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PROBIOTIC PROPERTIES OF LACTIC ACID BACTERIA ISOLATED FROM MARE'S MILK

Annotation: *This work aims to identify and study the probiotic potential of lactic acid bacteria isolated from mare's milk. One of the main probiotic properties of the obtained strains is resistance to acidic pH media and bile acid salts, resistance to various types of antibiotics, adhesive ability and high antagonistic activity against pathogenic and opportunistic microorganisms. This article presents the study and isolation of lactic acid bacteria from mare's milk and presents the results of studying the biological properties of isolated strains and their identification. During the study, 98 isolates isolated from mare's milk samples were selected based on the biological properties of the strains. Some probiotic properties were determined using a total of 98 isolates, but the study continued with 41 strains. The most active strains that showed probiotic properties were selected from them, and the DNA model of the strains (deoxyribonucleic acid) was used in the genetic molecular diagnosis of microorganisms. When replicating the 16s gDNA zone, common bacterial primers for lactic acid bacteria were used. Based on 16s ribosomal gDNA analysis and carbohydrate profile, they were identified as Lactobacillus (L.) plantarum, L. Paracasei and L. casei.*

Key words: *mare's milk, probiotic potential, lactic acid bacteria, strains, resistance, antibiotics, adhesive ability, antagonistic activity.*

Introduction

Functional nutrition products are a kind of probiotic composition. The concentrations of nutrients present in functional nutrition products and having a regulating effect on the functions and reactions of the macroorganism are close to optimal, physiological, and therefore such products must be taken to maintain the immune system [1, 2].

After the worldwide COVID-19 pandemic, the demand for therapeutic and preventive products has increased significantly. As you know, COVID-19 is a pandemic disease that has paralyzed social life and the economy around the world since the end of 2019 from which more than 5.5 million people have died so far. COVID-19 causes respiratory diseases, including pneumonia, and also affects many other organ systems, including the body's immune and digestive systems [3,4]. Therefore, the search for natural sources for the development of functional products with immunostimulating properties is an urgent task at the current time [5].

Mare's milk is a very valuable drink that can replace cow's milk. It is known that consumption of cow's milk in many cases causes digestive tract disorders in some people, especially allergy to cow's milk mediated by immunoglobulin, is one of the most common food allergies among the population, especially in infants [6, 7].

In addition, mare's milk has biophysical and biochemical properties [9], which bring it closer to human milk than to cow's milk [10,11,12]. The similar content of total protein and the optimal ratio of casein and whey protein, which can be an important factor in determining its allergenicity and richness of essential nutrients [8,13,14], confirm the potential of using horse milk as a substitute for breast milk. This may be especially important in certain scenarios to meet the nutritional needs of people with high sensitivity to cow's milk proteins. The popularity of mare's milk as a full-fledged dairy product is increasing everywhere and is used in the therapeutic and preventive direction of nutrition, since mare's milk contains many live probiotic microorganisms useful for humans to restore the normal functioning of the digestive tract [15, 16].

Probiotic biologically active additives are a complex of living microorganisms that are used for food additives, and, as a rule, are distributed through the pharmacy network. - Alimentary probiotics are living beneficial microorganisms that enrich food products (dairy, meat, flour, beverages), which play the role of a supplement to nutrition [6, 12]. According to the modern concept of functional nutrition, food should be not only a source of basic nutrients, but also other biologically active and

necessary substances for the body, for which positive clinical efficacy has been proven and which do not cause harm with constant consumption by healthy people [17].

Lactic acid bacteria and bifidobacteria, as reported in the literature, are probiotics with beneficial properties for health [3-6, 8, 10]. According to the data of the working group of the Food and Agriculture Organization of the United Nations (UN) [13] and the World Health Organization (WHO) [16], probiotic bacteria are defined as living microorganisms that are useful when ingested in adequate quantities. In this regard, the isolation and study of the properties of probiotic microorganisms in mare's milk is an urgent task [18].

Methods and materials

The objects of research were mare milk obtained in private farms of IP "Sadygul", Zhambyl region, Zhambyl district, village of Karakemer and IP "Dzhumabaev N.N.", Almaty region, Karasay district. Milk samples were selected according to established standards. In total, 120 samples of mare's milk were studied, which were selected from two regions of Kazakhstan. The studies used standard methods of biochemical and microbiological studies, as well as genetic methods, in particular 16S-ribosomal DNA. The acceptance of fresh mare's milk was carried out in accordance with ST RK 1005-98 [ST RK 1005-98 «Mare's milk. Procurement requirements»]. Laboratory selection was carried out in the reference laboratory of dairy products at Kaznau by excluding gram-negative and catalase-positive bacterial isolates from the experiment.

Isolation of lactic acid microorganisms. Samples of mare's milk were used to isolate microorganisms. All samples were sequentially diluted in sterile phosphate buffer saline solution (1X; pH 7.0; PBS), seeded with MRS-agar and M17-lactose agar for the isolation of lactobacilli and lactococci and pink Bengal agar for yeast. Cups with MRS-agar and M17-lactose agar were incubated at 37°C for 48 hours, while yeast cultures were incubated at 30 °C for 48 hours. Colonies were randomly selected and then purified. Staining of isolates (simple staining for yeast, gram staining for lactobacilli) of bacteria was analyzed for catalase activity using 2% (i/v) hydrogen peroxide. The isolated isolates were stored in an appropriate broth with a 15% glycerin content at -20°C for further testing.

The resistance of pure crops to acidity and bile acid salts. Inoculation was performed on an agar nutrient medium from active cultures by the method of bar plates (bar layout). The agar cups were kept at a temperature of 30° C for yeast and at a temperature of 37° C for lactobacilli and lacto cocci for 16-18 hours. After that, they were suspended in a sterile PBS solution. McFarland was set to 0.5 for yeast and 1.0 for lactic acid bacteria. Each isolate, prepared accordingly, was inoculated into a broth brought to pH 2.0, 4.0 and 7.0. After that, they were incubated at 30° C (yeast) and 37° C (lactic acid bacteria) for 24 hours. At the beginning of incubation and after 3 and 24 hours, samples of selected yeast and bacterial strains were taken aseptically and sequentially diluted in sterile PBS. Then their number was determined by counting plates. Resistance to bile acid salts was determined by inoculation of microorganisms carried out on an agar culture medium containing 0.3% bile (Tryptone Bile Salts Agar,) as described above.

Tests for antibiotic sensitivity. Tests for the sensitivity of bacteria to antibiotics were determined by the method of disk diffusion. Accordingly, strains stored at -80°C were activated twice in the appropriate broth with incubation duration of 16 and 18 hours at appropriate temperatures. The concentrations of these cultures were adjusted according to McFarland to 1.0. Petri dishes with a diameter of 9 cm were used for susceptibility tests, and agar medium was poured into Petri dishes to a depth of 4 mm. The suspension of microorganisms was evenly distributed over the surface of the medium with a sterile swab. Discs with antibiotics (enrofloxacin 5 mcg/disc, erythromycin 15 mcg/disc, gentamicin 10 mcg/disc, penicillin G10 IU/disc, tetracycline 30 mcg/disc, vancomycin 30 mcg/disc, trimethoprim-sulfamethoxazole 25 mcg/disc,) were placed on the inoculated bacteria medium at a distance of 10 mm from the edge of the Petri dish and 15 mm apart. The diameters of the inhibition zones around the discs were measured after 16-20 hours of incubation at an appropriate temperature. The sensitivity of the strains to antibiotics was determined by the obtained diameters of the inhibition zones. All inhibition zones were measured and evaluated for antibiotic sensitivity.

Adhesive properties of probiotic cultures. To determine the hydrophobic, i.e. adhesive properties of the cell surface, a test of microbial adhesion to hydrocarbons was performed. Cultures with triple activation were centrifuged for 10 minutes at 5000 rpm. After removing the supernatants,

the precipitate was washed 3 times with sterile PBS. The precipitate is dissolved in the same solution as at 600 Nm in the range of 0.4-0.6. The results obtained are accepted as Odb. The percentage of adhesive activity was calculated as follows.

Adhesion % =[(ODb-ODa)/(ODb]×100

Electrophoresis of the genomic DNA sample was performed in 35 ml of gel so that the agarosis was about 0.7% (W/v) in total. The electrophoresis process was carried out at 65 volts for 1.5-2 hours. Replication and sequence analysis of isolates using polymerase chain reaction (PCR) of the 16s rrna zone was performed on a Techne TC3000 heat exchanger using a total PCR mixture of 50 µl. During replication of the 16s region of gDNA, common bacterial primers pA (forward) 5'-AGA GTT TGA TCC TGG CTC AG-3' and pE' (reverse) 5'-CCG TCA ATT CCT TTG AGT TT-3' (primer sequence obtained by Edwards et al.1989) were used. The electrophoresis of 16s rDNA PCR fragments is made from a gel prepared with an agarose ratio of 1% on Thermo Minicell®Primo EC320, and the fragment size is calculated using the DNA marker O'generulertm 100-bp.

The statistical analysis was carried out using the Minitab 17 (ANOVA) program. Differences between the groups in which the study was conducted were revealed by the Turkey test P<0.05. The studied samples were conducted in parallel with the average and the probability of deviation from the standard.

Results and discussion

In this study, a total of 98 isolates were isolated from mare's milk and the probiotic potential was determined. Laboratory selection was carried out in the reference laboratory of dairy products at Kaznau by excluding gram-negative and catalase-positive bacterial isolates from the experiment. In addition, since some strains lost their viability during the purification and storage stages, the study continued using 12 yeasts, 16 laboratory cocci and 13 lactobacilli; a total of 41 isolates.

Some using a total of 98 isolates. Most isolates were negatively affected by pH 2.0; however, they retained their viability at pH 4.0 and 0.3% bile salt. Most of the laboratory isolates were sensitive to penicillin and tetracycline.

Resistance to acidic conditions of microorganisms was studied at three different pH values (pH 2.0, 4.0 and 7.0). Most isolates showed sensitivity to pH 2.0, but all microorganisms studied at pH 4.0 remained viable. While the absorption capacity of 8 yeast isolates increased at pH 2.0, the absorption capacity of 4 isolates remained the same after 3 and 24 hours. Accordingly, 11 laboratory cocci increased at pH 2.0, while 5 remained unchanged. Indicators of the absorption capacity of lactobacilli have retained their initial values. All isolates increased at pH 7.0. The results of the conducted counting of the selected microorganisms showed similar trends.

The tolerance properties of the same microorganisms to bile salt were also determined. The optical density values of all yeasts and lactococci increased in the medium containing bile salts after 3 and 24 hours, but no changes were observed in lactobacilli. The results of resistance to acidic conditions of microorganisms are shown in Fig. 1 and 2.

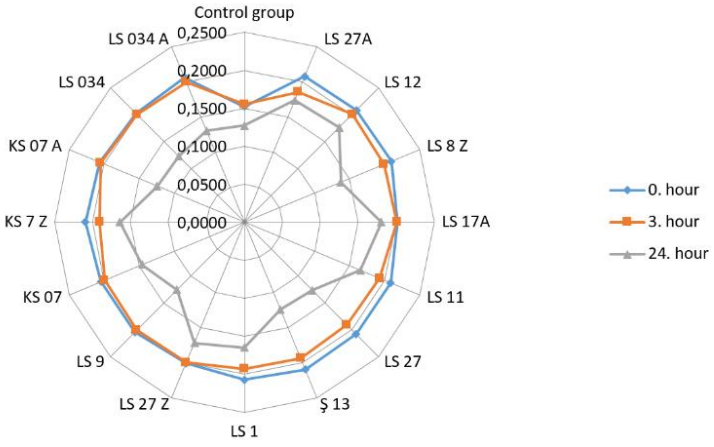

Figure 1 – Results of resistance to acidic conditions of pH 2 microorganisms

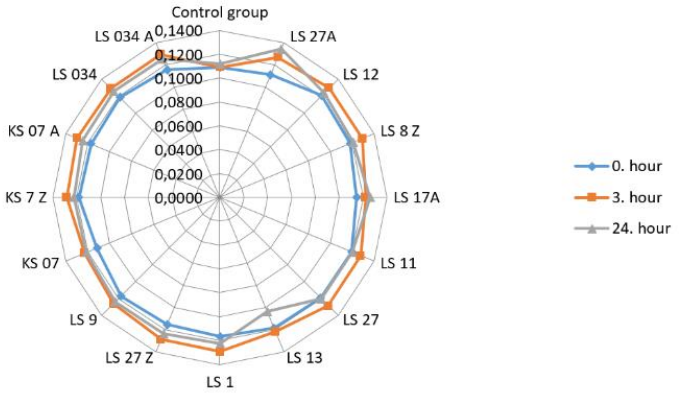

Figure 2 – Results of resistance to acidic conditions of pH 4 microorganisms

Sensitivity of isolates to antibiotics. To determine their sensitivity to antibiotics, 16 LAB lactococci and 13 lactobacilli were studied. Most laboratory cocci strains were sensitive to penicillin, tetracycline and vancomycin, but resistant to trimethoprim-sulfamethoxazole and enrofloxacin. All lactobacillus strains (100%) were sensitive to penicillin, but resistant to gentamicin. Most strains were sensitive to tetracycline, but resistant to vancomycin. The antibiotic sensitivity of selected laboratory isolates by their sensitivity status is shown in Table 1.

Table 1 – Sensitivity of isolates to antibiotics

Antimicrobial Agent	Disk Content	Strain						
		KS 07	LS 1	KS 2	LS 012	KS 13	LS 08	LS 034
Penicillin G (P10)	10 units	41-41	39-37.5	40-41.5	34.5-32	27.5-29	38.5-39.5	37-38.5
Gentamicin (CN 10)	10 μg	13-15	-	-	11-12	10-9.5	15.5-13.5	15-12.5
Vancomycin (VA 30)	30 μg	-	28-26.5	-	20-21	21.5-22.5	26-24.5	-
Enrofloxacin (ENR 5)	5 μg	15.5-16	14-12	25-26	17.5-16	21.5-23	22.5-21.5	18.5-19
Erythromycin (E 15)	15 μg	33.5-34	14-13	39-41	30.5-30.5	26.5-26.5	37.5-35	33-32.5
Tetracycline (TE 30)	30 μg	36.5-34.5	30-33	39-37	29-31	33-33	37.5-35	31-32.5
Trimethoprim (SXT 25)	25 μg /	-	-	-	23-25	-	28-29	11-11.5

*Note: *TE 30 – Tetracycline (TE 30, Contents dose in disk 30 μg); E 15 – Erythromycin (E 15, Contents dose in disk 15 μg); ENR 5 – Enrofloxacin (ENR 5; Contents dose in disk 5 μg); P 10 – Penicillin G (P10; Contents dose in disk 10 units); SXT 25 – Trimethoprim (SXT 25; Contents dose in disk 25 μg); CN 10 – Gentamicin (CN 10; Contents dose in disk 10 μg); VA 30 – Vancomycin (VA 30; Contents dose in disk 30 μg).*

The most important characteristic feature of probiotic bacteria is their adhesive ability. As a result of the study, it was found that the vast majority of lactococcal and yeast strains have high adhesive activity. However, Lactobacilli showed very high adhesive activity (71%). It was found that 8% of lactococcal strains and 2% of yeast strains exhibit high adhesive activity. The adhesive activity of the selected laboratory isolates is shown in Table 2.

Common bacterial primers pA (direct) 5'-AGAGTTTGATCCTGGCTCAG-3' and pE' (reverse) were used for amplification of the 16s rDNA region 5'- CCG TCA ATT CCT TTG AGT TT-3'. As a result of gel electrophoresis of a genomic DNA sample, concentrations of probiotic strains showed 900-1000 base pairs. In the electrophoresis buffer Thermo OWL EASYCAST B2 (USA) was painted with marker paint prepared with the addition of bromophenol blue, the results of the staining process were photographed using the UviTec DBT-08 gel imaging system under the ultraviolet light of the gel Fig. 3.

Table 2 – Adhesive activity of selected isolates

Strain	Hydrophobicity of lactobacillus in 600 nm		Hydrophobicity of lactobacillus was read with Toluene in 600 nm		calculating with the formula
Number of parallels	1	2	1	2	
LS 03	0.597	0.594	0.319	0.408	38.95886
LS 07A	0.404	0.402	0.049	0.080	83.99504
KS 6	0.560	0.559	0.100	0.063	85.43342
LS 27	0.548	0.551	0.172	0.135	72.06551
LS 027A	0.566	0.565	0.076	0.113	83.28912
LS 27 Z	0.548	0.549	0.113	0.121	78.6691
KS 12	0.588	0.586	0.468	0.448	21.97615
LS 13	0.465	0.467	0.121	0.118	74.35622
LS 034	0.564	0.562	0.067	0.091	85.96803
LS 034 Z	0.543	0.541	0.401	0.397	26.38376
LS 14	0.562	0.563	0.563	0.560	0.177778
LS 07	0.404	-	0.205	-	49.25743
LS 7 Z	0.431	0.430	0.125	0.110	72.70616
LS 17 Z	0.556	0.558	0.293	0.316	45.33214

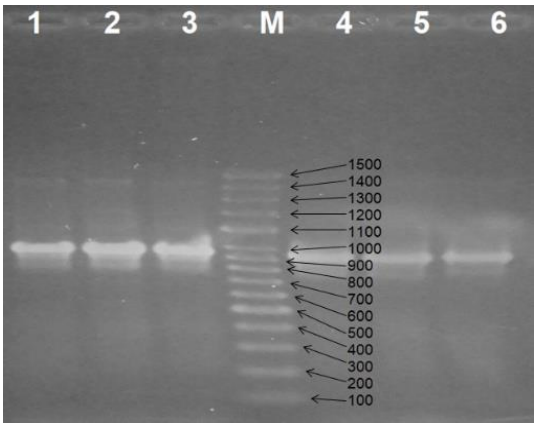

Figure 3 – The result of gel electrophoresis of a sample of the DNA genome of probiotic strains

As a result of the conducted PCR DNA sequence analysis studies, it was revealed that the dominant species of lactic acid bacteria in mare's milk are Lactobacillus (L.) plantarum, Lactobacillus casei and Lactobacillus paracasei. It was found that 41 lactic acid bacteria isolated from mare's milk of 6 strains have probiotic activity, identified as Lactobacillus (L.) plantarum, Lactobacillus casei and Lactobacillus paracasei ssp.

Conclusion

As a result of the conducted studies of the microflora of mare's milk, selected from 2 regions of Kazakhstan, it was possible to identify and identify 6 homofermentative probiotic strains of lactic acid bacteria. They have been identified and classified as Lactobacillus (L.) plantarum, Lactobacillus casei and Lactobacillus paracasei spp. and which are tolerant to bile salts and gastric juice and antibiotics. Thus, isolated and identified strains of lactic acid bacteria with probiotic properties can be recommended for inclusion in the collection of microorganisms in Kazakhstan and the creation of starter cultures used in the production of functional fermented milk products.

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The authors declare that there is no conflict of interest.

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БИЕ СҮТІНЕН ОҚШАУЛАНҒАН СҮТ ҚЫШҚЫЛДЫ БАКТЕРИЯЛАРЫНЫҢ ПРОБИОТИКАЛЫҚ ҚАСИЕТТЕРІ

Бұл жұмыстың мақсаты-бие сүтінен оқшауланған сүт қышқылды бактерияларының пробиотикалық әлеуетін анықтау және зерттеу. Алынған штамдардың негізгі пробиотикалық қасиеттерінің бірі-қышқыл pH орталарына және өт тұздарына төзімділік, антибиотиктердің әртүрлі түрлеріне төзімділік, адгезия қабілеті және патогендік және оппортунистік микроорганизмдерге қатысты жоғары антагонистік белсенділік. Бұл мақалада бие сүтінен сүт қышқылды бактерияларын зерттеу және оқшаулау ұсынылған және оқшауланған штамдардың биологиялық қасиеттерін зерттеу және оларды анықтау нәтижелері келтірілген. Зерттеу барысында штаммдардың биологиялық қасиеттері негізінде бие сүтінің үлгілерінен оқшауланған 98 изолят таңдалды. Кейбір пробиотикалық қасиеттер барлығы 98 изолятты қолдану арқылы анықталды, бірақ зерттеу 41 штамммен жалғасты. Олардың ішінен пробиотикалық қасиеттері бар ең белсенді штамдар таңдалды және микроорганизмдердің генетикалық молекулалық диагностикасында штаммдардың ДНҚ моделі (дезоксирибонуклеин қышқылы) қолданылды. гДНҚ-ның 16s аймағын репликациялау кезінде сүт қышқылы бактерияларына арналған жалпы бактериялық праймерлер қолданылды. Талдау мен көмірсулар профилінің 16s рибосомалық гДНҚ негізінде олар Lactobacillus (L.) plantarum, L. Paracasei және L. casei ретінде анықталды.

Түйін сөздер: *бие сүті, пробиотикалық потенциал, сүт қышқылды бактериялар, штамдар, төзімділік, антибиотиктер, адгезиялық қабілет, антагонистік белсенділік.*

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ПРОБИОТИЧЕСКИЕ СВОЙСТВА МОЛОЧНОКИСЛЫХ БАКТЕРИЙ, ВЫДЕЛЕННЫХ ИЗ КОБЫЛЬЕГО МОЛОКА

Цель данной работы является идентификация и изучение пробиотического потенциала молочнокислых бактерий, выделенных из кобыльего молока. Одним из основных пробиотических свойств полученных штаммов является устойчивость к кислым средам pH и солям желчных кислот, устойчивость к разным видам антибиотиков, адгезивная способность и высокая антагонистическая активность по отношению к патогенным и условно-патогенным микроорганизмам. В данной статье представлено изучение и выделение молочнокислых бактерий из кобыльего молока и представлены результаты изучения биологических свойств выделенных штаммов и их идентификации. В ходе исследования было отобрано 98 изоляты, выделенных из образцов кобыльего молока на основе биологических свойств штаммов. Некоторые пробиотические свойства были определены с использованием в общей сложности 98 изолятов, но исследование продолжалось с 41 штаммами. Из них были отобраны самые активные штаммы, проявившие пробиотические свойства и в генетической молекулярной диагностике микроорганизмов, использовалась модель ДНК штаммов (дезоксирибонуклеиновая кислота). При репликации зоны 16s rДНК использовались общие бактериальные праймеры для молочнокислых бактерий. На основе 16s рибосомальной rДНК анализа и углеводного профиля их идентифицировали как Lactobacillus (L.) plantarum, L. Paracasei и L. casei.

Ключевые слова: *кобылье молоко, пробиотический потенциал, молочнокислые бактерии, штаммы, резистентность, антибиотики, адгезивная способность, антагонистическая активность.*

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