

Two stomach-originated *Lactobacillus* strains improve *Helicobacter pylori* infected murine gastritis

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Abstract

AIM: To investigate the potential anti-*Helicobacter pylori* (*H. pylori*) and anti-inflammation *in vivo* effects of two *Lactobacillus* strains from human stomach.

METHODS: Forty *H. pylori* infected Balb/c mice were randomly divided into 4 groups: proton pump inhibitor and antibiotics triple treated group, *Lactobacillus fermenti* (*L. fermenti*) treated group, *Lactobacillus acidophilus* treated group and normal saline control group. Ten uninfected mice were also included as blank control group. The infection of *H. pylori* was detected by rapid urease tests, Giemsa staining and bacterial culture. The colonization of *H. pylori* was assessed in bacterial density score and gastric inflammation was assessed in histological score. The colonization of *L. fermenti* was performed by fluorescent probe.

RESULTS: Histopathologic evaluation showed significant release of mucosal inflammation in gastric antrum and gastric body in *Lactobacillus* treated groups and triple treated group. *H. pylori* eradication rate in both *Lactobacillus* treated groups and triple treated group were higher than normal saline control group. *Lactobacillus* treated groups and triple treated group showed significant decrease of *H. pylori* bacterial density.

CONCLUSION: Both *Lactobacillus* strains have a significant anti-*H. pylori* activity; *L. fermenti* displays more efficient antagonistic activity *in vivo* against *H. pylori* infection.

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Key words: *Helicobacter pylori*; *Lactobacillus fermenti*; Murine gastritis; *Lactobacillus acidophilus*; Therapy

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a spiral-shaped, Gram-negative microaerophilic stomach rod that infects over 50% of the population around the world^[1-3], which is considered to be the most important etiological agent of chronic gastritis, peptic ulcer, gastric cancer and mucosa-associated lymphoid tissue^[1,4,5]. So far, the eradication of

H. pylori depends on the combination of antibiotics and acid suppression drugs, with an efficiency of 80%-90%. Unexpectedly, symptoms of 10%-20% patients remain unimproved or reinfected due to the incomplete eradication or antibiotics resistance^[5,6]. Moreover, the side effect of using multi-antibiotics is obvious, such as antibiotic associated diarrhea, enteric dysbacteriosis, pseudo-membranous colitis, and so on, which lower the curative effect and treatment compliance. Thus attention has been drawn to seek for any alternative method which can eradicate or inhibit *H. pylori* infection efficiently without antibiotics associated side effects.

As mentioned above, over 50% of the population are infected by *H. pylori*, while only a few of them suffer from *H. pylori* associated disease. Some published data showed that when the density of *H. pylori* in gastric antrum drops to less than 10^5 , it cannot lead to the formation of gastric and duodenum ulcer^[7,8]. It is believed that the severity and activity of gastritis is related to the number of bacteria in stomach.

Recently, some reports have suggested that exogenous *lactobacilli* have some inhibitory effects on *H. pylori* infection^[9,10], and the colonization of *H. pylori* has a close relationship with the number of *lactobacilli*. It is also reported that *H. pylori* colonization can be inhibited in *lactobacillus* strain *L. salivarius*-fed mice^[11,12]. Some studies showed that probiotics isolated from dairy products or human feces have suppressive effects on *H. pylori* infection^[9,13]. Similar results from some clinical trials also support the inhibitory effects of some microbial ecological agents on *H. pylori* associated diseases^[14,15].

However, results of clinical trials from some separate groups did not make a definite conclusion. Some studies showed that it is effective to prevent and treat *H. pylori* infected diseases by administering *lactobacillus* or fermented dairy products^[13,16,17]. Some believe that probiotics have no effect in treating *H. pylori* infected diseases because they are live microbes, and their living environment may change depending on what kinds of food the host takes^[18]. The possibilities include inappropriate methods to assess the anti-*H. pylori* activity, different strains of probiotics and different origins of strains^[12,19-21]. Therefore, more detailed studies on more stomach originated probiotic strains will help draw a more definite conclusion.

In the present study, we isolated two stomach originated *lactobacillus* strains, and screened their potential anti-*H. pylori* activity and anti-inflammatory effects on mouse model of *H. pylori*-associated Balb/c gastritis. We found that *lactobacillus* strain *Lactobacillus fermenti* (*L. fermenti*) could adhere to gastric epithelium and displayed antagonistic activity *in vivo* against *H. pylori* infection, thus significantly improving the *H. pylori*-associated Balb/c gastritis in mice.

MATERIALS AND METHODS

Bacterial stains and culture conditions

H. pylori standard strain SS1 (Sydney strain 1) was kindly

provided by the Infectious Disease Institute of the Chinese Center for Disease Control. The two *lactobacillus* strains *L. fermenti* (CCTCC M 206110) and *Lactobacillus acidophilus* (*L. acidophilus*) (strain LC) were isolated by us from gastric biopsy materials of patients who received endoscopic examination in the Gastroenterology Department of the Second Xiangya Hospital, Hunan, China.

H. pylori was resuscitated and inoculated into brain heart infusion broth containing 10% sheep blood, incubated under microaerophilic conditions (5% O₂, 85% N₂, 10% CO₂) at 37°C for 3-5 d. Both *lactobacillus* strains were cultured in Man-Rogosa-Sharpe (MRS) broth, and incubated in anaerobic box at 37°C for 48 h. The concentration of bacteria was regulated to 1×10^9 CFU/mL by turbidimetry.

H. pylori infection of Balb/c mice

Sixty specific-pathogen-free 6-8-wk-old Balb/c mice (a male to female ratio of 1:1) were obtained from the Central Animal Facility of Wuhan. They were housed according to relevant Chinese national legislation, fed a commercial diet, and were given water and libitum, except as otherwise stated. *H. pylori* infections by the SS1 strain were induced as follows: freshly prepared aliquots (0.4 mL of 1×10^9 CFU/mL) of *H. pylori* SS1 strain in brain heart infusion broth were administered to 50 mice *via* orogastric inoculation 3 times a week (days 1, 3 and 5) at a 1-d interval between inoculations for 2 wk. Accordingly, the other 10 non-infected control animals were inoculated with the same volume of plain brain heart infusion broth. At the end of the 1st and 2nd wk, 5 animals receiving *H. pylori* strain SS1 were sacrificed by carbon dioxide inhalation, and tests were made to confirm whether they were infected with SS1 strain successfully.

The following groups of animals were included in the study: *H. pylori*-infected mice treated by *L. fermenti* (*L. fermenti* group, $n = 10$), *H. pylori*-infected mice treated by *L. acidophilus* (*L. acidophilus* group, $n = 10$), *H. pylori*-infected mice treated by proton pump inhibitor (PPI) and antibiotics (triple group, $n = 10$). The control groups were *H. pylori*-infected mice treated by normal saline solution (NS group, $n = 10$). The non-infected mice (blank control group, $n = 10$) were also included. The quantity of *lactobacillus* was regulated to 0.5 mL of 1×10^9 CFU/mL per mouse for *L. fermenti* and *L. acidophilus* groups, and PPI and antibiotics were 0.25 mL of 0.4 mg/mL pantoprazole (Zhongmei East China Pharmaceutical Group Corporation in Hangzhou, 060923), 0.25 mL of 20 mg/mL clarithromycin (HuiRen Pharmaceutical Group Corporation in Jiangxi, 0510019), and 0.1 mL of 50 mg/mL ampicillin (North China Pharmaceutical Group Corporation, 060307) per mouse for the triple group. The treatment lasted 10 d, and all animals were sacrificed by carbon dioxide inhalation 4 wk later.

All the methods for the assessment of *H. pylori* colonization and identification in gastric samples as well as evaluation of gastritis will be described in detail below.

Assessment of *H. pylori* colonization and histopathologic analysis of gastric tissue samples

The stomach of each mouse was removed and dissected longitudinally, the antrum and body were divided and treated separately. One-third of each longitudinal strip was fixed in 10% formalin-buffered saline, embedded in paraffin, and processed for histopathologic analysis. The other two strips were used for rapid urease test and *H. pylori* culture, respectively. Four sections were cut from each paraffin block specimen. Two sections of them were stained with hematoxylin-eosin (HE) for evaluation of gastric inflammation, the other 2 sections were used for Giemsa staining for the assessment of *H. pylori* colonization. The bacterial density was scored from 0 to 4 as follows: Score 0, no bacteria; score 1, 1-2 bacteria on average/crypt; score 2, 3-10 bacteria on average/crypt; score 3, 11-20 bacteria on average/crypt; and score 4, > 20 bacteria on average/crypt^[22]. The pathologic characteristics were graded for the degree of neutrophil and mononuclear cell infiltration in the antrum and body as follows: Score 1, mildly multifocal; score 2, mildly widespread or moderately multifocal; score 3, mildly widespread and moderately multifocal or severely multifocal; score 4, moderately widespread; score 5, moderately widespread and severely multifocal; and score 6, severely widespread^[8]. Histopathologic evaluation was performed with no prior knowledge of the identity of the samples by the histopathologist.

Determination of urease activity

Urease activity was determined by a method based on the commercial rapid urease test (Sanqiang Biochemical Industry Corporation in Fujian, China) with a sensitivity of 10^2 bacteria. Following the instruction of the product, each strip of stomach antrum and body was homogenized and placed in 1 mL of the reaction solution [1 g of urea/mL (wt/vol) containing 850 µg phenol red/mL (wt/vol) as a pH indicator]. The solution became pink or red or dark red within 5 min as positive result, still yellow as negative.

H. pylori culture

Each sample of stomach antrum and body was homogenized and suspended in 500 µL brain heart infusion broth containing 10% sheep blood, and 100 µL was inoculated onto brain heart infusion agar plates and cultured under microaerophilic conditions at 37°C for 5-7 d. Gram's staining and urease activity were detected.

Standard of *H. pylori* infection

When at least two of the three tests of rapid urease test, *H. pylori* culture and Giemsa staining for the assessment of *H. pylori* colonization, appeared positive, *H. pylori* infection could be diagnosed.

Standard of *H. pylori* eradication

When the three tests of rapid urease test, *H. pylori* culture

and Giemsa staining of gastric mucosa samples were all negative, the eradication was confirmed to be successful.

Labeling of bacteria with fluorescent probe

A 100 µmol/L stock solution of fluorescence-labeled molecular probe (cFDA-SE, Invitrogen Corporation) was prepared, which was first dissolved in 90 µL dimethyl sulfoxide and then further diluted in ethanol (1 mL; reagent grade). This solution was then filter sterilized (0.2-µm-pore-size Acrodisc filter; Gelman) before being aliquoted and stored at -20°C.

L. fermenti was grown overnight at 37°C in MRS broth. The bacterial culture was centrifuged at 3000 r/min for 10 min, and the pellet was washed twice in sterile phosphate-buffered saline (PBS). The concentration of bacteria was regulated to 1×10^{10} CFU/mL prior to labeling with 50 µm cFDA-SE at 37°C for 20 min. Fluorescent labeling was terminated by pelleting the bacteria, washing twice in PBS to remove excessive cFDA-SE, and resuspending the pellet in PBS.

Animals

A group of 30 mice were administered orally with approximately 10^9 cFDA-SE-labeled *L. fermenti* by orogastric intubation. The other group of 6 mice that had been orally fed with sterile PBS served as controls. The food and water intake for the experimental and control mice was measured daily. Groups of 6 mice each were sacrificed at 2, 4, 8, 12 and 24 h after cFDA-SE. The stomach and duodenum of each mouse was divided and dissected longitudinally, any visible residual food particles were removed. Both specimens were examined for the adherence of cFDA-SE-labeled *L. fermenti*. PBS 150 µL was added to each 1.0 cm of the tissue and microbes from the mucosal surface were dislodged by a plunger from a syringe (1.0 mL), then fixed with formaldehyde (0.75%, vol/vol) prior to the detection under fluorescence microscope (Nikon E80, Japan).

Fluorescent microscopic detection

Enumeration of cFDA-SE-labeled *L. fermenti* was conducted under a fluorescent microscope (Nikon E80, Japan) at a 488-nm excitation wavelength. Upon excitation at 488 nm under the fluorescent microscope, cFDA-SE gave a maximal emission signal in the green at 518 nm.

Statistical analysis

Statistical analyses were performed using SPSS for Windows, version 13.0. Results were expressed as measurement data and enumeration data. For statistical comparisons, *t* test and χ^2 test were performed and $P < 0.05$ was considered statistically significant.

RESULTS

Evaluation of mucosal inflammation in stomach

Histopathologic evaluation revealed significant improvement of mucosal inflammation in gastric antrum and

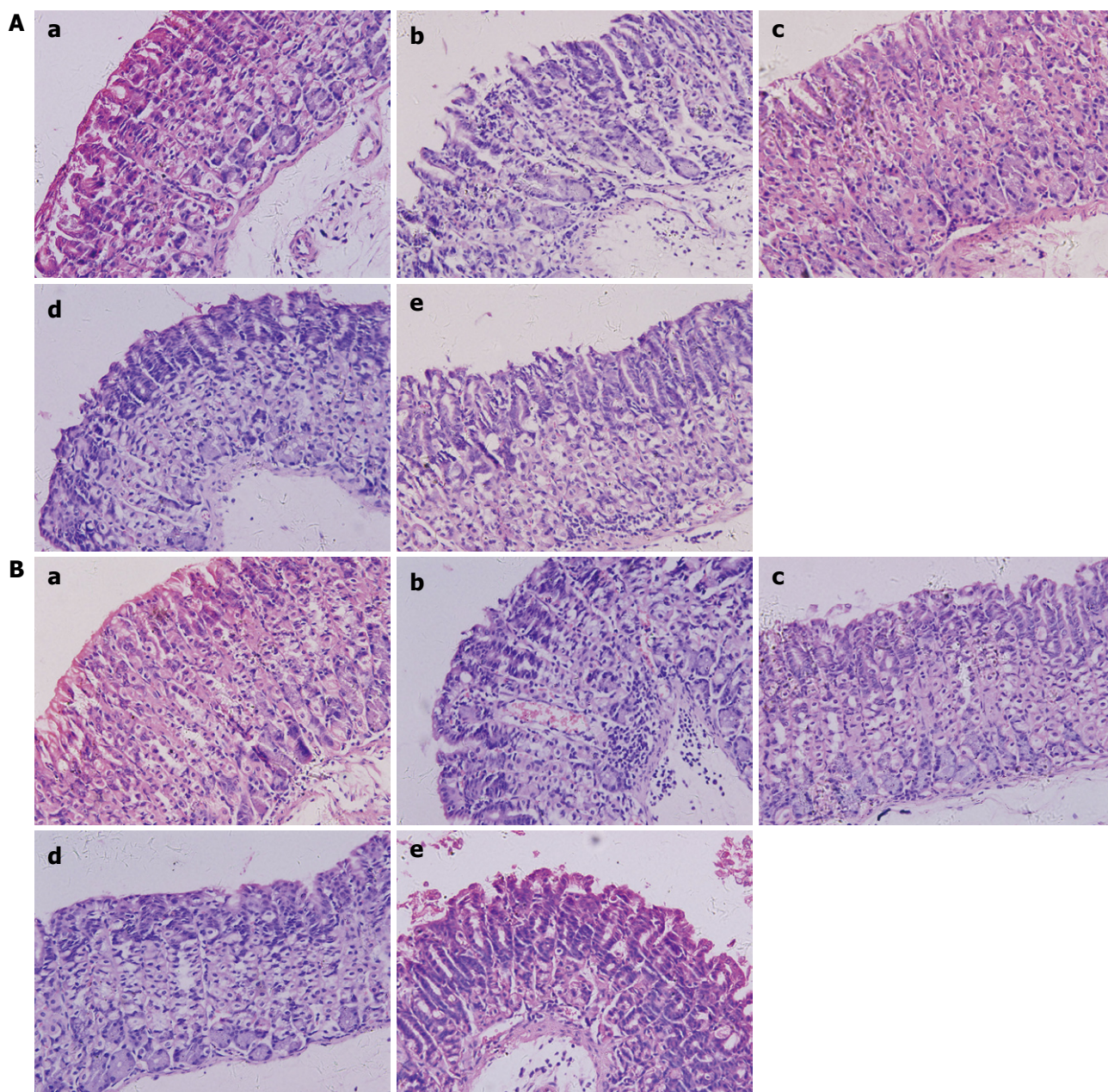


Figure 1 Histological image of gastric antrum and gastric body. A: Histological image of gastric antrum. a: Normal group, normal mucosa, no mucosal erosion; b: NS group, extensive inflammation in the mucosal layer, massive mixed cell infiltration (mainly mononuclear); c: Triple group, slight inflammatory cell infiltration; d: *L. fermenti* group, a few incomplete mucosa; e: *L. acidophilus* group, slight inflammation with moderate cell infiltration, (HE, light microscope, × 200); B: Histological image of gastric body. a: Normal group, normal mucosa, no mucosal erosion; b: NS group, extensive inflammation in mucosal layer, even in submucosal layer with massive mixed cell infiltration; c: Triple group, a few incomplete mucosa, slight inflammatory cell infiltration; d: *L. fermenti* group, almost normal mucosa, slight cell infiltration; e: *L. acidophilus* group, moderate inflammation with cell infiltration in submucosa (HE, light microscope, × 200).

gastric body both in lactobacillus treated as well as triple treated groups. The score of pathologic characteristics of the gastric mucosa also showed obvious decrease. The score of *L. fermenti* treated group was significantly lower than normal saline control group, and almost similar as triple treated group and blank control group. The score of *L. acidophilus* treated group was also lower than normal saline control group and close to triple treated group. The score of *L. acidophilus* group in gastric body was similar to the blank control group, while it was higher in gastric antrum (Figures 1 and 2).

Effects of lactobacillus on *H. pylori* eradication rate

The *H. pylori* infection rate in normal saline control group indicated that the model was made successfully while the rate of *H. pylori* infection in blank control group suggested that there was no pollution in environment and diet. We found a significant increase of *H. pylori* eradication rate in lactobacillus treated groups and triple treated group. The eradication rate of *H. pylori* in *L. fermenti* treated group was higher than the normal saline control group and close to that in PPI triple treated group. The eradication rate of *H. pylori* in *L. acidophilus* treated group was higher than

Table 1 Infection and eradication rate of *Helicobacter pylori* ($n = 10$) (%)

Treatment	Infection rate		Eradication rate	
	Gastric antrum	Gastric body	Gastric antrum	Gastric body
Triple group	30.0 ^a	30.0 ^a	70.0 ^a	70.0 ^a
<i>L. fermenti</i> group	30.0 ^a	40.0 ^a	70.0 ^a	60.0 ^a
<i>L. acidophilus</i> group	50.0	50.0	50.0	50.0
NS group	80.0	80.0	20.0	20.0
Blank control group	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a

^a $P < 0.05$ vs normal saline group.

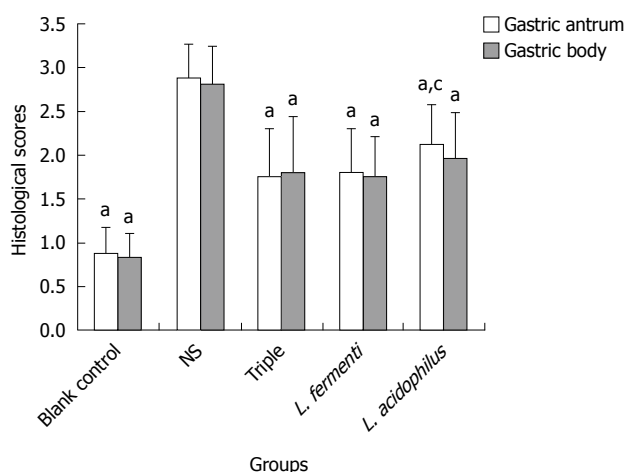


Figure 2 Histological score of all groups (mean ± SD, $n = 10$). ^a $P < 0.05$ vs NS group. The highest score was 2.88 ± 0.38 in gastric antrum and 2.82 ± 0.42 in gastric body in NS group. ^c $P < 0.05$ vs blank control group.

the normal saline control group, but without significant statistical difference (Table 1).

Effects of *Lactobacillus* on *H. pylori* colonization

Lactobacillus treated groups and triple treated group showed significant decrease of *H. pylori* bacterial density. In *L. fermenti* treated group, the bacterial density was lower than the normal saline control group and close to PPI triple treated group. The bacterial density in *L. acidophilus* treated group is lower than the normal saline control group, however it was statistically insignificant (Figure 3).

Detection of *Lactobacillus L. fermenti* colonization

The fluorescence intensity indicated the colonization of *Lactobacillus* at various time points after orogastric intubation. The bacteria were not uniformly labeled by cFDA-SE, probably due to the physiological status of the bacteria at the time of incubation with cFDA-SE (Figure 4).

DISCUSSION

H. pylori is a spiral-shaped, gram-negative organism that commonly causes chronic infections and is usually acquired in childhood^[23]. Patients with complications (e.g.

gastritis, ulcer and malignancy) should have the organism eradicated. So far, *H. pylori* can be typically eradicated using a combination of certain antibacterial agents and antacid treatment, which resulted in dramatic reduction in ulcer recurrence and associated complications. The efficacy of recommended eradication regimens is approximately 80%. In our study, standard *H. pylori* SS1 strain was used to colonize the stomach of Balb/c mouse, with an infection rate of 80.0% in gastric antrum and gastric body. This leads to the development of gastric mucosa inflammation closely mimicking human *H. pylori* associated gastritis. The eradication efficacy of *H. pylori* in our study was around 70.0% based on typical triple therapy, which proved that the mouse model of *H. pylori* infection was made successfully and could be established as an animal model to assess anti-*H. pylori* activities of potential therapeutic agents.

A recent clinical trials demonstrated that the addition of Will yogurt to triple therapy increased the *H. pylori* eradication rate by per-protocol (PP) analysis^[14]. Another clinical study showed that some probiotics, such as *L. johnsonii* LA1, could be used as an adjuvant of antibiotic-antacid treatment to prevent the reemergence of *H. pylori* infection in humans^[19]. It indicated that the addition of probiotics to PPI-based triple therapy could increase the likelihood of successful *H. pylori* eradication.

In our study, histopathologic changes on *H. pylori* SS1 mouse model showed a significant release of mucosal inflammation in both gastric antrum and gastric body in *Lactobacillus* strain *L. fermenti* and *L. acidophilus* treated groups and triple treated group. While the histological score of *L. acidophilus* treated group was higher than *L. fermenti* treated group and triple treated group. It showed that oral administration of *L. fermenti* strain could alleviate the gastric inflammation in *H. pylori*-infected Balb/c mouse model, with less significance in strain *L. acidophilus* treated group.

Accordingly, *Lactobacillus* treated groups and triple treated group showed a significant decrease of *H. pylori* bacterial density, with an eradication rate of *H. pylori* 70.0% in gastric antrum and 60.0% in gastric body in *L. fermenti* treated group, which was obviously higher than the normal saline control group and close to that in the triple treated group. The eradication rate of *H. pylori* in *L. acidophilus* treated group was 50.0% in gastric antrum and gastric body, being higher than the normal saline control group, but without statistical difference. It indicates that different *Lactobacillus* strains have various anti-*H. pylori* effects due to different *H. pylori* density and *H. pylori* eradication rate.

Our data from the SS1 mouse model with reference to *H. pylori* colonization were in agreement with the histopathological changes among different groups. *L. fermenti* can inhibit the colonization of *H. pylori* in gastric mucosa so as to improve its inflammatory injury. In the *L. fermenti* strain group, we observed a significant reduction of *H. pylori* colonization in the gastric mucosa

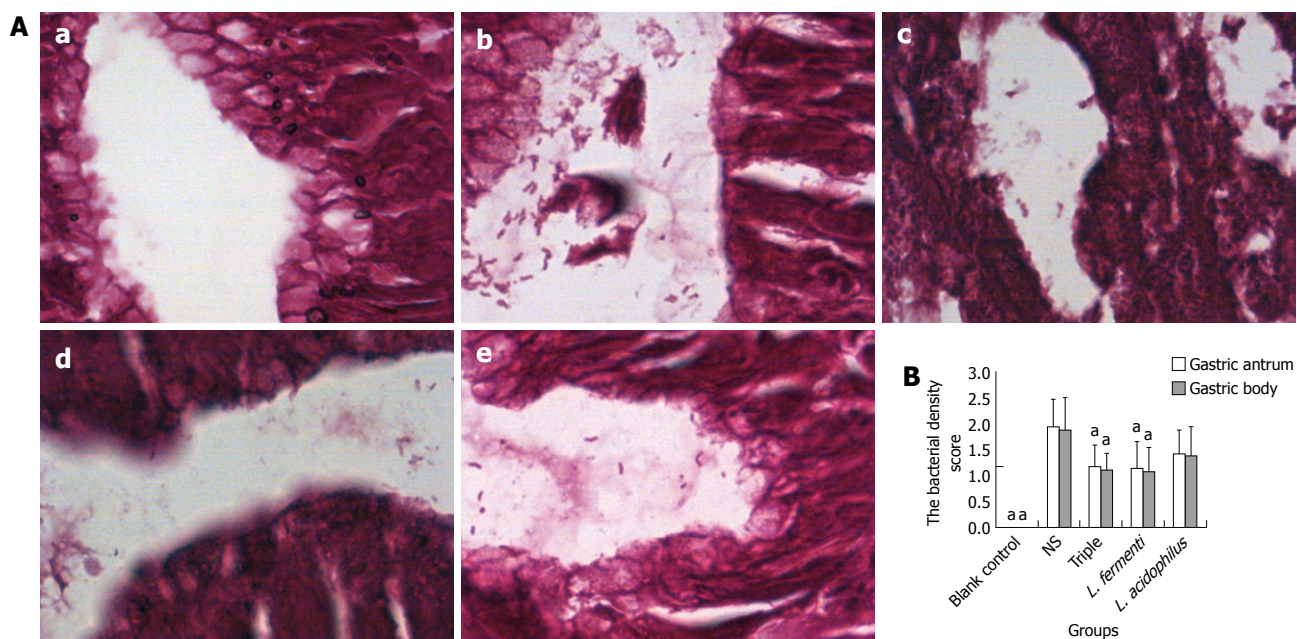


Figure 3 Bacterial density and bacterial density score of *H. pylori*. A: Bacterial density of *H. pylori*. a: Normal group, no detectable *H. pylori*; b: NS group, over 10 *H. pylori* were detected in one field of view; c: Triple group; d: *L. fermenti* group; e: *L. acidophilus* group, (Giemsa, light microscope, × 1000). B: Bacterial density score of *H. pylori*. mean ± SD, n = 10. ^aP < 0.05 vs NS group. The highest score was 1.95 ± 0.52 in gastric antrum and 1.88 ± 0.63 in gastric body in NS group.

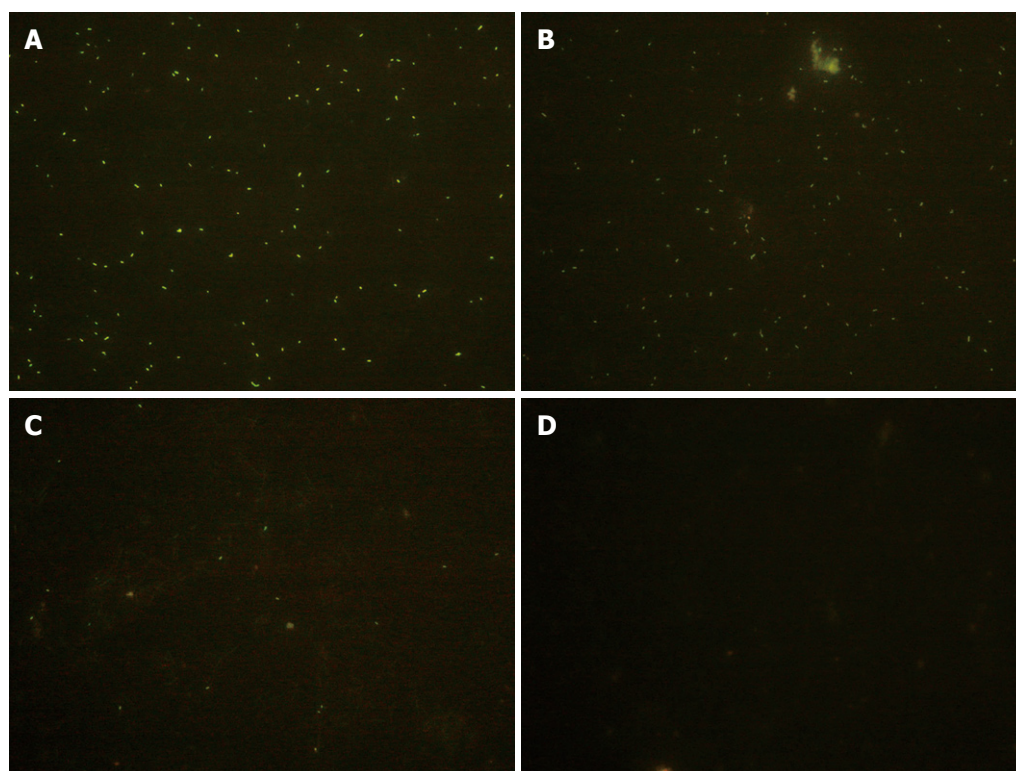


Figure 4 Fluorescent microscopic detection of *L. fermenti* labeled with cFDA-SE. A: 2 h, a diffusely distributed cFDA-SE labeled bacteria under fluorescent microscopy; B: 4 h, slightly reduced in comparison with 2 h; C: 8 h, cFDA-SE labeled bacteria reduced obviously; D: Blank control group, no cFDA-SE labeled bacteria was detected.

throughout the entire observation period. Continuous administration of *L. acidophilus* did not reduce the *H. pylori* colonizing numbers over this experimental period. These indicate that some strains of lactobacillus can

colonize in gastric mucosa and exhibit anti-*H. pylori* colonization. These strains due to their differences in species and specificity can lead to different anti-*H. pylori* activities. Detection of living bacteria under fluorescent

microscope provided evidence of colonization of the stomach and duodenum by *L. fermenti*. The appearance of *L. fermenti* supports the idea that they come from the supplement.

As it is known, lactobacilli are components of the normal intestinal flora of healthy humans which exert antagonistic activities against pathogens^[7,9,10,16]. In particular, it has been reported that the primary microorganisms associated with the stomach belong to the genus *Lactobacillus*. *Lactobacillus* earn particular capacity to survive and develop in an acidic environment and live as an indigenous bacterium in gastric mucosa, which can effectively inhibit the colonization of *H. pylori*^[11,12,15,21]. Our study also adds evidence that *H. pylori* can live together with other bacteria and maintain a dynamic equilibrium state. In another word, it is rational to prevent and control *H. pylori* infection by regulating the balance of flora in stomach. Thus *Lactobacillus* can be a choice to replace antibiotics or as an adjuvant to antibiotics in treating *H. pylori*-infected diseases.

In conclusion, both *Lactobacillus* strain *L. fermenti* and *L. acidophilus*, which are isolated from human gastric mucosa, showed significant anti-*H. pylori* activity, while strain *L. fermenti* displayed more efficient antagonistic activity *in vivo* whose efficacy is close to the standard triple therapy, thus significantly improving the *H. pylori*-associated Balb/c gastritis. It would be of great interest to further explore the role of such probiotic strains in the complex regulation of anti-*H. pylori* activities and screen for more efficient clinical potential agents.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) are considered to be the most important etiological agents of chronic gastritis. The eradication of *H. pylori* depends on the combination of antibiotics and acid suppression drugs. Unfortunately, the side effects of antibiotics reduce the curative effect and treatment compliance. Probiotics provides an alternative method which can inhibit *H. pylori* infection efficiently without antibiotics associated side effects.

Research frontiers

Lactobacilli earn particular capacity to survive and develop in an acidic environment and live as an indigenous bacterium in gastric mucosa, which can effectively inhibit the colonization of *H. pylori*. Different strains display different effects on *H. pylori* infected gastritis.

Innovations and breakthroughs

The study showed that *H. pylori* has some interactions with other microbes in stomach. They live together and maintain a dynamic equilibrium state. Both the strains of *Lactobacillus* isolated from human gastric mucosa showed significant anti-*H. pylori* activity while *Lactobacillus fermenti* (*L. fermenti*) displayed more efficient antagonistic activity *in vivo*. This study also provides evidences of colonization of the stomach and duodenum by *L. fermenti*.

Applications

The study provides a new clue for the therapy of *H. pylori* associated diseases, which could be prevented and treated by regulating the balance of flora in stomach. Thus *Lactobacillus* can be a choice to replace antibiotics or as an adjuvant to antibiotics in treating *H. pylori*-infected diseases.

Peer review

In this paper, the authors investigated the potential effects of anti-*H. pylori* activity and anti-inflammation *in vivo* of two *Lactobacillus* strains from stomach. And they found that both *Lactobacillus* strains showed significant anti-*H. pylori*

activity, and *L. fermenti* displayed more efficient antagonistic activity *in vivo* against *H. pylori* infection. This paper gave us a new point of view on the interaction of gastric microbiology and also provided a new clue for *H. pylori* treatment.

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