

Full Paper

Identification and characterization of the dominant lactic acid bacteria from kurut: The naturally fermented yak milk in Qinghai, China

Zhihong Sun, Wenjun Liu, Wa Gao, Mei Yang, Jiachao Zhang, Lan Wu, Junguo Wang, Bilige Menghe, Tiansong Sun, and Heping Zhang*

Key Laboratory of Dairy Biotechnology and Engineering, Education Ministry of P. R. China,
Inner Mongolia Agricultural University, Hohhot 010018, P. R. China

(Received May 13, 2009; Accepted September 11, 2009)

Forty-three samples of kurut, a kind of traditional naturally-fermented yak milk, were collected in Qinghai, China. One hundred and forty-eight lactic acid bacterial (LAB) strains were isolated and identified from the kurut samples according to phenotypic characterization and 16S rRNA gene sequence analysis. Among them, 52 isolates belonged to the *Lactobacillus* strains. Ninety-six isolates were resolved to coccoid LAB. The results showed that these isolates belonged to five genera and thirteen different species and subspecies. Moreover, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* were the predominant population in these samples. This paper systematically studied the composition of LAB in kurut, which may provide raw data for further study involving probiotics strain selection and starter culture design for industrialization production of traditional fermented milk in the future.

Key Words—identification; kurut; lactic acid bacteria; 16S rRNA gene sequence

Introduction

The Tibetan tribe living in Qinghai of Northwest China, which lies in the Qing-Tibetan highlands at an average elevation of 3,000 m, has a conserved rich traditional heritage that revolves around yak. Yak thrive in treeless uplands, including plains, hills, and mountains, from as low as 1,600 m up to the limit of vegetation at about 5,400 m (Cai, 1992). Kurut, the traditional naturally-fermented yak milk, is one of the staple foods of the Tibetan nomadic people. Consumption of kurut is a part of Tibetan tribe's culture. It is a common custom for the Tibetan people to prepare their own fer-

mented milks using traditional methods in Qinghai. Kurut is produced using raw yak milk from the plateau breed of yaks. To produce acidity, alcohol and flavor to the desired level, kurut is made by fermentation in a special big tung-made jar for at least 7–8 days at ambient temperature around 10–25°C. A special characteristic of kurut is the presence of alcohol and lactic acid, just like kefir and koumiss.

Kurut was popular among most regions of Qinghai, and was considered as sacred food by Tibetan people. Moreover, kurut was the most important indigenous fermented milk product, and of considerable economic and dietary importance to the people of Qinghai as reported by Cai (1985) and Cao et al. (2004). Given the growing public awareness on the importance of food nutrition and quality, knowledge of the microbiological distribution of kurut was of great significance for further development of its processing into high quality consumer products. Some of the major fermentation processes are based on the use of

* Address reprint requests to: Dr. Heping Zhang, The Key Laboratory of Dairy Biotechnology and Engineering, Education Ministry of P. R. China, 306 Zhaowuda Street, Hohhot, China, 010018.

Tel: 86-471-4319940 Fax: 86-471-4300122
E-mail: hepingdd@vip.sina.com

lactic acid bacteria (LAB). The presence of fermentative LAB is crucial to the intrinsic properties of fermented dairy products (Ehrmann et al., 2002; Tamine and Robinson, 1988). LAB have a long history of safe use, especially in the dairy industry. They play a major role in the production of fermented food, such as the key flavor, texture and preservative qualities (Ahmad and Irene, 2007; Atrih et al., 2001; Messens and Vuyst, 2002). Recently, some of them have been completely sequenced (Altermann et al., 2005; Guchte et al., 2006; Pridmore et al., 2004). Therefore, comparative genomic analysis can help us to understand the genetic and biological diversity in LAB (Makarova and Koonin, 2007; Makarova et al., 2006; Morita et al., 2008).

As yet, information on microbiological composition of kurut is scant. Thus, it is necessary to select LAB strains with beneficial function and stabile fermentation properties from the traditional fermented milk products, and then use them to design the starter cultures for commercial production. Based on this point, this study was undertaken to enumerate, isolate, identify and characterize the dominant microorganisms from kurut samples obtained from Qinghai, using the conventional method and 16S rRNA sequence analyses.

Materials and Methods

Sample collection. A total of 43 samples of kurut were collected from scattered households in Qinghai Province of China with temperature ranging from 10.8°C to 39.3°C. At each location, approximately 500 ml kurut were taken aseptically into sterile glass bottles after thorough mixing in the bulk kurut container. The pH values of these samples were determined at the sampling site using a calibrated portable pH-meter (pH100, Extech, USA). The kurut was collected within 15 min at ambient temperatures, and kept on ice during a 2 h transport. The operation for collection of all kurut samples was completed within 48 h. The microbiological analysis was carried out immediately after the samples arrived at the laboratory.

Reference strains and culture medium. *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus delbrueckii* subsp. *bulgaricus* JCM 1002 and *Leuconostoc mesenteroides* subsp. *dextranicum* AS 1.0017 (CGMCC, Center of General Microorganism Culture Collection, China) were used as control strains.

TPY (Trypticase Peptone Yeast broth, DIFCO, BD

Bioscience, USA), MRS (Man Rogosa Sharpe broth, Fluka, USA) solid and semisolid culture, LB (Lactic Broth, DIFCO, BD Bioscience), BCP (Plate Count Agar with Brom Cresol Purple, Eiken Chemical Co., Ltd., Japan) and PDA agar culture (Potato Dextrose Agar, Nissui Pharmaceuticals Co., Ltd., Japan) were used in the experiments.

Enumeration of microorganisms. The 10^{-1} dilution was made by diluting 1 ml of kurut within 9 ml of physiological saline (0.9% NaCl). Further tenfold serial dilutions, ranging from 10^{-5} to 10^{-9} , were prepared and the LAB counts were determined using MRS agar incubated anaerobically at 30°C for 3 days according to the pour plate method described by Kozaki et al. (1992). MRS plates were incubated anaerobically at 30°C using anaerobic jars together with the BBL (Baltimore Biological Laboratory, GasPak 100 Anaerobic system, BD Bioscience) GasPak EZ Anaerobic Pouch. The counts of yeasts were determined using PDA agar, acidified to pH 3.5 with 10% sterilized tartaric acid and incubated at 25°C for 5 days. Coliforms were enumerated using the most probable number (MPN) method described by the Association of Official Analytical Chemists (AOAC, 1997) Method No. 966.24.

Isolation and preservation of microorganisms. One milliliter of the kurut sample was transferred aseptically into 10 ml sterile litmus milk (DIFCO, BD Bioscience) and incubated at 30°C. After coagulation of the milk, one loop of the milk was streaked onto MRS and TPY agar plate, and then incubated at 30°C in an anaerobic gas-jar (BBL GasPak 100 Anaerobic system, BD Bioscience) for 48–72 h. A single representative colony was selected from the agar plates and examined microscopically. Gram-positive, catalase-negative bacterial isolates were purified and the stocks frozen in 10% (w/v) skim milk broth were stored at –80°C. Lyophilization of isolates was performed for longer storage.

Conventional identification of LAB. 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 (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 was performed according to the method described by Kozaki et al. (1992). Twenty-six kinds of carbohydrates were tested. Approaches followed in the phenotypic differentiation were according to the information supplied in Bergey's Manual (Kandler and Weiss, 1986).

16S rRNA sequencing and molecular identification. 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). Briefly, collected cells of LAB were suspended in 500 µl of TE (10 mM Tris hydrochloride, 1 mM EDTA) buffer (pH 8.0). The cells were lysed by addition of 50 µl of 10% sodium dodecyl sulfate and 10 µl of proteinase K solution (10 mg/ml). Then the mixture was incubated at 55°C for 1 h. After that, 10 µl NaCl (5 M) and 100 µl CTAB/NaCl (10%, w/w) were added to the digested beads followed by a 10-min incubation at 65°C. Deproteinization was done by extraction of one volume phenol-chloroform-isoamyl alcohol (25 : 24 : 1) and chloroform-isoamyl alcohol (24 : 1) twice. Finally, DNA was precipitated by adding 0.1 volume of 3 M sodium acetate to the water phase followed by 1 volume of ice dimethyl carbinol. The DNA was collected, washed and dissolved in 20 µl sterile ultrapure water.

The 16S rRNA gene were amplified using primers 16S-FA (GCAGAGTTCTCGGAGTCA CGAAGAGTTTG ATCCTGGCTCAG) and 16S-RA (AGCGGATCACTTC ACACAGGACTACGGCTACCTTGTTACGA) as described by Scarpellini et al. (2002) with some modifications. Nucleotides 1 to 21 of both 16S-FA and 16S-RA are the specific sequencing primers (underlined). The approximately 1,500 bp sequence between these two primers corresponds to the nucleotide numbers, 27 through 1515, based on the 16S ribosomal DNA sequence of *Escherichia coli* (Scarpellini et al., 2002).

16S rRNA genes of LAB were amplified in a MJ Research RTC-200 thermocycler (Biotech International Perth, Australia). Each sample contained 1 × Taq buffer (TaKaRa, China), 1.5 mM MgCl₂, 0.2 µM of each dNTP, 10 pmol of each primer, 10 ng template bacterial DNA and 1.0 U Ex TaqTM polymerase (TaKaRa, China). The reaction conditions were as follows: 94°C for 5 min, 94°C for 1 min, 58°C for 1 min, 72°C for 2 min, 30 cycles, and then 72°C for 10 min, 4°C for 30 min.

Reaction products were resolved by electrophoresis in 1.0% agarose gels and visualized by ethidium bromide staining. The interested PCR product was isolated

from the agarose gel using a Huashun Gel Extraction Kit (Huashun, China). The purified PCR fragments were used for sequencing by 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 gene of isolated strains which were available in the GenBank database were compared. Sequence alignments were done by using the MEGA software (version 4.0). Phylogenetic trees were constructed by applying the method of Nei and Gojobori (1986) and by using the MEGA software (Tamura et al., 2007).

Results

Enumeration of microorganisms

As shown in Table 1, the average pH value of the fermented milk samples from three different regions were 4.3 ± 0.7 , 3.9 ± 0.2 and 4.0 ± 0.3 . The low pH and low environmental temperature of kurut might ensure the safety of the production process against contamination of microorganisms and contribute to the development of the specific flavor.

The microbial counts of kurut samples are presented in Table 1. The distribution of LAB and yeasts is shown according to their levels of viable counts. The LAB dominated the microbial population of kurut and the viable counts ranged from 5.74 to 10.29 log₁₀ cfu g⁻¹, with a mean value of 8.69 ± 0.88 log₁₀ cfu g⁻¹. Moreover, the kurut samples had yeast counts with values ranging from 5.32 to 9.30 log₁₀ cfu g⁻¹, with an average of 7.54 ± 0.75 log₁₀ cfu g⁻¹. Differences in the counts of lactic acid bacteria and yeasts were observed among Hainan, Haixi and Haibei regions. The count of lactic acid bacteria in Haibei was slightly higher than that in Haixi, but 20 times higher than that in Hainan. The yeast count in Hainan was about 2 times higher than that in Haixi, and 3 times higher than that in Haibei.

The mean coliform count was 18 MPN/100 ml with a range of 3–51 MPN/100 ml. Obviously, the counts of coliforms (MPN) in all detected samples were lower than 90 MPN/100 ml, which conformed as the National Standard of the People's Republic of China for Yoghurt (GB 2746-1999) (Guo, 2003) (Table 1). The MPN of coliforms may be considered as an indicator of con-

Table 1. General features of kurut samples in three different regions of Qinghai.

Samples*	Location	Temperature (°C)	pH value	LAB (Log ₁₀ cfu g ⁻¹)	Yeasts (Log ₁₀ cfu g ⁻¹)	Coliforms MPN
1 to 11	Hainan	21.5±9.9 (13.0–39.3)	4.3±0.7 (3.5–6.0)	7.74±0.92 (5.74–8.74)	7.90±1.15 (5.32–9.30)	20 (4–36)
12 to 24	Haixi	14.1±2.5 (10.8–18.9)	3.9±0.2 (3.8–4.2)	8.98±0.32 (8.4–9.47)	7.48±0.57 (6.42–8.36)	17 (4–51)
25 to 43	Haibei	16.1±4.1 (12.1–27.8)	4.0±0.3 (3.6–4.5)	9.05±0.72 (7.49–10.29)	7.37±0.52 (5.63–8.19)	16 (3–41)
Average		16.9±6.4	4.0±0.4	8.69±0.88	7.54±0.75	18

*represents 43 different sites located in 16 different villages in three region of Qinghai.

tamination by microorganisms of fecal and other enteric pathogens. Thus, this result proved that the kurut samples were safe.

Conventional identification of LAB

A total of 148 bacterial strains isolated from kurut in Qinghai were considered as presumptive LAB based on their Gram positive and catalase-negative properties.

Isolates resolved to LAB by Gram positive status, absence of catalase and oxidase activity, and cell morphology were screened simultaneously. By means of phenotypic tests, they were divided into eight groups (Table 2). All of the isolates were able to ferment glucose but not rhamnose, starch or glycogen.

Fourteen isolates were identified as *Lactobacillus* (*Lb.*) *helveticus*-group. They grew well at 20°C and 45°C, and produced DL-lactic acid, and most of them could utilize glucose, sucrose, mannose, maltose, galactose, lactose, and fructose. The 12 DAP-positive facultatively heterofermenters isolates were identified as *Lb. plantarum*. They could all grow in the presence of 4.0% NaCl, and ferment most of sugars except rhamnose, starch, inositol and glycerol. Twenty-three isolates were found to be closely related to *Lb. delbrueckii* subsp. *bulgaricus*. They had no meso-DAP in their cell walls and produced D-lactic acid.

Only three heterofermentative bacillus strains were isolated in all kurut samples and identified as *Lb. fermentum*. The strains of *Lb. fermentum* could grow on MRS containing 4.0% NaCl and pH 9.0, and were DAP negative and arginine positive. They were able to ferment ribose, glucose, mannose, fructose, galactose, sucrose, maltose, lactose, melibiose, and raffinose.

Thirteen cocci were identified as *Enterococcus* (*En.*) group. They could grow at 10°C, 40°C and pH 9.0, and

produce NH₃ from arginine. Most of them could utilize glucose, ribose, mannose, fructose, galactose, maltose, cellobiose, lactose, trehalose, esculin, salicin, amygdalin, etc. Twenty cocci were identified as *Lactococcus* (*Lc.*). They could grow at 10°C, but not 50°C. Moreover, they produced L-lactic acid.

In the present study, 12 heterofermentative coccoid strains were identified as *Leuconostoc* (*Leu.*) group on the basis of their sugar fermentation test. The remaining 51 coccoid isolates were classified as *Streptococcus* (*Strep.*) according to their sugar fermentation and biochemical properties.

16S rRNA sequence identification and phylogenetic analysis

To confirm the species, the nucleotide sequences of the 16S rRNA gene of all the isolates were analyzed and determined by the BLAST program on NCBI (<http://www.ncbi.nlm.nih.gov/>). The obtained sequences (continuous stretches of approximately 1,400 bp) were deposited in GenBank and assigned the following accession numbers: FJ749291–FJ749404, FJ915675–FJ915706, FJ915627, and FJ915628.

16S rRNA gene sequences of isolates showed more than 99.8% similarity to that of the reference strains. Fifty-two rod isolates from kurut were accurately designated to 5 species, namely *Lb. helveticus* (13 strains), *Lb. suntoryeus* (1 strain), *Lb. fermentum* (3 strains), *Lb. plantarum* (12 strains), and *Lb. delbrueckii* subsp. *bulgaricus* (23 strains). Moreover, 96 cocci were characterized as *En. durans* (7 strains), *En. faecalis* (5 strains), *En. faecium* (1 strain), *Lc. lactis* subsp. *lactis* (15 strains), *Lc. lactis* subsp. *cremoris* (5 strains), *Leu. lactis* (8 strains), *Leu. mesenteroides* subsp. *mesenteroides* (4 strains), and *Strep. thermophilus* (51 strains).

Phylogenetic tree analysis was performed to show

Table 2. Taxonomic properties of LAB isolated from kurut.

Characteristics	Groups ^a							
	1	2	3	4	5	6	7	8
No. isolates	14	12	23	3	13	20	12	51
Lactic acid isomer ^b	DL	DL	D	DL	L	L	D	L
Shape	rod	rod	rod	rod	cocci	cocci	cocci	cocci
Gas from glucose	0/14 ^c	0/12	0/23	3/3	0/13	0/20	12/12	0/51
meso-DAP	0/14	12/12	0/23	0/3	NT ^d	NT	NT	NT
Growth at 10°C	NT	NT	NT	NT	13/13	20/20	12/12	0/51
15°C	0/14	12/12	0/23	0/3	NT	NT	NT	NT
20°C	14/14	12/12	23/23	3/3	NT	NT	NT	NT
40°C	NT	NT	NT	NT	13/13	15/20	10/12	51/51
45°C	14/14	0/12	23/23	3/3	NT	NT	NT	NT
50°C	NT	NT	NT	NT	0/13	0/20	0/12	51/51
4% NaCl	0/14	12/12	0/23	3/3	13/13	15/20	12/12	11/51
6.5% NaCl	NT	NT	NT	NT	13/13	15/20	0/12	0/51
pH 3.5	14/14	12/12	0/23	1/3	1/13	0/20	0/12	0/51
pH 4.5	14/14	12/12	23/23	3/3	11/13	20/20	5/12	18/51
pH 9.0	0/14	0/12	23/23	3/3	13/13	18/20	4/12	17/51
NH ₃	0/14	0/12	0/23	3/3	13/13	14/20	0/12	0/51
Acid from								
Arabinose	0/14	7/12	0/23	2/3	0/13	0/20	4/12	0/51
Xylose	0/14	8/12	0/23	2/3	0/13	0/20	3/12	0/51
Ribose	5/14	12/12	0/23	3/3	13/13	15/20	10/12	2/51
Mannose	12/14	12/12	0/23	3/3	13/13	20/20	12/12	51/51
Fructose	11/14	12/12	23/23	3/3	13/13	20/20	12/12	48/51
Galactose	14/14	12/12	0/23	3/3	13/13	20/20	12/12	51/51
Sucrose	14/14	12/12	8/23	3/3	6/13	9/20	12/12	51/51
Maltose	14/14	11/12	4/23	3/3	11/13	15/20	12/12	0/51
Cellobiose	4/14	12/12	3/23	1/3	13/13	20/20	3/12	0/51
Lactose	11/14	12/12	23/23	3/3	13/13	20/20	12/12	51/51
Trehalose	5/14	12/12	0/23	2/3	13/13	20/20	4/12	0/51
Melibiose	2/14	11/12	0/23	3/3	5/13	0/20	7/12	4/51
Raffinose	2/14	11/12	1/23	3/3	0/13	0/20	9/12	6/51
Melezitose	3/14	10/12	1/23	0/3	4/13	0/20	0/12	0/51
Dextrin	2/14	12/12	0/23	0/3	0/13	15/20	0/12	1/51
Inulin	1/14	4/12	0/23	0/3	0/13	0/20	0/12	0/51
Mannitol	2/14	12/12	1/23	0/3	7/13	11/20	3/12	0/51
Sorbitol	1/14	12/12	0/23	0/3	4/13	0/20	0/12	0/51
Inositol	1/14	0/12	0/23	0/3	0/13	3/20	0/12	0/51
Esculin	1/14	12/12	0/23	0/3	13/13	20/20	7/12	51/51
Salicin	5/14	12/12	0/23	0/3	13/13	20/20	11/12	2/51
Amygdalin	1/14	12/12	0/23	0/3	13/13	20/20	12/12	0/51

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

^a Groups 1 to 8 were identified as *Lb. helveticus*, *Lb. plantarum*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. fermentum*, *Enterococcus*, *Lactococcus*, *Leuconostoc* and *Streptococcus*. ^b L: L-lactic acid, DL: DL-lactic acid, D: D-lactic acid. ^c Number of positive strains/total number of strains. ^dNT: Not tested.

the relationship of 16S rRNA gene sequences between the representative isolates and related type strains by using MEGA software (version 4.0) (Fig. 1). The phylogenetic tree was constructed by a neighbor-joining

phylogenetic tree and estimated by bootstrap analysis for 500 replications using the same program (Tamura et al., 2007), and consisted of seven clusters belonging to different genera. All isolates were designated to

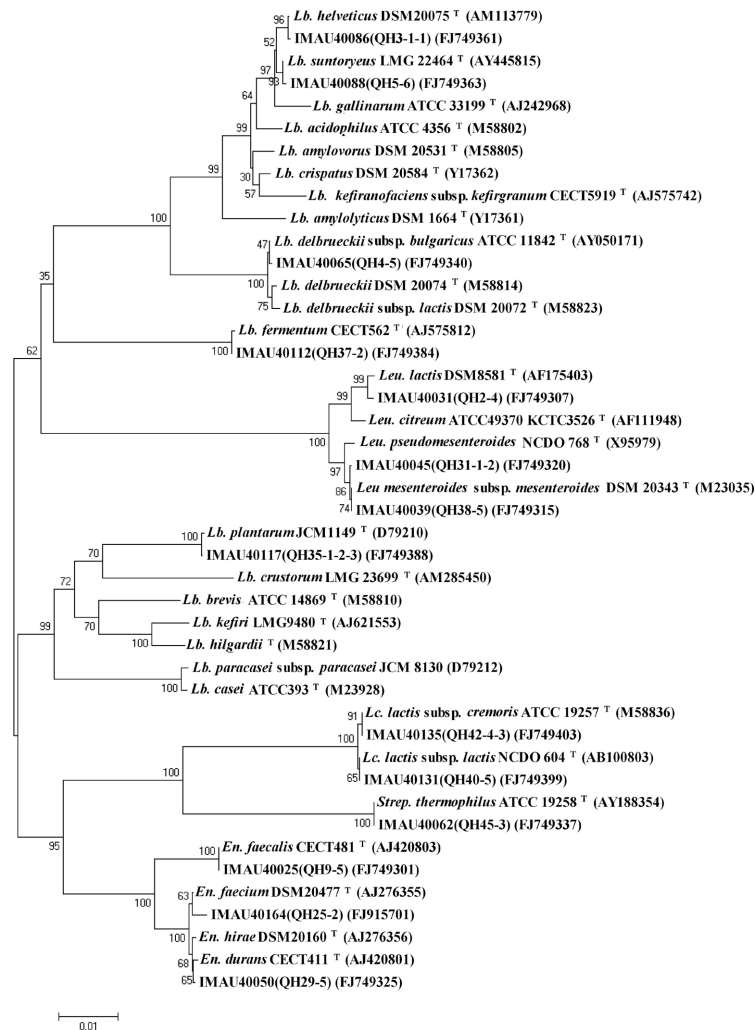


Fig. 1. Phylogenetic tree based on 16S rRNA sequence analyses, showing the phylogenetic placement of representative strains isolated from kurut.

seven clusters belonging to different genera. As for lactobacilli, 52 strains were clustered into three great groups containing five species. *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. helveticus* and *Lb. suntoryeus* clustered into one great group. Moreover, *Lb. fermentum* and *Lb. plantarum* formed two separate groups. Ninety-six coccoid strains were also clustered into three great groups containing four genera and eight species.

Discussion

In Qinghai, the history of kurut making goes back hundreds of years, and rich microbial resources have been handed down. As for kurut, fermentation is of a symbiotic nature and depends on the action of two

distinct types of microorganisms, LAB and yeasts. The major microbial composition of kurut is LAB. It was reported that the indigenous microbiota plays a major fermentation role in affecting the aroma, texture and acidity of the product as well as being of some health benefits to human beings (Duan et al., 2008; Montanari et al., 1996). In the present study, the counts of LAB and yeast were 8.69 ± 0.88 and $7.54 \pm 0.75 \log_{10} \text{cfu g}^{-1}$ in kurut samples, respectively. The results were compared with findings of similar studies on naturally fermented milks from various parts of the world. Both LAB and yeasts contents in kurut were much higher than those of the other types of traditional naturally fermented milk (Abdelgadir et al., 2001; An et al., 2004; Botes et al., 2007; Duan et al., 2008; Gadaga et al., 2000; Mathara et al., 2004; Naersong et al., 1996; Wa-

tanabe et al., 2008). The composition of yak milk and environmental factors may be the major reason influencing the characteristics of the predominant native microbiota in kurut (Zhang et al., 2008).

Potential health or nutritional benefits possible from some species of LAB have been reported, such as improving the nutritional value of food, controlling intestinal infections, improving digestion of lactose and controlling serum cholesterol levels (Gilliland, 1990; Holzapfel et al., 1998; Ishibashi and Yamazaki, 2001). As the kurut is the major dairy product consumed daily, large numbers of viable LAB from kurut are consumed daily by the local people in Qinghai. Thus, it may make a beneficial contribution to local people's health. As the advantages exhibited by probiotics are known by more and more people, more attention has been focused on the usage and safety of these beneficial strains. The first step is to identify and characterize them accurately. The characterization of LAB used to depend on cell morphologic and sugar-fermentation characters during a biochemical test (Stiles and Holzapfel, 1997; Vandamme et al., 1996). Currently, with the rapid development of biotechnology, more and more molecular technology has been applied in genetic diversity research in LAB. DNA-based techniques, such as DNA-DNA hybridization analysis, rRNA homology analysis, plasmid profiling and randomly amplified polymorphic DNA analysis have been introduced to characterize LAB (Catzeddu et al., 2006; Mohania et al., 2008; Sugimoto et al., 2008; Wang et al., 2008). In our research, we used physiological and molecular methods together.

In this study, we revealed the LAB composition of kurut by conventional and molecular genetic methods. One hundred and forty-eight representative strains isolated from the original fermented yak milk were classified and characterized through phylogenetic analysis based on their 16S rRNA gene sequences. The results revealed that the predominant lactic acid bacteria in kurut from Qinghai were *Lb. delbrueckii* subsp. *bulgaricus* and *Strep. thermophilus*. The current results were different to those in previous reports described by Watanabe et al. (2008). It is possibly because the specific environmental factors of Qinghai contributed to the composition of LAB in these samples. Wu (2001) reported that environmental factors such as elevation, dry climate, low temperature, scant oxygen, low atmospheric pressure, strong sunlight and long sunlight radiation may also contribute to the

different result. However, our results were also different to the original reports about kurut microbiologic composition from Tibet described by Yu et al. (2009), which found dominant LAB were *Lb. fermentum*, *Lb. casei*, *Enterococcus*, and *Lactococcus* in kurut from Tibet. The major reason for this difference is the different sample collecting season, which lead to different ambient temperatures in the production of these samples. In the literature of Yu et al. (2009), samples were collected in autumn. The production and preservation temperature was low; thus, the mesophilic LAB comprised the major population. However, in this study, the samples were collected in summer, so the predominant population was thermophilic strains, such as *Streptococcus thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*.

Lactobacillus usually predominated in the traditional naturally-fermented milk (An et al., 2004; Gadaga et al., 2001; Isono et al., 1994; Kamber, 2008; Naersong et al., 1996; Uchida et al., 2007). In this study, *Lactobacillus* was also considered as dominant LAB strains, which accounted for 35.1% of the total isolates. *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. plantarum*, especially *Lb. helveticus*, was isolated with high frequency in most traditional fermented milk such as koumiss (Watabe et al., 1998; Watanabe et al., 2008), Qula cheese (Duan et al., 2008), Parmigiano Reggiano cheese (Gala et al., 2008), and goats' milk cheeses (Martin-Platero et al., 2009). However, the count of *Lb. helveticus* in kurut was lower than that in koumiss, while the count of *Lb. delbrueckii* subsp. *bulgaricus* in kurut was higher than that in koumiss (Watabe et al., 1998; Watanabe et al., 2008). In addition, *Strep. thermophilus* is rarely isolated from Qula and Parmigiano Reggiano cheese (Duan et al., 2008; Gala et al., 2008). It is well known that different raw milk and different production technology of dairy products determined the various microorganism compositions and different nutritional values. Thus, kurut is a special dairy product in Qinghai, China, with indigenous LAB microflora and high nutritional value.

Similar to our result, *Strep. thermophilus* were the predominant isolates from Tarag according to Watanabe et al. (2008). In addition, about 8% of the isolate strains were identified as *Leuconostoc* species. *Leuconostoc* strains may contribute to the development of flavor quality attributes of kurut. *Leuconostoc* species generally showed a weak competitive ability during fermentation of milk and lower adaptation to

milk (Hammes and Vogel, 1995; Mathara et al., 2004). The high percentage of *Leuconostoc* strains isolated from kurut samples could be explained by the complete and rich nutrients provided by yak milk (Medina et al., 2001).

This study aimed to determine the composition of LAB and to clarify their physiological and chemical characteristics presented in kurut from Qinghai, China, by using conventional and molecular biology-based methods. We confirmed that the major lactic acid bacterial flora in kurut are *Lactobacillus* and *Streptococcus*. The physiological, chemical and sugar fermentation characteristics of LAB were also studied in this research. It will provide some raw data and strain resources for further study involved in probiotics strain selection and starter culture design concerning the industrialized production of traditional fermented milk.

Acknowledgments

This research was supported by Natural Science Foundation of China (Grant No. 30660135, 30800861), Hi-tech Research and Development Program of China (863 Program) (Grant No. 2006AA10Z345, 2007AA10Z353), New Century Excellent Talent Planning of Education Ministry of P. R. China (NCET-06-0269) and the earmarked fund for Modern Agro-industry Technology Research System of China.

References

- Abdelgadir, W. S., Hamad, S. H., Moller, P. L., and Jakobsen, M. (2001) Characterisation of the dominant microbiota of Sudanese fermented milk Rob. *Int. Dairy J.*, **11**, 63–70.
- Ahmad, F. M. and Irene, K. P. (2007) Isolation of lactic acid bacteria from Malaysian foods and assessment of the isolates for industrial potential. *Biol. Tech.*, **98**, 1380–1385.
- Altermann, E., Russell, W. M., Azcarate-Peril, M. A., Barrangou, R., Buck, B. L., McAuliffe, O., Souther, N., Dobson, A., Duong, T., Callanan, M., Lick, S., Hamrick, A., Cano, R., and Klaenhammer, T. R. (2005) Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. *Proc. Natl. Acad. Sci. USA*, **102**, 3906–3912.
- An, Y., Adachi, Y., and Ogawa, Y. (2004) Classification of lactic acid bacteria isolated from chigee and mare milk collected in Inner Mongolia. *Anim. Sci. J.*, **75**, 245–252.
- AOAC (1997) Official Method 966.24. In Official Methods of Analysis, 16th ed., 3rd revision, Association of Official Analytical Chemists, Washington, USA.
- Atrih, A., Rekhif, N., Moir, A. J. G., Lebrihi, A., and Lefebvre, G. (2001) Mode of action, purification and amino acid sequence of plantaricin C19, an anti-*Listeria* bacteriocin produced by *Lactobacillus plantarum* C19. *Int. J. Food Microbiol.*, **68**, 93–104.
- Botes, A., Todorov, S. D., Von Mollendorff, J. W., Botha, A., and Dicks, L. M. T. (2007) Identification of lactic acid bacteria and yeast from boza. *Process Biochem.*, **42**, 267–270.
- Cai, L. (1985) Domestic Animal Breeds and Their Ecological Characteristics in China, ed. by Pieliu, C., China Agricultural Press, Beijing, pp. 45–49.
- Cai, L. (1992) China Yak, ed. by Cai, L., China Agriculture Press, Beijing, 254 pp.
- Cao, Y., Gan, W., Ye, Z., Yu, H., Huang, B., Jiang, Y., and Bian, L. (2004) Investigation on yak milk products and their traditional process craft of Daocheng country. In Proceedings of Fourth International Congress on Yak, Research Institute of Animal Science and Veterinary Medicine of Ganzi Prefecture Chengdu, P. R. China, pp. 4–67.
- Catzeddu, P., Mura, E., Parente, E., Sanna, M., and Farris, G. A. (2006) Molecular characterization of lactic acid bacteria from sourdough breads produced in Sardinia (Italy) and multivariate statistical analyses of results. *Syst. Appl. Microbiol.*, **29**, 138–144.
- Duan, Y., Tan, Z., Wang, Y., Li, Z., Li, Z., Qin, G., Huo, Y., and Cai, Y. (2008) Identification and characterization of lactic acid bacteria isolated from Tibetan Qula cheese. *J. Gen. Appl. Microbiol.*, **54**, 51–60.
- Ehrmann, M. A., Kurzak, P., Bauer, J., and Vogel, R. F. (2002) Characterization of lactobacilli towards their use as probiotic adjuncts in poultry. *J. Appl. Microbiol.*, **92**, 966–975.
- Gadaga, T. H., Mutukumira, A. N., and Narvhus, J. A. (2000) Enumeration and identification of yeasts isolated from Zimbabwean traditional fermented milk. *Int. Dairy J.*, **10**, 459–466.
- Gadaga, T. H., Mutukumira, A. N., and Narvhus, J. A. (2001) The growth and interaction of yeasts and lactic acid bacteria isolated from Zimbabwean naturally fermented milk in UHT milk. *Int. J. Food Microbiol.*, **68**, 21–32.
- Gala, E., Landi, S., Solieri, L., Nocetti, M., Pulvirenti, A., and Giudici, P. (2008) Diversity of lactic acid bacteria population in ripened Parmigiano Reggiano cheese. *Int. J. Food Microbiol.*, **125**, 347–351.
- Gilliland, S. E. (1990) Health and nutritional benefits from lactic acid bacteria. *FEMS Microbiol. Rev.*, **7**, 175–188.
- Guchte, M. V. D., Penaud, S., Grimaldi, C., Barbe, V., Bryson, K., Nicolas, P., Robert, C., Oztas, S., Mangenot, S., Couloux, A., Loux, V., Dervyn, R., Bossy, R., Bolotin, A., Batto, J.-M., Walunas, T., Gibrat, J.-F., Bessières, P., Weissenbach, J., Ehrlich, S. D., and Maguin, E. (2006) The complete genome sequence of *Lactobacillus bulgaricus* reveals extensive and ongoing reductive evolution. *Proc. Natl. Acad. Sci. USA*, **103**, 9274–9279.
- Guo, B. (2003) Bacteria in yoghurt. In Yoghurt, 1st ed., Chemical Industry Press, Beijing, pp. 68–69.
- Hammes, W. P. and Vogel, R. F. (1995) The genus *Lactobacillus*. In The Genera of Lactic Acid Bacteria, Vol. 2, ed. by Wood,

- B. J. B. and Holzapfel, W. H., Blackie Academic & Professional, London, pp. 19–54.
- Holzapfel, W. H., Haberer, P., Snel, J., Schillinger, U., and Huis in't Veld, J. H. J. (1998) Overview of gut flora and probiotics. *Int. J. Food Microbiol.*, **41**, 85–101.
- Ishibashi, N. and Yamazaki, S. (2001) Probiotics and safety. *Am. J. Clin. Nutr.*, **73**, 465s–470s.
- Isono, Y., Shingu, I., and Shimizu, S. (1994) Identification and characteristics of lactic acid bacteria isolated from Masai fermented milk in Northern Tanzania. *Biosci. Biotechnol. Biochem.*, **58**, 660–664.
- Kamber, U. (2008) The manufacture and some quality characteristics of kurut, a dried dairy product. *Int. J. Dairy Technol.*, **61**, 146–150.
- Kandler, O. and Weiss, N. (1986) Genus *Lactobacillus*. In Bergey's Manual of Systematic Bacteriology, Vol. 2, 8th ed., ed. by Sneath, P. H. A., Mair, N. S., Sharpe, M. E., and Holt, J. G., Williams and Wilkins Co., Baltimore, pp. 1209–1234.
- Kozaki, M., Uchimura, T., and Okada, S. (1992) Experimental Manual of Lactic Acid Bacteria, Asakurasyoten, Tokyo, pp. 29–72.
- Makarova, K. S. and Koonin, E. V. (2007) Evolutionary genomics of lactic acid bacteria. *J. Bacteriol.*, **189**, 1199–1208.
- Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., Pavlov, A., Pavlova, N., Karamychev, V., Polouchine, N., Shakhova, V., Grigoriev, I., Lou, Y., Rohksar, D., Lucas, S., Huang, K., Goodstein, D. M., Hawkins, T., Plengvidhya, V., Welker, D., Hughes, J., Goh, Y., Benson, A., Baldwin, K., Lee, J.-H., Díaz-Muñiz, I., Dosti, B., Smeianov, V., Wechter, W., Barabote, R., Lorca, G., Altermann, E., Barrangou, R., Ganesan, B., Xie, Y., Rawsthorne, H., Tamir, D., Parker, C., Breidt, F., Broadbent, J., Hutkins, R., O'Sullivan, D., Steele, J., Unlu, G., Saier, M., Klaenhammer, T., Richardson, P., Kozyavkin, S., Weimer, B., and Mills, D. (2006) Comparative genomics of the lactic acid bacteria. *Proc. Natl. Acad. Sci. USA*, **103**, 15611–15616.
- Martin-Platero, A. M., Maqueda, M., Valdivia, E., Purswani, J., and Martinez-Bueno, M. (2009) Polyphasic study of microbial communities of two Spanish farmhouse goats' milk cheeses from Sierra de Aracena. *Food Microbiol.*, **26**, 294–304.
- Mathara, J. M., Schillinger, U., Kutima, P. M., Mbugua, S. K., and Holzapfel, W. H. (2004) Isolation, identification and characterisation of the dominant microorganisms of *Kule naoto*: The Maasai traditional fermented milk in Kenya. *Int. J. Food Microbiol.*, **94**, 269–278.
- Medina, R., Katz, M., Gonzalez, S., and Oliver, G. (2001) Characterization of the lactic acid bacteria in ewe's milk and cheese from northwest Argentina. *J. Food Prot.*, **64**, 559–563.
- Messens, W. and Vuyst, L. D. (2002) Inhibitory substances produced by *Lactobacilli* isolated from sourdoughs—A review. *Int. J. Food Microbiol.*, **72**, 31–43.
- Mohania, D., Nagpal, R., Kumar, M., Bhardwaj, A., Yadav, M., Jain, S., Marotta, F., Singh, V., Parkash, O., and Yadav, H. (2008) Molecular approaches for identification and characterization of lactic acid bacteria. *J. Dig. Dis.*, **9**, 190–198.
- Montanari, G., Zambonelli, C., Grazia, L., Kamesheva, G. K., and Shigaeva, M. K. (1996) *Saccharomyces unisporus* as the principal alcoholic fermentation microorganism of traditional koumiss. *J. Dairy Res.*, **63**, 327–331.
- Morita, H., Toh, H., Fukuda, S., Horikawa, H., Oshima, K., Suzuki, T., Murakami, M., Hisamatsu, S., Kato, Y., Takizawa, T., Fukuoka, H., Yoshimura, T., Itoh, K., O'Sullivan, D. J., McKay, L. L., Ohno, H., Kikuchi, J., Masaoka, T., and Hattori, M. (2008) Comparative genome analysis of *Lactobacillus reuteri* and *Lactobacillus fermentum* reveal a genomic island for reuterin and cobalamin production. *DNA Res.*, **15**, 151–161.
- Naersong, Tanaka, Y., Mori, N., and Kitamoto, Y. (1996) Microbial flora of "Airag", a traditional fermented milk of Inner-Mongolia in China. *Anim. Sci. Technol.*, **67**, 78–83.
- Nei, M. and Gojobori, T. (1986) Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. *Mol. Biol. Evol.*, **3**, 418–426.
- Pridmore, R. D., Berger, B., Desiere, F., Vilanova, D., Barretto, C., Pittet, A. C., Zwahlen, M. C., Rouvet, M., Altermann, E., Barrangou, R., Mollet, B., Mercenier, A., Klaenhammer, T., Arigoni, F., and Schell, M. A. (2004) The genome sequence of the probiotic intestinal bacterium *Lactobacillus johnsonii* NCC 533. *Proc. Natl. Acad. Sci. USA*, **101**, 2512–2517.
- Scarpellini, M., Mora, D., Colombo, S., and Franzetti, L. (2002) Development of genus/species-specific PCR analysis for identification of *Carnobacterium* strains. *Curr. Microbiol.*, **45**, 24–29.
- Stiles, M. E. and Holzapfel, W. H. (1997) Lactic acid bacteria of foods and their current taxonomy. *Int. J. Food Microbiol.*, **36**, 1–29.
- Sugimoto, S., Abdullah, A. M., and Sonomoto, K. (2008) Molecular chaperones in lactic acid bacteria: Physiological consequences and biochemical properties. *J. Biosci. Bioeng.*, **106**, 324–336.
- Tamine, A. Y. and Robinson, R. K. (1988) Fermented milks and their future trends: Technological aspects. *J. Dairy Res.*, **55**, 281–307.
- Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.*, **24**, 1596–1599.
- Uchida, K., Hirata, M., Motoshima, H., Urashima, T., and Arai, I. (2007) Microbiota of 'airag', 'tarag' and other kinds of fermented dairy products from nomad in Mongolia. *Anim. Sci. J.*, **78**, 650–658.
- Vandamme, P., Pot, B., Gillis, M., Vos, P. D., Kersters, K., and Swings, J. (1996) Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiol. Rev.*, **60**, 407–438.
- Wang, J. G., Chen, X., Liu, W. J., Yang, M., and Zhang, H. P.

- (2008) Identification of *Lactobacillus* from koumiss by conventional and molecular methods. *Eur. Food Res. Technol.*, **227**, 1555–1561.
- Watabe, J., Ikeda, N., Mizutani, J., Sato, N., Jin, S., Hirai, T., and Ariga, H. (1998) Comparison of microbiological and chemical characteristics among types of traditionally fermented milk in inner Mongolia in China and Calpis sour milk (Sannyuu). *Milk Sci.*, **47**, 1–7.
- Watanabe, K., Fujimoto, J., Sasamoto, M., Dugersuren, J., Tumursuh, T., and Demberel, S. (2008) Diversity of lactic acid bacteria and yeasts in Airag and Tarag, traditional fermented milk products of Mongolia. *World J. Microbiol. Biotechnol.*, **24**, 1313–1325.
- Wu, T. (2001) The Qinghai-Tibetan plateau: How high do Tibetans live? *High Alt. Med. Biol.*, **2**, 489–499.
- Yu, J., Sun, Z., Liu, W., Zhang, J., Sun, T., Bao, Q., and Zhang, H. (2009) Rapid identification of lactic acid bacteria isolated from home-made fermented milk in Tibet. *J. Gen. Appl. Microbiol.*, **55**, 181–190.
- Zhang, H., Xu, J., Wang, J., Menghebilige, Sun, T., Li, H., and Guo, M. (2008) A survey on chemical and microbiological composition of kurut, naturally fermented yak milk from Qinghai in China. *Food Control*, **19**, 578–586.
- Zhang, W. Y., Yun, Y. Y., Sun, T. S., Menghe, B., and Zhang, H. P. (2008) Isolation and identification of dominant microorganisms involved in naturally fermented goat milk in Haixi region of Qinghai, China. *Ann. Microbiol.*, **58**, 213–217.
- Zhu, H., Qu, F., and Zhu, L. H. (1993) Isolation of genomic DNAs from plants, fungi and bacteria using benzyl chloride. *Nucleic Acids Res.*, **21**, 5279–5280.