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Rapid Identification of Bifidobacteria in Dairy Products by Gene-targeted Species-specific PCR Technique and DGGE

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ABSTRACT : In this paper, a rapid and reliable gene-targeted species-specific polymerase chain reaction (PCR) technique based on a two-step process was established to identify bifidobacteria in dairy products. The first step was the PCR assay for genus *Bifidobacterium* with genus specific primers followed by the second step, which identified the species level with species-specific primer mixtures. Ten specific primer pairs, designed from nucleotide sequences of the 16-23S rRNA region, were developed for the *Bifidobacterium* species including *B. angulatum*, *B. animalis*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. infantis*, *B. longum*, *B. minimum*, *B. subtile*, and *B. thermophilum*. This technique was applied to the identification of *Bifidobacterium* species isolated from 6 probiotic products, and four different *Bifidobacterium* spp. (*B. bifidum*, *B. longum*, *B. infantis*, and *B. breve*) were identified. The findings indicated that the 16S-23S rDNA gene-targeted species-specific PCR technique is a simple and reliable method for identification of bifidobacteria in probiotic products. PCR combined with Denaturing Gradient Gel Electrophoresis (DGGE) for identification of the bifidobacteria was also evaluated and compared with the gene-targeted species-specific technique. Results indicated that for fermented milk products consistency was found for both species-specific PCR and PCR-DGGE in detecting species. However, in some lyophilized products, the bands corresponding to these species were not visualized in the DGGE profile but the specific PCR gave a positive result. (**Key Words :** *Bifidobacterium* spp., Species-specific PCR, DGGE)

INTRODUCTION

Members of the genus *Bifidobacterium* are Gram-positive, pleomorphic and strictly anaerobic bacteria, and major constituents of the human intestinal microflora as well as of other warm-blooded animals. It is believed that *Bifidobacterium* species are important in maintaining intestinal health because they contribute to a beneficial microbiota in the intestinal tract (Venema and Maathuis, 2003). Thus, many attempts have been made to increase the number of *Bifidobacterium* cells in the intestinal tract by supplying certain bifidobacteria strains or food ingredients that stimulate the growth of bifidobacteria (Vaughn and Mollet, 1999; Chen et al., 2004; Chen et al., 2005; Chen et al., 2006). Detecting and identifying various species of bifidobacteria with rapid method is often important for quality control of probiotic products.

Historically, species-specific identification methods for the genus *Bifidobacterium* have been based on carbohydrate fermentation profiles, cell-wall analysis or on their key enzyme fructose-6-phosphate phosphoketolase involved in hexose degradation (Scardovi and Trovatelli, 1965; Scardovi et al., 1979; Roy et al., 1994). All these methods are culture-dependent, labor-intensive and time consuming. Moreover, identification based on phenotypic traits does not always provide clear-cut results. A further disadvantage is the fact that not all microorganisms can be cultured on growth media, making it impossible to isolate and identify a significant number of microbial species (O'Sullivan, 1999). In recent years several molecular tools have been proposed for bifidobacterial identification without isolation and enumeration. Various polymerase chain reaction (PCR) methods have been developed using species-specific primers designed with wide-spread genes for lactic acid bacteria (Charteris et al., 1997; Tilsala-Timisjärvi and Alatossava, 1997; Ventura and Zink, 2002; Grand et al., 2003; Venema and Maatthiuis, 2003; Kwon et al., 2005; Kobayashi and El-Sawy, 2007). Nowadays, they mostly rely on amplification and comparison of the 16S rDNA derived primers allowed detection of *Bifidobacterium* spp. in fecal and food samples. The use of primers targeting different

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Table 1. Product label information of the different bifidobacteria products and identifications obtained by PCR-based DGGE and species-specific PCR

Product	Microorganisms declared on the labels	Bifidobacteria count (log CFU/ml)	PCR-DGGE	Specific PCR
Fermented milk products				
Bioyogurt AB	*B. longum*	8.3±0.2	*B. longum*	*B. longum*
Pro-life bifido	*B. bifidum*	8.5±0.4	*B. bifidum*	*B. bifidum*
High-quality	*B. longum*	7.8±0.3	*B. longum*	*B. longum*
Lyophilized products				
DOS-DOPHILUS	*B infantis*	5.1±0.4		*B infantis*
	B. longum		*B. longum*	*B. longum*
OLICO	*B. bifidum*	4.3±0.3		*B. bifidum*
	B. longum		*B. longum*	*B. longum*
Bio-Lac capsule	*B. breve*	4.6±0.1	*B. breve*	*B. breve*
	B. longum		*B. longum*	*B. longum*

Table 2. Reference strains used in this study and the nucleotide sequence accession numbers for the 16S-23S rRNA

Reference strains	Strain[a]	Accession No.
Bifidobacterium angulatum	ATCC 27535	M84775
Bifidobacterium animalis	ATCC 25527	U09858
Bifidobacterium bifidum	ATCC 29521	M84777
Bifidobacterium breve	ATCC 15698	X70972
Bifidobacterium catenulatum	ATCC 27539	M84784
Bifidobacterium infantis	ATCC 15697	M84783
Bifidobacterium longum	ATCC 15708	M84781
Bifidobacterium minimum	ATCC 27538	AY377414
Bifidobacterium subtile	ATCC 27537	AY377423
Bifidobacterium thermophilum	ATCC 25525	U10151

[a] Source of cultures: ATCC, American Type culture Collection.

regions of the 16S rDNA led to simultaneous detection of several isolates of *Bifidobacterium* spp. However, among the primers reported for identification purpose, the primer based on the *B. longum* sequence would recognize *B. catenulatum* (Kok et al., 1996). In addition, most of species-specific PCR methods have only been performed to detect single colonies (Kok et al., 1996; Matsuki et al., 1998) or fecal samples (Germond et al., 2002). Although Fasoli et al. (2003) applied species-specific PCR methods to probiotic products, only 5 primers were reported for discrimination of *Bifodobacterium* spp. including *B. bifidum*, *B. breve*, *B. infantis*, *B. longum* and *B. lactis*.

In order to achieve a rapid and reliable identification of species, PCR combined with Denaturing Gradient Gel Electrophoresis (DGGE) proved to be a useful analytical method for the investigation of complex microbial populations without previous separation of the individual inhabitants (Muyzer and Smalla, 1998). This method has been successfully used to evaluate bacterial composition of probiotic products (Fasoli et al., 2003), to analyze probiotic products claimed to contain bifidobacteriam (Masco et al., 2005), to identify probiotics in South African products (Theunissen et al., 2005) and to differentiate *Lactobacillus* spp. present in the gastrointestinal tract (Walter et al., 2000). On the other hand, certain studies (Felske et al., 1998; Fasoli et al., 2003; Theunissen et al., 2005) indicated that PCR-DGGE did not reveal microorganisms present in complex microflora at a level lower than 1% of the total microbial population.

Since directly identification of *Bifidobacterium* spp. from food products become an important quality issue, we compared the performance of two culture-independent approaches, including species-specific PCR method and PCR-DGGE, to identify the composition of dairy products labeled as containing *Bifidobacterum* directly from samples. In this present study, a novel series of species specific primers were designed that extend the number of probiotic *Bilfidobacterium* spp. to ten. Among these 10 primers, *B. minimum*, *B. subtile*, and *B. thermophilum* were never been identified by this species-specific PCR methods. In parallel, all products were also subjected to a PCR-DGGE analysis.

MATERIALS AND METHODS

Probiotic products

Six different commercially available Taiwan probiotic products which declared *Bifidobacterium* spp. were collected and analyzed. These products included 3 fermented milk and 3 probiotic lyophilized preparations in tablet and powder forms (Table 1). The fermented milk products were purchased from local supermarkets, whereas the lyophilized products were obtained from pharmacies. All probiotic products were tested prior to the expiry date that was indicated on the product labels.

Bacterial strains and culture conditions

The 10 references strains of *Bifidobacterium* species, which were summarized in Table 2, were obtained from Bioresource Collection and Research Center (Food Industry Research, Hsinchu, Taiwan). All bifidobacteria were cultured anaerobically in MRS broth (Difco, Detroit, ME, USA) containing 0.05% L-cysteine hydrochloride at 37°C for 16 h. The final bacterial counts were about 10^9 cell ml^{-1}.

Table 3. *Bifidobacterium* species and specific primer sets based on 16S-23S rRNA sequences

Target group	Primer	Sequence (5' to 3')	Length (bp)	Target site	Product size (bp)	Location
Bifidobacterium	P0	GAGAGTTTGATCCTGGCTCAG	21		1,427	16S rRNA gene
	Lm3	CGGGTGCTNCCCACTTTCATG	21			
B. angulatum	Bang F	GGCTAGAACTCAAAGACATT	20	64-83	442	16S rRNA gene
	Bang R	GGCACTTCCGCCTCAGAG	18	488-505		
B. animalis	Bani F	GCTACAACTCAAAGCATTAC	20	33-52	550	16S-23S rRNA
	Bani R	GTACTTCCGCCTCAGCGATG	20	563-582		intergenic spacer
B. bifidum	Bbif F	CGGCCGCAAGGCTAAAACT	20	55-75	479	16S rRNA gene
	Bbif R	CCGCGACGTTGCTGATTC	18	516-533		
B. breve	Bbre F	TCACACCGCATGGTGTGTTG	20	183-202	852	16S rRNA gene
	Bbre R	GATATCGCGTCTCAGCGA	18	991-1,008		
B. catenulatum	Bcat F	CGGGCAGTGGATAGGGATAG	20	135-154	382	16S rRNA gene
	Bcat R	TTAAGAACCTTCTGGGCGGC	20	495-516		
B. infantis	Bin F	AGGATACGTTCGGCGTC	17	27-46	377	16S rRNA gene
	Bin R	CGCAAGATTCCTCTAGCA	18	385-405		
B. longum	Blon F	GGCCGCAAGATTCCTCTAG	18	103-120	224	16S rRNA gene
	Blon R	CCTCGGCGGTCTCCCGTGA	19	308-326		
B. minimum	Bmin F	CGAGGATCTCAAGCTTCCCG	20	12-31	435	16S rRNA gene
	Bmin R	ACGGGATCTCGCGCACGGT	19	428-446		
B. subtile	Bsub F	AAGACTACGAGGTCAAG	17	38-54	225	16S rRNA gene
	Bsub R	TGTGCTCGTCGACCTGAGAT	20	243-262		
B.thermophilum	Bthe F	GACGGCGAAGACAATTTT	18	982-999	453	16S rRNA gene
	Bthe R	AGCAGAACTGGTCA	15	1,420-1,434		

Bifidobacterial counts

For lyophilized products were suspended in 10 ml of sterile saline solution (0.85% (m/v) NaCl) supplemented with 0.05% L-cysteine and vortexed for 10 min. For fermented milk products, 1 ml of each sample was diluted in 9 ml of sterile saline solution (0.85% (m/v) NaCl) supplemented with 0.05% L-cysteine and blended thoroughly. One ml samples were plated onto the different media, in triplicate. Plates of LP-MRS agar (GasPak System; Oxoid Unipath Ltd. Basingstoke, Hampshire, England) were incubated anaerobically (72 h at 37°C) before enumeration of the bifidobacteria. The population, in colony-forming units (CFU), and the characteristics of the colonies were recorded.

DNA extraction

For the fermented milk products and reference cultures, 1 ml of each sample was centrifuged under 7,500×g for 10 min at 4°C and collected bacterial cells. The bacterial pellets were washed twice with 2 ml of water and subjected to DNA extraction using Blood and Tissue Genomic DNA Extraction Minipred System (VIOGENE-BIOTEK Co., Taipei, Taiwan). For lyophilized products, 1 g of the product was dissolved in 10 ml of a sterile saline solution (0.85% (m/v) NaCl). DNA extraction and purification was followed the procedure previously described for fermented milk products.

Genus-specific PCR

The sequences of the *Bifidobacterium* spp. primers P0 (Di Cello and Fani, 1996) and Lm3 (Kaufmann et al., 1997) used in the present study are reported in Table 3. DNA from various strains and probiotic products were extracted as described previously. PCR was performed in a DNA thermal cycler (Mastercycler Gradient 5331, Eppendorf, NY, USA). The reaction mixture (50 μl) for PCR contained 10 μM each primer, 20 mM Tris HCl (VIOGENE-BIOTEK Co., Taipei, Taiwan), 10 mM KCl (VIOGENE-BIOTEK Co.), 2 mM $MgCl_2$ (VIOGENE-BIOTEK Co.), 200 μM each deoxyribonucleotide triphosphate (VIOGENE-BIOTEK Co.), 50 ng extract bacteria DNA and 1.5 U Taq DNA polymerase (Yeastern Biotech, Taipei, Taiwan). The amplification proceeded for an initial denaturation of 5 min at 94°C, 34 cycles of 30 sec at 94°C for denaturation, 30 sec at 55°C for annealing, and 45 sec at 72°C for extension. The final cycle was 72°C for 10 min, and samples were cooled down to 4°C. Amplified products were run on a 3% agarose gel (Nippon Gene Co., Tokyo, Japan), stained with ethidium bromide (Fluka & Riedel, St. Gallen, Switzerland) and visualized under UV light. A 100-bp DNA ladder and 1Kb DNA ladder was used as a molecular mass marker.

Species-specific PCR

PCR primers used for these studies are shown in Table 3. Primers used for the detection of *Bifidobacterium* spp. were based on the regions of the 16S-23S rDNA retrieved from GenBank (www.ncbi.nlm.nih.gov). The Primer3 software was used to design the primers. The reaction mixture (50 μl) and species-specific PCR derived from the 16S-23S rDNA were performed as described above except annealing

Table 4. List of bifidobacteria and non-bifidobacterium strains and the results of PCR tests using specific primer sets

Species	P0/ Lm3	Bang F/ Bang R	Bani F/ Bani R	Bbif F/ Bbif R	Bbre F/ Bbre R	Bcat F/ Bcat R	Bin F/ Bin R	Blon F/ Blon R	Bmin F/ Bmin R	Bsub F/ Bsub R	Bthe F/ Bthe R
B. angulatum	+	+	-	-	-	-	-	-	-	-	-
B. animalis	+	-	+	-	-	-	-	-	-	-	-
B. bifidum	+	-	-	+	-	-	-	-	-	-	-
B. breve	+	-	-	-	+	-	-	-	-	-	-
B. catenulatum	+	-	-	-	-	+	-	-	-	-	-
B. infantis	+	-	-	-	-	-	+	-	-	-	-
B. longum	+	-	-	-	-	-	-	+	-	-	-
B. minimum	+	-	-	-	-	-	-	-	+	-	-
B. subtile	+	-	-	-	-	-	-	-	-	+	-
B.thermophilum	+	-	-	-	-	-	-	-	-	-	+
Lactobacillus bugaricus	-	-	-	-	-	-	-	-	-	-	-
Straptococcus thermophilus	-	-	-	-	-	-	-	-	-	-	-
L. acidphilus (A)	-	-	-	-	-	-	-	-	-	-	-
L. casei (C)	-	-	-	-	-	-	-	-	-	-	-
L. brevis	-	-	-	-	-	-	-	-	-	-	-
L. plantarum	-	-	-	-	-	-	-	-	-	-	-
L. rhamnosus	-	-	-	-	-	-	-	-	-	-	-
Leuconostoc mesenteroides subsp. *cremoris*	-	-	-	-	-	-	-	-	-	-	-
Leu.mesenteroides subsp. *dextranicum*	-	-	-	-	-	-	-	-	-	-	-

temperature. The annealing temperature was 56°C for Bmin and Bsub. 57°C for Bani and Bbif. 58°C for Bbre. Bcat. and Bthe. 60°C for Bin. as well as 62°C for Bang and Blon.

Sequencing of PCR-amplified 16S-23S rDNA region

All species-specific PCR products amplified with primers designed for *Bifidobacterium* spp. were sequenced for species-specific confirmation. The PCR products were cleaned with Concert Rapid PCR Purification system (QIAquick Gel Extraction Kit, Qiagen Inc., CA. USA) and DNA concentration was checked on a spectrophotometer (Thermo-Spectronic. Rochester. New York. USA). Sequencing was carried out at the Genedragon Co. (Taipei, Taiwan) using a 373A automated gene sequencer (Applied Biosystems. CA. USA). The obtained DNA sequences were aligned in Vector NTI Advance 9 (Invitrogen Co., CA, USA) and the hierarchy of similar sequences received.

Denaturing gradient gel electrophoresis

DNA amplification : The PCR amplification of approximately 200 bp of V3 region of the 16S rRNA gene was obtained using the primers 336f (5'ACT CCT ACG GGA GGC AGC AG3') and 518r (5'GTA TTA CCG CGG CTG CTG CTG GCA C3')(Muyzer et al., 1995).

The PCR products were generated using an initial denaturation step of 5 min at 94°C followed by 34 cycles of denaturation at 94°C for 30 sec. annealing at 56°C for 30 sec and elongation at 72°C for 45 sec. A final chain extension at 72°C for 10 min was done. Amplified products were run on a 3% agarose gel. stained with ethidium bromide and visualized under UV light.

Denaturing gradient gel electrophoresis : The PCR fragments were separated by denaturing gradient gel electrophoresis (DGGE) using the BioRad DCode Universal Mutation Detection System (Bio-Rad Laboratories. CA. USA). Separation of the PCR amplicons was obtained by the direct application of 35 μl of PCR products onto 8% (w/v) polyacrylamide gels in 50× TAE buffer containing a linear denaturant gradient of between 40% and 60%. The 100% denaturing solution contained 40% formamide (J. T. Baker. Phillipsburg, NJ, USA) and 7.0 M urea (J. T. Baker). Electrophoresis was performed with a constant voltage of 130 V at 60°C for 6 h. the gel was stained with ethidium bromide for 30 min and the fragments were visualized under UV light.

The DGGE reference markers were composed by mixing equal amounts of amplicons obtained from 10 *Bifidobacterium* species.

Assessment of detection limit of species-specific PCR method and PCR-DGGE

To verify the detection limit of species-specific PCR method and PCR-DGGE, serial 10-fold dilutions of pure cultures of *B. angulatum*, taken as reference species, were prepared. DNA from each sample was extracted. amplified and run in species-specific PCR method and PCR-DGGE using the protocols described above.

RESULTS AND DISCUSSION

Gene-targeted species-specific PCR

Genus-specific PCR : Primers P0 and Lm3 were used to

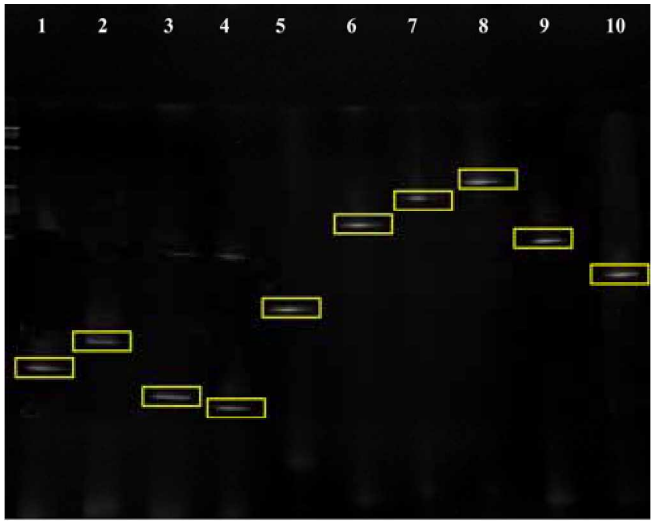

Figure 1. DGGE profiles of the PCR products originating from the reference strains used in this study. Lane 1: *B. angulatum*; Lane 2: *B. bifidum*; Lane 3: *B. longum*; Lane 4: *B. infantis*; Lane 5: *B. animalis*; Lane 6: *B. breve*; Lane 7: *B. catenulatum*; Lane 8:*B. minimum*; Lane 9: *B. subtile*; Lane 10: *B. thermophilum*; Lane 11: mix samples.

amplify the 16S rRNA gene from genomic DNA of *Bifidobacterium* spp. in this study. Genus-specific PCR results showed that a PCR product was obtained from all 10 references *Bifidobacterium* spp. with 1,427 bp in size, while no PCR product was found for other species (Table 4). This result proved that primers P0 and Lm3 could be used as a specific bifidobacterial genus-specific primer pair.

In order to determine if all probiotic products encompassed the *Bifidobacterium* genus, a bifidobacterial genus-specific primer pair (P0 and Lm3) was applied. Results demonstrated that a PCR product was obtained from all 6 probiotic products with 1,427 bp in size. To verify whether the primer set correctly detected target genus, the PCR products were sequencing. After alignment was carried out in BLAST, 10 sequences generated from genus-specific primers designed for identification *Bifidobacterium* spp. showed 98-99% homology with the sequences which were retrieved from Genbank accession numbers. This finding indicated that all 6 probiotic products were composed of *Bifidobacterium* spp. Certain studies also used this same genus-specific primer pair to successfully identify the *Bifidobacterium* genus in gastrointestinal and in human fecal samples (Ventura et al., 2001; Germond et al., 2002).

Species-specific PCR : Based on the comparison of the nucleotide sequences, ten pairs of self-design bifidobacteria species-specific primers, all targeting the 16S-23S rDNA sequences, were tested for detection of species *B. angulatum*, *B. animalis*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. infantis*, *B. longum*, *B. minimum*, *B. subtile*, and *B. thermophilum*, and the additional bacterial strains as further negative controls. These *Bifidobacterium* species cover most of the bifidobacterial species that have been identified in food products (Masco et al., 2005; Theunissen et al., 2005). Results indicated that all ten novel primer pairs were shown a good identification for respected species (Table 4). The sizes of PCR products were varied in length from 225 bp to 852 bp. In order to verify the species-specific PCR results, PCR-amplified 16S-23S rDNA region were sequenced. After alignment was carried out in BLAST, 10 sequences generated from species-specific primers designed for identification of 10 different *Bifidobacterium* spp. showed 90-96% homology with the sequences which were retrieved from Genbank accession numbers.

The presence of *Bifidobacterium*, declared on the labels of all the products, was examined. The specificity of each primer pair was tested on 6 DNA samples from 6 different products. Results indicated (Table 1) that four different *Bifidobacterium* spp. (*B. bifidum*, *B. longum*, *B. infantis*, and *B. breve*) were identified from 6 products. In addition, *Bifidobacterium longum* was the *Bifidobacterium* species most frequently detected in the products and was found in the products 1, 3, 4, 5 and 6. Consistency was found between the species detected by species-specific PCR and those declared on the label for all the products.

DGGE analysis

Differentiation of Bifidobacterium spp. by DGGE analysis : The results obtained by DGGE analysis on reference strains are shown in Figure 1. The expected 240-bp (including 42 GC clamp) PCR fragments were successfully amplified from all reference strains. As reported, *B. angulatum* (lane 1), *B. bifidum* (lane 2), *B. longum* (lane 3), *B. infantis* (lane 4), *B. animalis* (lane 5), *B. breve* (lane 6), *B. catenulatum* (lane 7), *B. minimum* (lane 8), *B. subtile* (lane 9) and *B. thermophilum* (lane 10) gave specific patterns in the DGGE gel that could be easily used

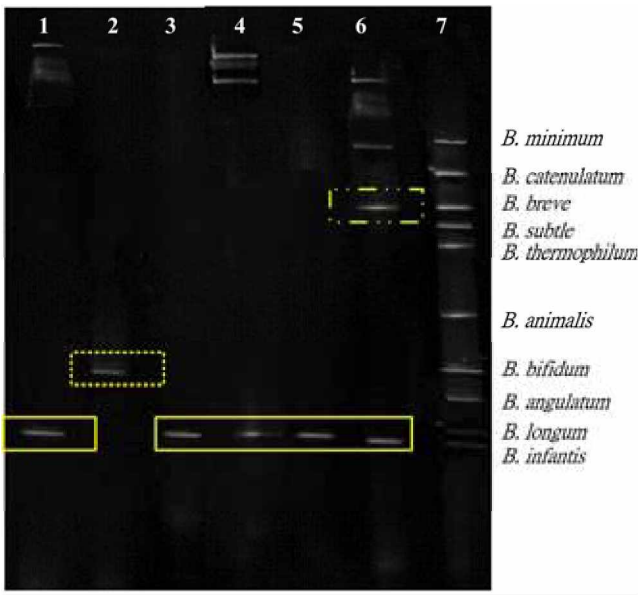

Figure 2. DGGE analysis of the PCR products generated by PCR from market products. Lane 1: sample 1; Lane 2: sample 2; Lane 3: sample 3; Lane 4: sample 4; Lane 5: sample 5; Lane 6: sample 6; Lane 7: marker.

for identification purposes. *B. longum* and *B. infantis* presented more than one DGGE band due to the amplification of multi-copies of the rRNA gene that contained differences detectable by DGGE as previously described by Cocolin et al. (2001).

DGGE analysis of the market products : The DGGE patterns obtained by PCR-based DGGE analysis of the 3 fermented milk products and the 3 lyophilized products are shown in Figure 2. Product 1 did contain *B. longum* as was stated on the label. *B. bifidum* was detected in the product 2 as was declared on the label. The product 3 was found to contain *B. longum* as declared on the label of the product. The DGGE profiles of product 4 revealed the presence of one *Bifidobacterium* spp., which was identified as *B. longum*. *B. infantis* observed by species-specific PCR was not detected in this product by DGGE, although the presence of this *Bifidobacterium* spp. was stated on the label. The product 5 was found to contain *B. longum* as was indicated on the label. However, *B. bifidum*, as claimed on the label, could not be detected in this product. Product 6 was detected both *B. longum* and *B. breve* as declared on the label of the product.

The products 1, 2, 3 and 6 did contain all the *Bifidobacterium* spp. that specified on the product label, whereas, the product 4 and 5 did not contain the exact *Bifidobacterium* spp. as indicated on the label. It should be noted that two third of the lyophilized products examined did not contain the probiotic microbial composition specified on the product label. There were 2 possibilities to explain this phenomenon. The strains labeled on the products were not present or the DGGE method failed to

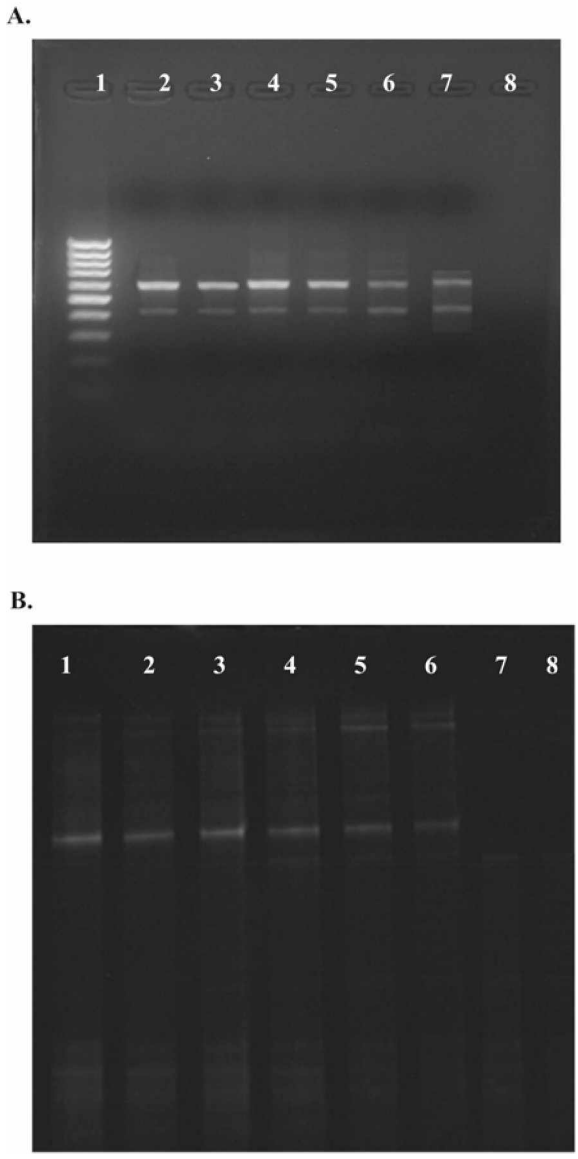

Figure 3. Detection limits of the species-specific PCR (A) and DGGE (B) for *B. angulatum*. (A). Lane 1: Molecular weight marker 100-bp DNA ladder; Lanes 2-8 contain the following amounts of *B. angulatum*: 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, and 0 cells, respectively. (B). Lanes 1-8contain the following amounts of *B. angulatum*: 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 cells, respectively.

detect those stains. In this case, the lyophilized products analyzed in this study have been proven possessing all bifidobacterial stains indicated on the label by species-specific PCR. Thus, in agreement with previous studies (Fasoli et al., 2003; Temmerman et al., 2003), this data indicated that the low viable cells of dried probiotics (Table 1) might lower the sensitivity of DGGE. The possible explanations might be that the cell numbers of certain strains were lower than the detection limit of DGGE or that high quantities of competitor templates were present (Cocolin et al., 2000). Theunissen et al. (2005) defined that

the presence of a DGGE band represents a LAB population above a minimum threshold value of 10^5 cells/ml and thus identifies only the predominant LAB populations in these starters. Moreover, species present at higher populations in the mixture will give greater amounts of template DNA, and, therefore, should have a higher probability of detection (Prakitchaiwattana et al., 2004). Ouwehand and Salminen (1998) claimed that a concentration below the detection limit is considered insufficient to exert beneficial effects on human health.

Above results indicated that for fermented milk products consistency was found for both species-specific PCR and PCR-DGGE in detecting species. However, in some lyophilized products, the bands corresponding to these species were not visualized in the DGGE profile but the specific PCR gave a positive result. These results suggested that, in samples containing multiple *Bifidobacterium* species, PCR-DGGE might fail to detect some *Bifidobacterium* species.

Detection limit and reproducibility of PCR assay and PCR-DGGE

B. angulatum was used as reference strain to indicate the lowest bacterial concentration at which bands could be visualized using PCR assay and PCR-DGGE. This species could be detected by PCR assay and PCR-DGGE at a minimum concentration of 10^1 CFU/ml and 10^3 CFU/ml, respectively (Figure 3). These detection limit values confirmed our above results. PCR assay shows higher sensitivity than PCR-DGGE in samples containing multiple *Bifidobacterium* species. This DGGE values were correlated well with the DGGE detection limit observed by Theunissen et al. (2005). However, the detection limit was increased to 10^7 CFU/ml when application of DGGE in yogurt samples (Temmerman et al., 2003).

Repeated DNA extracted from the same probiotic sample, as well as from different batches of the same analog, resulted in reproducible DGGE profiles. Similarly, Repetitive species-specific PCR reactions were performed using the same DNA and this resulted in reproducible results.

CONCLUSIONS

The choice of an identification method depends on the aim of investigation. PCR-DGGE can detect all species using one primer set in one test, which can decrease the workload and save the identification time for the investigation of complex microbial populations, but its low detection limit needs to be considered. Whereas, species-specific PCR is only capable of detecting single or a few species in one trial, but it is sensitive and reliable for the investigation of complex microbial populations.

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