OnLine Journal of Biological Sciences

Original Research Paper

Fermentation Conditions of Lactobacilli for the Production of Lactose-Free Starter Culture

[1,2,3]Madina K. Imanbayeva, [4]Zhaksylyk K. Masalimov, [5]Alexander Y. Prosekov, [5]Irina S. Milentyeva and [4]Utemurat Z. Sagyndykov

[1]*Department of General Biology and Genomics, L.N. Gumilyov Eurasian National University, Astana, Kazakhstan*
[2]*Department of Medicine, Astana Medical University, Astana, Kazakhstan*
[3]*School of Sciences and Humanities, Nazarbayev University, Astana, Kazakhstan*
[4]*Department of Biotechnology and Microbiology, L.N. Gumilyov Eurasian National University, Astana, Kazakhstan*
[5]*Department of Bionanotechnology, Kemerovo State University, Kemerovo, Russia*

Article history
Received: 11-07-2023
Revised: 02-01-2024
Accepted: 11-01-2024

Corresponding Author:
Madina K. Imanbayeva
Department of General Biology and Genomics, L.N. Gumilyov Eurasian National University, Astana, Kazakhstan
Email: imanbaeva_madina@inbox.ru

Abstract: This research aims to determine the fermentation conditions and viability in a medium simulating the gastrointestinal tract and to examine the antagonistic potential of *Lactobacillus acidophilus* (*Lb. acidophilus)* and *Lactobacillus rhamnosus* (*Lb. rhamnosus)* in producing a lactose-free starter. Our study examined Lactic Acid Bacteria (LAB). The sources of *Lb. acidophilus* strains are camel milk (shubat), *Lb. rhamnosus* mare milk (koumiss), traditional homemade dairy foods in Kazakhstan. *Lb. acidophilus* and *Lb. rhamnosus* are resistant to gastric juice acidity at a pH above 3, therefore they will be able to pass through the stomach intact. The cell populations remained $\geq 10^6$ CFU mL. When *Lb. acidophilus* was treated at pH 3.0-5.0, the cell population was significantly higher ($p \leq 0.05$). Simulated gastrointestinal transit was studied by assessing NaCl resistance. The cell counts in the medium with 5% NaCl were significantly higher ($p \leq 0.05$) than those in the medium with 2% NaCL. *Lb. acidophilus* and *Lb. rhamnosus* showed growth corresponding to ~7 log CFU at 5-7% NaCl. *Lb. acidophilus* and *Lb. rhamnosus* were tested for their antibacterial activity by agar-well diffusion test. Three common pathogens, *E. coli, S. marcescens,* and *S. Typhimurium* were used for the investigation of antagonistic activity. *Lb. acidophilus* and *Lb. rhamnosus* showed significantly high inhibition zone ranges ($p \leq 0.05$) from 10-12 mm against *E. coli* and *S. marcescens*. A medium zone of inhibition 5.0-6.0 mm against *S. typhimurium.* For co-fermentation, pasteurized milk inoculated with *Lb. acidophilus* and *Lb. rhamnosus* cells were fractionated into 25 mL portions and placed in 50 mL conical tubes. It was spread on a nutrient medium of Petri dishes and incubated for 24 h at 37°C. *Lb. acidophilus* and *Lb. rhamnosus* starter culture reached a significant cell population of about ~8 logs Colony Forming Unit (CFU/mL) during co-culture fermentation, pH in Pasteurized Milk (PM) rapidly decreased to 3.0.

Keywords: Lactobacilli, Probiotic, Starter Culture

Introduction

The aim of this study was to determine the viability of lactobacilli in a gastrointestinal tract simulated environment and to investigate the antagonistic potential of probiotic starter bacteria *Lb. acidophilus and Lb. rhamnosus*. To determine a suitable fermentation period for mixed cultures of *Lb. acidophilus* and *Lb. rhamnosus* to achieve low pH values in pasteurized milk.

The original contribution of the study is the use of a probiotic product containing domestic microorganisms will correct dysbiosis processes that have arisen against the background of the underlying disease or milk sugar deficiency. The use of autological lactobacilli preparations is comparable in effectiveness to the use of preparations of commercial lactobacilli strains.

Low-lactose and lactose-free dairy products are classified as functional foods, which provide adequate

nutrition for people with lactase deficiency. Their functionality is also enhanced by the probiotic potential of microbial cultures. Strains of LAB isolated from traditional homemade dairy foods in Kazakhstan; from solid fermented dairy products (Kurt, irimshik), liquid cow's milk (ayran, homemade butter), mare's milk (koumiss), and camel's milk (shubat).

The obtained research data on the study of microorganism cultures with probiotic potential are scientifically substantiated and expand the understanding of biological and technological properties of lactic acid bacteria cultures, allowing for the optimization of methodological approaches in the design of starter. On the basis of a comparative evaluation of biological and technological properties of microorganisms bacterial compositions based on *Lb. acidophilus* and *Lb. rhamnosus* were produced and a starter was developed.

The significance of probiotic cultures and their beneficial effects in dairy products as mass consumption products, the use of which aims to best fulfill the needs of special dietary products. Fermentation of yogurt starter based on probiotic cultures, better metabolized: Complete utilization of lactose and efficient degradation of galactose. Also, today the prescription of infant formulae supplemented with probiotic strains has become more frequent (Zhang *et al*., 2021). Ice cream is the most favorable for the inclusion of probiotic strains since the viability of probiotics during production and storage is higher compared to other dairy products (Mohammadi *et al*., 2011). However, the study shows that fermentation of ice cream blended with probiotics is recommended as a preferred method over including probiotics in the ice cream mix without further fermentation (Gao *et al*., 2021). The use of probiotic strains is not limited to fermented milk products, yogurt, and cheese, but there are a number of other products that contain probiotics. For example, butter. There is some data showing that high levels of saturated fatty acids in butter are linked to many cardiovascular diseases and diabetes. Probiotic bacteria decrease cholesterol levels (Pimpin *et al*., 2016).

The experiments conducted by us in the framework of our project, aimed at the implementation of the program "strategy Kazakhstan 2050" from 1.09.23 of the message of the president of the Republic of Kazakhstan, will allow the agrarian sector of the Republic of Kazakhstan to achieve food and environmental sustainability. The laboratory of microbiology and biotechnology of the Astana branch of "Kazakh Research Institute of Processing and Food Industry" LLP has now created a laboratorian starter for manufacturing lactose-free lactic acid products. The distinction from foreign analogs is that the preparation is based on microorganisms isolated in the territory of the Republic of Kazakhstan. This fact has a certain economic effect, as it allows to save costs on the import and transportation of expensive inoculants. After completing all the research experiments in the implemented project, the economic efficiency of the use of a domestic starter will be calculated.

Scientists from higher educational universities of the Republic of Kazakhstan and research institutes are actively studying and using starter cultures to produce preventive and medicinal dairy products.

In the presented study we identified the predominant species of lactic acid bacteria isolated from Kazakh national dairy foods were strains of the genus *Lactobacillus acidophilus, rhamnosus, plantarum, fermentum, paracasei,* and *bulgaricum*. Similar bacterial strains were identified in the teamwork of scientists of Buketov Karaganda University, in co-operation with scientists of the Medical University of Karaganda and Nazarbayev University of Astana. They also applied methods to study the biological and probiotic properties of isolated bacterial strains (adhesion to erythrocytes, antibacterial activity against indicator microorganisms, and analysis of morpho-cultural properties of isolates) (Amirkhanova *et al*., 2021).

Moreover, one of the works of Kazakhstani scientists in the field of microbiological diversity of shubat and ayran was conducted with the support of the JSC "center for international programs" and international scholarship "Bolashak" of the ministry of education and science of the Republic of Kazakhstan. As a result of research work, acid resistance, tolerance to bile acid salts, and antibiotic resistance of isolated strains of microorganisms were analyzed (Zhadyra *et al*., 2021).

A recent study published by Kazakhstani scientists on "Effects of low lactose mare's milk yogurt consumption on gut microbiota function" was funded under the scientific program of the ministry of education and science of the Republic of Kazakhstan "development of technology using new strains of beneficial microorganisms, enzymes, nutrients, and other complexes in the production of special dietary foods" INN BR10764998. National scientists studied the effect of low-lactose yogurt on the intestinal microbiota of 15 male and female mongrel rats suffering from lactose intolerance. The laboratory rats had a normal diet for 7 days, but for the following 4 weeks, probiotic yogurt (low lactose yogurt) was included in the diet. Fecal samples of the animals before and after the diet (first collection) were examined. A control collection of stool samples was examined on day 28. The microbial community was then determined and quantified using the 16S rRNA method which allowed to carry out genetic identification of microorganisms. The resulting starter is based on lactobacillus, Bifidobacterium, and Streptococcus strains. Sour milk yogurt made from a mixture of mare and cow milk with a low lactose content of 0.002% was used as substrate. This product was fermented for 8 h, at 38°C. As a result, the low lactose sour milk product had a

characteristic sour milk flavor and a clot of 1100 T. So eventually consumption of low lactose fermented milk products resulted in a change in microbial biodiversity where an increase in the microbial population of *Heliobacteraceae, Eubacterium, Muribaculaceae, Lactobacillaceae* and others was observed. Lactose-free yogurt contributed to the decrease in the population of *Ruminococcaceae, Peptostreptococcaceae*. Thus, the developed starter was aimed at correcting intestinal dysbacteriosis in laboratory rats suffering from lactase deficiency (Samat *et al*., 2022).

Based on the literature analysis, our group of scientists studied the fermentation conditions of lactobacilli isolated from domestic raw materials to produce lactose-free starters under laboratory conditions. The research we conducted does not require ethical approval as the research has not been conducted on animals or humans. At this stage, the members of the research group have developed a laboratory starter. We are working on writing an application to the ministry of education and science of the Republic of Kazakhstan for financial assistance to continue the current study.

Lactose malabsorption is a common condition worldwide characterized by decreased lactase activity. Lactose is a milk sugar that is hydrolyzed by the enzyme lactase into the monosaccharide's glucose and galactose (Di Stefano *et al*., 2002; Suchy *et al*., 2010; Scrimshaw and Murray, 1988).

Symptoms of lactose intolerance are a chronic gastrointestinal disorder characterized by pain, diarrhea, abdominal bloating, weight loss, flatulence, and abdominal cramps (Krieger-Grübel *et al*., 2020; Ratajczak *et al*., 2021). Lactose intolerance affects about 75% of the world's population and can be associated with various genetic factors. The global prevalence of lactose intolerance is 2-5% in Northern Europe (Scandinavia, Germany, UK), 17% in Finland and Northern France, and about 50% in South America and Africa. In North American adults (79% of Native Americans, 75% of African Americans, 51% of Hispanics and 21% of Caucasians). 90-100% in Southeast Asia, including the Republic of Kazakhstan (Heine *et al*., 2017). In addition, many studies have shown that avoiding milk and dairy products can increase the risk of bone fractures, osteoporosis, and nutrient deficiencies. However, dairy products are one of the main sources of calcium, potassium, vitamin D, B vitamins, and high-quality protein (Leis *et al*., 2020).

Shokryazdan *et al*. (2017) state that bacteria, yeasts, and fungi can be used as probiotics. In particular, IBCs can be selected as probiotic cultures. According to other researchers, starter bacteria (*Lb. acidophilus, Lb. plantarum, Lb. rhamnosus, Bifidobacterium breve, B. lactis, B. longum, and Streptococcus thermophiles*) induce B-galactosidase activity which naturally reduces lactose content, making the product suitable for people with lactose intolerance (Hemarajata and Versalovic, 2013; Ahmed et al., 2013). Probiotic products should contain live microorganisms. A combination of different populations of microorganisms related to lactobacilli species have traditionally been used as probiotics in fermented milk products (De Vrese and Schrezenmeir, 2008).

Innocente *et al*. (2016) reported that probiotic fermented foods should contain at least 10^7 CFU/g of live bacteria. Probiotics have now been shown to improve lactose metabolism. The criteria for selecting probiotics are to reduce pH, and lactose content and increase lactic acid concentration. They should have biological activity against opportunistic microorganisms and high viability to colonize the gastrointestinal tract (Lim *et al*., 2015).

In the 1970s the idea of probiotics was first proposed by Russian Nobel Prize winner Ilya Mechnikov, known for his book life extension. The term "probiotic" was first coined by W. Collat as "a microbial population favorable to the intestinal microflora". Fuller then listed criteria specific to probiotics. Later, a fuller proposed a definition of probiotics as "a preparation of live microorganisms consumed by humans that induces beneficial effects by qualitative or quantitative effects on the gut microflora". Floch MH, Madsen KK, and Jenkins DJ then expanded the definition to "Probiotics are human-associated microorganisms consumed either with food or as a supplement to improve health". The definition of probiotics was last updated in 2018 by the US National Institute of Health: The national centre for complementary and Integrative Health. The title was reworded as follows: "Probiotics are live microorganisms that provide health benefits when consumed, usually by improving gut flora." Products marketed as probiotics include dairy products and products that are not consumed orally, such as skin creams" (Shokryazdan *et al*., 2017; De Vrese and Schrezenmeir, 2008).

The present study describes the potential of the newly developed probiotic fermented sour milk starter. Our results are based on sourdough which was fermented with two lactic acid bacteria and isolated from Kazakhstan's homemade sour milk products kurt, irimshik, cow, mare, and camel milk. The study subjects were identified by 16rRNA genetic analysis and the construction of a phylogenetic tree. Two isolates of *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* can be practically used as probiotic cultures in sourdough production because of their beneficial acidifying capacity. The cultures studied had significant resistance to low pH and 5-7% NaCl. The studies conducted and results obtained demonstrate the ability of *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* to remain viable in gastric acid and bile salt tolerance (ph and NaCL treatment), to possess antagonistic activity against opportunistic microorganisms. The antagonistic activity of *Lactobacillus acidophilus* and *Lactobacillus*

rhamnosus demonstrated a high range of inhibition zones of 5-6 mm against all indicator bacteria: *E. coli*, *S. marcescens*, *S. Typhimurium*.

It is well recognized that live, active probiotics should be present in a ratio of 10^8-10^9 CFU/mL. However, it is currently a great challenge to maintain the viability of probiotic cultures. Despite this, *Lactobacillus acidophilus* and *Lactobacillus rhamnoses* showed high viability when co-cultured and the starter had an adequate pH.

Materials and Methods

Extraction and Counting

In June 2020, twelve samples were collected from Zhibek Zholy village located in the Arshali district of Akmola oblast. Around 100 mL of each sample was collected and placed in a sterile glass bottle. The samples were then taken to our laboratory and further isolated and microbiologically analyzed for LAB. Sixteen strains of LAB were isolated from solid fermented dairy products (Kurt, Irimshik), cow's milk (ayran, homemade butter), mare's milk (koumiss), and camel's milk (shubat).

Microorganisms were isolated in a Lamsystem laminar box. We prepared MRS agar HiMedia, nutrient medium MRS agar is sterilized at t 120°C under pressure 1A in autoclave "Steam Sterilizer VK-75-01". LAB colonies were cultured in the thermostat TS-200 SPU, produced by JSC "Smolensk SPU SKTB" at 37°C for 48 h. Cooled to room temperature 37°C, then distributed in sterile Petri dishes 15-20 mL each in three repetitions. In this method, the number of bacteria per unit volume of the sample is reduced by serial dilution before the sample is spread over the surface of the agar cup. Be sure to mix the tubes with nutrient broth before each serial transfer. Transfer 0.1 mL of the last three dilutions (10-5, 10-6, 10-7) into each of the three nutrient agar cups and label the cups.

Using a sterile dispenser "Dispenser lenpipet light 1000 mL" (manufacturer Thermo Fisher Scientific, Finland), drop the test suspension into the Petri dish, distributing the cell suspension over the surface of the petri dish. Evenly spread 0.1 mL samples of cow's milk (ayran, homemade butter), mare's milk (koumiss), and camel's milk (shubat) and crushed irimshik and kurt in a porcelain mortar over the entire surface of one of the nutrient media. Agar dishes until the medium no longer looks wet. Sowings were done with different pipettes (tips). The pipette is held strictly vertically. Droplets are not rubbed but can be slightly enlarged by slightly rocking the petri dish. The cultures are incubated and then the number of colonies grown is determined. After sowing the petri dishes are placed in the thermostat with lids downwards. Colonies of lactic acid bacteria are cultured in the thermostat TS-200 SPU produced by Smolensk JSC "SKTB SPU" at a temperature of 37°C for 48 h. Then we count the obtained lab cells.

Solid objects like "Kurt" and "irimshik" were rubbed with a porcelain mortar and pestle. The process of rubbing "Kurt" and "irimshik" with porcelain mortar and pestle was as follows. The hardness and rough surface of the porcelain mortar and pestle provide a perfect grinding surface. It is ideal for grinding hard objects such as irimshik and Kurt. The pestle and mortar should be rinsed with distilled water after each use. Grind the irimshik and Kurt to a fine powder and repeat this process until the powder is completely dissolved. We recommend that the pestle and mortar be thoroughly cleaned before first use. Wetted with physiological NaCL solution. Cycloheximide at a concentration of 0.01 was added to MRS to inhibit fungal growth.

Bacterial isolation was carried out according to Abosereh *et al*. (2016) with some modifications: Successively diluted samples (one milliliter from each sample starting from 10^7, 10^8, 10^9) were examined on MRS agar. The plates were incubated under anaerobic conditions at 37°C for 48 h. *Lactobacillus* colonies were randomly selected and numbered. This process was repeated three times to obtain well-separated purified colonies. The strains were then phenotypically examined by morphology and Gram staining.

Identification and Phylogenetic Analysis

Strains were identified using the 16S rRNA gene amplified by PCR using the direct primer 8f 5’-AgAgTTTgATCCTggCTCAg- 3 and 806R- 5’ ggACTA CCAgggTATCTAAT. PCR amplification included prolonged denaturation at 92°C for 3 min; 32 cycles: 95°C for 3 sec, 55°C for 40 sec, 72°C for 60 sec; final elongation for 10 min at 72°C. The primers we used in this study are specific to all bacteria. They are universal and designed to classify related groups of lactic acid bacteria based on sequencing results.

The PCR program was performed using a gene Amp PCR system 9700 thermal cycler (applied biosystems). PCR products were purified from unbound primers by enzymatic method using exonuclease I (ferments) and shrimp alkaline phosphatase (ferments). The sequencing reaction was performed using the big dye terminator v3.1 cycle sequencing kit (applied biosystems) according to the manufacturer's instructions, followed by fragment separation on a 3730×l DNA Analyzer (applied biosystems). The sequences obtained were identified in the gene bank using the BLAST algorithm. Mega software version 6.0 was used to generate phylogenetic trees using the Neiighbor-Joining (NJ) algorithm (Wang *et al*., 2016). This method allowed the identification of bacteria isolated from Kazakhstan home-made dairy products. Further identification of bacteria allowed the prediction of phylogenetic relationships.

Antimicrobial Activity

The antimicrobial activity of *Lb. acidophilus* and *Lb. rhamnosus* was assessed by agar diffusion methods (prick method) as described by Reuben *et al*. (2019). By means of a sterile pipette, a number of small drops of diluted culture medium are dispensed onto a sterile glass substrate. Subsequently, each drop is observed under a microscope. This drop is extracted with a sterile capillary pipette to fresh medium. The individual microorganism present in the drop starts multiplying to yield a pure culture. The indicator strains used in this study were *S. Typhimurium, S. marcescens*, and *E. coli.* The inhibitor tests were carried out in three replicates.

Lactobacilli species were inoculated into MRS broth at 37°C for 24 h under anaerobic conditions. One hundred microliters of 10^7 CFU/mL target strains were distributed on MRS agar and left to set in the refrigerator for 4 h to avoid premature growth of the strain. The dishes were incubated at 37°C for 24 h. The antagonistic activity of the probiotic strains was evaluated by the diameter (mm) of the growth inhibition zone (Ratajczak *et al*., 2021; Heine *et al*., 2017). The high inhibition zone was 10-5.5 mm, the medium inhibition zone was 5-8.9 mm and the low inhibition zone was 1-4.9 mm.

pH Treatment and Viability

The pH tolerance was tested according to the methodology described by (Lopes *et al*., 2020). To one milliliter of probiotic strains, 9 mL of sterile distilled water was added to obtain the initial dilution. Serial dilutions were made separately for *Lb. acidophilus* and *Lb. rhamnosus*. The initial pH values of the MRS medium were adjusted to 3.0, 4.0, 5.0, 6.4, and 8.5 using 1 mol/L HCl and 1 mol/L NaOH. PH was measured on a Mettler Toledo SevanCompact instrument. The tested probiotic cultures were incubated at 37 °C for 48 h, 72 h and 120 h. All cell concentrations were presented as log CFU/mL. The number of CFU was calculated using Eq. 1:

$$M = \frac{a * 10n}{V} \quad (1)$$

where, Eq.1:

M = The number of cells in 1 mL
A = The average number of colonies that grew from dilution
V = The volume of the suspension ml
10^n = The dilution

The use of this method makes it possible to assess the resistance of isolated cultures of microorganisms to samples simulating transit through the gastrointestinal tract. Entering the organism, probiotic microorganisms are exposed to various stress factors, so an important selection criterion is their survival and successful passage through the upper digestive tract. Resistance to bile is one of the most important properties of microorganisms introduced in probiotics. In the small intestine, probiotics are exposed to bile acids and pancreatic enzymes.

NaCL Treatment and Viability

The ability of LAB to survive under different salt concentrations was studied in MRS broth and agar. The tolerance to NaCL was determined according to methods by Do Amaral Santos *et al*. (2014). One milliliter of samples was mixed with 9 mL of distilled water to take an initial dilution. Serial dilutions were made separately for *Lb. acidophilus* and *Lb. rhamnosus*. Thus, 1 mL suitable dilution *Lb. acidophilus* and *Lb. rhamnosus* were mixed with the MRS broth with different concentrations of NaCl (2, 5, 7%) and inoculated at 37°C for 48, 72, and 120 h. Viable counts of probiotic bacteria were obtained by spreading 1 mL of the dilution mixed with the melted MRS agar. The MRS plates containing 0.1% (w/v) cysteine-HCl allowed differential count and the colonies presented of color white. Plates were incubated under anaerobic conditions at 37°C for 48 h. Then colonies enumeration was expressed as log CFU/mL.

The number of CFU was calculated by the Eq. 2:

$$M = \frac{a * 10n}{V} \quad (2)$$

where, Eq.2:

M = The number of cells in 1 mL
A = The average number of colonies that grew from dilution
V = The volume of the suspension mL
10^n = The dilution

Strains are resistant to the secretory fluids of the gastrointestinal tract, which is one of the main indicators of probiotic quality, due to the fact that most lactobacilli are inactivated when exposed to bile and low gastric pH. Probiotics must survive passing through the low pH of gastric juice and bile acids in order to reach the intestinal tract, colonize the host epithelium, and exert beneficial effects. The obtained results of resistance of isolates to stress factors indicate the ability of cultures promising as inoculums to survive under unfavorable conditions in the upper gastrointestinal tract.

Statistical Analysis

Significant differences of means ($p \leq 0.05$) were compared through independent Student's t-test by using SPSS 23 (IBM Corp., Armonk, New York, USA). Statistical analyses were carried out using Microsoft Excel 2010.

Independent Student's t-test was used to test the statistical significance between the mean values of microbial growth during the study of resistance of isolated microbial cultures to samples simulating gastrointestinal transit (pH, NaCl). Cell count data were obtained from three independent experiments. $p<0.05$ was considered to indicate a significant difference. Independent sampling showed an equal variance of 0.000. The confidence interval showed a plausible difference between the obtained values of microbial growth.

In our study, $p<0.05$ rejects the null hypothesis which argues that there is a statistically considerable difference in the growth of microorganisms simulating gastrointestinal transit.

Results

Extraction and Counting

The isolated IBC strains were identified using 16S rRNA. These 16S rRNA sequences were collated to construct a phylogenetic tree and phylogenetic position of the strains studied. The 16S rRNA gene sequence (about 1,400 bp) was determined and examined using the NCBI BLAST software (http://www.ncbi.nlm.nih.gov) for their closest relatives/the reference strains with homology greater than or equal to 99%. The genetic identification process established that the cultures studied belong to a taxonomic group such as *Lactobacillus* (Fig. 1).

The phylogenetic trees show *Lactobacilli species* (Lb.) including (*Lb. fermentum, Lb. gorillae, Lb. durianis, Lb. agilis, Lb. equi, Lb. alimentarius, Lb. zymae, Lb. saniviri, Lb. rhamnosus, Lb.paracasei. , Lb. zeae, Lb. algidus, Lb. dextrinicus, Lb. acidophilus, Lb. equicursoris*).

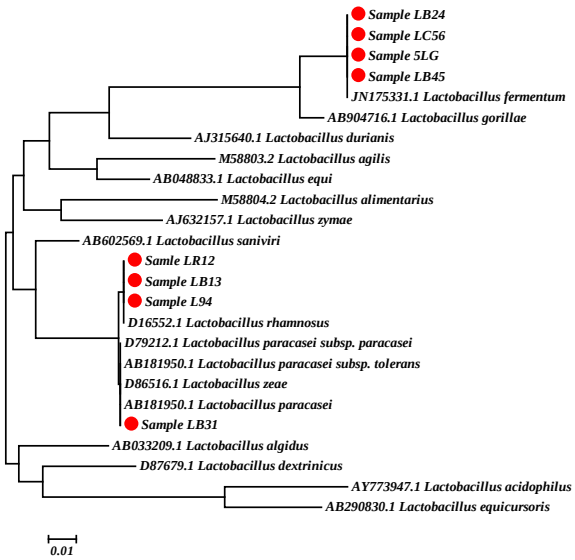

Fig. 1: Phylogenetic tree based on 16S rRNA sequences showing the position of *Lb. acidophilus* and *Lb. rhamnosus*

Table 1: Antagonistic activity of 16 strains of isolated lactic acid bacteria from traditional Kazakh home milk products

Strains	Identifications	I	II	III
Lb 1	*Lb. fermentum*	++	+	++
Lb 2	*Lb. gorillae*	+	++	++
Lb 3	*Lb. durianis*	++	+	++
Lb 4	*Lb. agilis*	+	-	-
Lb 5	*Lb. equi*	-	++	+
Lb 6	*Lb. alimentarius*	++	+	++
Lb 7	*Lb. zymae*	-	-	+
Lb 8	*Lb. saniviri*	+	+	-
Lb 9	*Lb. rhamnosus*	+++	++	+++
Lb 10	*Lb. paracasei paracasei*	++	+	+
Lb 11	*Lb. paracasei tolerans*	-	+	+
Lb 12	*Lb. zeae*	+	-	++
Lb 13	*Lb. algidus*	-	+	-
Lb 14	*Lb. dextrinicus*	++	+	+
Lb 15	*Lb. acidophilus*	+++	++	+++
Lb 16	*Lb. equicursoris*	++	-	+

+: Low zone of inhibition (1-4.9 mm), ++: Middle zone of inhibition (5-8.9 mm), +++: High zone of inhibition (10-5.5 mm), -: No inhibition. Indicators, I: *E. coli*, II: *S. typhimurium*, III: *S. marcescens*

We collected seventeen samples of bacteria from six traditional Kazakh domestic dairy products. Their colonies (2-6 mm in diameter) were oval or rounded in shape, smooth, flat-surfaced colonies with a shiny, white appearance on MRS agar. *Lactobacilli* isolates were identified by examination of colony characteristics and cell morphology.

Identification and Phylogenetic Analysis

Their probiotic properties: Antagonistic activity against pathogens, biocompatibility, high viability, and lactose utilization activity were investigated in our previous work (Arynova *et al*., 2020). Based on the results obtained, we settled on two strains*: Lb. acidophilus* and *Lb. rhamnosus.* The ability of Lb. acidophilus and *Lb. rhamnosus* to ferment lactose was investigated using the Cole ferricyanide method (Cole, 1933). The lactose content in PM with cultured *Lb. acidophilus* and *Lb. rhamnosus* was 3.1% (data not shown). In traditional Kazakh domestic dairy products, *Lb. acidophilus* and *Lb. rhamnosus* were identified as the dominant species and were present in all samples collected.

Antimicrobial Activity

The antagonistic activity of the sixteen probiotic strains was studied by inoculation on an agar medium. The antagonistic activity of MCB against the three indicator pathogens varies greatly and the results are presented in Table 1. *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* had high to medium-range inhibitory activity against all 3 indicator pathogens *S. typhimurium*, *S. marcescens*, and *E. coli*.

The high inhibition zone range was evident in *Lactobacillus acidophilus* against *E. coli* and *S. marcescens* from 10-12 mm. The average zone of inhibition against *S. Typhimurium* was 6 mm. *Lactobacillus rhamnosus* was also effective against all indicator bacteria. *Lactobacillus rhamnosus* showed a high zone of inhibition against *E. coli*, while *S. marcescens* was 10 mm. An average zone of inhibition was shown against *S. typhimurium* 5 mm.

pH Treatment and Viability

The effect of pH on the growth properties of the potential probiotic cultures *Lb. acidophilus* and *Lb. rhamnosus* was carried out as described under 'materials and methods'. One of the most significant characteristics of a lactose-free starter as a probiotic is its resistance to the acidity of gastric juice. Gastric pH ranges from pH 4.0-6.0. The ability of potential probiotic strains to survive under acidic conditions is a mandatory criterion for the production of probiotic dairy products (Abosereh *et al*., 2016).

It is assumed that a good probiotic should have the ability to withstand pH in the range of 3.5-4.5, as it is used to assess the acid resistance of the probiotic culture (Kim *et al*., 2019).

As pH is an important criterion of bacterial function and viability, we studied the effects of different pH values (3.0, 4.0, 5.0, 6.4, 8.5). The evolution of cell populations in *Lb. acidophilus* and *Lb. rhamnosus* cultivation is shown in Figs. 3-4.

Lb. acidophilus and *Lb. rhamnosus* shows good growth at pH 3.0. The cell populations remained ≥06 CFU mL during 72 h of incubation. When *Lb. acidophilus* was treated at pH 3.0, 4.0, and 5.0, the cell population was significantly higher than in the treatment group at pH 6.4, 8.5 ($p \leq 0.05$).

Figures 2-3 *Lb. acidophilus* a population of 6.38-6.52 log CFU was achieved. *Lb. rhamnosus* from 6.35-6.38 log CFU, the increase was statistically significant ($p \leq 0.05$) during fermentation from 24-72 h at pH 4.0. Rapid cell growth at pH 3.0 was observed in *Lb. acidophilus*, reaching from 6, 67-6, 72 log CFU at 72 h of fermentation ($p \leq 0, 05$). However, at pH 4.0 from 48-72 h and pH 5.0 from 48-72 h, the cell population decreased slightly but was not statistically significant ($p \geq 0.05$).

Lb. acidophilus and *Lb. rhamnosus* did not grow at a pH above 5.0. The number of viable cells decreases rapidly in the pH range of 6.4-8.5. At the end of the study, according to the initial counts Lb. acidophilus decreased by~1.4 log CFU, *Lb. rhamnosus*~1.6 log CFU.

NaCL Treatment and Viability

In order to select possible probiotic candidates, we investigate their tolerance to different salt concentrations.

The salt tolerance of probiotic strains is one of the important technological properties in the production of probiotic dairy products. Osmotic stress can cause pronounced inhibition of bacterial growth. High concentrations of NaCL cause a decrease in the adhesion capacity of the functional groups of lactobacilli and increase cell membrane damage (Ma *et al*., 2020).

In this study, *Lb. acidophilus* and *Lb. rhamnosus* were studied and their ability to grow at different concentrations of salts (2, 5, 7% NaCL). The results of cell growth are shown in Figs. 4-5. *Lb. acidophilus* and *Lb. rhamnosus* showed a slight increase in cell population at 24, 48, and 72 h in 2% NaCL, which were not statistically different ($p \geq 0.05$). The cell counts in the medium with 5% NaCL was significantly higher than those in the medium with 2% NaCL ($p \leq 0.05$). The tested strains showed the best survival rates at 5 and 7% NaCl. *Lb. acidophilus* and *Lb. rhamnosus* showed growth corresponding to ~7 log CFU at 5-7% NaCl for 72 h

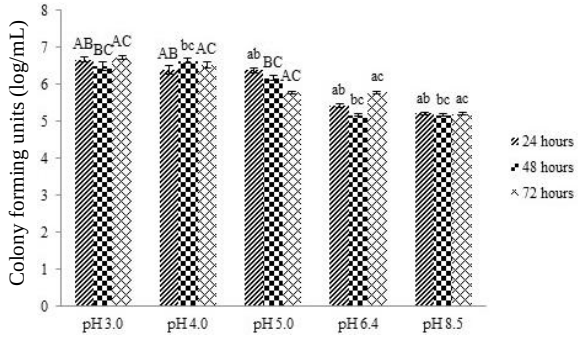

Fig. 2: Changes in the number of viable cells (differences between data after 24, 48, and 72 h, in log CFU mL of Lb. acidophilus at different pH values. Columns indicate the standard deviation from the mean value. Indicates a significant difference ($p \leq 0.05$) using the student's t-test. Different capitals indicate significant differences between cell populations at different indication times under the same stress conditions

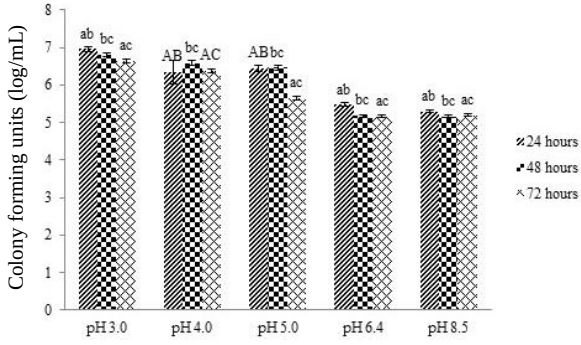

Fig. 3: Changes in the number of viable cells (differences between data after 24, 48, and 72 h, in log CFU ml of *Lb. rhamnosus* at different pH values. Columns indicate the standard deviation from the mean value. Indicates a significant difference ($p \leq 0.05$) using Student's test. Different capitals indicate significant differences between cell populations during different indication times under the same stress conditions

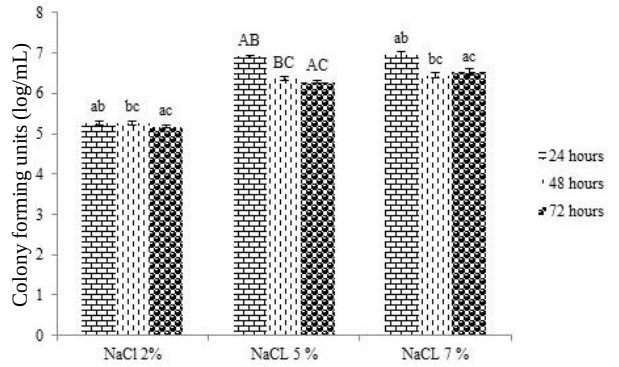

Fig. 4: Changes in the number of viable cells (differences between data after 24, 48, and 72 h, in log CFU ml of *Lb. acidophilus* at different NaCl concentrations. Columns indicate the standard deviation from the mean value. Indicates a significant difference ($p \leq 0.05$) using Student's t-test. Different capitals indicate significant differences between cell populations during different indication times under the same stress conditions

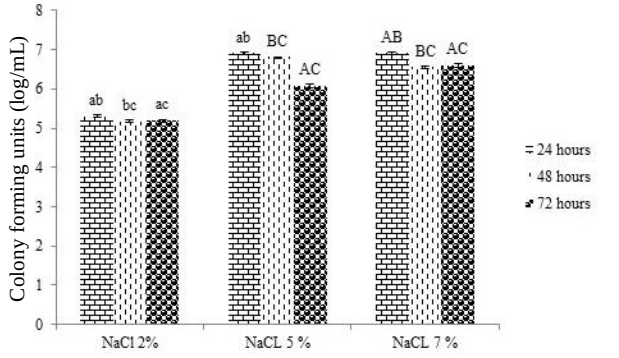

Fig. 5: Changes in the number of viable cells (differences between data after 24, 48, and 72 h, in log CFU mL of *Lb. rhamnosus* at different concentrations of NaCl. Columns indicate the standard deviation from the mean value. Indicates a significant difference ($p \leq 0.05$) using the student's t-test. Different capitals indicate significant differences between cell populations during different indication times under the same stress conditions

Co-Fermentation of Lb. Acidophilus and Lb. Rhamnosus

The quantification of one strain from the other was determined according to the methodology (Schiraldi *et al*. 2002). Cell growth was followed during experiments by measuring absorbance at 600 nm on a Beckman DU 640 Spectrophotometer (Milan, Italy). Cell counting by direct microscopic analysis was not always reliable, cell density estimate can be affected by an error. However, throughout the experiment, a few samples were extruded through a syringe needle (2-4 times), and diluted, and the number of cells per unit volume was counted in a Burker chamber using an optical microscope. The two Lactobacillus strains were recognized by morphological characteristics. Fermentation was carried out on a Sartorius Biostat® A MO UniVessel® Glass 1L 230V fermenter. The quantities of *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* during co-fermentation are shown in Fig. 6. At the beginning of co-fermentation, ~7 log CFU/mL of *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* were inoculated in 500 mL of Pasteurised Milk (PM). The fermentation process in co-culture was carried out for 24 h. The viable cell populations of the test strains increased slowly over 24 h and reached 8 log CFU/mL, showing an increase of 1 log CFU/mL. At 8 h of co-fermentation, their growth was above 7 log CFU/mL. The growth of *Lactobacillus rhamnosus* ranged from 7.47-7.77 log CFU/mL after 20 h. *Lactobacillus acidophilus* showed a population of 8 log CFU/mL at the end of co-cultured fermentation.

The acidification rate during fermentation of a mixed culture in PM is shown in Fig. 7. After 4 h, the pH values in the PM substrate with the incubated strains drop. Co-culture of *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* contributed to the pH drop from 6-3 after 24 h.

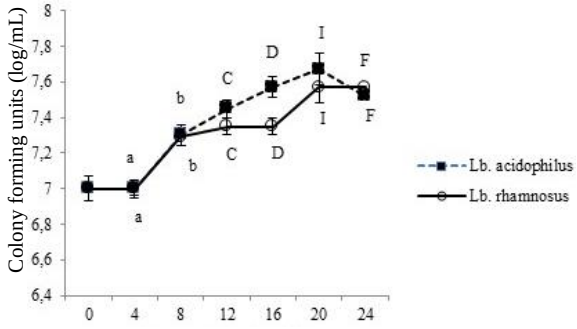

Fig. 6: Number of cell populations during co-fermentation in PM. Different uppercase letters indicate significant differences; lowercase letters indicate non-significant differences

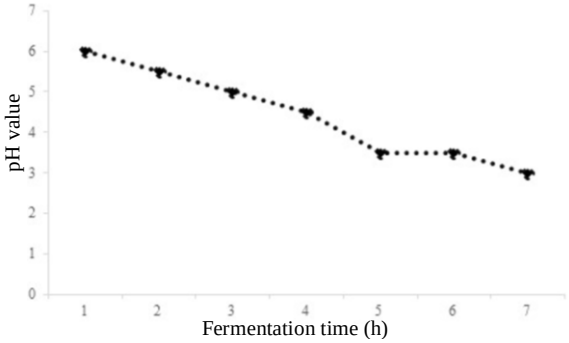

Fig. 7: Acidification rate during co-fermentation by 12 h a dense clot was formed which remained stable until the co-fermentation process was completed

In co-fermentation, *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* showed an increase in their population from ~7 log CFU/mL to ~8 log CFU/mL at 12-, 16-, 20- and 24-h fermentation than at 4 h fermentation, these differences were significant ($p \leq 0.05$). Thus, MCBs achieved a significant number of viable cell populations by 24 h fermentation compared to 12, 16, and 20 h fermentations.

Discussion

Wang *et al*. (2020) reported that probiotic bacteria must have the ability to survive and pass through the gastrointestinal tract. Thus, this study was carried out to evaluate several probiotic characteristics of *Lb. acidophilus* and *Lb. rhamnosus*.

Three common conditionally pathogenic microorganisms, *S. typhimurium*, *S. marcescens*, and *E. coli*, were used to investigate their antagonistic activity, carried out by in vivo diffusion methods. All two strains showed strong antimicrobial activity against *E. coli*. However, the inhibition was more pronounced in *Lb. acidophilus* than in *Lb. rhamnosus* (Fig. 2).

Fei *et al*. (2018) found that L. acidophilus inhibited not only the growth of Gram-negative bacteria but also the growth of Gram-positive bacteria such as *L. monocytogenes*, *S. aureus*, and *S. hemolyticus*. Similar observations have been made by Mpofu *et al*. (2016) *Lb. rhamnosus* can inhibit several pathogens. Rajoka *et al*. (2017) report that *Lb. rhamnosus* has a higher zone of inhibition against *E. coly* (8-13 mm diameter).

Lb. acidophilus and *Lb. rhamnosus* are lactic acid-producing bacteria. Georgieva *et al*. (2015); and Do Amaral Santos *et al*. (2014) also identified that the bactericidal activity of *Lb. acidophilus* with respect to Gram-negative pathogens may be related to the production of lactic acid as a result of carbohydrate glycolysis. Our studies showed that *Lb. acidophilus* and *Lb. rhamnosus* has the broadest spectrum of activity and zones of inhibition (with a range of high and medium diameters from 5-12 mm).

These strains were further investigated for resistance to pH and NaCL and their ability to grow under various acidic stresses and salt concentrations.

Kailasapathy and Chin (2000) in their work reported that pH values between 3.5 and 4.5 are good environmental conditions for populations of potential probiotic organisms. The survival of *Lb. acidophilus* and *Lb. rhamnosus* was based on pH values; low pH increases their survival. Figure 3 shows the survival of *Lb. acidophilus* and *Lb. rhamnosus* at 3.0, 4.0, 5.0, 6.4 and 8.5 for 72 h of incubation. At pH 3.0-4.0, survival was higher compared to pH 6.4-8.5 for all strains studied ($p \leq 0.05$). The results showed that all strains tested were highly resistant to acidic conditions. Lim *et al*. (2015); Rathore *et al*. (2012) studied the survival of *Lb. rhamnosus* and *Lb. acidophilus* under acidic conditions showed the highest survival (pH 1.5 - 3.0, pH 3.0 - 4.0). Zuljan *et al*. (2014) report in their experiments that an acidic environment plays an important role in bacterial growth.

An acidity tolerance in the pH range of 3-4, suitable for probiotic cultures, is sufficient for successful passage through the stomach and the environment of the human gastrointestinal tract. These results are in agreement with most of the data presented by other authors as well (Dinçer and Kıvanç, 2021; Innocente *et al*., 2016).

Lb. acidophilus and *Lb. rhamnosus* were tested for their ability to grow in the presence of NaCl (2, 5, 7%). Two strains were tolerant to NaCl concentrations. *Lb. acidophilus* and *Lb. rhamnosus* reached ~6 Log CFU/mL at 48 h.

Other researchers Vinderola *et al*. (2002); Wang *et al*. (2020) have also observed a high level of bacterial growth at 7-5% NaCL, so the growth of *Lb. acidophilus* was increased by 7-5% NaCl among the probiotic strains used in this study Thus, 7-5% NaCl stimulated the growth of strains.

Ma *et al*. (2020) write that NaCL is widely used in food production, but in probiotic products, high salt concentration affects cell viability, metabolism, and physiological functions of isolates.

Son *et al*. (2018) have suggested that probiotic products require a bacterial suspension (1×10^7 CFU/mL) to have an effect on the digestive system. The co-fermentation process for the production of fermented probiotic products is shown in Fig. 6. The highest level of *Lb. acidophilus* and *Lb. rhamnosus* cell population above 8 logs CFU/mL was observed at the end of co-fermentation (Fig. 6). The observed number of probiotic bacteria is in agreement with similar studies carried out by previous authors (Skryplonek *et al*., 2019).

Conclusion

Our study examined LAB isolated from traditional homemade dairy foods in Kazakhstan; specifically, up to sixteen strains of lactobacilli were identified via advanced genetic techniques known as "16sRNA sequencing". Up to sixteen strains of Lactobacilli species were identified via advanced genetic techniques known as "16sRNA sequencing". Gene amplified by PCR using the direct primer 8f 5'- AgAgTTTgATCCTggCTCAg- and 806R- 5' ggACT ACCAgggTATCTAAT. The PCR program was performed using a GeneAmp PCR System. The sequencing reaction was performed using the BigDye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequences obtained were identified in the gene bank using the BLAST algorithm. Mega software version 6.0 was used to generate phylogenetic trees using the Neighbor-Joining (NJ) algorithm. The above method enabled us to identify the biodiversity of lactic acid bacteria existing in Kazakhstan home-made dairy products (fermented cow, mare, camel milk, irimshik, kurta, home-made butter). The lactobacilli strain we have identified we are considering for further selection as lactose-free probiotics and want to examine them as valuable resources.

The present study describes the antagonistic activity and tolerance to acid and salt concentrations of *Lb. acidophilus* and *Lb. rhamnosus* isolated from traditional Kazakh domestic dairy products. The cultures investigated had significant resistance to low pH and 5-7% NaCl. Based on these results, it can be concluded that *Lb. acidophilus* and *Lb. rhamnosus* are potential probiotic cultures and can be used as a starter for dairy products. A cell population of *Lb. acidophilus* and *Lb. rhamnosus* of 8 logs CFU/mL and a pH value of 3.0 was achieved within 24 h during the co-culture process.

Development of starters by mixing *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* should become domestic fermented dairy products to meet consumer demand and market expansion in Kazakhstan. Based on the results of the research it is planned to conduct pilot testing of starters on the territory of Akmola region, Arshala district, Zhibek Zholy settlement based on the firm "Voskhod-2004" LLP. At the current moment, there is an agreement (contract) and in the future, an act of completed work on testing of starters for lactose-free lactic acid products will be obtained. Next, it is planned to implement this starter for industrial purposes so far only on the territory of the Akmola region, but with the prospect of neighboring regions.

In perspective, the area of our research will be focused on the immobilization of probiotic strains *Lactobacillus acidophilus* and *Lactobacillus rhamnoses*. For stability of probiotic viability using spray drying.

Acknowledgment

The project received financial support from the Ministry of education and Science of the Republic of Kazakhstan government funding influenced greatly the implementation of the research project. Laboratory equipment, nutrient media, and reagents were purchased with funding. At the expense of financing the research work samples were collected: Cow's milk, camel's milk, Kurt, irimshik, and homemade butter from the Arshali district of Akmola Region.

Funding Information

The research has been financially supported by the Ministry of education and Science of the Republic of Kazakhstan. Under the project "Technology of production of lactose-free fermented dairy products". On the priority direction "life and health sciences" under the budget program 217 "Development of Science", subprogram 101 "program-targeted funding for subjects of scientific and/or scientific-technical activity".

The author Madina Kairtaevna Imanbayeva received scientific support from the Astana branch of "KazNII of processing and food industry". The authors state that no funds, grants, or other support were received in the preparation of this manuscript. The amount of funding was 6 million.

Author's Contributions

Madina K. Imanbayeva: Isolation and genetic identification of lactic acid bacteria by 16S rRNA method isolated from Kazakhstan home-made dairy products. Literature reviewed, processing of the obtained research results, written and edited of this article.

Zhaksylyk K. Masalimov: Conducting the scientific study and processing the obtained research results on pH and NaCL treatment and viability section. Contributed to the writing of the original article.

Alexander Y. Prosekov: Statistical processing of the obtained research results. Provided advice for the experiment on the co-fermentation section of Lb. *acidophilus* and *Lb. rhamnosus*.

Irina S. Milentyeva: Written the Introduction chapter, advised in providing methods for experiments on pH and NaCL treatment and viability section.

Utemurat Z. Sagyndykov: Conducting scientific research and processing of the obtained research results on antagonistic activity, co-fermentation of *Lb. acidophilus* and *Lb. rhamnosus* section.

Ethics

The research we have conducted does not involve ethical approval, as the research has not yet been conducted on animals or humans. At this stage, members of the research group have developed a laboratory inoculum.

References

Abosereh, N. A., Abd El Ghani, S., Gomaa, R. S., & Fouad, M. T. (2016). Molecular identification of potential probiotic lactic acid bacteria strains isolated from Egyptian traditional fermented dairy products. *Biotechnology*, *15*(1/2), 35. https://doi.org/10.3923/biotech.2016.35.43

Ahmed, Z., Wang, Y., Ahmad, A., Khan, S. T., Nisa, M., Ahmad, H., & Afreen, A. (2013). Kefir and health: A contemporary perspective. *Critical Reviews in Food Science and Nutrition*, *53*(5), 422-434. https://doi.org/10.1080/10408398.2010.540360

Amirkhanova, Z., Akhmetova, S., Kozhakhmetov, S., Kushugulova, A., Bodeeva, R., Issina, Z., & Tusbayev, M. (2021). Screening of antimicrobial and adhesive activity of lactobacilli isolated from the national food products from different districts of the Karaganda region (Kazakhstan). https://doi.org/10.3889/oamjms.2021.7053

Arynova, R. A., Muslimov, N. Z., Imanbayeva, M. K., Zhaimayev, B. K., & Kadyrbekova, L. K. (2020). A consortium of starter cultures with lactose-utilizing and probiotic properties technology of production of delactosed sould-milk products. *EurAsian Journal of BioSciences*, *14*(1), 459-465. https://www.proquest.com/openview/919ba4165c89162dabbda62a59fa15ce/1?pq-origsite=gscholar&cbl=2042720

Cole, S. W. (1933). The determination of reducing sugars by titration of ferricyanide. *Biochemical Journal*, *27*(3), 723.https://doi.org/10.1042/bj0270723

De Vrese, M., & Schrezenmeir, A. J. (2008). Probiotics, prebiotics and synbiotics. *Food Biotechnology*, 1-66. https://doi.org/10.1007/10_2008_097

Di Stefano, M., Veneto, G., Malservisi, S., Cecchetti, L., Minguzzi, L., Strocchi, A., & Corazza, G. R. (2002). Lactose malabsorption and intolerance and peak bone mass. *Gastroenterology*, *122*(7), 1793-1799. https://doi.org/10.1053/gast.2002.33600

Dinçer, E., & Kıvanç, M. (2021). *In vitro* evaluation of probiotic potential of Enterococcus faecium strains isolated from Turkish pastırma. *Archives of Microbiology*, *203*(6), 2831-2841. https://doi.org/10.1007/s00203-021-02273-y

Do Amaral Santos, C. C. A., da Silva Libeck, B., & Schwan, R. F. (2014). Co-culture fermentation of peanut-soy milk for the development of a novel functional beverage. *International Journal of Food Microbiology*, *186*, 32-41. https://doi.org/10.1016/j.ijfoodmicro.2014.06.011

Fei, Y., Li, L., Zheng, Y., Liu, D., Zhou, Q., & Fu, L. (2018). Characterization of *Lactobacillus* amylolyticus L6 as potential probiotics based on genome sequence and corresponding phenotypes. *LWT*, *90*, 460-468. https://doi.org/10.1016/j.lwt.2017.12.028

Gao, J., Li, X., Zhang, G., Sadiq, F. A., Simal-Gandara, J., Xiao, J., & Sang, Y. (2021). Probiotics in the dairy industry Advances and opportunities. *Comprehensive Reviews in Food Science and Food Safety*, *20*(4), 3937-3982. https://doi.org/10.1111/1541-4337.12755

Georgieva, R., Yocheva, L., Tserovska, L., Zhelezova, G., Stefanova, N., Atanasova, A., ... & Karaivanova, E. (2015). Antimicrobial activity and antibiotic susceptibility of *Lactobacillus* and Bifidobacterium spp. intended for use as starter and probiotic cultures. *Biotechnology and Biotechnological Equipment*, *29*(1), 84-91. https://doi.org/10.1080/13102818.2014.987450

Heine, R. G., AlRefaee, F., Bachina, P., De Leon, J. C., Geng, L., Gong, S., ... & Rogacion, J. M. (2017). Lactose intolerance and gastrointestinal cow's milk allergy in infants and children-common misconceptions revisited. *World Allergy Organization Journal*, *10*, 1-8. https://doi.org/10.1186/s40413-017-0173-0

Hemarajata, P., & Versalovic, J. (2013). Effects of probiotics on gut microbiota: Mechanisms of intestinal immunomodulation and neuromodulation. *Therapeutic Advances in Gastroenterology*, *6*(1), 39-51. https://doi.org/10.1177/1756283X12459294

Innocente, N., Biasutti, M., Rita, F., Brichese, R., Comi, G., & Iacumin, L. (2016). Effect of indigenous *Lactobacillus rhamnosus* isolated from bovine milk on microbiological characteristics and aromatic profile of traditional yogurt. *LWT-Food Science and Technology*, *66*, 158-164. https://doi.org/10.1016/j.lwt.2015.10.031

Kailasapathy, K., & Chin, J. (2000). Survival and therapeutic potential of probiotic organisms with reference to *Lactobacillus acidophilus* and *Bifidobacterium spp*. *Immunology and Cell Biology*, *78*(1), 80-88. https://doi.org/10.1046/j.1440-1711.2000.00886.x

Kim, J. A., Bayo, J., Cha, J., Choi, Y. J., Jung, M. Y., Kim, D. H., & Kim, Y. (2019). Investigating the probiotic characteristics of four microbial strains with potential application in feed industry. *PloS One*, *14*(6), e0218922. https://doi.org/10.1371/journal.pone.0218922

Krieger-Grübel, C., Hutter, S., Hiestand, M., Brenner, I., Güsewell, S., & Borovicka, J. (2020). Treatment efficacy of a low FODMAP diet compared to a low lactose diet in IBS patients: A randomized, cross-over designed study. *Clinical Nutrition ESPEN*, *40*, 83-89. https://doi.org/10.1016/j.clnesp.2020.09.020

Leis, R., Castro, M.J., Lamas C., Picans, R., Couce, M.L. (2020). Effects of prebiotic and probiotic supplementation on lactase deficiency and lactose intolerance: A systematic review of controlled trials. *Nutrients*, 12(5):1-13. https://doi.org/10.3390/nu12051487

Lim, P. L., Toh, M., & Liu, S. Q. (2015). Saccharomyces cerevisiae EC-1118 enhances the survivability of probiotic *Lactobacillus rhamnosus* HN001 in an acidic environment. *Applied Microbiology and Biotechnology*, *99*, 6803-6811. https://doi.org/10.1007/s00253-015-6560-y

Lopes, L. A. A., Carvalho, R. D. S. F., Magalhães, N. S. S., Madruga, M. S., Athayde, A. J. A. A., Portela, I. A., ... & Stamford, T. C. M. (2020). Microencapsulation of *Lactobacillus acidophilus* La-05 and incorporation in vegan milks: Physicochemical characteristics and survival during storage, exposure to stress conditions and simulated gastrointestinal digestion. *Food Research International*, *135*, 109295. https://doi.org/10.1016/j.foodres.2020.109295

Ma, J., Wang, W., Sun, C., Gu, L., Liu, Z., Yu, W., ... & Hou, J. (2020). Effects of environmental stresses on the physiological characteristics, adhesion ability and pathogen adhesion inhibition of *Lactobacillus* plantarum KLDS 1.0328. *Process Biochemistry*, *92*, 426-436. https://doi.org/10.1016/j.procbio.2020.02.001

Mohammadi, R., Mortazavian, A. M., Khosrokhavar, R., & da Cruz, A. G. (2011). Probiotic ice cream: Viability of probiotic bacteria and sensory properties. *Annals of Microbiology*, *61*, 411-424. https://doi.org/10.1007/s13213-010-0188-z

Mpofu, A., Linnemann, A. R., Nout, M. J., Zwietering, M. H., Smid, E. J., & den Besten, H. M. (2016). Inactivation of bacterial pathogens in yoba mutandabota, a dairy product fermented with the probiotic *Lactobacillus rhamnosus* yoba. *International Journal of Food Microbiology*, *217*, 42-48. https://doi.org/10.1016/j.ijfoodmicro.2015.09.016

Pimpin, L., Wu, J. H., Haskelberg, H., Del Gobbo, L., & Mozaffarian, D. (2016). Is butter back? A systematic review and meta-analysis of butter consumption and risk of cardiovascular disease, diabetes, and total mortality. *PLoS One*, *11*(6), e0158118. https://doi.org/10.1371/journal.pone.0158118

Rajoka, M. S. R., Mehwish, H. M., Siddiq, M., Haobin, Z., Zhu, J., Yan, L., ... & Shi, J. (2017). Identification, characterization and probiotic potential of *Lactobacillus rhamnosus* isolated from human milk. *LWT*, *84*, 271-280. https://doi.org/10.1016/j.lwt.2017.05.055

Ratajczak, A. E., Rychter, A. M., Zawada, A., Dobrowolska, A., & Krela-Kaźmierczak, I. (2021). Lactose intolerance in patients with inflammatory bowel diseases and dietary management in prevention of osteoporosis. *Nutrition*, *82*, 111043. https://doi.org/10.1016/j.nut.2020.111043

Rathore, S., Salmerón, I., & Pandiella, S. S. (2012). Production of potentially probiotic beverages using single and mixed cereal substrates fermented with lactic acid bacteria cultures. *Food Microbiology*, *30*(1), 239-244. https://doi.org/10.1016/j.fm.2011.09.001

Reuben, R. C., Roy, P. C., Sarkar, S. L., Alam, R. U., & Jahid, I. K. (2019). Isolation, characterization and assessment of lactic acid bacteria toward their selection as poultry probiotics. *BMC Microbiology*, *19*, 1-20. https://doi.org/10.1186/s12866-019-1626-0

Samat, K., Nurislam, M., Zhanel, P., Dmitriy, B., Dinar, T., Indira, U., ... & Almagul, K. (2022). Effects of low lactose mare's milk yogurt consumption on gut microbiota function. *Functional Foods in Health and Disease*, *12*(8), 455-464. https://doi.org/10.31989/ffhd.v12i8.981

Scrimshaw, N. S., & Murray, E. B. (1988). The acceptability of milk and milk products in populations with a high prevalence of lactose intolerance. *The American Journal of Clinical Nutrition*, *48*(4), 1142-1159. https://doi.org/10.1093/ajcn/48.4.1142

Shokryazdan, P., Faseleh Jahromi, M., Liang, J. B., & Ho, Y. W. (2017). Probiotics: From isolation to application. *Journal of the American College of Nutrition*, *36*(8), 666-676. https://doi.org/10.1080/07315724.2017.1337529

Skryplonek, K., Dmytrów, I., & Mituniewicz-Małek, A. (2019). Probiotic fermented beverages based on acid whey. *Journal of Dairy Science*, *102*(9), 7773-7780. https://doi.org/10.3168/jds.2019-16385

Son, S. H., Jeon, H. L., Yang, S. J., Sim, M. H., Kim, Y. J., Lee, N. K., & Paik, H. D. (2018). Probiotic lactic acid bacteria isolated from traditional Korean fermented foods based on β-glucosidase activity. *Food Science and Biotechnology*, *27*, 123-129. https://doi.org/10.1007/s10068-017-0212-1

Schiraldi, C., Adduci, V., Maresca, C., Giuliano, M., Lamberti, M., Carteni, M., … & De Rosa, M. (2002). High cell density cultivation of probiotics and lactic acid productions. *Biotechnol Bioen, 20:82(2),* 213-222. https://doi.org/10.1002/bit.10557

Suchy, F. J., Brannon, P. M., Carpenter, T. O., Fernandez, J. R., Gilsanz, V., Gould, J. B., ... & Wolf, M. A. (2010). National institutes of health consensus development conference: Lactose intolerance and health. *Annals of Internal Medicine*, *152*(12), 792-796. https://doi.org/10.7326/0003-4819-152-12-201006150-00248

Vinderola, C. G., Costa, G. A., Regenhardt, S., & Reinheimer, J. A. (2002). Influence of compounds associated with fermented dairy products on the growth of lactic acid starter and probiotic bacteria. *International Dairy Journal*, *12*(7), 579-589. https://doi.org/10.1016/S0958-6946(02)00046-8

Wang, D., Liu, W., Ren, Y., De, L., Zhang, D., Yang, Y., ... & Menghe, B. (2016). Isolation and identification of lactic acid bacteria from traditional dairy products in Baotou and Bayannur of Midwestern Inner Mongolia and q-PCR analysis of predominant species. *Korean Journal for Food Science of Animal Resources*, *36*(4), 499. https://doi.org/10.5851/kosfa.2016.36.4.499

Wang, J., Da, R., Tuo, X., Cheng, Y., Wei, J., Jiang, K., ... & Han, B. (2020). Probiotic and safety properties screening of Enterococcus faecalis from healthy Chinese infants. *Probiotics and Antimicrobial Proteins*, *12*, 1115-1125. https://doi.org/10.1007/s12602-019-09625-7

Zhadyra, S., Han, X., Anapiyayev, B. B., Tao, F., & Xu, P. (2021). Bacterial diversity analysis in Kazakh fermented milks Shubat and Ayran by combining culture-dependent and culture-independent methods. *LWT*, *141*, 110877. https://doi.org/10.1016/j.lwt.2021.110877

Zhang, X., Hao, X., Wang, H., Li, X., Liu, L., Yang, W., ... & Bora, A. F. M. (2021). The effects of *Lactobacillus* plantarum combined with inulin on the physicochemical properties and sensory acceptance of low-fat Cheddar cheese during ripening. *International Dairy Journal*, *115*, 104947. https://doi.org/10.1016/j.idairyj.2020.104947

Zuljan, F. A., Repizo, G. D., Alarcon, S. H., & Magni, C. (2014). α-Acetolactate synthase of Lactococcus lactis contributes to pH homeostasis in acid stress conditions. *International Journal of Food Microbiology*, *188*, 99-107. https://doi.org/10.1016/j.ijfoodmicro.2014.07.017