Research Article

Differences in the Biodiversity of the Fecal Microbiota of Infants With Rotaviral Diarrhea and Healthy Infants

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Abstract

Background: Rotaviral diarrhea (RD) has been associated with the biodiversity of the fecal microbiota in infants; however, the differences in the biodiversity of the fecal microbiota between infants with RD and healthy (H) infants have not been clearly elucidated.

Objectives: This study aimed to reveal the changes in the biodiversity of the fecal microbiota of infants with RD.

Patients and Methods: For this study, 30 fecal samples from 15 RD infants and 15 H infants were collected. The biodiversity of the fecal microbiota from the two groups was compared via polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and gene sequencing.

Results: The Shannon-Weaver index showed that the biodiversity of the fecal microbiota from the RD infants was significantly lower (P < 0.05) than that from the H infants. All fifteen RD infants were grouped into one cluster and were separated from the H infants by the un weighted-pair group method, with the arithmetic average (UPGMA) clustering algorithm. In addition, when compared with the healthy infants, the communities of the dominant microbes, *Lactobacillus* and *Bifidobacterium*, in the fecal microbiota from the RD infants have obviously changed.

Conclusions: With regard to improving the understanding of the differences in the biodiversity of the fecal microbiota between RD infants and H infants, the findings of this study can provide a possible basis to reveal the relationship between RD and intestinal microbiota.

Keywords: Rotaviral Diarrhea, Fecal, Microbiota, Infants, Biodiversity, Denaturing Gradient Gel Electrophoresis, DGGE

1. Background

Rotaviral diarrhea (RD) is the most common cause of gastroenteritis in children (1, 2). Almost all children younger than 5 years old will have suffered from RD, with > 500,000 deaths, > 2 million hospitalizations, and > 25 million clinic visits each year (3, 4). The rotavirus can infect the intestinal epithelium villi cells and lead to watery diarrhea (5); furthermore, RD can cause intestinal dysbacteriosis, and destroy the microbial barrier, making the diarrhea more serious (6, 7).

Previous reports have shown that some diseases, especially intestinal diseases, are associated with the intestinal microbiota, and healthy fecal microbiota are increasingly becoming essential to the maintenance of human health (8, 9). The biodiversity of the fecal microbiota in patients with several diseases, including cow's milk protein allergy, celiac disease, inflammatory bowel disease, and ulcerative colitis, have been revealed (10-13). In addition, Ma et al. discovered that there was an imbalance in the fecal microbiota in patients aged 2 to 4 years old with viral diarrhea (adenovirus, norovirus, rotavirus, and astrovirus) (14). Although infants are the most susceptible group, the researchers have overlooked studies on the changes of the biodiversity of the fecal microbiota in infants with RD.

2. Objectives

Thirty subjects were included in this study, fifteen of which were untreated RD infants (mean age, 90 days old; range, 90 - 300 days old) from the children's hospital of Harbin city. The RD infants were diagnosed by the children's hospital of Harbin city, and the rotavirus (RV) antigens from the fecal samples were detected via specific enzyme immunoassay methods using the RIDASCREEN kit (R-Biopharm, Germany), and confirmed by specific PCR reactions (15). Fifteen of the subjects were healthy (H) infants (mean age, 200 days old; range, 90 - 300 days old) chosen from volunteers in Harbin city. All of the infant subjects were born in natural childbirth, on a breastfeeding diet, and their parents were healthy, without history of illness. In addition, none of the individuals ingested

probiotics (including yogurt) or antibiotics within 4 weeks before the sampling.

3. Patients and Methods

3.1. Fecal Sampling

Thirty fecal samples were collected from the RD infants and H infants during November of 2011 in Harbin. All of the fecal samples were transferred into sterile cryotubes and stored at -80°C until further analysis.

3.2. Extraction of Total DNA and PCR Amplification

Approximately 200 mg (wet weight) of the thawed fecal samples were weighed in order to extract the total DNA (13), using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. The DNA was amplified with the V3 universal primers BA-GC-338f and UN518r for the bacteria, primers Lac1 and Lac2-GC for the *Lactobacillus*, and primers Bif164-GC-f and Bif662-r for the *Bifidobacterium*. All of the PCR amplification protocols, as described in previous reports, are shown in Table 1 (16-18).

3.3. Analysis of Fecal Microbiota by Denaturing Gradient Gel Electrophoresis

The denaturing gradient gel electrophoresis (DGGE) scheme was performed by using a DCode apparatus (Bio-Rad, Richmond, CA, USA) at 60°C and employing 8% polyacrylamide gel with a denaturing range of 30% – 55% for the total bacteria, 30% – 50% for the *Lactobacillus*, and 45% – 55% for the *Bifidobacterium*. Gel electrophoresis of the total bacteria, *Lactobacillus,* and *Bifidobacterium* was run at 20V for 10 minutes, and again at 70V for 18 hours, 70V for 16 hour, and 85V for 16 hour, respectively (16-18). The gels were visualized under UV light after staining them with gene finder (0.5 μg mL^{-1}) and taking photographs.

3.4. Gene Sequencing for the Bands

The bands in the gels were excised and soaked in 50 μL of TE buffer at 4°C overnight to obtain a solution containing the DNA. A PCR program was executed with the same primers without the GC-clamp, and sequenced at the Beijing Genomics Institute (BGI, Beijing, China). The sequences were identified by the BLASTN algorithm in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/).

3.5. Statistical and Clustering Analysis

The similarities between the two groups were analyzed using the Dice coefficient and the un weighted-pair group method, with the arithmetic average (UPGMA) clustering algorithm using Quantity One software (Bio-Rad) (19). The biodiversity of the bacteria was calculated by the number of bands and by the Shannon–Weaver index of biodiversity (H′), according to previous reports (12, 21). All of the data analyses were performed using the SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA), and statistical significance was established at P < 0.05.

4. Results

The DGGE images of the 30 fecal samples (fifteen RD infants and fifteen H infants) were obtained by applying PCR-DGGE technology (Figure 1). The 30 fecal samples were divided into two clusters using the UPGMA (Quantity One software); all 15 fecal communities of the RD individuals were placed into cluster I, and separated from all 15 fecal communities of the H infants (cluster II) (Figure 2).

The number of DGGE bands, Shannon-Weaver, and Dice similarity coefficients (both intragroup and intergroup) were calculated, and the details of the results are given in Table 2. The results showed that the biodiversity of the fecal microbiota from the RD infants (Mean ± SD = 6.6 ± 1.92, Shannon-Weaver = 2.03 ± 0.12) was significantly lower (P < 0.05) than that from the H individuals (Mean ± SD = 8.8 ± 0.94, Shannon-Weaver = 2.24 ± 0.34). The index of similarity coefficient based on the DGGE profiles ranged from 30.9% to 94.6% (average 68.54 ± 13.33%) in the RD infants, from 46.7% to 78.9% (average 65.47 ± 8.34%) in the H infants, and averaged 33.16 ± 8.23% between the two groups. The UPGMA analysis (Figure 2) also revealed that the intragroup similarities within the RD and H groups were significantly higher (P < 0.05) than those calculated between the two groups.

Table 1. Primers Based on the 16S rDNA Sequences[a,b,c,d]

| Target Bacteria	Prime	Sequence (5′ to 3′)
Total Bacteria	BA-GC-338f ,UN518r	ACTCCTACGGGAGGCAGCAGATT ACC GCG GCTGCT GG
Lactobacillus	Lac1, Lac2-GC	AGCAGTAGGGAATCTTCCAATTTCACCGCTACACATG
Bifidobacterium	Bif164-GC-f, Bif662-r	GGGTGGTAATGCCGGATGCCACCGTTACACCGGGAA
GC[d]		CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG

[a]The reaction of total bacteria was performed using the following conditions: 92°C for 2 minutes and 30 cycles of 92°C for 1 minute, 55°C for 30 seconds and 72°C for 1 minute, and a final extension at 6°C for 72 minutes.
[b]The reaction was performed using the following conditions: 94°C for 2 minutes and 35 cycles of 94°C for 30 seconds, 61°C for 1 minute and 68°C for 1 minute. The reaction was terminated with an extension step of 7 minutes at 68°C.
[c]The reaction was performed using the following conditions: 95°C for 5 minutes and 35 cycles of 95°C for 1 minute, 62 °C for 20 seconds and 68 °C for 40seconds, final extension at 6°C for 7 minutes.
[d]All GC primers contained a 40 bp GC-clamp sequence at their 5′ end to prevent the complete denaturation of the amplicons.

Figure 1. DGGE Fingerprints of Fecal Microbiota from RD Infants and H Infants

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
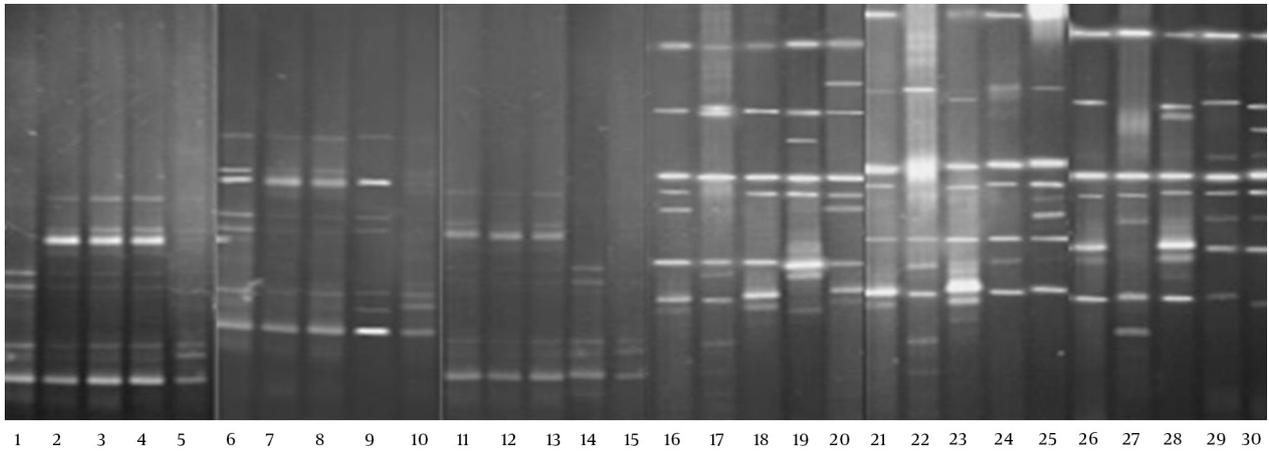
Lanes 1 – 15, RV infants; lanes 15 – 30, H infants.

Figure 2. Clustering of DGGE Profiles of Fecal Microbiota from RD Infants and H Infants Using Dice's Coefficient and the UPGMA
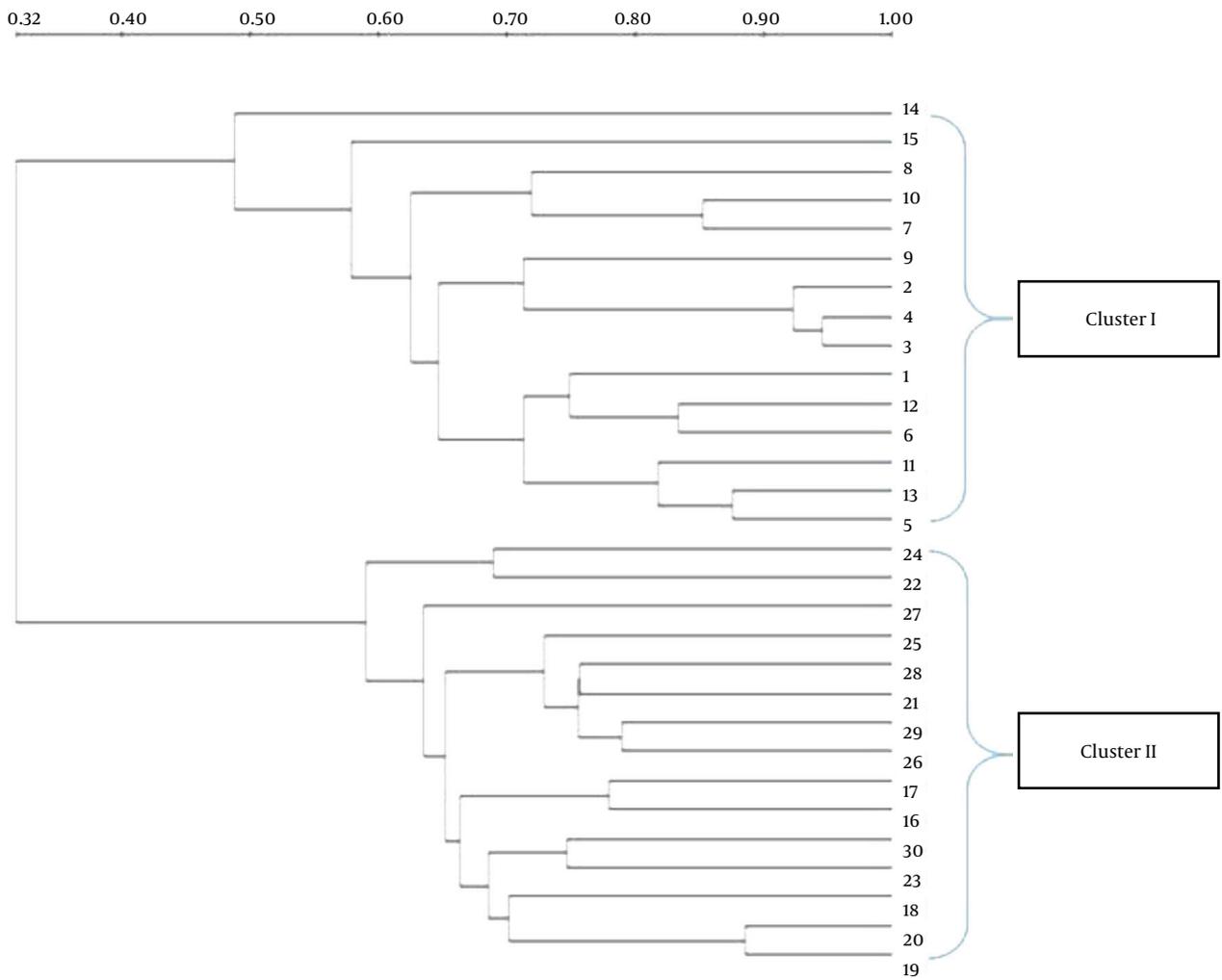
Lanes 1 – 15: RV infants; lanes 15 – 30: H infants.

Table 2. Microbiota Diversity and Similarity of the RD and H Groups[a,b,c]

| Group	Microbiota Diversity (Mean ± SD)		Microbiota Similarity (Mean H′ or H max′ ± SD)	
	DGGE bands	Shannon-Weaver	Intragroup	Intergroup
RV	6.6 ± 1.92	2.03 ± 0.12	68.54 ± 13.33	33.16 ± 8.23
H	8.8 ± 0.94	2.24 ± 0.34	65.47 ± 8.34	NA
p	0.00022	0.0038	NA	NA

Abbreviation: NA, not available.
[a]Number of DGGE bands produced by each sample.
[b]Dice similarity coefficients comparing DGGE band profiles within individuals of each group.
[c]Dice similarity coefficients comparing DGGE band profiles between each RD infant and H infant.

Figure 3. DGGE Profiles of the 16S rRNA Gene Fragments of the *Lactobacillus* spp
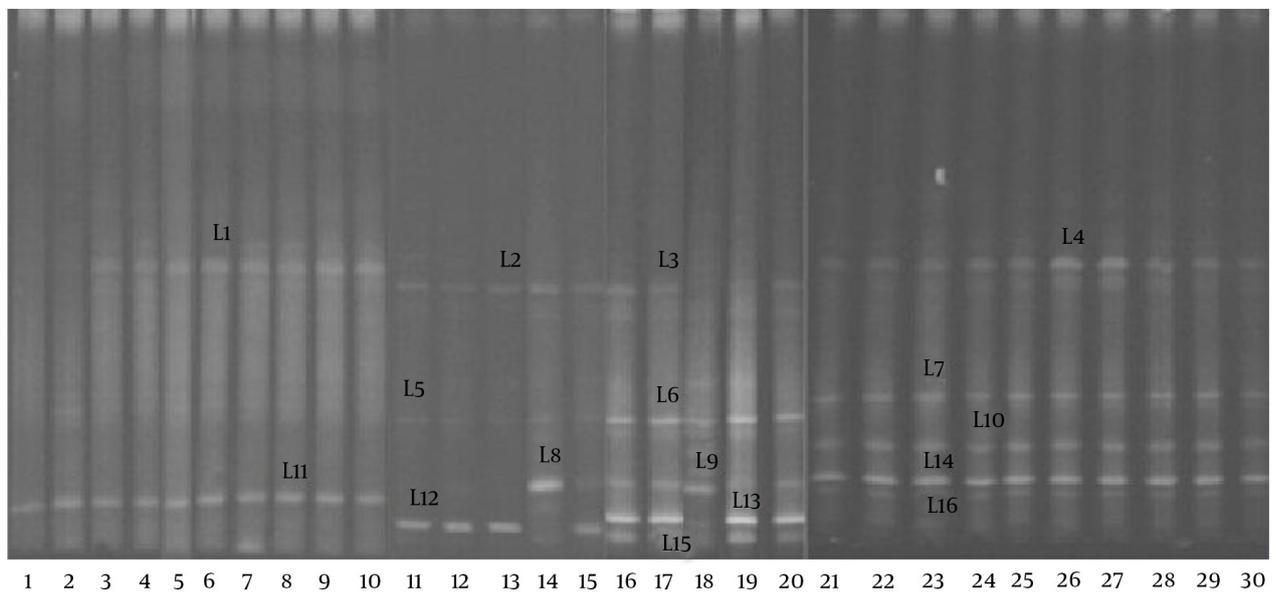
Lanes 1 – 15, RD infants; lanes 15 – 30: H infants.

Gene sequencing technology was used to define the microbiota based on the sequence similarity to their closest neighbor in the NCBI. The results of the sequence alignment are displayed in Appendix 1, and showed that *Escherichia coli*, *Bacteroides vulgatus*, *Enterococcus faecium*, *Clostridium*, *E. fergusonii*, *C. sardiniense*, and *Peptostreptococcus anaerobius* were the dominant fecal microbiota of the RD infants. Moreover, *Bacteroides, Bifidobacterium, Lactobacillus, Proteobacteria bacterium, Clostridium,* uncultured bacterium, and *E. coli* were the dominant fecal microbiota of the H infants.

The DGGE profiles (Figure 3) showed that the biodiversity of the *Lactobacillus* group in the fecal microbiota of the RD infants (mean 2.1 bands) was significantly lower

(P < 0.05) than that of the H group (mean 4.6 bands). The gene sequencing results of the bands in the DGGE profiles (Table 3) indicated that a significant reduction in the *L. helveticus*, *L. acidophilus*, and *L. fermentum* was found in the fecal microbiota of the RD infants.

The DGGE profiles (Figure 4) showed that the fecal microbiota of the RD infants (average 2.1 bands) had a significantly lower level of biodiversity (P < 0.05) than the fecal microbiota of the H infants (average 5.3 bands). The bands were identified with gene sequencing, the details of which are shown in Table 3. The results indicated that the *B. infantis*, *B. longum*, *B. bifidum*, and *B. adolescentis* in the fecal microbiota of the RD infants were significantly decreased (P < 0.05).

Table 3. The Frequency of the *Lactobacillus* Species, Identified by Sequencing the DGGE bands Amplified from the Fecal DNA of the RD and H Groups, Using the *Lactobacillus* and *Bifidobacterium* Group Specific Primers[a,b,c]

Amplicon ID	Closest relative (Accession number)	Size, bp	H = 15, No. (%)	RD = 15, No. (%)
L1, L2, L3, L4	*L. brevis (jx003598)*	1360	13 (87)	13 (87)
L5, L6, L7	*L. helveticus (fj749441)*	1462	15 (100)	3 (20)
L8, L9, L10	*L. acidophilus (ay763430)*	1475	15 (100)	2 (13)
L11, L12, L13, L4	*L. crispatus (jq805668)*	1503	14 (93)	14 (93)
L15, L16	*L. fermentum (gq455406)*	1523	12 (80)	0 (0)
B1, B2, B3, B4, B5	*B. breve (gu942826)*	294	12 (80)	12 (80)
B6, B7, B8, B9, B10	*B. catenulatum (ab846175)*	591	15 (100)	15 (100)
B11, B12, B13	*B. infantis (bif16srrge)*	642	14 (93)	0 (0)
B15, B17, B18	*B. longum (hq591348)*	1369	11 (73)	0 (0)
B16, B21	*B. breve (ay172656)*	1401	6 (40)	0 (0)
B14, B19, B20, B22	*B. adolescentis (hq259739)*	1460	12 (80)	5 (33)
B23, B24, B25	*B. bifidum (aj311604)*	1469	14 (93)	0 (0)

[a]The marks corresponding to the DGGE bands shown in Figures 3 and 4.
[b]The sequence identity was ≥ 99%.
[c]Significant differences between RD infants and H infants: $P < 0.05$.

Figure 4. The DGGE Profiles of the 16S rRNA Gene Fragments of the *Bifidobacterium* spp
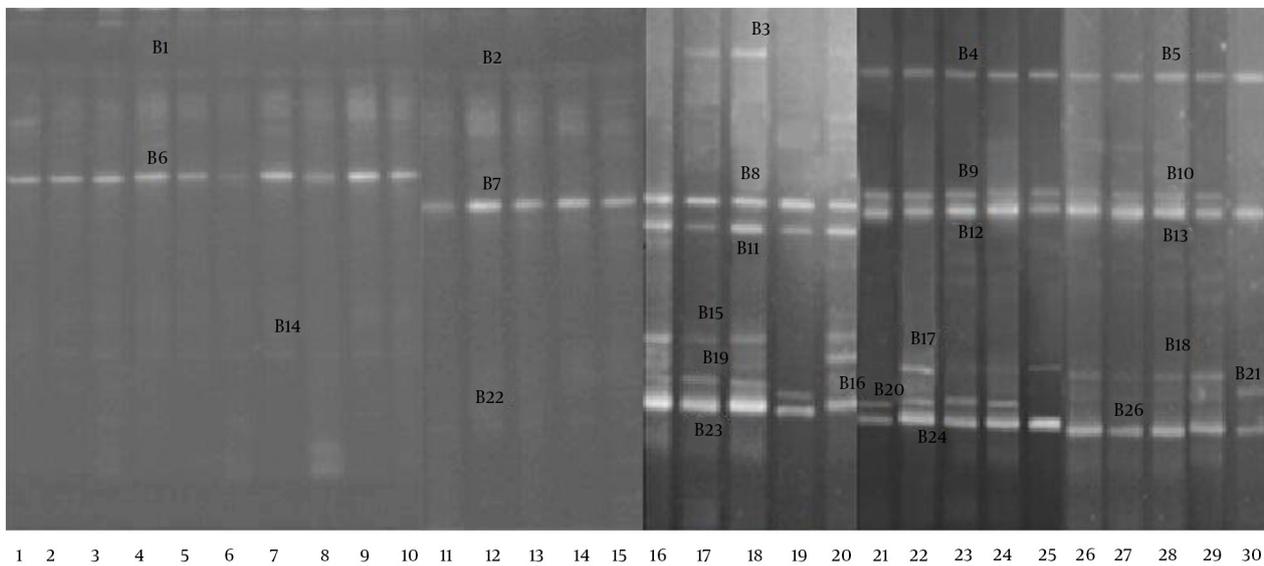
Lanes 1 – 15, RD infants; lanes 15 – 30, H infants.

5. Discussion

The complex intestinal microbial flora harbored by individuals has long been proposed to contribute to intestinal health and disease, and the intestinal microbiota is increasingly considered to be a symbiotic partner in the maintenance of health (9). Studies have shown that many diseases are associated with the intestinal microbiota, and healthy fecal microbiota have become increasingly necessary for the maintenance of human health (8, 21). As a common gastrointestinal disease via viral infection, RD has been considered to be closely related to prominent changes in the gastrointestinal microbiota (22).

Many factors can influence the biodiversity of the fecal microbiota, including genetics, age, gender, feeding, and region (8, 23, 24). Therefore, in order to reduce the differences caused by these factors, the infants chosen for our study were within the same parameters of age range, sex ratio, area, delivery, and feeding. In addition, the infants in this study were between 90 and 300 days old, and during this period the intestinal microbiota are colonizing and forming, and more easily influenced by certain stimulations. The intestinal microbiota of infants is not less complicated when compared with the intestinal microbiota of adults, which provided better feasibility to analyze the differences in the biodiversity of the fecal microbiota between the RD infants and H infants using PCR-DGGE. Our results indicated that the fecal microbial communities of the RD infants were clustered together with higher similarity in the coefficients of the UPGMA; these fecal microbial communities were significantly different from those present in the H infants.

Most bowel diseases can lead to a reduction in the biodiversity of the fecal microbiota (10, 12, 13). In our study, the biodiversity of the fecal microbiota of the RD infants was significantly decreased, and the composition had also changed. The Bacteroides species are the main group in human colonic microbiota, and have a beneficial effect on the prevention of intestinal colonization (25). *bacteroides* was discovered in the dominant fecal microbiota of the H infants, while *B. vulgatus* appeared as the dominant fecal microbiota of the RD infants. Therefore, we inferred that the composition of the *Bacteroides* changed due to the rotavirus infection.

Lactobacillus and *Bifidobacterium* play important roles in the intestinal microbiota, and have many benefits to human health (26, 27). *Lactobacillus* can synthesize peptidoglycan, with functional anti-inflammatory activity (28), and *Bifidobacterium* has a key effect on carbohydrate metabolism in the large bowel, suppressing enteritis in animal models (29). Some researchers have used *Lactobacillus* and *Bifidobacterium* for the prevention and treatment of certain intestinal diseases (7, 30, 31). However, *Lactobacillus* and *Bifidobacterium* vary in genus, and a further selection of these species was necessary. Therefore,

the differences in these two groups of fecal microbiota between the RD infants and H infants were discussed. These results suggested that *L. helveticus, L. acidophilus, L. fermentum. B. infantis, B. longum, B. bifidum, and B. adolescentis* were considered to be more conducive to mediating the imbalance of the intestinal microbiota of the RD infants.

Enterococcus faecium, as a pathogenic bacteria, can cause serious nosocomial infections, and is found to be associated with intestinal diseases (13, 32). In addition, *E. coli* and *Clostridium* have been proven to play important roles in diarrhea and inflammatory processes (33, 34). When compared with the H infants, *E. faecium*, *E. coli*, and *Clostridium* in the fecal microbiota of the RD infants showed obvious advantages.

PCR-DGGE is one of the main ways to assess the biodiversity of the fecal microbiota, in which, the uncultured microorganisms can be identified by the traditional culture-based method (35). In our study, we first analyzed the biodiversity of the dominant fecal microbiota in infants, and found that the harmful microbes were increased in the fecal microbiota of the RD infants. Contrarily, the beneficial microbes were reduced, especially the *Lactobacillus* and *Bifidobacterium*. In order to improve the understanding of the differences in the members of these two groups, the biodiversity of the *Lactobacillus* and *Bifidobacterium* were revealed via PCR-DGGE using specific primers. However, some researchers have suggested that the DGGE should not be used for quantitative biodiversity analysis (36).

As a supplement, real-time PCR will be used to measure the contents of the microbiota accurately for further research, and linked with metabonomics to explore the connections between the intestinal flora and metabolomics. Moreover, using the PCR-DGGE techniques, we only analyzed the dominant microbiota, and the total biodiversity information of the fecal microbiota cannot be measured. Therefore, we speculate that there should be more differences in the biodiversity of the fecal microbiota between the RD infants and H infants, and our results have provided an important idea and reference for further studies. The next research focus of our team will be to compare the differences in the biodiversity of the fecal microbiota between the two groups using an Illumina MiSeq platform to obtain comprehensive information.

In conclusion, in this study, we showed a comprehensive view of the fecal microbiota in RD infants by using PCR-DGGE and gene sequencing, which revealed the differences in the biodiversity of the dominant microbiota, *Lactobacillus* and *Bifidobacterium* in the fecal microbiota of RD infants and H infants. These results suggested that the intestinal microbiota of RD infants exhibited important changes, providing significant information about the relationship between RD and intestinal microbiota.

Appendix 1. The Sequencing Results of Every Band in the DGGE Profiles[a]

Band Number	Species	Closest relative ID in NCBI	Size, bp	Identities, %
1-1,2-4,3-4,4-4,6-4,7-4,8-4, 9-3,11-4,12-4,13-4,14-1	*Clostridium* spp.	JN605802	664	100
1-2,2-5,3-5,4-5,6-5,7-5,8-5, 9-4,11-5,12-5,13-5,14-2	*C. sardiniense*	FJ546743	681	99
1-3,2-6,3-6,4-6,5-3,6-5,7-6,8-6,9-5,10-1,11-6,12-6,13-614-3,15-1	*Escherichia fergusonii*	HE612113	1211	100
1-4,2-7,3-7,4-7,5-4,6-7,7-7,8-7,9-6,10-2,11-7,12-7,13-7, 14-4,15-2	*Enterococcus faecium*	JQ778275	1473	99
1-5,2-8,3-8,4-8,5-5,6-8,7-8,8-8,9-7,10-311-8,12-8,13-8,14-5,15-3,17-6,22-5,27-7	*E. coli*	HQ622348	1511	100
2-1,3-1,4-1,6-1,7-1,8-1,9-1, 11-1,12-1,13-1	*P. anaerobius*	GQ496449	300	100
2-2,2-3,3-2,3-3,4-2,4-3,5-1, 5-2,6-2,6-3,7-2,7-3,8-2,8-3,9-2,11-211-3,12-2,12-3,13-2,13-3	*Bacteroides vulgatus*	JF298877	540	99
16-1,17-1,18-1,19-1,20-1,21-1,23-1,24-1,25-1,26-1,27-1,28-1,29-1,30-1	*Clostridium* spp.	AM774623	354	99
16-2,17-2,18-2,19-2,20-3,21-1,22-2,23-2, 24-2,25-2,26-2,28-2,28-3,29-2,30-2	Uncultured bacterium	JF256482	644	100
16-3,17-3,18-3,19-4,20-4,21-3,22-2,23-3,24-3,25-3,26-3,27-2,28-4,29-4,30-5	*Bacteroides* spp.	AM117579	821	100
16-4,18-4,19-5,20-5,21-4,23-4,24-4,25-4,26-4,27-3,28-5,29-5,30-6	*Bifidobacterium* spp.	AM117580	845	99
16-6,17-4,18-5,19-6,20-7,21-5,22-3,23-5,24-5,25-6,26-5,28-6,29-7,30-8	*Lactobacillus* spp.	JH164960	936	99
16-7,17-6,18-6,20-8,21-5,22-5,23-7,24-7, 25-7.26-6,27-5,28-8,29-8,30-8	*B. infantis*	HQ591348	1369	99
16-5,20-6,25-5,27-4,29-6,30-7	*P. bacterium*	AXYJ01000008	1222	100

[a] i - i´ stands for the i´ th band of the i th lane in Figure 1.

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