

## RESEARCH ARTICLE

# Can gut microbiota throughout the first 10 years of life predict executive functioning in childhood?

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## Funding information

Academy of Finland, Grant/Award Number: Decision 295741; Nederlandse Organisatie voor Wetenschappelijk Onderzoek, Grant/Award Numbers: 575-25-009, 016.Vici.185.038; Jacobs Foundation Advanced Research Fellowship

## Abstract

Animal models suggest that the gut microbiota can influence cognitive development and functioning via various pathways. In line with that, a first human study found associations between infant fecal microbiota composition and cognition at 2 years of age. This longitudinal study investigated whether fecal microbiota composition in infancy and childhood is associated with executive functioning in childhood. We followed healthy individuals from birth to their 10th year of life. Executive functioning was assessed using the Digit Span working memory test at 10 years of age and the ecologically valid Behavior Rating Inventory for executive functioning at 8 and 10 years. Stool samples were collected at month 1, 3 and 4 as well as at 6 and 10 years. The V4 region of the 16S ribosomal RNA was analyzed to determine microbial composition at the genus level. Using established statistical techniques for microbiota analysis, we did not find associations between fecal microbiota composition and executive functioning after accounting for breastfeeding, maternal education, child sex and age. Our study results are most compatible with the absence or only a weak relationship between infant and childhood fecal microbiota composition and executive functioning in childhood in healthy community children.

## KEYWORDS

children's mental health, cognitive development, longitudinal study, microbiome

## 1 | INTRODUCTION

The gut-brain axis is a complex bidirectional network where the gut and the brain are connected via various pathways (Mayer et al., 2014). The gut microbiota, the ecosystem of microorganisms in the intestinal lumen, is a critical part of this network. It can generate endocrine-, neurocrine- and immune-related signals that can shape the development and functioning of the central nervous system (de Weerth, 2017; Mayer, 2011). In the present study, we investigated whether infant and childhood fecal microbiota (FM) composition as a measure of the distal gut microbiota can predict individual variation in executive functioning (EF) in childhood. Identifying and describing possible relationships between the (early) FM and EF is a necessary first step toward devel-

oping prevention, diagnostic and intervention strategies targeting the microbial ecosystem in the gut. The following paragraphs will elucidate the importance of EF and why the FM is a relevant study variable for psychologists researching child cognitive development (Sarkar et al., 2018).

EF comprises cognitive functions central to goal-directed, efficient and adaptive behavior (Huizinga & Smidts, 2010). These include inhibition, shifting, self-monitoring, planning, attention and working memory. Proper EF is crucial for every-day-life and academic achievement (Huizinga & Smidts, 2010; Huizinga et al., 2018). Disruption of EF has negative effects on health outcomes and is itself part of several psychiatric disorders, such as attention deficit hyperactivity disorder, bipolar disorder and schizophrenia (Testa & Pantelis, 2009). Thus, proper

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development of EF is crucial for the quality of life of the individual. EF emerges as the output of various neural networks during the first years of life (Goldstein & Naglieri, 2014) and continues to develop during childhood, adolescence and even adulthood (Best & Miller, 2010). These neural networks rely on the development of frontal and posterior cerebral cortex and subcortical regions (Goldstein & Naglieri, 2014). The plasticity of these networks is maximal early in life (Diamond, 2013). As extensively reviewed (Borre et al., 2014; Varier et al., 2020), the colonization of the intestines by microbes occurs simultaneously with and influences this pivotal period of brain development. For example, experimental rodent studies revealed that the gut microbiota affects social behavior, cognitive performance and neurobiology in brain regions related to learning and memory (Ohland et al., 2013; Savignac et al., 2015; Vázquez et al., 2015; Wang et al., 2015) and that there exists a time window in early life for such effects to take place (Buffington et al., 2016; Sudo et al., 2004). Therefore, it is necessary to study the gut microbiota in infancy in relation to EF later in life to disentangle such early programming effects (Borre et al., 2014). In sum, EF is important for the quality of life of the individual. It develops early in life and continues to develop throughout adulthood. The gut microbiota may influence both the early development and current functioning of the brain.

At present, there is a lack of human developmental studies that focus on the relationship between the FM and cognitive functioning. Studies that found associations focused on temperament (Aatsinki et al., 2019), attention to emotional faces (Aatsinki et al., 2020) or social behaviors related to autism (Laue et al., 2020). One study provided indirect support for a potential relationship between the early FM and later cognitive functioning as antibiotic treatment in the first 2 years of life was related to worse cognitive functioning at 11 years (Slykerman et al., 2019). Earlier, the first human developmental study (Carlson et al., 2018) examined whether FM around 1 year of age is related to cognitive functioning and global and regional brain volumes at 1 and 2 years of age in 89 typically developing infants. Cluster analysis of bacterial abundances identified three groups that significantly predicted cognition at 2 but not 1 year of age. Lower alpha diversity was related to higher cognitive functioning at 2 years of age. Furthermore, FM was weakly related to regional brain volumes at 1 ( $N = 46$ ) and 2 ( $N = 27$ ) years of age, as well as brain functional connectivity ( $N = 39$ ) (Carlson et al., 2018; Gao et al., 2019). A limitation of this study was that there was only one measurement of the FM, while infants show rapid shifts in microbial composition and diversity. Hence, multiple sample time points are necessary to distinguish temporary shifts in composition from more stable characteristics (Bäckhed et al., 2015; de Muinck & Trosvik, 2018; Meij et al., 2016). The finding that higher alpha diversity was associated with lower cognitive scores (Carlson et al., 2018) might indicate that low alpha diversity at around 1 year of age is beneficial for infant cognitive development or that there are underlying environmental factors that cause lower alpha diversity and have a positive influence on cognitive development. For instance, previous research found that breastfed infants have a more stable FM composition that is dominated by the genus *Bifidobacterium* and is therefore less diverse (Stewart et al., 2018). Breastfeeding also has been positively associated with cognitive functioning in previous studies (Kim & Choi, 2020).

Based on the outlined preclinical studies and the first human study (Carlson et al., 2018; Gao et al., 2019), we hypothesized (1) that FM composition in infancy and childhood is associated with childhood EF, (2) that there is a negative association between alpha diversity in infancy and childhood EF and (3) that (infant) FM samples can be grouped into clusters of community similarity that are differentially associated with EF. In the context of hypothesis 2, we also investigated whether alpha diversity in childhood was related to childhood EF. Finally, we explored whether microbiota compositional change over time (volatility) is associated with EF in childhood.

To investigate our hypotheses, we took the dynamic nature of the FM into account by analyzing stool samples obtained at 5 different time points, from here on referred to as T1–T5. Three stool samples per child were collected in infancy at months 1, 3 and 4 (T1–T3). Two more samples were collected at 6 and 10 years of age (T4–T5). We furthermore collected questionnaire and test measurements of EF at 8 and 10 years of age and tested our hypotheses using diverse complementary statistical and machine learning approaches as described in our preregistration (<https://aspredicted.org/uc98s.pdf>). This study represents a further important step in translating findings from animal studies into human research. It is the first study that examined the relationship between FM and EF beyond the age of toddlerhood and spanning a period of 10 years.

## 2 | MATERIALS AND METHODS

### 2.1 | Participants

Participants are children from the 193 healthy mother-infant dyads from the ongoing longitudinal BIBO study that started in the third trimester of pregnancy and is ongoing (Beijers et al., 2011). Mothers were recruited on a voluntary basis during late pregnancy as they responded to flyers that were spread among midwife practices in the cities of Nijmegen, Arnhem and surrounding areas. Inclusion criteria were an uncomplicated singleton pregnancy, no drug use and no current physical or mental health problems. Furthermore, all infants were healthy, born at full term ( $\geq 37$  weeks), and with a 5-min APGAR score  $\geq 7$ . Out of 220 women, eight were excluded due to medical reasons such as preterm birth. Another 19 women discontinued the study within the first 3 postpartum months due to personal circumstances. When the children were 10 years old, 177 mother-child dyads were still participating. Table 1 shows demographic variables for the 156 study participants that had complete data for this study. Table 2 shows participant numbers at the different time points. All mothers gave written informed consent, and the ethical Committee of the Faculty of Social Sciences, Radboud University Nijmegen approved the study (ECG/AvdK/07.563, ECG300107, ECG13012012, SW2017-1303-497, SW2017-1303-498).

### 2.2 | Procedure

Mothers completed demographic questionnaires in the third trimester of pregnancy ( $M = 37.4$ ,  $SD = 1.4$  weeks) as well as questionnaires

**TABLE 1** Demographic characteristics of subjects

Characteristic	N = 156 <sup>1</sup>
Maternal age	32.8 (30.3, 34.7)
Maternal ethnicity (Caucasian)	96.9%
Birthweight	3,618 (3,232, 3,931)
Smoking during pregnancy	2 (1.5%)
Alcohol during pregnancy	21 (15%)
Delivery mode	
Assisted vaginal	15 (9.9%)
Cesarean section	8 (5.3%)
Vaginal	128 (85%)
Gestational length (days)	282 (275, 287)
Firstborn	69 (44%)
Maternal education	
Secondary education	30 (19.2%)
College or university	126 (80.8%)
Child sex	
Female	71 (46%)
Male	85 (54%)
Child's age at FM collection*	
T1	28 (27, 28)
T2	82 (75, 89)
T3	112 (107, 119)
T4	6.06 (6.01, 6.15)
T5	10.11 (9.96, 10.21)
Antibiotics	
Birth-T1	1 (1%)
Birth-T2	2 (1.8%)
Birth-T3	2 (2.3%)
5–6 years	22 (17%)
9–10 years	6 (4.5%)
Child's age at EF collection	
8 years	8.04 (8.02, 8.10)
10 years	10.13 (10.00, 10.22)

Note: Missing values for any of the listed variables were omitted to calculate shown descriptive statistics.

Abbreviations: EF, executive functioning; FM, fecal microbiota.

<sup>1</sup>N (%); Median (IQR).

\*T1–T3, age in days; T4–T5, age in years.

**TABLE 2** Sample size per time point of stool sample collection and outcome variable

Time	DS F	DS BW	DS LNS	BRIEF
T1	135	132	131	144
T2	125	123	122	131
T3	123	121	120	129
T4	139	137	136	144
T5	146	146	145	146

Abbreviations: BW, backwards; BRIEF, behavior rating inventory of executive functioning; DS, Digit Span; F, forwards; LNS, letter-number sequencing.

about the delivery and the infant immediately after birth. Information about breastfeeding was obtained weekly through diaries (0–6 months) and through monthly health interviews (0–12 months). When the children were 8 and 10 years old, mothers filled in the behavior rating inventory of executive function (BRIEF) questionnaire. The Digit Span test scores were obtained during a home visit at 10 years.

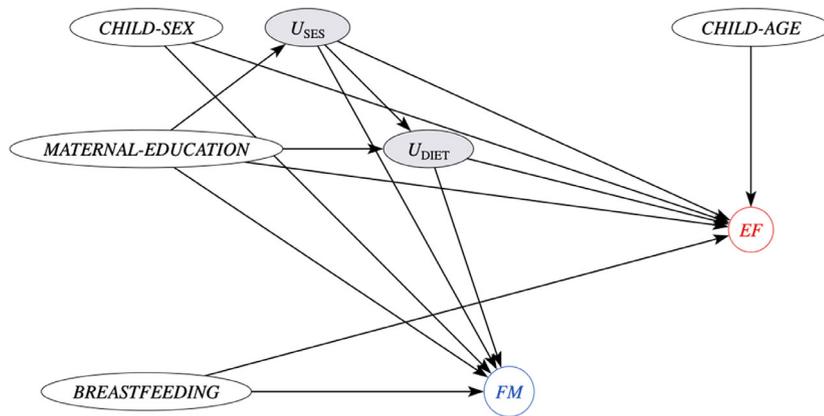
## 2.3 | Measures

### 2.3.1 | Fecal samples

We instructed parents to collect fecal samples at nine time points postpartum as well as one sample at 6 and 10 years of age. Due to financial constraints, we could use only three out of nine samples in infancy. After collection and temporary storage at  $-20^{\circ}\text{C}$  at home, samples were transported in coolers and later stored at  $-80^{\circ}\text{C}$ . Next, they were processed at the Microbiology Laboratory at Wageningen University as described in a previously published protocol (Gu et al., 2018; Ramiro-Garcia et al., 2018) (Supporting Information 1). Note that 16S rRNA sequencing was carried out on an Illumina HiSeq2000 sequencing platform at Eurofins Genomics, Germany. We utilized the NG-Tax 2.0 pipeline (Poncheewin et al., 2020) to process amplicon sequence variants. Only reads with matching barcodes were kept. Amplicon sequence variants were obtained by assigning reads to each sample based on distinguishable barcodes. The SILVA\_132\_SSU 16S rRNA gene reference database was used for taxonomic assignment (Quast et al., 2012).

### 2.3.2 | Digit Span (forwards, backwards and letter-number sequencing)

The Digit Span is part of the Wechsler Intelligence scale for children and measures working memory (Petrosko, 1975). For the Digit Span tests, a trained instructor reads out numbers and the child has to repeat these either in the given order (Digit Span forwards) or backwards (Digit Span backwards). The maximum score for each subtest is 14. For the Digit Span letter-number sequencing test, the instructor reads out a number of letters and numbers. The child has to memorize and give back the numbers in ascending order, and then the letters in alphabetical order. The maximum score for Digit Span letter-number sequencing is 30 points. For all Digit Span tests, each level of difficulty consists of two trials whereby each correct trial is worth 1 point. As soon as both trials are answered incorrectly, the test is finished. Previous research suggests that the different Digit Span tests measure different aspects of working memory functioning (Clair-Thompson & Allen, 2013; Gerton et al., 2004; Rosenthal et al., 2006). This was also reflected by low-moderate correlation strength among our Digit Span subscores ( $r < 0.41$ ). As norms were only available for the total score at the time of our preregistration, we used the raw subscores and corrected for age and gender within the analysis.



**FIGURE 1** Directed acyclic graph based on literature review. Grey variables with a leading U reflect unmeasured variables, for example: SES, unobserved socioeconomic status; FM, fecal microbiota; EF, executive functioning. The graph applies to each time point of FM measurement and each EF measurement separately

### 2.3.3 | Behavior rating inventory of executive function

Designed to achieve high ecological validity (Gioia & Isquith, 2004), the BRIEF was frequently utilized in clinical practice and research settings to measure daily life EF in children (Huizinga & Smidts, 2010). The BRIEF consists of 75 items that measure eight scales: Inhibition, Shift, Emotional Control, Initiate, Working Memory, Plan/Organize, Organization of Materials, and Monitor. Based on these scales, the age and gender normed T-scores for the Metacognition and Behavior Regulation Indices can be obtained. It is advised to use these subscales when the T-scores between them differ significantly (Huizinga & Smidts, 2010). That was not the case in our sample. Therefore, we calculated age and gender normed T-scores for the total score of the BRIEF as a general indicator of EF. A higher BRIEF score indicates lower EF. Note that we used the mean of the 8- and 10-year scores for the random forest models. In the linear models that relate alpha diversity or volatility to EF, we applied a multilevel structure for the repeated measurement.

### 2.3.4 | Confounding variables

Figure 1 shows a directed acyclic graph (Williams et al., 2018) based on our literature review. Directed acyclic graphs graphically depict potentially confounding variables of the association between an exposure (here FM) and an outcome (EF). They furthermore provide a set of rules to identify variables that reduce or induce bias when being adjusted for in a statistical model (Cinelli et al., 2020). The rationale behind our assumed graph (Figure 1) is as follows: socioeconomic status, age and sex can influence EF measurements (Cuevas et al., 2014; Grissom & Reyes, 2019) and FM (Bolnick et al., 2014; Bowyer et al., 2019; de Muinck & Trosvik, 2018). As we have no direct measurement of socioeconomic status, maternal education serves as a proxy in our study and might itself also influence both FM (e.g., via diet) and EF of the child. Age of the child during the EF task was included as it is expected to increase the precision of the effect of interest (Cinelli et al., 2020). Age and child sex were left out for the models that included the BRIEF scores as these were normed by age and sex. Breastfeeding is a strong driver of the gut microbiota in infancy, while there is contradictory research about

a relationship with later EF (Belfort et al., 2016; Rochat et al., 2016). For the analyses that include the childhood FM, diet is a confounding factor that we cannot adjust for. We might partially mitigate a potential bias under the assumption that maternal education has an effect on the child's diet (Cinelli et al., 2020). However, confounding effects of diet in childhood cannot be entirely ruled out given our data. In infancy, variation in diet is reflected in the amount of received breastfeeding. We considered other variables not shown in the graph: Gestational age and birthweight are not expected to influence EF unless the infant was born preterm or has low birthweight (<2500 g) (Houdt et al., 2019). To infer the total effect of the FM under the assumed directed acyclic graph model, all variables shown in the graph have to be included. However, several covariate structures were explored to also give room to other candidate directed acyclic graphs (e.g., a graph where breastfeeding is not related to EF). These did not lead to different conclusions.

## 2.4 | Statistical analysis

We performed our analyses in R (Team, 2020) version 4.0.2 and Stan (Carpenter et al., 2017) version 2.21.0. We used the packages microbiome (Shetty & Lahti, 2019) and phyloseq (McMurdie & Holmes, 2013) to process microbiome data in R. Per time point and outcome variable, data from all mother-infant dyads that provided fecal samples and the outcome variable were used. Among those subjects, there was very little missingness in the covariates (education: 0.7%–1.7%, age: 0.8%–2.4%). We performed complete case analyses in these cases.

### 2.4.1 | Code availability

The code corresponding to all statistical analyses is publicly available (<https://doi.org/10.5281/zenodo.5026029>).

### 2.4.2 | Random forest regression

The Random Forest algorithm (Breiman, 2001) is invariant to scaling of inputs, computationally efficient, appropriate for high dimensional data, able to predict nonlinear relationships and thus well suited to

analyze microbiome data (Belk et al., 2018; Loupe, 2014; Namkung, 2020). For each outcome and time point of microbiota determination, we fitted a random forest model using the ranger package (Wright & Ziegler, 2017) with relative abundances. First, we tuned the hyperparameters `mtry` and `sample.fraction` using the package `tuneRanger`, which uses the mean squared error as out of bag error. Next, 10× fourfold cross-validation was performed to estimate Pearson correlations of predicted values and leave-out values. To obtain a distribution of Pearson correlations under the null hypothesis, we performed the same procedure (incl. hyperparameter tuning) after permutation of the outcome variable (1000 permutations). We used the median Pearson correlation of the cross-validation procedure to obtain the *p*-value. Since we tested 20 random forest models for significance, we accounted for multiple testing using the Benjamini–Hochberg procedure (Benjamini & Hochberg, 1995). We also explored the Random Forest algorithm for feature selection as described in Bommert et al. (2020). Briefly, random forest accuracy was evaluated by the set of top scoring taxa that were identified in a fourfold cross-validation, and then used to train a new model on the whole data set. This was performed for each outcome and time point separately.

#### 2.4.3 | Bayesian linear models

We fitted Bayesian robust linear models to regress EF on Shannon diversity and volatility, respectively. Shannon diversity is a commonly used measure of alpha diversity. Experiments with alternative alpha diversity indices (observed richness, Chao1, inverse Simpson) yielded similar results. For volatility, we calculated intrasubject Aitchison distance sequentially resulting in four volatility scores for each individual: T1–T2, T2–T3, T3–T4 and T4–T5. This allowed us to determine whether volatility in infancy between infancy and childhood and in childhood is associated with EF. Covariates were included for both Shannon and volatility models as described. Maternal education was modeled using an ordinal regression approach as described by McElreath (2020) to respect the ordinal nature of this variable (Liddell & Kruschke, 2018). We used `cmdstanr` (Gabry & Cesnovar, 2020) to fit the models. The `cmdstanr` package utilizes the probabilistic programming language Stan (Carpenter et al., 2017). Stan estimates parameters using the Hamiltonian Monte Carlo (HMC) method. All continuous predictors were standardized to ease interpretability and setting prior distributions for the parameters. Priors were set based on prior predictive simulations such that the parameter space was only mildly restricted and the same priors could be used across all models. A Gaussian prior with a mean of 0 and a standard deviation of 0.5 was used for all  $\beta$  coefficients across all models. Assigning a prior probability to the effect centered at 0 and constraining the model to not consider highly unrealistic slope sizes results in a more conservative model compared to the classical approach (Gelman & Tuerlinckx, 2000; Gelman et al., 2012). Note that changing prior distributions to more or less constrictive priors did not influence the results. Posterior predictive checks and residual plots were used to evaluate appropriateness of the model. Finally, we evaluated correct functioning of the HMC method by screening

chain plots and diagnostic parameters such as divergent transitions and `rhat4` values (Gabry et al., 2019).

#### 2.4.4 | Partitioning around Medoids

We applied the Partitioning around Medoids clustering algorithm to the centered-log-ratio transformed genus level abundances (Aitchison distance) (Gloor et al., 2017) using the R package `cluster`. For each time point, we determined whether clustering is present based on prediction strength (Tibshirani & Walther, 2005) and the Silhouette Index utilizing functions from the packages `cluster` and `fpc`. Both measures are absolute measures of cluster strength that indicate support for clustering when they fall above a predefined threshold. The Calinski–Harabasz index is a relative measure of cluster strength that reflects which number of clusters is most likely (assuming that clusters are present). We considered clusters to be valid if either Prediction Strength is  $\geq 0.9$  or Silhouette Index is  $\geq 0.5$  as recommended by Koren et al. (2013).

### 3 | RESULTS

Figure 2 shows the distributions for all outcome variables including their means, ranges, medians and interquartile ranges. In the following sections, we describe the results per analysis method.

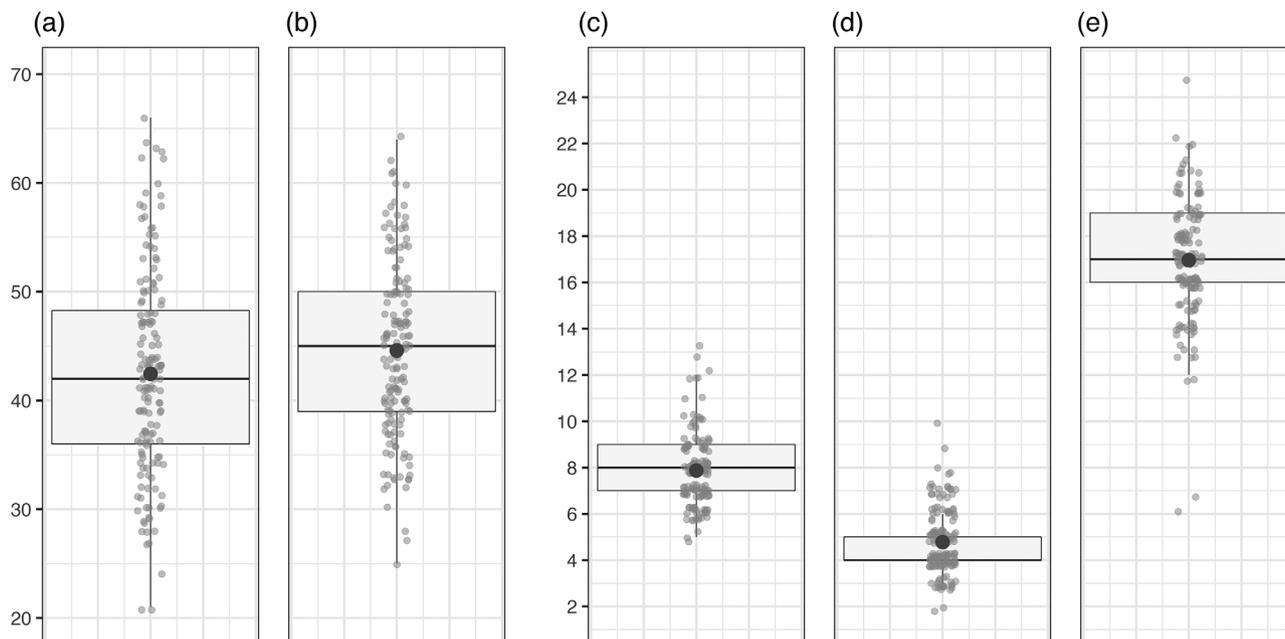
#### 3.1 | Predicting EF from genus level abundances: Random forest regression

Table 3 shows the median correlation between the random forest predictions and the leave-out values with the corresponding *p*-values and *q*-values for each model. If relative abundances at genus level are associated with EF, we would expect a significant correlation between the random forest predictions and the known outcome. Only two models yielded significant results: Predicting the combined BRIEF scores and Digit Span backwards from the samples obtained at T1 and T2, respectively. However, after applying the Benjamini–Hochberg procedure to correct for multiple testing, they no longer remain significant. In general, the correlation coefficients vary closely around zero, indicating that it was not possible to predict EF scores based on genus level relative abundances at any time point. In a separate exploratory analysis, we used feature selection prior to fitting the final models to avoid potential overfitting to uninformative features. This approach did not change results meaningfully (Table S1).

#### 3.2 | Bayesian linear models

##### 3.2.1 | Associations between Shannon diversity and EF

Figures 3 and 4 summarize 20 linear models by showing the posterior distributions of the slope coefficients for alpha diversity and the

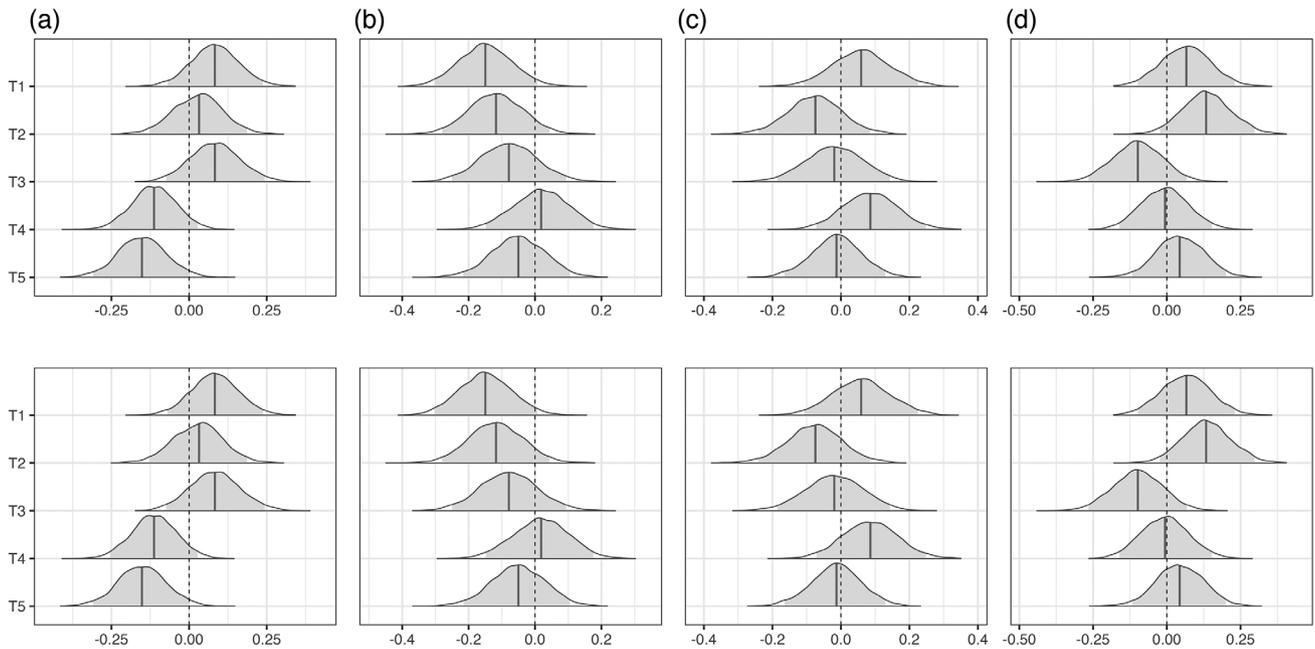


**FIGURE 2** For each outcome, a boxplot is shown on the first layer. On a second layer, the corresponding single data points are shown in light grey as well as a larger black point depicting the mean. Small random noise is added to the single data points to avoid overplotting. (a) BRIEF (8 years), (b) BRIEF (10 years), (c) Digit Span forwards, (d) Digit Span backwards and (e) Digit Span letter-number sequencing

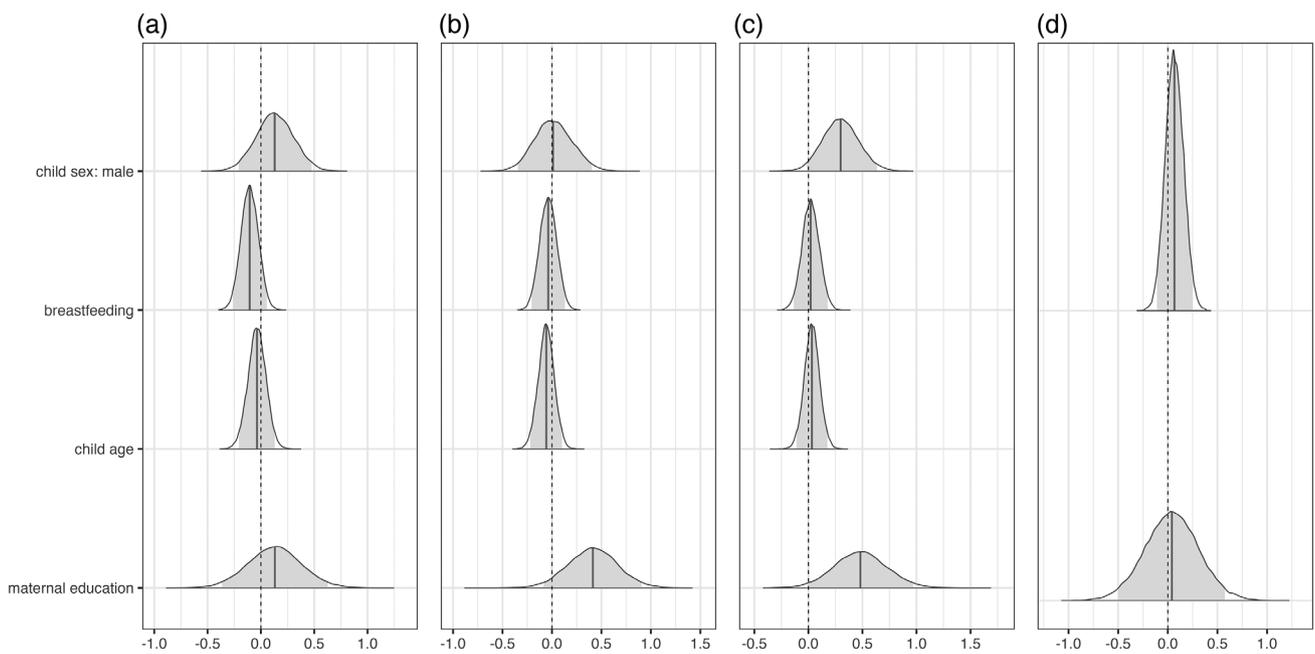
**TABLE 3** Correlation between random forest prediction and real data

Outcome	Time point	Median	Mean	SD	<i>p</i>	<i>q</i>
DS forwards	T1	-0.14	-0.14	0.14	.94	0.85
DS backwards	T1	-0.07	-0.09	0.13	.77	0.83
DS LNS	T1	0.17	0.14	0.17	.10	0.74
BRIEF	T1	0.20	0.18	0.11	.04	0.74
DS forwards	T2	0.03	0.00	0.15	.46	0.79
DS backwards	T2	0.25	0.24	0.15	.01	0.49
DS LNS	T2	0.02	0.00	0.15	.52	0.79
BRIEF	T2	-0.13	-0.11	0.13	.90	0.83
DS forwards	T3	-0.01	0.02	0.17	.60	0.83
DS backwards	T3	-0.12	-0.10	0.19	.87	0.83
DS LNS	T3	0.03	0.04	0.14	.48	0.79
BRIEF	T3	-0.06	-0.06	0.11	.74	0.83
DS forwards	T4	0.06	0.06	0.15	.38	0.79
DS backwards	T4	-0.13	-0.10	0.14	.88	0.83
DS LNS	T4	0.09	0.09	0.12	.25	0.75
BRIEF	T4	0.05	0.04	0.14	.44	0.79
DS forwards	T5	0.03	0.00	0.14	.46	0.79
DS backwards	T5	0.14	0.12	0.12	.13	0.74
DS LNS	T5	0.05	0.05	0.11	.39	0.79
BRIEF	T5	0.17	0.17	0.13	.08	0.74

Abbreviations: BRIEF, behavior rating inventory of executive functioning; DS, Digit Span; LNS, letter-number sequencing.



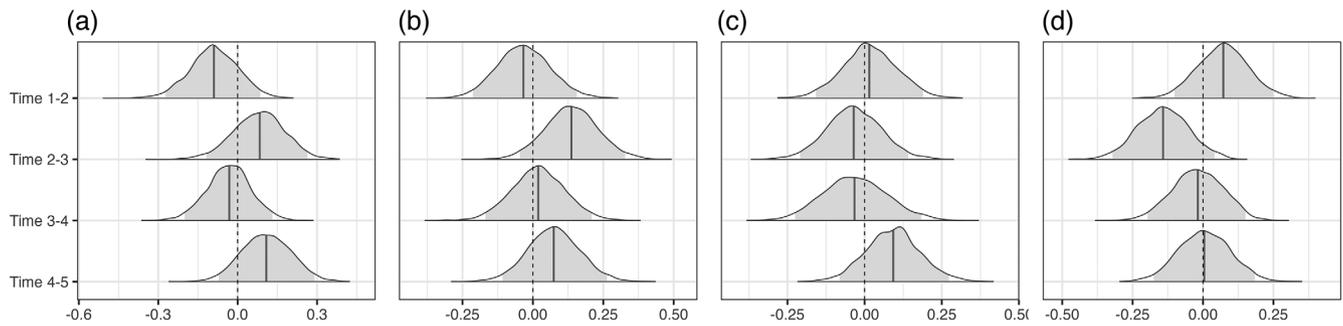
**FIGURE 3** Posterior distributions (x-axis) of the beta coefficients of alpha diversity (Shannon) for each outcome (a–d) and time point (y-axis) when stool samples were obtained. The grey areas reflect the 95% credible intervals of the estimates. (a) Digit Span forwards, (b) Digit Span backwards, (c) Digit Span letter-number sequencing and (d) BRIEF



**FIGURE 4** Posterior distributions of the beta coefficients of the covariates for each outcome averaged over all time points of microbiota sampling. The grey areas reflect the 95% credible intervals of the estimates. (a) Digit Span forwards, (b) Digit Span backwards, (c) Digit Span letter-number sequencing and (d) BRIEF

covariates, respectively. Figure 3 corresponds to our hypothesis that alpha diversity is associated with EF. For every slope, we evaluate the proportion of the distribution that lies above or below zero. The higher this proportion, the more confident we can be that the relationship is positive or negative, respectively. We conclude that the association is positive or negative with confidence, if the 95% credible interval (CI) (grey area) excludes zero. Despite two subfigures (Figure 3c T1 and

Figure 3b T5) indicating otherwise, this was not the case for any slope parameter (Tables S2–S21 for exact CIs). In addition to looking at the 95% CI, we can also evaluate consistency in the most likely direction of the association within infancy or childhood across our EF measures. If the most likely direction is consistent, this could be interpreted as evidence for an association between alpha diversity and EF. For example, the relationship between alpha diversity and Digit Span backwards



**FIGURE 5** Posterior distributions (x-axis) of the beta coefficients of volatility for each outcome (a–d) and time point pair (y-axis) when stool samples were obtained. Grey areas reflect the 95% credible intervals of the estimates. (a) BRIEF, (b) Digit Span forwards, (c) Digit Span backwards and (d) Digit Span letter-number sequencing

(B) is consistently estimated to be most likely negative in infancy. However, we can observe consistency in the opposite direction across the three infant time points for Digit Span forwards (C). Thus, the direction is not consistent across EF measures. In sum, we cannot observe evidence for an association between alpha diversity and EF using linear models. Among the covariates (Figure 4), the model is confident that maternal education is positively associated with Digit Span letter-number sequencing. Also, for Digit Span backwards this relationship is likely ( $P[\beta_{\text{edu}} > 0] = 0.95$ ). Sex and breastfeeding were not associated with EF scores, although there is a tendency ( $P[\beta_{\text{male}} > 0] = 0.97$ ) for boys to score higher on Digit Span letter-number sequencing. Note that these results do not differ depending on the alpha diversity index chosen (Figures S1–S4).

### 3.2.2 | Associations between volatility and EF

Figure 5 summarizes our exploratory analyses (not preregistered) that investigate a potential association between microbiota volatility (Figure S5) and EF in childhood (Tables S22–S37 for exact CIs). Each curve depicts a posterior distribution of a slope corresponding to the association between volatility at the given time point and EF in childhood. Applying the same criteria as outlined in the former section where we used Bayesian linear models, we do not observe a relationship between microbiota volatility and EF. For time pair T2–T3, the direction of the association is most likely positive for three out of four EF measures (note that BRIEF must be interpreted inversely). This might indicate a positive relationship between volatility in that specific infant time window and EF in childhood that could potentially be identified with higher samples sizes. Similarly, for T4–T5, we observe that the majority of the posterior distribution indicate a positive slope across the three-digit span measures.

### 3.3 | Identifying clusters of FM composition: Partitioning around Medoids

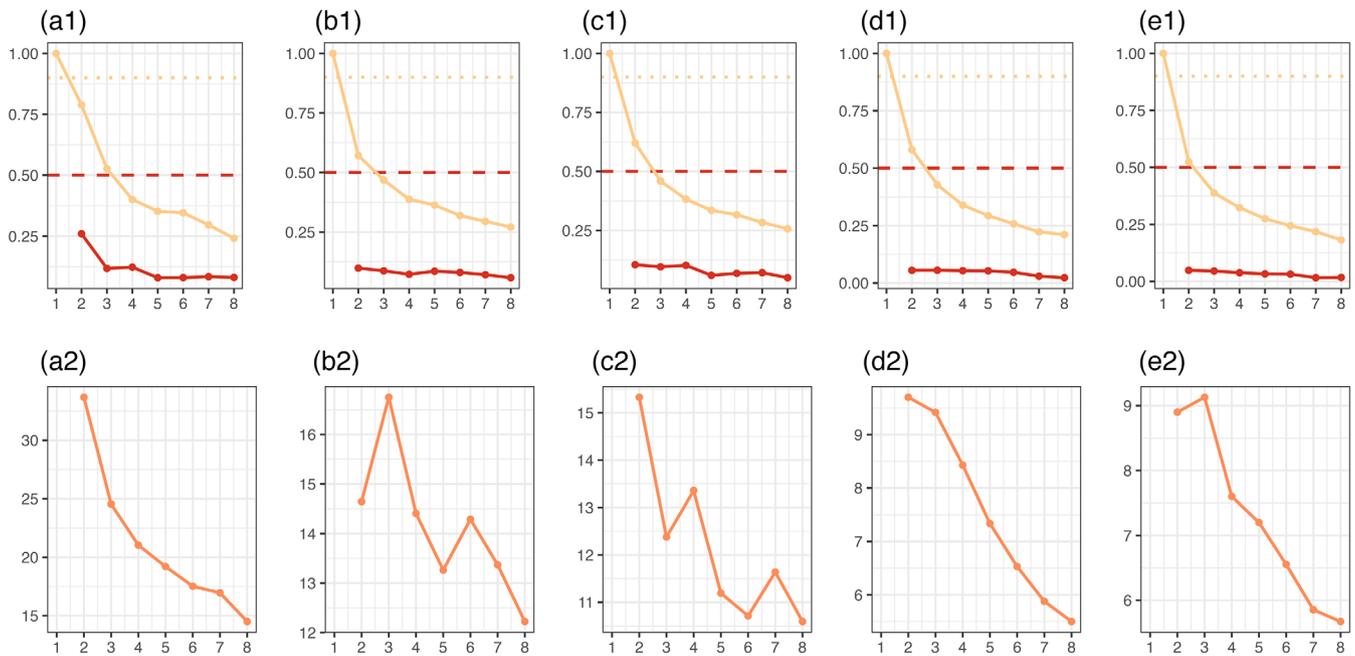
Neither Prediction Strength nor Silhouette Index indicated the presence of clustering at any of the five microbiota assessment moments

(Figure 6). This was also the case when we used the cluster algorithm on all infant or childhood samples at once. Therefore, no follow-up analyses comparing EF between clusters were warranted. Note that we preregistered the *k*-means algorithm rather than Partitioning around Medoids. The *k*-means algorithm led to similar results. We presented Partitioning Around Medoids here as this makes our analyses more comparable to previous research (Carlson et al., 2018).

## 4 | DISCUSSION

This study examined the relationship between infant and childhood FM composition and childhood EF. Our results did not reveal any consistent associations between FM composition and EF. The random forests algorithm, which is able to detect complex nonlinear relationships, was unable to predict EF from genus level relative abundances at any given time point. In line with that, Bayesian robust linear models found no association between alpha diversity or volatility and EF. Finally, we did not find that infant microbiota composition can be described by clusters based on the Partitioning Around Medoids method.

There are several potential explanations for the absence of statistical associations between FM composition and EF in our data. Considering that we explored the data using diverse complementary statistical approaches and also used five different time points of microbiota sampling that we could relate to our outcomes, the most likely explanation might be that there is no or only a weak relationship between the FM composition at the genus level and EF in childhood as assessed by our methods (parent questionnaires and Digit Span tests). That does not mean that gut microbes may not play a role in EF in humans in general. It is possible that the effects of the microbiota on EF become apparent when looking at high-risk populations as opposed to a low-risk healthy population as in our study. Our hypotheses were mainly based on animal models that have identified associations between the gut microbiota and cognitive functioning (Sarkar et al., 2018). In these models, higher risk is induced by an intervention (e.g., stressors or antibiotics). At the same time, animal models have far less variation in the gut microbiota (Lagkouvardos et al., 2016). Therefore, animal studies may more easily reveal effects that in the human population are obscured by the large variability in environments and the more complex



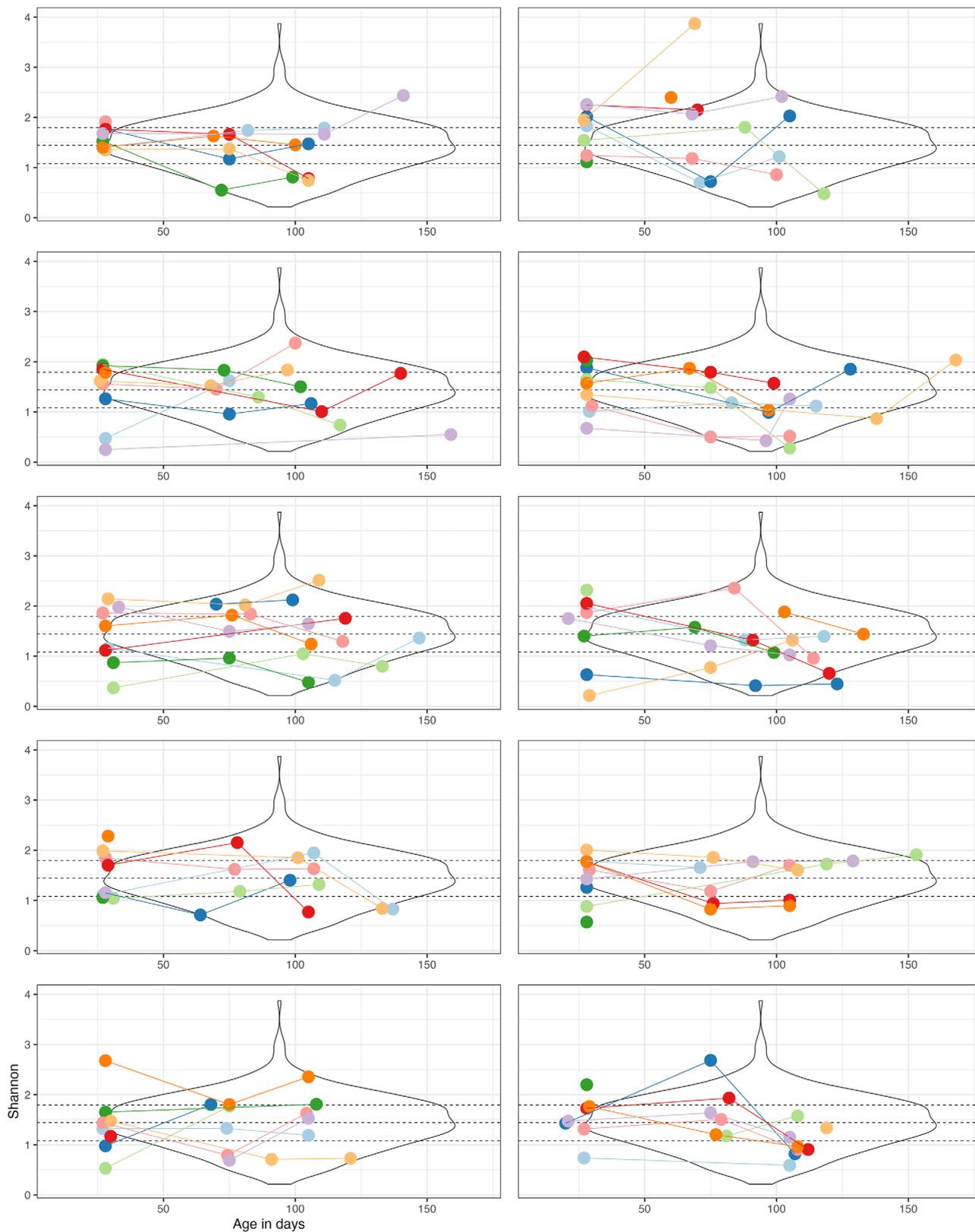
**FIGURE 6** The y-axis shows absolute (a1–e1) and relative (a2–e2) measures of cluster strength for the stool samples obtained at T1 (a), T2 (b), T3 (c), T4 (d) and T5 (e). The x-axis shows the number of clusters the calculation is based on. Silhouette index and prediction strength are indicated in red and yellow, respectively, including their predefined thresholds. Neither of these measures exceeds the threshold. Therefore, the Calinski–Harabasz index (a2–e2) will not be interpreted

microbial ecosystems. Also, the natural gut microbial ecosystem in infancy is highly variable even within infants (de Muinck & Trosvik, 2018), as was also observed in our data throughout the first 10 years of life when looking at volatility (Figure S5) or Shannon alpha diversity (Figures 7 and 8). Correlation coefficients between alpha diversity values over all time points ranged between 0.22 and 0.29, indicating high intraindividual variability. To find an association between any individual variable that is highly variable over time might require very large sample sizes. Indeed, a large-scale study showed that effect sizes of FM-covariate associations are often surprisingly small, requiring very large sample sizes to be identified (Falony et al., 2016).

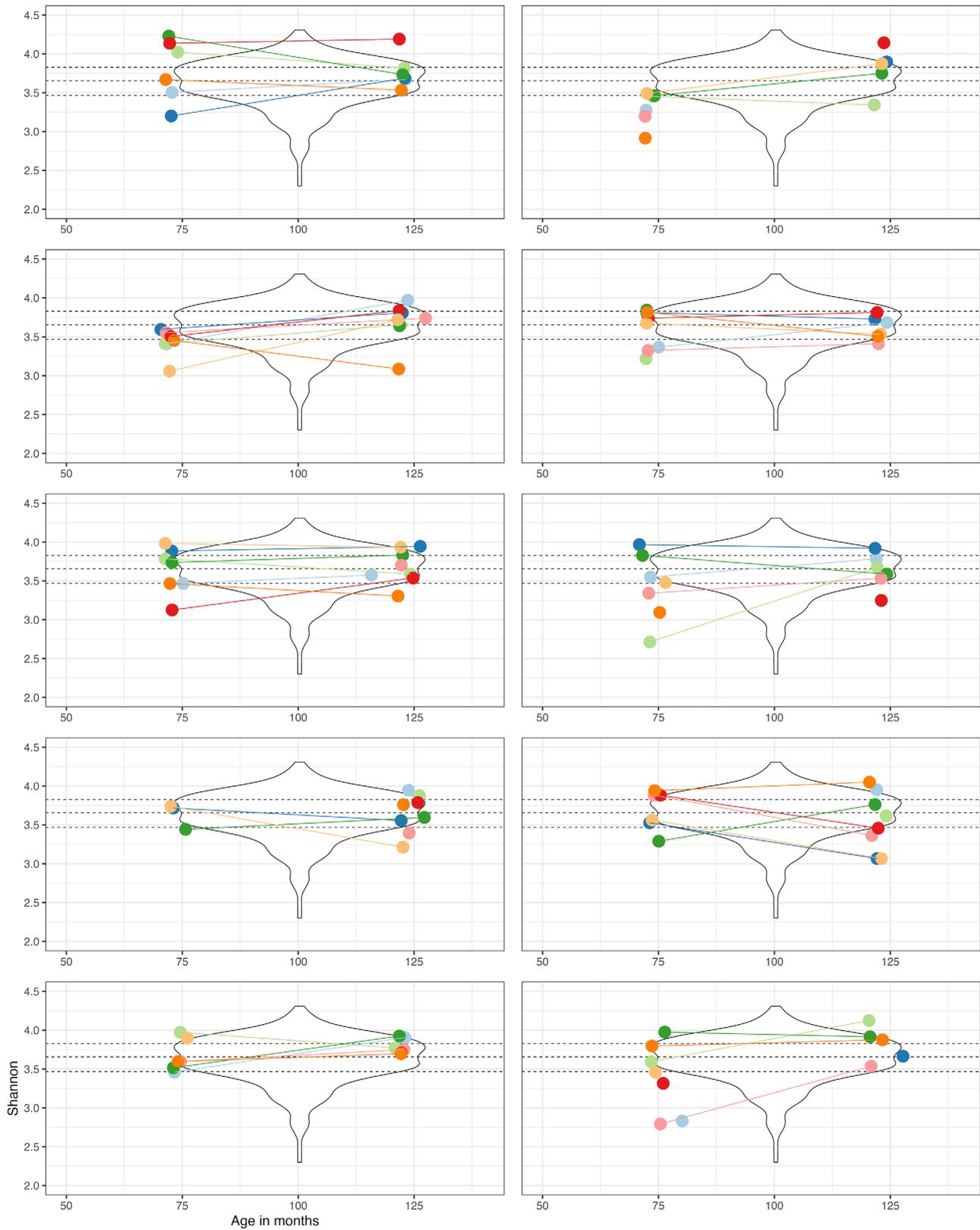
The absence of clustering in our data (determined with Partitioning around Medoids) as opposed to the data of, for example, Carlson et al. (2018) illustrates other important challenges in microbiome studies regarding cross-study comparisons, some of which have been discussed recently (Moreno-Indias et al., 2021). These challenges can include different choices regarding the sequenced region of the 16S gene or the pipeline used to process the sequencing data. For example, the V2 region has been shown to have higher resolution for lower-rank genera than the V4 region (Bukin et al., 2019). Other challenges arise because researchers choose different statistical methods or apply them differently. For instance, we applied thresholds to define the presence of clustering based on Koren et al. (2013). According to these thresholds, there would have been no clustering in the data of Carlson et al. (2018) either. As a final example, a Dirichlet Multinomial Mixture Model (Holmes et al., 2012), which we fitted as part of another project on the data of this study, can identify three clusters in infancy and four clusters in childhood (exploratory pairwise comparisons between

these clusters can be found in the Supporting Information). These examples illustrate that a lack of standardization of the many necessary analysis choices in microbiome studies makes a cross-study comparison difficult and in many cases impossible (Moreno-Indias et al., 2021).

Limitations of our study are that we could not take into account the functionality of the gut microbial ecosystem or look at species or even strain level. Relating (predicted) microbial neuroactive metabolites directly to EF was beyond the scope of this study but would be informative as the metabolites reflect an important pathway through which bacteria exert effects on the host. Also, it is possible that only single species or strains of a genus are associated with EF while the genus is not. Furthermore, given the high variability of the FM, partially caused by known uncontrolled variables such as diet (childhood), time of defecation, stool consistency and others, our sample size is too small to be confident that there is not a weak association between genus level FM and EF. Note, however, that the sample size is large compared to the earlier human studies (Carlson et al., 2018; Gao et al., 2019). Lastly, our study sample consisted of highly educated women with uncomplicated pregnancies and giving birth to healthy, full-term infants. This limits the generalizability of our findings. Strengths of the current study include using a longitudinal design over a long-time span. This allowed us to determine FM at three distinct time points in infancy and at 6 and 10 years of age in childhood. Repeated microbiota sampling makes our findings more robust compared to studies that analyzed a single sample. Furthermore, we used the Digit Span memory task in combination with the ecologically valid BRIEF questionnaire to measure different aspects of EF. The repeated measurement of the BRIEF resulted in a more robust estimation of daily life EF. Finally, we



**FIGURE 7** Alpha diversity (Shannon) for each sample in infancy. On each plot maximal eight infants (maximum eight colors) are shown to enable tracking the paths of the individuals without cluttering. The three dashed lines represent the 25%, 50% and 75% quantiles of all Shannon values in infancy. The violin plot in the background shows the corresponding whole distribution of Shannon diversity of our infant samples



**FIGURE 8** Alpha diversity (Shannon) for each sample in childhood. On each plot maximal eight children (maximum eight colors) are shown to enable tracking the paths of the individuals without cluttering. The three dashed lines represent the 25%, 50% and 75% quantiles of all Shannon values in childhood. The violin plot in the background shows the corresponding whole distribution of Shannon diversity of our childhood samples

utilized different complimentary and sophisticated statistical methods to evaluate our hypotheses while accounting for important confounding variables.

## 5 | CONCLUSIONS

In conclusion, we did not find a relationship between infant or childhood fecal microbiota composition and executive functioning in childhood. Future studies might benefit from a higher taxonomic resolution than the genus level, repeated assessments and larger sample sizes, as well as the addition of the (predicted) functional assessment of the gut microbial ecosystem.

## ACKNOWLEDGMENTS

The BIBO study was supported by a Netherlands Organization for Scientific Research VIDI grant (575-25-009, to C de Weerth), a Jacobs Foundation Advanced Research Fellowship (to C de Weerth) and a Netherlands Organization for Scientific Research VICI grant (016.Vici.185.038, to C de Weerth). Leo Lahti was supported by the Academy of Finland (decision 295741).

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

Henrik Andreas Eckermann performed the literature search, statistical analysis and wrote the manuscript. Yangwenshan Ou carried out wet lab analyses and analyzed the microbiota data until the generation of ASV tables. Leo Lahti performed the statistical analysis. Carolina de Weerth designed the study and collected the data. All authors contributed to the writing of the manuscript, critically reviewed and revised the manuscript, and approved the final manuscript as submitted.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on reasonable request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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## SUPPORTING INFORMATION

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**How to cite this article:** Eckermann, H. A., Ou, Y., Lahti, L., & de Weerth, C. (2022). Can gut microbiota throughout the first 10 years of life predict executive functioning in childhood? *Developmental Psychobiology*, 64, e22226. <https://doi.org/10.1002/dev.22226>