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COMPARATIVE ANTIMICROBIAL ACTIVITY OF SOME METABIOTICS SYNTHESIZED BY LACTIC ACID BACTERIA

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Abstract

This paper presents the comparative characteristics of the antimicrobial activity of selected lactic acid bacteria strains and antibiotics.

The metabiotics of probiotic lactic acid bacteria inhibited the growth of pathogenic, conditionally pathogenic bacteria, different etiology antibiotic resistant bacteria such as Salmonella sp., E. coli, Proteus mirabilis Pasteurella spp., Clostridium sp., Streptococcus sp., Staphylococcus aureus, Shigella sp., Yersinia enterocolitica, Bacillus cereus with different efficiency depending on pathogens isolation sources.

It was shown that bacteriocins of lactic acid bacteria in the same concentration did not affect growth of the commensal microbiota *strains, belonging to different genera and species. Lactobacillus and Enterococcus genera showed high sensitivity to investigated antibiotics (about 70%).*

Among all studied LAB strains of Enterococcus genus, some strains were shown to synthesize polysaccharides. The antimicrobial activity of isolated polysaccharides from Enterococcus faecium К Э-14, Enterococcus faecium К Э-5, Enterococcus lactic acid bacteria. Sp. К Э-9, Enterococcus lactic acid bacteria sp. К Э-6 strains was investigated. It was found that only polysaccharides isolated from Enterococcus faecium К Э-14 and Enterococcus faecium К Э-5 strains show an antimicrobial effect. The Enterococcus faecium КЭ-5 (MDC 9662) lactic acid bacteria strain was selected which produce protein-like substances and disaccharide polymers with antimicrobial activity, consist of glucose and galactose. The growth suppression of different Kl. pneumonia and St. pneumonia strains causing pneumonia by antimicrobial preparations of lactic acid bacteria was shown. The highest antimicrobial activity (100%) was observed when the antimicrobial preparations obtained after cultivation of lactic acid bacteria strains of the Enterococcus genus. The activity depends on the source of isolation of pathogens from a patient. The selected strains can be recommended for the creation of probiotic preparations with targeted purposes.

Keywords *LAB, exopolysaccharides, bacteriocins, antimicrobial activity, antibiotics.*

Introduction

The concept of foods that were developed specifically to promote health or reduce the risk of disease was introduced over the last decade. The concept of biofunctional foods is generally used when the desirable biological, medical, or physiological effect is exerted by microorganisms, in particular by lactic acid bacteria. The health benefits of microorganisms can be exerted either directly through the interactions of ingested live microorganisms with the host (probiotic effect), or

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indirectly by ingestion of the microbial metabolites (metabiotics) synthesized during fermentation (bioactive effect) [*Joshi V, 2015; Cuvas-Limón R et al., 2016*]. They contribute to maintaining human health, prevention and treatment of several gastrointestinal disorders such as infectious diarrhea, antibiotic-related diarrhea, acute or chronic diarrhea, irritable bowel syndrome or Crohn's disease, intestinal inflammation, certain allergies, dysbacteriosis, diabetes, etc.).

The development of concepts for the use of probiotics now also includes the production and use of metabiotics (biopreparations, pharmacobiotics), which are structural components of cells and/or metabolic products of probiotic microorganisms. Metabiotics of lactic acid bacteria (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, which can be used for creation and production of the new drugs for the prevention and treatment of chronic human diseases and can be included in different products composition [*Shenderov B, 2013*].

The use of probiotic preparations for the prevention and treatment of diseases, the selection of promising strains for the design of new probiotics is especially important at present. The isolation of new strains of endemic LAB and their investigations were carried out by us during the last decade. It was shown that some of them possessed basic probiotic properties, in particular, the ability to adhere to the epithelium of the mucous of the gastrointestinal tract, high resistance to proteolytic enzymes and viability after the influence of pH and bile; they had higher antioxidant activity and resistance to antibiotics, inhibited the growth of multidrug-resistant bacteria, food contaminating microorganisms of different taxonomic groups *Salmonella sp., E.coli, Proteus mirabilis Pasteurella spp., Clostridium sp., Streptococcus sp., Staphylococcus aureus, Shigella sp., Yersinia enterocolitica, Bacillus cereus* [*Tkhruni F et al., 2013; 2014; Israyelyan A et al., 2016*].

Selective pressure exerted by the use of antibiotics in both human and animal populations over the past several decades has led to the emergence of multidrug-resistant bacterial populations that are resistant to many commercially available drugs [*Levin B, 2001*]. In the WHO European Region, the resistance of some pathogens now reaches over 50. Methicillin-resistant *Staphylococcus aureus* is a significant public health problem worldwide [*Ken-ichi Okuda et al., 2013*]. *Proteus mirabilis* causes 90% of *Proteus* infections and can be considered a community-acquired infection. *Proteus vulgaris* and *Proteus penneri* are easily isolated from individuals in long-term care facilities and hospitals and from patients with underlying diseases or compromised immune systems [*D'Andrea M et al., 2011*]. Some of the more problematic drug-resistant pathogens encountered today include methicillin-resistant *Staphylococcus aureus*, multidrug-resistant *Streptococcus pneumoniae*, and vancomycin-resistant *Enterococcus* spp. among the gram-positive bacteria and multidrug-resistant *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa* among the gram-negative bacteria [*Barlow M, 2009*]. For the last time resistance to antimicrobial drugs (antibiotics) is a common characteristic in the world of bacteria. In the interaction between bacteria, genetic material is transferred from one bacterium to another and also genes coding for resistance to a certain antibiotic may be passed on to other bacterial species.

The growing problem of the prevalence of pathogenic bacteria resistant to antibiotics, motivated to search alternative natural microbial preparations, including on the basis of probiotic lactic acid bacteria and their bacteriocins. Non-antibiotics such as phytochemical flavonoids (galangin, quercetin and baicalein), bacteriocins, metabiotics and peptides of LAB represent bactericides with a broad range of activity and are excellent candidates for development of new prophylactic and therapeutic substances to complement or replace conventional antibiotics [*Kristiansen J et al., 2010*].

LAB are a heterogeneous group of bacteria found widely in nature. They colonize the gastrointestinal and urogenital tracts of humans and animals and are present in foods such as dairy products, fermented meats, fruits and vegetables. Many LAB species are generally recognized as

safe and several LAB species have received a Qualified Presumption of Safety status given by the European Food Safety Authority. The LAB are natural and profitable inhabitants in many environments (gastrointestinal tract, several foods), strains with resistance to antibiotics would not be detrimental to the wellbeing of humans or animals. However, there is some concern that antibiotic resistance in LAB could then be transferred to possibly pathogenic bacteria species, complicating the treatment of a disease or infection and lead to the spread of antibiotic-resistant bacteria. Lactic acid bacteria have been proposed as and are used as probiotic strains playing an important role in food production and health maintenance during last decade. Probiotics are defined as "live microorganisms, which when consumed in adequate amounts confer a health benefit on the host". Probiotics can affect all host mucosal surfaces, including the mouth and gastrointestinal tract, the upper respiratory tract, or the urogenital tract [*Cuvas-Limón R et al., 2016*].

Probiotic of LAB can be used as starter culture for biofunctional food production, as well as for obtaining of metabiotics. The LAB are also intentionally added to several probiotic products because of their potential health benefits (Fig. 1). Metabiotics are the structural components of probiotic microorganisms and/or their metabolites and/or signaling molecules with a determined (known) chemical structure. Metabiotics have some advantages because of their exact chemical structure, well dosed, very safe and long shelf-life; can optimize host-specific physiological functions, regulator, metabolic and/or behavior reactions

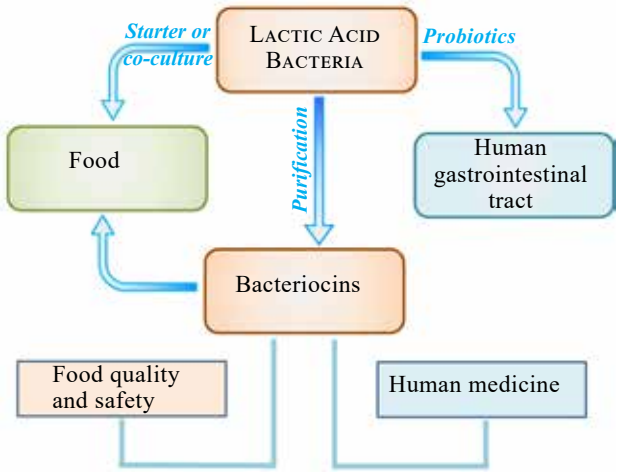

FIGURE 1. *Use of lactic acid bacteria*

connected with the activity of host indigenous microbiota [*Shenderov B, 2013*].

to the study of microflora of traditional fermented dairy products and creation of new products based on isolated bacteria (solitary or associated) [*Karimpour F et al., 2023*]. Traditional fermented dairy products are the result of biotechnological processes. This is one of the most practical and widespread methods for the preservation of microorganisms' biodiversity and use of their organoleptic and nutritive properties in the products [Bokulich N et al., *2015*].

Extensive research has been held on the identification of matsoon microflora in various regions of the Caucasus. In Armenia and Artsakh, the application of the medicinal properties of plants and fermented milk products in the treatment of a number of diseases (bronchitis, diarrhea, wound healing, etc.) has been passed from generation to generation.

From this point of view, the isolation of starter LAB cultures from various fermented milk products, the selection of LAB, which can be producers of biologically active substances and used for the prevention and treatment of a number of diseases are of great interest.

MATERIAL AND METHODS

Object of study: More than 300 strains of LAB were isolated by us from fermented dairy products made of milk of different domestic animals (goats, donkeys, buffalo, sheep, and cows) from the rural households of different high mountain regions of the Republic of Armenia and Artsakh. The results of LAB genotyping using API test, RAPD PCR and 16S RNA gene sequencing method have shown that the genetic biodiversity of LAB strains in the regions of Armenia and Artsakh is mainly represented by several species of *Lactobacillus* and *Enterococcus* genera [*Israyelyan A et al., 2016*].

Growth media: For cultivation of LAB strains the following nutrient media were used: **No 1.** MRS (agar and broth (Merck (Germany), ISO (Italy), HiMedia (India)). **No 2**. Milk (1.5 % of fat). **No 3**. The nutrient media prepared on the basis of curd whey with addition of the following salts ((%) ± 0.2, volume of whey 100 ml): $(NH_4)_2SO_4$-0.8; KH_2PO_4-0.1; $MgSO_4$-0.2; yeast extract-0.3; pepton-0.3; $MnSO_4$-0.05; CH

$_3$COONa·3H $_2$O-0.2)) [*Tkhruni F et al., 2015*]. LAB strains maintained as frozen stock at -20°C in the MRS broth containing 40% Glycerol.

Lactic acid bacteria strains of *Lactobacillus rhamnosus* 20-12 (MDC 9631), *L. plantarum* 66 (MDC 9619), *Enterococcus faecium* KE-5 (MDC 9662), *Ent. durans* KE-6 (MDC 9665) *Ent. faecium* KE-9 (MDC 9664), *Ent. durans* AA 11-6 (MDC 9767), *Ent. faecium* AA 20-2 (MDC 9768), and other strains of *Lactobacillus* genus used in the presented work, were taken from the Culture Collection of the Microbial Depository Center (MDC) of SPC "Armbiotechnology" NAS RA.

Species identification was confirmed by 16S rDNA gene sequencing method using universal primers for *Enterobacteriaceae,* and marker Genladder (100 bp, plus 1.5 kb, GENAXXON, Bioscience) [*Weisburg W et al., 1991*]. 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).

Test cultures: Conditionally pathogenic Gram-positive *Bacillus subtilis* G 17-89 and Gram-negative *Salmonella typhimurium* G 38, *Esherichia coli* K12, from the Microbial Culture Collection of the Laboratory of Probiotics Biotechnology at the SPC "Armbiotechnology" NAS RA, were used. Bacteria were grown in Nutrient agar (Himedia, India) at pH 7.2 for 16 hours and at 37°C, then harvested and suspended in the Nutrient broth until the cell count reach to 2.2x10^6 *CFU/ml*.

Antibiotic resistant conditionally pathogenic bacteria *E. coli, Staph. aureus, Ps. aeruginosa, Pr. Mirabilis, Pr. Vulgaris and Kl. pneumonia* and *St. pneumonia* strains causing pneumonia *were* isolated by infectionists from infected patients in the Stepanakert Center for Hygiene and Epidemiology, Artsakh during 2010-2022.

Gut microbiota pathogenic bacteria, such as G-negative *Salmonella enteritidis, S.typhimurium Salmonella* spp., *Pseudomonas aeruginosa*, *Klebsiella pneumonia, Proteus mirabilis*, G-positive *Staphylococcus epidermidis* and *Staph. aureus* were isolated from infected patients in the "Nork" Infections Hospital (Yerevan, RA), food contaminating pathogenic bacteria *Pseudomonas aeruginosa, Staph. aureus*, *Esherichia coli* were isolated from different food products in the National Bureau of Expertise (Yerevan, RA) during 2010-2015. Antibiotic resistance of isolated pathogenic bacteria was determined in the Research Institute of Epidemiology, Virology and Medical Parasitology after A.B. Alexanian, MOH RA, Yerevan, Armenia [*Melik-Andreasyan G et al., 2013*]. Antibiotic resistant pathogenic bacteria were grown in appropriate selective media. Isolated bacteria stored in the microorganism depository of the same Institute.

G-positive *Listeria monocytogenes,* G-negative *Yersinia pestis*, *Yersinia pseudotuberculosis* and *Yersinia enterolitica* as well as other Museum cultures of especially dangerous pathogens (EDP) were investigated in the "Center for Prophylaxis for Especially Dangerous Infections" (Yerevan, RA) during 2012-2014. The cultures were grown on solid selective nutrient media at the conditions specified for each culture. *L. monocytogenes, Yersinia pestis* and *Yersinia pseudotuberculosis* were grown overnight respectively on sugar and nutrient agar pH 7.3 at 37°C. The cells were harvested and suspended until the cell count reach to 1.0x10^9 *CFU/ml*. *Yersinia enterolitica* was cultivated during 24 hours on MPA pH 7.3 at 37°C, harvested and suspended until the cell count reach to 1.0x10^6 *CFU/ml*.

Cultivation of especially dangerous test cultures:

a) Vibrio cholerae M-75: one-day culture cultivated on MPA pH=7.8 at 37°C, cell count 2.2x10^6 *CFU/ml*;

b) Yersinia pestis 3344*:* two-day culture cultivated on MPA pH=7.5 at 28°C, cell count 1.0x10^6 *CFU/ml*;

c) *Brucella suis* 1330: one-day culture cultivated Brucella Agar Base add sterile 5% v/v inactivated Horse Serum pH=7.5 at 37°C, cell count 3x10^6 *CFU/ml*;

d)F.tularensis 234: one-day culture cultivated on MPA. pH=7.5 at 37°C, cell count 1.0x10^6 *CFU/ml.*

e) Bacillus antracoides and Bacillus antrahx: one-day culture cultivated on MPA pH=7.0 at 37°C, cell count 2.6x10^6 *CFU/ml*.

Each culture grown on solid medium was washed off with sterile physiological solution and transferred to the test tube. Concentration of test culture in the stock solution was adjusted to cell count 1.9 x10^9 *CFU/ml*. The concentration of each microorganism in suspension was controlled by Tarasevich's optical standard [*Innis M et al., 1999*].

All experiments were performed according to the sanitary-epidemiological protocols approved for Center for Prophylaxis for Especially Dangerous Infections [*National Committee for Clinical Laboratory (CL) Standards, 2006; MUK Guidelines 4.2.2495-09 RF, 2010*].

Obtaining of cell-free culture broth: Single colonies of LAB were grown in 5 *ml* of MRS broth (37°C, 24 *h*) and then were transferred into 100 *ml* Erlenmeyer flask containing 50 *ml* of MRS broth, and incubated overnight at 37°C in the thermostat. At the end of culture growth, cell concentration achieved 7.0±2x10^9 *CFU/ml* (of titration) and pH reduced to 3.5-4.2. Culture broth was centrifuged at 6.000 *g* during 20 *min* and cell free culture liquid (CFC) was obtained. CFC liquids were concentrated 5±0.5 times (concentrated supernatants x5) on a rotary evaporator at 50°C and residual pressure 0.01 MPa, pH-4.5-5.0.

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°C, residual pressure 0.01 MPa.

Determination of resistance to antibiotics: To determine resistance of isolated human gut microbiota pathogens to antibiotics, the method with antibiotic standard disks was applied. Each strain was inoculated into appropriate broth, incubated at 30°C or 37ºC for 16 *hrs*. By spread plate technique the cultures were inoculated in the Petri plates using sterile swab. The antibiotic discs of Amikacin 30 μg, Ofloxacin 5 μg, Ceftazidime 30 μg, Doxycyclin 10 μg, Ciprofloxacin 5 μg, Gentamicin 120 μg, Cefalotin 30 μg, Cefazolin 30 μg, Azithromicin 15 μg, Cefuroxime 30 μg, Augmentin 30 EG 10 B (Oxoid, UK) were placed in the plates. Agar plates with antibiotic disks were then incubated at 37ºC for 24 h. The diameters of the inhibition zones were measured. The results were expressed as sensitive (+) and resistant (-).

Determination of antimicrobial activity. Antimicrobial activity of samples (aliquots of 100 μl) was assessed by spot-on-lawn method, measuring the size of the inhibition zone (diameter) of test culture growth (Ø, mm) after 24 *h* incubation in the thermostat at 30°C. The antimicrobial activity was calculated and expressed in arbitrary units (*AU/ml*) [*Parente E et al., 1995*].

High-performance liquid chromatography (HPLC): Fractions with bactericidal activity, obtained after gel filtration method, were purified by HPLC method with application of various systems (Semi-preparative "Avex ODS" C_{18} column (8 by 250 mm, Waters and Shimadzu, Japan); Shimadzu LC-20 analytical C_{18} column (4.6 by 250 *mm*, Symmetry, USA, with a detector Diode array SPD-20 a, auto-sampler). The eluent consisted of bi-distilled water, trifluoroacetic acid and acetonitrile (HPLC "SIGMA", USA)) [*Aslam M et al., 2011*]. Sample injection volume was 100 μl. Elution monitored at different wavelengths ranged from 190 *nm* to 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, have been selected.

Isolation of the exopolysaccharides (EPS) was carried out according to the method, described by Bajpai et al. (2016). The exopolysaccharides 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 test culture.

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

Results

More than 60 strains of human pathogens were isolated from infected patients (feces, urine, wounds, blood, throat, etc.) in the Infection Hospitals of Yerevan and Stepanakert. The antibiotic resistance of 23 pathogenic strains to 12 widely used in practice in Armenia antibiotics: aminoglycosides - amikacin, azithromicin, gentamicin, beta-lactams - augmentin, amoxicillin, cefalotin, oxacillin, cefazolin, ceftazidime, cefuroxime, doxycyclin, tetracyclin,

Table 1

Comparative data of resistance of pathogenic bacteria to different groups of antibiotics

Groups of antibiotics	Antibiotics	Strains				ARS (n)
		Resistant		Sensitive		
		abs.	%	abs.	%	
Amino-glycosides	amikacin	12	52.2	11	47.8	23
	azithromicin	13	59.1	9	40.9	22
	gentamicin	3	20.0	12	80.0	15
Beta-lactams	augmentin	5	100	0	0	5
	cefalotin	13	81.2	3	18.7	16
	oxacillin	7	77.8	2	22.9	9
	cefazolin	13	56.5	10	43.5	23
	ceftazidime	11	50	11	50.6	22
	cefuroxime	11	47.8	12	52.2	23
	doxycyclin	18	78.3	5	21.7	23
Fluoro-quinolones	ofloxacin	4	17.4	19	82.6	23
	ciprofloxacin	4	18.2	18	81.8	22

Notes: *ARS - Antibiotic resistance strains*

quinolones - ofloxacin, ciprofloxacin was investigated by us during 2012-2021 [*Melik-Andreasyan G et al., 2013; Israyelyan A et al., 2015*]. The antibiotic resistance of 8 *Salmonella* spp. strains, 4 *Staphylococcus* spp. strains, 3 *Pseudomonas aeruginosa* strains, 3 *Proteus spp*. and 4 *E. coli* strains was investigated. Summary data are shown in table 1.

The results show that resistance to antibiotics varied from 100 to 10 % across almost all examined pathogenic bacteria and mostly depends from genera and species belonging of strains.

Staph. aureus and E. coli possesses high sensitivity to investigated antibiotics, while strain of *Salmonella* genus shown high resistance to antibiotics. The studied strains showed high sensitivity to fluoroquinolones - ofloxacin and ciprofloxacin - 82.6 and 81.8%, respectively and were highly resistant to beta-lactams - augmentin and cefalotin, cefazolin, ceftazidime, cefuroxime - 100, 81.3, 77.8, 56.5, 50.0, 47.8, correspondingly. Microorganisms reveal high resistance to doxycycline - 78.3%, less to aminoglycosides - 59.1% to azithromicin and 52.2% to amikacin respectively. Exception from this group is gentamicin to which was susceptible 80% of the studied strains.

It was shown that the partially purified compounds from *Lactobacillus rhamnosus* 20-12 (Bacillus Coagulans 1−1470 Da and Bacillus Coagulans 2−670 Da) and *L.acidophilus* 1991 (BCN −1100 Da), isolated from dairy products made of cow milk, display high antimicrobial activity. Results of chromatograms and spectrograms suggest that these compounds have peptide bonds. Based on several biochemical characteristics (molecular mass, pI, heat stability, protease sensitivity) bacteriocin BCN1 and bacteriocin BCN 2 can be considered as class II bacteriocins [*Tkhruni F et al., 2020*].

The influence of BCN 2 of *L. rhamnosus* 20-12 (MDC 9631) on the growth of pathogenic bacteria, isolated from infected patients (various sources, such as blood, feces, saliva and urine) is presented in fig. 2. As can be seen from the given results, the bacteriocins BCN 2 of LAB *L. rhamnosus* 20-12 strain inhibited the growth of pathogenic bacteria *E. coli, S. aureus, Clostridium sp., Salmonella sp.,*

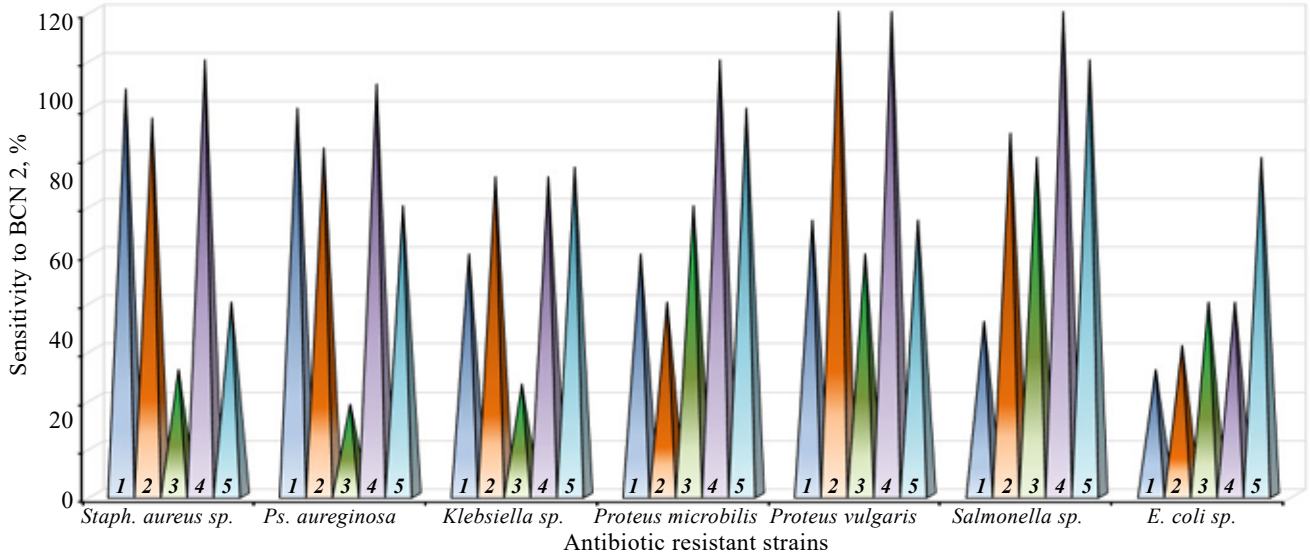

Figure 2. *Influence of bacteriocin BCN 2 on multidrug resistant pathogens isolated from different sources: Blood (**1**), Feces (**2**), Urine (**3**), Saliva (**4**), Wound (**5**).*

Streptococcus sp., Pasteurella sp., P. aureginosa and *E. cloacae* with different efficiency depending on pathogens isolation sources.

It should be noted that there are differences in the sensitivity of *Listeria, Clostridium, Propionibacterium, enterococcus sp.,* and oral *streptococcus sp.* to the action of LAB, described by other authors. Above all, they explain it with the presence of specific receptor proteins required for binding to bacteriocins and their transport into the bacteria [*Vuist L, Neysens P, 2005*].

Table 2 shows the results of inhibition of the growth of different bacteria of *Salmonella* genus by using of *Lactobacillus rhamnosus* 20-12 bacteriocins with different antimicrobial activity by the method of serial dilutions in the solution depending on incubation time. The presented results obtained during first hours of incubation. It is shown that BCN 2 demonstrates higher efficacy toward various strains of *Salmonella* species compared to BCN 1. Several *Salmonella* strains were antibiotic resistant, and the BCN 2 inhibited their growth with higher efficacy compared to other strains of the same species. This fact is essential since at present using of antibiotics in the treatment of infectious diseases of gut leads to the development of a great number of antibiotic resistant pathogenic bacteria.

The resistance of Gram-negative bacteria is attributed to the particular nature of their cell membrane (mechanism of their action described for bacteriocin involved phenomenon of adsorption). However, the literature data on the antagonistic activity of LAB against *Streptococcus sp.* bacteria are single scattered reports.

Previously it was shown that the probiotic culture *Enterococcus faecium* L3 possessed diverse ability to inhibit the growth of *Streptococcus sp.* belonging to different genera [*Yermolenko E et al., 2006*] (Table 3).

Both bacteriocins 1 and 2 inhibited the growth of EDP bacteria from different depository with high efficiency. Particularly large zones of growth inhibition were registered for the *Francisella tularensis* bacteria, which may be connected with high sensitivity of the cell wall of these bacteria to these bacteriocins. The efficacy depends on genus specificity, intraspecific

Table 2

The intraspecific differences of growth inhibition of bacteria of *Salmonella* genus by BCN 1 and BCN 2

Gram-negative bacteria	n	Bacteriocins							
		BCN 1				BCN 2			
		80.0 AU		56.0 AU		28.0 AU		14.0 AU	
		Ø, *mm*	%	Ø, *mm*	%	Ø, *mm*	%	Ø, *mm*	%
*S.enteritidis**	3	lawn	0	11±2	100	8±1	100	5±1	100
S.enteritidis	3	2±0.5	30	2±1	30	4±0.5	30	lawn	0
*S.typhimurium**	2	lawn	0	11±2	100	7±1	100	5±1	100
S.typhimurium	3	6±0.5	60	13±1	60	9±1	60	lawn	0
*Salmonella sp.**	2	2±0.5	50	12±1	100	10±1	100	6±1	100
S.enteritica	4	lawn	0	12±1	60	8±1	60	2±1	60

Notes: **antibiotic-resistant strains, n - number of strains, lawn- bacterial growth in form of lawn, absence growth inhibition.*

Table 3

The growth inhibition of EDP bacteria by BCN 1 and BCN 2

EDP bacteria	n	Bacteriocins							
		BCN 1				BCN 2			
		80.0 AU		56.0 AU		28.0 AU		14.0 AU	
		Ø, mm	%	Ø, mm	%	Ø, mm	%	Ø, mm	%
F. tularensis 234	2	20±1	100	20±1	100	18±1	100	15±1	100
V. cholerae 54	6	12±1	100	12±1	100	8±1	100	8±1	100
Y. pestis 3344	2	10±1	100	10±1	100	8±1	100	4±0.5	100
Br. suis 2	1	Lawn	0	Lawn	0	Lawn	0	lawn	0

Notes: *n - number of strains, lawn- bacterial growth in form of lawn, absence growth inhibition*

Table 4

Dependence of bactericidal activity of *L. rhamnosus* 20-12 bacteriocin BCN 2 (750 *AU/ ml*) on incubation time

Test cultures	**Incubation time, (*hours*)**					
	1		**3**		**24**	
	Cont.	Exp.	Cont.	Exp.	Cont.	Exp.
B. antracoides	$1.5x10^4$	none	$3.0x10^4$	none	$1.0x10^7$	none
V. cholerae 2590	$1.5x10^4$	none	$2.0x10^4$	none	$5.0x10^6$	none
B. subtilis G17-38	$6.0x10^5$	none	$8.0x10^5$	none	$2.0x10^7$	none
S. typh G38	$1.2x10^4$	none	$5.5x10^4$	none	$3.0x10^7$	none
Y. pestis 3344	$2.0x10^5$	$1.3x10^3$	$1.0x10^6$	none	$4.0x10^7$	none

Note: *None - absence of growth*

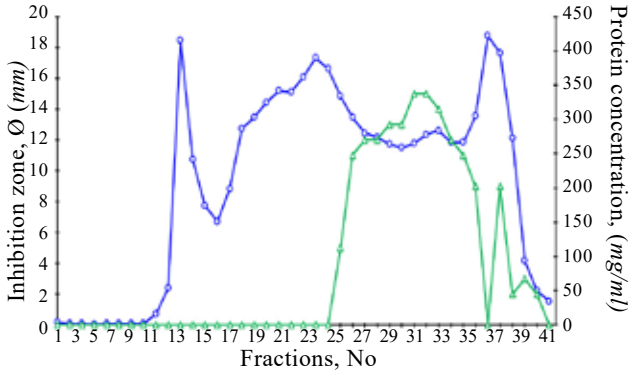

Figure 3. *The results of purification of the Clinical laboratory supernatant (CFCx5) of Ent. faecium KE-5. Green line - antimicrobial activity of fractions after gel filtration, Blue line - protein concentration of fractions after gel filtration*

belonging of bacteria, and concentrations of the BCN. BCN possesses a bactericidal or bacteriostatic mode of action on sensitive cells. These differences being greatly depend on its concentrations. Low concentrations show bacteriostatic effect. The method of serial dilutions showed that in liquid growth medium containing the BCN 2 inhibition of growth of the studied pathogens starts after 3 hours of incubation. Similar results were obtained for the BCN 2 (Table 4).

5-fold concentrated Clinical laboratory (CFCx5) of different LAB strains were purified by gel filtration and the fractions of protein nature that had antimicrobial activity against gram-positive and gram-negative test cultures were obtained. The results of purification of the supernatant of *Ent. faecium* KE-5 are shown in fig. 3.

The 5-fold concentrated Clinical laboratory (CFCx5) were obtained by us after growing of some lactic acid bacteria isolated from fermented milk of various domestic animals. CFCx5 then were purified by gel filtration and the fractions of protein nature with antimicrobial activity were combined and antimicrobial biological preparations (AMP) were obtained. Antimicrobial biological preparations were tested for their ability to inhibit the growth of certain antibiotic-resistant strains of *Klebsiella pneumonia* (n=15), *Ps. aeruginosa* (n = 15), *Staph. aureus* (n = 15), *Pr. vulgaris* (n = 15), *E. coli* (n = 20), *Pr. mirabilis* (n = 18), *Salmonella tuph* [*Israyelyan A et el., 2015; 2016*]. Preliminary data are shown in table 5. It was shown that the highest antimicrobial activity (100%) against several strains of *Kl. pneumonia* and *St. pneumonia* causing pneumonia was observed when the antimicrobial preparations obtained after cultivation of LAB strains of the *Enterococcus* genus. The lowest activity of protein-like fractions from strains isolated from fermented milk of cows (20-50%) was determined [*Israyelyan A et al., 2016*]. As can be seen from the above mentioned data, the effectiveness of growth inhibition by strains of the genus *Enterococcus* isolated from fermented milk of different animals differs depending on the type of animals. The highest antimicrobial activity (100%) against *Klebsiella pneumonia* strains was observed when the antimicrobial preparations was obtained

Table 5

The influence of LAB metabiotic (AMP, 100 *AU/ml*) on the growth of conditionally pathogenic bacteria, (%)

LAB isolation sources, milk	The source of metabiotics	Bacteria, isolated from patients					
		Staph.aureus	*Ps. aeruginosa*	*Pr. mirabilis*	*Klebsiella pneumonia*	*Pr. vulgaris*	*E. coli*
		n=15	n=15	n=18	n=25	n=10	n=20
Donkey	*Ent. faecium* KE 5	100.0	100.0	66.0	100.0	75.0	60.0
	Ent. sp. KE 3	100.0	100.0	66.0	40.0	25.0	33.0
Goat	*Ent. faecium* KAP-1	50.0	100.0	50.0	100.0	33.0	100.0
	Ent. faecium KA-3	50.0	100.0	100.0	100.0	66.0	100.0
Buffalo	*L. helveticus* KG_5	50.0	100.0	100.0	100.0	66.0	100.0
	Ent. sp. KG	50.0	100.0	100.0	100.0	66.0	66.0
Sheep	*Ent. faecium* KV-15	100.0	100.0	100.0	100.0	33.0	66.0
	Ent. sp. KV-15	0.0	50.0	50.0	100.0	66.0	33.0
Cow	*Ent. durans* K13	25.0	58.0	29.0	0.0	0.0	15.0
	Ent. faecium M14	0.0	50.0	50.0	0.0	0.0	50.0

Note: *n - number of strains*

after cultivation of various strains of the *Enterococcus*, isolated from fermented milk of goat and donkey. It can be assumed that the high antimicrobial activity of the strains may be the result of the total action of metabolic products (protein-like substances, EPS, etc.) synthesized during the cultivation of these strains [*Israyelyan A et al., 2016*].

It is known that in the process of growth, some lactic acid bacteria synthesize exopolysaccharides [*Bajpai V et al, 2016*]. The ability of some strains of the *Enterococcus* genus with probiotic properties to synthesize exopolysaccharides with antimicrobial activity was studied, 21 strains with probiotic properties were selected. It was shown, that the antimicrobial activity of the strains depends on the composition of the nutrient medium, the time and the temperature of cultivation. However, not all exopolysaccharides had the ability to inhibit the growth of the studied bacteria. The highest antimicrobial activity was observed when the exopolysaccharide, isolated after cultivation of the strain *Ent. faecium* KE-5 was used. The other exopolysaccharides isolated from the studied *Ent. durans* KE-6 and *Ent. faecium* KE-9 strains did not have the ability to suppress the growth of the test culture.

It has been shown that the antimicrobial activity of strains of the genus *Enterococcus* is due to the synthesis of different metabiotics, such as protein-like substances, exopolysaccharides (EPS). Table 6 shows the results of investigation.

From the preliminary data it appears that 5fold concentrated supernatants and EPS from *Enterococcus* KE 14 strain have higher efficacy compared to antibiotics. The obtained results showed the effectiveness of suppressing the growth of pathogenic bacteria (*St. pneumonia* n=33) by the obtained exopolysaccharide from the *Enterococcus sp.* KE 14 strain compared with the used antibiotics and exopolysaccaharides isolated from the Clinical laboratory after cultivation of other strains of the *Enterococcus* genus.

By using the HPLC analytical method, it was shown that exopolysaccharide, isolated from Clinical laboratory of probiotic LAB, consisted of glucose and galactose molecules, which is characteristic of exopolysaccharides of most lactic acid bacteria. It is possible that this could be associated with the active centers in the glucose configurations in the exopolysaccharide composition, which requires further research.

Table 6

Comparative effect of antimicrobial action of metabiotics and antibiotics against St. pneumonia

LAB strains/ antibiotics	Active substance	*St. pneumonia* (n=33)		
		R	I	S
Ent. durans KE 13	Exopolysaccharides	0	0	100.0
Ent. durans K 13	Antimicrobial preparations	0	0	100.0
Ent. faecium KE 14	Exopolysaccharides	33.33	66.67	0
Ent. faecium KE 14	Aimicrobial preparations	7.6	0	92.4
Aminoglycosides	Amikacin, Gentamycin, Tobramycin, Neomycin	75.75	6.07	18.18
Cephalosporins	Cephtriaxone, Cephatoxime, Cephalexin, Cefixim, Cefuroxime, Cephazolin	18.1	48.4	33.5
Quinolones	Ciprofloxacin, Levofloxacin Pefloxacin, Ofloxacin, Nalidix acid	33.33	33.33	33.33
Penicillins	Amoxicillin, Ampicillin, Oxacillin, Penicillin, Augmentin	35.0	30.0	35.0
Macrolides	Erythromycin, Clarithromycin, Azitromycin	33.3	45.45	21.25
Others group	Chloramphenicol, Polymyxin, Fosfomycin, Co-trimoxazole, Sulfamethoxazole/Trimethorim	51.5	27.2	21.3

Notes: *n - number of strains, R-resistant, I-intermediate, S-sensitive*

Comparative characteristics of the influence of 10% exopolysaccharide solution, 5 fold concentrated supernatants and protein-like antimicrobial preparations relative to two pneumonia-causing bacteria is shown in fig. 4.

As can be seen from results presented in fig. 4, the effectiveness of suppressing the growth of various bacteria with different metabiotics obtained after purification of the Clinical laboratory of *Ent. faecium* KE-5 strain depends on the species of pathogenic bacteria. Protein-like substances are more effective than other substances produced by studied strain. It can be assumed that the resistance of gram-negative bacteria is attributed to the particular nature of their

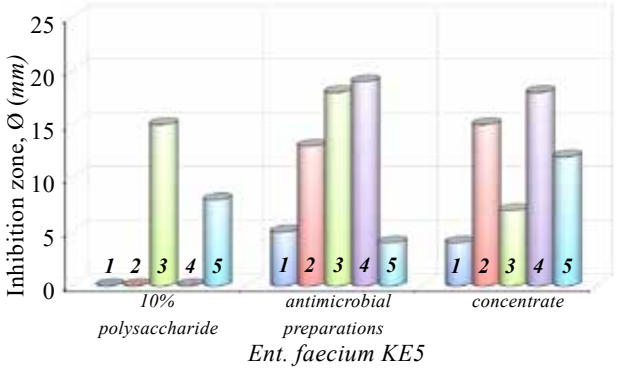

Figure 4. *Growth suppression of different pneumonia causing strains by antimicrobial preparations isolated from the Clinical laboratory of Ent. faecium KE-5 strain: Kl. pneumonia fl 357 (**1**), St. pneumonia fl 1456 (**2**), Kl. pneumonia fl 1231 (**3**), St. pneumonia fl 399 (**4**), St. pneumonia fl 400 (**5**)*

cell membrane; the mechanism of action described for bacteriocin involved a phenomenon of adsorption of bacteriocins on the cell wall.

As can be seen from the data presented, protein-like substances synthesized by strains of the genus *Enterococcus* suppress the growth of pathogenic strains with different efficiency, and their effectiveness depends on the nature of the pathogenic bacterium used. It can be assumed that the high antimicrobial activity of the strain *Ent. faecium* KE-5 may be the result of the total action of the exopolysaccharide and protein-like substances formed during its cultivation.

The antimicrobial activity of the 10% solution of antimicrobial preparations obtained after growing of *Ent. faecium* KE-5 compared to antibiotics was investigated. Comparative characteristics of their influence are shown in fig. 5.

Comparing the data obtained, it can be seen that the suppression of the growth of pathogenic strains by antimicrobial preparations (10%) is comparable to the effect of some antibiotics (Cefixime, Clarithromycin).

Comparison of the effectiveness of exopolysaccharides has shown that some strains of *Enterococcus* genus synthesize exopolysaccharides inhibited the growth of *Salmonella.* Out of the studied 19 strains of the *Enterococcus* genus that synthesize exopolysaccharides, only 3 strains have the ability to inhibit the growth of bacteria of the *Salmonella* genus. While isolated strains of the genus *Lactobacillus* synthesize an insignificant amount of polysaccharides. *L. plantarum* 66 and *L. plantarum* 65 strains also synthesize exopolysaccharides which do not inhibit the growth of *Salmonella*.

Study of the growth inhibition of 23 *Pr. mirabilis* strains, isolated from the urine, mucus and throat of sick patients by antimicrobial biological preparations of lactic acid bacteria showed different effectiveness: *L. rhamnosus* 2012 - 39%, *Enterococcus faecium* K 13 - 13%. Strains *L. rhamnosus* 2012 and *L. acidophilus* 1991 did not significantly suppress the growth of *Klebsiella* bacteria.

Discussion

Thus, it is obvious that different strains

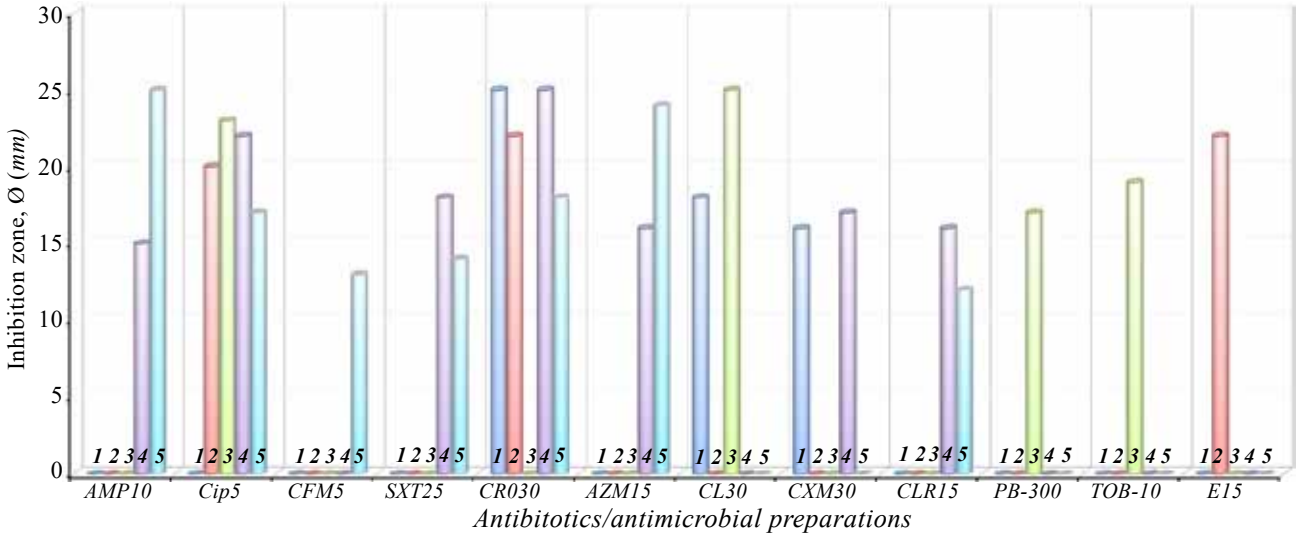

Figure 5. *Growth inhibition of different strains causing pneumonia by antibiotics and antimicrobial preparations: Kl. pneumonia fl 357 (**1**), St. pneumonia fl 1456 (**2**), Kl. pneumonia fl 1231 (**3**), St. pneumonia fl 399 (**4**), St. pneumonia fl 400 (**5**)*

synthesize different substances with antimicrobial activity (bacteriocins, exopolysaccharides) and demonstrate different effectiveness regarding to different pathogenic bacteria, depending on the source of their isolation, concentration of antimicrobial substances, time of action, genus and species belonging of pathogens. Strains of the *Enterococcus* KE-5 and *Enterococcus* KE-13 genera are promising for further research.

Conclusion

Metabiotics of LAB can contain bacteriocins with antimicrobial activity against multidrug-resistant bacteria and other low molecular weight antimicrobial molecules, exopolysaccharides, peptides with various activities, which can be used for creation and production of the new drugs for the prevention and treatment of chronic human diseases and can be included in different products composition. There are enough drugs on the probiotics market. Therefore the study of their biological properties and the identification of biotechnological potential are relevant and open up new prospects for obtaining various biological products.

Comparing the obtained research results, it is obvious that the manifestation of the antimicrobial activity of the selected strains of the *Lactobacillus* and *Enterococcus* genera differs and depends on the type of the pathogenic bacterium, the source of its isolation, the nature of the antimicrobial substance of the synthesized LAB and the type of bacteriocins.

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