# *Bacillus coagulans* TCI803 confers gastroesophageal protection against *Helicobacter pylori*-evoked gastric oxidative stress and acid-induced lower esophageal sphincter inflammation

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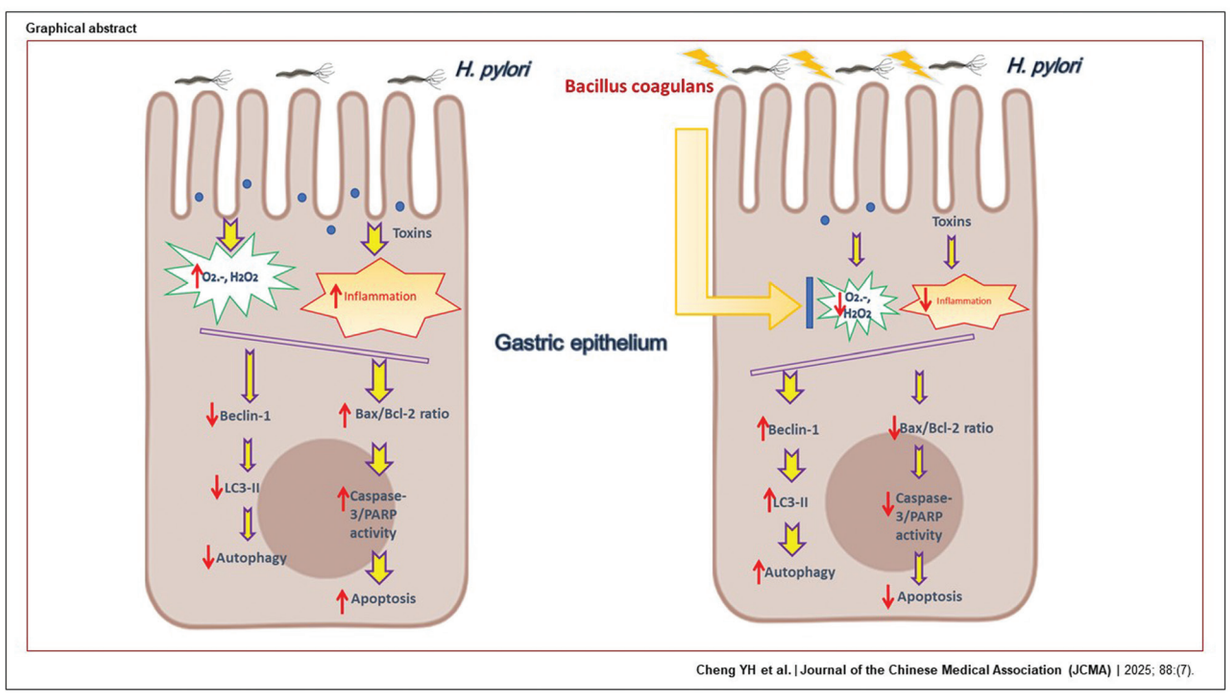
## Abstract

**Background:** Probiotic *Bacillus coagulans* (BC) may have an impact on gastrointestinal protection. This study was designed to investigate the BC effects on *Helicobacter pylori* (*H. pylori*) induced gastric inflammation in mice and acid-induced lower esophageal sphincter (LES) dysfunction in rats. We determined the oxidative stress/apoptosis/autophagy signaling pathways in *H. pylori*-induced gastric inflammation and HCl-evoked LES inflammation.

**Methods:** *H. pylori*-induced gastric inflammation was used in 40 mice and HCl-evoked LES inflammation in 40 Wistar rats. Western blot, immunohistochemistry and cytokine array were used to determine the pathophysiologic mechanisms.

**Results:** *H. pylori* increased leukocyte infiltration-mediated inflammation and the expression levels of gastric cytokines, 3NT/4HNE-mediated oxidative stress, and Bax/Caspase3-mediated apoptosis, but decreased Beclin-1/LC3-II-mediated autophagy in the mice gastric mucosa. BC treatment decreased inflammation, cytokines release, oxidative stress, and apoptosis, and reversed autophagy in *H. pylori*-infected gastric mucosa. To replace the antibiotic therapy, BC TCI803 was selected to inhibit *H. pylori* infection for commercial interests. Saline esophageal infusion evoked an increase in LES pressure and efferent vagus nerve activity during the emptying



phase. However, HCl dysregulated LES motility esophageal infusion by a decrease in threshold pressure, intercontraction interval and an increase in efferent vagus nerve activity. BC treatment significantly recovered the level of threshold pressure, intercontraction interval, and depressed the enhanced efferent vagus nerve activity. In vitro LES wire myography data displayed that HCl-treated LES significantly decreased the contractile response to acetylcholine. BC treatment significantly restored the contractile response to acetylcholine in LES wire myography. LES after HCl stimulation significantly increased leukocyte infiltration-mediated inflammation, whereas BC treatment effectively reduced the leukocyte infiltration-mediated inflammation in the HCl-treated LES.

**Conclusion:** BC via anti-oxidation and anti-inflammation confers gastroesophageal protection against *H. pylori* involved oxidative stress/inflammation/apoptosis/autophagy signaling in mice with gastric inflammation and HCl-induced LES dysregulation and inflammation.

**Keywords:** Apoptosis; Autophagy; *Bacillus coagulans*; *Helicobacter pylori*; Lower esophageal sphincter

**Lay Summary:** Probiotic *Bacillus coagulans* TCI803 (BC) may have impact on gastrointestinal protection. This study found that the BC protective effects on *Helicobacter pylori* (*H. pylori*) induced gastric inflammation in mice and acid-induced lower esophageal sphincter (LES) dysfunction in rats. We demonstrated that BC reduced the oxidative stress/apoptosis/autophagy signaling pathway in *H. pylori*-induced gastric inflammation and HCl-evoked LES inflammation. In summary, we suggest that BC via anti-oxidation and anti-inflammation confers gastroesophageal protection against *H. pylori* involved oxidative stress/inflammation/apoptosis/autophagy signaling in mice with gastric inflammation and HCl-induced LES dysregulation and inflammation.

## 1. INTRODUCTION

*Helicobacter pylori* (*H. pylori*) is a gram-negative bacterium, which can reside in the gastric mucosa and is one of the most prevalent bacterial infections worldwide.[1–5] *H. pylori* has been classified as a class 1 carcinogen by the World Health Organization's International Agency for Research on Cancer for causing gastric carcinoma and mucosa-associated lymphoid tissue lymphoma.[1] The epithelial cells response to *H. pylori* infection is a complex process reflecting the interactions among several factors, including bacterial virulence factors, specific receptor-linked signaling pathways, and the host immune response.[1–5] The virulence factors secreted by *H. pylori* activate the oxidative stress signaling pathway mediated apoptosis and autophagy and chronic inflammatory response in the epithelial cells.[6–8] Although patients infected with *H. pylori* develop an inflammatory response by recruiting and activating immune cells, the infection, unless treated with antibiotics, persists throughout the life of infected individuals. Early and successful infection treatment has been shown to prevent *H. pylori*-induced gastric disorder and overall medical costs. To date, many antibiotic-containing therapies have been recommended as the first-line and rescue therapy for treating *H. pylori* infection.[9,10] However, the emergence of an increasing number of antibiotic-resistant *H. pylori* strains has led to a decline in the eradication rates. Thus, a potential strategy for developing a non-antibiotic *H. pylori* therapy is required.

Gastroesophageal reflux disease (GERD) is one of the common diseases of the upper gastrointestinal system involving the pathological reflux from the gastric lumen to esophageal tract. The most important factor for GERD pathogenesis could be the inflammation and dysregulation of the lower esophageal sphincter (LES).[11] The LES basal tone is primarily myogenic and can be modulated by enteric motor neurons, the parasympathetic and sympathetic extrinsic nervous system, and several neurohumoral substances.[12] LES motility dysfunction associated with inflammation is an important factor in GRED pathogenesis, esophageal dysmotility, and esophageal hypersensitivity.[13] Dysregulated LES activity in GERD often causes recurrent, angina-like, retrosternal chest pain, after excluding a cardiac cause. GERD patients are usually treated with medical, endoscopic, and surgical therapeutics.[13]

Probiotics are a potential strategy to prevent and treat many gastrointestinal diseases[14] using several gastrointestinal protective effects including increasing the mucus layer, protecting the gastrointestinal epithelium integrity[15], and downregulating the expression of inflammatory cytokines.[16] *Bacillus coagulans* (BC) has been reported to effectively inhibit *H. pylori* to some extent, with rare adverse events, and thus may reduce the burden of antibiotic resistance.[17] Shinde et al[18] reported that supplementation with probiotic BC spores ameliorates gut inflammation in an inflammatory bowel disease mouse model. In addition, BC treatment had a protective effect on acute hepatic injury by a significant improvement in liver function, inflammation, and hepatocyte necrosis, similar to the well-known hepatoprotective agent silymarin.[19] Among several BC, BC clones, TCI803 has been tested in preliminary data displaying higher resistance to high temperature, gastric acid, and bile salt and could smoothly pass the gastric route and stable proliferation. In addition, to replacing antibiotic therapy, the BC TCI803 was selected to inhibit *H. pylori* infection for commercial interests. We previously indicated that the use of anti-*H. pylori* treatment could inhibit *H. pylori*-enhanced reactive oxygen species (ROS) production mediated Bax/Bcl-2-mediated apoptosis but promote *H. pylori*-related Beclin-1/LC3 II-mediated autophagy leading to gastric protection.[8] Therefore, we suggest that the anti-*H. pylori* effect using BC may regulate the oxidative stress, inflammatory cytokines, and the interaction between apoptosis and autophagy in *H. pylori*-related diseases. In addition, we also determined the BC protective effect on HCl-induced LES motility in the rat in vitro and in vivo.

## 2. METHODS

### 2.1. TCI83 strain isolation and identification

Lactobacillus BC TCI803 were obtained from TCI Co., Ltd. (Taipei, Taiwan). The plate was directly coated with natural fermented milk (MRS + 0.5% cystine), cultured in an anaerobic

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environment at 37°C for 2 days. The colonies on the plate were selected for streaking. A single colony was used for colony PCR, and lactic acid bacteria 16S were amplified using colony PCR. The PCR products were analyzed and compared using NCBI BLAST. The bacterial strain was identified and confirmed to be BC. The isolated strains were named BC TCI803 and cultured in MRS broth containing 0.5% cystine for 2 days at 37°C. The cultured broth was mixed with the same volume of 50% glycerol and stored in −80°C for long-term preservation.

### 2.2. The BC TCI803 strain manufacturing process

The MRS medium with some modification was used for BC TCI803: 30 g/L yeast peptone, 30 g/L glucose, 1 g/L $MgSO_4$-$7H_2O$, 3 g/L $KH_2PO_4$, 6 g/L $K_2HPO_4$, 2 g/L sodium acetate, 1.7 g/L citric acid, 0.1 g/L manganese sulfate and 1 g/L polysorbate 80. After sterilization (121°C, 20 minutes), BC TCI803 was added, and fermentation was then carried out (2 days at 37°C). After fermentation was terminated, the bacterial mud was collected through centrifugation and mixed with preservation agents for freeze drying. The freeze-dried product was ground, sieved, and packaged. The probiotic powder was sampled to confirm the specification and stored at −20°C for further use.

### 2.3. *H. pylori* strain

A *cagA-/vacA*-positive clarithromycin-sensitive strain of *H. pylori* was obtained from gastric biopsy specimens from a patient with a duodenal ulcer, after obtaining informed consent from the National Taiwan University Hospital. The standard strain ATCC 43504 was used as the internal control. A properly executed written informed consent in the Chinese language was obtained from each subject before entering the subject into the trial. Each investigator provided a copy of the IRB-approved informed consent to every subject and a signed copy was maintained in the subject's record file. Attention was directed to the basic elements that were required to be incorporated into the informed consent under ICH guideline for GCP and in accordance with the latest version of the Declaration of Helsinki (adopted by the 18th World Medical Assembly, Helsinki, Finland, 1964 and amended by the 64th WMA General Assembly, Fortaleza, Brazil, October 23). The protocol was approved by the Clinical Trial/Research Ethics Committee of National Taiwan University Hospital with the approval number (NTUH-REC No.: 201304065RIND). The blood agar plate was prepared using brain heart infusion (BHI) agar with 10% sheep blood, 1% IsoVitalex, and antibiotics. The *H. pylori* suspension concentration was adjusted to about $10^5$ CFU/L and cultured on the blood agar plates at 37°C under microaerophilic conditions (5% $O_2$, 10% $CO_2$, 85% $N_2$) in duplicate. After 3 to 5 days, the situation of bacterial growth was observed and analyzed in the agar plates. Test strains were grown as described previously[8] and stored at −80°C until use. The medium is prepared using secondary filtered water and mixed with different BC concentrations to prepare different medium concentrations, after 3 days of strain culture. The groups were compared with the control group, and the growth of different BC strains was observed to determine whether the concentration of this group has a bacteriostatic effect.

### 2.4. Anti-*H. pylori* experiment (disc diffusion)

First, *H. pylori* was thawed and cultured from the frozen state. The *H. pylori* suspension concentration was collected and adjusted to about $10^5$ CFU/L, and then moved to 37°C microaerophilic conditions (5% $O_2$, 10% $CO_2$, 85% $N_2$), BHI agar containing 10% sheep blood, 1% IsoVitalex additive, and brain heart extract agar (BHI agar). The medium was prepared using secondary filtered water and mixed with different BC TCI803 *Lactobacillus bacillus* product concentrations to prepare different concentrations (5, 50, and 500 μg/10 μL) of medium. After 3 days of strain culture, the groups were compared with the control group. The growth of different BC TCI803 *Lactobacillus pylori* colonies was observed to determine whether the concentration of this group has a bacteriostatic effect. A positive control amoxicillin disc was used (BD, Franklin Lakes, NJ, USA).

### 2.5. *H. pylori*-infected mouse model

Specific-pathogen-free male BALB/c mice aged 5 to 6 weeks were purchased from the BioLASCO Taiwan Co. Ltd. (Taipei, Taiwan) and housed at the Experimental Animal Center, National Taiwan Normal University, at a constant temperature and with a consistent light cycle (lights on from 07:00 to 18:00). Food and water were provided ad libitum. All surgical and experimental procedures were approved by the Institutional Animal Care and Use Committee of National Taiwan Normal University (Approval number 110006 on April 23, 2021) and were in accordance with the guidelines of the National Science Council of Republic of China and ARRIVE guidelines. The mouse model was modified from our previous study.[8] Specifically, bacteria were recovered at 37°C for 3 days under microaerophilic conditions, transferred to Brucella broth supplemented with 5% fetal bovine serum, 1% IsoVitalex, and antibiotics, and maintained for 48 hours. The concentration was adjusted to about $10^{11}$ bacteria/L for inoculation. Mice (n = 40 each) were divided into five groups for duplicate experiments. While mice in group A (uninfected control) received distilled water only, four groups (B-E) of mice were inoculated intragastrically two times on successive days with 0.5 mL of bacterial suspension. Two weeks after inoculation, mice in group B (infected control) received only distilled water for 14 days, whereas mice in group C (antibiotics therapy) were treated twice daily for 7 days with 0.5 mL of distilled water containing ampicillin (200 μg/mL, A0166, Sigma-Aldrich, Saint Louis, MO, USA). Mice in groups D and E were post-treated with 0.5 mL of distilled water containing low-dose BC (5 mg/kg) or high-dose BC (20 mg/kg) for 2 weeks after *H. pylori* inoculation.

Four weeks after treatment, the mice were sacrificed using anesthesia with 0.5 mL of 50% urethane. The mouse stomachs were resected and longitudinally divided into two parts for histological and biochemical examination. Gastritis was graded by a pathologist without knowledge of the treatment protocol, according to the updated Sydney system.[20] The presence of *H. pylori* was identified by histology and bacterial culture as described previously.[8]

### 2.6. Gastric acid test

The stomach was exposed before the anesthesia sacrifice. The gastric pylorus was clamped with a clamp, and the gastric juice collected after 2 hours and 3000 ×g was detached for 5 minutes. Gastric volume, acidity and pH were measured. Total acidity was titrated in 0.1 N NaOH and pH was measured with a pH meter.

### 2.7. Serum Gastrin-1 measurement

Gastrin-1 was measured using an ELISA assay (Mouse Gastrin ELISA Kit; EBL Biotechnology, Taipei City, Taiwan, A3733, Antibodies.com).

### 2.8. Animal surgery for LES pressure measurement

Adult male Wistar rats weighing 220 to 240 g were anesthetized with urethane (1.2 g/kg intraperitoneally). Body temperature was maintained at 36.5°C to 37.0°C using an infrared light and monitored with a rectal thermometer. PE50 catheters were placed in the left carotid artery to measure heart rate and arterial blood pressure (ABP) using an ADI system (PowerLab/16S; ADI Instruments, Pty Ltd, Castle Hill, Australia) with a transducer (P23 1D; Gould-Statham, Quincy, CA), and in the left femoral vein for tested drug or anesthetic

administration when needed. Middle line laparatomy was performed to expose lower esophagus, LES, and one vagus nerve branch near LES. The trachea was exposed via a midline cervical incision and then intubated with an endotracheal tube for airway protection. A heparinized cannula was inserted into the brachial artery and connected to an external transducer for constant blood pressure monitoring. Body temperature was kept at 36.5°C to 37°C using an infrared light and monitored using a rectal thermometer. Heart rate was determined using a tachograph triggered by the arterial pulsations. All hemodynamic responses were recorded using a Gould polygraph (RS3400) with a transducer (P23 1D; Gould-Statham). Catheters are placed in the left femoral artery for continuous blood pressure recordings.

### 2.9. Grouping

The 40 rats were divided into following groups (n = 8 in each group): Control group with saline infusion, HCl stimulation with HCl infusion, HCl stimulation followed by posttreatment of 5 mg/kg BC (post-low BC), HCl stimulation followed by posttreatment of 20 mg/kg BC (post-high BC), pretreatment of 5 mg/kg BC followed by HCl stimulation (pre-low BC) and pretreatment of 20 mg/kg BC followed by HCl stimulation (pre-high BC). The BC was orally fed to the rat once daily.

### 2.10. Intraesophageal solution perfusion

A long flexible PE50 tube was passed into the esophagus for intraluminal saline or HCL perfusion. The tube tip was positioned at the distal esophagus, just proximal to the LES. In the control group, normal saline; and in the experimental group, 0.36 N hydrochloric acid (HCl, pH = 2) was perfused via the intraesophageal tube into the distal esophagus lumen near LES. Normal saline and 0.36 N HCl, diluted in normal saline (0.9% sodium chloride), were given at a rate of 0.1 mL/min using an infusion pump (Infors AG, CH-4103;

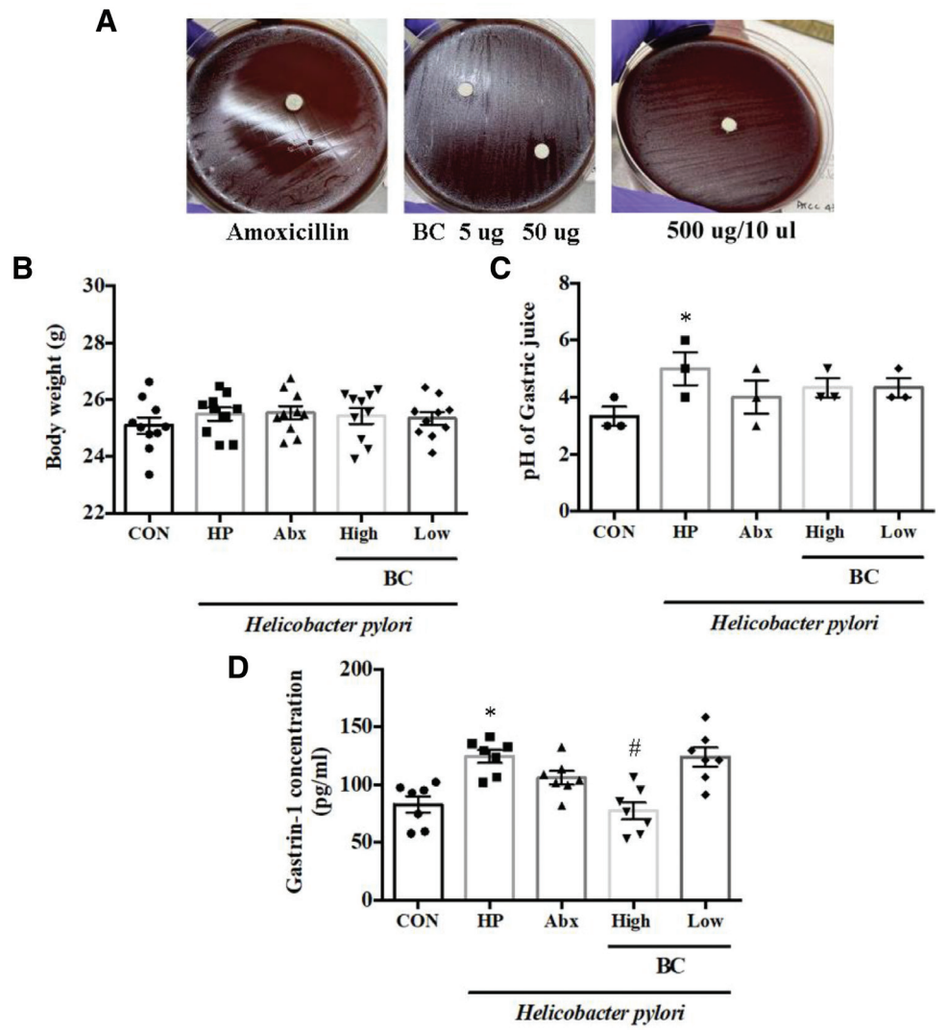


**Fig. 1** The anti-HP growth by different concentration of BC or amoxicillin was determined with the disc diffusion method (A). The baseline parameters of body weight (B, n = 10 in each group), pH of gastric juice (C, n = 3 in each group), and Gastrin-1 concentration (D, n = 7 in each group) among these five groups. The level of body weight is similar among these five groups. The pH level is significantly increased in the HP group vs CON group, whereas the pH level was reduced in antibiotics (Abx) treated group and BC treated groups. The Gastrin-1 level is significantly elevated in HP group vs CON group, whereas the Gastrin-1 level is significantly decreased in high BC group with HP infection vs *H. pylori* group. Each column with a vertical line represents mean ± SEM. *$p < 0.05$, compared to the CON group; #$p < 0.05$, compared to HP group. BC = *Bacillus coagulans*; HP = *H. pylori*; SEM = SE of the mean.

Bottmingen, Switzerland). LES pressure was identified as a high-pressure zone between the stomach and intra-abdominal esophagus.[21] The infuscate was drained out extra-corporally via a larger caliber PE catheter, which was placed with one end at the stomach cardia and the other end penetrated although the gastric wall to outside the body, to avoid the confounding factors attributable to the HCL effect on the stomach. The interval between BC treatment and HCl stimulation was 1 hour.

### 2.11. Recording the activity of vagus nerve innervating LES

The recording technique has been reported previously[22,23] with some modifications. Briefly, recordings from the multifiber preparations were made by placing the vagal nerve fibers near LES in one pair of thin bipolar stainless steel electrodes. The vagus nerves and electrodes were continuously bathed in a pool of warm paraffin oil (37°C) to prevent drying. Briefly, recordings from the multifiber preparations, which contain only efferent nerve activity and remove afferent sensory nerve activity by crushing the

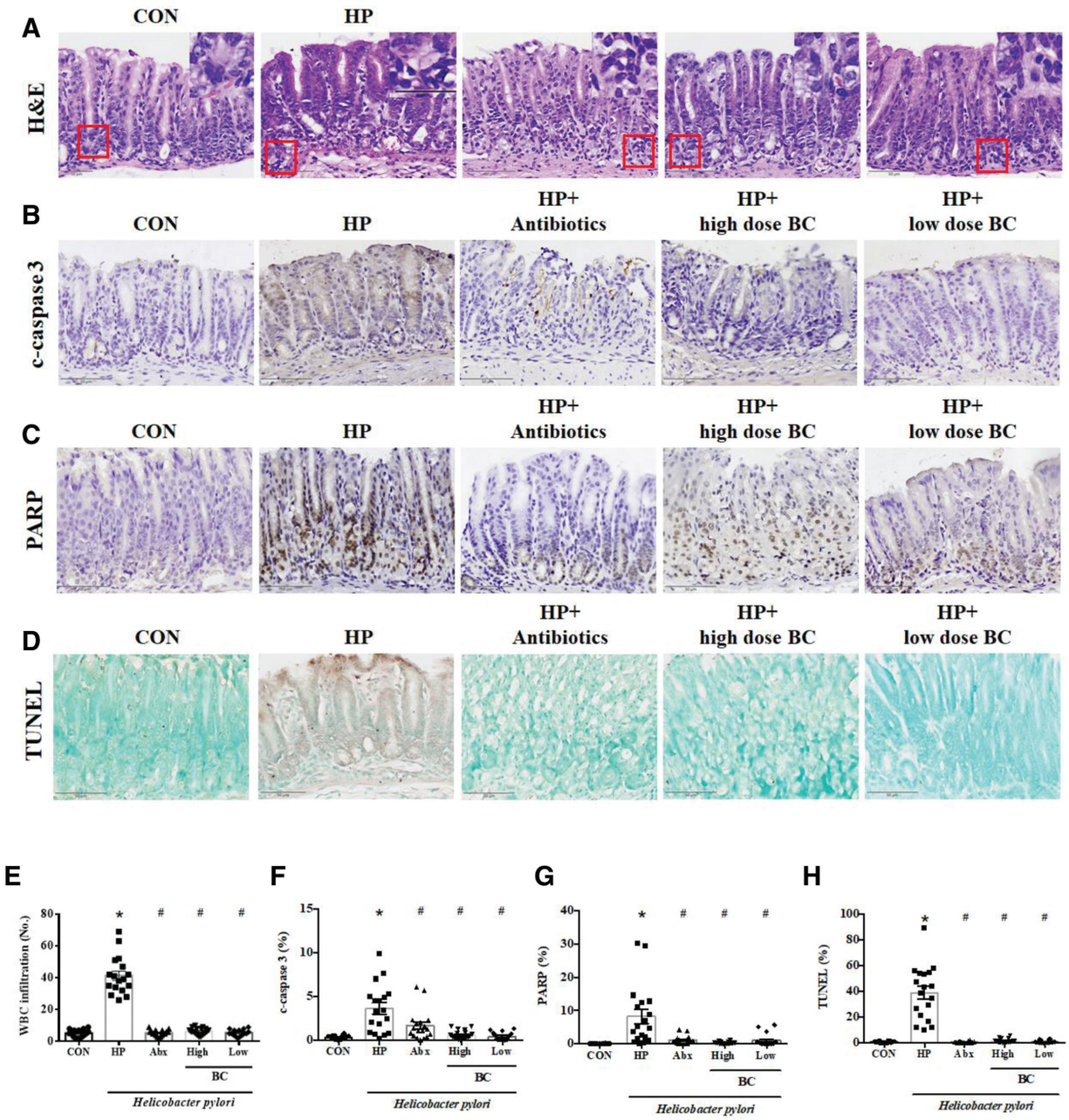


**Fig. 2** Effect of BC on the degree of leukocyte infiltration by H&E stain (A), c-Caspase3 expression (B), PARP expression (C), and TUNEL stain (D) by IHC in the HP-infected stomach of mice. The level of leukocyte infiltration, c-Caspase3, PARP, and TUNEL expression in HP-infected mice is significantly increased vs uninfected control (CON) mice. Antibiotics (Abx) and high- and low-dose BC treatment significantly decreased WBC infiltration (E), c-Caspase3 expression (F), PARP (G), and TUNEL (H) expression vs HP mice. Each column with a vertical line represents mean ± SEM with n = 18 in each group. *$p < 0.05$, compared to the CON group; #$p < 0.05$, compared to HP group alone. BC = *Bacillus coagulans*; HP = *H. pylori*; IHC = immunohistochemistry; SEM = SE of the mean.

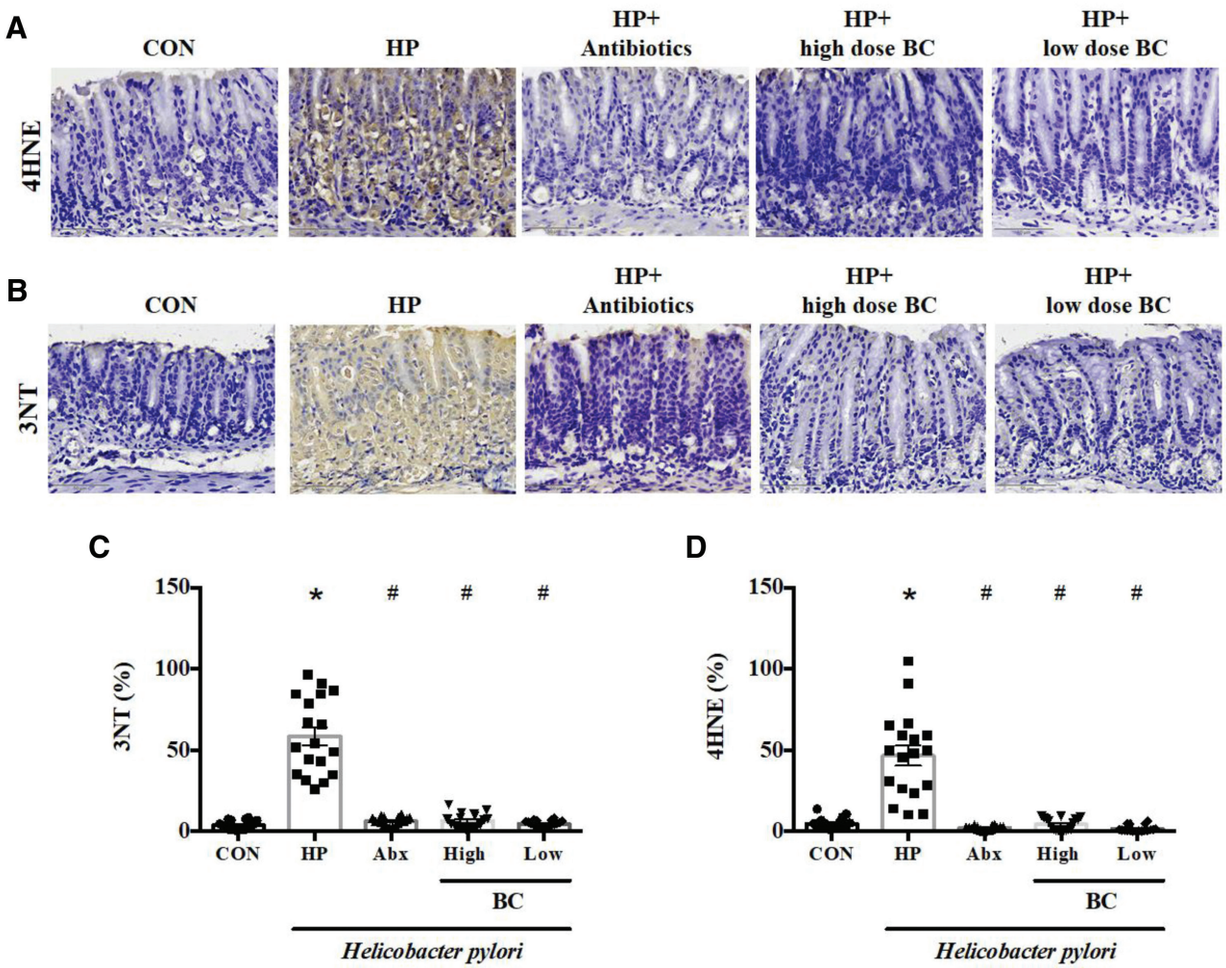


**Fig. 3** Effect of BC on 4HNE and 3NT-mediated oxidative stress in the stomach of mice. The 4HNE expression in HP-infected mice is significantly increased vs uninfected control (CON) mice (A and D). Antibiotics (Abx) and high- and low-dose BC treatment significantly decreased 4HNE expression vs HP mice. The 3NT expression in HP mice is significantly increased vs uninfected CON mice (B and C). Abx and high- and low-dose BC treatment significantly decreased 3NT expression vs HP mice. Each column with a vertical line represents mean ± SEM (n = 18). *$p < 0.05$, compared to the CON group; #$p < 0.05$, compared to HP group alone. BC = *Bacillus coagulans*; HP = *H. pylori*; SEM = SE of the mean.

proximal end near LES, were made by nerve fiber placement in one pair of bipolar stainless steel electrodes. The electrical signals were amplified 20 000-fold and filtered (high-frequency cutoff 3000 Hz, low-frequency cutoff 30 Hz) using a Grass model P511 AC preamplifier, fed into a window discriminator (WPI 121) and continuously displayed on an iWorx 214 data recorder (IX-214; iWorx Systems, Inc.). The neural activity that exceeded the threshold level was then transformed into a spike number.

### 2.12. In vitro LES wire myography

The wire myographic technique used to explore the LES response to various vasoactive agents was performed as previously described with some modifications.[24] Briefly, after anesthesia (3% isoflurane and 100% oxygen), the LES was carefully dissected in Krebs-Henseleit (KH) buffer (118 mmol/L NaCl, 3.4 mmol/L KCl, 1.2 mmol/L $KH_2PO_4$, 1.2 mmol/L $MgSO_4$, 25 mmol/L $NaHCO_3$, 11 mmol/L glucose, and 1 mmol/L $CaCl_2$, bubbled with 95% $O_2$-5% $CO_2$ at pH 7.4) and cut into 2-mm segments. The LES rings were then carefully mounted in a wire myograph (620M; Danish MyoTechnology, Aarhus, Denmark) using two stainless steel wires (with a diameter of 25 μm). The muscles were equilibrated for 60 minutes at 37°C, and then gradually stretched with up to a 20-mN force. After normalization, the muscle rings were left to equilibrate for another 30 minutes, and then tested for contraction using acetylcholine (Ach, $10^{-9}$-$10^{-3}$ mol/L).

### 2.13. In situ detection of oxidative stress, inflammation, apoptosis, and autophagy

Since increased oxidative stress might be associated with inflammation, autophagy and apoptosis, we evaluated the hematoxylin & eosin (H&E) stain, 3-nitroyrosine (3NT), and 4-hydroxynonenal (4HNE) expression for oxidative stress, Beclin-1 and LC3-II staining for autophagy, and Caspase3, poly (ADP-ribose) polymerase (PARP) and terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL), respectively, in paraffin-embedded gastric tissue sections. Tissue sections obtained from 10% formalin fixation and paraffin embedding were deparaffinized, rehydrated, and stained with H&E or immunohistochemically. For 3NT or 4HNE staining, tissue sections were incubated with rabbit anti-nitrotyrosine IgG antibodies (NITT12-A; Alpha Diagnostic) and rabbit anti-4HNE antibodies (Alpha Diagnostic) stained

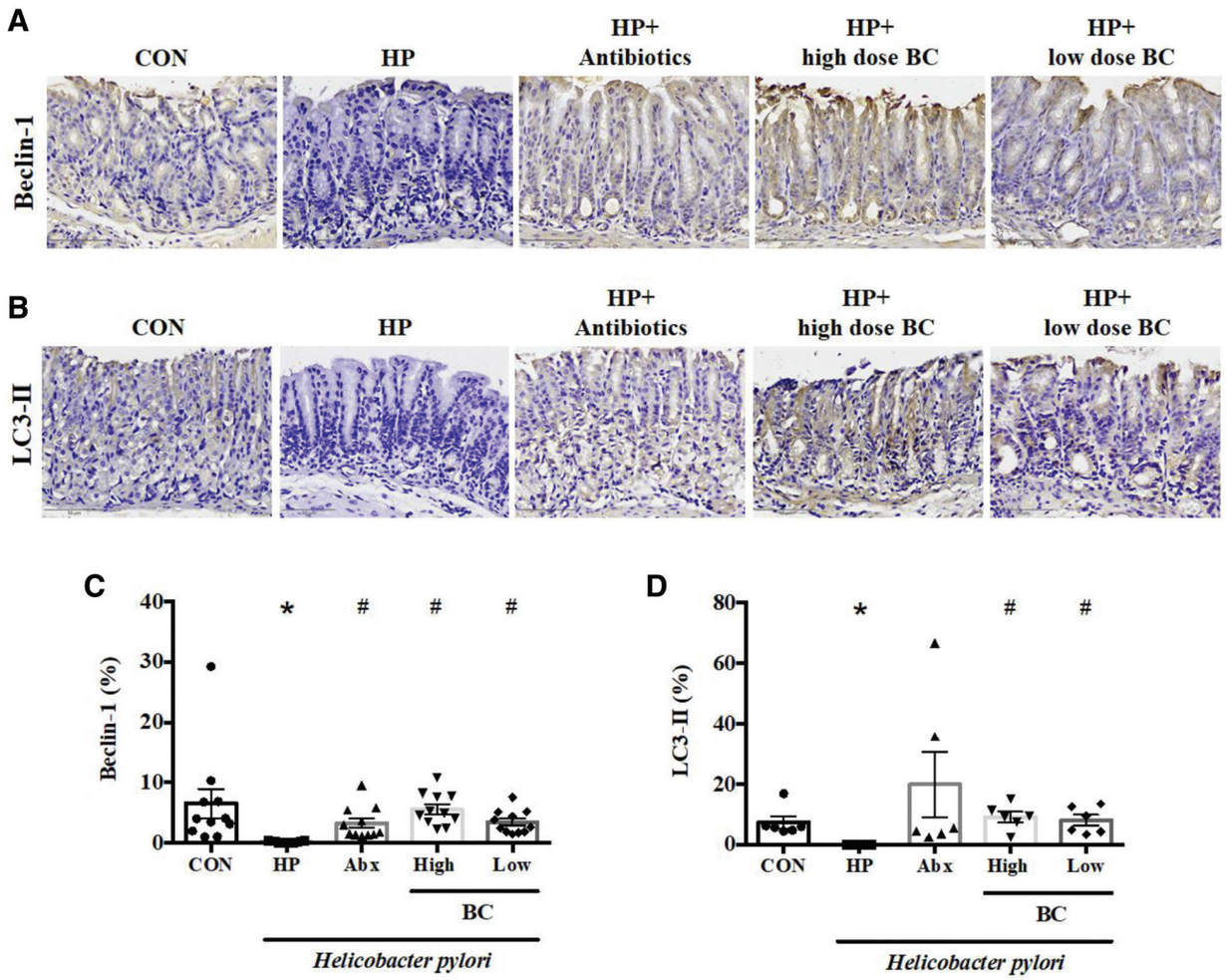


**Fig. 4** Effect of BC on Beclin-1 (A) and LC3-II (B) mediated autophagy in the stomach of mice. The expression of Beclin-1 (C) and LC3-II (D) in HP-infected mice is significantly decreased vs uninfected control (CON) mice. Antibiotics (Abx) and high- and low-dose BC treatment significantly increased Beclin-1 and LC3-II expression vs HP mice. Each column with a vertical line represents mean ± SEM (n = 10 in each group). *$p < 0.05$, compared to the CON group; #$p < 0.05$, compared to HP group alone. BC = *Bacillus coagulans*; HP = *H. pylori*; SEM = SE of the mean.

with avidin-biotinylated horseradish-peroxidase using a commercially available kit (ABC Elite; Vector Laboratories).[8] For Beclin-1 or LC3-II staining, tissue sections were incubated overnight at 4°C with mouse anti-rat Beclin-1 antibody (BD Biosciences; San Jose, CA, 1:100) or LC3-II (Cell Signaling Technology, Inc., 1:100), cleaved Caspase3 (CPP32/Yama/Apopain; Upstate Biotechnology, Lake Placid, NY, 1:100), cleaved PARP (Cell Signaling Technology, Inc., 1:100). Subsequently, biotinylated secondary antibodies (Dako, Botany, NSW, Australia) were applied, followed by incubation with streptavidin-conjugated horseradish-peroxidase (Dako). The chromogen used in this study was Dako Liquid diaminobenzidine. Twenty high-power (×400) fields were randomly selected from each gastric section, and the brown deposits/total section area value for 3NT, 4HNE, caspase3, PARP, Beclin-1 or LC3-II stain was analyzed with Adobe Photoshop 7.0.1 image software.

TUNEL was performed according to a previously described method.[8] Briefly, 5-μm-thick gastric tissue sections were prepared, deparaffinized, and stained using the TUNEL-ABC method. Twenty high-power (×400) fields were randomly selected from each section, and the number of apoptotic cells was counted. Finally, the apoptotic cells/(apoptotic cells and methyl green-stained cells) value was calculated.

### 2.14. Statistical analysis

All values were expressed as mean ± SE of the mean (SEM). The one-way analysis of variance (ANOVA) and Duncan multiple-range tests were used to examine differences among groups in the immunohistochemical staining results from various mouse groups. Differences with $p < 0.05$ were considered significant. Graphing and statistical analysis were performed using the SigmaPlot 12.0 software (Systat Software, Inc., Chicago, IL).

## 3. RESULTS

### 3.1. BC effect on *H. pylori* growth and baseline parameters in *H. pylori*-infected mice

We first examined the BC effect on *H. pylori* growth compared to the positive amoxicillin-treated disk control with the disc diffusion method. As shown in Fig. 1A, we found that BC at 5 μg/10 μL did not inhibit *H. pylori* growth, however, 50 and 500

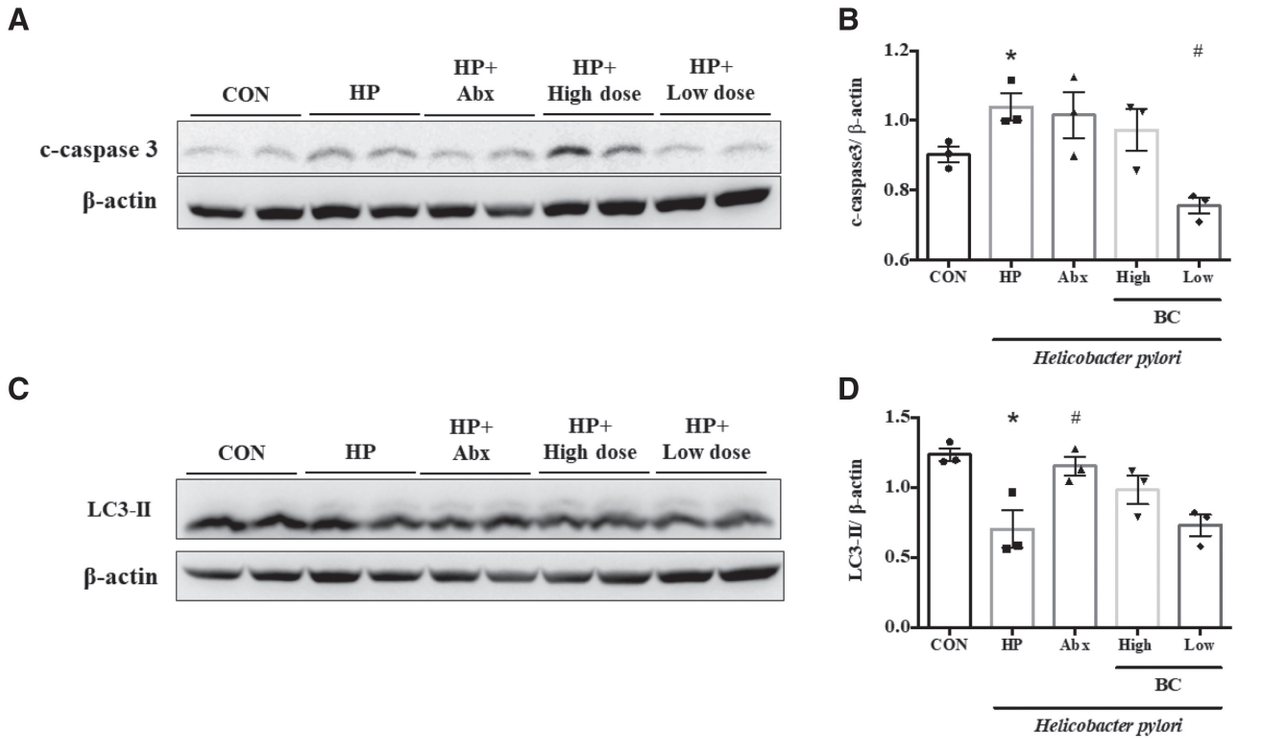


**Fig. 5** Effect of BC on c-Caspase3-mediated apoptosis (A) and LC3-II-mediated autophagy (C) in the stomach of mice by western blot analysis. Statistical analysis demonstrates that the expression of c-Caspase3 (B) is significantly increased in HP-infected mice compared to uninfected control (CON) mice. In contrast, the expression of LC3-II (D) is significantly decreased in HP-infected mice compared to uninfected control (CON) mice. Antibiotics (Abx) and high- and low-dose BC treatment significantly decreased c-Caspase3 expression but significantly enhanced LC3-II expression vs HP mice. Each column represents mean ± SEM (n = 3 in each group). *$p < 0.05$, compared to the CON group; #$p < 0.05$, compared to HP group alone. BC = *Bacillus coagulans*; HP = *H. pylori*; SEM = SE of the mean.

μg/10 μL partially inhibited *H. pylori* growth around the margin area by 20% to 30% inhibition. We then evaluated the baseline parameters among these five groups of mice. It has been shown that the body weight was similar among these five groups of animals (Fig. 1B). However, the gastric juice pH was significantly increased in the *H. pylori*-infected group (HP) vs CON group (Fig. 1C). The BC treatment with low- or high-dose seemed to decrease the gastric juice pH value but not significantly as compared to HP group, respectively. In addition, the serum Gastrin-1 level was significantly elevated in the HP group vs CON group, whereas the increased Gastrin-1 concentration was depressed in the low- or high-dose BC treated group (Fig. 1D). However, low-dose BC treatment shows similar value with that of HP.

### 3.2. BC effect on inflammation and apoptosis formation in *H. pylori*-infected mice

As shown in Fig. 2, the leukocyte infiltration degree (Fig. 2A) and the c-Caspase3 expression level (Fig. 2B), cleaved PARP (Fig. 2C), and TUNEL (Fig. 2D) by immunohistochemistry (IHC) were found to be markedly increased in *H. pylori*-infected stomach compared to those for CON group. The statistical data displayed that the expression in leukocyte infiltration (Fig. 2E), c-Caspase3 (Fig. 2F), PARP (Fig. 2G), and TUNEL (Fig. 2H) in HP group was significantly increased in HP group as compared to CON group. The Abx group, low-dose BC group, and high-dose BC group treatments all significantly decreased leukocyte infiltration (Fig. 2E), c-Caspase3 (Fig. 2F), PARP (Fig. 2G), and TUNEL (Fig. 2H) expression vs HP group. These data suggest that BC has exerted an anti-inflammatory and anti-apoptotic effect against *H. pylori* infection.

### 3.3. BC treatment effect on *H. pylori*-enhanced 3NT and 4HNE oxidative stress

We explored two oxidative stress biomarkers, 3NT and 4HNE, among these five groups. The 4HNE immunostaining intensity in the stomach of *H. pylori*-infected mice (Fig. 3A–D) was higher than that in uninfected CON mice. Fig. 3D also indicated that antibiotics, low-dose BC, or high-dose BC treatment could significantly and efficiently decrease 4HNE staining in the stomach of *H. pylori*-infected mice. Importantly, we observed that the administration of low- or high-dosage BC did not enhance 4HNE staining or induce morphologic changes in the stomach of uninfected mice, suggesting the absence of toxicity at this BC dose.

Similarly, the 3NT immunostaining intensity in the stomach of *H. pylori*-infected mice (Fig. 3B) was stronger than that in uninfected CON mice. Fig. 3C also demonstrated that antibiotics, low-dose or high-dose BC treatment, were able to significantly and efficiently reduce 3NT staining in the stomach of *H. pylori*-infected mice. Importantly, we observed that the administration of low- or high-dosage BC did not enhance 3NT staining or induce morphologic changes in the stomach of uninfected mice, suggesting the absence of toxicity at this BC dose.

### 3.4. BC effect on *H. pylori*-mediated Beclin-1 and LC3-II-mediated autophagy of gastric mucosa in the mouse model

In the present study, we investigated *H. pylori*-depressed Beclin-1/LC3-II expression by IHC, the crosstalk among different cell death pathways, and the possible effect of BC on these pathways in the gastric mucosa of the mouse model. Specifically, in the *H. pylori*-infected group, the expression of Beclin-1

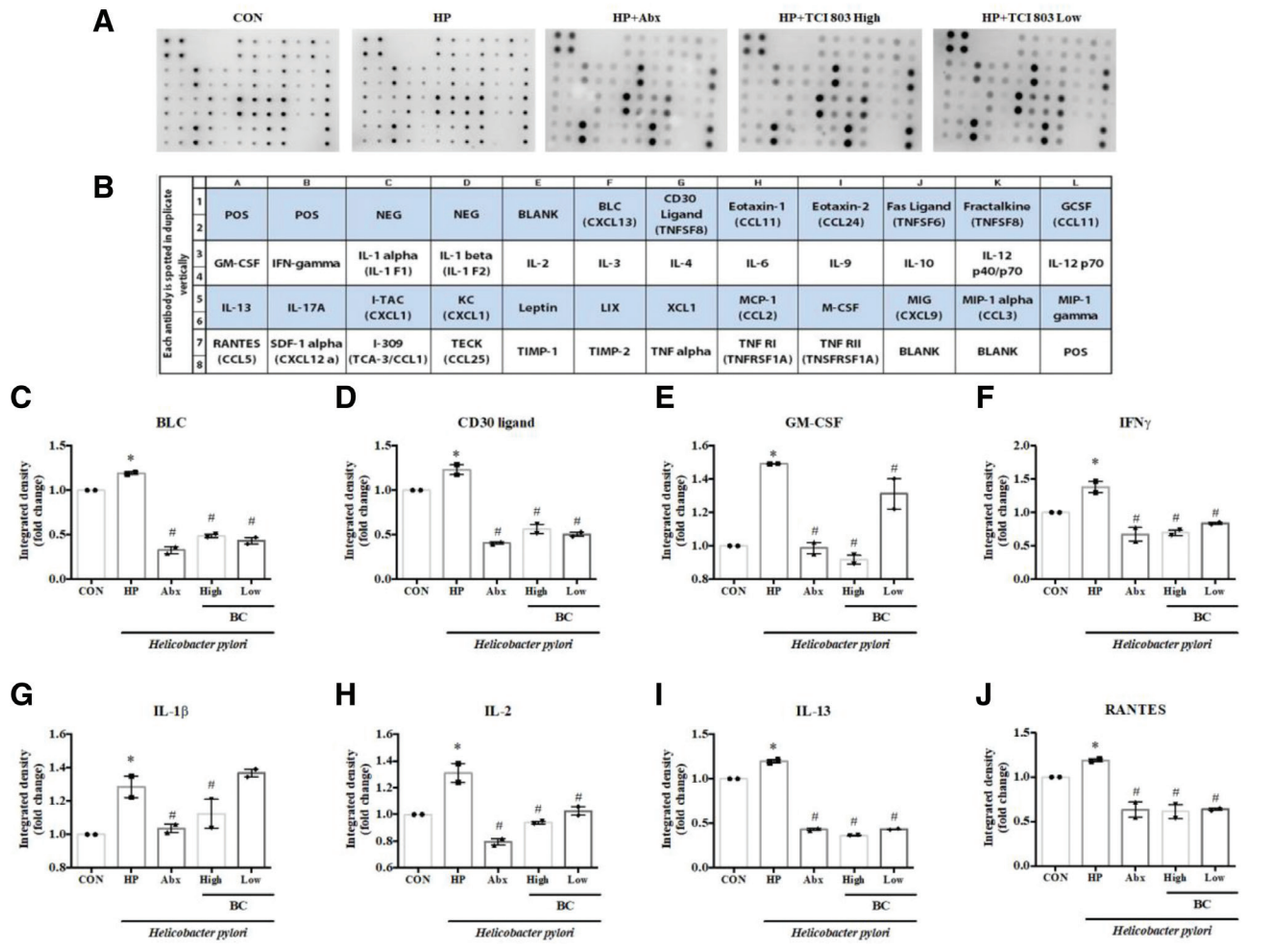


**Fig. 6** Effect of BC on the multiple inflammatory cytokines array of the mice stomach in response to HP infection of five groups of mice. A, The gastric cytokine profiles in five groups of mice stomach. B, The matched points of inflammatory cytokine antibodies are indicated. The mean changes (indicated by ratios of control) of 8 cytokines of BLC (C), CD30 ligand (D), GM-CSF (E), IFNγ (F), IL-1β (G), IL-2 (H), IL-13 (I), and RANTES (J) determination in uninfected control (CON), HP-infected, antibiotics treatment (Abx), and high- and low-dose BC treatment. Each column with a vertical line represents mean ± SEM (n = 2 in each group). *$p < 0.05$, compared to the CON group; #$p < 0.05$, compared to HP group alone. BC = *Bacillus coagulans*; HP = *H. pylori*; SEM = SE of the mean.

(Fig. 4A) and LC3-II (Fig. 4C) was markedly decreased compared to that observed in the epithelial and submucosal layers of the stomach from HP-infected mice in uninfected CON mice. Statistical data indicated that the significantly lower expression of Beclin-1 (Fig. 4B) and LC3-II (Fig. 4D) was observed in HP group vs CON group. In the low- or high-BC treatment group, both doses of BC were found to significantly recover the Beclin-1 and LC3-II stains as well as antibiotics-treated group in the gastric mucosa after *H. pylori* infection.

In Fig. 5, we determined the BC effect on c-Caspase3-mediated apoptosis (Fig. 5A) and LC3-II-mediated autophagy (Fig. 5C) in the stomach of mice by western blot analysis. Statistical data demonstrated that the c-Caspase3 expression (Fig. 5B) was significantly increased in HP mice compared to CON mice. In contrast, the LC3-II expression (Fig. 5D) was significantly decreased in HP mice compared to CON mice. Antibiotics and high- and low-dose BC treatment significantly decreased c-Caspase3 expression but significantly enhanced LC3-II expression vs HP mice. These results demonstrated that BC treatment was able to significantly depress *H. pylori*-enhanced apoptosis but significantly restore *H. pylori*-decreased autophagy formation in the stomach of infected mice.

### 3.5. BC effect on *H. pylori*-induced multiple gastric cytokine profile

Fig. 6A, B depicts multiple cytokines determination using the cytokine antibody array in the gastric tissue homogenate. The mean changes (indicated by ratios of control) in 8 BLC cytokines (Fig. 6C), CD30 ligand (Fig. 6D), GM-CSF (Fig. 6E), IFNγ (Fig. 6F), IL-1β (Fig. 6G), IL-2 (Fig. 6H), IL-13 (Fig. 6I), and RANTES (Fig. 6J) determination were determined in uninfected CON, HP, antibiotics treatment, and high- and low-dose BC treatment. The eight cytokines were significantly increased after *H. pylori* infection. Antibiotics or BC treatment significantly depressed the increase in these eight cytokines and chemokines.

### 3.6. Effect of BC treatment on HCl dysregulated LES motility and vagus nerve activity in vivo

As shown in Fig. 7A, we developed an experimental setup for simultaneous measurement of LES pressure and efferent vagus nerve activity in response to the esophageal infusion of saline or HCl to a threshold volume during the infusion and emptying periods in the rat. Based on the model, we can delineate the several parameters for simultaneous recording of ABP, LES pressure, efferent vagus nerve activity in the Fig. 7B. Although

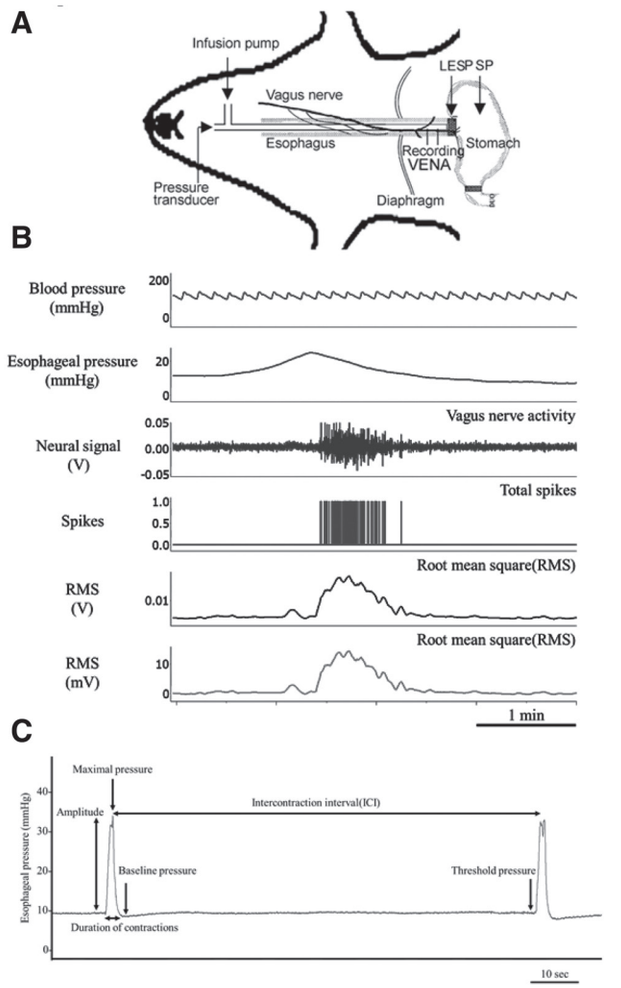


**Fig. 7** A, Schematic of experimental setup for measurement of LESP and VENA in the rat. B, The original parameters for simultaneous recording of arterial blood pressure, LESP, neural signals of VENA, spikes, and RMS. C, The LES activity evaluated by esophageal pressure is determined by the maximal pressure, baseline pressure, amplitude, duration of concentrations, ICI, and threshold pressure. ICI = intercontraction intervals; LESP = lower esophageal sphincter pressure; RMS = root mean square; VENA = vagus efferent nerve activity.

most studies of LES recording included a recording taken of the esophagus orad and caudad of the LES to show that the recording device is actually in the LES, our study using a marked PE tubing to clearly locate in the area close to the LES by opening the abdominal cavity. This confirmed that the recording device is actually in the LES. We determined the LES activity by the esophageal parameters with the maximal pressure, baseline pressure, amplitude of contraction, duration of concentrations, intercontraction intervals and threshold pressure in the anesthetized rats (Fig. 7C). According to our LES recording, we found that in response to liquid infusion the LES pressure was first increased (contraction) and followed by a decrease in LES pressure (relaxation) as shown in Fig. 7C.

Fig. 8 demonstrates the typical graph of blood pressure (red color), LES pressure (blue color), amplified vagus nerve activity (green color), integrated spike and voltage of vagus nerve activity in the control (Fig. 8A), post-high BC (Fig. 8B), post-low BC (Fig. 8C), pre-low BC (Fig. 8D), and pre-high BC (Fig. 8E) rat. The original parameters for simultaneous recording of ABP, LES pressure, neural signals, spikes, and root mean square (RMS) were indicated. The LES activity evaluated by esophageal pressure was measured by the maximal pressure, baseline pressure, amplitude, duration of concentrations, intercontraction intervals, and threshold pressure.

As shown in Fig. 9, our results indicated that BC on effect HCl dysregulated LES motility and vagus nerve activity among these five groups of rats (n = 8 in each group). We found that HCl stimulation significantly decreased the level of threshold pressure, intercontraction interval, and increased vagus nerve activity in the rats. BC treatment at low- or high-dose significantly increased threshold pressure and intercontraction interval and decreased vagus nerve activity implicating BC's improving HCl-enhanced LES activity.

### 3.7. BC treatment effect on HCl dysregulated LES myography and inflammation in vitro

Fig. 10A demonstrates the LES muscle preparation for myography determination. As shown in Fig. 10B, we determined the BC effect on acetylcholine-induced isolated LES muscle contraction in six groups of rats. After HCl stimulation, the isolated LES muscles significantly decreased the contractile response to acetylcholine challenge compared to the control LES muscle. Low and high pretreatment or posttreatment BC doses significantly restored HCl-depressed contractile response to acetylcholine challenge vs HCl stimulation alone.

Fig. 10C demonstrates the histologic and morphologic change in LES after HCl irritation and BC treatment. Our data showed that HCl stimulation markedly disrupted the esophageal mucosa integrity (green arrows) and increased the leukocyte infiltration (red arrows) in the LES section vs non-HCl-treated CON group, implicating that HCl destroys esophageal mucosa and triggers inflammation in the LES. Importantly, low- and high-dose BC treatment decreased the degree of impairment, leukocyte infiltration, and inflammation in the HCl-treated LES.

## 4. DISCUSSION

Due to antibiotic resistance or low compliance, the current antibiotic-based therapies for treating *H. pylori* infections are not absolutely effective. We previously reported that up to 95% of *H. pylori* infections in the mouse model were eradicated after anti-*H. pylori* treatment using a combination of catechins and sialic acid,[8] a potential non-antibiotic alternative therapy for treating *H. pylori* infection. Our data found that BC (probiotics) can be additional or alternative treatments for *H. pylori*. BC provides benefits 30% *H. pylori* inhibition, lowers pH of gastric juice and gastrin secretion, reduces oxidative stress, cytokines, and apoptosis process, increases autophagy, and preserves LES function. In the present study, we found that BC has partial anti-*H. pylori* effect on bacteria cultures in vitro for 30% inhibition and in mice in vivo. In addition, pre- or post-BC treatment confers protection against HCl-induced LES dysfunction in rats.

BC TCI803 *Lactobacillus* has been reported with the goal of inhibiting *H. pylori* growth and reducing infections. One previous report demonstrated that BC treatment could improve growth performance and alleviate diarrhea in piglets challenged with enterotoxigenic *Escherichia coli* via improving intestinal mucosal barrier integrity, which was possibly associated with intestinal microbiota and immune status impairment.[25] In a clinical trial, BC could effectively exhibit antagonistic activity against *H. pylori*, with rare adverse events, and thus may reduce the antibiotic resistance burden.[17] Preliminary research results for BC TCI803 in cellular isolated experiments with gastric *H. pylori* inhibition can enhance gastric mucin secretion to enhance protection from ulcers, and reduce gastric inflammation genes

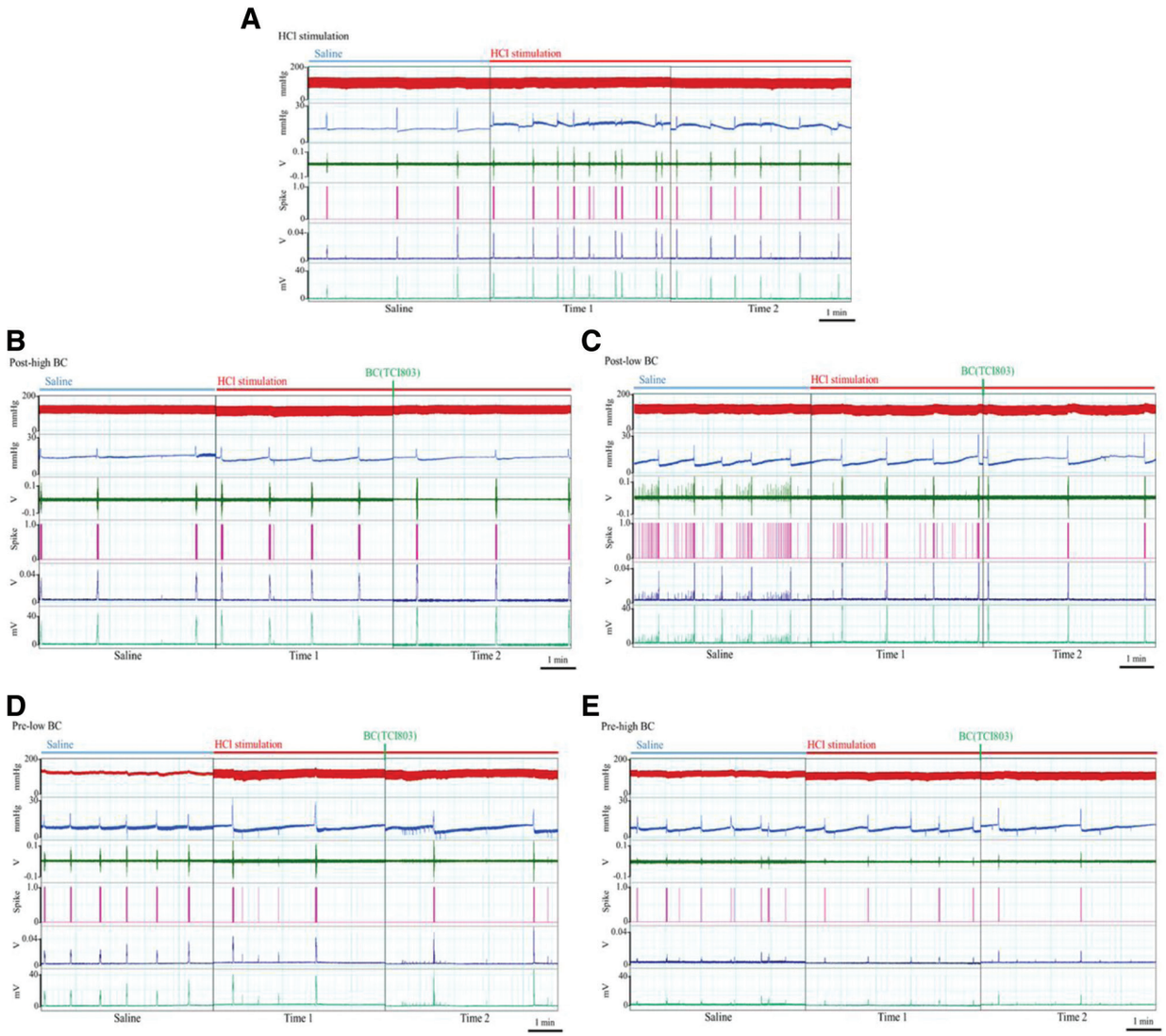


**Fig. 8** Typical graph of blood pressure (red color), LES pressure (blue color), amplified vagus nerve activity (green color), integrated spike and voltage of vagus nerve activity in the control (A), post-high BC (B), post-low BC (C), pre-low BC (D), and pre-high BC (E) rat. The original parameters for simultaneous recording of arterial blood pressure, LES pressure, neural signals, spikes, and RMS are demonstrated. The LES activity evaluated by esophageal pressure is determined by the maximal pressure, baseline pressure, amplitude, duration of concentrations, intercontraction intervals, and threshold pressure. BC = *Bacillus coagulans*; LES = lower esophageal sphincter; RMS = root mean square.

IL-18, TNFα, IL16, IL12A, and IL3 performance to reduce the degree of gastric inflammation (unpresented data). BC may have a preventive or therapeutic effect on *H. pylori* infection in mice. Our data in Fig. 6 depict that 8 BLC cytokines and chemokines, CD30 ligand, GM-CSF, IFNγ, IL-1β, IL-2, IL-13, and RANTES were upregulated in the HP group, whereas antibiotics treatment, high- and low-dose BC treatment significantly suppressed the increased release of eight cytokines and chemokines implicating the BC effect on reducing *H. pylori*-enhanced inflammatory cytokines release. In addition, our present results found that BC TCI803 *Lactobacillus* treatment efficiently reduced apoptosis and oxidative stress and increased autophagy relative to the *H. pylori* infection. These data indicate that BC has a preventive and therapeutic effect on gastric inflammation in mice infected with *H. pylori*.

Increased ROS production from mitochondria or other intracellular compartments may induce apoptosis, or autophagy—by activating caspases, or lysosomal proteases, respectively.[8] In this study, we consistently found an increase in two oxidative stress markers, 3NT and 4HNE, expression associated with an increased number of infiltrated leukocyte upon *H. pylori* infection. These events subsequently led to the activation of Bax/Caspase3/apoptosis signaling and the inhibition of Beclin-1/LC3-II-mediated autophagy in the inflammatory stomach, which was consistently demonstrated in our previous study.[8] Our previous research,[8,26] further indicated that catechins combined with sialic acid were able to effectively eradicate *H. pylori* colonization, reverse gastric epithelial cell damage, and significantly reduce the ROS production and Bax/Bcl-2-mediated apoptosis, but enhance Beclin-1-mediated autophagy. In the present study, our data showed that low and high doses of BC treatment partly inhibited *H. pylori* growth but could depress 3NT and 4HNE expression, decrease leukocyte infiltration and Bax/Caspase3/PARP PARP-mediated apoptosis and recover Beclin-1/

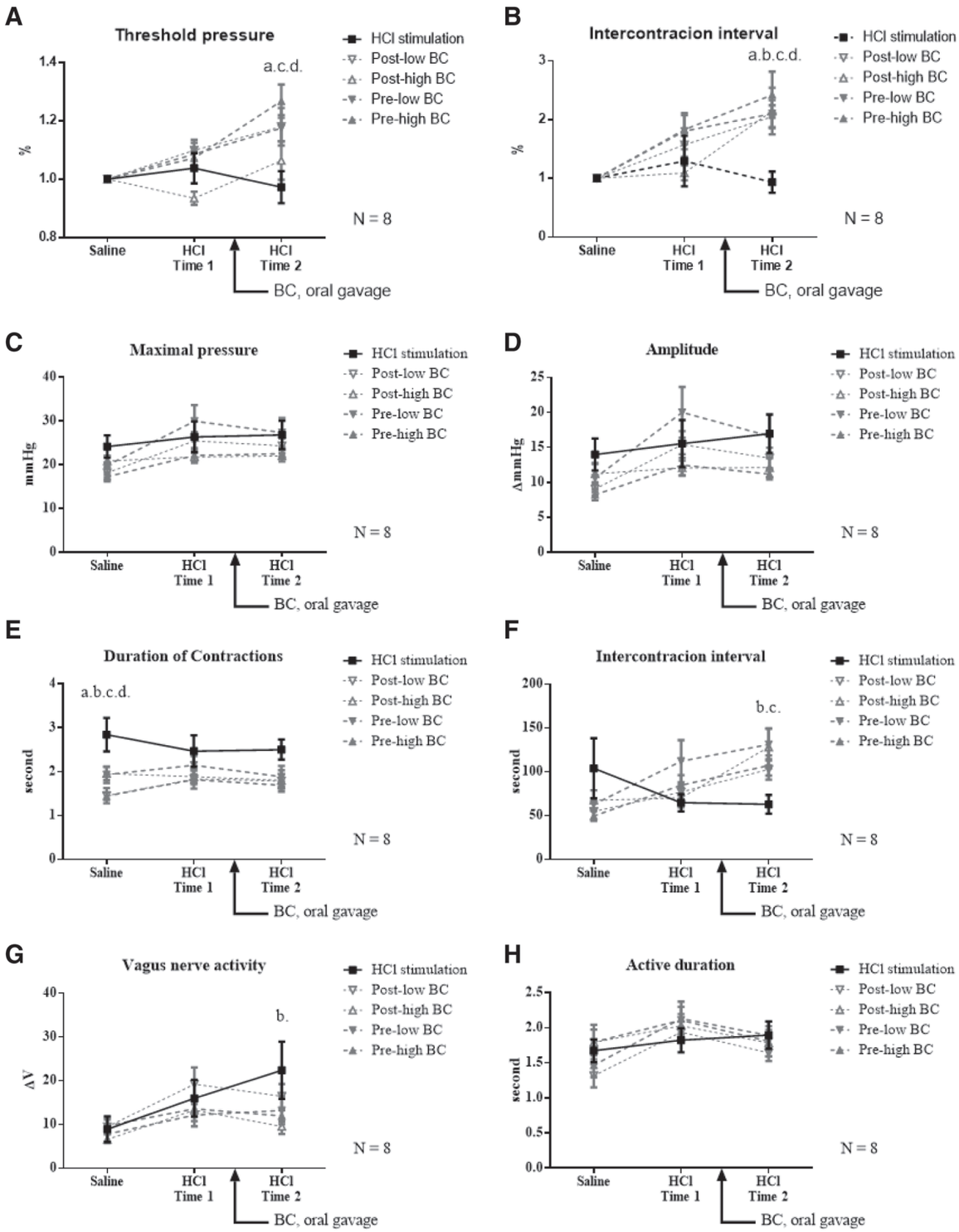


**Fig. 9** The effect of BC on HCl dysregulated LES motility and vagus nerve activity among these five groups of rats. A, % change of threshold pressure; B, % change of intercontraction interval; C, maximal pressure; D, amplitude; E, duration of contractions; F, intercontraction interval; G, vagus nerve activity; and H, active duration. HCl stimulation decreased threshold pressure, intercontraction interval, and increased vagus nerve activity. BC treatment at low or high dose significantly increased threshold pressure and intercontraction interval and decreased vagus nerve activity. HCl stimulation: intraesophageal perfusion of HCl without BC treatment; pre-low BC: pretreatment of low dose of BC with HCl stimulation; pre-high BC: pretreatment of high dose of BC with HCl stimulation; post-low BC: posttreatment of low dose of BC with HCl stimulation; post-low BC: posttreatment of high dose of BC with HCl stimulation. Data are expressed as mean SEM (n = 8 each group). (a, $p < 0.05$ post-low BC vs HCl stimulation by two-way ANOVA. b, $p < 0.05$ Post-high BC vs HCl stimulation by two-way ANOVA. c, $p < 0.05$ pre-low BC vs HCl stimulation by two-way ANOVA. d, $p < 0.05$ pre-high BC vs HCl stimulation by two-way ANOVA. ANOVA = analysis of variance; BC = *Bacillus coagulans*; LES = lower esophageal sphincter; SEM = SE of the mean.

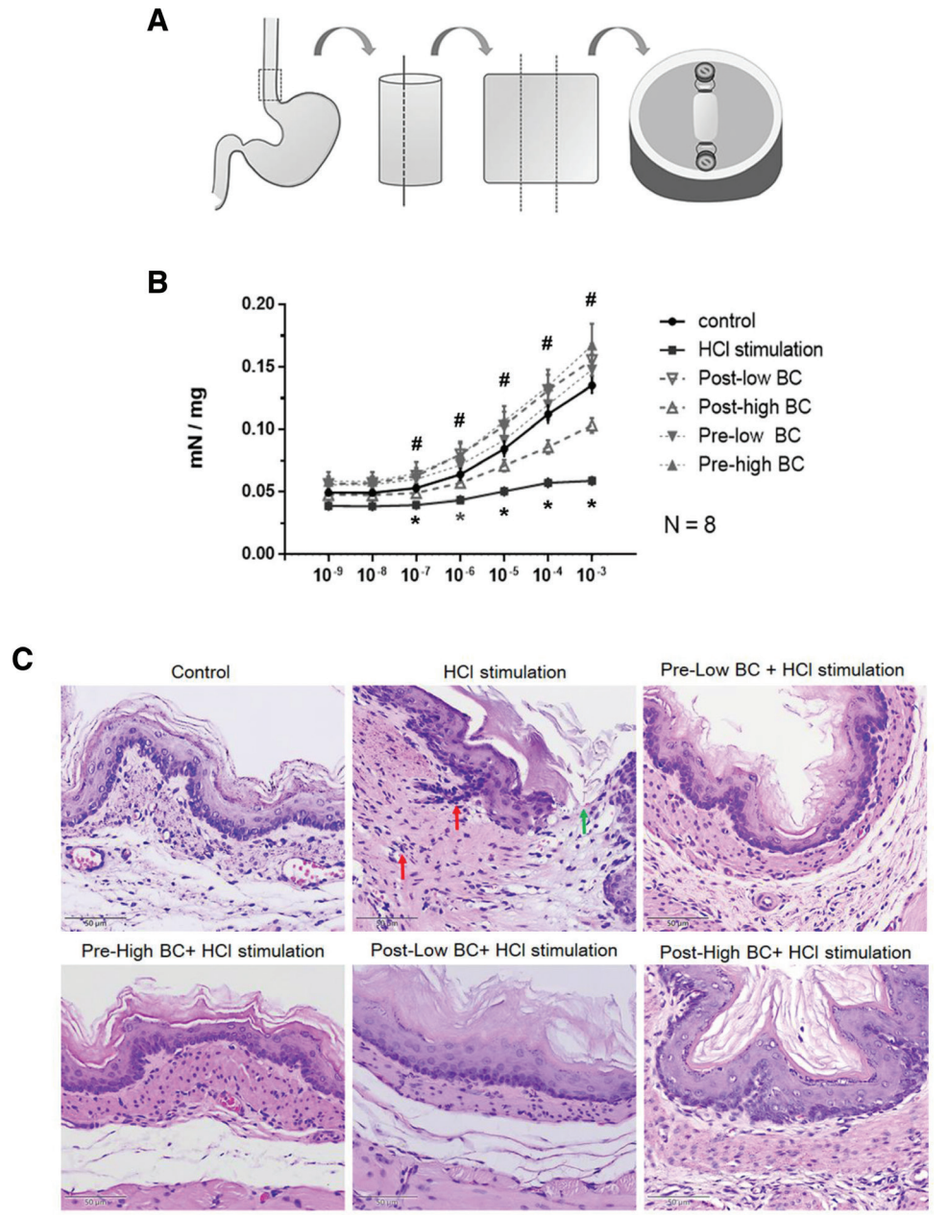


**Fig. 10** A, The preparation of LES muscle for myography determination. B, Effect of BC on acetylcholine-induced isolated LES muscle contraction in six groups of rats. After HCl stimulation, the isolated LES muscles significantly decreased the contractile response to acetylcholine challenge compared to control LES muscle. Pretreatment or posttreatment of low and high doses of BC significantly restored HCl-depressed contractile response to acetylcholine challenge vs HCl stimulation alone. Data are expressed as mean ± SEM (n = 8). *$p < 0.05$ compared to the CON group; #$p < 0.05$ compared to HCl stimulation group alone. C, H&E stains demonstrated the histologic and morphologic changes of LES after HCl irritation and BC treatment. HCl stimulation markedly disrupted the esophageal mucosa integrity (green arrows) and increased the leukocyte infiltration (red arrows) in the LES section vs non-HCl-treated CON group, implicating HCl triggering inflammation in the LES. Importantly, low and high dose of BC treatment decreased the degree of leukocyte infiltration and inflammation in the HCl-treated LES. BC = *Bacillus coagulans*; LES = lower esophageal sphincter; SEM = SE of the mean.

LC3-II-mediated autophagy in the *H. pylori*-infected stomach. According to these findings, we suggest that the BC suppression mechanism for *H. pylori*-triggered oxidative stress and apoptosis signaling may be modulated in the following three ways: (1) the number of bacteria on the gastric epithelial surface decreases because of the anti-adhesive and antimicrobial properties of BC, (2) the ROS production and apoptotic formation are downregulated and autophagy is upregulated by BC treatment and (3) the direct antioxidant activity of BC. In Fig. 5B, our result displayed that high dose of BC reduced the level of apoptosis less than a low dose of BC possibly due to the different levels of gut microbiota composition and metabolites affecting the gut barrier function. The BC probiotics may regulate gut epithelial homeostasis and promote health via their surface compounds. High doses of BC may release more surface layer proteins, flagella, pili, and capsular polysaccharides, which constitute microbial-associated molecular patterns and specifically bind to pattern recognition receptors to regulate signaling pathways, to produce cytokines or to affect apoptosis, thereby affecting inflammation and the gut epithelium function. We also suggest the effects of

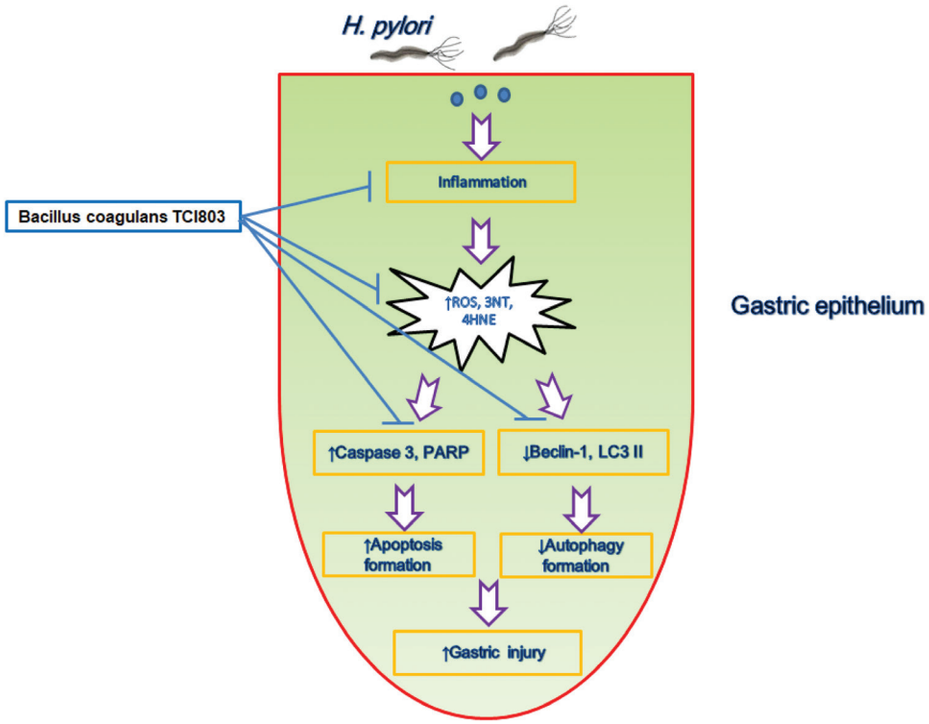


**Fig. 11** A proposed mechanism by which BC inhibits *H. pylori*-enhanced Caspase3/PARP PARP-mediated apoptosis and inhibited Beclin-1/LC3 II-mediated autophagy. BC through anti-inflammation and antioxidant activity to reduce *H. pylori*-enhanced apoptosis and decreased autophagy to ameliorate gastric injury. BC = *Bacillus coagulans*.

increased metabolites (such as secreted proteins, organic acids, indole, extracellular vesicles, and bacteriocins) from high doses of BC probiotics on host receptors and gut epithelial barrier function leading to higher apoptosis in Fig. 5B.

The LES works like a "two-way valve" to regulate esophageal sphincter activity. It allows passage of esophageal contents into the stomach with swallow (swallow-induced LES relaxation) and passage of gastric contents into the esophagus with belching and vomiting (transient LES relaxation). Dysregulated LES relaxation leads to achalasia and possibly other esophageal motor disorders and LES incompetence (too much relaxation) leading to gastroesophageal reflux. Therefore, normal LES activity regulated by the efferent vagus nerve is required for esophageal emptying. Jiang et al[22] suggested that the vagus nerve-mediated LES relaxation is mediated through the longitudinal muscle contraction-induced activation of mechanosensitive inhibitory LES motor neurons. Our present model at the first time displayed that a high LES pressure associated with the excited efferent vagus nerve activity suggesting a possible role of vagus nerve-mediated LES activity (Figs. 8A and 9G). Human and animal studies show that LES relaxation associated with swallow, balloon distension of the esophagus, and electrical stimulation of the vagus nerve is associated with LES cranial displacement.[22] Similarly, transient LES relaxation, the major GERD mechanism, such as belching and vomiting, is associated with localized contraction of the distal esophagus longitudinal muscle, resulting in LES cranial displacement.[27] HCl stimulation (PH = 2 used in our study) mimicked the gastric acid reflux to esophagus may induce the functional change in LES pressure and relaxation, which may occur in GERD-related symptoms. We therefore simultaneously recorded the LES pressure and efferent vagus nerve activity in response to intraesophageal HCl infusion in our developed model. We found that HCl significantly decreased mechanical stimulation (esophageal infusion of saline) of threshold pressure and intercontraction interval and activated efferent vagus nerve activity in Fig. 9 implicating an alteration in LES activity by HCl stimulation (mimicked gastric acid stimulation). Meanwhile, our data also found in the acetylcholine-induced isolated LES muscle contraction experiment, after HCl stimulation, the isolated LES muscle contractile response significantly decreased in response to acetylcholine challenge vs control LES muscle. This information implicates an alteration in LES contractility during gastric acid stimulation. Pretreatment or post-treatment with low and high-dose BC significantly restored HCl-depressed contractile response to acetylcholine challenge vs HCl stimulation alone. These results suggest that BC at low or high doses efficiently recovered the changes in LES activity and vagus nerve activity and subsequently LES motility restoration toward normal status.

Exposure to acidic gastric content like HCl due to LES malfunction leads to acute reflux esophagitis resulting in esophageal epithelial cells disruption and LES activity dysregulation. A previous report indicated that decreased LES motility in a GERD rat model may be mediated by reductions in serotonin and acetylcholine signaling.[21] Using the expression of antioxidant and inflammatory-related markers by western blot, Lee et al[28] found the increased ROS, peroxynitrite (ONOO-), and thiobarbituric acid reactive substance (TBARS) in the esophagus tissue of rats with acute reflux esophagitis. In addition, the use of Curcumae longae Rhizoma extract through the inactivation of NF-κB effectively increased antioxidant-related factors and reduced inflammatory protein leading to acute reflux esophagitis improvement.[28] The LES basal tone is primarily myogenic and can be modulated by enteric motor neurons, the parasympathetic vagus nerve, the sympathetic extrinsic nervous system, and several neurohumoral substances.[12] Dysregulated

LES motility is a critical factor in GERD pathogenesis, esophageal dysmotility, and esophageal hypersensitivity.[29] Because of the importance of regulating esophageal sphincter, the in vivo LES activity and in vitro isolated LES have been widely tested in GERD experiments. Our present data used both in vivo and in vitro experiments clearly demonstrated that low and high-dose BC during preventive or therapeutic treatment could improve HCl dysregulated LES activity and decrease leukocyte infiltration in the damaged esophageal tissue. However, a more detailed mechanism requires further experiments to determine.

As mentioned by Elmaliklis et al,[30] there are several potential mechanisms of action by which probiotics may benefit functional constipation. First, probiotics may modify the gastrointestinal microbiota, which is known to be altered in constipation. We will explore the detailed mechanism in future. Second, probiotic metabolites may alter gut function, including sensation and motility. Our data with LES activity have indicated the preventive and therapeutic effects on LES activity. Third, some probiotics increase the production of lactate and short-chain fatty acids reducing luminal pH, which, as some researchers have proposed, may enhance colonic peristalsis and shorten whole gut transit time.[31] In Fig. 1C, D, our results evidenced the reduced gastric pH and Gastrin-1 release in the HP group with BC treatment. Another systematic review and meta-analysis of six randomized controlled trials, showed that probiotics increased stool frequency and had beneficial effects in Asian children.[32] The results of these studies are in accordance with our animals study demonstrating probiotics with gastroesophageal protection.

The etiological association between *H. pylori* infection and GERD is still under debate. However, most patients with GERD need to receive proton-pump inhibitors (PPI) treatment for a long period, with either continuous or intermittent intake. PPIs may alter the gastric milieu by contributing increased inflammatory cytokine IL-1β and ammonia ($NH_4$).[33] With *H. pylori* infection, the long-term use of PPI may cause a shift from antrum-predominant gastritis to corpus-predominant gastritis with atrophic change, and consequently increase the risk for developing gastric cancer.[33] Eradication of *H. pylori* may markedly improve the gastritis in long-term PPI users.[34] In this occasion, the use of BC has two beneficial effects, decreasing the need for long-term treatment with PPI and also decreasing the corpus-dominant gastritis by the inhibition of *H. pylori* activity.

The antagonism of probiotics against *H. pylori* is achieved through a series of direct or indirect interactions, including secreting antibacterial substances, competing inhibition, enhancing mucous barriers, and regulating immunity.[35,36] Probiotic cosupplementation to antibiotic therapies is reported in several studies, presenting a moderate reduction in drug-related side effects and a promotion in positive treatment outcomes.[37] For example, probiotics have anti-*H. pylori* activity[38–40] and reduce antibiotic-related side effects[41] using the reduction in urease activity mechanism mediated by short-chain fatty acids, an enhancement of the stomach acidic environment, damage to the *H. pylori* strain cell wall, and *H. pylori* colonization inhibition in the gastric mucosa.[42,43] In addition, probiotics depressed the inflammatory response induced by IL-8 after an *H. pylori* infection.[44–46]

Gut microbiota is a group of microorganisms inhabiting in the mammalian gastrointestinal tract and this microbial community is host-specific and susceptible to both exogenous and endogenous modifications.[47] The gut microbiota was identified in immune and metabolic health regulation, but also in influencing several types of diseases, including motility disorders, neurodegenerative disease, cerebrovascular accidents, and neuroimmune-mediated disorders.[48] For example, metabolites secreted by certain microbes, generated by microbial digestion of dietary components or by transformation of host-derived factors can be sensed through various receptors and pathways to alter gut integrity and host health.[49] Probiotic supplements like BC may compete with gut microbiota to alter the host gastrointestinal microbiota following probiotic and drug uptake.[37] As mentioned in our previous report,[50] because *H. pylori* resistance to amoxicillin is generally low, an optimized high-dose dual therapy consisting of proton-pump inhibitors and amoxicillin could be an effective first-line or rescue therapy. We compared the respective contribution of BC or amoxicillin on *H. pylori* infection in the present study. The concomitant use of alternative therapy like probiotics BC with amoxicillin may have the potential to provide additive or synergistic effects against *H. pylori* infection via gut microbiota or gut barrier, however, we did not test these combined effects of BC and amoxicillin in the present study. We will verify these combined efficacy and mechanism in basic and clinical studies in the future.

In conclusion, we showed that *H. pylori* infection enhanced oxidative stress, apoptosis, and inflammatory cytokines expression but depressed autophagy-related proteins expression in mice. The administration of BC efficiently attenuated *H. pylori*-enhanced gastric oxidative stress, apoptotic cell death, and inflammatory cytokines expression but preserved autophagy-related mechanisms as indicated in Fig. 11. In addition, our data found that HCl dysregulated LES motility and evoked inflammation in the LES tissue of rats. BC treatment could improve the LES motility and inflammation. These results implicated that the preventive and therapeutic efficacy of BC treatment can confer gastroesophageal protection against *H. pylori*-evoked gastric oxidative stress and apoptosis and acid-dysregulated LES motility in rodents.

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