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A STUDY OF LACTIC ACID BACTERIA AND PROSPECTS FOR THEIR APPLICATION

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The comparative biodiversity of lactic acid bacteria (LAB) isolated from fermented dairy products produced in the southern regions of Armenia made from the milk of some domestic farm animals has been shown. Strains with probiotic properties belonging to *Lactobacillus* and *Enterococcus* genera were selected. LAB isolated from dairy products from different regions of Artsakh were mainly represented by strains of the genus *Enterococcus*. It has been shown that some strains of LAB during the growth process are able to synthesizing simultaneously different metabiotics – bacteriocins, polysaccharides, arginine, have bile salt hydrolase (BSH) activity which leads to the breakdown of bile salts and the taurine and glycine amino acids are formed as a result. The selected strains can be used for the production of functional foods.

Biodiversity – lactic acid bacteria – metabiotics – polysaccharides – arginine – fermentative activity

Ցույց է տրվել կաթնաթթվային բակտերիաների (ԿԹԲ) համեմատական կենսաբազմազանությունը, որոնք մեկուսացված են Հայաստանի հարավային շրջանների որոշ ընտանի գյուղատնտեսական կենդանիների կաթից արտադրված թթու կաթնամթերքից: Ընտրվել են *Lactobacillus* և *Enterococcus* ցեղերին պատկանող պրոբիոտիկ հատկություններով օժտված շտամները: Արցախի տարբեր շրջանների թթու կաթնամթերքից մեկուսացված կաթնաթթվային բակտերիաները հիմնականում ներկայացված են *Enterococcus* ցեղի շտամներով: Ցույց է տրվել, որ ԿԹԲ-ների որոշ շտամներ աճման ընթացքում միաժամանակ սինթեզում են տարբեր մետաբիոտիկներ՝ բակտերիոցիններ, բազմաշաքարներ, արգինին, դրսևորում են լեղու աղերի հիդրոլազի (BSH) ակտիվություն, ինչը բերում է լեղու աղերի ճեղքմանը, և արդյունքում առաջանում են տաուրին և գլիցին ամինաթթուները: Ընտրված շտամները կարող են օգտագործվել ֆունկցիոնալ մթերքների արտադրության համար:

Կենսաբազմազանություն – կաթնաթթվային բակտերիաներ – մետաբիոտիկներ – պոլիսախարիդներ – արգինին – ֆերմենտատիվ ակտիվություն

Показано сравнительное биоразнообразие молочнокислых бактерий (МКБ), выделенных из кисломолочных продуктов, произведенных в южных регионах Армении из молока некоторых домашних сельскохозяйственных животных. Отобраны штаммы с пробиотическими свойствами родов *Lactobacillus* и *Enterococcus*. МКБ, выделенные из кисломолочных продуктов из разных регионов Арцаха, в основном были представлены штаммами рода *Enterococcus*. Показано, что некоторые штаммы МКБ в процессе роста синтезируют одновременно разные метабиотики – бактериоцины, полисахариды, аргинин; обла-

дают активностью гидролазы желчных солей (BSH), что приводит к расщеплению солей желчных кислот с образованием аминокислот таурина и глицина. Отобранные штаммы могут быть использованы для производства продуктов функционального питания.

Биоразнообразие – молочнокислые бактерии – метабиотики – полисахариды – аргинин – ферментативная активность

Fermented milk products have long been associated with various demographic groups and are an integral part of their ethnicity. In recent years, special attention has been paid to the study of the microflora of national traditional fermented dairy products and creation of new ones based on isolated bacteria (solitary or associated). This is one of the most practical and widespread methods for preserving the biodiversity of microorganisms and using their organoleptic and nutritional properties. The experience of the widespread use of probiotics in the food industry as a whole confirms the history of safe human consumption of symbiont microorganisms of the intestinal microflora, to which belong most of probiotics. In fact, they lack the ability to cause infectious and invasive diseases in consumers.

Many traditional fermented milk products have been and are being produced in Asia, Africa, the Middle East, Northern and Eastern Europe and are currently used by many scientists to study their microflora and create new probiotic products. The traditional fermented milk product in regions with a cold climate contains mainly mesophilic bacteria, while thermophilic bacteria, which mainly include representatives of the genera *Lactobacillus* and *Streptococcus* prevail in regions with a hot, subtropical and tropical climate. Analyzing some data on LAB biodiversity of some southern regions with hot climatic conditions, the advantage of the presence of lactic acid bacteria, mainly of the *Lactobacillus* genus is evident [3, 7, 9, 17].

Extensive studies on the identification of matsun microflora in various regions of the Caucasus have been carried out in recent years. The microflora of matsun - the Caucasian traditional fermented milk product includes various species of microorganisms and their symbiotic associations. Georgian scientists isolated and described matsun bacteria: *Lactobacillus delbrueckii subsp. lactis, Lactobacillus acidophilus, Lactobacillus casei, Streptococcus thermophilus, Lactococcus lactis subsp. lactis, Lactobacillus lactis subsp. diacetylactis, Lactobacillus lactis subsp. cremoris, Enterococcus durans, Lactobacillus delbrueckii* [4]. The *Lactobacillus bulgaricus* strain was not detected. In Armenia, strains of acidophilic lactobacilli have been used for production of yogurt, which is fermented mainly by strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, while the microflora of matsun contains various types of microbes. A number of researchers have differently described the microbiota of matsun: *Lactobacillus delbrueckii* subsp. *lactis, Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus acidophilus, Streptococcus thermophilus, Lactococcus lactis, Lactococcus lactis* subsp. *cremoris, Geotrichum candidum, Saccharomyces*, *Candida*, and other yeast species [1].

In the scientific literature there are practically no data on the probiotic properties of LAB isolated from milk or matsun made of milk of different domestic animals (goats, sheep, buffaloes, donkeys, cows) from various households of the Southern regions of Transcaucasia. The authors showed that *Lactococcus* and *Enterococcus* species were common in many samples, in particular of those of Artsakh.

From the Microbial Culture Collection of the Microbial Depository Center (MDC) of the SPC "Armbiotechnology" NAS RA, the LAB strains, isolated from the

fermented milk of sheep, donkeys, buffaloes, cows, goats from different regions of Armenia and Artsakh were investigated [4, 10]. Some of them were shown to have probiotic properties [10, 18]. The 16S RNA gene sequencing method revealed that LAB strains isolated from the fermented milk of cows, were mainly represented by several species of genera *Lactobacillus* and *Enterococcus*; LAB strains isolated from the fermented milk of buffalo were mainly represented by strains of the genus *Lactobacillus*: *Lactobacillus plantarum, Lactobacillus casei, Lactobacillus rhamnosus, Lactobacillus acidophillus, Lactobacillus lactis*. The strains of LAB isolated from samples of donkey and goats milk were mainly represented by strains of the genus *Enterococcus*: *Enterococcus faecium, Enterococcus durans, Enterococcus faecalis*.

Thus, the comparative investigation of biodiversity of lactic acid bacteria (LAB) isolated from fermented dairy products produced in the southern regions made from the milk of some domestic farm animals was of interest.

Materials and methods. **Microbial strains and growth media.** The endemic LAB cultures were isolated from various samples of fermented dairy products produced from the milk of some domestic farm animals from rural households of several regions of the Republic of Armenia (RA) and Artsakh. Samples were collected in sterile small bottles and stored at 4^0C in the laboratory until they were used in experiments. Serially diluted samples were spread on MRS agar (Merck, Germany) and hydrolyzed milk agar (1.2 % w/v) and cultivated at 37^0C. Different morphotypes of colonies were selected and obtained pure cultures were characterized according to standard methods for lactic acid bacteria. Pure cultures of LAB were maintained as frozen stocks at -20^0C in the MRS broth containing 40 % glycerol. Before use, they were transferred twice into the appropriate medium and incubated during 48 hours under temperature controlled conditions in thermostat at 37^0C.

Inoculum preparation and obtaining of cell-free culture broth. Single colonies were grown in 5 ml of MRS broth (37^0C, 24 h) and then were transferred into 100 ml-Erlenmeyer's flask containing 50 ml of nutrient media and incubated overnight at 37^0C for 24 hours in the thermostat. For cultivation of LAB strains, the following nutrient media were used: **No. 1**. The nutrient media prepared on the basis of curd whey with addition of the following salts: $(NH_4)_2SO_4$-0.8; KH_2PO_4-0.1; $MgSO_4$-0.2; yeast extract-0.3; pepton-0.3; $MnSO_4$ -0.05; $CH_3COONa \cdot 3H_2O$ -0,2 [Tkhruni et al., 2015]. **No. 2** MRS agar and broth (Merck (Germany), ISO (Italy), HiMedia (India)). 50 ml of the obtained inoculum was transferred into 800 ml of media in 1L Erlenmeyers flask and grown at 37^0C for 48 hour in the thermostat. At the end of culture growth, cell concentration achieved 7.2 $x10^8$ CFU/ml (of titration) and pH reduced to 3.5-4.2. After the end of growth, culture broth was centrifuged at 5,000 rpm during 20 min and the obtained cell free culture broth (CFC broth) was used for future purification.

Purification of the CFC broth. CFC broth of LAB strains was purified by the standard method [19]. Survival of LAB in the conditions close to those in the intestine (influence of digestive enzymes, pH in the range of 3.0-8.0) was checked according to the generally accepted method. Determination of ability to adhesion was carried out, antioxidant activity of LAB was determined according to Guidelines MU 2.3.2.2789 -10 [20].

Isolation of protein-like substances with antimicrobial properties from the concentrated CFC liquid was carried out using the gel filtration method. Concentrated CFC liquids were fractioned on the column with Sephadex G-25 superfine resin (1.5 x 50 cm, vol. 100 ml). Each fraction (n=45) eluted from the column, was tested for

antibacterial activity against test cultures *S. typhimurium* G 38 and *B. subtilis* 17-89. Fractions displaying bactericidal properties were pooled and vacuum evaporated at a temperature of 50-55^{0}C, residual pressure 0.01 MPa.

High-performance liquid chromatography (HPLC). Fractions, obtained after gel filtration method, were purified by HPLC method with application of various systems (Semi-preparative "Avex ODS" C_{18} column (8 by 250mm, Waters and Shimadzu, Japan); Shimadzu LC-20 analytical C_{18} column (4.6 by 250 mm, Symmetry, USA, with a detector Diode array SPD-20a, auto-sampler). The eluent consisted of bi-distilled water, trifluoroacetic acid (TFA) and acetonitrile (HPLC "SIGMA", USA)). Sample injection volume: 100 µl. Elution was monitored at different wavelength range of 190-400 nm. Detection was performed at 210, 254, 280 nm wavelengths. Fractions eluted from the column were freeze-dried, dissolved in 150 µl of bi-distilled water and tested for antibacterial activity. Fractions, showing maximal antimicrobial activity were selected.

Determination of amino acid. To determine the amino acid content, high-performance liquid chromatography (HPLC), systems (Semi-preparative "Avex ODS" C18 column (8 by 250 mm, Waters and Shimadzu, Japan) was used; Shimadzu LC-20 analytical C18 column (4.6 by 250 mm, Symmetry, USA, with a detector Diode array SPD-20 a, auto-sampler).

Isolation of the exopolysaccharides (EPS) was carried out according to the method, described by Bajpai et al. [2]. The polysaccharides were obtained in the form of a dry powder and tested for the presence of antimicrobial activity. A 10 % solution was used and 100 µl of the tested samples was added to the surface of nutrient agar pre-inoculated with gram-negative *B. subtilis* 17-89.

BSH production was carried out according to the method, described by Lorena R. et al. [13].

Statistical analysis. Microsoft Word 10 and Microsoft Office Excel 2010 applications have been used, the data obtained are valid for $p < 0.05$.

Results and Discussion. Among all studied strains of LAB, the 100 strains of several species of *Lactobacillus* and *Enterococcus* genera from the MDC collection, were selected. It was shown that the selected strains were thermophilic and grew well at pH 8-9. The results of study of main probiotic properties of strains [20] are summarized in Table 1.

Table 1. Characterization of strains by the presence of the main probiotic properties

LAB	Characteristics								
	Activity			Resistance to					Adhesion
	AU/ml	AoA	BSH	Antibiotics	t°C, (42°C)	Bile (0.2-0.5%)	pH 2-3	pH 8-9	
Number of strains studied	96	74	72	62	72	72	78	74	76
%	37.5	30.6	36.8	40.3	77.8	62.5	25	98.6	33.3

Legend: AU/ml - antimicrobial activity
AOA – antioxidant activity
BSH –bile salt hydrolase

Currently, the properties of probiotic strains synthesizing metabiotics (structural components of probiotic microorganisms), which can have functional purposes, are of

interest. According to the modern classification, the main categories of functional nutrition products are lactic acid bacteria and products of their metabolism [16].

Study of antibacterial properties shown, that preparations, obtained from culture liquid (CL) of LAB strains, partially purified by ion-exchange chromatography(AMP), inhibited the growth of multi-resistant bacteria, isolated from patients and animals, with different efficiency. In the same way they also inhibited food spoilage microorganisms of various taxonomic groups of *Salmonella spp., E. coli, Proteus mirabilis, Pasteurella spp., Clostridium spp., Streptococcus spp., Staphylococcus aureus, Shigella spp., Yersinia enterocolitica, Bacillus cereus* [11, 19]. The effectiveness of growth inhibition depends on the species specificity of the strains and, in terms of their antimicrobial activity, is not inferior to the action of antibiotics. Strains of the genus *Enterococcus* isolated from the fermented milk of cows are inferior in antimicrobial activity to strains isolated from the fermented milk of sheep and donkeys. It should be noted that most of the studied strains did not have proteolytic activity.

It is known that LAB synthesize various amino acids during growth, but not all strains synthesize arginine. Arginine is a partially replaceable amino acid. In healthy adults arginine is produced by the body in sufficient quantities. At the same time, in children and teenagers, as well as in the elderly and sick people, the level of arginine synthesis is often insufficient. Arginine is a substrate of NO-synthases in the synthesis of nitric oxide (NO), which is a local tissue hormone with multiple effects from anti-inflammatory to vascular effects and stimulation of angiogenesis. The screening of the ability of LAB strains to synthesize L-arginine showed significant intra-species differences. It was shown that some *Lactobacillus* and *Enterococcus* strains were able to synthesize L-arginine (mg/ml) in 48-hour growth. It was shown that among 10 strains of *L. rhamnosus* species, 60% of strains were able to synthesize L-arginine. Two strains of *Lactobacillus rhamnosus* 20-12 and *Lactobacillus plantarum* 66 showed increase of L-arginine during growth in both nutrient media (MRS, milk). It was shown that *L. rhamnosus* 20-12 and *L. plantarum* 66 had potential probiotic activity and synthesized a protein-like substance (bacteriocin). Results of chromatograms and spectrograms of AMP, obtained from *L.rhamnosus* 20-12, indicate that these potential bacteriocins (BCN1 and BCN2) have peptide bonds. Spectrograms demonstrate that molecular weight of BCN 1 and BCN 2 are 1.427 Da and 602.6 Da, respectively and they can be considered as class II bacteriocins [19].

Various researchers have shown that lactobacilli have a great potential for the synthesis of exopolysaccharides (EPS). Some of them promote the selective growth of LAB and bifidobacteria, thereby playing a positive role in the microbiota and the host immune system [6]. Recently, EPS produced by LAB attract more and more attention, mainly, due to their health benefits [6, 12, 15].

The overall antimicrobial activity and the ability to synthesize polysaccharides of some strains of the genus *Lactobacillus* and *Enterococcus* was studied. The results are represented in Table 2. The comparative overall antimicrobial activity of and the quantitative synthesis of polysaccharides obtained after cultivation of the strains is higher in strains of the genus *Enterococcus*.

Among all studied 21 strains of *Enterococcus* genus, isolated from the donkey milk, some strains synthesized exopolysaccharides, the synthesis of which does not depend on the composition of the nutrient medium, but depends on the temperature of the strain growth. All strains synthesized exopolysaccharides in different amounts from 2 to 15g/l during 48-hour of growth. The analytical HPLC method showed that exopolysaccharides isolated from the culture liquid of probiotic *Ent. faecium* strains consisted of glucose and galactose molecules. However, not all isolated exopolysaccharides had the antimicrobial

activity. Our preliminary result showed that the highest yield of exopolysaccharides was observed in *Ent. faecium* KE-5 strains isolated from donkey milk. It was shown that that *Ent. faecium* KE-5 strains also had the antioxidant activity and synthesized a protein-like substance (bacteriocin). Thus, the data show the presence of a number of properties in the studied strain *Ent. faecium* KE-5 - the production of exopolysaccharides and bacteriocins, the synthesis of arginine and the ability to suppress the growth of a number of pathogenic bacteria from patients, which indicates the prospects of this strain in the creation of new functional products.

Table 2. Characterization of the antimicrobial activity of supernatants (CFCx5) and polysaccharides (test culture S. typhimurium G -38, 48h., 30^0C)

Genus of the strains	Number of the strains	Polysaccharides			Total antimicrobial activity of the supernatants (x5), AU
		%	n	Antimicrobial activity (AU/ml)	
Lactobacillus	13	8	4	1300	20000
Enterococcus	21	10-15	5	2500	27000

A number of authors have shown that some types of probiotics *Bifidobacterium* and *Lactobacillus*, which have the antimicrobial activity, produce bile salt hydrolase (BSH) [13]. Such probiotics containing active BSH increase the production of bile salts from cholesterol in their colonized area, thereby reducing the cholesterol problems. As a result of breaking down bile salts, the amino acids taurine and glycine are formed. The activity of BSH of probiotic LAB strains was studied when in a nutrient medium containing 1% bile. 72 Strains from the fermented milk of goats, donkeys, buffaloes, cows were studied, of which 35% had the ability to break down bile salts. According to the results of conducted research, strains were selected that hydrolyzed bile salts with the formation of crystals after 48-hour growth at a temperature of 37 ºC. It was shown by HPLC that the breakdown of bile salts resulted in amino acids taurine and glycine formation in the CL. It was shown that the quantitative formation of taurine and glycine during deconjugation of bile salts by *Ent. faecium* AA-20-2 and *Ent. durans* AA-11-6 strains differed from the parameters of the studied *Ent. faecium* KE-5 and *Ent faecalis* AG-32-6 strains. The probiotic strains *Ent. faecium* AA 20-2 showed the largest increase in glycine, while *Ent. durans* AA 11-6 showed the largest increase in the amino acid taurine. The studied strains after cultivation in a nutrient medium containing 10% bile were selected. The results showed that the resistance of 10% bile led to an increase in the content of taurine and glycine that indicates an increase in BSH activity in the selected strains. The probiotic properties of strains and the ability to break down cholesterol allow to use them for the production of biofunctional food products. Table 3 shows the names of the selected strains and metabiotics synthesized by the strains in the process of growth.

Table 3. Characteristics of LAB metabiotics producers

LAB strains	Nutrient medium		Metabiotic in CL, mg/ml
	Nutr. med. No. 1	MRS broth	
L.rhamnosus 20-12	0.4	2.5	*arginine*
L.plantarum 66	0.4	2.0	*arginine*
Ent. faecium KE-14	40	ND	*polysaccharide*
Ent. faecium KE-5	40	ND	*polysaccharide*
Ent. durans AA-11-6	ND	2.10	*taurine*
Ent. faecium AA-20-2	ND	1.25	*glycine*

The β-galactosidase activity of LAB isolated from the national fermented product matsun was studied. It was shown that some endemic lactic acid bacteria isolated from cow's milk matsun samples had β-galactosidase activity. Three strains *of L.fermentum* 27-6, *L.delbrueckii ssp. lactis* 17-13, *L. mesenteroides ssp. cremoris* G-6 with β-galactosidase activity were selected from 370 studied lactic acid bacteria. These strains differ in the activity of β-galactosidase and the amount of residual lactose in milk. *L. fermentum* 27-6 strain has high β-galactosidase activity and high residual amount of lactose, while *L. mesenteroides ssp. cremoris* G-6 with low β-galactosidase activity has high residual amount of lactose under the same conditions [5].

Thus, the study of bacteria of the genera *Lactobacillus* and *Enterococcus* isolated from fermented milk of different animals and selected for probiotic properties can be recommended for their use to create functional food products.

Conclusion.

In the Microbial Culture Collection of the Microbial Depository Center (MDC) at the SPC "Armbiotechnology" NAS RA, contains cultures of lactic acid bacteria (LAB) isolated milk and other dairy products made from buffalo, goat, cow, donkey milk. The selected strains synthesize metabiotics (bacteriocins, arginine and polysaccharides) in the process of growth, have β-galactosidase and BSH activity. Research in this direction opens up the prospect for targeted studies in the field of creating new functional food products with broader therapeutic and prophylactic properties.

Ethical Approval. This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest. The authors declare that they have no conflict of interest.

Data availability. All data generated or analyzed during this study are included in this published article and available from the corresponding author on reasonable request.

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