Identification of lactic acid bacteria in traditional fermented yak milk and evaluation of their application in fermented milk products

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ABSTRACT

In this study, 53 strains of lactic acid bacteria (LAB) isolated from Xueo, a traditional fermented yak milk in the western Sichuan Plateau of China, were identified and their use in fermented milk was evaluated. All gram-positive and catalase-negative strains were divided into 6 groups at the level of 87% similarity using amplified ribosomal DNA restriction analysis. These groups were identified as 6 species using 16S rDNA sequence analysis and *atpA* gene analysis. The dominant LAB strains in Xueo were *Enterococcus durans*, *Lactobacillus fermentum*, and *Lactobacillus paracasei*, accounting for 45.3, 22.6, and 17.0% of isolates, respectively. Milk fermented with most of the representative strains was high in quality, exhibiting relatively high viscosity, moderate acidity, good sensory quality, and high counts of viable LAB. Fermented milk of *E. durans* SCA16 and *L. fermentum* SCA52 achieved the highest scores for overall sensory quality. Most strains displayed antimicrobial activity against at least 1 of 9 spoilage microorganisms. Lactic acid was the main factor inhibiting the growth of spoilage bacteria, and H₂O₂ was also inhibitory to some extent. Excluding the influence of acid and H₂O₂, strains SCA52 (*L. fermentum*) and SCA7 (*Lactobacillus plantarum*) were antagonistic against some of the indicators, suggesting that the 2 strains may produce a bacteriocin-like substance. Therefore, the development of superior strains isolated from Xueo to ferment milk with similar flavor and texture to Xueo is expected.

Key words: fermentation characteristic, lactic acid bacteria, Xueo, fermented yak milk

INTRODUCTION

Fermented dairy products have been popular for thousands of years in Tibetan regions of western Sichuan, China. Xueo is a homemade, viscous, fermented yogurt-like food prepared with yak milk, the main breeds of which are the Maiwa yak in Aba Prefecture

and the Jiulong yak in Ganzi Prefecture. Given the special characteristics of Xueo and the complex environmental factors characterizing this region, such as low temperatures (annual mean temperature <8°C), high elevation (mean 3,500 m), low oxygen levels, low atmospheric pressure, and lengthy sunlight radiation periods, the unique microbial resources in Xueo merit investigation (Zhang et al., 2008).

The traditional technique for the production of Xueo is very simple. The milk is boiled for 10 min, cooled to about 30°C, and then placed in a container. A small amount of previously produced Xueo is added to the container as a starter, and the mixture is incubated in a warm location (~30°C) until it curdles. The end product has a unique flavor along with a smooth and viscous texture, and does not deteriorate when stored at room temperature for up to 10 d. Xueo is often consumed directly or mixed with honey or sugar. This delicious and readily preserved food has been produced and consumed for thousands of years in the minority prefectures of China, such as Tibet, Sinkiang, and Inner Mongolia, and in the Himalayan regions of India, Nepal, and Bhutan (Dewan and Tamang, 2006).

Currently, starters containing mainly *Lactobacillus bulgaricus* and *Streptococcus thermophilus* are used widely to improve fermentation quality in the industrial production of yogurt. However, the use of these starters limits the organoleptic variation of the end products (Leroy and Vuyst, 2004). Thus, this research sought to contribute to the selection and application of a new starter with desirable qualities based on traditional fermented milk.

The purpose of this study was to identify and screen the lactic acid bacteria (LAB) isolated from Xueo made in the western Sichuan Plateau, and to evaluate the use of these LAB species in a starter culture for producing Xueo-flavored yogurt.

MATERIALS AND METHODS

LAB Isolation

The LAB used in this study were isolated from 12 Xueo samples collected from Ganzi and Aba Tibetan

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Autonomous Prefectures, Sichuan, China, in August 2008. All samples were transported to the laboratory in an ice box and analyzed immediately.

Four diverse media and cultural conditions were applied. de Man, Rogosa, and Sharpe (MRS) agar (De Man et al., 1960), Elliker agar (Elliker et al., 1956), KF *Streptococcus* agar (Devriese et al., 1983), and *Leuconostoc* selective medium (LUSM; Benkerroum et al., 1993) were used to culture lactobacilli at 37°C, lactococci at 30°C, enterococci at 37°C, and leuconostocs at 25°C, respectively. Ten grams of each sample was added to 90 mL of sterile physiological saline and homogenized for 10 min. One milliliter of each sample was aspirated into different media to isolate the corresponding LAB. All pure isolates were stored at -75°C in nutrient broth (Difco, Sparks, MD) supplemented with 10% dimethyl sulfoxide (vol/vol).

The Gram stain status, morphological characteristics, and catalase activity of the isolates were determined after incubation on MRS agar for 24 h. The gram-positive and catalase-negative strains were subjected to amplified ribosomal DNA restriction analysis (ARDRA).

Amplified Ribosomal DNA Restriction Analysis

DNA Extraction. Strains were harvested for DNA extraction and purification after cultivating them in MRS broth at 37°C for 18 h. Total DNA was extracted using the Bacterial Genomic DNA Extraction kit (Tiangen Co. Ltd., Beijing, China). The DNA concentration and quality were determined with a spectrophotometer.

ARDRA. The 16S rDNA of the isolates was amplified by PCR using the primers P1 (8-27f: 5'-AGAGTTTGGATCCTGGCTCAG-3') and P2 (1520-1541r: 5'-AAGGAGGTGATCCAGCCGCA-3') (Wilmutte et al., 1993). The amplified products were digested with 4 restriction endonucleases: *Hae*III, *Msp*I, *Hinf*I, and *Taq*I (MBI Fermentas, Vilnius, Lithuania). A mixture including 0.5 µL of enzyme (10 IU/µL), 2 µL of buffer, 4 µL of PCR amplified product, and 3.5 µL of distilled water was digested for 3 h at 37°C (*Taq*I at 65°C). Digested fragments were separated on 3% agarose gels. The NT-SYS program (Applied Biostatistics Inc., New York, NY) was used to calculate the distance and the unweighted pair group method with arithmetic mean (UPGMA) method was used to cluster.

16S rDNA Sequence Analysis

Eleven representative isolates were chosen to determine the 16S rDNA gene sequences using an ABI 3730 sequencer (Applied Biosystems, Foster City, CA). The 16S rDNA nucleotide sequence data were deposited in the DDBJ/EMBL/GenBank nucleotide sequence data-

bases with the serial accession numbers AB602930 to AB602939 and AB605428 for the strains SCA2, SCA17, SCA16, SCA5, SCA36, SCA22, SCA34, SCA7, SCA39, SCA52, and SCA33, respectively. Multiple sequences were assembled and aligned with the Clustal W program (Thompson et al., 1994). Type strain sequences were compared using the BLAST tool in the GenBank database (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). A phylogenetic tree was constructed using MEGA 4.0 under the Kimura 2-parameter model (Tamura et al., 2007) and bootstrapped with 1,000 replicates.

atpA Gene Analysis

The *atpA* genes of the isolates were amplified using the primers *atpA*-1 (5'-TAYRTYGGKGAYGGDA-TYGC-3') and *atpA*-2 (5'-CCRCGRTHARYTTH GCYTG-3'; Naser et al., 2005) and amplifications were checked on 1% agarose gels. The gene fragments (about 1,100 bp) were cut and then purified with a UNIQ-10 Spin kit (Sangon, Shanghai, China), and 10 µL of purified product was cloned into pGEM-T (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. Positive clones were sequenced (Sangon). The accession numbers of the *atpA* gene sequences are AB618965 to AB618969 for the strains SCA2, SCA17, SCA16, SCA7, and SCA39. Phylogenetic trees based on the *atpA* genes were constructed using the same method as that used for the 16S rDNA sequence analysis.

Phenotypic Features

Representative isolates of the different groups in the ARDRA dendrogram were chosen to determine the phenotypic features including morphological, physiological, and biochemical characteristics. Gas production from glucose and growth in 3.0 and 6.5% NaCl was tested in MRS broth at 30°C for 48 h. Growth at different temperatures, including 5, 10, 45, and 50°C was observed after incubating the isolates in MRS broth for 7 d. Growth at pH 3.0, 3.5, 4.0, 7.0, and 9.6 was detected after incubating the isolates in MRS broth at 30°C for 7 d (Yang et al., 2010).

Antimicrobial Activity

The antimicrobial activities of the isolates were determined according to the agar well diffusion assay described by Vinderola et al. (2002). Potato dextrose agar and beef extract peptone agar were used to determine antifungal activity and antibacterial activity, respectively. Nine food spoilage and pathogenic microorganisms, including *Escherichia coli* ATCC 35218, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* CMCC

63501, *Bacillus megaterium* CICC 23692, *Pseudomonas aeruginosa* ATCC 27853, *Micrococcus luteus* CICC 10209, *Saccharomyces cerevisiae* CICC 31482, *Aspergillus flavus* CICC 2003, and *Rhizopus oryzae* CICC 3073 were used as the indicator microbes. The LAB cell-free supernatants were neutralized with NaOH and incubated at 80°C for 15 min to examine inhibition of pH and H₂O₂ on the indicators (Nazef et al., 2008). The bacterial inhibition zone was recorded after incubating the bacteria at 37°C for 24 h and fungi at 28°C for 48 to 72 h.

Fermentation Characteristics of Representative Strains in Fermented Milk

Fermentation Milk Manufacture. Reconstituted milk with 12% TS content was used to make yogurt. Milk was heated at 95°C for 10 min, cooled to 30°C (similar fermentation temperature to that of Xueo), inoculated with 2% starter culture, which was subcultured 20 times in milk with a single strain, and then incubated at 30°C. The incubation ended when the samples reached pH 4.7, and fermentation time was recorded. The samples were immediately cooled and stored at 4°C for 12 h to finish maturation.

Fermentation Characteristics. One gram of each sample was serially diluted in 9 mL of sterile physiological water, and a 1-mL dilution (10^{-5} – 10^{-7}) was used to determine viable LAB counts according to the description of Terzic-Vidojevic et al. (2007). Acidity was determined by titration with 0.1 N NaOH using phenolphthalein as the indicator and the results were expressed as Thorner degrees (°T). Viscosity was determined at 5°C with an NDJ-1 rotational viscometer (Shanghai Changji Geological Instruments Co. Ltd., Shanghai, China) based on the instruction manual. All tests were performed in triplicate for 1, 5, 10, 15, and 20 d. Flavor compounds were analyzed after storage at 4°C for 24 h. The lactate and acetate concentrations in the yogurt were determined by HPLC according to the method described by Zhang et al. (2008). Diacetyl and acetaldehyde contents were determined based on the method described by Ao et al. (2005).

Sensory Evaluation. Ten panelists (age, 18–52 yr) were selected and trained for 8 h to evaluate plain yogurt based on International Organization for Standardization methods (ISO, 1993, 2008). Panelists evaluated the characteristics of the fermented yogurt with different LAB strains using a 10-point scale (Hashim et al., 2009): appearance (1 = no curd; 10 = smooth yogurt gel without whey), texture (1 = water; 10 = plain yogurt with 15% NDM), flavor (1 = yogurt with unsatisfactory odor; 10 = yogurt with satisfactory, fer-

mented flavor), and overall quality (1 = poor quality; 10 = excellent quality).

Statistical Analyses. Data on fermentation characteristics and sensory quality of yogurt fermented with representative strains were subjected to ANOVA, and the Tukey test was used to identify differences ($P < 0.05$) between means using the GLM procedure of SAS (SAS Institute Inc., Cary, NC).

RESULTS

Xueo Samples and LAB Isolation

Table 1 lists the LAB isolated from Xueo. All strains were collected in August 2008 from sites on the western Sichuan Plateau between the elevations of 1,350 and 3,560 m, with local daily temperatures fluctuating from 7 to 25°C. The pH values of the samples ranged from 3.6 to 4.2.

Colonies ($n = 109$) that differed in color, shape, and transparency were selected from 4 media. Fifty-three strains were identified preliminarily as LAB on the basis of gram positivity, catalase negativity, and lactic acid production. These strains were collected from MRS ($n = 23$), KF *Streptococcus* ($n = 20$), Elliker ($n = 7$), and *Leuconostoc* selective ($n = 3$) media.

ARDRA

Figure 1 presents a comparison of ARDRA profiles based on the restriction fragments of 16S rRNA by *MspI*, *HinfI*, *TaqI*, and *HaeIII*. All isolates were divided into 11 restriction patterns (C1 to C11) according to dissimilar restriction fragments. In contrast to *MspI*, *HinfI*, and *TaqI*, *HaeIII* achieved relatively higher discrimination in the classification of the 16S rRNA of the isolates. Representative strains SCA2 ($n = 13$), SCA17 ($n = 8$), SCA16 ($n = 3$), SCA36 ($n = 1$), SCA5 ($n = 2$), SCA52 ($n = 12$), SCA7 ($n = 2$), SCA39 ($n = 2$), SCA34 ($n = 6$), SCA33 ($n = 3$), and SCA22 ($n = 1$) from different clusters were selected for further study.

16S rRNA Sequence Analysis

Figures 2 and 3 present phylogenetic trees that give the relative positions of the isolates. Phylogenetic analysis divided the representative strains into 6 groups (A to F). Groups A (SCA2, SCA17, and SCA16) and B (SCA36 and SCA5) were assigned to the genus *Enterococcus*. The 16S rRNA sequences in group A strains showed more than 99.2% similarity to *Enterococcus hirae* and *Enterococcus durans*. Strains in

Table 1. Lactic acid bacteria isolated from Xueo fermented yak milk and their collections

Sample	Collection place	Elevation (m)	Temperature ¹ (°C)	Sample pH value	Strain
A	Rongbacha, Ganzi prefecture	3,500	7–19	3.7	SCA1, SCA2, SCA3, SCA4
B	Sexidi, Ganzi prefecture	3,200	10–13	3.6	SCA5, SCA6, SCA7, SCA8
C	Yajiang, Ganzi prefecture	2,700	13–25	4.0	SCA9, SCA10, SCA11, SCA12, SCA13, SCA14
D	Kangding, Ganzi prefecture	3,450	11–17	3.8	SCA15, SCA16, SCA17, SCA18, SCA19
E	Tagong pasture, Ganzi prefecture	3,560	9–19	3.9	SCA20, SCA21, SCA22, SCA23, SCA24, SCA25
F	Bamei, Ganzi prefecture	2,800	8–18	4.0	SCA26, SCA27, SCA28, SCA29, SCA30, SCA31
G	Hongyuan, Aba prefecture	3,400	10–23	4.1	SCA32, SCA33
H	Aba pasture, Aba prefecture	3,400	7–19	4.2	SCA34, SCA35, SCA36, SCA37, SCA38
I	Maerkang, Aba prefecture	2,700	12–20	3.9	SCA39, SCA40, SCA41, SCA42, SCA43
J	Wenchuan, Aba prefecture	1,350	12–19	3.8	SCA44, SCA45, SCA46, SCA47
K	Axi pasture, Aba prefecture	3,470	10–23	3.8	SCA48, SCA49, SCA50
L	Ruoergai, Aba prefecture	3,400	9–24	3.8	SCA51, SCA52, SCA53

¹Local daily temperature of sample collection in August 2008.

group B were closely related to *Enterococcus faecalis*. The single strain (SCA22) in Group F was placed in the genus *Streptococcus* and showed the closest relationship with *Strep. thermophilus*. Strains in groups C, D, and E were assigned to the genus *Lactobacillus*. Group C strain SCA52 was grouped with *Lactobacillus fermentum*. Strains SCA7 and SCA39 in group D showed equal similarity to *Lactobacillus plantarum* and *Lactobacillus pentosus*. The 16S rRNA sequence of strain SCA7 showed 99.4% similarity to *L. plantarum* and *L. pentosus*, and that of strain SCA39 showed 100% similarity to *L. plantarum* and 99.9% similarity to *L. pentosus*. Strains SCA33 and SCA34 in group E were closely related to *Lactobacillus paracasei* on the phylogenetic tree.

atpA Gene Sequence Analysis

Figure 4 shows a phylogenetic tree based on the *atpA* gene sequences of the *E. durans* group. Three strains (SCA2, SCA16, and SCA17) in group A were closely related to *E. durans*. They showed more than 99.7% *atpA* gene-sequence similarity to *E. durans* and less than 90.9% similarity to the closest species, *E. hirae*. Figure 5 shows a phylogenetic tree based on the *atpA* gene sequences of the *L. plantarum* group. Strains SCA7 and SCA39 in group D formed a distinct cluster with *L. plantarum* and their *atpA* gene sequences showed more than 99% similarity to *L. plantarum*, less than 96% similarity to *Lactobacillus paraplantarum*, and 92% similarity to *L. pentosus*.

Figure 1. Comparison of amplified ribosomal DNA restriction analysis (ARDRA) profiles based on the restriction fragments of 16S rDNA by *HaeIII*, *MspI*, *HinFI*, and *TaqI*. The dendrogram was constructed by the unweighted pair group method with arithmetic mean (UPGMA). Only the representative strains of each cluster are listed.

Figure 2. Phylogenetic tree showing the relative position of representative strains SCA2, SCA17, SCA16, SCA36, SCA5, and SCA22 isolated from Xueo based on the 16S rDNA sequences. Bootstrap values (1,000 replicates) are indicated at branch nodes. *Bacillus subtilis* was used as an outgroup. *La.* = *Lactococcus*; *B.* = *Bacillus*; *S.* = *Streptococcus*; *E.* = *Enterococcus*; *W.* = *Weissella*; Knuc = nucleotide substitution rate.

Phenotypic Properties

Tables 2 and 3 list the morphological, physiological, and biochemical characteristics of the representative strains isolated from Xueo. All strains grew in 3.0% NaCl, at pH 4.0 to 7.0, and at 10 to 45°C. Strains in groups A, B, and F were homofermentative cocci that formed L-lactic acid and did not produce gas from glucose. Strains in groups A and B grew in 6.5% NaCl, and at pH 9.6, but those in group F did not. Strains in groups D and E were homofermentative rods that

did not produce gas from glucose. Strains in group D formed DL-lactic acid and those in group E formed L-lactic acid. Strains in group C were heterofermentative rods that produced gas from glucose, formed DL-lactic acid, and grew in 6.5% NaCl and at pH 3.0.

Antimicrobial Activities

Table 3 shows the antimicrobial activities of the representative strains isolated from Xueo against food

Figure 3. Phylogenetic tree showing the relative position of representative strains SCA33, SCA34, SCA7, SCA39, and SCA52 isolated from Xueo based on the 16S rDNA sequences. Bootstrap values (1,000 replicates) are indicated at branch nodes. *Bacillus subtilis* was used as an outgroup. L. = *Lactobacillus*; B. = *Bacillus*; Knuc = nucleotide substitution rate.

spoilage and pathogenic microorganisms. All representative strains showed antimicrobial activity against at least 1 of the 9 indicator microbes, but no strain inhibited undesirable fungi (*Saccharomyces cerevisiae*, *Aspergillus flavus*, and *Rhizopus oryzae*). The untreated

cell-free supernatants of half of the representative strains effectively inhibited *B. megaterium*, *B. subtilis*, and *M. luteus*. The SCA52 (*L. fermentum*), SCA7 (*L. plantarum*), and SCA34 (*L. paracasei*) strains showed stronger inhibition (80%) of the indicator bacteria.

Figure 4. Neighbor-joining phylogenetic tree based on the *atpA* gene sequences of the *Enterococcus durans* group. Bootstrap values (1,000 replicates) are indicated at branch nodes. *Streptococcus thermophilus* was used as an outgroup. *S.* = *Streptococcus*; *E.* = *Enterococcus*; Knuc = nucleotide substitution rate.

Heating at 80°C for 10 min decreased the antibacterial activity of strains SCA16, SCA5, SCA39, and SCA34, and neutralization with NaOH terminated the antibacterial activity of most strains. However, the SCA52 strain continued to effectively inhibit *E. coli*, *Staph. aureus*, and *P. aeruginosa*, and the SCA7 strain exhibited antibacterial activity against *Staph. aureus*, *B. subtilis*, and *M. luteus* after heating and neutralization.

Fermentation Characteristics

Table 4 lists the fermentation characteristics of representative strains. Yogurts fermented with isolates from different groups differed significantly ($P < 0.05$) in fermentation characteristics, viable LAB counts, and sensory quality. Fermentation times ranged from 3.17 to 5.48 h. Acidity ranged from 63.40 to 87.63°T, and strains in group D (*L. plantarum*) produced more lactic acid (>80°T) than other strains. In this experiment,

Lactobacillus strains showed stronger post-acidification (8.72 to 13.87°T) than *Enterococcus* (5.8 to 9.7°T) and *Streptococcus* (8.68°T) strains. Ninety percent of samples had high viscosity (>10³ mPa·s). Yogurt fermented with SCA2 (*E. durans*) acquired the highest viscosity and that fermented with SCA33 (*L. paracasei*) showed the lowest viscosity. The average viable LAB counts of samples remained high (>10⁷ cfu/g). With regard to sensory quality, strains in group A achieved the highest scores for appearance and texture, whereas strain SCA52 in group C achieved a higher score for flavor. Strains SCA16 and SCA52 had higher (>8.0) scores for overall sensory quality based on smooth appearance, viscous texture, and satisfying flavor.

DISCUSSION

Following the appearance of domesticated yaks in Tibet, fermented yak milk became an important daily

Figure 5. Neighbor-joining phylogenetic tree based on the *atpA* gene sequences of the *Lactobacillus plantarum* group. Bootstrap values (1,000 replicates) are indicated at branch nodes. *Streptococcus thermophilus* was used as an outgroup. *S.* = *Streptococcus*; *L.* = *Lactobacillus*; Knuc = nucleotide substitution rate.

Table 2. Characteristics of representative strains¹

Characteristic	Group A			Group B		Group C	Group D		Group E		Group F
	SCA2	SCA17	SCA16	SCA36	SCA5	SCA52	SCA7	SCA39	SCA34	SCA33	SCA22
No. of isolates	13	8	3	1	2	12	2	2	3	6	1
Shape	Cocci	Cocci	Cocci	Cocci	Cocci	Rod	Rod	Rod	Rod	Rod	Cocci
Gram stain	+	+	+	+	+	+	+	+	+	+	+
Catalase reaction	–	–	–	–	–	–	–	–	–	–	–
Gas from glucose	–	–	–	–	–	+	–	–	–	–	–
Fermentation type	Homo	Homo	Homo	Homo	Homo	Hetero	Homo	Homo	Homo	Homo	Homo
Optical form of lactate	L(+)	L(+)	L(+)	L(+)	L(+)	DL	DL	DL	L(+)	L(+)	L(+)
Growth at temperature:											
5°C	w	w	w	w	w	w	w	w	–	–	–
10°C	+	+	+	+	+	+	+	+	w	w	w
45°C	+	w	+	+	w	w	+	+	w	+	+
50°C	–	–	–	–	–	–	–	–	–	–	w
Growth in NaCl:											
3.00%	+	+	+	+	+	+	+	+	+	+	+
6.50%	+	w	+	+	+	+	+	+	–	–	–
Growth at pH:											
3.0	–	–	–	w	–	+	+	+	–	+	–
3.5	w	–	–	w	–	+	+	+	+	+	w
4.0	+	+	+	+	+	+	+	+	+	+	+
7.0	+	+	+	+	+	+	+	+	+	+	+
9.6	+	+	+	+	+	–	–	–	–	–	–

¹+ = positive; w = weakly positive; – = negative; Homo = homofermentation; Hetero = heterofermentation.

Table 3. Antimicrobial activities of the representative strains against food spoilage and pathogenic microorganisms¹

Indicator microorganism	Group A			Group B			Group C			Group D			Group E			Group F																	
	SCA2			SCA17			SCA16			SCA36			SCA5			SCA52			SCA7			SCA39			SCA34			SCA33			SCA22		
	C	H	N	C	H	N	C	H	N	C	H	N	C	H	N	C	H	N	C	H	N	C	H	N	C	H	N	C	H	N			
<i>Escherichia coli</i>	-	-	-	-	-	-	-	-	-	++	++	+	++	++	+	++	++	+	++	++	-	-	-	+	+	-	-	-	-	-	-		
<i>Staphylococcus aureus</i>	-	-	-	-	-	-	-	-	-	++	++	++	++	++	+	++	++	+	++	++	+	+	-	+	+	-	-	-	-	-	-		
<i>Bacillus subtilis</i>	+	+	-	+	+	-	-	-	-	++	++	-	++	++	-	++	++	-	++	++	+	+	+	++	++	-	-	-	-	-	-		
<i>Bacillus megaterium</i>	+	+	-	+	+	+	-	-	-	++	++	+	++	++	-	++	++	-	++	++	+	+	+	++	++	+	+	+	+	+	+		
<i>Pseudomonas aeruginosa</i>	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	-	-	-	-	-	-		
<i>Micrococcus luteus</i>	-	-	-	-	-	-	-	-	-	++	++	++	++	++	+	++	++	+	++	++	+	+	+	++	++	+	+	+	+	+	+		
<i>Saccharomyces cerevisiae</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
<i>Aspergillus flavus</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
<i>Rhizopus oryzae</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		

¹C = control, without any treatment; H = heating cell-free broth at 80°C for 10 min; N = neutralization with NaOH; +++ = d (diameter of inhibition zone) >10 mm; ++ = 5 mm < d < 10 mm; + = 0 < d < 5 mm; - = no inhibition.

food product in this region and has remained such for thousands of years (Airdengcaicike, 2010). To store and preserve excess yak milk, Tibetans follow the traditional and ancient method of making Xueo. The fermentation process depends on naturally occurring microorganisms in yak milk. The indigenous microbiota of this environment has been preserved in numerous subcultures and provides the distinct flavor and viscous texture of Xueo. No previous study has intensively investigated the characteristics of LAB isolated from Chinese Xueo. These microflora were described in detail due to the high fermentation quality and nutritive value of the fermented milk.

In the present study, all strains were analyzed using the ARDRA method. Then, the representative strains were selected by different 16S rDNA restriction patterns digested by HaeIII, MspI, HinfI, and TaqI (Figure 1). The LAB strains isolated from Xueo were divided into 11 clusters by ARDRA and 6 species were identified by 16S rDNA and *atpA* gene sequence analyses. Phylogenetic analysis of 16S rDNA sequences identified strains in groups B, E, and F as *L. fermentum*, *E. faecalis*, and *Strep. thermophilus*, respectively. Strains in group A showed equal similarity to *E. hirae* and *E. durans* on the phylogenetic tree and over 99.2% 16S rDNA sequence similarity to the 2 species. Previous reports have determined that 16S rDNA sequences have limited discriminating power for identifying the species in the *E. durans* group (*E. durans*, *E. hirae*, *Enterococcus villorum*, *Enterococcus munditii*, and *Enterococcus faecium*), and complicated methods, such as amplified fragment length polymorphism, pulsed-field gel electrophoresis, and transfer DNA-PCR, have been used as alternatives (Devriese et al., 2002). Naser et al. (2005) distinguished these species by comparing *atpA* gene sequences. In this study, 6 closely related *Enterococcus* species were categorized distinctively (Figure 4) by phylogenetic analysis of *atpA* genes and the strains in group A were identified exclusively as *E. durans*. Strains in group D formed a cluster with *L. plantarum* and *L. pentosus*, and their 16S rRNA sequences showed over 99.4% similarity to the 2 species. *L. plantarum*, *L. pentosus*, and *L. paraplantarum* have closely related genotypes and highly similar phenotypes, complicating their discrimination by the conventional method (Torriani et al., 2001). In the present study, *atpA* gene sequence analysis unambiguously identified the strains of group D as *L. plantarum*. Following the 16S rDNA analysis, strains SCA33 and SCA34 clustered together, and the typical strain *L. paracasei* was the most closely related species, which was supported by more than 99.5% similarity in the 16S rRNA gene sequence analysis. The other typical strains *Lactobacillus casei* and *Lactobacillus rhamno-*

Table 4. Fermentation characteristics of representative strains in fermented milk¹

Item	Strain	Fermentation characteristic					Sensory quality ³			
		Fermentation time ⁴ (h)	Acidity ⁵ (°T)	PA ⁶ (°T)	Viscosity ⁷ (10 ³ mPa·s)	Viable LAB ² [log (cfu/g)]	Appearance	Texture	Flavor	Overall quality
Group										
A	SCA2	4.12 ^d	67.52 ^d	6.72 ^e	6.66 ^a	9.69 ^{ab}	8.00 ^{ab}	8.07 ^a	7.63 ^{ab}	7.70 ^{ab}
	SCA17	4.52 ^c	65.62 ^{de}	6.57 ^e	4.63 ^c	9.48 ^b	7.87 ^{abc}	8.00 ^{ab}	7.77 ^{ab}	7.87 ^{ab}
	SCA16	4.33 ^{cd}	63.40 ^f	5.80 ^f	6.18 ^b	9.31 ^{bc}	8.16 ^a	8.10 ^a	7.83 ^{ab}	8.03 ^a
B	SCA5	5.23 ^b	64.93 ^e	8.45 ^d	4.35 ^c	9.57 ^{ab}	7.67 ^{bcd}	7.73 ^{bdc}	6.60 ^c	7.17 ^{dc}
	SCA36	5.48 ^b	65.47 ^{de}	8.88 ^d	3.63 ^d	9.51 ^{ab}	7.23 ^e	7.73 ^{bcd}	5.70 ^d	6.77 ^d
C	SCA52	4.31 ^{cd}	70.87 ^c	8.72 ^d	4.67 ^c	8.93 ^{cd}	7.83 ^{abc}	7.90 ^{abc}	8.03 ^a	8.00 ^a
D	SCA7	3.76 ^e	83.07 ^b	12.82 ^b	1.59 ^f	8.67 ^d	7.18 ^e	7.67 ^{dc}	7.77 ^{ab}	7.73 ^{ab}
	SCA39	3.17 ^f	87.63 ^a	13.87 ^a	1.82 ^f	9.91 ^a	7.33 ^{cd}	7.57 ^d	7.67 ^{ab}	7.60 ^b
E	SCA33	4.34 ^{cd}	71.37 ^c	9.20 ^c	0.82 ^g	7.71 ^e	6.77 ^f	6.23 ^e	4.73 ^e	4.30 ^e
	SCA34	4.10 ^d	73.43 ^c	9.70 ^c	1.00 ^g	8.80 ^d	7.47 ^{dce}	7.83 ^{abcd}	7.50 ^b	7.67 ^{ab}
F	SCA22	4.22 ^{cd}	67.90 ^d	8.68 ^d	2.28 ^e	8.61 ^d	7.60 ^{bcd}	7.73 ^{bcd}	7.73 ^{ab}	7.53 ^{bc}
SEM		0.07	0.57	0.11	0.08	0.08	0.08	0.06	0.09	0.08

^{a-g}Means in the same column with different superscript letters are significantly different ($P < 0.05$).

¹Means of 3 replicates.

²Viable lactic acid bacteria (LAB) was the average value for 20 d.

³1 = poor quality to 10 = excellent quality.

⁴The time from inoculation to pH 4.7.

⁵Acidity was determined after storing at 4°C for 12 h.

⁶PA = post-acidification, the increased acidity during storage at 5°C for 20 d, expressed as Thorner degrees (°T).

⁷Viscosity was the average value for 20 d.

sus showed similarities of <98.9%. Therefore, strains in group C belonged to *L. paracasei*.

The dominant LAB strains in naturally fermented milk were *Lactococcus lactis* in Rome (Zamfir, et al., 2006), *L. paracasei* and *L. lactis* in Himalayan ethnic fermented milk products (Dewan and Tamang, 2007), and *Leuconostoc mesenteroides* and *E. faecium* in Serbia (Jokovic et al., 2008). In our study, the dominant LAB strains in Xueo were *E. durans* (45.3%), *L. fermentum* (22.6%), and *L. paracasei* (17.0%). *E. faecalis*, *L. plantarum*, and *Strep. thermophilus* were present in lower frequencies. Different raw materials and environmental conditions have contributed to the unique microflora in these fermented milks and have influenced the texture and flavor of the end products.

The acidity, texture, and sensory quality of fermented milk are strongly related to the starter culture. In this study, different species appeared to have distinct functions in fermented milk. Enterococci, especially *E. durans*, were isolated from almost all samples and produced yogurts with high score on appearance and texture. Previous studies have demonstrated that enterococci have numerous functions, such as taste formation through proteolysis, enterocin production to inhibit other bacteria, and the formation of viscous material to improve texture, which have led to their use in many food production applications (Foulquié Moreno et al., 2006). In this study, yogurts fermented with *E. durans* acquired high viscosity (Table 4), and

this quality has been associated with the exopolysaccharide produced by LAB strains (Patrignani et al., 2006). Many papers have reported that exopolysaccharide enhances the texture and viscosity of products, improves their functional properties, and may protect cells against detrimental environmental conditions and phage infection (Broadbent et al., 2003; Purwandari et al., 2007). Exopolysaccharide may, thus, be one reason for the highly viscous texture of Xueo and its failure to deteriorate when stored at room temperature in its original environment for more than 10 d. The dominant lactobacilli in Xueo were *L. fermentum* and *L. paracasei*. Although moderate acidity and weak post-acidification contribute to product stability and long shelf-life, most lactobacilli in fermented milk are characterized by relatively higher acidification rates and strong post-acidification (St-Gelais et al., 2009). In this experiment, yogurts fermented with strains SCA52 (*L. fermentum*), SCA33 (*L. paracasei*), and SCA34 (*L. paracasei*) had appropriate acidity (70.87 to 73.43°T) and weaker post-acidification, which are important selection criteria for yogurt starter cultures (De Vuyst et al., 2003). Because it produced a more volatile organic aroma through heterofermentation, yogurt fermented with SCA52 achieved a high score for flavor. Yogurts fermented with the isolates in this study also maintained higher viable LAB counts during storage at 4°C. Lactic acid bacteria have many beneficial effects on humans, such as balancing of intestinal flora and enhancement of the immune

Table 5. Comparatively analyzed properties of Xueo, marketed yogurts, and the yogurts fermented with selected strains (average value $\pm$ SD)

Item	SCA52	SCA16	Xueo ¹	Marketed yogurt ²
Flavor compound				
Lactate (g/100 g)	0.87 $\pm$ 0.12	0.52 $\pm$ 0.10	0.93 $\pm$ 0.23	0.91 $\pm$ 0.22
Acetate (g/100 g)	0.48 $\pm$ 0.08	0.24 $\pm$ 0.11	0.53 $\pm$ 0.32	0.13 $\pm$ 0.10
Acetaldehyde (mg/L)	18.72 $\pm$ 1.10	15.68 $\pm$ 0.89	20.03 $\pm$ 1.52	6.73 $\pm$ 1.32
Diacetyl (mg/L)	1.21 $\pm$ 0.18	0.89 $\pm$ 0.07	1.33 $\pm$ 0.87	0.51 $\pm$ 0.27
Titrateable acidity ³ ($^{\circ}$ T)	73.36 $\pm$ 2.25	65.22 $\pm$ 1.76	92.43 $\pm$ 10.23	82.36 $\pm$ 3.23
Viscosity (Pa·s)	4.37 $\pm$ 0.31	6.68 $\pm$ 0.52	5.45 $\pm$ 2.42	3.06 $\pm$ 1.56
Sensory score ⁴	7.93 $\pm$ 0.26	8.02 $\pm$ 0.19	ND ⁵	8.05 $\pm$ 0.37

¹Data were the average value of 12 samples and determined after collection.

²Four plain set-style yogurt samples were collected from the market and fermented with *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. Data were the average value of 4 samples.

³Expressed as Thorner degrees ($^{\circ}$ T).

⁴Scale: 1 = poor quality to 10 = excellent quality.

⁵Not determined.

system, but only some LAB can withstand the adverse gastrointestinal environment to deliver these benefits (Huppertz et al., 2004). Generally, yogurt that confers these health effects contains more than 10^6 cfu/g of viable LAB at the time of consumption (Vanderhoof and Young, 1998). In our study, yogurt contained more than 10^7 cfu/g of viable LAB during storage, indicating that it could provide health benefits to consumers.

Microbial contamination causes large losses during the production and storage of dairy products. The increased demand for natural foods without chemical preservatives also makes it important to select LAB with good antimicrobial and fermentation properties (Cleveland et al., 2001). Lactic acid bacteria usually produce some antimicrobial substances, such as lactic acid, H_2O_2 , and bacteriocins, which inhibit the growth of spoilage organisms (Reid and Burton, 2002). Excluding the influence of acid and H_2O_2 , strains SCA52 (*L. fermentum*) and SCA7 (*L. plantarum*) inhibited some indicators (Table 3), suggesting that they produced a bacteriocin-like substance. Previous studies have isolated various bacteriocins from LAB, such as nisin from *Lactococcus lactis* ssp. *lactis* and plantarin from *L. plantarum* (Settanni and Corsetti, 2008), but bacteriocin production by *L. fermentum* has rarely been reported. The chemical structure and characteristics of the bacteriocin-like substance produced by SCA52 require further study.

Analyses were conducted based on flavor components, viscosity, and sensory quality to clarify the contribution of SCA52 and SCA16 to the properties of Xueo that distinguish it from other marketed yogurts (Table 5). Flavor components of fermented milk are the end products of bacterial metabolism. The main flavor compounds presented in fermented milk are acetaldehyde, diacetyl, lactate, and acetate (Marshall, 1987; Chammas et al., 2006). In the present study, the

concentrations of acetaldehyde, diacetyl, and acetate in yogurt fermented with SCA52 were similar to Xueo and higher than those in marketed yogurts. Although a special Xueo flavor was formed by the mutual actions of microorganisms, SCA52 (*L. fermentum*) presented the main flavor compounds, and the viscosity of Xueo may have been formed by *E. durans* (SCA16). Compared with currently marketed yogurts, those fermented with SCA16 and SCA52 had a different flavor, one that received a positive sensory quality score. The special flavor and viscous texture of Xueo may be the main reason why the ethnic people in this region like this product. Thus, mixing selected strains as starters for developing a Xueo-flavored yogurt should be considered.

CONCLUSIONS

The dominant LAB in Xueo, a traditional fermented milk product, were *E. durans*, *L. fermentum*, and *L. paracasei*. The special flavor and texture of yogurts depended on the mutual action of these LAB. *Enterococcus durans* probably contributes to the texture of Xueo and *Lactobacillus* mainly produces acidity and contributes to the product's unique flavor. Using artisanal strains of LAB to produce similar Xueo-flavored yogurt should be considered.

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