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Alterations of Intestinal Microbiome by Antibiotic Therapy in Hospitalized Children

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The administration of antimicrobial agents leads to an ecological imbalance of the host–microorganisms relationship, and it causes a rapid and significant reduction in the microbial diversity. The aim of the current study was to evaluate the impact of antibiotic therapy on intestinal microbiota of children between 3 and 12 years of age. The fecal samples were collected from hospitalized children ($n=31$) and from healthy untreated children ($n=30$). The presence of bacteria and their quantities were assessed by culture-based methods and quantitative polymerase chain reaction (qPCR). By culture method, in the children receiving antibiotics, a low recovery of *Bifidobacterium* spp. (54.8%), *Bacteroides* spp./*Parabacteroides* spp. (54.8%), *Clostridium* spp. (35.5%), and *Escherichia coli* (74.2%) was observed compared with the children without antibiotic therapy (100%, 80%, 63.3%, and 86.6%, respectively). By qPCR, the children receiving antibiotics showed a lower copy number for all microorganisms, except to *Lactobacillus* spp. ($p=0.0092$). In comparison to the nontreated children, the antibiotic-treated children showed a significantly lower copy number of *Bifidobacterium* spp. ($p=0.0002$), *Clostridium perfringens* ($p<0.0001$), *E. coli* ($p=0.0268$), *Methanobrevibacter smithii* ($p=0.0444$), and phylum *Firmicutes* ($p=0.0009$). In conclusion, our results obtained through qualitative and quantitative analyses, demonstrate that antibiotic therapy affect the intestinal microbiome of children.

Introduction

THE GASTROINTESTINAL TRACT of human and animal is a complex ecosystem inhabited by the climax community of several aerobic, anaerobic, and facultative bacteria.[1] In humans, the intestinal microbiota is enriched with anaerobic bacteria, such as *Bacteroides* spp., *Bifidobacterium* spp., *Eubacterium* spp., *Clostridium* spp., *Lactobacillus* spp., *Fusobacterium* spp., Gram-positive cocci, and methanogenic archaea such as *Methanobrevibacter smithii* and *Methanosphaera stadtmanae*.[2,3]

The initial colonization of infant microbiota is determined by several factors, that is, physiology and anatomy of the intestinal tract, bile salts, peristalsis, pH, and immunomodulation.[4] In addition, the composition of microbiota is dependent on the mode of delivery (vaginal delivery vs. caesarean section), feeding regime (breastfeeding vs. formula feeding), and exposure to antimicrobials.[5,6] The microbiota at birth is less diverse and the phyla *Proteobacteria* and *Actinobacteria* are predominant, but as the age progresses with the intake of solid food, the microbiota gets diversified and other phyla such as *Firmicutes* and *Bacteroidetes* also appear.[7]

Perturbation in the intestinal microbiota may occur due to changes in diet, diseases (*i.e.*, obesity, allergy, and colon cancer), and by the use of antimicrobial agents. The administration of antimicrobial agents decreases bacterial colonization and dramatically modifies intestinal microbiota; as a consequence, antibiotic-resistant microorganisms may increase in numbers, and this disturbance can occasion antibiotic-associated diarrhea.[8–10]

The exposure of intestinal microbiota to antimicrobials can reduce its microbial diversity to a variable extent depending on antibiotic spectrum, dosage, duration of treatment, route of administration, and the pharmacokinetic and pharmacodynamic properties of the drug.[11,12] However, the microbiota is relatively resilient and returns to the original state within several weeks postantibiotic therapy; but sometimes, the resilience may not be complete, and depending on the individuals, may result in an altered microbiome.[13–15]

The consequence of exposure to antibiotics is the emergence of antimicrobial-resistant bacteria in the commensal

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species. The intestinal microorganisms such as *Bacteroides* spp., *Clostridium* spp., *Escherichia coli*, and others become resistant to antibiotics by acquiring antibiotic resistance genes, in a consequence of excessive or improper use of antibiotics.[16]

In this study, the occurrence of *Clostridium* spp., *Bacteroides* spp., *Parabacteroides* spp., *Bifidobacterium* spp., and *E. coli* in hospitalized children treated with antibiotic therapy was verified by using a culture-based technique. In addition, using real-time polymerase chain reaction (PCR), we determined the presence of *Bacteroides fragilis*, *Bacteroides vulgatus*, *Parabacteroides distasonis*, *Parabacteroides merdae*, *Clostridium perfringens*, *Clostridium difficile*, *Lactobacillus* spp., and *M. smithii*, and the members of the phyla *Bacteroidetes* and *Firmicutes*.

Materials and Methods

Clinical samples and laboratory procedures

Fresh fecal samples were obtained from 31 children who were hospitalized and being treated with antibiotics (beta-lactams, quinolones, macrolides, glycopeptides, linezolid, lincosamide, folic acid antagonistic, or aminoglycosides) at the Institute of Children (Hospital das Clinicas). In a similar way, the control fecal samples were from 30 healthy children of private and municipal schools of Sao Paulo city, SP, Brazil who had not received any antibiotic treatment in the past 3 months (control group). The male and female children of all races, aged between 3 and 12 years, and without existing diarrhea were selected for this study. The fecal samples were collected from February 2012 through March 2013 in sterile vials and immediately stored at −80°C until further use. The study was approved by the Ethics Committee of the Biomedical Sciences Institute, University of Sao Paulo (No. 1058/CEP).

A portion of fecal material was directly streaked onto plates containing specific selective media *Bacteroides* Bile Esculin Agar (BBE) for the *B. fragilis* group, Cycloserine Cefoxitin Fructose Agar (CCFA) supplemented with 5% blood for *Clostridium* spp., *Bifidobacterium* modified agar[17] (using Reinforced *Clostridial* Medium as base, dextrose, and L-cysteine) for *Bifidobacterium* spp., and MacConkey agar for *E. coli*. The streaked plates containing BBE, CCFA, and *Bifidobacterium* agar media were incubated in anaerobic conditions for five days and MacConkey agar in aerobic conditions for 24 hr at 37°C.

Identification of bacterial species

To confirm bacterial species, four characteristic colonies for each microorganism were subcultured in blood or Luria Bertani agar medium. The bacterial DNA of *Bacteroides* spp., *Parabacteroides* spp., *Bifidobacterium* spp., and *E. coli* was extracted by using QIAamp DNA Mini Kit (Qiagen), following the manufacturer's instructions. The *Bacteroides* spp. and *Parabacteroides* spp. were identified by multiplex-PCR,[18] whereas *Bifidobacterium* spp.[19] and *E. coli*[20] by conventional PCR using 16S rRNA-specific primers. The PCR reactions were performed in the final volumes of 25 µl containing 1X PCR buffer, 50 mM $MgCl_2$, 0.2 mM dNTP mix, 0.4 mM of each primer, 0.5 U platinum *Taq* polymerase

TABLE 1. MICROORGANISMS AND SPECIES-SPECIFIC 16S rRNA GENE-TARGETED OLIGONUCLEOTIDES USED IN THIS STUDY

Microorganisms	*Oligonucleotides 5′ → 3′*	*Tm (°C)*	*Amplicon (bp)*	*References*
Bifidobacterium spp.	F: GCG TGC TTA ACA CAT GCA AGT C R: CAC CCG TTT CCA GGA GCT ATT	60	125	40
Bacteroides fragilis	F: TCR GGA AGA AAG CTT GCT R: CAT CCT TTA CCG GAA TCC T	63	162	41
Bacteroides vulgatus	F: GCA TCA TGA GTC CGC ATG TTC R: TCC ATA CCC GACT TTA TTC CTT	60	287	42
Parabacteroides distasonis	F: TGC CTA TCA GAG GGG GAT AAC R: GCA AAT ATT CCC ATG CGG GAT	60	80	41
Parabacteroides merdae	F: AGG GTG CGT AGG TGG TGAT R: TTC ACC GCT ACA CCA CGC	60	122	41
Clostridium perfringens	F: TCA TCA TTC AAC CAA AGG AGC AAT CC R: CCT TGG TAG GCC GTT ACC C	55	105	43
Clostridium difficile	F: ATT AGG AGG AAC ACC AGT TG R: AGG AGA TGT CAT TGG GAT GT	56	307	44
Escherichia coli	F: AGA AGC TTG CTC TTT GCT GA R: CTT TGG TCT TGC GAC GTT AT	57	120	45
Lactobacillus spp.	F:AGC AGT AGG GAA TCT TCC A R: ATT YCA CCG CTA CAC ATG	56	380	40
Methanobrevibacter smithii	F: AGG TAC TCC CAG GGT AGA GG R: TCC CTC ACC GTC AGA ATC G	59	92	46
Phylum *Bacteroidetes*	F: TGG TAG TCC RCR CDG TAA ACG ATG R: ATG TTC CTC CGC TTG TGC	60	150	This study
Phylum *Firmicutes*	F: TRA AAC TYA AAG GAA TTG ACG R: ACC ATG CAC CAC CTG TC	61	155	47

Strains used to the standard curve construction: *B. fragilis* ATCC 25285; *B. vulgatus* ATCC 8482; *P. distasonis* ATCC 8503; *P. merdae* ATCC 43184; *C. perfringens* ATCC 13124; *C. difficile* VPI 10468 *Bifidobacterium bifidum* ATCC 1696; *E. coli* ATCC 25922, and *M. smithii* ATCC 35061.

(Invitrogen), and 1 ng DNA. The PCR products were separated on 1% agarose gel electrophoresis stained with 0.5 μg/ml ethidium bromide and photographed under UV light. The *Clostridium* species was identified by using an API 20A Kit (bioMérieux).

Quantification of bacteria by real-time PCR assay

The bacterial DNA was extracted from the fecal samples collected with the help of QIAamp DNA Stool Mini Kit (Qiagen) following the manufacturer's instructions. The DNA concentration was determined by spectrophotometer (NanoDrop 2000; Thermo Scientific), and 10 μl of each DNA sample was used to assess integrity by 1% agarose gel electrophoresis. PCR assays were performed using species-specific primers for 16S rRNA gene sequences (Table 1). The PCR reactions were performed in final volumes of 20 μl containing: 2X SYBR® Green PCR Master Mix (GoTaq qPCR Master Mix; Promega Corporation), 5 μM of each primer and 2 ng of fecal DNA. Amplification reactions were performed in a Rotor Gene 6000 (Corbett Life Science) thermocycler using the following cycle: initial denaturation at 95°C for 10 min; 40 cycles of 95°C for 15 sec and annealing temperature suitable for each primer pair for 60 sec. The absolute quantification was determined by plotting standard curve using serially diluted bacterial DNA. A dissociation curve was used to analyze the presence of primer dimer. The samples showing efficiency between 0.9 and 1.0 were considered for analysis.

Statistical analyses

All statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Software). The copy numbers of the 16S rRNA gene per gram of feces were compared by Mann–Whitney *U*-test. The experiment reproducibility and bacterial isolation were assessed by Spearman's test (r) and Fisher's test, respectively. One-way ANOVA was used to compare the effect of antibiotics treatment on the quantity of microorganisms. $p<0.05$ were considered as statistically significant.

Results

The culture-based methods using selective media revealed that children were harboring at least one species of the *B. fragilis* group, *Clostridium* spp., *Bifidobacterium* spp., and *E. coli*. The occurrence of bacterial species in children treated with antibiotics was lower than in children without antibiotic therapy. Of the 31 antibiotic-treated children, 17 (54.8%) were found to harbor *Bacteroides/Parabacteroides* species, 23 (74.2%) *E. coli*, 11 (35.5%) *Clostridium* spp., and 17 (54.8%) *Bifidobacterium* spp. All children without antibiotic treatment were found to harbor *Bifidobacterium* spp., 24 (80%) *Bacteroides/Parabacteroides*, 19 (63.3%) *Clostridium* spp., and 26 (86.6%) *E. coli*. The prevalence of *Bacteroides vulgatus*, *Bifidobacterium adolescentis*, and *Bifidobacterium infantis* was significantly different between the groups (Table 2).

The quantitative analysis by real-time PCR revealed that the occurrence of the *Bifidobacterium* spp., *C. perfringens*, *E. coli*, *M. smithii*, and phylum *Firmicutes* was lower in children with antibiotic treatment than children not treated with antibiotics ($p<0.05$), except to *Lactobacillus* spp., which showed a high copy number. However, there was no significant difference in the quantities of *B. fragilis*, *B. vulgatus*, *P. distasonis*, *P. merdae*, *C. difficile*, and phylum *Bacteroidetes* in both groups (Table 3).

Table 2. Bacterial Species Identified in Feces from 31 Antibiotic-Treated and 30 Control Children by Culture Method

Microorganisms	*Antibiotic-treated (n=31) Prevalence,*[b] n *(%)*	*Control (n=30) Prevalence,* n *(%)*	p[a]
B. fragilis group			
B. vulgatus	6 (19.3)	21 (70)	**<0.0001**
B. fragilis	1 (3.2)	2 (6.6)	0.612
B. ovatus	1 (3.2)	1 (3.3)	1.000
B. caccae	2 (6.4)	1 (3.3)	1.000
B. stercoris	0	2 (6.6)	ND[c]
B. thetaiotaomicron	1 (3.2)	0	ND[c]
P. distasonis	12 (38.7)	6 (20)	0.160
P merdae	1 (3.2)	1 (3.3)	1.000
Clostridium spp.			
Clostridium butyricum/ Clostridium beijerinckii	8 (25.8)	8 (26.6)	1.000
C. perfringens	0	8 (26.6)	ND[c]
C. clostridioforme	1 (3.2)	1 (3.3)	1.000
C. innocum	3 (9.6)	4 (13.3)	0.707
C. paraputrificum	2 (6.4)	0	ND[c]
C. difficile	1 (3.2)	1 (3.3)	1.000
C. glycolicum	0	2 (6.6)	ND[c]
C. sporogenes	0	1 (3.3)	ND[c]
C. baratii	0	1 (3.3)	ND[c]
Clostridium sp.	5 (16.1)	4 (13.3)	1.000
Bifidobacterium spp.			
Bifidobacterium adolescentis	8 (25.8)	25 (83.3)	**<0.0001**
Bifidobacterium infantis	5 (16.1)	12 (40)	**0.048**
Bifidobacterium breve	12 (38.7)	0	ND[c]
E. coli	23 (74.1)	26 (86.6)	0.335

[b]Prevalence reflects the number of positive samples by culture-based technique.
[a]Fisher's test was applied. Significance levels in bold ($p<0.05$).
[c]ND: Without sufficient positive sample to perform the Fisher's test.

Spearman's test revealed a weak ($r<0.4$) to moderate ($r=0.40$–0.59) correlation of microorganisms in both groups. However, there was a significant correlation between the phylum *Firmicutes* and *C. perfringens* ($r=0.63$; $p=0.0010$) and *B. fragilis* and *P. distasonis* ($r=0.61$; $p=0.0002$) in children treated with antibiotics.

When we compared the effect of the number of antibiotics (drugs) used as therapy versus the number of microorganisms, the number of *Bifidobacterium* spp. and *P. distasonis* were found to be statistically significant ($p=< 0.05$). For the phylum *Bacteroidetes*, there was no decrease in their number even when more than three drugs had been taken (Table 4). The relationship between the effect of the classes of antibiotics used in children and the microbial number was also evaluated, and no statistically significant values were observed (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/mdr).

Table 3. Bacterial Prevalence and Quantification Verified in Feces of Antibiotic-Treated and Control Children by qPCR

	Antibiotic-treated (n=31)	*Control* (n=30)	*Total* (n=61)	p
Presence of genus, species, or phylum,[a] n (%)				
Bifidobacterium spp.	29 (93.5)	30 (100)	59 (96.7)	0.4918
Lactobacillus spp.	31 (100)	27 (90)	58 (95)	0.1128
B. vulgatus	31 (100)	30 (100)	61 (100)	ND[b]
B. fragilis	29 (93.5)	30 (100)	59 (96.7)	0.4918
P. distasonis	30 (96.7)	29 (96.6)	59 (96.7)	1
P. merdae	31 (100)	29 (96.6)	60 (98.3)	0.4918
C. perfringens	31 (100)	29 (96.6)	60 (98.3)	0.4918
C. difficile	26 (83.8)	29 (96.6)	55 (90.1)	0.1953
E. coli	31 (100)	30 (100)	61 (100)	ND[b]
M. smithii	19 (61.2)	28 (93.3)	47 (77)	**0.0050**
Bacteroidetes	31 (100)	30 (100)	61 (100)	ND[b]
Firmicutes	31 (100)	30 (100)	61 (100)	ND[b]
Quantitative determination (log_{10} copies/g feces)[c]				
Bifidobacterium spp.	4.62 (4–9.59)[d]	7.29 (6.51–8.95)	6.52 (4.43–9.59)	**0.0002**
Lactobacillus spp.	5.91 (5.53–8.86)[d]	5.52 (4.87–6.95)	5.71 (5.17–8.86)	**0.0092**
B. vulgatus	4.29 (3.08–8.74)	7.12 (5.91–8.88)	6.81 (4.10–8.88)	0.0901
B. fragilis	4.69 (3.18–8.11)	6.6 (5.29–8.53)	5.83 (3.96–8.53)	0.0055
P. distasonis	4.12 (3.02–8.28)	3.82 (2.96–7.86)	3.98 (3–8.28)	1
P. merdae	4.58 (3.53–8.74)	7.27 (5.52–8.38)	6.43 (3.9–8.74)	0.1700
C. perfringens	4.37 (3.6–6.35)[d]	5.84 (5.47–6.76)	5.45 (3.88–6.76)	**<0.0001**
C. difficile	1.67 (0.75–5.51)	3 (2–5.85)	2.75 (0.95–5.85)	0.0700
E. coli	5.89 (4.85–9.61)[d]	7.68 (7.11–9.28)	7.54 (5.47–9.61)	**0.0268**
M. smithii	4.29 (0–8.22)[d]	4.51 (4.05–8.98)	4.44 (3.56–8.98)	**0.0444**
Bacteroidetes	8.9 (7.72–10.54)	8.93 (8.47–9.78)	8.92 (8.33–10.54)	0.3634
Firmicutes	7.86 (7.44–8.84)[d]	8.42 (8.11–9.14)	8.13 (7.77–9.14)	**0.0009**

Significance levels in bold ($p<0.05$).
[a]Values noted as number (percentage), Fisher's exact test.
[b]ND: Without sufficient positive samples to perform the Fisher's exact test.
[c]Data are presented as median (interquartile range); differences among two groups are compared using Mann–Whitney test.
[d]$p<0.05$ indicated significant differences as compared with the control group.
qPCR, quantitative polymerase chain reaction.

Differences at gender were observed in *Bifidobacterium* spp. copy number in control children. Specifically, control girls had significantly higher *Bifidobacterium* spp. levels than control boys ($p<0.05$). Further stratification of the bacterial copy number by gender revealed significantly higher *Lactobacillus* spp., *E. coli*, *C. perfringens*, *M. smithii*, and phylum *Firmicutes* levels in control girls compared with the antibiotic-treated girls ($p<0.05$); there was difference in *B. fragilis*, *C. perfringens*, and phylum *Firmicutes* levels between control and antibiotic-treated boys. For the other

Table 4. Antimicrobial Effect on Quantities of Microorganisms

	Number of antibiotics vs. bacterial quantification (log_{10})			
Microorganisms	*1 drug, Pr[a]=12*	*2 drugs, Pr[a]=10*	*>3 drugs, Pr[a]=9*	p
Bifidobacterium spp.	5.72 (4.93–8.95)	4.50 (4.17–9.59)	4.01 (3.63–6.95)	**0.0282**[b]
Lactobacillus spp.	5.84 (5.50–8.86)	6.00 (5.63–8.44)	5.70 (5.55–7.00)	0.9114
B. fragilis	6.24 (4.57–8.11)	3.84 (2.17–8.08)	5.03 (3.11–7.86)	0.2762
B. vulgates	6.78 (4.21–8.40)	3.86 (3.07–8.64)	4.29 (2.91–8.74)	0.6994
P. distasonis	5.22 (4.11–7.99)	3.25 (2.51–6.48)	3.14 (3.31–8.56)	**0.0243**[c]
P. merdae	5.83 (4.51–8.40)	4.96 (3.66–8.74)	3.52 (3.31–8.56)	0.1672
C. perfringens	5.38 (4.68–6.34)	3.79 (3.61–6.35)	3.85 (3.61–5.98)	0.2862
C. difficile	3.16 (1.33–4.76)	2.31 (0.74–5.51)	1.00 (0.00–4.97)	0.3907
E. coli	7.24 (5.78–9.61)	7.24 (4.84–9.38)	5.10 (4.89–8.84)	0.3977
M. smithii	4.42 (4.06–8.22)	4.36 (0.00–7.74)	0.00 (0.00–5.26)	0.1984
Phylum *Bacteroidetes*	8.86 (8.57–9.82)	7.72 (6.28–9.61)	9.11 (9.02–10.54)	0.2153
Phylum *Firmicutes*	8.14 (7.82–8.70)	7.80 (7.46–8.84)	7.79 (7.15–8.38)	0.3021

Data are presented as log_{10} median (interquartile range); differences among three groups are compared using Kruskal–Wallis test (Dunn's post-test), $p<0.05$. Significance levels in bold ($p<0.05$).
[a]Prevalence (Pr) reflects the number of positive children taking antibiotics.
[b]$p<0.05$ indicated significant differences as compared with the one-drug and >3-drug groups.
[c]$p<0.05$ indicated significant differences as compared with the one-drug and two-drug groups.

microorganisms evaluated, no statistically significant differences were observed (Supplementary Table S2).

In a logistic regression, the variables eligible for the final model were *Lactobacillus* spp., *P. distasonis*, *M. smithii*, *Firmicutes*, and *C. perfringens*. The logistic regression analysis showed that *Lactobacillus* spp. and *P. distasonis* are significantly associated with antibiotic therapy group; *M. smithii*, *Firmicutes*, and *C. perfringens* are associated with control children (Supplementary Table S3). These results confirm the findings obtained by univariate analysis.

Discussion

The antibiotic therapy can have various undesirable effects on host intestinal microbiota. It can decrease the number of beneficial microbes and promote colonization of potentially pathogenic bacteria.[21,22] In addition, it can stimulate antibiotic resistance and stabilize the antibiotic-resistant population of the microbiota.[23]

Qualitative culturing was used to identify and characterize strains with specific traits, and to determine their viability in the intestinal ecosystem. Culture-based technique is the gold standard for the isolation of the selected bacterial group and it can help elucidate the host–microbiota interaction.[24,25]

In addition to qualitative culturing, real-time PCR was also used for a better determination of bacterial number in each children group. The obtained results were used to verify the decrease in the diversity and bacterial number of the microbiome in the children treated with antibiotics. Species-specific primers used in quantitative PCR (qPCR) have shown good reproducibility, sensitivity, and specificity; however, significant differences between the culture and PCR were observed for *Lactobacillus* spp. by Million *et al.*[24] In this study, PCR showed much more sensitivity than culture to detect differences in *B. fragilis*, *C. perfringens*, and *E. coli*.

The species of genus *Bifidobacterium* are commonly found in the intestinal microbiota. In this study, a high prevalence of *B. adolescentis* and *B. infantis* was observed in the children without antibiotics treatment than those treated with antibiotics (Table 2). Interestingly, *Bifidobacterium breve* was not observed in the control group; however, *B. breve* was found in 38.7% of the antibiotic-treated children possibly due to its resistance toward antimicrobials.[26–28] Moreover, species of *Bifidobacterium* are commonly resistant to several antimicrobials, and *B. breve* is considered for carrying different resistance markers against several antibiotics.[27,28]

Antimicrobial resistance can also be observed in beneficial bacteria (probiotics), especially in *Lactobacillus* spp. as observed in this study (Table 3). It is of interest due to the fact that they can become a reservoir of resistance markers.[29]

Several studies have reported similar prevalence of *B. fragilis*, *B. vulgatus*, and *P. distasonis* in human fecal microbiota.[30,31] In this study, *B. vulgatus* was found in high prevalence in the nontreated children than the treated ones (70% vs. 19.3%, respectively), whereas *P. distasonis* was observed more in the antibiotic-treated children (in 20% control children vs. 38.7% antibiotic-treated). This suggested that the species *B. fragilis* and *B. vulgatus* are sensitive and *P. distasonis* is resistant toward antibiotic therapy. This observation was similar to that reported by Nakano *et al.*[32] and Boente *et al.*[33]

The *Clostridium* species, by interacting with other members of microbiota, play a crucial role in intestinal homeostasis such as in biosynthesis of essential nutrients (vitamins K and B12), bile biotransformation, and carbohydrate degradation.[34] In this study, *Clostridium butyricum/Clostridium beijerinckii* were found in both antibiotic-treated (25.8%) and control (26.6%) children; and *C. perfringens* (26.6%) was more prevalent in the children without antibiotic therapy. Since *C. perfringens* is a member of resident microbiota, this was expected, as this supports the findings of Samb-Ba *et al.*[35] showing that *C. perfringens* is the most commonly found bacterium in individuals between 5 and 20 years of age with (32.7%) and without (53.5%) diarrhea.

C. difficile, however, was rarely found in either of the evaluated groups (Table 2). It is known that this microorganism causes diarrhea in infants of ≤2 years of age. In this study, the children evaluated were of ≥3 years without diarrhea; therefore, a low amount of this bacterium is expected and is in accordance with other reports.[36,37] Moreover, one strain was detected in the children using antibiotics and a different one was obtained from the control group (Table 2).

Since *Enterobacteriaceae* are the main facultative bacteria found in gut microbiota, the presence of *E. coli* in both control (86.6%) and antibiotic-treated (74.1%) groups is as expected and in accordance with Garcia *et al.*[38]

The real-time PCR showed significant differences in the prevalence of *Bifidobacterium* spp., *C. perfringens*, *E. coli*, *Lactobacillus* spp., *M. smithii*, and the phylum *Firmicutes* between the two groups (Table 3). Earlier studies have shown that the use of antimicrobials decreases the intestinal bacterial numbers, mainly of enterobacteria, enterococci, and anaerobic bacteria.[8,39] Similarly, our results showed a decrease in bacterial number after antibiotic treatment. Moreover, the antibiotic associations appeared to have no influence on bacterial numbers as determined by qPCR, which can be explained, as this technique detects small amount of DNA and not viable bacteria. Furthermore, the influence of antimicrobials on quantities of microorganisms was observed in *Bifidobacterium* spp. and *P. distasonis* with a statistical significance (Table 4).

It is known that commensal bacteria in the community and hospital environment carry many types of resistance genes.[1] The high copy numbers of *Lactobacillus* spp. observed in antibiotic-treated children suggest the presence of some resistance mechanism (not addressed in this study).

Conclusion

In conclusion, our results indicate that the antibiotic therapy qualitatively as well as quantitatively affect intestinal microbiota in children.

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Disclosure Statement

No competing financial interests exist.

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SUPPLEMENTARY TABLE S1. THE EFFECT OF CLASSES OF ANTIBIOTICS AGAINST THE LEVELS OF MICROORGANISMS

	Antibiotics vs. bacterial quantification (log_{10})						
Microorganisms	*Beta-lactam, Pr[a]=11*	*Sulfonamide, Pr[a]=3*	*Beta-lactam+ Macrolide, Pr[a]=3*	*Beta-lactam+ Glycopeptide, Pr[a]=3*	*Beta-lactam+ Aminoglycoside, Pr[a]=3*	*Others,[b] Pr[a]=8*	p
Bifidobacterium spp.	7.14 (5.38–8.95)	5.03 (4.82–5.63)	4.62 (4.44–6.69)	4.11 (3.83–4.96)	4.34 (4.04–9.59)	4 (3.56–6.95)	0.1332
Lactobacillus spp.	5.86 (5.27–8.86)	5.81 (5.7–5.97)	6.09 (5.88–6.42)	5.95 (5.94–6.12)	6.48 (6.21–8.44)	5.67 (5.54–7)	0.8801
Bacteroides fragilis	6.88 (4.83–8.11)	3.23 (2.93–5.51)	3.73 (3.7–4.08)	2.32 (2.23–4.08)	3.96 (3.68–7.72)	5.39 (2.99–7.86)	0.2139
Bacteroides vulgatus	7.25 (5.97–8.64)	4.1 (3.82–7.35)	3.07 (2.78–4.29)	3.25 (3.1–4.11)	5.73 (5.43–8.74)	7.13 (2.85–8.7)	0.2898
Parabacteroides distasonis	5.35 (4.35–7.99)	3.98 (3.73–7.9)	4.12 (3.86–5.64)	2.46 (2.18–3.57)	2.65 (2.62–3.39)	4.21 (2.99–8.28)	0.0934
Parabacteroides merdae	6.07 (4.42–8.4)	4.79 (4.5–6.61)	4.53 (4.24–8.74)	3.89 (3.65–5.24)	7.55 (7.25–8.02)	3.43 (3.22–8.56)	0.4205
Clostridium perfringens	5.38 (4.54–5.97)	5.83 (5.53–6.34)	3.61 (3.59–4.74)	3.64 (3.62–4.37)	3.78 (3.75–6.35)	4 (3.52–5.98)	0.5066
Clostridium difficile	2.61 (1.23–4.76)	3.19 (2.89–3.69)	2.81 (2.51–3.12)	0.84 (0.54–4.97)	2.9 (2.6–5.51)	1.02 (0–4.97)	0.8243
Escherichia coli	7.28 (6.48–9.61)	9.22 (8.92–9.35)	4.81 (4.6–5.27)	5.47 (5.27–8.52)	4.89 (4.7–7.69)	5.18 (5–9.38)	0.3248
Methanobrevibacter smithii	4.28 (4–8.22)	0 (0–4.92)	4.99 (4.69–7.74)	4.82 (4.51–5.61)	4.6 (4.3–4.64)	0 (0–5.26)	0.2857
Phylum *Bacteroidetes*	8.9 (8.28–9.82)	8.59 (8.38–8.81)	8.59 (8.38–8.81)	6.64 (6.34–7.62)	9.29 (9.09–9.61)	9.08 (8.9–10.54)	0.2149
Phylum Firmicutes	8 (7.83–8.7)	8.37 (8.08–8.44)	8.37 (8.08–8.44)	7.86 (7.69–8.33)	7.15 (7–8.84)	7.9 (7.56–8.38)	0.4636

Data are shown as log_{10} median (interquartile range); differences among six groups are compared using Kruskal–Wallis test (Dunn'spost-test), $p < 0.05$.
[a]Prevalence (Pr) reflects the number of positive children taking antibiotics.
[b]Other antibiotic associations: Beta-lactam+Macrolide+Aminoglycoside+Oxazolidone+Drug to *Mycobacterium*.

SUPPLEMENTARY TABLE S2. UNIVARIATE ANALYSIS OF THE BACTERIAL ASSOCIATION WITH ANTIBIOTIC THERAPY BY GENDER

	Antibiotic-treated (n=*31*)		*Control* (n=*30*)		*Total* (n=*61*)		
Variables	*Pr*[a]	*Median (interquartile range)*	*Pr*[a]	*Median (interquartile range)*	*Pr*[a]	*Median (interquartile range)*	p
Girls		(*n*=14)		(*n*=19)		(*n*=33)	
Bifidobacterium spp.	13	4.32 (4.12–8.14)[b]	19	7.64 (7.18–8.95)[c]	32	6.95 (4.62–8.95)	**<0.0001**
Lactobacillus spp.	14	5.92 (5.54–6.48)[b]	16	5.08 (4.68–6.95)	30	5.71 (4.97–6.95)	**0.0302**
B. fragilis	14	5.22 (3.8–8.08)	19	6.52 (5.31–8.53)	33	5.92 (4.08–8.53)	0.1960
B. vulgatus	14	7.05 (3.79–8.74)	19	7.22 (6.33–8.88)	33	7.22 (4.38–8.88)[c]	0.6229
P. distasonis	14	4.57 (2.99–8.28)	19	3.86 (3.33–7.86)	33	4.27 (3.25–8.28)	0.9274
P. merdae	14	4.26 (3.32–8.56)	18	7.53 (6.96–8.13)	32	7.16 (4.02–8.56)	0.0714
E. coli	14	5.19 (4.89–9.61)[b]	19	7.58 (7.06–8.88)	33	7.19 (5.58–9.61)	**0.0114**
C. perfringens	14	4.6 (3.74–6.34)[b]	18	5.86 (5.37–6.59)	32	5.45 (4.37–6.59)	**0.0038**
C. difficile	11	1.5 (0.3–5.51)	19	3.05 (2.35–5.85)	30	2.75 (1.51–5.85)	0.0903
M. smithii	9	4 (0–5.61)[b]	18	7.53 (6.96–8.13)	27	4.52 (3.75–8.98)	**0.0214**
Bacteroidetes	14	9.04 (8.05–10.54)	19	8.84 (8.37–9.78)	33	8.85 (8.33–10.54)	0.9274
Firmicutes	14	7.79 (7.23–8.38)[b]	19	8.41 (7.99–9.14)	33	8.06 (7.47–9.14)	**0.0179**
Boys		(*n*=17)		(*n*=11)		(*n*=28)	
Bifidobacterium spp.	16	5.66 (3.99–9.59)	11	6.51 (5.52–8.43)	27	6.13 (4.43–9.59)	0.3715
Lactobacillus spp.	17	5.86 (5.55–8.86)	11	5.7 (5.27–6.27)	28	5.71 (5.45–8.86)	0.2396
B. fragilis	15	4.69 (2.32–8.11)[b]	11	7.79 (5.61–8.35)	26	5.61 (3.21–8.35)	**0.0127**
B. vulgatus	17	4.11 (2.91–8.02)	11	6.63 (5.64–8.37)	28	6.13 (3.05–8.37)	0.2396
P. distasonis	16	3.98 (3.05–7.99)	10	4.27 (2.07–7.52)	26	3.87 (2.88–7.99)	0.6213
P. merdae	17	5.19 (3.89–8.74)	11	6.25 (3.82–8.38)	28	5.7 (3.83–8.74)	0.9625
E. coli	17	7.20 (4.7–9.38)	11	7.91 (7.57–9.28)	28	7.66 (5.42–9.38)	0.4517
C. perfringens	17	4.13 (3.45–6.35)[b]	11	5.84 (5.57–6.76)	28	5.40 (3.77–6.76)	**0.0073**
C. difficile	14	2.81 (0.77–4.97)	10	3.42 (1.21–5.13)	24	2.74 (0.76–5.13)	0.5248
M. smithii	10	4.28 (0–8.22)	10	4.39 (3.98–8.37)	20	4.28 (0–8.37)	0.7211
Bacteroidetes	17	8.81 (7.8–9.82)	11	9.05 (8.86–9.73)	28	8.92 (8.37–9.82)	0.1581
Firmicutes	17	7.99 (7.77–8.84)a	11	8.68 (8.19–9.01)	28	8.23 (7.9–9.01)	0.0073

Significance levels in bold ($p<0.05$).
Data are presented as $\log_{10}$ median (interquartile range); differences among two groups are compared using Mann–Whitney test, $p<0.05$.
[a]Prevalence (Pr) reflects the number of positive samples by qPCR assay.
[b]$p<0.05$ indicated significant differences as compared with the control group.
[c]$p<0.05$, indicated significant differences between girls and boys as found in control and total groups.
qPCR, quantitative polymerase chain reaction.

SUPPLEMENTARY TABLE S3. VARIABLES ASSOCIATED WITH ANTIBIOTICS USED BASED ON THE MULTIPLE LOGISTIC REGRESSION (LOGISTIC REGRESSION ANALYSIS USING qPCR RESULTS, $N=61$ CHILDREN)

Microorganisms	*OR (95% CI)*	p
Lactobacillus spp.[a]	9.85 (1.62–59.88)	**0.013**
P. distasonis[a]	2.53 (1.09–5.88)	**0.031**
M. smithii[b]	0.45 (0.22–0.91)	**0.025**
Firmicutes[b]	0.09 (0.01–0.86)	**0.037**
C. perfringens[b]	0.12 (0.02–0.61)	0.011

Significance levels in bold ($p<0.05$).
[a]Associated with the antibiotic group.
[b]Associated with the control group.