



Maternal prenatal stress is associated with the infant intestinal microbiota



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Summary Maternal prenatal stress has been often associated with infant physical development and health, as well as psychological functioning and behavior. However, the mechanisms underlying these relations remain elusive. The goal of the present study was to prospectively investigate the development of the intestinal microbiota as a potential pathway linking maternal prenatal stress and infant health. The development of the infant intestinal microbiota was followed over the first 110 days after birth in a healthy cohort of 56 vaginally born Dutch infants. Additionally, the relation between infant intestinal microbiota and gastrointestinal and allergic symptoms was examined. Results showed that maternal prenatal stress, i.e., either reported stress or elevated basal maternal salivary cortisol concentrations or both, was strongly and persistently associated with the infants' microbiota composition as determined by a phylogenetic microarray. Infants of mothers with high cumulative stress (i.e., high reported stress and high cortisol concentrations) during pregnancy had significantly higher relative abundances of Proteobacterial groups known to contain pathogens (related to *Escherichia*, *Serratia*, and *Enterobacter*), and lower relative abundances of lactic acid bacteria (i.e., *Lactobacillus*, *Lactococcus*, *Aerococcus*) and Bifidobacteria, altogether characteristics of a potentially increased level of inflammation. Furthermore, this aberrant colonization pattern was related to more maternally reported infant gastrointestinal symptoms and allergic reactions. In conclusion, clear links were found between maternal prenatal stress and the infant intestinal microbiota and health. Although causality cannot be concluded, the results suggest a possible mechanism by

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which maternal prenatal stress influences the offspring development. These results suggest a potential for bacterial interventions to enhance offspring health and development in pregnant women with stress.

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1. Introduction

Although the underlying mechanisms remain unclear, an increasing number of studies link maternal prenatal stress to infant physical development and health, and psychological functioning and behavior. Stress during pregnancy predisposes to premature birth and low birth weight (Mulder et al., 2002; Beydoun and Saftlas, 2008), eczema (Sausenthaler et al., 2009), asthma (Cookson et al., 2009), and respiratory, general and skin illnesses (Beijers et al., 2010). Regarding psychological functioning and behavior, children of prenatally stressed mothers often show more impulsivity, anxiety problems, ADHD symptoms, and worse cognitive and psychomotor development (Beydoun and Saftlas, 2008). Recently, the development of the infant gut microbiota has been put forward as a possible factor underlying the links between maternal prenatal stress and infant development (Beijers et al., 2014). Rhesus monkey infants whose mothers had experienced stress during late pregnancy, in the form of repeated exposure to an acoustic startle, had lower levels of Bifidobacteria and Lactobacilli and more diarrheic symptoms than the infants of non-stressed mothers (Bailey et al., 2004). Also, in adult mice a social stressor (i.e., social disruption) provoked a decrease in the relative abundance of the genus *Bacteroides* together with an increase in the relative abundance of the genus *Clostridium* (Bailey et al., 2011). The goal of the present study is to investigate the relation between maternal prenatal stress (i.e., reported stress and cortisol concentrations) and the development of infant intestinal microbiota and health in the first 110 days of life in humans.

The intestinal microbiota are known to play an important role in the maturation of an infant's gastro-intestinal tract, immunity, metabolism, as well as the hypothalamic-pituitary-adrenal system (Sudo et al., 2004; Dimmitt et al., 2010; Bäckhed, 2011). An aberrant acquisition of intestinal bacteria or a reduced complexity of the microbiota may delay immune maturation or alter the development of the immune system and stress responses (Sudo et al., 2004; Adlerberth and Wold, 2009; Sekirov et al., 2010). Bacterial colonization of the infant gut is thought to begin in utero (Gosalbes et al., 2013), and to accelerate dramatically during and after delivery, and during the first months of life (Palmer et al., 2007; Fallani et al., 2010). Microbes from the mother and, to a lesser extent, of the environment are thought to be the first colonizers of the infant's gut (Tannock et al., 1990; Gosalbes et al., 2013). After the initial establishment of the intestinal microbiota during the first year of life, the microbiota begins to stabilize to a unique individual composition, continuing to develop gradually throughout childhood and adolescence. To what extent the early colonization dictates later development and finally the stable adult composition, is currently unknown. Due to the intimate interaction between the developing intestinal

microbiota and the immune system, the early-life development of the intestinal microbiota may have long-lasting consequences (Bäckhed, 2011).

Distortions in the intestinal microbiota are associated with a wide range of diseases, including the risk of diarrheal illness, food allergy, inflammatory diseases (atopic diseases and inflammatory bowel disease), irritable bowel syndrome, obesity, and diabetes (Sekirov et al., 2010). Furthermore, as is the case with irritable bowel syndrome, gut-related diseases can develop or worsen during stressful periods (O'Mahony et al., 2009; De Palma et al., 2014). This may be due to the bidirectional communication between the central nervous system (CNS) and the gut (brain-gut axis; Dinan and Cryan, 2012), where both the autonomic nervous system (ANS) and the hypothalamic pituitary adrenal (HPA) axis play important roles (Rhee et al., 2009). When the HPA axis is activated in reaction to stress, cortisol is produced as an end product. In rats, experimentally increased cortisone levels in pregnant females resulted in lower levels of total bacteria and gram negatives in the intestine of the pups (Schiffman et al., 1993). This suggests that cortisone may influence the maternal microbiota, and thereby the transmission of bacteria to offspring. In humans it is as yet unknown if maternal prenatal psychological stress and cortisol concentrations are related to the infant gut microbiota.

The goal of the present human study is to prospectively investigate the relation between maternal prenatal stress and the development of infant intestinal microbiota and health in the first 110 days of life. A limitation of the Rhesus monkey study of Bailey et al. (2004) is that the intestinal microbiota analyses were carried out with traditional culturing approaches and were not able to show the more complex microbiota signatures. The present study avoids this limitation by using a high-throughput phylogenetic microarray (Rajilic-Stojanovic et al., 2009).

2. Methods

2.1. Participants and procedure

This project is part of an ongoing longitudinal study in which 192 children are followed from the third trimester of pregnancy on. Infants were healthy, born at full term (≥ 37 weeks) and had a 5-min APGAR score ≥ 7 . Inclusion criteria were an uncomplicated, singleton pregnancy, clear understanding of the Dutch language, no drug use and no current physical health problems (see Beijers et al., 2010 or Tollenaar et al., 2011 for more details). All mothers gave written informed consent, and the study was approved by the Ethical Committee of the Faculty of Social Sciences, Radboud University Nijmegen (ECG/AvdK/07.563).

Fecal samples at 9 time points were available for investigating the development of the infant intestinal microbiota

from birth until ± 110 days of life (see [De Weerth et al., 2013](#) for more details). For the current study, due to financial constraints, we made a selection of participants and fecal samples. Five out of the nine fecal sampling time points were selected. The mean (SD) of the postnatal collection days of these samples were: 6.7 (0.7), 12.5 (4.0), 24.8 (8.9), 83.8 (19.4), and 112.3 (15.4). Participants were selected based on reported prenatal maternal stress and anxiety. For five variables that measured maternal reported prenatal stress and anxiety, a median score was computed. Participants who scored above the median on 4 or 5 variables were selected and categorized as the 'high maternal prenatal stress group' ($N = 48$, 25% of the total sample). Participants who scored above the median on a maximum of 2 variables were selected and categorized as the 'low maternal prenatal stress group' ($N = 39$, 20.3% of the total sample). Next, participants were excluded if the infant was missing three or more out of the five fecal samples (in both groups 10 participants were excluded for this reason). Children born with cesarean delivery ($N = 4$, all in high maternal prenatal stress group) were also excluded. Seven participants were excluded because the microarray did not pass the quality check of high reproducibility ($>97\%$ Pearson's coefficient between two HiChip analyses) ([Rajilic-Stojanovic et al., 2009](#)). This resulted in a final group of 56 participants (low prenatal stress $N = 28$, high prenatal stress $N = 28$). The groups were significantly different on all of the five prenatal stress and anxiety and three postnatal stress and anxiety questionnaires, but did not differ on the maternal prenatal cortisol variables or the demographic characteristics (gender, birth weight, 5 min APGAR score, maternal age, prenatal smoking, prenatal alcohol consumption, gestational age, infant use of antibiotics, duration of breastfeeding).

2.2. Measurements

Mothers filled out questionnaires on demographics, lifestyle (i.e., alcohol and smoking), and stress and anxiety in the third trimester of pregnancy ($M = 35.29$ weeks, $SD = 1.22$). The five stress and anxiety variables that were used in the present study are described below.

2.2.1. General anxiety

A Dutch translation of the state anxiety subscale of the State-Trait Anxiety Inventory (STAI; [van der Ploeg et al., 1981](#); [Spielberger, 1983](#), α between 0.90 and 0.96) was used to measure maternal prenatal anxiety. This subscale consists of 20 items scored on a 4-point scale. An example item is: 'I feel calm'. Higher scores indicated more anxiety.

2.2.2. Pregnancy-related anxiety

The pregnancy-related anxieties questionnaire-revised (PRAQ-R; [Van den Bergh, 1990](#)) was used to measure pregnancy-related anxiety. Two subscales of this questionnaire scored on a 5-point scale, were used for analysis: PRAQ1. Fear of giving birth (three items; α between 0.79 and 0.83), PRAQ2. Fear of bearing a handicapped child (four items; α between 0.87 and 0.88). A higher score indicated more experienced anxiety.

2.2.3. Daily hassles

A Dutch questionnaire (Alledaagse Problemen Lijst, APL, [Vingerhoets et al. \(1989\)](#), with test-retest reliabilities between 0.76 and 0.87) was used to measure the occurrence and intensity of daily hassles. The questionnaire consists of 49 items for which participants have to indicate whether or not the daily hassle has occurred in the past 2 months and how much it bothered them on a 4-point scale. A mean valence rating of the 49 items was computed by dividing the sum of total (negative) valence divided by the frequency of daily hassles. A higher value indicated more negativity.

2.2.4. Pregnancy-related daily hassles

To measure pregnancy-related daily hassles, a Dutch translation of the Pregnancy Experience Scale (PES) was used ([DiPietro et al., 2004](#), α between 0.91 and 0.95). This questionnaire consists of 43 items and measures maternal appraisal of pregnancy related daily hassles. Participants have to rate pregnancy-related experiences (e.g., body changes during pregnancy) on two 5 point scales: one indicates the extent to which the experience was felt as a hassle, and the other indicates the extent to which the experience was felt as an uplift. The ratio of these ratings was computed by the sum of intensities of hassles divided by the sum of intensities of uplifts. A higher value indicated a larger negative emotional valence toward pregnancy.

2.2.5. Maternal prenatal cortisol

Around week 37 of pregnancy ($M = 37.37$ weeks, $SD = 1.68$) mothers collected 5 saliva samples on 2 consecutive days to determine cortisol concentrations. Samples were taken at awakening (day 1 = 07:48, $SD = 00:56$, day 2 = 07:56, $SD = 01:02$), 30 min after awakening, at 12:00, 16:00 and 21:00 h. The cortisol concentrations at each sampling time strongly correlated between the days. To obtain one cortisol concentration per sampling time, the mean raw cortisol concentrations over the two days were calculated (for more details see [Tollenaar et al., 2011](#)). To determine the total cortisol secretion during the day, the Area Under the Curve with respect to the Ground (AUCg) was calculated over these means ([Pruessner et al., 2003](#)).

2.2.6. Infant gastrointestinal and allergic symptoms

During the first three months, mothers were asked to report on infant gastrointestinal symptoms and allergic reactions on a monthly basis, by means of a semi-structured interview (for more details see [Beijers et al., 2010](#)). Mothers were asked if their infant had shown health symptoms during the past month, using yes-or-no items. Note that mothers reported about allergic reactions and not on diagnoses of allergies by physicians. Thereafter, the reported symptoms were coded using the International Classification of Primary Care (ICPC; [Lamberts and Wood, 1987](#)). Following the ICPC, the gastrointestinal symptoms reported by the mothers included: vomiting (D10), diarrhea (D11), constipation (D12), rectal bleeding (D16), gastroenteritis presumed infection (D73), disease digestive system, other (D99).

2.2.7. Confounders

Maternal age and educational level, parity, and infant birth weight were not associated with prenatal reported stress

or cortisol concentrations and were therefore not included in the analyses. Potential confounders that were included in the analyses were breastfeeding during the study period, and postnatal maternal stress and anxiety when the infant was 3 months old. A significant proportion of the infants were not breastfed at each time point: 20% at age 7 days, 21% at 14 days, 30% at 28 days, 39% at 80 days, and 44% at 110 days. Therefore, breastfeeding was included as an explanatory variable in all models. Postnatal maternal stress and anxiety was assessed by means of the perceived stress scale (Cohen et al., 1983), the state anxiety subscale of the STAI (van der Ploeg et al., 1981; Spielberger, 1983) and the daily hassles questionnaire (Dutch questionnaire Alledaagse Problemen Lijst, APL; Vingerhoets et al., 1989). Antibiotic use was very rare; at each time point of fecal sampling, maximally one infant had been given antibiotics, and this was found not to confound the analyses, so antibiotic use was not controlled for.

2.2.8. Collection of fecal samples and microbiota analysis

Parents collected the fecal samples of their infant at home and stored them at -20°C . The samples were transported in coolers with freezing cartridges or dry ice and stored at -20°C , and later at -80°C , until further processing at the Microbiology Laboratory of Wageningen University. DNA was extracted with the repeated bead-beating method as described by Salonen et al. (2010). The microbiota composition was analyzed twice with the phylogenetic microarray, the Human Intestinal Tract Chip (HITChip) (Rajilic-Stojanovic et al., 2009), which detects and quantifies over 1000 species-level phylotypes, with a specific focus on bacteria residing in the human intestine. The microarray consists of oligonucleotide probes for two hyper-variable regions (V1 and V6) on the 16S rRNA gene, allowing the identification, quantification and phylogenetic positioning of cultured and uncultured bacterial phylotypes. The DNA was amplified with PCR using the universal bacterial primers T7prom-Bact-27-for and Uni-1492-rev, and transcribed to RNA, which was labelled and hybridized on the microarray. The signal intensities of the oligonucleotide probes were translated into abundances of 1038 species-level phylotypes, 130 genus level-taxa, and 23 phylum-level taxa and clostridium clusters using the fRPA pre-processing algorithm (Lahti et al., 2013). The microarray has been shown to correspond to ca. 200 000 sequencing reads (Claesson et al., 2009). The microbiota data were transformed into relative abundances by dividing the signal intensities of each taxon by the total intensity of the sample.

2.3. Statistical analyses

All statistical analyses were conducted with R (R Development Core Team, 2012), using the libraries nlme (Pinheiro et al., 2011) and vegan (Oksanen et al., 2013). To identify the most important maternal stress indicators to be utilized in further analyses, we first modeled the (log-transformed) relative abundance of each genus-level bacterial group, with each of the stress indicators separately as an explanatory variable, and controlling for breastfeeding and postnatal maternal stress. The stress indicators used

were: the 5 separate questionnaire variables (raw scores), the sum of the 0–1 questionnaire scores (i.e., below or above the median) for the 5 prenatal stress variables (values ranging from 0 to 5), and the 5 separate cortisol concentrations (raw values) and the AUC_G. This was done separately for each sampling age, and for all sampling ages together (using linear mixed effects models, with the individual as the random factor, with the function lme in the package nlme in R). Based on these models, the number of genus-level groups significantly associated at any sampling age with each stress indicator was calculated (Fig. 1). For further analyses, we selected the two stress indicators with the strongest association with the microbiota: the sum of the 0–1 questionnaire scores, and the 12:00 cortisol concentration.

We estimated the amount of inter-individual variation in the total genus-level microbiota attributable to prenatal stress using permutational multivariate analysis of variance (Oksanen et al., 2013) (Fig. 2), controlling for breastfeeding and postnatal maternal stress by including these variables in the model. Including the relative abundances of all bacterial groups significantly associated with either stress indicator at any time point (101 genera, 78% of the microbiota), we performed a principal coordinates analysis (PCoA), using Manhattan distances (a measure of beta diversity). Based on the species-level data, we calculated the microbial alpha diversity as the inverse Simpson diversity index, over all species and within phyla.

3. Results

3.1. Correlations between prenatal stress and anxiety variables

None of the reported stress and anxiety variables was significantly correlated with the cortisol variables, indicating that cortisol and reported stress represent independent measures of stress. The questionnaire-based stress variables were weakly to moderately correlated (r ranging from 0.22 to 0.74), and the cortisol concentrations measured at different times of day were weakly to strongly correlated with each other (r ranging from 0.15 to 0.87).

3.2. Comparison of the different stress indicators

Based on the number of significantly associated bacteria, the sum of the stress questionnaire scores and the cortisol concentration measured at noon were most strongly associated with the infant microbiota (Fig. 1). Of the individual questionnaire scores, the fear of a handicapped child (PRAQ2) had the strongest association with the infant microbiota, but not as strong as the sum of the questionnaire scores. We therefore selected the sum of questionnaire scores and the 12:00 cortisol concentration as measures of reported stress and cortisol concentration for further analyses. There was a weak positive correlation between the reported stress and the cortisol concentration ($r=0.25$, $p<0.001$). Both indicators were significantly associated with the relative abundances of over 60% of the bacterial genus-level groups at one or more time points during the first four months of the infants' life. A major part (78%) of the microbial groups

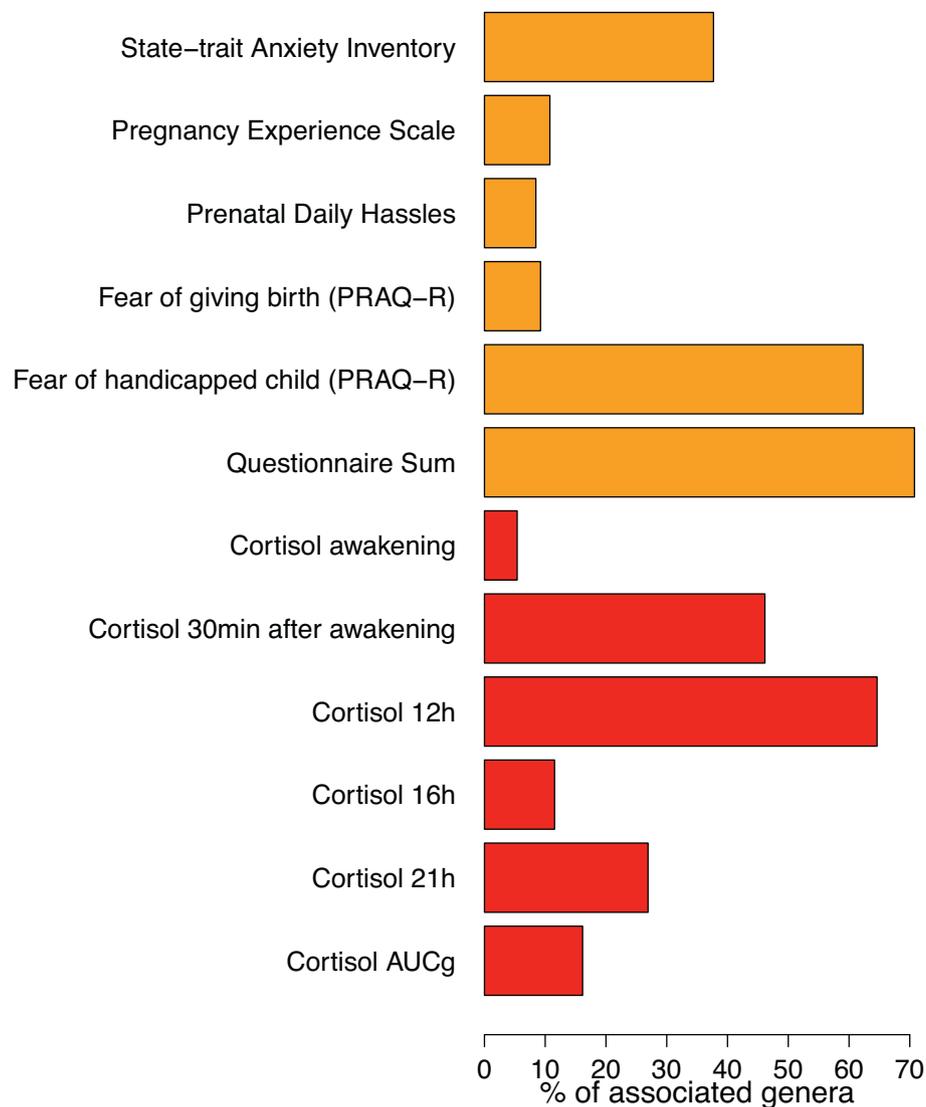


Fig. 1 Percentage of genus-level bacterial groups significantly associated with the different maternal prenatal stress indicators at any sampling age (see Section 2).

were associated with either reported stress or cortisol concentration.

The magnitude of the associations between the total infant microbiota and the prenatal stress (sum of questionnaires) and noon cortisol concentration were similar (Fig. 2). The effects of both prenatal stress indicators appeared comparable to or higher than those of breastfeeding, and were usually higher than the effects of postnatal maternal stress. The effects of prenatal stress on the infant microbiota were modest over the first month, peaked at 80 days, and were still clearly evident at 110 days.

As both stress indicators had similar associations with the microbiota, we combined the reported stress and the cortisol measure, forming a 'prenatal cumulative stress index': low reported stress+low cortisol concentration=low cumulative stress; low reported stress+high cortisol concentration, or high reported stress+low cortisol concentration=moderate cumulative stress; high reported stress+high cortisol concentration=high cumulative stress.

See Table 1 for the descriptive statistics of these three groups.

Based on the temporal dynamics and the associations with the two chosen stress indicators (i.e., sum of the questionnaire scores and noon cortisol), we grouped the stress-associated bacterial genera into clusters within which the bacteria behaved uniformly (Table 2).

3.3. Prenatal cumulative stress is a major driver of inter-individual variation in infant microbiota

The major source of inter-individual variation in the infant microbiota was the ratio between a group of proteobacteria (*Escherichia*, *Enterobacter*, *Serratia*; referred to as bacterial group PRO1), and a group of lactic acid bacteria (*Lactobacillus*, *Lactococcus*, *Aerococcus*; group LAB) and Actinobacteria (*Bifidobacterium*, *Collinsella*, *Eggerthella*; group ACT1) (Fig. 3). There was a negative

Table 1 Descriptive statistics for infants and mothers included in the present study.

	Low adversity (N = 18)	Moderate adversity (N = 24)	High adversity (N = 14)	p
Demographics				
Gender of infant				
Male	11	15	8	
Female	7	9	6	
Birth weight (gram; mean \pm SD, range)	3563.39 \pm 601.72 (2810–4600)	3567.38 \pm 512.77 (2645–4730)	3731.86 \pm 287.47 (3280–4150)	
5 min APGAR score (mean \pm SD, range)	9.65 \pm 0.61 (8–10)	9.55 \pm 0.74 (7–10)	9.57 \pm 0.85 (7–10)	
Maternal age (years; mean \pm SD, range)	32.42 \pm 3.10 (26.30–36.90)	32.38 \pm 3.36 (24.90–40.10)	32.46 \pm 3.70 (26.30–38.40)	
Prenatal smoking				
Yes	0	1	0	
No	18	23	14	
Prenatal alcohol consumption				
Yes	2	3	2	
No	16	21	12	
Infant use of antibiotics ^a				
Yes	1	1	0	
Breastfeeding month 0–4 (weeks; mean \pm SD, range) ^b				
Low	9.28 \pm 6.66, 0–16	9.52 \pm 6.37, 0–16	10.77 \pm 6.69, 0–16	
Gestational age (days; mean \pm SD, range)				
Low	281.33 \pm 8.97, 260–293	281.21 \pm 7.36, 267–296	283.14 \pm 7.52, 271–295	
Postnatal experienced stress ^c (mean \pm SD, range)				
Low	23.39 \pm 3.79, 17–30	26.54 \pm 6.57, 17–45	28.15 \pm 5.74, 20–40	<0.10
Postnatal anxiety ^c (STAI; mean \pm SD, range)				
Low	25.72 \pm 4.86, 20–36	29.67 \pm 10.18, 20–58	32.23 \pm 10.18, 20–58	
Postnatal daily hassles ^c (mean \pm SD, range)				
Low	0.77 \pm 0.39, 0–1.4	1.16 \pm 0.48, 0.2–2.7	1.15 \pm 0.43, 0–1.8	<0.05
Maternal stress variables; mean \pm SD, range				
Prenatal anxiety (STAI)				
Low	25.28 \pm 4.98, 20–41	31.08 \pm 8.38, 20–51	35.21 \pm 6.33, 23–46	<0.001
Pregnancy related daily hassles (PES)				
Low	0.20 \pm 0.10, 0–0.40	0.39 \pm 0.23, 0.20–1	0.52 \pm 0.32, 0.10–1.20	<0.001
Prenatal daily hassles (APL)				
Low	0.76 \pm 0.35, 0–1.30	1.18 \pm 0.50, 0.30–2.40	1.27 \pm 0.32, 0.60–1.80	<0.001
Fear of giving birth (PRAQ-R)				
Low	4.14 \pm 1.53, 3–8	6.17 \pm 3.50, 3–15	7.43 \pm 3.27, 3–15	<0.01
Fear handicapped child (PRAQ-R)				
Low	6.11 \pm 1.32, 4–9	8.42 \pm 2.95, 4–15	10.21 \pm 3.31, 5–15	<0.001
Cortisol awakening (nmol/L)				
Low	16.30 \pm 4.38, 9.50–24	15.37 \pm 3.73, 8.90–22.2	18.11 \pm 4.78, 12.60–27	
Cortisol awakening + 30 min (nmol/L)				
Low	20.74 \pm 5.21, 9.8–31.5	20.95 \pm 5.09, 11.3–31	24.29 \pm 6.90, 13.3–38	

Table 1 (Continued)

	Low adversity (N = 18)	Moderate adversity (N = 24)	High adversity (N = 14)	p
Cortisol 12:00 h (nmol/L)	13.13 ± 2.03, 9.6–16.1	14.78 ± 3.28, 7.6–19.7	19.78 ± 2.57, 17.1–25	<0.001
Cortisol 16:00 h (nmol/L)	11.02 ± 1.87, 6.9–14.9	12.14 ± 2.27, 7.8–16.1	15.06 ± 2.62, 11.6–20.9	<0.001
Cortisol evening (nmol/L)	8.50 ± 1.51, 5.50–10.80	9.01 ± 2.44, 4–14.80	11.55 ± 3.24, 6–18.2	<0.01
Cortisol AUCg ^d (nmol/L)	45.87 ± 7.09, 30–58.10	49.73 ± 9.01, 31–66.70	63.08 ± 10.18, 50.5–84.10	<0.001

Note: One way ANOVA's showed no significant differences between the groups unless otherwise indicated.

^a The infant in the low prenatal adversity group received antibiotics during month 1, the infant in the moderate prenatal adversity group received antibiotics during month 2.

^b 1 infant from the low prenatal adversity group, 5 infants from the moderate prenatal adversity group, and 2 infants from the high prenatal adversity did not receive any breastfeeding at all.

^c Postnatal stress and anxiety measured at 3 months.

^d Area under the curve with respect to the ground.

correlation between the relative abundances of PRO1 and LAB ($r = -0.23$, $p < 0.001$), which corresponded to the first principal coordinate (PC 1), and between PRO1 and ACT1 ($r = -0.40$, $p < 0.0001$), which corresponded to the second PC (PC2) (Fig. 3). The infants in the low cumulative stress group were characterized by high relative abundance of LAB (69%, 377%, 305%, and 39% higher in the low cumulative stress, as compared to the high cumulative stress group at ages 7, 14, 28, and 110 days, respectively) and ACT1 (23%, 29%, 66%, 53% higher in the low cumulative stress group at ages 7, 14, 80, and 110 days). The infants in the high cumulative stress group tended to localize in the *Escherichia*–*Enterobacter*-end of the microbiota gradient (Fig. 3), with 853%, 256%, 1244%, and 699% higher abundances in the high cumulative stress group at ages 14, 28, 80, and 110 days, respectively. The sum of the first two principal coordinates (indicating a high relative abundance of PRO1 and low relative abundance of LAB and ACT1) was strongly associated with prenatal cumulative stress (Fig. 3). Infants in the high cumulative stress group, with the combined effect of high maternal prenatal reported stress and cortisol concentration, had the

highest summed PC scores, and those in the low cumulative stress group had the lowest scores. Infants with only one prenatal cumulative stress factor, either high cortisol concentration or high reported stress, had an intermediate microbiota, suggesting a relatively linear association between cumulative prenatal stress and infant microbiota.

3.4. Temporal dynamics of the infant microbiota

We selected the low and the high cumulative stress groups and the bacterial groups most strongly associated with prenatal stress to illustrate the differences in the temporal dynamics (Fig. 4). In the low prenatal cumulative stress group, the overall diversity of the infants' microbiota decreased during the first four months of life, and was associated with the gradual establishment of dominant bacteria (mainly *Bifidobacterium*) (Fig. 4, blue lines; Fig. 5). However, the diversities within Actinobacteria, Proteobacteria, and Clostridia, the most abundant groups in the infant microbiota, increased with time (Fig. 4, blue lines). During

Table 2 Grouping of the stress-associated genera, based on similar temporal dynamics and associations with the stress indicators.

Group (CODE)	Genera
Lactic acid bacteria (LAB)	<i>Lactobacillus</i> , <i>Lactococcus</i> , <i>Aerococcus</i>
Actinobacteria 1 (ACT1)	<i>Actinomycetaceae</i> , <i>Bifidobacterium</i> , <i>Collinsella</i> , <i>Eggerthella</i>
Actinobacteria 2 (ACT2)	<i>Atopobium</i> , <i>Corynebacterium</i> , <i>Micrococcaceae</i>
Proteobacteria 1 (PRO1)	<i>Escherichia</i> , <i>Serratia</i> , <i>Haemophilus</i> , <i>Proteus</i> , <i>Enterobacter</i>
Proteobacteria 2 (PRO2)	<i>Aeromonas</i> , <i>Alcaligenes</i> , <i>Anaerobiospirillum</i> , <i>Aquabacterium</i> , <i>Bilophila</i> , <i>Campylobacter</i> , <i>Desulfovibrio</i> , <i>Helicobacter</i> , <i>Leminorella</i> , <i>Methylobacterium</i> , <i>Novosphingobium</i> , <i>Oceanospirillum</i> , <i>Oxalobacter</i> , <i>Sutterella</i> , <i>Xanthomonadaceae</i>
Clostridia (CLO)	<i>Anaerotuncus</i> , <i>Anaerostipes</i> , <i>Anaerovorax</i> , <i>Bulleidia</i> , <i>Clostridium</i> , <i>Catenibacterium</i> , <i>Coprobaecillus</i> , <i>Coprococcus</i> , <i>Dialister</i> , <i>Dorea</i> , <i>Eubacterium</i> , <i>Faecalibacterium</i> , <i>Lachnobacillus</i> , <i>Lachnospira</i> , <i>Megamonas</i> , <i>Megasphaera</i> , <i>Mitsuokella</i> , <i>Papillibacter</i> , <i>Peptococcus</i> , <i>Peptostreptococcus</i> , <i>Phascolarctobacterium</i> , <i>Roseburia</i> , <i>Ruminococcus</i> , <i>Sporobacter</i> , <i>Subdoligranulum</i>

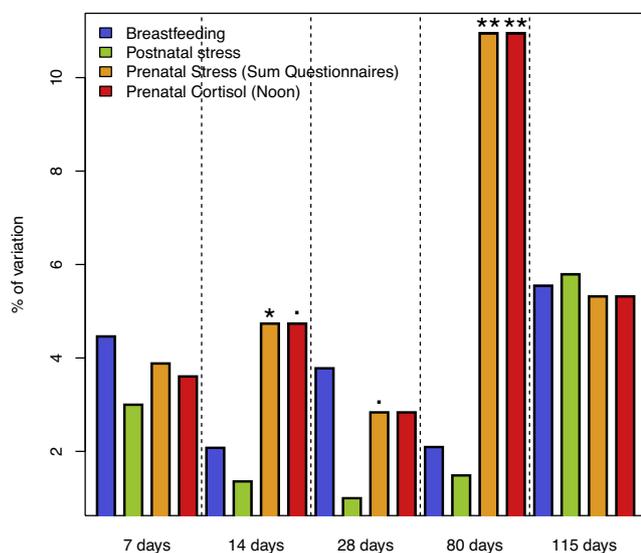


Fig. 2 Percentage of inter-individual variation in the composition of the total microbiota attributable to differences in breastfeeding, maternal postnatal stress, and maternal prenatal reported stress and cortisol (based on Permutational multivariate analysis of variance). Asterisks indicate a significant contribution to the inter-individual variation: · $p < 0.1$; * $p < 0.05$; ** $p < 0.01$.

the first month, the intestinal microbiota of the infants with low prenatal cumulative stress was characterized by a high abundance of streptococci and PRO1, which were gradually replaced by ACT1, LAB, *Clostridium* spp. (CLO), and Proteobacteria Group 2 (PRO2) (including low-abundance Proteobacteria, such as *Sutterella*; see Table 2). Similar patterns were evident at the phylum level (Fig. 5A).

In the high cumulative stress group, the temporal development was different (Fig. 4, red lines). The overall diversity was consistently higher, due to more even relative abundances of different bacterial groups (Fig. 5), but the diversities within Actinobacteria and Proteobacteria were lower than in the low stress group. Furthermore, the relative abundance of PRO1 (and the total abundance of Proteobacteria, Fig. 5) was higher, and correspondingly the abundances of ACT1, ACT2, LAB, CLO and PRO2 were lower. The abundance of *Akkermansia* also differed significantly between the groups, as it declined dramatically in the high cumulative stress group after the first month and remained low thereafter (Fig. 4, red lines). The total abundance of Bacilli remained relatively high throughout the study period, whereas it declined considerably in the low cumulative stress group (Fig. 5).

Although we did not have sufficient power to properly investigate the potential influence of breastfeeding interacting with prenatal stress, we checked the results also separately for the breastfed and non-breastfed infants (data not shown). Compared to the observed association of prenatal stress and the infant microbiota, breastfeeding had a minor influence on the microbiota. Also, the microbiota association with stress was comparable between breastfed and non-breastfed infants. Therefore, we conclude that in this cohort, breastfeeding did not confound the interpretation of the results.

3.5. Prenatal cumulative stress predisposes to gastrointestinal and allergic symptoms

Gastrointestinal symptoms (e.g., diarrhea, gastroenteritis, presumed infection and constipation) were more prevalent during the first three months of life in the high cumulative stress group (38%), as compared to the low cumulative stress group (22%). By the age of three months, 43% of the infants in the high cumulative stress group, and none in the low cumulative stress group, had shown allergic reactions. Note that due to the small sample sizes, these differences in symptom frequency were non-significant based on χ^2 -test ($p > 0.05$). The health differences between the groups appeared attributable to the differences in the intestinal microbiota. The infants with gastrointestinal symptoms had lower (albeit non-significantly) relative abundances of LAB (on average 0.5% of total microbiota) and *Akkermansia* (0.1%) than the infants without gastrointestinal symptoms (2% and 0.5%, respectively). Infants with allergic reactions by the age of three months had consistently lower abundances of LAB (0.5% vs. 2%, non-significant difference) and ACT1 (15% vs. 60%, $p < 0.001$), lower abundance of *Akkermansia* (only during the first month of life: 0.7% vs. 2%, $p < 0.05$), and higher abundances of PRO1 (30–50% vs. <10%, $p < 0.001$) than the infants without allergic reactions.

4. Discussion

4.1. Maternal prenatal stress is associated with altered infant microbiota

We characterized the temporal dynamics of the human infant microbiota during the early stage of succession, using a phylogenetic microarray covering the majority of intestinal bacteria, in a cohort of 56 healthy, vaginally born infants, with varying exposure to maternal prenatal stress. Both maternal reported stress and salivary cortisol concentrations during late pregnancy were associated with dramatic shifts in the infant microbiota, which persisted until the end of the follow-up period at 16 weeks of age, even after correcting for breastfeeding and maternal postnatal stress. However, maternal reported stress and cortisol concentrations during pregnancy were only moderately correlated, suggesting that they tap into two different aspects of prenatal stress. Our secondary analyses showed that a combination of high reported stress and high cortisol concentrations (cumulative stress) was related to increased abundance of Proteobacteria such as *Escherichia* and *Enterobacter*, and decreased abundance of lactic acid bacteria and Actinobacteria. Most strikingly, such a colonization pattern was associated with maternally reported gastrointestinal symptoms and allergic reactions in the infant. The changes in the microbiota appear surprisingly universal, considering that the same pattern, a reduced number of Bifidobacteria and Lactobacilli, was found in rhesus monkeys prenatally exposed to a very different type of stressor (acoustic stress; Bailey et al., 2004).

We envisage three main mechanisms through which maternal prenatal cortisol concentrations may influence the infant intestinal microbiota. First, cortisol is known to control bile acid production in the liver, and regulate cholesterol

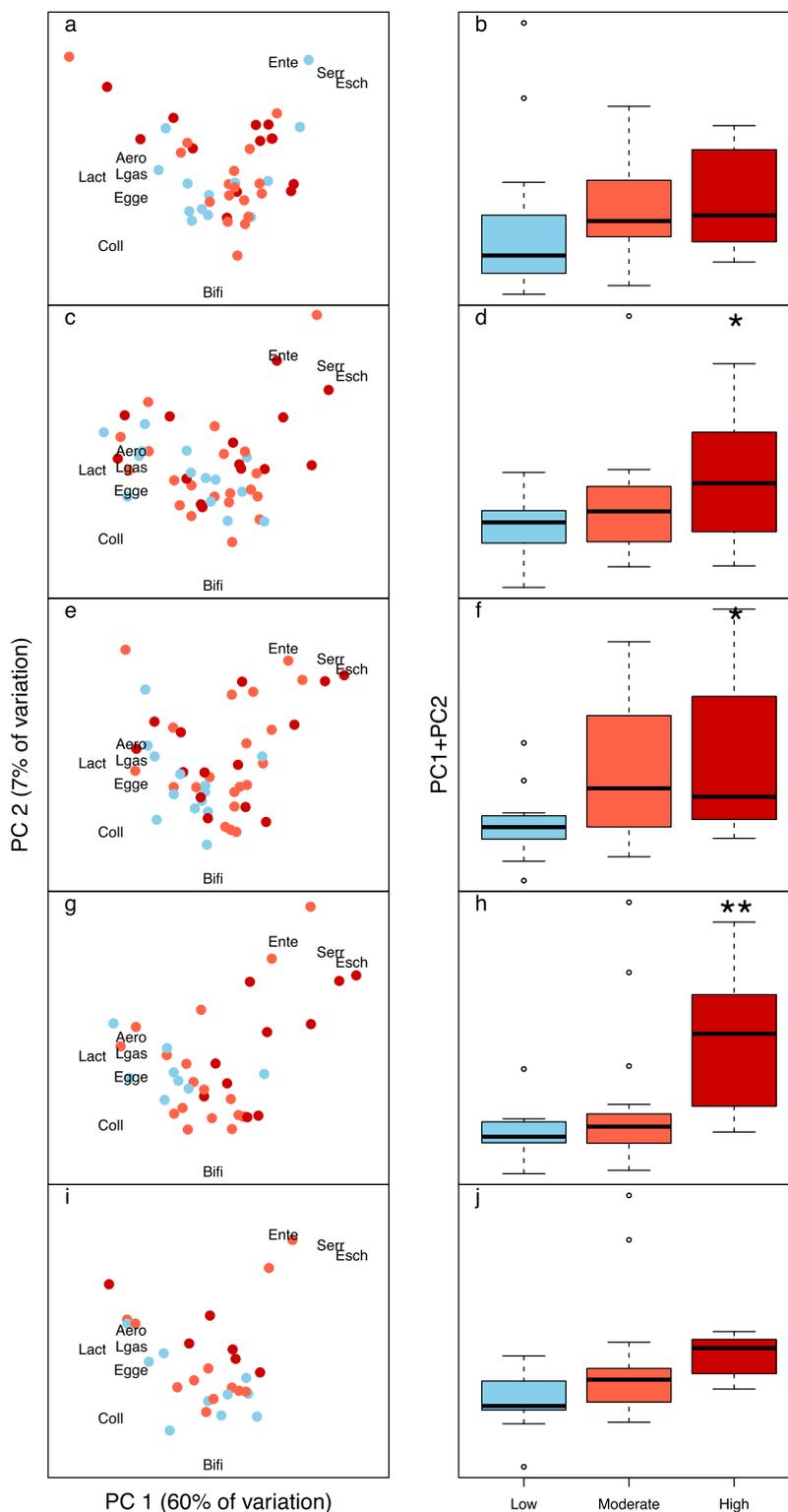


Fig. 3 Associations between microbiota principal coordinates (beta diversity) and prenatal stress. Samples collected at different ages are shown in different panels for clarity. Light blue = low prenatal cumulative stress; light red = moderate cumulative stress; dark red = high cumulative stress (based on both questionnaires and cortisol, see text). The bacterial groups shown are *Enterobacter* (Ente), *Serratia* (Serr), *Escherichia* (Esch), *Aerococcus* (Aero), *Lactococcus* (Lact), *Eggerthella* (Egge), *Collinsella* (Coll), *Bifidobacterium* (Bifi) and those related to *Lactobacillus gasseri* (Lgas). (a and b) Samples taken at 7 days of age; (c and d) 14 days; (e and f) 28 days; (g and h) 80 days; (i and j) 110 days. Asterisks indicate a significant difference from the low cumulative stress group: * $p < 0.05$; ** $p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

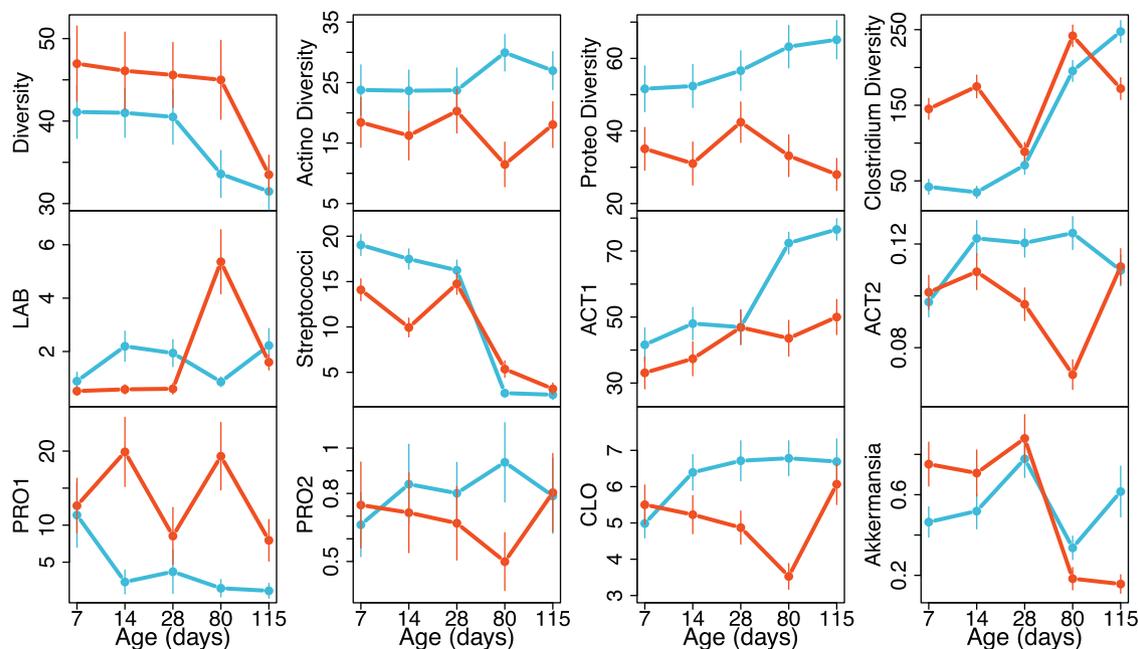


Fig. 4 Development of the microbiota alpha diversity (inverse Simpson index, top row graphs) and the relative abundances (mean \pm SE) of the significantly associated microbial groups (remaining graphs, see Table 2) in infants born to mothers with high (red) and low (blue) cumulative stress during pregnancy. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and bile acid homeostasis (Rose and Herzig, 2013), plausibly thus directly influencing the maternal microbiota (Islam et al., 2011; Salonen and de Vos, 2014). High maternal cortisol may result in increased bile acid production, which could interfere with the natural development of the maternal intestinal microbiota during pregnancy (Koren et al., 2012). This could influence the transmission of microbiota

from mother to infant (e.g., transfer of Bifidobacteria: Tannock et al., 1990). Second, maternal cortisol can cross the placenta and increase the fetal concentrations of cortisol (Duthie and Reynolds, 2013). This in turn could affect the development of the fetal HPA axis, resulting in higher infant basal cortisol concentrations and cortisol reactivity after birth (Tollenaar et al., 2011). Cortisol can affect the immune

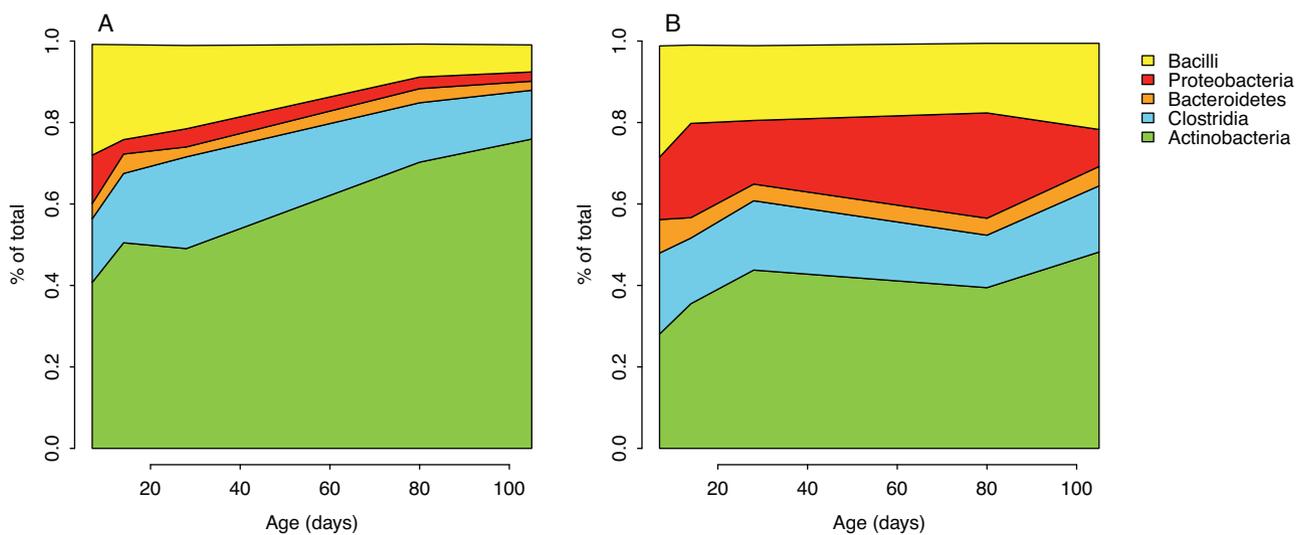


Fig. 5 Average relative abundances of phylum-level bacterial groups (Firmicutes divided into Bacilli and Clostridia) during the study period in the low cumulative stress infants (A) and high cumulative stress infants (B). Bacilli includes the bacterial groups LAB and streptococci, Proteobacteria includes the groups PRO1 and PRO2, and Actinobacteria includes the groups ACT1 and ACT2. Members of the phylum Bacteroidetes were not strongly associated with prenatal stress and were therefore not further analysed. The phylum Verrucomicrobia (including only the genus *Akkermansia*) is not shown as its relative abundance of the total microbiota is very small (see Fig. 4).

cells in the gut, change the permeability of the gut, disrupt the barrier function, and potentially influence the gut microbiota (Cryan and Dinan, 2012). Third, glucocorticoids in breast milk are correlated with salivary glucocorticoids in rhesus monkeys (Sullivan et al., 2011). Therefore, breast milk could be an additional potential vehicle by which mothers with higher prenatal concentrations of cortisol, and that continue to have high cortisol concentrations postnatally, transfer cortisol to the infant, affecting in turn the infant HPA axis and/or infant gut and finally, the infant intestinal microbiota. Although as yet hypothetical, the described pathways may explain how the maternal prenatal cortisol concentrations are related to the infant microbiota, and as such may constitute a basis for future research.

Reported prenatal stress, in the absence of elevated cortisol, had a similar impact on the infant microbiota as elevated cortisol in the absence of reported stress. One possible explanation is that, despite their normal levels of cortisol, mothers experiencing stress/anxiety transferred an altered microbiota to their infants, or exposed their infants to elevated cortisol concentrations via increased placental transfer due to reduced functioning of the 11β -HSD2 enzyme (O'Donnell et al., 2012). Furthermore, maternal psychological stress could affect her physiology without direct involvement of the HPA axis, e.g., through maternal lifestyle (e.g., diet, sleep) or the immune system (Beijers et al., 2014). Hypothetically, mothers experiencing stress during pregnancy may have problems sleeping. Disturbed sleep produces inflammatory activation which may affect placental trophoblast invasion, affecting in turn embryo implantation and development. This may lead to abnormalities in the development of the fetal intestines, possibly finally affecting postnatal colonization by bacteria. Although this chain of events has yet to be determined, evidence for several of the steps has already been found (Beijers et al., 2014). Furthermore, stress influences the immune system that is in constant interaction with the intestinal microbiota and may directly affect the intestinal environment, especially with respect to inflammation. This pathway may lead to a selection of inflammation-tolerant bacteria, such as the enterobacteria (Lupp et al., 2007), which includes the genera *Escherichia*, *Enterobacter*, and *Serratia*.

We found only a weak correlation between maternal reported stress and cortisol, which suggests that they measure different kinds of prenatal stress. Summing these variables led to the strongest associations with the infant microbiota, indicating that the accumulation of feelings of stress/anxiety and high cortisol concentrations has a stronger relation with the infant microbiota than these stress variables independently. This is in line with the cumulative risk hypothesis (Rutter, 1979). Although the sum of the reported stress/anxiety variables included in our study showed the strongest relation with the infant intestinal microbiota, a stress/anxiety variable that seems to be especially important is the subscale 'fear of having a handicapped child', representing pregnancy-specific anxiety. Accordingly, pregnancy-specific anxiety was a strong predictor of infant cortisol reactivity in a larger cohort from this study (Tollenaar et al., 2011), as well as of infant baseline cortisol in other studies (Gutteling et al., 2005).

4.2. Health consequences of the aberrant microbiota

In early infancy, there appears to be a competitive balance between two groups of facultative anaerobic bacteria, the lactic acid bacteria and the Proteobacteria, the latter including the genera *Escherichia*, *Enterobacter* and *Serratia*. All known species belonging to the genus-level groups *Escherichia*, *Serratia*, and *Enterobacter* in the HITChip phylogeny can cause infections, particularly in susceptible individuals such as infants. Moreover, these are Gram-negative organisms, which produce lipopolysaccharide (LPS), an inflammatory endotoxin that has been implied in inflammation in a variety of metabolic diseases (Cani et al., 2012). Moreover, it has been suggested that LPS play a role in stress responses (Black, 2002). A dominance of *Escherichia* and related organisms has been observed in infants treated with antibiotics (Fallani et al., 2010; Fouhy et al., 2012), in preterm infants later developing necrotizing enterocolitis (Normann et al., 2013), and in infants later developing colic (De Weerth et al., 2013). A dominance of enterobacteria in early infancy has been linked with allergy and eczema risk (Gosalbes et al., 2013), while lactic acid bacteria or Bifidobacteria appear to protect from allergies (Kuitunen et al., 2012). Furthermore, *Bifidobacterium infantis* has been shown to attenuate stress responses, and enteropathogenic *E. coli* to exacerbate them in infant mice (Sudo et al., 2004), suggesting that maternal prenatal stress may influence the HPA activity of the infant via the intestinal microbiota.

A low abundance of Lactobacilli and Bifidobacteria has been associated with increased crying in infants (De Weerth et al., 2013). Bifidobacteria, dominant members of the healthy infant microbiota (Fallani et al., 2010), are often not detected in prematurely born infants (Normann et al., 2013), in infants treated with antibiotics (Fouhy et al., 2012), or in infants born via caesarean section (Biasucci et al., 2010), suggesting that bacteria belonging to this genus are highly sensitive to environmental disturbances and are habitat specialists. In a recent paper (Subramanian et al., 2014) several species of *Bifidobacterium* and *Lactobacillus* were found to be associated with healthy microbiota development in children.

To conclude, *Bifidobacterium* may be a reliable indicator of a healthy infant microbiota. The colonization pattern observed in the infants whose mothers had experienced stress during pregnancy resembles the patterns previously reported in infants with compromised health. Indeed, infants in the high cumulative stress group had a higher risk of gastrointestinal symptoms throughout the study period, and most strikingly, a higher risk of developing allergic reactions during the first three months of life. Health symptoms were also associated with a reduced level of *Akkermansia muciniphila*. *Akkermansia muciniphila* is a mucus-degrading intestinal symbiont, residing in the mucus layer in close contact with the host (Belzer and de Vos, 2012). It is known to improve intestinal barrier function and reduce LPS levels in mice fed a high-fat diet (Everard et al., 2013) and thought to have a protective and anti-inflammatory influence (Belzer and de Vos, 2012).

5. Conclusion

In conclusion, maternal prenatal stress, based on questionnaires or on elevated basal cortisol concentrations, or on both, was strongly and persistently associated with the infants' microbiota composition and colonization pattern. Furthermore, the altered colonization pattern appeared to predispose the infants to gastrointestinal symptoms and allergic reactions. This is the first study to show a link between maternal prenatal stress, and the infant intestinal microbiota in humans. These results suggest that offspring health may be improved by modifying the intestinal microbiota during pregnancy, especially in women with stress.

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Conflict of interest statement

None declared.

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