RESEARCH ARTICLE

Association between intestinal bacterial carriage, biomarkers of environmental enteric dysfunction, and stunting in rural Malawian children [version 1; peer review: awaiting peer review]

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Abstract

Background. Available data from murine studies suggest that intestinal bacteria may have a role in modulating growth phenotypes in the host. We investigated the prevalence of four gut bacteria known in murine models to impair growth (*Bifidobacterium longum, Faecalibacterium prausnitzii, Dorea formicigenerans,* and *Akkermansia muciniphila*), the level of fecal biomarkers of environmental enteric dysfunction (EED) and stunting in rural Malawian children.

Methods. DNA and protein were extracted from fecal samples of rural Malawian children (aged 1-59 months) at a baseline cross-sectional survey in the Mangochi district of Malawi conducted within the framework of the Macrolides Oraux pour Réduire les Décès avec un Oeil sur la Résistance (MORDOR) trial. Intestinal carriage of bacteria was measured by PCR. Neopterin (NEO), myeloperoxidase (MPO), and alpha-1 antitrypsin (AAT), biomarkers of EED, were measured by an enzyme-linked immunosorbent assay (ELISA) test. Height-for-age Z (HAZ) score <-2 defined stunting. Tests of proportions and regression models were used to explore the relationship between bacterial carriage, EED, and stunting.

Results. Fecal samples from 613 children were available for laboratory analyses. *F. prausnitzii* and *D. formicigenerans* were prevalent in over 70% of children while *B. longum* was the least prevalent. *B. longum* carriage in younger children was associated with elevated EED

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biomarkers. Two thirds of children had elevated NEO, 33% had elevated MPO, and 16% had elevated AAT. Stunting was found in 38%. No significant associations were found between EED biomarkers or intestinal bacteria carriage and stunting.

Conclusion. Intestinal carriage of these four bacteria was not associated with stunting in Malawian children. Carriage was also not associated with EED, nor EED biomarker levels associated with stunting. Further factors acting in concert are necessary to impact EED, perturb growth, and alter gut bacterial carriage.

Keywords
Biomarker, environmental enteric dysfunction, intestinal bacterial carriage, qPCR, stunting

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Introduction

According to emerging evidence from murine studies, gut bacteria have a role in modulating growth phenotypes in the host. A study that looked into whether a growth phenotype could be passed down through a combination of diet and gut bacteria reported links between a weight loss phenotype and a combination of a Malawian diet and a kwashiorkor microbiota[1]. Another study, which looked at the interaction between gut microbiota development and undernutrition, reported links between gut microbiota and growth characteristics in a murine model[2]. *Bifidobacterium longum, Faecalibacterium prausnitzii,* and *Dorea formicigenerans* were found to be related with weight gain in mice. Another study utilizing a mouse model reported an inverse relationship between intestinal carriage of *Akkermansia* muciniphila and body weight[3]. Further studies in humans have reported relationships between the gut microbiota and growth characteristics. In Bangladeshi, intestinal bacterial diversity was persistently reduced in malnourished children compared to healthy children. Also, relative abundance of *Bifidobacterium* was reported to be lower in malnourished children compared to healthy children[4]. Furthermore, *A. muciniphila* abundance was shown to be lower in overweight or obese Swedish pre-school children[5].

The mechanisms by which the gut bacteria can mediate growth are not well known. One proposed mechanism is the preservation of intestinal integrity, which improves nutrient absorption after digestion. This hypothetical mechanism is better explained by studies of environmental enteric dysfunction (EED), a subclinical condition of the gut defined by structural alterations in the intestinal epithelium and underlying chronic inflammatory responses that may result in loss of gut integrity and influence growth[6–8]. In EED, alterations in gut microbiota assembly and function cause structural changes in the intestinal epithelium, allowing bacteria or microbial antigens from the lumen to enter the systemic circulation and cause persistent immunological activation. This then impacts linear growth, either by suppressing growth hormones or redirecting nutrients away from growth and toward more immunological metabolism[9,10]. A multi-site, longitudinal study that examined the relationship between the fecal biomarkers of EED (myeloperoxidase (MPO), neopterin (NEO), and alpha-1 anti-trypsin (AAT) and linear growth in infants living in low-income settings of South Asia, Sub-Saharan Africa and Latin America showed that high levels of these biomarkers predicted a decline in infant length-for-age z (LAZ) scores[11]. Similarly, in a case-control study assessing a relationship between MPO or AAT and growth impairment in rural Brazilian children (aged between 6-26 months), higher fecal levels of both biomarkers were associated with growth faltering[12].

The present study investigated the prevalence of *Akkermensia muciniphila, Bifidobacterium longum, Dorea formicigenerans,* and *Faecalibacterium prausnitzii* in rural Malawian children. We investigated the link between intestinal carriage of these bacteria, fecal biomarkers of EED, and stunting in rural Malawian children to understand if the growth mediating effects of these bacteria observed in mouse models are also observed in the human population.

Methods

Ethical considerations

The MORDOR trial was conducted in accordance with the Declaration of Helsinki. It was approved by the London School of Hygiene and Tropical Medicine Ethics Committee (UK) (reference number 6500) and the College of Medicine Research Ethics Committee (Malawi) (P.02/14/1521). Information and consent forms were translated into local languages (Yao and Chichewa) prior to their approval by the local ethics committee. Consent was first obtained at the community level through discussions with the village chief and community elders who then verbally indicated whether the community was willing to participate. Written, informed consent (by thumbprint or signature) was then obtained from the parent or legal guardian of each child for inclusion before they participated in the study. All parents and guardians were informed of their freedom to withdraw their child from the study at any time without a requirement to give a reason(s).

Study design

This study made use of fecal samples, and demographic and anthropometry data gathered during a baseline assessment of the prevalence of carriage of macrolide resistant enteropathogens undertaken as part of a multi-country randomized controlled trial named "MORDOR" (NCT02047981). The trial study design and protocol have been previously published[13,14]. Briefly, a total of 1,090 children residing in 30 clusters within the Mangochi district were enrolled into the baseline survey. All enrolled children were aged 1 to 59 months and weighed at least 3.8kg. Sample and data collection took place between May and July 2015. Details of fecal sample collection have been described in previous studies[15,16]. This study is based off the first named author's PhD thesis[17].

Laboratory methods

Total, genomic DNA was isolated from 250mg fecal sample using the commercially available PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc, Carlsbad, CA, now a part of Qiagen, Germany). As a quality control measure, DNA yield was quantified in 10% of samples using the Qubit dsDNA HS assay kit (Invitrogen, CA, USA). Endpoint qPCR was performed to detect the presence of the following bacteria: *Akkermensia muciniphila, Bifidobacterium longum Dorea formicigenerans,* and *Faecalibacterium prausnitzii.* Each PCR reaction used a 1:10 diluted DNA sample to negate any unwanted effects of high DNA yield. The qPCR was performed using a Rotorgene-Q instrument (Qiagen, Hilden, Germany) where samples were run in a 72-well rotor format. The qPCR assays were designed and performed using the microbial DNA qPCR assay platform (Qiagen). The total volume of a single PCR reaction was 12.5µl and each reaction contained 6.25µl of microbial qPCR master mix (Qiagen), a commercial microbial DNA qPCR assay containing primers (10µM), and a 5'-hydrolysis probe (5 µM) targeting the bacterial 16S rRNA gene and 3µl template DNA in an aqueous solution. A no template control and a microbial DNA positive control (Qiagen) were included on each run. Thermal cycling conditions for the assay were 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 2 minutes.

The endpoint qPCR results were classified as positive or negative using a method by Pickering *et al*.,[18]

To quantify fecal biomarkers of EED, commercially available enzyme-linked immunosorbent assay (ELISA) kits for MPO (Immundiagnostik, Germany), AAT (Immundiagnostik), and NEO (Genway, CA, USA) were used for the quantification of each biomarker[16].

Height/length measurements

The SECA Leicester height measure (Chasmors Ltd., UK) was used to measure height for children who were ≥2 years of age and able to stand whereas the SECA 417 infantometer (Chasmors Ltd.) was used to measure recumbent length for children who were <2 years of age or unable to stand unaided. Both height and length were measured to within 0.1 cm. All measurements were taken in triplicate by field nurses.

Statistical analysis

To describe the prevalence of carriage of defined bacteria, the proportions of fecal samples with a positive qPCR result for each of the defined bacteria were calculated. The biomarkers of EED were assessed individually or were combined to form a composite EED score as described by Kosek *et al.,*[11] with minor modifications as detailed elsewhere[16]. The distribution of the biomarkers of EED was determined using the median (IQR) concentration of the fecal biomarkers. Published cut-off values for each of the three biomarkers[11,19] were used to determine proportions of fecal samples with elevated biomarkers. The World Health Organization (WHO) 2006 Child Growth Standards were used for the calculation of height-for-age Z (HAZ) scores[20]. Participants with out-of-range HAZ scores, (>6 or <-6) were not included in the subsequent analyses as such scores are biologically implausible. HAZ scores were used in analyses as continuous variables or categorized into not stunted or stunted, defined as having a Z score of <-2. Fisher's exact or Pearson's chi-square tests were used to explore associations between bacterial carriage and sex, age, biomarkers of EED, and stunting. Age and sex adjusted regression models were also used to explore the relationship between bacterial carriage, biomarkers of EED, and stunting.

Results

Baseline characteristics of participants

The baseline survey of prevalence of carriage of macrolide resistant enteropathogens enrolled 1,090 children, of which only 709 (65%) returned fecal samples. Of the 709 fecal samples, 613 fecal samples had sufficient volume for the analyses of intestinal bacterial carriage while 523 of the 613 fecal samples had sufficient remaining volume for the analyses of fecal biomarkers of EED. Characteristics of all children who were included in the final analyses of this study and those not included are shown in Table 1. Participant sex was comparable between all participants who were not included the analyses and those included in the bacterial carriage and EED biomarker analyses whilst differences were seen for age and height between children included in the analyses of bacterial carriage and EED biomarkers and all participants not included in the analyses.

Prevalence of bacterial carriage and biomarker distribution

F. prausnitzii was the most prevalent bacterium, appearing in 98% of the samples (Table 2). *D. formicigenerans* was present in 79% of the samples, *A. muciniphila* was found in 43% of the samples, and *B. longum* was present in just over one third of the fecal samples. Fecal samples were stratified by sex and age to compare bacterial carriage. There was no difference in the prevalence of *F. prausnitzii*, *B. longum* and *A. muciniphila* between male and female children, however, a higher proportion of fecal samples from male children were positive for *D. formicigenerans* compared to the female children (Table 2). *B. longum* was most prevalent in fecal samples from the youngest age group while *D. formicigenerans, F. prausnitzii,* and *A. muciniphila* were more prevalent in the older children (Table 2).

Table 1. Characteristics of participants who were included in the analyses as compared to all participants not included in the analyses.

Variable	All participants excluded in the analyses	Participants included in bacterial carriage analyses	P value	Participants included in EED[+] biomarkers analyses	P value
Number of participants	469	613		523	
Male sex N (%)	222 (47.33)	299 (48.78)	0.664 ¥	264 (50.5)	0.24¥
Height, cm	81.90 (12.79)	84.5 (12.1)	0.007*	85.0 (11.6)	0.005*
Age, months	27.74 (16.21)	31.1 (16.4)	0.0008*	32.0 (16.0)	<0.001*

Data are presented as mean (SD) unless otherwise stated.

**Denotes P values obtained from Student's t-test while ¥denotes P values obtained from sample proportion test.*

[+] Environmental enteric dysfunction (EED).

Of the 523 fecal samples that were available for the measurement of fecal biomarkers of EED, 488 had sufficient sample volume for MPO ELISA, 421 for NEO, and 495 for AAT. Four hundred and twenty one samples had results for all the three markers. A larger proportion of children [77% (324/421)] had elevated NEO levels relative to published reference values (NEO <=70 nmol/L, AAT <=0.27 mg/g and MPO <=2000 ng/ml)[11,19]. Approximately, one third of the children had elevated MPO levels and levels of fecal AAT were elevated in 16% of the children (Table 3). The comparison of biomarker concentrations by sex did not show any differences in MPO and AAT concentrations; however, NEO concentration was higher in female children compared to male children (Table 3). There were significant differences in biomarker distribution among the five age groups. Median fecal concentration for all the three biomarkers was higher in younger children compared to older children (Table 3).

Association between bacterial intestinal carriage and biomarkers of EED

Logistic regression analysis examining the relationship between bacterial carriage and biomarker concentration did not find any association between fecal carriage of *A. muciniphila* or *F. prausnitzii* and any of the three individual biomarkers of EED. However, fecal carriage of *B. longum* was associated with increased odds of having elevated biomarker concentrations (vs normal concentrations), whilst fecal samples that were positive for *D. formicigenerans* were 70% less likely to have elevated MPO concentration (Table 4).

B. longum carriage in children younger than 24 months was associated with fecal concentrations of MPO and NEO but not in the older children (Table 5). Elevated MPO concentration was found in fecal samples of children aged between 0-12 months who were positive for *B. longum* while children

Table 2. Prevalence of bacterial carriage across all samples, by age, and by sex.

Variable	Carriage in all samples (N=613)	Carriage by age in months						Carriage by sex		
		1-12 (n=104)	13-24 (n=134)	25-36 (n=120)	37-48 (n=144)	49-60 (n=111)	P value	Female (n=314)	Male (n=299)	P value
B. longum positive % (n)	29 (179)	82 (85)	46 (61)	17 (17)	8 (12)	4 (4)	<0.001[a]	31 (96)	28 (83)	0.5[a]
A. muciniphila positive % (n)	43 (264)	26 (27)	48 (64)	53 (63)	42 (60)	45 (50)	0.001[a]	42 (132)	44 (132)	0.65[a]
D. formicigenerans positive % (n)	79 (483)	42 (44)	81 (109)	88 (105)	85 (122)	93 (103)	<0.001[a]	75 (235)	83 (248)	0.019[a]
F. prausnitzii positive % (n)	98 (603)	95 (99)	99 (132)	100 (120)	99 (143)	98 (109)	0.06b	98 (307)	99 (296)	0.34[b]

[a]*Denotes P values obtained from Pearson's chi-square test,*

[b]*Denotes P values obtained from Fisher's Exact test*

Table 3. Biomarker distribution across all samples, age, and sex.

Variable	*Proportion with elevated biomarkers in all samples, % (n)	Age in months						Sex		
		1-12	13-24	25-36	37-48	49-60	P value	Female	Male	P value
MPO+ (ng/ml)	22 (106/488)	4255 (13165)	971 (1778)	580 (1077)	402 (757)	290 (357)	<0.001[a]	610 (1383)	518 (1629)	0.91[b]
		N=68	N=104	N=86	N=114	N=86		N=242	N=246	
NEO+ (nmol/L)	76 (324/425)	2101 (3103)	402 (1379)	133 (295)	134 (348)	63 (153)	<0.001[a]	0.06 (0.14)	0.08 (0.15)	0.21[b]
		N=55	N=99	N=85	N=108	N=81		N=244	N=251	
AAT+ (mg/g)	16 (81/495)	0.25 (0.64)	0.1 (0.2)	0.06 (0.11)	0.06 (0.10)	0.03 (0.05)	<0.001[a]	140 (505)	201 (1044)	0.02[b]
		N=66	N=105	N=90	N=117	N=85		N=210	N=211	

**Normal values for the biomarkers were based on values reported in the literature (NEO <=70 nmol/L, AAT <=0.27 mg/g and MPO <=2000 ng/ml)[11,19,21]*

Biomarker concentration are presented as median (IQR)

[a]*Denotes P value obtained from Kruskal-walis test*

[b]*Denotes P values obtained from Wilcoxon rank sum test*

+ *Neopterin (NEO), myeloperoxidase (MPO), and alpha-1 antitrypsin (AAT).*

Table 4. Association between bacterial carriage and individual biomarkers of environmental enteric dysfunction (EED).

Variable	MPO[+]				AAT[+]				NEO[+]			
	Normal	Elevated	OR (95%CI)	P value[a]	Normal	Elevated	OR (95%CI)	P value[a]	Normal	Elevated	OR (95%CI)	P value[a]
B. longum, n (%)	60/345 (17)	59/104 (57)	2.6 (1.4,4.6)	0.002	72/380 (19)	47/74 (64)	2.6 (1.4,5.1)	0.004	7/122 (6)	98/295 (33)	4.7 (2.1,12.3)	0.0004
A. muciniphila, n (%)	159/345 (46)	41/104 (39)	0.9 (0.5,1.5)	0.619	176/380 (46)	27/74 (37)	0.8 (0.4,1.3)	0.354	61/122 (50)	133/295 (45)	0.8 (0.5,1.3)	0.407
D. formicigenerans, n (%)	307/345 (89)	66/104 (64)	0.3 (0.2,0.6)	0.001	328/380 (86)	48/74 (65)	0.7 (0.4,1.4)	0.298	112/122 (92)	244/295 (83)	0.7 (0.3,1.5)	0.427
F. prausinitzii, n (%)	340/345 (99)	102/104 (98)	0.9 (0.1,7.6)	0.89	374/380 (98)	73/74 (99)	2.5 (0.3,53.6)	0.454	121/122 (99)	291/295 (99)	0.5 (0.03,4)	0.585

CI= confidence interval, OR=odds ratio.

n (%) = Number of fecal samples (proportion) positive.

[a]Odds ratio (95% CI) and P values were obtained with logistic regression after adjusting for age and sex. Using the Bonferroni correction for multiple testing, associations with a P value less than 0.0125 were considered statistically significant.

[+] Neopterin (NEO), myeloperoxidase (MPO), and alpha-1 antitrypsin (AAT).

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Table 5. **Association between *B. longum* carriage and biomarkers of environmental enteric dysfunction (EED) stratified by age.**

	MPO[+]		AAT[+]		NEO[+]		MPO[+]		AAT[+]		NEO[+]	
Age (months)	**Normal**	**Elevated**	**Normal**	**Elevated**	**Normal**	**Elevated**	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**
	[¥]n=23	n=44	n=34	n=31	n=4	n=50						
0 – 12 [*]n (%)	13 (56)	40 (91)	23 (68)	27 (87)	3 (75)	40 (80)	7.4 (1.9,34.5)	**0.006**	3.02 (0.81,13.1)	0.112	1.4 (0.02,14)	0.804
	n=72	n=30	n=79	n=24	n=17	n=80						
12 – 24 n (%)	29 (40)	16 (53)	30 (38)	16 (67)	1 (6)	41 (51)	1.7 (0.7,4.3)	0.268	3.1 (1.2,8.6)	0.023	17.5 (3.26,324.8)	**0.007**
	n=73	n=10	n=79	n=8	n=28	n=53						
24 – 36 n (%)	7 (10)	2 (20)	6 (8)	3 (38)	0	7 (13)	2.1 (0.3,11.8)	0.427	11.9 (1.6,121)	0.019	2.8 (0.03,14.2)	0.991
	n=177	n=20	n=188	n=11	n=73	n=112						
36 – 60 n (%)	11 (6)	1 (5)	13 (7)	1 (9)	3 (4)	10 (9)	0.68 (0.04,11.8)	0.727	1.28 (0.06,8.5)	0.822	2.4 (0.7,11.3)	0.204

[*]n (%) = Number (proportion) positive for B. longum

[¥]Number with normal or elevated biomarker concentration

CI= confidence interval, OR=odds ratio. [α]Odds ratio (95% CI) and P values were obtained with logistic regression after adjusting for and sex. Using the Bonferroni correction for multiple testing, associations with a P value less than 0.0125 (highlighted in bold) were considered statistically significant.

[+] Neopterin (NEO), myeloperoxidase (MPO), and alpha-1 antitrypsin (AAT).

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aged between 12-24 months who were positive for *B. longum* were more likely to have elevated NEO concentration. *D. formicigenerans* carriage was not associated with any individual biomarker in any of the age groups (Table 6).

Bacterial carriage and composite EED score were analyzed by age and sex adjusted logistic regression. Composite EED scores were categorized into low and high scores centered around the mean score (1.73). *B. longum,* but not the other three bacteria species, was associated with the composite EED score. Fecal samples that were positive for *B. longum* were more likely to have a high composite EED score (Table 7).

Bacterial carriage and biomarkers of EED were not associated with stunting

Height measurements and demographic (age and sex) data were available for all participants whose fecal samples were assayed for bacterial carriage (n=613) and 488 of those whose samples were assayed for individual biomarkers of EED. For the complete data set, mean (SD) HAZ score was -1.6 (1.5) with 38% (229/607) of the children stunted. There was no relationship between bacterial carriage and HAZ or stunting. Similarly, none of the individual EED biomarkers or the composite EED score were associated with HAZ or stunting (Table 8).

Discussion

The current study examined the prevalence of four bacteria previously linked with growth in mouse models, as well as the severity of intestinal inflammation and permeability in rural Malawian children. It also investigated the links between bacterial carriage, increased biomarker concentrations, and stunting. *F. prausnitzii* was found in practically all children while *B. longum* was more common in younger children and *A. muciniphila* and *D. formicigenerans* were more common in older children. Fecal concentrations of MPO, NEO, and AAT reduced with age.

The high prevalence of carriage of *F. prausnitzii* reported in the current study is consistent with earlier findings demonstrating that this bacterium is one of the most prevalent and prolific intestinal bacteria in the human gut[22]. This bacterium is present at low levels in infants throughout the first six months of life, increasing slowly in abundance from seven months of life and doubling during the second year of life[23]. Furthermore, *F. prausnitzii* was identified as an important age discriminatory bacterium and proved its low abundance in early life in studies that employed a machine-learning-based approach to define a healthy gut microbiota of Bangladeshi or Malawian children[2,4]. The increase in *D. formicigenerans* carriage with age in our study population is comparable with earlier studies that used molecular approaches to demonstrate intestinal carriage[2,4,23]. The higher prevalence of *B. longum* in younger children is compatible with breast feeding as a source of *B. longum*[24–26], which is present in breast-milk[27,28].

Our data show that younger children had elevated levels of fecal AAT, MPO, and NEO, which is comparable to published findings from resource limited settings[19,21]. The reasons for the negative association between age and fecal levels of AAT, MPO, and NEO are not unknown; however, some authors have proposed that breastfeeding may be a factor because AAT and NEO are present in minute concentrations in breast milk[19,29,30] whereas MPO is thought to be produced from the activation of infant mucosal neutrophils by alpha-lactalbumin and tryptophan as an immune response. Both alpha-lactalbumin and tryptophan are constituents of breastmilk[31,32]. Nonetheless, there is insufficient evidence to determine whether the association between these biomarkers and age is physiological or pathological.

Our study also showed a positive relationship between fecal carriage of *B. longum* and raised biomarkers of EED, which is uncommon given that this bacterium has been demonstrated to have several benefits in the humans. This positive correlation could be attributed to confounding by other factors such as breastfeeding and enteric infections, which were not evaluated. Breastfeeding and enteric infections have been associated with fecal levels of AAT, NEO, and MPO, and MPO and AAT respectively in Bangladeshi infants[19]; therefore more research is needed to investigate the effect of breastmilk, its constituents, and enteric infections on biomarker concentration, gut bacteria, and implications for gut health.

We found no relationship between bacterial carriage or fecal biomarkers of EED and stunting. Some of the important aspects of EED are bacterial composition, intestinal inflammation, and permeability and prior studies have reported or predicted links between high levels of MPO, AAT, or NEO and growth impairment[11,12,33]. Inconsistent findings between these biomarkers and growth outcomes have been previously reported by George *et al.,*[34] who did not find a link between raised baseline fecal levels of AAT, MPO, or NEO and stunting, or between baseline composite EED score and stunting. Furthermore, Campbell and colleagues[35] did not find any relationship between composite EED score and HAZ score at 18 months of follow-up. The majority of studies that have demonstrated links between the biomarkers of EED and growth were longitudinal, used bigger sample sizes, and were conducted in children under the age of two[11,19,33]. There is currently no evidence demonstrating a link between bacterial carriage, assessed by qPCR, and stunting in humans. Given that there are many other factors that influence child growth, the relationship between bacterial carriage, intestinal inflammation or permeability, and stunting may be obscured by other factors such as, food insecurity, enteric infections, diarrheal diseases, social-economic status, maternal education, or other growth determinants.

One of the limitations for the current study was the availability of fewer fecal samples for analysis, which limited the generalizability of results. Furthermore, the lack of data for some demographic factors reduced the number of potential confounders that might have been used in the analysis. Data on socio-economic status, breastfeeding, water quality, sanitation, and hygiene indicators may have enriched the analysis.

Table 6. **Association between *D. formicigenerans* carriage and biomarkers of environmental enteric dysfunction (EED) stratified by age.**

	MPO[+]		AAT[+]		NEO[+]		MPO[+]		AAT[+]		NEO[+]	
Age (months)	**Normal**	**Elevated**	**Normal**	**Elevated**	**Normal**	**Elevated**	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**
0 – 12	[¥]n=23	n=44	n=34	n=31	n=4	n=50	0.27 (0.09,0.77)	0.018	0.34 (0.12,0.92)	0.037	0.38 (0.02,3.23)	0.42
[*]n (%)	16 (70)	17 (39)	21 (62)	11 (35)	3 (75)	27 (54)						
12 – 24	n=72	n=30	n=79	n=24	n=17	n=80	0.91 (0.28,3.28)	0.88	0.98 (0.29,3.97)	0.97	0.32 (0.02,1.86)	0.30
n (%)	61 (85)	25 (83)	66 (84)	20 (83)	16 (94)	67 (84)						
24 – 36	n=73	n=10	n=79	n=8	n=28	n=53	1.04 (0.15,21.03)	0.97	0.86 (0.13,17.4)	0.90	1.08 (0.20,4.83)	0.92
n (%)	66 (90)	9 (90)	71 (90)	7 (88)	25 (89)	48 (91)						
36 – 60	n=177	n=20	n=188	n=11	n=73	n=112	0.23 (0.07,0.84)	0.019	1.12 (0.19,21.38)	0.92	0.85 (0.25,2.54)	0.77
n (%)	164 (93)	15 (75)	170 (90)	10 (91)	68 (93)	102 (91)						

[*]*n (%) = Number (proportion) positive for D. formicigenerans*

[¥]*Number with normal or elevated biomarker concentration*

CI= confidence interval, OR=odds ratio. [α]Odds ratio (95% CI) and P values were obtained with logistic regression after adjusting for sex. Using the Bonferroni correction for multiple testing, associations with a P value less than 0.0125 were considered statistically significant.

[+] *Neopterin (NEO), myeloperoxidase (MPO), and alpha-1 antitrypsin (AAT).*

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Table 7. Association between bacterial carriage and composite environmental enteric dysfunction (EED) score.

Variable	Composite EED score			
	Low (n=175)	High (n=214)	OR (95%CI)	P value[a]
B. longum, n (%)	9 (5)	90 (42)	7.0 (3.3,16.3)	<0.001
A. muciniphila, n (%)	83 (47)	96 (45)	1.0 (0.6,1.6)	0.98
D. formicigenerans, n (%)	162 (93)	169 (79)	0.7 (0.3,1.5)	0.06
F. prausinitzii, n (%)	172 (98)	212 (99)	1.4 (0.2,12.6)	0.73

CI= confidence interval, OR=odds ratio.

n (%) = Number of fecal samples (proportion) positive.

[a] Odds ratio (95% CI) and P values were obtained with logistic regression after adjusting for age and sex. Using the Bonferroni correction for multiple testing, associations with a P value less than 0.0125 were considered statistically significant.

Table 8. Association between bacterial carriage, biomarkers of environmental enteric dysfunction (EED) and height-for-age Z (HAZ) or stunting.

Variable	HAZ		Stunting	
	Coefficient (95%CI)[1]	P value[1]	OR (95% CI)[2]	P value[2]
B. longum	-0.26 (-0.64,0.12)	0.175	0.99 (0.62,1.57)	0.97
A. muciniphila	-0.24 (-0.53,0.03)	0.083	1.21 (0.86,1.69)	0.27
D. formicigenerans	-0.08 (-0.44,0.28)	0.656	1.18 (0.76,1.85)	0.47
F. prausnitzii	-0.01 (-1.1, 1.08)	0.985	1.13 (0.30, 5.38)	0.86
MPO[+]	-0.34 (-0.68,-0.01)	0.046	1.06 (0.65,1.77)	0.81
AAT[+]	-0.22 (-0.60,0.16)	0.26	0.73 (0.42,1.26)	0.26
NEO[+]	-0.19 (-0.51,0.13)	0.25	1.01 (0.64,1.58)	0.97
Composite EED score	-0.07 (-0.38,0.24)	0.67	0.89 (0.56,1.40)	0.61

CI= confidence interval, OR=odds ratio

[1] Coefficients (95% CI) and P values obtained from a linear regression analysis after adjusting for age and sex.

[2] OR (95% CI) and P values obtained from a logistic regression analysis after adjusting for age and sex

[+] Neopterin (NEO), myeloperoxidase (MPO), and alpha-1 antitrypsin (AAT).

** Of all the 613 participants for qPCR, six had out of range HAZ scores while of the 488 participants whose samples were assayed for individual biomarkers of EED, 5 had out of range HAZ scores. These were excluded in this analysis.*

Conclusions

In conclusion, our findings demonstrate a significant prevalence of *F. prausnitzii* and *D. formicigenerans* in fecal samples from older rural Malawian children, which is consistent with previous studies. The current study also found elevated levels of AAT, MPO, and NEO in the age group matching when children are breastfed. Future studies should determine whether the association between age and the fecal levels of these biomarkers in children is normal or pathological. We found an association between *B. longum* carriage and biomarkers of EED and future studies should corroborate this by sequencing *B. longum* strains carried by children in this population to determine if there are any genetic differences with the strains that have been identified as beneficial in other settings. The current study found no relationship between bacterial carriage, intestinal inflammation, intestinal permeability and stunting, implying that the selected bacteria do not contribute to EED or growth in this Malawian population. More studies are needed to determine other factors that influence growth and their association with EED. Such studies could look at risk factors for EED by identifying enteropathogens in food, water, and fecal samples and examining

how the enteropathogens affect the gut microbiota, gut function and subsequent growth. Furthermore, such studies might look into how exclusive breastfeeding and complementary feeding alter gut microbiota composition, gut function, and ultimately linear growth.

Data availability

Underlying data

Figshare: Association between intestinal bacterial carriage, biomarkers of environmental enteric dysfunction and stunting in rural Malawian children.

https://doi.org/10.6084/m9.figshare.20178065.v1[36]

This project contains the following underlying data:

- Intestinal bacterial carriage_EED_stunting_Chaima et al associated dataset.xlsx. (description of data in file).

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References

1. Smith MI, Yatsunenko T, Manary MJ, *et al.*: **Gut microbiomes of Malawian twin pairs discordant for kwashiorkor.** *Science.* 2013; **339**(6119): 548–554.
PubMed Abstract | Publisher Full Text | Free Full Text
2. Blanton LV, Charbonneau MR, Salih T, *et al.*: **Gut bacteria that prevent growth impairments transmitted by microbiota from malnourished children.** *Science.* 2016; **351**(6275).
PubMed Abstract | Publisher Full Text | Free Full Text
3. Schneeberger M, Everard A, Gómez-Valadés AG, *et al.*: **Akkermansia muciniphila inversely correlates with the onset of inflammation, altered adipose tissue metabolism and metabolic disorders during obesity in mice.** *Sci Rep.* 2015; **5**: 16643.
PubMed Abstract | Publisher Full Text | Free Full Text
4. Subramanian S, Huq S, Yatsunenko T, *et al.*: **Persistent gut microbiota immaturity in malnourished Bangladeshi children.** *Nature.* 2014; **510**(7505): 417–421.
PubMed Abstract | Publisher Full Text | Free Full Text
5. Karlsson CL, Onnerfält J, Xu J, *et al.*: **The microbiota of the gut in preschool children with normal and excessive body weight.** *Obesity (Silver Spring).* 2012; **20**(11): 2257–2261.
PubMed Abstract | Publisher Full Text
6. Keusch GT, Denno DM, Black RE, *et al.*: **Environmental enteric dysfunction: pathogenesis, diagnosis, and clinical consequences.** *Clin Infect Dis.* 2014; **59 Suppl 4**(Suppl 4): S207–212.
PubMed Abstract | Publisher Full Text | Free Full Text
7. Fagundes-Neto U, Viaro T, Wehba J, *et al.*: **Tropical enteropathy (*environmental enteropathy*) in early childhood: a syndrome caused by contaminated environment.** *J Trop Pediatr.* 1984; **30**(4): 204–209.
PubMed Abstract | Publisher Full Text
8. Campbell DI, Murch SH, Elia M, *et al.*: **Chronic T cell-mediated enteropathy in rural west African children: relationship with nutritional status and small bowel function.** *Pediatr Res.* 2003; **54**(3): 306–311.
PubMed Abstract | Publisher Full Text
9. Prendergast AJ, Humphrey JH, Mutasa K, *et al.*: **Assessment of Environmental Enteric Dysfunction in the SHINE Trial: Methods and Challenges.** *Clin Infect Dis.* 2015; **61 Suppl 7**: S726–S732.
PubMed Abstract | Publisher Full Text | Free Full Text
10. Lunn PG: **The impact of infection and nutrition on gut function and growth in childhood.** *Proc Nutr Soc.* 2000; **59**(1): 147–154.
PubMed Abstract | Publisher Full Text
11. 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**(2): 390–396.
PubMed Abstract | Publisher Full Text | Free Full Text
12. Guerrant RL, Leite AM, Pinkerton R, *et al.*: **Biomarkers of Environmental Enteropathy, Inflammation, Stunting, and Impaired Growth in Children in Northeast Brazil.** *PLoS One.* 2016; **11**(9): e0158772.
PubMed Abstract | Publisher Full Text | Free Full Text
13. Hart JD, Samikwa L, Sikina F, *et al.*: **Effects of Biannual Azithromycin Mass Drug Administration on Malaria in Malawian Children: A Cluster-Randomized Trial.** *Am J Trop Med Hyg.* 2020; **103**(3): 1329–1334.
PubMed Abstract | Publisher Full Text | Free Full Text
14. Doan T, Arzika AM, Hinterwirth A, *et al.*: **Macrolide Resistance in MORDOR I - A Cluster-Randomized Trial in Niger.** *N Engl J Med.* 2019; **380**(23): 2271–2273.
PubMed Abstract | Publisher Full Text | Free Full Text
15. Chaima D, Pickering H, Hart JD, *et al.*: **Up to Four Biannual Administrations of Mass Azithromycin Treatment Are Associated with Modest Changes in the Gut Microbiota of Rural Malawian Children.** *Preprints.* 2020; 2020100138.
Publisher Full Text
16. 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.** *medRxiv.* 2021; 2021.2001.2019.21250144.
Publisher Full Text
17. Chaima D: **Characterization of the fecal microbiota of rural Malawian children, associations with biomarkers of environmental enteric dysfunction and the impact of a mass drug administration program.** Doctoral thesis, University of London, London School of Hygiene and Tropical Medicine. 2020.
Publisher Full Text
18. Pickering H, Holland MJ, Last AR, *et al.*: **Evaluation of a *Chlamydia trachomatis*-specific, commercial, real-time PCR for use with ocular swabs.** *Parasit Vectors.* 2018; **11**(1): 102.
PubMed Abstract | Publisher Full Text | Free Full Text
19. McCormick BJJ, Lee GO, Seidman JC, *et al.*: **Dynamics and Trends in Fecal Biomarkers of Gut Function in Children from 1-24 Months in the MAL-ED Study.** *Am J Trop Med Hyg.* 2017; **96**(2): 465–472.
PubMed Abstract | Publisher Full Text | Free Full Text
20. de Onis M, Garza C, Onyango AW, *et al.*: **[WHO growth standards for infants and young children].** *Archives de pediatrie: organe officiel de la Societe francaise de pediatrie.* 2009; **16**(1): 47–53.
Publisher Full Text
21. Naylor C, Lu M, Haque R, *et al.*: **Environmental Enteropathy, Oral Vaccine Failure and Growth Faltering in Infants in Bangladesh.** *EBioMedicine.* 2015; **2**(11): 1759–1766.
PubMed Abstract | Publisher Full Text | Free Full Text
22. Qin J, Li R, Raes J, *et al.*: **A human gut microbial gene catalogue established by metagenomic sequencing.** *Nature.* 2010; **464**(7285): 59–65.
PubMed Abstract | Publisher Full Text | Free Full Text
23. Hopkins MJ, Macfarlane GT, Furrie E, *et al.*: **Characterisation of intestinal bacteria in infant stools using real-time PCR and northern hybridisation analyses.** *FEMS Microbiol Ecol.* 2005; **54**(1): 77–85.
PubMed Abstract | Publisher Full Text
24. Khonsari S, Suganthy M, Burczynska B, *et al.*: **A comparative study of bifidobacteria in human babies and adults.** *Biosci Microbiota Food Health.* 2016; **35**(2): 97–103.
PubMed Abstract | Publisher Full Text | Free Full Text
25. Odamaki T, Kato K, Sugahara H, *et al.*: **Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study.** *BMC Microbiol.* 2016; **16**: 90.
PubMed Abstract | Publisher Full Text | Free Full Text
26. Yatsunenko T, Rey FE, Manary MJ, *et al.*: **Human gut microbiome viewed across age and geography.** *Nature.* 2012; **486**(7402): 222–227.
PubMed Abstract | Publisher Full Text | Free Full Text
27. Martín R, Jiménez E, Heilig H, *et al.*: **Isolation of Bifidobacteria from Breast Milk and Assessment of the Bifidobacterial Population by PCR-Denaturing**

Gradient Gel Electrophoresis and Quantitative Real-Time PCR. *Appl Environ Microbiol.* 2009; **75**(4): 965–9.
PubMed Abstract | Publisher Full Text | Free Full Text

28. Peirotén A, Arqués JL, Medina M, *et al.*: **Bifidobacterial strains shared by mother and child as source of probiotics.** *Benef Microbes.* 2018; **9**(2): 231–238.
PubMed Abstract | Publisher Full Text

29. Chowanadisai W, Lönnerdal B: **Alpha(1)-antitrypsin and antichymotrypsin in human milk: origin, concentrations, and stability.** *Am J Clin Nutr.* 2002; **76**(4): 828–833.
PubMed Abstract | Publisher Full Text

30. Matsubara Y, Gaull GE: **Biopterin and neopterin in various milks and infant formulas.** *Am J Clin Nutr.* 1985; **41**(1): 110–112.
PubMed Abstract | Publisher Full Text

31. Tsopmo A, Diehl-Jones BW, Aluko RE, *et al.*: **Tryptophan Released From Mother's Milk Has Antioxidant Properties.** *Pediatr Res.* 2009; **66**(6): 614–618.
PubMed Abstract | Publisher Full Text

32. Elisia I, Tsopmo A, Friel JK, *et al.*: **Tryptophan from human milk induces oxidative stress and upregulates the Nrf-2-mediated stress response in human intestinal cell lines.** *J Nutr.* 2011; **141**(8): 1417–1423.
PubMed Abstract | Publisher Full Text

33. Kosek MN, MAL-ED Network Investigators: **Causal Pathways from Enteropathogens to Environmental Enteropathy: Findings from the MAL-ED Birth Cohort Study.** *EBioMedicine.* 2017; **18**: 109–117.
PubMed Abstract | Publisher Full Text | Free Full Text

34. 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**(6): 1117–1124.
PubMed Abstract | Publisher Full Text | Free Full Text

35. Campbell RK, Schulze KJ, Shaikh S, *et al.*: **Biomarkers of Environmental Enteric Dysfunction Among Children in Rural Bangladesh.** *J Pediatr Gastroenterol Nutr.* 2017; **65**(1): 40–46.
PubMed Abstract | Publisher Full Text | Free Full Text

36. Chaima D: **Association between intestinal bacterial carriage, biomarkers of environmental enteric dysfunction and stunting in rural Malawian children.** *Figshare.* Dataset. 2022.
http://www.doi.org/10.6084/m9.figshare.20178065.v1