Gut Microbiota in Mexican Children With Acute Diarrhea

An Observational Study

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Background: Acute diarrhea is the second leading cause of preventable mortality and morbidity in children worldwide. This study aimed to identify the main pathogens associated with acute diarrhea and to describe changes in gut microbiota in Mexican children.

Methods: This single-center observational study included 30 children (6 months to 5 years old) with acute diarrhea who were referred to the Instituto Nacional de Pediatría of Mexico City and 15 healthy volunteers (control group). Stool samples at day 0 (D0) and day 15 (D15) were collected for identification of microorganisms (reverse transcriptase-polymerase chain reaction analyses with xTAG gastrointestinal pathogen panel multiplex assay) and microbiota analysis (16S gene amplification sequencing). Prescription decisions were made by the treating clinician.

Results: The main pathogens identified were norovirus and *Campylobacter jejuni* (20% each). The majority of patients (n = 24) were prescribed *Saccharomyces boulardii* CNCM I-745 for treatment of acute diarrhea. Diarrheic episodes resolved within 1 week of treatment. Compared with D15 and control samples, D0 samples showed significantly lower alpha diversity and a clear shift in overall composition (beta diversity). Alpha diversity was significantly increased in *S. boulardii*-treated group between D0 and D15 to a level similar to that of control group.

Conclusions: In these children, acute diarrhea was accompanied by significant alterations in gut microbiota. *S. boulardii* CNCM I-745 treatment may facilitate gut microbiota restoration in children with acute diarrhea, mostly through improvements in alpha diversity.

Key Words: acute diarrhea, children, gut microbiota, probiotics, *Saccharomyces boulardii* CNCM I-745

(*Pediatr Infect Dis J* 2021;40:704–709)

Acute diarrhea (AD) is the second leading cause of death in children under 5 years and the main cause of malnutrition. Despite being both preventable and treatable, there are nearly 1.7 billion cases of diarrhea globally in young children and around 525,000 have fatal consequences every year.[1]

In Mexico, the Epidemiological Surveillance System registered 4–6 million of cases of AD per year between 2008 and 2017.[2] While the introduction of a rotavirus vaccine (2006–2009) substantially reduced the incidence of AD and diarrhea-related deaths among children in Latin America,[3] an ecologic study showed that in 2016, there were still 4.8 million cases of AD in Mexico.[4] The study showed that the etiologic agent was unknown in 79.2% of cases; in 10% of the cases, diarrhea was due to intestinal amebiasis and fewer than 2.5% of cases were due to typhoid and paratyphoid fevers (2.1% and 1.5%, respectively), other protozoal intestinal diseases (1.5%), other bacterial foodborne pathogens (0.5%), giardiasis (0.5%), shigellosis (0.3%) or other *Salmonella* infections (0.3%).[4]

Over recent decades, many studies have focused on understanding human microbiome and its association with disease. We now know that this complex community of microorganisms is indispensable for maintaining a healthy state[5]; however, few studies have addressed gut microbiota dysbiosis (an altered microbiota composition) in AD. In an attempt to improve the management of acute gastroenteritis in children, the European Society for Pediatric Gastroenterology, Hepatology and Nutrition 2020 guidelines proposed an active treatment with probiotics, in addition to oral rehydration solution as first-line treatment for children with acute gastroenteritis.[6] *Saccharomyces boulardii*, *Lactobacillus rhamnosus GG* and *Lactobacillus reuteri* DSM 17938 are the most studied and commonly prescribed probiotics for children with AD. Several studies have shown that *S. boulardii* is an effective treatment for AD against viral, bacterial and protozoan causes in both developing and developed countries.[6,7]

The aim of our study was to describe the main pathogens associated with AD in children under 5 years old in Mexico City and to examine the modifications in their gastrointestinal microbiota before and after receiving prescribed treatment.

METHODS

Study Design and Study Population

This single-center observational study included children (male and female) from 6 months to 5 years old with AD who had not received any treatment, were healthy before developing diarrhea and were referred as ambulatory patients to the Instituto Nacional de Pediatría between January and November 2019 and 15 healthy volunteers (both genders) from 6 months to 5 years old (control group [CG]), without any previous medication (antibiotic or probiotic) nor any gastrointestinal symptom. For this study, we used the definition of diarrhea provided by the World Health Organization (3 or more loose or liquid stools per day or more frequent passage than is normal for the individual, in 24 hours and lasting ≤14 days).[1]

Obese children who had a body mass index (BMI) >95th percentile or chronic conditions, such as immunodeficiency, food allergy or cancer and those who had previously received treatment for the diarrheal episode, including antibiotics, antidiarrheal drugs, probiotics or anti-inflammatory drugs, were excluded from the study due to potential intrinsic microbiota alterations.

Accepted for publication February 11, 2021
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This study, including editorial support, was funded by Instituto Nacional de Pediatría of Mexico and Biocodex.
The authors have no conflicts of interest to disclose.
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Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's website (www.pidj.com).

ISSN: 0891-3668/21/4008-0704
DOI: 10.1097/INF.0000000000003128

Sample Collection

All patients with AD provided 2 fecal samples at day 0 (D0) when the diagnosis were established: 1 for pathogen panel analysis and 1 for microbiota analysis. After sample collection, the clinician prescribed appropriate standard of care treatment for AD. Another fecal sample for microbiota analysis was collected at day 15 (D15). Individuals in the CG provided 1 fecal sample for the microbiota analysis.

Pathogen Panel Analysis

The sample for pathogen panel analysis was sent to a virology laboratory, where it was screened for the presence of 15 microorganisms by multiplex polymerase chain reaction (PCR) analysis. Multiple reverse transcriptase-PCR analyses with the xTAG gastrointestinal pathogen panel multiplex assay (Luminex) (Luminex Corp, Austin, TX, USA) were conducted following the manufacturer's protocol (see Text, Supplemental Digital Content 1, http://links.lww.com/INF/E344). The panel included: adenovirus 40/41; rotavirus A; norovirus GI/GII; *Salmonella* spp.; *Campylobacter* spp. (*C. jejuni*, *C. coli* and *C. lari*); *Shigella* spp. (*S. boydii*, *S. sonnei*, *S. flexneri* and *S. dysenteriae*); *Clostridium difficile* toxin A/B; Shiga toxin-producing, enterotoxigenic *Escherichia coli* O157; *Yersinia enterocolitica*; *Vibrio cholerae*; *Giardia lamblia*; *Entamoeba histolytica* and *Cryptosporidium* spp. (*C. parvum* and *C. hominis*).

Microbiota Analysis

DNA Extraction and Preparation of 16S Ribosomal RNA Gene Amplicon Libraries

After collection, fecal samples were immediately transported to the laboratory facilities in ice-filled coolers. Aliquots of 200 mg were stored at –80 °C until processing. Bacterial DNA was extracted from the samples using NucleoSpin 96 Soil (Macherey-Nagel, Bethlehem, PA, USA). The bead-beating lysis step was conducted using a Vortex-Genie 2 horizontal mixer at 2700 revolutions per minute for 5 minutes. At least 1 positive control (ZymoBIOMICS Microbial Community Standard; Zymo Research, Irvine, CA, USA) and 1 negative control were included with each batch of samples.

Polymerase Chain Reaction

The V3–V4 hypervariable region of bacterial 16S ribosomal RNA gene was amplified by PCR using the forward primer with Illumina adapters attached as follows: Illumina adapter and S-D-Bact-0341-b-S-17: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGC CTACGGGNGGCWGCAG-3′ and Illumina adapter and S-D-Bact-0785-a-A-21: 5′-GTCTCGT GGGCTCGGAGATGTGTATAAG AGACAGGACTACHVGGGTATCTAATCC-3′.

The following PCR conditions were used: 98 °C for 30 seconds, 25 × (98 °C for 10 seconds, 55 °C for 20 seconds, 72 °C for 20 seconds), 72 °C for 5 minutes; amplification was verified by electrophoresis. Indices were added in a subsequent PCR using the Nextera Index Kit V2 (Illumina, San Diego, CA, USA) with the following program: 98 °C for 30 seconds, 8 × (98 °C for 10 seconds, 55 °C for 20 seconds, 72 °C for 20 seconds), 72 °C for 5 minutes; attachment of indices was verified by electrophoresis. Products from the nested PCR were pooled based on band intensity and the resulting amplicon library cleaned with magnetic beads.

Sequencing and Data Analysis

DNA concentrations in the pooled libraries were measured by fluorometry. DNA sequencing was conducted using an Illumina MiSeq sequencer and the MiSeq Reagent Kit V3 (Illumina, San Diego, CA, USA) for 2 × 300-bp paired-end sequencing. An adjusted Divisive Amplicon Denoising Algorithm 2 pipeline was used for bioinformatics processing of the sequence data into the amplicon sequence variant (ASV) abundance table.[8] Alpha diversity (ie, variance within a particular sample) was assessed using the Shannon index, which measures the number of species and differences between the abundance of species. Beta diversity (ie, variance between samples) was assessed using UniFrac distances, which uses phylogenetic data to compare samples and principal coordinates analyses. Calculations of alpha diversity were done on rarefied data, all other analyses on relative abundances of the non-rarefied abundance table.

Statistical Analysis

Descriptive statistics were used for demographic variables. When performing statistical testing on multiple hypotheses, the Benjamini-Hochberg method was used to control for a false discovery rate (FDR) of 10%. In our results, we report the "FDR-adjusted *P* value," which indicates the FDR at which a given hypothesis would be called significant using the Benjamini-Hochberg method. Parametric statistical tests (Student's *t* test, Pearson correlation) were applied for "normal" distributed data only (visually inspected); otherwise, a nonparametric test was used (Wilcoxon-Mann-Whitney *U* test, permutational multivariate analysis of variance or Spearman correlation). For alpha diversity analysis, paired and unpaired Wilcoxon tests were used. ASVs were summarized at the genus level and only genera with a prevalence of ≥20% were included in the analysis.

Ethics Approval and Consent to Participate

The study was performed in accordance with the requirements of Good Clinical Practices and the Declaration of Helsinki. The study protocol was approved by the ethics committee of the Instituto Nacional de Pediatría of Mexico City (Registro 022/2016). Written informed consent was obtained from the parents or legal guardian of the patient before inclusion in the study.

RESULTS

Study Population

Thirty children with AD (20 males and 10 females) and 15 healthy controls (8 males and 7 females) were enrolled in this study. The median patient age was 16 months (range 8–68 months) and the median CG age was 24 months (range 6–64 months).

The mean Z score for weight/length and BMI of the patients were –0.77 and –0.97 kg, respectively, and of the CG were 0.13 and –0.36, respectively. We found statistically significant differences in the mean Z score for weight/length (Table 1). Of the 30 patients with AD, 11 (36.6%) were classified as mildly malnourished (BMI Z score >–2 to <–1) and 4 (13.33%) as moderately malnourished (BMI Z score >–3 to <–2); none of the patients had severe malnutrition (BMI Z score <–3). All children from the CG had a normal nutritional status.

Twenty-three of the 30 patients with AD (76.7%) reported mucus and 7 (23.3%) had blood in the stools. Reported signs and symptoms included fever in 20 patients (66.7%), abdominal pain in 27 patients (90.0%) and vomiting in 19 (63.3%). The median for duration of diarrhea was 4 days (range 1–10 days). None of the children with diarrhea required hospital admission.

According to type of birth, 13 (43.3%) patients were born by vaginal delivery, and 17 cesarean section (56.6%), in the CG 7 children were born by vaginal delivery and 8 cesarean section. The type of feeding the patients received were 15 breast-feeding, 4 formula and 11 mixed; in the CG 6 children breast-feeding, 1 formula and 8 mixed. No statistically significant differences were found.

Identified Pathogens

The principal etiologic pathogens identified by reverse transcriptase-PCR are summarized in Table 2. The most frequently

TABLE 1. Demographic Characteristics of Study Participants

	Patient Group (N = 30)	Control Group (N = 15)	
Variable	Median	Median	*P*
Age (mo)	16	24	0.129
Range	(8–68)	(6–64)	
Sex			
Females	10	7	0.384
Males	20	8	
	Patient Group Mean	Control Group Mean	*P*
Weight/length	–0.77 (SD 1.8)	0.13 (SD 0.49)	0.043
Z BMI	–0.97 (SD 0.87)	–0.36 (SD 0.84)	0.192
Type of Birth	Patient Group Frequency	Control Group Frequency	*P*
Vaginal delivery	13	7	0.832
Cesarean Section	17	8	
Type of Feeding	Patient Group Frequency	Control Group Frequency	*P*
Breast-feeding	15	6	0.529
Formula	4	1	
Mixed	11	8	

N indicates number.

identified pathogens were norovirus and *C. jejuni* (20% each), followed by *C. difficile* (17%) and rotavirus (13%); the pathogenic agent was not identified in 23% of samples.

Treatment of Acute Diarrhea

Investigators prescribed the most appropriate treatment for AD based on their clinical judgment. Twenty-four patients received *S. boulardii* CNCM I-745 (400 mg for 6 days) and 6 received zinc (20 mg for 10 days), diosmectite (9 g for 3 days) or gelatin tannate (750 mg for 3 days). The diarrheic episode resolved within 1 week in all treated patients.

Microbiota Analysis

Overall Taxonomic and Alpha Diversity Analyses

A total of 75 fecal samples from 30 diarrheic patients and 15 from the CG (1 per child) were subjected to 16S recombinant DNA sequencing. Of the 75 AD samples collected, 74 were properly purified and sequenced, generating an average of 56,428 reads per sample after quality filtering (interquartile range: 43,219–65,748). Taxonomic relative abundance of phyla and genera were evaluated in D0, D15 and CG samples (see Figure, Supplemental Digital Content 2, http://links.lww.com/INF/E345 and Figure, Supplemental Digital Content 3, http://links.lww.com/INF/E346).

A total of 9 phyla were detected. The predominant phyla were *Firmicutes* (55%), *Actinobacteria* (16%), *Bacteroidetes* (13%) and *Proteobacteria* (12%); *Verrucomicrobia*, *Fusobacteria*, *Epsilonbacteraeota*, *Cyanobacteria*, *Tenericutes* and others accounted for 4%. The most abundant genus was *Bifidobacterium* (16%), followed by *Faecalibacterium* (14%), *Bacteroides* (10%) and *Blautia* (9%).

Alpha diversity, as reflected by observed ASVs, is decreased in patient samples at D0 compared with samples from CG ($P = 0.045$) and significantly increased between D0 and D15 ($P = 0.017$) to a level similar to that measured in CG ($P = 0.82$) (Fig. 1A). When considering the Shannon index, more suitable and reliable to estimate alpha diversity[9] similar results are obtained (D0 vs. CG, $P = 0.0076$; D0 vs. D15, $P = 0.0076$; D15 vs. CG, $P = 0.77$) (Fig. 1B), indicating that the microbial alpha diversity of patients was restored to a "healthy" level at D15.

TABLE 2. Main Pathogens Identified by Multiplex Polymerase Chain Reaction Analysis

Microorganism, n (%)	N = 30
Norovirus	6 (20)
Campylobacter jejuni	6 (20)
Clostridium difficile toxin A/B	5 (17)
Rotavirus	4 (13)
Enterotoxigenic *Escherichia coli*	3 (10)
Adenovirus	2 (7)
E. coli O157	2 (7)
Shigella spp.	2 (7)
Salmonella spp.	1 (3)
Cryptosporidium spp.	1 (3)
Giardia lamblia	1 (3)
Negative for the tested pathogens	7 (23)

N indicates number; ssp., species.

Beta Diversity and Taxa Relative Abundance Analyses

The overall microbial community composition was compared by calculating the beta diversity using UniFrac distances (Fig. 1C). Significant differences in beta diversity were observed in patients between D0 and D15 ($P = 0.001$), as well as between patients on D0 and CG ($P = 0.001$). No significant difference was found between D15 and CG ($P = 0.211$). These observations are in accordance with the principal coordinates' analysis since a clear shift of the patient samples towards the CG samples was observed on D15.

Twenty-five genera had statistically significant differential abundance (FDR ≤ 0.1) between D0 and D15. When comparing the relative abundance between the D0 patient samples and the CG group, 43 genera had statistically significant differential abundance (FDR ≤ 0.1). Finally, no significant differences in abundance of genera were detected between patients on D15 and the CG after adjusting for multiple testing.

Regarding the most relevant genera, there was a significantly lower abundance of *Roseburia* and *Faecalibacterium* in D0 versus D15 ($P = 0.0014$ and $P = 0.0028$, respectively) and between D0 and CG samples (*Roseburia* $P = 0.014$, Fig. 1D; *Faecalibacterium* $P = 0.0018$, Fig. 1E) and of *Blautia* in D0 versus CG samples ($P = 0.0032$, Fig. 1F), while the abundance of *Streptococcus*, *Escherichia* and *Bacteroides* were significantly greater in D0 versus D15 samples ($P = 0.03$, $P = 0.039$ and $P = 0.014$, respectively) and between D0 and CG samples ($P = 0.0043$, $P = 0.028$ and $P = 0.046$, respectively; Fig. 1G–I). There were no significant differences in the abundance of any of these genera between D15 patient samples and CG samples (P value range: 0.073–0.91).

S. boulardii CNCM I-745 Treatment Analysis

Similar beta diversity was detected in samples from *S. boulardii*-treated patients at D15 and CG, whereas significant differences in beta diversity were observed between D15 samples from *S. boulardii*-treated versus non-*S. boulardii*-treated patients ($P = 0.021$; Fig. 2A). No statistically significant differences in taxa abundance were found between *S. boulardii*-treated and *S. boulardii*-untreated patients due to the limited number of patients.

Compared with CG patients, alpha diversity at D0 was significantly lower in *S. boulardii*-treated patients ($P = 0.049$) but not in non-*S. boulardii*-treated patients. Alpha diversity was not modified between D0 and D15 in the samples of patients that did not receive the *S. boulardii* treatment (paired comparison, $P = 0.31$)

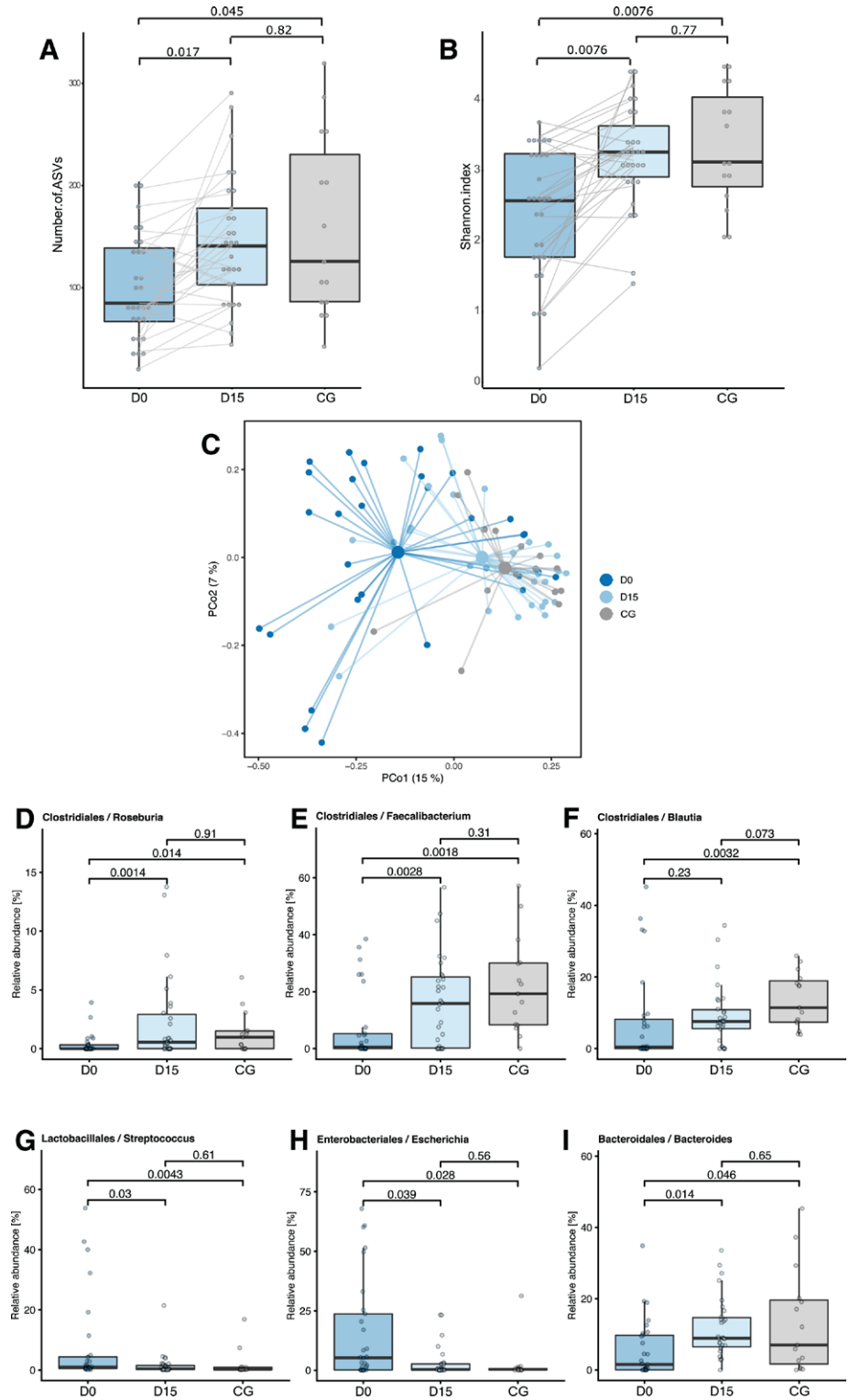

FIGURE 1. Microbiota analysis of D0, D15 and CG samples. Representation of alpha diversity using number of (A) ASVs (richness) and (B) Shannon index. Gray lines indicate D0 and D15 samples from the same patient; boxes indicate IQR; horizontal line within box indicates median; whiskers indicate 1.5 × IQR. C: Beta diversity by PCo plot using generalized UniFrac distances in D0, D15 and CG samples. Large dots indicate arithmetic mean position of the points belonging to the test group. D–I: Relative abundance of the most relevant genera in D0, D15 and CG samples: (D) *Roseburia*; (E) *Faecalibacterium*; (F) *Blautia*; (G) *Streptococcus*; (H) *Escherichia*; (I) *Bacteroides*. PCo1 (15%) and PCo2 (7%). IQR indicates interquartile range; PCo, principal coordinates analysis. full color online

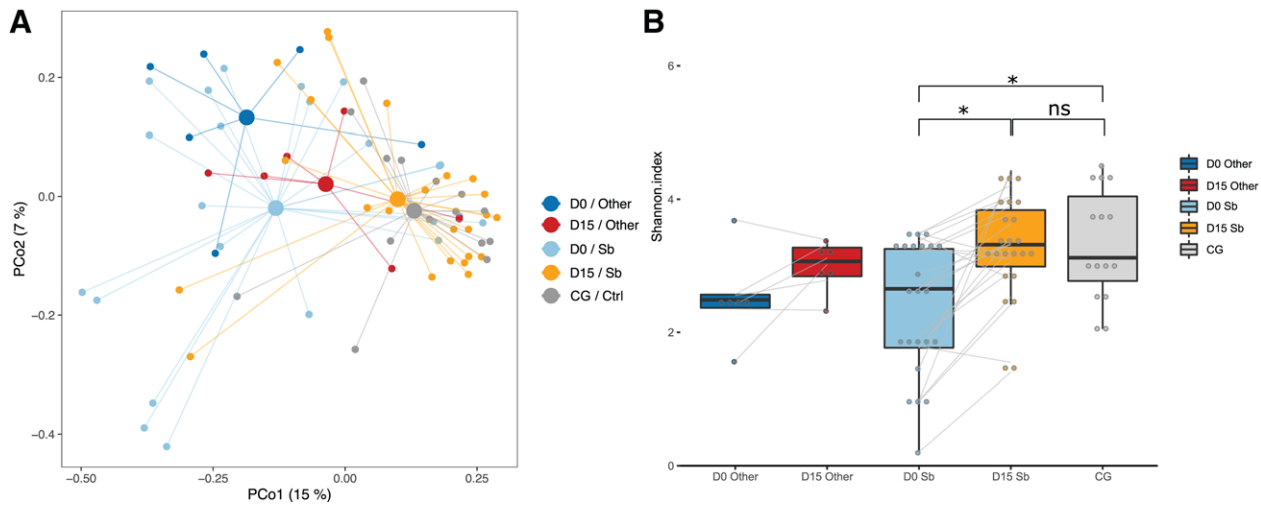

FIGURE 2. Effect of Sb treatment on beta diversity. A: PCo plot of generalized UniFrac distances in D0 and D15 samples (with vs. without Sb treatment) and CG samples. Large dots indicate arithmetic mean position of points belonging to the test subgroup. B: Alpha diversity by Shannon index. *P* values are adjusted using FDR correction. Gray lines indicate D0 and D15 samples from the same patient; boxes indicate IQR; horizontal line within box indicates median; whiskers indicate 1.5 × IQR. IQR indicates interquartile range; ns, not significant; PCo, principal coordinates analysis; Sb, *Saccharomyces boulardii* CNCMI-745. full color online

(Fig. 2B). Interestingly, *S. boulardii* treatment restored alpha diversity (paired comparison D0 and D15, $P = 6 \times 10^{-5}$) to a level similar to the CG.

DISCUSSION

In our study, we found that the most common pathogens in Mexican children with AD were norovirus and *C. jejuni* and that diarrheal episodes were associated with significant changes in microbiota diversity.

Despite the small size of the study population, our findings provide updated epidemiologic data revealing the evolution in the pathogens associated with AD that are consistent with other studies using PCR analysis. In a 2013 case-control multicenter study conducted in developing countries, the main causes of AD among children younger than 5 years were rotavirus, *Cryptosporidium* spp., enterotoxigenic *E. coli* and *Shigella* spp.[10] Similarly, an enteric multicenter study of moderate-to-severe diarrhea in children under 5 years in Africa and Asia found that *Shigella* spp., enteroinvasive *E. coli*, adenovirus 40/41, rotavirus, *Cryptosporidium* spp. and enterotoxigenic *E. coli* were highly prevalent and strongly associated with diarrhea.[11] However, in recent years, there has been an epidemiologic predominance of other viral agents (eg, enterovirus and norovirus) associated with AD.[12–14] This may be due to the introduction of the rotavirus vaccine, which has been associated with a decreased incidence of rotavirus-related diarrhea.[3] Data from 2014 to 2016 showed a 48.5% reduction in the number of rotavirus cases in Mexico, with 1299 cases reported in 2016.[4] In addition, emergency department visits due to rotavirus were reduced by 77.7% between 2007 and 2016.[4] Similarly, our study showed that a relatively low proportion of cases had rotavirus as the etiologic pathogen.

Antibiotic overuse in Mexico is high,[15] and is an important risk factor that can significantly modify the gastrointestinal microbiota, facilitating infection by opportunistic pathogens such as *Clostridium* spp.[16,17] Interestingly, few cases of AD-associated with *E. histolytica* or *G. lamblia* were found in our study, probably due to the improvements in sanitation measures in Mexico. Our study population comprised patients who were admitted to a tertiary level hospital, which could differ from the general population in Mexico.

Few studies have addressed gut microbiota dysbiosis in AD.[18,19] Treatment-wise, a study analyzing the fecal microbiota of children receiving probiotics due to infectious AD (Fecal microbiota analysis of children receiving probiotics due to acute infectious diarrhea study) explored microbiota diversity and the dynamics of recovery in healthy children and those with AD due to rotavirus after *S. boulardii* administration.[18] This study found low microbiota diversity in children during the first days of AD and increased diversity after 10 and 30 days of probiotic treatment. In addition, differences in the microbiota were observed between healthy children and those with AD in the first days but not in the last days of probiotic treatment. This indicates a transition in the microbiota from a diseased to healthy state over time.[18] Similarly, a Taiwanese study comparing the microbiota of 20 children with severe or complicated acute viral gastroenteritis found that microbiota diversity was significantly lower in patients with acute gastroenteritis than in CG, particularly in those with rotavirus infection.[20] Although malnutrition could alters the microbiota, and a proportion of our study population with AD presented some nutritional deficiency, at the D15, all patients showed the same effect of restauration.

To our knowledge, our study is the first to analyze the gut microbiota of Mexican children with AD. We have demonstrated that there are significant differences in microbial diversity between children during the early phase of the diarrheic episode (D0) and CG. This finding was expected due to the course of the disease and the fact that any pathologic state is associated with decreased microbiota diversity.[21]

Our taxonomic relative abundance analyses revealed that the most abundant phylum was *Firmicutes* and the most abundant genus was *Bifidobacterium*. This is consistent with a previous metagenomic analysis of microbiota from patients with diarrhea in India, in which *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* were identified across all samples.[22] Changes in gastrointestinal microbial composition due to diseases, such as AD, could impact future outcomes in children and has been related to dysbiosis,

characterized by high abundance of *Firmicutes*, an increase of *Proteobacteria* and fewer members of the *Bacteroidetes*.[17,23] This association between dysbiotic gut microbiota and bad prognosis has previously been suggested in other syndromes, such as irritable bowel syndrome.[24] It should be noted that, although a change in microbiota is often related to some diseases, this change is not necessarily the direct cause of disease; rather, it could be one of multiple risk factors.

Decreases in *Roseburia* and *Faecalibacterium* genera and increases in *Escherichia*, *Streptococcus* and *Bacteroides* in children with AD are consistent with modifications to the gut microbiota described in previous studies on diarrheic children.[25,26] Decreased levels of *Blautia* have also been reported.[18,25] These alterations of the gut microbiota compared with CG or patients who had recovered from AD could constitute a hallmark of the disease in children. However, AD-associated dysbiosis is likely to depend on the etiology of diarrhea.[27]

Our study investigated the dynamics of gut microbiota in children with AD before and after their recovery, following probiotic treatment with *S. boulardii*. Of the 30 children with AD, 24 received *S. boulardii* treatment and recovered by D15. Microbial diversity was similar in post-treatment (D15) and CG samples. Although this analysis should be considered exploratory, we can hypothesize that *S. boulardii* treatment facilitates the restoration of microbiota in children with AD, mostly by enhancing alpha diversity. The possible mechanisms of *S. boulardii* could include interference with pathogen attachment, interaction with normal microbiota, inactivation of toxins, antisecretory effects via normalization of the transcellular transport of chloride with reduced loss of sodium and water and immunomodulatory effects.[28]

In conclusion, our study demonstrated significant changes in gut microbiota diversity in Mexican children during diarrheal episodes and suggests that *S. boulardii* CNCM I-745 could facilitate microbiota restoration in children with AD. Although more studies are required.

ACKNOWLEDGMENTS

The authors thank Sarah Greig, PhD, of Springer Healthcare Communications, who provided English language editing services.

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