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Lactic Acid Bacteria Diversity of Koumiss Samples*

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Abstract: In this study lactic acid bacteria (LAB) diverstiy of koumiss samples were inverstigated. A total number of 22 koumiss samples were obtained from the pastures of the Naryn region of Kyrgyzstan Republic. Lactic acid bacteria and yeast counts of samples were determined. The identification of LAB strains from koumiss samples was carried out with the PCR, VITEC 2 Compact, and an automated mass spectrometry (MS) microbial-identification system using matrix assisted laser desorption ionization time-of-flight (MALDI-TOF). *Lactobacillus helveticus, Lactobacillus kefiri, Leuconostoc mesenteroides, Lactobacillus paraplantarum, Leuconostoc mesenteroides spp cremoris* were determined as lactic acid bacteria species. Bacterium like *Leuconostoc sp.* which is rarely met in koumiss has been identified on the genetic level using PCR. Information from these results could advance our understanding of koumiss fermentation, and also help improve the quality of koumiss.

Keywords: Koumiss, LAB, PCR, MALDI-TOF-MS, Kyrgyzstan

Kımız Örneklerinde Laktik Asit Bakteri Çeşitliliği

Özet: Kırgızistan Cumhuriyetinin Narın bölgesi yaylalarından toplam 22 adet kımız örneği temin edildi. İncelenen örneklerde laktik asit bakterisi (LAB) ve maya sayıları araştırıldı. Kımız örneklerinden elde edilen LAB suşlarının identifiye edilmesinde PCR, VITEC 2 Compact, MALDI-TOF kullanıldı. Çalışmada laktik asit bakterileri olarak *Lactobacillus helveticus, Lactobacillus kefiri, Leuconostoc mesenteroides, Lactobacillus paraplantarum, Leuconostoc mesenteroides spp cremoris* suşları tespit edildi. *Leuconostoc spp.* cinsine ait kımızlardan sık tespit edilemeyen mikroorganizmalar da PCR ile cins düzeyinde belirlendi. Araştırmadan elde edilen sonuçlar kımız fermentasyon sürecini daha iyi anlamamıza olanak sağlarken, standart niteliklerde kımız üretiminde seçilecek starter kültürler konusunda yardımcı olacaktır.

Anahtar Kelimeler: Kımız, LAB, PCR, MALDI-TOF-MS, Kırgızistan

1. Introduction

Koumiss, which originates from traditional fermentation of mare's milk, is a very popular dairy product for the people of Mongolia, Kazakhstan, Kyrgyzstan and some regions of Russian Federation (1).

The koumiss has a long history. The fact that it has beneficial effects on people's health and that it is pleasant beverage is known since time immemorial (2-4). Scientists have always interested in koumiss which is made of mare's milk it contains valuable food substances and probiotic microorganisms. Koumiss is mostly made in Kyrgyzstan, Kazakhstan, Mongolia and in some parts of China and Russia (1, 2, 5, 6). Koumiss is mostly made of mare's milk. It can be made of camel's and cow's milk too, and koumiss which is made of camel's milk is called shubat. Just milked milk is strained through a fine sieve into "chanach", "saba" and cask. (7). More than 10% of old koumiss made in the previous year or freshly prepared koumiss is added to fresh mares'milk and churned with stick called "bishkek" (8). The longer it is churned the tastier it becomes. If milk is added when it is warm, koumiss will be a bit sour. Therefore it must be added when it becomes cold. The vessel, where koumiss prepared have to be washed periodically in 6-7 days, than it's dried and smoked to prevent from the contamination and foreign cultures. The most difficult problem was to preserve the fermenting agent of koumiss made this year till the next year. To get fermenting agent of koumiss requires certain experience, our ancestors would ferment milk with "korongo" and "urp". Urp is sediment that sinks to the bottom of chanach in autumn. It is like curds. It was wrapped in gauze and dried. "Korongo" is usually collected from the edge of the dish

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where koumiss is made and is used to ferment milk the next year. But to ferment milk with the help of "korongo" is weaker than with the help of "urp" (9).

Utility of koumiss depends on chemical and bacterial composition. The chemical profile of koumiss depend not only on milk but also on microbial community. They play great role in increasing food substances, useful functionality and appearance of aroma specific to koumiss. Its microbiological resource is very rich and koumiss may vary depending on in what geographical area, in which climate it is made and temperature change during fermentation (10).
Consumption of koumiss is beneficial for enhancing innate immunity and treating tuberculosis and cardiovascular disease, improves the body's alimentary canal, metabolism, circulatory and nervous systems, blood-forming organs, functions of kidneys, endocrine glands and the immune system (11, 12). The procedure for the traditional preparation of the koumiss in China and in Kyrgyzstan is mainly similar and shown at Figure 1 (13).

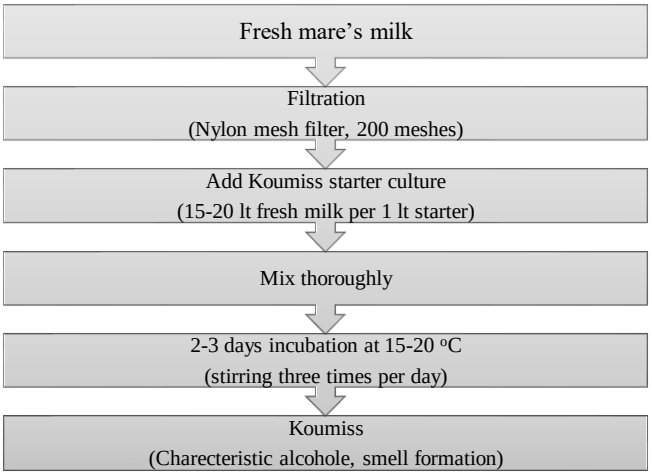

Figure 1: The traditional procedure for Koumiss preparation (13)

Depending on the geographical region where koumiss is made, its ingredients and fermenting microbiota differ, Lactic acid bacteria (LAB) and yeasts were proven to be the main components in koumiss starter (13-16).
Previous studies showed that Lb. helveticus was the most abundant species, which is in accordance with a previous study (17). As Mo *et al* (18) stated in cultured milk products Lb. helveticus was dominant, but several species typically present in dairy foods; for example *Leu. mesenteroides* were not detected by culture. According to another source, it was clear that Leuconostoc sp. was active at the end of fermentation (19).
The quality of koumiss is depends on fermentation procces, microbial community plays main role in fermentation. This research work is devoted to lactic acid bacteria isolated from total 22 different koumiss samples made in 10 different summer pastures of Naryn (Taman-Karagai, Dangi, Er-Alysh, Ajydar uyuk, Archaly, Ardakty, Too-jailoo, Joon, Oro bashy, Kyrk choro) which is located at an altitude of 2500 m above sea level. Isolated bacteria were identified with VITEK 2 compact, MALDI-TOF and Leuconostoc sp. PCR.

2. Materials and methods

2.1. Sampling

Total 22 koumiss samples were collected aseptically from different parts of Naryn, mountainous region of Kyrgyzstan, located at an altitude of more than 2500 m in summertime (May-June, 2018). Each sample was collected from separate traditional producer. A total of 100 mL koumiss samples were taken into sterile 100 mL tubes and brought to the laboratory under the cold chain.

2.2. Enumeration and isolation of LAB and yeast

The pour-plate method was applied to enumerate total LAB counts in the dairy samples. Briefly, 1 g of homogenized sample was aseptically diluted in 9 mL of sterile Ringer solution 1/4 strength. Following preparation of serial 10-fold dilutions, 1 mL of appropriate dilutions was mixed with molten de De Man Rogosa and Sharpe agar, Yeast Extract Glucose Chloramphenicol agar was parallelly inoculated in petri dishes and were incubated at 30oC for LAB, yeast was put at 25 oC into incubator for 5 days (20). Colonies with distinct morphologies (e.g., color, shape, and size) were randomly selected, streaked on the appropriate solid medium, and their Gram staining and catalase reactions were analyzed.

2.3. Isolation of LAB and yeasts

Colonies that have grown were counted and morphologically different LAB colonies were streaked. To obtain the pure culture, repetitive streaking was done that there was the only colony from each petri bowl, for further research they were taken to preserve in cryo test tube at below 18 ºC.

2.4. Identification with VITEK 2

Total 21 colonies were grown on blood agar plates for 48 h at 37 °C. A single colony from each isolate was picked and transferred to a new blood agar plate. After another incubation period of 24 h at 37 °C, the colonies were suspended in a solution of 3 ml of 0.45% saline. A turbidity of 0.5-0.63 McFarland standard using VITEK DensiCHEK Plus (bioMérieux, Nürtingen, Germany) was established. After morphological analysis of bacteria had been determined, the card suitable for Gram-Positive Anaerobic Cocci was chosen and Bacteria were identified with a VITEK 2 system (bioMérieux, Nürtingen, Germany) (21).

2.5. Identification with MALDI-TOF MS

Bacteria were grown on blood agar plates for 48 h at 37 °C stated previously. Subsequently, single colonies were picked

and plated on a 96-well steel target. Bacteria were dried in a laboratory workbench for 10 min and then overlaid with a 1 µl matrix-solution (α-Cyano-4-hydroxycinnamic acid, Bruker Daltonik, Bremen, Germany) in an organic solvent. Analyses were performed using a microflex MALDI-TOF MS system, using flexControl software 3.1 (Bruker Daltonik, Bremen, Germany). A bacterium colony is placed in a special slide of equipment, data inside is compared with library data (21).

2.6. Identification with 16 S RNA gene sequencing

Genomic DNA was extracted from the samples using the InstaGene (Bio-RAD, USA) according to the manufacturer's protocol, 3 to 5 colonies of LAB grown in blood agar medium are mixed with 1 ml of sterilized distilled water in ependof test tube and whirl wounded. Composition of bacterial cell is centrifuged at 13000 rpm for three minutes. Pellet is removed (with the help of pipette). 100 µL of is added to the sediment. (warning: magnetic mixture must be possible), mixed for 8 seconds. Test tubes are kept at 56 oC for 30 minutes. After mixing for 8 seconds, they are boiled at 100 oC. They are whirlwounded at 13000 rpm, centrifuged for 3 minutes and the supernatant obtained is DNA sample.

Bacterial 16S rRNA of *Leuconostoc sp.* was amplified using Fermentas Taq DNA polymerase (Fermentas, Genmark) and the LeuF (5'-CGA AAG GTG CTT GCA CCT TTC AAG-3') and LeuR (3'-TTT GTC TCC GAA GAG AAC A-5') primers (22).

Total genomic DNA was extracted from the isolates using the InstaGene, BioRAD. Next, 50 µL purified DNA was used as the template for PCR amplification of the 16S rRNA gene using an automatic thermal cycler (ThermoScientific, FINLAND) and the primers LeuF (5'-CGA AAG GTG CTT GCA CCT TTC AAG-3') and LeuR (3'-TTT GTC TCC GAA GAG AAC A-5'). Each 50-µL PCR contained 5 µL of DNA template (100 ng/µL), 5 µL of 10× PCR buffer (Thermo Scientific), 8 µL MgCl2 (25mmol), 5 µL of dNTPs (200 µmol, Fermentas, Genmark), 1 µL of primer LeuF (10 pmol/µL), 1 µL of primer LeuR (100 pmol/µL), 0.5 µL of Taq DNA polymerase (1 U/µL, Fermentas, Genmark), and 24.5 µL of triple-distilled water. The PCR was conducted as follows: 94°C for 5 min; followed by 30 cycles of 94°C for 30 sec., 55°C for 30 sec, and 61°C for 1 min; followed by 72°C for 2 min (22).

2.7. Statistical analysis

All data were collected from koumisss samples were expressed as mean ± standard deviation (SD). One way ANOVA was applied to compare pastures. Statistically significant differences between sample groups were evaluated with Duncan test. Pearson correlation analysis and Student's t-test were performed with the SPSS software (version 26, SPSS/IBM, Chicago, IL).

3. Results

3.1. LAB and yeast loads in koumiss samples

The LAB and yeast counts in Koumiss samples are given in Table 1.

Table 1: Sample, the pasture names where samples were taken, LAB and yeast counts per ml Koumiss

Code	Pasture name	LAB count*	Yeast count*	pH
1-1	Ajydar uyuk	6.89±0.006 ^{d}	6.29±0.011 ^{i}	4.03^{b}
1-2	Ajydar uyuk	6.26±0.015 ^{j}	6.23±0.016 ^{j,k}	3.98^{c,d}
1-3	Ajydar uyuk	7.00±0.006^{b,c}	6.21±0.018 ^{k}	3.67^{u}
1-4	Ajydar uyuk	6.51±0.011 ^{f}	6.42±0.011 ^{f}	3.87^{k}
2-1	Archaly	6.42±0.031 ^{h}	6.37±0.018 ^{g,h}	3.66^{v}
2-2	Archaly	6.52±0.009 ^{f}	6.39±0.02 $^{f, g}$	3.55^{y}
2-3	Archaly	6.80±0.006 ^{e}	6.69±0.006 ^{c}	3.98^{d,c,e}
3-1	Ardakty	7.02±0.009 ^{b}	5.79±0.016 ^{o}	3.92^{g}
3-2	Ardakty	6.15±0.018 ^{k}	6.69±0.004 ^{c}	3.77^{s}
3-3	Ardakty	7.00±0.004^{b,c}	6.00±0.002 ^{n}	3.86^{m}
3-4	Ardakty	6.45±0.015 ^{h}	6.83±0.014 ^{a}	3.77^{s}
3-5	Ardakty	6.91±0.002 ^{d}	6.35±0.016 ^{h}	3.89^{i}
3-6	Ardakty	5.13±0.063 ^{o}	5.40±0.02 ^{p}	3.85^{n}
3-7	Ardakty	6.27±0.016 ^{j}	6.52±0.009 ^{e}	3.71^{t}
4-1	Dangi	6.96±0.015 ^{c}	6.61±0.004 ^{d}	3.97^{e,d}
5-1	Er-Alysh	7.02±0.016 ^{b}	6.39±0.031^{f,g,h}	3.89^{j}
6-1	Jon	6.96±0.009 ^{c}	6.27±0.016 ^{i}	3.97^{f,e}
7-1	Kyrk choro	7.08±0.026 ^{a}	6.07±0.018 ^{m}	3.85^{n}
8-1	Oro bashy	6.69±0.007 ^{f}	5.97±0.006 ^{n}	3.83^{o}
8-2	Oro bashy	6.36±0.015 ^{i}	6.39±0.013^{f,g}	3.89^{j}
9-1	Taman-Karagai	7.10±0.010 ^{a}	6.74±0.004 ^{b}	4.32^{a}
10-1	Too jayloo	6.82±0.007 ^{e}	6.57±0.013 ^{d}	3.91^{h}

*The number of microorganisms is defined as log cfu/ml.
$\bar{X}$ is average value, SE is a standard error
Difference between number of values ($P < 0.05$).

As seen in Table 1, LAB number values were form 5.13 log cfu/ml to 7.10 log cfu/ml, Wurihan et al. (19) scientists found out that it was from 5.45 log cfu/ml to 6.78 log cfu/ml. Yeast were from 6.83 log cfu/ml to 4.53 log cfu/ml, values got were close to the results of other authors (13).

The highest LAB number values were 7.10±0.01 log10 cfu/ml-7.08±0.03 log10 cfu/ml that stayed unaffected in koumiss samples brought from pastures like Karagai and Kyrk choro of Naryn oblast. The lowest number values 5.13±0.06 log10 cfu/ml was determined in the samples brought from Ardakty pasture. Both the highest number values of yeast was determined in koumiss samples from Ardakty pasture and the lowest number values 5.40±0.02 log10 cfu/ml were determined in Ardakty pasture.

The fact that pH of samples was decreasing can be explained with the appearance of organic acids. With the growth of LAB in koumiss, lactic acid, acetic acid and butyric acids appear. pH of koumiss decreases from 6.13 to 3.59 for 84 hours, during the first 48 hours it considerably changes (19). In the sample with the highest pH 4.32 brought from Karagai pasture it is determined that the number of LAB was 7.10±0.01 log10 cfu/ml while the number of yeast was 6.74±0.01 log10 cfu/ml. As previous researchers highlighted in koumiss fermentation first LAB grows then growth of yeast is followed. In some dairy products, yeast consumes lactic acid. Bacterial growth may also be stimulated by the amino acids and vitamins produced by the yeast (23). It's habitual to divide the koumiss fermentation stage into three-

the strongest, moderate and light (saamal) which depends on persistence of lactic acid in koumiss. Light (saamal) koumiss is a bit sour due to Streptococcus thermophilus and *Str. cremoris* acidification (pH 4.5-5.0). In moderate koumiss contains Lactobacillus bacteria (*L. acidophilus, L. plantarum, L. casei, L. fermentum*), with restricted acidification properties that lower the pH 4.5-3.9 at the end of the process, lactose and lactic acid ratio is 50 %. Koumiss becomes strong due to growth process of LAB (*Lactobacillus bulgaricus, Lactobacillus rhamnosus*) which makes sour substance of koumiss pH 3.6-3.3 and lactose and lactic acid ratio is 80-90 % (24, 25).

3.2. VITEK 2 results

The results of analysis made using VITEK 2 compact are shown in the following table 2. Some strains haven't been determined as they didn't match the library basic data.

Table 2: The results got using VITEK 2 compact

Code	Pasture name	Identification
1-4	Ajydar uyuk	*Anaerococcus prevotii*
1-4	Ajydar uyuk	*Leuconostos mesenteroides spp.cremoris*
2-2	Archaly	*Kocuria rosea*
2-3	Archaly	*Leuconostos mesenteroides*
2-3	Archaly	*Staphylococcus warneri*
2-3	Archaly	*Kocuria kristinae*
3-2	Ardakty	*Anaerococcus prevotii*
3-4	Ardakty	*Anaerococcus prevotii*
5-1	Er-Alysh	*Anaerococcus prevotii*
8-1	Oro bashy	*Anaerococcus prevotii*
8-2	Oro bashy	*Leuconostos mesenteroides spp.cremoris*

VITEK 2 compact is identified 11 strains of bacteria, including the LAB; 1 strain *Leuconostos mesenteroides* and 2 strain *Leuconostos mesenteroides spp.cremoris*, as well as the strain of saprophytic bacteria: *Anaerococcus prevotii, Kocuria rosea, Kocuria kristinae* and *Staphylococcus warneri*. There are currently four reagent cards available for the identification of different organism classes as we use only GN-Gram-negative fermenting and non-fermenting bacilli some samples (Ardakty (3-2-1, 3-3-1), Taman-Karagai (9-1-1, 9-1-2, 9-1-3), Ajydar uyuk (1-2), Ajydar uyuk (1-3)), a total 7 strains were not identified.

3.3. MALDI-TOF results

A total 21 strains were transferred to MALDI-TOF the results of which are shown in table 3. In the result 7 strains of Lactobacillus species, 2 strains of Leuconostoc sp., 5 strains of *Staphylococcus sp., and Acinebacter sp., Cupriavidus sp., Enterococcus sp., Micrococcus sp., Prevotella sp.* were determined. Based on the results it is observed that level of bacteria types like *Lactobacillus paraplantarum, Prevotella intermedia* and *Streptococcus dysgalactiae sp* similarity was low.

Table 3: The results got using MALDI-TOF

Code	Pasture name	Strains	Similarity (%)
1-4	Ajydar uyuk	*Leuconostoc mesenteroides*	99.9
1-2	Ajydar uyuk	*Lactobacillus kefiri*	99.9
1-1	Ajydar uyuk	*Staphylococcus saprophyticus*	99.9
1-1	Ajydar uyuk	*Cupriavidus pauculus*	99.9
2-1	Archaly	*Lactobacillus paraplantarum*	50
2-3	Archaly	*Leuconostocmesenteroides*	99.9
3-5	Ardakty	*Lactobacillus kefiri*	99.9
3-7	Ardakty	*Staphylococcus saprophyticus*	99.9
1-6	Ardakty	*Lactobacillus helveticus*	99.9
4-1	Dangi	*Micrococcus luteus/lylae*	99.9
4-1	Dangi	*Staphylococcus equorum*	99.9
4-1	Dangi	*Streptococcus dysgalactiae spp equisimilis*	50
5-1	Er-Alysh	*Staphylococcus equorum*	99.9
5-1	Er-Alysh	*Staphylococcus equorum*	99.9
5-1	Er-Alysh	*Enterococcus saccharolyticus*	79.5
6-1	Joon	*Acinebacteri woffii*	52.8
7-1	Kyrk choro	*Lactobacillus kefiri*	99.9
8-1	Oro bashy	*Lactobacillus kefiri*	99.9
9-1	Taman-Karagai	*Prevotella intermedia*	50
10-1	Too jayloo	*Lactobacillus paraplantarum*	50

3.4. PCR results

Identified by VITEK compact and MALDI-TOF apparatus Leuconostoc sp. bacterium again was identified using PCR. Because of this bacterium consist in dairy product rarely and in koumiss acquires at the end of fermentation (18, 19).

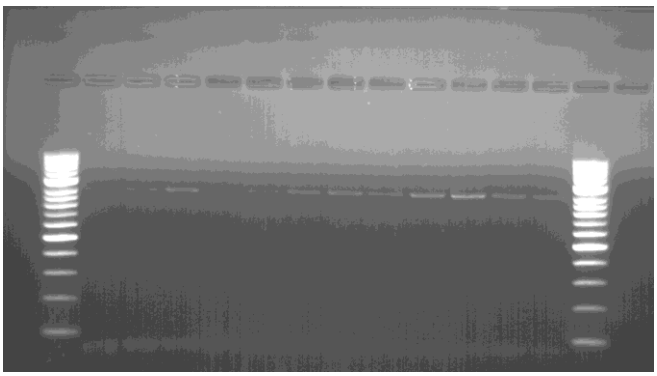

Figure 2. PCR product for the nine species of typical Leuconostoc with specific primers. Lane M, 1 kb Ladder DNA (Sigma, USA)

The specificity of the primers was confirmed by PCR using chromosomal DNA extracted from Leuconostoc species, found in koumiss (Figure 2). The LeuF and LeuR primers were able to detect specifically the typical Leuconostoc species, providing PCR products with the expected size (976 bp). No amplification was obtained for strains of all the other species tested.

4. Discussion

Composition of LAB and yeast of koumiss samples were studied and compared with literary sources. It has been found out that number of LAB is between 7.10 log cfu/ml and 5.13 log cfu/ml, number of yeast is between 6.83 log cfu/ml and 4.53 log cfu/ml. It is clear that dairy products should contain at list 108 cfu live probiotic LAB (26).

Strains belonging to LAB in koimiss have been identified using by VITEK 2 Compact and MALTI-TOF MS are *Lactobacillus kefiri, Lactobacillus helveticus, Lactobacillus paraplantarum, Leuconostoc mesenteroides, Leuconostoc mesenteroides spp.cremoris.* That bacterium like *Leuconostoc mesenteroides* can be rarely met had been also mentioned. What should be highlighted is the identification of strain *Leuconostoc mesenteroides spp.cremoris*, but it wasn't mentioned in the sources. Bacterium like *Leuconostoc* which is rarely met in koumiss and cannot be met in other dairy products has been identified on the genetic level using PCR. Data of bacteria got using PCR and express analyses have been proved.

In this research were found bacteria not belong to LAB, saprophytic bacteria like *Anaerococcus prevotii, Kocuria rosea, Kocuria kristinae, Staphylococcus warneri, Staphylococcus equorum, Staphylococcus saprophyticus, Acinebacter iwoffii, Cupriavidus pauculus, Enterococcus saccharolyticus, Micrococcus luteus/lylae, Prevotella intermedia and Streptococcus* dysgalactiae spp equisimilis have been determined. There can be contamination starting from milking the mare till the final ready koumiss, an udder of the mare, personal hygiene of the one who milks and cleanliness of the dishes used are important. Sanitary norms and hygiene standards should be kept; taste and quality depend on the food which is prepared without contamination with other cultures. While preparing koumiss Kyrgyz people clean the dishes in a timely manner and smoke to get rid of other microorganisms.

In conclusion information and data about LAB of koumiss made in Kyrgyzstan can be proposed to scientists and can be used in industry.

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