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Associations among rearing environment and the infant gut microbiome with early-life neurodevelopment and cognitive development in a nonhuman primate model (*Macaca mulatta*)

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Abstract

Early gut microbiome development may impact brain and behavioral development. Using a nonhuman primate model (*Macaca mulatta*), we investigated the association between social environments and the gut microbiome on infant neurodevelopment and cognitive function. Infant rhesus monkeys ($n = 33$) were either mother-peer-reared (MPR) or nursery-reared (NR). Neurodevelopmental outcomes, namely emotional responsivity, visual orientation, and motor maturity, were assessed with the Primate Neonatal Neurobehavioral Assessment (PNNA) at 14–30 days. Cognitive development was assessed through tasks evaluating infant reward association, cognitive flexibility, and impulsivity at 6–8 months. The fecal microbiome was quantified from rectal swabs via 16S rRNA sequencing. Factor analysis was used to identify "co-abundance factors" describing patterns of microbial composition. We used multiple linear regressions with AIC Model Selection and differential abundance analysis (*MaAsLin2*) to evaluate relationships between co-abundance factors, microbiome diversity, and neuro-/cognitive development outcomes. At 30 days of age, a gut microbiome co-abundance factor, or pattern, with high *Prevotella* and *Lactobacillus* ($\beta = -0.88$, $p = 0.04$, AIC Weight = 68%) and gut microbiome alpha diversity as measured by Shannon diversity ($\beta = -1.33$, $p = 0.02$, AIC Weight = 80%) were both negatively associated with infant emotional responsivity. At 30 days of age, being NR was also associated with lower emotional responsivity (Factor 1 model: $\beta = -3.13$, $p < 0.01$; Shannon diversity model: $\beta = -3.77$, $p < 0.01$). The infant gut microbiome, along with early-rearing environments, may shape domains of neuro-/cognitive development related to temperament.

Introduction

Infancy is a critical period when early-life conditions shape developmental trajectories with consequences for later-life health.[1] The gut microbiome, a commensal community of bacteria and other microorganisms that reside in the gastrointestinal tract, is central to human and nonhuman primate growth and development. The first few years of life are sensitive periods for the development of the gut microbiome. Initially sterile, the infant gut is gradually colonized by maternal and environmental microbiota during birth, breastfeeding, and from environmental microbiota.[2,3] Because of its low microbial diversity – often described as a "blank slate" after birth – the infant gut microbiome is particularly sensitive to such exposures. Previous research in human and nonhuman primates suggests that the infant gut microbiome is shaped by infant diet,[4] early social environments,[5] and exposure to antibiotics,[6] among other factors.[7] In humans, the infant gut microbiome continues to develop until it reaches a stable, adult-like state at approximately 2–3 years of age.[8]

The assembly and maturation of the gut microbiome occurs concordantly with the development of the host, including neurodevelopment and cognitive development in the first few years of life via the gut-brain axis.[9] Evidence for this co-development comes from germ-free mice, which exhibit different behavioral and cognitive outcomes than their counterparts. Compared to "colonized" mice, germ-free mice exhibit reduced anxiety-like behavior[10,11] and increased motor activity,[12] suggesting that the gut microbiota holds a role in brain function. In line with the rodent literature, observational studies in humans have identified correlational

relationships between the microbiome and cognition. For example, in a large cohort study, gut microbiome composition from 3–6 months of age was associated with fine motor skills and communication, personal, and social skills at 3 years.[12] Human studies have also explored the role of specific taxa in this association. In U.S infants, *Faecalibacterium* abundance and greater alpha diversity were associated with lower cognitive scores on the Mullen Scales of Early Learning at one year of age.[13] Another study found that in rural China, scores on Bayley Scales of Infant Development were positively associated with *Faecalibacterium*, *Sutterella,* and *Clostridium* abundance, while scores were not significantly associated with alpha diversity in this same study.[14] However, studies with human cohorts thus far lack data on the influence of different early-life social environments because such studies are complex and difficult to carry out in a controlled manner. Rodent studies provide careful control, but the majority of studies are carried out in environments that do not recapitulate human experiences; moreover, rodent models differ substantially from humans in a number of physiological, neurological, and behavioral traits.[15]

Rhesus macaques (*Macaca mulatta*) can fill these gaps. As an evolutionarily and translationally relevant animal model, macaques have been widely utilized to investigate infant growth and development because they allow the opportunity to study the relationships between early-life environments and developmental outcomes in a controlled manner.[15,16] Rhesus macaques are a widely dispersed and adaptable primate, second only to humans in their global population size and widespread distribution. Rhesus macaques live in large troops of both sexes, largely composed of related females and immigrant males.[17] Infants are highly attached to their mothers until 1 month of age, after which they gradually socialize more with peers and become increasingly distant from their mothers; by 4–5 months, play with peers becomes a main form of social interaction, growing more complex with time.[16,18,19]

Research in the past two decades has illustrated typical patterns of neurodevelopment, cognitive development, and the early-life social factors that shape these patterns in captive rhesus macaques. For instance, a descriptive analysis of cognitive development found that nursery-reared (NR) infants, which were reared with other infants by human caregivers in a highly enriched environment but absent species-typical caregiver interactions, had no gross cognitive differences as compared to mother-peer-reared (MPR) infants (though MPR infants showed greater initial reactivity to stimuli).[20] Additional work with this population of infant rhesus macaques found that NR and MPR infants differed in gut microbiome composition across early development. Specifically, though NR and MPR did not differ at birth, MPR infants had higher *Bacteroides*, *Clostridium,* and lower *Bifidobacterium* at Day 14; higher *Lactobacillus* and *Streptococcus* at Day 30; higher *Lactobacillus*, *Bacteroides*, *Clostridium*, and *Prevotella*, as well as lower *Bifidobacterium* and *Streptococcus* at Day 90; and lastly, no differences at Day 180.[5] Given the evidence in rodent literature highlighting links between the gut and brain, as well as the observed associations between early-life microbiomes and later cognitive outcomes in large-scale epidemiological studies,[12–14,21] it is of interest to delineate how the infant gut microbiome is associated with neuro and cognitive development. Therefore, this study investigates the relationships between the infant gut microbiome, early neurodevelopment, and cognitive development in rhesus macaques as a function of early social and caregiving experiences. We hypothesize that gut microbiome composition and diversity, in conjunction with early-rearing environments, may partly shape infant neurodevelopment and cognitive development in the first year of life. In captive NR and MPR infant macaques, we measured the gut microbiome via 16S rRNA sequencing of infant rectal swabs on Day 14, Day 30, and Day 180. In the first 30 days of life, we assessed neurodevelopment by measuring infant reactivity via the Primate Neonatal Neurobehavioral Assessment.[22] We assessed cognitive development by measuring a) reward association and cognitive flexibility by using a black/white discrimination (BW) and reversal (BWR) task, respectively, and b) impulsivity by using an Object Detour Reach task.[20,23] Utilizing controlled, experimental conditions not possible in humans, this study will contribute to our understanding of relationships between the composition of the gut microbiome, neurodevelopment, and cognition in sensitive periods of development as a function of early-rearing environment.

Methods

Subjects

This research was approved by the NICHD Animal Care and Use Committee and adhered to the American Society of Primatologists Principles for the Ethical Treatment of Nonhuman Primates. The sample included 33 infant rhesus macaques (*M. mulatta*), all of which were born and reared at the Laboratory of Comparative Ethology in Poolesville, Maryland, USA. Subjects were pseudo-randomly assigned to one of two rearing conditions: mother-peer rearing (MPR) or nursery rearing (NR). Sex-balancing was ensured to the degree possible. Because early rearing was a primary predictor of outcomes and the rearing of nonhuman primates occurred in different settings, researchers were aware of group allocation at all stages.

MPR infants were born and reared in indoor/outdoor pens in social groups consisting of their mothers, adult females (8–10), half-siblings (3–5), and one adult male. MPR infants were breastfed *ad libitum* from birth to approximately 8 months of age when they were relocated to be housed with NR infants in another part of the facility. MPR infants were also continuously exposed to foods their mothers ate, including commercial monkey chow (#5045; Purina, St Louis, MO), seeds, nuts, fruits, and other foraged items. NR infants were reared indoors by human caregivers with other infants and had visual and auditory contact with peers daily. From Day 37, NR infants were randomly assigned to either peer rearing, where they spent 24 h per day in contact with three other same-aged peers (peer groups were sex-balanced), or surrogate peer rearing, where they lived in single cages with cloth surrogates and had two hours of same-age peer contact daily. Because the microbiome composition did not differ between peer-reared and surrogate peer-reared (see supplementary information in [5]), nor did overall early cognitive development,[20] we combined these sub-groups into a single group for analyses, "NR." NR infants were formula-fed (Similac Advance Complete Nutrition formula, Chicago, IL) until Day 180, after which they ate monkey chow and foraged seeds, nuts, and fruits. Rearing protocols have been extensively described elsewhere.[16,20]

Biospecimen collection

Our analysis centers on rectal swabs collected around the time of neurodevelopmental and cognitive assessments (Days 14, 30, and 180). Swabs were collected during routine neonatal assessments to prevent unnecessary separation of infants from social groups and/or mothers. Samples were collected between 0900–1100 h

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by gently washing the exterior surface of the infant rectum with a sterile saline solution and gauze, then gently inserting a sterile swab and spinning the swab three times in each direction and against the walls of the rectum before extraction (BD CultureSwab, Becton, Dickinson, and Company, Franklin Lakes, NJ). Microbiome sampling was conducted on a standardized schedule for all infants based on their date of birth (e.g., postnatal days 14 and 30). If the sampling or assessment day fell on a weekend, the collection date was shifted to either Friday or Monday (to accommodate for Saturday or Sunday, respectively). Rectal swabs were then placed on dry ice and stored at −80°C until they were shipped and analyzed at the Bailey Laboratory at Nationwide Children's Hospital in Columbus, Ohio. Due to institutional constraints in 2015 that affected the project schedule, samples were unavailable for each infant and each age (Supplemental Table S1a–b shows the number of samples available by age and by neurodevelopmental/cognitive assessment).

Microbiome analysis

The QIAamp Fast DNA Stool Mini Kit (Qiagen, Germantown, MD) was used to extract DNA from rectal swabs for microbiome analysis; slight modifications were made to the manufacturer's instructions. Swabs were incubated at 37°C for 45 min in lysozyme buffer (22 mg/ml lysozyme, 20 mM Tris-HCl, 2 mM E5/3/23TA, 1.2% Triton-x, pH 8.0), then bead-beat for 150 s with 0.1 mm zirconia beads. Samples were incubated at 95°C for 5 min with InhibitEX Buffer, then incubated at 70°C for 10 min with Proteinase K and Buffer AL. Following this step, the QIAamp Fast DNA Stool Mini Kit isolation protocol was followed, beginning with the ethanol step. DNA was quantified with the Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA) using the dsDNA Broad Range Assay Kit (Carlsbad, CA). Samples were standardized to at least 5 ng/µl before being sent to the Molecular and Cellular Imaging Center in Wooster, OH, for library preparation. Amplified polymerase chain reaction libraries were sequenced from both ends of the 250 nt region of the V4–V5 16S rRNA hypervariable region using an Illumina MiSeq, Illumina, Inc. (San Diego, CA). Illinois Mayo Taxonomy Operations for RNA Database Operations (IM-TORNADO-2) workflow integrated with Mothur V1.40.0 was utilized for quality (> 30), and operational taxonomic unit binning of paired-end reads using Mothur and Greengenes (version 13.8) databases.[24]

Experiment 1: early-life neurodevelopment

Infants were given the Primate Neonatal Neurobehavioral Assessment (PNNA) to assess early neurodevelopment. The PNNA was administered on 14 and 30 ± 2 days (details about the PNNA can be found in previous work[20,22]). As with the microbiome sampling, PNNA assessments were conducted on a standardized schedule for all infants based on their date of birth (e.g., postnatal days 14, 30), and if the sampling or assessment day fell on a weekend, the collection date was shifted to either Friday or Monday (to accommodate for Saturday or Sunday, respectively). The PNNA assessed infants on 52 reflexes, behaviors, and developmental milestones by a trained researcher. For each item, infants were scored on a scale from 0 (absent), to 1 (weak) and 2 (strong) based on their reaction to stimuli or behavior. Half-values were also possible. From the PNNA, we calculated three developmental domains. Emotional responsivity was a composite measure of behavioral reactivity or emotional responsivity and was computed as the sum of the following measures: Irritability (amount of distress), Consolability (ease of researcher to console infant), Struggle During Test (amount of movement/wriggling), and Predominant State (amount of vigilance and agitation). The visual orientation score was the sum of Visual Orientation, Visual Follow, Duration of Looking, and Attention Span. Motor maturity was the sum of Head Posture in the prone position, Head Posture in the supine position, Response Speed, Coordination, and Labyrinthine Righting.

Experiment 2: cognitive development

We administered tasks developed specifically for infant macaques to assess infant cognitive development.[20,23,25] Procedures for acclimating infants to cognitive testing have been described previously.[20] The main period of cognitive testing was 6-8 months of age. Scores (i.e., % correct response) on cognitive tests were averaged over all sessions; these averages or means were then utilized in the main statistical analyses. In this analysis, we focus on three measures of infant cognition – reward association, cognitive flexibility, and impulsivity, all detailed below. Cognitive training and assessments were initiated on the Monday closest to the infant's postnatal at 120 days of age. Tasks were presented to infants in the following order: B/W Discrimination, B/W Reversal, and ODR, and each infant progressed to the next task once the criteria for passing the preceding task were achieved.

Reward association was evaluated with the Black/White Discrimination Task (BW). In this task, infants' abilities to associate a color (black/white) with a food reward were tested. This task involved infants having to push aside one of two blocks (one black, one white) to receive a treat placed underneath the box in a well. For each infant, one color (black or white) was always associated/rewarded with a treat, whether presented on the left or right side. Infants had 60 s in each trial to make a choice, and criterion was reached when the infant made a certain number of correct responses (23 out of 25 trials in a session were correct, or 32 out of 35 correct responses over two days of testing). Averages of % correct responses ("average % correct") were used in analyses to measure infant reward association ability.[20]

Cognitive flexibility was measured with the Black/White Reversal task (BWR). This task measured how quickly infants could reverse a previously learned response from the Discrimination task. The colored block previously associated with a treat was "reversed" to the other color – the previously unrewarded block was now always rewarded, whether on the right or left side of the test board. Infants had 60 s to complete the task. Criterion was 23 out of 25 correctly answered trials. Averages of % correct responses ("average % correct") were utilized as measures of infant cognitive flexibility.[20]

Impulsivity was measured with the Object Detour Reach ("ODR") task, which involved placing a clear plastic box with a treat inside in front of the infant, with an opening to the side of the box. An infant could "bonk" the box by incorrectly reaching for the treat through the front or could make the "correct" choice by reaching into the opening on the side of the box to obtain the treat. We calculated the z-scores of the average percentages of "bonks" (errors where the infant used a straight line-of-sight reach when the box was open to one side), of straight errors (where the infant used an incorrect side detour when the box was open to the front), and of side errors (where the infant used an incorrect side detour when the box was open to one side). We summed these three z-scores to create a composite "impulsivity" measure. Infants that displayed these errors were deemed to exhibit greater impulsivity.[23,26] We also ran analyses with the average percent correct

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responses of the ODR task as the outcome, presented in the supplementary material (Supplementary Table S4–5).

Statistical analysis

Our statistical analysis involved a multi-pronged exploratory approach: 1) exploring the within- and between-sample diversity in the microbiome data via alpha and beta diversity analyses, respectively; 2) using dimensionality reduction to collapse the microbiome data into "co-abundance factors"; 3) examining bivariate relationships between "co-abundance factors," alpha diversity of the microbiome, and neurodevelopmental/cognitive outcomes via Spearman's correlations (corrected for multiple comparisons); 4) assessing relationships between microbiome variables and neurodevelopmental and cognitive outcomes in multiple linear regression models separated by age group, while controlling for covariates (infant sex, growth rate); 5) comparing the predictive power of rearing environment vs. microbiome-related characteristics through information theory approaches; and, 6) exploring additional relationships between individual microbial taxa and outcomes of interest via FDR-corrected Multivariate Association with Linear Models (*MaAsLin2*).[27] Analyses were conducted in R using *phyloseq* (version 1.46),[28] *psych* (version 2.4.1),[29] *ccrepe* (version 1.1.3),[30] *vegan* (version 2.6.4),[31] and *MaAsLin2* (version 1.16.0) packages[27]; read counts were normalized through total sum scaling prior to analysis.[32] We ran analyses such that every independent variable temporally preceded outcomes of interest. The sample size was based on data availability since not all infants had completed the experimental tasks successfully, and not all infants had a fecal swab available at certain time points. Samples with missing data were omitted pairwise to maximize sample size for each analysis. In Experiment 1, the sample sizes were as follows: 18 infants had both Day 14 fecal samples and neurodevelopmental measures (emotional responsivity, visual orientation, and motor maturity), and 19 infants had both Day 30 fecal samples and neurodevelopmental measures. The sample sizes for Experiment 2 were as follows: 18 infants had both Day 180 fecal samples and reward association scores, 15 had both Day 180 fecal samples and cognitive flexibility scores, and 20 had both Day 180 fecal samples and impulsivity scores.

Alpha and beta diversity

We calculated alpha diversity to describe within-sample microbial diversity. We selected the Shannon diversity index as our alpha diversity measure because of its robusticity in small sample sizes.[33] We tested for sex- and rearing environment-based differences in alpha diversity, co-abundance factors, and neurodevelopmental and cognitive outcomes with Mann-Whitney U tests and utilizing Cliff's Delta as the effect size estimates. We conducted a permutational analysis of variance (PERMANOVA) to measure differences in overall gut microbiome composition according to variables of interest (i.e., rearing environment, growth rate, and infant sex). We utilized Bray-Curtis dissimilarity as the beta diversity metric for the PERMANOVAs; Bray-Curtis quantifies the dissimilarity between samples (with values ranging from 0, meaning that two samples share all species and 1, which indicates that two samples do not share any species). We used the R *adonis2* function (*vegan* package) for PERMANOVA, with the standard 999 permutations.[31] Principal Coordinates Analysis (PCoA) plots were used to visualize beta diversity analyses in two-dimensional space.

Principal components analysis (PCA) to generate microbiome "co-abundance factors"

To evaluate infant gut microbiome composition on a continuous scale and to reduce the dimensionality of the microbiome data, we obtained co-abundance factors describing groupings of bacterial taxa in the infant gut microbiome following previously published protocols.[12] Briefly, we determined Spearman's correlation coefficients for the top 10 most abundant taxa (based on mean relative abundance) for the following age groups: Days 14, 30, and 180. Using Spearman's correlation matrices, we conducted Principal Components Analysis (PCA; with varimax rotation) using the *psych* package in R. For all groups, a 3-factor solution was determined using the scree plot method. Factor/Principal Component loadings were used to generate factor scores. Individual factor scores, which we name Factors 1, 2 and 3 (for all ages), were utilized as independent variables in multiple linear regression models, with neurodevelopmental and cognitive measures as outcomes.

Spearman's correlations

We calculated Spearman's correlations between covariates, microbiome variables (co-abundance factors and Shannon diversity), and neurodevelopmental and cognitive variables. We also calculated *p*-values adjusted for the False-Discovery Rate.

Multiple linear regression models and AIC model selection

We ran separate multiple linear regressions by time point, with microbiome alpha diversity (Shannon diversity), "co-abundance" Factors 1, 2 and 3, and rearing environment predicting PNNA and cognitive scores. Each model included infant sex and infant growth rate (g/day) as covariates. Following previous protocol,[12] we opted out of using corrections for multiple comparisons in the multiple linear regression models (e.g., Bonferroni), in that these adjustments would be too conservative for our approach.[33] We checked that each model met regression assumptions beforehand by examining data linearity, normality of residuals, and homoscedasticity and by looking for data points with high leverage with diagnostic plots; each model met the assumptions within reason. We used AIC model selection to identify the best-fit model among models with rearing environment, alpha diversity, and both rearing environment and alpha diversity as predictors.

Multivariate association with linear models (MaAsLin2)

To investigate possible associations between less abundant taxa not captured by co-abundance groupings (following previously established methods[12]), we utilized Multivariate Association with Linear Models (*MaAsLin2*) to assess the relationship between individual genus-level taxa and cognitive and neurodevelopmental outcomes. *MaAsLin2* employs general linear models to assess associations between specific microbial taxa and variables of interest while controlling for false discoveries, using *p* and *q* values (FDR-adjusted *p*-values). Using our normalized microbiome data, we ran the *MaAsLin2* analysis with the following parameters: minimum abundance = 0; minimum prevalence = 10%; normalization = none; transformation = none; standardize = false; *q*-value threshold = 0.25. We selected the program's default *q*-value threshold of 0.25 because of our exploratory aims and because of our inclusion of covariates in the models and the additional load they have on the burden of multiple testing. We included various cognitive and neurodevelopmental outcomes as the "fixed effects" in each model. All models also included covariates – rearing environment, infant sex, and growth rate. To avoid reporting

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non-meaningful relationships in our small sample sizes, we only report statistically significant associations for taxa found at detectable levels in greater than 10 subjects.

Results

Descriptive statistics

This analysis used data from 33 infant rhesus macaque subjects. Most infants did not have a fecal swab for all three time points. For example, a portion (12/20) of the infants with data at Day 180 did not have any fecal swabs for either Day 14 or Day 30. Moreover, 11 of the 18 infants with Day 14 samples did not have a fecal swab for Day 180, and 11 of the 19 infants with Day 30 samples similarly did not have a Day 180 swab (Supplementary Table S1a–b). Forty-two percent of the infant subjects included in this analysis were were raised in an MPR environment (14/33), and 48% (16/33) were female (Table 1).

MPR and NR infants differed in markers of early neurodevelopment in the first 30 days of life. In the Day 14 and Day 30 age groups, MPR infants had higher emotional responsivity scores than their NR peers, indicative of greater emotionality (Day 14: Cliff's Delta = 0.84, $p < 0.01$; Day 30: Cliff's Delta = 2.67, $p < 0.01$). NR infants had higher visual orientation scores in Day 14 and Day 30 age groups (Day 14: Cliff's Delta = -0.86, $p < 0.01$; Day 30: Cliff's Delta = −0.81, $p < 0.01$); however, there were no significant differences in motor maturity by rearing environment at either age group (Day 14: Cliff's Delta = 0.27, $p = 0.35$; Day 30: Cliff's Delta = 0.27, $p = 0.33$). Infant sex was not statistically associated with either emotional responsivity scores or with visual orientation (effect sizes not shown; $p > 0.05$). Surrogate- and peer-reared infants did not differ in most neurodevelopmental and cognitive outcomes, except that at Day 30, SPR infants had higher visual orientation (Cliff's Delta: −1.0, $p = 0.02$), and at Day 180, SPR infants had lower cognitive flexibility (Cliff's Delta = 0.77, $p = 0.03$; Supplemental Table S6).

MPR and NR infants did not differ regarding later cognitive outcomes, except for one measure on the Object Detour Reach or impulsivity task. In the Day 180 age group, MPR infants had lower percent correct responses than NR infants (Cliff's Delta = −0.68, $p = 0.01$); this measure does not necessarily encapsulate impulsivity but instead captures the number of correct trials where the animal was given up to 60 s to correctly complete the trial in as many attempts as it wanted. We note that for these later cognitive outcomes, fewer infants completed the tasks, and as a result, smaller sample sizes of data were available for statistical analysis (Supplementary Table S1a–b).

Fecal microbiome composition and co-abundance factors

Figures 1–3 show taxonomic variation in the fecal microbiome across age groups and rearing environments. There were qualitative age-and-rearing-environment-specific patterns of taxonomic composition. In MPR infants, at Days 14, 30, and 180, the predominant phylum was Firmicutes at each time point (41.6 ± 8.2%, 43.7 ± 16.6%, and 57.9 ± 20.0%, respectively). In NR infants, the predominant phylum at Day 14 and 30 were Actinobacteria (36.5 ± 28.5% and 41.4 ± 30.7%, respectively), and the predominant phylum at Day 180 was Firmicutes (61.3 ± 20.3%). At the family level, MPR infants had gut microbiota enriched in Prevotellaceae at Days 14 and 30 (27.6 ± 13.6 and 28.8 ± 14.0%, respectively); at Day 180, Ruminococcaceae was the predominant bacterial family in the MPR group (19.3 ± 10.1%). In NR infants, Bifidobacteriaceae was predominant at Days 14 and 30 (34.5 ± 28.3% and 37.7 ± 33.3%, respectively); at Day 180, however, Prevotellaceae was predominant (28.6 ± 16.9%). At the genus level, age-specific trends mirrored those at the phylum and family levels. In MPR infants, *Prevotella* was the predominant genus at Days 14 (27.6 ±13.6%), 30 (28.8 ± 13.9%,) and 180 (18.8 ± 7.9%). In contrast, NR infants exhibited gut microbiota enriched in *Bifidobacterium* at Days 14 (34.6 ± 28.3%) and 30 (37.7 ± 33.3%); at Day 180, *Prevotella* was the most abundant genus in NR infants (28.6 ± 16.9%; Figs 1–3).

At each age group, factor analysis yielded three co-abundance factors, or general patterns of fecal microbiome composition. These general patterns of fecal microbiome composition tended to vary in representation of key bacterial genera such as *Prevotella, Lactobacillus*, and *Bifidobacterium*, which were also variably abundant across ages and rearing environments (Figs 1–3). At Day 14, Factor 1 had high *Catenibacterium* and *Lactobacillus* (40% of variance); Factor 2 represented low *Bifidobacterium* and high *Blautia* (36% of variance); Factor 3 represented low *Prevotella* (Prevotellaceae family; 24% of variance). At Day 30, Factor 1 was heavily loaded by *Prevotella* (Paraprevotellaceae family) and *Lactobacillus*, as well as low *Faecalibacterium* (41% of variance); Factor 2 had high *Eubacterium*, *Blautia,* and low *Catenibacterium* (37% of variance); Factor 3 had high *Collinsella*, *Ruminococcus* and low *Bifidobacterium* (22% of variance). At Day 180, Factor 1 had high *Roseburia*, *Blautia,* and low *Ruminococcus* and *Prevotella* (36% of variance); Factor 2 had low *Bifidobacterium* and low abundance of an unclassified genus (32% of variance); Factor 3 had high *Faecalibacterium*, and low *Prevotella*, *Oscillospira*, and *Lactobacillus* (32% of variance). The factor loadings for each factor are represented in Supplemental Tables 2a–c.

Alpha and beta diversity

Microbiome alpha diversity varied by age and rearing environment but not by infant sex. Infants across the two rearing conditions differed in their overall community composition, and this held across every age group. Specifically, Shannon diversity in the gut microbiome increased with age (ANOVA: F-statistic = 10.84, $p < 0.01$). Monkeys who were NR had significantly lower Shannon diversity on Day 14 (Cliff's Delta = 0.79, $p < 0.01$), Day 30 (Cliff's Delta = 0.60, $p = 0.02$), but not at Day 180 (Cliff's Delta = 0.29, $p = 0.30$; Supplementary Figures S1a–c).

Figures 4–6 show Principal Coordinates Analysis (PCoA) plots of beta diversity estimates and results of the PERMANOVA models. According to PERMANOVA, rearing environment was associated with microbiome beta diversity, or overall fecal microbiome composition, at Day 14 ($R^2 = 0.16$, $p = 0.01$), Day 30 ($R^2 = 0.17$, $p = 0.01$), and Day 180 ($R^2 = 0.13$, $p = 0.01$); rearing environment differences in beta diversity were also found in this cohort in 2019.[5] Infant sex was not associated with microbiome composition at any time point ($p > 0.05$; Figs 4–6).

Correlations between microbiome characteristics and neurodevelopmental/cognitive outcomes

After exploring Spearman's correlations between variables and FDR adjustment of p-values, we found that Shannon diversity and Factor 2 at Day 14 were positively correlated ($\rho = 0.79$, $p < 0.01$; Fig 7), suggesting that this co-abundance factor was associated with greater microbial diversity. We also observed that Shannon diversity and Factor 1 at Day 30 were positively associated ($\rho = 0.87$, $p < 0.001$; Fig 8). There were no statistically significant

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Table 1. Sample characteristics by infant's age at microbiome sampling (mean [standard deviation] unless noted;* indicates % [proportion of sample])

	Day 14	Day 30	Day 180	All subjects ($n = 33$)
N	18	19	20	33
Rearing style (% MPR)*	38.8 (7/18)	38.8 (9/19)	40 (8/20)	42.42 (14/33)
Infant sex (% female)*				
MPR	42.8 (3/7)	42.8 (3/9)	50 (4/8)	
NR	63.6 (7/11)	63.63 (6/10)	50 (6/12)	
Total	55.5 (10/18)	47.37 (9/19)	50 (10/20)	48.48 (16/33)
Growth rate (g/day)				
MPR	4.88 (1.01)	4.98 (0.85)	5.11 (0.69)	
NR	13.08 (6.57)	13.42 (6.82)	7.30 (1.82)	
Cliff's delta (*p*-value)	−0.82 (**$p < 0.01$**)	−0.80 (**$p < 0.01$**)	−0.70 (**$p = 0.01$**)	
Total	9.89 (6.53)	9.42 (6.51)	6.43 (1.82)	8.23 (5.23)
Emotional responsivity				
MPR	4.43 (1.48)	4.44 (1.37)		
NR	1.72 (1.13)	1.78 (1.18)		
Cliff's delta (*p*-value)	0.84 (**$p < 0.01$**)	2.67 (**$p < 0.01$**)		
Total	2.78 (1.83)	3.04 (1.84)		3.15 (1.94)
Visual orientation				
MPR	4.85 (2.23)	5.25 (1.91)		
NR	9.68 (2.71)	9.42 (2.71)		
Cliff's delta (*p*-value)	−0.86 (**$p < 0.01$**)	−0.81 (**$p < 0.01$**)		
Total	7.81 (3.46)	7.44 (3.14)		8.16 (3.47)
Motor maturity				
MPR	9.86 (0.57)	9.83 (0.56)		
NR	9.59 (0.42)	9.58 (0.44)		
Cliff's delta (*p*-value)	0.27 ($p = 0.35$)	0.27 ($p = 0.33$)		
Total	9.69 (0.49)	9.70 (0.50)		9.755 (0.61)
Reward association (average % correct)				
MPR			81.85 (8.67)	
NR			81.19 (5.89)	
Cliff's delta (*p*-value)			0.15 ($p = 0.64$)	
Total			81.41 (6.68)	81.51 (6.26)
Cognitive flexibility (average % correct)				
MPR			57.11 (6.85)	
NR			58.23 (6.99)	
Cliff's delta (*p*-value)			−0.22 ($p = 0.63$)	
Total			58.01 (6.73)	58.69 (6.60)
Impulsivity (average % correct)				
MPR			93.40 (4.90)	
NR			98.11 (2.48)	
Cliff's delta (*p*-value)			−0.68 (**$p = 0.01$**)	
Total			96.22 (4.24)	96.35 (4.22)

(*Continued*)

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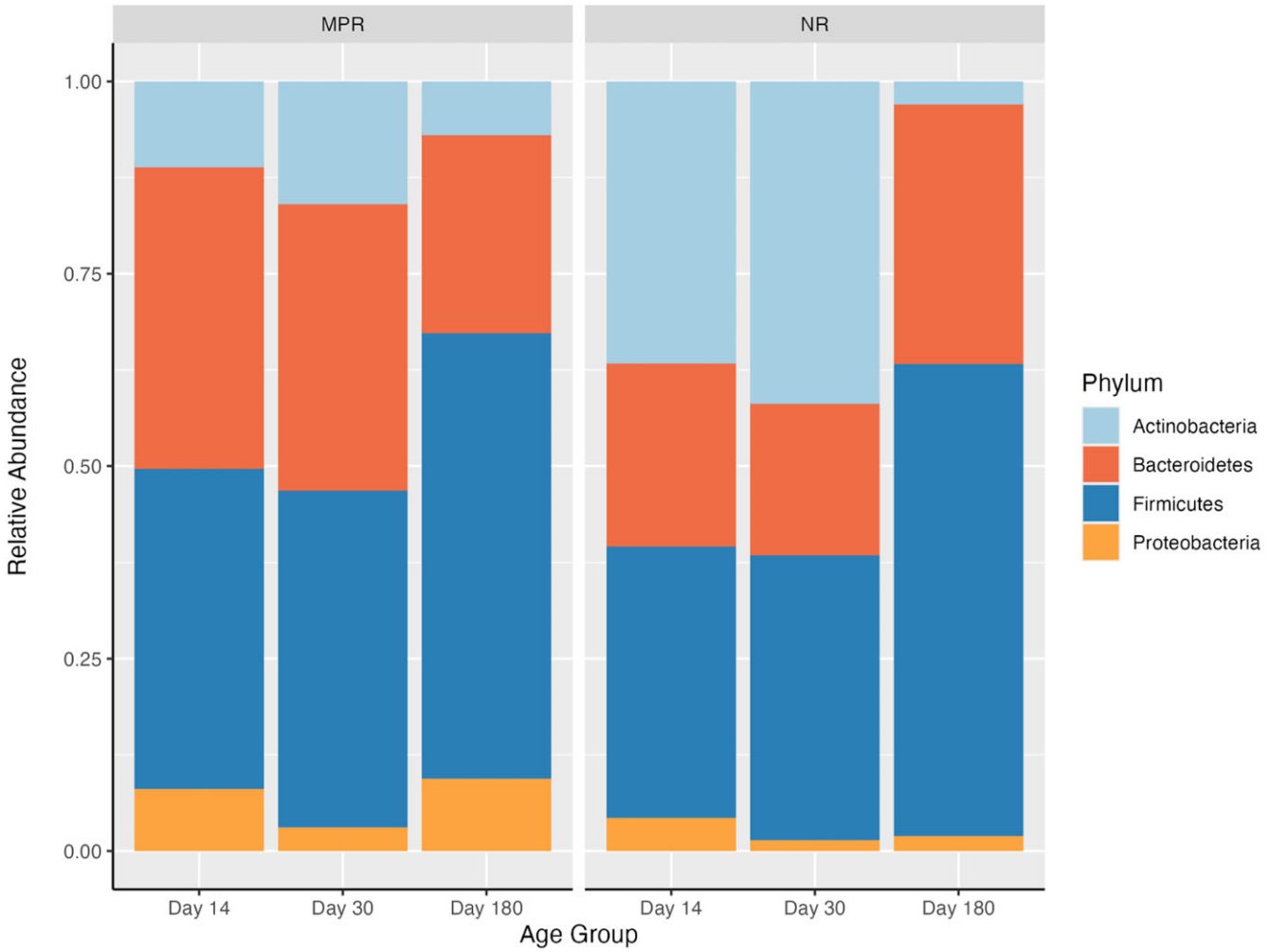
Table 1. (*Continued*)

	Day 14	Day 30	Day 180	All subjects ($n = 33$)
Impulsivity (composite measure, summed *z*-scores)				
MPR			−0.27 (2.32)	
NR			−0.41 (1.71)	
Cliff's delta (*p*-value)			−0.083 ($p = 0.79$)	
Total			−0.35 (1.92)	−0.01 (2.29)



[1]Day 14, MPR: *n* = 7; Day 30, MPR: *n* = 9; Day 180, MPR = 8; Day 14, NR: *n* =11; Day 30, NR: *n* = 10; Day 180, NR: *n* = 12)

Figure 1. Gut microbiome composition at phylum level (top 4 phyla)[1].

correlations between any co-abundance factor nor Shannon diversity with any neurodevelopmental or cognitive developmental outcome (Figures 7–9).

Multiple linear regression models and AIC model selection

Our regression models and AIC model selection procedure, in general, demonstrated that rearing environment was more often significantly associated with neuro and cognitive developmental outcomes than the composition and diversity of the infant fecal microbiome (Tables 2–5). Being NR was associated with lower emotional responsivity, higher visual orientation, and lower motor maturity in early infancy; however, there were no rearing-based differences in reward association, cognitive flexibility, or impulsivity. While most microbiome co-abundance factors and Shannon diversity across different ages were not significantly associated with

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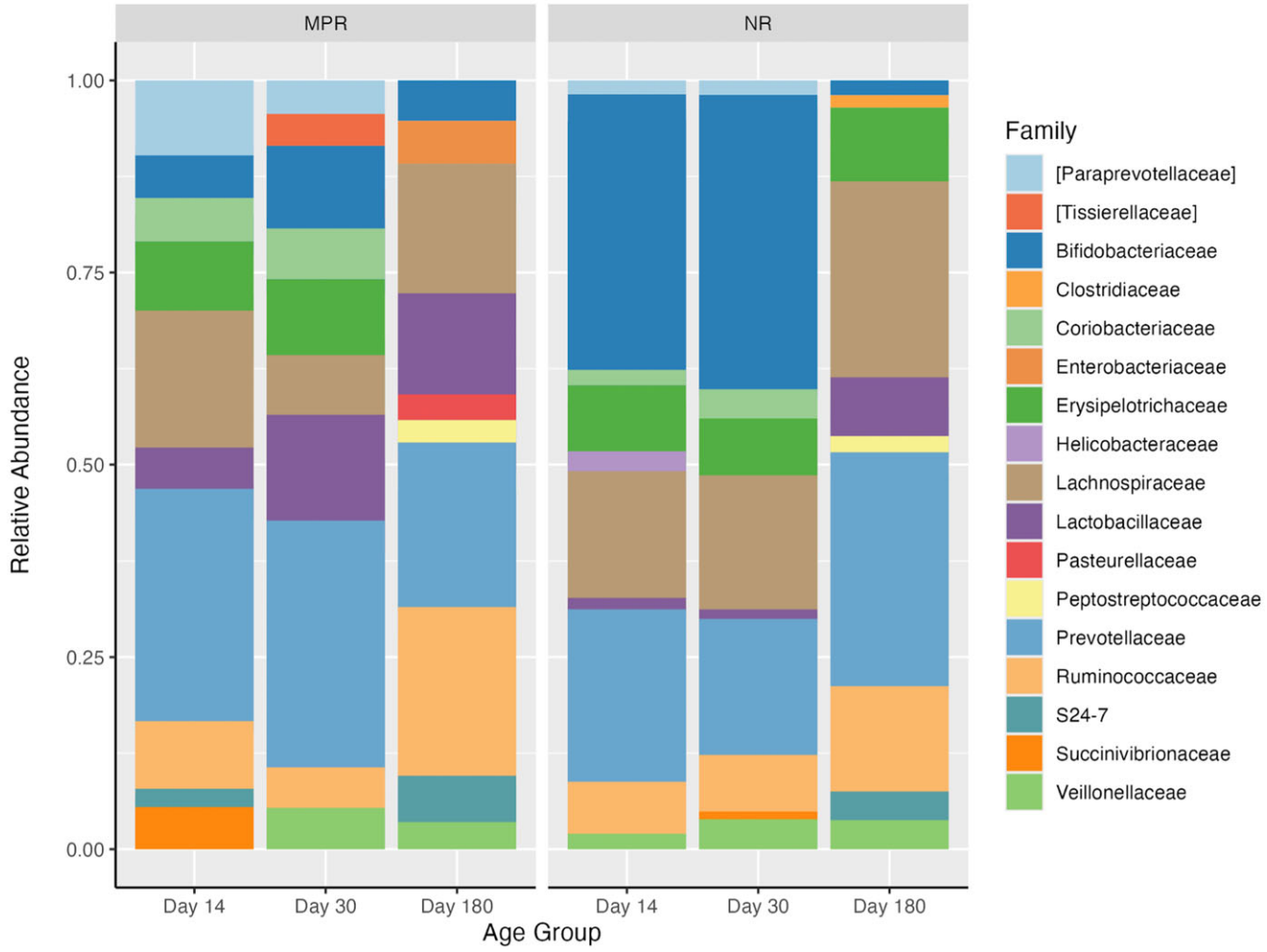

[1]Day 14, MPR: *n* = 7; Day 30, MPR: *n* = 9; Day 180, MPR = 8; Day 14, NR: *n* =11; Day 30, NR: *n* = 10; Day 180, NR: *n* = 12)

Figure 2. Gut microbiome composition at family level (top 10 families)[1].

neuro-/cognitive developmental outcomes, we found that fecal microbiome composition and diversity at 30 days of age were associated with emotional responsivity (Tables 2–5).

Experiment 1: early infancy gut microbiome and neurodevelopment

In Experiment 1, infant fecal microbiome composition was only associated with infant emotional responsivity at 30 days, while rearing environment was linked to differences in almost every neurodevelopmental outcome. Specifically, we found that at Day 14, there were no significant associations between microbiome characteristics and neurodevelopmental outcomes. However, at Day 14, being NR as opposed to MPR was associated with lower emotional responsivity ($\beta = -2.73$, $p = 0.01$; Table 2) and lower motor maturity ($\beta = -0.54$, $p < 0.01$; Table 2) while accounting for other covariates and microbiome co-abundance factors. At Day 14, in most of the models we ran, being NR was associated with higher visual orientation (Factor 1 model: $\beta = 4.47$ (95% CI: 0.23- 8.12), $p = 0.04$; Factor 2 model: $\beta = 3.76$ (95% CI: 0.05-7.5), $p = 0.05$); however, in one model, where Factor 2 was the independent variable, being NR was not significantly associated with visual orientation and the confidence interval included zero (Factor 2 model: $\beta = 3.29$ (95% CI: −0.98-7.6), $p = 0.11$). Therefore, the averaged 95% confidence interval (Rearing environment (NR): 95% CI: 0.98-8.12) shown in Table 2 passes through zero, even though the p-value indicates statistical significance. We emphasize that the effect size, the beta coefficient, is relatively consistent across models and that this points to a relationship between the rearing environment and visual orientation in the Day 14 age group.

At Day 30, Factor 1 (a pattern with high *Prevotella* and *Lactobacillus*, low *Bifidobacterium* and *Faecalibacterium*; $\beta = -0.88$, $p = 0.04$) and being NR ($\beta = -3.13$, $p < 0.01$) were negatively associated with emotional responsivity (Table 2). The model with Factor 1 and rearing environment as predictor variables was the "best-fit" model and accounted for 68% of the total predictive power in the model set (AIC Weight = 68%). Shannon diversity at Day 30 was also negatively associated with emotional responsivity, such that a 1-unit increase in Shannon diversity at Day 30 corresponded to a ~ 1.33 unit decrease in emotional responsivity score ($\beta = -1.33$, $p = 0.02$, AIC Weight = 80%; Table 4).

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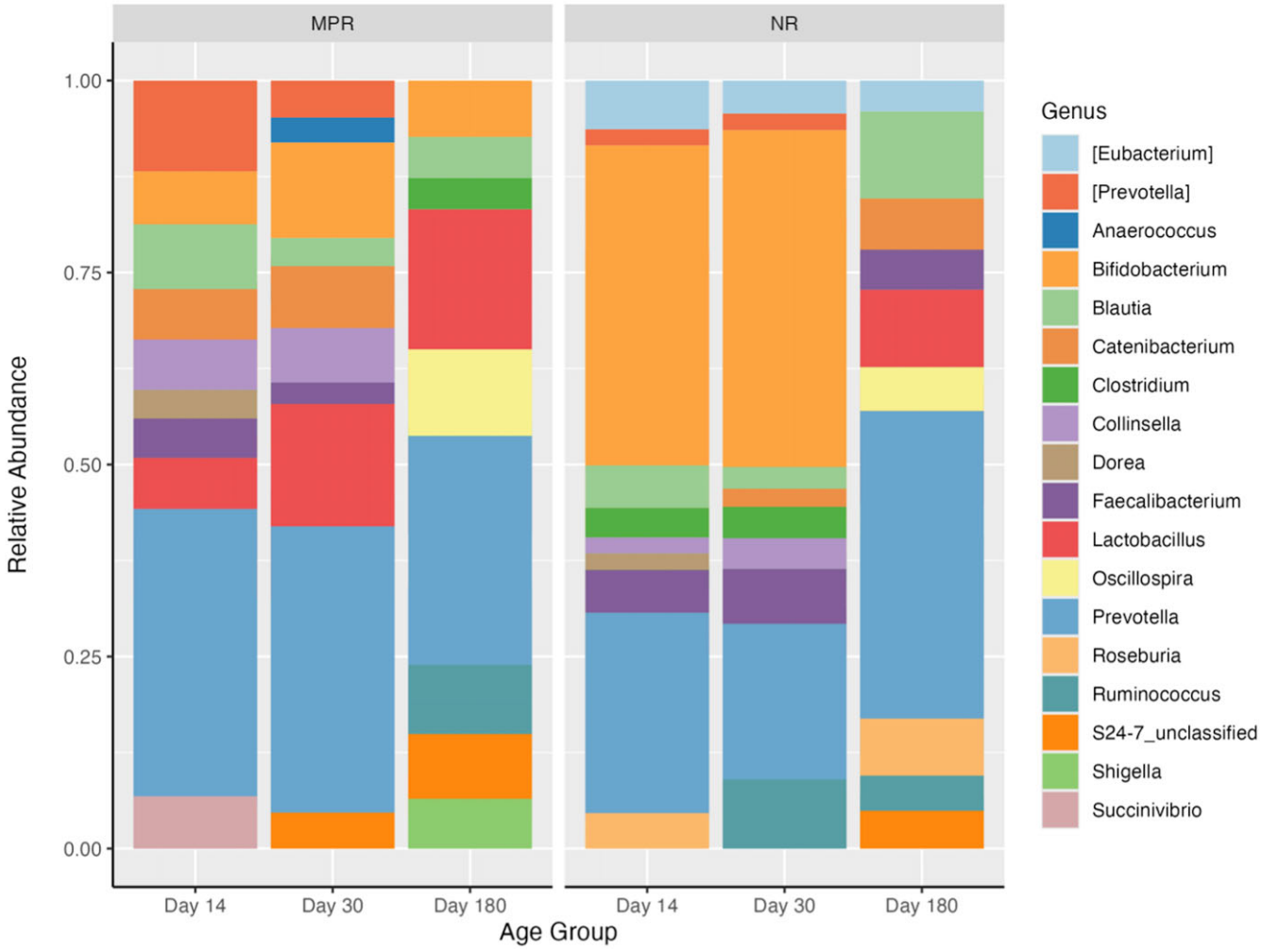
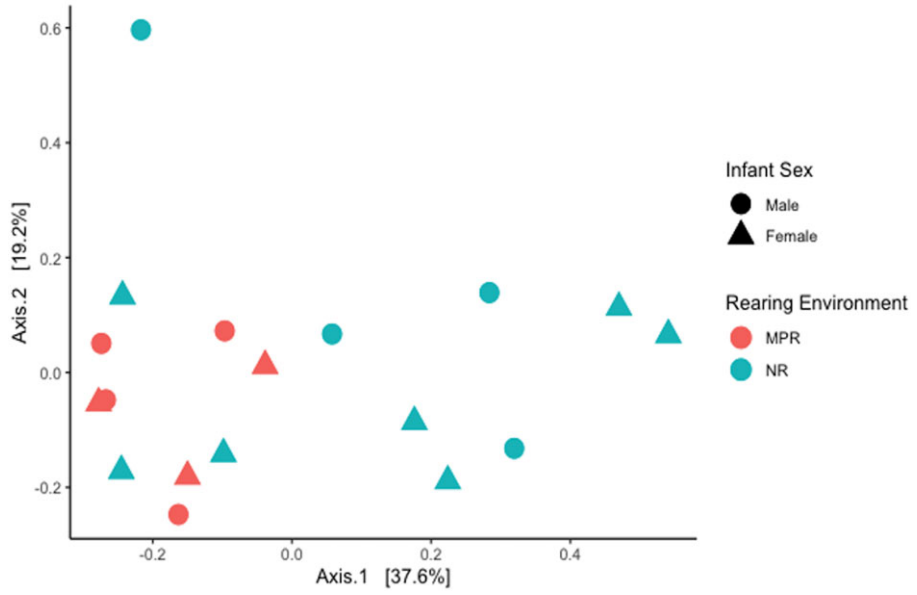


[1]Day 14, MPR: *n* = 7; Day 30, MPR: *n* = 9; Day 180, MPR = 8; Day 14, NR: *n* = 11; Day 30, NR: *n* = 10; Day 180, NR: *n* = 12)

Figure 3. Gut microbiome composition at genus level (top 10 genera)[1].



[1]PERMANOVA results: Rearing Environment: R^2 = 0.16; *p* = 0.01; Infant Sex: R^2 = 0.04; *p* = 0.74

Figure 4. Day 14 (MPR: *n* = 7; NR: *n* = 11; total: *n* = 18)[1].

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Table 2. Multiple linear regression models with microbial co-abundance factors predicting neurodevelopment (Experiment 1)[1]

	Emotional responsivity		Visual orientation		Motor maturity	
	β (95% CI)	*p*	*β* (95% CI)	*p*	*β* (95% CI)	*p*
Day 14[2]						
Factor 1	−0.22 (−1.14–0.69)	0.61	0.41 (−1.41–2.23)	0.63	−0.12 (−0.44–0.2)	0.43
Factor 2	0.25 (−0.69–1.19)	0.57	−0.48 (−2.36–1.39)	0.59	0.14 (−0.18–0.47)	0.35
Factor 3	0.6 (−0.18–1.38)	0.12	−0.29 (−1.99–1.4)	0.72	−0.14 (−0.43–0.14)	0.30
Infant sex	−0.42 (−1.97–1.1)	0.55	−0.43 (−3.58–2.52)	0.18	−0.16 (−0.73–0.44)	0.18
Growth rate	0.02 (−0.13–0.17)	0.81	0.14 (−0.15–0.45)	0.54	0.03 (−0.02–0.09)	0.54
Rearing environment (NR)	**−2.73 (−4.99–−0.39)**	**0.01**	**3.74 (−0.98–8.12)**	**<0.01**	**−0.54 (−1.33–−0.36)**	**<0.01**
Day 30[3]						
Factor 1	**−0.88 (1.71–−0.05)**	**0.04**	−0.23 (−2.06–1.60)	0.79	0.15 (−0.21–0.51)	0.38
Factor 2	−0.48 (−1.28–0.33)	0.22	0.58 (−0.99–2.15)	0.44	0.23 (−0.07–0.52)	0.12
Factor 3	0.41 (−0.25–1.08)	0.20	0.51 (−0.79–1.81)	0.41	−0.06 (−0.33–0.20)	0.63
Infant sex	−0.70 (−1.96–0.55)	0.25	−0.06 (−2.60–2.48)	0.88	−0.15 (−0.65–0.34)	0.52
Growth rate	0.04 (−0.09–0.18)	0.54	0.12 (−0.15–0.40)	0.36	0.04 (−0.02–0.09)	0.21
Rearing environment (NR)	**−3.13 (−4.93–−1.34)**	**<0.01**	2.92 (−0.75–6.59)	0.12	−0.50 (−1.22–0.21)	0.18

[1]Models for each Factor were run separately. Coefficients, confidence intervals and *p*-values of covariates (infant sex, growth rate and rearing environment) reflect the mean of their respective values across all models with Factor 1, 2 and 3 as predictors.
[2]Day 14: MPR: $n = 7$; NR: $n = 11$; total: $n = 18$.
[3]Day 30: MPR: $n = 9$, NR: $n = 10$; total: $n = 19$.

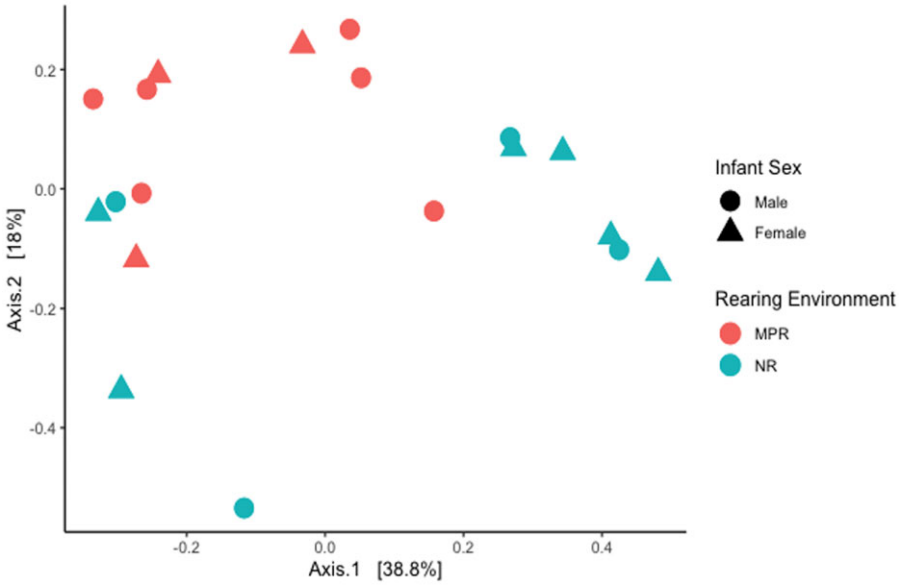

Figure 5. Day 30 (MPR: $n = 9$; NR: $n = 10$; total: n=19)[1].

[1]PERMANOVA results: Rearing Environment: $R^2 = 0.17$; $p = 0.01$; Infant Sex: $R^2 = 0.03$; $p = 0.86$

When we compared different models by using AIC model selection, "rearing" models in which only rearing environment was included as a predictor were consistently the best-fit models for predicting infants' early neurodevelopment (Supplemental Table S3*a-b*). However, "rearing and microbiome" models were best-fit among models predicting emotional responsivity at Day 30 (Factor 1: AIC Weight = 68%; Shannon diversity: AIC Weight = 80%; neurodevelopment; Supplemental Table S3a–b), suggesting that the gut microbiome and rearing environment together provide the greatest amount of predictive power among all models in explaining infant emotional responsivity at this age.

Experiment 2: late infancy gut microbiome and cognitive development

We did not observe statistically significant relationships between the microbiome, rearing environment, and cognitive outcomes in Experiment 2. Neither microbial co-abundance factors nor Shannon diversity were significantly associated with either

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Table 3. Multiple linear regression models with microbial co-abundance factors predicting cognitive outcomes (Experiment 2)[1]

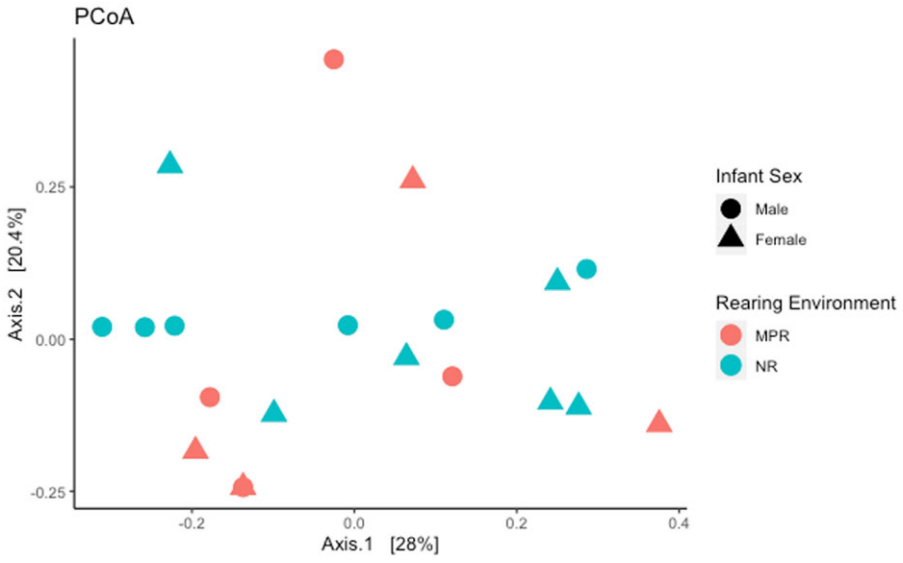
	Reward association[2]		Cognitive flexibility[3]		Impulsivity (composite) [4]	
	β (95% CI)	*p*	β (95% CI)	*p*	β (95% CI)	*p*
Day 180						
Factor 1	−3.28 (−7.53–0.97)	0.12	2.59 (−2.25–7.43)	0.26	−0.30 (−1.57–0.97)	0.62
Factor 2	−1.13 (−5.86–3.59)	0.61	−4.65 (−12.13–2.83)	0.20	−0.20 (−1.38–0.96)	0.71
Factor 3	3.15 (−1.83–8.13)	0.19	−4.98 (−11.03–1.07)	0.10	0.32 (−0.83–1.48)	0.56
Infant sex (Female)	−2.32 (−8.79–3.30)	0.61	1.28 (−2.54–13.83)	0.38	0.53 (−1.69–2.75)	0.61
Growth rate (g/day)	0.16 (−2.88–2.57)	0.65	1.97 (−2.79–3.82)	0.51	0.89 (−0.67–0.84)	0.81
Rearing environment (NR)	−0.09 (−11.28–8.24)	0.76	2.12 (−10.68–13.05)	0.60	−0.31 (−2.87–2.27)	0.80

[1]Models for each Factor were run separately. Coefficients, confidence intervals and p-values of covariates (infant sex, growth rate and rearing environment) reflect the mean of their respective values across all models with Factor 1, 2 and 3 as predictors.
[2]Reward Association: MPR: $n = 6$; NR: $n = 12$; total: $n = 18$.
[3]Cognitive flexibility: MPR: $n = 3$; NR: $n = 12$; total: $n = 15$.
[4]Impulsivity: MPR: $n = 8$; NR: $n = 12$; total: $n = 20$.

Table 4. Multiple linear regression models with Shannon diversity predicting neurodevelopment (Experiment 1)

	Emotional responsivity		Visual orientation		Motor maturity	
	β (95% CI)	*p*	*β* (95% CI)	*p*	*β* (95% CI)	*p*
Day 14[1]						
Shannon diversity	0.22 (−1.47–1.91)	0.78	−0.43 (−3.79–2.93)	0.79	0.08 (−0.51–0.68)	0.77
Infant sex	−0.54 (−1.97–0.88)	0.43	−0.32 (−3.17–2.52)	0.81	−0.16 (−0.66–0.34)	0.51
Growth rate	0.03 (−0.11–0.17)	0.63	0.12 (−0.16–0.40)	0.36	0.04 (−0.01–0.09)	0.11
Rearing environment (NR)	**−2.70 (−4.81–−0.61)**	**0.02**	3.60 (−0.57–7.78)	0.08	−0.50 (−1.24–0.24)	0.17
Day 30[2]						
Shannon diversity	**−1.33 (−2.45–−0.21)**	**0.02**	1.10 (−1.38–3.59)	0.36	0.09 (−0.42–0.60)	0.72
Infant sex	−0.95 (−2.06–0.16)	0.09	−0.05 (−2.50–2.41)	0.97	−0.11 (−0.62–0.39)	0.64
Growth rate	0.06 (−0.05–0.18)	0.27	0.13 (−0.13–0.38)	0.31	0.04 (−0.01–0.09)	0.12
Rearing environment (NR)	**−3.77 (−5.48–−2.06)**	**$p < 0.01$**	**3.81 (0.03–7.59)**	**0.05**	−0.52 (−1.29–0.26)	0.18

[1]Day 14: MPR: $n = 7$; NR: $n = 11$; total: $n = 18$.
[2]Day 30: MPR: $n = 9$, NR: $n = 10$; total: $n = 19$.



[1]PERMANOVA results: Rearing Environment: $R^2 = 0.13$; $p = 0.01$; Infant Sex: $R^2 = 0.06$; $p = 0.29$

Figure 6. Day 180 (MPR: $n = 8$; NR: $n = 12$; total: $n = 20$)[1].

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Table 5. Multiple linear regression model with Shannon diversity predicting cognitive outcomes (Experiment 2)

	Reward association[1]		Cognitive flexibility[2]		Impulsivity (composite)[3]	
	β (95% CI)	p	β (95% CI)	p	β (95% CI)	p
Day 180						
Shannon diversity	−3.84 (−12.08–4.39)	0.33	−2.21 (−14.63–10.21)	0.70	0.70 (−1.44–2.85)	0.50
Infant sex	−1.54 (9.44–6.34)	0.68	1.26 (−8.06–10.59)	0.77	0.75 (−1.29–2.79)	0.44
Growth rate	0.53 (−2.16–3.23)	0.67	0.79 (−2.43–4.00)	0.60	−0.004 (−0.76–0.75)	0.99
Rearing environment (NR)	−3.15 (−13.89–7.58)	0.54	−2.24 (−21.32–16.82)	0.80	0.04 (−2.73–2.81)	0.98

[1]Reward Association: MPR: $n = 6$; NR: $n = 12$; total: $n = 18$.
[2]Cognitive flexibility: MPR: $n = 3$; NR: $n = 12$; total: $n = 15$.
[3]Impulsivity: MPR: $n = 8$; NR: $n = 12$; total: $n = 20$.

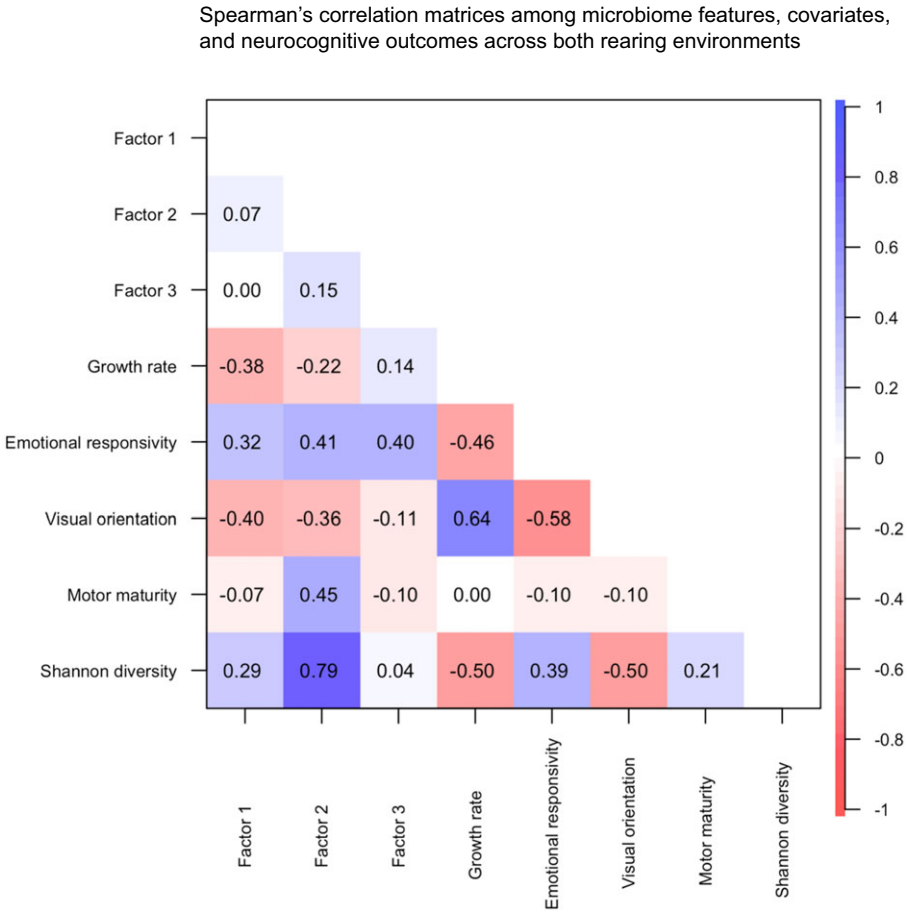

Figure 7. Day 14 (MPR: $n = 7$; NR: $n = 11$; total: $n = 18$).

reward association, cognitive flexibility, and impulsivity; rearing environment was also not associated with these variables (Tables 3,5). Being NR was significantly associated with higher average % correct responses on the Object Detour Reach task (Supplementary Table S4; $\beta = 5.97$, $p = 0.02$; $\beta = 5.02$, $p = 0.05$). However, this "average % correct" measure simply represents the number of correct trials in a 60 s interval and is not an accurate reflection of impulsivity like the composite impulsivity measure (which incorporates the total number of all possible impulsive responses *before* getting the trial correct). Therefore, we conclude that while NR infants persisted more to get the Object Detour Reach trial correct, rearing environment was not necessarily associated with infant impulsivity in this group of subjects.

Differential abundance analysis via MaAsLin2: associations between the abundance of microbial taxa and neurodevelopmental/cognitive outcomes

Emotional responsivity and cognitive flexibility were associated with several specific microbial genera. For Experiment 1, infants with greater abundance of fecal microbial taxa such as *Enterococcus* (coefficient = -0.0012, $q = 0.144$; significance at $q < 0.25$) and *Campylobacter* (coefficient = −0.0004, $q = 0.156$) had lower scores

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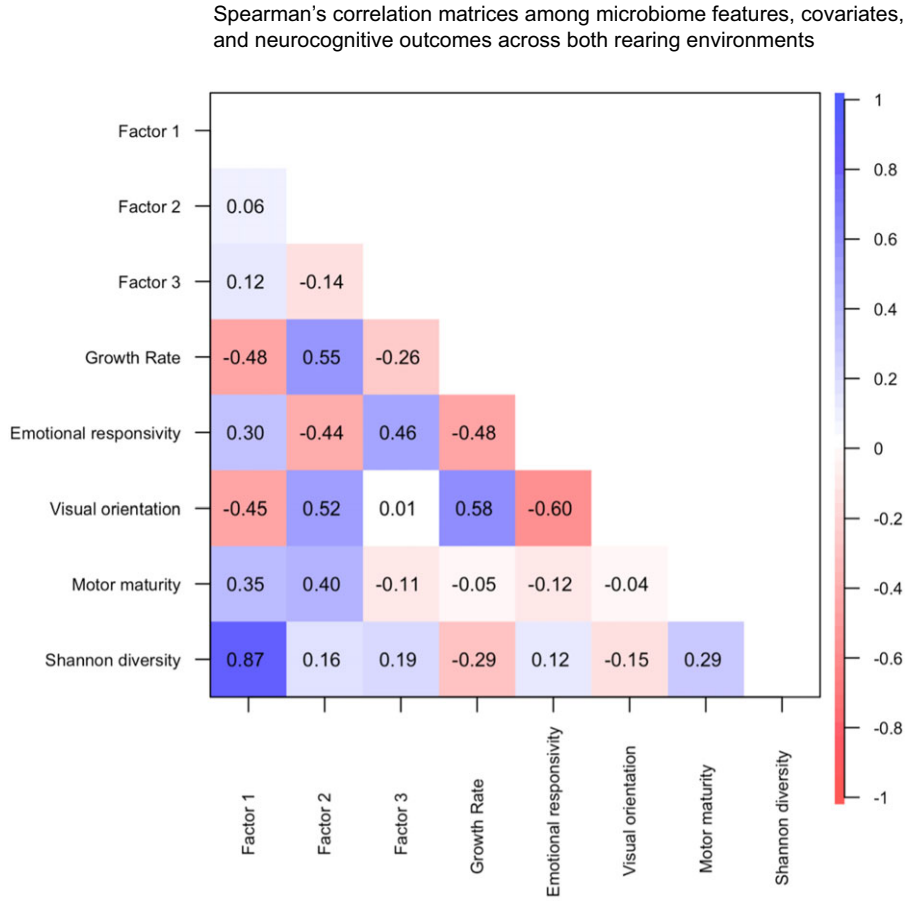

Figure 8. Day 30 (MPR: $n = 9$; NR: $n = 10$; total: n=19).

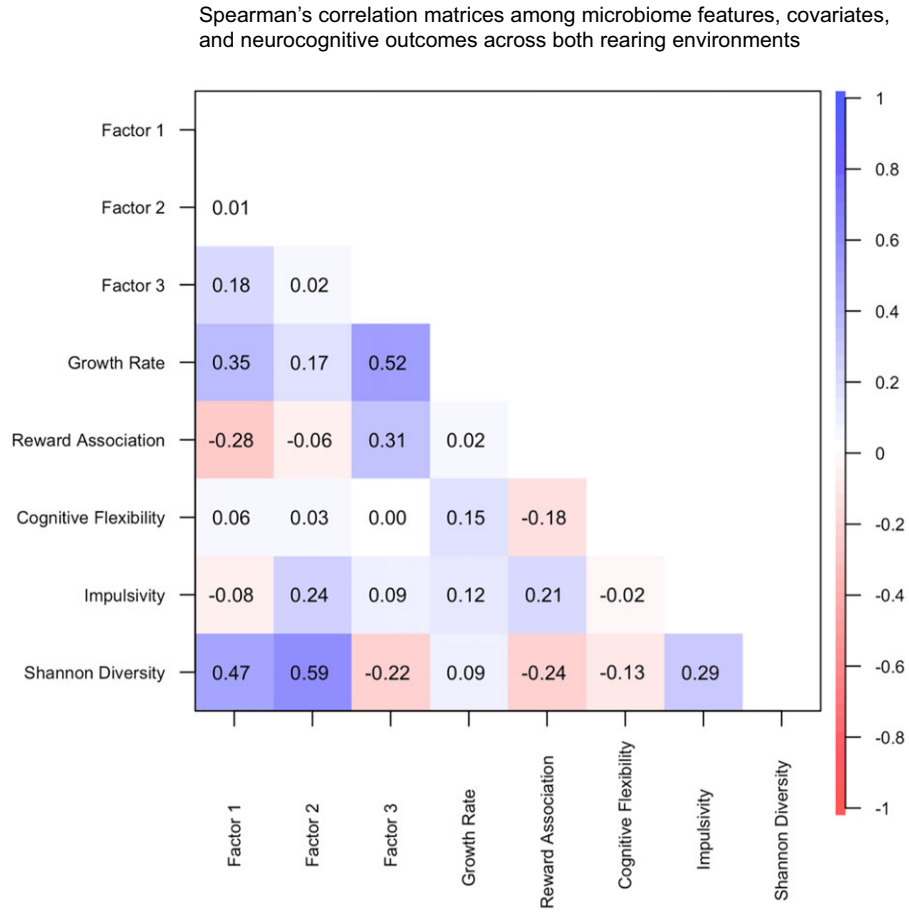

Figure 9. Day 180 (MPR: $n = 8$; NR: $n = 12$; total: $n = 20$).

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Table 6. *MaAsLin2* analysis results: associations between gut microbiome taxa at different ages postpartum and cognitive and neurodevelopmental measures across both rearing environments[1] (Total: $n = 33$)

Feature		Age group	Fixed effect	Coefficient	Standard error	N	*N* not 02	p^3	q[4]
Family	Genus								
Enterococcaceae	Enterococcus	Day 30	Emotional responsivity	−0.00124	0.00036	19	4	0.00426	0.14435
Tissierellaceae	Anaerococcus	Day 30	Emotional responsivity	−0.02490	0.00745	19	7	0.00485	0.14435
Tissierellaceae	Finegoldia	Day 30	Emotional responsivity	−0.00300	0.00083	19	4	0.00277	0.14435
Tissierellaceae	Peptoniphilus	Day 30	Emotional responsivity	−0.00915	0.00266	19	3	0.00402	0.14435
Alcaligenaceae	Sutterella	Day 30	Emotional responsivity	−0.00097	0.00030	19	5	0.00575	0.14435
Pasteurellaceae	Actinobacillus	Day 30	Emotional responsivity	−0.00002	0.00001	19	2	0.00388	0.14435
Pasteurellaceae	Aggregatibacter	Day 30	Emotional responsivity	−0.00105	0.00031	19	4	0.00479	0.14435
Ruminococcaceae	unclassified	Day 30	Emotional responsivity	−0.00015	0.00005	19	2	0.00670	0.14834
Bifidobacteriaceae	Alloscardovia	Day 30	Emotional responsivity	−0.00030	0.00010	19	2	0.00723	0.14986
Lachnospiraceae	Shuttleworthia	Day 30	Emotional responsivity	−0.00012	0.00004	19	3	0.00788	0.14986
Ruminococcaceae	Cellulosibacter	Day 30	Emotional responsivity	−0.00112	0.00039	19	3	0.01225	0.15641
Mogibacteriaceae	Mogibacterium	Day 30	Emotional responsivity	−0.00109	0.00037	19	4	0.01055	0.15641
Campylobacteraceae	Campylobacter	Day 30	Emotional responsivity	−0.00041	0.00014	19	3	0.01217	0.15641
Lachnospiraceae	Lactonifactor	Day 30	Emotional responsivity	−0.00021	0.00008	19	3	0.02105	0.20651
Lachnospiraceae	Pseudobutyrivibrio	Day 30	Emotional responsivity	−0.00019	0.00007	19	3	0.02330	0.21251
Streptococcaceae	Streptococcus	Day 180	Cognitive flexibility	−0.00084	0.00026	20	16	0.00872	0.11553
Veillonellaceae	Dialister	Day 180	Cognitive flexibility	−0.00131	0.00041	20	18	0.01004	0.12788
Lactobacillaceae	Lactobacillus	Day 180	Cognitive flexibility	−0.00566	0.00190	20	20	0.01393	0.16449
Ruminococcaceae	Butyrococcus	Day 180	Cognitive flexibility	−0.00025	0.00009	20	19	0.01851	0.20357

[1]All models adjusted for growth rate (g/Day), infant sex (Male, Female), and rearing environment (MPR, NR).
[2]Number of subjects with detectable feature; for brevity, we only show results for taxa present in greater than 10 subjects.
[3]significance at <0.05.
[4]FDR Adjusted *P*-Value; significance at $q < 0.25$.

of emotional responsivity regardless of rearing condition at Day 30 (Table 6). For Experiment 2, higher abundance of *Butyrococcus* (coefficient = −0.00025, $q = 0.204$), *Streptococcus* (coefficient = −0.00084, $q = 0.115$), and *Lactobacillus* (coefficient = −0.0057, $q = 0.164$) at Day 180 were negatively associated with cognitive flexibility. We did not observe any statistically significant associations between specific taxa at Day 14, nor with any other neurodevelopmental or cognitive measure besides emotional responsivity and cognitive flexibility.

Discussion

In this study, we investigated how patterns of gut microbiome composition and diversity predicted infant neurodevelopment in early infancy (Experiment 1) and cognitive development in mid to late infancy (Experiment 2) following controlled exposure to differing early social environments. Overall, we found that rearing environment was more often significantly associated with most of the repertoire of neurodevelopmental and cognitive outcomes in the multiple regression models; through an information theory approach, we also demonstrate that rearing-environment-driven models were most often the best-fit models. However, a gut microbiome pattern high in *Prevotella* and *Lactobacillus* and alpha diversity at 30 days of age was linked to emotional responsivity in early infancy; additionally, several models including both rearing environment and gut microbiome features (co-abundance factors, alpha diversity) were "best-fit" to the data. In all, this study is, to the best of our knowledge, the first study to investigate rearing environment, the infant gut microbiome, and neurodevelopment and cognition in infant rhesus macaques. This study provides novel findings showing that the gut microbiome's composition and diversity may partially explain infant emotional responsivity in addition to rearing condition; these results may have implications for the development of psychobiotic interventions. Because this study is not designed to test causality, we suggest that future research with larger sample sizes could include formal mediation analyses with interaction terms to determine whether the gut microbiome acts as a physiological link between early-rearing environments and neurodevelopmental and cognitive outcomes.

Through an exploration of taxonomic composition (Figs 1–3) by age and rearing environment, we found several age- and rearing-specific taxonomic patterns that were similar to previous work. The family Bifidobacteriaceae and the genus *Bifidobacterium* were highly abundant in infants who were NR and at 14 and 30 days of age (Figs 1–3). This was noted in previous research with this cohort, which found that NR infants at 14 and 30 days had greater *Bifidobacterium* abundance than MPR infants.[5] These

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differences may be attributed to the fact that the commercial formula given to the NR infants has plant-derived galacto-oligosaccharides that favor the growth of bifidobacteria,[34–36] a feature that may have sustained a relatively high abundance of *Bifidobacterium* in NR infants at both 14 and 30 days. *Prevotella* was the most abundant genus in MPR infants in all age groups (Figs 1–3). *Prevotella* is thought to play roles in fiber digestion in humans[37] and rhesus macaques alike.[38] MPR infants in this study had continuous access to nuts, seeds, and commercial monkey chow that their mothers ate; this nutritional environment may contribute to the relatively high *Prevotella* exhibited by MPR infants across age groups.

At 30 days of age (roughly equivalent to 4 months in humans), being NR and harboring a gut microbiome pattern with high *Prevotella* and *Lactobacillus* and low *Faecalibacterium* was associated with lower emotional responsivity. In addition, when we examined taxa-specific associations, several bacterial taxa (e.g., *Campylobacter*, *Enterococcus,* and genera from *Lacnospiraceae* and *Bifidobacteriaceae)* in the infant gut at 30 days of age were associated with emotional responsivity, but were not significantly associated other neurodevelopmental measures (Table 6). Our finding that features of the infant primate gut microbiome predict infant emotional responsivity, specifically, is in line with the literature and may reflect a relationship between the early infant microbiome and infant temperament. In humans, infant temperament forms the basis of social and emotional health. Temperament in infancy is associated with emotional and behavioral characteristics in childhood, including hyperactivity/inattention scores and emotional difficulties.[39] The PNNA was developed as a laboratory assessment of newborn macaque temperament, and its structure mirrors that of the Neonatal Behavioral Assessment Scale.[22] Emotional responsivity is a composite of irritability, consolability, struggle during the test, and predominant state. Emotional responsivity reflects an individual's ability to regulate arousal in response to external stimuli and to move from high arousal to lower arousal state[40]; emotional responsivity may reflect characteristics of individual temperament.[41]

The specific pattern of high *Prevotella* and *Lactobacillus* and high alpha diversity being linked to emotional responsivity warrants explanation, yet current literature shows that these relationships are still being uncovered. For instance, *Prevotella* and *Lactobacillus* species are reported to be a common dominant feature of rhesus macaque and other nonhuman primate microbiomes,[42,43] but also vary substantially between individuals.[44] *Prevotella* appears to play an important role in maintaining a healthy overall structure of the gut microbiome, at least in human populations.[45,46] *Lactobacillus* species, on the other hand, have probiotic properties and are common to macaque and human milk microbiomes alike,[42–44] possibly playing an integral role in the microbial exchange network in breastfeeding mother-infant dyads.[50] In this sample and in previous research with this cohort,[5] *Lactobacillus* and *Prevotella* are higher in the gut microbiome of MPR infants than in NR infants (Supplemental Figure S2d), a finding that may be related to aggregate differences in nutritional and social environments across the experimental rearing conditions. Sociability is also positively associated with the relative abundance of *Prevotella* among adult macaques, [51] suggesting that *Prevotella* species may be transmitted through early-life environmental factors, such as feeding mode and social contact. High *Prevotella* and *Lactobacillus* abundances seem to be linked to reduced emotional problems in early human development. For instance, human toddlers with a low abundance of *Prevotella* show higher levels of sadness, a temperamental trait that contributes to the broader domain of negative affectivity.[43] Human infants with a low abundance of *Lactobacillus* have greater negative affectivity [52]; supplemented as a probiotic, *Lactobacillus* reduced symptoms of anxiety and depression in human adults.[53] Reduced abundances of *Prevotella* have also been associated with other emotional problems in humans, including increased odds of internalizing disorder symptoms and autism in childhood.[54,55] The mechanisms by which taxa act remain unclear, but researchers have speculated that they may include interaction with host immune systems, production of short-chain fatty acids, and regulation of host metabolism.[56,57]

Greater taxonomic diversity of the infant gut microbiome is associated with lower emotional responsivity in this sample. Alpha diversity tends to increase with age in infant macaques and humans alike and is thought to reflect a state of gut microbial maturation.[58] Similar to our findings, Aatsinki and colleagues[59] found that alpha diversity measured at two and half months was inversely associated with negative emotionality and fear reactivity in six-month-old infants. Gut microbiome alpha diversity has also been found to be positively associated with concurrent fear behavior in twelve-month-old human infants.[60] Gut microbial diversity may influence temperament indirectly, as several studies have shown that it may predict aspects of brain structure and functional connectivity in infancy.[13,42,61] Caution is warranted, as is further research, because other studies have reported no associations between alpha diversity and temperament across infancy.[52,54,61]

That both the microbiome and rearing environment are together associated with emotional responsivity suggests that even when accounting for the complexities of nutritional, social, immunological, and environmental factors, the infant gut microbiome may still be partially associated with variation in infant temperament. Differences between these rearing conditions are likely to contribute to variation in the development of the gut microbiome, which is supported by our results here and in previous work.[5] There are several potential avenues by which early-life environments may contribute to variation in the gut microbiome that are pertinent to our study. For both humans and macaques, breastmilk and formula feeding have been shown to have distinct influences on the development of the gut microbiome.[62,63] Furthermore, bottle feeding of pumped breastmilk may lead to reduced co-occurrence of microbiota between breastmilk and infant stool as pumping can affect the composition of the breastmilk microbiome and potentially prevent the transfer of maternal (breast) skin microbes to the infant gut. Research in both humans and primates also points to the influence of the social environment, non-maternal allocaregivers, and social partners on the developing gut microbiome via vertical and horizontal transmission pathways,[64–69] which may potentially explain some of the microbial differences we observed between rearing conditions and the finding that both gut microbiome composition and rearing environment were significantly associated with one aspect of neurodevelopment (i.e., emotional responsivity).

Together, these results point to a model where early-life microbiomes converge with early social environments to influence infant neurodevelopment and cognition. This study contributes to the growing translational science literature on the link between the gut microbiome and neuro-/cognitive development in early life by presenting these results in a tractable nonhuman primate model. We note, however, that the specific physiological mechanisms underlying our reported associations are unclear and require further investigation. The gut microbiome is connected to the brain through immune, endocrine (e.g., glucocorticoid hormones,

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such as cortisol), and neural (e.g., the vagus nerve) pathways[56,70,71] Therefore, future research in nonhuman primate models and humans alike could additionally assess hormonal and immune biomarkers in addition to gut microbiome and cognitive data to further elucidate these physiological mechanisms and probable causal pathways.

Our study also has implications for translational science that aims to identify interventions for improving outcomes after early-life adversity. Our results suggest that the composition of the gut microbiome may impact infant outcomes jointly and independently from the contribution of early-life environments (indicated by rearing condition) on infant temperament and cognitive development. Work from experimental manipulation of rearing environment in other animal species points to potential mechanisms and opportunities for interventions. Several studies of piglets support the hypothesis that psychobiotic interventions that supplement the diet with fiber and pre/probiotics may accelerate the maturation of the gut microbiome in ways that support infant growth, increased expression of intestinal neurotransmitters, and reduced incidence of post-weaning problems, such as diarrhea.[72,73] Such findings provide support for additional research aimed at evaluating the use of psychobiotic interventions to support the development of the gut microbiome of infants affected by early-life adversity. This body of work also supports the development of new milk substitutes that may more closely mirror the components of milk that contribute to the normative development of the gut microbiome.

This study has several limitations that warrant discussion. First, the sample sizes for our analysis were relatively small (< 50 individuals); not all infants could be given cognitive assessments, so many infants with microbiome samples did not yield cognitive data. The small sample sizes in our analysis likely reduced statistical power and thus may limit the generalizability of our results. Additionally, while the experimental rearing environments in our study can be considered a proxy for infant diet, we do not have available data on specific dietary intakes of individual infants and thus cannot control for dietary variation that may have influenced the development of the gut microbiome. To address this limitation, future research could include detailed measures of infant nutrient intake in addition to neurodevelopmental/cognitive measures and fecal samples or a formula-supplemented group of MPR infants to control for dietary confounders.

Conclusion

Through a multi-faceted methodological approach, we examined the relationship between the infant gut microbiome and neurodevelopment and cognitive development in captive rhesus macaques subject to two differing early social/rearing environments. We found that along with being exposed to a nursery-rearing environment as opposed to mother-peer rearing, an infant gut microbiome pattern with a high abundance of *Prevotella* and *Lactobacillus*, as well as higher alpha diversity, are both associated with lower emotional responsivity in infant macaques. Our results suggest that infant gut microbiome composition and diversity uniquely explain a part of infant neurodevelopmental outcomes, even when accounting for the aggregate differences in diet, environment, and social contact across experimental rearing conditions. This further points to a potential role of the microbiome in shaping infant temperament in conjunction with early-rearing environment that could be teased apart in future research designed to test potentially mediatory and causal relationships.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S2040174424000400.

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Competing interests. The authors report no conflicts of interest.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals (American Society of Primatologists Principles for the Ethical Treatment of Nonhuman Primates) and have been approved by the institutional committee (NICHD Animal Care and Use Committee).

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