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Fecal microbiome composition and stability in 4- to 8-year old children is associated with dietary patterns and nutrient intake☆,☆☆,★

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Abstract

How long-term dietary intake shapes microbiota composition and stability in young children is poorly understood. Herein, the temporal variability in stool microbiota composition in relation to habitual dietary patterns of 4- to 8-year-old children (n=22) was investigated. Fecal samples were collected at baseline, 6 weeks and 6 months. Bacterial composition and volatile fatty acids were assessed by 16S rRNA sequencing and gas-chromatography, respectively. Nutrient intake was assessed using 3-day food diaries and dietary patterns were empirically derived from a food frequency questionnaire. Using a factor loading of >0.45 for a food group to be a major contributor to the overall dietary pattern, two dietary patterns were found to be associated with distinct microbiome composition. Dietary Pattern 1 (DP1), characterized by intake of fish, protein foods, refined carbohydrates, vegetables, fruit, juice and sweetened beverages, kid's meals and snacks and sweets, was associated with higher relative abundance of Bacteroidetes, *Bacteroides* and *Ruminococcus* and lower abundance of *Bifidobacterium*, *Prevotella*, *Blautia* and *Roseburia*. Dietary Pattern 2 (DP2), characterized by intake of grains, dairy and legumes, nuts and seeds, was associated with higher relative abundance of Cyanobacteria and *Phascolarctobacterium* and lower abundance of *Dorea* and *Eubacterium*. Fruit and starchy foods were present in both patterns, but were more associated with DP1 and DP2, respectively. Temporal stability of microbiota over a 6-month period was associated with baseline dietary patterns. Understanding how dietary intake contributes to microbiota composition and stability in early life in important for dietary recommendations and designing clinical interventions for microbiota-associated diseases.

Keywords: Dietary pattern; microbiota; nutrients; children

1. Introduction

The human gastrointestinal (GI) microbiota is a complex community containing an estimated 500 species and approximately 3 million bacterial genes, which benefit the host by providing protection against pathogens and programming the immune response [1–3]. Recent research has revealed that the GI microbiota is also involved in brain development and cognitive processes and microbial dysbiosis has been associated with various cognitive disorders, including Autism Spectrum Disorder [4–8]. Diet is one of the most influential factors in determining the composition of the gut microbiota. It is postulated that diet-induced microbial changes associated with modern dietary habits are associated with diseases, such as inflammatory bowel disease, allergies or other autoimmune diseases [9]. David and colleagues showed that dramatic changes in dietary fat, protein, and fiber intakes over 5-days rapidly modified the composition and function of the human GI microbiota [10]. Longer-term dietary patterns are more associated with stability of the microbiota [11,12]. Studies in humans and other mammals have shown that the gut microbiota composition can be clustered based on habitual diet (herbivore, omnivore or carnivore) [13], moreover individuals following a vegetarian diet have higher phylogenetic diversity compared to diet patterns rich in meat or mixed diets [14]. Additionally, diet-induced changes in microbiota composition can lead to increased risk of developing certain diseases (e.g., inflammatory bowel diseases), whereas a healthier long-term dietary pattern may be more beneficial in promoting a microbial profile that could potentially protect against diseases [15].

The microbiota undergoes rapid changes in the first years of life [16]. Although previous studies have suggested that the gut microbiota becomes relatively stable and resembles that of an adult at 3 years of age, other studies suggest that the gut microbiota might have a more prolonged development, lasting into pre-adolescence [17–21]. Given that the GI microbiota can potentially contribute to the development of diseases in genetically at-risk individuals, it is important to understand whether specific long-term dietary patterns

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are associated with a more beneficial microbiota that could show a higher resiliency to environmental challenges. Therefore, the goal of this longitudinal observational study was to investigate the GI microbial composition of children aged 4-8 years and determine whether associations exist between microbial composition and specific dietary patterns. We hypothesized that children with an eating pattern containing fiber-rich foods such as fruits, vegetables, legumes, and whole grains, would be associated with a more diverse microbiota which would be more stable over time, whereas a diet high in animal products and processed foods would be associated with less beneficial bacterial composition (e.g., butyrate producers, bacterial species known to protect against pathogens) and would be more variable over time.

2. Materials and methods

2.1. Participants and baseline questionnaire

Children between 4 and 8 years-of-age were recruited in the Champaign-Urbana, IL area between April 2016 and September 2016. Subjects were recruited as part of a larger study currently being conducted in the laboratory. Data presented herein was selected to serve as a pilot analysis to investigate the impact dietary patterns on the GI microbiota in children. All subjects were free of functional digestive disorders, had not used antibiotics, probiotics or prebiotic in the 3 months prior to enrollment in the study, did not take any medication and did not follow any special diet (e.g., gluten-free/casein-free diet). Parents completed a baseline questionnaire including questions regarding their child's age, gender, early feeding practices, mode of delivery, nutritional supplement use, height and weight. The height, weight and BMI of the participants were converted to percentiles according to the CDC growth charts for both male and female participants. Questionnaires at the 6-week and 6-month time points included questions on newly introduced medications and changes in gastrointestinal health in order to control for potential impact on changes in GI microbiota. Participants provided oral assent and their legal guardians provided written consent in accordance with the ethical standards of the Institutional Review Board of the University of Illinois at Urbana-Champaign.

2.2. Fecal sample collection, DNA extraction and 16S rRNA sequencing and analysis

For each subject, freshly-voided morning stool samples were collected at enrollment (baseline), 6-weeks and 6-months post-baseline for the analysis of the fecal microbiota and volatile fatty acids (VFA) concentrations. Stool samples were collected into a plastic commode specimen collection system (Fisher Scientific, Waltham, MA). Parents were provided with gloves and a sterile spoon to transfer ~5 to 10 g of freshly-voided fecal material into a sterile 50 mL conical tube. All samples were immediately placed in the participant's freezer (-20°C) until transported to the laboratory. All samples were stored in the laboratory at -80°C prior to analysis.

Microbial DNA was extracted from stool using a bead beating method followed by a combination of QIAamp Fast DNA Stool Mini Kit (Qiagen, Valencia, CA) and the FastPrep-24 System (MP Biomedicals, Carlsbad, CA), as previously described [22]. DNA concentration was measured using a NanoDrop 1000 spectrophotometer. Amplification of the V3 to V4 regions (ca. 430 bp) of 16S rRNA gene was performed using dual-index paired-end sequencing approach using primers F357 and R805 [23]. The AccuPrime™ Taq DNA Polymerase System (Life Technologies, Grand Island, NY) was used for PCR amplification in a DNAEngine (Bio-Rad, Hercules, CA). The amplicons were mixed in equimolar concentration and sequenced at the W. M. Keck Center at the University of Illinois, Urbana-Champaign. Paired-end sequencing (2 x 300 bp) was performed with an Illumina MiSeq (Illumina, Inc., San Diego, CA) using version 3 chemistry.

The 16S rRNA sequences were processed and analyzed using the QIIME 1.9.1 bioinformatics package [24]. Briefly, sequences were demultiplexed and clustered into operational taxonomic units (OTUs) using the closed-reference OTU picking algorithm with default parameters against the Greengenes 13_8 reference OTU database at a 97% similarity level. Singletons and OTUs with an abundance lower than 0.005% were removed [25] prior to rarefying the OTU table to a sampling depth of 39,140 sequences per sample using the alpha_rarefaction command. The rarefied PTU table was used for subsequent analysis.

2.3. VFA analysis

Sample preparation and analysis for VFA analyses was performed as previously described [22]. VFA production was calculated as VFA concentrations of substrate-containing tubes minus the VFA content of blank tubes divided by substrate weight expressed on a dry matter basis [22].

2.4. Dietary patterns

A revised version of the semi-quantitative Youth and Adolescent Food Frequency Questionnaire (YAQ) containing 156 food items was completed by the parents to estimate their child's usual food and beverage intake over the past year [26]. Parents completed the YAQ at all three sample collection time points. However, for purpose of this analysis only baseline dietary patterns were included. The questionnaire was modified for parent report as previously described [26]. The original YAQ has been tested for reproducibility and validity [27]. The questionnaire has five possible responses ranging from "never or less than once per month" to "more than 4 times per week". The 156 foods items were grouped *a priori* into 13 food groups (e.g. fruits, vegetables, grains, sweets) (Supplemental Table 1) using methods similar to those described in previous studies of dietary patterns and disease [28]. To estimate the number of servings of any food group, each response was converted to the corresponding frequency factor (Harvard T.H Chan School of Public Health Nutrition Department's Food Group Serving Table) and summed over all the food items to get the average servings of a specific food group per day.

2.5. 3-day food diary

To monitor short-term nutrient intake prior to fecal sample collection, parents were asked to report all types and quantities of food and beverages consumed by their children during the three-day (two week days, one weekend day) period prior to each fecal sample collection. Parents were trained by a member of the research team on how to record their child's meal on the food record. Additionally, parents were provided with a detailed description of how to keep a food record. After collection of the food record a member of the research team followed up with the parent if additional detail was needed. The food diary data were analyzed using the Nutrition Data System for Research (NDSR, Minneapolis, MN, 2014) software.

2.6. Assessment of gastrointestinal symptoms

Parents completed a questionnaire assessing the severity of their child's GI symptoms, including constipation, diarrhea, stool smell, flatulence and abdominal pain in an adapted version of the Gastrointestinal (GI) Severity Index [29]. Scores for each item were summed to determine an overall severity score. Additionally, stool consistency was assessed using the Bristol Stool Chart [30].

2.7. Statistical analysis

Descriptive statistics (means and frequencies) were generated for all demographic and epidemiologic variables of study participants. Dietary patterns data derived from the YAQ were derived using Principal Component Analysis and Factor Analysis with Varimax rotation and factor scores were calculated as previously described [31] Scores for dietary patterns were calculated for each participant and stratified as either falling above or below the median. All subsequent data presented in the manuscript was analyzed for significant differences between the two groups (above vs. below median).

Differences in microbial community structure were evaluated with principal coordinate analysis (PCoA) and permutational multivariate analysis (PERMANOVA) of variance using weighted and unweighted UniFrac distance in the QIIME software. Differences between the groups for each dietary pattern for all at the baseline sample collection were analyzed using Wilcoxon Rank Sum Test. Categorical variables were analyzed for significant differences using Chi-Square Test.

Longitudinal dynamics in the microbiota composition and changes in dietary intake derived from the 3-day food record were analyzed for significant differences between the two groups (above vs. below median) using generalized linear mixed models. Normally distributed data were analyzed using the PROC MIXED procedure and non-normally distributed outcomes were analyzed using PROC GLIMMIX procedure. Each model for the microbiota analysis was controlled for factors known to affect the gut microbiota composition including age, gender, BMI, introduction of medication, changes in GI health, season of sample collection and dietary fiber intake as well as nutrients and food groups whose intake changed significantly over the 6 months study period. Correlations between specific food groups and bacterial abundance were assessed using Spearman correlation coefficient. Data are expressed as mean ± SD. Level of significance was set at $P \leq 0.05$ and $P \leq 0.10$ was considered a trend. All data were analyzed using SAS version 9.4 (SAS Institute, Cary, NC).

3. Results

3.1. Participant characteristics

A sample of 22 subjects was selected for analysis for this manuscript. All three samples were collected from 20 subjects; however, 2 subjects failed to provide a sample at the 6-months sample collection. Thus, 22 samples were collected for baseline and 6 weeks and 20 samples were collected for the 6-month time point. The study population demographics are shown in Table 1. The mean age of