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Short Communication

Identification and characterization of the dominant lactobacilli isolated from koumiss in China

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Koumiss, a fermented dairy product, is a traditional drink of normande cattle breeders. It is a mildly alcoholic, sour-tasting fermented drink that is usually made from the raw milk of mares (Watanabe et al., 2008). It is produced from mare milk by a mixed lactic-acid and alcoholic fermentation by lactic acid bacteria (LAB) and yeasts. Making and drinking koumiss have a history of more than thousands years in Mongolia, Kazakhstan, Kyrgyzstan, and some regions of Russia and China (Danova et al., 2005). For centuries, koumiss has been considered as not only a kind of food, but also a complete nutriment with medicinal properties (Ishii and Konagaya, 2002). In China and Mongolia, Mongolian people have created the "koumiss therapy" which combined traditional medicine with koumiss consumption to help in the treatment of hepatitis, chronic ulcers, tuberculosis, etc. (Hasisurong et al., 2003).

As the largest grassland of China, Xinjiang, Inner Mongolia, and Qinghai have the biggest number of horses. People living in these places traditionally make and drink koumiss as part of their diet. However, there have been very few analyses of the *Lactobacillus* occurring in the koumiss of Inner Mongolia and Qinghai in China, especially in Xinjiang. Therefore the identification of lactobacilli in the traditional fermented koumiss will yield valuable knowledge.

This study reports on the isolation and characterization of the dominant lactobacilli isolated from 46 samples of koumiss which were collected from different individual households in Xinjiang, Inner Mongolia and Qinghai of China. To accurately identify and characterize the lactobacilli, several methods were used such as traditional bacterial classification methods based on morphology, physiology and biochemistry and 16S rRNA sequence analysis. Simultaneously, the phylogenetic distance between *Lactobacillus helveticus* group was estimated by analyzing the partial 16S rRNA, *tuf*, *Hsp60* and *pheS* gene sequences.

In this study, a total of 46 koumiss samples were aseptically collected from nomadic families of Xinjiang, Qinghai and Inner Mongolia Autonomous Region of China. The pH values of samples were measured with a pH meter (pH100, Extech, USA). Samples (10 ml) of each product were mixed with 90 ml of 0.85% (w/v) sterile physiological saline. Decimal dilution of milk samples were made in sterile 0.85% NaCl solution. Serial dilutions of samples (10^{-1}–10^{-8}) were plated in triplicate on the appropriate media. Plate count agar with

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Brom Cresol Purple (BCP agar, Nissui Pharmacy, Tokyo, Japan) was incubated at 30°C for 48 h for enumeration the total LAB under anaerobic conditions. Cycloheximide at a concentration of 0.01% (v/v) was added to BCP agar plate to prevent fungal growth. The counts of yeasts were determined using Potato Dextrose agar (PDA, Nissui Pharmacy), acidified to pH 3.5 with 10% sterilized tartaric acid and incubated at 25°C for 5 days.

Colonies with distinct morphological differences such as color, shape and size were selected and purified by streaking at least three times on MRS agar (DIFCO, Detroit, MI, USA). Positive lactobacilli isolates were indicated by a yellow clear zone around the colonies. Gram-positive, catalase-negative bacterial isolates were purified and frozen stocks were made in 10% (w/v) skim milk stored at −80°C.

LAB isolates were tested for NH_3 production from arginine, nitrate reduction, motility, dextran production, gas production from glucose, salt tolerance, growth temperature and pH ranges (Kozaki et al., 1992; Zhang et al., 2008). The presence of meso-DAP in the bacterial cell wall was determined with reference to the schemes outlined by Mathara et al. (2004). The type and amount of D and L isomers of lactic acid produced from glucose was assayed in modified MRS broth using a commercial kit (Hoffman La Roche Diagnostic, Mannheim, Germany). The determination of the carbohydrate fermentation patterns were performed according to the method described by Kozaki et al. (1992). Approaches followed in the phenotypic differentiation were according to the information supplied in Bergey's manual (Hammes and Vogel, 1995).

Genomic DNA of the tested strains was extracted from 5 ml of culture at 37°C in MRS broth by the CTAB (cetyltrimethylammonium bromide) method (Zhu et al., 1993). The primers of 16S rRNA and *PheS* gene had some modifications from the primers described by Scarpellini et al. (2002) and Naser et al. (2005), respectively (shown in Table 1). The fragments of *tuf* gene and *Hsp60* were amplified by using primers TUF1/TUF2 (Ventura et al., 2003) and H729/H730 (Goh et al., 2000), respectively (shown in Table 1). Each reaction mixture contained 1× *Taq* buffer (TaKaRa Biotechnology, Dalian, China), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM of each primer, 50 ng template bacterial DNA and 1.0 U Ex *Taq*™ polymerase (TaKaRa Biotechnology), and 50 μl deionized water. The PCR mixture was incubated in a MJ Research RTC-200 thermocycler (Biotech International, Perth, Australia), with an initial denaturation step at 94°C for 5 min, 30 cycles of 94°C for 30 s, 58°C for 1 min, 72°C for 1 min.

Reaction products were resolved by electrophoresis in 1.0% agarose gels and visualized by ethidium bromide staining. The PCR product of interest was isolated from the agarose gel using a Huashun Gel Extrac-

Table 1. Oligonucleotide primers used in this study.

Primer	Oligonucleotide sequence (5′-3′)	Annealing temperature (°C)
16S-FA[a]	GCAGAGTTCTCGGAGTCACGA*AGAGTTTGATCCTGGCTCAG*	58
16S-RA	AGCGGATCACTTCACACAGGA*CTACGGCTACCTTGTTACGA*	
TUF-1	GATGCTGCTCCAGAAGA	52
TUF-2	ACCTTCTGGCAATTCAATC	
PheS-21FA[b]	cgccagggttttcccagtcacgacCAYCCNGCHCGYGAYATGC	35
PheS-22RA	agcggataacaatttcacacaggaCCWARVCCRAARGCAAARCC	
H729	cgccagggttttcccagtcacgacGAIIIIGCIGGIGA(T/C)GGIACIACIAC	37
H730	agcggataacaatttcacacagga(T/C)(T/G)I(T/C)(T/G)ITCICC(A/G) AAICCIGGIGC(T/C)TT	
16S-FS	GCAGAGTTCTCGGAGTCACGA	
16S-RS	AGCGGATCACTTCACACAGGA	
M13-F	cgccagggttttcccagtcacgac	
M13-R	agcggataacaatttcacacagga	

[a] Nucleotides 1 to 21 of both 16S-FA and 16S-RA are the specific sequencing primers, respectively (underlined). Nucleotides (italic) are 16S rRNA gene universal primers (Scarpellini et al., 2002). [b] Nucleotides 1 to 24 of PheS-21FA, PheS-22RA, H729 and H730 are the M13 forward and reverse sequencing primers, respectively (lower-case).

tion Kit (Huashun, China). The purified PCR fragments were used for sequencing using the corresponding sequencing primers.

DNA sequencing was performed by Shanghai Sangni Biosciences Corporation. The consensus sequences were obtained from two reads of 16S rRNA gene using DNAstar software 7.1.0. The consensus sequences of the 16S rRNA, *tuf*, *Hsp60*, and *pheS* genes of isolated strains were compared with the sequences available in the GenBank database. A phylogenetic tree was constructed according to the neighbor-joining method by using MEGA software; the Tamura-Nei substitution model was chosen for analyzing nucleotide sequences (Tamura et al., 2007). Bootstrap analysis was performed with 500 replications.

The temperatures of koumiss collected from different areas were around 20°C , as shown in Table 2. The pH value of the fermented milk samples ranged between 3.01 and 4.08 (Table 2). A total of 46 koumiss samples were analyzed for microbiological load. The total numbers of LAB in the tested koumiss samples ranged from $10^{6.98}$ to $10^{8.09}$ CFU ml^{-1}, while the average values of yeast counts ranged from $10^{4.99}$ to $10^{6.49}$ CFU ml^{-1} (Table 2).

Koumiss fermentation is of a symbiotic nature and depends on the action of two distinct types of microorganisms, LAB and yeasts. Our experimental results indicated that the average counts of LAB in koumiss were much higher than that of the yeasts. Many researchers have demonstrated the invariable presence of large amounts of yeasts and the dominance role of LAB in koumiss samples (Naersong et al., 1996; Uchida et al., 2007; Watanabe et al., 2008). According to Narvhusa and Godaga (2003), wide distribution of LAB can lead to the specific profiles of organoleptic compounds in the milk ecosystem that are important not only for koumiss. On the other hand, strong competitiveness of LAB is necessary for bringing about the positive health-promoting effects, such as capabilities of immunity and lowering cholesterol (Gilliland, 1990; Montanari et al., 1996).

A total of 171 bacterial strains which were isolated from koumiss in Xinjiang (119), Inner Mongolia (41) and Qinghai (11) were considered as presumptive LAB because of their Gram-positive and catalase-negative properties. The isolates were screened for cell morphology and divided into seven groups according to the results of phenotypic tests (Table 3). All of the isolates fermented glucose but not rhamnose, starch or glycogen.

One hundred fourteen isolates were identified as *Lactobacillus helveticus* group. They grew well at 45°C, produced DL-lactic acid, and most of them could utilize glucose, mannose, fructose, galactose, sucrose, maltose and lactose. The 27 DAP-positive facultatively heterofermentative isolates were identified as *L. plantarum* group. They could all grow in the presence of 4.0% NaCl, and ferment most sugars except rhamnose, starch, glycogen and inositol. Eighteen isolates were found to be closely related to the *L. casei* group. They had no DAP in the cell wall and produced L-lactic acid. Only seven heterofermentative strains were isolated in all koumiss samples and were considered as belonging to the *L. fermentum* group. These strains could grow on MRS containing 4.0% NaCl, and were DAP negative and arginine positive. Most, if not all of them, were able to ferment arabinose, ribose, glucose, fructose, galactose, maltose, lactose, melibiose, and

Table 2. General features of koumiss samples.

Koumiss sample	Sampling location		Temperature (°C)	pH value	Log CFU ml^{-1} sample	
	Province	City			LAB	Yeasts
1 (n=5)	Inner Mongolia	Xilinhaote	20.86±2.07	3.82±0.21	6.98±0.13	4.99±0.27
2 (n=4)	Inner Mongolia	Zhenglan	19.93±1.52	3.94±0.08	7.46±0.11	5.04±0.32
3 (n=7)	Inner Mongolia	Abaga	20.82±1.10	3.74±0.26	7.72±0.18	5.94±0.42
4 (n=4)	Xinjiang	Wusu of Yili	23.88±2.07	3.82±0.21	7.96±0.16	5.93±0.30
5 (n=8)	Xinjiang	Nileke of Yili	17.48±3.07	3.86±0.14	7.20±0.13	5.46±0.71
6 (n=5)	Xinjiang	Sailim Lake of Boetala	19.98±1.69	4.08±0.26	7.23±0.12	5.67±0.44
7 (n=7)	Xinjiang	Xinyuan of Yili	19.73±1.97	3.87±0.24	7.32±0.22	5.84±0.47
8 (n=4)	Xinjiang	Babanrigaole	19.2 ±1.16	4.02±0.41	7.33±0.19	5.92±0.49
9 (n=2)	Qinghai	Delingha	19.40±0.20	3.01±0.02	8.09±0.12	6.49±0.43

Data represent the means (±*SD*) of number of samples (n).

Table 3. Taxonomic properties of LAB from koumiss.

Characteristics	Groups[a]						
	1	2	3	4	5	6	7
No. isolates	114	27	18	2	7	2	1
Lactic acid isomer	DL	DL	L	D(L)	DL	DL	D
Gas from glucose	0/114[b]	0/27	0/18	0/2	7/7	0/2	0/1
meso-DAP	0/114	27/27	0/18	0/2	0/7	0/2	0/1
Growth at 15°C	0/114	27/27	18/18	0/2	4/7	0/2	0/1
20°C	114/114	27/27	18/18	2/2	7/7	2/2	1/1
45°C	114/114	0/27	18/18	2/2	2/7	2/2	1/1
4% NaCl	0/114	27/27	18/18	2/2	6/7	2/2	0/1
pH 3.5	114/114	27/27	18/18	2/2	0/7	2/2	0/1
pH 4.5	114/114	27/27	18/18	2/2	7/7	2/2	1/1
pH 9.0	0/114	0/27	0/18	0/2	2/7	0/2	1/1
NH_3	0/114	0/27	0/18	0/2	7/7	0/2	0/1
Acid from							
Arabinose	0/114	17/27	0/18	0/2	6/7	0/2	0/1
Xylose	0/114	19/27	0/18	0/2	3/7	0/2	0/1
Ribose	2/114	27/27	18/18	0/2	7/7	0/2	0/1
Mannose	102/114	27/27	18/18	2/2	1/7	2/2	0/1
Fructose	86/114	27/27	18/18	2/2	7/7	2/2	1/1
Galactose	114/114	27/27	18/18	2/2	7/7	2/2	0/1
Sucrose	114/114	27/27	18/18	2/2	3/7	2/2	1/1
Maltose	114/114	26/27	18/18	2/2	7/7	2/2	0/1
Cellobiose	5/114	27/27	18/18	0/2	1/7	2/2	0/1
Lactose	98/114	27/27	16/18	2/2	5/7	2/2	1/1
Trehalose	67/114	27/27	18/18	0/2	1/7	2/2	0/1
Melibiose	11/114	23/27	0/18	2/2	7/7	2/2	0/1
Raffinose	9/114	26/27	0/18	2/2	4/7	2/2	0/1
Melezitose	15/114	20/27	18/18	0/2	0/7	0/2	0/1
Dextrin	15/114	25/27	18/18	2/2	0/7	0/2	0/1
Inulin	9/114	10/27	18/18	0/2	0/7	0/2	0/1
Mannitol	6/114	27/27	18/18	0/2	1/7	0/2	0/1
Sorbitol	9/114	27/27	18/18	0/2	0/7	0/2	0/1
Inositol	9/114	0/27	10/18	0/2	0/7	0/2	0/1
Esculin	9/114	27/27	18/18	0/2	0/7	2/2	0/1
Salicin	14/114	27/27	18/18	0/2	0/7	2/2	0/1
Amygdalin	9/114	27/27	18/18	0/2	0/7	2/2	0/1

All strains fermented glucose. No strains fermented rhamnose, starch or glycogen.

[a] Groups 1 to 7 were identified as *L. helveticus*, *L. plantarum* group, *L. casei* group, *L. kefiranofaciens*, *L. fermentum*-group, *L. acidophilus*, and *L. delbrueckii*. [b] Number of positive strains/total number of strains.

raffinose. The remaining isolates were classified as *L. kefiranofaciens*, *L. delbrueckii* and *L. acidophilus* according to their sugar fermentation and biochemical properties.

To confirm the species, the nucleotide sequences of the 16S rRNA gene of all the tested strains were analyzed and determined. The sequences obtained were deposited in GenBank and assigned the following accession numbers: FJ749565–FJ749723, FJ749725–FJ749729, FJ749732, FJ749733, EF536364, EU183494, EU715321, FJ172344, and FJ459815.

Based on the analysis of 16S rRNA genes, most of isolates were accurately identified to the species and subspecies level. The sequence distance between tested isolates and relative type strains were determined to be less than 0.003, that is, the homology ra-

Table 4. Species and numbers of LAB identified from koumiss.

Species of isolates	Number of isolates		
	Xinjiang	Inner Mongolia	Qinghai
Lactobacillus helveticus	99	12	3
Lactobacillus casei	4	14	
Lactobacillus plantarum	10	10	7
Lactobacillus kefiranofaciens	2		
Lactobacillus fermentum	1		
Lactobacillus acidophilus	2		
Lactobacillus diolivorans		2	
Lactobacillus kefiri		1	
Lactobacillus reuteri		2	
Lactobacillus pontis	1		
Lactobacillus delbrueckii			1
Total	119	41	11

tios were above 99.7%. The results of BLAST showed that the *L. casei* group isolates were highly homologous (99.9%) with *L. casei* ATCC 393, *L. paracasei* subsp. *paracasei* JCM 8130 and *L. zeae* ATCC 15820. Similarly, *L. plantarum* group and *L. kefiri* group isolates were highly homologous (99.9%) with the related type strains, too.

One hundred sixty-four of the 171 isolates belong to 6 phenotypical groups, namely, *L. helveticus* group (114 strains), *L. casei* group (18 strains), *L. plantarum* group (27 strains), *L. kefiranofaciens* (2 strains), *L. acidophilus* (2 strains), and *L. delbrueckii* (1 strain) matched well to the results of genetic analysis; 7 other strains from the defined *L. fermentum* group were clearly differentiated into 5 species, *L. fermentum* (1 strain), *L. diolivorans* (2 strains), *L. kefiri* (1 strain), *L. reuteri* (2 strains), and *L. pontis* (1 strain). Phylogenetic analysis revealed that the representative isolates and related type strains mainly consisted of two clusters. As depicted in Fig. 1, 171 *Lactobacillus* strains were clustered into 2 large groups and 6 smaller groups containing 11 species. *L. diolivorans*, *L. kefiri*, *L. casei* group, *L. plantarum* group, *L. fermentum*, *L. reuteri* and *L. pontis* clustered into one large group; moreover, *L. delbrueckii*, *L. kefiranofaciens* subsp. *kefirgranum*, *L. acidophilus* and *L. helveticus* formed another large group.

Within *Lactobacillus* isolated, three groups of species appeared as dominant: *L. helveticus*, the *L. casei* group and the *L. plantarum* group in Xinjiang, Inner Mongolia, and Qinghai, respectively. In general, *L. helveticus* is recognized as thermophilic bacteria, whilst the *L. casei* group and *L. plantarum* group belong to the mesophilic ones (Bernardeau et al., 2008; Gatti et al., 2003; Khedid et al., 2009). In this survey, their prevalence in corresponding milk samples was found quite relevant to climatic conditions since the around 20°C ambient temperature in the latter two local regions tends to favor the proliferation of mesophilic bacteria. Similarly, the linkage between ambient temperature and microbiota content in fermented milk was also justified by other studies (Mckay and Baldwin, 1990; Soomro et al., 2002).

Concerning the minor LAB found in koumiss samples, *L. acidophilus*, *L. delbrueckii*, and *L. kefiranofaciens*, have been frequently reported in various dairy products (An et al., 2004; Gadaga et al., 2001; Isono et al., 1994; Khedid et al., 2009; Naersong et al., 1996; Uchida et al., 2007). Strains of this group were most relevant to texture properties of the fermented milk for production of acid, folic acid, and exopolysaccharide, and synthesis of vitamins during growth (Forssen et al., 2000). Of the remaining five heterofermentative species that were identified as *L. fermentum*, *L. diolivorans*, *L. kefiri*, *L. reuteri*, and *L. pontis*, they were obviously isolated with relatively low frequency not only in koumiss but also in other fermented dairy products (Khedid et al., 2009; Mas et al., 2002; Tornadijo et al., 1995).

For accurate distinction between intraspecific variations, partial sequences of 16S rRNA, *tuf*, *Hsp60* and *pheS* gene of 32 randomly selected *L. helveticus* strains were analyzed. The sequences determined in this study have been deposited in the NCBI database with accession numbers from FJ825030 to FJ825125.

This allows better evaluation of the discriminatory power of the 16S rRNA gene for species identification when compared with the *tuf*, *Hsp60* and *pheS* genes included in the identification scheme. The major topology of the phylogenetic trees (Fig. 2A, B and C) constructed from the partial *tuf*, *Hsp60* and *pheS* gene sequences was similar to that constructed from the 16S rRNA gene sequences (Fig. 2D). However, *tuf*, *Hsp60* and *pheS* gene were found to provide better resolution for the *L. helveticus* species group, with lower interspecies sequence similarities compared to that obtained with the 16S rRNA gene. Our results show that the analysis of *tuf*, *Hsp60*, especially *pheS* partial gene sequences, effectively allows closely related *L. helveticus* species group to be differentiated at a higher discrimination level than that possible with

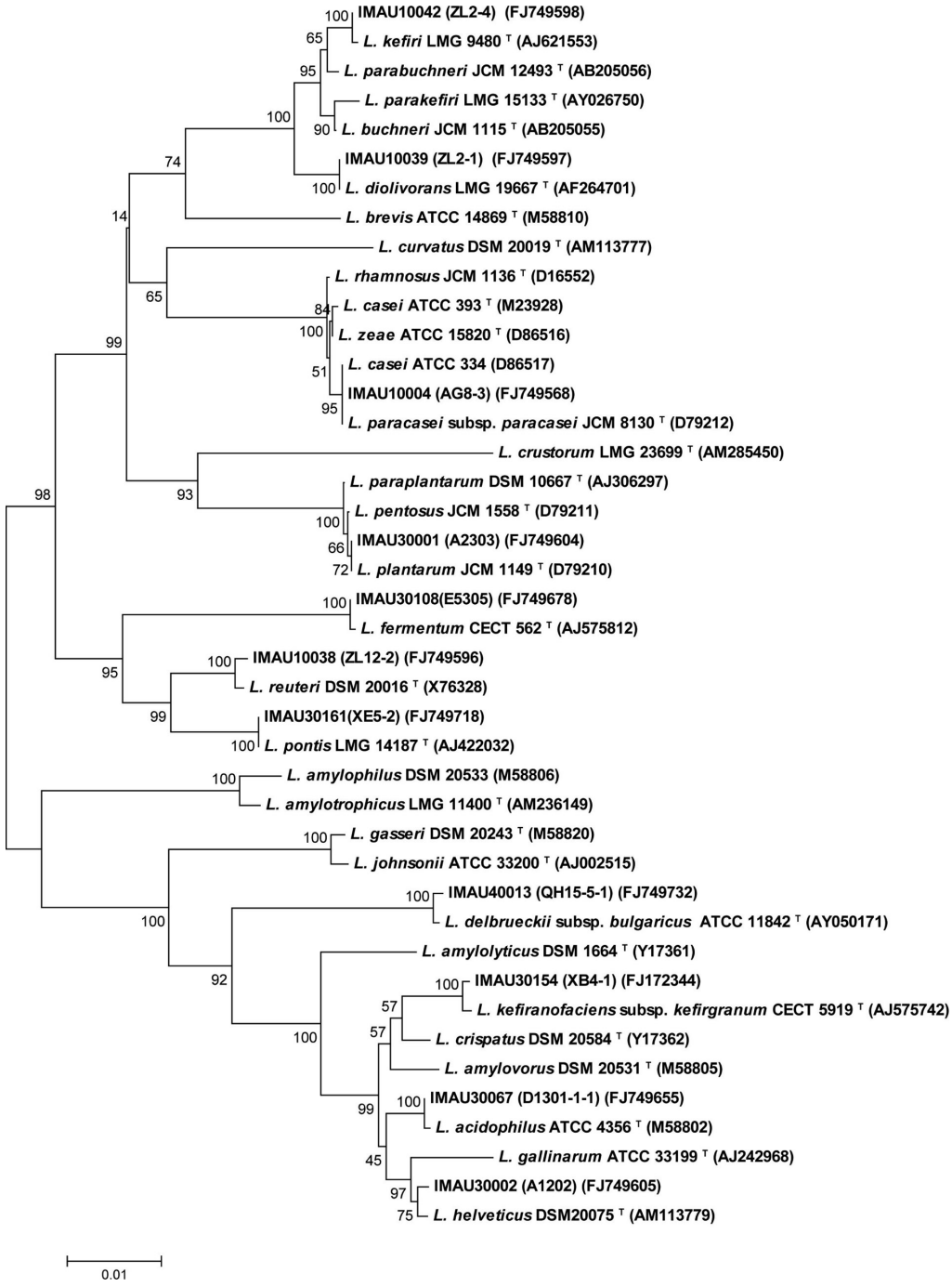

Fig. 1. Phylogenetic tree based on 16S rRNA sequence analyses, showing the phylogenetic placement of representative strains isolated from koumiss.

16S rRNA gene sequence comparisons.

During identification, 16S rRNA analysis definitely discriminates the lactobacilli isolates to species level while typing studies by sequencing *tuf*, *Hsp60*, and *pheS* genes were found more helpful in elucidating intraspecies relationships within a set of *L. helveticus*. Our observation is well consistent with the standpoint that 16S rRNA does not clarify the taxonomy of closely related *Lactobacillus* species (Schleifer and Ludwig, 1996). Given that identification based on metabolic traits is not reliable nowadays, there is growing interest in the usage of molecular methods for improving its quality and efficacy. Currently, much molecular technology has been applied in genetic diversity re-

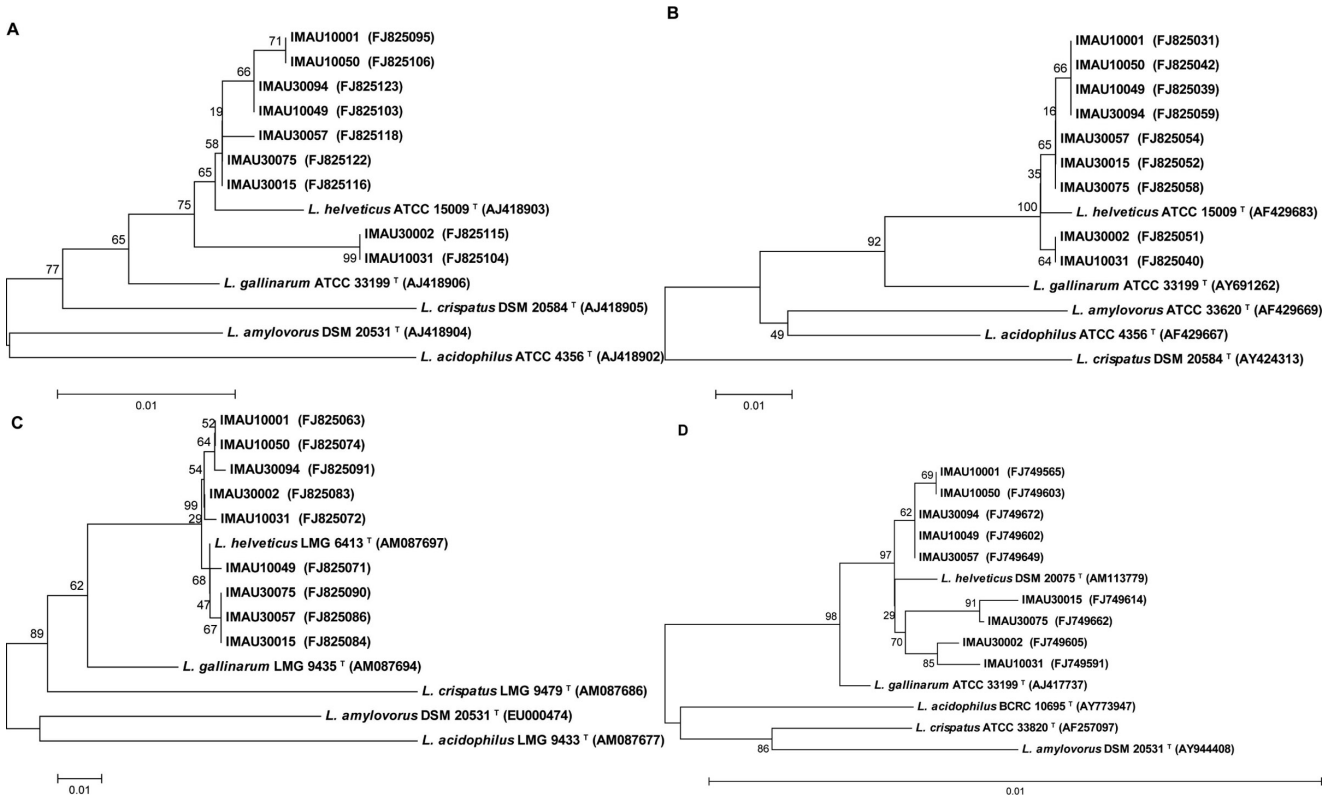

Fig. 2. (A–D) Phylogenetic trees indicated *Lactobacillus helveticus* isolates based on sequences of *tuf* (A), *Hsp60* (B), *pheS* (C) and 16S rRNA (D) gene.

search in *Lactobacillus*, such as DNA–DNA hybridization analysis, plasmid profiling and randomly amplified polymorphic DNA analysis (Catzeddu et al., 2006; Mohania et al., 2008; Sugimoto et al., 2008). For us, confirmation and full characterization of specific isolates may help to reveal the putative probiotics that exhibit desired properties and this would be an interesting finding in traditionally fermented home-made koumiss.

Generally, each kind of milk sample has its in-house microbiological composition. Indeed, differences in the distribution patterns of various lactobacilli groups were observed among samples from three geographically distant regions, in which the number of identified species ranged from 3 to 7. It is known that, during traditional fermentation of milk, contamination associated with *Lactobacillus* rapidly occurs from dairy utensils and dust (Feresu and Muzondo, 1990). In such cases, we can assume that different species distributed in samples mainly originate from production technology, which finally shape the various microbial profiles of the fermented milk.

This study aimed at determining the composition of lactobacilli and strain characteristics found in koumiss from Xinjiang, Inner Mongolia, and Qinghai in China, using conventional and molecular methods. Although there are many similar reports on the microbiota of the koumiss in Inner Mongolia and Mongolia (An et al., 2004; Danova et al., 2005; Watanabe et al., 2008), we believe that this paper provides significant data on the complex microbiota of koumiss in China, especially Xinjiang. The authors hope that the results of this study can offer useful information for further research on the traditional fermented milk.

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