Use of *tuf*-gene based primers for the PCR detection of probiotic *Bifidobacterium* species and enumeration of bifidobacteria in fermented milk by cultural and real-time QPCR methods

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Dear Editor:

We would like to submit the manuscript entitled “Use of tuf-gene based primers for the PCR detection of probiotic Bifidobacterium species and enumeration of bifidobacteria in fermented milk by cultural and quantitative real-time PCR methods” for the publication in Journal of Food Science. We hereby certify that this paper consists of original unpublished work and that each author meets the criteria for authorship and assumes the corresponding responsibility.

Sincerely,

Sen-Je Sheu

For Peer Review

1 Running title

2 ***Tuf* gene-based PCR detection of probiotic *Bifidobacterium* species**

3

4 Title

5 Use of *tuf*-gene based primers for the PCR detection of probiotic 6 *Bifidobacterium* species and enumeration of bifidobacteria in 7 fermented milk by cultural and quantitative real-time PCR 8 methods

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24 **ABSTRACT**

25 Due to the increasing use of bifidobacteria in probiotic products, it is essential to
26 establish a rapid method for the qualitative and quantitative assay of the bifidobacteria in
27 commercial products. In this study, partial sequences of the *tuf* gene for 18 *Bifidobacterium*
28 strains belonging to 14 species were determined. Alignment of these sequences showed that
29 the similarities among these *Bifidobacterium* species were 82.24-99.72%. Based on these *tuf*
30 gene sequences, six primer sets were designed for the polymerase chain reaction (PCR)
31 assay of *B. animalis* subsp. *animalis*, *B. animalis* subsp. *lactis*, *B. bifidum*, *B. breve*, *B.*
32 *longum* subsp. *infantis*, *B. longum* subsp. *longum*, and the genus of *Bifidobacterium*,
33 respectively. These *Bifidobacterium* species are common probiotic species present in dairy
34 and probiotic products. When each target *Bifidobacterium* spp. were assayed with the
35 designed primers, PCR product with expected size was generated. In addition, for each
36 target species, more than 70 bacterial strains other than the target species, including strains
37 of other *Bifidobacterium* species, strains of *Lactobacillus* spp., *Enterococcus* spp. and other
38 bacterial species, all generated negative results. PCR assay with primers specific to *B.*
39 *animalis* subsp. *lactis* and *B. longum* subsp. *longum* confirmed the presence of these
40 Bifidobacterium species in commercial yogurt products. In addition, for each product,
41 enumeration of the bifidobacteria cells by culture method with BIM-25 agar and the
42 quantitative real-time PCR (qPCR) showed similar cell counts. Such results indicated that
43 within 15 days storage (4°C) after manufacture, all the bifidobacteria cells originally present
44 in yogurt products were viable and culturable during the storage.

45 **Keywords:** probiotics, *Bifidobacterium* spp., *tuf* gene, real-time PCR.

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48 **Introduction**

49 The genus *Bifidobacterium* is common inhabitant of intestinal tract of human and
50 animal. *Bifidobacterium* strains with probiotic functions, such as *B. animalis* subsp.
51 *animalis*, *B. animalis* subsp. *lactis, B. longum* subsp. *infantis*, *B. longum* subsp. *longum*, *B.*
52 *breve,* and *B. bifidum,* in combination with other lactic acid bacteria (LAB), have been
53 widely used in dairy, probiotic products and feed supplements. Many studies have revealed
54 that these *Bifidobacterium* strains contribute the beneficial effects on human and animal
55 health (Saarela and others 2000; Ouwehand and others 2002; Reid and others 2003; Telabi
56 and others 2008; Lopez and others 2010). Because of the increasing use of bifidobacteria in
57 probiotics and feed supplements, correct identification and enumeration of viable cell counts
58 in products are important, not only to the regulatory agencies, but ultimately to the
59 consumer. In this study, we thus tried to establish a rapid method for the qualitative and
60 quantitative assay of the bifidobacteria cells in probiotic products.

61 Traditional methods for the identification of *Bifidobacterium* rely on the phenotypic
62 characteristics of biochemical, morphological and selective culture tests (Solano-Aguilar
63 and others 2008). These methods are time-consuming and labor-intensive. Recently,
64 genotypic method has become an alternative to phenotypic method. For some
65 *Bifidobacterium* spp. in addition to the 16S rRNA gene (Mullie and others 2003), 16S and
66 23S rRNA gene and the rRNA intergenic spacer region (ISR) (Kwon and others 2005),
67 several other genes, such as phosphoketoase (Cleusix and others 2010), chaperonin 60
68 (Desai and others 2009), and *recA* (Masco and others 2006) genes, have been used as target
69 genes. For most of the *Bifidobacterium* spp., 16S rDNA and 16S-23S rDNA internally
70 transcribed spacer (ITS), have been the most common targets used for PCR primers
71 designing (Tilsala-Timisjarvi and Alatossava 1997; Matsuki and others 1999; Kwon and

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72 others 2005). However, the high similarities of 16S rDNA sequences among closely related
73 species make it difficult to develop highly specific primers to differentiate species. Besides,
74 the divergent 16S rDNA sequences among *rrn* operons of a single organism remain problem
75 (Acinas and others 2004; Lin and others 2004). On the other hand, the ITS of the 16S-23S
76 rRNA gene may be included for a more detailed analysis of *Bifidobacterium* species
77 because these sequences are less conserved than the 16S rRNA gene sequence (Ventura and
78 Zink 2003). Youn and others (2007) evaluated 37 published *Bifidobacterium* primer sets
79 designed from 16S rDNA, 23S rDNA, ITS and repetitive DNA sequences of various
80 *Bifidobacterium* species and found that only part of these primer sets showed the expected
81 specificity. Recently, several mono-copy target genes, including *tuf* (Ventura and others
82 2003; Solano-Aguilar and others 2008), *recA* (Kullen and others 1997), *Idh* (Roy and Sirois
83 2001) and *hsp60* genes (Zhu and Dong 2003), revealed high divergence in LAB species and
84 could serve as alternative molecular markers.
85 The elongation factor Tu (*tuf*) gene has recently been used for the designing of
86 gene-based method to detect bacteria, such as *Enterococcus* and *Staphylococcus* (Ke and
87 others 1999; Martineau and others 2001), some *Lactobacillus* spp. (Chavagnat and others
88 2002; Sheu and others 2009), closely related *Lactobacillus* species, such as the members of
89 *Lactobacillus casei* group; and also, as a marker to differentiate *B. animalis* subsp. *lactis*
90 from *B. animalis* subsp. *animalis* (Ventura and others 2003). Since *tuf* gene sequences for
91 some *Bifidobacterium* spp. were not revealed in Genbank yet, in this study, we determined
92 the *tuf* gene sequences for 18 *Bifidobacterium* strains belonging to 14 species, including
93 those of the sequences not revealed, and deposited the sequences determined to the
94 GenBank. Based on these *tuf* gene sequences, species-specific primers were then designed
95 for the PCR detection of five potentially probiotic *Bifidobacterium* species. Meanwhile, two

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96 of the most common *Bifidobacterium* species, i.e., *B. animalis* subsp. *lactis* and *B. longum*
97 subsp. *longum*, present in commercial yogurt products, were indentified and their cell
98 numbers were determined by cultural method and the qPCR method.

99 **Materials and methods**

100 **Bacterial strains and culture conditions**

101 A total of 75 bacterial strains used in this study and their sources are listed in Table 1.
102 These strains include 32 strains of 21 *Bifidobacterium* spp. and subspecies, 54 strains of 22
103 *Lactobacillus* spp.; 6 strains of *Enterococcus* spp. and other bacteria spp. including those of
104 *Enterobacteriaceae* and some food pathogenic bacteria. LAB were cultured in deMan
105 Rogosa Sharpe (MRS) (Merck, Darmstadt, Germany) broth containing 0.05% L-cysteine
106 hydrochloride at 37 ℃ for 24 h under anaerobic condition (BBL GasPak, Becton Dickinson,
107 Cockeysville, MD). Strains other than LAB were grown aerobically in trypic soy broth
108 (Merck) at 37 ℃ for 24 h.

109 **Sequencing of the partial *tuf* gene of *Bifidobacterium* species**

110 The *tuf* gene sequences for some *Bifidobacterium* spp., i.e., *B. adolescentis*, *B. animalis*
111 subsp. *animalis*, *B. bifidum*, *B. longum* subsp. *longum*, *B. breve*, *B. catenulatum* and *B.*
112 *longum* subsp. *infantis*, available in the GenBank database of the National Center for
113 Biotechnology Information (NCBI), were retrieved and compared with the Clustal W
114 program (http://workbench.sdsc.edu). Afterwards, universal primers, i.e., a forward primer
115 (Bifseq-tF) 5'-GCCCACATCGAGTACCAG-3'and a reverse primer (Bifseq-tR)
116 5'-CCACCGACGTCACCGGCG-3' were selected. These primers were then used for the
117 amplification of the region flanking by these primer for 18 Bifidobacteria strains used in this
118 study. All the PCR products were electrophoresed and purified with the Viogene Gel-M Kit

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119 (Viogene, Taipei, Taiwan) according to the manufacturer's protocol. The purified products
120 were sequenced with the ABI 377 automatic sequencer (Perkin-Elmer, Applied Biosystems,
121 CA, USA). The partial *tuf* gene sequences determined for 18 *Bifidobacterium* strains
122 representing 14 species were then deposited in GenBank under the accession number from
123 FJ549338 to FJ549355.
124

125 **Sequence alignments**

126 Alignment of the sequences determined for *tuf* gene and those available in GenBank
127 were done by using the Multiple Sequence Alignment of the Clustal W program. The
128 similarities for nucleotide sequences were calculated and converted into a distance matrix
129 by the DNADIST program using the default models (http://workbench.sdsc.edu).

130 **Preparation of DNA for PCR assay**

131 Bacterial genomic DNAs were prepared using the Blood & Tissue Genomic DNA
132 Extraction Miniprep System for Bacteria (Viogene, Taipei, Taiwan) according to the
133 manufacturer's protocol. Bacterial strains were grown in the appropriate medium under the
134 conditions as described above. Cells collected (7000×g for 5 min) from 0.3 ml culture broth
135 were washed with 1ml of TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) and pelleted
136 (7000×g for 5 min). The pellet was suspended in 0.3 ml lysis buffer, followed by addition of
137 20 µl mutanolysin (1000 U/ml, Sigma, St. Louis, Mo.), 20 µl lysozyme (20 mg/ml, Sigma),
138 and 20 µl RNase (2 mg/ml, Sigma). The mixture was incubated at 37 ℃ for 90 min
139 followed by the addition of 200 µl EX buffer (Viogene) and 30 µl proteinase K (10 mg/ml,
140 Sigma). After incubation at 65 ℃ for another 90 min, total DNA was extracted according to
141 the manufacturer's manual. Afterward, genomic DNA was eluted with 100 µl double

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142 deionized water.

143 **PCR primers and amplification conditions**

144 Primers for the detection of six common *Bifidobacterium* spp. and subspecies
145 including *B. animalis* subsp. *animalis*/ *B. animalis* subsp. *lactis*, *B. bifidum, B. breve*, *B.*
146 *longum* subsp. *infantis*, *B. longum* subsp. *longum*, and the genus of *Bifidobacterium* (Table 2)
147 were designed by multiple alignments of the *tuf* gene sequences using the Clustal W
148 program. Sequences of the primers were then compared with all the sequences retrieved
149 from the GenBank database (http://www.ncbi.nlm.nih.gov) using blast program. Specificity
150 of each primer set was then confirmed by PCR assay with DNAs from all strains of the
151 *Bifidobacterium* and non-*Bifidobacterium* species (Table1). For PCR detection of
152 *Bifidobacterium*, 25 µl PCR reaction buffer (10 mM Tris–HCl pH 8.8, 1.5 mM $MgCl_2$, 50
153 mM KCl, 0.1% Triton X-100) containing 200 µM of each deoxynucleoside triphosphate
154 (dNTP), 1 µM of each the primer, 0.4 U of ProZyme II (Protech, Taipei, Taiwan), and 1 µl
155 of target DNA were used. PCR was carried out in a Robocycler® temperature thermal cycler
156 (Stratagene, Cedar Creek, Tex.). For primer sets designed for *Bifidobacterium* genus and
157 species (Table 2), each PCR cycle was performed at 94 ℃ for 35 s, annealing temperature
158 for each primer set (Table 2) for 35 s and 72 ℃ for 40 s, respectively, for a total of 35 cycles.
159 Each initial denaturation was performed at 94 ℃ for 5 min and final extension was at 72
160 ℃for 7 min. The amplicons were then electrophoresed with a 3.5% agarose gel and
161 visualized by staining with ethidium bromide (0.5 µg/ml).

162 **Evaluation of the PCR detection limit for *Bifidobacterium* spiked in milk samples**

163 Bifidobacteria cells such as *B. animalis* subsp. *lactis* BCRC 17394 and *B. longum* subsp.

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164 *longum* BCRC 11847, respectively, were serially diluted with sterile water. Then, 10 μl of
165 each cell suspension (N× 10^5 or 10^4, 10^3, 10^2, 10^1 CFU per 10 μl, N equal to 1~9) was
166 spiked to 1 ml pasteurized whole milk. For DNA extraction, 0.1 ml of the spiked milk was
167 mixed with 0.9 ml TE buffer and votexed for 30 s. Cells were collected (7000×g for 5 min)
168 and washed again with TE buffer. Total DNA was extracted according to the procedures
169 described earlier until the step of incubation with proteinase K (65 ℃, 90 min) and clear
170 lysate was obtained. Afterward, DNA was extracted with phenol-chloroform method.
171 DNA obtained was then suspended in 10 μl double deionized water and subjected to PCR.
172 The amplicons were electrophoresed with a 3.5% agarose gel and visualized by staining
173 with ethidium bromide (0.5 μg/ml).

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174 **Enumeration of viable cell numbers in yogurt by cultural method and comparison of**
175 **the results with those from the qPCR method**

176 Three brands of yogurt products purchased from local supermarkets were used as
177 samples. All samples were purchased within the expiration date. After purchasing, these
178 products were stored at 4°C and bifidobacteria cells were assayed and counted immediately
179 or after storage at 4°C for several days. In general, three samples for each product were
180 assayed. Total counts of LAB and *Bifidobacterium* in these products were determined by
181 counting the CFU in 1 ml of the serial dilutions after spreading 100 μl of these dilutions,
182 respectively, on MRS and the *Bifidobacterium* iodoacetate medium 25 (BIM-25) agar
183 (Difco) plates (Muñoa and others 1988). For both media, cells were incubated at 37°C for
184 24~48h under anaerobic condition using Gas generating kit BR0056A (Oxoid, Basingstoke,
185 UK). For DNA extraction, 0.1 ml yogurt sample was mixed with 0.9 ml TE buffer and
186 votexed for 30 s. Cells were collected (7000×g, 5 min) and washed again with TE buffer.

187 Then, total DNA was extracted according to the procedures described earlier. Afterwards,
188 10 μl DNA was subjected to PCR.
189 In addition, the *Bifidobacterium* spp. determined by PCR were also subjected to qPCR
190 using the SYBR Green PCR Master kit (No. 4369155, Applied Biosystems, Foster City, CA,
191 USA). The kit was supplied at a 2× concentration. PCR conditions for amplification with
192 primers Bani-tF/Bani-tR (for *B. animalis* subsp. *lactis*) and BloF/BloR (for *B. longum* subsp.
193 *longum*), were 95℃ for 5 min, 35 cycles of 95℃ for 15 s, 63℃ for 15s, 72℃ for 45s and
194 95℃ for 5 min, 35 cycles of 95℃ for 15 s, 70℃ for 50 s, respectively, and both with a final
195 extension step for 7 min. A dissociation stage was followed for the melting curve analysis
196 after the final extension step. The dissociation analysis determines the melting temperature
197 (T_m) of amplification products generated during PCRs. Melting curves were obtained for all
198 samples indentified as positive for *B. animalis* subsp. *lactis* and *B. longum* subsp. *longum*.
199 For standard curve, serial dilutions of target *Bifidobacterium* species (N$\times 10^1$ ~ N$\times 10^6$ cells
200 per 10μl) were spiked into 1ml of the pasteurized whole milk and 0.1ml of the sample were
201 subjected to DNA extraction followed by qPCR according to the conditions described
202 above.
203
204 **Results and Discussion**
205 **Alignment of the sequences in partial *tuf* genes among different *Bifidobacterium***
206 **species**
207 Many commercial probiotics including yogurt, heath foods, and feed supplements,
208 contain bifidobacteria cells. Because in comparison with *Lactobacillus* spp., bifidobacteria
209 are more sensitive to oxygen and less tolerant to acid (Shah 2000), it is important to

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210 establish a rapid method for the quantitative and qualitative assay of the bifidobacteria
211 products. Based on the *tuf* gene sequences among different Bifidobacterium species, we thus
212 attempted to establish PCR based methods for the quality assurance of the probiotic
213 products. Since some of the *tuf* gene sequences, for example, those of *B. boum, B. cuniculi,*
214 *B. gallinarum, B. globosum*, *B. indicum, B. magnum, B. minimum, B. subtile* and *B.*
215 *thermophilum* etc, have not been previously revealed in GenBank, in this study, partial *tuf*
216 gene sequences for these bacterium species were sequenced and aligned with those available
217 in GenBank. Results in Table 3 show that the sequence similarities for the 770 bp regions of
218 these 14 *Bifidobacterium* species were from 82.24 to 99.72%. It was observed that the
219 similarity for the sequenced 770 bp *tuf* gene between the two strains of *B. longum* subsp.
220 *longum* was 99.74%. Such similarity was close to those based on the *hsp60* gene (about
221 99%) reported for this *Bifidobacterium* species (Zhu and Dong, 2003). Also, the similarity
222 of *tuf* gene sequences between the two strains of *B. bifidum* was 99.72%. Therefore,
223 sequences of the strains within the same species or subspecies are highly conserved. On the
224 other hand, the sequence similarity between *B. longum* subsp. *infantis* and *B. longum* subsp.
225 *longum*, which are two of the three subspecies of *B. longum*, was 96.13%. These two
226 subspecies might be discriminated by carefully designed primers as shown in Table 2. The
227 three subspecies of *B. longum* have recently been reclassified as *B. longum* subsp. *longum*;
228 subsp. *infantis* and subsp. *suis* (Mattarelli and others 2008).

229 **Specificity and detection limits of the PCR primers**

230 Based on the *tuf* gene sequences, 5 species-specific primer sets, and one genus-specific
231 primer set, were designed for the detection of *B. animalis* subsp. *animalis/ B. animalis* subsp.
232 *lactis*, *B. bifidum*, *B. breve*, *B. longum* subsp. *infantis,* and *B. longum* subsp. *longum,*
233 respectively, and the genus of *Bifidobacterium* spp. (Table 2, Fig 1). Although these primes

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234 were designed from regions which may allow the designing of species specific primers,
235 specificity of each primer set was further assayed with all 75 bacterial strains including 32
236 strains of 21 *Bifidobacterium* spp. and subspecies, strains of 22 *Lactobacillus* spp., strains of
237 6 *Enterococcus* spp., and other strains, such as strains of *Enterobacteriaceae*, *Streptococcus*
238 *thermophilus*, and some common food pathogens (Table 1). Strains other than the target
239 organisms shown in Table 1 did not generate any false positive result. For each primer set,
240 the *Bifidobacterium* strains assayed representing 21 *Bifidobacterium* spp. and subspecies,
241 which were higher in numbers than those reported for the development or evaluation of the
242 PCR primers specific for *Bifidobacterium* species detection (Kwon and others 2005; Youn
243 and others 2008). The strains we used include all the strains of *Bifidobacterium* spp. which
244 fulfilling the FAD/WHO guidelines for probiotic use (Reid and others 2006), and all the
245 *Bifidobacterium* species which have been used in probiotic products, and feed supplements.
246 The potentially probiotic *Bifidobacterium* species generally used include *B. animalis* subsp.
247 *animalis*, *B. animalis* subsp. *lactis*, *B. bifidum*, *B. breve*, *B. longum* subsp. *infantis,* and *B.*
248 *longum* subsp. *longum* etc. (Saurela and others 2000; Ouwehand and others 2002; Reid and
249 others 2003; Telabi and others 2008; Lopez and others 2010) (Table1). The primers we
250 designed thus allowed the detection of the *Bifidobacterium spp*. in commercial probiotic
251 products. Recently, it has been reported that bifidobacteria strains exhibiting probiotic
252 properties belong to the species of *B. adolescentis, B. animalis, B. bifidum*, *B. breve*, *B.*
253 *longum* subsp. *infantis,* and *B. longum* subsp. *longum*, it can be noted that those species are
254 not phylogenetically related, thus underlining the strain specificity of those characteristics
255 (Felis and Dellaglio 2007). As described earlier, Youn and others (2008) have evaluated 37
256 previously reported PCR primers sets designed to amplify 16S rDNA, 23S rDNA, intergenic
257 spacer (ITS) regions or repetitive DNA sequences of various *Bifidobacterium* species and

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258 found that only 10 of these 37 primer sets showed specificity for *B. adolescentis*, *B.*
259 *angulatum*, *B. pseudocatenulatum*, *B. breve*, *B. bifidum*, *B. longum* subsp. *infantis* and *B.*
260 *dentium*. With this regard, the primers reported here offer an alternative for detection of the
261 potentially probiotic *Bifidobacterium* species.

262 The detection limits for PCR assay of *B. animalis* subsp. *lactis* and *B. longum* subsp.
263 *longum,* two of the most common probiotic species present in probiotic products, were
264 N×10^3 CFU per ml whole milk, respectively (data not shown). Such detection limit was in
265 general below the bifidobacteria levels of 10^4 to 10^6 CFU/ml (Table 4) usually present in the
266 commercial yogurt products. Therefore, the *Bifidobacterium* species in commercial yogurt
267 could be identified without the preculture step.

268 **Detection and enumeration of bifidobacteria cells in commercial yogurt products**

269 Three yogurt products available in markets were assayed for the presence of
270 *Bifidobacterium* species and the total cell counts of LAB and bifidobacteria.
271 *Bifidobacterium* species in these products were identified with PCR method followed by
272 enumeration of the bifidobacteria cells by cultural methods. PCR assay with primers
273 specific to *B. animalis* subsp. *lactis* and *B. longum* subsp. *longum* confirmed the presence of
274 these *Bifidobacterium* species in samples (Table 4). Total culturable counts of LAB
275 determined with MRS agar plates were 8.12, 8.22 and 8.51 log CFU/ml, respectively, and
276 total bifidobacteria counts determined with BIM-25 agar plates were between 6.15±0.21 log
277 CFU/ml for *B. lactis* in sample No.1, 5.93±0.18 and 4.68±0.11 log CFU/ml for *B. longum*,
278 respectively, in sample No.2 and No.3. The viable and culturable cell counts for LAB in
279 samples No. 1-3, and for bifidobactria in sample No. 1 were close to those labeled on the
280 products. For sample No.2 and No.3, however, bifidobacteria counts in samples were not

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281 labeled (Table 4). Since only one Bifidobacterium species was labeled and identified in each
282 of these three yogurt products, the cell counts determined by BIM-25 agar represented the
283 viable counts of *B. animalis* subsp. *lactis*, and *B. longum* subsp. *longum*, respectively, in
284 these samples.

285 For all the yogurt samples, bifidobacteria counts were determined not only by the
286 cultural method, but also by qPCR method. QPCR allowed the enumeration of both viable,
287 culturable and unculturable cells (Kramer and others 2009). By this way, the bifidobacteria
288 counts originally present in yogurt product may be determined. Results shown in Table 4
289 indicate that the bifidobacteria counts determined by qPCR and the cultural method using
290 BIM-25 agar were about the same. Such results imply that almost all the bifidobacteria cells
291 originally present in yogurt samples were viable and culturable. QPCR method may
292 complement the plate count method which considered only the culturable part of the
293 population (Kramer and others 2009). In a separate study, we have purchased three yogurt
294 products and enumerated the bifidobacteria counts 5, 10 and 15 days, after manufacture, the
295 viability and culturability of bifidobacteria in these yogurt products were not affected if they
296 were stored at 4℃ (Table 5). Although some probiotics can exert their beneficial health
297 effects even if the bacterial cells are dead (Jijon and others 2004; Lin and others 2006), it is
298 generally considered that the minimum viable LAB numbers required for a probiotic to
299 provide a healthy benefit are 10^7 CFU/ ml (Ross and others 2005; Jayamanne and Adams
300 2006). Some probiotic products may not meet such criterion.

301 **Conclusion**

302 The *tuf* genes are useful for the design of species-specific PCR primers. In this study,
303 we determined the partial *tuf* gene sequences for some *Bifidobacterium* spp. including those
304 not available in GenBank. Alignment of the *tuf* gene sequences for these *Bifidobacterium*

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305 spp. allowed us to develop PCR primer sets for the detection of the genus of
306 *Bifidobacterium* and the potentially probiotic Bifidobacterium species. Using these primers,
307 bifidobacteria cells in probiotic products could be identified. Furthermore, though the use of
308 cultural method with BIM-25 agar and the qPCR method, the viable and culturable
309 bifidobacteria counts and the total bifidobacteria counts originally present in yogurt could
310 be determined. Since a high percentage of the fermented milk and probiotic products have
311 incorrect labels (Coeuret and others 2004; Perea and others 2007), which highlights the poor
312 compliance to standards by many probiotic products, this study offers an effective method
313 to inspect the quality of probiotic products.
314

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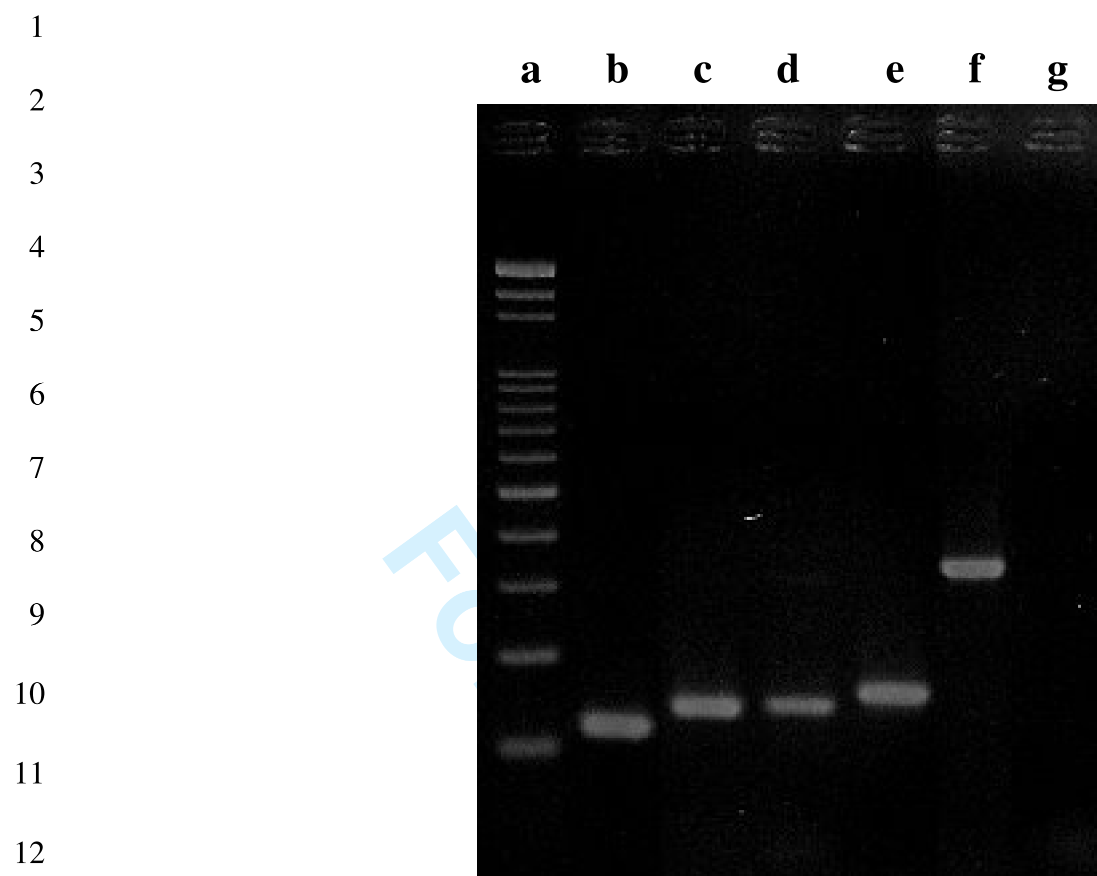

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13 Fig 1 PCR products amplified from five *Bifidobacterium* species using *tuf* gene
14 based primers. Experimental conditions were as those described in Methods.
15 The primers used were as those shown in Table 2. Lane a, 100-bp ladder;
16 lanes b~f, PCR products amplified from strains of *B. lonum* subsp. *infantis*
17 BCRC 14602, *B. lonum* subsp. *longum* BCRC 11847, *B. breve* BCRC 14632,
18 *B. animalis* subsp. *lactis* BCRC 17394 and *B. bifidum* BCRC 11844,
19 respectively; lane g, negative control.

1 Table 1. Bacterial strains used in this study.

Species	Source[a]	Bani-tF/ Bani-tR	Bbif-tF/ Bbif-tR	Bbre-tF/ Bbre-tR	Binf-tF/ Binf-tR	BloF/ BloR	Bif-tF/ Bif-tR
Bifidobacterium adolescentis	BCRC 14607	-	-	-	-	-	+
B. angulatum	BCRC 15971 BCRC 14668	-	-	-	-	-	+ 2
B. animalis subsp. *animalis*	CCUG 48185	+	-	-	-	-	+
B. animalis subsp. *lactis*	BCRC 17394 CCUG 37979T CCUG 33397, BLa 1-3	+ 6	-	-	-	-	+ 6
B. bifidum	BCRC 11844, BCRC 14613	-	+ 2	-	-	-	+ 2
B. boum	BCRC 14677	-	-	-	-	-	+
B. breve	BCRC 14632	-	-	+	-	-	+
B. catenulatum	BCRC 14667	-	-	-	-	-	+
B. cuniculi	BCRC 14672	-	-	-	-	-	+
B. dentium	BCRC 14662	-	-	-	-	-	+
B. gallinarum	BCRC 14679	-	-	-	-	-	+
B. globosum	BCRC 14663	-	-	-	-	-	+
B. indicum	BCRC 14674	-	-	-	-	-	+
B. longum subsp. *infantis*	BCRC 14602	-	-	-	+	-	+
B. longum subsp. *longum*	BCRC 11847 BCRC 14664 BLg 1-3	-	-	-	-	+ 5	+ 5
B. magnum	BCRC 14676	-	-	-	-	-	+
B. minimum	BCRC 14666	-	-	-	-	-	+
B. pullorum	BCRC 14678	-	-	-	-	-	+
B. pseudolongum	BCRC 16013	-	-	-	-	-	+
B. subtile	BCRC 14660	-	-	-	-	-	+
B. thermophilum	BCRC 14669	-	-	-	-	-	+
Lactobacillus spp.		-	-	-	-	-	-

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(20 species including *Lactobacillus acidophilus* BCRC 10695*, L. agilis* BCRC 12931*, L. casei* BCRC 10697*, L. crispatus* BCRC 14618*, L. delbrueckii subsp. delbrueckii* BCRC 12195*, L. farciminis* BCRC 14043, *L. fermentum* BCRC 12190 *, L. gasseri* BCRC 14619*, L. helveticus* BCRC 12936*, L. jensenii* BCRC 12939*, L. johnsonii* BCRC 17474*, L. murinus* BCRC 14020*, L. paracase* BCRC 12248*, L. pentosus* BCRC 11503*, L. plantarum* BCRC 10069, *L. reuteri* BCRC 14625*, L. ruminis* BCRC 14620*, L. rhamnosus* BCRC 10940*, L. salivarius sp. salicinius* BCRC 12574*, L. zeae* BCRC 17269.)

(continued)

Species	Source[a]	Bani-tF/ Bani-tR	Bbif-tF/ Bbif-tR	Bbre-tF/ Bbre-tR	Binf-tF/ Binf-tR	BloF/ BloR	Bif-tF/ Bif-tR
Enterococcus spp.		-	-	-	-	-	-
(6species including *E. avium* BCRC 14728, *E. durans* BCRC 10790, *E. faecalis* BCRC 12298, *E. faecium* BCRC 10067, *E. gallinarum* BCRC 15477, *E. casseliflavus* BCRC 14926.)							
Others		-	-	-	-	-	-
Streptococcus thermophilus BCRC 12257, *Bacillus cereus* BCRC 10603, *Brevibacterium linens* BCRC 10029, *Carnobacterium divergens* BCRC 14042, *Citrobacter freundii* BCRC 12292, *Enterobacter aerogenes* BCRC 10370, *Escherichia coli* BCRC 12653, *Lactococcus lactis* subsp. *lactis* BCRC 14041, *Leuconostoc mesenteroides* BCRC 14047, *Listeria monocytogenes*, BCRC 14848, *Burkholderia cepacia* ATCC 25416, *Salmonella typhimurium* ATCC 14028, *Sporalactobacillus inulins* BCRC 14647, *Staphylococcus aureus* BCRC 10780, *Yersinia enterocolitica* BCRC 10807.							

2 [a] ATCC, American Type Culture Collection, Manassas, Virginia, USA; BCRC, Bioresources
3 Collection and Research Center, Hsin-Chu, Taiwan. CCUG: Culture Collection, University of
4 Göteborg, Göteborg, Sweden. CB: Commercial bifidobacteria strain. Strains of BLa1-3 and BLg1-3
5 are those isolated from commercial probiotic products.

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6 Table 2. Specific primers used in this study.

Species	Primers	Sequences (5'→3')	Location within gene	Product Size (bp)	Accession No.[a]	Annealing Temperature	Reference
B. animalis subsp. *animalis*/ *B. animalis* subsp. *lactis*	Bani-tF Bani-tR	TCACGACAAGTGGGTTGCCA GTTGATCGGCAGCTTGCCG	316-335 493-475	178	FJ549339	60℃	this study
B. bifidum	Bbif-tF Bbif-tR	GTCAGGTGGGTGTCCCGCGT ATGCCGACGATCTCGACCGG	135-154 516-497	382	FJ549340	60℃	this study
B. breve	Bbre-tF Bbre-tR	CTGGCCGTCAACACTCCG TGGCCACGCTCGACAGCT	482-499 645-628	164	FJ549343	62℃	this study
B. longum subsp. *infantis*	Binf-tF Binf-tR	ATCCGTCCGACCCAGACC CTCGACATCCTCACGGCC	515-532 637-620	123	FJ549355	63℃	this study
B. longum subsp. *longum*	BloF BloR	GTATCCGTCCGACCCAGCAG GGTGACGGAGCCCGGCTTG	513-532 673-655	161	FJ549349	63℃	Sheu et al.
Bifidobacterium spp.	Bif-tF Bif-tR	GTCCGTGACCTCCTCGAC GTGGAAGGTCTCGATGGAG	224-241 562-544	339	FJ549338	60℃	this study

7 [a]The accession numbers of *tuf* gene were those from GenBank database.

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8 Table 3. Comparison of nucleotide sequence identities for the sequenced *tuf* genes[a] among different *Bifidobacterium* species[b].

Strain no.	Strain	% Sequence identity for strain no.																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	*B. adolescentis*	—	90.18	90.92	88.84	92.21	90.58	88.96	89.91	91.65	90.39	85.44	90.13	88.32	88.33	90.96	87.08	89.29	90.27
2	*B. animalis* subsp. *animalis*		—	96.54	90.24	92.66	90.27	88.96	92.17	90.63	92.89	85.06	89.99	89.08	89.09	92.26	86.94	90.11	90.40
3	*B. animalis* subsp. *lactis*			—	93.12	93.51	95.07	94.32	96.21	95.39	95.92	95.32	94.73	88.76	88.77	92.13	87.73	89.93	88.84
4	*B. bifidum* BCRC 11844				—	99.72	87.05	93.45	90.58	91.45	89.93	85.48	94.14	92.55	92.56	88.09	86.82	85.62	86.54
5	*B. bifidum* BCRC 14613					—	90.39	99.72	92.69	93.74	93.43	88.19	97.76	97.76	97.76	91.44	89.55	89.63	90.21
6	*B. boum*						—	86.93	87.87	87.97	88.58	86.81	86.61	86.45	86.46	88.08	88.56	89.54	97.62
7	*B. breve*							—	88.52	89.71	90.77	84.08	95.45	96.40	96.40	88.52	85.44	87.37	87.07
8	*B. cuniculi*								—	92.55	93.45	86.53	90.95	88.79	88.80	91.16	88.32	87.83	87.37
9	*B. gallinarum*									—	91.06	85.39	90.59	90.00	90.01	89.75	87.04	87.33	88.24
10	*B. globosum*										—	85.47	90.88	92.28	90.29	91.86	86.28	88.92	88.26
11	*B. indicum*											—	85.34	83.90	83.92	82.24	85.77	86.51	86.77
12	*B. longum* subsp. *infantis*												—	96.13	96.13	89.43	87.00	88.72	86.12
13	*B. longum* subsp. *longum* BCRC 11847													—	99.74	88.81	84.89	87.78	86.43
14	*B. longum* subsp. *longum* BCRC 14664														—	88.82	84.90	87.79	86.44
15	*B. magnum*															—	85.62	89.37	87.43
16	*B. minimum*																—	89.31	88.55
17	*B. subtile*																	—	89.82
18	*B. thermophilum*																		—

9 [a] Partial sequences of the *tuf* genes were obtained as described in Material and Methods.

10 [b] Data in the upper right triangle represent DNA sequence identities of the *tuf* genes in *Bifidobacterium* strains.

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11 Table 4. Labels and PCR detection as well as enumeration cells in commercial yogurt products.

Product no.	Species and cell numbers (log CFU/ml or g) labeled	Total culturable LAB counts (log CFU/ml) determined on MRS agar	PCR		Total culturable bifidobacteria count (log CFU/ml) determined on BIM-25 agar
			Species-specific PCR	qPCR[a] (log CFU/ml)	
No. 1 Yogurt	*B. lactis* (> 6.00) Total LAB > 8.00	8.51±0.23	*B.animalis/ B. lactis*	6.14±0.32	6.15±0.21
No. 2 Yogurt	*L. acidophilus* *B. longum* *L. bulgaricus* *S. thermophilus* Total LAB > 8.00	8.22±0.14	*B. longum*	5.67±0.23	5.93±0.18
No. 3 Yogurt	*L. acidophilus* *B. longum* *L. bulgaricus* *S. thermophilus* Total LAB > 8.00	8.12±0.18	*B. longum*	4.51±0.22	4.68±0.11

12 [a]Species specific primers for *B. lactis* and *B. longum* were used for qPCR

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13 Table 5. Comparison of bacterial enumeration in yogurt products obtained by plate count method and qPCR through storage at 4℃ for
14 15 days.

Yogurts (species)	Day 5		Day 10		Day 15	
	Plate count with BIM-25 agar (CFU/ml)[a]	qPCR (CFU/ml)[b]	Plate count with BIM-25 agar (CFU/ml)[a]	qPCR (CFU/ml)[b]	Plate count with BIM-25 agar (CFU/ml)[a]	qPCR (CFU/ml)[b]
No.1						
B. lactis	6.73 ± 0.58	6.93 ± 0.39	6.54 ± 0.08	6.14 ± 0.32	6.57 ± 0.30	6.76 ± 0.19
No.2						
B. logum	5.29 ± 0.50	5.67 ± 0.23	5.30 ± 0.30	5.47 ± 0.21	5.11 ± 0.45	5.64 ± 0.29
No.3						
B. longum	4.62 ± 0.22	4.51 ± 0.22	4.22 ± 0.13	4.58 ± 0.26	4.26 ± 0.19	4.29 ± 0.14

15 [a]Each value in the table represents the mean value ± standard deviation (SD) from three trials.
16 [b]Number of bacterial cells per ml determined by molecular quantification.

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