Early-life gut microbiota and attention deficit hyperactivity disorder in preadolescents

Andrea E. Cassidy-Bushrow[1,2,*], Alexandra R. Sitarik[1], Christine Cole Johnson[1,2], Tisa M. Johnson-Hooper[3,4], Zeinab Kassem[1], Albert M. Levin[1], Susan V. Lynch[5], Dennis R. Ownby[6], Jannel M. Phillips[4,7], Germaine J.M. Yong[5], Ganesa Wegienka[1,2], Jennifer K. Straughen[1,2]

[1]Department of Public Health Sciences, Henry Ford Hospital, Detroit, MI

[2]Center for Urban Responses to Environmental Stressors, Wayne State University, Detroit, MI

[3]Department of Pediatrics, Henry Ford Hospital, Detroit, MI

[4]Center for Autism and Developmental Disabilities, Henry Ford Hospital, Detroit, MI

[5]Department of Medicine, University of California, San Francisco, CA

[6]Division of Allergy and Clinical Immunology, Department of Pediatrics, Augusta University, Augusta, GA

[7]Department of Psychiatry and Behavioral Health Services, Division of Neuropsychology, Henry Ford Hospital, Detroit, MI

Abstract

Gut microbiota maturation coincides with nervous system development. Cross-sectional data suggest gut microbiota of individuals with and without attention deficit hyperactivity disorder (ADHD) differs. We hypothesized that infant gut microbiota composition is associated with later ADHD development in our on-going birth cohort study, WHEALS. Gut microbiota was profiled using 16S ribosomal RNA and the internal transcribed spacer region 2 (ITS2) sequencing in stool samples from 1 month and 6 months of age. ADHD was defined by parent-reported or medical record doctor diagnosis at age 10. A total of 314 children had gut microbiota and ADHD data; 59 (18.8%) had ADHD. After covariate adjustment, bacterial phylogenetic diversity (p = 0.017) and bacterial composition (unweighted UniFrac p = 0.006, R^2 = 0.9%) at age 6 months were associated with development of ADHD. At 1 month of age, 18 bacterial and 3 fungal OTUs were associated with ADHD development. At 6 months of age, 51 bacterial OTUs were associated with ADHD; 14

*Corresponding Author: Andrea E. Cassidy-Bushrow, Department of Public Health Sciences, Henry Ford Hospital, 1 Ford Place, 5C, Detroit, MI 48202; Telephone: (313)874-6097; Fax: (313)874-6656; acassid1@hfhs.org.
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of the order *Lactobacillales*. Three fungal OTUs at 6 months of age were associated with ADHD development. Infant gut microbiota is associated with ADHD development in pre-adolescents. Further studies replicating these findings and evaluating potential mechanisms of the association are needed.

Introduction

Attention deficit hyperactivity disorder (ADHD) is a complex neurodevelopmental disorder hallmarked by hyperactivity, inattention, and impulsivity at a developmentally inappropriate level.[1] Studies of the gut-brain axis suggest the gut microbiome influences neurodevelopment.[2–4] The gut microbiome develops rapidly over the first 3 years of life concurrently with synaptogenesis, myelination and synaptic refinement of the central nervous system, and produces a vast range of bioactive metabolites that could influence neurodevelopment.[2, 5–7] Studies of the role of the gut microbiome in neurodevelopment could enhance our understanding of the etiology of neurodevelopmental disorders as well as identify targets for intervention.

Most studies of gut microbiota and ADHD have been relatively small, and cross-sectional, precluding investigation of a temporal relationship. In a study of 19 adolescents and adults with ADHD and 77 healthy controls, ADHD was associated with higher abundance of *Bifidobacterium*.[8] In a cross-sectional study, children with ADHD had lower levels of *Faecalibacterium*, *Dialister* and *Sutterella* than controls.[9] Children with ADHD had relatively lower abundance of *Bacteroides coprocola* and higher abundances of *B. uniformis, B. ovatus* and *Sutterella stercoricanis* in a cross-sectional study of 30 Taiwanese children with ADHD and 30 healthy controls (mean age 8.4 years).[10] Finally, in Chinese children aged 6–12 years (17 cases with ADHD and 17 controls), cases had significantly lower abundance of *Faecalibacterium* and *Veillonellaceae* and significantly higher levels of *Odoribacter* and *Enterococcus.*[11] Less is known, however, about the infant gut microbiome and subsequent risk of childhood ADHD. In a randomized trial of *Lactobacillus rhamnosus* GG supplementation during the first 6 months of life, no supplemented children developed ADHD by age 13 years.[12] In this same trial, *Bifidobacterium* species were lower in children who later developed ADHD.[12]

Given that neurodevelopment and gut microbiome development are rapid during early life, studies of the association of the infant gut microbiota with neurodevelopmental disorders in childhood are needed. This study sought to examine if early-life gut microbiota composition (at 1 month and 6 months of age) is associated with ADHD in preadolescence.

Methods

Study Population

The Wayne County Health, Environment, Allergy and Asthma Longitudinal Study (WHEALS) recruited pregnant women aged 21–49 years, second trimester or later, with due dates from September 2003 through December 2007, who were seeing a practitioner at any of 5 clinics in the Henry Ford Health System, and were living in a predefined

geographic area that included the city of Detroit and surrounding suburban areas to establish a birth cohort.[13, 14] Mothers were interviewed in the clinic during pregnancy and postpartum interviewer-administered questionnaires were completed at child age 1, 6, 12, and 24 months. Research clinic visits were conducted at 2 and 10 years of age. Study protocols were approved by the Henry Ford Health System Institutional Review Board. The mother provided written, informed consent at the prenatal visit, and at the age 10 year visit the guardian (primarily the mother) provided written, informed consent and the child provided written, informed assent.

Definition of ADHD and Neurotypical Development

At the age 10-year visit, the caregiver reported if the child had ever been diagnosed with ADHD, autism spectrum disorder, Asperger's syndrome, or sensory processing disorder. Medical chart abstraction was also completed to obtain these diagnoses. We previously reported near perfect agreement between caregiver-reported and medical record ADHD diagnosis in WHEALS,[15] thus children were considered to have ADHD if either source indicated the child had ADHD. Children were considered neurotypical (NT) if they did not have a caregiver-reported or medical record identified autism spectrum disorder, Asperger's, ADHD, or sensory processing disorder diagnosis.

Stool Specimens

At infant ages 1 and 6 months, stool samples were collected from soiled diapers and stored at −80°C. Detailed information on DNA extraction methods are presented elsewhere.[16]

Polymerase Chain Reaction Conditions and Library Preparation for Sequencing

For bacterial sequencing, the V4 region of the 16S ribosomal RNA gene was amplified using Illumina Nextseq (San Diego, CA).[17] For fungal sequencing, the internal transcribed spacer region (ITS) 2 of the ribosomal RNA gene was amplified using the primer pair fITS7 (5'-GTGARTCATCGAATCTTTG-3') and ITS4 (5'-TCCTCCGCTTATT GATATGC-3') and processed using Illumina MiSeq according to previously published protocols.[18]

Sequence Data Processing and Quality Control

Bacterial paired-end sequences were assembled using FLASH v1.2.7 requiring a minimum base pair overlap of 25bp and demultiplexed by barcode using QIIME (Quantitative Insights Into Microbial Ecology, v1.8). Quality filtering was performed using USEARCH v8.0.1623 to remove reads with > 2 expected errors. Quality reads were dereplicated at 100% sequence identity, clustered at 97% sequence identity into operational taxonomic units (OTUs), filtered of chimeric sequences by UCHIME,[19] and mapped back to resulting OTUs using UPARSE;[20] sequence reads that failed to cluster with a reference sequence were clustered *de novo.* Taxonomy was assigned to the OTUs using the Greengenes database v13_5.[21] Sequences were aligned using PyNAST,[22] and FastTree 2.1.3[23] was used to build a phylogenetic tree. Resulting sequence reads were normalized by multiply rarefying to 60,000 reads per sample as described previously[24] to assure reduced data were representative of the fuller data for each sample. The rarefed OTU table was used for all subsequent analyses.

Fungal sequences were quality trimmed (Q score, < 25) and removed of adaptor sequences using cutadapt.[25] Paired-end sequences were assembled, demultiplexed by barcode, clustered into OTUs at 97% identity and filtered of chimeras using similar methods as described for 16S amplicons. Taxonomy was assigned using UNITE v7.0.[26] Resulting sequence reads were normalized by multiply rarefying to 1,000 reads per sample to assure reduced data were representative of the fuller data for each sample.

A total of 580 children had at least 1 stool sample in the final rarefied OTU table; of these, 323 (56%) had data to classify neurodevelopment. We excluded 9 children with autism spectrum disorder, Asperger's syndrome, or sensory processing disorder. A total of 431 stool samples (229 1-month and 202 6-month) from 314 children had bacterial gut microbiota measured; only 209 of these (107 1-month and 102 6-months) produced an ITS2 amplicon permitting fungal identification.

In the analytical dataset, stool specimens from the 1- and 6-month visits were collected at a mean ± standard deviation of 39 ± 19 days and 205 ± 32 days, respectively. Throughout, "1 month" and "6 months" are used as labels of the intended time period of sample collection.

Covariates

During the prenatal interview, the mother self-reported race, date of birth, marital status, household income, education, parity, smoking history and exposure to environmental tobacco smoke. Maternal medical records were abstracted to obtain maternal height and weight, prescription antibiotic and antifungal use, delivery mode, birth weight, and gestational age at delivery.[27]

Statistical Analysis

Basic parametric (independent samples t-test and chi-square test) and non-parametric (Fisher's exact test and Kruskal-Wallis test) tests were used to compare maternal and child characteristics between children with and without ADHD. Linear regression was used to test for the association of alpha diversity metrics (richness, Pielou's evenness, Faith's phylogenetic diversity [bacterial only], and Shannon's diversity) with ADHD. The association of infant gut microbiota composition with ADHD was evaluated using permutational multivariate analysis of variance (PERMANOVA) with the R package vegan (R Foundation, Vienna, Austria).[28, 29] Unweighted and weighted UniFrac were tested for bacterial composition and Canberra and Bray-Curtis for fungal composition.[30, 31] Because of the small number of children with ADHD and the unbalanced design (large number of NT children), we were concerned about violating the homogeneity of dispersions assumption inherent to PERMANOVA. Homogeneity of dispersion by group was evaluated using the betadisper function in vegan. Differential abundance of OTUs between ADHD and NT children was tested using zero-inflated negative binomial models (pscl package)[32] or negative binomial models (MASS package)[33] in cases of non-convergence or model-fitting issues. Only OTUs detected in 5% of samples were included for testing. P-values were corrected using the false discovery rate;[34] false discovery rate corrected p < 0.05 was considered significant. All models were fit unadjusted and adjusted for a pre-specified set of potential confounders (exact age at stool sample collection, maternal body mass index,

prenatal antifungal use, maternal smoking during pregnancy, child sex, mode of delivery, gestational age at delivery, parity, and breastfeeding status). Sex-specific effects were tested using interaction terms (interaction $p < 0.1$ was considered significant).[35] For all main effect analyses, $p<0.05$ was considered significant.

Results

Among the 314 WHEALS children with gut microbiota and neurodevelopment data, 59 (18.8%) had ADHD. Boys were more likely to develop ADHD (Table 1; $p = 0.001$). Mothers of children who developed ADHD were more likely during pregnancy to have used antifungals ($p = 0.001$), to have exposure to environmental tobacco smoke ($p = 0.048$), to be younger ($p = 0.031$) and to have a larger body mass index ($p = 0.023$) (Table 1).

Early-Life Bacterial and Fungal Alpha Diversity Metrics and ADHD

Bacterial alpha diversity metrics in 1-month samples were not associated with ADHD (Figure 1, Supplemental Table 1; all p 0.57). At 6 months of age, both before and after covariate adjustment, Faith's phylogenetic diversity was higher in children who developed ADHD (Figure 1, Supplemental Table 1; both $p = 0.017$). No other bacterial alpha diversity metrics in 6-month samples were associated with ADHD (Figure 1, Supplemental Table 1; all p 0.17).

Twenty-three (59%) children with ADHD and 84 (44%) NT children had 1-month fungal data (i.e., fungal ITS2 sequences). The fungal amplification rate was not statistically significantly different between the two groups ($p = 0.13$). Similarly, in 6-month stool samples, 23 (61%) children with ADHD had fungal data compared to 79 (48%) NT children ($p = 0.23$). Prior to covariate adjustment, fungal richness was higher ($p = 0.046$) at 6 months of age in children who developed ADHD; after covariate adjustment, this was slightly attenuated and no longer statistically significant (Figure 2, Supplemental Table 1; $p = 0.083$).

The association between all alpha diversity (both bacterial and fungal) metrics and ADHD did not differ by child sex (all interaction p 0.17).

Early-Life Gut Microbiota Compositional Differences and ADHD

Bacterial microbiota composition was associated with development of ADHD (Table 2; Supplemental Figure 1). Bacterial composition at 6 months of age reached statistical significance before covariate adjustment (PERMANOVA; $p = 0.012$ and $p = 0.034$, for unweighted and weighted UniFrac, respectively); after covariate adjustment, only the unweighted UniFrac remained statistically significant (PERMANOVA; $p = 0.006$), explaining 0.9% of the variability. When dispersions of unweighted UniFrac at 6 months were compared between groups, the null hypothesis of homogeneous dispersions was rejected ($p=0.036$), with dispersions being smaller in ADHD relative to NT children (average distance to median=0.38, 0.40, respectively). The association between gut microbiota composition and ADHD did not differ by child sex (all interaction p 0.12).

Specific bacterial and fungal OTUs at 1 and 6 months of age associated with ADHD development after covariate adjustment are presented in Table 3 (unadjusted results are

presented in Supplemental Table 2). At 1 month of age, 18 bacterial OTUs reached statistical significance after covariate adjustment and false discovery rate correction: 6 with lower abundance (including 2 *Lachnospiraceae* OTUs), and 12 with higher abundance in children who developed ADHD (including *Collinsella*, *Akkermansia*, and *Dorea* OTUs). At 6 months of age, 51 bacterial OTUs reached significance; *Enterococcus* and *Ruminococcus* OTUs were relatively depleted in children who developed ADHD, while *Dorea* and *Blautia* OTUs were relatively enriched. At the order level, 14 of these 51 OTUs were *Lactobacillales* (lactic acid bacteria), all but one of which was significantly depleted in children who developed ADHD.

After covariate adjustment, a total of 6 fungal OTUs were associated with ADHD development, 3 at each time point (Table 3; unadjusted results are presented in Supplemental Table 2). Specifically, all three significant fungal OTUs at 1 month (*Rhodotorula mucilaginosa*, *Candida orthopsilosis*, and *Malassezia globose*) were in lower abundance in children who developed ADHD. At 6-months of age, 2 fungal OTUs (1 *Candida* and 1 *Saccharomyces* OTU) were in lower abundance in children who developed ADHD while 1 (*Trichosporon OTU*) was relatively enriched.

Discussion

Our results indicate that the early-life gut microbiota is associated with ADHD at age 10 years. Specifically, children with ADHD had significantly higher phylogenetic diversity and distinct bacterial compositions at 6 months of age, characterized by a relative depletion of lactic acid bacteria, including *Enterococcaceae* and *Lactobacillales*.

Lactic acid bacteria are a diverse group of bacteria characterized by the primary end-product of carbohydrate metabolism being lactic acid[36] and impact health through metabolism regulation, defense against infection, and immune activation.[37] In a mouse model of dextran sulfate sodium-treated mice, administration of *Enterococcus faecalis* 2001 reversed neuroinflammation in the hippocampus and depressive symptoms.[38] Neuroinflammation is a potential mechanistic factor in ADHD.[39] Additionally, some *Lactobacillus* species are capable of producing γ-Aminobutyric Acid (GABA)[40]; lower GABA levels are associated with ADHD.[41] Thus, the relative depletion of lactic acid bacteria in the gastrointestinal tracts of children at 6 months of age who later developed ADHD could be related to neuroinflammatory effects or reductions in GABA; however, this requires further study.

To our knowledge, no other study has examined the early-life gut microbiome with later development of ADHD, making direct comparisons to other studies difficult. Children with ADHD in the current study had significantly higher bacterial phylogenetic diversity (measured as Faith's diversity) than NT children at 6 months of age. Previous studies of cross-sectional associations of gut microbiota alpha diversity metrics and ADHD have been mixed; 3 studies showed no differences in alpha diversity, another showed decreased Shannon diversity, and another study showed higher Shannon diversity and Chao index values, but lower Simpson index values, in children with ADHD compared to controls.[8–11] Diversity in the infant gut increases with age.[42] It is possible, although not part of the current analysis, that the association we found between higher bacterial diversity at 6 months of age

and ADHD in the current study may actually reflect that children with ADHD experienced precocious gut maturation. Of note, in a study of Bangladeshi infants through age 2 years, two *Dorea* species were part of the 24 age-discriminant taxa identified;[43] *Dorea* OTUs at both 1 month and 6 months of age were associated with ADHD development in the current study, and thus may suggest that aberrant gut microbiota maturation in early life may influence later ADHD development. Future studies that specifically investigate early maturation of the gut microbiota with neurodevelopment are needed.

Compositionally, previous cross-sectional studies have implicated *Bacteroides, Bifidobacterium, Dialister, Enterococcus, Faecalibacterium, Odoribacter, Sutterella*, and *Veillonellaceae*, as being in differential abundance comparing those with ADHD to healthy controls.[8–11] In the current study, the early life abundance of *Campylobacteraceae, Enterococcaceae, Eubacteriaceae, Ruminococcaceae*, and *Staphylococcaceae* was associated with ADHD in preadolescence. The inconsistent findings across these studies and our own likely relate to the study design (cross-sectional vs. longitudinal) and the timing of gut microbiota measurement (mean age of stool collection ranged from 8 to 27 years, compared to 1 and 6 months of age in the current study).

Several mechanisms by which the gut microbiome could impact neurodevelopment have been postulated, including upregulation of proinflammatory cytokines, altered mitochondrial function, blood-brain barrier or gut permeability changes, and stimulation of afferent vagal nerves by peptides produced by gut microbiota.[3] The gut microbiome may also influence available nutrients essential for the developing nervous system.[44] Cowan et al suggested there are critical windows of gut microbiome development that correspond with neurodevelopment.[44] In our study, the association of the gut bacterial microbiota with ADHD was more consistent at 6 months of age than 1 month of age, suggesting that exposures in later infancy (e.g., complementary food introduction) may influence or enhance risk of disease. Further study of the gut microbiota over more frequent time points in early life will be needed to identify specific critical windows of development.

A small body of literature in humans supports the role of the early-life microbiome in various aspects of neurodevelopment, including achievement of developmental milestones and variations in brain structure and function. Sordillo et al measured the infant gut microbiome (at 3 to 6 months of age) and the Ages and Stages Questionnaire, 3rd edition, at age 3 years in children participating in the VDAART trial; 4 bacterial co-abundance groups were identified.[45] *Clostridiales*-dominated co-abundance scores were significantly associated with greater odds of communication skills and personal and social skills delays at 3 years of age whereas *Bacteriodetes*-dominated co-abundance scores were associated with greater odds of fine motor skills delays.[45] Children with ADHD have weaker social skills[46] and fine motor control,[47] thus, these findings are consistent with an early influence of the gut microbiota on ADHD risk. In a study of 39 1-year-olds, higher Faith's phylogenetic diversity was statistically significantly associated with functional connectivity in several brain regions.[48] A number of studies have shown altered functional connectivity between various brain regions in those with and without ADHD.[49, 50] In a study of 89 healthy children, gut microbiota was measured at age 1 year and the Mullen Scales of Early Learning was administered along with structural brain magnetic resonance imagining at

ages 1 and 2 years.[51] Higher alpha diversity at age 1 year was associated with an overall lower Mullen cognitive score as well as lower scores on the visual reception and expressive language scales at 2 years of age and with higher brain volumes in the precentral gyrus, left amygdala and right angular gyrus.[51] Children with ADHD have deficits in language, including expressive language.[52] Together, these data support a role for the gut microbiota in altering specific aspects of early development and brain structure and function that may precede diagnosis of ADHD.

Little is known about fungal microbiota and ADHD, with the exception of a case study suggesting that *Candida* infection worsened ADHD symptoms.[53] A challenge in profiling the fungal microbiome is that DNA extraction steps need to be modified due to difficulties with fungal cell lysis.[54] Although successful fungal amplification rates were relatively modest in our study despite making such modifications, we did detect fungal OTU differences in children with and without ADHD. Children with ADHD had a significant depletion of 3 fungal OTUs at 1 month (*R. mucilaginosa*, *C. orthopsilosis*, and *M. globosa*) while at age 6 months 2 fungal OTUs were depleted (a *Candida* and a *Saccharomyces OTU*) and 1 was enriched (*Trichosporon OTU*.). The relative depletion of some *Candida* in the early life gut of children with ADHD was unexpected, given that *Candida* overgrowth, particularly *C. albicans*, is associated with other neurodevelopmental disorders, including autism spectrum disorder.[55, 56] Of note, *Rhodotorula* may strengthen intestinal barrier function and provide a probiotic effect,[57] thus its relative depletion in early life may plausibly influence neurodevelopment. Additional studies are needed to confirm these findings.

Our findings may have implications for prevention of ADHD beginning in very early life. In a randomized trial, Pärtty et al found that among children supplemented with *L. rhamnosus* GG during the first 6 months of life, none developed ADHD by age 13 years.[12] However, there is a paucity of randomized trial data on this topic.[58] Observational study data, such as ours, may be critical to identify potential targets for intervention. In the current study we utilized 16S technology to identify potential compositional differences in the early-life gut microbiota of children who later developed ADHD. This does not provide information on the function of these organisms, which may be the best targets for intervention (e.g. production of short chain fatty acids, metabolism of omega-3 polyunsaturated fatty acids[59]). Future studies that utilize technologies such as metagenomics, metabolomics and/or metatranscriptomics[59, 60] to understand the functional changes in the early-life gut are needed to design potential interventional trials for prevention of ADHD.

Our definition of ADHD was based on medical record or caregiver-reported doctor diagnosis. While we have previously shown high agreement between medical record and caregiver-reported ADHD diagnosis,[15] we do not have data on ADHD subtype (inattentive, hyperactive, or combined), severity, or age at diagnosis, which could be differentially related to microbiota. We were able to adjust for a number of potential confounding factors (e.g., breastfeeding and mode of delivery). Some of these factors, however, may be on the causal pathway, thus models may be over-adjusted.[61] ADHD is heritable,[62] but we do not have data on family history of ADHD. It is possible we are missing an important confounding or effect modifying variable. Because we only have antibiotic information in early life

on a subset of children, we did not include that as a covariate. In a previous publication of factors impacting early life microbiota in WHEALS children[16], we failed to detect an association with infant antibiotic use, which was likely due to the small sample size of children with this covariate. While antibiotic use is known to impact gut microbiota, several studies suggest antibiotic use in early life is not associated with ADHD,[63, 64] thus it is unlikely we missed an important confounder in this analysis. However, future studies with more complete data on both prenatal and early-life antibiotic use should examine these relationships further, including careful investigation by antibiotic timing and class. Although our measurement of the infant gut microbiota at 1 and 6 months of age aligns with the shift from predominantly a facultative anaerobe composition in the first few weeks of life to a strict anaerobe composition at around 6 months of age,[65] even with two early life measurements, we may be missing key time points and inferences about key developmental windows should not be made from our study. However, the current study does provide compelling evidence suggesting the need for future studies with multiple stool sample collection over infancy/early childhood. In their recent review on gut microbiota and ADHD, Checa-Ros et al noted the considerable differences in associations of the gut microbiota and ADHD across studies;[59] we also found some inconsistent findings between our work and previous studies. Previous studies have relied on cross-sectional study designs, and thus alterations in the gut microbiota in those studies could reflect differences due to ADHD treatment, differences in dietary patterns between children with and without ADHD, or reverse causality (changes in the gut microbiota caused by ADHD).[66] We found evidence that the assumption of homogenous dispersions was not met. However, for unbalanced designs, PERMANOVA tests are typically too *conservative* if the larger group has greater dispersion,[67] so despite not meeting the homogeneity assumption, results are unlikely to be a false positive.

A strength of the current study is the longitudinal design, with measurement of the gut microbiota preceding ADHD diagnosis by years, and thus differences in the gut microbiota found in our study are not due to ADHD treatment. As described in Checa-Ros et al, future studies with standardized protocols leveraging high-resolution techniques such as shotgun metagenomics are needed to reduce heterogeneity in results across different studies.[59]

In summary, the early-life gut microbiota is associated with ADHD in preadolescence. Future studies replicating these findings and evaluating potential functional differences are needed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding:

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Impact:

- Cross-sectional studies suggest the gut microbiota of individuals with and without ADHD differ

- We found evidence that the bacterial gut microbiota of infants at ages 1 month and 6 months of age is associated with ADHD at age 10 years

- We also found novel evidence that the fungal gut microbiota in infancy (ages 1 month and 6 months) is associated with ADHD at age 10 years

- This study addresses a gap in the literature in providing longitudinal evidence for an association of the infant gut microbiota with later ADHD development

Figure 1.
Gut bacterial microbiota alpha diversity metrics at 1 and 6 months of age in children who later developed Attention Deficit Hyperactivity Disorder (ADHD) or were neurotypical (NT). Data are mean±standard deviation. Unadjusted (p_{unadj}) and adjusted (p_{adj}) p-values are presented; adjusted analyses included exact age at stool sample collection, maternal prenatal body mass index, prenatal antifungal use, maternal smoking during pregnancy, child sex, mode of delivery, gestational age at delivery, parity, and breastfeeding status.
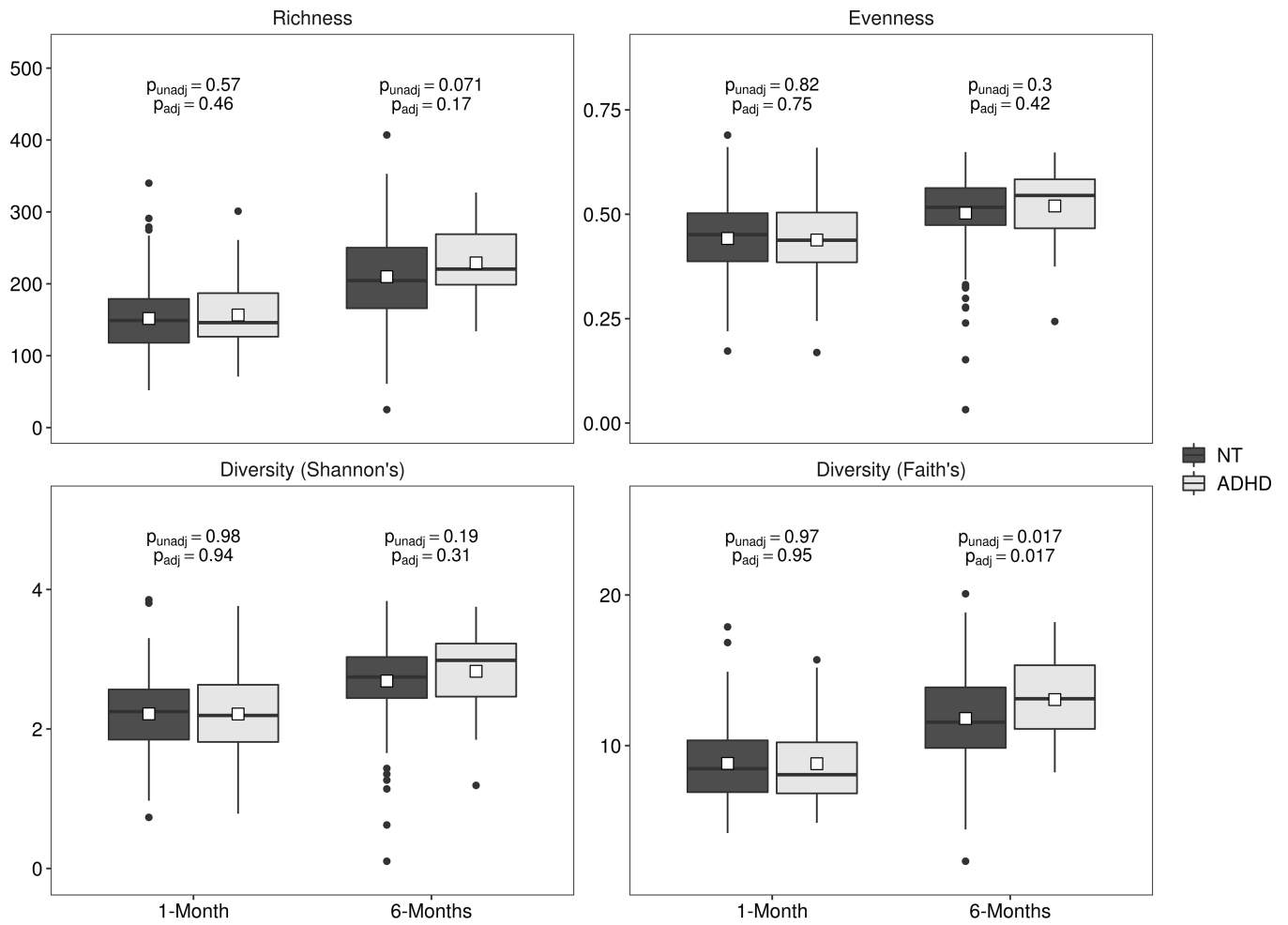
Figure 2.
Gut fungal microbiota alpha diversity metrics at 1 and 6 months of age in children who later developed Attention Deficit Hyperactivity Disorder (ADHD) or were neurotypical (NT). Data are mean±standard deviation. Unadjusted (p_{unadj}) and adjusted (p_{adj}) p-values are presented; adjusted analyses included exact age at stool sample collection, maternal prenatal body mass index, prenatal antifungal use, maternal smoking during pregnancy, child sex, mode of delivery, gestational age at delivery, parity, and breastfeeding status.
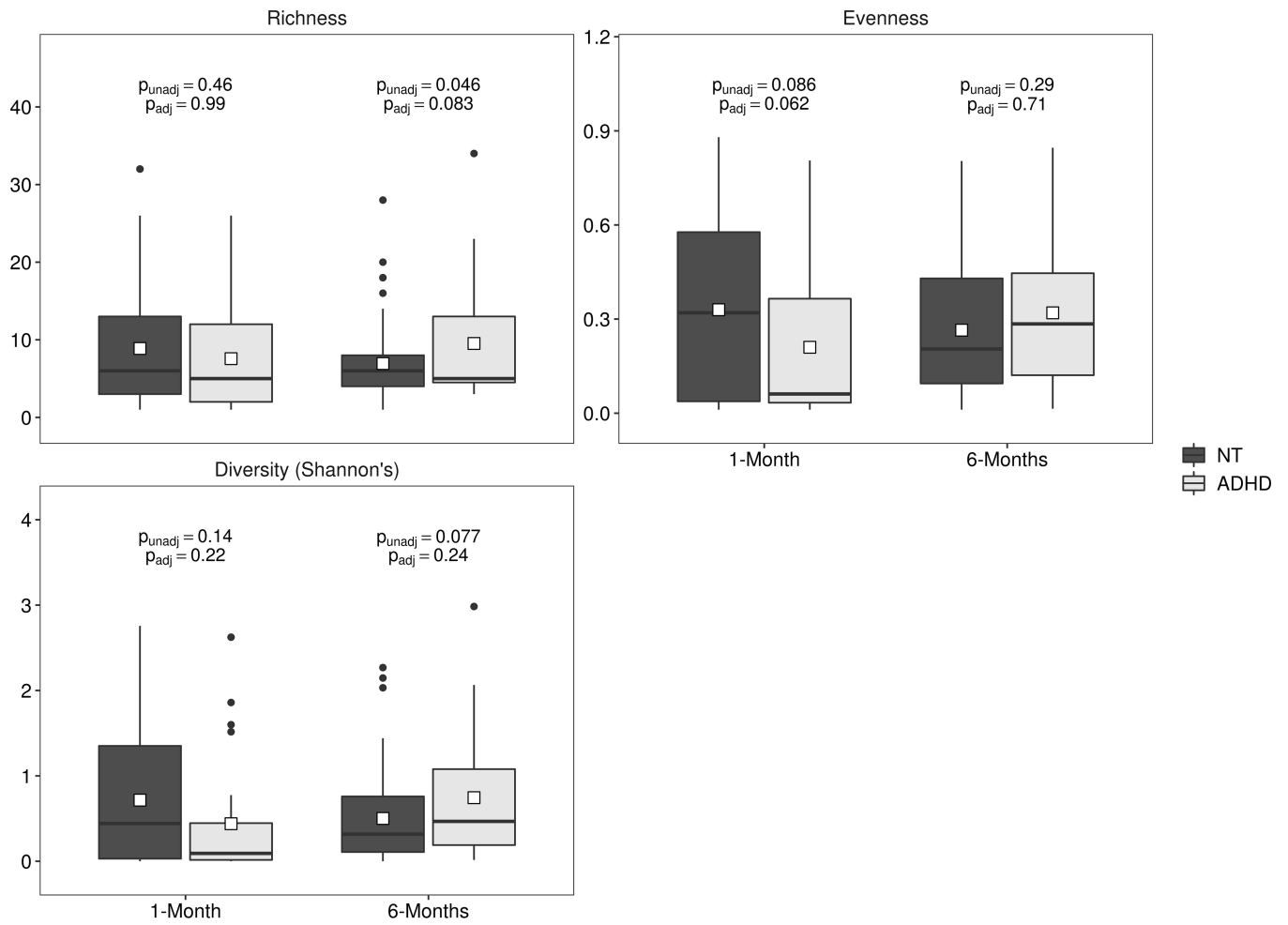
Table 1.

Descriptive characteristics of children with attention deficit hyperactivity disorder (ADHD) or neurotypical (NT) development through age 10 years and early-life gut microbiota measurements.

Covariate	NT (N=255)	ADHD (N=59)	P
Maternal Characteristic	N (%), Mean ± SD, or Median [Q1; Q3]		
Age at delivery (years)	30.6 ± 5.34	28.8 ± 5.64	0.031[a]
Race			0.749[b]
White	65 (25.5%)	13 (22.0%)	
Black	155 (60.8%)	36 (61.0%)	
Mixed/Other	35 (13.7%)	10 (16.9%)	
Marital status			0.113[b]
Unmarried	86 (33.7%)	27 (45.8%)	
Married	169 (66.3%)	32 (54.2%)	
Household income			0.243[c]
<$20,000	26 (10.2%)	11 (18.6%)	
$20,000–<$40,000	54 (21.2%)	13 (22.0%)	
$40,000–<$80,000	58 (22.7%)	12 (20.3%)	
$80,000–<$100,000	36 (14.1%)	9 (15.3%)	
$100,000	44 (17.3%)	4 (6.78%)	
Refused to answer	37 (14.5%)	10 (16.9%)	
Education			0.127[c]
<High school diploma	10 (3.92%)	1 (1.69%)	
High school diploma	36 (14.1%)	11 (18.6%)	
Some college	112 (43.9%)	33 (55.9%)	
Bachelor's degree	97 (38.0%)	14 (23.7%)	
Smoked during pregnancy			0.794[b]
No	235 (92.2%)	54 (91.5%)	
Yes	20 (7.84%)	5 (8.47%)	
Prenatal ETS exposure			0.048[b]
No	202 (79.2%)	39 (66.1%)	
Yes	53 (20.8%)	20 (33.9%)	
Prenatal antibiotic use			1.000[b]
No	101 (41.2%)	23 (41.1%)	
Yes	144 (58.8%)	33 (58.9%)	
Prenatal antifungal use			0.001[b]
No	203 (82.9%)	35 (62.5%)	
Yes	42 (17.1%)	21 (37.5%)	
BMI from first prenatal visit (kg/m^2)[d]	30.2 ± 7.59 (N=253)	33.0 ± 8.67 (N=58)	0.023[a]
Prenatal indoor dog(s)			0.864[b]

Covariate	NT (N=255)	ADHD (N=59)	P
No	187 (73.3%)	42 (71.2%)	
Yes	68 (26.7%)	17 (28.8%)	
Prenatal indoor cat(s)			0.805[b]
No	210 (82.4%)	50 (84.7%)	
Yes	45 (17.6%)	9 (15.3%)	
Child Characteristic			
Mode of delivery			0.370[b]
Vaginal	169 (66.5%)	35 (59.3%)	
C-section	85 (33.5%)	24 (40.7%)	
Firstborn			0.139[b]
No	159 (62.4%)	30 (50.8%)	
Yes	96 (37.6%)	29 (49.2%)	
Gestational age at delivery (weeks)[d]	39.0 [38.0; 40.0] (N=254)	39.0 [38.0; 40.0] (N=58)	0.238[e]
Preterm birth (gestational age <37 weeks)			0.780[b]
No	236 (92.9%)	53 (91.4%)	
Yes	18 (7.09%)	5 (8.62%)	
Birthweight (g)[d]	3388 ± 607 (N=243)	3308 ± 613 (N=52)	0.391[a]
Low birth weight (<2,500 g)			0.776[c]
No	224 (92.2%)	49 (94.2%)	
Yes	19 (7.82%)	3 (5.77%)	
Child sex			0.001[b]
Male	126 (49.4%)	44 (74.6%)	
Female	129 (50.6%)	15 (25.4%)	
Breastfeeding status at 1-month			0.629[b]
Formula feeding	54 (21.3%)	12 (21.1%)	
Mixed feeding	156 (61.4%)	38 (66.7%)	
Breastfeeding	44 (17.3%)	7 (12.3%)	
Breastfeeding status at 6-months			0.971[c]
Formula feeding	54 (21.3%)	12 (21.1%)	
Mixed feeding	178 (70.1%)	41 (71.9%)	
Breastfeeding	22 (8.66%)	4 (7.02%)	
Exact age at 1-month stool sample collection (months)[d]	1.09 [0.89; 1.45] (N=190)	1.12 [0.86; 1.56] (N=39)	0.666[e]
Exact age at 6-month stool sample collection (months)[d]	6.32 [5.99; 7.11] (N=164)	6.68 [6.00; 7.33] (N=38)	0.498[e]
Child age at 10-year visit (years)[d]	9.96 ± 0.75 (N=255)	9.97 ± 0.74 (N=50)	0.940[a]

BMI, body mass index; ETS, environmental tobacco smoke; Q1, 1st quartile; Q3, 3rd quartile; SD, standard deviation.

[a] Calculated by independent samples t-test.

[b] Calculated by the chi-square test.

[c]Calculated by Fisher's exact test.

[d]Continuous covariate with missing data; sample size is presented.

[e]Calculated by the Kruskal-Wallis test.

Table 2.

Difference in stool beta diversity comparing children with ADHD to NT children.

Time	Metric	Unadjusted			Adjusted[a]		
		N	p-value	R^2	N	p-value	R^2
Bacterial							
1-month	Unweighted UniFrac	229	0.50	0.004	216	0.27	0.005
	Weighted UniFrac	229	0.79	0.002	216	0.59	0.003
6-months	Unweighted UniFrac	202	0.012	0.009	190	0.006	0.009
	Weighted UniFrac	202	0.034	0.012	190	0.057	0.012
Fungal							
1-month	Canberra	107	0.76	0.008	102	0.76	0.008
	Bray-Curtis	107	0.61	0.007	102	0.67	0.007
6-months	Canberra	102	0.22	0.011	96	0.36	0.011
	Bray-Curtis	102	0.57	0.007	96	0.53	0.008

ADHD, attention deficit hyperactivity disorder; NT, neurotypical.

[a] Adjusted for exact age at stool sample collection, maternal body mass index, prenatal antifungal use, maternal smoking during pregnancy, child sex, mode of delivery, gestational age at delivery, parity, and breastfeeding status.

Table 3:

Bacterial and fungal OTUs significantly associated with ADHD by age at time of stool sample.[a]

OTU	Taxonomy[b]	Estimate[c]	p-value	FDR corrected p-value
1-month bacterial				
OTU_1685	*Dorea*	−16.4	4.10E-06	5.24E-04
OTU_68	*Pseudoramibacter_Eubacterium.*	−11.9	1.17E-10	5.95E-08
OTU_104	*Lachnospiraceae*	−9.7	7.12E-04	2.27E-02
OTU_806	*Lachnospiraceae*	−7.6	4.33E-07	1.11E-04
OTU_241	*Prevotella*	−5.7	1.10E-05	6.04E-04
OTU_152	*Veillonella*	−4.8	9.37E-07	1.60E-04
OTU_1347	*Megasphaera*	1.5	1.18E-05	6.04E-04
OTU_130	*Clostridiaceae*	3.1	7.61E-04	2.29E-02
OTU_45	*Lactococcus*	3.5	1.90E-04	6.93E-03
OTU_1329	*Bacteroides*	3.5	1.50E-03	4.25E-02
OTU_138	*Streptococcus*	3.7	2.81E-04	9.58E-03
OTU_523	*Dorea*	4.2	1.16E-05	6.04E-04
OTU_949	*Erysipelotrichaceae*	4.3	1.87E-04	6.93E-03
OTU_509	*Dorea*	5.3	8.98E-06	6.04E-04
OTU_391	*Akkermansia muciniphila*	6.8	3.69E-05	1.71E-03
OTU_292	*Corynebacterium kroppenstedtii*	7.5	6.99E-06	5.98E-04
OTU_242	*Comamonadaceae*	9.9	1.53E-04	6.50E-03
OTU_1492	*Collinsella aerofaciens*	13.8	7.02E-06	5.98E-04
6-months bacterial				
OTU_57	*Lachnospiraceae*	−8.8	2.51E-05	1.02E-03
OTU_144	*Ruminococcus*	−7.4	2.66E-04	6.33E-03
OTU_1430	*Bacteroides*	−5.7	1.88E-04	5.76E-03
OTU_318	*Lachnospiraceae*	−5.3	1.69E-03	2.62E-02
OTU_533	*Lachnospiraceae*	−4.9	4.02E-11	4.55E-09
OTU_1563	*Enterococcus*	−3.7	4.69E-07	2.90E-05
OTU_1443	*Streptococcus*	−3.5	2.06E-03	3.05E-02
OTU_636	*Lachnospiraceae*	−3.3	8.88E-04	1.66E-02
OTU_128	*Ruminococcus gnavus*	−2.2	8.19E-07	4.64E-05
OTU_986	*Streptococcus*	−2.2	2.29E-04	6.08E-03
OTU_1134	*Enterococcus*	−2.2	2.28E-03	3.30E-02
OTU_437	*Enterococcaceae*	−2.1	1.26E-05	6.12E-04
OTU_360	*Peptostreptococcaceae*	−2.1	2.70E-04	6.33E-03
OTU_1604	*Enterococcaceae*	−2.1	2.40E-03	3.33E-02
OTU_1659	*Enterococcus*	−2.1	2.51E-03	3.35E-02
OTU_1278	*Enterococcus*	−2	1.69E-03	2.62E-02
OTU_923	*Enterococcus*	−1.9	2.04E-04	5.78E-03

OTU	Taxonomy[b]	Estimate[c]	p-value	FDR corrected p-value
OTU_772	*Enterococcus*	−1.9	2.64E-04	6.33E-03
OTU_1238	*Lactobacillales*	−1.9	1.56E-03	2.55E-02
OTU_157	*Clostridiales*	−1.8	2.32E-05	1.02E-03
OTU_1149	*Enterobacteriaceae*	−1.7	9.05E-04	1.66E-02
OTU_395	*Bifidobacterium*	−1.7	2.47E-03	3.35E-02
OTU_37	*Staphylococcus*	−1.6	6.97E-04	1.39E-02
OTU_41	*Ruminococcus*	−1.5	3.95E-04	8.95E-03
OTU_1161	*Enterococcaceae*	−1.5	1.45E-03	2.47E-02
OTU_950	*Ruminococcus gnavus*	−1.5	2.65E-03	3.47E-02
OTU_208	*Erysipelotrichaceae*	−1.4	2.01E-03	3.03E-02
OTU_9	*Enterococcus*	−1.3	7.21E-04	1.40E-02
OTU_77	*Coprobacillus*	−1.2	1.57E-03	2.55E-02
OTU_1067	*Clostridiales*	1.1	1.16E-03	2.06E-02
OTU_1540	*Enterobacteriaceae*	1.3	5.35E-04	1.10E-02
OTU_810	*Serratia marcescens*	1.6	4.78E-06	2.50E-04
OTU_756	*Dorea*	1.7	3.06E-03	3.78E-02
OTU_781	*Streptococcus*	1.9	5.09E-04	1.08E-02
OTU_883	*Blautia*	2.4	4.28E-04	9.39E-03
OTU_1343	*Peptostreptococcaceae*	2.7	1.64E-09	1.60E-07
OTU_1411	*Clostridiales*	3.5	3.81E-15	5.18E-13
OTU_1089	*Clostridiales*	3.6	2.76E-03	3.55E-02
OTU_791	*Lachnospiraceae*	3.8	1.52E-04	4.92E-03
OTU_167	*Lachnospiraceae*	3.8	1.95E-04	5.76E-03
OTU_1587	*Lachnospiraceae*	3.8	2.35E-03	3.32E-02
OTU_356	*Lachnospiraceae*	4	2.47E-07	1.68E-05
OTU_520	*Ruminococcus*	4	2.88E-03	3.63E-02
OTU_1259	*Ruminococcaceae*	4.9	2.54E-05	1.02E-03
OTU_429	*Peptoniphilus*	5.3	9.09E-05	3.09E-03
OTU_189	*Lachnospiraceae*	5.5	8.08E-05	2.89E-03
OTU_1490	*Dialister*	5.7	1.18E-03	2.06E-02
OTU_410	*Ruminococcaceae*	5.8	3.10E-15	5.18E-13
OTU_76	*Erysipelotrichaceae*	6.4	4.00E-29	1.36E-26
OTU_82	*Dorea*	8.4	5.58E-05	2.11E-03
OTU_47	*Bacteroides*	10.7	2.33E-04	6.08E-03

1-month fungal

OTU	Taxonomy	Estimate	p-value	FDR corrected p-value
OTU_7	*Rhodotorula mucilaginosa*	−4.4	4.81E-04	1.25E-02
OTU_411	*Candida orthopsilosis*	−8.8	1.13E-03	1.47E-02
OTU_13	*Malassezia globosa*	−1.9	2.05E-03	1.78E-02

6-months fungal

OTU	Taxonomy[b]	Estimate[c]	p-value	FDR corrected p-value
OTU_1093	*Candida*	−3.5	1.93E-05	3.09E-04
OTU_1375	*Saccharomyces*	−3.1	1.87E-04	1.50E-03
OTU_9	*Trichosporon*	11.0	6.38E-03	3.40E-02

ADHD, attention deficit hyperactivity disorder; FDR, false discovery rate; OTU, operational taxonomic units.

[a]No overlapping OTUs in 1- and 6-month samples.

[b]Most specific taxonomic rank available is noted.

[c]Estimate from zero-inflated negative binomial or standard negative binomial model, after adjusting for exact age at stool sample collection, maternal body mass index, prenatal antifungal use, maternal smoking during pregnancy, child sex, mode of delivery, gestational age at delivery, parity, and breastfeeding status. Interpreted as the difference in the log OTU abundance, comparing children with ADHD to neurotypical children (i.e., positive values imply higher abundance in children with ADHD, while negative values imply lower abundance in children with ADHD).