

Received: 31.7.2023
Revised: 1.9.2023
Accepted: 10.9.2023
Published: 12.9.2023

Potravinárstvo Slovak Journal of Food Sciences
vol. 17, 2023, p. 777-787
<https://doi.org/10.5219/1906>
ISSN: 1337-0960 online
www.potravinarstvo.com
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Comparative characterization of strains of lactic acid bacteria isolated from Kazakhstan mare's milk and koumiss to create probiotic preparation

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ABSTRACT

The most widely used probiotics that benefit human and animal health are lactic acid bacteria (LAB) derived from milk and dairy products. Therefore, this study aimed to investigate the probiotic properties of LAB strains isolated from Kazakhstan mare's milk and koumiss (fermented mare's milk) samples. A total of 24 LAB strains were isolated to test their probiotic properties. Based on analysis of probiotic properties, the strains 3K, 7K, 9K, 10K and 11K were identified by 16S rDNA sequence analysis. According to PCR analysis, three strains (3K, 7K, 9K) were assigned to the species *Limosilactobacillus fermentum* and the remaining two strains (10K and 11K) were assigned to the species *Lactocaseibacillus paracasei*. In summary, the high biological potential of the strain *Lactocaseibacillus paracasei* 10K was identified as having probiotic property, which suggests its possible use as a promising candidate.

Keywords: mare's milk, koumiss, LAB, probiotic properties.

INTRODUCTION

In recent years, probiotics have been used extensively for the prevention of gastrointestinal disorders in human and animals [1]. In this sense, functional food supplements, including pro-, pre- and synbiotics, are gaining increasing attention as an environmentally sound strategy to improve health. Meanwhile, probiotic bacterial strains should have a set of characteristics that allow them to compete with pathogenic and conditionally pathogenic microorganisms [2]. *Lactobacilli* and *Bifidobacteria* are beneficial human and animal gut bacteria with therapeutic functions [3].

Milk and fermented milk products contain large amounts of bacteria with probiotic properties, which positively affect the maintenance of the body's intestinal system [4]. Recently, mare's milk, widely represented in the diet of the population of Kazakhstan, is now being actively used as a product with healthy ingredients in a naturally digestible form. Koumiss is a sour milk drink made mainly from mare's milk by fermentation with a special starter [5]. Scientific studies revealed high koumiss activity in treating gastric and duodenal ulcers, chronic gastritis and enterocolitis [6], [7]. In this regard, production of probiotics with strains derived from koumiss needs to be expanded as a highly therapeutic and dietary product, contributing to enhancing the human and animal's immune system. The main microbiota involved in making koumiss are lactic acid bacteria (LAB) [8], [9]. For instance, *L. helveticus* NS8 was investigated as potential strain isolated from koumiss, which might benefit health [10]. Probiotic preparation with *Lactobacillus casei* Zhang (LcZ) derived from Chinese koumiss, has been proved to positively affect human intestinal microbiota [11]. Nevertheless, a few studies still exist on strains derived from Kazakhstan koumiss, their characterizations, and probiotic properties.

One of the first and important steps in finding and selecting a strain promising for use in the food industry is determining its taxonomic identity. Correct identification of the strain at the species level allows the researcher to understand its safety, origin, habitat and physiological characteristics of the isolated microorganism. Therefore, the study was aimed to isolate active strains of lactic acid bacteria from Kazakhstan mare's milk and koumiss samples and evaluate their probiotic properties.

Scientific Hypothesis

The hypothesis of the study is to select potential LAB isolated from mare's milk and koumiss for use as probiotics and to pay attention to properties such as acid and bile tolerance, antimicrobial effect, etc. that allow LAB strains to be considered as probiotic candidates.

MATERIAL AND METHODOLOGY

Samples

Samples of raw mare's milk ($n = 6$) and koumiss ($n = 6$) were obtained from mares aged four and a half years of Zhaby, Kazakh and Mugalzhar breeds. The farms are located in the foothills of Talgar district (Tuzdybastau and Panfilov villages) and Karasai district (Almaty region, Kazakhstan). The horses were kept under standard conditions, with the same management conditions. The horses were provided with clean water and fed pasture grass. The samples of mare's milk and koumiss were collected during horse lactation (September 2021) in the morning. The raw 1000 mL and fermented dairy products of each sample was taken into flasks and were immediately placed on ice, stored at 4 °C for further analysis. Sampling of milk and dairy products was carried out according to the procedure of GOST 26809.1-2014 [12].

Chemicals

The De Man, Rogosa, and Sharpe (MRS) broth (Merck, Germany), was used for LAB isolation; gram staining was performed using a special Gram staining kit (Merck, Germany); different antibiotic disks (levomycin, neomycin, tetracycline, streptomycin and erythromycin) (Scientific Research Centre of Pharmacotherapy, St. Petersburg, Russia) were used for antibiotic susceptibility test.

Instruments

The morphological and cultural properties of the lactic acid bacteria were studied using a Micros MC-300 electron microscope (Austria). DNA concentrations were determined using the Qubit™ dsDNA HS Assay Kit (Life Technologies, Oregon, USA) on a fluorimeter, Qubit 2.0. PCR amplification was performed on a GeneAmp PCR System 9700 amplifier (Bio-Rad, USA).

Laboratory Methods

Laboratory researches were carried at the Kazakh National Agrarian Research University (Almaty, Kazakhstan). The isolation of lactic acid bacteria strains was done by culture characteristics and macroscopic analysis [13], acid and bile resistance [14], antibiotic resistance was done by disc diffusion method [15] and the agar diffusion method was employed for antimicrobial effects [13], and molecular identification were investigated by 16S rRNA gene via PCR analysis [16].

Description of the Experiment

Sample collection and isolation of LAB: Lactic acid bacteria from samples were isolated by inoculating 10 mL of each milk sample into De Man, Rogosa, and Sharpe (MRS) broth (Merck, Germany) and incubated at 37 °C for 24 h. The morphological and cultural properties of the lactic acid bacteria were studied using a Micros MC-300 electron microscope. Colonies were transferred to MRS agar and incubated in a refrigerator at 4°C for further study (GOST 33951-2016) [13].

Resistance to acid and bile: Isolates were selected according to the characteristics of the cultures. Isolates forming colonies with formed off-white pinhead colonies characteristic of *Lactobacillus* spp. were selected. The tolerance of the cultures to different concentrations of acid and bile salt were tested. To determine the acid tolerance of the tested strains, hydrochloric acid (HCl) was added to MRS liquid medium seeded with the tested bacteria, setting the pH between 6 and 2; then cultured at 37 °C for 24 h. Determination of resistance to NaCl was carried out by inoculating cultures in liquid MRS medium with different salt contents (2, 4, 6, and 8%), in which the bacteria were cultured at 37 °C for 36 hours.

Resistance to bile was determined by adding daily culture to MRC broth containing 0.3, 0.4, and 0.5% bile concentrate. Bacterial growth was analysed by counting viable colonies after 2 and 4 hours of culturing, following inoculation in agar medium at 37 °C for 48 hours. For this purpose, 0.1 ml of a solution that contained a particular previously isolated strain was added to 10 ml of MRS broth, then inoculated for 24 hours at 37 °C [14].

Antibiotic susceptibility test and antimicrobial effect of isolated bacterial cultures: The choice of antibiotics was based on the natural resistance of LAB and the different classes and mechanisms of action of

antibacterial drugs. To determine strains' sensitivity to antibiotics, standard disks impregnated with standard solutions of amphenicols – levomycin (30 µg), polyketide – tetracycline (30 µg), aminoglycoside antibiotics – streptomycin (30 µg) and neomycin (30 µg), macrolide – erythromycin (15 µg) was used. Milk agar was a nutrient medium for lactic acid bacteria [15]. One-day cultures grown at optimum temperature were used in the experiments in the form of cell suspension in the amount of 1 billion/ml, based on the calculation of 0.1 ml of suspension per one Petri dish. After seeding the dishes with the tested strain cultures, discs impregnated with antibiotic were placed on the surface of the nutrient medium. Cultivation was carried out for 72 hours at 37 °C. The sensitivity of lactic acid bacteria to antibiotics was determined by measuring the diameter of the growth suppression zone.

The agar diffusion method was used to determine the antagonistic activity of the bacteria [13]. The *Sarcina flava*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* (*E.coli*) cultures are opportunistic bacteria. In the body, it is permanently present in the intestinal tract, therefore, they were used as indicator bacteria. *Salmonella Dublin* was selected as the causative agent of gastrointestinal infectious diseases. Test cultures were grown on media with the optimal composition for each species: agar wort mixed with MPA in a 1:1 ratio. 0.1 ml of suspension was added to the melted and cooled medium, mixed and poured into Petri dishes. Wells with a diameter of 10 mm were cut in the layer of nutrient medium, into which a liquid suspension of daily cultures of the studied lactic acid bacteria was added and placed in the thermostat at 37 °C for 24 hours. After 24 hours, the zones of growth suppression of the test cultures by lactic acid bacteria were measured. The strains that showed the strongest antagonistic properties by the maximum number of relevant indicators with wide zones of inhibition were selected for further studies.

Molecular identification of LAB strains: Genetic study of microbial isolates was based on nucleotide sequence analysis of the 16S rRNA gene. DNA extraction was performed according to the standard protocol (the PureLink Genomic DNA Kit, Promega, USA). Qualitative DNA assessment was performed by electrophoresis on 1% agarose gel.

Universal primers were used to determine the nucleotide sequence of 16S rRNA gene: forward 8F (5'-AGAGTTTTGATCCTGGCTCAG-3') and reverse 806R (5' GGACTACCAGGGTATCTAAT-3') [16]. The PCR reaction was performed in a total volume of 20 µl. The PCR conditions were as follow as: stage 1 – 5 min at 95 °C – 1 cycle; stage 2 – 30 sec at 95 °C, 40 sec at 55 °C, 50 sec at 72 °C – 30 cycles; stage 3 – 10 min at 72 °C – 1 cycle.

To assess the efficiency of PCR, the amplification products were visualized on 1% agarose gel. The separation of gene fragments was performed using an automatic genetic analyzer. The obtained nucleotide sequence was compared with nucleotide sequences from international GenBank databases (NCBI: <https://www.ncbi.nlm.nih.gov/genbank>). Phylogenetic trees were constructed via MEGA 6 software. Alignment of nucleotide sequences was performed using ClustalW algorithm. The Neighbor-Joining (NJ) method was used to construct phylogenetic trees.

Number of samples analyzed: We analyzed a total of 12 samples.

Number of repeated analyses: All measurements were performed two times.

Number of experiment replication: All experiments were performed in three replications.

Statistical Analysis

Data were presented as mean (\pm) standard deviation. SPSS version 25 (IBM Corporation, New York, USA) was used to perform all statistical analysis. The statistical comparison analysis was done using Student's t-test. Statistically significant data were considered when $p < 0.05$.

RESULTS AND DISCUSSION

The beneficial properties of koumiss are due to the unique composition of mare's milk, which, unlike cow's milk, is closer in composition to human milk [17]. Nowadays koumiss is used not only for treatment of pulmonary tuberculosis, but also gastrointestinal diseases, non-specific lung diseases, some diseases of cardiovascular and nervous systems [18]. The huge needs of the nascent food industry and health care institutions demanded increased production of mare's milk and koumiss. Therefore, in the present work, mare's milk and koumiss are chosen as research objects to fill the gaps in potential probiotic research.

In this study, a total of 24 bacterial isolates were isolated on MRS agar after 24 hours incubation at 37 °C. They were marked with the letters 'K' (koumiss) and 'M' (mare). Isolates 1M, 2M, 3M, 4M, 5M, 6M, 7M, 8M, 9M, 10M and 11M were isolated from four mare's milk samples. Isolates 1K, 2K, 3K, 4K, 5K, 6K, 7K, 8K, 9K, 10K, 11K, 12K and 13K were isolated from six samples of koumiss. In a study by Jin et al. (2021), 114 strains of lactic acid bacteria were also isolated from raw mare's milk and their probiotic traits were tested [19]. The biochemical characteristic of all strains was examined. All strains were found to be facultative anaerobes and all

were Gram-positive, catalase-negative and immobile. 2M, 3M, 6M, 7M, 11M and 3K, 7K, 9K, 10K, 11K were bacilliform. 1M, 4M, 5M, 8M, 9M, 10M and 1K, 2K, 4K, 5K, 6K, 8K, 12K, 13K were identified as cocci forming bacteria.

Requirements for probiotic strains include resistance to low pH of gastric juice and bile, antagonism to opportunistic and pathogenic flora, etc. [20], [21]. The high acidity of gastric juice is known to kill most bacteria and viruses, which prevents them from multiplying and spreading. Thus, the survival of lactic acid bacteria when exposed to human gastric juice is a critical factor [22]. Subsequently, all strains were evaluated for viability at different pH values (6, 4, and 2) for 24 hours of cultivation. Based on the obtained results (Table 1), strains were able to grow at pH 6 ($p \leq 0.05$). At pH 4, 11 strains grew, of which 4 were isolated from mare's milk samples and 7 from koumiss samples. However, 3 strains could not grow at pH 2 for 24 hours of cultivation. Only 8 strains (2M, 3M, 8M, 3K, 7K, 9K, 10K and 11K) were able to grow at pH 2 ($p \leq 0.05$). According to a study by Azat et al. survival at pH 3.0 is considered the optimal acid tolerance for probiotic strains [23]. The difference in results may be because the strains' acid regulatory mechanisms failed to maintain their intracellular pH and internal acidification decreased enzyme activity, damaging certain proteins and DNA, leading to death. However, the pH value (2.0) used in the current study to choose potentially probiotic strains is very selective and it guarantees the isolation of very acid-tolerant strains. There is a lack of studies reporting resistance of koumiss LAB strains to low pH values. Previous study on Mongolian koumiss observed that only two strains showed normal growth at pH 2.0 [24]. These data potentially indicate that the strains tested in our research are the most acid-tolerant and can be used as promising probiotic strains.

Bile enters the duodenal section of the small intestine, causing the death of a large number of bacteria, as cell membranes composed of lipids and fatty acids are very sensitive to destruction by bile acid salts. In this regard, the effectiveness of probiotic microorganisms depends on their resistance to bile acids [25]. Therefore, bile salt tolerance has often been used as the most important criterion for the selection of active strains suitable for use as probiotics [26]. All strains were also tolerant to 0.3% and 0.4% bile. Moreover, among the 8 strains that were acid tolerant strains such as 3M, 3K, 7K, 9K, 10K and 11K had better tolerance to 0.5% bile ($p \leq 0.05$) (Table 1). Strains of *Lactobacillus* spp. isolated from milk and curd were resistant and showed maximum growth at 0.8% bile salt concentration in the study by Kasimin et al. [27].

Hydrochloric acid is produced by special parietal cells from the glands of the stomach. The main functions of hydrochloric acid are protein digestion, antibacterial action, etc. Sodium chloride (NaCl) is the main source of hydrochloric acid formation of gastric juice [28]. NaCl is an inhibiting substance that can inhibit the growth of certain types of bacteria. Some strains of lactic acid bacteria are resistant to NaCl, so it was important to test the tolerance of LAB to NaCl. All strains could tolerate concentrations of 2% NaCl, and only some strains were resistant to concentrations of 4% and 6% sodium chloride. Among all strains, those such as 2M, 3M, 3K, 7K, 9K, 10K, and 11K showed better tolerance compared to other strains ($p \leq 0.05$) (Table 1). Our results agreed with those in the research by Kasimin et al., where most strains isolated from milk and dairy products were not resistant to media containing more than 6.5% NaCl [27].

Antimicrobial activity is one of the most important factors in selecting effective and novel probiotics [29]. The antimicrobial action of LAB is supported by the production of several substances, such as organic acids, hydrogen peroxide, low molecular weight antimicrobials and bacteriocins [30]. The results of the strains' antagonistic abilities are shown in Table 2. We can conclude that strains 7K, 10K, and 11K have the most pronounced antagonistic properties, and their use as probiotic strains is quite appropriate.

All strains intended for probiotic use must be investigated to establish the sensitivity to the appropriate range of antimicrobial agents relevant for humans or animals [31]. The antibiotic resistance of isolated strains is shown in Table 3. LAB that are studied in our analysis were relatively resistant to the following antibiotics: erythromycin and tetracycline, which disrupt protein synthesis but also the genome replication processes of microorganisms. Erythromycin actively penetrates through the cell membrane of bacteria and binds irreversibly to the subunits of bacterial ribosomes, thus inhibiting protein synthesis of the pathogen. Growth inhibition was observed with erythromycin in the following strains: 3M, 3K, 7K. Only three strains 7K, 10K and 11K showed significant resistance ($p \leq 0.05$). Notably, the 3M strain was the most sensitive to this antibiotic. A similar result was obtained by Guo et al. where 33 *Lactobacillus* strains were tested for antibiotic resistance [32]. All *Lactobacillus* spp. strains were found to be resistant to vancomycin but sensitive to erythromycin and gentamicin. Aryantini et al. investigated the safety and probiotic characteristic of *Lactobacillus* spp. isolated from fermented mare's milk, where strains were sensitive to ampicillin and streptomycin but resistant to erythromycin [33]. In summary, strains 7K, 9K, 10K, and 11K were the most resistant to the above antibiotics.

Table 1 Growth performance of isolated strains in milk hydrolysate containing bile and sodium chloride.

No	Strain notation	pH value			Bile, %			NaCl, %			
		6	4	2	0.3	0.4	0.5	2	4	6	8
1	1M	+	-	-	+	+	-	+	-	-	-
2	2M	+	+	+	+	+	-	+	+	-	-
3	3M	+	+	+	+	+	+	+	+	+	+
4	4M	+	-	-	+	-	-	+	+	-	-
5	5M	+	+	-	+	+	-	+	+	-	-
6	6M	+	-	-	+	-	-	+	+	+	-
7	7M	+	-	-	+	-	-	+	+	+	-
8	8M	+	+	+	+	+	-	+	+	+	-
9	9M	+	-	-	+	+	-	+	-	-	-
10	10M	+	-	-	+	-	-	+	-	-	-
11	11M	+	-	-	+	-	-	+	+	-	-
12	1K	+	-	-	+	+	-	+	-	-	-
13	2K	+	-	-	+	+	-	+	-	-	-
14	3K	+	+	+	+	+	+	+	+	+	+
15	4K	+	-	-	+	+	-	+	+	-	-
16	5K	+	-	-	+	-	-	+	-	-	-
17	6K	+	+	-	+	-	-	+	-	-	-
18	7K	+	+	+	+	+	+	+	+	+	+
19	8K	+	-	-	+	+	-	+	+	-	-
20	9K	+	+	+	+	+	+	+	+	+	+
21	10K	+	+	+	+	+	+	+	+	+	+
22	11K	+	+	+	+	+	+	+	+	+	+
23	12K	+	-	-	+	+	-	+	-	-	-
24	13K	+	+	-	+	-	-	+	+	+	-

Note: «+» - growth; «-» - no growth; * - ($p \leq 0.05$).

Table 2 Antagonistic activity of lactic acid bacteria strains.

No	Strains	Culture test, (mm)				
		<i>Sarcina flava</i>	<i>Bacillus subtilis</i>	<i>Staphylococcus aureus</i>	<i>E. coli</i>	<i>Salmonella dublin</i>
1	2M	-	5.4 ±0.2	-	-	-
2	3M	-	9.7 ±0.1	-	10.1 ±1.2*	-
3	8M	-	-	-	8.7 ±0.3	-
4	3K	11.7 ±0.2	-	8.9 ±0.3*	-	-
5	7K	5.5 ±0.2*	3.7 ±1.1	4.1 ±0.2	5.8 ±0.4	-
6	9K	8.8 ±0.3	7.0 ±0.3	-	8.2 ±0.2	-
7	10K	11.7 ±0.1*	11.2 ±0.1*	12.2 ±0.1	15.3 ±0.1*	12.2 ±0.1*
8	11K	10.2 ±0.3*	10.3 ±0.1*	10.2 ±0.3	11.4 ±1.1*	11.7 ±0.5*

Note: Clear zones were measured in mm. Results represent the mean ± standard deviation of three replicates. Strains 2M, 3M, 8M isolated from of horse milk, strains 3K, 7K, 9K, 10K and 11K isolated from koumiss; “-” – no activity; * - ($p \leq 0.05$).

Table 3 Study of antibiotic resistance of LAB strains.

No	Strains	Antibiotics, suppression zones (mm)				
		Levomyacin 30 mc	Neomycin 30 mc	Tetracycline 30 mc	Streptomycin 30 mc	Erythromycin 15 mc
1	3M	14.3 ±0.6	16.8 ±0.2	25.8 ±0.1	41.6 ±0.2*	47.1 ±0.3
2	3K	12.2 ±0.1	18.3 ±0.1	22.4 ±0.3	24.1 ±0.2*	35.3 ±0.2
3	7K	R*	14.5 ±0.5	33.6 ±0.1	25.1 ±0.4	33.8 ±0.3
4	9K	11.8 ±0.05	16.5 ±0.3	R	26.3 ±0.05	28.7 ±0.3
5	10K	R*	7.9 ±0.1*	R*	17.2 ±0.6	15.3 ±0.4*
6	11K	R*	R	R	15.9 ±0.1*	16.1 ±0.1*

Note: The suppression zones were measured in mm. The results are the mean value ± standard deviation of the three replicates. R – stable; * - ($p \leq 0.05$).

In our study, genetic identification of five strains (3K, 7K, 9K, 10K, and 11K) was performed by direct nucleotide sequence determination of the 16S rRNA gene fragment, followed by comparison of nucleotide identity with sequences deposited in the international Gene Bank database (NCBI: <https://www.ncbi.nlm.nih.gov/genbank>), and construction of phylogenetic trees with nucleotide sequences of reference strains. The results of sample amplification are shown in Figure 1. The results of phylogenetic analysis of 16S rRNA gene sequences in the strains are presented on a phylogenetic tree. As it can be seen from the data presented, strains can be classified into two species, one of which is the species *Limosilactobacillus fermentum*, whose nucleotide sequences are characterized by 100% similarity (Figure 2-4). The second cluster includes a strain of *Lacticaseibacillus paracasei* whose 16S rRNA gene sequence similarity was 100% (Figure 5-6). To date, Wu et al. identified the both species in Inner Mongolian koumiss samples [34]. Furthermore, Pan et al. proved the cholesterol-reducing effect of the *Limosilactobacillus fermentum* SM-7 strain derived from koumiss in mice [35].

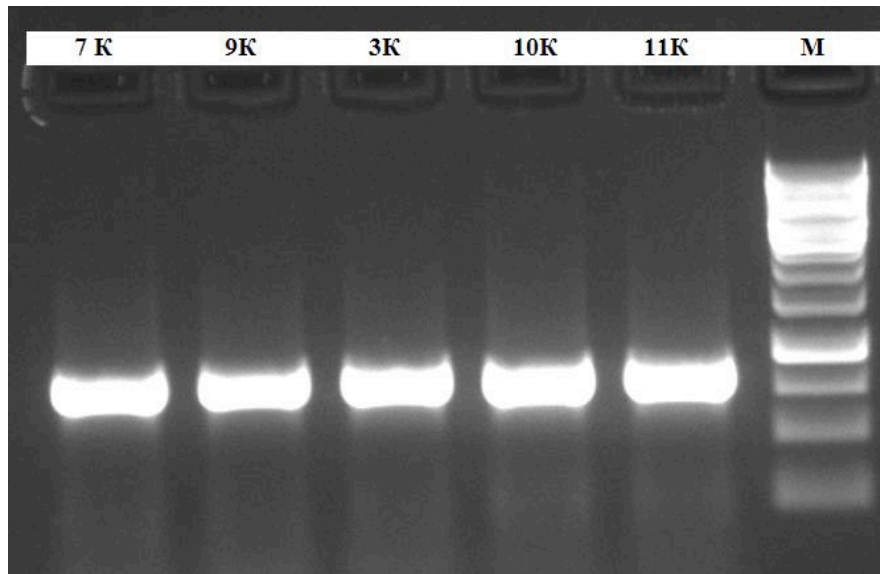


Figure 1 PCR electrophoresis pattern of the amplification products of the 16S rRNA fragment of the DNA gene. (M) 100bp Plus DNA molecular weight marker.

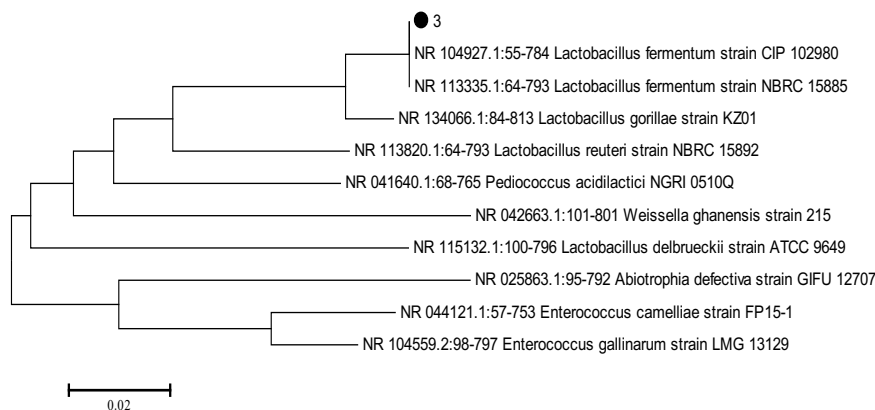


Figure 2 Phylogenetic tree based on analysis of 16S rRNA fragment structures showing the kinship of strains of the genus *Limosilactobacillus fermentum*.

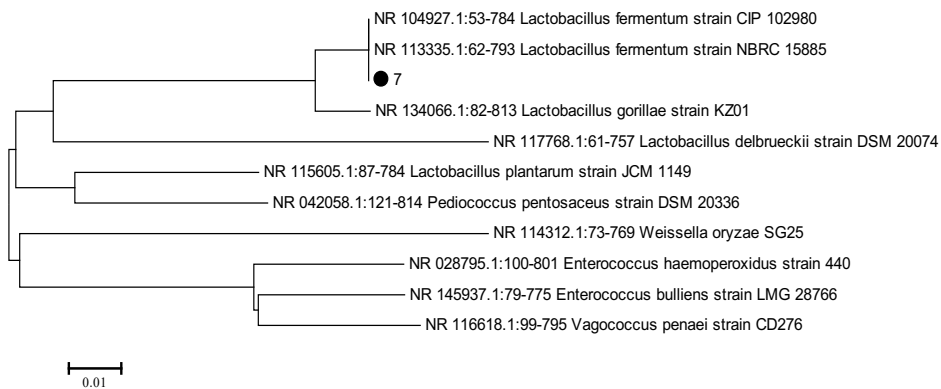


Figure 3 Phylogenetic tree based on analysis of 16S rRNA gene fragment structures showing the relationship between strains of the lactic acid bacteria genus *Limosilactobacillus fermentum*.

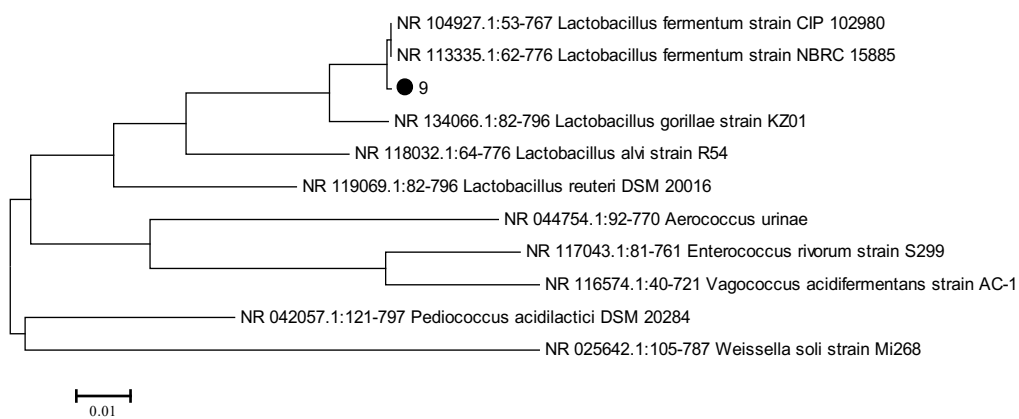


Figure 4 Phylogenetic tree based on the analysis of 16S rRNA fragment structures showing the relationship between strains of the lactic acid bacteria genus *Limosilactobacillus fermentum*.

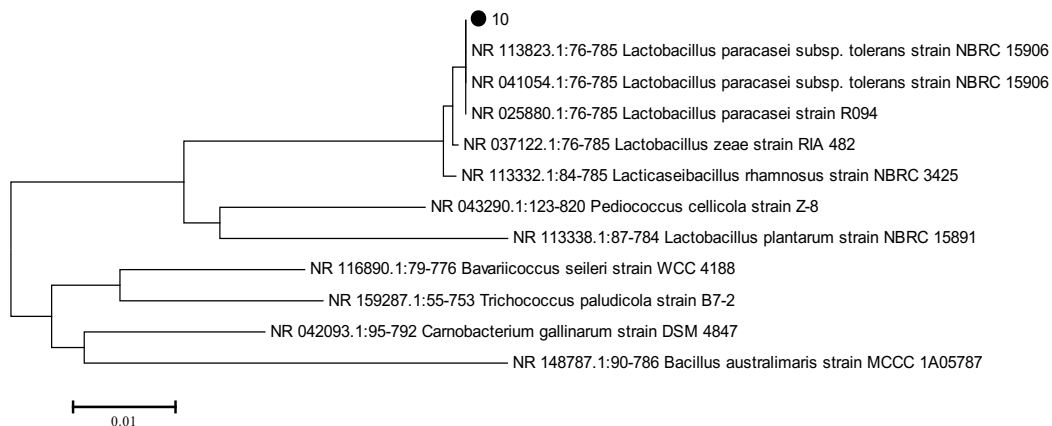


Figure 5 Phylogenetic tree based on the analysis of 16S rRNA fragment structures showing the relatedness of the lactic acid bacteria genus *Lacticaseibacillus paracasei* strains.

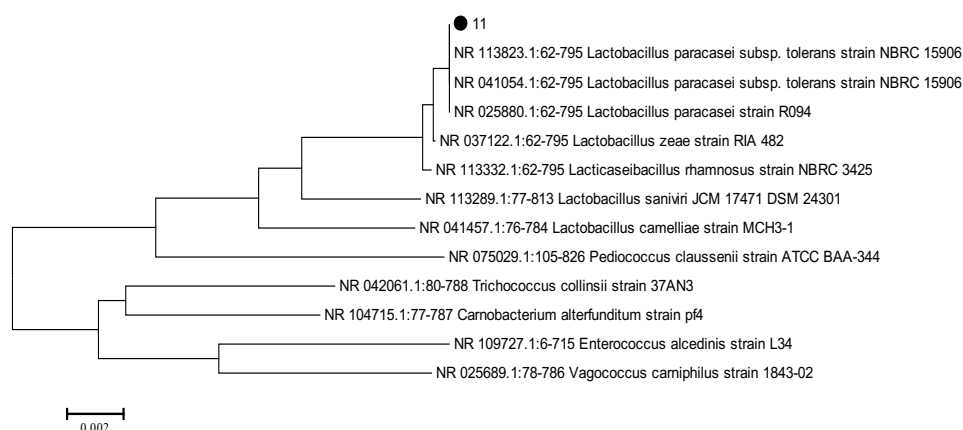


Figure 6 Phylogenetic tree based on 16S rRNA fragment structure analysis showing the relationship between strains of the genus *Lacticaseibacillus paracasei*.

CONCLUSION

The current research is the first step in studying beneficial *Lactobacillus* strains derived from Kazakhstan mare's milk and koumiss, which ensure further in-depth study. The results showed that the isolated novel LAB strains, especially *Lacticaseibacillus paracasei* 10K strain, possess several important probiotic properties such as bile and acid tolerance, antibiotic resistance and antimicrobial action. This makes it possible to recommend isolated strains of LAB as potential probiotics for applications in the food and pharmaceutical industries. However, *in vivo* studies of the quantitative and qualitative effects of strains on growth and immunity of organisms are needed.

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Funds:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgments:

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Conflict of Interest:

No potential conflict of interest was reported by the author(s).

Ethical Statement:

This article does not contain any studies that would require an ethical statement.

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