Certain probiotic properties of lactic acid bacteria from the Iranian dairy product "Richal"

F. Karimpour [1, 2], F. N. Tkhruni[1], K. J. Karapetyan[1], S. H. Razavi [3]

[1]-Scientific and Production Center "Armbiotechnology" of the National Academy of Sciences of Armenia, Yerevan.
[2]- Yasuj University of Medical Sciences, Food &Drug and Social Determinants of Health Research Center, KohgiloyehvaBoyerahmad, Yasuj, Iran.
[3]-Professor. Dr. in Food & Biotechnology Process Engineering, Head of center of excellence application of modern technologies for producing foods and drinks functional University of Tehran, Iran.
Correspondence to Farzad Karimpour: saverzida@yahoo.com

Abstract: Traditional dairy products have lacto acid bacteria and source of probiotics, In our country, there are different kinds of traditional dairy products which are produced from sheep and goat milk such as drinking yoghurt, yoghurt, kashk, gharaghooroot, cheese, etc, so many authors mentions about beneficially of probiotics properties for human being.The new potential probiotic lactic acid bacteria (LAB) from traditional Iranian dairy beverage Richal were isolated and investigated. The Richal, technology of which is to ferment milk in bags of sheep, goat skin tanned with the addition of special local herbs and salt, was used. The morphological and physiological properties, resistance to various concentrations of bile (0,2-1.0%), the range of pH (2-10), resistance to enzymes (pepsin, trypsin), the rate of growth in skim milk was studied. Selected LAB had different properties. 17 strains were selected with promising probiotic properties: fermented milk for 4-16h, were resistant to bile, enzymes, possess antimicrobial properties and had a high growth rate. The results testified that isolated new perspective strains can be used as basis for obtaining the new products of functional nutrition significance.
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1.Introduction

For the past years there is great interest towards probiotics. On the fact it is connected with the modern state of antibiotic resistance stipulating search for new alternative antibiotics with more physiological and safe means for routine maintenance and treatment of infections additionally, with elaboration of new technologies which allow to create active and safe preparations of probiotics. Probiotics are natural preparations consisting of stable living cultures of microorganisms or products of metabolism which have diverse pharmacological properties. The most frequently used microorganisms in the probiotic preparations are different strains of lactic acid bacteria (LAB)and bifidobacteria [1]. Analyses of the data in literature testify that the bifidobacteria which together with other anaerobic bacteria make the main part of norm flora, in the unfavorable conditions disappears from the bowels first of all. Elimination and considerable lowering of their amount in the gastrointestinal tract leads to deep imbalance of intestines, a profound disturbance of digestion and all kinds of metabolism. On the background of bifidoflora deficiency the pathogenic properties of the staphylococcus, fungus of the Candida genus are actively manifested [2].Literatures showed that probiotics must meet certain requirements and have predetermined properties, in particular, they must be commensal to the human organism, must have plantation potential, i.e. be preserved in the intestine until there achieved the positive action (be resistant to the bile acids, antimicrobial substances produced by the indigenous micro flora, be good adhesive to the epithelium of the corresponding mucous membranes, be resistant to a number of enzymes of the gastrointestinal tract and produce the antibiotic like substances[3]. Useful impact on the host organism must be confirmed by laboratory investigations and clinical observations. An important properties of the probiotic strains are manifestation of activity at low pH, as decrease of the pH media leads to death of many pathogenic microorganisms. Analyses of literature show that LAB may grow in a wide range of pH=2,0-10,0 [4]. For the past few years the source for isolation of the probiotic strains of LAB has been different food products. LAB can be found in milk, meat and other dairy products as well as in fermented drinks. At present it has been proved that the LAB, isolated from Greek cheese, salted cheeses of Armenia,

Mongolian Yogurt, Irish curds etc. are capable to suppress wide range of microorganisms which cause spoilage of food products [5]. Dairy products may differ depending on the region of their production. That depends on the indigenous micro flora which in its turn reflects climatic conditions of the region [6]. Main purpose of this work was isolation of strains of lactic acid bacteria from the transnational Iranian product Richal and investigation of their probiotic properties.

2Materials and methods

Collection of samples: Strains of the lactic acid bacteria which were isolated from different samples of the Iranian dairy product .Richal produced in natural farms of the Northern Iran, There are produced three different dairy products of Richal which differ by their organoleptic properties and technology of production. Hereinafter we set forth description of their production:

(Richal massti) – milk with fat 3.5% is boiled and put local starter so left for ripening at the room temperature for 6-10 hours and keep in cooling condition(yoghurt), after that put the yoghurt into a sheep, goat leather (Mashk) and add salt, herbs included, mint, wild celery and chicory. Ripening of the fermented milk takes place during 3-4 days at room temperature. From such sample there were separated 30 colonies of LAB which differ from each other by their morphology and physiological features[7].

(Richal Shiri) – heated whole milk the 3.5% fat with local starter is put into a sheep, goat leather (Mashk) and salt, the herbs are added. Ripening of the milk takes place during 3-4 days. From such a sample there were separated 11 colonies of LAB which differ from each other by their morphology and physiological features[7].

(Richal Dooghi) – milk with fat 3.5% is boiled and put local starter so left for ripening at the room temperature for 6-10 hours the (yoghurt), then mixed water and put into the skin bags (Mashk). The Mashk is shacked together with that mixture during 2 hours for separation of the butter. The butter is removed, there is received a product in the Mashk which is called Doogh "drinking yougurt". After it we add salt and the local herbs and put it into the Mask again for 3-4 days for ripening at room temperature. From this sample there were separated 36 colonies of LAB which differ from each other by their morphology and physiological features[7].Out of three samples of the Richals there were isolated 77 strains of lactic acid bacteria.

Objects of investigations: Separation of clear cultures from the samples of the dairy product Richal was carried out by the way of cultivations of serial dilutions of the selected samples on the solid nutrient media MRS and medium with hydrolyzed milk (with content of 1,2% of agar), by method of exhaustive stroke with purpose of gaining single colonies according to the accepted in the practice methods [8]. The separated LAB cultures were selected according to their morphological and physical characteristics and, as it was shown by the researches, they were mainly represented by bacill - shaped and cocci shaped cells of different sizes. As a criterion for preliminary selection of the isolated LAB strains was taken their possibility to ripen the milk as well as speed of ripening the milk after input of some amount of LAB. The separated new strains were numbered. Museum cultures of LAB were stored at -20C0 in sterile skim with 40% glycerin[9].

Conditions of growth :The following nutrient media were used: MRS broth (Merck) and 10 % dry skim milk. The overnight inoculums of the culture inputted into the nutrient media in the amount of 10% from the volume of the media. The cultures were cultivated at 37oC and 42oC during 24, 48 and 72 hours [10]. Titer of the culture after the growth was diluted4time defined by measurements of its optical density on the spectrometer Hitachi U-1100 at 590 nm.

Obtaining of supernatants: The cultural liquid (CL), obtained after growing of the researched microorganisms was centrifuged for separation of the biological mass at 4000 r/pm during 30 minutes.

Antimicrobial activity of the supernatants: At pH=6,0 was defined on the tested cultures with using the method of diffusion into agar. The activity was estimated by measuring the size of the zones of suppression of the tested cultures growth (Ø, mm) after 40 hours of incubation in the thermostat at 300C [11].

Test cultures: For definition of antimicrobial properties of the supernatants of LAB the gram-negative conditionally pathogenic bacteria *Salmonella typhimurium G-38* and gram-positive bacteria *B. subtilis 17-89,* contained in the collection of the "Laboratory of Microbial preparations" of the Scientific and Productional Center of Armbiotechnology, were used.

Definition of sensitivity to bile and pH ferments: The isolated strains of LAB were incubated in a nutrient media MRS broth (Merck) with content of definite amount of bile during 24 hours at 37o C in a thermostat [12].Survival of LAB in the conditions close to those as in intestine (influence of digestive enzymes, pH in the range 3,0-8,0) was checked according to the generally accepted

method [3]. pH of the definite volume of the media was changed with 1N hydrochloric acid (HCl) or 40% solution of NaOH. Determination of sensivity of LAB to enzimes was carried out according to the method [13]. For this purpose single colonies of each strain were incubated in the nutrient media of MRS broth (Merck) with content of the corresponding ferment with the final concentration of 0,5 mg/ml. There were used the following chemical agents in the work: Trypsin, Pure from bovine pancreas 3x, activity 2500 NFU/mg (HIMEDIA), Pepsin, Extra pure (1:3000), (HIMEDIA). After two hours of incubation at 370C activity of the ferments was stopped by heating up to 1000C during 5 minutes. pH of the researched batches reached up to 5,5-6,0 and checked the residual antibacterial activity. Survival of the LAB was estimated by the change of the optical density at 590 nm.

3.Results and discussion: Out of the traditional Iranian product - dairy drinking yogurt Richal - were isolated and investigated new potential probiotic lactic acid bacteria. Richal was used in the process of work technology of which includes fermentation of milk in sacks from sheep skin with addition of special local herbs and salt. The selected strains were gram-positive and non-sporogenous. They were represented by cocci-, and bacill -shaped forms. Stability to bile is one of the most important properties of the microorganisms included into the content of probiotics. Bile stipulates death of great amount of bacteria as their cell membranes consisting of lipids and fat acids are very sensitive to salts of bile acids. In this connection efficiency of the probiotic microorganisms depends on their stability to bile [3]. For definition of the of stability of secondary metabolites of LAB to bile, the influence of different concentrations of bile on the growth of the investigated LAB have been carried out (Table 1). The probiotic microorganisms, passing through the gastrointestinal tract, are subject to change of the environment acidity as different parts of intestines differ by pH environment. An important feature of the probiotic strains is also manifestation of its activity at low pH, as because decrease of the pH environment leads to death of many pathogenic microorganisms [3]. Analyses of literature show that LAB may grow in a wide range of pH = 2,0-10,0 [4]. Results of the LAB growth at different value of pH, as well as its stability to different concentrations of bile are summarized in the Table 1.

Table 1. Growth of LAB strains at different meaning of bile and pH

Strains	Check (without treating)	Investigated properties						
		Bile, %			Value of pH			
		0.2	0.4	1	2	5	7-8	9-10
	Optical density (590 nm)							
LAB sp. F4	0.6	1.2	0.34	0.12	0.22	0.6	0.4	0.21
LAB sp. F14	1.2	0.37	0.23	0.16	0.19	1.0	0.04	0.04
LAB sp. F15	1.4	0.95	0.73	1.4	0.4	1.4	1.5	1.4
LAB sp. F18	1.4	1.0	0.95	1.4	0.41	1.3	1.4	1.3
LAB sp. F22	1.0	1.0	0.59	0.76	0.24	0.95	0.62	0.15
LAB sp. F48	0.8	0.95	0.85	0.66	0.2	1.0	0.44	0.11
LAB sp. F60	1.2	0.87	0.8	0.87	0.6	1.25	1.4	1.2
LAB sp. F61	1.3	0.83	0.85	1.0	0.31	1.2	1.3	1.3
LAB sp. F62	1.2	0.95	0.9	1.2	0.21	1.1	1.2	1.2
LAB sp. F63	1.2	0.98	0.9	0.9	0.28	1.15	1.3	1.2
LAB sp.. F64	1.2	0.9	0.82	0.83	0.29	1.2	1.3	1.2
LAB sp. F66	1.4	1.1	0.8	1.2	0.42	1.4	1.4	1.4
LAB sp. F67	1.4	1.2	0.9	1.4	0.37	1.4	1.4	1.4
LAB sp. F69	1.15	0.7	0.9	0.68	0.3	1.0	1.2	1.2
LAB sp. F72	0.9	1.2	1	1.4	0.39	1.4	1.4	1.4
LAB sp. F73	0.64	0.75	0.22	0.32	0.22	0.64	0.8	0.75
LAB sp. F78	1.2	0.85	0.85	1.0	0.37	1.2	1.4	1.4

As it can be seen from the set forth results low concentrations of bile (0,1-0,2%) didn't essentially influence on the growth of the investigated bacteria. Their high concentrations (1%), inhibited growth of the bacteria (*LAB sp.* F4, *LAB sp.* F14, *LAB sp.* F73). The investigated bacteria made three groups on their ability to grow at different values of pH. Group of bacteria growing only in the medium with pH = 5.0 (*LAB sp.* F41, *LAB sp.* F14, *LAB sp.* F22, *LAB sp.* F73). The second group of LAB grew at pH =5.0-8.0. The third group of the

isolated LAB grew at pH = 9.0-10.0 as well (all the LAB except for *LAB sp.* F 48 and *LAB sp.* F 73).Thus all the investigated LAB strains preserve their survival ability at the physiological concentrations of bile (correspondingly 0,2% - 0,4%). But they differ on their ability to survive in the medium with different values of pH. One on the important properties of the bacteria used in the capacity of probiotics must be its stability to the impact of protheolitic enzymes [3]. With purpose of definition of sensivity of the investigated LAB to digestive enzymes, they were subject to fermentation treatment in the conditions corresponding to each enzyme. Results of researches of LAB sensivity to ferment of intestine are set forth in the Table 2.

Table . Growth of lactic acid bacteria in the presence of digestive enzymes

Strains	Check		Enzymes, (0.5 mg/ml)			
			Tripsine		Pepsine	
	Optical density (590 nm)					
	2h	24h	2h	24h	2h	24h
LAB sp. F4	0.9	1.4	0.37	0.87	0.35	0.77
LAB sp. F14	0.5	1.2	0.36	0.68	0.47	0.64
LAB sp. F15	0.7	1.4	0.68	1.2	0.48	1.2
LAB sp. F18	0.7	1.4	0.53	1.2	0.46	1.0
LAB sp. F22	0.46	1.3	0.3	0.77	0.35	0.85
LAB sp. F48	0.44	1.2	0.31	0.66	0.27	0.7
LAB sp. F51	0.48	1.3	0.37	0.85	0.33	0.8
LAB sp. F61	0.46	1.2	0.46	0.85	0.32	0.8
LAB sp. F62	0.48	1.2	0.37	0.77	0.27	0.7
LAB sp. F63	0.44	1.1	0.36	0.85	0.42	0.8
LAB sp. F64	0.48	1.2	0.42	0.85	0.42	0.85
LAB sp. F66	0.66	1.4	0.6	1.2	0.61	1.0
LAB sp. F67	0.68	1.4	0.6	1.2	0.61	1.0
LAB sp. F69	0.46	1.2	0.7	0.75	0.51	0.75
LAB sp. F72	0.77	1.4	0.62	1.2	0.75	0.9
LAB sp. F73	0.32	1.2	0.21	0.58	0.17	0.5
LAB sp. F78	0.5	1.2	0.35	1.0	0.36	0.71

Thus, probiotic microorganisms as well as products of their metabolism, passing through different parts of the intestine, are subject to impact of different enzymes of the intestine [14], it can be admitted that when using *LAB sp.*F15 *LAB sp.*F18 *LAB sp.*F 66 *LAB sp.* F 67 as the ingredients of the products of functional nutrition, they won't be subject to impact of these enzymes. The researchers were interested whether or not the supernatants gained after growth of the isolated LAB are capable to suppress growth of some gram-positive and gram-negative bacteria on the example of *S.typhimurium G-38* и *B.subtilis 17-89.*Definition of the antimicrobial activity of the supernatants of the investigated 5 LAB showed that some of them suppressed growth of the gram-positive and gram-negative bacteria. It can be accepted that these bacteria synthesize substances with antimicrobial activity. Results of definition are set forth in Table 3.

Proceed from the gained results it can be seem that the isolated bacteria differ from each other on manifestation of antimicrobial properties. Out of 17 bacteria only 5 strains suppressed growth of the investigated test cultures with clearly expressed zone of growth suppression in the shape of bacterium growth lysis. According to classification by Klaenhammer [15], on the indices of stability to bile, range of growth at different meaning of pH, antimicrobial activity the most perspective is the strain of bacteria LAB sp. F15. At present there are carried out works on investigation of the productive

characteristics of the strain for its use in creation of a new probiotic product of functional nutrition.

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Table . Antimicrobial activity of the supernatants of LAB

Strains	Test cultures, Ø mm	
	S.typhimurium G-38	*B.subtilis 17-89*
LAB sp. F51	8±0,5	7±0.3
LAB sp. F48	8±0,5	13±1,0
LAB sp. F67	12±1.0	8±0.2
LAB sp. F22	7±0.3	13±1,0
LAB sp. F15	16±1.0	17± 1.0

Corresponding Author: Farzad Karimpour saverzida@yahoo.com Scientific and Production Center "Arm biotechnology" of the National Academy of Sciences of Armenia, Yerevan. /Yasuj University of Medical Sciences, Food &Drug and Social Determinants of Health Research Center, KohgiloyehvaBoyerahmad, Yasuj, Iran.

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