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REGULAR ARTICLE

Microflora identification of fresh and fermented camel milk from Kazakhstan

Shynar Akhmetsadykova[1,2], Almagul Baubekova[2], Gaukhar Konuspayeva[3,4*], Nurlan Akhmetsadykov[1,2] and Gérard Loiseau[5]

[1]*Kazakh National Agrarian University, 8 av. Abai 050013 Almaty, Kazakhstan*
[2]*Scientific and Production Enterprise Antigen Co. Ltd., 4 Azerbayev str., 040509, Almaty region, Kazakhstan*
[3]*Camel Range and Research Center, P.O.Box 761, Al-Kharj, Kingdom of Saudi Arabia*
[4]*Kazakh National al-Farabi University, Av. Al-Farabi, 050040, Almaty, Kazakhstan*
[5]*UMR Qualisud, CIRAD, TA B-95/16, 73, rue J.-F. Breton, 34398 Montpellier Cedex 5, France*

Abstract

In Kazakhstan where Bactrian camel, dromedary camel and their hybrids are cohabiting within same farms, the consumption of camel milk is very popular because its medicinal and dietary properties. This milk is consumed under fermented form, called *shubat*. *Shubat* is still very often made on a small scale in the steppe with a fermentation step driven by wild bacteria. Camel milk and *shubat* were sampled from 4 regions with high number of camel population. As the whole, 26 samples were obtained from 13 selected farms representing the variability of the farming system. Isolated LAB strains were identified by method of a polymorphism determination of 16S ribosome DNA. PCR with using two different pairs of amorces (338f/518r; W001/23S1) was done. Majority of microflora were cocci in a both milk products. The following microorganisms were identified: *Enterococcus durans ; Enterococcus faecalis; Enterococcus faecium; Lactobacillus casei; Lactobacillus casei subsp. casei; Lactobacillus curvatus; Lactobacillus kefiri; Lactobacillus paracasei; Lactobacillus sakei; Lactococcus lactis subsp. lactis; Leuconostoc mesenteroides*. Diversity of microorganisms in a both products was similar, but percentage of each microorganism changed during fermentation process. Yeast biodiversity in *shubat* was studied by using denaturing gradient gel electrophoresis (DGGE). Target DNA bands were identified according to the reference species scoring. Comigrating bands present in the DGGE profiles were resolved by species-specific PCR. The dominant yeasts in both products included *Kazakhstania unispora, Saccharomyces cerevisiae* and *Kluyveromyces marxianus.* Frequently isolated yeast species were *Dekkera bruxellensis* and more rarely *Galactomyces geotrichum*. The results of microflora identification in these products provide a theoretical foundation for developing starter cultures.

Key words: Camel, Fermented camel milk (*shubat*), LAB, Yeast, PCR, DDGE, Kazakhstan

Introduction

Shubat, which is made from unpasteurized fresh camel milk, is the most popular fermented dairy beverage in Kazakhstan. This traditional fermented product is widely consumed also in Mongolia, Uzbekistan, Turkmenistan and some regions of Russia (Konuspayeva and Faye, 2011). For centuries, *shubat* has been regarded not only as an essential food, but also as a nutriment and a medicinal remedy (Urazakov et Bainazarov, 1974; Mal et al., 2000; Mohamad et al., 2009; Konuspayeva et al., 2003; Yagil et Creveld, 2000; Djangabilov et al., 2000; Chuvakova et al., 2000). Lactic acid bacteria (LAB) and yeasts were proven to be the main components in fermentation process. They play detrimental role to the safety of dairy products. Moreover, the benefits of *shubat* are mainly attributable to these microorganisms which not only were reported to play a major fermentative role on the aroma, texture, and acidity of this product, but also play a major therapeutic role on improvement of digestion properties, against diarrhea and responsible for antimicrobials properties (Puzyrevskaya et al., 2000; Saubenova et al., 2002). The specific microflora of *shubat* directly depends from fresh milk, utilized starters and fermentation conditions (Serikbayeva et al., 2005). In particular, differences in microflora composition of conventional starters originating

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*Corresponding Author

Gaukhar Konuspayeva
Camel and Range Research Center, P.O. Box 322, Al-Jouf, Sakaka, Saudi Arabia

Email: konuspayevags@hotmail.fr

from the respective family environment will result in *shubat* quality instability. Nowadays studying microflora of traditional fermented dairy products as *shubat* and creation of starters is very important. To obtain the *shubat* of better quality and to produce this traditionally fermented product on the industrial level with high quality control starter cultures should be developed. The first step of such ambitious project is the identification of the main microflora strains available in *shubat* of different origin which is the objective of the present paper.

Materials and Methods

Dairy products sampling

Four regions (Almaty, South Kazakhstan, Kyzylorda and Atyrau) of the Kazakhstan were selected according to their importance of camel livestock. As the whole, 13 farms were selected representing the variability of the farming system in the retained regions and overall producing *shubat* with different known organoleptic quality. Each sample (n=26, i.e. two samples per farm) was aseptically transferred to a 500 ml sterile bottle, transported in ice-box until the laboratory and stored at 4°C.

Microorganisms and growth conditions

LAB strains were isolated on the nutritive media M17 and MRS (Biokar Diagnostics, France) and yeasts on the Saburo media (Himedia, India). The transfers were repeated until to get pure colonies. The pure colony was inoculated in the respective media and conserved at 4°C after incubation at 37°C for LAB and 25°C for yeasts, 48 hours. For long term maintenance of isolates, stock cultures were stored at - 20°C in 30% (v/v) glycerol, with 70% (v/v) M17, MRS and Saburo broth, respectively.

Preliminary identification of microorganisms

The pure strains were characterized by coloration Gram (reagent kit "Color Gram2-E" BioMérieux, France), catalase tests (ID color catalase ID-ASE Biomérieux France) and oxydase tests (Oxydase reagent Biomérieux, France).

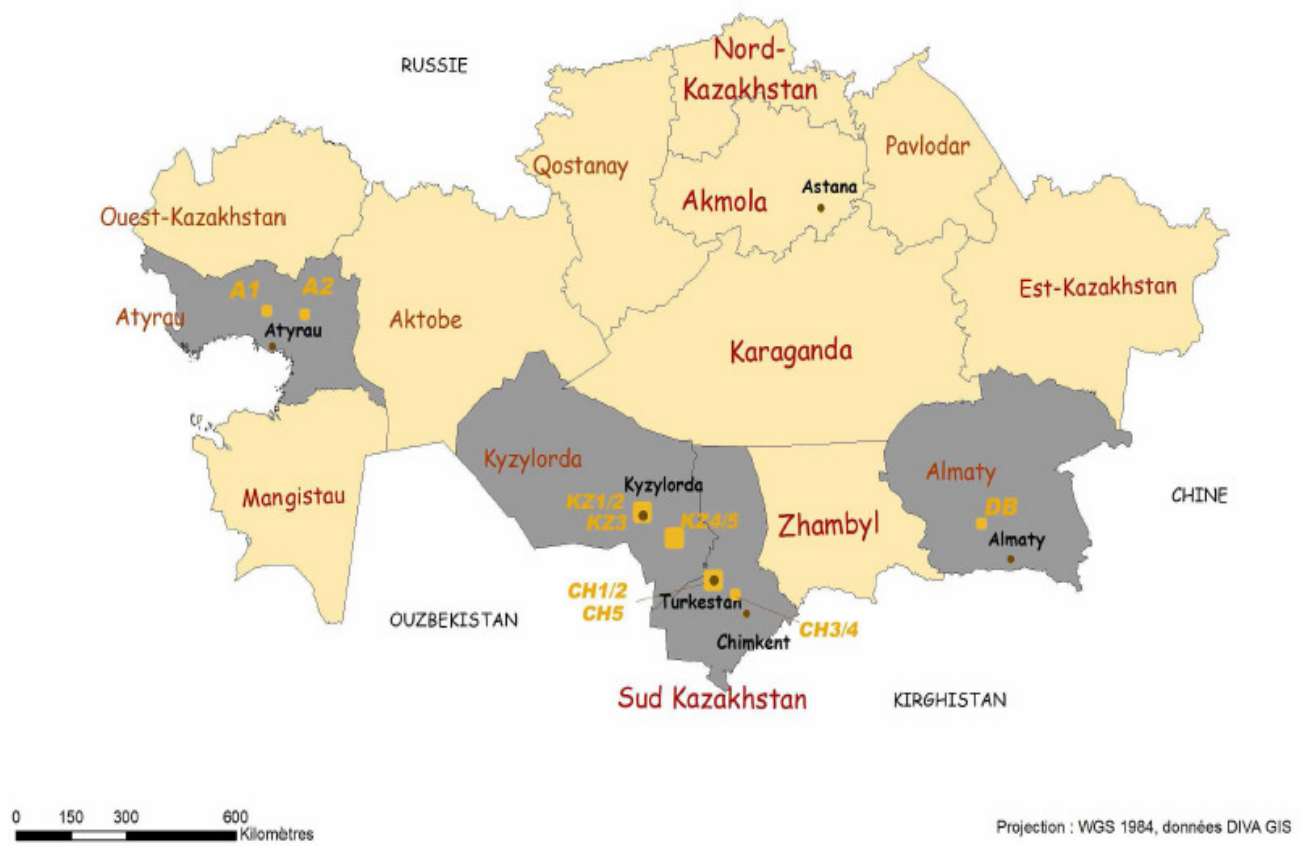

Figure 1. Map of Kazakhstan, showing the locations of Almaty, Atyrau, Kyzylorda and South Kazakhstan sampled regions.

DNA extraction

Bacterial DNA extraction was done according to the manual method described by Leesing (2005). Extraction of the yeasts DNA was achieved by using commercial Wizard kit (Promega, France). The DNA extracted was then stored at -20°C. Existence and purity of DNA was verified by electrophoresis in 0.8% (w/v) agarose gel (Promega, France) in TAE 1X buffer.

Amplification of DNA by PCR

The method of a polymorphism determination of 16S ribosome DNA was used. The PCR samples were prepared by performing 2 successive PCR using a DNA Peltier thermal cycler PTC-100 (MJ Research Inc., USA). Firstly, a 237-bp fragment of the 16S rDNA including the V3 region (in *Escherichia coli*, which corresponds to position (338-534) was amplified with primers 338f (5'-ACTCCTACGGGAGGCAGCAG-3') and 518r (5'-ATT ACC GCG GCT GCT GG-3') (Sigma-Genosys, France). Secondly, amorces which amplifies the intergenic region (ITS: Internal Transcribed Spacer) between the regions coding RNA16S and RNA 23S (Turpin et al., 2011). A 1500-bp fragment was amplified with the primers W001 (5'- AGA GTT TGA TCM TGG CTC-3') and 23S1 (5'- CNC GTC CTT CAT CGC CT-3'). The PCR reaction mixtures and the 2 above amplification programs were the same as described previously (Ampe et al., 1999; Leesing, 2005) and (Turpin et al., 2011), respectively.

Yeast biodiversity in shubat was studied using polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) fingerprinting. Target DNA bands were identified according to the reference species scoring, constructed in this study. Comigrating bands present in the DGGE profiles were resolved by species-specific PCR. For DNA amplification, two primers were used: NL1 (GCCATATCAATAAGCGGAGGAAAAG) and LS2 (ATTCCCAAACAACTCGACTC) (Sigma-Genosys, France), respectively.

The sizes and quantities of PCR products were determined by 1% (w/v) agarose gel QA TM (Q-Biogene, USA) electrophoresis in comparison with a standard containing DNA fragments of defined length.

Purification and Sequencing of PCR bands

The corresponded bands were excised from the denaturing gels with sterile scalpel. The amplicons of PCR were purified with Wizard PCR Preps DNA Purification system kit (Promega, France) and stored at -20°C. Sequencing was done by EUROFINS GENOMICS enterprise. Sequence annotation and database searches for similar sequences were performed by using BLAST at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) to determine the closest known relative species (Altschul et al., 1990).

Results

From the 26 *shubat* samples, 138 strains of microflora were isolated and among them only 37 LAB strains (Table 1) and 12 yeasts strains were identified. The majority of microflora among the 138 isolated strains was cocci (109), 17 bacilli and 12 yeasts. The percentage of similarity for the 37 LAB strains with their affiliations was above 80 % in all the cases except *Enterococcus faecium* (NC_017960.1) which was 81% only (Table 1).

The preponderance of cocci in lactic microflora of camel milk has been already reported by other authors (Grillet, 2006; Kacem et al., 2002). Khedid et al. (2009) listed the dominant species of camel milk as *Lactococcus lactis* subsp. *lactis* (17.5%), *Lactobacillus helveticus* (10%), *Streptococcus salivarius* sub sp. *thermophilus* (9.2%), *Lactobacillus casei* subsp. *casei* (5.8%), *Lactobacillus plantarum* (5%) and *Leuconostoc mesenteroides* subsp. *mesenteroides* (4.2%).

The predominance of enterococci in microflora of *shubat* in our results is in accordance with results of Zadi-Karam and Karam (2005) who, after analyzing eight samples of raw camel milk from eight different animals in five farms of Timimoune and Bechar (South-western Algeria) regions, found 35% of enterococci, *Lc. lactis* ssp *diacetylactis* (28.4%), *Lc. lactis* ssp *cremoris* (4.9%), *Lc. lactis* ssp *lactis* (1.2%), *Leuconostoc lactis* (7.4%), *Leuconostoc dextranicum* (4.9%) and *Lactobacillus plantarum* (18.5%). The presence of enterococci can also be caused by poor hygiene during milking (Khedid et al., 2009, Martin and Mundt, 1972 cited by Stiles and Holzapfel, 1997). For many authors, the presence of enterococci is evidence of possible fecal contamination and therefore a risk to consumers because although these strains are known for their low virulence, they pose serious health problems due to the emergence of many antibiotic-resistant strains, for example strains of *E. faecalis* (Giraffa et al., 2000 cited by Khedid et al., 2009). However, the positive role of these cocci in the development of quality of fermented dairy products should not be forgotten. For example, the proteolytic properties of these strains lead to the

release of casein amino acid precursors of molecules involved in the flavor of cheese (Urbach, 1995 cited by Khedid et al., 2009). Enterococci produce enterocins which have a specific inhibitory activity against some pathogenic bacteria (Sabia et al., 2002). It was also reported that *E. faecalis* produce anti-listeria bacteriocins in milk and cheese. Enterococci contribute significantly to the development of organoleptic properties of cheese mature (Litopoulou-Tzanetaki, 1990) and have a beneficial effect on the growth of other lactic acid bacteria in their proteolytic activity that promotes intense gas production by strains of *Leuconostoc* and lactic acid production by lactococci, enterococci that's why it is used very often in cheese production in the Mediterranean countries (Macedo et al., 1995; Jovanovic and Sandine-Levata, 1996 cited Zadi-Karam et al., 2011).

Also, five yeasts species were identified in *shubat.* Among them, *Kazakhstania unispora, Saccharomyces cerevisiae* and *Kluyveromyces marxianus* (*Candida kefyr*) were predominant. More rarely isolated yeasts species were *Dekkera bruxellensis* (*Brettanomyces*) and *Galactomyces geotrichum.*

Table 1. Phylogenetic affiliations of LAB isolates recovered in *shubat* from four regions in Kazakhstan.

No.	Closest 16S rRNA sequence in Gene bank	Accession no.	Similarity,%	Affiliation
1	*Leuconostoc mesenteroides subsp. mesenteroides*	NC_016805.1	92	*Firmicutes*
2	*Leuconostoc mesenteroides subsp. mesenteroides*	NC_016805.1	100	*Firmicutes*
3	*Leuconostoc mesenteroides subsp. mesenteroides*	NC_016805.1	97	*Firmicutes*
4	*Enterococcus durans*	S000004741	98	*Firmicutes*
5	*Enterococcus durans*	S000004741	98	*Firmicutes*
6	*Enterococcus durans*	S000004741	99	*Firmicutes*
7	*Enterococcus durans*	S000004741	100	*Firmicutes*
8	*Enterococcus faecalis*	NC_004668.1	90	*Firmicutes*
9	*Enterococcus faecalis*	NC_018221.1	99	*Firmicutes*
10	*Enterococcus faecalis*	NC_018221.1	96	*Firmicutes*
11	*Enterococcus faecalis*	NC_018221.1	99	*Firmicutes*
12	*Enterococcus faecium*	NC_017960.1	81	*Firmicutes*
13	*Enterococcus faecium*	NC_017960.1	95	*Firmicutes*
14	*Enterococcus faecium*	NC_017960.1	99	*Firmicutes*
15	*Enterococcus faecium*	S000002717	99	*Firmicutes*
16	*Enterococcus faecium*	NC_017960.1	99	*Firmicutes*
17	*Enterococcus faecium*	S000002717	99	*Firmicutes*
18	*Enterococcus faecium*	NC_017960.1	98	*Firmicutes*
19	*Enterococcus faecium*	S000002717	100	*Firmicutes*
20	*Enterococcus faecium*	NC_017960.1	98	*Firmicutes*
21	*Enterococcus hirae*	NC_018081.1	99	*Firmicutes*
22	*Enterococcus hirae*	NC_018081.1	99	*Firmicutes*
23	*Lactobacillus buchneri*	NC_018610.1	99	*Firmicutes*
24	*Lactobacillus buchneri*	NC_018610.1	93	*Firmicutes*
25	*Lactobacillus casei*	S000004550	98	*Firmicutes*
26	*Lactobacillus casei*	S000008152	100	*Firmicutes*
27	*Lactobacillus casei*	HE970764.1	98	*Firmicutes*
28	*Lactobacillus casei*	S000008152	96	*Firmicutes*
29	*Lactococcus lactis subsp. cremoris*	NC_017949.1	99	*Firmicutes*
30	*Lactococcus lactis subsp. lactis*	NC_017486.1	98	*Firmicutes*
31	*Lactococcus lactis subsp. lactis*	NC_017486.1	98	*Firmicutes*
32	*Lactococcus lactis subsp. lactis*	NC_017486.1	100	*Firmicutes*
33	*Lactobacillus sakei subsp. sakei*	NC_007576.1	99	*Firmicutes*
34	*Lactobacillus sakei*	S000261305	100	*Firmicutes*
35	*Lactobacillus sakei subsp. sakei*	NC_007576.1	95	*Firmicutes*
36	*Lactobacillus sakei subsp. sakei*	NC_007576.1	95	*Firmicutes*
37	*Lactobacillus sakei subsp. sakei*	NC_007576.1	100	*Firmicutes*

Saccharomyces cerevisiae has also been isolated by Njage et al. (2011) in African fermented camel milk (*suusac*). Gadaga et al. (2007) also founded *Saccharomyces cerevisiae* and *Candida kefyr* in *amasi* - naturally fermented cow milk from Zimbabwe.

The yeast *Dekkera bruxellensis* (*Brettanomyces*) is usually regarded as a contamination organism in wine production and distilleries. But in production of beer and sourdough it is a desirable member of microflora which plays a key role in the spontaneous fermentation and food flavor (Stender et al., 2001; Blomqvist et al., 2010). The yeast *Geotrichum candidum* which was identified in our study is appearing in the early stages of ripening on soft and semi-hard French cheeses. Its lipases and proteases promote flavor development, and its amino-peptidases reduce bitterness imparted by low-molecular-weight peptides in cheese (Marcellino et al., 2001).

Njage et al. (2011) also identified species belonging to the genera *Rhodotorula, Cryptococcus, Candida, Trichosporon, Geotrichum* and *Issatchenkia* which weren't founded in our study. Perhaps it's depending of relatively few *shubat* samples taken for this study. Geographic factors, specific natural fermentation processes and hygienic practices could play an important role on the yeast biodiversity in dairy products (Njage et al., 2011).

Conclusion

This study revealed the high biodiversity of microflora available in fermented camel milk. In the perspectives, the identification of the remaining isolated LAB strains should be done to give a definitive idea of microflora diversity in the fermented camel milk in Kazakhstan. This step is essential for selecting in a second step, specific strains according to their role in fermentation process of camel milk. It is expected in that sense, after proper testing, to conduct fermentation with specific starter allowing special flavor and taste of the final product. It is the objective of our further investigations.

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