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Isolation, Identification and Analysis of Probiotic Properties of *Lactobacillus* spp from Traditional Yoghurts in North of Iran

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Lactic Acid Bacteria (LAB) are widely distributed in nature and occur naturally as indigenous micro flora in raw milk, yoghurt, etc. They are gram positive bacteria that play an important role in many food fermentation processes. During of 2011 and 2012, a total of 50 yoghurt samples were collected from different parts of Guilan province (Northern Iran). The samples were collected in sterile universal tubes and kept cool until they could be taken to the laboratory where they were kept at 4°C for further use. The samples were aseptically weighed and homogenized. From each sample, a 1:10 dilution was subsequently made using peptone water followed by making a 10 fold serial dilution. The 0.1 mL from each dilution was then sub cultured, in duplicate, into the M17 and MRS agar used for isolating LAB Strains with gram positive and catalase negative reactions were finally used for further identification. As many as 17 gram-positive non-spore-bearing bacilli were identified, of which 4 strains (*Lactobacillus acidophilus, Lactobacillus plantarum*, *Lactobacillus case*i and *Lactobacillus brevis*) produced the highest tolerance against acid, tolerance against bile salts as well as antimicrobial properties.

Key words: Traditional Yoghurt, *Lactobacillus*, Antimicrobial Properties, Probiotics, Northern Iran.

The *Lactobacillus* genus consists of a genetically and physiologically diverse group of rod-shaped, Gram-positive, non-spore forming, non-pigmented, catalase negative and microaerophilic to strictly anaerobic[1,2]. Selection lactic acid bacteria (LAB) that have widespread use in probiotic fermented food production and are considered as preparations include antibiotic tolerance as well as the generally recognized as safe (GRAS) organisms and can be safely used for medical and veterinary applications[3,4]. In the food industry, LAB is widely used as starter cultures and has been cited to be part of human micro biota[5,6]. In raw milk and dairy products such as yoghurts, cheeses, and fermented milks, *Lactobacilli* are naturally present or added intentionally, for technological reasons or to generate a health benefit for the consumer and yoghurt is one of the best-known foods that contain probiotics[3,7]. Probiotics are live microorganisms that provide beneficial effects to the consumer if found in a desirable number in their intestine. Currently, probiotic products are commercially available the world over[8]. Bacteria in this study belong to *Lactobacillus* family that is highly important in food industry. Their functional properties include, inducing and boosting immune system, decreasing cholesterol level, decreasing gastrointestinal infections and diarrhea, and

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decreasing cancer. These bacteria could inhibit pathogenic bacteria by producing compounds such as fatty acids, various types of acids and peptidic compounds such as bacteriocin[9,10,11]. One of the disadvantages of probiotic bacteria is that they make the dairy products non-tolerant against ecological conditions and give an undesirable aroma and taste to them[12]. Therefore, isolation of potentially probiotic bacteria from traditional dairy products not only removes the probiotic bacteria of characteristic properties, but also can prove an efficient approach as how to market traditional probiotic products of Iran and introduce native probiotic straits. Furthermore, since these strains are isolated from dairy products, they might be more adaptable and survive longer in dairy products than bacteria with non-dairy origins[13]. Investigation on survival of probiotic bacteria in gastrointestinal tract is one of the important factors in selection of probiotic strains[14]. Factors such as tolerance under acidic conditions, tolerance against bile salts and antagonistic characteristic against pathogenic bacteria were used during this study in order to identify the probiotic potential of the isolated strains[15,16]. Although *Lactobacilli* show a high impact on effective protection to human health, there is obvious evidence those Lactobacilli from different origins possess probiotic properties at different levels[17]. In order to survive and colonize in the gastrointestinal tract, the bacteria should express high tolerance to acidic media and bile and should be able to adhere to the intestinal surfaces[18]. The antibiotic resistance of pathogenic bacteria is an increasing medical problem and raises the question of antibiotic resistance among desired robotic strains. Therefore, the antibiotic susceptibility test therefore should be incorporated for the safety assessment of the desired property of the promising probiotic *Lactobacilli*. In the country, there are different kinds of traditional dairy products which are produced from sheep and goat milk such as drinking yoghurt, yoghurt and cheese, etc. In comparison with the commercial species, composition of lactic acid bacteria is more varied and in constant in these products[19]. The aim of the present study was isolation and identification of *Lactobacilli* from yoghurt in order to constitute an original collection of *Lactobacilli* strains.

MATERIALS AND METHODS

Samples

A total of 50 different traditional yoghurts collected from Northern of Iran. All samples were transferred to the laboratory under refrigeration and stored at 4°C until their analysis.

Isolation of Bacteria

A number of bacterial strains were isolated from such food origins by the dilution agar method. Briefly; a sample was mixed with normal saline to appropriate dilutions. A volume of 0.1 ml of the dilutions was plated on MRS agar and incubated in anaerobic conditions at 37°C for 48 h. Isolated colonies were taken randomly for purification. The purified colonies were tested for Lactobacilli by microscopic examination with gram stain and catalase production. The gram-positive, catalase-negative rods were selected for further studies. The bacteria *Lactobacillus* spp., were isolated from yoghurt samples by using modified MRS broth and MRS agar media. Additionally, 0.05% cysteine was added to MRS to improve the specificity of this medium for isolation of *Lactobacillus*. The pH of the media was adjusted to 6.5. One 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. The cultures were subjected to five subculture at 37°C under low pH (pH 4.5) and anaerobic condition in the presence of 10% CO_2 to remove unwanted bacteria. After subcultures, the bacterial culture was streak onto MRS agar media at pH 4.8. Finally, the single colony of *Lactobacillus* was isolated by observing their colony morphology and some biochemical tests (Gram staining, catalase, endospore and motility test) and the culture were maintained in MRS broth at pH 5.5. The isolated bacteria were identified as *Lactobacillus* spp. by observing their morphological characteristics and by means gram staining, motility test, catalase test, endospore test, milk coagulation activities, 0.4% bacteriostatic phenol tolerance test and 1-10% NaCl tolerance test. MRS broth containing inhibitory substances such as 0.4% phenol and 1-10% NaCl were inoculated with 1% (v/v) 24 h active culture of *Lactobacillus* and incubated (anaerobically) for

24 h at 37°C in the presence of 10% CO_2. For the determination of optimal growth and pH of *Lactobacillus*, 1% (v/v) fresh over night culture of *Lactobacillus* were inoculated into MRS broth with varying pH ranging from 2.5-8.5. The pH were adjusted with concentrated acetic acid (99%) and 5 N NaOH. The inoculated broths were incubated in anaerobic condition 24 h at 37°C in the presence of 10% CO_2. After 24 h of incubation growth of the bacteria were measured using a spectrophometer, reading the optical density at 560 nm (OD) against the un-inoculated broth.

Bile tolerance test

Firstly, the screening for bile tolerance was carried out by growing the isolated *Lactobacilli* in MRS broth containing 0.3% of bile salt for 24 h under anaerobic conditions at 37°C. Culture broths with turbidity more than 0.5 units at 600 nm were classified as bile tolerant strains. These strains were selected for exposure to broths containing higher concentrations of 0.5 and 1.0%(w/v) of bile salt. The survival rate of each strain was expressed as the percentage of viable cells in the presence of bile salt compared to that without bile salt. The experiment was performed in triplicate and the mean values were calculated.

Acid tolerance test

The isolated *Lactobacilli* were subjected to primary screening for acid tolerance in MRS broth adjusted to pH 2.5 with 1N HCl for 90 min at 37°C. The determination of survival was performed by single streaking on MRS agar plates, and the growth was observed after 24-48 h after anaerobic incubation at 37°C. Isolates which were growing on the agar were considered to be acid tolerant strains. These strains were selected and cultivated in MRS broth under anaerobic atmosphere at 37°C. Cultures (107-108cfu/ml) were inoculated in 10 ml of 0.05 M sodium phosphate buffer adjusted to pH 2.0, 3.0, and 7.0 with 1 N HCl. Samples were incubated at 37°C for 2 h. Cells were serially adjusted to 10-fold dilution by phosphate buffer pH 7.0. The dilution was plated on MRS agar for determination of viable cells after 48 h of incubation. The survival rate was calculated as the percentage of colonies grown on MRS agar compared to the initial cell concentration. Each experiment was performed in triplicate.

Antibacterial activities of the strains of *Lactobacillus spp*

Antimicrobial effects of *Lactobacillus* isolates on *Staphylococcus aureus* PTCC1431, *Salmonella typhi* PTCC 1639 and *Escherichia coli* PTCC 1399 were determined by agar diffusion method. The tested bacteria were obtained from microbial collection center of the Scientific and Industrial Researches Organization in Iran. The tested bacteria were incubated in Nutrient broth at 37°C for 24 h. Approximately 105-107 cfu/ml of the bacteria to be evaluated for sensitivity (indicator bacteria) were inoculated (1%) into 20 ml of Nutrient agar and poured in the Petri dishes. For determination of antibacterial activity of the *Lactobacillus* spp., MRS medium containing only 0.2% glucose was used. Ten milliliters of broth was inoculated with each strain of *Lactobacillus* spp. and were incubated at 37°C for 48 h. After incubation, a cell-free solution was obtained by centrifuging (6000 g for 15 min) the culture, followed by filtration of the supernatant through a 0.2 µm pore size cellulose acetate filter. Some of the supernatants were neutralized with 1 N NaOH to pH 6.5, and the inhibitory effect of the hydrogen peroxide was eliminated by the addition of catalase (5 mg/ml). Unneutralized (general inhibitory effect) and neutralized (bacteriocin and bacteriocin-like metabolites) supernatants of the strains of *Lactobacillus* spp. were checked for antibacterial activity against pathogenic bacteria in inoculated nutrient agar. Then 100 ml of cell free supernatants was poured in 8 mm diameter sealed wells cutin the Nutrient agar. Once solidified, the dishes were stored for 2 h in a refrigerator. The inoculated plates were incubated for 24 h at 37°C, and the diameter of the inhibition zone was measured with calipers in millimeters.

RESULTS

Seventeen presumptive strains of *Lactobacillus* were isolated from 50 traditional different yoghurts. All isolates were catalase-negative, gram positive and oxidase negative rods producing no gas from glucose. Based on survival rate of the isolates under acidic conditions, 5 strains were designated as susceptible, 8 as moderate

Table 1. Identification of *Lactobacillus* species based on biochemical and morphological tests

Isolates	Growth at						Sugar Fermentation											
	15°C only	45°C only	15 and 45°C	Catalase	Motility	NH^3 from arginine	Cellobiose	Lactose	Mannitol	Raffinose	galactose	Melebiose	Sucrose	Maltose	Mannose	Trehalose	Sorbitol	Asculin
L. acidophilus	-	+	-	-	-	-	+	+	-	-	+	-	+	+	+	-	-	+
L. Plantarum	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
L. casei	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
L. brevis	+	-	-	-	-	+	-	+	-	+	+	+	+	+	+	-	+	-

Table 2. Inhibition of tested bacteria by *Lactobacillu*s spp. isolates by agar diffusion method

Isolates	Diameter zone of Inhibition (mm)		
	Tested microorganisms		
	*S. typhi*PTCC1639	*E. coli* PTCC1399	*S.aureus*PTCC1431
L. acidophilus	9	10	9
L. Plantarum	8.5	9	8
L. casei	7.5	8	7.5
L. brevis	8	9	8.5
Mean±SD	8.25±0.64	9±0.81	8.25±0.64

tolerant and the 4 remaining strains as good tolerant. Based on tolerance of species to bile salts they were classified as four groups including tolerant strains (equal delay growth or less than 15 minutes), highly tolerant strains (delay growth between 15 to 40 minutes), poorly tolerant strains (delay growth between 40 to 60 minutes) and susceptible strains (delay growth more than 60 minutes)20. So, based on this classification 2 strains were designated as tolerant, 4 as highly tolerant, 8 as poorly tolerant and 3 as susceptible. Finally, only 4 out of 17 strains with a potentially good capacity of resisting acidic conditions and tolerating bile salts were selected for final identification. These isolates were identified as *L. acidophilus, L. Plantarum* and *L. casei* and *L.* brevisby observing their colony morphology, physiological and as well as some biochemical characteristics. Their characteristics have shown in Table 1. A total of 4 *Lactobacillus* spp. were tested for their antimicrobial activity against *Staphylococcus aureu*s PTCC 1431, *Salmonella typhi* PTCC 1639 and *Escherichia coli* PTCC 1399. The antimicrobial activity of *Lactobacillus* spp. Are given in Table 2. All of them exhibited antimicrobial activity against tested bacteria. Moreover, *L. acidophilus* had highest antimicrobial activity against to tested microorganisms. In general, bacteria with an average inhibition power of 8.46 mm produced a good capacity for inhibiting of pathogenic bacteria.

DISCUSSION

Produced yoghurts in traditional way, are carriers of potentially probiotic bacteria, which their addition to industrial products increase their features and enhance marketability. These bacteria play an important role in human health by improving gastrointestinal system and boosting immune system. Mezaini *et al*, reported that twenty LAB strains which were isolated from Algerian dairy milk were screened for their antagonistic activity against *L.innocua*, *E.faecalis*, *B. cereus*, *B.subtilis*, *S. aureus*, *S. epidermitidis*, *E. coli* and *S.typhimurium*. Among these bacteria *S.thermophilus* T2 strain showed a wide inhibitory spectrum against all the gram positive[21]. Some

researcher found that the antimicrobial effect of *Lactobacillus* sp. have been higher than *Streptococcus* sp[22]. Alexander *et al*, reported that 192 strains of lactic acid bacteria were isolated from 5 samples of artisanal minas cheese. The results of direct inhibition test indicated that 48 strains inhibited the *in vitro* growth of the indicator microorganisms: *S. aureus* and *Listeria monocytogenes*[23]. Aroutcheva *et al*., revealed that no correlation was found between bacteriocin activity, lactic acid and hydrogen peroxide production. They found that 3 *Lactobacillus* strains produced H_2O_2 but did not demonstrate any inhibitory effect[11]. Yuksekdag *et al*, reported that *Lactococcuslactis* sub sp. *cremoris* Z20S strain produced maximum lactic acid but did not produce H_2O_2. Moreover, the strain had an inhibitory effect against *S. aureus* but noinhibitory effect against *E. coli* and *P. aeruginosa*[22]. In a study by Tadesse *et al*, LAB involved in the fermentation of traditional beverages had an antimicrobial property against various food-borne pathogens and the inhibitory products were extracellular and diffusible. The observed inhibitory property of LAB was influenced by the medium they grew in[24]. The good probiotics should present their antimicrobial actions particularly to the pathogens in the GI system. In this study, *Staphylococcus aureus*, *Salmonella typhi* and *Escherichia coli* were used as the tested bacteria because they are occasionally found as food borne microorganisms that might cause gastroenteritis. The results revealed that the antibacterial activity of the three selected *Lactobacilli* could inhibit all test pathogenic bacteria however at different inhibition levels as shown in Table 2. *L. acidophilus* showed the most antibacterial potency to *E. coli*. The production of organic acid and hydrogen peroxide by *Lactobacilli* was reported to inhibit both gram positive and gram negative bacteria, whereas bacteriocin affects only the growth of gram positive bacteria[25]. Probiotic bacteria go through intensively acidic conditions of stomach and are exposed to intestinal bile salts before reaching to intestine and having any positive effect[26]. During this study, 4 strains tolerant to acid and bile salts were isolated from 50 samples of traditional yoghurts and as these strains were isolated from dairy products so it insures that they could be safely used in dairy products while survive for a desired period within them. In Italy, 63 strains of *Lactobacillus* were isolated from one type of traditional cheese, of which only 3 strains produced a high tolerance against acidic conditions and bile salts. In another study, 6 out of 88 *Lactobacillus* strains isolated from un pasteurized milk and cheese were tolerant against acidic conditions and bile salts[27]. According to the obtained results, great number of non-probiotic strains may die out while exposed to unfavorable acidic conditions or to bile salt, as in this study only 4 out of 17 isolated *Lactobacillus* strains produced a good tolerance against acidic conditions or bile salts. In this study, the concentration as much as 0.3% was applied to evaluate tolerance rate to bile salts, which is paramount to average concentration of bile salts in human gastrointestinal tract. In an investigation by Chateau *et al*, on the impact of bile salts on growth of 38 *Lactobacillus* strains, half of the studied strains were influenced in a slow process by 0.3% bile salts concentration. While they displayed an under-hourly growth delay to reach 0.3 optical absorption in 600 nanometer wavelength at MRS broth liquid medium compared to control culture without any bile salt[20]. During our study, also delayed growth of the treated strains by bile salts was more evident than that of control culture. In this study the supernatant culture of bacteria of *Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus plantarum* and *Lactobacillus brevis* produced inhibitory effects against pathogenic bacteria. Ogunbanwo *et al*, investigated the microbial activity and bacteriocin production of two probiotic strains namely *Lactococcus plantarum* and *Lactobacillus brevis* on some pathogens. The results included the inhibitory effect on bacillus cereus (8-10mm), *Escherichia coli* (6-8mm), *Yersinia enterocolitica* (6-7mm)[28]. Another study found that application of supernatant of bacteria such as *Lactobacillus fermentum*, *Lactobacillus casei*, and *Lactobacillus acidophilus* and *Lactococcus lactis* has an inhibitory effect on a wide range of pathogenic bacteria[29]. In our study, metabolites produced by these bacteria, which were isolated using centrifuge, were able to inhibit pathogenic bacteria. The highest inhibitory effect (10mm) belonged to *Lactobacillus acidophilus*, which was against *E. coli* ; whereas the lowest inhibitory effect (7.5mm) belonged to *Lactobacillus casei*, which were

against *S. typhi* and *S.aureus*. According to this study, strains of *Lactobacillus* isolated from traditional yogurts, which were tolerant against acidic conditions and bile salts and had good antimicrobial effects, could be used widely in production of industrial products and native probiotic strains, so contribute to enhance health in the society. Of course, owing to diversity among the species, phenotypic and biochemical identification is not a meticulous and efficient method to identify these species, and for a better identification molecular methods should be considered.

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