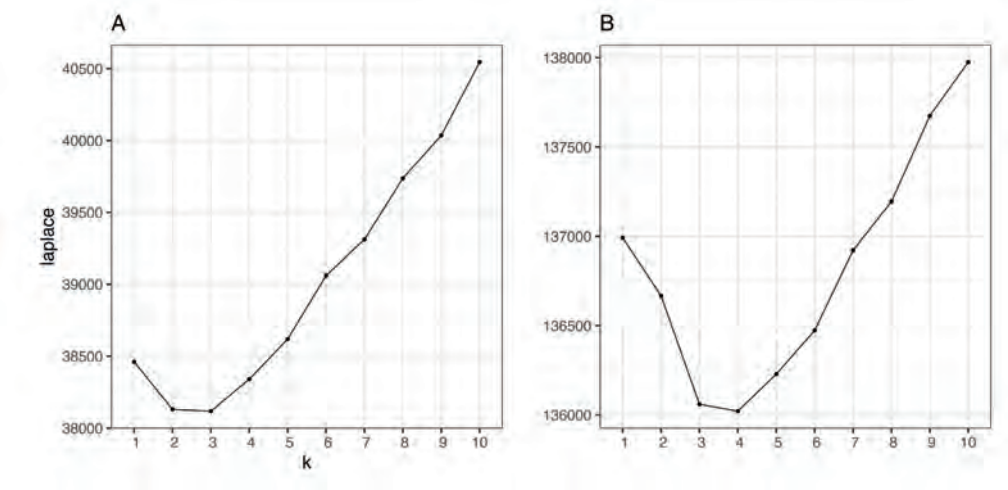

Figure 5.14: Laplace approximation for the Dirichlet Multinomial Mixture Models for infancy (A) and childhood (B). The lowest value indicates which number of cluster results in the best model fit.

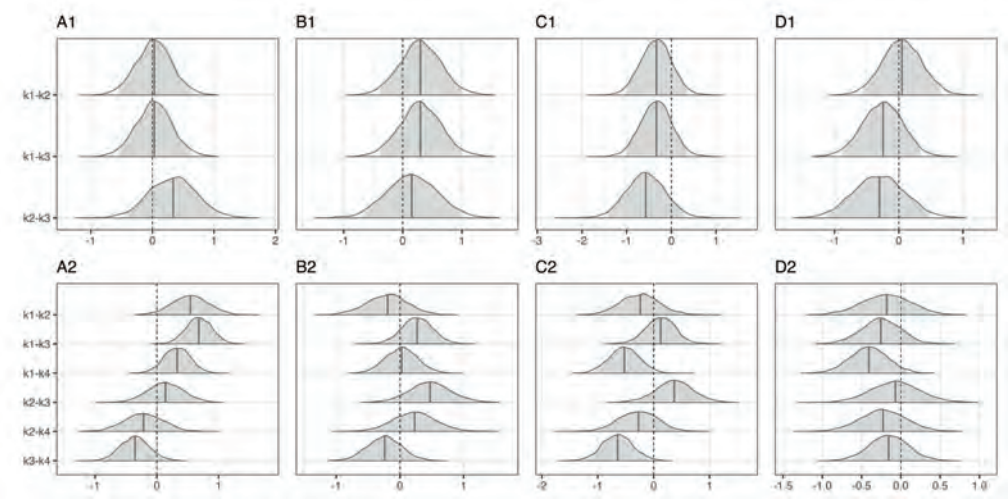

Figure 5.15: Pairwise comparisons of outcome variables between Dirichlet Multinomial Mixture Model clusters. A = Digit Span Forwards. B = Digit Span Backwards. C = Digit Span Letter Number Sequencing. D = BRIEF. The top row (1) refers to the clusters in infancy. The bottom row (2) refers to clusters in childhood.

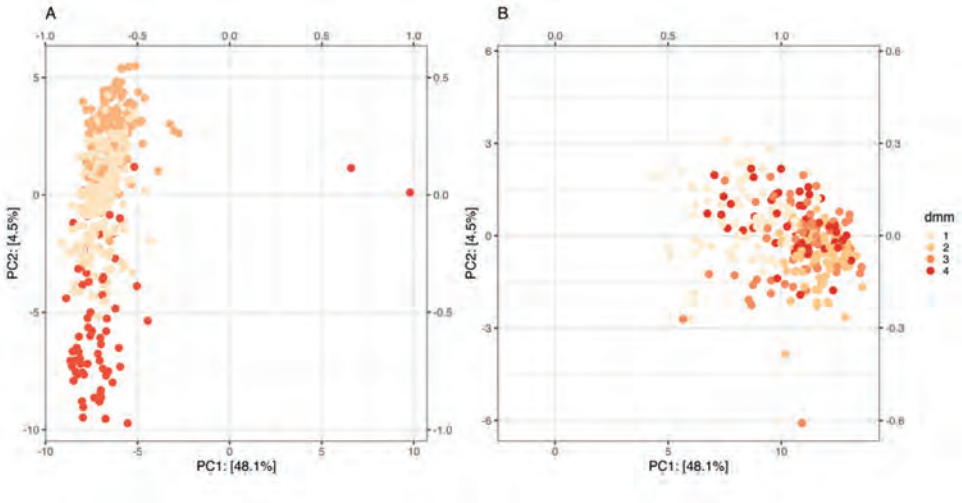

Figure 5.16: Principal Component Analysis (Aitchison distance metric) of all fecal samples graphically split by infancy (A) vs childhood (B) showing the clustering according to the Dirichlet Multinomial Mixture Models approach. Note that graphical representation of beta diversity differs depending on which distance metric is used.

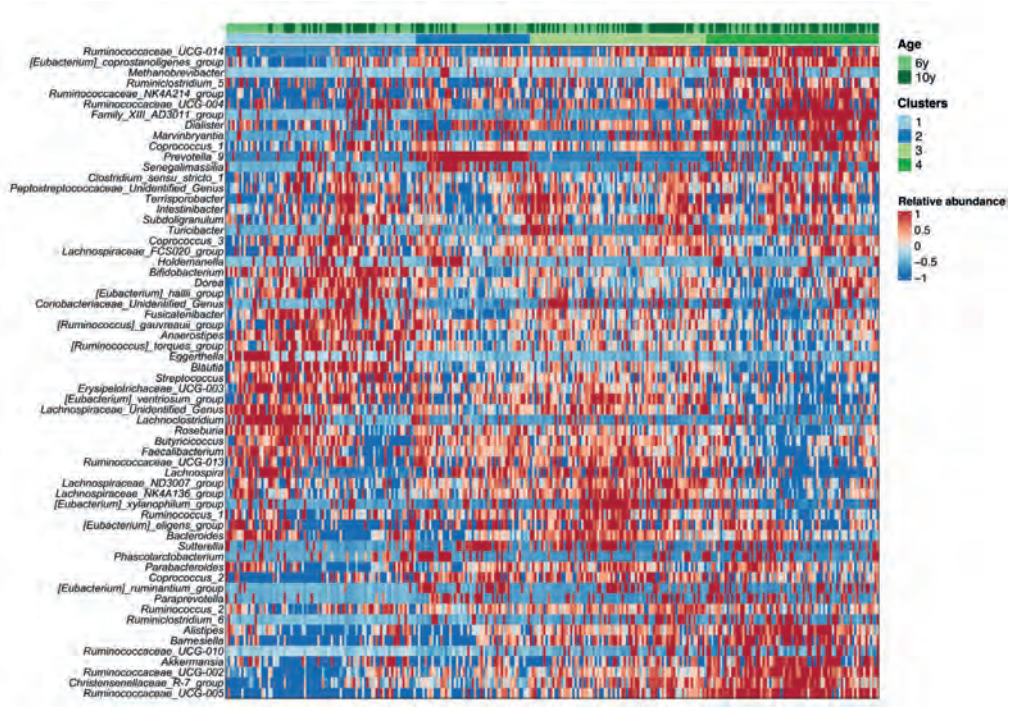

Figure 5.17: Scaled centered-log-ratio transformed abundances of the 50 most discriminating genera between clusters for each subject. The values have been scaled to zero mean and unit variance in order to highlight differences in the variation relative to the mean level within each taxonomic group. The horizontal bars at the top indicate the cluster assignment and volunteer age at the sample collection. The color scale has been limited to the interval [-1, 1].

5.7.3 Supplementary Tables

Table 5.4: Correlation between Random Forest prediction and real leave-out data.

Outcome	Time Point	Median	Mean	SD
DS Forwards	T1	-0.06	-0.07	0.06
DS Backwards	T1	0.00	0.01	0.09
DS LNS	T1	0.04	0.03	0.09
BRIEF	T1	0.09	0.08	0.08
DS Forwards	T2	0.00	-0.01	0.09
DS Backwards	T2	0.12	0.12	0.07
DS LNS	T2	-0.01	-0.01	0.09
BRIEF	T2	0.04	0.04	0.11
DS Forwards	T3	0.00	-0.02	0.12
DS Backwards	T3	-0.11	-0.11	0.09
DS LNS	T3	-0.10	-0.10	0.08
BRIEF	T3	0.07	0.05	0.12
DS Forwards	T4	0.08	0. 09	0.09
DS Backwards	T4	-0.19	-0.20	0.09
DS LNS	T4	-0.09	-0.07	0.09
BRIEF	T4	0.06	0.05	0.09
DS Forwards	T5	0.00	-0.02	0.10
DS Backwards	T5	0.02	0.03	0.07
DS LNS	T5	-0.10	-0.11	0.08
BRIEF	T5	0.10	0.09	0.11

Table 5.5: Parameter estimates for the model using Shannon diversity calculated from samples collected at T1 as predictor and Digit Span Forwards as outcome variable.

Parameter	Estimate	SD	95% CI
intercept_female	-0.200	0.207	-0.600 : 0.214
intercept_male	-0.074	0.206	-0.496 : 0.328
age	-0.044	0.080	-0.211 : 0.106
breastfeeding	-0.102	0.076	-0.248 : 0.048
education	0.106	0.244	-0.360 : 0.594
shannon	0.083	0.077	-0.070 : 0.238
sigma	0.737	0.060	0.629 : 0.866

Table 5.6: Parameter estimates for the model using Shannon diversity calculated from samples collected at T1 as predictor and Digit Span Backwards as outcome variable.

Parameter	Estimate	SD	95% CI
intercept_female	-0.413	0.215	-0.837 : 0.001
intercept_male	-0.491	0.208	-0.900 : -0.083
age	-0.073	0.078	-0.230 : 0.079
breastfeeding	0.020	0.079	-0.136 : 0.170
education	0.475	0.241	0.006 : 0.949
shannon	-0.150	0.080	-0.303 : 0.007
sigma	0.772	0.066	0.656 : 0.911

Table 5.7: Parameter estimates for the model using Shannon diversity calculated from samples collected at T1 as predictor and Digit Span Letter Number Sequencing as outcome variable.

Parameter	Estimate	SD	95% CI
intercept_female	-0.495	0.213	-0.919 : -0.077
intercept_male	-0.244	0.211	-0.664 : 0.158
age	0.027	0.073	-0.120 : 0.170
breastfeeding	0.034	0.076	-0.110 : 0.184
education	0.503	0.247	0.016 : 0.995
shannon	0.059	0.084	-0.108 : 0.225
sigma	0.763	0.064	0.649 : 0.897

Table 5.8: Parameter estimates for the model using Shannon diversity calculated from samples collected at T1 as predictor and BRIEF as outcome variable.

Parameter	Estimate	SD	95% CI
intercept	0.025	0.218	-0.405 : 0.455
breastfeeding	0.044	0.086	-0.123 : 0.212
education	-0.059	0.268	-0.582 : 0.467
shannon	0.066	0.084	-0.098 : 0.230
sigma	0.716	0.065	0.599 : 0.856

Table 5.9: Parameter estimates for the model using Shannon diversity calculated from samples collected at T2 as predictor and Digit Span Forwards as outcome variable.

Parameter	Estimate	SD	95% CI
intercept_female	-0.265	0.213	-0.686 : 0.161
intercept_male	-0.037	0.206	-0.441 : 0.393
age	-0.027	0.082	-0.190 : 0.131
breastfeeding	-0.070	0.076	-0.223 : 0.079
education	0.102	0.247	-0.391 : 0.579
shannon	0.032	0.080	-0.128 : 0.186
sigma	0.729	0.066	0.615 : 0.872

Table 5.10: Parameter estimates for the model using Shannon diversity calculated from samples collected at T2 as predictor and Digit Span Backwards as outcome variable.

Parameter	Estimate	SD	95% CI
intercept_female	-0.479	0.212	-0.887 : -0.058
intercept_male	-0.307	0.197	-0.685 : 0.081
age	-0.068	0.079	-0.226 : 0.087
breastfeeding	-0.038	0.081	-0.196 : 0.117
education	0.397	0.238	-0.085 : 0.860
shannon	-0.118	0.083	-0.280 : 0.044
sigma	0.767	0.068	0.646 : 0.912

Table 5.11: Parameter estimates for the model using Shannon diversity calculated from samples collected at T2 as predictor and Digit Span Letter Number Sequencing as outcome variable.

Parameter	Estimate	SD	95% CI
intercept_female	-0.556	0.220	-1.011 : -0.152
intercept_male	-0.152	0.218	-0.599 : 0.257
age	0.027	0.073	-0.119 : 0.172
breastfeeding	0.033	0.077	-0.117 : 0.185
education	0.496	0.245	0.027 : 0.976
shannon	-0.075	0.080	-0.241 : 0.085
sigma	0.713	0.062	0.602 : 0.847

Table 5.12: Parameter estimates for the model using Shannon diversity calculated from samples collected at T2 as predictor and BRIEF as outcome variable.

Parameter	Estimate	SD	95% CI
intercept	-0.058	0.215	-0.494 : 0.361
breastfeeding	0.092	0.084	-0.076 : 0.257
education	0.042	0.259	-0.466 : 0.545
shannon	0.133	0.082	-0.023 : 0.297
sigma	0.716	0.068	0.593 : 0.858

Table 5.13: Parameter estimates for the model using Shannon diversity calculated from samples collected at T3 as predictor and Digit Span Forwards as outcome variable.

Parameter	Estimate	SD	95% CI
intercept_female	-0.395	0.232	-0.843 : 0.057
intercept_male	-0.180	0.224	-0.624 : 0.268
age	0.011	0.083	-0.153 : 0.168
breastfeeding	-0.081	0.084	-0.248 : 0.075
education	0.228	0.259	-0.300 : 0.734
shannon	0.083	0.080	-0.074 : 0.244
sigma	0.758	0.066	0.641 : 0.903

Table 5.14: Parameter estimates for the model using Shannon diversity calculated from samples collected at T3 as predictor and Digit Span Backwards as outcome variable.

Parameter	Estimate	SD	95% CI
intercept_female	-0.366	0.233	-0.826 : 0.080
intercept_male	-0.311	0.213	-0.730 : 0.125
age	-0.076	0.082	-0.235 : 0.089
breastfeeding	-0.068	0.086	-0.229 : 0.101
education	0.323	0.255	-0.175 : 0.827
shannon	-0.079	0.087	-0.250 : 0.094
sigma	0.795	0.069	0.674 : 0.945

Table 5.15: Parameter estimates for the model using Shannon diversity calculated from samples collected at T3 as predictor and Digit Span Letter Number Sequencing as outcome variable.

Parameter	Estimate	SD	95% CI
intercept_female	-0.456	0.220	-0.906 : -0.038
intercept_male	-0.159	0.216	-0.606 : 0.267
age	0.041	0.074	-0.105 : 0.184
breastfeeding	0.012	0.079	-0.147 : 0.170
education	0.430	0.249	-0.057 : 0.929
shannon	-0.019	0.085	-0.186 : 0.143
sigma	0.727	0.063	0.614 : 0.864

Table 5.16: Parameter estimates for the model using Shannon diversity calculated from samples collected at T3 as predictor and BRIEF as outcome variable.

Parameter	Estimate	SD	95% CI
intercept	-0.162	0.223	-0.614 : 0.286
breastfeeding	0.125	0.087	-0.046 : 0.296
education	0.151	0.268	-0.376 : 0.686
shannon	-0.098	0.085	-0.266 : 0.068
sigma	0.702	0.069	0.581 : 0.849

Table 5.17: Parameter estimates for the model using Shannon diversity calculated from samples collected at T4 as predictor and Digit Span Forwards as outcome variable.

Parameter	Estimate	SD	95% CI
intercept_female	-0.222	0.204	-0.625 : 0.175
intercept_male	-0.139	0.195	-0.517 : 0.253
age	-0.061	0.079	-0.226 : 0.083
breastfeeding	-0.143	0.073	-0.289 : 0.002
education	0.159	0.231	-0.293 : 0.609
shannon	-0.113	0.072	-0.254 : 0.027
sigma	0.717	0.062	0.611 : 0.849

Table 5.18: Parameter estimates for the model using Shannon diversity calculated from samples collected at T4 as predictor and Digit Span Backwards as outcome variable.

Parameter	Estimate	SD	95% CI
intercept_female	-0.365	0.221	-0.802 : 0.071
intercept_male	-0.425	0.207	-0.838 : -0.019
age	-0.053	0.076	-0.202 : 0.101
breastfeeding	-0.056	0.077	-0.207 : 0.099
education	0.388	0.240	-0.096 : 0.851
shannon	0.019	0.083	-0.148 : 0.176
sigma	0.784	0.064	0.670 : 0.920

Table 5.19: Parameter estimates for the model using Shannon diversity calculated from samples collected at T4 as predictor and Digit Span Letter Number Sequencing as outcome variable.

Parameter	Estimate	SD	95% CI
intercept_female	-0.487	0.216	-0.920 : -0.069
intercept_male	-0.243	0.215	-0.680 : 0.164
age	0.030	0.068	-0.106 : 0.167
breastfeeding	-0.021	0.075	-0.166 : 0.139
education	0.501	0.239	0.037 : 0.971
shannon	0.086	0.081	-0.071 : 0.248
sigma	0.721	0.059	0.618 : 0.854

Table 5.20: Parameter estimates for the model using Shannon diversity calculated from samples collected at T4 as predictor and BRIEF as outcome variable.

Parameter	Estimate	SD	95% CI
intercept	-0.080	0.225	-0.515 : 0.377
breastfeeding	0.055	0.085	-0.112 : 0.223
education	0.079	0.266	-0.465 : 0.575
shannon	-0.006	0.080	-0.161 : 0.153
sigma	0.724	0.066	0.607 : 0.861

Table 5.21: Parameter estimates for the model using Shannon diversity calculated from samples collected at T5 as predictor and Digit Span Forwards as outcome variable.

Parameter	Estimate	SD	95% CI
intercept_female	-0.138	0.217	-0.565 : 0.294
intercept_male	-0.125	0.211	-0.539 : 0.303
age	-0.054	0.080	-0.212 : 0.098
breastfeeding	-0.125	0.077	-0.277 : 0.021
education	0.067	0.245	-0.405 : 0.551
shannon	-0.152	0.078	-0.309 : 0.002
sigma	0.773	0.061	0.666 : 0.903

Table 5.22: Parameter estimates for the model using Shannon diversity calculated from samples collected at T5 as predictor and Digit Span Backwards as outcome variable.

Parameter	Estimate	SD	95% CI
intercept_female	-0.440	0.216	-0.874 : -0.021
intercept_male	-0.446	0.196	-0.840 : -0.076
age	-0.018	0.075	-0.166 : 0.129
breastfeeding	-0.047	0.080	-0.206 : 0.106
education	0.487	0.242	0.031 : 0.974
shannon	-0.050	0.081	-0.216 : 0.105
sigma	0.800	0.063	0.693 : 0.933

Table 5.23: Parameter estimates for the model using Shannon diversity calculated from samples collected at T5 as predictor and Digit Span Letter Number Sequencing as outcome variable.

Parameter	Estimate	SD	95% CI
intercept_female	-0.485	0.216	-0.931 : -0.085
intercept_male	-0.184	0.198	-0.572 : 0.192
age	0.040	0.070	-0.097 : 0.181
breastfeeding	0.044	0.074	-0.103 : 0.193
education	0.478	0.237	0.019 : 0.955
shannon	-0.013	0.073	-0.165 : 0.130
sigma	0.743	0.059	0.636 : 0.869

Table 5.24: Parameter estimates for the model using Shannon diversity calculated from samples collected at T5 as predictor and BRIEF as outcome variable.

Parameter	Estimate	SD	95% CI
intercept	-0.019	0.214	-0.451 : 0.381
breastfeeding	0.023	0.081	-0.138 : 0.184
education	-0.006	0.258	-0.498 : 0.517
shannon	0.043	0.081	-0.117 : 0.200
sigma	0.717	0.063	0.601 : 0.851

Table 5.25: Parameter estimates for the model using volatility calculated between samples 1-2 as predictor and Digit Span Forwards as outcome variable.

Parameter	Estimate	SD	95% CI
intercept_female	-0.320	0.220	-0.756 : 0.105
intercept_male	-0.123	0.220	-0.561 : 0.321
age	-0.036	0.089	-0.212 : 0.135
breastfeeding	-0.081	0.087	-0.253 : 0.093
education	0.208	0.260	-0.293 : 0.727
volatility	-0.090	0.092	-0.273 : 0.086
sigma	0.732	0.070	0.605 : 0.884

Table 5.26: Parameter estimates for the model using volatility calculated between samples 1-2 as predictor and Digit Span Backwards as outcome variable.

Parameter	Estimate	SD	95% CI
intercept_female	-0.357	0.218	-0.790 : 0.062
intercept_male	-0.344	0.211	-0.746 : 0.081
age	-0.102	0.086	-0.281 : 0.056
breastfeeding	-0.002	0.090	-0.172 : 0.174
education	0.357	0.253	-0.133 : 0.849
volatility	-0.034	0.094	-0.213 : 0.156
sigma	0.780	0.074	0.644 : 0.941

Table 5.27: Parameter estimates for the model using volatility calculated between samples 1-2 as predictor and Digit Span Letter Number Sequencing as outcome variable.

Parameter	Estimate	SD	95% CI
intercept_female	-0.536	0.225	-0.998 : -0.108
intercept_male	-0.240	0.219	-0.666 : 0.195
age	0.020	0.081	-0.144 : 0.175
breastfeeding	0.050	0.089	-0.126 : 0.227
education	0.504	0.255	-0.012 : 1.014
volatility	0.015	0.089	-0.158 : 0.189
sigma	0.751	0.072	0.624 : 0.906

Table 5.28: Parameter estimates for the model using volatility calculated between samples 1-2 as predictor and BRIEF as outcome variable.

Parameter	Estimate	SD	95% CI
intercept	-0.081	0.228	-0.533 : 0.363
breastfeeding	0.057	0.093	-0.123 : 0.246
education	0.086	0.281	-0.474 : 0.634
volatility	0.072	0.089	-0.101 : 0.249
sigma	0.695	0.074	0.567 : 0.856

Table 5.29: Parameter estimates for the model using volatility calculated between samples 2-3 as predictor and Digit Span Forwards as outcome variable.

Parameter	Estimate	SD	95% CI
intercept_female	-0.504	0.229	-0.967 : -0.076
intercept_male	-0.231	0.231	-0.668 : 0.238
age	-0.028	0.096	-0.221 : 0.157
breastfeeding	-0.016	0.090	-0.191 : 0.156
education	0.323	0.267	-0.206 : 0.858
volatility	0.083	0.097	-0.119 : 0.264
sigma	0.749	0.077	0.620 : 0.922

Table 5.30: Parameter estimates for the model using volatility calculated between samples 2-3 as predictor and Digit Span Backwards as outcome variable.

Parameter	Estimate	SD	95% CI
intercept_female	-0.287	0.241	-0.753 : 0.197
intercept_male	-0.290	0.221	-0.719 : 0.153
age	-0.123	0.089	-0.304 : 0.055
breastfeeding	-0.046	0.091	-0.229 : 0.131
education	0.270	0.265	-0.253 : 0.773
volatility	0.137	0.095	-0.046 : 0.328
sigma	0.771	0.077	0.638 : 0.943

Table 5.31: Parameter estimates for the model using volatility calculated between samples 2-3 as predictor and Digit Span Letter Number Sequencing as outcome variable.

Parameter	Estimate	SD	95% CI
intercept_female	-0.491	0.240	-0.994 : -0.046
intercept_male	-0.180	0.237	-0.640 : 0.292
age	0.033	0.084	-0.126 : 0.199
breastfeeding	0.014	0.089	-0.157 : 0.193
education	0.400	0.268	-0.133 : 0.924
volatility	-0.035	0.089	-0.209 : 0.142
sigma	0.749	0.074	0.618 : 0.911

Table 5.32: Parameter estimates for the model using volatility calculated between samples 2-3 as predictor and BRIEF as outcome variable.

Parameter	Estimate	SD	95% CI
intercept	-0.132	0.226	-0.570 : 0.333
breastfeeding	0.036	0.095	-0.152 : 0.222
education	0.157	0.278	-0.424 : 0.689
volatility	-0.142	0.091	-0.321 : 0.041
sigma	0.677	0.076	0.545 : 0.844

Table 5.33: Parameter estimates for the model using volatility calculated between samples 3-4 as predictor and Digit Span Forwards as outcome variable.

Parameter	Estimate	SD	95% CI
intercept_female	-0.478	0.233	-0.919 : -0.015
intercept_male	-0.243	0.240	-0.721 : 0.230
age	-0.059	0.099	-0.260 : 0.124
breastfeeding	-0.099	0.089	-0.275 : 0.080
education	0.323	0.277	-0.212 : 0.887
volatility	-0.031	0.084	-0.200 : 0.131
sigma	0.741	0.078	0.605 : 0.916

Table 5.34: Parameter estimates for the model using volatility calculated between samples 3-4 as predictor and Digit Span Backwards as outcome variable.

Parameter	Estimate	SD	95% CI
intercept_female	-0.212	0.253	-0.708 : 0.272
intercept_male	-0.329	0.239	-0.808 : 0.141
age	-0.129	0.092	-0.314 : 0.046
breastfeeding	-0.049	0.090	-0.220 : 0.134
education	0.263	0.281	-0.280 : 0.816
volatility	0.019	0.096	-0.168 : 0.209
sigma	0.768	0.079	0.632 : 0.950

Table 5.35: Parameter estimates for the model using volatility calculated between samples 3-4 as predictor and Digit Span Letter Number Sequencing as outcome variable.

Parameter	Estimate	SD	95% CI
intercept_female	-0.404	0.245	-0.896 : 0.066
intercept_male	-0.163	0.253	-0.656 : 0.342
age	0.047	0.088	-0.130 : 0.225
breastfeeding	-0.035	0.092	-0.217 : 0.148
education	0.342	0.279	-0.198 : 0.888
volatility	-0.033	0.104	-0.225 : 0.183
sigma	0.751	0.078	0.616 : 0.921

Table 5.36: Parameter estimates for the model using volatility calculated between samples 3-4 as predictor and BRIEF as outcome variable.

Parameter	Estimate	SD	95% CI
intercept	-0.202	0.242	-0.680 : 0.271
breastfeeding	0.082	0.102	-0.118 : 0.275
education	0.221	0.294	-0.363 : 0.794
volatility	-0.018	0.091	-0.197 : 0.151
sigma	0.698	0.079	0.561 : 0.864

Table 5.37: Parameter estimates for the model using volatility calculated between samples 4-5 as predictor and Digit Span Forwards as outcome variable.

Parameter	Estimate	SD	95% CI
intercept_female	-0.249	0.240	-0.732 : 0.211
intercept_male	-0.129	0.231	-0.572 : 0.337
age	-0.080	0.096	-0.279 : 0.093
breastfeeding	-0.102	0.089	-0.281 : 0.068
education	0.140	0.270	-0.389 : 0.690
volatility	0.108	0.094	-0.072 : 0.291
sigma	0.764	0.068	0.644 : 0.908

Table 5.38: Parameter estimates for the model using volatility calculated between samples 4-5 as predictor and Digit Span Backwards as outcome variable.

Parameter	Estimate	SD	95% CI
intercept_female	-0.314	0.249	-0.831 : 0.164
intercept_male	-0.449	0.235	-0.939 : 0.004
age	-0.077	0.083	-0.248 : 0.079
breastfeeding	-0.057	0.086	-0.224 : 0.112
education	0.410	0.269	-0.122 : 0.960
volatility	0.074	0.093	-0.108 : 0.263
sigma	0.779	0.070	0.659 : 0.931

Table 5.39: Parameter estimates for the model using volatility calculated between samples 4-5 as predictor and Digit Span Letter Number Sequencing as outcome variable.

Parameter	Estimate	SD	95% CI
intercept_female	-0.446	0.236	-0.919 : 0.023
intercept_male	-0.163	0.225	-0.604 : 0.281
age	0.045	0.081	-0.117 : 0.207
breastfeeding	0.003	0.085	-0.166 : 0.166
education	0.391	0.262	-0.136 : 0.906
volatility	0.093	0.089	-0.081 : 0.273
sigma	0.760	0.071	0.637 : 0.917

Table 5.40: Parameter estimates for the model using volatility calculated between samples 4-5 as predictor and BRIEF as outcome variable.

Parameter	Estimate	SD	95% CI
intercept	-0.147	0.236	-0.618 : 0.303
breastfeeding	-0.020	0.091	-0.197 : 0.161
education	0.177	0.283	-0.386 : 0.751
volatility	0.004	0.093	-0.175 : 0.184
sigma	0.690	0.074	0.562 : 0.847

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Chapter 6

General Discussion

Preclinical evidence showed that stress, specifically maternal prenatal stress and early maternal separation, drastically affect the gut microbiota of the offspring in rodents. The gut microbiota in turn impacts brain development and functioning as well as behavior via the microbiota-gut-brain-axis. However, there is still a lack of human studies investigating these links. The general aim of this thesis was to translate insights garnered from three themes of experimental animal studies to human research using data from existing longitudinal studies.

Within Theme 1, we investigated whether there are relations between maternal prenatal and postnatal stress and features of the maternal and infant gut microbiota during and after pregnancy and in early life, respectively (Chapter 2). To achieve this, we obtained diverse measures of stress from 165 mother-infant-dyads at several time points in pregnancy and postnatally. The questionnaires included the EPDS, STAI and PSS-10, pregnancy specific stress questionnaires such as the PRAQR2 and the PSAS as well as biological measures of stress (hair cortisol and cortisone). Maternal stool samples were collected at 18 and 32 weeks of gestation and at eight months postpartum. The gut microbiota of the infants was obtained at two, six and 12 weeks and at eight months of age. Looking at several features of the gut microbiota (alpha diversity, beta diversity, relative abundances, and volatility), we found strong evidence that prenatal and postnatal maternal stress is associated with the maternal and infant gut microbiota, albeit not in a uniform manner for most features. Namely, we found both positive and negative associations with alpha diversity, depending on the time the stress or the microbiota was sampled. We also found associations with beta diversity and individual bacterial species and phyla. Importantly, we found exclusively positive associations between measures of stress and infant microbiota volatility (T. Bastiaanssen et al., 2021), in line with previous research.

Within Theme 2, we looked at the relation between early life stress and the infant gut microbiota using two studies. In the first study (Chapter 3), we investigated whether the entrance into center-based-childcare at around 10 weeks of age is associated with gut microbiota composition over the course of one month. For this we compared infants who entered childcare to those cared for at home taking a stool sample before childcare entrance and around one month later. Specifically, we looked at alpha- and beta diversity as well as relative abundances. Our results indicated that entering childcare had no or only small effects on the infant gut microbiota four weeks after entrance. In the second study (Chapter 4), we explored whether a destressing early life intervention influences microbiota development in the first year of life using data from a randomized controlled trial. The early life intervention consisted of a daily one-hour long session of skin-to-skin (SSC) contact between mother and infant in the first five postnatal weeks. Specifically, we looked at alpha- and beta diversity, relative abundances at genus level, a microbiota maturation index (Subramanian et al., 2014) and volatility. We found that the infants in the treatment group had significantly lower microbiota volatility in early infancy. Furthermore, their gut microbiota was less mature compared to the control group at one year of age. The latter effect was partially mediated by breastfeeding duration.

Within Theme 3 we explored whether we could find relations between the gut microbiota in infancy and childhood and executive functioning in childhood (Chapter 5). To achieve this, we collected stool samples from children at one, three and four months as well as at six and 10 years of age and measured executive functioning using a parent report and the Digit Span test as part of the Wechsler Intelligence Scale for children. Specifically, we looked at alpha- and beta diversity, relative abundances, potential clusters of gut microbiota composition and microbiota

volatility between all the time points. Our results were most compatible with no or only a weak association between any of the investigated gut microbiota features and executive functioning at any time point.

6.1 Main conclusions

6.1.1 Theme 1: Maternal stress and offspring microbiota development

- We found strong evidence that maternal prenatal and postnatal stress is associated with the infant gut microbiota in the first year of life in healthy Dutch mother-infant dyads.
- The direction of the associations appears to vary depending on the time point of sampling of stress measures and the gut microbiota.
- Infant microbiota volatility is likely consistently positively associated with maternal stress.

6.1.2 Theme 2: Early life stress and microbiota development

- The entrance into center-based childcare at three months of age in healthy Dutch infants does not appear to affect the infant gut microbiota composition in a uniform way.
- An early life skin-to-skin intervention in the first five weeks of life is associated with lower infant microbiota volatility in the first 5 weeks of life, as well as lower microbiota age at one year of age in healthy Dutch infants.

6.1.3 Theme 3: Early microbiota development and learning and memory

- There is likely no or only a weak association between the gut microbiota composition measured at genus level in infancy and at six and ten years of age and executive functioning measured at eight and 10 years of age using maternal report and a digit span task in healthy Dutch children.

6.2 Challenges and future directions: Towards a gold standard in microbiota-gut-brain-axis research

Our results from prospective studies in low-risk Dutch children add important insights to the accumulating human research that studies the microbiota-gut-brain-axis. These include the presence of an association between prenatal and postnatal maternal stress and features of the gut microbiota in infants in the first year of life, the lack of an association between the early entrance to center-based childcare and the gut microbiota of infants at three months of age, the

effect of a skin-to-skin contact intervention on microbiota development, and the lack of a strong association between the gut microbiota and executive functioning as measured by parent report and a cognitive task.

We discussed research-specific results, challenges, and future directions within the discussion section of each chapter. In this general discussion, we take a bird's-eye view of the research on the human microbiota-gut-brain axis, discussing challenges that are relevant across study topics in this field. Raising awareness and addressing these challenges should be prioritized to advance our understanding of the microbiota-gut-brain axis in the future.

A central issue is the lack of replicability in human microbiota-gut-brain axis research. Replicability involves achieving consistent outcomes in multiple studies addressing the same scientific inquiry, each using its unique dataset (Committee on Reproducibility and Replicability in Science et al., 2019). This term is often used interchangeably with reproducibility, which is defined as "obtaining consistent results using the same input data; computational steps, methods, and code; and conditions of analysis" (Committee on Reproducibility and Replicability in Science et al., 2019). Both replicability and reproducibility are crucial for gaining confidence in the validity and generalizability of findings and for advancing our scientific theories.

Several factors make replicability challenging in microbiome studies, including the complexity of the gut microbiota and variations between studies in stool sample collection and storage methods (Bartolomaeus et al., 2021; Z. J. Wang, 2018), DNA extraction (Bartolomaeus et al., 2021), the sequenced region of the 16S rRNA gene (Bukin et al., 2019), data processing pipelines that convert raw genetic sequences to amplicon sequence variants or operational taxonomic units, and the choice and application of statistical models and methods to control for bias in observational studies. Discussing all these factors is beyond the scope of this thesis. Therefore, in the following paragraphs we will focus on three key factors that limit replicability in microbiota-gut-brain axis research.

6.2.1 From animal to human research: Adding complexity to a dynamic complex system demands power

There are many hurdles when attempting to integrate findings from experimental animal research into human research. One significant hurdle is the increased intra- and interindividual variance of the gut microbiota in natural environments (Delaroque et al., 2022). In animal studies, variance can be controlled through the use of genetically identical animals, administration of identical diets, and a highly controlled living environment. In microbiome studies, added variance is particularly challenging because microbiomes are inherently complex and dynamic systems that constantly evolve and change depending on interactions between microbes and their environments (Ji et al., 2020). This leads to relatively high intraindividual variance compared to other outcome variables. For instance, total microbial cell counts (microbial load) varied between 1.1×10^8 and 1.6×10^{11} cell counts per gram of feces within individuals over just one week (Vandeputte et al., 2017). Furthermore, unwanted technical variation is added due to the measurement process, increasing total variation (Fachrul et al., 2022). All these factors combined can obscure the effects of a single environmental variable and result in small effect sizes for individual variables in association with the human gut microbiota (Falony et al., 2016).

Unsurprisingly, it has been concluded that microbiome studies must use relatively high sample sizes (Falony et al., 2016). For example, Falony et al. (2016) estimated that the required sample size to achieve 80% power at $P < 0.05$ when studying the association between the gut microbiota and obesity (case-control design; 16S rRNA gene amplicon sequencing) is 1730. While this sample size could be reduced to 1070 when several covariates are included (in this specific case stool quality, age, and gender), a comparable sample size is still reached only by few microbiota-gut-brain axis studies. To our knowledge, Falony et al. (2016) was one of the first to provide these recommendations on sample size requirements for microbiome studies based on power analysis. Recent developments allow all microbiome researchers to conduct their own power analyses. For example, Rahman et al. (2023) designed the tool *Evident*, which can calculate effect sizes for a wide range of metadata variables—including birth mode, antibiotic use, and socioeconomic factors—to assist in performing power calculations for new studies. Furthermore, new tools to simulate microbiome data enable a customized power analysis (Y. Gao et al., 2023).

Other recommendations to cope with the outlined properties of microbiome data include collecting samples from the same (small) communities who are more likely to share comparable environments and lifestyle variables (Sarkar et al., 2018). However, this would limit the generalizability of findings. Controlling factors partially responsible for high intraindividual variance to increase power is possible for shorter intervention studies. For instance, Delaroque et al. (2022) conducted a controlled-feeding study in which participants followed a controlled diet as inpatients for 11 days. They showed that controlling the diet helped to significantly reduce intraindividual variance and enabled the study of the effect of a food additive on the gut microbiota. However, this approach is not applicable for many study aims and infeasible for longer-lasting developmental studies.

Another way to increase power is to use longitudinal within-subject designs. While this remains applicable in microbiome studies, we noted that intraindividual correlation between microbiota features such as alpha diversity or relative abundances is relatively low over time when employing a sampling structure typically used in developmental studies in the social sciences (i.e., sampling every few months or years and at varying time intervals). Accordingly, longitudinal models often performed similarly or sometimes worse than models fit per time point (as evaluated by crossvalidation prediction accuracy). This reflects the complexity and resulting high intraindividual variability, as well as the technical variation introduced by the measurement process. To better enable the longitudinal modeling process and truly harness power benefits from within-subject designs, we believe that higher sampling frequency and evenly spaced time intervals (e.g., monthly, weekly, or daily) are necessary in longitudinal studies. Longitudinal models have been successfully utilized with such a sampling structure (see e.g. DiGiulio et al. (2015) or Shenhav et al. (2019)). A possible compromise for very long-lasting studies, such as our BIBO study, could be to sample yearly but take several samples on consecutive days per measurement round. A microbial profile based on several samples collected over around a week should help to create a less noisy representation of the gut microbiota composition of an individual at a given time and also allows to estimate short term volatility of the ecosystem.

In summary, the dynamic complex nature of the gut microbiome and added technical variation demand high sample sizes and hinder the longitudinal modeling process when there are single snapshots and long and uneven time intervals between samples. We therefore recommend to use the recently developed tools for power analysis in microbiome studies to ensure sufficiently

powered studies in the future. Furthermore, more dense time series data with monthly, weekly or even daily sampling would help to harness power benefits that can come with longitudinal within-subject designs.

6.2.2 Choice and application of statistical methods

In Chapter 5, we discussed how both the choice and application of a statistical method can lead to different results. In the specific example we encountered, we used the partitioning around mediods (PAM) algorithm as recommended by Koren et al. (2013) to group infant microbiota samples into clusters based on their bacterial relative abundances. The absolute measures of cluster strength as applied in Koren et al. (2013) suggested that there were no clusters, while a different method- a generative model for microbial metagenomics (Dirichlet Multinomial Mixtures)- suggested that clusters were present. Furthermore, applying PAM using a much less strict evaluation procedure, as applied in Carlson et al. (2018), would have also suggested that clusters were present. Additional variation in results from clustering methods arises from different choices of the applied distance metric (Shi et al., 2022). Thus, different configurations of variables such as distance metric, clustering model and evaluation criteria of the model are all common practice and accepted, manifesting lack of standardization in the field of microbiome research, which in turn hinders replicability.

Lack of standardization is not limited to unsupervised machine learning tasks such as clustering. For example, Nearing et al. (2022) compared 14 differential abundance testing methods on 38 16S rRNA gene datasets, each containing two distinct sample groups (e.g., diseased vs. healthy). Despite all the tested methods being commonly used and accepted in the microbiota-gut-brain-axis field, they yielded markedly different results (Nearing et al., 2022; L. Yang & Chen, 2022). Furthermore, each of these different methods can be applied differently, e.g. by performing different pre-processing steps (rarefaction vs no rarefaction, relative abundances vs centered-log-ratio transformed abundances etc.). Thus, lack of replicability can arise from the choice and application of the statistical method.

Analogously to these two examples (clustering and differential abundance analysis), for any modeling task there is a variation of accepted and common statistical models available that can be applied in different manners. A common recommendation to tackle this problem is to use a consensus approach based on multiple accepted methods to help ensure robust biological interpretations (Nearing et al., 2022). An example of this was presented in Chapter 4, where we attempted to detect bacterial genera that are differentially abundant between the treatment and the control group (skin-to-skin and care as usual). We carried out three differential abundance analysis methods (MaAsLin2 (Mallick et al., 2021), ANCOMBC (Lin & Peddada, 2020) and LinDA (Zhou et al., 2022)) and indicated their overlap in Figure 4.5. As statistical analyses can become increasingly automated, this consensus approach is indeed feasible and recommendable under current circumstances. Nevertheless, comparing and reporting alternative analyses for each feature of the gut microbiota remains tedious, time consuming, and results in large analysis reports. Also, different methods may be differently suited for modeling particular aspects of the data, such that disagreements in results do not necessarily mean the result was noise rather than signal. Therefore, it would be highly desirable to reach a gold standard for the analyses

of the different microbiota features. How can we reach such a gold standard?

One challenge that has hindered the field from achieving this was the inability to simulate microbiome data sets using different kinds of generative models. Most published statistical methods were benchmarked using a single generative model that was underlying the statistical method proposed. Unsurprisingly, the proposed statistical method then performed better than others to model the data. Nearing et al. (2022) attempted to circumvent this issue by using many real datasets and looking for robustness of findings between methods as an evaluation criterium as to which methods perform well. However, as the ground truth in real datasets remains unknown, robustness could also mean that certain methods are robust in responding to noise. Therefore, a significant milestone for the field has been the recent development of tools that make the simulation of microbiome data readily available to the research community (Y. Gao et al., 2023). The miaSim R/Bioconductor package allows researchers to simulate microbiome data based on diverse assumptions and commonly employed models. Specifically, data can be simulated based on MacArthur's consumer-resource model, the stochastic logistic model, Hubbell's neutral model, and the generalized Lotka-Volterra model alongside various derivatives (Y. Gao et al., 2023). Developers of methods are now able to benchmark their method against others using various generative models. This should not only help to improve method development but to also understand strengths and weaknesses of existing models better, hopefully paving the way to a gold standard of analysis methods.

However, relying solely on simulations still entails the risk that the simulated data does not correspond to data that we would encounter in a real-world experiment under the assumed ground truth (Pelto et al., 2024). To circumvent this problem, Pelto et al. (2024) created a sophisticated evaluation procedure for differential abundance analysis methods. They split 54 microbiome datasets repeatedly randomly into an exploration and a validation set. For each method, they evaluated how well the analysis on the exploration set agreed with the analysis on the validation set using several evaluation metrics. They applied a similar procedure using a between dataset approach. By employing this within and between dataset comparison approach, they were able to identify strengths and weaknesses of the individual methods. Interestingly, from the 13 differential abundance analysis methods tested, the elementary methods provided more consistent results without unnecessarily compromising sensitivity. Specifically, linear regression (MaAsLin2) or non-parametric methods (Wilcoxon test or a ordinal regression) performed comparably well (Pelto et al., 2024).

To sum up, variability in the choice and application of statistical methods impedes replicability. Until the field defines a gold standard of analysis methods for different microbiota features, testing results for robustness to the choice of methods (using a consensus approach) is recommended. Recently published tools that aid the simulation of microbiome data as well as increasingly sophisticated benchmark studies that employ within and between dataset replication tests should help reach a gold standard.

6.2.3 Defining, identifying, and handling confounders

One of the main advantages of experimental animal research is that it allows a causal interpretation of findings. In human research, we are often limited to the use of observational studies due

to ethical considerations. For example, we would not be able to conduct a randomized controlled trial where humans are randomly assigned to experience traumatic life events in childhood to study how the gut microbiota is related to this stressor. It remains a challenge across scientific disciplines to go beyond the reporting of mere associations in such settings. The reason for that is that third variables that influence both the predictor of interest and the outcome may cause the estimate (that quantifies the direction and magnitude of the relation) to be biased (Hernan & Robins, 2020). For example, if that third variable is unequally distributed along the predictor space (e.g., between a disease and a control group) and not being accounted for, the estimate will be biased in magnitude or even direction.

There are various strategies to prevent bias from confounding in an estimate. These include matching, instrumental variables, propensity score matching, regression discontinuity design, G-methods, and others (Hernan & Robins, 2020). All these methods require that the applicant has expert knowledge to reason how the variables could be causally related. This reasoning process typically results in one or several alternative theoretical models that can be visualized, for example using directed acyclic graphs. The established theoretical model(s), in combination with data-driven approaches, in turn help to define a confounding variable (Cinelli et al., 2020; Hernan & Robins, 2020).

The correct identification of confounding variables is crucial to reduce bias in estimates obtained from observational studies. Drastic differences in whether and how researchers define and identify confounding variables and how they account for them, hinder replicability. In the social sciences, it is very common to identify confounding variables as those that are correlated with both the predictor of interest and the outcome. Also, variables that have been correlated to predictor and outcome in previous studies are defined as confounders. Then these variables are included in statistical models, such as a linear regression model. This wide-spread approach is not a valid approach to reduce bias because a correlation can also arise if both the predictor and the outcome cause variation in the third variable (this is referred to as a *collider*) (Cinelli et al., 2020; Hernan & Robins, 2020; Wysocki et al., 2022). Including a collider can induce bias in the estimate rather than reduce or prevent it. Similarly, a third variable can also correlate with the predictor and outcome if it mediates the effect of the predictor on the outcome. In that case the estimate will also be biased if the mediator is included in, e.g., a regression analysis.

A similar approach is common in bio-medical research, whereby the correlation analysis is often replaced by feature selection methods such as Random Forest feature selection (Bommert et al., 2020) or model selection methods using information criteria. These approaches can identify whether the provided variables improve the prediction accuracy of the employed model. However, whether one employs correlation analyses, machine learning models, or other data-driven methods, these tools alone are unable to distinguish between different types of relationships in observational studies (as of the time of writing this thesis) (Wysocki et al., 2022).

For example, Vujkovic-Cvijin et al. (2020) used a sophisticated and yet purely data driven approach to identify confounding variables with respect to the gut microbiota and diseases. They identified alcohol consumption and stool quality as confounders because these variables were often unequally distributed between disease- and control group while also being related to gut microbiota composition. They recommend performing matching to distribute these variables equally between disease and control groups in future research. However, this data

driven approach is not able to distinguish whether any third variable is a mediator or a common cause of the microbiota and the studied disease using observational data. For instance, it is possible that certain bacteria influence stool quality (e.g., an extreme case would be a pathogenic bacterium inducing diarrhea). Stool quality could in turn contribute to or even define other health issues (such as irritable bowel syndrome). In such a case, matching patients of that health issue and the control group by stool quality, can lead to bias in the effect estimate. Because stool quality undoubtedly influences the quantified relative abundances of bacteria in stool samples by affecting the measurement procedure (Tito et al., 2024), there seems to be no good solution to this problem at the moment and either way some bias may remain. Possibly, moving from relative microbiome profiling to quantitative approaches, such as combining sequencing data with flow cytometry measurements of fecal microbial load (which quantifies the total number of microbial cells in a sample; (Vandeputte et al., 2017)), can reduce the bias that stool quality would cause (Tito et al., 2024). Independent of how this question resolves, it is important to note that data-driven approaches alone may not define which variables are possible confounders and how these should be handled.

Summarizing, there are diverse practices as well as recommendations on how to identify and handle confounding variables in observational studies within and beyond the microbiota-gut-brain-axis research field. These differences are problematic because they hinder comparability and replicability of results. Furthermore, several common practices are prone to lead to biased results, such as purely data-driven approaches to define which variable is a confounder. Data-driven approaches must be combined with causal justification to define confounding variables (Wysocki et al., 2022). Causal reasoning will inevitably result in different theoretical models across research labs. Therefore, it is crucial to engage in collective debate and refine these models over time. As statistical analyses increasingly occur within automated pipelines, testing different theoretical models becomes more feasible. Consequently, researchers should assess and report whether their results hold under varying causal assumptions. This practice will help ensure the replication of results despite differing theoretical frameworks across research labs.

6.3 Thesis reflections

The motivation for this thesis stemmed from a desire to integrate findings from experimental animal research about the microbiota-gut-brain axis with human research using ongoing longitudinal studies. Through comprehensive analysis of data aligned with three different themes of animal research, this study has provided valuable insights and knowledge. Notably, prenatal and postnatal maternal stress was positively associated with infant microbiota volatility. Furthermore, the infant microbiota appeared relatively unaffected by a potential early life stressor, specifically the entrance to center-based childcare at 10 weeks of age. An early skin-to-skin intervention was associated with lower microbiota volatility and slower microbiota maturation in the first year of life. Lastly, our data indicated a weak or absent association between the gut microbiota measured at the genus level throughout the first 10 years of life and executive functioning as assessed by parental reports and a cognitive task at 10 years of age. These findings point at the relevance of early life factors such as maternal stress and skin-to-skin contact for the development of the infant gut microbiota, with possible long-term implications for health.

Reflecting on this journey, conducting high-quality microbiota-gut-brain axis research demands substantial interdisciplinary knowledge. Next to discipline-specific knowledge (in this case Developmental Psychobiology) it requires expertise in various microbiome-related methods, from sample collection and DNA extraction to the processing of raw genetic sequences and statistical analysis of complex data. It is challenging and inefficient for each microbiome researcher to master all these skills at a high level. Instead, microbiota-gut-brain axis research necessitates collaborative interdisciplinary efforts. Furthermore, after more than a decade of hype and excitement, it is crucial to reflect on the acquired knowledge and the many realizations about methodological challenges. Building on this foundation, this field offers tremendous opportunities to improve diagnosis and treatment options for various poorly understood conditions. This thesis contributed knowledge about early life environmental factors influencing the gut microbiota and highlighted methodological challenges in microbiota-gut-brain axis research. We hope these contributions will inform and support future studies in this evolving field.

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Chapter 7

Appendices

7.1 Nederlandse samenvatting

Preklinisch onderzoek bij knaagdieren heeft aangetoond dat stress, met name prenatale maternale stress en vroege scheiding van de moeder, de darmbacteriën van het nageslacht drastisch beïnvloedt. De darmbacteriën beïnvloedt op zijn beurt de hersenontwikkeling en -functie, evenals gedrag via de microbiota-darm-hersen-as. Er is echter nog steeds een gebrek aan menselijke studies die deze verbanden onderzoeken. Het algemene doel van dit proefschrift was om inzichten uit drie thema's van experimentele dierstudies te vertalen naar menselijk onderzoek, gebruikmakend van gegevens uit bestaande longitudinale studies.

7.1.1 Thema 1: Maternale stress en ontwikkeling van de microbiota bij het nageslacht

Binnen Thema 1 onderzochten we of er verbanden waren tussen pre- en postnatale maternale stress en kenmerken van de maternale en kind darmbacteriën tijdens en na de zwangerschap en in de vroege levensjaren (Hoofdstuk 2). Hiervoor verzamelden we diverse maten van stress bij 165 moeder-kind-paren op verschillende tijdstippen tijdens de zwangerschap en na de geboorte. Daaronder waren maten van depressie (EPDS), angst (STAI), algemene stress (PSS-10), zwangerschaps-specifieke angst (PRAQR2 en de PSAS) en biologische stressmaten (haarcortisol en cortison). Maternale ontlastingsmonsters werden verzameld bij 18 en 32 weken zwangerschap en acht maanden postpartum. De darmbacteriën van de baby's werd verzameld op twee, zes en twaalf weken en op acht maanden. Door te kijken naar verschillende kenmerken van de darmbacteriën (alfa-diversiteit, beta-diversiteit, relatieve hoeveelheden en volatiliteit), vonden we sterk bewijs dat prenatale en postnatale maternale stress geassocieerd is met de maternale en kind darmbacteriën, zij het niet op een uniforme manier voor de meeste kenmerken. We vonden namelijk zowel positieve als negatieve verbanden met alfa-diversiteit, afhankelijk van het tijdstip waarop de stress of de microbiota werd gemeten. We vonden ook verbanden met beta-diversiteit en individuele bacteriesoorten en -phyla. Belangrijk is dat we uitsluitend positieve

verbanden vonden tussen stressmaten en volatiliteit van de kind microbiota, in overeenstemming met eerder onderzoek.

7.1.2 Thema 2: Vroege stress en ontwikkeling van de microbiota

Binnen Thema 2 onderzochten we het verband tussen vroege stress en de kind darmbacteriën met behulp van twee studies. In de eerste studie (Hoofdstuk 3) onderzochten we of het beginnen met de kinderopvang op ongeveer 10 weken geassocieerd is met de samenstelling van de darmbacteriën gedurende een maand. Hiervoor vergeleken we zuigelingen die naar de kinderopvang gingen met zuigelingen die thuis werden verzorgd, waarbij een ontlastingsmonster werd genomen vóór het beginnen met de kinderopvang en ongeveer een maand later. We keken specifiek naar alfa- en beta-diversiteit en relatieve hoeveelheden bacteriën. Onze resultaten gaven aan dat de kinderopvang geen of slechts kleine effecten had op de darmbacteriën van de zuigelingen vier weken na het begin. In de tweede studie (Hoofdstuk 4) onderzochten we of een vroege interventie, die mogelijk stress verlagende effecten heeft, de ontwikkeling van de microbiota in het eerste levensjaar beïnvloedde, gebruikmakend van gegevens uit een gerandomiseerde gecontroleerde studie. De vroege interventie bestond uit een dagelijkse sessie van één uur huid-op-huid (SSC) contact tussen moeder en kind in de eerste vijf postnatale weken. We keken specifiek naar alfa- en beta-diversiteit, relatieve hoeveelheden bacteriën op genusniveau, een microbiota-maturatie-index en volatiliteit. We vonden dat de zuigelingen in de behandelingsgroep significant lagere microbiota-volatiliteit hadden in de vroege kindertijd. Bovendien leek hun darmbacteriën trager te ontwikkelen in vergelijking met de controlegroep op éénjarige leeftijd. Dit laatste effect werd gedeeltelijk verklaard door de duur van de borstvoeding.

7.1.3 Thema 3: Vroege ontwikkeling van de microbiota en leren en geheugen

Binnen Thema 3 onderzochten we of er verbanden zijn tussen de darmbacteriën in de vroege kindertijd en de kindertijd en executief functioneren in de kindertijd (Hoofdstuk 5). Hiervoor verzamelden we ontlastingsmonsters van kinderen op één, drie en vier maanden, evenals op zes en tien jaar en maten we het executief functioneren met behulp van een ouderrapport en de cijferreekstest als onderdeel van de Wechsler Intelligentietest voor kinderen. We keken specifiek naar alfa- en beta-diversiteit, relatieve hoeveelheden, potentiële clusters van darmbacteriën-samenstelling en volatiliteit tussen alle tijdspunten. Onze resultaten waren het meest in overeenstemming met geen of slechts een zwak verband tussen de onderzochte kenmerken van de darmbacteriën en executief functioneren op elk tijdstip.

Door middel van een uitgebreide analyse van data, afgestemd op drie verschillende thema's van dieronderzoek, heeft deze proefschrift waardevolle inzichten en kennis opgeleverd. Met name pre- en postnatale maternale stress was positief geassocieerd met de volatiliteit van de microbiota van het kind. Bovendien leek de kind microbiota relatief onaangetast door een mogelijke vroege stressfactor, namelijk het beginnen met kinderopvang op 10 weken leeftijd. Een vroege huid-op-huid-interventie was geassocieerd met lagere volatiliteit en langzamere maturatie van de microbiota in het eerste levensjaar. Ook gaven onze gegevens aan dat er geen of alleen een

zwak verband was tussen de darmbacteriën gemeten op genusniveau gedurende de eerste 10 levensjaren en het executief functioneren, zoals beoordeeld door ouderrapporten en een cognitieve taak op 10-jarige leeftijd. Deze bevindingen wijzen op het belang van vroege levensfactoren zoals maternale stress en huid-op-huid contact voor de ontwikkeling van de kind darmbacteriën, met mogelijke lange termijn implicaties voor de gezondheid. Ten slotte wijst dit proefschrift op belangrijke methodologische uitdagingen in het veld van de microbiota-darm-hersen-as die de reproduceerbaarheid van bevindingen beperken. De uitdagingen omvatten de dynamische complexe aard van de darmbacteriën, de keuze en toepassing van statistische methoden en hoe confounders in observationele studies gedefinieerd, geïdentificeerd en behandeld kunnen worden.

7.2 Research Data Management Statement

This thesis utilized data from several studies including the BIBO, BINGO, SKIPPY and the SMILEY study. FAIR principles were used for the research data management for all studies.

7.2.1 Ethics

The BINGO, BIBO, SKIPPY and SMILEY studies were conducted in accordance with the 1964 Declaration of Helsinki and its later amendments. The study protocols were approved by the ethics committee of the Social Science faculty of Radboud University, Nijmegen, the Netherlands, with the following approvals: BINGO study (ECSW2014-1003-189, ECSW-2018-034), BIBO study (SW2017-1303-497, SW2017-1303-498, ECSW-2018-067, ECG 300107), SKIPPY study (ECSW2015-2311-358) and SMILEY study: (SW2017-1303-497, ECSW-2019-051 and ECSW2020-021). Written informed consent was obtained from all participants regarding the use and storage of their data. Participants were informed that they could discontinue the study at any moment without providing a reason. This research was supported by the European Union's Horizon 2020 Eat2beNice grant (728018 to C. de Weerth and A. Arias Vásquez), a Jacobs Foundation Advanced Research Fellowship (to C. de Weerth), and a Netherlands Organization for Scientific Research VENI grant (016.195.197 to R. Beijers), VIDI grant (575-25-009 to C. de Weerth), and VICI grant (016.Vici.185.038 to C. de Weerth).

7.2.2 FAIR principles

7.2.2.1 Findable

All data from the BINGO, BIBO, SKIPPY and SMILEY studies, including raw, cleaned, and master data, are stored on the secure network drive of the Donders Institute for Brain, Cognition, and Behavior. The pseudonymization keys are stored in subfolders specific to each study. Physical data, including paper data and informed consent forms, are stored in the locked archive of the department of Cognitive Neuroscience at the Donders Institute.

7.2.2.2 Accessible

The data and research documentation are only accessible to researchers involved in the BINGO, BIBO, SKIPPY and/or SMILEY projects, and to the Developmental Psychobiology (DPB) lab manager. The data are not freely available as we did not obtain participant consent for public online depository storage. Researchers may request access to the data with a methodologically sound proposal directed to carolina.deweerth@radboudumc.nl. Upon approval, requestors must sign a data transfer agreement or research collaboration agreement, depending on the collaboration level. Anonymous processed data will be shared, and researchers are expected to analyze the data and/or publish the results within two years. Data availability statements were added to the published papers whenever possible.

7.2.2.3 Interoperable

Documentation on the setup of the BINGO, BIBO, SKIPPY and SMILEY studies can be found in their respective folders and published papers. The study protocol of the SKIPPY study was preregistered (Netherlands Trial Register: NTR5697; 13/03/2016) and published (Cooijmans et al., 2017). As the raw, cleaned, and master data, along with syntax, output, and documentation files for the papers presented in the current thesis, are stored on the secure network drive of the Donders Institute, the results are reproducible and interoperable.

7.2.2.4 Reusable

All data will be stored for at least 20 years from the moment of data collection and can be reused within this period, as stated in the informed consent forms.

7.2.3 Privacy

The privacy of the participants in the BINGO, BIBO, SKIPPY and SMILEY studies was ensured by pseudonymization. The pseudonymization keys, which link codes to personal data, are stored on the secure network drive of the Donders Institute in separate folders for each study. These keys are only accessible to the DPB lab manager and researchers involved in the projects, depending on their roles. Since the BINGO study may continue, and the SKIPPY and BIBO studies are ongoing longitudinal studies, the pseudonymization keys have not been destroyed to allow for future contact with participants for new measurement waves. If data is shared, no personal data (e.g., addresses, videos with participant faces) will be shared.

7.3 List of publications

Eckermann, H. A., Lugones, M., Abdala, D., Roge, H., & De Weerth, C. (2024). Maternal early life and prenatal stress in relation to birth outcomes in Argentinian mothers. Developmental Psychobiology, 66(5), e22502. https://doi.org/10.1002/dev.22502

Eckermann, H. A., Meijer, J., Cooijmans, K., Lahti, L., & De Weerth, C. (2024). Daily skin-to-skin contact alters microbiota development in healthy full- term infants. Gut Microbes, 16(1), 2295403. https://doi.org/10.1080/ 19490976.2023.2295403

Eckermann, H. A., Ou, Y., Lahti, L., & Weerth, C. (2022). Can gut microbiota throughout the first 10 years of life predict executive functioning in childhood? Developmental Psychobiology, 64(3). https://doi.org/10. 1002/dev.22226

Hermes, G. D. A., Eckermann, H. A., de Vos, W. M., & de Weerth, C. (2020). Does entry to center-based childcare affect gut microbial colonization in young infants? Scientific Reports, 10(1), 10235. https://doi.org/10. 1038/s41598-020-66404-z

Houtman, T. A., Eckermann, H. A., Smidt, H., & De Weerth, C. (2022). Gut microbiota and BMI throughout childhood: The role of firmicutes, bacteroidetes, and short-chain fatty acid producers. Scientific Reports, 12(1), 3140. https://doi.org/10.1038/s41598-022-07176-6

Vacaru, S. V., Brett, B. E., Eckermann, H., & De Weerth, C. (2023). Determinants of maternal breast milk cortisol increase: Examining dispositional and situational factors. Psychoneuroendocrinology, 158, 106385. https: //doi.org/10.1016/j.psyneuen.2023.106385

7.4 Acknowledgements

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Next, I want to thank my promoter, Leo. Your guidance and availability were invaluable. Without your affirmation and direction, this project would not have been possible. I particularly enjoyed and benefited from our collaboration on numerous workshops, summer schools, and the machine learning challenge.

I also want to extend my heartfelt thanks to the entire DPB-lab group. I benefited immensely from the work of both past and present group members who designed studies and worked tirelessly to collect data. Without your efforts, this thesis would not have been possible. Specifically, I want to thank Irene, Roseriet, Hellen, Nina, Stefania, Bonnie, and Nicole. Your understanding and support were truly appreciated.

Furthermore, I am grateful to my collaborators from Wageningen University. Thank you, Gerben Hermes, for the critical discussions at the beginning of my journey. I also want to thank Yangwenshan Ou, Wasin Poncheewin, and Prokopis Konstanti. Without your help, I would not have been able to process genetic sequencing data for my lab group.

Lastly, I want to thank my entire family, especially my grandparents, parents, and siblings, for working hard to put me in the privileged position I started my professional career in. I could not have continued my career when I became ill without your help. You were my safety net, and now I can walk again by myself. I will be forever grateful to you and will strive to support you and the next generation, as I have been supported.

Thank you, Milica, for your love and for truly seeing me. With you by my side, I am better at everything I do. I hope we will celebrate many more successes together.

Finally, thank you, God, for carrying me through the darkest moments when I couldn't continue alone.

7.5 Curriculum Vitae

Henrik Eckermann was born in Kevelaer, Germany, on December 4th, 1987. He obtained his Bachelor of Science degree in Psychology from the Medical School Berlin in 2013. He received the Scientific Research Award from the Medical School Berlin for his Bachelor's thesis titled 'Biome depletion, immune regulation, and psychiatric disorders: How microorganisms influence mental health.' In 2018, he obtained his MSc degree (cum laude) in the Research Master Behavioral Science at Radboud University. His Master's thesis examined the relationship between bacterial relative abundances and cognitive functioning in healthy children. In 2019, he began his PhD candidacy at the Donders Institute under the supervision of Prof. dr. Carolina de Weerth and Prof. dr. Leo Lahti, focusing on the development of the human gut microbiota in relation to environmental variables and cognition during childhood, as outlined in this thesis.

7.6 PhD portfolio of Henrik Eckermann

Department: Cognitive Neuroscience
PhD period: 01/09/2019 – 31/05/2024
PhD Supervisor: Prof. dr. C. de Weerth
PhD Co-supervisor: Prof. dr. L. Lahti

Table 7.1: PhD Portfolio

Year	Title	Organizer	Duration
Conferences			
2019	Summer School Microbial Community Modeling	KU Leuven	28 hours
2019	Gut Day	University of Amsterdam	16 hours
2021	Analysis of Skin Microbiome and Metabolome	Savitribai Phule Pune University	13 hours
2021	Microbiome Data Analysis Workshop	Hasselt University	24 hours
2023	International Society of Psychoneuroendocrinology	ISPNE	21 hours
Courses			
2021	Bayesian Regression Modelling for Biologists	Oxford University	16 hours
2021	Donders Graduate School - Scientific Integrity Course	Donders Institute	7 hours
2021	Bayesian Networks	Radboud University	28 hours
Supervision			
2020	Timothy Houtman	Master Behavioral Sciences	6 months
2022	Jennifer Meijer	Master Biomedical Sciences	6 months
2023	Eva Spetter	Master Biomedical Sciences	6 months
Teaching			
2020	DondeRs	Donders Institute	16 hours
2021	Summer School Brain, Bacteria and Behavior	Radboud University Medical Center	21 hours
2022	Summer School Brain, Bacteria and Behavior	Radboud University Medical Center	21 hours

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