Title

The effect of legume supplementation on the gut microbiota in rural Malawian infants aged 6 to 12 months

Journal

American Journal of Clinical Nutrition, 111(4)

ISSN

0002-9165

Authors

Ordiz, M Isabel
Janssen, Stefan
Humphrey, Greg
et al.

Publication Date

2020-04-01

DOI

10.1093/ajcn/nqaa011

Peer reviewed

The effect of legume supplementation on the gut microbiota in rural Malawian infants aged 6 to 12 months

M Isabel Ordiz,[1] Stefan Janssen,[2] Greg Humphrey,[2] Gail Ackermann,[2] Kevin Stephenson,[1] Sophia Agapova,[1] Oscar Divala,[3] Yankho Kaimila,[3] Ken Maleta,[3] Caroline Zhong,[1] Rob Knight,[2,4,5,6] Indi Trehan,[1,7] Phillip I Tarr,[1] Brigida Rusconi,[1] and Mark J Manary[1,3,8]

[1]Department of Pediatrics, Washington University at St. Louis, St. Louis, MO, USA; [2]Department of Pediatrics, University of California San Diego, La Jolla, CA, USA; [3]Department of Community Health, College of Medicine, University of Malawi, Malawi; [4]Department of Computer Science and Engineering, University of California San Diego, La Jolla, CA, USA; [5]Department of Bioengineering, University of California San Diego, La Jolla, CA, USA; [6]Center for Microbiome Innovation, University of California San Diego, La Jolla, CA, USA; [7]Department of Paediatrics and Child Health, University of Malawi, Blantyre, Malawi; and [8]USDA/Agricultural Research Service Children's Nutrition Research Center, Houston, TX, USA

ABSTRACT

Background: Common bean and cowpea contain about 25% protein and 25% fiber, and are recommended as complementary foods in sub-Saharan Africa.

Objective: The objective of this study was to determine if a daily legume supplement given to Malawian infants aged 6 to 12 mo alters the 16S configuration of the fecal microbiota as read out by amplicon sequence variants (ASVs).

Methods: This study was conducted within the context of a randomized, double-blind, controlled clinical trial to assess whether cowpea or common bean supplementation reduced intestinal permeability or increased linear growth. There were 2 village clusters in which the study was conducted. Fresh stool collections were flash frozen from 236 infants at ≤6 time points. The stools were sequenced using Earth Microbiome project protocols and data were processed using Qiime and Qiita, open-source, validated software packages. α-diversity was measured using the Faith's test. The 16S configuration was characterized by determining the weighted UniFrac distances of the ASVs and comparing them using permutational multivariate ANOVA.

Results: Among the 1249 samples analyzed, the α-diversity of the fecal microbiome was unchanged among subjects after initiation of legume supplementation. Neither cowpea nor common bean altered the overall 16S configuration at any age. The 16S configuration differed between children with adequate and poor linear growth aged from 6 to 9 mo, but no specific ASVs differed in relative abundance. The 16S configuration differed between children with normal and abnormal intestinal permeability at 9 mo, but no specific ASVs differed in relative abundance. Among categorical characteristics of the population associated with different 16S configurations, village cluster was most pronounced.

Conclusion: Legume supplementation in breastfed, rural African infants did not affect the structure of the gut microbial communities until the children were aged 9 mo. This trial was registered at clinical trials.gov as NCT02472262. *Am J Clin Nutr* 2020;111:884–892.

Keywords: legumes, fecal microbiota, African infants, cowpea, *Veillonella spp*, *Lactobacillus mucosae*

Introduction

Suboptimal child growth and development, chiefly embodied as stunting, is a key indicator of chronic, functional disability. Reducing stunting rates has been the focus of a myriad of clinical investigations, including efforts to improve complementary feeding and gut health. One intervention trial recently demonstrated improved linear growth in a population of infants vulnerable

Supported by the United States Agency for International Development (USAID), as part of Feed the Future, the US government's global hunger and food security initiative, under the terms of Cooperative Agreement No. EDH-A-00-07-00005-00, and the Children's Discovery Institute of Washington University and St. Louis Children's Hospital. The opinions expressed herein are those of the authors and do not necessarily reflect the views of USAID or the US government. The funders played no role in the study design, data collection, data analyses, or writing of the manuscript.

MJM is an associate editor of AJCN and has played no role in the journal's evaluation of the manuscript.

Supplemental Tables 1–3 and Supplemental Figures 1 and 2 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

Present address for SJ: Algorithmic Bioinformatics, Faculty of Biology and Chemistry, Justus Liebig 32 University Giessen, Germany.

MIO and SJ: are considered as first authors; BR: is considered as a senior author.

Address correspondence to MJM (e-mail: manary@kids.wustl.edu).

Abbreviations used: ASV, amplicon sequence variant; EBI, European Bioinformatics Institute; L: M test, lactulose: mannitol test; ΔLAZ, change in length-for-age z-score; 16S, 16S rRNA gene amplicon sequencing.

Received September 14, 2019. Accepted for publication January 21, 2020.

First published online February 11, 2020; doi: https://doi.org/10.1093/ajcn/nqaa011.

to stunting using cowpea supplementation (1), which provides dietary protein and fiber.

Gut microbial communities are a determinant of human health. The diet, and dietary fiber in particular, are primary determinants of the gut microbiota (2, 3) and dietary supplementation can alter the gut microbiota. Descriptive studies of the gut microbiota using 16S rRNA gene amplicon sequencing (16S) have identified configurations that are associated with normal linear growth (4).

Here, extensive fecal 16S sequencing of healthy rural African children aged from 6 to 12 mo participating in a legume supplementation trial was used to test the following hypotheses: *1*) introduction of a substantial amount of fiber-rich legume into the daily diet at age 6 mo will reconfigure the fecal microbiota, and *2*) children with either poor linear growth and/or increased intestinal permeability will have different 16S configurations than those with adequate linear growth and/or normal intestinal permeability.

Methods

Participants

All children aged 5.5 to 6.5 mo resident in the Masenjere or Limela village clusters in southern Malawi were eligible to participate in a supplementary legume feeding study of 6-mo duration, which has been previously reported (1). Of the 291 participants who completed the trial, a randomized subset of 236 children were selected for fecal 16S sequencing at 6 time points. Exclusion criteria for the primary clinical trial were the presence of acute malnutrition, receiving supplemental food from another intervention program, a chronic noninfectious disease, or a congenital abnormality. Ethical approval was obtained from the University of Malawi College of Medicine Research and Ethics Committee and the Human Research Protection Office at Washington University in St. Louis. The study was explained to each child's caregiver by a native Malawian Chichewa-speaking nurse and verbal and written consent were obtained. Caregivers who could not sign their names documented consent with a thumbprint.

The trial was conducted from July 2015 to October 2016 (1, 5). The residents of these villages lived in houses made from mud and hatch, with extremely limited access to electricity; their water source was from boreholes and shallow wells. They practiced subsistence farming, where maize was the main crop harvested annually after the rainy season. During the first year of life, all the infants were breastfed ad libitum and consumed corn porridge as the primary complementary food.

Study design

This was a 24-wk prospective, randomized, double-blinded, controlled clinical trial in which the intervention groups received a daily ration of cowpea or common bean, whereas the control group received corn-soy blend flour. The study protocol has been previously published (5) and the trial is registered at clinicaltrials.gov as NCT02472262. The primary outcomes were change in length-for-age z-score (ΔLAZ) and reduction in the percentage of excreted lactulose after oral challenge, a test of intestinal permeability. The primary outcomes have been previously reported (1). Secondary outcomes were differences in the 16S configuration of the fecal microbiota among children receiving different complementary foods or with differing linear growth or intestinal permeability. Of the 291 children who completed the primary trial, 236 were randomly selected using a spreadsheet random number generator (Excel, Microsoft), and these children had extensive fecal 16S assessment to determine the secondary outcomes related to the microbiota. It was necessary to create the subset for analyses of 16S configuration due to resource constraints.

Participation

At the first visit, the children's anthropometry was measured and sociodemographic and health information were collected. Subjects were then given a 6-wk supply of flour. Caregivers were educated by study nurses on how to prepare and feed the flour to their infants.

A fresh fecal sample was collected at the time of enrollment, which was transferred to cryovials that were then immersed and stored in liquid nitrogen. Stool was transferred weekly from the liquid nitrogen to a −80°C freezer. A dual sugar (lactulose: mannitol, L: M) test was also performed in a rigorous manner, as previously described (6).

Anthropometric measurements, a symptom and compliance survey, and stool collection were repeated when each child was aged 6.5, 7.5, 9, 10.5, and 12 mo, whereas the L: M test was repeated when each child was aged 9 and 12 mo. Intervention flour was distributed every 6 wk by study nurses.

Study foods

Cowpea and common bean were chosen as complementary food interventions for this population because they contain 25% protein and were not routinely consumed by this population. Cowpeas and common beans were purchased at local markets, roasted to an internal temperature of 120–130°C for 45 min, and milled into flour (7). The control group received a traditional complementary food of extrusion cooked corn and soybean flour. All groups received 40% of the recommended complementary food intake from the supplements, 80 kcal/d aged from 6 to 9 mo and 120 kcal/d aged from 9 to 12 mo. The cowpea and common bean contained 21% and 28% fiber, respectively, whereas the control supplement had 12% fiber (8). Study nurses instructed caregivers to add either the legume or the control flour to the child's serving of porridge once daily, though several children in the study preferred to eat the legume flour plain. Compliance was assessed with random home visits.

Two 24-h dietary recalls were conducted on random subsets of 50 subjects in October 2015 and February 2016, which were times of relative food abundance and scarcity, respectively, to assess dietary intake. During all of the regular follow-up visits, each caretaker was asked about the habitual family consumption of cowpea and common bean to determine how much the habitual diet might be confounding the intervention.

Fecal DNA extraction, 16S sequencing, and bioinformatic analysis

The Earth Microbiome project protocols were followed to perform 16S rRNA assessment (9). Briefly, DNA was extracted using the DNeasy® PowerSoil® kit (Qiagen) using QIAcube following the manufacturer's instructions, and the V4 region

of the 16S rRNA gene was amplified using barcoded primers (10). PCR was performed in triplicate for each sample and V4 paired-end sequencing (10) was performed using Illumina MiSeq (Ilumina). Raw sequencing data from 4 MiSeq runs were uploaded to Qiita (11). Within Qiita, Illumina reads were demultiplexed and quality controlled using the defaults, as provided by QIIME1 1.9.1 (12). A total of 150 nucleotide forward reads were used to generate the primary amplicon sequence variant (ASV) table using Deblur (13); the resulting table was filtered to exclude samples with <5000 reads. Phylogeny was reconstructed via SEPP against the Greengenes 13.8 reference database. The resulting tree was used to compute Faith's α-diversity (15) and weighted UniFrac (14) distances for ß-diversity. Weighted UniFrac distance matrices were calculated in R (16) using the phyloseq version 1.26.1 (QIIME1) (17) package. Taxonomic labels of ASVs were assigned based on the ribosomal database project (Michigan State University) classifier algorithm with kmer size 8 and 100 bootstrap replicates, with species-level assignment only performed for 100% matches, against the ribosomal database project database 16 within the dada2 package version 1.10.1 (QIIME1) and confirmed by manual comparison using the basic local alignment search tool for nucleotides (US National Library of Medicine) for ASVs identified as significantly different. To compare with previous analyses, annotation at 97% for species is also reported for significant ASVs (**Supplementary Table 1**). ASV sequences are provided in **Supplementary Table 2**. All raw sequence data can be found in the European Bioinformatics Institute (EBI) under accession number EBI: ERP106623.

Statistical analyses

Anthropometric indices were calculated based on the WHO's 2006 Child Growth Standards using Anthro v 3.1 (WHO). Clinical and demographic data corresponding to each 16S-sequenced sample were tabulated by complementary food group. These data were compared using 1-factor ANOVA and the Fisher's exact test 2×3 to determine if there were differences between the dietary groups of this subset. A P <0.05 was considered to be significant.

Change in linear growth was assessed over 4 intervals, aged from 6 to 7.5 mo, >7.5 to 9 mo, >9 to 10.5 mo, and >10.5 to 12 mo, and determined by calculating ΔLAZ. A ΔLAZ < −0.2 per 6-wk period was designated as poor linear growth, in accordance with the linear growth rates described as faltering in a recent meta-analysis, and ΔLAZs ≥ −0.2 per 6-wk period were deemed as adequate linear growth (18). Intestinal permeability was categorized as in the primary clinical trial report as lactulose excretion <0.2% being normal and lactulose ≥0.2% being abnormal.

Linear mixed effect models via q2-longitudinal's "linear mixed effects" command (19) was used to test whether first difference and baseline difference of *1*) Faith's Phylogenetic Diversity Index differed between the intervention groups and *2*) the weighted UniFrac distances of the 16S configuration differed between the intervention groups. These analyses used the following fixed effects: age, sex, and village cluster. Dietary intervention and time slope were random effects. The results are displayed as volatility plots with spaghetti representations of each child's 16S sequence to emphasize the longitudinal nature of the data. Only subjects with data available at each time point (136 individuals) were kept prior to genus-level collapse for feature selection. Features of interest were identified by a random forest classifier. Temporal trends of ASVs were visualized with volatility plots (q2-longitudinal plugin). The data was further divided based on the 9-mo time point when legume intake was increased. For each feature we used an interaction model with month, intervention, sex, and village as fixed effects, and individual and month as random effects. Depending on the feature, models were simplified and only significant interactions were kept.

PERMANOVA, a multivariate ANOVA with permutations, was used to identify differences between the weighted UniFrac distances from the different categorical designations of 16S configurations: children with adequate or poor linear growth, children with normal or abnormal intestinal permeability, children with or without access to clean water, children resident in Masenjere or Liemla, and children with a history of some or no antibiotic usage. For linear growth and intestinal permeability, comparisons were made only between children of a similar age. Two-tailed P values <0.05 were considered significant. The differential abundances in ASVs were identified using ANCOM (analysis of composition of microbes) in Qiime2–version 2018.11 (12).

Results

After sequencing, ASV assignment, and filtering of these 1416 samples, 1249 samples were analyzed for the 16S profile (**Supplemental Figure 1**). Among the clinical, anthropometric, environmental, and demographic characteristics in each of the 3 dietary supplement groups, only roofing material differed (**Table 1**). The number of stools sequenced at different ages is shown in Supplemental Figure 1, demonstrating comprehensive, longitudinal sampling throughout the course of participation.

Compliance was assessed with 537 home visits. No flour remained in the home at 6% of these visits, and the amount of flour remaining was, on average, 90% of what was expected based on correct use. Caregivers reported missing a daily ration of legume flour at 7% of the visits.

Among the control group during the 6-mo participation period, 22/76 caretakers reported consuming cowpea ≥1 time, and 4/76 control caretakers reported consuming cowpea weekly; 29/76 caretakers reported consuming common bean ≥1 time, and 10/76 reported consuming common bean weekly.

The 10 ASVs with the largest relative abundances identified in these samples are shown in **Figure 1**; the groups aged 7.5 mo and 10.5 mo are displayed as examples. There were no differences is Faith's Phylogenetic Diversity for the 3 supplementary foods (linear mixed effects model, z statistic = 0.69 and P = 0.43 for common bean, z statistic = 0.64 and P = 0.73 for cowpea, model includes age, sex, village cluster, and subject, **Figure 2**). No differences in the 16S configuration were caused by legume feeding (z statistic = −1.61 and P = 0.11 for common bean, z statistic = −0.015 and P = 0.99 for cowpea, model includes age, sex, village cluster, and subject, **Figure 3**). The 11 ASVs came from 8 genera, these genera were also tested

TABLE 1 Characteristics of Malawian children receiving cowpea or common bean[1,2]

	Cowpea ($n = 82$)	Common bean ($n = 78$)	Control group ($n = 76$)	P
Female	41 (50)	37 (47)	35 (46)	0.86
Age, mo	5.8 ± 0.2	5.8 ± 0.2	5.8 ± 0.3	0.99
Father living in home	67 (82)	68 (87)	63 (83)	0.65
Siblings	2.4 ± 1.9	2.5 ± 1.9	2.4 ± 2.0	0.93
Clean water source	60 (73)	58 (74)	51 (67)	0.58
Animals sleep with the child	25 (30)	30 (38)	20 (26)	0.26
Mother washes hands often when preparing food	46 (56)	46 (59)	39 (51)	0.65
Midupper arm circumference, cm	14.2 ± 0.9	14.0 ± 0.7	14.1 ± 1.0	0.35
Weight-for-length, z-score	0.5 ± 0.9	0.3 ± 0.8	0.4 ± 0.9	0.35
Length-for-age, z-score	−1.2 ± 1.2	−1.1 ± 1.0	−1.2 ± 1.0	0.41
Weight-for-age, z-score	−0.5 ± 0.9	−0.5 ± 0.8	−0.5 ± 0.9	0.99
Diarrhea in past week	12 (15)	12 (15)	10 (13)	0.95
Fever in past week	28 (34)	24 (31)	21 (28)	0.67
Radio in home	34 (41)	24 (31)	25 (33)	0.33
Bicycle in home	41 (50)	38 (49)	35 (46)	0.86
Roof made of metal	23 (28)	25 (32)	9 (12)	0.01
Breastfed	81 (99)	78 (100)	76 (100)	1.0
Village, Limela	32 (39)	35 (45)	35 (46)	0.63

[1] Values are means ± SD or *n* (%).
[2] Comparisons made with Fisher's exact test 2 × 3 for categorical characteristics and with 1-factor ANOVA for continuous characteristics.

using the linear mixed effects action to determine if their relative abundance was affected by legume feeding. Consuming cowpea caused a higher relative abundance of Bifidobacterium during the ages of >9 to 12 mo than consuming common bean or control feeding (z statistic = 2.49 and $P = 0.013$), lower relative abundances of *Prevotella* during the ages of 6 to 12 mo (z statistic = −2.39 and $P = 0.02$), and lower relative abundances *Escherichia/Shigella* during the ages of 6 to 9 mo (z statistic = −2.97 and $P = 0.003$). These weighted UniFrac comparisons were similar whether the 4 control children who were reported to consume cowpea at least weekly were excluded or included.

From the ages of 6 to 7.5 and >7.5 to 9 mo, children with poor linear growth had a different 16S configuration than those with adequate linear growth (pseudo F = 2.74, $P = 0.042$ and pseudo F = 3.38, $P = 0.016$, respectively). The most abundant ASVs are shown in **Figure 4**. No ASVs were identified by ANCOM as differing in relative abundance for children with adequate growth and faltering growth.

Aged 9 mo, the 16S configuration differed between children with normal and abnormal intestinal permeability (pseudo F = 4.32, $P = 0.006$). No differences were found between children with normal and abnormal intestinal permeability aged 6 and 12 mo. No ASVs were identified by ANCOM as differing in relative abundance when children aged 9 mo with normal and abnormal intestinal permeability were compared.

The use of clean water within Limela was associated with differences in 16S configuration (PERMANOVA t-statistic = 3.76, P = 0.018).

Of the 103 courses of antibiotics received by the children, 84 (82%) were trimethoprim/sulfamethoxazole and 12 (12%) were amoxicillin. Children who received a course of antibiotics did not have a different 16S configuration when compared with children who did not receive antibiotics (PERMANOVA t-statistic = 2.42).

The 16S configuration of the microbiota differed between children from Masenjere and Limela (PERMANOVA t-statistic = 35.7, $P = 0.001$). The population characteristics of these 2 village clusters differed in that children from Masenjere reported better hand sanitation, had more access to clean water, were more likely to keep animals in the home, had better access to health care services, utilized more antibiotics, were wealthier, and had greater intestinal permeability (**Supplemental Table 3**). Differential abundance by ANCOM identified multiple ASVs that differed based on village; a higher relative abundance of ASVs matching members of the genera *Collinsella*, *Olsenella, Prevotella,* and *Dialister* spp. was seen in Masenjere. In Limela, ASVs mapping to the genera *Veillonella*, and 1 ASV for *Megasphaera elsdenii* and *Lactobacillus mucosae* were significantly higher (**Supplemental Figure 2**).

Discussion

The introduction of cowpea as a daily complementary food from the ages of 6 to 12 mo in rural Malawian children did not alter the 16S configuration of the fecal microbiota, as represented by a weighted UniFrac matrix using a longitudinal analyses method. Cowpea supplementation improved linear growth in this population from the ages of 6 to 9 mo. Coincident with this differential linear growth, the 16S configuration differed between children with adequate growth and faltering growth. Intestinal permeability was associated with the 16S configuration at the age of 9 mo.

Although the sample size was large and taken from individuals at multiple points in time, the study is limited in that complete microbiome data were not obtained. Shotgun metagenomics covering the whole genome was not performed, but rather targeted sequencing of the 16S rRNA gene was employed. Thus, differences in the metabolic capacity of the microbiota as a whole

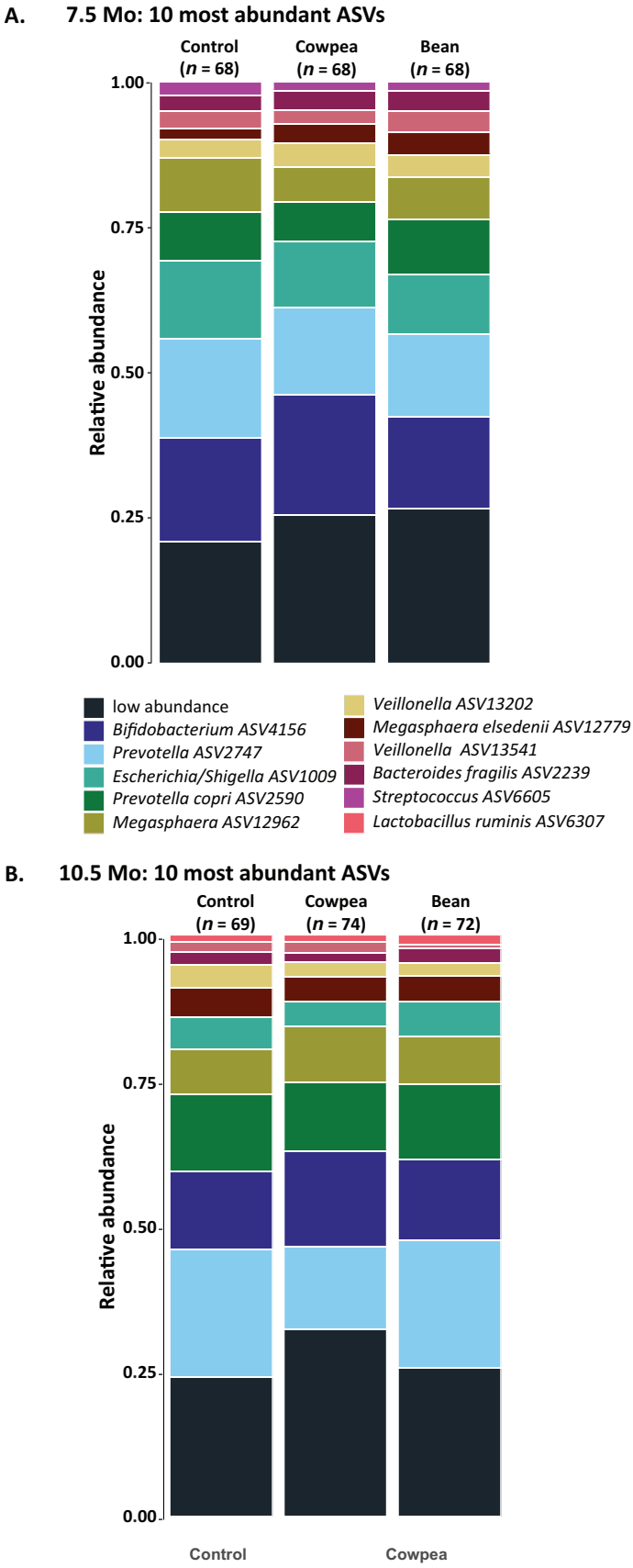

FIGURE 1 Mean relative abundance of the most common amplicon sequence variants in the fecal microbiota of Malawian infants at different ages divided by food intervention. A) aged 7.5 mo and B) aged 10.5 mo.

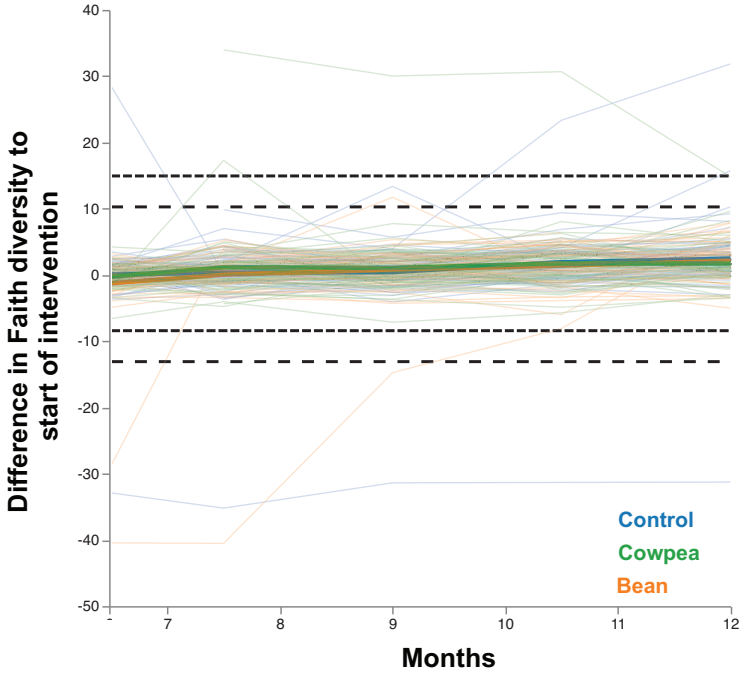

FIGURE 2 Faith's diversity of 16S fecal samples from Malawian infants according to their age and food consumed during the study (control, cowpea, common bean). Data presented as a spaghetti plot over age from 6.5 to 12 mo. Comparisons of diversity within each of the supplement groups did not vary significantly over time (linear mixed effects model, $P = 0.43$ for common bean, $P = 0.73$ for cowpea, model includes age, sex, and village cluster as fixed effects).

with the introduction of cowpea were not elucidated. Therefore, our data does not enable the opportunity to describe how within a species some community members may affect other members, as has been done previously with *Bacteroides fragilis* (20). As the microbiota demonstrate considerable diversity in settings across the world, our findings cannot be generalized outside of the rural sub-Saharan African context. Likewise, our study focused on breastfed infants aged from 6 to 12 mo and cannot be generalized

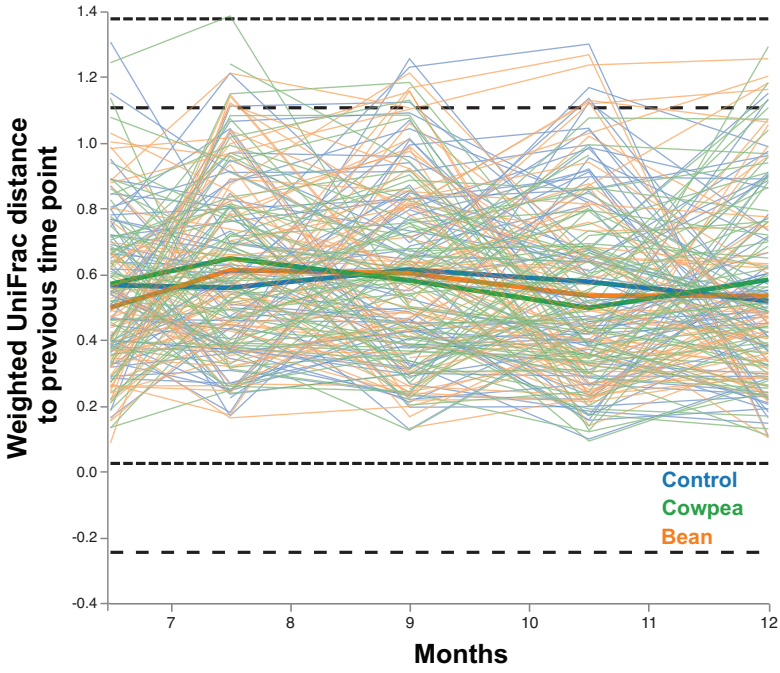

FIGURE 3 Comparison of ß-diversity of fecal 16S configurations measured using weighted UniFrac distances for Malawian children aged 6 to 12 mo receiving 1 of 3 supplementary foods: control, cowpea, or common bean. Configuration of sequences compared with the most recent previous sequence from the same child, children were measured aged 6, 6.5, 7.5, 9, 10.5, and 12 mo (linear mixed effects model, $P = 0.58$ for common bean, $P = 0.85$ for cowpea).

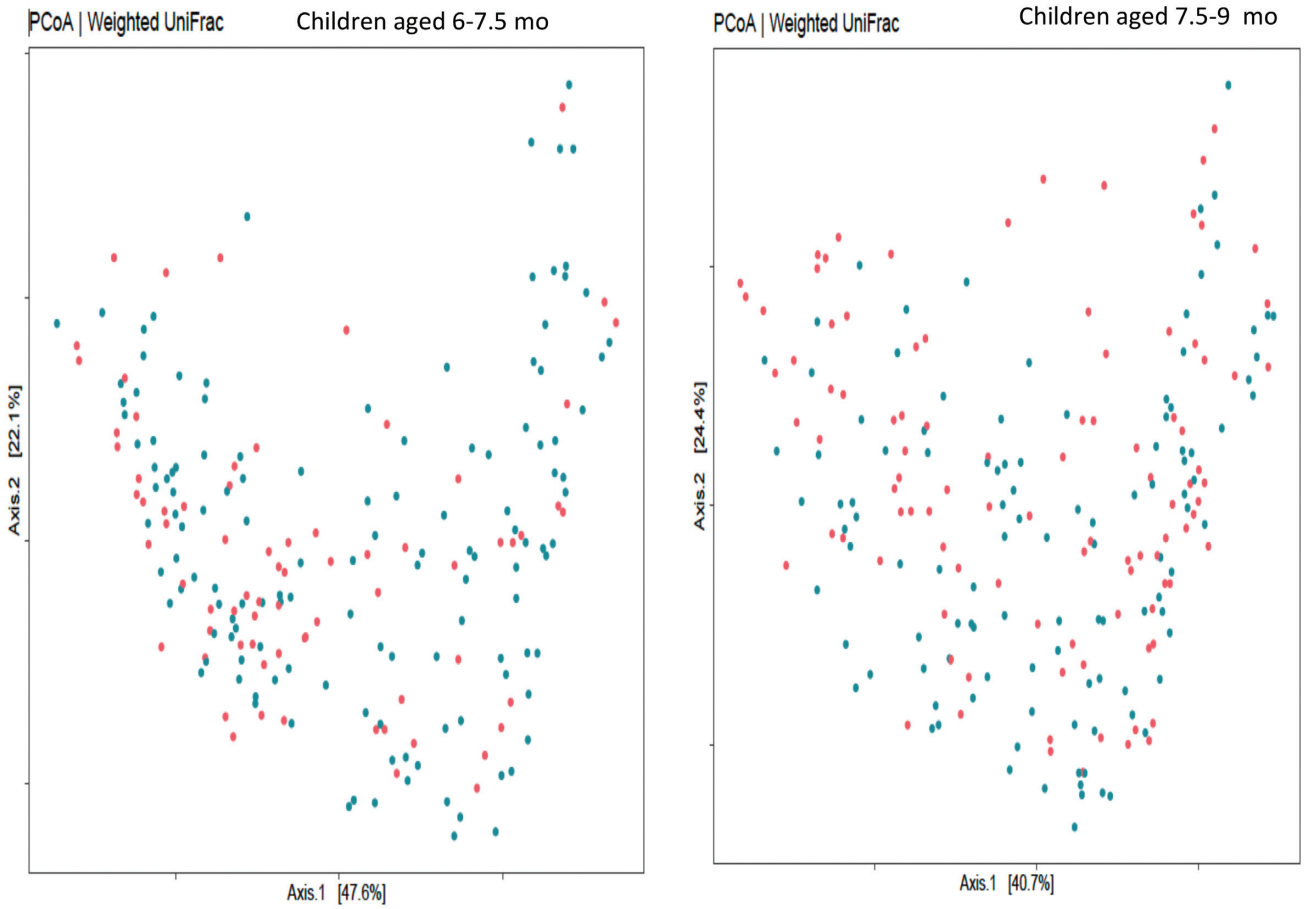

FIGURE 4 Microbial communities clustered using principal coordinates analysis (PCoA) of a weighted UniFrac matrix, plot compares children with adequate linear growth (blue dots) and faltering growth (red dots) aged 6 to 7.5 mo and >7.5 to 9 mo. A Δ length-for-age z-score (Δ LAZ) < −0.2/6 wk was designated as poor linear growth and ΔLAZ ≥ −0.2/6 wk was deemed as adequate. 16S configurations of growth from the ages of 6 to 7.5 mo and >7.5 to 9 mo differed when compared by PERMANOVA ($P = 0.04$, $P = 0.01$, respectively).

to individuals outside of this demographic. In this study we did not observe the expected effect of a reconfiguration of the microbiota with a dietary supplement. One potential explanation for this would be noncompliance with the intervention, however, our in-home surveys and inventories indicate compliance was excellent.

The inability of a "substantial" amount of dietary fiber to cause discernable changes in the 16S configuration is in contrast to what has been observed in other populations and animal models (21). When dietary interventions reconfigured the gut microbiota, these changes were seen over periods of weeks and months, which this study thoroughly encompasses (22). All of the children studied were breastfed ad libitum. Breast milk contains large amounts of prebiotics in the form of human milk oligosaccharides and lactose, which comprise over 50% of its dry matter content. This attests to the prominent role that breast milk plays in establishing and maintaining the configuration of the microbiota, and the stability it confers to the infant gastrointestinal tract in low-resource settings. This also speaks to the importance of microbiota working in synergy with the infant intestine for maturation and survival (23, 24).

When children aged 6 and 7.5 mo were divided into categories of adequate growth and faltering growth, different 16S configurations were noted. The differences were subtle in that shifts in relative abundance of specific ASVs were not seen, and the principle coordinate analyses plots appear very similar (Figure 4). This alteration of 16S configuration was coincident with cowpea increasing linear growth. Certainly, both linear growth and the configuration of the gut microbiota are complex, essential biological processes that must impact each other. While this study provides no information about the pangenome of each species, gene expression, and/or metabolite production, it might be that cowpea is acting synergistically with a favorable 16S configuration to affect linear growth.

This study also identified provocative data at the level of community of residence. Specifically, village clusters were distinguished by different 16S fecal configurations, which was the most pronounced difference in 16S configurations observed in this study. Masenjere infants had greater lactulose permeability, more acute malnutrition, and poorer linear growth than Limela infants (25). Paradoxically, Masenjere had cleaner water, a higher rate of maternal handwashing, was wealthier, with better access to health care, receives more antibiotics, and had greater dietary energy intake than Limela. The elevation of Limela is about 740 meters and Masenjere 106 meters. The lower elevation in Masenjere allows a humid tropical climate 12 mo a year,

which is associated with continuous malaria transmission. In contrast, the higher elevation in Limela allows a dry, cool season, with average daily temperatures of ∼15°C, which interrupts malaria transmission. We cannot determine from our data which attributes of Masenjere and Limela confer benefits to children, although differences in the gut microbiota could well play an important role. Malaria infection is known to affect the gut microbiota composition in mammalian hosts (26). In a Malian cohort, the reduction of *Plasmodium falciparum* infection was associated with a high abundance of *Bifidobacterium, Streptococcus, Escherichia,* and *Shigella*. In this instance, modest wealth and good sanitation practices are not enough to ameliorate the constraints on linear growth imposed by geography. The finding that geography is an important determinant of 16S configuration was demonstrated in a large study from China (27).

The increased relative abundance of *Veillonella spp* and *Megasphaera elsdenii* in Limela, the village where inhabitants had better clinical outcomes, warrants particular comment. These genera belong to the class previously referred to as *Negativicutes*, which was overrepresented in preterm infants who did not develop necrotizing enterocolitis (i.e., these organisms were protective). *Veillonella* primarily, and to a much lesser extent *Megasphaera*, were components of the putatively protective *Negativicutes* populations in neonatal intensive care units in St. Louis, Oklahoma City, and Louisville (28). *Veillonella* was also associated with protection against necrotizing enterocolitis and with healthy growth in infants born preterm in a subsequent cohort study in Durham (29). Recent investigation of the role of small proteins made by *Veillonella spp* suggest they have a role in the bacterial defense against phages (30). Finally, *Veillonella* is associated with successfully finishing the Boston marathon, and conferred increased exercise performance on mice (31).

A greater relative abundance of *Lactobacillus mucosae* was found in Limela where there is less intestinal permeability; this is consistent with laboratory studies that show this organism strengthens the mucosal barrier by enhancing tight junctions and reduces adhesion of pathogenic enteric bacteria in animal models (32).

In conclusion, extensive 16S sequencing of fecal samples from a trial of cowpea supplementation in rural African infants did not reveal a microbiota signature for stunting. Observations concerning *Lactobacillus mucosae,* thought to enhance tight junction integrity, from this trial may fuel further hypothesis testing regarding environmental enteric dysfunction. The association of the *Veillonella* genus in the feces with health benefits in several populations warrants further investigation.

The authors' responsibilities were as follows—KS, SA, KM, IT, RK, and MJM: designed the research; KS, SA, OD, YK, MIO, CT, and IT: conducted the clinical research and collected the samples; SJ, GH, GA, and RK: sequenced and processed the 16S data; SJ, KS, SA, OD, YK, MIO, BR, IT, and MJM: analyzed data; MIO, SJ, BR, and CZ: created the figures and graphically analyzed the data; SJ, BR, PIT, IT, RK, MIO, and MJM: interpreted the data; MIO, SJ, BR, and MJM: wrote the first draft of the manuscript; MJM: has primary responsibility for the final content; and all authors: read and approved the final manuscript. The authors report no conflicts of interest.

References

1. Stephenson KB, Agapova SE, Divala O, Kaimila Y, Maleta KM, Thakwalakwa C, Ordiz MI, Trehan I, Manary MJ. Complementary feeding with cowpea reduces growth faltering in rural Malawian infants: a blind, randomized controlled clinical trial. Am J Clin Nutr 2017;106:1500–7.
2. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling AV, Devlin AS, Varma Y, Fischbach MA, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature 2014;505:559–63.
3. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, Magris M, Hidalgo G, Baldassano RN, Anokhin AP, et al. Human gut microbiome viewed across age and geography. Nature 2012;486:222–27.
4. Robertson RC, Manges AR, Finlay BB, Prendergast AJ. The human microbiome and child growth – first 1000 days and beyond. Trends Microbiol 2019;27:131–47.
5. Trehan I, Benzoni NS, Wang AZ, Bollinger LB, Ngoma TN, Chimimba UK, Stephenson KB, Agapova SE, Maleta KM, Manary MJ. Common beans and cowpeas as complementary foods to reduce environmental enteric dysfunction and stunting in Malawian children: study protocol for two randomized controlled trials. Trials 2015;16:520.
6. Galpin L, Manary MJ, Fleming K, Ou CN, Ashorn P, Shulman RJ. Effect of *Lactobacillus* GG on intestinal integrity in Malawian children at risk of tropical enteropathy. Am J Clin Nutr 2005;82:1040–45.
7. Ngoma TM, Chimimba UK, Mwangwela AM, Thakwalakwa C, Maleta KM, Manary MJ, Trehan I. Effect of cowpea flour processing on the chemical properties and acceptability of a novel cowpea blended maize porridge. PLoS One 2018;13:e0200418.
8. Borresen EC, Zhang L, Trehan I, Nealon NJ, Maleta KM, Manary MJ, Ryan EP. The nutrient and metabolite profile of 3 complementary legume foods with potential to improve gut health in rural Malawian children. Curr Dev Nutr 2017;1:e001610.
9. Gilbert JA, Jansson JK, Knight R. The Earth Microbiome project: successes and aspirations. BMC Biol 2014;12:69.
10. Walters W, Hyde ER, Berg-Lyons D, Ackermann G, Humphrey G, Parada A, Gilbert JA, Jansson JK, Caporaso JG, Fuhrman JA, et al. Improved bacterial 16S rRNA gene (V4 and V4-5) and fungal internal transcribed spacer marker gene primers for microbial community surveys. mSystems 2015;1:e00009–15. eCollection 2016 Jan-Feb.
11. Gonzalez A, Navas-Molina JA, Kosciolek T, McDonald D, Vázquez-Baeza Y, Ackermann G, DeReus J, Janssen S, Swafford AD, Orchanian SB, et al. Qiita: rapid, web-enabled microbiome meta-analysis. Nat Methods 2018;15:796–8.
12. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 2010;7:335–6.
13. Amir A, McDonald D, Navas-Molina JA, Kopylova E, Morton JT, Zech Xu Z, Kightley EP, Thompson LR, Hyde ER, Gonzalez A, et al. Deblur rapidly resolves single-nucleotide community sequence patterns. mSystems 2017;2:e00191–16.
14. Lozupone C, Lladser ME, Knights D, Stombaugh J, Knight R. UniFrac: an effective distance metric for microbial community comparison. ISME J 2011;5:169–72.
15. Faith DP. Conservation evaluation and phylogenetic diversity. Biol Conserv 1992;61:1–10.
16. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 2013;8(4):e61217.
17. R Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2014 URL http://www.R-project.org/
18. Roth DE, Krishna A, Leung M, Shi J, Bassani DG, Barros AJD. Early childhood linear growth faltering in low-income and middle-income countries as a whole-population condition: analysis of 179 demographic and health surveys from 64 countries (1993–2015). Lancet Glob Health 2017;5:e1249–57.
19. Bokulich NA, Dillion MR, Zhang Y, Rideout JR, Bolyen E, Li H, Albert PS, Caporaso JG. q2-longitudinal: longitudinal and paired sample analyses of microbiome data. mSystems 3:e00219–18.
20. Wagner VE, Dey N, Guruge J, Hsiao A, Ahern PP, Semenkovich NP, Blanton LV, Cheng J, Griffin N, Stappenbeck TS, et al. Effects of a gut

pathobiont in a gnotobiotic mouse model of childhood undernutrition. Sci Transl Med 2016;8:366ra164.
21. Makki K, Deehan EC, Walter J, Bäckhed F. The impact of dietary fiber on gut microbiota in host health and disease. Cell Host Microbe 2018;23:705–15.
22. Uhr GT, Dohnalova L, Thaiss CA. The dimension of time in host–microbiome interactions. mSystems 2019 4:e00216–18.
23. Davis JC, Lewis ZT, Krishnan S, Bernstein RM, Moore SE, Prentice AM, Mills DA, Lebrilla CB, Zivkovic AM. Growth and morbidity of Gambian infants are influenced by maternal milk oligosaccharides and infant gut microbiota. Sci Rep 2017;7:40466.
24. Charbonneau MR, O'Donnell D, Blanton LV, Totten SM, Davis JC, Barratt MJ, Cheng J, Guruge J, Talcott M, Bain JR, et al. Sialylated milk oligosaccharides promote microbiota-dependent growth in models of infant undernutrition. Cell 2016;164:859–71.
25. Kaimila Y, Pitman RT, Divala O, Hendrixson DT, Stephenson KB, Agapova S, Trehan I, Maleta K, Manary MJ. Development of acute malnutrition despite nutritional supplementation in Malawi. J Pediatr Gastroenterol Nutr 2019;68:734–7.
26. Yooseph S, Kirkness EF, Tran TM, Harkins DM, Jones MB, Torralba MG, O'Connell E, Nutman TB, Doumbo S, Doumbo OK, et al. Stool microbiota composition is associated with the prospective risk of *Plasmodium falciparum* infection. BMC Genomics 2015;16:631.
27. He Y, Wu W, Zheng HM, Li P, McDonald D, Sheng HF, Chen MX, Chen ZH, Ji GY, Zheng ZD, et al. Regional variation limits applications of healthy gut microbiome reference ranges and disease models. Nat Med 2018;24:1532–5.
28. Warner BB, Deych E, Zhou Y, Hall-Moore C, Weinstock GM, Sodergren E, Shaikh N, Hoffmann JA, Linneman LA, Hamvas A, et al. Gut bacteria dysbiosis and necrotising enterocolitis in very low birthweight infants: a prospective case-control study. Lancet 2016;387:1928–36.
29. Younge NE, Newgard CB, Cotten CM, Goldberg RN, Muehlbauer MJ, Bain JR, Stevens RD, O'Connell TM, Rawls JF, Seed PC, et al. Disrupted maturation of the microbiota and metabolome among extremely preterm infants with postnatal growth failure. Sci Rep 2019;9:8167.
30. Sberro H, Fremin BJ, Zlitni S, Edfors F, Greenfield N, Snyder MP, Pavlopoulos GA, Kyrpides NC, Bhatt AS. Large-scale analyses of human microbiomes reveal thousands of small, novel genes. Cell 2019;178:1245–59.e14.
31. Scheiman J, Luber JM, Chavkin TA, MacDonald T, Tung A, Pham LD, Wibowo MC, Wurth RC, Punthambaker S, Tierney BT, et al. Meta-omics analysis of elite athletes identifies a performance-enhancing microbe that functions via lactate metabolism. Nat Med 2019;25:1104–09.
32. Watanabe M, Kinoshita H, Nitta M, Yukishita R, Kawai Y, Kimura K, Taketomo N, Yamazaki Y, Tateno Y, Miura K, et al. Identification of a new adhesin-like protein from *Lactobacillus mucosae* ME-340 with specific affinity to the human blood group A and B antigens. J Appl Microbiol 2010;109:927–35.