Up to four biannual administrations of mass azithromycin treatment are associated with modest changes in the gut microbiota of rural Malawian children

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Abstract

Community-level mass treatment with azithromycin has been associated with a mortality benefit in children. However, antibiotic exposures result in disruption of the gut microbiota and repeated exposures may reduce recovery of the gut flora. We conducted a nested cohort study to examine associations between mass drug administration (MDA) with azithromycin and the gut microbiota of rural Malawian children aged between 1-59 months. Fecal samples were collected from the children prior to treatment and 6 months after two or four biannual rounds of azithromycin treatment. DNA was extracted from fecal samples and V4-16S rRNA sequencing used to characterize the gut microbiota. *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* were the dominant phyla while *Faecalibacterium* and *Bifidobacterium* were the most prevalent genera. There were no associations between azithromycin treatment and changes in alpha diversity, however, four biannual rounds of treatment were associated with increased abundance of *Prevotella*. The lack of significant changes in gut microbiota after four biannual treatments supports the use of mass azithromycin treatment to reduce mortality in children living in low- and middle-income settings.

Introduction

Azithromycin is a broad-spectrum, macrolide antibiotic characterized by a long intra- and extra-cellular half-life. Its use is indicated in the treatment of atypical pneumonia, skin and soft tissue infections and sexually transmitted infections[1]. The World Health Organization (WHO) recommends mass azithromycin treatment at the community level as one of the key strategies for the elimination of trachoma as a public health problem[2]. Studies of mass azithromycin distribution for trachoma control in endemic areas indicate that mass treatment has secondary effects, which include reductions in child morbidity and mortality[3-6]. Recently, a multi-site trial, conducted in Malawi, Tanzania and Niger reported lower mortality rates in children (under 5 years of age) who received mass azithromycin treatment compared to children who received a placebo[7]. The specific mechanism through which azithromycin reduced mortality in children is not understood, however, several studies have reported a reduction in the community burden of nasopharyngeal carriage of *Streptococcus pneumoniae*[8-10] and a reduction in the

abundance of *Campylobacter* spp. in the gut[11]. Additionally, reduced risks of diarrhea and acute lower respiratory infections related to azithromycin mass drug administration (MDA) for trachoma control have been reported in Tanzanian children[4,5]. Thus, azithromycin may reduce morbidity and mortality by reducing carriage of pathogenic bacteria.

Available evidence indicates that azithromycin treatment causes alterations in the gut microbiota that can be measured in the weeks immediately following treatment. Recent randomized, placebo-controlled trials of Parker *et al.*[12] and Wei *et al.*[13] characterized the intestinal microbiota of Indian and Danish infants respectively, at baseline and 14 days after a 3-day course of azithromycin and reported changes in intestinal microbiota. Both studies reported a decrease in alpha diversity in fecal samples of treated children compared to those who received placebo. Additionally, Parker *et al.*[12] reported a decrease in relative abundance of *Proteobacteria* and *Verrucomicrobia* while Wei *et al.*[13] reported a decrease in the abundance of *Actinobacteria* after treatment in fecal samples of treated children compared to placebo. Alterations in the gut microbiota have also been reported more than 6 months after exposure to antibiotics, suggesting that the short-term changes may persist for a longer period of time. An observational study by Korpela *et al.*[14], which characterized the fecal microbiota of Finnish children aged 2-7 years with varying durations of exposure to azithromycin, clarithromycin, penicillin or no antibiotic exposure over a 24-months span, showed a decrease in microbial richness in children treated with macrolides compared to those treated with penicillin or without exposure to antibiotics. Additionally, a significant decrease in the relative abundance of *Actinobacteria* and an increased abundance of *Bacteroidetes* and *Proteobacteria* was found in children who had used macrolide antibiotics within 6 months of sample donation. However, Wei *et al.*[13] reported no significant differences in alpha diversity and taxonomic composition between the treatment and control groups 4 years after treatment. To date, it is not clear how long the azithromycin-related changes in the gut microbiota persist. A study investigating the intestinal microbiota of three individuals over a period of 10 months covering two courses of the antibiotic ciprofloxacin reported loss of diversity and a change in community composition that occurred within 3 to 4 days of

initiating a course[15]. Approximately 1 week after the end of each treatment course, the return to a pre-treatment state was still incomplete.

In the current study, we investigated the association between azithromycin treatment and changes in the fecal microbiota of rural Malawi children 6 months after 2 or 4 biannual azithromycin distributions. We explored the diversity and composition of the intestinal microbiota in children who received azithromycin versus children who received placebo.

Materials and methods

Study design

This study was nested within a survey of prevalence of carriage of macrolide resistant enteropathogens conducted within the Childhood Mortality Reduction after Oral Azithromycin in Malawi (MORDOR-Malawi) trials (NCT02047981), carried out in the Mangochi district. Briefly, in the MORDOR-Malawi study, 30 clusters, defined as the catchment area of a Health Surveillance Assistant (HSA) (approximately 1,000 total population), were randomly selected to receive 4 biannual rounds of MDA with either azithromycin or placebo. All children aged 1-59 months and weighing $\geq$3.8kg were eligible for treatment at each of 4 biannual mass distributions. Fecal sample collection took place in the 30 clusters during the baseline survey (May-July 2015) and at 12-month and 24-month surveys (April-June 2016 and 2017 respectively), approximately 6 months after the second and fourth treatment rounds (**Figure 1**). At each survey, children aged 1-59 months were randomly selected for participation in sample collection using custom software on Android devices. The target for recruitment at each survey was 40 children in each of the 30 clusters, to recruit a total of 1200 children.
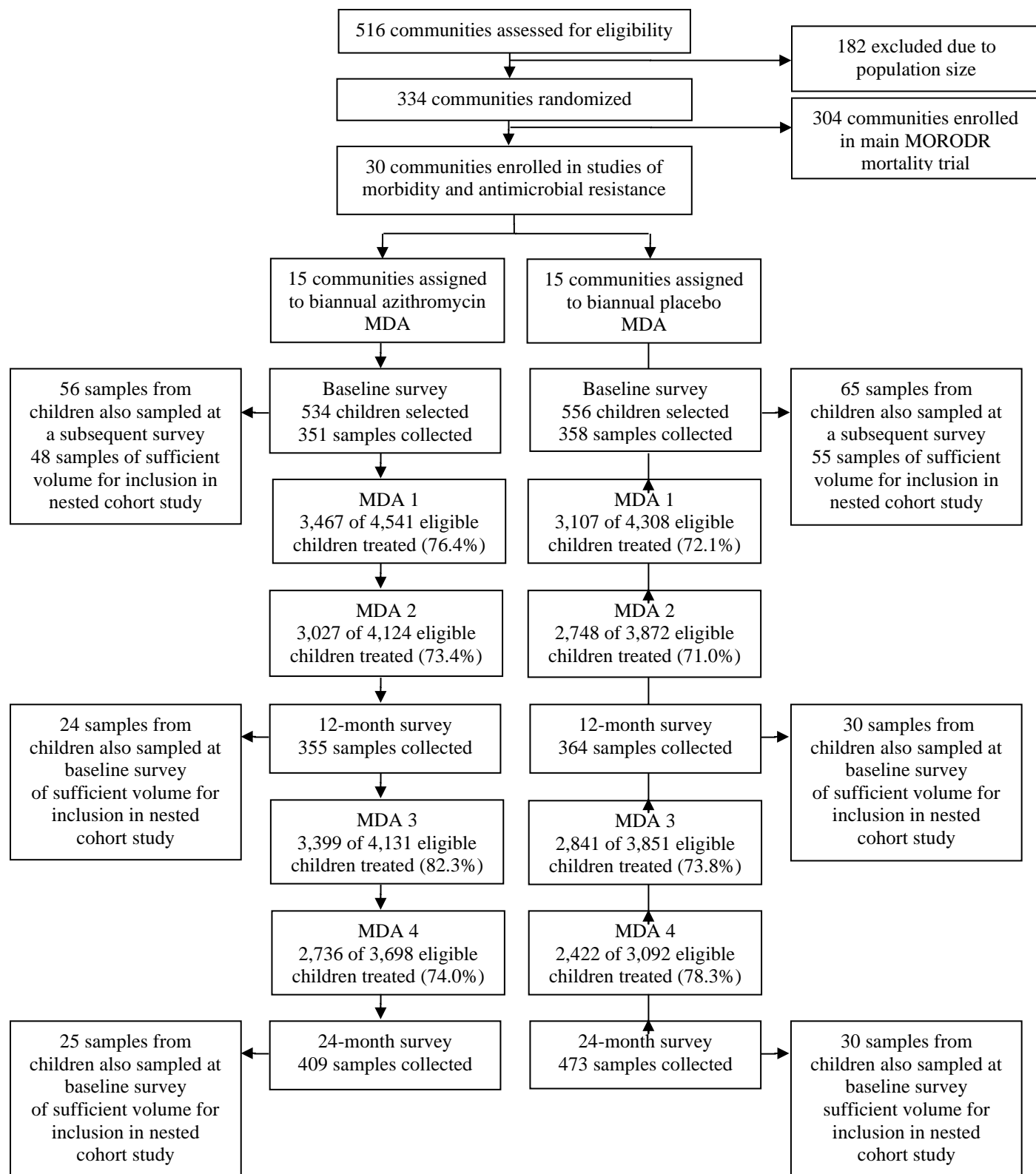
Figure 1. Study flow. *Study flow for the cross sectional survey of carriage of antimicrobial resistance and selection of samples for the nested cohort study*

A retrospective nested cohort study was then conducted within the framework provided by the three cross-sectional surveys described above. Of the recruited children who provided fecal samples at the baseline cross-sectional survey, 121 were also sampled, by chance, at an additional survey thereby providing the potential to generate longitudinal pairs of samples (before and after treatment). Baseline samples from 103 such children had sufficient volume remaining for 16S rRNA gene sequence analysis and were therefore included in the nested cohort study (**Figure 1**). Fifty-four of these children were sampled at the baseline and 12-month surveys and 55 children were sampled at the baseline and 24-month surveys (**Figure 1**).

Fecal sample collection

Fecal samples from participating children were collected by their mothers or guardians, who were provided with sterile fecal collection bottles (Wheaton, UK) and given verbal instruction, by a nurse, on how to collect the sample. Fecal samples were returned to the field team as soon as possible after collection. These samples were put in a cool box with ice packs until the end of the day (not more than 8 hours after collection) and were brought to the laboratory where they were stored at -80°C.

V4 16S rRNA gene sequencing

Samples were thawed at room temperature and 250mg amounts weighed into sterile Eppendorf tubes. Total, genomic DNA was then extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc, Carlsbad, CA, now a part of Qiagen, Germany). Indexed primers 515F (515F_Indexed; 5'-adapter-GTGCCAGCMGCCGCGGTAA-3') and 806R (806R_Indexed; 5'-adapter-GGACTACHVGGGTWTCTAAT-3')[16] were used to amplify the V4 region of the bacterial 16S rRNA gene in the extracted DNA. The total volume of a single PCR reaction was 25µl and each reaction contained 10µl 2.5× Quantabio 5prime HotMasterMix (Quantabio, Beverly, MA, USA), 10µM primers (SIGMA, UK), 4µl template DNA and 8µl PCR grade water (Qiagen). Amplification took place on a Veriti™ 96 well thermal cycler (Applied Biosystems, UK) using the following thermal cycling conditions: 94°C for 3 minutes, 35 cycles of 30 seconds at 94°C, 30 seconds at 50°C and 45 seconds at 72°C, then a final extension of 10 minutes at 72°C. Each PCR run included a mock bacterial community (composed of DNA from

Hemophilus influenza, *Moraxella catarrhalis* and *Staphylococcus epidermis*), which acted as a positive control, and a no-template control. Amplicon size and PCR efficiency were verified by gel electrophoresis after which amplicons were purified using 0.6 v/v AMPure XP beads (Beckman Coulter, CA, USA) and 70% ethanol.

A second PCR was then performed to barcode each amplicon thereby enabling downstream multiplexing. The total volume for a single reaction of index PCR was 49μl. Each 49μl PCR reaction contained 20μl 2.5× Quantabio 5prime HotMasterMix (Quantabio, Beverly, MA, USA), 2μM MID Illumina primers[17], 22μl of amplicon and 5.5μl of PCR grade water (Qiagen). Cycling conditions were as follows: 94°C for 3 minutes followed by 5 cycles of 10 seconds at 94°C, 30 seconds at 58°C and 45 seconds at 72°C, then 10 minutes at 72°C.

Amplicons were purified, quantified on a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) and thereafter pooled in equimolar amounts. The MiSeq v3 reagent kit (Illumina Inc., San Diego, CA, USA) was used to prepare the DNA library for cluster generation and sequencing. The DNA library was denatured with a sodium hydroxide solution (0.2N NaOH) and then diluted with hybridization buffer (HT1) to a final loading concentration of 15pM. It was then spiked with 10% PhiX control, which served as an internal control for low-diversity libraries, heat-denatured at 96°C for 2 minutes and assayed by 2×300bp paired-end sequencing on the Illumina MiSeq platform for a total of 600 cycles using a standard protocol[18].

Sequence processing

FastQ files, containing raw sequence data, were processed in QIIME 2 software (version 2018.6)[19]. Barcoded sequences were demultiplexed using the *demux* function in QIIME 2. Poor quality reads were filtered out and chimeras were removed.

The 16S rRNA sequences were clustered *de novo* into OTUs at ≥ 97% identity. Taxonomy was assigned to the OTUs using a naïve Bayes classifier pre-trained on the SILVA 16S database[20]. To exclude spurious OTUs, only bacterial OTUs identified to the genus level, with sequences more than 0.005% of the total number of sequences[21] and a frequency of more than 0.01% in any sample were retained in the analyses.

Statistical analysis

All statistical analyses were performed in R[22]. Shannon (H) and Simpson (D) diversity indices were calculated using the phyloseq package[23]. Differences in the distribution of parametric data between groups were tested using Student's t-test or ANOVA while the Wilcoxon-rank or Kruskal-Wallis tests were used for non-parametric data. The sample proportion test was used to compare proportions between groups. To determine bacterial phylogenetic distance between samples, weighted and unweighted UniFrac distance matrices were calculated using the phyloseq R package. The phylogenies used to calculate the UniFrac distances were computed using RAxML v8.2.11147 from a variable sites alignment using a generalized time-reversible (GTR) + gamma model. To compare bacterial community compositional differences between groups, PERMANOVA with 1000 permutation tests was run on weighted and unweighted UniFrac distances and principal coordinate analysis (PCoA) plots were used to visualize differences in UniFrac clustering by groups.

Ethical considerations

The MORDOR-Malawi study was approved by the London School of Hygiene and Tropical Medicine ethics committee in the UK (study number 6500) and the College of Medicine Research Ethics Committee in Malawi (study number 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 indicated the willingness, or unwillingness, of the community to participate through verbal consent. Written, informed consent (by thumbprint or signature) was then obtained from the parent or guardian of each child before recruitment. During the consenting process, all parents and guardians were informed of their freedom to withdraw their child from the study at any time without giving reason for doing so. All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki.

Results

Baseline demographics

A baseline survey of prevalence of carriage of macrolide resistant enteropathogens enrolled 1090 children, however, of these, only 709 (65%) returned fecal samples. Of these 709 children, 103 (9%) were included in the nested cohort study that examined the fecal microbiota using 16S rRNA sequence analyses. One baseline sample consistently failed to amplify and could not be sequenced; 102 samples were retained for analysis after quality filtering. The age and sex of children whose fecal samples were included in the analysis of the nested cohort compared to all children enrolled in the cross-sectional survey are shown in **Table 1**. The proportion of male children was comparable between those whose fecal samples were included in this nested study and all enrolled participants. However, children whose fecal samples were included in the nested cohort were younger compared to all enrolled participants.

Table 1 Baseline demographics of participants whose samples were included in the nested cohort study of 16S rRNA sequencing versus all participants who provided fecal samples for the cross sectional survey of antimicrobial resistance

Participant characteristic	Children included in nested cohort	All enrolled children	P value
Number of participants	102	1090	
Male sex N (%)	52 (51%)	487 (45%)	0.304¥
Mean (SD) age in months	22.95 (13.54)	29.59 (16.42)	<0.001*
Age range in months	2 - 56	1 - 59	

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

Number of sequencing reads, characteristics and taxon abundance at baseline

One hundred and two baseline samples were retained for analysis after quality filtering. The baseline dataset generated a total of 1,685,792 reads with an average read depth per sample of 16,527±11,071. A total of 9 phyla, 16 classes, 25 orders, 43 families and 117 classified and 6 unclassified genera were identified from the V4-amplicon sequence reads. Seven genera (*Faecalibacterium, Streptococcus, Bacteroides, Haemophilus, Subdoligranulum, Bifidobacterium* and *Escherichia-Shigella*) were present in approximately 60% of the samples and together accounted for 46% of the whole bacterial community. After rarefaction to 1000 reads with 1000 permutations, 69 genera belonging to 7 phyla, *Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, Tenericutes* and *Verrucomicrobia* were retained. Out of the 7 identified phyla, *Firmicutes, Bacteroidetes, Proteobacteria* and *Actinobacteria* were the most dominant and accounted for 64%, 14%, 9% and 7% of the total bacterial community respectively (**Figure 2A**). Seven genera (*Faecalibacterium, Streptococcus, Bacteroides, Haemophilus, Subdoligranulum, Bifidobacterium* and *Escherichia-Shigella*) were present in approximately 62% of the samples and together accounted for 46% of the whole bacterial community; *Faecalibacterium* was prevalent in 81% of the samples making it the most prevalent genus. *Bifidobacterium* was prevalent in 51% of the samples and had the highest number of reads, accounting for 15% of the total reads (**Figure 2B**).
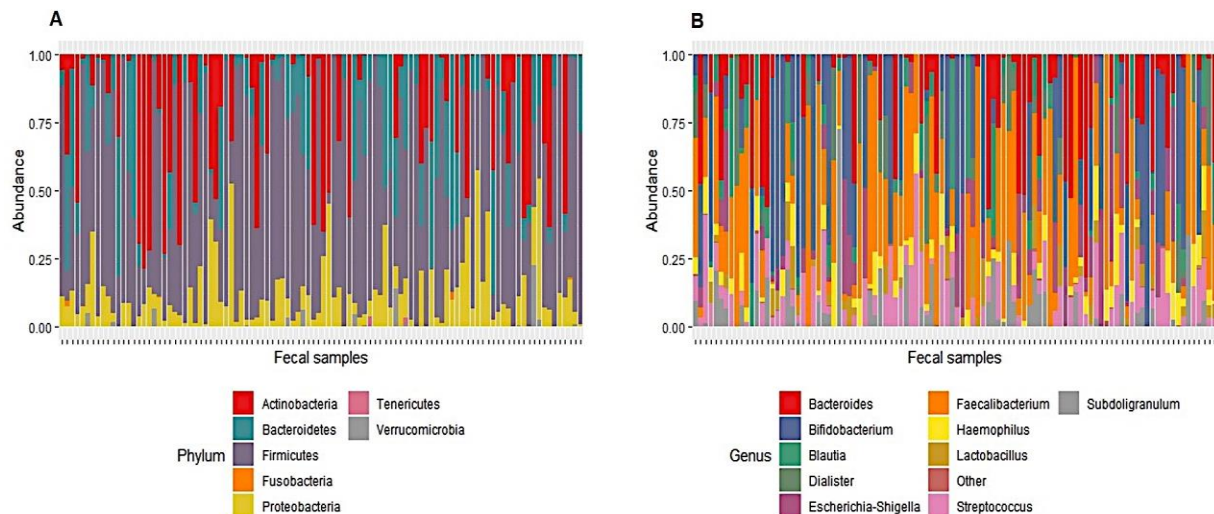
***Figure 2. Relative abundance of major taxa found in fecal samples at baseline**. A. Major phyla. The proportion of total number of reads for each phylum in a sample represents phylum abundance after rarefaction to 1000 reads. B. Major genera. The proportion of total number of reads for each genus in a sample represents genus abundance after rarefaction to 1000 reads. The stacked bar plot only shows the 10 most abundant genera. Genera with relative abundance < 1% were grouped as "Other".*

<u>Definition of datasets and baseline demographics of the datasets</u>

Samples from 54 children who were sampled at baseline and at the 12-month survey, which was conducted 6 months after 2 rounds of biannual treatment, were included in the final analysis. The data from these samples are referred to as the "BL vs 2MDA dataset". Samples from 55 children who participated in baseline sampling and sampling at the 24-month survey, conducted 6 months after 4 rounds of biannual treatment, were included in the final analysis. This data set is referred to as the "BL vs 4MDA dataset".

Within the BL vs 2MDA dataset, 30 children received placebo and 24 received azithromycin treatment while within the BL vs 4MDA dataset, 30 children received placebo and 25 received azithromycin treatment (**Figure 1**). Age and sex of children were comparable between azithromycin and placebo arms in both data sets **(Table 2)**.

Table 2 Comparison of baseline participant characteristics between treatment groups for the BL vs 2MDA and BL vs 4MDA datasets

	Treatment arm		
Participant characteristics	Azithromycin	Placebo	P value
BL vs 2MDA dataset			
Number of participants	24	30	
Child male sex N (%)	14 (58%)	15 (50%)	0.74$^{¥}$
Mean (SD) age, months	27 (14)	24.3 (15)	0.51 *
Treatment rounds received n (%)			
0	1 (4%)		
1	10 (42%)		
2	13 (51%)		
BL vs 4MDA dataset			

Number of participants	25	30	
Child male sex N (%)	13 (52%)	16 (53%)	0.99¥
Mean (SD) age, months	18.3 (10.6)	19 (10.8)	0.82*
Treatment rounds received n (%)			
0		3 (12)	
1		3 (12)	
2		6 (24)	
3		4 (16)	
4		9 (36)	

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

Alpha diversity measures of the datasets at baseline

We examined the distribution of alpha diversity indicated by Shannon and Simpson indices in the BL vs 2MDA and BL vs 4MDA datasets. Within the BL vs 2MDA dataset, Shannon and Simpson indices were different between azithromycin and placebo arms at baseline, however, both diversity indices were similar between the two treatment groups at baseline within the BL vs 4MDA dataset **(Table 3)**.

Table 3 Alpha diversity distribution between treatment groups

Dataset	Time-point	Shannon diversity index			Simpson diversity index		
		Azithromycin	Placebo	P value	Azithromycin	Placebo	P value
BL vs 2MDA	Baseline	2.4 (1.9,2.5)¥	2 (1.6,2.2)¥	0.02	0.9(0.8,0.9)¥	0.8 (0.7,0.9)¥	0.01
	12 months	2.2(2,2.4)¥	2.2 (1.7,2.4)¥	0.71a	0.8 (0.8,0.9)¥	0.8 (0.7,0.8)¥	0.72a
BL vs 4MDA	Baseline	2.0 (0.5)α	2.1 (0.5)α	0.69b	0.8 (0.7,0.8)¥	0.8 (0.7,0.8)¥	0.61
	24 months	2.2 (0.5)α	2.2(0.4)α	0.97b	0.8 (0.8,0.9)¥	0.8 (0.8,0.9)¥	0.91a

¥*Median (25th,75th quartile)*

α*Mean (SD)*

a*P value calculated by Wilcoxon rank sum test*

b*P value calculated by Student's t-test*

No change in alpha diversity measures observed following 2 and 4 treatment rounds

The BL vs 2MDA dataset was used to explore changes in microbiota diversity 6 months after 2 rounds of azithromycin MDA. At the 12-month survey, no differences in alpha diversity, measured by Shannon and Simpson diversity indices, were found between the azithromycin and placebo treatment arms (**Table 3**). Similarly, there was no difference in Shannon or Simpson diversity index distribution between baseline and 12-month fecal samples within the azithromycin arm (**Table 4**).

The BL vs 4MDA dataset was used to assess the effect of up to 4 rounds of biannual azithromycin treatment on fecal microbiota diversity 6 months after the last treatment round. A comparison of fecal microbiota diversity between azithromycin and placebo arms after 4 rounds of azithromycin had been distributed showed no differences in Shannon and Simpson diversity indices (**Table 3**). A comparison of Shannon and Simpson diversity indices between fecal samples collected baseline and 24-month surveys in the azithromycin group in the unadjusted analysis showed differences between the two time-points but no differences were observed after adjusting for age and sex (**Table 4**).

Table 4 Alpha diversity distribution within the azithromycin treatment group

Dataset	Time-point	Shannon index	P value[c]	P value[d]	Time-point	Simpson index	P value[c]	P value[d]
BL vs 2MDA	Baseline	2.1(1.9,2.4)[¥]			Baseline	0.8 (0.77,0.88)[¥]		
	12 months	2.2(1.9,2.4)[¥]	0.88[a]		12 months	0.8 (0.78,0.87)[¥]	0.99[a]	
BL vs 4MDA	Baseline	1.8 (0.5)[α]			Baseline	0.77 (0.66,0.83)[¥]		
			0.005[b]	0.37			0.33[a]	0.99
	24 months	2.2 (0.5)[α]			24 months	0.82 (0.78,0.87)[¥]		

[¥]*Median (25th,75th quartile)*

[α]*Mean (SD)*

[a]*P value calculated by Wilcoxon rank sum test*

[b]*P value calculated by Student's t-test*

[c]*Denotes unadjusted P values while* [d]*denotes P values calculated from a linear regression model after adjusting for age and sex*

Modest changes in microbiota composition following 2 and 4 treatment rounds

Within the BL vs 2MDA dataset, PERMANOVA analysis using unweighted (R^2 =0.01, F=0.69, P=0.85) and weighted (R^2 =0.01, F=0.47, P=0.89) UniFrac distances did not detect differences in overall microbiota composition between azithromycin and placebo treatment arms following two rounds of treatment. Similarly, there were no differences in microbiota composition between the fecal samples collected at the baseline and 12-month surveys in the azithromycin group based on weighted (R^2 =0.03, F=1.39, P=0.21) and unweighted (R^2 =0.03, F=1.35, P=0.14) UniFrac distances.

Within the BL vs 4MDA dataset, PERMANOVA analysis did not detect any differences in overall fecal microbiota composition between azithromycin and placebo groups after four treatment rounds, based on weighted (R^2 =0.005, F=0.24, P=0.97) and unweighted (R^2 =0.015, F=0.79, P=0.73) UniFrac distances.

A comparison of fecal microbiota composition between baseline and 24-month within the azithromycin arm, after distribution of 4 rounds of azithromycin showed significant differences based on unweighted (**Figure 3a)** and weighted (**Figure 3b**) UniFrac distances. The relative abundances of taxa at baseline and after 4 rounds of treatment within the azithromycin arm were compared and visualized using stacked bar plots at phylum and genus levels. Crude comparisons of relative abundance at the phylum level showed a higher relative abundance of *Bacteroidetes* after treatment [median (IQR) of 0.38 (0.31,0.45) at the 24-month survey vs 0.21 (0.08,0.40) at the baseline survey, Wilcoxon P=0.004]. *Actinobacteria* was shown to be more abundant in fecal samples collected at baseline in the azithromycin arm [median (IQR) of 0.08 (0,0.47) at baseline vs 0.01 (0,0.02) at the 24-month survey, Wilcoxon P=0.014]. At the genus level, *Bifidobacterium* and *Streptococcus* were more abundant in samples collected at baseline compared to the 24-month survey samples, while *Prevotella* was more abundant in fecal samples collected at the 24-month survey (**Table 5** and **Figure 4a**).

To test whether the crude differences observed between the baseline and 24-month surveys in the azithromycin group could be due to treatment or natural changes in the microbiota, we then compared the baseline and 24-month samples within the placebo arm. A comparison of microbiota composition between baseline and 24-month surveys within the placebo group also showed significant differences based on unweighted (**Figure 3c**) and weighted (**Figure 3d**) UniFrac distances. Relative abundance of the phylum *Actinobacteria* was shown to be higher in samples collected at baseline than at 24-month [median (IQR) of 0.14 (0.00 - 0.31) at the baseline survey vs 0.0001 (0.00 - 0.01) at the 24-month survey, Wilcoxon P=0.001]. At the genus level, *Bifidobacterium* was shown to be more abundant in fecal samples collected at baseline compared to 24-month samples while *Prevotella* was more abundant in samples collected at the 24-month survey (**Table 5** and **Figure 4b**).
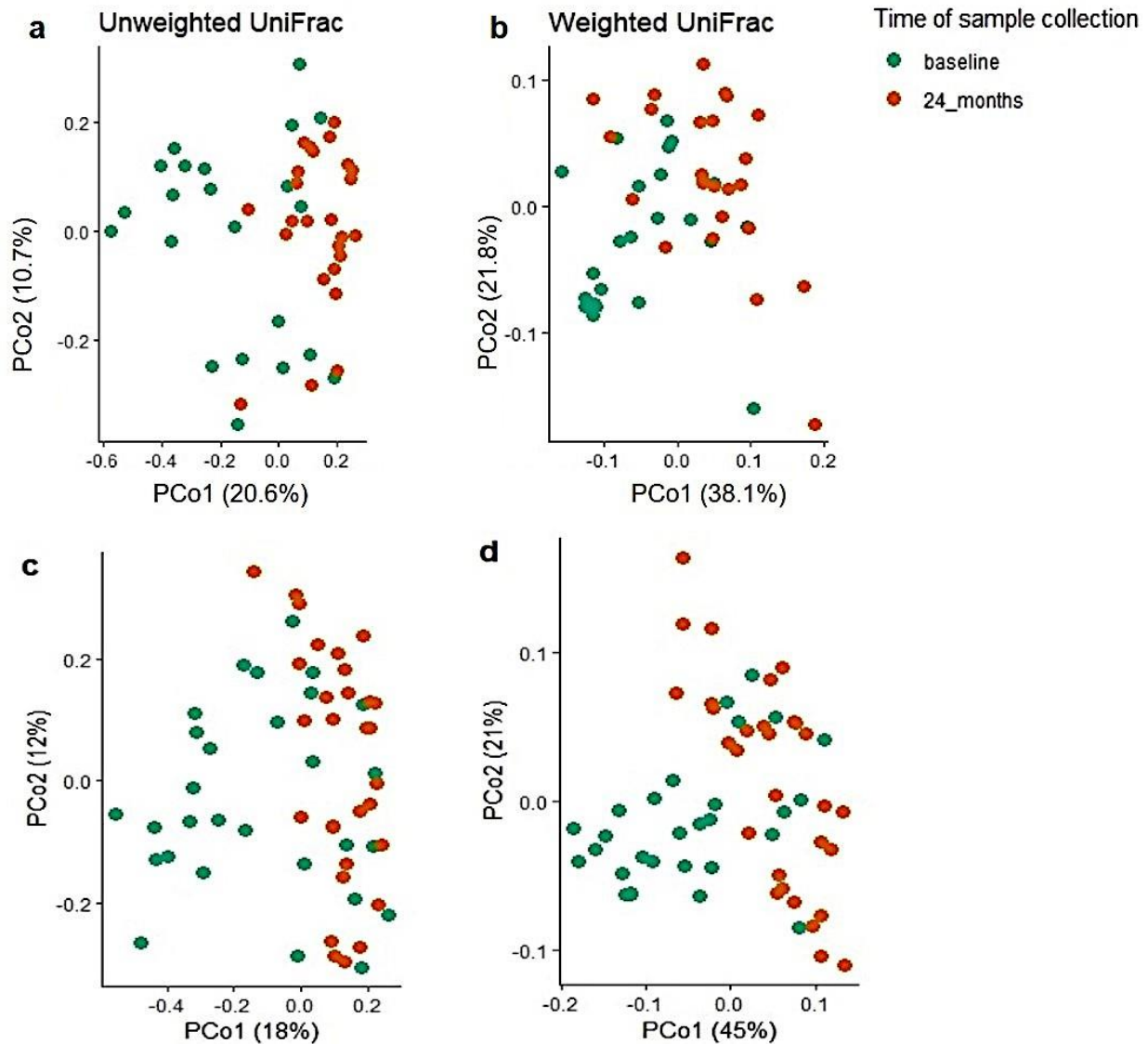
Figure 3 Overall fecal microbiota composition differences between baseline and 24-month fecal samples in the azithromycin (a and b) and placebo (c and d) arms. *(a) PCoA plot of unweighted UniFrac distances [PERMANOVA, R^2 =0.1, F=5.09, P=0.001] (b) PCoA plot of weighted UniFrac distances [PERMANOVA, R^2=0.1, F=5.1, P=0.001], (c) PCoA plot of unweighted UniFrac distances [PERMANOVA, R^2 =0.07, F=4.01, P=0.001] (d) PCoA plot of weighted UniFrac distances [PERMANOVA, R^2 =0.16, F=11.87, P=0.001]. In all the PCoA plots above (a-d), PCo1 explained the most variation and baseline fecal samples clustered in the negative space of PCo1 while 24-month fecal samples clustered in the positive space of PCo1.*

Generalized linear mixed models, adjusted for age, sex and time since last treatment with the participant as a random effect, were then used to assess the longitudinal

differences in the relative abundance of individual genera by treatment arm. The analyses showed no significant differences in the relative abundance of *Streptococcus* between the baseline and 24-month surveys in the azithromycin arm. There were also no significant differences in the relative abundance of *Bifidobacterium* between the baseline and 24-month surveys in both the azithromycin and placebo arms. However, azithromycin treatment was associated with increased relative abundance of *Prevotella* at the 24-month survey; samples collected at the 24-month survey in the azithromycin arm had 30% increased odds of relative abundance of *Prevotella* compared to samples collected at the baseline survey within the same azithromycin arm **(Table 5)**. No longitudinal differences were shown in the placebo arm in the adjusted analysis (**Table 5**).
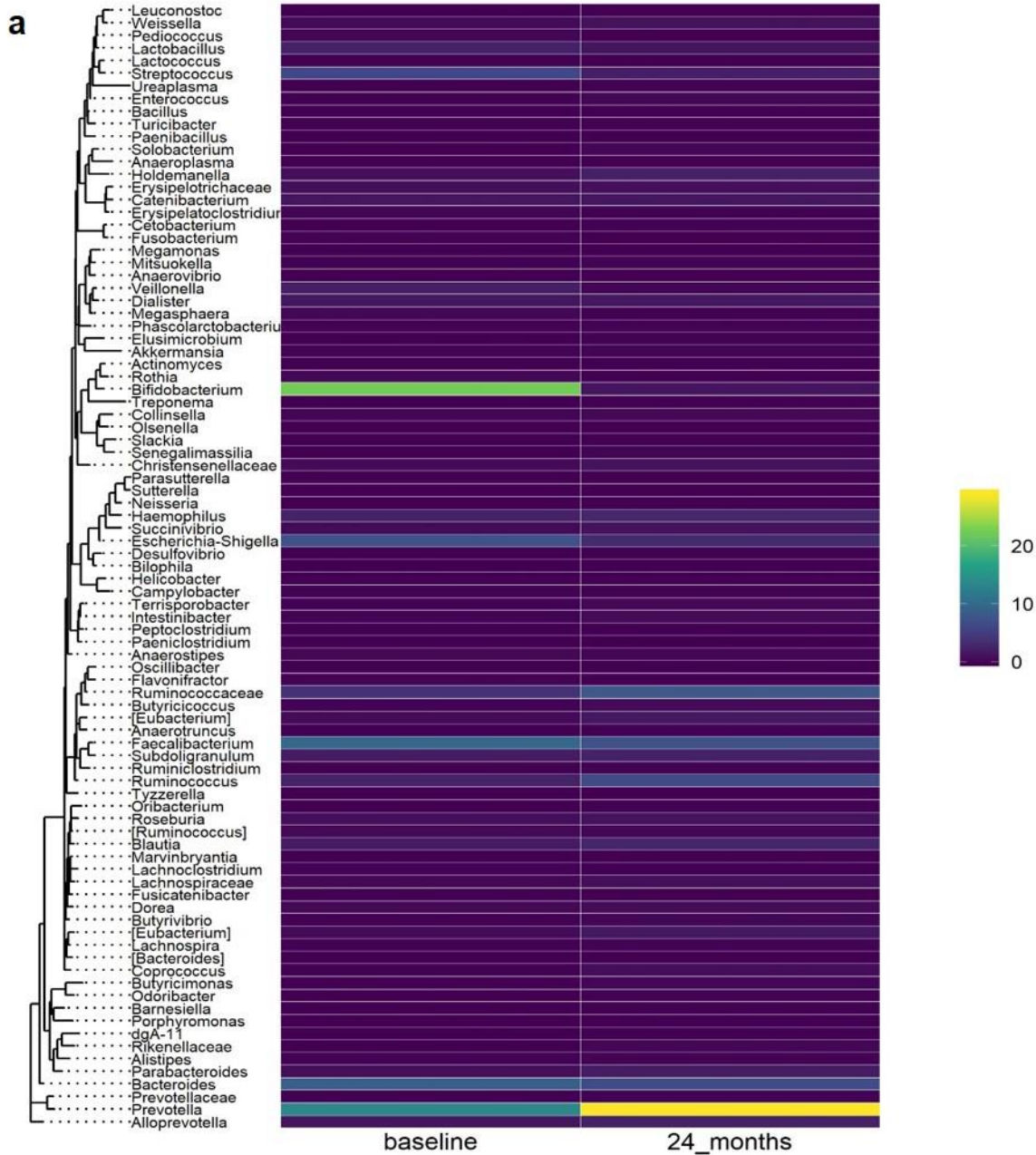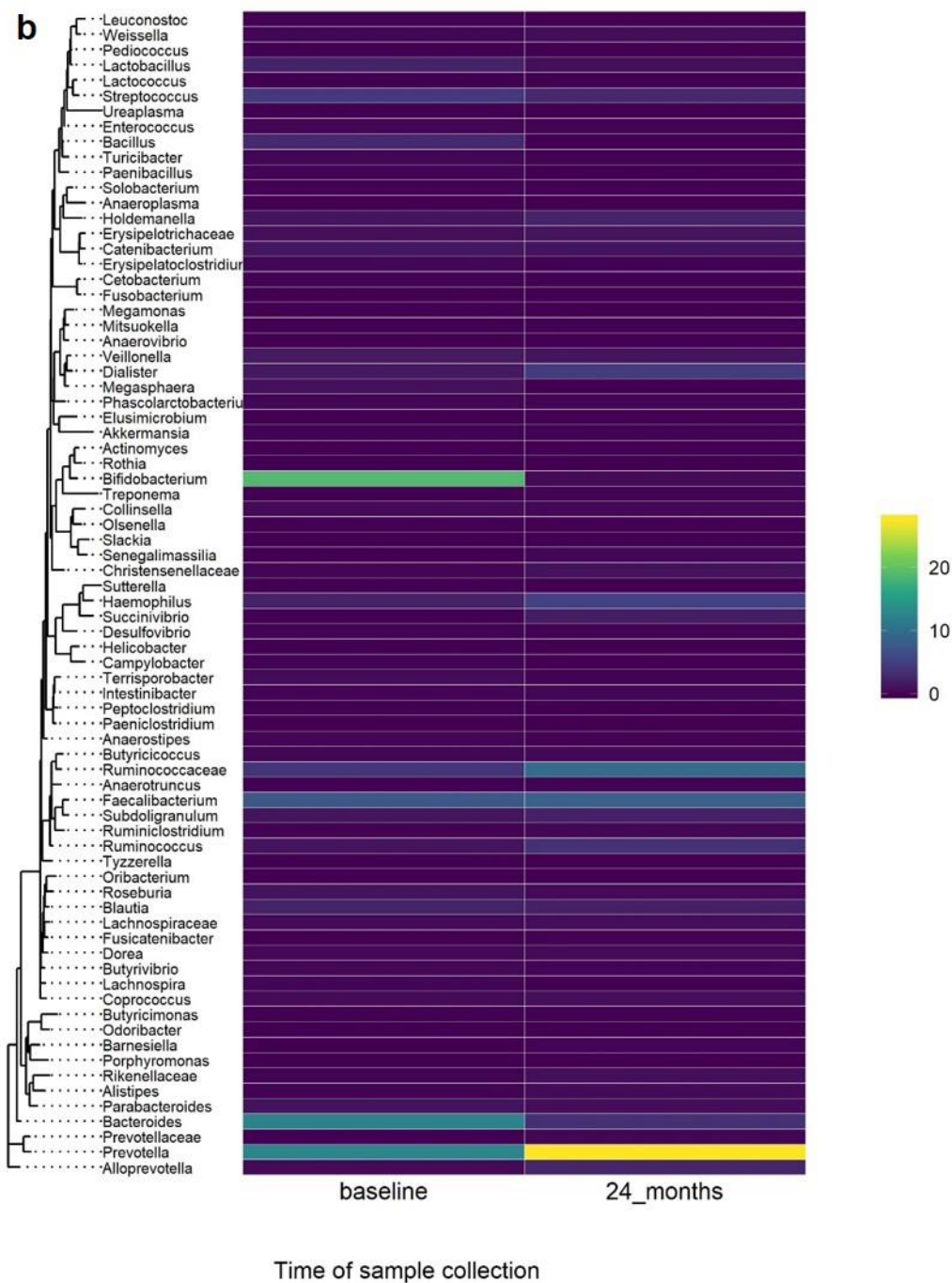
Figure 4. Heatmap showing relative abundance of genera in baseline and 24-month fecal samples by treatment arm. (a) Azithromycin arm, (b) Placebo arm.

Table 5 Longitudinal comparison of relative abundance of individual genera in the azithromycin and placebo arms within the BL vs 4MDA dataset

Treatment arm	Genera	Timeline	Coefficient (95% CI)[a]	P value[a]	OR (95% CI)[b]	P value[b]
Azithromycin	*Bifidobacterium*	Baseline				
		24 months	-3.9 (-5.9,-1.9)	<0.01	1.2 (0.5,3.2)	0.71
	Prevotella	Baseline				
		24 months	1.5 (0.6,2.4)	0.002	1.3 (1.0,1.8)	0.05
	Streptococcus	Baseline				
		24 months	-40 (-70,-9)	0.012	1.7 (0.9,3.2)	0.1
Placebo	*Bifidobacterium*	Baseline				
		24 months	-3.3 (-4.7,-1.8)	<0.01	1.9 (0.5,7.4)	0.34
	Prevotella	Baseline				
		24 months	1.3 (0.6,1.9)	0.0002	1.2 (0.6,1.9)	0.4

CI= confidence interval, OR=odds ratio. [a]Coefficient (95% CI) and P-values obtained with unadjusted linear regression model. [b]OR (95%CI) and P-values obtained with generalized linear mixed models adjusted for age, sex and time since last treatment with the participant as a random effect.

Discussion

We examined associations between 2 or 4 biannual azithromycin treatment rounds and changes in intestinal microbiota diversity and composition in children resident in treated

communities. Azithromycin administration was not associated with changes in alpha diversity but was weakly associated with changes in gut microbiota composition after 4 biannual treatment rounds.

The lack of an effect of azithromycin treatment on microbiota diversity is consistent with data by Wei *et al.*[13], although the follow-up period in the present study was shorter. In the study by Wei *et al.*[13], Danish children aged 12-36 months were prescribed a 3-day course of azithromycin or placebo and the gut microbiota was characterized in fecal samples collected from each child at 4 years of follow-up. The study reported no significant differences in alpha diversity (measured by observed richness and Shannon index) between the treatment and placebo groups.

Conflicting results on alpha diversity have been recently reported by the MORDOR trial in Niger[24]. A reduction in inverse Simpson and Shannon diversity indices in children who received azithromycin compared to children who received placebo was detected 6 months after 2 biannual treatment rounds. The discrepancy in the findings between that study and our own could be attributed to differences in sequencing methods. In the present study, we performed V4-16S rRNA sequencing while the study in Niger performed whole genome sequencing, which has higher resolution to detect differences.

Four biannual azithromycin treatment rounds in the present study were weakly associated with increased abundance of *Prevotella,* detectable 6 months after the last treatment round. While this finding has not previously been reported, it is consistent with a recent study that reported an increased abundance of *Bacteroidetes,* the phylum to which *Prevotella* belongs*,* in children who received macrolides within the 6 months preceding sample collection[14]. *Prevotella* is a gram-negative commensal bacterium found at mucosal sites of the respiratory tract, gut, and oral cavity. Reduced abundance of this bacterium has been associated with Crohn's disease in pediatric patients[25]. Therefore, a trend towards increased abundance of *Prevotella* 6 months after 4 biannual azithromycin treatment rounds may suggest potential long-term beneficial effects of mass azithromycin treatment to the gut, although further studies from additional study sites would be needed to validate this finding.

The absence of large-scale changes in the gut microbiota 6 months after 2 or 4 biannual azithromycin treatment rounds suggests that any effects of azithromycin mass treatment on the gut microbiota may last days or weeks after treatment but not months. Our observation that there were modest changes in gut microbiota composition 6 months after 4 biannual rounds of azithromycin treatment but not 6 months after 2 biannual rounds suggests that the long-term effects of azithromycin MDA on the gut microbiota may be dependent on the number of treatments.

Our study has several limitations. The study was reliant on samples collected in larger cross-sectional surveys and participation in those surveys was low. At the baseline survey, 1090 children were enrolled and provided with stool sample collection kits. However, only 709 (65%) returned stool samples. Similar participation rates were seen at the 12- and 24-month surveys. This may have resulted in selection bias, particularly if families of children who participated had different health-seeking behaviors than those families of children who chose not to participate. Furthermore, the samples included in the present nested cohort analyses were selected based on the availability of longitudinal pairs (baseline and 12 months or baseline and 24-month), which may also be prone to selection bias. Another limitation was that not all of the participants who were sampled received antibiotic as scheduled at each treatment round. While it is unusual to achieve 100% treatment coverage in MDA programs, having participants miss treatment might have contributed to the magnitude of the effect of azithromycin on the gut microbiota reported by this study.

Author contributions

Conceptualization, SB, KK, RB and MH; Methodology, DC, HP, JH and JDH; Formal Analysis, DC and HP; Resources, SB, KK, RB and MH; Data Curation, DC, JDH and KK; Writing – Original Draft Preparation, DC; Writing – Review & Editing, DC, HP, SB, KM, KK, RB and MH; Supervision, JH, SB, KK, RB and MH; Project Administration; KK and RB; Funding Acquisition, SB, KK, RB and MH

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Conflicts of interest

None to declare

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