Antibacterial activity of lactobacilli probiotics on clinical strains of Helicobacter pylori

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**ABSTRACT**

**Objective(s):** Treatment of Helicobacter pylori infection by common drugs may be associated with several problems such as antimicrobial resistance to commonly used antibiotics and side effects of employed drugs. Therefore, exploration of non-chemical compounds which are safer than chemical ones is becoming important as an alternative therapy. The purpose of this study was to evaluate the effects of lactic acid bacteria (LAB) against clinical strains of H. pylori.

**Materials and Methods:** Study of antibacterial effects of LAB against H. pylori strains included: evaluation of LAB effects as well as its cell-free supernatant (CFS) to reduce the number of H. pylori, and to examine the effects of CFS to inhibit the urease activity of H. pylori. The anti-adhesion effect of LAB on adherence of H. pylori to epithelial cell line was also evaluated.

**Results:** Evaluation of the anti H. pylori effect of LAB depended on the strain of H. pylori strain Lactobacillus. However, CFS of LAB reduced significantly the growth of all H. pylori strains. Also, urease activity of H. pylori strains was inhibited by CFS of LAB demonstrating that their organic acid may have a role in this inhibition. The significant anti-adhesion effect of LAB on adherence of H. pylori was also observed.

**Conclusion:** Presence of LAB and/or their CFS can reduce the count of H. pylori, inhibit the urease activity of H. pylori, and reduce adhesion of H. pylori to epithelial cell line. This may be important for the impact of H. pylori colonization in the host stomach.

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**Introduction**

Bacterial interference between the natural flora and the pathogens was proposed in the 20th century and following this finding, it was investigated for the control of infections. The investigators have demonstrated that the probiotics such as lactobacilli can prevent the growth of a wide range of human and animal microbial species which are their pathogens. Of them; the effects of Lactic Acid Bacteria (LAB) on Helicobacter pylori may be noted (1, 2). H. pylori has been known as a major cause of chronic gastritis, peptic ulcer disease and stomach cancer (3, 4). Bacterial virulence factors help H. pylori to invade the host stomach, cause disease and evade the host defenses. Expression of several virulence factors such as urease, adhesion factors, vacuolating cytotoxin A, and the cytotoxin-associated gene A, are associated with pathogenicity of H. pylori (5). The standard triple therapy regimen for treatment of H. pylori infection was widely used throughout the world (6). This treatment consisted of a proton pump inhibitor (PPI) plus two antibiotics (clarithromycin and amoxicillin) (7). The success of this treatment regimen has become progressively decreased in recent years. Non-compliance and the emergence of antimicrobial-resistant strains of H. pylori are considered as the major factors contributing to treatment failure (8). Regarding the side effects of antibiotics in majority of the cases and emergence of resistant bacteria in the stomach of the infected patients, use of living microbial agents, such as LAB (as probiotics bacteria), may be a useful alternative in preventing the symptoms of H. pylori infection via inhibiting its growth by competing with this pathogens (9). Probiotics are define as live microorganisms, which when administered in enough amount give a health benefit on the host. Probiotics have diverse mechanisms for inhibition of pathogenic bacteria, e.g. nutrient competition, production of inhibitory compound (bacteriocins, organic acids, biosurfactant,...), immunostimulation and competition for binding sites (10).

The purpose of present study was to investigate the inhibitory effects of six species of LAB and their CFS against growth, urease (in vitro) and adhesion to epithelial cell line of eight clinical strains of H. pylori which were selected according to their diverse antibiotic resistance profiles.

**Materials and Methods**

Bacterial strains and culture conditions: H. pylori strains used in this study were selected according to their susceptibility profile that were in H. pylori Lab at Alzahra University (Table 1). They were grown at 37 °C for 48 hr under microaerobic conditions by Gas pack C (Merck) on Brucella Agar containing 5-10% defibrinated sheep blood supplemented with vancomycin (10 μg/ml), polymyxin (2.5 IU/ml) and amphotericin B (2 μg/ml). Antibiotic susceptibility testing of H. pylori strains was performed according to agar disk-diffusion procedure (11). For this purpose, bacterial suspension (McFarland tube No 4) corresponding to approximately 9×10⁸ CFU/ml were plated on Muller Hinton Agar (MHA) (Merck)

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Table 1. *Helicobacter pylori* strains used in this study

| H. pylori strains | Antibiotic resistance profile | Sex-age-pathology |
|------------------|-----------------------------|-------------------|
| 1 HP1            | Met, Cly, Amox, Amp, FR, E, ATH | TE, CIP, CRO, CFM | Male-14-GG |
| 2 HP13           | Met, Cly, Amox, Amp, TE, E, ATH | CIP, FR, CRO, CFM | Female-13-GG |
| 3 HP70           | Met, Cly, Amox, Amp, TE, E, ATH | FR, CRO, CFM | Female-62-GG |
| 4 HP153          | Met, Cly, Amox, Amp, TE, FR, E, ATH, CFM | CIP, CRO | Female-24-GG |
| 5 157            | Met, Cly, Amox, Amp, FR, E, ATH | TE, CIP, CRO, CFM | Female-4-GG |
| 6 23F            | Met, Cly, Amox, Amp, TE, CIP, E, CFM, CRO, FR, ATH | - | Male-9-GG |
| 7 AS923          | Met, Cly, Amox, Amp, CIP, E, FR, ATH | TE, CRO, CFM | F |
| 8 PR             | Met, Cly, Amox, Amp, TE, CIP, CFM, CRO, FR, E | ATH | Male-4-GG |

CG: Chronic Gastritis
Amo: Amoxicillin [25 µg/disk], TE: Tetracycline [30 µg/disk], Ampicillin [10 µg/disk], CIP: Ciprofloxacin [5 µg/disk], Ath: Azithromycin [15 µg/disk], E: Erythromycin [15 µg/disk], CRO: Ceftriaxone [30 µg/disk], CFM: Cefixime [5 µg/disk], FR: Furazolidone [100 µg/disk], Met: Metronidazole [5 µg/disk], Cly: Clarithromycin [15 µg/disk]

containing 5% defibrinated sheep blood without antibiotic. Antibiotics disks corresponded to amoxicillin [25 µg/disk], tetracycline [30 µg/disk], ampicillin [10 µg/disk], ciprofloxacin [5 µg/disk], azithromycin [15 µg/disk], erythromycin [15 µg/disk], ceftriaxone [30 µg/disk], cefixime [5 µg/disk], furazolidone [100 µg/disk], Metronidazole [5 µg/disk] and Clarithromycin [15 µg/disk] purchased from Padtan Tab were placed on the plates and incubated at 37 °C in a microaerobic condition for 72 hr; then their inhibition zone diameters were examined. The zones of inhibitions were interpreted according to those of the previously described protocol (12-15).

LAB corresponded to *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus rhamnosus* ATCC 7469, *Lactobacillus reuteri* ATCC 23272, *Lactobacillus fermentum* ATCC 9338, *Lactobacillus plantarum* ATCC 8014 and *Lactobacillus casei* ATCC 39392 that were provided from Iranian Research Organization for Science and Technology (IROST). These LAB were selected since their antibacterial effects on the most photogenic bacteria has been confirmed in our previous researches (10). LAB were cultured in DeMan-Rogosa-Sharpe (MRS) broth for 48 hr at 37 °C. CFS of LAB were prepared from Padtan Tab were cultured in RPMI 1640 (Dacell) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 1% PenStrep (100 U/ml penicillin and 100 µg/ml streptomycin, bioidea). The cells were maintained at 37 °C and 5% CO2 in a humidified environment. The day before of experiment, the cells were seeded into tissue culture plates to form a monolayer overnight. At the start of each experiment, the cell culture medium was replaced with RPMI 1640 with 3% serum and without antibiotics.

**Effect of lactobacilli CFS on *H. pylori***

**Agar diffusion assay**

*H. pylori* suspensions equivalent to 2 McFarland, were plated on Mueller-Hinton agar plates containing 5% defibrinated sheep blood without antibiotics. Wells (diameters, 6 mm) were drilled into the agar using the sterile Pasteur pipettes. They were filled with 100 µl CFS of LAB. Plates were incubated for 72 hr under microaerobic conditions at 37 °C, and the diameters of inhibition zones around the wells were measured according to the previously described protocol (16).

**Liquid culture assay**

*H. pylori* suspension (10⁶ CFU/ml) were prepared in brain heart broth without antibiotics and were incubated under microaerobic conditions at 37 °C with CFS (equal volume); MRS broth medium was used as the control. The viability of *H. pylori* after 48 hr was evaluated by determination of the viable count (CFU) on MHA agar containing 5% defibrinated sheep blood plates following incubation at 37 °C under microaerobic conditions (16).

**Effect of lactobacilli CFS on urease activity of *H. pylori* strains**

The urease activity of *H. pylori* strains

Urease activity was determined in medium containing, phosphate buffer, urea and 0.012% phenol red (as indicator of pH). The principle of this analysis was the production of ammonia due to hydrolysis of urea, which was measured by absorbance of produced color at 550 nm by the spectrophotometer. Optimization of the reaction conditions concerning urea concentration, pH of reaction buffer and temperature for urease activity of *H. pylori* was performed before analysis of urease activity. For this purpose, the optimum effect of urea concentration (15 to 25 mg/ml), pH (5.8 to 7.8 pH), and temperature (37 to 55 °C) was examined by response
surface methodology (RSM) (17). In all cases, 50 ul of H. pylori cells, grown in the Brucella broth for 48 hr at 37 °C (initial number of bacteria: 10⁸ CFU/ml) was added to 1 ml of urease reaction buffer and the color absorbance was measured at 550 nm after incubation for 1 hr (18-20).

The inhibitory effect of lactobacilli CFS on urease activity of H. pylori

For analysis of urease inhibitory effects by LAB, H. pylori cells incubated in Brucella broth at 37 °C (initial number: 10⁸ CFU/ml) for 48 hr, were mixed with various concentrations of CFS (10 to 40%) and incubated at 37 °C for 30, 60, and 120 min. The result color was read at 550 nm by spectrophotometer according to the previously described protocol (16). MRS broth was used as control of reaction. Also, the effect of CFS that could be neutralized by NaOH (1 mol/lit) to 7.0±0.01 and acids such as acetic acid (Merck) and lactic acid (Merck) on urease activity were evaluated (21).

Effect of LAB on the count of H. pylori strains by co-culture

H. pylori strains also LAB were recovered from plates and were suspended in BHB with 10 % (v/v) horse serum and MRS broth, respectively (McFarland No. 1 and 0.5 respectively). Equal volumes of each suspension was combined and incubated at 37 °C at atmosphere containing 5% CO₂. After 48 hr incubation at 37 °C under microaerobic conditions, the number of CFU was evaluated on MHA agar containing 5% defibrinated sheep blood which was selective for the growth of H. pylori containing 5 µg/ml trimethoprim, 2.5 U/ml polymyxin B, 10 µg/ml vancomycin and 5 µg/ml penicillin (Sigma-Aldrich) (the LAB were sensitive to these antibiotics) (22).

Effect of LAB on adhesion of H. pylori to AGS cell line

Bacterial suspensions were prepared from the culture plates of H. pylori strains and adjusted to McFarland tube No 3. After centrifugation, bacterial cells were suspended in RPMI 1640 with 3% serum. As well, bacterial suspensions were prepared from the overnight culture plates of LAB and adjusted to McFarland tube no 0.5; after centrifugation, bacterial cells were suspended in RPMI 1640 with 3% serum. Epithelial cells grown in 48-well plates were infected with both H. pylori strains and LAB at an multiplicity (MOI) of 100 for each species. After 3 hr of incubation, the cells were washed three times with phosphate-buffered saline (PBS) to remove any unbound bacteria. The host cells were lysed by treatment with Brucella broth containing 10% fetal bovine serum (FBS) for 30 min, and then all cells from the wells were scrubbed. The number of CFU for H. pylori was determined by serial dilution and spreading on appropriate selective medium as previously described after incubation for 4 to 7 days (23).

Statistical analysis

All of the experiments were performed in triplicate samples and error bars represent standard deviations. Differences with a P-value below 0.05 were considered statistically significant by paired T-Test. Normality of the data and the statistical analysis was performed using Minitab 17.

Figure 1. (A-H) Count of Helicobacter pylori strains (log₁₀ CFU/ml) in presence of cell free supernatant (CFS) of lactobacillus bacteria and lactobacillus bacteria alone after 48 hr incubation at 37 °C (equal volume, initial concentration of bacteria were 10⁸ CFU/ml). [1: MRS Broth Medium; 2: Lactobacillus acidophilus ATCC 4356; 3: Lactobacillus rhamnosus ATCC 23272; 4: Lactobacillus reuteri ATCC 23272; 5: Lactobacillus fermentum ATCC 9338; 6: Lactobacillus plantarum ATCC 8014; 7: Lactobacillus casei ATCC 39392] *indicate significant differences from the control (MRS Broth Medium) by T-Test (P<0.05)

Results

Effect of LAB and their CFS on the count of H. pylori

The count of H. pylori strains (Log₁₀ CFU/ml) in the presence of CFS of LAB and LAB are shown in Figure 1 (A-H). Results demonstrated that the reduction effect of CFS was higher than LAB. In the case of CFS was between 1-6 log but in the case of LAB was not more than 1 log. In the case of CFS of L. acidophilus ATCC 4356 and L. casei ATCC 39392 a reduction of 6-log was observed in H. pylori 23F but only a reduction of 1 log was observed in case H. pylori 23F in presence of L. ruteri ATCC 23272.

Based on Figure 1 (A-H), CFS of L. acidophilus, L. rhamnosus, L. reuteri and L. casei had a decreasing effect on the growth of every 8 strains of H. pylori but L. fermentum and L. plantarum had this effect on the growth of 4 and 6 strains of H. pylori respectively. Also this effect on LAB included 5 strains of H. pylori in presence of L. ruteri and L. casei, 3 strains in presence of L. plantarum and 2 strains in presence of other LAB (Table 2).

Effect of lactobacilli CFS on the urease activity of H. pylori

To perform this test, the optimized conditions
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For urea concentration, pH of reaction buffer and temperature were obtained using RSM method. They were 15%, 5.8 and 46-55 °C for urea concentration, pH, and temperature, respectively. The anti-urease activity effect of CFS was higher at concentrations of 30-40% than other concentrations, but the same effect was observed in these two concentrations. Figure 2 shows the results of the anti-urease activity effect of CFS in the concentration of 30% after 120 min. The result showed that CFS of *L. acidophilus* ATCC 4356, *L. reuteri* ATCC 23272 and *L. fermentum* ATCC 9338 reduced urease activity of all eight *H. pylori* strains. However, *L. rhamnosus* ATCC 7469, *L. plantarum* ATCC 8014 and *L. casei* ATCC 39392 were effective only on urease activity of six out of eight *H. pylori* strains. This inhibition effect was lost in pH of 7.0, indicating that this effect may be mediated by organic acid present in CFS. As the control, we tested the effect of various concentrations of acetic acid and lactic acid (10, 20 and 30%) on urease activity of *H. pylori* and we obtained similar results as above. The most effective inhibitory effect was observed for *H. pylori* HP70 such that its initial absorbance of 0.963 at 550 nm was changed to 0.122, 0.2 and 0.208 after 120 min in presence of CFS obtained from *L. fermentum* ATCC 9338, *L. reuteri* ATCC 23272 and *L. acidophilus* ATCC 4356 respectively (Figure 2).

**Effect of LAB on the adhesion of *H. pylori* to AGS cell line**

By counting the number of adherent *H. pylori* to host cell AGS, the percentage of adherence was analyzed. We observed that adherence in the *H. pylori* HP1 and AS923 (20%) were inhibited by all of the LAB. However, *L. rhamnosus* ATCC 7469 had anti-adherent effect against five *H. pylori* strains, and *L. acidophilus* ATCC 4356 and *L. reuteri* ATCC 23272 had anti-adherent effect against four *H. pylori* strains. Adherence of *H. pylori* HP1 and AS923 was reduced in presence of all LAB but in the cases of *H. pylori* hp13 and 23F no decrease was found. Figure 3 showed CFU (log₁₀ CFU/ml) of adherent bacteria to AGS in the presence and in absence of LAB.

**Discussion**

Treatment of *H. pylori* infection is often effectuated by routine triple therapy regimen and if the infecting strain is resistant, its successful eradication would be compromised (24). Nowadays, the rate of treatment failures is rising and the major cause for this would be bacterial resistance to frequently prescribed antibiotics.

### Table 2. Reduction (%) of *Helicobacter pylori* in presence of cell free supernatant (CFS) and lactobacillus bacteria

| LAB Strains of *H. pylori* % (n=8) | CFS | LAB |
|---------------------------------|-----|-----|
| *L. acidophilus* ATCC 4356      | 100% (8) | 25% (2) |
| *L. rhamnosus* ATCC 7469        | 100% (8) | 25% (2) |
| *L. reuteri* ATCC 23272         | 100% (8) | 62.5% (5) |
| *L. fermentum* ATCC 9338        | 50% (4) | 25% (2) |
| *L. plantarum* ATCC 8014        | 75% (6) | 37.5% (3) |
| *L. casei* ATCC 39392           | 100% (8) | 62.5% (5) |

Figure 2. Effect of lactobacilli cell free supernatant (CFS) (30% concentration) on urease activity of *Helicobacter pylori* strains by measuring the absorbance of ammonium at 550 nm after 120 min. Differences with a P-value below 0.05 were considered statistically significant by paired T-test.

Figure 3. Number (log₁₀ CFU/ml) of adherent *Helicobacter pylori* on AGS cell line in the presence and in absence of lactobacillus bacteria after 3 hr incubation at 37°C. Data represent mean±standard deviation (SD) from 3 independent experiments (error bar). *indicate significant differences from the control (MRS Broth Medium) T-Test (P<0.05)
so, it is important to know the pattern of this resistance. Furthermore, the antibiotic resistance patterns vary between countries and within different regions of the same country (25, 26). The major pattern of antibiotic-resistance in *H. pylori* strains used in the present study was resistance to metronidazole, clarithromycin, amoxicillin, ampicillin, erythromycin, furazolidone, and azithromycin (Table 1). Studies of researchers in Iran recommended fluorquinolones for the treatment of *H. pylori* infection because of the inadequacy of the common antibiotics (27, 28). This resistance pattern reflects the importance of antibiotic use in our country, especially in children (11). The results of a study on 218 isolates of *H. pylori* gathered from 985 dyspeptic patients during 2010-2017 in Iran, showed a considerable increase in resistance to presently used antibiotics such as metronidazole, ofloxacin, tetracycline, and clarithromycin (29). This high resistance to antibiotics, requires alternative ways to reduce the rates of *H. pylori* infection.

Probiotics bacteria are prescribed for the treatment of many gastrointestinal disorders, ranging from diarrhea to *H. pylori* infection (15). Researchers demonstrated an increase in the rate of *H. pylori* eradication due to the use of probiotics along with triple therapy (30). The in vivo studies have shown that eradication rate of *H. pylori* infection by drugs can be improved by administration of lactobacilli and also due to reducing gastric mucosal inflammation (31-34). The decrease of 6-log of *H. pylori* was reported in presence CFS of LAB after 24 hr (35). Complete inhibition of *H. pylori* in vitro was observed in the case of co-incubation with probiotics (*L. rhamnosus* and *L. acidophilus*) at ratios of 1:10 and 1:100 (36). Takeda et al. reported that inhibitory activity of probiotic bacteria against *H. pylori* was strain-dependent so that *L. paracasei* strain 06TCa19 and *L. plantarum* strain 07MR044 exhibited potent abilities to inhibit the growth of *H. pylori* in co-culture method (22), that detected in the present study. Antagonistic effect of LAB related to resource competition, production different low molecular weight substances (e.g. diactyl, acetaldehyde, hydrogen peroxide, etc.); production of different organic acid (e.g. lactic, propionic, succinic and acetic); pH lowering effect and production of bacteriocin and bacteriocin-like substances that are antibacterial (23). The bacteriocin and bacteriocin-like substances that are antibacterial may be different from lactic acid (45). In our study, CFS of probiotics bacteria could decrease urease activity but no inhibitory effect was found on urease activity when pH of CFS was neutralized with NaOH. Therefore, organic acids in CFS of LAB play an important role in the inhibition of urease activity of *H. pylori* strains. Also, inhibition effect of urease by acetic acid and lactic acid was observed in our work. The urease activity of *H. pylori* co-cultured with lactobacilli supernatants decreased (20). Lactic acid of *L. fermentum* UCO-979C obtained from human gut could inhibit the urease enzyme of *H. pylori* strains (35). However, the bacteriocin of *L. plantarum* and *L. acidophilus* could inhibit or reduce the urease activity in Proteus spp (47).

Inhibitory effects of LAB on adherence of *H. pylori* strains to AGS cell line may reflect their in vivo effect that can help to prevent infection in an early stage of *H. pylori* colonization. The anti-adherence effects of probiotics can be produced by bacterial competition for binding sites on epithelial cells where at the same time the antimicrobial substances are also secreted by probiotic bacteria. *L. reuteri* possesses the cell surface proteins that inhibit *H. pylori* to bind to glycolipids receptor in vitro (48), also lactobacilli act directly on *H. pylori* by an effectors molecule that is released into the medium. This effectors molecule acts on *H. pylori* by inhibiting expression of the adhesion-encoding gene *sabA* (23). Animal studies demonstrated that prior colonization by *H. pylori*, probiotics can prevent *H. pylori* infection.
in germ-free mice (49). In our study, it seems that due to simultaneous exposure of *H. pylori* and LAB to AGS, competition between them to bind to the host cells may be produced, which is one of the inhibitory mechanisms.

**Conclusion**

Treatment of *H. pylori* using synthetic compounds is associated with several problems such as the high cost of medications, post-treatment bacterial resistance, and adverse side effects. Therefore, exploration of some safer and non-chemical anti-*H. pylori* compounds are becoming important as an alternative therapy against *H. pylori* infections. Probiotic bacteria can inhibit *H. pylori* by immunological and non-immunological mechanisms. Our study reviewed the non-immunological mechanisms that showed the anti-*H. pylori* effect of probiotics bacteria depends on the strain of *H. pylori* and LAB. As lactobacillus can reduce the growth of *H. pylori*, inhibit the activity of its urease, and reduce adhesion *H. pylori* to cell line, they can play an important role in preventing colonization of stomach by *H. pylori*.

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**Conflicts of Interest**

The authors declare that there are no conflicts of interest.

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