Research Article

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Probiotic potential comparison of *Lactobacillus* strains isolated from Iranian traditional food products and human feces with standard probiotic strains

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Abstract

BACKGROUND: Traditional fermented products are a rich source of microorganisms which may have remarkable probiotic properties even more significant than probiotic strains of human origin. In this study three *Lactobacillus plantarum* and one *Lactobacillus fermentum* strains, isolated from either Iranian traditionally fermented products or children's feces, identified with molecular methods and selected based on high acid resistance, were investigated for their probiotic properties *in vitro* and compared with standard probiotic strains of the species; *L. plantarum* ATCC 14917, *L. fermentum* PTCC 1744 and *L. acidophilus* ATCC 4356.

RESULTS: Most of the isolates showed a high survival rate under gastrointestinal tract conditions and *L. plantarum* strains displayed a moderate ability to adhere to human colon adenocarcinoma cell line, HT-29. Neutralized cell free culture supernatants of *L. plantarum* strains were capable of inhibiting pathogens. Almost all of the strains were resistant to vancomycin and streptomycin and susceptible to other clinically relevant antibiotics. Isolated strains exhibited low to moderate autoaggregation (Auto-A), co-aggregation (Co-A) and hydrophobicity, following a strain specific manner. None of the strains invaded into HT-29 cells while strain PF11 could significantly decrease the number of adhering pathogenic bacteria. Most of the strains increased apoptosis of HT-29 cells, though they had no effect on human umbilical vein endothelial cells (HUVECs).

CONCLUSION: Favorable probiotic properties of strains PL4 and PF11 along with their anticancer activity imply their potential for clinical or technological applications. However, further *in vitro*/*in vivo* investigations are recommended.
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Keywords: *Lactobacillus plantarum*; *Lactobacillus fermentum*; traditionally fermented products; anticancer activity

INTRODUCTION

Probiotics are known as non-pathogenic microorganisms and their administration in sufficient amounts can positively influence the health of consumers [as reported in 2006 by the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO)]. Probiotics health benefits include: competition with pathogens for receptor binding, providing of nutrients and gut colonization; enhancement of mucosal barrier function; promotion of innate and adaptive immune responses; elaboration of bacteriocins; reduction in the level of serum cholesterol and decreased risk of colon cancer.[1,2]

Even though it is better to use the strains with human origin, detection of the strains in traditional food products with better physiological characteristics compatible with probiotic properties, has persuaded the manufacturers to use them. Isolation and screening of microorganisms from natural sources are the most powerful means to obtain useful and genetically-stable strains for industrially-important products. Screening the probiotic potential of each specific strain individually is necessary because it is generally agreed that probiotic characteristics are strain specific.

Tarkhineh or Tarhana is made and consumed as a part of the everyday diet in the majority of western parts of Iran. It usually consists of cracked wheat, yogurt, and vegetables that are fermented together. Horreh is another traditional fermented food belonging to south-western provinces of Iran. Kardeh (Biarum carduchorum), wheat flour and Doogh (Ayran) are mixed together and after 2 days of incubation in a warm place, steamed cooked rice and onion (fried in ghee) are added to it. Lighvan cheese is a popular Iranian

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local cheese, witnessing a long history of safe use. It is produced from sheep milk and there is not any heating process or starter addition involved in its manufacturing steps. Its natural microbial flora consists of inherent lactic acid bacteria with specific properties.

Lactic acid bacteria (LAB) are one of the major groups of probiotic bacteria and among them lactobacilli are the most popular genera isolated from all portions of the human gastrointestinal tract (GIT)[3] and applied as adjunct cultures in various types of food products or in therapeutic preparations. To determine the probiotic potential of a specific selected strain, many properties should be investigated *in vitro*. Characteristics such as resistance to gastric acidity and pancreatic secretions, adhesion to intestinal epithelial cells, antimicrobial activity, inhibition of the adhesion of pathogenic bacteria and antibiotic resistance are all considered relevant in tracing the probiotic profile of a specific strain.[4] Numerous studies evaluated the probiotic potential of several *Lactobacillus* strains, isolated from different food products and could segregate strains with promising probiotic properties similar to or even better than the reference probiotic strains.[5–7]

The aim of this study was to apply *in vitro* tests to evaluate the probiotic potential of *Lactobacillus* strains, isolated from different sources such as human feces and traditionally produced food products including Tarkhineh, Horreh and Lighvan cheese, selected based on the higher acid resistance among the isolated strains and to compare them with commercially available probiotic and standard strains.

MATERIAL AND METHODS

Bacterial strains and growth conditions

Table 1 illustrates the strains included in this study. These strains were previously isolated from Lighvan cheese, Tarkhineh, Horreh and human feces and identified with biochemical and molecular methods. The strains were chosen according to the higher acid (pH 2.5) resistance, compared to the isolated strains. Three strains including *L. plantarum* ATCC 14917, *L. acidophilus* ATCC 4356 and *L. fermentum* PTCC 1744 were used as reference strains with proven *in vitro* probiotic properties. *Lactobacilli* were cultured on Man Rogosa Sharpe (MRS) agar medium (Liofilchem, Roseto degli Abruzzi, Italy) at 37 °C under microaerophilic conditions. Pathogen strains including *Escherichia coli* O157:H7ATCC 43895 and *Salmonella typhimurium* ATCC 14028 were grown on Tryptone soya agar (TSA) medium (Oxoid, Basingstoke, UK) and propagated at 37 °C under aerobic conditions.

Survival under the conditions simulating human GIT

Resistance to gastrointestinal juices was evaluated by monitoring bacterial growth in two solutions simulating the conditions that bacteria encounter during transit through the GIT. Pepsin of pig stomach mucous membrane (Sigma-Aldrich, Milan, Italy) and pancreatin of pig pancreas (Sigma-Aldrich) were separately resuspended in sterile saline solution (0.5% *w/v*), and their pH values were adjusted to 3.0 and 7.5, respectively. Intestinal solution was supplemented with 0.45% (*w/v*) oxgall (Sigma-Aldrich). Then, 15 µL of bacterial suspension (10^8 CFU/mL) was added to 1000 µL of simulated gastric or pancreatic juices. Bacterial counting was performed in the samples taken at 0, 90 and 180 min of incubation in the first stage and at 0, 120, and 240 min in the second. Assays were performed in triplicate.

Table 1. *Lactobacillus* strains and their origins

Strain	Origin	Strain identity[a]
L. plantarum	Lighvan cheese	PL4
L. plantarum	Tarkhineh	PT10
L. plantarum	Human feces	PF11
L. plantarum	ATCC 14917	PATCC
L. fermentum	Horreh	FH19
L. fermentum	PTCC 1744	FPTCC
L. acidophilus	ATCC 4356	AATCC

[a] The codes represent *Lactobacillus* species and their origins, e.g. PL4 indicates *L. plantarum* isolated from Lighvan cheese.

Antimicrobial activity

Agar well diffusion method (AWDM) was carried out according to Santini *et al.*[7] The inhibitory effect of cell-free culture supernatants (CFCS) (neutralized or not) on *E. coli* O157:H7 and *S. typhimurium* was investigated. The diameters of inhibition zones around the wells were measured and expressed as antimicrobial activity of CFCS.

Evaluation of pathogenicity factors; gelatinase and hemolysin production and bile salt deconjugation

Production of gelatinase and hemolytic activity were determined following the method described by Han *et al.*[8]

For bile salt deconjugation test, overnight culture of the strains was spotted onto MRS agar plates containing 0.5% (*w/v*) taurodeoxycholic acid (TDCA, Sigma-Aldrich). After 48 h of microaerophilic incubation, different colony morphology from the control MRS plates indicated bile salt hydrolysis (BSH).

Resistance to antibiotics

Disk diffusion method (from the Clinical and Laboratory Standards Institute 2003) was used to determine antibiotic susceptibility of the strains. Antibiotics with different mechanisms of action were used including: inhibitors of cell wall synthesis – penicillin G (P, 10 IU), ampicillin (AMP, 10 µg/disk), vancomycin (VA, 30 µg/disk); inhibitors of nucleic acid synthesis – rifampicin (R, 30 µg/disk); inhibitors of protein synthesis – chloramphenicol (C, 30 µg/disk), streptomycin (S, 10 µg/disk) and tetracycline (TE, 30 µg/disk).

Cell surface characteristics

Auto-aggregation (Auto-A), co-aggregation (Co-A) and cell surface hydrophobicity were determined to show the cell surface characteristic and some phenotypical specialties. Auto-aggregation test was performed according to the method described by Tomás *et al.*[9] Briefly, 1 mL of each bacterial suspension was vortexed and the absorbance of an aliquot of upper suspension at 600 nm, before initial absorbance (Abs_{t0}) and after 5 h incubation in room temperature, was measured. The results were expressed as Auto-A%.

$$\text{Auto} - \text{A}\% = \Delta\text{Abs}/\text{Abs}_{t0} \times 100$$

Co-A was determined as reported by Handley *et al.*[10] *Escherichia coli* O157:H7 and *S. typhimurium* ATCC 14028 were the Co-A partners for assessing the Co-A ability of the strains. Absorbance of mixed and separate bacterial suspensions was measured and Co-A rate was calculated using the following equation:

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$$\text{co-A}\ (\%) = \frac{\{[(Ax + Ay)/2]\ A(x + y)\}}{(Ax + Ay)/2)} \times 100$$

where *A* represents absorbance, *x* and *y* represent each of the two strains in the control tubes, and $(x + y)$ represents their mixture.

Cell surface hydrophobicity was determined according to Solieri *et al.*[6] Bacterial adhesion to xylene, an aromatic hydrocarbon, confirmed the hydrophobic phenotype of the strains.

Adhesion of the strains to the epithelial cell line (HT-29)

To evaluate adhesion capacity of the strains, a human colon adenocarcinoma cell line (HT-29), was used to simulate normal small intestinal villous cells. Cells were seeded at the concentration of 5×10^5 cell/well and grown in RPMI (Gibco, Thermo Scientific, Illkirch, France), supplemented with 10% inactivated fetal bovine serum and penicillin G/streptomycin (5000 IU/mL, 5000 µg/mL, respectively) and incubated at 37 °C in a humidified atmosphere with 5% CO_2 to reach 80% confluency (2–3 days). Twenty-four hours before experiment, the cells were washed with phosphate-buffered saline (PBS) (pH 7.4) and fresh media without antibiotics were added. Overnight cultures of *Lactobacillus* strains were washed once with PBS, resuspended in complete media (CM) without antibiotics and added to the cells with a final concentration of 10^8 CFU/mL. After 4 h of incubation, in order to remove non-adherent bacteria, the cell layer was washed twice with PBS. Cells with adherent bacteria were treated with trypsin (0.5%) for 10 min and adherent bacteria were enumerated by plating serial dilutions on MRS agar plates. Bacterial adhesion capacity was calculated as the number of adhered bacteria relative to the total number of bacteria added.

Invasion of epithelial cell line (HT-29) by the strains

Bacterial suspensions were added to HT-29 monolayers (cell media replaced with the antibiotic free one, 24 h before), with aforesaid concentrations and after 3 h of incubation, cells were washed twice to remove non-adherent bacteria, and then, CM containing 150 µg/mL gentamycin was added and followed by 2 h of incubation to efface the monolayer surface. Afterward, cell monolayers were washed three times with PBS and the cells were lysed by addition of 0.1% triton-X100. The remaining suspensions with invasive bacteria, were diluted and plated on MRS agar plates. Bacterial invasion capacity was calculated as the number of invasive bacteria relative to the total number of bacteria added.

Pathogen adhesion inhibition

Escherichia coli O157:H7 and *S. typhimurium* were used to examine the pathogen adherence inhibition (PAI) by *Lactobacilli*. Exclusion test was used to assess PAI according to the methodology described by Tareb *et al.*[11]

Cytotoxicity and anticancer activity

Confluent monolayer of HT-29 cell line and human umbilical vein endothelial cell (HUVEC) were treated with *Lactobacillus* bacterial suspensions (10^8 CFU/mL) for 24 h, and then, cells were harvested, as described earlier and used for flow cytometry assessment. Apoptosis and necrosis were studied using Annexin V-FITC Kit (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions and analyzed using flow cytometer (BD FACSCalibur). Cells cultured in lactobacilli-free medium served as controls. Data analysis was conducted using FlowJo software, version 7.6.1 (Tree Star, Ashland, OR, USA).

Statistical analysis

Data are presented as mean ± standard deviation (SD) of three independent experiments with duplicate determinations. Statistical analysis was performed using SPSS v.21.0 (IBM Corp., Armonk, NY, USA). The results of *in vitro* transit tolerance in the upper GIT were analyzed using two-tailed paired *t*-test. Statistical significance between groups was assessed by a one-way analysis of variance (ANOVA), followed by the Tukey test for multiple comparisons with controls. Correlations were measured by bivariate Pearson correlation tests. The *P* value less than 0.05 was considered statistically significant. Graphs were prepared using Microsoft Office Excel.

RESULTS AND DISCUSSION

Survival under conditions simulating the human GIT

Table 2 illustrates the viable count (log CFU/mL) and survival rates of the *Lactobacillus* strains pre- and post-treatment in gastric and pancreatic juices. *Lactobacillus acidophilus* ATCC 4356, a known probiotic strain, showed the lowest acid tolerance (21% survival rate) and its survival rate was significantly different from the others. *Lactobacillus plantarum* strains revealed 1 to 2-log cycles reduction in their viability and the strain PT10 was the most resistant one. *Lactobacillus fermentum* FH19 exhibited the highest survival rate (96% – 0.2 log reduction in viability) after 180 min in simulated gastric juice.

Almost all the strains were unaffected by the pancreatic juice and none of them displayed loss of viability more than 1 log cycle, except strain FPTCC which showed the least survival rate (73%). Survival rates higher than 95% was for the strains PL4, PATCC and AATCC. Reductions > 1 log CFU were statistically significant at $P < 0.05$.

The first criterion for the selection of a candidate probiotic strain to be beneficial for the host health is tolerance to the harsh conditions such as lysozyme-containing saliva, acidic juice of stomach which contains pepsin, bile and pancreatin in the upper intestine.[12] Campana *et al.*[13] reported that tolerance to GIT transit is a strain specific capacity. *Lactobacillus acidophilus* ATCC 4356 lost its viability more than 4-log cycles and showed the least resistance to simulated gastric juice which is in accordance with the results of Ortakci and Sert.[14] Charteris *et al.*[15] found that some strains of *L. fermentum* are intrinsically acid resistant. In the present study, *L. fermentum* FH19 showed the highest resistance to simulated gastric juice while *L. fermentum* PTCC 1744 (Strain PATCC) was not that much tolerant. Although strain specific acid resistance genes are very effective, the low pH of the habitat can be important too, as pH value of Horreh (strain FH19 isolation source) ranges between 3 and 4. *Lactobacillus plantarum* strain PF11 retained its viability after exposure to GIT conditions, supporting pervious findings that reported high resistance of *Lactobacilli* of human and animal origin in simulated gastrointestinal juices.[16]

These *Lactobacillus* strains are more resistant to bile salts and enzymatic treatments than to low pH values, being consistent with the results of Lee *et al.*[17] Several researchers have claimed that bile salt tolerance is related to BSH activity. In our study, most of the strains could not deconjugate bile salt, but were able to survive it. These results are in accordance with Moser and Savage[18] reporting that bile salt resistance has nothing to do with BSH activity.

Antimicrobial activity

Table 3 presents the diameter of inhibition zones formed by the neutralized CFCS of *Lactobacillus* strains against the two bacterial

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Table 2. Effects of simulated gastric and pancreatic juices on the viability of *Lactobacillus* strains

Strains	Gastric juice (log CFU/mL)				Pancreatic juice (log CFU/mL)			
	0 min	90 min	180 min	%SR[1]	0 min	120 min	240 min	%SR[1]
PL4	5.07 ± 0.33^{a}	3.62 ± 0.73^{a}	$3.11 \pm 0.25^{a**}$	61	6.07 ± 0.32^{a}	5.31 ± 0.96^{a}	6.02 ± 0.36^{a}	99
PT10	4.87 ± 0.12^{a}	3.83 ± 0.27^{a}	$3.56 \pm 0.36^{ab*}$	77	5.45 ± 0.15^{ab}	4.91 ± 0.72^{a}	4.79 ± 0.4^{b}	74
PF11	5.03 ± 0.21^{a}	$4.02 \pm 0.15^{a*}$	$3.14 \pm 0.32^{a*}$	62	5.7 ± 0.55^{ab}	5.23 ± 1.05^{a}	4.82 ± 0.62^{b}	83
PATCC	5.93 ± 0.28^{a}	$4.77 \pm 0.15^{ab**}$	$4.12 \pm 0.28^{b**}$	69	5.82 ± 0.15^{ab}	5.75 ± 1.3^{a}	5.64 ± 0.15^{ab}	96
FH19	5.52 ± 0.61^{a}	5.43 ± 0.25^{bc}	5.37 ± 0.65	96	5.02 ± 0.05^{b}	4.61 ± 0.55^{a}	4.1 ± 0.25^{c}	85
FPTCC	5.81 ± 0.52^{a}	$4.61 \pm 0.51^{ac*}$	$3.08 \pm 0.55^{a*}$	52	5.31 ± 0.23^{b}	4.06 ± 0.55^{a}	$3.9 \pm 0.28^{c**}$	73
AATCC	5.23 ± 0.6^{a}	$2.15 \pm 0.75^{***}$	$1.2 \pm 0.57^{***}$	21	5.7 ± 0.21^{ab}	5.58 ± 0.51^{a}	5.45 ± 0.25^{ab}	95

Values are mean ± standard deviation. Different lowercase letters in the same column differ significantly ($P < 0.05$). Viable counts of each strain at 90, 180, 120 and 240 min were compared with that of 0 min. Those that differ significantly $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).
[1]%SR stands for the percent of survival rate, calculated by dividing the final viable population (CFU/g) by initial viable population (CFU/g) of the test organism inoculated in simulated gastric and pancreatic juices.

Table 3. Inhibition zones (mm) of neutralized cell-free culture supernatants (CFCS) of *Lactobacillus* strains against pathogenic microorganisms

Pathogens	Diameter of pathogen inhibition (mm)						
	PL4	PT10	PF11	PATCC	FH19	FPTCC	AATCC
Escherichia coli O157:H7	14	14	10	15	N	N	N
Salmonella typhimurium ATCC 14028	11	15	11	15	N	N	N

N, no inhibition zone was observed.

pathogens *E. coli* O157:H7 and *S. typhimurium*. CFCS of all the strains (with original acidic pH) showed moderate antibacterial effect and could inhibit the growth of both pathogens (data not shown) but when neutralized, only CFCS of *L. plantarum* strains were found to be effective. Most of the effective strains had moderate inhibitory potential with the exception of PT11 which demonstrated weak anti-bacterial activity according to the diameters of inhibition zones created.

LAB can synthesize several metabolites including lactic acid, ethanol, acetic acid, succinic acids and hydrogen peroxide (H_2O_2) in different amounts, depending on their fermentation pathways. These products are responsible for the antibacterial effect of *Lactobacillus* strains. Lactic acid as the main product of sugar fermentation by *Lactobacillus* strains acts as a permeator of the outer membrane of gram negative bacteria and is responsible for low intracellular pH,[19] such that CFCS of all *Lactobacillus* strains could inhibit the growth of pathogenic indicator microbes in this study. But when neutralized CFCS was used, only *L. plantarum* strains showed inhibitory properties. Most probably, bacteriocins are responsible for this kind of inhibitory activities. Numerous plantaricins have been described in the literature[20,21] and further investigations are required to determine the kind and nature of these bacteriocin substances.

Evaluation of pathogenicity factors

Results of gelatinase test revealed that none of the strains could hydrolase gelatin and hemolytic activity was not observed. No bile salt deconjugation activity was observed with the exception of two types of strains PATCC and AATCC. TDCA had slight inhibitory effect on the growth of all the strains and smaller colonies were observed in comparison to the control ones. BSH may be a desirable trait because of lowering of serum cholesterol but can be harmful as the products of this reaction are toxic undesirable deconjugated bile salts.[22]

Resistance to antibiotics

The susceptibility of *Lactobacillus* strains to high-consumption antibiotics was examined. Almost all of the isolates were resistant to vancomycin and streptomycin, and sensitive to penicillin, ampicillin, rifampicin and chloramphenicol (Table 4).

Although *Lactobacilli* have a long history of safe use as microbial adjunct nutrition, their safety evaluation should not be neglected, as resistant strains may become the source for the spread of antibiotic resistance genes. Resistance to streptomycin and vancomycin was expected and also reported by several studies including Goldstein *et al.*,[23] and Solieri *et al.*[6] Fortunately, none of the strains in our study was resistant to chloramphenicol and tetracycline while their corresponding transferable resistant genes among lactobacilli are emerging.[24]

Adhesion of the strains to HT-29 cells

Adherence of *Lactobacillus* strains was measured by incubating them with confluent HT-29 monolayers for 4 h and then the adherent bacteria was enumerated. The strain PF11 showed the highest adherence potential to HT-29 cells. In general, most of the strains revealed intermediate adhesion capacity (2.3–6%), except the two *L. fermentum* strains that adhered quite low to the cells. Adhesion capacity of *L. acidophilus* ATCC 4356 was less than that of three strains (Table 5).

In order to manifest the beneficial effects, adhesion and colonization of the probiotic bacteria in the GIT of the host, is necessary.[25] Maragkoudakis *et al.*[26] reported adhesion rates of 2.6–14.4% to intestinal cells for well-known probiotic LAB. In our study, most of the strains showed good adhesion properties with the average value of 3.2 ± 1.9%. High adhesion ability

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Table 4. Antibiotic susceptibility patterns of *Lactobacillus* strains

Strains	*C* (μg)	Zone diameter (mm)						
		PL4	PT10	PF11	PATCC	FH19	FPTCC	AATCC
Penicillin	10	36 (S)	30 (S)	32 (S)	33 (S)	40 (S)	37 (S)	27 (S)
Ampicillin	10	41 (S)	37 (S)	36 (S)	40 (S)	38 (S)	40 (S)	31 (S)
Vancomycin	30	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)
Rifampicin	5	32 (S)	30 (S)	31 (S)	30 (S)	31 (S)	33 (S)	37 (S)
Tetracycline	30	26 (S)	23 (S)	0 (R)	20 (I)	25 (S)	22 (S)	24 (S)
Chloramphenicol	30	32 (S)	34 (S)	32 (S)	30 (S)	33 (S)	31 (S)	37 (S)
Streptomycin	10	0 (R)	0 (R)	16 (I)	0 (R)	0 (R)	0 (R)	0 (R)

C, concentration; R, resistant; I, intermediate; S, susceptible.

Table 5. Percent hydrophobicity, percent adhesion and percent invasion of *Lactobacillus* strains to HT-29 cells

Strains	Adhesion to HT-29 (%)	Invasion to HT-29 (%)	Hydrophobicity (%)
PL4	4.6 ± 1.0^{ab}	0.002	15.7 ± 1.4^{ac}
PT10	2.3 ± 1.2^{ab}	0.001	37.9 ± 5.2^{bc}
PF11	6.0 ± 1.8^{b}	0.045	8.3 ± 3.2^{a}
PATCC	4.6 ± 1.1^{ab}	0.005	33.4 ± 7.8^{bc}
FH19	1.7 ± 1.5^{a}	0.021	15.3 ± 3.7^{a}
FPTCC	0.6 ± 0.5^{a}	0.003	62.0 ± 6.7
AATCC	2.4 ± 1.4^{ab}	0.0001	30.1 ± 5.4^{bc}

Values represented as mean ± standard deviation. Different lowercase letters in the same column differ significantly ($P < 0.05$).

of the strain PF11 was expected, as it was of human origin. There are limited reports on the adhesion of *L. fermentum* strains to the human epithelial cells. In our study, *L. fermentum* strains showed the least adhesion ability, supporting the findings of Asahara *et al.*[27] who reported the highest and lowest adhesion ability to Caco-2 cells of the standard strains *L. plantarum* ATCC 14917 (equivalent to strain PATCC) and *L. fermentum* ATCC 14931 (equivalent to strain FPTCC), respectively. The results of our study indicate that adhesion ability in lactobacilli might be species-specific which is in accordance with that of Li *et al.*[28] reporting a strong species specificity of the subcellular adhesion-promoting factors of *Lactobacilli*.

Cell surface characteristics

Auto-A was determined with sedimentation experiments. The results showed that most of the strains had intermediate auto-aggregating phenotype and Auto-A values ranged between 13% and 63% (suspension showing both a precipitate and turbidity) (Fig. 1). In most cases, aggregation is related to achieving an adequate mass to form biofilms, adhering to the mucosal surfaces and thus, persisting in the GIT of the host and utilizing their functions.[29] In our study, when analyzing the characteristics of all the strains belonging to different species, a slight statistically significant correlation between Auto-A and attachment to HT-29 was observed (Fig. 2(a)) whereas a remarkable significant correlation existed between Auto-A and adhesion of the strains belonging to *L. plantarum* species suggesting that the latter are more potent in occupying the GIT niches. This result was in accordance with that of Li *et al.*[28] who reported species specificity of the adhesion potential.

Analysis of Co-A potential of *Lactobacillus* strains revealed that all investigated strains could aggregate well with indicator

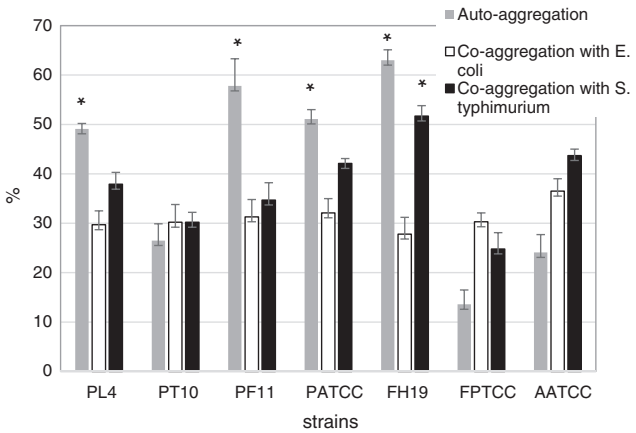

Figure 1. Percent of auto-aggregation (Auto-A) and co-aggregation (Co-A) of *Lactobacillus* strains. *Significantly different from others in each test.

strains tested (Co-A ≥ 24%). As shown in Fig. 1, the majority of strains showed higher Co-A abilities with *S. typhimurium*, in comparison to *E. coli* O157:H7. Among them, the strain FH19 exhibited the highest Co-A value with *S. typhimurium*. Strain AATCC demonstrated the most effective phenotype regarding its Co-A ability with *E. coli*. However, no significant difference was observed between the strains for their Co-A ability with *E. coli*.

Co-A of probiotic strains with a potential pathogen may play an important role in eliminating pathogens from the GIT via several mechanisms including forming a barrier that prevent colonization by pathogenic bacteria and producing antimicrobial substances in very close proximity to pathogens.[30] Vizoso *et al.*[31]

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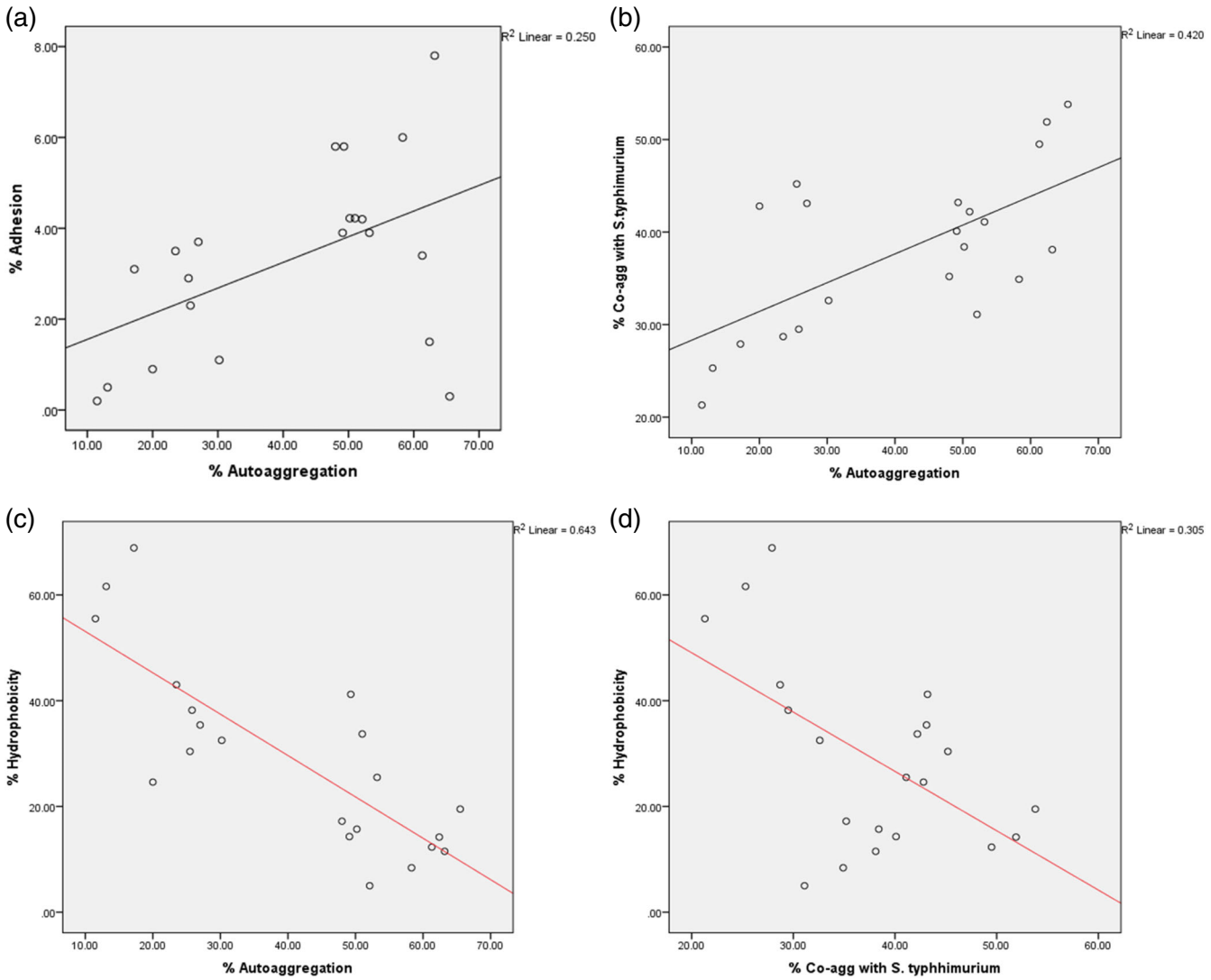

Figure 2. Correlation graphs represent association between cell surface characteristics. (a) Association between auto-aggregation (Auto-A) and adhesion of the strains to human colon adenocarcinoma cell line, HT-29 ($P < 0.05$); (b) association between Auto-A and co-aggregation (Co-A) with *Salmonella typhimurium* of the strains ($P < 0.01$); (c) association between Auto-A and hydrophobicity of the strains ($P < 0.01$); (d) association between Co-A with *S. typhimurium* and hydrophobicity of the strains ($P < 0.01$).

proved that not necessarily a strong inclination to Auto-A would result in strong Co-A property, but usually strains with a high Co-A ability also show a high Auto-A. This was also supported by our findings, because the strains with the highest Co-A scores also auto-aggregated strongly. In the present study, there was a significant positive correlation between Auto-A and Co-A with *S. typhimurium* (Fig. 2(b)). This might be explained by the extremely higher surface total charge (positive plus negative) of *S. typhimurium* than that of *E. coli* O157:H7, as reported by Ukuku and Fett.[32] This would also explain the negative relationship observed between hydrophobicity of *Lactobacillus* strains and Co-A ability of them with *S. typhimurium* (Fig. 2(d)) and might be the reason why *Lactobacillus* strains with more hydrophilic phenotypes co-aggregated more efficiently with *S. typhimurium*.

As shown in Table 5, cell-surface hydrophobicity is a strain specific character, in the case of these strains. *Lactobacillus fermentum* strain FPTCC showed the highest level of hydrophobicity (62%) while *L. fermentum* strain FH19 demonstrated a hydrophilic phenotype. A significant difference in hydrophobicity was observed, ranging from 8.3 to 62% (Table 5).

Cell-surface hydrophobicity was determined in order to find out its correlation with the ability to adhere to HT-29 cells. Hydrophobicity is a physico-chemical property that would facilitate the first contact between the microorganism and the host cells. This interaction is weak and reversible and a strong effective adhesion would be achieved with subsequent more specific mechanisms.[33] There was no significant correlation between adhesion to epithelial cells and hydrophobicity of *Lactobacillus* strains, studied in this research. Similar results were found by Zago *et al.*,[34] confirming that hydrophobicity values do not correlate with adhesion properties. Amazingly, in our study, hydrophobicity correlated significantly negative with Auto-A ability of the studied strains (Fig. 2(c)). Furthermore, statistically positive correlation was observed between Auto-A and adhesion to HT-29 cells. The explanation could be that electrostatic power and cell surface charges originated from proteins, glycoproteins, teichoic, lipoteichoic acids and exopolysaccharides on the cell wall surface of bacteria play the

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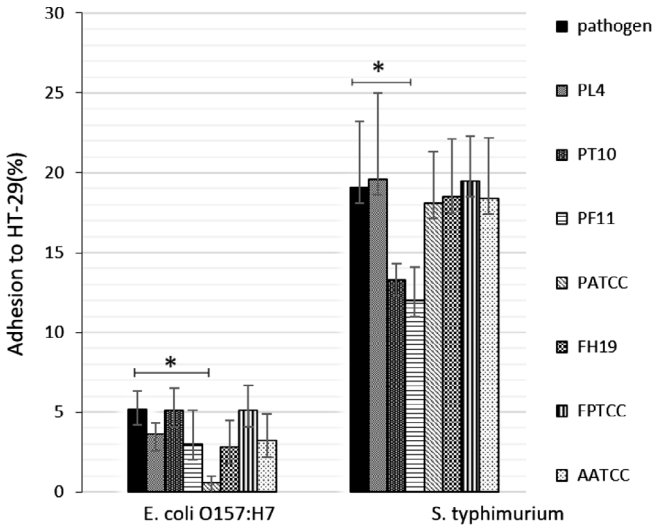

Figure 3. Adhesion of *Escherichia coli* O157:H7 and *Salmonella typhimurium* ATCC 14028 to human colon adenocarcinoma cell line, HT-29, pretreated with *Lactobacillus* strains. Black columns show percent adhesion of the pathogens to HT-29 cells but the pattern filled columns represent percent adhesion of the pathogens to the pretreated HT-29 cells with each *Lactobacillus* strain. The data are expressed as the percent of adherent bacteria ± standard deviation.

main role in Auto-A and biofilm formation to facilitate more and stronger attachment to HT-29 cells. In line with this explanation, Dickson and Koohmaraie[35] also declared that bacterial attachment to any surface is related to surface charges on both the cells and the substratum and Granato *et al.*[36] reported that pH influences the binding of lactobacilli to Caco-2 cells.

Invasion of HT-29 cells by the strains

Table 5 shows the invasion rates of *Lactobacillus* strains into HT-29 cells which were nearly zero indicating that the strains had no invasiveness into the cells.

The effect of *Lactobacillus* strains on the pathogen adhesion to HT-29 cells

Lactobacillus strains were examined for their potential to impair the adherence of two bacterial pathogens; *E. coli* O157:H7 and *S. typhimurium*, to HT-29. Figure 3 shows adhesion of pathogens to HT-29 cells, pretreated with each *Lactobacillus* strain. The adhesion of *E. coli* O157:H7 and *S. typhimurium* to confluent monolayers of HT-29 cells was found to be 6.2 and 19%, respectively. Pre-treatment of HT-29 cells with *Lactobacillus* strains caused a different and strain specific impact on each pathogen adhesion. Although most of the strains inhibited the adhesion of bacterial pathogens, only the strains PATCC and PF11 significantly decreased the adherence of *E. coli* and *S. typhimurium*, respectively.

Adhesion and invasion to epithelial cells by enteric pathogens play an important role in the pathogenesis of the disease. Intervention with the ability of bacteria to adhere to epithelial cells can prevent intestinal injury and improve clinical outcomes. In the present study, we showed that most of the strains interfered with the adhesion of two pathogens. In addition to competitive exclusion, other mechanisms might intervene in the pathogen adhesion inhibition. Induction of mucin and biosurfactants production by some strains of *Lactobacillus* can also interfere with the adhesion of pathogens to intestinal cells.[30] In our study, the inhibition of adhesion might result from a specific mechanism, since it occurs at different levels for each couple of pathogen strain and *Lactobacillus* one.

Cytotoxicity

The effect of *Lactobacillus* strains in the induction of apoptosis and necrosis in HUVEC (normal human cells) and HT-29 cells (cancerous cell line) was measured using annexin V and 7AAD, which bind strongly to phosphatidylserine and exclusively stains cells with interrupted cell membrane, respectively.

As shown in Fig. 4, treatment with different *Lactobacillus* strains had no effect on HUVEC, supporting the findings of Kalani *et al.*[37] Although no statistically significant effect was observed by some cases, most of the strains left an apoptotic effect on HT-29 cells. As shown in the dot plots (Fig. 5), while the strain PATCC significantly increased the necrosis, other strains such as PL4 and PT10 induced substantial late apoptosis. Early apoptosis was significantly induced when HT-29 cells were treated with the strains PF11 and AATCC.

The anti-carcinogenic property of *Lactobacillus* strains has been reported by some researchers and many studies have concentrated on the effect of probiotics on cancerous cells and tumor size.[38,39] Apoptotic effect on epithelial origin cancer cell line is of great importance because such cells are less sensitive to anticancer drugs.[40] The mechanisms involved in anticancer characteristics of probiotics consist of changing the metabolic activities of gut microflora and colon physicochemical condition, removing the carcinogens, producing anti-tumorigenic or anti-mutagenic substances and boosting the immunity of the host.[38]

CONCLUSION

Altogether, according to the resistance to simulated GIT conditions, capability of antimicrobial activity, Auto-A and Co-A

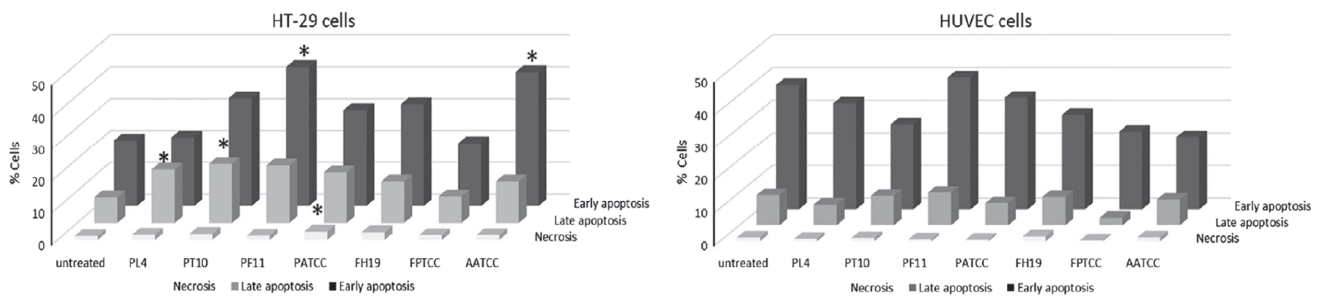

Figure 4. Flowcytometric analysis of human colon adenocarcinoma cell line, HT-29 and human umbilical vein endothelial cells (HUVECs) after 24 h incubation with *Lactobacillus* strains. *Significantly different from the untreated cells.

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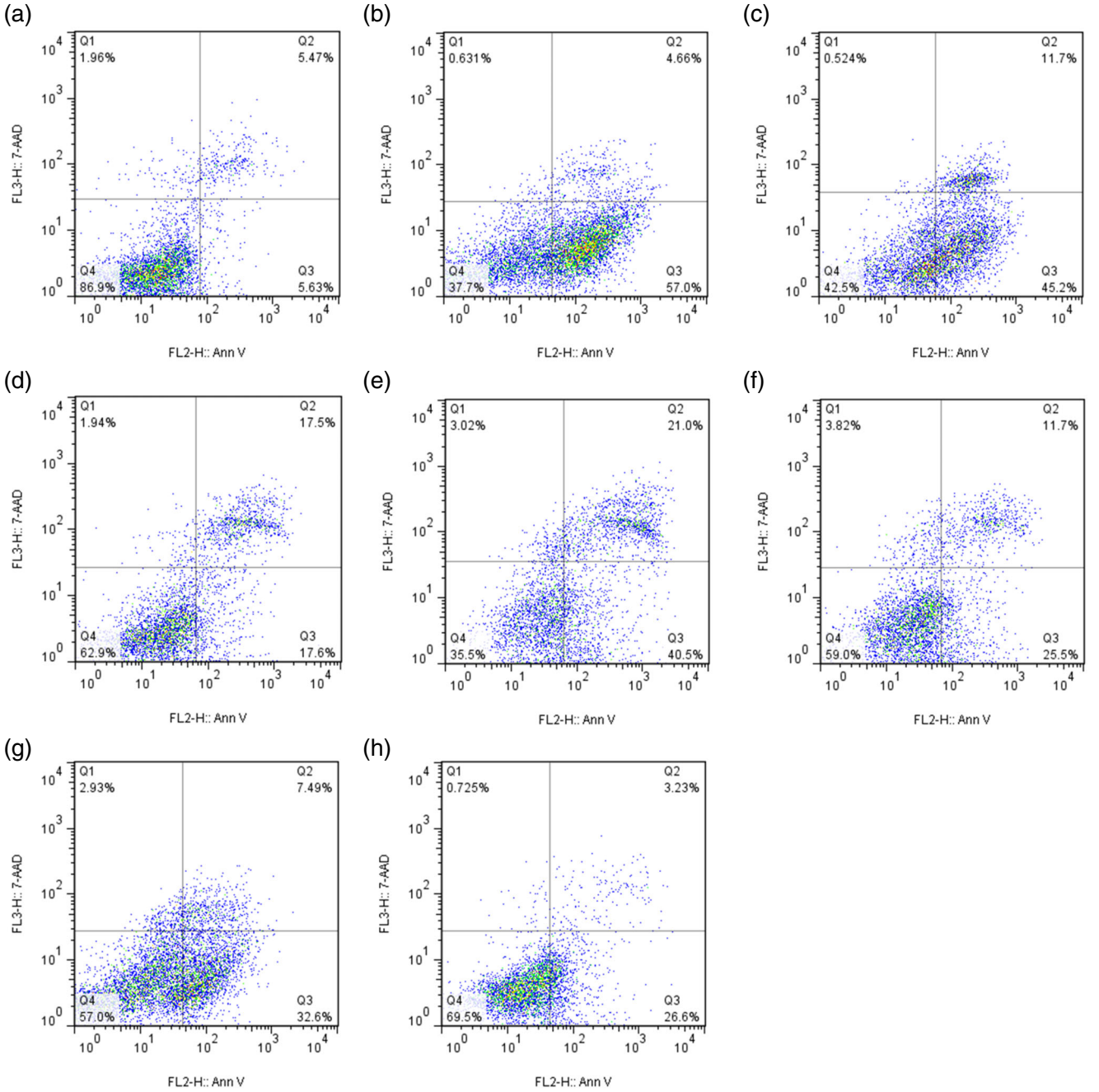

Figure 5. Dot plots of Annexin V/7-AAD flow cytometry. Human colon adenocarcinoma cell line, HT-29 was subjected to *lactobacillus* strains for 24 h. (a) Control cells cultured in lactobacilli-free medium; (b) cells treated with the strain AATCC; (c) cells treated with the strain PF11; (d) cells treated with the strain PL4; (e) cells treated with the strain PT10; (f) cells treated with the strain PATCC; (g) cells treated with the strain FH19; (h) cells treated with the strain FPTCC.

properties, adhesion to epithelial cells and anticancer effects, we can conclude that *L. plantarum* strains had a comparatively higher potential than *L. fermentum* strains for practical application. In most cases, *L. plantarum* strains were equal or better than *L. acidophilus* ATCC 4356. Probiotic potential characterization of these strains, once again proved that food-associated *Lactobacillus* strains might have a significant probiotic potential. In most experiments, except cell surface characteristics which complied a strain specific manner, there was no remarkable difference between the strains and their homolog standards. Further investigations are suggested to make the strains PL4 and PF11 applicable for clinical or technological purposes.

Ethical issues

No ethical issues were promulgated.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

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