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Molecular Studies of Fecal Anaerobic Commensal Bacteria in Acute Diarrhea in Children

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ABSTRACT

Background and Objective: The commensal bacterial flora of the colon may undergo changes during diarrhea, owing to colonization of the intestine by pathogens and to rapid intestinal transit. This study used molecular methods to determine changes in the composition of selected commensal anaerobic bacteria during and after acute diarrhea in children.

Materials and Methods: Fecal samples were obtained from 46 children with acute diarrhea in a rural community during an episode of acute diarrhea, immediately after recovery from diarrhea, and 3 months after recovery. DNA was extracted and quantitative polymerase chain reaction using SYBR green and genus- and species-specific primers targeting 16S rDNA were undertaken to quantitate the following groups of bacteria: *Bifidobacterium* spp., *Bifidobacterium longum* group, *Bacteroides-Prevotella* group, *Bacteroides fragilis, Lactobacillus acidophilus* group, *Faecalibacterium prauznitzii*, and *Eubacterium rectale*, relative to amplification of universal bacterial domain 16S rDNA.

Results: Bacteria belonging to the *Bacteroides-Prevotella-Porphyromonas* group, *E rectal*e, *L acidophilus*, and *F prauznitzii* groups were low during acute diarrhea compared with their levels after recovery from diarrhea. The pattern was similar in rotavirus diarrhea and nonrotavirus diarrhea. Administration of amylase-resistant maize starch as adjuvant therapy was associated with lower levels of *F prauznitzii* at the time of recovery but did not lead to other changes in the floral pattern.

Conclusions: Specific classes of fecal bacteria are lower during episodes of acute diarrhea in children than during periods of normal gastrointestinal health, suggesting specific alterations in the flora during diarrhea. ***JPGN 46:514–519, 2008.*** **Key Words:** Anaerobes—*Bacteroides*—Bifidobacteria—Children—Commensal bacteria—Diarrhea—Lactobacilli—Quantitative polymerase chain reaction. © 2008 by European Society for Pediatric Gastroenterology, Hepatology, and Nutrition and North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition

The human gastrointestinal tract plays host to a large number of bacteria, consisting of more than 400 species, their total number exceeding the number of cells in the human body. The large majority of these bacteria are anaerobic. These bacteria play an important role in human health by producing nutrients, preventing colonization of the gut by potential pathogens, and affecting immune responses (1,2). Alterations in the bacterial flora, such as during administration of antibiotics, may lead to diarrhea (3). Several bacterial species, derived from normal residents of the gut or from natural foods such as yogurt, are used as probiotics to hasten recovery from diarrhea. Bacteria of the *Lactobacillus* genus and *Bifidobacterium* genus, in particular, can prevent infective diarrhea in experimental animals and in children (4–6).

Acute diarrhea is an important cause of childhood mortality and morbidity in developing countries (7). During diarrhea the intestinal milieu is altered, and overgrowth of the intestine by pathogens is likely to change the normal bacterial flora in the gut. Acute diarrhea may sometimes eventuate in persistent diarrhea and malnutrition (8), usually attributed to changes in the epithelial cells lining the intestine (9); it is conceivable that persistence of diarrhea can actually be due to changes in the normal bacterial flora of the gut. Previous studies that examined the gastrointestinal flora in acute diarrhea using conventional culture techniques indicated that the fecal anaerobic flora are significantly reduced in number in acute diarrhea (10–12), leading to a relative predominance of aerobic bacteria. In one study, *Bacteroides*, *Bifidobacterium*, *Lactobacillus*, and

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Eubacterium were found to be lower during diarrhea. Anaerobic bacteria are difficult to cultivate, in particular bifidobacteria, for which several media are used for culture. Recent techniques to examine the anaerobic bacteria use molecular approaches that target 16S rDNA (13).

The management of acute gastroenteritis is based on adequate hydration using glucose-based oral rehydration solution (ORS) and early refeeding (14). In addition to these measures, administration of amylase-resistant starch to children with diarrhea hastened recovery (15). It has been suggested that this occurs through fermentation of the starch to short-chain fatty acids by colonic bacteria. Production of short-chain fatty acids, such as butyrate, from unabsorbed carbohydrate is one of the significant contributions of the colonic flora to human health. Inasmuch as amylase-resistant starch reaches the colon and is available as a substrate for bacterial nutrition and growth, it is possible that it may also have a prebiotic effect and selectively stimulate the growth of beneficial commensal bacteria.

The present study used real-time polymerase chain reaction (PCR) targeting the 16S rDNA to quantitatively compare selected fecal commensal bacteria in children with acute gastroenteritis: during, immediately after, and 4 months after an episode of acute diarrhea. Given that bifidobacteria and lactobacilli have probiotic effects and are known to affect the course of diarrhea (16), we specifically targeted these bacterial groups, whereas *Bacteroides* were included for comparison, being another major fecal anaerobe group that may sometimes also have a deleterious effect on intestinal health. We also examined the effect of short-term administration of amylase-resistant high-amylose maize starch (HAMS) during diarrhea on the selected fecal bacteria; to do this, the 2 major human fecal butyrate-producing bacteria, *Eubacterium rectale* and *Faecalibacterium prauznitzii* (17), were additionally targeted.

MATERIALS AND METHODS

Study Participants and Study Design

The study was undertaken in a block of 20 villages covered by the Community Health and Development Department of the Christian Medical College, Vellore. A trial of ORS acceptability is ongoing in these villages. In each village, primary health care is administered by a community health worker who resides in the village. Children with acute diarrhea receive their first assessment and care from this health worker, who dispenses ORS to children with no dehydration or mild dehydration, whereas the parents of a child with severe dehydration are advised to take the child to the nearest health center. In 10 of the villages, health workers were provided with regular ORS packets, whereas in the other 10 villages, health workers were provided ORS with adjuvant HAMS, which has a high content of amylase-resistant starch, in separate sachets to be added to the ORS at the time of reconstitution. Each village also had a trained volunteer who was responsible for detection and reporting of diarrhea cases in children to the base team. The health volunteers were informed about the study and were given stool sampling kits to be provided to parents of the children under survey. When a child had acute diarrhea and the parent was willing to comply with the study, the health volunteer immediately informed the base team via telephone, and provided instructions and a kit to the parent to collect a stool sample at the earliest. Simultaneously, a person was dispatched from the base hospital to the appropriate village to collect the stool sample, which was brought back immediately to the laboratory. Children 3 months to 5 years of age affected with acute diarrhea (defined as >3 episodes of watery stools in 24 hours) and with mild dehydration were recruited for this study. A second group of student volunteer health informants in each village identified the cases and reported them to the investigating team by telephone. The health informants were provided with stool collection kits that included a screw-capped plastic tube, tissue napkins, and a wooden spatula. Three samples of feces were collected from each affected child: the first sample during the diarrheal episode, the second being the first formed stool after cessation of diarrhea, and the third at 3 months after the index episode of diarrhea. All of the samples were collected by the team member within 2 hours of being passed and were transported immediately to the laboratory in a cold box and stored at $-80^{\circ}C$ to be processed in batches. A medical research officer from the investigating team visited the village and interviewed the parents of each child to obtain demographic information including age, sex, mode of delivery, and age at weaning. The study was approved by the Research Committee of the Christian Medical College, Vellore.

DNA Extraction and PCR

DNA was extracted from 200 to 250 mg (wet weight) of feces using the QIAamp DNA stool mini kit (QIAgen, Germany) (18), eluted in a final volume of 200 μL and stored at $-20^{\circ}C$.

Oligonucleotide primers were targeted at the 16srRNA gene (rDNA) sequences of the bacterial species or groups shown in Table 1. Primers were also used to amplify a conserved 16S rDNA sequence present in all bacteria (universal primer set, recognizing domain bacteria) (19), the amplification of which served as the denominator against which the amplifications of the other bacteria were compared. All of the primer sequences were derived from the previously published studies mentioned in Table 1 (20–22) with the exception of the primer sets for *E rectale* and *F prauznitzii*. The latter were designed using PRIMER3 software, using sequences retrieved from the ribosomal database project II (RDP-II), and screened using the BLAST program of the National Center for Biotechnology Information. The PCR primers against *Bacteroides-Prevotella-Porphyromonas* have been shown to amplify target bacteria from the genera mentioned, whereas the *Bifidobacterium* genus–specific primers amplified total *Bifidobacterium* species. The *Bifidobacterium longum* primers detected a small group of bifidobacteria, including *B infantis*, *B longum*, *B pseudolongum*, and *B suis*. Similarly, the *L acidophilus* primers amplified rDNA from a closely related group of lactobacilli, including *L acidophilus*, *L amylovorus, L amylolyticus, L crispatus, L gasseri, and L johnsonii. Eubacterium rectale* and *Faecalibacterium prauznitzii* primer sets were

TABLE 1. *Primers used in study*

Target organism	Primer	Sequence (5′-3′)	Product size, bp	Reference
Universal	Forward	TCCTACGGGAGGCAGCAGT	466	Nadkarni et al, 2002 (19)
	Reverse	GGACTACCAGGGTATCTAATCCTGTT		
Bacteroides-Prevotella-Porphyromonas	Forward	GGT GTC GGC TTA AGT GCC AT	140	Malinen et al, 2005 (21)
	Reverse	CGG ACG TAA GGG CCG TGC		
Bacteroides fragilis	Forward	GAA AGC ATT AAG TAT TCC ACC TG	176	Malinen et al, 2003 (20)
	Reverse	CGG TGA TTG GTC ACT GAC A		
Bifidobacterium genus	Forward	TCG CGT CCG GTG TGA AAG	243	Malinen et al, 2005 (21)
	Reverse	CCA CAT CCA GCA TCC AC		
Bifidobacterium longum group	Forward	CAG TTG ATC GCA TGG TCT T	106	Malinen et al, 2003 (20)
	Reverse	TAC CCG TCG AAG CCA C		
Lactobacillus acidophilus	Forward	AGA GGT AGT AAC TGG CCT TTA	391	Malinen et al, 2003 (20)
	Reverse	GCG GAA ACC TCC CAA CA		
Eubacterium rectale	Forward	AAG GGA AGC AAA GCT GTG AA	200	
	Reverse	TCG GTT AGG TCA CTG GCT TC		
Faecalibacterium prauznitzii	Forward	GGA GGA TTG ACC CCT TCA GT	203	
	Reverse	CTG GTC CCG AAG AAA CAC AT		

species specific. The primers were synthesized by Genosys, SiGMA (Bangalore, India).

A gradient PCR was performed initially to standardize the PCR conditions. PCR amplification was performed with initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 61°C for 30 seconds, and extension at 72°C for 30 seconds. PCR products were analyzed on agarose gel electrophoresis for the specific band of the amplified product. After standardization, these conditions were then used to perform quantitative PCR (qPCR) to quantify bacterial levels from fecal samples. Quantification of bacterial DNA was performed using the Chromo4 real time PCR system (Biorad, USA), using SYBR Green master mix (Eurogentec, Belgium). All of the PCR reactions were performed in duplicate in a volume of 20 μL, using 96-well full skirt clear PCR microplate and PCR strip caps (AXYGEN Scientific, USA). Melting curve analysis was performed from 40° to 95°C with a plate read step after every 1°C, and held at a particular temperature for 10 seconds to check the specificity of the product formed. The Opticon 3.1 software (Biorad) plots the rate of change of the relative fluorescence units (RFU) with reference to time (T) (-d (RFU)/dT) on the y axis versus the temperature on the x axis, with the curve peaking at the melting temperature (Tm), and melting curve analysis was always done to check the specificity of the amplification. Quantification was based on the fluorescence intensity obtained from the intercalated SYBR Green dye. The cycle number at which the signal was first detected—the threshold cycle (Ct)—correlated with the original concentration of DNA template. DNA copy was not expressed as absolute number but was expressed by the relative cycle threshold at which DNA for each target was detected relative to the cycle threshold at which universal bacterial DNA was detected after amplification. This relative quantification is done automatically by the Opticon 3.1 software and expressed as relative fold difference compared with the reference (universal) amplicon.

The specificity of the primers was checked by performing PCR using standard strains of representative bacteria from each bacterial group, and on the results of *in silico* PCR. Strains used to check specificity of the PCR included *Bifidobacterium adolescentis* CIP64.59, *Bifidobacterium angulatum* CIP104167, *Bifidobacterium bifidum* CIP56.7, *Bifidobacterium breve* CIP64.69, *Bifidobacterium infantis/longum* CIP64.67, *Bifidobacterium lactis* CIP105265, *Bifidobacterium longum* CIP64.62, *B. longum* CIP64.63, *Bacteroides uniformis* ATCC8492, *Clostridium perfringens* ATCC13124, *Lactobacillus acidophilus* ATCC4356, *Bacteroides fragilis* NCTC 9343, *B. sporogenes, Clostridium perfrin*gens NCTC 8346, *Enterococcus faecalis* ATCC 51299, *Eubacterium rectale* DUN-128, and *Faecalibacterium prauznitzii*. The *Bifidobacterium*-type strains were grown at 37°C under anaerobic conditions in M20 broth or *Bifidobacterium* broth (HiMedia). The other organisms were cultured on enriched blood agar. Bacterial colonies were removed, washed with phosphate-buffered saline, and resuspended in phosphate-buffered saline by adjusting against MacFarland tubes. Resuspended bacteria were serially diluted (log dilutions from 10^7 to 10^0) and added to control fecal samples. Fecal samples were also tested for rotavirus (the single most common cause of acute gastroenteritis in this age group) antigen using a commercially available enzyme-linked immunosorbent assay.

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism version 4.0 (*www.graphpad.com*). Relative fold differences of the selected bacteria were expressed as median values (interquartile range). Significance of differences between groups was tested using the Kruskal-Wallis analysis of variance with the Dunn multiple comparison test for post-hoc analysis of differences between individual groups. Two-tailed $P < 0.05$ were considered statistically significant.

RESULTS

A total of 46 children (30 male, 16 female) were enrolled into the study. They ranged in age from 3 months to 5 years, with a median age of 12 months. All but 3 of the children had been weaned and were a median of 6 months after weaning. Of these children, 13 had positive results for rotavirus by enzyme-linked immunosorbent assay. The median socioeconomic status (23) was IV (range III–V), indicating a relatively homogeneous low to

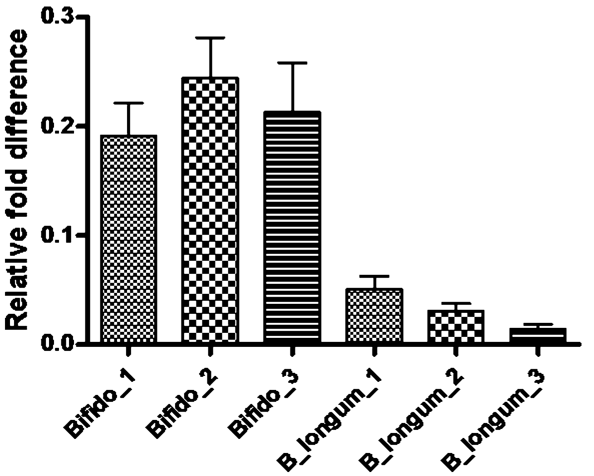

FIG. 1. Fecal *Bifidobacterium* group (Bifido) and *Bifidobacterium longum* (*B longum*) organisms during diarrhea (1), immediately after (2), and 3 months later (3). Bars represent mean (SEM). None of the differences was statistically significant.

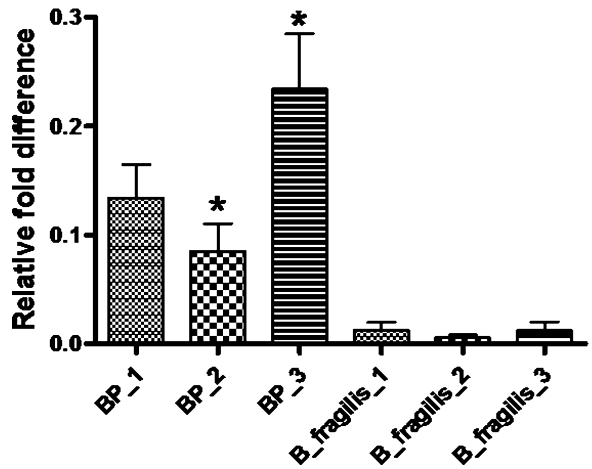

FIG. 2. Fecal *Bacteroides-Prevotella-Porphyromonas* group (BP) and *B. fragilis* (*B. fragilis*) organisms during diarrhea (1), immediately after (2), and 3 months later (3). Bars represent mean (SEM). $^{*}P < 0.01$ when comparing healthy period to period during and after diarrhea.

low-to-middle socioeconomic group. Of the total 46 children in the study, 23 received standard therapy with ORS and early refeeding, and the other 23 received ORS together with early refeeding and supplemental HAMS on the first day of diarrhea.

As shown in Figure 1, the relative levels of *Bifidobacterium* species and *B longum* group were not significantly different between children during diarrhea, immediately after diarrhea, or during a period without diarrhea. By contrast, relative levels of *Bacteroides-Prevotella-Porphyromonas* species were significantly lower during and immediately after acute diarrhea than during the period without diarrhea (Fig. 2). The relative levels of *Bacteroides fragilis* were not significantly different during diarrhea, immediately after diarrhea, or during a period of normal health without diarrhea.

Levels of *Eubacterium rectale* were significantly lower during and immediately after diarrhea than during a diarrhea-free period of normal health (Fig. 3). *Faecalibacterium prauznitzii* were also significantly less abundant during or immediately after diarrhea than during normal health. The *Lactobacillus acidophilus* group showed an interesting phenomenon, increasing immediately upon cessation of diarrhea with subsequent reduction in number during normal health. There was no major difference in bacterial predominance between the children positive for rotavirus and those who were negative for rotavirus (data not shown). Table 2 shows the characteristics of children receiving HAMS supplements on Day 1 compared with those receiving ORS alone. There was no major difference in fecal bacteria between the children receiving HAMS and those receiving only conventional therapy, with the exception of *Faecalibacterium prauznitzii*, which was significantly lower in the HAMS group at the time of recovery from diarrhea (Table 3).

DISCUSSION

The present study indicates that there are subtle changes in the composition of the fecal anaerobic bacterial flora during acute diarrhea in children. In particular, the numbers of *Bacteroides-Prevotella* group, the predominant fecal anaerobic bacteria, were lower during acute diarrhea. Lower levels of *Eubacterium rectale* and *Faecalibacterium prauznitzii* were also noted during diarrhea. By contrast, there were no significant differences between diarrheal periods and healthy periods in the relative levels of *Bifidobacterium* species. To date, quantitative studies on

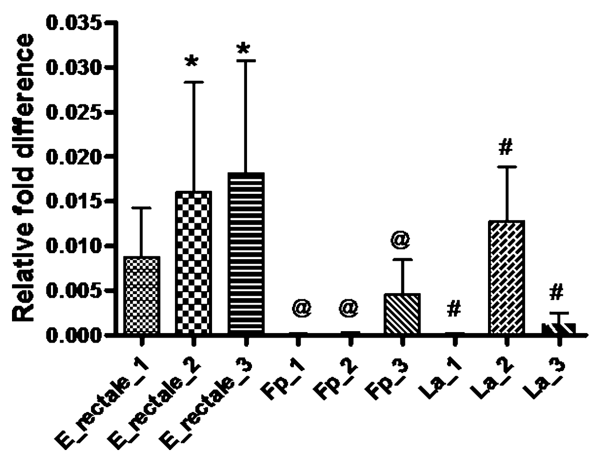

FIG. 3. Fecal *Eubacterium rectale* (*E rectale*), *Faecalibacterium prauznitzii* (Fp) and *Lactobacillus acidophilus* (La) organisms during diarrhea (1), immediately after (2), and 3 months later (3). Bars represent mean (SEM). $^{*}P < 0.01$ when comparing healthy period to period during and after diarrhea. $^{@}P < 0.01$ for diarrhea compared to nondiarrheal period. $^{\#}P < 0.01$ for diarrhea compared to nondiarrheal period.

TABLE 2. *Characteristics of children in study*

	HAMS	Control	P
n	23	23	
Age, mo	15.7 (2.6)	15.9 (2.3)	NS
Sex	12 M, 11 F	18 M, 5 F	NS
Diarrhea duration, days	4.7 (0.7)	5.3 (0.6)	NS
Antibiotic use	2	2	NS
Months since weaning	5.5 (0.4)	6.0 (0.6)	NS
Delivery by cesarean section	2	3	NS
Socioeconomic status	IV	IV	NS

Values mean (SEM). Socioeconomic status was determined by use of the modified Kuppuswamy scoring system (23).

the human gut microbiota in acute diarrhea have been done exclusively through culture-based methods. These have important limitations, primarily related to the fastidious nature of the gut microbiota. The advent of real-time PCR methods targeting 16S rDNA prompted this reexamination of specified fecal anaerobic commensal bacteria in acute diarrhea.

Limited information is available on the commensal bacterial flora of the gut in acute diarrhea. An early study from this institution (10) noted that there was a marked shift in fecal bacteria from predominance of anaerobes (which normally outnumber aerobes by a factor of ≥10) to a predominance of aerobes during acute diarrhea in children. This was attributed to an overgrowth of the gut by pathogens and to an alteration in the redox environment of the colon. A study from Kenya confirmed the marked reduction in anaerobes and found that there were also lower short-chain fatty acid concentrations in the feces during acute diarrhea (11). The same group reported that *Bacteroides*, *Bifidobacterium*, *Lactobacillus,* and *Eubacterium* were fewer during acute diarrhea than after recovery (12). In the present study, there was no apparent disturbance of *Bifidobacterium* species, in contrast to the culture-based study from Kenya. Bifidobacteria are difficult to grow, and *B adolescentis* in particular may require special media for its culture.

The significantly lower levels of the *Bacteroides-Prevotella-Porphyromonas* group and of *Eubacterium rectale* and of the *Faecalibacterium prauznitzii* species during acute diarrhea may be important in the pathophysiology of diarrhea. Each component of the anaerobic bacterial flora contributes to fermentation in the gut. Metabolic interactions between different groups of bacteria are important in the entire process of carbohydrate fermentation. Studies using gnotobiotic animals show that the capacity of *Bacteroides* to use carbohydrate is enhanced by cooperation with *Bifidobacterium* and *Lactobacillus* (24). *Bacteroides* populations have been shown to be susceptible to changes in pH (being reduced in numbers at lower colonic pH) and peptide supply (25).

Another observation in the present study was that *Lactobacillus* was increased in the feces when the children were recovering from diarrhea. Levels were low

TABLE 3. *Comparison of fecal bacteria in children receiving either HAMS ORS or standard therapy*

	During diarrhea			After diarrhea			Normal health		
	HAMS	Control	P	HAMS	Control	P	HAMS	Control	P
Bifidobacterium spp.	0.1251 (0.0306, 0.2191)	0.1443 (0.0201, 0.4113)	NS	0.2062 (0.1586, 0.3206)	0.2075 (0.0056, 0.6654)	NS	0.1873 (0.0668, 0.3360)	0.03285 (0.0118, 0.3076)	NS
Bifidobacterium longum	0.00235 (7.29E-05, 0.0703)	0.02683 (7.52E-05, 0.1115)	NS	0.006631 (0.00045, 0.0160)	0.0098 (0.00039, 0.1025)	NS	0.0016 (0.00047, 0.0262)	0.00159 (0.00038, 0.0077)	NS
Bacteroides-Prevotella	0.07862 (0.0104, 0.1521)	0.01429 (0.0024, 0.1606)	NS	0.0317 (0.0071, 0.0678)	0.01664 (0.0108, 0.0410)	NS	0.1257 (0.0400, 0.5248)	0.07257 (0.0212, 0.3408)	NS
Bacteroides fragilis	9.62E-05 (5.66E-06, 0.0110)	0.000214 (8.06E-06, 0.00027)	NS	9.12E-05 (3.83E-06, 0.0016)	0.000162 (6.20E-06, 0.0013)	NS	0.00031 (5.01E-05, 0.0026)	0.001134 (0.00014, 0.01543)	NS
Eubacterium rectale	5.32E-05 (1.67E-05, 0.0002)	1.70E-05 (9.55E-07, 0.00017)	NS	3.73E-06 (2.12E-07, 3.73E-05)	5.54E-06 (8.08E-08, 0.00076)	NS	0.00040 (2.63E-05, 0.0074)	0.00011 (4.68E-05, 0.0018)	NS
Faecalibacterium prauznitzii	4.521E-06 (1.34E-08, 2.40E-05)	1.27E-06 (2.46E-09, 1.29E-05)	NS	5.491E-07 (2.11E-07, 3.34E-05)	2.45E-05 (7.42E-07, 0.00013)	0.035	0.00018 (4.80E-06, 0.0020)	3.96E-05 (1.62E-05, 0.00011)	NS
Lactobacillus acidophilus	1.35E-06 (6.96E-09, 6.24E-06)	1.608E-07 (6.10E-011, 2.95E-06)	NS	4.753E-05 (3.39E-05, 0.00099)	0.00012 (1.22E-05, 0.00032)	NS	2.26E-06 (6.42E-08, 3.53E-05)	4.57E-06 (1.25E-08, 2.43E-05)	NS

Median (interquartile range) of relative difference values of the various bacteria are shown. In instances in which the decimal value is low, exponential values are shown as E-05, and so forth. This is indicative of low abundance bacterial groups or species.

during the phase of diarrhea, increased significantly just after recovery, but then again declined to extremely low levels. The reason for this phenomenon was not investigated in this study, but it could be related to dietary changes and perhaps an increase in milk or yogurt intake during diarrhea. The bacterial changes were similar in the subgroup of children positive for rotavirus, and it is likely that changes were secondary to alterations in colonic milieu rather to any specific effect of the pathogen.

There were no differences between children who received HAMS and those who did not, except for a reduction in *Faecalibacterium prauznitzii* in the former group. Again, there is no obvious explanation for this, but the fact that it occurred immediately after recovery suggests that it may be related to the adjuvant HAMS, which was the only ongoing intervention at that time that was different between the 2 groups. HAMS has been noted to shorten diarrhea, and its effect is attributed to colonic fermentation to short-chain fatty acids. The lack of effect of HAMS on fecal bacterial composition suggests that it did not have an obvious prebiotic effect. However, children in the present community-based study received small amounts of starch, compared with dehydrated infants in the previous hospital-based study (15), who received greater quantities of the starch. This may be one reason for the lack of a discernible prebiotic effect. This is corroborated by another study that failed to find any alteration in counts of *Eubacterium*, *Clostridium,* or *Ruminococcus* after the intake of resistant starch in healthy human volunteers (26). It is, of course, possible that HAMS does not have a prebiotic effect at all but may still serve as a substrate for short-chain fatty acid production.

In summary, alterations in the fecal anaerobic commensal bacterial populations are noted during acute diarrhea in children. Further studies are needed to examine the impact of these alterations on disease course and complications in acute diarrhea.

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