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Authors

Doan, Thuy
Arzika, Ahmed M
Ray, Kathryn J
et al.

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Gut Microbial Diversity in Antibiotic-Naive Children After Systemic Antibiotic Exposure: A Randomized Controlled Trial

Thuy Doan,[1,2] Ahmed M. Arzika,[5] Kathryn J. Ray,[1] Sun Y. Cotter,[1] Jessica Kim,[1] Ramatou Maliki,[5] Lina Zhong,[1] Zhaoxia Zhou,[1] Travis C. Porco,[1,2,3] Benjamin Vanderschelden,[1] Jeremy D. Keenan,[1,2] and Thomas M. Lietman[1,2,3,4]

[1]Francis I. Proctor Foundation, [2]Department of Ophthalmology, [3]Department of Epidemiology and Biostatistics, and [4]Medical Sciences, University of California–San Francisco; and [5]The Carter Center Niger, Republique du Niger

Background. Antibiotic exposure can alter the gut microbiome. We evaluate the effects of azithromycin on the gut microbiome diversity of children from an antibiotic-naive community in Niger.

Methods. A population-based sample of 80 children aged 1–60 months in the Dosso region of Niger was randomized to receive a single dose of either oral azithromycin or placebo. Fecal samples were collected immediately before treatment and 5 days after treatment for 16S rRNA gene sequencing. The prespecified outcome was α-diversity (inverse Simpson's α-diversity index), with secondary outcomes of β and γ Simpson's and Shannon's diversities.

Results. At 5 days after treatment, 40 children aged 1–60 months were analyzed in the azithromycin-treated group and 40 children in the placebo-treated group. Diversity of the gut microbiome was significantly lower in the treated group (inverse Simpson's α-diversity, 5.03; 95% confidence interval [CI], 4.08–6.14) than in the placebo group (6.91; 95% CI, 5.82–8.21; $P = .03$). Similarly, the Shannon's α-diversity was lower in the treated group (10.60; 95% CI, 8.82–12.36) than the placebo group (15.42; 95% CI, 13.24–17.80; $P = .004$). Simpson's community-level (γ) diversity decreased with azithromycin exposure from 17.72 (95% CI, 13.80–20.21) to 10.10 (95% CI, 7.80–11.40; $P = .00008$), although β-diversity was not significantly reduced (2.56, 95% CI, 1.88–3.12; to 2.01, 95% CI, 1.46–2.51; $P = .26$).

Conclusions. Oral administration of azithromycin definitively decreases the diversity of the gut microbiome of children in an antibiotic-naive community.

Clinical Trials Registration. NCT02048007.

Keywords. randomized controlled trial; gut microbiome; children; azithromycin; antibiotics.

Although the use of antibiotics has saved millions of lives, the practice also selects for antibiotic-resistant microbes, potentially limiting our ability to control previously treatable and emerging infections [1]. Further, antibiotic exposure could alter the host's commensal microbiota with potential health consequences [2–6]. Decreased diversity of the gut microbiome from antibiotic intake has been associated with increased susceptibility to *Clostrium difficile*–related diarrhea [6, 7]. Similarly, dysbiosis of the gut microbiome has been linked to the increased risk of asthma, diabetes, and obesity [8–10].

Multiple observational studies in adults have suggested that antibiotic exposure reduces the phylogenetic bacterial diversity of the human gut microbiome (Supplementary Table 1) [4, 10, 11]. This association between lower gut microbiome diversity and antibiotic exposure has also been observed in pediatric populations, although recent longitudinal studies have shown conflicting results [2, 3, 12]. Observational studies, regardless of sample size, are subjected to confounding biases. To evaluate the effects of antibiotic exposure on the pediatric gut microbiome diversity, we performed a double-masked, randomized controlled clinical trial in a relatively antibiotic-naive community in Niger.

METHODS

Study Setting and Design

We obtained ethical approval for the study from the University of California–San Francisco Committee for Human Research and the Ethical Committee of the Niger Ministry of Health. We obtained oral consent from guardians of children. No incentives were offered for participation in this study. The study was undertaken in accordance with the Declaration of Helsinki. This study is a double-masked, randomized controlled clinical trial with a prespecified primary outcome conducted in Dosso Region, Niger (clinical trial registration

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Correspondence: T. M. Leitman, 513 Parnassus Ave, Room S309, Medical Sciences, University of California–San Francisco, San Francisco, CA 94143-0412 (Tom.Lietman@ucsf.edu).

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NCT02048007). The study community was rural and agricultural, in an area with relatively high coverage of vaccination but considerable malnutrition. Further, its residents had not received any mass antibiotic distribution within the previous 6 years; hence the children enrolled in the study were antibiotic naive. In this trial, we randomly assigned 87 healthy children aged 1–60 months from a single village to either placebo or a single dose of oral azithromycin (Pfizer, New York, NY) (height-based dosing to roughly 20 mg/kg). The randomization was generated by T. C. P., implemented by S. Y. C., and concealed until assignment. Healthcare personnel directly observed both antibiotic and placebo being taken by study subjects (Figure 1). The primary outcome was the gut microbial inverse Simpson's α-diversity at the genus level determined with 16S rRNA gene sequencing. Eighty children were selected for the 16S rRNA gene sequencing because we estimated that 40 children per arm would provide >80% power to detect a 2/3 standard error effect size in the primary outcome of inverse Simpson's α-diversity, assuming a 2-sided alpha of 0.05. Assuming from preliminary results that the standard deviation of inverse Simpson's in the population would be approximately 3.0, this would suggest a detectable effect size of 2.0 (the units of inverse Simpson's are effective numbers).

Sample Processing and Sequencing

Rectal samples were collected in the field at baseline (day 0) and at 5 days after treatment (day 5). Examiners changed into clean gloves and placed the swab 1–3 cm into the anus, rotating 360 degrees and repeating until stool was visible on the swab. Swabs were immediately placed into a Stool Nucleic Acid Collection and Transport Tube containing Norgen Stool Preservative (Norgen, Ontario, Canada). Samples were immediately placed on ice in the field and transported to the study center for storage at −20°C while in Niger and shipped to the University of California–San Francisco for long-term storage at −70°C. Samples were de-identified and placed in a random order for subsequent library preparation and sequencing. Researchers performing the assays were masked. DNA was extracted from the fecal samples using the Norgen stool DNA isolation kit (Norgen, Ontario, Canada) as per manufacturer's instructions. Concentration of DNA was quantified using the Qubit dsDNA HS Assay Kit (ThermoFisher Scientific, Waltham, MA) and adjusted to 15 ng/uL. The gut bacterial community was assessed by deep sequencing the V3–V4 hypervariable regions of the 16S rDNA gene. Library preparation was performed by SeqMatic (Fremont, CA) per Illumina16S metagenomic sequencing library preparation protocol [13–15]. All samples collected on day 5 were run in duplicates.

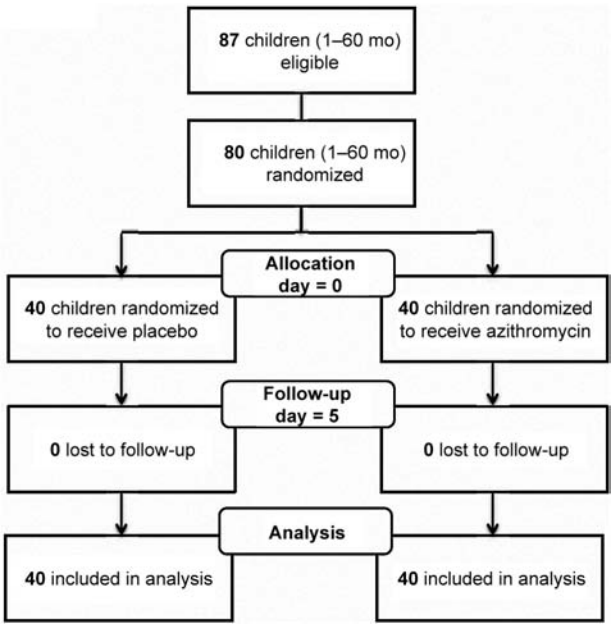

Figure 1. Trial profile. Eighty of the eighty-seven healthy and eligible children aged 1–60 months from a single village were randomly assigned to either the placebo or azithromycin treated group, with samples from 80 children subjected to 16S rRNA gene analysis. No children were lost to follow-up.

Analysis of 16S rDNA and Statistical Methods

Sequencing reads were analyzed using the BaseSpace 16S Metagenomics App, which is a pipeline that uses the Ribosomal Database Project (RDP) algorithm and the full GreenGenes reference database to assign taxonomy to each sequencing read [16]. After filtering reads as previously described [16, 17], 66 696 024 high-quality sequences of 600 base pairs were recovered from 80 samples (averaging 234 845 sequences per sample).

To assess sample collection, preparation, and sequencing reproducibility of the diversity measure, we used 1 duplicate aliquot of extracted DNA for each of the 80 study participants and 1 duplicate field swab for each of 11 study participants. Interclass correlation coefficients (ICC) between the duplicate aliquots and duplicate field samples were calculated using the R package "ICC." The primary outcome of this study was prespecified as α-diversity (inverse Simpson's) at the genus level within microbiome samples collected from children 5 days after treatment with azithromycin or placebo. Shannon's α-diversity was also compared as a sensitivity analysis. The β and γ diversities (Simpson's and Shannon's) were each assessed using a permutation procedure in R. Equations used to estimate diversity are shown in Supplementary Table 2 [18, 19]. We computed the difference in means of diversities by arm—that is, $\Delta = \left|\overline{\alpha}_{placebo} - \overline{\alpha}_{abx}\right|$. The permutation based P values for testing $H_0 : \Delta = 0$ were computed as the proportion of permutations with Δ larger than the observed Δ value for n = 10 000. Analyses were implemented in the R software environment (http://cran.r-project.org/), version 3.1.3. Diversity measures were calculated using the R package "vegetarian."

Table 1. Baseline Characteristics Between Treatment Groups

Characteristic	Placebo (n = 40)	Antibiotics (n = 40)	Total (n = 80)	*P* value[a]
Sex, no.				
Male	13	18	31	.40
Female	27	22	49	
Age, y, median (IQR)	3 (2–4)	2.65 (1.7–4)	3 (2–4)	.78
Child currently breastfeeding?				
Yes	10	14	24	.47
No	30	26	56	
Child eating solid food?				
Yes	36	37	73	1.00
No	4	3	7	

[a]Fisher's exact test was used for categorical variables. Wilcoxon rank-sum test was used for continuous variables.
Abbreviation: IQR, Interquartile range.

RESULTS

Of 87 eligible children, 80 were included in this study. Forty were randomized to receive a 1-time dose of azithromycin at baseline (day 0), and 40 were randomized to receive placebo (Figure 1). The pretreatment characteristics of the study groups are described in Table 1. In both groups, the median age was 3 years, with a range of 1–60 months. There were no statistically significant differences in age, sex, breastfeeding, or solid food intake between the 2 study groups. Duplicate aliquots taken from the same fecal sample allowed estimation of a within-sample ICC of 0.91 (95% CI, .86–.94), and duplicate field samples obtained from the same child allowed estimation of a within-child, between-sample ICC of 0.92 (95% CI, .75–.98) (Table 2).

The 16S rRNA gene analysis of 80 fecal samples identified 760 genera. As depicted in Figure 2A, variability in the abundance of the gut microbial composition between children at baseline and after azithromycin treatment exists. In this population, the most common genera on day 0 were *Faecalibacterium*, *Blautia*, *Bifidobacterium*, *Succinivibrio*, *Ruminococcus*, *Roseburia*, *Escherichia*, *Clostridium*, *Megasphaera*, and *Anaerovibrio*, accounting for 61% of the filtered reads (Figure 2, left panel). On day 5, a similar fraction of the reads (62%) were composed of *Faecalibacterium*, *Blautia*, *Ruminococcus*, *Veillonella*, *Roseburia*, *Prevotella*, *Clostridium*, *Anaerovibrio*, *Bifidobacterium*, and *Peptoniphilus* (Figure 2, right panel). Both time points were enriched for *Faecalibacterium* (18%–24% of all the sequences) and *Blautia* (7%–10%) and depleted of *Bacteroides*, the predominant genus in the gut microbiome of Westerners [20, 21].

At baseline, all diversity indices were not statistically significant between treatment groups (Table 3). Five days after a single oral dose of azithromycin, we found that the inverse Simpson's α-diversity index was decreased in the antibiotic-treated group (5.03; 95% CI, 4.08–6.14) compared with the placebo group (6.91; 95% CI, 5.82–8.21; $P = .03$). The sensitivity of this prespecified outcome was determined using Shannon's diversity, which also showed there was a reduction in diversity after azithromycin treatment (10.60; 95% CI, 8.82–12.36) compared with placebo (15.42; 95% CI, 13.24–17.80; $P = .0004$). Similarly, the γ-diversity, the total richness in each treatment group, was significantly higher in the placebo-treated group than in the azithromycin-treated group (Table 4).

To determine the degree of community differentiation after azithromycin treatment, we compared the β-diversities between the 2 treatment groups. Here, we defined β-diversity as the ratio between γ- and α-diversity [18, 19]. Five days after baseline, the β-diversity did not decrease significantly with azithromycin intake, measuring 2.01 (95% CI, 1.46–2.51) in the azithromycin group and 2.56 (95% CI, 1.88–3.12; $P = .26$) in the placebo-treated group (Table 4). Similar results were seen with Shannon's β-diversity (Table 4).

We next used linear discriminant analysis effect size (LEfSe), an established biomarker discovery tool, to detect differentially abundant bacterial genera between the treatment groups [22]. Using this algorithm, we identified 51 genera whose abundance differed between placebo-treated and azithromycin-treated arms (with a threshold of $P < .001$) (Figure 3). Overall, 1 dose of azithromycin led to the depletion of more bacterial genera (43/51, 84.3%) than it did to the enrichment of bacterial genera (8/51, 15.7%), consistent with the finding of decreased α- and γ-diversity (Figure 2B and 2C, Table 4). The niches left open by the lack of those genera were occupied by Gram-positive anaerobes, predominantly *Blautia* and *Dorea*, intestinal commensal organisms within the bacterial class *Clostridia*. At 5 days after treatment, azithromycin did not alter the abundance of *Clostridium*.

Table 2. Reproducibility of Samples

Type of Duplicates	α-Diversity	Number of Duplicates	ICC	95% CI
Duplicate aliquots taken from same fecal samples	Simpson (inverse)	80	0.91	(.86–.94)
	Shannon (exponential: $e^{-H\alpha}$)	80	0.91	(.86–.94)
Duplicate field sample taken from same participant	Simpson (inverse)	11	0.92	(.75–.98)
	Shannon (exponential: $e^{-H\alpha}$)	11	0.91	(.72–.98)

Sequencing reliability was evaluated using duplicate aliquots taken from all samples tested (n = 80). Sample collection reliability was analyzed in 11 patients. Simpson's α-diversity index $(H_\alpha = \sum_{i=1}^{M} Pi^2)$; Shannon's α-diversity index $(H_\alpha = \sum_{i=1}^{M} \ln(p_i)p_i)$.
Abbreviations: CI, confidence interval; ICC, intraclass correlation coefficient.

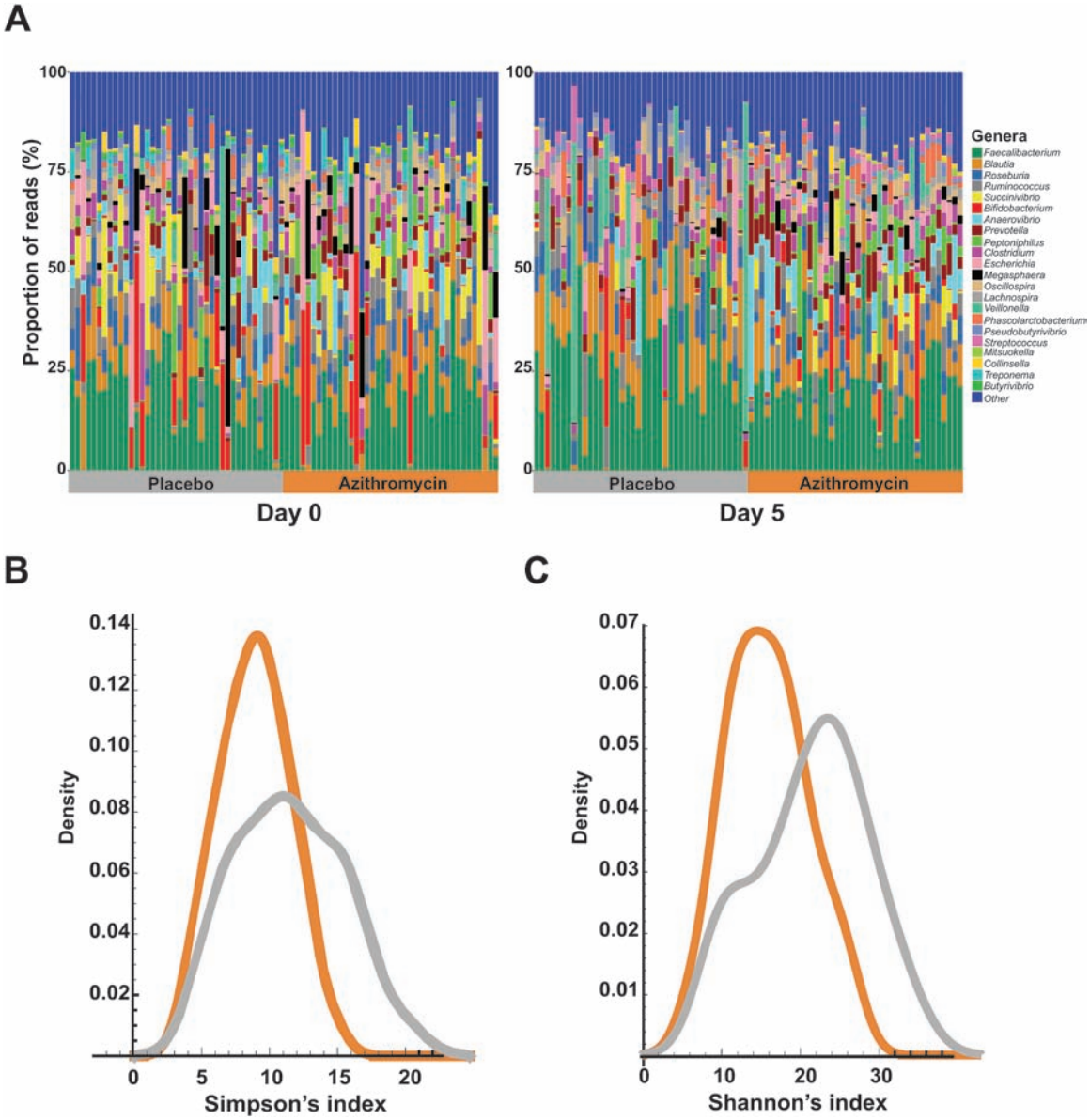

Figure 2. The gut microbial composition (*A*) of children and alpha-diversity indices (*B, C*) between treatment groups. *A*, The 10 most abundant genera were depicted for each sample between treatment groups at baseline and at 5 days after treatment. Distributions of the Simpson's index (*B*) and the Shannon's index (*C*) for the placebo-treated (gray lines) and azithromycin-treated (orange lines) groups.

Table 3. Diversity Indices by Treatment Group at Baseline

	Diversity	Azithromycin Effective Number (95% CI)		Placebo Effective Number (95% CI)		*P* value*
Alpha	Simpson (inverse)	7.07	(5.93–8.15)	7.42	(6.38–8.84)	.69
	Shannon (exponential: $e^{-H\alpha}$)	14.88	(12.89–16.86)	16.47	(14.33–18.83)	.32
Beta	Simpson (inverse)	2.36	(1.74–2.94)	2.07	(1.57–2.57)	.51
	Shannon (exponential: $e^{-H\beta}$)	2.28	(1.86–2.53)	1.97	(1.64–2.18)	.21
Gamma	Simpson (inverse)	16.33	(13.82–18.35)	15.42	(12.51–17.67)	.66
	Shannon (exponential: $e^{-H\gamma}$)	33.96	(29.71–34.93)	32.46	(28.07–33.71)	.54

Alpha, beta, and gamma diversity indices were calculated for each treatment group as described by Jost [18, 19].

Abbreviation: CI, confidence interval.

Table 4. Diversity Indices by Treatment Group at Day 5

	Diversity	Azithromycin Effective Number (95% CI)		Placebo Effective Number (95% CI)		*P* value
Alpha	Simpson (inverse)	5.03	(4.08–6.14)	6.91	(5.82–8.21)	.03
	Shannon (exponential: $e^{-H\alpha}$)	10.60	(8.82–12.36)	15.42	(13.24–17.80)	.0004
Beta	Simpson (inverse)	2.01	(1.46–2.51)	2.56	(1.88–3.12)	.26
	Shannon (exponential: $e^{-H\beta}$)	2.07	(1.68–2.31)	2.32	(1.85–2.57)	.40
Gamma	Simpson (inverse)	10.10	(7.80–11.40)	17.72	(13.80–20.21)	.00008
	Shannon (exponential: $e^{-H\gamma}$)	21.97	(18.09–23.33)	35.76	(30.44–36.67)	<.0001

Alpha, beta, and gamma diversity indices were calculated for each treatment group as described by Jost [18, 19].

Abbreviation: CI, confidence interval.

DISCUSSION

The choice of azithromycin as the antibiotic for this study was based on its use in a larger study of its effect on childhood mortality (Mortality Reduction after Oral Azithromycin [MORDOR], Bill and Melinda Gates Foundation [BMGF] no. 1032340). Azithromycin is a broad-spectrum azalide that reversibly binds to the bacterial ribosome and inhibits protein synthesis [23]. It has a long half-life with excellent tissue penetration and is primarily used for the treatment of ocular, respiratory, enteric, and genitourinary infections. In this double-masked, randomized controlled trial, we showed that healthy children treated with 1 dose of azithromycin had a reduction in the intestinal microbial α-diversity 5 days after treatment, whether measured as Simpson's or Shannon's diversity. Further, azithromycin caused community-level alterations, as evidenced in the reduction of γ-diversity. This finding reflects the overall reduction in the gut bacterial diversity of the treatment group compared with the placebo group. The absence of a change in the β-diversity between the 2 treatment groups indicates that the differences in bacterial composition of the children within the 2 arms are similar. This is the expected result if azithromycin has comparable effect on diversity across children.

Multiple observational studies in adults have suggested that antibiotic exposure reduces phylogenetic bacterial diversity of the human gut microbiome (Supplementary Table 1) [4, 10, 11]. This observation was suggested in a randomized controlled clinical trial by Zaura et al, in which 30 healthy adults in Sweden and 44 healthy adults in the United Kingdom were treated with various antibiotics (clindamycin, ciprofloxacin, amoxicillin, and minocycline) or placebo [24]. Diversity was one of a large number of outcomes in this trial and was not prespecified. These authors found that bacterial richness, an α-diversity index, was significantly reduced up to 2 months after 1 treatment (multiple doses) of antibiotics. In the pediatric population, however, the correlation between the gut microbiome diversity and antibiotics intake was less clear because cross-sectional studies have shown conflicting results (Supplementary Table 1) [2, 3, 12]. In an observational study of 142 Finnish children, Korpela et al [3] found a significant reduction in bacteria richness that persisted over 6 months, whereas Bokulich et al [12] did not see

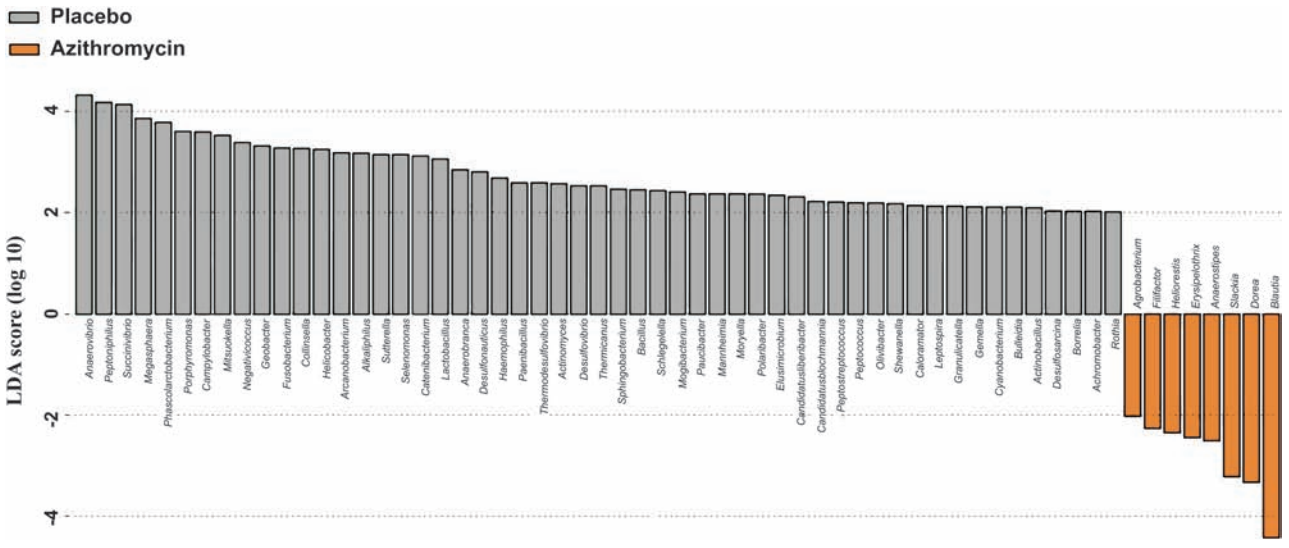

Figure 3. Differentially abundant genera between the placebo- and azithromycin-treated children. Genera are shaded gray when significantly more abundant in the placebo group ($P < .001$) and shaded orange when significantly more abundant in the azithromycin-treated group. Abbreviation: LDA, linear discriminant analysis.

a short-term reduction in phylogenetic diversity or Shannon equitability of the gut microbiome in 43 healthy infants from the United States. Here, we have proven that oral azithromycin causes a reduction in intestinal microbiome diversity in the pediatric population.

We observed that the core gut microbiome in healthy Nigerien children shared some features of the African population and some features of the Western world. Specifically, it is enriched for *Faecalibacterium*, seen in high abundance in children and adults of the Western population, and *Blautia*, *Ruminococcus,* and *Treponema species*, seen in high abundance in African populations [20, 21]. *Bacteroides species* were minimally represented in the gut of these children, although all were vaginally delivered [2, 25]. Previous studies have shown that this signature was associated with C-section delivery [2, 25, 26]. The discrepancy suggests geographic location as a factor that influences children's microbial composition and diversity [27, 28]. We also found that *Bifidobacterium spp.* represented the third most abundant genera, consistent with the signature found in the gut microbiome of breastfed children in the developed world [8].

Observational studies have suggested that disruption of the gut microbiome from antibiotics may be long-lasting, up to 1 year in adults and 2 years in children [3, 29]. The resilience of the gut microbiome, however, may be correlated with its initial state [30]. Because the children enrolled in this study were vaginally delivered, initially breastfed with natural transition to solid food, and were unexposed to antibiotics prior to this study, it could be argued that the gut microbiome of these children was maximized for diversity [2, 5, 12, 26]. In the Niger population, exposure to azithromycin rapidly led to the reduction of multiple genera, including *Lactobacillus*, generally considered to be beneficial. We did not find a transient enrichment of *Clostridum*, a genus that contains >100 species that are both beneficial and pathogenic (ie, *C difficile*) to humans.

This work is limited by its study population of young children in a community of rural Niger, and results may not be generalizable to other pediatric populations. An antibiotic-naive community in the resource-rich countries would be exceptional. Our results provide evidence for the reduction of the gut microbial diversity within an individual following antibiotic intake in a pediatric population. Although observational studies have suggested such disruption in the gut microbiome of children in Finland and the United States, a randomized controlled trial would be necessary to show causation. Another limitation is stool was sampled only at day 5, not over the longer periods necessary to provide insight into the resilience of the gut microbiome. This is particularly relevant for areas in Africa and underdeveloped countries where mass antibiotics are distributed in annual intervals for the prevention of infectious diseases [31].

In conclusion, the pediatric gut microbiome is sensitive to antibiotic exposure. A single dose of antibiotic can result in a significant reduction in bacterial diversity. Continued research regarding the role of antibiotic use in the alterations of the gut microbiome will help to better understand the risks and benefits of antibiotic distribution to promote childhood health.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No potential conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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