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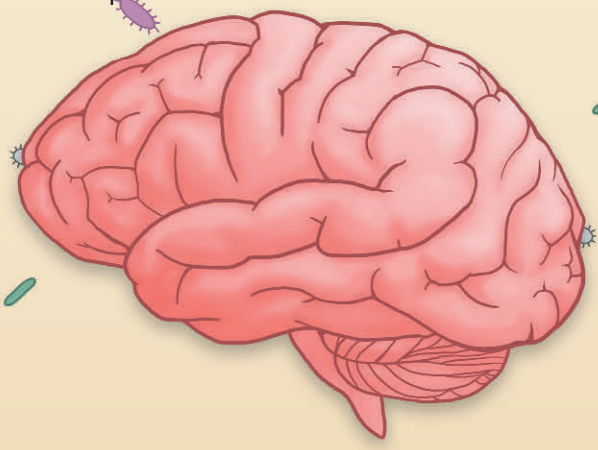

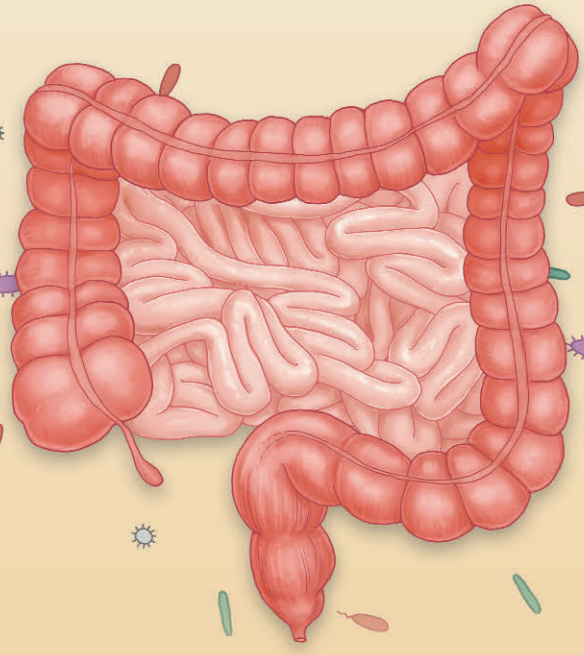

MATERNAL PRENATAL STRESS, INFANT MICROBIOTA, BRAIN, AND BEHAVIORAL DEVELOPMENT

The FinnBrain Birth Cohort Study

Anna-Katariina Aatsinki

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA - SER. D OSA - TOM. 1531 | MEDICA - ODONTOLOGICA | TURKU 2021

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To my loved ones

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ABSTRACT

The gut microbiota and its manipulation have been shown to affect behavior and neurodevelopment in rodents. Likewise, maternal prenatal stress is known to influence offspring health and development as well as gut microbiota composition in rodents and non-human primates. However, how infant fecal microbiota is associated with infant behavioral and brain developmental phenotypes or with exposure to prenatal stress remains largely unknown.

The first aim of this dissertation was to explore how infant fecal microbiota associates with temperament, emotional attention, and amygdala volume, all of which may relate to later socioemotional and behavioral development. The second aim was to investigate if maternal prenatal chronic psychological distress and chronic cortisol levels, which are measures of prenatal stress in this study, associate with infant fecal microbiota composition and diversity. The studies were conducted in the prospective, general population-based FinnBrain Birth Cohort Study.

First, the results showed that early fecal microbiota composition was associated with temperament traits and attention bias towards fearful faces. Specifically, *Bifidobacterium*, *Stroptococcus*, and *Atopobium* were positively associated with positive emotionality, whereas *Bifidobacterium* was negatively and *Clostridium* was positively associated with greater attention bias towards fearful faces. Both temperament and attention bias towards faces showed an interaction by sex regarding fecal microbiota composition. The left amygdala volume as well as negative emotionality and fear reactivity were negatively associated with fecal microbiota diversity. Second, maternal prenatal stress associated with fecal microbiota composition, including increases in abundances of genera within the Proteobacteria phylum and decreases in *Lactobacillus* abundance.

This dissertation argues that infant fecal microbiota associates with later brain and behavioral phenotypes and encourages future longitudinal and mechanistic studies. Likewise, we corroborate some earlier findings regarding maternal prenatal stress and infant fecal microbiota.

KEYWORDS: prenatal stress; infant; gut; microbiota; emotional attention; amygdala; temperament; gut-brain axis

TURUN YLIOPISTO
Lääketieteellinen tiedekunta
Kliininen laitos
Psykiatria
ANNA-KATARIINA AATSINKI: Äidin raskaudenaikainen stressi, lapsen mikrobisto, käyttäytymisen ja aivojen kehitys
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TIIVISTELMÄ

Eläintöiden perusteella on ehdotettu, että suolistomikrobisto vaikuttaa aivojen toimintaan ja käyttäytymiseen. Lisäksi jyrsijöillä ja kädellisillä on osoitettu, että äidin raskaudenaikainen stressi vaikuttaa jälkeläisten kasvuun ja terveyteen sekä suolistomikrobiston koostumukseen. Vielä ei kuitenkaan täysin ymmärretä, että liittyykö äidin raskaudenaikainen stressi lapsen suolistomikrobiston koostumukseen tai liittyykö lapsen suolistomirkobiston koostumus varhaiseen käyttäytymiseen tai aivojen kehittymiseen ihmisillä.

Tässä väitöskirjassa kartoitettiin FinnBrain-syntymäkohorttitutkimuksessa lapsen varhaisen mikrobiston yhteyksiä temperamenttiin, kasvoihin ja kasvojen ilmeisiin kohdistuvaan kognitiiviseen tarkkaavuuteen sekä mantelitumakkeen kokoon. Lisäksi väitöskirjassa tutkittiin äidin raskauden aikaisen pitkäaikaisten psyykkisten oireiden ja kortisolipitoisuuksien – joita käytettiin raskaudenaikaisen stressin mittareina tässä tutkimuksessa – yhteyksiä lapsen suolistomikrobiston koostumukseen ja monimuotoisuuteen.

Lapsen suolistomikrobiston koostumus oli yhteydessä temperamenttiin ja varhaiseen pelokkaisiin kasvoihin kohdistuvaan tarkkaavaisuuteen. *Bifidobacterium*, *Streptococcus*, *Atopobium* bakteerisuvut olivat yhteydessä positiiviseen emotionaalisuuteen, ja toisaalta *Clostridium* ja *Bifidobacterium* suvut olivat yhteydessä pelokkaisiin kasvoisiin kohdistuvaan tarkkaavaisuuteen. Sukupuoli vaikutti suolistomikrobiston ja temperamentin sekä lapsen kasvoihin kohdistuvan tarkkaavaisuuden välisiin yhteyksiin. Suolistomikrobiston vähäisempi monimuotoisuus oli yhteydessä suurempaan vasemman mantelitumakkeen kokoon ja voimakkaampaan negatiiviseen emotionaalisuuteen ja pelkoreagoivuuteen. Äidin raskaudenaikaisten stressi oli yhteydessä Proteobakteereihin kuuluvien sukujen ja maitohappobakteerien pitoisuuksiin.

Väitöskirjan löydökset tukevat väitettä, että suolistomikrobisto on yhteydessä aivojen kehitykseen ja käyttäytymiseen, mutta löydökset eivät vielä kerro taustalla olevista syy-seuraussuhteesta. Äidin raskaudenaikaisen stressin yhteydet lapsen suolistomikrobistoon vahvistivat jo aiemmin raportoituja löydöksiä.

AVAINSANAT: stressi, mikrobisto, suoli-aivoakseli, tarkkaavaisuus, temperamentti, mantelitumake

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Abbreviations

ADHD	Attention-deficit hyperactivity disorder
ASD	Autism spectrum disorder
BMI	Body mass index
CLR	Centered log ratio
C-section	Cesarean section
DP	Probabilities of disengagement
EPDS	Edinburgh Postnatal Depression Scale
FDR	False discovery rate
GABA	*gamma*-aminobutyric acid
GI	Gastrointestinal
GM	Gut microbiota
GWK	Gestational week
HCC	Hair cortisol concentration
HPA	Hypothalamus-pituitary-adrenal
IBQ-R SF	Infant Behavior Questionnaire Revised Short Form
IBS	Irritable bowel syndrome
IgA	Immunoglobulin A
MRI	Magnetic resonance imaging
OTU	Operational taxonomic unit
PERMANOVA	Permutational Analysis of Variation
PPD	Prenatal psychological distress
PRAQ-R2	Pregnancy-Related Anxiety Questionnaire Revised 2
QIIME	Quantitative Insights into Microbial Ecology
SCFA	Short-chain fatty acid
SCL	Symptom Checklist
SNRI	Serotonin-norepinephrine reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Aatsinki, A.K., Lahti, L., Uusitupa, H.M., Munukka, E., Keskitalo, A., Nolvi, S., O'Mahony, S., Pietilä, S., Elo, L.L., Eerola, E., Karlsson, H., Karlsson, L. Gut microbiota composition is associated with temperament traits in infants. *Brain, Behavior, and Immunity*, 2020; 80: 849-858.

II Aatsinki, A.K.*, Kataja E.L.*, Munukka, E., Lahti, L., Keskitalo, A., Korja, R., Nolvi, S., Häikiö, T., Tarro, S., Karlsson, L., Karlsson, H. Infant Fecal Microbiota Composition and Attention to Emotional Faces. *Emotion*, 2020; Advance online publication.

III Aatsinki, A.K.*, Tuulari J.T.*, Munukka, E., Lahti, L., Keskitalo, A., Uusitupa, H.M., Nolvi, S., Scheinin, N., Saunavaara, J., Parkkola, R., Lewis, J., Hashempour, N., Lehtola, SJ., Karlsson, L., Karlsson, H. Infant Left Amygdala Volume Is Negatively Associated with Fecal Microbiota Diversity. Submitted.

IV Aatsinki, A.K., Keskitalo, A., Laitinen, V., Munukka, E., Uusitupa, H.M., Lahti, L., Kortesluoma, S., Mustonen, P., Rodrigues A.J., Coimbra, B., Huovinen, P., Karlsson, H., Karlsson, L. Maternal prenatal psychological distress and hair cortisol levels associate with infant fecal microbiota composition at 2.5 months of age. *Psychoneuroendocrinology*, 2020; 119:104754.

*These authors had equal contribution

1 Introduction

The human gut has been attributed to several factors of health during human history and across cultures. In antique Greece, there was a prevalent view that all diseases arise from the gut, and they were ameliorated with dietetic regimes. In 16th-century Great Britain, doctors thought that the stomach was a source of "nervous sympathy" or energy due to the dense nervous innervations (Miller, 2018). By then, the doctors were alarmed, because intestinal problems seemed to have become more prevalent and more widespread across all the societal classes. Before the industrial revolution, intestinal problems were a characteristic of the well-being of the upper class, who had the luxury of overthinking and physical inactivity (Miller, 2018). The increased prevalence of intestinal problems was attributed to an altered diet in the urban environment, excessive consumption of strong tea and white bread, which was an especially pronounced problem in working-class women (Miller, 2018). This unbalance in the nervous sympathy, in turn, was thought to lead to extensive mental and emotional burden, hysteria, and mania and other types of misery.

During the Victorian times, the understanding of microbes was increasing. In 1898, D. R. Brower wrote about melancholia and intestinal microbiota in the Journal of American Medical Association (JAMA) (Brower, 1898). He suggested that low stomach acid content might promote microbial growth in the intestines and lead to a higher production of toxic products due to inadequate detoxification pathways in melancholic patients (Brower, 1898). On the other hand, in the early 1900's, Elie Metchnikoff observed a connection between longevity and consumption of fermented milk products known to contain probiotics in the Bulgarian population (Bested *et al.*, 2013). There was a great interest in the variety of bacterial products, such as drinks, capsules, and powders, which were tested even in clinical populations according to the scientific standards of those days (Bested *et al.*, 2013). Interestingly, Lactobacillus products were reported to alleviate symptoms of melancholic patients already back in 1910 (Phillips, 1910).

However, the "bacteriomania" of the early 20th century tailed off, and for the majority of 20th century, the bacteria were simply viewed as pathogens. However, the view was challenged during late 20th century and early 21st century, when the

scientific and medical community was alarmed with increasing rates of obesity (Vuorela *et al.*, 2011; Ogden *et al.*, 2012; Ng *et al.*, 2014), asthma (Lundbäck *et al.*, 2016), inflammatory bowel disease (Alatab *et al.*, 2020) among other non-communicable diseases. Often, the predisposition for these diseases is embedded in the early life, and the increased burden of non-communicable disease has been coupled with urbanization (Ezzati *et al.*, 2005) and the increasing use of antibiotic treatments (Klein *et al.*, 2018) among other factors. Interestingly, these factors or closely related factors (Allender *et al.*, 2011) are known to affect microbial populations (McCall *et al.*, 2020) in the human body or their immediate surroundings. Likewise, microbial disruptions may have more deleterious effects on infants, when the immune system is not fully developed. This observation has led to the theory that disappearing microbiota in our environment, and subsequent changes in human microbiota might contribute to the changing burden of disease (Blaser and Falkow, 2009).

Both the increased understanding of human microbiota characteristics and the relations to immune system functioning and metabolism as well as an increased appreciation of the immunological and neuroendocrinological underpinnings of psychiatric conditions have brought the intestinal microbiota back to the psychiatric and neuroscience discussions with momentum (Cryan *et al.*, 2019). Experimental animal studies have shown that the lack of microbiota or manipulation of microbiota in early life can impact rodent physiology, behavior, and brain chemistry (Bravo *et al.*, 2011; Diaz Heijtz *et al.*, 2011; Hoban *et al.*, 2018; Cryan *et al.*, 2019).

However, despite a wealth of experimental research on the so-called "microbiota-gut-brain axis," there is still a limited amount of research on humans. Studies in clinical populations, such as in patients with a major depressive disorder and Parkinson's disease, show alterations in gut microbiota composition (Scheperjans *et al.*, 2015; Kelly *et al.*, 2016; Cryan *et al.*, 2019), although the cross-sectional studies neither imply causation nor reveal underlying mechanisms. The risk for neurological and psychiatric disorders is often embedded in early life, and the early life and prenatal environment and exposures participate in the programming of the infant brain and behavior (O'Donnell and Meaney, 2017). The variation in the early brain and behavioral phenotypes may be indicative of different developmental trajectories, which may precede psychiatric disorders later in life (Qin *et al.*, 2014). However, despite the potential importance of early life brain and behavioral development, how early microbiota characteristics are related to brain and behavioral phenotypes in typically developing infants are largely unknown.

Likewise, maternal prenatal stress is one of the important contributors to child health and development (Sourander, 2016). Animal studies have shown that

maternal stress leads to alterations in offspring gut microbiota composition, and it has been hypothesized that the alterations in gut microbiota composition or function might be one of the mechanisms mediating the association between maternal prenatal stress and infant health and development (Hartman *et al.*, 2018). Whether prenatal maternal stress is related to early microbiota characteristics is still poorly understood in human infants. First, this study aimed at exploring how infant fecal microbiota is associated with brain developmental phenotypes. Second, this study aimed at investigating how prenatal maternal distress is associated with infant fecal microbiota characteristics. Understanding the biological underpinnings of prenatal distress exposure as well as early life brain developmental phenotypes may help to understand microbiota-gut-brain-axis functioning in infants and help to develop early preventive interventions.

2 Review of the Literature

2.1 Microbial Landscapes of the Human Body

The majority of surfaces in human body are colonized by microbial cells including bacteria, viruses, archaea, and fungi. Bacteria are the most abundant in biomass and hereafter microbiota mainly refers to bacteria, although viruses, including bacteriophages, might outnumber the bacteria (Gilbert *et al.*, 2018). The genetic material provided by bacterial cells in the gut outnumbers human genetic material a hundred-fold (Gilbert *et al.*, 2018). This rich community varies in composition depending on the body site, surrounding environment, and number of other internal and external factors (Falony *et al.*, 2016; Zhernakova *et al.*, 2016). The most studied body site thus far is the gut, and the most abundant members of gut microbiota belong to the Bacteroidetes and Firmicutes phyla, but the Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia phyla are also prevalent at least in western populations (Huttenhower *et al.*, 2012). However, there is a large body of unknown species in the gut, especially in the less studied non-western populations (Pasolli *et al.*, 2019). Variation in the gut microbiota is mostly derived from environmental sources, and the effect of genetics on gut microbiota composition is limited (Rothschild *et al.*, 2018). Nevertheless, certain taxa are highly heritable, such as *Christensenella* (Goodrich *et al.*, 2014), although the exact connection to genetic variation of this taxa has remained elusive (Waters and Ley, 2019).

In addition to extensive intra-individual variation, the gut microbiota presents both spatial and temporal variation. First, the intestinal microbiota differs along the length of the gastrointestinal (GI) tract (Tropini *et al.*, 2017), which is affected by the nutrient concentrations, pH, oxygen levels, gut transit time, and immune factors. The microbial density increases from the proximal to the distal gut, as the stomach content contains 10^1 microbial cells per gram, while the colon content contains 10^{12} microbial cells per gram (Sekirov *et al.*, 2010). Second, the microbiota in the gut differs cross-sectionally, i.e., the less abundant mucosal microbiota is distinct from the more abundant luminal microbiota (Ringel *et al.*, 2015; Tropini *et al.*, 2017). Regarding temporal variation, the adult gut microbiota is personalized and finger-print-like in stable conditions, although it is rapidly

affected by factors such as infections and dietary and environmental changes (Faith *et al.*, 2013; David *et al.*, 2014). There is also diurnal variation in gut microbiota, which is dependent both on host genetic elements determining the circadian rhythm as well as light exposure (Wu *et al.*, 2018), timing of feeding (Thaiss *et al.*, 2014), and quality of nutrient intake (Zarrinpar *et al.*, 2014) (Nobs *et al.*, 2019).

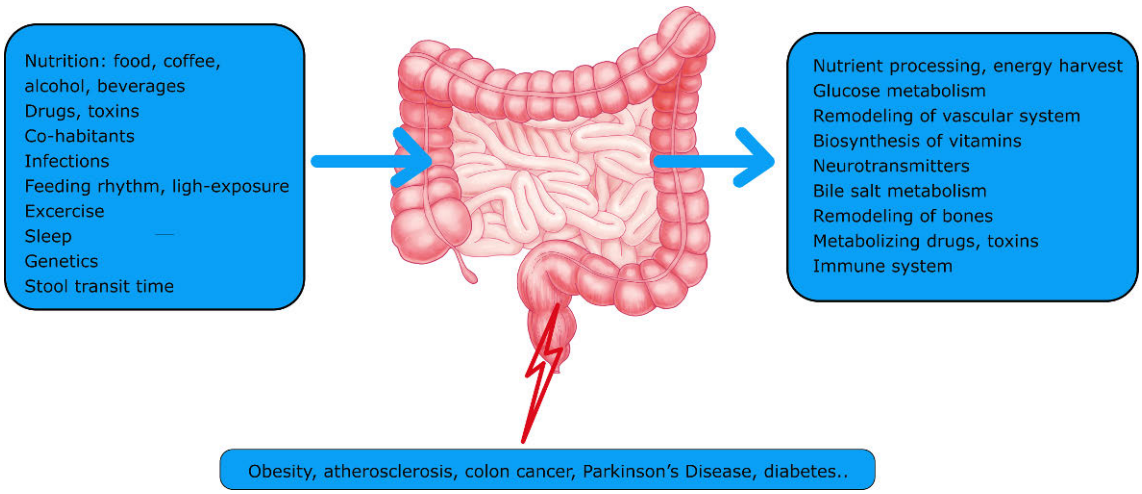

Figure 1. Gut microbiota is affected by multiple intrinsic and external factors and influences physiological processes and pathogenesis.

The gut microbiota is sometimes called the hidden organ (Fig. 1). The gut microbiota affects major physiological processes starting from immune system functioning, digestion of nutrients, and energy harvest (Hehemann *et al.*, 2010); host glucose metabolism (Vrieze *et al.*, 2012); remodeling of the vascular system (Reinhardt *et al.*, 2012) and bones (Sjögren *et al.*, 2012); biosynthesis of vitamins (Huttenhower *et al.*, 2012); neurotransmitters (Koenig *et al.*, 2011); bile salt metabolism (Sayin *et al.*, 2013); and metabolizing toxins and drugs (Rekdal *et al.*, 2019). As the human gut contains a large number of microbial cells, which are necessary to compliment host physiological processes, the host immune system needs to tolerate the complex microbial ecosystem (Maynard *et al.*, 2012). Many components, including the gut epithelium and gut secretions, participate in this process in addition to the immune system. First, there is the gut epithelium as a physical barrier, which prevents bacterial translocation to the systemic circulation (Wells *et al.*, 2011). In addition to that, gut secretions, such as immunoglobulins, antimicrobial peptides, and bilayer mucosa, inhibit the microbial overgrowth and/or prevent microbes from penetrating to the epithelial surface (Johansson *et al.*, 2008). Further, the gut microbiota interacts with both the innate and the adaptive

immune system. During the early life, the gut microbiota is necessary for the development of lymphoid structures, which are needed for appropriate adaptive immune responses (Bouskra *et al.*, 2008). The gut microbiota promotes the normal development of certain natural killer cells that are part of the innate immune system (Olszak *et al.*, 2012). Moreover, different gut microbes and/or their secretions can affect both the proinflammatory and the anti-inflammatory T-cell subpopulations, which participate in the microbial defense, intestinal barrier damage repair, and hence determine the overall immune homeostasis and also extra-intestinal immune reactions (Wu *et al.*, 2010; Lee *et al.*, 2011). The gut microbiota can also influence the secretion of soluble immune mediators, such as cytokines, chemokines, immunoglobulins (Kawamoto *et al.*, 2012) as well as antimicrobial peptides (Cash *et al.*, 2006). Consequently, the connections between the gut microbiota and host immune system functioning are bidirectional and complex (Maynard *et al.*, 2012).

Traditionally, characterization of gut microbiota has been performed with selective culturing methods, which is feasible for detecting, i.e., single pathogens. A modern version of culturing, so called "culturomics," facilitates multiple culturing media to mimic the original environment, and hence can capture species difficult to characterize otherwise (Lagier *et al.*, 2018). However, these methods are rather laborious to use in large-scale studies, and molecular methods are nowadays widely used to study microbiota. All bacteria and archea have a 16S small subunit ribosomal RNA gene, which is highly evolutionarily conserved but contain hypervariable regions (Morgan and Huttenhower, 2014). By amplifying these hypervariable regions with polymerase chain reaction followed by sequencing, one can have an estimate of proportions of detected bacteria (Morgan and Huttenhower, 2014). However, the 16s rRNA sequencing is prone to biases starting from sample collection, processing, choice of primers, and processing of sequencing data (Morgan and Huttenhower, 2014). Likewise, distinction of highly similar species is often not feasible, which often limits the reliable taxonomic annotation to genus level (Morgan and Huttenhower, 2014). In addition to the restricted taxonomic interpretation, the 16s rRNA sequencing does not inform about the functional potential. Shotgun sequencing of either the whole DNA (i.e., metagenomics) or RNA (i.e., metatranscriptomics) would yield information on the functional potential at the species- or gene level or the actual gene expression levels, but these methods do not come without biases or technical challenges (Morgan and Huttenhower, 2014). Additionally, the functional output of microbiota can be assessed with proteomic and targeted or untargeted metabolomic assessment that utilize chromatography and mass spectrometry (Morgan and Huttenhower, 2014). It has been argued that the functional output might be more

important regarding the microbiota-host interaction than the taxonomic profiles (Heintz-Buschart and Wilmes, 2018).

Molecular methods have been essential for studying the epidemiology of microbial communities in disease. Indeed, with the help of the modern molecular methods, gut microbiota characteristics and function have been implicated as a causative agent in the development of metabolic disease (Koh and Bäckhed, 2020), allergy (Feehley *et al.*, 2019); and also plays a mechanistic role in the pathogenesis of several conditions such as multiple sclerosis (Cekanaviciute *et al.*, 2017), colon cancer (Garrett, 2015), atherosclerosis (Jonsson and Bäckhed, 2017), and Parkinson's disease (Sampson *et al.*, 2016; Baizabal-Carvallo and Alonso-Juarez, 2020) (Fig. 1). Additionally, information about the underlying mechanisms may help to develop new interventions.

Although the gut microbiota-targeted therapies are still in their infancy, the fecal microbiota transplants are becoming more and more prevalent, and the most established indication is *Clostridium difficile* infection. Although the donors undergo rigorous screening, the potential risk is to transfer undetected disease risks in the form of bacterial communities. Aside from fecal microbiota transplantation, the gut microbiota has provided useful treatment targets as well as biomarkers for personalized medicine. For example, a dietary intervention that is designed to target specific microbial populations has helped to develop more successful regimes for both adults with insulin resistance (Zeevi *et al.*, 2015) as well as children with malnutrition (Gehrig *et al.*, 2019). Likewise, targeting gut microbiota has the potential to help to mitigate individual drug responses, such as the reduced efficacy and adverse effects related to levodopa-treatment in Parkinson's disease patients (Rekdal *et al.*, 2019). However, the application of the microbiota-targeted therapies is not yet widespread, although the preliminary results are promising.

2.1.1 Developing Microbiota

Early life is a turbulent time for microbiota development. The development of microbiota starts from birth, although there is contradictory evidence regarding the presence of bacterial DNA in the placenta or in the fetus, which is traditionally considered sterile (Aagaard *et al.*, 2014; Kuperman *et al.*, 2020; Olomu *et al.*, 2020; Rackaityte *et al.*, 2020). Right after delivery (<24h), the infant feces is characterized by a high diversity of species, but this is transient, and the following microbiota composition is characterized by low diversity and colonization with facultative anaerobic bacteria (Ferretti *et al.*, 2018). However, the facultative anaerobes, such as *Enterobacteriaceae*, are gradually replaced by strictly anaerobic bacteria, such as *Bifidobacterium* and *Lactobacillus* during the first weeks of life (Bäckhed *et al.*, 2015; Ferretti *et al.*, 2018). Vaginally born breastfed infants are

colonized with the genera *Bifidobacterium* and *Bacteroides* and show a reduced diversity of fecal microbiota, whereas formula-fed infants have a higher diversity, and caesarean section-(C-section)-born infants have higher relative abundances of *Enterobacteriaceae*, *Veillonella*, *Haemophilus*, *Staphylococcus*, and *Strepotococcus* (Yatsunenko *et al.*, 2012; Bäckhed *et al.*, 2015; Ferretti *et al.*, 2018; Stewart *et al.*, 2018). After the cessation of breastfeeding, the microbiota diversity and Firmicutes abundance increase (Stewart *et al.*, 2018). During the first three years of life, the Proteobacteria, Actinobacteria, are decreasing, and Firmicutes and Bacteroidetes are increasing in relative abundance (Bäckhed *et al.*, 2015; Stewart *et al.*, 2018). The later development during school age and teenage years is not as well understood. During later childhood and preadolescence, the microbiota still has lower diversity with a higher *Bifidobacterium* abundance (Hollister *et al.*, 2015; Cheng *et al.*, 2016) compared to adults. Hence, it seems that the microbiota undergoes rapid early development, but continues to evolve towards adult-like microbiota thorough childhood.

The development of microbiota is associated with prenatal, obstetric, environmental, and infant factors (Fig. 2). Maternal prenatal health factors, such as antibiotic intake (Zhang *et al.*, 2019), gestational weight gain (Baumann-Dudenhoeffer *et al.*, 2018), pre-pregnancy obesity (Stanislawski *et al.*, 2017; Singh *et al.*, 2019), diet (Lundgren *et al.*, 2018), and medical conditions such as gestational diabetes (Su *et al.*, 2018; Wang *et al.*, 2018), inflammatory bowel disease (Torres *et al.*, 2019; Van Der Giessen *et al.*, 2019), and preeclampsia (Hu *et al.*, 2019) associate with differences in infant microbiota composition. Maternal vaginal and fecal microbiota seem to transiently change during pregnancy (Koren *et al.*, 2012; Aagaard *et al.*, 2014; DiGiulio *et al.*, 2015; Rasmussen *et al.*, 2020). These changes potentially impact the energy harvesting capacity of the gut microbiota (Koren *et al.*, 2012) and improve immune tolerance (Blaser and Dominguez-Bello, 2016), which are necessary processes for optimal fetal development and successful pregnancy. The importance of maternal microbiota colonization was highlighted by a rodent model, as a pregnant dam was colonized with non-pathogenic microbes, which affected the innate immune system of the offspring (Gomez de Agüero *et al.*, 2016). The prenatal colonization ameliorated the inflammatory response during early postnatal colonization of the offspring (Gomez de Agüero *et al.*, 2016). In all, maternal prenatal microbial and immune dysregulation seem to affect infant immune development and potentially future microbial colonization (Gomez de Agüero *et al.*, 2016; Hu *et al.*, 2019).

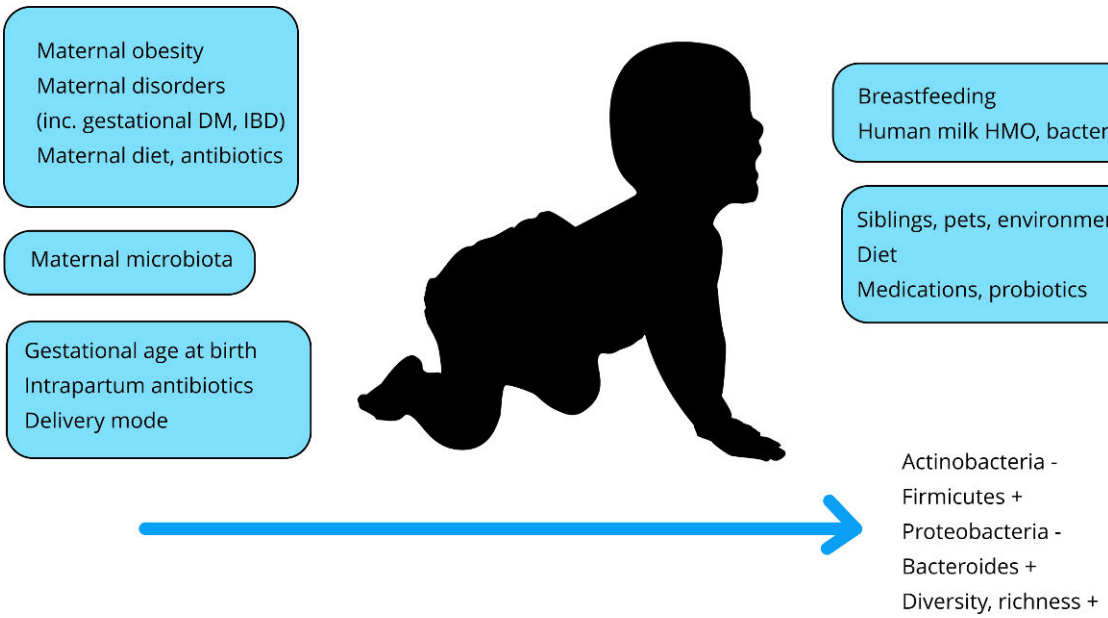

Figure 2. Infant gut microbiota goes thorough rapid colonization during early childhood and the microbiota shifts from the dominance of facultatively anaerobic bacteria to anaerobic bacteria. Alterations in infant fecal microbiota are associated with maternal and obstetric factors as well as nutrition, medications, and surroundings. The maternal fecal microbiota is a potential source of initial colonizers for infant microbiota.

Birth mode, duration of gestation, and infant nutrition have been associated with long-term differences in infant gut microbiota (Ardissone *et al.*, 2014; Bäckhed *et al.*, 2015; De Leoz *et al.*, 2015; Wampach *et al.*, 2018; Fouhy *et al.*, 2019; Reyman *et al.*, 2019; Shao *et al.*, 2019; Galazzo *et al.*, 2020; Le Goallec *et al.*, 2020). It seems that birth by caesarean section decreases *Bacteroides* abundance and potentially disrupts the vertical transmission of maternal gut/rectal *Bacteroides* species to the infant (Rutayisire *et al.*, 2016; Stewart *et al.*, 2018; Shao *et al.*, 2019). In a recent preprint article, Mitchell et al. show that C-section born infants had high initial abundance of *Bacteroides*, which readily decreased after the first week, and the *Bacteroides* strains were less often from maternal origin (Mitchell *et al.*, 2020). Despite the wealth of evidence, it has been also hypothesized that the effects of birth mode on infant microbiota might be attributed to confounding factors, such as maternal obesity, breastfeeding, onset of labor, and antibiotic administration rather than the birth mode itself (Stinson *et al.*, 2018). Nevertheless, Mitchell et al. did not observe associations between C-section and any potential confounders (Mitchell *et al.*, 2020), and the pivotal role of contact to maternal fecal microbiota during the delivery was highlighted in a recent fecal microbiota transplantation study by Korpela and colleagues (Korpela *et al.*, 2020). They

showed that early maternal fecal microbiota transplantation restored the typical *Bacteroides* and *Bifidobacterium* colonization in C-section-born infants (Korpela *et al.*, 2020). The evidence hence cements the role of delivery mode on infant fecal microbiota development, which may have important developmental implications.

In addition to delivery, early nutrition is likewise important for the development of fecal microbiota. This was illustrated recently, when Stewart et al. showed in a large TEDDY-cohort that the driver of microbiota maturation is the cessation of breastfeeding (Stewart *et al.*, 2018). On the other hand, even a brief ingestion of formula during infancy may cause small changes in the infant microbiota (Forbes *et al.*, 2018). Human milk contains components important for the intestinal microbiota, such as indigestible oligosaccharides, which act as prebiotic antimicrobial and antiadhesive agents, and they affect epithelial and immune cell function (Bode, 2015). Further, breast milk as well as surrounding areolar skin contain microbes. The human milk oligosaccharides (Zivkovic *et al.*, 2011; Ramani *et al.*, 2018) as well as breast milk and areolar microbiota (Pannaraj *et al.*, 2017) affect the composition of infant fecal microbiota.

Interestingly, factors affecting microbiota composition, such as duration of gestation, antibiotic treatments, early nutrition, and mode of delivery are associated with child development and varied health outcomes (Owen *et al.*, 2005; Li *et al.*, 2013; Mueller *et al.*, 2015; Miller *et al.*, 2018; Zou *et al.*, 2020). It has been suggested that alterations in the infant gut microbiota could be one mediating factor behind these observations. Indeed, alterations in early gut microbiota communities have been linked with later obesity (Korpela *et al.*, 2017), asthma (Stokholm *et al.*, 2018), recurrent infections (Reyman *et al.*, 2019), and development of type 1 diabetes (Vatanen *et al.*, 2018). Preliminary reports have showed some evidence for a causative role of microbiota in certain pediatric conditions. For instance, C-section may increase *Enterococcus* and *Klebsiella* in favor of *Bifidobacterium*, and the same changes associate with respiratory infection probability (Reyman *et al.*, 2019). Further, C-section (Tun *et al.*, 2018), breastfeeding (Forbes *et al.*, 2018), and prenatal antibiotic treatments (Zhang *et al.*, 2019) may affect the development of becoming overweight or gaining adiposity via microbiota alterations. Furthermore, the microbiota has been shown to be a mechanistic factor in the development of food allergy, and a healthy microbiota protects against anaphylactic response to a cow's milk allergen (Feehley *et al.*, 2019). In all, emerging studies show that the development of the microbiota, the immune system, and future health is a complex system that is affected by multiple interrelated prenatal and early life factors. Despite the complexity, the early microbiota is a potential intervention target as suggested by the preliminary mediation analyses in human populations as well as experimental models.

2.2 Microbiota-Gut-Brain Axis

As the role of microbiota as a key regulator of immunity and metabolism is starting to be more and more evident, the relationships between brain functioning, behavior, and microbiota have started to unravel (Cryan *et al.*, 2019). Rodent models have shown that a lack of microbiota in early life, i.e., germ-free conditions lead to altered behavior (Diaz Heijtz *et al.*, 2011), social functioning (Desbonnet *et al.*, 2014), and differences in fear-related neurobiological responses and behavior (Hoban *et al.*, 2018). Likewise, an absence of microbiota leads to alterations in the levels of neurochemicals including differences in neurotransmitter and neurotrophic factor levels (Bercik *et al.*, 2011; Clarke *et al.*, 2013), increased permeability of the blood-brain barrier (Braniste *et al.*, 2014), and differences in gene expression regulation in brain areas relevant for learning from emotionally salient information (Stilling *et al.*, 2015; Hoban *et al.*, 2017). Moreover, the absence of microbiota may even override the genetic influences on social behavior (Gacias *et al.*, 2016). The germ-free conditions do not have a practical human counterpart, but the differences in behavior and brain-related phenotypes are not limited to germ-free conditions. Additionally, dietary interventions and non-absorbing antibiotic and probiotic treatments have been shown to cause behavioral and neurochemical changes (Sudo *et al.*, 2004; Desbonnet *et al.*, 2010; Messaoudi *et al.*, 2010; Bravo *et al.*, 2011; Burokas *et al.*, 2017; Schmidtner *et al.*, 2019). Interestingly, some of the behavioral or brain-related changes related to microbiota disruptions are reversible by colonization with microbiota during early life (Diaz Heijtz *et al.*, 2011; Buffington *et al.*, 2016; Leclercq *et al.*, 2017) but not necessarily anymore during adulthood (Neufeld *et al.*, 2011) or adolescence (Ogbonnaya *et al.*, 2015). This indicates that the early life microbiota is also important for the behavioral outcomes.

2.2.1 Experimental Models Suggest Bi-Directional Gut-Brain Communication Via Multiple Mechanisms

The microbiota-gut-brain-axis functioning acts via multiple physiological routes (Fig. 3). As mentioned earlier (Chapter 2.1), the microbiota and immune system have a tight crosstalk, as immune system functioning modulates gut microbiota and gut microbiota modulates multiple aspects of both the innate and adaptive immune system (Huh and Veiga-Fernandes, 2020). As can be expected, immune system functioning plays a critical role in brain functioning, including cognition, (Zhu *et al.*, 2011; Brombacher *et al.*, 2017), social behavior (Filiano *et al.*, 2016), and brain connectivity (Zhan *et al.*, 2014). The immune system functioning modulation via altering the microbiota is hence a potent route of action in the microbiota-gut-brain-axis communication.

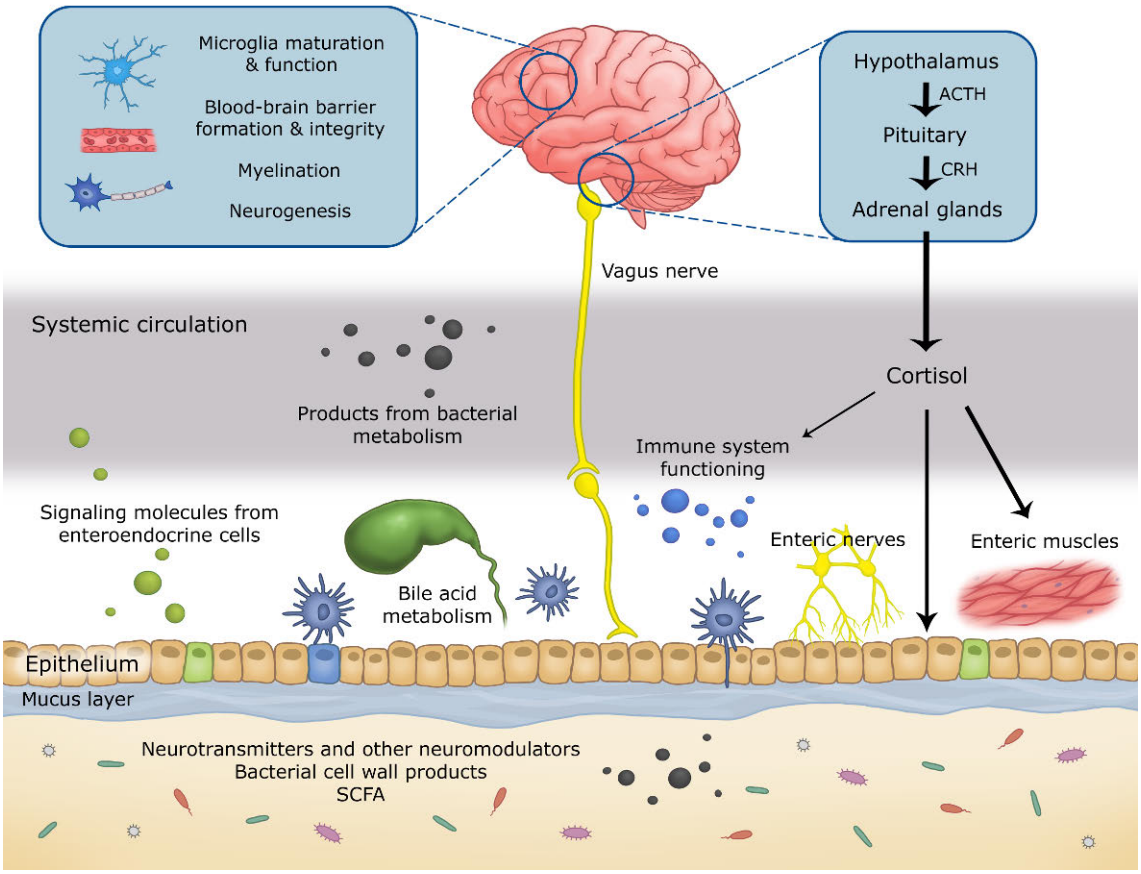

Figure 3. Compromised gut microbiota can affect neurodevelopmental processes, including microglial maturation and function, blood-brain barrier integrity, myelination, and neurogenesis in germ-free models. The microbiota-gut-brain-axis signaling may be mediated via neuronal signaling, immune system functioning, and metabolites from microbiota or host-microbe interactions, e.g., tryptophan. On the other hand, the immune system, the autonomic nervous system, and HPA-axis functioning can affect the intestinal microbiota composition. ACTH: adrenocorticotrophic hormone; CRH: corticosteroid-releasing hormone; SCFA: short-chain fatty acid.

Further, the microbiota or the products of microbiota-host interactions may affect the nervous systems innervating the GI tract. First, the autonomic nervous system controls the gut motility, permeability, secretions, bile metabolism, immune system functioning, and mucus production, which have potential to modulate the microbiota composition (Calcagni and Elenkov, 2006; Wehrwein *et al.*, 2016). The vagus nerve is an important part of the autonomic nervous system. It consists of 80% of afferent neurons conveying hormonal, chemical, mechanical, and nociceptive information derived from a variety of different receptors in the intestines to the central nervous system (Berthoud *et al.*, 2004; Egerod *et al.*, 2018).

The importance of the vagus nerve in gut-brain signaling has been studied in subdiaphragmatic deafferentation and vagus nerve-stimulation studies. Rodents subjected to subdiaphragmatic deafferentation, i.e., rodents deprived from visceral bottom-up signaling from the intestines exhibit decreased anxiety-like behavior. The behavioral changes are concurrent with changes in limbic system neurotransmitter levels (Klarer *et al.*, 2014). Subdiaphragmatic vagotomy increases dopamine levels and impairs attentional control of associative learning (Klarer *et al.*, 2018). On the other hand, vagus nerve stimulation has a variety of central effects, including central reward system activation (Han *et al.*, 2018), increased neural plasticity, increases in serotonin and noradrenalin levels, and simultaneous antidepressant-like behavioral effects (Grimonprez *et al.*, 2015). This underlines the potential role of sensory signaling, regardless of the microbiota composition or modulation, in behavior.

In addition to the vagotomy and vagus nerve-stimulation studies, the role of the vagus nerve in the microbiota-gut-brain-axis signaling has been studied, as several effects of microbiota modulation depend on the intact vagus nerve (Bercik *et al.*, 2011; Bravo *et al.*, 2011). The gut microbiota can activate the vagal afferents and sensory nuclei in the brain stem (Muller *et al.*, 2020). Although the exact mechanism of action is unknown, it has been shown that microbiota metabolites, such as indole (Jaglin *et al.*, 2018), short-chain fatty acids (Lal *et al.*, 2001; De Vadder *et al.*, 2014; Goswami *et al.*, 2018), and certain microbes themselves activate the vagus nerve or interneurons (Perez-Burgos *et al.*, 2013; Perez-Burgos *et al.*, 2014). This can happen directly or via epithelial cells (Muller *et al.*, 2020) or secondary neuromodulators (Jameson *et al.*, 2020).

The enteric nervous system consists of a submucosal and myenteric plexus and, in conjunction with autonomic nervous system, it controls the gut motility and fluid movement. The enteric nervous system interacts with sympathetic ganglia and hence potentially connects with the central nervous system (Furness, 2012). The gut microbiota is critical for the maturation of the enteric nervous system, potentially via serotonin signaling in the gut (De Vadder *et al.*, 2018). The enteric nervous system displays various receptor types, which are sensitive for microbial metabolites (Mao *et al.*, 2013; Hyland and Cryan, 2016). On the other hand, normal enteric nervous system functioning is necessary to maintain microbiota without excessive proinflammatory effects (Rolig *et al.*, 2017). Despite the abovementioned connections, colonic innervation density is not necessarily related to differences in intestinal microbiota composition (Golubeva *et al.*, 2015). An interesting notion from a developmental perspective is that enteric nervous system neuronal density decreases after infancy (Burns and Thapar, 2013). Based on this, it could be hypothesized whether this reduction may play a role in the critical

developmentally sensitive time during infancy, when gut-microbiota interventions may reverse otherwise subsequent behavioral outcomes.

Enteroendocrine cells in the epithelium are responsible for sensing the luminal nutrient content and secrete hormones in response to them (Moran-Ramos *et al.*, 2012), and the effect of diet on enteroendocrine cells is dependent on the microbiota (Ye *et al.*, 2019). The enteroendocrine cells have direct connections with neurons, and the secreted hormones may affect the central nervous system functioning, such as food intake (Latorre *et al.*, 2016). Enterocromaffin cells produce serotonin in large quantities, and the biosynthesis is promoted by gut microbiota (Yano *et al.*, 2015). These enterocromaffin cells may synapse with vagal afferents (Bellono *et al.*, 2017), and hence, potentially signal to the central nervous system.

Apart from controlling serotonin production, the gut microbiota has long been known to produce and respond to signaling molecules that also function as neurotransmitters (Lyte and Ernst, 1992, 1993; Yano *et al.*, 2015; Hata *et al.*, 2017; Kennedy *et al.*, 2017). Interestingly, it seems that certain microbes may utilize the neurotransmitter *gamma*-aminobutyric acid (GABA) or serotonin as a growth substrate (Strandwitz *et al.*, 2018; Fung *et al.*, 2019), but it can be expected that the interactions between neurotransmitters and microbes are even more diverse. Furthermore, the microbial product, short-chain fatty acid acetate, crosses the intestinal barrier as well as blood-brain barrier and increases central GABA production (Frost *et al.*, 2014). However, it is not conclusively known how the neurotransmitters affect microbiota, and how microbiota affect the neurotransmitter levels, and in turn, what are the central effects of these interactions (Jameson *et al.*, 2020).

Apart from neurotransmitters, the microbiota is known to metabolize and utilize amino acids and hence have an impact on the subsequent absorption of amino acids (Neis *et al.*, 2015). The most interesting amino acids are the branched-chain amino acids, as they are more abundant in gut microbes compared to other amino acids (Neis *et al.*, 2015), and they potentially play a role in neurotransmitter synthesis and food intake (Sperringer *et al.*, 2017). The role of amino acids was studied in a rodent model investigating a ketogenic diet and epilepsy. A ketogenic diet is a dietary intervention long known to alleviate refractory epilepsy, but the mechanism has been only speculated. The treatment effect was recently shown to depend on the gut microbiota (Olson *et al.*, 2018), and specifically, the effect of a ketogenic diet is mediated by a decrease in *gamma*-glutamyl amino acid both in the gut lumen and systemic circulation (Olson *et al.*, 2018). This indicates that microbiota-directed interventions may have effects in the central nervous system via alterations in amino acid metabolism.

Bile acids are synthesized in the liver and metabolized by gut microbiota to secondary bile acids, but the gut microbiota also inhibits the bile acid synthesis (Sayin *et al.*, 2013). Interestingly, some bile acid metabolizing microbes are associated with social behavior in a rodent model (Golubeva *et al.*, 2017). The potential downstream effects may be mediated via activation of farnesoid X receptor (FXR) and Takeda G protein-coupled receptor 5 that stimulate the release of fibroblast growth factors and glucagon-like peptide 1, which both can signal to the central nervous system (Mertens *et al.*, 2017).

The most investigated group of microbial products is the short-chain fatty acids (SCFA), such as butyrate, propionate, acetate, and formate, which are fermentation products of complex carbohydrates (Dalile *et al.*, 2019). Increased fiber intake increases the fecal SCFA levels and especially butyrate and abundances of carbohydrate degraders and SCFA producers, such as bifidobacteria and *Eubactrium rectale* (Baxter *et al.*, 2019). Nonetheless, it is challenging to interpret whether increased fecal concentrations of SCFA are due to increased production, decreased absorption, or a combination thereof. They are absorbed to the portal and systemic circulation and may cross the blood-brain barrier and thus have both local and systemic effects. SCFAs activate free fatty acid receptors, and G-protein-coupled receptors and inhibit histone deacetylases (Dalile *et al.*, 2019), and hence modulate immunity, metabolism, and epigenetic programming (Krautkramer *et al.*, 2016). Different SCFA have different structural properties, and they differ regarding receptor binding, and hence may have different functional effects (Dalile *et al.*, 2019). However, drawing distinct conclusions about specific SCFA is problematic, as the majority of studies concentrate on one individual SCFA or supplement a cocktail of multiple SCFA. Nevertheless, evidence suggests that both butyrate and propionate supplementation enhances the blood-brain barrier integrity (Braniste *et al.*, 2014; Hoyles *et al.*, 2018), whereas butyrate enhances intestinal barrier integrity (Peng *et al.*, 2009). Locally, propionate and butyrate activate vagal afferents (Lal *et al.*, 2001; De Vadder *et al.*, 2014; Goswami *et al.*, 2018), and hence, may influence central functions, such as food intake (Goswami *et al.*, 2018). Centrally, butyrate and propionate may alter gene expression affecting neurotransmitter levels (Nankova *et al.*, 2014). Likewise, a SCFA cocktail containing propionate, butyrate, and acetate restored defective microglia in germ-free mice (Erny *et al.*, 2015). Despite the supposed positive effects, administration of a mix of acetate, propionate, and butyrate may also exacerbate the clinical and inflammatory phenotype of a rodent model predisposed to Parkinson's disease (Sampson *et al.*, 2016). Hence, although SCFAs are putative intervention targets, the relationships between their humoral, immunological, and neural pathways and host developmental outcomes need further unraveling (Dalile *et al.*, 2019).

Aside from microbial products, bacterial cell wall and outer membrane molecules can have a potential to affect the developing brain and behavior (Arentsen *et al.*, 2018; Gonzalez-Santana and Diaz Heijtz, 2020). A bacterial cell wall consists of multilayered peptidoglycans in Gram-positive bacteria, whereas in Gram-negative bacteria, the peptidoglycan layer is relatively thin and surrounded by an outer membrane containing lipopolysaccharides. Peptidoglycans, lipopolysaccharides, and other molecular motifs are sensed by pattern recognition receptors (i.e., Toll-like receptors and cytosolic NOD-like receptors) that are part of the innate immune system (Clarke *et al.*, 2010; Gonzalez-Santana and Diaz Heijtz, 2020). Parts of the cell wall and outer membrane, such as polysaccharides, may have direct neuronal effects (Mao *et al.*, 2013). Peptidoglycan transporters are even expressed in the developing brain, and peptidoglycan-sensing molecules are sensitive for microbiota modulation (Swann *et al.*, 2020). Interestingly, the bacterial cell wall may cross the placenta to the fetal brain and lead to neuronal proliferation and subsequent cognitive deficits (Humann *et al.*, 2016).

The hypothalamus-pituitary-adrenal (HPA) axis is the major neuroendocrine system controlling the output of cortisol. Cortisol is a key regulator of glucose, protein, and lipid metabolism and immune responses (Bellavance and Rivest, 2014; Horowitz and Zunszain, 2015), and hence may affect the microbiota composition. The HPA-axis functioning is intertwined with the gut microbiota, as germ-free mice have an exaggerated HPA-axis response that can be attenuated by early colonization with *Bifidobacteria* (Sudo *et al.*, 2004). Although not thoroughly investigated, pro-inflammatory reactions to gut pathogens may activate the HPA axis, and the HPA-axis response to inflammation is dependent on blood-brain barrier endothelial cells encoding Toll-like receptor 4 (Gosselin and Rivest, 2008).

In all, there is wealth of evidence on the microbiota-gut-brain-axis signaling (Cryan *et al.*, 2019). The proposed mechanisms include immune, metabolic, and neuronal pathways that affect functions such as food intake, social behavior, fear processing, and cognition. However, the evidence for the direct or indirect signaling pathways is still under vigorous research. Understanding the exact mechanisms behind microbiota-gut-brain-axis communication is necessary for future development of interventions.

2.2.2 Observational Studies Suggest Microbiota-Gut-Brain Axis Communication in Humans

While building the body of literature on rodent gut-brain-axis functioning, it is imperative to translate the rodent findings to humans. Irritable bowel syndrome (IBS) and other functional bowel disorders are most prominently viewed as disorders of the gut-brain axis. The heterogenous pathophysiology of IBS includes

alterations in gastrointestinal motility, visceral hypersensitivity, altered gut microbiota, and intestinal permeability as well as immune activation (Simrén and Tack, 2018). Different fecal microbiota community types in IBS patients seem to associate with differences in brain structure (Labus *et al.*, 2017). It has been hypothesized that functional gastrointestinal disorders could be preceded by infantile colic, however, the literature, nevertheless, is mixed in terms of the longitudinal findings (Partty *et al.*, 2013; Zeevenhooven *et al.*, 2018; Sjölund *et al.*, 2020). The hypothesized pathophysiology of infantile colic also includes alterations in gut microbiota, visceral sensitivity, permeability, and inflammation (Rhoads *et al.*, 2009; Eutamène *et al.*, 2017; Pärtty *et al.*, 2017; Zeevenhooven *et al.*, 2018; Loughman *et al.*, 2020b). Albeit controversial, it has been suggested by several studies that infantile colic or other infant regulatory problems might be related to socioemotional as well as cognitive development, especially when persistent (Rautava *et al.*, 1995; Rao *et al.*, 2004; Hemmi *et al.*, 2011; Clara *et al.*, 2017). Specifically, infant colic might be related to later negative emotional reactivity (Canivet *et al.*, 2007). In all, it is important to note that infantile colic usually resolves after the first four months of life (Zeevenhooven *et al.*, 2018), and the literature related to self-limited and persistent infantile colic and later outcomes is mixed in terms of somatic, cognitive, and behavioral outcomes (Zeevenhooven *et al.*, 2018). Although colic and excessive crying are common problems causing distress to the families, due to the usually benign and self-resolving nature and unconclusive evidence related to the long-term outcomes of these conditions, this Review of the Literature focuses on other behavioral, cognitive, and emotional phenotypes.

Regarding psychiatric disorders, effort has been put to study major depressive disorder and gut microbiota. However, the microbiota alterations observed in major depressive disorder patients are divergent from study to study, although typically increases in the genera *Blautia, Clostridium, Klebsiella, Parabacteroides*, and decreases in *Bifidobacterium, Dialister, Escherichia, Faecalibacterium,* and *Ruminococcus* are noted (Cheung *et al.*, 2019). A recent large population-based study that investigated the associations between gut microbiota and reported major depressive disorder diagnosis and quality of life found potential metabolic signatures related to major depressive disorder / quality of life assessments (Valles-Colomer *et al.*, 2019). *Coprococcus* and *Dialister* were reported to be lower in major depressive disorder patients, and on the other hand, abundances of the SCFA-producers *Faecalibacterium* and *Coprococcus* were positively associated with quality of life. Using a computational approach, they revealed that microbes capable of synthesizing certain dopamine metabolites were associated with quality of life (Valles-Colomer *et al.*, 2019). The insight into this potential pathway hopefully inspires further mechanistic work. Unfortunately, although having access

to a rich metadata including dietary factors and other medications (Falony *et al.*, 2016), these potential important confounders were not considered in the study design. A recent, albeit possibly underpowered, study suggested that the association between major depressive disorder and the variation in overall community composition may be confounded by lifestyle factors, such as alcohol consumption, diet, and geography (Vujkovic-Cvijin *et al.*, 2020). Nonetheless, despite the potential confounders, the microbiota has been suggested to be one mechanistic agent in rodent studies. A fecal microbiota transplantation of human microbiota seems to transfer some behavioral changes including anhedonia as well as anxiety-like behavior, as the recipient rodents showed less exploration in an open field and open arms in an elevated plus maze (Kelly *et al.*, 2016), although the effects of microbiota cannot be differentiated from other factors in the stool.

In addition to clinical depression, other psychiatric disorders are suggested to associate with microbiota as well. Acute infections are speculated to increase the risk for acute mania (Yolken *et al.*, 2016), and in similar vein, there is preliminary evidence that bipolar disorder might be related to gut microbiota diversity, as euthymic patients have higher alpha diversity than depressed adult patients (Bengesser *et al.*, 2019). Moreover, schizophrenia, also often comorbid with gastrointestinal problems, is characterized by altered in gut microbiota community that are able to increase glutamate and decrease glutamine and GABA in the hippocampus in a fecal microbiota transplantation model (Zheng *et al.*, 2019). Nonetheless, literature related to bipolar and psychotic disorders is more limited than the literature related to major depressive disorder.

The rodent models on autism-like phenotypes and gut microbiota have given rise to several studies investigating fecal microbiota of the pediatric and adolescent patients with autism spectrum disorder (ASD) (Bundgaard-Nielsen *et al.*, 2020). The studies, albeit heterogenic regarding methodology and age groups, have yielded some consistent results regarding the differential abundance of gut-residing taxa. It seems that the overall microbiota composition differs between ASD patients and healthy controls, and there is an increase in abundance of taxa from the Proteobacteria phylum and *Bacteroides* genus and a decrease in the *Dialister* genus in ASD-patient fecal microbiota (Bundgaard-Nielsen *et al.*, 2020). However, autism is typically comorbid with gastrointestinal symptoms and adjusting for the bowel movements may be an important confounder often lacking from the study designs (Vujkovic-Cvijin *et al.*, 2020). Moreover, a similar agreement has not been found regarding attention-deficit hyperactivity disorder (ADHD) (Bundgaard-Nielsen *et al.*, 2020).

Apart from the abovementioned distinct disorders, recent studies have reported associations between fecal microbiota and socioemotional and cognitive functioning as well as brain structural and functional differences during infancy or

childhood (Table 1). Temperament, i.e., the individual differences in reactivity and self-regulation (see Chapter 3.1), has been associated with a variety of differences in early microbiota composition and diversity (Christian *et al.*, 2015; Wang *et al.*, 2020). *Bacteroides*, which is related to the delivery mode (Stewart *et al.*, 2018), has been implicated with cognitive development with partially mixed results. The receptive and expressive language skills in two-year-olds have been positively associated with *Bacteroides* dominance in infant microbiota when adjusting for birth mode and breastfeeding among other covariates (Carlson *et al.*, 2018). Likewise, functional reactivity to sad faces in the prefrontal cortex in 5- to 11-year-olds is reported to have a positive association with *Bacteroides* dominance in fecal microbiota (Callaghan *et al.*, 2019). On the other hand, poorer fine motor development at the age of three years and more self-rated negative emotions and parent-rated emotional problems during later childhood and preadolescence are reported to associate with increased *Bacteroides* abundance in early life and later childhood or preadolescence (Carlson *et al.*, 2018; Callaghan *et al.*, 2019; Sordillo *et al.*, 2019).

In addition to *Bacteroides*, *Clostridium*, and *Prevotella* have been reported to associate with behavioral and emotional development during childhood. An increased abundance of *Clostridium*-annotated taxa has been linked with increased externalizing and/or internalizing problems in two-year-olds, poorer communication, personal, and social skills in three-year-olds but more self-rated positive emotions during later childhood and preadolescence (Michels *et al.*, 2019; Sordillo *et al.*, 2019; Loughman *et al.*, 2020b). A decreased abundance of *Prevotella* in two-year-olds' fecal microbiota was associated with internalizing and/or externalizing problems at the age of two years but not with temperament during the first year of life (Loughman *et al.*, 2020a).

Furthermore, increased alpha diversity has been linked with reduced functional connectivity between the amygdala and thalamus and between the anterior cingulate cortex and anterior insula and increased functional connectivity between the supplementary motor area and the inferior parietal lobule at the age of one year (Gao *et al.*, 2019). Further, greater alpha diversity at the age of one year is associated with worse expressive language and visual reception capabilities at the age of two years and larger volumes of left precentral gyrus and right angular gyrus at the age of two years (Carlson *et al.*, 2018; Gao *et al.*, 2019).

Unfortunately, only one study thus far has facilitated a measure of functional output of gut microbiota in the assessment, which is beneficial, as the functional output is likely more important for host outcomes than mere taxonomic differences (Heintz-Buschart and Wilmes, 2018). Loughman and colleagues reported no associations between SCFA concentrations in feces and infant behavioral problems (Loughman *et al.*, 2020a). Likewise, only a recent preprint has facilitated a more

in-depth sequencing approach to study infant gut microbiota and neurodevelopment. Bonham et al. concluded that microbial potential to metabolize GABA and glutamate was related to cerebellum microstructure and overall cognitive development. Genes related to GABA synthesis, which were mostly found in *E. coli* and different *Bacteroides* species, were negatively associated with cerebellar volumes and cognitive function, while genes related to GABA degradation, mostly seen in *E. coli*, were also related negatively to cognitive function (Bonham *et al.*, 2020). On the other hand, both glutamate synthesis and degradation seemed to negatively associate with cognitive development, and the genes were dispersed among variety of taxa, including *B. longum* and *F. prausnitzii* (Bonham *et al.*, 2020).

The studies investigating different aspects of socioemotional, behavioral, or brain development show high variability in the assessment of phenotypes as well as the age range of the study subjects. Likewise, and potentially consequently, the preliminary results show great divergence regarding microbial signatures, although the abundances of *Bacteroides*, *Clostridium*, and *Prevotella* seem to repeatedly associate with child socioemotional or brain developmental phenotypes, although with partially differing effects of direction. In all, drawing inferences about the potential microbiota-gut-brain-axis functioning in the context of socioemotional and brain development during infancy ARE problematic. Although animal work on the microbiota-gut-brain axis has shed light on multiple routes of action, there is a paucity of studies showing how this translates to humans and especially infants. There are several studies investigating the associations between fecal microbiota composition and neurological disorders, such as multiple sclerosis and amyotrophic lateral sclerosis, which bridge the gap between the associations and the potential mechanisms linking the associations (Cekanaviciute *et al.*, 2017; Blacher *et al.*, 2019). Unfortunately, similar rigorous study designs have not been utilized when studying associations between fecal microbiota and cognitive, emotional, or behavioral development in early life, at least to date. This issue could be alleviated by utilizing functional output of gut microbiota (Heintz-Buschart and Wilmes, 2018) in addition to gut microbiota composition characterization. Similarly, describing the role of nutrition, medication, physical activity, and other factors affecting the potential microbiota-gut-brain-axis functioning would be informative. The more thorough investigation could also infer the external factors potentially underlining the certain associations accounted for the altered microbiota-gut-brain-axis functioning. Integrating these aspects in the future studies would enhance the reliability and interpretation of the biological significance of the findings.

Table 1. Summary of studies published in 2015-2020 investigating behavioral, cognitive, and brain structural or functional phenotypes and fecal microbiota profile during infancy or childhood in a general population. Infantile colic is not included, as it is typically self-resolving, and the evidence regarding long-term outcomes of infantile colic is unconclusive.

Phenotype	Microbiota	Age	Sample	Main findings	Reference
Temperament (mother-rated Early Childhood Behavior Questionnaire)	Pyro-sequencing	18–27 mo	77 (41 boys)	Surgency was associated positively with alpha diversity. In boys, subscales of Surgency were positively associated with alpha diversity, and abundances of Dialister, Rikenellaceae, and Parabacteroides. Subscales of Surgency were associated also with beta diversity. In girls, Effortful Control was associated negatively with alpha diversity, and fear reactivity was associated with differences in beta diversity and an increase in Rikenellaceae. Dietary patterns were unrelated to temperament-microbiota associations.	Christian et al. (2015)
Behavioral problems (parent-rated Child Behavior Checklist [CBCL]-questionnaire)	16S rRNA	Fecal microbiota: 1, 6, 12 mo, Behavioral phenotype: 2 y	201	Children scoring over the cut-off point in Internalizing, Externalizing, or Total Problems had decreased Prevotella abundance at 12 mo. Behavioral outcomes were not associated with SCFA concentrations. Temperament was not associated with Prevotella abundance.	Loughman et al. (2020)
Cry/fuss time, behavioral problems (CBCL)	16S rRNA	Cry/fuss and fecal microbiota assessment before 3 mo of age, reassessment after 4 wk, CBCL at 2 y	118 (63 boys)	*Bifidobacterium, Clostridium, Lactobacillus*, and *Klebsiella* associated with colic severity. Baseline microbiota composition predicted future crying/fuss time with 65% accuracy. Several *Clostridium* OTUs were enriched in children scoring over the cut-off point in Internalizing, Externalizing, or Total Problems, however many OTUs from the Bifidobacterium genera were alternately enriched and depleted.	Loughman et al. (2020)
Temperament (primary caregiver-rated Infant Behavioral Questionnaire Revised)	16S rRNA	12 mo	51 (20 boys)	Soothability was positively correlated with the abundance of *Bifidobacterium* genera. Cuddliness was negatively correlated with the abundance of *Hungatella* genera.	Wang et al. (2020)

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Phenotype	Microbiota	Age	Sample	Main findings	Reference
Stress (hair cortisol, heart rate variability, negative and positive emotions, emotional problems)	16S rRNA	8–16 y	61	Decreased abundance of Firmicutes (esp. *Closridiales*) and *Phaslarctobacterium*, increased *Bacteroides* and *Parabacteroides* in high-stress groups.	Michels et al. (2019)
Functional reactivity in brain networks to fearful faces (fMRI)	16S rRNA	fMRI 5–11 y, fecal sample 3.95 y after scan	16 (4 boys)	Bacteroides associated positively with reactivity in the medial prefrontal cortex. *Lachnospiraceae* associated positively with the left lateral, medial prefrontal cortex, precuneus/cerebellum, and negatively with the post-central gyrus	Callaghan et al. (2019)
Resting-state fMRI scans	16S rRNA	1 y	91 (16 boys)	Alpha diversity was negatively associated with functional connectivity between the amygdala and thalamus and between the anterior cingulate cortex and anterior insula. Alpha diversity was also associated positively with functional connectivity between the supplementary motor area and the inferior parietal lobule.	Gao et al. (2019)
Cognitive development (Mullen scales of early learning), global and regional brain volumes (MRI)	16S rRNA	Fecal microbiota: 1 y, Cognitive assessment and brain imaging: 1, 2 y	89 (49 males)	Infants with *Faecalibacterium*-community type had lower cognitive performance as compared to infants with *Bacteroides*-community type. At 1 y, the right superior occipital gyrus was largest in the *Bacteroides*-community type and the smallest in *Ruminococcaceae*-community type. At 2 y, the left and right caudate nuclei were smallest in the *Bacteroides* community-type and largest in the *Ruminococcaceae*-community type. At 2 y, alpha diversity was negatively associated with cognitive performance and positively with the left precentral gyrus and right angular gyrus	Carlson et al. (2018)
Development (Ages and Stages Questionnaire, 3RD edition)	16S rRNA	ASQ-3 3 y, microbiota assessment 3–6 mo	309 (170 males)	Infants with *Clostridiales*-dominated microbiota showed poorer communication, personal, and social scores. Infants with *Bacteroides*-dominated microbiota showed poorer fine motor scores.	Sordillo et al. (2019)

2.3 Emotional Reactivity, Self-Regulation, and Attention

It is well understood that the risk for developing psychiatric or somatic conditions is embedded during the early life (Weiss and Wagner, 1998; Copeland *et al.*, 2015; O'Donnell and Meaney, 2017; Hur *et al.*, 2019). Although the disease risk is modified during the whole life span, early life is a developmentally sensitive time-window, when exposures have a disproportionately larger effect (Gabard-Durnam and McLaughlin, 2019). Likewise, early life developmental trajectories may indicate risk factors for later psychopathology. Hence, studying early life intermediate phenotypes may potentially lay the ground for more of a profound understanding of the pathogenesis and the development of preventive strategies.

2.3.1 Infant Reactivity and Self-Regulation Relate to Later Health and Development

Temperament is a psychobiological construct that describes the individual tendencies regarding reactivity and self-regulation as conceptualized by Mary K. Rothbart (Rothbart, 1981). The temperament is described in terms of negative emotionality, surgency/positive emotionality, and self-regulation/orienting. Negatively emotional individuals express negative emotions, such as sadness, anger and fear, whereas positively emotional individuals have a tendency to express positive emotions, higher activity levels, and have high-intensity pleasure (Gartstein and Rothbart, 2003). Self-regulation refers to the ability to regulate emotion, cognition, and behavior, which may be expressed by an extended time of interaction with or attention to a single object, expression of enjoyment, or reduced distress when held by a caregiver or expressions of enjoyment related to a low-intensity stimulus during infancy (Gartstein and Rothbart, 2003).

Temperament is relatively stable overtime, but the temperament constructs appear differently in various developmental stages and become more complex and nuanced thorough the development (Gartstein *et al.*, 2017). Newborns express distress tendencies (Rothbart, 2007), and 2-3-month-olds start to present approach tendencies with smiling, laughter, and motor activity as well as anger and frustration (Rothbart, 2007). Fear reactivity, i.e., distress to novel stimuli, are expressed around the age of six to ten months (Rothbart, 2007). Despite the relative stability, approximately little less than half of the variation in temperament traits is not explainable by prior assessment in childhood (Komsi *et al.*, 2006; Bornstein *et al.*, 2019) potentially reflecting maturation and experience. Temperament is related, for example, to the functioning of the HPA axis and autonomic nervous system (Laurent *et al.*, 2012; Dougherty *et al.*, 2013; Mackrell *et al.*, 2014; Kolacz *et al.*, 2016) and often are presented as sex differences with girls exhibiting more fear reactivity (Else-Quest *et al.*, 2006).

Temperament is associated with the prenatal and postnatal environment as well as genetic makeup. Temperament traits are heritable, and a recent genome-wide association study concluded that up to 48% of variation in temperament is explained by the variation in a range of single nucleotide polymorphisms that relate to synaptic plasticity, neurotransmission, associative conditioning, and stress reactivity (Zwir *et al.*, 2018). Of note, environmental factors still play a substantial role compared to genetic factors in shaping the temperament traits as elucidated by a twin study (van Wijk *et al.*, 2019). The environmental factor extent to the *in utero* environmental and prenatal factors that are related to temperament include maternal dietary factors, medication, toxin exposure, and psychological distress (Gustafsson *et al.*, 2016; Nolvi *et al.*, 2016; Stroustrup *et al.*, 2016; Gartstein and Skinner, 2018; Erickson *et al.*, 2019). Likewise, infant gestational age and maturity at birth may relate to the later development of temperament (Kaitz *et al.*, 2017). Postnatal factors also play an important role. Temperament has been linked with human milk composition and breastfeeding practices, which are related to maternal characteristics (de Lauzon-Guillain *et al.*, 2012; Jonas *et al.*, 2015; Hahn-Holbrook *et al.*, 2019). Naturally, parental care and characteristics as well as the child-parent interaction are important in shaping infant temperament (Gartstein *et al.*, 2018).

Temperament is an interesting early phenotype, as some of its characteristics may be related to later risk for health and disease (Stifter and Moding, 2018; Faith *et al.*, 2019). Most notably, temperament traits may precede later socioemotional development and problems. Most consistently, negative emotionality is related to later psychopathology, and especially the traits of behavioral inhibition or fear reactivity (substantially overlapping terms) are a risk for internalizing problems (Pérez-Edgar and Guyer, 2014; Kostyrka-Allchorne *et al.*, 2020). Likewise, reduced regulatory capacity is a related risk for externalizing problems, and activity levels, a trait related to positive emotionality, may increase the risk of ADHD and conduct problems (Abulizi *et al.*, 2017; Einziger *et al.*, 2018; Frick *et al.*, 2019). It must be noted that the effect sizes linking main dimensions (i.e., surgency, negative emotionality, self-regulation) to broad, heterogenous phenotypes, such as psychopathology, are small (Kostyrka-Allchorne *et al.*, 2020). However, individual traits, such as activity level and fear reactivity may potentially be prodromal of ADHD and internalizing problems, respectively, especially when persistent or extreme, although more longitudinal research is still needed (Pérez-Edgar and Guyer, 2014; Einziger *et al.*, 2018). On the other hand, temperament traits are not only vulnerability factors, but may also indicate vantage sensitivity for a positive environment (Pluess and Belsky, 2013). A child expressing a high negative emotionality, for instance, can lead to better socioemotional and behavioral outcomes with good quality care, whereas children with low negative emotionality do not show such sensitivity (Pluess and Belsky, 2009).

2.3.2 Emotional Attention Biases Associate with the Trajectories of Socioemotional Development

Early self-regulation is grounded in the infant's ability to engage, disengage, and shift gaze from stimuli (Rothbart *et al.*, 2011; Posner *et al.*, 2014). Attention is a crucial component in information processing and is an important "gate-keeper" limiting the information that is further processed. Self-regulation is important in regulation of affective states, and shifting attention from distressing signals can ameliorate the affective states in infants (Harman *et al.*, 1997). Typically, infants show a disproportionate preference to view faces during infancy, and especially faces with angry or fearful facial expressions during the first year of life (Peltola *et al.*, 2009; Nakagawa and Sukigara, 2019). The development of threat-related attention concurs with the ability to express fear reactivity. Both angry and fearful faces are signals of threat, although they are crucially different, as angry faces are a direct source of threat, whereas fearful faces illustrate an indirect threat in the environment (Taylor and Whalen, 2014). Thereof, seeing fearful faces elicits increased attention to the surroundings and better memory function (Taylor and Whalen, 2014).

Attention has strong neurobiological connections. An alerting network activates to generate and maintain a vigilant state (Petersen and Posner, 2012). The attention processes during infancy are rooted in several key brain regions related to an orienting network (Petersen and Posner, 2012), which are interconnected with brain regions related to processing of emotionally salient information, such as the amygdala (Tamietto and De Gelder, 2010). Later during childhood, an executive attention network takes a more dominant role in resolving conflicting thoughts, emotions, and behavioral responses that are not in line with an individual's goals (Petersen and Posner, 2012). On top of the key brain regions and circuits, attentional profiles are related to autonomic nervous system functioning (Mateus *et al.*, 2018; Woody *et al.*, 2019).

Individual differences in attention may affect how one experiences the surrounding environment, and it may have an impact on the trajectories of socioemotional development. Attention biases have been described in adult and child populations with anxiety disorders, and modulation of attention may be used as an intervention for anxiety, which suggests a mechanistic role of attention biases in anxiety (Bar-Haim *et al.*, 2007; Dudeney *et al.*, 2015; Roy *et al.*, 2015). However, how early attentional profiles relate to later psychopathology, and whether the attention biases are indicative of certain trajectories of socioemotional development, are still under research. The greater attention bias towards faces is related better to social functioning later during toddlerhood. Namely, increased time-gazing to faces (Peltola *et al.*, 2018) as well as a prolonged first look followed by a greater disengagement from fearful faces (Grossmann *et al.*, 2018) have been associated with later prosocial behavior. Attention biases also associate with the

parent-child relationship, as greater attention bias towards fearful faces associates with better attachment security later (Peltola *et al.*, 2015).

These preliminary results of early attentional biases and anxiety disorder are somewhat counterintuitive regarding the direction of the effects. Anxiety usually associates with an increased bias towards threat, but may also manifest as decreased attention to threat (Bar-Haim *et al.*, 2007, 2010). Furthermore, children with a distress disorder present a bias towards threat, but children with a fear disorder present a bias away from threat (Waters *et al.*, 2014). Likewise, there is no conclusive evidence that attention biases would be directly associated with emotional reactivity, and especially fear reactivity, which is considered a risk factor for later anxiety (Fu and Pérez-Edgar, 2019). However, a subtype of children with high fear reactivity in low-threat situations show bias away from the threat (Morales *et al.*, 2015). This suggests that subtypes of children with internalizing problems have different attentional profiles to threat signals, and general fear-processing characteristics do not linearly associate with the attention to threat.

Even if the attention to threat or emotional attention in general does not link with temperament traits in a straight-forward manner, a growing body of literature shows that attentional profiles may be important mediating- or moderating factors in socioemotional development in conjunction with other individual- and environmental factors (Morales *et al.*, 2016). Infants with behavioral inhibition, who also showed greater attentional bias to threat during childhood, were more likely to present social withdrawal during adolescence (Pérez-Edgar *et al.*, 2010). Likewise, an attention bias to a threat has been associated with later shyness only in the case of internalizing symptoms (Hummel *et al.*, 2017). Hence, attentional biases are important early life intermediate phenotypes that may be indicative of later socioemotional outcomes, especially when persistent or in conjunction with other risk factors for later psychopathology (Burris *et al.*, 2019).

Despite the connections to early self-regulation and later socioemotional development, relatively little is known about what environmental or individual characteristics influence the individual attentional processes. Parental characteristics affect the infant environment, and it is well-established that infants of mothers with psychological distress pre- and postpartum show a greater bias for threat (Kataja *et al.*, 2018, 2019). Preliminary studies have suggested that infant iron deficiency and prenatal maternal DHA supplementation may associate with infant attention (Colombo *et al.*, 2016; East *et al.*, 2018). Genetic variation related to serotonin metabolism has been linked with attention to emotional faces, although there is conflicting results (Forssman *et al.*, 2014; Kataja *et al.*, 2020). Contrary to temperament, prominent sex differences have not been reported in attention biases.

2.3.3 The Amygdala is the Central Structure for Learning from Emotionally Salient Stimuli

The amygdala is a central structure located within the temporal lobes that has a complex structure and functionally distinct nuclei in adults (Qin *et al.*, 2012). The amygdala volume peaks during preadolescence, although it reaches relatively high structural maturity already during the prenatal period (Ulfig *et al.*, 2003; Uematsu *et al.*, 2012). The amygdala connections to other limbic regions are relatively stable during development, whereas connections to cortical areas, including the prefrontal cortex increase before adolescence (Gabard-Durnam *et al.*, n.d.), and the intrinsic connectivity in the amygdala increases during childhood (Qin *et al.*, 2012). Amygdala volumes and microstructure are heritable (approximately 34-59% for volumes), but the effect of genotype interaction with early environment has a greater influence on infant amygdala volumes than the effects of genotype (Ong *et al.*, 2019; Satizabal *et al.*, 2019). Males typically have a larger amygdala with higher gray matter density than females across ages (Ruigrok *et al.*, 2014).

The amygdala is an important structure in learning from both positive and negative experiences, and it reallocates attention and increases vigilance for relevant information, especially with emotional valence (Sander *et al.*, n.d.; LeDoux, 2003; Tottenham and Sheridan, 2009; Hur *et al.*, 2019). Amygdala activation is also important in recognition of emotional expressions in faces (Sergerie *et al.*, 2008; Wang *et al.*, 2017). Amygdala volumes have been linked with psychopathology and neurodevelopmental disorders, as a larger amygdala has been reported in obsessive-compulsory disorder and anxiety, whereas a smaller amygdala has been reported in patients with autism, ASD, and conduct problems (Mana *et al.*, 2010; Schumann *et al.*, 2011; Qin *et al.*, 2014; Norman *et al.*, 2016; Rogers and De Brito, 2016; Hoogman *et al.*, 2017; Van Rooij *et al.*, 2018). As the amygdala shows relatively early maturity, it has been hypothesized that the variation in amygdala structure would associate with socioemotional functioning. Indeed, larger amygdala volumes have been related to less behavioral problems, less anxiety symptoms, lower impulse control, less negative emotionality, but also to an increased behavioral response to a non-social fearful stimulus during childhood (Holmes *et al.*, 2012; Dennison *et al.*, 2014; Qin *et al.*, 2014; Hanson *et al.*, 2015; Cismaru *et al.*, 2016; Rogers *et al.*, 2017; Graham *et al.*, 2018; Warnell *et al.*, 2018). Alongside the structural changes, greater amygdala activation and connectivity to other brain regions have been implicated in negative emotionality, behavioral inhibition as well as in facial recognition in adults characterized as behaviorally inhibited during childhood (Schwartz *et al.*, 2003; Blackford *et al.*, 2011; Roy *et al.*, 2014; Kann *et al.*, 2017; Thomas *et al.*, 2019). Not only the cross-sectional differences, but also the developmental trajectory of the amygdala is important regarding the socioemotional development. This is illustrated by a finding that negative emotionality is linked with reduced growth of the

amygdala during preadolescence, which is the period of rapid amygdala development as compared with positively emotional subjects (Filippi *et al.*, 2020).

Albeit being heritable to some degree, amygdala structure and functioning are related to different prenatal and early life exposures. Most notably, the amygdala is considered stress-responsive (Tottenham and Sheridan, 2009; Raineki *et al.*, 2019) and in line with this, prenatal maternal cortisol and cytokine levels as well as maternal psychological distress have been associated with enlarged amygdala and/or greater amygdala connectivity in a sex-specific manner (Buss *et al.*, 2012; Acosta *et al.*, 2019; Graham *et al.*, 2019). Furthermore, maternal SSRI use is linked with an enlarged right amygdala (Lugo-Candelas *et al.*, 2018). Regarding the postnatal environment, good parenting quality has been linked with a smaller amygdala (Bernier *et al.*, 2019), but on the other hand, also neonatal invasive exposures and childhood deprivation in the form of poverty have been associated with a smaller amygdala (Luby *et al.*, 2013; Chau *et al.*, 2019). Other potential factors that may affect early brain development include, but are not limited to, maternal nutrition/weight status as well as alcohol, tobacco, and drug abuse during pregnancy (Pulli *et al.*, 2019). Interestingly, a preliminary report on breastfeeding showed no associations between breastfeeding and brain volumes, although breastfed infants showed higher connectivity in certain structures (Blesa *et al.*, 2019). Although correlations between neural structures and socioemotional development do not imply causation, certain experimental models support the mechanistic role of the amygdala in socioemotional problems (Raineki *et al.*, 2019). The cumulative evidence from humans underline the potential importance of the amygdala in socioemotional development and suggest that it is malleable for early life exposures making it an interesting intermediate phenotype.

It must be noted that development of psychopathology happens in tandem with individual heritable dispositions, altered neurochemistry, behavioral patterns, and exposure to trauma and stressful events. However, as the foundations for mental health are embedded during early life (O'Donnell and Meaney, 2017; al-Haddad *et al.*, 2019), studying early risk factors increases the understanding of the development of psychopathology. In repeated experiments, early temperament and especially negative emotionality and its subscales, have been be related to later risk for psychopathology (Pérez-Edgar and Guyer, 2014). Early attention, and especially threat-related attention biases are important modulators of socioemotional development, especially in the context of temperament disposition (Burris *et al.*, 2019; Hur *et al.*, 2019). The amygdala, the relevance detector of human brain, is important in learning from emotionally salient stimuli and for reallocating attention to biologically relevant signals (Tottenham and Sheridan, 2009; Hur *et al.*, 2019). However, how these intermediate phenotypes of socioemotional development are related to early life environmental exposures are still under investigation.

2.4 Maternal Prenatal Stress

Pregnancy is a major life event accompanied with turbulent physiological changes including increased gonadal and glucocorticoid hormone levels. The psychological and physiological changes may predispose some women for psychological distress, including anxiety, depressive symptoms as well as pregnancy-specific anxiety, collectively called prenatal psychological distress, PPD. Depressive and anxiety symptoms are common during pregnancy, as approximately 10-20% of mothers present potentially clinically significant depressive symptoms, and similar estimates are provided for anxiety symptoms (Teixeira *et al.*, 2009; Figueiredo and Conde, 2011; Korja *et al.*, 2018). The depressive and anxiety symptoms are comorbid, and approximately 9.5% of pregnant women suffer from overlapping anxiety and mild to severe depressive symptoms (Falah-Hassani *et al.*, 2017). However, it seems that consistently high symptoms of either depression or anxiety are less concurrent than milder symptoms (Korja *et al.*, 2018). Both anxiety and depressive symptoms are not specific to pregnancy, whereas some pregnant women report fears and worries related to the delivery, the health of the child, and their own changing appearances. While some degree of worries is part of a natural adaptation to pregnancy, pregnancy-specific anxiety is related to adverse pregnancy outcomes, such as spontaneous preterm delivery (Kramer *et al.*, 2009). Pregnancy-specific anxiety is a partially independent phenomenon, but high levels of pregnancy-specific anxiety may provoke subsequent general anxiety symptoms (Huizink *et al.*, 2014). Further, acute (e.g., a natural disaster, a death or illness of close family members) and chronic (e.g., caregiving stress, unemployment, homelessness) stressful life events as well as stress derived from partnership dysfunction and daily hassles may increase maternal prenatal stress. Consequently, prenatal stress is a complex phenomenon, but the current literature review will mostly concentrate on the PPD and biological measures of prenatal stress. However, it is important to acknowledge that both the stressful events and psychological distress may have an impact on child development, although the effects may be different regarding the prenatal stress type (Simcock *et al.*, 2017), and likewise, different PPD symptom domains may associate differently with child socioemotional development (Szekely *et al.*, 2020)(Nolvi *et al.*, 2016). It is essential to note that exposure to a prenatal stressful event that is mild to moderate in magnitude may not relate to adverse brain developmental outcomes (Laplante *et al.*, 2008), and exposure to moderate maternal PPD may even accelerate cognitive development at least in socioeconomically advantaged populations (DiPietro *et al.*, 2006). Maternal PPD is a co-morbidity with other risk factors related to pregnancy and child development, such as low socioeconomic status, smoking, domestic violence, and single parenthood as well as medical conditions such as preeclampsia (Cripe *et al.*, 2011; Korja *et al.*, 2018).

2.4.1 Maternal Prenatal Stress Affects Infant Health and Development

Maternal PPD is a common phenomenon decreasing the sense of wellbeing and quality of life in the mother-to-be. Additionally, it has been associated also with birth outcomes as well as later child development. Maternal PPD, especially pregnancy-specific anxiety, has been associated with lower birth weight and gestational age (Grigoriadis *et al.*, 2013; Bussières *et al.*, 2015). The effects of maternal PPD on birth outcomes seem to be more pronounced outside high-income settings (Bussières *et al.*, 2015). Maternal PPD has been associated with an increased prevalence of offspring health problems, including inflammatory (Korhonen *et al.*, 2019) and metabolic disorders, such as recurrent infections (Korhonen *et al.*, 2019), asthma (van de Loo *et al.*, 2016), atopic disease (Andersson *et al.*, 2016), and obesity (Burgueño *et al.*, 2020).

Likewise, maternal PPD has been associated with several aspects of child brain development (Goodman *et al.*, 2011; Kingston *et al.*, 2012; Tarabulsy *et al.*, 2014; Madigan *et al.*, 2018; Walsh *et al.*, 2019), and it has been concluded that maternal PPD may account for up to a two-fold increase in childhood psychiatric disorders (O'Donnell *et al.*, 2014a). Differences in socioemotional functioning and psychological wellbeing have been suggested to start already *in utero*, as fetal reactivity to vibroacoustic stimuli is decreased in the context of maternal depression (Emory and Dieter, 2006; Reissland *et al.*, 2018). Postnatally, maternal PPD is associated with differences in temperament, increased behavioral problems, and colic/crying, and the effects are usually stronger in socioeconomically disadvantaged families compared to more well-off counterparts (Madigan *et al.*, 2018). Exposure to maternal PPD has been associated with a minor negative impact on cognitive development (Kingston *et al.*, 2012), and likewise, exposure to prenatal stressful life events (Tarabulsy *et al.*, 2014) and postnatal psychological distress seem to play a role regarding cognitive capacity (Kingston *et al.*, 2012). Psychiatric conditions, especially affective disorders, often first appear during adolescence or young adulthood (Torikka *et al.*, 2014), and maternal PPD increases the risk for adolescent anxiety and depression independent from postnatal psychological distress (Davis and Sandman, 2012; Pearson *et al.*, 2013). Again, the effect of maternal prenatal depression on adolescent depression is more pronounced in socioeconomically disadvantaged groups (Pearson *et al.*, 2013).

Both rodent and human studies have suggested prenatal distress has sex-specific effects on child development (Hodes and Epperson, 2019). Prenatal stress, both psychological and physiological, is associated with an increased number of female fetuses compared to male fetuses (Walsh *et al.*, 2019). Female fetuses are at a greater risk for reduced growth and preterm birth, but on the other hand, male fetuses are at a greater risk for still-birth (Hodes and Epperson, 2019). Various

studies have suggested prenatal stress interaction by sex on childhood brain developmental outcomes, as males have been suggested to be more prone to impulsivity, conduct problems, and impairments in cognitive development, but on the other hand, females are suggested to show more anxiety and depressive symptoms when exposed to maternal PPD (Goodman *et al.*, 2011). The paradigm called vulnerability-viability tradeoff suggests that although female fetuses have compensatory mechanisms (i.e., growth restriction, preterm birth) for prenatal stress, they show vulnerability for later problems, especially in fear processing and anxiety (Sandman *et al.*, 2013). However, it must be noted that not all meta-analyses findings support that maternal prenatal stress would interact with infant sex regarding obstetric outcomes (Cherak *et al.*, 2019).

The notion that how person perceives stress is heritable (Wüst *et al.*, 2000) raises the question of how genetic factors influence prenatal stress transmission. In a sample of women conceiving with IVF, Rice and colleagues showed that maternal genetic makeup did not influence the relationship between maternal PPD and child antisocial behavior and anxiety symptoms, as the association was evident both in unrelated and related dyads. On the other hand, the link between maternal PPD and child ADHD was evident only in the genetically related dyads. Despite that this is a special population and the generalizability may be limited, the findings support the notion that *in utero* exposure to depression is a significant predictor of child socioemotional development, but not necessarily ADHD, regardless of maternal genotype. Partial support for the same notion is offered by adoption studies and investigating paternal prenatal symptoms. Kerr et al. showed in an adoption study that both a biological mother's pre-pregnancy depression (i.e., history of major depressive disorder) and a biological mother's prenatal depressive symptoms independently associate with increased child-externalizing symptoms (Kerr *et al.*, 2013). Pre-pregnancy major depressive disorder indicates a potential genetic susceptibility to depression, as early-onset and recurrent major depressive disorder is associated with a higher genetic risk score than a single-episode, transient major depressive disorder (Wray *et al.*, 2018). Thus, it seems that both genetic susceptibility to depression and pregnancy-specific depressive symptoms affect the child's socioemotional development (Kerr *et al.*, 2013). Similar results were yielded from a general population cohort, as Lahti et al. reported that prenatal depressive symptoms and pre-pregnancy depression independently associates with an increase in externalizing problems (Lahti *et al.*, 2017). Interestingly, pre-pregnancy depression seemed to protect from a child's internalizing symptoms (Lahti *et al.*, 2017). In a similar vein, paternal prenatal depressive symptoms have not been associated with infant psychiatric problems or adolescent depression (Pearson *et al.*, 2013; Lahti *et al.*, 2017) partially supporting the notion that exposure to *in utero* expression is a significant predictor of child psychopathology independent of the parental genetic susceptibility.

There is ample experimental evidence showing that genetic variation related to dopamine, noradrenaline, serotonin, and GABA metabolism affects how prenatal stress influences offspring health and development (Abbott *et al.*, 2018). Similar effects have been observed in human samples investigating candidate genes, and hence, genetic factors may influence the differential susceptibility for prenatal stress (Belsky and Pluess, 2009; Abbott *et al.*, 2018). The maternal serotonin transporter gene (5-HTTLPR) genotype affects the relationship between prenatal stressful events and child autism spectrum disorder (Hecht *et al.*, 2016). Likewise, the child's 5-HTTLPR genotype affects the relationship between maternal prenatal anxiety and child emotion recognition and dysregulation (Tiemeier *et al.*, 2012), and the variation in the brain-derived neurotrophic factor gene affects the relationship between prenatal maternal anxiety and a child's internalizing problems (O'Donnell *et al.*, 2014b). Further, both variation in a gene encoding an enzyme involved in dopamine metabolism (COMT) and in a gene encoding the dopamine receptor affect the relationship between maternal PPD and prenatal stress and ADHD symptoms (Grizenko *et al.*, 2012; O'Donnell *et al.*, 2017). These results highlight that the prenatal stress effects on a child's socioemotional and brain development are not entirely due to genetic influences but are modulated by genetic makeup.

2.4.2 Multiple Interrelated Pathways Mediate the Effects of Prenatal Stress to Child Health and Development

Exposure to stressful events as well as psychological distress associates with a host of physiological changes including alterations in HPA-axis functioning and an increase in proinflammatory cytokines (Osborne *et al.*, 2018; Mustonen *et al.*, 2019; Nazzari *et al.*, 2020). Prenatal stress is also related to epigenetic alterations. Maternal PPD is associated with placental epigenetic profile, namely increased methylation of a gene encoding glucocorticoid receptor and 11β-hydroxysteroid dehydrogenase enzyme, which regulates the fetal cortisol exposure (Conradt *et al.*, 2013). Maternal PPD may increase fetal exposure to cortisol via the 11β-hydroxysteroid dehydrogenase functioning and participates in programming of the infant's HPA-axis functioning and brain development (Davis *et al.*, 2011; Kim *et al.*, 2016). Consequently, markers of HPA-axis functioning, such as hair, saliva, and serum cortisol and cortisone, are often used as biological markers of stress. However, not all stressor associate with altered HPA-axis functioning, for example, only maternal prenatal depressive symptoms that are chronic and increasing associate with increased long-term HPA-axis functioning (Mustonen *et al.*, 2018, 2019). Moreover, maternal immune system functioning is shown to associate with offspring brain development in rodent models (Shin Yim *et al.*, 2017; Reed *et al.*, 2020), and epidemiological findings show that maternal infections can increase the

risk for psychopathology (Davies *et al.*, 2020). Maternal increased proinflammatory cytokine content antenatally associate with changes in amygdala structure and functioning, which mediate the impact of prenatal inflammation on an infant's socioemotional functioning (Graham *et al.*, 2018). Likewise, maternal prenatal inflammation may associate with birth outcomes and cognitive- and behavioral development, but the results thus far are inconclusive (Nazzari and Frigerio, 2020). The proposed mechanism of how maternal inflammation may affect infant outcomes include the direct effects of cytokines on fetal development and/or effects of circulating cytokines on placental inflammation and subsequent fetal immune dysregulation (Bronson and Bale, 2014; Monk *et al.*, 2019). Maternal lifestyle factors, such as nutrition, sleep, exercise, and exposure to toxic chemicals may also affect the transmission of prenatal stress in conjunction with epigenetic changes as well as HPA-axis functioning and immunological pathways (Gomes Da Silva *et al.*, 2016; Trujillo *et al.*, 2018; Kaartinen *et al.*, 2019; Lindsay *et al.*, 2019; Lavonius *et al.*, 2020; Padula *et al.*, 2020).

Thus, it can be concluded that experiencing mild to moderate levels of PPD are common, and this may have a strong implication to the somatic and psychological development of the offspring. However, it is relatively premature to conclude how different PPD modalities and depressive-, anxiety-, and pregnancy-specific anxiety symptoms associate with different aspects of child development. Second, it seems that some of the associations between maternal PPD and infant behavioral and emotional development are unrelated to the genetic make-up, although genetic factors may affect the differential susceptibility to PPD. Third, PPD is related to changes in the HPA-axis and immune system functioning and epigenetic alterations, but no marker seems to serve as universal prenatal stress biomarker.

2.4.3 Prenatal Stress and Microbiota Alterations

In addition to immune dysregulation, altered HPA-axis functioning in placental functioning via epigenetic changes and changes in microbiota could participate in mediating the prenatal stress effects to the offspring. Primarily, Bailey and colleagues showed in non-human primates that prenatal stress decreases lactic acid bacteria in the offspring, and specifically, that early gestation stress decreases *Lactobacillus*, whereas stress during both early and late gestation decreases *Lactobacillus* and *Bifidobacterium* (Bailey *et al.*, 2004). Rodent studies have shown that prenatal stress alters the maternal as well as offspring microbiota and causes alterations in offspring behavior, physiology, and neurobiology (Golubeva *et al.*, 2015; Jašarević *et al.*, 2015, 2017; Gur *et al.*, 2019). Interestingly, the effects of maternal prenatal stress on offspring behavior and brain inflammation are

mediated via maternal immune signaling and are dependent on the maternal microbiota (Chen *et al.*, 2020). Further, maternal prenatal stress increases placental tryptophan and serotonin dependent on the microbiota (Chen *et al.*, 2020). Likewise, it has been shown that alterations in maternal vaginal microbiota caused by prenatal stress partially mediate the effects of prenatal stress on offspring microbiota, stress reactivity, and hypothalamus gene expression, i.e., offspring inoculation with vaginal fluids from non-stressed dams was not able to ameliorate the effects of prenatal stress on offspring completely (Jašarević *et al.*, 2018). This illustrates that microbiota- and immune signaling-dependent changes beginning already *in utero* are potentially more important in defining the future microbiota and stress- and neuroendocrine phenotype of the offspring than the initial colonizers of vaginal microbiota at birth.

In humans, there is preliminary evidence that maternal prenatal fecal microbiota might be altered in distressed mothers (Hechler *et al.*, 2019), although we could not corroborate that finding in a small sample of mothers from the FinnBrain Birth Cohort Study (Aatsinki *et al.*, 2018). It has been suggested that maternal PPD might be associated with bacterial vaginosis during pregnancy (Culhane *et al.*, 2001), although not all studies corroborate this finding (Paul *et al.*, 2008). A study from South Africa showed that mothers exposed to domestic violence had alterations in fecal microbiota including a higher proportion of *Lactobacillaceae* (Naudé *et al.*, 2019). In the first study on human infants, Zijlmans, Korpela, and colleagues showed that exposure to maternal prenatal stress, defined as a combination of elevated maternal late pregnancy saliva cortisol and PPD, associates with increased Proteobacteria and decreased lactic-acid bacteria colonization in infants (Zijlmans *et al.*, 2015). Despite methodological differences, that finding was partially corroborated by Naude et al. in a South African sample, as they showed that maternal PPD was associated with a slower decline in Gammaproteobacteria (within the phylum Proteobacteria) in infant fecal microbiota. Parallel to the alterations in microbiota composition, infants, who were exposed to maternal prenatal depression, had decreased fecal immunoglobulin A (IgA) in a large population from the CHILD-cohort (Kang *et al.*, 2020). IgA contributes to the intestinal immunity and microbiota colonization patterns (Wells *et al.*, 2011; Donaldson *et al.*, 2018; Kang *et al.*, 2020). Interestingly, this was not observed in the context of postnatal depression, and breastfeeding did not influence the association (Kang *et al.*, 2020).

The wealth of animal studies suggest that: a) maternal prenatal stress causes alterations in both maternal vaginal and gut microbiota; b) maternal prenatal stress causes alterations in offspring microbiota, behavior, and physiology; c) certain changes in offspring behavior and neurobiology related to prenatal stress are dependent on maternal microbiota and immune signaling; and d) vaginal

microbiota partially contributes to the transmission of prenatal stress on offspring. There is preliminary support from human studies that maternal prenatal stress is associated with changes in maternal microbiota and in a child's fecal microbiota and intestinal immunity. However, the methodology is diverse, and replication in independent samples are needed. Likewise, whether the observed alteration in infant microbiota has clinical significance regarding later health and development is currently unknown.

2.5 Summary of Current Literature

It has been suggested that the prenatal and early life environment influences the child's future health and development, as infancy is a developmentally highly sensitive period (Barker, 2004; Codagnone *et al.*, 2019; Monk *et al.*, 2019). Likewise, later psychopathology is often preceded by different developmental phenotypes, including temperament traits related to reactivity and self-regulation as well as biased attention to threat stimuli. These phenotypes are suggested to have biological underpinnings, such as differences in the structure and function of the amygdala, which is the central nervous system structure that participates in the processing of emotionally and biologically relevant stimuli. Early gut microbiota composition has been shown to affect behavioral and neurobiological development in animal models, and it has been suggested that early microbiota could be related to the programming of the psychopathology. However, human studies, especially on typically developing infants, are scarce. Likewise, sex differences in socioemotional development have been proposed, and early life microbiota depletion may have sex-specific neurodevelopmental effects in rodents, but similar phenomena in human infants are not thoroughly reported.

Prenatal stress and maternal subjective well-being are important regarding the health and development of the child. However, the exact mechanisms are still relatively elusive, although immunological, epigenetic changes, and HPA-axis functioning likely play a role. It has been proposed that changes in infant microbiota could mediate the potential health effects, yet the evidence of prenatal stress relations to infant microbiota composition is relatively limited.

3 Aims

The aim of this study was to explore how the infant fecal microbiota composition and diversity are associated with infant brain developmental outcomes. Likewise, we explored whether infant fecal microbiota associates with infant brain development in a sex-specific manner. Furthermore, the study also aimed to investigate how maternal prenatal stress associates with infant fecal microbiota composition and diversity.

Specifically, the aims were:

I. To explore how infant fecal microbiota composition and diversity at the age of 2.5 months associates with infant temperament at the age of 6 months. The aim was to assess sexually dimorphic associations between infant fecal microbiota and temperament.

II. To explore whether infant fecal microbiota composition and diversity at the age of 2.5 months associates with infant emotional attention at the age of 8 months, and whether the associations show a sex-interaction.

III. To explore whether infant fecal microbiota composition and diversity at the age of 2.5 months associates with infant amygdala volumes around the age of one month, and whether the associations show a sex-interaction.

IV. To investigate how maternal chronic prenatal psychological distress and hair cortisol concentrations, as measures of prenatal stress, associate with infant fecal microbiota composition and diversity.

4 Materials and Methods

4.1 Study Design and Participants

The participants were part of FinnBrain Birth Cohort Study, which is a prospective, observational birth cohort focusing on the effects of prenatal and early life exposures and child health and development. The study was conducted in South-Western Finland and recruitment occurred between December 2011 and April 2014. The cohort consists of 3808 participating mother-infant dyads and 2623 fathers. The cohort represents the general Finnish population, although the prevalence of young, multiparous, and smoking mothers as well as preterm births is lower than in the general population (Karlsson *et al.*, 2018).

Participants were recruited to the FinnBrain Birth Cohort Study after the first ultrasound examination at gestational week (gwk) 12 at three maternity welfare clinics at Turku University Hospital in the Southwest Finland Hospital District and the Åland Islands in Finland. Study participants in this thesis were the mothers and their infants, although fathers were also recruited. All mothers received mailed or online questionnaires three times during pregnancy and 3 and 6 months postpartum. The FinnBrain Birth Cohort Study consisted of different study visits and sampling protocols aligning with different aims of the cohort. Mothers were recruited to the different study visit via email and phone contacts. A subset of mothers donated hair samples around gwk 24 (see 4.6.2). Fecal samples from the infant subjects in the present study were collected around the age of 2.5 months either in an early nutrition study visit or in a pediatric study visit. Subset of infants underwent neonatal MRI (see 4.6) or eye-movement tracking (see 4.5, Fig. 4).

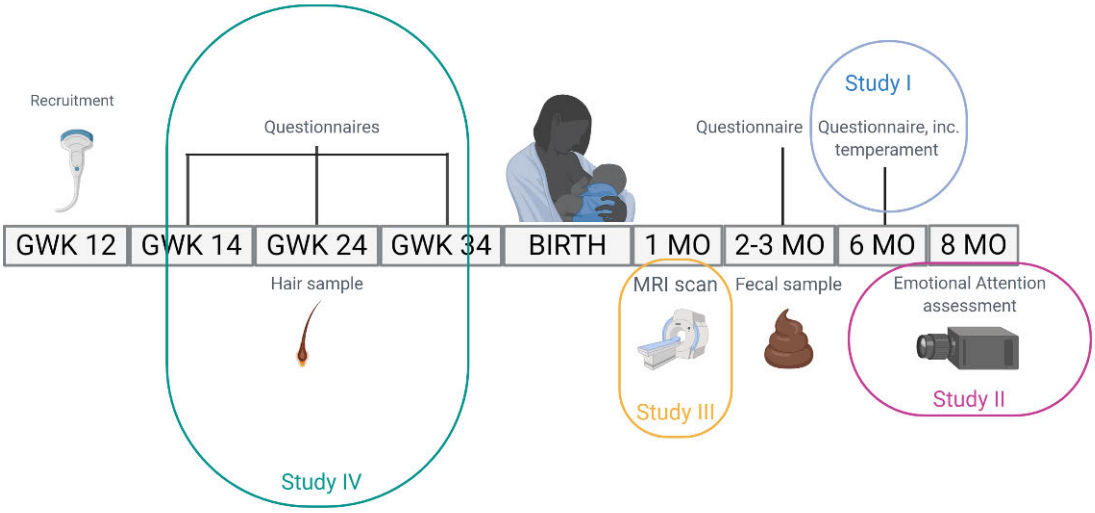

Figure 4. Schematic timeline of the Studies I-IV.

The target population for the pediatric study visit were mothers with elevated and low prenatal psychological distress and/or prenatal SSRI/SNRI use. Depressive symptoms (Edinburgh Postnatal Depression Scale, EPDS), general anxiety (Symptom Check List-90/anxiety subscale, SCL-90), and pregnancy-related anxiety (Pregnancy-Related Anxiety Questionnaire Revised 2, PRAQ-R2) were measured at gwk 14, 24, and 34. Mothers were assigned as elevated PPD, if she scored twice above cut-point (EPDS > 11, SCL-90 > 9, PRAQ-R2 > 32 for elevated PPD) in any of the questionnaires or reported use of SSRI/SNRI during pregnancy. On the other hand, the mother was assigned as low PPD, if she scored below cut-points in all measures (EPDS < 7, SCL-90 < 5, PRAQ-R2 < 25 for low PPD) and reported no SSRI/SNRI use. The target population for the breastfeeding and early nutrition study visit was the whole cohort population.

4.2 Fecal Samples

4.2.1 Sample Collection and Processing

Parents collected the infant fecal samples at approximately 2.5 months (see Tables 2, 5, 7) of age at their homes. Parents were instructed both orally and by written tutorials to collect the fecal material into sterile collection tubes, to immediately store the samples in their household refrigerators or freezers, and to deliver the samples to the study center as soon as possible after the collection using coolers.

Parents were also requested to mark the date and time of the sample taking. Samples were immediately homogenized, divided into aliquots, and frozen at -80˚C in the research facilities. Only those delivered within 48 h were included in the analyses. Due to variation in sample collection times, age as months during the sample collection was included in the adjusted analyses.

4.2.2 Sample Sequencing and Data Processing

After samples were delivered to the research facilities and processed, and DNA was extracted from the weighed, homogenized fecal pellets with a semi-automated GXT stool extraction kit (Hain Lifescience, Nehren, Germany). Prior to the extraction, mechanical lysis was performed by bead-beating the samples in glass bead tubes with MOBIOPowerLyzer™ 24 Bench Top Bead-Based Homogenizer. The DNA concentrations were measured with a Qubit 2.0 dsDNA HS assay kit (Life Technologies, USA), after which the DNA was stored at -80°C until sequencing.

The sequencing libraries for the NGS-based gut microbiota composition analysis were generated in a single PCR with custom dual-indexed primers containing the adapter and specific index sequences required for sequencing. Briefly, the V4 area of the bacterial 16S rRNA gene was amplified using the KAPA HiFi PCR kit (KAPA Biosystems, Massachusetts, USA) with in-house generated primers. Forward and reverse primer sequences were 5'-AATGATACGGCGACCACCGAGATCTACAC-i5-TATGGTAATT-GT-GTG CCAGCMGCCGCGGTAA-3' and 5'-CAAGCAGAAGACGGCATACGAGAT -i7- AGTCAGTCAG-GC-GGACTACHVGGGTWTCTAAT-3', respectively, where i5 and i7 represent the sample-specific index sequences.

The PCR products were purified with Agencourt AMPure XPMagnetic beads (BeckmanCoulter, Inc., USA) on a DynaMag™-96 magnetic plate (Life Technologies, USA). The PCR product length and DNA integrity were checked with TapeStation (Agilent Technologies Inc., USA), and the final DNA concentrations of the purified products were measured with Qubit 2.0 dsDNA HS assay kit (Life Technologies, USA). The products were then mixed in equal concentrations to generate a 4 nM library pool, which was denatured, diluted into a final concentration of 4 pM, and spiked with 25% denatured PhiX control (Illumina, USA) for sequencing. Sequencing was done with 2 × 250 bp paired-end reads on MiSeq system (Illumina, USA) using a MiSeq v3 reagent kit (Illumina, USA). Raw reads across the samples sequenced with the Illumina MiSeq 250bp paired-end sequencing were used as input for the data analysis.

Of the delivered samples (n = 517), only the samples that were collected and preprocessed according to the instructions given and successfully

sequenced were included in the analyses (n = 446, 86%). The quality of the raw sequences was checked with the FastQC program (v. 0.10.1; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), after which the downstream analyses were performed using Quantitative Insights Into Microbial Ecology (QIIME) pipeline (v. 1.9) (Caporaso *et al.*, 2010; Kuczynski *et al.*, 2012). The sequence reads were filtered with a quality score acceptance rate of ≥ 20, resulting in 41k–1,052k reads per sample (total: 73,222k, mean: 178,156, SD: 109k). Chimeric sequences were filtered out using USEARCH (v. 6.1), and operational taxonomic units (OTUs) were picked using UCLUST algorithm with 97% sequence similarity (Edgar, 2010). OTUs representing less than 0.05% of the total sequence count were removed. Annotations for the OTUs were derived from the GreenGenes database (DeSantis *et al.*, 2006).

4.3 Self-reported and Register-based Measures

4.3.1 Background Information and Register Data

Self-report prenatal questionnaires were obtained at gwk 14, 24, and 34. At gwk 14, parental background data, including the level of education categorized as (i) university education, i.e., tertiary level academic/ general education; (ii) vocational tertiary education; and (iii) secondary or lower level education, and the intake of selective serotonin reuptake inhibitor or serotonin-norepinephrine reuptake inhibitors (SSRI/SNRI intake) were reported (Karlsson et al., 2018). Information on the duration of exclusive and partial breastfeeding was collected through postnatal follow-up questionnaires, and infant medication intake was reported at fecal sample collection. Breastfeeding at 2.5 months was categorized as never breastfed, breastfeeding ceased, partial breastfeeding, and exclusive breastfeeding in accordance with Stewart et al. (Stewart *et al.*, 2018). Information about maternal pre-pregnancy body mass index (BMI; kg/m^2); the duration of gestation (preterm < 37 gwk; term 37-41; post-term ≥ 41 gwk); antibiotic intake during the neonatal period, birth weight (g), and height (cm); and the mode of delivery (all C-section vs. all vaginal) was collected from the National Birth Registry provided by the National Institute for Health and Welfare (www.thl.fi).

4.3.2 Maternal Psychological Distress

Prenatal maternal depression and anxiety symptoms were measured with the EPDS (Cox *et al.*, 1987; Gibson *et al.*, 2009) and SCL- 90 (Derogatis *et al.*, 1973; Holi *et al.*, 1998), which were also reported at three-month postpartum. Stress related to

everyday life was measured with Daily Hassles (Korpela *et al.*, 2008), which consists of both a positive and a negative scale and having four points in which the subjects rate their worries (a-scale, "negative") or delights (b-scale, "positive") related to social relationships, work, finances, household matters, news and media, and substance use (i.e., tobacco, alcohol, drugs). PRAQ-R2 (Huizink *et al.*, 2016) was used to measure pregnancy-related worries and anxiety related to the fear of giving birth, worries about bearing a physically or mentally handicapped child, and concerns about the mother's own appearance.

4.3.3 Temperament

Infant temperament was assessed using the maternal reports of Infant Behavior Questionnaire Revised Short Form (IBQ-R SF) at the age of 6 months (Putnam *et al.*, 2014), when the fear tendencies start to emerge (Rothbart, 2007). The IBQ-R is a reliable and valid measure for infant temperament evaluation and consists of 91 items. In each question, mothers are asked to assess their infant's behavior in different everyday situations based on the past two weeks. The questionnaire was comprised of three main dimensions: negative emotionality, containing subscales of distress to limitations, fear, sadness and a reversed scale of falling reactivity; regulation/orienting with subscales of perceptual sensitivity, low intensity pleasure, cuddliness, duration of orienting and soothability; and surgency/positive emotionality with subscales of activity level, smiling and laughter, high intensity pleasure, approach, and vocal reactivity. Refer to the earlier literature section for a detailed description of the individual subscales (Gartstein and Rothbart, 2003; Putnam *et al.*, 2014). Both individual item scores and subscale total scores ranged between 1 and 7. Cronbach's Alpha across subscales ranged from 0.65 to 0.84 and for main dimensions Cronbach's alphas were 0.88 for surgency (0.65-0.78 for its subscales), 0.85 for negative emotionality (0.72-0.81), and 0.80 for regulation/ orienting (0.73-0.84).

4.4 Emotional Attention

4.4.1 Eye-movement Tracking Assessment

Emotional attention was assessed at the infant age of 8 months (± 2 weeks) from due date, when there is a normative tendency to prefer fearful faces over neutral or happy faces (Peltola *et al.*, 2009). Emotional attention was assessed during a study visit that was conducted by psychologists or advanced psychology students. Eye tracking (EyeLink1000+ SR Research Ltd, Toronto, Ontario, Canada) with a sampling frequency of 500Hz and a face-distractor paradigm (Peltola *et al.*, 2009)

with emotional faces (e.g., scrambled, neutral, happy, and fearful faces) and distractors (e.g., geometric shapes) was used to study infant attention bias for faces and fear. A set of 48 trials were presented, 12 trials per condition with each emotion and the scrambled-face control picture comprising of 18 photographs of each woman with two different women presented and 12 scrambled-face pictures in a semi-random order.

Prior to every measurement, a five-point calibration procedure with an audiovisual animation with rapidly appearing, rotating animated pictures, either of a duck or a barking dog sequentially presented in five locations on the screen, was used. The calibration could be repeated before actual testing and also during measurement when necessary. During eye tracking, the child sat on the caregiver's lap at the distance of 50-70 cm from the eye tracker facing the computer screen. The eye-tracking laboratory was dimly lit, and the researcher sat on an independent host computer next to the infant-parent dyads but was separated by a curtain to avoid interference. Small breaks were allowed during measurement if necessary. The parent was instructed not to comment on the emotional content of the faces but otherwise not prevented from talking to the infant.

A brief animation was shown before each trial to capture the attention of the infant to the center of the screen. After the infant fixated on the animated stimulus (depicted as a red circle in Fig. 2), as judged by the experimenter monitoring the infant via a host computer, the experiment was started. During the experiment, first, a picture of a face or a scrambled-face stimulus was shown in the center of the screen for 1000 ms (Fig. 2). Then, with a 1000-ms onset asynchrony, a salient lateral distractor being a checkerboard or circles appeared on either the left or right side of the face with a visual angle of 13.6° for 3000 ms simultaneously with the face. One trial lasted for 4000 ms. The sizes of the emotion-depicting pictures and distractor stimuli were 15.4° × 10.8° and 15.4° × 4.3°, respectively. Once the infant's gaze was in the middle of the screen, the next trial was presented by the researcher. The order of the central stimuli was semi-randomized, with a constraint that the same stimulus was not presented more than three times in a row. The lateral stimulus was selected and presented randomly for each trial (see Fig. 5. for illustration and a description of the procedure in Kataja et al. (2018).

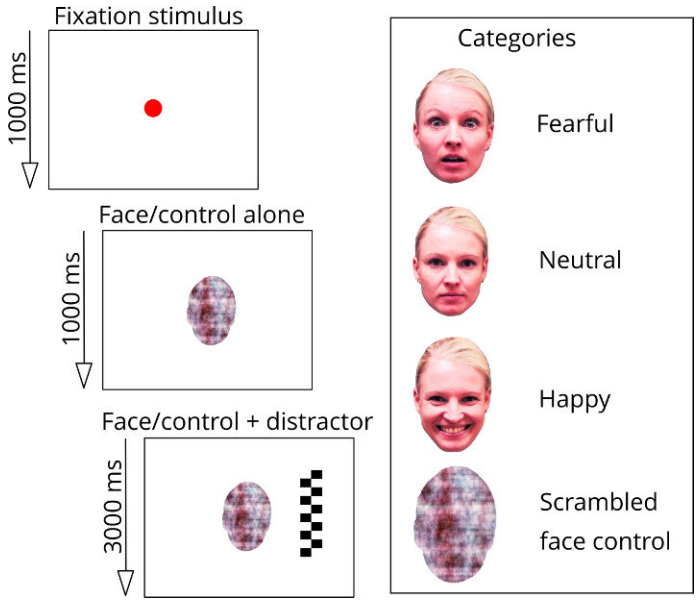

Figure 5. Illustration of the overlap paradigm used in the eye-tracking experiment to assess infant's attention to social signals of emotion. After the infant looked at a fixation stimulus in the center of the screen (red circle), a face or a scrambled face pattern and subsequently a high-contrast lateral distractor were presented. The probability of attention disengagement from the central to the lateral stimulus was analyzed from the eye-tracking data and used as a measure of attention to scrambled face patterns and neutral, happy, and fearful faces.

4.4.2 Preprocessing of Eye-Tracking Data

The trial data, comprising of timestamps for the onset times of central and lateral pictures and the xy coordinates of the participants' gaze position with 500 samples per second, were stored as text files and analyzed offline using a library of Matlab scripts (Mathworks, Natick, MA) (Leppänen, Forssman, Kaatiala, Yrttiaho, & Wass, 2015). We used the following quality control criteria based on prior studies (Leppänen et al., 2015) to retain trials for the analysis. First, trials had to have sufficiently long fixation on the central stimulus (i.e., > 70% of the time) during the time preceding gaze disengagement or the end of the analysis period (i.e., 1000 ms from the appearance of the lateral distractor). Secondly, trials had to have a sufficient number of valid samples in the gaze data (i.e., no gaps > 200 ms). Thirdly, trials had to have valid information about the eye movement from the central to the lateral stimulus (i.e., the eye movement did not occur during a period of missing gaze data). The proportion of invalid trials was 20.8% corresponding to previous infant studies using the same methodology in 7- to 11-month-old infants (21.6–28.2%) (Leppänen *et al.*, 2015)). On average, subjects provided 9.0 (SD ± 2.5) trials for control stimulus, 9.0 (SD ± 2.5) for a neutral face stimulus, 9.2 (SD ±

2.3) for happy face stimulus, and 9.3 (SD ± 2.4) for a fearful face stimulus in this sample (n = 131).

First, we calculated the probabilities of disengagement (DPs, time from the beginning of experiment to 2000 ms) separately for each stimulus condition (i.e., neutral, happy, fearful faces, and scrambled-face pictures). The DP is a ratio of the number of disengagement (in a given condition) / number of valid trials (in a given condition). A cut-off of 2000 ms was set as typically reactive saccades from central stimuli to lateral distractors appearing in this time window (Pyykkö, Ashorn, Ashorn, Niehaus, & Leppänen, 2019). Then, to investigate the infants' preference for faces vs. scrambled faces, we calculated a face-bias score. Following Yrttiaho, Forssman, Kaatiala, and Leppänen (2014) the "face bias" was calculated by contrasting the face condition (Happy, Neutral) to the scrambled-face condition (Control stimulus) using the following formula: *face-bias = p(saccade/Control Stimulus) - p(saccade/Happy&Neutral*) (Yrttiaho *et al.*, 2014). The fearful face condition was left out from the face-bias score to minimize the effect of emotional cues to infant face bias, as fearful faces have been shown to strongly suppress attention shifts to peripheral targets differently from neutral and happy faces at this particular age (Leppänen, 2016). Then, to investigate the differences in infants' fear bias, we calculated a fear-bias score. The "fear bias" was calculated by contrasting the fear condition (Fearful) to other face conditions (Happy, Neutral) using the following formula: *Fear bias = p(saccade/Neutral&Happy) - p(saccade/Fearful).*

4.5 Amygdala Volumes

Imaging was performed around the age of one month during natural sleep with a Siemens Magnetom Verio 3T scanner (Siemens Medical Solutions, Erlangen, Germany). The imaging protocol lasting 40 min included an Axial Dual Echo Turbo Spin Echo sequence, a sagittal 3D-T1 (T1-weighted MPRAGE) sequence, and diffusion tensor imaging (DTI) sequence, of which only T2-weighted images were used in the current study, as they provide a good contrast between the skull and the brain. The spatial resolution of both the T1 and T2 images was 1 × 1 × 1 mm, and the number of slices was 128 (Lehtola *et al.*, 2019). A time repetition of 12,070 ms and effective time echo times of 13 ms and 102 ms were used to produce both PD-weighted and T2-weighted images from the same acquisition. All the successful brain images were evaluated by a radiologist specializing in pediatric neuroradiology. If abnormalities were found, the families were offered an opportunity for a child neurological examination and consultation by an experienced pediatric neurologist. The sample in the current study was free from participants with incidental findings.

Raw MRI images were converted to a Neuroimaging Informatics Technology Initiative format using dcm2nii software to improve alignment, after which the images were converted to MINC format with MINC tools' version 1.5.1 developed at McConnell Brain Imaging Centre, Montreal, Canada. Manual segmentation of the amygdala was performed with Display software package version 2.0 with a brush size of 0.5 mm. Manual segmentation was performed in slice-by-slice manner one hemisphere at a time. Images were reviewed for three-dimensional consistency. Delineation of structure was double-checked after the segmentations were performed. Volumes of amygdala were automatically calculated with the minc tools' volume_stats function (Hashempour *et al.*, 2019; Lewis *et al.*, 2019).

4.6 Prenatal Psychological Distress and Hair Cortisol

4.6.1 Prenatal Psychological Distress

To assess the chronic symptom level across pregnancy, a categorical variable of each symptom scale (i.e., chronic EPDS, PRAQ-R2, SCL, and Daily Hassles negative subscale total score) was assigned. Subjects that scored above the selected cut-offs at the two measurement points were classified as having chronically elevated scores for each variable. A total score of 10 or more was used as a cut-off point for EPDS in order to present more clinically significant symptoms (Gibson *et al.*, 2009), while the median split was used for the other questionnaires. The median scores of the questionnaires in this subpopulation corresponded with those of the whole FinnBrain Birth Cohort population (Table 8) (Korja *et al.*, 2018).

4.6.2 Hair Cortisol Concentration Assessment

HCC samples were collected during a study visit at gwk 24 (mean = 24.6 gwk, SD ± 1.15, range 22.3–27.9 gwk). There were no differences in the maternal PPD questionnaires (i.e., chronic EPDS, PRAQ-R2, SCL, and Daily Hassles negative subscale) between the mothers with or without available hair samples (Wilcoxon rank-sum test, W = 1874-16469, FDR = 0.30-0.92). Hair samples were cut from a standardized area in the posterior vertex region of the head most proximal to the scalp. For analyses, a five cm segment weighing 5–15 mg was used to represent the past five months of pregnancy. The hair segments were washed in isopropanol for 3 min three times and finely minced using surgical scissors. 1.5 ml of methanol was added to each sample, and the samples were incubated at 55°C for 24 h. Samples were centrifuged at 10,000 rpm for 2 min, after which the supernatant was transferred to a new vial. Samples were dried from methanol in 60°C under a

constant stream of nitrogen. Finally, 0.15 ml of phosphate buffer was added, and 50 µl of each sample HCC assessment was performed by ELISA using an IBL International Cortisol Saliva kit (Mustonen *et al.*, 2019).

4.7 Statistical Analyses

Statistical analyses were performed with R (versions 3.5.0–3.6.1) software using phyloseq (McMurdie and Holmes, 2013), DESeq2 (Love *et al.*, 2014), vegan (Oksanen *et al.*, 2017), and microbiome R/Bioconductor packages. A microbiome package was used to calculate diversity (Shannon Index) and richness (observed species, Chao1, log transformed as needed) indexes, which describe the intra-individual microbial diversity or richness based on the OTU counts. The DESeq2 R package, which uses shrinkage estimation for dispersions and fold changes (log2 Fold Changes are reported) to perform the quantitative analysis of differential abundance, was used to identify bacterial signatures associated with the mother or infant phenotypes when adjusting for covariates (Love *et al.*, 2014). DESeq2 expects non-rarefied data and normalizes the count data following negative binominal distribution (Love *et al.*, 2014). It fits a generalized linear model to each taxa and tests statistical significance with the Wald test (Love *et al.*, 2014).

Subjects in Study I were clustered according to their fecal microbiota composition profiles using the Bray-Curtis distance based on the OTU counts and the Partitioning Around Medoids clustering method from the R package cluster (Maechler *et al.*, 2018). The optimal number of clusters were assessed using the gap statistics, and the R package indicspecies was used to detect the most discriminative OTUs among clusters (De Cáceres and Legendre, 2009). In the rest of the studies, which mostly have smaller samples sizes, the variation in the overall microbiota community composition across individuals, i.e., beta diversity was analyzed with the Permutational Analysis of Variation (PERMANOVA), which is a more common strategy for testing the variation in the overall microbiota community structure across individuals. The function adonis from the R package vegan (Oksanen et al., 2017) was used with Bray-Curtis dissimilarity and 999 permutations.

Associations between background, dependent, and main independent variables were investigated using Pearson's correlation coefficients, ANOVA or a two groups unpaired t-test, Wilcoxon's rank-sum test, Kruskal-Wallis H test, Spearman's rank correlation coefficient, and $\chi 2$ test depending on the variable type and distribution. Linear regression models were used to investigate cluster membership or diversity associations when adjusting for selected covariates. Likewise, potential sex interactions were investigated by including an interaction term to the model (cluster × sex or diversity × sex). In Study I, sex-stratified analyses were performed to screen sex-specific associations between fecal

microbiota composition and temperament traits in a similar manner as the previous study of the same topic (Christian *et al.*, 2015). In Study II, a potential sex interaction in the associations between microbiota and attention bias measures was investigated by including an interaction term in the DESeq2-model or the linear regression, when statistically significant associations were observed in the primary model. In Study III, a potential sex interaction in the associations between microbiota and volume of amygdala was investigated by including an interaction term in the DESeq2-model or the linear regression, when statistically significant associations were observed in the primary model.

In Study II, a random forest regression model was built with 501 trees using the randomForest R package (Liaw & Wiener, 2002) to check the robustness of genera-phenotype associations to the computational method. A matrix of centered log ratio (clr-) transformed genera abundances was used as an input, and the most important features were quantified as unit increase in Mean Squared Error. Sensitivity analysis were performed by including gestational age as a covariate in the models, as gestational age (i.e., premature, term, post-term) may cause an important variation both to the composition of the fecal microbiota (Fouhy *et al.*, 2019) and to the development of attention problems (Eryigit-Madzwamuse and Wolke, 2015). In Study III, the robustness of statistically significant findings, defined as $p < 0.05$, were validated excluding non-breastfed and C-section-born infants from the analyses, as they cause great variation to the fecal microbiota composition (Stewart *et al.*, 2018) in addition to a leave-one-out cross-validation test. In Study IV, a graded adjustment was performed by controlling additionally antibiotic use and postnatal maternal psychological symptoms. Graded adjustment was performed with the available postnatal psychological distress measures by including the corresponding postnatal scores from children aged three months whenever available, i.e., the models, which included prenatal EPDS or SCL as the main predictor, were additionally controlled for the corresponding postnatal measure.

In Study I, covariates were selected based on theoretical assumptions derived from existing literature and association analyses of the current data set due to the exploratory nature of the study. Based on earlier literature, we considered infant sex (Else-Quest *et al.*, 2006; Clarke *et al.*, 2013), mode of delivery (Adler and Wong-Kee-You, 2015; Rutayisire *et al.*, 2016), gestational age (Hill *et al.*, 2017; Fouhy *et al.*, 2019), infant age during fecal sampling (Bäckhed *et al.*, 2015; Stewart *et al.*, 2018), antibiotic treatments (Yassour *et al.*, 2016; Leclercq *et al.*, 2017), and breastfeeding (Jonas *et al.*, 2015; Stewart *et al.*, 2018) as covariates. A stepwise procedure was applied for genus-level differential abundance testing. First, the models were adjusted for a subset of the prespecified covariates such as age, sex, and mode of delivery and then controlled for with all the pre-specified covariates such as age, sex and mode of delivery, gestational age, antibiotic treatments, and

breastfeeding. Covariates included in the linear regression models assessing alpha diversity were sex, the mode of delivery, gestational age, the infant age during sampling, antibiotic treatments, and breastfeeding at 2.5 months of age. During the time of conducting Study I, there was no reliable *a priori* information on the covariate selection for cluster membership, and here, exclusively data-driven covariate screening was employed. The models utilizing fecal microbiota clusters were adjusted for covariates associated with cluster membership.

In Study II, covariate candidates associated with either the face or fear bias or alpha or beta diversity were included in the adjusted models in addition to infant sex, as one of the aims in Study II was to assess the interaction by sex. In Study III and IV, covariates were chosen *a priori*. Study III consisted of a smaller sample size, and hence, data-driven covariate selection might miss important covariates due to a low power. Infant sex, breastfeeding, delivery mode and age during fecal sampling, age from conception during scan, and intracranial volume were included as covariates in Study III (Clarke *et al.*, 2013; Stewart *et al.*, 2018; Pulli *et al.*, 2019). Study IV was confirmatory and included infant sex, breastfeeding, delivery mode, and age during fecal sampling as covariates.

In Study I, missing covariate data, such as birth weight, n = 2; antibiotic intake, n = 1; and mode of delivery, n = 1, was imputed with the R package mice (Buuren and Groothuis-Oudshoorn, 2011). In Studies II and III, subjects with missing data for covariates, for main independent, or for dependent variables were excluded. In Study IV, missing PPD questionnaire data (n = 398, population with at least 50% PPD questionnaire data available, 9% of the entire questionnaire matrix) (Table 8) was imputed with the R package missForest (Stekhoven and Bühlmann, 2011).

In Studies I, II, and IV, p-values were adjusted for multiple testing using the Benjamini & Hochberg method (R function p.adjust), which provides estimates of the False Discovery Rate (FDR). Due to the exploratory nature of Study I, findings with a FDR < 0.25 were considered as significant. In Study II and IV, findings with a FDR $\leq$ 0.01 and, when applicable, absolute log2 Fold Change > 1 were considered as significant, as they were confirmatory studies on infant self-regulation and fecal microbiota and prenatal maternal distress and infant fecal microbiota, respectively. In Study III, findings with $p < 0.05$ were considered significant due to the exploratory nature of the study.

4.8 Ethical Considerations

The studies have been approved by the Ethics Committee of the Hospital District of Southwest Finland. Written informed consent was obtained from all families, and mothers provided written informed consent on behalf of their infants. The Finnish legal regulations were followed in this study.

5 Results

5.1 Fecal Microbiota Composition

Five different bacterial phyla being Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, and Verrucomicrobia and 64 different genera were detected in the population (n = 445). The most abundant identified genera were *Bacteroides* (mean relative abundance = 21%, SD ± 0.3, range = 0-87%), *Bifidobacterium* (mean relative abundance = 19%, SD ± 0.2, range = 0-98%), *Veillonella* (mean relative abundance = 12%, SD ± 0.2, range = 0-98%), *Clostridium* (mean relative abundance = 7.8%, SD ± 0.2, range = 0-91%), and *Parabacteroides* (mean relative abundance = 3.3%, SD ± 0.09, range = 0-76; Fig. 6.) Further, the most prevalent genera (prevalence > 95%) were the unidentified genus in the *Enterobacteriaceae* family, *Bacteroides, Bifidobacterium, Veillonella, Clostridium, Streptococcus, Citrobacter, Staphylococcus*, and *Enterococcus*. The most abundant group was the unidentified genus from the *Enterobacteriaceae* family (mean relative abundance = 21%, SD ± 0.2, range = 0–97%). Moreover, genus level taxonomic annotation was not possible for 16 of the 64 detected genera (25%).

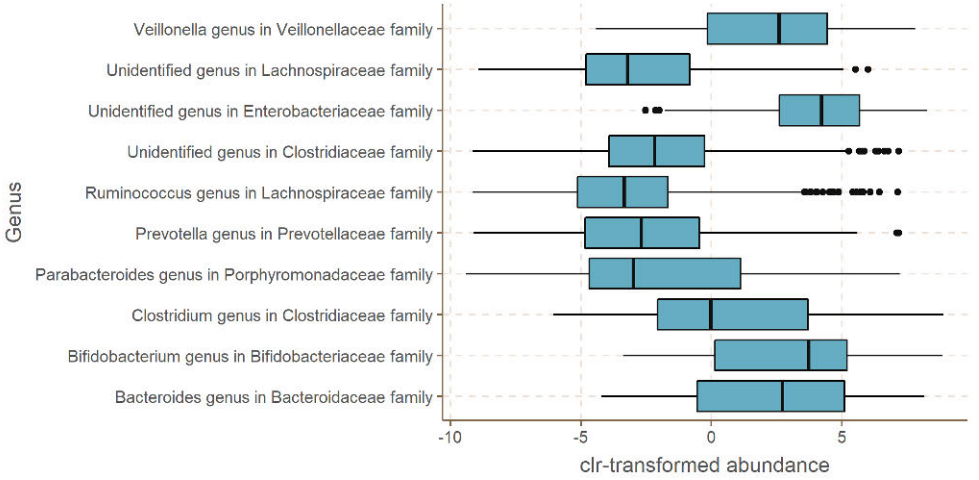

Figure 6. The distribution of ten most abundant genera in 2.5-month-old infants. For illustration, centered log ratio (CLR)-transformed abundances are used.

5.2 Infant Temperament Associates with Fecal Microbiota Composition and Diversity (Study I)

5.2.1 Participant Characteristics and Temperament

Sample characteristics and sex differences in the background and the temperament variables are presented in Table 2. Temperament traits were not associated with the other selected background factors, such as gestational age, the mode of delivery, the birth weight, the mother's education, the BMI, the age, breastfeeding at the age of 2.5 months, or antibiotics intake (FDR ≥ 0.42) except for sex.

Table 2. Study I participant characteristics and temperament traits by infant sex. Temperament traits are organized by main domains. Only FDR values < 0.25 are reported. Gestational weeks = gwk. From original Publication I.

Mean/Count (SD/%)		**Overall**	**Boys**	**Girls**	**FDR**
		n = 301	n = 159 (52.8%)	n = 142 (47.2%)	
Mothers age, years		30.8 (4.3)	31.0 (4.4)	30.6 (4.1)	
Mothers education, n					
	upper secondary	67 (22.3%)	33 (20.8%)	34 (23.9%)	
	vocational school	97 (32.2%)	49 (30.8%)	48 (33.8%)	
	tertiary education	128 (42.5%)	71 (44.7%)	57 (40.1%)	
	missing data	9 (3%)	6 (3.8%)	3 (2.1%)	
Gestational age, weeks		40.2 (1.4)	40.1 (1.5)	40.3 (1.2)	
Gestational stage					
	preterm, < 37 gwk	12 (4%)	9 (5.7%)	3 (2.1%)	
	early term, < 39 gwk	36 (12%)	23 (14.5%)	13 (9.2%)	
	full term, < 40 gwk	69 (22.9%)	37 (23.3%)	32 (22.5%)	
	late term, < 42 gwk	93 (30.9%)	42 (26.4%)	51 (35.9%)	
	post term, ≥ 42 gwk	91 (30.2%)	48 (30.2%)	43 (30.3%)	
	missing data	0 (0%)	0 (0%)	0 (0%)	
Birth weight, g		3622.5 (455.0)	3680.0 (482.6)	3558.1 (414.1)	0.15
	missing data	1 (0.3%)	0 (0%)	1 (0.7%)	
Vaginal delivery, n		248 (82.4%)	133 (83.6%)	115 (81%)	
	missing data	2 (0.7%)	0 (0%)	2 (1.4%)	

Mean/Count (SD/%)		Overall	Boys	Girls	FDR
Breastfeeding, n					
	exclusive breastfeeding	236 (78.4%)	121 (76.1%)	115 (81%)	
	partial breastfeeding	47 (15.6%)	30 (18.9%)	17 (12%)	
	ceased before 2.5 months age	13 (4.3%)	7 (4.4%)	6 (4.2%)	
	no breastfeeding	5 (1.7%)	1 (0.6%)	4 (2.8%)	
	missing data	0 (0%)	0 (0%)	0 (0%)	
Antibiotic treatments, n		37 (12.3%)	26 (16.4%)	11 (7.7%)	0.15
	missing data	1 (0.7%)	0 (0%)	1 (0.7%)	
Infant age during sampling, days		65.2 (13.4)	64.8 (13.3)	65.7 (13.5)	
Surgency /positive emotionality		4.8 (0.7)	4.8 (0.7)	4.8 (0.7)	
	activity level	4.5 (1.0)	4.5 (1.0)	4.5 (1.0)	
	smiliness	6.1 (0.8)	6.2 (0.7)	6.0 (0.8)	
	high intensity pleasure	4.3 (1.2)	4.3 (1.1)	4.3 (1.2)	
	vocational reactivity	4.4 (1.0)	4.3 (1.1)	4.4 (1.0)	
Negative emotionality		3.1 (0.8)	3.1 (0.8)	3.1 (0.8)	
	distress to limitations	3.5 (1.1)	3.5 (1.1)	3.5 (1.1)	
	fear	2.6 (1.2)	2.4 (1.2)	2.7 (1.1)	0.14
	falling reactivity	5.2 (1.0)	5.2 (0.9)	5.2 (1.0)	
	sadness	3.6 (1.1)	3.5 (1.2)	3.6 (1.1)	
Regulation /Orienting		5.3 (0.6)	5.3 (0.6)	5.4 (0.6)	
	cuddliness	5.8 (0.8)	5.8 (0.9)	5.9 (0.7)	
	soothability	6.1 (0.7)	6.1 (0.7)	6.1 (0.7)	
	duration of orienting	4.3 (1.3)	4.2 (1.2)	4.3 (1.3)	
	low intensity pleasure	5.2 (1.0)	5.1 (1.0)	5.3 (1.0)	
	perceptual sensitivity	3.9 (1.6)	3.8 (1.6)	3.9 (1.6)	

5.2.2 Clusters in Infant Fecal Microbiota Associate with Temperament

The cluster analysis of fecal microbiota composition suggested the presence of three distinct community types (Fig. 7). The five most discriminating OTUs for each cluster were *V. dispar* with three different OTU's annotated as *V. dispar*; *Enterobacteriaceae*; *Clostridium neonatale*, named as *V. dispar*-cluster, 27.9%; *Bacteroides* (×4); *Bacteroides fragilis*, named as *Bacteroides*-cluster, 34.0%; *Bifidobacterium*; *Enterobacteriaceae* (×4), named as *Bifidobacterium*/ *Enterobacteriaceae*-cluster, 38.4%. The *Bacteroides*-cluster had the highest and

the *V. dispar*-cluster had both the lowest microbiota richness (Kruskall-Wallis H test $\chi 2 = 39$, FDR < 10-8) and diversity (Kruskall-Wallis H test $\chi 2 = 50$, FDR = <10-10).

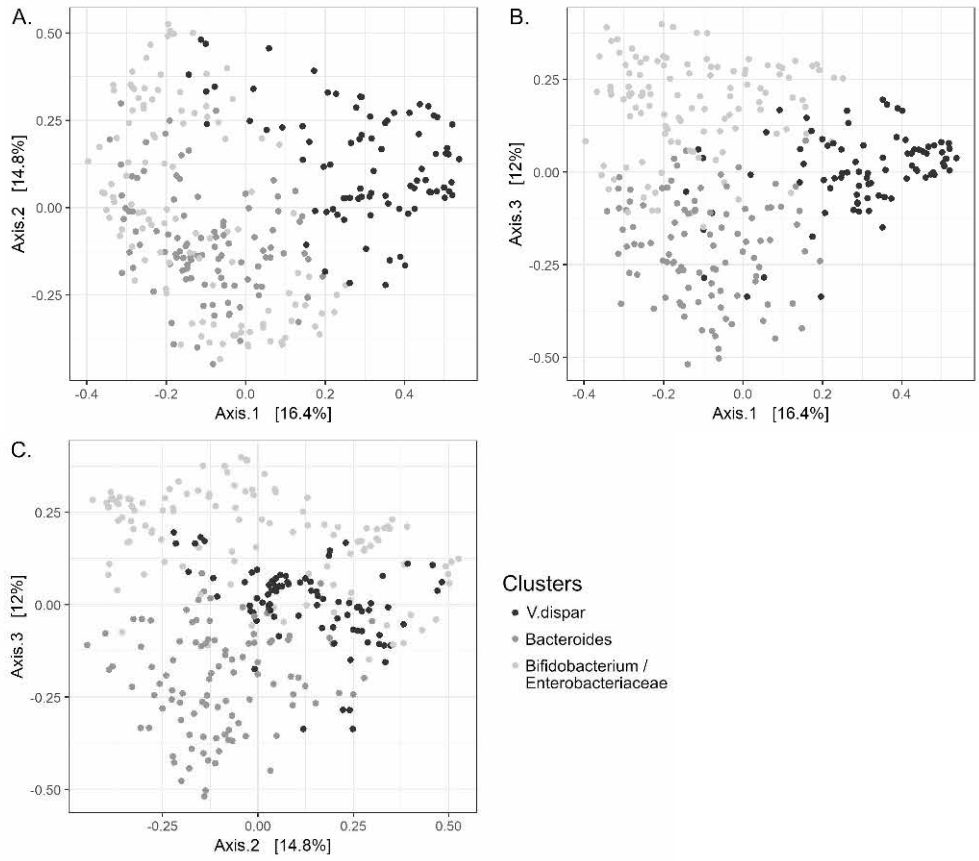

Figure 7. Three GM clusters (*V. dispar, Bacteroides, Bifidobacterium / Enterobacteriaceae*) were identified in the study population. Clusters illustrated here in PCoA plot in three dimensions: A) axes 1 and 2, B) axes 1 and 3, and C) axes 2 and 3. From original Publication I.

The clusters correlated to some of the temperament traits, as the *Bifidobacterium*/*Enterobacteriaceae*-community presented with the highest scores and the *Bacteroides*-cluster with the lowest scores in the temperament trait of regulation (Kruskall-Wallis H test $\chi 2 = 5.8$, FDR = 0.23), and subscales of high intensity pleasure (Kruskall-Wallis H test $\chi 2 = 6.3$, FDR = 0.23), cuddliness (Kruskall-Wallis H test $\chi 2 = 7.9$, FDR = 0.20), and duration of orienting (Kruskall-Wallis H test $\chi 2 = 7.5$, FDR = 0.20). Regarding the background factors, the *Bacteroides*-cluster had the lowest proportion (3%), and the *V. dispar*-cluster had

the highest proportion (32.1%) ($\chi2$ = 27.9, FDR < 10-5) of infants born with C-section. There was a higher share of females in the *Bacteroides*-cluster (59.4%, $\chi2$ = 9.1, FDR = 0.057).

The *Bacteroides*-cluster was associated negatively with the main dimension of regulation (β = -0.18, FDR = 0.17, adjusted R^2 = 0.023; Table 3) and its subscale duration of orienting (β = -0.51, FDR = 0.008, adjusted R^2 = 0.020; Table 3) when contrasted with the *Bifidobacterium*/*Enterobacteriaceae*-cluster and adjusting for sex and the mode of delivery. In addition, the *V. dispar*-cluster was negatively associated with regulation (β = -0.22, FDR = 0.09, adjusted R^2 = 0.023) and cuddliness (β = -0.29, FDR = 0.009, ΔR^2 = 0.016; Table 3) in comparison with the *Bifidobacterium/Enterobacteriaceae*-cluster.

When contrasted with the *Bifidobacterium*/*Enterobacteriaceae*-cluster, the *V. dispar*-cluster was differentially associated with the duration of orienting (sex × cluster interaction, FDR = 0.16) between boys and girls. Additionally, sex differences were noted in the associations between *V. dispar*- and *Bacteroides*-cluster in surgency (FDR = 0.16), regulation (FDR = 0.07), duration of orienting (FDR = 0.03), and cuddliness (FDR = 0.03). Further, there was a sex difference in the association between the *Bifidobacterium*/*Enterobacteriaceae*- and the *Bacteroides*-cluster in fear reactivity (FDR = 0.16) (Table 3).

5.2.3 Fecal Microbiota Alpha Diversity Associates with Infant Temperament

Neither alpha diversity nor richness were associated with any of the temperament traits in the unadjusted analyses. When adjusted for gestational age, infant age, sex, mode of delivery, breastfeeding, and antibiotics intake, diversity was negatively associated with negative emotionality (β = -0.17, FDR = 0.17, adjusted R^2 = 0.016) and fear reactivity (β = -0.27, FDR = 0.17, adjusted R^2 = 0.032) with small effect sizes (Table 3). No sex differences were observed. Richness was not associated with temperament traits in the adjusted models.

Table 3. Linear regression models for each temperament trait and fecal microbiota composition parameter. All main dimensions being surgency, regulation and negative emotionality, and fear reactivity were investigated in the regression models as well as temperament traits associating in the exploratory analyses. Duration of orienting, cuddliness, and high intensity pleasure were included in the regression models, because they showed associations with clusters. Richness was not associated with temperament traits (p-value > 0.2). [a] Linear regression models assessing clusters as main independent variable were adjusted for infant, sex, and mode of delivery, as those covariates had different distribution among clusters. [b] Linear regression models assessing diversity as main independent variable were adjusted for gestational age, infant age, infant sex, mode of delivery, antibiotics intake, and breastfeeding status. [c] Estimates and FDR reported for interaction term (cluster x sex or Shannon Index x sex). From original Publication I.

Adjusted regression models		**Surgency**	**Regulation**	**Negative emotionality**	**Fear reactivity**	**Duration of Orienting**	**Cuddliness**	**High Intensity Pleasure**
Clusters[a]								
Bifidobacterium/Enterobacteriaceae - Bacteroides	β	-0.07	-0.18	-0.20	-0.25	-0.51	-0.08	-0.16
	FDR	0.57	0.17	0.27	0.27	0.08	0.57	0.27
Bifidobacterium/Enterobacteriaceae - V.dispar	β	-0.16	-0.22	-0.07	-0.12	-0.30	-0.29	-0.20
	FDR	0.27	0.09	0.59	0.57	0.27	0.09	0.27
V.dispar - Bacteroides	β	0.09	-0.03	-0.13	-0.13	-0.21	-0.21	-0.01
	FDR	0.57	0.76	0.51	0.57	0.51	0.51	0.95
Diversity[b]	β	0.00	0.05	-0.17	-0.27			
	FDR	0.97	0.71	0.17	0.17			
Sex-interactions								
Clusters[c]								
Bifidobacterium/Enterobacteriaceae - Bacteroides	β	0.29	0.24	0.29	0.64	0.35	-0.14	0.09
	FDR	0.37	0.37	0.38	0.16	0.54	0.74	0.74
Bifidobacterium/Enterobacteriaceae - V.dispar	β	-0.14	-0.23	0.10	0.15	-0.79	-0.13	0.02
	FDR	0.74	0.38	0.74	0.74	0.16	0.74	0.95
V.dispar - Bacteroides	β	0.43	0.46	0.19	0.49	1.14	1.14	0.08
	FDR	0.16	0.07	0.70	0.37	0.03	0.03	0.77
Diversity[c]	β	0.01	0.06	0.01	0.02			
	FDR	0.96	0.96	0.96	0.96			

5.2.4 Fecal Microbiota Composition Associates with Infant Temperament

When controlling for infant age at the time of sample collection, infant sex and mode of delivery, the temperament trait surgency was associated negatively with the genus *Atopobium* and positively both with the genera *Bifidobacterium* and *Streptococcus* (Fig. 8, Table 4). Regulation was positively associated with *Erwinia*. Negative emotionality and fear reactivity were positively associated with *Erwinia*, *Rothia*, and *Serratia* with fear reactivity additionally correlating positively with *Peptinophilus* and *Atopobium* (Table 4, Fig. 5). When controlling for sex, mode of delivery, gestational age, infant age during sampling, antibiotic treatments, and breastfeeding status at 2.5 months of age, only the positive associations between *Erwinia* and regulation (log2 Fold Change = 1.07, FDR = 0.0087, Table 4) and *Streptococcus* and surgency (log2 Fold Change = 0.63, FDR = 0.18, Table 4) remained.

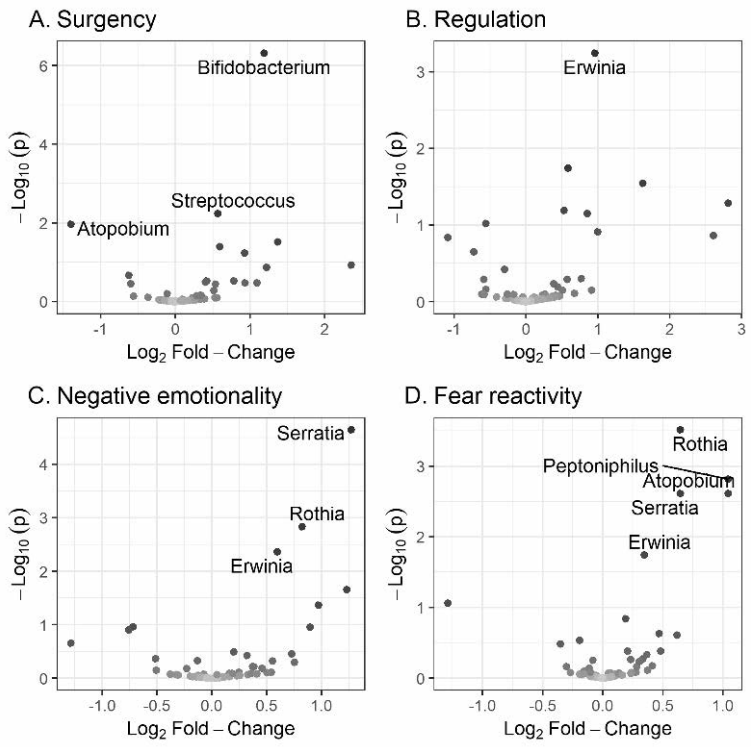

Figure 8. A volcano plot on the association between A) surgency, B) regulation, C) negative emotionality, D) fear reactivity, and genera in the whole population when controlling for infant age, sex, and birth mode. X-axis is binary logarithm of abundance fold change (log2 Fold Change) and Y-axis stands for decimal logarithm of p-value. Only the associations with FDR < 0.25 are labeled. From original Publication I.

Table 4. Genera associated with the temperament. A step-wise adjustment strategy was used when assessing temperament-genus associations. First, the analyses were adjusted for infant age, sex, and mode of delivery[A]. The second analyses were adjusted for infant age, sex, mode of delivery, gestational age, breastfeeding, and antibiotics intake (full model)[B]. Null-findings are not presented. From original Publication I.

	Genus	Baseline Mean Abundance	Log2 Fold Change	FDR
Surgency[a]				
	Atopobium	23	-1.4	0.247
	Streptococcus	4584	0.6	0.200
	Bifidobacterium	85872	1.2	$3.4x10^{-5}$
Surgency: full model[b]				
	Streptococcus	4584	0.6	0.177
Regulation[a]				
	Erwinia	96	1.0	0.040
Regulation: full model[b]				
	Erwinia	96	1.1	0.009
Negative emotionality **[a]**				
	Erwinia	96	0.6	0.099
	Rothia	71	0.8	0.050
	Serratia	51	1.3	0.002
Fear reactivity [a]				
	Erwinia	96	0.3	0.247
	Rothia	71	0.6	0.021
	Serratia	51	0.6	0.042
	Peptoniphilus	93	1.0	0.042
	Atopobium	23	1.0	0.042

5.2.5 Sex-Specific Associations

OTU-temperament associations showed differential findings in analyses stratified by infant sex when controlling for the mode of delivery, gestational age, infant age during sampling, antibiotic treatments, and breastfeeding status at 2.5 months of age. Boys presented associations with surgency and several OTUs annotated as *Bifidobacterium* (data shown in the original publication). Further, in boys, regulation was negatively associated with several OTUs annotated as *Veillonella* and positively with *Bifidobacterium* and *Clostridiaceae*. In girls, both negative and positive associations with fear reactivity and *Veillonella* were noted (data shown in the original publication). Sex-stratified analyses did not show any associations at the genus level.

5.3 Infant Fecal Microbiota Composition Associates with Attention to Fearful Faces (Study II)

Sample characteristics are presented in Table 5. Nine infants with missing data in the covariates were excluded from the analyses resulting in a final sample size of 122 (boys, n = 65, 53%). The delivery mode was associated with the Shannon Index (unadjusted, p = 0.02), Chao1 (unadjusted, p = 0.001) as well as beta diversity (unadjusted, p = 0.003). The Shannon Index was associated with breastfeeding (unadjusted, p = 0.01) and age at fecal sampling (unadjusted, p = 0.02).

All infants disengaged their attention most probably from control pictures, following neutral, happy, and fearful faces. There was also an age-typical bias for faces (i.e., face bias) and for fear (i.e., fear bias) across the whole sample population. Boys and girls did not differ in any of the attention variables with all unadjusted p-values > 0.15 for two-sided t-tests. Fear bias was positively associated with maternal depressive symptoms at gwk 34 (r = 0.22, p = 0.01). Face bias was not related to any of the pre-selected background variables (i.e., maternal age and education, BMI, prenatal SSRI/SNRI use, depressive symptoms at the end of pregnancy, infant gestational age at birth, birth length and height, age at fecal sampling, breastfeeding status, infant antibiotic intake, and mode of delivery).

Variables associated with either Shannon Index, Chao1, face or fear biases were included as covariates. In addition, as we aimed to assess sex-interactions, infant sex was included as a covariate. Hence, mode of delivery, breastfeeding, infant age at fecal sampling and maternal depressive symptoms at the end of pregnancy and infant sex were included as covariates.

Table 5. Mother and infant characteristics in Study II. From original Publication II.

Variable	Categories / units	n = 131
Mother's age at birth	mean (SD) years	31 (4)
Mother's education	n (%)	
	secondary or primary level	39 (29.8)
	vocational tertiary	43 (32.8)
	university	47 (35.9)
	missing data	2 (1.5)
Mother's SSRI/SNRI use in the beginning of pregnancy	n (%)	
	no	123 (93.9)
	yes	5 (3.8)
	missing data	3 (2.3)
Infant's sex	n (%)	
	boy	69 (52.7)
	girl	62 (47.3)
Mother's BMI in the beginning of pregnancy	n (%)	
	BMI <25 (normal weight)	78 (59.5)
	BMI 25-30 (overweight)	38 (29)
	BMI >30 (obese)	14 (10.7)
	missing data	1 (0.8)
Gestational weeks at birth	mean (SD) weeks	40 (1)
Prematurity	n (%)	
	Premature (< 37 gestational weeks)	2 (1.5)
	Term (37–42 gestational weeks)	100 (76.3)
	post term (≥ 42 gestational weeks)	29 (22.1)
Birth weight	mean (SD) grams	3632 (456)
Mode of delivery	n (%)	
	C-section	25 (19.1)
	vaginal	105 (80.2)
	missing data	1 (0.8)
Breastfeeding at the time of fecal sampling	n (%)	
	never breastfed	2 (1.5)
	breastfeeding ceased	7 (5.3)
	partial breastfeeding	19 (14.5)
	exclusive breastfeeding	99 (75.6)
	missing data	4 (3.1)
Infant's antibiotic courses	n (%)	0 (0)
Infant's age at the fecal sampling	mean (SD) days	69 (14)
EPDS total score sum	mean (SD)	
	at the beginning of pregnancy	5 (5)
	at the end of pregnancy	5 (5)

BMI: Body Mass Index; kg/m^2
EPDS: Edinburgh Postnatal Depression Scale

5.3.1 Diversity and Fear and Face Bias

Neither Shannon Index (fear bias, p = 0.70: face bias, p = 0.30), Chao1 (fear bias, p = 0.34, face bias, p = 0.41), nor beta diversity (fear bias, p = 0.23: face bias, p = 0.96) were associated with fear or face-bias scores. Further, no sex interaction with either face (Shannon Index unadjusted, p = 0.26; Chao1 unadjusted, p = 0.85 for interaction terms) or fear bias (unadjusted, p = 0.57; Chao1 unadjusted, p = 0.19 for interaction terms) was observed.

5.3.2 Genus-Level Associations with Fear and Face Bias

Abundances of *Lactobacillus* from the phylum Firmicutes, *Bifidobacterium* from the phylum Actinobacteria, *Prevotella* from the phylum Bacteroidetes, and *Haemophilius* from the phylum Proteobacteria were negatively associated with fear bias (Table 6, Fig. 9). The *Clostridium* genus from phylum Firmicutes was positively associated with fear bias (Table 6, Fig. 9). Fear bias did not show a sex interaction with genera (FDR > 0.015). The random forest model showed that the five most important features related to fear bias were *Bifidobacterium, Actinomyces, Clostridium, Collinsella*, and *Parabacteroides.*

Table 6. Genera associated with fear bias when adjusting for mode of delivery, breastfeeding, sex, age at sampling, and EPDS at the end of pregnancy.

Genus	Baseline Mean Abundance (counts)	log2 Fold Change	FDR	95% Confidence Interval
Bifidobacterium	56593	-5.3	0.0003	-7.8 to -2.8
Prevotella	38651	-5.7	0.0010	-8.8 to -2.7
Lactobacillus	2598	-6.5	0.0003	-9.4 to -3.5
Clostridium	41276	5.4	0.0010	2.5 to 8.2
Haemophilus	1216	-6.8	0.0010	-10.4 to -3.2

EPDS: Edinburgh Postnatal Depression Scale

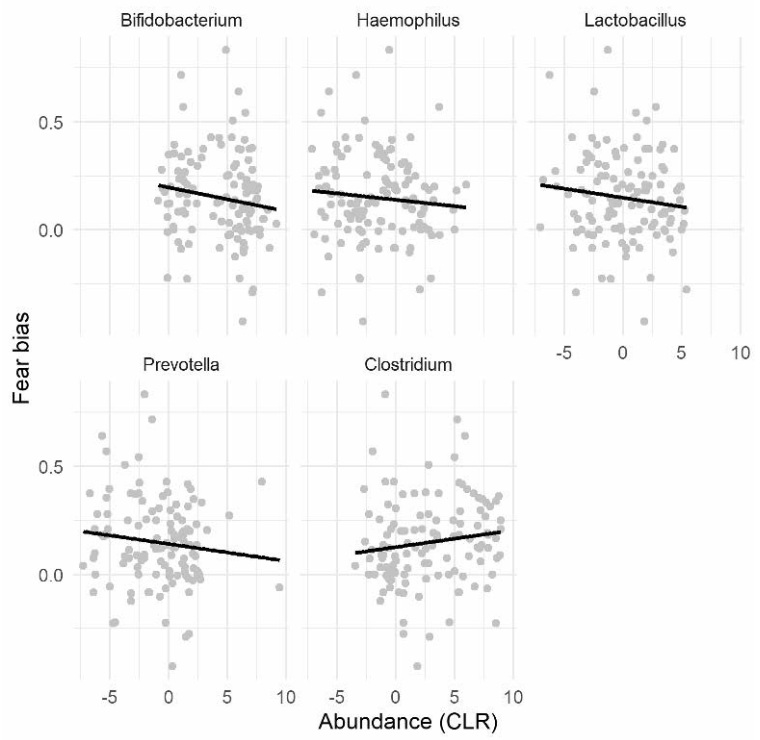

Figure 9. Fear bias associations with genera. For illustration, CLR-transformed abundances are used. From original Publication II.

Face bias was not associated with the tested genera, but *Clostridium* (log2 Fold Change = -9, FDR = 0.0017), *Parabacteroides* (log2 Fold Change = -13, FDR = 0.0009), an unidentified genus in the *Lachnospiraceae* family (log2 Fold Change = 12, FDR = 0.0017), *Collinsella* (log2 Fold Change = 18, FDR = 0.00003), and *Citrobacter* (log2 Fold Change = 8, FDR = 0.0047) showed a sex interaction with face bias (Fig. 10).

5.3.3 Sensitivity Analyses

Including prematurity as a covariate, the association patterns regarding log2 Fold Change direction and value of FDR stayed the same, except that additionally a new association between fear bias and *Enterococcus* (log2 Fold Change = -6, FDR = 0.0078) was observed.

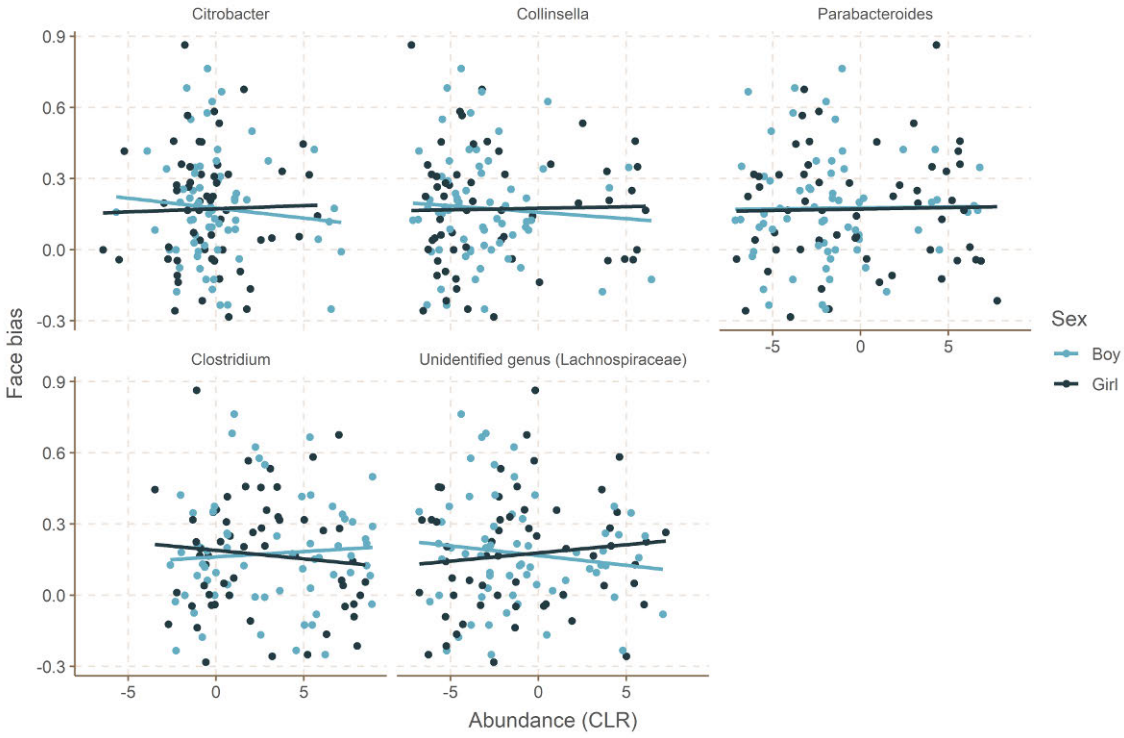

Figure 10. Genera interaction by sex with face bias. For illustration, CLR-transformed abundances are used. From original Publication II.

5.4 Neonatal Amygdala Volume Associates Negatively with Fecal Microbiota Alpha Diversity (Study III)

In Study III, the mean age of the mothers was 29 ± 5 years and mean pre-pregnancy body mass index was 24 (SD ± 4). Eighteen mothers (32%) had prescription medication during the 3rd trimester, and nine mothers reported SSRI/SNRI use during pregnancy (16%). Two mothers (4%) reported smoking, and four mothers (7%) reported alcohol consumption at the end of the pregnancy, and none reported illicit drug use. Mean gestational age was 40 ± 1 weeks, all infants were born normal weight (3449 ± 385 grams), and three infants were delivered with caesarean section (5%). Most infants were breastfed during stool sampling with exclusive breastfeeding: 77%, mixed feeding: 16%, no breastfeeding: 3.5%, and those with missing information: 3.5%. Five infants (9%) had received antibiotic treatment at the neonatal ward. Four subjects were missing covariate data and were excluded from the analyses resulting in a sample size of n = 52.

There were no zero-order associations between infant amygdala volumes and alpha diversity metrics (all, $p > 0.17$). A small, negative association was detected between infant left amygdala volume and alpha diversity ($n = 52$, $\beta = -0.0043$, $p = 0.034$, adjusted for infant sex, breastfeeding, delivery mode, and age during fecal sampling, age from conception during scan, and intracranial volume, Fig.11 as well as in the subpopulation of vaginally born breastfed infants ($n = 48$, $\beta = -0.0049$, $p = 0.017$ when adjusting for intracranial volume, exclusive vs. mixed feeding, infant sex, age during sampling, and age from conception during scan). The age during fecal sampling and age from conception during scan were not correlated thus not creating a collinearity problem. A leave-one-out cross-validation test further indicated that the left but not right amygdala volume significantly improves predictions on gut microbiota diversity in leave-out samples. No interaction with child's sex was observed ($p > 0.3$). No associations were detected between right amygdala volume and diversity ($p = 0.95$) or between richness and amygdala volumes ($p > 0.6$) in similarly constructed linear models. Amygdala volumes were neither associated with beta diversity ($p = 0.21$) nor genus abundances when adjusted for the same covariates.

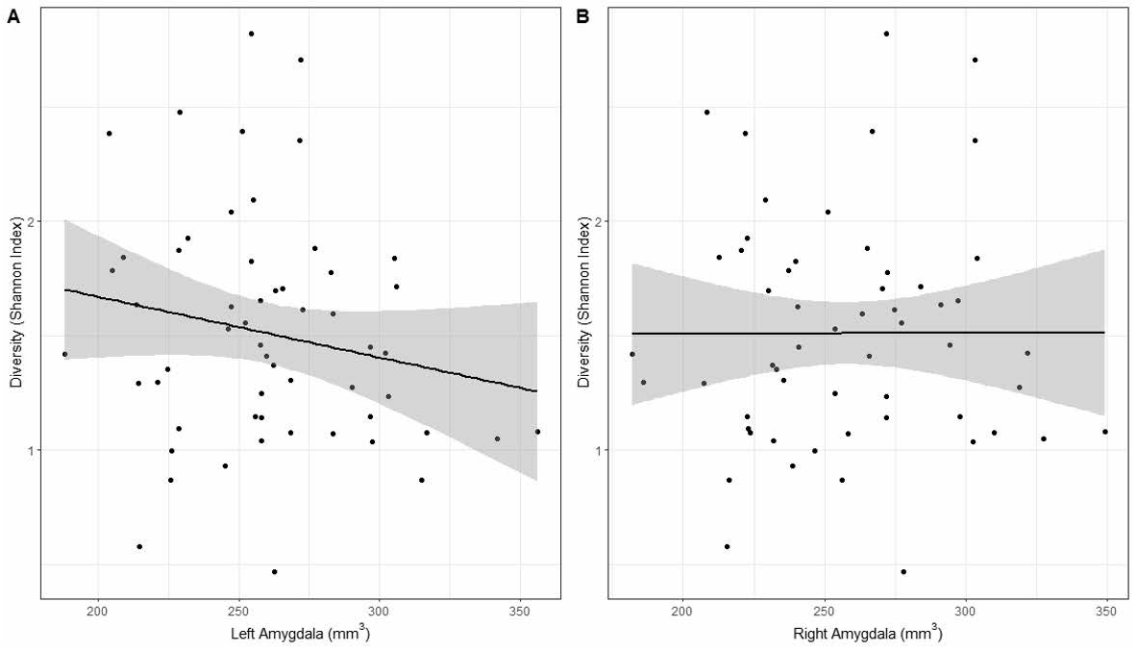

Figure 11. Microbiota alpha diversity associates negatively with left (A) but not with right (B) amygdala volume. The grey areas depict 95% confidence intervals. From original Publication III.

5.5 Maternal Prenatal Stress and Hair Cortisol Concentration Associates with Infant Fecal Microbiota Composition (Study IV)

5.5.1 Participant Characteristic and Descriptive Analyses

Sample characteristic are presented in Table 7 and 8. C-section was more prevalent in the chronically elevated SCL group (FDR = 0.02, $\chi 2 = 9.8$). None of the other selected background variables such as breastfeeding, infant age and sex, infant antibiotic use, mother's SSRI/SNRI use, gestational age at birth, birth weight and height, mother's education, the number of previous deliveries, and maternal pre-pregnancy BMI) differed from the other maternal PPD groups such as chronic EPDS, PRAQ-R2, SCL, and the Daily Hassles negative subscale, (FDR $\geq$ 0.1 for all) or HCC (FDR 0.70-1).

Table 7. Mother and infant characteristics included in the statistical analyses in Study IV that had the complete background information as well as adequate prenatal questionnaires (n = 398) and/or HCC (n = 115, total population in the analyses n = 399). From original Publication IV.

		Whole population	Boy	Girl
		n = 399	n = 204	n = 195
Mother's age	mean (SD), years	30.8 (4.4)	30.9 (4.3)	30.8 (4.5)
Mother's education, count (%)	secondary or primary level	91 (22.8%)	42 (20.6%)	49 (25.1%)
	vocational tertiary	126 (31.6%)	66 (32.4%)	60 (30.8%)
	university	165 (41.4%)	86 (42.2%)	79 (40.5%)
	missing data	17 (4.3%)	10 (4.9%)	7 (3.6%)
SSRI/SNRI use during 1st trimester, count (%)	no	361 (90.5%)	185 (90.7%)	176 (90.3%)
	yes	18 (4.5%)	8 (3.9%)	10 (5.1%)
	missing data	20 (5%)	11 (5.4%)	9 (4.6%)
Mother's prepregnancy BMI	mean (SD), kg/m^2	24.4 (4.6)	23.9 (4.3)	25.0 (4.8)
Gestational age	mean (SD), gwks	40.1 (1.4)	40.0 (1.5)	40.2 (1.2)
Prematurity, count (%)	preterm	14 (3.5%)	11 (5.4%)	3 (1.5%)
	term	281 (70.4%)	143 (70.1%)	138 (70.8%)
	post term	104 (26.1%)	50 (24.5%)	54 (27.7%)
Birth weight	mean (SD), grams	3,626.7 (464.7)	3,699.1 (490.0)	3,551.1 (425.0)
Mode of delivery, count (%)	C-section	68 (17%)	33 (16.2%)	35 (17.9%)
	vaginal	331 (83%)	171 (83.8%)	160 (82.1%)
breastfeeding status, count (%)	none	5 (1.3%)	2 (1%)	3 (1.5%)
	ceased	19 (4.8%)	10 (4.9%)	9 (4.6%)
	partial	62 (15.5%)	35 (17.2%)	27 (13.8%)
	exclusive	313 (78.4%)	157 (77%)	156 (80%)
Infant antibiotic courses, count (%)	no	355 (89%)	174 (85.3%)	181 (92.8%)
	yes	44 (11%)	30 (14.7%)	14 (7.2%)
Age during fecal sampling	mean (SD), days	64.3 (13.4)	63.8 (12.7)	64.9 (14.2)

Table 8. Mean questionnaire sum scores and proportion of missing data for each measurement point and number of subjects scoring above cut-off in at least two measurement points (n = 398) in Study IV. From original Publication IV.

		EPDS	SCL	PRAQ	Daily Hassles negative scale
gwk 14	median (range)	4 (0–26)	2 (0–30)	21 (10–45)	12 (6–19)
	missing data	5%	5%	71%	11%
gwk 24	median (range)	4 (0–21)	2 (0–28)	21 (10–44)	12 (6–18)
	missing data	2%	2%	2%	8%
gwk 34	median (range)	3 (0–20)	2 (0–25)	21 (10–47)	11 (6–19)
	missing data	4%	4%	4%	10%
postnatal	median (range)	4 (0–19)	1 (0–17)	-	-
	missing data	11%	11%		
Cut-off		10	median	median	median
Chronically high*	count (%)	30 (7.5%)	120 (30.2%)	41 (10.3%)	140 (35.2%)

* Subjects scoring above the cut-off in two or more prenatal measurements

5.5.2 Associations between the Maternal Chronic PPD, HCC, and Infant Fecal Microbiota Composition

5.5.2.1 Chronic PPD Symptoms

Abundances of genera from the gram-negative Proteobacteria phylum showed associations with chronically elevated maternal chronic PPD symptoms when adjusting for selected covariates such as infant age and sex, the mode of delivery, and breastfeeding. The Daily Hassles negative subscale associated positively with *Erwinia, Haemophilus,* and *Serratia*; SCL associated positively with *Campylobacter, Citrobacter*, and *Serratia*; EPDS associated negatively with *Desulfovibrio* and positively with *Citrobacter* and *Serratia*; and PRAQ-R2 associated positively with *Camplylobacter, Serratia,* and *Haemophilus* (FDR < 0.01, Fig. 9) when adjusted for infant age and sex, the mode of delivery, and breastfeeding by including the variable as a covariate.

The genera *Veillonella, Finegoldia, Dialister, Dorea,* and *Coprococcus* belonging to Gram-positive Firmicutes as well as *Actinomyces* and *Rothia* belonging to Gram-positive Actinobacteria showed positive associations with maternal chronic PPD measures (Fig. 12). *Akkermansia* (the only genus belonging to phylum Verrucomicrobia), *Pseudoramibacter, Phaslarctobacterium, Megamonas, Megasphera, Eubacterium, Epulopiscium, Anaerotruncus, Pseudoramibacter Eubacterium* (phylum Firmicutes), *Paraprevotella, Parabacteroides, Odoribacter* (gram-negative Bacteroidetes) as well as *Slackia, Actinobaculum*, and *Propionibacterium* (phylum Actinobacteria) showed only negative associations with maternal chronic PPD symptoms (Fig. 12). *Butyricimonas* and *Prevotella* (phylum Bacteroidetes) were positively associated with EPDS (Fig. 12). *Staphylococcus*

(phylum Actinobacteria) was positively associated with SCL and negatively with EPDS (Fig. 12) when adjusted for infant age and sex, the mode of delivery, and breastfeeding by including the variable as a covariate.

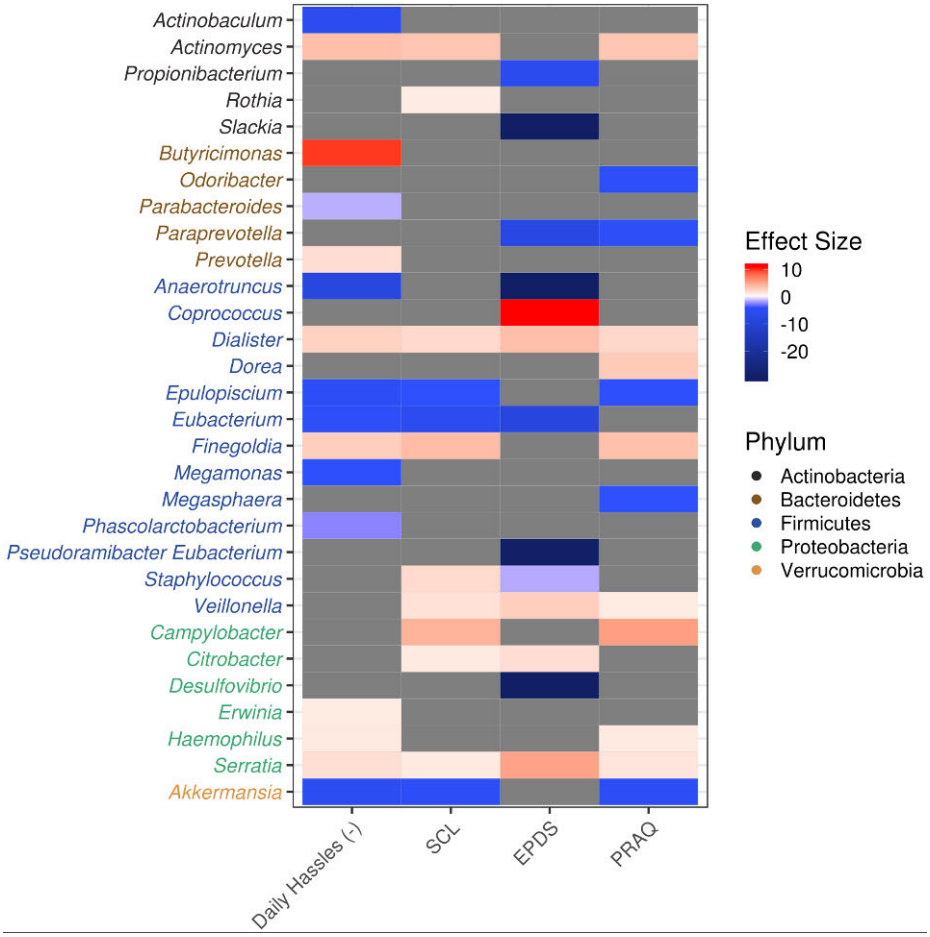

Figure 12. Log2 Fold Changes of genera significantly (FDR < 0.01, absolute log2 Fold Change > 1) associated with chronic PPD symptoms. Gray depicts non-significant findings. From original Publication IV.

Graded adjustment with infant antibiotic intake lead to only minor alterations, as the association between *Dialister* and EPDS as well as *Megasphaera* and PRAQ-R2 attenuated (FDR > 0.01 and when applicable, absolute log2 Fold Change ≤1). Adjusting for postnatal maternal EPDS attenuated the EPDS associations with *Eubacterium*, *Citrobacter*, and *Dialister*, however, the majority of the observed associations between EPDS and genera remained unchanged regarding the direction of the log2 Fold Change and the significance level. Adjusting for postnatal maternal SCL attenuated the observed SCL associations with *Actinomyces*, *Rothia*, *Serratia*, and *Dialister*.

5.5.2.2 Hair Cortisol Concentration

Maternal HCC was associated negatively with the genera *Slackia* and *Actinobaculum* (phylum Actinobacteria), *Paraprevotella*, and *Butyricimonas* (phylum Bacteroidetes), *Citrobacter* (phylum Proteobacteria) as well as *Ruminococcus*, *Phascolarctobacter*, *Anaerotruncus*, *Enterococcus*, and *Lactobacillus* (phylum Firmicutes) (Fig. 13). Graded adjustment with infant antibiotic intake did not change the abovementioned associations as indicated by the direction of the log2 Fold Change and the significance level.

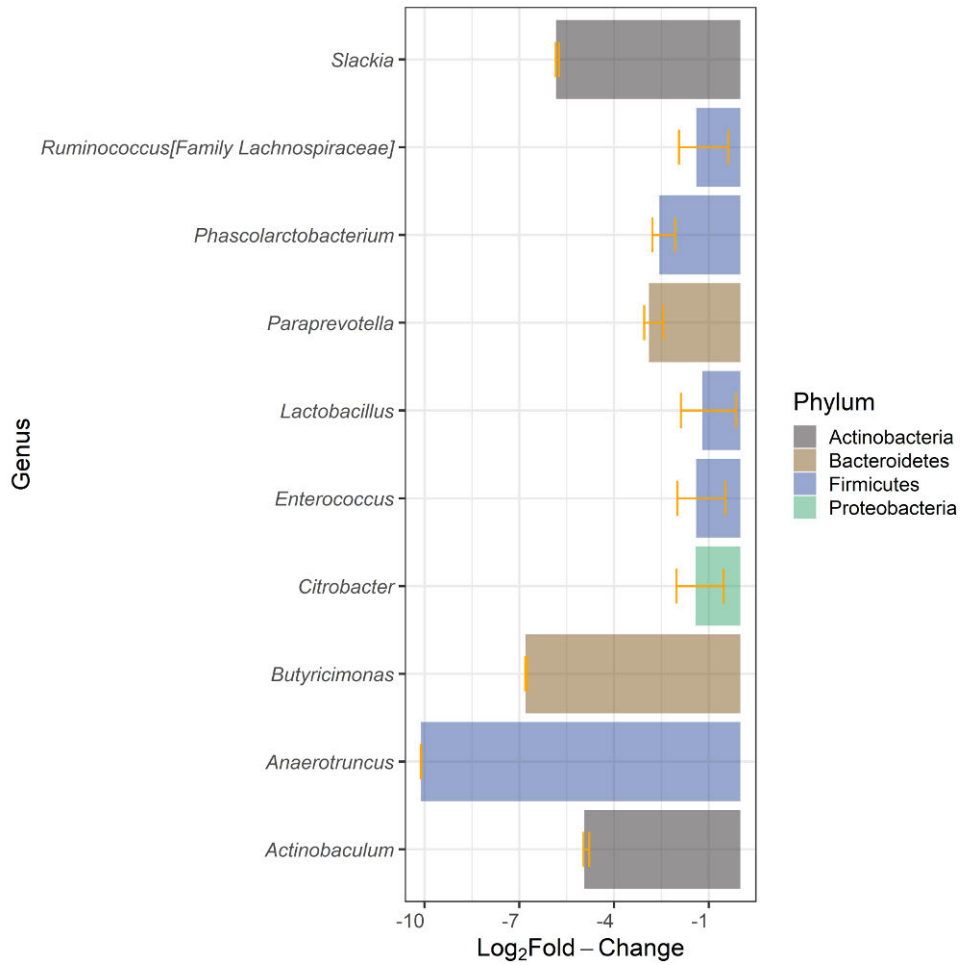

Figure 13. Log2 Fold Changes of genera abundances of genera associated with natural logarithm of HCC as a continuous variable (FDR < 0.01, absolute log2 Fold Change > 1). Error bars show unadjusted 95% confidence intervals of the Log2 Fold Changes derived from normally distributed log2 Fold Change standard errors from DESeq2 model. From original publication IV.

5.5.2.3 Associations between maternal prenatal stress and infant fecal microbiota diversity

Neither maternal HCC of approximately the past five months of pregnancy nor any of the chronic PPD symptom variables were associated with the infant fecal microbiota alpha (Shannon Index and richness, FDR $\geq$ 0.27) or beta diversity when adjusted for infant age and sex, the mode of delivery, and breastfeeding by including the variable as a covariate (FDR $\geq$ 0.97).

5.6 Summary of the Results

- These exploratory results suggest that higher fecal microbiota diversity in infants around the age of 2-3 months could associate with lower fear reactivity and negative emotionality at the age of six months and a smaller left amygdala volume around the age of one month.
- In the exploratory analyses, infant fecal microbiota clusters were associated with temperament, and specifically the infants in the *Bacteroides*-cluster had lower scores of self-regulation and its subscale duration of orienting compared to the infants in the *Bifidobacterium/Enterobacteriaceae*-cluster. Infants in the *V. dispar*-cluster had lower scores in self-regulation and cuddliness in comparison to infants in the *Bifidobacterium/Enterobacteriaceae*-cluster.
- Fecal microbiota composition was associated with temperament traits and emotional attention but not with amygdala volumes.
    - Specifically, in the exploratory analyses regarding temperament, surgency was positively associated with *Atopobium*, *Bifidobacterium*, and *Streptococcus*; regulation was positively associated with *Erwinia*; negative emotionality and fear reactivity were positively associated with *Erwinia*, *Rothia* and *Serratia*; and fear reactivity associated positively with *Peptinophilus* and *Atopobium*.
    - *Bifidobacterium* was negatively associated and *Clostridium* was positively associated with fear bias.
- Sex-interaction was observed regarding temperament and disengagement from faces.
    - Infant microbiota clusters showed interaction by sex regarding temperament traits regulation, surgency, duration of orienting, cuddliness, and fear reactivity in the exploratory analyses. Sex-specific associations between infant microbiota OTU's and the temperament traits surgency, regulation, and fear reactivity were noted.
    - Face bias but not fear bias showed a sex interaction with *Clostridium*, *Parabacteroides*, *Collinsella*, and *Citrobacter*.
- Maternal chronic PPD was associated with increased abundances of genera belonging to the Proteobacteria phylum, and other phyla presented associations with mixed directions.
- Maternal HCC was associated with several genera, e.g., negatively with *Lactobacillus*.

6 Discussion

In this thesis, the main aims were to first explore the associations between early fecal microbiota composition and diversity and infant brain developmental phenotypes such as temperament, emotional attention, and amygdala volumes. The second aim was to investigate how maternal prenatal stress, i.e., chronic PPD and prenatal long-term HPA-axis functioning associates with infant microbiota composition and diversity. The results drawn from the prospective FinnBrain Birth Cohort Study corroborate certain findings from previous literature, and additionally illustrate novel findings potentially related to early microbiota-gut-brain-axis functioning.

6.1 Infant Fecal Microbiota Associations with Brain Development

Early microbiota alterations impact the behavior and neurochemistry at least in rodents (Hoban *et al.*, 2016, 2018; Stilling *et al.*, 2018; Chu *et al.*, 2019; Cryan *et al.*, 2019), and preliminary human studies show that early life microbiota composition or the factors known to affect microbiota composition are associated with behavioral outcomes and psychopathology (Sampson and Mazmanian, 2015; Lavebratt *et al.*, 2019; Loughman *et al.*, 2020a). Nevertheless, the studies in human infants are scarce and deploy a variety of socioemotional and brain phenotypes. Infant temperament is a psychobiological phenotype that is associated with later psychopathology, and the attentional bias to threat acts in conjunction with other risk factors to associate with the socioemotional development (Burris *et al.*, 2019). The amygdala is an important neural structure, which participates in reallocating the attention to emotionally salient information (Tuulari *et al.*, 2020), and it reaches structural maturity early (Ulfig *et al.*, 2003). Furthermore, the amygdala volume and connectivity are potentially associated with a later risk for psychopathology (Qin *et al.*, 2014). The presented results indicate that an infant fecal microbiota characteristic may associate with these developmentally important phenotypes albeit with modest effect sizes. The modest effect sizes are expected due to the multifactorial background of these phenotypes, and for instance, the effect size between fecal microbiota diversity and left amygdala volume is comparable with

that of the prenatal maternal prenatal psychological distress and amygdala volume (Lehtola *et al.*, 2020). The findings from Studies I and III had a less conservative correction for multiple comparison, and thus false-positive findings are more likely than in the Study II. Nevertheless, Study III showed an association between fecal microbiota composition and infant emotional attention, which is a part of the broader construct of self-regulation.

Early self-regulation reportedly predicts better cognitive development and reduces the risk for later psychopathology (Canals *et al.*, 2011; Gartstein and Skinner, 2018). Infants in the *Bifidobacterium / Enterobacteriaceae*-cluster scored higher in self-regulation than infants in the *Bacteroides*- or the *V. dispar*-cluster in our exploratory analyses. The previous work by Carlson et al. noted that the *Bacteroides*-dominated community type at the age of one year would predict better cognitive development at the age of two years (Carlson *et al.*, 2018), and the report seems contradictory to ours, but it must be acknowledged that the results may be sensitive for the timing. However, the preliminary results suggest that a *Bacteroides*-cluster membership associated with lower scores in duration of orienting, and in turn, higher scores in early duration of orienting, which is part of orienting/regulation scale, may be associated with later autism (Zwaigenbaum *et al.*, 2005). This underlines the need for longitudinal, developmentally sensitive studies.

Surgency predicts more extraversion and better self-regulation during toddlerhood (Komsi *et al.*, 2006), potentially resulting in greater social competence (Hayden *et al.*, 2006), and is associated with CNS connectivity in areas related to emotion processing (Hanford *et al.*, 2018) and HPA-axis functioning (Turner-Cobb *et al.*, 2008). In Study I, surgency was suggested to associate with higher abundances of the genera *Bifidobacterium* and *Streptococcus* and a lower abundance of the genus *Atopobium*, although some of the findings attenuated in the stepwise adjustment and should be therefore treated with some caution. Previously, Christian et al. reported associations between several subscales loading to surgency and higher abundance of *Parabacteroides*, *Dialister*, and *Rikenellaceae* only in boys (Christian *et al.*, 2015). The microbiota signatures associating with surgency among toddlers are different from the current study in infants, which are not surprising, as both the temperament assessment and fecal sampling are from different timepoints with differing typical developmental characteristics (Posner *et al.*, 2014; Stewart *et al.*, 2018). However, in the current sample, boys showed associations between several *Bifidobacterium*-annotated OTUs and surgency, partially corroborating the sex-specific associations related to surgency albeit with different taxa than in toddlers. This may be related to the general sex differences in temperament, and the finding that boys typically show more surgency (Else-Quest *et al.*, 2006).

Negative emotionality and fear reactivity, which are temperament traits related to a higher risk for later psychiatric disorders (Nigg *et al.*, 2004; De Pauw and Mervielde, 2010; Sayal *et al.*, 2014), were associated positively with abundances of the genera *Erwinia, Rothia,* and *Serratia,* and fear reactivity was also associated with *Peptinophilus* and *Atopobium* in the explorative analyses. Loughman et al. showed that low *Prevotella* abundance and carriage associated with behavioral problems in a sample of two-year-olds (Loughman *et al.*, 2020a). Although behavioral problems are usually associated with early temperament, the fecal microbiota *Prevotella* carriage was only associated with a lower likelihood of behavioral problems and not the temperament traits (Loughman *et al.*, 2020a). This may imply that different microbiota signatures associated with behavioral problems are not necessarily associated with the potential antecedes of behavioral problems, in this context, the temperament traits. The same may be true for the opposite, as fecal microbiota characteristics related to temperament traits may prove not to be associated with later psychopathology, although this needs to be addressed in future studies. Likewise, even though negative emotionality has been considered a risk factor for later psychopathology, it is also a differential susceptibility factor that may help an individual to benefit from the environment (Pluess & Belsky, 2013), which further emphasizes the complexity of potential mechanisms underlying these observed associations.

An important aspect of early self-regulation is the ability to direct attention to and away from stimuli (Rothbart *et al.*, 2011; Posner *et al.*, 2014). In the Study II, the genera *Lactobacillus, Prevotella,* and *Bifidobacterium* were negatively and *Clostridium* was positively associated with attention bias for fearful faces. Heightened fear bias is a part of typical infant development, and therefore it is very difficult to estimate the level of "normal" and "abnormal" fear biases especially during infancy at the time of its developmental peak (Peltola *et al.*, 2009). However, in some longitudinal infant studies, attention biases for negative information have been found to predict a risk of self-regulation difficulties (Nakagawa and Sukigara, 2012; Morales *et al.*, 2016), especially if consistently high or connected with other risk factors, such as negative emotionality. In a previous study, increased initial attention to and subsequent greater disengagement from fearful faces (i.e., signals of threat in the environment) at the age of seven months was positively associated with prosocial behavior later in life (Grossmann *et al.*, 2018). Therefore, it may be that a low initial orientation towards but a longer dwelling time on fearful faces are unfavorable for socioemotional development. Additionally, the normative bias towards fearful faces during infancy (Peltola *et al.*, 2009) may even predict positive outcomes such as better attachment security in the future (Peltola *et al.*, 2015). Then again, a higher attention bias towards threat at the age of five years was reportedly associated with more internalizing

symptoms in the presence of temperamental behavioral inhibition (Nozadi *et al.*, 2016). Hence, variation in the tendency to disengage from signals of fear may predict differences in socioemotional development especially when combined with other risk factors (Morales *et al.*, 2015). In addition to the assessment of additional risk factors for maladaptive socioemotional development, such as temperament traits, future studies should assess emotional attention longitudinally, as persistently biased attention might be more important for socioemotional development in comparison to transient differences in its magnitude (Pérez-Edgar *et al.*, 2010; Morales *et al.*, 2016).

Administration of *Bifidobacterium* and *Lactobacillus* strains is often associated with changes in rodent behavior (Desbonnet *et al.*, 2008; Bercik *et al.*, 2010; Savignac *et al.*, 2013). In humans, gut microbiota-targeted interventions with pre- or probiotic products, including supplementation with *Bifidobacterium* and *Lactobacillus* strains, have induced changes in self-reported emotional processing, outcomes ranging from reduced cognitive reactivity, a sad mood, and a reduced attentional vigilance to negative information (Schmidt *et al.*, 2015; Steenbergen *et al.*, 2015), and differential activation patterns in brain areas related to emotional processing (Tillisch *et al.*, 2013; Schmidt *et al.*, 2015; Steenbergen *et al.*, 2015; Allen *et al.*, 2016; Pinto-Sanchez *et al.*, 2017; Bagga *et al.*, 2018). Further, probiotic and prebiotic intervention studies in adults have reported altered activity in brain regions related to emotional processing, including the amygdala (Tillisch *et al.*, 2013; Pinto-Sanchez *et al.*, 2017). This illustrates that microbiota modulation may have a potentially beneficial influence on emotional processing and on the underlining neural structures, and the *Bifidobacterium* and *Lactobacillus* may have therapeutic potential as so called "psychobiotics," a term coined by Ted Dinan and John Cryan (Dinan *et al.*, 2013).

A recent study also suggested that in a sample of 5- to 11-year-olds, the fecal microbiota characteristics, including *Lachnospiraceae* from the Clostridiales order, were positively associated with the activation of the prefrontal cortex in response to sad faces (Callaghan *et al.*, 2019), but comparisons cannot be made due to differences in age range. Likewise, in a previous study conducted with a toddler population of three-year-olds, poorer personal, social, and communication skills were associated with a higher abundance of Clostridiales taxa (Sordillo *et al.*, 2019). Moreover, another study reported a link between increased Clostridiales abundance and autism spectrum disorder (De Angelis *et al.*, 2013). Hence, *Bifidobacterium, Lactobacillus, Clostridium*, and *Prevotella* may be important regarding socioemotional or brain functioning, and the current study is the first to address the association between fecal microbiota, such as those from the genera *Lactobacillus, Bifidobacterium*, and *Clostridium,* and the attention to fearful faces.

Our preliminary results suggested that fecal microbiota alpha diversity is associated negatively with left amygdala volume as well as fear reactivity and negative emotionality, but interestingly not with infant attention to fearful faces. Both larger amygdala volume and fear reactivity may increase the risk for future anxiety (Qin *et al.*, 2014; Hur *et al.*, 2019), whereas attentional biases can modulate the risk for anxiety in the context of temperamental disposition (Buzzell *et al.*, 2017). Our finding regarding amygdala volume is contrary to the previous report that stated a positive correlation between fecal microbiota diversity and amygdala volume in one-year-olds (Carlson *et al.*, 2018). Early on, microbiota diversifies during the first year of life due to cessation of breastfeeding and a more varying diet (Stewart *et al.*, 2018), and hence the direction of the association may be related to the dietary characteristics, as our sample was mostly breastfed. However, the role of early microbiota diversity in relation to later health and development is yet unspecified. Theoretically, a more diverse ecosystem would be more resilient and efficient (Larsen and Claassen, 2018), and a higher diversity usually associates with better health outcomes in adults (Menni *et al.*, 2017). However, high early fecal microbiota diversity is not likely to be preferable regarding socioemotional development, as an infant's intestines usually harbor a minimally diverse community. Regarding cognitive development, this paradigm was supported by Carlson et al., who reported that diversity is negatively associated with cognitive development (Carlson *et al.*, 2018). It is unknown whether the diverse ecosystem is composed of multiple potentially proinflammatory species, or whether the diverse gut microbiota stimulates the relatively immature immune system improperly, and hence leads to changes in brain development. In all, microbiota diversity can be viewed as a robust summary metric of overall microbiota composition in the context of early microbiota and development. Future research should focus on more functionally relevant assessment of microbiota in longitudinal designs.

Microbiota in early life has been shown to have male-specific effects on the CNS serotonergic system in rodents (Clarke *et al.*, 2013). Likewise, there is support that females show higher and more rapidly developing self-regulatory capacities and express more fear reactivity, whereas males typically show more surgency and specifically its subscale activity during childhood (Else-Quest *et al.*, 2006). Although the probability to disengage from face or fearful faces is not different among girls and boys, the maternal postnatal anxiety may affect the overall disengagement probabilities differently for girls and boys (Kataja *et al.*, 2019). Girls exposed to maternal postnatal anxiety were more easily disengaged from faces, regardless of the expression, whereas boys exposed to maternal postnatal anxiety were less likely to disengage from faces (Kataja *et al.*, 2019). Likewise, males usually have a larger amygdala (Ruigrok *et al.*, 2014). Thus, temperament traits seem to be more dependent on infant sex than emotional

attentional profiles based on the current literature. Consequently, it is not surprising that the microbiota cluster showed a sex interaction with regulation, surgency, and fear reactivity, and sex seemed to modify the associations between OTUs and temperament traits, while the associations between genera abundances and the probability to disengage from fearful faces showed no interaction by sex. However, how sex modifies the relations between amygdala volume and the early microbiota is still ambiguous, as the sample sizes were most likely limited to detect effect modification in Study III.

We observed a sex interaction with infant attention to faces in general (i.e., a face bias) including with the genera *Clostridium, Parabacteroides,* an unknown genus in *Lachnospiraceae* family, *Collinsella,* and *Citrobacter*. A face bias associated positively with *Clostridium* and negatively with *Citrobacter* in boys and the opposite in girls. It can be speculated whether boys with elevated *Clostridium* abundance is associated with overall higher tendency to fixate to both faces and fearful faces while in girls, the association between *Clostridium* abundance and attention is only apparent when a stronger emotional stimuli is presented. That being the case, it could reflect that boy's attentional properties may be more sensitive for early life microbiota alterations or an unmeasured factor that is a cause of *Clostridium* abundance. The preliminary findings support that the associations between early microbiota and brain development are sex-specific, which is in line with the findings from Christian et al., who showed sex-specific associations (Christian *et al.*, 2015). This indicates that infant sex is an important confounder regarding the early microbiota-gut-brain-axis functioning. Depending on the research question and the phenotype, both interaction and effect modification by sex could be specifically addressed, as they may occur independently (VanderWeele, 2009).

6.2 Maternal Prenatal Stress and Infant Fecal Microbiota Composition

Previous human and animal studies have associated prenatal stress with changes in infant microbiota. As could be expected based on the prior reports, the results from Study IV suggest that higher levels of prenatal cortisol exposure were related to decreased abundances of potentially health-promoting microbes such as *Lactobacillus* (Mu *et al.*, 2018). On the other hand, maternal chronic PPD was associated with increased abundances of potentially opportunistic Gram-negative genera within the Proteobacteria phylum (Jiang *et al.*, 2015). However, the *Bifidobacterium* genus was not associated with PPD or HCC. Hence, the findings corroborate some, but not all, results from existing animal models (Bailey *et al.*, 2004) and human studies (Zijlmans *et al.*, 2015; Naudé *et al.*, 2019).

In Study IV, infants exposed to the maternal PPD symptoms had increased abundances of genera that include several pro-inflammatory and even potentially pathogenic bacteria, such as *Serratia, Haemophilus, Citrobacter,* and *Campylobacter* from the Proteobacteria phylum as well as *Veillonella* and *Finegoldia* from the Firmicutes phylum, which harbors LPS in their outer surface (Antunes *et al.*, 2016). These findings were in line with previous evidence, since increased abundances of genera belonging to Gram-negative Proteobacteria have been associated with exposure to maternal prenatal stress (Zijlmans *et al.*, 2015; Naudé *et al.*, 2019). LPS is a widely studied endotoxin, and it has been suggested to influence the development of psychopathology (Rudzki and Szulc, 2018; Gonzalez-Santana and Diaz Heijtz, 2020). Increased Proteobacteria levels have been reported, for example, in patients with depression (Jiang *et al.*, 2015), and further rodent models show that colonization with pathogens, such as *Campylobacter jejuni*, may increase anxiety-like behavior (Goehler *et al.*, 2008). This could serve as an interesting indicator that the observed alterations in the fecal microbiota in this study could mediate, to some extent, the observed health effects of PPD in the offspring. Likewise, the bacterial antigenic macromolecules may be one part of the signaling cascade implicated here, although this remains to be investigated by further longitudinal research.

As expected, we found evidence that infants with low levels of exposure to maternal PPD had increased abundances of potentially health-promoting microbes, including *Akkermansia* (Cani and de Vos, 2017), and infants exposed to low levels of maternal HCC had increased abundances of *Lactobacillus* (Mu *et al.*, 2018). Intervention studies have shown that probiotic treatments with *Bifidobacterium* and *Lactobacillus* species are able to alleviate self-reported distress and affect cortisol excretion (Messaoudi *et al.*, 2010). Experimental models support the associations found in humans, as the colonization with *Lactobacillus* and/or *Bifidobacterium* have been demonstrated to normalize both the HPA-axis functioning (Sudo *et al.*, 2004) and depressive-like behavior (Messaoudi *et al.*, 2010). Our observations are in line with a previous human study (Zijlmans *et al.*, 2015), which indicated that lactic acid bacteria abundance may serve as a potential marker for healthy infant microbiota and optimal prenatal conditions regarding PPD.

At the time of writing, HCC has not been used in the context of maternal prenatal stress and infant fecal microbiota before, as the study by Zijlmans, Korpela, and colleagues utilized late pregnancy saliva cortisol. It should be noted that while there is an increase in overall cortisol levels towards the end of pregnancy, the HPA-axis reactivity is attenuated, and therefore saliva cortisol may have limited value as an indicator of long-term maternal prenatal cortisol homeostasis (de Weerth and Buitelaar, 2005). Thus, obtaining a stable cortisol measure, such as HCC, is potentially a relevant approach when we aim to assess

long-term cortisol secretion during a developmentally important time such as pregnancy (Hoffman *et al.*, 2016; Mustonen *et al.*, 2018, 2019), although the method compromises temporal dissection of cortisol fluctuations.

The exact mechanisms of how the prenatal cortisol levels influence fetal development remain poorly understood. In animal models, prenatal stress is mediated via changes in the maternal microbiota and immune signaling molecules with concurrent changes in placental serotonin and tryptophan (Chen *et al.*, 2020). In humans, antenatal corticosteroid treatment may increase fetal gastrin production (Costalos *et al.*, 2003) and inflammatory responses in preterm infants (Rautava *et al.*, 2016). Hence, even though the literature on the prenatal glucocorticoid exposure is scarce, it can be interpreted that prenatal glucocorticoid exposure may affect early gut physiology and immunity, and this may be reflected in the fecal microbiota composition.

In addition to the changes in HPA-axis functioning, immune system functioning is an important part of prenatal stress with physiological changes. IgA is a crucial factor in mucosal immunity by taking part in the modulation of microbial colonization in the gut (Planer *et al.*, 2016). Recently, Kang et al. suggested a potential mechanism linking maternal PPD with infant fecal microbiota, as they reported that maternal depressive symptoms are associated with reduced fecal secretory IgA content in infants (Kang *et al.*, 2020). It can be speculated that maternal stress could result in immune dysregulation and concurrent IgA depletion in the infant gut alongside with a bloom of certain opportunistic microbes such as members within Proteobacteria. Hence, the current and previous reports are in line with this finding by Kang and colleagues.

6.3 Strengths and Limitations

These studies included several common strengths and limitations. First, the population is drawn from a geographically restricted area in southwestern Finland and the associations might be different from other kind of samples. The cohort is mainly healthy and skewed towards a higher socioeconomic status (Karlsson *et al.*, 2018), and the results drawn from more socioeconomically disadvantaged, high-risk, or clinical samples might differ from the current ones (Raizada and Kishiyama, 2010). Regarding prenatal stress, it has been noted that the effects between maternal PPD on infant and birth outcomes are stronger in low- and middle-income countries, and on the other hand, higher maternal education may buffer against the effects of maternal PPD on offspring psychopathology (Pearson *et al.*, 2013; Bussières *et al.*, 2015). Hence, it could be hypothesized that at least the prenatal stress and microbiota associations would be larger in effect size in a more high-risk setting than reported here. Likewise, socioeconomic deprivation

may associate with poorer infant psychosocial development, and greater variability in infant self-regulation might produce a more nuanced or differential association between fecal microbiota and the behavioral and emotional phenotypes (Poulain *et al.*, 2020).

The 16S rRNA amplicon sequencing does not permit the strain-level or functional capacity assessment, which may be more informative than taxonomic profiling (Savignac *et al.*, 2014; Heintz-Buschart and Wilmes, 2018). 16S rRNA sequencing is prone to biases that may explain some of the observed discrepancies in the results, such as the high proportion of unidentified members of the *Enterobacteriaceae* family with a 20% relative abundance. Phylogenetic relations influence the colonization patterns in the infant gut and a close relatedness increases the likelihood for future colonization in a nepotistic manner (Darcy *et al.*, 2020). Incorporating phylogenetic measures might influence the inferences especially in longitudinal designs (Darcy *et al.*, 2020). Updating the data-processing pipeline by changing QIIME to QIIME2 (Bolyen *et al.*, 2019) and the Greengenes to the SILVA (Quast *et al.*, 2012) or to another more updated database could improve the coverage and accuracy of read mapping. However, whether that would affect the overall qualitative conclusions of this thesis is debatable (Moossavi *et al.*, 2020). Likewise, sample collection and storage methods may affect the microbiota composition, so that the Firmicutes abundance increases and Proteobacteria and Bacteroidetes abundances decrease when left to ambient room temperature for a span of several days and weeks (Song *et al.*, 2016). Without a preservative, such as 95% ethanol, the compositional changes start to accumulate after one week (Song *et al.*, 2016), and immediately freezing to -80˚C or storing the sample in 95% ethanol has been recommended to enhance the detection of more subtle signals (Song *et al.*, 2016). Nevertheless, it seems that the refrigeration without preservative retains the majority of individual sample characteristics and does not cause a great variance in the community composition (Song *et al.*, 2016). If there is a large and significant variation in technical aspects in a given set of samples, one remedy for this issue might be to adjust for individual technical variables such as storage time.

It is well-established that infant microbiota goes thorough developmental stages in early infancy (Stewart *et al.*, 2018), and longitudinal sampling might offer a better temporal dissection of how the preceding and the current fecal microbiota composition associate with the infant developmental outcome. Previous rodent studies show that the alterations in the gut microbiota composition during early life may cause behavioral changes that are not reversible with the manipulation of the microbiota during adulthood (Neufeld *et al.*, 2011). This supports the theory that early life is an important time window, when microbiota alterations may have a larger effect on the development of an individual (Codagnone *et al.*, 2019; Gabard-

Durnam and McLaughlin, 2019). Thus, it could be hypothesized that early alterations in both socioemotional phenotype and microbiota are essential for their developmental trajectories. However, human studies with longitudinal fecal sampling in this area are still scarce, and the first studies with longitudinal sampling show only an association between behavioral problems and the microbiota signatures closest to the behavioral assessment and not the preceding early life fecal microbiota composition (Loughman *et al.*, 2020a). On the other hand, another study from the same group shows an association between early fecal microbiota composition and later behavioral problems with no concurrent sampling (Loughman *et al.*, 2020b). Thus, when interpreting the results, it should be noted that they only describe a snapshot of associations during infancy. Although early life fecal microbiota is essential for later behavior, concurrent sampling might reveal additional insights regarding the longitudinal development of the microbiota. The lack of concurrent sampling is a limitation for Study III, where the brain imaging occurred before the fecal sampling. However, it seems more putative that the early life microbiota exposure affects the amygdala development (Hoban *et al.*, 2018; Stilling *et al.*, 2018), and it is well possible that the preliminary association might account for the residual confounding of common prenatal or early-life exposures or genetic variation.

Further, future studies should consider investigating the potential mediators and moderators. The maternal diet and antibiotic intake might both have an impact on the infant microbiota and may potentially mediate the association between maternal PPD and HCC and infant microbiota composition (Buffington *et al.*, 2016; Savage *et al.*, 2018; Champagne-Jorgensen *et al.*, 2020). Likewise, the early microbiota associations with temperament, emotional attention, and amygdala volume might be related to differential developmental trajectories that are influenced by prenatal cortisol, toxins, medication exposure, or a shared genetic variation for instance. Research shows that the influence of genetic variation on temperament and amygdala structure is notable, but still the other factors, such as environmental or epigenetic factors, influence the variation in temperament and amygdala volumes and hence offer a plausible possibility that the intestinal communities could likewise influence these phenotypes (Zwir *et al.*, 2018; Ong *et al.*, 2019; Satizabal *et al.*, 2019). Genetically informed study designs, such as twin studies or in-vitro fertilization studies, could help in dissecting the influence of genetic background on the observed associations (Rice *et al.*, 2010). Building of the literature will likely aid future studies to investigate the most relevant mediators and moderators of the association and to choose the correct confounders and hence lead to more precise models and estimates (VanderWeele, 2019).

Moreover, assessment of maternal microbiota and immune system functioning during pregnancy, infant/placental methylation profile, and breast milk

composition might elucidate the potential biological pathways mediating the effects of prenatal stress on the infant microbiota (Di Benedetto *et al.*, 2019; Monk *et al.*, 2019). Vertical transmission of microbes from the mother to the infant may have a role to play in mediating the association between prenatal stress and the infant's microbiota. As shown in a human study (Hechler *et al.*, 2019) and rodent studies (Jašarević *et al.*, 2017, 2018), stress can alter the maternal vaginal and gut microbiota composition. Likewise, prenatal stress elicits changes in offspring only in mice with regular microbiota colonization and exposure to vaginal microbiota mediates some of the effects of prenatal stress on the offspring gut microbiota at least partially (Jašarević *et al.*, 2018). Hence, future studies should include assessment of the maternal prenatal microbiota or in the immune system functioning profile. Further, the biological pathways mediating the potential associations between infant brain development and early microbiota in humans are likewise unknown. Whether these include alterations in intestinal or systemic immune system functioning, differences in circulating metabolites or other signaling molecules from host-microbiota interactions, altered vagal signaling, or other mechanisms remain to be investigated by future research.

6.4 Future perspectives

The current findings from the healthy Finnish population indicate that there are associations, albeit modest, between early microbiota composition and diversity and an infants' phenotypes that are important regarding future socioemotional and brain development. However, future studies are needed to replicate the observations in independent samples and to investigate potential mechanisms underlying the observed associations. Likewise, the importance regarding future health and developmental outcomes is unknown, although one can have tentative speculations regarding early *Bacteroides, Clostridium, Bifidobacterium*, and Proteobacteria abundances and later psychopathology based on the findings from the FinnBrain Birth Cohort and other cohorts (Carlson *et al.*, 2018; Callaghan *et al.*, 2019; Sordillo *et al.*, 2019; Loughman *et al.*, 2020b). For instance, an increased abundance of species belonging to the Proteobacteria phylum has been linked with depression in adults (Jiang *et al.*, 2015; Kelly *et al.*, 2015). The predictive value of the infants' phenotypes could potentially be strengthened by combining different phenotypes known to associate with psychopathology or by assessing the persistence of the phenotypes (Morales *et al.*, 2015, 2016). Future studies should consider the potential biological pathways mediating the associations, including microbial metabolites and short-chain fatty acids.

Exposure to prenatal stress has been associated with increased risk for psychopathology as well as inflammatory and metabolic conditions (Andersson *et*

al., 2016; van de Loo *et al.*, 2016; Burgueño *et al.*, 2020). It is tempting to hypothesize that the observed fecal microbiota signatures in the infants with exposure to maternal chronic PPD, such as increased abundances of potentially inflammatory genera from Proteobacteria phylum, could mediate the effects of prenatal stress on an infant's future health. Similar fecal microbiota alterations have been previously associated with future adverse health outcomes such as asthma (Stokholm *et al.*, 2018). However, it could be that the observed microbiota alterations do not have causal role, and prudent animal and longitudinal human studies including elements of gut microbiota and immune system functioning, nutrition, maternal medications, prenatal, and early life medication, and chemical exposure are warranted.

Stress-related psychiatric disorders, such as affective disorders, are an increasing public health problem (Torikka *et al.*, 2014), and maternal PPD is an important factor affecting offspring mental health from childhood to adulthood (Pearson *et al.*, 2013). Understanding the exact mechanisms linking the prenatal exposure to stress and later health is an important step to develop targeted interventions for at-risk groups. Alterations in fecal microbiota composition are associated with developmentally important phenotypes and prenatal stress exposure, which encourage future studies.

7 Conclusions

Infant fecal microbiota composition associates with temperament traits such as negative emotionality, fear reactivity, surgency, and regulation in a sex-specific manner in exploratory analyses. Likewise, infant fecal microbiota composition associates with disengagement probabilities from fearful faces, which is a measure of early emotional attention. The genera associated with these brain developmental phenotypes included *Bifidobacterium, Bacteroides, Clostridium, Veillonella*, and taxa within Proteobacteria phylum. The exploratory analyses suggest that infant fecal microbiota alpha diversity associates negatively with amygdala volume as well as negative emotionality and fear reactivity. Hence, early fecal microbiota signatures associate with infant phenotypes, which are important regarding emotion processing and may be implicated in the development of psychopathology.

Maternal chronic PPD and chronic cortisol levels during approximately the first half of pregnancy, markers of prenatal stress, were associated with alterations in fecal microbiota composition but not diversity. The observed signatures were increased abundances of genera in the Proteobacteria phylum and genera harboring lipopolysaccharide in their outer membrane as well as a decreased abundance of *Akkermansia* and *Lactobacillus* following the prenatal stress exposure. The question whether the observed alterations in an infant's microbiota are deterministic for later health remains unanswered.

These findings contribute to the understanding of potential microbiota-gut-brain-axis connection in infants, although it must be acknowledged that the field is still in its infancy, and future studies are needed for replication and mechanistic insight. The cross-sectional nature of these studies calls for a longitudinal follow-up of both fecal microbiota and socioemotional development. The selected infant phenotypes have been used seldom or never prior to these studies, which underlines simultaneously the novelty and importance of the work but also the cautiousness that is required when interpreting the results. The observations from this cohort population of young infants offer interesting hypotheses for future studies.

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