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Gut microbiota composition is associated with temperament traits in infants

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A B S T R A C T

Background: One of the key behavioral phenotypes in infancy are different temperament traits, and certain early life temperament traits have been shown to precede later mental health problems. Differences in the gut microbiota composition (GMC) have been suggested to link with neurodevelopment. For example, toddler temperament traits have been found to associate with differences in GMC; however, studies in infants are lacking although infancy is a rapid period of neurodevelopment as well as GM development. Thus, we aimed to investigate association between infant GMC and temperament.

Methods: The study population (n = 301, 53% boys) was drawn from the FinnBrain Birth Cohort Study. Stool samples were collected from the 2.5-month-old infants and sequenced with 16S Illumina MiSeq platform. GMC taxonomic composition (at Genus and OTU level), observed sample clusters, diversity and richness were investigated in relation to the maternal reports of Infant Behavior Questionnaire -Revised (IBQ-R) at the age of 6 months.

Results: Three sample clusters (*Bifidobacterium/Enterobacteriaceae, Bacteroides, V. Dispar)* based on GMC were identified, of which *Bifidobacterium/Enterobacteriaceae*–cluster presented with higher scores on the IBQ-R main dimension regulation and its subscale duration of orienting compared to *Bacteroides*-cluster. The clusters associated with temperament in a sex-dependent manner. The IBQ-R main dimension surgency (positive emotionality) was associated positively both with genus *Bifidobacterium* and *Streptococcus*. Alpha diversity had a negative association with negative emotionality and fear reactivity.

Conclusion: This is the first study demonstrating associations, but not causal connections, between GMC and temperament in young infants in a prospective design.

1. Introduction

The gut microbiota is a densely populated microbial ecosystem that is a central component in host physiology and metabolism (Cani, 2018; Lynch and Pedersen, 2016). During the past decade aberrations in the gut microbiota composition (GMC) and diminished diversity have been linked to a wide range of somatic disorders, as well as brain-related conditions such as Parkinson's disease (Scheperjans et al., 2015), depression (Kelly et al., 2016), autism (Kang et al., 2013) and attention deficit hyperactivity disorder (ADHD) (Aarts et al., 2017; Jiang et al., 2018). The microbial colonization of the human gastrointestinal tract takes place in parallel with the neurodevelopment during one of the critical developmental windows in early life (Borre et al., 2014). Both the gut and the brain go through rapid changes during early postnatal period (Borre et al., 2014). Disruption during the early colonization process can lead to the impairment in hypothalamus-pituitaryadrenal gland (HPA)-axis functioning (Sudo et al., 2004), maturation of microglia (Erny et al., 2015), brain cytokine profile, blood-brain barrier integrity and consequently, alterations in behavior (Leclercq et al., 2017).

Recent studies indicate that the gut microbes play essential roles in the neurodevelopment and control of behavior (Rogers et al., 2016; Sampson and Mazmanian, 2015). Gut microbiota is capable of communicating with the central nervous system (CNS) via the vagal nerve

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Table 1
Study participant characteristics and temperament traits by infant sex. Temperament traits are organized by main domains. Only FDR-values < 0.25 are reported. Gestational weeks = gwk, Not available = NA.

Mean/Count (SD/%)		Overall	Boys	Girls	FDR
		n = 301	n = 159 (52.8%)	n = 142 (47.2%)	
Mothers age, years		30.8 (4.3)	31.0 (4.4)	30.6 (4.1)	
Mothers education, n					
	Upper secondary	67 (22.3%)	33 (20.8%)	34 (23.9%)	
	Vocational school	97 (32.2%)	49 (30.8%)	48 (33.8%)	
	Tertiary education	128 (42.5%)	71 (44.7%)	57 (40.1%)	
	NA	9 (3%)	6 (3.8%)	3 (2.1%)	
Gestational age, weeks		40.2 (1.4)	40.1 (1.5)	40.3 (1.2)	
Gestational stage					
	preterm, < 37 gwk	12 (4%)	9 (5.7%)	3 (2.1%)	
	early term, < 39 gwk	36 (12%)	23 (14.5%)	13 (9.2%)	
	full term, < 40 gwk	69 (22.9%)	37 (23.3%)	32 (22.5%)	
	late term, < 42 gwk	93 (30.9%)	42 (26.4%)	51 (35.9%)	
	post term, ≥ 42 gwk	91 (30.2%)	48 (30.2%)	43 (30.3%)	
	NA	0 (0%)	0 (0%)	0 (0%)	
Birth weight, g		3622.5 (455.0)	3680.0 (482.6)	3558.1 (414.1)	0.15
	NA	1 (0.3%)	0 (0%)	1 (0.7%)	
Vaginal delivery, n		248 (82.4%)	133 (83.6%)	115 (81%)	
	NA	2 (0.7%)	0 (0%)	2 (1.4%)	
Breastfeeding, n					
	Exclusive breastfeeding	236 (78.4%)	121 (76.1%)	115 (81%)	
	Partial breastfeeding	47 (15.6%)	30 (18.9%)	17 (12%)	
	Cessation before 2.5 months age	13 (4.3%)	7 (4.4%)	6 (4.2%)	
	No breastfeeding	5 (1.7%)	1 (0.6%)	4 (2.8%)	
	NA	0 (0%)	0 (0%)	0 (0%)	
Antibiotic treatments, n		37 (12.3%)	26 (16.4%)	11 (7.7%)	0.15
	NA	1 (0.7%)	0 (0%)	1 (0.7%)	
Infant age during sampling, days		65.2 (13.4)	64.8 (13.3)	65.7 (13.5)	
Surgency/positive emotionality		4.8 (0.7)	4.8 (0.7)	4.8 (0.7)	
	Activity level	4.5 (1.0)	4.5 (1.0)	4.5 (1.0)	
	Smiliness	6.1 (0.8)	6.2 (0.7)	6.0 (0.8)	
	High intensity pleasure	4.3 (1.2)	4.3 (1.1)	4.3 (1.2)	
	Vocational reactivity	4.4 (1.0)	4.3 (1.1)	4.4 (1.0)	
Negative emotionality		3.1 (0.8)	3.1 (0.8)	3.1 (0.8)	
	Distress to limitations	3.5 (1.1)	3.5 (1.1)	3.5 (1.1)	
	Fear	2.6 (1.2)	2.4 (1.2)	2.7 (1.1)	0.14
	Falling reactivity	5.2 (1.0)	5.2 (0.9)	5.2 (1.0)	
	Sadness	3.6 (1.1)	3.5 (1.2)	3.6 (1.1)	
Regulation/Orienting		5.3 (0.6)	5.3 (0.6)	5.4 (0.6)	
	Cuddliness	5.8 (0.8)	5.8 (0.9)	5.9 (0.7)	
	Soothability	6.1 (0.7)	6.1 (0.7)	6.1 (0.7)	
	Duration of orienting	4.3 (1.3)	4.2 (1.2)	4.3 (1.3)	
	Low intensity pleasure	5.2 (1.0)	5.1 (1.0)	5.3 (1.0)	
	Perceptual sensitivity	3.9 (1.6)	3.8 (1.6)	3.9 (1.6)	

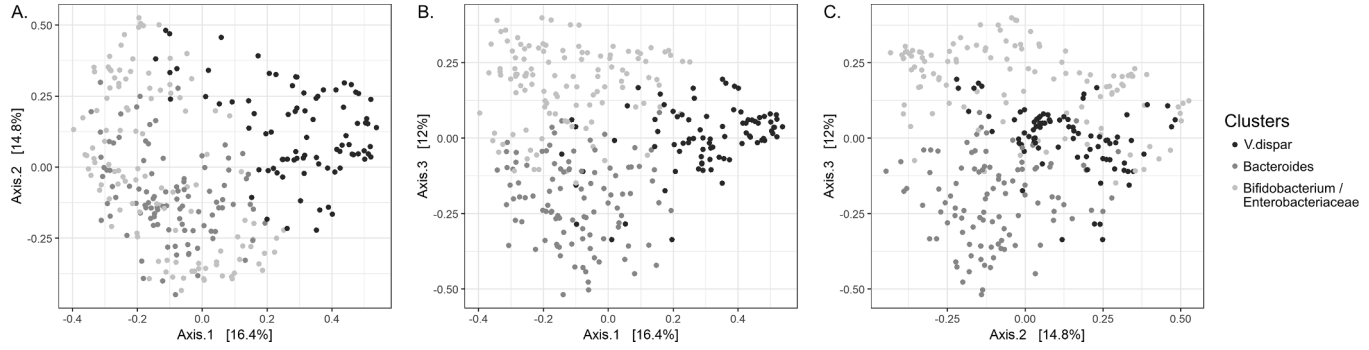

Fig. 1. Three GM clusters (*V. dispar, Bacteroides, Bifidobacterium/Enterobacteriaceae*) were identified in study population. Clusters illustrated here in PCoA plot in three dimensions: A) axes 1 and 2; B) axes 1 and 3; and C) axes 2 and 3.

(Bravo et al., 2011), and both the cytokine (Dantzer et al., 2000; van Dam et al., 1992; Xu et al., 2016) and neurotransmitter (O'Mahony et al., 2015) production and thus, potentially participates in behavioral regulation. Sub-optimal gut microbial colonization during the early developmental windows may be detrimental for some neurodevelopmental outcomes (Rogers et al., 2016; Sampson and Mazmanian, 2015).

In humans, differences in the behavior can be assessed by temperament (Rothbart, 2007) which refers to the biologically-based individual variation in behavior, more specifically, activity, affectivity and self-regulation (Rothbart, 2007). Temperament is considered to be rather stable feature of an individual, although maturation processes are also reflected in the changes in temperament traits and especially in the development of self-regulation (Montroy et al., 2016). Importantly, some temperament traits are shown to precede mental health problems

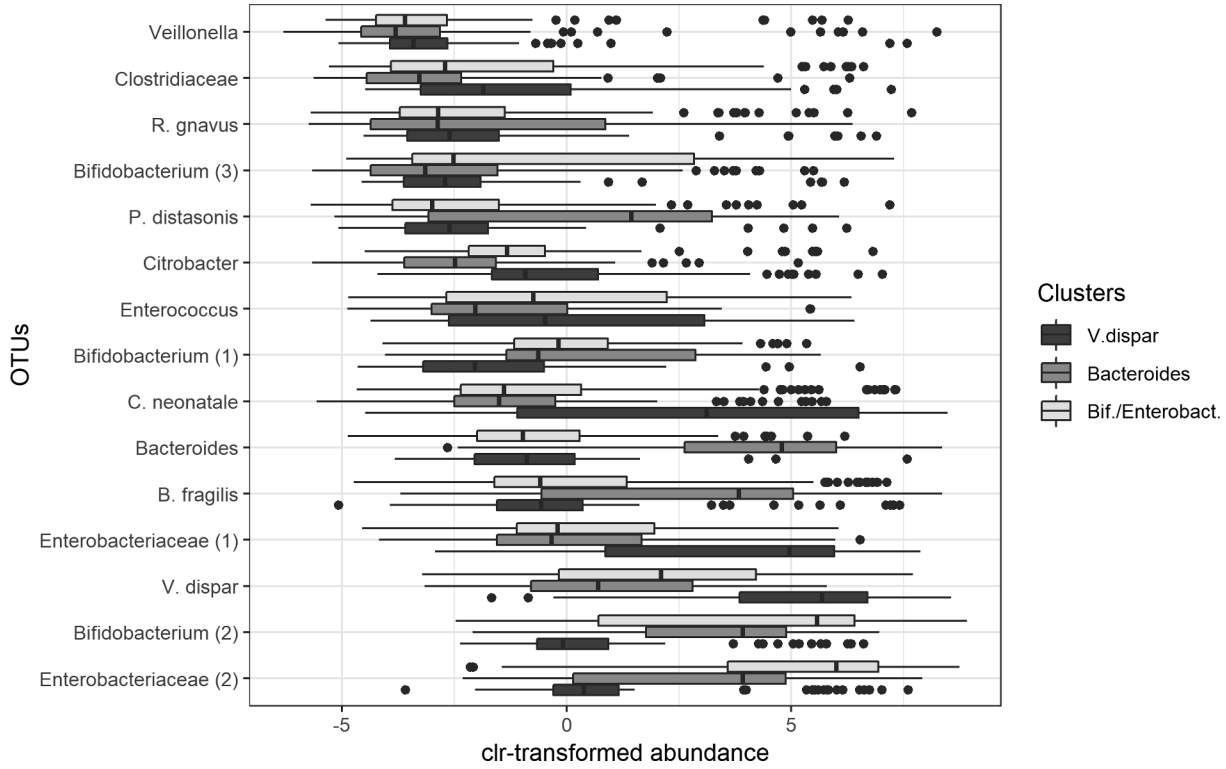

Fig. 2. Compositional differences in the 15 most abundant OTUs (clr-transformed abundance) between the clusters.

(Sayal et al., 2014). For instance, higher negative emotionality early in life is related to an increased risk of developing anxiety disorder (De Pauw and Mervielde, 2010) depressive symptoms (Compas et al., 2004), ADHD (Gomez and Corr, 2014) and autism (Visser et al., 2016). Child temperament traits are also suggested to be linked with differences in HPA-axis functioning, for instance, higher levels of cortisol have been reported in individuals with the temperament trait of higher fear reactivity (Talge et al., 2008) further communicating the physiological basis of temperament.

To our knowledge, regardless of the increasing evidence linking GMC and behavior, relatively few studies have investigated the possible correlation between GMC and temperament traits in humans. Christian et al. (2015) reported that the main dimension of surgency/positive emotionality was associated with greater gut microbiota phylogenetic diversity in toddlers at 18–27 months of age (Christian et al., 2015). Interestingly, the findings were mostly sex-specific: surgency was associated with higher alpha and beta diversity, alongside with higher abundances of *Dialister*, *Rikenellaceae*, *Ruminococcaceae* and *Parabacteroides* among boys, whereas fear reactivity was positively associated with *Rikenellaceae* among girls GMC (Christian et al., 2015). However, prospective human data investigating the associations between GMC and behavioral phenotypes during infancy, which is the most rapid period of neurodevelopment, are largely lacking and given the involvement of gut microbiota in the development of different behaviors, studies of this type are urgently needed.

The aim of this study is to extend the current research by investigating associations between gut microbiota and temperament in infancy. We aimed to study whether its composition (profile, genera, OTU's), alpha diversity and richness at the age of 2.5 months associate with infant temperament at the age of 6 months. Based on the previous literature, we were interested in the main dimensions (regulation, surgency, negative emotionality) and fear reactivity *a priori*, but also conducted exploratory analyses on all the temperament scales. We further hypothesized that early infant GMC, alpha diversity and richness would be associated with temperament traits in a sex-dependent manner.

2. Methods and Materials

2.1. Study design and demographics

The study population (n = 301, 159 boys and 142 girls) was drawn from the FinnBrain Birth Cohort Study (Karlsson et al., 2018) conducted in southwest Finland. The study has been approved by the Ethics Committee of the Hospital District of Southwest Finland. All mothers provided written informed consent on behalf of their children. Subjects with both successfully analyzed stool sample approximately at 2.5 months of age and a returned temperament questionnaire at 6 months were included in the analysis. The exact age during sampling was provided. Data collection occurred from 2013 until 2016.

Mothers provided information about their age, ethnicity, level of education (1: basic to upper secondary level, 2: vocational school diploma, 3: lower degree level tertiary education to doctorate or equivalent diploma), infant antibiotic intake and duration of exclusive and partial breastfeeding in months. Maternal reports of breastfeeding were categorized as no breastfeeding, cessation before 2.5 months of age, partial breastfeeding at the age of 2.5 months, and exclusive breastfeeding at the age of 2.5 months. Information about birth weight (g), height (cm), duration of gestation (weeks), antibiotics intake during neonatal period, and the mode of delivery [all caesarian (C)-section vs. all vaginal] was collected from National Birth Registry provided by the National Institute for Health and Welfare (www.thl.fi).

2.2. Infant temperament

Infant temperament was assessed using the maternal reports of Infant Behavior Questionnaire Revised Short Form (IBQ-R SF) at the age of 6 months (Putnam et al., 2014). The IBQ-R is a reliable and valid measure for infant temperament evaluation and consists of 91 items. In each question, mothers are asked to assess their infant's behavior in different everyday situations based on the past two weeks. The questionnaire is comprised of three main dimensions: negative emotionality (containing subscales distress to limitations, fear, sadness and reversed

Table 2
Cluster characteristics and temperament traits. Only FDR-values < 0.25 are reported. NA = not Available, gwk = gestational weeks.

Mean/Count (SD/%)		Bacteroides	V.Dispar	Bifidobacterium/Enterobacteriaceae	FDR
		n = 101	n = 84	n = 116	
Mothers age, years		30.9 (4.7)	30.9 (4.7)	30.7 (3.5)	
Mothers education, n					
	Upper secondary	24 (23.8%)	20 (23.8%)	23 (19.8%)	
	Vocational school	36 (35.6%)	17 (20.2%)	44 (37.9%)	
	Tertiary education	39 (38.6%)	44 (52.4%)	45 (38.8%)	
	NA	2 (2%)	3 (3.6%)	4 (3.4%)	
Gestational age, weeks		40.2 (1.2)	40.3 (1.5)	40.0 (1.5)	
Gestational stage					
	preterm, < 37 gwk	2 (2%)	3 (3.6%)	7 (6%)	
	early term, < 39 gwk	12 (11.9%)	9 (10.7%)	15 (12.9%)	
	full term, < 40 gwk	21 (20.8%)	23 (27.4%)	25 (21.6%)	
	late term, < 42 gwk	38 (37.6%)	18 (21.4%)	37 (31.9%)	
	post term, ≥ 42 gwk	28 (27.7%)	31 (36.9%)	32 (27.6%)	
	NA	0 (0%)	0 (0%)	0 (0%)	
Birth weight, g		3589.7 (445.0)	3610.1 (474.0)	3660.0 (450.8)	
	NA	1 (1%)	0 (0%)	0 (0%)	
Vaginal delivery, n		96 (95%)	57 (67.9%)	95 (81.9%)	0.00001025
	NA	2 (2%)	0 (0%)	0 (0%)	
Breastfeeding, n					
	Exclusive breastfeeding	75 (74.3%)	66 (78.6%)	95 (81.9%)	
	Partial breastfeeding	17 (16.8%)	15 (17.9%)	15 (12.9%)	
	Cessation before 2.5 months age	6 (5.9%)	3 (3.6%)	4 (3.4%)	
	No breastfeeding	3 (3%)	0 (0%)	2 (1.7%)	
	NA	0 (0%)	0 (0%)	0 (0%)	
Antibiotic treatments, n		7 (6.9%)	12 (14.3%)	18 (15.5%)	
	NA	1 (1%)	0 (0%)	0 (0%)	
Infant age during sampling, days		65.8 (13.7)	65.0 (13.7)	64.8 (12.9)	
Girls, n		60 (59.4%)	35 (41.7%)	47 (40.5%)	0.0464
Surgency/positive emotionality		4.7 (0.8)	4.7 (0.7)	4.8 (0.7)	
	Activity level	4.6 (1.0)	4.5 (1.0)	4.5 (1.0)	
	Smiliness	6.0 (0.8)	6.0 (0.7)	6.2 (0.8)	
	High intensity pleasure	4.4 (1.1)	4.2 (1.2)	4.4 (1.2)	0.23
	Vocational reactivity	4.3 (1.1)	4.3 (1.1)	4.4 (1.0)	
Negative emotionality		3.0 (0.8)	3.1 (0.8)	3.2 (0.8)	
	Distress to limitations	3.4 (1.0)	3.5 (1.1)	3.5 (1.1)	
	Fear	2.5 (1.2)	2.4 (1.1)	2.6 (1.2)	
	Falling reactivity	5.2 (1.0)	5.2 (1.0)	5.1 (0.9)	
	Sadness	3.5 (1.2)	3.5 (1.1)	3.7 (1.1)	
Regulation/Orienting		5.3 (0.6)	5.3 (0.6)	5.5 (0.6)	0.23
	Cuddliness	5.9 (0.8)	5.7 (0.8)	5.9 (0.8)	0.20
	Soothability	6.0 (0.7)	6.1 (0.7)	6.1 (0.7)	
	Duration of orienting	4.0 (1.4)	4.2 (1.1)	4.5 (1.2)	0.20
	Low intensity pleasure	5.1 (1.1)	5.2 (0.9)	5.2 (0.9)	
	Perceptual sensitivity	3.8 (1.7)	3.6 (1.5)	4.0 (1.6)	

scale of falling reactivity), regulation/orienting (subscales perceptual sensitivity, low intensity pleasure, cuddliness, duration of orienting and soothability) and surgency/positive emotionality (subscales activity level, smiling and laughter, high intensity pleasure, approach, vocal reactivity). Both individual item scores and subscale total scores range between 1 and 7. Cronbach's Alpha across subscales ranged from 0.65 to 0.84 and for main dimensions Cronbach's alphas were 0.88 for surgency (0.65–0.78 for its subscales), 0.85 for negative emotionality (0.72–0.81) and 0.80 for regulation/orienting (0.73–0.84).

2.3. Infant gut microbiota analysis

Parents collected the stool samples at 2.5 months of age at their homes into collection tubes, following both oral instructions upon recruitment and written instructions provided with sampling equipment. Parents were instructed to store the samples immediately at +4 °C, mark the date and time of the sample taking, and bring the sample to the laboratory as soon as possible and within 24 h after which the DNA was extracted from the samples. The samples were processed as previously described (Rintala et al., 2018). The MiSeq platform (Illumina, San Diego, CA) was used for 16S rRNA-sequencing of the V4 area. The quality of the raw reads was checked with FastQC (v. 0.10.1) after which the downstream analysis was carried out using QIIME (v.1.9) (Bokulich et al., 2013; Caporaso et al., 2010; Kuczynski et al., 2012). Reads were first quality filtered requiring at least 20 Phred quality score, resulting in 48 k–1063 k reads per sample (total: 54 476 k, mean: 183 k, sd: 116 k). Chimeric sequences were filtered out using usearch (v.6.1 against the GreenGenes database (v. 13.08) (DeSantis et al., 2006). Operational Taxonomic Units (OTUs) were picked using UCLUST with 97% sequence similarity and OTUs with less than 0.05% of total sequence count were removed. Annotations for the OTUs were derived from the GreenGenes database. (Additional details in Supplemental information, Methods and Materials.)

2.4. Statistical analyses

The statistical analyses were performed with R 3.5.0. software (R Core Team, 2017). Alpha diversity indices were calculated with *phyloseq* R package (McMurdie and Holmes, 2013). Shannon Index was measured to present the diversity and Chao1 to present the richness. Further statistical testing on richness was performed with Chao1, and this was log-transformed in order to obtain approximately normally distributed values for the statistical analyses. Principal Coordinates Analysis (PCoA) was conducted with *microbiome* package in R (Lahti et al., 2012–2017).

To assess broad characteristics of GMC, the subjects were clustered

Table 3
Linear regression models for each temperament trait and GMC parameter. All main dimensions (surgency, regulation, negative emotionality) and fear reactivity were investigated in the regression models as well as temperament traits associating in the exploratory analyses. Duration of orienting, cuddliness and high intensity pleasure were included in the regression models because they showed associations with clusters. Richness was not associated with temperament traits (p-value > 0.2). [a] Linear regression models assessing clusters as main independent variable were adjusted for infant, sex and mode of delivery, as those covariates had different distribution among clusters. [b] Linear regression models assessing diversity as main independent variable were adjusted for gestational age, infant age, infant sex, mode of delivery, antibiotics intake and breastfeeding status. [c] Estimates and FDR reported for interaction term (cluster × sex or Shannon Index × sex).

Adjusted regression models		Surgency	Regulation	Negative emotionality	Fear reactivity	Duration of Orienting	Cuddliness	High Intensity Pleasure
Clusters[a]								
Bifidobacterium/Enterobacteriaceae - Bacteroides	β	−0.07	−0.18	−0.20	−0.25	−0.51	−0.08	−0.16
	FDR	0.57	0.17	0.27	0.27	0.08	0.57	0.27
Bifidobacterium/Enterobacteriaceae - V. dispar	β	−0.16	−0.22	−0.07	−0.12	−0.30	−0.29	−0.20
	FDR	0.27	0.09	0.59	0.57	0.27	0.09	0.27
V. dispar - Bacteroides	β	0.09	−0.03	−0.13	−0.13	−0.21	−0.21	−0.01
	FDR	0.57	0.76	0.51	0.57	0.51	0.51	0.95
Diversity[b]	β	0.00	0.05	−0.17	−0.27			
	FDR	0.97	0.71	0.17	0.17			
Sex-interactions								
Clusters[c]								
Bifidobacterium/Enterobacteriaceae - Bacteroides	β	0.29	0.24	0.29	0.64	0.35	−0.14	0.09
	FDR	0.37	0.37	0.38	0.16	0.54	0.74	0.74
Bifidobacterium/Enterobacteriaceae - V. dispar	β	−0.14	−0.23	0.10	0.15	−0.79	−0.13	0.02
	FDR	0.74	0.38	0.74	0.74	0.16	0.74	0.95
V. dispar - Bacteroides	β	0.43	0.46	0.19	0.49	1.14	1.14	0.08
	FDR	0.16	0.07	0.70	0.37	0.03	0.03	0.77
Diversity[c]	β	0.01	0.06	0.01	0.02			
	FDR	0.96	0.96	0.96	0.96			

according to their GMC profiles using Bray-Curtis distance based on the OTU counts and the Partitioning Around Medoids (PAM) clustering method from the R package *cluster* (Maechler et al., 2018). The optimal number of clusters were assessed using the gap statistics. The R package *indicspecies* was used to detect the most discriminative OTUs among clusters (De Caceres and Legendre, 2009).

The dependent variables were temperament traits. The main independent variables were the following GMC parameters: OTU's, genera, diversity, richness or clusters. Covariates were selected based on theoretical assumptions derived from existing literature and association analyses of the current data set. Covariates included in the analysis were sex (when analyzing overall sample), the mode of delivery, gestational age, infant age during sampling, antibiotic treatments and breastfeeding at 2.5 months of age.

Associations between dependent and independent variables were investigated using Wilcoxon's rank-sum test, Kruskal–Wallis H test and Spearman's rank correlation coefficient. χ^2 test was used to analyze associations between categorical independent variables (birth mode, breastfeeding, child sex, antibiotic treatments, clusters). Linear regression models were built for the four *a priori* hypothesis (main dimensions of surgency, negative emotionality, regulation and the subscale of fear reactivity) and additionally the subscales that showed correlations in our exploratory analyses with the independent variables. In these regression models, GMC parameters (clusters and diversity) were the main independent variables and were to be modeled regardless the results of the unadjusted analyses. Sex differences in the associations between GMC parameters (clusters, diversity) and temperament traits were investigated by including an interaction term (diversity or cluster × sex) in the regression models.

The *DESeq2* R package, which uses shrinkage estimation for dispersions and fold changes to perform quantitative analysis of differential expression, was used to identify bacterial signatures with statistically significant association with temperament traits when adjusting for covariates (Love et al., 2014). The non-rarefied data limited to the core members (OTU's representing less than 0.1% abundance and with less than 5% prevalence were excluded) was used for statistical analysis. Due to the sex-dependent associations in the current study, the associations between GMC (genera, OTU's) and temperament were explored in boys and girls separately as suggested by previous literature (Christian et al., 2015) to inspect if the association profiles are different by sex.

P-values were adjusted for multiple testing using the Benjamini & Hochberg method (R function p.adjust), which provides estimates of the False Discovery Rate (FDR). We report findings with FDR < 0.25 as significant. Missing values of birth weight (n = 2), antibiotic intake (n = 1) and mode of delivery (n = 1) were imputed with the R package *mice* (van Buuren and Groothuis-Oudshoorn, 2011).

3. Results

3.1. Participant characteristics and temperament

Mean gestational age was 40.2 weeks and 12 subjects were born preterm (Table 1.). Boys had higher birth weight (W = 13060, FDR = 0.14) as well as frequency of antibiotic treatments (χ^2 = 4.4, FDR = 0.14, Table 1) than girls.

Girls showed higher fear reactivity in comparison to boys (W = 9292, FDR = 0.14, Table 1). Temperament traits were not associated with the other selected background factors (gestational age, the mode of delivery, birth weight, mother's education, BMI and age, breastfeeding at the age of 2.5 months or antibiotics intake) (FDR ≥ 0.42 Table 1).

3.2. Clusters in infant gut microbiota

The cluster analysis of GMC suggested the presence of three distinct community types (Fig. 1). The five most discriminating OTUs for each cluster were *V. dispar* (three different OTU's annotated as *V. dispar*), *Enterobacteriaceae, Clostridium neonatal e* (named as *V. dispar*-cluster, 27.9%), *Bacteroides* (×4) and *Bacteroides fragilis* (named as *Bacteroides* -cluster, 34.0%), and *Bifidobacterium*, *Enterobacteriaceae* (×4) (named as *Bifidobacterium/Enterobacteriaceae* -cluster, 38.4%) (Fig. 2). *Bacteroides*-cluster had highest and *V. dispar* -cluster had both lowest microbiota richness (Kruskall-Wallis H test χ^2 = 39, FDR < 10^{-8}) and

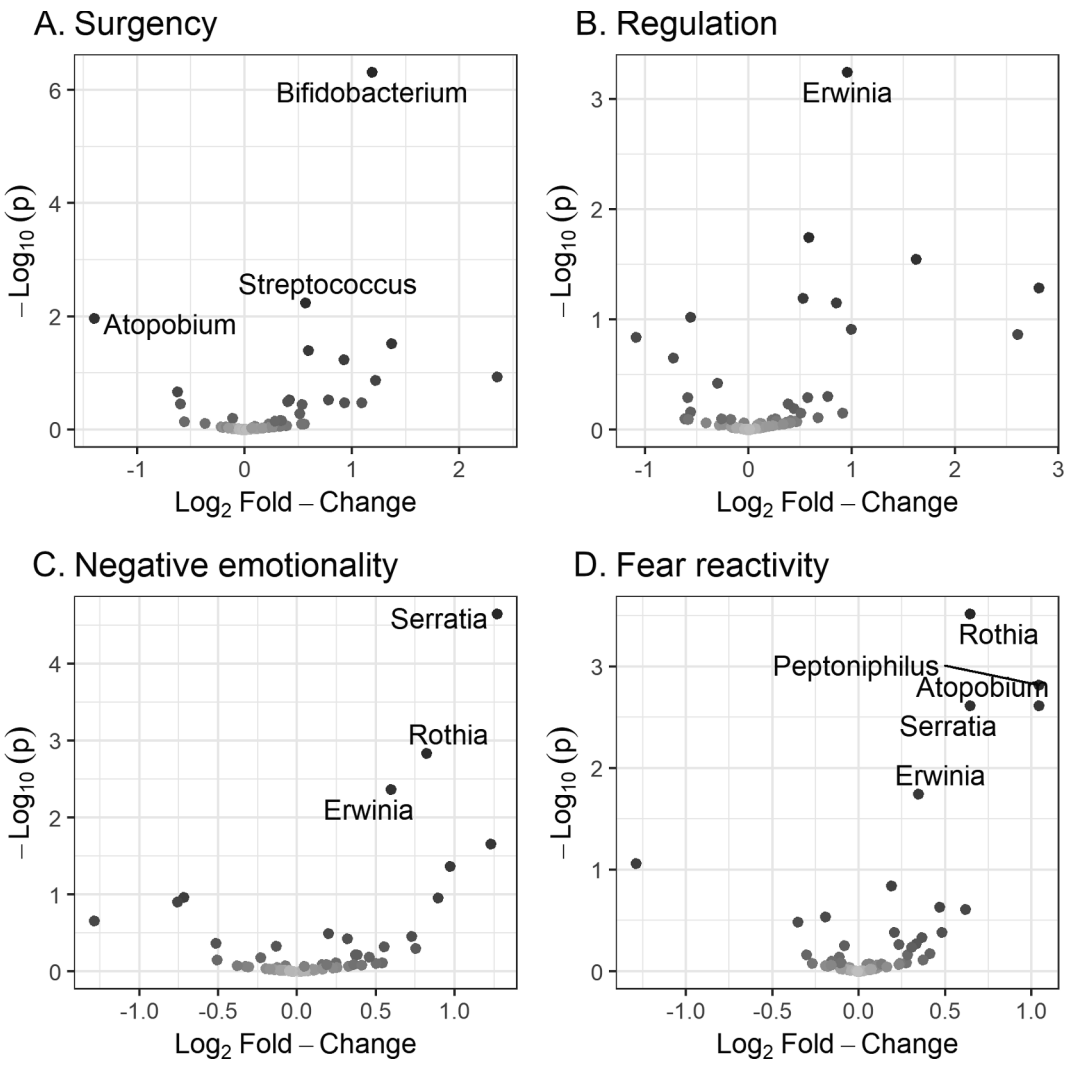

Fig. 3. A volcano plot on the association between A) surgency, B) regulation C) negative emotionality D) fear reactivity and genera in whole population when controlling for infant age, sex and birth mode. X-axis is binary logarithm of abundance fold change ($\log_2$ FC) and Y-axis stands for decimal logarithm of p-value. Only the associations with FDR < 0.25 are labeled.

diversity (Kruskall-Wallis H test $\chi^2 = 50$, FDR = $< 10^{-10}$).

3.2.1. Clusters and infant temperament

The clusters correlated to some of the temperament traits: *Bifidobacterium/Enterobacteriaceae*-community presented with the highest scores and *Bacteroides*-cluster with the lowest scores in temperament trait of regulation (Kruskall-Wallis H test $\chi^2 = 5.8$, FDR = 0.23), and subscales high intensity pleasure (Kruskall-Wallis H test $\chi^2 = 6.3$, FDR = 0.23), cuddliness (Kruskall-Wallis H test $\chi^2 = 7.9$, FDR = 0.20) and duration of orienting (Kruskall-Wallis H test $\chi^2 = 7.5$, FDR = 0.20) (Table 2).

Regarding the background factors, *Bacteroides*-cluster had lowest proportion (3%) and *V. dispar* -cluster highest proportion (32.1%) ($\chi^2 = 27.9$, FDR $< 10^{-5}$) of infants born with C-section (Table 2). There was higher share of females in the *Bacteroides*-cluster (59.4%, $\chi^2 = 9.1$, FDR = 0.057). No other significant differences existed between the clusters regarding the distribution of the covariates (Table 2).

In linear regression analyses, *Bacteroides* –cluster was associated with the main dimension of regulation ($\beta = -0.18$, FDR = 0.17, adjusted R2 = 0.023, Table 3.) and its subscale duration of orienting ($\beta = -0.51$, FDR = 0.008, adjusted R2 = 0.020, Table 3.) when contrasted with *Bifidobacterium/Enterobacteriaceae* -cluster and adjusting for sex and the mode of delivery. In addition, *V. dispar* -cluster was negatively associated with regulation ($\beta = -0.22$, FDR = 0.09, adjusted R2 = 0.023) and cuddliness ($\beta = -0.29$, FDR = 0.009, ΔR2 = 0.016, Table 3.) in comparison with *Bifidobacterium/Enterobacteriaceae* -cluster.

As three clusters were identified, two reference categories for the sex × cluster term were used to investigate the presence of sex differences in the regression model. When contrasted with the *Bifidobacterium/Enterobacteriaceae* -cluster, the *V. dispar*-cluster was differentially associated with duration of orienting (sex × cluster interaction FDR = 0.16) between boys and girls. Additionally, sex differences were noted in the associations between *V. dispar* – and *Bacteroides*-cluster in surgency (FDR = 0.16), regulation (FDR = 0.07), duration of orienting (FDR = 0.03) and cuddliness (FDR = 0.03). Further, there was a sex difference in the association between *Bifidobacterium/Enterobacteriaceae*- and *Bacteroides*-cluster in fear reactivity (FDR = 0.16) (Table 3).

3.3. Alpha diversity and infant temperament

Alpha diversity (mean Shannon Index = 1.7, SD = 0.5) was associated with breastfeeding status (Kruskall-Wallis H test $\chi^2 = 21.6$, FDR = 0.006), the mode of delivery (Kruskall-Wallis H test $\chi^2 = 5.1$, FDR = 0.08) and mother's age ($\rho = 4.0 \times 10^6$, FDR = 0.08). Richness

Table 4
Genera associated with the temperament. [a]Adjusted for infant age, sex and mode of delivery. [b]Adjusted for gestational age, sex, mode of delivery, infant age, breastfeeding and antibiotics intake.

Genus	Baseline Mean Abundance	Log_2 Fold-Change	FDR
Surgency[a]			
Atopobium	23	−1.4	0.247
Streptococcus	4584	0.6	0.200
Bifidobacterium	85,872	1.2	3.4×10^{-5}
full model[b]			
Streptococcus	4584	0.6	0.177
Regulation[a]			
Erwinia	96	1.0	0.040
full model[b]			
Erwinia	96	1.1	0.009
Negative emotionality[a]			
Erwinia	96	0.6	0.099
Rothia	71	0.8	0.050
Serratia	51	1.3	0.002
Fear reactivity[a]			
Erwinia	96	0.3	0.247
Rothia	71	0.6	0.021
Serratia	51	0.6	0.042
Peptoniphilus	93	1.0	0.042
Atopobium	23	1.0	0.042

(mean Chao1 = 763.7, SD = 299.1) was associated with breastfeeding status (Kruskall-Wallis H test $\chi^2 = 12.1$, FDR = 0.06). Neither alpha diversity nor richness were associated with any of the temperament traits in the unadjusted analyses. When adjusted for gestational age, infant age, sex, mode of delivery, breastfeeding and antibiotics intake, diversity was associated with negative emotionality (β = −0.17, FDR = 0.17, adjusted R2 = 0.016) and fear reactivity (β = -0.27, FDR = 0.17, adjusted R2 = 0.032) (Table 3). No sex differences were observed. Richness was not associated with temperament traits in the adjusted models.

3.4. Gut microbiota composition and infant temperament

3.4.1. Genus level

Screening for GMC-phenotype associations revealed several associations. When controlling for infant age at the time of sample collection, infant sex and mode of delivery, the temperament trait surgency was associated negatively with genus *Atopobium* and positively both with genus *Bifidobacterium* and *Streptococcus* (Fig. 3, Table 4.). Regulation was positively associated with *Erwinia.* Negative emotionality and fear reactivity were positively associated with *Erwinia, Rothia* and *Serratia*, fear reactivity additionally correlating positively with *Peptinophilus* and *Atopobium* (Table 4, Fig. 3). When controlling for sex, mode of delivery, gestational age, infant age during sampling, antibiotic treatments and breastfeeding status at 2.5 months of age, only the association between *Erwinia* and regulation (binary logarithm of abundance fold change, log_2 FC = 1.07, FDR = 0.0087, Table 4.) and *Streptococcus* and surgency (log_2 FC = 0.63, FDR = 0.18, Table 4) remained.

3.4.2. OTU level

Screening of GMC-phenotype associations among OTUs revealed different profiles between boys and girls, when controlling for the mode of delivery, gestational age, infant age during sampling, antibiotic treatments and breastfeeding status at 2.5 months of age. Boys presented associations with surgency and several OTUs annotated as *Bifidobacterium* (Supplemental information, Table S1). Further, in boys, regulation was negatively associated with several OTUs annotated as *Veillonella* and positively with *Bifidobacterium* and *Clostridiaceae*. In girls, both negative and positive associations with fear reactivity and *Veillonella* were noted (Supplemental information, Table S1).

4. Discussion

Accumulating preclinical studies show that gut microbiota influences neurodevelopment during the early critical developmental time window, which is reflected in long-term changes in behavior. Associations between temperament and GMC in toddlerhood has been reported in one study (Christian et al., 2015), but the associations in infants remain unreported. Temperament is behavioral and developmentally important phenotype and here we noted that temperament and GMC are already associated during early infancy. The current study suggested that infants harboring GMC distinguished by both *Bifidobacterium* and *Enterobacteriaceae* and genus *Erwinia* associated positively with regulation. Higher abundance of *Bifidobacterium* and *Streptococcus* was associated positively with surgency, *i.e.* positive emotionality. Higher fear reactivity was related to lower diversity.

4.1. Gut microbiota clusters and infant neurodevelopment

First, we detected three GMC clusters among the infants that differed by temperament. The clusters were characterized by either *V. dispar, Bacteroides* and *Bifidobacterium/Enterobacteriaceae*, which seemed to reflect the differences in the mode of delivery that is in line with previous literature, as for example *Bacteroides* is a typical inhabitant of vaginally born infants (Bäckhed et al., 2015; Rutayisire et al., 2016; Stewart et al., 2018). Further, previous work by Carlson *et al.* noted that the *Bacteroides*-dominated community type at the age of one year would predict better cognitive development at the age of two years (Carlson et al., 2017). The report by Carlson *et al.* seems contradictory to our finding of *Bacteroides*-dominated cluster associating with lower regulation, as even very early regulation reportedly predicts better cognitive development (Canals et al., 2011). However, research on the relations between GMC and infant early temperament traits are in fact lacking, hampering the comparability with earlier studies. Additionally, existing information on, e.g, higher levels of early duration of orienting (part of orienting/regulation scale) being associated with later autism (Zwaigenbaum et al., 2005), further underlines the need for longitudinal, developmentally sensitive studies.

4.2. Temperament and diversity

Our result that greater gut microbiota diversity is associated with lower negative emotionality and fear reactivity can possibly translate into more favorable emotional development as negative emotionality and its aspects such as fear reactivity predicts development of anxiety and other internalizing symptoms later in life (Kopala-Sibley et al., 2016; Pérez-Edgar and Guyer, 2014). Previous work on infant temperament and GMC suggested that greater phylogenetic diversity is associated with higher surgency *i.e.* positive emotionality in toddlers (Christian et al., 2015). Typically, lower gut microbiota diversity is associated with active breastfeeding and increasing diversity is noted during gut microbiota maturation when children are older and nutrition becomes more heterogeneous (Bäckhed et al., 2015; Stewart et al., 2018), which could partially explain why associations were noted only in the adjusted model in our population of young infants.

4.3. Gut microbiota composition and infant temperament

We observed several associations between genera and temperament. Specifically, surgency was associated with higher abundance of genera *Bifidobacterium* and *Streptococcus* and lower abundance of genus *Atopobium*. Previously, Christian *et al.* reported associations between several subscales loading to surgency and higher abundance of *Parabacteroides*, *Dialister* and *Rikenellaceae* only in boys (Christian et al., 2015). The bacterial signatures associating with surgency among toddlers are different in our study, which is not surprising given the vast variation in the developing GMC early in life (Bäckhed et al., 2015).

Surgency predicts more extraversion and better self-regulation in toddlerhood (Komsi et al., 2006), potentially resulting in greater social competence (Hayden et al., 2006) and is associated with CNS connectivity in areas related to emotion processing (Hanford et al., 2018) and HPA-axis functioning (Turner-Cobb et al., 2008). In turn, gut microbiome has been shown to be involved in HPA-axis development (Sudo et al., 2004) and CNS connectivity (Gao et al., 2019; Tillisch et al., 2013) and upcoming mechanistic studies should consider the HPA-axis and CNS emotion processing areas as potential domains of bridging gut microbiota and behavior.

Negative emotionality and fear reactivity, temperament traits related to the higher risk for later psychiatric disorders (De Pauw and Mervielde, 2010; Nigg et al., 2004; Sayal et al., 2014), were also associated positively with genera *Erwinia, Rothia and Serratia,* and fear reactivity specifically was also associated with *Peptinophilus* and *Atopobium*. Interestingly, *Serratia* abundance is reportedly associated with high maternal prenatal stress symptoms (Zijlmans et al., 2015), and in turn prenatal stress symptoms have been associated with higher infant negative emotionality also in the context of this cohort study (Nolvi et al., 2016). However, even though negative emotionality has been considered a risk factor for later psychopathology, it is also a differential susceptibility factor that may help individual benefiting from the environment (Pluess & Belsky, 2013), which further emphasize the complexity of potential mechanisms underlying these observed associations.

4.4. Sex differences in temperament and gut microbiome associations

Finally, different association profiles between OTU's and temperament were noted in girls and boys and the interrelations of GMC and temperament also presented with sex differences as illustrated by the significant sex × cluster interactions. Several associations between *Bifidobacterium* OTU's and surgency in boys were found. However, boys typically show more surgency and its subscales (Else-Quest et al., 2006; Willoughby et al., 2015) as reported by parents and observed in laboratory settings (Gagne et al., 2013). Additionally, boys may be differentially susceptible for the effects of gut microbiota on brain development (Clarke et al., 2013; Jašarević et al., 2016) as early disruptions in gut microbiota colonization reportedly have male-specific effects on CNS serotonergic system (Clarke et al., 2013). Our findings emphasize the need to include infant sex as one key covariate and potential source of confound.

4.5. Limitations of the study

Certain limitations in the current study should be noted. Temperament was reported only by the mother and a laboratory-based assessment of the infant temperament could possibly reveal different aspects of the phenotype. The temperament reported by mother is also influenced by her temperament and other characteristics (Bayly and Gartstein, 2013), which might increase bias in the maternal reports. Additionally, parental reports might encase gender bias, however, the meta-analysis by Else-Quest et al. also includes many observational studies indicating that there are gender differences in temperament traits, as is also reported by single empirical studies (Gagne and Hill Goldsmith, 2011; Planalp et al., 2017; Willoughby et al., 2015), so we expect that sex differences in temperament are not completely due to a rater bias. Both temperament and GMC were assessed only at a single time point and further studies should involve serial and concurrent sampling and measurements to elucidate the associations between early life GMC and developmental trajectories of early life temperament and potential development of psychopathology. Additionally, the future studies should consider factors potentially contributing to the association between infant temperament and GMC, e.g. breast milk components (Nolvi et al., 2018).

The current study was performed using 16S rRNA sequencing which offers comprehensive taxonomic profiling, but other methods, such as shotgun sequencing, which sequences entire genomes of all present organisms, could offer better resolution on a functional strain level. Additionally, the 16S profiles do not describe the functional properties of gut microbiota and metabolomic profiling including, for example, the assessment of short chain fatty acids, would offer mechanistic insight in metabolites potentially inducing the behavioral differences (Sarkar et al., 2018). However, experimental studies are required for establishing mechanisms underlying the associations.

4.6. Conclusion

Our study shows, in a relatively large sample, that infant GMC at the age of 2.5 months is associated with temperament at the age of 6 months. As temperament traits might precede psychopathology and present as resilience factors, the results may have implications on early prevention of child mental health problems in the future (Dinan et al., 2013). Our study benefits from large sample size of homogeneous genetic population and prospective design. The results can help in formulating hypotheses for studying potential mechanisms and causal associations, but currently they only imply associations between gut microbiota and behavior in a single point of development in infancy. Thus, future research should further elucidate if, and how, infant GMC at different phases of colonization can shape temperament development and health outcomes.

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HK, LK, HMU, EM, AKA conceptualized the study. HMU, EM, AKA, AK participated in the data collection. The original draft was written by AKA and reviewed and edited by HK, LK, HMU, EM, LL, SN, AK, PS, LLE, SOM and EE. Statistical analysis and visualization were done by AKA, supported by LL. QIIME pipeline was provided by SP. Project administration was done by LK and HK. Supervision was provided by LK, LL and EM. Funding was provided by HK, LK, EE, HMU. All authors accepted the final version of manuscript.

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Appendix A. Supplementary data

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