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Regular Article

Microbial exposure at birth and the development of behavioral temperament during the first three years of childhood

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Abstract

We investigated cross-sectional and longitudinal associations between neonate microbial exposure and emerging behavioral temperament measures at the ages of 1, 2, and 3 years. Infants and mothers ($n = 335$) were extracted from the Kuopio Birth Cohort Study. Temperament was assessed using the Infant Behavioral and Early Childhood Behavioral Questionnaires. Microbial samples were collected from oral cavity at birth and the bacterial profiles were assessed using 16S rRNA gene sequencing. Microbial diversity was characterized using alpha and beta diversity metrics. Analyses were performed for the most abundant genera. The sample was analyzed as a whole, as well as divided into subgroups representing no antibiotic use during birth ($n = 198$) and those with antibiotic use during birth ($n = 137$). No significant associations were observed between microbial profiles and behavioral measures after Bonferroni corrections. Nevertheless, our pre-correction results indicated an association between increased behavioral temperament surgency in the first year and beta diversity (high abundance of *Bacteroides*, *Faecalibacterium* and *Blautia*, low abundance of *Lactobacillus*) in the antibiotic use group. Additionally, pre-corrections, a high relative abundance of *Staphylococcus* was associated with increased surgency through years 1, 2, and 3 in the no antibiotics group, prompting consideration into a possible link between antibiotic use and emerging behavioral temperament.

Keywords: infant behavioral temperament; intrapartum antibiotics; microbiota

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Introduction

Childbirth is an event often characterized by the appearance of an offspring exposed to fluids of various origin (amniotic, vaginal etc.), and it is with these fluids the neonate first encounters an array of microbial organisms that will likely influence his/her development (Heijtz et al., 2011). Generally, a neonate is considered to exit the womb in a sterile state, thereafter quickly encountering millions of microorganisms from the immediate surroundings, although some findings have questioned whether the initial exposure to microbes may already have begun within the amniotic sac (Aagaard et al., 2014; Bearfield et al., 2002; Dzidic et al., 2018; Rosenblatt et al., 2015).

From the moment of birth, humans develop a symbiotic relationship with a variety of bacteria, bacteriophage, fungi, protozoa and viruses, collectively called the microbiome (Dunn et al., 2017). These are primarily located in the gastrointestinal tract, on the skin conjunctiva, and within the oral cavity (Sender et al., 2016). Following the initial microbial exposure at birth, a proportion of the prevalent microorganisms encountered proceed further toward colonization of the newborn host (Sampaio-Maia & Monteiro-Silva, 2014). The oral cavity serves as an initial entry point to colonization of both the oral and gut microbiota in infants, and likely serves to influence the composition of the initial gut microbiome as well (Costello et al., 2013; D'Agostino et al., 2022; Dal Bello & Hertel, 2006; Dominguez-Bello et al., 2010; Xiao et al., 2020).

Samples taken from neonate skin and oral mucosa directly after birth show various bacteria capable of initiating the colonization process are already present (Dominguez-Bello et al., 2010). Moreover, it has previously been demonstrated that the mode of delivery creates an effect upon the types of bacteria to seed the microbiome (Biasucci et al., 2008; Dominguez-Bello et al., 2010; Nelun Barfod et al., 2011; Penders et al., 2006).

Evidence suggests that children delivered vaginally exhibit bacterial strains similar to their mother's vaginal microbiota, whilst cesarean section (CS) deliveries manifest strains similar to their mother's skin microbiota or those in the hospital environment

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(Chu et al., 2017; Dominguez-Bello et al., 2010; Holgerson et al., 2011; Madan et al., 2016). The routine use of prophylactic antibiotics during CS deliveries are thought to contribute to the presence of maternal skin and nosocomially acquired strains upon the neonate (Kaan et al., 2021). The early colonizing strains are considered to have a significant impact on future health, helping to prime the mucosal immune system and further reducing vulnerability to a variety of diseases later in life, including those that affect psychological development and eventual mental health (Guarner & Malagelada, 2003; Heijtz et al., 2011; Salminen & Isolauri, 2006; Turroni et al., 2008).

There currently exist a number of proposed mechanisms thought to explain how the developing microbiome affects the emerging behavioral temperament of an infant. These range from a neural perspective, involving neurotransmitters sourced from gut bacteria, to the production of pro-inflammatory cytokines that influence brain function, as well as the production of neuroactive metabolites such as short-chain fatty acids (Kim & Shim, 2023). Metabolites are products created when bacteria metabolize nutrients in areas of the body, and can affect the central nervous system as they are able to penetrate the blood brain barrier and directly impact central nervous system development and function (Rutsch et al., 2020). These pathways cohesively create a complex system inducing a bi-directional communication network between the central nervous system and the gut microbiome ecosystem - forming the proposed pathway whereby the microbiome influences the development of behavioral temperament - known as the gut-brain axis (Wang et al., 2018). The gut-brain axis is susceptible to modification from these pathways, thereby disrupting communication, further impacting both physical and mental health of an individual (Alving-Jessep et al., 2022).

A cross-sectional study by Rothenberg and associates using a sample of 46 three-year-old children found a number of varying correlations between gut sourced bacterial genera and infant mental and psychomotor neurodevelopmental indices (Rothenberg et al., 2021). Another cross-sectional study reported an association between surgency and a greater phylogenetic diversity for 77 infants between 18 and 27 months of age (Christian et al., 2015). By contrast, a recent longitudinal pilot study ($n = 34$) assessing a variety of brain imaging and fear behavior outcomes in infants reported no association between fear behavior as measured by the infant behavior questionnaire fear subscale and the gut microbiome in infants, sampled at one month and one year of age (Carlson et al., 2021). As the majority of infant temperament studies have explored the developing stages of the microbiome using gut samples usually retrieved from ≥1 month of age (as in the aforementioned studies), research using a time-point in the earliest part of neonate microbial exposure is currently scarce, with one study reporting upon gut microbiome data from weeks 1-3 (Fox et al., 2021). As bacteria present in the first moments of birth partly proceed to colonize the oral and subsequent gut microbiota, the present study sample seemed ideal for exploring the presently underexplored first postnatal moments. The aim of our study was therefore to explore whether neonate bacterial exposure at birth had comparable effects upon behavioral outcomes as results from studies using later microbiome samples - primarily from gut microbiota - have reported.

Methods

Study population

The present study utilizes data collected as part of the Finnish Kuopio Birth Cohort (KuBiCo) Study (www.kubico.fi). KuBiCo investigates the effects of genetics and pregnancy-related environmental, lifestyle, and psychological risk factors on the health of the mother and their offspring. KuBiCo is a co-operation project between the University of Eastern Finland, Kuopio University Hospital, and the Finnish Institute for Health and Welfare; it is an epidemiological study initiated in 2012 with an aim to collect data from approximately 10,000 mother-child pairs from the eastern and central Finnish population of approximately 1 million inhabitants. A detailed description of KuBiCo has been given elsewhere (Huuskonen et al., 2018). The participation rate was approximately 37% of all pregnant women in the region during 2012–2017. The cohort has been analyzed and established as representing the Finnish pregnant population (Huuskonen et al., 2018). Participants gave their written informed consent after receiving a full explanation of the study. The protocol of KuBiCo has been approved by the research ethics committee of the Central Finland Health Care District (8.12.2011, K-S SHP Dnro 18U/2011).

Selection of the study population

All children from KuBiCo with an oral cavity microbial sample collected immediately after birth and who were equal to or more than four years by the end of the year 2017 were invited to attend a five-year clinical follow-up ($n = 2041$). Microbial samples were sequenced and analyzed from those who attended the five-year follow-up ($n = 464$). Of these 464 children, 372 had at least one of the three behavioral questionnaires completed during the first three years. As mode of delivery has been shown to alter the density and counts of oral microbial species in neonates (Blum et al., 2022), we chose to select only vaginal delivery births from this sample resulting in a sample size of $n = 335$. Furthermore, as the administration of intrapartum antibiotics, regardless of delivery mode, leads to alterations to the emergent microbiome (Dierikx et al., 2020), we chose to create subgroups reflecting antibiotic use (AB use; $n = 137$) and no antibiotic use (No AB use; $n = 198$) for the analyses.

Child temperament measures

The Infant Behavior Questionnaire (IBQ) and the Early Childhood Behavior Questionnaire (ECBQ) are widely used measures of infant temperament (Putnam et al., 2006; Rothbart, 1981). The IBQ (revised, very short form, IBQ-VFS) was used to measure the temperament of the infants at the age of 12 months, while the ECBQ (very short form, ECBQ-VSF) was used for children at 2 and 3 years of age. The IBQ-VFS consists of 37 questions employing a 1–7 Likert scale with a 'not applicable' option in addition. The ECBQ-VSF has 36 questions and was scored similarly to the IBQ. These measures provide three broad dimensions of behavior temperament scores: surgency, effortful control, and negative affect (NEG), and were completed by the mother.

Surgency/Positive Emotionality (SUR) is defined as the tendency to experience and express positive emotions, seek out stimulation and novelty, and approach new people and situations. Effortful control (EFF) is defined as the ability to regulate one's attention and emotions and inhibit dominant responses in favor of subdominant responses. NEG is defined as the tendency to experience and express negative emotions such as fear, sadness, and anger (De Pauw, 2016), with low levels of EFF reported to predispose toward a later range of psychopathology, such as anxiety and attention deficit hyperactivity disorder (Nigg, 2006).

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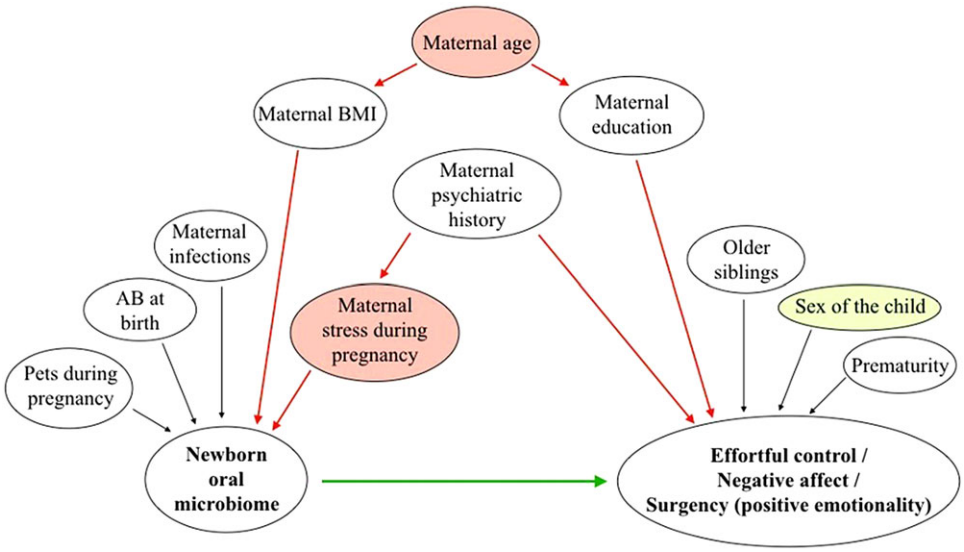

Figure 1. The causal directed acyclic graph (DAG) used to identify the minimal sufficient adjustment set when estimating the effect of the neonatal oral microbiome on behavior temperament (green arrow). The variables and associations included in the DAG were based on subject-matter knowledge and literature search. The biasing paths (red arrows) were blocked by adjusting the subsequent models for maternal stress during pregnancy and maternal age. Additionally, models were chosen to be adjusted for the sex of the child to yield a more precise estimate.

The scales result in a total score whereby higher scores indicate more surgency/NEG behaviors, or else better effortful control.

Covariates

Causal directed acyclic graphs (DAGs) were used to identify the minimal sufficient adjustment set when estimating the effect of the newborn oral microbiome on behavior temperament (Figure 1). Potential covariates and associations between them, and the exposure and outcome, were defined based on subject-matter knowledge and literature search. The biasing paths were blocked by adjusting the subsequent models for maternal stress during pregnancy and maternal age. Additionally, models were chosen to be adjusted for sex of the child to yield a more precise estimate. Maternal depression scores were obtained from third trimester Edinburgh Postnatal Depression Score (EPDS) assessment, with participants responding online between 28th and 44th gestational weeks and eight weeks after delivery. The EPDS is a 10-item 4-level Likert-scale questionnaire used for screening postpartum depression in clinics (range 0–30) (Cox et al., 1987). Maternal age and child sex data were obtained from the hospital birth register.

Microbial sampling, DNA extraction, and purification

Oral swab samples were taken in the delivery room immediately after birth, prior to palpation of the oral palate. Samples were collected by a midwife swirling a flocked swab (ESwab™, Copan, Brescia, Italy) from both inner cheeks of the newborn.

The microbial DNA was extracted using the protocol introduced as 'method 1' in a previous study (Yuan et al., 2012) with minor modifications. Here we used 300 µl of the amies buffer solution as the sample volume for DNA extraction and added 30 µl of mutanolysin (5KU/ml, Sigma-Aldrich Co., USA) prior the first incubation. Further modifications from the original protocol included use of 30 µl of Proteinase K (20 mg/ml, Qiagen, Valencia, CA), 300 µl of AL buffer (Qiagen, Valencia, CA) and addition of 300 µl of ethanol prior to DNA purification with the QIAmp DNA mini kit (Qiagen, Valencia, CA) following manufacturer instructions. Per every 100 processed samples, two Eswab blanks (from two clean Eswabs using 300 µl of amies liquid solution), two positive controls (known bacterial and fungal mock communities), and two negative reagents controls were processed along with the actual samples.

Bacterial 16S rRNA gene amplification and amplicon sequencing

The DNA extracted from oral swab and control samples was shipped frozen to the sequencing service partner LGC Genomics (Germany), who did the library preparation and sequencing, similar to the protocol described elsewhere (Jayaprakash et al., 2017). The V4-V5 region of the bacterial 16S rRNA gene was amplified in a nested approach to reduce input of eukaryotic DNA using pre-amplification with 341F-1061R (Andersson et al., 2008; Herlemann et al., 2011) prior to the final amplification with 515FY-926R. DNA amplicons were sequenced on Illumina MiSeq platform with V3 chemistry resulting in paired-end reads with a length of 300 bp each. The sequence libraries were demultiplexed using Illumina's bcl2fastq v1.8.4 software and the barcode and primer sequence removal and sorting performed with custom Python v2.7.6 script. Adapter sequences were removed from the 3′ end of reads with a proprietary script discarding reads shorter than 100 bp.

Bioinformatics

The 16S amplicon data was analyzed by standard dada2 pipeline version 1.8 (Callahan et al., 2016). Taxonomy was assigned using SILVA database version 138 (Quast et al., 2013). Phylogenetic tree was constructed in QIIME version 1.9.1 (Caporaso et al., 2010). The downstream post processing included removal of non-bacterial (for 16S), singletons, mitochondria, eukaryote and chloroplast amplicon sequence variants (ASVs). We included sequencing of oral swab blank samples, as well as negative and reagent controls from DNA extraction (alongside bacterial and fungal mock communities). The bacterial mock community is a cell suspension containing seven bacterial strains (including *Staphylococcus aureus*, *Streptomyces californicus*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Sphingomonas faeni*, and *Mycobacterium mucogenicum*). Negative and reagent controls were utilized primarily in the quantitative PCR analyses, to control the process of DNA extraction, alongside the positive controls (mock communities). Oral swab sample blanks were used to flag

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and remove contaminant ASVs from the bacterial sequencing data. We applied function isContaminant of the Decontam package version 1.2 (Davis et al., 2018), using prevalence method and a rather stringent probability threshold 0.5. This method compares prevalence of detection of an ASV in controls versus real samples and flags ASVs that occur at higher prevalence in the controls, as to be expected from reagent and other contaminants. In this decontamination step we used 12 swab blank samples as controls to compare ASV prevalence in controls versus actual samples. Those blanks most closely resemble the reagents constituents and processing steps of the actual samples, since they were processed, extracted, amplified, and sequenced alongside the oral swab samples. Using this approach, a total of $n = 35$ bacterial ASVs with <1000 sequence reads were removed prior to downstream analyses (Supplement 1).

Alpha diversity measures microbiome diversity within a single sample and reflects community heterogeneity, typically described by relative abundance and total number of species (D'Agostino et al., 2022). The alpha diversity measures Chao1, observed species, and Shannon index were calculated using *estimate_richness* -function in R-package *phyloseq* version 1.38.0 (McMurdie & Holmes, 2013), applying rarefaction values of 1002 sequences (*rarefy_even_depth* -function in *phyloseq*). The fourth alpha diversity metric Faith's phylogenetic diversity was computed using *pd*-function in R-package *picante* version 1.8.2 (Kembel et al., 2010).

Beta diversity (β-diversity) measures similarity or dissimilarity between two communities and is used to study associations between environmental variables and microbial composition, providing a statistical comparison between two samples (D'Agostino et al., 2022). Phylogenetically informed variation between pairs of samples (beta diversity) in the bacterial community was evaluated with Generalized UniFrac distances calculated using R-package *GUniFrac* version 1.7 (Chen et al., 2012) with midpoint rooted tree and $\alpha = 1$ for abundance weighted beta diversity.

Statistical analysis

The behavioral questionnaires generating three behavioral temperament subscales Effortful control (EFF)/NEG/Surgency/Positive Emotionality (SUR) for three time-points (years 1, 2, 3) of the infants' development were tested for associations with the neonates' bacterial exposure measures of alpha and beta diversity at birth. Principal component (PC) analysis was used to reduce the dimensionality of the four alpha diversity metrics (describing the diversity of the bacterial exposure within individual) into two PCs, alpha PC1 and alpha PC2, while still preserving maximum amount of information. Principal coordinate (PCo) analysis of generalized unifrac distances was used to generate principal coordinates beta PCo1 and beta PCo2. Scree plots were used for both PCA and PCo to determine the number of components or coordinates chosen. Pearson correlation coefficients between the relative abundances of each bacterial genus and PCo1 and 2 were calculated to better understand how bacterial community composition impacts the beta diversity principal coordinates.

Linear regression models were used to test for cross-sectional effects of alpha and beta diversity metrics (alpha PC1 and 2, beta PCo 1 and 2) on behavior temperament EFF, NEG, and SUR at year 1. Models were adjusted for sex of the child, maternal age, and 3rd trimester EPDS score, and fitted for the whole sample as well as in subgroups of those who received antibiotics during birth ($n = 137$) and those who did not ($n = 198$). Any missing data in maternal age and EPDS score were mean imputed while multiple imputations by chained equations with $m = 5$ imputations were used to account for any missing year 1 EFF/NEG/SUR values. Sex of the child, maternal age, EPDS score, and EFF/NEG/SUR values at year 2 or 3 (when available) were included in the imputation models. As a sensitivity analysis, complete case data ($n = 281$) with non-missing year 1 EFF/NEG/SUR values available were analyzed.

Linear mixed models with random intercepts for each newborn (id) were used to test for longitudinal effects of alpha and beta diversity metrics on repeatedly measured behavior temperament EFF/NEG/SUR at years 1, 2, and 3, and fitted with R-package *lme4* version 1.1.27.1 and *lmerTest* version 3.1.3. Inclusion of random intercepts allows each newborn to have their own intercept and a slope parallel to others. This can be interpreted as estimating individual levels of the average behavioral subscale score while assuming the relationship with the predictors as being the same for each newborn. Alpha and beta diversity measures as well as continuous time were included as fixed effects. Models were additionally adjusted for fixed effects of the sex of the child, maternal age, and 3rd trimester EPDS score, and fitted for the whole sample as well as in subgroups of antibiotic use during birth. Any missing outcome values were handled within the mixed model framework.

As a secondary analysis, the linear regression models and linear mixed models were used to test effects of the most abundant individual genera on behavior temperament at year 1, or repeatedly measured behavior temperament at years 1, 2, and 3. Bacterial genera with median relative abundance *>0* were included to target only dominant genera in the swab samples. Covariate adjustments were identical to those of primary analyses.

In all analyses, Bonferroni corrections taking into account the number of predictors and the number of models run were used to counteract the multiple comparison problem. Models with alpha and beta diversity metrics were tested at the significance level of *0.0014* (4 bacterial features, 3 outcomes EFF/NEG/SUR, 3 models for full sample and 2 antibiotic use subgroups), whereas genera level analyses were tested at the significance level of *0.0007* (8 microbiome features, 3 outcomes EFF/NEG/SUR, 3 model for full sample and two antibiotic use subgroups).

The structure and approach to all methodology in this study was performed in adherence with the STORMS (Strengthening The Organizing and Reporting of Microbiome Studies) checklist guidelines for microbiome analyses in human microbiome research (Mirzayi et al., 2021); the completed STORMS checklist is available for inspection in its entirety in Supplement 2.

The code used to perform the statistical analyses was written in R and is deposited in GitHub: https://github.com/maijupesonen/Microbial_exposure_at_birth_and_temperament.

Results

Population characteristics

The mean age of mothers involved in the study was 31.2 years with a mean gestational length at birth of 279 days (39 weeks + 6 days). Maternal third trimester EPDS score had a mean value of 4.1, and 151 (*51%*) of the infants were female. Missing values for maternal age (*3%* missing) and third trimester EPDS score (*21%* missing) were mean imputed when included as covariates in the regression models. Behavioral subscale scores from years 1 to 3 ranged from 5.0 to 5.4, and the distribution of the behavioral subscale scores across years 1–3 grouped by sex of the child were comparable

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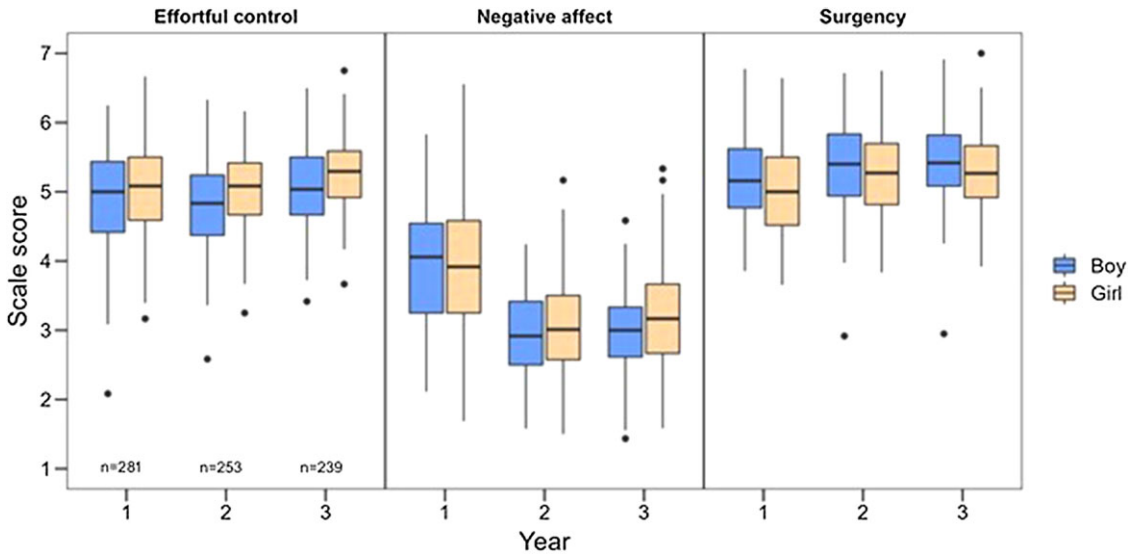

Figure 2. Distributions of the observed behavior temperament scores effortful control, negative affect, and surgency/positive emotionality over 1, 2, and 3 years of age grouped by sex of the child. The numbers of non-missing subscale score values per year are given at the bottom of effortful control panel and are the same across subscales.

(Figure 2) Girls had on average slightly higher EFF scores in comparison to boys across ages of 1–3 years, whereas boys had on average slightly higher SUR scores in comparison to girls at all ages. NEG scores showed largest change over time in both sexes being highest at age 1 and decreasing after that. The proportion of missing values for behavioral subscale scores increased over time with *16%* being missing at year 1, *24%* at year 2, and *29%* at year 3. Cronbach's alpha for reliability was 0.75, 0.78, and 0.83 for the SUR, EFF, and NEG subscale, respectively, suggesting an acceptable to good internal consistency of the items measuring the same characteristic.

Microbial composition

In total, we observed 22 unique bacterial phyla and 370 unique bacterial genera (unassigned taxa excluded). Most of the observed genera were rare and present only in 1–5 newborns. Four phyla (*Firmicutes*, *Actinobecteriota*, *Bacteroidota*, *Proteobacteria*), and eight genera (*Lactobacillus*, *Staphylococcus*, *Corynebacterium*, *Prevotella*, *Anaerococcus*, *Bacteroides*, *Stenotrophomonas*, *Streptococcus*) were present in most samples (defined as at least *50%* of the samples having the taxa present at some non-zero relative abundance). Typically, a sample was dominated by 1-3 genus. The most prevalent genera alone, *Lactobacillus*, constituted at least *54%* of the entire microbiome in *50%* of the samples, whereas all eight of the most prevalent genera constituted at least *78%* of the entire microbiome in *50%* of the samples (Supplement 3, Figure S1).

Microbial exposure effects on behavioral temperament

Based on scree plots (Supplement 3, Figure S2), two first PCs of alpha diversity metrics (alpha PC1 and 2) and two first principal coordinates of the generalized unifrac distance matrix (beta PCo1 and 2) were chosen, resulting in a total of *98.3%* of the variation in alpha diversity and *62.6%* of the variation in beta diversity explained. Behavioral temperament outcomes were tested for associations with these four bacterial exposure measures of alpha and beta diversity. As shown in Figure 3, alpha PC1 captured richness (total number of species in the community) while alpha PC2 captured evenness.

Of the 330 genera detected in swab samples (after rarefaction, unassigned taxa excluded), the relative abundances of 12 genera correlated *(>|0.4|)* with at least one of the beta diversity principal axes. The full correlation results for relative abundances of each genus and beta PCo1 and 2 are presented in Supplement 4. Beta PCo1 was positively associated with *Lactobacillus*, and negatively associated with *Bacteroides*, *Faecalibacterium* and *Blautia*, while beta PCo2 was positively associated with *Gardnerella* and *Corynebacterium*, and negatively associated with *Faecalibacterium* and *Blautia*.

Beta PCo1 could thus be interpreted as reflecting exposure to *Lactobacilli* dominant ("healthy") vaginal microbiota vs. other sources (faecal, mixed vaginal microbiota), whereas beta PCo2 is reflecting exposure to vaginal microbiota dominated by genera other than *Lactobacilli*, suggesting bacterial imbalance potentially caused by bacterial vaginosis (Supplement 3, Figure S3).

When each behavioral subscale was tested for associations with alpha PC1 and 2, together with beta PCo1 and 2 output at year one, we found an uncorrected significant negative association between beta PCo1 and SUR ($\beta = -0.99$, $p = 0.02$) for the antibiotic use (AB use) group only, suggesting that increased surgency is associated with a higher abundance of *Bacteroides*, *Faecalibacterium* and *Blautia*, and a lower abundance of *Lactobacillus* (Figure 4); however, no significant associations were observed after Bonferroni corrections (Table 1).

In the group of mothers not receiving AB during birth, the association between beta PCo1 and SUR was not significant ($\beta = 0.18$, $p = 0.70$). As a sensitivity analysis, we refitted the full sample model (both AB groups combined) adding an interaction term between AB use and beta PCo1. The interaction between AB use and beta PCo1 was significant at the uncorrected 0.05-level ($\beta = -1.36$, $p = 0.031$), supporting the finding that the effect of beta PCo1 on surgency is different between the AB use groups (results not shown).

No significant associations were found when repeatedly measured behavioral subscales at 1, 2, and 3 years of age were tested for associations with alpha PC1 and PC2, and beta PCo1 and 2 (Table 2).

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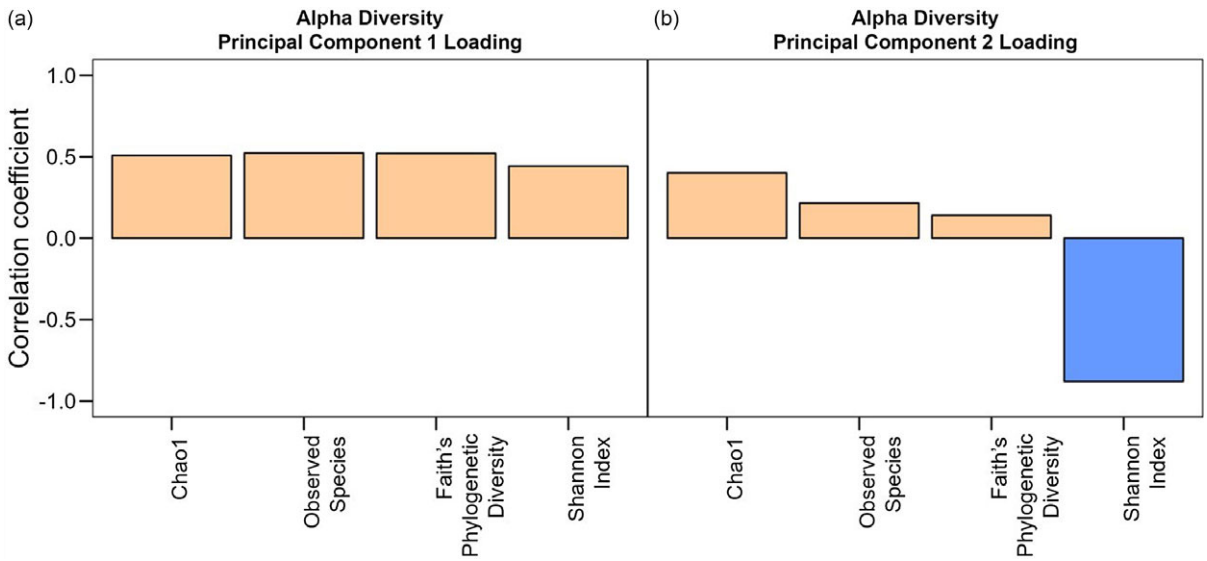

Figure 3. Loadings of alpha diversity metrics on two first principal components. Positive (orange) and negative (dark blue) loadings of Chao1, observed species, Faith's phylogenetic diversity, and Shannon index on alpha diversity principal component 1 (panel a) and principal component 2 (panel b).

Bacterial genera analyses

Secondary analyses were performed to study associations between individual bacterial genera, the behavior temperament, and its development during the first three years with the most prevalent bacterial genera. Rare genera were removed to retain the dominant genera reflecting overall oral microbiome composition. The genera considered included *Lactobacillus*, *Streptococcus*, *Staphylococcus*, *Corynebacterium*, *Stenotrophomonas*, *Bacteroides*, *Prevotella*, and *Anaerococcus*. No significant associations were found between the tested bacterial genera and the three behavior temperament subscales at year 1. (Table 3). A positive association between repeatedly measured surgency at ages of 1, 2, and 3 and relative abundance of *Staphylococcus* was observed in the no antibiotic use during birth group ($\beta = 1.19$, $p = 0.02$), however, becoming non-significant after Bonferroni correction (Table 4).

The sensitivity analyses for behavior temperament at year 1 models which were performed on the complete case data ($n = 281$) produced identical results, the one exception to this being an uncorrected negative association found between *Anaerococcus* genus and NEG at year 1 ($\beta = -3.93$, $p = 0.03$, data not shown).

Discussion

The present study explored the associations between bacterial exposure profiles at birth and behavioral temperament assessed during infancy and early childhood. To the best of our knowledge, this is the first study performed addressing a newborn's initial bacterial exposure and infant temperament during the first three years of childhood. We tested associations between bacterial alpha and beta diversity measures against three behavioral temperament subscales (EFF, NEG, and SUR) for infants in year one alone and longitudinally across years 1, 2, and 3. No significant associations were found after Bonferroni corrections.

The use of neonatal bacterial exposure profiles for predicting behavioral outcomes is still considered an emergent field of research. In this study, prior to corrections for multiple comparison testing, the behavioral subscale surgency at the age of one was negatively associated with beta PCo1 (high abundance of *Bacteroides*, *Faecalibacterium* and *Blautia*, low abundance of *Lactobacillus*) for the group using antibiotics during childbirth. Surgency is a behavioral temperament dimension associated with expressions of positive emotions, reward seeking, and a high activity level, often linked with sociability and lack of shyness (Holmboe, 2016). Our pre-correction observations are comparable to a recent study investigating the gut microbiome of infants at four different time-points during the first year of an infant's life, whereby an association between surgency and beta diversity at the earliest time-point (1–3 weeks) for 23 infants was reported (Fox et al., 2021). Moreover, Fox et al., identified specific genera from the gut microbiome (*Bifidobacterium*, *Lachnospiraceae*, and *Collinsella*) as being positively associated with the surgency scale at age 12 months (Fox et al., 2021). By contrast, Aatsinki et al., found a higher abundance of *Bifidobacterium* and *Streptococcus,* and a lower abundance of *Atopobium* at age 2.5 months, were associated with greater surgency scores, also measured using the Infant Behavior Questionnaire in 6-month-old infants (Aatsinki et al., 2019). Another study found that surgency scores were associated with a unique microbiota community profile in gut microbiota composition (beta diversity) for boys only, using the stool-sourced gut microbiome samples of 77 children between 18 and 27 months of age (Christian et al., 2015). However, neither of these studies appear to have adjusted for intrapartum antibiotic use.

Although non-significant post-correction, the observed association in the antibiotic use group accentuates the potential formative role of intrapartum antibiotic exposure upon the developing microbiome, with the significance of the association further emphasized by our neonatal sample being exclusively composed of vaginal delivery births. Roesch et al. (2017) reported that intrapartum antibiotic administration modified vaginal microbiota composition by balancing the dominance of *Lactobacillus* and thus increasing the microbial diversity (Roesch et al., 2017). In line with this, we observed that the group with antibiotic use - thought to be a consequence of antibiotic prophylaxis for group B streptococcal colonization in pregnant women (Lyytikainen et al., 2003) - had lower beta PCo1 values, on average, in comparison to the group without intrapartum antibiotics. Additionally, these low beta PCo1 values were

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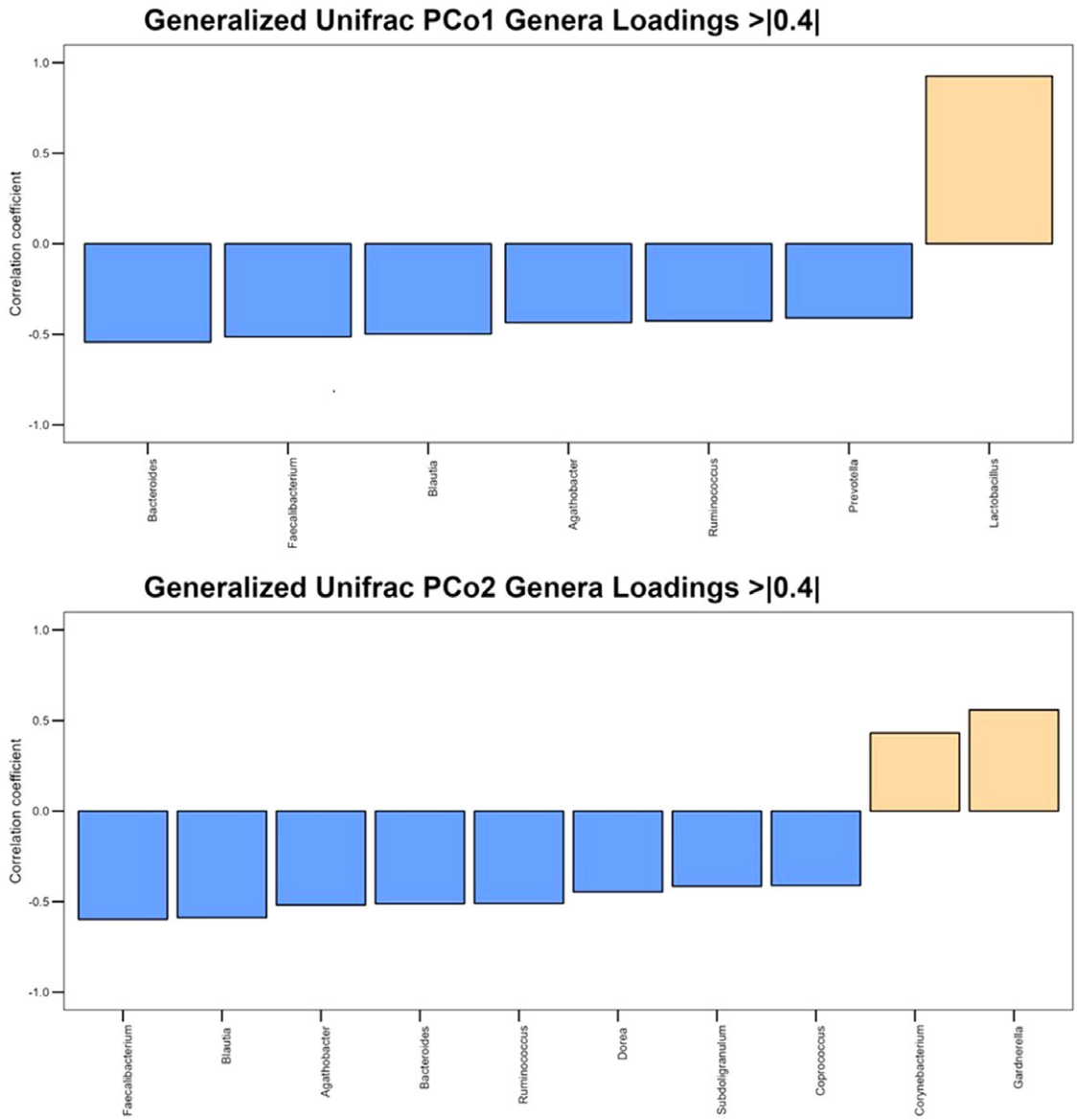

Figure 4. Strongest correlations between the two first generalized unifrac principal coordinates and bacterial genera. Positive (orange) and negative (blue) correlations between bacterial genera (unidentified genera filtered out) and principal coordinate 1 (panel a) and 2 (panel b) computed from the generalized unifrac distance matrix. For both principal coordinates, only the most strongly correlated genera (>|0.40|) were considered and plotted in an ascending order.

associated with a higher relative abundance of *Bacteroides* and lower relative abundance of *Lactobacillus*. We cannot exclude the possibility, however, that the vaginal microbiota in women treated or untreated with antibiotics may have differed to begin with. Alternately, the results may imply that higher relative exposure to fecal microbes over vaginal microbes is associated with surgency, due to fecal microbes being proposed as more likely to contribute to the infant gut microbiota development in vaginally delivered infants (Mitchell et al., 2020). One reason why the association is seen only in the antibiotic exposed group may be owing to differences in microbial exposure reflected by beta PCoA1.

Our group with no intrapartum antibiotic use displayed a pre-correction association between the *Staphylococcus* genus abundance at birth and surgency across years 1–3. *Staphylococcus*, together with *Lactobacillus*, *Propionibacterium*, and *Streptococcus*, are often the most abundant genera in neonates across all body sites (Chu et al., 2017). It is feasible to consider the lack of exposure to intrapartum antibiotics sustained the abundance of these genera (whereas exposure to antibiotics could have resulted in their decrease), yet the association with surgency shown here is novel. Loughman et al., reported a lower abundance of *Prevotella* in gut microbiota at 12 months was associated with an increase of behavioral problems at 2 years of age, surmising that recent exposure to antibiotics was the best predictor of decreased *Prevotella* (Loughman et al., 2020). These findings elicit further attention into potential mechanisms linking intrapartum antibiotic use and the emerging infant behavioral temperament.

The majority of previous microbiota research on infants and children have utilized gut microbiome profiles, usually obtained from stool samples (Aatsinki et al., 2019; Carlson et al., 2021; Loughman et al., 2020), whereas a microbial profile sourced from the first oral cavity exposure is infrequently used (Simpson et al.,

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Table 1. Linear regression models with behavior temperament effortful control (EFF)/negative affect (NEG)/surgency/positive emotionality (SUR) at year 1 as a dependent variable and alpha and beta diversity measures as independent variables

	All Imputed ($n = 335$)	AB use ($n = 137$)	No AB use ($n = 198$)
EFF			
Alpha PC1	0.02 (0.04)	0.06 (0.06)	0.01 (0.05)
Alpha PC2	−0.11 (0.09)	−0.11 (0.15)	−0.13 (0.14)
Beta PCo1	0.09 (0.32)	0.33 (0.47)	0.07 (0.46)
Beta PCo2	0.20 (0.27)	−0.06 (0.39)	0.41 (0.40)
NEG			
Alpha PC1	−0.04 (0.05)	−0.02 (0.07)	−0.04 (0.07)
Alpha PC2	0.11 (0.13)	0.15 (0.21)	0.02 (0.19)
Beta PCo1	−0.49 (0.41)	−0.37 (0.60)	−0.41 (0.61)
Beta PCo2	0.03 (0.39)	−0.11 (0.51)	0.32 (0.59)
SUR			
Alpha PC1	−0.04 (0.04)	−0.10 (0.05)	0.02 (0.05)
Alpha PC2	0.01 (0.09)	0.13 (0.13)	−0.14 (0.14)
Beta PCo1	−0.39 (0.32)	−0.99 (0.42)*	0.18 (0.46)
Beta PCo2	−0.17 (0.26)	−0.32 (0.34)	0.17 (0.37)

Models were adjusted for sex of the child, maternal age, and 3rd trimester EPDS score, and fitted for the whole sample as well as in subgroups of antibiotic use during birth (AB). The results are reported as *estimate (standard error)*. **denotes a significance after Bonferroni correction (threshold for significance 0.0014), *denotes a *p*-value <0.05.

Table 2. Linear mixed models with random intercepts for repeatedly measured behavior temperament effortful control (EFF)/negative affect (NEG)/surgency/positive emotionality (SUR) at years 1, 2, and 3. alpha- and beta-diversity measures as well as continuous time were included as fixed effects

	All Imputed ($n = 335$)	AB use ($n = 137$)	No AB use ($n = 198$)
EFF			
Alpha PC1	0.01 (0.03)	0.01 (0.04)	0.02 (0.04)
Alpha PC2	−0.10 (0.07)	−0.07 (0.11)	−0.14 (0.10)
Beta PCo1	0.06 (0.24)	0.03 (0.35)	0.14 (0.34)
Beta PCo2	0.18 (0.21)	0.02 (0.29)	0.32 (0.31)
NEG			
Alpha PC1	−0.02 (0.03)	0.02 (0.05)	−0.04 (0.05)
Alpha PC2	0.06 (0.09)	−0.04 (0.12)	0.12 (0.13)
Beta PCo1	−0.36 (0.29)	0.13 (0.41)	−0.56 (0.41)
Beta PCo2	0.02 (0.25)	−0.29 (0.34)	0.35 (0.38)
SUR			
Alpha PC1	−0.02 (0.03)	−0.07 (0.04)	0.03 (0.03)
Alpha PC2	−0.06 (0.07)	0.02 (0.11)	−0.17 (0.09)
Beta PCo1	−0.17 (0.22)	−0.67 (0.36)	0.28 (0.30)
Beta PCo2	−0.12 (0.20)	−0.16 (0.30)	0.06 (0.27)

Models were additionally adjusted for fixed effects of the sex of the child, maternal age, and 3rd trimester EPDS score, and fitted for the whole sample as well as in subgroups of antibiotic use during birth (AB). The results are reported as *fixed effect estimate (standard error)*. **denotes a significance after Bonferroni correction (threshold for significance 0.0014), *denotes a *p*-value <0.05.

2020). Even though microbiota sourced from another location may cause uncertainties regarding the reliability of comparisons with previous literature, colonization of the gut has been charted back from initial oral colonization (D'Agostino et al., 2022; Dominguez-Bello et al., 2010; Olsen & Yamazaki, 2019; Xiao et al., 2020), hence the emerging microbiome trajectory and its effect upon behavioral characteristics in early life retains plausibility.

Strengths of this study include the exclusive focus on infants born via vaginal delivery and the use of bacterial samples reflecting actual exposure at birth, the inclusion of longitudinal behavior measures up to three years of age, as well as the conservative statistical approach applied. Furthermore, a fundamental element in approaching observational studies is the incorporation of confounder assessment. In this study, DAGs were used to identify

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Table 3. Linear regression models with behavior temperament effortful control (EFF)/negative affect (NEG)/surgency/positive emotionality (SUR) at year 1 as a dependent variable and most abundant individual bacterial genera as independent variables

	All Imputed (*n* = *335*)	AB use (*n* = *137*)	No AB use (*n* = *198*)
EFF			
Lactobacillus	−0.16 (0.15)	−0.20 (0.24)	−0.08 (0.22)
Streptococcus	0.12 (0.73)	0.15 (0.99)	−0.40 (1.02)
Staphylococcus	0.28 (0.53)	0.11 (0.70)	0.20 (0.80)
Corynebacterium	−0.43 (0.63)	−0.13 (0.84)	−0.21 (0.97)
Stenotrophomonas	0.05 (0.97)	−0.37 (1.04)	4.48 (4.45)
Bacteroides	−0.13 (0.82)	0.21 (1.06)	−0.68 (1.41)
Prevotella	−0.41 (0.68)	−0.54 (0.92)	0.09 (1.02)
Anaerococcus	0.02 (1.31)	−3.38 (2.12)	2.25 (1.77)
NEG			
Lactobacillus	−0.15 (0.21)	−0.17 (0.30)	−0.14 (0.30)
Streptococcus	0.31 (0.83)	−0.04 (0.98)	0.42 (1.34)
Staphylococcus	−0.38 (0.69)	0.18 (0.94)	−1.35 (1.15)
Corynebacterium	0.65 (0.81)	0.03 (1.00)	1.84 (1.33)
Stenotrophomonas	−1.46 (1.31)	−1.86 (1.28)	4.73 (6.29)
Bacteroides	0.29 (1.16)	0.87 (1.31)	−0.70 (2.01)
Prevotella	−0.60 (0.97)	−1.27 (1.20)	0.27 (1.50)
Anaerococcus	−3.18 (1.75)	−4.12 (2.52)	−2.83 (2.45)
SUR			
Lactobacillus	−0.07 (0.14)	−0.09 (0.22)	−0.08 (0.19)
Streptococcus	0.17 (0.50)	−0.21 (0.68)	0.84 (0.98)
Staphylococcus	0.46 (0.52)	−0.33 (0.68)	1.20 (0.74)
Corynebacterium	−0.31 (0.56)	0.40 (0.72)	−0.73 (0.90)
Stenotrophomonas	0.54 (0.90)	0.89 (0.97)	3.32 (4.16)
Bacteroides	0.51 (0.79)	0.96 (0.92)	−0.48 (1.32)
Prevotella	−0.47 (0.84)	0.07 (1.00)	−1.12 (1.13)
Anaerococcus	0.52 (1.18)	−0.81 (1.79)	0.49 (1.65)

Models were adjusted for sex of the child, maternal age, and 3rd trimester EPDS score, and fitted for the whole sample as well as in subgroups of antibiotic use during birth (AB). The results are reported as *estimate (standard error)*. **denotes a significance after Bonferroni correction (threshold for significance 0.0007), *denotes a *p*-value <0.05.

the minimal sufficient adjustment set when estimating the effect of the newborn microbial exposure on behavior temperament. Our use of DAGs for the determination of confounding adjustment is a method encouraged and endorsed within the STORMS checklist guidelines for microbiome analyses in human microbiome research (Mirzayi et al., 2021). Limitations in this study, as with the majority of studies in this area (Aatsinki et al., 2019; Carlson et al., 2021; Christian et al., 2015; Kelsey et al., 2021), is the use of parent rated IBQ and ECBQ questionnaires to assess infant behavioral temperament; the parent reported data may be prone to bias. Furthermore, our analysis is constricted by the singular microbiota sample obtained moments after birth. As the newly introduced bacterial species rapidly change whilst the microbiome develops throughout the gastrointestinal tract (Dashper et al., 2019; Stewart et al., 2018) caution is required when comparing our results with studies performed at later stages of infant development. Additionally, emerging evidence now suggests prenatal inflammation may contribute to offspring temperament traits, which would necessitate maternal infections processed as potential confounders rather than as an element affecting the neonate microbiome only, as analyzed in the present study (Serrano et al., 2024).

Although the various gut-brain axis pathways are proposed as the prime mechanisms for the developing microbiome's effect upon behavioral temperament (Alving-Jessep et al., 2022), Kelsey et al. (2021) also discuss the likelihood that the link between taxa diversity and behavioral temperament is mediated by functional brain network connectivity (Kelsey et al., 2021). It is therefore likely that additional factors beyond the developing neonate microbiome, such as environmental influences and genetic predispositions, contribute to behavioral outcomes during early childhood. For example, in contrast to other temperament traits, the family environment also appears to play an important role in shaping individuals' surgent behavioral traits (Holmboe, 2016).

In conclusion, this study investigated the association between initial bacterial exposure profiles at birth and behavioral outcomes in early childhood. Although no significant associations were

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Table 4. Linear mixed models with random intercepts for repeatedly measured behavior temperament effortful control (EFF)/negative affect (NEG)/surgency/positive emotionality (SUR) at years 1, 2, and 3. Most abundant individual bacterial genera as well as continuous time as fixed effects

	All Imputed ($n = 335$)	AB use ($n = 137$)	No AB use ($n = 198$)
EFF			
Lactobacillus	−0.02 (0.12)	−0.08 (0.18)	0.03 (0.16)
Streptococcus	0.27 (0.42)	0.33 (0.51)	−0.11 (0.73)
Staphylococcus	0.50 (0.39)	0.38 (0.50)	0.51 (0.60)
Corynebacterium	−0.14 (0.47)	−0.19 (0.61)	0.25 (0.74)
Stenotrophomonas	0.13 (0.75)	−0.24 (0.78)	3.30 (3.18)
Bacteroides	0.13 (0.61)	0.11 (0.73)	0.17 (1.10)
Prevotella	−0.01 (0.51)	0.33 (0.68)	−0.05 (0.78)
Anaerococcus	0.05 (0.95)	−2.64 (1.41)	1.79 (1.36)
NEG			
Lactobacillus	−0.21 (0.14)	−0.06 (0.21)	−0.34 (0.20)
Streptococcus	0.62 (0.51)	0.39 (0.62)	0.56 (0.88)
Staphylococcus	−0.13 (0.46)	0.38 (0.58)	−0.75 (0.73)
Corynebacterium	−0.16 (0.56)	−0.80 (0.72)	0.48 (0.90)
Stenotrophomonas	−1.22 (0.90)	−1.11 (0.92)	−1.21 (3.84)
Bacteroides	−0.36 (0.73)	0.48 (0.87)	−2.11 (1.33)
Prevotella	−0.51 (0.62)	−1.06 (0.81)	−0.29 (0.95)
Anaerococcus	−1.97 (1.15)	−0.38 (1.66)	−2.90 (1.66)
SUR			
Lactobacillus	0.06 (0.11)	−0.06 (0.19)	0.13 (0.14)
Streptococcus	0.55 (0.39)	0.21 (0.54)	0.97 (0.62)
Staphylococcus	0.67 (0.36)	0.21 (0.53)	1.19 (0.51)*
Corynebacterium	−0.21 (0.44)	−0.04 (0.65)	−0.21 (0.64)
Stenotrophomonas	0.78 (0.70)	0.98 (0.83)	0.37 (2.72)
Bacteroides	0.63 (0.57)	0.64 (0.78)	0.47 (0.94)
Prevotella	0.51 (0.48)	0.73 (0.72)	0.32 (0.67)
Anaerococcus	1.33 (0.89)	0.93 (1.50)	1.34 (1.17)

Models were additionally adjusted for fixed effects of the sex of the child, maternal age, and 3rd trimester EPDS score, and fitted for the whole sample as well as in subgroups of antibiotic use during birth (AB). The results are reported as *fixed effect estimate (standard error)*. **denotes a significance after Bonferroni correction (threshold for significance 0.0007), *denotes a *p*-value <0.05.

observed after Bonferroni corrections, the pre-correction findings indicate a possible link between intrapartum antibiotic use and the emerging infant behavioral temperament. Further studies are needed addressing the impact of the earliest periods of bacterial exposure at birth to oral colonization and subsequent trajectory towards gut microbiome development. Furthermore, our findings underscore the need for methodologically rigorous research in this emerging field necessary to explore the role of the neonate microbiome in behavioral development.

Understanding the complex interplay between the neonatal microbiome and behavioral outcomes could provide valuable insights and potentially inform interventions for promoting positive behavioral health in children.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0954579424001676.

Data availability statement. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contributions. MP performed the statistical analyses with contributions from AC and SML. KB, JP, LK-N, and SML designed the original cohort study this study is a part of. AC wrote the first draft of the manuscript with contributions and comments from MP and SML. All the authors reviewed and commented on subsequent drafts of the manuscript for important intellectual content.

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