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BIODIVERSITY STUDY OF THE YEAST IN FRESH AND FERMENTED CAMEL AND MARE'S MILK BY DENATURING GRADIENT GEL ELECTROPHORESIS

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ABSTRACT

Shubat and *Koumiss* are traditional fermented products widely consumed in Kazakhstan and Central Asia. Microflora of those fermented products is composed of bacteria and yeasts, which could have deep impacts on beverages quality. Although, the known presence of yeast in such fermented foods, few studies on yeast microflora of *Shubat* and *Koumiss* were available. The aim of the study was to identify the main indigenous yeasts in these traditional beverages and additionally, the differences between these 2 types of fermented products.

Five samples of fresh and fermented camel and mare's milk from tank were collected in 5 different farms, located in South of Kazakhstan. Farms were selected because of their importance of production of fermented milk at the regional level and the reputation of these products among the consumers. Yeast biodiversity in *Shubat* and *Koumiss* was studied using denaturing gradient gel electrophoresis (DGGE). Target DNA bands were identified according to the reference species ladder, constructed in this study. Co-migrating bands present in the DGGE profiles were resolved by species-specific PCR.

Galactomyces geotrichum, Kluyveromyces marxianus, Kazachstania unispora, Saccharomyces cerevisiae, Dekkera anomala, Dekkera bruxellensis yeast were identified. Some yeasts were not identified by used primers. In all samples of *Shubat* and *Koumiss* the dominant yeast species were *Kazakhstania unispora, Kluyveromyces marxianu*. Other species were not present in all samples.

Key words: Camel milk, DGGE, fermented milk, koumiss, shubat, yeast

Central Asia is famous for its tradition to consume fermented milk products known for their curative and preventive qualities which have been used for centuries (Doreau and Boulot, 1989; Konuspayeva *et al*, 2003). Based on the current research, useful properties of fermented milk products to some extent are connected to the properties of milk compound and their bacterial population. It is admitted that the biodiversity of traditional national fermented milk products in Kazakhstan was poorly studied, but constitutes a special scientific and technological interest.

Shubat and *Koumiss* are traditional drinks widely consumed in Kazakhstan (Faye and Konuspayeva, 2012). *Shubat* is home made fermented camel milk especially consumed on South and West Kazakhstan, in the arid and semi-arid regions of the country. *Koumiss* is a low alcohol (below 5%), slightly acidic dairy product which is made from mare's milk. It is the main dairy product which was drunk by nomads. Traditionally, both products are processed from raw milk through fed-batch fermentation process. Microflora of *shubat* and *koumiss* are composed of bacteria and yeast, which could have deep impacts on milk quality. Lactic acid bacteria and yeast were proven to be the main components in *Shubat* and K*oumiss* starters (Hao *et al*, 2010). Although, the known presence of yeast in fermented foods (mainly studied in champagne wine), little researches were achieved on milk products. Especially, few studies on yeast microflora of *shubat* and *koumiss* were done. Yet, it is widely known that eukaryotic communities

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play an important role in food fermentation (Rahman *et al*, 2009).

With traditional methods of identification of yeast (culture methods), few types have been identified. So, the aim of present study was to identify yeast profiles in traditional non-conventional dairy products by using molecular biology tools.

Materials and Methods

Samples of fermented milk

Five samples of fresh and fermented camel and mare's milk from tank were collected from 5 different farms, located in South Kazakhstan. The farms were selected because of their importance in production of fermented milk at the regional level and the reputation of these products among the consumers.

The samples were kept at 4°C after sampling until the laboratory, then stored at -20°C until analysis.

Extraction of total DNA from fermented drinks

After thawing of the raw and fermented milk samples, total DNA extraction was the protocol of El Sheikha *et al* (2010). Extracted samples were verified by electrophoresis and loaded in to 0.8% agarose gels in 1 × TAE buffer (40 mM Tris–HCl, pH 7.4, 20 mM sodium acetate, 1.0 mM Disodium–EDTA; Eppendorf, Wesseling-Berzdorf, Germany) with a molecular weight ladder (Supercoiled DNA Ladder 16.21 kb; Invitrogen, USA). After running at 100 V for 30 min, the gels were stained for 30 min in ethidium bromide solution (50 µg/mL; Promega, Charbonnières-les-bains, France), rinsed for 20 min in distilled water, then observed and photographed on UV trans-illuminator, using a black and white camera (Scion Co., USA) and Gel Smart 7.3 system software (Clara Vision, Les-Ulis, France).

PCR-DGGE protocol

The D1/D2 region of the 26S rRNA gene was amplified by PCR using universal primers NL1GC (5´-GCG GGC CGC GCG ACC GCC GGG ACG CGC GAG CCG GCG GCG GGC CAT ATC AAT AAG CGG AGG AAA AG-3´) (the GC clamp is underlined) and a reverse primer LS2 (5´-ATT CCC AAA CAA CTC GAC TC–3´; Sigma), amplifying a 250 bp fragment (Kurtzman and Robnett, 1998; Cocolin *et al*, 2000). A 30 bp GC clamp (Sigma) was added to the forward performed primers. PCR was performed in a final volume of 50 µl containing 0.2 µM of each primers, all the deoxyribonucleotide triphosphate (dNTPs) at 200 µM, 1.5 mm $MgCl_2$, 5 µM 10x of reaction Taq buffer, $MgCl_2$-free (Promega), 1.25 UI Taq-polymerase (Promega) and 2 µl of the extracted DNA. The amplification was carried out as follows: initial denaturation at 95°C for 5 min, 30 cycles of 95°C for 60 s, 52°C for 2 min, 72°C for 2 min and a final extension at 72°C for 7 min.

Five microlitres of the amplified mixture was analysed in a 2% w/v agarose gel with TAE 1 x buffer (40 mM Tris-HCL, pH 7.4 20 mM sodium acetate, 1.0 mM Disodium-EDTA), stained with ethidium bromide (Promega) 50 µg/ml in TAE 1x and quantified by using standard (DNA mass ladder 100 bp; Promega).

For PCR products sequence specific separation, DGGE analysis with the Dcode^{TM} Universal Mutation Detection System (BioRad, Hercules, CA, USA) were then carried out, using the procedure first described by Muyzer *et al* (1993) and improved by Leesing (2005).

Samples containing approximately equal amounts of PCR amplicon were loaded into 8% w/v polyacrylamide gel (acrylamide: bisacrylamide 37.5:1, Promega) using a denaturing gradient from 30% to 60% of urea and formamide (100% corresponds to 7 mole urea and 40% w/v formamide) increasing in the direction of the electrophoresis run. The gel electrophoresis was done at 20 V for 10 min and then at 80 V for 12 h. After the run, the gels were stained for 20 min in distilled water and photographed under UV illuminator with the Gel Smart 7.3 system (Clara Vision, Les-Ulis, France).

Identification of DGGE bands

Detected bands were cut from the DGGE gel with a sterile scalpel. DNA of each band was then eluted in 100 µL TE buffer at 4°C overnight. One-hundred µL of DNA eluted from each band was purified and sent for sequencing as described above. The amplicons of PCR were purified with Wizard PCR Preps DNA Purification system kit (Promega) and stored at -20°C.

Sequencing was done by EUROFINS GENOMICS enterprise (Paris, France). Sequence annotation and database searches for similar sequences were performed using BLAST (Altschul *et al*, 1997) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) to determine the closest known relative species.

Results and Discussion

The PCR-DGGE technique is widely employed in microbial ecology because it is able to provide

fingerprint of bacterial community in a sample after direct DNA extraction. DGGE method is a rapid and efficient method for the identification and is suitable to study the food microflora. DNA samples extracted from fermented food produced DNA bands on DGGE gels that have sufficient intensities to be analysed by sequencing. Traditional methods are enabling to characterise microorganisms for which selective enrichments and culturing are problematic or impossible, thereby eliminating entire populations from consideration. At reverse, PCR can amplify dead cells that can be detected as specific bands in the DGGE gels, evidenced yeast populations not viable in the samples. As the whole, 8 clear DNA stripes belonging to different species appeared (Fig 1).

Among these 8 DNA stripes, 7 yeast species were identified with % identity of 97-100% (Table 1). In all samples of *Shubat* and K*oumiss*, the dominant yeast species were *Kazakhstania unispora* and *Kluyveromyces marxianus*. These strains are particularly active to metabolise lactate (Lachance and Starmer, 1998). The other species were not present in the whole samples. Rahman *et al* (2009) reported that *Kluyveromyces marxianus* was predominant genera in *shubat*.

In addition to these 2 species mentioned above, *Saccharomyces cerevisiae* was present in raw and fermented milk (mare and camel), but if it was found in all *koumiss* samples, it appeared rarely in *shubat* ($^1/_5$ sample). The presence of *Saccharomyces cerevisiae* in *koumiss* leads to specific organoleptic properties of the product because it produces ethanol.

Galactomyces geotrichum was found and in all samples of *shubat* in 2 samples of *koumiss* only and *Galactomyces geotrichum* strain is able to deaminate glutamic and aspartic acid as well as tryptophan, leucine, methionine and phenylalanine. The catabolism of amino acids by *Galactomyces geotrichum* strains can produce alcohols and volatile sulfide, methanethiol and various S-methyl thioesters which are important for flavour development (Wyder, 2001).

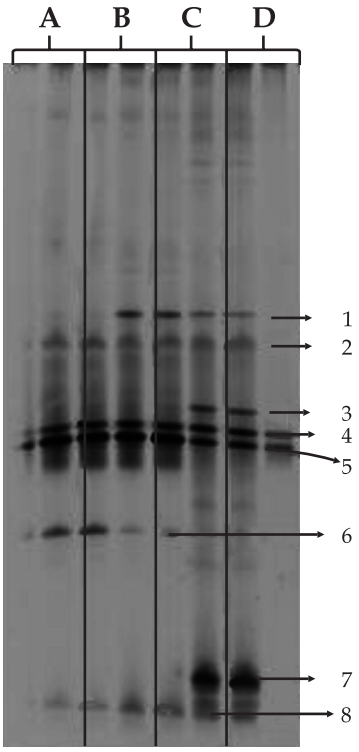

Fig 1. DGGE profiles of PCR amplicons of the domain D1 of 26S rDNA that represent the yeasts biodiversity in samples of milk products (A – *Koumiss*, B – mare's milk, C – *Shubat*, D – camel milk).

In some samples ($^2/_5$) of *shubat*, *Dekkera anomala* species was present with varying intensity patterns. *Dekkera bruxellensis* was rarely encountered in both beverages, and was observed in the bottom of the gel. *Dekkera anomala* and *Dekkera bruxellensis* are regarded generally as contaminant found worldwide and are responsible for milk and red wine spoilage (Cousin, 1982; Barbin *et al*, 2008). Probably these 2 yeast species in *Shubat* and K*oumiss* indicated the presence of spoilage in our samples.

Table 1. Yeast species occurring in dairy products (ND: Non determined).

№	Yeast species	Products (% identity)			
		Camel milk	Shubat	Mare's milk	Koumiss
1	*Galactomyces geotrichum*	ND	100	100	ND
2	*Kluyveromyces marxianus*	98	98	97	97
3	*not identified*	not identified	not identified	ND	not identified
4	*Kazachstania unispora*	97	97	97	97
5	*Kluyveromyces marxianus*	99	99	100	100
6	*Saccharomyces cerevisiae*	ND	97	99	99
7	*Dekkera anomala*	ND	100	ND	ND
8	*Dekkera bruxellensis*	ND	100	97	97

A Identical nucleotides percentage in the sequence obtained from the DGGE band and the sequence found in GenBank.

Among the 10 samples of fermented products and the 5 samples of raw camel milk 1 stripe was not identified. Probably, another primer would be necessary for identification.

Some microorganisms did not give a band in the gel underlining a population less than 10^4 cfu mL^{-1}, defined as the sensitivity of the PCR-DGGE protocol used (Cocolin *et al*, 2000). Comparing 2 methods (traditional and PCR), differences in the biodiversity can be detected.

Generally, yeasts occur in both raw and pasteurised milks, but at a low and insignificant size of populations (Fleet, 1990). Population less than 10^3 cells mL^{-1} were mostly reported but, occasionally, count higher than 10^4 cells mL^{-1} was reported by Cocolin *et al* (2002). Such yeasts rarely grow in milk during refrigerated storage and are quickly overgrown by psychrotrophic bacteria. However, yeast growth might occur in milk where bacterial growth has been inhibited by residual antibiotics (Cousin, 1982; Bishop and White, 1986).

Yeasts in fermented products are often presented as secondary microflora but they contribute with lactic acid bacteria to flavour and aroma of the final product (Shori, 2011). The yeasts play a leading role in the development of characteristic taste and aroma because of their ability to ferment carbon sources, releasing ethanol and carbon dioxide (Koroleva, 1991). For instance, Fonseca and Heinzle (2008) reported that *Candida krusei* has been used with dairy starter cultures to maintain the activity of LAB and, as such increased their longevity. Lore *et al* (2004) and Abdelgadir *et al* (2008) isolated some important yeast species that could improve fermentation to produce different fermented camel milk products. Gariss (Sudanese fermented camel milk), Suusac (Kenyan fermented camel milk) and *Shubat* have been reported to have different yeast species.

Conclusion

The yeast biodiversity could explain partly the variability in sensory properties of the final fermented products. In consequence, the present investigation could be a preliminary step for identifying the candidate microorganisms in starters prepared at industrial scale for dairy sector interested by non-conventional milk in Central Asia.

The position of bands is indicated by numbers that correspond to species of yeast: 1: *Galactomyces geotrichum*; 2: *Kluyveromyces marxianus;* 3: not identified; 4: *Kazachstania unispora*; 5: *Kluyveromyces marxianus;* 6: *Saccharomyces cerevisiae*; 7 *Dekkera anomala;* 8: *Dekkera bruxellensis*.

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