A THESIS FOR THE DEGREE OF
MASTER OF SCIENCE IN FOOD AND NUTRITION

Strain Specific Detection and Quantification of Probiotic *Bifidobacterium bifidum* BGN4 in Mouse Model and Positive Effect of *B. bifidum* BGN4 on Intestinal Microflora

Bifidobacterium bifidum BGN4 특이 검출을 통한
장관 내 균주 정량 및 장내 균 총 개선효과

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Abstract

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Bifidobacterium species have been used in the production of functional foods based on their well-known beneficial effects on intestinal health. *B. bifidum* BGN4 is a commercialized probiotic strain with proven evidence in clinical trials but still lacks a DNA-based identification method. This study aimed to develop a specific polymerase chain reaction (PCR) primer set for strain specific identification of *B. bifidum* BGN4 using whole genome sequences and applied research. Consequently, a primer set targeting 200 bp was developed. The developed primer was applied to detect and quantify *B. bifidum* BGN4 strain in the fecal sample after feeding ICR mouse and bio-distribution of this

strain was analyzed at each position of the intestinal tract. Strong viability of *B. bifidum* BGN4 was identified by analysis of gastrointestinal contents and mouse fecal samples. Also, intake of *B. bifidum* BGN4 promoted the quantity and viability of *Bifidobacterium* genus in GI tract. This study revealed that the administration of *B. bifidum* BGN4 promoted the colonization of not only *B. bifidum* BGN4 but also the other *Bifidobacterium* strains.

Key words : *Bifidobacterium bifidum* BGN4, polymerase chain reaction, colonization

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List of Abbreviations

BGN4 : *Bifidobacterium bifidum* BGN4

PCR : Polymerase chain reaction

GI : Gastrointestinal

RAPD : Random amplified polymorphic DNA

qPCR : Quantitative real time PCR

KCTC : Korean Collection for Type Cultures

MRS : Mann-Rogosa-Sharpe

NCBI : National Center for Biotechnology Information

C_T : Threshold cycle

LG : Live cell gavage

DG : Dead cell gavage

MP : Mixed with the powdered commercial diet

XRE : Xenobiotic response element

EMA : Ethidium monoazide

1. Introduction

The health promoting effects of various probiotics have been studied and several lactic acid bacteria and bifidobacteria are used in probiotic products to meet its growing demands. The intake of probiotics has been proved to modulate the composition of the intestinal microflora, alleviate allergic symptoms and irritable bowel disease [1, 2]. Furthermore, the effects of probiotics on the promotion of immunologic functions and improvement of lactose tolerance are reported in many previous studies [3, 4].

Bifidobacterium bifidum BGN4 (BGN4) is a strain that had been isolated from the fecal sample of a healthy breast-fed infant [5]. This strain inhibits the growth of several cancer cell lines and lowers the production of allergy-related cytokines from mouse cells [6, 7]. Furthermore, BGN4 showed the highest adhesion capacity among various *Bifidobacterium* species, which is a desirable property for its colonization in gastrointestinal (GI) tracts [8]. The ability to adhere to intestinal cells may play a critical role on the colonization and the expression of the probiotic properties of the administered strains in a host intestine [11]. In double-blind, randomized placebo-controlled

human trials the products containing BGN4 significantly lowered the prevalence of atopic dermatitis in infants and also significantly lowered the pain scores and increased the bowel movement comfortably in irritable bowel syndrome patients [9, 10]. To understand more deeply, understanding on how the intestinal colonization can contribute to clinical results of the probiotics is needed. Although various researches investigated the survival capacity and adhesion of probiotics using in cell experiments, in vivo experiments are far more instructive. However, there were no appropriate methods for analysing the viability of bacteria in mouse model.

Recently, many studies have been researched about probiotic bacteria in strain levels and the necessity of strain specific detection methods are increasing [11-17]. However culture-based methods can not be used to identify the administered bacteria at a strain level and there are many drawbacks such as similarity in physiological properties [18]. Random amplified polymorphic DNA polymerase chain reaction (RAPD PCR), phage related sequence analysis, multiplex PCR and DNA sequence homology based method, such as KEGG also employed specific identification of a certain probiotic strains [12, 15, 19]. Among them, RAPD PCR has been widely used to identify bacteria at a strain level. However, this method requires a large number of bacterial cultures for analysis. Additionally, reproducibility was quite low and the detection failure of RAPD

method was reported [13, 18]. The aim of the present study was to establish a new rapid and accurate method for strain-specific detection using NCBI genome sequence comparison.

Furthermore it has been demonstrated that fluorescence in situ hybridization (FISH) method has been a tool for the analysis of bacterial ecosystem analysis [20]. However it is unsuitable for the quantitative analysis of bacteria [1]. Therefore the present study was conducted using quantitative real time PCR (qPCR) for the analysis of intestinal microflora.

Although various health promoting effects of BGN4 have been reported, the change of intestinal microflora, retention time and distribution of GI tracts after the administration of this strain have not been fully assessed. For selection and efficacy evaluation of probiotic strains, it is necessary to know their bio-distribution. Therefore, we tried develop strain specific detection and quantification method for BGN4 and applied this method to identify the retention time and bio-distribution of BGN4 in fecal samples after administration of this strain using mouse model. Finally, we also observed the effect of the administration of BGN4 on the composition of microbial flora in fecal samples after daily administration.

2. Materials and methods

2.1. Microorganism and culture conditions

The bacterial strains used in this work are listed in Table 1. They were obtained from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea) and Food Microbiology Lab., Department of Food and Nutrition in Seoul National University (Seoul, Korea).

The experimental bacterial strains were grown in de-Mann-Rogosa-Sharpe (MRS) medium supplemented with 0.05% (w/v) L-cysteine · HCl (Sigma-Aldrich Co., LLC., USA) under anaerobic conditions at 37℃ for 18 h.

Table 1. Bacterial strains used in this study

No.	Bacterial strains
1	*Bifidobacterium bifidum* BGN4
2	*B. bifidum* KCTC 3202
3	*B. bifidum* KCTC 3418
4	*B. bifidum* KCTC 3440
5	*B. longum* RD01
6	*B. longum* RD03
7	*B. longum* RD47
8	*B. longum* RD65
9	*B. longum* RD72
10	*B. longum* BORI
11	*B. adolescentis* KCTC 3567
12	*B. angulatum* KCTC 3236
13	*B. animalis* KCTC 3219
14	*B. breve* KCTC 3419
15	*B. infantis* KCTC 3249
16	*B. catenulatum* KCTC 3221
17	*B. thermophilum* KCCM 12097
18	*B. pseudocatenulatum* G4 KCTC 3223
19	*Lactobacillus acidophilus* KCTC 3164
20	*L. acidophilus* KCTC 3145
21	*L. plantarum* KFRI 708
22	*L. rhamnosus* KCTC 3237
23	*L. casei* KFRI 699
24	*L. cremoris* ATCC 19257
25	*L. bulgaricus* KCTC 3186
26	*L. brevis* GABA 100
27	*Lactococcus lactis* subsp. *lactis* KCTC 2013
28	*Enterococcus faecium* KCTC 13225
29	*E. faecalis* KCTC 2012
30	*Streptococcus salivarius* subsp. *thermohilus* KCTC 3779

2.2. Strain-specific primer design

A whole genome sequence of BGN4 was manually checked for sequence homologue by using National Center for Biotechnology Information (NCBI) nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn).

A strain specific primer set was designed using Primer BLAST from the NCBI Website (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The "PCR product size" was set to between 80 bp and 200 bp for real time PCR and "Database" and "Organism" were set as "nr" and "bacteria" respectively. Furthermore, Primer BLAST was used to confirm that the primers were complimentary with the target sequence but not with other strains. Primer dimers and mismatches were checked and primers were synthesized by Bionics (Seoul, Korea).

The specificity of primer set was monitored by conventional PCR using genomic DNA from 18 *Bifidobacterium* strains including 3 *Bifidobacterium bifidum* strains and other 12 reference strains.

2.3. DNA extraction

Genomic DNA of pure culture bacteria (1 ml) was extracted using MG™ Cell SV (Doctor Protein, Korea). Extraction was performed according to the manufacturer's instructions and the total bacterial DNA was eluted with 200 ㎕ of sterile water. DNA extracts were aliquoted and stored at −20℃.

The fecal samples (200mg) of each group were used for DNA extraction. DNA from stools and contents of GI tracts except stomach sample were extracted according to modified QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK) protocols to improve the yield of the DNA extraction. The optimized condition was selected after conducting DNA extraction under the various conditions (Table 2). The lysis temperature was increased to 100℃ and 1 ml lysozyme with TE buffer (50 mg/ml) was added before sonication for 4 min (50/60 Hz, 230V, 40 Amps; Qsonica, Newtown, CT. U.S.A). Contents of stomach were diluted 1/10 using 0.9% (w/v) saline and then used for the extraction of genomic DNA using MG™ Cell SV (Doctor Protein, Korea). Extraction was performed according to the protocols.

When the data were analyzed, the dilution rate was multiplied. DNA samples were stored at −20℃ until further processing.

Table 2. The optimized conditions for detecting BGN4

NO.	METHOD	Temp (℃)	Time (Minutes)	C_T[a]
1	ASL[b]	95	5	34.26
2	ASL	95	10	31.98
3	ASL	95	30	ND[c]
4	ASL	100	5	29.43
5	ASL	100	10	32.97
6	Lysozyme (50 mg/ml)[d]	37	30	20.63
7	Lysozyme (50 mg/ml)	37	30	21.96
	ASL	95	5	
8	Lysozyme (50 mg/ml)	37	45	22.53
	ASL	95	5	
9	Lysozyme (50 mg/ml)	37	30	22.56
	ASL	95	10	
10	Lysozyme (50 mg/ml)	37	45	21.95
11	Lysozyme (50 mg/ml)	37	30	22.07
	ASL	100	5	
12	Sonication[e] with lysozyme (50 mg/ml)			19.94
	ASL	100	5	
13	Lysozyme (50 mg/ml)	37	30	22.68
	Sonication			
14	Sonication with PBS 1 ml	–	–	20.82
15	Sonication with ASL 1 ml	–	–	23.42

[a]The C_T values of each condition were determined by real time PCR. BGN4-free feces were spiked with 9 log CFU/ml BGN4 suspension. DNA of each sample was extracted following the modified methods.

[b]ASL means buffer ASL which is a lysis buffer of QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK).

[c]ND: No detection

[d]The supplementary lysozyme digestion step was added.

[e]Sonication conditions : Amp 38 %, 4 min, pulse 1 s

2.4. Standard curves

2.4.1. Pure culture standard curve

To construct the pure culture standard curve for the real time PCR, DNA was extracted from 1 ml of BGN4 suspension (9 log CFU/ml). The standard curve was prepared by serial tenfold dilution of this DNA extract. The exact number of BGN4 (CFU/ml) in the standards was determined by spreading on MRS agar plates. The plates were incubated anaerobically for 24 h at 37℃.

2.4.2. Feces-based standard curve

For the feces-based standard, precise amounts of BGN4 dilutions were spiked to feces. The 200 mg of feces used for the preparation of the standard were from mice feces free of BGN4. BGN4-free feces were spiked with BGN4 suspension from 2 log CFU/ml to 9 log CFU/ml. DNA from these mixtures was extracted for the feces-based standard curve. The exact number of added BGN4 (CFU/ml) was determined by the same method as for the pure culture standard.

2.5. Experimental animals

All animal study designs and procedures were approved by the local animal ethics committee at the Seoul National University (approval number: SNU-160426-6-1). Also animals were maintained and treated as a guideline of Institute of Laboratory Animal Resource (Seoul National University, Seoul, Korea).

Five-week-old male ICR mice (n=89, Central Lab. Animal Inc., Seoul, Korea) were used in the current study. The animals were maintained in a temperature controlled environment (22±2℃) with relative humidity of 50±10% and the light was maintained on a 12 h light/dark cycle. All animals were acclimatized for a week prior to being randomly assigned into their respective treatment groups based on their body weight. They were fed a commercial diet, AIN-93G (TD. 94045, Harlan Teklad) and tap water ad libitum in this period.

2.6. Lyophillization of bacteria

Freeze-dried BGN4 cells were provided from Bifido Inc., Hongcheon, Gangwon 250-804, Korea.

2.7. In vivo study design and sample collection

2.7.1. Retention of BGN4 in mouse gut and intestinal microflora analysis

2.7.1.1. Study design

Live cell gavage (LG) group mice (n=7) were daily ingested for 1 week with 0.2 ml of 0.9% (w/v) saline containing 0.01 g of 12 log CFU/g of lyophillized BGN4 cells. Dead cell gavage (DG) group mice (n=7) were ingested with dead BGN4 cells which were treated by heating at 70℃ with air exposure for 48h. In addition, 7 mice were fed the same amount of BGN4 cells mixed with the AIN-93G commercial diet (MP group). Mice were daily fed lyophillized BGN4 cells with 5 g of powdered AIN-93G for 1 week. Control group mice (n=7) of each treatment were orally administered daily with equal volume of saline alone or consumed only powdered AIN-93G.

2.7.1.2. Sample collection

To study intestinal retention time of BGN4 and its effects on gut microbiota, fecal samples of each group were collected for 7 days post successive administration for 7 days. The fecal samples (200 mg) were used for DNA extraction as described above and aliquots of DNA were used in quantification. To analyze the effects of BGN4 colonization on the dominant microbial composition, fecal levels of total bacteria, *Lactobacillus* and *Bifidobacterium* were quantified. The number of these bacteria was compared between control and BGN4-administered mice.

2.7.2. Bio-distribution of BGN4 in mouse GI tracts

2.7.2.1. Study design

To study bio-distribution of BGN4 in the GI tract axis, 63 healthy mice were fed with a single dose of 0.2 ml of 0.9% (w/v) saline containing 0.01 g of 12 log CFU/g of lyophillized BGN4 cells by gavage. Mice were sacrificed by CO2 inhalation followed by cervical dislocation at 3, 6, 9, 12, 15, 18, 21, 24 h post inoculation (n=6-7 mice per time point).

2.7.2.2. Sample collection

The samples of stomach, small intestine, cecum, and large intestine were excised and contents of each time point (approximately 0.1-0.5 g) were immediately weighed for conversion followed by quantification. Samples were collected separately and stored at -70℃ until further processing. Also, fecal samples of each time point were collected for comparing the amount of BGN4.

2.8. Quantitative assay

The concentration of BGN4 in each sample was calculated by comparing the threshold cycle (C_T) of the sample with that of the standard curve according to the instrument manual [14]. The concentration of total bacteria, *Lactobacillus* and *Bifidobacterium* were calculated using relative quantification method of real time PCR because dilution factors were different between protocols of DNA extraction from pure cultures and from feces. The oligonucleotide primers and PCR conditions used for the assessment of the amount of BGN4, total bacteria, *Lactobacillus* and *Bifidobacterium* are listed in Table 3. For real time PCR, SYBR Green qPCR mix (2X) (Takara, Japan) was used and the samples were amplified by Thermal Cycler Dice Real Time System Single (Takara, Japan).

Table 3. Primer sequences used in this study

Target bacteria	Primer sequence	Annealing(℃)	Extension(℃)	Cycle	Product size (bp)	Reference
B. bifidum BGN4	F-BGN4 5'-TTCGAGCGACTTCAGTTGAC-3' R-BGN4 5'-TTGCATTTCAGTCACCCACC-3'	63	72	40	200	This study
Universal	F-Uni 5'-GTGSTGCAYGGYYGTCGTCA-3' R-Uni 5'-ACGTCRTCCMCNCCTTCCTC-3'	60	No	40	147-178	[21]
Bifidobacterium spp.	F-Bifid 5'-TCGCGTCYGGTGTGAAAG-3' R-Bifid 5'-GGTGTTCTTCCCGATATCTACA-3'	60	No	40	128	[21]
Lactobacillus spp.	F-Lac 5'-AGCAGTAGGGAATCTTCCA-3' R-Lac 5'-CACCGCTACACATGGAG-3'	60	No	40	341	[22]

3. Statistical analysis

Data are expressed as mean ± standard deviation. Time-dependent BGN4 changes in the feces and contents of the GI tracts after treatments were analyzed using SPSS ver 22.0 software (SPSS Inc.), which were subjected to a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Within given region, differences between two groups were determined with the student's t-test. P values of <0.05 were classified as statistically significant.

4. Results

4.1. Search for potential BGN4 specific sequence and primers

When the whole genome sequence data was compared with those of various *Bifidobacterium* strains, a 671bp, including Xenobiotic Response Element (XRE) type transcriptional regulator gene, was highly specific to BGN4 (GenBank accession No. NC_017999, 1392770-1393440). The primer set for strain specific detection was designed targeting this sequence. A final product size of this primer set was 200 bp which was an appropriate size for real time PCR analysis. Selected primer set was confirmed to be complimentary with the target sequence but not with the other strains. In addition, we checked for mismatches and dimers of the primers.

Specificity test for this primer set by conventional PCR using 30 reference strains proved that a single band of 200 bp was obtained only from BGN4 DNA (Fig. 1). Furthermore, florescence intensities were positive only for BGN4 in real time PCR analysis. In silico PCR amplification (http://insilico.ehu.es/PCR/) against all reported *Bifidobacterium* genomes also showed no predicted amplicon.

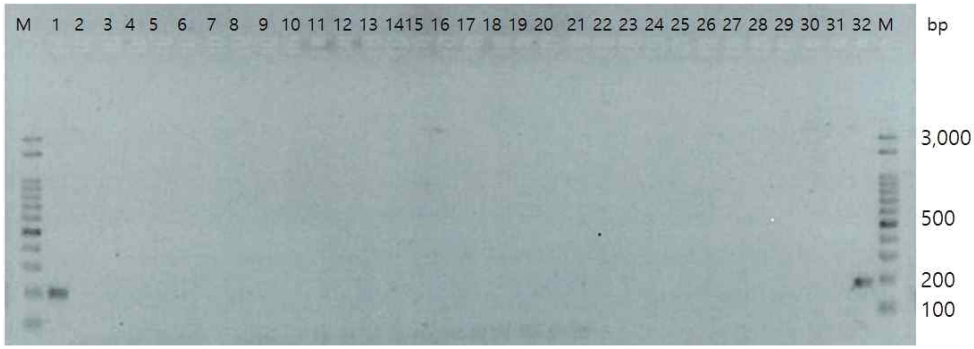


Fig. 1 Conventional PCR using BGN4 specific primer sets

Lane 1-32: BGN4, KCTC 3202, 3418, 3440, *B. longum* RD01, RD03, RD47, RD65, RD72, BORI, *B. adolescentis* KCTC 3567, *B. angulatum* KCTC 3236, *B. animalis* KCTC 3219, *B. breve* KCTC 3419, *B. infantis* KCTC 3249, *B. catenulatum* KCTC 3221, *B. thermophilum* KCCM 12097, *B. pseudocatenulatum* G4 KCTC 3223, *Lactobacillus acidophilus* KCTC 3164, KCTC 3145, *L. plantarum* KFRI 708, *L. rhamnosus* KCTC 3237, *L. casei* KFRI 699, *L. cremoris* ATCC 19257, *L. bulgaricus* KCTC 3186, *L. brevis* GABA 100, *Lactococcus lactis* subsp. *lactis* KCTC 2013, *Enterococcus faecium* KCTC 13225, *E. faecalis* KCTC 2012, *Streptococcus salivarius* subsp. *thermohilus* KCTC 3779, dimer and BGN4 respectively.

M : 3,000bp size marker.

4.2. Standard curves and detection limits

Standard curves were assembled by plotting cycle threshold (C_T) versus equivalent log cell numbers (Fig. 2). The PCR efficiencies of each standard, determined by the slope of the standard curves, were calculated based on the equation [23]. Amplification efficiencies E were approximately 99% for each standard. Based on qPCR results, the detection limit of pure culture standard was 2 log CFU/ml. When BGN4 were added to the fecal samples at concentration of 3−9 log CFU/g, detection limit was 5 log CFU/g feces.

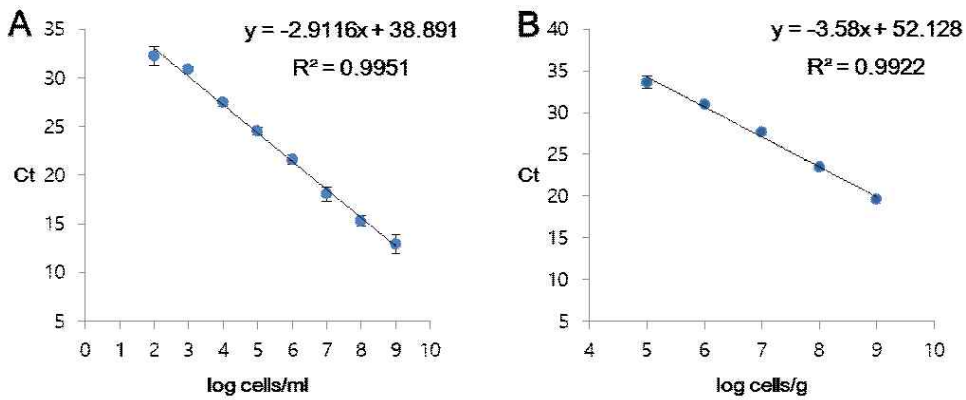


Fig. 2 Standard curves and detection limits

(A) Standard curve obtained from serial dilution of BGN4 genomic DNA, (B) Standard curve obtained from fecal-based standard spiked with serial dilution of BGN4. Each datum dot represents the average cell count from triplet. Error bars indicate standard deviations.

4.3. Retention of BGN4 in mouse gut

To estimate the retention of BGN4 in mouse gut, the amount of BGN4 in the fecal samples were quantified following 7 days (Fig. 3). The BGN4 cell numbers were below the detection limit in every sample prior to administration. The maximum cell count was 10.06 (±0.46) log CFU/g and 10.19 (±0.31) log CFU/g at day 1 (24 h post administration) for each group. Detected cell counts decreased continuously and reached to 5.23 (±0.35) log CFU/g and 6.21 (±0.58) log CFU/g at day 7, respectively.

The cell counts of MP group were significantly higher than that of LG group at every time points, except for the 1st day. The difference between the two experimental groups was getting widen from day 2 to day 7 (Fig. 3).

BGN4 cell counts in feces of live cell gavage group (LG) were higher than dead cell treatment group (DG) in general. DG group and LG group showed significant differences at day 2, day 3 and day 4 (Fig. 4).

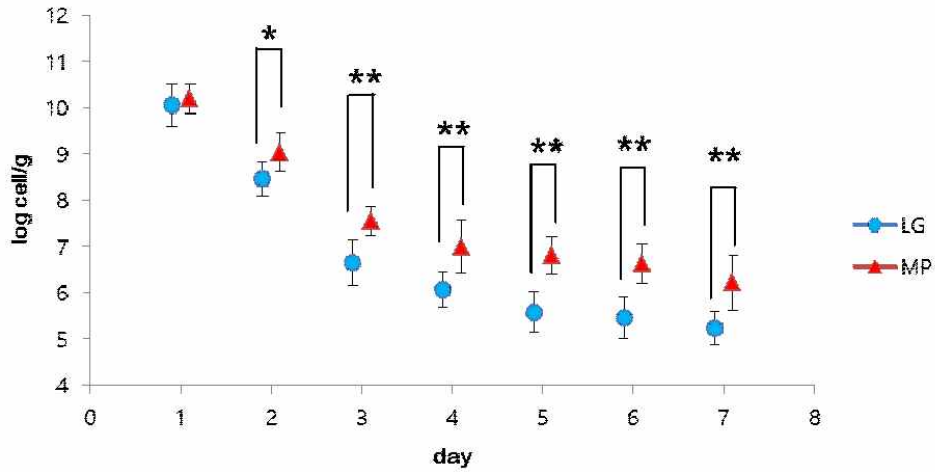


Fig. 3 Retention time and cell counts in feces

Fecal samples of each group were analyzed using real time PCR and target cell count was quantified. Each datum dot represents the average cell count from 7 rats. Error bars indicate standard deviations. Student's t test was used to determine the difference between two groups. * $P< 0.05$, ** $P< 0.01$.

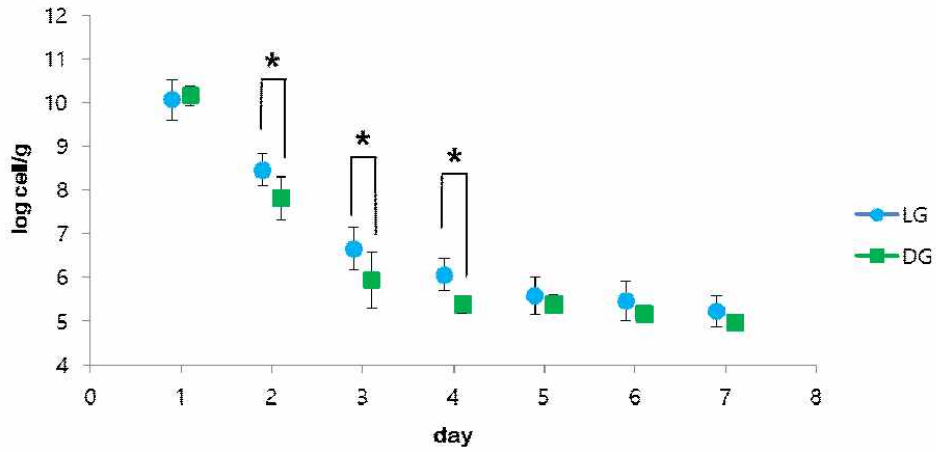


Fig. 4 Retention time and cell counts of BGN4 after the administration of live or dead cells

Fecal samples of each group were analyzed using real time PCR and target cell count was quantified. Each datum dot represents the average cell count from 7 rats. Error bars indicate standard deviations. Student's t test was used to determine the difference between two groups. * $P< 0.05$.

4.4. Cell counts comparison between single serving group and successive ingestion group

In the single serving group, mice were ingested a single dose of 0.2 ml of 0.9% (w/v) saline containing 0.01 g of 12 log CFU/g of lyophilized BGN4 cells. In a successive oral administration group, they had the same amount of BGN4 cells for 7 days.

The number of BGN4 in the feces of mice ingested a single serving was confirmed to be 5.58 (±0.47) log CFU/g at 3 h and reached maximum of 10.27 (±0.08) log CFU/g at 9 h. Cell numbers subsequently decreased to 5.35 (±1.25) log CFU/g at 24 h. On the other hand, the number of BGN4 in the feces of mice ingested for 7 days was confirmed as 7.75 (±0.6) log CFU/g at 3 h and reached maximum of 10.37 (±0.02) log CFU/g at 12 h. At last, 7.52 (±0.35) log CFU/g cell number was detected at 24 h.

There was no significant difference between two groups at 6, 9, and 21 h post administration. However, there were significant differences between two groups at 3, 12, 15, 18 and 24 h post administration ($P < 0.05$ at 18 h and $P < 0.01$ at 3, 12, 15 and 24 h). At 27 h, 5.68 (±0.88) log CFU/g was detected only in the successive administration group. (Fig. 5).

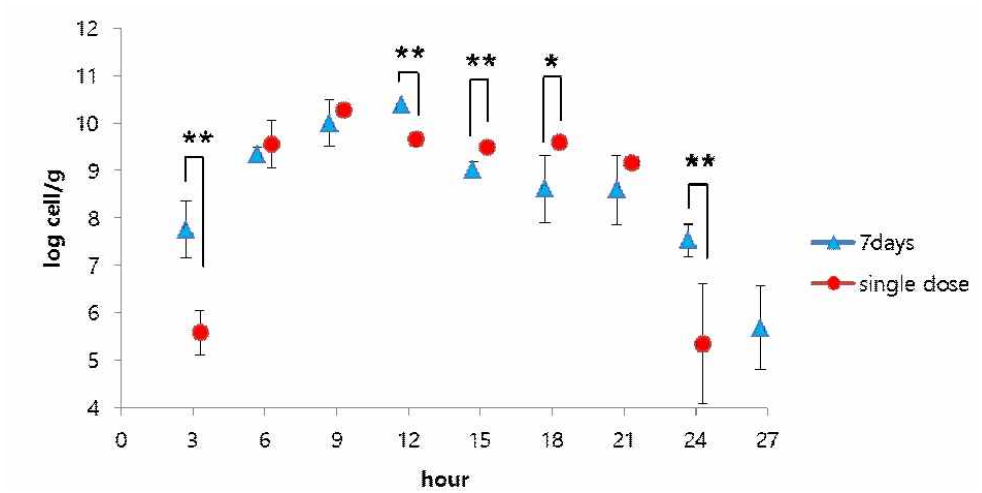


Fig. 5 Cell counts comparison between the single serving group and the successive ingestion group

▲ : a group administered for 7 days, ● : a group administered single dose. Fecal samples of each group were analyzed using real time PCR and target cell count was quantified. Each datum dot represents the average cell count from 7 rats. Error bars indicate standard deviations. Student's t test was used to determine the difference between two groups. * $P< 0.05$, ** $P< 0.01$.

4.5. The analysis of the composition of fecal microflora

Total bacteria and *Lactobacillus* was not significantly different between the control and the administered group at every time points. On the other hand, the number of *Bifidobacterium* was increased considerably in the LG group (Fig. 6). The fecal *Bifidobacterium* level was under the detection limit in the control group and in all groups prior to administration. However, after administration of BGN4 (LG-BGN4), *Bifidobacterium* cell counts were higher than 10 log CFU/g at every time points except for day 2 (9.42±0.57 CFU/g).

Though the cell counts of BGN4 decreased gradually after probiotic treatment, *Bifidobacterium* still maintained at a high level until 7 days post administration in LG group. In case of DG group, BGN4 cell count tended to decline as shown in LG group. However, no significant increase of *Bifidobacterium* was detected in DG group (Fig. 6).

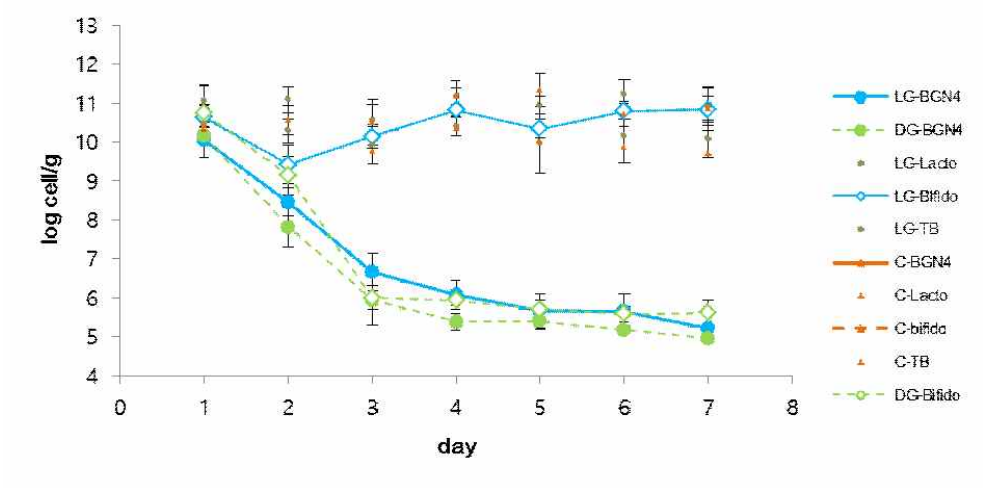


Fig. 6 Fecal microbial composition analysis

Absolute and relative qPCR quantification were conducted to analyze dominant and subdominant microbes in fecal samples. Each datum dot represents the average cell count from 7 rats. Error bars indicate standard deviations.

4.6. The bio-distribution of BGN4 in mouse GI tracts

The bio-distribution of ingested BGN4 in the longitudinal gut axis such as stomach, small intestine, cecum, large intestine and feces are shown in Fig. 7. The BGN4 numbers were below detection limit in every sample prior to administration of BGN4.

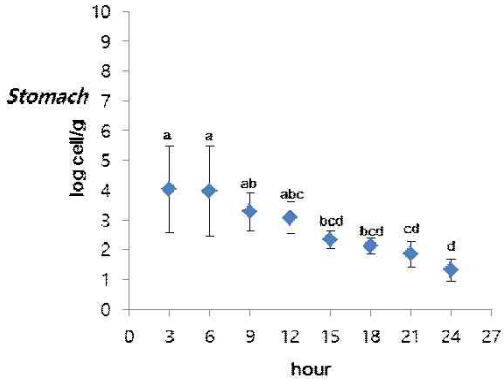
In stomach contents (Fig. 7A), the cell counts of BGN4 were the highest at 3 h after the gavage conducted, which was 4.02 (±1.46) log CFU/g.

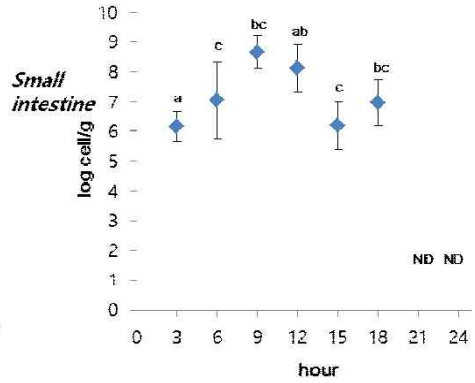
In the small intestine (Fig. 7B), the cell counts were the highest at 9 h after gavage (8.66±0.54 log CFU/g). Interestingly, BGN4 cell numbers were under the detection limit at 21 h and 24 h in the small intestine after gavage.

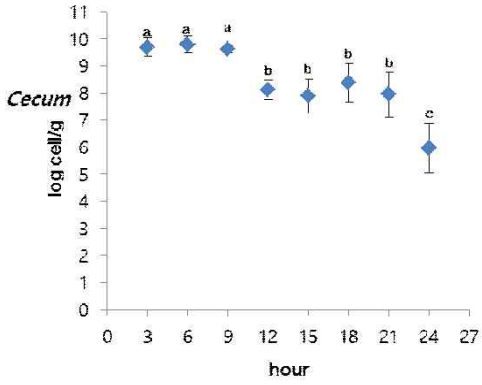
From 3 h after gavage to 21 h, BGN4 cell counts in cecum were maintained at a high level, with a cell count of ≥ 7 log CFU/g. At 24 h, 5.95 (±0.91) log CFU/g was detected (Fig. 7C).

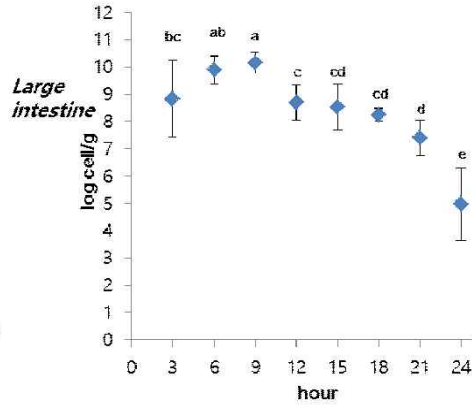
The maximum cell count in large intestine was 10.15 (±0.38) log CFU/g at 9 h post gavage. Like fecal samples, BGN4 cell counts in the large intestine luminal contents continuously decreased until 24 h with 4.96 (±1.31) log CFU/g (Fig. 7D).

In the fecal samples, BGN4 cell counts were sharply increased from 5.58 (±0.47) log CFU/g at 3 h to 10.22 (±0.08) log CFU/g with a maximum level at 9 h (Fig. 7E).


Stomach
log cell/g
hour
a a ab abc bcd bcd cd d


Small intestine
log cell/g
hour
a c bc ab c bc
ND ND


Cecum
log cell/g
hour
a a a b b b b c


Large intestine
log cell/g
hour
bc ab a c cd cd d e

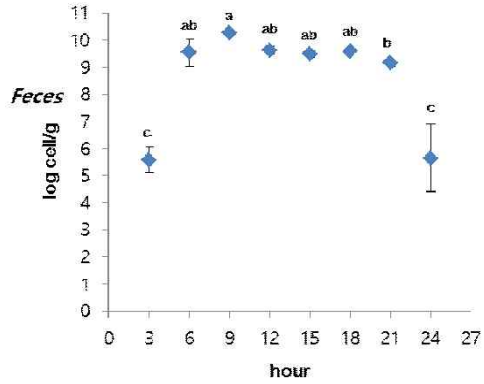

Feces
log cell/g
hour
c ab a ab ab ab b c

Fig. 7 The bio-distribution of BGN4 in mouse GI tracts

Each datum dot means the average cell count (log cell counts per gram intestinal contents or feces) from 7 rats. Error bars indicate standard deviations. Statistical significance was determined by the Duncan's multiple range test. Different superscript letters in each panel imply significant differences.

5. Discussion

BGN4 has been reported as promising probiotic bacteria with many positive research results. To achieve the expected effects, BGN4 has to survive in a harsh GI tract environment. The survival capacity, the distribution, and the passage tendency of BGN4 must be verified at the strain level because various probiotic characteristics are strain-dependent [24]. Therefore the survival capacity in the GI tract and the luminal colonization of BGN4 warrants a thorough investigation. Furthermore, there were no observations on how the intake of live or dead BGN4 differently influences the balance of the mouse intestinal microflora. In this study, we observed the alteration of the intestinal microflora composition after administration of BGN4 for 7 days using real time PCR.

However, there was no applicable method for detecting bacterial cell counts of BGN4 for quantification. Selective medium for the differentiation of BGN4 from other *Bifidobacterium* strains was absent [25]. Therefore we established a strain specific detection method based on DNA sequence to quantify target bacteria in feces and GI tract contents by using real time PCR.

The detection limits of our standard curves were quite similar to those in previously reported real time PCR assay for species or strain specific detection [12,14].

Next, we analyzed how the different dietary methods could affect the retention time and viability of BGN4. To our knowledge, *Bifidobacterium* is reported to be less acid resistant than *Lactobacillus*, especially under a gastric juice environment [26]. However the acidic environment of the stomach is likely to be neutralized by foods. Thus, the ingested probiotic bacteria may survive easily in the stomach when consumed together with foods [27]. Likewise, the consumption method of the MP group in which the BGN4 was mixed with diet might have promoted the viability and the colonization of BGN4.

The feeding periods of BGN4 may also be related to the viable cells of BGN4 detected in the fecal samples. When the feces of successive oral administration for 7 days were compared with a single gavage, BGN4 accumulates and colonizes in the gut efficiently in the successive administration. Previously, it was also shown that oral administration for 7 days could increase cell counts at a higher level in feces than the single dose group [12]. Moreover, each group has the highest cell counts at 9 h

and 12 h after oral administration, which coincides well with the physiological transit time of mice [28].

Furthermore, the passage and the colonization of BGN4 were assessed in the GI tracts and feces after administration. This in vivo study is more informative than a simulation study of an artificial gastric environment to demonstrate the survival capacity of probiotic bacteria [29–32]. In the stomach, there were less than 4 log CFU/g cells on average, probably because the environment of the stomach is harsh for probiotic bacteria to survive or colonize. Interestingly, BGN4 was not observed in the small intestine at 21 and 24 h post single administration. The bile and the pancreatic enzymes could affect the viability of BGN4 in the small intestine. Moreover, the intestinal peristalsis play important roles in preventing bacterial colonization in the small intestine [33]. As well known, the probiotic *Bifidobacterium* localize preferably in the cecum and the colon where is strictly anaerobic. The maximum cell counts of the cecum and the large intestine were high in comparison with other sessions of GI tracts. According to data obtained, the cecum was found to be the principle habitat for *Bifidobacterium* in mice. Cell adhesion and the colonization of BGN4 in the cecum and colon may vary depending on various environmental factors such as pH, nutrients, and the presence of proteolytic enzymes

and lipoteichoic acids in the bacterial cells [8]. At 9 h, BGN4 cell counts in the stool samples and the large intestine were slightly higher than the number originally administered which result imply successful colonization and proliferation of BGN4 in the mouse GI tracts. It has been shown that bifidobacteria survived passage to the large intestine, with 29.7% of ingested cells being able to reach the colon [34].

Substantiating a health benefit related to the consumption of probiotics, one commonly considered parameter is whether a specific probiotic strain can have impact on the composition of host microflora [35]. According to the previous studies, live probiotic cells can modify the gastrointestinal microflora directly, whereas the component of dead cells can only exert an anti-inflammatory response in the GI tract and act as biological response modifiers [36]. In this respect, we monitored the change of intestinal microflora after the administration of live or dead BGN4. Interestingly, before the administration, we could not detect *Bifidobacterium* in the fecal samples using real time PCR because the cell counts were under the detection limit level. However, according to the previous studies, *Bifidobacterium* and *Lactobacillus* were reported to be abundant in mouse fecal samples [37, 38]. This discrepancy might have come from the difference of mice breeding facilities [39]. In other words, the

different mice strains may have different microbial composition in their guts. Furthermore, diet composition may differently affect gut microflora [40].

Surprisingly, microbial composition of mouse fecal samples was changed only when the mice administered live BGN4 cells. The numerical increase of *Bifidobacterium* in feces after administration was only detected in the LG group although BGN4 cells decreased time-dependently in both the LG group and the DG group. This finding disagrees with the existing study that the normal intestinal microbes have a strong resistance against the colonization by newly ingested bacteria [41]. Previously, probiotic bacteria affected the intestine environment if their population reaches a minimum level of 6 log CFU/g to 8 log CFU/g [41]. Therefore, the ingestion of 10 log CFU/g BGN4 for 7 days might have overcome the resistance against the colonization of the newly administered bacteria. The intestinal microflora of the LG group mice might have been changed by the high dose administrations. Since *Bifidobacterium* reduce the intestinal endotoxin level and enhance the mucosal barrier function, this result could have a positive meaning [42].

6. Conclusion

In this research, the strain specific detection and quantification method for BGN4 was established through comparative genomics. Using this method, the retention time, the retention amount, the bio-distribution, and changes of intestinal microflora after adminstration were identified. In summary, this study would demonstrate that probiotic properties of BGN4 can be promising, as it survived in mouse GI tracts for 7 days. Furthermore, BGN4 could positively change the composition of microflora by increasing the number of *Bifidobacterium*. To broaden our knowledge about probiotic properties of BGN4, investigations are needed to identify the biological mechanism of interactions between BGN4 and gut microflora.

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국문초록

*Bifidobacterium*은 장내 환경에 유익한 영향을 주어 기능성 식품으로 자주 이용되어왔다. 상업 균 주인 *Bifidobacterium bifidum* BGN4의 경우 다양한 임상 연구를 통하여 그 효과가 입증되었으나 유전체를 이용한 검출 방법이 부재하여 균 주를 특이적으로 검출해 낼 수 있는 방법이 미비하였다. 따라서 이번 연구를 통해 *B. bifidum* BGN4의 유천체를 다른 유산균들과 비교하여 특이적으로 검출할 수 있는 방법을 수립하였다. 프라이머 세트는 real time PCR 분석에 이용될 수 있도록 최종 증폭 산물의 사이즈를 200 bp로 하여 설계되었으며, 이는 ICR mouse의 분변과 장관 내 내용물에서 해당 균주를 빠르고 정확하게 검출 해 내는 것에 사용되었다. 분석 결과 *B. bifidum* BGN4 의 경우 장관내의 생존능이 비교적 강한 것으로 확인되었다. 또한 *B. bifidum* BGN4의 장관 내 흐름을 가시적으로 확인하였으며, 해당 균주를 섭취한 후의 변속 균 총을 확인 하였다. 그 결과 생 균 상태의 *B. bifidum* BGN4를 섭취할 경우 *B. bifidum* BGN4뿐만 아니라 장관 내 *Bifidobacterium*의 우점화를 크게 향상 시키는 긍정적인 효과를 확인할 수 있었다.

주요어 : *Bifidobacterium bifidum* BGN4, 중합효소연쇄반응, 우점화

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