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Investigation of the Properties of Endemic LAB Strains of Genus Enterococcus Isolated From Samples of Cow Matsoon

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Abstract: *It was shown, that strains of lactic acid bacteria genus of Enterococcus, isolated from the sample of cow matsoon from different regions of Nagorno Karabakh Republic, are comply with the requirements of the WHO to probiotics and can be used as basis for obtaining of the new products of functional nutrition significance. It was shown, that during co-cultivation strains Enterococcus with different combinations, the increase in antimicrobial activity are not for all combinations of Enterococcus .*
Keywords: *endemic strains of Enterococcus, fermentation, probiotics, antimicrobial activity.*

I. Introduction

Traditional foods have a unique place in diets of the consumer today, associated with different demographic groups and have been an integral part of their ethos. It is amply evident that traditional foods of a particular country or region may not entirely suit people in other countries or regions.. It is one of the most practical, economic and widely applied empirical methods for preserving and often enhancing organoleptic and nutritional quality of fresh food. Nature of fermented products varies from one region to another. It depends on the local indigenous microflora, which in its turn reflects the climatic conditions of the area. Thus, traditional fermented milk in regions with a cold climate contained mesophilic bacteria such as Lactococcus and Leuconostoc spp., thermophilic bacteria which include mostly Lactobacillus and Streptococcus, prevailed in regions with a hot, subtropical or tropical climate [1,5].

Great variety of ecological and geographical conditions of South Caucasian Republics promoted development of unique associations of lactic acid bacteria (LAB) and yeasts in homemade dairy products. Matsoon was considered as an analog of yoghurt and is prepared from milk of cow, sheep, goat and buffalo. Traditional Armenian Matsoon (syn. mazun, matsoni, matsun), as well as the soft and hard cheeses is produced using the home made starters, so called "spontaneous starters", microbiology content and biotechnological characteristics of which are unknown. Its commercial and, especially health beneficial value is poorly studied. Hence, it can be considered as a potential pool of the strains with probiotic and antimicrobial activity against pathogens causing human and animal diseases, as well as food-spoilage microorganisms [5]. It was shown that isolated from the sample of cow matsoon from different regions of Republic of Armenia LAB of the genus Lactobacillus had high antibacterial activity[7,13]. The aim of this study was investigation of the properties of new strains of LAB genus Enterococcus isolated from matsoon from different regions of Republic of NKR with antimicrobial properties for the creation of new complex preparation. At the same time it was shown that the some strains genus Enterococcus produced bacteriocins. So, Enterococcus faecium CRL35, a strain isolated from regional Argentinean cheese, produces a bacteriocin called enterocin CRL35 which inhibited the growth of Listeria monocytogenes . From horse milk (mare) isolated strain of LAB Ent. durans, which inhibited the growth of E. coli, Staphilococcus Aureus, Listeria innocua. It is known that strains of this type inhibit the growth of, and are in a symbiotic relationship with bacteria of the genus Lactobacillus, Lactococcus, Bifidobacterium. [8,9,11,14]. It is these nontoxigenic antimicrobial peptides that are of the greatest medical interest. The same authors point out that studies of antimicrobial peptides or their combination will allow the creation of drugs for the therapy and prevention of infectious pathology.

II. Materials And Methods

Microbial strains and growth media. Samples were collected in sterile small bottles and stored at 4^{o}C 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 in thermostat at 37^{o}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^{o}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^{o}C.

Genera and species belonging of some selected strains was determined by the application of different methods of identification and LAB genotyping (API 50 CH, 16S rRNA sequencing, GS-PCR, RAPD PCR). The culture

collections is in the Department of Center of Microbial Depository (CMD). Selected LAB strains deposited with the CMD at the SPC "Armbiotechnology" NAS RA. Lactobacillus jensenii M14-MDC 9634, Enterococcus durans P13-MDC 9635, Enterococcus durans M42-MDC 9636, Enterococcus durans M44-MDC 9637, Enterococcus faecium M22-MDC 9638. The culture collections is in the Department of Center of Microbial Depository (CMD).

The strains of multidrug resistant human pathogens were isolated from infected patients (feces, urine, wounds, blood, throat, etc) in the Infection Hospitals of Stepanakert and antimicrobial activity were investigated in the "Center for Hygiene and Epidemiology", Stepanakert, NKR. It was used S.typhimurium G-38, B.subtilis 17-89 strains Laboratory of Microbial Preparations of SPC "Armbiotechnology" Yerevan, RA.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 (phys. solution) at $2.2x10^6$ CFU ml^{-1}.

Obtaining of cell-free culture broth. Single colonies were grown in 50 ml of MRS broth and incubated overnight at 37°C for 24 hours. Obtained inoculum was transferred into 1000 ml of MRS broth and grown at 37°C for 24 hour. At the end of culture growth cell concentration achieved (7±2) x 10^8 CFU ml^{-1} (of titration) and pH reduced to 3.5- 4.2. Culture broth was collected after 24 hrs and centrifuged at 6,000 rpm during 20 min. Cell free culture broth of LAB strains was concentrated 5 times and purified by gel filtration method (Sephadex G25) [3].

Determination of antimicrobial activity. The spot-on-lawn method on the test culture Salmonella typhimurium G-38 and Bacillus subtilis 17-89 inoculated 20 мкл in the solid medium was applied. The antimicrobial activity is expressed in arbitrary units (AU ml^{-1}) [10].

III. Results And Discussion

More than 150 cultures of LAB were isolated from various samples matsoon from different regions of NKR. Colonies of isolated LAB differed by color, shape and size when inoculated in agar media (MRS and/or hydrolyzed milk). Strains study at separate and joint cultivation in milk (3,2 %) showed a change in the total acidity of from 80 to 120 ° T to, within 12 days of storage in the refrigerator $+4^0$C. LAB strains were mainly presented by coccoid cells (about 86 %),which may be due to the climatic conditions of the southern region.Isolated colonies of strains of LAB grows in MRS and fermented milk 7-8 hours at a temperature of 30 and 37^0C. The main probiotic characteristics of selected LAB strains were investigated according to the methods described [3,6]. The results of which are shown in Table 1. As seen from the results of investigation probiotic properties selected strains of genus, Enterococcus are different.

Table 1. Comparative characteristics of probiotic properties of strains of some genus Enterococcus

Characteristics	**Strains of LAB**			
	Ent. faecium M22	**Ent. durans M42**	**Ent. durans M44**	**Ent. durans P13**
Resistance to proteolytic enzymes, (%)	42	58	41	35
Stability to bile, 0,1-1.0, (%)	27	62	66	72
Survival at pH, 2.0-9.0 (%)	60	40	45	25
NaCL tolerance, 2-10 %	35	55	45	25
Antibiotics (19) resistance, (%)	45	80	60	55
Ability to adhesion	-	+	++	+
Antioxidant activity, (%)	50	40,5	20	100
Proteolytic activity	-	+	+	+

A number of authors showed that non-pathogenic strains of Enterococcus faecium and other Enterococcus species isolated from healthy humans and animals, as well as from probiotic and food products, secrete second-class bacteriocins as a rule. For studying of antibacterial substance produced of Enterococcus , bacteria were grown in MRS media 48 hour.37^0C. Results was shown in table 2 as seen from the data at the pH=6.0 had growth inhibition of test culture and antimicrobial activity of culture liquid (CL) was higher at the co- cultivation. According to Kleinhammer, one of the main approaches to the detection of bacteriocins is the determination of antimicrobial activity at pH = 6.0-7.0, which indicates the protein-like nature of the antimicrobial substance.

The strains of Enterococcus M42 has the greatest resistance to 19 antibiotics tested than other strains and suppresses the growth of gram negative bacteria with high efficiency. Three strains of Ent. durans were selected for work. Table 2 shows the results of purification of CL after cultivation of the studied bacteria. As can be seen from the results obtained, the greatest activity at pH = 6.0 with the co-cultivation of Ent.durans P13 + Ent. durans M44, which may be due to an increase in the synthesis of protein-like substances .

Table 2. Antimicrobial activity of CL Ent. Durans(purification by gel filtration Sephadex G25) pH 6.0.

Type of Grown of LAB	**LAB**	**Antimicrobial fraction**	**Salmonella typhimurium G-38**	**Bacillus subtilis G17-89**
			AU/ml	**AU/ml**
Separately	**Ent..durans M42**	20-25	1500	1600
	Ent. durans M44	15-18	800	1200
	Ent.durans P13	19-25	500	400
Co- cultivation	**Ent.durans P13+ Ent. durans M44**	11-20	1000	2400
	Ent.duransP13+ Ent.durans M42	18-22	1000	1200
	Ent.duransM44+ Ent.durans M42	18-25	700	1200

The results are of antimicrobial activity supernatant of LAB investigated purification by gel filtration Sephadex G25 are shown in Table 2. It was shown the highest antimicrobial activity (2400 AU/ml) were at the co-cultivation two strains of Ent.durans P13+ Ent. durans M44 in contrast to separate cultivation.The increasing could be resulting synergism of products of metabiotics of bacteria with antimicrobial activity.

Purification of culture broth, obtained after growth of some selected strains of LAB by the method of gel filtration have shown that the output of the fraction with antimicrobial properties differ in volume and content of dry substances. Figure 1 shows the appearance of the antimicrobial fraction by the example of the strains of Ent. durans P13 and Ent. durans M44.In figure 1 and 2 shows that Ent. durans P13 is different Ent. durans M44 from antimicrobial fractions. Ent. durans P13 has three antimicrobial fractions but Ent. durans M44 has one big antimicrobial fraction. However, when co-cultivation, the antimicrobial activity is represented by fractions № 11-26, in contrast to the fractions obtained when the strains under study were separately grown (№ 8-23 and 19-25)

Fig 1 Ent. durans P13

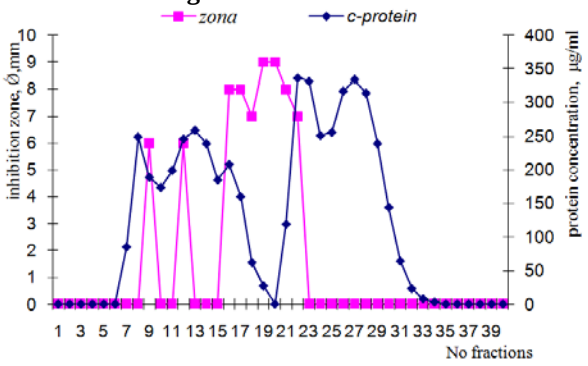


Fig 2 Ent. durans M44

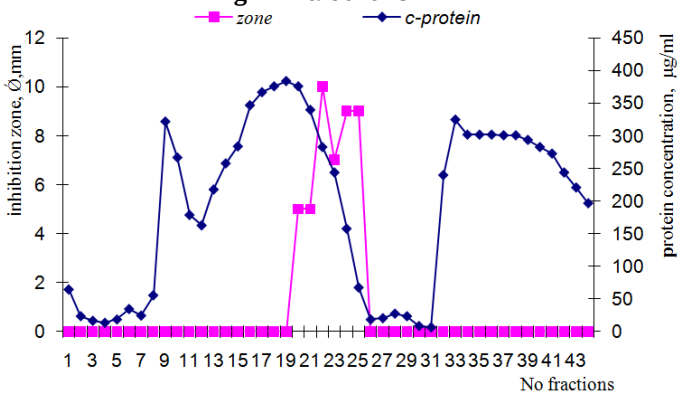
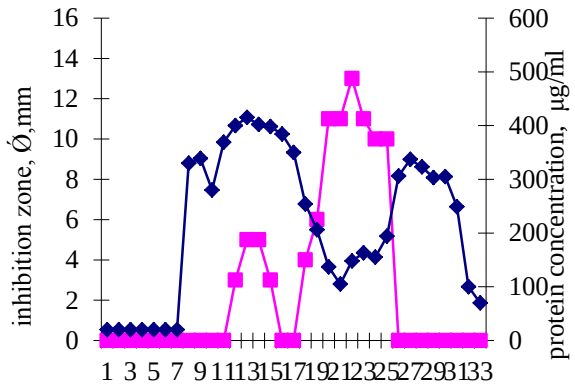


Fig 3 Ent.durans P13 +Ent. duransM44



We have previously published data about the growth suppressing preparation derived from the cultural fluids of genus Lactobacillus strains [7,13].

The investigation of antimicrobial activity of antimicrobial preparations obtained from of culture broth Ent.durans K13, Ent.durans M44 against antibiotic resistant bacteria against, was of interest.

Comparative results of influence of fraction with antimicrobial activity obtained after co-cultivation LAB genus strains Enterococcus against antibiotic resistant bacteria are presented in the Table 3 . As can be seen partially purified preparations from different strains of LAB possessed different antimicrobial activity.

Table 3. Comparative antimicrobial activity partially purified preparations of strains genus Enterococcus against antibiotic resistant bacteria(pH 6.0), %

Antibiotic resistant strains	**N**	**partially purified preparations (200 AU/ml)**			
		Ent.faeciumM14	**Ent.duransM42**	**Ent.duransM44**	**Ent.durans P13**
		%	%	%	%
Staph. aureus sp.	28	0.0	0.0	0.0	25.0
Ps. aeruginosa	21	50.0	0.0	50.0	58.0
Pr. mirabilis	23	50.0	20.0	50.0	29.0
Klebsiella sp.	8	0.0	50,0	0	0.0
Pr. vulgaris	7	0.0	0.0	0	0.0
E.coli sp.	15	50.0	40.0	50.0	15.0

N-number of investigated antibiotic resistant bacteria

Thus, our experimental data shown, that isolated 3strains of of strains genus Enterococcus are able to produce substances with antimicrobial activity with high inhibitory activity against antibiotic-resistant strains Ps. aeruginosa. This can be resulting from differences in nature and amount of antibacterial compounds, produced by strains genus Enterococcus. Above all, this can be explained with the presence of specific receptor proteins required for binding to bacteriocins and their transport into the bacteria.

A number of studies show that biodiversity LAB matsun samples differs and depends on the area where they are isolated .So in different cheeses, shows that in the samples mainly contain the following kinds of LAB L. plantarum, L. brevis, L. paraplantarum and Ent. faecium, and yogurt, L. bugaricus, L. delbruecki and S. termophilus, Ent. faecium in Georgian yoghurt samples was isolated L. acidophillus, L. casei, S. termophilus, L. lactis, L. diacetylactis, Ent. durans but have not met the bacteria L. bulgaricus [4].Our findings show that isolated LAB from samples of matsun of Nagorno Karabakh contain LAB Ent. faecium, Ent.durans, which exhibit probiotic properties and the ability to inhibit the growth of some antibiotic-resistants strains.

On the basis of experimental data, the LAB strains can be used as basis for obtaining the new products with antimicrobial and health promoting effects, as well as for obtaining of partially purified preparations .

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