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EDITED BY

Leon Morales-Quezada,
Spaulding Rehabilitation Hospital, United States

REVIEWED BY

Ancuta Lupu,
Grigore T. Popa University of Medicine and
Pharmacy, Romania
Vasile Valeriu Lupu,
Grigore T. Popa University of Medicine and
Pharmacy, Romania

*CORRESPONDENCE

Khitam Muhsen
✉ kmuhsen@tauex.tau.ac.il

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Relationships of the gut microbiome with cognitive development among healthy school-age children

Yelena Lapidot¹, Maayan Maya¹, Leah Reshef², Dani Cohen¹,
Asher Ornoy^{3,4}, Uri Gophna² and Khitam Muhsen^{1*}

¹Department of Epidemiology and Preventive Medicine, School of Public Health, the Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel, ²The Shmunis School of Biomedicine and Cancer Research, Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel, ³Adelson School of Medicine, Ariel University, Ariel, Israel, ⁴Department of Medical Neurobiology, The Hebrew University Hadassah Medical School, Jerusalem, Israel

Background: The gut microbiome might play a role in neurodevelopment, however, evidence remains elusive. We aimed to examine the relationship between the intestinal microbiome and cognitive development of school-age children.

Methods: This cross-sectional study included healthy Israeli Arab children from different socioeconomic status (SES). The microbiome was characterized in fecal samples by implementing 16S rRNA gene sequencing. Cognitive function was measured using Stanford-Binet test, yielding full-scale Intelligence Quotient (FSIQ) score. Sociodemographics and anthropometric and hemoglobin measurements were obtained. Multivariate models were implemented to assess adjusted associations between the gut microbiome and FSIQ score, while controlling for age, sex, SES, physical growth, and hemoglobin levels.

Results: Overall, 165 children (41.2% females) aged 6–9 years were enrolled. SES score was strongly related to both FSIQ score and the gut microbiome. Measures of α -diversity were significantly associated with FSIQ score, demonstrating a more diverse, even, and rich microbiome with increased FSIQ score. Significant differences in fecal bacterial composition were found; FSIQ score explained the highest variance in bacterial β -diversity, followed by SES score. Several taxonomic differences were significantly associated with FSIQ score, including *Prevotella*, *Dialister*, *Sutterella*, *Ruminococcus callidus*, and *Bacteroides uniformis*.

Conclusions: We demonstrated significant independent associations between the gut microbiome and cognitive development in school-age children.

KEYWORDS

gut microbiome, children, healthy, school age, cognitive development, socioeconomic status

1. Introduction

The gut microbiome is important in health and disease (1, 2). The microbiota is increasingly recognized for its ability to influence the nervous system and several complex behaviors of the host, by modulation of neurodevelopment through the microbiome-gut-brain axis (3, 4).

The gut-brain axis is characterized by a bidirectional communication between the gut and the brain, that might modify both gastrointestinal and nervous systems function, influencing emotion and cognition (4). Preclinical and clinical studies showed that variations in microbiota composition contribute to various cognitive states, including

functional brain connectivity, depression, stress, anxiety, and autism spectrum disorder (5–12). The mechanisms underlying these relationships are not fully understood, but a compelling hypothesis is that gut microbiota variation during childhood with vulnerable neurodevelopmental window, might influence both mental and cognitive outcomes (13).

The first years of life are characterized by intense structural and functional changes in the brain and thus are critical for neurodevelopmental plasticity (14). Intriguingly, these changes occur simultaneously with dynamic intestinal microbiome alterations, thus raising the possibility of dialogue between the microbes that inhabit the gastrointestinal tract and the brain in early life (15, 16). Animal models demonstrated that early life gut microbiome influences later neurodevelopment (17). However, evidence from human populations remains limited and focused merely on infancy, demonstrating the association between the intestinal microbiome with both temperament (18, 19) and cognitive performance (12, 20). Evidence suggests substantial functional and taxonomic differences in the gut microbiota of healthy children compared to those of adults, suggesting that the development of gut microbiome may continue into school age and more slowly than previously thought and that the gut microbiota of children may be more malleable to environmental factors than that of adults (21, 22). Moreover, neurodevelopment remains an ongoing critical process during the early to middle childhood years (23), nevertheless the association between the microbiome and cognitive development during school age remains elusive.

Environmental exposures, including socioeconomic status (SES) play a critical role in both intestinal microbiome (24, 25) and neurologic development (26, 27). Moreover, iron deficiency anemia, a main risk factor for diminished neurodevelopmental and cognitive abilities in children (28–30), was linked with the gut microbiome, in both animal models and human studies (31–33). However, the interconnection between the microbiome, environmental exposures, and cognitive function in childhood is not fully clear. To address these gaps, we examined the association between the intestinal microbiome and cognitive development of school-age children, with the possible intermediating effect of environmental exposures, including sociodemographics, physical growth, and nutritional status. Our working hypothesis was that the gut microbiome might be related to cognitive development of healthy school-age children, independent of potential confounders.

2. Materials and methods

2.1. Study population and design

This cross-sectional study focused on a population under transition, the Israeli Arab population, the main ethnic minority in Israel. This population comprises 20% of the Israeli population (34, 35), while 75% are Jews and 5% belong to other population groups. The Arab population has lower educational levels and SES compared to the Jewish population (35, 36), but there is an ongoing improvement in the educational level and

health indicators among Arabs. Access to care is universal in Israel, due to the universal health insurance law (37).

This study was conducted in 2007–2009, in three Arab villages in Hadera sub-district. In 2007, there were about 153,000 Muslim Arab residents living in this region, with 3,921 live births (34). One village had approximately 14,000 residents during the study period and the other two villages had about 10,000 residents each. According to the Central Bureau of Statistics, one village belonged to cluster 2 SES, one belonged to cluster 3 SES, and the third village belonged to cluster 4 SES. The clusters are on a scale of 1–10, the lower the index, the lower the SES (36). At the national level, these villages are of low and intermediate SES levels (36). Given the SES differences across the villages, they were referred herein as village A = high SES, village B = intermediate SES, and village C = low SES. The drinking water supply in these villages is piped, and all households are connected to the national electricity company similar to the rest of the country. Connection to the internet and cable television is also available.

In this cross-sectional study, we examined the gut microbiome in archived stool samples obtained from healthy children who participated in a study on gastrointestinal tract infections. Briefly, in 2003–2004, a cohort of 289 healthy children aged 3–5 years from three villages of different SES were recruited. In 2007–2009, a follow-up was performed among 196 children at age 6–9 years (38, 39). Overall, 176 children who provided stool samples had sufficient material for 16S rRNA sequencing. Eight children were excluded due to medical conditions that might affect cognitive function directly (thalassemia minor, type-1 diabetes, Glucose-6-phosphate dehydrogenase deficiency with anemia, major heart defect, panhypopituitarism, hemophilia, and significant developmental delay). Three additional children with missing IQ scores were omitted from the analysis, thus leaving 165 participants in the analysis.

2.2. Data collection

Information on household and socioeconomic characteristics was obtained via personal interviews with the mothers, by trained Arabic-speakers interviewers. The questionnaire included information on age, sex, the village of residence, maternal education, maternal age, paternal education, monthly family income, number of persons living in the household, and number of rooms in the household. Crowding index was calculated by dividing the number of people living in a household by the number of rooms in a household. Data were collected on early life determinants e.g., birth weight, breastfeeding, and daycare center attendance.

2.3. Current hemoglobin levels

Blood collected by finger lancing was used for hemoglobin measurement using a portable hemoglobinometer (Hemocue Hb

201+, Sweden). Hemoglobin was assessed as an indicator of nutritional status.

2.4. Anthropometric measurements

Anthropometric measurements were performed by trained registered nurses. Body weight was measured to the nearest 0.1 kilogram using an analog scale (calibrated before use), and height (to the nearest 0.1 centimeter) with a stadiometer. Information on anthropometric measurements in early childhood (ages 18–30 months) was obtained from medical records. *Z* scores of height for age (HAZ), weight for height (WAZ), and body mass index for age (BMIZ) were calculated using Epi/Info software [Center for Disease Control and Prevention, Atlanta, Georgia (CDC)] based on the 2,000 CDC growth reference curves. BMI was calculated as weight (kg)/height (m)².

2.5 . Socioeconomic status (SES)

Multiple SES indicators were examined: (1) community SES rank as classified by the Israel Central Bureau of Statistics, (2) household socioeconomic characteristics: (a) maternal education, (b) paternal education, (c) crowding index, and (d) reported family income. We used these variables to generate a composite SES score, based on confirmatory factor analysis. The analysis was implemented using “Principal Axis” method, including rotation with “varimax” (*r* package psych). Since maternal education was significantly correlated with parental education (Pearson’s $r = 0.46$), we included only maternal education level in the analysis. The selected variables were tested with Bartlett’s test of homogeneity of variances (p -value < 0.0001) and Kaiser–Meyer–Olkin factor adequacy resulting in adequate scores for all selected variables: village of residence = 0.7, crowding index = 0.7, maternal education = 0.7, reported family income = 0.68. The newly generated SES score was composed of a combination of the standardized loadings, based on the correlation matrix of the selected variables ([Supplementary Figure S1](#)).

2.6. Assessment of cognitive function

Cognitive function was measured by Intelligence Quotient (IQ) score using Stanford–Binet–5th edition (SB5) test, performed by a trained Arabic speaking psychologist (39). The following parameters were assessed: full-scale IQ (FSIQ), non-verbal IQ and verbal IQ. The test was performed at standard conditions, lasting 45–60 min. The SB5 was scored with the *SB5 Scoring Pro*, a Windows®-based software program. Since FSIQ is highly correlated with non-verbal and verbal IQ (Pearson’s correlation $r = 0.95$ and $r = 0.94$ respectively), FSIQ score was selected as the main outcome variable in this study. The psychologist was masked to background information of the participants.

2.7. Samples collection, DNA extraction and bacterial DNA amplification

Fresh stool samples were obtained from the children using collection plastic cups and transferred on ice to the laboratory at Tel Aviv University. Samples were divided stored at -80°C until testing. All samples underwent a single thaw prior to DNA extraction. DNA was extracted from 180 to 220 mg of fecal material from each sample using the QIAamp® Fast DNA Stool Mini Kit (Qiagen, Valencia, CA) following the manufacturer’s instructions (40) and stored at -20°C until shipment to the Sequencing Core at the University of Illinois. Genomic DNA was prepared for sequencing using a two-stage amplicon sequencing workflow (41). Initially, genomic DNA was amplified via PCR using primers targeting the V4 region of microbial 16S ribosomal RNA (rRNA) genes. The primers, 515F modified and 806R modified (42), contained 5’ linker sequences compatible with Access Array primers for Illumina sequencers (Fluidigm, South San Francisco, CA). The PCR assays were performed in a total volume of 10 μl using MyTaq™ HS 2X Mix (Bioline) with primer concentrations at 500 nM. Thermocycling conditions were as follows: 95°C for 5 min (initial denaturation), followed by 28 cycles of 95°C for 30 s, 55°C for 45 s, and 72°C for 30 s. One microliter of the PCR product from each reaction was transferred to the second-stage PCR assay. Each second-stage reaction was conducted in a final volume of 10 μl using MyTaq HS 2X mix, and each well contained a unique pair of Access Array primers containing Illumina sequencing adapters, single index sample-specific barcode, and linker sequences. Thermocycling conditions were as follows: 95°C for 5 min (initial denaturation), followed by 8 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Libraries were pooled and purified using 0.6 \times concentration of AMPure XP beads to remove short fragments below 300 bp. Pooled libraries were loaded onto a MiniSeq sequencer (Illumina, San Diego, CA) with 15% phiX spike-in and paired-end 2×153 base sequencing reads.

2.8. Statistical analyses

Quality control analysis of demultiplexed sequences was performed using the Deblur (43) workflow, following the construction of a phylogenetic tree (mafft-fasttree) and taxonomy assignment with QIIME2 (44). The quality process with Deblur uses sequence error profiles to obtain putative error-free sequences, referred to as “sub” operational taxonomic units (s-OTU). Taxonomic composition was assigned to the s-OTUs using a pre-trained Naive Bayes classifier, trained on the Greengenes (45) 13_8 99% OTUs. Downstream analysis was conducted using R version 4.0.3. Diversity analysis was calculated at rarefaction depth of 11,158. Bacterial α -diversity, which quantifies the intra-sample diversity, i.e., the distribution of species abundances in a given sample, was estimated using Shannon’s diversity and Pielou’s evenness indexes (46) and compared across independent variables using multivariate

analysis of variance (ANOVA) tests. β -diversity, which measures dis-similarities between samples (46), was calculated using the Bray-Curtis dissimilarity index, the Jensen-Shannon divergence (JSD), and the phylogenetic weighted and unweighted Unifrac distances. Permutational multivariate analysis of variance (PERMANOVA) was used to test differences in overall microbiome composition [vegan; Adonis (47)], implementing a multivariate model with the covariates: age, sex, SES score, hemoglobin levels, HAZ at age 18–30 months and current BMIZ scores. The Analysis of Composition of Microbiomes (48) (ANCOM) was applied for the identification of differentially abundant features in association with FSIQ scores, with the false discovery rate (FDR) level set to 0.05. ANCOM uses a linear framework to statistically detect features whose composition varies across FSIQ scores, while controlling for other covariates of interest (a linear model comprised of the abovementioned covariates). A feature was considered significantly varying in composition across an independent variable of interest at a detection level of ≥ 0.6 , meaning that the feature composition varied across the independent variable with respect to 60% of reference features. Non-parametric Spearman's correlation coefficient was used to evaluate the association between α -diversity indices and FSIQ scores.

Differences in demographic characteristics across the study villages were examined using one-way ANOVA for continuous variables, the Kruskal–Wallis H test for rank-based variables and the χ^2 test for categorical variables. Post-hoc pairwise comparisons were conducted using Games–Howell test, including multiple comparisons correction with FDR.

2.9. Ethical Approval

The Institution Review Board of Hillel Yaffe Medical Center (approval number 6/2005, year of approval 2005) and the Ethics Committee of Tel Aviv University approved the study (approval year 2018). Written informed consent was obtained from the parents of the participants.

3. Results

3.1. Demographic characteristics of the study participants

Data from 165 children (41.2% females) who provided stool specimens and underwent a cognitive assessment were included in the analysis. The participants' mean age was 7.8 years, [SD = 0.9], with significant differences between the villages ($p = 0.019$). The composite SES score ranged from -2.1 to 4.6 [mean 2.1 (SD = 1.4); **Supplementary Figure S1B**] and was profoundly different between the villages ($p < 0.0001$; **Supplementary Figure S2A**). Children from village C (low SES) had significantly worse SES indicators than children from villages A/B, but there were no significant differences between the villages in early life determinants, e.g., birth weight, breastfeeding, and attending a daycare. HAZ scores in infancy were significantly lower ($p = 0.026$) in children from village C (low SES) than children from villages A/B (high/intermediate SES). The mean BMIZ score at school age was higher among children from village C compared to villages A/B ($p < 0.001$) (**Table 1**). The mean FSIQ of

TABLE 1 Characteristics of the participants by the village of residence ($N = 165$).

	Villages A/B (intermediate/high SES)	Village C (low SES)	p value
Number of participants, (%)	100 (60.6%)	65 (39.4%)	–
Age, years, mean (SD)	7.9 (0.9)	7.6 (0.8)	0.019
Sex, females, N (%)	40 (40.0%)	28 (43.1%)	0.818
Household crowding index ^a , mean (SD)	1.4 (0.6)	2.6 (1.3)	<0.001
Household monthly income ^b			<0.001
Above average	16 (16.0%)	3 (4.6%)	
Average	34 (34.0%)	8 (12.3%)	
Below average	50 (50.0%)	54 (83.1%)	
Father education, years, mean (SD)	11.3 (3.4)	8.3 (3.5)	<0.001
Maternal education, years, mean (SD)	11.2 (3.5)	6.5 (3.7)	<0.001
SES score ^c , mean (SD)	3.0 (0.9)	0.8 (1.0)	<0.001
Birth weight (kg), mean (SD)	3.2 (0.5)	3.4 (0.5)	0.225
Breastfeeding, yes, N (%)	98 (98.0%)	57 (87.7%)	0.09
Age of introducing solid foods, months, mean (SD)	6.0 (2.9)	5.9 (2.6)	0.741
Daycare center attendance in early life, N (%)	20 (20.0%)	12 (18.8%)	0.697
Current hemoglobin level (g/dl), mean (SD)	12.6 (0.9)	12.5 (1)	0.567
Height for age z-score (age 18–30 months), mean (SD)	0.10 (0.80)	−0.20 (0.80)	0.026
Weight for age z-score, (age 18–30 months), mean (SD)	0.00 (0.9)	0.20 (1.0)	0.275
BMIZ score ^c , mean (SD)	0.20 (1.0)	0.82 (0.9)	<0.001
Full scale IQ score	105.0 (9.2)	89.2 (12.3)	<0.001

BMIZ, body mass index z score; SD, standard deviation; SES, socioeconomic status.

^aHousehold crowding: Number of people living in the household/Number of rooms in the household.

^bHousehold income: Household income as compared to the national average.

^cIndividual level socioeconomic status score—a composite score based on confirmatory factor analysis including village of residence, maternal education, household crowding, and household income.

this cohort was 98.8 [SD = 13.1] points. FSIQ score was lower among children from village C compared to villages A/B (**Supplementary Figure S2B**).

A significant positive association was found between the composite SES score and FSIQ scores ($p < 0.0001$ by ANOVA; **Supplementary Figure S3A** and Spearman's $r = 0.61$, $p < 0.0001$; **Supplementary Figure S3B**). FSIQ score was not correlated with hemoglobin level, nor with WAZ score at age 18–30 months (**Supplementary Figures S4A,B**). Significant associations were found between HAZ at age 18–30 months with FSIQ (Spearman's $r = 0.22$, $p = 0.004$) and between current BMIZ scores and with FSIQ (Spearman's $r = -0.17$, $p = 0.025$), **Supplementary Figures S4C,D**).

Based on these results and existing knowledge regarding the environmental effects of SES on both the microbiome (2, 49–51) and FSIQ (16, 52, 53), we examined the association between FSIQ score and fecal microbiome alterations, while adjusting for covariates that were associated with microbial alterations and FSIQ score.

3.2. The association between FSIQ score and bacterial α -diversity

Bacterial α -diversity as estimated by the Shannon's diversity index followed a normal distribution (**Supplementary Figure S5**). We found a significant positive association between Shannon's diversity and FSIQ score (**Figure 1A**). A multivariate analysis of variance model that adjusted for sex, age, SES score, hemoglobin level, HAZ at age 18–30 months and current BMIZ scores on bacterial diversity (**Figure 1B**), showed that FSIQ and sex were significantly associated with Shannon's diversity index ($F = 6.16$, $p = 0.014$ and $F = 4.89$, $p = 0.029$, respectively). A multivariate analysis that included FSIQ score as the dependent variable, showed a strong positive association between fecal α -diversity and FSIQ ($F = 9.73$, $p = 0.002$; **Figure 1C**). In this model, SES scores had the strongest association with FSIQ score ($F = 97.91$, $p < 0.0001$). Hemoglobin level was significantly associated with FSIQ score ($F = 3.94$, $p = 0.049$; **Figure 1D**). Bacterial α -diversity and FSIQ score were positively linearly correlated (Person's $r = 0.20$, $p = 0.015$; **Figure 1E**).

These associations were further strengthened by an estimation of bacterial α -diversity with Pielou's evenness index. There was a strong association between FSIQ score and increased species evenness (**Supplementary Figure S6A**), with FSIQ being the strongest and the only significant variable associated with altered species evenness ($F = 11.32$, $p < 0.0001$; **Supplementary Table S1A**). This model showed a borderline significant association between hemoglobin level and Pielou's evenness index ($F = 2.91$, $p = 0.089$). A multivariate model showed a significant relationship between FSIQ score and species evenness ($F = 17.41$, $p < 0.0001$), but the effect of SES ($F = 91.13$, $p < 0.0001$) on FSIQ score was stronger (**Supplementary Table S1B**). Significant positive linear correlation between FSIQ score and species evenness (Spearman's $r = 0.24$, $p = 0.002$; **Supplementary Figure S6C**).

3.3. The association of gut microbiome composition and FSIQ score

We found significant differences in fecal bacterial composition, as measured by the Bray–Curtis dissimilarity index ($F = 10.79$, $R^2 = 0.06$, $p = 0.001$; **Figure 2A**, **Supplementary Table S2A**), the phylogenetic unweighted and weighted UniFrac distance matrixes ($F = 5.04$, $R^2 = 0.03$, $p = 0.001$, and $F = 8.59$, $R^2 = 0.05$, $p = 0.001$ relatively; **Figure 2B**, **Supplementary Tables S2B, S2C**), and by the JSD ($F = 7.40$, $R^2 = 0.04$, $p = 0.001$; **Figure 2C**, **Supplementary Table S2D**). All multivariate models included the covariates age, sex, SES score, hemoglobin, HAZ and BMIZ scores.

FSIQ score explained the highest variance in bacterial β -diversity as measured by the Bray–Curtis dissimilarity index, followed by the SES score ($F = 10.79$, $R^2 = 0.06$, $p = 0.001$, and $F = 5.17$, $R^2 = 0.03$, $p = 0.001$, respectively), the phylogenetic unweighted UniFrac distance matrix ($F = 5.04$, $R^2 = 0.029$, $p = 0.001$, and $F = 3.33$, $R^2 = 0.019$, $p = 0.001$, respectively), the weighted UniFrac distance matrix ($F = 8.59$, $R^2 = 0.048$, $p = 0.001$, and $F = 4.35$, $R^2 = 0.024$, $p = 0.001$, respectively) and the JSD ($F = 15.99$, $R^2 = 0.085$, $p = 0.001$, and $F = 7.4$, $R^2 = 0.039$, $p = 0.001$, respectively).

We found a significant association of weaker magnitude, between the participant's age, sex, and BMIZ score, and bacterial composition, in some β -diversity measurements. Age was significantly associated with the Bray–Curtis dissimilarity index ($F = 2.13$, $R^2 = 0.012$, $p = 0.014$), the weighted UniFrac ($F = 2.82$, $R^2 = 0.016$, $p = 0.003$) and the JSD ($F = 2.64$, $R^2 = 0.014$, $p = 0.011$). BMIZ was significantly associated with bacterial composition when measured by the Bray–Curtis dissimilarity method ($F = 1.72$, $R^2 = 0.010$, $p = 0.03$) and the JSD ($F = 1.94$, $R^2 = 0.010$, $p = 0.046$), while sex was significantly associated the weighted UniFrac index ($F = 1.88$, $R^2 = 0.011$, $p = 0.043$).

The multivariate model using the JSD method explained the overall highest amount of variance 16.6% of the variation in bacterial composition (**Figure 2D**). The remaining models explained a smaller amount of β -diversity variation: 12.8%, 11.8% and 8.3% for the Bray–Curtis dissimilarity, the weighted UniFrac and the unweighted UniFrac, respectively.

3.4. Taxonomic alterations associated with FSIQ score

In agreement with the profound differences of bacterial composition, we found significant associations between the relative abundance of several bacterial genera, with adjustment for age, sex, SES score, hemoglobin, HAZ and BMIZ scores (**Figure 3A**). Genus *Prevotella* was detected at the highest detection level ($W_{\text{stat}} = 706$), followed by *Dialister* ($W_{\text{stat}} = 675$), *Sutterella* ($W_{\text{stat}} = 637$), *Ruminococcus callidus* ($W_{\text{stat}} = 609$), *Bacteroides uniformis* ($W_{\text{stat}} = 605$) and Lachnospiraceae ($W_{\text{stat}} = 553$). At a lower detection level, there was an association with *Bacteroides*, *Prevotella copri*, *Oscillospira* and *Clostridium* (**Supplementary Table S3**). FSIQ scores were inversely associated

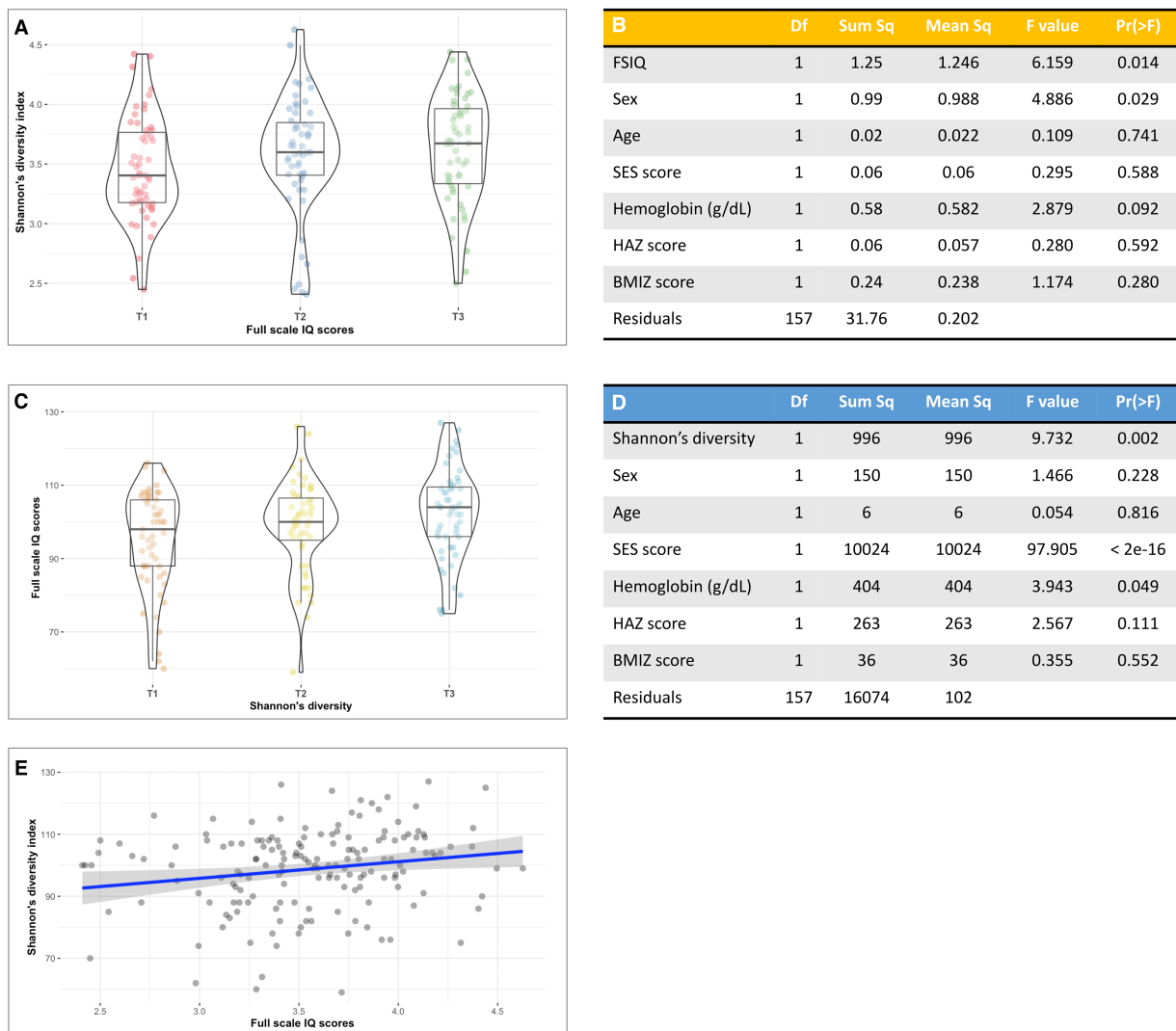


FIGURE 1

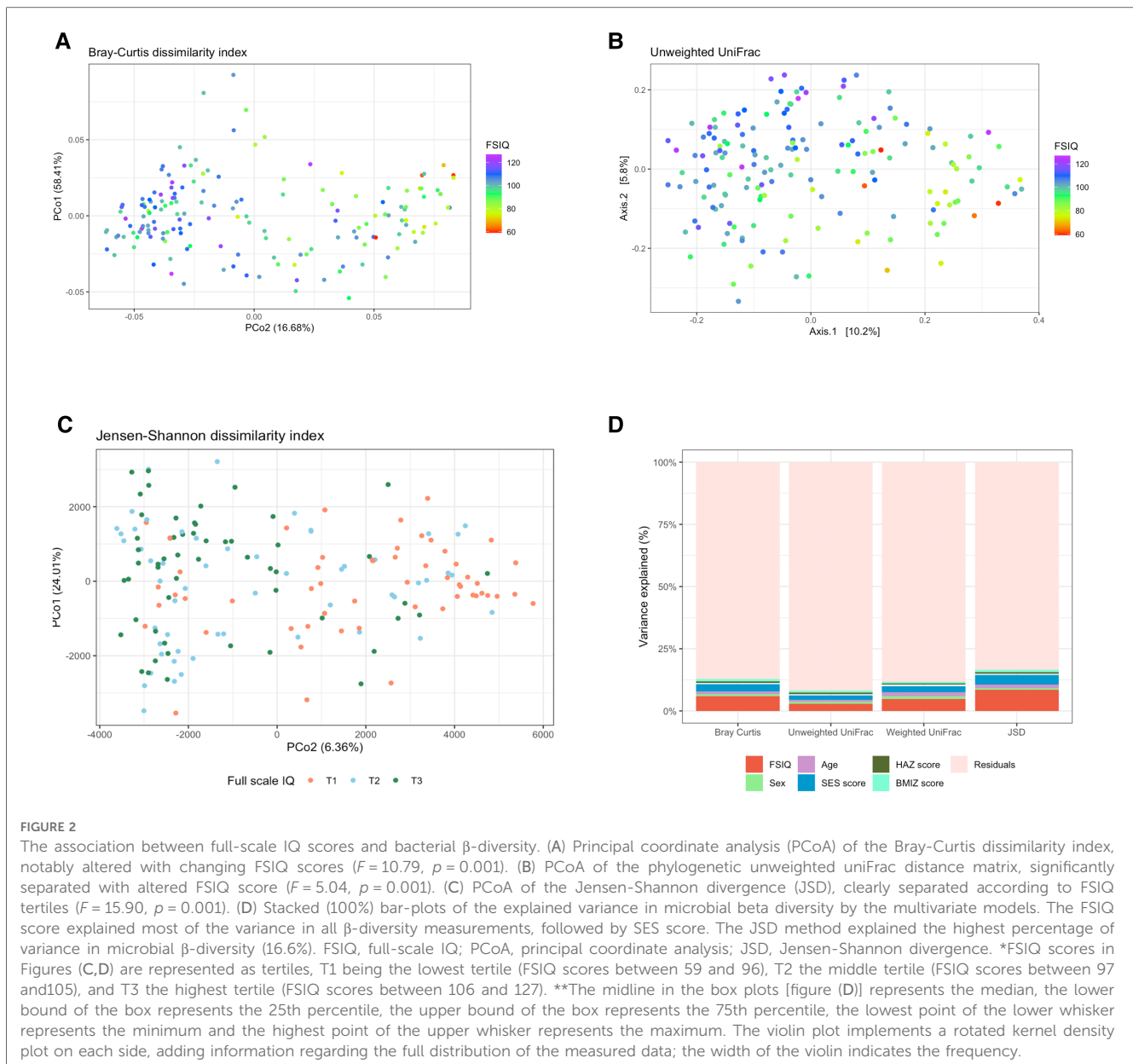
The association between full-scale IQ scores and bacterial α -diversity. (A) Box-violin plots of microbial diversity, measured by Shannon's diversity index, across tertiles of FSIQ scores, showing a significant increase in microbial α -diversity with increased FSIQ ($p = 0.014$). (B) Results of a multivariate analysis of variance displaying the association between FSIQ score and covariates of interest with bacterial diversity. FSIQ score and sex were significantly associated with Shannon's bacterial α -diversity index ($F = 6.16$, $p = 0.014$ and $F = 4.89$, $p = 0.029$, respectively). (C) Box-violin plots of FSIQ scores across tertiles of Shannon's diversity index, show a significant increase in FSIQ scores with increased bacterial diversity ($p = 0.002$). (D) Results of a multivariate analysis of variance displaying the association between the individuals' gut Shannon's diversity and covariates of interest with FSIQ scores. Bacterial α -diversity and SES scores were strongly associated with FSIQ score ($F = 9.73$, $p = 0.014$ and $F = 97.91$, $p = 0.029$, respectively), while hemoglobin levels had a more delicate albeit significant association ($F = 3.94$, $p = 0.049$). (E) The correlation between Shannon's α -diversity index and FSIQ score; Pearson's $r = 0.20$, $p = 0.015$. SES, socioeconomic status; FSIQ, full-scale IQ; HAZ, height for age z-score at age 18–30 months; BMIZ, body mass index z-score at age 6–9 years. *The x axis in figures (A,C) represents tertiles, T1 = lowest tertile, and T3 = highest tertile. **The mid line in the box plots [figures (A,C)] represents the median, the lower bound of the box represents the 25th percentile, the upper bound of the box represents the 75th percentile, the lowest point of the lower whisker represents the minimum and the highest point of the upper whisker represents the maximum. The violin plot implements a rotated kernel density plot on each side, adding information regarding the full distribution of the measured data; the width of the violin indicates the frequency.

with the relative abundance of *Prevotella* (including species *Prevotella copri*), *Dialister* and *Sutterella*, while *Ruminococcus callidus*, *Bacteroides uniformis* and Lachnospiraceae were characterized by a positive association with FSIQ levels (Figures 3B–I).

An unadjusted analysis revealed significant correlations between FSIQ scores and *Prevotella* (Spearman's $r = -0.42$, $p < 0.0001$), *Dialister* (Spearman's $r = -0.19$, $p = 0.012$), *Sutterella* (Spearman's $r = -0.2$, $p = 0.01$), the species *Ruminococcus callidus*

(Spearman's $r = 0.27$, $p = 0.001$), and *Bacteroides uniformis* (Spearman's $r = 0.41$, $p < 0.0001$; Supplementary Figures S7A–E).

Since a significant percentage of bacterial variance was explained by SES scores, we performed a stratified analysis by village of residence. We found a consistent bacterial variation associated with FSIQ score in all villages, thus independent from SES and adjusted for the aforementioned covariates (Figures 4A, B). Among children from village C (low SES), *Bacteroides uniformis* was the most strongly associated species with FSIQ



score ($W_{\text{stat}} = 700$), followed by *Prevotella* ($W_{\text{stat}} = 635$), the species *Clostridioforme* ($W_{\text{stat}} = 607$), including the lower taxonomic levels Clostridiales ($W_{\text{stat}} = 581$) and *Clostridium* ($W_{\text{stat}} = 567$), *Veillonella dispar* ($W_{\text{stat}} = 567$), *Bacteroides* ($W_{\text{stat}} = 557$) and *Ruminococcus torques* ($W_{\text{stat}} = 539$). Importantly, FSQ score was positively associated with the relative abundance of *Bacteroides*, including *Bacteroides uniformis*, *Clostridium*, including species *Clostridioforme*, *Ruminococcaceae* including *Ruminococcus torques* and *Veillonella dispar*, while *Prevotella* was inversely associated with FSQ score (Figure 4C).

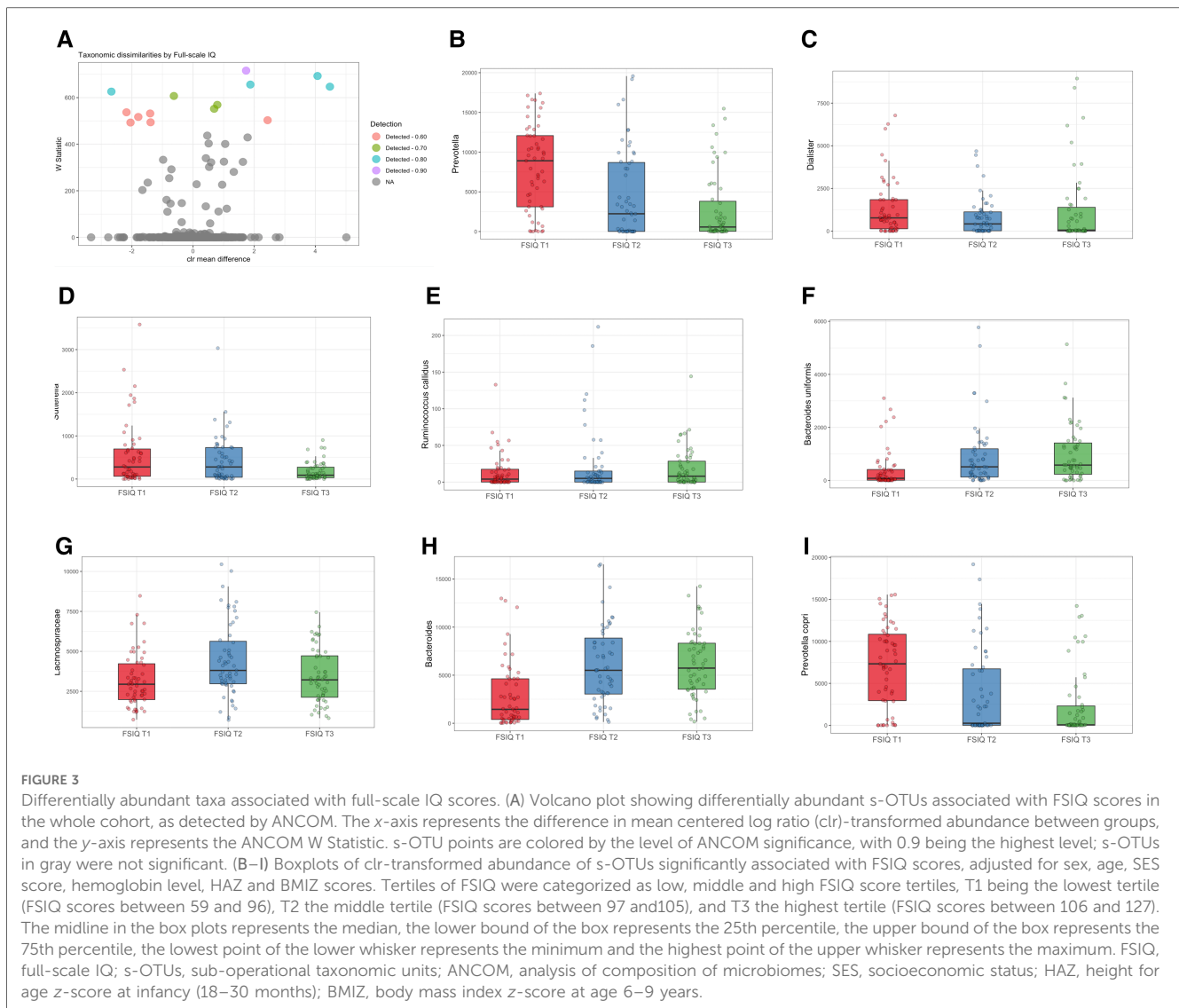
FSQ score of children from the higher SES villages (A/B), was associated with altered relative abundance of *Faecalibacterium prausnitzii* ($W_{\text{stat}} = 643$), *Oscillospira* ($W_{\text{stat}} = 587$), *Coprococcus* ($W_{\text{stat}} = 562$) and *Catenibacterium* ($W_{\text{stat}} = 520$). The full results of the stratified ANCOM analysis are presented in Supplementary Table S4. Notably, *Coprococcus*, *Ruminococcaceae*, including genus

Oscillospira were positively associated with increased FSQ score, while *Coriobacteriaceae*, *Faecalibacterium prausnitzii* and *Catenibacterium* levels were depleted with increasing FSQ score (Figure 4D).

4. Discussion

We characterized the cognitive development of school-age children, in association with intestinal microbiome diversity and composition and environmental exposures, including SES, a major factor that influences both cognitive development and the gut microbiome.

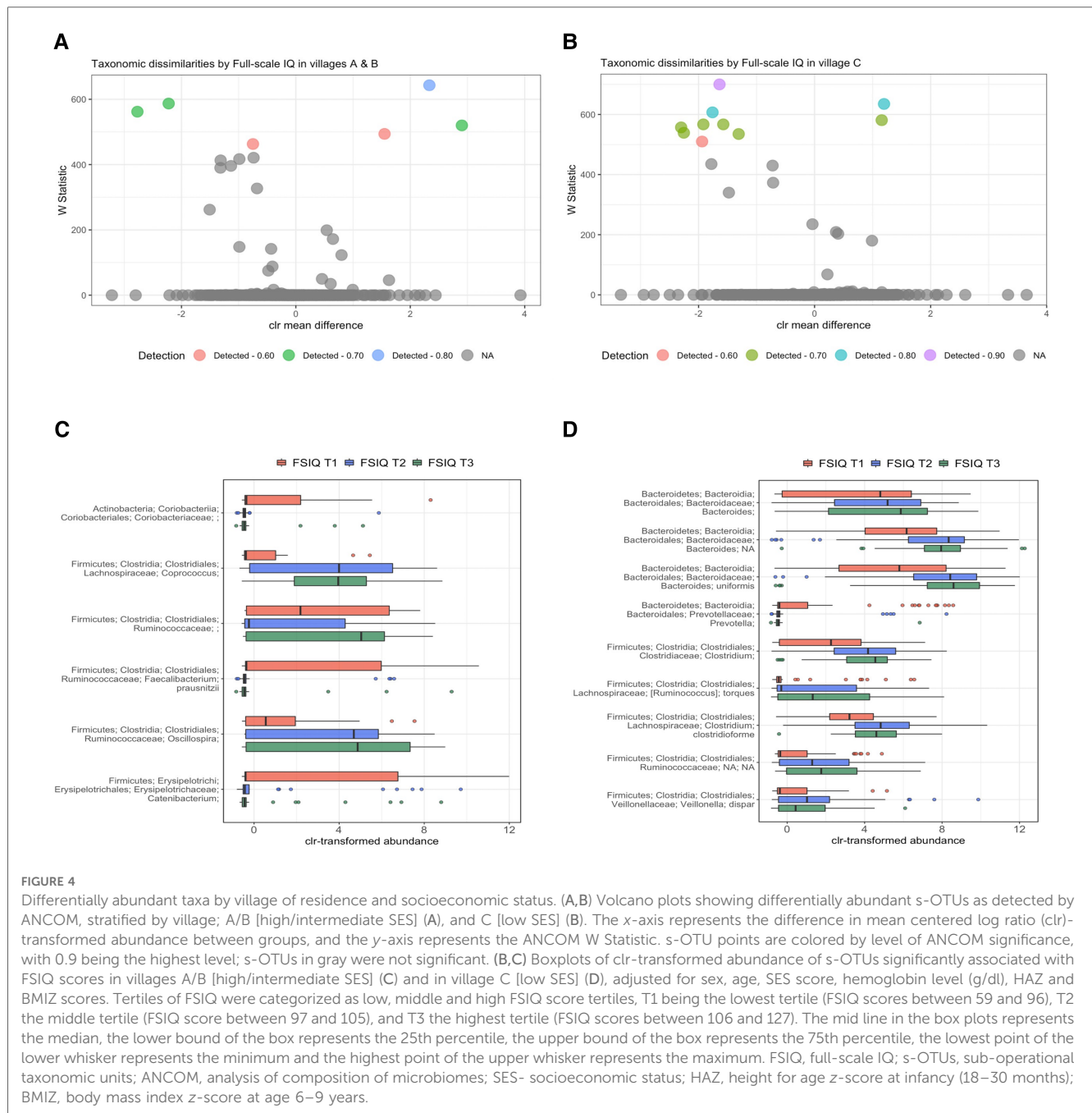
We found a significant association between microbial α -diversity, measured by both Shannon's diversity and Pielou's evenness indices and FSQ scores. There was a progressive



increase in both diversity indices with increased FSIQ score. The Shannon diversity index accounts for both richness and evenness of s-OTUs and largely mirrors the evenness findings of this cohort. In general, lower values of Shannon diversity index indicate less diversity, thus the intestinal microbiome of children with lower FSIQ scores was less diverse, less even, and less rich compared to children with higher scores. Correspondingly, Pielou's evenness considers the number of species and the relative abundance of species in a sample. We found a positive relationship between evenness and FSIQ score. Lower values of Pielou's index represent less even distributions of species, thus implying potential dominance of some species in the gut. Therefore, in our study, lower FSIQ scores were associated with less evenness of species inhabiting the human gut. We also found significant differences in the intestinal microbiome composition according to FSIQ score. These associations were observed even after adjustment for SES and nutritional status, measured by hemoglobin levels, BMIZ and HAZ scores.

A limited number of studies examined the associations of the gut microbiome diversity and composition with children's

developments, mainly between ages 2 of 3 years, (20, 54, 55). These ages are usually characterized by profound changes in the gut microbiome until it stabilizes. Unlike our study, Carlson et al. (20) in their study of 89 children aged 1–2 years demonstrated that greater α -diversity was associated with poorer cognitive performance. Streit et al. studied 323 children aged 45 months and showed weak negative correlations between alpha diversity, as measured by Faith phylogenetic diversity index and FSIQ (correlation coefficient ranged between -0.10 and -0.14), but such differences were not observed for other indices of alpha diversity when adjusting for confounders and multiple comparisons (54). Rorthenberg and colleagues studied 46 children from rural China, and reported no significant association between alpha diversity and cognitive development at age 3 years (55). The negative associations between alpha diversity and cognitive development in prior studies (20, 54), might be unexpected, since higher α -diversity usually indicates a more mature, adult-like community, while reduced α -diversity is commonly associated with poor health outcomes, including metabolic and inflammatory bowel diseases (1, 56). The



differences between our finding of a positive relationship between alpha diversity and others (20, 54) showing inverse associations might be related to discrepancies in the study population, specifically, our study included school-age children that likely have adult-like stable microbiomes, while other studies mainly included infants and pre-school children, characterized by a microbiome that is still evolving.

Our model was adjusted for SES and we observed a strong and significant association between SES and cognitive performance. These results are in line with existing evidence, demonstrating the profound influence of SES on cognitive development, and behavioral outcomes (57), and on the gut microbiome at school age (25). This complex interplay between environmental

exposures, the intestinal microbiome, and individual neurodevelopment emphasizes the need for tailored developmental programs and policies that are designed to alleviate SES-related disparities in cognitive performance in children.

We found significant associations between the intestinal microbiome composition and FSIQ score, which were consistent in four distance measures for quantifying β -diversity: the binary Bray-Curtis, the JSD, and the phylogenetic weighted and unweighted UniFrac. Cognitive performance explained the highest percentage of variance in all methods, followed by the SES score. Age was significantly, yet more finely associated with microbial composition. Overall, JSD was the most sensitive

method capturing 16.6% of the variance. While the FSIQ score explained the most variance, SES score was an important determinant of β -diversity, suggesting that these factors independently influence both the intestinal microbiome and cognitive performance. The composition of the gut microbiome is a complex trait, with the quantitative variation in the microbiome affected by a large number of host and environmental factors, each of which may have only a small additive effect, making it difficult to identify the association for each separate item (58, 59). Falony et al. reported significant relationships between previously unidentified factors such as red blood cell count and hemoglobin levels and fecal bacterial composition (58), while in our study the association between hemoglobin level and beta diversity was not statistically significant, possibly due to the smaller sample size. Our model explained 16% of the variance in microbial composition, a relatively high percentage, nonetheless indicating the need to explore additional contributions for example from dietary habits, stochastic effects, and/or biotic interactions.

While alpha and beta diversity capture complex variation across the community, we observed significant differences in the relative abundance of specific genera and species in association with FSIQ score. High relative abundance of the genera *Prevotella*, *Dialister* and *Sutterella* was associated with the lower cognitive performance tertiles, while *Bacteroides* were positively correlated with elevated FSIQ score. Interestingly, Carlson et al. (20) described a strong association between high levels of *Bacteroides* with the highest level of cognitive performance at 2 years old. Similarly, Tamana et al. (60) showed that *Bacteroidetes*-dominant gut microbiome and higher relative abundance of genus *Bacteroides* in late infancy were associated with enhanced neurodevelopment by the age of 2 years among Canadian children, mainly among males (60). It was also found that *Bacteroidetes*-dominant microbiome was enriched with numerous metabolic functions including sphingolipid metabolism and glycosphingolipid biosynthesis. Furthermore, genes involved in metabolism of folate, biotin, pyruvate, vitamin B6, lipoic acid and fatty acid biosynthesis were enriched in *Bacteroidetes*-dominant microbial composition (60). The intestinal microbiome at 6–9 years is substantially different and more diverse compared to age two years, thus our study adds new knowledge regarding potential involvement of the gut microbiome with cognitive function at school age and not only in early life, when the gut microbiome is less mature, and still affected by early life factors, such as delivery mode (61). Collectively these findings support the idea of microbiota gut-brain-axis during childhood.

Nutrition and dietary patterns are considered major determinants of cognitive performance in children and adolescents (62, 63). For example, iron deficiency anemia is linked to lower neurodevelopmental achievements (28). We measured hemoglobin levels as a proxy for iron deficiency anemia, and included this parameter in all multivariable models, suggesting that the gut microbiome was associated with cognitive function regardless of hemoglobin levels. Additional nutritional factors might influence cognitive performance and academic achievements, including B vitamins, e.g., folate, vitamin B12 (64–66), and overall dietary

patterns (63). Diet in turn affects the development of the intestinal microbiome in early life (67). We also found altered composition of the gut microbiome in relation to dietary intake of polyunsaturated fatty acids in a different cohort of Arab children aged 10–12 years (68). Our study lacks information on dietary intake of this cohort. Therefore, the associations between intestinal microbiome and cognitive function in children should be further explored in large-scale prospective studies, while deciphering potential mechanistic role of dietary intake and nutritional status in such associations and possibly intervention studies including probiotic or prebiotic supplementation to assess the association with the microbiome and cognitive performance.

Our study has some limitations. First, this is a cross-sectional study design, thus the directionality of the observed association between the gut microbiome and cognitive development remains to be determined in prospective studies. Second, diet is one of the main determinants of the gut microbiome and affects the (development) of cognitive (dys)function (24), and we did not obtain detailed dietary questionnaires for the participants. In the current cohort, early life dietary exposures were relatively similar and characterized by a high prevalence of breastfeeding, a similar age of first exposure to solid foods, and a late entrance to a daycare. Moreover, since all participants belong to the same ethnic group, they share common dietary practices, mainly a high prevalence of home-cooked, traditional meals and a diet rich in fruit, vegetables, and legumes. Nevertheless, at school-age, there are uncontrolled dietary exposures, that might have an important association with the child's microbiome and cognitive function.

We used archived stool samples that were collected during 2007–2009. There might have been changes over time, that might affect both cognitive development and the gut microbiome. The SES rank of the study villages remained stable during over one decade (69). Conversely, changes in diet and nutritional status were documented (70, 71), which might influence both the gut microbiome and cognitive development. Although, these changes should not affect the observed association between the gut microbiome and cognitive development in our study, future studies using specimens reflecting an up-to-date host-microbiome-environment interaction are needed.

The strengths of the current study include a relatively large cohort of healthy school-age children, with a defined geographic, ethnic, and cultural background, yet divergent SES. Geographic residency and ethnicity are strong modulators of the intestinal microbiome (72), thus the associations demonstrated in the current study are independent from these important confounders. Moreover, our results were adjusted for potential confounders that affect both the microbiome and FSIQ score.

5. Conclusions

We demonstrated significant associations between the gut microbiome and cognitive development in healthy school age children, independent of SES. Future longitudinal studies are

needed to understand the directionality of the associations and mechanisms that might explain these relationships.

Data availability statement

Data generated in the framework of the study cannot be made publicly available due to legal and ethical restrictions. Aggregate anonymized data might be available upon request from the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the Institution Review Board of Hillel Yaffe Medical Center and the ethics committee of Tel Aviv University. Written informed consent was obtained from the parents of the participants.

Author contributions

The authors contributed to the study as follows: YL, study concept, analysis and interpretation of data, drafting of the manuscript, and statistical analysis. LR study design, laboratory methods, data analysis, critical revision of the manuscript for important intellectual content. MM study concept, acquisition of data and data management. DC study design, supervision, acquisition of data and samples, critical revision of the manuscript for important intellectual content. UG study design, critical revision of the manuscript for important intellectual content. AO study design, critical revision of the manuscript for important intellectual content and acquisition of funding. KM study concept and design, acquisition of data and samples, study supervision, contributed to writing of the manuscript, and acquisition of funding. All authors had access to the study data, reviewed, and approved the final version of the manuscript. All

authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fped.2023.1198792/full#supplementary-material>.

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