The Role of Gut Microbiota Diversity in Infant Attentional Processing of Emotional Faces at the Age of Eight Months

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ABSTRACT

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In recent years a lot of interest has been put in linking neuroscience and microbiology together as we start to understand the importance of the bacteria to our brain health and concept of gut-brain axis have been launched. Gut microbiota can influence neurodevelopment in several mechanisms including immunological, endocrine, neural and metabolic pathways. Germ-free rodent studies show that microbiota is necessary for normal stress responsivity, fear-related behavior, anxiety-like behaviors, sociability and cognition in sex specific manner. Recent human studies demonstrate that gut microbiota diversity is associated with temperament, cognitive performance and differences in amygdala-thalamus connectivity, which is central in processing emotional information. In addition, human probiotic intervention studies have suggested that probiotics intake reduce negative mood and might help in preventing depression.

Infant is selecting information from sensory input by orienting attention. Already newborns' attention is biased towards faces. Another attention bias emerging during second half of first year is the heightened bias towards faces expressing fear, called fear bias. Attention bias for fear can be seen for example in a slower or less probable disengagement of attention from fearful or angry faces as compared to happy or neutral faces. Deviations in early emotion processing from facial expressions may influence the trajectories of socio-emotional development.

The data for this master thesis was a sub-sample (n=126) from FinnBrain Birth Cohort Study. Aim was to investigate the associations between early gut microbiota alpha diversity/richness and infant attention to emotional faces. The possible sex differences in these associations were also investigated. The gut microbiota diversity was assessed at 2.5 months of age and analyzed with 16s rRNA sequencing. Attention to emotional faces was assessed with eye tracking using age-appropriate face-distractor paradigm microbiota at the age of eight months.

In this master thesis no associations between gut microbiota diversity and attention to emotional faces was found. Probability of disengagement was lowest for fearful faces. The only significant correlation was found between fear bias and gut microbiota richness in boys but not in girls, but there was not statistically significant interaction between the sex and gut microbiota in a linear regression model controlling for mode of delivery and breastfeeding. This data encourages for further human studies to illustrate the potential underlying neural structures of emotional attention and gut microbiota in sex-specific manner.

Keywords: microbiota, gut-brain-axis, brain development, face processing, threat processing, attention mechanism, emotional attention

TIIVISTELMÄ

Saija Tarro

Suolistomikrobiston diversiteetin yhteys 8-kuukauden ikäisen vauvan kasvojenilmeisiin suuntautuvaan tarkkaavaisuuteen

Viime aikoina on kiinnostuttu yhä enemmän neurotieteiden ja mikrobiologian yhdistämisestä, kun ymmärrys bakteerien merkityksestä aivoterveydellemme on lisääntynyt ja uusi käsite suoli-aivo –yhteys on lanseerattu. Suoliston mikrobisto voi vaikuttaa hermoston kehitykseen useilla mahdollisilla mekanismeilla, kuten immunologisilla, endokriinisilla, hermostollisilla ja aineenvaihdunnallisilla väylillä. Tutkimukset mikrobivapailla jyrsijöillä ovat osoittaneet, että mikrobisto on välttämätön normaalille stressireagoinnille, pelko- ja ahdistuskäyttäytymiselle, sosiaalistumiselle ja kognitiolle sukupuolispesifein tavoin. Viimeaikaiset ihmistutkimukset osoittavat, että suoliston mikrobiston monimuotoisuus on yhteydessä temperamenttipiirteisiin, kognitiiviseen kehitykseen ja eroavaisuuksiin amygdalan ja thalamuksen yhteyksissä, mitkä kaikki liittyvät tunnepitoisen tiedon käsittelyyn aivoissa. Lisäksi, ihmisillä tehtyjen probiootti-interventiotutkimusten perusteella on esitetty, että probiootit voivat vähentää negatiivista mielialaa ja siten mahdollisesti auttaa masennuksen ehkäisyssä.

Vauvat valikoivat aistien kautta välittyvää tietoa suuntaamalla tarkkaavaisuuttaan. Jo vastasyntyneiden tarkkaavaisuuden fokus on suuntautunut kasvoihin. Toinen puolueellisuusvinouma joka ilmenee toisella vuosipuoliskolla, on korkeampi tarkkaavaisuuden fokus kasvoihin, jotka ilmentävät pelkoa, tätä kutsutaan pelkovinoumaksi. Pelkovinouma näkyy esimerkiksi tarkkaavaisuuden hitaampana tai vähemmän todennäköisenä irrottautumisena pelokkaista tai vihaisista kasvoista verrattuna iloisiin tai neutraaleihin kasvoihin. Poikkeamat varhaisessa tunnepitoisen tiedon käsittelyssä voivat vaikuttaa sosio-emotionaalisen kehityksen kulkuihin.

Pro gradu -tutkielman aineistona toimi osaotos (n = 126) FinnBrain –syntymäkohorttitutkimuksesta. Tarkoitus oli tutkia varhaisen suolistomikrobiston monimutoisuuden/lajimäärän ja vauvan kasvojenilmeisiin suuntautuvan tarkkaavaisuuden välisiä yhteyksiä. Mahdollisia sukupuolieroja näissä yhteyksissä tutkittiin myös. Ulostenäyte kerättiin 2.5 kuukauden iässä ja analysoitiin 16s rRNA sekvensointimenetelmällä, jonka avulla monimuotoisuus määritettiin. Kasvojenilmeisiin suuntautuva tarkkaavaisuus määritettiin silmänliikemenetelmällä käyttäen ikätasoista kasvo-häiriöärsyketehtävää käyttäen 8 kuukauden iässä.

Tässä pro gradu työssä ei löytynyt tilastollisesti merkitseviä yhteyksiä suolistomikrobiston diversiteetin ja kasvoihin suuntautuvan emotionaalisen tarkkaavaisuuden välillä. Katseen irrottamisen todennäköisyys oli pienin pelokkaiden kasvojen tilanteessa. Ainut tilastollisesti merkittävä korrelaatio oli pelkovinouman ja suolistomikrobiston lajimäärän yhteys pojilla, mutta ei tytöillä. Kuitenkaan sukupuoli-suolistomikrobisto –interaktio ei ollut tilastollisesti merkitsevä lineaarisessa mallissa, jossa synnytystapa ja imetystapa oli huomioitu. Tämä aineisto kannustaa laajempiin ihmistutkimuksiin, jossa voitaisiin tutkia tarkemmin tunnepitoisen tarkkaavaisuuden ja suolistomikrobiston yhteyksiä huomioiden sukupuolen mahdollinen vaikutus yhteyteen.

Avainsanat: mikrobiomi, suoli-aivoyhteys, aivojen kehitys, kasvojenilmeisiin suuntautuva tarkkaavaisuus, uhkaärsykkeiden prosessointi, tarkkaavaisuusmekanismit, tunnepitoinen tarkkaavaisuus

Contents

1 Introduction

The importance of gut microbiota, i.e. the community of microorganisms in the intestine, to our general health has been known for a long time (Cani, 2018; Dinan et al., 2018) as over a century ago Metchnikoff theorized that health could be improved and even senility delayed by manipulating the intestinal microbiota with bacteria found in sour milk (Mackowiak, 2013). In recent years our understanding on the role of gut microbiota on human brain function and behavior has broadened and term microbiota-gut-brain axis has been put forward (Cryan et al., 2019). Exploration of gut-brain connections can be traced back to early psychologists William James and Carl Lange who proposed that emotional response might be directly modulated by signals transmitted from the viscera to the brain (Eisenstein, 2016). Nowadays microbiota-gut-brain axis research is a fast growing field investigating bidirectional communication between the gut and brain (Hooks, Konsman, & O'Malley, 2018; Mayer, Knight, Mazmanian, Cryan, & Tillisch, 2014).

One of the key topics in microbiota research currently is the associations between gut microbiota and mental health (Christian, 2019). There has been a great deal of interest in the associations between gut microbes and psychological processes, especially the interrelations between bacteria and stress regulation systems (Mayer et al., 2014). Further, gut microbiota is linked to the development of normal social functioning (L. Desbonnet, Clarke, Shanahan, Dinan, & Cryan, 2014). Also, few studies related to the role of bacteria in cognition are made, mostly in rodents. (Carlson et al., 2018; Fröhlich et al., 2016; Savignac, Tramullas, Kiely, Dinan, & Cryan, 2015).

Germ-free rodent studies are pointing to an ability of gut microbiota to influence the brain development in a long-lasting manner (Lieve Desbonnet et al., 2015; Heijtz et al., 2011; Sudo et al., 2004). Further, the results show that early disruptions in developing microbiota persist even after the microbiota recolonization in adulthood (Neufeld, Kang, Bienenstock, & Foster, 2011). Thus, gut microbiota could be seen as one important environmental factor in brain "developmental programming", affecting the development of brain structure and function during a sensitive brain developmental period (Heijtz et al., 2011).

The effect of gut microbiota on brain development has been mainly studied in rodents and only a few studies in humans have been published (e.g. Lieve Desbonnet et al., 2015). Preliminary human studies suggest that gut microbiota diversity is associated with temperament, cognitive performance and differences in amygdala-thalamus connectivity during early childhood (A. K. Aatsinki et al., 2019;

Carlson et al., 2018; Christian et al., 2015). It is nowadays widely thought that a diverse microbiota is beneficial for health (Rouse & Anglero-diaz, 2018), although some critical perspectives on increased diversity being only beneficial have also been put forward (Hooks et al., 2018; Shade, 2017). Additionally, a recent study in infants found that higher alpha gut microbiota diversity (i.e. intra-individual diversity) was associated with lower scores in Mullen Early Learning Cognitive Composite (ELC) at 2 years of age and therefore suggest, that higher levels of bacterial diversity may not always be associated with benefits in cognitive development (Carlson et al., 2018). Taken together, these results indicate that microbiota diversity in infants might have more ambiguous associations with later development compared to adults.

Attention is an important cognitive process and attention can be seen as a way to cognitively control uncertainty (Mackie, Van Dam, & Fan, 2013). Human attention system is biased toward faces from birth onwards (Reynolds & Roth, 2018) and face bias can be seen as an early marker of social development (Peltola, Yrttiaho, & Leppänen, 2018). In addition to face bias, later during first year emerging bias to fearful faces (Peltola, Hietanen, Forssman, & Leppänen, 2013; Peltola, Leppänen, Mäki, & Hietanen, 2009) is an important step in developing the ability to process and respond appropriately to social stimuli. Infants orient attention to salient stimuli and it provides the first means of self-regulation for the developing infant (Posner & Rothbart, 2007). Many psychiatric disorders are related to difficulties in attentional processes (Trivedi, 2006). Problems in controlling attention when processing socio-emotional signals could underlie the cognitive, emotional and behavioral problems seen for example in anxiety disorders (Kataja, 2018). Thus, it is important to understand the biological mechanisms behind as deviations in early emotion processing may influence the trajectories of social-emotional development. It is suggested, that gut microbiota could be part of "the unconscious system" regulating behavior (Dinan, Stilling, Stanton, & Cryan, 2015).

However, most of the studies on the influence of gut microbiota to neurodevelopment are preclinical animal models and clinical studies are mostly done in adult populations. Much more human studies are needed to show the role of microbiota in attentional processes. It is suggested, that there might be an early life sensitive period for the effects of gut microbiota on behavior (Sudo et al., 2004). Therefore, studies with children and/or infants need to be conducted to investigate the role of early microbiota diversity in emotional attention, and particularly processing emotional faces and signals of threat.

2 Infant attention

2.1 Development of attention in infancy

Attention is a fundamental cognitive function critical for perception, language, and memory (Posner, Rothbart, & Voelker, 2016). Thus, attention is such an important psychological construct, that several models of attention have been developed (Burk, Blumenthal, & Maness, 2018). In 1990, neuroscientists Posner and Petersen proposed a unique perspective on attention as they divided attention into three networks, each representing a different set of attentional processes and having discrete anatomical basis in the brain (M. Posner, 1990). These three networks are alerting network, orienting network and executive network (Petersen & Posner, 2012). The development of attentional networks are shaped by genes and experiences through the actions of caregivers and the culture (Posner & Rothbart, 2007). Even though the network model of attention was launched when neuroimaging was only in it's infancy, brain imaging data is also supporting the presence of different attention networks (Posner & Rothbart, 2007). Alerting network is involved in maintaining sensitivity to incoming stimuli, orienting is the selection or prioritization of information from sensory input and executive control includes the mechanisms for monitoring and resolving conflict among thoughts, feelings and responses (Rothbart & Posner, 2015).

The orienting network involves adjusting attention according to a source of sensory signals, which might be overt (with eye-movements) or covert (with no eye movements) (Posner & Rothbart, 2007). The orienting component of attention is suggested to be mainly responsible for perceptual processing during first year of life, and provides the first means of self-regulation for the developing infant (Posner & Rothbart, 2007) until the maturation of the executive network and more controlled attention by 4 years of age (Posner, Rothbart, Sheese, & Voelker, 2012). However, some parts of executive attention network related to the ability of resolving conflicts, are present already in infancy, such as the anterior cingulate cortex (ACC), but these parts do not exercise their full control over other networks until about 4 years of age (Posner et al., 2016). An electroencephalogram (EEG) study have reported that ACC is activated by error detection already at 7 months, although the ability of the infant to take action based on the errors is suggested to be present only later (Berger, Tzur, & Posner, 2006). There are several important brain regions involved in the orienting system for visual events including frontal eye fields and areas of the superior and inferior parietal lobe (Posner et al., 2012, 2016). Superior parietal lobe is specifically associated with orienting to new location after a cue and temporal parietal junction to uncued location and lesions of the parietal lobe and superior temporal lobe have

been related to difficulties in orienting (Rothbart & Posner, 2015). Attentional function is modulated by different neurotransmitters, each linked with different attentional networks (Burk et al., 2018; Posner & Rothbart, 2007). Cholinergic systems emerging from the basal forebrain involving superior parietal lobe appear to play a critical role in orienting attention (Posner et al., 2012). The alerting network is modulated by the brain's norepinepherine system and executive network by dopamine from the ventral tegmental areas (Posner et al., 2012).

Attention plays an important role in self-regulation (Posner & Rothbart, 2007). It is suggested that the ability to flexibly control attention is essential for maintaining psychological well-being (Bardeen, Daniel, Hinnant, & Orcutt, 2017). Infants orient attention to interesting visual and auditory stimulus, which can make their distress to over-stimulation disappear (Posner & Rothbart, 1998). Indeed, caregivers usually calm infants by distracting their attention, i.e. bringing their attention to other stimuli in order to reduce distress. The ability to focus and shift attention have also been seen as a way for adults to experience less negative affect (Posner & Rothbart, 1998, 2007). Adult functional MRI brain imaging data suggests that distraction reduces the activity in amygdala, they key emotion processing area in the brain (Posner et al., 2012). The functions of the orienting attention network downregulates the activity of the amygdala already in infancy (Posner et al., 2012). Thus, the regulation by orienting in stressful situations is indicating the vital role of attention mechanisms already on the very early self-regulation (Posner et al., 2012). Disengagement can be also seen as an important ability to downregulate symphatetic nervous system and habituate to a stressful environment (Bardeen et al., 2017).

As orienting of attention is central in early self-regulation, the development of the orienting network is very important for the psychological wellbeing (Posner & Rothbart, 2007). Problems in controlling attention when processing socio-emotional signals could underlie the cognitive, emotional and behavioral problems seen for example in anxiety disorders (Kataja, 2018). Many psychiatric disorders, such as schizophrenia, mood disorders and obsessive-compulsive disorder (OCD) are related to deficits in attentional processes (Trivedi, 2006). Likewise, autism is linked to the orienting network of attention as persons with autism spectrum disorder show deficits in orienting towards faces (Posner & Rothbart, 2007), a feature of attention that is prevalent among typically developing individuals (Reynolds & Roth, 2018). Individual differences in temperament, i.e. children's innate individual differences in self-regulation and emotional reactivity (Rothbart, 2011) in infancy might also reflect the maturation of neural attention networks as attentional capacities are important regarding self-regulation, a major aspect of temperament (Posner & Rothbart, 2007). For example

temperamental effortful control reflects the efficiency of executive control network. (Rothbart & Posner, 2015).

2.2 Attention bias to faces

Face perception can be seen as a specific form of attention (Haist & Anzures, 2017; Hung, 2011), and it can be divided into attention orienting and attention holding (Leppänen, 2016). Faces are important to us as humans as they reveal relevant social information about identity, age, gender and emotions (Simion & Di Giorgio, 2015). Thus, face processing can be seen as an important perceptual ability, the cornerstone of human interaction, and already newborns can discriminate faces from other objects (Haist & Anzures, 2017). Infants orient preferentially toward faces (Simion & Di Giorgio, 2015). Orienting preference for faces may be an early marker of social development as it is linked with the development of empathy and responsivity to others's needs (Peltola et al., 2018). Face processing capabilities develope from infancy to adolescence (Haist & Anzures, 2017; Mondloch, Geldart, Maurer, & Le Grand, 2003). Age-related improvements can be seen for example in longer fixations towards faces in complex dynamic scenes (Leppänen, 2016). Attention holding in infants is measured by calculating the duration of total looks at the target area. Both attention holding and attention orienting measures show that 6-month-olds start to prioritize faces even more, a tendency called face bias. (Leppänen, 2016). As early as 3 months, infants can already discriminate among facial emotional expressions when pictures of emotional faces are shown (Bornstein, Arterberry, Mash, & Manian, 2011; Hung, 2011). Five-month-olds still hold attention similarly to neutral and emotional faces, but around 7 months infants start to hold attention more selectively for emotional faces, especially fearful faces (Leppänen, 2016; Peltola et al., 2013).

The basic cortical face processing systems in the brain include fusiform face area (FFA), occipital face area (OFA), and posterior superior temporal sulcus (pSTS) (Haist & Anzures, 2017). According to dual route model of face processing, faces are processed through both a subcortical face-detection route involving the superior colliculus, pulvinar and amygdala and cortical face identification route (Johnson, 2005). Evidence demonstrate the important role of subcortical route in rapid processing of visual threat and its activity further modulates cortical face processing networks (Johnson, Senju, & Tomalski, 2015). Johnson (2005) suggests that early preference for faces would be largely controlled by subcortical structures as subcortical route seems to be more developed around the time of birth (see also Johnson, Senju, & Tomalski, 2015). Development of frontoparietal networks controlling

eye movents and prioritization of sensory inputs during the second half of the first year could be related strengthening of the attentional bias for faces (Leppänen, 2016).

2.3 Attention bias to fearful faces

Fear as a basic emotion is emerging during the first year of life (Graham et al., 2016). During the time when infant starts to move and actively explore the environment, it is important to acquire ability to use caregivers' nonverbal emotional signals to learn about potentially threatening situations. Recognizing the emotional signals of so called universal facial expressions is a skill most humans have. Also infants at the second half of the first year start to attend more to fearful facial expressions – this well-evidenced feature of infant attention is often called as an attention bias for fear or threat. (Leppänen & Nelson, 2009; Peltola et al., 2013). Attention bias for threat can be seen for example in a slower or less probable disengagement of attention from fearful or angry faces as compared to happy or neutral faces. (Kataja et al., 2018; Peltola et al., 2013, 2009). Among adults, enhanced orienting of attention to threat-related facial cues and difficulties in disengaging from fearful facial expressions are related to high trait anxiety (Leppänen & Nelson, 2009).

Studies which have recorded event related potentials (ERPs) to presentation of facial expressions, have observed larger negative-central (Nc) component amplitudes for fearful than happy faces in 7-month-old infants. Negative-central component is thought to reflect attentional orienting to salient stimuli or general attentional arousal. (de Haan, Belsky, Reid, Volein, & Johnson, 2004; Leppänen, Moulson, Vogel-Farley, & Nelson, 2007). Similar findings were not found with 5-month-olds, but Nc was of similar magnitude for fearful and happy faces. This suggests that the attentional bias for threat emerges between 5 and 7 months of age (Peltola et al., 2009).

Cortical generators of the Nc have been suggested to be located in prefrontal regions, mainly anterior cingulate cortex (ACC), which is known to have an important role in controlling the direction and maintaining the focus of attention (Peltola et al., 2009). During infancy, the processing of fear is suggested to depend mostly on amygdala. During adulthood also hippocampus and frontal areas play role in fear processing. (Gunnar, Hostinar, Sanchez, Tottenham, & Sullivan, 2015; Leppänen & Nelson, 2009). It is not known how the functional connectivity of amygdala and cortical networks exactly develops (Peltola et al., 2009). However it s suggested that some connectivity of amygdala and ACC already exist at 6-months of age as Graham et al. (2016) found that stronger amygdala connectivity to anterior cingulate predicted higher fear combined with advanced cognitive development. Leppänen & Nelson (2009) conclude, that key components of emotion-processing

networks are established soon after birth, but the fine-tuning of this network continues during postnatal development.

3 Gut microbiota

The term microbiome refers to micro-organisms and their genetic material in the body and the term microbiota means populations of microorganisms in the different parts of the body, for example intestinal (gut) microbiota (Yang et al., 2016). Both terms 'microbiota' and 'microbiome' are in literature used interchangeably as if they were synonyms. In this thesis mostly term microbiota is used. Human gut microbiota is a huge collection of different kind of bacteria, viruses, fungi and archaea with approximately 100 times more genes than in the human genome (Sender, Fuchs, & Milo, 2016). In bacterial taxonomy, the main levels in their descending order are: domain (bacteria), phylum (e.g. Proteobacteria), class (e.g. y-Proteobacteria), order (e.g. Enterobacteriales), family (e.g. Enterobacteriaceae), genus (e.g. *Escherichia*), species (e.g. *E. coli*) (Hardy, 2003). Firmicutes (such as Clostridium, Enterococcus, Lactobacillus, and Ruminococcus) and Bacteroidetes (Bacteroides and Prevotella), are the most common bacteria phyla living in the gut in addition to Actinobacteria, and Proteobacteria phyla (Bäckhed et al., 2015; Turnbaugh et al., 2007; Yang et al., 2016). The number and abundance of microbes within certain body habitat (e.g. intestine) is called diversity, which can be measured within sample (alpha diversity) or between samples (beta diversity) (Huttenhower et al., 2012).

3.1 Gut microbiota development in infancy

It is suggested, that infants, (especially vaginally delivered), acquire their indigenous microbiome primarily from the mother (Rautava, 2016). Infants gut microbiota is less diverse than adults (Stewart et al., 2018; Yang et al., 2016). However, gut microbiota of formula-fed infants is more diverse than breastfed infants (Dinan et al., 2015). Gut microbiota composition after birth is influenced by many factors, such as mode of delivery, antibiotic use, diet, and other environmental factors such as presence siblings and furry pets (Dinan et al., 2015; Stewart et al., 2018). Bacteroides, Bifidobacterium and Escherichia/Shigella genera are usually most abundant members of the newborns' gut microbiota (Bäckhed et al., 2015). Breastfeeding explains most of the variance during early life and is associated with lower diversity and dominance of Bifidobacterium (Stewart et al., 2018). During the first years of life the gut microbiota is evolving into a more complex and adult-like

configuration with higher alpha diversity and lower beta diversity (i.e. inter-individual diversity) until age of 3 years (Bäckhed et al., 2015; Yatsunenko et al., 2012). Two key factors affecting the early colonization of gut microbiota composition is the mode of delivery and cessation of breast milk (Bäckhed et al., 2015; Stewart et al., 2018).

3.2 Microbiota-Gut-Brain axis and neurodevelopment

The postnatal period is critical for brain development as considerable amount of morphological development, cell differentiation, and acquisition of function takes place during that period (Al-Asmakh, Anuar, Zadjali, Rafter, & Pettersson, 2012). Similarly, microbiota undergoes rapid process of development after birth and establishes symbiotic relationship with the host early in life (Borre et al., 2014a). Thus, it has been hypothesized that early life disturbances in the developing gut microbiota can impact neurodevelopment and potentially lead to adverse mental health outcomes later in life (Borre et al., 2014a). Therefore, understanding the parallel development and communication of gut and brain opens new avenues for early life interventions during a period when the gut microbiota is malleable to change (Christian, 2019).

The gut–brain axis consists of a bidirectional communication network where gut microbiota is playing a major role (Borre et al., 2014a; Evrensel & Ceylan, 2017; Hoban et al., 2016; S.M., Z., & P., 2013). The network monitors gut functions and connects them to cognitive and emotional centres of the brain (Rogers et al., 2016). Gut microbiota can influence brain functions and neurodevelopment in several possible mechanisms (L. Desbonnet et al., 2014; Heijtz et al., 2011; Hoban et al., 2018; Sudo et al., 2004) including immunological (such as production of cytokines), endocrine (hypothalamic-pituitary-adrenal, HPA axis), neural (vagus nerve) and metabolic pathways (Grenham, Clarke, Cryan, & Dinan, 2011; Yang et al., 2016).

A neural pathway from gut to brain includes afferent and efferent nerves of parasympathetic vagus nerve. Vagus nerve is suggested to play a major role in communication between gut microbes and centrally mediated behavioral effects (Bravo, Forsythe, Chew, Escaravage, & Savignac, 2011). Bacteria in the gut stimulate the production of peptides which activate afferent endings of the vagus nerve (Yang et al., 2016). The signals are sent to CNS, affecting behavior and efferent neural activity (Yang et al., 2016). Vagal afferents are also thought to be responsible of “gut-feelings” (Yang et al., 2016). Also pro-inflammatory cytokines can activate vagus nerve and transmission of inflammatory

signals is thought to be a key mechanism how the brain receives information regarding systemic inflammation. (Yang et al., 2016).

Gut microbiota influence the levels of pro-inflammatory cytokines and anti-inflammatory cytokines, which is one possible mechanism, as cytokine levels have been shown to affect behavior in rodents and alter neurochemistry (Miller, Haroon, Raison, & Felger, 2013). Some bacteria (e.g. Enterobacteriaceae and Pseudomonadaceae) can maintain low-grade systemic pro-inflammatory state whereas some bacteria, like Lactobacillus, may be more inflammatory protective (Yang et al., 2016). In fact, ingestion of probiotics (e.g. Bifidobacterium and Lactobacillus) and prebiotics (e.g. oligosaccharides) can reduce pro-inflammatory cytokines and increase anti-inflammatory cytokines (Dinan et al., 2018).

Gut microbiota can influence the hypothalamic–pituitary–adrenal (HPA) axis functioning, the major stress regulation system, directly by influencing cortisol secretion and the normal development of stress response and indirectly, influencing pro-inflammatory cytokines which can activate HPA axis (Sudo et al., 2004; Yang et al., 2016). By manipulating microbiota composition and activity, HPA axis responsiveness can be altered as well (Rees, 2014). HPA axis development is also dependent on specific bacteria colonizing the gut at a specific time point (O'Mahony, Clarke, Dinan, & Cryan, 2017). HPA-axis functioning is linked to cognitive processes, such as attention (Vedhara, Hyde, Gilchrist, Tytherleigh, & Plummer, 2000). It is suggested that children with ADHD have lower levels of cortisol than controls and HPA-axis function might be dysregulated in ADHD (Isaksson, Nilsson, Nyberg, Hogmark, & Lindblad, 2012).

In addition to neural pathways, immune and endocrine mechanisms, one possible mechanism for gut bacteria to influence brain development, is through neurotransmitters and bacterial metabolites (O'Mahony et al., 2017). GABA, the main inhibitory neurotransmitter in the human brain is produced by gut bacteria, in addition to 5HT and DA (Dinan et al., 2015; O'Mahony et al., 2017). Especially Lactobacillus and Bifidobacteria produce GABA by metabolizing dietary glutamate (Barrett, Ross, O'Toole, Fitzgerald, & Stanton, 2012). Despite microbiota produces vast amount of neurotransmitters, it's unknown to which extent the neurotransmitters are absorbed to systemic circulation or if they cross the blood-brain barrier to affect neurodevelopment (Jones, 1982).

3.3 Studying the impact of gut microbiota to brain development and function

There are four basic methodology categories in gut-brain research: 1. comparisons of behaviors in germ-free rodents with conventionally colonized or specific pathogen-free rodents, 2. studies of normally colonized rodents which are treated with antibiotics, 3. probiotic animal and human studies, 4. experimental alteration of gut microbiota (Hooks et al., 2018).

3.3.1 Preclinical studies on germ-free animals

Preclinical experiments with germ-free (GF) animals, i.e. animals (mostly rodents) that do not have microbiota, are important in order to understand the effects of gut microbiota on brain functions and behavior (Evrensel & Ceylan, 2017). In addition, they allow the study of the impact of e.g. probiotic on the gut-brain axis in isolation (J. F. Cryan & O'Mahony, 2011). Rodents follow similar gut microbiota colonization than humans, even though the GI tract function and anatomy differs from humans (Grenham et al., 2011). GF animal studies show the ability of microbiota to influence multiple aspects of neurodevelopment (Borre et al., 2014a). For example, microbiota is necessary for normal stress responsivity via hypothalamic–pituitary–adrenal (HPA) axis (Sudo et al., 2004), fear-related behaviours (Hoban et al., 2018), anxiety-like behaviors (Heijtz et al., 2011; Neufeld et al., 2011), sociability and cognition (John F. Cryan & Dinan, 2015; L. Desbonnet et al., 2014; Luczynski et al., 2016).

Interestingly, Hoban et al. (2018) found increased neural activity in the amygdala and impairments in fear conditioning in GF mice. In addition, functional microbiota during critical windows of neurodevelopment is important for appropriate prefrontal cortical myelination (Hoban et al., 2016). Germ-free mice have also reduced levels of cortical and hippocampal brain-derived neurotrophic factor (BDNF), an important protein associated with neuroplasticity, learning and memory (Mayer et al., 2014; Sudo et al., 2004). However, the changes might be gender specific as it is suggested that neurochemical and endocrine effects of germ-free environment are only evident in male rodents (J. F. Cryan & O'Mahony, 2011). Indeed, Neufeld et al. (2011) found that female mice show a reduction in anxiety behavior and increase in BDNF. Also the role of microbiota in social development is gender specific as Desbonnet et al. (2014) showed that GF mice exhibit autism-like traits, such as impairments in sociability and social cognition and these effects are are much more common in males.

If GF mice are exposed to the gut microbiota (e.g. Bifidobacteria infantis), early enough during postnatal development, the impairments of HPA axis functioning might be mitigated and thus it is suggested that there might be an early life sensitive period for the effects of gut microbiota on behavior (Sudo et al., 2004). In conclusion, germ-free animal studies show us important aspects of complete absense of gut microbiota on behavior. There is increasing need to understand the mechanisms how enteric microbiota can alter behavior. As most of the rodent studies are done with males, it is even more important to consider gender specific effects.

3.3.2 Child microbiota-gut-brain studies

Child gut-brain studies mostly discuss the role of gut microbiota in neurodevelopmental disorders, mostly ADHD and autism. ADHD and autism are multi-factorial disorders triggered by genetic and many environmental factors (Cenit, Nuevo, Codoñer-Franch, Dinan, & Sanz, 2017; Curran et al., 2015). Gut microbiota is seen as an important environmental factor as many environmental factors (such as delivery mode, gestation age and type of feeding) linked with the risk of developing attention-deficit hyperactivity disorder (ADHD) are also known to influence the early gut microbiota composition (Cenit et al., 2017). Similarly, delivery by C-section is found to increase odds of autism spectrum disorder diagnosis when compared to vaginal delivery (Curran et al., 2015). One study comparing the gut microbiota composition of treatment-naive children with ADHD and healthy controls, found a significant decrease in the *Faecalibacterium* genus in children with ADHD compared to HC (Jiang et al., 2018). Children with autism spectrum disorders (ASD) have been reported to have higher gut microbiota diversity compared to controls (Finegold et al., 2010).

Only recently few human child studies have reported associations between gut microbiota and cognition/emotions processing (A. K. Aatsinki et al., 2019; Carlson et al., 2018; Christian et al., 2015). A study in toddlers by Christian et al. (2015) shows that gut microbiota diversity is associated with child temperament, i.e. children's innate individual differences in self-regulation and emotional reactivity (M. K. Rothbart, 2011). Specifically, Christian et al. (2015) found an association between gut microbiota beta diversity and fear reactivity among girls. Temperament was associated with infant gut microbiota also in the FinnBrain Birth Cohort Study. Aatsinki et al. (2019) found that greater alpha diversity was associated with lower negative emotionality and fear reactivity. Further, the genus abundance of *Bifidobacterium* and *Streptococcus* were associated with temperamental positive affectivity.

The first human infant study to demonstrate associations between gut microbiota and cognitive performance was done only recently by Carlson et al. (2018). They found that higher alpha diversity was associated with lower scores in Mullen Early Learning Cognitive Composite (ELC) at 2 years of age. Further, Gao et al. (2019) provide initial evidence that diversity of the gut microbiome in infancy is associated with functional connectivity of neural circuits, which are critical for fear processing and cognitive development. Specifically, they found that alpha diversity was strongly associated with weaker functional connectivity between amygdala and thalamus and between anterior cingulate cortex (ACC) and anterior insula (Gao et al., 2019). In addition to amygdala, the microbiota has also effects to other brain circuits which are relevant to fear and anxiety (Gao et al., 2019). Preliminary findings in FinnBrain Birth Cohort study also demonstrate, that gut microbiota is associated with left amygdala volume (Aatsinki et al., submitted).

3.3.3 Probiotic studies

Gut microbes may have important effects on cognitive and emotional processing via many pathways. There is growing interest in the possibility of modulating the microbiota-gut-brain axis with different psychobiotics, living microorganisms that benefit the host's mental health (Dinan, Stanton, & Cryan, 2013). Even though most of the evidence comes from animal models, probiotic intervention studies with adult populations have suggested that probiotics intake reduce negative mood and might help in preventing depression (Dinan et al., 2018). A study investigating the effect of two probiotic strains (*Lactobacillus helveticus R0052 and Bifidobacterium longum R0175*) on both humans and rats showed reduced anxiety in animals and beneficial psychological effects with a decrease in urinary cortisol in humans (Messaoudi et al., 2011).

In a study where participants received 4-week multispecies probiotics supplementation, reduced overall cognitive reactivity to sad mood was reported (Steenbergen, Sellaro, van Hemert, Bosch, & Colzato, 2015). Tillisch et al. (2013) were the first ones to show in humans that intake of fermented milk product with probiotics can modulate brain activity in an emotional faces attention task. They used functional magnetic imaging (fMRI) to measure rapid, preconscious and conscious brain responses to emotional stimuli before and after 4-week intervention in healthy women. They reported changes in gut microbiome composition and reduced neural response in a network of areas including somatosensory cortex, insula and parahippocampal gyrus to angry and fearful facial expressions. These findings suggest, that gut microbiota can alter emotional processing. Similarly, Bagga et al.

(2018) reported in their study, that 4-week multi-strain probiotics supplementation improved self-reported behavioral measures of positive affect, cognitive reactivity and memory performance in healthy volunteers. In addition, they found changes in gut microbiota composition (Bagga et al., 2018). Allen et al. (2016) showed, that even intake of a single probiotic strain (*B. longum* 1714) can have an effect on stress and memory.

In addition to probiotics, modulation of microbiota-gut-brain axis with prebiotics, nondigestible food ingredients that promote the growth of beneficial bacteria, have been studied and they also might have a role in reducing anxiety and depressive symptoms (Mayer et al., 2014; Schmidt et al., 2015). Schmidt's study group (2015) gave 45 healthy volunteers one of two prebiotics (fructooligosaccharides, FOS, or Bimuno®-galactooligosaccharides, B-GOS) or a placebo (maltodextrin) for 3 weeks. BGOS prebiotic intake was associated with significantly decreased salivary cortisol awakening response compared with placebo. Thus it may be concluded that prebiotics could also have an effect on HPA activity in the same way than probiotic strains do directly.

In all, the intervention studies described above have increased our understanding on the interactions of gut-microbiota-brain axis on the human brain and behavior. It seems that the composition and functioning of the gut microbiota is important for brain development and the development of stress and emotion regulation systems. However, no data exists on whether infant attention system functioning is related to the characteristics of gut microbiota. This data is needed to understand the mechanisms behind the attention system development and also to increase our possibilities to support the developing brain in important domains.

4 Aims and research questions

The aim of this study is to explore the associations between the infant gut microbiota diversity/richness and attention to emotional faces. The possible sex differences in these associations are also investigated. In addition to investigating the probability of disengagement from different emotional expressions, a measure of "fear bias", indicating the influence of fearful faces vs. neutral and happy faces on attention disengagement, is also used. This study is exploratory in nature as no previous studies exist and thus associations between gut microbiota and all disengagement probability measures and fear bias will be explored.

Specifically, research questions were:

1. Is there an association between 2.5-month-old infant's gut microbiota alpha diversity/richness and attention to emotional faces at the age of 8 months?

Hypothesis:

Based on preliminary findings from FinnBrain Birth Cohort Study on fear reactivity, it could be expected that gut microbiota alpha diversity is positively associated with fear bias and disengagement probability from fearful face. However, as solid study background is missing, no specific hypothesis can be set. If the association between gut microbiota and emotional attention variables is found it can be in any direction.

2. Are there any sex differences in these associations?

Hypothesis:

Gut microbiota seems to influence neurodevelopment in gender specific manner. Preclinical animal models show the effect mostly on males. As Cryan & O'Mahony (2011) state, most of the neurochemical and endocrine effects are only evident in male rodents. In addition, attention patterns might be differently related to other measures in boys and girls. In an infant study by Kataja et al. (2019) it was found that maternal anxiety was associated with probability of disengagement from faces to distractors differently for boys and girls. Therefore, it can be assumed, that there might be sex-specific effects and boys might be more predisposed to the deviations in gut microbiota.

5 Materials and methods

5.1 Study design and participants

The participants for this study were infants from an ongoing FinnBrain Birth Cohort Study (www.finnbrain.fi), a population-based pregnancy Cohort located in South-Western Finland (Karlsson et al., 2018). The aim is to study prospectively the effects of early life environment on child neurocognitive development and later mental and somatic health (e.g. depression, anxiety and cardiovascular illness). Families were recruited between December 2011 and April 2015 at the first ultrasound visit at the maternity clinics. 66% of the invited mothers (n=3808) and fathers or partners (n=2623) agreed to participate in the study. The total number of children in the study is n=3837.

The subjects for this sub study belong partly to the Focus Cohort, i.e. a nested case-control population within the main Cohort. Focus Cohort was established in order to compare subjects exposed to prenatal stress with their non-exposed controls. Focus Cohort members were selected using mothers' questionnaire data from gestational weeks 14, 24 and 34 on depressive symptoms, general anxiety and pregnancy-related anxiety. 27% (n=710) of the whole Cohort members were controls with low prenatal stress and 20% (n=509) were subjects with high prenatal stress. Also some infants outside the Focus Cohort, who had participated to a MRI or a pediatric visit, are included. The Ethics Committee of the Hospital District of Southwest Finland have approved the FinnBrain study protocol.

Stool samples (n=516) were taken approximately at 2.5 months of age. The exact age of sampling was also provided. At the infant age of eight months (corrected for prematurity), infants and their mothers were invited to a study visit as part of the Child Development and Parental Functioning lab visits. Out of n=390 attempted eye tracking, n=363 infants provided satisfactory data for eye-movement tracking experiment (Kataja, 2018). Subjects with both stool sample and eye-movement tracking data create the final study population (n=140, 75 boys and 65 girls). For data analysis 14 subjects were excluded from the data: 9 which did not have successful eye-movement tracking data and 5 subjects which were missing important covariate information: mode of delivery and breastfeeding status. Final study sample is thus n=126, 67 boys and 59 girls.

5.2 Study visits: Disengagement probability and fear bias

Infant attention to emotional faces was measured with eye tracking and an age appropriate emotional overlap paradigm (Peltola, Leppänen, Palokangas, & Hietanen, 2008) assessing attention disengagement probability from centrally presented faces (neutral, happy or fearful face or a scrambled face control picture) towards lateral distractors. Monocular eye movement data (right eye) were collected using EyeLink 1000+ (SR Research Ltd, Canada) at 8 months. Three emotion conditions: neutral, happy, fearful and control with scrambled face were used. Emotional face or a scrambled face was presented in the center of the screen and a salient lateral distractor appeared to right or left side of the stimulus after 1000 ms from onset (overlap paradigm). The face and distractor were presented together for 3000 ms. Based on the averages per condition and per participant (continuous disengagement probability variable, ranging between 0-1) the probability to disengage from centrally presented face or scrambled face to lateral distractor was measured. Based on this data, disengagement probability was calculated per each condition. In addition, the difference between

infant's tendency to disengage from a fearful condition and neutral/happy condition was calculated as an index of fear bias ((DP neutral + DP happy/2) – DP fearful). Thus, the infants who had higher probability to disengage from happy/neutral condition compared to fearful condition, have a higher fear bias. (Kataja, 2018).

5.3 Gut Microbiota

2.5-month-old-infant's stool sample data from FinnBrain study was used. Parents collected the stool sample approximately at the age of 2.5 months following oral and written instructions. Parents were instructed to collect the samples to collection tubes and store the samples immediately at 4˚C and brought to laboratory within 24 hours.

In order to measure the abundance of different microbes in a stool sample DNA sequencing was done using 16S ribosomal RNA (rRNA) gene V4 region and using Illumina MISeq approach. In sequencing the nucleic acid sequence of the gene is identified. 16S gene is widely used marker in microbiome analysis to quantify microbial structure and diversity because it is universal across bacteria and archaea and it is phylogenetically informative. Based on 16S sequences it is possible to derive the microbial taxa. Although this is well-established method of comparing sample phylogeny and taxonomy, it has its limitations as many microbes have multiple copies of the gene in their genome, which means that variation in 16S gene can be caused both by genomic variation and variation in the abundance of organisms. (Kembel, Wu, Eisen, & Green, 2012) Gut microbiota contains 100 times the number of genes of the human genome. (Eckburg et al., 2005) QIIME (v.1.9) was used for downstream processing of sequencing data (Caporaso et al., 2010). Chimeric sequences were filtered out using usearch (v.6.1 against the GreenGenes database (v. 13.08). From sequencing so called operational taxonomic units (OTUs) are grouped using UCLUST on the basis of 97% DNA sequence similarity compared to other sequences in the community. (Claesson, Clooney, & O'Toole, 2017; Schloss & Westcott, 2011). Annotations for the OTUs were derived from GreenGenes database.

5.4 Covariates

Mothers provided feeding information by postnatal questionnaires. Breastfeeding variable was categorized based on the age at stool sample as exclusive breastfeeding, no breastfeeding, partial breastfeeding and cessation before the age of stool sample. Data on birth weight (g), height (cm), duration of gestation (weeks) and the mode of delivery (categorized as caesarian C-section and vaginal delivery) was collected from National Birth Registry provided by the National Institute for Health and Welfare (www.thl.fi) (Karlsson et al., 2018).

5.5 Statistical analyses

Statistical analysis was mainly carried out using tools implemented in the R package (R Core Team, 2018), which is a lingua franca in statistics (Wallace et al., 2015). Also using RStudio (integrated development environment for R programming) and tidyverse package it is easy to visualize the data analysis. An R package tidyverse is needed in addition to base R as it includes a collection of functions, data, and documentation. (Wickham & Grolemund, 2017). Alpha diversity indices (Shannon Index, species richness) were calculated with microbiome R package (Leo & Shetty, 2017). Shannon index is commonly used index to characterize species diversity in a community (Shen, Zhang, Zhu, Zhang, & He, 2008). Also IBM SPSS statistics was used partly in data analysis.

The main dependent variable was is fear bias, which was defined as the ratio of the average odds to disengage from the happy and neutral conditions to the odds to disengage from fearful condition (Kataja, 2018). The main independent variables were the following gut microbiota parameters: alpha diversity and richness. Covariates which are expected to associate with both independent and dependent variables were selected based on theoretical assumptions derived from existing literature, are sex, delivery mode and feeding information (Stewart et al., 2018). Covariates included in this analysis were sex, mode of delivery and breastfeeding status at the actual age of stool sample.

Significance level (alpha) was defined on .05 level to control for type I error, i.e. mistakenly rejecting the H0 when it is true (Cohen, 1992). Shapiro-Wilk test was used to test if the variables are normally distributed. Shannon index (gut microbiota alpha diversity) was normally distributed as well as observed species (richness) and fear bias. However, disengagement probability (DP) variables were not normally distributed. The results of Shapiro-Wilk tests are given in Table 1.

Table 1. Shapiro-Wilk test results for independent and dependent variables.

Variable	Shapiro-Wilk test W	P-value
Shannon index	0.99	0.75
Observed species	0.98	0.12
Fear Bias	0.99	0.2
DP Fearful Condition	0.96	0.002
DP Control Condition	0.85	<0.001
DP Happy Condition	0.95	<0.001
DP Neutral Condition	0.96	<0.001

Two-sample t-tests were used to compare the means of boys and girls for normally distributed variables (richness, diversity and fear bias). Mann-Whitney U test was used for DP variables.

Associations between dependent and independent variables were investigated using Pearson correlation coefficient. Chi-square test was used to determine the correlations between categorical variables. Linear regression models were built with gut microbiota parameters (diversity, richness) and a priori chosen covariates as the independent variables. In the main linear model sex was taken into account as a covariate. In order to study sex differences in the associations between gut microbiota and infant attention patterns, a stepwise linear regression models were built with two new additional variables: richness * child sex and diversity * child sex. This was done to see if sex interaction effect exists, i.e. if child sex influences the relationship between independent and dependent variables.

6 Results

6.1 Participant Characteristics

Mean gestational age was 40.1 weeks and 2 babies were born preterm (36.6 and 36.9 gwk). Boys had higher birth weight than girls (t= 2.02, p= 0.045). Most of the babies were born vaginally. Most of the babies were exclusively breastfed at the time of the stool sample. Only one girl was not breastfed at all. There were no major differences between boys and girls in breastfeeding pattern (p= 0.71) or birth mode (p= 0.91). (Table 2)

Table 2. Clinical characteristics of the mothers and children in the study presented as mean (standard deviation, SD) or count (percentages, %).

Variable		Overall n = 126	Boys n = 67	Girls n = 59
Mothers				
Maternal pre-pregnancy BMI (body mass index)	mean (SD)	24.9 (4.9)	24.0 (4.6)	25.9 (5.1)
Mothers age, years	mean (SD)	30.7 (4.1)	30.7 (3.7)	30.7 (4.6)
Children				
Gestational age, weeks	mean (SD)	40.1 (1.2)	40.1 (1.2)	40.1 (1.2)
Birth weight, g	mean (SD)	3648.5 (451.8)	3723.9 (447.8)	3562.9 (444.6)
Birth lenght, cm	mean (SD)	51.0 (2.0)	51.6 (1.9)	50.4 (1.9)
Breastfeeding	count (%)			
	No breastfeeding	1 (0.8)	0 (0)	1 (1.7)
	Exclusive breastfeeding	99 (78.6)	52 (77.6)	47 (79.7)
	Cessation before sample age	7 (5.6)	4 (6)	3 (5.1)
	Partial breastfeeding	19 (15.1)	11 (16.4)	8 (13.6)
Birth mode	count (%)			
	Vaginal	102 (81)	55 (82.1)	47 (79.7)
	C-section	24 (19)	12 (17.9)	12 (20.3)
Infant age at stool sample collection, months	mean (SD)	2.29 (0.48)	2.29 (0.43)	2.30 (0.53)

6.2 Disengagement probability and fear bias

Disengagement probabilities (DP) meaning the probability that infant look shifts towards distractor were highest for the control stimulus and lowest for the fearful condition. No significant differences

between boys and girls were found (Fear bias p= 0.9, DP Fearful p= 0.28, DP Neutral p= 0.39, DP Happy p= 0.32, DP Ctrl p= 0.30). (Table 3)

Table 3. The distribution of disengagement probabilities (DP), fear bias (odds ratio), and microbiota variables of diversity (Shannon index) and richness (observed species) as means (SD).

Variable	Overall	Boys	Girls
DP Ctrl	0.80 (0.22)	0.83 (0.19)	0.77 (0.24)
DP Neutral	0.63 (0.26)	0.66 (0.24)	0.60 (0.29)
DP Happy	0.62 (0.27)	0.65 (0.25)	0.59 (0.29)
DP Fearful	0.48 (0.28)	0.51 (0.27)	0.45 (0.29)
Fear bias	0.15 (0.20)	0.15 (0.20)	0.15 (0.22)
Diversity	1.52 (0.45)	1.47 (0.43)	1.58 (0.47)
Richness	99.27 (20.79)	97.03 (21.18)	101.81 (20.20)

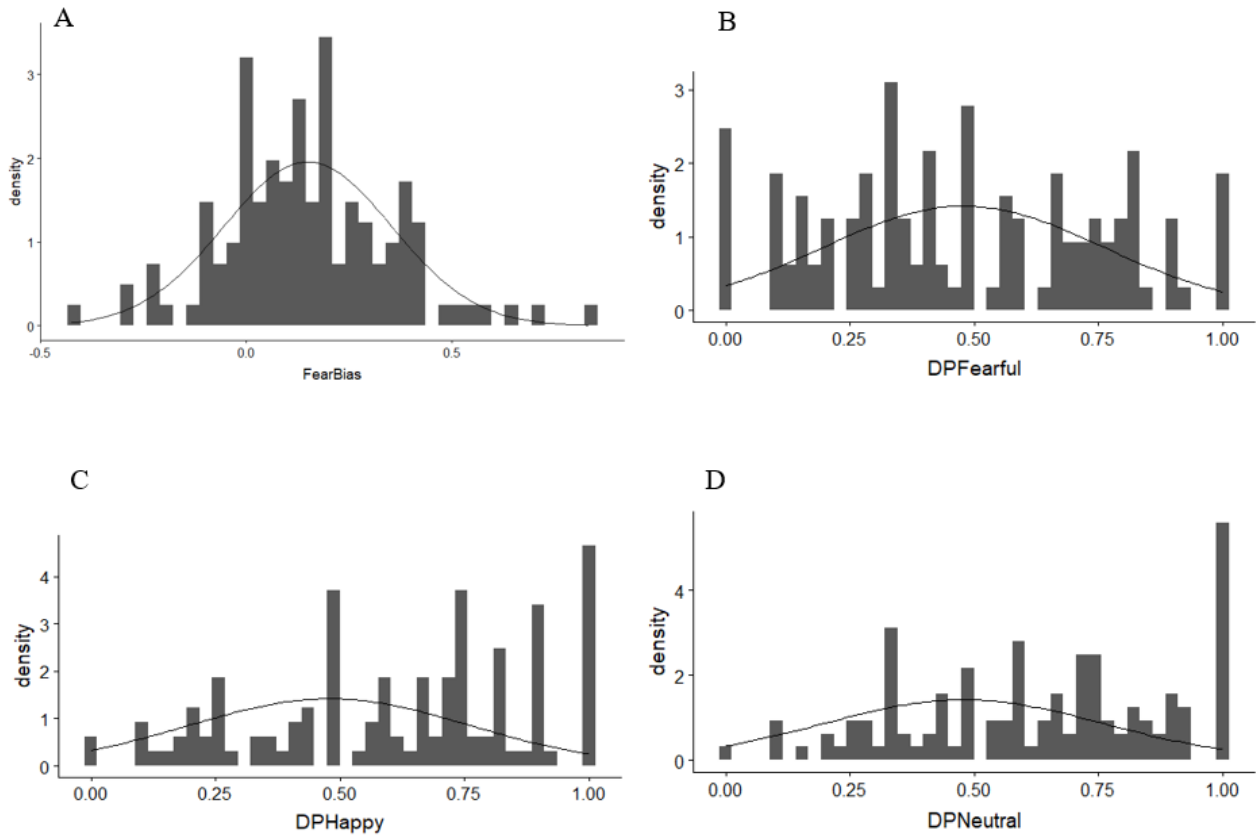

Figure 1 A-D. Histogram for fear bias (A), histogram for Disengagement probability (DP) in the fearful condition (B), histogram for Disengagement probability (DP) in the happy condition (C) and histogram for Disengagement probability (DP) in the neutral condition (D).

6.3 Diversity and richness

Shannon diversity index (i.e. relative phylotype abundance) and species richness (i.e. count of the number of species in a fecal sample) was slightly higher, but not statistically significantly, among girls than boys (diversity p=0.17, richness p=0.19, Table 3). Richness and diversity were associated with birth mode:

- Richness: Kruskall-Wallis H test $\chi 2 = 7.06$, p=0.008
- Diversity: Kruskall-Wallis H test $\chi 2 = 5.42$, p=0.02

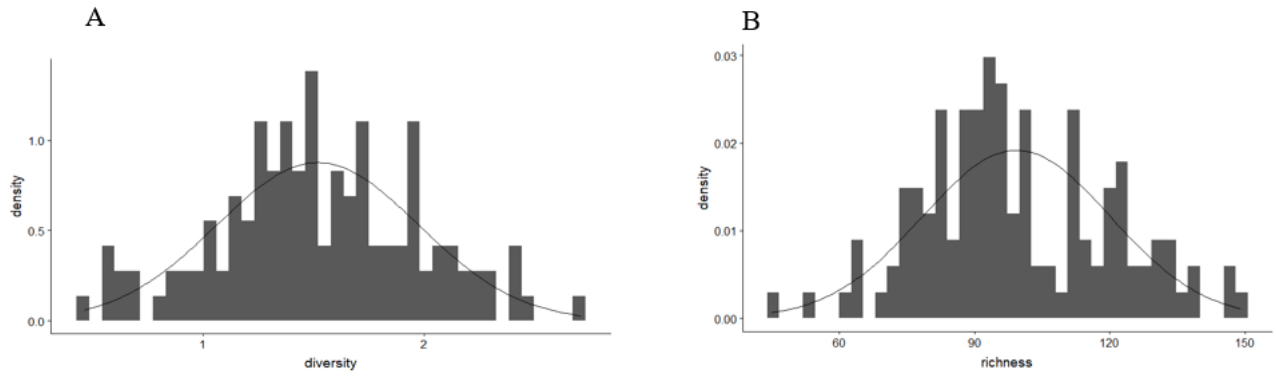

Figure 2 A-B. Histogram for diversity (A), Histogram for richness (B).

6.4 Correlations

Associations between gut microbiota variables (Shannon diversity index, observed species) and eye tracking variables (fear bias/disengagement probability) were examined first with scatter plots including linear regression lines (Figure 3). Figure 3 shows the relationships between gut microbiota and eye tracking variables separately for boys (red) and girls (blue).

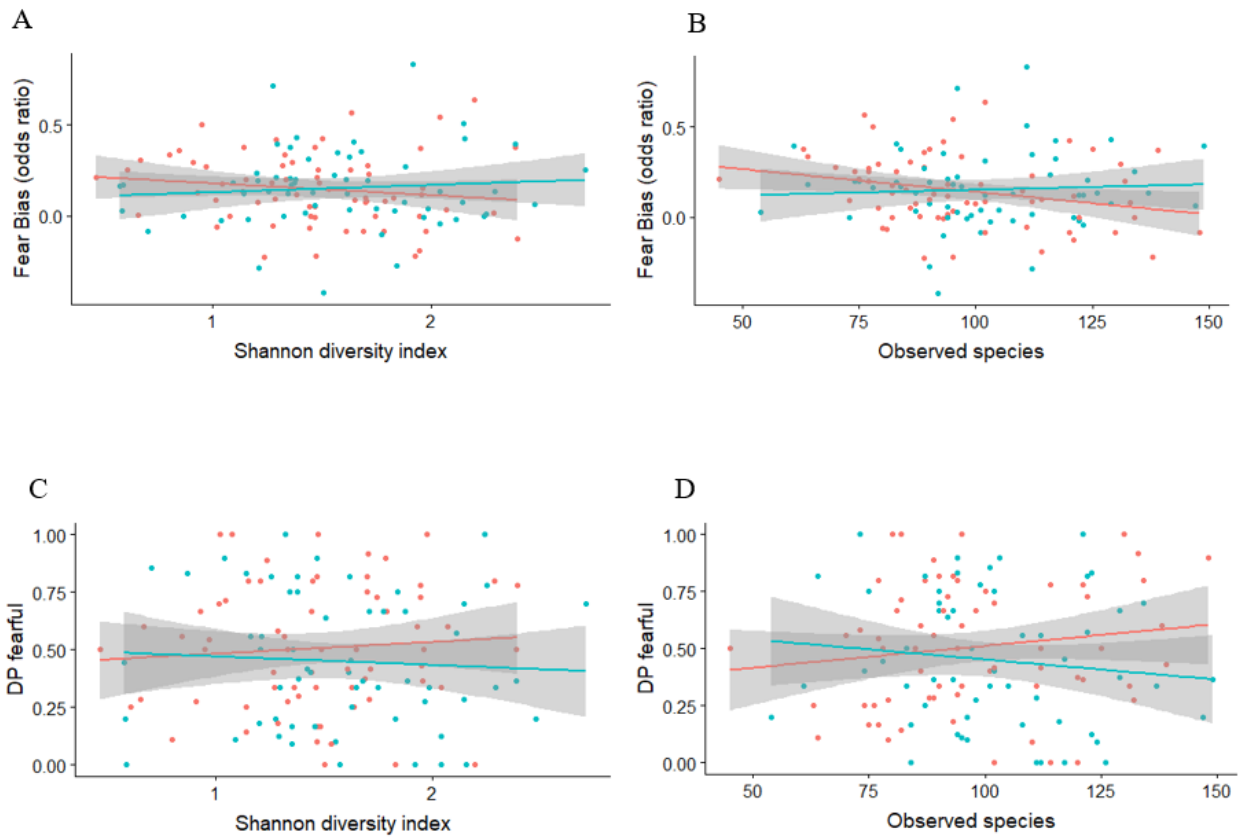

Figure 3: A–D: Scatter plots with linear regression line. Scatter plot of diversity and fear bias (A), scatter plot of richness and fear bias (B), scatter plot of diversity and disengagement probability (DP) fearful condition (C), scatter plot of richness and disengagement probability (DP) fearful condition (D).

No statistically significant correlations were found between gut microbiota variables and fear bias/disengagement probabilities. The largest correlation coefficient was observed between fear bias and richness (-0.11 Pearson correlation coefficient), but it was not statistically significant (p=0.21). Equally, all other correlations between dependent microbiota variables and independent eye-movement tracking variables were insignificant (p-values above 0.2, Table 4).

Table 4. Pearson correlation coefficient and p-values

Variable	**rho**	**P-value**
Fear bias – Diversity	-0.02	0.79
Fear bias – Richness	-0.11	0.21
DP Happy – Diversity	-0.03	0.71
DP Happy – Richness	-0.11	0.22
DP Neutral – Diversity	-0.01	0.92

DP Neutral – Richness	-0.04	0.64
DP Fearful – Diversity	-0.003	0.97
DP Fearful – Richness	0.01	0.92
DP Ctrl – Diversity	0.06	0.50
DP Ctrl – Richness	-0.06	0.49

Correlations were also examined separately for boys and girls. Among boys, a low but significant correlation between fear bias and gut microbiota richness was found ($\rho = -0.27$, $p = 0.025$).

6.5 Regression analysis

Linear regression models did not show any associations between gut microbiota and eye tracking variables. Neither alpha diversity nor richness were associated with fear bias or disengagement probabilities from fearful faces (p-values above 0.24, Table 5). Adjustment was done for sex, mode of delivery and breastfeeding. Coefficient of determination (R^2) shows only a minor effect between independent and dependent variables.

Table 5. Linear regression models for eye tracking variable fear bias and disengagement probabilities (DP) and gut microbiota alpha diversity and richness.

Dependent variable	**Independent variable**	**β**	**P-value**	**R^2**
Fear Bias	Diversity	0.0001	0.998	1.3 %
Fear Bias	Richness	-0.0011	0.24	2.5 %
DP Fearful	Diversity	-0.0059	0.92	2.3 %
DP Fearful	Richness	0.0003	0.79	2.4 %

6.6 Sex interaction in linear regression model

The other research question was to investigate if there were any sex differences in the associations of gut microbiota and emotional attention. Based on scatter plots on diversity/richness and fear bias/disengagement probability fearful condition, small sex interaction might be possible. In the main linear model child sex was taken into account as covariate. In order to calculate sex interaction, two new variables were created: richness * child sex and diversity * child sex.

Based on stepwise linear regression model, weak evidence for sex interaction was found and it was clearest between richness and fear bias (interaction term p=0.079). For diversity and fear bias adding sex interaction term also changed the p-value towards more significant value (p=0.196).

7 Discussion

In this study, the associations between the infant's gut microbiota diversity and attention to emotional faces, specifically to fearful faces, were investigated. Contrary to our hypotheses, no associations between gut microbiota diversity and emotionally directed attention in infants were observed. The only statistically significant correlation was found between fear bias and gut microbiota richness in boys ($\rho = -0.27$, $p = 0.025$), but the interaction between child's sex and gut microbiota failed to reach significance ($p = 0.079$) in a linear regression model controlling for the mode of delivery and breastfeeding.

7.1 Gut microbiota diversity and infant attention to emotional faces

Despite the gut microbiota's critical role in neurodevelopment, we currently have little knowledge regarding the interrelations between gut microbiota and cognitive, attentional processes early in life. Infants have attention bias toward faces from the very first days and during second half of first year infants normally develop a tendency to orient and maintain attention towards fearful faces, which is a well-established infant phenotype called fear bias (see e.g., Leppänen, 2016). In this study validated measures for infant attentional biases, disengagement probability ratio and fear bias, were used as markers of emotional attention. It was found that disengagement probability ratio was highest for the control stimulus and lowest for the fearful condition, which is in line with previous findings (Peltola et al., 2013, 2008). As part of typical development, babies under 1 year of age look more intensively at fearful faces and do not shift attention so easily from fearful faces as compared to happy and neutral faces (Kataja, 2018; Peltola et al., 2013, 2008). However, it is not clear, whether strong fear bias is favorable for later socio-emotional development. Some findings suggest that heightened fear bias might signal difficulties in socio-emotional processing (Kataja, 2018). Kataja (2018) reported heightened fear bias in 8-month-old infants, whose mothers had elevated depressive symptoms during pregnancy (either increasing or decreasing). On the other hand, there is evidence, that smaller age-typical fear bias might be associated with insecure attachment (van IJzendoorn, Puura, Forssman, Leppänen, & Peltola, 2015). So differences in disengagement probabilities and fear bias can have

predictive value in terms of later development. Thus, understanding the factors affecting emotional attention is important as problems in controlling attention might underlie development of psychiatric conditions. As suggested, gut microbiota could play an important role in brain development Humans live in a symbiotic relationship with gut microbiota and it is suggested that in the absence of bacteria humans would not have developed the current level of cognitive performance (Dinan et al., 2015). HPA-axis and serotonergic system development are dependent on gut microbiota (Clarke et al., 2013; Sudo et al., 2004). Both HPA-axis and serotonergic systems are linked to emotional activity and thus it might be suggested, that gut microbiota could be part of "the unconscious system" regulating behavior (Dinan et al., 2015).

This study used a novel perspective on linking the infant gut microbiota diversity to emotional attention and as no previous literature exists, this study was exploratory in nature. However, an association between gut microbiota alpha diversity and fear bias and disengagement probability from fearful face, was expected as previous findings from FinnBrain Birth Cohort Study indicate an association between gut microbiota alpha diversity and temperamental fear reactivity (A.-K. Aatsinki et al., 2019). Also, Gao et al. (2019) found that alpha diversity is associated with functional connectivity between amygdala and thalamus and anterior cingulate cortex and anterior insula; brain areas which play important role in processing threat related information. Contrary to this, our data did not yield evidence for a link between the selected gut microbiota measures and emotionally directed attention and fear processing in infants.

7.2 Statistical power in the study

This study consisted of sample of 126 infants. As the variance in infant emotional attention phenotypes are affected by several factors, it might be that statistical power was inadequate to detect an effect even there is one. Anyhow the size of the effect in this case might be relatively modest and thus the size of the sample should be even higher in order to detect an effect. In a wide population-level analysis of gut microbiome variation Falony et al. (2016) conclude that total gut diversity is not yet fully covered. Even combining microbiome data from almost 4000 individuals and observing total western gut microbiota richness would require sampling an estimated additional 40739 individuals (Falony et al., 2016). Further, they estimated the sample size needed to evaluate microbiota compositional changes associated to obesity and found that taking sex, age and Bristol stool scores as covariates, the estimated sample size would be about 535 with a power of 80%. Power of 80 % is

suggested as desired power by Cohen (1992). However, as there are no previous studies on the topic and we don't know the expected effect size (Cohen's d), it was not possible to calculate sample size needed for sufficient statistical power. In addition, the attentional bias for fear is a complex phenomenon, and variables measuring it in infancy (fear bias and disengagement probability) are multifactorial. Hence, future studies should consider larger sample size to increase statistical power and/or clarifications to the dependent and independent variables in order to decrease noise.

7.3 Confounding factors

Breastfeeding and delivery mode are significant factors associated with the microbiota structure (Stewart et al., 2018). In TEDDY study breastfeeding was associated with higher levels of Bifidobacterium species and vaginal delivery was associated with higher levels of Bacteroidetes species (Stewart et al., 2018). Further, Bacteroidetes were also associated with increased gut diversity (Stewart et al., 2018). Thus, in this study child sex, mode of delivery and breastfeeding status at the stool sample age was included as covariates in linear regression model. Richness and diversity were associated with birth mode, but not with breastfeeding status. However, approximately 81 % of babies were born vaginally, so the distribution was not even. Also 94 % of babies were breastfed at least partially and only one infant did not receive any breastmilk. The information about feeding in addition to breastmilk was not known. So it was not possible to take into account the effect of feeding pattern as a whole. Also other factors, such as siblings and furry pets could have been taken into account as TEDDY study found that household exposures were found to be associated with differences in the microbiome profiles in early life (Stewart et al., 2018). In addition to this, infant antibiotic use was not controlled for.

7.4 Sex differences in gut microbiota and neurodevelopment

The weak evidence for sex interaction and correlation between richness and fear bias in boys is consistent with rodent studies, where microbiota has been suggested to affect brain development in sex-specific manner (Clarke et al., 2013; Jašarević, Morrison, & Bale, 2016). Early disruptions in gut microbiota reportedly have male-specific effects on CNS serotonergic system and thus boys might be specifically sensitive for the altered microbiota (Clarke et al., 2013). Further, neurodevelopmental deficits studied with GF mice are specific to males in which there are higher incidence rates of neurodevelopmental disorders relative to females (L. Desbonnet et al., 2014). In general, previous

studies have shown sex-related differences in gut microbiota composition and the alpha diversity appears to be greater in females (Kim, Unno, Kim, & Park, 2019). Thus, child's sex is an important covariate in studying effects of gut microbiota to neurodevelopment.

7.5 Limitations of the study

Certain limitations in the current study should be mentioned. Both gut microbiota and eye movement measures were assessed only at a single time point and further studies should consider adding longitudinal sampling to deepen the understanding of gut microbiota and early life neurodevelopmental trajectories.

The current study concentrated on a very narrow perspective on gut microbiota and diversity might be too crude summary metric of gut microbiota as it does not characterize the underlining gut microbiota with enough precision. As Shade (2017) suggest, microbial diversity calculations have many biases and they can be considered only as rough approximations. Diversity is not an absolute value and different methods give slightly different picture of a community (Shade, 2017). The diversity metric used in this thesis, Shannon diversity index, assesses relative phylotype abundance. Additionally, the measures used in this study do not describe the functional properties of gut microbiota. Thus, assessment of gut microbiota metabolites, such as short chain fatty acids which are produced from the fermentation of dietary fiber, would offer deeper understanding on how the metabolites possibly influence the brain and behavior (Dinan et al., 2018).

Also the age group studied gives more challenges into measuring diversity as with infants undergo rapid gut microbiota colonization and gut microbiota maturates during the first year of life into a more complex and adult-like configuration with higher alpha diversity and lower beta diversity until age of 3 years (Bäckhed et al., 2015; Yatsunenko et al., 2012). Thus it would be interesting to investigate the associations of gut microbiota diversity and emotionally directed attention later in development. Some limitations in this study could be caused also by the long time distance between fecal microbiota sample and eye-tracking measurements, because it might have been possible that gut microbiota was already different at the age of 8 months.

8 Conclusions

This thesis investigated novel information on the associations between emotionally directed attention and gut microbiota diversity. Even though no clear associations were found, the results of this thesis encourage for further human studies to illustrate the potential underlying neural structures of emotional attention and gut microbiota. Studying the neonatal gut microbiota alpha diversity and its association with emotionally directed attention processing is an important step towards understanding the role of early microbiota in emotion regulation development. As the ability to flexibly control attention is essential for maintaining psychological well-being and for habituating to stressful environments, it is important to understand the potential biological mechanisms behind the attentional control mechanisms. Understanding how early gut microbiota associates with mental health opens new ways for interventions in at-risk populations (Borre et al., 2014b). Hence, future studies should measure gut microbiota composition in addition to gut microbiota diversity. These results describe only a snapshot of associations during infancy and future follow-up is needed to investigate the later integration of gut microbiota and emotional attention development.

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