Prenatal maternal stress and infant gut microbiota

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Master's Thesis

Psychology

Faculty of Medicine

May 2020

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Faculty Faculty of Medicine	Degree Programme Department of Psychology and Logopedics	
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Title Prenatal maternal stress and infant gut microbiota		
Subject/Study track Psychology		
Level Master's Thesis	Month and year May 2020	Number of pages 42

Abstract

OBJECTIVES. The association between prenatal maternal stress and adverse health and developmental offspring outcomes has been long known but explanations for this association remain insufficient. One of the most recent suggestions is gut microbiota. Only a few studies with many limitations have concentrated on the association between prenatal stress and offspring gut microbiota. The aim of this study is to conduct a large scale study with follow-up covering the whole infancy, and to test whether the association differs between girls and boys.

METHODS. This study's sample consists of 825 mothers and their infants from HELMi cohort. Prenatal maternal stress is measured with self-report questionnaire, and infant gut microbiota from fecal samples. 16S rRNA sequencing is used in analyzing the microbiota.

RESULTS. High stress group had lower alpha-diversity than low stress group at 3 weeks. No differences were found in richness and beta-diversity. Several phylum, family, and genus level bacteria were associated with prenatal stress. Regarding sex differences, no differences were found in richness or in alpha- or beta-diversity. However, in phylum, family, and genus level bacterial relative abundances, more associations were found in boys than in girls.

CONCLUSION. Overall the findings in this study were contradicting compared to previous findings. There was indication that there is no clear association between prenatal stress and infant overall microbiota composition. Also, the association regarding bacterial abundances could decline over age, and the association might be stronger in boys. However, not very consistent conclusions can be made based on research conducted so far.

Keywords
prenatal maternal stress, infant, gut microbiota

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Tiedekunta Lääketieteellinen tiedekunta	Koulutusohjelma Psykologian ja logopedian osasto	
Tekijä Viitaharju, Janika Sofia		
Työn nimi Äidin raskaudenaikainen stressi ja lapsen suolistomikrobiomi		
Oppiaine/Opintosuunta Psykologia		
Työn laji Pro gradu -tutkielma	Aika Toukokuu 2020	Sivumäärä 42

Tiivistelmä

TAVOITTEET. Yhteys äidin raskaudenaikaisen stressin ja lapsen terveydellisten ja kehityksellisten haitallisten seurausten välillä on tunnettu jo pitkään, mutta selitykset yhteydelle ovat olleet riittämättömiä. Yksi uusimmista selityksistä on suolistomikrobiomia. Tutkimuksia raskaudenaikaisen stressin ja lapsen suolistomikrobiomin yhteydestä on tehty vasta muutamia, ja niihin on liittynyt huomattavia puutteita. Tämän tutkimuksen tavoitteena on lisätä tietoa äidin raskaudenaikaisen stressin ja lapsen suolistomikrobiomin yhteydestä vauvan ensimmäisen elinvuoden aikana laajamittaisella seurantatutkimuksella ja selvittää onko yhteys erilainen tytöillä ja pojilla.

MENETELMÄT. Aineistona käytetään HELMi-kohortista valittua 825 äitiä ja heidän HELMi-kohorttiin kuuluvia lapsiaan. Äidin raskaudenaikaista stressiä mitataan itsearviointilomakkeella, ja lapsen suolistomikrobiomia ulostenäytteistä. Mikrobiomin analysoinnissa käytetään 16S rRNA sekvensointia.

TULOKSET. Vauvoilla, joiden äidit kokivat paljon stressiä raskausaikana, oli pienempi mikrobiomin alfa-diversiteetti kolme viikkoa syntymän jälkeen. Eroja rikkaudessa ja beta-diversiteetissä ei löytynyt. Useat pääjakso, heimo ja luokka tasoilla mitatut bakteerit olivat yhteydessä raskaudenaikaiseen stressiin. Vertailtaessa yhteyksiä tytöillä ja pojilla, eroja ei löytynyt rikkaudessa, tai alfa- tai beta-diversiteetissä. Vertailtaessa yhteyksiä pääjakso, heimo ja luokka tason bakteereihin, pojilla löytyi enemmän yhteyksiä kuin tytöillä.

JOHTOPÄÄTÖKSET. Pääsääntöisesti tämän tutkimuksen tulokset olivat ristiriitaisia aiempien tutkimusten kanssa. Stressillä ei näyttänyt olevan yhteyttä vauvan kokonaisvaltaiseen mikrobiomin rakenteeseen. Eri bakteerien osalta yhteys näytti olevan vahvempi lähempänä syntymää, ja vahvempi pojille kuin tytöille. Nykyisen tietämyksen valossa ei vielä kuitenkaan voida tehdä tarkkoja johtopäätöksiä.

Avainsanat
raskaudenaikainen, stressi, suolistomikrobiomi

Ohjaaja
Kati Heinonen-Tuomaala

Säilytyspaikka
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1. INTRODUCTION

A large proportion of pregnant women experience psychological distress: it has been estimated that as much as 20% experience mood, anxiety, and related disorders reflecting high distress (Bennett, Einarson, Taddio, Koren & Einarson, 2004; Fairbrother, Janssen, Antony, Tucker & Young, 2016; Heron, O'Connor, Evans, Golding & Glover, 2004) and even bigger proportion is suggested to experience milder distress below clinical threshold (Van den Bergh et al., 2017). It has been noticed that maternal prenatal stress predisposes the child later in life to many health adversities, including physical and mental health problems, as well as poorer motor and cognitive performance (Beydoun & Saftlas, 2008; Flanigan, Sheikh, DunnGalvin, Brew, Almqvist & Nwaru, 2018; Glover, O'Donnell, O'Connor & Fisher, 2018; O'Mahony, Clarke, Dinan & Cryan, 2017; Van den Bergh et al., 2017; Ziljmans, Beijers, Riksen-Walraven & Weerth, 2016).

Many mechanisms linking prenatal stress and child outcomes have been suggested, including elevated cortisol levels, activated immune system, and mother's altered health behaviors (Beijers, Buitelaar & de Weerth, 2014; Glover et al., 2018). However, these explanations remain insufficient and new explanations are needed (Beijers et al., 2014). One of the most recent suggestions is gut microbiota. The suggestion rises from the notion of a microbiota-gut-brain axis (MGBA-axis): a two-way communication pathway between the brain and the gut (Cryan & Dinan, 2012). It has been found that via the MGBA-axis stress may alter microbiota composition (Cresci & Bawden, 2015; Cryan & Dinan, 2012; Gur & Bailey, 2016) and microbiota, in turn, is associated with several health consequences (Aureli et al., 2011; Fujimura, Slusher, Cabana & Lynch, 2010; Wu & Wang, 2019).

Regarding the gut microbiota as a linking mechanism for the association between prenatal stress and child outcomes, it is suggested that prenatal stress alters mother's microbiota that then transfers into the child (Beijers et al., 2014; Gosalbes et al., 2013). It is suggested that maternal microbiota may be transferred into the fetus in at least three ways: during the pregnancy via the placenta or amniotic fluid and during delivery when the fetus is in touch with the mother's vaginal and fecal microbiota (Beijers et al., 2014; Gosalbes et al., 2013). Thus, the child may be predisposed to dysfunctional microbiota already before birth (Beijers et al., 2014; Gosalbes et al., 2013). Dysfunctional microbiota may alter the infant's development and predispose to many adverse health consequences since the gut microbiota, immune system, gastrointestinal tract, and metabolism mature at the same time (Milani et al., 2017).

Based on literature search, there are only a few studies focusing on the association between prenatal maternal stress and offspring gut microbiota: five animal studies and three human studies. Also, the methodologies have varied highly, sample sizes have been relatively small, and follow-ups short. The aim of this study is to add knowledge on the association between maternal self-reported prenatal stress and the infant gut microbiota during the first year after birth.

1.1 Prenatal maternal stress

On a large scale stress means a stressful situation or a stressor, evaluations considering the situation/stressor, and physiological and behavioral responses (Beijers et al., 2014). Prenatal stress has been measured for example as exposure to major life events (eg. natural disasters), stressful life events, daily hassles, pregnancy related distress, and depression or anxiety symptoms, and with biomarkers such as cortisol, CRH, and ACTH (Beydoun & Saftlas, 2008).

The wide variety of stress measures makes it difficult to estimate the prevalence of prenatal stress. However, of the pregnant women approximately 3.9%-20.4% are estimated to experience clinically relevant depressive symptoms (Bennett et al., 2004; Fairbrother et al., 2016; Heron et al., 2004), and about 15% with anxiety and related disorders (Fairbrother et al., 2016; Heron et al., 2004). Even bigger number is assumed to experience psychological distress below clinical threshold (Van den Bergh et al., 2017) reflecting the phenomenon relevant to many pregnant women.

1.1.1 Prenatal maternal stress and offspring outcomes

Prenatal maternal stress has widely been associated with many different adverse offspring outcomes. There is evidence that prenatal stress alters the fetal brain development and the immune system, which predisposes the infant to different diseases and developmental problems (Ruiz & Avant, 2005).

Existing literature has shown that higher prenatal stress is associated with fetal growth restriction and the infant's lower birthweight, shorter gestational length, and premature birth (Beydoun & Saftlas, 2008; Glover et al., 2018). It has also been associated with increased risk to general illnesses, digestive illnesses, asthma, eczema/dermatitis, wheeze, and allergic rhinitis in toddlerhood and childhood (Flanigan et al., 2018; Ziljmans, Beijers, Riksen-Walraven & Weerth, 2016).

Prenatal stress has also been associated with the offspring's mental health consequences in infancy, childhood, and even in early adulthood, and with developmental problems. Higher prenatal stress has been associated with the offspring's difficult temperament (more crying, and difficulties in feeding, sleeping, and soothing) and with more motor and cognitive developmental problems (lower performance) in infancy (Glover et al., 2018; Van den Bergh et al., 2017). Also, prenatal stress has been associated with more problems in attention, aggressive behavior, conduct disorder, ADHD, autism, depression, anxiety, and with cognitive and motor developmental problems in childhood (Glover et al., 2018; O'Mahony et al., 2017; Van den Bergh et al., 2017). Further, association regarding increased risk for depression, anxiety, and schizophrenia has been found to be evident even in early adulthood (Glover et al., 2018; O'Mahony et al., 2017).

Association between prenatal stress and adverse offspring outcomes may be different in girls and in boys (Glover et al., 2018; Sutherland & Brunwasser, 2018; Van den Bergh et al., 2017). Association with developmental problems, anxiety, and affective disorders are suggested to be stronger in girls and associations with ADHD, conduct disorder, and respiratory illnesses stronger in boys (Sutherland & Brunwasser, 2018).

1.2. Possible mechanisms linking prenatal maternal stress to offspring outcomes

1.2.1 Traditional explanations

The association of prenatal maternal stress with adverse offspring outcomes has been explained in several different ways. Perhaps the most known suggested mechanism is maternal hypothalamic-pituitary-adrenal axis (HPA-axis). It is suggested that prenatal stress activates mother's HPA-axis, which leads to increased levels of cortisol in the placenta (Beijers et al., 2014). Cortisol may be transferred into the fetal blood circulation and affect the fetal brain development (Beijers et al., 2014). Maternal stress reaction does not necessarily elevate mother's cortisol levels but the fetus still may be exposed to an excessive amount of cortisol via the 11b-HSD2 enzyme (Glover et al., 2018). Prenatal stress may cause a downregulation of the 11b-HSD2 enzyme that controls the transfer of maternal cortisol into the fetal blood circulation (Glover et al., 2018). Another possible mechanism for the association is that prenatal stress activates the maternal "fight or flight" system that leads to increased amounts of adrenaline and noradrenaline that may increase fetal catecholamine release and the supply of nutrients in the placenta (Beijers et al., 2014). Also, increased catecholamine levels may predispose the offspring to increased reactivity for stressful

events (Beijers et al., 2014). Another suggested mechanism is that prolonged prenatal stress leads to poor maternal immunity and more frequent infections, which increases the amount of pro-inflammatory cytokines (Beijers et al., 2014; Ruiz & Avant, 2005). Pro-inflammatory cytokines may affect the fetal development in the placenta or cross the placenta into the fetus (Beijers et al., 2014). Also, the fetus's reactions to prenatal environment may be one mechanism linking prenatal stress to offspring outcomes (Beijers et al., 2014). Finally, mother's altered health behavior, postnatal environment, and the offspring's genetic vulnerabilities have been proposed as mechanisms linking prenatal stress and offspring outcomes (Beijers et al., 2014; Glover et al., 2018). In regard of health behaviors, there is evidence that stress is associated with unhealthy eating patterns, decreased physical activity, increased substance abuse, and poor sleep quality that are known to affect fetal development (Beijers et al., 2014). Regarding the postnatal environment maternal prenatal and postnatal mood are correlated (Heron et al., 2004) and postnatal mood may alter the interaction between the mother and the child, which may affect the child's mental health later in the future (Glover et al., 2018). The role of genetic vulnerabilities has not yet been studied widely and, thus, cannot be ruled out as one possible mechanism (Beijers et al., 2014).

Even though many explaining mechanism for the association between prenatal stress and adverse offspring outcomes have been proposed existing explanations still remain insufficient (Beijers et al., 2014). Thus, new explanations are needed.

1.2.2 New explanation: gut microbiota

It is suggested that prenatal stress may alter mother's intestinal microbiota and lead to subclinical inflammation in vaginal microbiota (Beijers et al., 2014). Maternal microbiota may be transferred into the fetus and predispose the fetus to pathological microbiota already before birth (Beijers et al., 2014). This suggestion is supported by the fact that bacteria have been found in umbilical cord blood and in infants' first stool, meconium (Gosalbes et al, 2013). Also, in three animal studies (Gur et al., 2017; Gur et al., 2019; Jašarević et al., 2017) prenatal stress has been found to alter maternal gut microbiota and in one of them (Jašarević et al., 2017) also maternal vaginal microbiota. The changes in microbiota were found to correlate with offspring microbiota. Also, one human study (Naudé et al., 2020) found that prenatal stress alters both maternal and infant gut microbiota but the correlation of them was not assessed.

There are two suggested mechanisms by which maternal microbiota may be transferred into the fetus already in the utero. First, maternal microbiota may travel into mother's blood circulation from where it may travel into the placenta (Gosalbes et al., 2013). From the placenta microbiota can travel into the fetal blood circulation and into the fetal gut (Gosalbes et al., 2013). Second, microbiota may travel into the fetus via amniotic fluid (Gosalbes et al., 2013) that fetuses often swallow (Beijers et al., 2014). When maternal microbiota is transferred into the fetus it alters the development of the gut microbiota and immunity, which may predispose the infant to many adverse health consequences later in life (Gosalbes et al, 2013).

The fetus is further exposed to maternal pathological microbiota during birth. During vaginal delivery the fetus is in touch with mother's vaginal and fecal microbiota (Gosalbes et al., 2013). In cesarean delivery, however, the fetus is in touch with the microbiota on mother's skin (Greenhalgh, Mayer, Aagaard & Wilmes, 2016). Indeed, it has been found that the microbiota of vaginally born infants resembles mother's vaginal microbiota and the microbiota of infants born by cesarean section is more similar to mother's skin microbiota (Greenhalgh et al., 2016).

1.3. Gut microbiota

Human microbiota consists of bacteria, archaea, eukarya, and viruses (Dave, Higgins, Middha & Rioux, 2012). So far most of the studies have concentrated only in bacteria (Hooks, Konsman & O'Malley, 2019). Microbiota is found in nasal passages, oral cavity, skin, stomach, bowel, and urogenital system but especially large numbers of microbiota are in gastrointestinal tract (Dave et al., 2012). In addition to being found all over our body the fact that the amount of microbial genes outnumber our own genes (Dave et al., 2012) points out how relevant microbiota may be on our health.

This study concentrates on the bacteria of gut microbiota. The bacteria of the gut play many important functions on our health and metabolism: they are a part of the intestinal wall, resist colonization, absorb and product nutrients, interact with the immune system, protect against xenobiotics, and suppress inflammatory processes (Aureli et al., 2011). Gut microbiota also functions together with the brain via the MGBA-axis (Cryan & Dinan, 2012). The gut and the brain communicate via sympathetic and parasympathetic autonomic nervous systems, neuroendocrines (eg. cortisol), and neuroimmune system (eg. cytokines) in a bidirectional way (Cryan & Dinan, 2012). This means that the brain can affect gut microbiota and vice versa. For example the HPA-

axis controls the secretion of cortisol that affects immune cells and cytokines, composition of gut microbiota, and gut permeability (Cryan & Dinan, 2012). Gut microbiota, in turn, can alter cytokines that can affect brain functioning (Cryan & Dinan, 2012).

1.3.1 The development of gut microbiota

Gut microbiota starts to develop already in utero (Greenhalgh et al., 2016). In infancy, gut microbiota composition is defined by low diversity, low individual stability, and high inter-individual variation (Wang, Monaco & Donovan 2016). Gut microbiota composition develops gradually and reaches adult like composition at about three years of age (Matamoro, Gras-Leguen, Le Vacon, Potel & de La Cochetiere, 2013). Although gut microbiota composition keeps changing through the whole lifetime depending from age, diet, environment, ethnicity, and geographical location etc. (Cresci & Bawden, 2015; Greenhalgh et al., 2016) the basis is created during the first three years and especially the first year of life is considered as a particularly important timeframe (Matamoro et al., 2013).

Many factors can affect gut microbiota composition in the first years of life. In the utero important factors are for example mother's diet and antibiotic use (Greenhalgh et al., 2016). At birth the mode of delivery and gestational age are important factors for the development of gut microbiota (Greenhalgh et al., 2016; Wang et al., 2016). Premature birth and delayed birth have been associated with lower bacterial diversity (Greenhalgh et al., 2016; Wang et al., 2016), and lower abundance of genera *Bacteroides*, *Lactobacillus*, and *Bifidobacterium* (Greenhalgh et al., 2016). After birth important factors affecting the development of offspring gut microbiota are mother's diet and medication (via breastfeeding), mode of feeding (breastfeeding versus formula), infant's transfer to solid food, diet and medication, and the presence of other children and pets in the household (Matamoro et al., 2013).

As mentioned before, also in adulthood many factors can affect gut microbiota composition. In addition to previously mentioned factors, also stress has been considered as one significant factor to alter gut microbiota composition (Cresci & Bawden, 2015).

1.3.2 Stress and gut microbiota

Both physical stress due to exercise or illness and psychological stress may affect gut microbiota via the MGBA-axis (Cresci & Bawden, 2015; Cryan & Dinan, 2012). The mechanisms by which stress affects gut microbiota are not yet entirely known. However, for example the HPA-axis (Cresci & Bawden, 2015; Cryan & Dinan, 2012), gastrointestinal physiology, and hormonal changes (Gur & Bailey, 2016) have been proposed to play a role in the transition of stress to gut microbiota composition. It has been suggested that stress reaction activates the HPA-axis that releases glucocorticoid hormones and noradrenaline that, in turn, alter gastrointestinal physiology (Gur & Bailey, 2016). Gastrointestinal physiology, in turn, determines microbial composition (Gur & Bailey, 2016). There is also evidence that gastrointestinal physiology induced changes in gut microbiota may be mediated through immunity (Cong, Henderson, Graf & McGrath, 2015). Both animal and human studies suggest that the HPA-axis activation increases gut permeability, which activates the immune system, which, in turn, can change gut microbiota composition (Cong et al, 2015). Further, one possibility is that stress reaction releases neuroendocrines that may have a direct effect on gut microbiota by increasing bacterial growth (Gur & Bailey, 2016).

Studies in rodents have shown that stress exposure, usually transfer of the cage or maternal separation, increases bacterial growth, alters the overall microbial community (Gur & Bailey, 2016), and may decrease microbial richness and diversity (Bailey et al., 2010). Stress has been associated with for example lowered relative abundance of genera *Bacteroides* (Bailey et al., 2011) and *Lactobacillus* (Gur & Bailey, 2016), and increased abundance of genus *Clostridium* (Bailey et al., 2011) and species *Citrobacter Rodentium* (Bailey et al., 2010).

1.3.3 Gut microbiota and health

The inter-individual variation in gut microbiota composition is so huge that it may not be possible to determine a normal healthy gut microbiota (Dave et al., 2012). There is no consensus among researchers even on whether lower or higher bacterial diversity is considered better on our health. (Hooks et al., 2019). However, it is thought that there may be some common indicators on healthy gut microbiota (Hooks et al., 2019. For example in adult populations low abundance of phyla Proteobacteria and Firmicutes to phylum Bacteroidetes ratio has been proposed as one sign (Shin, Whon & Bae, 2015). In adult and pediatric populations overall microbiota composition and/or relative abundances of different bacteria have been associated with for example atopy, asthma,

coeliac disease, type I and II diabetes, HIV, irritable bowel syndrome (IBS), inflammatory bowel diseases (IBD), necrotizing enterocolitis (NEC), obesity, rheumatoid arthritis, cancer, and mental disorders (Fujimura et al., 2010; Van Ameringer, Turna, Patterson, Pipe, Mao, Anglin & Surette, 2019; Wu & Wang, 2019).

In infancy gut microbiota, immune system, gastrointestinal tract, and metabolism mature at the same time and, thus, it is suggested that early gut microbiota would be associated with later health issues (Milani et al., 2017). Aberrations in several bacteria and low bacterial diversity in infancy have been associated for example with NEC, IBD, atopy, eczema, asthma, obesity, and autism in childhood and even later in life (Wang et al., 2016). Increased abundance of phylum Proteobacteria, and decreased abundance of genera *Bifidobacterium* and *Lactobacilli* at the age of 2 weeks have been associated with colic at the age of 6 weeks (de Weerth, Fuentes, Puylaert & de Vos, 2013). Genera *Bifidobacterium* and *Lactobacillus* have been noticed to protect against atopy (Gosalbes et al., 2013). Also, a low amount of genus *Bifidobacterium* and a high amount of class *Clostridia* in infancy have been associated with development of atopic disease at the age of 2 years (Milani et al., 2017). Reduced bacterial diversity and a high amount of species *Escherichia coli* have been associated with eczema (Gosalbes et al., 2013). Low abundance of *Bifdobacterium adolescentis, Clostridium difficile,* and several *Lactobacilli* have been associated with allergy development during the first five years of life (Sjögren, Jenmalm, Böttcher & Sverremark-Ekström, 2009). Early microbial dysbiosis caused by maternal antibiotic use has been associated with increased risk to suffer from asthma (Gosalbes et al., 2013). Low number of *Bifidobacterium* and antibiotic use have been associated with obesity in childhood (Kalliomäki, Collado, Salminen & Isolauri, 2008; Milani et al., 2017).

In adult populations some bacteria have also found to be probiotic. For example different *Lactobacillus* strains may shorten the duration of gastroenteritis, decrease the risk for respiratory infections, constipation and bloating (Aureli et al., 2011), and relieve visceral pain (Cryan & Dinan, 2012). There is also evidence that different *Lactobacillus* strains may relieve symptoms of major depressive disorder (Wallace & Milew, 2017). Further, also *Bifidobacterium* strains may decrease the risk of respiratory infections and constipation (Aureli et al., 2011), and relieve visceral pain (Cryan & Dinan, 2012).

1.4 Prenatal maternal stress and offspring gut microbiota

On the basis of systematic literature search (Appendix 1 and 2) so far only eight studies have concentrated on the association between maternal prenatal stress and offspring gut microbiota. Five of them are animal studies (Golubeva et al., 2015; Bailey, Lubach & Coe, 2004; Gur, Palkar, Rajasekera, Allen, Niraula, Godbout & Bailey, 2019; Gur, Shay, Palkar, Fisher, Varaljay, Dowd & Bailey, 2017; Jašarević, Howard, Misic, Beiting & Bale, 2017) and three with humans (Hu et al., 2019; Naudé et al., 2020; Ziljmans, Korpela, Riksen-Walraven, de Vos & de Weerth, 2015).

The association between prenatal stress and offspring gut microbiota has been studied in different bacterial taxonomic levels from phylum to species. Result are not very consistent but all in all it seems that prenatal stress is somehow associated with offspring gut microbiota.

1.4.1 Animal studies

Of the previously mentioned five animal studies (Appendix 1) three are with mice (Gur et al., 2019; Gur et al., 2017; Jašarević, et al., 2017), one with rats (Golubeva et al., 2015), and one with monkeys (Bailey et al., 2004). All of them are experimental studies in which stress is manipulated by researcher by different stressful events for example being chained or exposed to threatening odors or loud sounds etc. Only in one study (Bailey et al., 2004) cortisol levels were checked to see if the manipulation really worked. Three of the five animal studies are about late prenatal stress (Golubeva et al., 2015; Gur et al., 2019; Gur et al., 2017), one about both early and late prenatal stress (Bailey et al., 2004), and one about early prenatal stress (Jašarević et al., 2017). The study and control group sizes have differed between 5-24 subjects. In microbiota analyses all studies but one have used 16S rRNA sequencing method (Golubeva et al., 2015, Gur et al., 2019; Gur et al., 2017; Jašarević et al., 2017). Three of the studies have assessed microbiota at only one time point: Gur et al. (2019) and Gur et al (2017) 60-70 days after birth, and Golubeva et al. (2015) four months after birth. Two studies have assessed microbiota at several time points: Jašarević et al. (2017) three times during the first 28 days after birth and Bailey et al. (2004) four times during the first 24 weeks after birth.

The most consistent results are about the association between prenatal stress and offspring microbial beta- (diversity within samples) and alpha-diversity (diversity within individuals) and relative abundance of genus *Lactobacillus*. The association between prenatal stress and offspring

beta-diversity has been assessed in two studies (Gur et al., 2019; Gur et al., 2017). Prenatal stress was found to be associated with offspring beta-diversity in both studies. Association with alpha-diversity has been assessed in three studies. None of them (Golubeva et al., 2015; Gur et al., 2019; Jašarević et al., 2017) found differences between the stressed and control rodents' offspring microbial alpha-diversity. Regarding the relative abundance of genus *Lactobacillus*, three studies (Bailey et al., 2004; Golubeva et al., 2015; Jašarević et al., 2017) found that offspring exposed to prenatal stress has lowered relative abundance of genus *Lactobacillus* compared to non-exposed offspring.

In addition to previously mentioned findings there are also many that are highly inconsistent. All of the following associations have been found only in one study, reflecting the high inconsistency related to this field of study. At phylum level exposure to prenatal stress has been associated with lowered relative abundance of Firmicutes and Bacteroidetes in female mice (Gur et al., 2017). At family level prenatal stress exposure has been associated with offspring's lowered relative abundance of *Streptococcaceae* (Golubeva et al., 2015) in rats and *S24-7, Rikenellaceae*, and *Bifidobacteriaceae* in female mice (Gur et al., 2017). At genus level exposure to prenatal stress has been associated with lowered abundance of *Bifidobacterium* (Bailey et al., 2004) and in male mice *Bacteroides* and *Parabacteroides* (Gur et al., 2019). Prenatal stress exposure has also been associated with elevated relative abundance of genera *Oscillibacter*, *Anaerotruncus*, and *Peptococcus* (Golubeva et al., 2015).

There has also been some indication for differing associations between female and male offspring but the results are inconsistent. Jašarević et al. (2017) found 27 sex specific bacteria that were found both in female and male offspring but with different abundances. They found that prenatal stress exposure affects the male offspring more than female offspring. Male offspring's gut microbiota composition regarding genus level *Odoribacter, Desulfovibrio, Flexispira*, and *Mucispirillum* changes to resemble female offspring's microbiota composition (Jašarević et al., 2017). Gur et al. (2019, 2017) studied male and female mice separately, and the studies found different kind of associations in male and in female. In male mice prenatal stress exposure was associated with lowered relative abundance of genera *Bacteroides* and *Parabacteroides*. In female mice exposure to prenatal stress was associated with lowered abundance of phyla Firmicutes and Bacteroidetes, and families *S24-7, Rikenellaceae*, and *Bifidobacteriaceae*. However, no statistical tests were conducted to assess if the differences are statistically significant so these findings should be interpreted cautiously.

1.4.2 Human studies

The three existing human studies (Appendix 2) have been conducted in the USA (Hu et al., 2019), the Netherlands (Ziljmans et al., 2015), and South Africa (Naudé et al., 2020). Since geographical location and ethnicity affect our microbiota (Cresci & Bawden, 2015) it may affect the comparability of the results. Compared to animal studies stress measures in human studies are very different. The three human studies have all used different self-report questionnaires and in one study (Ziljmans et al., 2015) also cortisol levels were measured. Both Hu et al. (2019) and Ziljmans et al. (2015) used validated questionnaires measuring general anxiety and pregnancy related anxiety, and in addition to this Hu et al. (2019) measured stressful life events, perceived prenatal stress, and symptoms of depression, and Ziljmans et al. (2015) daily hassles and pregnancy related daily hassles. Compared to these Naudé et al. (2020) used very different measures of stress: experiences of intimate partner violence, posttraumatic stress disorder, violence, depression, and symptoms of psychological stress. Of the three human studies all are about late prenatal stress: Hu et al. (2019) measured stress at the second trimester, Naudé et al. (2020) at the second or the third trimester, and Ziljmans et al. (2015) at the third trimester. The amount of subjects have varied between 56 and 101. All studies but one used 16S rRNA sequencing method to analyze microbiota (Hu et al., 2019; Naudé et al., 2020). Follow-ups have been relatively short: only one to five samples maximum 28 weeks after birth. All the studies used widely known factors affecting gut microbiota composition or development as confounders but the selected confounders varied between studies.

Also regarding human studies results are inconsistent. The most consistent findings are about the association between prenatal stress and offspring bacterial diversity and family *Enterobacteriaceae* in meconium. Hu et al. (2019) found that pregnancy related anxiety is significantly associated with increased genus level beta-diversity and has a nearly significant trend with increased alpha-diversity. Also Ziljmans et al. (2015) found a significant association between increased alpha-diversity and their measure of cumulative prenatal stress. However, Naudé et al. (2020) found that none of their different measures of stress are associated to changes in beta-diversity. Regarding family *Enterobacteriaceae* in meconium both Hu et al. (2019) and Naudé et al. (2020) found that their measure of stress is associated with elevated relative abundance.

Especially inconsistent findings are regarding phylum Proteobacteria. Ziljmans et al. (2015) found that high cumulative prenatal stress is associated with elevated relative abundance of Proteobacteria but Hu et al. (2019) found that pregnancy related anxiety is associated with lowered relative

abundance of Proteobacteria. However, the studies used very different measurements of stress and Hu et al. (2019) studied only meconium in contrary to Ziljmans et al. (2015) who did 110 days follow-up.

In addition to previously mentioned associations there are findings that have been found only in one study. Naudé et al. (2020) found that intimate partner violence is associated with higher relative abundance of genus *Weissella* 4-12 weeks after birth. 20-28 weeks after birth they found that higher amount of symptoms of psychological distress is associated with decreased relative abundance of family *Veillonellaceae*. Hu et al. (2019) found that prenatal maternal pregnancy related anxiety is associated with elevated relative abundance of undefined genus of family *Enterobacteriaceae* in meconium.

Only one study (Ziljmans et al., 2015) assessed the association between prenatal stress and offspring microbial profiles. They found that cumulative prenatal stress is associated with a combination of elevated relative abundance of a group of Proteobacteria and lowered relative abundance of groups of *Lactic acid bacteria* and Actinobacteria.

In addition to associations at single time points two studies have assessed the association of prenatal stress and microbial developmental trajectories regarding overall alpha-diversity, diversity within different bacterial groups, and relative abundances of different bacterial groups. Ziljamans et al. (2015) compared groups of low and high cumulative prenatal stress and found that overall diversity declines during 110 days follow-up in both of the groups but it is constantly higher in the high prenatal stress group. They found also that developmental trajectories in diversity within phylum Proteobacteria are opposite in low and high prenatal stress group. In high stress group the diversity of Proteobacteria declines over time compared to low stress group in which diversity is higher already at the beginning and continues to increase during the follow-up. Diversity in phylum Actinobacteria remains almost the same during the follow-up but is constantly lower in the high stress group. Diversity in genus *Clostridium* increases during the follow-up in low stress group but compared to that in high stress group the diversity is higher at the beginning but lower at the end of the follow-up.

Regarding findings about developmental trajectories of bacterial relative abundances Ziljamans et al. (2015) found that groups of Actinobacteria, Proteobacteria, and *Clostridium* have same kind of pattern: in low cumulative stress group the offspring relative abundance elevates early to a certain level and stays there to the end of the 110 day follow-up, but in high cumulative stress group

relative abundance first declines dramatically and only later reaches the same abundance as in low stress group. They also found that in another group of Proteobacteria relative abundance declines faster and lower in low cumulative prenatal stress group than in the high group, and in another group of Actinobacteria relative abundance increases during the follow-up in both groups but more in the low stress group. The relative abundance of genus *Akkermansia* is almost the same during the follow-up in low stress group but in high stress group the abundance is higher at the beginning, then declines dramatically, and finally remains considerably lower than in low stress group. The relative abundance of genus *Streptococcus* declines in both groups at the same level but is higher at the beginning of the follow-up in low stress group. Also Naudé et al. (2020) studied developmental trajectories with a smaller subgroup. They found that intimate partner violence exposure is associated with longitudinal increase in family *Enterobacteriaceae* and earlier elevated genus *Weissella*, and higher psychological distress is associated with slower decline of class *Gammaproteobacteria*.

Sex differences have not yet been studied in humans. However, Naudé et al. (2020) concluded that offspring gender is associated with bacterial alpha-diversity in some analyses and was included as a confounder. On the basis of this it seems that it might be possible that sex differences in the association between prenatal stress and offspring gut microbiota could be found also in humans.

There is some indication that different measures or aspects of stress may be associated with different kind of microbial changes. Hu et al. (2019) found that only pregnancy related anxiety, not symptoms of depression or general anxiety, perceived prenatal stress or stressful life events, is associated with gut microbiota. Ziljmans et al. (2015) found that cumulative stress has the strongest association. They also found that of the individual measures fear of bearing a handicapped child has the strongest association compared to general anxiety, fear of giving birth, daily hassles, pregnancy related daily hassles, or maternal prenatal cortisol. Naudé et al. (2020) found that many measures are associated with microbial changes but in different ways. Of their measures intimate partner violence has strongest associations, compared to symptoms of psychological distress, symptoms of depression, and posttraumatic stress disorder.

1.5. Research questions and hypotheses

The association between prenatal maternal stress and infant gut microbiota in humans has been studied only in three studies with very different methodologies and the results have been

inconsistent. Therefore, the aim of this study is to add knowledge on this topic and to cover the gap of large scale studies and follow-up's across infancy. Further, none of the previous studies has tested whether the association is different in girls and in boys. The aim of this study is to test:

1. Is maternal self-reported prenatal stress in the third trimester associated with infant gut microbiota richness, alpha-diversity and beta-diversity at 3 and 6 weeks and 3, 6, 9, and 12 months after birth?
2. Is maternal self-reported prenatal stress in the third trimester associated with infant gut microbiota phylum, family, and genus level bacterial relative abundances at 3 and 6 weeks and 3, 6, 9, and 12 months after birth?
3. Is the association different depending on the sex of the infant?

Since this field of study is still very new and the literature is scarce and highly inconsistent, no hypotheses are set.

2. METHODS

2.1 Study design

This study used the HELMi (Health and Early Life Microbiota) birth cohort. 1587 families were recruited from the general population mainly in the capital region of Finland between February 2016 and March 2018. Study was approved by the ethical committee of The Hospital District of Helsinki and Uusimaa (263/13/03/03 2015) and performed in accordance with the principles of the Helsinki Declaration. Inclusion criteria were healthy and term babies born at gestational weeks 37-42 and no known congenital defects. 1149 families consented. After exclusion (preterm birth), withdrawal or missing gender data, 1055 infants were included in the study. More information about the HELMi cohort is available in Korpela et al. (2019).

Mothers were invited to fill the internet based self-report stress questionnaire in the third trimester of pregnancy and rate the questions regarding experiences of the last trimester. Of those mothers who answered the stress questionnaire, and reported date of answering the questionnaire and infant's date of birth (n=1047), mothers answered the questionnaire on average 13 days before delivery (range from 62 days before to 105 days after delivery). In the current study mothers who answered stress questionnaire over 30 days after delivery were excluded.

In the current study fecal samples were collected at six different time points: 3 and 6 weeks, and 3, 6, 9, and 12 months after birth. Parents were asked to collect the samples at home and freeze them at -20°C. After this parents were asked to transport samples to laboratory where the samples were freezed at -80°C.

2.2 Participants in the current study

Of the included 1055 participants who met the inclusion criteria in the HELMi cohort, 230 participants were excluded in this study due to different criteria: family dropped out after answering the stress questionnaire (n=98), mother's missing stress questionnaire answer (n=3) or missing date of answering the stress questionnaire (n=2), missing information in infant's gender (n=2) or time of birth (n=3), family did not return any fecal sample (n=105), and infant's antibiotic consumption before the first fecal samples (n=17). After this all remaining mothers answered the stress questionnaire within 30 days after delivery so no further exclusions were necessary. After this the final sample size was 825. Further, all following fecal samples were excluded after reports of antibiotic use during the first year. All participants that had at least one fecal sample were included. Final sample sizes for different time points were: 3 weeks n=567, 6 weeks n=560, 3 months n=537, 6 months n=672, 9 months n=540, and 12 months n=405.

2.2.1 Attrition analysis

Of the 1055 participants included into the HELMi cohort, 825 included and 230 excluded participants were compared on several background variables (Table 1). Comparisons were conducted either with the chi-square test of independence (categorical variables) or with a one-way analysis of variance (ANOVA; continuous variables). Post-hoc analyses were conducted with the column proportions test with Bonferroni correction. Significance level $p<.05$ was used. There were no differences in any of the background variables related to the infant: birthweight (in cm), mode of delivery (C-section – vaginal), sex (girl – boy), or presence of other children (infant shared time and space with other children: not at all – only part time – permanently). In mothers no differences were found in age (in years), alcohol consumption before or during pregnancy (yes – no), or smoking before pregnancy (yes – no). However, differences were found in education (high – low; high education defined as tertiary level studies or degree) [$\chi^2(1)=5.06$, $p<.05$], smoking during pregnancy

(yes – no) [$\chi^2(1)=5.16$, $p<.05$], and antibiotic consumption during delivery (yes – no – I do not know) [$\chi^2(2)=7.51$, $p<.05$]. Included mothers compared to excluded smoked during pregnancy less frequently ($p<.05$) and were more frequently highly educated ($p<.05$). Regarding antibiotic consumption during delivery included mothers compared to excluded significantly more frequently did not consume antibiotics or did not know (p-values <.05). There was no difference in 'yes' answers.

2.2.2 Comparisons between study groups

Mothers in different stress groups (description in section 2.3) were compared on several background variables (Table 2). Comparisons were conducted either with the chi-square test of independence (categorical variables) or with a one-way ANOVA (continuous variables). Post-hoc analyses were conducted with the column proportions test with Bonferroni correction. Significance level $p<.05$ was used. No differences were found in mothers' education, smoking before or during pregnancy, or alcohol consumption during pregnancy. However, differences were found in mothers age [$F(2,822)=3.10$, $p<.05$] and antibiotic consumption during delivery [$\chi^2(4)=12.93$, $p<.05$]. Based on post-hoc analyses mothers in high stress group compared to low stress group were older ($p<.05$) but no differences were found between low and moderate, and moderate and high stress groups. In antibiotic consumption during delivery mothers in high stress group compared to low stress group more frequently consumed antibiotics ($p<.05$) and less frequently did not consume antibiotics ($p<.05$). No differences were found between low and moderate, and moderate and high stress groups. Also, no differences were found in 'I do not know' answers. Further, there were no differences in infant's mode of birth, sex, birthweight, duration of exclusive or any type of breastfeeding (in months), beginning to consume solid foods (in months), or in presence of pets in infant's household (yes – no). Differences were only found in presence of other children [$\chi^2(4)=29.73$, $p<.001$]. Infants whose mothers were in high stress group compared to moderate stress group, and moderate stress group compared to low stress group, shared time and space permanently with other children more frequently ($p <.05$). No differences were found in those who shared time and space only part of the time or not at all.

Also girls and boys were compared on several background variables (Table 3). There were total 403 girls and 422 boys. No differences were found in any background variables related to the mother. Further, there were no differences in girls and boys in mode of birth, duration of exclusive or any

type of breastfeeding, beginning to consume solid food, or presence of pets in infant's household. Differences were only found in presence of other children [$\chi^2(2)=9.65$, $p<.01$]. Girls compared to boys more frequently shared time and space with other children permanently ($p<.05$) and less frequently only part of the time ($p<.05$). No differences were found in those who did not share space and time with other children at all.

2.3 Maternal prenatal stress

Maternal prenatal stress was measured with a short internet based self-report questionnaire. Mothers were invited to rate the questionnaire based on the last trimester. Stress was reported on the basis of five following questions: 1. How much stress you have experienced related to your work or studies?, 2. How much stress you have experienced related to your relationship?, 3. How much stress you have experienced related to your household chores/child care/family), 4. How much stress you have experienced related to your pregnancy?, 5. Have you felt low or depressed during your pregnancy? Items were rated on visuo-analogical-scales scores ranging from 0 to 100. All participants answered to each of the five questions. The mean of the five stress measures was calculated for each participant. Due to skewed distribution (0.66) square root transformation was used (scores ranging from 0 to 10). The reliability of the scale was .70. Mothers' reports ranged from 0 to 9.37.

For the group analyses three groups were generated. All three groups are used in group analyses in richness alpha- and beta-diversity. Later in group analyses of bacterial relative abundances only low and high stress groups are compared. Using the transformed mean measures the lowest 25% were defined as low stress group (n=209, mean=2.83, sd=0.79), highest 25% as high stress group (n=206, mean=7.07, sd=0.66), and the rest as moderate stress group (n=410, mean=4.96, sd=0.64). Groups' mean stress measures differed statistically significantly [$F(2,822)=1995.65$, $p<.001$)] and based on post-hoc analyses with Bonferroni correction every group differed statistically significantly compared to other two groups ($p<.001$).

Table 1

Descriptive statistics for all included participants in the HELMi cohort and separately for included and excluded participants in this study

Variable	Total Mean(sd)/n(%)	Included Mean(sd)/n(%)	Excluded Mean(sd)/n(%)	Difference between included and excluded F(df)/χ2(df)
Infant				
Birthweight	3559.4(436.3)	3552.0(436.7)	3586.1(434.5)	1.10(1,1053)
C-section n(%)	177(16.8)	132(16.0)	45(19.6)	1.64(1)
Girl n(%)[a]	520(49.4)	403(48.8)	117(51.3)	0.44(1)
Other children n(%)				3.30(2)
Only part time	34(3.2)	25(3.0)	9(3.9)	
Permanently	540(51.2)	412(49.9)	128(55.7)	
Mother				
Age	32.9(4.1)	33.0(4.0)	32.4(4.4)	3.76(1,1053)
Alcohol during pregnancy n(%)	172(16.3)	129(15.6)	43(18.7)	1.23(1)
Antibiotics during delivery n(%)[b]				7.51(2)*
Yes	369(35.0)	280(33.9)	89(38.9)	
No	663(62.8)	532(64.5)	131(57.2)	
High education n(%)	930(88.2)	737(89.3)	193(83.9)	5.06(1)*
Smoked before pregnancy n(%)	125(11.8)	96(11.6)	29(12.6)	0.16(1)
Smoked during pregnancy n(%)	7(0.7)	3(0.4)	4(1.7)	5.16(1)*

Note. sd = standard deviation

[a] n=1053, missing information from two excluded participants

[b] n=1054, missing information from one excluded participant

*p<.05

Table 2

Descriptive statistics for all participants and separately for stress groups

Variable	All participants Mean(sd)/n(%)	Low stress group Mean(sd)/n(%)	Moderate stress group Mean(sd)/n(%)	High stress group Mean(sd)/n(%)	Difference between stress groups F(df)/χ^2(df)
Infant					
Any type of breastfeeding	10.8 (2.4)	10.8 (2.5)	10.9 (2.4)	10.7 (2.5)	0.75(2,808)
Birthweight	3552.0(436.7)	3516.3(423.8)	3584.1(441.4)	3524.3(437.7)	2.22(2,822)
C-section n(%)	132(16.0)	30(14.6)	67(16.2)	35(17.0)	0.48(2)
Exclusive breastfeeding	4.4 (1.9)	4.4 (1.8)	4.3 (1.9)	4.3 (2.0)	0.13(2,808)
Girl n(%)	403(48.8)	98(46.9)	203(49.5)	102(49.5)	0.43(2)
Other children n(%)					29.73(4)***
Only part time	25(3.0)	3(1.4)	13(3.2)	9(4.4)	
Permanently	412(49.9)	79(37.8)	207(50.5)	126(61.2)	
Pets n(%)	288(34.9)	80(38.8)	133(32.1)	75(36.4)	3.01(2)
Solid foods	5.5(0.8)	5.5(0.9)	5.5(0.8)	5.4(0.8)	0.16(2,822)
Mother					
Age	33.0(4.0)	32.6(3.9)	32.9(4.1)	33.5(3.8)	3.10(2,822)*
Alcohol during pregnancy n(%)	129(15.6)	30(14.4)	67(16.3)	32(15.5)	0.42(2)
Antibiotics during delivery n(%)					12.93(4)*
Yes	280(33.9)	60(28.7)	133(32.3)	87(42.2)	
No	532(64.5)	147(70.3)	267(65.1)	57.3)	
High education n(%)	738(89.3)	179(86.9)	369(89.1)	190(92.2)	3.13(2)
Smoked before pregnancy n(%)	96(11.6)	21(10.2)	46(11.1)	29(14.1)	1.72(2)
Smoked during pregnancy n(%)	3(0.4)	1(0.5)	1(0.2)	1(0.5)	0.34(2)

Note. sd = standard deviation.

*p<.05, **p<.01, ***p<.001

Table 3

Descriptive statistics separately for girls and boys

Variable	Girls Mean(sd)/n(%)	Boys Mean(sd)/n(%)	Difference between girls and boys F(df)/χ2(df)
Infant			
Any type of breastfeeding	10.9(2.4)	10.8(2.5)	0.20(1,808)
Birthweight	3513.3(425.3)	3588.9(444.7)	6.22(1,823)*
C-section n(%)	62(15.4)	70(16.6)	0.22(1)
Exclusive breastfeeding	4.4(1.9)	4.3(1.9)	1.20(1,808)
Other children n(%)			9.65(2)**
Only part time	7(1.7)	18(4.3)	
Permanently	220(54.6)	192(45.5)	
Pets n(%)	140(34.7)	148(35.1)	0.01(1)
Solid foods	5.5(0.9)	5.4(0.8)	3.86(1,820)
Mother			
Age	33.0(4.0)	33.0(3.9)	0.02(1,823)
Alcohol during pregnancy n(%)	64(15.9)	65(15.4)	0.04(1)
Antibiotics during delivery n(%)			1.98(2)
Yes	129(32.0)	151(35.8)	
No	266(66.0)	266(63.0)	
High education n(%)	360(89.3)	377(89.3)	0.00(1)
Smoked before pregnancy n(%)	43(10.7)	53(12.6)	0.72(1)
Smoked during pregnancy n(%)	3(0.7)	0(0.0)	3.15(1)

Note. sd = standard deviation.

*p<.05, **p<.01

2.4 Microbiota analysis

Bacterial DNA was extracted using repeated bead beating method (Salonen et al., 2010). Microbiota composition was analyzed with 16S rRNA gene amplicon sequencing, using regions V3-V4 (primers 341F/758R). Sequencing was done with Illumina MiSeq and Illumina HiSeq relying on Illumina protocol, except for library preparation, that was done with dual index TrusSeq-tailed 1-step amplification (Raju et al., 2018). The sequencing was done at the sequencing unit of the Institute for Molecular Medicine Finland (FIMM), Helsinki, Finland. The preprocessing (quality filtering, chimera removal, taxonomic annotation) was done with R package mare, relying on USEARCH (Edgar, 2010), as described in Mare guide (Korpela, 2016). After this forward and reverse reads were pooled, 21 nucleotides were removed to match primer length, and the reads were truncated to length of 150 bases (Korpela et al., 2018). Default settings for minimum quality score and maximum expected errors were used (Korpela, 2016). Reads below 0.001% prevalence were removed.

2.5 Statistical analyses

R version 3.5.1 was used for all the analyses. R packages vegan (Oksanen et al., 2019) and mare (Korpela, 2016) were used. Richness and alpha-diversity measures were calculated based on operational taxonomic units (OTU). Alpha-diversity index inverse Simpson was used. Analyses of beta-diversity were performed with permutational multivariate analysis of variance (PERMANOVA) with 5000 permutations and stress was used as both continuous and categorical variable. Analyses of richness and alpha-diversity were performed with multivariate analysis of variance (MANOVA) and stress was used as a categorical variable. Further analyses were performed with analysis of variance (ANOVA). In analyses of beta- and alpha-diversity and richness sex differences and interaction of prenatal stress and infant were assessed. Analyses of differences in phylum, family, and genus level bacterial relative abundances were performed using mare functions GroupTest and CovariateTest that both use MASS for generalized linear models with negative binomial distribution, and p-value correction for false discovery rate (FDR). In both analyses same criteria were used: readcount cutoff 3000, outlier cutoff 3, and minimum prevalence 0.3. The number of bacterial taxa fulfilling these criteria are presented in Table 4. Analyses were also performed separately for boys and girls but due to limitations of mare package statistical comparisons between girls and boys or analyses of interaction of prenatal stress and infant sex

could not be performed. Five selected confounders (description in section 2.5.1) were used in all GroupTest and CovariateTest analyses. Due to a large amount of analyses significance level $p<.01$ was used for all analyses. This has also been proposed by many authors (Hooks et al., 2019).

Table 4

The number of bacterial taxa fulfilling the criteria in GroupTest and CovariateTest

	Bacterial taxon		
Timepoint	Phylum	Family	Genus
3 weeks	4	18	28
6 weeks	4	17	28
3 months	4	17	30
6 months	5	24	46
9 months	5	20	40
12 months	5	21	43

2.5.1 Confounders

Both theoretical and statistical approaches were used for the selection of confounders. First possible confounders were selected based on previous knowledge of factors affecting microbiota development or composition. Then based on PERMANOVA with 999 permutations, five largest predictors were selected as confounders. Mode of delivery, presence of other children, mother's antibiotic use during delivery (answers I do not know were handled as missing information), and fecal sample's sequencing platform were selected for all time points. For time points from 3 months to 12 months diet was included. Variable that best reflected the infant's diet varied at different time points. Breastfeeding was selected for all time points, expect for 6 months. For 6 months consumption of solid foods was selected.

3. RESULTS

3.1 All participants

3.1.1 Prenatal stress and overall infant gut microbiota composition

No significant associations (prenatal stress as a continuous variable) or differences between stress groups (prenatal stress as a categorical variable) were found in beta-diversity at any time point at phylum, family, or genus level. However, in alpha-diversity and richness differences were found at 3 weeks [Wilk's λ=.97, $F(2,561)=7.37$, $p<.001$]. When 3 week time point was further analyzed significant group differences were found only in diversity [$F(1,562)=8.88$, $p<.01$]. Based on post-hoc analyses (with Bonferroni correction), high stress group compared to low stress group had significantly lower diversity ($p<.01$).

3.1.2 Prenatal stress and infant relative abundances of bacteria

Linear association and group differences in relative abundances of bacteria were assessed at phylum, family, and genus levels. First, linear associations were assessed. At 3 weeks higher maternal prenatal stress was associated with infant's higher relative abundance of family *Coriobacteriaceae* ($p<.001$) and its genera *Collinsella* ($p<.001$) and *Slackia* ($p<.001$), and with family *Porphyromonadaceae* ($p<.001$) and its genus *Barnesiella* $p<.001$). Also, higher prenatal stress was associated with lower relative abundance of family *Enterococcaceae* ($p<.01$) and its genus *Enterococcus* ($p<.01$). At 6 weeks higher prenatal stress was associated with higher relative abundance of family *Coriobacteriaceae* ($p<.001$) and its genera *Collinsella* ($p<.001$) and *Slackia* ($p<.001$). No significant associations were found at 3 months. At 6 months higher prenatal stress was associated with lower relative abundance of genus *Proteus* ($p<.01$). At 9 months higher prenatal stress was associated with higher relative abundance of genus *Dialister* ($p<.001$) and lower relative abundance of genus *Salmonella* ($p<.001$). Finally, at 12 months higher prenatal stress was associated with higher relative abundance genera *Butyrivibrio* ($p<.001$) and IncertaeSedis of family *Ruminococcaceae* ($p<.001$), and with lower relative abundance of genus *Klebisella* ($p<.01$).

Next, group comparisons for low and high stress groups were assessed. No significant group differences were found at 3 weeks or 3 months. However, at 6 weeks infants whose mothers belonged to high stress group compared to low stress group had higher relative abundance of genus *Collinsella* ($p<.01$). At 6 months high stress group infants had higher relative abundance of genus

Salmonella (p<.001). However, at 9 months high stress group infants had lower relative abundance of genus *Salmonella* (p<.01). Finally, at 12 months high stress group infants had higher relative abundance of genus level IncertaeSedis of family *Ruminococcaceae* (p<.001), and lower relative abundance of genus *Klebsiella* (p<.01). Results are presented in Table 5.

3.2 Sex differences and separate analyses for girls and boys

3.2.1 Prenatal stress and sex differences in overall microbiota composition

No significant associations or differences were found between infant sex and infants' beta-diversity at bacterial phylum, family, or genus level at any time point. Also, no interaction of prenatal stress and infant sex on infants' beta-diversity were found at any bacterial taxonomic level at any time point. Further, no sex differences or interaction of prenatal stress and infant sex were found in alpha-diversity at any time point.

3.2.2 Prenatal stress and relative abundances of bacteria separately for girls and boys

Linear association and group differences in relative abundances of bacteria were assessed at phylum, family, and genus levels separately for girls and boys. First, linear associations were assessed. At 3 weeks in girls higher maternal prenatal stress was associated with infant's lower relative abundance of family *Veillonellaceae* (p<.01) and its genus *Veillonella* (p<.01). In boys higher prenatal stress was associated with higher relative abundance of phylum Actonibacteria (p<.001), its family *Bifidobacteriaceae* (p<.001), and its genus *Bifidobacterium* (p<.001). Higher prenatal stress was also associated with higher relative abundance of genus *Faecalibacterium* (p<.001). At 6 weeks in girls higher prenatal stress was only associated with higher relative abundance of family *Coriobacteriaceae* (p<.001). In boys higher prenatal stress was associated with higher relative abundance of phylum Actinobacteria (p<.001), its family *Bifidobacteriaceae* (p<.001), and its genus *Bifidobacterium* (p<.001). At 3 months in girls higher prenatal stress was associated with lower relative abundance of family *Lactobacillaceae* (p<.01) and its genus *Lactobacillus* (p<.01). In boys higher relative abundance was associated with higher relative abundance of genera *Collinsella* (p<.001) and *Slackia* (p<.001). At 6 months in girls higher prenatal stress was associated with higher relative abundance of phylum Verrucomicrobia (p<.001), its family *Verrucomicrobiaceae* (p<.001), and its genus *Akkermansia* (p<.001). In boys higher prenatal

stress was associated only with higher relative abundance of genus *Atopobium* (p<.01). At 9 months in girls no significant associations were found. However, in boys higher prenatal stress was associated with lower relative abundance of genus Salmonella (p<.01). Finally, at 12 months in girls higher prenatal stress was associated with lower relative abundance of family *Coriobacteriaceae* (p<.01). In boys higher prenatal stress was associated with higher relative abundance of family *Lactobacillaceae* (p<.001) and its genus *Lactobacillus* (p<.01). Also, higher prenatal stress was associated with higher relative abundance of genus level IncertaeSedis (of family *Ruminococcaceae*) (p<.001).

Next, group comparison for high and low stress groups were conducted separately for girls and boys. At 3 weeks girls whose mothers belonged to high stress group higher relative abundance of genera *Collinsella* (p<.001) and *Salmonella* (p<.001) than girls whose mothers belonged to low stress group. Also, high stress group girls had lower relative abundance of genus *Faecalibacterium* (p<.001). High stress group boys had higher relative abundance of phylum Actinobacteria (p<.001), its family *Bifidobacteriaceae* (p<.001), and its genus *Bifidobacterium* (p<.001). Also, they had higher relative abundance of genus *Faecalibacterium* (p<.0001), and lower relative abundance of phylum Proteobacteria (p<.01) and genus *Blautia* (p<.0001). At 6 weeks high stress group girls had higher relative abundance of family *Enterococcaceae* (p<.01) and its genus *Enterococcus* (p<.01). Also, they had higher relative abundance of genus *Salmonella* (p<.001). High stress group boys had higher relative abundance of phylum Actinobacteria (p<.001). At 3 months no group differences were found either in girls or in boys. At 6 months high stress group girls had lower relative abundance of family *Bacillaceae* (p<.01) and its genus *Bacillus* (p<.01), and lower relative abundance of family *Lactobacillaceae* (p<.01) and its genus *Lactobacillus* (p<.001). High stress group boys had higher relative abundance of genera Atopobium (p<.001) and *Salmonella* (p<.01), and lower relative abundance of genus *Faecalibacterium* (p<.001). At 9 and 12 months no significant group differences were found in girls. However, at 9 months high stress group boys had lower relative abundance of genus *Salmonella* (p<.001). And at 12 months they had higher relative abundance of family *Lactobacillaceae* (p<.001) and its genus *Lactobacillus* (p<.01). Results are presented in Table 5.

Table 5

Linear associations and group differences in phylum, family and genus level bacterial relative abundances

		All participants			Girls			Boys		
Time point	Bacterial taxon	Linear estimate	Group difference estimate	Fold change	Linear estimate	Group difference estimate	Fold change	Linear estimate	Group difference estimate	Fold change
3 weeks	Phylum									
	Actinobacteria	0.005	0.011	1.159	-0.116	-0.568	0.566	0.055***	0.170***	1.186
	Proteobacteria	-0.001	-0.036	0.965	0.067	0.243	1.275	-0.217 ˚	-1.340**	0.262
	Family									
	Bifidobacteriaceae	0.003	0.005	1.145	0.371	-0.055	-1.289	0.059***	0.218***	1.244
	Coriobacteriaceae	0.335***	0.682	1.978	0.002 ˚	1.515	4.550	0.112	0.366	1.441
	Enterococcaceae	-0.277**	-1.207˚	0.299	0.059	-0.931	0.394	-0.248	-1.186	0.305
	Porphyromonadaceae	0.417***	1.015	2.760	0.878	-0.198	0.820	0.294	1.095	2.989
	Veillonellaceae	-0.166 ˚	-0.707	0.493	-0.314**	-1.003	0.367	-0.059	-0.572	0.564
	Genus									
	Barnesiella	0.448***	1.005	2.733	0.006	-0.158	0.854	0.226	0.138	0.979
	Bifidobacterium	0.003	0.005	1.145	-0.009	-0.055	-1.289	0.059***	0.218***	1.244
	Blautia	0.006	-0.080	0.923	0.001	0.087	1.091	-0.121	-1.762***	0.556
	Collinsella	0.454***	0.789	2.201	0.002 ˚	1.762***	5.826	0.075	-0.007	0.993
	Enterococcus	-0.28**	-1.207 ˚	0.299	0.442	-0.931	0.394	-0.248	-1.186	0.630
	Faecalibacterium	0.026	-0.234	0.791	-0.248	-2.063***	0.127	0.506***	1.913***	5.353
	Salmonella	0.169	0.927	2.527	0.230	2.223***	9.232	0.002	-0.054	1.897
	Slackia	0.415***	1.497 ˚	4.469	0.249	0.839	2.313	0.056	-0.142	0.868
	Veillonella	-0.162 ˚	-0.709	0.492	-0.310**	-1.026	0.358	-0.043	-0.501	1.092
6weeks	Phylum									
	Actinobacteria	-	-0.175	0.839	-0.105	-0.536	0.585	0.015***	0.110***	1.116
	Family									
	Bifidobacteriaceae	0.006	0.035	1.595	0.000	-0.002	0.753	0.023***	0.068	1.546

	Coriobacteriaceae	0.410***	1.077.	2.936	0.464***	0.348	1.416	0.268	0.912	2.490
	Enterococcaceae	0.050	0.202	1.224	0.224	1.821**	6.176	0.036	0.054	1.056
	Genus									
	Bifidobacterium	0.006	0.035	1.595	0.000	-0.002	0.753	0.023***	0.068	1.546
	Collinsella	0.480***	1.871**	6.496	0.294	-0.210	0.811	-	0.582	1.789
	Enterococcus	0.050	0.202	1.224	0.224	1.821**	6.176	0.036	0.054	1.056
	Salmonella	0.101	0.423	1.526	0.274	2.173***	8.785	-	-0.414	0.661
	Slackia	0.443***	0.904	2.469	0.503 ˚	-0.376	0.687	-	0.519	1.680
3 months	Family									
	Lactobacillaceae	-0.098	-0.338	0.713	-0.342**	-1.271	0.281	0.097	0.479	1.614
	Genus									
	Collinsella	-0.049	0.113	1.119	0.249	-0.443	0.642	0.561***	1.177	3.244
	Lactobacillus	-0.098	-0.338	0.713	-0.342**	-	-	0.097	0.479	1.614
	Slackia	-0.054	0.059	1.060	0.231	-0.521	0.594	0.538***	1.029	2.800
6 months	Phylum									
	Verrucomicrobia	0.060	0.511	1.667	0.572***	1.698 ˚	5.461	0.061	0.128	1.137
	Family									
	Bacillaceae	-0.103	-0.555	0.574	-0.193 ˚	-1.303**	0.272	-0.014	0.025	1.026
	Lactobacillaceae	-0.155	-0.745	0.475	-0.235 ˚	-1.561**	0.210	-0.079	0.000	2.000
	Verrucomicrobiaceae	-	0.511	1.667	0.572***	1.698 ˚	5.461	-	0.128	1.137
	Genus									
	Akkermansia	-	0.511	1.667	0.572***	1.698	5.461	-	0.128	1.137
	Atopobium	0.180	0.757	2.132	-0.053	-0.043	0.958	0.442**	2.312***	10.092
	Bacillus	-0.103	-0.555	0.574	-0.193	-1.303**	0.272	-0.014	0.025	0.983
	Faecalibacterium	-0.001	0.098	1.102	-0.073	0.930	-	-0.355	-2.512***	0.081
	Lactobacillus	-0.155	-0.745	0.475	-0.235˚	-1.561***	0.210	-0.079	0.000	2.000
	Proteus	-0.184**	0.192	1.212	-0.119	-0.117	0.890	-	0.251	1.286
	Salmonella	0.137 ˚	0.927***	2.528	0.239	0.728	2.071	0.168	0.961**	2.614
9 months	Genus									
	Akkermansia	-	-0.465	0.628	-	-0.172	0.842	-	-0.285	0.752
	Dialister	0.336***	0.906	2.473	0.131	-0.008	0.992	-	0.287	1.332

	Salmonella	-0.239***	-0.925**	0.397	-0.200 ˚	-0.555	0.574	-0.325**	-1.558***	0.211
12 months	Family									
	Coriobacteriaceae	-0.045	-0.212	0.809	-0.275**	-1.190 ˚	0.304	0.043	0.240	1.272
	Lactobacillaceae	0.054	0.236	1.267	-0.095	-0.390	0.677	0.705***	4.073***	58.727
	Genus									
	Butyrivibrio	0.298***	0.601	1.824	-	0.146	1.157	0.319 ˚	0.351	1.420
	Klebsiella	-0.245**	-1.259**	0.284	-0.290 ˚	-1.300	0.272	-0.161	-0.774	0.461
	Lactobacillus	0.054	0.236	1.267	-0.095	-0.390	0.677	0.705***	4.073***	58.727
	IncerateSedis of family Ruminococcaceae	0.396***	1.539***	4.661	-	0.008	1.008	0.537***	1.742	5.711

Note. All of the phylum, family, and genus level bacteria that had at least one significant finding (linear association or group difference) in any of the analyses (all participants, girls, boys) are presented for each time point. Dashes indicate that information was not available. The measures of prenatal stress were square root transformed so the estimates (effect sizes) here are only directional.

˚p<.05, **p<.01, ***p<.001

4. DISCUSSION

The purpose of this study was to add knowledge about the association between prenatal maternal stress and infant gut microbiota. This study contributes to current knowledge by a large scale study with longer follow-up than previously. This is also the first human study to assess if the associations differ between girls and boys.

Perhaps the most interesting finding of this study was that in beta-diversity no association with prenatal stress as a continuous variable nor differences between low and high stress group were found. Previously also Naudé et al. (2020) human study found no association between infant beta-diversity and prenatal stress. However, Hu et al. (2019) human study and Gur et al. (2017, 2019) studies with mice found prenatal stress to be associated with beta-diversity. Further, in this study differences in alpha-diversity between stress groups were found only at 3 weeks: high stress group had lower alpha-diversity than low stress group. This finding is contradicting compared to previous findings since in previous human studies there is evidence of association between higher prenatal stress and higher alpha-diversity (Hu et al., 2019; Ziljmans et al., 2015). Also, in animal studies no differences in stressed and non-stressed rodents' offspring microbial alpha-diversity has been found (Golubeva et al., 2015; Jašarević et al., 2017). Interestingly, it is not even yet clear if higher or lower diversity is considered as a sign of healthy gut microbiota (Hooks et al., 2019).

In phylum, family, and genus level analyses of bacterial relative abundances prenatal stress was found to be associated with several bacteria at different taxonomic levels. Prenatal stress was associated with different bacteria at different time points with an inconsistent pattern. Also, there seemed to be more connections closer to birth than later: for each time point from 3 weeks to 12 months the number of bacteria that had at least one significant finding (linear association or group difference) were 16, 9, 4, 11, 3, and 6, and the number of bacteria that had concordant (significant finding in both linear and group analysis) findings 4, 2, 0, 1, 1, and 4. When compared to the number of bacteria filling the minimum prevalence and abundance criteria the proportions of significantly associated bacteria are respectively 32%, 18%, 8%, 15%, 5%, and 9%, and 8%, 4%, 0%, 1%, 2%, and 6%. These differences between time points were not significantly tested but the descending trend could indicate that the association might be stronger closer to birth and decline over age.

All in all, in phylum, family, and genus level analyses of bacterial relative abundances there was not very much consistency. Not all significant findings are discussed here but instead a few interesting

findings are pointed out. The most consistent finding in this study was the association between prenatal stress and higher relative abundance of phylum Actinobacteria, its family *Bifidobacteriaceae*, and its genus *Bifidobacterium* in boys. This association was evident the first 6 weeks: at 3 weeks all above mentioned bacteria had concordant findings, and at 6 weeks there was concordant finding in Actinobacteria, and linear associations in the other two. This finding is interesting since previously *Bifidobacterium* has been considered as a beneficial bacteria (eg. Gosalbes, et al., 2013; Kalliomäki et al., 2008). This finding is not in line with the idea of gut microbiota to be a linking mechanism between prenatal stress and adverse infant outcomes. Interestingly, Ziljmans et al. (2015) found prenatal stress to be associated with group of Actinobacteria (of which one bacteria was *Bifidobacterium*), only the association was other way round: higher prenatal stress was associated with lower relative abundance of group of Actinobacteria. Also Bailey et al. (2004) study with monkeys found that stressed monkeys' offspring had lower relative abundance of *Bifidobacterium*.

There were also other concordant findings: at 3 weeks in boys with higher relative abundance of genus *Faecalibacterium*, at 6 weeks in all participants higher prenatal stress was associated with higher relative abundance of genus *Collinsella*, at 6 weeks in boys with higher relative abundance of genus *Atopobium*, at 9 months in all participants and in boys with lower relative abundance of genus *Salmonella*, and at 12 months in all participants with higher relative abundance of IncertaeSedis of family *Ruminococcaceae* and lower relative abundance of genus *Klebsiella*, and in boys with higher relative abundance of family *Lactobacillaceae* and genus *Lactobacillus*. However, as can be seen, in these associations there is not consistency over time. Compared to previous studies the finding regarding *Lactobacillaceae* and *Lactobacillus* were contradicting. Ziljmans et al. (2015) found higher prenatal stress to be associated with lower relative abundance of group of *Lactic acid bacteria* (of which one bacteria was *Lactobacillus*). Same kind of results have also been found on several animal studies (Bailey et al., 2004; Golubeva et al., 2015; Gur et al., 2017; Jašarević et al., 2017). Also contradicting, Ziljmans et al. (2015) found higher prenatal stress to be associated with lower relative abundance of Actinobacteria, of which one bacteria was *Collinsella*.

Especially conflicting findings were about genus *Salmonella*. First, at 3 and 6 weeks there was a group difference in girls: high stress group girls had higher relative abundance of *Salmonella*. Then, at 3 months no linear association or group differences were found. At 6 months there again there were group differences: in all participants and in boys high prenatal stress group infants had higher relative abundance of *Salmonella*. Then surprisingly at 9 months there were both linear associations

and group differences in all participants and in boys: higher prenatal stress was associated with lower relative abundance of *Salmonella*, an opposite finding compared to previous time points. Then again, at 12 months no linear association or group differences were found. At most of the time points there seemed to be a positive association between prenatal stress and *Salmonella*. However, these associations were not concordant and they alternated between all participants, girls and boys, and between time points from positive and negative associations to no associations at all. More consistent findings about *Salmonella* would have been interesting, since previously it has been considered as pathogenic bacteria, at least in adult populations (Yan et al., 2004).

This was the first human study to assess if the association between prenatal stress and infant gut microbiota is different in girls and in boys. No differences in richness, or alpha- and beta-diversity were found between boys and girls, and no interaction of prenatal stress and infant sex was found. As in Gur et al (2017, 2019), also in this study analyses of bacterial relative abundances were conducted separately for boys and girls. There seemed to be more significant findings in boys than in girls. In boys there were significant findings in 13 bacteria and concordant findings in 8 of them. In girls there were 13 and 0 findings, respectively. This might indicate that the association between prenatal stress and infant gut microbiota could be stronger in boys. However, due to methodological limitations differences between boys and girls or interaction of prenatal stress and infant sex could not be statistically tested in analyses of bacterial relative abundances. This means that no conclusion about statistical differences between boys and girls can be made. This is certainly a gap that should be covered in the future.

The strengths of this study are higher sample size and longer follow-up than in previous studies. However, the fact that the mothers included in this study versus excluded were more highly educated might have an effect on the results. Almost 90% of the included mothers had high education, which certainly does not reflect the situation in Finland. Also, of the excluded about 84% had high education so the problem applies to the whole cohort, not just the sample in this study. The higher proportion of highly educated mothers could affect the results in this study since high socioeconomic status may affect the amount of stress, thus, lowering the variance in stress measures, and diet, that can affect gut microbiota (Cresci & Bawden, 2015). However, on the other hand reports of stress questionnaire were highly variable in this study. No diet information was available.

Another strength of this study is the selection of confounding variables relying both on theoretical and statistical approaches. However, the amount of confounding variables was limited to five due to

methodological limitations. But on the other hand at most of the time points, five confounders was sufficient. There are several other possible confounding variables that could not be included here, for example medication, diet, gastrointestinal transit time, inflammation markers etc. (Valles-Colomer, Falony, Vieira-Silva & Raes, 2019). Further, postnatal mood was not assessed and thus could not be controlled and since prenatal and postnatal mood are highly correlated, as previously mentioned, it would be an important variable to control. Also, as mentioned earlier one of the limitation of this study is that differences between boys and girls in bacterial phylum, family, and genus level analyses could not be statistically tested. However, this was the first human study to conduct separate analyses. Further sex differences and interaction of prenatal stress and infant sex could be assessed in overall microbiota composition analyses, which can be considered as a strength of this study.

To conclude, as previously, also in this study some associations were found between prenatal maternal stress and infant gut microbiota. However, not very consistent conclusions can be made based on research conducted so far. Based on this study it seems that there is no clear association with overall microbiota composition, association regarding bacterial abundances might be stronger closer to birth and then decline over age, and the association might be stronger in boys than in girls. However, the results were highly inconsistent, limited due to methodological issues, and by most of the part inconsistent with previous findings. Also, there still remain several defects in this field of study. It is not known what kind of gut microbiota is good on our health, or is it even possible to determine a healthy microbiota due to huge individual variation (Dave et al., 2012). There are several technical and methodological challenges in analyzing microbiota composition, and it is not even known if fecal samples really are representative of intestinal microbiota actually living in our intestines (Dave et al., 2012). Also, most of the studies so far have concentrated only on bacteria of gut microbiota leaving out eukarya, archaea, and viruses (Hooks et al., 2019). Further, due to a huge amount of different bacteria numerous statistical tests are needed. Even though p-value corrections were used it still might be possible that there is a relatively high probability for significant findings to be statistical coincidences. However, this field of study is still very new. Only a few studies have been conducted and with different methodologies. Also, most of the studies have concentrated on differences in bacterial relative abundances at specific time points and bacterial profiles developmental trajectories associated with prenatal stress have been studied only little. Perhaps later with a lot more studies and more consistent methodologies an association could be found between prenatal maternal stress and infant gut microbiota.

References

Aureli, P., Capurso, L., Castellazzi, A. M., Clerici, M., Giovannini, M., Morelli, L., Poli, A., Pregliasco, F., Salvini, F. & Zuccotti, G. V. (2011). Probiotics and health: an evidence-based review. *Pharmacological Research, 63* (5), 366-376. https://doi.org/10.1016/j.phrs.2011.02.006.

Bailey, M. T., Dowd, S. E., Galley, J. D., Hufnagle, A. R., Allen R. G. & Lyte, M. (2011). Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. *Brain, Behavior, and Immunity, 25* (3), 397-407. https://doi.org/10.1016/j.bbi.2010.10.023

Bailey, M. T., Dowd, S. E., Parry, N. M. A., Galley, J. D., Schauer, D. B. & Lyte, M. (2010). Stressor exposure disrupts commensal microbial populations in the intestines and leads to increased colonization by *Citrobacter rodentium. American Society for Microbiology Journals.* https://doi.org/10.1128/IAI.00862-09

Bailey, M. T., Lubach, G. R. & Coe, C. L. (2004). Prenatal stress alters bacterial colonization of the gut in infant monkeys. *Journal of Pediatric Gastroenterology and Nutrition, 38* (4), 414-421.

Beijers, R., Buitelaar, J. K. & de Weerth, C. (2014). Mechanisms underlying the effects of prenatal psychosocial stress on child outcomes: beyond the HPA-axis. *European Child & Adolescent Psychiatry, 23*, 943-956. doi:10.1007/s00787-014-0566-3

Bennett, H., Einarson, A., Taddio, A., Koren, G. & Einarson, T. (2004). Prevalence of depression during pregnancy: Systematic review. *Obstetrics & Gynecology, 103* (4), 698-709. doi: 10.1097/01.AOG.0000116689.75396.5f

Beydoun, H. & Saftlas, A. F. (2008). Physical and mental health outcomes of prenatal maternal stress in human and animal studies: a review of recent evidence. *Paediatric and Perinatal Epidemiology, 22* (5), 438-466. https://doi.org/10.1111/j.1365-3016.2008.00951.x

Cresci, G. A. & Bawden, E. (2015). Gut microbiome, what we do and don't know. *Nutrition in Clinical Practice, 30* (6), 734-746.

Cong, X., Henderson, W. A., Graf, J. & McGrath, J. M. (2015). Early life experiences and gut microbiome: the brain-gut-microbiota signaling system. *Advances in Neonatal Care, 15* (5), 314-323. doi: 10.1097/ANC.0000000000000191

Cryan, J. F. & Dinan, T. G. (2012). Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nature Reviews Neuroscience, 13,* 701-712.

Dave, M., Higgins, P. D., Middha, S. & Rioux, K. P. (2012). The human gut microbiome: current knowledge, challenges, and future directions. *Translational Research, 160* (4), 246-257. https://doi.org/10.1016/j.trsl.2012.05.003

de Weerth, C., Fuentes, S., Puylaert, P. & de Vos, W. M. (2013). Intestinal microbiota in infants with colic: development and specific signatures. *Pediatrics, 131* (2), 550-558. https://doi.org/10.1542/peds.2012-1449

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics, 26* (19), 2460-2461. https://doi.org/10.1093/bioinformatics/btq461

Fairbrother, N., Janssen, P., Antony, M. M., Tucker, E. & Young, A. H. (2016). Perinatal anxiety disorder prevalence and incidence. *Journal of Affective Disorders, 200,* 148-155. https://doi.org/10.1016/j.jad.2015.12.082

Flanigan, C., Sheikh, A., DunnGalvin, A., Brew, B. K., Almqvist, C. & Nwaru, B. I. (2018). Prenatal maternal psychosocial stress and offspring's asthma and allergic disease: a systematic review and meta-analysis. *Clinical & Experimental Allergy, 48* (4), 403-414.

Fujimura, K. E., Slusher, N. A., Cabana, M. D. & Lynch, S. V. (2010). Role of the gut microbiota in defining human health. *Expert Review of Anti-infective Therapy, 8* (4), 435-454. https://doi.org/10.1586/eri.10.14

Glover, V., O'Donnell, K. J., O'Connor, T. G. & Fisher, J. (2018). Prenatal maternal stress, fetal programming, and mechanisms underlying later psychopathology - a global perspective. *Development and Psychopathology, 30,* 843-854. doi:10.1017/S095457941800038X

Golubeva, A. V., Crampton, S., Desbonnet, L., Edge, D., O'Sullivan, O., Lomasney, K. W., Zhdanov, A. V., Crispie, F., Moloney, R. D., Borre, Y. E., Cotter, P. D., Hyland, N. P., O'Halloran, K. D., Dinan, T. G., O'Keeffe, G. W. & Cryan, J. F. (2015). Prenatal stress-induced alterations in major physiological systems correlate with gut microbiota composition in adulthood. *Psychoneuroendocrinology, 60,* 58-74. https://doi.org/10.1016/j.psyneuen.2015.06.002

Gosalbes, M. J., Llop, S., Vallès, Y., Moya, A., Ballester, F. & Francino, M. P. (2013). Meconium microbiota types dominated by lactic acid or enteric bacteria are differentially associated with maternal eczema and respiratory problems in infants. *Clinical & Experimental Allergy, 43* (2), 198-211. https://doi.org/10.1111/cea.12063

Greenhalgh, K., Meyer, K. M., Aagaard, K. M. & Wilmes, P. (2016). The human gut microbiome in health: establishment and resilience of microbiota over a lifetime. *Environmental Microbiology, 18* (7), 2103-2116. doi:10.1111/1462-2920.13318

Gur, T. L. & Bailey, M.T. (2016). Effects of stress on commensal microbes and immune system activity. *Microbial Endocrinology: Interkingdom Signaling in Infectious Disease and Health. Advances in Experimental Medicine and Biology, 874*, 289-300.

Gur, T. L., Palkar, A. V., Rajasekera, T., Allen, J., Niraula, A., Godbout, J. & Bailey, M. T. (2019). Prenatal stress disrupts social behavior, cortical neurobiology and commensal microbes in adult male offspring. *Behavioural Brain Research, 359*, 886-894. https://doi.org/10.1016/j.bbr.2018.06.025

Gur, T. L., Shay, L., Palkar, A. V., Fisher, S., Varaljay, V. A., Dowd, S. & Bailey, M. T. (2017). Prenatal stress affects placental cytokines and neurotrophins, commensal microbes, and anxiety-like behavior in adult female offspring. *Brain, Behavior and Immunity*, *64*, 50-58. https://doi.org/10.1016/j.bbi.2016.12.021

Heron, J., O'Connor, T. G., Evans, J., Golding, J. & Glover, V. (2004). The course of anxiety and depression through pregnancy and the postpartum in a community sample. *Journal of Affective Disorders, 80* (1), 65-73. https://doi.org/10.1016/j.jad.2003.08.004

Hooks, K.B., Konsman, J.P. & O'Malley, M.A. (2019). Microbiota-gut-brain research: a critical analysis. *Behavioral and Brain Sciences 42*, e60: 1–53. doi:10.1017/S0140525X18002133

Hu, J., Ly, J., Zhang, W., Huang, Y., Glover, V., Peter, I., Hurd, Y. L. & Nomura, Y. (2019). Microbiota of newborn meconium is associated with maternal anxiety experienced during pregnancy. *Developmental Psychobiology, 61*, 640–649. doi: 10.1002/dev.21837

Jašarević, E., Howard, C. D., Misic, A. M., Beiting, D. P. & Bale, T. L. (2017). Stress during pregnancy alters temporal and spatial dynamics of the maternal and offspring microbiome in a sex-specific manner. *Scientific Reports, 7* (article 44182). doi:10.1038/srep44182

Kalliomäki, M., Collado, M. C., Salminen, S. & Isolauri, E. (2008). Early differences in fecal microbiota composition in children may predict overweight. *The American Journal of Clinical Nutrition, 87* (3), 534-538. https://doi.org/10.1093/ajcn/87.3.534

Korpela, K. (2016). mare: Microbiota Analysis in R Easily. R package version 1.0.

Korpela, K., Blakstad, E. W., Moltu, S. J., Strømmen, K., Nakstad, B., Rønnestad, A. E., Brække, K., Iversen, P. O., Drevon, C. A. & de Vos, W. (2018). Intestinal microbiota development and gestational age in preterm neonates. *Scientific Reports, 8*, 2453. https://doi.org/10.1038/s41598-018-20827-x

Korpela, K., Dikareva, E., Hanski, E., Kolho, K-E., de Vos, W. M. & Salonen, A. (2019). Cohort profile: finnish health and early life microbiota (HELMi) longitudinal birth cohort. *BMJ Open, 9 (6).* doi: 10.1136/bmjopen-2018-028500

Milani, C., Duranti, S., Bottacini, F., Casey, E., Turroni, F., Mahony, J., Belzer, C., Palacio S. D., Montes, S. A., Mancabelli, L., Lugli, G. A., Rodriguez, J. M., Bode, L., de Vos, W., Gueimonde, M., Margolles, A., van Sinderen, D. & Ventura, M. (2017). The first microbial colonizers of the human gut: composition, activities, and health implications of the infant gut microbiota. *Microbiology and Molecular Biology Reviews, 81* (4) e00036-17; doi: 10.1128/MMBR.00036-17

Naudé, P. J. W., Claassen-Weitz, S., Gardner-Lubbe, S. & Botha, G., Kaba, M., Zar, H. J., Nicol, M. P. & Stein, D. J. (2020). Association of maternal prenatal psychological stressors and distress with maternal and early infant faecal bacterial profile. *Acta Neuropsychiatrica*. https://doi.org/10.1017/neu.2019.43

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O' Hara, R. B., Simpson G. L., Solymos, P., Stevens, M. H. H., Szoecs, E. & Wagner, H. (2019). ve gan: Community Ecology Package. R package version 2.5-6. https://CRAN.R-project.org/packag e=vegan

O'Mahony, S. M., Clarke, G., Dinan, T. G. & Cryan, J. F. (2017). Early-life adversity and brain development: is the microbiome a missing piece of the puzzle? *Neuroscience, 342*, 37-54. https://doi.org/10.1016/j.neuroscience.2015.09.068

Raju, S. C., Lagström, S., Ellonen, P., de Vos, W. M., Eriksson, J. G., Weiderpass, E. & Rounge, T. B. (2018). Reproducibility and repeatability of six high-throughput 16S rDNA sequencing protocols for microbiota profiling. *Journal of Microbiological Methods, 147*, 76-86. https://doi.org/10.1016/j.mimet.2018.03.003

Ruiz, R. J. & Avant, K. C. (2005). Effects of maternal prenatal stress on infant outcomes: a synthesis of the literature. *Advances in Nursing Science, 28* (4), 345-355.

Salonen, A., Nikkilä, J., Jalanka-Tuovinen, J., Immonen, O., Rajilić-Stojanović, M., Kekkonen, R. A., Palva, A. & de Vos, W. M. (2010). Comparative analysis of fecal DNA extraction methods with phylogenetic microarray: Effective recovery of bacterial and archaeal DNA using mechanical cell lysis. *Journal of Microbiological Methods, 81* (2), 127-134. https://doi.org/10.1016/j.mimet.2010.02.007

Shin, N-R., Whon, T. W. & Bae, J-W. (2015). *Proteobacteria*: microbial signature of dysbiosis in gut microbiota. *Trends in Biotechnology, 33* (9), 496-503. https://doi.org/10.1016/j.tibtech.2015.06.011

Sutherland, S. & Brunwasser, S. M. (2018). Sex differences in vulnerability to prenatal stress: a review of the recent literature. *Current Psychiatry Reports, 20* (102). https://doi.org/10.1007/s11920-018-0961-4

Sjögren, Y. M., Jenmalm, N. C., Böttcher, M. F., Björkstén, B. & Sverremark-Ekström, E. (2009). Altered early infant gut microbiota in children developing allergy up to 5 years of age. *Clinical & Experimental Allergy, 39* (4), 518-526.

Valles-Colomer, M., Falony, G., Vieira-Silva, S. & Raes, J. (2019). Practical guidelines for gut microbiome analysis in microbiota-gut-brain axis research. *Behavioral and Brain Sciences, 42*, E78. doi:10.1017/S0140525X18002881

Van Ameringer, M., Turna, J., Patterson, P., Pipe, A., Mao, R. Q., Anglin, R. & Surette, M. G. (2019). The gut microbiome in psychiatry: a primer for clinicians. *Depression & Anxiety, 36* (11), 1004-1025. https://doi.org/10.1002/da.22936

Van den Bergh, B. R. H., Van den Heuvel, M. I., Lahti, M., Braeken, M., de Rooij, S. R., Etringer, S., Hoyer, D., Roseboom, T., Räikkönen, K., King, S. & Schwab, M. (2017). Prenatal developmental origins of behavior and mental health: The influence of maternal stress in

pregnancy. *Neuroscience & Behavioral Reviews*. In press. https://doi.org/10.1016/j.neubiorev.2017.07.003

Wallace, C. J. K. & Milew, R. (2017). The effects of probiotics on depressive symptoms in humans: a systematic review. *Annals of General Psychiatry, 16* (article 14). Published online. doi:10.1186/s12991-017-0138-2

Wang, M., Monaco, M. H. & Donovan, S. M. (2016). Impact of early gut microbiota on immune and metabolic development and function. *Seminars in Fetal and Neonatal Medicine, 21* (6), 380-387. https://doi.org/10.1016/j.siny.2016.04.004

Wu, Z. A. & Wang, H. X. (2019). A systematic review of the interaction between gut microbiota and host health from a symbiotic perspective. *SN Comprehensive Clinical Medicine, 1* (3), 224-235. https://doi.org/10.1007/s42399-018-0033-4

Yan, S. S., Pendrak, M. L., Abela-Ridder, B., Punderson, J. W., Fedorko, D. P. & Foley, S. L. (2004). An overview of *Salmonella* typing: public health perspectives. *Clinical and Applied Immunology Reviews, 4* (3), 189-204.

Ziljmans, M. A. C., Korpela, K., Riksen-Walraven, J. M., de Vos, W. M. & de Weerth, C. (2015). Maternal prenatal stress is associated with the infant intestinal microbiota. *Psychoneuroendocrinology, 53*, 233-245. https://doi.org/10.1016/j.psyneuen.2015.01.006

Ziljmans, M. A. C., Beijers, R., Riksen-Walraven, M. J. & de Weerth, C. (2016). Maternal late pregnancy anxiety and stress is associated with children's health: a longitudinal study. *Stress, 20* (5), 495-504, doi: 10.1080/10253890.2017.1348497

Appendix

Appendix 1

Characteristics of animal studies

Reference	Study population	Study design	Prenatal stress measure	Offspring microbiota measure
Bailey et al 2004	7 infant monkeys of early prenatally stressed dams, 8 infant monkeys of late prenatally stressed dams, 9 control infant monkeys of non-stressed dams.	Experimental, longitudinal	Manipulated stress five times per week for six weeks at the first or third trimester, success of the manipulation was checked with cortisol measures	Enumeration by fecal culture, fecal samples at 2 days, and at 2, 8, 16 and 24 weeks after birth
Golubeva et al 2015	6 stressed rats and their 9 pups, 5 control rats and their 8 pups	Experimental, longitudinal	Manipulated stress at the last week of pregnancy	16S rRNA sequencing, fecal samples 4 months after birth
Gur et al 2017	9 stressed mice and their 16 female pups, 6 control mice and their 16 female pups	Experimental, longitudinal	Manipulated restraint stress daily at E10-E16	16S rRNA sequencing, fecal samples 60-70 days after birth
Gur et al 2019	9 stressed mice and their 15 male pups, 6 control mice and their 14 male pups	Experimental, longitudinal	Manipulated restraint stress daily at E10-E16	16S rRNA sequencing, fecal samples 60-70 days after birth
Jašarević et al 2017	8 prenatally stressed mice and their one female and one male pup, 5 control mice and their one female and one male pup	Experimental, longitudinal	Manipulated stress at first week of pregnancy	16S rRNA sequencing, fecal samples at postnatal days 2, 6 and 28

Note. Systematic literature search on prenatal maternal stress and its association with offspring gut microbiota was conducted using three different databases: Scopus, PubMed and Google Scholar. Different combinations of following search terms were used: ‘maternal’, ‘prenatal’, ‘during pregnancy’, ‘stress’, ‘distress’, ‘anxiety’, ‘depression’, ‘infant’, ‘child’, ‘offspring’, ‘gut’, ‘intestinal’, ‘microbiota’, ‘microbiome’.

Appendix 2

Characteristics of human studies

Reference	Study population	Study design	Prenatal stress measure	Offspring microbiota measure	Confounders
Hu et al., 2019	USA, 75 moms from ethnic minorities, and their term infants (25% born via cesarean section)	Longitudinal, cohort study	EPDS, STAI, PRAQ-R, PSS-14, PERI at second trimester	16S rRNA, fecal samples during the first 48h after birth	Maternal ethnicity, age, education, marital status, time of sampling, delivery mode, antibiotic use during pregnancy and delivery
Naudé et al., 2020	South Africa, 101 moms from two low socioeconomic communities and their offspring. 101 infants at birth, 69 infants at 4-12 weeks after birth and 36 infants at 20-28 weeks.	Longitudinal, cohort study	SRQ-20, BDI-II, MPSS, IPV at second or third trimester	16S rRNA fecal samples right after birth, 4-12 weeks and 20-28 weeks after birth	Maternal BMI, breastfeeding, area, maternal HIV status, maternal education, gender of infant depending of the analysis
Ziljamans et al., 2015	Dutch/Holland, 28 low stress mothers and 28 highly stressed mothers, and their healthy vaginally born infants	Longitudinal, cohort study	STAI, PRAQ-R (PRAQ1 and PRAQ2), daily hassles, pregnancy related daily hassles, prenatal cortisol	Phylogenetic microarray, the Human Intestinal Tract Chip, fecal samples averagely at 6.7, 12.5, 24.8, 83.8 and 112.3 days after birth	Breastfeeding, postnatal maternal stress and anxiety 3 months after delivery

Note. Systematic literature search on prenatal maternal stress and its association with offspring gut microbiota was conducted using three different databases: Scopus, PubMed and Google Scholar. Different combinations of following search terms were used: ‘maternal’, ‘prenatal’, ‘during pregnancy’, ‘stress’, ‘distress’, ‘anxiety’, ‘depression’, ‘infant’, ‘child’, ‘offspring’, ‘gut’, ‘intestinal’, ‘microbiota’, ‘microbiome’.