Infant Fecal Microbiota Composition and Attention to Emotional Faces

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Abstract

The gut microbiota has been suggested to influence neurodevelopment in rodents. Preliminary human studies have associated fecal microbiota composition with features of emotional and cognitive development as well as differences in thalamus-amygdala connectivity. Currently, microbiota-gut-brain axis studies cover heterogenous set of infant and child brain developmental phenotypes, while microbiota associations with more fine-grained aspects of brain development remain largely unknown. Here (N=122, 53% boys), we investigated the associations between infant fecal microbiota composition and infant attention to emotional faces, as bias for faces is strong in infancy and deviations in early processing of emotional facial expressions may influence the trajectories of social-emotional development. The fecal microbiota composition was assessed at 2.5 months of age and analyzed with 16S rRNA gene sequencing. Attention to emotional faces was assessed with an age-appropriate face-distractor paradigm, using neutral, happy, fearful, and scrambled faces and salient distractors, at eight months of age. We observed an association between a lower abundance of *Bifidobacterium* and a higher abundance of *Clostridium* with an increased "fear bias", i.e. attention towards fearful vs. happy/neutral faces. This data suggests an association between early microbiota and later fear bias, a well-established infant phenotype of emotionally directed attention. However, the clinical significance or causality of our findings remains to be assessed.

Keywords: microbiota, gut-brain axis, emotional attention, brain development, infant

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Facial expressions depicting emotion, such as fear, convey biologically salient information (e.g. signaling of a threat in the environment), and their processing is prioritized in the visual systems. Human visual attention system is biased towards faces from the very first days of life, with this bias for faces developing in interaction with individual characteristics and environmental exposures (Reynolds & Roth, 2018). In addition to a general face bias, another well-established example of attention bias in infancy is a heightened bias towards faces expressing fear emerging between five and seven months of age, at a time when other fear-related behavior starts to emerge (Leppänen, Cataldo, Bosquet Enlow, & Nelson, 2018; Nakagawa & Sukigara, 2012; Peltola, Leppänen, Mäki, & Hietanen, 2009; Peltola, Yrttiaho, & Leppänen, 2018). Interestingly, common psychiatric disorders, such as anxiety and depression, involve biased attention towards negative emotional stimuli, such as faces signaling fear, anger, or sadness (Armstrong & Olatunji, 2012; Bar-Haim, Lamy, Pergamin, Bakermans-Kranenburg, & Van Ijzendoorn, 2007), indicating an altered functioning of the attention systems when processing salient emotional signals. Little is known of individual preceding factors leading to altered phenotypes during development.

In parallel with the normative bias towards fearful stimuli in infancy, it has also been suggested that variance in early emotional attention may indicate differential trajectories of social-emotional development (Hummel, Premo, & Kiel, 2017; Peltola et al., 2018). For example, lower overall engagement to social cues, namely eye fixation, is related to the phenotype of autism (Klin, Shultz, & Jones, 2015; Zwaigenbaum et al., 2005). A flexible orienting of attention in infancy (e.g. engaging and disengaging from stimuli) is considered as the first means of infant self-regulation aiding in the regulation of affective states (Bridgett, Burt, Edwards, & Deater-Deckard, 2015; Posner, Rothbart, Sheese, & Voelker, 2014; Rothbart, Sheese, Rueda, & Posner, 2011). On one hand, the bias towards fearful faces is

common in infancy (Peltola et al., 2009) and may even predict better attachment security in the future (Peltola, Forssman, Puura, van IJzendoorn, & Leppänen, 2015). However, on the other hand, infant's enhanced attention to threat may mediate the risk for anxiety symptoms later in childhood particularly when concurrent with other risk factors, such as behavioral inhibition, a trait related to negative emotionality (Nozadi et al., 2016). Overall, attentional biases are important factors affecting socioemotional development (Morales, Fu, & Pérez-Edgar, 2016).

Interestingly, a wealth of rodent literature has suggested that gut microbiota may affect neurodevelopment and behavior via the functioning of the gut-brain axis (Cryan et al., 2019). Possible routes include alterations in the immune system functioning and neural connections (most notably the vagus nerve) as well as via metabolites produced in host-microbiota interaction (Cryan et al., 2019). Rodent studies have likewise indicated that early life microbiota alterations may cause irreversible changes in behavior. For example, Neufeld et al. showed that early perturbations in microbiota cause anxiety-like behavior in rodents, which persists even after the microbiota recolonization in adulthood (Neufeld, Kang, Bienenstock, & Foster, 2011). In humans, alterations in the fecal microbiota have been observed to link with psychiatric disorders, such as depression (Jiang et al., 2015), as well as neurodevelopmental conditions, such as autism (Kang et al., 2018). Despite the lack of longitudinal human studies to illustrate potential causal relationships, arising studies in infants have reported associations between certain fecal microbiota profiles and aspects of brain development (Aatsinki et al., 2019; Carlson et al., 2018; Christian et al., 2015; Loughman et al., 2020; Sordillo et al., 2019). For example, *Bacteroides* dominance has been associated with enhanced cognitive development during the first two years of life (Carlson et al., 2018). In addition, *Clostridiales* class dominance has been associated with poor communication, personal, and social skills at the age of three years (Sordillo et al., 2019).

Furthermore, reduced fecal microbiota diversity in one-year-olds has been associated with other aspects of infant brain development, such as better cognitive development (Carlson et al., 2018) and stronger amygdala-thalamus connectivity (Gao et al., 2019). We reported previously that the reduced microbial diversity was associated with the main domain of temperamental negative emotionality and its subscale fear reactivity while the abundance of *Erwinia* genus was positively associated with self-regulation (Aatsinki et al., 2019).

Given that emotional attention may predict differences in social-emotional development (Morales et al., 2016), whether and how the fecal microbiota composition is associated with the emotional attention in infancy remains an important, yet understudied, topic. Emotional attention in infancy and especially the more general "face bias" as well as a specific, infant-typical "fear bias" have strong biological bases in human development (Haist & Anzures, 2017; Leppänen & Nelson, 2009; Marsh, 2016). Thus, when investigating the early predispositions for developing self-regulation and risk for psychopathology, these aspects of infant's emotional attention may serve as an important intermediate phenotype.

In the present study, we aimed to explore whether and how infant early fecal microbiota composition and diversity associate with later attention to faces and emotional facial expressions. Fecal microbiota composition on genus level, interindividual diversity (i.e. beta diversity) as well as intraindividual diversity and richness (i.e. alpha diversity) were assessed from fecal microbiota samples. Richness describes the number of unique species and diversity additionally considers their abundance distribution and species dominance, i.e. the uniformity of the taxa abundances, in the fecal sample. Emotional attention was measured using eye tracking and an age-appropriate face-distractor paradigm (Peltola et al., 2009), with neutral, happy, and fearful faces together with scrambled faces and abstract distractors as stimuli. A low likelihood to disengage from faces (towards distractors) may describe prolonged dwelling in certain stimuli, while greater likelihood may describe more flexible

attention shifting, which is important for emotion regulation (Leppänen et al., 2011). Finally, we aimed at investigating the potential sex interactions as the gut microbiota has shown sex-specific effects on brain development in rodent models (Clarke et al., 2013) and early life exposures may associate with emotional attention in a sex-specific manner (E. L. Kataja et al., 2019). The previous literature is insufficient regarding the connections between the infants' emotional attention and fecal microbiota in order to hypothesize the exact directions of the effects between fecal microbiota composition or diversity and emotional attention.

Materials and Methods

To study the possible associations between infants' microbiota profile and attention to emotional faces, fecal samples and eye tracking were obtained from healthy infants as a part of the FinnBrain Birth Cohort Study, a general population pregnancy cohort (Karlsson et al., 2018). Previous study linking cognitive functioning to infant fecal microbiota characteristics with a sample of 89 infants found a correlation between alpha diversity and infant cognitive development scores with $R^2 = 0.085$ ($f^2 = 0.093$) (Carlson et al., 2018). Hence, to achieve 90% power with 0.05 alpha level to detect the same $f^2 = 0.093$ effect size, a sample size of 116 is needed for two-tailed linear regression (Faul, Erdfelder, Lang, & Buchner, 2007). However, as there are no previous studies with a similar phenotype and the study is exploratory in nature, estimation of the potential effect size and the power analysis must be treated with caution, especially in the case of interaction analyses, which were not reported in the abovementioned study. The study was approved by the Ethics Committee of the Hospital District of Southwest Finland. Written informed consent was obtained from all families, and mothers provided written informed consents on behalf of their infants.

Study Subjects

The FinnBrain Birth Cohort study embeds a nested case-control design that is enriched with prenatally distressed mothers (elevated levels of depressive and anxiety symptoms, highest ca. 20% from the original cohort of n=3808) and their controls (lowest ca. 27 % from the original cohort of n=3808), i.e. the Focus Cohort (Karlsson et al., 2018).(E.-L. Kataja et al., 2018). The study population for the current study was drawn from this Focus Cohort background population and comprised of subjects who provided both the fecal sample and the eye movement tracking measurement. The fecal samples were collected as part a of a pediatric visit: mothers from an unselected subsample of Focus Cohort were contacted via emails and phone and ultimately a total of 200 infants provided a fecal sample at the age of 2.5 months. Similarly, infants from the Focus Cohort that participated the Child Development and Parental Functioning Lab visit and provided valid eye movement tracking data were eligible for this study (n=421) (E.-L. Kataja et al., 2018). Finally, a total of 131 (boys =69, 52%) subjects with overlapping measurements comprised the current study population. Of note, 89 (68 %) subjects in the current study were included in our previous study on infant temperament and fecal microbiota (Aatsinki et al., 2019). The main reasons for incomplete overlap were constrains in cohort resources and logistics.

Background information

Self-report questionnaires were obtained at gestational week (gwk) 14, 24, and 34 and three months postpartum (see below). At the gwk 14, parental background data, including level of education (categorized as (i) university education, i.e., tertiary level academic/general education; (ii) vocational tertiary education; (iii) secondary level education) and intake of selective serotonin reuptake inhibitor or serotonin–norepinephrine reuptake inhibitor (SSRI/SNRI intake) were reported (18). Edinburgh Postnatal Depression Scale [EPDS (Cox, Holden, & Sagovsky, 1987)] and Symptom Checklist-90, anxiety subscale [SCL-90 (Derogatis, Lipman, & Covi, 1973)] were measured at gwks 14, 24, 34 and three months

postpartum. Information on the duration of exclusive and partial breastfeeding was collected from postnatal follow-up questionnaires, and infant medication intake was reported at fecal sample collection. The categories for breastfeeding at 2.5 months of age were never breastfed, breastfeeding ceased, partial breastfeeding, and exclusive breastfeeding. Information about maternal pre-pregnancy body mass index (BMI; kg/m2), the duration of the gestation (gwks: preterm <37 gwk; term 37–41; post term ≥ 42 gwk), antibiotic intake during the neonatal period, birth weight (g) and height (cm), and the mode of delivery (all caesarean (C-) section vs. all vaginal) was collected from the National Birth Registry provided by the National Institute for Health and Welfare (www.thl.fi).

Fecal Sample Collection and Microbiota Analyses

Fecal samples (age 2.5 months) were collected by the parents as previously reported (Aatsinki et al., 2019). In order to analyze the bacterial profiles of the infant fecal microbiome, V4 region of the bacterial 16S rRNA gene was analyzed (Aatsinki et al., 2019). The operational taxonomic unit (OTU) table was generated using QIIME pipeline (v. 1.9) as previously reported (Caporaso et al., 2010; Rintala et al., 2018). The sequence reads were filtered with a quality score acceptance rate of ≥ 20, resulting in 41 k - 1052 k reads per sample (total: 73,222 k, mean: 178,156 k, sd: 109 k).

Eye Tracking of Emotional Attention

Emotional attention was assessed at the infant age of eight months (+/- two weeks) from due date. The due date was determined with an ultrasound assessment immediately after which the study subjects were recruited to the FinnBrain Birth Cohort Study. The due date was reported by the mother to the recruiting study nurse and checked from the ultrasound visit documents by the nurse. On average, the fecal sampling and eye tracking were 5.8 months (range =_4.3–7.9, sd = 0.59) apart. Emotional attention was assessed during a study visit that

was conducted by a psychologist or advanced psychology student as part of Child Development and Parental Functioning Lab assessment. Eye tracking (EyeLink1000+ SR Research Ltd, Toronto, Ontario, Canada) with a sampling frequency of 500 Hz, and a face-distractor paradigm (Peltola et al., 2009) with emotional faces (scrambled, neutral, happy, and fearful faces) and distractors (i.e., geometric shapes) were used to study infant attention bias for faces and fear. A set of 48 trials were presented, 12 trials per condition (each emotion and the scrambled face control picture), comprising of 18 photographs of each woman (two different women were presented) and 12 scrambled-face pictures in a semi-random order.

Prior to every measurement, a five-point calibration procedure with an audiovisual animation (rapidly appearing, rotating animated pictures, either of a duck or a barking dog) sequentially presented in five locations on the screen was used. The calibration could be repeated before actual testing and also during measurement when necessary. Small breaks were allowed during measurement if necessary. During eye tracking, the child sat on the caregiver's lap at a distance of 50–70 cm from the eye tracker facing the computer screen. The eye-tracking laboratory was dimly lit and the researcher sat by an independent host computer next to the infant-parent dyads, but was separated from them by a curtain to avoid interference. The parent was instructed not to comment on the emotional content of the faces but otherwise not prevented from talking to the infant. The assessment was monitored by a researcher and the parent would have been re-instructed in the case of a deviation from the original instructions.

A brief animation was shown before each trial to capture the attention of the infant to the center of the screen. After the infant fixated on the animated stimulus (depicted as a red circle in Figure 1), as judged by the experimenter monitoring the infant via a host computer, the experiment was started. During the experiment, first, a picture of a face (or a scrambled-face stimulus) was shown in the center of the screen for 1,000 ms (Figure 1). Then, with a 1,000-ms onset asynchrony, a salient lateral distractor (checkerboard or circles) appeared on

either the left or right side of the face (a visual angle of 13.6°) for 3,000 ms, simultaneously with the face. One trial lasted for 4,000 ms. The sizes of the emotion-depicting pictures and distractor stimuli were 15.4° x 10.8° and 15.4° x 4.3°, respectively. Once the infant's gaze was focused to the middle of the screen, the next trial was presented by the researcher. The order of the central stimuli was semi-randomized, with a constraint that the same stimulus was not presented more than three times in a row. The lateral stimulus was selected and presented randomly for each trial (see Figure 1. for illustration and a description of the procedure in Kataja et al., 2018).

Preprocessing of Eye-Tracking Data

The trial data, comprising of timestamps for the onset times of central and lateral pictures and the xy coordinates of the participants' gaze position (500 samples per second) were stored as text files and analyzed offline using a library of Matlab scripts (Mathworks, Natick, MA) (Leppänen, Forssman, Kaatiala, Yrttiaho, & Wass, 2015). We used the following quality control criteria based on prior studies (Leppänen et al., 2015) to retain trials for the analysis. First, trials had to have sufficiently long fixation on the central stimulus (i.e., >70% of the time) during the time preceding gaze disengagement or the end of the analysis period (i.e., 1,000 ms from the appearance of the lateral distractor). Secondly, trials had to have a sufficient number of valid samples in the gaze data (i.e., no gaps >200 ms). Thirdly, trials had to have valid information about the eye movement from the central to the lateral stimulus (i.e., the eye movement did not occur during a period of missing gaze data). The proportion of invalid trials was 20.8% corresponding to previous infant studies using the same methodology in 7- to 11-month-old infants (21.6–28.2%, Leppänen et al., 2015). On average, subjects provided 9.0 (sd 2.5) trials for control stimulus, 9.0 (sd 2.5) for neutral face, 9.2 (2.3) for happy face and 9.3 (2.4) for fearful face in this sample (n=131).

First, we calculated the probabilities of disengagement (DPs, time from the beginning of experiment to 2,000 ms) separately for each stimulus condition (i.e., neutral, happy, and fearful faces, and scrambled-face pictures). The DP is a ratio of the number of disengagement (in a given condition) / the number of valid trials (in a given condition). A cut-off of 2,000 ms was set as typically reactive saccades from central stimuli to lateral distractors appear in this time window (Pyykkö, Ashorn, Ashorn, Niehaus, & Leppänen, 2019). Then, to investigate the infants' preference for faces vs. scrambled faces we calculated a face bias score. Following Yrttiaho, Forssman, Kaatiala, and Leppänen (2014), the "face bias" was calculated by contrasting the face condition (Happy, HA, Neutral, NE) with the scrambled face condition (Control stimulus, CS) using the following formula: *face-bias = p(saccade/CS) - p(saccade/HA&NE)* (Yrttiaho et al., 2014). The fearful face condition was left out from the face bias score to minimize the effect of emotional cues to infant face bias [as fearful faces have been shown to strongly suppress attention shifts to peripheral targets when compared to neutral and happy faces at this particular age; (Leppänen, 2016)]. Then, to investigate the differences in infants' fear bias we calculated a fear bias score. The "fear bias" was calculated by contrasting the fear condition (Fearful, FE) to other face conditions (Happy, HA, Neutral, NE) using the following formula: *Fear bias = p(saccade/NE&HA) - p(saccade/FE)*.

Statistical Analyses

Statistical analyses were performed with R 3.6.1 software using *phyloseq* (McMurdie & Holmes, 2014) and *microbiome* (Lahti & Shetty, n.d.) R/Bioconductor packages. Alpha diversity (Shannon index) and Chao1 richness were calculated with the microbiome R package based on OTU-level resolution. Covariate or confounder candidates (maternal age and education, BMI, prenatal SSRI/SNRI use, prenatal end-of-pregnancy depressive symptoms, infant gestational age at birth, birth length and height, age at fecal sampling, breastfeeding status, infant antibiotics intake, and mode of delivery) potentially associating

with fecal microbiota composition and/or infant attention to emotional faces were selected based on previous literature (Fu & Pérez-Edgar, 2019; E.-L. Kataja et al., 2018; Stewart et al., 2018; Vatanen et al., 2018). Covariate associations with the normally distributed main dependent variables (fear and face bias, Shapiro-Wilk test $p>0.05$ for both) were investigated with Pearson's correlation coefficients, ANOVA or two groups unpaired t-test. Covariate associations with the main independent variables (Shannon Index and Chao1, Shapiro-Wilk test $p<0.05$ for both) were investigated with Spearman's rank correlation coefficients, Kruskall-Wallis rank sum test or Wilcoxon rank sum test. Covariate associations with beta diversity (i.e. interindividual variation in community composition) was quantified with PERMANOVA from R package *vegan* (Oksanen et al., 2017) with rarefied OTU counts and Bray-Curtis dissimilarity using 999 permutations. Beta diversity describes how different individuals' microbiota communities are from one another. Covariate candidates associated with either the face or fear bias or alpha or beta diversity were included in the adjusted models (see Results) in addition to infant sex, as we aim at studying the interaction by sex.

To search for associations between infant Shannon Index, Chao1, and fear and face bias, a linear regression model was applied after confirming that the dependent variables (fear bias and face bias) were normally distributed. Chao1 (skewness = 0.30, kurtosis = 2.78) was log-transformed. Beta diversity was tested with PERMANOVA (Oksanen et al., 2017) as described above. Differences in genus abundances were calculated with DESeq2 (Love, Huber, & Anders, 2014), which uses negative binomial generalized linear modes and reports $\log_2$-Fold Changes (LFC). Only genera with >50% prevalence were included in the differential abundance test in order to focus on the core microbiota and reduce multiple testing. To validate the observed taxa that associated with the dependent variable, a random forest regression model was built with 501 trees using the *randomForest* R package (Liaw & Wiener, 2002). Abundance matrix of clr-transformed genera with >50% prevalence was used

as input and the most important features were quantified as unit percentage increase in the Mean Squared Error. Sensitivity analyses were performed by including gestational age as a covariate in the models, as gestational age (premature, term, post term) may cause important variations both to the composition of the fecal microbiota (Fouhy et al., 2019) and to the development of attention problems (Eryigit-Madzwamuse & Wolke, 2015). The analyses were adjusted for multiple comparisons with the Benjamini-Hochberg method, and findings with False Discovery Rate (FDR) ≤0.01 and, when applicable, absolute LFC > 1 were considered statistically significant.

Results

Descriptives

Sample characteristics are presented in Table 1. Most infants were born vaginally in term and were breastfed during the fecal sample collection (Table 1). Nine infants with missing data in covariates were excluded from analyses, resulting in a final sample size of 122 (Boys, n=65, 53%).

The most abundant and prevalent genera (n=122) were in descending order as follows: unidentified genus in *Enterobacteriaceae* family, *Bacteroides*, *Bifidobacterium*, *Veillonella*, and *Clostridium* (prevalence 100% and the mean relative abundance ≥9% for all). Delivery by C-section was associated with lower Shannon Index (mean=1.6, SD=0.5, unadjusted p=0.02) and Chao1 (mean=126, SD=26; unadjusted p= 0.001) as well as beta diversity (unadjusted p=0.003). Shannon Index was associated with breastfeeding (exclusively breastfed infants had lowest Shannon Index, unadjusted p=0.01) and age at fecal sampling (oldest subjects having higher Shannon Index, unadjusted p=0.02).

All infants disengaged their attention most probably when shown control pictures (M=0.80, SD=0.21), following neutral (M=0.62, SD=0.26), happy (M=0.62, SD=0.27), and

fearful faces (M=0.47, SD=0.28). There was also an age-typical bias for faces (face bias, M=0.18, SD=0.24) and for fear (fear bias, M=0.15, SD=0.20) across the whole sample. Boys and girls did not differ in any of the attention variables (all unadjusted p-values > 0.15 for the two-sided t-tests). Fear bias was positively associated with maternal depressive symptoms at gwk 34 (r=0.22, p=0.01). Face bias was not related to any of the pre-selected background variables (i.e., maternal age and education, BMI, prenatal SSRI/SNRI use, depressive symptoms at the end of pregnancy, infant gestational age at birth, birth length and height, age at fecal sampling, breastfeeding status, infant antibiotics intake, and mode of delivery).

Variables associated with either Shannon Index, Chao1, or face or fear biases were included as covariates. In addition, as we aimed to assess sex interactions, infant sex was included as a covariate. Hence, mode of delivery, breastfeeding, infant age at fecal sampling, maternal depressive symptoms at the end of pregnancy, and infant sex were included as covariates.

Diversity and Fear and Face Bias

Neither Shannon Index (fear bias p=0.70, face bias p=0.30), Chao1 (fear bias p=0.34, face bias p=0.41) nor beta diversity (fear bias p=0.23, face bias p=0.96) were associated with fear or face bias scores despite of the mode of delivery, breastfeeding, infant sex, fecal sampling age and depressive symptoms at gwk 34. Further, no sex interaction with either face (Shannon Index unadjusted p=0.26, Chao1 unadjusted p=0.85 for interaction terms) or fear bias (unadjusted p=0.57, Chao1 unadjusted p=0.19 for interaction terms) was observed.

Genus Level Associations with Fear and Face Bias

When adjusted for the mode of delivery, breastfeeding status, depressive symptoms at gwk 34, and infant sex and age at fecal sampling, the abundances of *Lactobacillus* from the phylum Firmicutes, *Bifidobacterium* from phylum Actinobacteria, *Prevotella* from phylum

Bacteroidetes, and *Haemophilius* from phylum Proteobacteria were negatively associated with fear bias (Table 2, Fig. 2.). *Clostridium* genus from phylum Firmicutes was positively associated with fear bias (Table 2, Fig. 2.). Fear bias did not show sex interaction with genera (FDR >0.015). The random forest model showed that the five most important features related to fear bias were *Bifidobacterium, Actinomyces, Clostridium, Collinsella,* and *Parabacteroides*.

Face bias was not associated with the tested genera, but *Clostridium* (LFC=-9, FDR= 0.0017), *Parabacteroides* (LFC=-13, FDR= 0.0009), unidentified genus on *Lachnospiraceae* family (LFC=12, FDR=0.0017), *Collinsella* (LFC=18, FDR= 0.00003), and *Citrobacter* (LFC=8, FDR=0.0047) showed sex interaction with face bias (Supplementary Fig. 1).

Sensitivity Analyses

Including prematurity as a covariate, the association patterns regarding LFC direction and the value of FDR remained the same, except that additionally a new association between fear bias and *Enterococcus* (LFC=-6, FDR=0.0078) was observed.

Discussion

Gut microbiota has been suggested to influence behavior and emotion processing in rodents, but the translatability of the findings has been questioned (Hooks, Konsman, & O'Malley, 2019). In this study, we show as the first line of evidence that an early snapshot of fecal microbiota composition is associated with infant attention to emotional faces including a preference for faces expressing fear. The face-distractor paradigm, used in our study, has been used in several independent studies to assess orienting responses to faces depicting different emotions and also the influence of faces and emotional expressions on attention shifts between faces and distractors (Fu & Pérez-Edgar, 2019; Leppänen, 2016). We failed to find associations between emotional attention and microbial diversity, but our findings show

interesting associations between infant fecal microbiota genus abundances and early emotional attention, and specifically the fear processing, a component of self-regulation and emotional processing that may affect future social-emotional development.

More specifically, in the current study, the genera *Lactobacillus, Prevotella*, and *Bifidobacterium* were negatively and *Clostridium* was positively associated with attention bias for fearful faces. Heightened fear bias is a part of typical infant development, and therefore it is very difficult to estimate the level of "normal" and "abnormal" fear bias especially in infancy at the time of its developmental peak. However, in some longitudinal infant studies, attention biases for negative information have been found to predict a risk of self-regulation difficulties (e.g., Morales et al., 2016; Nakagawa & Sukigara, 2012), especially if consistently high or connected with other risk factors, such as negative emotionality. In a previous study, (using a paired-face paradigm with neutral and emotional faces and long stimulus presentation times), increased initial attention to and greater disengagement from fearful faces (i.e., signals of threat in the environment) at the age of seven months was positively associated with prosocial behavior later in life (Grossmann, Missana, & Krol, 2018). Therefore, it may be that a low initial orientation towards but longer dwell times on fearful faces are unfavorable for socioemotional development. Additionally, the normative bias towards fearful faces in infancy (Peltola et al., 2009) may even predict positive outcomes, such as better attachment security in future (Peltola et al., 2015). Then again, higher attention bias towards threat at the age of five years was reportedly associated with more internalizing symptoms in the presence of temperamental behavioral inhibition (Nozadi et al., 2016). Hence, variation in the tendency to disengage from signals of fear may predict differences in social-emotional development especially when combined with other risk factors (Morales, Pérez-Edgar, & Buss, 2015). In addition to the assessment of additional risk factors for maladaptive socioemotional development, such as temperament, future studies

should assess emotional attention longitudinally, as persistent biased attention might be more important for socioemotional development in comparison to transient differences in its magnitude (Morales et al., 2016; Pérez-Edgar et al., 2010).

Interestingly, recent gut microbiota targeted interventions with pre- or probiotic products, including supplementation with *Bifidobacterium* and *Lactobacillus* strains, have induced changes in (self-reported) emotional processing, outcomes ranging from reduced cognitive reactivity, sad mood, and reduced attentional vigilance to negative information (Schmidt et al., 2015; Steenbergen, Sellaro, van Hemert, Bosch, & Colzato, 2015), and differential activation patterns in brain areas related to emotional processing (Allen et al., 2016; Bagga et al., 2018; Pinto-Sanchez et al., 2017; Schmidt et al., 2015; Steenbergen et al., 2015; Tillisch et al., 2013). Further, intervention studies in adults have reported altered activity in brain regions related to emotional processing, including the amygdala (Pinto-Sanchez et al., 2017; Tillisch et al., 2013), which also relates to the processing of emotional facial expressions, particularly fear (Leppänen & Nelson, 2012; Marsh, 2016). A recent study also suggested that in a sample of 5- to 11-year-olds, the fecal microbiota characteristics, including *Lachnospiraceae* from *Clostridiales* order, were positively associated with the activation of the prefrontal cortex in response to sad faces (Callaghan et al., 2019), but comparisons cannot be made due to differences in age range. Thus, the current study is the first to address the potential role of abundant members of fecal microbiota, such as genera *Lactobacillus* and *Bifidobacterium,* in decreasing the attention to fearful faces, even though more research is needed on the role of early attentional biases and gut microbiota alterations on later developmental outcomes.

Abundant *Clostridium* genus was the only genus associated with the increased attention to fearful faces. Interestingly, in a previous study conducted with a toddler population of three-year-olds, poorer personal, social, and communication skills were

associated with a higher abundance of *Clostridiales* taxa (Sordillo et al., 2019). Moreover, another study reported a link between increased *Clostridiales* abundance and autism spectrum disorder (De Angelis et al., 2013). The dynamic changes in the microbiota during the first years of life (Stewart et al., 2018) make the comparisons between ages difficult. Despite this, we still observed similar fecal microbiota characteristics associated with social-emotional processing as previous studies in older populations (De Angelis et al., 2013; Sordillo et al., 2019).

Reduced microbial alpha diversity has been associated with higher fear reactivity (Aatsinki, 2019) as well as an increased connectivity in the left amygdala with the thalamus in infants (Gao et al., 2019), which is interesting as the amygdala is a central structure in the processing of emotionally salient information (Schumann, Bauman, & Amaral, 2011) and specifically fear (Marsh, 2016; Marsh et al., 2008). Based on these previous reports concerning fecal microbiota alpha diversity (Aatsinki et al., 2019; Carlson et al., 2018; Gao et al., 2019), one could have expected to observe an association between infant fecal microbiota alpha diversity and attention to fearful faces, but no such correlations were observed. This might be due to a larger sample size in comparison to the previous studies, or younger population with a majority of exclusively breastfed infants (Bäckhed et al., 2015), which reportedly decreases the alpha diversity. Further, diversity does not necessarily characterize the underlining fecal microbiota communities in this age group as well, i.e. individuals may have similar diversity values, but different metabolic effects of the gut microbiota communities. Hence, we did not find associations between fecal microbiota diversity and emotional attention but we observed only associations with the genera composition of the fecal communities. However, these previous results combined with the current report suggest that early fecal microbiota may associate with future emotional and cognitive development, and differences in attention to social-emotional cues may be one mechanism mediating the

deviant behavioral phenotypes, even though the exact microbiota function and hence the mechanism remains elusive.

Due to the fact that microbiota has been suggested to affect neurodevelopment in a sex-specific manner (Clarke et al., 2013) and that there is support for females showing higher and more rapidly developing self-regulatory capacities than males during childhood (Else-Quest, Hyde, Goldsmith, & Van Hulle, 2006), sex interaction with fear bias could have been anticipated. However, we did not note any sex-interaction regarding disengagement from fearful faces but observed a sex interaction with infant attention to faces in general (i.e., face bias) including genera *Clostridium, Parabacteroides,* unknown genus in *Lachnospiraceae* family, *Collinsella*, and *Citrobacter*. However, the sample size might be too limited to detect the hypothesized sex differences in the associations. Additionally, we did not observe genera associations with the probability to disengage from faces, which might be related to the ambiguous role of infant attention to faces regarding the infant development. On one hand, engaging with faces (i.e., higher face bias) has been shown to be essential for social-emotional development and future social behavior as the early attention bias to faces was associated with later helping responses and lower callous-unemotional behaviors (Peltola et al., 2018). On the other hand, a flexible shifting of attention between faces and lateral distractor stimuli in the face-distractor paradigm has, in turn, been found to be related to higher parent-reported infant soothability, which is one aspect of self-regulation (Leppänen et al., 2011).

In rodents, the absence of microbiota affects social interaction and differential gene activation in the amygdala after social interaction (Stilling et al., 2018) and reduces learning from fearful experiences and alters the transcriptional profile in amygdala (Hoban et al., 2018). The connections between the gut and the brain are at least to some extent mediated by the vagus nerve, as *Bifidobacterium longum* administration has been reported to ameliorate

chronic gut inflammation and reduce anxiety-like behavior via the vagus nerve (Bercik et al., 2011). Similarly, probiotic treatment with certain *Lactobacillus rhamnosus* strain changes fear-related behavior and expression of GABA-receptors in the amygdala, prefrontal cortex, and hippocampus (Bravo et al., 2011), but these effects are not present in vagotomised mice. Then again, the behavioral profile may affect how an individual interacts with others and the environment that can affect the transmission of gut microbiota species, which may happen in repeated social contact (Brito et al., 2019; Moeller et al., 2016). However, the social transmission of gut microbiota is a more important factor in older children, and the mother is the main source of microbiota transmission for infants in comparison to other family members (Korpela et al., 2018). Hence, the exact mechanisms mediating the reported associations in humans remain largely unknown, but our results are an encouraging example of progress for future human studies aimed at illustrating the underlying neural structures of emotional attention and self-regulation with respect to fecal microbiota composition.

Our study describes an association between fecal microbiota profile and emotional attention, which are both developmental processes (Vatanen et al., 2018), and the study would have benefited from a longitudinal assessment of fecal microbiota. However, previous rodent studies show that the alterations in the gut microbiota composition during early life may cause behavioral changes that are not reversible with the manipulation of the microbiota and immunity in the adulthood (Neufeld et al., 2011). This supports the theory that early life is an sensitive time window, when microbiome can have greater influence in the development of an individual (Codagnone et al., 2019). Thus, it could be hypothesized that early alterations in both emotional attention and microbiota are essential for their developmental trajectories. However, good quality longitudinal human studies in this area are still scarce. Thus, when interpreting our results, it should be noted that they only describe a snapshot of associations during infancy. In the future, intervention and longitudinal studies are needed in

order to better understand how the microbiota and the emotional attention / self-regulation developmental trajectories are related to each other, as well as their potential significance for later social-emotional development.

Certain limitations should be considered while interpreting the results. The 16S rRNA amplicon sequencing does not permit the strain-level or functional capacity assessment, which may be more informative than taxonomic profiling (Heintz-Buschart & Wilmes, 2018; Savignac, Kiely, Dinan, & Cryan, 2014). Additionally, we focused the analyses on high-prevalence genera as both the low prevalence and low abundance taxa may show potentially spurious associations that are not biologically significant. The random forest model supported the link between fear bias and *Bifidobacterium* and *Clostridium*, and the fact that *Prevotella*, *Haemophilus* and *Lactobacillus* were not as important features as the aforementioned may be related to the unadjusted nature of random forest regression. We included maternal prenatal depressive symptoms into our models as they are known to affect both fecal microbiota composition (Zijlmans, Korpela, Riksen-Walraven, de Vos, & de Weerth, 2015) and emotional attention (E.-L. Kataja et al., 2018) in infants. Further, the manner in which the other prenatal factors, such as glucocorticoid (Cortese, Lu, Yu, Ruden, & Claud, 2016; Vázquez, Neal, Patel, Kaciroti, & López, 2012) or SSRI/SNRI (Erickson et al., 2019; Ramsteijn, Jašarević, Houwing, Bale, & Olivier, 2020) exposure, influence the association between emotional attention and fecal microbiota, was not within the reach of the current study design and sample. Additionally, potential mediators or moderators such as breast milk composition (Di Benedetto, Bottanelli, Cattaneo, Pariante, & Borsini, 2019) or other maternal characteristics remain to be studied. The effect sizes are small, but this can be expected as the development of emotionally oriented attention is multifactorial (Fu & Pérez-Edgar, 2019).

Conclusion

To conclude, we report novel findings describing the associations between attention to emotional faces and early fecal microbiota composition in a study population of infants. The heightened attention bias towards fearful faces seems to be associated with a decreased abundance of *Bifidobacterium* and an increased abundance of *Clostridium*, which are abundant members of the infant fecal microbiota. We corroborate findings from earlier literature on the potential role of probiotic bacteria (Allen et al., 2016; Bagga et al., 2018; Steenbergen et al., 2015) and *Clostridium* species (De Angelis et al., 2013; McGeachie et al., 2016) on the emotional processing and brain development. However, our results describe a single time point and require replication in independent cohorts and in future longitudinal studies of infant fecal microbiota and social-emotional development.

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Data statement

Due to Finnish federal legislation, the research data cannot be made available online, but data can potentially be shared with Material Transfer Agreement. Requests can be directed to the Board of the FinnBrain Birth Cohort Study. Please contact Linnea Karlsson (linnea.karlsson@utu.fi).

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Table 1. Mother and infant characteristics.

Variable	**Categories / unites**	**n = 131**
Mother's age at birth	mean (SD) yeas	31 (4)
Mother's education	n (%)	
	secondary or primary level	39 (29.8)
	vocational tertiary	43 (32.8)
	university	47 (35.9)
	missing data	2 (1.5)
Mother's SSRI/SNRI use in the beginning of pregnancy	n (%)	
	no	123 (93.9)
	yes	5 (3.8)
	missing data	3 (2.3)
Infant's sex	n (%)	
	boy	69 (52.7)
	girl	62 (47.3)
Mother's BMI in the beginning of pregnancy	n (%)	

	BMI <25 (normal weight)	78 (59.5)
	BMI 25-30 (overweight)	38 (29)
	BMI >30 (obese)	14 (10.7)
	missing data	1 (0.8)
Gestational weeks at birth	mean (SD) weeks	40 (1)
Prematurity	n (%)	
	Premature (< 37 gestational weeks)	2 (1.5)
	Term (37-42 gestational weeks)	100 (76.3)
	post term (≥ 42 gestational weeks)	29 (22.1)
Birth weight	mean (SD) grams	3632 (456)
Mode of delivery	n (%)	
	C-section	25 (19.1)
	vaginal	105 (80.2)
	missing data	1 (0.8)
Breastfeeding at the time of fecal sampling	n (%)	
	never breastfed	2 (1.5)

	breastfeeding ceased	7 (5.3)
	partial breastfeeding	19 (14.5)
	exclusive breastfeeding	99 (75.6)
	missing data	4 (3.1)
Infant's antibiotic courses	n (%)	0 (0)
Infant's age at the fecal sampling	mean (SD) days	69 (14)
EPDS total score sum	mean (SD)	
	at the beginning of pregnancy	5 (5)
	at the end of pregnancy	5 (5)

BMI: Body Mass Index; kg/m2

EPDS : Edinburgh Postnatal Depression Scale

Table 2. Genera associated with fear bias when adjusting for mode of delivery, breastfeeding, sex, age at sampling and EPDS at the end of pregnancy.

Genus	**Baseline Mean Abundance**	**log_2-FoldChange**	**FDR**	**95% Confidence Interval**
Bifidobacterium	56593	-5.3	0.0003	-7.8 to -2.8
Prevotella	38651	-5.7	0.0010	-8.8 to -2.7
Lactobacillus	2598	-6.5	0.0003	-9.4 to -3.5
Clostridium	41276	5.4	0.0010	2.5 to 8.2
Haemophilus	1216	-6.8	0.0010	-10.4 to -3.2

EPDS: Edinburgh Postnatal Depression Scale

Figure 1. Illustration of the overlap paradigm used in the eye-tracking experiment to assess infant's attention to social signals of emotion. After the infant looked at a fixation stimulus in the center of the screen (red circle), a face or a scrambled face pattern and subsequently a high-contrast lateral distractor were presented. The probability of attention disengagement from the central to the lateral stimulus was analyzed from the eye tracking data and used as a measure of attention to scrambled face patterns and neutral, happy, and fearful faces. The neutral, happy, and fearful faces are presented in a previous publication (E.-L. Kataja et al., 2018).

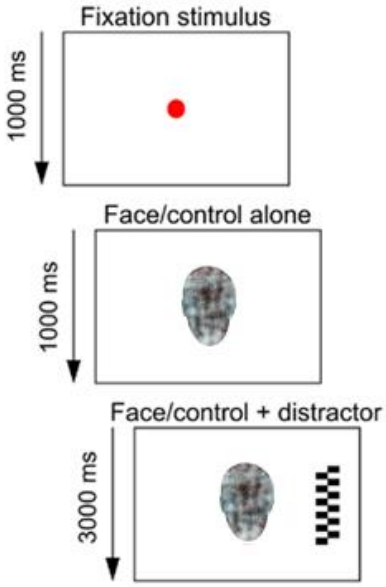

Figure 2. Fear bias associations with bacterial genera. For illustration, CLR-transformed abundances are used.