Isolates of *Lactobacillus plantarum* and *L. reuteri* display greater antiproliferative and antipathogenic activity than other *Lactobacillus* isolates

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**Abstract**

**Purpose.** Lactic acid bacteria (LAB) have been associated with many beneficial effects in human digestive physiology. The aim of this study was to evaluate such effect, including attachment, antiproliferation and anti-pathogenic/antibacterial/antimicrobial properties of LAB isolated from healthy humans.

**Methodology.** Thirteen isolates, obtained from fecal samples of healthy individuals, were identified by phenotypic and molecular methods. Human colon adenocarcinoma cell line HT-29 and the cell proliferation kit II (XTT) assay were used for examination of the *Lactobacillus* adherence and antiproliferative activity, respectively. In addition, the inhibitory effect of *Lactobacillus* isolates against pathogenic bacteria was examined.

**Results.** Out of 13 *Lactobacillus* isolates, 5 (38 %) isolates were non-adhesive, 4 (31 %) were adhesive and 4 (31 %) were strongly adhesive. Amongst the isolated lactobacilli, *L. reuteri* showed the highest degree of inhibitory effect against the attachment of the enteropathogens. The XTT assay showed that 3 different isolates had the strongest antiproliferative activity with the maximum effect observed by *L. plantarum* isolates.

**Conclusion.** Our results described that different *Lactobacillus* species isolated from normal fecal samples had different degrees of antiproliferative and anti-pathogenic/antibacterial/antimicrobial activities. However, no isolates showed all of the examined properties concurrently, suggestive that a combination of *Lactobacillus* species is needed for an active biological defense system.

**INTRODUCTION**

The colonic microbiota has an effective role in the human digestive physiology and plays an important role in maintaining homeostasis of the large bowel and modulating the host immune response [1].

Lactic acid bacteria (LAB) are part of the human intestinal microbiota, which are considered as health beneficial probiotics. The suggestive valuable effects in humans, as reported by some investigators, included; countering colon cancer, serum cholesterol and competition with pathogenic microorganisms [2, 3]. Competition with pathogenic microorganisms, including *Escherichia coli*, *Salmonella*, *Shigella* and *Staphylococcus aureus* makes LAB an attractive microorganism for combating gastrointestinal illnesses and bacterial food borne diseases [4]. Such anti-pathogenic/antibacterial/antimicrobial effects have been attributed to the production of organic acids, inhibitory enzymes, hydrogen peroxide, bacteriocin, competition for adhesion sites and prevention of pathogen growth [5].

Anticancer and antiproliferative properties of LAB have also been proven in humans and animals. LAB are capable of inhibiting mutagen and carcinogen formation, inactivation and removal of cancerogenic substances, modification of colonic microbiota, improvement of the host’s immune response, and regulation of cellular proliferation and differentiation [6].

The HT-29 cell line (human colonic adenocarcinoma cells) is used as a suitable model for experiments related to gut...
epithelium [7]. Because of their structural and differentiation similarity to enterocytes, they can be used for screening and understanding of anticanccer effects.

The aim of this research was to evaluate the Lactobacillus attachment to HT-29 cells, their anti proliferation effect and their potential for counteracting the adhesion of enteropathogenic bacteria to this cell line.

METHODS

Bacterial isolates and growth conditions

The isolates were collected from fecal samples of healthy individuals aged between 2 and 40 years old in Tehran, Iran. Inclusion criteria were: lack of use of any antibiotic therapies over 6 months prior sampling, and no gastrointestinal (GI) disorders or consumed probiotic at the time of sampling. The isolates were identified by phenotypic methods as described previously [8], after which they were kept in −80 °C in de Man, Rogosa and Sharpe (MRS) broth with 20 % glycerol.

Molecular Identification of Lactobacillus genus and species

DNA was extracted using Bacterial Genomic DNA Purification Kit (Roche, Germany) and Identification of the Lactobacillus genus was done by PCR with 16S rRNA specific primers for LactoF 5′-TGGAACACGARTGCTAAATACCG-3′ and LactoR 5′-GTCATTGTGGAGGATCCC-3′ using the following condition: initial denaturation step of 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s and 62 °C for 1 min [9].

The species identification was done by multiplex PCR using species specific primers (Table S1, available with the online Supplementary Material) in the following PCR condition: initial denaturation step of 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 51 °C for 40 s, 72 °C for 30 s and final extension 72 °C for 7 min [10].

Cell line and adhesion assay

HT-29 cell culture (NCBI Code: C466) was used in the adhesion assay. HT-29 cells were cultured in RPMI medium supplemented with 10 % (v/v) fetal bovine serum (Thermo-Gibco, USA) and antibiotics (100U ml⁻¹ penicillin, 100mg ml⁻¹ streptomycin) incubated with 5 % CO₂ and 95 % air at 37 °C. The culture medium was changed every intermittent day and maintained for at least four days until reaching to suitable confluency (70–90 %). A suspension of 500 µl of Lactobacillus (10⁷ c.f.u. ml⁻¹) with 500 µl of either of enteropathogenic E. coli (EPEC)(ATCC 4388), enteroaggregative E. coli (EAEC)(PTCC 1533), Salmonella Typhi (ATCC 19430) and Shigella dysenteriae (PTCC 1188107) containing 10⁷ c.f.u. ml⁻¹ were added to each well separately followed by incubation for 2 h at 37 °C in 5 % CO₂. Finally, the wells were washed three times with PBS to remove free bacteria followed by treatment with 0.5 ml of 0.5 % (v/v) Triton X-100 for 5 min. To evaluate the bacterial attachments, serial dilutions of mixtures were cultured on MRS and violet red bile (VRB) agar, after which Lactobacillus and enteropathogen bacteria (c.f.u. ml⁻¹) were counted, respectively. Each assay was done in triplicate and the average of mean±SD was reported. Significant differences were tested by analysis of variance (ANOVA).

Inhibition of adhesion of the enteropathogen bacteria to HT-29 cells

The ability of the Lactobacillus isolates to inhibit the enteropathogen adhesion was performed according to previous method [13]. Briefly, 2×10⁶ cells per well were seeded in a 12-well plate and medium was changed every intermittent day and maintained for at least four days until reaching to suitable confluency (70–90 %). A suspension of 500 µl of Lactobacillus (10⁷ c.f.u. ml⁻¹) with 500 µl of either of enteropathogenic E. coli (EPEC)(ATCC 4388), enteroaggregative E. coli (EAEC)(PTCC 1533), Salmonella Typhi (ATCC 19430) and Shigella dysenteriae (PTCC 1188107) containing 10⁷ c.f.u. ml⁻¹ were added to each well separately followed by incubation for 2 h at 37 °C in 5 % CO₂. Finally, the wells were washed three times with PBS to remove free bacteria followed by treatment with 0.5 ml of 0.5 % (v/v) Triton X-100 for 5 min. To evaluate the bacterial attachments, serial dilutions of mixtures were cultured on MRS and violet red bile (VRB) agar, after which Lactobacillus and enteropathogen bacteria (c.f.u. ml⁻¹) were counted, respectively. Each assay was done in triplicate and the average of mean±SD was reported. Significant differences were tested by analysis of variance (ANOVA).

Antiproliferation effect of Lactobacillus isolates on HT-29 cells

To determine antiproliferation activity of Lactobacillus, XTT [2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] assay was used as described previously [14]. HT-29 cells a concentration of 2×10⁴ cells ml⁻¹ with 200 µl RPMI1640 supplemented medium were seeded in 96-well plates. After 24 h, the cells were washed twice with PBS followed by addition of Lactobacillus isolates, at a multiplicity of infection (MOI) of 100 alone (100 : 1) to each well. They were then incubated for 16 h at 37 °C in 5 % CO₂. After the incubation time, the cell viability was checked by trypan blue. Then, 100 µl of RPMI medium and 50 µl of XTT reagent was added to each well followed by incubation. After 6 h, formazan absorbance was assayed at 450 nm. The proliferation percentage of cells was calculated as follows: A₄₅₀nm test−A₄₅₀nm control / A₄₅₀nm control × 100, where A₄₅₀nm test: HT-29 cells treated with Lactobacillus and A₄₅₀nm control: HT-29 cells alone. XTT assay was done in triplicate. Results are reported as the average of mean±SD and analysed with unpaired t-test.
RESULTS

Isolation and identification of Lactobacillus species
In this study, all isolates were confirmed by phenotypic properties. Thirteen isolates were confirmed at the genus level by PCR using 16S rRNA primers followed by identification of 13 isolates at the species level by multiplex PCR. The results showed 6 isolates belonged to L. plantarum, 3 L. brevis, 2 L. rhamnosus, 1 L. delbruecki and 1 L. reuteri.

Cell line and adhesion assay
Lactobacillus adhesion ability to HT-29 cells was examined by light microscopy after Gram stain. Overall, 5 (38 %) isolates were nonadhesive, 4 (31 %) isolates were adhesive and 4 (31 %) isolates were strongly adhesive. The adherence scores of these 13 isolates are shown in Fig. 1. In comparison, L. reuteri and L. brevis 02 exhibited the highest and lowest adherence capacity to HT-29 cells, respectively.

Inhibition of adhesion of the enteropathogen bacteria to HT-29 cells
The results showed that 13 Lactobacillus species had different effects on enteropathogens adhesion to HT-29 cells (Table 1). L. reuteri showed the highest degree of inhibitory effect for EPEC (56 %), EAEC (54 %), Salmonella Typhi (53 %) and Shigella dysenteriae (49 %) (P<0.05). The other isolates were unable to display significant differences in their ability to prevent adhesion of enteropathogen bacteria to the HT-29 cells (P>0.05).

Antiproliferation effect of Lactobacillus isolates on HT-29 cells
It has been suggested that a 20 % inhibition of proliferation of HT-29 cells could be used to report a significant antiproliferative effect [14]. Amongst the 13 Lactobacillus isolates, L. plantarum 03 followed by 2 different isolates showed the maximum antiproliferative effects (P<0.001). The other 10 isolates showed less than 20 % or no effect on the proliferation of HT-29 cells. (Fig. 2)

DISCUSSION
In recent years, Lactobacillus protective effect against bacterial infections, especially enteropathogens has attracted great attention. Some investigators even have suggested anti-cancer capabilities of LAB [15, 16]. Specific aspects of these properties were investigated here.

Our study described thirteen isolates confirmed at the species level, including 6 isolates belonging to L. plantarum, 3 isolates L. brevis, 2 L. rhamnosus, 1 L. delbruecki and 1 L. reuteri. The distribution of isolates were 8 (62 %) and 5 (38 %) different Lactobacillus species from men and women, respectively. Moreover, all three isolates of L. brevis were isolated from men.

Our study indicated that 31 % of the Lactobacillus isolates showed strong adhesion, 4 isolates (31 %) were adhesive and 5 isolates (39 %) were nonadhesive. This is contrary to the report by others [1] who found >57 % adhesion and an absence of nonadhesive Lactobacillus isolates. Such variation was expected since diverse ecology of human bacterial populations can dictate the presence of different species of Lactobacillus with dissimilar biological activities.

Inhibitory effects of the 13 Lactobacillus isolates against enteropathogens adhesion to HT-29 cells showed that 5 of the isolates displayed inhibitory actions. However, only L. reuteri showed a significant degree of inhibition (P<0.05). Similar to our results, Lee and colleagues [17] have also reported the reduced attachment of E. coli caused by Lactobacillus. Such reduction, however, was caused by L. rhamnosus.

Our results suggest that amongst the 13 Lactobacillus isolates, 3 (23 %) different isolates showed strong antiproliferative effect. Orlando and colleagues [18] have reported that use of commercially known L. rhamnosus LGG caused the inhibition of proliferation and induction of apoptosis in colon cancer cells. Rohani and colleagues [8] described that 30 % of all Lactobacillus isolates showed antiproliferative effect on Caco-2 cells. Lactobacillus antitumour effects have been shown through reducing tumour size and cell viability [15]. The exact mechanism of action for these effects are not known. Binding interaction of Lactobacillus with tumour cell membrane is under investigation in this laboratory [19].

In the present study, L. reuteri showed the highest adherence score to HT-29 and, as expected, the highest degree of inhibitory effect against EPEC, EAEC, Salmonella Typhi and Shigella dysenteriae in comparison with other species of Lactobacillus. However, no antiproliferation activity against HT-29 was observed by L. reuteri, suggesting that the prevention of adhesion of pathogenic bacteria to HT-29 cells may be indicative of the occupation of the HT-29 cell’s membrane by L. reuteri which, in turn, prevented other bacterial species to attach.

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**Fig. 1.** Adhesion of Lactobacillus isolates to HT-29 cell line. The adherence score was determined by the number of adherent bacteria in 20 random microscopic fields of view. The results are given as average values of three different experiments standard deviation.
Interestingly, the comparative analysis of adhesion, anti-pathogenic/antibacterial/antimicrobial and antiproliferative effects of *Lactobacillus* isolates in the present study suggested that none of these isolates could exhibit all of the valued probiotic properties simultaneously. Perhaps different biological factors, environmental conditions and different by-products produced by these bacteria are the reasons for this reliance.

**Conclusion**

Lactobacilli have probiotic characteristics and health promoting effects. In short, our results described that different *Lactobacillus* species isolated from normal fecal samples were capable of adhesion to HT-29 cells, with different degrees of anti-pathogenic/antibacterial/antimicrobial and antiproliferative effects. Using a combination of LAB seems to provide valuable health benefits in areas of antipathogenic and antitumour proliferation activities.

### Table 1. Inhibition of adhesion of enteropathogen bacteria by *Lactobacillus* isolates (mean ±SD)

<table>
<thead>
<tr>
<th>Strains</th>
<th>EPEC (c.f.u. per well ×10^5)</th>
<th>EAEC (c.f.u. per well ×10^5)</th>
<th>Salmonella Typhi (c.f.u. per well ×10^5)</th>
<th>Shigella dysenteriae (c.f.u. per well ×10^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.57±0.29</td>
<td>3.11±0.31</td>
<td>2.31±0.22</td>
<td>2.14±0.19</td>
</tr>
<tr>
<td>L. brevis01</td>
<td>2.53±0.43</td>
<td>3.00±0.32</td>
<td>2.29±0.23</td>
<td>2.00±0.54</td>
</tr>
<tr>
<td>L. delbrueckii</td>
<td>1.92±0.22</td>
<td>2.31±0.16</td>
<td>1.87±0.21</td>
<td>1.68±0.31</td>
</tr>
<tr>
<td>L. reuteri</td>
<td>1.12±0.33*</td>
<td>1.43±0.12*</td>
<td>1.08±0.19*</td>
<td>1.10±0.41*</td>
</tr>
<tr>
<td>L. plantarum01</td>
<td>2.56±0.61</td>
<td>3.00±0.33</td>
<td>2.29±0.19</td>
<td>2.12±0.22</td>
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<tr>
<td>L. plantarum02</td>
<td>2.55±0.4</td>
<td>2.98±0.37</td>
<td>2.30±0.31</td>
<td>2.13±0.21</td>
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<tr>
<td>L. plantarum03</td>
<td>2.00±0.22</td>
<td>2.49±0.16</td>
<td>1.63±0.19</td>
<td>1.76±0.22</td>
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<tr>
<td>L. brevis02</td>
<td>2.27±0.33</td>
<td>2.71±0.4</td>
<td>1.83±0.4</td>
<td>1.81±0.33</td>
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<tr>
<td>L. rhamnosus01</td>
<td>2.81±0.71</td>
<td>3.45±0.38</td>
<td>2.59±0.27</td>
<td>2.81±0.4</td>
</tr>
<tr>
<td>L. rhamnosus02</td>
<td>2.54±0.15</td>
<td>3.10±0.11</td>
<td>2.28±0.18</td>
<td>2.10±0.2</td>
</tr>
<tr>
<td>L. plantarum04</td>
<td>2.69±0.41</td>
<td>3.34±0.82</td>
<td>2.49±0.85</td>
<td>2.43±0.76</td>
</tr>
<tr>
<td>L. plantarum05</td>
<td>2.53±0.17</td>
<td>3.00±0.22</td>
<td>2.30±0.18</td>
<td>2.11±0.3</td>
</tr>
<tr>
<td>L. brevis03</td>
<td>2.10±0.43</td>
<td>2.63±0.64</td>
<td>1.77±0.38</td>
<td>1.88±0.41</td>
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<tr>
<td>L. plantarum06</td>
<td>2.71±0.61</td>
<td>3.42±0.44</td>
<td>2.56±0.32</td>
<td>2.64±0.21</td>
</tr>
</tbody>
</table>

Significant difference was tested by analysis of variance (ANOVA).

*Indicates means which were significantly different from the control value (P<0.05).

### References


2. Živković M, Miljković MS, Ruas-Madiedo P, Markelc MB, Veljović K et al. EPS-SJ exopolisaccharide produced by the strain *Lactobacillus paracasei* subsp. *paracasei* BGSJ2-8 is involved in adhesion to epithelial intestinal cells and decrease on *E. coli* association to Caco-2 cells. Front Microbiol 2016;7:286.


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**Fig. 2.** Antiproliferation effect of *Lactobacillus* isolates on HT-29 cells. The results are reported as average values of three experiments ±standard deviation and analysed with unpaired t-test. P<0.05(*), P<0.001(**).


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