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DISCOVERY OF *Brevibacterium* PREDOMINATING IN FECAL SAMPLES FROM THREE CHILDREN WITH PERSISTENT DIARRHEA NEGATIVE FOR COMMON PATHOGENS

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ABSTRACT

Acute diarrhea is a common disease in children under 5 years old and can develop into persistent diarrhea, greatly affecting the children's health. Although advanced techniques had been used to diagnose and detect common pathogens in hospitals, however, 40% of cases are negative for the pathogens. In this study, to investigate dominant bacteria in stool samples of three persistent-diarrheal children with unidentified pathogenic agents, the V3 and V68 regions of the 16S rRNA gene were amplified from fecal bacterial metagenomic DNA, separated on DGGE gel, and the dominant DNA bands were sequenced. As a result, the V3 and V68 regions of bacteria in persistent diarrheal children were less diverse and different from the corresponding DNA bands of the indicator strains. Sequence analysis and similarity comparison of six DNA bands of V3 regions and seven DNA bands of V68 regions showed that two V3 sequences (of 160 bp) derived from two samples were novel and did not match any genes from the non-redundant database, but they shared 93.75% similarity to each other. The four V3 sequences left derived from all three samples were the most similar (94.53-100%) with the corresponding genes of *Brevibacterium*. Six of the seven V68 sequences derived from dominant DNA bands of all three samples were the most similar (from 99.4% to 100%) to the corresponding genes of referent strains belonging to the genus *Enterococcus.* In sample D3, a sequence of the V68 region possessed 100% identity to the *E. faecalis* ATCC 19433 strain. This is the first study report that *Brevibacterium* was

the dominant bacteria in the gastrointestinal microflora of children with persistent diarrhea although the bacterial genus has been reported to cause dangerous diseases in humans with immunodeficiency.

Keywords:*16S rRNA, DGGE, V3, V68, persistent diarrhea.*

INTRODUCTION

In Vietnam, diarrhea is one of the 10 most common diseases and ranks 4th in mortality (Nguyen *et al.*, 2017). In the most recent study at the National Children's Hospital, diarrhea mainly affects children under 2 years old (accounting for 77.5%) (Pham *et al.*, 2024). The majority of diarrheal illnesses are acute in duration (<14 days); however, a subset of illnesses - known as persistent diarrhea - begin acutely and then persist 14 days or longer, thereby resulting in a disproportionate share of diarrhea-related morbidity and mortality.Acute diarrhea can progress to persistent diarrhea with a rate of 2.8-5.3% cases, depending on the locality, causing a great impact on the child's health. Although National Children's Hospital has applied real-time PCR that is capable of detecting 24 common diarrheal pathogens, the proportion of children with persistent diarrhea negative for pathogens is still high (Vu *et al.*, 2016). In this case, bacterial dysbiosis, typically dominant bacteria, in the gastrointestinal tract is believed to be related to persistent diarrhea (Kieser *et al.*, 2018).

To investigate intestinal microbial imbalance and find dominant bacteria in stool samples of children with diarrhea, molecular biology methods are considered the most appropriate to be able to survey all bacterial communities, including both the culturable and unculturable bacteria. Denaturing gradient gel electrophoresis (DGGE) is one of the common molecular fingerprinting techniques that can helpto quickly analyze changes in microflora under the influence of external factors or diseases (Westaway *et al.*, 2021) and has been applied to detect bacteria causing diarrhea in the previous study based on hypervariable regions of the 16S rRNA gene(Mai *et al.*, 2006, Nguyen *et al.*, 2024). The 16S rRNA gene (over 1.5 kb) is divided into nine hypervariable regions labeled V1 to V9, interspersed with eight conservative regions. Region V3 is the most suitable region for analysis of microbial diversity using the DGGE method (Yu, Morrison, 2004), but the region V6-V8 (designated as V68 in this study) is the gene segment capable of detecting bacteria related to diarrhea such as *Clostridium* and *Staphylococcus*(Yang *et al.*, 2016). Both regions have been used to survey microbial diversity related to allergies, eczema, irritable bowel syndrome, and diarrhea (Mirsepasi-Lauridsen *et al.*, 2018). Therefore, in this study, we used the DGGE method to analyze the V3 and V68 regions of bacteria in stool samples of three children with persistent diarrhea negative to 24 common pathogens to investigate dominant bacteria that may be related to diarrhea or an imbalance of gastrointestinal bacteria.

MATERIALS AND METHODS

Materials

Bacteria extracted from stool samples of three children with prolonged diarrhea (D1-

D3) and a healthy child (C1)were kept at -80^{o}C at the Genetic Engineering Laboratory, Institute of Biotechnology.

The DNAs of V3, V68 regions derived from strains including *E. coli* HM 3175, *E. coli* ATCC27117, *B. aureus, B. subtilis* 168, *Staphylococcus aureus*, *Salmonella enterica* Typhymurium (*S.* Typhimurium), *S.* Enteritidis ATCC 13076, *Listeria fermentum*, *Enterococcus faecium*had been amplified (Nguyen *et al.*, 2023)and kept at -20^{o}C to be used as indicators and positive control in DGGE analysis.

Extraction of bacterial metagenomic DNAs and amplification of 16S rRNA metagenes, V3, V68 regions

The bacterial samples from -80^{o}C were thawed on ice. Then, metagenomic DNAs of the bacteria were extracted using a biochemical method and the GeneJET genomic DNA purification kit (Thermo Scientific, USA) to obtain diverse metagenomic DNAs (Nguyen *et al.*, 2024, Nguyen *et al.*, 2023). Then, the same amount of metagenomic DNAs extracted by two methods was mixed to serve as a template for PCR.

The metagene 16S rRNAs of bacteria were amplified from the bacterial metagenomic DNAs by PCR using primers 27-F, 1527-R (Table 1). The amplified genes were used as templates for amplification of the V3, V68 regions using primers BA338F-GC, UN518R (Ariefdjohan *et al.*, 2010, Fei *et al.*, 2016), and primers U968F-GC, L1401R (Mai *et al.*, 2006) (Table 1), respectively, whereas GC-clamp in the primers BA338F-GC, U968F-GC was 5'- CGC CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG -3'. PCR was conducted in a reaction of 25 µl consisting of 2.5 µl PCR buffer 10x, 2.0 µl dNTP (2 mM), 1.0 µl of each primer (10 µM), 5.0 µl DNA template (2 ng/µl), 0.25 µl DreamTaq DNA polymerase (5 U/µl, Fermentas). Tough-up PCR program described by Nguyen et al.(Nguyen *et al.*, 2024) was used for the PCR.The PCR products were checked by electrophoresis on 0.8% agarose gel.

Table 1. List of primers for amplification of 16S rRNA gene and V3, V68 regions

Genes	PCR size	Primers	Primer sequences
16S rRNA	1500 bp	27F	5′-GAG TTT GAT CCT GGC TCAG-3′
		1527R	5′-AGA AAG GAG GTG ATC CAGCC-3′
V3 region	180 bp	BA338F-GC	5'-GC-clamp GAC TCC TAC GGG AGG CAG CAG-3'
		UN518R	5'-ATT ACC GCG GCT GCT GG-3‘
V68 region	433 bp	U968F-GC	5′-GC-clamp GAA CGC GAA GAA CCT TAC-3’
		L1401R	5′-GCG TGT GTA CAA GAC CC-3’

Analysis of V3, V68 regions by DGGE

The amplified V3 and V68 regions were analyzed by DGGE as described by Hovda et al.(Hovda *et al.*, 2007) on the VS20-DGGETC system (Cleaver Scientific Ltd, England). The DGGE gel contained 8% polyacrylamide and gradient concentrations

of denaturant urea and deionized formamide, in which the denaturant gradient from 35 to 70% was applied for analysis of the V3 region, the denaturant gradient of 40-60% was adopted for analysis of V68 region. Electrophoresis was conducted in TAE buffer (Eppendorf AG, Hamburg, Germany) at 60^{o}C, 20V for 10 minutes then 70V for 18 hours. After electrophoresis, the gel was stained with SYBR-Gold (S11494, Thermo Scientific, USA) diluted 10000 times in TAE buffer, pH 7.6 for 10 minutes, then observed under ultraviolet light. The dominant DNA bands observed on the gel were cut to harvest DNAs for sequencing.

Sequencing and analysis of V3, V68sequences

The dominant DNA bands of each sample were excised from the gel and transferred to tubes containing 30 µl of deionized water, kept at 4^{o}C for one hour then at -80^{o}C overnight. The DNA from the samples was extracted by low-speed centrifugation at 500 rpm for 3 minutes. The DNA was diluted 10^{-4}, 10^{-5} times before being used as a template for PCR to re-amplify the corresponding V3 and V68 regions by primers without GC-clamp. The PCR products were purified by the MEGAquick-spin plus total fragment DNA purification kit (Intron, Korea) and sequenced on an ABI 3100 system using primers UN518R and L1401R forthe Sanger method. Sequencing chromatograms were observed using Chromas software. The sequences were aligned against first non-redundant sequences, then reference RNA sequences (refseq_rna) in the GenBank using the BLASTN tool. The sequences were compared by ClustalW.

RESULTS

Extraction of metagenomic DNA of bacteria in the fecal samples and amplification of 16S rRNA metagene, V3, V68 regions

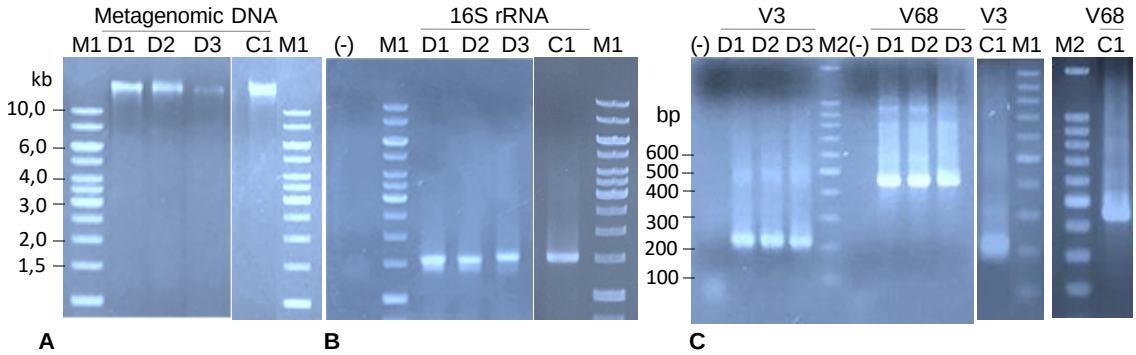

Figure 1. Analysis of bacterial metagenomic DNA and PCR products of 16S rRNA metagene, V3, V68 regions by electrophoresis in the agarose gel 0.8%.

A: Analysis of metagenomic DNA extracted from bacteria in fecal samples from three persistent diarrheal children and a healthy child; B: PCR products amplified 16S rRNA metagene from corresponding metagenomic DNA; C: PCR products amplified V3, V68 regions from 16S rRNA metagene. (-): Negative control of PCR without DNA template; M1: DNA ladder 1 kb (Fermentas); M2: DNA ladder 100 bp (Intron, Korea); D1-D3: Samples from persistent diarrheal children; C1: Samples from a healthy child.

With the aim to preliminary investigate dominant bacteria in stool samples of persistent-diarrheal children with unidentified pathogenic agents, in this studywe selected the samples from three children at six months old with persistent diarrhea (excluding other infections leading to persistent diarrhea),including: D1 (a girl, hospital admission code 230314776, diarrhea for 20 days), D2 (a girl, hospital admission code 230585587, diarrhea for 28 days), and D3 (a boy, hospital admission code 230590030, diarrhea for 21 days). A healthy child (a boy hospital admission code 230506865), who was free of gastrointestinal diseases and had not used antibiotics within one month before sample collection, was recruited as control for this study. All stool samples had been tested to be negative for 24 common diarrheal pathogens by real-time PCR using the QIAstat-Dx Gastrointestinal Panel 2 kit (QIAGEN) at the Department of Molecular biology for Infectious diseases, National Children's Hospital. To reduce the PCR inhibitors from stool samples, the bacteria from stools were extracted as describe by Nguyen et al. (Nguyen *et al.*, 2024) and are available for metagenomic DNA extraction.

Metagenomic DNAs of bacteria in the fecal samples from three children with persistent diarrhea and a healthy child were successfully extracted (Figure 1A), exhibited by high molecular weight (higher than 10 kb of DNA ladder) and slight degradation. The DNA content extracted from the healthy child was higher than that from diarrheal children. The ratios A_{260}/A_{280} and A_{260}/A_{230} of the metagenomic DNA samples all reached over 1.8. The metagene 16S rRNAs of ~ 1.5 kb of bacterial communities in the fecal samples were successfully amplified (Figure 2B) to be used as a template to amplify the V3, V68 regions. As expected, the V3 regions of about 200 bp and V68 regions of approximately 500 bp were successfully amplified (Figure 1C) from all C1, D1-D3 samples.

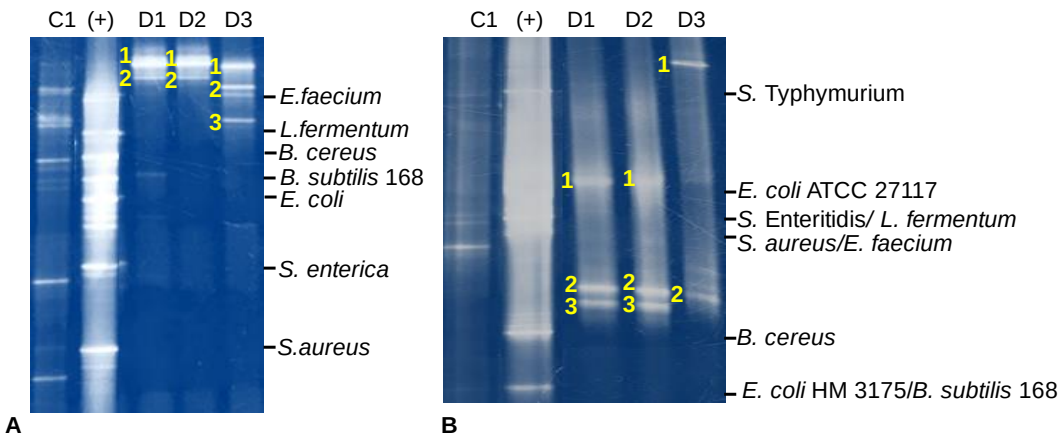

Figure 2. Analysis of V3 (A), V68 (B) regions by DGGE on polyacrylamide gel 8%.(+): Positive control including V3, V6-8 regions derived from indicator strains indicating on the right side of the picture; S. enterica: DNA of S. Enteritidis and S. Typhimurium belong to the species S. enterica; 1, 2, 3: the order of dominant DNA bands that were cut and harvested for sequencing.

Analysis of V3, V68 regions by DGGE

The DGGE analysis of the V3 and V68 regions (Figure 2) showed that although the size of the V3 (~ 200 bp) and V68 (~ 500 bp) regions amplified from C1, D1-D3 samples were the same (Figure 1C), corresponding to the regions, but on the denaturing gel, V3 DNA of different strains were separated in different melting domains. According to the DGGE principle, sequences with higher GC ratios possess high melting temperatures, so they migrate longer distances on the gel. The V3 DNA region was better separated on the DGGE gel than the V68 DNA region (Figure 2), typically in the positive control sample. The V3 DNA regions of seven different bacteria in the positive control sample separated into seven DNA bands on the gel, while the V68 DNA regions separated into six observable bands.

Among the three disease samples, samples D1 and D2 showed the same DNA fingerprints that were different from the fingerprints of C1 and D3 samples in both V3 and V68 regions. The V3 region of sample C1 was separated into 6 clear DNA bands on the gel (Figure 2A) while this region derived from diseased samples was separated into a maximum of four DNA bands. The V68 region of sample C1 separated into many DNA bands, only some bands of them were bright on the gel, while the V68 regions of diseased samples generated 3 clear dominant DNA bands on the gel. This result indicated that bacteria in control sample C1 weremore diverse than bacterial communities in disease samples D1-D3. Remarkably, samples D1 and D2 did not have any DNA bands of V3, V68 regions corresponding to the bands of positive control strains including *E. coli*, *B. aureus*, *B. subtilis* 168, *S. aureus*, *S.* Typhymurium, *S.* Enteritidis ATCC 13076, *L. fermentum*, *E. faecium*. Sample D3 had a small, faint band corresponding to the V3 region of *E. faecium* (Figure 2A). The dominant DNA bands that were exhibited by sharp, clear bands, including seven V3 DNA bands and eight V68 DNA bands (Figure 2), were excised from the gel for sequencing.

Sequence analysis of the dominant V3, V68 regions

A total of 15 DNA samples of V3 and V68 regions were sequenced. However, two samples, named V3D3.3 (V3 region of sample D3, 3rd DNA band on the gel in Figure 2A), and V68D3.1, were defective during sequencing. Among the 13 successful sequenced DNA fragments, the six V3 sequences had sizes from 128 to 160 nucleotides and seven V68 DNA sequences comprised 391-398 nucleotides. Results of BLASTN showed that two DNA sequences of V3D2.2 and V3.D3.1 did not match any genes from the NR database. However, the sequences of the two genes shared a 93.75% similarity (Figure 3).

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V3D2.2     1    GGTACCGTCATTGTCGCTTCTTATACGCTGCTCCGTTTCATACCGAAAAAACAATTTAACCACACCCGGGGGCTTCGTGC  80
V3D3.1     1    GGTTACGTCATTGTCTCTTCTTATACGCTGCTTCTTTTCCTACCGAAAAAACAACTTTACCACACCCGGGGGCTTCGTGC  80
                ***:.********** *************** * ****.************** **:**********************

V3D2.2     81   CTGGGCGGCCGTGCTGCATCAGGATTGCCTCCGTTGGCTAAGATTCCCCTCCGACGGCACCCGGAAGAATCCCCCCGTAA  160
V3D3.1     81   CTGGGCGGCCGTGCCGCATTAGGATTGCCTGCGTTGGCTAAGATTCCCCTCCGACGGCACCCGGAAGAATCCCCCCGTAA  160
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Figure 3. Sequences comparison of sample V3D2.2 and V3D3.1

All four V3 DNA sequences of samples V3D1.1, V3D1.2, V3D2.1, and V3D3.2 hadthe most similarity (94.53-100%) with the gene 16S rRNA of *Brevibacterium iodinum* DSM 20626 (NCBI number NR_026241.1) (Table 2). Looking back to Figure 2A, the V3D1.1 (sequence was 100% identical to the gene code NR_026241.1 of *B. iodinum* DSM 20626) and V3D1.2 (sequence was 98.41% identical to the gene code NR_026241.1) were the separated bands on the gel. Thus, the V3D1.2 gene may be derived from other strains of the*Brevibacterium* genus. However, this result indicated that all the disease samples D1, D2, and D3 harbored *Brevibacterium* and the genus *Brevibacterium* was detected to be the most dominant in the bacterial community in the feces of the children with persistent diarrhea based on analysis of the V3 region.

The sequences of the V68 region in all three samples were the most similar (from 99.4% to 100%) to the 16S rRNA gene of referencestrains belonging to the two genera *Enterococcus* and *Staphylococcus* (Table 2). Although V68D1.1, V68D1.2, and V68D1.3 bands were separated at different positions in the gel, all three sequences were the most identical with the 16S rRNA gene of *Enterococcus faecium* ATCC 19434. So*Enterococcus* was the dominant genera in fecal sample D1. In contrary, two DNA bands of D2 samples possessed sequences similar to the 16S rRNA gene of *Enterococcus*, the sequence oftheV68D2.2 band had the highest similarity (99.75%) to the 16S rRNA gene of *Staphylococcus pasteuri* ATCC 51129. In sample D3, the DNA sequence of the second band was 100% similar to the *E. faecalis* ATCC 19433 strain. Taking all into account, the genus *Enterococcus* was the most dominant bacterial community in the feces of the children with persistent diarrhea based on analysis of the V68 region. *Staphylococcus* was found in sample D2.

Table 2. Sequences comparison of V3, V68 DNA regions with the most similar gens in NCBI

No	DNA bands	Size (Nu)	The most identical genes	Cove-rage	E value	Identities	Location (Nu)	Nt substitution	Gene code
1	V3D1.1	130	The16S rRNA gene of *Brevibacterium iodinum* DSM 20626	100%	6e-61	100.00%	324-453	No	NR_026241.1
2	V3D1.2	126		100%	6e-61	98.41%	324-449	G281A, G284C, G387T, C400T, C403T	
3	V3D2.1	132		100%	7e-61	97.62%	324-449		
4	V3D3.2	128		100%	1e-53	94.53%	324-451	G335A, A355G, T368A, G387T, C400T, C403T, C417G	
5	V68D1.1	393	The16S rRNA gene of *Enterococcus faecium* ATCC 19434	100%	0.0	100.00%	960-1352	No	NR_115764.1
6	V68D1.2	384		100%	1e-152	91.91%	960-1342	31 substitutions*	
7	V68D1.3	398		100%	0.0	99.50%	960-1358	T1248C, C1355-	

8	V68D2.1	391		100%	0.0	100.00%	960-1350	No	
9	V68D2.2	397	The16S rRNA gene of *Staphylococcus pasteuri* ATCC 51129	100%	0.0	99.75%	919-1315	T974C	NR_114435.1
10	V68D2.3	394	The16S rRNA gene of *E. faecium* ATCC 19434	100%	0.0	99.49%	960-1353	T1249C, C1263T	NR_115764.1
11	V68D3.2	393	The16S rRNA gene of.*E. faecalis* ATCC 19433	100%	0.0	100.00%	960-1353	No	NR_115765.1

Nucleotide substitutions*: C965A, A976C, C977A, G986C, C1014T, C1020T, C1023G, C1104T, C1138T, C1140A, G1151C, A1162T, T1180G, C1209T, C1214T, A1216C, A1220C, C1225T, A1227C, C1243G, C1250G, C1258G, C1263T, T1274C, C1275A, T1276A, T1277A, C1285T, C1287T, C1311T, C1313G

DISCUSSION

Persistent diarrhea accounts for a significant portion ofmortality cases caused by diarrhea, but its origin remains controversial. Persistent diarrhea is usually developed from acute diarrhea with an infectious disease associated with intestinal colonization by pathogenic bacteria. The colonization could be promoted by many factors, such as malnutrition, exposure to antibiotics, etc., to result in intestinal bacterial dysbiosis. Thus, the identification of infected pathogens in persistent children is difficulty (Vu *et al.*, 2016). In this study, three children at six months old with persistent diarrhea were checked to be negative with 24 common diarrheal pathogens comprised of 14 bacteria, including *Vibrio vulnificus*, *V. parahaemolyticus*, *V. cholerae*, *Campylobacter spp.* (*C. jejuni*, *C. upsaliensis*, *C. coli*), *Salmonella spp.*, *Clostridium difficile* (*tcdA/tcdB*), *Yersinia enterocolitica*, Enteroaggregative *E. coli* (EAEC), Enterotoxigenic *E. coli* (ETEC), Shiga-like toxin-producing *E. coli* (STEC), Shiga toxin-producing *E. coli* (*STEC*) serumO157:H7, Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC)*/Shigella* and*Plesiomonas shigelloides.* Supported by this result, the dominant DNA bands of V3, V68 regions derived from sample D1-D3 did not correspond to the bands of indicator strains including *E. coli*, *B. aureus*, *B. subtilis* 168, *S. aureus*, *S.* Typhymurium, *S.* Enteritidis ATCC 13076, *L. fermentum*, *E. faecium* in positive control (Figure 2). Only a band of sample D3 immigrated to the melting point of the V3 region derived from *E. faecium.*

Analyzing sequences of the dominant V3 and V68 DNA bands indicated that the genus *Brevibacterium*, *Enterococcus* presented in all D1-D3 fecal samples, while *Brevibacterium* was detected by analyzing V3 DNA region, *Enterococcus* was identified through analysis of the V68 region. The DNA of the 16S rRNA V68 region of *Staphylococcus* was detected in sample D2. The V3, and V68 regions used to be applied for the detection of dominant bacteria related to diarrhea. A previous study showed that the V3-V5 region was suitable for detecting *Klebsiella*, V1-V3 was suitable for detecting *Escherichia* and *Shigella* bacteria, the V68 region was

suitable for detecting *Clostridium* and *Staphylococcus*(Yang *et al.*, 2016). To the authors' best knowledge, this is the first time *Brevibacterium* was detected in the fecal samples of children with persistent diarrhea. According to a report in 2021, the genus *Brevibacterium* consists of 50 species, commonly found in vast environments such as soil, fermented foods, and the skin of animals and humans. These bacteria are considered to rarely cause disease, but there are some reports revealed that *Brevibacterium* was an opportunistic pathogen causing bacteremia with severe clinical features in immunocompromised patients with central venous catheters (Benson, Tatem, 2021, Nguyen and Nand, 2024). The discovery of this bacterium as the dominant bacterium in stool samples of children with prolonged diarrhea is noteworthy because the genus of bacteria used to cause disease in humans.

Enterococcus is considered to be a beneficial bacteria, capable of adsorbing viruses such as Norovirus on the cell surface, limiting the growth of viruses (Almand *et al.*, 2017). *E. faecium* SF68 is also used as a probiotic to treat acute diarrhea due to antibiotic resistance very effectively(Greuter *et al.*, 2020). However, with a number over 50 species, some species are still related to the ability to cause gastrointestinal diseases (Chung, Le, 2022). This study also found DNA of *Staphylococcus pasteuri* bacteria in stool sample D2. This species is considered a benign bacterium. The representative strain of *S. pasteuri* RSP-1 (KACC 91660P) has been shown to inhibit the growth of antibiotic-resistant food poisoning bacteria, especially *S. aureus*, which causes diarrheal dehydration in humans (Hong *et al.*, 2018). In nature, there is much evidence that beneficial bacteria such as *Bifidobacterium* quickly increase proportion to be dominant in the digestive system (naturally or supplemented) to restore the intestinal microflora to help children recover from diarrhea (Wong *et al.*, 2019, Yu, 2023). Therefore, the presence of bacteria, including *Enterococcus* and *Staphylococus*, in the stools of children with persistent diarrhea in this study may be a natural mechanism to help children gradually restore their intestinal microflora as supported by previous studies in the case of *Enterococcus*(Krawczyk *et al.*, 2021, Nguyen *et al*., 2024).

CONCLUSION

Using PCR-DGGE and sequencing methods to analyze the V3, V68 regions of the bacterial 16S rRNA gene in stool samples of children with persistent diarrhea negative for common pathogenic agents, the genera *Brevibacterium*, *Enterococcus* were detected in all 3 samples and *Staphylococcus* was present in one sample. This is the first study to discover *Brevibacterium* as the dominant bacteria in the fecal bacterial community of children with persistent diarrhea that needs to be noted and considered for the possibility of causing opportunistic diseases in children.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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