Simultaneous detection of 37 *Lactobacillus* species using a real-time PCR assay based on whole-genome sequence analysis

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Abstract

Background

*Lactobacillus* species are used as probiotics and play an important role in fermented food production. However, use of 16S rRNA gene sequences as standard markers for the differentiation of *Lactobacillus* species offers a very limited scope, as several species of *Lactobacillus* share similar 16S rRNA gene sequences. In this study, we developed a rapid and accurate method based on comparative genomic analysis for the simultaneous identification of 37 *Lactobacillus* species that are commonly used in probiotics and fermented foods.

Results

To select species-specific sequences or genes, a total of 143 *Lactobacillus* complete genome sequences were compared using Python scripts. In 14 out of 37 species, species-specific sequences could not be found due to the similarity of the 16S–23S rRNA gene. Selected unique genes were obtained using comparative genomic analysis and all genes were confirmed to be specific for 52,478,804 genomes via *in silico* analysis; they were found not to be strain-specific, but to exist in all strains of the same species. Species-specific primer pairs were designed from the selected 16S–23S rRNA gene sequences or unique genes of species. The specificity of the species-specific primer pairs was confirmed using reference strains, and the accuracy and efficiency of the real-time polymerase chain reaction (PCR) with the standard curve were confirmed. The real-time PCR method developed in this study is able to accurately differentiate species that were not distinguishable using the 16S rRNA gene alone. This Real-time PCR method was designed to detect 37 *Lactobacillus* species in a single reaction. The developed method was then applied in the monitoring of 19 probiotics and 12 dairy products. The applied tests confirmed that the species detected in 17 products matched those indicated on their
labels, whereas the remaining products contained species other than those appearing on the label.

**Conclusions**

The method developed in this study is able to rapidly and accurately distinguish different species of *Lactobacillus*, and can be used to monitor specific *Lactobacillus* species in foods such as probiotics and dairy products.

**Background**

*Lactobacillus* is a Gram-positive, non-spore-forming, rod-shaped, catalase-negative genus of bacteria that often grows best under microaerophilic conditions. *Lactobacillus* belongs to the family *Lactobacillaceae* and consists of 170 species and 17 subspecies [1]. Human and animal gastrointestinal tracts harbor a variety of *Lactobacillus* species, including *L. plantarum*, *L. rhamnosus*, *L. fermentum*, and *L. casei* [1], while species such as *L. gasseri*, *L. vaginalis*, *L. crispatus*, *L. iners*, and *L. jensenii* are known to exist in the vagina [2]. They have a high tolerance to acidic environments and are typically used as starter cultures for fermented foods such as kimchi, yogurt, and cheese [1]. *Bifidobacterium* and *Lactobacillus* species are among the most commercially used lactic acid bacteria (LAB) in probiotic products [3]. In particular, *L. acidophilus*, *L. casei*, *L. rhamnosus*, *L. plantarum*, and *L. paracasei* are often used in probiotic products in combination with other *Lactobacillus* species.

Probiotics are gut health promoting bacteria that are generally recognized as safe and are known to provide beneficial effects on host health [4, 5]. In recent years, the probiotic product market has expanded proportionately with an increased interest in gut health [6, 7]. Despite the widespread use of probiotic products to improve human health, there is increasing concern among consumers regarding the quality and the label claims of commercial probiotic products [3]. In terms of functionality and safety, it is very important
that probiotic products contain well-documented probiotic strains that are accurately displayed on the label. However, reports have shown that the LAB species present in some commercial probiotic products do not match those represented on the label [8–10].

The traditional methods used to study microbial communities, such as morphological and physiological characteristics, protein profiling, carbohydrate fermentation patterns, and counts on selective media, are time-consuming and often produce ambiguous outcomes [11, 12]. To achieve the reliable and rapid identification of bacterial species, molecular methods such as 16S rRNA gene sequencing, metagenome sequencing, and denaturing gradient gel electrophoresis (DGGE) have been increasingly applied. 16S rRNA sequencing is commonly used for bacterial identification, including the identification of *Lactobacillus* species [13–15]. Metagenome sequencing and DGGE based on 16S rRNA gene sequences are useful analytical methods for investigating complex microbial communities without previous isolation of individual bacteria [16–18]. However, 16S rRNA gene sequences in many *Lactobacillus* species are too similar to be readily distinguished. In particular, closely related species within the *L. acidophilus* group (*L. acidophilus*, *L. gallinarum*, and *L. helveticus*), the *L. casei* group (*L. casei*, *L. paracasei*, and *L. rhamnosus*), the *L. plantarum* group (*L. plantarum*, *L. paraplantarum*, and *L. pentosus*), and the *L. sakei* group (*L. sakei*, *L. curvatus*, and *L. graminis*) are notoriously difficult to distinguish by 16S rRNA gene sequences [19, 20]. For example, the 16S rRNA gene sequence of the *L. casei* group and that of the *L. sakei* group have more than 98.7% similarity between species [19, 20].

In this study, we designed species-specific primer pairs targeting the 16S–23S rRNA gene and species-unique genes, and developed a detection method for 37 *Lactobacillus* species by a single reaction of real-time polymerase chain reaction (PCR). The developed real-time PCR assay was successfully applied to commercial probiotics and dairy products. We have also confirmed that this assay has the ability to determine the composition of
Lactobacillus species present in a product, as well as the presence of species not stated on the label.

Results

Selection of species-specific sequences and primer designs

The species-specific primer pairs of 37 Lactobacillus were designed from unique gene or 16S–23S rRNA region (Table 1). The similarities of the 16S–23S rRNA regions among Lactobacillus species were verified in silico and 23 Lactobacillus species were distinguished with each primer pair designed in the 16S–23S region. Some Lactobacillus species are difficult to distinguish using the 16S–23S rRNA region alone due to the small number of single-nucleotide polymorphisms. Therefore, unique genes of 14 Lactobacillus species were obtained using comparative genomics (Table 2). A membrane protein was found in four L. acidipiscis genomes, but was not present in other species of Lactobacillus. Adenylosuccinate lyase and leucine-rich repeat protein were detected as the specific genes in L. amylovorus and L. parabuchneri, respectively. In L. paraplantarum, L. plantarum, L. pentosus, and L. helveticus, MFS (Major Facilitator Superfamily)-type transporter YcnB, LPXTG-motif cell wall anchor domain protein, GHKL domain-containing protein, and decarboxylate/amino acid:cation Na\(^+\)/H\(^+\) symporter family protein were detected as the specific genes to each respective species. We also confirmed the specificity of unique genes using BLAST. The unique genes did not match any of the 52,478,804 sequences found in the NCBI database outside of the target species (Table 3). However, some genomes of L. casei contained unique genes of L. paracasei. The presence of unique genes in some, but not all, L. casei strains suggests that the genome information given for the strains is incorrect. These L. casei strains were found to be more similar in the 16S rRNA gene to L. paracasei than to the L. casei described in a previous
study [21]. Also, one genome of *L. gallinarum* contained a unique gene of *L. helveticus*. To clarify the problem of *L. gallinarum* strain, we further performed a genomic analysis of *L. helveticus* and *L. gallinarum*. The result showed that a *L. gallinarum* strain containing a unique gene of *L. helveticus* was more similar to other strains of *L. helveticus* (Fig. 1).

**Specificity of designed primer pairs**

To confirm whether primer pairs were species-specific for the identification of each *Lactobacillus* species, conventional PCR assays were performed with 37 *Lactobacillus* reference strains. For each of the primer pairs, the amplification product was exclusive to each target strain with a high specificity. The results of the conventional PCR assays confirmed 100% specificity for all *Lactobacillus* species.

**Specificity and accuracy of the developed real-time PCR assay**

The accuracy and efficiency of the real-time PCR assay were validated using the template DNA of the *Lactobacillus* reference species. All primer pairs exhibited a linear relationship over the range of 0.005 to 50 ng. The slopes for the specific primer pairs of *L. acetotolerans*, *L. casei*, *L. parabuchneri*, and *L. lindneri* were −3.209, −3.284, −3.207, and −3.595, respectively, and the $R^2$ values were 1, 0.999, 1, and 0.985, respectively (Fig. 2). The $R^2$ and slope values of the remaining primer pairs are shown in Table 4.

The specificities of all 37 *Lactobacillus* reference strains were evaluated for each species-specific primer pair. A non-template was used as a negative control, and the template DNA of 37 *Lactobacillus* reference stains was used as a positive control for each primer pair. All genomic DNA from *Lactobacillus* species yielded detectable amplicon signals for each primer pair, whereas none of the non-target *Lactobacillus* species generated any signals at all (Fig. 3). The $C_T$ ranges were 9.0 to 15.0 for each *Lactobacillus* species (Table 5). Thus, all primer pairs were considered specific for the detection of an individual *Lactobacillus*.
species. To verify the accuracy of the assay, a primer pair targeting the 16S rRNA gene was used as an IPC; the amplification of the target region was observed within the $C_t$ value range of 5.7 to 9.1 for all tested Lactobacillus species.

**Application of the developed real-time PCR assay in probiotics and dairy products**

The real-time PCR assay was applied to identify Lactobacillus species from commercial probiotics and dairy products. A total of 31 products were evaluated using the real-time PCR assay we have developed, and the assay results were compared with the probiotic label claims. Probiotic products were tagged as P1 to P19, whereas dairy products were designated as D1 to D12. As a result of the validation process, 17 products were confirmed to match their label claims (Table 6). However, the label claims of four products (P14, P15, P17, and P18) identified L. helveticus but contained L. acidophilus, and three products (P14, P15, and P17) contained L. paracasei instead of the L. casei indicated on the label. In one product (P16), we detected additional Lactobacillus species that were not listed on the label. We were also able to identify the Lactobacillus species from products labeled with the compound LAB. Our real-time PCR results confirmed that these products contained either L. acidophilus and L. delbrueckii or L. paracasei and L. helveticus.

**Discussion**

A variety of methods have been used to identify LAB in foods or in the environment. The most representative method is a conventional method consisting of phenotypic and biochemical tests, which have limitations in accuracy among isolates possessing similar physiological specificities and fermentation profiles at the species level [22, 23]. To overcome these difficulties, several genotype-based methods such as DGGE and metagenome sequencing have been developed [23]. In addition, metagenome sequencing based on the 16S rRNA gene is a common approach in investigating microbial communities
but is limited to distinguishing similar species [24]. Because metagenome sequencing remains a time-consuming process and requires specialized equipment and techniques, it is unsuitable for analyzing a large number of samples. To combat this, we have developed a real-time PCR assay that can rapidly and easily analyze *Lactobacillus* communities in fermented foods and potentially environmental samples.

PCR is generally considered to be a rapid, sensitive, and time-saving method for the detection of bacterial species [25–27]. The accuracy of PCR is determined by the specificity of the primer pairs used. The 16S rRNA gene is considered a marker gene for bacterial genotypic analysis and is useful for the accurate identification of bacteria [12, 28]. Studies focusing on the identification of *Lactobacillus* have mainly used PCR-based molecular analysis by primer pair targeting variable regions of the 16S rRNA gene sequences [23, 29]. However, for closely related species such as the members of the *L. casei*, *L. sakei*, *L. plantarum*, and *L. acidophilus* groups, each of which has a 16S rRNA gene similarity of more than 98% [30–32], only species-specific PCR primer pairs could sufficiently differentiate species.

To overcome the limitations of the 16S rRNA gene, we developed 37 *Lactobacillus* species-specific primer pairs based on 16S–23S rRNA gene analysis and comparative genome analysis. Species-specific primer pairs were designed to have a small amplicon size (~260 bp) to increase amplification efficiency and detect *Lactobacillus* species present in processed foods. The specificities of the species-specific primer pairs were confirmed using the 37 *Lactobacillus* species, and amplification was observed only in the target species DNA without any cross-reactivity. Also, it was confirmed that species such as the *L. casei* group, *L. acidophilus* group, and *L. plantarum* group, which are not distinguished by the conventional identification method, were differentiable using the species-specific primer pairs. According to the CODEX guidelines, the slope values of −3.1 to −3.6 are
considered to indicate a high PCR efficiency. The coefficient value of determination should be at least 0.98 to be considered viable data [33]. Therefore, these results demonstrate that the developed real-time PCR assay provides high accuracy and efficiency.

The developed real-time PCR assay was used to assess probiotics and dairy products. Using this assay, 17 products were determined to contain the *Lactobacillus* species advertised on the label. In the remaining products, the species indicated on the labels were either replaced with or contaminated by another species. For example, *L. acidophilus* was replaced by *L. helveticus* and *L. casei* was replaced by *L. paracasei* in four probiotic products. Though these products were produced by different companies, the same strains were identified. As described above, *L. acidophilus* belongs to the same group as *L. helveticus*, and *L. casei* belongs to the same group as *L. paracasei*. The likely reason a label names species other than the one detected is misidentification [20, 34]. In one product, additional *Lactobacillus* species that were not indicated on the label were detected by real-time PCR. These were detected at much higher C\(_t\) values than the *Lactobacillus* species indicated on the label, suggesting that such strains were only present in low concentrations [35]. We were also able to accurately identify the species contained in products labeled compound LAB. In all of these products, we detected *L. acidophilus* and *L. delbrueckii* or *L. helveticus* and *L. paracasei*. These results confirm that our real-time PCR assay can detect all species of *Lactobacillus* contained in these products.

Many researchers have provided evidence that the advertised contents of commercial probiotic products containing LAB are significantly different from the actual contents [25, 34]. Lewis et al. (2016) reported that only one of the 16 commercial probiotic products corresponded exactly with the *Bifidobacterium* species claimed on the label [5]. In addition, some products are inconsistent from one lot to another. These results indicate
inadequate that quality control for these products.

Conclusion

In this study, we developed specific primer pairs using comparative genomics to identify *Lactobacillus* accurately and rapidly at the species level, then applied this technology in a real-time PCR assay that can detect 37 *Lactobacillus* species in a single reaction. The developed real-time PCR method was able to accurately discriminate species that were not distinguishable by the conventional identification method. To verify the developed real-time PCR assay, we compared the label claims of probiotics and dairy products with the *Lactobacillus* species detected using the real-time PCR method. The real-time PCR assay that we have developed was successfully applied to commercial probiotic and dairy products, and showed that some products did not accurately match the *Lactobacillus* species listed on their labels. Thus, this assay will be helpful for monitoring the reliability of commercial probiotic and dairy product labels. In addition to its application in probiotic products, the assay can be applied to identify *Lactobacillus* communities in various food or environmental samples.

Methods

**Bacterial strains and probiotic and dairy products**

The *Lactobacillus* reference strains were obtained from the Korean Collection for Type Cultures (KCTC; Daejeon, South Korea; https://kctc.kribb.re.kr/) and the Korean Agricultural Culture Collection (KACC; Jeonju, South Korea; http://genebank.rda.go.kr/) (Table 7). All reference strains were cultured in Lactobacilli MRS Broth (Difco, Becton & Dickinson, Sparks, MD, USA) at 30°C for 48 h under anaerobic conditions. The probiotic and dairy products tested in this study were obtained from various markets around the world (South Korea, United States, and Canada). The samples used in this study included
19 probiotic products (10 capsule-form pharmaceuticals and 9 powder-form food supplements) and 12 dairy products manufactured by 19 different companies. All products were labeled with bacterial species or LAB compounds.

**DNA extraction**

All *Lactobacillus* reference strains were grown in MRS broth at 30°C for 48 h under anaerobic conditions. The cultured cells were harvested by centrifugation at 13,600 × g for 5 min, after which the supernatant was removed. Genomic DNA was extracted using a bacterial genomic DNA extraction kit (Intron Biotechnology, Seongnam, South Korea) according to the manufacturer’s instructions. Total genomic DNA from the probiotic and dairy products was extracted using a DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the method described in a previous study [36]. DNA concentration and purity were determined by absorbance using a MaestroNano® spectrophotometer (Maestrogen, Las Vegas, NV, USA).

**Identification of *Lactobacillus* species-specific regions and primer designs**

In total, 143 complete genome sequences of 37 different *Lactobacillus* species were obtained from the National Center for Biotechnology Information (NCBI; ftp://ftp.ncbi.nlm.nih.gov/genomes/) database (Additional file 1: Table S1). The 16S–23S rRNA regions, including the intergenic spacer regions, of 143 strains were extracted from the *Lactobacillus* complete genomes using a script written in the Python language, and the extracted regions were aligned using the Geneious program ver. 11.1.2 (Biomatters Limited, Auckland, New Zealand). According to the alignment results, primer pairs were designed on the basis of species-specific sequences in the 16S–23S rRNA gene. Some *Lactobacillus* species are difficult to distinguish at the species level because of the high degree of similarity in their 16S-23S rRNA gene sequences. For these species, we have developed species-specific primer pairs from unique genes that exist only in the target
species obtained through comparative genomic analysis.

The genome sequences of target species were blasted against the complete genome of target species using the UBLAST function of USEARCH program ver. 9.0 [37], with 80% cutoff identity. The genes that showed a significant match with the genomes of all target species were considered as core genes of target species. Those genes were then blasted against all of the Lactobacillus complete genomes except the target species using the UBLAST function of USEARCH program (ver. 9.0) with default parameter settings of 50% cutoff identity [37]. Genes that found no match to all genomes of the non-target species were identified as potential unique genes. The identified potential unique genes were verified using the Basic Local Alignment Search Tool (BLAST) for 52,478,804 sequences including Lactobacillus genomes. Genes found only in the target species were determined to be unique genes, and species-specific primer pairs were designed from these genes. To verify the presence of genomic DNA from Lactobacillus species, primer pairs were designed from the conserved regions of 37 Lactobacillus species in the 16S rRNA gene sequence and used as an internal positive control (IPC). All primer pairs were designed using Primer Designer (Scientific and Educational Software, Durham, NC, USA) and synthesized by Bionics Co. Ltd. (Seoul, South Korea).

**Specificity of species-specific primer pairs**

PCR assays were performed to confirm the specificity of the designed species-specific primer pairs. The specificity was evaluated using 37 Lactobacillus reference strains. PCR products were amplified using the following conditions in a thermocycler (Astec, Fukuoka, Japan): 94°C for 10 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 72°C for 5 min. The 25 µL reaction mixtures contained 20 ng of template DNA of a Lactobacillus reference strain, 0.5 unit of Taq DNA polymerase (TaKaRa BIO Inc., Tokyo, Japan), and species-specific primer pairs. The optimal concentration of each species-
specific primer pair obtained from the experiments is shown in Table 1. The amplification products were confirmed by electrophoresis on a 2% agarose gel, and the product bands were visualized under a UV transilluminator (Vilber Lourmat, Marne La Vallee, France).

**Development of real-time PCR assay**

The real-time PCR assays were performed on the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the following conditions: 95°C for 2 min, followed by 30 cycles of 95°C for 5 s and 60°C for 30 s. The melting curve data were generated using one cycle of 95°C for 15 s, 60°C for 1 min, 95°C for 30 s, and 60°C for 15 s. The amplification mixture with a final volume of 20 µL for real-time PCR assays included 2X LeGene SB-Green Real-Time PCR Master Mix (LeGene Biosciences, San Diego, CA, USA), template DNA, and species-specific primer pairs at optimal concentrations shown in Table 1. To evaluate the analytical accuracy of the real-time PCR assay, a standard curve was constructed using serial dilutions (50 to 0.005 ng) of genomic DNA from *Lactobacillus* reference strains in triplicate. The specificities of the species-specific primer pairs were tested using 20 ng of DNA extracted from 37 *Lactobacillus* reference strains. Real-time PCR amplifications of IPC were also confirmed with 37 *Lactobacillus* reference strains. The results of the real-time PCR were confirmed using 7500 Software V2.3 (Applied Biosystems).

**Application of the developed real-time PCR assay in probiotic and dairy products**

We designed a validation test to detect 37 *Lactobacillus* species with real-time PCR in a single reaction using primer pairs. Each well of a reaction plate contained each primer pair and IPC for the simultaneous detection of 37 *Lactobacillus* species. Briefly, 20 ng of product DNA and 2X Master Mix (LeGene Biosciences) were added to each well of the reaction plate containing species-specific primers. Then, real-time PCR was performed in the 7500 Real-Time PCR system (Applied Biosystems). The real-time PCR conditions were
similar to those described in “Development of real-time PCR assay” section.

Declarations

Acknowledgments

Not applicable.

Authors’ contributions

EK, SMY, BL, SHP, and HYK designed this study. EK, SMY, BL, and HYK performed experiments, analyzed data and wrote the manuscript. SHP, BR, and HK reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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### Tables

Table 1. Information of primer pairs designed for this study

| Species          | Target gene        | Primer name | Sequence (5¢–3¢)                        |
|------------------|--------------------|-------------|----------------------------------------|
| **IPC**<sup>a</sup> | 16S–23S region     | IPC-F       | CAA CGC GAA GAA CCT<sup>+</sup>         |
|                  |                    | IPC-R       | CCA ACA TCT CAA CGA<sup>+</sup>         |
| *L. gasseri*     | 16S–23S region     | Gasseri-F   | TCA AGA GCT GTT AAG<sup>+</sup>         |
|                  |                    | Gasseri-R   | CTA TCG CTT CAA GTG<sup>+</sup>         |
| *L. rhamnosus*   | 16S–23S region     | Rhamnosus-F | GCC GAT CGT TGA CGT<sup>+</sup>         |
|                  |                    | Rhamnosus-R | CAG CGG TTA TGC GAT<sup>+</sup>         |
| *L. brevis*      | 16S–23S region     | Brevis-F    | GGG CAA CGA AGC AAG<sup>+</sup>         |
|                  |                    | Brevis-R    | TTC CAA TCG TGT GCA<sup>+</sup>         |
| *L. sakei*       | 16S–23S region     | Sakei-F     | TCG AAC GCA CTC TCG<sup>+</sup>         |
|                  |                    | Sakei-R     | CGA AAC CAT CTT TCA<sup>+</sup>         |
| *L. johnsonii*   | 16S–23S region     | Johnsonii-F | AGA GAG AAA CTC AAC<sup>+</sup>         |
|                  |                    | Johnsonii-R | CCT TCA TTA ACC TTA A<sup>+</sup>       |
| *L. jensenii*    | 16S–23S region     | Jensenii-F  | AGT TCT TCG GAA TGG<sup>+</sup>         |
|                  |                    | Jensenii-R  | GCC GCC TTT TAA ACT T<sup>+</sup>       |
| *L. fermentum*   | Unique gene        | Fermentum-F | GAC CAG CGC ACC AAG<sup>+</sup>         |
|                  |                    | Fermentum-R | AGC GTA GCG TTC GTG<sup>+</sup>         |
| *L. plantarum*   | Unique gene        | Plantarum-F | GCT GGC AAT GCC ATC<sup>+</sup>         |
|                  |                    | Plantarum-R | TCT CAA CGG TTG CTG<sup>+</sup>         |
| *L. paracasei*   | Unique gene        | Paracasei-F | CAA TGC CGT GGT TGT<sup>+</sup>         |
|                  |                    | Paracasei-R | GCC AAT CAC CGC ATT<sup>+</sup>         |
| *L. paraplantarum* | Unique gene      | Paraplantarum-F | TTA TTC AAG CCG TCG<sup>+</sup>     |
|                  |                    | Paraplantarum-R | TCG CTG GTG CTA ATG<sup>+</sup>     |
| *L. casei*       | Unique gene        | Casei-F     | CCA CAA TCC TTG GCT<sup>+</sup>         |
|                  |                    | Casei-R     | GCT TGA GGC GAT TGT<sup>+</sup>         |
| *L. curvatus*    | 16S–23S region     | Curvatus-F  | ACT CTC ATT GAA TTA<sup>+</sup>         |
|                  |                    | Curvatus-R  | CCC GTG TTG GTA CTA<sup>+</sup>         |
| *L. acidophilus* | 16S–23S region     | Acidophilus-F | CCT TTC TAA GGA AGC<sup>+</sup>     |
|                  |                    | Acidophilus-R | ACG CTT GGT ATT CCA<sup>+</sup>     |
| *L. salivarius*  | 16S–23S region     | Salivarius-F | TAC ACC GAA TGC TTG<sup>+</sup>         |
|                  |                    | Salivarius-R | AGG ATC ATG CGA TCC<sup>+</sup>         |
| L. reuteri       | 16S–23S region | Reuteri-F  | GAT TGA CGA TGG ATC |
| L. coryniformis | 16S–23S region | Coryniformis-F | CAT CCC AGA GTG ATA |
| L. farciminis   | Unique gene    | Farciminis-F | ACG AAT CCG GCA GTCA |
| L. zymae        | 16S–23S region | Zymae-F     | TCG GCA GTG TGA CAT |
| L. pentosus     | Unique gene    | Pentosus-F  | GGG GTA TCG ATT CGA |
| L. crustorum    | 16S–23S region | Crustorum-F  | CAA TTG CGC TCT TTC |
| L. mucosae      | 16S–23S region | Mucosae-F   | GCT AAA GCA AGC GCA |
| L. buchneri     | 16S–23S region | Buchneri-F  | CAA GTC GAA CGC GTC |
| L. helveticus   | Unique gene    | Helveticus-F | CTA CTT CGC AGG CGT |
| L. amylovorus   | Unique gene    | Amylovorus-F | CAA GCA CGA TTG GCA |
| L. heilongjiangensis | 16S–23S region | Heilongjiangensis-F | GCT TCA TGA ATC GGA |
| L. parabuchneri | Unique gene    | Parabuchneri-F | TAA ACT ACG ATG ATG |
| L. acidipiscis  | Unique gene    | Acidipiscis-F | TCC AAG TCC GAC ACC |
| L. sanfranciscensis | Unique gene   | Sanfranciscensis-F | TGG AAC TGA TAC GCG |
| L. ruminis      | 16S–23S region | Ruminis-F   | GGC CAA TTC CTC CAA |
| L. agilis       | 16S–23S region | Agilis-F    | CAT AAA CAT CAT GCG |
| L. delbrueckii  | 16S–23S region | Delbrueckii-F | CAT GTG CAG ACA TGC |
| L. amylophilus  | 16S–23S region | Amylophilus-F | CTC TGA AGT GCC ATG |
| L. kunkeei      | 16S–23S region | Kunkeei-F   | GAA CGA CTT CAC AAG |
| L. amylophilus  | 16S–23S region | Amylophilus-R | CGC CAT CTT TCA AAC |
| Species                 | Region       | Forward Primer          | Reverse Primer         |
|------------------------|--------------|-------------------------|------------------------|
| L. acetotolerans       | 16S–23S      | Acetotolerans-F         | Acetotolerans-R        |
|                        | region       | GAT TAC CTT CGG GTA T   | TCA TGT GAT CTC TCC T  |
| L. lindneri            | Unique gene  | Lindneri-F               | Lindneri-R             |
|                        |              | CGG CGT TCT CGA GGA     | CAT CCG GCG TCC TTC T  |
| L. gallinarum          | Unique gene  | Gallinarum-F             | Gallinarum-R           |
|                        |              | AAC TGG CGG TTA TCG A   | CAC AGC AGG AAC CAT T  |
| L. amylyticus          | 16S–23S      | Amylyticus-F             | Amylyticus-R           |
|                        | region       | TTC GGT AGT GAC GTT T   | TCA AGC AAG TGC CAT C  |

^aIPC, internal positive control.

^bconc., concentration.

Table 2. Characteristics of unique genes to each species
| Species                | Gene name                                                                 |
|------------------------|---------------------------------------------------------------------------|
| *L. sanfranciscensis*  | Acetyltransferase                                                          |
| *L. acidipiscis*       | Membrane protein                                                           |
| *L. fermentum*         | Mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase                    |
| *L. amylovorus*        | Adenylosuccinate lyase                                                     |
| *L. pentosus*          | GHKL domain-containing protein                                             |
| *L. plantarum*         | LPXTG-motif cell wall anchor domain protein                                 |
| *L. helveticus*        | Dicarboxylate/amino acid:cation Na+/H+ symporter family protein            |
| *L. farcininis*        | DUF262 domain-containing protein                                           |
| *L. parabuchneri*      | Leucine-rich repeat protein                                                |
| *L. paraplantarum*     | MFS-type transporter YcnB                                                  |
| *L. gallinarum*        | LacI family transcriptional regulator                                       |
| *L. casei*             | Putative truncated melibiose symporter                                     |
| *L. paracasei*         | Cation transport ATPase                                                    |
| *L. lindneri*          | Accessory Sec system protein Asp2                                           |

Table 3. The BLASTN results of unique genes
| Species               | Description                                      | Identity (%) | Target species match |
|----------------------|--------------------------------------------------|--------------|----------------------|
| L. sanfranciscensis  | L. sanfranciscensis TMW 1.1304                    | 99           | L. sanfranciscensis   |
| L. acidipiscis       | L. acidipiscis strain ACA-DC 1533                 | 99.58        | L. acidipiscis       |
| L. fermentum         | L. fermentum strain B1 28                        | 100          | L. fermentum         |
| L. amylovorus        | L. amylovorus DSM 20531                          | 100          | L. amylovorus        |
| L. pentosus          | L. pentosus strain DSM 20314                      | 100          | L. pentosus          |
| L. plantarum         | L. plantarum strain IDCC3501                      | 100          | L. plantarum         |
| L. helveticus        | L. helveticus isolate NWC_2_3                     | 100          | L. helveticus        |
| L. farcininis        | L. farcininis KCTC 3681                          | 100          | L. farcininis        |
| L. parabuchneri      | L. parabuchneri strain FAM21731                   | 99.97        | L. parabuchneri      |
| L. paraplantarum     | L. paraplantarum strain DSM 10667                 | 100          | L. paraplantarum     |
| L. gallinarum        | L. gallinarum DSM 10532                          | 100          | L. gallinarum        |
| L. casei             | L. casei subsp. casei ATCC 393                    | 100          | L. casei             |
| L. paracasei         | L. paracasei ATCC 334                            | 100          | L. paracasei         |
| L. lindneri          | L. lindneri strain TMW 1.481                      | 100          | L. lindneri          |

Table 4. Slope, $R^2$, and efficiency of *Lactobacillus* reference strain in the real-time PCR assay

| Species  | Slope     | $R^2$  | Efficiency (%) |
|----------|-----------|--------|----------------|
| L. gasseri | -3.214    | 0.999  | 104.701        |
| L. rhamnosus | -3.362    | 0.998  | 98.35          |
| L. brevis   | -3.444    | 1      | 95.158         |
| L. sakei    | -3.212    | 1      | 104.797        |
| L. johnsonii | -3.214    | 0.999  | 104.701        |
| L. jensenii | -3.328    | 0.996  | 99.764         |
| L. fermentum  | -3.56     | 0.995  | 90.955         |
| L. plantarum  | -3.221    | 0.995  | 104.396        |
| L. paracasei  | -3.305    | 0.98   | 100.694        |
| Primer name       | Detected species          | Ct value | Tm (°C)  |
|-------------------|---------------------------|----------|----------|
| L. paraplantarum  | -3.256                    | 0.998    | 102.822  |
| L. casei          | -3.284                    | 0.999    | 101.612  |
| L. curvatus       | -3.485                    | 0.999    | 93.617   |
| L. acidophilus    | -3.506                    | 1        | 92.845   |
| L. salivarius     | -3.564                    | 1        | 90.809   |
| L. reuteri        | -3.342                    | 0.999    | 99.161   |
| L. coryniformis   | -3.217                    | 0.989    | 104.578  |
| L. farciminis     | -3.386                    | 0.991    | 97.39    |
| L. zymae          | -3.5                     | 0.997    | 93.073   |
| L. pentosus       | -3.292                    | 0.999    | 101.251  |
| L. crustum        | -3.438                    | 0.999    | 95.366   |
| L. mucosae        | -3.478                    | 0.986    | 93.886   |
| L. buchneri       | -3.411                    | 0.993    | 96.424   |
| L. helveticus     | -3.230                    | 0.998    | 103.98   |
| L. amylovorus     | -3.582                    | 0.993    | 90.167   |
| L. heilongjiangensis | -3.462                   | 1        | 94.458   |
| L. parabuchneri   | -3.207                    | 1        | 105.049  |
| L. acidipiscis    | -3.528                    | 0.984    | 92.075   |
| L. sanfranciscensis | -3.229                  | 0.999    | 104.034  |
| L. ruminis        | -3.295                    | 1        | 101.153  |
| L. agilis         | -3.508                    | 1        | 92.795   |
| L. delbrueckii    | -3.31                     | 0.999    | 100.479  |
| L. amylophilus    | -3.481                    | 0.984    | 93.768   |
| L. kunkeei        | -3.571                    | 0.998    | 90.568   |
| L. acetotolerans  | -3.209                    | 1        | 104.92   |
| L. lindneri       | -3.559                    | 0.982    | 90.972   |
| L. gallinarum     | -3.346                    | 0.999    | 98.989   |
| L. amylolyticus   | -3.552                    | 0.996    | 91.209   |

Table 5. Specificity results of the real-time PCR assay
| Name                  | Species                  | Accession 1 | Accession 2 | 11.258 | 79.329 | 5.762 | 85.319 | 11.139 | 82.441 | 6.450 | 84.193 | 6.583 | 81.378 | 4.260 | 88.582 | 10.715 | 82.027 | 12.012 | 80.746 | 10.884 | 82.306 | 10.739 | 82.513 | 13.832 | 82.686 | 12.383 | 79.308 | 14.905 | 81.806 | 9.142 | 83.439 | 13.638 | 84.793 | 10.678 | 80.465 | 7.546 | 82.568 | 11.603 | 84.268 | 12.467 | 82.012 | 11.598 | 83.109 | 11.606 | 82.206 | 12.087 | 79.059 | 11.256 | 82.037 | 11.922 | 81.205 | 9.377 | 81.604 | 10.743 | 81.566 | 10.273 | 79.814 | 9.724 | 82.341 | 11.758 | 82.095 | 8.621 | 83.114 | 10.943 | 82.733 | 8.542 | 83.217 | 11.912 | 82.031 | 12.910 | 79.917 | 10.132 | 78.138 | 11.694 | 83.460 |
Table 6. Results of application test of the developed real-time PCR assay to commercial probiotic and dairy products

| Name | Label claim | Detected species |
|------|-------------|------------------|
| P1   | *L. plantarum* | *L. plantarum* |
| P2   | *L. rhamnosus* | *L. rhamnosus* |
| P3   | *L. acidophilus* | *L. acidophilus* |
| P4   | *L. delbrueckii, L. paracasei* | *L. delbrueckii, L. paracasei* |
| P5   | *L. acidophilus, L. rhamnosus* | *L. acidophilus, L. rhamnosus* |
| P6   | *L. acidophilus, L. rhamnosus* | *L. acidophilus, L. rhamnosus* |
| P7   | *L. acidophilus, L. delbrueckii* | *L. acidophilus, L. delbrueckii* |
| P8   | *L. acidophilus, L. plantarum, L. reuteri* | *L. acidophilus, L. plantarum, L. reuteri* |
| P9   | *L. acidophilus, L. plantarum, L. reuteri* | *L. acidophilus, L. plantarum, L. reuteri* |
| P10  | *L. acidophilus, L. fermentum, L. plantarum* | *L. acidophilus, L. fermentum, L. plantarum* |
| P11  | *L. acidophilus, L. brevis, L. casei, L. delbrueckii, L. paracasei, L. plantarum, L. salivarius* | *L. acidophilus, L. brevis, L. casei, L. delbrueckii, L. paracasei, L. plantarum, L. salivarius* |
| P12  | *L. acidophilus, L. casei, L. gasseri, L. paracasei, L. plantarum, L. reuteri, L. rhamnosus* | *L. acidophilus, L. casei, L. gasseri, L. paracasei, L. plantarum, L. reuteri, L. rhamnosus* |
| P13  | *L. rhamnosus* | *L. helveticus* |
| P14  | *L. acidophilus, L. casei, L. rhamnosus* | *L. helveticus, L. rhamnosus* |
| P15  | *L. acidophilus, L. casei, L. rhamnosus* | *L. helveticus, L. rhamnosus* |
| P16  | *L. rhamnosus* | *L. helveticus* |
| P17  | *L. acidophilus, L. casei, L. plantarum, L. rhamnosus* | *L. helveticus* |
| P18  | *L. acidophilus, L. paracasei, L. rhamnosus, L. salivarius* | *L. helveticus* |
| P19  | *L. delbrueckii, L. plantarum, LAB mixed powder* | *L. delbrueckii, L. plantarum, LAB mixed powder* |
| D1   | *L. acidophilus, L. casei* | *L. acidophilus, L. casei* |
| D2   | *L. delbrueckii, L. rhamnosus* | *L. delbrueckii, L. rhamnosus* |
| D3   | *L. delbrueckii, L. rhamnosus* | *L. delbrueckii, L. rhamnosus* |
| D4   | *L. delbrueckii, L. rhamnosus* | *L. delbrueckii, L. rhamnosus* |
| D5   | *L. rhamnosus, LAB* | *L. rhamnosus, LAB* |
| D6   | *LAB, probiotic LAB* | *LAB, probiotic LAB* |
| D7   | *Compound LAB* | *Compound LAB* |
| D8   | *LAB* | *LAB* |
| D9   | *LAB* | *LAB* |
| D10  | *LAB* | *LAB* |
| D11  | *LAB* | *LAB* |
| D12  | *LAB* | *LAB* |

LAB, lactic acid bacteria.
Table 7. *Lactobacillus* reference strains used in this study

| Species            | Strain no.  |
|--------------------|-------------|
| *L. gasseri*       | KCTC³ 3163  |
| *L. rhamnosus*     | KCTC 3237   |
| *L. brevis*        | KCTC 3498   |
| *L. sakei*         | KCTC 3603   |
| *L. johnsonii*     | KCTC 3801   |
| *L. jensenii*      | KCTC 5194   |
| *L. fermentum*     | KACC⁵ 11441 |
| *L. plantarum*     | KACC 11451  |
| *L. paracasei*     | KACC 12361  |
| *L. paraplantarum* | KACC 12373  |
| *L. casei*         | KACC 12413  |
| *L. curvatus*      | KACC 12415  |
| *L. acidophilus*   | KACC 12419  |
| *L. salivarius*    | KCTC 3600   |
| *L. reuteri*       | KCTC 3594   |
| *L. coryniformis*  | KACC 12411  |
| *L. farcinis*      | KACC 12423  |
| *L. zymae*         | KACC 16349  |
| *L. pentosus*      | KACC 12428  |
| *L. crustorum*     | KACC 16344  |
| *L. mucosae*       | KACC 12381  |
| *L. buchneri*      | KACC 12416  |
| *L. helveticus*    | KACC 12418  |
| *L. amylovorus*    | KACC 12435  |
| *L. heilongjiangensis* | KACC 18741 |
| *L. parabuchneri*  | KACC 12363  |
| Species               | Accession |
|----------------------|-----------|
| L. acidipiscis       | KACC 12394|
| L. sanfranciscensis  | KACC 12431|
| L. ruminis           | KACC 12429|
| L. agilis            | KACC 12433|
| L. delbrueckii       | KACC 12420|
| L. amylphilus        | KACC 11430|
| L. kunkeei           | KACC 19371|
| L. acetotolerans     | KACC 12447|
| L. lindneri          | KACC 12445|
| L. gallinarum        | KACC 12370|
| L. amylolyticus      | KACC 12374|

a KCTC, Korean Collection for Type Cultures.

b KACC, Korean Agricultural Culture Collection.

Figures
Figure 1

Pan-genome distribution across Lactobacillus gallinarum and L. helveticus. Each ring represents L. gallinarum and L. helveticus strain and each layer displays the pan-genome distribution. The blue and black rings represent the genomes of L. gallinarum and L. helveticus, respectively.
Examples of real-time PCR standard curves, amplification curves and melting curves: (A) L. acetotolerans standard curve between 50 and 0.005 ng ($y = -3.209x + 14.197$, $R^2 = 1$, left), amplification plot (middle) and melt curve (right); (B) L. casei standard curve ($y = -3.284x + 17.817$, $R^2 = 0.999$, left), amplification plot (middle), melt curve (right); (C) L. parabuchneri standard curve
Specificities of species-specific primer pairs against 37 Lactobacillus species: (A) specificity of L. acetotolerans specific primer pair, amplification curve: L. acetotolerans KACC 12447; (B) specificity of L. casei specific primer pair, amplification curve: L. casei KACC 12413; (C) specificity of L. parabuchneri specific primer pair, amplification curve: L. parabuchneri KACC 12363; and (D) specificity of L. lindneri specific primer pair, amplification curve: L. lindneri KACC 12445.
Supplementary Files

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