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Full Length Research Paper

Lactic acid bacteria biodiversity in raw and fermented camel milk

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Abstract

Consumption of fermented camel milk, named shubat, is very popular in Central Asia and especially in Kazakhstan where it is known for its medicinal and dietary properties. To identify lactic acid bacteria (LAB) camel milk and shubat were sampled from 4 regions of Kazakhstan with important camel's population. In total, 26 dairy samples from 13 selected farms representing the variability of the farming system in the country were collected. Isolated strains were identified by genotypic approach including PCR using three different pairs of primers (338f/518r; W001/23S1; Lac1/Lac2/Lac3) and 16S rDNA gene sequencing. Three genus were in majority: Lactococcus, Lactobacillus and Enterococcus. 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. Identification of camel milk and shubat microflora provides a theoretical foundation for developing starter cultures by using local LAB strains for industrial production of traditional fermented milk products.

Key words: camel milk, shubat, LAB, PCR, 16 S rDNA

INTRODUCTION

Camel milk and traditional fermented camel milk called shubat are valuable source of food for people living in steppe and arid areas of central Asia (Faye and Konuspayeva, 2012). These products are widely consumed in Kazakhstan and it is an important part of Kazakh people diet (Konuspayeva and Faye, 2011). Camel milk and shubat microflora plays major fermentative role in the aroma, texture and acidity; therapeutic role on improvement of digestion properties and responsible for antimicrobials properties (Arab et al. 2014).

Nowadays, interest for camel milk and shubat microflora is increasing. Different methods, like biochemical (Khelid et al. 2009) and 16S rDNA sequence analysis (Rahman et al. 2010; Wu et al. 2011) were used. Methods based on the use of rRNA genes (rDNA) can analyze microflora on the basis of sequence diversity (Randazzo et al. 2002). By using this technique, it is easy to study and compare microbial communities in different feed like fermented dairy products. The most part of microflora diversity studies on different fermented products are established by using of PCR techniques (Van Hoorde et al. 2008; Vernile et al., 2008; Gaglio et al., 2014). Especially, using of PCR primers that target the 16S/23S rRNA (Berthier and Dusko-Ehrlich, 1998). This technique 16S rRNA gene sequencing was used for LAB identification in different dairy fermented products using different types of milk such yak, mare, goat, and cow milk (Yu et al., 2011; Bao et al., 2012).

Up to now, farmers are preparing shubat by using

ancestral techniques, which are an important part of the tradition. The diversity in microflora composition of conventional starters originating from the respective family environment will result in shubat quality variability (Serikbayeva et al. 2005). Consequently, studying the microflora of traditional fermented dairy products as shubat is useful for organizing industrial production of traditional fermented products with local strains. It is one important step in development of camel milk processing (Yateem et al. 2008; Ashmaig et al. 2009)

After a preliminary identification of some of the microflora isolated from shubat samples reported elsewhere (Akhmetsadykova et al., 2014), the aim of the present paper was to study the microbiological biodiversity by providing the identification of the entire population of the spontaneous microflora in camel milk and shubat by using PCR-based methods and 16S rDNA sequence analysis for the further starters' production.

MATERIALS AND METHODS

Sampling procedure

As the whole, 13 camel farms producing shubat by traditional way and representing the variability of the camel farming system were selected in four regions of the country: Almaty (one farm), South Kazakhstan (five farms), Kyzylorda (five farms) and Atyrau (two farms). Those regions were selected for their importance in camel stock. Selected farms reared the two species of large camelids (*Camelus dromedarius* and *Camelus bactrianus*) and their hybrids. In each farm, two samples were collected: fresh bulk milk and shubat prepared with the same milk. As the different species cohabited in the same farms, it was not possible to distinguish the milk according to the species. For each sample, has been assigned a code number according to region, farm and dairy sample type: Almaty (AL); South Kazakhstan (SK); Kyzylorda (KZ); Atyrau (AT); camel milk (M) and shubat (SH). For example, SKSH1 – shubat sample from the first farm situated in the South Kazakhstan region. Each sample (n=26) was aseptically transferred to 500 ml sterile bottle, transported in cold-box (4°C) until Almaty, Kazakhstan, then frozen and transferred in France for identification analysis.

Identification steps

The identification process was achieved in the Qualisud laboratory, CIRAD, Montpellier, France. The different strains of lactic acid bacteria were identified by achieving a five-step analytical procedure described below:

Step 1: Isolation and growth conditions

LAB strains were isolated from sample by using wire loops on the M17 and MRS agar (Biokar Diagnostics, France). After the incubation period (48 h, 37°C), single colonies that had different morphological traits were sub-cultured. Cells were maintained at −20°C in the culture broth supplemented with 30% glycerol.

Step 2: Preliminary identification

The strains were characterized by Gram's staining (reagent kit "Color Gram2-E" BioMérieux, France), catalase tests (ID color catalase ID-ASE Biomérieux, France) and oxidase tests (Oxidase reagent Biomérieux, France).

Step 3: DNA extraction and storage

Bacterial DNA extraction was achieved according to the manual method described by Leesing (2005). The extracted DNA was 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 under UV light after ethidium bromide staining.

Step 4: Amplification of DNA by PCR

Each DNA sample was amplified 3 times by using different pairs of primers:
primers 338f (5'-ACTCCTACGGGAGGCAGCAG-3') and 518r (5'-ATT ACC GCG GCT GCT GG-3') (Sigma-Genosys, France) (Leesing, 2005);
primers W001 (5'- AGA GTT TGA TCM TGG CTC-3') and 23S1 (5'- CNC GTC CTT CAT CGC CT-3') (Sigma-Genosys, France) (Turpin et al., 2011);
primers Lac1 (5'-AGCAGTAGGGAATCTTCCA-3') or Lac3 (5'-AGCAGTAGGGAATCTTCCA-3') and reverse primer Lac2 GC (5'CGCCCGGGGCGCGCCCCGGGCGGCCCGGGGGC ACCGGGGGATTYCACCGCTACACATG-3') (Sigma-Genosys, France) (Santos et al., 2011).

Step 5: Purification and Sequencing of PCR bands

Agarose gel electrophoresis was performed to visualize amplified DNA fragments and to excise corresponded bands with sterile scalpel under UV light after ethidium bromide staining. 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.

RESULTS AND DISCUSSION

In total, 130 strains were isolated from 26 dairy samples.

Table 1. Phylogenetic affiliations of 48 LAB strains isolated from raw camel milk and shubat from four regions of Kazakhstan.

Strain's origin	Species	DNA sequence identity 16S rDNA gene (%)	Specificity of PCR primers			
			338f/518r	W001/23S1	Lac1, Lac3	Lac2,
SKM1	Leuconostoc mesenteroides	92	-	+	-	
SKM3	Leuconostoc mesenteroides	100	+	+	-	
SKSH1	Leuconostoc mesenteroides	97	+	-	-	
SKM2	Leuconostoc mesenteroides	98	+	+	-	
SKSH2	Enterococcus durans	99	+	+	+	
SKSH1	Enterococcus durans	98	+	+	-	
SKSH2	Enterococcus durans	99	+	-	-	
KZSH2	Enterococcus durans	95	+	+	-	
SKSH5	Enterococcus durans	98	+	+	+	
SKSH1	Enterococcus durans	95	+	-	-	
SKSH2	Enterococcus durans	97	+	-	-	
KZSH2	Enterococcus durans	88	-	+	-	
SKM5	Enterococcus durans	99	+	-	-	
SKM1	Enterococcus durans	99	+	+	+	
SKSH3	Enterococcus durans	98	-	+	+	
SKSH5	Enterococcus durans	98	+	+	-	
SKSH5	Enterococcus durans	98	-	+	-	
KZSH2	Enterococcus faecalis	92	+	+	-	
SKSH1	Enterococcus faecalis	99	+	+	-	
SKSH1	Enterococcus faecalis	100	+	+	-	
ATSH1	Enterococcus faecalis	99	+	+	-	
SKM4	Enterococcus faecalis	100	+	+	-	
ALSH1	Enterococcus faecium	90	+	+	+	
ATSH3	Enterococcus faecium	99	+	+	-	
SKSH5	Enterococcus faecium	99	+	+	-	
ATM2	Enterococcus faecium	98	+	+	-	
SKSH2	Enterococcus faecium	99	+	+	-	
SKSH4	Enterococcus faecium	99	+	+	-	
SKM2	Enterococcus faecium	99	+	+	-	
SKM3	Enterococcus faecium	99	+	+	+	
KZM5	Enterococcus faecium	99	+	-	-	
KZM5	Enterococcus faecium	98	+	+	-	
KZSH1	Enterococcus faecium	81	+	-	-	
SKM2	Enterococcus faecium	99	+	-	-	
KZSH2	Enterococcus faecium	98	+	+	-	
SKSH1	Lactobacillus kefiri	99	+	-	+	
SKM2	Lactobacillus bucheri	93	+	+	-	
KZSH2	Lactobacillus casei	99	+	+	-	
ALSH1	Lactobacillus casei	100	+	+	-	
KZSH2	Lactobacillus casei	99	+	-	-	
KZM5	Lactobacillus casei subsp. casei	98	+	+	-	
SKSH4	Lactobacillus casei subsp. casei	99	+	+	-	
SKSH1	Lactobacillus sakei	100	+	+	-	
KZM4	Lactobacillus sakei	100	+	+	-	
SKSH1	Lactobacillus sakei	95	+	+	-	
SKM3	Lactobacillus sakei	100	+	+	-	
KZSH2	Lactococcus lactis subsp. lactis	99	+	-	-	
SKM3	Lactococcus lactis subsp. lactis	100	+	+	-	

All these strains were tested by Gram's staining, oxidase and catalase tests. After laboratory screening, 118 strains were Gram positives, oxidase and catalase negative and non-spore forming bacteria which were considered as lactic acid bacteria strains. The majority of isolates were cocci (70%).

All 118 LAB strains were taken for molecular identification analysis. PCR amplification results showed existence of microorganisms (26) which could not be identified by used primers.

From 118 strains, 44 were identified only until genera: Lactococcus (18); Lactobacillus (14); Leuconostocs (7)

and Enterococcus (5). Other 48 strains were identified until species. Most of them were identified by using primers 338f/518r and W001/23S1. In contrast, the primers Lac1, Lac2, Lac3 failed to produce an amplicons in all tested strains, except 7 strains (Table 1).

The percentage of similarity for 48 LAB strains with their affiliations showed high identity 81%－100%. The rDNA sequences demonstrated similarity with 16S rDNA sequences of members of the Enterococcus, Lactobacillus, Lactococcus, Leuconostoc genera in GenBank.

Results cannot give full information about diversity of LAB in these products and about microflora diversity per region. However, it supplies some idea about microflora of camel milk and shubat from Kazakhstan which was not investigated deeply yet. The identification was achieved on mixed milk in farms with heterogeneous camel population; it was not possible to present results by species and to determine a potential specific difference. According to research results, Enterococcus and Lactococcus genus seem to be dominating in camel milk and shubat. Similar results on preponderant cocci microorganisms of camel milk compared to other species' milk have been already reported in the literature (Dalmasso et al; 2008; Ashmaig et al., 2009; Khedid et al., 2009; Rahman et al., 2010). This is opposite to mare's milk and koumiss microflora where bacilli, especially Lactobacillus strains are dominant (Wang et al., 2008; Hao et al., 2010; Sun et al., 2010). In Ititu, a traditional Ethiopian fermented camel milk, Seifu et al. (2012) found 58 % Lactobacillus, 25% Lactococcus and 17% Enterococcus, but the identification of their 146 strains was based on biochemical tests only. In shubat collected from 7 Bactrian samples in China, Rahman et al. (2009), using biochemical test (API 50 CHL) and molecular method (16s rDNA gene sequencing PCR amplification and by using pA and pH primers), identified 48 LAB isolates where Lactobacillus end Enterococcus were predominant.

In our study, 26 isolates could not be identified with the used primers. To study these not identified strains and research microbiological richness of these traditional dairy products other more appropriate primers should be found or be designed (Schleifer et al., 1995; Heilig et al., 2002; Odamaki et al., 2011). It is admitted that accurate identification of Lactobacillus species can be accomplished by reference to 16S rRNA gene sequences. However, species-specific, PCR primers that target the 16S-23S rRNA spacer region are available for a limited number of Lactobacillus species (Tannock, 1999).

In the present study, one of the dominating genus Enterococcus group was presented by 3 different species such E. faecium, E. durans and E. faecalis. The predominance of enterococci, especially Enterococcus faecalis, in camel milk microflora was also reported by Benkerroum, et al. (2003) in Morocco, and by Jans et al. (2012) in Kenya. But, for many authors, 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 could pose serious health problems especially because the emergence of antibiotic-resistant strains, for example strains of E. faecalis (Khedid et al. 2009). However, the positive role of these cocci in the development of quality of fermented dairy products should not be forgotten. Indeed, the proteolytic properties of these strains lead to the release of casein amino acid precursors of molecules involved in the flavor of cheese (Khedid et al. 2009; Zadi-Karam and Karam, 2011). Also, a specific inhibitory activity of enterococci was showed against some pathogenic bacteria (Sabia et al. 2002).

However, the difference between species milk microflora diversity could be due to geographical, environmental and milk composition (Aziz et al. 2009).

CONCLUSION

This study emphasized the high biodiversity of microflora available in fermented camel milk in Kazakhstan. The identification of the remaining isolated LAB strains should be done to give a definitive idea of microflora diversity in this product. Moreover, the link between microflora population and variation factors as species or regions could be investigated to understand the variability in organoleptic properties of the different shubat samples. And the further studies will be very important step in creation of starters and probiotics based on local lactic acid bacteria strains as it is expected through the commercialization project "Starters for manufacturers of national fermented milk products" in Kazakhstan.

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