Preventive and therapeutic effect of Lactobacillus paracasei ZFM54 on Helicobacter pylori-induced gastritis by ameliorating inflammation and restoring gastric microbiota in mice model

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Helicobacter pylori is the most prevalent pathogen causing chronic gastritis, gastroduodenal ulcers, and gastric tumors and is asymptptomatically present in >50% of the world’s population. This research is focused on investigating the effect of Lactobacillus paracasei ZFM54 (CCTCC NO:2016667) on attenuating H. pylori-induced gastritis. H. pylori ZJC03 isolated from a patient with gastritis harbored the virulence genes of vacA and cagA and was highly resistant to metronidazole (MIC > 256 µg/mL). In vitro analysis revealed that the potential anti-H. pylori characteristics of L. paracasei ZFM54 in terms of 65.57 ± 1.87% survival rate in simulated gastric juice at a pH of 2.0, 69.00 ± 2.73% auto-aggregation, 30.28 ± 2.24% co-aggregation, 70.27 ± 2.23% urease inhibition, and 57.89 ± 1.27% radical scavenging. In H. pylori infected mice, L. paracasei ZFM54 pre- and post-treatment reduced the levels of malondialdehyde in liver tissues to 0.71 ± 0.04 nmol/mgprot (p < 0.05) and 0.70 ± 0.06 nmol/mgprot (p < 0.05), respectively. Glutathione levels were increased to 1.78 ± 0.02 µmol/gprot (p < 0.05) and 1.76 ± 0.52 µmol/gprot (p < 0.05), respectively. L. paracasei ZFM54 significantly inhibited H. pylori-mediated inflammation observed in gastric mucosal repair and downregulated the mRNA expression of pro-inflammatory cytokines IFN-γ, IL-1β, and IL-6 (p < 0.01). Importantly, L. paracasei ZFM54 increased Firmicutes and Actinobacteria and decreased the relative abundance of bacterial taxa belonging to Campylobacterota and Proteobacteria. With the preventive and therapeutic administration of L. paracasei ZFM54, significant reductions in the average relative abundance of genera Helicobacter, Muribaculum, Staphylococcus,
Introduction

_Helicobacter pylori_ is the only bacterium reported to cause cancer and has been grouped as a class I carcinogen by the International Agency for Research on Cancer (IARC). _H. pylori_ infection is the main risk factor for gastric duodenal ulcer, tumor, and lymphoma. Approximately, half of the population on the earth is infected with _H. pylori_ (1). A high prevalence of _H. pylori_ infection was reported in developing countries with approximately 80% prevalence in China and some states of South America and Eastern Europe (2). The major virulence genes of _H. pylori_ are vacuolating cytotoxin A (vacA) and cytotoxin-associated gene A (cagA), which are closely related to pathogenesis (3). VacA exists in almost all _H. pylori_, including the signaling region (s1 and s2 alleles) and the middle region (m1 and m2 alleles), while cagA, regarded as a virulence marker, is positive in 60–70% of _H. pylori_ strains (4). Treatment of _H. pylori_ infection includes two antibiotics and a proton pump inhibitor (PPI), but the emergence of antibiotic resistance in _H. pylori_ presents a great challenge (5). Side effects of antibiotics (nausea, vomiting, and diarrhea) often lead to patients’ non-compliance. Recurrence of infection after treatment is also a threat, especially in high prevalence areas (6). The declining rate of successful treatment due to antibiotic resistance, side effects, and patients’ non-compliance prompts researchers to seek alternative therapy to control the risk of _H. pylori_ infection and associated gastroduodenal ulcers and tumors (7).

_Lactobacillus_ species are the most commonly studied genera to have potential probiotic characteristics and have been extensively studied to control _H. pylori_ infection (8, 9). In Deng et al.’s study, _H. pylori_ could only infect specific pathogen-free mice, while pre-gavage with _L. paracasei_ FJ86111.1 inhibited the colonization of _H. pylori_ in gastric tissue (10). _L. paracasei_ HP7 associated with extracts of _Perilla frutescens_ and _Glycyrrhiza glabra_ inhibited _H. pylori_ with virulence genes such as _alpA_, _cagA_, _fluA_, and _ureA_ and significantly reduced the infection rate of _H. pylori_ in mice (11). _L. paracasei_ strain 06TCa19 was shown to suppress _H. pylori_-mediated inflammation cytokines of IL-8 and regulated the activation of normal T-cell expression and secretion in MKN45 cells (12).

The mechanism of action of _Lactobacillus_ has been investigated, but the exact underlying mechanism is largely unknown. _Lactobacillus_ has been demonstrated to inhibit the urease enzyme activity of _H. pylori_ in _in vitro_, _in vivo_, and clinical trials (9). Inhibition of urease would not allow _H. pylori_ to colonize the gastric mucosa and further protect from _H. pylori_-mediated inflammation. Secretion of antimicrobial substances (H_2_O_2_, lactic acid, bacteriocin, etc.), competition for adhesion receptors, co-aggregation ability, strengthening epithelial barrier, and immunomodulation are the main actions of _Lactobacillus_ against pathogens (13, 14). _H. pylori_ also induced oxidative damage to the host using its multiple antigens such as lipopolysaccharides and the type IV secretory system (15). As a consequence, neutrophils produce free radicals to combat _H. pylori_ and cause the accumulation of different reactive oxygen species (ROS), which subsequently damage the host’s cellular membranes producing malondialdehyde (MDA), which is a hallmark of an _H. pylori_ infection (16). Furthermore, the production of anti-oxidant metabolites such as glutathione (GSH) and butyrate helps _Lactobacillus_ block free radicals with glutathione peroxidase (GSH-Px) (17). Not all _Lactobacilli_ possess probiotic potential, and even the differences occur within the same species; therefore, the characteristics of newly identified strains should be studied in detail.

The present study study is aimed at investigating the potential of _L. paracasei_ ZFM54 (CCTCC NO:M 2016667) to ameliorate gastric inflammation, oxidative stress, and microbiota dysbiosis in the mice model by administering _L. paracasei_ ZFM54 before (prevention) and after (therapy) _H. pylori_ challenge. The probiotic potential of _L. paracasei_ ZFM54 was first examined in _in vitro_ experiments to get insight into the strain’s efficacy to withstand the harsh gastric environment and inhibition against _H. pylori_.

Materials and methods

Bacterial culture

_L. paracasei_ ZFM 54 isolated from healthy infant’s feces was stored at the China Center for Type Culture Collection (Wuhan, China) and sub-cultured in DeMan–Rogosa–Sharpe (MRS) broth at 37°C for 24 h. Cell-free supernatant (CFS) was obtained by centrifuging at 8,000 × g at 4°C for 15 min.
H. pylori ZJC03 (CCTCC NO: M 20211218) was provided by the Zhejiang University School of Medicine and was previously isolated from patients with gastritis. H. pylori was inoculated on Columbia Blood Agar (CBA) broth supplemented with sheep’s blood (7%, v/v) (Hopebio, Qingdao, China) and antibiotics (10 mg/mL vancomycin, 5 mg/mL amphotericin, 2.5 units/mL polymyxin B, and 5 mg/mL trimethoprim) (Sangon Biotech, Shanghai, China). The plates were kept in an anaerobic box containing a micro-aerobic pack (Anaeropack-MicroAero, Mitsubishi, Japan) and incubated at 37°C for 48–72 h. For liquid culture, the bacteria were scraped off and sub-cultured in Brain Heart Infusion (BHI) broth in microaerophilic condition at 37°C for 48 h.

Simulated gastric juice tolerance assay

The simulated gastric juice tolerance assay of L. paracasei ZFM54 was detected according to Charteris et al. (18) with some modifications. Bacterial cells were centrifuged (4°C, 8,000 × g) for 10 min and suspended in an equal volume of PBS (pH 7) after 18–20 h of culture. The pH of 1 g/L pepsin (Sigma Aldrich, USA) was set to 2, 3, and 4, respectively. A mixture of 100 µL cell suspension, 300 µL NaCl solution (0.5% w/v), and 1 mL pepsin with different pH was cultured at 37°C. Aliquots were removed at 0 h, 2 h, and 4 h, diluted, and enumerated for viability by plate count. The survival rate (%) was calculated as below:

\[
\text{Survival rate} \%(\%) = \frac{\text{No. of cells survived}}{\text{No. of initial viable cells inoculated}} \times 100, \quad (1)
\]

Auto-aggregation assay

Auto-aggregation of L. paracasei ZFM54 was assayed as reported by Ahire et al. (19) with slight modifications. The initial optical density of the cell suspension at 600 nm was set to 0.5 and incubated at 37°C for 4 h. The OD_{600} was recorded every 1 h. The auto-aggregation rate (%) was measured by the following formula:

\[
\text{Auto-aggregation} \%(\%) = \frac{A_0 - A_t}{A_0} \times 100, \quad (2)
\]

where A_0 is the absorbance at 0 h, and A_t is the absorbance at time t = 1, 2, 3, and 4 h.

Virulence genes

Genomic DNA of H. pylori ZJC03 was extracted by genome extraction kits (Takara, Beijing, China) according to the manufacturer’s instructions. Virulence genes of cagA, vacA s1/s2, and vacA m1/m2 were amplified, and the primers were synthesized (Sangon, Shanghai, China) and are listed in Table 1.

Antibiotic sensitivity assay

Susceptibility of H. pylori ZJC03 to amoxicillin, metronidazole, tetracycline, and clarithromycin was evaluated by the epsilometric method (E-test) (20). The E-test strip containing antibiotics was attached to the surface of the agar medium containing H. pylori (10^8 CFU/mL) and cultured in a microaerophilic tank (85% N_2, 10% CO_2, and 5% O_2) at 37°C for 48 h–72 h. The minimal inhibitory concentration (MIC) was determined by the point of the antibacterial ring and the strip.

Co-aggregation assay

Co-aggregation ability was assayed according to Kos et al. (21). The cells were washed three times with PBS (pH 7.0), and the initial optical density was set to 0.50 at 600 nm. Same volumes of L. paracasei ZFM54 (10^6 CFU/mL) and H. pylori ZJC03 (10^6 CFU/mL) cell suspension were mixed, vortexed for 10 s, and incubated at 37°C for 4 h. OD_{600} was measured every 1 h. The co-aggregation rate (%) was measured by the following formula:

\[
\text{Co-aggregation} \%(\%) = \left(\frac{Ax + Ay}{Ax + Ay} - 2\right) \times \frac{At}{Ax + Ay} \times 100, \quad (3)
\]

where Ax is the absorbance of L. paracasei ZFM54 at 0 h, Ay is the absorbance of H. pylori at 0 h, and At is the absorbance of the mixture at 1, 2, 3, and 4 h.

| Primer | DNA sequence (5’-3’) | DNA size (bp) |
|--------|----------------------|---------------|
| cagA   | F TTAGCACCAACCACAAACCGAAG | 183 |
|        | A CTTCCTAAACTTGGGAGATTCC |   |
| vacA s1/s2 | F ATGGAATCACAACAAACACAC | 259/286 |
|        | A CTGCTTGAAGCGCCAAAC |   |
| vacA m1/m2 | F CAATCTGTCCAATCAGCGAG | 567/642 |
|        | A GGTCAAAAATATTTCAAGG |   |

E: forward; R: reverse.
Anti-H. pylori activity in vitro

As previously described by Hirano et al., antibacterial activity was determined by the Oxford cup assay with slight modifications (22). H. pylori ZJC03 cell culture in a semi-liquid BHI medium containing 7% sheep’s blood was poured onto agar plates in sterilized Oxford cups. After solidification, the Oxford cups were pulled out to form wells, and 100 µL of L. paracasei ZFM54 cell (10⁹ CFU/mL) in phosphate-buffered solution (PBS) and CFS was added into different wells.

Urease inhibition assay

Urease activity was used as the activity index of H. pylori ZJC03 by the phenol red method (23) with modification. In a 96-well microtiter plate, the bacterial cultures were added: (i) 40 µL H. pylori cells (10⁸ CFU/mL) and 10 µL L. paracasei ZFM54 cells (10⁹ CFU/mL), (ii) 40 µL H. pylori cells (10⁸ CFU/mL) and 10 µL L. paracasei ZFM54 CFS, and (iii) 40 µL H. pylori cells (10⁸ CFU/mL) and 10 µL PBS. The 96-well microtiter plate was incubated at 37°C for 48 h under microaerophilic condition (85 N₂, 10 CO₂, and 5% O₂). Urease indicator (0.9% NaCl, 20 mM NaCl, 14 µg/mL phenol red, pH 6.8) was added to each well, and the absorbance was measured at 540 nm. The inhibition rate was calculated by the following formula:

\[
\text{Urease inhibition (\%)} = \frac{\text{Urease activity of H. pylori} - \text{Urease activity of sample}}{\text{Urease activity of H. pylori}} \times 100.
\]

Free radical scavenging activity

The anti-oxidant activity was assessed by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity according to Saravanakumar et al. (24) with some modifications. About 1 mL L. paracasei ZFM54 cell culture (10⁹ CFU/mL) and 1 mL CFS were cultured in combination with 1 mL DPPH ethanol solution (0.2 mM) respectively. Then, 1 mL DPPH mixed with 1 mL saline was used as the control. All tubes were vortexed and placed in the dark at room temperature for 30 min. The absorbance was measured at 517 nm. The DPPH scavenging activity (%) was calculated by the following formula:

\[
\text{Scavenging activity (\%)} = \frac{A_1 - A_2}{A_1} \times 100,
\]

where \(A_1\) is the absorbance of the control and \(A_2\) is the absorbance of the sample.

Animal model

The animal experiment was conducted in accordance with the ethical guidelines for animal use and with permission from Shanghai Public Health Clinical Center Animal Ethics Committee. Specific pathogen-free C57BL/6 mice (6-week-old, 18–20 g) were obtained from Sino-British SIPPR/BK Lab Animal Ltd. (Shanghai, China), and the experiment was carried out in the Laboratory Animal Department of Shanghai Public Health Clinical Center. Mice were adapted to the controlled condition (24 ± 2°C room temperature and 50 ± 5% relative humidity) for a week prior to the experiment. All mice were grouped into two experimental approaches including preventive administration and therapeutic administration, as seen in Figure 1. Six mice in each subgroup were randomly sacrificed for sampling and testing.

Preventive trials

Mice in the control group (normal mice) were free to eat and drink without infection. On a group of 54 mice, with only L. paracasei ZFM54 (10⁹ CFU/mL) was administrated for 21 days without H. pylori challenge. After 21-day intrastragical administration of L. paracasei ZFM54, mice in the groups of Hp (negative control) and Hp+Hp (prevention with L. paracasei ZFM54) were challenged with H. pylori ZJC03 (10⁸ CFU/mL, 400 µL) three times (every other day). After infection, all mice were fed with a normal diet for 35 days and finally sacrificed by cervical dislocation.

Therapeutic trials

Mice in the control group (normal mice) were free to eat and drink without H. pylori ZJC03 infection. Mice in the groups of Hp (negative control), Hp+5 (therapy with L. paracasei ZFM54), and Hp+TT (therapy with triple therapy) were challenged intrastragically with H. pylori (10⁸ CFU/mL, 400 µL) three times a week for 21 days. Then, mice in the groups of Hp+5 and Hp+TT were fed intrastragically with 400 µL L. paracasei ZFM54 (10⁹ CFU/mL) for 28 days and triple therapy for 10 days, respectively. Triple therapy (TT) consists of PPI (5.2 mg/kg), amoxicillin (260 mg/kg), and clarithromycin (65 mg/kg).

MDA and GSH assays

Liver tissues of mice were homogenized and centrifuged at 4000 × g for 10 min. The MDA level was determined using thiobarbituric acid (TBA) assay kit according to the manufacturer’s instructions. One molecule of MDA reacts with two molecules of TBA to form thiobarbituric acid reactive substance (TBARS) at high temperature and acidic conditions.
FIGURE 1
Grouping of C57BL/6 mice for prevention and therapeutic models. (A) The preventive groups of control (normal mice without prevention and infection in the preventive group), Hp (mice infected with *H. pylori* without prevention in the preventive group), 54 (only treated with *L. paracasei* ZFM54), and 54+Hp (pretreatment with *L. paracasei* ZFM54 before *H. pylori* challenge). (B) The therapeutic groups of control' (normal mice without prevention and infection in the therapeutic group), Hp' (mice infected with *H. pylori* without treatment in the therapeutic group), Hp +54 (treatment with *L. paracasei* ZFM54 after *H. pylori* challenge), and Hp+TT (treatment with triple therapy after *H. pylori* challenge).

and was measured at 532 nm. The following formula was used to calculate MDA content in liver tissues:

\[
MDA (\text{nM}) = \frac{\text{measured OD value} - \text{controlled OD value}}{\text{standard OD value} - \text{blank OD value}} \times \text{Cs} \times \text{Cp}, \tag{6}
\]

where Cs is the standard concentration (10 nmol/mL), and Cp is the protein concentration of samples before being measured (mgprot/mL).

Tissue GSH level was determined using the GSH assay kit by the manufacturer’s instructions. The reaction of 5, 5-Dithiobis(2-nitrobenzoic acid) (DTNB) with GSH takes place to produce a yellow chromogen, and the absorbance is measured at 405 nm. The calculation formula is as follows:

\[
\text{GSH (mM)} = \frac{\text{measured OD value} - \text{blank OD value}}{\text{standard OD value} - \text{blank OD value}} \times \text{Cs} \times \text{Cp} \times \text{dilution factor of sample}, \tag{7}
\]

where Cs is the standard concentration (20 mM), Cp is the protein concentration of samples before being measured (mgprot/mL), and the dilution factor of samples is 2 times.

**Hematoxylin and eosin (H&E) staining**

Histopathological alterations of gastric mucosa in preventive and therapeutic groups were evaluated by H&E staining. Gastric tissues of mice were fixed in 4% paraformaldehyde for 48 h at room temperature, were dehydrated, waxed, embedded, and sectioned according to Ahire et al. (19). H&E images were observed under a NIKON Eclipse Ci microscope assisted with a NIKON digital sight DS-Fi2 (NIKON Corporation, Tokyo, Japan).

**Real-time quantitative polymerase chain reaction (RT-qPCR)**

The mRNA expression of IL-1β, IFN-γ, IL-6, and IL-10 in the stomach tissues in the preventive and therapeutic groups was quantitatively analyzed by RT-qPCR (25). Total RNA from gastric tissue was extracted by TRIzol reagent (Servicebio, Wuhan, China) and reversed-transcribed into single-strand cDNA by RevertAid First Strand cDNA Synthesis Kit according to the manufacturer’s instruction (Thermo Scientific, Vilnius, Lithuania). qPCR was conducted using FastStart Universal SYBR Green Master (Rox) (Servicebio, Wuhan, China) with the primers of inflammatory cytokines (Table 2). The cycling program consisted of initial denaturation at 95°C for 10 min followed by 40 cycles at 95°C for 15 s, 60°C for 15 s, 60°C for 60 s, and 95°C for 15 s. The gene expression was calculated using the 2^-ΔΔCt_ method, with glyceraldehyde-3-phosphate dehydrogenase (GADPH) as the reference gene (21).

**16S rRNA sequencing**

The V3-V4 region of the 16S rRNA gene of gastric microbiota was sequenced using the QIIME pipeline (26). According to SILVA 138/16s database, paired-end reads were
TABLE 2 Primers used for inflammatory cytokines.

| Gene   | Primers | DNA sequences (5’-3’)            | Segment length (bp) | RefSeq ID          |
|--------|---------|----------------------------------|---------------------|--------------------|
| GAPDH  | S       | CTCGTTCCCTAGACAAATG              | 133                 | NM_008084.2        |
|        | A       | TGGGCTCAATGAGGGTCGT              |                     |                    |
| IFN-γ  | S       | CTCAAGTGGCGATATGTCGAAGG          | 251                 | NM_008337.4        |
|        | A       | TGGCATCAACATTTGCAAAATC           |                     |                    |
| IL-1β  | S       | TCAAATCTCCCAGCAGCACATC           | 206                 | NM_008361          |
|        | A       | CGTCACACACAGCGTATTCAT            |                     |                    |
| IL-6   | S       | CCCCACATTGCAATGCTCTCC            | 141                 | NM_031168.2        |
|        | A       | CGCAGTTGGCCATGCTGAC             |                     |                    |
| IL-10  | S       | TTTCACACTTGGGTTGTC              | 106                 | NM_010548.2        |
|        | A       | AATGCTCTGATTTCTGGGG             |                     |                    |

Inferred by DADA2, linked to tags, and clustered to Amlocipon Sequence Variant (ASV) with 100% similarity. Alpha diversity-related community richness (Chao index and Ace index) and community diversity (Shannon index and Simpson index) were analyzed. Beta diversity analysis was performed by principal coordinate analysis (PCoA) based on unweighted-uniFrac distances. The difference between the classes and the features such as organism, clades, and ASV was analyzed by the linear discriminant analysis effect size (LEfSe) method (LDA > 4.0). The relative abundance differences of the genus Helicobacter between the Hp group and the preventive or therapeutic groups were further analyzed.

Statistical analysis

All results are expressed as mean value ± standard deviation (SD). Graphs of in vitro indexes, MDA, GSH, and inflammatory cytokines were drawn using GraphPad Prism 9.0, and the differences between the two groups were compared by Tukey’s test or Student’s t-test. The differences in gastric microbiota were analyzed using the Wilcoxon rank-sum test. A p-value < 0.05 was considered statistically significant.

Results

pH tolerance, auto-aggregation, and co-aggregation properties of L. paracasei ZFM54

Probiotic strains need to resist low pH to survive and colonize in the host’s stomach. The survival rate of L. paracasei ZFM54 was 65.57 ± 1.87%, 88.37 ± 1.19%, and 97.99 ± 0.50% at pH 2, 3, and 4 for 4 h, respectively (Figure 2A). These findings indicated that L. paracasei ZFM54 can normally grow and meet the requirements for probiotic growth in vivo. The auto-aggregation ability of L. paracasei ZFM54 was checked, which is the ability to interact non-specifically with own cells. The auto-aggregation rate was 29.60 ± 2.73% after 1 h, which gradually increased to 45.80 ± 1.75% after 2 h and 69.00 ± 2.73% after 4 h (Figure 2B). L. paracasei ZFM54 was also able to co-aggregate with H. pylori ZJC03 in vitro at 6.23 ± 1.23% after 1 h, 17.78 ± 1.02% after 2 h, and 30.28 ± 2.24% after 4 h, respectively (Figure 2C).

Virulence genes and antibiotic susceptibility of H. pylori ZJC03

As seen in Figure 3, virulence genes of cagA, vacA s1/s2, and vacA m1/m2 were all expressed in H. pylori ZJC03. E-test results showed that H. pylori ZJC03 was sensitive to clarithromycin, amoxicillin, and tetracycline with the MIC values of 0.80 µg/mL, 0.016 µg/mL, and 0.023 µg/mL, respectively. However, H. pylori ZJC03 was not sensitive to metronidazole, with an MIC value exceeding 256 µg/mL.

Anti-H. pylori activity of L. paracasei ZFM54

The cells of L. paracasei 54 showed stronger anti-H. pylori ability compared to CFS. The inhibition zone of the cells was 11.25 ± 0.78 mm, and the zone of the CFS was 9.02 ± 0.20 mm (Table 3). L. paracasei ZFM 54 cells inhibited urease activity of H. pylori ZJC03 with a rate of 70.27 ± 2.23%, while CFS showed an increased inhibition rate of 89.51 ± 0.72%. L. paracasei ZFM54 exhibited strong radical scavenging ability in DPPH scavenging assay. L. paracasei ZFM 54 cells showed remarkable oxidative activity calculated to be 57.89 ± 1.27%, and CFS was 51.36 ± 0.78%.
MDA and GSH levels in liver

The MDA level in liver tissue of *H. pylori*-infected mice in the preventive group (0.85 ± 0.05 nmol/mgprot) was higher than that of the control group (*p* < 0.05) (Figure 4A). Pretreatment with *L. paracasei* ZFM54 significantly reduced MDA to 0.71 ± 0.04 nmol/mgprot (*p* < 0.05), thus protecting the mice mucosal layer from oxidative damage of ROS. Treatment with *L. paracasei* ZFM54 also significantly reduced the MDA level to 0.70 ± 0.06 nmol/mgprot (*p* < 0.01), when MDA contents were also significantly reduced in mice treated with triple therapy (TT) (0.71 ± 0.04 nmol/mgprot, *p* < 0.01) (Figure 4B).

GSH content in liver tissues of *H. pylori*-infected mice significantly decreased (1.30 ± 0.05 µmol/gprot) compared to control (1.59 ± 0.10 µmol/gprot, *p* < 0.05) (Figure 4C), while significant increased level of GSH was observed in mice pretreated with *L. paracasei* ZFM54 (1.78 ± 0.02 µmol/gprot, *p* < 0.05), treated with *L. paracasei* ZFM54 (2.09 ± 0.15 µmol/gprot, *p* < 0.01), and triple therapy (TT) (2.12 ± 0.29 µmol/gprot, *p* < 0.01) (Figure 4D).
TABLE 3 Anti- \( H. \) pylori activity, urease inhibition, and DPPH scavenging of \( L. \) paracasei 54.

| Assay          | Bacteria/Sample | Anti-\( H. \) pylori activity (mm) | Urease inhibition(%) | DPPH scavenging (%) |
|---------------|-----------------|------------------------------------|----------------------|----------------------|
|               | Cells of \( L. \) paracasei 54 | 11.25 ± 0.78                      | 70.27 ± 2.23         | 57.89 ± 1.27         |
|               | CFS of \( L. \) paracasei 54     | 9.02 ± 0.20                       | 89.51 ± 0.72         | 51.36 ± 0.78         |

**FIGURE 4** Levels of MDA and GSH during prevention and therapy. (A) MDA levels in preventive groups. (B) GSH levels in preventive groups. (C) MDA levels in therapeutic groups. (D) GSH levels in therapeutic groups. Data are shown as the mean ± SD (\( n = 6 \)). Significant differences between two groups based on Student’s \( t \)-test, *\( p < 0.05 \), **\( p < 0.01 \).

Histopathological changes of gastric tissues

Mice in the control and control’ groups exhibited normal appearance of gastric mucosal morphologically, including the normal glands and structure of gastric epithelial cells, and little inflammatory infiltration in gastric epithelium (Figure 5). In contrast, the gastric mucosa of \( H. \) pylori-infected mice (Hp and Hp’) showed irregular arrangement of mucosal glands, enlarged and hyperchromatic nuclei, and severe infiltration of inflammatory cells in the lamina propria. \( L. \) paracasei ZFM54 did not induce mucosal inflammation and other pathological changes in stomach. Inflammatory infiltration was significantly inhibited in mice pretreated with \( L. \) paracasei ZFM54 before \( H. \) pylori challenge (54+Hp). As expected, no obvious signs of tissue lesion and inflammation were also observed in the
therapeutic groups of L. paracasei ZFM54 (Hp+54) and triple therapy (Hp+TT). These observations suggest that L. paracasei ZFM54 can inhibit or even reverse the injury process and protect gastric mucosa.

mRNA expression level of inflammatory cytokines

The levels of pro-inflammatory cytokines (IFN-γ, IL-1β, and IL-6) and anti-inflammatory cytokine (IL-10) were significantly altered after H. pylori infection, as displayed in Figure 4. In groups of Hp and Hp', the upregulation levels ranged between 3.00 ± 0.18 and 6.55 ± 1.86 log fold change of the INF-γ gene after H. pylori challenge, respectively (Figures 6A,E). IL-1β gene expression was significantly stimulated to 1.54 ± 0.36 and 1.53 ± 0.26 log folds, respectively (Figures 6B,F). Another pro-inflammatory cytokine, IL-6, was also increased to 2.11 ± 0.30 and 3.80 ± 0.24 log folds, respectively (Figures 6C,G). All pro-inflammatory cytokines were notably downregulated in L. paracasei ZFM54 prevention (54+Hp) and therapy (Hp+54) groups compared to probiotically unprotected mice (p < 0.01). No changes in the anti-inflammatory cytokine IL-10 were observed in either group (Figures 6D,H).

Difference analysis of gastric microbiota

To compare the gastric microbial composition in various groups of mice, we randomly selected the gastric contents for 16S high-throughput sequencing and taxonomic classification analysis. H. pylori infection increased the abundance and diversity of gastric microbiota by analysis of the Ace, Chao, Shannon, and Simpson indices of alpha diversity (Figures 7A,B). PCoA analysis showed apparent clustering of gastric microbiota in different groups based on genus-level unweighted-uniFrac distances (Figures 7C,D). Preventive and therapeutic administration of L. paracasei ZFM54 improved gastric microbiota structure to near normal levels.

At the phylum level, it was found that the gavage of L. paracasei ZFM54 significantly increased the relative abundances of Firmicutes and Actinobacteriota and significantly decreased the abundance of Bacteroidota and Proteobacteria (Figures 8A,B). Instead, triple therapy treatment promoted the growth of Bacteroidota and Proteobacteria (Figure 8B). At the genus level, the relative abundances of bacterial groups such as Lactobacillus, Veillonella, and Bacillus in the H. pylori group decreased significantly, while Helicobacter, Lachnospiraceae_NK4A136_group, Sphingomonas, Staphylococcus, Alloprevotella, and Prevotellaceae_UCG-001 increased significantly (Figures 8C,D). Compared with infected mice, L. paracasei ZFM54 prevention and treatment significantly decreased the relative abundance of Helicobacter, Lachnospiraceae_NK4A136_group, Staphylococcus, and Prevotellaceae_UCG-001 and increased Lactobacillus, Veillonella, and Thermus. Although triple therapy treatment significantly inhibited the growth of gastric Helicobacter, it resulted in a significant increase in relative abundances of Sphingomonas, Bacteroides, Streptococcus, Oscillibacter, and Desulfovibrio (Figure 8D). The relative abundance of Helicobacter of 54, 54+Hp, Hp+54, and Hp+TT groups was lower than that of the H. pylori group (Hp and Hp', p < 0.01) (Figures 8E,F).

Gastric microbiota in the normal, Hp infection, and intervention groups were compared using LEfSe. Abundant bacterial taxa observed in the Hp group were Lachnospiraceae, Helicobacteraceae, and Prevotellaceae, most of which belong to gram-negative human opportunistic pathogens (Figure 9A). Bacterial taxa were observed in abundance in pretreatment with L. paracasei ZFM54 including Sphingomonadaceae and Oscilloridae. According to the analysis of the Wilcoxon rank-sum test, significant reductions in the average relative abundance of Muribaculaceae, Staphylococcus, Lachnospiraceae_NK4A136_group, and Prevotellaceae_UCG-001 were observed in the preventive group of L. paracasei ZFM54 on genus level compared to the Hp group (Figure 9B). In therapeutic groups (Figure 9C), 16 bacterial taxa were identified to be in abundance in the H. pylori group including Helicobacteraceae, Lachnospiraceae, Oscilloridae, and Prevotellaceae. Lachnospiraceae were observed in significant abundance in treatment with L. paracasei ZFM54 (Hp+54), while Sphingomonadaceae were abundant in triple therapy group (Hp+TT). In addition, significant reductions in average relative abundance of Muribaculaceae, Alloprevotella, and Oscillibacter were observed by L. paracasei ZFM54 treatment (Hp+54) (Figure 9D), while Helicobacter, Lachnospiraceae_NK4A136_group, and Muribaculaceae were significantly reduced by triple therapy treatment (Hp+TT) on the genus level using the Wilcoxon rank-sum test (Figure 9E).

Discussion

Usually, host innate and adaptive immune responses are stimulated shortly after H. pylori colonization of gastric epithelial cells (27). Various clinical complications of H. pylori infection can be attributed to changes in bacterial genotypic and phenotypic characteristics. Infection with vacA and cagA-positive H. pylori leads to severe gastric inflammation and a high risk for cancer development (28). H. pylori ZJC03 in this study was previously isolated from a patient with gastritis, which has virulence genes of vacA and cagA and is highly resistant to metronidazole. Lactobacillus strains are among the most prominent members of probiotics, mostly normal residents of different regions of human body. Our previous study examined the prebiotic properties of L. paracasei ZFM54 by genome
sequence analysis and related prebiotic properties, including antagonism against \textit{H. pylori} ZJC03, antibiotic susceptibility, and no adverse effects during acute oral toxicity in mice (29–31), suggesting that \textit{L. paracasei} ZFM54 can be a potential candidate for use in the health or diet field.

For an effective strain to be called a probiotic, it must be able to survive in a hostile gastric environment (32). To reach host epithelial tissues of stomach, the acidic pH (1–2) in stomach is the foremost challenge to overcome. The ingested food buffers the acidic environment to pH 3, so it is considered as the optimum pH for the survival of a potential probiotic strain (33). \textit{L. paracasei} ZFM 54 exhibited good tolerance as it showed considerable growth at low pH (2.0). Sornsenee et al. found that the survival rates of eight \textit{L. paracasei} strains isolated from fermented palm sap in Thailand were 40–60% at pH 2 for 4 h (34). \textit{L. paracasei} L1 was highly resistant to low pH (viability of 98.73% at pH 2 for 3 h) and was fed as probiotics to improve the intestinal microflora of chicken (35). The auto-aggregation ability of a probiotic strain is another important factor correlated with the bacterium’s ability to form biofilm, which is an important property of probiotic to prevent pathogenic microorganisms from invading the host (1). The auto-aggregation rate of \textit{L. paracasei} ZFM 54 rose to 69.00 ± 2.73% after 4 h. The 2- and 24-h auto-aggregation values of \textit{L. paracasei} L1 were 20.4 ± 2.3% and 47.2 ± 0.8%, respectively (35). Co-aggregation is considered a key strategy for \textit{Lactobacillus} strains to prevent pathogenic bacteria from adhering to biological surfaces (36). Barache et al. analyzed that \textit{L. paracasei} FB1 showed stronger co-aggregation with \textit{S. aureus} (28.34% ± 2.56%) after 2 h of incubation, followed by \textit{E. coli} (23.39 ± 2.12%) and \textit{L. monocytogenes} (20.10 ± 2.04%) (37). \textit{L. paracasei} ZFM 54 displayed a co-aggregation rate of over 30% and exhibited their capability to agglutinate \textit{H. pylori} in vitro.

Inhibition of urease enzyme is an important ability of \textit{Lactobacillus} to decrease \textit{H. pylori} density in the host and inhibit dysbiosis. \textit{H. pylori} uses urease enzyme to survive in the acidic environment of the stomach by utilizing urease enzyme activity to convert urea into ammonia, thus raising the pH and...
FIGURE 6
Expression levels of mRNA for pro-inflammatory or anti-inflammatory cytokines in preventive and therapeutic groups. (A–D) IFN-γ, IL-1β, IL-6, and IL-10 mRNA expression levels in the preventive group. (E–H) IFN-γ, IL-1β, IL-6, and IL-10 mRNA expression levels in the therapeutic group. Data are shown as the mean ± SD (n = 6). Significant differences between two groups based on Student’s t-test, **p < 0.01, ***p < 0.001.
creating a favorable environment for its growth and colonization (38). Infection with *H. pylori* stimulates phagocytic cells at inflammation sites, producing ROS to combat *H. pylori* (17). The increased level of ROS is detrimental for host tissues, causes lipid peroxidation, and produces MDA. MDA is a biomarker of *H. pylori* infection (17). GSH, a thiol-based anti-oxidant, is reduced in *H. pylori* infection, which is naturally present in all cells and contributes in defending against oxidative injury. As observed in this study, *L. paracasei* ZFM 54 significantly reduced *H. pylori*-mediated MDA level in mice liver tissues, while significantly increasing the GSH level in preventive and therapeutic studies. *L. paracasei* ZFM 54 appears to have potential anti-oxidation ability *in vivo* as well, validating the anti-oxidation potential shown *in vitro* experiment.

*H. pylori* infection stimulates adaptive immune response mainly through the activation of T-helper cells. Th1 cells further produce cytokines IL-2, IL-22, and IFN-γ with subsequent stimulation of neutrophil infiltration and macrophage activation (39). *H. pylori* modulate pro-inflammatory and regulatory immune responses, which stimulate the activation of IL-1β, a strong pro-inflammatory cytokine which promotes gastric tumor cancer through genetic polymorphism (40). Consistent results in this study were observed through *H. pylori*-mediated enhanced level of pro-inflammatory cytokines (IFN-γ, IL-1β,
and IL-6) and severe infiltration of inflammatory cells in gastric mucosa. Prevention and therapy with *L. paracasei* ZFM 54 significantly downregulated the pro-inflammatory cytokines in gastric mucosa. Previously, similar results were obtained in pretreatment with *L. plantarum* ZDY 2013 against *H. pylori* in mice (41). *L. plantarum* ZDY 2013 showed a significant
increase in IL-10 against *H. pylori* in AGS cell lines (42). No increase in the level of anti-inflammatory cytokine IL-10 was observed in this research, suggesting the different mechanisms to be involved in reducing inflammation. Other mechanisms responsible for the protective effect may be contributed by competitive exclusion of *H. pylori* or inhibition by secreting antimicrobial substances or blocking urease activity, which further hinder *H. pylori* colonization.

The microbiota in normal healthy stomach has been reported to be mainly composed of Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria, and the most abundant genera have been reported to include *Streptococcus, Prevotella,* and...
Fusobacteria, Veillonella, and Rothia (43, 44). Previous reports provided evidence of reduced bacterial diversity and an increase in the bacterial taxa belonging to the phyla Campilobacterota and Proteobacteria in subjects with H. pylori-positive infection (43). Preventive and therapeutic experiments with L. paracasei ZFM 54 significantly suppressed the relative abundance of H. pylori, while increasing the abundance of bacterial taxa belonging to the phyla Firmicutes and Actinobacteriota. Interestingly, treatment with triple therapy reduced high levels of inflammation cytokines and MDA, but the LEfSe analysis showed increased abundance of Rodentibacter and Escherichia-Shigella Mucispirillum, members of which are opportunistic human pathogens in the stomach. It is also suggestive of disruption of gastric microbiota due to antibiotics. Suppression of acid by PPI triggers dysbiosis in gastric microbiota composition (45). An increased abundance of non-H. pylori bacteria such as Escherichia-Shigella, Nitrospirae, and Burkholderia were found in patients with gastric cancer (46).

Conclusion

The strain L. paracasei ZFM 54 demonstrated remarkable probiotic characteristics owing to its ability to survive in the simulated gastric acid, auto-aggregation, and hydrophobic activities. The potential inhibition against H. pylori and urease enzyme, anti-oxidizing, and co-aggregation activities further validate the efficacy of the strain to be effective against H. pylori. During preventive and therapeutic experiments in the H. pylori-induced gastritis mouse model, L. paracasei ZFM 54 can effectively reduce inflammation, MDA level, and inhibition of microbiota dysbiosis. The use of L. paracasei ZFM 54 against H. pylori challenge would be more advantageous over antibiotics, as it more favorably improves gastric microbiota caused by H. pylori infection. The potential to prevent and treat H. pylori inflammation is highly indicative of its use not only as a prophylaxis but also as a therapeutic agent to eradicate H. pylori.

Data availability statement

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

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Ethics statement

The animal study was reviewed and approved by mice in vivo. Experiments was conducted under approval of by Shanghai Public Health Clinical Center Laboratory Animal Welfare and Ethics Committee with protocol No. 2019-A003-01.

Author contributions

PL and QG: conceptualization and supervision. QZ, NQ, and BX: methodology, investigation, and data curation. QZ and NQ: writing—original draft preparation. QZ and ZX: writing—review and editing. QG: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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