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Rapid Identification of *Lactobacillus* and *Bifidobacterium* in Probiotic Products Using Multiplex PCR

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Abstract Lactic acid bacteria (LAB) are beneficial for the gastrointestinal tract and reinforce immunity in human health. Recently, many functional products using the lactic acid bacteria have been developed. Among these LAB, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Bifidobacterium longum*, and *Bifidobacterium bifidum* are frequently used for probiotic products. In order to monitor these LAB in commercial probiotic products, a multiplex PCR method was developed. We designed four species-specific primer pairs for multiplex PCR from the 16S rRNA, 16S-23S rRNA intergenic spacer region, and 23S rRNA genes in *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Bifidobacterium longum*, and *Bifidobacterium bifidum*. Using these primer pairs, 4 different LAB were detected with high specificity in functional foods. We suggest that the multiplex PCR method developed in this study would be an efficient tool for simple, rapid, and reliable identification of LAB used as probiotic strains.

Keywords: *Bifidobacterium*, lactic acid bacteria, *Lactobacillus*, multiplex PCR, probiotic product

Probiotics are defined as microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host [10, 11]. There is currently a growing interest in probiotics that have been suggested or shown to provide beneficial effects on health, that is, to promote a healthy state of the gastrointestinal tract and immune system, and reduce the risk of disease. In addition, probiotics may be helpful in minimizing allergy and reducing colon cancer [3, 7, 11, 12]. Because of these reasons, probiotic products are expected to be widely used in the future. *Lactobacillus* and *Bifidobacterium* are the most commercially used species of lactic acid bacteria (LAB) in the probiotic products [13]. Among these LAB, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Bifidobacterium longum*, and *Bifidobacterium bifidum* are mainly used with functional foods that are circulated in the commercial markets worldwide. Nevertheless, labeling of some of those products is misleading or not detailed [5]. Recently, various genotype-based methods have been adopted as useful ways for identification of bacteria [1]. Single PCR, using primers that target variable regions in universal genes, such as the 16S rRNA, 16S-23S rRNA intergenic spacer region (ISR), or 23S rRNA, have successfully detected and identified the LAB [8, 15, 18]. Moreover, the multiplex PCR methodologies, using primers that target different genes, have been shown to be a useful tool to quickly identify multiple bacteria in a single reaction [9, 17]. These are a few earlier reports to demonstrate that *Lactobacillus* and *Bifidobacterium* were detected using single PCR, but there is no study to show that the above bacteria were detected by a single reaction using multiplex PCR assay [2].

In this study, we developed a multiplex PCR assay system for the detection and identification of LAB in commercial probiotic products.

Materials and Methods

Bacterial Strains and Culture Conditions

Twenty-two bacterial strains used in this study are listed in Table 1, and they were used for the verification of specific primers designed in this study. Strains from the Korean Collection for Type Cultures (KCTC) were used to identify species. Lactic acid bacteria were cultured on MRS broth (Hardy Diagnostics, Santa Maria, CA, U.S.A.) at 37°C in an anaerobic condition (AnaeroPack•Anaero, Mitsubishi Gas Chem., Tokyo, Japan).

Probiotic Products

Nine ingredient-labeled probiotic products were collected from markets in Korea. They included 4 powder-form food

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Table 1. List of bacterial strains used in this study.

Reference strains		Accession No.
Lactobacillus paracasei subsp. *paracasei*	KCTC 3510	AB126872, AB237507
Lactobacillus fermentum	KCTC 3112	AF302116, AF182720
Lactobacillus reuteri	KCTC 3594	L23507, AF182723
Lactobacillus salivarius subsp. *salicinius*	KCTC 3600	M59054, AB102859
Lactobacillus acidophilus	KCTC 3164	AY773947, AB092634
Lactobacillus gasseri	KCTC 3163	AF519171, AF182721
Lactobacillus casei	KCTC 3109	AB239468, AF182729
Lactobacillus rhamnosus	KCTC 3237	AF243146, AF182730
Lactobacillus plantarum	KCTC 3104	AB104855, AB092637
Lactobacillus johnsonii	KCTC 3801	AB236694, AF074860
Lactobacillus brevis	KCTC 3498	AF090328, AB102858
Lactobacillus delbrueckii subsp. *bulgaricus*	KCTC 3635	AY050171, AB102856
Lactobacillus sakei	KCTC 3598	M58829, U97137
Bifidobacterium longum	KCTC 3128	M58739, M84781, U09792
Bifidobacterium longum	KCTC 3421	U10152, U09832
Bifidobacterium breve	KCTC 3220	AB006658, DQ167806
Bifidobacterium pseudocatenulatum	KCTC 3223	AB125917
Bifidobacterium catenulatum	KCTC 3358	M58732, U09522
Bifidobacterium infantis	KCTC 3249	AB125903, M84783, U09792
Bifidobacterium animalis	KCTC 3126	AB027536, AY225132
Bifidobacterium bifidum	KCTC 3418	M84777, U09831, U09517
Bifidobacterium adolescentis	KCTC 3216	M58729, DQ108596

The nucleotide sequences accession numbers of the 16S rRNA, 16S-23S rRNA ISR, and flanking 23S rRNA used for the design of the species-specific multiplex PCR primer set.
KCTC is Korean Collection for Type Cultures.

supplements, 1 capsule-form pharmaceutical, and 4 different kinds of liquid yogurts manufactured by six different companies. All products were refrigerated or stored at room temperature by following the manufacturers' suggestions.

DNA Extraction

Genomic DNA from a strain was extracted using a DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Genomic DNAs from the probiotic products were extracted directly by using a QIAamp DNA Stool Mini Kit (Qiagen). Ten ml of lysis buffer of the QIAamp DNA Stool Mini Kit was added to 1 g of probiotic products, vortexed vigorously for lysis, and 2 ml each from 10 ml of the probiotic product lysate was processed according to the manufacturer's protocol. The amount and purity of DNA were determined by absorbance at 260 nm and 280nm using a UV-spectrophotometer (Model UV-1700, Shimadzu, Tokyo, Japan) and by a Qubit Fluorometer (Invitrogen, Eugene, OR, U.S.A.)

Primer Design and Multiplex PCR Assay

The new species-specific primers for identification of *L. rhamnosus*, *L. acidophilus*, and *B. bifidum* were designed based on the 16S rRNA, 16S-23S rRNA intergenic spacer region, or 23S rRNA genes in LAB. Those sequences were obtained from the National Center for Biotechnology Information (NCBI) (Table 1). The species-specific primer for *B. longum* was designed according to Matsuki *et al.* [14]. Location of the PCR primers, expected size of PCR products, and sequences are displayed in Fig. 1 and Table

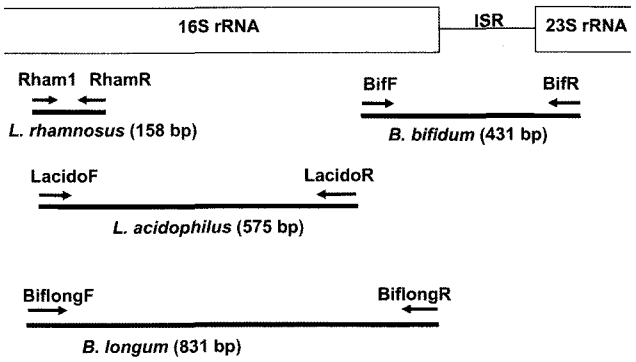

Fig. 1. Alignment of the approximate location of the PCR primer and amplicon sizes of each PCR product; multiplex PCR primers used were Rham1, RhamR, BifF, BifR, LacidoF, LacidoR, BiflongF, and BiflongR (see Table 2).
Species-specific primers were designed from the regions of identity within the 16S rRNA, 16S-23S rRNA intergenic spacer region, and 23S rRNA sequences from *L. rhamnosus* (Accession No. AF243146), *L. acidophilus* (Accession No. AY773947), *B. longum* (Accession No. M58739), and *B. bifidum* (Accession No. U09831). ISR, intergenic spacer region. Arrows indicate the direction of primers.

Table 2. Oligonucleotide primers used in this study.

Primer	Primer sequence (5'→3')	Length (bp)	Reference
Rham1	GTCGAACGAGTTCTGATTATTG	22	This study
RhamR	GAACCATGCGGTTCTTGGAT	20	This study
BifF	ATTTGAGCCACTGTCTGGTG	20	This study
BifR	CATCCGGGAACGTCGGGAAA	20	This study
LacidoF	CACTTCGGTGATGACGTTGG	20	This study
LacidoR	CGATGCAGTTCCTCGGTTAAGC	22	This study
BiflongF	TTCCAGTTGATCGCATGGTC	20	[14]
BiflongR	GGGAAGCCGTATCTCTACGA	20	[14]

2. Genomic DNA prepared as described above was used as a template for PCR amplification. The reaction mixture in 25 μl volume contained 1 unit of *Taq* DNA polymerase (TaKaRa, Shiga, Japan), 2 mM $MgCl_2$, and 0.2 mM each of dATP, dCTP, dGTP, and dTTP, and concentrations of template DNA and optimized concentrations of primer pairs are shown in Table 4. Each PCR product was amplified by the following conditions: initial template denaturation step for 5 min at 95°C, followed by 30 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 63°C, and elongation for 30 sec at 72°C. The final extension step was 5 min at 72°C. The amplification products were analyzed by electrophoresis on a 2% agarose gel containing 0.5 μg/ml of ethidium bromide, and the DNA bands were visualized under UV illumination and photographed with a digital camera (Model COOLPIX 4300, Nikon, Tokyo, Japan) [16].

Sequence Analysis of PCR Products

PCR products of probiotic samples were extracted from agarose gel using a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The pMD18-T vector (TaKaRa) was used to clone the amplified DNA fragments and transformed into *Escherichia coli* strain DH5α. *E. coli*, containing the recombinant plasmid, was cultivated and a plasmid DNA was purified. The sequencing of the amplified DNA fragment was carried

Table 3. Specificity of primer pairs designed for multiplex PCR on various LAB strains.

Bacterial species	Specificity of primer pairs			
	LacidoF/LacidoR (575 bp)	Rham1/RhamR (158 bp)	BiflongF/BiflongR (831 bp)	BifF/BifR (431 bp)
Lactobacillus paracasei subsp. *paracasei*	–	–	–	–
Lactobacillus fermentum	–	–	–	–
Lactobacillus reuteri	–	–	–	–
Lactobacillus salivarius subsp. *salicinius*	–	–	–	–
Lactobacillus acidophilus	+	–	–	–
Lactobacillus gasseri	–	–	–	–
Lactobacillus casei	–	–	–	–
Lactobacillus rhamnosus	–	+	–	–
Lactobacillus plantarum	–	–	–	–
Lactobacillus johnsonii	–	–	–	–
Lactobacillus brevis	–	–	–	–
Lactobacillus delbrueckii subsp. *bulgaricus*	–	–	–	–
Lactobacillus sakei	–	–	–	–
Bifidobacterium longum (KCTC 3128)	–	–	+	–
Bifidobacterium longum (KCTC 3421)	–	–	+	–
Bifidobacterium breve	–	–	–	–
Bifidobacterium pseudocatenulatum	–	–	–	–
Bifidobacterium catenulatum	–	–	–	–
Bifidobacterium infantis	–	–	–	–
Bifidobacterium animalis	–	–	–	–
Bifidobacterium bifidum	–	–	–	+
Bifidobacterium adolescentis	–	–	–	–

+: positive result, –: negative result.

out with an ABI PRISM 3700 DNA analyzer (Perkin Elmer, Wellesley, MA, U.S.A.). The DNA sequences were analyzed using the Vector NTI software Version 7 (Invitrogen, San Francisco, CA, U.S.A.).

RESULTS AND DISCUSSION

Specificity of Primers

Novel species-specific primers for identification of *L. acidophilus*, *L. rhamnosus*, and *B. bifidum* were designed from 16S rRNA, 16S-23S rRNA intergenic spacer region, and 23S rRNA sequences for *L. rhamnosus* (Accession No. AF243146), *L. acidophilus* (Accession No. AY773947) and *B. bifidum* (Accession No. U09831). The specificity of the 4 species-specific primer pairs was evaluated by PCR with genomic DNAs purified from the reference strains, which are listed in Table 1. All species-specific primer pairs produced a single PCR product with an expected product size, representing species-specificity of the designed primers (Table 3). Therefore, these primers seemed to be appropriate for identification of LAB in probiotic products using multiplex PCR.

Validation of the Multiplex PCR to the Reference Strains

In order to optimize the multiplex PCR condition, the ratio of applied species-specific primer pairs was investigated. It is highly likely that the presence of more than one primer pair in the multiplex PCR may produce not only unexpected PCR products, but also result in poor sensitivity. All the primer pairs in multiplex PCR should amplify PCR products with similar intensity for their respective targets. Therefore, equimolar primer concentrations (10 μM) were initially used in the multiplex PCR. When there was unacceptable amplification, the concentration ratio of primers in the reaction was modified by increasing the concentration of primer that generates a DNA with weak intensity and decreasing the concentration of primer that generates a DNA with strong intensity [6]. Using the multiplex PCR with optimized concentration ratio of primer pairs (Table 4), the amplified products from a mixture of genomic DNAs from four reference strains are shown in the lane R of Fig. 2. As expected, the multiplex PCR product was amplified to the size of 158 bp for the introduced genes in *L. rhamnosus*, 431 bp in *B. bifidum*, 575 bp in *L. acidophilus*, and 831 bp in *B. longum* (lane R in Fig. 2).

Table 4. Template DNA and primer pairs concentrations of multiplex PCR.

Reference strains	Template DNA (ng)	Primer name	Concentration (μM)
Lactobacillus rhamnosus	12.5	Rham1	13
		RhamR	13
Bifidobacterium bifidum	12.5	BifF	8
		BifR	8
Lactobacillus acidophilus	12.5	LacidoF	1.7
		LacidoR	1.7
Bifidobacterium longum	12.5	BiflongF	3
		BiflongR	3

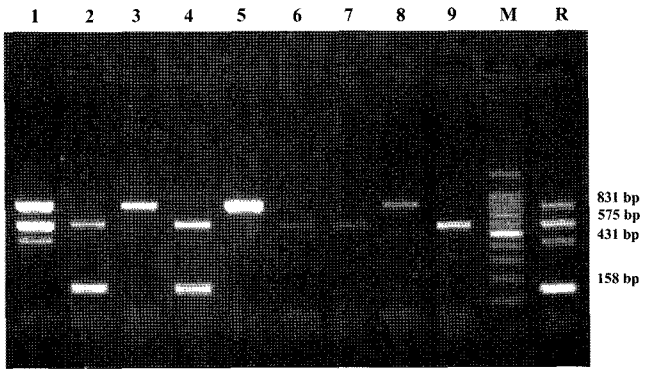

Fig. 2. Agarose gel electrophoreses of PCR products from multiplex PCR assays.
Lanes: M, 100 bp DNA ladder marker (code 3407, Takara, Korea); R, reference strains (*L. acidophilus*, *L. rhamnosus*, *B. longum* and *B. bifidum*); 1–9, genomic DNAs directly extracted from 9 probiotic products.

Application of the Multiplex PCR to Identification of Probiotic Isolates

The genomic DNAs were isolated from the probiotic products using the QIAamp DNA Stool Mini Kit. A total of 30 ng to 3 μg of genomic DNA were extracted from 1 g each of probiotic products. The specific primer pairs were used to identify LAB isolated from 9 probiotic samples. As shown in Fig. 2, the PCR products were identified with *L. rhamnosus*, *B. bifidum*, *L. acidophilus*, and *B. longum*. Table 5 summarizes whether the multiplex PCR product was present or not. Probiotic product 1 showed the PCR products of 831 bp in *B. longum*, 575 bp in *L. acidophilus*, and 431 bp in *B. bifidum* (lane 1 in Fig. 2). Probiotic products 2 and 4 showed the PCR product of 575 bp corresponding to *L. acidophilus* and 158 bp corresponding to *L. rhamnosus* (lanes 2 and 4 in Fig. 2). Probiotic products 3, 5, and 8 showed the PCR products of *B. longum* (831 bp) (lanes 3, 5, and 8 in Fig. 2), and probiotic products 6, 7, and 9 showed the PCR products of *L. acidophilus* (575 bp) (lanes 6, 7, and 9 in Fig. 2).

The PCR products amplified by these species-specific primers were sequenced, and their sequences were compared with those of reference strains. As expected, the sequences obtained from PCR products were identical with strains *L. rhamnosus* (Accession No. AF243146), *L. acidophilus* (Accession No. AY773947), *B. longum* (Accession No. M58739), and *B. bifidum* (Accession No. U09831).

We assayed 9 commercial probiotic products by multiplex PCR analysis and the results were compared with their

Table 5. Application of the multiplex PCR method on the LAB strains found in the probiotic products.

Samples	*L. rhamnosus*	*B. bifidum*	*L. acidophilus*	*B. longum*
A company Probiotic product 1	-	+	+	+
B company Probiotic product 2	+	-	+	-
C company Probiotic product 3	-	-	-	+
D company Probiotic product 4	+	-	+	-
E company Probiotic product 5	-	-	-	+
F company Probiotic product 6	-	-	+	-
F company Probiotic product 7	-	-	+	-
F company Probiotic product 8	-	-	-	+
F company Probiotic product 9	-	-	+	-

+: positive result, -: negative result.
Probiotic product 1~probiotic product 9 correspond to Fig. 2.

label claims. Six probiotic products were identical to the label claims. However, the product 2 contained *L. rhamnosus* and *L. acidophilus* instead of the *L. acidophilus* claimed, and the product 4 contained *L. acidophilus* and *L. rhamnosus* instead of the *L. rhamnosus* claimed. These two strains had been misidentified in a previous report [4]; however, our multiplex PCR method successfully distinguished these strains using specific primers. We also found that the product 8 contained *B. longum* instead of the bifidobacteria claimed.

In conclusion, we identified 2 species of *Lactobacillus* and 2 species of *Bifidobacterium* from probiotic products by using multiplex PCR analysis and verified these by DNA sequencing. Taken together, the multiplex PCR method could distinguish four different species of LAB in a single reaction and reduce experimental time and cost compared with single or duplex PCR. Thus, this method would be helpful in monitoring the reliability of the probiotic products labeling system.

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