Development of Real-Time PCR Assay to Specifically Detect 22 Bifidobacterium Species and Subspecies Using Comparative Genomics

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Bifidobacterium species are used as probiotics to provide beneficial effects to humans. These effects are specific to some species or subspecies of Bifidobacterium. However, some Bifidobacterium species or subspecies are not distinguished because similarity of 16S rRNA and housekeeping gene sequences within Bifidobacterium species is very high. In this study, we developed a real-time polymerase chain reaction (PCR) assay to rapidly and accurately detect 22 Bifidobacterium species by selecting genetic markers using comparative genomic analysis. A total of 210 Bifidobacterium genome sequences were compared to select species- or subspecies-specific genetic markers. A phylogenetic tree based on pan-genomes generated clusters according to Bifidobacterium species or subspecies except that two strains were not grouped with their subspecies. Based on pan-genomes constructed, species- or subspecies-specific genetic markers were selected. The specificity of these markers was confirmed by aligning these genes against 210 genome sequences. Real-time PCR could detect 22 Bifidobacterium specifically. We constructed the criterion for quantification by standard curves. To further test the developed assay for commercial food products, we monitored 26 probiotic products and 7 dairy products. Real-time PCR results and labeling data were then compared. Most of these products (21/33, 63.6%) were consistent with their label claims. Some products labeled at species level only can be detected up to subspecies level through our developed assay.

Keywords: Bifidobacterium, real-time PCR, pan-genome, whole-genome sequence, probiotic, comparative genomics, identification, detection method

INTRODUCTION

Probiotics are living microorganisms that provide health benefits such as improving digestive health and preventing infectious diarrhea, irritable bowel syndrome, and inflammatory bowel disease of hosts (O’Callaghan and van Sinderen, 2016; Floch, 2018; Shehata et al., 2019). Health benefits of probiotics are species- or strain- specific. Not all lactic acid bacteria are considered as
probiotics (Pinto-Sanchez et al., 2017). *Bifidobacterium* is one important member of probiotics that has benefits such as anti-cancer effects (Inoue et al., 2009) and reducing cholesterol level (Zhang et al., 2016) for the host. *Bifidobacterium* is Gram-positive, non-motile, and catalase-negative lactic acid bacterium that survives in the intestine of human. *Bifidobacterium* species in human gut microbiota include *Bifidobacterium longum*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium adolescentis*, *Bifidobacterium dentium*, and *Bifidobacterium pseudocatenulatum* (Junick and Blaut, 2012). *Bifidobacterium pseudolongum* and *Bifidobacterium thermophilum* previously considered to be of animal origin have been isolated from baby feces and human adults, respectively (von Ah et al., 2007; Turroni et al., 2009; Junick and Blaut, 2012).

*Bifidobacterium* species have universally different functions according to subspecies. For instance, *Bifidobacterium animalis* subsp. *lactis* has a strong anti-inflammatory effect to improve the immune system (Weizman et al., 2005), whereas *B. animalis* subsp. *animalis* cannot grow in milk (Masco et al., 2004). *B. longum* also has different types of glycolytic enzymes according to its subspecies (LoCascio et al., 2010; Lewis et al., 2016). Hence, differentiating *Bifidobacterium* subspecies is necessary. Furthermore, presenting correct species in probiotic products is critical for providing correct information to consumers and claiming health benefits of the product (Shehata et al., 2019). Recently, some studies have shown mislabeling issues such as absence of some species, inaccurate taxonomy information, and undeclared species (Lewis et al., 2016; Morovic et al., 2016) of commercial probiotic products. However, there is no reliable detection method to distinguish different species and subspecies of *Bifidobacterium*.

Polymerase chain reaction (PCR)-based methods have been widely used to detect bacterial strains in probiotics, dairy products, meat products, and seafood (Binetti et al., 2008; Cammà et al., 2012; Kim et al., 2019). In particular, the 16S rRNA gene has been used as a useful target gene for bacterial identification. However, the resolution of this gene among closely related species is low (Junick and Blaut, 2012). To differentiate *Bifidobacterium* species, more distinguishable identification markers need to be found because 16S rRNA genes of *Bifidobacterium* species share high similarities (mean, 95%) (Ventura et al., 2006; Junick and Blaut, 2012). Housekeeping genes such as *recA* (Ventura and Zink, 2003), *tuf* (Ventura and Zink, 2003), *atpD* (Ventura et al., 2004a), *groEL* (Zhu et al., 2003), and *groES* (Ventura et al., 2004b) have been used as alternative genetic markers for the discrimination of *Bifidobacterium*. Although these genes have been demonstrated to have a relatively higher resolution than 16S rRNA gene, similar species and subspecies are still indistinguishable. Thus, those genes can only be applied to limited species (Lawley et al., 2017).

Whole-genome sequencing (WGS) is a powerful method for identifying unique genes through bioinformatics (Chen et al., 2010; Mellmann et al., 2017). Comparative genomics has been performed for pathogenic bacteria and lactic acid bacteria using various algorithms (Lugli et al., 2017; Zhang et al., 2019). But, studies on development of specific primers of probiotic species based on comparative genomics have not been widely conducted.

The objective of the present study was to develop a real-time PCR assay using comparative genomics known to be able to detect highly specific genetic markers and bacterial strains very quickly. A brief description of the method is as follows: specific genetic markers were selected using comparative genomics from 210 *Bifidobacterium* genomes, and species- or subspecies-specific primers were designed based on identified markers. Real-time PCR assay was then applied for quantitative identification of 22 *Bifidobacterium* species, which is mainly found in intestine of human and food samples such as probiotic or dairy products and difficult to differentiate by conventional methods. Furthermore, label claims of commercial probiotics and dairy products were verified using the developed real-time PCR assay.

### MATERIALS AND EQUIPMENT

#### Bacterial Strains

Forty-one *Bifidobacterium* species or subspecies strains, 11 *Lactobacillus* species, 1 *Lactococcus* species, and 2 *Enterococcus* species obtained from Korean Agricultural Culture Collection (KACC, Jeonju, South Korea), Korean Collection for Type Cultures (KCTC, Daejeon, South Korea), and Korean Culture Center of Microorganisms (KCCM, Seoul, South Korea) were used to confirm the specificity of the developed real-time PCR (Table 1).

#### Equipment and Software

Anvi′o, Bacterial Pan Genome Analysis pipeline (BPGA), USEARCH, and Basic Local Alignment Search Tool (BLAST) software were used for comparative genomics to select specific genetic genes for *Bifidobacterium* species or subspecies. 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, United States) and 7500 software were used for the specificity and accuracy of species- or subspecies-specific primers.

### METHODS

#### Cultivation and Genomic DNA Extraction of *Bifidobacterium* Strains

*Bifidobacterium* strains were cultured in *Bifidobacterium* broth (MB cell, Seoul, South Korea) and BL broth (MB cell, Seoul, South Korea) at 37°C for 48 h under anaerobic condition. Other lactic acid bacterial strains were cultured in MRS broth (Difco, Becton Dickinson, Sparks, MD, United States) at 30°C for 48 h under anaerobic condition (Kim et al., 2020). All strains were stored in 30% (v/v) glycerol (Bioshop, Burlington, ON, Canada) at −80°C until use. To extract genomic DNA, all cultured bacterial cells were collected by centrifugation at 16,200 × g for 3 min. DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) was used to extract total genomic DNAs from all strains following the manufacturer’s protocol for Gram-positive bacteria. Purity and concentration of extracted bacterial DNA were measured using a MaestroNano® spectrophotometer (Maestrogen, Las Vegas, NV, United States).
TABLE 1 | List of strains used in this study.

| Species | Strain number |
|---------|---------------|
| Bifidobacterium animalis subsp. animalis | KACC 16637 |
| Bifidobacterium animalis subsp. lactis | KACC 16638 |
| Bifidobacterium animalis subsp. lactis | LI 001941 |
| Bifidobacterium animalis subsp. lactis | LI 001942 |
| Bifidobacterium animalis subsp. lactis | LI 000026 |
| Bifidobacterium animalis subsp. lactis | LI 000004 |
| Bifidobacterium animalis subsp. lactis | LI 000019 |
| Bifidobacterium animalis subsp. lactis | LI 000062 |
| Bifidobacterium breve | KACC 16639 |
| Bifidobacterium breve | KCTC 3419 |
| Bifidobacterium breve | LI 000070 |
| Bifidobacterium longum subsp. infantis | KCTC 3249 |
| Bifidobacterium longum subsp. infantis | LI 000033 |
| Bifidobacterium longum subsp. infantis | LI 000261 |
| Bifidobacterium longum subsp. infantis | LI 000262 |
| Bifidobacterium longum subsp. suis | KACC 16649 |
| Bifidobacterium longum subsp. longum | KCCM 11953 |
| Bifidobacterium bifidum | LI 000175 |
| Bifidobacterium bifidum | KCTC 3418 |
| Bifidobacterium bifidum | KCTC 3440 |
| Bifidobacterium bifidum | LI 000058 |
| Bifidobacterium bifidum | LI 000061 |
| Bifidobacterium bifidum | LI 000063 |
| Bifidobacterium gallicum | KACC 16645 |
| Bifidobacterium thermacidophilum | KACC 16653 |
| Bifidobacterium thermacidophilum | KACC 16674 |
| Bifidobacterium thermophilum | KACC 20600 |
| Bifidobacterium corneforme | KACC 16642 |
| Bifidobacterium asteroides | KACC 16635 |
| Bifidobacterium adolescentis | KACC 16634 |
| Bifidobacterium pseudolongum | KACC 16667 |
| Bifidobacterium pseudodolongum | KACC 16666 |
| Bifidobacterium cuniculi | KACC 16643 |
| Bifidobacterium gallinarum | KACC 16646 |
| Bifidobacterium scardovii | KACC 16672 |
| Bifidobacterium pseudocatenulatum | KCTC 3223 |
| Bifidobacterium angulatum | KCTC 3236 |
| Bifidobacterium dentium | KACC 16644 |
| Bifidobacterium tsuruimiense | KACC 16645 |
| Bifidobacterium catenulatum | KACC 16640 |
| Bifidobacterium catenulatum | KACC 16648 |
| Lactobacillus gasseri | KCTC 3163 |
| Lactobacillus rhamnosus | KCTC 3237 |
| Lactobacillus casei | KACC 12413 |
| Lactobacillus delbrueckii | KACC 12420 |
| Lactobacillus acidophilus | KACC 12419 |
| Lactobacillus helveticus | KACC 12418 |
| Lactobacillus fermentum | KACC 11441 |
| Lactobacillus paracasei | KACC 12427 |
| Lactobacillus plantarum | KACC 11451 |
| Lactobacillus reuteri | KCTC 3594 |
| Lactobacillus salivarius | KCTC 3600 |
| Lactococcus lactis | KACC 19376 |
| Enterococcus faecium | KCTC 13225 |
| Enterococcus faecalis | KCTC 3206 |

Genomic DNA Extraction of Commercial Products

Commercial products used in this study are listed in Table 2. These products were classified from A1 to A26 for probiotic products and from B1 to B7 for dairy products. Twenty-six probiotic products and 7 dairy products were purchased from markets worldwide (South Korea: 16, United States: 7, Canada: 8, United Kingdom: 1, Italy: 1). These probiotic products included 18 capsules, 7 powders, and 1 chewable. Total genomic DNAs of probiotic products were extracted according to a previous study (Kim et al., 2017). One-hundred milligrams of probiotic product were aliquoted and dissolved in 300 μL of lysis buffer following the manufacturer’s instruction (DNeasy Blood and Tissue kit, Qiagen). Purity and concentration of extracted probiotic DNAs were measured as previously mentioned.

Comparative Genomic Analysis of Bifidobacterium Species or Subspecies

All Bifidobacterium genome sequences were downloaded from the National Center for Biotechnology Information.

TABLE 2 | Type, form, and country of purchase in probiotic and dairy products.

| Products | Type | Form | Country |
|----------|------|------|---------|
| A1 | Probiotic product | Capsules | Canada |
| A2 | Probiotic product | Chewable | South Korea |
| A3 | Probiotic product | Capsules | United States |
| A4 | Probiotic product | Capsules | United States |
| A5 | Probiotic product | Capsules | United States |
| A6 | Probiotic product | Powder | South Korea |
| A7 | Probiotic product | Capsules | Canada |
| A8 | Probiotic product | Powder | South Korea |
| A9 | Probiotic product | Capsules | United States |
| A10 | Probiotic product | Capsules | Canada |
| A11 | Probiotic product | Capsules | Canada |
| A12 | Probiotic product | Capsules | Canada |
| A13 | Probiotic product | Capsules | United States |
| A14 | Probiotic product | Powder | South Korea |
| A15 | Probiotic product | Powder | South Korea |
| A16 | Probiotic product | Powder | South Korea |
| A17 | Probiotic product | Capsules | United Kingdom |
| A18 | Probiotic product | Capsules | United States |
| A19 | Probiotic product | Powder | South Korea |
| A20 | Probiotic product | Capsules | Italy |
| A21 | Probiotic product | Capsules | Canada |
| A22 | Probiotic product | Capsules | United States |
| A23 | Probiotic product | Capsules | Canada |
| A24 | Probiotic product | Capsules | Canada |
| A25 | Probiotic product | Capsules | Canada |
| A26 | Probiotic product | Powder | South Korea |
| B1 | Dairy product | Yogurt | South Korea |
| B2 | Dairy product | Yogurt | South Korea |
| B3 | Dairy product | Yogurt | South Korea |
| B4 | Dairy product | Yogurt | South Korea |
| B5 | Dairy product | Yogurt | South Korea |
| B6 | Dairy product | Yogurt | South Korea |
| B7 | Dairy product | Yogurt | South Korea |
(NCBI), including 110 complete genomes, 52 scaffolds, and 31 contigs (Supplementary Table S1). To avoid drawing incorrect conclusions from the genomic analysis due to mislabeled genomes, a total of 210 Bifidobacterium genomes were evaluated using phylogenetic trees based on pan and core genes. A phylogenetic tree based on the pan-genome was constructed using Anvi’o version 6.0 publically available software according to the workflow for pan-genomics (Eren et al., 2015). Genome sequences obtained from NCBI were stored in Anvi’o storage for genomes to build a genome database. Pan-genome analysis was performed with the genome database. A phylogenetic tree was constructed according to pan gene cluster frequencies. Also, a phylogenetic tree based on core genes was constructed using BPGA version 1.3. The core genes were aligned using MUSCLE in BPGA, and a neighbor-joining phylogenetic tree was constructed. To select Bifidobacterium species- or subspecies-specific genetic markers, the core genome common to each species or subspecies was selected. Core genomes were then compared to explore candidate genetic markers using BPGA version 1.3 with default identity value (Chaudhari et al., 2016). Final candidates for species- or subspecies-specific genetic markers were verified using BLAST against 57,122,612 sequences, including sequences of other lactic acid bacteria. Then 22 genetic markers and 210 genome sequences were aligned with UBLAST algorithm with USEARCH version 9.0 (Edgar, 2010). The alignment of genetic markers to genomes is shown in a heatmap (Figure 2).

Also, the presence/absence of genes is easily skewed when the selected genetic marker is variable, so for all genetic markers their locations were verified, such as whether they are located in prophage genomes and plasmids or are really part of the core genome of that species using PlasmidFinder version 2.1 and BLAST analysis. Species- and subspecies-specific primers were designed based on selected genetic markers using Primer Designer (Scientific and Education Software, Chapel Hill, NC, United States). All 22 primer pairs were developed to be less than 200 bp in size to increase the amplification efficiency (Kim et al., 2020) suitable for the application of processed food products. All primers were synthesized by Bionics (Seoul, South Korea).

### Comparative Genomic Analysis of Bifidobacterium

Species- or subspecies-specific genetic markers were selected using comparative genomic analysis for 210 Bifidobacterium genomes. Candidate genetic markers for targets were selected by comparing core genomes with non-target pan-genome. To select specific genetic markers for the target, candidate genetic markers were blasted against 57,122,612 sequences, including sequences of other lactic acid bacteria. A phylogenetic tree was constructed based on the pan-genome for Bifidobacterium. Phylogeny showed that most genomes (n = 208) shared the same lineage according to their species or subspecies type (Figure 1). In contrast, B. longum subsp. infantis CCUG 52486 and 157F were more closely related to B. longum subsp. longum group than to B. longum subsp. infantis. The phylogenetic tree constructed by core genomes also showed the same clusters, where these two B. longum subsp. infantis genomes were clustered into B. longum subsp. longum (Supplementary Figure S2).

A total of 372,743 genes yielded a pan-genome of 21,669 genes. The core genome had 250 genes. The accessory genome had 15,429 genes. The unique genome had 7,170 genes. The unique genome was divided into genetic markers common to the same species or subspecies. The specificity of identified genetic markers was confirmed by BLAST. Most of these genomes (208/210, 99.05%) shared 90–100% sequence identities within genetic markers of the same species or subspecies and 0–50% sequence identities against other species. Information of these genes is shown in Table 3. These identified genetic markers shared more than 90% sequence identities against each target genome except two B. longum subsp. infantis strains. These two strains were...
FIGURE 1 | Pan-genomic phylogenetic tree of the *Bifidobacterium*. The figure shows that each ring represents *Bifidobacterium* genome and each layer displays the pan-genome distribution. The dark and bright colors of each ring indicate the presence and absence of core genes, respectively.

TABLE 3 | The accession number and information of species- or subspecies-specific genetic markers.

| Target species                  | Species- or subspecies-specific genetic markers                  | Accession no. |
|---------------------------------|------------------------------------------------------------------|---------------|
| B. *animals* subsp. *animals*   | Hypothetical protein                                             | API62648.1    |
| B. *animals* subsp. *lactis*    | Sel1 repeat family protein                                       | WP004218390.1 |
| B. *breve*                      | Serine hydrolase                                                 | WP014443379.1 |
| B. *longum* subsp. *infantis*   | ABC transporter permease                                          | WP012576966.1 |
| B. *longum* subsp. *suis*       | Glycosyl hydrolase, BNR repeat-containing protein                | KFI72947.1    |
| B. *longum* subsp. *longum*     | Bacterial Ig-like domain-containing protein                      | WP013141462.1 |
| B. *bilidum*                    | Conserved hypothetical protein containing Ig-like domain         | ADO53681.1    |
| B. *gallicum*                   | Adhesin isopeptide-forming adherence domain-containing protein   | WP052296095.1 |
| B. *thermacidophilum*           | Hypothetical protein                                             | KFI99790.1    |
| B. *thermolophilum*             | RelA/SpoT domain containing protein                              | AGH40345.1    |
| B. *coryneforme*                | Hypothetical protein                                             | WP038459169.1 |
| B. *asteroides*                 | Conserved repeat domain protein with Cna protein B-type          | AFU70840.1    |
| B. *adolecentis*                | MFS transporter                                                  | WP011743138.1 |
| B. *pseudolongum*               | Hypothetical protein                                             | WP022857512.1 |
| B. *cuniculi*                   | Hypothetical protein                                             | WP033518587.1 |
| B. *gallinarum*                 | ATP-binding protein                                              | WP081929610.1 |
| B. *scardovii*                  | DNA helicase                                                     | KFI95242.1    |
| B. *pseudocatenulatum*          | Hypothetical protein                                             | WP004223713.1 |
| B. *angulatum*                  | Type 2 lantipeptide synthetase LanM                               | WP052946496.1 |
| B. *dentium*                    | Cna B-type domain-containing protein                             | WP003837636.1 |
| B. *tsurumiense*                | BspA family leucine-rich repeat surface protein                  | WP026842738.1 |
| B. *catenulatum*                | Transcriptional regulator                                        | WP003833517.1 |
classified into \textit{B. longum} subsp. \textit{longum} according to our pan-genome analysis (Figure 2). The genetic marker for \textit{B. longum} subsp. \textit{infantis} such as ABC transporter permease (accession no. WP012576966.1) was present in 7 out of 9 strains (except CCUG 52486 and 157F). Instead, \textit{B. longum} subsp. \textit{infantis} CCUG 52486 and 157F had a bacterial Ig-like domain-containing protein (WP013141462.1), a genetic marker for \textit{B. longum} subsp. \textit{longum}. We confirmed that in these two \textit{B. longum} subsp. \textit{infantis} genomes, the genetic marker of \textit{B. longum} subsp. \textit{longum} was not present in their plasmids but on the chromosome, by blasting the contigs against the reported plasmid sequences. As well as, all genetic markers identified in this study were not located in plasmids or phage proteins and present in chromosome, meaning that these genetic markers are not variable and are part of the core genome. Based on these results, species- or subspecies-specific primers were designed and used for further studies (Table 4).

**Specificity and Quantification of the Developed Real-Time PCR Assay**

The specificity of the developed real-time PCR assay was confirmed with 41 \textit{Bifidobacterium} strains and 14 non-\textit{Bifidobacterium} strains. As a result, all primer sets specific for each \textit{Bifidobacterium} species/subspecies in silico showed detectable amplicons, with \textit{Ct} values between 11 and 16 against target strains, whereas those from all non-targets did not generate any positive signal (Figure 3 and Supplementary Table S2). To quantify the number of bacteria and to confirm the accuracy of real-time PCR, a standard curve was obtained using template DNA of \textit{Bifidobacterium} at a range of $8 \times 10^5$ to $8 \times 10^9$ CFU/mL in triplicates. This range included the number of bacteria labeled on probiotic products used. Slope for standard curves of \textit{B. animalis} subsp. \textit{lactis}, \textit{B. bifidum}, \textit{B. breve}, and \textit{B. longum} subsp. \textit{infantis} mainly used in probiotic products were $-3.499$, $-3.134$, $-3.275$, and $-3.552$, respectively. All $R^2$ values (correlation coefficients) were $\geq 0.997$ (Figure 4). Results of the slope, $R^2$ value, and efficiency of remaining primers are shown in Supplementary Table S3. According to the efficiency of quantitative real-time PCR, $R^2$ values $\geq 0.98$ are considered as reliable (Broeders et al., 2014). Thus, the real-time PCR developed in this study was confirmed to be highly accurate and efficient.

**Monitoring of Probiotic and Dairy Products Using the Real-Time PCR Developed**

Commercially available probiotic and dairy products were used to verify whether the real-time PCR developed in this study could be applicable to quantify and identify probiotics in food products (Supplementary Figure S1). A total of 33 commercial probiotic and dairy products containing \textit{Bifidobacterium} were monitored. Obtained results were compared with product label claims. Results of 21 products were identical to their label claims. In particular, probiotic strains of eight products that were only labeled at the species level such as \textit{B. longum} and \textit{B. animalis} were able to be analyzed up to subspecies level using our real-time PCR assay (Table 5). For the remaining four products (B4 to B7) labeled as “Lactic acid bacteria or \textit{Bifidus},” this real-time PCR assay was able to detect \textit{Bifidobacterium} at the subspecies level. Based on the standard quantitative curve for each \textit{Bifidobacterium} species or subspecies obtained by plotting \textit{Ct} values against the number of bacteria per reaction, the number of \textit{Bifidobacterium} species or subspecies present in the food products was estimated to be within the range of $8 \times 10^5$ to $8 \times 10^9$ CFU/mL. Thus, the real-time PCR method developed in this study could accurately detect and quantify \textit{Bifidobacterium} strains contained in probiotic and dairy products at species level and subspecies level.

**DISCUSSION**

\textit{Bifidobacterium} subspecies (\textit{B. animalis} subsp. \textit{animalis} or \textit{B. animalis} subsp. \textit{lactis} and \textit{B. longum} subsp. \textit{longum} or
**TABLE 4 | Primer information used in this study.**

| Target species                  | Primer name  | Sequence (5′-3′)                  | Size (bp) |
|---------------------------------|--------------|-----------------------------------|-----------|
| B. animalis subsp. animalis     | Animalis-F   | CAG ACC TCG CCG ATG AGC TA        | 110       |
|                                 | Animalis-R   | ATA TCC GGC TTG ATG ACC TG        |           |
| B. animalis subsp. lactis      | Lactis-F     | ACC TCA CCA ATC GGC TGT TC        | 137       |
|                                 | Lactis-R     | GAT CCG CAT GGT GGA ACT CT        |           |
| B. breve                        | Breve-F      | TCA TCA CGG CAA GGT CAA GA        | 111       |
|                                 | Breve-R      | GCC CAG AAC AGC TGG AAC AA        |           |
| B. longum subsp. infantis      | Infantis-F   | ATG ATG GGC TGC CAC CTA TG       | 132       |
|                                 | Infantis-R   | CGG TGA CCG TCA ATG TAT CT        |           |
| B. longum subsp. suis          | Suis-F       | CAA GCC GGA TAT GCT CTT TG        | 130       |
|                                 | Suis-R       | GAG GAT GCT GGC ATG CTG TC        |           |
| B. longum subsp. longum        | Longum-F     | GTG TGG ATT ACC TGC CTA TG C       | 179       |
|                                 | Longum-R     | GTG GCC AAC TCT TGG AAC CT        |           |
| B. bifidum                      | Bifidum-F    | CGG TCA CGG TCA ATG CTA TC        | 102       |
|                                 | Bifidum-R    | TGA ACT GCC CTA CTA CTA CCA TA    |           |
| B. gallicum                     | Gallicum-F   | TCA CCA TCA CCA CCT CAC           | 182       |
|                                 | Gallicum-R   | GGT CCA TTG TCA CCA CCA C       |           |
| B. thermacidophilum             | Thermacidophilum-F | CTT TCA CCA CCA ACC ACC AG   | 116       |
|                                 | Thermacidophilum-R | GCC GCC GCA ATT GCC AC ACC AC    |           |
| B. thermophilum                 | Thermophilum-F | CGG ATG CCG ATG CAG ATG AA       | 109       |
|                                 | Thermophilum-R | TGT CAT CCG ACG CTT CCA GA       |           |
| B. coryneforme                  | Coryneforme-F | TAA ATT CGT CCC CGG TTT GC        | 144       |
|                                 | Coryneforme-R | TCC TCA TCC TCC TCC ACA ATT ACC |           |
| B. asteroides                   | Asteroides-F | GCC GTG GTC ACC ACA CTA TC        | 130       |
|                                 | Asteroides-R | GGG CAC TAT GTC ATT CTA TG        |           |
| B. adolescentis                 | Adolescents-F | GCT GAT TGC TGC GCT GTA CC       | 135       |
|                                 | Adolescents-R | AAA CCA COG AGT AGC CCT CC       |           |
| B. pseudolongum                 | Pseudolongum-F | CAA GCC CAT CAA CTG GTG CA       | 120       |
|                                 | Pseudolongum-R | ACG TCG TGC TGC TGC TAT C       |           |
| B. cuniculi                     | Cuniculi-F   | TGA AGG AAA CAC CGA CCA TC        | 127       |
|                                 | Cuniculi-R   | ACC TCC TCC TCA GGC TTG AC       |           |
| B. gallinarum                   | Gallinarum-F | CGA CGA AAC ATT AGC CAT CC        | 163       |
|                                 | Gallinarum-R | ATG AAA TCC ACT TGC CCA CC        |           |
| B. scardovii                    | Scardovii-F  | CGC AGG CAC TGC CTA TAC CA       | 102       |
|                                 | Scardovii-R  | GCC GTA AGC TCT CAG TAC CA       |           |
| B. pseudocatenulatum            | Pseudocatenulatum-F | ACC TAC GAT TTG TCC TCT TCC      | 173       |
|                                 | Pseudocatenulatum-R | CTC CAG CAA AGG CAA CGA AC       |           |
| B. angulatum                    | Angulatum-F  | TGC GGA TAC CAT CGA AGA AC        | 101       |
|                                 | Angulatum-R  | TGC GGA ACA CCA ACC ATG TG C      |           |
| B. dentium                      | Dentium-F    | GCC ACC GCT TCC ATT AT            | 123       |
|                                 | Dentium-R    | GGA GAT GGC TGC CTT AGA TT        |           |
| B. tsurumiense                  | Tsurumiense-F | TGC GGT TCA ACC AAG CTT AC        | 167       |
|                                 | Tsurumiense-R | TCG TCG TCA CCA GAT TCT C        |           |
| B. catenulatum                  | Catenulatum-F | GCC CAA CGC AGT AGT CTA TA        | 106       |
|                                 | Catenulatum-R | TAG GGC ACC TGG ATG CTA TA       |           |

*B. longum subsp. infantis* are known to be similar to each other. However, these subspecies have different functions such as having ability to grow in milk or expressing enzymes (Masco et al., 2004). To distinguish these species or subspecies, previous studies have targeted marker genes such as 16S rRNA and tuf genes. However, it is difficult to distinguish subspecies by using these genes because of their highly similar sequences (Tannock et al., 2013; Kurakawa et al., 2015). Some researchers have screened specific genes through genomic analysis to distinguish *Bifidobacterium* subspecies. Lawley et al. (2017) have reported the identification of functional gene targets for the differentiation of *B. longum* subsp. *longum* and *B. longum* subsp. *infantis* based on comparative genomic analysis. However, these functional genes they identified showed some limitations. For example, *B. longum* subsp. *infantis* specific sialidase gene (accession no. ACJ53406.1) was limited to some *B. longum* subsp. *infantis* strains, but not all subspecies. It was also found to be present in *B. bifidum*. In addition, *B. longum* subsp. *longum* specific
FIGURE 3 | Specificity of species- and subspecies-specific primer pairs which were mainly used in probiotic products against 41 strains. (A) Specificity of B. animalis subsp. lactis specific primer pair, amplification curve: B. animalis subsp. lactis KACC 16638, LI 001941, LI 001942, LI 000026, LI 000004, LI 000019, and LI 000062; (B) Specificity of Bifidobacterium bifidum specific primer pair, amplification curve: B. bifidum KCTC 3418, KCTC 3440, LI 000058, LI 000061, and LI 000063; (C) Specificity of Bifidobacterium breve specific primer pair, amplification curve: B. breve KACC 16639, KCTC 3419, and LI 000070; (D) Specificity of B. longum subsp. infantis specific primer pair, amplification curve: B. longum subsp. infantis KCTC 3249, LI 000033, LI 000261, and LI 000262.

kinase gene (accession no. AAN24115.1) was present in many Bifidobacterium species such as B. adolescentis and B. dentium. Because of the limited number of genomes (n = 2) used in their analysis, these identified genes could not be applied to distinguish all Bifidobacterium species.

To overcome limitations of previous studies, we identified genetic markers with large-scale Bifidobacterium genome sequences (n = 210). All genetic markers obtained through comparative genomic analysis were confirmed to be specific by in silico analysis. We also confirmed that some genomes deposited in NCBI were misclassified. Previous studies have also reported that taxonomy information for similar species in the NCBI is incorrect (Kim et al., 2020). For Bifidobacterium, this is the first report to confirm the incorrect classification of genomes in NCBI. Inaccuracies of genomic information may contribute to difficulty in developing methods to distinguish Bifidobacterium. Our results suggest that B. longum subsp. infantis CCUG 52486 and 157F are B. longum subsp. longum.

The real-time PCR method developed in this study showed high specificity and accuracy. However, the limited information of some species, such as Bifidobacterium coryneforme, Bifidobacterium cuniculi, and B. longum subsp. suis were available in the NCBI (only one or two representatives), thus, we can only include the small number of genomes for those strains. This method was also successfully applied to monitoring of probiotic products. It correctly identified Bifidobacterium species contained in all products. We were also able to analyze these strains up to subspecies level labeled in probiotic products as B. animalis and B. longum, allowing us to better understand the presence of strains contained in probiotic products. A previous study (Patro et al., 2016) using shotgun next-generation sequencing has shown that nine out of ten probiotic products are consistent with their label claims. One product, which was misidentified, contained B. longum subsp. longum instead of B. longum subsp. infantis. They found that these strains were frequently mislabeled in other products.
FIGURE 4 | Real-time PCR amplification plots and standard curves which were mainly used in probiotic products for quantitative evaluation. (A) B. animalis subsp. lactis amplification plot (left), standard curve between 20 and 0.002 ng ($y = -3.564x + 18.03$, $R^2 = 0.998$, Eff% = 90.788, right); (B) B. bifidum amplification plot (left), standard curve ($y = -3.438x + 17.713$, $R^2 = 0.998$, Eff% = 95.359, right); (C) B. breve amplification plot (left), standard curve ($y = -3.448x + 18.169$, $R^2 = 0.998$, Eff% = 94.987, right); (D) B. longum subsp. infantis amplification plot (left), standard curve ($y = -3.312x + 17.727$, $R^2 = 0.998$, Eff% = 100.424, right).
| Products | Label claim | Detected species or subspecies |
|----------|-------------|--------------------------------|
| A1       | B. longum   | B. longum subsp. longum        |
| A2       | B. bifidum, B. longum | B. bifidum, B. longum subsp. longum |
| A3       | B. animalis subsp. lactis | B. animalis subsp. lactis |
| A4       | B. animalis subsp. lactis | B. animalis subsp. lactis |
| A5       | B. animalis subsp. lactis | B. animalis subsp. lactis |
| A6       | B. animalis subsp. lactis | B. animalis subsp. lactis |
| A7       | B. bifidum, B. breve, B. longum | B. bifidum, B. longum subsp. longum |
| A8       | B. animalis subsp. lactis, B. bifidum | B. animalis subsp. lactis, B. bifidum |
| A9       | B. animalis subsp. lactis, B. bifidum | B. animalis subsp. lactis, B. bifidum |
| A10      | B. breve, B. longum subsp. longum | B. breve, B. longum subsp. longum |
| A11      | B. animalis subsp. lactis, B. bifidum, B. breve | B. animalis subsp. lactis, B. bifidum, B. breve |
| A12      | B. breve, B. longum, B. longum subsp. infantis | B. breve, B. longum subsp. longum, B. longum subsp. infantis |
| A13      | B. animalis subsp. lactis, B. breve, B. longum | B. animalis subsp. lactis, B. breve, B. longum subsp. longum |
| A14      | B. animalis subsp. lactis, B. bifidum, B. breve | B. animalis subsp. lactis, B. bifidum, B. breve |
| A15      | B. animalis subsp. lactis, B. bifidum, B. breve | B. animalis subsp. lactis, B. bifidum, B. breve |
| A16      | B. animalis subsp. lactis, B. bifidum, B. breve | B. animalis subsp. lactis, B. bifidum, B. breve |
| A17      | B. animalis subsp. lactis, B. bifidum, B. breve, B. longum | B. animalis subsp. lactis, B. bifidum, B. breve, B. longum subsp. longum |
| A18      | B. animalis subsp. lactis, B. bifidum, B. breve, B. longum | B. animalis subsp. lactis, B. bifidum, B. breve, B. longum subsp. longum |
| A19      | B. animalis subsp. lactis, B. bifidum, B. breve, B. longum | B. animalis subsp. lactis, B. bifidum, B. breve, B. longum subsp. longum |
| A20      | B. animalis subsp. lactis, B. bifidum, B. breve, B. longum | B. animalis subsp. lactis, B. bifidum, B. breve, B. longum subsp. longum |
| A21      | B. animalis subsp. lactis, B. bifidum, B. breve, B. longum subsp. longum | B. animalis subsp. lactis, B. bifidum, B. breve, B. longum subsp. longum |
| A22      | B. animalis subsp. lactis, B. bifidum, B. breve, B. longum subsp. longum, B. longum subsp. infantis | B. animalis subsp. lactis, B. bifidum, B. breve, B. longum subsp. longum, B. longum subsp. infantis |
| A23      | B. animalis subsp. lactis, B. bifidum, B. breve, B. longum subsp. longum, B. longum subsp. infantis | B. animalis subsp. lactis, B. bifidum, B. breve, B. longum subsp. longum, B. longum subsp. infantis |
| A24      | B. animalis subsp. lactis, B. bifidum, B. breve, B. longum subsp. longum, B. longum subsp. infantis | B. animalis subsp. lactis, B. bifidum, B. breve, B. longum subsp. longum, B. longum subsp. infantis |
| A25      | B. animalis subsp. lactis, B. bifidum, B. breve, B. longum, B. longum subsp. infantis | B. animalis subsp. lactis, B. bifidum, B. breve, B. longum, B. longum subsp. infantis |
| A26      | B. animalis subsp. lactis, B. bifidum, B. breve, B. longum, B. longum subsp. infantis | B. animalis subsp. lactis, B. bifidum, B. breve, B. longum, B. longum subsp. infantis |
| B1       | B. animalis subsp. lactis | B. animalis subsp. lactis |
| B2       | B. animalis subsp. lactis | B. animalis subsp. lactis |
| B3       | B. animalis subsp. lactis | B. animalis subsp. lactis |
| B4       | Bifidus, Lactic acid bacteria | B. animals subsp. lactis |
| B5       | Bifidus, Lactic acid bacteria | B. animals subsp. lactis |
| B6       | Lactic acid bacteria | B. animals subsp. lactis |
| B7       | Lactic acid bacteria | B. animals subsp. lactis |
In conclusion, genetic markers were identified to distinguish different Bifidobacterium species and subspecies through comparative genomics based on their whole-genome sequences. Although Bifidobacterium species are commonly used in probiotic and dairy products, it is still difficult to distinguish all Bifidobacterium species by conventional detection methods. This study designed specific primers from these identified genetic markers. A real-time PCR assay was developed in this study to accurately and rapidly detect 22 Bifidobacterium in a single 96-well plate. The developed real-time PCR assay can be used to monitor commercial probiotic and dairy products. Our assay can also be used to verify the reliability of claims of probiotic and dairy products. Furthermore, it can be applied to identify Bifidobacterium communities in various food products and environmental samples.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

H-BK, EK, S-MY, and H-YK designed the experiment. H-BK, EK, and S-MY confirmed primer specificity and performed application tests using real-time PCR. H-BK, EK, and M-JK prepared a draft manuscript. H-BK, EK, SL, and H-YK reviewed and edited the manuscript. All authors read and approved the final manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.02087/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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