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ORIGINAL ARTICLE

Investigation of new exopolysaccharides produced by strains of donkey milk

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ABSTRACT

Background and Objectives: It has been shown that strains of the genus *Enterococcus* isolated from donkey milk from different regions of the Republic of Armenia have antimicrobial activity, synthesize different polysaccharides and produce disaccharide polymers (glucose and galactose). The quantitative synthesis of polysaccharides (8-15%) depends on the composition of the nutrient medium, temperature and growing time.
Materials and Methods: Species identification of LAB strains was confirmed by 16S rDNA gene sequencing method using universal primers LAB strains. The methods used for extraction, purification and detection of exopolysaccharides are based on the method of Sørensen et al. (2022). The antibacterial activity of EPS was investigated by agar diffusion assay. Determination of the immunostimulating property was carried out using the ELISA method.
Results: The antimicrobial activity of the polysaccharide and antimicrobial protein-like fractions of the genus *Enterococcus* strains depends on its concentration, time of interaction with the test culture, and the species of the pathogenic bacteria.
Conclusion: The obtained results were shown that strains isolated from fermented donkey milk that are capable of synthesizing two substances of different nature with high antimicrobial properties during the growth process are promising for further research and application for their use as probiotics and biopreparations in pharmaceuticals.

Keywords: Exopolysaccharides; Protein-like fractions; Interaction; Donkey milk; Antimicrobial activity

INTRODUCTION

In recent years, much attention has been paid to products of biologically active compounds synthesized by lactic acid bacteria (LAB) isolated from the milk of different animals (1, 2).

Matsoon (national product of Armenia) is characterized by more abundant microflora representing different microbial species and their symbiotic associations. It is essentially important that the microbial composition of Matsoon starters and of the final product itself significantly differs from various Caucasian areas but is very stable and characteristic for the region originated. Due to the region and milk used, the qualitative and quantitative composition of Matsoon microflora significantly differs. It permits

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to isolate new microbial forms and their associations adapted and stabilized for many years to certain ecological and geographical conditions (3).

Currently the development of concepts for the use of probiotics also includes the production and use of metabiotics. Metabiotics of LAB can contain bacteriocins with antimicrobial activity against multidrug-resistant bacteria and other low molecular weight antimicrobial molecules, short-chain fatty acids, exopolysaccharides, antioxidants, different proteins, including enzymes, peptides with various activities, amino acids, and others (4, 5). In recent years, we have been focused on the research of the metabiotics of synthesized LAB. Metabiotics are structural components of probiotic microorganisms and their metabolites, or signal molecules with a certain chemical structure that are formed during their growth.

Antimicrobial peptides such as bacteriocins produced by some bacteria, can serve as the basis for serious consideration as an alternative to traditional antibiotics. These molecules have significant activity against other bacteria (including strains resistant to antibiotics), are stable, and can have a narrow or wide range of activity. Bacteriocins can even be made in intestines by probiotic bacteria and struggle with intestinal infections without affecting microflora harmlessly (6).

Various researchers showed that lactic acid bacteria (LAB) have great potential for the synthesis of exopolisaccharides (EPS). Some of them contribute to the selective growth of lactic acid bacteria and bifidobacteria by playing a role in the microbiota and the host's immune system (7, 8). EPS, produced by the LAB, attracts more and more attention, mainly due to their advantages for health, such as immune stimulation, antimutagenic activity, increased content of antioxidants and antitumor activity, and reducing cholesterol levels (9-11).

Different researchers have shown that lactobacilli have great potential for the synthesis of exopolysaccharides; however, the functions of these biopolymers are not fully understood, although there are enough studies on their use in food (12, 13). The exopolysaccharides are thought to play a significant role in the colonization of lactic acid bacteria to various ecosystems by facilitating the colonization of bacteria to intestinal mucosa, thus enhancing the immunity of the host (14). Nowadays, the exopolysaccharides are used as bio-thickeners due to their stabilizing, emulsifying, or gelling properties, especially in the food industry (15). Recently, EPS produced by LAB have received increasing attention, mainly because of their health benefits,in particular, immune stimulation, antimutagenicity, antioxidants, and the antitumor activity of fermented dairy products prepared with EPS-producing LAB.

In recent decades, the immunomodulatory potential of EPSs has received a lot of scientific consideration. Many in vitro studies have demonstrated that EPSs produced by different LAB species have immunomodulatory ability (16). The phosphate group (a good inducer of the immune response) plays a critical role and characterizes the immunomodulatory effects of EPSs. Phosphate molecules can activate various immune cells (such as macrophages and lymphocytes) and initiate immune responses. According to these results, it can be speculated that EPS generated under acid stress may exhibit stronger immunological properties (17). The immunoregulatory function of EPS strongly correlates with the chemical structure and configuration of these bacterial exopolysaccharides. Another health-promoting function of EPSs produced by LAB are cholesterol-lowering effects. In an in vitro assay, EPSs produced by *L. plantarum* BR2 showed cholesterol-lowering properties (45%). Based on animal and in vitro experiments, several hypotheses to explain the cholesterol-lowering mechanism of EPSs have been proposed, including bile removal, anabolism, cholesterol conversion, and co-precipitation effects (18, 19). LAB-EPS can improve intestinal health by adhering to intestinal epithelial cells, restoring impaired intestinal barrier function, and inhibiting biofilm formation by bacterial pathogens. LAB-EPS has also been found to regulate the immune system by boosting the proliferation of T/B lymphocytes, natural killer cell tumoricidal activity, mitogenic activity, mononuclear cell phagocytic capacity, and inducing cytokines, therefore enhancing the host immune response to pathogens (20, 21).

EPS produced by LAB varies widely in structure, and understanding these differences is key to assessing EPS functionality. In this study, 201 LAB strains were evaluated for heteropolysaccharide production, with the following LAB strains evaluated for EPS production: *Lactobacillus* (L.) *delbrueckii* subsp. *bulgaricus*, L. *delbrueckii* subsp. lactis, *L. helveticus, L. casei, L. paracasei, L. rhamnosus, Enterococcus (Ent.) faecalis, Ent. faecium*, and *Streptococ-*

cus thermophilus. This study highlighted the great structural diversity and molecular weight differences (from 8 kDa to 5000 kDa) of heteropolysaccharides (22, 23). The results have shown that the most frequently encountered species are *Lactobacillus paracasei, Lactococcuslactis,* and *Carnobacterium maltaromaticum,* less *Leuconostoc, Enterococcus, Streptococcus,* and yeast *Kluyveromyces marxianus* (24). We have shown that the LAB strains were mainly presented with several species of *Enterococcus* genus. The isolated LAB from fermented milk of different domestic animals (cow, sheep, donkey, buffalo, goat) and hand-selected LAB were mainly represented by the genus *Enterococcus.* It was shown that the supernatant selected strains inhibit the growth of gram-negative, gram-positive bacteria, and some pathogenic bacteria (25).

The data of the study of the properties of the LAB isolated from domestic animal milk (in particular) are limited in the literature. The donkey's milk is indispensable for the treatment of pneumonia, bronchitis, and diseases of respiratory paths (26, 27).

The aim of this work is to study the probiotic properties and the ability to synthesize polysaccharides-EPS and AMP (antimicrobial preparations) -containing bacteriocins (protein-like substances) by endemic strains of LAB of the genus *Enterococcus,* isolated from fermented donkey milk.

MATERIALS AND METHODS

Microbial strains and growth media. The endemic LAB cultures were isolated from various samples of donkey's milk from rural households of several regions of Armenian highlands. Samples were collected in sterile small bottles and stored at 4°C in the laboratory until they were used in experiments. A serially diluted sample was spread on MRS agar (Merck, Germany) and hydrolyzed milk agar (1.2% w/v) and cultivated at 37°C. 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°C in the MRS broth containing 40% glycerol. Before use, they were transferred twice into the appropriate medium and incubated during 48 hours in temperature controlled conditions in thermostat at 37°C. The strains *Enterococcus* and *Lactobacillus* were taken from Microbial Culture Collection of the Microbial Depository Center (MDC) at the SPC "Armbiotechnology" NAS RA. Species identification of LAB strains was confirmed by 16S rDNA gene sequencing method using universal primers LAB strains, and marker Gen ladder (100 bp, plus 1.5 kb, GENAXXON, Bioscience). Nucleotide sequence of the obtained amplified 16S rDNA was determined by "MACROGEN" (Korea). Strain identification was performed using the online BLAST software (www.ncbi.nlm.nih.gov/BLAST).

Inoculum preparation and obtaining of cell-free culture broth. The strains were grown in five ml of MRS broth (37°C, 24 h) and then transferred into a 100 ml Erlenmeyer's flask containing 50 ml of nutrient media and incubated overnight at different values of temperature and time in the thermostat.

For cultivation of LAB strains, the following nutrient media were used: Media№1. The nutrient media prepared on the basis of curd whey (28). Media№2. MRS and broth (Merck (Germany), ISO (Italy), Hi-Media (India)).

Growing LAB. 50 ml of the obtained inoculums were transferred into 100 ml of media in a 0.5L Erlenmeyer flask and grown at different values of temperature and time in the thermostat. At the end of culture, growth cell concentration achieved $(7 \pm 2) \times 10^8$ 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 for 20 min and obtained cell-free culture broth (CFCx5), which was used for future purification.

Obtaining the culture liquid. The 16-24-hour inoculant of the LABs will be added in the amount of 10% in test tubes containing the nutrient medium prepared on the basis of milk serum with a volume of 200 ml. During combined cultivation of LAB, the strains were grown under anaerobic conditions in 100-ml volume Erlenmeyer flasks containing 50 ml of liquid medium (5% v/v) at 37°C for 48 hrs. At the end of growth, culture liquid was centrifuged at 6,000 g for 20 min at +4°C, and the cell-free culture liquid (CFC liquid) was used for future purification.

Purification of CFC liquids and isolation of protein fractions. CFC liquids were concentrated 5 ± 0,5 times (CFCx5) on a rotary evaporator at 50°C and residual pressure 0.01 MPa (DM = 8-12%). Concentrated supernatants were treated with cooled ethanol

(96%) to the extent of 30-50% saturation with constant stirring using a magnetic stirrer (29, 30). Samples, treated with ethanol, were kept for 24 h at +40°C for precipitation and then centrifuged at 10,000 g at +4°C for 10 min. Ethanol was removed by evaporation until reaching the initial volume of CFC liquid. Obtained partially purified CFCx5 liquids were fractioned by gel filtration method on the column with Sephadex G-25(1.5×57 cm, resin vol: 100 mL). Fractions displaying bactericidal properties were pooled and vacuum evaporated at temperature 50-55°C, residual pressure 0.01 MPa. Aliquots (N = 30-50) of 5 ml were collected during gel-filtration purification of each cell-free culture broth (CFCx5) to check the density of each aliquot (230-260-320 nm, mg/ml, 2800 WV/VIS by spectrophotometer). The content of dry matter (DM) of the obtained partially purified antimicrobial preparation reached 30% (IFT 10HP Reichert Refractometer).

Isolation and purificationof exopolysaccharides. For isolation of EPS, cell-free culture broth was treated with 10% trichloroacetic acid and kept in the refrigerator at +4°C for 24 hours. Then the sediment was removed by centrifugation at 6.000 g during 20 min. The cooled ethanol (96%) in a ratio of 1:3 was added to supernatants and kept in the refrigerator at +4°C for 24 hours. After which sediment (exopolysaccharide) was removed from supernatants by centrifugation at 6.000 g for 20 min. Obtained EPS was kept in the thermostat at 50°C until it reached a constant mass. Dry powder of EPS was resolved in sterile water and 20% EPS liquids (5 mL) were passed through a column filled with Sephadex G50 resin (Sigma Aldrich) (1.5×57 cm, volume: 100 mL). The elution was carried out by sterile distilled water. Each fraction (n=45) eluted from the column, was tested for antibacterial activity on *B. subtilis* G 17-89 and *S. typhimurium* G 38 test cultures. EPS concentration of fractions was detected on 2800 Scanning UV/visible Spectrophotometer (Cole Parmer USA) at 200-600 nm). Fractions displaying bactericidal properties were pooled and vacuum evaporated at a temperature of 55°C, residual pressure 0.01 MPa. Obtained EPS preparation has antimicrobial activity at 500 -700 AU/ml, pH=4.2-5.0 depending on the strain.

Test cultures. Conditionally pathogenic gram-positive *Bacillus subtilis* G 17-89 and gram-negative *Salmonella typhimurium* G38 from the collection of bacteria of RA NAS "Armbiotechnology" were used. Bacteria were grown on nutrient agar (Himedia, India) at pH 7.2 for 16 hours and at 37°C. Pathogenic bacteria were isolated from different patients from Infection Hospitals (Armenia and Nagorno Karabakh). Antibiotic resistance of pathogenic strains was determined in the Institute of Epidemiology, Microbiology and Parasitology of Ministry of Health of Armenia and in the Stepanakert Center for Hygiene and Epidemiology. Bacteria were grown on Nutrient agar (Himedia, India) at pH 7.2 for 16 hours and at 37°C, then harvested and suspended in the Nutrient broth at 2.2 $\times 10^6$ CFU/ml.

Determination of the immunostimulating. Property was carried out using the ELISA method (31).

RESULTS

The comparative results of synthesis polysaccharide by probiotic strains of the genus *Lactobacillus* and *Enterococcus* are shown in Table 1.

As can be seen, the synthesis of polysaccharides and their antimicrobial activity are higher in strains of the genus *Enterococcus* isolated from the milk of different domestic animals (sheep, donkey, buffalo, goat) from the various regions of RA. Despite the same high antimicrobial activity, the isolated polysaccharides of the strains have different efficiency in inhibiting the growth of test cultures. To evaluate the properties of polysaccharides, selected strains isolated from donkey milk of the genus *Enterococcus* with probiotic properties synthesized polysaccharides.

Fig. 1 shows the results of the yields of polysaccharides (EPS) and the antimicrobial activity of concentrates of supernatants CFCx5 (AMP) as an example strains *Ent. faecium* KE-5 and *Ent.durans* KE-6 under different growing conditions (nutrient medium, different growing temperatures).

The greatest antimicrobial activity concentrates of the studied strains occurs after growing at 42°C temperature, but increased polysaccharide synthesis is observed in the studied strains when grown at 30°C. This pattern was also obtained for other strains of this genus isolated from the milk of different animals. Studies have shown that adding №1 0.5-1% peptone, 0,5% tryptone to the nutrient medium significantly increases the synthesis of polysaccharide of all studied strains with high antimicrobial activity.

Table 1. The antimicrobial activity of supernatants (CFCx5) and polysaccharides (test culture *S. typhimurium* G-38, 48h, 30°C, medium №1)

Genus of the strains	N	The polysaccharides			Total antimicrobial activity of the supernatants (x5), AU/ ml
		Yield,%	Total antimicrobial activity, AU/ ml	Growth inhibition of test culture,%	
Lactobacillus	17	5-10	1500-2000	30-40	20000
Enterococcus	21	10-15	1500-3500	60-70	25000

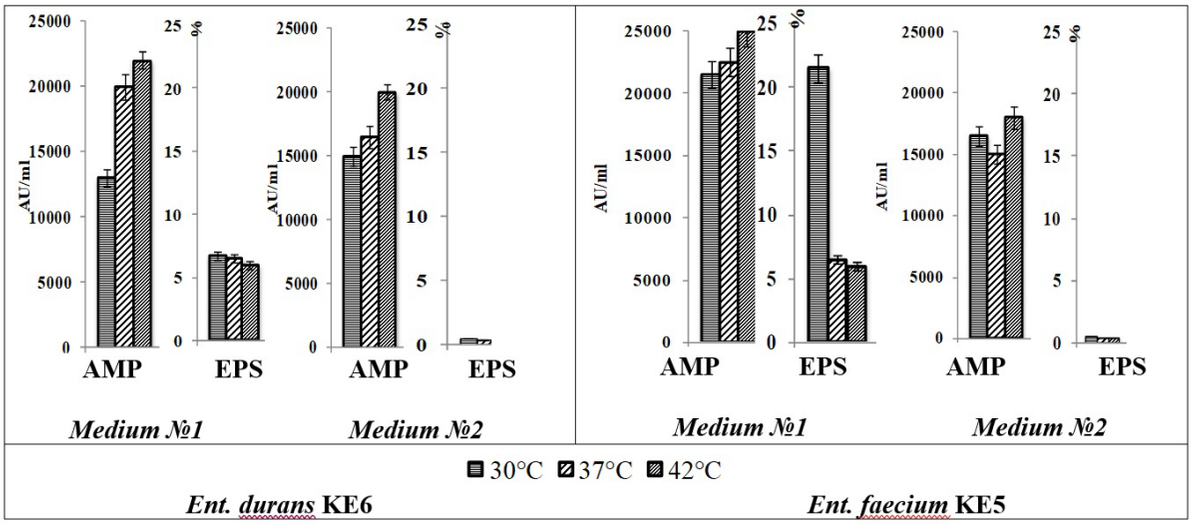

Fig. 1. Antimicrobial activity of supernatants (CFCx5) and yield of polysaccharides in different medium

Adding lactose concentrations (2-8%) and increasing ammonium sulfate to 1.5% did not lead to increase in antimicrobial activity and the synthesis of polysaccharides. A number of authors such as Sørensen et al., reported that different strains, depending on the composition of the nutrient medium, synthesize different amounts of polysaccharides.

We investigated the antimicrobial activity in the concentrated supernatants (CFCx5) of strains containing protein-like substances (AMP) after the purification by gel filtration method on Sephadex G-25. Purification and isolation of polysaccharides were carried out using the gel filtration method on Sephadex G-50. Isolated polysaccharides from the supernatants (CFCx5) of the strains *Ent. durans* KE-6 and *Ent. faecium* KE-9 did not show antimicrobial activity unlike the strain *Ent. faecium* KE5, but the AMP of these strains suppresses the growth of test cultures with high antimicrobial activity. We investigated the ability of some strains with probiotic properties to synthesize polysaccharides by using the HPLC analytical method. It was shown that polysaccharide, isolated from cultural liquid of investigated probiotic strains, consists of glucose and galactose molecules, which is the characteristic of polysaccharides in most lactic acid bacteria (32).

Previously it was shown that the AMP (100 AU/ml) of some probiotic strains of the genus *Enterococcus* and *Lactobacillus* with antimicrobial activity were tested for their ability to inhibit the growth of antibiotic-resistant strains of *Klebsiella pneumonia* (n = 15), *Ps. aeruginosa* (n = 15), *Staph. aureus* (n = 15), *Pr. vulgaris* (n = 15), *E. coli* (n = 20), and *Pr. mirabilis* (n = 18). It was shown that the biological metabiotics of different LAB strains inhibited the growth of pathogenic bacteria with different efficiency, and it depends on sources of pathogen isolation. As a result *Ent. faecium* KE-5 (100 AU/ml) which suppresses the growth of the studied antibiotic-resistant strains with almost the same efficiency (90%) was selected.

The results of the study showed that purified polysaccharides of the genus *Enterococcus* of species faecium inhibit the growth of bacteria causing pneu-

monia with varying efficiency at the concentrations used (10, 20%) depending on the source of their isolation (Table 2).

Inhibition of the growth of pathogenic bacteria with obtained polysaccharides (100 AU/ml) is depended on the species of bacteria too. It is possible that this could be associated with the active centers in the glucose configurations in the polysaccharide composition, which requires further research. A number of scientists such as Israyelyan et al. suggest that the biological specificity of polysaccharides is associated with the structure of the polysaccharides. Protein-like substances of strain *Ent. faecium* K-5 (500 AU/ml) were introduced into a water solution with a *Salmonella typhimurium* G-38 culture, a drop in the titer of *S. typhimurium* G-38 was observed in 5 hours by 7log10 degree at a drug 500 AU/ml and by 3 log10 degree at an activity of 100 AU/ml within 1 hour of exposure (Fig. 2).

Determination of the dependence of the influence of the polysaccharide on time and its concentration in a solution with a culture of *S. typhimurium* G-38 showed that a 10% aqueous solution of the polysaccharide strain solution *Ent. faecium* KE-5 inhibits the growth of *S. typhimurium* G-38 strain by 5log10 degree in 5 hours. Microscopy showed that in this case there is a change in the morphology of *Salmonella* cells (Fig. 2).

The growth inhibitory effect of *S. typhimurium* G-38 depends on the concentration of the polysaccharide. For comparison, we previously published data showing that protein-like substances (bacteriocins) of strains of the genus *Lactobacillus* and *Enterococcus* exhibit antimicrobial properties at a concentration of 100 AU/ml for 1 hour (24).

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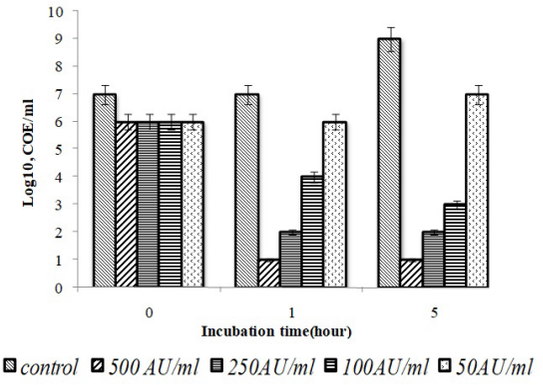

Fig. 2. *Salmonella typhimurium* G-38 growth inhibition by different concentrations of polysaccharide of *Ent. faecium* KE-5 strain

epithelium of the oral mucosa under the microscope addition polysaccharides of strains *L. rhamnosus* 2012 and *L. plantarum* 66. Polysaccharides isolated from the CL of strains *Ent. faecium* KE-5 and *Ent. durans* KE-6 stimulates the adhesion of lactic acid bacteria *L. rhamnosus* 2012 and *L. plantarum* 66. But polysaccharides obtained from strains of *Ent. faecium* KE-13 and *Ent. faecium* KE-14 do not significantly affect the manifestation of adhesion.

Immunological properties were conducted using cytokines (IL-10, IL-6, IL-8) by the ELISA method and using the test system: ZAO Vector-Best (Novosibirsk), OOO Cytokin (St. Petersburg). The immunomodulatory properties of a particular strain were determined by the spectrum and intensity of cytokine production. Interleukin-8 is known as a typical anti-inflammatory cytokine. Preliminary positive results were obtained in the model of blood erythrocytes on the stimulation of interleukin-8 secretion by polysaccharides of the *Ent. faecium* KE-5 strain. The

Table 2. Inhibition of growth of some pathogenic bacteria by polysaccharide of strains *Ent. faecium* (Ø,mm)

№	Polysaccharide	Solution %	*Kl.pneumonia* c-5	*St. pneumonia* fl-67	*St. pneumonia* fl-24	*St. pneumonia* fl-76
	Strains		Blood	Mucus of the nasopharynx	Sputum	Mucus of the nasopharynx
1.	*Ent. faecium* KE-13		19 ± 1	19 ± 1	20 ± 1	15 ± 2
2.	*Ent. faecium* KE-5	20	18 ± 1	20 ± 1	20 ± 1	22 ± 1
3.	*Ent. faecium* KE-14		17 ± 2	22 ± 1	20 ± 1	17 ± 2
4.	*Ent. faecium* KE-13		16 ± 2	10 ± 2	10 ± 2	10 ± 2
5.	*Ent. faecium* KE-5	10	19 ± 2	19 ± 1	20 ± 1	20 ± 1
6.	*Ent. faecium* KE-14		18 ± 1	15 ± 2	16 ± 2	10 ± 2

polysaccharide of the strain of *Ent. faecium* KE-5 showed the greatest immunomodulatory activity compared to other studied polysaccharides of strains *L. rhamnosus* 2012, *L. brevis* AG 31-9, *Ent. durans* KE-6, *Ent. faecium* KE-13.

Of interest was the possibility of interaction and influence of two purified metabiotics (polysaccharides, AMP) synthesized by the selected strains of LAB isolated from different donkey milk in vitro for their further joint use as biopreparations in pharmaceuticals. For this purpose, the isolated bacterial polysaccharides in the form of aqueous solutions were mixed in vitro in a ratio of 1:1 (by volume) at a temperature of 30°C, pH 4.0-4.5, contact time 5 min. AMP of different strains (N = 5) of the genus *Enterococcus* (activity is 1100-3900 AU/ml) and EPS of the strains *L. rhamnosus* 2012 (activity is 500 AU/ml) were used (Table 3).

Inhibition of test culture growth when containing a mixture of protein-like substances (AMP) of the genus *Enterococcus* and different polysaccharides of different strains in a given proportion in a solution leads to a decrease in the antimicrobial activity index to varying degrees. It is possible that the growth suppression effect on *S. typhimurium* G 38 bacteria with polysaccharides isolated from different origins strains of donkey milk (genus *Enterococcus* and *Lactobacillus*) is interacted with the structural features of AMP (protein-like substances) and polysaccharides.

DISCUSSION

For the first time the comparative difference in synthesis and antimicrobial activity of strains of the genus *Enterococcus* and *Lactobacillus* isolated from fermented milk of different animals was shown, besides this was shown that strains of the genus *Enterococcus* are more promising for their further use as probiotics, since they at the same time synthesize two metabiotics (bacteriocins and EPS) with antimicrobial properties. The dependence of polysaccharide synthesis on the composition of the medium used and the temperature of cultivation was shown, which was also noted by a number of authors such as Sørensen et al. These properties do not depend on the genus affiliation of the strains. The obtained data on the interaction of AMP and polysaccharides indicate a possible different mechanism of interaction of metabolic products in vitro, possibly due to the structural features of the polysaccharide and the type of lactic acid bacteria. Of particular interest is the study of the interaction of metabiotics (AMP and EPS) synthesized by LAB strains in the culture liquid. Interaction can be associated with the physicochemical, structural, mechanical properties of polysaccharides and protein-like substances, which is the subject of our further studies using HPLC methods, X-ray structural analysis and electron microscopy, determination of their molecular weights and the features of their effect on the cell membrane.

CONCLUSION

The obtained results showed that strains isolated from fermented donkey milk are capable of synthesizing two substances of different nature with high antimicrobial properties during the growth process, which are promising for further research and application for their use as probiotics and biopreparations in pharmaceuticals.

Table 3. Interaction of AMP of genus *Enterococcus* with different EPS (in vitro)

№	AMP	AMP *Enterococcus*	AMP *Enterococcus* +EPS *L. rhamnosus* 2012	%	AMP *Enterococcus* +own EPS	%
		AU/ml *S. typhimurium* G 38			AU/ml *S. typhimurium* G 38	
1.	*Ent. faecium* KE5	3900	1600	59↓	2600	33↓
2.	*Ent. faecium* KE13	1100	1000	10↓	1000	10↓
3.	*Ent. faecium* KE14	2500	2400	4↓	1800	28↓
4.	*Ent.faecium* KE18	2400	3000	20↑	2000	17↓
5.	*Ent.durans* KE6	1200	500	59↓	500	59↓

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