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Development of Strain-Specific Primers for Identification of *Bifidobacterium bifidum* BGN4

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Bifidobacterium bifidum BGN4 (BGN4) has many proven beneficial effects, including anti-allergy and anticancer properties. It has been commercialized and used in several probiotic products, and thus strain-specific identification of this strain is very valuable for further strain-dependent physiological study. For this purpose, we developed novel multiplex polymerase chain reaction (PCR) primer sets for strain-specific detection of BGN4 in commercial products and fecal samples of animal models. The primer set was tested on seven strains of *B. bifidum* and 75 strains of the other *Bifidobacterium* species. The BGN4-specific regions were derived using megaBLAST against genome sequences of various *B. bifidum* databases and four sets of primers were designed. As a result, only BGN4 produced four PCR products simultaneously whereas the other strains did not. The PCR detection limit using BGN4-specific primer sets was 2.8×10^1 CFU/ml of BGN4. Those primer sets also detected and identified BGN4 in the probiotic products containing BNG4 and fecal samples from a BGN4-fed animal model with high specificity. Our results indicate that the PCR assay from this study is an efficient tool for the simple, rapid, and reliable identification of BGN4, for which probiotic strains are known.

Keywords: Multiplex PCR assay, *Bifidobacterium bifidum* BGN4, probiotics, fecal

Introduction

Bifidobacterium is one of the most widely used probiotic bacteria [1–4]. It is believed that *Bifidobacterium* species are important in maintaining intestinal health because they contribute to beneficial microbiota in the intestinal tract, including the prevention of intestinal colonization by pathogenic bacteria [5, 6]. Thus, the beneficial effects of *Bifidobacterium* intake in the form of probiotics have been focused on in previous studies [7, 8].

Bifidobacterium bifidum BGN4 was isolated from healthy breast-fed infants as a probiotic strain that contributed to intestinal health. Several health-associated characteristics from in vivo and in vitro experiments substantiate the fact that *B. bifidum* BGN4 can inhibit cancer and boost the host immune system. For instance, the anticarcinogenic polysaccharide isolated from *B. bifidum* BGN4 inhibited the growth of cancer cell lines [9]. Furthermore, *B. bifidum* BGN4 is reported to have a potent adhering activity with respect to Caco-2 cells [10]. In vivo intervention studies suggest that *B. bifidum* BGN4 administration alleviates allergic reactions elicited by ovalbumin in a mouse model and in infants at high risk of allergy during the first year of life [11, 12].

B. bifidum BGN4 is now being used to produce various commercial probiotic products. The consumption of the product results in the improvement of intestinal microbiota, such as increase of the viable number of *Bifidobacterium* and reduced number of *Escherichia coli*, *Bacteroides*, and *Clostridium* in the fecal sample during the intake period [13]. However, to establish a specific monitoring system for *B. bifidum* BGN4 in clinical fecal samples from a subject fed *B. bifidum* BGN4, *B. bifidum* BGN4-specific PCR primers are

required. For this reason, we developed *B. bifidum* BGN4-specific PCR primer sets that will be used to verify the quality and presence of this strain in probiotic products and fecal samples for both laboratory and industrial applications.

Strain-specific identification was carried out in previous studies by specific culture practices, and then DNA fingerprinting or enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies [14–16]. However, these methods are time-consuming and need a practiced hand to perform them. PCR-based identification methods using strain-specific primers are simple, rapid, and accurate methods. These methods have been established for several industrially valuable probiotic strains [17–19]. To verify the efficacy of probiotics in clinical tests, it is necessary to detect specific probiotics in clinical fecal samples or dairy products using strain-specific primer sets; however, few studies have identified strain-specific PCR primer sequences [20, 21].

Ribosomal RNA genes and the intergenic region-based detection assay, such as PCR, have been designed to detect bacteria at the species level [22–24]. However, the 16S rRNA gene sequence is not a suitable PCR primer target for strain level identification because of the highly conserved character of this gene. Therefore, we designed strain-specific primers based on the complete genome sequence of *B. bifidum* BGN4, which we determined in a previous study [25; GenBank Accession No. CP001361]. The whole genome of *B. bifidum* BGN4 was compared with other deposited genomes of *Bifidobacterium* strains to discover a *B. bifidum* BGN4-specific region. The specific region sequences were used to develop PCR primers.

In this study, we developed *B. bifidum* BGN4-specific primer sets and evaluated the *B. bifidum* BGN4 detection limit using these primers sets to confirm their application in fecal samples and dairy products.

Materials and Methods

Bacterial Strains and DNA Preparation

The various *Bifidobacterium* strains and other lactic acid bacteria used in this study are listed in Table 1. These strains were freshly cultured in Lactobacilli MRS broth (MRS; BD Biosciences, USA) supplemented with 0.05% L-cysteine·HCl (Sigma-Aldrich, USA) at 37°C for 15 h. Then, 500 µl of the sample mixture was used for DNA isolation. DNA was extracted from the cell pellet using the DNeasy Blood and Tissue kit (Qiagen, Germany) according to the manufacturer's instructions and stored at −20°C until use.

Oligonucleotide Primers

Bioneer Co. (Korea) synthesized all oligonucleotide primers used in this study. MegaBLAST was used to align genome sequences of various *Bifidobacterium* strains to detect *B. bifidum* BGN4-specific regions. Four PCR primer sets were designed using the sequences in those regions. The primer sequences are shown in Table 2 along with their corresponding GenBank accession numbers and predicted product sizes. The primer pairs were

Table 1. Bacterial strains used in this study.

Bacterial strains	No. of isolate (n = 96)
Bifidobacterium spp.	82
B. bifidum BGN4	1
B. adolescentis ATCC 15703	1
B. angulatum ATCC 27535	1
B. animalis ATCC 25527	1
B. breve ATCC 15700	1
B. catenulatum ATCC 27539	1
B. dentium ATCC 27534	1
B. infantis ATCC 15697	1
B. longum ATCC 15707	1
B. pseudocatenulatum ATCC 27919	1
B. subtile ATCC 27537	1
B. thermophilum ATCC 25525	1
B. bifidum KCTC 3202	1
B. bifidum KCTC 3418	1
B. bifidum KCTC 3281	1
B. bifidum KCTC 3357	1
B. bifidum KCTC 3440	1
B. bifidum ATCC 15521	1
Others	64
Non-*Bifidobacterium* spp.	14
L. sakei subsp. *sakei* ATCC 31063	1
L. brevis ATCC 14869	1
L. salivarius subsp. *salicinius* ATCC 11742	1
L. acidophilus ATCC 04356	1
L. casei ATCC 00393	1
L. rhamnosus GG	1
Streptococcus thermophilus ATCC 12020	1
Lactococcus lactis subsp. *cremoris* ATCC 19257	1
Leuconostoc mesenterioides subsp. *mesenteroides* ATCC 27258	1
Lactococcus lactis subsp. *lactis* ATCC 11454	1
Pediococcus acidilactici ATCC 33314	1
Enterococcus faecium ATCC 27270	1
Weissella confusa ATCC 10881	1
Enterococcus faecalis ATCC 19433	1

Table 2. Sequences of the *B. bifidum* BGN4-specific PCR primers along with their corresponding position in the BGN4 genome.

Primer	Description	Sequence (5′ → 3′)	Position[a]	Amplicon size (bp)	Reference
Primer4	Forward primer	GGACGCGCTGATCGTCTCGGTGACGACCG	1427078 - 1427106	362	This study
	Reverse primer	TGAGCGACAGCTGGCACGTGAACATCGAGGC	1427409 - 1427439		
Primer6	Forward primer	GTCGCATGCGCTGATTGCGGTCTCATGCGC	1425725 - 1425754	847	This study
	Reverse primer	AGGGCTGGAAATGCACCGGCATCAGCGGGC	1426542 - 1426571		
Primer7	Forward primer	GCCGGTGCATTTCCAGCCCTTGTCGGCGG	1426552 - 1426580	540	This study
	Reverse primer	CGATCAGCGCGTCCGACCTCAAGGCCATGG	1427062 - 1427091		
Primer10	Forward primer	TGTCCGTCTGCCGGAGGGAGCATGAAGTC	1424839 - 1424867	752	This study
	Reverse primer	GGACGCAAAGACGACCGCCATCAAGGGTTC	1425561 - 1425590		

[a]The positions are numbered based on the *Bifidobacterium bifidum* BGN4 complete genome sequence (GenBank Accession No. CP001361).

designed to have a similar melting temperature (T_m) and to yield products that differed from each other by >100 bp, which could be resolved by 3% low-melting-point agarose gel (Sigma-Aldrich, USA) electrophoresis (Table 2). The BLAST program was used to ensure that the proposed primers were complementary with the target strain, but not with other strains.

Amplification of Multiplex PCR and Analysis of its Products

Our multiplex PCR amplification system using 50 µl of reaction mixture was as follows: The PCR mix was composed of 33.9 µl deionized distilled water, 5 µl 2× GC buffer II (Takara, Japan), 5 µl dNTP mixture (2.5 mM, Takara), 0.5 µl each primer (100 µM), 2.5 U LA Taq (Takara), and 5 µl template DNA. PCR amplification was performed using the GenAmp9700 thermal cycler (Applied Biosystem, USA). Target amplification was initiated at 95°C for 3 min, followed by 30 cycles of 3-step cycles of denaturation at 95°C for 3 min, annealing at 72°C for 90 sec, extension at 72°C for 1 min, and a final extension of 72°C for 10 min. Amplified PCR products were analyzed by gel electrophoresis on a 3% low-melting-point agarose gel containing the Loading Star (Dynebio, Korea) and visualized by ultraviolet light using ethidium bromide staining.

Sensitivity and Specificity Assays

The sensitivity of the multiplex PCR was tested using a serial dilution of *B. bifidum* BGN4 prepared from overnight cultures in MRS. The bacteria were serially diluted (2.8×10^7, 2.8×10^6, 2.8×10^5, 2.8×10^4, 2.8×10^3, 2.8×10^2, 2.8×10^1, 2.8×10^0 CFU/ml) in phosphate-buffered saline (PBS). DNA was isolated from 500 µl of each dilution and subsequently tested using the multiplex PCR assay.

The multiplex PCR specificity was determined by examining the ability to detect and distinguish *B. bifidum* BGN4 among 82 strains of *Bifidobacterium*, and 14 non-*Bifidobacterium* strains.

Fecal Sample Collection and DNA Extraction

Seoul National University's Institutional Animal Care and Use Committee approved all animal care procedures prior to experiment initiation. Two-week-old C57BL/6 mice, which are the general animal model for the study of probiotic effect of lactic acid bacteria [26], were purchased from Central Laboratory Animal Inc. (Korea) and divided into two groups of five mice. Mice were housed in a standard animal laboratory with a 12:12 h light:dark cycle and allowed free access to water.

B. bifidum BGN4, obtained from Research Institute Bifido Co. Ltd. (Korea), was mixed with the mouse AIN-93G diet. Mice in the *B. bifidum* BGN4 group were administered *B. bifidum* BGN4 bacterial powder. Mice in the sham group received no bacteria. As a positive control, *B. bifidum* BGN4-treated mice were fed 0.2% of lyophilized *B. bifidum* BGN4 (1.0×10^9 CFU/g) via a diet pellet. Mice were fed the experimental bacterial powders for 2 weeks.

The fecal samples were collected on days 1, 3, 7, 10, and 14 after feeding. The total genomic DNA from each fecal sample was extracted using the QIAamp DNA stool mini kit (Qiagen) according to the manufacturer's instructions.

Probiotic Products and DNA Extraction

In this study, four commercially available probiotic products collected from various retail stores in Korea, were analyzed. In addition to *B. bifidum* BGN4 products, the choices of other products were also based on the number of different bacterial

Table 3. Overview of five probiotic products used in multiplex PCR analyses.

Products (Freeze-dried products)	Organism(s) stated on product label
I	*Bifidobacterium bifidum* BGN4, *B. longum*, *Lactobacillus acidophilus*
II	*Enterococcus faecium*, *L. plantarum*, *L. acidophilus*, *Streptococcus thermophilus*, *B. longum*, *B. breve*
III	*E. faecium*, *L. acidophilus*, *L. rhamnosus*, *B. longum*, *B. bifidum*
IV	*L. casei*, *L. rhamnosus*, *L. plantarum*, *B. lactis*
V	*L. plantarum*, *L. rhamnosus*, *B. lactis*, *L. acidophilus*, *B. bifidum*, *B. breve*, *S. thermophilus*

groups claimed on the product label. As shown in Table 3, the products investigated contained 3–6 bacterial strains.

One gram of commercial probiotic product was resuspended in 1 ml of PBS to remove saccharides. Then, pellets were collected by centrifugation at 4,000 ×*g* for 30 min. Bacterial DNAs were extracted using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions.

Results

Establishment of the Multiplex Primer Set for *B. bifidum* BGN4 Identification

Novel multiplex PCR primer sets for probiotic *Bifidobacterium bifidum* BGN4 identification were constructed from molecular chaperone sequences (GenBank Accession No. BBB_1207; primer 6-forward and primer 10-reverse), hypothetical proteins (GenBank Accession No. BBB_1208; primer 7-forward), and putative secreted transglycosylase genes (GenBank Accession No. BBB_1209; primer 4 pair) through comparison of genome sequences of *Bifidobacterium* strains. Other primer sequences, like primer 6-reverse, primer 7-reverse, and primer 10-forward, were located in non-functional protein sequences of *B. bifidum* BGN4 (Table 2). The *B. bifidum* BGN4-specific primer sets produced four amplicons at each expected size of 362, 540, 752, and 847 bp (Fig. 1). They clearly discriminated *B. bifidum* BGN4 from other lactic acid bacteria because the strain-specific amplicon of *B. bifidum* BGN4 revealed four amplicons.

Specificity of the *B. bifidum* BGN4 Primer Sets

To verify whether the primers were available for strain-specific identification of *B. bifidum* BGN4, the PCR assay was performed with 96 lactic acid bacteria, including 82 *Bifidobacterium* and 14 non-*Bifidobacterium* strains. The primers produced the four expected amplicons only from *B. bifidum* BGN4. They did not interfere with each other and the multiplex PCR correctly detected and discriminated *B. bifidum* BGN4 from other tested bifidobacteria and LAB. No other strains produced four amplicons simultaneously, although *B. bifidum* KCTC3440 and *Bifidobacterium* sp. RD10 produced three amplicons in the PCR product (Table 4). Furthermore, 14 non-*Bifidobacterium* strains did not produce any amplicons. Thus, using our technique, *B. bifidum* BGN4 could be distinguished from other *Bifidobacterium* species.

Detection Limits of the Multiplex PCR

Fig. 2 shows the sensitivity of the *B. bifidum* BGN4-specific PCR method using 10-fold serially diluted *B. bifidum* BGN4 DNA templates. The PCR was performed with genomic DNA of *B. bifidum* BGN4 extracted from different bacterial concentrations of 2.8×10^7, 2.8×10^6, 2.8×10^5, 2.8×10^4, 2.8×10^3, 2.8×10^2, 2.8×10^1, and 2.8×10^0 CFU/ml. Four *B. bifidum* BGN4-specific bands were evident over 2.8×10^1 CFU/ml and, therefore, the detection limit for this multiplex PCR assay in pure culture was determined as 2.8×10^1 CFU/ml.

Application of the Multiplex PCR for Identifying *B. bifidum* BGN4 from Probiotic Products

The primer sets we established were applied to identify

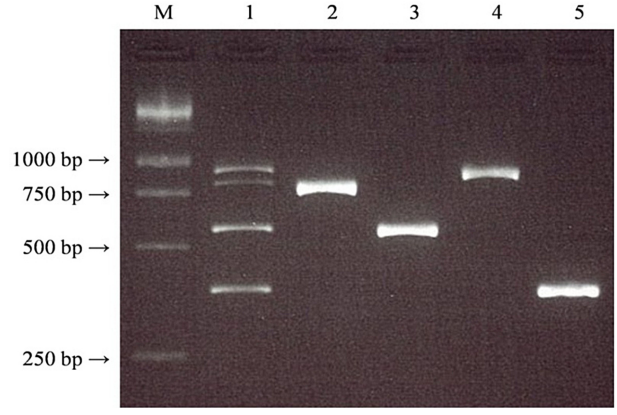

Fig. 1. Comparison of single target versus multiplex PCR using *B. bifidum* BGN4 from pure culture (MRS broth).
The PCR products were separated on a 3% low melting-point agarose gel. Lane M, 1kb DNA ladder; Lane 1, 4 primers combined; Lane 2, primer 4; Lane 3, primer 6; Lane 4, primer 7; and Lane 5, primer 10.

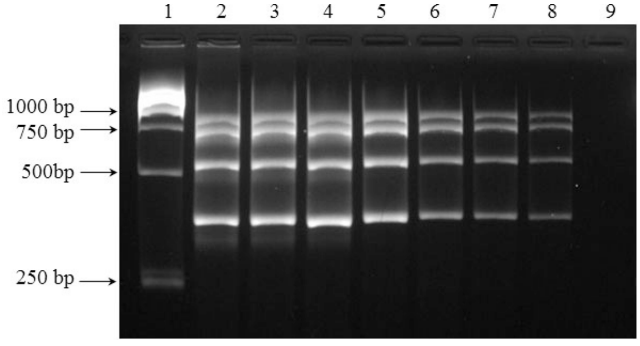

Fig. 2. Determination of the detection limit of *B. bifidum* BGN4 specific multiplex PCR.
Lane 1, 1 kb DNA ladder; Multiplex PCR with DNA from *B. bifidum* BGN4 at the following cell concentration: Lane 2, 2.8×10^7 CFU/ml (370 ng); Lane 3, 2.8×10^6 CFU/ml (37 ng); Lane 4 (3.7 ng), 2.8×10^5 CFU/ml (370 pg); Lane 5, 2.8×10^4 CFU/ml (37 pg); Lane 6, 2.8×10^3 CFU/ml (3.7 pg); Lane 7, 2.8×10^2 CFU/ml (0.37 pg); Lane 8, 2.8×10^1 CFU/ml (0.037 pg); and Lane 9, 2.8×10^0 CFU/ml (0.0037 pg).

Table 4. Results of PCR using *B. bifidum* BGN4-specific primer sets.

Bacterial strains	Primer4	Primer5	Primer6	Primer7	Bacterial strains	Primer4	Primer5	Primer6	Primer7	Bacterial strains	Primer4	Primer5	Primer6	Primer7
BGN4	+	+	+	+	RD22	-	-	-	-	RD79	-	+	-	+
ATCC15703	-	-	-	-	RD24	-	-	-	-	RD88	-	-	-	-
ATCC27535	-	-	-	-	RD25	-	-	-	-	RD90	+	-	-	-
ATCC25527	-	-	-	-	RD26	-	-	-	-	RD91				+
ATCC15700	-	-	-	-	RD28	-	-	-	-	RD93	+	-	-	-
ATCC27539	-	-	-	-	RD29	-	-	-	-	RD98	+	-	-	-
ATCC27534	-	-	-	-	RD30	-	-	-	-	RD102	-	-	-	-
ATCC15697	-	-	-	-	RD32	-	-	-	-	RD115	-	-	-	-
ATCC15707	-	-	-	-	RD34	-	-	-	-	RD117	-	-	-	-
ATCC27919	-	-	-	-	RD35	-	-	-	-	RD120	-	-	-	-
ATCC27537	-	-	-	-	RD37	-	-	-	-	RD122	+	-	-	-
ATCC25525	-	-	-	-	RD38	-	-	-	-	RD123	-	-	-	-
KCTC3202	-	-	-	-	RD39	-	-	-	-	RD125	-	-	-	-
KCTC3418	-	-	-	-	RD40	-	-	-	-	RD128	+	-	-	-
KCTC3281	-	-	-	-	RD41	-	-	-	-	RD156	+	-	-	-
KCTC3357	-	-	-	-	RD42	-	-	-	-	RD161	-	-	-	-
KCTC3440	+	+	+	-	RD43	-	-	-	-	RD163	-	-	-	-
ATCC15521	-	-	-	-	RD46	-	-	-	-	RD181	-	-	-	-
RD01	-	-	-	-	RD48	-	-	-	-	ATCC31063	-	-	-	-
RD02	-	-	-	-	RD50	+	-	-	-	ATCC14869	-	-	-	-
RD05	-	-	-	-	RD51	-	-	-	-	ATCC11742	-	-	-	-
RD06	-	-	-	-	RD53	-	-	-	-	ATCC04356	-	-	-	-
RD08	+	+	-	-	RD54	-	-	-	-	ATCC00393	-	-	-	-
RD09	+	+	-	-	RD55	-	-	-	-	GG	-	-	-	-
RD10	+	+	-	+	RD56	-	-	-	-	ATCC12020	-	-	-	-
RD11	-	-	-	-	RD58	-	-	-	-	ATCC19257	-	-	-	-
RD14	-	-	-	-	RD59	-	-	-	-	ATCC27258	-	-	-	-
RD15	-	-	-	-	RD62	-	-	-	-	ATCC11454	-	-	-	-
RD16	-	-	-	-	RD64	-	-	-	-	ATCC33314	-	-	-	-
RD19	-	-	-	-	RD65	-	-	-	-	ATCC27270	-	-	-	-
RD20	+	-	-	-	RD66	-	-	-	-	ATCC10881	-	-	-	-
RD21	-	-	-	-	RD71	-	-	-	-	ATCC19433	-	-	-	-

B. bifidum BGN4 from commercial probiotic products. Five probiotic products claiming to contain several probiotic strains, including *B. bifidum* BGN4, were tested (Table 3). Amplicons corresponding to *B. bifidum* BGN4 were not found in the other probiotic products (Fig. 3A). Our multiplex PCR system did not detect amplicons corresponding to *B. bifidum* BGN4 in the probiotic products containing different *B. bifidum* strains (products III and V, lanes 3 and 5, Fig. 3A). Only the product claiming to contain *B. bifidum* BGN4 (Lane 1, Fig. 3A) produced *B. bifidum* BGN4-specific amplicon.

Multiplex PCR Application for Identifying *B. bifidum* BGN4 from Fecal Matter

In order to estimate whether the multiplex PCR system is a reliable tool for the detection of *B. bifidum* BGN4 from various samples, we tried to detect *B. bifidum* BGN4 from mouse feces. Fresh murine fecal samples were collected at 1, 3, 7, 10, and 14 days after diet feeding as described in the Materials and Methods section. Among these, *B. bifidum* BGN4 was detected from all fecal samples in the *B. bifidum* BGN4 group (Figs. 3B–3F lanes 1–5) by *B. bifidum* BGN4-specific multiplex PCR assay. Amplicons corresponding to

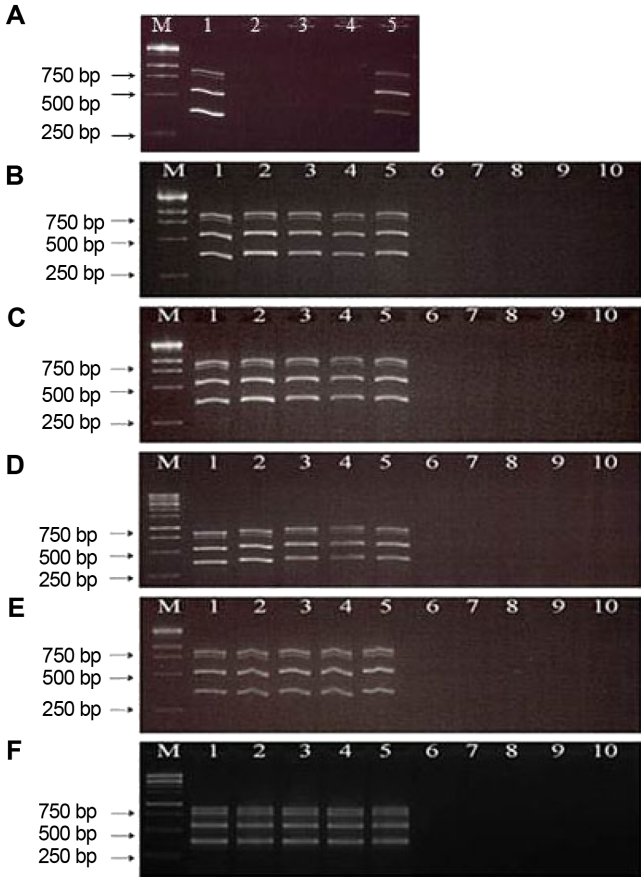

Fig. 3. Detection of *B. bifidum* BGN4 from commercial dairy products (**A**) and mice fecal samples (**B**–**F**) by multiplex PCR. (**A**) Lane M, 1 kb DNA ladder; Lane 1, product I; Lane 2, product II; Lane 3, product III; Lane 4, product IV; and Lane 5, product V. (**B**–**F**) *B. bifidum* BGN4-specific PCR products from each 5 mice fecal samples at the 1st (**B**), 4th (**C**), 7th (**D**), 10th (**E**), and 14th (**F**) days after ingestion of diet with *B. bifidum* BGN4 (BGN4 group, Lanes 1–5) or without BGN4 (placebo group, Lanes 6–10).

B. bifidum BGN4 were not found in the control group (Figs. 3B–3F lanes 6–10).

Discussion

Although conventional microbiological methods such as biochemical and genotypical methods for identifying *Bifidobacterium* strains are reliable, these methods need several days to complete. PCR is generally considered to be a time-saving and sensitive method for the detection of bacterial species. In previous studies, PCR-based strain-specific identification using strain-specific primers was reported for several probiotics and fecal samples [18, 19]. This technique is a valuable means to identify strains in commercialized products and to monitor the population of probiotics in humans or animals in clinical studies.

Therapeutic benefits of *Bifidobacterium bifidum* BGN4 application have been shown in different randomized, controlled clinical trials [12]. Kim *et al.* [12] clearly demonstrate that *B. bifidum* BGN4-specific PCR assays are necessary for effective and accurate identification of this strain, not only from pure cultures, but also from fecal samples after oral administration. This study satisfied these needs (Tables 1 and 3).

Four PCR primer pairs for *B. bifidum* BGN4 (primer 4, primer 6, primer 7, and primer 10 pair) were designed based on *B. bifidum* BGN4-specific sequences, including partial molecular chaperone, hypothetical protein, and putative secreted transglycosylase. Among them, the primer 4 pair was not selective enough for the other tested *Bifidobacterium* strains (14.8% positive). In contrast to primer 4 pair, the other three primer pairs (primer 6, primer 7, and primer 10 pair) were better suited for the specific detection of *B. bifidum* BGN4. Nevertheless, *B. bifidum* BGN4-specific primers clearly discriminated BGN4 from other *B. bifidum* strains, such as *B. bifidum* KCTC3202, *B. bifidum* KCTC3418, *B. bifidum* KCTC3281, *B. bifidum* KCTC3357, *B. bifidum* KCTC3440, and *B. bifidum* ATTC15521, because the four amplicons of *B. bifidum* BGN4 were not detected simultaneously with other *B. bifidum* strains.

In *B. bifidum* BGN4 from pure culture, the sensitivity of the *B. bifidum* BGN4-specific PCR assay for direct detection was tested. Although *B. bifidum* BGN4 at 2.8×10^1 CFU/ml showed very weak bands in the detection limit assay, the four amplicons were successfully amplified at this concentration. Therefore, the detection limit was determined to be 2.8×10^1 CFU/ml. This sensitivity is comparable to other *Bifidobacterium*-specific PCR assays reported [27].

In conclusion, for BGN4 strain-specific identification, we created a specific PCR system targeting the *B. bifidum* BGN4-specific sequence, based on partial molecular chaperone, hypothetical protein, and putative secreted transglycosylase gene sequences. The system developed in this study is a valuable tool to monitor the *B. bifidum* BGN4 content of probiotic products and in human specimens, where the accuracy and specificity of the identification is of the utmost importance. The results in this study suggest that the next step is to combine this method with real-time PCR and propidium monoazide to identify the viable cells of BGN4 in complex microbiota compositions, as suggested for other probiotic strains [17].

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