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1 **Development of a rapid SNP-typing assay to differentiate *Bifidobacterium animalis* subsp.**
2 ***lactis* strains used in probiotic-supplemented dairy products**

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10 **Running Title:** Novel SNP-based method for differentiating BAL strains

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14 **Keywords:** *Bifidobacterium animalis* subsp. *lactis*, probiotic, SNPs minisequencing, molecular
15 subtyping.

17 **Abstract**

18 Identification at the genus, species, and strain level is desirable when a probiotic
19 microorganism is added to foods. Strains of *Bifidobacterium animalis* subsp. *lactis* (BAL) are
20 commonly used worldwide in dairy products supplemented with probiotic strains. However, strain
21 discrimination is difficult, given the high degree of genome identity (99.975%) between different
22 genomes of this subspecies. Typing of monomorphic species can be efficiently carried out by
23 targeting informative single nucleotide polymorphisms (SNPs). Findings from a previous study
24 analyzing both reference and commercial strains of BAL identified SNPs that could be used to
25 discriminate common strains into 8 groups.

26 This paper describes development of a minisequencing assay based on the primer extension
27 reaction (PER) targeting multiple SNPs that can allow strain differentiation of BAL.
28 Based on previous data, six informative SNPs were selected for further testing and a multiplex
29 preliminary PCR was optimized to amplify the DNA regions containing the selected SNPs.
30 Extension primers (EPs), annealing immediately adjacent to the selected SNPs, were developed and
31 tested in simplex and multiplex PER to evaluate their performance. Twenty-five strains belonging
32 to 9 distinct genomic clusters of *B. animalis* subsp. *lactis*, were selected and analyzed with the
33 developed minisequencing assay, simultaneously targeting the six selected SNPs. Fragment analysis
34 was subsequently carried out in duplicate and demonstrated the assay gave rise to 8 specific
35 profiles, separating the most commonly used commercial strains. This novel multiplex PER
36 approach provides a simple, rapid, flexible SNP-based subtyping method for proper
37 characterization and identification of commercial probiotic strains of BAL from fermented dairy
38 products. To assess the usefulness of this method, DNA was extracted from yogurt manufactured
39 with and without the addition of *B. animalis* subsp. *lactis* BB-12. Extracted DNA was then
40 subjected to the minisequencing protocol resulting in a SNP profile matching the profile for the
41 strain BB-12.

42

INTRODUCTION

43

44 *Bifidobacterium lactis* was originally isolated as a moderately oxygen tolerant species from
45 a commercial yogurt sample (Meile et al., 1997). *B. animalis* subsp. *lactis*, as it was later re-
46 classified (Masco et al., 2004), exhibits certain technological advantages such as acid- and oxygen
47 tolerance when compared to other species within the same genus. These characteristics allow for
48 successful inclusion in commercial food products (Matsumoto et al., 2004, Simpson et al., 2005,
49 Vernazza et al., 2006). Health promoting benefits have also been attributed to strains of this
50 subspecies, including reduced gut transit time and immune modulation (Marteau et al., 2002, Veiga
51 et al., 2010, Waller et al., 2011). Since at the present time, health benefits attributed to probiotic
52 microorganisms are considered to be strain-specific (FAO/WHO, 2002), health benefits attributed
53 to a strain via clinical studies may not be extrapolated to other strains of the same species or sub-
54 species. Because of this it is critical for suppliers of probiotics and manufacturers of probiotic-
55 containing product to be able to verify the strain of *B. animalis* subsp. *lactis* claimed is, in fact, the
56 strain actually present in the product. Historically this has not been a simple task due to the
57 phenotypic and genotypic similarity of different strains. Methods for strain level differentiation
58 such as Pulsed Field Gel Electrophoresis (PFGE), Randomly Amplified Polymorphic DNA-PCR
59 (RAPD-PCR), and Multi Locus Sequence Typing (MLST) based on conserved gene sequences do
60 not have sufficient discriminatory power to properly differentiate strains of this subspecies (Roy
61 and Sirois, 2000, Jian et al., 2001, Ventura and Zink, 2003, Briczinski and Roberts, 2006).

62 The complete genomes of several strains of *B. animalis* subsp. *lactis* have been sequenced,
63 including most widely used commercial strains (Barrangou et al., 2009, Kim et al., 2009, Garrigues
64 et al., 2010, Sun et al., 2010, Bottacini et al., 2011, Chervaux et al., 2011, Stahl and Barrangou,
65 2012). This work revealed the genomes of these strains exhibit remarkable homogeneity despite
66 unique chronological and geographical isolations complicating strain identification and
67 differentiation. Only recently, a unique strain containing novel genetic content was sequenced and
68 described (Loquasto et al., 2013).

69 By comparing the genome sequences of two strains of *B. animalis* subsp. *lactis*, Briczinski
70 et al were able to develop a SNP-INDEL-based method to differentiate strains (Briczinski et al.,
71 2009). This SNP/INDEL typing scheme provided a reliable method to differentiate strains among
72 this subspecies. However, to perform the analysis, each of 6 informative loci had to be individually
73 amplified using PCR, electrophoresed, purified and sequenced, a time consuming and expensive
74 process.

75 One approach to make the SNP analysis more rapid is minisequencing based on single
76 nucleotide primer extension reactions (PERs). Such an approach has been widely applied in the last
77 few years for rapid differentiation of some human pathogens including *Vibrio* species, *Listeria*
78 species, the *Brucella* genus, common clinically encountered mycobacterial species as well as
79 common *Salmonella* serotypes (Gopaul et al., 2008, Dalmaso et al., 2009, Ben-Darif et al., 2010,
80 Dalmaso et al., 2010, Wang et al., 2010). PER assays have also been developed for typing of
81 *Listeria monocytogenes* and *Escherichia coli* O157 (Van Stelten et al., 2010, Haugum et al., 2011,
82 Lomonaco et al., 2011) as well as for rapid identification/differentiation of *Lactobacillus casei* and
83 *Lactobacillus plantarum* (Huang et al., 2011b, a). Therefore the purpose of the present work was to
84 design and develop a multiplex minisequencing assay, able to rapidly differentiate different groups
85 of *B. animalis* subsp. *lactis* strains in pure culture and when present as a single strain in yogurt
86 products.

87

88 MATERIALS AND METHODS

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90 **Strains and DNA extraction.** To test the ability of the method to correctly differentiate *B. animalis*
91 subsp. *lactis* (BAL), 25 BAL strains representing 9 distinct genotypic groups were selected for use
92 in this study (Table 1). All strains were grown for 18 hours in 10 ml of MRSC broth consisting of
93 MRS (de Man et al., 1960) supplemented with 0.05% L-cysteine hydrochloride. Cells were

94 harvested and genomic DNA was extracted using the Wizard Genomic DNA Purification Kit
95 (Promega, Madison, WI), according to the manufacturer's instructions

96 The ability of this method to distinguish *B. animalis* subsp. *lactis* from other species of
97 *Bifidobacterium* commonly added to probiotic containing dairy products was evaluated by analysis
98 of frozen cultures of *Bifidobacterium longum* subsp. *infantis* (BB-02) and *Bifidobacterium longum*
99 subsp. *longum* (BB-46) (Chr. Hansen, Denmark). DNA was extracted from the pure cultures of
100 each organism alone, when combined together (1:1) and when mixed with BB-12 (1:1:1) using the
101 PowerFood Microbial DNA Isolation Kit (MoBio, Carlsbad, CA). Following isolation, the DNA
102 was subjected to multiplex preliminary PCR and minisequencing.

103 In both cases DNA was quantified by spectrophotometry (Biophotometer 6131, Eppendorf AG,
104 Hamburg, Germany) and stored at -20°C before use.

105

106 **Primer design.** Six loci containing SNPs able to discriminate between different BAL groups as
107 described in Briczinski et al., (2009) were selected as targets for this study. Sequences of internal
108 fragments in these loci were aligned using Mega 5.0 software (Tamura et al., 2007) and six SNPs
109 that were deemed able to differentiate among *B. animalis* subsp. *lactis* were selected for further
110 testing (Table 2). Primers for the preliminary multiplex PCR were designed approximately 200-300
111 nt upstream and downstream from the diagnostic SNPs (Table 2). Additionally, primers were
112 designed so that the amplification products had different sizes, to allow visualization following
113 multiplex PCR.

114 For the multiplex PER, extension primers (EPs) were designed immediately adjacent,
115 upstream or downstream, to the selected SNP (Table 2). To allow efficient separation during
116 capillary electrophoresis, two of the six EPs were designed with poly (dT)s tails attached to their 5'
117 ends (Table 2). All EPs were evaluated individually prior to testing in multiplex. Primers were
118 synthesized by the Penn State Genomics Core Facility and Integrated DNA Technologies
119 (Coralville, IA).

120

121 **Preliminary and Multiplex PCR.** Specificities of primers targeting the loci of interest were
122 assessed by singleplex PCR using DNA from DSM 10140 and BI-04. Amplifications were
123 performed in a final volume of 50 μ l containing 75 mM Tris-HCl (pH 8.8), 1 unit of Recombinant
124 Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA), 0.2 mM each of dATP, dCTP, dGTP,
125 dTTP (Pharmacia, Uppsala, Sweden), 5 μ M of each primer, 1.5 mM MgCl₂, 0.5 mM and 250 ng of
126 DNA template. Once primer specificity was verified, multiplex PCR was performed on DNA
127 extracted from all strains of interest in order to detect potentially undesirable pairings of primers.
128 Multiplex PCR reactions were carried out as described above with 5 μ M of each primer except for
129 INDEL2_F/R, which was used at 10 μ M. Amplifications were performed using a Mastercycler®
130 pro thermocycler (Eppendorf North America, Inc., Hauppauge, NY, USA) beginning with 5 min at
131 95°C followed by 30 cycles at 95°C for 30 s, 58°C for 1 min, 72°C for 2 min and a final extension
132 of 72°C for 7 min. Amplicons were resolved by electrophoresis on a 2.0% agarose gel (Invitrogen,
133 Carlsbad, CA, USA) and visualized on a UV transilluminator and images captured by an
134 AlphaImager 3300 gel documentation system (Alpha Innotech, San Leandro, CA).

135

136 **Primer extension reaction (PER).** Multiplex PCR products were used as templates for subsequent
137 PERs following enzymatic clean-up to remove unincorporated dNTPs and primers. To clean up the
138 samples, 2 μ l of Exo-Sap (Affymetrix, Santa Clara, CA, USA) was added to 5 μ L of PCR products
139 and incubated at 37°C for 1 h and then heated to 80°C for 15 min to inactive residual enzyme.

140 EPs were first tested individually to evaluate performance and validate their molecular size.

141 Simplex PERs were performed in a total volume 10 μ L according to the SNaPshot multiplex Kit
142 protocol (Applied Biosystems, Foster City, California, USA) with the following minor
143 modifications: 3 μ L of purified preliminary PCR products; 3 μ L of SnaPshot Multiplex Ready
144 Reaction Mix and the EP at a final concentration of 0.2 μ M.

145 Subsequently, multiplex PERs were performed using 0.1 μ M each of sequencing primers
146 EP1, EP3, EP4, and EP6 and 0.6 μ M of sequencing primers EP2 and EP5, respectively. The
147 concentration of each primer was chosen based on results from the preliminary assays. The
148 minisequencing reactions consisted of 25 cycles with the following conditions: denaturation at 96°C
149 for 10 s, annealing at 50°C for 5 s and extension at 60°C for 30 s. Reactions were performed using a
150 Mastercycler® pro thermocycler (Eppendorf North America, Inc., Hauppauge, NY, USA)

151 The final volume of the PER (10 μ L) was treated post-extension with 1 unit of Calf
152 Intestinal Alkaline Phosphatase (CIAP) (Fermentas, Burlington, California, USA) and incubated at
153 37°C for 1 h followed by 15 min at 75°C. This was carried out to remove the 5'-phosphoryl group
154 of the [F]ddNTPs of un-incorporated [F]ddNTPs, that could otherwise co-migrate with the extended
155 primers and produce a high background signal.

156 Finally, 1 μ L of the post-extension purified PER product was mixed with 24.6 μ L of Hi-Di
157 formamide and 0.4 μ L of GeneScan 120 LIZ size standard (Applied Biosystems, Foster City,
158 California, USA). Each multiplex minisequencing reaction was analyzed with an ABI 310 Genetic
159 Analyzer (Applied Biosystems, Foster City, CA, USA), at the Penn State Genomics Core Facility
160 (University Park, PA, USA). Electropherograms were evaluated with PeakScanner 1.0 software
161 (Applied Biosystems, Foster City, CA, USA), available for free at
162 <http://www.lifetechnologies.com/order/catalog/product/4381867>.

163

164 **Discriminatory Index.** The discriminatory power of the method (Hunter and Gaston, 1988) with
165 95% confidence interval was calculated by EpiCompare version 1.0 (Ridom, Münster, Germany).

166

167 **Product production and analysis.**

168 To test the applicability of the method for the analysis of probiotic-supplemented dairy products,
169 total genomic DNA was also directly extracted from yogurt samples. A yogurt-based strawberry
170 smoothie was manufactured with and without the addition of *B. animalis* subsp. *lactis* BB12 at a

171 level of ca. 1.30×10^8 CFU/g. These products are manufactured as part of a previous (Merenstein
172 et al., 2011) and another ongoing study. Compositional details are outlined in Table 3. Viable
173 counts of *B. animalis* subsp. *lactis* were determined by pour-plating on MRS-NNPL (Laroia and
174 Martin, 1991) with growth under anaerobic conditions at 37°C for 72 hours. Total DNA was
175 extracted from both products using MoBio PowerFoods DNA extraction kit according to
176 manufacturer instructions. Subsequently, isolated DNA was subjected to minisequencing analysis,
177 as detailed above. In addition, DNA extracted from each product was also evaluated using the sub-
178 species specific primers Bflact2/5 (Ventura et al., 2001) and genus-specific primers Lm3/26
179 (Ventura and Zink, 2002).

180

181

RESULTS

182

183 **Primer design and PCR.** Analysis of the alignment of the BAL sequences obtained from
184 Briczinski et al., (2009) confirmed the combination of selected SNPs could provide profiles specific
185 for 8 BAL groups (Table 1). Primers tested in simplex reactions produced amplicons of the
186 expected size (Figure 1). All evaluated strains yielded amplicons of the expected size (368 bp for
187 igr6, 730 bp for igr9, 490 bp for Balat_0051, 418 bp for Balat_0710, 627 bp for *glcU*, and 511 bp
188 for INDEL2). Amplicons from reactions with DSM 10140 and BI-04 are shown in Figure 1.

189 After verification in simplex reactions, the primers were combined and used in multiplex PCR.

190 Evaluation of the PCR amplicons from multiplex reactions using DSM 10140 and BI-04 are shown
191 in lanes 1-6 and 9-14 of Figure 1, respectively. Analysis of non-BAL species BB-02 and BB-46,
192 revealed 3 or 2 amplicons, respectively (data not shown).

193 **Minisequencing.** For all BAL strains, simplex minisequencing gave rise to a peak of the expected
194 color green (A), black (C), blue (G) and red (T) specific to the fluorescently labeled [F]ddNTP
195 incorporated at the diagnostic SNP site. Following optimization, multiplex PERs were performed
196 on DNA extracted from all BAL strains giving rise to 8 specific patterns (Figure 2, Table 1).

197 Representative chromatograms for each of the eight distinct BAL_SNP_Groups are shown in
198 Figure 2. The SNP-specific extension primers were designed for simultaneous annealing and single
199 nucleotide extension, and their length ranged in size from 17 bp to 39 bp to allow simultaneous
200 resolution via capillary electrophoresis. Following optimization, the preliminary multiplex PCR
201 products served as template for subsequent multiplex PER reactions, performed using the mixture
202 of the six extension primers. All evaluated strains gave the expected profiles, giving rise to 8
203 specific patterns (Figure 2, Table 1). With the exception of BAL_SNP_7, the PER data all
204 corresponded to one SNP profile of the corrected Briczinski et al. typing scheme (Table 1).
205 BAL_SNP_7 contained both Briczinski groups 12 and 13, because the PER was unable to resolve a
206 difference in a string of “G’s”.

207 Minisequencing for the two non-BAL species, BB-02 and BB-46, did not give rise to any
208 peaks, regardless of whether they were pure or in a mixture. When combined with BAL strain BB-
209 12 the expected chromatogram was obtained (data not shown).

210 **Differentiation of Strains.** Using one isolate for each of the profiles listed in Table 1 a
211 Discriminatory Index of 0.97 was calculated with a 95% confidence interval of .74-.90. This
212 indicates that the method provided a 97% chance that analysis of any two randomly selected strains
213 of this closely related strain collection would be placed into two different groups.

214 **Utilization of the method to assess a yogurt product.** The minisequencing protocol was applied
215 to drinkable strawberry yogurt products manufactured with and without *B. animalis* subsp. *lactis*
216 BB-12. Viable bifidobacterial cells counted on MRS-NNLP agar were present at a level of $1.65 \times$
217 10^8 CFU/ml in the yogurt drink manufactured with BB-12 addition and was undetectable in yogurt
218 drink manufactured without BB-12. PCR analysis of total DNA extracted from yogurt drink
219 samples containing BB-12 using genus- and subspecies-specific primers resulting in amplicons of
220 appropriate size indicating presence of DNA from the organism. Analysis of DNA from yogurt
221 manufactured without BB-12 did not result in amplification (data not shown). Minisequencing
222 applied to DNA from yogurt manufactured with BB-12 resulted in the profile of AGTTTG,

223 appropriately corresponding to BAL_SNP_3, which contains *B. animalis* subsp. *lactis* BB-12 (see
224 Table 2).

225

226

DISCUSSION

227 Detection of minisequencing products may require as little as 18 min compared with the 2.5
228 hours required for capillary electrophoresis of sequencing products. Minisequencing also yields
229 results that are simple to analyze and interpret. Additionally, by developing a multiplex preliminary
230 PCR assay to simultaneously amplify all the loci of interest, the time and expense required to
231 complete the analysis would be reduced. Identification and differentiation of specific strains of *B.*
232 *animalis* subsp. *lactis* has traditionally been difficult and unreliable due to the high degree of
233 relatedness between strains of interest. PFGE has been considered the “gold-standard” for the
234 differentiation of bacterial strains (FAO/WHO, 2002) however, PFGE does little to resolve strains
235 of this monomorphic subspecies (Mayer et al., 2007, Briczinski et al., 2009). In this work, a
236 SNP/INDEL typing scheme developed in our lab (Briczinski et al., 2009), were adapted for
237 minisequencing analysis. Notably, minisequencing can be used only to discriminate those
238 species/strains for which DNA sequence data is already available. The incorporation of new SNPs
239 in existing SNP-typing schemes would likely be required to differentiate newly defined strains or
240 groups of strains. Such SNPs would have to be identified relying on traditional sequencing data.
241 Over the last few years minisequencing approaches have been successfully developed for
242 identification or characterization of bacterial species belonging to a variety of different genera
243 (Gopaul et al., 2008, Dalmaso et al., 2009, Ben-Darif et al., 2010, Wang et al., 2010, Lomonaco et
244 al., 2011).

245 Previous work with *B. animalis* subsp. *lactis* identified 14 distinct genomic clusters among a
246 collection of 24 strains of *B. animalis* subsp. *Lactis* (Briczinski et al., 2009). However, the current
247 analysis resolved the same collection of strains into 8 genomic clusters. In particular, during the
248 current study some strains (Table 1) did not give rise to the expected SNP profile reported by

249 Briczinski et al (2009). Re-sequencing of the DNA from that study revealed sequence data
250 transposition errors. Re-analysis of the corrected sequence data, revealed 9 groups were observed
251 by Briczinski et al. (2009).

252 Discriminatory Power (D) expresses the probability that two unrelated strains randomly
253 sampled in the analyzed population will be assigned a different type by the typing system.
254 Therefore, only one isolate for each profile was used for the calculation. The assay developed
255 herein was able to discriminate 8 out of 9 previously identified distinct genomic clusters (Briczinski
256 et al., 2009) and therefore yielded a discriminatory power of 0.97. The PER method develop herein
257 is not able to resolve groups 12 and 13, originally separated based on the differing number of “G’s”
258 (7 or 8) at the locus Balat_0141 (Briczinski et al., 2009). However, the sequence surrounding this
259 SNP in groups 12 and 13 in both the forward and reverse direction is identical, and thus these two
260 groups were combined into the BAL_SNP_7 group in this work. Direct analysis of a drinkable
261 strawberry yogurt demonstrated the utility of this method to detect a single strain of *B. animalis*
262 subsp. *lactis* present in a yogurt smoothie. The product made without bifidobacteria showed no
263 amplification using the six preliminary PCR primers highlighting the lack of cross-reactivity with
264 traditional yogurt cultures and which was further confirmed by the lack of a positive reaction with
265 genus and subspecies-specific primers. The lack of cross-reactivity with cultures of *B. longum*
266 subsp. *longum* and *B. longum* subsp. *infantis* was also confirmed, as the preliminary PCR did not
267 show amplification for all targeted genes and therefore that sample would not be subjected to the
268 minisequencing assay (which would result in no peaks anyway). This method could be used to
269 identify a culture supplier’s own strain in customer’s products. This method may also be used to
270 lend credence to clinical trials, when *B. animalis* subsp. *lactis* is being used as an intervention. The
271 assay developed herein can be employed to prove that recovered strains are the intervention strain
272 and not a strain already present in the panelist.

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CONCLUSIONS

Traditionally, identification of *B. animalis* subsp. *lactis* strains has been difficult and time consuming. In the method described here, we were able to directly purify all six PCR products from a single multiplex PCR reaction prior to minisequencing, resulting in a savings of reagents and time. Additionally, we demonstrated the value of this method in the identification of BAL_SNP_Groups directly from a yogurt product without plating on expensive selective media. The method described here can be used in research laboratories, clinical trials evaluating the efficacy of *B. animalis* subsp. *lactis*, or in commercial products in order to identify commercial strains utilized in the industry.

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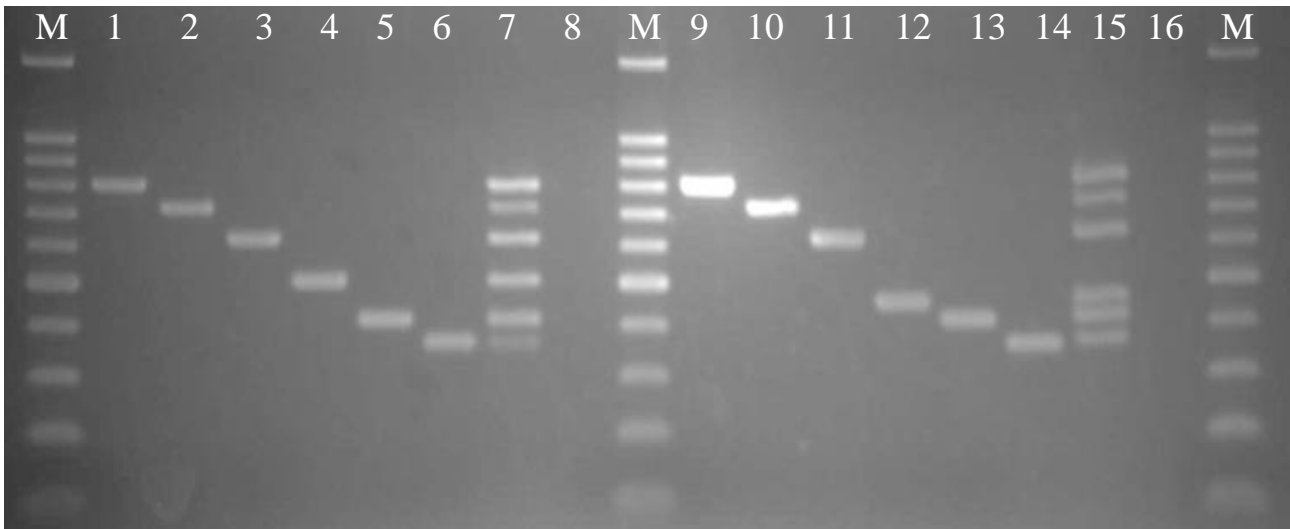
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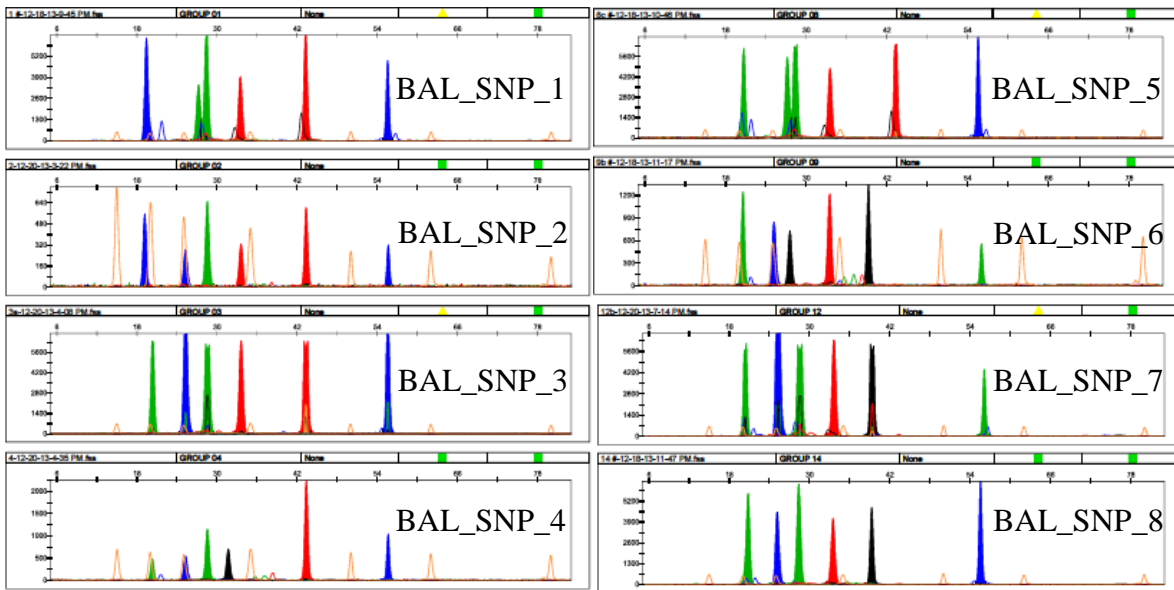
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460 **Figure 1** – Preliminary multiplex amplification of the 6 *Bifidobacterium animalis* subsp. *lactis*
461 genomic regions: igr6, igr9, Balat_0051, Balat_0710, *glcU* and INDEL2 in strains DSM 10140 and
462 B1-04.
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467 Figure 1 Legend- M- 100 bp DNA Ladder; (Primer/Locus) Lane 1- DSM 10140/Balat_0051; Lane
468 2- DSM 10140/igr9; Lane 3- DSM 1014/*glcU*; Lane 4- DSM 10140/INDEL2; Lane 5- DSM
469 10140/Balat_0710; Lane 6- DSM 10140/igr6; Lane 7- Multiplex; Lane 8- Negative Control; Lane
470 9- B1-04/ Balat_0051; Lane 10- B1-04/igr9; Lane 11- B1-04/*glcU*; Lane 12- B1-04/INDEL2; Lane
471 13- B1-04/Balat_0710; Lane 14- B1-04/igr6; Lane 15- Multiplex; Lane 16- Negative Control

472 **Figure 2** – Representative chromatograms for each of the specific profiles obtained by multiplex
473 primer extension reaction (PER) of strains of *Bifidobacterium animalis* subsp. *lactis*.
474 Minisequencing peaks colors correspond to green (A), black (C), blue (G) and red (T).
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Table 1 – Description of the 25 *Bifidobacterium animalis* subsp. *lactis* strains analyzed in this study and the resulting 8 SNP profiles.

Strain ¹	Corrected Briczinski et al., (2009) Groups ³	Minisequencing profile	SNP profile # (This Work)
DSM 10140	1	GATTTG	BAL_SNP_1
RB 1280	2	GGTTTG	BAL_SNP_2
RB 1573	3	AGTTTG	BAL_SNP_3
RB 4052	3	AGTTTG	BAL_SNP_3
RB 4536	3	AGTTTG	BAL_SNP_3
RB 7339	3	AGTTTG	BAL_SNP_3
RB 9321	3	AGTTTG	BAL_SNP_3
Bb-12	3	AGTTTG	BAL_SNP_3
RB 5851²	3	AGTTTG	BAL_SNP_3
RB 4753²	3	AGTTTG	BAL_SNP_3
RB 1791²	4	AGTCTG	BAL_SNP_4
RB 7239	4	AGTCTG	BAL_SNP_4
HN019	8	AATTTG	BAL_SNP_5
RB 4825	9	AGGTCA	BAL_SNP_6
RB 5251	9	AGGTCA	BAL_SNP_6
B1-04	9	AGGTCA	BAL_SNP_6
RB 5859²	9	AGGTCA	BAL_SNP_6
RB 3046²	9	AGGTCA	BAL_SNP_6
RB 5422²	9	AGGTCA	BAL_SNP_6
RB 1281	12	AGTTCA	BAL_SNP_7
RB 5733	12	AGTTCA	BAL_SNP_7
RB 8613	12	AGTTCA	BAL_SNP_7
RB 9632	12	AGTTCA	BAL_SNP_7
RB 0171	13	AGTTCA	BAL_SNP_7
ATCC 27536	14	AGTTTCG	BAL_SNP_8

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¹RB strains were obtained from the strain collection at Penn State University. DSM 10140 was obtained from The German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. ATCC 27536 was obtained from the American Type Culture Collection, Manassas, Virginia. Bb-12 was obtained from Chr. Hansen, Milwaukee, Wisconsin. HN019 and B1-04 were obtained from DuPont Nutritional and Health, Madison, Wisconsin.

²During this study the indicated strains did not give rise to the expected SNP profile reported by Briczinski et al. (2009). Re-sequencing of the DNA from that study revealed data transposition errors. The correct groupings are given in the second column of this table (³).

492 **Table 2** – PCR and extension primers (EPs), fragment sizes and allelic locations for the 6 *Bifidobacterium animalis* subsp. *lactis* genomic regions
 493 and the 6 SNPs interrogated in this study.

	Primer	Primer sequences (5'–3')	Fragment Size (bp)	Allelic location on strain DSM 10140 (GenBank: CP001606)
Preliminary PCR ¹	Balat_0051F	5'-GAGACCGTCATCGAGTCC -3'	783	53234-53723
	Balat_0051R	5'-CACTGAGCGAGTAATCACAGGA -3'		
	igr6F	5'-TGCGAGATGAAACGGATATG-3'	368	686118-687770
	igr6R	5'-ACCTTCGTATGGTTCGAGCTG-3'		
	Balat_0710F	5'- GCATTGGTTCAGGGTCTGTT -3'	418	839211-839628
	Balat_0710R	5'- GTAGCCCCAATGGTTCGAAT -3'		
	INDEL2_F	5'- AACCGTCTGCTGCTGTTTCT -3'	511/457	902690-903183
	INDEL2_R	5'- GGTGGCTTTCTGCCAAT -3'		
	<i>glcU</i> _F	5'- GTCCGGCGCATTGTAGATAG -3'	627	1259721- 1260328
	<i>glcU</i> _R	5'- CGTACAACGATTTGGCATTG -3'		
	igr9_F	5'-GTTTCGAGGCCTGGTATTGA -3'	730	1636223-1636935
	igr9_R	5'-ATCACGTGGTTGCCTTGC -3'		
	Primer extension reaction (PER)	EP1 (Balat_0051)	5'- CGGCGCCCACCGCCTCA-3'	17+1
EP2 (igr9)		5'- GTTGCCGTTCTGCTTCAG -3'	18+1	1636587-1636604
EP3 (INDEL2) ²		5'- TGTGTTCCGACATCATCTTCGACC -3'	24+1	902969-902946
EP4 (<i>glcU</i>)		5'- ACAACGCAATGAGCATGCCCATGTGAAG -3'	28+1	1260287-1260314
EP5 (Balat_0710)		5'- (18T)CGCGCTCGTCGCCGGCGTGG -3'	38+1	839408-839427
EP6 (igr6)		5'- (33T)CTAGTAAAGATGTGCCGGCA-3'	53+1	686979 -686998

494 ¹These regions represented intergenic regions 6 and 9 (igr6 and igr9); a transposase (Balat_0051); a hypothetical membrane protein (Balat_0710); a
 495 putative glucose uptake permease (*glcU*); and an insertion/deletion in the long-chain fatty acid-CoA ligase (INDEL2).

496 ²Primer was designed in a reverse position

497 **Table 3-** Product composition of strawberry yogurt drink evaluated for *B. animalis* subsp.
498 *lactis* content.

Component	Composition in Yogurt Drink
Fat	1.0%
Milk Solids Non-Fat	6.8%
Sucrose	6.1%
Corn Syrup Solids	6.4%
Pectin	0.4%
Strawberry Puree	3.0%
Total Solids	23.7%

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