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# Biodistribution of a Promising Probiotic, *Bifidobacterium longum* subsp. *longum* Strain BBMN68, in the Rat Gut

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*Bifidobacterium longum* subsp. *longum* BBMN68, isolated from centenarians in Guangxi, China, has been proved to be a promising probiotic strain for its health benefits. In this study, the biodistribution of this strain in the rat gut was first investigated using the quantitative real-time PCR assay and propidium monoazide. Strain-specific primers were originally designed based on the BBMN68 genome sequence. Healthy rats were orally inoculated with either a single dose of BBMN68 ($10^{10}$ colony-forming units/kg), or with one dose per day for 7 days and bacterial concentrations were analyzed in detail from the intestinal contents and feces of four different gut locations, including stomach, small intestine, colon, and rectum. Results indicated that strain BBMN68 could overcome the rigors of passage through the upper gastrointestinal tract and transiently accumulate in the colon, even though survival in the stomach and small intestine was not high. A good level of BBMN8 could stay *in vivo* for 72 h following a 7-day oral administration, and a daily administration is suggested for a considerable and continuous population of BBMN68 to be maintained in the host intestine.

**Keywords:** *B. longum* BBMN68, *in vivo*, qPCR, retention time, survival

## Introduction

There has been a recent upsurge in demand for probiotics in response to the large number of reports on their health-promoting functions [33]. The intake of probiotics as dietary supplements has been proved effective in modulating the gut microbiota [36], enhancing the immune system [10], and reducing the levels of cholesterol and blood pressure [35]. Therapeutically, probiotics can also alleviate lactose intolerance and diarrhea [24], prevent allergic diseases and irritable bowel syndrome, even certain cancers [37].

To achieve these beneficial effects, a minimum level of $10^6$–$10^8$ viable probiotic cells per gram of intestinal contents is required [17]. Thus, to reach a sufficiently therapeutic number, probiotic bacteria must be resistant to low pH in the stomach, bile salts in the small intestine, and various digestive enzymes during passage through the gastrointestinal (GI) tract [4]. The ability of probiotic bacteria to survive and accumulate in the GI tract varies considerably amongst species, and even between strains [5]. For selection and efficacy evaluation of probiotic strains, it is therefore necessary to know their biodistribution (survival and retention) in the GI tract.

Numerous studies have been conducted *in vitro* using simulated gut conditions to investigate the survival of probiotic bacteria in the GI tract [2, 4, 13, 16, 27]. However, *in vivo* studies are usually much more informative. To monitor a probiotic strain *in vivo*, a strain-specific method of identification is required. Moreover, it should also be able to distinguish viable from non-viable cells. The development of quantitative PCR (qPCR) methods using strain-specific primers has allowed the detection of individual probiotic strains in colonic contents or feces [28]. This technique, in conjunction with propidium monoazide (PMA) treatment, also allows the discrimination of viable cells from dead cells [7]. The PMA-qPCR technique has

previously been used effectively to analyze the survival of probiotics *in vivo* [7-9].

For PMA-qPCR, strain-specific primers that can distinguish the target strain from other bacteria are vital [28]. The 16S rDNA gene is often used as a target region for primer design [18–20]. However, this region can only be used for discrimination at the genus or species level owing to high sequence conservation [34]. Recently, the design of strain-specific primers has been accomplished using the random amplified polymorphic DNA (RAPD) technique [1, 8, 9, 14, 34]. This technique is useful for identifying strain-specific regions when the genome of the target strain is unavailable [14]. However, it lacks reproducibility and requires a large number of bacterial cultures for analysis [31]. Additionally, the indirect identification of bacteria is time-consuming [1]. In the current study, a new method for designing strain-specific primers was developed based on the available genetic information of a probiotic bacterial strain.

*Bifidobacterium longum* subsp. *longum* BBMN68 is a newly identified potential probiotic strain isolated from centenarians in Guangxi, China [41]. Our previous research has shown that BBMN68 can significantly boost the innate and acquired immune response in mice [41], enhance murine intestinal function [40], and promote the *in vitro* immune responses of murine macrophages [42]. The full-length genome sequence of this strain is available (GenBank: NC_014656), and subjected to KEGG annotation (http://www.genome.jp/kegg/genes.html).

Although previous studies have outlined the many probiotic properties of BBMN68, its survival and retention in the GI tract have not been determined. Therefore, the aim of the current research was to illustrate the survival and retention of ingested BBMN68 along the murine GI tract. For this purpose, a BBMN68 strain-specific primer for PMA-qPCR that would allow the identification and enumeration of viable BBMN68 *in vivo* was designed. To the best of our knowledge, this is the first examination of the passage of viable bifidobacteria through the digestive tract, and the first using genome annotation for strain-specific primer design.

## Materials and Methods

### Bacterial Strains and Culture Conditions

A total of 24 reference strains were included in the current study (Table 1). Bifidobacteria were incubated at 37°C for 12 h in de Man-Rogosa-Sharpe (MRS) broth (BD, MD, USA) supplemented with 0.05% (w/v) L-cysteine-HCl (Amresco LLC, Solon, OH, USA). Manipulations of bifidobacteria were carried out in Hungate tubes initially sparged with 99.9 vol.% $N_2$ to maintain an anaerobic environment. For colony counting, *B. longum* BBMN68 was cultivated on MRS agar (BD, MD, USA) for 48 h under anaerobic conditions maintained using an Anaerocult oxygen-depleting system (Merck, Darmstadt, Germany). Lactobacilli, streptococci, and lactococci were semi-aerobically cultured for 12 h at 37ºC in MRS broth, M17 broth (Oxoid, UK), and at 30ºC in M17 supplemented with 0.5% (w/v) glucose (Sinopharm Chemical Reagent Co., Ltd), respectively.

### Strain-Specific Primer Design

A total of 1876 genes from the BBMN68 genome were manually checked for sequence orthologs in the KEGG gene database (http://www.genome.jp/kegg/genes.html). Genes with identity below 40% were regarded as highly specific to BBMN68. FASTA sequences of these genes were then used as templates for specific primer design using Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) from the National Center of Biotechnology Information Website. The "PCR product size" was set to between 100 and 800 bp, while "Database" and "Organism" were "nr" and "bacteria," respectively. Default settings were used for all other parameters. Primers were then checked for mismatches and dimers using the Primer Premier 5 software (Premier Biosoft) and were synthesized by Invitrogen.

The specificity of candidate primers was first verified by conventional PCR using genomic DNA from 24 reference strains. Genomic DNA was extracted using a TIANamp Bacteria DNA Kit (Tiangen Biotech Ltd., China). PCR amplification was performed using a PTC-200 thermocycler (MJ Research, MA, USA) with 20 µl reaction mixture containing 1× Pfu PCR MasterMix (Tiangen

**Table 1.** Reference strains used in this study.

| | |
|---|---|
| *Bifidobacterium longum* subsp. *longum* | BLS[a], BLL1[a], BLL3[a], BLL4[a], BLL5[a], BLL6[a], BLL7[a], BLL9[a], BLL10[a], BLL12[a], BLL13[a] BB46[b], BB536[e], NCC2705[c], BBL[e] |
| *Bifidobacterium animalis* subsp. *lactis* | BB12[d] |
| *Bifidobacterium breve* | BLL11[a] |
| *Bifidobacterium adolescentis* | BBMN23[b] |
| *Streptococcus thermophiles*[e], *Lactobacillus casei*[e], *Lactobacillus delbrueckii* subsp. *bulgaricus*[e], *Lactococcus lactis* subsp. *lactis*[e], *Lactococcus lactis* subsp.*cremoris*[e], *Lactobacillus rhamnosus*[e] | |

The strains were obtained from the following sources: [a]Feces of healthy infants; [b]Feces of healthy centenarians; [c]Nestle Research Center, Lausanne, Switzerland; [d]Chr. Hansen's collection of dairy cultures, China; [e]Commercial fermented milk products.

Biotech Ltd., China), 0.5 μM each primer, 1 μl of template DNA, and 8 μl of water. The cycler conditions were as follows: initial denaturation at 94°C for 30 sec, followed by 30 cycles of 63°C for 10 sec, 72°C for 20 sec, and 72°C for 5 min. PCR amplicons were visualized by agarose gel electrophoresis.

### Animals

A total of 130 female Sprague Dawley rats (6–8 weeks old, body weight 240 ± 40 g) obtained from Vital River Laboratories were used in this study. Animals were maintained in a temperature-controlled environment (22 ± 2°C) with a 12 h light/dark cycle and with free access to water and rodent chow purchased from Vital River Laboratories. All animals were treated according to the China Laboratory Animal Care guidelines, and all experiments were approved by the Ethics Committee of China Agricultural University.

### Study Design and Sample Collection

For gavage, *B. longum* BBMN68 was cultured in MRS broth overnight, and cells were harvested by centrifugation at 1,700 ×*g* for 10 min. Cell pellets were washed with PBS buffer (pH 7.4) and resuspended in 0.9% (w/v) saline to a concentration of $1.0 \times 10^{10}$ colony-forming units (CFU) in 200 μl ($5.0 \times 10^{10}$ CFU/ml). Oral administration began after an acclimatization period of 7 days.

To study the biodistribution of BBMN68 in the gut, 120 healthy rats (260 ± 6 g) were fed with a single dose of BBMN68 ($1.0 \times 10^{10}$ CFU/kg body weight) by gavage. Rats were sacrificed by $CO_2$ inhalation followed by cervical dislocation at 10 min, 0.5, 1, 2, 3, 6, 9, 12, 18, 24, 30, and 48 h post-inoculation ($n = 10$ rats per time point). Approximately 0.3–0.5 g of the contents of the stomach, small intestine, colon, and rectum were collected separately at each time point.

To study the retention time of BBMN68 *in vivo*, 10 rats (260 ± 6 g) were fed $1.0 \times 10^{10}$ CFU/kg body weight BBMN68 each day for 7 days. Fecal samples were collected from each rat at 0, 1, 2, 3, 4, 6, 9, 11, 13, and 14 days post-gavage. Samples were weighed and immediately homogenized in anaerobic diluent [3] at a ratio of 1/6 (sample/diluent). Then, 400 μl of each diluted sample was used for DNA extraction, 200 μl was used for total cell count detection, and 200 μl was used for viable cell count detection after PMA treatment.

### PMA Treatment and DNA Extraction

Fecal samples and pure cultures of BBMN68 were treated with 70 μM PMA according to the method of Nocker *et al*. [23]. Samples on ice were then incubated for 5 min in the dark and then light-exposed for 4 min at a distance of about 20 cm from two 500 W halogen light sources. Samples were shaken occasionally to guarantee homogeneous light exposure. The PMA-treated cell pellets were preserved at −80°C until DNA extraction was performed. DNA was extracted from pure cell pellets (with or without PMA treatment) using the method described by Fujimoto *et al*. [7]. DNA was stored at −20°C until use.

### Quantitative PCR

A Techne Quantica real-time PCR detection system (Techne, Staffordshire, UK) was used for quantitative PCR analysis. Each sample was run in triplicate and contained 10 μl of SYBR Premix Ex *Taq* II (Tli RNaseH Plus; Takara, Japan), 0.5 μM each primer, 1 μl of diluted template DNA, 0.4 μl of Rox (Takara, Japan), and 7.6 μl of water. The cycler conditions were as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 65°C for 8 sec, and 72°C for 20 sec. A melting curve analysis was carried out at the end of each qPCR run to verify the amplification product. A 10-fold dilution series of DNA extracted from BBMN68 was prepared to generate a standard curve. The corresponding cycle threshold values ($C_t$) were plotted against the logarithm of each of these known concentrations of bacterial cells ($10^3$–$10^9$ CFU/ml). To determine the assay detection limit, fecal samples were spiked with a 10-fold serial dilution of BBMN68 at concentrations ranging from $10^4$–$10^{10}$ CFU/g feces prior to DNA extraction. Regression lines were generated between the actual number of viable BBMN68 added to feces and that determined by qPCR with/without PMA treatment. The amplification efficiency for the pure culture, determined by the slope of the standard curves, was calculated based on the equation $E = (10^{-1/slope}–1) \times 100$.

### Statistical Analysis

Results are expressed as the mean ± standard deviation. Changes in the BBMN68 concentration following ingestion were analyzed using SPSS ver. 13.0 software (SPSS Inc.), which were subjected to a one-way analysis of variance followed by the LSD-Dunn test. *P* values of <0.05 were classified as statistically significant.

## Results

### Search for Potential BBMN68-Specific Genes

KEGG database analysis of the BBMN68 genome showed that four out of the 1,876 genes were highly specific (<40% sequence similarity) to BBMN68. These genes were BBMN68_483, BBMN68_549, BBMN68_564, and BBMN68_1003, and all were predicted to code for hypothetical proteins of unknown function. A total of 37 primer sets were designed to amplify these gene regions, of which only five were confirmed to be negative for mismatches and dimers and therefore appropriate for PCR analysis (listed in Table 2).

### Specificity of the Primer Sets

Specificity testing of the candidate primers by conventional PCR using the 24 reference strains showed that a single band of approximately 190 bp was obtained from *B. longum* BBMN68 DNA using the p549-2 primer pair (Fig. 1), whereas nonspecific amplification was observed for the other four primer sets (data not shown). The qPCR assay using the primer pair p549-2 also gave a positive result for

**Table 2.** Primers designed based on the BBMN68 genomic sequence.

| Primer | Position | Gene definition | Identity (%) | Amplicon (bp) | Oligonucleotide sequence (5′-3′) |
|---|---|---|---|---|---|
| p483-7 | 483 | Hypothetical protein | <0.352 | 158 | F: TCGGGCGAAACGGCGATAGC<br>R: CTGACGCCGTGTCCGTAGGC |
| p549-2 | 549 | Hypothetical protein | <0.385 | 190 | F: GCGCAGGTCCGCAGCAACTA<br>R: TGGCTCCGGCTGCTATTGCG |
| p549-6 | 549 | Hypothetical protein | <0.385 | 167 | F: CGATTCCTGCCGGGGAGACG<br>R: GGCTCCGGCTGCTATTGCGG |
| p564-10 | 564 | Hypothetical protein | <0.384 | 455 | F: AAGAAGTGCGCGCGAAGGCC<br>R: AGCACGGCAGCCGCATACTT |
| p1003-1 | 1003 | Hypothetical protein | <0.381 | 100 | F: CGGCACTACTTGACAAGCG<br>R: CTCCAACGGGAACCGACATC |

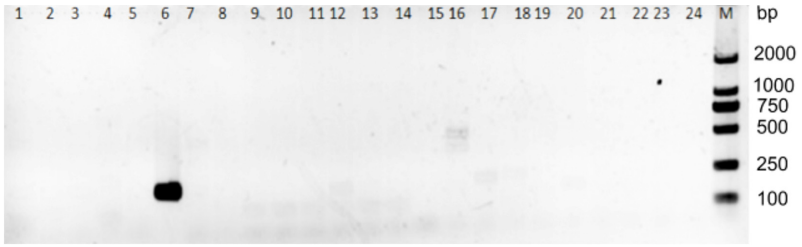


**Fig. 1.** Conventional PCR using BBMN68-specific primer pair p549-2.
Lanes 1–24: BLS, BLL1, BB12, BB23, BB46, BBMN68, NCC2705, BB536, BLL3, BLL4, BLL5, BLL6, BLL7, BLL9, BLL10, BLL11, BLL12, BLL13, *Streptococcus thermophilus*, *Lactobacillus casei*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, and *Lactobacillus rhamnosus*, respectively. M: 2,000 bp size marker.

*B. longum* BBMN68, with negative amplification observed for any of the non-target *Bifidobacterium* strains. Furthermore, we tested seven DNA samples extracted from the feces of healthy rats, which were unlikely to contain BBMN68, and found no amplification products (data not shown).

### Standard Curves and Limit of Detection for Fecal Samples

The amplification plot generated a slope of −3.255, with a correlation coefficient of 0.996 and an efficiency of 102% (Fig. 2). When BBMN68 was added to feces at concentrations of 4–10 log CFU/g, viable cells were detectable at levels of 5–10 log CFU/g feces (r = 0.9925; Fig. 3A). Fecal samples that were not treated with PMA (total cells) showed the same detection limit (r = 0.9963; Fig. 3B).

### Survival and Retention of *B. longum* BBMN68 in Rat GI Tract

The biodistribution of ingested BBMN68 in the rat GI tract is shown in Fig. 4. The total or viable BBMN68 numbers were below the detection limit in every sample prior to gavage. In the stomach contents (Fig. 4A), the total cell count and viable cell count of BBMN68 were highest at 10 min (immediately after the gavage was conducted), at which time the viable count was 8.45 (±0.47) log CFU/g, accounting for 44.7% of the total cell count.

In the small intestine (Fig. 4B), the maximum viable count

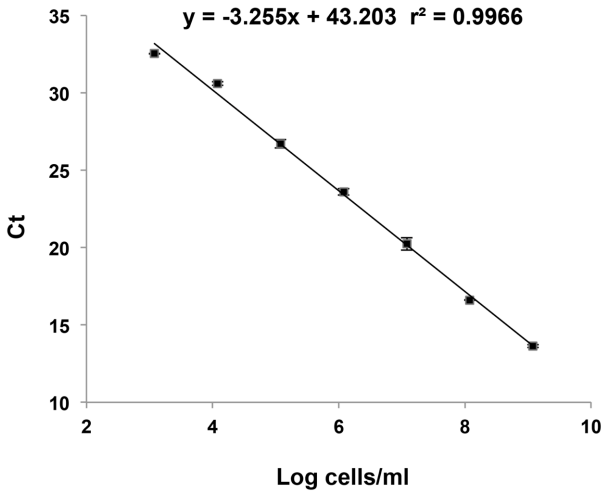


**Fig. 2.** Standard curve obtained from serially diluted BBMN68 genomic DNA.
$C_t$ values are the average of three replicates. Error bars represent standard deviation.

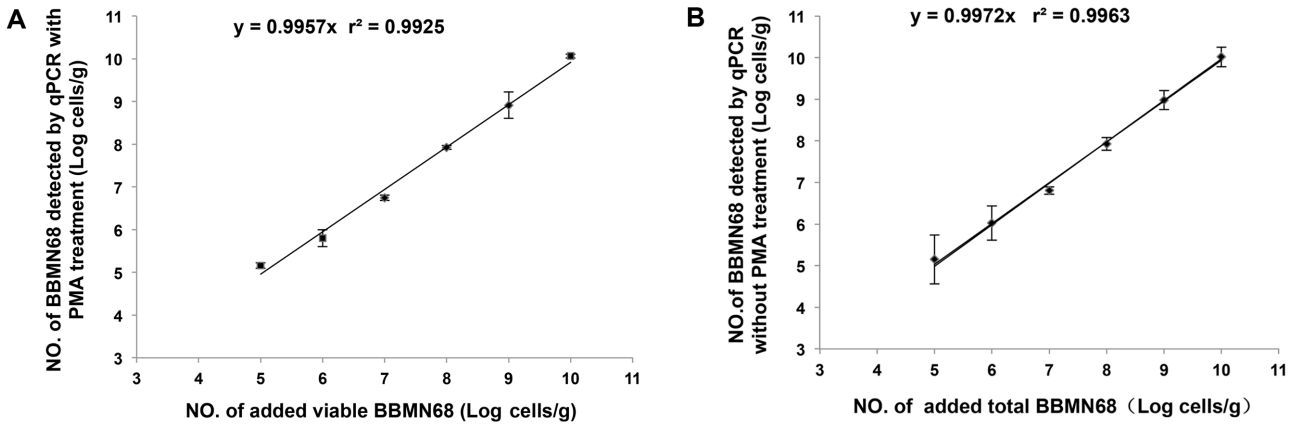


**Fig. 3.** Correlation between the number of viable BBMN68 cells added to fecal samples and that determined by quantitative real-time PCR (qPCR).

(**A**) With PMA treatment. (**B**) Without PMA treatment. Error bars represent standard deviations from three independent trials.

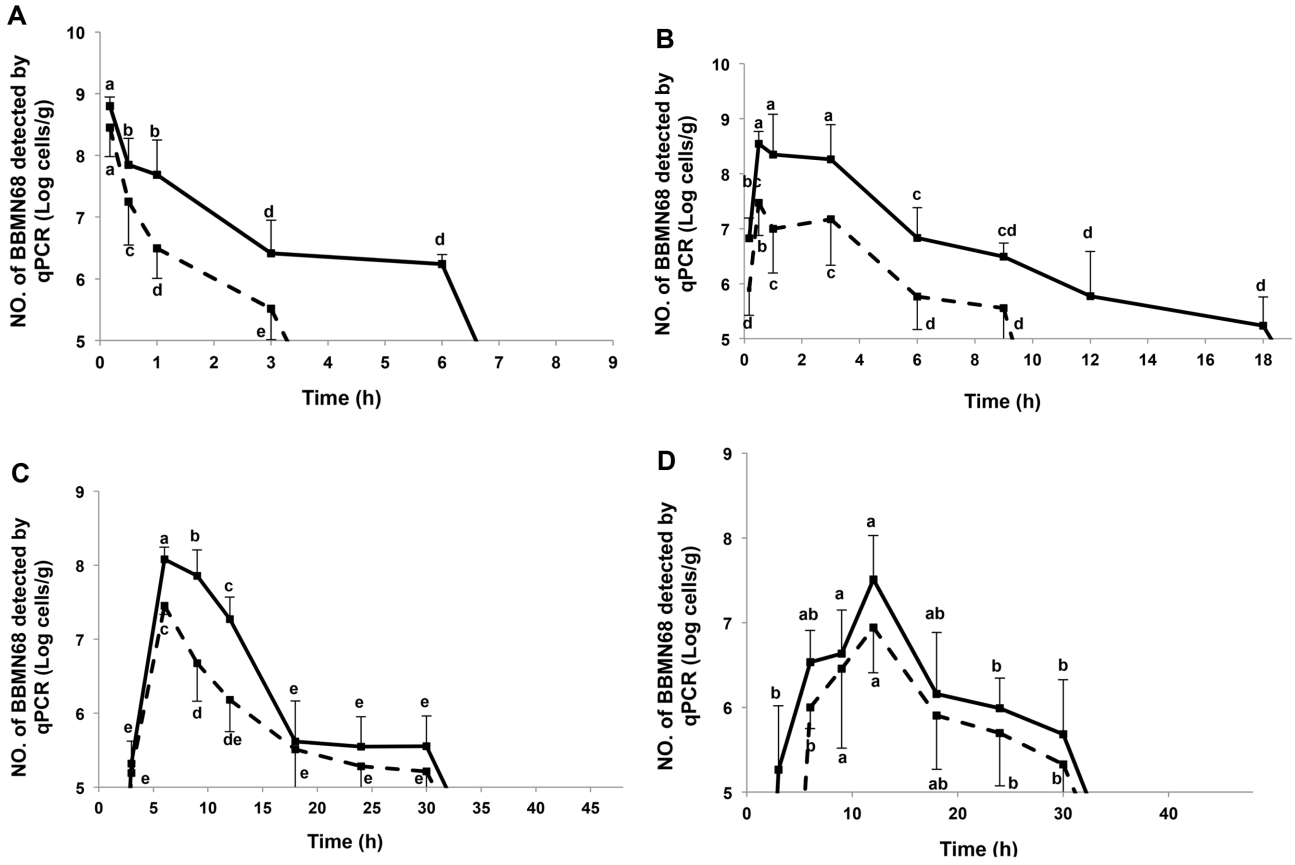


**Fig. 4.** Survival of BBMN68 in the GI tract.

(**A**) Stomach. (**B**) Small intestine. (**C**) Colon. (**D**) Rectum. Black line: total cell number (viable plus dead); dashed line: viable cell number. Each datum point represents the average viable count from ten rats. Error bars indicate standard deviations. Different superscript letters in each panel for the same day indicate significant differences.

was recorded at 0.5 h post-gavage, with a cell count of 7.47 (±0.59) log CFU/g . The highest rate of survival was 11.7% at 10 min post-gavage. BBMN68 cells were not detected in the colon until 3 h post-gavage (Fig. 4C). The

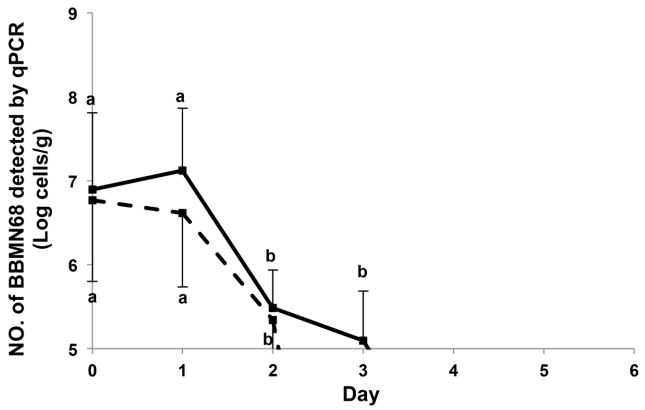


**Fig. 5.** Retention of BBMN68 in the rat gastrointestinal tract following a 7-day oral administration regimen.

Solid line: total cell number (viable plus dead); dashed line: viable cell number. Each datum point represents the average viable count from ten rats. Error bars indicate standard deviations. Different superscript letters in each panel for the same day indicate significant differences.

maximum total cell count and viable cell count in the colon was 8.08 (±0.16) log CFU/g and 7.45 (±0.1) log CFU/g, respectively, at 6 h post-gavage. The maximum survival in the colon was 79.4% at 18 h.

For rectum samples (Fig. 4D), the maximum total cell count and viable cell count were recorded at 12 h post-gavage, at which time viable cell numbers reached 6.94 (±0.54) log CFU/g. The maximum survival of BBMN68 was 66.1% at 9 h post-gavage.

### Retention of Gavaged *B. longum* BBMN68 in Rat Gut

The retention of BBMN68 in the gastrointestinal tract following 7 days of oral administration is shown in Fig. 5. The total and viable BBMN68 cell numbers were below the detection limit in every sample prior to gavage. The maximum viable cell count was 6.8 log CFU/g at day 0, with similar survival rates observed on both days 1 and 2 post-administration (74.1% and 72.4%, respectively). Viable cell counts reduced to 5.5 log CFU/g at 2 days post-gavage, and were below the detection limit by 3 days post-gavage. Total cell counts dropped below the level of detection by 4 days post-gavage.

## Discussion

To achieve a therapeutic level within the host, a probiotic bacterium must be able to survive in the gastrointestinal tract. *B. longum* BBMN68 is a human intestinal bacterium that has recently been characterized as a promising probiotic [40]. However, its survival and retention *in vivo* following consumption are not known. In this study, the biodistribution of *B. longum* BBMN68 in the GI tract was analyzed using a murine model combined with daily oral administration. To detect BBMN68 in intestinal contents and feces, a specific and quantifiable method that could also distinguish viable and dead cells was needed. In previous studies, culture plating was used for calculating bacterial survival [29, 32]. However, this approach does not support precise and reproducible results [6]. Moreover, it cannot be used for identifying BBMN68 because no selective medium is available for differentiating between bifidobacterial species [26]. Real-time qPCR is widely used for efficiently quantifying probiotic strains in fecal samples. Moreover, the combination of qPCR with PMA treatment can successfully target viable bacteria without harming the bacterial cells [22]. Thus, PMA-qPCR was applied in the current study for enumeration of BBMN68 *in vivo*.

This study also confirmed that genome annotation is a good way of identifying strain-specific genes that can be used as unique amplification targets for qPCR analysis. Using BBMN68 genome data that had previously been annotated in the KEGG database [12, 39], genes unique to strain BBMN68 were easily targeted through homolog analysis in the database. An identity threshold of 0.4 (< 40% identity) was selected for identification of unique genes. The designed primers proved highly specific for BBMN68, with no reaction products for 15 closely related strains of the same subspecies. The limit of detection for the qPCR assay was similar to that of Fujimoto *et al*. [9], but the upper limit was extended to $10^{10}$ cells per gram of feces. In contrast to the RAPD technique, this new procedure for strain-level primer design was much quicker and simpler for identifying unique genes in the target strain, and avoided the need for large-scale bacterial isolation, gel pattern analysis, and strain identification. The only limitation of this method is a lack of homology data for some species, although it should be useful for most microbial species from the GI tract. However, the increasing number of sequenced genomes from environmental microbial species means that this limitation is becoming less of an issue.

Studies on the survival and colonization of probiotics in the GI tract can give an indication of the potential probiotic attributes of a bacterial strain and its function efficacy. Given that these properties are often strain specific, tests must be conducted at the strain level [30]. For bifidobacterial strains, almost all previous survival and retention data are based on simulated gastric conditions or fecal samples, which can provide no more than an estimate of the situation in the GI tract. In the present study, for the first

time, the biodistribution of *B. longum* subsp. *longum* strain BBMN68 was evaluated in detail during its transit through the stomach, small intestine, colon, and rectum *in vivo*. Survival data for these four GI loci indicated that BBMN68 can survive passage through the upper GI tract, and transiently accumulate in the colon.

Previous studies have reported that the acid tolerance of bifidobacteria is generally weak, with the exception of *Bifidobacterium animalis* [21]. The current results for BBMN68 were consistent with this finding, with a 45% decrease in viable cells observed at 15 min post-administration. Izquierdo *et al*. [13] showed that *B. longum* strain NCC2705 had a high survival rate (96%) following incubation for 90 min in stimulated gastric conditions (pH 2.5), whereas commercial strains such as *B. longum* BB 536 and SP 07/3 showed weaker survival rates of about 76% and 78%, respectively. Bile and pancreatic enzymes are likely to affect the viability of BBMN68 in the small intestine. The highest observed survival was 11.7% at 3 h post-gavage, which indicated a further loss of viability of BBMN68. However, the viable cell count maintained an effective level of over 6 log CFU/g in the 6 h following gavage, indicating some bile resistance. Previous studies *in vitro* investigated the growth of many *B. longum* strains. However, in the current study, general growth rates were not included for difficult manipulation. Although it is hard to make a comparison between BBMN68 and other strains, an overview of the *in vivo* biodistribution of BBMN68 was obtained in the current study. It is clear that the tolerance of *B. longum* varies among strains, and BBMN68 does not appear to be a highly resistant *B. longum* strain. However, the probiotic character of a strain is not likely to be based on a single attribute, and the potential of a probiotic for future application should be evaluated based on its various properties [13].

In the analysis of survival data from the colon, the level of viable cells soared between 3 and 6 h post-inoculation, and maintained a functional concentration of over 6 log CFU/g until 12 h post-inoculation. This trend probably indicates a transient colonization by BBMN68, with preferential accumulation of cells at this site. This result is consistent with a previous postulation that the ecological niche of bifidobacteria is in the distal GI tract [11], which provides a suitable pH and non-digestible carbon sources for bifidobacterial growth [25].

The retention of BBMN68 *in vivo* was also examined. Based on the results of the rectum, this strain appeared to be maintained *in vivo* for 30 h after a single oral administration. This indicated that viable BBMN68 could transiently accumulate in the rat intestine although it was eliminated once the gavage was discontinued. For introduced probiotics, stable colonization in such a short time period might be difficult as the normal intestinal microbiota may exert resistance to their colonization [38]. It was reasonable to find that compared with the single dose group, oral feeding for 7 days could prolong the retention of BBMN68 in the gut to 3 days. During this time, viable BBMN68 maintained a functional level of 6 log CFU/g for 1 day, better than strain N7 with a level of 3–6 log CFU/g during the first 24 h [15]. As mentioned above, probiotics cannot exert beneficial effects unless their population reaches a minimum of 6–8 log CFU/g of intestinal content. Thus, we postulated that a much more elevated level of viable BBMN68 would likely be obtained in the intestine if a high concentration of cells was administered daily.

Given our results, it would appear that BBMN68 can still be considered a promising probiotic strain, as it remained viable in the murine GI tract for almost 3 days. Future study will focus on the promotion of BBMN68 tolerance of GI tract conditions by co-administration of prebiotics or genetic manipulation, with the aim of improving the survival of BBMN68 in the GI tract.

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