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Probiotic properties of endemic strains of lactic acid bacteria

Flora N. Tkhruni [1]
Kristina J. Karapetyan [1]
Svetla T. Danova [2]
Svetoslav G. Dimov [3]
Farzad A. Karimpour [1]

Authors' addresses:
[1] Scientific and Production Center "Armbiotechnology" NAS, Laboratory of Microbial Preparations, Yerevan, Armenia.
[2] Institute of Microbiology "Stephan Angeloff", Bulgarian Academy of Sciences, Sofia, Bulgaria.
[3] Department of Genetics, Sofia University «St. Kliment Ohridsky», Sofia, Bulgaria.

Correspondence:
Flora Nubar Tkhurini
Scientific and Production Center "Armbiotechnology" NAS,
Laboratory of Microbial Preparations,
14 Gyurjyan Str., 0056 Yerevan,
Republic of Armenia.
Tel.: +374-10-536285
e-mail: floranub@yahoo.com

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ABSTRACT
Strains of lactic acid bacteria (LAB) isolated from various samples of matsun, yogurt and salted cheese from natural farms of Armenia were studied. They have high antimicrobial and probiotic activities, growth rate and differ by their resistance to enzymes. Supernatants of LAB retain bactericidal activity at pH 3.0-8.0 and inhibit growth of various microflora. The application of different methods of identification and LAB genotyping (API 50 CH, 16S rRNA sequencing, GS-PCR, RAPD PCR) showed that isolated LAB evidenced a 99.9% similarity with *L. rhamnosus*, *L. plantarum* and *L. pentosus* species and coccoid forms of *Streptococcus* and *Enterococcus* species. It can be concluded, that some strains of lactic acid bacteria, isolated from dairy products from natural farms of Armenia, can be properly used for biopreservation of some foodstuffs. On the basis of experimental data, the LAB can be used as basis for obtaining the new products of functional nutrition.

Key words: Lactic acid bacteria, probiotics, bactericidal activity, 16S rRNA sequencing

Introduction

The development of new additives based on probiotic microorganisms and produced biologically active substances, is a strategic direction of national policy in many developed countries. Probiotics are used for the prevention of gastrointestinal diseases, especially among children and young animals, and for correction of the intestinal microflora after therapy with antibiotics.

Probiotics are defined as live microorganisms that may beneficially affect the host upon ingestion by improving the balance of the intestinal microflora. Several *Lactobacillus* species, *Bifidobacterium* species and yeasts are accepted as probiotics. The selection criteria for probiotic lactic acid bacteria (LAB) include: safety, viability, resistance to acid and bile, adherence to gut epithelial tissue, ability to colonize the gastrointestinal tract, production of antimicrobial substances, ability to stimulate a host immune response and the ability to influence metabolic activities such as vitamin production, cholesterol assimilation and lactose activity. The *Lactobacillus* and *Bifidobacterium spp.* are prominent members of the commensally intestinal flora and are the commonly studied probiotics bacteria. Oral addition of viable *Lactbacillus acidophilus* of human origin, led to a significant decline of three different fecal bacterial enzymes (beta glucuronidase, azoreductase and nitroreductase). All these enzymes cause reduced lactose intolerance alleviation of some diarrheas, lowered blood cholesterol, increased immune response and prevention of cancer. Some LAB strains of possess health-promoting properties, such as the potential to combat gastrointestinal pathogenic bacteria as *Helicobacter pylori, Escherichia coli,* and *Salmonella* (Fernándes de Palencia et al., 2008). However, LAB are further known to produce bioactive molecules such as ethanol, formic acid,

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fatty acids, hydrogen peroxide, diacetyl, reuterin, and reutericyclin. Many strains also produce bacteriocins and bacteriocin-like molecules that display antibacterial activity (Atta et al., 2009; Schnürer & Magnusson, 2005).

LAB are found in milk, meat and fermented products, as well as in fermented vegetables and beverages, inhibiting the growth of pathogenic and deteriorating microorganisms, maintaining the nutritive quality and improving the shelf life of foods. They have also been used as flavor and texture producers. Whereas a food fermentation process with LAB is traditionally based on spontaneous fermentation or backslopping, industrial food fermentation is nowadays performed by the deliberate addition of LAB as starter cultures to the food matrix. This has been a breakthrough in the processing of fermented foods, resulting in a high degree of control over the fermentation process and standardization of the end products. Recently, the use of functional starter cultures, a novel generation of starter cultures that offers functionalities beyond acidification, is being explored (Leroy & De Vuyst, 2004; Corsetti et al., 2012).

The nature of fermented products is different from one region to another. This depends on the local indigenous microflora, which in turn reflected the climatic conditions of the area. Thus, traditional fermented milk in region with a cold temperature climate contained mesophilic bacteria such as *Lactococcus* and *Leuconostoc spp.*, while thermophilic bacteria, which include mostly *Lactobacillus* and *Streptococcus*, prevailed in regions with a hot, subtropical or tropical climate (Corsetti et al., 2012).

Throughout centuries, in Armenia have been preparing protein-rich traditional fermented products known with significant physiological, in particular, antagonistic and antioxidant activities. Fermented dairy products are mostly made from cow, buffalo, sheep's milk or mixtures thereof. The aim of this work was isolation and study of LAB from matsun, yogurt and salted cheese (Motal, Chechil, sheep and cow milk) selected from natural farms from different regions of Armenia.

Materials and Methods

Sampling, bacterial cultures and media

LAB strains were isolated from different samples of matsun, yogurt and traditionally homemade semi-hard and salted cheese, made of ovine, cow milk from individual households in different regions of Armenia. Samples were collected in sterile small bottles and stored at 4°C in the laboratory until they were used in experiments.

Serially dilutes samples from matsun and cheeses were spread plated onto MRS and hydrolyzed milk agar plates (1.2% w/v) and cultivated at 37°C. Different morphotypes of colonies were selected and pure cultures were obtained. The cultures were characterized according to methods for lactic acid bacteria (De Roissart & Luquet, 1994).

The LAB strains were maintained as frozen stocks at -20°C in the plastic tubes containing 40% glycerol. Before use, LAB strains were transferred twice into the appropriate medium and incubated during 48 hours in temperature controlled conditions at 37°C.

Antimicrobial activity of LAB

The spot-on-lawn method on the test-culture inoculated in the solid MPA medium was applied. Antimicrobial activity was assessed by measuring the size of the inhibition area of test culture growth (Ø in mm) after 24 h incubation in thermostat at 30°C (Ten Brinnk et al., 1991).

Cell-free supernatants and test cultures

Cells-free supernatants of LAB cultures in MRS broth (Merck, USA) were collected and were concentrated 3 times by evaporation on a rotary evaporator at 50°C for 15 min.

To determine antimicrobial properties of supernatants, conditionally pathogenic bacteria from the collection of the Laboratory of Microbiological Technologies of SPC "Armbiotechnology" NAS RA, as well as test cultures from the collection of Laboratory of Microbiology and Biotechnology, Sofia University «St. Kliment Ohridsky» (Bulgaria), and yeasts from the company Sibio-93 OOD, Plovdiv (Bulgaria) were used. Incubation of the test cultures were carried out at the culture medium and temperature conditions, appropriate for each culture.

Biochemical characterization of new isolates (API 50 CH test)

API 50 CH test (bioMerieux, Marcy, I'Etoile, France) was used together with API 50 CHL medium for identification of *Lactobacillus* and related species, while the API 50 CHB/E medium was applied for identification of *Bacillus, Enterobacteriaceae, Vibrionaceae* and related to them species. Results were processed by a special software called «Identification software for *Lactobacillus*» (Delarras et al., 1979).

Isolation of DNA and PCR for 16S rRNA sequencing

A modified method of Delley et al. (1990) was applied. Exponential LAB cultures grown in MRS broth

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(for 16-20 h) at 37°C were centrifuged at 10000 rpm for 5-7 min and washed with 1M NaCl.

To amplify the DNA from isolated LAB, the method of 16S PCR was employed using universal primers for *Enterobacteriaceae,* and marker Genladder (100 bp, plus 1.5 kb, GENAXXON, Bioscience) (Weisburg et al., 1991).

Applied primer pairs were: fD1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and rD1 (5'-TAA GGA GGT GAT CCA GGC-3'). For the polymerase chain reaction was used a standard kit (puReTaq Ready-To-Go PCR kit, Amercham). To perform the PCR reaction, primers (0.6 μg), $MgCl_2$ (10 mM, 3.75 μg) and DNA (1 μg) were added to the kit, and the total volume was adjusted to 25 μg with distilled water. Amplification was conducted in a Thermal Cycler (Perkin, Elmer 2400, CA, USA). The program for amplification was as follows: initial denaturation at 94°C for 5 min, 35 cycles: denaturation at 94°C for 1 min, "annealing" DNA renaturation at 56°C for 1.15 min, extension at 72°C for 1.15 min, and final extension at 72°C for 5 min.

DNA gel electrophoresis of the investigated LAB was carried out by using an agarose gel (0.8% agarose, Seakem GTG in Trisborat buffer, TBB). The gel was stained in ethidium bromide for 5 min, then washed with water and photographed. Nucleotide sequence of the obtained amplified 16S rDNA was determined by "MACROGEN" (Korea). The results were analyzed using the program Lasergene (1999). To determine species belonging to LAB, sequences of DNA bases of the investigated LAB were compared with 16S rDNA sequences of the known species of microorganisms from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Determination of stability

The stability of the LAB to the action of bile was determined by standard methods. Survival of the LAB to conditions, similar to those in the gastrointestinal tract (the influence of digestive enzymes and pH in the range 3.0-8.0) were tested according to the generally accepted method. Determination of the sensitivity of the LAB to the enzymes was determined by the method, described by Fernándes de Palencia et al. (2008).

Samples of cell-free supernatants were exposed to different temperatures from 30 to 100ºC for 5 min and autoclaved at 121ºC for 20 min, cooled and assayed for bactericidal activity. The sensitivity to different pH was estimated by adjusting the pH of supernatant to pH 3, 4, 5, 6, 7, 8, 9 and 10 with 1N NaOH and 4N HCl. After 120 minutes of incubation at room temperature (29±1ºC), pH of samples were adjusted to 5.0 and bactericidal activity was tested against the indicator strains (Fernandes et al., 2008; Thornton, 1996).

Effect of enzymes on the antibacterial activity of supernatants

The supernatants were assessed to determine their sensitivity to various proteolytic enzymes. Samples were treated with proteinase-K (5 mg/mL, 36 unit/mg, Sigma), trypsin (5 mg/mL, Pure from bovine pancreas 3x, activity 2500 NFU/mg, HIMEDIA), pepsin (5 mg/mL, Extra pure 1:3000, HIMEDIA), pronase E (5 mg/mL, from *Streptomyces griseus*, activity 4 unit/mg, Sigma), catalase (5 mg/mL, 2860 unit/mg). 200 μl of preparations were added to 20 μl of each enzyme (final concentration 0.5 mg/ml enzyme) and incubated at 37°C for 2 h and then boiled at 100°C for 5 min. After incubation pH of samples were adjusted to 5.0 and residual activity was tested by spot on lawn method (Thornton, 1996).

Resistance of LAB to antibiotics

The resistance of LAB to antibiotics was determined by disk-diffusion method. The following BBL™ disks with antibiotic were used: amoxicillin 30 mg (AmC-30), erythromycin 15 mg (E-15), tetracycline 30 mg (Te-30), chloramphenicol 30 mg (C-30), penicillin 10 mg/EUI (P-10), streptomycin 10 mg (S-10), enrofloxacin 5 mg (ENO-5) and ciprofloxacin 10 mg (CIP-10), all from Becton Dickinson & Co (BD).

Results and Discussion

More than 140 cultures of the LAB were isolated by us from several samples of different cheese, matsun yogurt, from nine regions of the Armenia. All LAB strains were correspondingly numerated. Isolated LAB strains were examined microscopically for cellular morphology. Results of microscopic analysis showed that the LAB presented mainly rod-shaped and coccoid cells of different sizes. The criteria for pre-screening of isolated new strains of LAB were the ability of strains to ferment the milk and bactericidal activity at pH 6.0. Selected LAB were able to ferment the milk for different time intervals: 80% for 16 h, and the rest for 40 h. Only one strain (No. 103) can ferment milk for 8 h. For further tests have been selected strains which were able to ferment milk up to 16 hours.

The results of these studies have shown that the investigated strains differ in the needs of certain sugars, which is displayed in

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the difference of staining intensity of API 50 CH medium after incubation. Computerized data processing as an example of some strains (according to the taxonomic analysis and classification of microorganisms in the homologous group) showed that the strain No. 65 had 99.9% similarity with *L. plantarum*, strain No. 66 had 99.9% similarity with *L .plantarum*, strain No.103 showed 78% similarity with *Str. lactis*, and strain No.109 was 99.9% similar with *L. rhamnosus.* For identification of strains No. 64 and 103 were used primers specific for enterococci and streptococci. The results showed that the strain No. 64 has genetic identity with enterococci, and strain No. 103 has identity with *Streptococcus*. In parallel, the usage of selective medium for the cultivation of enterococci also confirmed their genetic identity with enterococci.

For determination of the genera and species of selected strains, 16S RNA sequencing was used. The 16S ribosomal DNA sequence was determined by direct sequencing. Universal primers for *Enterobacteriacea* and Genladder marker (100 bp) were used. The genetic analysis showed that the isolated strains are closely related to the LAB strains. Results are presented as a dendrogram (Figure 1). The diagram revealed that the bacterial strains are closely related to each other.

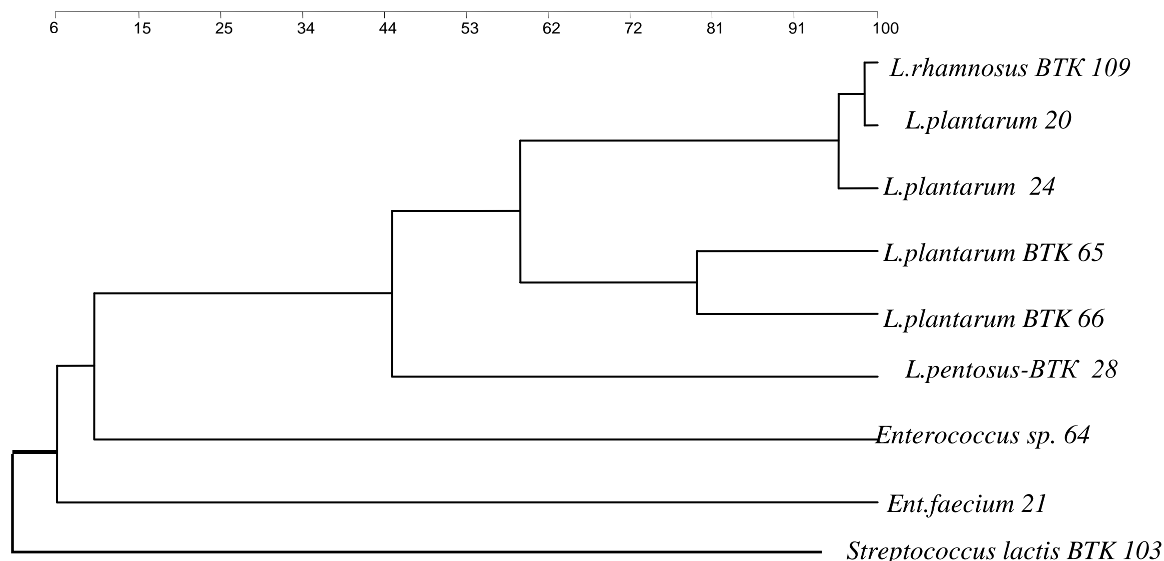

Figure 1. *A dendrogram showing related positions of some isolated LAB strains on the basis of RAPD PCR data*.

One of the important properties of the bacteria used as probiotics and functional food products is that they must be resistant to the influence of proteolytic enzymes, bile. Since different sections of the intestine are characterized by different value of pH, probiotic microorganisms passing through the gastrointestinal tract are exposed to changes in acidity of the environment. With this purpose, the survival of isolated LAB at different pH values was investigated. The stability of the studied strains to the action of several enzymes and to different concentrations of bile was demonstrated. Results of comparative evaluation of probiotic properties of isolated strains of LAB are presented in Table 1. As it seen, the investigated strains exhibited different sensitivity to antibiotics and were able to inhibit the growth of antibiotic-resistant strains of *E. coli.*

Supernatants, obtained after growth of LAB maintained its antibacterial activity at pH 3.0 – 6.5. The strains also differ by their sensitivity to the proteolytic enzimes.

All strains of LAB were resistant to the antibiotics S-10, ENO-5 and CIP-10. *L. plantarum* 65 and *L. plantarum* 66 were sensitive to AmC-30, E-15, Te-30, P-10, S-10, and stable in presence of ENO-5 or S-10. *L. pentosus* 28 showed low stability to tested antibiotics.

Thus, our preliminary results indicated, that investigated strains and their supernatants possess all the properties, appropriate to the requirements for probiotics.

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Table 1. *Comparative evaluation of probiotic properties of isolated LAB.*

Characteristics		Supernatants of investigated LAB					
		L. pentosus 28	*Enterococcus sp.* 64	*L. plantarum* 65	*L. plantarum* 66	*Str. lactis* 103	*L. rhamnosus* 109
		Growth inhibition zone, Ø (mm)					
Resistance to enzymes	Control	26,0±2	24,0±2	30,0±2	24,0±2	22,0±2	30,0±2
	Pepsin	22,0±2	10,0±1	24,0±2	20,0±1	abs	28,0±2
	Trypsin	22,0±2	14,0±1	27,0±2	22,0±2	abs	22,0±2
	Proteinase K	22,0±2	20,0±1	20,0±1	18,0±1	18,0±1	24,0±2
	Pronase E	20,0±1	18,0±1	28,0±2	20,0±1	18,0±1	32,0±2
	Katalase	24,0±2	20,0±1	28,0±2	20,0±1	20,0±1	27,0±2
Stability to bile (%)	0,0	18,0±1	14,0±1	14,0±1	18,0±1	20,0±1	20,0±2
	0,1	20,0±1	18,0±1	16,0±1	14,0±1	24,0±2	24,0±2
	0,2	26,0±2	16,0±1	18,0±1	16,0±1	28,0±2	22,0±2
	0,5	20,0±1	14,0±1	16,0±1	20,0±1	28,0±2	24,0±2
	1,0	22,0±2	20,0±1	20,0±2	22,0±2	22,0±2	24,0±2
Activity at pH 3.0-8.0	3,0	26,0±2	24,0±2	18,0±1	22,0±2	22,0±2	26,0±2
	4,0	22,0±2	20,0±1	16,0±1	12,0±1	16,0±1	30,0±2
	5,0	46,0±2	28,0±2	20,0±1	20,0±1	16,0±1	24,0±2
	6,0	34,0±2	24,0±2	24,0±1	12,0±1	16,0±1	24,0±2
	7,0	abs	abs	11,0±1	abs	10,0±1	6,0±1
	8,0	abs	abs	9,0±1	abs	10,0±1	5,0±1
Resistance to antibiotics (mg)	AmC	40,0±2	30,0±2	30,0±2	30,0±2	20,0±1	20,0±1
	E	40,0±2	25,0±2	28,0±2	30,0±2	25,0±2	15,0±1
	Te	40,0±2	25,0±2	22,0±2	20,0±1	22,0±2	abs
	C	30,0±2	15,0±1	28,0±2	25,0±2	30,0±2	20,0±1
	P	40,0±2	15,0±1	22,0±2	12,0±1	25,0±1	abs
	S	2,0±1	abs	abs	abs	15,0±1	8,0±1
	ENO	15,0±1	abs	abs	abs	17,0±1	15,0±1
	CIP	15,0±1	2,0±1	2,0±1	abs	abs	abs
Activity against *E. coli* K-12 (resistant to antibiotics)	*E. coli* K-12	20,0±1	20,0±1	20,0±1	21,0±1	10,0±1	20,0±1
	Sm+	24,0±2	20,0±1	20,0±1	20,0±1	20,0±1	20,0±1
	Km+	25,0±2	18,0±1	20,0±1	18,0±1	abs	22,0±2
	Ap+	18,0±1	20,0±1	18,0±1	20,0±1	abs	20,0±1
	Cm+	28,0±2	20,0±1	20,0±1	18,0±1	abs	24,0±2
	Tc+	20,0±1	18,0±1	10,0±1	18,0±1	20,0±1	abs

Legend: **Sm+**, Streptomycin resistant; **Km+**, Kanamycin resistant; **Ap+**, Ampicillin resistant; **Cm+**, Chloramphenicol resistant; **Tc+**, Tetracycline resistant; **abs**, absence of inhibitory effect on the test culture growth.

Lactic acid bacteria exert a strong antagonistic activity against many food-contaminating microorganisms as a result of the production of organic acids, hydrogen peroxide, diacetyl, inhibitory enzymes and bacteriocins (Atta et al., 2009). The spectrum of antimicrobial activity of the investigated LAB, their ability to inhibit the growth of several strains causing the spoilage of food products, was also studied. The results are shown in Table 2. Thus, our experimental data shown, that isolated strains of lactic acid bacteria inhibited the growth of different microorganisms – bacteria and yeasts, especially pathogenic bacteria. This can be resulting from differences in nature of antibacterial compounds, produced by LAB. Our experimental data are confirmed by literature information described by other authors in relation to different susceptibility of *Listeria, Clostridium, Propionobacteria, Enterococcus, Streptococci* towards various lactic acid bacteria.

Microorganisms, by virtue of their metabolic activities,

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contribute to the development of peculiar properties such as taste, aroma, visual appearance, texture, shelf life, and safety (Atta et al., 2009; Corsetti et al., 2012).

At first, fermented foods were obtained through spontaneous fermentation, but this kind of process often resulted in an uncontrollable fermentation, so different skills have been developed for controlling technical parameters during the fermentation processes. Actually, in some sectors of food industry the fermentation is controlled through the inoculation of selected starter cultures.

The LAB can be used as basis for obtaining new products that can be used as functional nutrition. On the basis of obtained data, by co-cultivation of certain investigated strains we have developed a new combination from strains that could be used for preparing of functional food. Data about co-cultivation of chosen best combination of LAB strains is shown in Table 3. As can be seen from the given data, during co-cultivation in milk, investigated strains *L. rhamnosus* 109 and *Streptococcus sp.* 103, *L. pentosus* 28 and *Streptococcus sp.* 103 had ripening time of 6-8 hours. On day 5, the acidity (Thorner degrees, °T) was 50-60°T (except *L. pentosus* 28 + *Streptococcus sp.* 103) when stored in a refrigerator at 4°C, while during separate fermentation, an increase in the acidity (150-220°T) for *L. rhamnosus* 109 and *Streptococcus sp.* 103 was detected. The acidity of the *L. pentosus* 28 increased to 270°T for the same time of storage. Storage of this product for 15 days did not alter its organoleptic characteristics.

Table 2. *Antimicrobial activity of supernatants of LAB strains.*

Microorganisms	Supernatants of LAB strains					
Conditionally pathogenic	*L. pentosus 28*	*Enterococcus sp. 64*	*L. plantarum 65*	*L. plantarum. 66*	*Streptococcus sp. 103*	*L. rhamnosus 109*
	Growth inhibition zone, Ø, mm					
S. typhimurium G -38	30.0±3	24.0±2	20.0*±1	18.0±1	25.0±2	32.0±2
E. coli K- 12	24.0±2	20.0±1	18.0*±1	20.0±1	20.0±1	25.0±2
E. coli 5009	ND	10.0±1	12.0±1	12.0±1	4.0±1	14.0±1
B. subtilis 17 -89	24.0±2	20.0*±1	16.0±1	16.0±1	22.0±2	28.0±2
B. subtilis sp. 1	30.0±2	24.0*±2	28.0±2	20.±1	18.0±1	22.0±1
B. subtilis sp2.	28.0±1	20.0*±1	30.0±2	28.0±2	20.0±1	28.0±2
B. thuringensis sp.	28.0±1	20.0±1	24.0*±2	20.0±1	ND	ND
B. mesenterius sp.	28.0±2	30.0±2	26.0±2	28.0±2	20.0±1	28.0±2
Pseudomonas sp.	20.0±1	22.0±2	20.0±1	16.0±1	ND	ND
Listeria innocua sp.	ND	4.0±1	4.0±1	6.0±1	6.0±1	12.0±1
St. aureus 5233	ND	absent	absent	absent	absent	18.0±1
L. brevis F 145	ND	6.0±1	4.0±1	absent	absent	12.0±1
Pathogenic						
Salmonella sp.	8.0±1	6.0±1	8.0±1	9.0±1	ND	ND
E. coli sp.	10.0*±1	10.0*±1	10.0*±1	10.0*±1	ND.	ND.
P. vulgaris sp.	11.0±1	absent	absent	10.0±1	ND	ND
E. cloacae sp. 1)	10.0±1	7.0*±1	absent	11.0±1	ND	ND
E. cloacae sp -2)	13.0±1	11.0±1	absent	11.0±1	ND	ND
Yeasts						
Rhodotorula 96	34.0±2	20.0*±1	12.0±1	16.0±1	30.0±1	absent
Rhodotorula C-1	32.0±1	10.0*±1	8.0*±1	6.0*±1	28.0±2	absent
Saccharomycopsis sp.151	14.0±1	absent	absent	absent	12.0±1	6.0±1
Kluyeromyces lactis 412	20.0±1	absent	absent	absent	10.0±1	4.0±1
Saccharomyces cerevisiae	24.0±2	absent	absent	absent	10.0*±1	abs.
Candida tropicalis 103	24.0±2	absent	absent	absent	20.0±1	10.0±1

Legend: * - secondary growth of bacteria; **absent** – lack of the effect on the growth of test-cultures; **ND** - not determined.

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Table 3. *Co-cultivation of some new strains of LAB.*

Strains	Inoculum (%)	Time of fermentation	Acidity, (°T)		Taste of the product
			1-st day	15-th day	
L. rhamnosus 109	5	24	70±5	150±10	sweet and sour
Streptococcus sp. 103	5	18	90±5	200±20	sweet and sour
L. pentosus- 28	5	20	70±5	250±20	sweet and sour
L. rhamnosus 109 + Streptococcus sp. 103	3+3	6	70±5	50±5	sweet
L. pentosus-28 + Streptococcus sp. 103	3+3	6	90±5	100±15	sweet and sour

Thus, some strains with probiotic properties, isolated from the matsun yugurt and salted cheese, are perspective for creation of the functional food products.

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