Development and application of tuf gene-based PCR and PCR-DGGE methods for the detection of 16 Bifidobacterium species

Sen-Je Sheu a, Hsin-Chih Chen a, Chien-Ku Lin b, Wen-Hsin Lin c, Yu-Cheng Chiang b, Wen-Zhe Huang a, Hau-Yang Tsen b,*

a Department of Food Science and Biotechnology, National Chung Hsing University, Taichung, Taiwan, ROC
b Department of Food Science and Technology, Hungkuang University, Taichung, Taiwan, ROC
c School of Pharmacy, China Medical University, Taichung, Taiwan, ROC

Abstract

A total of 16 Bifidobacterium species were assayed by polymerase chain reaction (PCR) and PCR—denaturing gradient gel electrophoresis (PCR–DGGE) methods targeted on a 770-bp region of the tuf gene. Based on this sequence, a genus-specific primer set and 12 primer sets for 12 Bifidobacterium species including those previously reported for six probiotic species were developed. On the other hand, when these 16 Bifidobacterium species were subjected to PCR–DGGE analysis, 13 product migration patterns were obtained. PCR products for strains in pairs of B. adolescentis/B. thermophilum, B. longum/B. magnum, and B. lactis/B. gallicum migrated the same distance on the DGGE gel. Combined with species-specific PCR primers specific to B. adolescentis, B. longum, and B. lactis, all of the 16 Bifidobacterium species could be identified. In addition, the subspecies of B. animalis, i.e., B. animalis and B. lactis, could be discriminated. This study indicated that the tuf gene is highly useful for the molecular detection of different Bifidobacterium species. Using the PCR and PCR–DGGE methods, 16 Bifidobacterium species, including those from probiotic products and those from other origins, could be rapidly identified.

1. Introduction

Rapid methods for the detection of Bifidobacterium strains with probiotic functions, such as B. animalis subsp. lactis (B. lactis), B. bifidum, B. breve, B. infantis, and B. longum subsp. longum (B. longum), have attracted the interest of many researchers. On the other hand, for Bifidobacterium species other than the commonly used probiotic species, such as B. adolescentis, B. cuniculi, B. gallicum, B. globosum, B. minimum, and B. subtilis, rapid methods, such as PCR and real-time PCR, for their detection are, thus far, limited. These methods can be used for the rapid screening and survey of Bifidobacterium species from different animals and environments.

For the molecular detection of Bifidobacterium species other than probiotic species, Nebra et al [1] developed DNA probes based on 16S rDNA sequence for the detection of B. dentium, B. animalis, B. astroides, B. coryneforme, B. cuniculi, B. globosum, B. magnum, B. minimum, and B. subtilis. However, only two probes were developed for these nine Bifidobacterium spp. The BDE probe is specific to B. dentium while the BAN probe cannot differentiate among the rest. On the other hand, using species-specific amplified ribosomal DNA restriction analysis, Ventura et al [2] discriminated among 16 Bifidobacterium spp., including probiotic isolates and isolates from different environments.

For molecular detection methods, rRNA and internal transcribed spacer genes have been the most widely used.
targets. However, the high degree of similarity between 16S rDNA sequences of closely related species makes it difficult to develop highly specific primers or probes for different species within the same genus. In addition, the divergent 16S rDNA sequences among \textit{rrn} operons of a single organism remain problematic [3–5]. Recently, several mono-copy target genes, such as \textit{tuf} [6], \textit{Idh} [7] and \textit{hsp} 60 genes [8], revealed high divergence in LAB species and might be used as alternative molecular markers.

Denaturing gradient gel electrophoresis (DGGE) is a technique used for the resolution of DNA fragments of the same size but with different sequences by the different endurance of the DNA fragments to the denaturant concentration [9]. Polymerase chain reaction (PCR) combined with DGGE has proven to be a useful method for the investigation of complex microbial populations without previous separation of the individual inhabitants [7,10,11]. Recently, DGGE has been employed in monitoring the microbial population dynamic of dairy products and has provided fast and reliable data [9]. For example, based on the 16-23S rRNA region, Hong and Chen [12] have developed species-specific PCR and DGGE methods for the identification of bifidobacteria in dairy products. For \textit{B. indicum}, Kopecný et al [13] combined 16S rDNA-based DGGE with real-time PCR for the detection of this species.

Since Ventura et al [6] analyzed the \textit{tuf} gene for 17 \textit{Lactobacillus} and 8 \textit{Bifidobacterium} species including \textit{B. longum} subsp. \textit{longum}, \textit{B. longum} subsp. \textit{infantis}, \textit{B. bifidum}, \textit{B. animalis} subsp. \textit{lactis}, \textit{B. catenulatum}, \textit{B. adolescentis}, \textit{B. breve}, and \textit{B. animalis} subsp. \textit{animalis}, and demonstrated that the \textit{tuf} gene is a reliable molecular clock for investigating evolutionary distances of lactobacilli and bifidobacteria, previously, we designed six PCR primer sets based on a 770-bp sequence of the \textit{tuf} gene for the detection of six commonly used probiotic species of \textit{Bifidobacterium} including \textit{B. longum}, \textit{B. animalis} subsp. \textit{animalis}/\textit{B. animalis} subsp. \textit{lactis}, \textit{B. bifidum}, \textit{B. breve}, and \textit{B. infantis} [14].

In this study, in an attempt to develop more primers for the detection of more \textit{Bifidobacterium} spp., not only those obtained from probiotic products but also those present in different animals and from different environmental origins, \textit{tuf} gene-based PCR and PCR–DGGE methods were employed for the detection of 16 \textit{Bifidobacterium} species we collected for this study. The advantages of utilizing the \textit{tuf} gene to discriminate among a number of \textit{Bifidobacterium} species include the high divergence present in the \textit{tuf} gene sequence and the superior resolution capability of PCR–DGGE to distinguish between even a few nucleotide differences. Such a study would also demonstrate that the \textit{tuf} gene is an ideal target for the molecular detection of \textit{Bifidobacterium} spp.

\begin{table}[h]
\centering
\caption{Reference bacterial strains used in this study}
\begin{tabular}{llll}
\hline
Species & Source & Species & Source \\
\hline
\textit{Bifidobacterium} \textit{adolescentis} & BCRC 14607 & \textit{Lb.} \textit{johnsonii} & BCRC 17474 \\
\textit{B. animalis} subsp. \textit{animalis} & BCRC 14668 & \textit{Lb.} \textit{murinus} & BCRC 14020 \\
 & CCUG 48185 & \textit{Lb.} \textit{paracasei} & BCRC 12248 \\
\textit{B. animalis} subsp. \textit{lactis} & BCRC 17394 & \textit{Lb.} \textit{pentosus} & BCRC 11503 \\
 & CCUG 33397, 37979 & \textit{Lb.} \textit{plantarum} & BCRC 10069 \\
\textit{B. bifidum} & BCRC 11844, 14613 & \textit{Lb.} \textit{reuteri} & BCRC 14625 \\
\textit{B. boum} & BCRC 14677 & \textit{Lb.} \textit{ruminis} & BCRC 14620 \\
\textit{B. breve} & BCRC 14632 & \textit{Lb.} \textit{rhamnosus} & BCRC 12094 \\
\textit{B. cuniculi} & BCRC 14672 & \textit{Lb.} \textit{salivarius} subsp. \textit{salicinius} & BCRC 12574 \\
\textit{B. gallinarum} & BCRC 14679 & \textit{Lb.} \textit{zea} & BCRC 17259 \\
\textit{B. globosum} & BCRC 14663 & \textit{Enterococcus} \textit{avium} & BCRC 14728 \\
\textit{B. indicum} & BCRC 14674 & \textit{E.} \textit{durus} & BCRC 10790 \\
\textit{B. longum} subsp. \textit{infantis} & BCRC 14602 & \textit{E.} \textit{faecalis} & BCRC 12298 \\
\textit{B. longum} subsp. \textit{longum} & BCRC 11847, 14664 & \textit{E.} \textit{faecium} & BCRC 10067 \\
\textit{B. magnus} & BCRC 14676 & \textit{E.} \textit{galilharum} & BCRC 15477 \\
\textit{B. minimum} & BCRC 14666 & \textit{E.} \textit{casseliflavus} & BCRC 14926 \\
\textit{B. subtilis} & BCRC 14660 & \textit{Streptococcus} \textit{thermophilus} & BCRC 12257 \\
\textit{B. thermophilum} & BCRC 14669 & \textit{Bacillus} \textit{cereus} & BCRC 10603 \\
\textit{Lactobacillus} \textit{acidophilus} & BCRC 10695 & \textit{Brevibacterium} \textit{linens} & BCRC 10029 \\
\textit{Lb.} \textit{agilis} & BCRC 12931 & \textit{Carnobacterium} \textit{dversgens} & BCRC 14042 \\
\textit{Lb. amylovorus} & BCRC 11648 & \textit{Citrobacter} \textit{frendii} & BCRC 12292 \\
\textit{Lb. brevis} & BCRC 12187 & \textit{Enterobacter} \textit{aerogenes} & BCRC 10370 \\
\textit{Lb. casei} & BCRC 10697 & \textit{Escherichia} \textit{coli} & BCRC 12653 \\
\textit{Lb. crispatus} & BCRC 14618 & \textit{Lactococcus} \textit{latis} \textit{subsp.} \textit{latis} & BCRC 14041 \\
\textit{Lb. delbrueckii subsp. delbrueckii} & BCRC 12195 & \textit{Leucosostoc} \textit{mesenteroides} & BCRC 14047 \\
\textit{Lb. faciminis} & BCRC 14043 & \textit{Listeria monocytogenes} & BCRC 14848 \\
\textit{Lb. fermentum} & BCRC 12190 & \textit{Pseudomonas} \textit{cepacia} & ATCC 25416 \\
\textit{Lb. gasseri} & BCRC 14619 & \textit{Salmonella} \textit{typhimurium} & ATCC 14028 \\
\textit{Lb. helveticus} & BCRC 12936 & \textit{Sporalactobacillus} \textit{inulin} & BCRC 14647 \\
\textit{Lb. jensenii} & BCRC 12939 & \textit{Staphylococcus} \textit{aureus} & BCRC 10780 \\
\textit{Lb. johnsonii} & BCRC 14607 & \textit{Yersinia} \textit{enterocolitica} & BCRC 10807 \\
\textit{Lb. johnsonii} & BCRC 14607 & \textit{Yersinia} \textit{enterocolitica} & BCRC 10807 \\
\hline
\end{tabular}
\end{table}

ATCC = American Type Culture Collection, Manassas, Virginia, USA; BCRC = Bioresources Collection and Research Center, Hsinchu, Taiwan; CCUG = Culture Collection, University of Go¨teborg, Sweden.
2. Methods

2.1 Bacterial strains and culture conditions

The bacterial strains used in this study and their sources are listed in Table 1. LAB were cultured in deMan Rogosa Sharpe (MRS) (Merck, Darmstadt, Germany) broth containing 0.05% L-cysteine hydrochloride at 37 °C for 24 hours under anaerobic conditions (BBL GasPak; Becton Dickinson and Co., Cockeysville, MD, USA). Strains other than LAB were grown aerobically.

2.2 Preparation of DNA for PCR assays

Bacterial genomic DNAs were prepared using the Blood & Tissue Genomic DNA Extraction Miniprep System for Bacteria (Viogene, Taipei, Taiwan) according to the methods described by Sheu et al [14].

2.3 PCR primers and amplification conditions

Primers for the PCR detection of *Bifidobacterium* spp. (Table 2) were designed by multiple alignment of 770-bp *tuf* gene sequences using the Clustal W program and compared with other sequences retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov) using blast program. The 770-bp *tuf* gene sequences for some *Bifidobacterium* spp., such as those of *B. boum*, *B. cuniculi*, *B. gallinarum*, *B. globosum*, *B. indicum*, *B. magnun*, *B. minimum*, *B. subtilis* and *B. thermophilum*, were those retrieved from our previous report with accession numbers from FJ549338 to FJ549355 [14]. The specificity of the primers was then confirmed by PCR assay with DNAs from *Bifidobacterium* and non-*Bifidobacterium* strains (Table 1). PCR conditions were as those described by Sheu et al [14], except for the use of different annealing temperatures as shown in Table 2.

2.4 DGGE analysis of PCR products

The 339-bp sequence located within the 770-bp region of the *tuf* gene was amplified using *Bif_tuf_F* and *Bif_tuf_R_GC* primers (*Bif_tuf_R_GC* modified by the addition of a GC clamp, i.e., CCAGCCGCCGCGCGCGCGCGCGGACGGGGGCAACCGGGGTGGAAGTGCTCGATGGAG to 5' position) and 5 μL of the PCR product was subjected to DGGE. DGGE was performed with the DCode™ Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA) utilizing 16-cm by 16-cm by 1-mm gels. For the PCR–DGGE of the *tuf* gene, separation of the amplicons was obtained with 6.4% (w/v) polyacrylamide gels containing 50–65% of denaturant gradient in the direction of electrophoresis. A 100% denaturant corresponds to 40% (v/v) formamide (Sigma-Aldrich, St. Louis, MO, USA) and 7.0 M urea (Amresco, Solon, OH, USA). Electrophoresis was performed with a constant voltage of 130 V at 60 °C for 6.5 hours in 1 × TAE buffer. After electrophoresis, the gel was

<table>
<thead>
<tr>
<th>Table 2 – Specific primers used in this study</th>
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<tr>
<td>Species</td>
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<tr>
<td>B. adolescens</td>
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<tr>
<td>B. adolescens</td>
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<tr>
<td>B. animalis/B. lactis</td>
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<tr>
<td>B. animalis/B. lactis</td>
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<tr>
<td>B. bifidum</td>
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<td>B. breve</td>
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<td>B. cuniculi</td>
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<td>B. cuniculi</td>
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<td>B. gallinarum</td>
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<td>B. gallinarum</td>
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<td>B. globosum</td>
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<td>B. globosum</td>
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<td>B. indicum</td>
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<td>B. indicum</td>
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<td>B. infantis</td>
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<td>B. infantis</td>
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<td>B. longum</td>
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<tr>
<td>B. longum</td>
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<td>B. minimum</td>
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<td>B. minimum</td>
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<tr>
<td>B. subtilis</td>
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<td>B. subtilis</td>
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a The accession numbers of the *tuf* gene used were obtained from the GenBank database.
stained with ethidium bromide (5 μg/mL in 1 x TAE buffer) for 5 minutes and visualized under UV light.

2.5. Evaluation of the PCR–DGGE sensitivity of Bifidobacteria spiked in milk samples

To evaluate the sensitivity of PCR–DGGE, B. lactis BCRC 17394 and B. longum BCRC 11847 were used as reference strains. Bacterial cells were serially diluted to N x 10^1, 10^2, 10^3, and 10^4 colony-forming units (cfu) per 10 μL (N = 1–9) with sterile water, respectively. Then, 10 μL of each cell suspension was spiked to 1 mL of pasteurized whole milk, respectively. For DNA extraction, 0.1 mL of the spiked milk was mixed with 0.9 mL of TE buffer and vortexed for 30 seconds. Cells were collected (7000g for 5 minutes) and washed again with TE buffer. Total DNA was extracted using phenol-chloroform method described earlier [14]. Finally, the DNA obtained was suspended in 10 μL of double deionized water, and then 10 μL of the DNA solution was subjected to PCR. Five μL of the PCR products was subjected to DGGE.

2.6. Comparison of PCR and PCR–DGGE for the detection of Bifidobacterium species in probiotic products

Six probiotic products including three yogurt and three lyophilized products purchased from local supermarkets were used as samples. After purchase, these products were stored at 4 °C and assayed immediately. In general, three samples of each product were assayed. The total counts of LAB and Bifidobacterium in these products were determined by counting the number of cells in 1 mL of the serial dilutions cultured on MRS and the Bifidobacterium iodoacetate medium 25 (BIM-25) agar plate [15], respectively. For PCR–DGGE, 0.1 g of sample was mixed with 1 mL of TE buffer and vortexed for 30 seconds. Cells were collected (7000g, 5 minutes) and washed again with TE buffer. Then, total DNA was extracted according to the procedures described earlier. Afterwards, 10 μL of DNA was subjected to PCR followed by DGGE analysis. As for PCR, the conditions described by Sheu et al [14] were used.

3. Results

3.1. Specificity for the PCR detection of seven Bifidobacterium species

Based on 770-bp tuf gene sequences, we have previously designed one genus-specific primer set and five primer sets for six common probiotic species and subspecies of Bifidobacterium. In this study, seven more primer sets were designed for the specific detection of B. adolescentis, B. cuniculi, B. gallinarum, B. globosum, B. indicum, B. minimum, and B. subtilis, respectively (Table 2, Fig. 1). This made up a total of 12 primer sets that allowed the detection of 13 Bifidobacterium species and subspecies. The specificity of each of these primer sets was determined by assay with 64 bacterial strains as listed in Table 1. Strains other than the target organisms did not generate any false-positive results.

3.2. PCR–DGGE detection of Bifidobacterium species and the detection limit

After PCR amplification with Bif_tuf_F/Bif_tuf_R_GC primers, the PCR products were subjected to DGGE analysis directly. The 16 Bifidobacterium species used in this study generated 13 product migration patterns (Fig. 2). All of the Bifidobacterium species could be clearly discriminated except for six species in three pairs: B. adolescentis/B. thermophilum, B. longum/B. magnum and B. lactis/B. gallinarum. Although the species in each of these pairs could not be discriminated by the PCR–DGGE method, through the combined use of primers specific to B. adolescentis, B. longum and B. gallinarum, three Bifidobacterium species (B. thermophilum, B. magnum and B. lactis) could be identified. The two subspecies of B. animalis, i.e., B. animalis and B. lactis, could not be discriminated by PCR [14] but could be identified by the combined use of PCR–DGGE and species-specific PCR (Fig. 2).

B. lactis and B. longum, both common probiotic species, were used to evaluate the detection limits of PCR–DGGE. For viable bifidobacteria, the detection limits of the PCR–DGGE method for these two species were N x 10^3 and N x 10^4 cfu/mL or cfu/g of milk sample, respectively (Fig. 3), which are similar to those obtained by Temmerman et al [16] who used 16S rRNA-based PCR–DGGE. For these strains, when PCR was used, the detection limit was N x 10^3 cfu/mL of sample [14]. These detection limits are generally below the levels of viable bifidobacterial cells (>10^6 cfu/mL, Table 3) in commercial probiotic products. Therefore, the Bifidobacterium species in these products could be identified without the preculture step. For the PCR and PCR–DGGE detection of Bifidobacterium species in non-probiotic samples, such as those from the gastrointestinal or feces of animals, fecal-polluted water, sewage
and other environmental samples [6,13,17], a pre-enrichment step may be required.

3.3. PCR and PCR–DGGE analyses of the probiotic products

Six probiotic products available in the market (3 yogurts and 3 powdered products) were assayed for the viable counts of LAB and bifidobacteria. Total viable counts of LAB determined in these samples were $10^8$–$10^9$ cfu/mL or cfu/g respectively, except for sample no. 6 which contained $10^5$–$10^6$ cfu/g of LAB.

The total counts of bifidobacteria for samples 1, 2 and 3 were $10^4$–$10^5$ cfu/mL. For sample no. 1, the bifidobacteria counts were close to those stated on the label of the product (Table 3). For products not labeled with bifidobacteria counts and species, i.e., sample no. 5 and no. 6, the viable counts of Bifidobacterium spp. were not determined due to the presence of cocci on BIM-25 agar. For these samples, our PCR–DGGE and PCR results indicated the presence of B. animalis (B. lactis and B. animalis) could be discriminated by the combined use of PCR and PCR–DGGE.

![Fig. 2](image2) PCR–DGGE analysis of the tuf gene amplicons from different Bifidobacterium species with genus-specific primers. Lanes a and i = reference ladders of 13 Bifidobacterium species; lanes b–h and j–o = B. infantis BCRC 14602, B. globosum BCRC 14663, B. lactis BCRC 17394, B. bifidum BCRC 11844, B. animalis BCRC 14668, B. breve BCRC 14632, B. minimum BCRC 14666, B. cuniculi BCRC 14672, B. longum BCRC 11847, B. boum BCRC 14677, B. subtilis BCRC 14660, B. indicum BCRC 14674 and B. adolescentis BCRC 14607, respectively.

![Fig. 3](image3) Detection limits for PCR–DGGE. Lanes a and g = reference ladders of Bifidobacterium species; lanes b–e and h–k = PCR–DGGE run from 10-fold dilutions ($N \times 10^6$, $10^5$, $10^4$ and $10^3$ cells/mL, respectively) of B. animalis subsp. lactis BCRC 17394 and B. longum subsp. longum BCRC 11847 in whole milk; lanes f and l = negative control.
Commercial probiotic products assayed in this study by conventional and molecular methods

<table>
<thead>
<tr>
<th>Product no.</th>
<th>Species and cell numbers (log cfu/mL or cfu/g) labeled</th>
<th>Count of total viable LAB (log cfu/mL) determined a</th>
<th>Count of total viable bifidobacteria (log cfu/mL) determined a</th>
<th>Detection of Bifidobacterium by PCR–DGGE</th>
<th>Species-specific PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 1 Yogurt</td>
<td>B. lactis (&lt;6.00) Total LAB &gt;8.00</td>
<td>8.51 ± 0.23</td>
<td>6.15 ± 0.21</td>
<td>B. lactis/B. gallinarum B. lactis/B. animalis</td>
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<tr>
<td>No. 2 Yogurt</td>
<td>L. acidophilus B. longum L. bulgaricus S. thermophilus Total LAB &gt;8.00</td>
<td>8.22 ± 0.14</td>
<td>5.93 ± 0.18</td>
<td>B. lactis/B. gallinarum B. lactis/B. animalis</td>
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<tr>
<td>No. 3 Yogurt</td>
<td>L. acidophilus B. longum L. bulgaricus S. thermophilus Total LAB &gt;8.00</td>
<td>8.12 ± 0.18</td>
<td>4.68 ± 0.11</td>
<td>B. lactis/B. gallinarum B. lactis/B. animalis</td>
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<tr>
<td>No. 4 Lyophilized product</td>
<td>L. acidophilus L. paracasei B. lactis E. faecium Total LAB &gt;9.62</td>
<td>9.75 ± 0.16</td>
<td>—</td>
<td>B. lactis/B. gallinarum B. lactis/B. animalis</td>
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<tr>
<td>No. 5 Lyophilized product</td>
<td>Bifidobacterium spp. Total LAB &gt;9.48</td>
<td>8.33 ± 0.31</td>
<td>—</td>
<td>B. lactis/B. gallinarum B. lactis/B. animalis</td>
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<tr>
<td>No. 6 Lyophilized product</td>
<td>ND* Total LAB &gt;6.00</td>
<td>5.44 ± 0.28</td>
<td>—</td>
<td>B. lactis/B. gallinarum B. lactis/B. animalis</td>
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</table>

a The viable counts were determined by MRS or BIM-25 agar; ND = LAB species were not declared on the label; — = the presence of the bacteria species or cell numbers were not determined.

4. Discussion

Many studies have shown that 16S rRNA gene-based PCR–DGGE is useful for the identification of LAB in probiotic products [10–12,16]. However, due to the multiple, heterogeneous rDNA operons, more than one band may appear for a single species after migration on the DGGE gel [10,11]. This makes it complicated and may interfere with the PCR–DGGE analysis for the identification of bacterial species. Based on a 770-bp region of the tuf gene, we designed 12 species-specific primer sets and one genus-specific primer for the identification of 13 Bifidobacterium species. Combined with the use of PCR–DGGE, all of the 16 Bifidobacterium spp. including the subspecies we collected for this study could be detected.

With regard to the detection specificity of our methods, 21 reference strains of 16 Bifidobacterium species and subspecies and 43 reference strains representing 43 non-Bifidobacterium species collected from culture collection centers, i.e., BCRC, ATCC and CCUG (Table 1), were used for assay. Although it was difficult for us to collect high numbers of different strains representing different Bifidobacterium species for this study, the detection of each target species, such numbers of Bifidobacterium species and non-Bifidobacterium species we tested were higher than those reported by others for the evaluation of the PCR primers specific for Bifidobacterium species [18,19]. In addition, since the specificity of PCR depends on the primer sequence while PCR–DGGE allows for the resolution of amplified DNA fragments of the same size but different sequences, the combined use of PCR and PCR–DGGE would allow us to determine the species of the Bifidobacterium strains. Moreover, the combination of PCR and PCR–DGGE enabled us to discriminate between the subspecies of Bifidobacterium species, such as B. animalis and B. lactis (Table 3). As for the PCR primers, for some Bifidobacterium species, the sizes of the PCR products may be too close to allow for multiplex PCR detection. Under such conditions, singlet PCR with a specific primer for each target organism can be used. If multiplex PCR is to be used, the selection of primer sets that generate PCR products of different sizes may be necessary.

In conclusion, this study confirmed that the tuf gene is an ideal target for designing molecular methods to detect Bifidobacterium species. The PCR and PCR–DGGE methods described in this report offer an alternative for the investigation of 16 Bifidobacterium species including those commonly used in probiotic products and those isolated from other origins.

Acknowledgments

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References


