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Psykiatrian laitos
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Vastuuhenkilöt: Linnea Karlsson, Anna-Katariina Aatsinki, Hasse Karlsson

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ABSTRAKTI

Taustatietoa

Äidin raskaudenaikainen psykiatrinen oireilu on erityinen terveysongelma, joka vaikuttaa sekä äidin että jälkeläisen hyvinvointiin. Tämä ilmiö, jota kutsutaan äidin raskaudenaikaisesti stressiksi, voi olla yhteydessä suoliaivoakselin kautta hormonaalisiin, metabolisiin ja immunologisiin muutoksiin niin äidissä kuin jälkeläisessäkin. Useat elintapoihin ja ympäristöön liittyvät asiat voivat vaikuttaa lapsen suolistomikrobiston koostumukseen ja hiljattain onkin huomattu, että äidin raskaudenaikainen stressi voi olla tässä yhtenä välittävänä tekijänä. Ilmiön taustalla vaikuttavista mekanismeista ei ole vielä paljoakaan tietoa, ja valtaosa tutkimustuloksista on peräisin eläintutkimuksista. Tutkimustiedon harvinaisuus alleviivaa tarvetta uusille tutkimuksille siitä, kuinka suolistomikrobiston koostumuksen muuttuminen vaikuttaa lapsen terveyteen myöhemmin elämässä.

Tutkimuksen tarkoituksena on määrittää, kuinka äidin raskaudenaikainen stressi ilmenee taaperon ulosteen bakteerikoostumuksessa.

Metodit

Tutkimusaineisto on peräisin laaja-alaisesta, Varsinais-Suomessa vuonna 2011 aloitetusta ja edelleen meneillään olevasta FinnBrain syntymäkohorttitutkimuksesta, jonka perimmäinen tarkoitus on tutkia raskaudenaikaisen stressin ja varhaiselle stressille altistumisen vaikutuksia lapsien terveyteen ja aivojen kehitykseen. Alkuperäinen kohortti sisältää noin 3800 perhettä, josta tämän tutkimuksen tutkimuspopulaatio käsittää 207:n perhettä.

Äidin raskaudenajan stressi, mukaan lukien masennus- ja ahdistuneisuusoireet, mitattiin seuraavilla kyselylomakkeilla: EPDS, SCL ja PRAQ-R2. Äidit täyttivät nämä lomakkeet kolmesti raskauden aikana. Oireiden kroonisuus määritettiin muodostamalla pisteytyksille rajat: äidit, jotka saivat 10 tai enemmän pisteitä vähintään kahdessa mittapisteessä sijoitettiin ”korkea EPDS” ryhmään; samalla tavalla äidit, jotka saivat mediaanin ylittävät pisteet vähintään kahdessa mittapisteessä, sijoitettiin ryhmään ”korkea SCL” ja ”korkea PRAQ”. Sitä vastoin äidit, joiden pisteet alittivat nämä rajat, sijoitettiin matalien oiretasojen ryhmiin.

Taaperoiden ulostenäytteet kerättiin 30 kuukauden iässä ja analysoitiin 16S rRNA-amplikon sekvensointimenetelmällä. Data-analyysi suoritettiin käyttämällä koodausohjelmistoa nimeltään Rstudio.

Tulokset

Kolmesta tutkimuksessa käytetystä kyselylomakkeesta vain ne, jotka mittasivat yleisiä psykiatrisia oireita, eli masennusta (EPDS) tai ahdistuneisuutta (SCL-90), tuottivat merkitseviä tuloksia. Korrelaatiota raskausspesifiseen ahdistuksen (PRAQ-R2) ja taaperon suoliston mikrobiomin välillä ei havaittu.

Tutkimuksessa havaittiin, että differentiaalisessa runsausanalyysissa *Agathobaculum*-, *Bifidobakteeri*- ja *Ruminococcus*-suvut olivat harvalukuisempia niillä taaperoilla, jotka olivat altistuneet äidin raskaudenaikaiselle stressille. Lisäksi *Ruminococcus.1* suvun määrä lisääntyi näiden taaperoiden ulostenäytteissä. Alfa- ja beeta monimuotoisuuksia analysoitaessa ei saatu tilastollisesti merkitseviä tuloksia mitattaessa yhteyttä äidin raskaudenaikaisen stressioireilun ja taaperon ulosteen bakteerien monimuotoisuuden välillä.

Yhteenveto

Tulokset heijastelevat sitä, että äidin raskaudenaikainen krooninen stressi, saattaa vaikuttaa syntyvän lapsen suolistomikrobiston muodostumiseen ja sitä kautta voi potentiaalisesti vaikuttaa lapselle myöhemmin kehittyviin sairauksiin. Tällaisen ilmiön taustalla yhtenä vaikuttavana tekijänä on suoliaivoakseli, joka toimii molempiin suuntiin aivojen ja suoliston välillä apunaan erilaiset hermoston osat, välittäjäaineet ja hormonit. On selvää, että lisää pitkittäistutkimuksia selvittämään syvällisemmin suoliaivoakselin toimintaa ja terapeuttisia hoitokohteita tarvittaisiin. Tutkimusten pitäisi keskittyä kausaalisiin yhteyksiin ja niiden taustalla vaikuttaviin mekanismeihin sekä tarkastella pitkäaikaisia terveysvaikutuksia äidin raskaudenaikaiselle stressille altistuneissa jälkeläisissä. Lisäksi interventiotutkimukset voisivat selvittää mahdollisia ennaltaehkäiseviä toimia ja hoitoja, joilla voitaisiin lieventää äidin raskaudenaikaisen stressin haitallisia vaikutuksia lapsen terveyteen.

Asiasanat

mikrobisto, suoliaivoakseli, prenataali stressi, taapero, suolisto

ABSTRACT

Background

Maternal psychiatric symptoms during pregnancy present a significant health challenge, impacting the well-being of both mother and offspring. This phenomenon, here referred to as maternal prenatal psychological distress (PPD), may be linked with alterations in hormonal, metabolic, and immunological processes through gut-brain axis in both the mother and the offspring. Several lifestyle and environmental factors reportedly affect child gut microbiota composition, a critical factor for overall health, and recently, it has been suggested that maternal PPD could be one of these key factors. However, data predominantly come from animal research while human studies remain limited. This scarcity underscores the need for more research into how altered gut microbiota in toddler may lead to health effects later in life.

The purpose is to study if and how maternal PPD is associated with toddler fecal bacterial composition in a large prospective human cohort.

Methods

The study population consisted of mothers and toddlers from the large FinnBrain Birth Cohort Study. Maternal depression, general and pregnancy-specific anxiety symptoms were measured using the following questionnaires: EPDS, SCL, and PRAQ-R2 three times during pregnancy (n=208). The classification of symptom chronicity was determined by established scoring thresholds: mothers achieving a score 10 or higher on at least two occasions were classified as "high EPDS"; similarly, scores above the median on two or three occasions led to classifications of "high SCL" and "high PRAQ". Conversely, mothers scoring below these thresholds in most instances were identified as having low symptom levels.
The toddler fecal samples were collected at the age of 30 months and analyzed with 16S rRNA amplicon sequencing. Data analyses were conducted with Rstudio.

Results

Among the three utilized questionnaires, only those evaluating general mental health demonstrated significant associations with gut microbiota composition. Conversely, the questionnaire intended to measure pregnancy-related anxiety did not yield significant results.

The genera *Agathobaculum*, *Bifidobacterium,* and *Ruminococcus* abundances were lower in toddlers exposed to the mother's chronic anxiety and depression during pregnancy. In addition, the abundance of the genus *Ruminococcus.1* was increased in infants exposed to chronic anxiety and depression prenatally. Interestingly, pregnancy-related anxiety (PRAQ-R2) didn't associate with toddler gut microbiota. Alpha or beta diversity wasn't associated with maternal prenatal symptoms.

Conclusion

The findings demonstrate that the mother's prenatal psychological symptoms may be associated with the composition of intestinal microbiota in the child, potentially influencing the diseases that the child may develop later in life. To further elucidate the role of the gut-brain-axis in the mediating this effect and to identify potential therapeutic targets, more longitudinal studies are required. These studies should focus on the causal relationships and mechanisms underlying the observed associations and evaluate the long-term health outcomes in children because of maternal PPD. Additionally, interventional research could explore preventive strategies and treatments to mitigate the adverse effects of PPD on child health.

Key words:

microbiota, gut-brain axis, prenatal stress, toddler, gut

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1 Introduction

Stress during pregnancy, including conditions such as anxiety, depressive symptoms, and pregnancy-specific anxiety, represents a significant challenge within our society. Clinically significant maternal prenatal psychological distress (PPD) symptoms affect approximately 10-20% of pregnant mothers (Figueiredo & Conde, 2011; Korja et al., 2018; Skouteris et al., 2009; Teixeira et al., 2009). PPD is associated with offspring's development and health, such as the increased risk for infections (Korhonen et al., 2019), obesity (Burgueño et al., 2020), asthma (A. Lee & Wright, 2016; van de Loo et al., 2016) as well as psychopathology and alterations in brain development (Bozkurt et al., 2017; Davis & Sandman, 2012; Dawson et al., 2021; Erickson et al., 2017; Glynn et al., 2018; Madigan et al., 2018; Pearson et al., 2013; Walsh et al., 2019). Maternal prenatal stress may lead to alterations in offspring intestinal microbiota, which is one potential underlying mechanism mediating the association between prenatal stress and child health and development (Rodriguez et al., 2021).

There are multiple hypotheses concerning the pathways through which prenatal stress affects the offspring's microbiota. The most supported hypothesis is vertical transmission, wherein prenatal stress alters the mother's vaginal, intestinal, and breast milk microbiota, which in turn is directly transferred to the offspring (Dominguez-Bello et al., 2010; Fernández et al., 2013; Ferretti et al., 2018; Mitchell et al., 2020). Beyond the vertical transmission of microbes, other promising mechanisms may include metabolites produced by the altered maternal microbiota, or even entire microbes, that migrate to the fetal circulation (Gomez de Agüero et al., 2016; Nugent & Bale, 2015; Perez-Muñoz et al., 2017a; Willyard, 2018a), disruptions in microbial tryptophan metabolism and serotonergic signaling (Galley et al., 2021; Gheorghe et al., 2019), altered SCFA production (Jašarević et al., 2017; Pessa-Morikawa et al., 2022), disturbances in the differentiation of TH17 cells (Choi et al., 2016; Ivanov et al., 2009; Kim et al., 2017), increased maternal infections (Estes & McAllister, 2016) and offspring's altered hypothalamus-pituitary-adrenal axis induced by maternal postnatal stress (Heim & Nemeroff, 2001).

Animal studies have shown that early and late gestation stress could reduce the abundance of *Bifidobacterium* and *Lactobacilli* in infant monkey intestines, as reported by Bailey and colleagues (Bailey et al., 2004). This is in line with findings from several rodent studies by Jašarević (2017, 2018) where prenatal-stress-exposed offspring had reduced relative abundance of *Lactobacilli* (Jašarević et al., 2017, 2018)and increased *Bacteroides* and *Clostridium* abundances in gut microbiota (Jašarević et al., 2015). Similarly, Zhang (2021) showed that offspring intestinal *Bacteroides* relative abundance was increased in mice exposed to prenatal stress (Z. Zhang et al., 2021). Moreover, Golubeva's (2015) rat model suggested that PPD may induce alterations in the gut microbiota that may persist into adulthood (Golubeva et al., 2015). According to this theory, rats subjected to prenatal stress, exhibit decreased abundances of *Lactobacillus* and other genera.

In humans, Zijlmans (2015) concluded that infants of mothers with high stress during pregnancy had a significantly increased abundance of *Proteobacterial* groups known to contain pathogens and a decreased relative abundance of *Bifidobacterium* and *Lactic acid bacteria* (Zijlmans et al., 2015). Exposure to prenatal distress has been observed to correlate with the reduction in infant *Bifidobacterium* levels also by Aatsinki et al., 2020b, Galley et al., 2023 and Jahnke et al., 2021. Further, a human study by Naude (2020) observed that infants exposed to maternal prenatal stress had a higher proportion of

opportunistic *Proteobacteria* (Naudé et al., 2020). These findings are in line with Wei (2022), who reported an association between maternal emotional symptoms during pregnancy and increased levels of *Proteobacteria* in the meconium of newborns (Wei et al., 2022). Similarly, Rojas et al. (2023) discovered that prenatal depression, anxiety, and cortisol levels were associated with variations in the alpha diversity of the gut microbiota and the abundance of numerous bacterial taxa in 3–4-year-old toddlers in a trimester-dependent manner (Rojas et al., 2023).

Aatsinki and colleagues (2020) from FinnBrain Birth Cohort Study reported an association between exposure to maternal chronic PPD and 2,5 months of age infants' decreased abundance of health-promoting *Lactobacillus* and increased abundances of potentially opportunistic genera within the *Proteobacteria* phylum and *Firmicutes* phylum (Aatsinki et al., 2020b). The bacterial flora goes through rapid changes during the first years of life (Singh & Mittal, 2020; Stewart et al., 2018), and bacterial diversity increases with age (Yatsunenko et al., 2012a).

This study aimed to investigate associations between maternal chronic PPD and 30-month-old toddler fecal microbiota composition and diversity in a large prospective human cohort. Focusing on chronic maternal prenatal symptoms of depression, general anxiety, and pregnancy-related anxiety, we extend the findings of previous human and animal studies which have indicated associations between maternal PPD and variations in infant microbiota. Given the developmental changes in bacterial flora over time, it is hypothesized that 30-month-old toddlers exposed to maternal chronic PPD might exhibit a different pattern of bacterial associations compared to younger infants.

Specially, this study hypothesizes that these toddlers would show a lower abundance of opportunistic bacterial genera. This hypothesis is partly based on the age-related differences observed in previous studies, suggesting that the associations found in younger infants might not directly apply to older toddlers. Reliable human studies in toddlers remain scarce, underlining the need for this research to replicate and extend the investigative framework established by Aatsinki et al. to older age groups.

2 Materials and methods

2.1 Study subjects

The FinnBrain Birth Cohort Study (Karlsson et al., 2018) was the source of the available data of the target study population. This cohort study is conducted in South-West Finland and aims to research prenatal and early life stress effects on child health and brain development. The original cohort includes 3.808 families of which the smaller subset (n=207) for this study was selected based on specific study designs.

The study was approved by the Ethics Committee of the Hospital District of Southwest Finland. Written consent was collected from all families. Parents gave consent on behalf of their children.

Questionnaires were collected from mothers at gestational weeks (gwk) 14, 24, and 34 as well as postnatally. Parental information on the level of mothers' education, and maternal self-report on SSRI/SNRI medicine use were investigated at the first time point.

Information about breastfeeding (exclusive or partial) was surveyed using postnatal questionnaires. The Finnish National Institute for Health and Welfare register (www.thl.fi) was used to assemble information about maternal pre-pregnancy BMI, the length of gestation (preterm < 37 gwk, term 37–41 gwk, post-term ≥ 41 gwk), the newborn's height and weight, and the mode of delivery (vaginal or C-section) (Aatsinki et al., 2020b). Information about toddler medication use was reported by the mother at the time of the fecal sample collection.

Initially, there were 208 toddler fecal samples. One of the fecal samples was excluded due to low sequencing depth (< 10 000). The whole analysis was made with 207 dyads, including mothers and their children, of which all background information and questionnaire results were available and represented in Table 1.

Table 1: Clinical characteristics of the study population.
This table summarizes the demographic characteristics of the 208 mothers and their descendants in this study.

	Mean/Count (SD/%) n = 208
Mother's age	
	31.2 (4.5)
Education divided into 3 classes, count (%)	
1: Secondary or primary level	**48 (23.1 %)**
2: Vocational tertiary	**65 (31.2 %)**
3: University	**89 (42.8 %)**
NA: Missing data	**6 (2.9 %)**
Mode of delivery, count (%)	
1: Vaginal	**152 (73.1 %)**
2: Sectional	**27 (13.0 %)**
NA: Missing data	**28 (13.5 %)**
Birth weight (g)	
	3578.6 (498.0)
Mother's prepregnancy BMI, count (%)	
	24.4 (5.1)
Gestational weeks at birth moment, count (%)	
	39.9 (1.4)
SSRI/SNRI use at pregnancy timepoint 1 and 3, count (%)	
0: No	**200 (96.2 %)**
1: Yes	**6 (2.9 %)**
NA: Missing data	**2 (1 %)**
SSRI/SNRI use at pregnancy timepoint 1 or 3, count (%)	
0: No	**182 (87.5 %)**
1: Yes	**11 (5.3 %)**
NA: Missing data	**1 (0.5 %)**
Infant antibiotic courses, count (%)	
0: No	**182 (87.5 %)**
1: Yes	**25 (12 %)**
NA: Missing data	**1 (0.5 %)**
Duration of lactation (categorical), count (%)	
0: Any exclusive breastfeeding yes/no	**21 (10.1 %)**
1: Fully breastfed	**182 (87.5 %)**
NA: Missing data	**5 (2.4 %)**

2.2 Questionnaires

Maternal prenatal depression symptoms were measured with the Edinburg Postnatal Depression Scale (EPDS) (Cox et al., 1987; Levis et al., 2020). Symptom Checklist-90 (SCL-90) (Holi et al., 1998) was used to assess maternal prenatal anxiety. These two self-reported questionnaires were reported three times during pregnancy and three months postpartum. Pregnancy-Related Anxiety Questionnaire-Revised 2 (PRAQ-R2) (Huizink et al., 2016) was completed three times during pregnancy and used to measure a mother's fear of giving birth, apprehension about bearing a child with a disability and worries about changes in her appearance.

This study aimed to detect the chronic PPD symptom level throughout the whole pregnancy and evaluate the impact on its toddler fecal microbiota. For this aim, EPDS scores were stratified based on total score 10 or more per timepoint. Subjects that scored over the cut-point twice during pregnancy were classified as "high EPDS" group. For SCL-90 and PRAQ-R2 the median was used as cut-off point. Subjects scoring over median in two or three timepoints were classified as "high SCL" and "high PRAQ", respectively. Subjects were classified to group low in each symptom domain if she scored below the cut-off point 2 or 3 times. The questionnaire-derived variables are presented in Table 2.

Table2: Distribution of questionnaire scores
This table resents the distribution of high and low scores for the EPDS, SCL and PRAQ-R2 among a sample of 208 mothers.

	Mean/Count (SD/%) n = 208
EPDS	
High	**26 (12.5%)**
Low	**166 (87.0%)**
NA: Missing data	**15 (7.2%)**
SCL	
High	**111 (53.4%)**
Low	**81 (38.9%)**
NA: Missing data	**15 (7.2%)**
PRAQ	
High	**90 (43.3%)**
Low	**112 (53.8%)**
NA: Missing data	**5 (2.4%)**

2.3 Fecal samples

Toddler fecal samples were collected by parents at the child age of 30 months. Stool samples were collected in sterile tubes and taken immediately after that to the freezer or refrigerator. The date and time of sample collection were recorded. The tubes were delivered within 48 hours using coolers to FinnBrain's office for further processing. Parents were given instructions both orally and in written format. Only samples arriving at the research facilities within 48 hours were sequenced (Song et al., 2016).

Bacterial DNA was extracted from the frozen stool samples and DNA was analyzed with 16S rRNA amplicon sequencing. Gene libraries were developed with PCR and custom-

designed primers as previously reported (Rintala et al., 2018). DADA2 pipeline was used to process the raw sequences and in addition, 225 forward and 125 reverse base pairs were used with default parameters. DECIPHER package and SILVA database were used for taxonomic annotations.

2.4 Statistical analysis

Data analyses were made with a program called R using packages phyloseq (McMurdie & Holmes, 2013), microbiome, and mia. The phyloseq was used to analyze microbiome census data and the microbiome was used for Principal Coordinates Analysis (PCoA).

Alpha diversity describes the diversity or richness of bacterial species in one sample, and it can be measured with "Observed richness" and "Shannon index". Alpha diversity differences were analyzed with both the t-test and the Wilcoxon test.

Beta diversity quantifies similarities and dissimilarities between samples based on the overall gut microbiota profile. Beta diversity was illustrated with PCoA (Principal Coordinate Analysis) for amplicon sequence variant (ASV) -level data using Bray-Curtis dissimilarity, Jaccard Index, and Aitchison distance, (i.e., the Euclidean Distance on CLR-transformed data). PERMANOVA (Permutational multivariate analysis of variance) was used to analyze differences in the overall beta diversity profile. PERMDISP2 from package vegan was used to analyze group differences in beta dispersion, i.e., differences in intergroup heterogeneity.

Differential abundance analyses test statistical differences in genus abundances between high and low symptom groups. It is recommended to use several methods across the dataset and search for variations and overlaps between those methods. Taxa-analysis of this study was performed using three different statistical tests: the Wilcoxon test using CLR-transformed abundances, DESeq2 (Love et al., 2014), and ALDEx2 (Fernandes et al., 2014). The adjusted p-value was considered statistically significant if $p>0,05$.

As part of the data preparation process, missing responses, i.e., NA values from EPDS, SCL, and PRAQ surveys, were excluded from the analyses.

2.5 Potential confounders and adjustments

The progression of the neonatal microbiome is influenced by many factors (Mackie et al., 1999; Singh & Mittal, 2020) such as the mode of delivery (Dominguez-Bello et al., 2016; Jašarević et al., 2018; Salminen, 2004), sex (Elderman et al., 2018; Fransen et al., 2017; Maurice et al., 2015; Miyoshi et al., 2018; Shobeiri et al., 2022; Vemuri et al., 2019; Yoon & Kim, 2021), breastfeeding status (Differding et al., 2020; Kumbhare et al., 2022; Z. Wang et al., 2020), mothers' education (Pearson et al., 2013b), mother's SSRI intake (Pawluski et al., 2023) along with children's early life antibiotic exposure (Fouhy et al., 2012; Penders et al., 2006; Yassour et al., 2016). These six covariates were chosen based on previous publications (Bäckhed et al., 2015; Stewart et al., 2018). By adjusting the results, the study aimed to determine which confounding factors influence the development of the toddler microbiome and to improve the validity of the findings.

We performed stepwise adjustment in the analyses. Two different models were constructed to describe the impact of confounders in alpha and beta diversity analyses.

Firstly, the sex, delivery mode, mothers' education, and children's antibiotic intake were adjusted. Secondly, breastfeeding status was also included in the adjustment, in addition to the previous four. Finally, a sensitivity analysis was performed to clarify the importance of SSRI intake. Mothers using SSRI were excluded from the analyses, to see if this influenced the results.

For differential abundance analysis, we used only DESeq for the adjusted analyses. Initially, DESeq recuired the removal of NA values from the PPD variables. Subsequently, we reconducted the DESeq analyses, further excluding NA values from the five previously identified confounding factors, alongside the earlier omitted PPD values. The analysis was further refined by simultaneously adjusting for these five confounding factors. Additionally, we performed a sensitivity analysis for SSRIs.

3 Results

3.1 Toddler Fecal Microbiota Composition in general

In the amplicon sequencing of all fecal samples, five main bacterial phyla were detected: *Firmicutes* (1.0), *Bacteroidetes* (0.96), *Actinobacteria* (0.98), *Proteobacteria* (0.94), and *Verrucomicrobia* (0.02). Within them, 77 different genera were identified of which *Bacteroides* (0.99), *Clostridium* (0.97), *Bifidobacterium* (0.89), and *Veillonella* (0.86) were the most abundant. These numbers represent the relative abundance of each bacterial phylum or genus as a proportion of the total bacterial community. Values range from 0 to 1, where 1 indicates the highest possible abundance relative to other bacteria in the sample, and values closer to 0 indicate lower abundance.

3.2 Association between the Maternal Chronic PPD and Toddler Fecal Microbiota Composition

There were no statistically significant findings while measuring ALDEx2 (Fig.6., Fig.7., Fig.8.).

While measuring DESeq2, in cases where mothers were categorized within the high EPDS group, analyses revealed a diminished abundance of *Bifidobacterium* ($p = 0.03$, Fig.1., Fig.2.) and *Agathobaculum* ($p = 0.04$, Fig.1., Fig.2.) in their toddlers, whereas *Ruminococcus.1* ($p = 0.03$, Fig.1., Fig.2.) was found to be more abundant relative to the control group. Conversely, when mothers scored high in SCL across pregnancy, there was statistically significant decrease in the abundance of *Bifidobacterium* ($p = 0.0000003$, Fig.3., Fig.4.) and *Ruminococcus* ($p = 0.00005$, Fig.3., Fig.4.) in their toddlers' fecal samples. PRAQ-R2 wasn't associated with toddler microbiome (Fig.5.).

In conclusion, out of the three different questionnaires used, only depression (EPDS) or anxiety (SCL-90), produced significant results. Pregnancy-related anxiety (PRAQ-R2) produced no association with toddler gut microbiota.

genus	log2FoldChange	padj
Bifidobacterium	1.4700230	0.0294036
Barnesiella	0.9576951	0.0320993
Ruminococcus.1	-2.7794017	0.0320993
Agathobaculum	3.0277978	0.0424297
Massilibacillus	5.8603019	0.0951315
Enterococcus	-0.7952483	0.1195328

Fig.1. DESeq2: EPDS-questionnaire: To obtain statistically significant results, the "log2FoldChange" column needs to be under -1 or over 1, and the adjusted p-value "padj" needs to be under 0.05. These conditions are met by *Bifidobacterium*, *Ruminococcus.1*, and *Agathobaculum*.

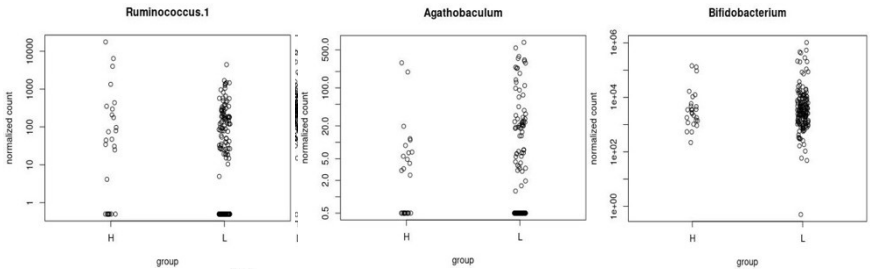

Fig.2. DESeq2: EPDS-questionnaire: Plot counts -graphs visualizing statistically significant microbes.

genus	log2FoldChange	padj
Bifidobacterium	1.7907271	0.0000003
Ruminococcus	1.4881255	0.0000504
Barnesiella	-0.6257119	0.0594136
Bacteroides	0.5551002	0.1074475
Sutterella	-1.0686979	0.2595075
Subdoligranulum	-2.3879845	0.2595075

Fig.3. DESeq2: SCL-questionnaire: Statistically significant conditions are met by *Bifidobacterium* and *Ruminococcus*.

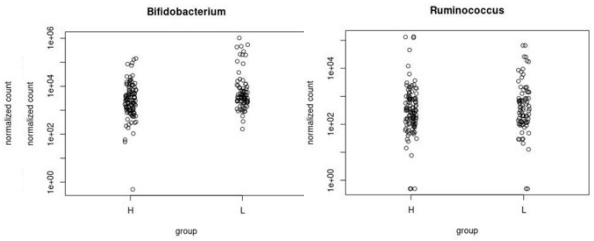

Fig.4. DESeq2: SCL-questionnaire: Plot counts -graphs visualizing statistically significant microbes.

genus	log2FoldChange	padj
Bifidobacterium	-0.5804671	0.4251356
Clostridium	-0.4752905	0.4251356
Barnesiella	-0.4701622	0.4251356
CAG-41	-0.9938733	0.4251356
Clostridium.2	-1.5038904	0.4251356
Eubacterium	-2.7162314	0.5432288

Fig.5. DESeq2: PRAQ-questionnaire: Statistically significant conditions aren't met.

genus	we.ep	we.eBH	wi.ep	wi.eBH
Massilibacillus	0.1241481	0.7566825	0.2126735	0.8211730
Lactococcus	0.1235190	0.8642661	0.1177182	0.8432019
TM7x	0.2230205	0.8529110	0.2193177	0.8643213
Unidentified_Genus.1	0.0755787	0.8769501	0.0609827	0.8704007
Clostridium_A	0.2687771	0.8944233	0.2333282	0.8774297
Lachnospira	0.1729825	0.7901573	0.3127249	0.8775800

Fig.6. ALDEx2: EPDS-questionnaire: "Wi.eBH", Wilcoxon test doesn't produce any significant results, because the minimum p-value is 0.82.

genus	we.ep	we.eBH	wi.ep	wi.eBH
Clostridium	0.0035019	0.2153732	0.0034604	0.2462986
Agathobacter	0.0383331	0.4489712	0.0197330	0.4392719
Faecalibacterium	0.0211522	0.3439637	0.0287667	0.4432240
Bifidobacterium	0.0095671	0.3058730	0.0318365	0.4772763
Alistipes	0.1061130	0.6531304	0.0397271	0.5257497
Barnesiella	0.0393581	0.5056533	0.0567425	0.5765195

Fig.7. ALDEx2: SCL-questionnaire: "Wi.eBH", Wilcoxon test doesn't produce any significant results, because the minimum p-value is 0.25.

genus	we.ep	we.eBH	wi.ep	wi.eBH
Cellulosilyticum	0.0526125	0.7616162	0.0240249	0.6154817
Unidentified_Genus.9	0.0664091	0.7230137	0.0378661	0.6493907
Senegalimassilia	0.0724582	0.7592677	0.0627057	0.7055006
Eubacterium	0.1159456	0.7512075	0.1314114	0.7127428
Escherichia	0.0698145	0.8307735	0.0419346	0.7210099
Akkermansia	0.2331020	0.8573990	0.2711826	0.8048100

Fig.8. ALDEx2: PRAQ-questionnaire: "Wi.eBH", Wilcoxon test doesn't produce any significant results, because the minimum p-value is 0.62.

3.3 Association between the Maternal Chronic PPD and Toddler Fecal Microbiota Diversity

There were no statistically significant differences in alpha diversity between PPD groups (t-test p-values: EPDS > 0.73, SCL > 0.40, PRAQ > 0.79, Wilcoxon test p-values: EPDS > 0.48, SCL > 0.93, PRAQ > 0.99, Fig.9., Fig.10., Fig.11.).

Moreover, there were no differences in beta diversity (PERMANOVA p-values: EPDS > 0.9, SCL > 0.079, PRAQ > 0.65) or beta dispersion (PERMDISP2 p-values: EPDS > 0.80, SCL > 0.84, PRAQ > 0.52, Fig.12., Fig.13., Fig.14., Fig.15.).

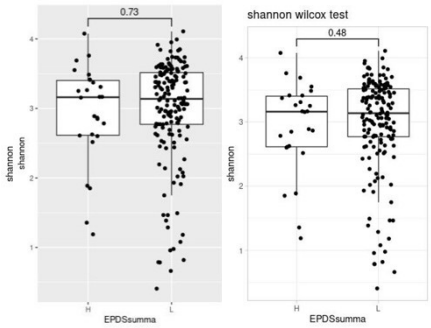

Fig.9. EPDS-questionnaire: Alpha diversity visualized with Boxplots. Shannon t-test and Shannon Wilcoxon test.

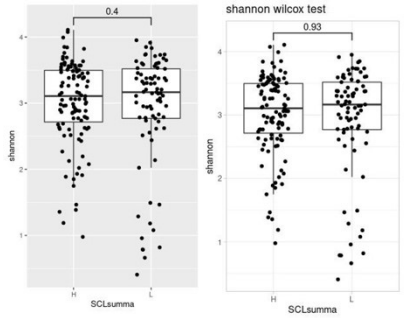

Fig.10. SCL-questionnaire: Alpha diversity visualized with Boxplots. Shannon t-test and Shannon Wilcoxon test.

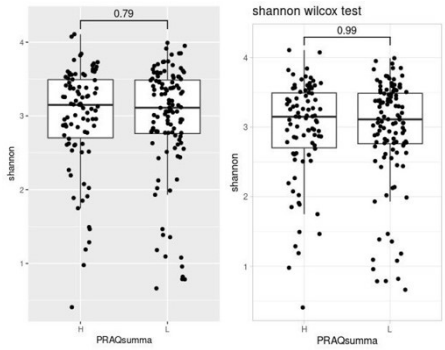

Fig.11. PRAQ-questionnaire: Alpha diversity visualized with Boxplots. Shannon t-test and Shannon Wilcoxon test.

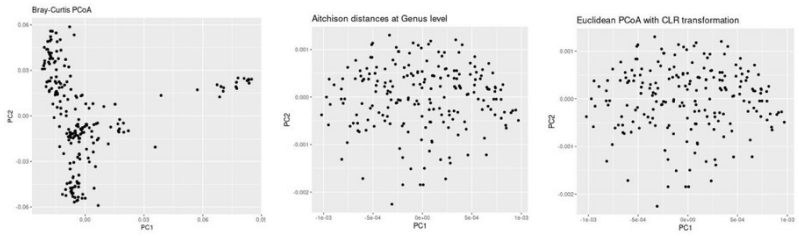

Fig.12. Beta diversity visualized with PCoA-plots. Bray-Curtis, Aitchison distance (i.e., the Euclidean Distance on CLR-transformed data), and Aitchison distances at the genus level.

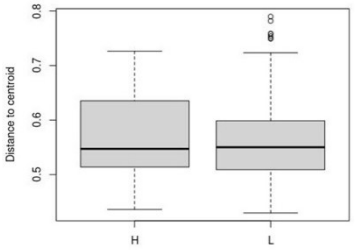

Fig.13. EPDS- questionnaire: Beta dispersion visualized with Boxplot.

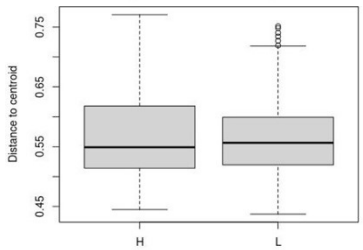

Fig.14. SCL-questionnaire: Beta dispersion visualized with Boxplot.

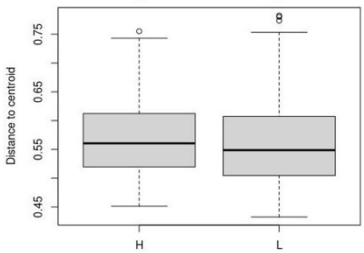

Fig.15. PRAQ-questionnaire: Beta dispersion visualized with Boxplot.

3.4 Confounding factors and adjustments

Alpha diversity was not associated with PPD when adjusted for confounders. SSRI-sensitivity analysis did not produce any significant results.

PPD variables did not associate with beta diversity when adjusted for confounders. Excluding SSRI-medicated mothers did not affect the results.

When the missing values of all five confounding factors were removed in addition to the NA values of the EPDS questionnaires (n = 162), the DESeq analysis revealed increased *Barnesiella* as the only significant genus ($p = 0.028$) in the EPDS high group. *Bifidobacterium*, *Ruminococcus.1*, and *Agathobaculum* lost their significance. After controlling the five selected variables, within the EPDS high group, no significant bacteria remained. When excluding SSRI-medicated mothers, *Veillonella* ($p = 0.00067$) and *Barnesiella* ($p = 0.034$) were increased in the high EPDS group.

Upon adjustment for five variables, the PRAQ high group was positively associated with *Ruminococcus .1* ($p = 0.020$) and negatively with *Ruminococcus* ($p = 0.020$). This finding is particularly intriguing, given the lack of statistically significant results for PRAQ in prior DESeq analyses. The SSRI sensitivity analysis had no impact on the results.

4 Discussion

This study aimed to examine how PPD is associated with toddler fecal microbiota composition and diversity. The results drawn from the prospective FinnBrain birth cohort study corroborate certain findings described in previous literature, as well as illustrate some new findings that may be related to the early functioning of the microbiota-gut-brain axis.

4.1 Maternal Chronic PPD is associated with four different bacterial genera in 30-months-old toddlers: *Bifidobacterium*, *Agathobaculum, Ruminococcus,* and *Ruminococcus.1*

Firstly, DESeq2 was the only analysis method producing significant results out of the three differential abundance analyses.

Secondly, among the three different questionnaires that measured long-standing chronic symptoms, only the EPDS and the SCL-90, which asses broader mental health issues including chronic depression and general anxiety, produced significant results. The PRAQ-R2, which specifically measures anxiety related to pregnancy, showed no association with toddler gut microbiota. It appears that chronic mental health issues, rather than transient pregnancy-related anxiety, are associated with alterations in the offspring's intestinal microbiota.

Thirdly, there were statistically significant differences between certain microbes, but not with the same microbes that were found in 2.5-month-old infants with almost the same study design (Aatsinki et al., 2020). A 30-month-old toddler's gut microbiota may differ in composition from the gut microbiota of a 2.5-month-old infant as a result of rapid changes in the microbiome during early childhood (Singh & Mittal, 2020; Stewart et al., 2018) and as a result of bacterial diversity increasing with age (Yatsunenko et al., 2012a). In this case, the association between two observed variables was found between different bacteria. The most important thing is that the association between maternal chronic PPD and the altered descendant gut microbiota was significant in both studies, albeit Aatsinki (2020) pointed out that PPD is positively associated with *Proteobacteria* and negatively associated with *Akkermansia* in 2,5 months infants. This study found out that PPD is negatively associated with *Bifidobacterium*, *Agathobaculum,* and *Ruminococcus* whereas positively associated with *Ruminococcus.1*, in 30-month-old toddlers.

The butyrate-producing *Ruminococcus* genus is beneficial for the intestinal barrier and belongs to the *Lachnospiraceae* family, which is part of the *Firmicutes* phylum. It is common for low microbial diversity to be characterized by a reduction in the number of *Lachnospiraceae* and *Ruminococcaceae* species, two of the most important anaerobic fermenters of butyrate and other SCFAs (Biddle et al., 2013; Vacca et al., 2020). Recent studies have shown that the lower *Lachnospiraceae* family abundance is linked with depression (Naseribafrouei et al., 2014), and a higher abundance of *Lachnospiraceae* is linked with anti-depressive and anti-inflammatory effects (Brinkman et al., 2011; Guida et al., 2018; Wong et al., 2016). Park and colleagues (2021) showed in a rat model that the *Lachnospiraceae* family was decreased in the feces of rats that experienced early-life stress, and therefore the lack of *Lachnospiraceae* family in the gut was involved in the induction of the anxiety-like behavior (Park et al., 2021). In this study, toddlers exposed to maternal chronic PPD had a lower abundance of *Ruminococcus* in their gut analyzed with DESeq2. This is in line with previous studies and one can speculate whether it will increase the risk of developing anxiety-like behavior.

The *Ruminococcus.1* genus belongs to the *Ruminococcaceae* family, which is also part of the *Firmicutes* phylum. As shown in Li's deer model (2022), forest musk deer with an early separation from their mothers had higher levels of *Ruminococcus.1* genus in their gut, however, the abundance of *Actinobacteria* decreased (Li et al., 2022), consistent with the results of the present study.

The *Agathobaculum* genus belongs to the *Ruminococcaceae* family, which is also part of the *Firmicutes* phylum. Previous findings from different mouse models have suggested that one species within the *Agathobaculum* genus could be a potential microbe-based treatment for Parkinson's disease and Alzheimer's disease (Go et al., 2021; D. W. Lee et al., 2022). Research has also noted a reduced abundance of the *Agathobaculum* in patients with Parkinson's disease and progressive multiple sclerosis (L. M. Cox et al., 2021; Kenna et al., 2021). In this study toddlers exposed to maternal chronic PPD demonstrated lower levels of *Agathobaculum* in their gut microbiota, which may suggest a possible increased risk for developing neurological conditions like Parkinson's disease or multiple sclerosis later in life. Further research is needed to confirm these associations and understand the implicants fully.

The *Bifidobacterium* belongs to the *Actinobacteria* phylum and is an important anti-inflammatory and immune-boosting bacterial genus. Some *Bifidobacterial* genera are used as probiotics because they positively affect the composition and functioning of the intestinal microbiota and have a wide range of beneficial host-health outcomes(Allen et al., 2016; Pinto-Sanchez et al., 2017; Tian et al., 2022a). It has been found in several mouse models that the *Bifidobacterium* genus reduces depressive behavior (Tian, Wang, et al., 2019; Tian, Zou, et al., 2019) and has an anxiolytic-like effect (H. M. Jang et al., 2018; H.-M. Jang et al., 2019; Tian et al., 2020). Two non-human studies have shown that the anxiolytic and antidepressant effects of the *Bifidobacterium* are related to suppressing gut microbiota dysbiosis (Guo et al., 2019; Han & Kim, 2019). Recent studies have focused primarily on animal models, however, there have been a few human studies that indicate that the *Bifidobacterium* genus reduces stress and depression scores, alters brain activity, and improves memory (Allen et al., 2016; Pinto-Sanchez et al., 2017; Tian et al., 2022a). In this study elevated maternal chronic PPD was negatively associated with beneficial *Bifidobacterium* in a toddler fecal sample, which may cause changes in brain and mental health development. This finding is in line with Bailey and colleagues (2004) who claimed that gestation stress can reduce the abundance of *Bifidobacterium* in infant monkey intestines (Bailey et al., 2004). The same association has been found in Zijlman's (2015) human study where infants of mothers with high stress during pregnancy had decreased relative abundance of *Bifidobacterium* (Zijlmans et al., 2015).

It is interesting that the *Proteobacteria* phylum, known to contain pathogens, was not able to produce any statistically significant results in this study, even though several human studies have shown the association between maternal stress and offspring altered *Proteobacteria* abundances. Zijlmans (2015), Naude (2020), and Aatsinki (2020) each reported an association between exposure to maternal stress and infants' increased abundances of potentially opportunistic genera within the *Proteobacteria* phylum (Aatsinki et al., 2020b; Naudé et al., 2020; Zijlmans et al., 2015). These findings are in line with Wei (2022), who found that maternal emotional symptoms during pregnancy increased the abundance of *Proteobacteria* in the meconium of newborns (Wei et al., 2022).

In conclusion, this study found that prenatal stress is associated with changes in the offspring microbiota, which were centered on *Actinobacteria* and *Firmicutes* phyla, in support of both human and animal studies previously published.

4.2 Adjustments of confounders and SSRI sensitivity: Significant and surprising outcomes

To distort the results, six potential confounding factors were selected based on previous publications (Bäckhed et al., 2015; Stewart et al., 2018) including sex, mode of delivery, breastfeeding status, children's antibiotic intake, mothers' education, and mothers' use of SSRIs during pregnancy. Children's diet during early life would have also been an important confounder to take into consideration (Brink et al., 2020; De Filippo et al., 2010), but the collected food diary data wasn't available when performing analyses. The maternal antibiotic intake (Champagne-Jorgensen et al., 2020; Tormo-Badia et al., 2014) and diet (Buffington et al., 2016; Li et al., 2019; Savage et al., 2018) would have also impacted the toddler microbiota but were not considered when completing adjustments. No significant outcomes were observed following the adjustment of alpha diversity. In contrast, the adjustment for beta diversity indicated that mode of delivery and antibiotic use, from the set of selected potential confounders, were significant variables affecting the study results. This is partly in line with Bokulich et al.'s (2016) article which claims that antibiotics, birth mode, and diet are the factors shaping microbiome maturation during early life (Bokulich et al., 2016). In the context of this study, neither breastfeeding, gender nor maternal education associated with beta diversity.

Upon adjusting the DESeq analysis, numerous significant findings were observed regarding the decreases and increases in the abundance of various bacteria. Intriguingly, this analysis also identified bacterial species not detected in prior analyses in this study. A notable observation emerged from the DESeq analysis of EPDS, which initially indicated a decrease in *Bifidobacterium* and *Agathobaculum* and an increase in *Ruminococcus.1* within the EPDS high group. However, after the exclusion of SSRI-medicated mothers from the dataset and reanalysis, an increase in *Veillonella* and *Barnesiella* bacteria was detected in the offspring of these mothers within the same EPDS high group. *Barnesiella* belongs to the Bacteroidetes phylum whereas *Veillonella* is part of the Bacillota phylum. In addition, the analysis of the EPDS high group, with the exclusion of missing values from all potential confounding factors, revealed a heightened bacterial load of *Barnesiella*. Both Jašarević (2015) and Zhang (2021) introduced mouse models where descendant intestinal *Bacteroides* relative abundances were increased in mice exposed to prenatal stress (Jašarević et al., 2015; Z. Zhang et al., 2021). After adjusting for all five potential confounders, the high EPDS group no longer retained any of the bacteria that were initially identified as significant.

In earlier sections of this paper, the high SCL group was associated with decreased levels of *Bifidobacterium* and *Ruminococcus*. Yet, upon removal of NA values from five selected variables in the analysis, the data revealed a significant increase in *Bacteroides* which suggests a possible causality. Upon considering all five potential confounding factors, the analysis yielded an unexpected result: a reduction in both *Bacteroides* and *Bifidobacterium* within the SCL high group. *Bacteroides* belongs to the Bacteroidetes phylum.

A notable and unexpected outcome was observed upon considering all five potential confounders: in the high PRAQ group, *Ruminococcus* levels decreased while *Ruminococcus.1* level increased. This result is particularly surprising given that previous analyses yielded no significant findings related to the PRAQ questionnaire.

4.3 Deciphering the pathways: Exploring how prenatal and postnatal stress impacts offspring gut microbiota

There has been growing attention in the field of gut microbiota, especially the gut-brain axis, over the last few decades. Among the researchers, this dynamic bidirectional communication between the gut microbiota and brain has become an important factor that influences human metabolism, neurodevelopment, immunity, and behavior. Gut-brain axis functions through three different pathways (Asadi et al., 2022). The neural pathway consists of the vagus nerve and the enteric nervous system, the endocrine pathway affects the hypothalamus-pituitary-adrenal axis, and the third is the immunological pathway.

Maternal stress may alter the gut and vaginal microbiota during pregnancy (Dawson et al., 2021; Doroftei et al., 2022; Hechler et al., 2019; Jašarević et al., 2015, 2017, 2018; M. Kimmel et al., 2022). Disruptions occurring either preconception or throughout pregnancy, which affect the dynamics of vaginal microbiota, may have consequential impacts on the microbial profiles transmitted from mother to child at the time of birth (Jašarević et al., 2015). It has been previously hypothesized that the maternal vaginal microbiota constitutes a primary reservoir for the pioneer microbial community that initially colonizes the neonate's gut (Dominguez-Bello et al., 2010). However, recent findings challenge this view, suggesting that during vaginal delivery, the colonization of the newborn is more likely to be sourced from the mother's rectal microbiota rather than the vaginal microbiota (Ferretti et al., 2018; Mitchell et al., 2020). Infants delivered via cesarean section exhibit a microbiota composition that more closely resembles human skin and most of the microbial transmission to these neonates is attributed to contact with individuals handling the infant (Dominguez-Bello et al., 2010).

The perspective on initial infant gut colonization during birth is challenged by emerging, albeit controversial, evidence supporting the existence of a prenatal microbiome (Perez-Muñoz et al., 2017a; Willyard, 2018a). Isolations of bacteria from human meconium (Ardissone et al., 2014; Gosalbes et al., 2013; Jiménez et al., 2005, 2008), umbilical cord blood (Jiménez et al., 2005), amniotic fluid (Bearfield et al., 2002; Rautava et al., 2012) and fetal membranes (Rautava et al., 2012; Steel et al., 2005) have been reported. Further studies have indicated morphological and sequencing evidence of a placental microbiome that shares similarities with the oral microbiome (Aagaard et al., 2014; Rackaityte et al., 2020; Stout et al., 2013). However, the hypothesis of placental colonization is subject to debate, as it might occur during labor due to disruptions in the placental barrier (Willyard, 2018b) and several studies have faced criticism for potential methodological limitations (Aagaard et al., 2014; Perez-Muñoz et al., 2017b).

The composition and functional dynamics of the maternal gut microbiome undergo modifications during pregnancy to accommodate the energy requirements of the developing fetus (Nugent & Bale, 2015). Factors such as maternal diet, stress levels, and exposure to infections can lead to alterations in the composition and functionality of the maternal gut microbiota, as well as in the availability of microbiota-derived metabolites.

These changes in metabolites may have programming effects on both the placenta and the fetal compartment (Gomez de Agüero et al., 2016; Nugent & Bale, 2015).

SCFAs are one essential group of metabolites derived from microbial fermentation and crucial for brain development, especially for microglia (Caetano-Silva et al., 2023; Erny et al., 2015, 2021). SCFAs may mediate the impact of maternal stress on the neurological development of offspring by distributing the maturation of microglia (M. C. Kimmel et al., 2024). This effect may occur in the developing fetus through transplacental transport and in the offspring due to a reduced number of colonizing microbes capable of producing SCFAs (Jašarević et al., 2017; Pessa-Morikawa et al., 2022).

Maternal microbial metabolites and the balance between pro-inflammatory TH17 cells and anti-inflammatory regulatory T cells play a critical role in fetal brain development and immune tolerance, with alterations potentially leading to structural, neurodevelopmental, and behavioral changes in the offspring (Choi et al., 2016; M. C. Kimmel et al., 2024). Animal models have demonstrated segmented filamentous bacteria's role in those maternal-fetal interactions, affecting TH17 cell differentiation (Ivanov et al., 2009; Kim et al., 2017; M. C. Kimmel et al., 2024). Additionally, maternal stress may exacerbate these effects by further altering the gut microbiota and immune responses, suggesting a complex interplay where stress potentially heightens the risk of adverse neurodevelopmental outcomes in the offspring (X. Lu et al., 2024).

Altered maternal intestinal metabolism of tryptophan due to prenatal stress, notably through the kynurenine pathway influenced by proinflammatory cytokines and specific gut microbes, plays a critical role in neurodevelopmental outcomes by affecting serotonergic signaling and serotonin availability in the offspring (Badawy, 2013; Galley et al., 2021; Gheorghe et al., 2019; Haq et al., 2021; M. C. Kimmel et al., 2024; KNOX, 1951; Salter & Pogson, 1985). This disruption is linked to changes in fetal and placental serotonin level, potentially mediated by microbial tryptophan metabolites like indoles that impact immune regulation and central nervous system inflammation through mechanisms such as AhR signaling in microglia and astrocytes (M. C. Kimmel et al., 2024; Ramsteijn et al., 2020; Rothhammer et al., 2018).

Infants born to mothers exhibiting depressive symptoms have been discovered to have diminished fecal concentrations of Secretory Immunoglobulin A (sIgA), a molecule pivotal to healthy gut mucosal immunity in infants (Kang et al., 2018). A reduction in slgA could potentially make infants more susceptible to infections and may affect their overall immune development, underscoring the importance of maternal health in early immune training (Rodriguez et al., 2021).

Breastfeeding constitutes an alternative pathway for the transmission of maternal microbes to the infant (Fernández et al., 2013). This process involves an entero-mammary pathway, wherein gut bacteria are transported to the mammary gland through lymphatic and blood circulation (Perez et al., 2007). Additionally, external contributions to the microbial composition of milk include bacteria from the subcutaneous skin of the breast and the oral cavity of the infant (Grice et al., 2009; Hunt et al., 2011; Ramsay et al., 2004).

Postpartum depression in mothers may affect maternal caregiving behaviors and the hypothalamus-pituitary-adrenal axis in children, potentially influencing the

developmental programming of brain health later in life (Heim & Nemeroff, 2001). Additionally, there is an ongoing dialogue between the hypothalamus-pituitary-adrenal axis and the gut microbiota, which may further influence these outcomes.

Chronic stress in individuals has been documented to compromise the organism's immunological response capabilities, leading to an increased prevalence of morbidity (Dragos & Tanasescu, 2010). Prenatal maternal infections have been implicated in affecting the neuropsychiatric development of offspring and therefore may potentially influence the composition of the child's gut microbiota (Estes & McAllister, 2016).

4.4 Limitations of the study

Statistical methods to analyze differential abundance (DA) in microbiome data are still in their infancy. To ensure robust biological interpretations, researchers should use a consensus approach based on multiple DA methods, because it's widely known that there is high variation across different DA tool results (Weiss et al., 2017). Out of the three DA analyses used in this study, DESeq2 (Love et al., 2014) was the only method producing significant results which caused more uncertainty in the results. Both the Wilcoxon test (CLR) and ALDEx2 produced insignificant outcomes, which makes the results of this study even more questionable because ALDEx2 is classified as the most reliable method of abundance analysis according to Nearing et al. (2022) (Nearing et al., 2022). As Nearing and colleagues pointed out, ALDEx2 identified only a relatively small number of ASVs as significant being quite accurate and conservative but having poor sensitivity (Nearing et al., 2022). ALDEx2 and Wilcoxon's tests did not produce a single false positive in Nearing's analyses, while DESeq2 performed poorly when evaluated from the false positive perspective (Nearing et al., 2022). However, according to Calgaro et al.'s study (2020), DESeq2 demonstrated the best performance and is the most recommended tool for DA analyses, whereas ALDEx2 showed lower statistical power, even though ALDEx2 was less likely to identify false positives (Calgaro et al., 2020). According to Hawinkel et al. (2019), the sensitivity increases with sample size and effect size for many methods except for DESeq2, and in addition, DESeq2 has poor specificity (Hawinkel et al., 2019). To conclude, it is quite unlikely to detect significant microbial characteristics in one dataset that overlap with significant ones in similar datasets using different DA methods. From this, it can be concluded that there will often be drastic differences in biological interpretations based on which DA tool is used and therefore the results of this current study should also be treated with caution.

Fecal samples were analyzed with 16S rRNA amplicon sequencing designed for taxonomic profiling, which does not permit functional capacity or strain-level evaluation. Instead of 16S rRNA amplicon sequencing, shotgun metagenome sequencing would give a higher resolution on the composition (Jovel et al., 2016). These two sequencing methods are used to assess microbial composition on the same sample, but both methods yield qualitatively and quantitatively comparable results (Laudadio et al., 2018; Shah et al., 2010). Shotgun metagenome sequencing allows deeper characterization of the microbiome complexity and also the identification of species and strains for each stool sample when compared to 16S rRNA amplicon sequencing, which reliably describes taxonomy on the genus level (Laudadio et al., 2018). Despite the advantages of shotgun metagenomics, 16S rRNA amplicon sequencing is the most widely used technology due to its low cost (Cottier et al., 2018). However, there are likely to be similar issues with analyses of metagenome sequencing data type compared to analyses with 16S rRNA

amplicon sequencing, due to its similar levels of sparsity and variability between samples.

We as humans are exposed to a wide range of stress exposures during our lives, the burden of which can be calculated using a variety of biological and psychological measurements. In the FinnBrain Birth Cohort Study mothers' hair samples were collected during pregnancy to measure hair cortisol concentration as a biological meter for PPD. It has been reported that elevated hair cortisol concentration is associated with depressive symptoms during pregnancy (Hoffman et al., 2016; Mustonen et al., 2018, 2019). However, collected hair cortisol concentration data was not used in the present study, unlike in the previous FinnBrain article (Aatsinki et al., 2020b). This study used only psychological questionnaires from a subjective perspective to measure PPD. Pregnant mothers experience depressive symptoms in different ways and different areas of life, which may result in different responses to questionnaires (Bygstad-Landro & Giske, 2018; Staneva et al., 2015). Enhancing the study's methodology by including diverse stress metrics, such as a hair cortisol concentration, alongside questionnaire data could have yielded a more robust understanding. Given that hair cortisol concentration is not a direct indicator of stress and that physiological and psychological stressors may influence differently biological processes, such an approach might have improved the reliability of the findings.

When construing the results, it should be borne in mind that the results only characterize a snapshot of associations during toddlerhood. Therefore, one potential factor that reduces the credibility of the results is that the bacterial composition was only determined at the one-time point. This makes the study vulnerable to wrong conclusions.

The gut microbiome and circadian rhythm interact closely (Frazier & Chang, 2020), which means that the time of day when the stool sample was collected may affect the sequencing results. Microbe-derived metabolites such as short-chain fatty acids (SCFAs) and bile acids (BAs) are probable mediators in this phenomenon. A preclinical study has also shown that the gut microbiota displays circadian rhythms and that the timing of food consumption can affect the function and composition of gut microbiota (Kaczmarek et al., 2017). Because the composition and function of the microbiota vary throughout the day and eating disturbs the normal composition, the time of stool sample collection is a potential limitation in this study. Thus, the daily and intra-day variation is quite stochastic, and the time itself may not be as important as the events preceding the sampling. It is important to remember that there may be some variation in the number of individual taxa, but the microbiome profile still resembles that of the individual.

Seasonal variation has also been reported to influence intestinal microbiota composition and diversity (Davenport et al., 2014). In that study, seasonal differences were found in both the abundance of distinct taxa and overall gut microbiome diversity. During the summer, there was an increased abundance of *Bacteroidetes;* during the winter, *Actinobacteria* abundance increased (Davenport et al., 2014). In this study, *Bifidobacterium*, which is defined as a healthy bacterium, was decreased in toddler microbiota if mother had the chronic PPD symptoms. *Bifidobacterium* belongs to the *Actinobacteria* phylum. As previously stated, *Actinobacteria* abundance may increase in the winter. This can distort results because toddler stool samples were collected at the age of 30 months, regardless of whether it was summer or winter. As opposed to this, it

is assumed that pregnant mothers are stressed regardless of the season, making the season irrelevant to the association between prenatal stress and toddler microbiota.

Even though the composition and functional potential of the human gut microbiota develops over the lifespan, kinship has been identified as a key covariate influencing the diversification of microbial communities. In their human study Valles-Colomer et al. (2023), found that time since cohabitation affected strain sharing more than genetics or age (Valles-Colomer et al., 2023). Furthermore, they (2021) noted that transmission rates were highest between sisters and mother-daughter pairs, decreasing with increasing daughter's age and being highest between cohabiting couples compared to those living apart (Valles-Colomer et al., 2021). In conclusion, our gut microbiome is greatly influenced by the people we live with and should be taken into consideration when analyzing the data.

Those who attended the most surveys of the FinnBrain birth cohort study were mainly healthy and well-off citizens (Karlsson et al., 2018), which must be considered when evaluating the results because the clinical samples taken from those with a lower socioeconomic status might differ from present ones (Raizada, 2010).

4.5 Strengths of the study

This study was part of a large prospective human cohort study, and the sample size (n = 207) of this study was comprehensive. However, an even larger sample size would potentially facilitate the perception of associations between maternal PPD and toddler fecal microbiota composition.

Using the Finish National Institute for Health and Welfare registry data strengthened this study (Karlsson et al., 2018).

The Finnish population is homogenous from environmental, genetic, and socioeconomic points of view (Virtaranta-Knowles et al., 1991). Because of this, the association found in this study can be generalized to the whole of Finland, even though the examinees are drawn from a geographically restricted area in southwestern Finland. There should be caution when generalizing the results internationally because for example the Finnish population is genetically quite separated even from the rest of Europe (Salmela et al., 2008). However, genetics is not the only persuasive factor, since the social structure, socioeconomic status, and the level of development in the community are also crucial factors defining microbiota (Bowyer et al., 2019; De Filippo et al., 2010; Gacesa et al., 2022; Yatsunenko et al., 2012b). It is noteworthy that Finland's structure of society closely resembles that of Europe.

Incorporating five potential confounders into the later analyses and conducting an SSRI sensitivity analysis enhanced the reliability of the results, thereby underscoring the transparency of the findings.

4.6 Future perspectives

The altered intestinal microbiota has been associated with metabolic syndrome (Dabke et al., 2019; Koren et al., 2012; P.-X. Wang et al., 2020), obesity (Asadi et al., 2022; Cuevillas et al., 2022; Ley, 2010; Patterson et al., 2016; Schwiertz et al., 2010),

inflammatory bowel disease (Chen & Wang, 2022; Glassner et al., 2020; Gophna et al., 2006; Joossens et al., 2011; Lepage et al., 2011; Mangin et al., 2004; Manichanh et al., 2006; Nishida et al., 2018), diabetes (Gurung et al., 2020; Larsen Nadja AND Vogensen, 2010; Patterson et al., 2016; Qin et al., 2012; Scheithauer et al., 2020; Tilg & Moschen, 2014; Wu et al., 2010), autoimmune diseases (Christovich & Luo, 2022; de Luca & Shoenfeld, 2018; Jiao et al., 2020; Xu et al., 2022) and other non-communicable diseases. In addition, gut microbiota could be the missing puzzle piece in the etiopathogenesis of psychiatric symptoms and disorders, because dysbiosis of the human microbiome has been linked with brain development (J. Lu & Claud, 2019; Morais et al., 2021; Socała et al., 2021; Tran & Mohajeri, 2021), temperament (Aatsinki et al., 2019; Christian et al., 2015; Kelsey et al., 2021; Y. Wang et al., 2020), behavior (Sarkar et al., 2018) and autism (Golubeva et al., 2017). The predisposition for these listed diseases has often taken root in early life. Hartman et al (2019) speculated that intestinal microbiota may be one suggested mechanism by which prenatal stress promotes postnatal developmental plasticity (Hartman et al., 2019).

To clarify, the prevalence of stress-related psychiatric and somatic conditions is emerging and at the time increasing the burden on public health (Torikka et al., 2014). Maternal antenatal depression is a crucial element especially affecting descendant mental health from childhood to maturity (Pearson et al., 2013c). Because of this, it is crucial to understand mechanisms linking prenatal stress exposure and health outcomes to be able to create preventive steps and evolve targeted interventions.

It has been proposed that several alterations in the intestinal microbiota can lead to mood disorders, and therefore therapies targeting the gut bacteria, for example, microbiota supplementation and transplantation with probiotics could be noteworthy mental health treatments for depression(Akkasheh et al., 2016; Kazemi et al., 2019; Saccarello et al., 2020; Schaub et al., 2022; Tian et al., 2022b; Ullah et al., 2022). There is evidence that some *Bifidobacterium* genera as probiotic treatments could protect against early-life stress-induced mood disorders and neuroendocrine alterations (Desbonnet et al., 2010; Fukui et al., 2018; Moya-Pérez et al., 2017; Zhu et al., 2022), thus probiotics may be able to serve a wider range of therapeutic purposes than previously thought.

Zhang Xuan's (2022) experimental procedure showed that newborns who experienced maternal mindfulness intervention during the mother's pregnancy had a higher abundance of beneficial *Bifidobacterium* and *Blautia* in their neonate meconium (X. Zhang et al., 2022). This study proved that alleviating maternal psychological distress can alter the infant meconium microbiota and potentially reduce possible gut-microbiome-mediated negative health effects in later life.

As part of the FinnBrain Birth Cohort Study, mothers were asked to complete questionnaires three times during pregnancy and three months after delivering their child as a psychological meter for PPD. However, collected questionnaire data from three months postpartum was not used in the present study, unlike in the previous FinnBrain article about 2.5-month-old infants (Aatsinki et al., 2020a). Considering the postnatal environment for 2.5-year-old toddlers is already its own research question, but it should be considered as a confounding factor in future studies when investigating associations between maternal prenatal psychological distress and toddler fecal microbiota.

Further studies are required to confirm the findings in independent samples and to investigate the potential mechanisms underlying the observed phenomenon. Studies investigating the biological pathways mediating the associations, including microbial metabolites and short-chain fatty acids, are needed.

4.7 Conclusion

This study aimed to examine if and how maternal prenatal psychological distress, measured with questionnaires, is associated with toddler fecal microbiota composition and diversity. According to toddler fecal microbiota composition, significant results were produced only with a single tool out of three used tools. Exposure to early-life maternal chronic stress was associated with an increased presence of *Ruminococcus.*1 in toddlers, while concurrently, the levels of *Agathobaculum*, *Bifidobacterium*, and *Ruminococcus* genera were observed to decrease. No link was found between maternal chronic PPD and the diversity of toddler fecal microbiota.

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