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Probiotic effect on *Helicobacter pylori* attachment and inhibition of inflammation in human gastric epithelial cells

HANYI SONG[1], LONG ZHOU[2], DONGYAN LIU[3], LIHUI GE[1] and YAN LI[1]

Departments of [1]Gastroenterology and [2]Orthopedics; [3]Medical Research Center, Shengjing Hospital of China Medical University, Shenyang, Liaoning 110004, P.R. China

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Abstract. *Helicobacter pylori (H. pylori)* is a major cause of chronic gastritis, gastric ulcers and gastric cancer. Recent studies have identified that probiotics are beneficial to human health due, in part, to their anti-*H. pylori* activities. Therefore, the present study investigated the antagonistic and local immunoregulatory activities of seven commercial probiotic strains and explored their mechanisms of actions. The human gastric epithelial cell line-1 (GES-1) was used to assess the effects of probiotics on the adhesion ability of *H. pylori*. GES-1 cells were infected with *H. pylori* plus lipopolysaccharide (*HP-LPS*) or the drug-resistant *H. pylori* strain (*HP021*) in the presence or absence of live probiotics. Following this, the growth rate and the adhesion ability of GES-1 cells were detected using MTT and urease activity assay. Toll-like receptor 4 (TLR4), NFKB inhibitor-α (IκBα) and nuclear factor (NF)-κB levels were measured by western blot analysis. The amount of interleukin (IL)-8 in the cell culture medium was determined by ELISA. Amongst the seven probiotic strains studied, live *Lactobacillus acidophilus (L. acidophilus)* and *Lactobacillus bulgaricus (L. bulgaricus)* inhibited *H. pylori* adherence to GES-1 cells most significantly. *L. bulgaricus* inhibited IL-8 production by GES-1 cells through modulation of the TLR4/IκBα/NF-κB pathway. Therefore, the present results suggested that consumption of food containing *L. acidophilus* and *L. bulgaricus* may be used as an adjuvant therapy for *H. pylori*-associated gastritis.

Introduction

Helicobacter pylori (H. pylori) was originally isolated from the gastric mucosa of patients with chronic active gastritis in 1983 with further study revealing that the pathogen itself caused the condition (1). *H. pylori* infects >50% of the world's population (2) and it frequently causes chronic active gastritis, gastroduodenal ulcers (3) and gastric cancer (4). *H. pylori* is occasionally associated with functional dyspepsia, unexplained iron deficiency anemia (5) and idiopathic thrombocytopenic purpura (6). The standard triple therapy used to treat *H. pylori* is a proton pump inhibitor (PPI) in conjunction with a 7-10 day course of two antibiotics. This protocol eradicates *H. pylori* infection in ~80% of patients (7). Bismuth-based quadruple therapies, including clarithromycin, amoxicillin and metronidazole, are another alternative treatment (8,9). However, some strains of *H. pylori* demonstrate antibiotic-resistance, which complicates treatment. In addition, the neurotoxicity of bismuth limits its use in the elderly and children (10). Copious use of antibiotics is not only accompanied by a variety of side effects, but also increases the risk of antibiotic resistance. Therefore, the development of safer and more effective new therapeutic agents targeting *H. pylori* is an important focus of current research. Recent studies have demonstrated that administration of oral probiotics is greatly beneficial for the treatment of *H. pylori* infection (11-13). The substances produced by probiotics significantly inhibit VacA and flaA virulence genesin *H. pylori* (12). Use of probiotics alongside drugs often reduces the drug side effects and attenuates the gastric mucosal inflammation (14,15). It has been reported that *Lactobacillus salivarius (L. salivarius)*, *Lactobacillus gasseri*, *Lactobacillus casei Shirota*, *Lactobacillus Johnsonii La1*, *Lactobacillus rhamnosus GG* and *Saccharomyces boulardii* have either anti-*H. pylori* activities or anti-inflammatory properties (16,17). The antagonistic activities of probiotics against *H. pylori* are strain-specific; however, their mechanisms of action remain unclear (18).

Currently, mixed strain probiotics are the most-widely studied (8). Different strains of probiotics may possess synergetic or antagonistic effects. In the present study, the anti-*H. pylori* activities of *Lactobacillus acidophilus (L. acidophilus)*, *L. salivarius*, *Clostridium butyricum (C. butyricum)*, *Bacillus licheniformis (B. licheniformis)*, *Bifidobacterium infantis (B. infantis)*, *Bifidobacterium longum* (*B. longum*) and *Lactobacillus bulgaricus (L. bulgaricus)* were evaluated. These probiotic strains are widely used in clinical settings to treat diarrhea and have been reported to improve the eradication rate of *H. pylori* in some clinical cases due to the secretion of antibacterial substances including lactic acid,

Correspondence to: Dr Yan Li, Department of Gastroenterology, Shengjing Hospital of China Medical University, 36 Sanhao Street, Heping, Shenyang, Liaoning 110004, P.R. China
E-mail: yanli0227@hotmail.com

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acetic acid and hydrogen peroxide (19,20). The present study objective was to evaluate the probiotic mechanisms of action against *H. pylori* with the results potentially providing a new theoretical basis for eradicating *H. pylori*.

Materials and methods

Bacterial strains and culture conditions. The probiotic strains used were as follows: *L. acidophilus*, *L. salivarius*, *C. butyricum*, *B. licheniformis*, *B. infantis*, *B. longum* and *L. bulgaricus*, purchased from Siliankang, Jinshuangqi, Changlekang and Taiwan Yaxin (Table I). The probiotics were cultured on De Man, Rogosa and Sharpe agar plates (MRS; Oxoid; Thermo Fisher Scientific, Inc.) at 37˚C in an anaerobic humidified environment for 24 h. Live bacteria cells were obtained by centrifuging the cultures and washing with sterile PBS three times. The precipitate were resuspended with RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.) and adjusted to $3x10^8$ colony-forming units (CFU)/ml.

H. pylori ATCC43504 strain was purchased from the American Type Culture Collection and the multidrug resistant *H. pylori* strain (HP021) was isolated from the gastric biopsy of a patient with chronic atrophic gastritis and confirmed to have resistance to clarithromycin and levofloxacin. *H. pylori* ATCC43504 strain expresses CagA and VacA proteins, which induces nuclear factor (NF)-κB and interleukin (IL)-8 expression (21). Both *H. pylori* strains were grown on Columbia blood agar plates supplemented with 7% defibrinated horse blood (Beijing Biotek Medical Device, Ltd.) for at least 3 days at 37˚C under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2). Normal human gastric epithelial GES-1 cells (American Type Culture Collection) were cultured at 37˚C in RPMI 1640 medium supplemented with 10% fetal bovine serum (Biological Industries) and 100 µg/ml penicillin/streptomycin in a humidified atmosphere with 5% CO_2. The medium was changed every other day. Before experiments were initiated, the cells were plated in 96-well plates at $5x10^3$ cells/well or $2.5x10^5$ cells/well in 6-well plates for 24 h in serum-free RPMI-1640 medium.

Cell-free supernatant (CFS) preparation and H. pylori ATCC43504–lipopolysaccharide (LPS) culture. Cultures of the various probiotic strains were grown in MRS broth (Oxoid; Thermo Fisher Scientific, Inc.) in an anaerobic humidified environment at 37˚C for 96 h. CFS from the probiotics was prepared by centrifuging (12,000 x g; 4˚C; 10 min) the respective MRS broths. CFS were filtered through a 0.2 µM filter and stored at -20˚C.

Culture broth from *H. pylori* ATCC43504 was centrifuged (8,000 x g; 4˚C; 10 min) and washed three times then resuspended in PBS. *H. pylori* lipopolysaccharide (HP-LPS) was obtained using an LPS extraction kit (Intron Biotechnology, Inc.) according to the manufacturer's instructions. HP-LPS concentrations were determined with a kinetic Limulus Amebocyte Lysate Assay kit (Xiamen Limulus Reagent Biotechnology Co., Ltd.).

GES-1 cell viability. Probiotics and CFS are toxic to GES-1 cells at high concentrations and during long incubations (17). To determine the optimal concentration and incubation time for each probiotic and CFS, GES-1 cells were infected with CFS and probiotics at multiplicities of infection (MOI) of 100 and 1,000 in antibiotic-free RPMI 1640 medium at 37˚C, 5% CO_2 for up to 8 h. Viable GES-1 cell numbers were determined by trypan blue staining following incubation for 2, 4, 6 and 8 h at 37˚C. Non-infected cell cultures served as controls. Cells that were not stained with trypan blue were counted as viable cells.

GES-1 proliferation. GES-1 cells were seeded into 96-well plates at $1.5x10^5$ cells/ml in 100 µl culture volume for 24 h. CFS (100 µl) and probiotics were then added to the GES-1 cells at 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8 and 10^9 CFU/ml. Each condition was performed in triplicate and cultured for 24 h. Cells were washed three times with sterile PBS solution then 20 µl of 4 mg/ml MTT reagents was added. The cells were cultured for another 4 h and then centrifuged for 5 min at 800 x g. The supernatant was discarded and 150 µl dimethyl sulfoxide was added. Absorbance was measured at 490 nm.

Assessment of adhesion ability. Adhesion ability of probiotics on GES-1 cells was determined as previously described by de Klerk *et al* (22). In brief, probiotics were suspended in RPMI-1640 medium to a concentration of $3x10^8$ CFU/ml. GES-1 cells cultured on slides in 6-well plates were infected with probiotics at a MOI of 100. Following 4 h of incubation, each well was washed three times with sterile PBS to remove any unbound bacteria. Cells were fixed with 4% paraformaldehyde at room temperature for 30 min and then the bacteria were Gram stained at room temperature for 5min. The cell monolayer was washed with tap water and the cells were observed at a magnification of x1,000 under light microscope oil immersion. The adherence of probiotics was calculated as previously described (23,24) and was as follows: Cell adhesion index=number of adhering probiotics/total number of cells in the field of view x100. Cell adhesion rate=number of cells adhered by probiotics/total number of cells in the field of view x100. Cell adhesion index indicates the number of probiotics adhering to each cell, and cell adhesion rate represents the proportion of cells adhered by probiotics in total cells.

Inhibition of H. pylori growth. *H. pylori* ATCC43504 and HP021 were evenly seeded on Columbia agar plates without antibiotics. Holes were introduced to the agar plates with a sterile oxford cup then the bottom of holes were sealed with 0.8% agar liquid. Live probiotics (120 µl at $3x10^9$ CFU/ml) were suspended in MRS broth and CFS (120 µl) was added to the holes in the plates. The plates were incubated under microaerophilic conditions for 72 h at 37˚C, and then the diameters of the inhibition zones were measured. PBS and MRS medium were used as negative controls.

Adhesion of H. pylori to GES-1 cells. For the infection studies, GES-1 cells were grown on microtiter plates to form a confluent monolayer. The concentration of each probiotic was adjusted to $1.5x10^7$ CFU/ml. *H. pylori* ATCC43504 and HP021 concentrations were then adjusted to $1.5x10^7$ CFU/ml. GES-1 cells were pre-treated with 50 µl of live probiotics for 2 h before infection (pre-treated group) or following infection

(post-treated group). GES-1 cells were infected for 2 h with 50 μl of live *H. pylori* ATCC43504 or HP021. Subsequently, each well was washed three times to remove any non-adherent *H. pylori*. Urease activity was determined using a modified phenol red method (18,25,26). In brief, 200 μl of urease test solution (20% [w/v] urea and 0.012% phenol red in phosphate buffer; pH 7.0) was added to each well of a microtiter plate. The plate was then incubated at 37˚C for 1 h. The absorbance at 550 nm was measured with a microtiter plate spectrophotometer (BioTek Instruments, Inc.). The adherence of *H. pylori* was calculated as described by Chen *et al* (18) and was as follows: Adherence=([Optical density experimental-optical density negative]/[optical density positive-optical density negative] x100.

The negative control contained only GES-1 cells and the positive control contained both GES-1 cells and *H. pylori*, which were used to establish 100% adherence.

ELISA for interleukin (IL)-8 detection. LPS is a pathogenic factor of *H. pylori* which can induce GES-1 cells to produce IL-8 (27). Probiotics with proven adhesive ability were added to GES-1 cells (MOI of 100) and incubated for 2 h. Following washing with PBS to remove bacilli, HP-LPS (final concentration of 7,000 endotoxin units [EU]/ml) was added and the cells were incubated for another 6 h. The final culture supernatants were centrifuged for 10 min at 12,000 x g and 4˚C to remove bacteria and cell debris. Supernatants were then aliquoted and stored at -80˚C. IL-8 concentration in the supernatant was determined using a commercially available ELISA kit (cat. no. VAL103; R&D Systems, Inc.). Absorbance values were measured at 450 nm using a microplate reader. Each sample was measured three times.

Preparation of cellular lysates and western blot analysis. GES-1 cells were pre-treated for 2 h with probiotics, followed by HP-LPS stimulation for 60 or 120 min at a final concentration of 7,000 EU/ml. Cytoplasmic and nuclear extracts were then isolated using a Nuclear Extract kit (Active Motif, Inc.). Protein concentrations were determined with an enhanced bicinchoninic acid protein assay kit.

Each extract was mixed with x5 loading buffer and boiled for 5 min. The extracted proteins were then aliquoted and stored at -80˚C. A total of 30 μg of protein was loaded into each lane, separated via 8% SDS-PAGE and then electrotransferred to a polyvinylidene difluoride membrane. Membranes were blocked at room temperature with 5% fat-free dried milk in Tris buffered saline containing 0.1% Tween-20 (TBST) for 2 h. Following this, membranes were incubated overnight at 4˚C with primary antibodies anti-toll-like receptor 4 (TLR4; cat. no. AF1478-SP; 1:1,000; R&D Systems, Inc.), anti-NF-κB p65 (cat. no. 8242P; 1:1,000; Cell Signaling Technology, Inc.), and anti-NFKB inhibitor-α (IκBα; cat. no. 4814P; 1:1,000; Cell Signaling Technology, Inc.). Anti-lamin B1 (cat. no. 66095-1-Ig; 1:5,000; ProteinTech Group, Inc.) and anti-GAPDH (cat. no. 60004-1-Ig; 1:2,000; ProteinTech Group, Inc.) were used to verify equal protein loading. Following washing three times in TBST, the membranes were incubated with horseradish peroxidase conjugated goat anti-rabbit (cat. no. ZB-5301; 1:10,000; OriGene Technologies, Inc.) and goat anti-mouse (cat. no. ZB-2305; 1:5,000; OriGene Technologies, Inc.) secondary antibodies at room temperature for 1 h.

The proteins were visualized using an enhanced chemiluminescence reagent (Beyotime Institute of Biotechnology). The integrated optical density (IOD) of each band was analyzed using Image-Pro Plus software v6.0 (National Institutes of Health.). Each TLR4 and IκBα band value was normalized as the ratio of the IOD to the GAPDH band. Each NF-κB p65 band value was normalized as the ratio of the IOD to the lamin B1 band.

Statistical analysis. Statistical analyses were performed by SPSS v17.0 software (SPSS, Inc.). Graphs were generated using GraphPad Prism 5.2 software (GraphPad Software, Inc.). All data were presented as the mean value ± standard deviation. Multiple comparisons were evaluated by one-way analysis of variance followed by the Student-Newman-Keuls and least significant difference post hoc tests. The Dunnett method was used for comparisons with the control group. $P<0.05$ was considered to indicate statistical significance.

Results

GES-1 cell viability and growth rate following incubation with probiotics and CFS. The morphological characteristics of the probiotics and *H. pylori* were first evaluated following Gram staining (Fig. 1). The probiotics in this study were all Gram-positive bacteria. *L. acidophilus* was chain rod shaped, *L. bulgaricus*, *B. longum* and *B. licheniformis* were long rod shaped, *B. infantis* and *L. salivarius* were short rod shaped. *C. butyricum* contained giant spore and was enlarged at one end in a drum-hammer shape. *H. pylori* was a gram-negative bacterium, which is S-shaped or spiral. Furthermore, the viability of GES-1 cells incubated with CFS and different titers of probiotics (MOI of 100 and 1,000) was determined by assessing the percentage of cells not stained with trypan blue following 2, 4, 6 and 8 h of incubation. At a MOI of 100, the cytotoxicity results indicated that the CFS of *L. acidophilus*, *L. bulgaricus*, *L. salivarius*, *C. butyricum*, *B. infantis* and *B. longum* were toxic to GES-1 cells. The CFS of *B. licheniformis* did not show any toxicity towards GES-1 cells with an average survival rate at 8 h of 96%. *L. acidophilus*, *L. bulgaricus*, *L. salivarius*, *Clostridium butyricum*, *Bifidobacterium infantis*, *Bifidobacterium longum* and *B. licheniformis* did not show any toxicity towards GES-1 cells after 4 h of incubation, with an average survival rate of 97% and no significant differences between these groups and the control (Fig. 2A). However, GES-1 cell viability decreased gradually after 4 h. At 6 h, the most significant decrease in viability was observed in *B. longum*-treated cells, which had a 92% survival rate. Notably, the viability of these cells was significantly lower compared with the cells treated with the other six bacteria ($P<0.05$; Fig. 2A). GES-1 cell viability following *B. licheniformis* treatment decreased at the slowest rate, with an average survival rate of 99.7%. This was significantly higher than the survival rate of the cells treated with other bacteria ($P<0.05$; Fig. 2A). There was no significant differences in the average GES-1 survival rates following treatment with *L. acidophilus*, *L. bulgaricus*, *L. salivarius*, *C. butyricum*, *B. longum* and *B. infantis* at 6 h. At 8 h, the

Table I. Details of probiotic strains.

Species	Strain	Product
Lactobacillus acidophilus	CGMCC0460.2	Siliankang
Bifidobacterium infantis	CGMCC0460.1	Siliankang
Bifidobacterium longum	NQ1501	Jinshuangqi
Lactobacillus bulgaricus	NQ2508	Jinshuangqi
Clostridium butyricum	CGMCC N0.0313-1	Changlekang
Bacillus licheniformis	CMCC63516	Zhengchangsheng
Lactobacillus salivarius	ATCC11741	Taiwan Yaxin

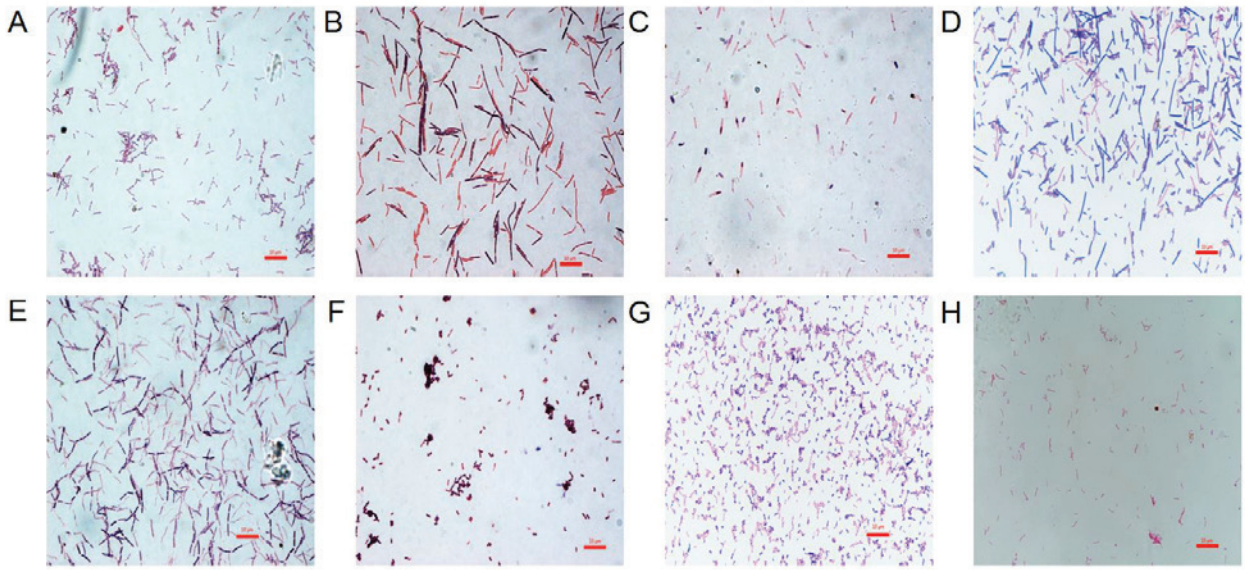

Figure 1. Morphological characteristics of various probiotic strains and *H. pylori*. (A) *Lactobacillus acidophilus*, (B) *Lactobacillus bulgaricus*, (C) *Clostridium butyricum*, (D) *Bifidobacterium longum*, (E) *Bacillus licheniformis*, (F) *Lactobacillus salivarius*, (G) *Bifidobacterium infantis* and (H) *H. pylori* gram staining (red or violet) micrographs under oil immersion (magnification, x1,000). *H. pylori*, *Helicobacter pylori*.

survival rate of the GES-1 cells decreased further with cells treated with *L. bulgaricus*, demonstrating significantly lower survival rate (82.3%) compared with the cells treated with the other six probiotics (P<0.05; Fig. 2A). Furthermore, the survival rate of cells treated with *B. licheniformis* was significantly increased (96.7%) at 8 h compared with cells treated with the other six bacteria (P<0.05; Fig. 2A).

At a MOI of 1,000, all seven probiotic strains exhibited no toxicity towards GES-1 cells at 2 h (Fig. 2B). However, GES-1 cell viability decreased following 4 h of incubation, with cells treated with *L. bulgaricus* (10.4% at 4 h) exhibiting significantly reduced viability compared with cells treated with the other six bacterial strains (P<0.05; Fig. 2B). GES-1 cell viability following *B. licheniformis* treatment demonstrated the slowest decrease, with the average survival rate of cells 100% at 4 h, which was significantly higher when compared with cells treated with the other bacterial strains (P<0.05; Fig. 2B). The viability of *B. licheniformis*-treated GES-1 cells decreased significantly after 6 h compared with the control group (P<0.05; Fig. 2B). Furthermore, the viability of these cells was 42.7% at 8 h, whereas GES-1 cells treated with the other six bacteria did not survive past 8 h. There were no significant differences between *L. acidophilus*-, *L. salivarius*-, *B. longum*-, *B. infantum*- and *C. butyricum*-treated cells (Fig. 2B). The probiotic half maximal inhibitory concentration (IC_{50}) for each group is listed in Table II, with *B. longum* demonstrating the lowest IC_{50} and *B. licheniformis* demonstrating the highest IC_{50}.

Adhesion of probiotics to GES-1 cells. Probiotics adhered to GES-1 cells with different cell adhesion indexes and cell adhesion rates (Table III; Fig. 3). Staining of GES-1 cells for each probiotic group varied because metabolites produced by different probiotics were able to change the pH values of the culture medium, thereby affecting viability and staining (28). The pH values of the culture medium were pH 4 (*L. acidophilus*), pH 5 (*L. bulgaricus*), pH 3.8 (*B. infantis*), pH 4.2 (*B. longum*), pH 4.8 (*L. salivarius*), pH 5.5 (*C. butyricum*) and pH 6.8 (*B. licheniformis*).

As indicated in Fig. 4, the cell adhesion index was consistent with the cell adhesion rate, with the highest to lowest results as follows: *L. acidophilus*>*L. bulgaricus*>

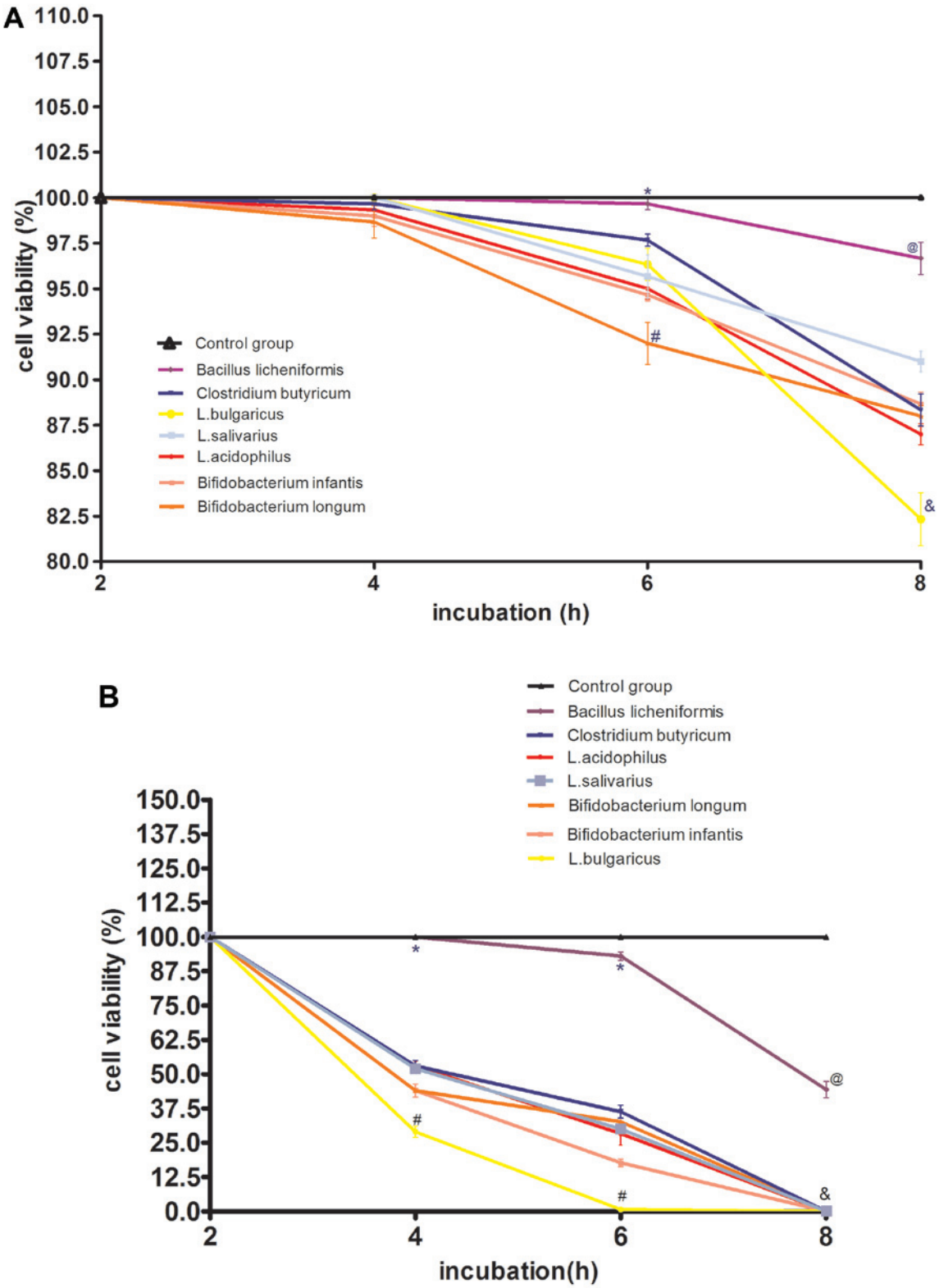

Figure 2. Effect of various probiotics strains on GES-1 cell viability. (A) GES-1 cell viability following culture with *L. acidophilus*, *L. bulgaricus*, *L. 0salivarius*, *Clostridium butyricum*, *Bifidobacterium longum*, *Bifidobacterium infantis or Bacillus licheniformis* at a MOI of 100 and (B) MOI of 1,000. (A) *P<0.05 vs. *L. acidophilus*, *L. bulgaricus*, *L. salivarius*, *Clostridium butyricum*, *Bifidobacterium longum*, *Bifidobacterium infantis*; $^{\#}$P<0.05 vs. *L. acidophilus*, *L. bulgaricus*, *L. salivarius*, *Clostridium butyricum*, *Bifidobacterium infantis*, *Bacillus licheniformis* and control group; $^{@}$P<0.05 vs. *L. acidophilus*, *L. bulgaricus*, *L. salivarius*, *Clostridium butyricum*, *Bifidobacterium infantis*, *Bifidobacterium longum* and control group; $^{\&}$P<0.05 vs. *L. acidophilus*, *L. salivarius*, *Clostridium butyricum*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bacillus licheniformis* and control group. (B) *P<0.05 cells vs. *L. acidophilus*, *L. bulgaricus*, *L. salivarius*, *Clostridium butyricum*, *Bifidobacterium longum*, *Bifidobacterium infantis*; $^{\#}$P<0.05 vs. *L. acidophilus*, *L. salivarius*, *Clostridium butyricum*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bacillus licheniformis* and control group; $^{@}$P<0.05 vs. *L. acidophilus*, *L. bulgaricus*, *L. salivarius*, *Clostridium butyricum*, *Bifidobacterium infantis*, *Bifidobacterium longum* and control group; $^{\&}$P<0.05 cells treated with *L. bulgaricus*, *L. acidophilus*, *L. salivarius*, *Clostridium butyricum*, *Bifidobacterium infantis*, *Bifidobacterium longum* vs. *Bacillus licheniformis* and control group. MOI, multiplicities of infection; *L*, *Lactobacillus*.

B. infantis>*B. longum*>*L. salivarius*> *B. licheniformis*> *C. butyricum*. There was no significant difference in the cell adhesion index between *C. butyricum* and *B. licheniformis* (Fig. 4A); however, there were significant differences between the other five bacterial groups when compared with each other (P<0.05; Fig. 4A). There was no significant difference in the cell adhesion rate between *L. bulgaricus* and *L. acidophilus*. In addition, no significant differences in the cell adhesion rate were observed between *B. longum*, *C. butyricum*, *B. licheniformis* and *L. salivarius* (Fig. 4B).

Live probiotics and CFS inhibit H. pylori growth. CFS from probiotic culture and live probiotics were screened for inhibitory effects against HP021 by measuring the diameters of inhibitory growth zones on agar plates (Table IV). Fresh MRS medium, used as a negative control, also exhibited significant activity against HP021 and formed inhibition zones of ~27 mm, which was larger than all the tested probiotics (P<0.05; Table IV). This suggested that there were likely unknown anti-HP substances in MRS medium. Probiotics consumed some of the anti-HP substances of MRS, and likely secreted some anti-HP substances. Live *L. bulgaricus* demonstrated significantly greater anti-*H. pylori* activity compared with the other live probiotics, with inhibition zones of ~25 mm (P<0.05; Table IV). Live *B. licheniformis* did not display any anti-*H. pylori* activity. By contrast, there were no significant differences in inhibition of *H. pylori* growth by live *L. acidophilus*, *B. infantis*, *B. longum*, *L. salivarius* and *C. butyricum*. The CFS from all the probiotics also inhibited *H. pylori* activities; however, there were no significant differences between the probiotic groups (Table IV). Similar results were observed when *H. pylori* ATCC43504 was used (data not shown).

Probiotics and CFS suppress adhesion of H. pylori to GES-1 cells. The urease activity of HP021 and ATCC43504 were examined to evaluate the effect of CFS and probiotics on HP021 and *H. pylori* ATCC43504 adherence to GES-1 cells. The adherence rate of *H. pylori* was 100% without probiotic or CFS treatment. Results indicated that pre- or post-treatment with *B. licheniformis* CFS did not reduce the adhesion of *H. pylori* to GES-1 cells compared with HP group (Fig. 5A). However, urease activity was significantly reduced following probiotic treatment, with the exception of *C. butyricum* and *B. licheniformis* treatment compared with the HP group (Fig. 5A). No significant differences between the two *H. pylori* strains were found (the data not shown). The adherence rate of *H. pylori* dropped to ~50% with *L. acidophilus* treatment. Compared with non-treated cells, the adherence rate of HP021 did not decrease with *C. butyricum* or *B. licheniformis* treatment (Fig. 5B). These results demonstrated that pre-treatment with *L. acidophilus*, *L. bulgaricus*, *L. salivarius*, *B. infantis* and *B. longum* inhibited the adherence of *H. pylori* to GES-1 cells.

Certain probiotics significantly affect IL-8 production in GES-1 cells. Under normal physiological conditions, GES-1 cells secrete a small amount of IL-8 (29). The addition of HP-LPS to GES-1 cells for 6 h significantly increased the IL-8 levels compared with non-treated cells (P<0.05; Fig. 6). No decrease in IL-8 secretion following treatment with the CFS of *B. licheniformis* was observed compared with HP-LPS group. However, following pre-treatment with *L. bulgaricus* and *L. salivarius*, there was a significant decrease in IL-8 secretion induced by HP-LPS compared with HP-LPS group (P<0.05; Fig. 6). Notably, IL-8 levels following HP-LPS plus *L. acidophilus*, *B. infantis* or *B. longum* treatment were higher compared with HP-LPS only treatment.

Probiotics attenuate the TLR4-NF-κB p65 signaling pathway following HP-LPS activation. To explore the molecular mechanism of *H. pylori* on GES-1 growth, expression levels of markers associated with inflammation, TLR-4, IκBα and NF-κB, p65 were detected. *H. pylori* ATCC43504 contains CagA protein that induces activation of NF-κB and IL-8 whereas HP021 does not contain CagA protein. Therefore, *H. pylori* ATCC43504 was selected for the NF-κB activation and IL-8 production experiment. *L. bulgaricus* reduced IL-8 production most significantly. Therefore, *L. bulgaricus* was selected for the NF-κB activation experiment. Treatment of GES-1 cells with 7,000 EU/ml of HP-LPS upregulated the expression of TLR-4, increased the degradation of IκBα and induced the translocation of NF-κB p65 into nucleus in a time-dependent manner (Fig. 7A and B). Pre-treatment for 2 h with viable *L. bulgaricus* (MOI of 100) significantly inhibited the effects of HP-LPS on the TLR4/NFκB pathway by increasing cytoplasmic IκBa and decreasing nuclear NF-κB p65 levels (Fig. 7C-E). The levels

Table II. GES-1 cell growth rate in the presence of various probiotic strains.

Probiotics	Half maximal inhibitory concentration (colony-forming units)
Bifidobacterium longum	$6.56x10^7$
Bifidobacterium infantis	$6.76x10^7$
Lactobacillus acidophilus	$8.57x10^7$
Lactobacillus salivarius	$1.55x10^8$
Lactobacillus bulgaricus	$2.09x10^8$
Clostridium butyricum	$3.0x10^8$
Bacillus licheniformis	$3.33x10^9$

Table III. Cell adhesion index and adhesion rate at the same concentration onto GES-1 cells.

Probiotic strain	Adhesion index (%)	Adhesion rate (%)
Lactobacillus acidophilus	891.2±24.35	56.6±8.35
Bifidobacterium infantis	394.4±31.00	37.8±7.12
Bifidobacterium longum	328.2±23.04	21.2±5.12
Clostridium butyricum	24.4±12.78	11.2±0.84
Bacillus licheniformis	38.00±7.97	17.8±4.60
Lactobacillus bulgaricus	499.2±27.83	50.0±8.12
Lactobacillus salivarius	229.8±22.19	20.6±5.41

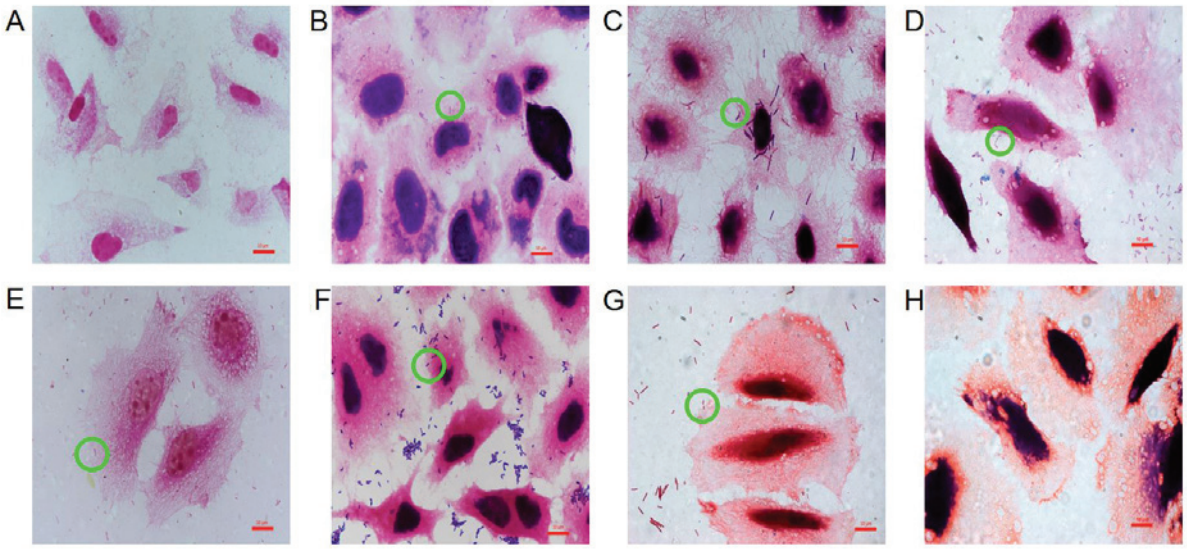

Figure 3. Binding of various probiotic strains to GES-1 cells. (A) Micrographs demonstrating gram staining of the control group with no probiotics. (B) GES-1 cells cultured with *Lactobacillus acidophilus*, (C) *Lactobacillus bulgaricus*, (D) *Lactobacillus salivarius*, (E) *Bifidobacterium longum*, (F) *Bifidobacterium infantis*, (G) *Bacillus licheniformis* or (H) *Clostridium butyricum* following Gram staining (red or violet). Green circles highlight probiotics that have adhered to GES-1 cells.

of NFκB and TLR-4 in *L. bulgaricus* pre-treatment 60 and 120 min groups were significantly lower than those of HP-LPS 60 and 120 mins groups, respectively (P<0.05; Fig. 7C and E). The level of IκBα in *L. bulgaricus* pre-treatment 120 min group was significantly higher than HP-LPS 120 min group (P<0.05; Fig. 7D). These results indicated that the TLR4/NF-κB signaling pathway may be a critical target for probiotics to alter GES-1 cell biological functions.

Discussion

The present study assessed the effects of live probiotics and their CFS on *H. pylori* growth, adherence to gastric epithelial cells and *H. pylori*-induced inflammation. The findings demonstrated that *L. acidophilus* and *L. bulgaricus* significantly inhibited the adherence of *H. pylori* to GES-1 cells and also decreased IL-8 production by GES-1 cells following stimulation with HP-LPS. In addition, *L. bulgaricus* inhibited the TLR4/IκBα/NFκB signaling pathway in a time-dependent manner.

H. pylori causes gastritis, as well as gastric and duodenal ulcers (2,30). In the past few decades, *H. pylori* treatment has improved due to the administration of PPI plus antibiotics. However, the emergence of drug-resistant *H. pylori* strains has complicated the eradication of the bacteria. Therefore, research is focusing on developing new strategies for treating *H. pylori*. Recent clinical trials have demonstrated that the administration of certain exogenous probiotics may improve the eradication of *H. pylori* (31). In addition, some probiotic strains can attenuate the mucosal inflammation induced by *H. pylori* (32). However, the molecular mechanism of these biological effects remains unclear.

The present study identified that the anti-*H. pylori* effects of live probiotics or their CFS were highly strain dependent. Seven probiotic strains commonly used in clinical settings were chosen. The *H. pylori* standard strain (ATCC43504), containing CagA and VacA virulence factors, and the clarithromycin and levofloxacin-resistant HP021 strain, originally isolated from the gastric mucosa of a patient, were also investigated. Results revealed that all seven probiotics significantly affected GES-1 cell viability following longer incubation times and higher MOIs. The CFS of *Lactobacillus* culture is a 96-h fermentation product containing high concentrations of acetic acid and other bactericidal substances. The present study determined that *Lactobacillus* CFS has a pH of 2, which was toxic to gastric epithelial cells *in vitro*. GES-1 cell death occurred within 2 h when treated with *L. acidophilus*, *B. infantis*, *B. longum*, *L. salivarius*, *L. bulgaricus* and *C. butyricum* CFS. Only *B. licheniformis* CFS had a pH of 7 therefore, was not considered toxic to GES-1 cells. Hence, only *B. licheniformis* CFS was selected for investigation into the adhesion of *H. pylori* to GES-1 cells and IL-8 production.

It is well established that adhesion to mucosal surfaces is a key step in the pathogenesis of *H. pylori* (33). The inhibition of *H. pylori* colonization by probiotics is strain specific. Chen *et al* (18) reported that CFS, live and dead *lactobacilli* inhibits *H. pylori* adhesion to SGC7901 cells. Hsieh *et al* (16) identified that *L. johnsonii* MH-68 and salicinius AP-32 effectively suppress *H. pylori* viability and reduce *H. pylori* colonization in the gastric mucosa of mice. Aiba *et al* (34) proved that *L. salivarius* is capable of producing a high amount of lactic acid and inhibiting the growth of *H. pylori*. In the present study, five probiotic strains adhered to GES-1 cells, with *L. acidophilus* displaying the strongest adhesive ability, followed by *L. bulgaricus*. However, *C. butyricum* and *B. licheniformis* did not demonstrate any notable adhesive ability. Further study is required to identify the mechanism of adhesion to the cell surface of *L. acidophilus* and *L. bulgaricus*. Pre-treatment with *L. acidophilus*, *L. bulgaricus*, *L. salivarius*, *B. infantis*

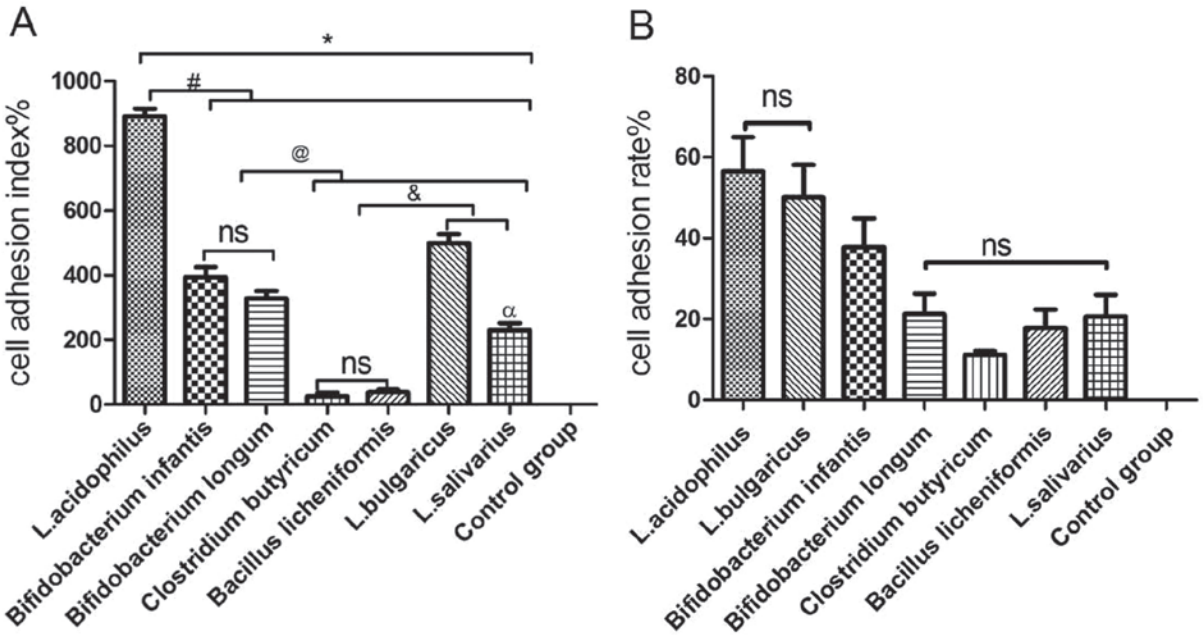

Figure 4. Adhesion of various probiotic strains to GES-1 cells. (A) Cell adhesion index of *L. acidophilus*, *L. bulgaricus*, *L. salivarius*, *Clostridium butyricum*, *Bifidobacterium longum*, *Bifidobacterium infantis* and *Bacillus licheniformis* to GES-1 cells. (B) Cell adhesion rate of *L. acidophilus*, *L. bulgaricus*, *L. salivarius*, *Clostridium butyricum*, *Bifidobacterium longum*, *Bifidobacterium infantis* and *Bacillus licheniformis* to GES-1 cells. *P<0.05 vs. control group; $^{\#}$P<0.05 vs. *L. acidophilus*; $^{@}$P<0.05 vs. *Bifidobacterium infantis* and *Bifidobacterium longum*; $^{\&}$P<0.05 vs. *Clostridium butyricum* and *Bacillus licheniformis*; $^{\alpha}$P<0.05 vs. *L. bulgaricus*. *L, Lactobacillus*; ns, not significant.

Table IV. HP021 growth in the presence of live probiotics and cell-free supernatant.

	Average zone of inhibition (mm)	
Probiotic strain	Live probiotic	Cell-free supernatant
Lactobacillus acidophilus	20.33±1.53	21.00±1.00
Bifidobacterium infantis	20.00±1.00	20.67±1.53
Bifidobacterium longum	20.67±0.58	21.91±0.58
Clostridium butyricum	22.33±0.58	21.33±0.58
Lactobacillus bulgaricus	25.33±0.58^{b}	22.33±0.58
Bacillus licheniformis	0	22.67±0.58
Lactobacillus salivarius	21.00±1.00	22.24±0.58
Man, Rogosa and Sharpe broth	27.00±1.00^{a}	27.00±1.00^{a}

^{a}P<0.05 vs. *L.acidophilus*, *L.bulgaricus*, *L.salivarius*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bacillus licheniformis* and *Clostridium butyricum*; ^{b}P<0.05 vs. *L. acidophilus*, *L. salivarius*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bacillus licheniformis* and *Clostridium butyricum*.

and *B. longum* reduced the ability of *H. pylori* to adhere to GES-1 cells to differing extents. The inhibitory effects of *L. acidophilus* and *L. bulgaricus* were greater than *L. salivarius*, *B. infantis* and *B. longum* at the same concentration. Notably, the CFS of *B. licheniformis* did not reduce the adhesion of *H. pylori* to GES-1 cells compared with the HP group. The inhibitory effect of probiotic pre-treatment against *H. pylori* adherence is likely mediated via increasing production of mucin (35) or competition to bind *H. pylori* adhesion sites by probiotics (36). The adhesion of *H. pylori* to GES-1 cells was not inhibited when cells were treated with probiotics post-infection. Therefore, it appears that probiotics cannot reduce the adhesion rate of *H. pylori* when it is already adhered to gastric epithelial cells. Therefore, probiotics may be more effective in a preventive rather than therapeutic role.

Certain probiotic strains produce bactericidal substances that are either secreted into the culture supernatant or expressed on the cell surface, which can significantly inhibit *H. pylori* (37). The agar plate diffusion assays performed in the present study determined that live bacteria and their CFS from *L. acidophilus*, *B. infantis*, *B. longum*, *L. bulgaricus*, *C. butyricum* and *L. salivarius* inhibited the growth of *H. pylori* ATCC43504 and HP021 strains. Live *B. licheniformis* did not appear to inhibit the growth of *H. pylori* ATCC43504 or HP021, but the CFS of *B. licheniformis* did demonstrate anti-*H. pylori* properties. A possible mechanism of action is the secretion of lactic acid by the probiotics, as the metabolic end products of lactic acid fermentation and organic acids are capable of interfering with the growth of pathogens. Notably, El-Adawi *et al* (37) demonstrated that lactic acid may be a potent antimicrobial. However, *B. licheniformis* is not a lactic acid-producing bacterium, and the nature of the antimicrobial substance contained within its CFS requires further characterization.

Human immunity plays an important role in the development of clinical diseases. Pro-inflammatory cytokine production occurs during *H. pylori* infection, with the inflammatory reactions potentially leading to chronic inflammation rather than eliminating *H. pylori* (38,39). The transcription factor NF-κB can be activated by IL-1β,

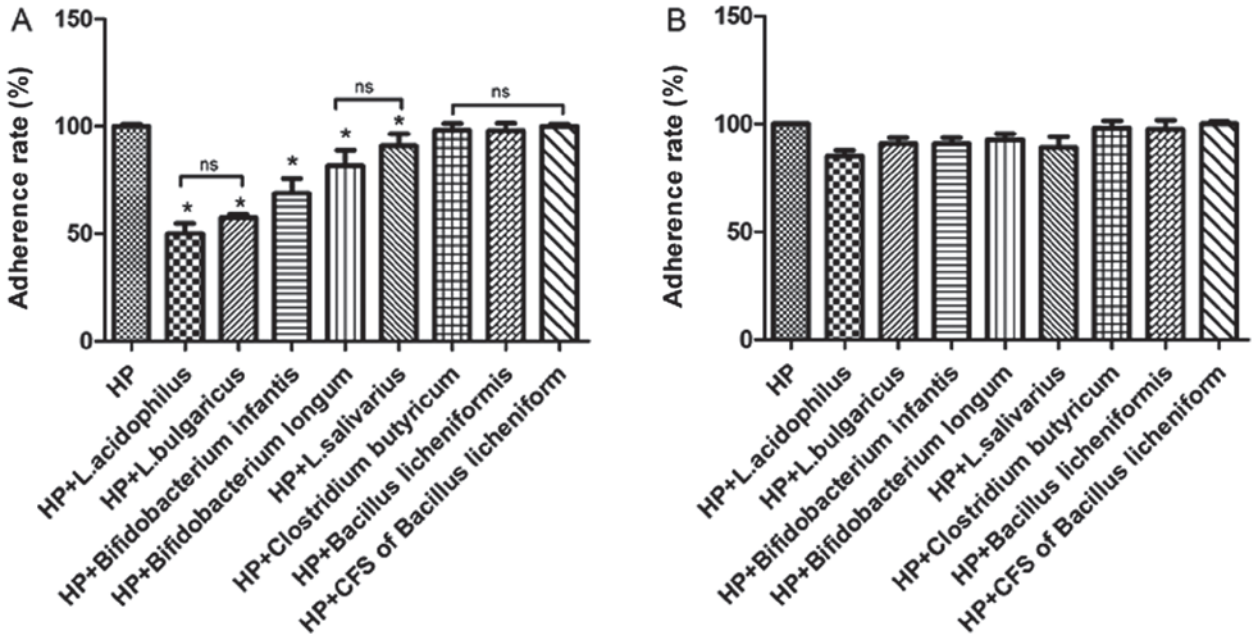

Figure 5. Adherence rate of HP to GES-1 cells in the presence of different probiotics at a MOI of 100. (A) Adherence rate of HP to GES-1 cells pretreated with *L. acidophilus*, *L. bulgaricus*, *L. salivarius*, *Clostridium butyricum*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bacillus licheniformis* and CFS of *Bacillus licheniformis*. (B) Adherence rate of HP to GES-1 cells treated with *L. acidophilus*, *L. bulgaricus*, *L. salivarius*, *Clostridium butyricum*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bacillus licheniformis* and CFS of *Bacillus licheniformis* following infection (post-treated). *P<0.05 vs. HP group. HP, *Helicobacter pylori; L*, *Lactobacillus*; CFS, cell-free supernatant; ns, not significant.

LPS, peptidoglycan and tumor necrosis factor-α during *H. pylori* infection (40). NF-κB is critical modulator of cytokine expression (41). TLRs are cell transmembrane and pathogen-associated molecular pattern receptors that have a central role in the recognition of microbial pathogens and may be a first line of immunity against *H. pylori* (42). HP-LPS-induced inflammation in gastric mucosa demonstrates similar pathological characteristics to the mucosal inflammation initiated by *H. pylori* infection (43). The present study determined that *H. pylori* infection induced TLR4 and IL-8 pro-inflammatory cytokine expression *in vitro*. The findings demonstrated that pre-treatment with viable *L. bulgaricus* for 2 h prevented TLR4 signaling and IL-8 production stimulated by HP-LPS. This strongly supports the hypothesis that certain soluble proteins secreted by *L. bulgaricus* and/or cell-bound components of *L. bulgaricus* exert inhibitory effects on the TLR4 signaling pathways in GES-1 cells. Other cytokines such as IL-1β, IL-10, IL-6 and Smad family member 7 are also involved in the response of *H. pylori* to epithelial cells (17,44). The present study suggested that suppression of the TLR4/NF-κB signaling pathway occurred in a time-dependent manner and was mediated through the stabilization of IκBα.

In conclusion, the present study identified that two probiotic strains, *L. acidophilus* and *L. bulgaricus*, were effective in reducing the *H. pylori* load. One possible mechanism of *L. bulgaricus* on *H. pylori* activity was implied to be via modulation of the TLR4/IκBα/NF-κB signaling pathway. Considering the safety and health function of probiotics, food containing *L. acidophilus* and *L. bulgaricus* may have potential as an adjuvant therapy for gastric diseases caused by *H. pylori*, and displays promise as a preventive measure against *H. pylori* infection.

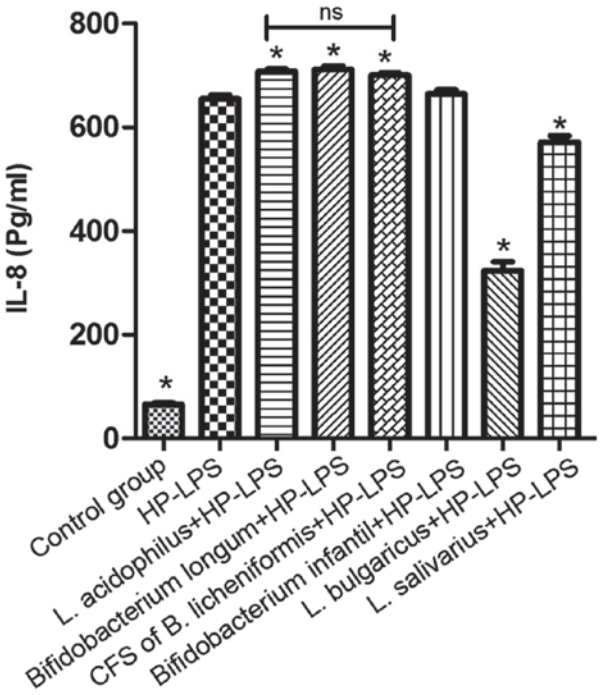

Figure 6. Effect of *L. acidophilus*, *L. bulgaricus*, *L. salivarius*, *Bifidobacterium infantis*, *Bifidobacterium longum* CFS of *Bacillus licheniformis* on IL-8 production following HP-LPS treatment. *P<0.05 vs. HP-LPS. HP, *Helicobacter pylori*; LPS, lipopolysaccharide; IL, interleukin; NS, not significant, *L, Lactobacillus*.

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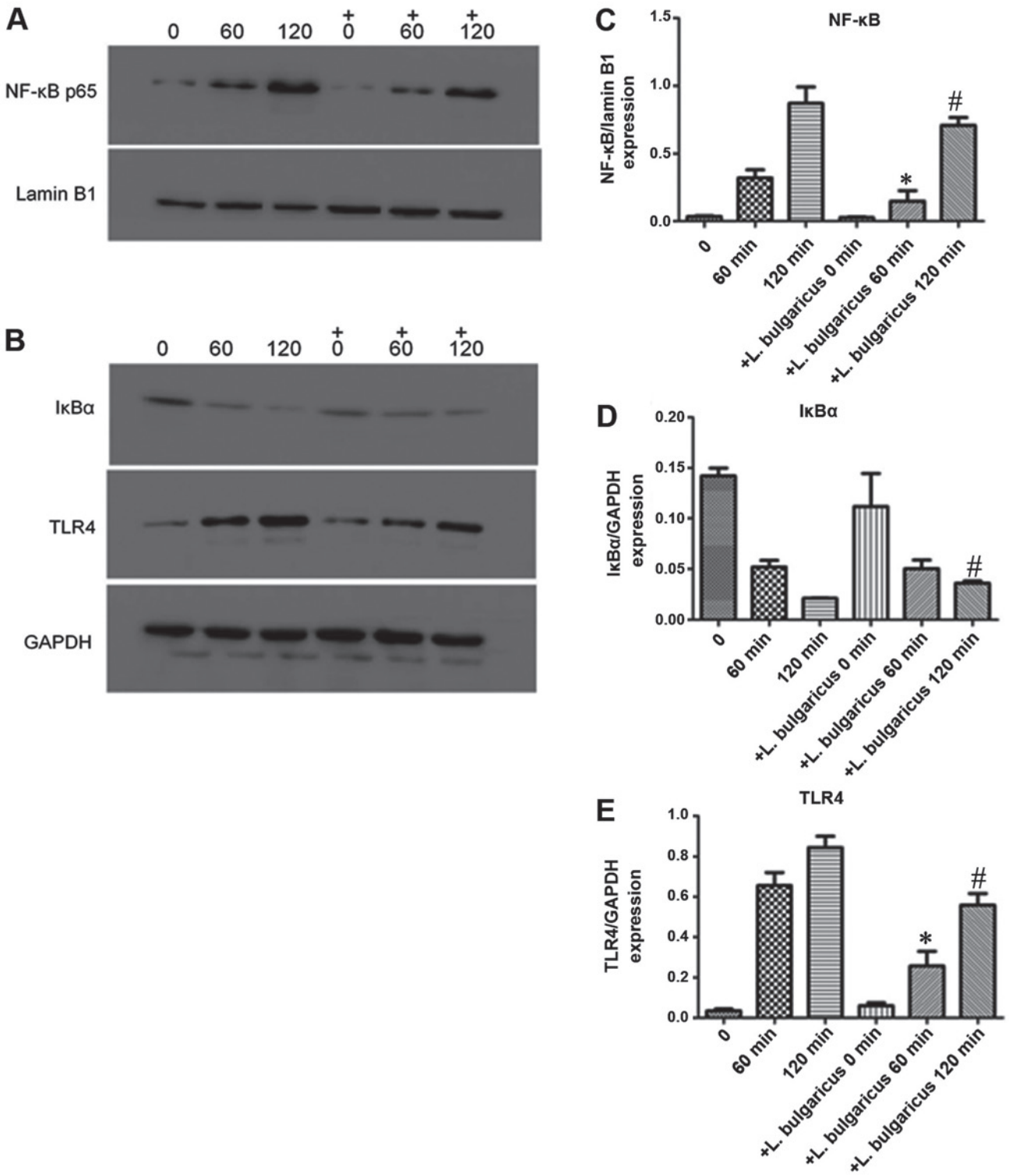

Figure 7. TLR4, IκBα and NF-κB p65 expression by GES-1 cells treated with HP-LPS in the absence or presence of live *L. bulgaricus*. (A) Representative immunoblots of NF-κB p65 compared with lamin B1 in nuclear extracts. (B) Representative immunoblots of TLR4 and IκBα compared with GAPDH in cytoplasm extracts. (C) Quantification of NF-κB p65, (D) IκBα and (E) TLR4 protein levels following different treatments. *P<0.05 vs. HP-LPS 60 min group; #P<0.05 vs. HP-LPS 120 min group. NF-κB, nuclear factor-κB; TLR4, toll-like receptor 4; IκBα, NFKB inhibitor-α; HP, *Helicobacter pylori*; LPS, lipopolysaccharide; *L*, *Lactobacillus*.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SHY, ZL and GLH performed the experiments and analyzed data. LY designed the experiment, analyzed data and wrote the manuscript. LDY analyzed data and was involved in discussions about the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The clinical study was approved by the Ethics Committee of Shengjing Hospital and the patient provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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