The Anti-*Helicobacter pylori* Effects *of Lactobacillus acidophilus, L. plantarum*, and *L. rhamnosus* in Stomach Tissue of C57BL/6 Mice

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Abstract

Background and Aim: Helicobacter pylori is one of the most common pathogenic bacteria in the human gut, and is also one of the most important factors that cause digestive disorders such as chronic inflammation, gastric ulcers, and even gastric cancer. Since the use of various antibiotics to treat *H. pylori* infection is associated with the development of resistance in this bacterium, the aim of this study was to determine the anti-*H. pylori* effects of *Lactobacillus acidophilus, L. plantarum*, and *L. rhamnosus* in the stomach tissue of C57BL/6 mice. *Materials and Methods:* In this experimental study, 70 mice in ten groups were evaluated from July to September 2017 in the microbiology laboratory of the School of Medicine, Alborz University of Medical Sciences, Karaj, Iran. After induction of *H. pylori* infection in mice with the standard strain of *H. pylori* (ATCC 43504), the infected mice were treated with drug and *Lactobacillus* species in different groups. Then, the anti-*H. pylori* effects of lactobacilli were evaluated by stool antigen test and tissue staining. *Results:* Based on ELISA results and histological findings, a reduction of inflammation was observed. The group which was only exposed to *L. rhamnosus* and the one which was exposed to all three strains of *Lactobacillus* showed the highest antimicrobial effect on *H. pylori*. *Conclusion:* According to the results of this study, probiotic bacteria including *L. acidophilus, L. plantarum*, and *L. rhamnosus* could be useful in the reduction of *H. pylori* infection in the mouse model.

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Introduction

Helicobacter pylori is a gram-negative spiral-shaped or curved-rod, motile and microaerophilic bacterium which is the cause of several acute and chronic digestive tract infections and even malignancies [1–3]. Infection caused by this bacterium is spread throughout the world and is prevalent among all age groups such that the World Health Organization (WHO) classified *H. pylori* as a group 1 carcinogen with the ability to cause gastric cancer [4]. The prevalence of *H. pylori* differs in various countries and even in separate populations of a country and is related to financial and social conditions of the infected population and also to their lifestyle and type of nutrition [5, 6]. In many developing countries, *H. pylori* is widespread in 80–90% of the adult population, such that eradication of *H. pylori* infection is considered to be a public health priority in developing and also developed countries [1, 7]. Many virulence factors such as the shape of spiral bacteria, adhesion factors, and urease enzyme are thought to be involved in ailments caused by this bacterium; consequently, it is of the utmost importance to timely diagnose and get rid of this bacterium, especially

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during the onset of malignancy and before stomach tissue becomes cancerous [8–10]. Nowadays, various treatment regimens containing many antimicrobial agents are proposed to treat *H. pylori*, among which triplet therapy (including a bismuth or proton pump inhibitor and two antibiotics that are usually metronidazole, amoxicillin, tetracycline, or clarithromycin) is currently being used [11]. Most of the studies which have been conducted to treat gastritis and *H. pylori* infection up to now relied on antibiotic treatments and these studies indicate that by using triplet therapy, 85–90% of drug-sensitive bacteria could be eliminated in a span of 2 weeks [12, 13]. It must be noted that in addition to the drug resistance, there are many flaws and deficiencies in triplet and quadruplet treatments. Given the deficiencies of these treatments and the role of *H. pylori* in the development of peptic ulcer, today, the role of probiotics in controlling *H. pylori* colonization in the stomach has drawn the attention of many leading scientists [14, 15]. Based on conducted experiments, probiotics which have antimicrobial effects due to their mineral acids and bacteriocins could hinder the proliferation of *H. pylori*. One of the most important probiotic bacteria groups is *Lactobacillus* which has the ability to affect the healing process of diseases caused by *H. pylori* [16, 17]. Regarding the antibacterial property of probiotics, the aim of the present study is to determine the anti-*H. pylori* effects of *Lactobacillus rhamnosus*, *L. acidophilus*, and *L. plantarum* strains in the stomach tissue of C57BL/6 mice.

Materials and Methods

Bacterial Strain and Culture Condition
H. pylori ATCC43504 were purchased from England and were transferred to the microbiological laboratory of Alborz University of Medical Sciences (Karaj, Iran). They were then cultured on Brucella agar with 7.5% of sheep blood, 10% of fetal bovine serum, and containing antibiotics (amphotericin B at 2 mg/L, polymyxin B at 0.25 mg/L, and vancomycin at 10 mg/L). The cultures were incubated under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2) with a type C gas pack at 37 °C for 5–7 days. Then, all the clear and spherical colonies were identified using Gram staining, catalase, oxidase, and rapid urease test. Standard *L. acidophilus* DSM (20079) and *L. plantarum* DSM (20174) strains were obtained from the Pasteur Institute (Tehran, Iran) and *L. rhamnosus* strain was isolated from organic honey in Mazandaran province (North of Iran) mountains. In the next step, bacteria were cultured on Man-Rogosa-Sharpe (MRS) agar and were incubated at 37 °C for 48 h under microaerophilic conditions using a type C gas pack. Microbial suspensions were produced as 10^9 CFU/mL doses from cultured bacteria using PBS solution.

Animal Preparation
Seventy male C57BL/6 mice (pathogen-free, 6–8 weeks old), weighing about 20–40 g at the beginning of evaluation, were purchased from the Razi institute of Karaj (Iran). All the animals were housed at the animal facility of Alborz University of Medical Sciences under standard conditions (food and water ad libitum at room temperature, 21 ± 3 °C, 12:12-h light/dark cycle, and 55% humidity).

Induction of H. pylori *Infection in C57BL/6 Mice*
All the animals were randomly divided into ten experimental groups. Nine groups were considered as treatment groups and one group as a control group (7 mice in each group) (Table 1). Suspension containing *H. pylori* (5×10^{10} CFU/mL) and PBS (1 mL) was made and was inoculated into the stomach using polyethylene tubes once daily for 1 month. Each animal received 1 mL of the prepared suspension.

Inoculation of Drugs and Lactobacilli to Animals
In all of the 9 treated groups, bismuth and omeprazole were inoculated during the first week after *H. pylori* inoculation. Then, in the second week, the mice were treated by gavage with the following: *L. acidophilus* (group 1), *L. plantarum* (group 2), *L. rhamnosus* (group 3), *L. acidophilus* and *L. plantarum* (group 4), *L. acidophilus* and *L. rhamnosus* (group 5), *L. rhamnosus* and *L. plantarum* (group 6), all three *Lactobacillus* strains (group 7), and clarithromycin (group 8). In group 9, only bismuth and omeprazole were inoculated.

H. pylori *Stool Antigen Test*
H. pylori stool antigen test (RIA test kit) was performed on mice in the control and infected groups 4, 8, and 12 weeks after infection with *H. pylori*. At 4 weeks after the last inoculation, 30 mg of animal stool were collected in extraction tubes and 1 mL of extraction solution was added to the tubes containing stool. For stool antigen identification, some of the animals in the control and infected groups were sacrificed, the gut section was cut open, and a few biopsies were performed on the body and pylorus of the stomach for histological analysis. ELISA was performed and the results were analyzed. Also, in weeks 8 and 12 one animal was sacrificed in both the control and infected groups and histological analysis was performed. ELISA results were evaluated based on the measured standards, and the interpretation of positive and negative results was done based on titration (titration <0.05 was considered as negative and >0.05 as positive results).

*Assessment of the Presence of* H. pylori *after Infection with* H. pylori *by PCR Method*
In this study, after extraction of DNA using a DNA extraction kit (QIAamp; Cat. No. 51304), *16SrRNA* and *ureC* genes of *H. pylori* were amplified by using specific primers (Table 2) under specific thermal conditions as follows: initial denaturation at 95 °C (3 min), second denaturation at 93 °C (45 s), primer annealing at 56 °C (30 s), extension at 72 °C (45 s), and final DNA extension at 95 °C (5 min), during 35 cycles.

Assessment of H. pylori *Infection and Histopathology Staining of Gastric Tissue Samples*
In order to evaluate *H. pylori* infection in the mice, histopathological examination was conducted by a pathologist. One mouse from each group was randomly selected and sacrificed 12 weeks after inoculation. After obtaining longitudinal cross-sections of the animals' stomachs, samples were fixed in 10% formalin and embedded in paraffin. Tissue samples were sent to the laboratory for pathological analysis. Tissues were stained using hematoxylin and eosin and Giemsa staining, and alterations in epithelial cells of the stomach were investigated in the control and infected groups following induction of infection and inflammation (Fig. 1).

Statistical Analysis
All data were analyzed with the Kruskal-Wallis test using SPSS version 21. $p < 0.05$ was considered statistically significant.
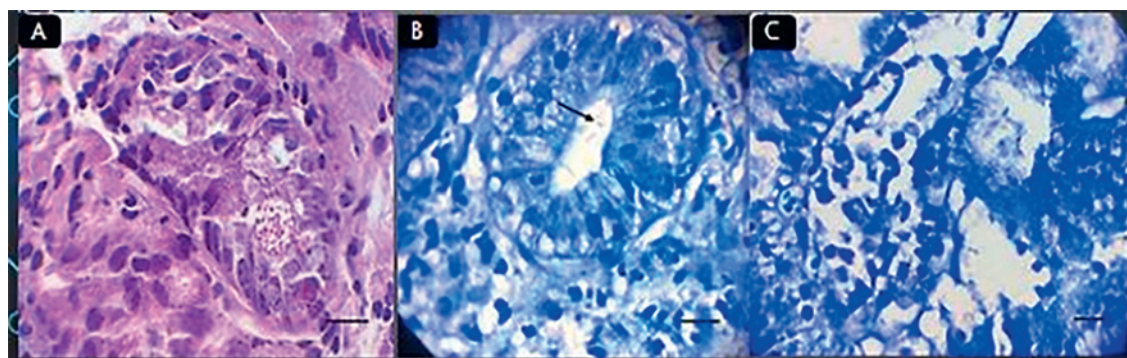
Fig. 1. A Stomach epithelium in control group stained with hematoxylin and eosin. Original magnification, ×40. **B** Stomach epithelium after infection in 8th week stained using Giemsa stain. Arrow points to bacterium. Original magnification, ×40. **C** Stomach epithelium after infection in first week stained with Giemsa stain. Original magnification, ×40.

Table 1. Drugs and *Lactobacillus* compounds used in different animal groups

Animal groups	Drug and microbial compounds
Control group	None
Treatment groups	
First	Bismuth + omeprazole during the first week + *L. acidophilus* during the second week
Second	Bismuth + omeprazole during the first week + *L. plantarum* during the second week
Third	Bismuth + omeprazole during the first week + *L. rhamnosus* during the second week
Fourth	Bismuth + omeprazole during the first week + *L. acidophilus* and *L. plantarum* during the second week
Fifth	Bismuth + omeprazole during the first week + *L. acidophilus* and *L. rhamnosus* during the second week
Sixth	Bismuth + omeprazole during the first week + *L. rhamnosus* and *L. plantarum* during the second week
Seventh	Bismuth + omeprazole during the first week + all three *Lactobacillus* strains during the second week
Eighth	Bismuth + omeprazole during the first week + clarithromycin during the second week
Ninth	Bismuth + omeprazole during the first week

**Table 2.** Specific primers for amplification of *16SrRNA* and *ureC* genes

Primers		Sequences	Size of PCR product (bp)	Ref. No.
16SrRNA	forward	ATAGACGGGGACCCGCACAAG	120	32
	reverse	TGGCAAGCCAGACACTCCA		
ureC	forward	AAGCTTTTAGGGGTGTTAGGGGTTT	294	33
	reverse	AAGCTTACTTTCTAACACTAACGC		

Results

Two weeks after exposure of mouse stomachs to drugs and bacteria, stool samples were obtained; the stool antigen test was performed and the results were compared to those collected before the exposure. The results were confirmed using PCR. The final results indicated that *H. pylori* infection improved after being exposed to the aforementioned compounds in the treatment groups and control group (Table 3). In the first treatment group, after exposing the cells to *L. acidophilus*, followed by Giemsa tissue staining (Fig. 2), irregular cell dressing, epithelial mucus maturation, and inflammation under the mucosa was observed under the microscope. According to the re-
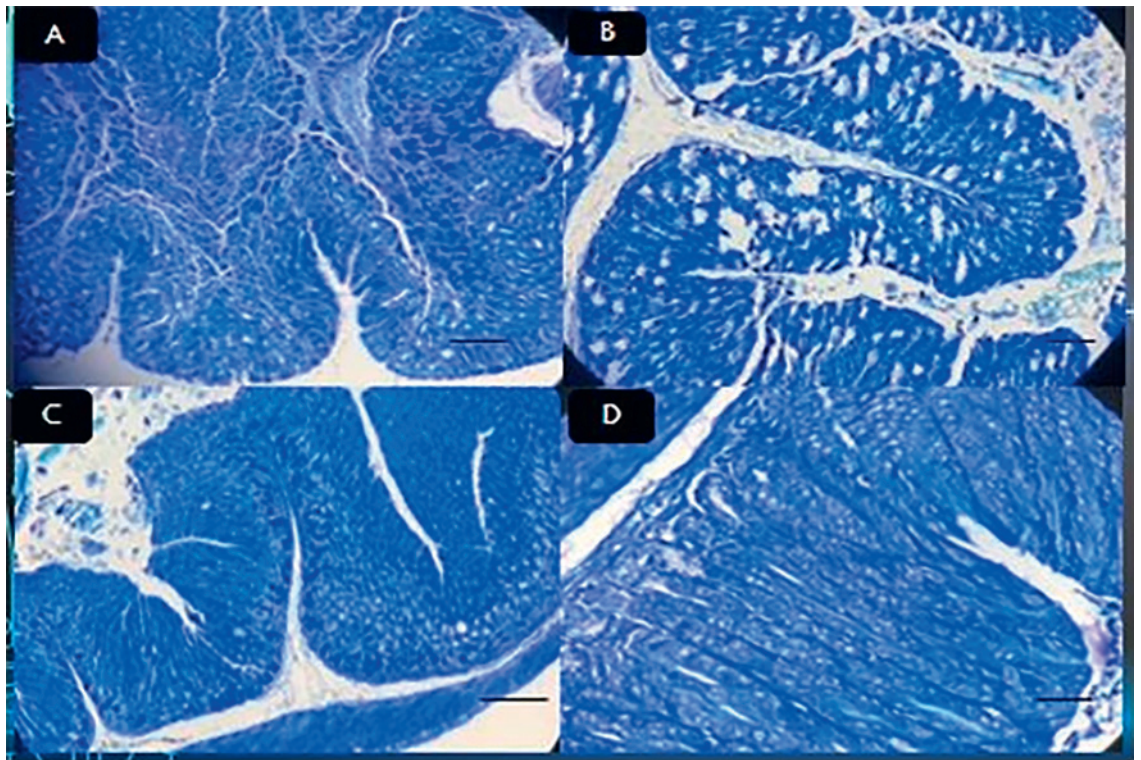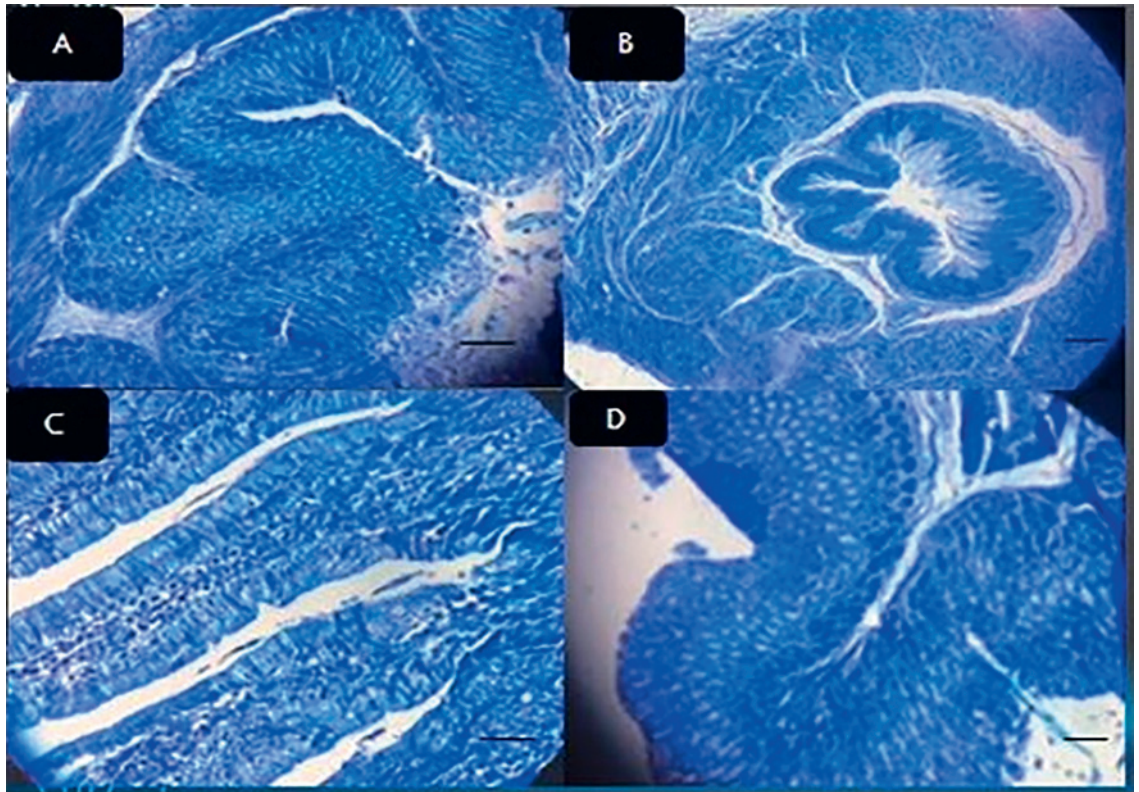
Fig. 2. A Stomach epithelium of mice after treatment with *L. rhamnosus* stained with Giemsa stain. Original magnification, ×40. **B** Stomach epithelium of mice after treatment with *L. acidophilus* stained with Giemsa stain. Original magnification, ×40. **C** Stomach epithelium of mice after treatment with *L. plantarum* stained with Giemsa stain. Original magnification, ×40. **D** Stomach epithelium of mice after treatment with all three lactobacilli stained with Giemsa stain. Original magnification, ×40.

Table 3. ELISA titration results (μg/mL) of mice in the nine treatment groups and the control group

	Mouse No. 1	Mouse No. 2	Mouse No. 3	Mouse No. 4	Mouse No. 5	Mouse No. 6	Mouse No. 7
After infection (control group)	0.225	0.230	0.525	0.852	1.101	1.011	1.012
After treatment with *L. acidophilus*	1.002	0.950	1.005	0.083	0.302	0.903	1.005
After treatment with *L. plantarum*	0.042	0.035l	0.048	0.040	0.032	0.00l	0.00
After treatment with *L. rhamnosus*	0.006	0.002	0.001	0.005	0.002	0.00	0.00
After treatment with *L. acidophilus* and *L. plantarum*	0.051	0.062	0.031	0.022	0.055	0.00	0.052
After treatment with *L. acidophilus* and *L. rhamnosus*	0.042	0.041	0.035	0.028	0.025	0.041	0.015
After treatment with *L. plantarum* and *L. rhamnosus*	0.028	0.015	0.018	0.005	0.019	0.00	0.00
After treatment with clarithromycin	0.025	0.052	0.016	0.059	0.010	0.012	0.030
After treatment with all three lactobacilli	0.008	0.005	0.00	0.014	0.008	0.006	0.00
After treatment with bismuth + omeprazole	0.800	0.078	0.856	1.020	1.015	1.013	0.090

sults of the tissue study in this group, the therapeutic group had the lowest anti-*H. pylori* effect on the elimination of *H. pylori* in gastritis (Table 1). The results of the PCR in this group indicated that the applied treatment method was not able to remove bacteria. In the second group, after being exposed to *L. plantarum*, the cells had a more regular appearance (Fig. 2). Also, the epithelial cells were decreased compared to the first group and the percentage of recovery was close to the third treatment group (Table 1). In the third treatment group, before the treatment and during the infection, cellular edema, swelling of the cell, cellular loosening, and chromatin accumu-

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Fig. 3. A stomach epithelium of mice after treatment with *L. plantarum* + *L. acidophilus* stained with Giemsa stain. Original magnification, ×40. **B** Stomach epithelium of mice after treatment with *L. rhamnosus* + *L. plantarum* stained with Giemsa stain. Original magnification, ×40. **C** Stomach epithelium of mice after treatment with *L. rhamnosus* + *L. acidophilus* stained with Giemsa stain. Original magnification, ×40. **D** Stomach epithelium of mice after treatment with clarithromycin (control group) stained with Giemsa stain. Original magnification, ×40.

lation as well as the presence of bacteria in the tissue was observed. The results indicated that the treatment was more effective in this group than in the other treatment groups, and the results of the ELISA represented a complete anti-*H. pylori* effect on gastritis caused by *H. pylori* compared with other groups (Table 1). Also, the confirmation of anti-*H. pylori* effect in the stomach tissue of the mouse was investigated by PCR method. In the fourth treatment group, after *L. acidophilus* and *L. plantarum* exposure, edema, swelling, cellular loosening, and chromatin accumulation in epithelial cells were observed and a slight improvement was achieved (Table 1; Fig. 3). A moderate recovery was achieved in the fifth treatment group. In the sixth treatment group, which was treated with *L. rhamnosus* and *L. plantarum*, there was a better recovery than in the fourth and fifth treatment groups (Table 1). Treatment with all three lactobacilli, which was performed in the seventh group, was shown to be the most effective treatment, right after the treatment group which was exposed to *L. rhamnosus* (Table 1; Fig. 2). Exposure to bismuth and omeprazole without any probiotics also had a moderate and lower recovery rate than the Lactobacillus groups (Table 1). In summary, all of the

groups which were treated with *L. rhamnosus* had the highest rate of *H. pylori* eradication and subsequent peptic ulcer recovery.

Discussion

The high rate of *H. pylori* resistance to antibiotics which are commonly used in treatment is a great challenge for treating and overcoming infections caused by this microorganism. Most studies which have been conducted concerning gastritis and *H. pylori* infections have used a drug combination consisting of proton pump inhibitors or bismuth and antibiotics, including metronidazole, amoxicillin, tetracycline, and clarithromycin [18]. In order to achieve effective results, the medications should be administered for 7–14 days. The results of these studies indicate that most often there is an 85–90% chance of eliminating this bacterium by triplet therapy (if the bacterium is sensitive to antibiotics), but the efficacy of these regimens decreases if the bacterium develops antibiotic resistance [19]. No doubt, as with many other combination therapies, there are numerous side effects with

H. pylori treatment. Due to the treatment complications and the increased antibiotic resistance, there is a great tendency to develop new alternative therapies. To limit side effects, using the probiotics in *H. pylori* management is taken into consideration. Although a large number of studies focused on treatment and eradication of *H. pylori* in gastric diseases, the effect of probiotic strains including *Lactobacillus* received far less attention. Considering the global requirement for the treatment of gastrointestinal diseases, particularly gastritis, caused by *H. pylori*, our goal was to investigate the antimicrobial effects of *L. plantarum*, *L. acidophilus*, and *L. rhamnosus* along with bismuth and omeprazole on gastritis caused by *H. pylori* infection in C57BL/6 mice. According to our results, *Lactobacillus* compounds were able to improve the gastritis process and decrease *H. pylori* infection. The group which received *L. rhamnosus* had the highest rate of reduction of *H. pylori*-induced inflammation. In addition, complete recovery of gastritis was observed. After that, the group which received all three *Lactobacillus* strains showed the highest anti-inflammatory effect, and the group which received *L. acidophilus* had the weakest outcome in microbial amelioration. Other groups exhibited an average anti-*H. pylori* effect and did not achieve complete recovery. Nevertheless, in all the tested groups, some improvements were observed in intestinal epithelial cells. Various studies have investigated the effect of probiotics in gastrointestinal infections [20–23] and most of them confirm the results that we have obtained regarding the effect of probiotics in gastrointestinal diseases, namely, gastritis. Sgouras et al. [24] (2004), investigated the potential antimicrobial effect of *L. casei* in C57BL/6 mice infected with *H. pylori* which was collected from patients suffering from gastritis and peptic ulcer. The group which received *L. casei* had a much higher *H. pylori* eradication rate. Sgouras et al. [25] (2005) also explored the antimicrobial and anti-inflammatory effect of *L. johnsonii* on gastritis in C57BL/6 mice. Their result indicated that in the primary stages of infection, *L. johnsonii* was able to mitigate gastritis induced by *H. pylori* by lowering chemical signals produced by inflammatory proteins which mobilize lymphocytes and neutrophils in lamina propria. Another study conducted by Cui et al. [26] (2010) also demonstrated that *Lactobacillus* strains could generate a marked reduction in *H. pylori* colonization in BALB/c mice. According to their results, mice which were infected with *H. pylori* and exposed to *Lactobacillus* strains showed a significant reduction in *H. pylori* compared to the control group that received saline. In addition, Sunanliganon et al. [16] (2012) investigated the antimicrobial effect of *L. plantarum* in mice that suffered from *H. pylori*-induced gastritis, and concluded that *L. plantarum* hinders the growth of *H. pylori*. Many other experiments have been conducted concerning antimicrobial and anti-inflammatory effects of *Lactobacillus* in infections caused by *H. pylori* [27, 28]. In some of these studies, it has been pointed out that *Lactobacillus* produces anti-inflammatory effects through balancing inflammatory and anti-inflammatory cytokines, increasing expression of interleukin 10, and reducing TNFα [29–31]. These results validate our findings in the present study.

Conclusion

Based on the findings of the current study, significant anti-*H. pylori* effects of *L. acidophilus*, *L. plantarum*, and *L. rhamnosus* can be had by introducing them as a supplement against *H. pylori* infection. Using these *Lactobacillus* species as pharmaceutical supplements along with proton pump inhibitors may have more beneficial effects on improvement of gastritis induced by *H. pylori*. In addition, the *Lactobacillus* species which have been used in our study may be able to reduce resistance to antibiotics that are commonly used in the treatment of *H. pylori*-induced gastritis, and therefore could have a tremendous role in treating gastritis in humans in the future.

Statement of Ethics

The experimental protocol was approved by the Animal Care Committee of Alborz University of Medical Sciences, Karaj, Iran.

Disclosure Statement

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