










Screening of Antimicrobial and Adhesive Activity of Lactobacilli Isolated from the National Food Products from Different Districts of the Karaganda Region (Kazakhstan)

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Abstract

BACKGROUND: It is a national priority to look for new probiotic bacteria with highly active biological properties to create a new generation of probiotics, ferments, therapeutic, and prophylactic fermented milk products, taking into account ethnocultural and regional characteristics.

AIM: The aim of the study is to assess probiotic properties of strains of lactobacilli (antimicrobial and adhesive), which are isolated from national lactic acid products from different districts of the Karaganda region (Kazakhstan).

MATERIALS AND METHODS: There were modern microbiological methods applied during the experiment. To determine the morpho-cultural properties, the following methods were used: Gram staining, a catalase test, serial dilutions. The Matrix Supported Laser Desorption/Ionization Flight Time Mass Spectrometry was used for identification, and the deferred-antagonism method was used to determine the antimicrobial activity. The buccal epithelial cells were used for the cell object as a test system to determine the adhesive activity.

RESULTS: In this experiment, 26 lactobacillus isolates were isolated from 68 samples of national lactic acid products produced in a traditional homemade way in different districts of the Karaganda region (Kazakhstan). As a result of the studies carried out on the cultural and morphological characteristics and identification by the mass spectrometer, the following lactobacilli were obtained: *Lactobacillus acidophilus* (two strains), *Lactobacillus delbrueckii* subsp. *bulgaricus* (two strains), *Lactobacillus rhamnosus* (seven strains), *Lactobacillus plantarum* (two strains), *Lactobacillus paracasei* (11 strains), and *Lactobacillus fermentum* (two strains). Twenty-six isolates of lactobacilli were tested for antimicrobial activity, 13 isolates of which showed an inhibitory effect, but the degree of antagonism varied among lactobacillus isolates. In general, the inhibitory activity of lactobacillus isolates was shown against the Gram-negative indicator microorganisms *Salmonella typhimurium* NCTC 12023, *Escherichia coli* NCTC 12923. The antibacterial activity was shown against the *Staphylococcus aureus* NCTC 12973 indicator microorganism in nine isolates of lactobacilli. Only six isolates of lactobacilli showed antifungal activity against the test strain of *Candida albicans* NCPF 3179. Out of 13 isolates of lactobacilli, nine isolates of medium and high activity competed for binding to buccal epithelial cells.

CONCLUSION: The obtained isolates from traditional dairy products are considered to be promising candidates and competitive isolates with some probiotic potential. This study calls for further researches to be made in this area.

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Introduction

The nutritional peculiarities of the population of Kazakhstan have a long history of traditional lactic acid products from milk of cows, mares, camels, and goats and are based on milk fermentation [1]. To date, the study of probiotic strains with highly active biological properties isolated from local Kazakh lactic acid products with a long history is of high priority [2]. Due to the variability of opportunistic bacteria manifested and an increase in antibiotic resistance [3], [4], a problem of food safety is particularly rampant as for the preservation of food by suppressing other microorganisms causing foodborne illness or food spoilage [3], [5], as well as changes in the biological properties of probiotic crops

during long-term storage as industrial crops in dairy industries [6]. The foregoing necessitates the search for new unique candidates for probiotics, in particular those with high antagonistic (capable of synthesizing metabolites with antimicrobial action with the generally recognized as safe status) adhesive properties.

The progress of the sciences engaged in studying microbiota confirms the nature of these microbes as a protective microflora with an expressed antagonistic activity against putrefactive and pyogenic opportunistic and pathogenic microorganisms.

Lactobacilli, being the representatives of the normal microbiota of the mucous membrane of humans and animals, play a key role in forming colonization resistance, which is one of the most important factors of

the body's anti-infectious defense system in improving the microbial balance. This prompted us to conduct this study, namely, the interest in the peculiarities of lactobacilli and their metabolites as the main components of starter cultures, being the most widely used microorganisms as probiotics.

The given study was carried out to assess the probiotic properties (antimicrobial and adhesive) of lactobacillus isolates isolated from national lactic acid products from different districts of the Karaganda region (Kazakhstan).

Methods

Materials

Sixty-eight samples of national lactic acid products were used for this research (Table 1). All of them were home-made in a traditional way and taken from different districts of the Karaganda region (Kazakhstan).

Table 1: National lactic acid products and number of samples from the regions

Districts	National lactic acid products (number of samples)						
	Ayran	Koumiss	Ashigan kozhe	Kurt	Suzbe	Cottage cheese	Brynza
Aktogay	2	3	2	4	3	3	-
Bukhar-Zhyrausky	-	2	-	2	2	2	2
Zhanaarkinsky	2	5	-	-	-	-	-
Karkaralinsky	3	4	2	3	2	3	-
Osakarovsky	-	1	-	2	-	3	4
Shetsky	-	-	-	3	2	2	-
Total	68						

Isolation and identification of lactobacilli isolates

The lactobacillus isolates were isolated from national lactic acid products by the standard plate method [7].

The serial dilutions were prepared from each product in sterile solution followed by MRS agar-based inoculation (deMan, Rogosa, Sharpe), which had been cultivated at 37°C for 2 days under anaerobic conditions.

After incubation, the isolated colonies were tested by conducting Gram stain and catalase tests. The rod-shaped, Gram-positive, and catalase-negative isolates were selected for the tests. Following that, some individual colonies were subcultured in the MRS broth for 48 ± 2 h at +37°C with a couple of control smears taken. The selected strains were stored in the MRS broth containing 20% glycerol and stored at -22°C for further analysis. Before each analysis, the isolates were activated in the MRS broth.

The pure cultures of lactobacilli were identified using Matrix Supported Laser Desorption/Ionization Flight Time Mass Spectrometry (MALDI-TOF MS) (MALDI-TOF MS, Bruker, Germany). The bacterial

spectra were compared with the MALDI-TOF MS biotype reference library [8].

The identification reliability criteria were applied by the following score values:

2.300–3.000 – highly probable identification of species; 2.000–2.299 – reliable identification of genus and probable identification of species; 1.700–1.999 – probable identification of genus; and 0–1.699 – identification failed.

Antimicrobial properties of lactobacilli isolates against indicator microorganisms

The study of the bactericidal and antifungal activity of antagonist strains in relation to test strains to opportunistic microorganisms of various groups was determined by the deferred-antagonism method [9].

The antimicrobial activity of the obtained isolates of lactobacilli was assessed using indicator microorganisms: *Staphylococcus aureus* NCTC 12973, *Escherichia coli* NCTC 12923, *Salmonella typhimurium* NCTC 12023, *Candida albicans* NCPF 3179 (the test strains from Laboratory of Human Microbiome and Longevity "National Laboratory Astana," Nazarbayev University (the city of Nur-Sultan, Kazakhstan).

The antimicrobial activity was assessed by no growth area of test strains around the colony of the tested isolates of lactobacilli. The studies were repeated 3 times with the results expressed as the arithmetic mean.

Adhesive activity of lactobacilli isolates

Buccal epithelial cells of a human were placed in a 7.2 pH phosphate buffer. These cells were washed 3 times by centrifugation (at 1000 rpm for 5 min). The control smears were prepared from the sediment. A sample was considered suitable for the research if at least 2–3 epithelial cells were found in each field of view during microscopy.

To study the adhesive activity, 800 µl of buccal cells' suspension and 600 µl of lactobacilli's strain suspension at the concentration of 10⁵ CFU/ml were added to a centrifuge tube. The contents of the tubes were incubated for 2 h at 37°C with intermittent stirring. After incubation, non-adsorbed lactobacilli were removed by washing them twice by centrifugation (at 1000 rpm for 3 min). The sediment was stained with gentian violet, microscopied, and the number of attached lactobacilli was counted [10], [11].

The result was expressed as the arithmetic mean of the number of lactobacilli attached to one buccal cell. The intensity of adhesive activity was assessed: Non-adhesive strains = 0; weakly adhesive = 1–5; medium adhesive = 5–10; and highly adhesive = above 10 [12].

Statistical analysis

The obtained results of antimicrobial and adhesive activity are presented as mean \pm standard error, obtained as a result of three-fold repetition of the experiment (Statistica 8.0 software was used) [13], [14].

Results

Morpho-cultural properties and identification of obtained isolates of lactobacilli

In total, 26 isolates of lactobacilli were selected from the national lactic acid products, which were traditionally homemade and taken from different districts of the Karaganda region. The studies carried out on morphological characters indicate their belonging to the genus of *Lactobacillus*, *Lacticaseibacillus*, *Lactiplantibacillus*, and *Limosilactobacillus* [15].

All isolated strains are Gram-positive with the cells located: Singly, in pairs, in the form of a chain, motionless, no spores are formed (Figure 1a-c), catalase-negative (lactobacilli do not have catalase; therefore, no gas formation was observed in the sample with hydrogen peroxide).

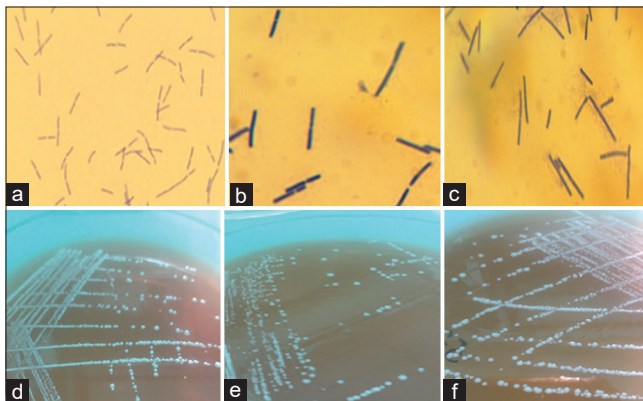


Figure 1: Microscopic image ($\times 100$) and MRS agar-based colony's morphology of isolated lactic acid bacillus isolates. (a), (d) *L. paracasei* isolates isolated from suzbe – 28; (b), (e) *L. fermentum* isolates isolated from ashigan kozhe -18; (c), (f) *L. acidophilus* isolates isolated from koumiss -52

The morphology of the colony of the isolated MRS agar-based isolates (Figure 1d-f) demonstrates white small, medium, convex with smooth edges, 1–3 mm in diameter, unpigmented ones, which grow well at 37°C for 48 h under anaerobic conditions.

In general, the lactic acid bacteria are identified in lactic acid products based on phenotypic, physiological, and biochemical criteria. However, these complex and laborious methods can underestimate the microbiological diversity of the food ecosystem [16].

The MALDI-TOF MS identification is a time efficient, economical, and reliable method [17]. The given studies have shown that the use of

MALDI-TOF-MS analysis has become part of the daily practical work of a microbiologist to identify lactobacilli, and even surpasses the previously used (classical) methods of microbiological identification in terms of speed and reliability.

As a result, the mass spectrometer identified the following in the presented samples: *Lactobacillus acidophilus* (two strains), *Lactobacillus delbrueckii* subsp. *bulgaricum* (two strains), *Lactobacillus rhamnosus* (seven strains), *Lactobacillus plantarum* (two strains), *Lactobacillus paracasei* (11 strains), and *Lactobacillus fermentum* (two strains).

In accordance with the score values, the results indicate the following accuracy of identification: (2.300–3.000) – in 11 strains; (2.000–2.299) – in 12 strains; (1.700-1.999) – in three strains.

Screening for antagonistic activity of lactobacilli isolates

Lactobacilli are known for their production of various antimicrobial compounds [18]. Microbial pathogens such as *S. aureus*, *E. coli*, *S. typhimurium*, and *C. albicans* in food items can cause spoilage and contribute to both foodborne diseases, drug resistance, and resistance to disinfectants [19].

In this regard, to determine the probiotic properties, all 26 isolated lactobacillus isolates were tested for antimicrobial activity against Gram-positive, Gram-negative, and yeast indicator microorganisms. Thirteen isolates had an inhibitory effect (Table 2 and Figure 2), but the degree of antagonism varied among these isolates.

Table 2: Antibacterial and antifungal activity of isolated isolates

Symbols used to indicate isolated lactobacillus isolates	<i>S. aureus</i> NCTC 12973	<i>E. coli</i> NCTC 12923	<i>S. typhimurium</i> NCTC 12023	<i>C. albicans</i> NCPF 3179
	Inhibition zones (diameter in mm)			
<i>L. rhamnosus</i> 1a	-	10.0 \pm 2.0	13.0 \pm 4.0	7.0 \pm 2.0
<i>L. rhamnosus</i> 2a	-	-	16.0 \pm 3.2	-
<i>L. rhamnosus</i> 3a	-	-	14.0 \pm 3.6	5.0 \pm 2.0
<i>L. rhamnosus</i> 1/1	15.0 \pm 1.0	7.0 \pm 1.5	10.0 \pm 0.5	5.0 \pm 0
<i>L. plantarum</i> 2/1	7.0 \pm 2.0	-	14.0 \pm 1.0	4.0 \pm 1.5
<i>L. paracasei</i> 28	8.0 \pm 1.0	6.0 \pm 2.3	9.0 \pm 2.0	-
<i>L. paracasei</i> 32	9.0 \pm 1.0	10.0 \pm 1.0	5.0 \pm 1.0	-
<i>L. paracasei</i> 34	9.0 \pm 1.5	11.0 \pm 1.5	9.0 \pm 0	-
<i>L. paracasei</i> 38	4.0 \pm 1.0	-	4.0 \pm 1.0	3.0 \pm 4.0
<i>L. fermentum</i> 38/1	10.0 \pm 1.1	9.0 \pm 1.0	13.0 \pm 0.5	3.0 \pm 0
<i>L. rhamnosus</i> 39	13.0 \pm 2.0	10.0 \pm 0	10.0 \pm 0.5	-
<i>L. rhamnosus</i> 40	-	9.0 \pm 0	7.0 \pm 0	-
<i>L. rhamnosus</i> -52	7.0 \pm 0.5	3.0 \pm 0	7.0 \pm 1.2	-

The diameters of inhibition zones (in mm) are presented as mean \pm SD, the research is repeated 3 times. *S. aureus*: *Staphylococcus aureus*, *E. coli*: *Escherichia coli*, *S. typhimurium*: *Salmonella typhimurium*, *C. albicans*: *Candida albicans*, *L. rhamnosus*: *Lactobacillus rhamnosus*, *L. plantarum*: *Lactobacillus plantarum*, *L. paracasei*: *Lactobacillus paracasei*, *L. fermentum*: *Lactobacillus fermentum*, *L. acidophilus*: *Lactobacillus acidophilus*.

The antibacterial activity against the indicator microorganism *S. aureus* NCTC 12973 was shown in nine isolates (the diameter of the retention zone varied, the lowest activity at 4 mm, and the high activity at 15 mm).

The antibacterial activity against *E. coli* NCTC 12923 was shown in nine isolates (the diameter of the retention zone varied from 10 to 16 mm).

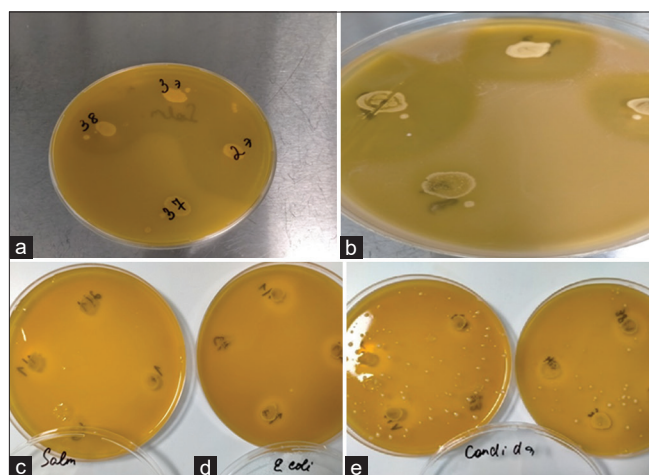


Figure 2: Inhibitory effect of isolated lactobacillus isolates to the following indicator microorganisms *S. typhimurium* NCTC 12023 (a), (c), *S. aureus* NCTC 12973 (b), *E. coli* NCTC 12923 (d), *C. albicans* NCPF 3179 (e)

The antibacterial activity against the ratio of *S. typhimurium* NCTC 12023 was detected in 13 isolates (the diameter of the retention zone varied with the lowest activity at 5 mm, and the highest activity at 16 mm).

Six strains of lactobacilli demonstrated antifungal activity against *C. albicans* NCPF 3179, the diameter of the retention zone varied from 3 to 7 mm.

The spectrum of antimicrobial activity of lactobacilli (*L. rhamnosus*, *L. plantarum*, *Lactobacillus casei*, *L. paracasei*, *L. acidophilus*, *L. fermentum*, and *Lactobacillus buchneri*) metabolites is directed against shigella, salmonella, clostridia, pseudomonads, staphylococci, streptococci, listeria, and some types of fungi [19], [20], [21], [22], [23], [24].

Thus, we have not found any dependence between manifestation of antimicrobial and antifungal activity of the isolated strains and a particular genus, species and subspecies of lactobacilli, inhibiting antimicrobial and antifungal action, which is a strain-specific property. This conclusion is consistent with the data presented by other researchers [25].

Adhesive activity of lactobacilli isolates

Adhesive activity allows a microbial cell to adapt to adverse environmental impacts and increase its population. Thus, adhesive capacity can be considered as a standard biomarker when selecting a potential probiotic [26].

In the present study, we have evaluated 13 probiotic strains of lactobacilli with antimicrobial activity for their ability to adhere to buccal cells.

In the course of these studies, it has been found that the adhesive ability to the buccal epithelial cells of 13 lactobacillus isolates under investigation have the following adhesive ability: High (6), medium (3), and low (4) (Figure 3).

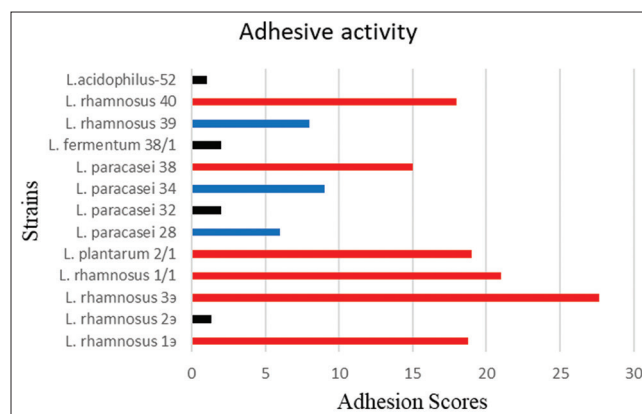


Figure 3: The number of lactobacillus isolates attached to one buccal cell: Up to 30 adhered bacterial cells – 1 point, 31 to 60 – 2 points, 61 to 90 – 3 points, 91 to 120 – 4 points, more than 121 – 5 points

The actual majority of the lactobacillus cell strains adheres to buccal epithelial cells and provides colonization. The highest adhesive activity was found in the isolates of *L. rhamnosus* 40, *L. paracasei* 38, *L. plantarum* 2/1, *L. rhamnosus* 1/1, *L. rhamnosus* 3e, and *L. rhamnosus* 1e.

The average adhesive activity is observed in the isolates of *L. rhamnosus* 39, *L. paracasei* 34, and *L. paracasei* 28.

The low adhesive activity is observed in the *L. acidophilus*-52, *L. fermentum* 38/1, *L. paracasei* 32, and *L. rhamnosus* 2e strains.

The differences observed in the adhesion capacity of lactobacilli strains suggest that this trait varies among probiotic strains. This conclusion is completely consistent with other researchers who also reported the ability of probiotics to adhere in the cell being highly dependent on the strain, species, and genus [24].

Discussion

In this study, 26 lactobacillus isolates were isolated from 68 samples of national lactic acid products, which were home-made in a traditional way and were taken from different districts of the Karaganda region (Kazakhstan).

As a result of these studies, which were carried out on the cultural and morphological characteristics, the mass spectrometer identified the following strains: *L. acidophilus* (2 strains), *L. delbrueckii* subsp. *bulgaricum* (2 strains), *L. rhamnosus* (7 strains), *L. plantarum* (2 strains), *L. paracasei* (11 strains), and *L. fermentum* (2 strains).

The antimicrobial activity against pathogens is an important attribute to consider when selecting potential probiotic strains to maintain a healthy microbial balance in the gastrointestinal tract [25].

They are able to produce substances with antibiotic activity in the process of their growth and development, the resulting antibiotic substance ensures the dominance of lactobacilli and suppression of opportunistic pathogenic microflora. Therefore, the use of lactobacilli with expressed antagonistic activity is of practical importance [27].

In the present study, 26 lactobacillus isolates were tested for antimicrobial activity. Thirteen isolates of which showed an inhibitory effect, but the degree of antagonism varied among lactobacillus isolates.

Basically, the inhibitory activity of lactobacillus isolates was shown against the Gram-negative indicator microorganisms *S. typhimurium* NCTC 12023, *E. coli* NCTC 12923, and *S. aureus* NCTC 12973. 9 lactobacillus isolates demonstrated some antibacterial activity.

The antifungal activity against the *C. albicans* NCPF 3179 test strain was detected only in six lactobacillus isolates. It is believed that this could be due to the high productivity of candida lysozyme, which acts as a protective factor.

Furthermore, 13 isolates of lactobacilli were additionally tested for adhesive activity *in vitro*.

The *in vitro* studies with various intestinal cell lines and mucin (high molecular weight glycoproteins containing acidic polysaccharides, are produced by epithelial cells of all animals and humans) have been widely used over the past decades to assess the adhesion capacity of probiotic bacteria.

The adhesive capacity of probiotic lactobacilli is usually studied *in vitro*. The most widely used models for assessing the adhesion of microorganisms are culture tissue's cells. To study the adhesion to intestinal epithelial cells, in particular, Caco-2, HT-29 cells, and mucus secreting HT-29 MTX cells [28], [29], [30], [31] are usually used.

The majority of probiotic foods are consumed orally, thus, it is possible that bacteria in these foods can also attach to the surfaces of the oral cavity [11].

In this regard, to study the adhesive properties, buccal epithelial cells were used. In this experiment, *L. rhamnosus* 40, *L. paracasei* 38, *L. plantarum* 2/1, *L. rhamnosus* 1/1, *L. rhamnosus* 3e, *L. rhamnosus* 1e, *L. rhamnosus* 39, *L. paracasei* 34, and *L. paracasei* 28 isolates competed for binding to buccal epithelial cells.

The obtained results confirmed the experimental data that 13 selected isolates of lactobacilli have antimicrobial activity, while nine isolates showed medium and high adhesive activity. The experiment showed that the lactobacillus isolates were promising probiotics, taking into account their probiotic data, namely, antimicrobial (the effect of resisting the opportunistic flora) and adhesive activity (the phenomenon of adhesion).

Conclusions

The isolated strains from traditional dairy products are promising candidates and competitive isolates with probiotic potential properties. They can play an important role in the food industry as starter cultures, cocultures or bioprotective crops to improve food quality and safety, or as probiotic therapeutic agents suitable for clinical practice.

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