Investigation of lactic acid bacteria isolated from domestic Iranian product Richal Masti

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Abstract: Fermentation of traditional foods, as a hurdle technology, is profitable in terms of food quality, preservation and Decontamination of toxins, often found in food. The category of dairy,Fermented milk products are cultured made from whole or skim, that require specific lactic acid bacteria to develop their characteristic flavor and texture. In Iran we have a lot of tradition fermented milk so this study is Morphological, isolation, characterization the occurrence of micro flora and lactic acid bacteria in a kind of Richal, the traditional dairy beverage used in the south of Iran. Richal is prepared in skin churn tanned container sheep and goat (Mashk). The beverage is produced by three method, the Richal Masti was prepared, which consists of special local herbal and salt with full fat yoghurt, kept in environmental condition some days for natural processing same as source. The prepared sample was transferred to the Institute of Arm Biotechnology in Armenia from Kohgiloyeh region in Iran.For the first time have been separated lactate bacteria (LAB) from the traditional Iranian drinking yoghurt Richal. There have been set forth results of microscopy investigations and estimation of the growth of the separated isolates of LAB on agar and broths MRS and milk. Out of 27 LAB have been selected which some strains have probiotic properties.
[Farzad Karimpour. **Investigation of lactic acid bacteria isolated from domestic Iranian product Richal Masti.** *Life Sci J* 2013;10(6s):513-516] (ISSN:1097-8135). http://www.lifesciencesite.com. 79

Key words: lactic acid bacteria, Richal Massti, probiotic, Kohgiloyeh, Mashk

1..Introduction

In history milk played a major role as a nutritional source and since 1900's golden era of industrial microbiology began. It was also economically significant because larger quantity of milk was processed daily in factories for the fermented food products. LAB were first isolated from milk and have since been found in such foods and fermented products as meat, milk products, vegetables, beverages and bakery products [1,2,3]. Lactic acid bacteria are widely distributed in nature. In general Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Lactospaera, Leuconostoc, Melissococcus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus and Weissella are recognized as LAB [4].Many traditional fermented milk products are made in Asia, Africa, the Middle East and Northern and Eastern Europe, and the microbiological characteristics of several of these products have been studied in the world. The nature of fermented products differs from one region to another, and depends mainly on the local indigenous micro flora, which in its turn reflects the climatic conditions of the area and obtained the scientific evidence of beneficial effects of fermented milk products containing specific probiotic strains [5].In production of salami sausage (Italy) lactic acid bacteria *Lactobacillus plantarum, L. Curvatus* producing bacteriocin-like substances were added to the sausage mass at various stages of ripening; in production of cheese, *Lactococcus lactis* and *E. faecalis* that formed bacteriocins which prevented growth of strain-producers of histamines were added to a starter. Strain *Enterococcus mundtii* GRL-35 synthesizes several bacteriocins some of which are used as biopreservatives. In spite of the fact that enterococcus do not appear as GRAS they have many positive properties that make it possible to use them in foodstuff production technology. Earlier we had publications on investigation of application of supernatants of *L.acidophilus 1991* and of other new LAB strains in processed cheese production technology with the purpose to extend its storage time [6, 7].

Out of different types of brine cheese there were separated isolates of LAB which represent types of *L.rhamnosus u L.plantarum, P.pentosus, L.faecium,* and *cocci of Streptococcus* and *Enterococcus* types. There was shown their perspective in their usage as biological conserving agent for a number of foods. The carried out analyses of different data testifies the great potential of LAB depending on the origin.In Iran 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. In comparison with the commercial species, composition of lactic acid bacteria is more varied and inconstant in these products.The aim of the present

study is isolation and identification of a large number of lactic acid bacteria from drinking yoghurt in order to constitute.One of the goals in this study is morphological, isolation, characterization the occurrence of micro flora and lactic acid LAB strains bacteria an original collection of Kohgiloyeh region in Richal as traditional dairy beverage used in the south of Iran. Richal is prepared in skin churn tanned container sheep and goat (Mashk). The produced by three different methods, the Richal was prepared, which consists of special local herbal and salt with full fat yoghurt, kept in environmental condition for some days for natural processing [6,8,9].

2 Materials and methods

Sample collection Isolation of lactobacilli. The samples were aseptically weighted and homogenazied. From each sample, a 1:10 dilution was subsequently made using peptone water followed by making a 10 fold serial dilution. 0.1 ml from each dilution was then subcultured, in duplicate, into the M17 and MRS agars (Merck, Germany) used for isolating LAB [10].Bacterial cultures and Medium. Strains of lactic acid bacteria were isolated from Richal Massti from Kohgiloyeh region, Iran. Isolation of pure LAB cultures was carried out by inoculation of the selected samples on agar nutrient MRS Medium and Medium with hydrolyzed milk (containing 1,2% agar) by one of the method generally accepted in laboratory practice aimed at obtaining of separate colonies. LAB cultures were grown in either milk or MRS broth (MERK, Germany or Himedia, India), in the amount of 10% of the Medium volume during 24 hours in temperature controlled conditions at 37oC. The LAB strains were maintained as frozen stocks: 1 ml of each grown culture was transferred to the plastic tubes containing 40% Glycerol, and then the cultures were stored in the freezer at -20oC. Before use, LAB strains were transferred twice into the appropriate Medium and incubated according to the respective growth conditions. After the growth, cell concentration achieved 7±2x108 CFU ml-1. As a criterion for preliminary selection of the isolated LAB was taken the ability of strains to ferment milk as well as the rate of milk ripening after inoculation of a certain amount of LAB, as well as presence of antimicrobial activity of supernatants obtained from isolate culture liquid (CL) after growing of the investigated microorganisms on the MRS nutrient broth for 48h, at 37 oC. Growth was assayed in MRS broth at 10, 15, 37 and 45o C as well as at pH of 4.4, 5.0, 8.6 and 9.0 incubated at 37 o C. Salt tolerance was tested with 6.5, 10 and 15% (w/v) NaCl in MRS broth.The acidity of the culture fluid was determined by Turner (oT).Obtaining of cell-free supernatant: Culture liquids obtained after growing of the researched microorganisms in MRS broth were centrifuged at 4000 rpm for 30 minutes to remove biomass. Determination of antimicrobial activity: Antimicrobial activities of supernatants from the investigated LAB were determined on test cultures using the methods, spot-on- lawn. The test-culture inoculated in the solid 0.7 % nutrient Medium. Antimicrobial activity was assessed by measuring the size of the inhibition area of test culture growth (Ø, mm) after 24 h incubation in thermostat at 30 oC. Test-cultures: To determine antimicrobial properties of supernatants conditionally bacteria from the collection of the Laboratory of Microbiological Technologies of the Scientific and Production Center "Armbiotechnology", National Academy of Sciences of the Republic of Armenia (SPC "Armbiotechnology" NAS RA) were used.Sensitivity of cell-free supernatant, pH and enzymes, bile.The dependence of antibacterial activity on pH was measured in the range of 2-10 by adding 1N hydrochloric acid (HCl) or 40% NaOH with further retention at room temperature for 4 h, followed by their activity determination [11,3]. Determination of the resistance to bile and enzymes were determined by standard methods [12, 13, 14,15, 16,3].

3.Results and discussion.

Results of the preliminary selection on the possibility of the separated colonies to grow in the different Medium at incubation in thermostats in temperature 37^0 C at the agar and broth are shown in the Table 1.

Table 1. Growth of separated LAB in different Medium (37^0 C, 48 h)

Agar								Broth			
MRS (Merk)		MRS (Himedia)		MRS (Merk)		MRS (Himedia)		MRS (Merk)		MRS (Himedia)	
N:	%	N:	%	N:	%	N:	%	N:	%	N:	%
27	90	27	90	27	90	27	90	27	90	27	90

Table 2.

Microscopy				Gram Staining				Catalase			
Cocci sp.		Bacillus sp.		Positive		Cocci sp.		Bacillus sp.		Positive	
N:	%	N:	N:	%	N:	N:	%	N:	N:	%	N:
27	90	3	27	90	3	27	90	3	27	90	3

It should be noted that this feature of growth on solid Medium was marked at growth of the separated of LAB at temperature 42^0 C during 48 hours. Some physiological indices of LAB are set forth in the Table 2.

Results of microscopic analyses showed that the colonies separated from the sample were presented mainly by gram positive bacteria (100%) on which cocci-shape ones made 90% and stick-shaped – 10%. Among the separated colonies 92% were catalase-negative and 8% catalase-positive bacteria. Presence of catalase-positive bacteria may be stipulated by taking place the process of fermentation in the leather bottle and they might have passed into the analyzed sample of the product [17,18]. Preparation of the initial sample for the process of fermentation is of almost importance. The process of preparation was carried out at different temperatures. Results of fermentation are set forth in the Table 3. From the results of the Table one can see that during the process of fermentation in milk 13,3% of colonies out of 30 equally fermented milk at 37 C^o and 42 C^o. Results at shown in table 3.At first the separated colonies were grown in milk. As it can be seen only 4 colonies fermented milk.(first option) At this method of inoculation there took place 100% of milk fermentation at 37 C^o and 96,6% at 42C^o (Overnight from MRS to Milk). At reinoculation of the colonies from milk to milk reduction of the colonies amount was observed: at 37C^o milk was fermented to 66% and at 42 C^o - 83, 3%. Comparing the received data one can see that for obtaining the initial isolate the best results were achieved at reinoculation from MRS into milk [1912, 15,20].

Table3. Fermentation of the milk with separated colonies at different ways of reinoculation and different temperatures of growing.

Temperature, ^oC		Growth of Colony in Milk				Overnight from MRS to Milk				Overnight from milk to milk			
		PH	Acidity, ^0T	N	%	PH	Acidity, ^0T	N	%	PH	Acidity, ^0T	N	%
37	30	4.2	122	4	13.3	4.9	105.4	30	100	4.9	108	18	66
42	30	3.9	143	4	13.3	4.9	95.66	29	96	4.9	72.6	25	83

100% of the milk reinoculation took place at this method of reinoculation at 37 C^o and 96, 6% at 42 C^o. The separated colonies which were reinoculated in the milk and again replaced into milk after growth at 37 C^o, fermented milk to 66% and at 42C^o to 83,3%. Comparing the received data one can see that for obtaining the initial isolate the best results were achieved at reinoculation from MRS into milk. The received after the growth initial isolates were checked for probiotic properties according to the accepted methods of researches. The results are set forth in Table 4.

Table4. Is evidence of probiotic properties at the researched strains.

Tests / Enzyme	Quantity	Isolates, %
Growth at Bile%	0.2	47±0.3
	0.4	47±0.5
	1	47±0.5
Trypsin mg/ml	0,5	41,2±0.3
Pepsin mg/ml	0,5	41,8±0.2
Antimicrobial activity (pH=6.0)	Salmonella typhimurium G-38	20.6±0.5
	Bacillus subtilis G17-89	79.4±0.4
	Bacillus thuringensis.69-6	85.3±0.1

As it can be seen from the data set forth in the Table 4 separated 30 strains of LAB demonstrated 47% of stability towards different concentrations of bile, 41% of stability towards trypsin and pepsin. They demonstrated antibacterial activity against gram-negative and gram-positive test bacteria and different degree of stability towards some antibiotics. Thus separated from the drinking yogurt Richal LAB isolates can be used for further investigations for presence of probiotic properties[12,15,20,21,22].

Acknowledgements: I would like to express my great appreciation to Dr.Flora Tkhruni also her colleagues then Professor Seyed Hadi Razavi, for their valuable and constructive suggestions during the planning, development of this research work.I wish to acknowledge the help provided by Yasuj University of Medical Sciences.

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4/2/2013