



Dietary Habits of 2- to 9-Year-Old American Children Are Associated with Gut Microbiome Composition

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ABSTRACT

Background The human gut microbiome is recognized as an important determinant of human health, yet little is known about how dietary habits are related to the microbiome in post-weaned, pre-pubescent children.

Objective The goal of this work was to link quantitative dietary intake with microbiome features in a diverse population of children consuming a predominantly Western diet.

Design This was a cross-sectional study.

Participants/settings English- or Spanish-speaking families with healthy children between the ages of 2 and 9 years were recruited from a community-based, early childhood learning center in suburban Los Angeles, California between June and September 2014.

Main outcome measures Children included in the analyses ($n=75$) contributed three fecal samples and three quantitative 24-hour dietary recalls using the multiple-pass method with an average of 5.7 days between samples. Microbial communities of each fecal sample were characterized using Illumina sequencing of the 16S ribosomal RNA gene. Dietary recalls were analyzed using the Automated Self-Administered 24-Hour Recall Dietary Assessment Tool.

Statistical analysis performed Associations between dietary factors and microbiome features were assessed using the Kruskal-Wallis test, Spearman rank correlations, or permutational multivariate analysis of variance. For demographic and health-related variables, χ^2 analyses were used to test for differences between age groups for categorical variables.

Results Our results show that age is correlated with three metrics of microbiome diversity ($P<0.05$) and is associated with both community structure ($P=0.0488$) and membership ($P=0.0002$). Several dietary food groups and nutrients were likewise associated with microbiome features. For example, consumption of non-whole-grain foods was associated with community structure ($P=0.0089$) and membership ($P=0.0057$), but not diversity ($P>0.05$). Likewise, the relative abundance of several bacterial taxa were linked to consumption of particular food groups and/or nutrients, as illustrated by the positive associations between total fruit ($P_{\text{false discovery rate}}<0.05$) and fiber ($P_{\text{false discovery rate}}<0.05$) consumption with the relative abundance of the *Lachnospira* genera.

Conclusions This hypothesis-generating study demonstrates that the composition of the child gut microbiome remains dynamic beyond the age of 3 years and responds to dietary differences across individuals. In particular, non-whole-grain foods fortified with vitamins and minerals appear to be associated with the composition of the microbiome. Future interventional or model organism-based studies will be needed to test these associations between diet and microbiome composition.

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THE HUMAN GUT HARBORS TRILLIONS OF MICROBIAL cells that encode a gene set 150 times larger than their human hosts.¹ Collectively known as the human gut microbiota, these microorganisms play a key role

in promoting and maintaining human health by performing essential functions throughout one's life. For example, the gut microbiota helps develop the immune system, aids in energy harvest from food, synthesizes vitamins, and protects from

invading pathogens.^{2,3} Despite these essential functions, the taxonomic composition⁴ and stability⁵ of each individual's microbiome is distinct. The individualized nature is related to a number of factors,⁶ but diet in particular has been suggested as one of the most important determinants of gut microbiome composition.⁷

The human gut microbiome is largely founded at birth,⁸ with delivery mode determining which microbes colonize first.⁹ After this initial colonization, development of the gut microbiome continues into infancy and is heavily affected by diet. For example, breastfed infants host different gut microbiomes than formula-fed infants.¹⁰ Once solid foods are introduced, the infant microbiome undergoes an abrupt shift in community composition, which ultimately leads to an adult-like community by about the age of 3 years.¹¹⁻¹⁵ Beyond this life stage and before adulthood, comparably little is known about how diet is related to the microbiome. Recently, Berding and colleagues¹⁶ were able to link broad-scale dietary patterns to gut microbiome composition and stability in a population of 4- to 8-year old American children. They found that children could be separated into two broad-scale dietary groups largely determined by consumption of fish, refined carbohydrates, vegetables, and convenience foods like juice and snacks. These dietary groups were associated with differences in microbiome composition, highlighted by differential abundance of key bacterial genera like the *Bacteroides*, *Prevotella*, and *Bifidobacterium*. In adulthood, both short-¹⁷ and long-term^{18,19} dietary habits are known to influence diversity and composition of the gut microbiome. Clearly, dietary habits are an important factor structuring the gut microbiome throughout life.

During the last 50 years, global dietary patterns have shifted due to a rapid transition to a "Western diet." A Western diet can be defined broadly by an increased consumption of refined re-fortified carbohydrates, high-fat animal products, and highly processed foods.²⁰ This shift in diet has been connected to the current and expanding obesity epidemic, with >65% of American adults being overweight or obese,²¹ resulting in an increase in heart disease,²² type 2 diabetes,²³ and multiple gastrointestinal diseases.²⁴ Dysbiosis of the human gut microbiome has been connected to all of the aforementioned diseases,²⁵⁻²⁷ suggesting that this dietary change has a detrimental effect on the microbiome–host relationship. Although it is unclear whether the dysbiosis is the cause or consequence of the disease state, dysbiosis effectively changes the composition of the gut microbiota, having potentially detrimental effects on human physiology.²⁸ The gut microbiome has also been suggested to play a role in these inflammatory diseases through the production of pro-inflammatory compounds that leak from the gut, causing chronic low-grade inflammation.^{29,30} Consumption of a Western diet has been shown to result in a less diverse gut microbiome when compared to a more traditional rural diet.^{13,31}

Seeing that diet is an important factor modulating the composition of the gut microbiota in infants and adults, a comprehensive understanding of these interactions across all life stages in healthy populations is needed. The purpose of this study was to examine the relationship between diet and gut microbiome composition of post-weaned, pre-pubescent American children aged 2 to 9 years. The primary objective was to link quantitative

RESEARCH SNAPSHOT

Research Question: Is diet associated with the diversity and composition of the gut microbiome in a diverse population of American children?

Key Findings: In this cross-sectional study that included 75 children between the ages of 2 and 9 years, several food groups and nutrients were linked to differences in gut microbiome composition. For example, consumption of non-whole-grain foods and some B vitamins were significantly linked to community structure and membership. A variety of bacterial taxa were also significantly correlated with consumption of particular food groups or nutrients.

dietary intake data with microbiome diversity, composition, and taxonomy to generate hypotheses for future intervention or model organism-based studies.

METHODS

Ethics

The Institutional Review Board at California State University, Northridge approved the study protocol (#1314-223). All adult participants provided written informed consent for their children to participate in the study and children provided verbal assent.

Data Availability

Raw sequence data, accompanying metadata, and all supplementary data are available online through figshare.³² Sequence data are also available through Qiita^{33,34} (QIITA: 12360).

Recruitment

Participants were recruited in the summer of 2014 at an early childhood education center in Canoga Park, CA, a suburb north of Los Angeles. Eligibility criteria for the study were the following: English- or Spanish-speaking parents, families with a child between the ages of 2 and 9 years, weaned from breastfeeding, and no known communicable diseases at the time of recruitment. Families were also excluded if in the 3 months before the screening interview, children had taken antibiotics, had excessive vomiting or diarrhea, or had blood or mucus in their stool. A brief survey collecting demographic and health information (ie, age, height, weight, and ethnicity) was administered after signed consent (<https://doi.org/10.6084/m9.figshare.7011272.v1>). Participants were asked to provide three fecal samples and accompanying dietary information (see below) within a 2-month time window. Upon completion of data collection, volunteers were given a \$20 gift card as compensation for their participation.

Microbiome Sampling, DNA Extraction, Polymerase Chain Reaction, and Sequencing

Fecal samples were collected by parents in the privacy of their homes using sterile, double-tipped swabs by swabbing toilet paper or diapers after use. This protocol is minimally invasive and has been used successfully in other

community-based research projects.^{4,5,35} Although feces may not provide the best representation of the spatial heterogeneity of the gut microbiome, it is the best and only option we have to study these communities in healthy individuals. Nucleic acids (DNA) were extracted directly from swabs using the MoBio PowerSoil DNA isolation kit following the manufacturer's protocol, with slight modifications that allow for robust yields.³⁶ Polymerase chain reaction (PCR) amplification of the variable region 4 of bacterial and archaeal 16S ribosomal RNA genes was performed using the barcoded primer set (515F/806R), PCR mixture conditions, and thermal cycling steps described previously.³⁷ PCR amplicons of triplicate reactions for each sample were quantified and pooled at approximately equal amounts, cleaned using a single-tube MoBio Ultraclean PCR Clean-up Kit (MoBio Laboratories), and sequenced using an Illumina MiSeq (v2, 2 × 150 bp) instrument.

Sequence Data Processing

Raw fastq files of 16S ribosomal RNA genes were processed using the UPARSE pipeline³⁸ with a few modifications. Briefly, a custom Python script was used to demultiplex and prepare sequence files for paired-end assembly and clustering. Paired sequences were assembled using UPARSE with the following parameters: `fastq_truncqual 3`, `fastq_maxdiffs 1`, `fastq_minovlen 20`, `fastq_minmergegelen 200`. Assembled sequences were filtered at a maxee value of 0.5, which means that, on average, only one nucleotide in every two sequences is potentially incorrect. Quality sequences were then dereplicated and singletons were removed. Operational taxonomic units (OTUs) were assigned with UPARSE at a 97% sequence identity threshold. Taxonomy was assigned to representative sequences of each OTU using the RDP classifier³⁹ with a confidence threshold of 0.5 against the Greengenes 13.8 database^{40,41} as implemented in QIIME, version 1.9.0.⁴² QIIME was also used to generate phylogenetic trees using FastTree⁴³ and to calculate various α -diversity (richness, phylogenetic diversity,⁴⁴ and the Shannon Diversity Index) and β -diversity (UniFrac⁴⁵) metrics. The two β -diversity metrics used, weighted and unweighted UniFrac, provide different insights into microbiome community composition. The unweighted UniFrac metric accounts only for the presence and absence of OTUs in each pair of samples and is considered to measure community membership. In contrast, the weighted UniFrac metric also accounts for the relative abundance of each OTU and is considered to measure community structure.

After quality filtering, a total of 3,810,822 sequences were generated across 349 samples. After rarefaction ($n=3,772$ sequences/sample), α -diversity metrics were calculated and averaged across the three samples per individual. To determine the average composition (β -diversity) and taxonomy across the three time points, we used the `collapse_samples.py` command in QIIME with the `-collapse_mode` option set to "mean" after rarefaction.⁴² β -diversity metrics and taxonomic summaries were generated using the collapsed OTU table.

Dietary Assessment

Children's dietary information and supplement intake was collected using a paper instrument with parents as proxies for children's intakes. Parents were considered the best

proxies for these young children, as they were with them during the day and were the ones to feed them and observe their eating habits. To assist with recall, parents were first asked to record a food list at home for the 24-hour recall period that was assessed in person at the early childhood learning center. The food list was used as a memory prompt for parents when collecting the actual 24-hour recall in person, which was interviewer-administered. A total of three, non-consecutive, quantitative, 24-hour recalls using the multiple-pass method were collected from each participant, one time weekly and included both weekdays and 1 weekend day. Twenty-four-hour dietary recalls were chosen to assess recent dietary intake because they have been shown to be a valid measure for this age group when compared to measures using doubly-labeled water.⁴⁶⁻⁴⁸ At the end of the 24-hour recall, a question was posed asking whether the child was taking any supplements and to indicate which one(s). The 24-hour recalls and supplement data were manually entered in the Automated Self-Administered 24-Hour Recall (ASA24) Dietary Assessment Tool, version 2016,⁴⁹ an electronic data collection and dietary analysis program. The ASA24 employs research-based strategies to enhance dietary recall using a respondent-driven approach allowing initial recall to be self-defined; association with the day's events; probes for frequently forgotten foods; repetition with minimal burden; reviews 24-hour day; and placement of foods with eating occasions. These steps were emulated in the interviewer-administered version of the 24-hour dietary recall with parents and the question regarding supplement use was reviewed at that time with the parent respondent. Individual-level nutrient and food group estimates from the ASA24 were compared to Dietary Reference Intakes as well as food group recommendations based on the 2015-2020 Dietary Guidelines for Americans.⁵⁰ Macronutrients were compared to the Acceptable Macronutrient Distribution Ranges (AMDRs) by age, and macro- (where appropriate) and micronutrients were compared to the Estimated Average Requirements (EARs) by age. Food group data were provided by the MyPyramid Equivalents database (version 2)⁵¹ and the Center for Nutrition Policy and Promotion's fruit database (03-04 version)⁵² and were calculated by the ASA24 program as described in the ASA24 researcher instructions guide.⁴⁹ Food groups calculated included grains in ounce equivalents (total, whole, and non-whole/refined), vegetables in cup equivalents (total of all groups, dark green, orange, white potatoes, other starchy vegetables, tomatoes and other starchy vegetables), fruits in cup equivalents (total of all groups, citrus fruits melons and berries, other, and whole), milk in cup equivalents (total of all groups, milk, yogurt, cheese), meat and beans in ounce equivalents (meat, poultry and fish together, meat, organ meats, frankfurters, sausage and luncheon meats, poultry, fish and shellfish high in n-3 fatty acids, fish and shellfish low in n-3 fatty acids, eggs, cooked dried peas and beans, soybean products [tofu and meat analogs]), and nuts and seeds. Disaggregated dietary data averaged across the three time points for healthy children ($n=75$) are available through figshare.³²

Statistical Analysis

The non-parametric Kruskal-Wallis test was used to determine whether the sex of a child was related to overall microbiome

diversity. Spearman rank correlations were used to investigate relationships between microbiome diversity and taxonomy with age and dietary features using the “corr.test” function in the R⁵³ package *psych*.⁵⁴ Correlation heatmaps were plotted using the *corrplot*⁵⁵ package, also in R. To determine whether dietary features are associated with community membership (unweighted UniFrac) or structure (weighted UniFrac), the “adonis” function (ie, permutational multivariate analysis of variance) with 9,999 permutations from the R *vegan*⁵⁶ package was used. Also in *vegan*, the “envfit” function was run with 9,999 permutations to find correlations between dietary features and gut microbiome composition in ordination space. Dietary variables with *P* values <0.05 were overlaid as directional vectors on non-metric multidimensional scaling plots to visualize correlations. Because of the exploratory nature of the study and nested structure of many dietary components (eg, whole grains are part of total grains), uncorrected and corrected (Benjamini and Hochberg false discovery rate correction⁵⁷) *P* values are presented when appropriate. For demographic and dietary variables, χ^2 analyses were used to test for differences between age groups for categorical variables. Percentages of recommended intakes by age are provided for the Dietary Reference Intakes,⁵⁸ AMDRs,⁵⁹ EARs, and Adequate Intakes and supplement use.⁵⁸

Sample Selection

In total, 130 individuals provided consent and at least one fecal sample and 24-hour dietary recall. Of these, 94 provided all three fecal samples and 24-hour dietary recalls. Because the focus of this study was to examine how dietary factors are associated with the gut microbiome in healthy children, the study population was filtered further to exclude subjects that self-reported conditions previously found to be associated with microbiome dysbiosis. Exclusion criteria included asthma; eczema; colitis; autism spectrum disorder, including pervasive developmental disorder not otherwise specified; and epilepsy. The remaining 75 children were included in the analyses presented here. The research protocol was to obtain microbiome samples and dietary data once weekly. However, the number of days between data collection varied due to participant convenience. As a result, the number of days between sample collections ranged from 1 to 42, with a mean of 5.7 (median=4) days.

RESULTS

Demographics and Dietary Intake

Table 1 shows the demographic characteristics of the analytical sample by age group. The majority of study participants were female (60%), of Hispanic ethnicity (50.7%), had family income <\$20,000/year (30.7%), participated in the Special Supplemental Nutrition Program for Women, Infants, and Children (88.9%), had a normal weight according to their body mass index percentile (62.7%), and had been breastfed (84%). The only difference between the age groups (2 to 3 years vs 4 to 9 years) in terms of their sociodemographic factors was that more children 4 to 9 years were in the lowest income category compared to those who were 2 to 3 years of age.

Table 2 shows the comparison of average dietary intake, including intake of dietary supplements by age group and the Dietary Reference Intakes, specifically the percent above and below the EARs.⁵⁸ All dietary data were assessed with respect

to meeting participants' age-specific requirements. Study participants reported the lowest intakes compared to EARs/Adequate Intakes for fiber, calcium, potassium, choline, and the fat-soluble vitamins, D, E, and K, with smaller differences for magnesium, phosphorus, and vitamins A and B-12.⁵⁸ Participants also reported dietary intakes in excess of the EARs/Adequate Intakes for all but two of the nutrients analyzed.⁵⁸ Excesses greater than two times the EARs were reported for >90% of study participants for: protein, iron, selenium, riboflavin, pyridoxine, and vitamin B-12. Macronutrient intakes were also compared to Dietary Reference Intakes using the AMDRs⁵⁹ by age group (data not shown). AMDRs were exceeded by 2.6% of study participants for protein, 6.7% for carbohydrates, 5.3% for total fat, and 82.7% for saturated fat. Intakes lower than AMDRs were reported for 28% of study participants for total fat.

A total of 37.3% of participants (n=28) reported taking some type of dietary supplement. These included multivitamin/multimineral supplements (60.7%), calcium (3.6%), n-3s (3.6%), zinc (3.6%), minerals alone (3.6%), and Pediasure (3.6%). Two participants reported taking supplements but did not specify which type (7.1%). One participant reported taking a probiotic supplement. Data for the relationship with the microbiome were analyzed with and without this individual. Because no differences were identified with this participant included, they were not removed from these analyses.

Microbiome

α -Diversity. With all three metrics tested (Shannon Index, phylogenetic diversity, and richness), diversity was positively associated with age, meaning that older children tended to have more diverse microbiomes than younger children (Table 3). In contrast, sex was not significantly associated with diversity in this population of children. To investigate which dietary components were associated with diversity of the child gut microbiome, dietary data were disaggregated and split into food groups (fruits, vegetables, grains, dairy, and proteins) and micro- and macronutrients. Protein consumption, split into plant and animal-based proteins, were the only food groups correlated with any of the α -diversity (Shannon Index, phylogenetic diversity, and richness) metrics (Table 3). When food groups were further split into subcategories, consumption of meat, poultry, and yogurt showed significant relationships with two of the three α -diversity metrics. With the exception of selenium ($P=0.014$) and niacin ($P=0.037$), which were correlated with the Shannon Index, macronutrients and micronutrients were not related to microbiome α -diversity (Table 4).

β -Diversity. Similar to what was observed with α -diversity, age was strongly associated with microbiome β -diversity, while sex was not (Table 3). Food groups that were significantly associated with microbiome structure (weighted UniFrac) included total fruit, total grain, and plant protein consumption (Table 3). In addition, total grain and total vegetable consumption were significantly associated with community membership (unweighted UniFrac). For both community structure and membership, consumption of non-whole grains was strongly associated with the variation observed in the grain food group. Tomato consumption was also strongly related to the variation with both β -diversity

Table 1. Demographics, program participation, and health information of 75 children recruited from a community-based, early childhood learning center in suburban Los Angeles, CA, by age group^a

Characteristic	Age Group		χ^2	P value
	2-3 y n (%) (n=30)	4-9 y n (%) (n=45)		
Sex			0.23	0.63
Female	17 (56.7)	28 (62.2)		
Male	13 (43.3)	17 (37.8)		
Race/ethnicity			5.93	0.20
White	10 (33.3)	5 (11.1)		
African American	1 (3.3)	1 (2.2)		
Hispanic	12 (40)	26 (57.8)		
Asian or Pacific Islander	2 (6.7)	3 (6.7)		
Other ^b	5 (16.7)	10 (22.2)		
Annual household income			11.72	0.008
<\$20,000	4 (16)	19 (45.2)		
\$20,000 to \$29,000	3 (12)	9 (21.4)		
\$30,000 to \$59,000	7 (28)	9 (21.4)		
≥\$60,000	11 (44)	5 (11.9)		
WIC^c			3.57	0.06
Yes	12 (40)	28 (62.2)		
No	18 (60)	17 (37.8)		
SNAP^d			0.26	0.61
Yes	4 (13.3)	8 (17.8)		
No	26 (86.7)	37 (82.2)		
BMI, ^e percentile^f			3.06	0.383
Underweight	1 (3.3)	4 (8.9)		
Normal weight	20 (66.7)	27 (60)		
Overweight	3 (10)	9 (20)		
Obese	6 (20)	5, 11.1		
Ever breastfed, n (%)			0.17	0.898
Yes	25 (83.3)	38 (84.4)		
No	5 (16.7)	7 (15.6)		

^aResults of Pearson's χ^2 test comparing various categorical variables across age groups are also presented.

^bOther race/ethnicity includes Armenian, East Indian, Spanish, and mixed race.

^cWIC=Special Supplemental Nutrition Program for Women, Infants and Children.

^dSNAP=Supplemental Nutrition Assistance Program.

^eBMI=body mass index; calculated as kg/m².

^fBMI percentiles were calculated using the Centers for Disease Control and Prevention growth chart data and calculation tools.⁶⁰

metrics. Yogurt, soy, and nut/seed consumption was associated with variation for community membership only. Using the “envfit” function in the R package *vegan*, many of these same food groups were significantly correlated with axes coordinates illustrating the directional pull of these factors in ordination space (Figure 1A and 1B). With regard to macronutrients, total protein, fat, and carbohydrate consumption were related to community membership

(Table 4). Several micronutrients, including a number of minerals and B vitamins, were also associated with community structure and membership (Table 4, Figure 1). The most likely dietary sources of B vitamins and minerals include fortified, non-whole grains and meats (Figure 2).

Taxonomy. A few genus-level taxa including *Ruminococcus* (positive) and *Blautia* (negative) were correlated with age

Table 2. Comparison of average dietary intake of 75 children recruited from a community-based, early childhood learning center in suburban Los Angeles, CA, by age group across three 24-hour dietary recalls using US Department of Agriculture's multiple-pass method Dietary Reference Intakes: Percent above and below the Estimated Average Requirements by age

Variable	Age Group		EAR ^a		Percent (%) above EAR ^b	Percent (%) below EAR ^c
	2-3 y Mean±SD ^d (min-max)	4-8 y Mean±SD (min-max)	2-3 y	4-8 y		
Energy (kcal)	1,369.6±314.2 (890.6-2,225.5)	1,467.6±334.9 (782.8-2,145.5)	—	—	—	—
Protein (g/kg/d)	3.43±1.01 (2.07-5.42)	2.90±1.05 (1.26-7.28)	0.87	0.76	96	—
Fat (g/d) ^e	47.3±14.9 (28.0-83.9)	49.2±13.3 (19.4-80.3)	—	—	—	—
Carbohydrates (g/d)	191.7±52.6 (131.2-361.0)	203.1±51.8 (117.3-332.9)	100	100	42.7	—
Fiber (g/d) ^f	13.9±4.5 (5.8-26.0)	14.3±6.0 (5.9-34.0)	19	25	—	90.7
Calcium (mg/d)	910.8±218.7 (533.4-1525.1)	845.6±271.1 (399.2-1519)	500	800	10.7	29.3
Copper (μg/d)	950±260 (610-1,690)	970±310 (440-1,770)	260	340	85.3	—
Iron (mg/d)	11.1±4.16 (5.53-19.9)	13.5±4.47 (7.10-24.7)	3	4.1	90.7	—
Magnesium (mg/d)	216.0±44.5 (138.8-310.4)	218.2±57.3 (122.2-337.6)	65	110	64	1.3
Phosphorus (mg/d)	1,006.3±203.0 (561.6-1,465.6)	1,037.9±243.2 (583.1-1,538.5)	380	405	82.7	2.7
Potassium (g/d) ^f	2.07±0.48 (1.30-3.62)	2.11±0.64 (1.19-3.52)	2.0	2.3	—	54.7
Selenium (μg/d)	64.7±16.5 (34.4-95.0)	78.5±22.4 (40.3-160.8)	17	23	94.7	—
Sodium (mg/d) ^f	1,898.8±461.6 (1,322.7-2,961)	2,275.1±581.6 (1,159-3,758)	800	1,000	69.3	—
Zinc (mg/d)	8.32±2.22 (5.36-14.6)	9.45±3.19 (4.53-20.1)	2.5	4	77.3	—
Vitamin A (RAE ^g) (μg/d)	450.2±156.3 (194.8-761.4)	438.6±175.7 (107.6-1,115.9)	210	275	68	4.0
Thiamin (B-1) (mg/d)	1.23±0.42 (0.58-2.58)	1.36±0.38 (0.69-2.33)	0.4	0.5	86.7	—
Riboflavin (B-2) (mg/d)	1.82±.43 (1.12-2.95)	1.85±.53 (0.92-3.20)	0.4	0.5	97.3	—
Niacin (B-3) (mg/d)	14.7±5.17 (5.36-28.6)	17.3±5.31 (8.66-31.00)	5	6	77.3	—
Pyridoxine (B-6) (mg/d)	1.52±0.58 (0.57-2.96)	1.62±0.57 (0.93-3.02)	0.4	0.5	92.0	—
Folate (B-9) (μg/d)	314.8±110.3 (123.5-616.4)	362.3±137.9 (182.2-715.0)	120	160	58.7	—
Vitamin B-12 (μg/d)	4.31±1.78 (1.22-8.67)	5.66±7.36 (0.93-52.36)	0.7	1.0	96	1.3

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Table 2. Comparison of average dietary intake of 75 children recruited from a community-based, early childhood learning center in suburban Los Angeles, CA, by age group across three 24-hour dietary recalls using US Department of Agriculture's multiple-pass method Dietary Reference Intakes: Percent above and below the Estimated Average Requirements by age (*continued*)

Variable	Age Group		EAR ^a		Percent (%) above EAR ^b	Percent (%) below EAR ^c
	2-3 y Mean±SD ^d (min-max)	4-8 y Mean±SD (min-max)	2-3 y	4-8 y		
Vitamin C (mg/d)	79.6±36.8 (15.3-173.1)	92.4±60.2 (23.9-361.0)	13	22	82.7	—
Vitamin D (μg/d)	5.77±2.55 (1.57-12.9)	5.05±2.36 (0.51-11.3)	10	10	—	96.0
Vitamin E (mg/d)	6.32±3.61 (2.02-15.4)	5.28±2.55 (1.33-12.7)	5	6	6.5	65.3
Vitamin K (μg/d) ^f	73.1±66.9 (18.3-307.1)	59.5±55.5 (9.98-294.2)	30	55	20	52.0
Choline (mg/d) ^f	210.7±60.8 (81.2-329.9)	222.8±74.1 (106.5-472.4)	200	250	—	60.0

^aEAR=Estimated Average Requirement.

^bPercent refers to proportion of participants with dietary intakes ≥ 2 times (200%) the EAR. Two times the EAR (200%) was chosen as the cutoff because $>54\%$ of the nutrients analyzed in the table had EAR $>100\%$.

^cPercent refers to proportion of participants with dietary intakes $<100\%$ of EAR.

^dSD=standard deviation.

^eTotal fat and saturated fat are compared to the Acceptable Macronutrient Distribution Ranges, which are 30% to 40% for children 1 to 3 years of age, 25% to 35% for children aged 4 to 18 years (total fat) and $<10\%$ for children of all ages (saturated fat).

^fAdequate Intake (AI) used for nutrients without EAR. For sodium, 30.7% of participants reported intakes $>100\%$ of the AI, but $<200\%$.

^gRAE=retinol activity equivalent.

(Figure 3A). Likewise, several genera were correlated with the consumption of specific food groups and subcategories (Figure 3A). For example, *Bacteroides*, the most abundant genera observed on average (Figure 4), was positively correlated with the amount of whole grains and negatively correlated with the amount of animal protein consumed. Many of the same macronutrients and micronutrients associated with microbiome β -diversity were also correlated with the abundance of several taxonomic groups (Figure 3B).

DISCUSSION

The goal of this study was to link quantitative dietary intake with gut microbiome features in a healthy population of post-weaned US children aged 2 to 9 years. Both diet and microbiome composition were averaged across three, non-consecutive time points of healthy subjects to approximate "recent intake" and minimize dietary and microbiome irregularities. This was the method used in this study to identify linkages between dietary intake and microbiome features in an understudied US demographic.

Other studies among young children assessing the relationship between the gut microbiome and dietary intake have used both 24-hour dietary recall information over 3 consecutive days⁶¹ or 3-day food records¹⁶ in addition to food frequency questionnaires, but in these studies assessment of dietary intake took place over longer time periods from 6 months up to 1 year. The present study was conducted over the summer months and thus, averaging 3 days of non-consecutive intake allowed for the estimation of recent dietary intake to be correlated with the fecal samples that were collected during the same time period.

The dietary intake of this study population is similar in some ways to the nation as a whole, and different in others. In a nationally representative study of children ages 2 to 11 years using a combined data set from What We Eat in America, National Health and Nutrition Examination Survey, and the School Nutrition Dietary Assessment Study, the authors demonstrated low intakes of vitamin D, calcium, and potassium and excess intakes for energy, carbohydrates, and sodium.⁶² In contrast, the population studied here had intakes of calcium at or just above the EAR. Hamner and colleagues⁶³ examined mineral intakes for 2-year old children in the National Health and Nutrition Examination Survey between 2003 and 2012. They showed that $\leq 2\%$ had usual intakes below the EAR for iron, calcium, and zinc with one in every two children exceeding the upper limit (UL) for zinc. Similarly, 36% of all participants in this study exceeded the UL for zinc and although intakes of iron exceeded the EAR by more than 3 times the requirement for 54.7% of participants, no one exceeded the UL for iron intake. Results from the recent Feeding Infant and Toddlers Study (FITS), which assessed dietary practices of children in the United States from birth to 48 months, demonstrated low intakes of potassium, fiber, vitamin D, and vitamin E, and high intakes of zinc, sodium, and saturated fats—similar to the results for this study.⁶⁴ When examining the AMDRs for macronutrient intake, low proportions of study participants demonstrated excesses. Although 28% of study participants had a value lower than the AMDR for total fat, 82.7% had saturated fat intake greater than the AMDR of $<10\%$. This means that while total fat intake may have been low for about one-third of study participants, the quality of their fat intake was poor, with many of them

Table 3. Results of permutational multivariate analysis of variance and Spearman rank correlations between selected demographic features and dietary food groups with gut microbiome community structure (weighted UniFrac), community membership (unweighted UniFrac), and three measures of diversity in a population of children aged 2 to 9 years (n=75) recruited from a community-based, early childhood learning center in suburban Los Angeles, CA^a

Factor	Weighted UniFrac		Unweighted UniFrac		Shannon Index		Phylogenetic Diversity		Richness	
	R ²	P value ^b	R ²	P value ^b	ρ	P value ^b	ρ	P value ^b	ρ	P value ^b
Age	0.027	0.0488	0.035	0.0002	0.379	<0.001	0.242	0.037	0.340	0.003
Sex	0.006	0.9329	0.014	0.3221	0.073 ^c	0.787	0.003 ^c	0.957	0.054 ^c	0.816
Fruit	0.033	<i>0.0169</i>	0.015	0.2242	0.021	0.856	-0.110	0.349	-0.057	0.628
Citrus/melon/berries ^d	0.043	<i>0.0071</i>	0.015	0.2365	-0.025	0.831	-0.131	0.262	-0.011	0.925
Other fruit	0.008	0.8034	0.017	0.1097	0.027	0.818	-0.130	0.268	-0.137	0.242
Grain	0.038	<i>0.0059</i>	0.023	0.0176	0.098	0.400	-0.070	0.550	0.011	0.924
Whole grain	0.021	0.1134	0.017	0.112	-0.088	0.451	-0.085	0.470	-0.117	0.317
Non-whole grain	0.037	<i>0.0089</i>	0.025	<i>0.0057</i>	0.141	0.226	-0.023	0.843	0.070	0.551
Vegetable	0.024	0.0746	0.021	0.0261	0.145	0.216	-0.015	0.901	0.027	0.816
Dark green	0.010	0.5985	0.013	0.5146	0.047	0.692	-0.052	0.657	0.019	0.872
Orange	0.008	0.7730	0.014	0.3535	-0.072	0.538	-0.136	0.245	-0.177	0.129
Potato (white)	0.024	0.0771	0.023	0.0144	0.002	0.983	0.048	0.684	0.045	0.699
Starchy ^e	0.020	0.1439	0.013	0.5212	0.024	0.841	-0.037	0.752	-0.033	0.779
Tomato	0.042	<i>0.0057</i>	0.021	0.0329	0.034	0.772	0.001	0.993	0.014	0.903
Other	0.010	0.6216	0.018	0.1108	0.080	0.493	-0.119	0.308	-0.060	0.609
Dairy	0.009	0.6928	0.011	0.7236	-0.036	0.758	-0.053	0.652	-0.129	0.270
Milk	0.019	0.1710	0.015	0.2694	-0.036	0.761	0.069	0.558	-0.033	0.782
Yogurt	0.014	0.3852	0.023	<i>0.0147</i>	-0.158	0.176	-0.311	<i>0.007</i>	-0.218	0.060
Cheese	0.013	0.4364	0.016	0.1923	0.035	0.769	-0.190	0.102	-0.157	0.179
Protein, animal^f	0.020	0.1506	0.019	0.0593	0.282	<i>0.014</i>	0.151	0.196	0.224	0.054
Meat ^g	0.020	0.1533	0.019	0.0809	0.301	<i>0.009</i>	0.142	0.224	0.225	0.052
Meat (no poultry or fish)	0.021	0.1233	0.012	0.5502	0.099	0.399	0.047	0.688	0.108	0.355
Organ meat	0.009	0.6454	0.012	0.5829	0.000	1.000	-0.081	0.492	-0.086	0.464
Processed meats ^h	0.017	0.2242	0.013	0.5007	0.025	0.829	0.010	0.931	0.060	0.606
Poultry	0.012	0.5182	0.017	0.1186	0.350	<i>0.002</i>	0.122	0.299	0.191	0.102
Seafood high fatty acids	0.008	0.7110	0.013	0.4767	-0.026	0.822	-0.097	0.408	-0.118	0.315
Seafood low fatty acids	0.019	0.1775	0.009	0.9808	-0.055	0.640	-0.014	0.906	-0.048	0.685
Eggs	0.009	0.7480	0.014	0.3919	0.060	0.609	0.103	0.378	0.139	0.235
Protein, plantⁱ	0.030	0.0301	0.019	0.0534	-0.308	<i>0.007</i>	-0.055	0.637	-0.137	0.239
Soy	0.014	0.3658	0.025	<i>0.0185</i>	-0.032	0.788	-0.059	0.617	-0.039	0.740
Nuts/seeds	0.023	0.0961	0.027	<i>0.0051</i>	-0.214	0.065	-0.204	0.078	-0.226	0.051
Legumes	0.025	0.063	0.017	0.1139	-0.057	0.630	0.200	0.086	0.102	0.383

^aExplained variance for permutational multivariate analysis of variance analysis is expressed as R² values for 9,999 permutations.

^bP values in italics remain significant after correction for multiple comparisons (corrected P≤0.05).

^cχ² values from Kruskal-Wallis test.

^dIncludes juices.

^eOther than potatoes, peas, and beans (legumes).

^fSum of M_MPF and eggs.

^gIncludes beef, pork, veal, lamb, game, organ meats, sausages, luncheon meat, poultry, and fish and shellfish.

^hFrankfurters, sausages, and luncheon meat.

ⁱSum of nuts, soy, and legumes (converted to ounces).

Table 4. Results of permutational multivariate analysis of variance and Spearman rank correlations between dietary nutrient intake and gut microbiome community structure (weighted UniFrac), community membership (unweighted UniFrac), and three measures of diversity in a population of children aged 2 to 9 years (n=75) recruited from a community-based, early childhood learning center in suburban Los Angeles, CA^a

Factor	Weighted UniFrac		Unweighted UniFrac		Shannon Index		Phylogenetic Diversity		Richness	
	R ²	P value ^b	R ²	P value ^b	ρ	P value ^b	ρ	P value ^b	ρ	P value ^b
kcal	0.027	0.0433	0.025	<i>0.0050</i>	0.041	0.7260	-0.092	0.4317	-0.036	0.7587
Protein	0.015	0.3076	0.022	<i>0.0261</i>	0.176	0.1311	0.010	0.9290	0.052	0.6602
%kcal from protein	0.017	0.2309	0.013	0.4127	0.157	0.1794	0.158	0.1754	0.137	0.2424
Fat	0.026	0.0642	0.021	<i>0.0267</i>	-0.022	0.8492	-0.105	0.3712	-0.070	0.5516
%kcal from fat	0.012	0.4957	0.015	0.2329	-0.084	0.4726	-0.077	0.5125	-0.092	0.4335
Carbohydrate	0.024	0.0782	0.023	<i>0.0167</i>	0.068	0.5604	-0.104	0.3723	-0.021	0.8595
%kcal from carbohydrates	0.011	0.6011	0.011	0.8244	-0.041	0.7277	-0.043	0.7160	-0.012	0.9206
Fiber	0.014	0.3613	0.015	0.2167	0.039	0.7415	0.074	0.5279	0.062	0.5950
Calcium	0.010	0.6201	0.013	0.5040	-0.088	0.4541	-0.134	0.2505	-0.207	0.0744
Copper	0.017	0.2294	0.022	0.0217	-0.183	0.1171	-0.137	0.2396	-0.145	0.2131
Iron	0.030	0.0278	0.019	0.0725	0.181	0.1211	0.089	0.4495	0.145	0.2160
Magnesium	0.016	0.2882	0.019	0.0704	-0.162	0.1657	-0.090	0.4436	-0.130	0.2655
Phosphorous	0.008	0.7882	0.016	0.206	-0.034	0.7709	-0.090	0.4442	-0.120	0.3036
Potassium	0.018	0.1859	0.018	0.0860	-0.035	0.7681	-0.059	0.6149	-0.069	0.5586
Selenium	0.019	0.1825	0.030	<i>0.001</i>	0.282	0.0141	0.068	0.5617	0.139	0.2345
Sodium	0.039	0.0057	0.031	<i>0.0004</i>	0.106	0.3664	-0.111	0.3423	0.004	0.9754
Zinc	0.033	0.0173	0.017	0.1509	0.059	0.6137	-0.015	0.8965	0.007	0.9558
Vitamin A (RAE^c)	0.012	0.512	0.011	0.7476	0.070	0.5507	0.007	0.9533	-0.020	0.8625
Thiamin (B-1)	0.038	0.0081	0.023	0.0156	0.141	0.2266	0.044	0.7088	0.070	0.5517
Riboflavin (B-2)	0.021	0.1199	0.018	0.1017	0.056	0.6312	0.010	0.9303	-0.021	0.8553
Niacin (B-3)	0.028	0.0427	0.026	<i>0.0037</i>	0.242	0.0367	0.078	0.5082	0.141	0.2265
Pyridoxine (B-6)	0.023	0.0913	0.020	0.0381	0.154	0.1882	0.017	0.8842	0.065	0.5803
Folate (B-9)	0.024	0.0729	0.021	0.0277	0.195	0.0930	0.047	0.6905	0.101	0.3877
Vitamin B-12	0.007	0.8135	0.013	0.5043	0.202	0.0815	0.085	0.4673	0.098	0.4010
Vitamin C	0.008	0.7752	0.017	0.1340	0.137	0.2398	-0.102	0.3844	0.044	0.7081
Vitamin D	0.012	0.4989	0.014	0.4098	-0.005	0.9664	0.075	0.5224	-0.030	0.7992
Vitamin E	0.010	0.6308	0.016	0.1887	0.029	0.8031	0.011	0.9220	0.018	0.8765
Vitamin K	0.011	0.5990	0.013	0.5342	0.026	0.8272	-0.068	0.5600	-0.022	0.8545
Cholesterol	0.013	0.4381	0.016	0.1766	0.164	0.1592	0.186	0.1107	0.174	0.1347
Saturated fatty acid	0.019	0.1700	0.015	0.2663	0.047	0.6862	-0.061	0.6027	-0.057	0.6272
Monounsaturated fatty acid	0.023	0.0920	0.021	0.0362	-0.060	0.6098	-0.104	0.3723	-0.083	0.4804
Polyunsaturated fatty acid	0.020	0.1436	0.022	0.0217	-0.079	0.5006	-0.124	0.2907	-0.076	0.5157
Choline	0.010	0.6771	0.015	0.2833	0.120	0.3041	0.121	0.2995	0.116	0.3198

^aExplained variance for permutational multivariate analysis of variance analysis is expressed as R² values for 9,999 permutations.

^bP values in italics remain significant after correction for multiple comparisons (corrected P≤0.05).

^cRAE=retinol activity equivalent.

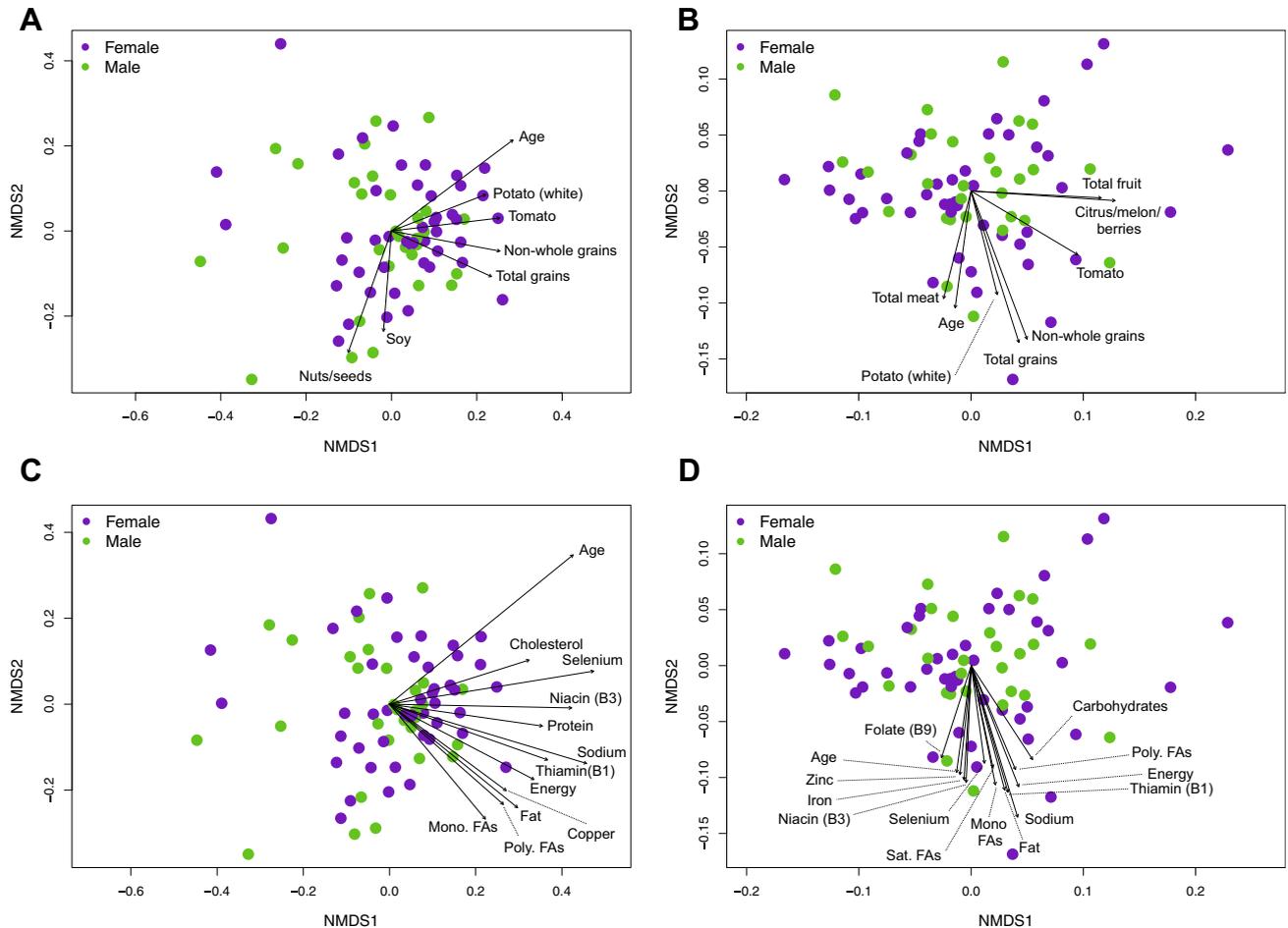


Figure 1. Consumption of specific food groups (A, B) and nutrients (C, D) were strongly associated with gut microbiome membership (unweighted UniFrac, A, C) and structure (weighted UniFrac, B, D) in children aged 2 to 9 years. Each point in each non-metric multidimensional scaling (NMDS) plot represents the average microbiome composition of one child across three time points and is colored based on sex of the child. Vectors depict significant correlations ($P \leq 0.05$) between specific dietary features and microbiome composition across the study population as determined from 10,000 permutations using the “envfit” function in R *vegan* package. Text files of “envfit” results are available online through figshare.³²

consuming animal fats as their primary source. Almost 91% of study participants had fiber intakes below recommendations as well. Berding and colleagues¹⁶ also showed lower intakes for fiber and high intakes of saturated fat in their study of children 4 to 8 years old when associating dietary patterns with microbiome composition. National intakes of protein for children aged 2 to 3 years and 4 to 8 years show average intakes around 14% to 16%, which tracks with the population in this study.⁶⁵ Sodium intake exceeded the UL for 78.7% of study participants.⁵⁸ This observation is similar to national studies that show children in the United States exceed recommended intakes of sodium, but have low intakes of fiber.^{66,67} This may also explain in part the high sodium intake, as many of the protein-rich foods included hot dogs, chicken nuggets, and pizza. Finally, intakes of B vitamins were high and exceeded ULs for niacin, vitamin B-6, and folate. One reason for this may be a high intake of ready-to-eat cereals. More than 46% of the families reported annual incomes <\$30,000 and 53.3% participated in the Special Supplemental Nutrition Program for Women, Infants, and Children. Ready-to-eat cereals and whole-grain

bread are components of the Special Supplemental Nutrition Program for Women, Infants, and Children food package, which may allow greater access than usual for the families in our study to these food items, which are often fortified with B vitamins.

One of the strongest factors related to microbiome features observed was a child’s age. Older children tended to have more diverse and more even gut communities than younger children, suggesting that assembly of the gut microbiome continues well into childhood. Likewise, community membership and structure were associated with age. Previous studies relying on high-throughput molecular techniques have suggested that by about the age of 3 years, a child’s microbiome reaches diversity levels proximate to those of an adult.^{13,15} Other more recent studies have observed continued development of the gut microbiome beyond 3 years of age, similar to what was observed here.^{16,68} From a human development perspective, early childhood represents a period of time when children are beginning to explore greater dietary diversity. They are becoming more independent in their eating habits and shifting from a higher fat diet

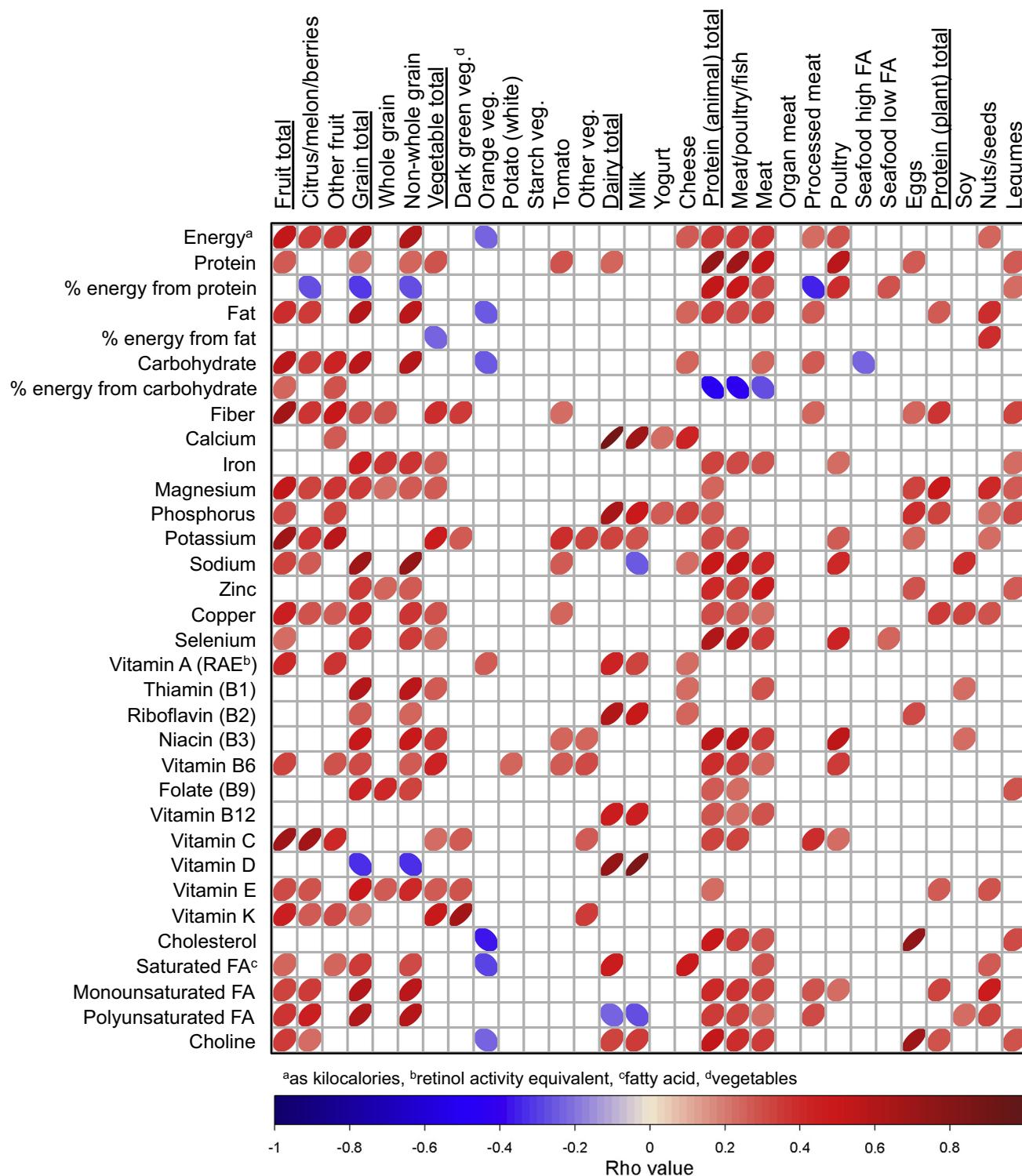
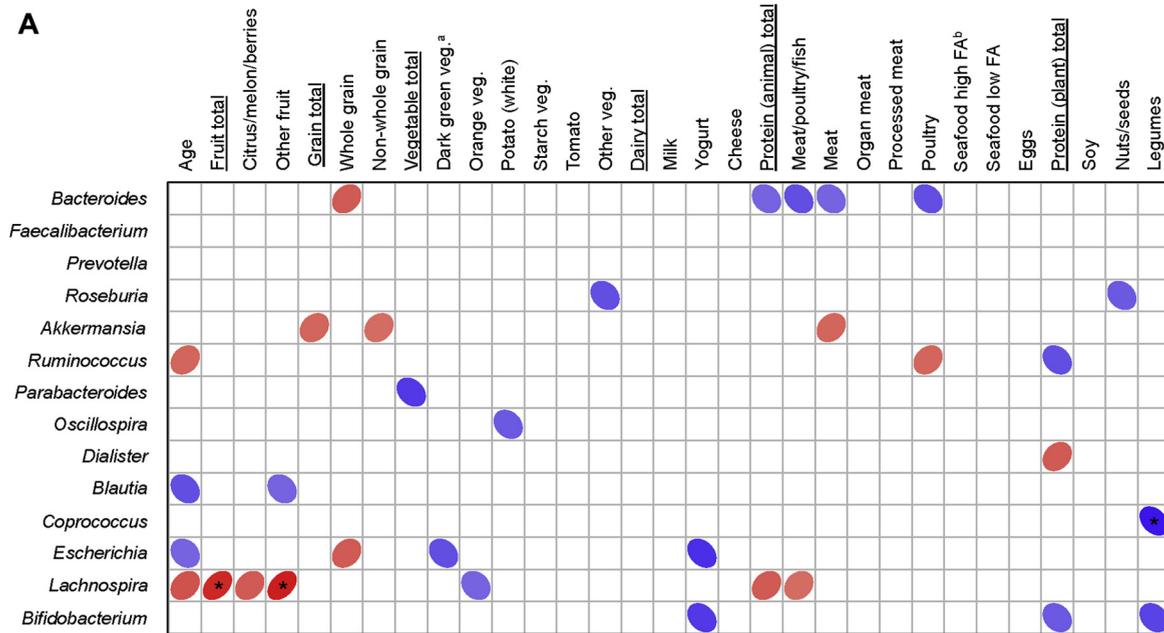
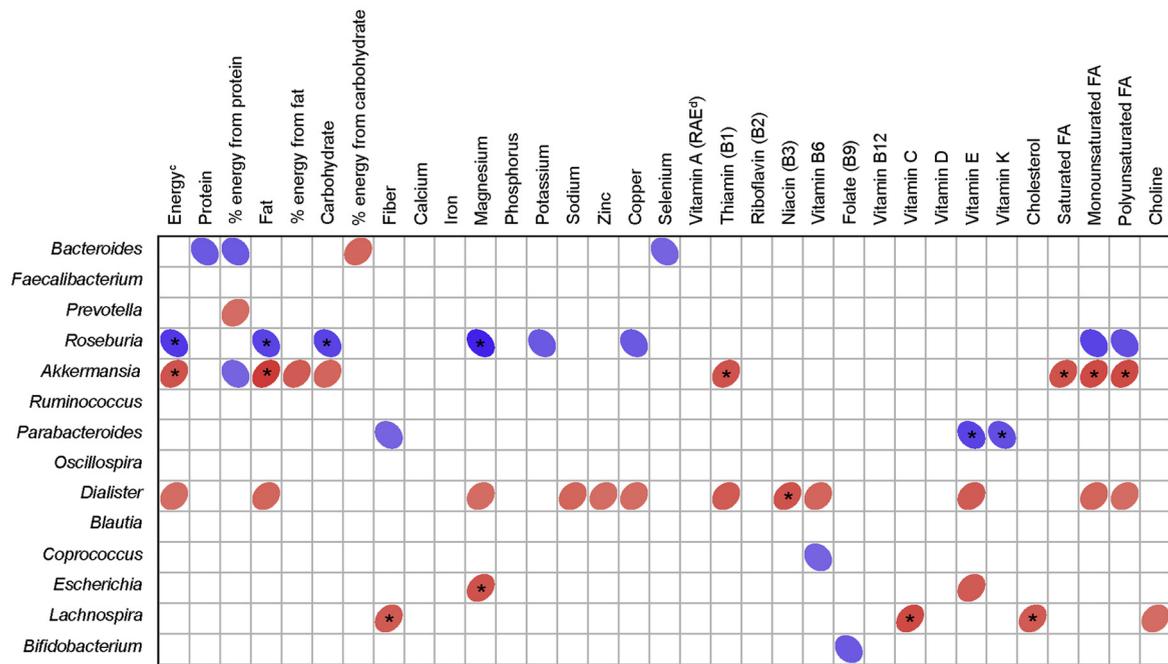


Figure 2. Spearman rank correlation matrix showing relationships between food group consumption and average nutrient intake in the study population. Red colors illustrate positive correlations while blue illustrate negative correlations. The width of each ellipse and shade of color is proportional to each ρ value with wider widths and lighter color shade indicating lower ρ values. Only correlations with P values < 0.05 are shown.

A



B



^avegetables, ^bfatty acid, ^cas kilocalories, ^dretinol activity equivalents

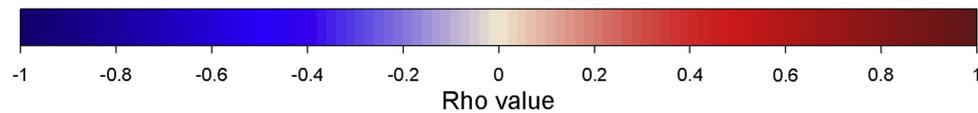


Figure 3. The relative abundance of several bacterial genera were correlated with consumption of several food groups (A) and with nutrient intake (B) in a diverse population of children aged 2 to 9 years. Age (A) was also correlated with several bacterial genera. Only correlations with uncorrected $P < 0.05$ are shown as ellipses, with asterisks indicating significance after false discovery rate correction (corrected $P \leq 0.05$). Red ellipses indicate a positive association, whereas blue ellipses indicate a negative correlation. The width of each ellipse is proportional to the strength of the correlation (ie, Spearman ρ values) with narrower ellipse indicating a more significant relationship (ie, a lower P value). Darker colors of red and blue indicate Spearman ρ values closer to 1 and -1, respectively.

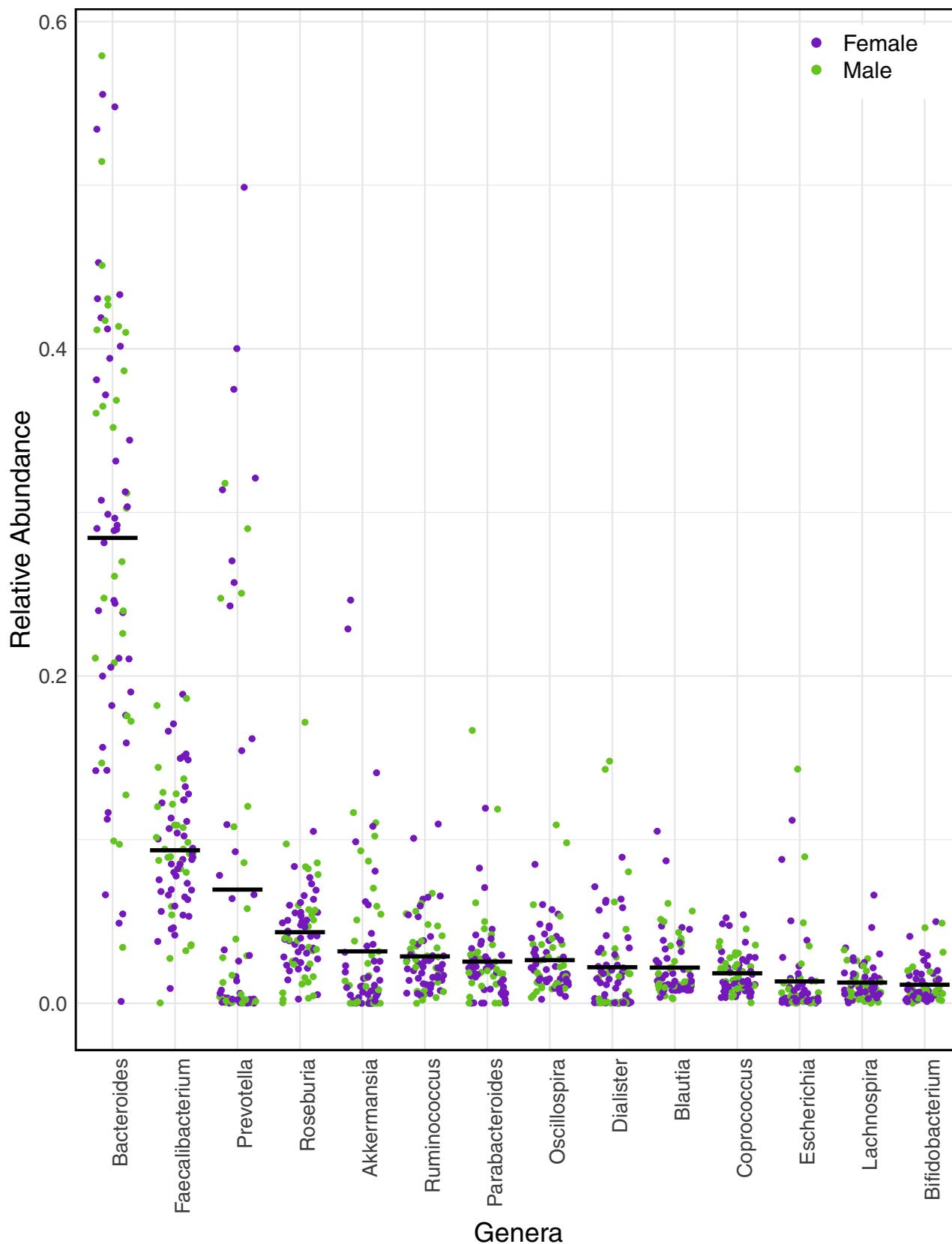


Figure 4. Taxonomic composition of the child gut microbiome. Each point represents the average abundance of each genera across three time points for each of 75 children. Bars illustrate mean values for each genera across the population. Only genera >1% on average are shown.

to one that has a greater proportion of carbohydrates and protein.⁶⁹ As children approach middle childhood, they continue to gain independence in their eating habits and may be more likely to purchase food on their own from school, corner stores, and fast-food outlets, further expanding their dietary diversity. With guidance from adults, this can be a stage when children learn healthy habits and continue to expand the numbers and types of healthy foods they consume to ensure healthy weight gain and prevention of later chronic diseases.⁶⁹ Taken together, results presented here demonstrate continued development of the gut microbiome beyond the age of 3 years, and because this is also an important developmental stage for humans, it is hypothesized that early and middle childhood may represent a crucial window when the gut microbiome might still be amenable to lasting manipulations through diet.

Consumption of several food groups were associated with different microbiome features. Interestingly, with one exception (ie, yogurt), animal-derived foods were not strongly associated with microbiome structure or membership. Instead, protein-rich animal products like meats were positively correlated with microbial diversity (as Shannon Index). When total protein consumption as a macronutrient was examined, there was no correlation with any of the α -diversity metrics, likely because this category includes all dietary sources of proteins regardless of whether they were plant- or animal-derived. Several previous studies have observed increases in microbiome diversity related to increased consumption of proteins.^{31,70-74} However, people in industrialized nations consuming a diet rich in animal proteins and fats, but poor in plant-based fibers (ie, a Western diet), generally have less diverse gut communities than people eating predominantly plant-based diets.^{13,31,75,76} Results observed here suggest that within populations consuming a Western diet, the source of proteins consumed, whether plant- or animal-based, may also be an important determinant of microbiome diversity.

Among the plant-derived foods, grain consumption, specifically non-whole grains, was one of the strongest factors associated with microbiome structure and membership. Grains, including non-whole grains, contain a mixture of indigestible starches and fibers that pass through the small intestine to the large intestine, where they are fermented into short-chain fatty acids by various members of the gut microbiome.⁷⁷ Non-whole-grain foods like breakfast cereals, loaf breads, and pasta, are often enriched with several B vitamins (eg, thiamin, riboflavin, and niacin) and minerals (eg, iron) that are lost during processing. Many of these same micronutrients were also strongly associated with microbiome structure and composition. Data from a recent study of the major contributors to energy intake of children aged 2 to 18 years in the past 21 years show that pizza, grain-based desserts, breads, pasta, and ready-to-eat cereals were among the top 10 contributors.⁷⁸ These foods were also among the top contributors to diets of the children studied here. Many of these grain-based products are fortified with B vitamins and may explain the high intakes observed. Dietary micronutrients are also known to be associated with the composition of the gut microbiome when deficient.⁷⁹⁻⁸¹ However, their relationship with the gut microbiome when consumed in excess is less understood. Whether the differences in microbiome features observed are related to the indigestible starches and fibers or the added micronutrients in these grain foods is unknown, but they do

provide intriguing targets for future interventions or model organism-based studies. B vitamins in particular are known to mediate numerous symbiotic interactions between eukaryotes and prokaryotes in a number of systems.⁸²⁻⁸⁴ For example, vitamin B-12 has been suggested to be a modulator of gut microbial ecology.⁷⁹

Other plant-based foods that were significantly associated with microbiome composition included fruits. Within fruits, the category that includes citrus, melons, and berries was associated with much of the variation in microbiome structure, but not membership. These fruits are significant sources of fiber, pectin, and polyphenols, dietary compounds that are known to be associated with the diversity of gut bacteria.⁸⁵⁻⁹¹ The only genus that was positively associated with fruit consumption here was *Lachnospira*, a member of the Firmicutes phylum that is known to contain pectin fermenting species.⁹² Other human observational studies have also found positive associations between *Lachnospira* abundance and consumption of fruits⁹³ and vegetables.⁶¹ Decreases in the relative abundance of members of the *Lachnospira* lineage have been observed in asthmatic children⁹⁴ and adults with gallstones⁹⁵ or human immunodeficiency virus,⁹⁶ suggesting some sort of beneficial role for this lineage. Future research should more directly address how members of the *Lachnospira* lineage interact with its human host.

Although an extremely diverse phylum, human-associated Bacteroidetes are considered ecological generalists able to consume a diversity of dietary and host-produced substrates.⁹⁷ On average, the two most abundant genera within the Bacteroidetes observed were *Bacteroides* and *Prevotella*. These genera are common members of the gut microbiome in a diversity of populations and their abundances have been linked to broad-scale dietary habits in several previous studies. For example, *Prevotella* are enriched in populations consuming fiber-rich diets like those in rural Africa and South America, whereas the *Bacteroides* are typically enriched in individuals consuming a Western diet.^{13,31,76} The abundance of *Prevotella* is also linked to a vegetarian diet in Western populations.¹⁸ In this study, children who consumed more whole grains and fewer meat products were enriched in *Bacteroides*, while *Prevotella* were not associated with consumption of any food groups. Both of these observations are in contrast to most previous studies as stated above. Linking these genera to more fine-scale dietary components, however, has proven difficult, likely because of diversity at the species and strain levels, as recently investigated by De Filippis and colleagues.⁹⁸ They found sub-genus oligotypes of *Prevotella* and *Bacteroides* that were associated with both plant-based and animal-based diets, suggesting the current paradigm regarding the abundances of these genera (ie, agrarian/vegetarian vs meat-based diets) may be an oversimplification. It is worth noting, however, that in isolation, species of these genera do have strikingly different responses to bile salts, with *Prevotella* spp generally being more sensitive than *Bacteroides* spp.⁹⁹ More fine-scale taxonomic resolution coupled with laboratory culturing experiments may be needed to better define the niche space of these common gut bacteria.

While the current study has identified dietary factors that are associated with features of the microbiome, this study does have limitations. Sample size is perhaps the most obvious limitation of this study, as it has been suggested that more than 40,000 humans would be needed to observe the

full diversity of the gut microbiome.¹⁹ From a microbiome perspective, this study has only examined taxonomic differences and inferred how this may be related to the function of these communities. A multi-omic approach that includes metagenomics, transcriptomics, and metabolomics would be able to better elucidate more direct relationship between diet and the microbiome. Furthermore, these correlative results should be interpreted with caution, as correlations do not determine causation. More controlled studies involving animal models may be able to more directly assess the linkages uncovered in this study. Ideally, dietary assessment would have included both a 3-day food record and a food frequency questionnaire to better estimate dietary habits over a longer period of time and to understand how the dietary data collected reflect typical dietary intake. However, given that study participants were recruited during the summer months in a community-based setting and parents were the primary caregivers during the time period of data collection and were with children for meals, averaging the three, non-consecutive 24-hour multiple pass dietary recalls that included weekdays and weekend days and using parents as proxy reporters was considered the most appropriate method to estimate both energy and nutrient intakes of these children. However, it is also important to acknowledge potential for bias associated with self-report of dietary intake. Overreporting and underreporting of dietary intake using parents as proxies for the age group under study is a common concern and has been documented.¹⁰⁰ Comparisons of parental estimates of energy intake compared to doubly labeled water have been found to be in congruence at the group level, but individual-level estimates often reveal under- or overreporting.^{101,102} Desirability bias may also cause parents to alter the foods they provide their children to make it appear they are offering healthier options when recording dietary intake.¹⁰³

In addition, while age is an important characteristic that may be key in better understanding the relationship between dietary intake and the gut microbiome, stratifying by age within this population to compare younger and older children would have limited the power of the results. Future studies should include a larger number of participants to be able to tease apart both the developmental differences that may be occurring in the microbiome due to changes in eating habits, as well as to better structure potential interventions to ensure healthy eating habits at the earliest stage possible.

CONCLUSIONS

Findings presented here suggest the importance of diet in structuring overall gut microbiome composition throughout early life. By taking an interdisciplinary approach, this study was able to identify key dietary features that may have important roles in structuring the child gut microbiome. In particular, the consumption of fortified, non-whole-grain foods enriched in vitamins and minerals was among the strongest factors associated with gut microbiome features.

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STATEMENT OF POTENTIAL CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

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DATA STATEMENT

Research data for this article, raw sequence data, accompanying metadata, supplementary data, averaged nutrition variables, tables, and figures are available at <https://figshare.com/s/805f2deead68cd2d14fc>.

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AUTHOR CONTRIBUTION

D. R. Herman and G. E. Flores designed research. D. R. Herman, J. Mercado, P. Argueta, N. Rhoades, U. Lopez, and G. E. Flores conducted research. D. R. Herman, J. Mercado, P. Argueta, N. Rhoades, U. Lopez, and G. E. Flores analyzed data. U. Lopez translated all documents into Spanish. D. R. Herman and G. E. Flores wrote the paper. All authors reviewed and commented on subsequent drafts of the manuscript.