






Associations between Gut Microbiota and Intestinal Inflammation, Permeability and Damage in Young Malawian Children

Emma Kortekangas , MD,¹ Yue-Mei Fan , PhD¹
David Chaima , PhD² Kirsi-Maarit Lehto, PhD¹,
Chikondi Malamba-Banda, MPhil,² Andrew Matchado, PhD,^{2,3}
Chilungamo Chingwanda, PhD,² Zhifei Liu, MPH,¹
Ulla Ashorn, PhD,¹ Yin Bun Cheung , PhD,⁴
Kathryn G. Dewey, PhD,³ Kenneth Maleta , PhD,² and
Per Ashorn, PhD^{1,5}

¹Center for Child, Adolescent and Maternal Health Research, Faculty of Medicine and Health Technology, Tampere University, Tampere 33014, Finland

²School of Public Health and Family Medicine, College of Medicine, University of Malawi, Blantyre, Malawi

³Department of Nutrition and Institute for Global Nutrition, University of California Davis, Davis, CA 95616, USA

⁴Program in Health Services & Systems Research and Centre for Quantitative Medicine, Duke-NUS Medical School, Singapore 169857, Singapore

⁵Department of Pediatrics, Tampere University Hospital, Tampere 33520, Finland

Correspondence: Emma Kortekangas, Tampere Center for Child, Adolescent and Maternal Health Research, Faculty of Medicine and Health Technology, Global Health, Tampere University, Arvo Ylpön katu 34, Arvo building, Tampere 33014, Finland. Tel: +358-3-355-111. Fax +358-3-213-4473. E-mail <emma.kortekangas@tuni.fi>.

ABSTRACT

Background: Environmental enteric dysfunction (EED) is common in low- and middle-income countries and associated with childhood undernutrition. The composition of gut microbiota has been implicated in the pathogenesis of EED. Our aim was to assess the associations between gut microbiota and EED biomarkers in rural Malawian children. We hypothesized that there would be an inverse association between microbiota maturity and diversity and fecal concentrations of EED biomarkers.

Methods: We used data from fecal samples collected at 6, 18 and 30 months from 611 children who were followed up during a nutrition intervention trial. The primary time point for analysis was 18 months. Microbiota data were obtained through 16S rRNA sequencing and variables included microbiota maturity and diversity, phylogenetic dissimilarity and relative abundances of individual taxa. EED biomarkers included calprotectin (marker of inflammation), alpha-1 antitrypsin (intestinal permeability) and REG1B (intestinal damage).

Results: There was an inverse association between microbiota maturity and diversity and fecal concentrations of all 3 EED biomarkers at 18 months ($p \leq 0.001$). The results were similar at 30 months, while at 6 months inverse associations were found only with calprotectin and alpha-1 antitrypsin concentrations. At 18 months, EED biomarkers were not associated with phylogenetic dissimilarity, but at 6 and 30 months several associations were observed. Individual taxa predicting EED biomarker concentrations at 18 months included several *Bifidobacterium* and *Enterobacteriaceae* taxa as well as potentially displaced oral taxa.

Conclusions: Our findings support the hypothesis of an inverse association between microbiota maturity and diversity and EED in rural Malawian children.

LAY SUMMARY

Chronic childhood undernutrition is an important public health concern that affects about 150 million children, mostly in low- and middle-income countries. Undernutrition is caused by insufficient nutrient intake and frequent infections, but there are also other underlying factors. One of these is a condition called environmental enteric dysfunction (EED), which is characterized by intestinal inflammation and damage without apparent clinical symptoms. EED is thought to be caused by the ingestion of pathogenic bacteria that leads to changes in the intestine such as increased permeability and decreased absorptive capacity. This might make the intestinal wall vulnerable to bacterial invasion and reduce the absorption of nutrients. Besides potentially pathogenic bacteria, there are many commensal bacteria in the gastrointestinal tract that have beneficial functions and that interact with the immune system. The aim of our study was to assess the associations between all these bacteria, that is the intestinal microbiota and biomarkers of EED. We used data from fecal samples collected from young children participating in a nutrition intervention trial in rural Malawi. Our findings support an inverse association between the diversity and maturity of the intestinal microbiota and biomarkers of EED. Additionally, we identified the differences at the level of individual bacterial taxa (groups of bacteria defined by genetic similarity) between participants with different levels of EED biomarkers. Due to the type of study, we cannot determine whether the observed associations represent a causal relationship between the intestinal microbiota and EED. This as well as the exact mechanisms behind these associations should be assessed in further studies.

KEYWORDS: gastrointestinal microbiome, environmental enteric dysfunction, child health, leukocyte L1 antigen complex, REG1B, alpha 1-antitrypsin

INTRODUCTION

Several studies have focused on environmental enteric dysfunction (EED) as a potential underlying factor of childhood undernutrition [1–5]. In EED, a high pathogen load caused by fecal–oral contamination is thought to lead to intestinal damage and permeability, bacterial translocation and intestinal and systemic inflammation, ultimately contributing to impaired growth and development [6–10]. The exact mechanisms are unclear, and more insights into the determinants and consequences of EED are needed to improve child health outcomes in low-income settings, in which children are at a high risk for undernutrition [11].

Recent studies suggest that intestinal microbiota composition, which has been linked with undernutrition, may be associated with EED [12, 13]. Based on experimental studies in rodents, ingested pathogens

could directly cause EED and a relative reduction in anti-inflammatory intestinal bacteria could aggravate the condition [14, 15]. On the other hand, EED could alter the microbiota composition, for example through immune activation [16]. However, few studies have investigated the associations between EED and microbiota in humans [15, 17].

The aim of the present study was to investigate how the gut microbiota is associated with markers of intestinal inflammation, permeability and damage (EED biomarkers) in rural Malawian children at 6, 18 and 30 months of age. We used data from 611 children who were followed up during and after a nutrition intervention trial (iLiNS-DYAD Malawi, NCT01239693) [18, 19]. We hypothesized that there would be an inverse association between microbiota diversity and maturity and EED biomarkers [13, 20]. Additionally, we conducted

secondary exploratory analyses on associations of beta diversity and taxon-level microbiota composition with those biomarkers.

MATERIALS AND METHODS

Study sample

The data for this study were collected during the iLiNS-DYAD trial, which was a randomized-controlled nutrition intervention trial conducted in 2011 to 2015 in a mostly rural area in the Mangochi district in southern Malawi. The main outcome of the trial was length-for-age Z-score (LAZ) at 18 months on which no positive effect of the intervention was found [18, 19]. There was also no consistent effect of LNS on the gut microbiota or EED, but LNS was associated with higher microbiota diversity at 18 months. (Z. Liu, submitted for publication and [21]) The trial enrolled 1391 pregnant women who were randomized to 1 of 2 intervention groups or a control group. The intervention groups received either a small-quantity lipid-based nutrient supplement (LNS) or multiple-micronutrient tablets (MMN) until 6 months after delivery while the control group received iron and folic acid during pregnancy and placebo for 6 months after delivery. The first 869 enrolled mother-child dyads were assigned to a complete follow-up scheme (288 in the LNS, 291 in the MMN and 290 in the control group), in which they were monitored closely during pregnancy and the first 18 months after birth and children in the LNS intervention group received LNS from 6 to 18 months. Most of these participants were additionally followed up until 30 months. The intended sample size of 864 was calculated based on the assumed effect size of the main outcome of the trial and an estimated loss to follow-up of up to 25% [19]. This sample size gave 72% power to detect a partial correlation of 0.1 for associations between microbiota maturity and diversity and EED biomarkers with a 2-sided type 1 error rate of 0.05.

Sample collection and processing

Data from fecal samples collected as described previously at 6, 18 and 30 months from children in the complete follow-up group were used for the current study [22]. Microbiota data were obtained through

16S sequencing of the frozen fecal samples using previously described methods [13, 23, 24]. Briefly, the samples were cryo-pulverized using liquid nitrogen, suspended and shaken in a bead beater (BioSpec Products, Bartlesville, OK) to mechanically disrupt bacterial cells. DNA was purified by centrifuging, precipitation and binding to a silica membrane that was washed with elution buffer (QIAquick column, Qiagen, Germantown, MD). The amount of DNA in all samples was normalized and the 16S V4 region of the bacterial DNA was amplified by PCR using primers with a barcode sequence unique to each sample [25]. After a second normalization step and pooling and purification of all samples, the amplicons were sequenced using an Illumina MiSeq instrument (version 2 chemistry, Illumina, San Diego, CA). Paired-end 250 bp reads were trimmed to 200 bp, combined and clustered according to 97% base pair identity using QIIME [26]. These clusters were each defined as an operational taxonomic unit (OTU) and sequences were aligned with PyNAST. The Ribosomal Database Project version 2.4 classifier was trained with a custom dataset of bacterial taxonomy and OTUs were assigned taxonomy mostly to genus or species level resolution [23, 27]. The relative abundance of each OTU in each sample was quantified by the number of sequence reads assigned to it. To exclude artefacts, OTUs were filtered with a threshold of 0.1% of sequencing reads in at least 2 samples. The V4-16S sequence data generated and analyzed for this study are available through the European Nucleotide Archive under the study accession number PRJEB29433.

For the measurement of EED biomarker concentrations, fecal samples were homogenized in extraction buffer and diluted. Calprotectin, alpha-1-antitrypsin and REG1B concentrations were measured from the sample supernatants using quantitative enzyme-linked immunosorbent assays (DH002 Fecal Calprotectin Assay, Hycult Biotech, Uden, The Netherlands; Human alpha1-Antitrypsin ELISA Kit, PromoCell GmbH, Heidelberg, Germany; and REG1B ELISA kit, TECHLAB, Inc., Blacksburg, VA, USA). For quality control, the first 100 measurements of calprotectin and alpha-1-antitrypsin were done in duplicate. All measurements of REG1B were done in duplicate and the mean of the 2 values was taken. If a value differed

from the mean by more than 15%, the measurement was repeated.

Variables

Microbiota variables included measures of microbiota maturity and diversity for primary analyses and beta diversity and relative abundances of bacterial taxa for secondary analyses. As a measure of microbiota maturity, the microbiota ages of the participants were estimated using a previously described Random Forests model [13, 24]. These microbiota ages were compared to the median of microbiota ages of an age-matched healthy reference cohort of Malawian children to obtain microbiota-for-age Z-scores (MAZ) [24]. For microbiota diversity, Shannon's diversity Index was calculated from rarefied OTU-counts (rarefied to 5000 reads) using the R package phyloseq [28]. In addition, weighted and unweighted UniFrac distances were calculated to assess phylogenetic dissimilarity (beta diversity) between samples. Relative abundances of individual bacterial taxa were measured as the number of reads assigned to each OTU standardized with cumulative sum-scaling.

Fecal concentrations of EED biomarkers were used as continuous variables. These included calprotectin (in $\mu\text{g/g}$) as a measure of intestinal inflammation. Calprotectin is an unspecific marker that has been widely used to diagnose and monitor inflammatory bowel diseases in children and that has been associated with geophagy and stunting in the context of EED [8, 29–32]. Alpha-1-antitrypsin (in mg/dl), a serine protease inhibitor that is excreted in the gut during protein loss enteropathy, was used as a measure of intestinal permeability; and REG1B (in $\mu\text{g/g}$), a gene involved in the regeneration of intestinal cells, was used as a measure of intestinal damage [2, 33, 34].

Statistical analysis

Variables that could confound the association between gut microbiota and EED biomarkers were included as covariates. These included season of fecal sample collection, exact age and sex of the child, delivery mode, maternal education, household-assets Z-score, ownership of domestic animals, source of drinking water, type of sanitary facility, randomization group and number of sequencing reads.

As primary analyses, we tested hypotheses on the association between gut microbiota diversity and maturity and markers of intestinal inflammation, permeability and damage. We also conducted exploratory, secondary analyses of other measures of microbial community composition. Because the sampling time points were 12 months apart and microbiota composition and levels of EED biomarkers change rapidly at this age, no longitudinal analyses were conducted. All analyses were cross-sectional (separate analyses for the 3 time points) and the main time point was 18 months. At this age, children are relatively mobile and no longer predominantly breast-fed, which increases potential exposure to pathogens in the living environment [29]. Analyses with 6 and 30 months data were additionally performed to examine whether the associations between microbiota and EED vary at different ages. At each time point, all available samples were analyzed and participants who missed a visit were included in the analyses at other time points.

The hypothesis that there is an inverse association between the diversity and maturity of fecal microbiota and concentrations of EED biomarkers was tested using linear regression models. Calprotectin, alpha-1-antitrypsin and REG1B were used as outcomes and MAZ-score and Shannon Index as predictors in fully covariate-adjusted models (forced-entry). Outcome variables were assessed for conformance to the normal distribution assumption by inspecting histograms. For calprotectin and alpha-1-antitrypsin, $\log(10)$ -transformed values were used in the analyses. Because breast-feeding has been found to be associated with both microbiota maturity and diversity and EED biomarkers, a sensitivity analysis was completed to assess interaction (at $p < 0.1$ for the interaction term) or confounding (at $>10\%$ change-in-estimate) by breast-feeding status at 18 months [35]. At 6 months, almost all children in this population are breast-fed and no data on breast-feeding were collected at 30 months. We also conducted a sensitivity analysis excluding children whose mothers were HIV-positive during pregnancy. HIV tests were not conducted on children.

Secondary analyses on beta diversity between participants with different levels of EED biomarkers assessed differences in weighted and unweighted

UniFrac distances with permutational analysis of variance (PERMANOVA). The models included all covariates aforementioned and assessed the marginal effect of calprotectin, alpha-1-antitrypsin and REG1B [36]. Pseudo p values were obtained through 1000 permutations.

To analyze associations at the level of individual bacterial taxa, Random Forests machine learning regression models were built to estimate how relative abundances of bacterial OTUs at 18 months predict the levels of calprotectin, alpha-1-antitrypsin and REG1B. OTUs were ranked based on their importance, which was measured as the mean decrease in prediction accuracy if the OTU was excluded from the model. Only OTUs present in at least 10% of the samples were included in the models. For the 10 highest ranked OTUs in each of the 3 models, differences in EED-biomarker abundances between participants with relative taxon abundances below and above median were compared with Mann–Whitney test and fdr -corrected p values were calculated using the Benjamini–Hochberg correction.

All analyses were carried out in STATA version 15 and R version 3.5.3.

Ethics approval

The iLiNS-DYAD study was approved by the College of Medicine Research and Ethics Committee, Malawi and the ethics committee of the Pirkanmaa hospital district, Finland. All participants provided informed consent at enrollment by signing or thumb printing a consent form.

RESULTS

There were 790 live-born children (including 8 sets of twins, who were excluded from the analyses) in the complete follow-up scheme, of which 611, 666 and 596 provided a fecal sample at 6, 18 and 30 months, respectively. Of these, complete data for predictors and at least 1 EED biomarker were available for 459, 578 and 532 at 6, 18 and 30 months, respectively. A total of 77 children died between birth and 30 months, 68 were otherwise lost to follow-up and 71 did not consent to additional follow-up beyond 18 months (Fig. 1). Participants included in the analysis had a median gestational age of 39.7 weeks and a birth length of 49.8 cm. Thirteen percent had access

to piped water and 10% had improved sanitary facilities. Excluded participants had lower maternal age, higher maternal education, lower gestational age and length at birth, and higher socioeconomic status compared to participants who provided microbiota and EED data (Table 1). The fecal concentrations of all 3 EED biomarkers decreased with age (Fig. 2, Supplementary Table S1).

Log-transformed concentrations of calprotectin and alpha-1-antitrypsin and the concentration of REG1B were inversely associated with MAZ-score and Shannon Index at 18 months (each $p \leq 0.001$; Table 2 and Fig. 3). The results were similar at 30 months (Supplementary Table S2). At 6 months, calprotectin was inversely associated with MAZ-score, alpha-1-antitrypsin was inversely associated with both MAZ-score and Shannon Index and REG1B was not associated with either (Supplementary Table S3). At 18 months, 8% of the children were no longer breast-fed. When breast-feeding status was added as an additional covariate to the models at 18 months, the point estimates changed by less than 10% and the interaction term for breast-feeding status was not statistically significant in any of the models ($p > 0.1$). An analysis that excluded children of HIV-positive mothers gave essentially identical regression coefficients to those in the main analysis for all outcomes (data not shown).

In secondary analyses on beta diversity, phylogenetic distance measured as UniFrac distance was not associated with any EED biomarker at 18 months (pseudo p values > 0.05). At 30 months both unweighted and weighted UniFrac distances were associated with all EED biomarkers with R^2 values ranging from 0.8% to 3.9%. At 6 months unweighted UniFrac distances were associated with concentrations of all EED biomarkers and weighted UniFrac distances were associated with calprotectin and alpha-1-antitrypsin concentration with R^2 values between 0.4% and 6% (Fig. 4).

Random Forests models on individual bacterial taxa (OTUs) predicting the concentration of EED biomarkers at 18 months explained 24.6%, 27.7% and 24.5% of the variation in the concentrations of calprotectin, alpha-1-antitrypsin and REG1B, respectively. Taxa that were among the 10 highest ranked predictors in at least 1 of the models included

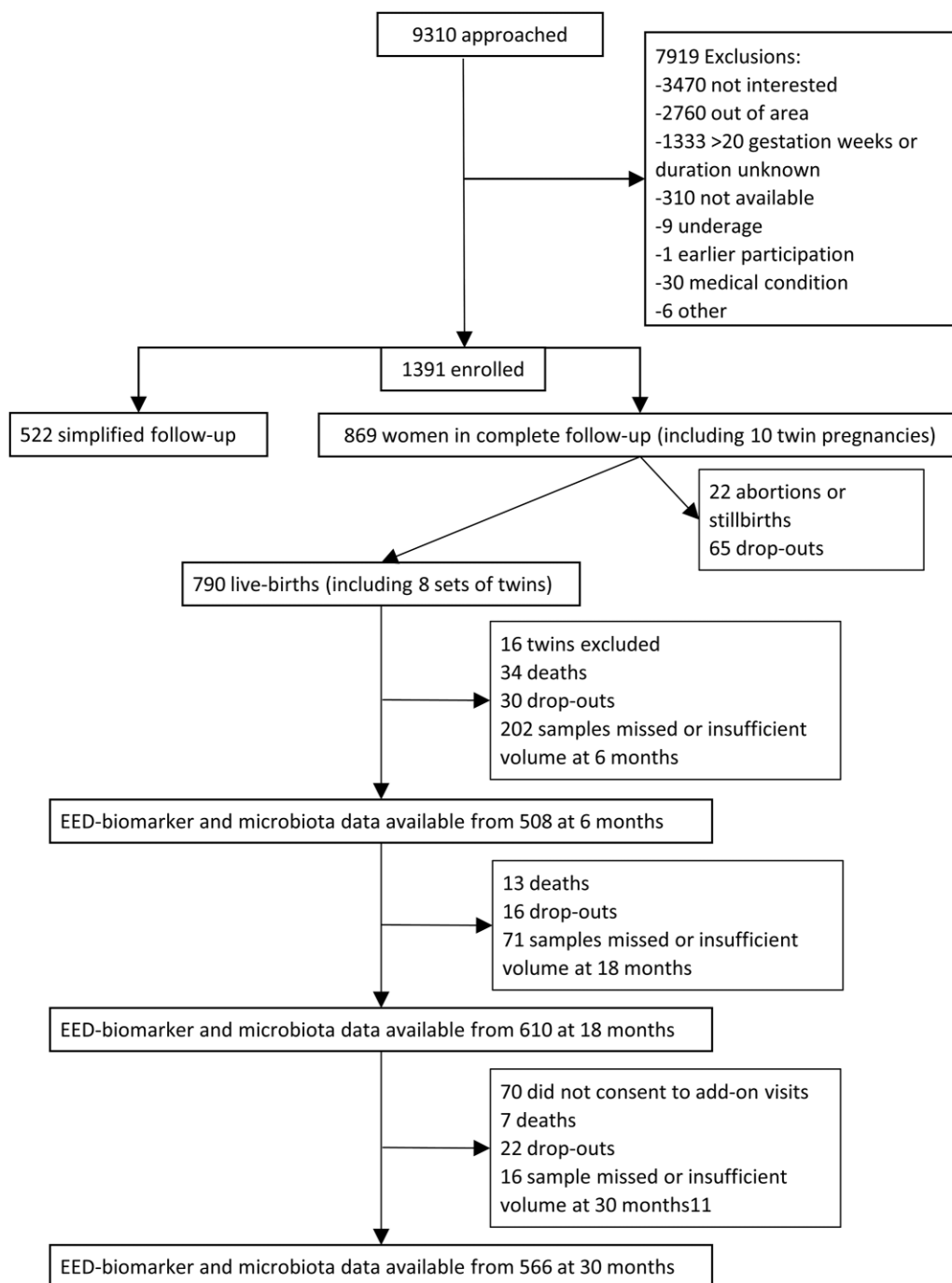


FIG. 1. Participant flow.

OTUs assigned to the bacterial orders of Actinomycetales, Bifidobacteriales, Bacteroidales, Clostridiales, Lactobacillales, Enterobacteriales and Pasteurellales. Out of all Bifidobacteriales taxa present at 18 months, 42% (5/12) were among the highest ranked predictors of EED biomarker

TABLE 1. Characteristics of included and excluded participants, median (inter-quartile range) or percentage

Characteristic	Included 610	Excluded 180	<i>p</i> value
Participants, <i>n</i>			
Maternal age at enrollment, years	24.9 (20.4; 29.5)	23.0 (19.3; 28.1)	0.02
Maternal education completed, years	3 (0; 6)	4 (1; 7)	0.03
Positive malaria RDT of the mother at enrollment	23%	23%	1.0
Mother HIV-positive at enrollment	12%	12%	0.82
Gestational age at birth, weeks	39.7 (38.7; 40.7)	39.2 (37.6; 40.3)	<0.001
Length at birth, cm	49.8 (48.5; 51.3)	49.0 (47.6; 50.3)	<0.001
LAZ at birth	-0.9 (-1.6; -0.3)	-1.4 (-2.2; -0.7)	<0.001
Household assets Z-score	-0.4 (-0.7; 0.1)	0.2 (-0.7; 0.8)	0.001
Source of drinking water is borehole, well, river, or lake (vs. piped)	87%	75%	<0.001
Type of sanitary facility is none or regular pit latrine (vs. ventilation improved pit latrine or water closet)	90%	91%	0.62

LAZ: length-for-age Z-score; RDT: rapid diagnostic test.

Included participants are those who had data on calprotectin and microbiota maturity and diversity available at 18 months. *p* values are obtained from Mann-Whitney test (continuous variables) or chi-square test (proportions).

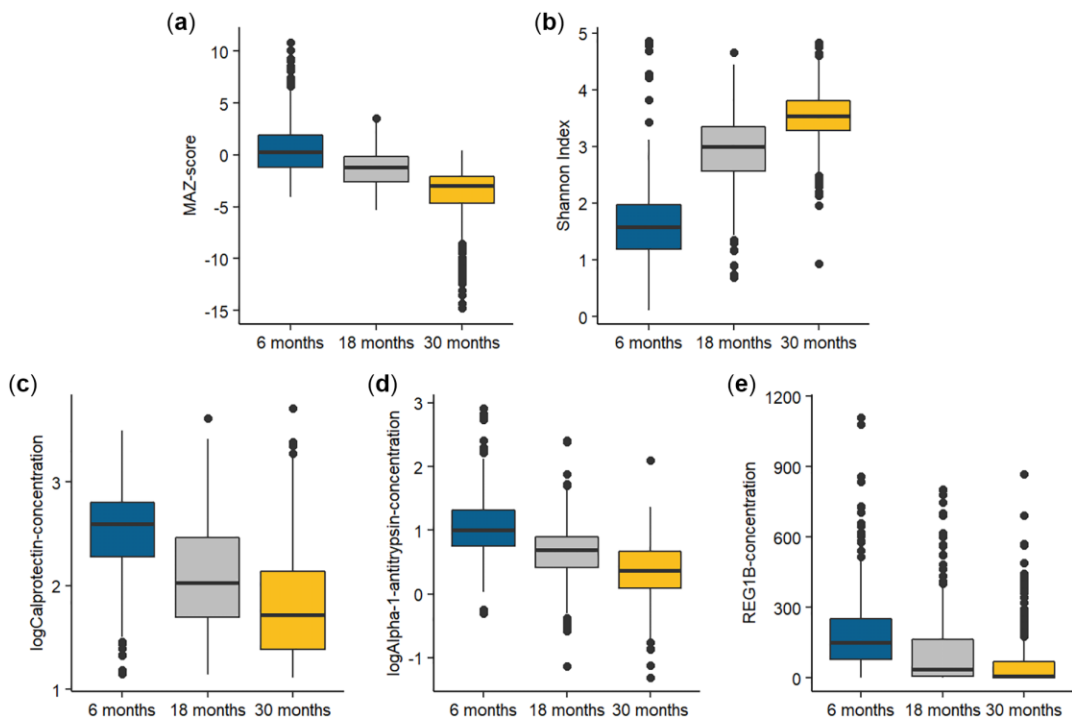
**FIG. 2.** Distribution of microbiota maturity and diversity variables and EED biomarkers.

TABLE 2. The association of microbiota maturity and diversity variables with fecal calprotectin, alpha-1-antitrypsin and REG1B at 18 months.

Predictor variable	Association between predictor and outcome variable, adjusted for covariates ^a								
	Log(calprotectin)			Log(alpha-1-antitrypsin)			REG1B		
	Regression coefficient (95% CI)	<i>p</i> value	<i>n</i>	Regression coefficient (95% CI)	<i>p</i> value	<i>n</i>	Regression coefficient (95% CI)	<i>p</i> value	<i>n</i>
MAZ-score	-0.07 (-0.09, -0.05)	<0.001	578	-0.08 (-0.1, -0.06)	<0.001	547	-25.14 (-31.75, -18.52)	<0.001	558
Shannon Index	-0.12 (-0.18, -0.05)	<0.001	578	-0.16 (-0.22, -0.11)	<0.001	547	-71.96 (-91.22, -52.70)	<0.001	558

Results from multivariable analysis. CI: confidence interval; MAZ: microbiota-for-age Z-score.

^aAdjusted for education level of the mother, household assets index, water source, sanitary facility, domestic animals, season, sex of the child, delivery mode, exact age, randomization group, sample processing pool and sequencing depth.

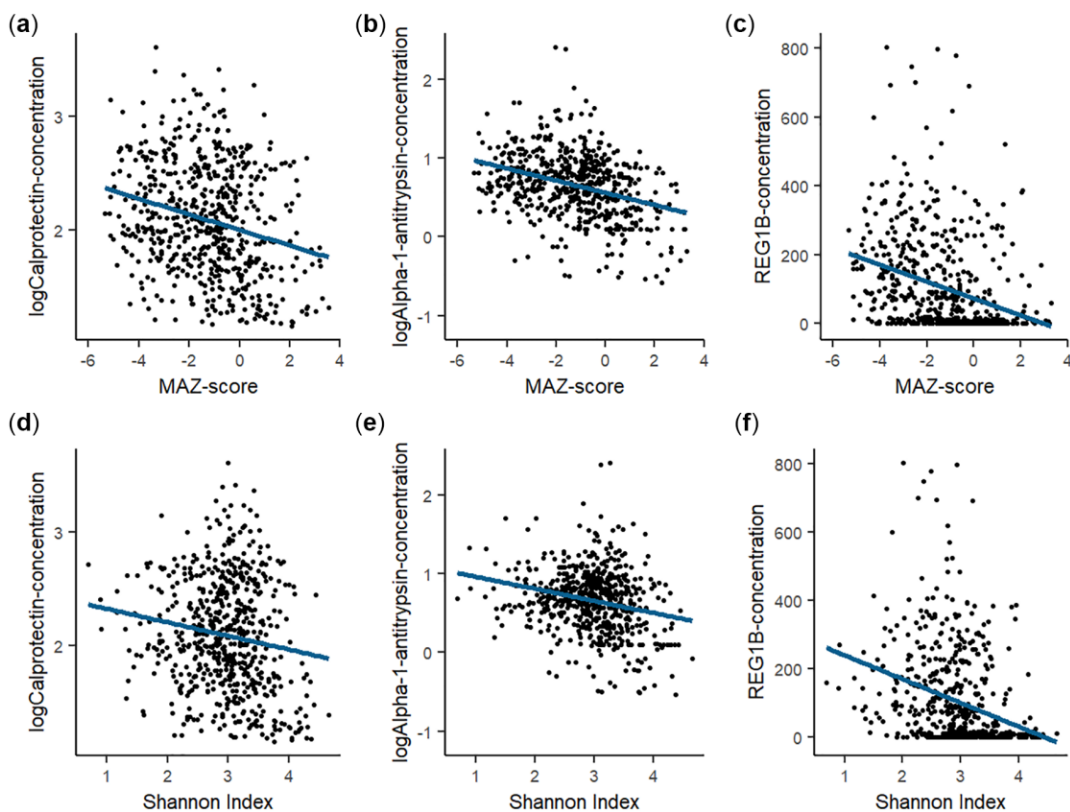


FIG. 3. The association of microbiota maturity and diversity variables with fecal calprotectin, alpha-1-antitrypsin and REG1B at 18 months. Scatterplots of MAZ-score (microbiota-for-age Z-score) (A-C) or Shannon Index (D-F) and EED biomarkers at 18 months with fitted (unadjusted) linear regression lines.

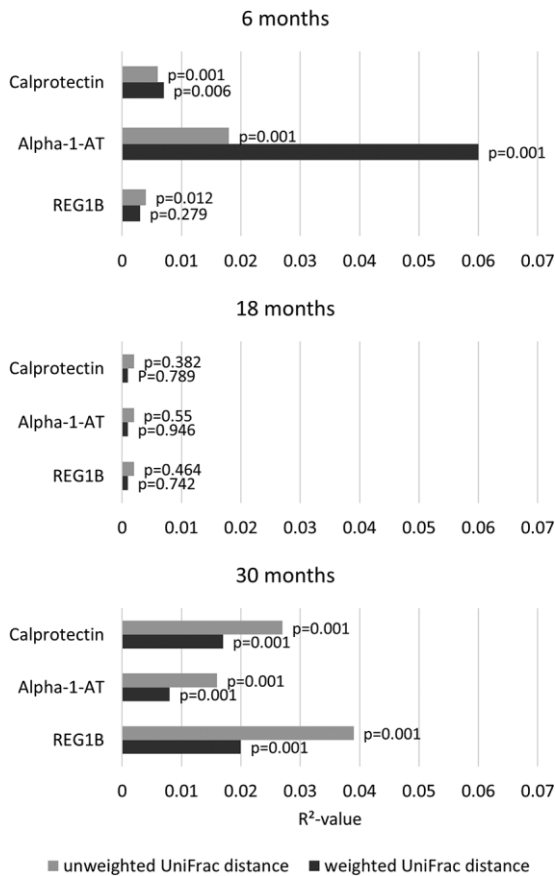


FIG. 4. Associations between EED biomarkers and phylogenetic dissimilarity. Bar chart of R^2 values from covariate-adjusted PERMANOVA models. Pseudo p values were obtained through 1000 permutations.

concentrations. Differences in EED biomarker concentrations by relative taxon abundance are shown in Fig. 5.

DISCUSSION

The aim of this study was to investigate the associations between biomarkers of EED and gut microbiota composition in rural Malawian children. Our findings support the hypothesis of an inverse association between the biomarkers calprotectin, alpha-1 antitrypsin and REG1B, and microbiota maturity and diversity at 18 months of age. The findings were similar in children aged 6 and 30 months. In secondary analyses, levels of EED biomarkers were associated with microbial beta-diversity measured as

UniFrac distances at 6 and 30, but not at 18 months. In addition, we identified bacterial taxa that predicted the levels of EED biomarkers, including several Bifidobacterium, Enterobacteriaceae, Dorea formicigenerans and Haemophilus taxa positively correlated and Prevotella and Oscillibacter taxa inversely correlated with EED biomarkers.

Our study is limited by the use of microbiota data from fecal samples as a proxy for gut microbiota composition, because EED is a condition of the small intestine and the microbiota composition changes along the gastrointestinal tract [37]. In our study population of relatively healthy children, it would not have been feasible nor ethical to perform endoscopies to obtain samples from the small intestine [38]. Even though previous studies with sampling from the small intestine found the differences between small intestinal and fecal microbiota, in those studies many bacterial taxa from the small intestine were also detected in feces and disease-associated changes occurred in both small intestinal and fecal microbiota [15, 37]. Thus, the observed differences in microbiota composition likely reflect EED-associated intestinal dysbiosis, but we cannot infer whether these differences are causally related to EED.

Further, we did not collect detailed information on nutrient or breast-milk intake at all time points [39, 40]. However, sensitivity analyses did not suggest confounding or interaction by breast-feeding status at 18 months and previous studies in this population have revealed relatively homogenous breast-feeding and complementary feeding practices [41, 42]. Therefore, we do not expect our conclusions to be significantly biased by differences in feeding patterns. Participants who were lost to follow-up and did not provide data for these analyses had on average lower gestational age and length at birth and a higher socioeconomic status than included study participants. Theoretically, this may reduce the external validity of our results. However, although the differences were statistically significant, they were very small and clinically probably insignificant. Therefore, we believe that the included study participants are representative of the study population. Finally, we used only calprotectin as a marker of intestinal inflammation and did not analyze other established

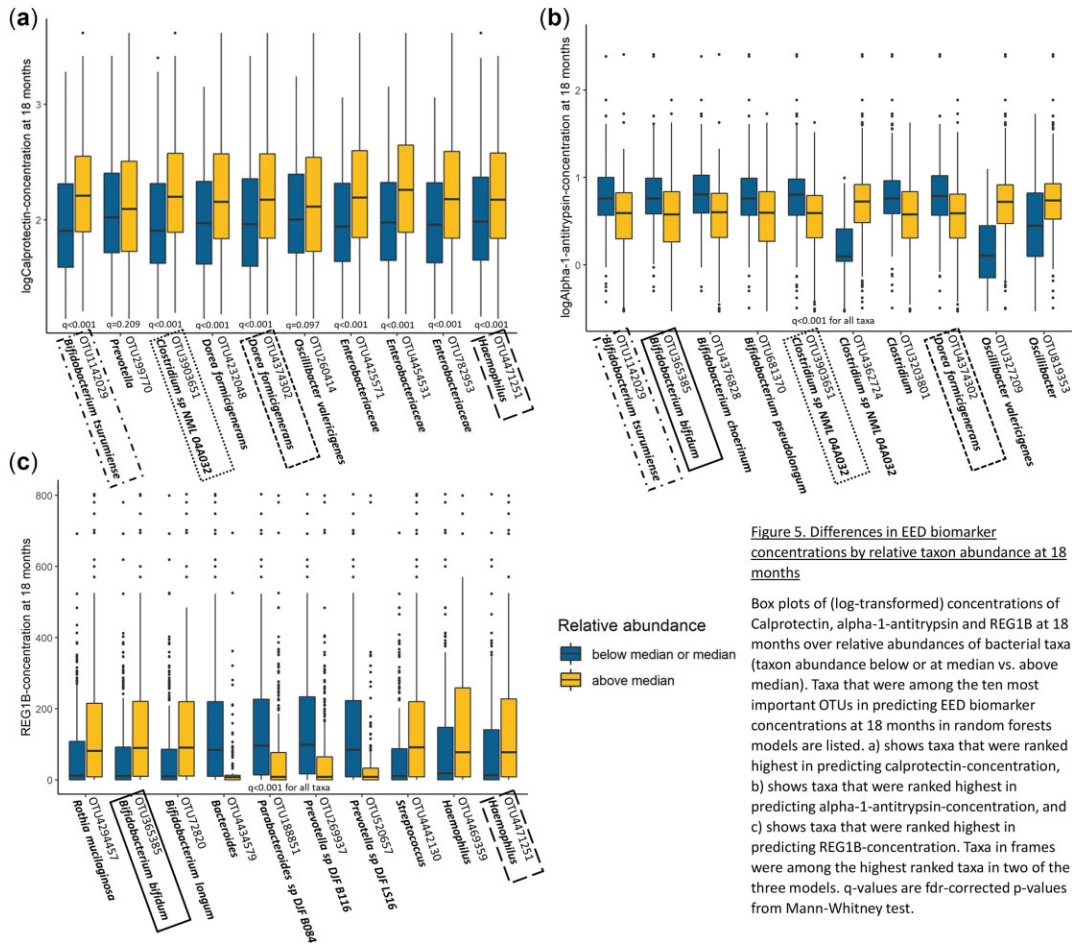


Figure 5. Differences in EED biomarker concentrations by relative taxon abundance at 18 months

Box plots of (log-transformed) concentrations of Calprotectin, alpha-1-antitrypsin and REG1B at 18 months over relative abundances of bacterial taxa (taxon abundance below or at median vs. above median). Taxa that were among the ten most important OTUs in predicting EED biomarker concentrations at 18 months in random forests models are listed. a) shows taxa that were ranked highest in predicting calprotectin-concentration, b) shows taxa that were ranked highest in predicting alpha-1-antitrypsin-concentration, and c) shows taxa that were ranked highest in predicting REG1B-concentration. Taxa in frames were among the highest ranked taxa in two of the three models. q-values are fdr-corrected p-values from Mann-Whitney test.

Fig. 5. Differences in EED biomarker concentrations by relative taxon abundance at 18 months. Box plots of (log-transformed) concentrations of calprotectin, alpha-1-antitrypsin and REG1B at 18 months over relative abundances of bacterial taxa (taxon abundance below or at median vs. above median). Taxa that were among the 10 most important OTUs in predicting EED biomarker concentrations at 18 months in Random Forests models are listed. (A) shows taxa that were ranked highest in predicting calprotectin concentration, (B) shows taxa that were ranked highest in predicting alpha-1-antitrypsin concentration and (C) shows taxa that were ranked highest in predicting REG1B concentration. Taxa in frames were among the highest ranked taxa in 2 of the 3 models. q values are fdr-corrected p values from Mann-Whitney test.

markers such as myeloperoxidase, which might have limited our ability to detect the differences in microbiota composition related to intestinal inflammation [43, 44].

The presented findings are mostly consistent with reports from the previous studies. A recent study found an inverse association between microbiota diversity and fecal neopterin concentration [45]. Otherwise, the association between microbiota diversity and maturity and EED biomarkers has not been

described before, but reduced microbiota diversity and maturity have been associated with malnutrition and reduced diversity has been associated with disease severity in patients with Crohn’s disease [13, 20, 46, 47]. One smaller study on EED conducted in Malawi measured the lactulose to mannitol ratio in young children and did not find associations with microbiota diversity. Further, the study reported the differences in relative abundances of bacterial taxa that were not found to predict EED biomarkers in

our study [17]. This discrepancy could be due to different sample processing and analysis methods, but it is also possible that the biomarkers used in our study capture different aspects of EED than the lactulose to mannitol ratio.

Regarding differences in bacterial taxon abundances, the finding of a positive association between several Bifidobacterium taxa and all 3 EED biomarkers is unexpected, because Bifidobacterium species have previously been associated with probiotic and anti-inflammatory properties [20, 48, 49]. However, the relative abundance of Bifidobacteria also decreases significantly with age and a higher abundance can thus reflect an EED-associated immaturity of the microbiota [24, 50]. This has previously been inversely associated with ponderal growth and might be attributed to a reduced fitness of certain Bifidobacteria strains [51, 52]. Our finding of a positive association between Enterobacteriaceae and calprotectin is in line with several studies linking Enterobacteriaceae with intestinal inflammation as well as HIV, which often leads to a form of enteropathy [20, 53–56]. We found both positive and negative associations between Clostridium taxa and EED biomarkers, in agreement with several studies reporting opposing functions of different Clostridium species [57]. Haemophilus, Streptococcus and Rothia mucilaginosa, which were positively associated with calprotectin and REG1B, are part of the oral microbiota [58, 59]. Decompartmentalization of the microbiota, that is oral taxa present in the distal gastrointestinal tract, has been associated with inflammation and stunting [15, 37, 53].

There are several plausible mechanisms by which the gut microbiota could cause or influence EED or by which EED could lead to changes in microbiota composition. Pathogenic bacteria could directly cause EED through mucosal damage or enteroinvasion and intestinal inflammation [60–62]. Though we could not directly assess the associations between pathogenic bacteria and EED biomarkers due to insufficient taxonomic resolution at species level, several EED-associated taxa were assigned to the family of Enterobacteriaceae, which includes pathogenic *Escherichia coli* and *Shigella* species [63]. Further, members of the intestinal microbiota could indirectly alleviate or aggravate EED through interactions with

the immune system. Potential mechanisms include production of anti-inflammatory short-chain fatty acids, strengthening of the epithelial barrier, and induction of immune cells [53, 64–67]. On the other hand, antimicrobial peptides and immunoglobulin A secreted by epithelial cells after sensing of bacteria can affect the microbiota composition [16, 68]. Of the EED biomarkers used in this study, calprotectin and alpha-1 antitrypsin have known antibacterial properties and REG1B is thought to be antibacterial based on similarities with other antimicrobial peptides, implying that these biomarkers could directly affect microbiota composition [34, 69–73]. Finally, changes in bile-acid metabolism have been described in EED and these changes could mediate influences of the gut microbiota on host metabolism [74, 75]. Thus, intestinal dysbiosis could be either a cause or a consequence of EED. It is also conceivable that EED-associated changes in microbiota composition constitute beneficial adaptations that mitigate the negative effects of the disease [76, 77].

Our findings support the hypothesis of an association between the gut microbiota composition and EED assessed by the biomarkers calprotectin, alpha-1 antitrypsin and REG1B in rural Malawian children. EED was found to be associated with reduced microbiota diversity and maturity and relative abundances of potentially pathogenic and oral bacterial taxa. However, the etiology of EED may vary in different settings and microbiota composition has been shown to differ by geographic location [78]. Therefore, the findings of our study are likely not generalizable to other regions. To advance the prevention and treatment of EED, further studies are needed to establish whether these associations are causal and whether similar patterns occur in different populations.

ACKNOWLEDGEMENTS

We thank Laura Blanton, Michael Barratt and Nicholas Griffin from Jeffrey Gordon's laboratory at Washington University in St. Louis for providing the 16S rRNA data sets used in these analyses. The trial was registered at clinicaltrials.gov as NCT01239693.

SUPPLEMENTARY DATA

Supplementary data are available at *Journal of Tropical Pediatrics* online.

FUNDING

This work was supported in part by the Finnish Funding Agency for Technology and Innovation [1509/31/2015]; and a personal grant by the Pediatric Research Foundation in Finland to EK. The original trial was supported in part by a grant to the University of California, Davis, from the Bill & Melinda Gates Foundation, with additional funding from the Office of Health, Infectious Diseases, and Nutrition, Bureau for Global Health, US Agency for International Development (USAID) under terms of Cooperative Agreement No. AID-OAA-A-12-00005, through the Food and Nutrition Technical Assistance III Project (FANTA), managed by FHI 360.

REFERENCES

- Weisz AJ, Manary MJ, Stephenson K, *et al.* Abnormal gut integrity is associated with reduced linear growth in rural Malawian children. *J Pediatr Gastroenterol Nutr* 2012;55:747–50.
- Kosek M, Haque R, Lima A, *et al.* Fecal markers of intestinal inflammation and permeability associated with the subsequent acquisition of linear growth deficits in infants. *Am J Trop Med Hyg* 2013;88:390–6.
- Campbell RK, Schulze KJ, Shaikh S, *et al.* Environmental enteric dysfunction and systemic inflammation predict reduced weight but not length gain in rural Bangladeshi children. *Br J Nutr* 2018;119:407–14.
- Richard SA, McCormick BJJ, Murray-Kolb LE, *et al.*, MAL-ED Network Investigators. Enteric dysfunction and other factors associated with attained size at 5 years: MAL-ED birth cohort study findings. *Am J Clin Nutr* 2019;110:131–8.
- Kaimila Y, Pitman RT, Divala O, *et al.* Development of acute malnutrition despite nutritional supplementation in Malawi. *J Pediatr Gastroenterol Nutr* 2019;1:734–737.
- Kelly P, Menzies I, Crane R, *et al.* Responses of small intestinal architecture and function over time to environmental factors in a tropical population. *Am J Trop Med Hyg* 2004;70:412–9.
- Paganelli M, Albanese C, Borrelli O, *et al.* Inflammation is the main determinant of low bone mineral density in pediatric inflammatory bowel disease. *Inflamm Bowel Dis* 2007;13:416–23.
- George CM, Oldja L, Biswas S, *et al.* Geophagy is associated with environmental enteropathy and stunting in children in rural Bangladesh. *Am J Trop Med Hyg* 2015;92:1117–24.
- Etheredge AJ, Manji K, Kellogg M, *et al.* Markers of environmental enteric dysfunction are associated with neurodevelopmental outcomes in Tanzanian children. *J Pediatr Gastroenterol Nutr* 2018;66:953–9.
- Lauer JM, Ghosh S, Ausman LM, *et al.* Markers of environmental enteric dysfunction are associated with poor growth and iron status in rural Ugandan infants. *J Nutr* 2020;150:2175–82.
- Harper KM, Mutasa M, Prendergast AJ, *et al.* Environmental enteric dysfunction pathways and child stunting: a systematic review. *PLoS Negl Trop Dis* 2018;12:e0006205.
- Smith MI, Yatsunenko T, Manary MJ, *et al.* Gut microbiomes of Malawian twin pairs discordant for kwashiorkor. *Science* 2013;339:548–54.
- Subramanian S, Huq S, Yatsunenko T, *et al.* Persistent gut microbiota immaturity in malnourished Bangladeshi children. *Nature* 2014;510:417–21.
- Brown EM, Wlodarska M, Willing BP, *et al.* Diet and specific microbial exposure trigger features of environmental enteropathy in a novel murine model. *Nat Commun* 2015;6:7806.
- Chen RY, Kung VL, Das S, *et al.* Linking the duodenal microbiota to stunting in a cohort of undernourished Bangladeshi children with enteropathy. *N Engl J Med* 2020;383:321–33.
- Hooper LV, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. *Science* 2012;336:1268–73.
- Ordiz MI, Stephenson K, Agapova S, *et al.* Environmental enteric dysfunction and the fecal microbiota in Malawian children. *Am J Trop Med Hyg* 2017;96:473–6.
- Ashorn P, Alho L, Ashorn U, *et al.* The impact of lipid-based nutrient supplement provision to pregnant women on newborn size in rural Malawi: a randomized controlled trial. *Am J Clin Nutr* 2015;101:387–97.
- Ashorn P, Alho L, Ashorn U, *et al.* Supplementation of maternal diets during pregnancy and for 6 months postpartum and infant diets thereafter with small-quantity lipid-based nutrient supplements does not promote child growth by 18 months of age in rural Malawi: a randomized controlled trial. *J Nutr* 2015;145:1345–53.
- Kolho K-L, Korpela K, Jaakkola T, *et al.* Fecal microbiota in pediatric inflammatory bowel disease and its relation to inflammation. *Am J Gastroenterol* 2015;110:921–30.
- Kamng'ona AW, Young R, Arnold CD, *et al.* Provision of lipid-based nutrient supplements to mothers during pregnancy and 6 months postpartum and to their infants from 6 to 18 months promotes infant gut microbiota diversity at 18 months of age but not microbiota maturation in a rural Malawian setting: secondary outcomes of a randomized trial. *J Nutr* 2020;150:918–28.
- Kortekangas E, Young R, Cheung YB, *et al.* A prospective study on child morbidity and gut microbiota in rural Malawi. *J Pediatr Gastroenterol Nutr* 2019;69:431–7.
- Ridaura VK, Faith JJ, Rey FE, *et al.* Gut microbiota from twins discordant for obesity modulate metabolism in mice. *Science* 2013;341:1241214.
- Blanton LV, Charbonneau MR, Salih T, *et al.* Gut bacteria that prevent growth impairments transmitted by microbiota from malnourished children. *Science* 2016;351:aad3311.

25. Caporaso JG, Lauber CL, Walters WA, *et al.* Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci U S A* 2011; 108: 4516–22.
26. Caporaso JG, Kuczynski J, Stombaugh J, *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 2010;7:335–6.
27. Wang Q, Garrity GM, Tiedje JM, *et al.* Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 2007;73: 5261–7.
28. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* 2013;8:e61217.
29. Morita T, Perin J, Oldja L, *et al.* Childhood mouthing of soil contaminated objects is associated with environmental enteropathy. *Trop Med Int Health* 2017;22:670–8.
30. Berni Canani R, Rapacciuolo L, Romano MT, *et al.* Diagnostic value of faecal calprotectin in paediatric gastroenterology clinical practice. *Dig Liver Dis Off* 2004;36: 467–70.
31. Liu J-R, Sheng X-Y, Hu Y-Q, *et al.* Fecal calprotectin levels are higher in rural than in urban Chinese infants and negatively associated with growth. *BMC Pediatr* 2012;12:129.
32. Fagerberg UL, Löf L, Lindholm J, *et al.* Fecal calprotectin: a quantitative marker of colonic inflammation in children with inflammatory bowel disease. *J Pediatr Gastroenterol Nutr* 2007;45:414–20.
33. Peterson KM, Guo X, Elkahlon AG, *et al.* The expression of REG 1A and REG 1B is increased during acute amebic colitis. *Parasitol Int* 2011;60:296–300.
34. van Beelen Granlund A, Østvik AE, Brenna Ø, *et al.* REG gene expression in inflamed and healthy colon mucosa explored by in situ hybridisation. *Cell Tissue Res* 2013; 352:639–46.
35. Mickey RM, Greenland S. The impact of confounder selection criteria on effect estimation. *Am J Epidemiol* 1989; 129:125–37.
36. Anderson MJ. A new method for non-parametric multivariate analysis of variance: non-parametric Manova for ecology. *Austral Ecol* 2001;26:32–46.
37. Vonaesch P, Morien E, Andrianonimiadana L, *et al.*, AfriBiota Investigators. Stunted childhood growth is associated with decompartmentalization of the gastrointestinal tract and overgrowth of oropharyngeal taxa. *Proc Natl Acad Sci U S A* 2018;115:E8489–98.
38. Chandwe K, Amadi B, Chipunza M, *et al.* Safety and ethics in endoscopic studies in children: evidence from the BEECH study in Zambia. *J Trop Pediatr* 2021;67: fmaa074.
39. Dorosko SM, MacKenzie T, Connor RI. Fecal calprotectin concentrations are higher in exclusively breastfed infants compared to those who are mixed-fed. *Breastfeed Med* 2008;3:117–9.
40. Levin AM, Sitarik AR, Havstad SL, *et al.* Joint effects of pregnancy, sociocultural, and environmental factors on early life gut microbiome structure and diversity. *Sci Rep* 2016;6:31775.
41. Vaahtera M, Kulmala T, Hietanen A, *et al.* Breastfeeding and complementary feeding practices in rural Malawi. *Acta Paediatr* 2007;90:328–32.
42. Kumwenda C, Hemsworth J, Phuka J, *et al.* Factors associated with breast milk intake among 9-10-month-old Malawian infants: factors associated with breast milk intake. *Matern Child Nutr* 2016;12:778–89.
43. Arndt MB, Richardson BA, Ahmed T, *et al.* Fecal markers of environmental enteropathy and subsequent growth in Bangladeshi children. *Am J Trop Med Hyg* 2016;95: 694–701.
44. D’Arcangelo G, Imondi C, Terrin G, *et al.* Is fecal calprotectin a useful marker for small bowel Crohn disease? *J Pediatr Gastroenterol Nutr* 2021;73:242–6.
45. Chaima D, Pickering H, Hart JD, *et al.* Fecal biomarkers of environmental enteric dysfunction and the gut microbiota of rural Malawian children: an observational study. *Heliyon* 2021;7:e08194.
46. Gevers D, Kugathasan S, Denson LA, *et al.* The treatment-naïve microbiome in new-onset Crohn’s disease. *Cell Host Microbe* 2014;15:382–92.
47. Gough EK, Stephens DA, Moodie EEM, *et al.* Linear growth faltering in infants is associated with *Acidaminococcus* sp. and community-level changes in the gut microbiota. *Microbiome* 2015;3:24.
48. Dinh DM, Ramadass B, Kattula D, *et al.* Longitudinal analysis of the intestinal microbiota in persistently stunted young children in South India. *PLoS ONE* 2016;11: e0155405.
49. Ismail IH, Boyle RJ, Licciardi PV, *et al.* Early gut colonization by *Bifidobacterium breve* and *B. catenulatum* differentially modulates eczema risk in children at high risk of developing allergic disease. *Pediatr Allergy Immunol* 2016;27:838–46.
50. Kortekangas E, Kamng’ona AW, Fan Y-M, *et al.* Environmental exposures and child and maternal gut microbiota in rural Malawi. *Paediatr Perinat Epidemiol* 2020;34:161–70.
51. Chen RY, Mostafa I, Hibberd MC, *et al.* A microbiota-directed food intervention for undernourished children. *N Engl J Med* 2021;384:1517–28.
52. Raman AS, Gehrig JL, Venkatesh S, *et al.* A sparse covarying unit that describes healthy and impaired human gut microbiota development. *Science* 2019;365:eaau4735.
53. Atarashi K, Suda W, Luo C, *et al.* Ectopic colonization of oral bacteria in the intestine drives TH1 cell induction and inflammation. *Science* 2017;358:359–65.
54. Ho TTB, Groer MW, Kane B, *et al.* Enteric dysbiosis and fecal calprotectin expression in premature infants. *Pediatr Res* 2019;85:361–8.

55. Gootenberg DB, Paer JM, Luevano J-M, *et al.* HIV-associated changes in the enteric microbial community: potential role in loss of homeostasis and development of systemic inflammation. *Curr Opin Infect Dis* 2017;30:31–43.
56. Sankaran S, George MD, Reay E, *et al.* Rapid onset of intestinal epithelial barrier dysfunction in primary human immunodeficiency virus infection is driven by an imbalance between immune response and mucosal repair and regeneration. *J Virol* 2008;82:538–45.
57. Schirmer M, Garner A, Vlamakis H, *et al.* Microbial genes and pathways in inflammatory bowel disease. *Nat Rev Microbiol* 2019;17:497–511.
58. Dewhirst FE, Chen T, Izard J, *et al.* The human oral microbiome. *J Bacteriol* 2010;192:5002–17.
59. Costalonga M, Herzberg MC. The oral microbiome and the immunobiology of periodontal disease and caries. *Immunol Lett* 2014;162:22–38.
60. Fahim SM, Das S, Gazi MA, *et al.* Association of intestinal pathogens with faecal markers of environmental enteric dysfunction among slum-dwelling children in the first 2 years of life in Bangladesh. *Trop Med Int Health* 2018;23:1242–50.
61. Kosek MN, Investigators M-EN, MAL-ED Network Investigators. Causal pathways from enteropathogens to environmental enteropathy: findings from the MAL-ED birth cohort study. *EBioMedicine* 2017;18:109–17.
62. Lee GO, McCormick BJJ, Seidman JC, *et al.* Infant nutritional status, feeding practices, enteropathogen exposure, socioeconomic status, and illness are associated with gut barrier function as assessed by the lactulose mannitol test in the MAL-ED birth cohort. *Am J Trop Med Hyg* 2017;97:281–90.
63. McLean K, Rosenthal CA, Sengupta D, *et al.* Improved species-level clinical identification of Enterobacteriaceae through broad-range *dnaJ* PCR and sequencing. *J Clin Microbiol* 2019;57:e00986–19.
64. Zheng D, Liwinski T, Elinav E. Interaction between microbiota and immunity in health and disease. *Cell Res* 2020;30:492–506.
65. Cervantes-Barragan L, Chai JN, Tianero MD, *et al.* *Lactobacillus reuteri* induces gut intraepithelial CD4(+)CD8 $\alpha\alpha$ (+) T cells. *Science* 2017;357:806–10.
66. Fujimura KE, Sitarik AR, Havstad S, *et al.* Neonatal gut microbiota associates with childhood multisensitized atopy and T cell differentiation. *Nat Med* 2016;22:1187–91.
67. Mulder IE, Schmidt B, Lewis M, *et al.* Restricting microbial exposure in early life negates the immune benefits associated with gut colonization in environments of high microbial diversity. *PLoS ONE* 2011;6:e28279.
68. Vaishnav S, Behrendt CL, Ismail AS, *et al.* Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. *Proc Natl Acad Sci U S A* 2008;105:20858–63.
69. Nakashige TG, Zhang B, Krebs C, *et al.* Human calprotectin is an iron-sequestering host-defense protein. *Nat Chem Biol* 2015;11:765–71.
70. Kehl-Fie TE, Chitayat S, Hood MI, *et al.* Nutrient metal sequestration by calprotectin inhibits bacterial superoxide defense, enhancing neutrophil killing of *Staphylococcus aureus*. *Cell Host Microbe* 2011;10:158–64.
71. Liu JZ, Jellbauer S, Poe AJ, *et al.* Zinc sequestration by the neutrophil protein calprotectin enhances *Salmonella* growth in the inflamed gut. *Cell Host Microbe* 2012;11:227–39.
72. Kaner Z, Ochayon DE, Shahaf G, *et al.* Acute phase protein α 1-antitrypsin reduces the bacterial burden in mice by selective modulation of innate cell responses. *J Infect Dis* 2015;211:1489–98.
73. Miyamoto Y, Akaike T, Alam MS, *et al.* Novel functions of human alpha(1)-protease inhibitor after S-nitrosylation: inhibition of cysteine protease and antibacterial activity. *Biochem Biophys Res Commun* 2000;267:918–23.
74. Ramírez-Pérez O, Cruz-Ramón V, Chinchilla-López P, *et al.* The role of the gut microbiota in bile acid metabolism. *Ann Hepatol* 2017;16:s15–20.
75. Semba RD, Gonzalez-Freire M, Moaddel R, *et al.* Environmental enteric dysfunction is associated with altered bile acid metabolism. *J Pediatr Gastroenterol Nutr* 2017;64:536–40.
76. De Filippo C, Cavalieri D, Di Paola M, *et al.* Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc Natl Acad Sci* 2010;107:14691–6.
77. Stražar M, Temba GS, Vlamakis H, *et al.* Gut microbiome-mediated metabolism effects on immunity in rural and urban African populations. *Nat Commun* 2021;12:4845.
78. Huus KE, Rodriguez-Pozo A, Kapel N, *et al.*, Afribiota Investigators. Immunoglobulin recognition of fecal bacteria in stunted and non-stunted children: findings from the Afribiota study. *Microbiome* 2020;8:113.