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ORIGINAL PAPER

Characterization of the dominant microflora in naturally fermented camel milk *shubat*

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Abstract This study aimed to characterize the dominant microflora in *shubat*, a special fermented product prepared from unheated two-humped camel milk. In the seven investigated samples of *shubat*, lactic acid bacteria and yeasts were the dominant microorganisms with the number ranging from log 6.8 to 7.6 cfu/ml and from log 4.3 to 4.7 cfu/ml, respectively. Using phenotypic and molecular methods, a total of 48 LAB isolates were identified as *Lactobacillus sakei*, *Enterococcus faecium*, *Lactobacillus helveticus*, *Leuconstoc lactis*, *Enterococcus feacalis*, *Lactobacillus brevis* and *Weissella hellenica*, whereas 15 yeast isolates were identified as *Kluyveromyces marxianus*, *Kazahstan uiosporus*, and *Candida ethanolica*. Results showed also that *Lactobacillus* and *Enterococcus* as well as *Kluyveromyces* were the predominant genera, with the most frequently isolated species being *Lactobacillus sakei* and *Enterococcus faecium* as well as *Kluyveromyces maxius*, respectively. This is the first report on the characterization of dominant microflora of *shubat* in China.

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Introduction

shubat is a special fermented product, prepared from unheated two-humped camel milk through indigenous fermentation process. Although it is more or less similar to yoghurt in appearance, there are important differences between these two products since *shubat* is liquid rather than creamy, sparking due to its CO_2 production and has a high degree of sourness (pH of ca. 3.8). In the Jungghar baisin region of Xinjiang in China, *shubat* have been widely used both as beverage and folk medicine. The indigenous populations have believed that fermented camel milk (*shubat*) is safe and even has medicinal properties such as antidiabetic, anti-cancer and anti-tuberculosis activities.

Traditionally, *shubat* is homemade by using a semi-continuous or fed-batch fermentation process that was handed down throughout generations. Whenever part of the product is withdrawn for consumption, a portion of raw camel milk is added to make up volume and this process of retrieval and replacement of milk continues for months. The spontaneous fermentation of unheated milk takes advantage of natural microflora inherent in milk and environmental contaminants. However, many reports about traditional dairy products have shown that they have unique and different microflora dependent on the production technology as well as on the ecological localities where they have been produced (Zamfir et al. 2006; Dewan and Tamang 2007). It is well known that specific characteristics of dairy products depend on the present microflora (Wouters et al. 2002; Leroy and De Vuyst 2004).

Therefore, this knowledge can contribute to improving the process of the *shubat* manufacture, as well as to the fermentation condition for obtaining the product of better quality. The present study aimed to isolate and identify predominant microflora in homemade *shubat* by using phenotypic and molecular methods, which can further developed starter cultures for camel milk fermentation. To the best of our knowledge, no similar investigation into microflora of *shubat* from two-humped camel milk in China has been carried out so far.

Materials and methods

Samples

Seven samples of *shubat* were collected from different nomadic families in Kanas and Brojin areas, the Jungghar baisin of Xinjiang in China. All the samples were homemade by traditional method in their gers (portable houses for nomads). Each *shubat* sample was aseptically transferred to a 200 ml sterile screw-capped bottle, transported immediately to the laboratories, and kept in refrigerator at 4°C before.

Enumeration and isolation of lactic acid bacteria and yeasts

After mixing 10 ml of each *shubat* sample was pipetted aseptically into 90 ml of sterile physiological saline (0.85% w/v). One hundred microlitre from tenfold dilutions of the samples was surface inoculated onto each of the following media: (1) MRS (Oxoid^{TM}) and tomato juice agar (TMJ, Oxoid^{TM}) incubated anaerobically for 48 h at 37°C for enumeration of LAB and (2) yeast extract-glucose-chloramphenicol-agar (YGCA, Merck^{TM}) incubated for 72 h at 25°C for enumeration of yeast. Colonies showing different appearance were randomly selected and further purified by successive streaking on MRS agar (for LAB) or PDA agar (for yeasts). For long term maintenance of isolates, stock cultures of LAB and yeasts were stored at −80°C in 20% (v/v) glycerol, with 80% (v/v) MRS and YPG broth (Oxoid^{TM}), respectively.

Identification of LAB isolates

A total of 48 LAB isolates were macro- and microscopically characterized. For Gram-positive, catalase-negative rods and cocci, growth at 10 and 45°C, and carbon dioxide production from glucose in MRS broth (with inverted Durham tubes) were further identification, according to the methods of Harrigan and McCance (1976). Lactic acid isomer was determined enzymatically using D-lactate and L-lactate dehydrogenase test kits (Roche Diagnostic, France), according to the manufacturer's instructions.

Carbohydrate fermentation patterns of LAB isolates were determined using the API 50 CHL (bioMérieux, France), which enabled identification of the isolates to species level. Based on API identification, representative isolates of LAB were selected for sequencing of 16S rDNA as previously described by Najjari et al. (2008). In brief, genomic DNA was extracted using Taq DNA polymerase kit Wizard DNA purification Kit as described with the manufacturer (promega, Madison, WI USA). The extracted DNA (2 µl) was amplified by using the primers pA 5′ AGAGTTTGATCC TGGCTCAG 3′ and pH 5′ GGCTACCTTGTTACGACT 3′. The reaction mixture (50 µl) consists of 50 ng of bacterial DNA, 60 pmol of each primer, deoxynucleoside triphosphate at a concentration of 200 pM, 2.5 U of Taq DNA polymerase, 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$ and enough sterile deionized water to bring the volume to 25 µl. The reactions were carried out in PCR Thermocycler (Bio-Rad Mycycler^{TM}) programmed as follows: 94°C for 3 min; 35 cycles of 94°C for 15 s, 59°C for 60 s and 72°C for 2 min and finally, 72°C for 10 min.

Identification of yeast isolates

Fifteen isolates of yeast were tested for their ability to assimilate various compounds by using the ID 32 API kits (bioMérieux, France). Based on API groups, representatives of yeast isolates were selected for amplification of 18S-28S ITS region using the ITS1 forward primer (5′-TCC GTA GGT GAA CCT GCG G-3′) and TS4 reverse primer (5′-TCC TCC GCT TAT TGA TAT GC-3′) (White et al. 1990). The genomic DNA were extracted by using DNA kits for yeasts (Tiangen Biotech, Beijing, China). For each 1 µl of the prepared DNA, 49 µl of the reaction mixture were added. This mixture contained 5 µl of 10× PCR buffer (Pharmacia Biotech, Uppsala, Sweden), 4 µl of 2.5 mmol of each dNTP (Pharmacia Biotech), 4 µl of 25 mmol $MgCl_2$, 3 µl of 50 pm of each of the primer, 0.5 of 1% (v/v) formamide, 0.25 µl of 2.5 U Taq polymerase (Pharmacia Biotech), and 30.25 ml of Milli Q water (Milli-Q Plus, Ultra Pure Water System, Millipore, Molsheim, France). The reactions were carried out in PCR Thermocycler (Bio-Rad Mycycler^{TM}). The PCR conditions were: initial denaturation at 94°C for 5 min, 35 cycles of 98°C/15 s, 59°C/60 s and 72°C/2 min with a final extension of 72°C/10 min.

DNA sequencing and sequence analysis

DNA fragments amplified by PCR were separated using 2% agarose gel electrophoresis and purified by QIAquick PCR Purification kit/250 (QIAGEN GmbH, Hilden,

Table 1 pH values and log counts of the *shubat* samples

Sample location	Kanas areas ($n = 4$)	Borjin areas ($n = 3$)
pH	4.1 ± 0.07	3.7 ± 0.15
Log (CFU/ml)		
$LAB_{on\ MRS}$	6.8 ± 0.52	7.3 ± 0.24
$LAB_{on\ TMJ}$	7.3 ± 0.55	7.6 ± 0.43
Yeasts	4.7 ± 0.14	4.3 ± 0.44

± Standard deviation

N Number of samples

Germany). The sequencing of purified products was performed by Sangon Company, Shanghai, China. The BLAST algorithm was used to determine the most related sequence relatives in the NCBI nucleotide sequence database (www.ncbi.nlm.nih.gov/blast).

Statistical analysis

All experiments were performed in triplicate and the results were expressed as the mean value ± the standard deviation. All microbiological counts were converted to the base-10 logarithm of cfus per milliliter (ml) of *shubat* samples (log cfu/ml), and from these, means and their standard deviations were calculated. Data were tested for statistical significance by the analysis of variance (ANOVA) using the Statistical Analysis System software (SAS version 9.00, SAS Institute, Inc. 2000), $P < 0.05$.

Results and discussion

Microbial counts and pH

Table 1 showed that lactic acid bacteria and yeasts constituted the predominant microflora of *shubat*. The four samples from Kanas areas contained LAB log 6.8 and 7.3 cfu/ml when determined on MRS and TMJ agar plates, respectively. Slightly higher LAB counts were observed in the three samples from Borjin areas (log 7.3 and 7.6 cfu/ml, enumerated on MRS and TMJ, respectively). Yeasts were detected in all *shubat* samples with counts ranging between log 4.3 and 4.7 cfu/ml (Table 1). The samples from Kanas areas had the higher yeast counts than those from Borjin areas. In addition, no mold and coliform bacteria were detected in final products of *shubat* (data not shown). The pH values of the seven investigated *shubat* samples collected from kanas and Brojin areas were found to be 4.1 and 3.7, respectively, indicating that *shubat* has a high degree of acidity.

The microbial flora in *shubat* was comparable to those reported in previous studies on *suusac* (traditional Kenyan fermented camel milk) and *Gariss* (traditional Sudanese fermented milk product) (Lore et al. 2005; Abdelgadir et al. 2008). Lore et al. (2005) reported that the LAB and yeast counts in *suusac* were log 6.8 and 6.8 cfu/ml, respectively, while Abdelgadir et al. (2008) indicated that LAB and yeast counts in *Gariss* were between log 7.76 and 8.66 cfu/ml for LAB and between log 6.05 and 7.79 cfu/ml for yeasts.

Identification of LAB

A total of 48 isolates were Gram-positive and catalase-negative rod or cocci, which were presumptively considered to be LAB isolates. According to their observed features such as microscopy appearance, CO_2 production from glucose, NH_3 production from arginine, the ability to grow under different temperature and salt conditions, these isolates were grouped into six groups (Table 2). By use of API 50 CH system, the representative isolates from group I, II, III, IV and V were clearly identified as *Lactobacillus sakei*,

Table 2 Phenotypic and molecular identification of lactic acid bacteria groups isolated from *shubat*

Groups[a]	Cell shape	NH_3 from arginine	Growth at (°C)		Growth in (% NaCl)			Lactate isomer	Identified by API 50 CH	Identified by 16S rDNA sequencing	
			15	45	4	6.5	10			Closest relative	Identity (%)
Homofermentative											
Group I	Rods	−	+	−	+	−	−	DL	*Lb. sake* (10)[b]	*Lb. sakei*	99 (5)[c]
Group II	Rods	−	−	+	+	−	−	DL	*Lb. helveticus* (5)	*Lb. helveticus*	99 (3)
Group III	Cocci	+	+	+	+	+	−	L(+)	*Ec. faecium* (5)	*Ec. feacium*	99 (5)
		+	+	+	+	+	+	L(+)	*Ec. feacalis* (3)	*Ec. feacalis*	99 (1)
Heterofermentative											
Group IV	Rods	+	+	−	+	+	+	DL	*Lb. brevis* (3)	*Lb. brevis*	99 (1)
Group V	Ovoid	−	+	−	−	−	−	D(−)	*Ln. lactis* (4)	*Ln. lactis*	99 (2)
Group VI	Cocci	−	+	−	+	+	−	D(−)	Unidentified (2)	*Ws. helleca*	99 (2)

[a] Classification based on morphological and physiological characteristics

[b,c] Number of isolates that underwent API CH50 and molecular identification, respectively

Lb. helvelticus, *Enterococcus feacium*, *Ec. feacalis*, *Lb. brevis* and *Leuconstoc lactis*, respectively. Unfortunately two isolates from group VI (Le5-1 and Le5-2) were not identified by API 50 CH system (Table 2).

A set of representative isolates from different phenotypic groups were selected for 16S rDNA sequencing. The resulting16S rDNA sequences were aligned with all sequences present in the GenBank database and resulted in the final identification of the isolates from *shubat*. The API identification of all the isolates belonging to group I, II, III, IV, V were confirmed by 16S rDNA sequencing at 99% of sequence similarity (Table 2), respectively. The two unidentified isolates from group VI were identified as *Weissella hellenica* with 99% sequence similarity.

Table 3 Biochemical (API) and molecular identification of yeasts isolated from *shubat*

Isolates	Identified by API systems	Identified by 16S rDNA sequencing	
		Closest relative	Identity (%)
LE2-3, Y2-1, M3-8, M3-9, YGC-X,Y2-3, Y-3-1, YGC2-2,YGC1-2	*Kluyveromyces marxianus*	*Kluyveromyces marxianus*	99
Y-HP, Y-HP-1, M3-1, Y-HP-3-2, Y-HP-2-3	*Kazachstania unispora*	*Kazachstania unispora*	99
YY-HP	*Candida ethanolica*	*Candida ethanolica*	99

Identification of yeast

Based on Carbohydrate assimilation profiling using the API ID 32C kit, the 15 yeast isolates from seven *shubat* samples belonged to *Kluyveromyces marxianus* (perfect form of *Candida kefyr*), *Kazachstania unispora* (senior name of *Saccharomyces unisporus*) and *Candida ethanolica*, which was confirmed by sequencing of the 26S rDNA at 99% of sequence similarity (Table 3).

Distribution of LAB and yeast microflora in *shubat*

As seen from Table 4, *Lactobacillus* clearly dominated the other genera (44% of total isolates), followed by *Enterococcus* (19%), *Kluyveromyces* (14%) and *Leuconostoc* (10%).

Among the LAB micrfora, *Lb. sakei* were the most frequently isolated species (26% of total isolates, Table 4), which was found in all the seven investigated samples except for BJ1 sample and dominant in 5 of them (sample KS2, KS3, KS4, BJ2 and BJ3). *Lb. helveticus* was isolated from 5 out of 7 samples and *Lb. brevis* was isolated from 3 samples. *Ec. faecium* was found in five *shubat* samples where it was predominant species in BJ1 sample. *Ec. feacalis* was isolated from KS1 and KS2 as well as BJ2 samples. *Ln. lactis* was isolated from four samples (*KS1*, *KS3*, *KS4*, and *BJ1*), whereas two strains of *Ws. hellenica* was isolated from same samples (BJ1). The yeast micrfora in *shubat* were found to be *Kluyveromyces marxianus* and *Kazachstania unispora* as well as *Candida ethanolica* with

Table 4 Distribution of lactic acid bacteria and yeast species isolated from *shubat* samples

Species	No. of identified strains								Frequency (%) in total
	KS1	KS2	KS3	KS4	BJ1	BJ2	BJ3	Total	
Lactbacillus								28	44
Lb. sakei	1	5	4	3	0	2	2	17	26
Lb. helveticus	0	2	2	2	1	1	0	8	13
Lb. brevis	1	1	0	0	1	0	0	3	5.0
Enterococcus								12	19
Ec. feacium	0	1	2	2	3	0	1	9	14
Ec. feacalis	1	1	0	0	0	1	0	3	5.0
Leuconostoc								6	10
Ln. lactis	1	0	2	2	1		0	6	10
Weissella								2	3.0
Ws. helleca	0	0	0	0	2	0	0	2	3.0
Kluyveromyces								9	14
Kl. marxianus	2	1	1	1	2	1	1	9	14
Kazachstania								5	8.0
Kz. unispora	1	1	1	0	0	1	1	5	8.0
Candida								1	2.0
Cd. ethanolica	0	0	0	0	1	0	0	1	2.0

Kl. marxianus being dominant yeast that was isolated from all the 7 samples (Table 4). *Kazachstania unispora* was detected from 5 samples, only one *Cd. Ethanolica* strain was isolated in BJ1 sample.

Abdelgadir et al. (2008) reported *Streptococcus bovis* group was predominant species in *Gariss*. Also *Lb. fermentum* were detected in high numbers, whereas *Ec. faecium* and *Lb. helveticus* were detected more occasionally in *Gariss*. Lore et al. (2005) reported that the frequently isolated species in *suusac* was *Ln. mesenteroides* (24% of total isolates), followed by *Lb. plantarum*. In our work, *Lactobacillus* clearly dominated the other LAB genera. However, *Lb. sakei* was the predominant species in *shubat*. To the best of our knowledge, it has never been reported on *Lb. sakei* present in fermented milks. Indeed, all the *Lb. sakei* isolates from *shubat* were homofermentative, fermenting lactose to produce lactic acid as sole product. This suggests their significant role in lactic fermentation of camel milk.

Enterococi are commonly isolated from dairy products, especially from Southern Europe (Giraffa 2003). Although the presence of enterococci in dairy products is controversial, many reports showed that enterococci play an important role in the ripening of cheeses due to their proteolytic and lypolitic activities and by the production of aromatic compounds from citrate (Moreno et al. 2006). This is a characteristic that would be of functional significance towards aroma development in *shubat*.

Weissella hellenica was isolated only from one sample meaning that these bacteria are probably present in low numbers. The species is occasionally detected in dairy products (Morea et al. 1998; Randazzo et al. 2006) and it is believed that their presence is a result of low sanitary conditions during the hand milking and handling.

Kluyveromyces marxianus and *Kazachstania unispora* have previously been isolated from indigenous fermented milk products such as *Gariss*, *suusac*, koumiss and kefir (Abdelgadir et al. 2001; Lore et al. 2005; Narvhus and Gadaga 2003; Shuangquan et al. 2004; Latorre-García et al. 2007). *Kluyveromyces marxianus* was isolated predominantly from Mongolian Airag and *Saccharomyces cerevisiae*, *Issatchenkia orientalis* and *Kazachstania unispora* were the predominant isolates from Mongolian Tarag (Watanabe et al. 2008). In the present study, *Kl. marxianus* and *Kz. unisporus* were frequently isolated and constitute the dominant yeast microflora of *shubat*. Surprisingly we did not detect *Saccharomyces cerevisiae*, *Issatchenkia orientalis* in all the seven *shubat* samples. Lachance indicated that all isolates of *Kl. marxianus* are capable of metabolizing lactose whereas *Kz. unisporus* isolates are lactose negative. Both species are able to metabolize lactate (Lachance 1998), Yeast growth in *shubat* is thus probably positively influenced by the metabolic activities of the LAB present (Narvhus and Gadaga 2003). *Candida ethanolica* was originally isolated from industrial fodder yeast cultivated on synthetic ethanol as the only source of carbon. This species differs from all recently accepted Candida species in not assimilating nitrate, not producing urease and not fermenting sugars. Thus, it is likely that the occurrence of *Candida ethanolica* in *shubat* does not have a functional significance in the fermentation process.

Conclusion

The present study has showed that the microflora in *shubat* comprises a combination of LAB and yeasts. The LAB were represented by *Lactobacillus*, *Enterococcus*, *Leuconostoc* and *Weissella*, with the most frequently isolated LAB being *Lb. sakei*, *Ec. feacium* and *Lb. helveticus*. Lactose-fremneting *Kl. marxianus* was found to be the predominant yeast species, the precise role of yeasts in *shubat* requires further study.

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