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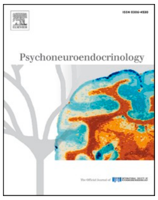

Maternal pre- and postnatal stress and maternal and infant gut microbiota features

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ABSTRACT

Background: Maternal stress can have short and long term adverse (mental) health effects for the mother and her child. Previous evidence suggests that the gut microbiota may be a potential mediator and moderator for the effects of stress via various pathways. This study explored the maternal microbiota trajectory during pregnancy as well as the association between pre- and postnatal maternal stress and features of the maternal and infant gut microbiota during and after pregnancy. In line with previous research, we hypothesized that maternal stress would be positively related to maternal and infant microbiota volatility and that infants of highly stressed mothers would show a relative increase in Proteobacteria and a relative decrease in *Bifidobacterium.*

Methods: We collected maternal stool samples at 18 and 32 weeks of pregnancy and 8 months postpartum. Infant stools samples were obtained at 2, 6 and 12 weeks and 8 months postpartum. All samples were analyzed using shotgun metagenome sequencing. We also collected several measures of maternal stress (self-reported depression, anxiety, and stress, and hair cortisol and cortisone), most at the same time points as the microbiota samples.

Results: Our data indicated that the maternal microbiota does not undergo drastic changes from the second to the third trimester of pregnancy but that the postpartum microbiota differs significantly from the prenatal microbiota. Furthermore, we identified associations between several stress measures and maternal and infant gut microbiota features at different time points including positive and negative associations with alpha diversity, beta diversity and individual microbial phyla and species relative abundances. Also, the maternal stress composite score, the perceived stress score and the log-ratio of hair cortisol and cortisone were all positively associated with infant microbiota volatility.

Conclusion: Our study provides evidence that maternal prenatal and postnatal stress is related to both the maternal and the infant microbiota. Collectively, this and previous studies indicate that maternal stress does not uniformly associate with most gut microbial features. Instead, the associations are highly time point specific. Regarding infant microbiota volatility, we have consistently found a positive association between stress and infant microbiota volatility. This warrants future research investigating this link in more depth.

1. Introduction

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

The gut microbiota can influence host-health for instance via the gut

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brain axis (Cryan et al., 2019). The maternal gut microbiota plays a special role. Recent evidence suggests that microbiota-derived metabolites influence the programming of the fetal intestine prenatally by reaching the fetus via the placenta, where they modulate gene expression related to host-microbe interactions, immunity, and epithelial function (Husso et al., 2023; Kaisanlahti et al., 2023; Pessa-Morikawa et al., 2022). Additionally, maternal gut microbiota-derived extracellular vesicles, which can cross the placenta, may impact fetal development (Husso et al., 2023; Kaisanlahti et al., 2023). Postnatally, the maternal gut microbiota serves as the primary source of microbes for the initial colonization of the infant gut through vertical transmission (Browne et al., 2022; Valles-Colomer et al., 2022, 2023; Van Daele et al., 2019; Wang et al., 2020). Accordingly, variables that affect the maternal microbiota, such as prenatal stress, may also directly or indirectly affect the health of the offspring (Kimmel et al., 2023) and should be explored and defined. In the following, we briefly summarize the status of the literature on maternal microbiota studies in relation to pregnancy and then maternal and infant microbiota studies in relation to prenatal and postnatal stress.

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

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

More studies examined the associations between maternal prenatal stress and the infant gut microbiota (Dutton et al., 2023; Galley et al., 2023; Mepham et al., 2023; Weiss and Hamidi, 2023; Zijlmans et al., 2015). Collectively, they identified associations between prenatal stress and infant microbiota features including alpha- and beta diversity, as well as relative abundances of individual genera. Besides several unique findings, five out of 10 studies found a positive association between prenatal stress and Proteobacteria (one study found the opposite; reviewed in Mepham et al. 2023). Also, four out of 10 studies found a negative association with *Bifidobacterium* (Dutton et al., 2023; Galley et al., 2023; Jahnke et al., 2021; Zijlmans et al., 2015), while one found the opposite (Weiss and Hamidi, 2023). Studies found both positive (Dutton et al., 2023; Rojas et al., 2023; Zijlmans et al., 2015) and negative (Galley et al., 2023; Jahnke et al., 2021) associations with alpha diversity. Prenatal stress was defined heterogeneously between studies and time points of stress measurement and stool sampling varied considerably, although most studies included an infant stool sample taken at around two to three months of age. These differences impede reproducibility of findings. Moreover, Rojas et al. (2023) and Van den Bergh et al. (2020) report measurement- and trimester-specific associations between prenatal stress and the infant gut microbiota and other outcome measures, respectively. This implies that reproducibility of findings may have been further hampered by the possibility that the effects of prenatal stress may be trimester specific. Lastly, most of these studies (nine out of 10) were limited to a taxonomic resolution at the genus level (due to the use of 16S rRNA sequencing) and only two out of 10 studies collected more than one infant stool sample (Dutton et al., 2023; Zijlmans et al., 2015).

In sum, there is a lack of studies focusing on the association between prenatal stress and the maternal microbiota during pregnancy. Across mother and infant microbiota studies, differences between studies related to the stress measurements utilized and the time points of stress and microbiota sampling necessitate a larger number of studies and standardization of research methodology to disentangle the association between prenatal stress and the maternal and infant gut microbiota. Moreover, studies are yet to investigate microbiota volatility (Bastiaanssen et al., 2021), even if repeated stool sampling was performed. Microbiota volatility can be defined as intra-individual compositional change over time and is quantified by the intra-individual Aitchison distance (beta diversity). This volatility measure has recently been positively linked to stress in male mice as well as young adults (Bastiaanssen et al., 2021). While it remains unclear whether stress causes a more volatile microbiota or vice versa, a recently published randomized controlled trial found that a skin-to-skin intervention between mothers and their infants significantly reduced microbiota volatility in the infants (Eckermann et al., 2024). For the skin-to-skin intervention naked infants (only wearing a diaper) were placed on the mother's bare chest for one hour daily in the first 5 weeks postpartum. This was compared to a care-as-usual group that provided significantly less skin-to-skin contact. More research is needed to determine whether volatility is a biomarker of stress, which factors influence it and how it relates to other health outcome measures. The present study on healthy low-risk mothers and their infants used shotgun metagenomic sequencing to analyze three stool samples from the mother, at 18 and 32 weeks of gestation and at eight months postpartum, and four infant stool samples in the first eight months of life. Maternal stress was measured at all these time points using different self-report questionnaires. Additionally, maternal stress was physiologically assessed by means of two hair cortisol samples reflecting chronic stress pre- and postnatally.

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

2. Methods

2.1. Participants

Participants are mother-infant dyads from the ongoing longitudinal SMILEY study (Study of MIcrobiota and Lifestyle in the Early Years), which investigates lifestyle and well-being of pregnant women in relation to infant development (Epstein et al., 2024; Lustermans et al., 2024). Pregnant women were recruited through the Baby & Child Research Center's network of midwifery practices in the Netherlands and via social media, between December 2019 and April 2021. Inclusion criteria were ≥ 18 years of age, mastery of the Dutch language, singleton pregnancy, and pre-pregnancy BMI ≤ 30, and exclusion criteria were severe obstetric complications, and (severe) mental or physical health issues (i.e. mental health problems that require psychiatric treatment and/or medication). Inclusion criteria for the infant were born at ≥ 37 weeks of pregnancy, birth weight of ≥ 2500 g, and a 5-min Apgar score of ≥ 7. The study was approved by the Ethics Committee of the Faculty of Social Sciences of the Radboud University under the blanket research line 'pregnancy-4years Developmental Psychobiology Lab' (SW2017–1303–497), including two amendments (ECSW-2019–051 and ECSW2020–021). In total, 160 participants enrolled in the study by providing written informed consent. The current study contains data from both measurements in pregnancy (18 and 32 weeks of gestation) and four measurements after birth (2, 6 and 12 weeks and 8 months postpartum). Supplementary Fig. S1 shows a flow-chart of participant numbers at each assessment moment. Note that some participants were excluded after completing one or more measurement rounds. Table 1 shows demographic data for all infant-mother dyads that were included in the current study (i.e. that provided at least one maternal or infant stool sample).

2.2. Procedure

This study is based on multiple assessments that took place at 18 and 32 weeks of gestation and 2, 6 and 12 weeks and 8 months postpartum. These time points were chosen in line with the goal of the SMILEY study (to study the relation between maternal stress during and after pregnancy, the maternal microbiota during pregnancy and the development of the infant microbiota in early life). Maternal stool sample collection had to occur at later stages in pregnancy as there is significant transmission between the maternal and infant microbiota at and closely after birth. Simultaneously, we tried to keep the burden on participants as low as possible and therefore avoided collecting samples very close to birth. Infant samples were chosen at commonly used time points compared to our other studies as well as other infant microbiota studies from other research groups to allow comparing results between studies (Willemsen et al., 2024; Ou et al., 2024). At each wave, participants were asked to complete online questionnaires measuring maternal stress and potential covariates (e.g. demographics, maternal diet, antibiotic use). Also, at 18 and 32 weeks of gestation and 8 months postpartum, mothers collected a fecal sample of themselves. At 2, 6 and 12 weeks and 8 months postpartum, mothers collected a fecal sample of their infant. For each fecal sample, a paper questionnaire was completed by the mother to assess maternal and/or infant health and medication use. In addition, around 32 weeks of gestation and 8 weeks postpartum a researcher collected a maternal hair sample for hormone assessments during a laboratory visit.

Table 1
Descriptives of demographics, maternal stress variables and covariates (N = 151).

	Mean (SD) / N (%)	Median [Min, Max]	Missing
Demographics			
Maternal age (in years)	32.0 (3.60)	32.0 [22.4, 41.7]	0 (0.0 %)
Maternal education			
High	130 (86.1 %)		0 (0.0 %)
Low/medium	21 (13.9 %)		
Parity			
First born	74 (49.0 %)		0 (0.0 %)
Infant sex			
Boy	71 (47.0 %)		9 (6.0 %)
Girl	71 (47.0 %)		
Infant birthweight (in grams)	3540 (414)	3530 [2570,4840]	9 (6.0 %)
Infant gestational age at birth (weeks)	40.1 (1.09)	40.0 [37.9, 42.7]	9 (6.0 %)
Infant age 2 weeks postpartum (days)	15.6 (7.06)	14.0 [10.0, 70.0]	34 (22.5 %)
Infant age 6 weeks postpartum (days)	46.3 (7.11)	45.0 [14.0, 84.0]	32 (21.2 %)
Infant age 12 weeks postpartum (days)	86.7 (10.8)	87.0 [14.0, 150]	17 (11.3 %)
Infant age 32 weeks postpartum (days)	248 (23.7)	242 [204,320]	42 (27.8 %)
Maternal gestational week 18 weeks gestation	19.5 (1.43)	18.7 [17.3, 24.6]	0 (0 %)
Maternal gestational week 32 weeks gestation	31.3 (1.19)	31.3 [29.1, 35]	1 (0.7 %)
Maternal stress variables			
EPDS 18 weeks gestation	4.52 (4.09)	3.00 [0, 21.0]	0 (0.0 %)
EPDS 32 weeks gestation	5.31 (4.38)	5.00 [0, 24.0]	1 (0.7 %)
EPDS 32 weeks postpartum	4.64 (4.67)	4.00 [0, 29.0]	44 (29.1 %)
STAI 18 weeks gestation	32.5 (8.86)	31.0 [20.0, 57.0]	0 (0.0 %)
STAI 32 weeks gestation	32.9 (8.80)	32.0 [20.0, 75.0]	1 (0.7 %)
STAI 32 weeks postpartum	31.8 (9.50)	31.0 [20.0, 80.0]	44 (29.1 %)
PSS−10 18 weeks gestation	11.0 (5.51)	11.0 [2.00, 28.0]	0 (0.0 %)
PSS−10 32 weeks gestation	11.9 (5.42)	11.0 [0, 27.0]	1 (0.7 %)
PSS−10 32 weeks postpartum	11.0 (5.84)	10.0 [0, 33.0]	45 (29.8 %)
PRAQR2-B 18 weeks gestation	5.91 (2.68)	5.00 [3.00, 15.0]	0 (0.0 %)
PRAQR2-B 32 weeks gestation	6.09 (2.51)	6.00 [3.00, 14.0]	1 (0.7 %)
PRAQR2-H 18 weeks gestation	8.56 (3.07)	8.00 [4.00, 15.0]	0 (0.0 %)
PRAQR2-H 32 weeks gestation	7.95 (2.91)	8.00 [4.00, 19.0]	1 (0.7 %)
PSAS-RSF-C 2 weeks postpartum	19.6 (4.28)	19.0 [12.0, 35.0]	10 (6.6 %)
PSAS 6 weeks postpartum	80.7 (17.5)	78.0 [53.0, 157]	10 (6.6 %)
PSAS-RSF-C 12 weeks postpartum	19.2 (4.55)	18.0 [11.0, 39.0]	12 (7.9 %)
Cortisol (log) 6–15 weeks gestation	0.120 (1.11)	0.250 [−3.48, 4.02]	43 (28.5 %)
Cortisol (log) 15–23 weeks gestation	0.628 (0.944)	0.645 [−2.39, 5.18]	40 (26.5 %)
Cortisol (log) 23–32 weeks gestation	1.23 (0.630)	1.21 [−0.863, 4.12]	40 (26.5 %)
Cortisol (log) 4–8 weeks postpartum	0.977 (0.971)	1.15 [−2.41, 2.72]	64 (42.4 %)
Cortisone (log) 6–15 weeks gestation	0.918 (0.946)	1.01 [−2.03, 2.49]	40 (26.5 %)
Cortisone (log) 15–23 weeks gestation	1.69 (0.836)	1.83 [−1.27, 3.02]	40 (26.5 %)
Cortisone (log) 23–32 weeks gestation	2.64 (0.502)	2.65 [−0.232, 3.67]	40 (26.5 %)
Cortisone (log) 4–8 weeks postpartum	2.04 (0.818)	2.16 [−0.412, 3.67]	60 (39.7 %)
Log-ratio Cortisol/Cortisone 6–15 weeks gestation	−0.842 (1.06)	−0.892 [−5.59, 2.70]	43 (28.5 %)
Log-ratio Cortisol/Cortisone 15–23 weeks gestation	−1.06 (0.739)	−1.16 [−2.76, 2.93]	40 (26.5 %)
Log-ratio Cortisol/Cortisone 23–32 weeks gestation	−1.41 (0.510)	−1.39 [−4.20, 1.37]	40 (26.5 %)
Log-ratio Cortisol/Cortisone 4–8 weeks postpartum	−1.14 (0.615)	−1.10 [−3.19, 0.171]	64 (42.4 %)

(*continued on next page*)

Table 1 (*continued*)

	Mean (SD) / N (%)	Median [Min, Max]	Missing
Other Covariates			
Pre-pregnancy BMI	23.0 (2.75)	22.5 [17.3, 30.4]	1 (0.7 %)
Physical activity 18 weeks gestation	4.09 (2.03)	4.00 [0, 11.0]	0 (0.0 %)
Physical activity 32 weeks gestation	3.79 (2.06)	4.00 [0, 14.0]	1 (0.7 %)
Dutch Healthy Diet index 18 weeks gestation	89.5 (16.7)	90.4 [47.3, 127]	0 (0.0 %)
Dutch Healthy Diet index 32 weeks gestation	87.3 (17.6)	90.1 [38.8, 122]	1 (0.7 %)
Presence of pet(s)			
No	76 (50.3 %)		0 (0.0 %)
Yes	75 (49.7 %)		
Maternal antibiotics use sample M1			
No	130 (86.1 %)		19 (12.6 %)
Yes	2 (1.3 %)		
Maternal antibiotics use sample M2			
No	144 (95.4 %)		6 (4.0 %)
Yes	1 (0.7 %)		
Maternal antibiotics use sample M3			
No	95 (62.9 %)		56 (37.1 %)
Yes	0 (0.0 %)		
Delivery mode			
vaginal	125 (82.8 %)		9 (6.0 %)
c-section	17 (11.3 %)		
Infant antibiotics use sample I1			
No	138 (91.4 %)		10 (6.6 %)
Yes	3 (2.0 %)		
Infant antibiotics use sample I2			
No	134 (88.7 %)		16 (10.6 %)
Yes	1 (0.7 %)		
Infant antibiotics use sample I3			
No	139 (92.1 %)		10 (6.6 %)
Yes	2 (1.3 %)		
Infant antibiotics use sample I4			
No	137 (90.7 %)		14 (9.3 %)
Yes	0 (0 %)		
Infant feeding sample I1			
Breastmilk	91 (60.3 %)		10 (6.6 %)
Formula	6 (4.0 %)		
Mixed	44 (29.1 %)		
Infant feeding sample I2			
Breastmilk	103 (68.2 %)		10 (6.6 %)
Formula	13 (8.6 %)		
Mixed	25 (16.6 %)		
Infant feeding sample I3			
Breastmilk	96 (63.6 %)		10 (6.6 %)
Formula	20 (13.2 %)		
Mixed	25 (16.6 %)		
Infant feeding sample I4			
Breastmilk	28 (18.5 %)		43 (28.5 %)
Formula	27 (17.9 %)		
Mixed	53 (35.1 %)		

2.3. *Measures*

2.3.1. *Fecal samples*

Mothers were instructed to collect a stool sample of themselves and their infant at different time points at home. After collection, they were instructed to store the sample immediately in their own freezer, at ± −20 °C. Participants brought the samples to the lab in a cool box with ice when visiting our lab or a researcher picked up the samples at home with a mobile ± −18 °C freezer. In the lab, the samples were first stored in a −20 °C freezer and within a few weeks switched to a −80 °C freezer, where they were kept until being sent to Baseclear (BaseClear BV, The Netherlands) for analysis (see supplementary methods for microbial DNA extraction and taxonomic profiling). The number of collected stool samples per time point are shown in supplementary Table S1. Among the infants, their mothers collected all four samples for 92 infants, three samples for 43 infants, two samples for eight infants, and one sample for one infant. Among the mothers, 97 provided all samples followed by 50 who provided two and four who provided only one sample.

2.3.2. *Hair cortisol*

Maternal hair cortisol and cortisone were used as biomarkers of physiological stress, capturing cortisol and cortisone secretion over prolonged periods. Hair grows on average one centimeter per month, providing a timeline of hormone exposure based on segment length (Sauve et al., 2007). Cortisol, a hormone released in response to stress, is deposited in hair follicles from blood circulation as hair grows, making hair cortisol a cumulative indicator of systemic cortisol levels over time (e.g., months). This reflects chronic stress levels, as opposed to the immediate, short-term fluctuations often measured in saliva or blood samples (Sauve et al., 2007).

Cortisol can be transformed into the inactive form cortisone through the action of the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) (Tomlinson and Stewart, 2001). The ratio of cortisone to cortisol serves as an indicator of cortisol metabolism mediated by 11β-HSD2 (Dötsch et al., 2001). Furthermore, metabolites of cortisol generated by gut microbiota function as inhibitors of 11β-HSD2 (Morris and Brem, 2019).

During the laboratory visit, a trained researcher collected a hair sample of around 100–150 hairs from the posterior vertex of the mothers' heads, as close as possible to the scalp. For the sample taken at 32 weeks of pregnancy, three segments of two centimeters (measured from the scalp) were analyzed. For the sample taken at 8 weeks postpartum, a 1 cm segment was analyzed. These segments reflect different periods of hormonal exposure prenatally and postnatally, as shown in Table 2, which also introduces abbreviations representing these periods for simplicity.

Each sample was packed individually in aluminum foil, placed in an envelope, and stored until all samples were collected. They were then sent for analysis to the Dresden LAB Service, Germany. The hormone concentrations of the samples were assayed using column-switching Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS). Due to COVID-19 restrictions, several lab visits had to be canceled, resulting in a relatively high number of missing hair sample data (Table 1).

2.3.3. *Stress questionnaires*

To capture a comprehensive picture of perinatal mental health, we included both general and perinatal-specific measures. General questionnaires (EPDS, PSS-10, STAI) assess overall symptoms of depression, stress, and anxiety, while perinatal-specific questionnaires (PRAQR2, PSAS) target unique challenges associated with pregnancy and postpartum periods, such as pregnancy-related anxieties and postpartum concerns about infant care. This dual approach ensures a nuanced assessment of mental health that reflects both general and context-specific symptoms across the perinatal period. Below we provide more details about the individual questionnaires.

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

Table 2
Number of hair cortisol samples, corresponding period of cortisol and cortisone production and abbreviations used throughout the manuscript for these samples.

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

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

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

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

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

2.3.3.6. Maternal stress composite. We created a composite stress score (maternal stress; MS) from three highly correlated (Supplementary Figs. S2-S3) questionnaires (EPDS, PSS-10, STAI) to reduce the complexity of the analysis and reporting. However, to alleviate the issue of reproducibility due to the use of different stress measurements, we also ran all analyses with the single stress measurements and reported if results differed meaningfully from the composite score. The PRAQR2 (prenatal) and the PSAS (postnatal) were left out of the MS variable because otherwise the composite score would represent a different measure pre- and postnatally.

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

2.4. Statistical analysis

The statistical analyses were performed in R (version 4.3.1) (R Core Team et al., 2022). All analyses were preregistered (https://doi.org/10.17605/OSF.IO/CKE84) and the code is openly available with a permanent DOI (https://doi.org/10.5281/zenodo.11611111). We explored missing data using the *naniar* and the *BaylorEdPsych* (LittleMCAR test) packages. We could not reject our assumption that data is not "missing not at random" (MNAR). Accordingly, missing covariates were imputed using predictive mean matching (m = 50) (Kleinke, 2017) using the *mice* (v3.16.0) package (Buuren and Groothuis-Oudshoorn, 2011). Deviations of results from complete case analyses are reported. Several features of the gut microbiome were examined, including alpha diversity, beta diversity, species and phylum level relative abundances and volatility. For alpha diversity, we calculated Faith and Shannon diversity indices using the *mia* (v1.11.4) package (Ernst et al., 2022). For beta diversity, we used Aitchison distance (Euclidean distances of centered-log-ratio transformed abundances) and we report if results deviate if Bray-Curtis is used instead. Volatility was calculated as described by Bastiaanssen et al. (Bastiaanssen et al., 2021). Briefly, centered-log-ratio transformation was applied to microbial abundance data. After that we calculated the Euclidean distance (i.e. Aitchison distance) between intra-individual subsequent samples (e.g. for infants, we calculated the distances between the samples I2 – I1, I3 -I2 and I4 – I3 for each infant). The resulting volatility measure reflects the change in the microbiota over time. For differential abundance analyses we used *MaAsLin2* (Mallick et al., 2020), which has performed well according to multilple benchmark studies (Nearing et al., 2022; Pelto et al., 2024). Results were corrected for multiple testing using the Benjamini–Hochberg procedure (Benjamini and Hochberg, 1995). All adjusted p-values can be found in the column q in the supplementary tables.

We used Bayesian robust linear models to investigate associations between the maternal stress variables and alpha diversity and volatility using the *brms* (v2.21.0) package (Bürkner, 2018) with default priors and a student t distribution for the response variable. Furthermore, the response variables were scaled before model fitting to improve model convergence. Accordingly, visualizations of the regression line also use the scaled response variable. In these Bayesian models we reject the null hypothesis if the 95 % highest probability density interval (HDI) excludes zero for non-directional hypotheses or if more than 95 % of the posterior distribution is larger or smaller than zero, respectively, for directional hypotheses (indicated by $P(\beta \geq 0)$ in text). For beta diversity analyses we applied PERMANOVA with the *adonis2* function from the *vegan* (v2.6.4) package (Oksanen et al., 2022).

To provide valid and reproducible testing of our hypotheses, we employed the following algorithm: For each microbiota feature, we fitted longitudinal models as well as models that span over a single time point. In the longitudinal models we accounted for non-independence by specifying random intercepts. To decide which model type will be used for hypothesis testing, we calculated model fit (mean squared error) using leave-one-out cross validation (Vehtari et al., 2017). The estimate reported in text refers to the β coefficient of the best fitting model and we indicate if the association refers to a specific time point.

Furthermore, to determine the model structure, we created directed acyclic graphs (DAG) for the infant and mother samples separately (Supplementary Figs. S4-S5). We tested conditional independence and adjusted the DAG if necessary. DAGs graphically represent the knowledge and assumptions of the analyst. Some of the assumptions can be tested (testing conditional independence). Given these assumptions, the DAG dictates which covariates must be included to answer the research questions of interest (Cinelli et al., 2020). While some covariates need to be included to reduce bias (i.e. physical activity, parity and maternal age and education for the maternal samples and delivery mode, gestational age and parity for the infant samples), others can optionally be included (in this case pBMI for the maternal samples and maternal education, feeding mode, gestational age, and child sex for the infant samples). We again used leave-one-out cross-validation (Vehtari et al., 2017) to decide whether such optional covariates should be included to improve model fit.

3. Results

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

3.1.1. Alpha diversity

We did not detect changes in the alpha diversity during pregnancy. However, we observed that Faith diversity decreased 8 months postpartum compared to at 18 ($\beta = -1.85$, 95 % HDI = [-2.39; -1.34], $P(\beta \geq 0) = 0$) and 32 ($\beta = -1.56$, 95 % HDI = [-2.07; -1.04], $P(\beta \geq 0) = 0$) weeks of gestation on average. A similar trend was observed when looking at the difference in Shannon diversity between 8 months postpartum and 18 ($\beta = -0.08$, 95 % HDI = [-0.16; 0.01], $P(\beta \geq 0) = 0.03$) and 32 ($\beta = -0.06$, 95 % HDI = [-0.14; 0.03], $P(\beta \geq 0) = 0.10$) weeks of gestation (Fig. 1).

3.1.2. Beta diversity and differential abundance analysis

Similarly, we did not find evidence that beta diversity is different between 18 weeks and 32 weeks of gestation. Beta diversity at eight months postpartum differs significantly compared to 18 weeks ($p = 0.01$) and the 32 weeks ($p = 0.04$) of gestation. However, effect sizes were very small ($R^2 \leq .01$) and there is no visible separation from maternal samples within pregnancy and postpartum (Supplementary Fig. S6). Looking at individual species, we only found a small increase in *Bifidobacterium pseudocatenulatum* between 18 and 32 weeks of gestation (FDR =.039). We found many individual species to be differentially abundant (FDR $\leq$ 0.1) between 32 weeks of pregnancy and eight months postpartum (supplementary Table S2). Mainly we observed a decrease in *Akkermansia muciniphila* and *Anaerostipes hadrus* as well as an increase in *Fusicatenibacter saccharivorans* and an unclassified species of *Ruminococcaceae*. Lastly, in the beta diversity models, we observed an interaction between parity and pBMI, such that pBMI was only associated with microbiota composition in multiparous mothers.

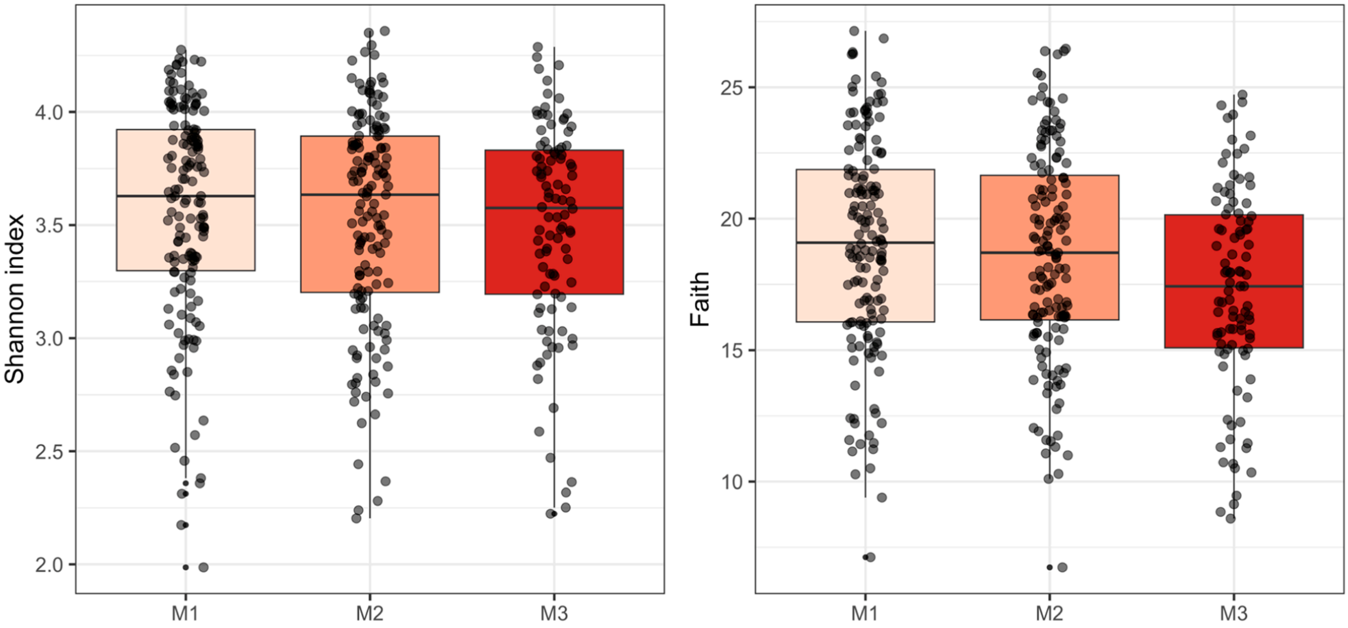

Fig. 1. Shannon and Faith alpha diversity across time points for maternal microbiota samples. Samples were taken at 18 (M1) and 32 (M2) weeks of pregnancy and at 8 months postpartum (M3). Alpha diversity dropped significantly from M1 and M2 to M3 for both indices.

3.2. How is maternal prenatal and postnatal stress related to the maternal gut microbiota during and after pregnancy?

3.2.1. Alpha diversity

Prenatally, our data indicated no or only a weak association between MS and the alpha diversity measures (Supplementary Fig. S7). However, we found a negative association between the PRAQR2-B (fear of giving birth) with both the Shannon ($\beta = -0.25$, 95 % HDI = [-0.39; -0.11], $P(\beta \geq 0) = 0$) and Faith index ($\beta = -0.12$, 95 % HDI = [-0.24; -0.01], $P(\beta \geq 0) = 0.02$) at 18 weeks but not 32 weeks of pregnancy (Fig. 2A).

Postnatally, we only detected an association between the maternal stress composite (MS) and alpha diversity when exploring non-linear associations by segmenting the predictor variable into low, medium and high stress. Specifically, we found that the difference in alpha diversity between the medium and high stress group varied closely around zero, indicating that these groups do not differ in alpha diversity. However, the low stress group had consistently lower alpha diversity than the medium and high stress groups. While the HDIs for most contrasts overlapped slightly with zero, the model indicated that Shannon index in low stress mothers is lower at 8 months postpartum ($\beta = -0.49$, 95 % HDI = [-0.89; -0.06], $P(\beta \geq 0) = 0.01$) compared to the medium stress group (Fig. 2B). The effect size for the contrast with the high stress

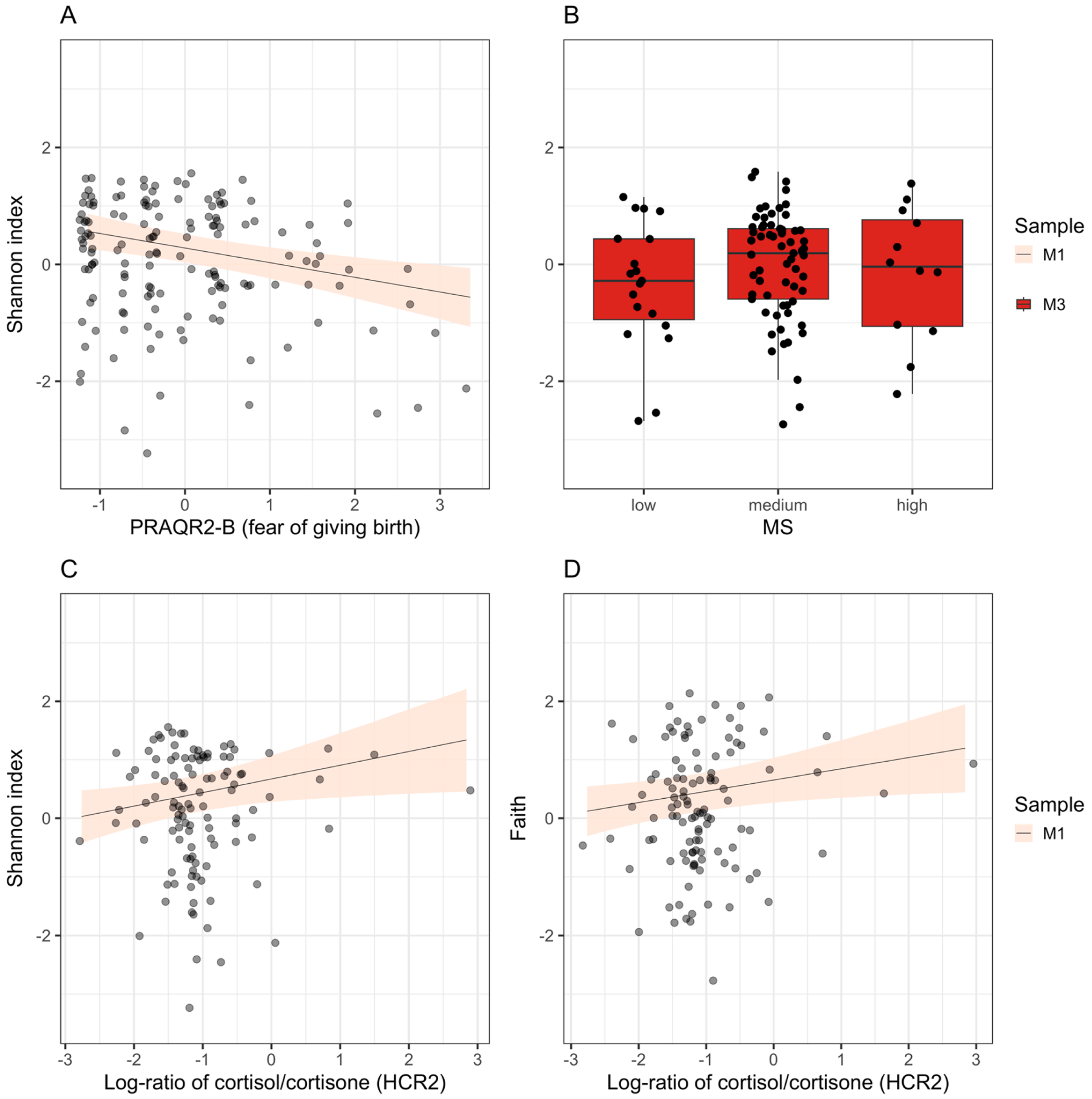

Fig. 2. Scaled Shannon alpha diversity for maternal microbiota samples obtained at 18 weeks of pregnancy (M1) and at 8 months postpartum (M3) plotted with maternal stress measures. For the plot with PRAQR2-B (A), we added random noise to avoid overplotting of the data points. The regression line and the colored shaded region illustrate our slope estimate including the 95 % credible interval. We used the median for all covariates to generate the posterior distribution for the slope parameter.

group is similar but the HDI is wider due to the smaller group sizes of low and high stress ($\beta = -0.46$, 95 % HDI = [-1.05; 0.13], $P(\beta \geq 0) = 0.06$)

For the time point at 8 months postpartum we also investigated whether the PSAS was related to alpha diversity and found that the directions of the estimates were in line with the association reported for the prenatal PRAQR2-B (fear of giving birth) for Shannon ($\beta = -0.10$, 95 % HDI = [-0.32; 0.12], $P(\beta \geq 0) = 0.19$) and Faith diversity ($\beta = -0.16$, 95 % HDI = [-0.36; 0.05], $P(\beta \geq 0) = 0.07$). However, we could not reject the null hypothesis for the PSAS.

Looking at the hair cortisol and cortisone concentrations, our models indicate no, or a weak association between most hormone measures and alpha diversity (Supplementary Fig. S7). However, the log-ratio of cortisol and cortisone (HCR2) was positively related to both alpha diversity measures at 18 weeks of gestation ($\beta = 0.23$, 95 % HDI = [0.03; 0.44], $P(\beta \geq 0) = 0.99$; Fig. 2C; $\beta = 0.19$, 95 % HDI = [0.02; 0.36], $P(\beta \geq 0) = 0.99$; Fig. 2D).

3.2.2. Beta diversity and differential abundance analysis

We did not find evidence that any of the stress variables was associated with beta diversity. In addition, there were only a few associations between the stress variables and individual species, including *Blautia* for the PSS-10 and *Bacteroides cellulosilyticus* for hair cortisone

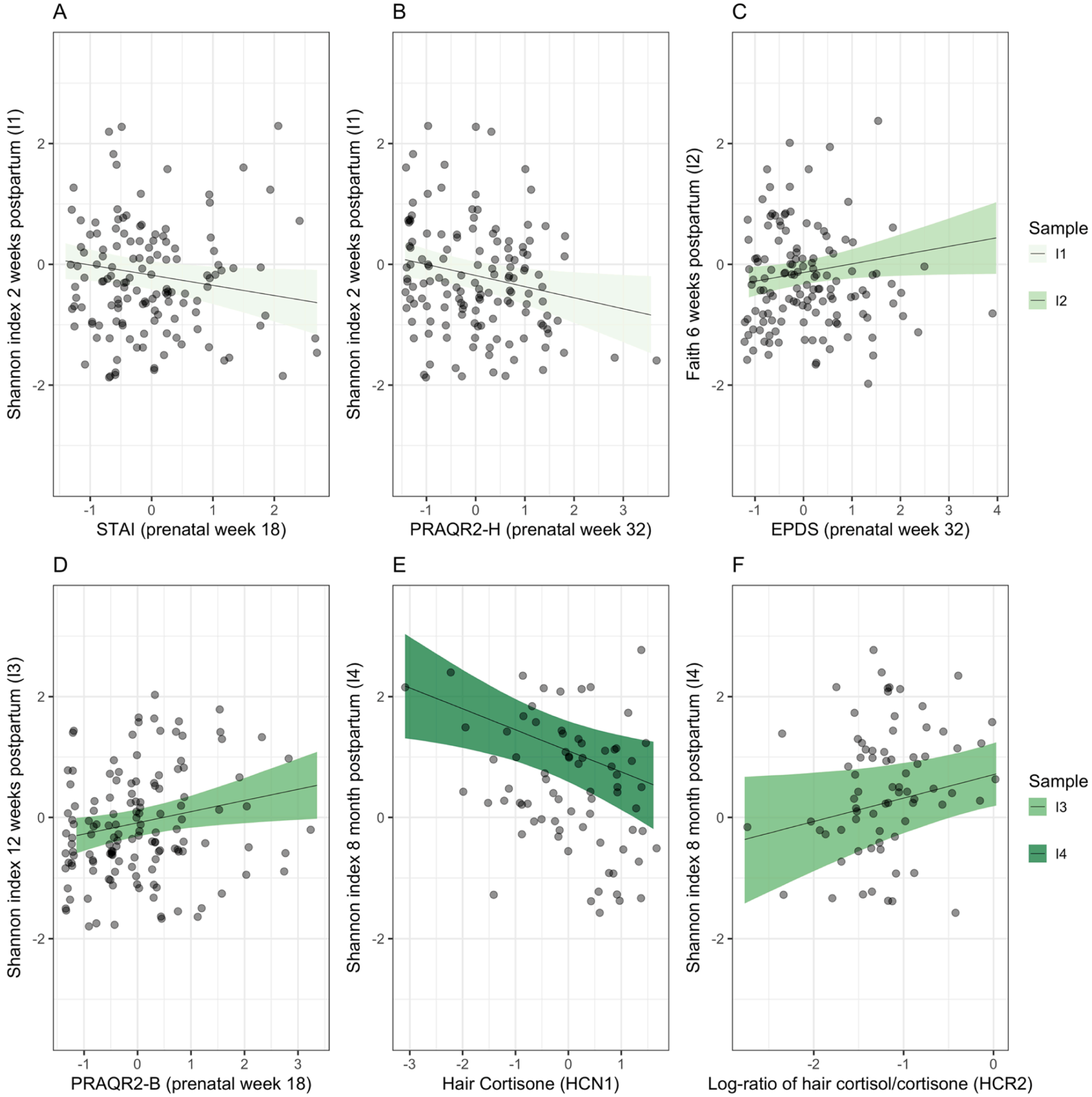

Fig. 3. Scaled alpha diversity of infant samples at different time points (green shades) plotted against different maternal stress measures. The regression lines and the colored shaded regions illustrate our slope estimates including their 95 % credible interval. We used the median for all covariates to generate the posterior distribution for the slope parameters. HCN1 = Hair cortisol reflecting maternal stress between week 6 and 15 of gestation, HCR2 = log-ratio of hair cortisol and cortisone reflecting stress between 15 and 23 weeks of gestation.

(HCN3). Supplementary tables S3-S4 list the coefficients of all the single species that were associated with any of the stress variables ($FDR \leq 0.1$) across all time points (supplementary Table S3) and per time point (supplementary Table S4).

3.2.3. *Volatility*

Lastly, MS was not associated with the maternal microbiota volatility between 18 and 32 weeks of pregnancy and 32 weeks of pregnancy and 8 months postpartum ($\beta = -0.02$, 95 % HDI = [-0.13; 0.10], $P(\beta \geq 0) = 0.38$), also not for any of the separate questionnaires (including PRAQR2 and PSAS) or hair cortisol/cortisone. The microbiota volatility from 32 weeks of pregnancy to 8 months postpartum was significantly higher than the volatility between the second and the third trimester ($\beta = 0.72$, 95 % HDI = [0.53; 0.90], $P(\beta \geq 0) = 1$) as would be expected due to the longer time interval between taking the samples.

3.3. *Does maternal pre- and postnatal stress associate with the infant gut microbiota development?*

3.3.1. *Alpha diversity*

While we did not find a significant association between the MS composite score measured at 18 weeks of pregnancy, the STAI was negatively associated with infant Shannon diversity at 2 weeks postpartum ($\beta = -0.17$, 95 % HDI = [-0.33; -0.01], $P(\beta \geq 0) = 0.02$). Also, the PRAQR2-H (fear of a handicapped child) at 32 weeks of pregnancy was negatively associated with Shannon diversity at 2 weeks postpartum ($\beta = -0.18$, 95 % HDI = [-0.34; -0.03], $P(\beta \geq 0) = 0.01$), see also Supplementary Fig. S8. In contrast, we found a positive association between MS ($\beta = 0.05$, 95 % HDI = [0.00; 0.11], $P(\beta \geq 0) = 0.97$) and specifically the EPDS ($\beta = 0.14$, 95 % HDI = [0.00; 0.28], $P(\beta \geq 0) = 0.98$) measured at 32 weeks of pregnancy and Faith diversity at 6 weeks postpartum and between PRAQR2-B (fear of giving birth) measured at 18 and 32 weeks of pregnancy and Shannon diversity at 12 weeks postpartum ($\beta = 0.18$, 95 % HDI = [0.03; 0.34], $P(\beta \geq 0) = 0.99$).

Furthermore, we identified a negative association between hair cortisone (HCN1) and Faith diversity at eight months postpartum ($\beta = -0.35$, 95 % HDI = [-0.61; -0.09], $P(\beta \geq 0) = 0.00$), with similar trends seen also for hair cortisone (HCN2) as well as when using Shannon diversity (see Supplementary Fig. S8). Also, the log-ratio of hair cortisol and cortisone (HCR2) was positively related to Shannon diversity at 8 months postpartum ($\beta = 0.39$, 95 % HDI = [0.02; 0.75], $P(\beta \geq 0) = 0.98$). Fig. 3 shows all associations where we rejected the null hypothesis.

Our data further indicated that there is no or only a weak association between postnatal maternal stress variables and alpha diversity at most time points in the infant samples (see Supplementary Fig. S9 for the corresponding β coefficients of all time points and stress measures). However, we found negative associations between Shannon diversity and EPDS ($\beta = -0.25$, 95 % HDI = [-0.46; -0.04], $P(\beta \geq 0) = 0.01$) and PSS-10 ($\beta = -0.22$, 95 % HDI = [-0.43; -0.01], $P(\beta \geq 0) = 0.02$) at 8 months postpartum. In line with that, comparing the groups of low and high MS we found that at 8 months postpartum, infants of low stress mothers had higher Shannon alpha diversity than infants of high stress mothers ($\beta = 0.96$, 95 % HDI = [0.14; 1.74], $P(\beta \geq 0) = 0.99$), see Fig. 4.

3.3.2. *Beta diversity and differential abundance analysis*

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

Several bacterial species were associated with the postnatal stress variables (supplementary Tables S5-S7 list all species and phyla related to any of the stress variables across time and at the separate time points ($FDR \leq 0.1$)). Among other associations, we found positive associations between *Bacteroides fragilis* and hair cortisol (HCS3 and HCS2). This association was present across all samples and at several stool sampling

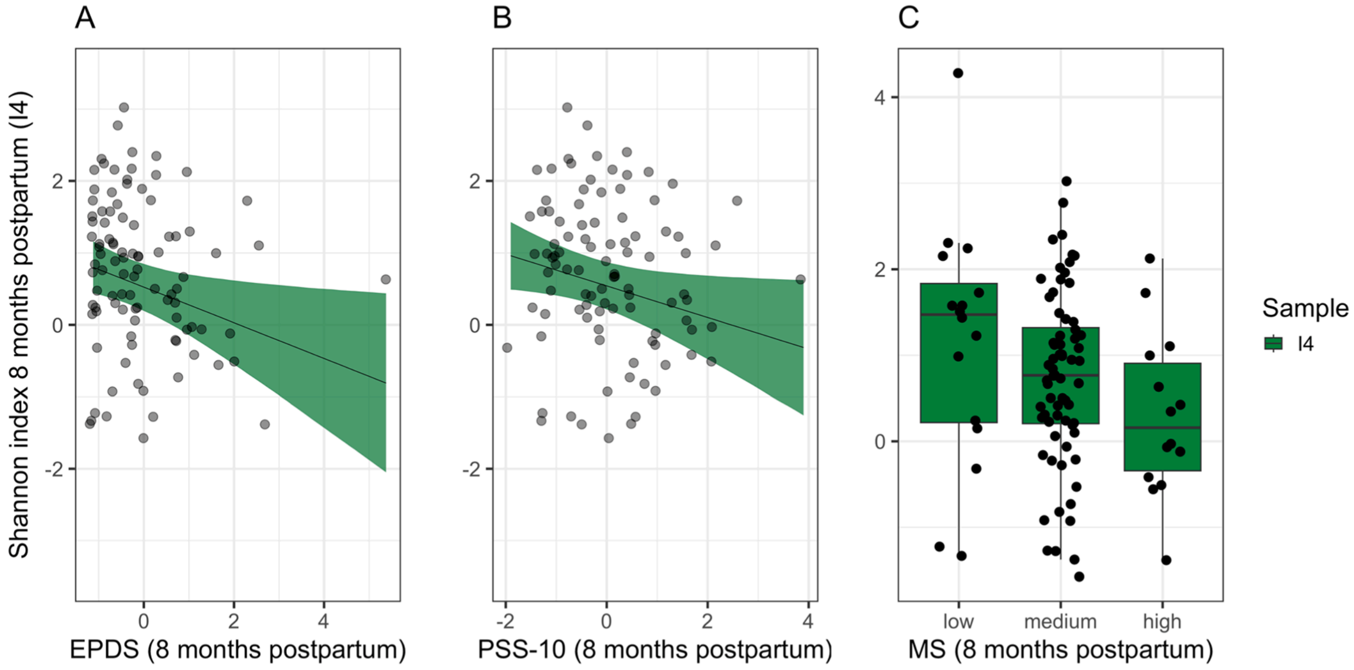

Fig. 4. Scaled Shannon alpha diversity of infant microbiota samples collected at 8 months postpartum plotted against different stress measures. The regression lines and the colored shaded regions illustrate our slope estimates including their 95 % credible interval. We used the median for all covariates to generate the posterior distribution for the slope parameters. MS = Maternal stress composite score.

time points individually. It became particularly strong when looking at the log-ratio of hair cortisol and cortisone (HCR3) and the infant sample at 6 weeks postpartum. Furthermore, we observed negative associations between *Flavonifractor plautii* and *Bacteroides ylanisolvens* and hair cortisol (HCS3) as well as *Bacteroides thetaiotaomicron* and hair cortisol (HCS2). We did not find evidence for a positive association between any of the stress variables and Proteobacteria or for a negative association with Lactobacilli or *Bifidobacterium*. Instead, the postnatal stress measures MS, EPDS and PSS-10 were positively associated with Firmicutes across all time points.

3.3.3. Volatility

We found a positive association between MS at two weeks postpartum and volatility between 2 and 6 weeks postpartum ($\beta = 0.18$, 95 % HDI = [0.05; 0.31], $P(\beta \geq 0) = 1.00$), see Fig. 5A. We found the same for all questionnaires individually. Also, the PSS-10 at 6 weeks postpartum was positively associated with volatility between 6 and 12 weeks postpartum, see Fig. 5B. Lastly, we found that the log-ratio of hair cortisol and cortisone (HCR2) was positively related to volatility between 12 weeks and eight months postpartum ($\beta = 0.29$, 95 % HDI = [-0.01; 0.57], $P(\beta \geq 0) = 0.97$), see Fig. 5C.

4. Discussion

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

4.1. Maternal microbiota during and after pregnancy

Our data supports previous findings indicating that the maternal microbiota does not undergo major changes between the second and the third trimester (DiGiulio et al., 2015; Yang et al., 2020). However, we did observe changes from pregnancy to eight months postpartum. Specifically, we detected a significant decrease in Faith alpha diversity at eight months postpartum as compared to the first and the second trimester. While we also observed that beta diversity was significantly different at eight months postpartum compared to the second trimester of pregnancy, the effect size of this finding does not warrant interpreting it as a major change. Nevertheless, the decrease in Faith alpha diversity as well as observed decreases in, for example, *Akkermansia muciniphila*, *Anaerostipes hadrus*, *Streptococcus thermophilus* and *Streptococcus salivarius* as well as increases in, for example, *Fusicatenibacter saccharivorans*, *Ruminococcaceae* and *Clostridiales bacterium* indicate that the microbiota postpartum differs significantly from the prenatal microbiota. The observed changes could be due to physiological changes induced by shifting from the pregnant to the non-pregnant state and to the induction of breastfeeding but could also be due to lifestyle changes related to the postpartum period, which is characterized by alterations in sleep, activity, diet, and other behavioral changes. While we collected prenatal data on lifestyle factors such as diet, physical activity, and sleep, we did not collect these variables at eight months postpartum, limiting our analysis in that regard. To further study the potential changes induced by pregnancy or the shift into the non-pregnant state, it would be interesting to include more follow up samples as well as samples from before conception and to collect data about lifestyle changes associated with the pregnancy and postpartum period. Moreover, we observed that pre-pregnancy body mass index (pBMI) was associated with microbiota composition in multiparous but not primiparous women across time. This is in contrast with previous research that found an association between pBMI and changes in the gut microbiota throughout pregnancy only in primiparous women (Kennedy et al., 2022). However, our data and analyses are not comparable to those of the previous study as they collected samples also in the first trimester

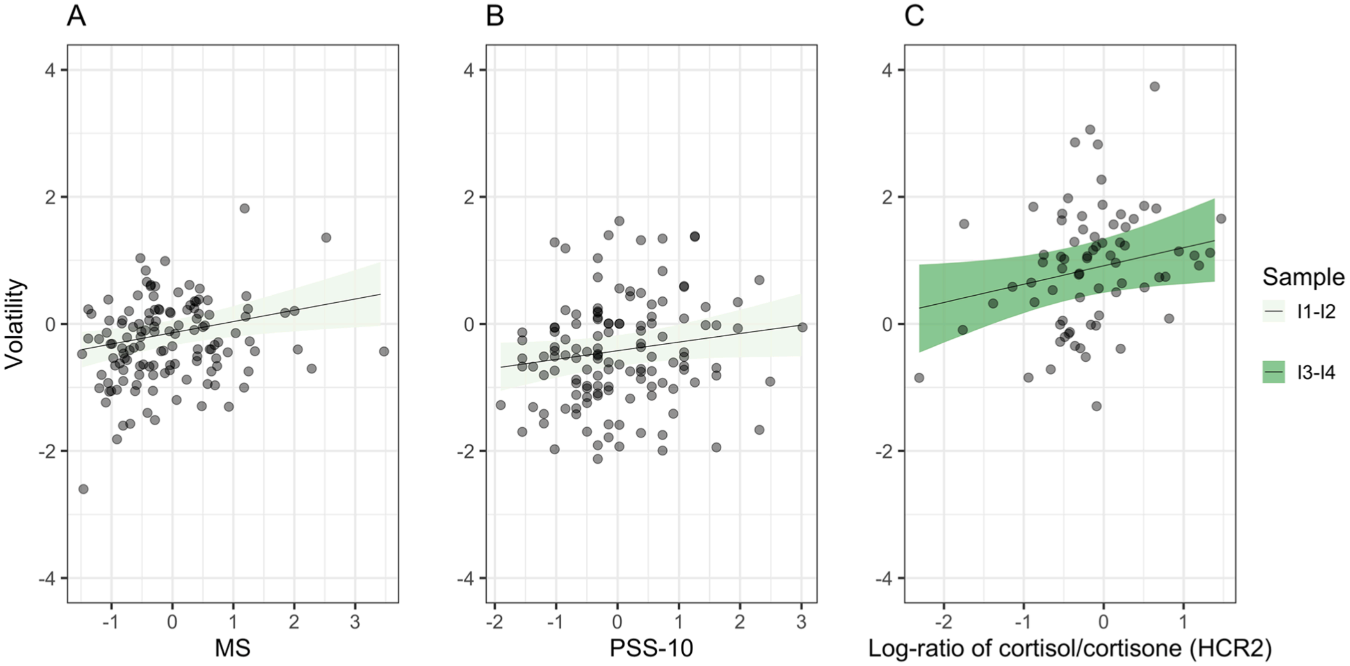

Fig. 5. Scaled infant microbiota volatility at different time point intervals (green shades) and the postnatal maternal stress composite score (MS) (A), PSS-10 (B) and log-ratio of hair cortisol and cortisone (HCR2) (C). The regression line and the colored shaded region illustrate our slope estimate including the 95 % credible interval. We used the median for all covariates to generate the posterior distribution for the slope parameter. HCR2 = log-ratio of hair cortisol and cortisone reflecting maternal stress between 15 and 23 weeks of gestation.

and included gestational weight gain in their statistical models. The potential interaction between parity and pBMI in relation to gut microbiota trajectories in the perinatal period warrants further research.

4.2. Maternal prenatal and postnatal stress and the maternal microbiota

We found limited evidence for an association between MS and alpha diversity. Namely, when comparing the low MS group with the medium or high MS group we found that only at eight months postpartum mothers reporting low levels of stress had slightly lower Shannon diversity than mothers reporting medium or high levels of stress. Interestingly, we did find that the PRAQR2-B (fear of giving birth) measured at 18 weeks pregnancy was negatively associated with alpha diversity at 18 weeks of pregnancy with both Shannon and Faith diversity. Similar results were observed at 32 weeks of pregnancy and therefore this association appeared to be more robust compared to the findings related to MS. The fact that we find different associations for MS and PRAQR2-B can be explained because of the limited overlap between those variables (Supplementary Figs. S2-S3) and the different time points the microbiota samples were obtained. Indeed, the PRAQR2-B (fear of giving birth) subscale assesses very specific prenatal anxieties whereas the MS assesses stress in general. Furthermore, we detected two associations between measures of maternal stress with individual microbial species. First, we found a positive association between the PSS-10 and *Blautia* throughout all time points. Second, we found a negative association between hair cortisone (HCN3) and *Bacteroides cellulosilyticus* at 32 weeks of pregnancy. Lastly, we could not reject our null hypothesis that maternal stress is not related to maternal microbiota volatility. Possible explanations include that there is indeed no association between maternal stress and microbiota volatility during pregnancy. Alternatively, it is possible that the stress experienced in our sample of highly educated women, was not severe enough to induce significant changes in the more stable adult maternal microbiota. It is also possible that increased volatility could be observed at earlier stages of pregnancy such as from the first to the second trimester. Altogether these findings provide evidence that maternal stress, specifically fear of giving birth, is associated with the maternal gut microbiota at different time points in the perinatal period.

4.3. Maternal prenatal and postnatal stress and the infant microbiota

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

Next to the associations with alpha diversity, we found that the PRAQR2-B (fear of giving birth), hair cortisol (HCS3) and hair cortisone (HCN3) were related to infant beta diversity at different time points. We also identified many individual species related to our stress measurements across time and at the individual time points (supplementary Tables S5-S7). Most notably, we found a positive association between infant *Bacteroides fragilis* and maternal hair cortisol in the second and third trimester (HCS2 and HCS3) that was present across time and at the individual time points. *Bacteroides fragilis* has been associated with reduced levels of aggressive behavior, emotional reactivity, externalizing behavior, sadness, and impulsivity as well as an increase in inhibitory control and lower reported incidents of family turmoil (Flannery et al., 2020). Next to that it was associated with lower reported incidents of family turmoil (Flannery et al., 2020). Also, animal studies found that *Bacteroides fragilis* has protective effects against pathogen-induced gut inflammation and an autism-spectrum-disorder phenotype induced by maternal immune activation (Hsiao et al., 2013). This means that our results are either in contrast with previous findings or, possibly, that the association between stress and *Bacteroides fragilis* is not linear, such that, e.g., while moderate levels of stress may be positively associated with it, more extreme levels of stress may lead to a relative decrease of this bacterium. Furthermore, we found a negative association between *Flavonifractor plautii* at week six and maternal stress as measured by hair cortisol in the third trimester (HCS3). *Flavonifractor plautii* has been negatively associated with the development of asthma later in life (Stokholm et al., 2018) and was able to strongly suppress Th2 immune responses in mice (Ogita et al., 2020). Furthermore, in line with our findings, *Flavonifractor plautii* has been previously reported to be negatively associated with a maternal stress composite score at six weeks postpartum (Dutton et al., 2023) (which was based on STAI, EPDS, PSS and scales assessing several types of trauma and PTSD). We also reported previously that *Flavonifractor* was increased in a group of infants that received a destressing skin-to-skin contact intervention in the first five weeks of their lives (Eckermann et al., 2024). Thus, our findings are in line with the literature and suggest that maternal physiological stress is related to a relative decrease in infant *Flavonifractor* at around six weeks postpartum. Lastly, in contrast to previous literature (Dutton et al., 2023; Galley et al., 2023; Jahnke et al., 2021; Mepham et al., 2023; Zijlmans et al., 2015), we did not find evidence for a positive association between prenatal stress and Proteobacteria or for a negative association with *Bifidobacterium*.

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

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

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

4.4. Strengths and limitations

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

5. Conclusion

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

Author note

The authors made the following contributions. Henrik Eckermann: Original Draft Preparation, Data Analysis, Review & Editing; Hellen Lustermans: Conceptualization, Data Collection, Review & Editing; Katariina Parnanen: Metagenomic taxonomic profiling, Review & Editing; Leo Lahti: Supervision, Review & Editing; Carolina de Weerth: Conceptualization, Review & Editing, Supervision.

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CRediT authorship contribution statement

Katariina Parnanen: Writing – review & editing, Formal analysis, Data curation. **Hellen Lustermans:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Henrik Andreas Eckermann:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Carolina de Weerth:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization. **Leo Lahti:** Writing – review & editing, Writing – original draft, Supervision, Software.

Declaration of Competing Interest

The author Carolina de Weerth is an Editorial Board Member for Psychneuroendocrinology and was not involved in the editorial review or the decision to publish this article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.psyneuen.2024.107273.

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