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Isolation and identification of cultivable lactic acid bacteria in traditional yak milk products of Gansu Province in China

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Various traditional fermented yak milk and raw milk foods could be considered as an abundant resource for obtaining novel lactic acid bacteria (LAB) with unique properties. Eighty-eight samples of yak milk products were collected from Gansu Province in China. Three hundred and nineteen strains of LAB isolated from these samples were identified by phenotypic methods, 16S rRNA gene sequence analysis and PCR-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) technology. Among the isolates, one hundred and sixty-four isolates (51.41% of the total) were classified under Lactobacilli, and one hundred and fifty-five (48.59%) belonged to cocci. All the isolates were classified to six genera (*Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, *Enterococcus* and *Weissella*) and twenty-one species. *Lactobacillus helveticus* (87 strains), *Leuconostoc mesenteroides* subsp. *mesenteroides* (49 strains), *Streptococcus thermophilus* (39 strains), *Lactobacillus casei* (31 strains) and *Lactococcus lactis* subsp. *lactis* (19 strains) were considered as the predominant populations in the yak milk products. The results showed that there were abundant genus and species LAB existing in yak milk products in Gansu Province in China. The obtained LAB pure cultures may be a valuable source for further starter selection.

Key Words——identification; isolation; lactic acid bacteria; PCR-DGGE; 16S rRNA gene sequence analysis; yak milk products

Introduction

Lactic acid bacteria (LAB) belong to a large family of fermentative Gram-positive bacteria which can ferment glucose with lactic acid as the major metabolic end product. LAB have a long history of safe use, especially in the dairy industry, and play a significantly role in the production of fermented dairy products (Airidengcaicike et al., 2010; Liu et al., 2004). Currently, the dairy industry is keen on exploring new possibilities for enlarging the diversity of dairy products in order to meet consumers' demands. Therefore, there is a growing interest in searching for potential starter microorganisms from various dairy products, such as raw milk products (Ouadghiri et al., 2009; Wouters et al., 2002)

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and many traditional dairy products like fermented yak milk, fermented goat milk, koumiss, butter, cheese, kefir, whey and qula (Badis et al., 2004; Chena et al., 2008; Duan et al., 2008; Kongo et al., 2007; Liu et al., 2009; Ouadghiri et al., 2005; Watanabe et al., 2008; Zhang et al., 2008a, b). Therefore, more and more basic work on isolation, genotypic and phenotypic studies was applied to wild LAB, which were isolated from raw milk and traditional fermented dairy products (Cogan et al., 1997; Wouters et al., 2002).

Gan-Nan autonomous region is located in one of the ten Zang communities (Tibetan nationality) in China. The average altitude of this area is over 3,000 m. It possesses abundant milk resources, especially yak milk. There are many kinds of yak milk products, such as raw milk, kurut (a naturally fermented yak milk product), butter (a traditional food staple on the Tsinghai-Tibet Plateau), and qula (a traditional Tibetan dried kurut solid with a granular shape). Owing to the shortage of vegetables, the local population of the Zang communities in Gansu Province has consumed much raw yak milk and traditional yak milk products to supply the nutritional needs for thousands of years. To extend the shelf-life of yak milk, fermented yak milk foods are widely produced by using traditional methods and naturally fermented in primitive utensils in this area. Kurut, qula and butter are three kinds of common naturally fermented yak milk products and are popular with Tibet herders for their rich nutrition as well as their unique flavor (Hu et al., 2010; Sun et al., 2010). The fermentation process of these products depends on the action of distinct types of microorganisms, yeasts and LAB (Coeuret et al., 2003). Abundant LAB resources were deposited and retained in these foods from generation to generation. Therefore, indigenous yak milk products could be considered as a good resource for obtaining novel LAB with desirable properties. To our knowledge, no information about LAB composition in the yak milk products of Gansu in China has been reported.

On the identification and characterization of LAB, conventional phenotypic properties may not provide sufficient basis for the reliable identification of LAB, although it is a useful tool for presumptive classification (Licitra et al., 2007). Recently, along with the fast development of molecular biology and increasing knowledge on genomic structures of LAB, many molecular typing techniques including 16S rRNA gene sequence analysis and DGGE have been developed (Ercolini 2004; Flórez and Mayo, 2006; Harun-ur-Rashid et al., 2007; Kongo et al., 2007; Licitra et al., 2007; Ogier et al., 2002). Now, the large database of 16S rRNA gene sequences enables us to identify LAB very easily (Rantsiou and Cocolin, 2006).

This paper reports the isolation, identification, and enumeration of the predominant LAB from 88 yak milk samples obtained from Gansu Province in China. All isolated LAB strains were identified on the basis of their phenotypic characterization, 16S rRNA gene sequence analysis and PCR-DGGE technologies. This study will provide useful information on the diversity of LAB flora in yak milk products in China. Moreover, the obtained LAB pure cultures will be valuable for the selection of starter cultures used in the dairy industry.

Materials and Methods

Collection of samples. Eighty-eight yak milk product samples including kurut, qula, raw yak milk, whey and butter were collected from 15 distributed villages located in Gan-Nan Zang Autonomous Region in Gansu Province in China during the period from September 4th to 12th, 2009. Detailed information on the samples is listed in Table 1. After detection of the pH value of yak milk products, samples were collected aseptically into sterile tubes, kept in an ice-box and transported to the laboratory for analysis.

LAB enumeration and isolation. One milliliter or 1 g of each product was mixed with 9 ml of 0.85% (w/v) sterile physiological saline. Serially diluted aliquots (10^{-1}–10^{-8}) of milk samples were prepared in sterile physiological saline (0.85% NaCl). The dilutions (10^{-5}–10^{-8}) were plated on appropriate BCP media (Plate Count Agar with Brom Cresol Purple, Eiken Chemical Co., LTD, Japan) in triplicate. After inoculation the plates were kept at 30°C for 2–3 days, and the total LAB counts under anaerobic conditions were counted on agar containing 0.01% (v/v) cycloheximide (Ishii, 2003).

For LAB isolation, appropriate dilution of each sample was plated on MRS (Man Rogosa Sharpe broth, Difco™) and M17 (Oxoid CM0785) culture agar plates, and then incubated anaerobically (BBL GasPak 100 Anerobic system, BD Biosciences, Sparks, MD, USA) at 30°C for 2–3 days. A representative single colony was randomly selected from the agar plates and transferred to TPY (Trypticase Peptone Yeast) broth for further identification. All isolates were initially examined

Table 1. Mean pH value, LAB count in various yak milk samples and sampling location in Gansu, China.

Sample type	Samples (*n*)	pH value	LAB count (lg CFU ml^{-1} or lg CFU g^{-1})	Sampling location in Gansu	
				Samples in each county	Sampling county (villages)[c]
Kurut	39	3.78±0.25[b] (3.54–4.50)	8.10±0.77[a] (6.03–9.97)	16	Luqu County (6)
				12	Xiahe County (5)
				9	Maqu County (2)
				2	Cooperation City (2)
Raw yak milk	15	4.42±0.48[a] (4.01–5.26)	7.96±0.67[a] (6.66–9.01)	6	Luqu County (6)
				5	Xiahe County (5)
				3	Maqu County (3)
				1	Cooperation City (1)
Yak milk qula	23	ND	7.9±0.89[a] (5.04–9.15)	11	Xiahe County (5)
				7	Luqu County (5)
				4	Maqu County (2)
				1	Cooperation City (1)
Yak milk whey	9	3.89±0.50[b] (3.50–4.50)	8.17±1.04[a] (5.64–8.98)	6	Luqu County (4)
				2	Maqu County (2)
				1	Xiahe County (1)
Yak milk butter	2	ND	7.01±0.15[b] (6.86–7.15)	2	Xiahe County (2)
Total	88			88	County/City (15)

[ab] Samples showing common superscript letters do not differ significantly ($p > 0.05$); ND: not detected; [c] The number of different villages sampled in each county in Gansu.

by Gram reaction, catalase reaction and cell morphology. Gram-positive, catalase-negative isolates were purified, and the frozen stocks in 10% (w/v) skim milk broth were stored at −80°C u. ntil use.

Molecular analysis of LAB. DNA extraction: All the strains grown in 5 ml TPY culture broth at 37°C were used for DNA extraction by a revised CTAB (cetyltrimethylammonium bromide) method (Airidengcaicike et al., 2010; Ausubel et al., 2005; Zhu et al., 1993). Briefly, collected cells of LAB were suspended in 500 µl TE buffer (pH 8.0, 10 mM Tris hydrochloride, 1 mM EDTA). Freezing and thawing were repeated three times in liquid nitrogen and a 65°C water bath. Then the cells were lysed by the addition of 50 µl 10% SDS (sodium dodecyl sulphate) and 15 µl proteinase K solution (10 mg ml^{-1}). After that, the mixture was incubated at 55°C for 10–12 h. Then, 10 µl NaCl (5 M) and 100 µl 10 mol L^{-1} CTAB/NaCl (4.1 g NaCl dissolved in 80 ml water, slowly adding 10 g CTAB) were added to the digested beads followed by a 20-min incubation at 65°C. Deproteinization was done by extraction of an equal volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and chloroform : isoamyl alcohol (24 : 1) twice. Finally, DNA was precipitated by adding 0.1 volumes of 5 M NaCl into the water phase followed by 1 volume of iced isopropyl alcohol. The DNA was collected and washed with 70% alcohol (v/v). Purified DNA was diluted to 100 ng μl^{-1} for further application.

16S rRNA sequence identification and phylogenetic analysis: All PCR amplifications of 16S rRNA genes of LAB were carried out on a PTC-200 Peltier Thermal Cycler (MJ Research Corporation), using 50 µl PCR buffer containing 15 pM of each primer, 2.5 U of r*Taq* DNA polymerase (TaKaRa Biomedical Technology, Dalian Co., Ltd., China), 5 µl of 10×PCR reaction buffer with Mg^{2+}, 4 µl of 2.5 mM dNTPs mix and 100–200 ng of template DNA. The amplification program consisted of 1 cycle at 94°C for 4 min; 30 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min; and finally 1 cycle at 72°C for 7 min.

16S rRNA genes were amplified using primers FA-27F (GCAGAGTTCTCGGAGTCACGAAGAGTTTGATCCTGGCTCAG) and RA-1495R (AGCGGATCACTTCACACAGGACTACGGCTACCTTGTTACGA) which were described by Scarpellini et al. (2002) with some modifications. Nucleotides 1 to 21 (underlined) of both FA-27F and RA-1495R are specific sequencing primers. The nucleotide numbering, 27 through 1515, was

based on the 16S rRNA gene sequence of *Escherichia coli* (Scarpellini et al., 2002). Positive products were purified using a commercial kit (TaKaRa, China) and the purified fragments were sequenced on an ABI Prism 3730XL DNA Analyzer by Shanghai Sangni Biosciences Corporation of China (Shanghai, China).

The consensus sequences were obtained from two reads of the 16S rRNA gene of the isolated strains using DNAstar software package version 7.1 (DNASTAR, Inc., Madison, WI). Gene sequence similarity searches were examined by comparing the obtained sequence with those in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1997). Phylogenetic analysis of 16S rRNA gene sequences was conducted by the neighbor-joining method using MEGA software package version 4.0 (Tamura et al., 2007). The 16S rRNA gene sequences of the isolates were deposited with the NCBI database with the accession numbers as HM058717 to HMO059029, HM217942 to HM217944, HM217953, HM217962, HM217964 and HM217967.

PCR-DGGE analysis for closely related LAB species and subspecies. The 16S rRNA gene sequences of some species, such as *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*, *Enterococcus faecium* and *Enterococcus faecalis*, shared high similarity (≥99%). Thus, it was difficult to discriminate them accurately only by 16S rRNA gene sequence analysis. So, *Lactococcus lactis* and *Enterococcus* isolates were also confirmed by PCR-DGGE technology. The variable V3 region of the 16S rRNA gene of *Lactococcus lactis* and *Enterococcus* group was amplified using the primers V3F (5′-CGCCCGCC GCGCGCGGCGGGCGGGGCGGGGGCACGGGGGC CTACGGAGGCAGCAG-3′) and V3R (5′-ATTACCGCG GCTGCTGG-3′) (Muyzer et al., 1993). PCR was performed in a total reaction volume of 50 μl containing 0.01 mM of each primer, 0.5 U of r*Taq* DNA polymerase (TaKaRa, China), 5 μl of 10× PCR reaction buffer with Mg^{2+}, 1 μl of 2.5 mM dNTPs mix and 1 μl of the template DNA. Amplification was achieved in 0.2 ml tubes by using a PTC-200 Peltier Thermal Cycler (MJ Research Corporation).

DGGE analysis of the V3 region PCR products was performed using a Dcode Universal Mutation Detection System (Bio-Rad, Hercules, USA). The 1-mm-thick 8% (w/v) polyacrylamide gel (acrylamide / diacrylamide ratio, 37.5 : 1) was made with a denaturing gradient range between 32% and 57% PCR products were applied to DGGE gels in aliquots with 35 μl per lane. The electrophoresis was performed at a constant voltage of 200 V for 4 h at 60°C in 1× TAE buffer. Then, the DNA bands were visualized by silver staining.

Results

Enumeration and isolation of LAB

The mean pH values and LAB counts in various yak milk samples are presented in Table 1. The pH values of three kinds of samples decreased in the following sequence raw yak milk, yak milk whey and kurut, and there was significant difference between raw yak milk (4.42 ± 0.48) and yak milk whey (3.89 ± 0.50). There was no difference in the pH values between yak milk whey and kurut (3.78 ± 0.25).

The LAB counts of 39 kurut samples ranged from 6.03 to 9.97 lg CFU ml^{-1}, with an average count of 8.10±0.77 lg CFU ml^{-1}. The LAB counts in 15 raw yak milk samples ranged from 6.66 to 9.01 lg CFU ml^{-1}, with an average count of 7.96±0.67 lg CFU ml^{-1}. As shown in Table 1, the average LAB counts among kurut, raw yak milk, qula and yak milk whey did not differ significantly. The average LAB counts (7.01 lg CFU ml^{-1}) of the two yak milk butter samples were slightly lower than those of other kinds of yak samples. In the present study, LAB count in most samples was larger than 7.9 lg CFU ml^{-1}. The results were similar to the findings of previous studies on naturally fermented milks from parts of various countries, as shown from Watanabe et al., 2008 and Sun et al., 2010. In 2008, Watanabe et al. reported that total concentration of LAB in Airag and Tarag in traditional fermented milk products of Mongolia were 7.78 and 8.35 lg CFU ml^{-1}, respectively (Watanabe et al., 2008). In 2010, Sun et al. found that LAB count of kurut in Qinghai in China ranged from 7.49 to 10.29 lg CFU ml^{-1}, with an average count of 8.69±0.88 lg CFU ml^{-1} (Sun et al., 2010). In our study, the count of LAB in qula varied a lot in the range from 5.04 to 9.15 lg CFU g^{-1}. This might be due to the different humidity in 23 qula samples.

Isolation of LAB

All the isolates were primarily confirmed as LAB since they manifested Gram-positive status, absence of catalase and oxidase activity. Finally, a total of 319 LAB strains were isolated from MRS and M17 agar media and purified from 88 samples (Table 2). Most of them were rod-shaped LAB (164 isolates, which

Table 2. Number of LAB isolated from various yak milk samples using MRS and M17 culture media.

	Kurut (39)[a]	Qula (23)	Raw milk (15)	Whey (9)	Butter (2)	Total LAB
Rod LAB	94 (68A+26B)[b]	32 (25A+7B)	9 (4A+5B)	22 (16A+6B)	7 (4A+3B)	164 (117A+47B) (51.41% in total)
Cocci LAB	72 (30A+42B)	18 (9A+9B)	47 (30A+17B)	14 (2A+12B)	4 (3A+1B)	155 (48.59% in total) (74A+81B)
Total LAB	166 (98A +78B)	50 (34A+16B)	56 (34A+22B)	36 (18A+18B)	11 (7A+4B)	319 (191A +128B)

[a] Sample numbers; [b] A, isolates from MRS agar; B, isolates from M17 agar.

accounted for 51.41%) and the remainder were cocci (155 isolates, which accounted for 48.59%). Moreover, there were more LAB isolates from MRS medium (191/319=59.9%) than from M17 medium (128/319=40.1%). Most rod-shaped LAB strains (117/164=71.3%) were isolated from MRS medium. On the other hand, most (81/155=52.3%) cocci LAB strains were isolated from M17 medium. Table 2 also shows that the number of cocci isolated from kurut was larger than those from the other samples.

16S rRNA gene sequence identification and phylogenetic analysis

To identify at the species level, the 16S rRNA gene sequences of the isolates were amplified and analyzed. It was found that the two selected primers (FA-27F and RA-1495R) were able to amplify the 16S rRNA gene fragment of each LAB isolate with an amplicon of approximately 1,500 bp. The consensus sequences (continuous stretches of approximately 1,450 bp) were obtained by DNAstar software. Then the sequences were compared with related bacterial sequences in the GenBank and the sequence similarities were checked using the BLAST program.

Phylogenetic analysis was performed to investigate the relationship of 16S rRNA gene sequences among the representative isolates and related type strains (Fig. 1). The isolates and related reference strains were mainly divided into four large clusters including six genera (*Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, *Enterococcus* and *Weissella*) (Fig. 1). Cluster I was the Lactobacilli group which was composed of six sub-clusters: sub-cluster I (*Lactobacillus kefiri*), sub-cluster II (*Lactobacillus diolivorans*), sub-cluster III (*Lactobacillus casei*), sub-cluster IV (*Lactobacillus plantarum)*, sub-cluster V (*Lactobacillus brevis*), and sub-cluster VI (*Lactobacillus uvarum*). Cluster II was composed of three sub-clusters: sub-cluster I (*Lactococcus raffinolactis* and *Lactococcus garvieae*), sub-cluster II (*Streptococcus*) and sub-cluster III *(Lactococcus lactis*). Cluster III was the *Weisselli* group. Cluster IV was *Leuconostoc* and the other part of the *Lactobacillus* group, which was composed of three sub-clusters: sub-cluster I (*Lactobacillus fermentum*), sub-cluster II (*Leuconostoc*) and sub-cluster III (*Lactobacillus delbrueckii* and *Lactobacillus helveticus*). Most isolates were identified at the species level by sequencing of the 16S rRNA gene, which showed more than 99% similarity with the reference strains.

Discrimination of closely related subspecies

It is well known that the 16S rRNA gene sequences of some species and subspecies share a very high similarity ($\geq$ 99%), such as *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis*, *Enterococcus faecium*, *Enterococcus faecalis* and *Enterococcus durans*. Thus, it is very difficult to discriminate them accurately only by 16S rRNA gene sequence analysis. In order to classify *Lactococcus lactis* and *Enterococcus* at the subspecies level, 25 isolates of the *Lactococcus lactis* group and 9 strains of the *Enterococcus* group were differentiated by PCR-DGGE (Figs. 2 and 3). From Fig. 2, we can see that migration distances of the V3 region of 16S rRNA gene in five LAB type strains in PCR-DGGE profile were different, which proved that PCR-DGGE technology was able to identify these five LAB strains (*Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus durans*) at the species or subspecies level. From Fig. 3, we can clearly see that some strains represented by G35-6, G45-4, G39-2, and G50-2 showed the same band size as type strain *Lactococcus lactis* subsp. *cremoris* ATCC19257. Other strains represent-

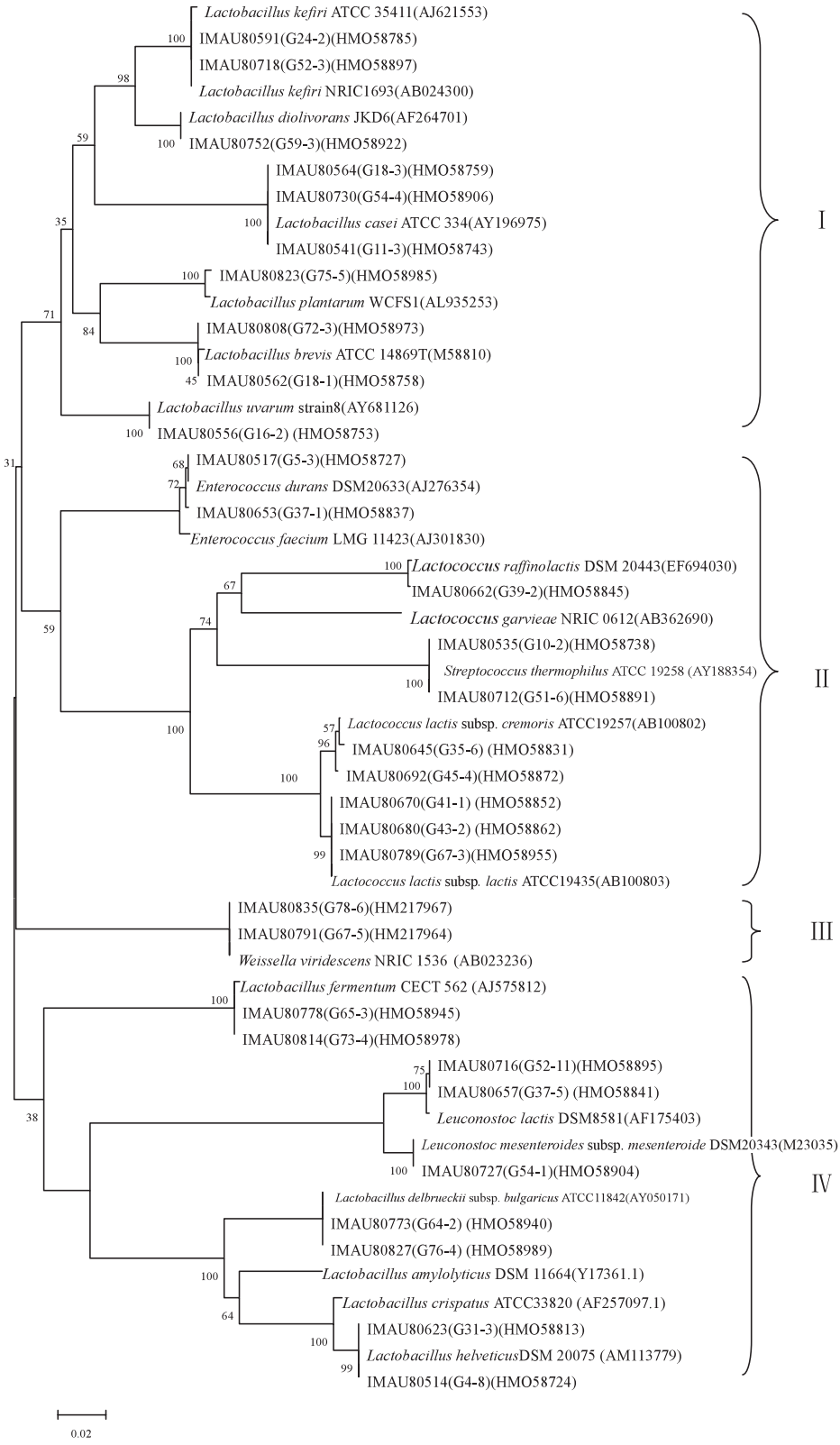

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence analyses, showing the phylogenetic placement of representative LAB strains isolated from five kinds of yak milk products in Gansu Province in China.

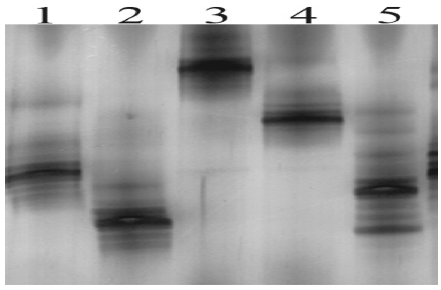

Fig. 2. PCR-DGGE profile of V3 region of 16S rRNA gene in type LAB strain of *Lactococcus lactis* group and *Enterococcus* group.
Lanes: 1, *Enterococcus durans* 19432; 2, *Enterococcus faecium* 19434; 3, *Lactococcus lactis* subsp *cremoris* 19257; 4, *Lactococcus lactis* subsp *lactis* 19258; 5, *Enterococcus faecalis* 19433.

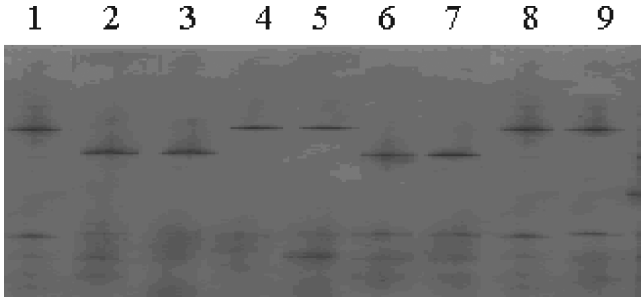

Fig. 3. DGGE profile of V3 region of 16S rRNA gene in *Lactococcus lactis* subsp. *lactis* and *cremoris*.
Lanes: 1, *Lactococcus lactis* subsp. *cremoris* ATCC19257; 2, *Lactococcus lactis* subsp. *lactis* ATCC 19435; 3, G41-1; 4, G35-6; 5,G45-4; 6, G67-3; 7, G44-3; 8, G39-2; 9, G50-2.

ed by G41-1, G67-3, and G44-3 showed the same band size as type strain *Lactococcus lactis* subsp. *lactis* ATCC 19435. Therefore, 25 isolates in the *Lactococcus* group could be classified into two subspecies accurately.

Finally, using PCR-DGGE technology, the isolates with high similarity in their 16S rRNA gene sequence were more accurately classified into four groups: *Lactococcus lactis* subsp. *lactis* (19 strains), *Lactococcus lactis* subsp. *cremoris* (6 strains), *Enterococcus durans* (8 strains) and *Enterococcus faecium* (1 strain).

Diversity of total LAB

All the isolates were successfully identified at the species level by the conventional method, 16S rRNA gene sequence analysis and PCR-DGGE technology (Table 3). According to the results shown above, these 319 isolates belonged to six genera (*Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, *Enterococcus* and *Weissella*) and twenty-one different species. The 164 rod isolates were designated as 13 species, namely *Lactobacillus brevis*, *Lactobacillus casei*, *Lactobacillus coryniformis* subsp. *torquens*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus diolivorans, Lactobacillus fermentum*, *Lactobacillus helveticus*, *Lactobacillus hilgardii, Lactobacillus kefiri, Lactobacillus plantarum, Lactobacillus rapi*, *Lactobacillus uvarum* and *Weissella viridescens*. The 155 cocci isolates were classified into 8 species: *Lactococcus lactis* (include 2 subspecies), *Lactococcus raffinolactis*, *Leuconostoc citreum, Leuconostoc lactis, Leuconostoc mesenteroides* subsp. *mesenteroides*, *Streptococcus thermophilus*, *Enterococcus durans* and *Enterococcus faecium*.

In addition, the results in Table 3 show that *Lactobacillus helveticus* (87 isolates, 27.27% of the total) and *Leuconostoc mesenteroides* subsp. *mesenteroides* (49 isolates, 15.36% of the total) can be considered as the predominant LAB in yak milk products. However, predominant LAB were different among yak milk samples. For example, in kurut samples (166 isolates), *Lactobacillus helveticus* (50 isolates, 50/166=30.12%), *Streptococcus thermophilus* (26 isolates, 26/166=15.7%) and *Leuconostoc mesenteroides* subsp. *mesenteroides* (21 isolates, 21/166=12.65%) were the dominant LAB. In raw yak milk samples, *Leuconostoc mesenteroides* subsp. *mesenteroides* (21 isolates, 21/56=37.5%) and *Lactococcus* were the dominant LAB. In yak milk whey, *Lactobacillus helveticus* (10 isolates, 10/36=27.78%) and *Lactobacillus casei* (6 isolates) were the dominant LAB. The dominant LAB in qula samples (50 isolates) was similar to that of kurut samples, with *Lactobacillus helveticus* (23 isolates, 23/50=46%), *Streptococcus thermophilus* (7 isolates) and *Leuconostoc lactis* (6 isolates) as dominant flora.

Discussion

Gansu Province is located on the plateau of China, which is famous for the breeding a large number of yaks by local Tibetans. Local herders make use of the raw yak milk to produce various home-made fermented yak milk products, such as butter, kurut, qula and whey. Yak milk butter extracted from yak milk by hand agitation is a traditional folk dairy product and is fermented by natural inoculation (Hu et al., 2010). Generally, the cream on the upper layer is removed from raw

Table 3. Distribution of LAB species in various traditional yak milk products in Gansu Province.

	Genus	Species	Kurut (39)[a]	Qula (23)	Raw milk (15)	Whey (9)	Butter (2)	Total LAB
Rod (164 isolates)	*Lactobacilli*	*Lactobacillus brevis*	1 (1A)	1 (1A)			2 (2B)	4
		Lactobacillus casei	17 (9A+8B)[b]	5 (1A +4B)	1	6 (1A+5B)	2 (1A +1B)	31 (31/319= 9.72%)
		Lactobacillus coryniformis subsp. *torquens*					2 (2A)	2
		Lactobacillus delbrueckii subsp. *bulgaricus*	11 (10A +1B)					11
		Lactobacillus diolivorans				1 (1A)		1
		Lactobacillus fermentum	6 (1A+5B)	1 (1A)	1	1 (1B)	1 (1B)	10
		Lactobacillus helveticus	50 (42A+8B) (50/166= 30.12%)	23 (19A+4B) (23/50=46%)	4	10 (6A+4B) (10/36= 27.78%)		87 (87/319= 27.27%)
		Lactobacillus hilgardii				1 (1A)		1
		Lactobacillus kefiri	4 (4A)			2 (2A)		6
		Lactobacillus plantarum		2 (1A+1B)	2 (2B)			4
		Lactobacillus rapi	1 (1B)					1
		Lactobacillus uvarum	4 (2A+2B)					4
	Weissella	*Weissella viridescens*			1 (1B)	1 (1B)		2
Cocci (155 isolates)	*Lactococcus*	*Lactococcus lactis* subsp. *cremoris*	4			2		6
		Lactococcus lactis subsp. *lactis*	9		8 (5A+3B)	2		19
		Lactococcus raffinolactis	5		8 (4A+4B)			13
	Leuconostoc	*Leuconostoc citreum*				1		1
		Leuconostoc lactis	6	6 (2A +4B)	4	2	1 (1A)	19
		Leuconostoc mesenteroides subsp. *mesenteroides*	21 (21/166= 12.65%)	1 (1A)	21 (15A+6B) (21/56= 37.5%)	5	1 (1A)	49 (49/319= 15.36%)
	Streptococcus	*Streptococcus thermophilus*	26 (26/166= 15.66%)	7 (3A+4B)	4	2		39
	Enterococcus	*Enterococcus durans*	1	4 (3A+1B)	1		2 (1A+1B)	8
		Enterococcus faecium			1			1
Total			166 (94 rod, 72 cocci)	50 (32 rod, 18 cocci)	56 (9 rod, 47 cocci)	36 (22 rod, 14 cocci)	11 (7 rod, 4 cocci)	319 (164 rod, 155 cocci)

[a] The number of samples; [b] A, isolates from MRS agar; B, isolates from M17 agar.

yak milk by agitation and the remaining yak skim milk is fermented by natural inoculation (Hu et al., 2010). Usually, raw milk kept at room temperature will spontaneously become sour due to the activity of LAB. So, herders usually heat raw milk, and add an appropriate amount of kurut as a starter into cooled processed milk, and then allow the mixture to undergo fermentation naturally into kurut. If the kurut can be separated from whey and then dried, it becomes qula, which is usually a granular dried kurut solid without a shaping process (Duan et al., 2008). By the above processes, various kinds of fermented milks and derived products were produced using naturally occurring LAB developed in Gansu, each with its own characteristic history.

In order to obtain rare, novel and various species of LAB with desirable properties, the following points during the experiment should be noted. (1) To obtain diverse samples that contain the diversity of LAB, a total of 88 samples from five kinds of yak milk products were collected in the season when the strong female yak produces the highest yield because of rich grass and sufficient water. (2) During the process of LAB isolation, LAB colonies were randomly picked using two kinds of separation media (MRS and M17 agar plates) with 30−300 colonies. It is reported that MRS and M17 medium were the most suitable media for the isolation and counting of LAB from dairy products (Ouadghiri et al., 2009). In this study, these two media were able to isolate the most LAB species found in the fermented dairy products. Some of the LAB species, for example, *Weissella viridescens* (2 strains) and *Lactobacillus rapi* (1 strain), were isolated only from M17 agar. *Lactobacillus diolivorans* (1 strain), *Lactobacillus hilgardii* (1 strain) and *Lactobacillus kefiri* (6 strains) were isolated only from MRS agar.

This paper firstl systematically analyzed the composition of LAB in various yak milk products in Gansu Province of China by conventional and molecular methods. Three hundred nineteen representative strains were isolated and identified through phylogenetic analysis based on their 16S rRNA gene sequences and PCR-DGGE technology. All the 319 isolates belonged to six genera and twenty-one species. The data indicates that LAB genera and species and their ratio existing in yak milk products are different from those of commercial yoghurt or cheese. The LAB species and diversity detected in this study were greater than those of previous studies which were conducted in other regions (Duan et al., 2008; Sun et al., 2010; Zhang et al., 2008a). This might be associated with the resource of samples, sample types, separation medium, separation technology and many other factors.

The results revealed that *Leuconostoc mesenteroides* subsp*. mesenteroides* (28.5%) and *Lactobacillus helveticus* (19.2%) were the predominant LAB in kurut samples. The results are similar to the results reported by Watanabe et al. in which *Lactobacillus helveticus* was the major LAB in traditional fermented Airag and Tarag in Mongolia (Watanabe et al., 2008). The current result for kurut is different from those in a previous report described by Sun et al. 2010, which found that *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* were the predominant population in kurut in Qinghai, China (Sun et al., 2010). The qula result (*Lactobacillus helveticus* were the predominant flora) in this research is different from the publication of Duan (Duan et al., 2008). In their study, *Leuconostoc mesenteroides* subsp. *mesenteroides* was the predominant population in yak milk qula cheese in Qinghai, China (Duan et al., 2008). This shows that the LAB microflora in yak milk products in the plateau region in China has regional differences.

Conclusion

This study was performed to isolate and identify LAB isolates accurately in traditional yak milk products which were collected from Gansu Province in China. The 319 LAB isolates in 88 yak milk products were successfully identified at the species and subspecies level by the conventional method, 16S rRNA gene sequence analysis and PCR-DGGE technologies. It will provide some raw data for further studies on the diversity of LAB and the predominant populations in the yak milk products in China. The potential use of these LAB isolates as starter culture is expected, and further studies will be needed about selected isolates and assessment of their effects on the quality of fermented products.

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References

Airidengcaicike, Chen, X., Du, X. H., Wang, W. H., Zhang, J. C., Sun, Z. H., Liu, W. J., Li, L., Sun, T. S., and Zhang, H. P. (2010) Isolation and identification of cultivable lactic acid bacteria in traditional fermented milk of Tibet in China. *Int. J. Dairy Technol.,* **63**, 437–444.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipmann, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids. Res.*, **25**, 3389–3402.

Ausubel, F. M., Moore, R. E., and Smith, J. G. (2005) Current Protocols in Molecular Biology. 4th ed., Beijing Science Press, Beijing, PP. 37–55.

Badis, A., Guetarni, D., Boudjema, B. M., Henni, D. E., and Kihal, M. (2004) Identification and technological properties of lactic acid bacteria isolated from raw goat milk of four Algerian races. *Food Microbiol.,* **21**: 579–588.

Chena, H. C., Wang, S. Y., and Chen, M. J. (2008) Microbiological study of lactic acid bacteria in kefir grains by culture-dependent and culture-independent methods. *Food Microbiol*., **25**, 492–501.

Coeuret V., Duberet, S., Bernardeau, M., Gueguen, M., and Vernoux, J. P. (2003) Isolation, characterisation and identification of *Lactobacilli* focusing mainly on cheeses and other dairy products. *Dairy Sci. Technol.,* **83**, 269–306.

Cogan, T. M., Barbosa, M., Beuvier, E., Bianchi-Salvadori, B., Cocconcelli, P. S., Fernandez, I., Gomez, M. J., and Gomez, R. (1997) Characterization of the lactic acid bacteria in artisanal dairy products. *J. Dairy Res.,* **64**, 409–421.

Duan, Y. H., Tan, Z. F., Wang, Y. P., Li, Z. W., Li, Z., Qin, G. Y., Huo, Y. P., and Cai, Y. M. (2008) Identification and characterization of lactic acid bacteria isolated from Tibetan Qula cheese. *J. Gen. Appl. Microbiol.,* **54**, 51–60.

Ercolini, D. (2004) PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *J. Microbiol. Methods,* **56**, 297–314.

Flórez, A. B. and Mayo, B. (2006) PCR-DGGE as a tool for characterizing dominant microbial populations in the Spanish blue-veined Cabrales cheese. *Intern. Dairy J.,* **16**, 1205–1210.

Harun-ur-Rashid, M., Kaname, T., Minoru, U., and Taku, M. (2007) Identification and characterization of dominant lactic acid bacteria isolated from traditional fermented milk Dahi in Bangladesh. *World J. Microbiol. Biotechnol.,* **23**, 125–133.

Hu, S. Q., Liu, H. W., Guo, S. S., Li, L., and Hou, Y. (2010) Flavor evaluation of yak butter in Tsinghai-Tibet Plateau and isolation of microorganisms contributing flavor. *Anim. Sci. J.,* **82**, 122–126.

Ishii, S. (2003) Study on the kumiss (airag) of mongolian nomads after severe cold in the winters of 2000 and 2001. *Milk Sci.,* **52**, 49–52.

Kongo, J. M., Ho, A. J., Malcata, F. X., and Wiedmann, M. (2007) Characterization of dominant lactic acid bacteria isolated from Sao Jorge cheese, using biochemical and ribotyping methods. *J. Appl. Microbiol.,* **103**, 1838–1844.

Licitra, G., Ogier, J. C., Parayre, S., Pediliggieri, C., Carnemolla, T. M., Falentin, H., Madec, M. N., Carpino, S., and Lortal, S. (2007) Variability of bacterial biofilms of the "tina" wood vats used in the Ragusano cheese-making process. *Appl. Environ. Microbiol.,* **73**, 6980–6987.

Liu, S. Q., Holland, R., and Crow, V. L., (2004) Esters and their biosynthesis in fermented dairy products: A review. *Int. Dairy J.,* **14**, 923–945.

Liu, W. J., Sun, Z. H., Zhang, J. C., Gao, W., Wang, W. H., Wu, L., Sun, T. S., Chen, W., Liu, X. M., and Zhang, H. P. (2009) Analysis of microbial composition in acid whey for dairy fan making in Yunnan by conventional method and 16S rRNA sequencing. *Curr. Microbiol.,* **59**, 199–205.

Ogier, J. C., Son, O., Gruss, A., Tailliez, P., and Delacroix-Buchet, A. (2002) Identification of the bacterial microflora in dairy products by temporal temperature gradient gel electrophoresis. *Appl. Environ. Microbiol.,* **68**, 3691–3701.

Ouadghiri, M., Amar, M., Vancanneyt, M., and Swings J. (2005) Biodiversity of lactic acid bacteria in Moroccan soft white cheese (Jben). *FEMS Microbiol. Lett.,* **251**, 267–271.

Ouadghiri, M., Vancanneyt, M., Vandamme, P., Naser, S., Gevers, D., Lefebvre, K., Swings, J., and Amar, M. (2009) Identification of lactic acid bacteria in Moroccan raw milk and traditionally fermented skimmed milk "Iben". *Appl. Microbiol.,* **106**, 486–495.

Rantsiou, K. and Cocolin, L. (2006) New developments in the study of the microbiota of naturally fermented sausages as determined by molecular methods: A review. *Int. J. Food Microbiol.,* **108**, 255–267.

Scarpellini, M., Mora, D., and Colombo, S. (2002) Development of genus/species-specific PCR analysis for identification of carnobacterium strains. *Curr. Microbiol .,***45**, 24–29.

Sun, Z. H., Liu, W. J., Gao, W., Yang, M., Zhang, J. C., Wu, L., Wang, J. G., Meng, H., and Zhang, H. P. (2010) Identification and characterization of the dominant lactic acid bacteria from kurut: The naturally fermented yak milk in Qinghai, China. *J. Gen. Appl. Microbiol.*, **56**, 1–10.

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.

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.

Wouters, J. T. M., Ayad, E. H. E., Hugenholtz, J., and Smit, G. (2002) Microbial from raw milk for fermented dairy products. *Int. Dairy J.,* **12**, 91–109.

Zhang, H. P., Xu, J., Wang, J. G., Meng, H., Sun, T. S., Li, H. P., and Guo, M. R. (2008a) 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., Meng, H., and Zhang, H. P. (2008b) Isolation and identification of dominant microorganisms involved in naturally fermented goat milk in Haixi region of Qinghai, China. *Ann. Microbiol.*, **58**, 213–218.

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.