Original Article

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In Vitro characterization of *Lactococcus lactis* strains Isolated from Iranian Traditional Dairy Products as a Potential Probiotic

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Abstract

Few studies have been reported regarding probiotic properties of *Lactococcus lactis* strains although they are extensively used as starter cultures in the production of dairy products. In this study 8 wild isolates of *Lactococcus lactis* were evaluated in vitro with regard to resistance to simulated gastric and intestinal juices, adherence ability to Caco-2 cells and HT29-MTX-E12 cell lines, anti-microbial activity, hydrophobicity and antibiotic susceptibility. The results revealed that all isolates had better survival after exposure to simulated gastrointestinal tract stresses in comparison to control probiotic *Lactobacillus rhamnosus* GG. Regarding adherence efficiency, almost all isolates exhibited similar adherence with control. Three isolates showed antibacterial activity against Gram-positive pathogens (*Staphylococcus aureus* and *Listeria monocytogenes*) through spot-agar method. Almost all isolates (seven out of eight) showed similar hydrophobicity to control probiotic. Regarding to antibiotic resistance, all isolates were susceptible to gentamicin, ampicillin, ciprofloxacin, erythromycin, tetracycline, penicillin, kanamycin and nitrofurantoin. Although, further investigations are necessary, it was concluded that strains derived from raw milk and home-made dairy products could be a remarkable reservoir for identification of new potential probiotic strains.

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1. Introduction

Functional foods containing safe microorganisms convey beneficial effects, named probiotics, have attracted much attention in the last decade. Probiotics are defined as live microorganisms which after consumption in adequate numbers can confer health benefits to the host [1].

Several well-known probiotic strains are of commercial interest and currently used in the production of different functional foods and in dietary supplements in the form of capsules and tablets. Lactobacilli and Bifidobacteria as autochthonous inhabitants of human's gastrointestinal tract have been extensively subjected in probiotic characterization assays. However, probiotic characteristics have been reported for other food-derived lactobacilli [2,3], other members of lactic acid bacteria (LAB), such as enterococci and lactococci [4,5], and non-LAB, such as *Escherichia coli* [6]. According to this, new bacterial isolates originating from food environment with superior probiotic characteristics have been reported frequently in recent years.

Lactococcus lactis is usually used as starter in manufacturing different dairy products. However, in recent years, several interesting evidences show *L. lactis* strains, alone or in combination with other probiotics, have the potential to be beneficial to human health and considered as probiotic [7-9].

There are many different characteristics expected from a candidate probiotic strain, for example safety, presenting antimicrobial activity, surviving in harsh conditions of upper part of human GIT, e.g. low pH and the presence of bile salts, and the ability to adhere to the

intestinal cells and mucus and interact correctly with gastrointestinal epithelia cells in order to confer any health effect [10-12]. So, in order to present a strain as probiotic, extensively deep analyses are usually necessary to investigate its viability and interaction with the human host.

The objective of this study was to test a number of LAB isolates that had been identified as *L. lactis* for probiotic characteristics via in vitro experiments. These strains have been isolated from raw milk and home-made dairy products (cheese and butter) [13]. The parameters examined to determine likely probiotic features of the isolates included the viability after exposing the bacteria in vitro to GIT conditions, test for adhesion to Caco-2 and HT29-MTX-E12 cells, antimicrobial activity, cell surface hydrophobicity and antibiotic susceptibility.

2. Materials and Methods

2.1 Bacterial strains and growth conditions

The 8 wild type bacterial strains were isolated from milk and traditional cheese and butter in Iran (the data have been described in our previous study) [13]. Strains used in the current study were identified as *L. lactis*, including AS1, SPT2, GC10, JP51, FK23, JP32, AS2 and DC103 isolates by 16s rRNA sequencing. M17 medium (Oxoid, Frankfurt, Germany) supplemented with 5% lactose, was used for cultivation of all isolates. Overnight incubation at 32°C was used for culturing of isolates. The commercial probiotic strains *Lactobacillus rhamnosus* GG (ATCC 53103) was used as a positive control (Ardeypharm GmbH, Herdecke, Germany).

Pathogenic indicator strains were Entrotoxigenic *Escherichia coli* (ETEC) strain H10407 (O78:H11), *Escherichia coli* (UPEC) strain 536 (O6:K15:H31), *Salmonella enterica* serovar typhimuirum SL1344, *Shigella flexneri* M90T, *Listeria monocytogenes* EGD and *Staphylococcus aureus* Cowan 1 (ATCC 12598). *S. aureus* and *L. monocytogenes* were grown in tryptic soy broth and brain heart infusion, respectively. Other strains were cultured in Luria Bertani broth. All strains were grown overnight at 37°C.

2.2. Resistance to simulated gastric and small intestine juices

Simulated gastric juice was prepared by dissolving 3 g l^{-1} pepsin (P7000 Sigma-Aldrich, Germany) in sterile salt solution (125 mM NaCl, 7 mM KCl, 45 mM $NaHCO_3$, pH= 3.0), and pH adjusted to 2.5 with 0.1 N HCl using gentle mixing. Great attention needed to be paid in order to prevent denaturation of pepsin. Simulated small intestinal juice was prepared by dissolving 3 g l^{-1} ox-gall (Fluca 70168, Germany) and 1 g l^{-1} pancreatin (P3292Sigma-Aldrich, Germany) in sterile salt solution (45 mM NaCl), and pH adjusted to 8.0 by 0.1 M NaOH. Both solutions were prepared freshly and filter-sterilized through a 0.22 µm membrane. Survival under simulated gastric and small intestinal conditions was determined as previously described [10]. Five milliliters of each bacterial culture were incubated overnight at 32°C. After that, cells were harvested, washed twice with 0.85 %w v^{-1} NaCl and re-suspended in 500 µL of the same solution. 100 µL of bacterial suspensions were added to 900 µL of simulated gastric or intestinal juice, so that initial populations ranged from 8.2 to 9.0 log CFU ml^{-1} and incubated at 37°C. Samples were withdrawn after 90 and 180 min from simulated gastric and intestinal juice, respectively. In order to determine viable cells, plate counts with M17 agar were done at time 0 and after incubation [10].

2.3. Adhesion to epithelial intestinal cell lines

2.3.1 Cell cultures

Caco-2 and HT29-MTX-E12 (E12) cells were grown in Dulbecco's modified Eagle Medium (DMEM) (56°C for 30 min) containing 10% v/v inactivated fetal bovine serum (PAA, Colbe, Germany) and without antibiotics. Both cell lines were cultured at 37°C in an atmosphere of 5 % v/v CO_2 in a CO_2 incubator. The experiments were performed in 24-well tissue culture plates. Caco-2 cells were seeded at a concentration of 4×10^5 cells/well and used after 24 h incubation when confluent growth was achieved (6.2×10^5 cells/well). HT29-MTX-E12 (E12) cells were seeded at a concentration of 6×10^4 cells/well and were used after 7 days when they had produced mucus. The culture medium (1 mL $cell^{-1}$) was replaced by fresh medium at days 3, 5 and 6 after seeding. For both cell lines, 1 h before adding the bacterial suspension DMEM was replaced by fresh medium. Mucus production after 7 days was confirmed by microscopic evaluation of stained cells. For this purpose E12 were seeded and cultivated on Nunc Lab-Tek Chamber Slides (Thermo Scientific) and stained by Periodic acid-Schiff [14].

2.3.2 Adhesion assay

Bacterial overnight cultures (18 h) incubated under appropriate conditions were used in this assay. Two hundred microliters of each bacterial overnight culture were used to inoculate 5 ml DMEM containing 10% fetal bovine serum and incubated 1 h at 37°C. After that, 25 µL of each culture were added to wells of a 24 well plate containing the confluent monolayer of Caco-2 or E12 cells and 1 mL DMEM (to reach the final concentration of 1-2 × 10^6 CFU of bacteria per well). After incubation for 90 min at 37°C and 5 %v/v CO_2, non-adherent bacteria were removed by washing the cells two times with 1 mL of phosphate buffer solution. Then, the cell monolayer was lysed by addition of 1 ml pre-warmed filter-sterilized 0.1% Triton X-100 and shacked for 15 min at room temperature. The number of colony forming units was determined after dilution and plating on M17-agar. Assays were carried out triplicate independently and in duplicate each time for each isolate.

2.4. Hydrophobicity of bacterial strains

Bacterial hydrophobicity was determined by the n-hexadecane test according to Lukic et al. [15] with slight modification. Briefly, 500 µL of 18 h cultures were washed once with 0.85 %w v^{-1} NaCl and re-suspended

in the same buffer to achieve OD_{600}= 0.4-0.5. In the following, 500 µL of n-hexadecane (Merck, Germany) was added to 2.5 mL of bacterial suspension. The mixture was mixed twice for 30 s with 30 s intermissions between mixing. The absorbance (OD_{600}) of the aqueous phase was measured after 1 h of incubation at room temperature (A_1), and compared with OD_{600} of bacterial suspension before mixing (A_0) with n-hexadecane. Hydrophobicity was calculated by Eq. 1:

$$\text{Hydrophobicity (\%)} = (1 - A_1/A_0) \times 100 \quad \text{Eq.1}$$

2.5. In vitro inhibition of pathogen growth

Inhibition of pathogen growth was determined with the agar spot and well diffusion methods [16, 17]. For the agar spot test, 5 µL of an overnight culture of each strain were spotted on the surface of M17 agar plates and incubated at 32°C temperatures for 24 h to allow growth of colonies. Overnight grown pathogen cultures were diluted in 0.85 %w v^{-1} NaCl until approximately 1×10^7 CFU ml^{-1}, and latter suspensions were used to inoculate (4%) 5 mL appropriate soft agar (7 g l^{-1} agar) and poured on the M17 agar plates containing the spots of the isolates on the surface. Before pouring the soft agar containing pathogen cultures, 5 µL of antibiotic solutions (100 µg ml^{-1} tetracycline for *L. monocytogenes* and *S. aureus* and 100 µg ml^{-1} gentamycin for Gram-negative pathogens) was spotted on M17 agar plates as positive control. The plates were incubated at 37°C overnight. Zones free of the pathogen around the *L. lactis* spots (from spot to the border of indicator growth), if any, were measured in millimeters. For isolates showing inhibitory effects, well diffusion assay was performed as follows.

In well diffusion assay, the supernatant of *L. lactis* isolates which had inhibitory effect in spot agar assay were applied. Supernatants of the isolates were prepared by centrifugation (6000 ×g, 5 min) of overnight grown cultures in M17 medium. The pH of the resulting supernatant was adjusting to pH 6.0 with 2.5 M NaOH, and passed through a 0.22 µm filter (Millipore Corporation, Germany).

In order to perform the analysis, appropriate dilution of pathogen cultures was inoculated into molten 50°C water agar (Oxoide, Germany) (14 g l^{-1} of agar in water), and then 5 ml of that was poured into plates already containing 15 ml of the same solidified water agar. After solidification of the layer containing the pathogen, wells (5 mm in diameter) were punched into the agar, and 90 µL of isolates' supernatants (obtained as indicated below), were pipetted into each well. The plates were kept at 4°C (for about 4 h) in order to ensure fluid diffusion into the agar. The probable pathogen inhibition is observable after incubation at 37°C overnight. The appropriate antibiotic was applied in a separate well as positive control (100 µg ml^{-1} tetracycline for *L. monocytogenes* and *S. aureus* and 100 µg ml^{-1} gentamicin for Gram-negative pathogens).

2.6. Antibiotic susceptibility

All isolates were included in antibiotic susceptibility tests against a selection of nine antibiotics including ampicillin, erythromycin, gentamicin, kanamycin, nitrofurantoin, penicillin, tetracycline, nalidixic acid, and ciprofloxacin were applied in a disc diffusion test (Oxoid, Germany). The assay was performed according to Noreen et al. [18] with slight modifications. A 10-fold diluted suspension of an overnight culture was spread uniformly (swabbing in 3 directions) on a freshly poured M17 agar plate (diameter 10 cm, containing 20 ml medium) using a sterile cotton swab. The plate was allowed to dry for 15 min and then the antibiotic containing discs were dispensed on the plate. Following 24 h incubation of the plates, inhibition zones around discs (from disc to the border of bacterial growth) were measured.

2.7. Statistical analysis

The statistical analyses were performed in GraphPad Prism version 6.0 (GraphPad Software, La Jolla, Canada). Results were expressed as the mean and standard error. Student's *t*-test was used to determine the significance of differences between each isolates and *L. rhamnosus* GG. Data were considered significantly different when the p-values were less than 0.05. All analysis were done in duplicate.

3. Results and discussion

Raw milk and traditional dairy products as rich reservoirs of novel LAB have been attracted high attention in the last decades. In this study, some potential probiotic characteristics were evaluated for some *L. lactis* strains, which were isolated and identified from raw milk and home-made dairy products (Cheese and butter) in Iran in a previous work [13]. *L. lactic* is one of the most commonly used cheese starter [19]. Application of a probiotic starter strain in yogurt and cheese production probably can eliminate the need for addition of an exogenous probiotic strain in order to manufacture healthy products. This would be of interest to the dairy industry. Although, traditionally, this species is not considered to be a natural inhabitant of the human GIT, some studies reported the presence of strains belonging to *L. lactic* in the flora of the human GIT [20].

3.1 Tolerance to simulated gastric and intestinal conditions

Resistance to lethal GIT conditions (low pH, presence of bile salts and digestive enzymes) has been indicated as an important parameter in characterization of candidate probiotic stains. According to the results (Table 1), simulated gastric juice (pH=2.5 and 3 g l^{-1} pepsin) was more lethal than the intestinal juice (3 g l^{-1} oxgall and 1 g l^{-1} pancreatin) for all isolates, except the isolates FK23 and JP51. The numbers of viable cells after 90 min exposure to gastric juice were 7.30 ± 0.09 for SPT2 and 4.76 ± 0.63 log CFU ml^{-1} for AS1. Survival of control strain *L. rhamnosus* GG was 2.23 ± 0.11 log CFU ml^{-1}.

Table 1. Viable counts of isolates and control (log CFU ml^{-1}) after exposure to simulated GIT condition (gastric and intestinal juice)

Isolates	0 min	Gastric juice (90 min) [a]	Intestinal juice (180 min) [b]
SPT2	8.18 ± 0.08 [a]	7.30 ± 0.09 [c]	7.71 ± 0.31 [c]
FK23	9.00 ± 0.04 [a]	6.26 ± 0.86 [b]	5.09 ± 0.69 [a]
JP51	8.76 ± 0.14 [a]	6.21 ± 0.68 [b]	4.55 ± 0.29 [a]
JP32	8.45 ± 0.65 [a]	5.20 ± 0.13 [b]	6.38 ± 0.32 [b]
AS2	8.91 ± 0.06 [a]	5.50 ± 0.32 [b]	7.45 ± 0.31 [c]
AS1	8.85 ± 0.13 [a]	4.76 ± 0.63 [b]	7.49 ± 0.60 [c]
DC103	9.04 ± 0.06 [a]	6.46 ± 0.18 [b]	6.39 ± 0.55 [b]
GC10	8.86 ± 0.12 [a]	5.76 ± 0.34 [b]	7.50 ± 0.51 [c]
L. GG	8.37 ± 0.09 [a]	2.23 ± 0.11 [a]	3.60 ± 0.65 [a]

[a] Simulated gastric juice is 3 g l^{-1} pepsin and pH 2.5
[b] simulated intestine juice is 3 g l^{-1} ox-gall, 1 g l^{-1} pancreatin and pH 8.0.
The results expressed as mean±SE, n=3, compared to *L. rhamnosus* GG (in columns). [a] no significant difference ($p \geq 0.05$) compared to *L. rhamnosus* GG. [b] $p<0.05$ and [c] $p \leq 0.01$

Table 2. Antibiotic susceptibility of *L. lactis* strains analyzed using agar-disc diffusion method

		The inhibition zone around discs (mm) for each antibiotic								
		Gentamicin	Kanamycin	Ampicillin	Ciprofloxacin	Erythromycin	Tetracyclin	Penicillin	Nitrofurantion	Nalidixic acid
Isolates	Concentration (µg)	10	30	10	5	15	30	10	300	30
SPT2		4	3	10	6	6	13	7	9	Re†
FK23		5	5	11	7	11	11	11	5	Re
JP51		7	7	15	7	15	13	13	8	Re
JP32		5	6	12	6	12	13	13	7	Re
AS2		4	3	10	6	6	13	7	9	Re
AS1		5	3	11	7	6	12	8	10	Re
DC103		4	4	11	5	11	12	11	4	Re
GC10		4	3	11	6	5	11	7	10	Re

†Re : resistance.
The Inhibition values (in mm) are the average of duplicates.

According to results, all isolates showed higher viability after exposure to gastric juice in comparison to *L. rhamnosus* GG, which was more considerable for isolate SPT2.

Survival under simulated intestinal condition was rather variable, ranging from 7.71 ± 0.31 log CFU ml^{-1} for SPT2 to 4.55 ± 0.29 log CFU ml^{-1} for JP51 (Table 1). Probiotic strain *L. rhamnosus* GG survived only with 3.60 ± 0.65 log CFU ml^{-1} under this condition. According to this observation six isolates survived significantly better than the established probiotic *L. rhamnosus* GG.

In this study none of the isolates could completely resist simulated gastric (pH 2.5 and pepsin) and intestinal (presence of ox-gall and pancreatin) conditions. Susceptibility of Lactococcus strains to the digestive system has been reported frequently [4, 21, 22]. Vinderola et al. [21] indicated strains of *L. lactis* as the second most sensitive strains, and Faye et al. [22] observed strain-dependency for *L. lactis* regarding sensitivity to intestinal conditions. Although, it has been generally assumed that Lactococcus strains are not resistant to stresses induces in the GIT, the results of this study revealed that susceptibility of almost all of the tested isolates (with the exception of the isolates FK23 and JP51) is significantly lower than for probiotic control strain *L. rhamnosus* GG under both simulated gastric and intestinal conditions.

In our study, *L. rhamnosus* GG showed about 6 log CFU ml^{-1} drop after 90 min exposure to simulated gastric juice with pH 2.5 which is comparable to the results of Prasad et al. [23] who reported a 7.6 log CFU ml^{-1} drop for *L. rhamnosus* GG after 3 h incubation at pH 3. Similarly, Succi et al. [24] reported 7.2 log CFU drop after 2 h incubation at pH 2. However, it is necessary to indicate that the composition of medium usually used in this test greatly affects the outcome of such assays. For example, Velez et al. [25] reported

highly gastric resistance for *L. rhamnosus* GG that could be a result of the presence of glucose in simulated juice supporting survival of bacteria [26]. Corcoran et al. [26] showed that the presence of glucose in gastric juice improves *L. rhamnosus* GG survival by 5.6 log CFU mL^{-1} (this is probably because the glucose is the most suitable substrate for bacteria and it could be metabolized efficiently), and Faye et al. [22] showed that the number of *L. rhamnosus* GG remained approximately constant in acidified MRS (pH 3.0). In addition , several studies showed that food matrix has a considerably positive effect on survival of strains in the gastrointestinal tract [12,22].

3.2 Adhesion to human epithelial intestinal cell line

Adhesion to cells of the intestinal epithelium is commonly viewed to be another important property of probiotics or even representing a prerequisite for successful colonization of the human gastrointestinal tract. However, probiotics rather adhere to the mucus covering the intestinal epithelium and do not directly bind to the epithelial cells. We have therefore not only employed a non-mucus producing cell line (Caco-2) but also the mucus producing cell line E12 (subclones of mucus-secreting HT29-MTX) in the adherence assays with the 8 isolates and control strain *L. rhamnosus* GG. The later cell line mimics much better the in vivo environment in the intestine because of mucin production.

In this study, adhesion to Caco-2 ranged between 15.1 ± 2.0% for GC10 and 49.5 ± 5.4% for AS2, and 24.6 ± 6.6% for control strain *L. rhamnosus* GG. According to Figure 1A, one isolate, AS2, exhibited significantly higher adhesion to Caco-2 cells in comparison to *L. rhamnosus* GG.

Adhesion to E12 cells ranged between 8.6 ± 1.6% for AS1 and 21.0 ± 5.4% for AS2, and 29.6 ± 5.6% for *L. rhamnosus* GG. According to Figure 1B, the isolates didn't show superior adhesion to E12 in comparison to *L. rhamnosus* GG.

In previous works, adherence of probiotic strain *L. rhamnosus* GG has been reported to reach 9.2% [5], 15.7% [17] and 34 % for Caco-2 cells [11], and 27% for HT29-MTX cells (HT29-MTX is the previous generation of E12) [27]. These results are comparable with the adherence efficiencies of *L. rhamnosus* GG observed in the current study (24.6 ± 6.6 % for Caco-2 and 11.5 ± 1.6 % for E12 cells). The slight variability in adherence to cell line in different studies is greatly depend to condition of analysis (such as well surface, configuration of flask, composition of medium used for culturing of cell line and bacteria, temperature through adhesion step, and the time of assay).

Cell line E12 is able to produce mucin MUC5AC and the trefoil proteins TFF1 and TFF3 in the adherent mucus layer, which are important in interaction of bacteria with mucus [28]. Presence of mucus binding proteins (MucBP) that mediate adhesion, have been indicated most abundantly in lactobacilli inhabiting the GIT [15]. In this study, it was found that adherence efficiency to E12 cells was higher than to Caco-2 cells for isolates FK23 and GC10. For other strains adhesion to E12 was lower than to Caco-2 which is probably due to a lack of suitable adhesion factors to mucin in these strains. Lukic et al. [15] showed that expression of aggregation factor AggL in *L. lactis* mediated binding to colonic mucus and reduced adherence to the ileum as well as to HT29-MTX cells. In contrast, mucin binding protein MbpL imparted affinity to gastric mucin proteins such as MUC5AC to HT29-MTX. Those authors further showed that expression of aggregation factor AggL was related to higher affinity to n-hexadecane [15]. However, the presence of AggL or MbpL in the test strains of this study was not investigated.

3.3. Bacterial hydrophobicity

In order to characterize cell surface hydrophobicity, evaluation of bacterial affinity to n-hexadecan was performed and the results presented in Figure 2. Affinity to n-hexadecan covered a wide range; the least affinity was observed for JP32 (5.13 ± 0.13%) and the highest affinity was recorded for strain GC10 (83.68 ± 1.55%). Hydrophobicity of *L. rhamnosus* GG was 69.63 ± 6.28%. There were no significant differences in hydrophobicity between isolates and *L. rhamnosus* GG, except for isolate JP32. Adhesion of bacteria is a complex process involving two essentially different mechanisms: specific and nonspecific binding [29]. Cell-surface charge and hydrophobicity considerably influence the strength of adhesion via nonspecific binding to hydrophobic surfactant lipids coating the mucus gel [15, 29].

However, the correlation of hydrophobicity and adhesion to intestinal cells/the intestinal mucus is still controversial. Xu et al. [30] showed bacterial cell affinity to organic solvent (xylene) was highly correlated with auto-aggregation and ability of adhesion to Caco-2 cells, and indicated that this parameter is a good indicator for screening of potential probiotics. Vinderola et al. [31] and Schillinger et al. [27] suggested cell surface hydrophobicity not to be a prerequisite for strong adhesion, but rather a physico-chemical property that facilitates the first contact between the microorganism and the host cell for subsequent specific binding [27].

In this study, evaluation of hydrophobicity of the 8 isolated strains revealed considerable differences between the strains.

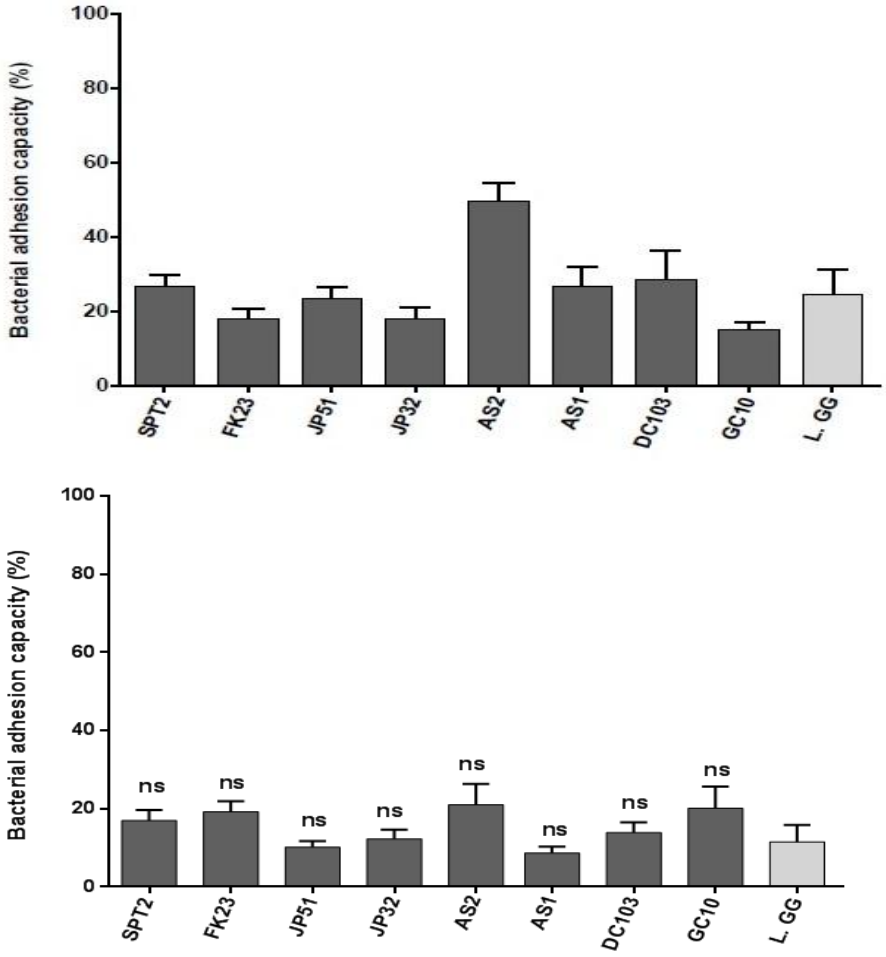

Figure 1. Adhesion capacity of 8 isolates of *L. lactis* and *L. rhamnosus GG* (control strain) to Caco-2 (A), and HT29-MTX-E12 (B) cells. Adhesion capacity is calculated as the percentage of adhered bacteria in relation to the total number of bacteria added. The results are expressed as mean ± SE, n=3. * $p < 0.05$ compared to *L. rhamnosus GG*. ns: no significance ($p \geq 0.05$) compared to *L. rhamnosus GG.*

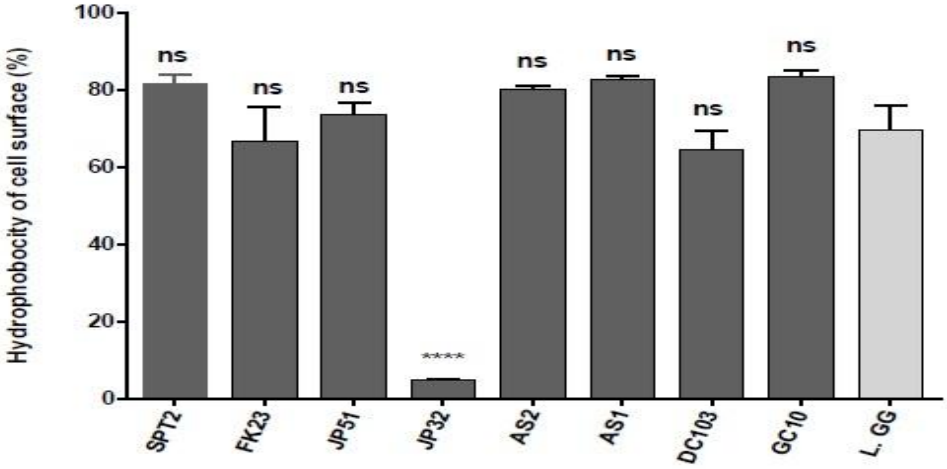

Figure 2. Hydrophobicity of 8 isolates and control strain *L. rhamnosus GG*. The results are expressed as mean ± SE, n=2. **** $p \leq 0.0001$ compared to *L. rhamnosus GG*. ns: no significance ($p \geq 0.05$) compared to *L. rhamnosus G*

In addition, *L. rhamnosus* GG showed 69.63 ± 6.28% affinity to n-hexadecane that is comparable to 62% reported by Schillinger et al. [27] for this strains using the same solvent. No clear correlation was found

between surface hydrophobicity and adhesion for all test isolates. Isolate JP32, however, with the lowest hydrophobicity (5.13 ± 0.13%) showed also moderately low adhesion to both cell lines (18.0 ± 2.9% to Caco-2 and 12.3 ± 2.4% to E12). However, it has to be taken into account that the bacterial cell's physicochemical situation and surface hydrophobicity depends to a great deal on environmental conditions such as composition of culture medium, pH and temperature [32].

3.4. In vitro inhibition of pathogen growth

One important property of probiotic bacteria is their ability to prevent and cure enteropathogenic infections of the host. Growth inhibition of pathogens by isolates was determined by the agar spot method. Tetracycline was employed as positive control against *L. monocytogenes* and *S. aureus* and exhibited inhibition zones with radius of 11-12 mm, and gentamicin caused inhibition zones with radius of 2 to 4 mm for Gram-negative pathogens. In the agar spot test, only 3 isolates of JP51, FK23 and DC103 were able to inhibit *L. monocytogenes* EGD and *S. aureus* Cowan 1. *L. monocytogenes* EGD was inhibited with radius of 13, 10 and 5 mm by JP51, FK23 and DC103, respectively. Also, *S. aureus* Cowan 1 was inhibited by JP51, FK23 and DC103 with radius of 13, 12 and 10 mm, respectively. According to this, the inhibitory potential of these isolates (except DC103 for inhibition of *L. monocytogenes* EGD) was similar to 100 µg ml^{-1} tetracycline. It seems that DC103 have higher activity against *S. aureus* Cowan 1 than *L. monocytogenes* EGD. None of the isolates showed antibacterial activity against Gram-negative indicator pathogens.

However, in well diffusion assay that were performed with pH-adjusted supernatants from test strains JP51, FK23 and DC103, no inhibitory activity was observed against *L. monocytogenes* EGD and *S. aureus* Cowan 1.

The capacity of some LAB strains to produce substances with inhibitory effects on the growth of other microbes is well known. Among them are strains of the Lactococcus genus which produce bacteriocins and bacteriocin-like compounds, such as nisin, which are effective against a range of Gram-positive bacteria [33]. In the current study, the agar spot assay revealed inhibition of *S. aureus* and/or *L. monocytogenesis* only by three isolates, though the corresponding neutralized supernatant had not inhibitory effect. This implies the inhibitory effect to be a result of organic acids produced by these isolates. Similar observations have been reported frequently in previous studies [3]. Gonzalez et al. [16] examined 125 Lactococcus strains isolated from cheese for inhibition of pathogens and reported almost all of them inhibited *S. aureus* CECT 240 and *L. monocytogenes* CECT 4031. However, neutralized supernatants from only 7 strains inhibited *S. aureus* and from only one strain inhibited *L. monocytogenes*.

3.5 Antibiotic susceptibility

Although, LAB are extensively used in production of fermented food products, these microorganisms have the potential ability to transfer antibiotic resistance. Therefore, evaluation of antibiotic resistance is strongly advised for all bacteria with application in food industry, especially probiotic strains [34].

In this work, we used discs already containing antibiotics. Table 2 shows the diameters of the inhibition zones caused by the antibiotics of the discs. Taken together, all isolates were susceptible to 8 antibiotics (gentamicin, ampicillin, ciprofloxacin, erythromycin, tetracyclin, penicillin, kanamycin and nitrofurantoin) out of 9 tested (all isolates were resistance to nalidixic acid). Nalidixic acid, is mainly a Gram-negative spectrum antibiotic with a minor activity against Gram-positive bacteria, and then resistance of *L. lactis* isolates to this antibiotic was expected. Therefore, there is no concern of antibiotic resistance characteristic transmission from tested probiotics to other pathogens.

4. Conclusions

The present study was designed to carry out a preliminary evaluation on probiotic properties among 8 isolates of *L. lactis* which originated from raw milk and home-made cheese and butter in Iran. The isolates, which survived best in simulated GIT juices and adhered efficiently to the cell lines were SPT2 and AS2 that seems to be comparable to probiotic strain *L. rhamnosus* GG. These strains showed similar hydrophobicity to probiotic strain of *L. rhamnosus* GG, however didn't exhibit antibacterial activity against pathogens assessed in this study. In addition, these strains showed almost similar antibiotic susceptibility pattern as that have been reported for most of lactococci strains. These 2 isolates are therefore promising candidates worth further evaluations including immuno-modulatory activities and analysis in animal models. The three strains FK23, JP51 and DC103, with significant antibacterial activity against Gram-positive pathogens are also worth to be tested for bio-preservatives in food products. These isolates showed lower survivability in intestinal juice compared to *L. rhamnosus* GG, and higher survivability in gastric juice. Comparable adherence to cell lines was observed in above three isolates compared to control. In addition, they were sensitive to Gram-positive spectrum antibiotics. Seven out of 8 isolates (except JP32) showed similar surface hydrophobicity to *L. rhamnosus* GG, however, it needs more studies in order find any correlation between adhesion process and cell hydrophobicity.

5. Acknowledgment

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6. Conflict of interest

The authors declare that there is no conflict of interest.

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