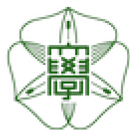

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Detection and quantification of viable bacteria using selectively membrane-permeable dye and PCR

Junji Fujimoto

2013

Detection and quantification of viable bacteria using selectively membrane-permeable dye and PCR

Junji Fujimoto

Doctoral thesis

2013

Graduate School of Environmental Science

Hokkaido University

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Chapter 1

General introduction

Increasing interest in healthy lifestyles has led to the use of probiotics in a variety of fermented milk products. *Lactobacillus* and *Bifidobacterium* strains are the most widely used probiotics, and there are many reports of their benefits to human health (Collins and Gibson 1999; Fuller 1991; Holzapfel, et al. 1998; Kleerebezem and Vaughan 2009; Leahy, et al. 2005; Ouwehand, et al. 2002). The basic requirement for probiotic bacteria to exert expected positive effects is to be alive (Fuller 1989); therefore, appropriate quantification methods are crucial (Klaenhammer and Kullen 1999). To determine the effectiveness of probiotics it is therefore essential to establish a specific method to identify them and measure their numbers.

The current method for detecting and identifying probiotics in fecal samples after ingestion of probiotics involves using both a selective medium and a strain-specific monoclonal antibody in an enzyme-linked immunosorbent assay (ELISA) (Yuki, et al. 1999) or strain-specific identification by randomly amplified polymorphism DNA fingerprinting (RAPD) (Williams, et al. 1990). These methods, however, require considerable time, labor, experience, and skill. Recently, a PCR-based method for strain-specific identification using a strain-specific primer set has been reported (Ahlroos and Tynkkynen 2009; Bunte, et al. 2000; Maruo, et al. 2006). This method is a powerful tool for identifying and enumerating specific strains from various kinds of materials.

However, the number of probiotics determined by the quantitative PCR method (qPCR) using strain-specific primer set included both viable and dead cells. For understanding probiotics accurately, it is important to be able to discriminate viable cells from dead cells in order to characterize the usefulness of probiotics. Recently, differentiation of viable and dead cells in samples with several types of bacteria has been accomplished by using a PCR-based method with

propidium monoazide (PMA) treatment, which selectively penetrates dead cells, which have compromised membrane integrity, but not viable cells with intact cell membranes (Bae and Wuertz 2009; Nocker, et al. 2007). PMA is a DNA-intercalating dye that enables covalent binding to DNA under bright visible light; this makes DNA insoluble and strongly inhibits PCR amplification. Subjecting a bacterial population to PMA treatment before PCR therefore results in selective amplification of DNA from viable cells with intact membranes.

The aim of this study is to establish aa suitable method for the detection and quantification of viable probiotics using selectively membrane-permeable dye and qPCR.

In the first step, I developed a PCR-based method for the identification and quantification of *Lactobacillus casei* strain Shirota (LcS) using an LcS-specific primer set (pLcS) derived from RAPD analysis. And, I evaluated the detection limit of LcS in feces by using pLcS and quantified LcS in the feces after ingestion of fermented milk containing LcS by using qPCR with pLcS (chapter 2).

I next developed a procedure for the detection and quantification of viable *Bifidobacterium breve* strain Yakult (BbrY) in feces by a PCR-based method. The procedure was based on the combined use of the DNA intercalating agent PMA and qPCR (PMA-qPCR), using strain-specific primers derived from RAPD analysis. I also investigated the application of this technique for enumerating viable BbrY in feces after the ingestion of fermented milk containing BbrY (chapter 3).

In the last step, I developed a PCR-based procedure for the detection and quantification of viable *Bifidobacterium bifidum* strain BF-1 (BF-1) in feces by the same method in Chapter 3. Additionally, I compared the changes in numbers of BF-1 viable cells determined by various methods (T-EMSM selective agar-based culture, ATP content, RT-qPCR targeting of *B. bifidum* specific 16S rRNA sequences, and PMA-qPCR) when the cells were subjected to moderately slow death by long-term cultivation and rapid death by artificial gastric juice treatment. I also applied this technique to quantify viable BF-1 cells in feces of subjects who had ingested fermented milk

containing BF-1 (chapter 4).

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Chapter 2

Identification and quantification of *Lactobacillus casei* strain Shirota in human feces with strain-specific primers derived from randomly amplified polymorphic DNA

2. 1. Introduction

Increasing interest in healthy lifestyles has led to the use of probiotics in a variety of fermented milk products. *Lactobacillus* strains are the most widely used probiotics, and there are many reports of their benefits to human health (Fuller 1991; Holzapfel, et al. 1998; Ouwehand, et al. 2002). *Lactobacillus casei* strain Shirota (LcS) has been used in fermented milk products for more than 70 years and is one of the most intensively studied probiotics. Its various benefits include improving the balance of intestinal microbes and volatile fatty acids in the gastrointestinal environment, antitumor action, activation of the immune system, and antimicrobial activity (Asahara, et al. 2001; Kanazawa, et al. 2005; Matsuki, et al. 2004; Nagao, et al. 2000; Spanhaak, et al. 1998; Walter, et al. 2001). To exert maximal beneficial effects on the host, probiotics should live and proliferate in the intestines, increasing the number of cells secreting beneficial factors such as volatile fatty acids, vitamins, and bacteriocins. Therefore, it is necessary to establish a specific method to identify the probiotics and measure their numbers to determine whether they can be effective.

The current method for detecting and identifying LcS in fecal samples after ingestion of LcS involves using both LLV (a selective medium for LcS) and a strain-specific monoclonal antibody in an enzyme-linked immunosorbent assay (ELISA) (Yuki, et al. 1999). This method, however, requires considerable time, labor, experience, and skill. In recent years, analytical methods based on molecular biological techniques have been established and used to detect and identify *Lactobacillus*. Species-specific primers that target the 16S rRNA gene or the 16S–23S rRNA intergenic spacer regions are effective for detecting and identifying strains at the species level (Tannock 1999; Tilsala-Timisjarvi and Alatossava 1997; Walter, et al. 2001). Methods used for the discrimination of

bacterial strains include randomly amplified polymorphic DNA (RAPD) analysis, pulsed-field gel electrophoresis, and ribotyping (Daud Khaled 1997; McCartney, et al. 1996). However, these methods require pure cultures of the bacterial strains to be analyzed, and are therefore not appropriate for DNA isolated directly from feces or food. In contrast, strain-specific primers have been developed for the direct detection and identification of probiotics using PCR. These primers were developed using the RAPD technique, subtraction hybridization (Bunte, et al. 2000; Maruo, et al. 2006; Tilsala-Timisjarvi and Alatossava 1998), and sequence data from the 16S-23S rRNA intergenic spacer regions, phage-related sequences, and the S-layer gene (Brandt and Alatossava 2003; Flint and Angert 2005; Saito, et al. 2004).

In this study, we developed a PCR-based method for the identification and quantification of LcS using an LcS-specific primer set (pLcS) derived from RAPD analysis. We evaluated the detection limit of LcS in feces by using pLcS and quantified LcS in the feces after ingestion of LcS by using pLcS to confirm the ability of LcS to proliferate in human intestines. The results from the pLcS method correlated well with those of the standard culture method.

2.2. Materials and Methods

2.2.1. Reference strains and culture conditions

The 167 bacterial strains (57 strains of *L. casei* and 110 other strains of bacteria commonly isolated from human feces; Table 1) were obtained from the Culture Collection of the Yakult Central Institute (YIT; Tokyo, Japan). Anaerobic bacteria were cultured at 37 °C for 1 or 2 days in modified GAM broth (Nissui, Tokyo, Japan) supplemented with 0.5% glucose. Lactic acid bacteria were cultured in MRS broth (Becton Dickinson, Sparks, MD, USA) at 37 °C for 1 day. For a quantitative PCR standard, the number of LcS was counted after the bacteria were stained with 4′, 6-diamino-2-phenylindole (DAPI) and mounted in VECTASHIELD mounting medium (Vector

Laboratories, Burlingame, CA, USA) (Takada, et al. 2004).

2.2.2. RAPD PCR analysis

Bacterial DNA for RAPD analysis was extracted as previously described, with some modification (Zhu, Qu and Zhu, 1993). Briefly, 0.5-ml aliquots of overnight cultures were pelleted by centrifugation at 20 000 × *g* for 5 min, and the cell pellet was suspended in 250 µl of extraction buffer (100 mM Tris-HCl, 40 mM EDTA [pH 9.0]) and 50 µl of 10% sodium dodecyl sulfate. Benzyl chloride (150 µl) was added to the suspension, and the mixture was vortexed vigorously at 50 °C for 30 min using a MicroIncubator M-36 (Taitech, Tokyo, Japan). Then, 150 µl of 3 M sodium acetate was added, and the mixture was cooled on ice for 5 min. After centrifugation at 20 000 × *g* for 10 min, the supernatant was collected and DNA was obtained by isopropanol precipitation. Finally, the DNA was diluted to 10 µg ml^{-1} in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). RAPD-PCR amplification was performed in a DNA Engine (MJ Research, Tokyo, Japan). Each reaction mixture (20 µl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1.5 U *Taq* DNA polymerase (Takara, Shiga, Japan), 1.5 µM RAPD primer, and 10 ng template DNA. The RAPD primers were: p1001, GTGAAGTAGG; p1002, CAATAGCCGT; p1003, CAGTACCCAC; p1004, AGGTAACCGT; p1005, CAGTACCTTC; p1006, GGTTAAAGCC; p1007, TCGACGATAG; p1008, AGCCAACGAA; p1009, GTTGCGGTCC; p1010, TGCGACTTAC; p1011, GTAGACAAGC; p1248, TGCCGAATTC; p1249, CGAACTAGAC; p1250, GGCTTAACAC; p1251, AAGACTGTCC; p1252, GCGGAAATAG; p1254, CCGCAGCCAA; p1255, CCGATCTAGA; p1280, GAGGACAAAG; p1281, AACGCGCAAC; p1282, GACGACTATC; p1284, GTCAACGAAG; p1285, AGCCAGTTTC; p1287, CGCATAGGTT; p1288, GGGGTTGACC; p1289, ACTTGCATCC; and p1292, CCCGTCAGCA (Akopyanz, et al. 1992; Daud Khaled 1997; Guillot and Mouton 1996; Zavaleta, et al. 1997). The amplification program consisted of 1 cycle of 94 °C for 2 min; 6 cycles of 94 °C for 30 s, 36 °C for

60 s, and 72 °C for 90 s; 30 cycles of 94 °C for 20 s, 36 °C for 30 s, and 72 °C for 90 s; and finally 1 cycle of 72 °C for 3 min. RAPD products were electrophoresed at 50 V in a 1.5% agarose gel.

2.2.3. Cloning and sequence analysis of RAPD products specific to LcS

Potential strain-specific RAPD markers were extracted from agarose gels with a SUPREC-01 gel extraction kit (Takara). The collected amplification products were cloned using a TA Cloning kit with the pCR 2.1 vector (Invitrogen, Leek, The Netherlands). The nucleic acid sequences of 5 clones of each potential strain-specific RAPD marker were determined by an ABI model 373A DNA sequencer with a Dye Terminator sequencing kit (Applied Biosystems, Foster, CA, USA).

2.2.4. Specificity of RAPD-derived primers

The specificity of the LcS-specific primer set (pLcS) was confirmed by PCR using DNA from 167 bacterial strains (Table 1). PCR amplifications were performed in a DNA Engine (MJ Research). Each reaction mixture (20 µl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1.5 U *Taq* DNA polymerase (Takara), 0.4 µM primers, and 10 ng template DNA. The amplification program consisted of 1 cycle of 94 °C for 2 min; 32 cycles of 94 °C for 20 s, 60 °C for 10 s, and 72 °C for 20 s; and finally 1 cycle of 72 °C for 3 min. PCR products were electrophoresed at 50 V in a 1.5% agarose gel.

2.2.5. Quantification of added LcS in fecal samples with quantitative PCR

Various concentrations of LcS ($10^{3.1}$ to $10^{9.6}$ per gram of feces) were added to 6 fecal samples that had been confirmed by the culture method and qPCR with pLcS to contain no LcS. The total concentration of intestinal microorganisms by DAPI was $10^{11.0 \pm 0.2}$ cells g^{-1}, (mean ± S.D.). Fecal samples were collected in individual sterile Faeces Containers (Sarstedt, Nümbrecht, Germany), refrigerated, and taken to the laboratory within 4 hours. The DNA was extracted from these mixed

feces and subjected to qPCR analysis using the pLcS.

2.2.6. Examination of fecal samples after ingestion

Informed consent was obtained from the volunteers who provided the fecal samples used in this study. The Ethical Committee of the Yakult Central Institute provided ethical clearance for this microbiological research study in accordance with the Helsinki Declaration. The study population comprised 14 healthy men (age range, 28 to 59 years; mean ± SD, 35.9 ± 7.9 years) who ingested a commercially available fermented milk product (Yakult 400; 80 ml) containing 10^{11} CFU LcS once daily for 7 days. Feces excreted on days 0 (before drinking the fermented milk product), 7, 9, 11, and 14 were collected in individual sterile Faeces Containers (Sarstedt), refrigerated, and taken to the laboratory within 4 hours. No subject ingested probiotic products, including the study product, during the 2 weeks before and 1 week after they drank the LcS-containing fermented milk product for the study.

2.2.7. Enumeration of LcS by the standard culture method

Counts (in CFU) of LcS were determined by using strain-specific LLV selective agar medium according to the method of Yuki, et al. (1999), with slight modification. Aliquots (0.1 ml) of 10-fold serial dilutions of feces (starting sample, 0.5 g) in 0.85% NaCl were spread on LLV agar and incubated aerobically at 37 °C for 72 h. For each fecal sample, we collected all colonies from the LLV plate inoculated with the highest dilution that yielded growth and subjected these colonies to real-time PCR analysis using the pLcS. The number of LcS per gram of feces (wet weight) was estimated from the number of colonies that were identified as containing LcS by PCR with pLcS. Some isolates were also analyzed by ELISA using the L8 monoclonal antibody (Yuki, et al., 1999) and RAPD analysis.

2.2.8. Extraction of DNA from fecal samples

The DNA from fecal samples was extracted using a Stool Mini Kit (Qiagen, Valencia, CA, USA). The 10-fold diluted fecal solution (200 µl) was pelleted by centrifugation at 20 000 × *g* for 8 min and washed 3 times with 1.0 ml phosphate-buffered saline to remove PCR inhibitors, and suspended in 550 µl Buffer ASL (Qiagen). The fecal suspension was heated to 70 °C for 5 min. The suspension was vortexed with glass beads (700 mg; 0.1 mm in diameter) and 500 µl buffer-saturated phenol using a FastPrep Fp120 (Bio 101, Irvine, CA, USA) at a speed setting of 6.5 m s^{-1} for 30 sec. We added 100 µl of 3 M sodium acetate (pH 4.8) to the suspension, which was kept on ice for 5 min and then centrifuged at 20 000 × *g* for 8 min. The supernatant (700 µl) was mixed with 700 µl Buffer ASL and an InhibitEX tablet (Qiagen). After centrifugation at 20 000 × *g* for 2 min, 550 µl of supernatant was mixed with 50 µl of 3 M sodium acetate (pH 4.8) and 600 µl isopropanol for DNA precipitation. The DNA was resuspended in 0.4 ml TE buffer including 4 µg RNase (Sigma-Aldrich Japan, Tokyo, Japan) and incubated at 37 °C for 15 min. The DNA solution was mixed with 400 µl of Buffer AL (Qiagen) and 400 µl of 100% ethanol. The lysate was trapped on a QIAamp spin column (Qiagen). After the column was washed with washing buffer (AW1 and AW2, Qiagen), DNA was eluted in 100 µl of Buffer AE (Qiagen).

2.2.9. Real-time PCR analysis

PCR amplification and detection of LcS were performed in an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The reaction mixture (20 µl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 500 µg/ml bovine serum albumin (Takara), a 1:75 000 dilution of SYBR Green I (Invitrogen), 0.4 U *Taq* DNA polymerase Hot Start version (Takara), 0.2 µM of each of the 2 specific primers, and 5 µl of template DNA diluted 10, -10^2-, or 10^3-fold. The amplification program consisted of 1 cycle of 94 °C for 5 min; 40 cycles of 94 °C for 20 s, 60 °C for 15 s, and 72 °C for 50 s; and finally 1 cycle of 72 °C for 180 s.

Fluorescence intensities were detected during the last step of each cycle. To distinguish the targeted PCR product from non-targeted PCR products (Ririe, et al. 1997), melting curves were obtained after amplification by slow heating from 60 to 95 °C in increments of 0.2 °C s^{-1} with continuous fluorescence collection.

2.2.10. Statistical methods

Pearson's correlation coefficients were used to determine the correlations between the number of added LcS and the counts obtained with qPCR in the experiment with added LcS, and between the CFU of LcS and the counts obtained with qPCR in the ingestion experiment. Simple linear regression was used to develop regression equations for statistically significant relationships. Mean differences in the concentrations of LcS for quantifying ingested LcS in feces were analyzed by the paired *t*-test.

2.3. Results

2.3.1. Screening for strain-specific RAPD markers

To identify a strain-specific PCR product for LcS, we tested a total of 27 RAPD primers on 57 *L. casei* strains. p1252 (5′ GCG GAA ATA G 3′) generated a 0.7-kb LcS-specific band (arrows, Figure 1). We determined the sequence of this LcS-specific PCR product (accession no. AB246299; Fig. 2), which we did not find in the DDBJ/GenBank/EMBL DNA database using BLAST and FASTA analyses.

2.3.2. Design of a specific RAPD-derived primer pair

To amplify an LcS-specific PCR product, we designed the primer set p1252-1F (GCG GAA ATA GTA GTG TGA CGA TC) and p1252-742R (GCG GAA ATA GGT GCA TAG GCG; Fig. 2) and used this primer set for PCR on 57 strains of *L. casei*. Six strains (YIT 0171, YIT 0209, YIT

0262, YIT 0289, YIT 0437, and LcS) yielded PCR products. These products were sequenced and aligned so that a new, more specific, target sequence for LcS could be identified (Fig. 2). We then designed a new set of LcS-specific primers (pLcS; pLcS-57F, CTC AAA GCC GTG ACG GTC, and pLcS-597R, ACG TGG TGC TAA TAA TCC TAG TG) and tested its specificity against DNA extracted from 167 bacterial strains, including the 57 strains of *L. casei* (Table 1). These primers exclusively supported PCR amplification of LcS template DNA, with no cross-reaction against nontarget microorganisms. The amplification product was 541 bp in length and had a melting temperature of 85.7 °C.

2.3.3. Quantitative PCR detection of LcS added to feces

We added LcS directly to fecal samples and analyzed the correlation between the number of added LcS and the value obtained with qPCR. We also determined the lower limit of detection of LcS in the feces. When $10^{4.6}$ to $10^{9.6}$ LcS were added per gram of feces, the qPCR gave very accurate results (r^2 = 0.999; Fig. 3). When $10^{4.1}$ LcS were added per gram of feces, 3 of 6 fecal samples yielded amplification products; when $10^{3.6}$ LcS were added, only 1 of 6 samples yielded an amplification product.

2.3.4. Quantitative detection of ingested LcS in feces by qPCR

We next used our quantitative PCR method to measure the number of LcS in the feces of subjects who drank a fermented milk product containing LcS. The number of LcS in the feces was maximal on day 7 (just after the ingestion of fermented milk product was discontinued) and decreased every day thereafter. LcS was detected in 6 of 14 subjects at $10^{5.3\pm0.5}$ g^{-1} (mean ± S.D., n=6) on day 0 (before ingestion). LcS was detected in all 14 subjects on day 7 ($10^{9.1\pm0.5}$ g^{-1}) and day 9 ($10^{7.4\pm1.1}$ g^{-1}). On day 11, LcS was detected in 11 of 14 subjects at $10^{6.2\pm1.1}$ g^{-1} (n = 13). On day 14, LcS was detected in 8 of the 14 subjects at $10^{5.5\pm0.5}$ g^{-1} (n = 8; Table 2).

2.3.5. Quantitative detection of ingested LcS with a culture method

We also quantified the number of LcS using the standard method of culturing them on LLV agar plates. LLV is a strain-specific selective medium. We then used PCR with pLcS to identify all colonies that appeared on the LLV agar plate of the highest fecal dilution that yielded growth. In total, 576 isolates from the LLV plates were subjected to PCR analysis; 336 (58.3%) of these isolates gave positive PCR results. In addition, 20 of the 576 isolates were randomly selected and evaluated by ELISA using a monoclonal antibody and RAPD analysis. Thirteen isolates were identified as LcS by all 3 methods, and the remaining 7 strains were confirmed as non-LcS by all 3 methods; thus, our qPCR method produced results for all samples that were consistent with those obtained with the standard method of LLV culture and ELISA. However, we found that the number of LcS in feces after ingestion of LcS was about 10 times higher when measured with qPCR than when measured as CFU on culture plates (Table 2). For example, on day 7 (after ingestion of LcS for 7 days), LcS was detected in the fecal samples of all 14 subjects at $10^{9.1\pm0.5}$ g^{-1} (mean ± S.D.) by qPCR, but at $10^{8.0\pm0.9}$ CFU $g^{-}1$ ($P < 0.001$, paired *t*-test). With this culture-based PCR method, LcS was isolated from 4 out of 14 subjects at $10^{3.7\pm0.3}$ g^{-1} (mean ± S.D., n=4) on day 0 (before ingestion). LcS was isolated from all subjects on day 7 ($10^{8.0\pm0.9}$ g^{-1}) and day 9 ($10^{6.1\pm1.3}$ g^{-1}). On day 11, LcS was isolated from 12 of 14 subjects at $10^{4.7\pm0.9}$ g^{-1} n=12), and on day 14, LcS was isolated from 5 of 14 subjects at $10^{3.1\pm0.7}$ g^{-1} (n=5; Table 2).

2.4. Discussion

Lactobacillus casei strain Shirota (LcS) is considered to be an excellent probiotic (Asahara, et al. 2001; Kanazawa, et al. 2005; Matsumoto, et al. 2006; Matsuzaki, et al. 2004; Nagao, et al. 2000; Spanhaak, et al. 1998). We developed an LcS-specific primer set (pLcS) derived from RAPD

analysis to identify and quantify LcS in the feces to evaluate the ability of LcS to proliferate in human intestines.

To our knowledge, few published studies have used a sufficient number of strains to confirm the specificity of strain-specific primers. In contrast, we tested the specificity of our primers against 167 bacterial strains, including 57 strains of *L. casei.* We also used strain-specific primers to confirm the absence of cross-reactive strains in the feces of the volunteers before they began drinking the fermented milk product containing LcS. The protocol of this study prohibited the ingestion of ordinary, commercially available fermentation milk products for 2 weeks before LcS ingestion; however, there were 3 fecal samples (subject G, K, M) in which LcS was detected by the qPCR method using pLcS ($>10^{4.6}$ / g^{-1}), although no LcS ($<10^{1.7}$ CFU g^{-1}) was detected by culturing on LLV agar plates in 14 fecal samples on day 0 (Table 2). This result suggested that some strains cross-reactive with pLcS existed in small numbers in these 3 fecal samples before the ingestion of LcS. Of the 576 colonies that grew on LLV agar plates from the feces of the volunteers who had ingested LcS, 336 (58.3%) were identified as LcS by qPCR using pLcS. In addition, we randomly selected identified 20 isolates from the above 576 colonies for analysis with ELISA and RAPD. Thirteen isolates were identified as LcS by all 3 methods we used, and the other 7 isolates were identified as non-LcS by the same methods. Therefore, ELISA using a monoclonal antibody, RAPD analysis, and PCR using pLcS were all able to accurately identify LcS isolates from feces. However, PCR using pLcS was easier and more rapid than the ELISA or RAPD methods for identifying colonies on LLV medium.

We had previously used the phenol and glass beads method to extract DNA from feces for qPCR (Matsuki, et al. 2004), but the detection limit of LcS in the feces was only $10^{5.7}$ per gram of feces using qPCR with pLcS (data not shown). Therefore, we modified the DNA extraction method and PCR protocol in order to increase the sensitivity of the specific PCR amplification. We used a QIAamp DNA Stool Mini Kit (Qiagen) to raise the quality of the DNA, and added bovine serum

albumin to the PCR buffer to stabilize *Taq* polymerase. This modification improved the detection limit of LcS in feces 10-fold, to $10^{4.6}$ per gram feces. The counts of LcS in feces by qPCR were highly and significantly correlated with the number of LcS added to fecal samples within the range of $10^{4.6}$ to $10^{9.6}$ per gram of feces ($r^2 = 0.999$, $P < 0.001$, Fig. 3).

The culture-based method and our quantitative PCR assay produced results that correlated well ($r^2 = 0.86$, $P < 0.001$, Fig. 4) for the number of LcS in the feces after the ingestion of the commercial fermented milk product containing LcS at 10^{11} CFU per bottle once daily for 1 week. However, we found that the number of LcS in the feces after the ingestion of LcS was significantly higher when measured with qPCR than when measured as CFU on culture plates. Essentially all bacteria with intact chromosomal DNA can be detected with the PCR method, whether they are alive or dead. Therefore, it is possible that the total number of LcS in feces measured by qPCR after ingestion of LcS (live cells plus dead cells) was 10 times higher than the number of living bacteria detected with the culture method. However, it is also possible that culture on LLV agar underestimates the number of living LcS cells because it has the inherent disadvantages of using selective culture media. For example, the sensitivity of LcS to antibiotics might have increased during the transition in the intestines, or the efficiency of its use of Lactitol, the selective growth factor in LLV, might have decreased.

Recently, it was shown that even dead LcS cells effectively stimulate macrophages to induce IL-12, because LcS has a rigid cell wall that is resistant to intracellular digestion (Shida, et al. 2006), and dead LcS cells can improve chronic inflammatory bowel disease by regulating the immune system (Matsumoto, et al. 2005). Therefore, we firmly believe that it is very meaningful to estimate the total cell number of LcS in the feces accurately. In previous studies using culture methods (Matsumoto, et al. 2006; Yuki, et al. 1999), the number of LcS in feces decreased to below the detection limit immediately after the ingestion of LcS was discontinued. We therefore believed that

LcS is eradicated from human intestines completely within 2 weeks at most after the ingestion of LcS is discontinued. In this study, we detected LcS in 6 of 14 subjects at $10^{5.3\pm0.5}$ g^{-1} (mean ± S.D., n=6) by pLcS and in 4 of 14 subjects at $10^{3.7\pm0.3}$ g^{-1} (n=4) by culture method on day 0, after the subjects had not ingested fermented products for 2 weeks. The detection frequency and number of LcS in feces before ingestion of LcS by qPCR were considerably larger than what was previously found with culture methods (Matsumoto 2006; Yuki, et al. 1999). Tuohy, et al. (2007) reported that LcS was present 1 week after fermented milk containing LcS was no longer ingested, indicating that LcS may multiply in the human intestinal tract for some time. Lee, et al. (2004) confirmed that LcS divides on the intestinal mucosa in mice on day 6 after administration of LcS labeled with 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester. Our results strongly suggest that LcS can live and proliferate in the intestines more than 7 days after ingestion of LcS. Although LcS has beneficial effects whether alive or dead, live cells can proliferate and secrete beneficial factors, and further analyses to accurately discriminate numbers of live cells of LcS in feces are needed to characterize the usefulness of LcS as a probiotic. We also need to know where in the gastrointestinal tract LcS changes from cultivable to noncultivable on LLV to confirm whether or not LcS exerts its probiotic effect in the intestines.

In conclusion, the LcS-specific PCR primers that we developed in this study enabled efficient and accurate identification of colonies that formed on LLV medium. The combination of quantitative PCR and our LcS-specific primers will make it possible to analyze quickly the number of LcS in numerous fecal specimens. In light of the increasing public interest in probiotics, we need to demonstrate the efficacy of our method in experiments with large numbers of test subjects, but we believe that the strain-specific primers we have described here will be powerful tools for future studies.

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Table 1 Bacterial strains used in this study

Group	Strains
Bacteroides spp.	*Bacteroides distasonis* YIT 6162^{T}(JCM 5825^{T}), *B. fragilis* YIT 6158^{T}(NCTC 9343^{T}), *B. ovatus* YIT 6161^{T}(JCM 5824^{T}), *B. thetaiotaomicron* YIT 6163^{T}(JCM 5827^{T}), *B. uniformis* YIT 6164^{T}(JCM 5828^{T}), *B. vulgatus* YIT 6159^{T}(ATCC 8482^{T})
Bifidobacterium spp.	*Bifidobacterium adolescentis* YIT 4011^{T}(ATCC 15703^{T}), *B. angulatum* YIT 4012^{T}(ATCC 27535^{T}), *B. animalis* YIT 4044^{T}(JCM 1190^{T}), YIT 4121(DSM 10140), *B. asteroides* YIT 4033^{T}(ATCC 25910^{T}), *B. bifidum* YIT 4039^{T}(IFO 14252^{T}), *B. boum* YIT 4091^{T}(JCM 1211^{T}), *B. breve* YIT 4010, YIT 4014^{T}(ATCC 15700^{T}), YIT 4065, *B. catenulatum* YIT 4016^{T}(ATCC 27539^{T}), *B. choerinum* YIT 4067^{T}(ATCC 27686^{T}), *B. coryneforme* YIT 4092^{T}(JCM 5819^{T}), *B. cuniculi* YIT 4093^{T}(JCM 1213^{T}), *B. dentium* YIT 4017^{T}(ATCC 27534^{T}), *B. gallicum* YIT 4085^{T}(JCM 8224^{T}), *B. gallinarum* YIT 4094^{T}(JCM 6291^{T}), *B. indicum* YIT 4083^{T}(JCM 1302^{T}), *B. longum* YIT 4018(ATCC 15697), YIT 4021^{T}(ATCC 15707^{T}), YIT 4082(JCM 1269), *B. magnum* YIT 4098^{T}(JCM 1218^{T}), *B. merycicum* YIT 4095^{T}(JCM 8219^{T}), *B. minimum* YIT 4097^{T}(JCM 5821^{T}), *B. pseudocatenulatum* YIT 4072^{T}(JCM 1200^{T}), *B. pseudolongum* subsp. *globosum* YIT 4101^{T}(JCM 5820^{T}), *B. pseudolongum* subsp. *pseudolongum* YIT 4102^{T}(JCM 1205^{T}), *B. pullorum* YIT 4104^{T}(JCM 1214^{T}), *B. ruminantium* YIT 4105^{T}(JCM 8222^{T}), *B. saeculare* YIT 4111^{T}(DSM 6531^{T}), *B. subtile* YIT 4116^{T}(DSM 20096^{T}), *B. thermophilum* YIT 4073^{T}(JCM 1207^{T})
Clostridium spp.	*Clostridium amylophilus* YIT 6167^{T}(DSM 10710^{T}), *C. celatum* YIT 6056^{T}(JCM 1394^{T}), *C. celerecrescens* YIT 6168^{T}(DSM 5628^{T}), *C. clostridioforme* YIT 6034^{T}(JCM 1291^{T}), *C. coccoides* YIT 6035^{T}(JCM 1395^{T}), *C. leptum* YIT 6169^{T}(DSM 753^{T}), *C. nexile* YIT 6170^{T}(ATCC 27757^{T}), *C. oroticum* YIT 6037^{T}(DSM 1287^{T}), *C. perfringens* YIT 6050^{T}(JCM 1290^{T}), *C. scindens* YIT 6171^{T}(JCM 6567^{T})
C. aerofaciens	*Collinsella aerofaciens* YIT 10235^{T}(ATCC 25986^{T})
Enterococcus spp.	*Enterococcus faecalis* YIT 2031^{T}(ATCC 19433^{T}), *E. faecium* YIT 2032^{T}(ATCC 19434^{T})
E. coli	*Escherichia coli* YIT 6044^{T}(JCM 1649^{T})
Eubacterium spp.	*Eubacterium biforme* YIT 6076^{T}(ATCC 27806^{T}), *E. ramulus* YIT 6172^{T}(DSM 3995^{T}), *E. rectale* YIT 6082^{T}(ATCC 33656^{T}), *E. xylanophilum* YIT 6173^{T}(ATCC 35991^{T}),
F. prausnitzii	*Faecalibacterium prausnitzii* YIT 6174(ATCC 27766)
Lactobacillus spp. (except *L. casei*)	*Lactobacillus acidophilus* YIT 0070^{T}(ATCC 4356^{T}), *L. amylophilus* YIT 0255^{T}(JCM 1125^{T}), *L. amylovorus* YIT 0211^{T}(JCM 1126^{T}), *L. bifermentans* YIT 0260^{T}(JCM 1094^{T}), *L. brevis* YIT 0076^{T}(ATCC 14869^{T}), *L. buchneri* YIT 0077^{T}(ATCC 4005^{T}), *L. coryniformis* subsp. *coryniformis* YIT 0237^{T}(JCM 1164^{T}), *L. crispatus* YIT 0212^{T}(JCM 1185^{T}), *L. delbrueckii* subsp. *delbrueckii* YIT 0080^{T}(ATCC 9649^{T}), *L. delbrueckii* subsp. *lactis* YIT 0086^{T}(ATCC 12315^{T}), *L. delbrueckii* subsp. *bulgaricus* YIT 0181^{T}(ATCC 11842^{T}), *L. fermentum* YIT 0081^{T}(ATCC 14931^{T}), *L. gallinarum* YIT 0218^{T}(JCM 2011^{T}), *L. gasseri* YIT 0192^{T}(DSM 20243^{T}), *L. helveticus* YIT 0083^{T}(ATCC 15009^{T}), *L. johnsonii* YIT 0219^{T}(JCM 2012^{T}), *L. malefermentans* YIT 0271^{T}(NRIC 1779^{T}), *L. oris* YIT 0277^{T}(NCFB 2160^{T}), *L. parabuchneri* YIT 0272^{T}(NRIC 1780^{T}), *L. paraplantarum* YIT 0445^{T}(DSM 10667^{T}), *L. pentosus* YIT 0238^{T}(JCM 1558^{T}), *L. plantarum* YIT 0102^{T}(ATCC 14917^{T}), *L. pontis* YIT 0273^{T}(DSM 8475^{T}), *L. reuteri* YIT 0197^{T}(JCM 1112^{T}), *L. rhamnosus* YIT 0105^{T}(ATCC 7469^{T}), *L. sakei* YIT 0247^{T}(JCM 1157^{T}), *L. salivarius* subsp. *salivarius* YIT 0104^{T}(ATCC 11741^{T}), *L. sharpeae* YIT 0274^{T}(JCM 1186^{T}), *L. vaginalis* YIT 0276^{T}(JCM 9505^{T}), *L. zeae* YIT 0078(ATCC 393)
Lb. casei	*L. casei* Y 88013, Y 91044, Y 92001, Y 93039, Y 93042, Y 93060, Y 94027,Y 96006, Y 97023, YIT 0003, YIT 0005, YIT 0006, YIT 0007, YIT 0009, YIT 0010, YIT 0011, YIT 0013, YIT 0014, YIT 0015, YIT 0018, YIT 0038, YIT 0047, YIT 0123(ATCC 27216), YIT 0128(ATCC 4646), YIT 0130, YIT 0144, YIT 0171, YIT 0180^{T}(ATCC 334^{T}), YIT 0209(NCDO 151), YIT 0210(ATCC 25599), YIT 0226, YIT 0262(JCM 1181), YIT 0289, YIT 0290, YIT 0295, YIT 0304, YIT 0305, YIT 0306, YIT 0309, YIT 0311, YIT 0322, YIT 0334, YIT 0338, YIT 0356, YIT 0358, YIT 0379, YIT 0393, YIT 0395, YIT 0396, YIT 0410, YIT 0419, YIT 0425, YIT 0437, YIT 9004, YIT 9009, YIT 9010, strain shirota
Lactococcus spp.	*Lactococcus garviae* YIT 2071^{T}(NCFB 2155^{T}), *L. lactis* subsp. *cremoris* YIT 2007^{T}(ATCC 19257^{T}), *L. lactis* subsp. *lactis* YIT 2008^{T}(ATCC 19435^{T}), *L. lactis* subsp. *hordiniae* YIT 2060^{T}(ATCC 29071^{T}), *L. plantarum* YIT 2061^{T}(ATCC 43199^{T}), *L. raffinolactis* YIT 2062^{T}(ATCC 43920^{T})
P. denticolens	*Parascardovia denticolens* YIT 4114^{T}(DSM 10105^{T})
Prevotella spp.	*Prevotella denticola* YIT 6131(JCM 8528), *P. melaninogenica* YIT 6039^{T}(ATCC 25845^{T}), *P. oralis* YIT 6127(JCM 6330), *P. oris* YIT 6134^{T}(JCM 8540^{T})
Propionibacterium	*Propionibacterium acnes* YIT 6165^{T}(JCM 6425^{T})
Ruminococcus spp.	*Ruminococcus bromii* YIT 6078^{T}(ATCC 27255^{T}), *R. callidus* YIT 6175^{T}(ATCC 27760^{T}), *R. gnavus* YIT 6176^{T}(ATCC 29149^{T}), *R. lactaris* YIT 6084^{T}(ATCC 29176^{T}), *R. obeum* YIT 6085^{T}(ATCC 29174^{T}), *R. productus* YIT 6141^{T}(JCM 1471^{T}), *R. schinkii* YIT 6177^{T}(DSM 10518^{T})
Sc. inoponatum	*Scardovia inopinatum* YIT 4115^{T}(DSM 10107^{T})
Streptococcus spp.	*Streptococcus thermophilus* YIT 2001, YIT 2021, YIT 2037^{T}(ATCC 19258^{T})

All strains were obtained from the Yakult Central Institute (YIT; Tokyo, Japan). The identification number for each strain is given in parentheses. ATCC, American Type Culture Collection (USA);

DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen (Germany); IFO, Institute for Fermentation (Japan); JCM, Japan Collection of Microorganisms (Japan); NCDO, National Collection of Dairy Organisms (UK); NCFB, National Collection of Food Bacteria (UK); NCTC, National Collection of Type Cultures (UK); NRIC, NODAI Culture Collection Center (Japan).

Table 2 Number of *Lactobacillus casei* strain Shirota (LcS) over 14 days in the feces of 14 volunteers who ingested a fermented milk product for 7 days.

Subject	Log$_{10}$ cells or CFU g^{-1} of feces									
	Day 0		Day 7		Day 9		Day 11		Day 14	
	qPCR[a]	CFU	qPCR	CFU	qPCR	CFU	qPCR	CFU	qPCR	CFU
A	<4.6[b]	<1.7[b]	9.3	7.9	6.4	5.3	5.7	<1.7	4.9	<1.7
B	<4.6	3.6	8.9	7.5	7.0	6.0	6.0	5.6	<4.6	<1.7
C	<4.6	<1.7	8.9	7.4	7.0	5.2	<4.6	<1.7	6.4	<1.7
D	<4.6	<1.7	9.6	8.7	7.9	6.6	6.2	4.9	5.7	2.0
E	4.9	3.9	9.7	9.2	7.8	6.9	6.1	5.6	<4.6	3.2
F	4.8	3.3	9.3	8.4	6.4	5.4	4.7	3.1	<4.6	<1.7
G	5.7	<1.7	9.6	9.2	9.3	8.4	7.3	5.6	<4.6	<1.7
H	<4.6	<1.7	8.0	6.1	5.6	3.1	4.7	3.7	<4.6	<1.7
I	<4.6	<1.7	9.8	8.9	7.7	6.6	5.3	4.1	5.3	3.6
J	<4.6	<1.7	9.2	7.9	9.2	7.4	8.2	5.4	6.1	<1.7
K	4.8	<1.7	8.9	7.8	7.4	6.4	6.6	5.4	<4.6	2.8
L	<4.6	<1.7	8.7	7.8	7.1	6.0	5.8	3.6	5.8	<1.7
M	5.4	<1.7	8.9	7.9	8.1	6.9	6.6	4.6	5.3	<1.7
N	5.9	4.1	8.9	7.2	6.3	4.8	7.4	4.3	4.9	3.7
No. of LcS positive subjects	6	4	14	14	14	14	13	12	8	5
mean[c]	5.3	3.7	9.1	8.0	7.4	6.1	6.2	4.7	5.5	3.1
SD[c]	±0.5	±0.3	±0.5	±0.9	±1.1	±1.3	±1.1	±0.9	±0.5	±0.7

[a] "qPCR", quantitative PCR.

[b] The lower limits of detection of the real-time PCR and the culture method were $10^{4.6}$ cells and $10^{1.7}$ CFU per gram of feces, respectively.

[c] "Mean" and "±S.D." were calculated only for subjects positive for LcS.

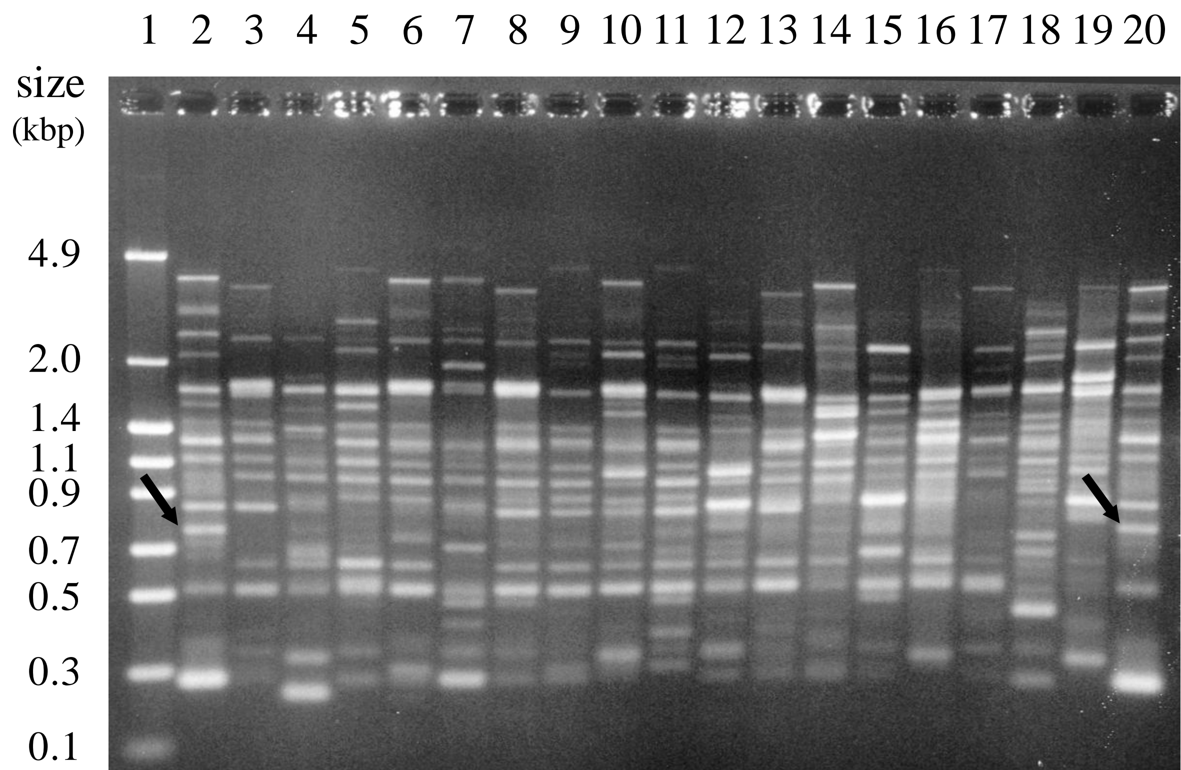

Fig. 1. RAPD patterns obtained from 18 *Lactobacillus casei* strains using the p1252 primer. Lane 1, pHY DNA size marker; lanes 2 and 20, *L. casei* strain Shirota (LcS); lanes 3 through 19, other strains of LcS. The arrows indicate a potential strain-specific band of LcS.

```
GCGGAAATAG TAGTGTGACG ATCGCAACTG ATGGTAAACA AACCGTGACC GGTGGGCTCA  60
AAGCCGTGAC GGTCAAACCA AACCAGGAAA TCAAGAAAGG AGATCAAATC GGAATTAGCG 120
AAAAGAACAT CACTCTTCAG CTTATCAAAA TCACGCAGCA GAGCGCTCCT GACGAGTCTT 180
CATCAACGTC ATCATTGTCA AGCACCTCAG AAAGCGTCTC TCAGGCACCA GGTTTATCTT 240
CAGAAAACTC TTCCTCCGCT TTCAGTTCAA CCGTTGCGGC AAGTTCAGCA CCTTTGCCAC 300
CAGTTGAGGA AGAATTAAAT CCTGCCTTTG CTTTACCAAA TGTGAAATAC ACGTATGCGA 360
CTACCTATGG ACTAGTTACA AGTGGAGGCG ATTTAACTGG TCAGGATTTC GATGCTAATA 420
AATTTCACGC TGCCTGGGTT AAGTATTTTT CTAATGGCGT TTTAGCAGAC AAAGAAAGCT 480
ACTCAATTGA TGCTGCTAAG AAAGCGGGCG TCAATCCCGC TCTAATTGCC GCTATCATGG 540
GAACTGAGTC CTCTTGGGGA ACCAGTGCAG CAGTACGTGG TGCTAATAAT CCTAGTGGTC 600
AGATGAGCGG GGGAACCATT ATTGCCTATC CATCCTTAGA AGGCGGAATT GATGCCACCG 660
GGAACACGTT GCATAACTTA GTTGTTACCC GTGGTTTGAA TACTGTCCAG AAGTTAGGCG 720
CCGCCTATGC ACCTATTTCC GC
```

Fig. 2. Nucleotide sequence of the 742-bp RAPD band (shown in Fig. 1) obtained from *L. casei* strain Shirota (LcS) using the p1252 primer. The primer regions used in the first screening for LcS-specific sequences are underlined. Strain-specific primer (pLcS) regions are boxed. The DDBJ/GenBank/EMBL accession number for the LcS-specific sequences is AB246299.

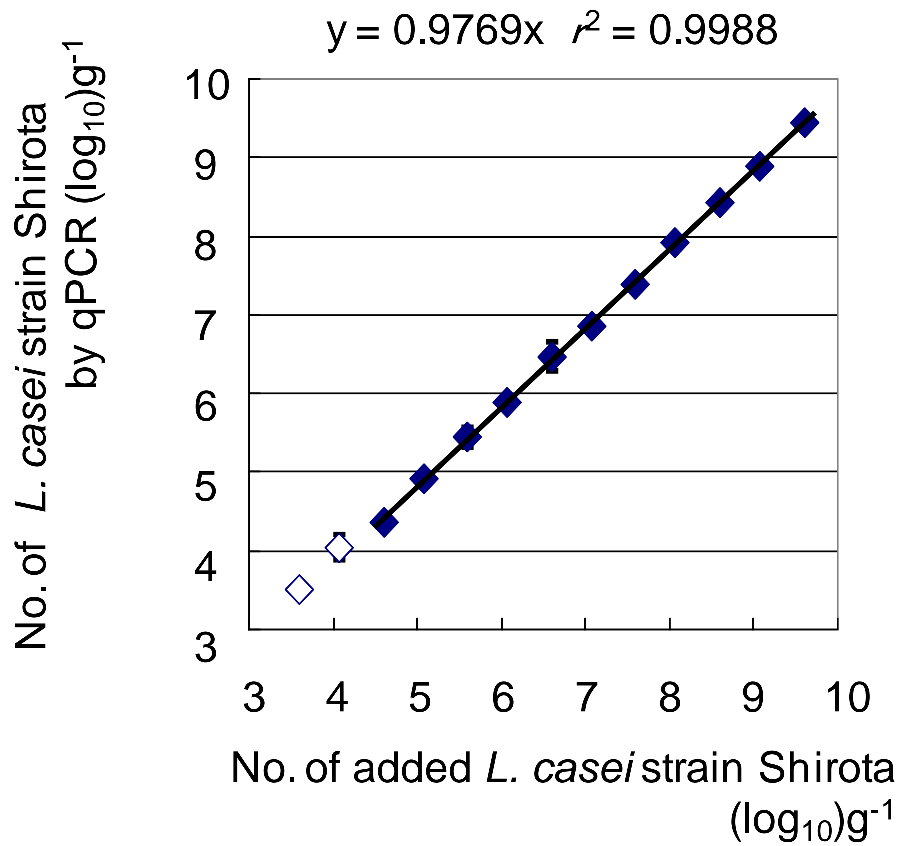

Fig. 3. Correlation between number of *L. casei* strain Shirota (LcS) added to fecal samples and that determined with quantitative real-time PCR (qPCR). The regression line was made between $10^{4.6}$ g^{-1} (filled diamonds) and $10^{9.6}$ g^{-1} as detected by qPCR, because amplification products were not always detected with an input of $10^{4.1}$ g^{-1} or less (open diamonds). The regression line was calculated with an intercept of 0. Bars, SD.

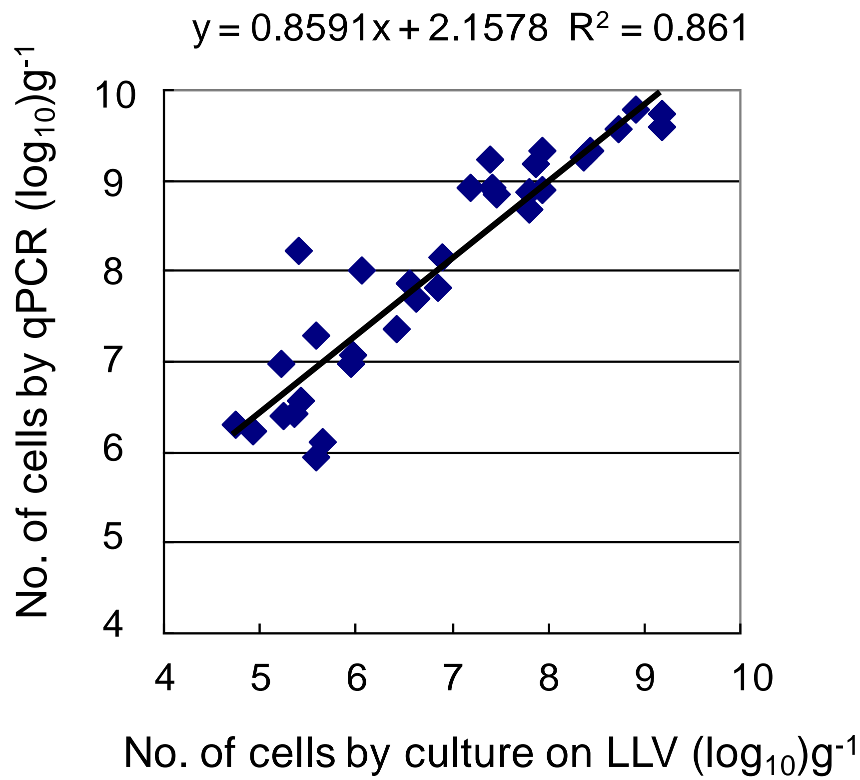

Fig. 4. Correlation between number of *L. casei* strain Shirota (LcS) obtained with the qPCR method and by culture on LLV for fecal samples collected after ingestion of fermented milk product. Only numbers of LcS above the qPCR detection limit ($>10^{4.6}$ g^{-1}) are shown.

Chapter 3

Identification and quantification of viable *Bifidobacterium breve* strain Yakult in human feces by using strain-specific primers and propidium monoazide

3.1. Introduction

The importance of a healthy lifestyle has created a great interest in probiotics. In the human gastrointestinal tract, bifidobacteria are a numerically important group of microorganisms that are considered to exert positive influences on biological activities related to host health (Collins and Gibson 1999; Kleerebezem and Vaughan 2009; Leahy, et al. 2005). *Bifidobacterium breve* strain Yakult (BbrY) has been used in fermented milk products for many years and is one of the most intensively studied probiotics. Some of the various benefits of BbrY include improving the balance of intestinal microbes by increasing the numbers of beneficial bacteria and decreasing the numbers of harmful bacteria, increasing the total amount of volatile fatty acids in the gastrointestinal environment, decreasing urinary mutagenicity, activating the immune system and anti-infectious activity, and acting as an anti-inflammatory bowel disease adjunct (Asahara, et al. 1999; Asahara, et al. 2004; Imaoka and Umesaki 2009; Ishikawa, et al. 2003; Kanamori, et al. 2009; Kanazawa, et al. 2005; Kato, et al. 2004; Matsumoto, et al. 2001; Shimakawa, et al. 2003; Sugawara, et al. 2006).

The basic requirement for probiotic bacteria to exert expected positive effects is to be alive (Fuller 1989); therefore, appropriate quantification methods are crucial (Klaenhammer and Kullen 1999). To determine the effectiveness of probiotics it is therefore essential to establish a specific method to identify them and measure their numbers.

The current method for detecting and identifying BbrY in fecal samples after ingestion of BbrY involves using both transgalactosylated oligosaccharide–carbenicillin (T-CBPC) selective agar medium for BbrY and strain-specific identification by randomly amplified polymorphism DNA fingerprinting (RAPD) (Williams, et al. 1990) or by enzyme-linked immunosorbent assay (ELISA)

using a monoclonal antibody (Shimakawa, et al. 2003). These methods, however, require considerable time, labor, experience, and skill for isolating BbrY. Recently, a PCR-based method for strain-specific identification using a strain-specific primer has been reported (Ahlroos and Tynkkynen 2009; Bunte, et al. 2000; Fujimoto, et al. 2008; Maruo, et al. 2006). This method is a powerful tool for identifying and enumerating specific strains from various kinds of materials.

We developed a strain-specific primer set for BbrY (pBbrY) by using a specific RAPD band sequence from BbrY. Using quantitative real-time PCR (qPCR) with pBbrY, we found that the number of BbrY in the feces after ingestion of BbrY was about 10 times higher than the number of living bacteria detected by the culture method. However, the number determined by the former method included both viable and dead cells. Although bifidobacteria have beneficial effects in both the live and dead state (Lopez, et al. 2010; Yasui, et al. 1999; Young, et al. 2004), live cells can proliferate and secrete beneficial factors. It is therefore important to be able to discriminate viable cells from dead cells in order to characterize the usefulness of BbrY as a probiotic.

Recently, differentiation of viable and dead cells in samples with several types of bacteria has been accomplished by using a PCR-based method with propidium monoazide (PMA) treatment, which selectively penetrates dead cells, which have compromised membrane integrity, but not viable cells with intact cell membranes (Bae and Wuertz 2009; García-Cayuela, et al. 2009; Nocker, et al. 2007). PMA is a DNA-intercalating dye that enables covalent binding to DNA under bright visible light; this makes DNA insoluble and strongly inhibits PCR amplification. Subjecting a bacterial population to PMA treatment before PCR therefore results in selective amplification of DNA from viable cells with intact membranes.

In this study we developed a procedure for the detection and quantification of viable BbrY in feces by a PCR-based method. The procedure was based on the combined use of the DNA intercalating agent PMA and qPCR, using strain-specific primers derived from RAPD analysis. We also demonstrated the application of this technique for enumerating viable BbrY in feces after the

ingestion of fermented milk containing BbrY.

3.2. Materials and methods

3.2.1. Reference strains and culture conditions

The 112 bacterial strains (30 strains of *B. breve* and 82 other strains of bacteria commonly isolated from human feces; Table 1) were obtained from the Culture Collection of the Yakult Central Institute (YIT and Y; Tokyo, Japan). Anaerobic bacteria were cultured at 37 °C for 1 or 2 days in GAM Broth, Modified "*Nissui*" (code 05433, Nissui, Tokyo, Japan) supplemented with 0.5% glucose. Lactic acid bacteria were cultured in MRS broth (Becton Dickinson, Sparks, MD, USA) at 37 °C for 1 day. For a quantitative PCR standard, the number of BbrY was counted after the bacteria had been stained with 4', 6-diamino-2-phenylindole (DAPI). Briefly, 500 µl of bacterial cells was collected and resuspended with 450 µl of PBS. Then 50 µl of formaldehyde (3.7% final concentration) was added to the bacterial suspension to fix the cells. The suspension was left overnight at 4 °C. Then, 10 µl fixed bacterial suspension was dropped onto each 1 × 1 cm well of an MAS-coated glass slide (Matsunami Glass Ind., Ltd., Osaka, Japan). The glass slide was left overnight at RT and washed once with 99.5% ethanol. After being air dried, bacterial cells were stained with 5 µl of DAPI and embedded in VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA, USA). Microscopic counts and image acquisition from glass slides were performed using a Q-550 FW system (Leica DM RXA2 and Q-Fluoro, Wetzlar, Germany). Ten fields were used, and a minimum of 100 cells were counted per field.

3.2.2. RAPD PCR analysis

Bacterial DNA for RAPD analysis was extracted by physical destruction and benzyl chloride purification, as previously described (Fujimoto, et al. 2008). RAPD-PCR amplification was performed using 27 RAPD primers, as described by Fujimoto, et al. (2008). RAPD products were

electrophoresed at 50 V in a 1.5% agarose gel.

3.2.3. Cloning and sequence analysis of RAPD products specific to BbrY

Potential strain-specific RAPD markers were extracted from agarose gels with a SUPREC-01 gel extraction kit (Takara, Shiga, Japan). The collected amplification products were cloned using a TA cloning kit with pCR 2.1 vector (Invitrogen, Leek, the Netherlands). The nucleic acid sequences of 8 clones of each potential strain-specific RAPD marker were determined with an ABI model 373A DNA sequencer with a Dye Terminator sequencing kit (Applied Biosystems, Foster, CA, USA).

3.2.4. Specificity of RAPD-derived primers

The specificity of the BbrY-specific primer set (pBbrY) was confirmed by PCR using DNA from 112 bacterial strains (Table 1). PCR amplifications were performed in a DNA Engine PTC-200 (MJ Research, Waltham, MA, USA). Each reaction mixture (20 μL) contained 10 mmol Tris-HCl (pH 8.3), 50 mmol KCl, 1.5 mmol $MgCl_2$, 200 μmol of each dNTP, 1.5 U *Taq* DNA polymerase (Takara), 0.3 μmol primers, and 10 ng template DNA. The amplification program consisted of 1 cycle of 94 °C for 2 min; 32 cycles of 94 °C for 20 s, 60 °C for 10 s, and 72 °C for 20 s; and finally 1 cycle of 72 °C for 3 min. PCR products were electrophoresed at 100 V in a 1.5% agarose gel.

3.2.5. PMA treatment

Pure culture of BbrY or fecal samples were treated with PMA, as described by Nocker, et al. (2007). Propidium monoazide (PMA, Biotium, Inc, CA, USA) was dissolved in 20% dimethyl sulfoxide (DMSO) to create a 20 mmol l^{-1} PMA stock solution and stored at –20 °C in the dark. An adequate amount of PMA stock solution was added to 500 µl pure culture of BbrY or 10-times diluted fecal solutions to make final concentrations of 5, 50, and 150 µmol l^{-1}. Following incubation at room temperature for 5 min in the dark with occasional mixing, triplicate samples were light

exposed for 1, 2, and 5 min at a distance of about 20 cm from two 500-W halogen light sources. After photo-induced cross-linking, cells were pelleted at 20,000 *g* for 4 min to remove the supernatant. PMA-treated samples were preserved at –80 °C until the DNA was extracted.

3.2.6. Quantification of added BbrY in fecal samples by quantitative PCR

Various concentrations of viable or heat-killed (incubated at 80 °C for 10 min) BbrY (10^4 to 10^9 cells per gram of feces) were added to 3 fecal samples containing no BbrY (confirmed by the culture method and qPCR with pBbrY). The total concentration of intestinal microorganisms by DAPI was $10^{10.7\pm0.4}$ cells g^{-1} (mean (±S.D).). Fecal samples were collected in individual sterile Faeces Containers (Sarstedt, Nümbrecht, Germany), refrigerated, and taken to the laboratory within 4 h. The DNA was extracted from these mixed feces and subjected to qPCR analysis using pBbrY.

3.2.7. Examination of fecal samples after ingestion

Informed consent was obtained from the volunteers who provided the fecal samples used. The Ethical Committee of the Yakult Central Institute provided ethical clearance for this microbiological research study in accordance with the Helsinki Declaration. The study population comprised 11 healthy volunteers (age range, 23–59 years; mean±S.D., 32.8±10.3 years) who ingested a commercially available fermented milk product (Bifine S™), containing $10^{10.7}$ CFU BbrY, once daily for 10 days. Feces excreted before and after drinking the fermented milk product were collected in individual sterile Faeces Containers (Sarstedt), refrigerated, and taken to the laboratory within 4 h. No subject ingested probiotic products, including the study product, during the 3 weeks before drinking the BbrY-containing fermented milk product for this study.

3.2.8. Enumeration of BbrY by the standard culture method

Counts (in CFU) of BbrY were determined by using strain-specific T-CBPC selective agar

medium. T-CBPC selective agar medium consists mainly of transgalactosylated oligosaccharide (TOS) as a growth factor, and carbenicillin (CBPC) and streptomycin as selective agents of *B. breve* strain Yakult (BbrY). We used commercially available TOS propionate agar medium (Yakult Pharmaceutical Industry Co., Ltd., Tokyo, Japan) supplemented with CBPC and streptomycin as a strain-specific selective culture medium. The medium contained (per liter) 10 g of trypticase, 1 g of yeast extract, 3 g of KH_2PO_4, 4.8 g of K_2HPO_4, 3 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4$, 0.5 g of L-cysteine, 15 g of sodium propionate, 10 g of TOS, 15 g of powdered agar, 5,000,000 units of streptomycin sulfate (Sigma Chemical, St. Louis, MO, USA) and 1000 µg of carbenicillin disodium salt (Sigma). Aliquots (0.1 ml) of 10-fold serial dilutions of feces (starting sample, 0.5 g) in 0.85% NaCl were spread on T-CBPC agar and incubated anaerobically at 37 °C for 72 h. For each fecal sample, we collected all colonies from the T-CBPC plate inoculated with the highest dilution that yielded growth and subjected these colonies to real-time PCR analysis using pBbrY. The number of BbrY per gram of feces (wet weight) was estimated from the number of colonies that were identified as containing BbrY by PCR with pBbrY. All isolates were also analyzed by RAPD analysis.

3.2.9. Extraction of DNA from fecal samples

The DNA from fecal samples was extracted by using a Stool Mini kit (Qiagen, Valencia, CA, USA), as previously described (Fujimoto, et al. 2008) with slight modification. The 10-fold diluted fecal solution (200 µl) was pelleted by centrifugation at 20,000 *g* for 5 min, washed 3 times with 1.0 ml phosphate-buffered saline to remove PCR inhibitors, and suspended in 600 µl Buffer ASL (Qiagen). The fecal suspension was heated to 70 °C for 5 min. The suspension was vortexed with glass beads (700 mg; 0.1 mm in diameter) and 500 µl buffer-saturated phenol using a FastPrep Fp120 (Bio 101, Irvine, CA, USA) at a speed setting of 6.5 m s^{-1} for 30 s. We added 100 µl of 3 mol l^{-1} sodium acetate (pH 4.8) to the suspension, which was kept on ice for 5 min and then centrifuged at 20,000 *g* for 8 min. The supernatant (700 µl) was mixed with 700 µl Buffer ASL and an InhibitEX

tablet (Qiagen). After centrifugation of the mixture at 20,000 *g* for 2 min, 550 µl of supernatant was mixed with 550 µl of Buffer AL (Qiagen) and 550 µl of 100% ethanol. The lysate was trapped on a QIAamp spin column (Qiagen). After the column was washed with washing buffer (AW1 and AW2, Qiagen), DNA was eluted in 100 µl of Buffer AE (Qiagen).

3.2.10. Real-time PCR analysis

PCR amplification and detection of all or live BbrY were performed in an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems), as previously described (Fujimoto, et al. 2008), with slight modification. The reaction mixture (20 µl) contained 10 mmol l^{-1} Tris-HCl (pH 8.3), 50 mmol l^{-1} KCl, 1.5 mmol l^{-1} $MgCl_2$, 200 µmol l^{-1} of each dNTP, 500 µg ml^{-1} bovine serum albumin (Takara), a 1:75,000 dilution of SYBR Green I (Invitrogen), 0.4 U *Taq* DNA polymerase Hot Start version (Takara), 0.3 µmol of each of the specific primers, and 5 µl of template DNA diluted 10-, 10^2-, or 10^3-fold. The amplification program consisted of an initial heating step at 94 °C for 5 min; 40 cycles of 94 °C for 20 s, 60 °C for 10 s, and 72 °C for 20 s; and a final extension step at 72 °C for 3 min. Fluorescence intensities were detected during the last step of each cycle. To distinguish the targeted PCR product from non-targeted PCR products (Ririe, et al. 1997), melting curves were obtained after amplification by slow heating from 60 to 95 °C in increments of 0.2 °C s^{-1} with continuous fluorescence collection.

3.2.11. Statistical methods

Pearson's correlation coefficients were used to determine the correlations between the number of added BbrY and the counts obtained with qPCR in the experiment with added BbrY. Simple linear regression was used to develop regression equations for statistically significant relationships. Mean differences in the concentrations of BbrY using qPCR were analyzed by the paired *t*-test. The data obtained to optimize the PMA treatment test were statistically analyzed by the variance analysis, and

the means were separated according to Tukey's HSD test with a significance level (*P*) of 0.05.

3.3. Result

3.3.1. Screening for strain-specific RAPD markers

To identify a strain-specific PCR product for BbrY, we tested a total of 27 RAPD primers on 30 *B. breve* strains. Primer p1285 (5′ AGC CAG TTT C 3′) generated a 1.1-kb BbrY-specific band (arrows, Fig. 1). We determined the sequence of this BbrY-specific PCR product (accession no. AB568490). As a result of the FASTA analysis in the DDBJ/GenBank/EMBL DNA database, we found that the partial sequence of BbrY-specific PCR product (position 106–860) had 80.3% similarity to the SalX-type ABC antimicrobial peptide transport system ATPase component of *Bifidobacterium longum* DJO10A (CP000605, position 1925211–1926284).

3.3.2. Design of a specific RAPD-derived primer pair

To amplify a BbrY-specific PCR product, we designed a candidate strain-specific primer set p1285-1F (AGC CAG TTT CGA GGT ATG GC) and p1285-1151R (AGC CAG TTT CCG AAG TTA CC) and used this primer set for PCR on 30 strains of *B. breve*. Five strains (YIT 4063, YIT 4064, YIT 4079, YIT 11888, and BbrY) yielded PCR products. These products were sequenced and aligned so that a new, more specific, target sequence for BbrY could be identified. We then designed a new set of BbrY-specific primers (pBbrY; pBbrY-F, ATG GCA AAA CCG GGC TGA A, and pBbrY-R, GCG GAT GAG AGG TGG G) and tested its specificity against DNA extracted from 112 bacterial strains, including the 30 strains of *B. breve* (Table 1). These primers exclusively supported PCR amplification of BbrY template DNA, with no cross-reaction against nontarget microorganisms. The amplification product was 313 bp long and had a melting temperature of 89.8 °C.

3.3.3. Quantitative PCR detection of viable or heat-killed BbrY with PMA treatment

To optimize the PMA treatments, viable and heat-killed BbrY cells were exposed to different concentrations of PMA (5, 50, or 150 μmol l^{-1}) and different photoactivation times (1, 2, or 5 min). Using qPCR did not result in significant differences in enumeration of viable BbrY under any conditions (all $10^{9.6}$ cells ml^{-1}). In the case of heat-killed BbrY, using qPCR without PMA treatment resulted in a reduced count of BbrY ($10^{8.6}$ cells ml^{-1}). The number of BbrY was significantly higher at a low PMA concentration (5 μmol l^{-1}) than at PMA concentrations of 50 and 150 μmol l^{-1} ($P <$ 0.05). Changes in photoactivation time did not influence the enumeration of heat-killed BbrY when qPCR with PMA treatment at 50 or 150 μmol l^{-1} was used. Moreover, there was no significant difference between PMA treatments with 50 and 150 μmol l^{-1} in the number of heat-killed BbrY. PMA treatment at 5 μmol l^{-1} was not sufficient to significantly decrease the number of heat-killed BbrY compared with no PMA treatment.

We confirmed that the number of heat-killed BbrY using qPCR with PMA treatment in 50 μmol l^{-1} PMA solution and with 2 min photoactivation ($10^{4.7±0.3}$ cells ml^{-1}) was significantly decreased ($P <$ 0.05) than without PMA treatment ($10^{8.6±0.1}$ log cells ml^{-1}) (Fig. 2).

3.3.4. Quantitative PCR detection of viable or heat-killed BbrY, with PMA treatment, added to feces

We confirmed that PMA treatment worked to reduce the numbers of dead cells for quantifying viable BbrY in the fecal samples. We added $10^{10.5}$ cells g^{-1} heat-killed BbrY directly to fecal samples. Following PMA/no PMA treatment, we extracted DNA and quantified BbrY by using qPCR with pBbrY. With heating, the number of BbrY by qPCR using pBbrY without PMA treatment was reduced slightly to $10^{9.8±0.1}$ cells g^{-1}, whereas the number of BbrY with PMA treatment was significantly reduced to $10^{5.5±0.5}$ cells g^{-1} ($P < 0.001$).

We next added viable BbrY directly to fecal samples in amounts from 10^4 to 10^9 g^{-1}. After PMA

treatment, we analyzed the correlation between the number of added BbrY and the value obtained with qPCR. We also determined the lower limit of detection of BbrY in the feces by qPCR with pBbrY and PMA treatment. When 10^5 to 10^9 viable BbrY were added per gram of feces, the qPCR gave accurate results ($r^2 = 0.9983$, $P < 0.001$; Fig. 3a). Fecal samples not treated with PMA had the same detection limit and correlation between added BbrY and the value obtained with qPCR ($r^2 = 0.9996$, $P < 0.001$; Fig. 3b).

3.3.5. Quantitative detection of ingested BbrY in feces

We used our quantitative PCR method with PMA treatment (with 50 μmol^{-1} PMA solution and 2 min photoactivation) to measure the number of BbrY in the feces of subjects who drank a fermented milk product containing BbrY. Before ingestion, the number of BbrY in the feces was below the detection limit (qPCR, < 10^5 cells g^{-1}; CFU, < 10^2 CFU g^{-1}) indicating that we used both the strain-specific qPCR method and the conventional culturing method in T-CBPC medium. After ingestion, BbrY was detected in all subjects at $10^{8.1\pm0.8}$ cells g^{-1} (mean±S.D.) by qPCR without PMA treatment and was detected in 10 of 11 subjects at $10^{7.5\pm1.0}$ cells g^{-1} by qPCR with PMA treatment. In addition, BbrY was isolated from all subjects at $10^{6.9\pm1.5}$ CFU g^{-1} by using a T-CBPC agar plate (Table 2).

3.4. Discussion

Numerous reports of PCR-based methods for strain-specific detection and enumeration have been recently published. Investigators designing strain-specific primer sets have used RAPD (Ahlroos and Tynkkynen 2009; Fujimoto, et al. 2008; Maruo, et al. 2006; Tilsala-Timisjarvi and Alatossava 1998), subtraction hybridization techniques (Bunte, et al. 2000), sequence analysis of AFLP markers (Sisto, et al. 2009), sequence data from the 16S-23S rRNA intergenic spacer regions, phage-related sequences, the S-layer gene (Brandt and Alatossava 2003; Flint and Angert 2005; Saito,

et al. 2004). In this study we confirmed the RAPD technique to be one of the best methods of developing strain-specific primers because, as noted by Briczinski, et al. (2009), it can compare the whole genome of many strains in detail to easily and rapidly find a strain-specific sequence.

Using both pBbrY and T-CBPC medium, we confirmed that BbrY and other strains with sequences identical to those of the target site of specific primer in their genomes did not exist in the volunteers' feces before they drank the fermented milk product containing BbrY.

We also quantified the number of BbrY by using the standard method of culture on T-CBPC selective agar medium. T-CBPC is a strain-specific selective medium. We then used PCR with pBbrY to identify some colonies that appeared on the T-CBPC agar plate at the highest fecal dilution that yielded growth. DNA extracted directly from 68 colonies that appeared on the T-CBPC agar plates was subjected to PCR analysis. Forty-eight isolates were identified as BbrY by using pBbrY and RAPD analysis, and the remaining 20 isolates were confirmed as non-BbrY by both methods. We have confirmed that PCR using pBbrY enabled us to identify the colonies on the T-CBPC medium efficiently and accurately. It also enabled us to reduce the need for the laborious process of identification that we had to use previously, including isolation and purification of isolates, RAPD or immunological methods using monoclonal antibody.

A DNA-intercalating dye, such as PMA, can be covalently linked to DNA by photoactivation and enables conventional PCR amplification of target DNA from viable but not dead cells. We attempted ethidium monoazide (EMA) treatment (Soejima, et al. 2008) to enumerate viable BbrY using qPCR, but the number of viable BbrY with EMA treatment exhibited a clear declining trend about 10 times lower (data not shown). EMA has been suggested as being toxic to some viable cells (Nocker, et al. 2006; Pan and Breidt 2007); we therefore chose to use PMA treatment with qPCR to accurately enumerate viable BbrY. We confirmed the optimal conditions of PMA treatment (50 µmol l^{-1} PMA, 5 min incubation, 2 min photoactivation) (Nocker, et al. 2007) for enumeration of viable BbrY by varying the conditions and examining the results. PMA treatment did not affect the

numbers of viable BbrY cells detected, but compared with non-PMA treatment it reduced the numbers of heat-killed cells detected (by about 1/10 000) (Fig. 2).

We have previously reported on a method of DNA extraction from feces for qPCR (Fujimoto, et al. 2008). The method includes glass-beads destruction, phenol extraction, degradation of RNA using RNase, and purification using the Stool Mini kit (Qiagen). This extraction method can remove a large amount of existing rRNA by processing RNase, and consequently the background fluorescence in qPCR is less. We have also confirmed that qPCR with a strain-specific primer using DNA extracted by this method can detect *Lactobacillus casei* strain Shirota at $10^{4.7}$ cells g^{-1} feces. In the present study, we reconfirmed that the detection limit of BbrY by qPCR without RNase treatment (10^5 cells g^{-1} feces) was not significantly different from our previous results.

We confirmed that the counts of BbrY in feces by qPCR with or without PMA treatment were highly and significantly correlated with the numbers of viable BbrY (Fig. 3). We also confirmed that heating at 80 °C for 10 min was effective in killing all the viable cells of BbrY. The number of heat-killed BbrY detected in the feces by qPCR with PMA treatment was 1/10,000 that detected without PMA treatment. Moreover, the number of BbrY detected by qPCR with PMA treatment did not change in fecal samples that had been supplemented with viable BbrY and preserved at –80 °C for 3 months.

By qPCR without PMA treatment, the total number of BbrY detected in the feces after ingestion of BbrY ($10^{8.1\pm0.8}$ cells g^{-1}) was more than 10 times the number of colony-formable bacteria detected by the culture-dependent method ($10^{6.9\pm1.5}$ CFU g^{-1}). On the other hand, by qPCR with PMA treatment, the number of viable BbrY detected in the feces after ingestion of BbrY was $10^{7.5\pm1.0}$ cells g^{-1}. Consequently, 40% of the total BbrY in the feces after ingestion was viable (as judged by the integrity of the cell membrane in terms of its permeability to PMA entry), and the number of viable BbrY was four times the number of viable cells counted by the culture-dependent method. It is possible that culture on T-CBPC agar underestimates the number of viable BbrY cells because it

has the inherent disadvantage of using selective culture media supplemented with antibiotics [even if the minimum inhibition concentrations of CBPC and streptomycin against BbrY were 6.25 μg ml^{-1} and > 5000 U ml^{-1}, respectively (data not shown)].

Precisely how the bifidobacteria function as beneficial probiotics is not yet resolved. However, it has been suggested that viable bifidobacteria exert their health-promoting effects on the host via their metabolism and metabolites. In contrast, cell components of *Bifidobacterium* unrelated to whether the cells are viable or dead have been proposed to stimulate immune function (Leahy, et al. 2005; Lopez, et al. 2010; Yasui, et al. 1999). Consequently, there is a need to separately enumerate viable and dead cells if we are to understand the role of BbrY as a probiotic.

In conclusion, the BbrY-specific PCR primer set that we developed enabled efficient and accurate identification of the colonies that formed on T-CBPC medium. We confirmed that the use of a combination of quantitative PCR with PMA treatment and our BbrY-specific primers quickly and accurately analyzed the number of viable BbrY in fecal samples. In light of the increasing public interest in probiotics, we need to demonstrate the efficacy of our method in experiments with large numbers of test subjects. However, we believe that the strain-specific primers with PMA treatment we have described here will be powerful tools for understanding BbrY as probiotics in future studies.

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Table 1. Bacterial strains used in this study

Bacteroides (*Bact.*) spp.	*Bact. distasonis* YIT 6162^{T}, *Bact. fragilis* YIT 6158^{T}, *Bact. ovatus* YIT 6161^{T}, *Bact. thetaiotaomicron* YIT 6163^{T}, *Bact. uniformis* YIT 6164^{T}, *Bact. vulgatus* YIT 6159^{T}
Bifidobacterium (*Bif.*) spp. (except *Bif. breve*)	*Bif. adolescentis* YIT 4011^{T}, *Bif. angulatum* YIT 4012^{T}, *Bif. animalis* YIT 4044^{T}, YIT 4121 (DSM 10140), *Bif. asteroides* YIT 4033^{T}, *Bif. bifidum* YIT 4039^{T}, *Bif. boum* YIT 4091^{T}, *Bif. catenulatum* YIT 4016^{T}, *Bif. choerinum* YIT 4067^{T}, *Bif. coryneforme* YIT 4092^{T}, *Bif. cuniculi* YIT 4093^{T}, *Bif. dentium* YIT 4017^{T}, *Bif. gallicum* YIT 4085^{T}, *Bif. gallinarum* YIT 4094^{T}, *Bif. indicum* YIT 4083^{T}, *Bif. longum* YIT 4018 (ATCC 15697), YIT 4021^{T}, YIT 4082 (JCM 1269), *Bif. magnum* YIT 4098^{T}, *Bif. merycicum* YIT 4095^{T}, *Bif. minimum* YIT 4097^{T}, *Bif. pseudocatenulatum* YIT 4072^{T}, *Bif. pseudolongum* subsp. *globosum* YIT 4101^{T}, *Bif. pseudolongum* subsp. *pseudolongum* YIT 4102^{T}, *Bif. pullorum* YIT 4104^{T}, *Bif. ruminantium* YIT 4105^{T}, *Bif. saeculare* YIT 4111^{T}, *Bif. subtile* YIT 4116^{T}, *Bif. thermophilum* YIT 4073^{T}
Bif. breve	*Bif. breve* YIT 4014^{T}, YIT 4015 (ATCC 15698), YIT 4023, YIT 4024, YIT 4043, YIT 4049 (ATCC 15701), YIT 4063, YIT 4064, YIT 4079, YIT 11016, YIT 11043, YIT 11044, YIT 11045, YIT 11046, YIT 11047, YIT 11049, YIT 11062, YIT 11063, YIT 11064, YIT 11065, YIT 11066, YIT 11067, YIT 11068, YIT 11069, YIT 11888, Y 91010, Y 91023, Y 94016, Y 94028, strain Yakult (YIT 12272)
Clostridium (*Cl.*) spp.	*Cl. celatum* YIT 6056^{T}, *Cl. perfringens* YIT 6050^{T}
Collinsella aerofaciens	*Collinsella aerofaciens* YIT 10235^{T}
Enterococcus (*Ent.*) spp.	*Ent. faecalis* YIT 2031^{T}, *Ent. faecium* YIT 2032^{T}
Escherichia coli	*E. coli* YIT 6044^{T}
Eubacterium (*Eu.*) spp.	*Eu. biforme* YIT 6076^{T}, *Eu. rectale* YIT 6082^{T}
Lactobacillus (*Lact.*) spp.	*Lact. acidophilus* YIT 0070^{T}, *Lact. amylophilus* YIT 0255^{T}, *Lact. amylovorus* YIT 0211^{T}, *Lact. bifermentans* YIT 0260^{T}, *Lact. brevis* YIT 0076^{T}, *Lact. buchneri* YIT 0077^{T}, *Lact. casei* YIT 0180^{T}, YIT 9029, *Lact. coryniformis* subsp. *coryniformis* YIT 0237^{T}, *Lact. crispatus* YIT 0212^{T}, *Lact. delbrueckii* subsp. *delbrueckii* YIT 0080^{T}, *Lact. delbrueckii* subsp. *lactis* YIT 0086^{T}, *Lact. delbrueckii* subsp. *bulgaricus* YIT 0181^{T}, *Lact. fermentum* YIT 0081^{T}, *Lact. gallinarum* YIT 0218^{T}, *Lact. gasseri* YIT 0192^{T}, *Lact. helveticus* YIT 0083^{T}, *Lact. johnsonii* YIT 0219^{T}, *Lact. malefermentans* YIT 0271^{T}, *Lact. oris* YIT 0277^{T}, *Lact. parabuchneri* YIT 0272^{T}, *Lact. paraplantarum* YIT 0445^{T}, *Lact. pentosus* YIT 0238^{T}, *Lact. plantarum* YIT 0102^{T}, *Lact. pontis* YIT 0273^{T}, *Lact. reuteri* YIT 0197^{T}, *Lact. rhamnosus* YIT 0105^{T}, *Lact. sakei* YIT 0247^{T}, *Lact. salivarius* subsp. *salivarius* YIT 0104^{T}, *Lact. sharpeae* YIT 0274^{T}, *Lact. vaginalis* YIT 0276^{T}, *Lact. zeae* YIT 0078 (ATCC 393)
Lactococcus (*Lc.*) spp.	*L. garviae* YIT 2071^{T}, *L. lactis* subsp. *cremoris* YIT 2007^{T}, *L. lactis* subsp. *lactis* YIT 2008^{T}, *L. lactis* subsp. *hordiniae* YIT 2060^{T}, *L. plantarum* YIT 2061^{T}, *L. raffinolactis* YIT 2062^{T}
Propionibacterium acnes	*Propionibacterium acnes* YIT 6165^{T}
Ruminococcus (*R.*) spp.	*R. bromii* YIT 6078^{T}, *R. lactaris* YIT 6084^{T}, *R. productus* YIT 6141^{T}
Streptococcus thermophilus	*Streptococcus thermophilus* YIT 2001, YIT 2021, YIT 2037^{T}

All strains were obtained from the Yakult Central Institute (YIT and Y ; Tokyo, Japan). The identification number for each strain other than type strains is given in parentheses. ATCC, American Type Culture Collection (USA); DSM, German Collection of Microorganisms and Cell Cultures (German); JCM, Japan Collection of Microorganisms (Japan).

Table 2. Number of *Bifidobacterium breve* strain Yakult in the feces of 11 volunteers who ingested a fermented milk product for 10 days.

subject	log cells or CFU g^{-1} of feces					
	Before ingestion			After ingestion		
	qPCR[a)]		CFU	qPCR		CFU
	without PMA	with PMA		without PMA	with PMA	
a	< 5.0[b)]	< 5.0	< 2.0[b)]	8.5	8.4	7.9
b	< 5.0	< 5.0	< 2.0	6.7	< 5.0	3.3
c	< 5.0	< 5.0	< 2.0	8.5	8.3	7.4
d	< 5.0	< 5.0	< 2.0	8.8	8.4	7.9
e	< 5.0	< 5.0	< 2.0	6.4	5.3	4.8
f	< 5.0	< 5.0	< 2.0	8.4	7.9	7.6
g	< 5.0	< 5.0	< 2.0	8.4	7.7	6.9
h	< 5.0	< 5.0	< 2.0	9.1	8.8	8.3
I	< 5.0	< 5.0	< 2.0	8.2	7.1	6.8
j	< 5.0	< 5.0	< 2.0	8.2	8.0	7.2
k	< 5.0	< 5.0	< 2.0	8.5	8.1	7.5
Mean[c)]	< 5.0	< 5.0	< 2.0	8.1	7.5	6.9
S.D.[c)]	-	-	-	0.8	1.0	1.5

[a] qPCR, quantitative real-time PCR.

[b] The lower limits of detection of quantitative real-time PCR (qPCR) and the culture method were 10^5 cells g^{-1} feces and 10^2 CFU g^{-1} feces, respectively.

[c] Mean and ±S.D. were calculated by using the detection limit of qPCR (10^5 cells g^{-1} feces) for subject b.

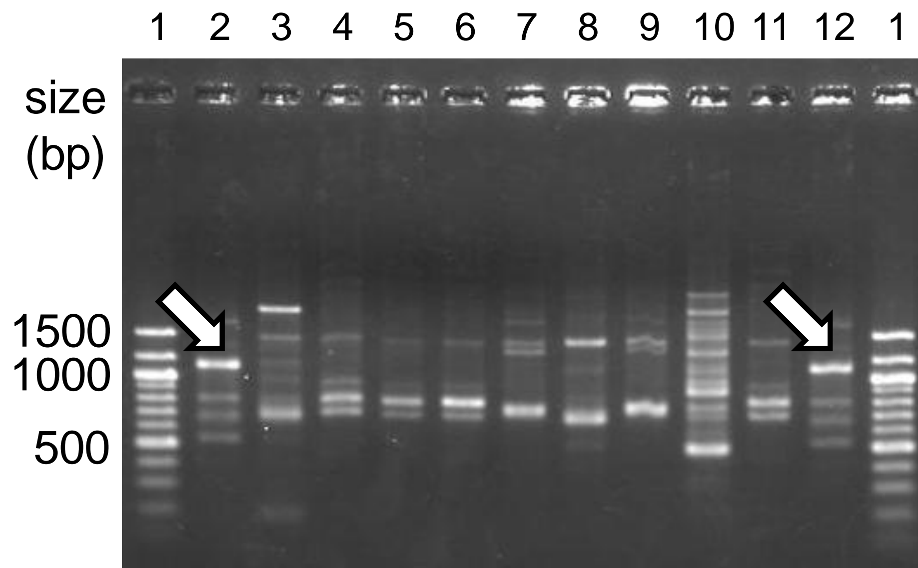


Figure 1. RAPD patterns obtained from 10 *Bifidobacterium breve* strains using p1258 primer. Lane 1, 100-bp DNA size marker; lanes 2 and 12, *B. breve* strain Yakult (BbrY); lanes 3 through 11, other strains of BbrY. Arrows indicate potential strain-specific bands of BbrY

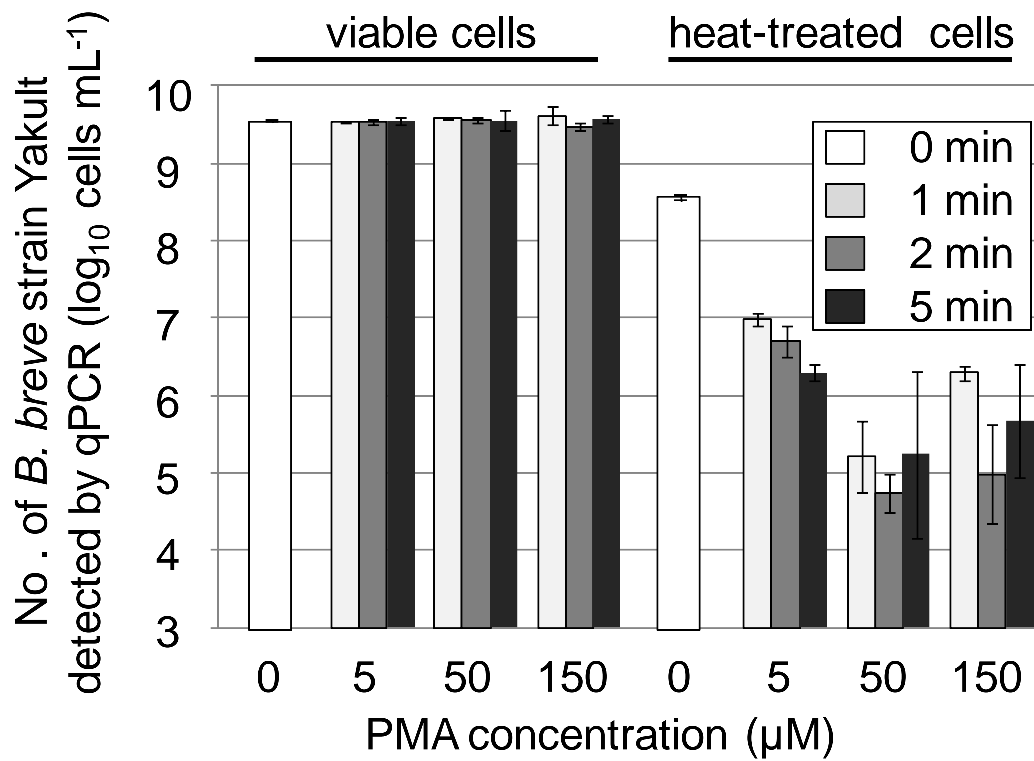
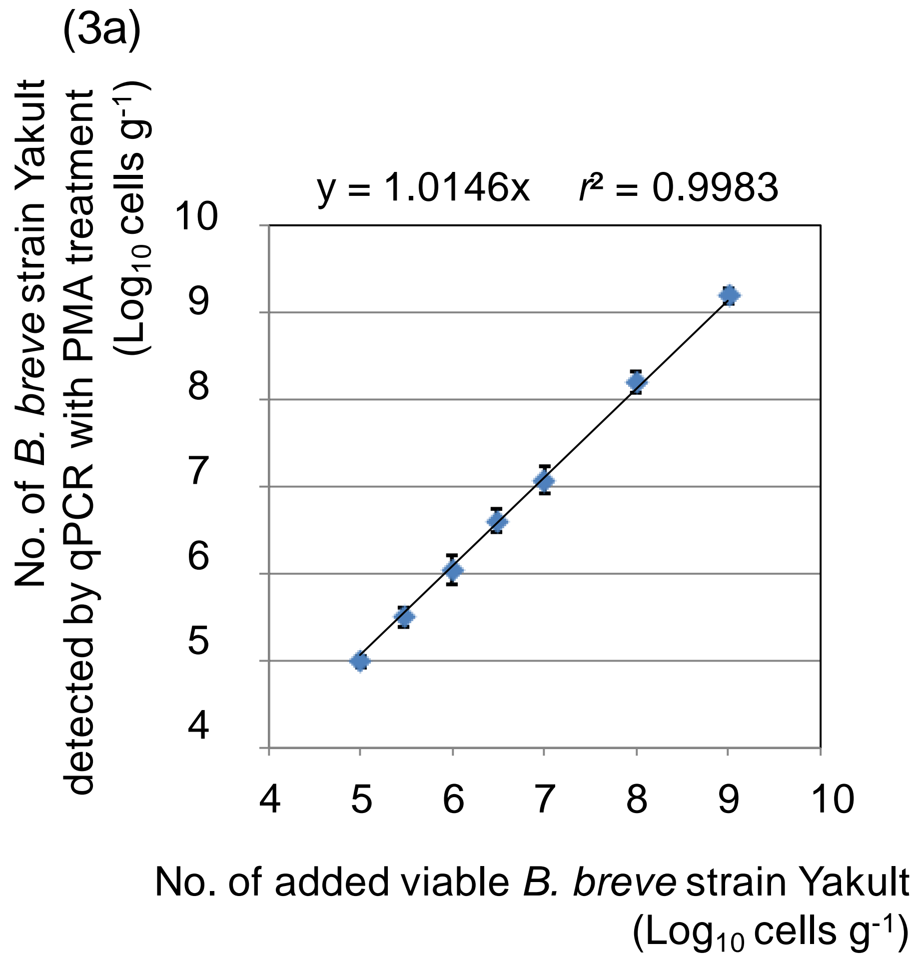


Figure 2. Amplification by quantitative real-time PCR of DNA from heat-killed (80 °C, 10 min) or unheated bacterial cells from pure culture of *Bifidobacterium breve* strain Yakult (BbrY) treated with different concentrations of PMA (5, 50, or 150 μmol l^{-1}) and with photoactivation times (1, 2, or 5 min).


(3a)
y = 1.0146x   r² = 0.9983
No. of B. breve strain Yakult detected by qPCR with PMA treatment (Log10 cells g-1)
No. of added viable B. breve strain Yakult (Log10 cells g-1)
4
5
6
7
8
9
10

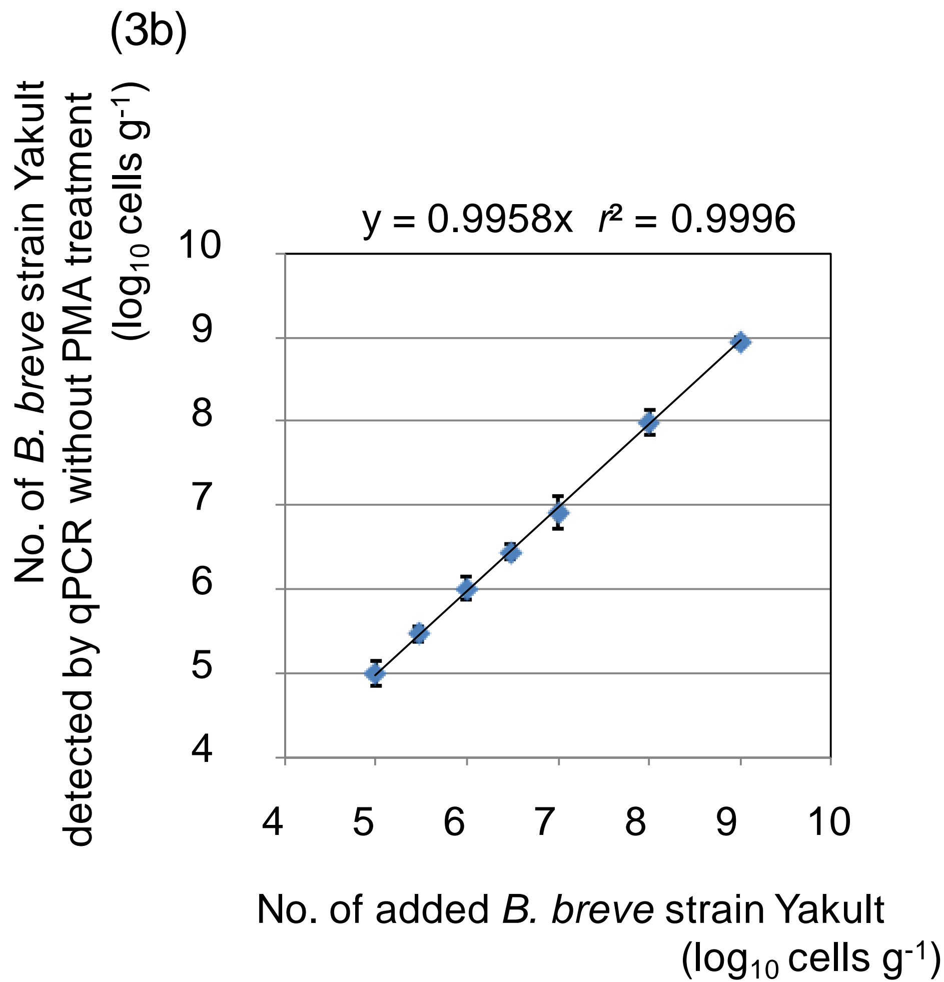


Figure 3. Correlation between number of viable *Bifidobacterium breve* strain Yakult added to fecal samples and that determined by quantitative real-time PCR (qPCR) with PMA treatment (Fig. 3a) and without PMA treatment (Fig. 3b). The regression line was made between 10^5 cells g^{-1} and 10^9 cells g^{-1}, as detected by qPCR. The regression line was calculated with an intercept of 0. Error bars represent standard deviations from three independent tests.

Chapter 4

Quantitative detection of viable *Bifidobacterium bifidum* BF-1 in human feces by using propidium monoazide and strain-specific primers

4.1. Introduction

In the human gastrointestinal tract, bifidobacteria are a numerically important group of microorganisms that are considered to exert positive influences on biological activities related to host health (Collins and Gibson 1999; Kleerebezem and Vaughan 2009; Leahy, et al. 2005; Turroni, et al. 2011). *Bifidobacterium bifidum* YIT 10347 (BF-1) was isolated from *B. bifidum* YIT 4007 as an oxygen-resistant strain, and is used as a starter culture for production of fermented milk products. The consumption of fermented milk containing BF-1 can improve gastric symptoms caused by *Helicobacter pylori* infection (Miki, et al. 2007), and BF-1 affects regulatory mechanisms in human cells, especially nuclear factor kappa B (NF-κB) expression, which is induced by *H. pylori* infection (Shirasawa, et al. 2010).

The generally accepted definition of probiotics was proposed by the Food and Agriculture Organization (FAO) World Health Organization (WHO): i.e., “Probiotics are live microorganisms which when administrated in adequate amounts confer a health benefit on the host” (FAO/WHO 2001). To determine the effectiveness of probiotics it is therefore essential to establish a specific method to identify them and measure their numbers (Klaenhammer and Kullen 1999).

First, we developed a strain-specific method for detecting and identifying BF-1 that was based on a conventional culture method. Because BF-1 is resistant to erythromycin and streptomycin (Sato and Iino 2010), we used selective agar containing transgalactosylated oligosaccharide-erythromycin-streptomycin (T-EMSM) followed by strain-specific identification of the colonies on the agar plate by random amplified polymorphism DNA (RAPD) fingerprinting (Williams, et al. 1990). Such culture-based methods, however, require considerable time, labor, experience, and skill.

Therefore, there has been increasing interest in the development of rapid PCR-based methods for strain-specific detection (Fujimoto, et al. 2008; Peng, et al. 2011; Xiang, et al. 2010) and cell viability determination (Nocker and Camper 2009; Nocker, et al. 2009; Taskin, et al. 2011; Varma, et al. 2009). Recently, a method for strain-specific quantification of viable cells was reported (Fujimoto, et al. 2011). This method uses a combination of intercalating dye, propidium monoazide (PMA), which selectively penetrates dead cells through their compromised cell membranes and covalently binds to the DNA under bright visible light, and strain-specific primers for quantitative PCR (qPCR).

Here, we developed a PCR-based procedure for the detection and quantification of viable BF-1 cells in feces. The procedure combines the use of PMA with qPCR using strain-specific primers designed from BF-1 specific sequences derived from an RAPD analysis. We used this method to examine changes in the membrane permeability of BF-1 cells in long-term cultivation and following artificial gastric juice treatment. We also successfully used the technique to quantify viable BF-1 cells in feces of subjects who had ingested fermented milk containing BF-1.

4.2. Materials and methods

4.2.1. Reference strains and culture conditions.

The 127 bacterial strains (30 strains of *B. bifidum* and 97 strains of other bacteria commonly isolated from human feces) (Table 1) were obtained from the Culture Collection of the Yakult Central Institute (YIT; Tokyo, Japan). Anaerobic bacteria were cultured at 37 °C for 1 or 2 days in GAM Broth, Modified “*Nissui*” (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 0.5% glucose. Lactic acid bacteria were cultured in MRS broth (Becton Dickinson, Sparks, MD, USA) at 37 °C for 1 day. Since it was necessary to know the cell numbers for a quantitative PCR standard, BF-1 cells were stained with 4', 6-diamino-2-phenylindole (DAPI) in VECTASHIELD Mounting

Medium (Vector Laboratories, Burlingame, CA, USA) and counted, as described by Fujimoto et al. (2008).

4.2.2. RAPD analysis.

Bacterial DNA for the RAPD analysis was extracted by physical disruption of cells and benzyl chloride purification, as previously described (Fujimoto, et al. 2008). PCR amplification was performed by using 27 RAPD primers, as described by Fujimoto et al. (2008). RAPD products were separated by electrophoresis at 50 V in a 1.5% agarose gel.

4.2.3. Cloning and sequence analysis of RAPD products specific to BF-1.

Potential strain-specific RAPD markers were extracted from the agarose gels with the use of a Gel Indicator DNA Extraction Kit (BioDynamics Laboratory, Tokyo, Japan). The extracted amplification products were cloned by using pTAC-1 vector and Jet Competent Cells (DH5α) (TA PCR Cloning Kit; BioDynamics Laboratory). The DNA sequences of 5 clones of each potential strain-specific marker were determined by using an AB3130XL DNA sequencer (Applied Biosystems, Foster, CA, USA) and a BigDye Terminator v3.1 sequencing kit (Applied Biosystems).

4.2.4. Specificity of RAPD-derived primers.

The BF-1-specific primer set (pBF-1) was designed from the potential strain-specific sequences identified by RAPD analysis. The specificity of this primer set was confirmed by PCR analysis of DNA from 127 bacterial strains (Table 1). PCR amplifications were performed in a PTC-200 DNA Engine (MJ Research, Waltham, MA, USA) as previously described by Fujimoto et al. (2008), with slight modification. Each reaction mixture (20 μl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 U *Taq* DNA polymerase (Takara Bio, Shiga, Japan), 0.4 μmol primers, and 10 ng template DNA. The amplification program consisted of 1 cycle of 94 °C for

2 min; 32 cycles of 94 °C for 20 s, 60 °C for 10 s, and 72 °C for 20 s; and 1 cycle of 72 °C for 3 min. PCR products were separated by electrophoresis at 100 V in a 1.5% agarose gel.

4.2.5. PMA treatment.

Pure cultures of BF-1 or fecal samples were treated with 50 µM PMA (Biotium, Inc, CA, USA) and photoactivation for 2 min, as described by Fujimoto et al. (2011). The PMA-treated cell pellets were preserved at −20 °C until DNA extraction was performed.

4.2.6. Quantification of ATP content of samples.

The ATP content of each sample tested was measured using an ATP-luciferase reaction kit (Lucifer HS; Kikkoman, Chiba, Japan) based on the firefly luciferin-luciferase reaction (Sakakibara, et al. 1997), and a luminescence-measuring instrument, TD-20/20 (Turner designs, Sunnyvale, CA, USA), according to the manufacturer's instructions. ATP content was measured as relative light units (RLU). To convert from RLU to colony forming units (CFU), we used the ratio (RLU/CFU) obtained from BF-1 that was cultured anaerobically at 37 °C for 24 h in modified MRS (m-MRS; glucose replaced with lactose) broth containing (per liter) 10 g BBL Trypticase peptone (BD, Sparks, MD, USA), 5 g yeast extract, 3 g Bacto tryptose (BD), 3.9 g $K_2HPO_4 \cdot 3H_2O$, 3 g KH_2PO_4, 2 g diammonium hydrogen citrate, 5 ml salt solution (11.5% (w/v) $MgSO_4 \cdot 7H_2O$, 3.09% $MnSO_4 \cdot 5H_2O$; 0.68% $FeSO_4 \cdot 7H_2O$), 1 g Tween 80, 1.7 g sodium acetate, 10 g lactose, 0.2 g L-cysteine $HCl \cdot H_2O$ (Watanabe, et al. 2008). The cells were harvested by centrifugation at 20,000 × *g* for 4 min at 4°C and suspended in 50 mM sodium phosphate buffer (pH 7.0). The samples were stored at −80 °C until further use. The CFU of BF-1 was determined by using m-MRS agar plate cultured anaerobically at 37 °C for 72 h.

4.2.7. Extraction of nucleic acid from BF-1 cells.

The bacterial culture (250 µl) was centrifuged at 20,000 × *g* for 5 min at 4 °C, and the pellet was suspended in 500 µl RNA*later* solution (Ambion, Austin, TX, USA) to stabilize the RNA. After incubation at 4 °C for 1 h, the solution was centrifuged at 20,000 × *g* for 5 min at 4 °C, and the pellet was stored at −80°C until nucleic acid extraction. The pellet was vortexed with 250 µl of extraction buffer (100 mM Tris, 40 mM EDTA disodium salt [pH 9.0]), 500 µl of TE-saturated phenol (Wako Pure Chemical Industries, Osaka, Japan), 50 µl of 10% SDS, and glass beads (700 mg; 0.1 mm in diameter : Shinmaru Enterprises, Osaka, Japan) by using a FastPrep 24 Instrument (MP Biomedicals Irvine, CA, USA) at a speed of 6.5 m s^{-1} for 30 s at room temperature. We added 100 µl of 3 M sodium acetate (pH 4.8) to the suspension and incubated it on ice for 5 min. The suspension was then centrifuged at 20,000 × *g* for 8 min at 4 °C, and the supernatant (400 µl) was mixed with 400 µl ice cold 100% isopropanol. After centrifugation at 20,000 × *g* for 8 min at 4 °C, the pelleted nucleic acids were washed in ice cold 70% ethanol and air dried prior to suspension in 250 µl of TE buffer (10 mM Tris, 1mM EDTA [pH 8.0]).

4.2.8. Quantification of BF-1 cells by using reverse transcription quantitative PCR and quantitative PCR.

For quantification of the viable BF-1 cells, the real-time reverse transcription–quantitative PCR (RT-qPCR) analysis was conducted in two-steps. In the first step, reverse transcription was performed with the use of a Takara RNA PCR kit (AMV) version 3.0 (Takara Bio). The template nucleic acids were diluted 1:50 before use. Each reaction mixture (10 µl) contained 1 µl of template nucleic acids, 1 × RT Buffer, 1mM dNTP mixture, 5 mM $MgCl_2$, 10 U RNase inhibitor, 2.5 U AMV reverse transcriptase XL, and 1 µM reverse primer (*B. bifidum* species–specific, BiBIF-2) (Matsuki, et al. 1999). The reaction was performed at 52 °C for 20 min, and then the reaction mix was heated to 95 °C for 10 min and quick-chilled on ice. In the second step, qPCR was performed with the use of an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems), as previously

described (Fujimoto, et al. 2011), with slight modifications. The reaction mixture (20 μl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 mg ml^{-1} bovine serum albumin (Takara Bio), a 1:75,000 dilution of SYBR Green I (Invitrogen), 0.4 U *Taq* DNA polymerase Hot Start version (Takara Bio), 0.4 μmol of each of the *B. bifidum* species–specific primers (BiBIF-F and BIBIF-R) (Matsuki, et al. 1999), and 5 μl of template cDNA that had been diluted 10-, 10^2-, or 10^3-fold. The amplification program consisted of an initial heating step at 94 °C for 5 min; 40 cycles of 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 50 s; and a final extension step at 72 °C for 3 min. Fluorescence intensities were detected during the last step of each cycle. To distinguish the targeted PCR product from non-targeted PCR products (Ririe, et al. 1997), after the amplification, RT-qPCR melting curves were obtained by continuously collecting fluorescence intensity measurements as the reaction mix was slow heated from 60 to 95 °C in increments of 0.2 °C/s.

The real-time quantitative PCR (qPCR) analysis was performed to quantify total BF-1 cells (without PMA treatment) or viable BF-1 cells (with PMA treatment) were performed by using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems), as described above as second step of RT-qPCR with slight modifications: The qPCR was performed with pBF-1, and the annealing temperature was changed to 60 °C for 10 s.

4.2.9. Long-term culture and artificial gastric juice treatment of BF-1 cells.

Long-term culture was performed as follows. After pre-incubation in m-MRS broth anaerobically at 37 °C for 24 h, *B. bifidum* BF-1 culture was inoculated into 4 ml of m-MRS broth (1% v/v) and cultured anaerobically at 37 °C for 1, 2, 4, 7, or 10 days.

Artificial gastric juice treatment was performed as follows. After pre-incubation at 37 °C for 24 h, the BF-1 culture was inoculated into 16 ml of m-MRS broth (1% v/v) and cultured anaerobically at 37 °C for 24 h. The BF-1 cells were harvested by centrifugation at 8,000 × *g* for 5 min at 4 °C and

suspended in 16 ml of artificial gastric juice. The artificial gastric juice was prepared by supplementing the basal medium (pH 2.8) with pepsin as follows: 900 ml of basal medium (5.0 g l^{-1} Trypticase peptone, 1.5 g l^{-1} mucin from porcine stomach (Wako Pure Chemical Industries), 5.0 g l^{-1} NaCl, 3.0 g l^{-1} $NaHCO_3$, 1.0 g l^{-1} KH_2PO_4, adjusted to pH 2.8 with 1N HCl) was autoclaved for 15 min at 115 °C and then supplemented with 100 ml of filter-sterilized 400 mg l^{-1} pepsin solution (pepsin 1:10,000 from porcine stomach mucosa; Wako Pure Chemical Industries). BF-1 cells suspended in artificial gastric juice were cultured aerobically at 37 ºC for 1, 2, or 3 h.

4.2.10.Quantification of BF-1 cells added to fecal samples.

Fecal samples were collected in individual sterile Faeces Containers (Sarstedt, Nümbrecht, Germany), refrigerated at 4 °C, and taken to the laboratory within 4 h. The total concentration of intestinal microorganisms by counting DAPI-stained cells (DAPI count) was $10^{11.1 \pm 0.2}$ (mean ± SD) g^{-1} feces (wet weight). We added viable or heat-killed (80 °C for 10 min) BF-1 cells at an estimated concentration (by DAPI count) of $10^{10.3}$ cells g^{-1} feces (wet weight) to 3 fecal samples (200 µl of 10-fold diluted suspension) (from different volunteers) containing no BF-1 that were confirmed by using culture method with a BF-1 strain-specific T-EMSM selective agar (describe below) and strain-specific qPCR with pBF-1. Then viable BF-1 cells were added to 2 fecal samples (200 µl of 10-fold diluted suspension) (from different volunteers) containing no BF-1 to give final concentrations ranging from $10^{4.3}$ to $10^{10.3}$ cells g^{-1}. After PMA treatment, the DNA was extracted as previously described (Fujimoto, et al. 2008) from each sample and subjected to qPCR analysis using the pBF-1.

4.2.11. Examination of fecal samples produced after ingestion of BF-1.

The study population comprised 12 healthy volunteers (age range, 25–60 years; mean ± SD, 45.5 ± 11.3 years). The subjects ingested a commercially available fermented milk product (BF-1™),

containing $10^{10.3}$–$10^{11.0}$ CFU BF-1 bottle^{-1}, once daily for 28 days. Feces excreted before and after ingestion of the fermented milk product were collected in individual sterile Faeces Containers (Sarstedt), refrigerated at 4 ℃ and taken to the laboratory within 4 h. No subject ingested probiotic products, including the study product, during the 2-week period before commencement of this study. Informed consent was obtained from all volunteers who provided fecal samples. The Ethics Committee of the Oriental Ueno Health Promotion Center (Tokyo, Japan) provided ethical clearance for this microbiological research study in accordance with the Helsinki Declaration.

4.2.12. Quantitative detection of ingested BF-1 in feces by qPCR.

The DNA from fecal samples and was extracted by using a Stool Mini kit (Qiagen, Valencia, CA, USA), as previously described (Fujimoto, et al. 2011). The qPCR method with PMA or without PMA was performed to quantify the viable or total (viable plus dead) BF-1 cells in the feces of subjects who drank fermented milk product containing BF-1, according to the method described previously (Fujimoto, et al. 2011), using pBF-1.

4.2.13. Quantitation of BF-1 cells by culturing on T-EMSM selective agar.

Counts (in CFU) of BF-1 were determined by using strain-specific T-EMSM selective agar plate that contained transgalactosylated oligosaccharide (TOS) as a growth factor, and erythromycin (EM) and streptomycin (SM) as selective agents for BF-1 (Klaenhammer and Kullen 1999). The T-EMSM selective agar was based on commercially available TOS propionate agar medium (Yakult Pharmaceutical Industry, Tokyo, Japan), and consisted of (per liter) 10 g trypticase, 1.0 g yeast extract, 3.0 g KH_2PO_4, 4.8 g K_2HPO_4, 3.0 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4$, 0.5 g L-cysteine, 15 g sodium propionate, 10 g TOS, 15 g powdered agar, 50 mg erythromycin (Wako Pure Chemical Industries), and 5,000,000 U streptomycin sulfate (Sigma Chemical, St. Louis, MO, USA). Aliquots (100 µl) of 10-fold serial dilutions of feces (starting sample, 0.5 g) in 0.85% NaCl were spread on T-EMSM

agar and incubated anaerobically at 37 °C for 72 h in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA; atmosphere comprising 91:6:3 $N_2/CO_2/H_2$). For each fecal sample, we isolated colonies from the T-EMSM plate inoculated with the highest dilution that yielded growth and subjected these colonies to PCR analysis using the pBF-1. The number of BF-1 g^{-1} feces was estimated from the number of colonies that were identified as BF-1 by PCR analysis with the pBF-1. All isolates were also analyzed by RAPD analysis.

4.2.14. Statistical methods.

Differences in the concentrations of BF-1 added to the fecal samples measured by qPCR were analyzed by the paired Student's *t* test. Simple linear regression was used to develop regression equations for statistically significant relationships. Pearson's correlation coefficients were used to determine the correlations between the number of BF-1 cells added to the fecal samples and the BF-1 cell counts determined by qPCR. The Tukey's method was used to determine the correlations between the numbers of BF-1 cells detected in the feces produced after the ingestion of a BF-1-containing fermented milk product by using three methods (T-EMSM selective agar-based culture, PMA-qPCR and qPCR without PMA). P values < 0.05 were classified as statistically significant.

4.3. Result

4.3.1. Screening for strain-specific RAPD markers.

To identify a strain-specific marker for BF-1, we tested a total of 27 RAPD primers on 30 *B. bifidum* strains and analyzed the specificity of the resultant PCR products. Primer OPA11 (5′ CAA TCG CCG T 3′) generated a 1.4-kb BF-1–specific PCR product (Fig. 1). We determined the sequence of this BF-1–specific PCR product (DDBJ accession no. AB748455).

4.3.2. Design of a specific RAPD–derived primer pair.

Based on the sequence of the BF-1-specific PCR product, we designed BF-1-specific primers (pBF-1; pBF-1f, 5' ATG GCA AAA CCG GGC TGA A 3', and pBF-1r, 5' GCG GAT GAG AGG TGG G 3'). We tested the specificity of these primers against DNA extracted from 127 bacterial strains, including 30 strains of *B. bifidum* (Table 1). The primers exclusively supported PCR amplification of BF-1 template DNA, with no amplification against non-target microorganisms (data not shown). The amplification product was 697-bp in length and had two melting temperatures of 89.3 °C (main) and 86.7 °C.

4.3.3. Quantitative PCR detection of viable or heat-killed BF-1 cells in feces.

When viable BF-1 was added to feces, the counts of BF-1 cells obtained by PMA-qPCR (50 μM PMA solution and 2 min photoactivation) were similar to those obtained by qPCR without PMA treatment (Fig. 2). In contrast, in the case of heat-killed BF-1 cells added to feces, the use of PMA-qPCR resulted in a significantly reduced count of BF-1 cells ($10^{6.8 \pm 0.3}$ cells ml^{-1}) ($P < 0.001$) compared to that obtained without PMA treatment ($10^{10.4 \pm 0.1}$ cells ml^{-1}) (Fig. 2).

4.3.4. Accuracy of PMA-qPCR method for quantitative determination of viable BF-1 cells in feces.

We added viable BF-1 directly to 200 μl of 10-fold diluted fecal samples in amounts ranging from $10^{4.3}$ to $10^{10.3}$ cells g^{-1} feces (wet weight) and determined the number of BF-1 cells by using PMA-qPCR with pBF-1. Amplification products were not always detected for samples with $10^{4.8}$ cells g^{-1} feces, so this amount was deemed to be below the limit of detection. When the amount of viable BF-1 cells added $10^{5.3}$ to $10^{10.3}$ cells g^{-1} feces (wet weight), the PMA-qPCR results significantly correlated with the number of added viable BF-1 cells ($r > 0.99$, $P < 0.001$; Fig. 3),

suggesting that the PMA-qPCR method accurately determined the number of viable BF-1 cells, without any inhibitions to amplify viable BF-1.

4.3.5. PMA-qPCR detection of viable BF-1 cells in long-term cultures and artificial gastric juice– treated cultures.

During long-term culture (10 days), the total number of BF-1 cells determined by DAPI count or RT-qPCR remained constant at about 10^{10} cells ml^{-1}, and the number determined by qPCR without PMA treatment decreased slowly to about 10^{9} cells ml^{-1}. In contrast, the numbers of viable BF-1 cells determined by PMA-qPCR, ATP contents that were converted to CFU, and by the culture method employing m-MRS agar all decreased dramatically to a similar final level of 10^{6} cells ml^{-1} or CFU ml^{-1} (Fig. 4A).

During the first hour of treatment with artificial gastric juice, the numbers of viable BF-1 cells determined by ATP content and by the m-MRS–based culture method decreased rapidly from $10^{9.4}$ to $10^{5.9}$ CFU ml^{-1} and from $10^{9.4}$ to $10^{4.2}$ CFU ml^{-1}, respectively, whereas the number of viable BF-1 cells determined by PMA-qPCR decreased slowly from $10^{9.9}$ to $10^{9.0}$ cells ml^{-1}. During the entire 3-h treatment, the number of viable BF-1 cells determined by PMA-qPCR decreased to 10^{6} cells ml^{-1}. In contrast the number of viable BF-1 cells determined by the m-MRS–based culture method decreased rapidly to 10^{2} CFU ml^{-1}, and the number of viable BF-1 cells determined by RT-qPCR targeting of BF-1 specific 16S rRNA sequences decreased slightly from $10^{9.9}$ to $10^{9.1}$ cells ml^{-1} (Fig. 4B).

4.3.6. Selectivity of BF-1 strain-specific T-EMSM selective agar.

To determine the performance of the T-EMSM selective agar, equal amounts of BF-1 cells sampled from BF-1 cells cultured overnight in m-MRS broth and fermented milk product containing BF-1 were separately inoculated onto T-EMSM agar plates or TOS propionate agar plates. In both the pure cultures and the fermented milk, BF-1 formed large, milky-white, smooth colonies on the

T-EMSM agar plate, and there was no significant difference between the number of the colonies on the T-EMSM agar plate and the TOS propionate agar plate (unpaired Student's *t* test) (Table 2). When fecal samples taken from subjects prior to ingestion of the fermented milk were inoculated onto T-EMSM agar plates, some colonies grew on the selective medium; however, these colonies could be distinguished from BF-1 colonies by size or color and were confirmed to be non-BF-1 by PCR using pBF-1 and RAPD analysis (data not shown).

4.3.7. Quantitative detection of ingested BF-1 in feces.

We used our PMA-qPCR method to measure the number of viable BF-1 cells in the feces of subjects who drank fermented milk product containing BF-1. Before ingestion of the fermented milk product, the number of BF-1 cells in the feces was below the detection limit for the strain-specific qPCR method without PMA treatment ($<10^{5.3}$ cells g^{-1}feces [wet weight]) and the conventional culturing method employing T-EMSM agar plate ($< 10^{2.0}$ CFU g^{-1}). After ingestion of the fermented milk, BF-1 was detected in the feces of all subjects at $10^{7.6 \pm 0.7}$ cells g^{-1} by qPCR without PMA treatment and $10^{6.2 \pm 0.4}$ cells g^{-1} by PMA-qPCR. In addition, BF-1 was isolated from all subjects at $10^{4.5 \pm 1.5}$ CFU g^{-1} by using a T-EMSM agar plate (Table 3).

4.4. Discussion

Due to the disadvantages of conventional microbiological methods, *e.g.* underestimation by using selective reagents , clumping bacterial cells, or need laborious process and skills, bacterial quantification based on PCR detection of nucleic acids is increasingly being used (Ahlroos and Tynkkynen 2009; Fujimoto, et al. 2008; Maruo, et al. 2006; Ndoye, et al. 2011). Here we confirmed that the RAPD technique is one of the best methods for developing strain-specific primers for use in qPCR analysis. As noted by Briczinski et al. (2009) and Fujimoto et al. (2011), RAPD can

comprehensively compare the whole genome of many strains to easily and rapidly find strain-specific sequences.

The use of DNA-based molecular detection tools for bacteria has been hampered by the inability to distinguish signals originating from viable and dead cells; however, recently, differentiation of viable and dead cells in samples with several types of bacteria has been accomplished by methods that PCR amplify DNA from cells that have been treated with ethidium monoazide (EMA) or PMA (Fittipaldi, et al. 2010; Kramer, et al. 2009; Meng, et al. 2010; Nocker, et al. 2010). However, EMA has been suggested as being toxic to some viable cells (Nocker, et al. 2006). When BF-1 cells that were heat-killed by treatment at 80 °C for 10 min (Fujimoto, et al. 2011) were introduced into feces, the number of BF-1 cells detected in the feces by PMA-qPCR was 1/10,000 that detected without PMA treatment (Fig. 2). This result confirmed that PMA-qPCR detected only viable cells from the negligible amount of amplification of dead cells. We also demonstrated the accuracy of PMA-qPCR by confirming that the counts of BF-1 in feces analyzed by PMA-qPCR were highly and significantly correlated with the numbers of viable BF-1 cells added to the fecal samples (Fig. 3).

We compared the changes in numbers of BF-1 viable cells determined by various methods (T-EMSM selective agar-based culture, ATP content, RT-qPCR targeting of *B. bifidum* specific 16S rRNA sequences, and PMA-qPCR) when the cells were subjected to moderately slow death by long-term cultivation and rapid death by artificial gastric juice treatment. After long-term cultivation, the number of viable BF-1 cells determined by PMA-qPCR was similar to the numbers determined by T-EMSM selective agar-based culture and ATP contents converted to CFU (Fig. 4A). In contrast, during treatment with artificial gastric juice, the number of viable BF-1 cells determined by PMA-qPCR, which is dictated by membrane permeability, began to decrease 1 h later than those determined by T-EMSM selective agar-based culture and ATP contents converted to CFU (Fig. 4B). The T-EMSM selective agar-based culture method has the inherent disadvantage of underestimation

of viable microbes, because the use of antibiotics or the cells clumping. The method for quantification of ATP content also has disadvantages; it cannot be used to target-specific bacterial strains in complex environmental samples, such as feces. In addition, contrary to our expectations, the RT-qPCR method, which targets *B. bifidum*-specific 16S rRNA sequences and is believed to quantify only viable cells in some bacterial species (Matsuda, et al. 2007; McKillip, et al. 1999), did not successfully discriminate between viable and dead cells of BF-1 cells (Fig. 4). Lahtinen et al. (2008) demonstrated that viable but nonculturable probiotics maintain high levels of rRNA and retain viable properties. In our study, the rRNA contents of BF-1 did not changed or experience a slight reduction after either long-term culture or treatment with artificial gastric juice, whereas other properties of viability (CFU assayed by T-EMSM selective agar-based culture, ATP content, and membrane integrity assayed by PMA-qPCR) were decreased. Our results suggest that the rRNA of BF-1 is retained after the cells lose their viability. Therefore, we consider the PMA-qPCR to be the best way to rapidly and accurately quantify viable BF-1 cells.

To exert the expected positive effects, the basic requirement for probiotic strains is that they remain alive in the digestive tract (Fuller 1989). Here, we demonstrated that ingested BF-1 was detectable as viable cells in human samples by using T-EMSM selective agar and RAPD fingerprinting. We used PCR with pBF-1 to identify 74 colonies that appeared on the T-EMSM agar plates at the highest fecal dilution that yielded growth. Forty-nine isolates that formed large, milky white colored, and smooth colonies were identified as *B. bifidum* by using the pBF-1 and RAPD analysis, and the remaining 25 isolates formed colonies smaller than those of BF-1; gray or ocher colored colonies were confirmed as non-BF-1 by both methods. Thus, PCR using the pBF-1 enabled us to identify BF-1 colonies on the T-EMSM selective agar efficiently and accurately. It also enabled us to reduce the laborious process of identification including isolation and purification of isolates, and RAPD analysis.

After subjects ingested fermented milk product containing BF-1, the total number of BF-1 cells

in their fecal samples determined by using qPCR without PMA treatment was $10^{7.6 \pm 0.7}$ cells g^{-1}, but the number of viable BF-1 cells determined by PMA-qPCR was $10^{6.2 \pm 0.4}$ cells g^{-1}. This result suggests that about 11% (the average BF-1 ratio of PMA-qPCR to qPCR in 12 subjects) of the total BF-1 cells in the feces were still viable after passing though the digestive tract, when viability was assayed in terms of integrity of the cell membrane. The number of viable BF-1 cells determined by PMA-qPCR was 50 times higher ($P < 0.01$) than that determined by the T-EMSM selective agar–based culture method, suggesting that the use of antibiotics in the culture-dependent method may lead to underestimation of the number of viable BF-1 cells, even when the minimum amount of antibiotics required for selection (erythromycin and streptomycin at 5000 μg ml^{-1} and 50 μg ml^{-1}, respectively [Sato and Iino 2010]) was used.

After the subjects ingested the fermented milk product containing BF-1 for 28 days, the number of BF-1 cells in feces was compared between subjects (Fig. 5). The number of viable BF-1 cells detected by the T-EMSM selective agar–based culture method ranged from $10^{2.0}$ to $10^{6.6}$ CFU g^{-1} among the various subjects; in contrast the numbers determined by PMA-qPCR method showed a smaller range of $10^{5.5}$ to $10^{6.9}$ cells g^{-1}. The numbers of viable BF-1 cells determined by PMA-qPCR in 5 of the 12 subjects (b, h, i, j and l) were more than 100 times higher ($P < 0.01$) than those determined by T-EMSM selective agar-based culture (dotted lines, Fig. 5). In contrast, the numbers of viable BF-1 cells determined by PMA-qPCR in the other seven subjects (a, c, d, e, f, g and k) were almost at the same level as those determined by T-EMSM selective agar-based culture (dashed lines, Fig. 5). Thus, inter-subject differences in recovery of viable BF-1 cells by T-EMSM selective agar-based culture may be due to the differences in the magnitude of increased membrane permeability (reflected in differences in the PMA-qPCR results) and decreased colony forming ability of BF-1 during its transit in the gastrointestinal tract in each individual. Further studies are required to understand the survival mechanisms of BF-1 in different compartments of the digestive tract and the influence of different parameters on these mechanisms.

In conclusion, the BF-1-specific PCR primer set, pBF-1, which we developed here, enabled efficient and accurate identification of colonies that formed on T-EMSM agar. We confirmed that combining PMA treatment of samples before DNA extraction and quantitative PCR with the pBF-1 could be used to quickly and accurately analyze the number of viable BF-1 cells in fecal samples. We believe that the PMA-qPCR methodology we have described here will be a powerful tool for understanding the use of BF-1 as a probiotic.

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Table 1 Bacterial strains used in this study

Bacteroides	*B. caccae* YIT 10226^T, *B. distasonis* YIT 6162^T, *B. eggerthii* YIT 10227^T, *B. fragilis* YIT 6158^T, *B. ovatus* YIT 6161^T, *B. thetaiotaomicron* YIT 6163^T, *B. uniformis* YIT 6164^T, *B. vulgatus* YIT 6159^T
Bifidobacterium *B. bifidum*	*B. bifidum* YIT 4013(ATCC 15696), YIT 4039^T, YIT 4042(ATCC 11863), YIT 4069, YIT 4070, YIT 10987, YIT 10988, YIT 10990, YIT 10991, YIT 10992, YIT 10993, YIT 10994, YIT 10995, YIT 10996, YIT 10997, YIT 10998, YIT 10999, YIT 11000, YIT 11001, YIT 11002, YIT 11003, YIT 11004, YIT 11005, YIT 11007, YIT 11008, YIT 11009, YIT 11010, YIT 11011, YIT 11012, YIT 10347(BF-1)
Not *B. bifidum*	*B. adolescentis* YIT 4011^T, *B. angulatum* YIT 4012^T, *B. animalis* subsp. *animalis* YIT 4044^T, *B. animalis* subsp. *lactis* YIT 4121^T, *B. asteroides* YIT 4033^T, *B. boum* YIT 4091^T, *B. breve* YIT 4014^T, *B. breve* YIT 12272, *B. catenulatum* YIT 4016^T, *B. choerinum* YIT 4067^T, *B. coryneforme* YIT 4092^T, *B. cuniculi* YIT 4093^T, *B. dentium* YIT 4017^T, *B. gallicum* YIT 4085^T, *B. gallinarum* YIT 4094^T, *B. indicum* YIT 4083^T, *B. longum* subsp. *infantis* YIT 4018^T, *B. longum* subsp. *longum* YIT 4021^T, *B. magnum* YIT 4098^T, *B. merycicum* YIT 4095^T, *B. minimum* YIT 4097^T, *B. pseudocatenulatum* YIT 4072^T, *B. pseudolongum* subsp. *globosum* YIT 4101^T, *B. pseudolongum* subsp. *pseudolongum* YIT 4102^T, *B. pullorum* YIT 4104^T, *B. ruminantium* YIT 4105^T, *B. saeculare* YIT 4111^T, *B. subtile* YIT 4116^T, *B. suis* YIT 4082^T, *B. thermophilum* YIT 4073^T
Blautia	*B. producta* YIT 6141^T
Clostridium	*C. acetobutylicum* YIT 10170^T, *C. bifermentans* YIT 10228^T, *C. butyricum* YIT 10073^T, *C. celatum* YIT 6056^T, *C. coccoides* YIT 6035^T, *C. perfringens* YIT 6050^T
Collinsella	*C. aerofaciens* YIT 10235^T
Enterococcus	*E. faecalis* YIT 2031^T, *E. faecium* YIT 2032^T
Escherichia	*E. coli* YIT 6044^T
Eubacterium	*E. biforme* YIT 6076^T, *E. rectale* YIT 6082^T
Lactobacillus	*L. acidophilus* YIT 0070^T, *L. amylophilus* YIT 0255^T, *L. amylovorus* YIT 0211^T, *L. bifermentans* YIT 0260^T, *L. brevis* YIT 0076^T, *L. buchneri* YIT 0077^T, *L. casei* YIT 0180^T, *L. coryniformis* YIT 0237^T, *L. crispatus* YIT 0212^T, *L. delbrueckii* subsp. *bulgaricus* YIT 0181^T, *L. delbrueckii* subsp. *delbrueckii* YIT 0080^T, *L. delbrueckii* subsp. lactis YIT 0086^T, *L. fermentum* YIT 0081^T, *L. gallinarum* YIT 0218^T, *L. gasseri* YIT 0192^T, *L. helveticus* YIT 0083^T, *L. johnsonii* YIT 0219^T, *L. malefermentans* YIT 0271^T, *L. oris* YIT 0277^T, *L. parabuchneri* YIT 0272^T, *L. paraplantarum* YIT 0445^T, *L. pentosus* YIT 0238^T, *L. plantarum* YIT 0102^T, *L. pontis* YIT 0273^T, *L. reuteri* YIT 0197^T, *L. rhamnosus* YIT 0105^T, *L. sake* YIT 0247^T, *L. salivarius* subsp. *salicinius* YIT 0089^T, *L. salivarius* subsp. *salivarius* YIT 0104^T, *L. sharpeae* YIT 0274^T, *L. vaginalis* YIT 0276^T, *L. zeae* YIT 0078^T
Lactococcus	*L. garviae* YIT 2071^T, *L. lactis* subsp. *cremoris* YIT 2007^T, *L. lactis* subsp. *hordiniae* YIT 2060^T, *L. lactis* subsp. *lactis* YIT 2008^T, *L. raffinolactis* YIT 2062^T, *L. plantarum* YIT 2061^T
Parascardovia	*P. denticolens* YIT 4114^T
Propionibacterium	*P. acnes* YIT 6165^T
Ruminococcus	*R. bromii* YIT 6078^T, *R. lactaris* YIT 6084^T
Scardovia	*S. inopinata* YIT 4115^T
Streptococcus	*S. thermophilus* YIT 2001, *S. thermophilus* YIT 2021, *S. thermophilus* YIT 2037^T

Table 2 Comparison of recoveries of BF-1 by T-EMSM selective media and non-selective medium (TOS propionate agar medium) (Log CFU ml^{-1})

	Pure culture of BF-1	Fermented milk containing BF-1
T-EMSM agar medium	9.34 ± 0.03	9.01 ± 0.05
TOS propionate agar medium	9.33 ± 0.02	8.97 ± 0.07

The data are means ± SD

Table 3 Number of BF-1 in the feces of 12 volunteers who ingested a fermented milk product containing BF-1 for 28 days

subject	Log cells g^{-1} feces or Log CFU g^{-1} feces (wet weight)					
	Before ingestion			After ingestion		
	qPCR			qPCR		
	Without PMA	With PMA	CFU	Without PMA	With PMA	CFU
a	< 5.3	< 5.3	< 2	6.8	5.5	4.9
b	< 5.3	< 5.3	< 2	8.1	6.2	3.3
c	< 5.3	< 5.3	< 2	8.0	6.5	5.1
d	< 5.3	< 5.3	< 2	8.0	6.6	6.6
e	< 5.3	< 5.3	< 2	8.2	5.9	4.8
f	< 5.3	< 5.3	< 2	8.5	6.6	5.3
g	< 5.3	< 5.3	< 2	7.8	6.4	6.1
h	< 5.3	< 5.3	< 2	8.2	5.7	2.8
i	< 5.3	< 5.3	< 2	6.7	6.0	2.0
j	< 5.3	< 5.3	< 2	7.6	5.9	3.7
k	< 5.3	< 5.3	< 2	7.7	6.9	6.5
l	< 5.3	< 5.3	< 2	5.9	5.8	2.9
Mean	< 5.3	< 5.3	< 2	7.6	6.2	4.5
SD	-	-	-	0.7	0.4	1.5

[a] The lower limits of detection of quantitative real-time PCR (qPCR) and the culture method were $10^{5.3}$ cells g^{-1} feces (wet weight) and $10^{2.0}$ CFU g^{-1} feces, respectively.

[b] PMA, propidium monoazide; qPCR, quantitative real-time PCR.

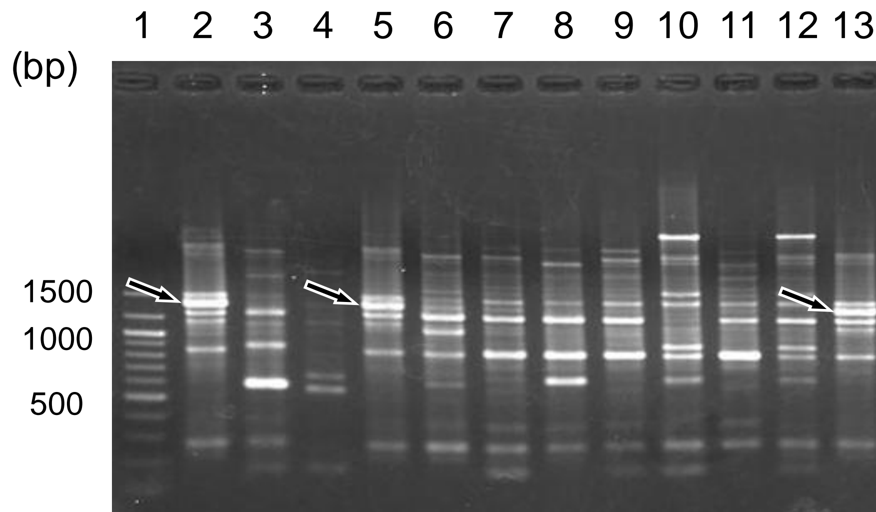


Fig. 1 RAPD patterns obtained from 10 *Bifidobacterium bifidum* strains with the use of OPA11 primer.

Lane 1, 100-bp DNA size marker; lanes 2, 5 and 13, *B. bifidum* BF-1; lanes 3, YIT 4013; lane 4, YIT 4039^{T}; lane 6, YIT 4042; lane 7, YIT 4069; lane 8, YIT 4070; lane 9, YIT 10990; lane 10, YIT 10994; lane 11, YIT 10996 ; lane 12 , YIT 10999. Arrows indicate bands corresponding to a BF-1-specific PCR product.

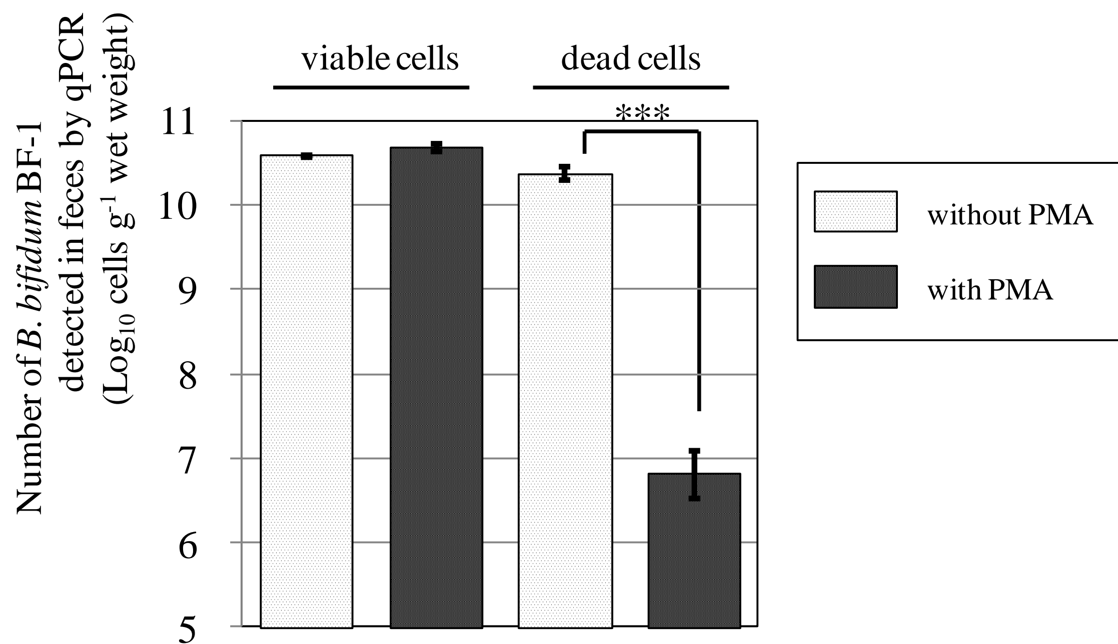


Fig. 2 Effect of PMA treatment on quantitative real-time PCR amplification of heat-killed BF-1 cells in human feces.

Viable or heat-killed BF-1 cells (80 ℃, 10 min) were introduced into human feces samples and the cells were quantitated by real-time PCR with or without PMA treatment. ***, $P < 0.001$, PMA treatment versus no PMA treatment. Data are mean ± SD (from 3 independent experiments).

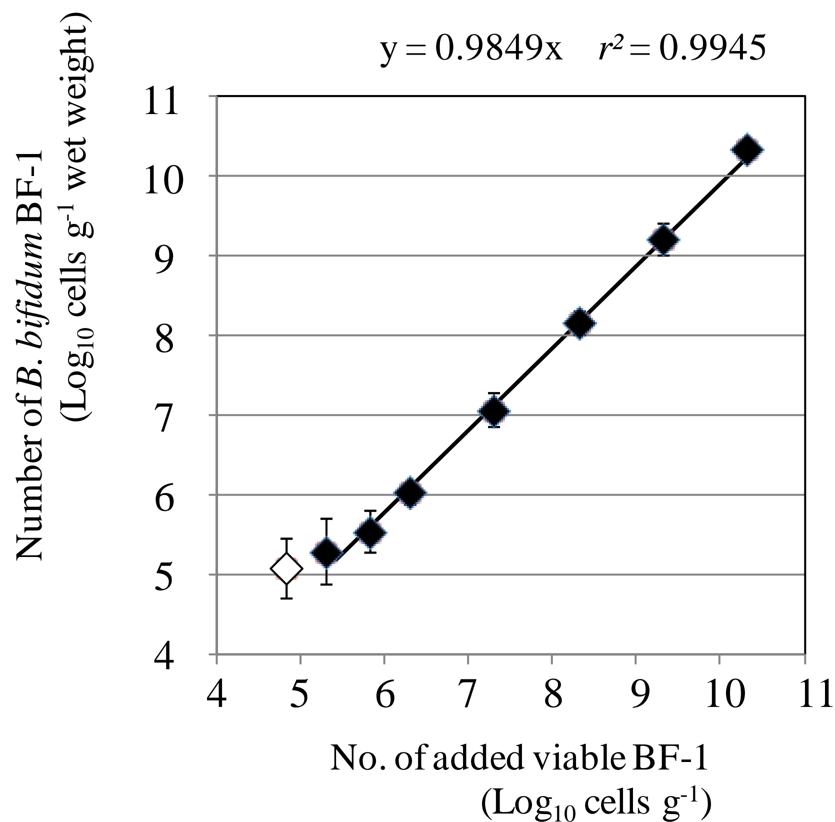
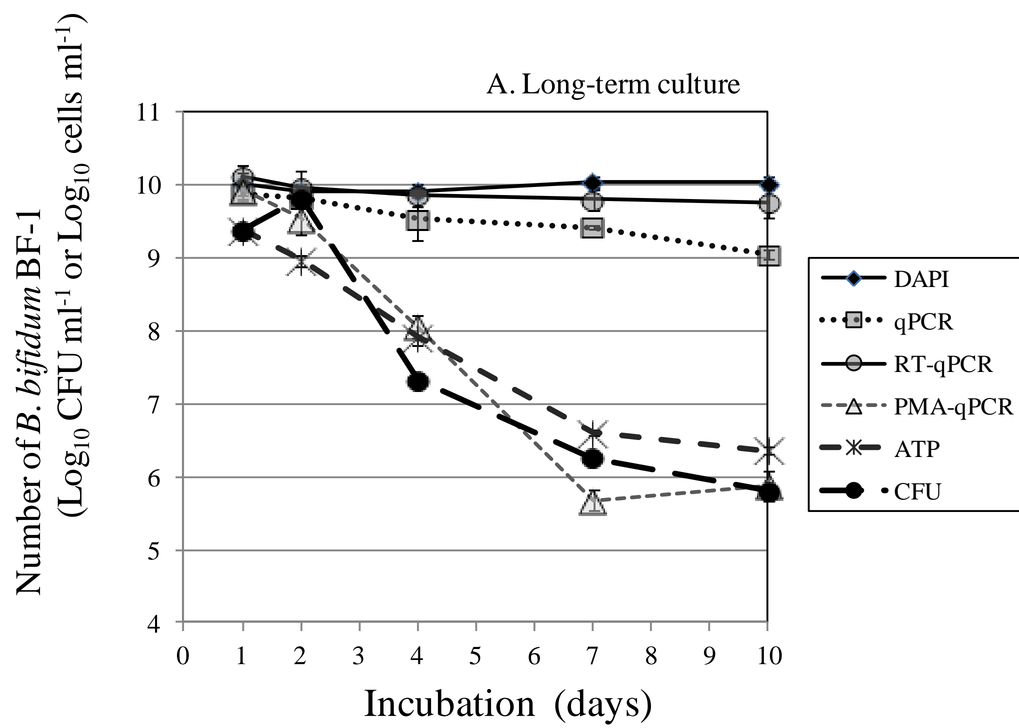


Fig. 3 Correlation between the number of viable BF-1 added to fecal samples and the number determined by quantitative real-time PCR (qPCR) with PMA.

The regression line was made between $10^{5.3}$ cells g^{-1} and $10^{10.3}$ cells g^{-1} (wet weight), as detected by qPCR, because amplification products were not always detected with an input of $10^{4.8}$ cells g^{-1} (open diamond). The regression line was calculated with an intercept of 0. Error bars represent standard deviations from three independent tests.



A. Long-term culture
Number of *B. bifidum* BF-1
(Log_{10} CFU ml^{-1} or Log_{10} cells ml^{-1})
11
10
9
8
7
6
5
4
0
1
2
3
4
5
6
7
8
9
10
Incubation (days)
DAPI
qPCR
RT-qPCR
PMA-qPCR
ATP
CFU

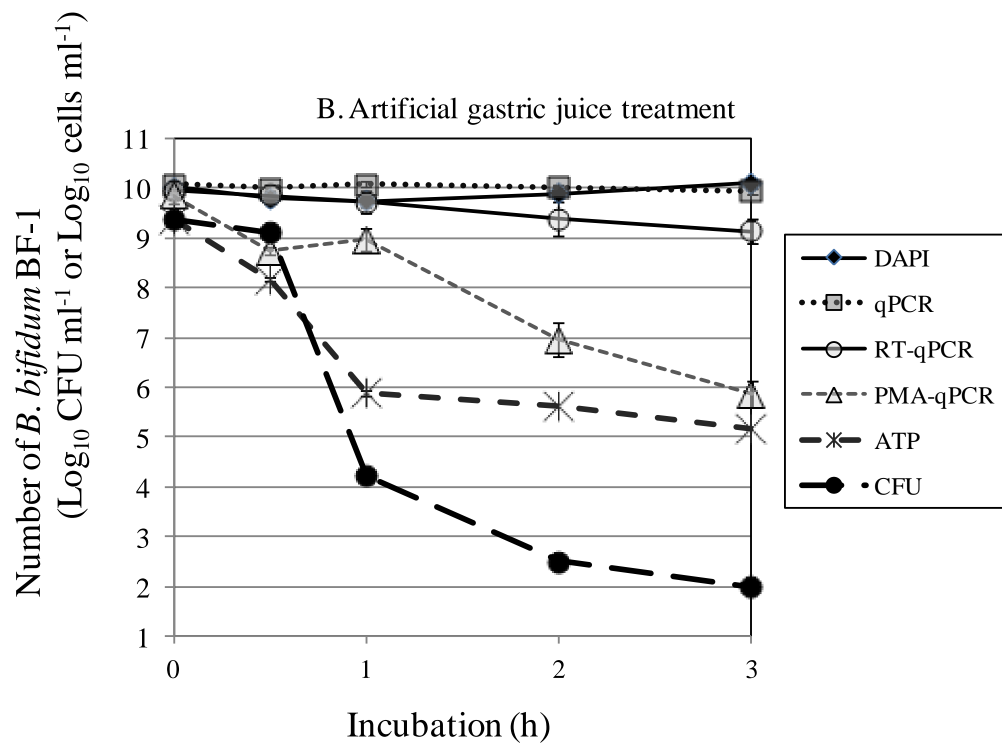


Fig. 4 Use of quantitative real-time PCR (qPCR) with PMA and other methods to determine the number of BF-1 during long-term culture in m-MRS broth or artificial gastric juice.

Cells were cultured long term (10 days) in m-MRS broth at 37 ºC (A) or treated with artificial gastric juice at 37 ºC for 3-h (B) and the cell numbers were assayed by various methods at the specified time points. The methods were DAPI count (DAPI); qPCR with pBF-1, with no PMA treatment of cells before DNA extraction (qPCR); RT-qPCR targeting of *B. bifidum* specific 16S rRNA sequences (RT-qPCR); qPCR with pBF-1, with PMA treatment of cells before DNA extraction (PMA-qPCR); ATP content as RLU and converted to CFU (ATP); culturing of cells on T-EMSM selective agar (CFU).

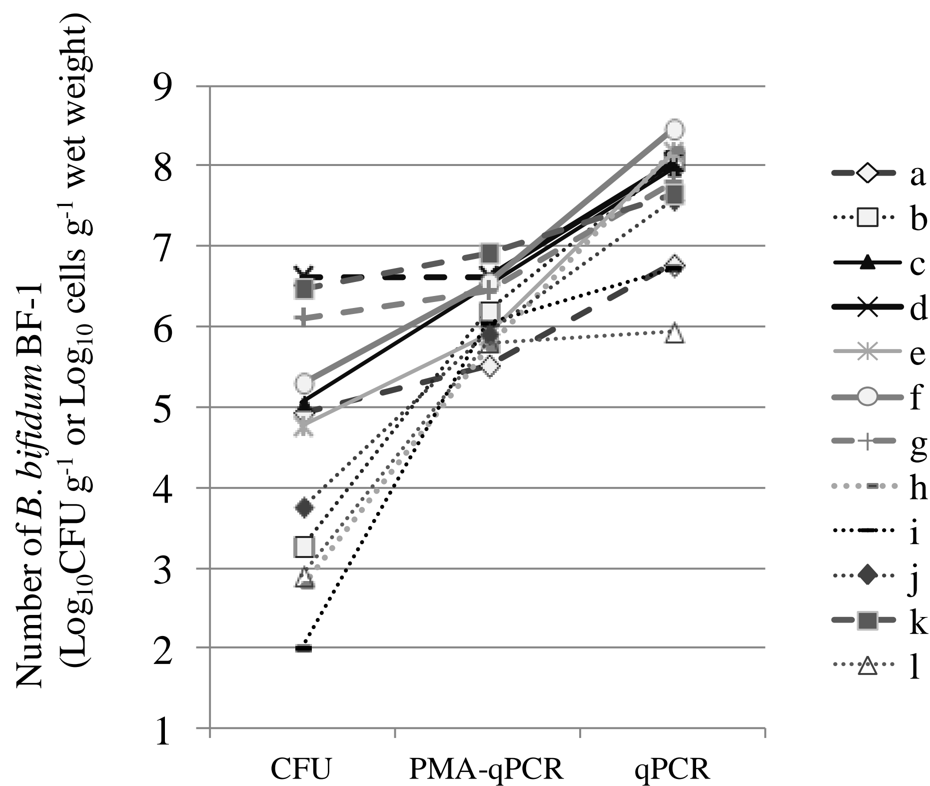


Fig. 5 Number of BF-1 in the feces of 12 volunteers who ingested a fermented milk product for 28 days

dashed line : the volunteer whose the number of viable BF-1 determined by the PMA-qPCR was 10 times less than that determined by T-EMSM selective agar-based culture.

dotted line: the volunteer whose the number of BF-1 determined by the PMA-qPCR was 100 times higher ($P < 0.01$) than that determined by T-EMSM selective agar-based culture.

Chapter 5

Comprehensive discussion

In this study, I established PCR-based method to detect, identify and quantify for three probiotics (*Lactobacillus casei* strain Shirota [LsS], *Bifidobacterium breve* strain Yakult [BbrY], and *Bifidobacterium bifidum* BF-1 [BF-1 cells]), using strain-specific primer sets. These primer sets were developed using randomly amplified polymorphic DNA (RAPD) technique, respectively. Numerous reports of PCR-based methods for strain-specific detection and enumeration have been published (Ahlroos and Tynkkynen 2009; Briczinski, et al. 2009; Flint and Angert 2005; Maruo, et al. 2006; Peng, et al. 2011; Sisto, et al. 2009). Here we confirmed that the RAPD technique to be one of the best methods for developing strain-specific primer set for use in quantitative PCR (qPCR), because RAPD can comprehensively compare the whole genomes of many strains to easily and rapidly detect strain-specific sequences.

The basic requirement for probiotic bacteria to exert expected positive effects in to be live (Fuller 1989); therefore I developed an appropriate quantification method for differentiation of viable and dead cells in samples. This method uses combination of intercalating dye, propidium monoazide (PMA) (which selectively penetrates dead cells through their compromised membranes and covalently binds to their DNA under bright visible light), and strain-specific primer set for quantitative PCR (qPCR). When probiotics (BbrY and BF-1 cells) that were heat killed by treatment at 80 °C for 10 min were introduced into fecal sample, the numbers of these probiotics detected in the feces by PMA-qPCR were about 1/10,000 of that detected without PMA treatment. These results demonstrated that PMA-qPCR detected only viable cells from the negligible amount amplification of dead cells.

I next compared the changes in the numbers of viable BF-1 cells determined by various methods when the cells were subjected to moderately slow death by long-term cultivation and rapid

death by artificial gastric juice treatment. After these treatments, the number of viable BF-1 cells by PMA-qPCR was decreased similar tendency to the numbers determined by selective agar-based culture and measurement of ATP contents. In contrast, the numbers of BF-1 by reverse transcription-qPCR targeting of 16S rRNA and qPCR without PMA treatment were not changed during these experiments. Therefore, I confirmed that PMA-qPCR to be the best way to rapidly and accurately quantify viable probiotic cells.

Finally, I investigated the enumeratation of the total numbers of probiotic cells in human fecal samples ingested fermented milk products containing probiotics by using qPCR without PMA treatment, viable probiotic cells (which had intact membranes) by using PMA-qPCR and colony-formable cells using selective agar-based culture. Through this experiment, I confirmed the number of viable probiotic cells determined by PMA-qPCR was significantly higher than that determined by the colony-formable cells on selective agar-medium.

In conclusion, I confirmed that combining PMA treatment of samples before DNA extraction and quantitative PCR with strain-specific primer set could be used to quickly and accurately analyze the number of viable probiotic cells in the fecal samples. I believe that the PMA-qPCR methodology I have developed will be a powerful tool in the future studies for understanding the use of probiotics.

In addition, the combination of PMA treatment and PCR-based techniques (DGGE, T-RFLP, metagenome analysis using next-generation sequencing, etc.) provides us new points of view to understand for microbial community profile for not only human feces but also various environmental substances, since PMA treatment is able to detect and quantify differentially viable from non-viable microorganisms. Furthermore, PMA-qPCR method which combined PMA treatment and strain-specific primers is able to monitor not only the fate of probiotics in human intestinal tracts but also the bacterial strains added in the process of bioaugmentation and soil improvement. I believe firmly that the novel method I have developed will be contributed to the progress of research study

in microbial ecology.

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Abstract in Japanese

ヒト腸管内には多様な細菌が存在し、複雑な微生物生態系 (腸内細菌叢) が形成され、宿主の健康と密接に関係している。その中でもプロバイオティクスは、“腸内細菌叢のバランスを改善し、宿主に有益な作用をもたらす生きた微生物” と定義され、予防医学の重要性が見直される今日、その重要性はますます増加している。糞便中のプロバイオティクスの検出には選択培地を用いた培養法とその後の菌株識別など多大の時間と熟練した操作が必要であり、多数の検体を処理することは困難であった。

そこで、本研究ではプロバイオティクスの簡便で正確な検出、識別、定量方法の開発を試みた。また、プロバイオティクスは腸管に生きて届くことが求められるため、定量的PCR (qPCR) と選択的膜透過性色素を組み合わせることで生きた菌体のみを定量する方法の開発を試みた。

はじめに、腸内環境改善、免疫刺激、抗腫瘍作用などが明らかにされているプロバイオティクスの一つである*Lactobacillus casei* シロタ株 (LcS) のPCRを用いた菌株特異的な検出・定量を可能にするため、菌株識別法のひとつであるRandomly Amplified Polymorphic DNA (RAPD) 法を用いて菌株特異的な遺伝子配列を検索した。その菌株特異的配列を用いてPCRプライマー (pLcS) を作製し、*L. casei* 57 菌株を含むヒト腸内からよく分離される157菌株に対して特異性を確認したところ、LcSからのみ増幅産物が得られ、特異性が確認できた。LcSの存在しないヒト糞便にLcSを添加し、DNAを抽出してqPCRで定量したところ、糞便1g (湿重量)あたり$10^{4.6}$ cells 以上で検出可能であり、添加した菌数とqPCRの定量値は$10^{4.6}$–$10^{9.6}$ cells/g でよく一致した ($r^2 = 0.999$, $P < 0.001$)。LcSを含む発酵乳を7日間飲用した糞便 (n = 14) からは、培養法で$10^{8.0\pm0.9}$ CFU/g (mean ± S.D.)、菌株特異的プライマーを用いたqPCR法で$10^{9.1\pm0.5}$ cells/g のLcSが検出され、培養法での検出菌数が明らかに少なかった ($P < 0.001$, paired *t*-test)。培養法での菌数が少なかった理由として選択培地に含まれる抗生剤の影響が考えられたが、qPCR法では生菌のみならず、死菌体を検出している可能性も考えられた。

次に、腸内環境改善、炎症性大腸炎改善効果や免疫刺激効果が確かめられている*Bifidobacterium breve* ヤクルト株 (BbrY) の菌株特異的プライマーを特異的RAPDバンドから作製し、*B. breve* 30菌

株を含む腸内細菌112菌株に対して特異性を確認した。さらに、生菌と死菌を分別定量するため、菌株特異的プライマーと選択的膜透過性色素であるpropidium monoazide (PMA) を組み合わせたPMA-qPCR法による生きているBbrYの検出・定量を試みた。PMAはイオン性色素であるため、生菌の細胞膜を透過できないが、膜完全性が壊れている死菌体には容易に透過する。また、DNA結合性色素であるため、細胞内に透過したPMAは2本鎖DNA間に侵入する。その後、PMAは強光を受けることで、DNA鎖同士を共有結合してPCR反応を阻害する。したがって、PMA処理は死菌体からのPCR増幅を阻害するが、生菌の定量には影響しない。BbrYを50 μM PMA中で5分間室温保存し、2分間強光照射することにより、生菌に比べて死菌体からのPCR増幅を1/10,000に抑制できることを確認した。生きたBbrYを糞便に添加したところ、10^5−10^9 cells/g で添加菌数とPMA-qPCR法の定量値が一致した ($r^2 = 0.9983$, $P < 0.001$)。BbrYを含む発酵乳を10日間飲用後の糞便 (n = 11) には培養法で$10^{6.9\pm1.5}$ CFU/g、PMA-qPCR法 (生菌)で$10^{7.5\pm1.0}$cells/g、通常のqPCR法 (生菌+死菌)で$10^{8.5\pm0.8}$ cells/g検出された。PMA-qPCR法を用いることで膜完全性を維持している生きたBbrYのみを培養することなく検出可能となった。

次に、*Helicobacter pylori*による胃炎の改善効果やヒトに対する免疫調整能が確認されているプロバイオティクスである*Bifidobacterium bifidum* BF-1株 (BF-1株) の菌株特異的プライマーを特異的RAPDバンドから作製し、*B. bifidum* 30菌株を含む腸内細菌127菌株に対して特異性を確認した。PMA-qPCR法は，BF-1株の加熱死菌体のPCR増幅を通常のqPCR法と比較して約1/10,000に抑制した。つぎに、長期培養 (10日間)、および人工胃液処理 (pH2.8、3時間) におけるBF-1株の膜完全性の変化を継時的にPMA-qPCR法で解析し、DAPIカウント、16S rRNAを標的とするRT-qPCR法、菌体内ATP量、およびBF-1株の選択培地による菌数と比較した。その結果、BF-1株は培養法、ATP法およびPMA-qPCR法では同様に菌数減少が観察されたが、DAPIカウント、通常のqPCR法およびRT-qPCR法では菌数変動は検出できなかった。これにより、PMA-qPCR法が優れた生菌検出法であることが証明された。ヒト糞便に生きたBF-1株を添加してPMA-qPCR法を行うと、添加菌数とPMA-qPCR法による菌数には$10^{5.3}$−$10^{10.3}$ cells/gの間で非常に高い相関がみられた (r >0.99, P <0.001)。BF-1株を含む発酵乳を26日間飲用後の糞便 (n = 12) から、培養法で$10^{4.5\pm1.5}$ CFU/g、PMA-qPCR法で$10^{6.2\pm0.4}$ cells/g、

qPCR法で$10^{7.6\pm0.7}$ cells/gのBF-1株がそれぞれ定量され、膜完全性を指標とするPMA-qPCR法により、コロニー形成能を指標とする培養法に比べ50倍量 ($P < 0.01$) のBF-1株が生きて腸内に存在することが確認された。

以上、本研究により、PMA処理と菌株特異的primerを用いたqPCR法による糞便中の生きたプロバイオティクスの迅速かつ正確な定量が可能となったことで、プロバイオティクスの理解を深め、プロバイオティクスの健康への影響の検証に大いに役立つことが期待される。

PMA処理は生菌と死菌の分別定量を可能にするため、PMA処理とPCRを利用した微生物検出法 (DGGE、T-RFLP、次世代シークエンサーを用いたメタゲノム解析など) を組み合わせることで、ヒト腸内細菌叢のみならず、多様な微生物群を含む様々な一般環境試料に対しても応用可能と考えられ、微生物群集の理解に対して新たな視点を与えることが期待される。さらに、PMA処理と菌株特異的プライマーを組み合わせたPMA-qPCR法はヒト消化管におけるプロバイオティクスの追跡のみならず、環境浄化や土壌改良におけるバイオオーグメンテーションの添加微生物の追跡への応用も期待される。この新しい微生物検出法が微生物生態学の発展に貢献することを期待する。