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

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

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Abstract

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

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

INTRODUCTION

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

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

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

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

MATERIALS AND METHODS

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

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

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

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

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

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

Preliminary and Multiplex PCR. Specificities of primers targeting the loci of interest were assessed by singleplex PCR using DNA from DSM 10140 and Bl-04. Amplifications were performed in a final volume of 50 µl containing 75 mM Tris–HCl (pH 8.8), 1 unit of Recombinant Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA), 0.2 mM each of dATP, dCTP, dGTP, dTTP (Pharmacia, Uppsala, Sweden), 5 µM of each primer, 1.5 mM $MgCl_2$, 0.5 mM and 250 ng of DNA template. Once primer specificity was verified, multiplex PCR was performed on DNA extracted from all strains of interest in order to detect potentially undesirable pairings of primers. Multiplex PCR reactions were carried out as described above with 5 µM of each primer except for INDEL2_F/R, which was used at 10 µM. Amplifications were performed using a Mastercycler® pro thermocycler (Eppendorf North America, Inc., Hauppauge, NY, USA) beginning with 5 min at 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 of 72°C for 7 min. Amplicons were resolved by electrophoresis on a 2.0% agarose gel (Invitrogen, Carlsbad, CA, USA) and visualized on a UV transilluminator and images captured by an AlphaImager 3300 gel documentation system (Alpha Innotech, San Leandro, CA).

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

EPs were first tested individually to evaluate performance and validate their molecular size. Simplex PERs were performed in a total volume 10 µL according to the SNaPshot multiplex Kit protocol (Applied Biosystems, Foster City, California, USA) with the following minor modifications: 3 µL of purified preliminary PCR products; 3 µL of SnaPshot Multiplex Ready Reaction Mix and the EP at a final concentration of 0.2 µM.

145 Subsequently, multiplex PERs were performed using 0.1 μM each of sequencing primers
146 EP1, EP3, EP4, and EP6 and 0.6 μM 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 μL) 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 μL of the post-extension purified PER product was mixed with 24.6 μL of Hi-Di
157 formamide and 0.4 μL 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

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

RESULTS

Primer design and PCR. Analysis of the alignment of the BAL sequences obtained from Briczinski et al., (2009) confirmed the combination of selected SNPs could provide profiles specific for 8 BAL groups (Table 1). Primers tested in simplex reactions produced amplicons of the expected size (Figure 1). All evaluated strains yielded amplicons of the expected size (368 bp for igr6, 730 bp for igr9, 490 bp for Balat_0051, 418 bp for Balat_0710, 627 bp for *glcU*, and 511 bp for INDEL2). Amplicons from reactions with DSM 10140 and Bl-04 are shown in Figure 1. After verification in simplex reactions, the primers were combined and used in multiplex PCR. Evaluation of the PCR amplicons from multiplex reactions using DSM 10140 and Bl-04 are shown in lanes 1-6 and 9-14 of Figure 1, respectively. Analysis of non-BAL species BB-02 and BB-46, revealed 3 or 2 amplicons, respectively (data not shown).

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

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

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

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

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

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

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

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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Figure 1 – Preliminary multiplex amplification of the 6 *Bifidobacterium animalis* subsp. *lactis* genomic regions: igr6, igr9, Balat_0051, Balat_0710, *glcU* and INDEL2 in strains DSM 10140 and Bl-04.

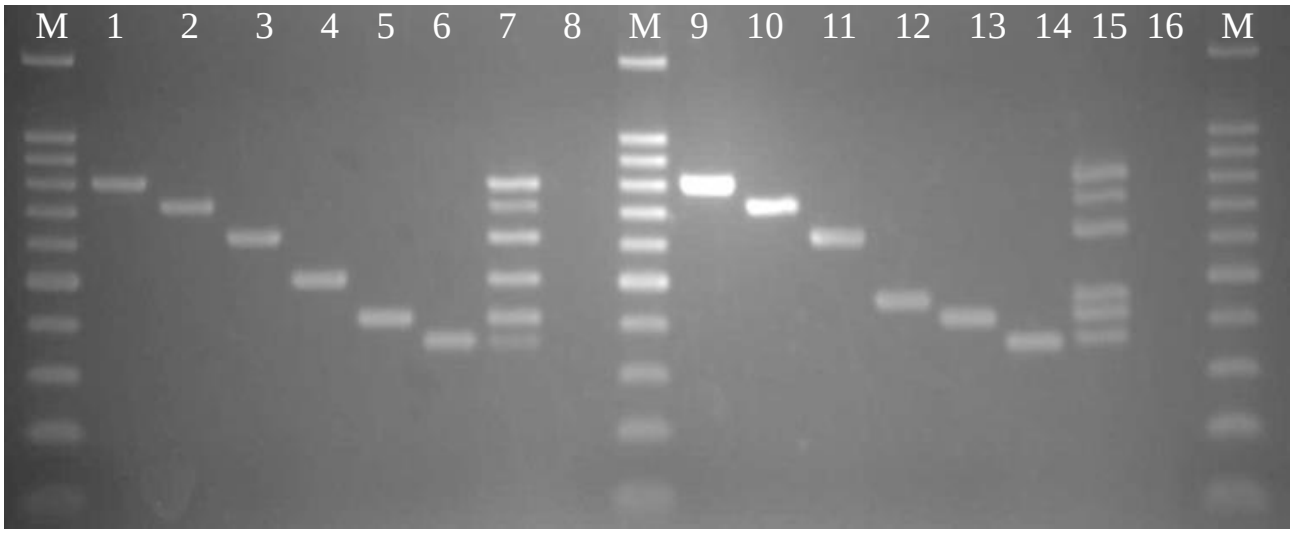

Figure 1 Legend- M- 100 bp DNA Ladder; (Primer/Locus) Lane 1- DSM 10140/Balat_0051; Lane 2- DSM 10140/igr9*;* Lane 3- DSM 1014/*glcU*; Lane 4- DSM 10140/INDEL2; Lane 5- DSM 10140/Balat_0710; Lane 6- DSM 10140/igr6; Lane 7- Multiplex; Lane 8- Negative Control; Lane 9- Bl-04/ Balat_0051; Lane 10- Bl-04/igr9; Lane 11- Bl-04/*glcU*; Lane 12- Bl-04/INDEL2; Lane 13- Bl-04/Balat_0710; Lane 14- Bl-04/igr6; Lane 15- Multiplex; Lane 16- Negative Control

Figure 2 – Representative chromatograms for each of the specific profiles obtained by multiplex primer extension reaction (PER) of strains of *Bifidobacterium animalis* subsp. *lactis*. Minisequencing peaks colors correspond to green (A), black (C), blue (G) and red (T).

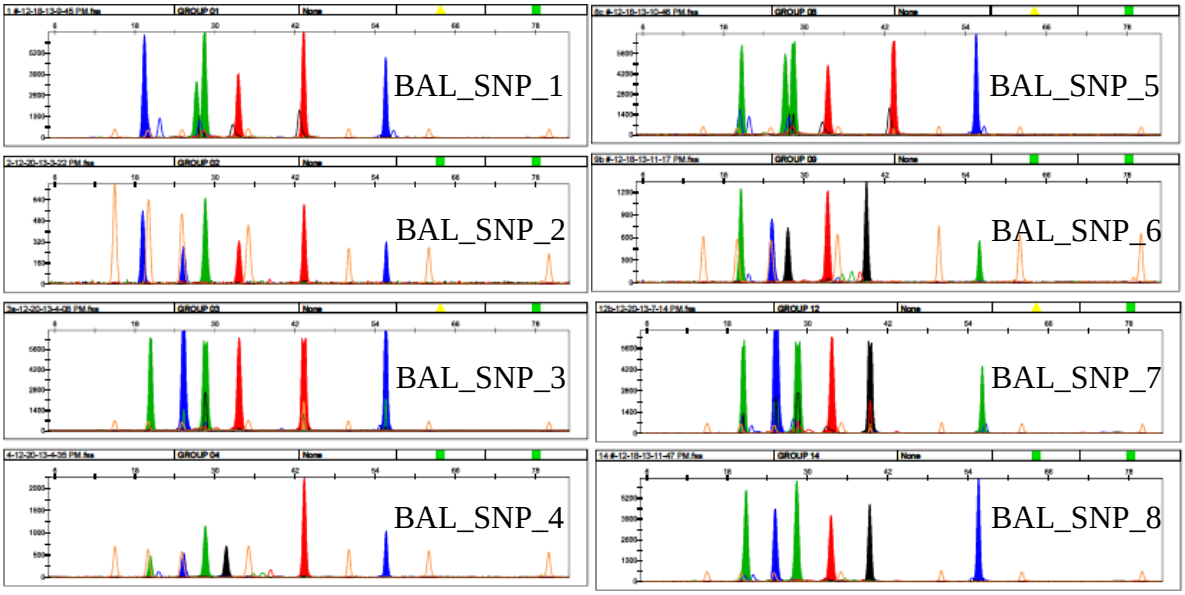

Table 1 – Description of the 25 *Bifidobacterium animalis* subsp. *lactis* strains analyzed in this study and the resulting 8 SNP profiles.

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 5851[2]	3	AGTTTG	BAL_SNP_3
RB 4753[2]	3	AGTTTG	BAL_SNP_3
RB 1791[2]	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
Bl-04	9	AGGTCA	BAL_SNP_6
RB 5859[2]	9	AGGTCA	BAL_SNP_6
RB 3046[2]	9	AGGTCA	BAL_SNP_6
RB 5422[2]	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	AGTTCG	BAL_SNP_8

[1] RB 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 Bl-04 were obtained from DuPont Nutritional and Health, Madison, Wisconsin.

[2]During 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]).

Table 2 – PCR and extension primers (EPs), fragment sizes and allelic locations for the 6 *Bifidobacterium animalis* subsp. *lactis* genomic regions 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'-ACCTTCGTATGGTCGAGCTG-3'		
	Balat_0710F	5'- GCATTGGTTCAGGGTCTGTT -3'	418	839211-839628
	Balat_0710R	5'- GTAGCCCCAATGGTCGAAT -3'		
	INDEL2_F	5'- AACCGTCTGCTGCTGTTTCT -3'	511/457	902690-903183
	INDEL2_R	5'- GGTTGGCTTTCTGCCAAT -3'		
	*glcU*_F	5'- GTCCGGCGCATTGTAGATAG -3'	627	1259721- 1260328
	*glcU*_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	53304-53320
	EP2 (igr9)	5'- GTTGCCGTTCTGCTTCAG -3'	18+1	1636587-1636604
	EP3 (INDEL2)[2]	5'- TGTGTTCCGACATCATCTTCGACC -3'	24+1	902969-902946
	EP4 (glcU)	5'- ACAACGCAATGAGCATGCCCATGTGAAG -3'	28+1	1260287-1260314
	EP5 (Balat_0710)	5'- (18T)CGCGCTCGTCGCCGGCGTGG -3'	38+1	839408-839427
	EP6 (igr6)	5'- (33T)CTAGTAAAGATGTGCGGGCA-3'	53+1	686979 -686998

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

[2]Primer was designed in a reverse position

Table 3- Product composition of strawberry yogurt drink evaluated for *B. animalis* subsp. *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%**