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Rapid discrimination of *Bifidobacterium animalis* subspecies by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry

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Abstract

Currently, the species *Bifidobacterium animalis* consists of two subspecies, *B. animalis* subsp. *lactis* and *B. animalis* subsp. *animalis*. Among these two subspecies, *B. animalis* subsp. *lactis* is especially important because it is widely used in the manufacture of probiotic dairy products. The application of these microbes in the food industry demands fast, accurate and low cost methods to differentiate between species and strains. Although various genotypic methods have been employed to discriminate between these two subspecies, they are not easily adapted for rapid identification in the industry. The purpose of this study was to evaluate the use of matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) to differentiate between the two subspecies of *B. animalis*, and for discrimination at strain level. We identify twenty-three strains of *B. animalis* at subspecies and strain level by genotypic methods and by proteomics using MALDI-TOF MS. The proteomics identification by MALDI-TOF was nearly identical to that obtained by genotypic identification using comparison of *tuf* and *atpD* genes sequences, and single-nucleotide polymorphisms (SNPs), insertions, and deletions (INDELs). We identified four protein markers, L1, L2, A1, and A2, which are useful for discriminating between both subspecies. Proteomics identification using MALDI-TOF MS was therefore an accurate method for discriminating and identifying these bacteria. Given the speed in which this method is achieved (~20 min including sample preparation), MALDI-TOF MS is promising as a tool for rapid discrimination of starter cultures and probiotics.

Keywords

Bifidobacterium animalis subsp. *lactis*; *Bifidobacterium animalis* subsp. *animalis*; MALDI-TOF; mass spectrometry

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1. Introduction

Bifidobacteria are natural inhabitants of the human and mammalian gastrointestinal tract (GIT) and have been widely used as probiotics in food products (Sanders et al., 1996, Gomes and Malcata, 1999; Turrone et al., 2009). One bifidobacterial species of considerable importance is *Bifidobacterium animalis*, which contains the two subspecies, *lactis* and *animalis*. Importantly, *Bifidobacterium animalis* subsp. *lactis* is commonly used as a probiotic in dairy products and infant foods, in part, due to its tolerance of the stressful conditions of both commercial culture production and the gastrointestinal tract (Meile et al., 1997, Prasad et al., 1998, Masco et al., 2005, 2007, Mättö et al. 2006, Li et al., 2010, Zacarias et al., 2011). To guarantee the quality, safety and correct labelling of products that contain probiotic microorganisms, it is important to quickly and accurately identify the microorganisms added at species and strain level (Saarela et al., 2000).

Traditional identification of bifidobacteria based on phenotypic tools are increasingly being replaced with molecular methods offering a more rapid analysis, higher reproducibility and more accurate results (Matsuki et al., 2002, Ventura et al., 2004). In the last decade, several molecular methods have been proposed to discriminate between the two subspecies of *B. animalis*, subsp. *animalis* and subsp. *lactis*, e.g., ribotyping (Mättö et al., 2004), specific PCR primers Bflact2-Bflact5 (Ventura et al., 2001), comparative sequence analysis of specific genes (Ventura et al., 2003, Masco et al., 2004), and the 16S-23S ITS region (Ventura et al., 2003), multiplex PCR (Ventura et al., 2002), multilocus sequence typing (MLST) (Delétoile et al., 2010), pulsed-field gel electrophoresis (PFGE) (Ventura et al., 2002, Mayer et al., 2007), randomly amplified polymorphic DNA (RAPD) analysis (Mayer et al. 2007, Křížová et al., 2008), protein fingerprinting FAFLP (Ventura et al., 2004), and repetitive element sequences techniques: ERIC-PCR (Ventura et al. 2002), BOX-PCR (Ventura et al., 2004), and REP-PCR (Křížová et al., 2008). While some of these techniques such as PFGE or RAPD-PCR, can differentiate strains to an extent, the high degree of genomic identity among *B. animalis* subsp. *lactis* strains, makes strain identification difficult (Mayer et al., 2007, Briczinski et al., 2009). Perhaps the most promising molecular method for differentiation between *B. animalis* strains is MLST analysis of nine polymorphic genetic loci (Briczinski et al., 2009). Although molecular methods are commonly used to identify bacteria, tools for very rapid identification are needed.

As an alternative to molecular methods, various mass spectrometric strategies have been examined to type bacterial species and strains (Mott et al., 2010, Sědo et al., 2011, Carbonnelle, et al., 2011). Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is of interest to clinical microbiologists, because of its powerful features that allow very rapid bacterial identification (taking roughly ~20 min), including clinically important microbes (Mandrell et al., 2005, Dieckmann et al., 2007, Barbuddhe et al., 2008, Benagli et al., 2011) and food microorganisms (Teramoto et al., 2007, Tanigawa et al., 2010, Albesharat et al., 2011, Angelakis et al. 2011). Moreover, this modality has been successfully applied to biomarker discovery and characterization of various bacterial agents, including bifidobacteria strains (Tanigawa et al., 2010, Sato et al., 2011).

The aim of the present work was to evaluate the accuracy and feasibility of MALDI-TOF MS to differentiate between the two subspecies of *B. animalis*, and for identification at strain level.

2. Material and Methods

2.1. Strains and growth conditions

A total of 23 *Bifidobacterium animalis* strains assigned as *B. animalis* subsp. *lactis* and *B. animalis* subsp. *animalis* were obtained from American Type Culture Collection (ATCC), Japan Collection of Microorganisms (JCM), University of California-Davis Viticulture and Enology Culture Collection (Davis, CA), University of Parma bacteria culture collection (Parma, Italy) or isolated from human faeces (Ruiz-Moyano et al. 2008) or dairy products (Table 1). To isolate *B. animalis* strains from dairy products, we used dairy products from seven different companies which contain *B. animalis* as starter culture. One g of each sample was taken aseptically, transferred to a sterile tube, 10-fold diluted with 1% sterile peptone water (Becton Dickinson, Sparks, MD), and homogenized. Serial 10-fold dilutions were prepared with 1% peptone water and inoculated on Raffinose Bifidobacterium agar (RB) prepared according to Hartemink et al. (1996). The plates were incubated 48 h at 37°C in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). Colonies with yellow colour and 3-4 mm of diameter were streaked onto RB agar. Finally, pure isolates were grown in deMan, Rogosa, Sharpe (MRS) media (Becton Dickinson, Sparks, MD) supplemented with 0.05% L-cysteine-HCl and stored at -80°C plus 50% glycerol. Prior to the assays all bacteria strains were subcultured twice in MRS broth supplemented with 0.05% L-cysteine-HCl and incubated at 37°C for 18 h in an anaerobic chamber.

2.2 Isolation of DNA

To get the genomic DNA 1 ml of each culture was centrifuged for 5 min at 10,000 rpm. The bacterial pellet was resuspended and incubated for 30 min at 37°C with enzymatic lysis buffer (20 mM Tris-Cl pH 8.0, 2 mM sodium EDTA, 1.2% Triton X-100, and 40 mg/ml lysozyme (Sigma, MO)), followed by an incubation with proteinase K and buffer for 30 min at 56°C. After enzymatic lysis was carried out, bacterial DNA was isolated from the samples using the DNeasy tissue kit (Qiagen, Valencia, CA) according to the instructions of the manufacturer. DNA quality and yield was checked using a Nanodrop (Wilmington, DE); the DNA was then stored at -20°C until further use.

2.3 Classification and identification of *Bifidobacterium animalis* strains by single-nucleotide polymorphisms (SNPs), insertions, and deletions (INDELs)

All strains were confirmed at genus and subspecies level by using PCR amplification of genes *tuf* and *atpD* as described by Ventura and Zink (2003), and Masco et al. (2004), respectively. Moreover, to discriminate the 23 *Bifidobacterium animalis* at strain level, intragenic regions of ten genes containing SNPs or INDELs (*igr 6*, *igr 8*, *igr 9*, *balat_0051*, *balat_0141*, *balat_0710*, *balat_0864*, *glcU*, *oxc*, and INDEL 2) were selected and amplified based on a previous study by Briczinski et al. (2009). PCR amplifications were performed on an Applied Biosystem 2720m Thermal Cycler (ABI, Mountain View, CA) using the Promega GoTaq Green Master Mix. PCR reactions (50 µL) were prepared according to the manufacturer protocol, and the resulting amplicons separated on a 1% agarose gel, followed by GelRed staining (Phenix Research Products, Candler, NC), and purification using a Qiagen QIAquick PCR Purification Kit (Valencia, CA). Sequencing was performed on an ABI 3730 Capillary Electrophoresis Genetic Analyzer using BigDye Terminator chemistries at the University of California Sequencing Center. Sequencing data for all loci was edited using BioEdit 7.0 (<http://www.mbio.ncsu.edu/BioEdit/BioEdit.html>) and aligned using CLUSTAL W (Thompson et al., 1994). The sequence of the genes *tuf* and *atpD* were checked by nucleotide-nucleotide BLAST of the NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to identify the strains. With respect to the ten genes which contained SNPs or INDELs, sequences from a strain that matched the sequence from the reference strain (*B. animalis* subsp. *lactis* DSMZ 10140) were assigned letter "A". Each

unique sequence from a strain that did not match with the reference strain was assigned a unique letter. These analyses provide an allelic profile for each strain and clustering of the strains in similar genotypes.

2.4 Sample preparation and identification by MALDI-TOF MS

MALDI-TOF mass spectrometry was performed for the 23 strains. The protocol developed by Bruker Daltonics (Bremen, Germany) was then used for the extraction of proteins, as described below.

A colony from 48 h bacterial culture on MRS agar (3 or 4 mg) was washed in 300 μ l of water and additionally in 900 μ l of absolute ethanol. Then the tube was centrifuged at 15000 rpm for 2 min, after which the supernatant was discarded, and the rest of ethanol was removed completely. Cells were dissolved by vortexing in 50 μ l of 70% formic acid (Sigma, MO) and additionally 50 μ l of acetonitrile and vortexing. The mixture was centrifuged at 15,000 rpm for 2 min to pellet the cell debris, and then the supernatant (containing the cell extract) was transferred to a new tube. One μ l of the culture extract was spotted on microscout target plates and allowed to air dry, then overlaid with 1 μ l of matrix solution containing 1.5 mg of α -cyano-4-hydroxynamic acid in 50% acetonitrile with 2.5% trifluoroacetic acid (TFA) in H₂O and dried under ambient conditions. Ten spectra for each strain were obtained and each sample was spotted in duplicate onto the MALDI target plate. The mass range was set from 2000 to 12000 m/z in positive linear mode by averaging of 200 laser shots on a MALDI-TOF MS (Microflex LRF mass spectrometer (Bruker Daltonics, Billerica, MA) analyzed by MALDI-bioTyper (MBT) software, Bruker Daltonics, GmbH, Bremen, Germany).

A cluster analysis with the m/z of the peaks list from each sample was carried out with similarity estimates by using the unweighted pair group method with arithmetic average (UPGMA), from which a dendrogram showing the relationships between *Bifidobacterium animalis* strains was obtained. The analysis was performed by using the NTSYS.PC package, version 2.0 (Rohlf F.J. NTSYS.PC. Numerical taxonomy and multivariate analysis system, version 2.0. New York, N.Y.: Applied Biostatistics Inc.; 1993.)

3. Results and Discussion

3.1- Classifications of the strains

All the strains in the study were confirmed as *Bifidobacterium animalis* subsp. *lactis* or *Bifidobacterium animalis* subsp. *animalis* using subspecies specific primers for the genes *tuf* and *atpD*. The sequencing of these two genes has been shown to differentiate between these two subspecies by Ventura and Zink (2003) and Masco et al. (2004). Twenty of the 23 *B. animalis* strains had the same sequence with both genes, and were identified as subsp. *lactis*, including all the strains isolated from dairy products. Only three strains, JCM 1190, JCM 11658 and ATCC 27672, were identified as subsp. *animalis* and showed different sequence for both genes. Moreover, the identification of three strains, *B. gallicum* JCM 8224, *B. animalis* subsp. *animalis* ATCC 27536 and JCM 7117, do not match with the previous identification for these strains (Table 1). However, in the case of ATCC 27536 and JCM 7117 several authors have already classified these strain as subsp. *lactis* (Ventura and Zink, 2003; Masco et al., 2004; Kwon et al., 2005).

3.2. Identification of *Bifidobacterium animalis* strains by single-nucleotide polymorphisms (SNPs), insertions, and deletions (INDELs)

The 23 strains were examined by this strain-specific typing method. The analysis based on ten loci previously identified by Briczinski et al. (2009) was able to resolve the 23 strains of

B. animalis into 12 different clusters (Table 2). The 20 *B. animalis* subsp. *lactis* strains clustered into 9 different groups. Of these 9 groups, 5 are possibly comprised of a single strain, and four contain more than one strain. Clusters containing more than one strain may include different strains that are not differentiated with the loci examined in this work, or they may represent different isolates of the same strain. This is not unusual as all but one of the strains in multistrain clusters were isolated from dairy products and human faeces. Other authors have shown significant genetic homogeneity of the strain ATCC 27536 with other *B. animalis* subsp. *lactis* used as commercial starter strains using RAPD-PCR and PFGE approaches (Mayer et al. 2007). Interestingly, all 3 strains of *B. animalis* subsp. *animalis* gave a unique allelic profile using this method.

In general the diversity between the strains of *B. animalis* subsp. *lactis* was low, and only two different sequence types were found for all the loci, except for *glcU* locus which showed the highest diversity exhibiting 5 different sequence types (Table 2). Three strains, S332, Y456 and JCM 7117, have a specific allele for the *glcU* locus, these results are in agreement with those determined by Briczinski et al. (2009). On the other hand, all *B. animalis* subsp. *animalis* strains have specific alleles for each locus, except for locus Balat_0051, where only two different sequence types were found, so it was the most conservative locus studied between both subspecies.

3.3 Discrimination by MALDI-TOF MS

Previous efforts have shown that MALDI-TOF analysis can detect slight sequence variations in cellular proteins, and can be useful for discriminating strains at the subspecies, or even at strain, level (Teramoto et al., 2007; Mott et al., 2010; Tanigawa et al. 2010; Sato et al., 2011). The 23 strains of *B. animalis* were profiled by proteomic analysis using the mass of the proteins extracted by the method described above. Sixty-one peaks were monitored in the range from m/z 2,000 to 11,000, a result which is similar to that obtained for clostridia (Mandrell et al., 2005), *Listeria* (Barbuddhe et al., 2008), mycobacteria (Hettick et al., 2006), *Lactococcus lactis* (Tanigawa et al., 2010), and other nonfermenting bacteria (Mellmann et al., 2008). Peaks with m/z 3956, 4000, 4047, 4059, 4859 and 4924 were excluded of the analysis as potential $[M+2H]^{2+}$ ions of the corresponding peaks 7910, 8000, 8090, 8117, 9700 and 9850 ($[M+H]^+$), respectively. The remaining peaks matched from 63.6%–100% (with 35 to 55 peaks matching) among the *B. animalis* strains. In addition, 11 (20%) and 8 (14.5%) peaks were specific for strains of subspecies *animalis* and *lactis* respectively. These results indicate that more than half of the proteins identified by MALDI-TOF MS are the same in both subspecies and reflect their close phylogenetic relationship (Ventura and Zink 2003; Masco et al., 2004). However, more than 30% of the peaks examined have different mass, and thus provide potential markers for subspecies discrimination in *B. animalis*. Similar results by MALDI-TOF MS were found by Tanigawa et al (2010) and Sato et al. (2011), who could differentiate the subspecies of *Lactococcus lactis* and *Bifidobacterium longum* respectively, with the comparison of the mass spectra of ribosomal proteins.

Table 3 summarizes the mass list of the 20 specific peaks found in the 23 *B. animalis* samples. Four peaks were identified as subspecies type-specific; two in subspecies *lactis* (L) and two in subspecies *animalis* (A), and represent potential markers for rapid identification of isolates at the subspecies level. Figure 1 shows a comparison of two representative spectra of the strains *B. animalis* subsp. *lactis* S149 (fig. 1a) and *B. animalis* subsp. *animalis* JCM 11658 (Fig. 1b) with subspecies potential biomarkers peaks designated as asterisks. Subspecies *lactis* type-specific biomarkers were observed at masses of 7998 and 8117 m/z (Table 3; L1, L2, respectively), whereas *animalis* type-specific peaks were noted at 7025 (A1) and 7910 (A2) m/z (Table 3; A1, A2 respectively). The intensity of the biomarker L1 was higher than the other three biomarkers. As a consequence peaks L1 was proved to be

effective and reliable biomarker for discrimination of *B. animalis* subspecies. Using these MALDI-TOF MS criteria, 20 of the 23 strains were identified as *B. animalis* subsp. *lactis* and three as *B. animalis* subsp. *animalis*. This result corresponds with the identification previously determined using the genes *tuf* and *atpD* above (Table 1). Subspecies discrimination of bacteria by MALDI-TOF MS has previously been shown for streptococci (Friedrichs et al., 2007) *Salmonellae* (Dieckmann et al., 2008) *Lactococcus lactis* (Tanigawa et al., 2010) and *Bifidobacterium longum* (Sato et al., 2011).

Eleven peaks in *B. animalis* subsp. *animalis* strains, and 8 in *B. animalis* subsp. *lactis* strains were strain-specific (Table 3). These differences in the mass of the peaks could be due to variations in the amino acid sequence of the protein(s) and are potentially useful for strain identification. To assess if strain discrimination was reliable, the whole peak list was then processed by UPGMA cluster analysis using a DICE coefficient. Figure 2 shows the resulting phylogenetic tree. The samples clearly divided into two major clusters at a similarity of 82,5%. Cluster A contained all the strains of subsp. *animalis*, whereas cluster L contained the strains of the subsp. *lactis*. The three strains of subsp. *animalis* included in the cluster A showed similarity below 92%, and were clearly differentiated at strain level by MALDI-TOF MS. However, the strains of subsp. *lactis* included in cluster L showed more similar spectra, although it was possible to group the 20 subsp. *lactis* strains in 8 groups. These results are in agreement with our genotypic identification, which showed a greater diversity in the allelic profiling of the subsp. *animalis* strains than in subsp. *lactis* strains. Only the strains identified in cluster 2 and 6 by SNP/INDEL analysis (Table 2) could not be differentiated with MALDI-TOF MS proteomics. In general, however, the proteomics profiling by MALDI-TOF MS demonstrated a significant discriminatory power for classification of *B. animalis* strains as the MALDI-TOF results produced nearly the same results as the most robust molecular method used to date for identification *B. animalis* strains (Briczinski et al., 2009).

In conclusion, we describe four potential protein biomarkers, by MALDI-TOF MS that are useful for accurate differentiation of *B. animalis* strains at the subspecies level. In addition, the MALDI-TOF proteomics analysis enabled differentiation of *B. animalis* isolates at strain level. Given that the MALDI-TOF MS methods are very fast (up to 20 min including protein extraction and sample preparation) this typing method would appear to be useful to monitor starter culture and probiotic strains (like *B. animalis*) in various clinical and commercial settings.

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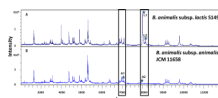


Figure 1. MALDI-TOF MS spectra of proteins of two *Bifidobacterium animalis* type strains. (a) *B. animalis* subsp. *lactis* S149, and (b) *B. animalis* subsp. *animalis* JCM 11658. The relative intensities of ions are shown on the y axis, and the m/z values of the ions are shown on the x axis. The m/z value is the mass to charge ratio. The asterisks indicate specific peaks (biomarkers) for each subspecies. The label L1 and L2 indicate subspecies *lactis* type-specific biomarkers, and A1 and A2 subspecies *animalis* type-specific biomarkers.

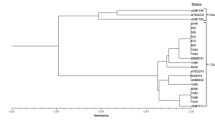


Figure 2. Phylogenetic tree of *B. animalis* strains based on protein profiling employing MALDI-TOF MS. Major clusters (A and L) are divided at a similarity of 82,5%. Cluster A contains *B. animalis* subsp. *animalis* strains, and cluster L *B. animalis* subsp. *lactis* strains.

Table 1

List of *Bifidobacterium* strains used in this study.

Code	Putative Species	Additional strain information ^a	Source	Species ^b
S149	<i>B. animalis</i> subsp. <i>lactis</i>	Ruiz-Moyano et al. 2008	Human faeces	<i>B. animalis</i> subsp. <i>lactis</i>
252	<i>B. animalis</i> subsp. <i>lactis</i>	University of Parma bacteria culture collection	Human faeces	<i>B. animalis</i> subsp. <i>lactis</i>
S306	<i>B. animalis</i> subsp. <i>lactis</i>	Ruiz-Moyano et al. 2008	Human faeces	<i>B. animalis</i> subsp. <i>lactis</i>
UCD 316	<i>B. animalis</i> subsp. <i>lactis</i>	Isolate	Human faeces	<i>B. animalis</i> subsp. <i>lactis</i>
JCM 8224	<i>B. gallicum</i>	ATCC 49850; DSMZ 20093	Human faeces	<i>B. animalis</i> subsp. <i>lactis</i>
S332	<i>B. animalis</i> subsp. <i>lactis</i>	Ruiz-Moyano et al. 2008	Human faeces	<i>B. animalis</i> subsp. <i>lactis</i>
358	<i>B. animalis</i> subsp. <i>lactis</i>	University of Parma bacteria culture collection	Human faeces	<i>B. animalis</i> subsp. <i>lactis</i>
DSMZ 10140	<i>B. animalis</i> subsp. <i>lactis</i>	JCM 10602	Dairy product	<i>B. animalis</i> subsp. <i>lactis</i>
Y450	<i>B. animalis</i> subsp. <i>lactis</i>	Isolate	Dairy products	<i>B. animalis</i> subsp. <i>lactis</i>
Y452	<i>B. animalis</i> subsp. <i>lactis</i>	Isolate	Dairy products	<i>B. animalis</i> subsp. <i>lactis</i>
Y454	<i>B. animalis</i> subsp. <i>lactis</i>	Isolate	Dairy products	<i>B. animalis</i> subsp. <i>lactis</i>
Y456	<i>B. animalis</i> subsp. <i>lactis</i>	Isolate	Dairy products	<i>B. animalis</i> subsp. <i>lactis</i>
Y463	<i>B. animalis</i> subsp. <i>lactis</i>	Isolate	Dairy products	<i>B. animalis</i> subsp. <i>lactis</i>
Y466	<i>B. animalis</i> subsp. <i>lactis</i>	Isolate	Dairy products	<i>B. animalis</i> subsp. <i>lactis</i>
Y470	<i>B. animalis</i> subsp. <i>lactis</i>	Isolate	Dairy products	<i>B. animalis</i> subsp. <i>lactis</i>
533	<i>B. animalis</i> subsp. <i>lactis</i>	University of Parma bacteria culture collection	Human faeces	<i>B. animalis</i> subsp. <i>lactis</i>
672	<i>B. animalis</i> subsp. <i>lactis</i>	University of Parma bacteria culture collection	Human faeces	<i>B. animalis</i> subsp. <i>lactis</i>
892	<i>B. animalis</i> subsp. <i>lactis</i>	University of Parma bacteria culture collection	Human faeces	<i>B. animalis</i> subsp. <i>lactis</i>
ATCC 27536	<i>B. animalis</i> subsp. <i>animalis</i>	JCM 1253; DSMZ 20105	Chicken faeces	<i>B. animalis</i> subsp. <i>lactis</i>
JCM 7117	<i>B. animalis</i> subsp. <i>animalis</i>	ATCC 27674	Rabbit faeces	<i>B. animalis</i> subsp. <i>lactis</i>
JCM 1190	<i>B. animalis</i> subsp. <i>animalis</i>	ATCC 25527; DSMZ 20104	Rat feces	<i>B. animalis</i> subsp. <i>animalis</i>
JCM 11658	<i>B. animalis</i> subsp. <i>animalis</i>	CGMCC 1.3003	-	<i>B. animalis</i> subsp. <i>animalis</i>
ATCC 27672	<i>B. animalis</i> subsp. <i>animalis</i>	ATCC 27672	Rat faeces	<i>B. animalis</i> subsp. <i>animalis</i>

^aThe original strain numbers are also noted, if known. JCM, Japan Collection of Microorganisms, ATCC, American Type Culture Collection; DSMZ, German Collection of Microorganisms and Cell Cultures; CGMCC, China General Microbiological Culture Collection Center.

^bSpecies determined by sequence the genes *tuf* and *atpD*.

Table 2

Allelic profiles of 23 *B. animalis* strains analyzed by single-nucleotide polymorphisms (SNPs), insertions, and deletions (INDELs).

Clusters	Strains	Subspecies	Locus											
			igr6	igr8	igr9	Balat_0051	Balat_0141	Balat_0710	Balat_0864	g/cU	oxc	INDEL2		
Cluster 1	DSMZ 10140	<i>lactis</i>	A	A	A	A	A	A	A	A	A	A	A	A
Cluster 2	S149, 252, 892, Y452, Y454	<i>lactis</i>	A	A	B	B	A	A	A	A	A	A	A	A
Cluster 3	S306, Y463, Y466, Y470	<i>lactis</i>	B	B	B	B	A	A	A	B	B	B	B	B
Cluster 4	UCD 316, JCM 8224, ATCC 27536	<i>lactis</i>	A	A	B	B	A	A	C	A	A	A	A	A
Cluster 5	S332	<i>lactis</i>	A	A	B	B	A	A	A	A	C	A	A	A
Cluster 6	358, 533, 672	<i>lactis</i>	A	A	B	B	B	B	A	A	A	A	A	A
Cluster 7	Y450	<i>lactis</i>	A	A	B	B	B	B	B	A	A	A	A	A
Cluster 8	Y456	<i>lactis</i>	A	A	A	B	A	A	A	A	A	D	A	A
Cluster 9	JCM 7117	<i>lactis</i>	B	A	B	B	A	A	B	B	B	E	A	A
Cluster 10	JCM 1190	<i>animalis</i>	C	C	C	B	C	C	D	C	C	F	C	C
Cluster 11	JCM 11658	<i>animalis</i>	D	D	D	B	D	D	E	D	D	G	D	C
Cluster 12	ATCC 27672	<i>animalis</i>	E	E	E	B	E	E	F	E	E	H	E	D

