Molecular identification and quantification of lactic acid bacteria in traditional fermented dairy foods of Russia

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ABSTRACT

Russian traditional fermented dairy foods have been consumed for thousands of years. However, little research has focused on exploiting lactic acid bacteria (LAB) resources and analyzing the LAB composition of Russian traditional fermented dairy foods. In the present study, we cultured LAB isolated from fermented mare and cow milks, sour cream, and cheese collected from Kalmykiya, Buryats, and Tuva regions of Russia. Seven lactobacillus species and the *Bifidobacterium* genus were quantified by quantitative PCR. The LAB counts in these samples ranged from 3.18 to 9.77 log cfu/mL (or per gram). In total, 599 LAB strains were obtained from these samples using de Man, Rogosa, and Sharpe agar and M17 agar. The identified LAB belonged to 7 genera and 30 species by 16S rRNA and *murE* gene sequencing and multiplex PCR assay. The predominant LAB isolates were *Lactobacillus helveticus* (176 strains) and *Lactobacillus plantarum* (63 strains), which represented 39.9% of all isolates. The quantitative PCR results revealed that counts of 7 lactobacilli species and *Bifidobacterium* spp. of 30 fermented cow milk samples ranged from 1.19 ± 0.34 (*Lactobacillus helveticus* in Tuva) to 8.09 ± 0.71 (*Lactobacillus acidophilus* in Kalmykiya) log cfu/mL of fermented cow milk (mean $\pm$ standard error). The numbers of *Bifidobacterium* spp., *Lb. plantarum*, *Lb. helveticus*, and *Lb. acidophilus* revealed no significant difference between the 3 regions; nevertheless, *Lactobacillus paracasei*, *Lactobacillus fermentum*, *Lactobacillus sakei*, and *Lactobacillus delbrueckii* ssp. *bulgaricus* exhibited different degrees of variation across 3 regions. The results demonstrate that traditional fermented dairy products from different regions of Russia have complex compositions of LAB species. The diversity of LAB might be related to the type of fermented dairy product, geographical origin, and manufacturing process.

Key words: traditional fermented dairy product, lactic acid bacteria, 16S rRNA gene, quantitative PCR

INTRODUCTION

Russia, the largest country in Europe, consists of 193 different ethnic groups and indigenous peoples. In the past, traditional fermented milk, cheese, and sour cream were staples in the diet of Russian people and were believed to have contributed to a long life expectancy. These dairy foods are mostly produced in summer and autumn every year when milk yields of mare and cow are high. Fermented cow milk is made by adding old yogurt as starter culture to fresh milk, followed by fermentation in a wooden cask or glass jar at ambient temperature (10–25°C) for 1 to 2 d until the desired degree of acidity is reached. The production of fermented mare milk is continuous; raw mare milk is first spontaneously fermented by environmental microorganisms in a large container at room temperature for 3 to 5 d. Then, after some of the fermented mare milk has been consumed, raw milk is added to the container and fermentation continues. Fermented mare milk contains a small amount of alcohol due to alcoholic fermentation by yeasts. Different from industrialized sour cream, Russian sour cream is a traditional dairy product extracted from milk by manual stirring and fermented by natural inoculation. Traditional cheese is made by adding calf abomasum powder as rennin into raw milk to coagulate the milk.

Lactic acid bacteria (LAB) are widely distributed in nature and occur naturally as indigenous microflora in fermented foods; the LAB play an essential role in the nutritious and organoleptic properties of traditional fermented milk products (Rhee et al., 2011). Undoubtedly, traditional fermented dairy foods in Russia contain a large array of valuable LAB resources (Watanabe et al., 2008).

In previous studies, our research group has systematically analyzed the diversity of LAB isolated from different traditional fermented foods (*qula*, congee, pickle, dairy fan, yak milk, goat milk, and so on) in minority areas of China and Mongolian (Liu et al., 2009; Sun

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Table 1. Samples, pH (range in parentheses), lactic acid bacteria (LAB) counts (log cfu/mL; range in parentheses), and sampling locations in Russia

Sample type	Location	No. of samples	Sample number	pH value	LAB count
Fermented mare milk	Kalmykiya	3	Ru8, Ru15, Ru21	3.70 ± 0.26 (3.51–4.05)	4.60 ± 1.51 (3.18–5.30)
	Tuva	2	Tw9, Tw57	3.90 ± 0.14 (3.80, 4.01)	6.73 (5.22, 8.24)
Sour cream	Tuva	2	Tw1, Tw2	ND ¹	6.86 (5.69, 8.02)
	Kalmykiya	6	Ru7, Ru25, Ru38, Ru40, Ru41, RuM1	ND	8.13 ± 0.22 (7.30–8.53)
Cheese	Kalmykiya	8	Ru3–Ru6, Ru24, Ru26, Ru30, Ru39	ND	8.10 ± 0.81 (7.16–9.05)
Fermented cow milk	Kalmykiya	24	Ru1, Ru2, Ru10, Ru14, Ru16, Ru17, Ru19, Ru22, Ru23, Ru27–Ru29, Ru31–Ru37, Ru42–Ru45, RuM2	4.25 ± 0.25 (3.80–4.82)	8.06 ± 0.50 (7.06–9.23)
	Buryats	63	ELS1–ELS10, ELS12–ELS18, ELS20–ELS23, ELS26–ELS28, ELS30–ELS37, ELS39–ELS42, ELSA1–ELSA5, ELSA9–ELSA12, ELSA14–ELSA18, ELSA20–ELSA30, ELSA32	4.31 ± 0.39 (3.75–5.03)	7.86 ± 1.33 (4.18–9.77)
	Tuva	51	Tw3–Tw8, Tw10–Tw21, Tw24–Tw56	4.12 ± 0.35 (3.63–4.88)	5.77 ± 1.46 (3.96–8.34)

¹ND = not detected.

et al., 2010; Yu et al., 2011a,b; Bao et al., 2012a; Liu et al., 2012; Yu et al., 2012). From LAB isolates, we have screened several excellent strains for use as probiotics for development of starter cultures, such as *Lactobacillus casei* Zhang (Wu et al., 2009; Zhang et al., 2010), *Lactobacillus plantarum* P-8 (Wang et al., 2013, 2014), and *Lactobacillus helveticus* H9 (Chen et al., 2014).

Currently, along with the industrialization of yogurt, fewer Russians are making traditional fermented dairy foods. To acquire LAB resources from Russian traditional fermented dairy foods, we isolated and characterized LAB from 159 samples that were collected from 3 regions in Russia. A total of 599 isolates were initially identified by 16S rRNA gene sequence, and then the *Lactobacillus* (*Lb.*) *delbrueckii* group and *Lb. plantarum* group were further classified by *murE* gene sequencing and multiplex PCR assay of *recA* gene. To obtain more accurate quantitative information of the dominate LAB, quantitative PCR (qPCR) methods were applied to reveal the diversity of predominant LAB in traditional fermented cow milk of Russia.

MATERIALS AND METHODS

Samples

One hundred fifty-nine samples of traditional fermented dairy foods were obtained from Kalmykiya, Buryats, and Tuva regions of Russia in September 2013; detailed information on the samples is shown in Table 1. The temperature of fermented milk samples at sampling ranged from 16.7 to 26.4°C with an average of 22.8°C. The pH values of those samples ranged

from 3.51 to 5.03. The temperature and pH values of cheese and sour cream samples were not measured. Samples were collected aseptically and transported to our laboratory in a 4°C vehicle-mounted refrigerator. Subsequently, those samples were stored at –20°C and analyzed within a few days.

Enumeration and Isolation of LAB

One milliliter or 1 g of sample was mixed with 9 mL of sterile physiological saline (0.85% NaCl) to make an initial dilution. Serial dilutions were made for each sample and 1 mL of the appropriate dilution was mixed with melted de Man, Rogosa, and Sharpe (MRS; Difco Laboratories, Detroit, MI) agar for enumeration of the total LAB by pour-plate method. Cycloheximide at a concentration of 0.01% (vol/vol) was added to the MRS plates to prevent fungal growth. Meanwhile, appropriate dilutions were plated onto MRS and M17 (Oxoid Ltd.) plates and incubated under anaerobic condition at 30°C for 48 to 72 h. Colonies with distinct morphological differences (color, shape, and size) were selected and purified by streaking on the same medium. The catalase and Gram reactions of all the isolates were assessed, and gram-positive, catalase-negative, nonmotile cocci and rods were preserved in milk containing 0.1% sodium glutamate and stored at –40°C.

Identification of LAB

Total genomic DNA of all isolates was extracted from overnight cultures by using a previously reported

method (Yu et al., 2009). For each strain, the purified DNA was used as a template for 16S rRNA gene amplification on the automatic thermal cycler (PTC-200, MJ Research, Waltham, MA). The primers of 16S rRNA gene were 16S-FA (GCAGAGTTCTCG-GAGTCACGAAGAGTTTGATCCTGGCTCAG) and 16S-RA (AGCGGATCACTTACACAGGACTACG-GCTACCTTGTTACGA) (Sun et al., 2010). The reaction mixtures and PCR amplifying procedure were described in previous study (Yu et al., 2012). *Lactobacillus delbrueckii* isolates determined by 16S rRNA gene sequences were further identified at the subspecies level based on *murE* gene sequencing. The primers of *murE* gene were *murE*-F (CTGAGCAGCCATACCCG) and *murE*-R (CCTTGCTTGAAGCCGTAG). Each sample contained 1 × *Taq* buffer (TaKaRa Bio Inc., Shiga, Japan), 1.5 mM MgCl₂, 0.2 μM of each dNTP, 10 pmol of each primer, 10 ng of template bacterial DNA, and 1.0 U of Ex *Taq*TM polymerase (TaKaRa Bio Inc.). The reaction conditions were as follows: 94°C for 5 min, 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, 30 cycles, and then 72°C for 10 min. The sequencing of 16S rRNA gene and *murE* gene was performed in Majorbio Bio-pharm Technology Corporation of China (Shanghai). The sequences were determined using the BLAST algorithm and were submitted to the National Center for Biotechnology Information (NCBI; <http://www.blast.ncbi.nlm.nih.gov>). MEGA version 6.0 software (<http://www.mega-software.net>; Tamura et al., 2013) was used to create phylogenetic trees based on the neighbor-joining method.

Strains in the *Lb. plantarum* group showed more than 99% similarity to the type strains of *Lb. paraplantarum*, *Lb. plantarum*, and *Lb. pentosus*. Therefore, a multiplex PCR assay was performed to further discriminate those strains with *recA* gene-based primers: paraF (5'-GTCACAGGCATTACGAAAAC-3'), pentF (5'-CAGTGGCGCGGTTGATATC-3'), planF (5'-CC-GTTTATGCGGAACACCTA-3'), and pREV (5'-TC-GGGATTACCAAACATCAC-3'). The PCR mixture and amplifications were performed as described by Torriani et al. (2001).

Quantification of Common LAB in Fermented Cow Milks

Total DNA of the samples was extracted according to methods described in Xu et al. (2014). All DNA samples were stored at -20°C until further experiments. To accurately detect the usual species and genera in traditional fermented cow milk, the common LAB species of *Lb. acidophilus*, *Lb. paracasei*, *Lb. delbrueckii* ssp. *bulgaricus*, *Lb. plantarum*, *Lb. fermentum*, *Lb. sakei*, *Lb. helveticus*, and *Bifidobacterium* genus were chosen

as targets and were subjected to enumeration by qPCR with specific primers as listed in Table 2. Those primers were designed by Primer Premier 5.0 program (Premier Biosoft International, Palo Alto, CA), which amplified a conserved region of the *pyrG* and *recA* genes. Quantitative PCR was performed in an ABI Step-One detection system (Applied Biosystems, Carlsbad, CA) using SYBR Premix Ex *Taq*II kits (TaKaRa Bio Inc.). The amplification reaction mixture and program were prepared as reported previously (Kwok et al., 2014). Corresponding type strains and *Bifidobacterium breve* ATCC 15700 were used for standard curve construction, the coefficient of determination (R^2) values of qPCR were greater than 0.99, and the efficiency value ranged from 95.5 to 102.1%. The statistical analyses were performed by using the PAST software (<http://folk.uio.no/ohammer/past/>; Hammer et al., 2001). Bacterial counts were expressed as mean values ± standard error and presented as box-plots. Mann-Whitney test with Bonferroni correction for multiple testing was used to evaluate the sample difference in a pairwise manner. Two sample groups with a corrected *P*-value < 0.05 were considered significantly different.

Nucleotide Sequence Accession Numbers

All sequences were deposited in the GenBank nucleotide databases under KC836519 to KC836532, KC836540 to KC836745, KF149397 to KF149648, and KJ026561 to KJ026699 numbers.

RESULTS

Enumeration and Isolation of LAB

Viable counts of the LAB present in the different fermented dairy foods are given in Table 1. Microbiological analyses showed the presence of LAB at levels ranging from 3.18 log cfu/mL (sample Ru21) to 9.77 log cfu/mL (sample ELS6). The LAB counts in 3 fermented mare milk samples, 6 sour cream samples, 8 cheese samples, and 24 fermented milk samples ranged from 3.18 to 5.30 log cfu/mL, 7.30 to 8.53 log cfu/mL, 7.16 to 9.05 log cfu/g, and 7.06 to 9.23 log cfu/mL, respectively. Total LAB counts in 63 fermented cow milk samples from Buryats ranged from 4.18 to 9.77 log cfu/mL. The LAB counts in 2 fermented mare milk samples, 2 sour cream samples, and 24 fermented milk samples from Tuva ranged from 5.22 to 8.24 log cfu/mL, 5.69 to 8.02 log cfu/mL, and 3.96 to 8.34 log cfu/mL, respectively.

Five hundred ninety-nine gram-positive and catalase-negative microorganisms were presumptively identified as LAB. The majority of isolated strains were rod-

Table 2. Quantitative-PCR primer information (R = reverse, F = forward) and predicted product size

Species	Primer sequence (5'-3')	Annealing temperature (°C)	Predicted PCR product size (bp)
<i>Lactobacillus acidophilus</i>	La-R TCCCTAAACAAATACCCAAG La-F GCAGGCTACCTTTACAACAC	55	205
<i>Lactobacillus paracasei</i>	Lc-R AGTGGTATGGGTCAAATGCT Lc-F CGGAAGATATGAAGAAGAAA	55	221
<i>Lactobacillus delbrueckii</i>	Ld-R TCTGGTCAACGCCTTGTTCC Ld-F CCGACCCAGCAGTCAGTTTC	59	208
<i>Lactobacillus plantarum</i>	Lp-R ATGTTGCGTTGGCTTCGTCT Lp-F AAAATCATGCGTGCGGGTAC	55	210
<i>Lactobacillus fermentum</i>	Lf-R TTCATCTGGCGAATTGCTTC Lf-F ACGGTTTCATTGACAACGACT	55	258
<i>Lactobacillus sakei</i>	Ls-R CAGCAAATACTAAGCCCTTG Ls-F GGTATCACAGATGCCACAAC	55	259
<i>Lactobacillus helveticus</i>	Lh-R CCAGGTGGTTCGTGCTCTTAA Lh-F TGACCGATCCGATCACTCTT	57	270
<i>Bifidobacterium</i> spp.	Bi-R TTGCGTTGATCACAGATTCA Bi-F CCGTACGGCAATCGCGATAT	56	550

shaped bacteria (401 isolates in total, with 208 isolates from MRS and 193 from M17 medium) and the remainder were cocci (198 isolates in total with 68 from MRS and 130 from M17 medium).

Identification of LAB

To confirm the species, partial 16S rRNA gene sequences (approximately 1,400 bp) of all isolates were determined and compared with related bacteria using the BLAST program at NCBI (<http://www.ncbi.nlm.nih.gov/>). Phylogenetic tree analysis was performed to show the relationship of 16S rRNA gene sequences between the representative isolates and type strains by MEGA software. As shown in Figure 1, representative strains IMAU92041, IMAU92037, IMAU90085, IMAU90033, IMAU95059, and IMAU90159 had 100% similarity to *Lb. parabuchneri*, *Lb. rapi*, *Lb. diolivorans*, *Lb. brevis*, *Lb. paracasei*, and *Lb. kefirifaciens*, respectively. Representative strains IMAU92115, IMAU95008, IMAU90047, IMAU95056, and IMAU92080 shared >99% homology with *Lb. kefir*, *Lb. sakei*, *Lb. curvatus*, *Lb. fermentum*, and *Lb. helveticus*, respectively. However, IMAU90088 appeared to be equally linked to both *Lb. plantarum* and *Lb. pentosus*, indicating that the 16S rRNA gene sequence did not give discriminatory results for this group. Moreover, isolate IMAU92020 grouped with 5 *Lb. delbrueckii* subspecies, and its 16S rRNA gene sequence showed a similarity of >99% to those subspecies. As shown in Figure 2, the homology of 16 representative isolates and their corresponding type strain ranged from 99 to 100%. Therefore, the 16S rRNA gene sequence analysis showed that the 599 isolates belonged to 7 genera (*Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, and *Vagococcus*), 1 species group, and 28 species: *Lb.*

plantarum group, *Enterococcus* (*E.*) *durans*, *E. faecalis*, *E. faecium*, *E. italicus*, *Lb. brevis*, *Lb. delbrueckii*, *Lb. diolivorans*, *Lb. fermentum*, *Lb. graminis*, *Lb. helveticus*, *Lb. kefirifaciens*, *Lb. kefir*, *Lb. parabuchneri*, *Lb. paracasei*, *Lb. rapi*, *Lb. sakei*, *Lactococcus* (*Lc.*) *garvieae*, *Lc. lactis* ssp. *cremoris*, *Lc. lactis* ssp. *lactis*, *Leuconostoc* (*Leu.*) *citreum*, *Leu. lactis*, *Leu. mesenteroides*, *Leu. pseudomesenteroides*, *Pediococcus* (*P.*) *parvulus*, *P. pentosaceus*, *Streptococcus* (*S.*) *parauberis*, *S. thermophilus*, and *Vagococcus teuberi* (Table 3).

The *murE* gene of 44 representative strains was sequenced to identify *Lb. delbrueckii* at the subspecies level. Approximately 500 bp of *murE* gene sequences were obtained and a phylogenetic tree was constructed using MEGA software (version 6.0; Figure 3). The results showed that 23 strains were identified as *Lb. delbrueckii* ssp. *lactis*, and 21 strains were closely related to *Lb. delbrueckii* ssp. *bulgaricus*. Multiplex PCR was used to distinguish the closely related species of the *Lb. plantarum* group. A total of 63 strains in *Lb. plantarum* group and type strain *Lb. plantarum* JCM 1149^T displayed a pattern characterized by the typical band of *Lb. plantarum* (318 bp; Figure 4).

Enumeration of Predominant LAB Using qPCR

The 7 lactobacilli species and *Bifidobacterium* spp. of 30 fermented cow milk samples from Kalmykiya, Buryats, and Tuva regions of Russia were quantified by qPCR. The composition of those species or genus in fermented cow milk of different regions is presented in Figure 5. The counts of 7 bacterial species and *Bifidobacterium* genus ranged from 1.19 ± 0.34 (*Lb. helveticus* in Tuva) log cfu/mL of fermented milk (mean $\pm$ standard error) to 8.09 ± 1.73 (*Lb. acidophilus* in Kalmykiya). The numbers of *Bifidobacterium* genus, *Lb.*

Figure 1. Neighbor-joining tree showing the phylogenetic relationships between 13 *Lactobacillus* (*Lb.*) species and the type strains based on 16S rRNA gene sequences. *Bacillus subtilis* was used as an outgroup.

Figure 2. Neighbor-joining tree showing the phylogenetic relationships between 16 cocci and the type strains of related genera based on 16S rRNA gene sequences. *Bacillus subtilis* was used as an outgroup. *E.* = *Enterococcus*; *Lc.* = *Lactococcus*; *Leu.* = *Leuconostoc*; *S.* = *Streptococcus*; *P.* = *Pediococcus*; *V.* = *Vagococcus*.

Table 3. Distribution of lactic acid bacteria (LAB) species in various fermented dairy products in 3 regions of Russia (Kalmykiya, Tuva, and Buryats)

LAB	Kalmykiya				Tuva			Buryats	Total
	Fermented mare milk (n = 3)	Cheese (n = 8)	Sour cream (n = 6)	Fermented cow milk (n = 24)	Fermented mare milk (n = 2)	Sour cream (n = 2)	Fermented cow milk (n = 51)	Fermented cow milk (n = 63)	
<i>Enterococcus (E.) durans</i>		2		1				2	5
<i>E. faecalis</i>								2	2
<i>E. faecium</i>		6					12	5	23
<i>E. italicus</i>				2					2
<i>Lactobacillus (Lb.) brevis</i>		2	1	2				1	6
<i>Lb. delbrueckii</i> ssp. <i>bulgaricus</i>	1			3			19	7	30
<i>Lb. delbrueckii</i> ssp. <i>lactis</i>				3				20	23
<i>Lb. diolivorans</i>		1		2					3
<i>Lb. fermentum</i>	1			3			7	6	17
<i>Lb. graminis</i>		1		1					2
<i>Lb. helveticus</i>	5	7		39	1	4	11	109	176
<i>Lb. kefiranoformans</i>	1			12			1	9	23
<i>Lb. kefir</i>		1		2				16	19
<i>Lb. parabuchneri</i>								1	1
<i>Lb. paracasei</i>		6	1	5			10	7	29
<i>Lb. plantarum</i>	1	12	5	20	2	3	14	6	63
<i>Lb. rami</i>								1	1
<i>Lb. sakei</i>							7	1	8
<i>Lactococcus (Lc.) garvieae</i>				2				1	3
<i>Lc. lactis</i> ssp. <i>cremoris</i>			2	4			1		7
<i>Lc. lactis</i> ssp. <i>lactis</i>	1		5	5			3	13	27
<i>Leuconostoc (Leu.) citreum</i>		1	1	1	1		12	3	19
<i>Leu. lactis</i>			3					11	14
<i>Leu. mesenteroides</i>	2		5	5	1		12	22	47
<i>Leu. pseudomesenteroides</i>	1	1	4	14			5		25
<i>Pediococcus parvulus</i>				1					1
<i>Pediococcus pentosaceus</i>				1					1
<i>Streptococcus parauberis</i>				1					1
<i>Streptococcus thermophilus</i>			1	3	2		11	3	20
<i>Vagococcus teuberi</i>		1							1
Total	13	41	28	132	7	7	125	246	599

Figure 3. Neighbor-joining tree showing the phylogenetic relationships between representative *Lactobacillus* (*Lb.*) *delbrueckii* strains and the type strains based on *murE* gene sequences. Bootstrap values based on 100 replications are given at the nodes.

Figure 4. Multiplex-PCR amplification patterns of *Lactobacillus* (*Lb.*) *plantarum* group. M = DL2000 DNA marker; 1 = *Lb. pentosus* ATCC 8041; 2 = *Lb. paraplantarum* ATCC 700211; 3 = *Lb. plantarum* ATCC 14917; 4 = IMAU90046; 5 = IMAU95001; 6 = IMAU90084; 7 = IMAU90088; 8 = IMAU92109; 9 = IMAU92168; 10 = IMAU92148; 11 = IMAU94200; 12 = IMAU95105; 13 = IMAU95030; 14 = IMAU95051 and 15 = IMAU95105.

plantarum, *Lb. helveticus*, and *Lb. acidophilus* revealed no significant difference between 3 regions, whereas *Lb. paracasei*, *Lb. fermentum*, *Lb. sakei*, and *Lb. delbrueckii* ssp. *bulgaricus* exhibited different degrees of variation across the 3 regions. The numbers of *Lb. paracasei* in Tuva and Kalmykiya were almost identical and were significantly higher than those in Buryats ($P < 0.01$). The fermented cow milks of Buryats and Kalmykiya had almost identical mean numbers of *Lb. delbrueckii* ssp. *bulgaricus*, which were significantly lower than those in Tuva ($P < 0.001$). Counts of *Lb. sakei* in fermented cow milk in Buryats were significantly lower than those in Tuva ($P < 0.05$) and counts of *Lb. fermentum* in Buryats were significantly higher than those in Tuva ($P < 0.05$).

Comparing the mean counts of 8 LAB species/genera in fermented cow milk, counts of *Lb. fermentum* and *Lb. acidophilus* were significantly higher than those of the other species, whereas counts of *Lb. helveticus* were lowest. All species were detected in almost every sample, except *Lb. helveticus* and *Bifidobacterium* spp. (data not shown).

DISCUSSION

Traditional fermented dairy foods have been consumed for thousands of years around the world. After a long period of natural selection, excellent LAB strains were handed down through generations by ethnic minority families. In recent years, there has been increasing interest in the biodiversity and exploitation of LAB in traditional fermented dairy products. Yet, to the best of our knowledge, this is the first study that identifies and quantifies LAB involved in traditional fermented dairy foods of Russia.

Based on viable counts of LAB, slight variations were observed among fermented cow milk, fermented mare milk, sour cream, and cheese in 3 regions of Russia. In

Kalmykiya, the mean numbers of LAB in fermented cow milk, cheese, and sour cream were almost identical and significantly higher than those in fermented mare milk. In Tuva, the mean numbers of LAB in fermented mare milk and sour cream were almost identical and were higher than those in fermented cow milk. For fermented cow milk, the mean numbers of LAB in Kalmykiya and Buryats were similar and significantly higher than those in Tuva, but the mean numbers of LAB in fermented mare milk of Tuva were significantly higher than those in Kalmykiya. The LAB in sour cream of Tuva were significantly lower than those in Kalmykiya. Thus, type of fermented dairy product and geographical origin may explain variations in LAB counts.

By pure culture method and gene sequencing technology, 599 LAB strains were isolated and identified as belonging to 7 genera and 30 species. In Kalmykiya, *Lb. helveticus* was the predominant species isolated from fermented mare and cow milks, *Lb. plantarum* was the predominant species in cheese, and *Lb. plantarum*, *Lc. lactis* ssp. *lactis*, and *Leu. mesenteroides* were the predominant species in sour cream. However, *Lb. helveticus* and *Lb. delbrueckii* ssp. *bulgaricus* were the predominant isolates in fermented cow milk of Buryats and Tuva, respectively. Because of the small number of fermented mare milk and sour cream samples in Tuva, we did not analyze the diversity of those samples. Overall, *Lb. helveticus*, as the predominant LAB component (representing 29.4% of all isolates), was isolated from all fermented dairy products except sour cream samples in Kalmykiya. Other species isolated at relatively high frequencies included *E. faecium*, *Lb. delbrueckii* ssp. *bulgaricus*, *Lb. delbrueckii* ssp. *lactis*, *Lb. fermentum*, *Lb. kefirifaciens*, *Lb. kefirii*, *Lb. paracasei*, *Lb. plantarum*, *Lc. lactis* ssp. *lactis*, *Leu. citreum*, *Leu. lactis*, *Leu. mesenteroides*, *Leu. pseudomesenteroides*, and *S. thermophilus*. In addition, a few other isolates were classified as *E. durans*, *E. faecalis*, *E. italicus*, *Lb. brevis*, *Lb. diolivorans*, *Lb. parabuchneri*, *Lb. rapi*, *Lb. sakei*, *Lc. garvieae*, *Lc. lactis* ssp. *cremoris*, *P. pentosaceus*, and *S. parauberis*. Those species have been isolated using culture methods from the other fermented dairy products such as Mongolian fermented dairy products (Watanabe et al., 2008; Yu et al., 2011b), Sudanese fermented camel milk (Abdelgadir et al., 2008), *Dahi* (Harun-ur-Rashid et al., 2007), and Chinese traditional fermented yak milk (Bao et al., 2012b). Furthermore, *Lb. graminis*, *P. parvulus*, and *V. teuberi* were detected only occasionally in fermented dairy products of Kalmykiya. To our knowledge, there are no reports on these species being previously isolated from fermented milks by culture-dependent methods. Whether those species represent contamination or play a technological role in fermented dairy products needs to be confirmed.

Figure 5. Box plots showing 7 *Lactobacillus* (Lb.) species and *Bifidobacterium* genus of fermented cow milk in different regions of Russia (Tuva, Buryats, and Kalmykiya). Bacterial amounts are expressed in Log copy number per milliliter or gram of dairy sample. Boxes show the median and 25th and 75th percentiles; the lower and upper adjacent hinges show the minimum and maximum values. Significant differences between sample pairs were evaluated by pairwise Mann-Whitney test with Bonferroni correction; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

In terms of species diversity, 8, 12, 10, and 23 LAB species were isolated from fermented mare milk, cheese, sour cream, and fermented cow milk, respectively, in

Kalmykiya. Moreover, fermented cow milk samples of Buryats and Tuva contain 20 and 14 LAB species. Interestingly, the numbers of lactobacilli species were

slightly higher than that of cocci species in all samples except sour cream samples. Three lactobacilli species and 7 cocci species were isolated from 3 sour cream samples in Kalmykiya. A previous study on LAB diversity in Romanian sour cream (Zamfir et al., 2006) also found that *Lc. lactis* ssp. *lactis*, *E. durans*, *E. faecium*, and *Leu. mesenteroides* were the predominant species. Comparing the LAB composition of fermented cow milk in 3 regions, we found 10 species were common LAB in 3 regions, 2 to 5 species were common LAB in 2 regions, 4 LAB species were only isolated from Buryats, and 6 species were only isolated from Kalmykiya. The results indicated that the same types of fermented dairy products in different regions have different microbial composition. Therefore, we deduced that the predominant LAB and species compositions varied with the raw milks, the types of fermented dairy products, and geographic origins. This finding is consistent with the results of previous studies. In 2008, Watanabe et al. (2008) used culture- and molecular-based methods to investigate microbial diversity in the traditional fermented milks *airag* (fermented mare milk) and *tarag* (fermented milk of cows, yaks, goats, or camels) in Mongolia. They suggested that the milk used to prepare the fermented milk products is the most important factor influencing the microbial diversity of traditional fermented milks in Mongolia. Zamfir et al. (2006) suggested that the biodiversity of the isolated strains is correlated with the type of product and technology applied.

Because the qPCR approach is economical, easy to perform, and, importantly, provides more accurate quantitative data, it has been used successfully for detecting and quantifying LAB species in various fermented foods (Furet et al., 2004; Neeley et al., 2005; Wieschebrock et al., 2011; Lu et al., 2015) and human gut (Haarman and Knol, 2006; Zhang et al., 2013; Kwok et al., 2014). In the present research, 7 typical lactobacilli in dairy products and *Bifidobacterium* spp. were quantified by qPCR to determine the numbers of predominant LAB species in traditional fermented cow milk. Differences in the predominant LAB in traditional fermented cow milk existed among 3 regions of Russia (Figure 5). The raw milk used and the environment may explain variations in predominant LAB.

Of the 7 LAB species identified, *Lb. acidophilus* and *Lb. fermentum* dominated in fermented cow milk (by qPCR). By qPCR, those 2 species were detected in almost every sample and counts reached 8.00 log cfu/mL of sample in 3 regions, whereas by culture method, only 16 *Lb. fermentum* were isolated from 11 fermented cow milk samples (total of 138 samples) and *Lb. acidophilus* was not detected in any samples. Furthermore, compared with the other 5 lactobacilli and *Bifidobacte-*

rium sp., counts of *Lb. helveticus* were lowest, ranging from 1.19 to 2.85 log cfu/mL in fermented cow milk of 3 regions, but the highest numbers of *Lb. helveticus* were isolated by culture (31.6% of isolates). This might be attributed to choice of medium, culture conditions, survival ability of strains, or other factors. Hence, we should improve the medium components and culture conditions to avoid the disadvantages of the pure culture method in the future research.

CONCLUSIONS

This report focused on the exploitation of LAB resources and analysis of LAB composition in Russian traditional fermented dairy foods by culture and qPCR. First, 599 strains were isolated and identified as belonging to 7 genera and 30 species or subspecies using pure culture and molecular biological techniques. *Lactobacillus helveticus* and *Lb. plantarum* were isolated at relatively higher frequencies than the other LAB species or subspecies. Differences in species distribution existed among various fermented dairy products from 3 regions of Russia, which might be related to their geographical origin and manufacturing process. In addition, a striking difference was found between the compositions of lactobacilli in fermented cow milks of 3 regions by qPCR. This research comprehensively documents the composition of the LAB in various types of traditional fermented dairy products in Russia.

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