Short communication: Identification and differentiation of bifidobacteria obtained from Ukraine

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ABSTRACT

Ten freeze-dried bifidobacterial strains used as probiotics in Ukrainian dairy foods, identified by the supplier as *Bifidobacterium adolescentis* (2), *Bifidobacterium bifidum* (2), *Bifidobacterium longum* (4), *Bifidobacterium animalis* (1), and *Bifidobacterium infantis* (1), were characterized. Following rehydration and anaerobic growth on de Man, Rogosa, and Sharpe-cysteine medium at 37°C for 72 h, single-colony isolates were picked and evaluated using PCR primers specific for the *Bifidobacterium* genus, for the supplier-identified species, and for *B. animalis* ssp. *lactis*. All isolates were identified as members of the genus *Bifidobacterium*; however, species-specific PCR revealed all 10 isolates were actually strains of *B. animalis* ssp. *lactis*. Further evaluation using pulsed-field gel electrophoresis was only able to separate a single strain (RT 09) from the other 9 strains evaluated. Application of genome-wide allelic profiling to the Ukrainian bifidobacterial strains revealed 4 distinct groups. Interestingly, 6 (60%) of the isolates fell into the same cluster as that containing the common commercial probiotic strain BB-12. Two of the strains (RT 02 and RT 09) were found to be in the same group as ATCC 27536 and one strain (RT 08) was in the same group as the RB 7239 (a previously evaluated commercial strain). One strain, RT 04, was placed on a unique branch. These results highlight the importance of employing routine typing of bifidobacterial isolates, demonstrate the utility of single nucleotide polymorphism/insertion-deletion polymorphism-based allelic typing in *B. animalis* ssp. *lactis* strain differentiation and further point to the limited genetic variability of *B. animalis* ssp. *lactis* strains and the worldwide distribution of a small number of commercial strains.

Key words: polymerase chain reaction, pulsed-field gel electrophoresis, single nucleotide polymorphism, *Bifidobacterium animalis* ssp. *lactis*

Short Communication

Probiotic bacteria are used extensively in dairy products in Ukraine (Kvasnikov, 1992; Bondarenko et al., 1998; Kovalenko; 2002). Many of these organisms are bifidobacteria that are added to fermented milk products for their putative health-promoting properties (Grigorov et al., 2002; Ott and Chagarovskiy, 2002). Because the Ukrainian strains analyzed in this work have been used for many years and were not widely available in the West, they could represent a source of new strains with potentially novel characteristics.

Given the lack of information on bifidobacterial isolates used in Ukraine, the objective of the present research was to identify 10 bifidobacterial cultures used in Ukraine as probiotics in fermented dairy products to the genus and species levels and to differentiate the cultures at the strain level using molecular methods.

The bifidobacterial strains evaluated in this work were obtained from a commercial starter culture supplier in Ukraine as freeze-dried stock cultures. Each strain was assigned a unique laboratory number beginning with RT when it was received in the Roberts research laboratory (The Pennsylvania State University, University Park). The Ukrainian strains studied in the work are available from the corresponding author for non-commercial purposes. Reference strains used in this work were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; Table 1).

All bifidobacterial strains were cultured in liver lactose broth (LLB; Lapierre et al., 1992) or de Man, Rogosa, and Sharpe (MRS) broth (Becton, Dickinson and Co., Sparks, MD) with 0.05% L-cysteine hydrochloride (Sigma, St. Louis, MO). When solidified media were required, 15 g/L of agar was added to LLB and MRS broth.

Duplicate sets of the 10 bifidobacterial isolates were obtained from the culture supplier as lyophilized preparations. The 2 preparations were stored at –70°C and analyzed separately. A vial containing an RT isolate was aseptically opened and a sample of the dried pow-

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Table 1. Microorganisms used in this research

Strain	Comment
Reference strain	Description
<i>Bifidobacterium adolescentis</i> ATCC ¹ 15703	Species reference
<i>Bifidobacterium animalis</i> ssp. <i>animalis</i> ATCC 25527	Subspecies reference
<i>B. animalis</i> ssp. <i>lactis</i> DSMZ ² 10140	Subspecies reference
<i>Bifidobacterium bifidum</i> ATCC 29521	Subspecies reference
<i>Bifidobacterium longum</i> ssp. <i>longum</i> ATCC 15708	Subspecies reference
<i>B. longum</i> ssp. <i>infantis</i> ATCC 15697	Subspecies reference
<i>B. animalis</i> ssp. <i>lactis</i> BI-04 ³	Common commercial strain
<i>B. animalis</i> ssp. <i>lactis</i> BI-07 ³	Common commercial strain
<i>B. animalis</i> ssp. <i>lactis</i> BB-02 ³	Common commercial strain
<i>B. animalis</i> ssp. <i>lactis</i> BB-12 ⁴	Common commercial strain
Ukrainian strain (as designated by the supplier)	Species (as determined in this study)
<i>B. adolescentis</i> (RT 01)	<i>B. animalis</i> ssp. <i>lactis</i>
<i>B. adolescentis</i> (RT 02)	<i>B. animalis</i> ssp. <i>lactis</i>
<i>B. animalis</i> (RT03)	<i>B. animalis</i> ssp. <i>lactis</i>
<i>B. bifidum</i> (RT 04)	<i>B. animalis</i> ssp. <i>lactis</i>
<i>B. bifidum</i> (RT 05)	<i>B. animalis</i> ssp. <i>lactis</i>
<i>B. infantis</i> (RT 06)	<i>B. animalis</i> ssp. <i>lactis</i>
<i>B. longum</i> (RT 07)	<i>B. animalis</i> ssp. <i>lactis</i>
<i>B. longum</i> (RT 08)	<i>B. animalis</i> ssp. <i>lactis</i>
<i>B. longum</i> (RT 09)	<i>B. animalis</i> ssp. <i>lactis</i>
<i>B. longum</i> (RT 10)	<i>B. animalis</i> ssp. <i>lactis</i>

¹ATCC = American Type Culture Collection (Manassas, VA).

²DSMZ = Deutsche Sammlung von Microorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany).

³Obtained from Danisco USA Inc. (Madison, WI).

⁴Obtained from Chr. Hansen A/S (Hørsholm, Denmark).

der was obtained by touching with a sterile inoculating loop moistened with LLB. These samples were transferred into sterile PCR tubes and stored frozen at -70°C before analysis by direct PCR. The remaining portion of the lyophilized powder was rehydrated with MRS-cysteine broth, and then a loopful of rehydrated culture was streaked for isolation onto fresh MRS-cysteine agar and incubated under anaerobic (5% H_2 , 10% CO_2 , and 85% N_2) conditions at 37°C for 72 h. After incubation, plates were evaluated for growth. White, shiny, convex colonies of similar size were considered pure culture. Stock culture was prepared for each isolate in either LLB or MRS (10% glycerol) and frozen at -70°C .

All strains were identified to the genus level by PCR using the *Bifidobacterium* genus-specific primer set lm26 and lm3 (Kaufmann et al., 1997). Briefly, fresh RT, ATCC, and DSMZ bifidobacteria stock cultures were cultured on solid media as described above. A portion of a single well-isolated colony was picked from the agar plate and transferred into PCR tubes, and cells were then lysed by microwaving PCR tubes containing the bifidobacterial cells for 7 min (750 W) at high power (Bollet et al., 1991).

The PCR reaction mix contained 12.5 μL Go Taq Green Master Mix (Promega Corp., Madison, WI), 1 μL of each primer pair [1 μM ; Integrated DNA Technologies Inc. (IDT), Coralville, IA], lysed bifidobacterial cells, and nuclease-free water in a total volume

of 25 μL . The negative control for the PCR reaction contained 25 μL of the amplification mixture without any sample. The positive control for all PCR reactions was *Bifidobacterium animalis* ssp. *lactis* DSMZ 10140^T.

Polymerase chain reaction conditions for identification of the *Bifidobacterium* genus were described by Kaufmann et al. (1997). Polymerase chain reaction amplicons were separated by electrophoresis (80–100 V) in 1% agarose gels, stained with ethidium bromide, and visualized using an AlphaImager 3300 Gel Documentation System (Alpha Innotech Corp., San Leandro, CA).

Polymerase chain reaction was used to assess the species (subspecies) level identity of each of the RT bifidobacteria isolates using primers specific for *Bifidobacterium adolescentis*, *B. animalis* ssp. *animalis*, *B. animalis* ssp. *lactis*, *Bifidobacterium longum* ssp. *longum*, *B. longum* ssp. *infantis*, and *Bifidobacterium bifidum*, as described by Matsuki et al. (1999) and Ventura et al. (2001). Positive controls for species-specific PCR were the appropriate ATCC or DSMZ type strain for the species being examined (Table 1). Amplicons were detected as described previously. All RT bifidobacterial isolates were also evaluated using the *B. animalis* ssp. *lactis*-specific primer set as described by Ventura et al. (2001b). The positive control for this reaction was *B. animalis* ssp. *lactis* DSMZ 10140^T.

Lyophilized material collected from the commercial cultures was retrieved for direct PCR as described

above. Cells were lysed using the microwave method and, following lysis, the PCR analyses as described above were conducted to determine if the species identified by the culture supplier could be detected. In addition, RT isolates were evaluated using the *B. animalis* ssp. *lactis*-specific primer set described by Ventura et al. (2001). Appropriate reference strains were included in each analysis.

All RT bifidobacteria isolates and *B. animalis* ssp. *lactis* DSMZ 10104^T were streaked on LL agar and incubated at 37°C under anaerobic conditions for 72 h before characterization by pulsed-field gel electrophoresis (PFGE) using the method described by Briczinski and Roberts (2006). Briefly, cells were suspended in agarose plugs and lysed and then the DNA was digested using *Xba*I or *Spe*I (Promega Corp.). Electrophoresis was performed using the CHEF Mapper System (Bio-Rad Laboratories Inc., Hercules, CA). The experiment was performed in duplicate.

Deoxyribonucleic acid was isolated from RT bifidobacterial strains as described by Vincent et al. (1998). Amplification, band extraction, sequencing, and allelic profile constructs for the RT bifidobacterial isolates were performed as described by Briczinski et al. (2009).

The RT bifidobacteria, grown in anaerobic incubators on LLB and MRS-cysteine agar (37°C, 72 h), formed white, convex, soft, shiny colonies with a well-defined round shape. The RT bifidobacterial strains grown in the anaerobic chamber on MRS-cysteine media formed larger, more uniform colonies than cells grown in the anaerobic incubator on LL.

Analysis of lyophilized RT bifidobacterial isolates revealed they were all members of the genus *Bifidobacterium* (data not shown). None of the RT bifidobacterial isolates showed a positive result for the species identified by the supplier when analyzed using the corresponding species-specific primers. Further analysis of the RT isolates by PCR using the *B. animalis* ssp. *lactis* species-specific primer pair revealed that all were isolates of *B. animalis* ssp. *lactis* (data not shown). Direct PCR, performed using the lyophilized powder from the Ukrainian bifidobacteria cultures as template DNA, resulted in detection of only *B. animalis* ssp. *lactis*.

In an initial effort to differentiate the Ukrainian bifidobacteria, the isolates were evaluated using PFGE. No differences were observed between RT bifidobacteria isolates and *B. animalis* ssp. *lactis* DSMZ 10104^T using *Xba*I and *Spe*I, with the exception of isolate RT 09 restricted with *Xba*I, where one additional band was observed (data not shown).

Allelic profiles were created after sequencing of SNP and insertion-deletion polymorphism (INDEL) regions (Table 2). The phylogenetic tree created using these allelic profiles allowed assignment of the RT strains

into specific groups. Single nucleotide polymorphism analysis revealed that the allelic profiles of 9 of the RT strains matched 3 distinct groups (Figure 1) described previously by Briczinski et al. (2009). The majority of RT strains (6) were clustered into a group that matched the commercial control strain *B. animalis* ssp. *lactis* BB-12. Two isolates (RT 02, RT 09) matched ATCC 27536, whereas RT 08 matched the previously characterized commercial strain RB 7239. The final isolate (RT 04) exhibited a unique SNP in Balat_0051 and was placed on a new branch (Figure 1).

Growth of the bifidobacterial isolates was better on MRS-cysteine media in the anaerobic chamber where these organisms formed more uniform and larger colonies than when grown on LLB in the anaerobic incubator. It appears that either the more strictly anaerobic conditions or composition of the growth medium influenced colony size. Culture maintenance and manipulations in the anaerobic chamber, along with growth on MRS-cysteine agar, resulted in better bifidobacterial growth. This was not unexpected, as bifidobacteria are sensitive to oxygen (de Vries and Stouthamer, 1969) and growth under oxidative stress differs among the bifidobacterial species (Shimamura et al., 1992). Meile et al. (1997) reported no growth of *B. longum* and *B. animalis* at elevated oxygen concentration, whereas *B. animalis* ssp. *lactis* tolerated oxygen. However, regardless of the incubation system used (anaerobic incubator or anaerobic chamber), only *B. animalis* ssp. *lactis* was recovered from the culture samples obtained from Ukraine.

Direct PCR was conducted using samples of the lyophilized culture as the source of template DNA to determine if the reason that only *B. animalis* ssp. *lactis* was recovered from commercial cultures was the inability to culture the target strains. Results obtained from this analysis confirmed that only *B. animalis* ssp. *lactis* was present in the samples. These data suggest that all 10 bifidobacterial isolates obtained from Ukraine contained high levels of *B. animalis* ssp. *lactis* and that, if the designated strains were present, the numbers were too small to detect using the conditions applied in this study. Based on these results, it appears that the typing methods used by the commercial supplier routinely resulted in assignment of the correct genus, but incorrect species.

No differences were detected between the RT bifidobacterial isolates and *B. animalis* ssp. *lactis* DSMZ 10104^T digested with *Xba*I and *Spe*I with the exception of RT 09 restricted with *Xba*I. As previously reported, the monomorphic nature of the *B. animalis* ssp. *lactis* genome limits the utility of PFGE for differentiation of strains of this organism (Barrangou et al., 2009; Briczinski et al., 2009).

Table 2. Allelic profiles of strains of *Bifidobacterium animalis* ssp. *lactis* based on comparative analysis of SNP and insertion-deletion polymorphism (INDEL) using the scheme of Briczinski et al. (2009)

Genome position in DSMZ ¹ 10140	SNP in noncoding regions				Nonsynonymous SNP in protein-coding regions						INDEL	
	687000	959934	1456329	1636606	53321	53378	159526	993008	1260073	1260380	902893	1512304
DSMZ 10140 ²	G	G	—	A	G	G	G	T	C	T	1	1
BL-04 ^{2,3}	A	A	G	G	A	G	—	C	G	T	2	2
RB 4825 ⁴	A	A	G	G	A	G	—	C	G	T	2	2
RB 5251	A	A	G	G	A	G	—	C	G	T	2	2
HN 019 ^{2,3,5}	G	G	—	A	A	G	G	T	C	T	1	1
V9 ^{2,5}	G	G	—	G	A	G	—	T	C	T	2	1
RB 4753	G	G	—	G	A	G	—	T	C	T	2	1
RB 1791	G	G	—	G	A	G	—	T	C	C	2	1
BB12 ^{2,3,5}	G	G	—	G	A	G	—	T	C	T	1	1
RT 01 ⁶	G	G	—	G	A	G	—	T	C	T	1	1
RT 03	G	G	—	G	A	G	—	T	C	T	1	1
RT 05	G	G	—	G	A	G	—	T	C	T	1	1
RT 06	G	G	—	G	A	G	—	T	C	T	1	1
RT 07	G	G	—	G	A	G	—	T	C	T	1	1
RT 10	G	G	—	G	A	G	—	T	C	T	1	1
RB 1573	G	G	—	G	A	G	—	T	C	T	1	1
RB 4052	G	G	—	G	A	G	—	T	C	T	1	1
RB 4536	G	G	—	G	A	G	—	T	C	T	1	1
RB 7339	G	G	—	G	A	G	—	T	C	T	1	1
RB 9321	G	G	—	G	A	G	—	T	C	T	1	1
BI-07 ³	A	A	—	G	A	G	—	C	G	T	1	2
RB 1281	A	A	—	G	A	G	—	C	G	T	1	2
RB 5733	A	A	—	G	A	G	—	C	G	T	1	2
RB 8613	A	A	—	G	A	G	—	C	G	T	1	2
RB 9632	A	A	—	G	A	G	—	C	G	T	1	2
ATCC 27536	G	A	—	G	A	G	—	T	C	T	1	1
RT 02	G	A	—	G	A	G	—	T	C	T	1	1
RT 09	G	A	—	G	A	G	—	T	C	T	1	1
RB 1280	G	G	—	G	G	G	—	T	C	T	1	1
RB 5851	G	A	—	G	A	G	—	T	C	T	1	1
RT 08	G	G	—	G	A	G	—	T	C	C	1	1
RB 7239	G	G	—	G	A	G	—	T	C	C	1	1
RB 5859	G	G	G	G	A	G	—	C	C	C	1	1
BB-02 ³	A	A	G	G	A	G	—	C	G	T	1	2
RB 3046	A	A	G	G	A	G	—	C	G	T	1	2
RB 5422	A	A	G	G	A	G	—	C	G	T	1	2
RT 04	G	G	—	G	A	A	G	T	C	T	1	1
RB 0171	A	A	—	G	A	G	G	C	G	T	1	2
ADO11 ^{2,5}	G	G	—	G	A	G	G	T	C	T	2	1
Gene name	Igr6	Igr7	Igr8	Igr9	Balat_0051	Balat_0051	Balat_0141	Balat_0864	glcU	glcU	INDEL2	INDEL3

¹DSMZ = Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany).²Boldface indicates a fully sequenced reference genome for *B. animalis* ssp. *lactis*.³Common commercial strain of *B. animalis* ssp. *lactis* BI-04 (Danisco USA Inc., Madison, WI), HN-019, BB-12 (Chr. Hansen A/S, Hørsholm, Denmark), BI-07 (Danisco USA Inc.), and BB-02 (Danisco USA Inc.).⁴RB represents a set of commercial strains of *B. animalis* ssp. *lactis* analyzed by Briczinski et al. (2009).⁵SNP profiles for HN-019, V9, and ADO11 were determined in silico.⁶RT represents a set of commercial *B. animalis* ssp. *lactis* isolates obtained from Ukraine and characterized in this study.

Figure 1. Cluster diagram placing the Ukrainian bifidobacterial isolates, designated RT, into the scheme of bifidobacterial isolates in the scheme of Briczinski et al. (2009). Common commercial strains (HN019, BI-04, BB-02, BI-07, ADO11, and BB-12) were included in the analysis for reference purposes.

Application of genome-wide SNP and INDEL analysis has recently been shown to be capable of differentiating isolates of *B. animalis* ssp. *lactis* (Briczinski et al., 2009). In the present study, SNP/INDEL analysis was able to separate RT strains into 4 distinct groups. Six strains had allelic profiles identical to the commonly used commercial strain BB-12. Single nucleotide polymorphism analysis of RT 02 and RT 09 revealed an allelic profile identical to that of ATCC 27536 (Table 2).

Strain RT 08 had an allelic profile identical to that of the previously characterized strain RB 7239. Strain RT 04 was found to have a unique profile and was placed in its own group. These results demonstrate the utility of SNP analysis for differentiation of strains of *B. animalis* ssp. *lactis*.

Knowing that bifidobacteria are anaerobic and that the sensitivity to oxygen differs dramatically, it is hypothesized that, if the initial determination of the

identity of bifidobacterial strains obtained from commercial supplier from Ukraine was correct, the RT strains may have been exposed to oxygen during culturing, and were contaminated with and then overgrown by *B. animalis* ssp. *lactis* during culture production by the Ukrainian supplier. Alternatively, it is possible the methods initially employed by the supplier to identify these organisms did not allow for accurate species-level identification.

Accurate identification to the species and strain level is a crucial control element when producing fermented dairy products containing probiotic bacteria and, in fact, the probiotic effects of bifidobacteria are considered to be strain-specific (FAO/WHO, 2002). In previous work, Huys et al. (2006) reported that 28% of commercial strains were misidentified at the genus or species level. Temmerman et al. (2003) noted mislabeling of strains contained in some preparations and also commented on the use of old or inappropriate nomenclature. The need for policies regulating microbiological quality and labeling of probiotic products and pharmaceutical preparations has been suggested by various authors (Sanders and Huis in't Veld, 1999; Szajewska et al., 2004; Harish and Varghese, 2006) and is highlighted and reconfirmed by this study.

Results obtained in this study revealed all 10 of the Ukrainian bifidobacterial cultures were correctly identified by the culture supplier at the genus level, but were misidentified at the species/subspecies level. All of the isolates evaluated were determined to be strains of *B. animalis* ssp. *lactis*. As expected, PFGE exhibited limited discriminatory power when applied to differentiation of this subspecies (Barrangou et al., 2009; Briczinski et al., 2009). Whole-genome SNP and INDEL analysis was able to separate the Ukrainian isolates into 4 separate groups and was found to be more discriminative than PFGE. Perhaps not surprisingly, 6 of the 10 Ukrainian strains evaluated were assigned to the group containing the well-known commercial strain *B. animalis* ssp. *lactis* BB-12 and 2 were assigned to the group containing the culture collection strain ATCC 27536. These results highlight the importance of effective strain identification and differentiation and clearly suggest that attention be given to ensure the identity of the bifidobacteria used in probiotic-containing dairy products to ensure continued consumer confidence in these products. It is recommended that manufacturers who are adding specific probiotics to products require adequate documentation of strain identity from the supplier and consider implementing procedures to verify the species or, where possible, the strain being added to the product. In addition, the results demonstrate the utility of SNP/INDEL-based allelic typing in *B. animalis* ssp. *lactis* strain differentiation and further

point to the limited genetic variability of *B. animalis* ssp. *lactis* strains and the worldwide distribution of a small number of commercial strains.

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