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Isolation and Identification of potentially probiotic bacteria from Traditional Dairy Products of Ardabil region in Iran

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ABSTRACT

Probiotics are alive and nonpathogenic microorganisms that have beneficial effects on their host's health. Traditional dairy products have been used for many centuries among the natives and are the main source of potentially probiotic bacteria. In this study a total 38 lactic acid bacteria strain were isolated by preliminary screening in PBS with pH=3. The second step was in vitro test and determining their potential as probiotic. Therefore, resistance to low pH 2.5 and evaluation of 0.3 %bile salt tolerance was performed. Results showed that all isolates were able to grow at low pH and were divided to four groups according to their growth delay in bile salt (0.3%). Identification of isolates was followed by biochemical and physiological tests and 12 Lactobacillus isolates were located in tree groups including Lactobacillus sakei, Lactobacillus casei and Lactobacillus plantarum. And 26 Enterococcus isolates werelocated in four groups including Enterococcus durans, Enterococcus faecium, Enterococcus faecalis and Enterococcus avium. Finally, antimicrobial activity of three different Lactobacillus and four different Enterococcusisolates (according to biochemical and morphological tests) were tested in pH=4(culture pH) and 6.5(neutralized pH) against three pathogenic bacteria including E.coli strain PTCC 1399, Yersinia entercolitica ATCC 1159 and Listeria innocua DSMZ 20649 by agar well diffusion method. All of tested isolates showed inhibitory zone against pathogenic bacteria in pH 4, but two Enterococcus isolates and two Lactobacillus isolates showed inhibitory zone in pH 6.5. In conclusion, present study showed that traditional dairy products of these regions can be used as a good source of potentially probiotic bacteria.

Key words: probiotic, dairy product, antibacterial activity.

INTRODUCTION

lactic acid bacteria (LAB) are widely used in fermented food production and are considered as generally recognized as safe (GRAS) organisms which is safely applied in medical and veterinary functions [1]. In the food industry, LAB is widely employed as starter cultures and has been indexed as part of human microbiota [2]. Yogurt, cheese and fermented milk products are

mentioned as the main food sources of probiotics [3]. The use of Lactic Acid Bacteria (LAB) in foods and food supplements has a long history and most strains are considered commensal microorganisms with no pathogenic potential. Their unique presence in intestinal epithelium and human gastrointestinal tract, and their traditional use in fermented foods and dairy products without remarcable problems prove their safety [4-5]. The aim of this study was to isolate the strains with high probiotic potentiality which may exist in traditional dairies for extra use as probiotic strains. The criteria used for *in vitro* selection of probiotic bacteria, in food preparations, which allow them to be established in the intestinal tract, include Bile tolerance and gastric juice resistance, which enable them to survive and grow to do their impressive action in the gastrointestinal tract (GIT). Although the range of tolerance required for maximum growth in the GIT is not known, it seems rational that the most bile and acid-resistant species should be selected. Production of antimicrobial compounds such as bacteriocins, acetic and lactic acid is another criterion for potentially probiotic bacteria which may take part in the inhibition of intestinal pathogenic bacteria [6-7]. For this purpose, different traditional dairy product samples were collected from different regions of Ardabil (Moghan and meshkinshahr) and used to isolate potential probiotic bacteria.

MATERIALS AND METHODS

Sample Collection

Due to their wide acceptance among the consumers of Ardebil, the dairy samples including yoghurt, cheese, gorout and shour, were collected from different regions. The samples were transferred immediately to the laboratory for microbiological analysis and stored aseptically in low refrigerator temperature (-4°C) to protect normal micro flora and avoid from contamination and deterioration.

Preliminary screening

In order to promptly isolate acid resistant bacteria from rich micro flora of dairy products, the preliminary screening in Phosphate Buffer Solution (PBS) with PH 3.0 was performed for three hours.

Isolation of Bacteria

Bacteria were isolated from dairy products by using MRS medium. Ten gram of each sample was dissolved into 100 ml of MRS broth at pH 6.5. After dissolving into MRS broth, they were shaken homogeneously and were incubated at 37°C for 24 h in aerobic condition. Anaerobic condition in the presence of 10% CO_2 was created for removal of unwanted bacteria. Finally, the single colony of bacteria was isolated by observing their colonial morphology and some physiological tests (Gram staining and catalase reaction).

Resistance to low pH (2.5)

Resistance of isolates for Gastric Juice (*in vitro*) was conducted according to the method of Pennacchia [8].The survival of isolates was compared in PBS at pH 6.5 and 2.5.

Bile tolerance

Isolates with most resistance to acid were selected for evaluation of bile tolerance. Bile tolerance was measured as described by Gilliland [9]. Briefly, growth was measured in MRS broth containing 0.3% bile salt in 7 h by spectrophotometer ($OD_{600\ nm}$) and bile salt -free MRS was used as control. Growth delay was employed as the measuring instrument for tolerance [9].

Identification of isolates

Identification of strains was performed by biochemical and morphological tests such as Growth test at 4 °C and 15 °C in tubes containing MRS broth and the fermentation of carbohydrates including salisin, arabinose, sucrose, inositol, maltose, monnose, manitol, cellobiose, raffinose , rhamnose , sorbitol , trehalose , fructose, lactose, galactose, xylose, and glucose and also sterile water was used as positive and negative controls [10]. At last coefficiency of isolates was determined with some standard *Lactobacillus* and *Enterococcus* strains.

Determinaion of Antimicrobial Activity

Some of acid and bile resistant isolates were assessed for their antibacterial activity against main three pathogenic bacteria using well-diffusion method. In short, 30 µl of fresh cultured supernatant of LAB were poured into 5mm in diameter wells of sof BHIagar(0/7%agar) including pathogenic bacteria. The inhibitory features were observed and Experiments were performed in triplicate. Indicator bacteria were *E.coli strain* PTCC 1399, *Yersinia Entercolitica* ATCC 1159 and *Listeria innocua* DSMZ 20649.

RESULTS AND DISCUSSION

Table1, the origin and number of cocci and rod-shape isolates after screening for acid tolerance

Number of isolates		Dairy Product
Bacilli	Cocci	
0	6	A(moghan gorout)
1	6	B(meshkin gorout)
4	2	C (meshkin shoure)
1	6	D(moghan cheese)
1	6	E(moghan yogurt)
5	0	F(moghan shoure)

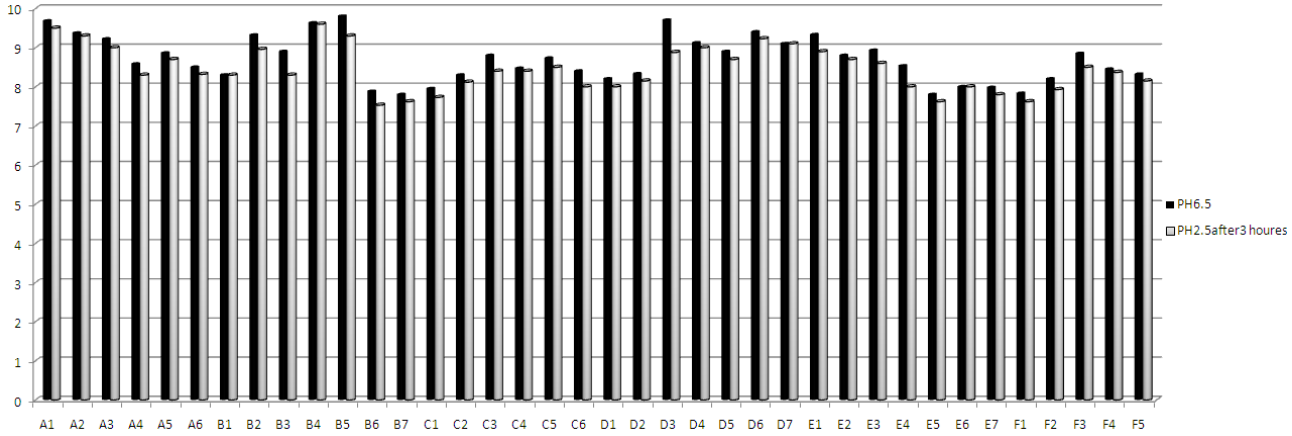

Fig 1. The survival rate of isolates before and after transferring to PBS with pH 2.5

Table 2, Grouping of isolates according to their growth delay in MRS containing 0.3% Bile salt

4 d <60	3 60<d< 40	2 40<d <15	1 d <15	Growth delay by minute
			+	A5-B6-B7-C1-C3-C4-D1-D2-D4-D5-E3-F2
		+		B1-B2-B4-B5-C2-C5-D3-F4-F5
	+			A2-A3-A4-B3-C6-D7-E2-E4F1-F3
+				A1-A6-D6-E1-E5-E6

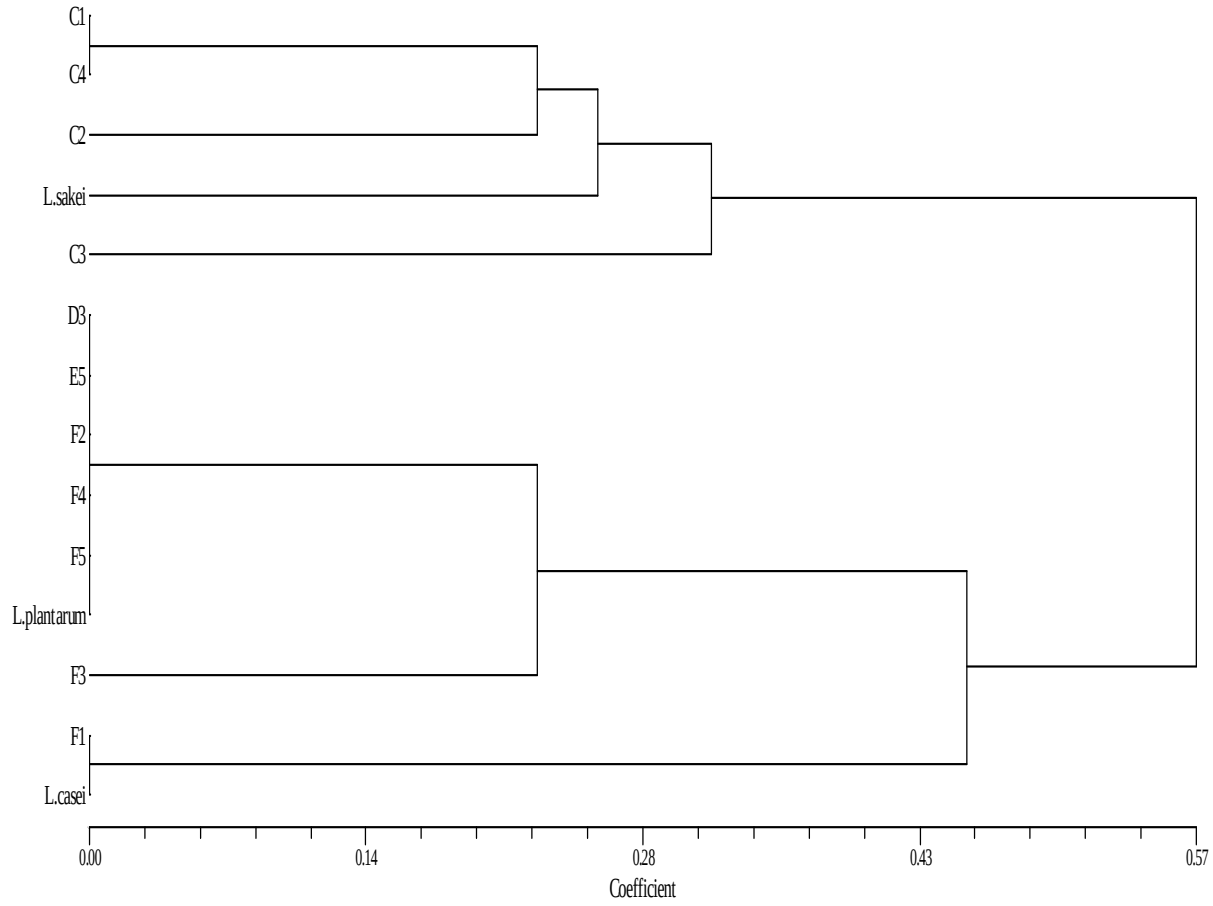

Fig2. Dendrogram of *Lactobacillus spp.* obtained from biochemical and morphological evaluation, after similarity calculation with NTSYS software and clustering with UPGMA.

Table 3, Antibacterial activity of selected *Enterococcus* and *Lactobacillus* isolates against three pathogenic bacteria

	Culture supernatant with neutralized pH 6.5			Culture supernatant with normal pH 4		
	E.coli PTCC 1399	*Yersinia Entercoliticaa* ATCC 1159	*Listeria innocua* DSMZ 20649	*E.coli* PTCC 1399	*Yersinia Entercol itica* ATCC 1159	*Listeria Innocua* DSMZ 20649
Lactobacillus (C1)	+	+	_	+	+	+
Lactobacillus(E5)	_	_	+	+	+	+
Lactobacillus(F1)	+	_	_	+	+	+
Enterococcus (A1)	+	_	+	+	+	+
Enterococcus (A3)	_	_	_	+	+	+
Enterococcus (D2)	+	+	_	+	+	+
Enterococcus (E1)	_	_	+	+	+	+

In this study, 38 LAB were isolated from six different traditional dairy products collected from two different regions of Ardabil province of Iran (Meshkinshahr and Moghan) according to their wide acceptance among the people of those regions(Table1). Preliminary screening in pH=3 let us to get rid of too many other bacteria existing in rich flora of dairy products. The single colony of bacteria were isolated according to their morphological an biochemical tests (gram and catalase reaction) and gram positive and catalase negative bacteria were isolated and stored in MRS broth culture in refrigerator temperature -80°C for testing and determining probiotic potential of isolates. For testing the resistance of isolates to acidic condition of gastric juice in *in*

vito, cfu/ml of isolates was determined by colony counting method in PBS with pH 2.5 after three hours and before transferring to pH 2.5 .

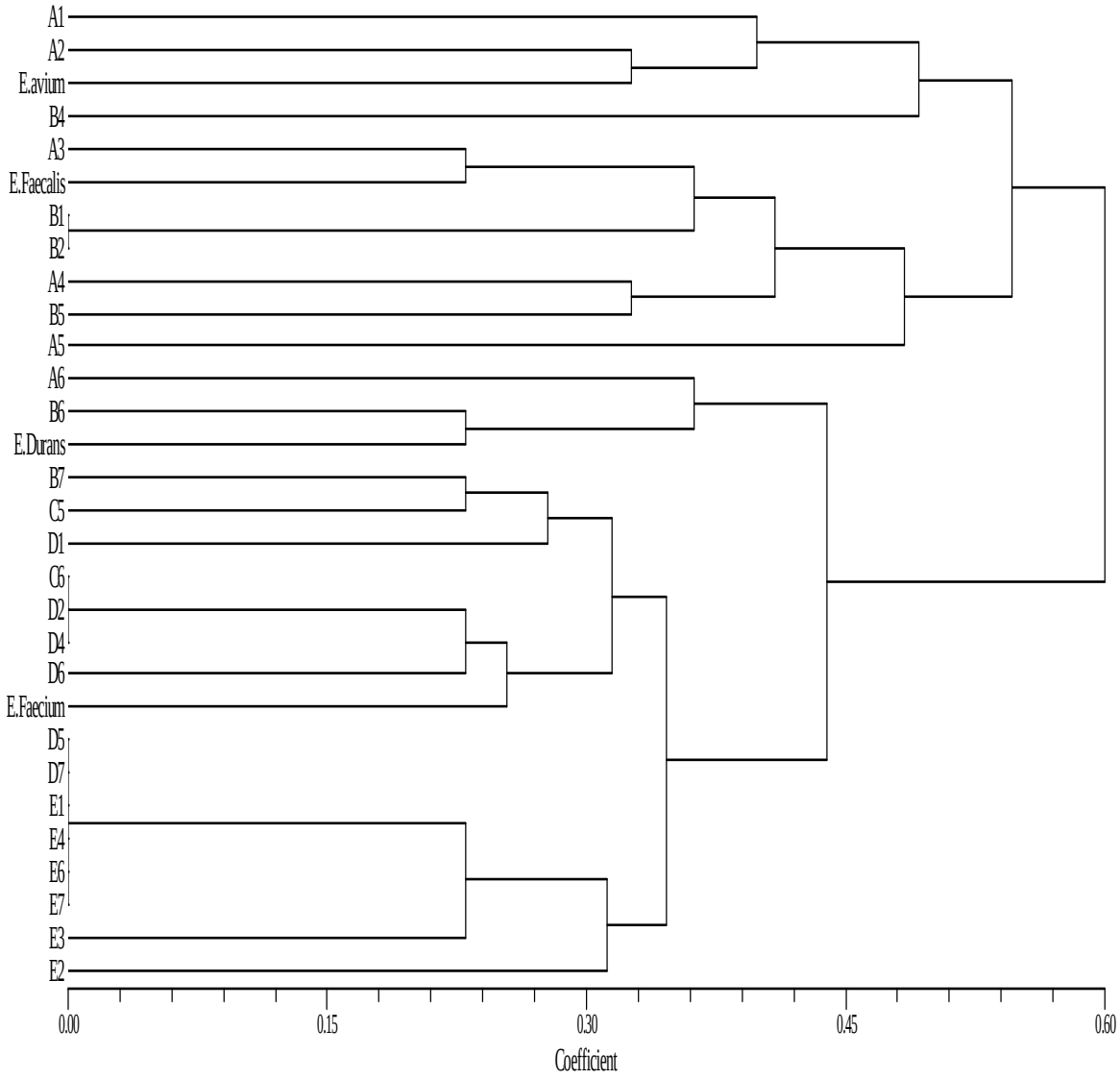

Fig. 3, Dendrogram of *Enterococus spp*. obtained from biochemical and morphological evaluation, after similarity calculation with NTSYS software and clustering with UPGMA.

The decrease of LAB colony forming unit (CFU) at the low pH(2.5) condition was lower than 1.0 log unit (Table 1). The result showed high ability of LAB isolates to survive in *in vitro* acidic condition of stomach.

The LAB survival in low pH is very important for bearing initial stress in the stomach [11]. At the application level, when LAB enter into human body, first constraint is gastric acid with very low pH level around 2- 3 [6,12-13].All acid resistant LAB isolates were moreover tested for bile tolerance according to their growth delay in MRS broth containing 0.3% bile salt and MRS broth without bile salt as a control. The growth delay ranged from 15min to more than one hour and isolates were located in four groups (Table2). As the result showed , 12 isolates had growth delay less than 15min and were resistant to bile salt. Other isolates showed different tolerance to 0.3% bile salt condition. Therefore, what is obvious is this that all LAB isolates were able to grow and survive at bile salt condition after seven hours. The survival at bile salt condition is one

of the main criteria for *in vitro* selction of potentially probiotic bacteria and critical points for the microbes. Because some of LAB are able to survive at bile salt condition. Hydrolyzes of bile salt decreases or eliminates the toxic effect of the bile salt to the LAB. Some LAB isolates are able to survive at bile salt. Identification of isolates was performed by morphological tests (gram and catalase reaction, growth at 4 °C and 15 °C and growth at 6.5% Nacl) and biochemical testes such as 17 different sugar fermentation pattern. Dendrogram of *Enterococus* and *Lactobacillus spp* obtained from biochemical and morphological evaluation, after similarity calculation with NTSYS software and clustering with UPGMA was drawn (fig 2and 3) . In general, in case of LAB, majority of the isolates (26 from 38 isolate) Were *Enterococus* with four different species identified *Enterococcus durans*(isolates A6, B6) *Enterococcus faecium*(isolates B7, C5, D1, D6, C6, D2, D4,D5, D7, E1, E2, E3, E4, E6, E7) *Enterococcus faecalis*(isolates A3, B1, B2, A4, A5, B5) and *Enterococcus avium*(isolates A1, A2, B4) and 12 isolates were *Lactobacillus spp* with three different species identified as *Lactobacillus sakei*(isolates C1, C2 and C4), *Lactobacillus casei*(isolates F3 and F1) and *Lactobacillus plantarum* (isolates D3, E5, F2, F4 and F5) in this study on the biodiversity of LAB from food related ecosystem also reported that the *Enterococus* strain dominated all ecosystems and consisted70% of LAB isolates.after determining the probiotic potentiall of isolates, antibacterial activity is one of the main features of probiotic bacteria. For this purpose, seven different LAB isolates (one representative from each group) were tested for their antibacterial activity in pH 4(culture pH) and 6.5(neutralized pH) against three pathogenic bacteria. The result showed that almost all of isolates were inhibitory against pathogenic bacteria in pH 4 and that may be because of low pH and acidic condition of culture. But in pH 6.5, some of them were inhibitory against some pathogenic bacteria (Table 3).

The capability of the probiotics present in Ardabil dairy products that inhibit the growth of pathogens confirms the health benefits on the consumption of these products. Our study suggests that probiotics are helpful in the protection and improvement of our intestinal flora. Consuming these traditional dairy products can help human healthcare and can also protect against occurrences of diarrhea, food poisoning and enteric infections [14-16].

CONCLUSION

In conclusion, this study showed that preliminary screening of dairy products microflora in low pH(3) is a rapid way and method for isolation of acid resistant LAB bacteria and may be useful for isolation of bile salt tolerant bacteria, because all acid resistant isolates in this study, could grow in 0.3% bile salt. Also biochemical, physiological and morphological tests showed that Enterococcus sp were domiant in compare with lactobacillus sp in traditional dairy products of these region. Whereas all isolates were resistant for acid and bile salt, so it can be stated that LAB isolated from traditional dairy products of these region can be used as potentially probiotic bacteria with promoting host-specific health

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