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Gut Microbiome among Children with Uncomplicated Severe Acute Malnutrition in a Randomized Controlled Trial of Azithromycin versus Amoxicillin

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Abstract. Antibiotics are routinely used as part of the management of severe acute malnutrition and are known to reduce gut microbial diversity in non-malnourished children. We evaluated gut microbiomes in children participating in a randomized controlled trial (RCT) of azithromycin versus amoxicillin for severe acute malnutrition. Three hundred one children aged 6 to 59 months with uncomplicated severe acute malnutrition (mid-upper arm circumference < 11.5 cm and/or weight-for-height Z-score < -3 without clinical complications) were enrolled in a 1:1 RCT of single-dose azithromycin versus a 7-day course of amoxicillin (standard of care). Of these, 109 children were randomly selected for microbiome evaluation at baseline and 8 weeks. Rectal swabs were processed with metagenomic DNA sequencing. We compared alpha diversity (inverse Simpson's index) at 8 weeks and evaluated relative abundance of microbial taxa using DESeq2. Of 109 children enrolled in the microbiome study, 95 were followed at 8 weeks. We found no evidence of a difference in alpha diversity between the azithromycin and amoxicillin groups at 8 weeks controlling for baseline diversity (mean difference -0.6, 95% CI -1.8 to 0.6, $P = 0.30$). Gut microbiomes did not diversify during the study. Differentially abundant genera at the $P < 0.01$ level included *Salmonella* spp. and *Shigella* spp., both of which were overabundant in the azithromycin compared with amoxicillin groups. We found no evidence to support an overall difference in gut microbiome diversity between azithromycin and amoxicillin among children with uncomplicated severe acute malnutrition, but potentially pathogenic bacteria that can cause invasive diarrhea were more common in the azithromycin group.
Trial Registration: ClinicalTrials.gov NCT03568643.

INTRODUCTION

Gut dysbiosis has been hypothesized to have a role in the etiology of severe acute malnutrition in children.[1] Because malnourished children may have asymptomatic infection due to suppressed immune systems, a broad-spectrum antibiotic, commonly amoxicillin, is routinely used as part of the management of uncomplicated severe acute malnutrition.[2] Antibiotics have been shown to disrupt the gut microbiome in children without acute malnutrition.[3,4] Quantifying changes in the gut microbiome after antibiotics in children with severe acute malnutrition may help elucidate the potential role of antibiotics in these children.

The evidence base for the use of amoxicillin in severe acute malnutrition management is mixed, with one trial in Niger showing no difference in nutritional recovery in children receiving amoxicillin compared with placebo and a trial in Malawi showing a benefit of amoxicillin for promoting recovery.[5,6] Single dose azithromycin has recently been shown to reduce all-cause mortality in some high mortality settings in sub-Saharan Africa, with the strongest effects in groups at higher risk of mortality, including undernourished children, suggesting azithromycin may be an acceptable alternative to amoxicillin for acutely malnourished children.[7,8] In a pilot trial evaluating azithromycin versus amoxicillin for severe acute malnutrition, we found no evidence of a difference in child growth, but there was a reduction in clinical signs of infection, including diarrhea and fever, among children who received azithromycin compared with amoxicillin.[9] Here, we evaluate the gut microbiome in children with severe acute malnutrition receiving azithromycin compared with amoxicillin in a subset of trial participants.

METHODS

Study setting. This study was conducted at six primary healthcare facilities in Boromo District, Burkina Faso. Participants were enrolled from June through October 2020, and the last follow-up visit occurred in December 2020. Burkina Faso is located in the Sahel of West Africa and experiences seasonal rainfall from approximately July through October, which corresponds to the high malaria transmission season and the malnutrition season. An annual harvest occurs at the end of the rainy season in November to December. The study was reviewed and approved by the Institutional Review Boards at the University of California, San Francisco and the Center de Recherche en Santé de Nouna. Written informed consent was obtained from the guardian of each child enrolled.

Participants. Participants were aged 6 to 59 months and met national guidelines for uncomplicated severe acute malnutrition (a mid-upper arm circumference of < 11.5 cm and/or weight-for-age Z-score, WHZ, < -3 SD, with no symptoms of infection or other clinical complications that would require an antibiotic or inpatient treatment). All participants had to have a sufficient appetite per a feeding test. Participants were excluded if they had received an antibiotic or treatment of severe acute malnutrition in the previous 7 days and if they had a congenital abnormality or chronic illness that would impair growth. Participants were recruited through routine malnutrition screening days at the enrollment facility and through community-based malnutrition screening.

Trial methods. Complete trial methods have been previously reported.[9,10] In brief, participants were randomized in

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a 1:1 fashion to a single, oral 20 mg/kg dose of azithromycin (Azithrin oral suspension 200 mg/5 mL, Strides Shasun Ltd., Bangalore, India) or a 7-day course of amoxicillin (80 mg/kg divided into two daily doses, amoxicillin syrup 250 mg/5 mL, Reyoung Pharmaceuticals, Shandong, China). Twice-daily amoxicillin for 7 days is standard of care per Burkinabè national guidelines. In addition to antibiotics, children received a standard package of interventions for uncomplicated severe acute malnutrition per national guidelines, which included ready-to-use therapeutic food, antimalarials if positive for malaria by rapid diagnostic test (RDT), antiparasitics, vitamin A supplementation, and any missing vaccinations per the child's government-issued health card.

At baseline, all children were weighed and measured, a baseline questionnaire related to socioeconomic status and feeding practices was administered, and all children were tested for malaria using an RDT (SD Bioline Malaria Ag P.f/Pan, Abbott Laboratories, Chicago, IL). All children were followed weekly for 4 weeks and then at 8 weeks.

Rectal swabs were collected in a random sample of 100 children (50 per arm) at baseline before study treatment or initiation of feeding program interventions and in the same children at 8 weeks after enrollment in the study. Rectal swabs were placed on RNA/DNA Shield (Zymo Research, Irvine, CA) to inactive pathogens and preserve nucleic acids. Samples were placed on ice in the field and then stored in a −80°C freezer in Nouna, Burkina Faso, until shipment to the United States for processing.

Metagenomic DNA sequencing. All laboratory personnel were masked to the child's treatment assignment and samples were processed in a random order. Nucleic acids from deidentified samples were extracted using the ZymoBIOMICS DNA/RNA Miniprep Kit per manufacturer's instructions. Sequencing libraries were prepared using the New England Biolabs' (NEB) NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA) and sequenced on the NovaSeq system (Illumina, San Diego, CA) using 150-nucleotide paired-end sequencing. Human reads were removed as previously described.[11] Nonhost reads were then quality filtered and another round of human reads was removed using very-sensitive-local mode of Bowtie2 v.2.2.4 as previously described.[12] Remaining nonhost reads were aligned to the National Center for Biotechnology Information nonredundant collection.

Statistical methods. We calculated alpha diversity using inverse Simpson's index and Shannon's index, expressed in effective number.[13] The sample size for the trial was based on the trial's primary efficacy and feasibility endpoints, as previously described.[10] For microbiome endpoints, assuming a mean inverse Simpson's diversity of 5 in the amoxicillin arm and standard deviation of 3, we estimated a minimum detectable effect of 1.7 with 50 samples per arm. We compared alpha diversity between treatment groups using a linear regression model with a term for baseline diversity and the randomized treatment assignment. Within-child changes in alpha diversity between baseline and 8 weeks were analyzed separately for the amoxicillin and azithromycin arms using a paired *t* test. DESeq2 (v.1.22.1) was used to compare the relative abundance of individual microbial taxa between the amoxicillin and azithromycin groups at the genus and species level at baseline and 8 weeks using a Benjamini–Hochberg correction of 5% for multiple comparisons. $P < 0.01$ after Benjamini–Hochberg correction was considered notable.

RESULTS

Of 301 children enrolled in the trial, 109 were randomly selected for a rectal swab (azithromycin $N = 55$; amoxicillin $N = 54$; Figure 1). Of these, 95 had a repeat rectal swab collected at 8 weeks (azithromycin $N = 49$; amoxicillin $N = 46$). Baseline characteristics were well balanced between the amoxicillin and azithromycin groups (Supplemental Table 1). Children randomized to azithromycin were a median of 15 months of age at enrollment (versus 14 months in the amoxicillin group) and 58% and 52% of enrolled children in the azithromycin and amoxicillin groups were female, respectively.

Mean alpha diversity expressed as inverse Simpson's index at baseline was 4.0 in the azithromycin arm and 5.1 in the amoxicillin arm. At 8 weeks, Simpson's diversity was 4.5 in the azithromycin arm and 5.3 in the amoxicillin arm. There was no significant difference in alpha diversity between baseline and 8 weeks in the azithromycin ($P = 0.24$) or amoxicillin ($P = 0.91$) arms. In a linear regression model with terms for randomized treatment assignment and baseline Simpson's diversity, there was no significant difference at 8 weeks in diversity between the azithromycin and amoxicillin groups (mean difference −0.6, 95% CI −1.8 to 0.6, $P = 0.30$; Figure 2).

The relative abundance for the most common bacterial genera were similar between groups (Figure 3A). Differentially abundant genera at 8 weeks at the $P < 0.01$ level included *Salmonella* spp. and *Shigella* spp., both of which were overabundant in the azithromycin compared amoxicillin groups (Supplemental Figure 1B), both of which were similar in abundance at baseline (Supplemental Figure 1A). At the species level, these differences appeared to be driven by *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, and *Salmonella enterica* (Figure 3B, Supplemental Figure 2). *Bacteroides cellulosilyticus* was overabundant in the azithromycin compared with amoxicillin group.

DISCUSSION

Overall, we found no evidence of a difference in the diversity of the gut microbiome in children with uncomplicated severe acute malnutrition 8 weeks after azithromycin or amoxicillin. In children without malnutrition in Burkina Faso, azithromycin and amoxicillin have previously been shown to lead to reduced gut bacterial diversity relative to placebo within 5 days of treatment, with a greater reduction in diversity observed with azithromycin compared with amoxicillin.[3] In Finland, an observational study showed a decrease in gut bacterial diversity with macrolides but not penicillins.[14] The present study suggests no evidence of a difference in gut microbial diversity between azithromycin and amoxicillin over 8 weeks, but in the absence of a comparison group that did not receive antibiotics, we are unable to comment on whether antibiotics alter the gut microbiome in children with severe acute malnutrition relative to no antibiotic use.

We found no evidence that gut microbiome diversity increased after treatment of severe acute malnutrition. Previous studies in twin pairs discordant for kwashiorkor have found transient increases in gut bacterial diversity during

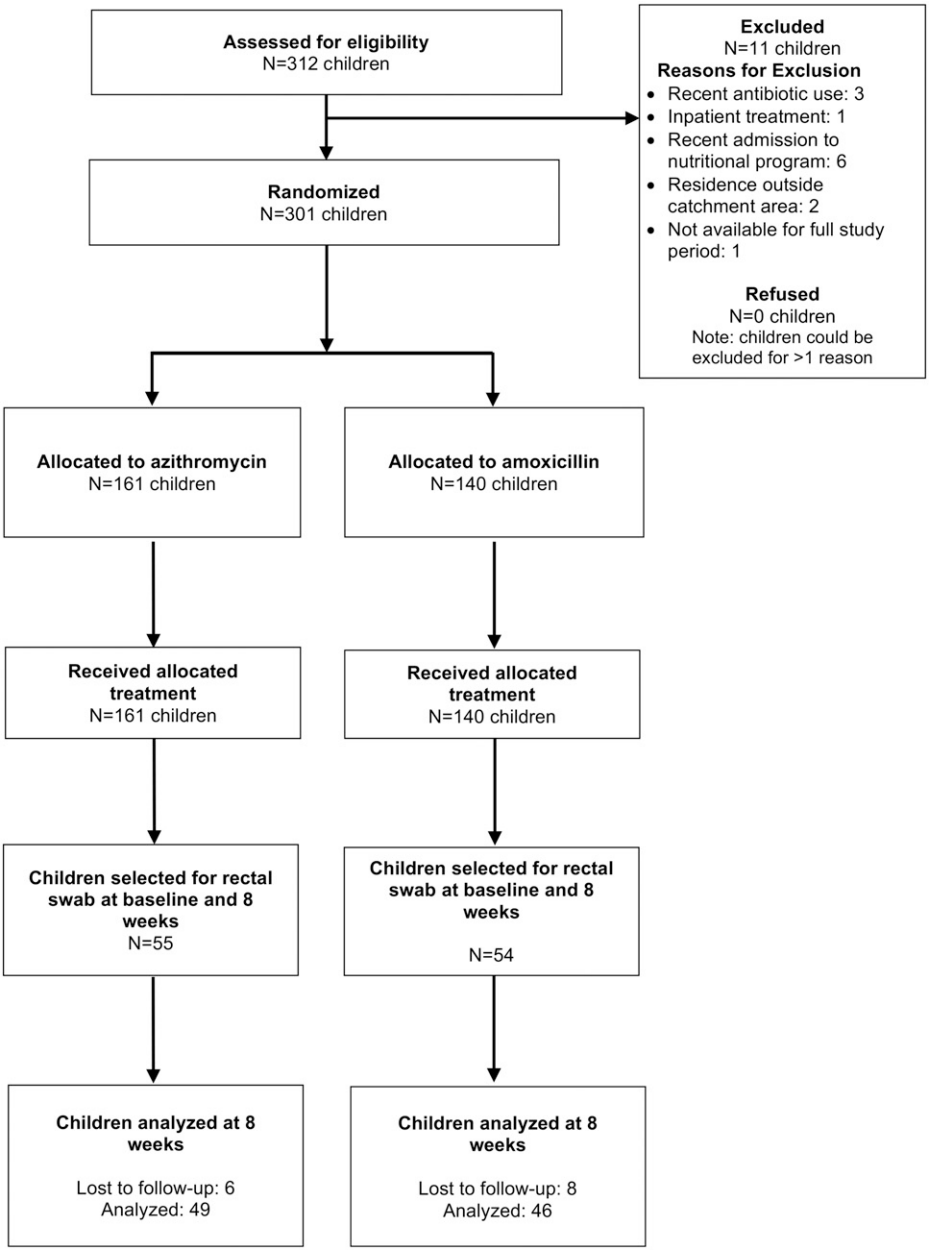

FIGURE 1. Flow chart showing enrollment of participants (CONSORT diagram).

receipt of ready-to-use therapeutic food (RUTF), but that these differences reemerge after RUTF is stopped.[1,15] Similar findings were observed in the gut virome of the twin pairs.[16] Although the current study did not include a non-malnourished comparator group and thus we cannot comment on differences in gut microbiomes between malnourished and non-malnourished children, the study adds to the evidence that an 8-week treatment program may not be sufficient to repair any dysbiosis associated with severe acute malnutrition.

Decreases in gut microbial diversity could be due to changes in either beneficial or potentially pathogenic bacteria. In the present study, *Salmonella* spp. and *Shigella* spp., both important causes of childhood diarrhea,[17] were over-abundant in the azithromycin group compared with amoxicillin at 8 weeks. Azithromycin is considered first-line therapy for *Shigella* spp. in children, and treatment with amoxicillin is not currently recommended due to resistance[18]; however most children with bloody diarrhea in the study area are treated with ciprofloxacin or metronidazole presumptively because laboratory diagnostic testing is not readily available.[19] Biannual mass azithromycin distribution has been shown to reduce *Shigella* spp. compared with placebo in population-based samples of children.[20] In the current study, resistance patterns in *Salmonella* spp. and *Shigella* spp. were not available, although there was no overall difference in the gut resistome or class-specific differences for resistance to macrolides or beta-lactams between arms.[21] Azithromycin was a single oral dose compared with a 7-day twice-daily course of amoxicillin. It is possible that a higher concentration or longer duration of treatment with amoxicillin led to reduced abundance of *Salmonella* spp. and *Shigella* spp. compared

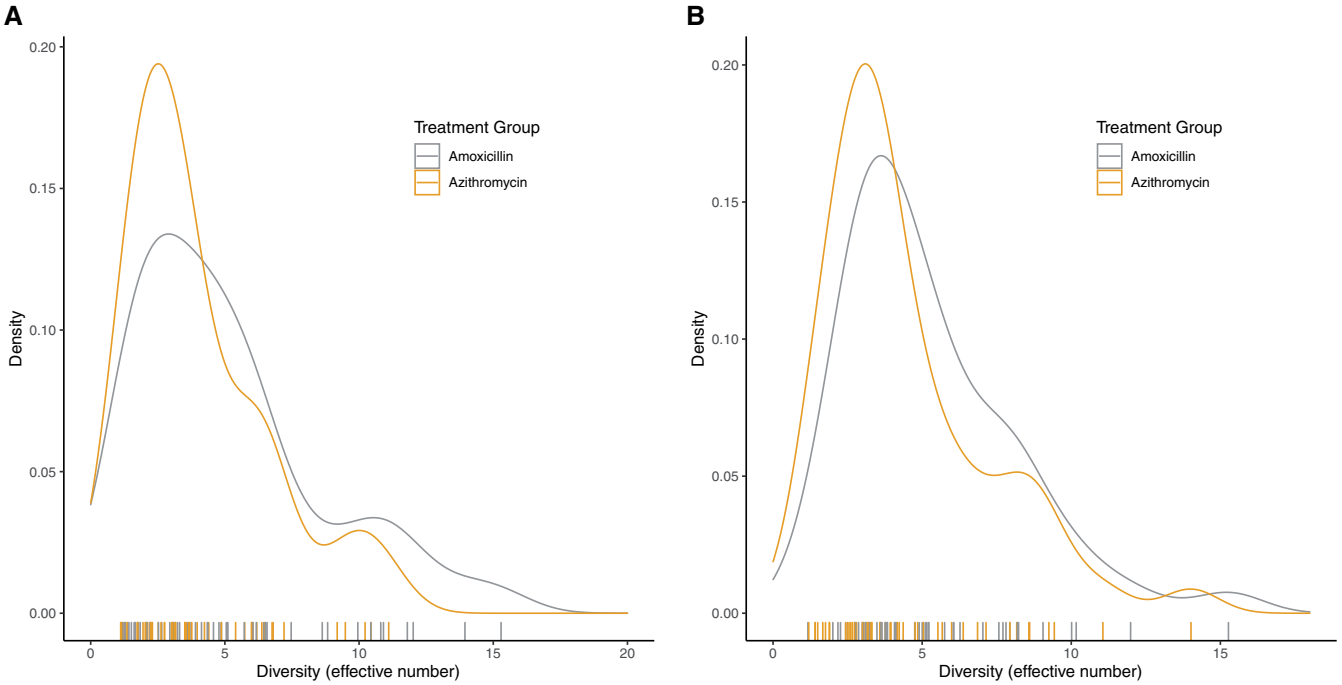

FIGURE 2. Gut diversity (alpha diversity using inverse Simpson's index) among children randomized to amoxicillin vs. azithromycin at baseline (**A**) and 8 weeks (**B**). Ticks on x-axis are actual values.

with azithromycin. The present study used unbiased deep sequencing to evaluate changes in the gut microbiome without prior assumptions about specific organisms. The gut microbiome contains numerous organisms, and any differences in individual taxa may be due to multiple comparisons, especially given the relatively modest sample size of the present study. In addition, taxa assignment is inherently dependent on the pipeline and reference data used and thus closely related species may be misidentified. Future studies could consider directed pathogen polymerase chain reaction and culture conditions specific for *Salmonella* spp. and *Shigella* spp. with specific hypotheses for those organisms. In the parent study, there was a decrease in clinical symptoms of diarrhea in the azithromycin arm compared with amoxicillin at 2 weeks after treatment, although the number of clinical diarrhea events was relatively rare.[9] Diarrhea and enteric infection are thought to be important contributors to malnutrition,[22] and although there was no difference between antibiotics in

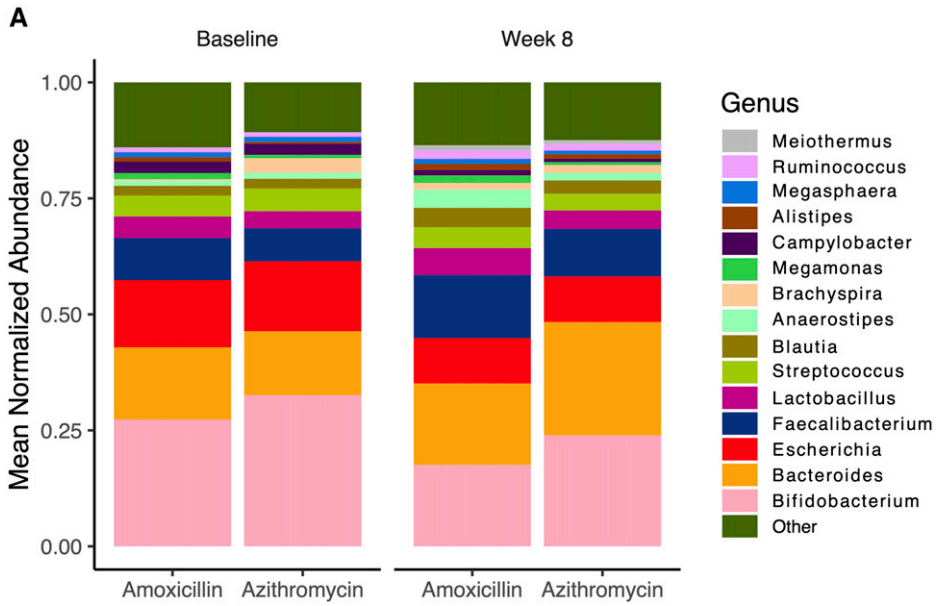

FIGURE 3. Mean relative abundance of the 15 most abundant genera at baseline and week 8 by treatment group (**A**) and heat map showing the distribution of the most differential species in the gut identified by DESeq2 (*t* false discovery rate < 0.01), arranged by hierarchical clustering, with overabundance indicated in red and underabundance in blue for each sample (**B**).

(Figure 3B continued on next page)

FIGURE 3. Continued.

growth or recovery outcomes in the parent trial,[9] understanding differences in antibiotic regimens on causes of invasive diarrhea is an important priority for optimizing treatment regimens.

Bacteroides cellulosilyticus was also overabundant in the azithromycin compared with azithromycin arm. *Bacteroides* spp. have been shown to be decreased in children with severe acute malnutrition compared with well-nourished children.[23] Other bacterial genera have been implicated to have a role in undernutrition, including *Lactobacillus* spp. and *Bifidobacterium* spp.[24] Although there were no difference in growth outcomes by antibiotic treatment in the parent trial, previous studies have shown short-term differences in growth among children receiving antibiotics compared with placebo in children with severe acute malnutrition.[5,6] Although in general antibiotics typically reduce gut microbial diversity,[12] if they are reducing pathogenic bacteria and allowing for proliferation of beneficial bacterial, this could be an additional pathway through which antibiotics benefit children with severe acute malnutrition.

The results of this study must be considered in the context of several limitations. First, all children in this study had severe acute malnutrition at baseline and received an antibiotic (azithromycin or amoxicillin). We are therefore unable to

comment on whether the gut microbiomes differed compared with healthy controls or on the gut microbiome of children with severe acute malnutrition who did not receive antibiotic treatment. Second, approximately 13% of children enrolled in the microbiome study were lost to follow-up at 8 weeks and thus did not have a rectal swab collected. If these children were different with respect to their microbiomes, this could have resulted in selection bias. Third, unbiased deep sequencing methods identify many organisms in the gut, and despite multiple comparisons correction, it is possible that some findings may be chance differences. Because of the randomized nature of the study interventions, any imbalances in organisms at baseline prior to treatment are by definition due to chance. Fourth, this study was done in a high malnutrition region of Burkina Faso. Microbiomes are affected by diet and other environmental conditions,[25] and the results of this study may not be generalizable to children in settings with very different food sources or pathogens.

In this randomized controlled trial of antibiotics for severe acute malnutrition, we found no evidence of an overall difference in diversity of the gut microbiome in children randomized to azithromycin compared with amoxicillin. Microbiome diversity did not increase over time, suggesting that any gut dysbiosis present at baseline may have persisted despite treatment of severe acute malnutrition. If the microbiome has a causal role in progression of and recovery from severe acute malnutrition, interventions that restore the gut microbiome may be helpful for these children.

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