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In vitro and *in vivo* inhibition of *Helicobacter pylori* by *Lactobacilllus paracasei* HP7

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The efficacy of standard therapeutic strategies for *Helicobacter pylori* (*H. pylori*) infection is decreasing over time due to the emergence of drug-resistant strains. As an alternative, the present study investigated the capacity of *Lactobacilllus paracasei (L. paracasei)* HP7, isolated from kimchi, to inhibit *H. pylori* growth. The effects of *L. paracasei* HP7 on *H. pylori* adhesion and *H. pylori*-induced inflammation were examined in AGS human gastric adenocarcinoma epithelial cells and a mouse model of *H. pylori* SS1 infection. *L. paracasei* HP7 reduced *H. pylori* adhesion to AGS cells and suppressed the inflammatory response in infected cells by downregulating interleukin-8. *H. pylori* colonization in the stomach of C57BL/6 mice was demonstrated by rapid urease test, and results showed significant decrease in mice post-treated with *L. paracasei* HP7. Additionally, *L. paracasei* HP7 decreased gastric inflammation and epithelial lesions in the stomach of *H. pylori*-infected mice. These results demonstrate that *L. paracasei* HP7 treatment can inhibit *H. pylori* growth and is thus a promising treatment for patients with gastric symptoms such as gastritis that are caused by *H. pylori* infection.

Keywords: *Lactobacilllus paracasei*, HP7, *Helicobacter pylori*, AGS cells, Kimchi

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Helicobacter pylori is a Gram-negative, spiral-shaped bacterium in stomach that is the major pathogen of chronic gastric inflammation [1] and stomach ulcers [2] and is related to increased risk of stomach cancer [3,4]. Removing *H. pylori* in the stomach by inoculating antibiotis can reduce *H. pylori*-related gastrointestinal diseases [5,6] and alleviate the risk of stomach cancers [7]. The standard recommended treatment for *H. pylori* therapy is triple combination therapy with two antibiotics-usually clarithromycin with amoxicillin or metronidazole-and a proton pump inhibitor, which reveals a successful eradication result in the beginning [8,9]. However, the efficacy of this triple therapy has decreased over time; the recent therapy rate of <80% is mainly due to an increase in the prevalence of *H. pylori* strains resistant to metronidazole and clarithromycin [10-12]. Furthermore, some patients reveal allergic side effects to antibiotics, which can occasionally cause adverse effects while failing to treat *H. pylori* [13]. Long-term inoculation of antibiotics to prevent *H. pylori* infection cannot be recommended. It is therefore important to develop new non-antimicrobial agents to treat *H. pylori* [14].

Lactic acid bacteria (*Lactobacillus* spp.) have been recommended as an additive agent in the standard recommended treatment for *H. pylori* therapy and can improve patient compliance by decreasing antimicrobial agents-associated side effects [15,16]. *Lactobacillus salivarius* was reported to inhibit *H. pylori* colonization

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in a mice experiment as evidenced by a decrease in *H. pylori*-specific IgG concentrations, while negative control mice were infected by *H. pylori* and revealed gastritis lesions [17]. In another study, intragastic treatment of a culture supernatant of *Lactobacillus acidophilus* revealed to inhibit *Helicobacter felis* infection [18,19]. Additionally, *L. acidophilus* culture supernatant had a partial but long-term inhibiting effect on *H. pylori* infection in humans [20].

In the present study, we are aimed to study that the lactic acid bacterium *Lactobacillus paracasei* HP7 isolated from kimchi, a fermented vegetable dish widely consumed in Korea, has inhibitory effects against *H. pylori in vitro* and *in vivo*.

Materials and Methods

Bacterial strains and culture conditions

L. paracasei HP7 was cultured at 35°C for 24 h in Man-Rogosa-Sharpe broth (Difco Laboratories, Detroit, MI, USA) composed of 0.2% dipotassium hydrogen phosphate, 0.5% sodium acetate, 0.8% meat extract, 0.1% Tween 80, 0.4% yeast extract, 2% D(þ)-glucose, 0.02% magnesium sulfate, 1% peptone from casein, 0.2% diammonium hydrogen citrate, and 0.004% manganese sulfate. *H. pylori* strain SS1 (B0890; Korean Collection for Type Cultures, Jeongeup, Korea) was cultured overnight at 37°C under microaerophilic conditions in brain-heart infusion broth containing 10% fetal bovine serum (FBS) and was allowed to grow to a density of ~2.0×10^9 CFU/mL. The cultured bacteria were then transferred to phosphate-buffered saline (PBS) before they were used to infect cells.

Cell culture

AGS human gastric adenocarcinoma epithelial cells (CRL-1739; American Type Culture Collection, Manassas, VA, USA) were cultured in Roswell Park Memorial Institute 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated FBS (Invitrogen, Carlsbad, CA, USA). Antibiotic-antimycotic (Gibco, Grand Island, NY, USA) was added if needed. For analysis of *H. pylori*-induced interleukin (IL)-8 production, antibiotics were not added to the culture medium.

Inhibition of *H. pylori* adhesion to AGS cells

AGS cells were seeded in 6-well tissue culture plates at 1×10^6 cells/mL in Ham's F-12 medium (Sigma-Aldrich) supplemented with 10% FBS and 1% antibiotic-antimycotic solution and cultured at 37°C in a humidified atmosphere of 95% air/5% CO_2 (v/v) for 16 h. When the cells reached 90% confluence, the medium was replaced with serum- and antibiotic-free F-12 medium. An overnight culture of *H. pylori* SS1 and *L. paracasei* HP7 was washed twice in sterile PBS and resuspended in Ham's F-12 medium. For co-culture of bacteria and gastric epithelial cells, *H. pylori* SS1 cells (1×10^7 CFU/mL) were added to wells containing 1×10^6 AGS cells at a cell ratio of 10:1 and incubated for 4 h in the absence or presence of *L. paracasei* HP7.

RNA preparation and real-time (RT)-PCR

Total cellular RNA was extracted using TRIzol reagent (Sigma-Aldrich), and 2 μg were reverse-transcribed using murine leukemia virus reverse transcriptase, 1 mM dNTP, and 0.5 μg/μL oligo (dT12-18). The cDNA was used as a template for RT-PCR to detect *H. pylori* 16S RNA as a measure of the *H. pylori* infection rate. The reaction was carried out on a QuantStudio 6 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex Taq (Takara Bio, Otsu, Japan), with glyceraldehyde 3-phosphate serving as an internal standard. Relative mRNA levels at each time point were compared with those in *H. pylori*-infected control AGS cells. Forward and reverse sequences of primers for amplifying the *H. pylori* 16S RNA gene were as follows: 5'-TCG GAA TCA CTG GGC GTA A-3' and 5'-TTC TAT GGT TAA GCC ATA GGA TTT CAC-3' [21].

Measurement of IL-8 levels

IL-8 released by AGS cells infected with *H. pylori* was detected by enzyme-linked immunosorbent assay (ELISA). AGS cells (2×10^4 cells/well) were seeded in 96-well plates; *L. paracasei* HP7 cells were added to the cell culture medium 30 min before *H. pylori* infection for 24 h. AGS cells cultured in the absence of *L. paracasei* HP7 cells served as a control. The culture supernatant was collected and IL-8 levels were measured with a sandwich ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Each sample was tested in triplicate.

Animals

Specific pathogen-free (SPF) male C57BL/6 mice weighing 20-24 g were purchased from Samtako Co. (Osan, Korea) and were maintained at the inspection facility of Wonkwang University (Iksan, Korea) for 1 week before experiments. Thereafter, the mice were maintained in an SPF barrier room with regulated temperature (23ºC±1ºC) and humidity (50±5%) on a 12:12-h light/dark cycle. The animals were fed a sterilized pellet diet (2 Mrad radiation) (Purina, Seoul, Korea) and sterilized water *ad libitum*. All studies were performed in accordance with the Guide for Animal Experimentation of Wonkwang University and were approved by the Institutional Animal Care and Use Committee of Wonkwang University (approval no. WKU 16-44).

Bacterial inoculation

H. pylori SS1 was incubated in brain-heart infusion broth containing 10% FBS overnight at 37ºC under a micro-aerophilic atmosphere and allowed to grow to a density of ~2.0×10^9 CFU/mL. Animals were intragastrically inoculated three times at 3-day intervals with *H. pylori* at 1.0×10^9 CFU in 0.5 mL broth. The challenged animals were confirmed as *H. pylori*-positive by stool antigen analysis using the Bioline *H. pylori* Ag kit (Standard Diagnostics, Suwon City, Korea) as previously described [22].

***In vivo* study protocol**

The inhibition of *H. pylori* growth by *L. paracasei* HP7 was also investigated in a mouse model. Mice were divided into four groups: negative control (group I, n=10); *H. pylori*-infected without *L. paracasei* HP7 treatment (group II, n=10); *L. paracasei* HP7-treated without *H. pylori* infection (group III, n=10); and *H. pylori*-infected with *L. paracasei* HP7 treatment (group IV, n=10). *L. paracasei* HP7 was orally administered at a daily dose of 2.0×10^7 CFU/kg/day/day during a 4-week treatment period. Animals were then sacrificed and their stomachs were dissected after euthanasia with ether. The stomach was opened along the greater curvature and washed with saline, and half of the glandular mucosa was scraped off for detection of colonizing *H. pylori*, while the residual portion was formalin-fixed and embedded in paraffin for histological analysis. *H. pylori* colonization was confirmed by the rapid urease test CLO as previously described [23]. Mucosal damage was evaluated according to established criteria [24].

Blood analysis

Blood samples were collected from the heart of sacrificed animals and centrifuged at 1000×*g* for 15 min at 4ºC; the plasma was stored at 80ºC until analysis. Serum titers of anti-*H. pylori* antibody were measured using the mouse anti-*H. pylori* antibody (IgG-1) ELISA kit (Cusabio Biotech, Wuhan, China) according to the manufacturer's instructions.

Statistical analysis

Values for all parameters under study were recorded for each experimental unit, and statistical analysis was performed using a general linear model. Values are reported as mean±standard deviation where appropriate. The Student's t test was used for pairwise comparisons. The incidence with 95% confidence interval was calculated with MiniTab (State College, PA, USA) statistical software package. A P value <0.05 was considered significant.

Results

***L. paracasei* HP7 inhibits *H. pylori* adhesion to AGS cells**

L. paracasei HP7 was screened with the agar well diffusion assay and was shown to have anti-microbial activity against *H. pylori* SS1 (inhibition zone diameter: 11.5-12 mm). To determine whether *L. paracasei* HP7 affects the adhesion of *H. pylori* to AGS cells, we examined *H. pylori* 16S RNA gene expression in AGS cells. HP7 reduced *H. pylori* adhesion by 65% relative to the control (P<0.05; Figure 1). These results demonstrate

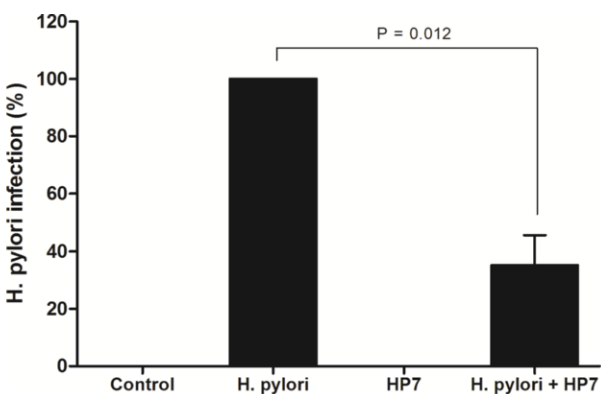

Figure 1. Inhibition of *H. pylori* adhesion to AGS cells by *L. paracasei* HP7 (HP7). AGS cells pre-treated with *L. paracasei* HP7 showed lower expression of *H. pylori* 16S RNA.

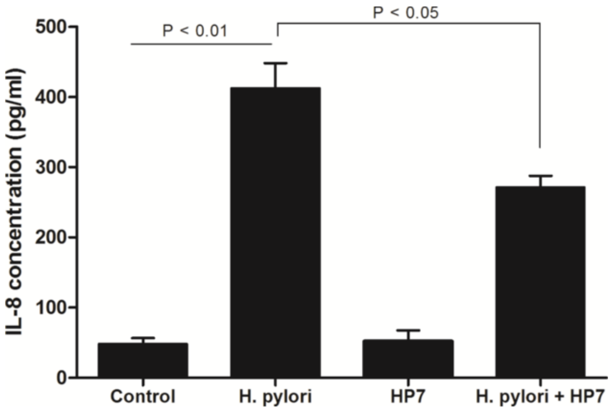

Figure 2. Inhibitory effect of *L. paracasei* HP7 (HP7) on *H. pylori*-induced IL-8 production. *L. paracasei* HP7 was added to a confluent layer of AGS cells in a 96-well plate 30 min before adding *H. pylori*. After incubation for 24 h, the culture supernatant was collected to measure the amount of released IL-8.

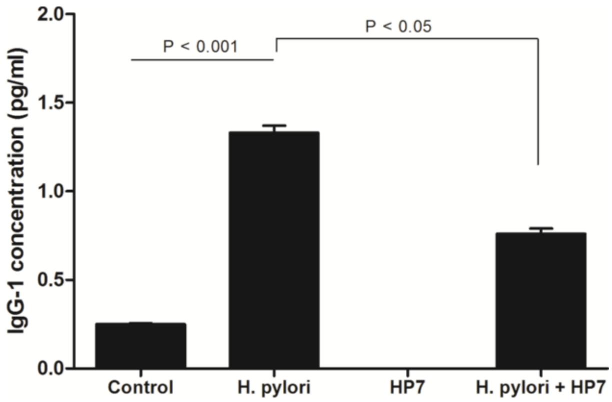

Figure 3. Suppression of *H. pylori* infection in C57BL/6 mice by treatment with *L. paracasei* HP7. Serum samples collected after sacrifice were evaluated for *H. pylori* IgG-1 by ELISA. *H. pylori* IgG-1 levels were decreased in the *H. pylori*/*L. paracasei* HP7 group as compared to the *H. pylori* infection group.

that HP7 can inhibit bacterial adhesion to gastric epithelial cells.

L. paracasei HP7 suppresses *H. pylori*-induced IL-8 production

To determine whether *L. paracasei* HP7 can block *H. pylori*-induced IL-8 production, AGS cells were left untreated or were pre-treated with *L. paracasei* HP7 prior to *H. pylori* infection, and IL-8 production was measured by ELISA. Pre-treatment of *H. pylori*-infected AGS cells with *L. paracasei* HP7 for 24 h decreased IL-8 level by 65.9% (from 410 to 270 pg/mL) relative to control cells (Figure 2).

L. paracasei HP7 decreases anti-*H. pylori* antibody titer in serum

To confirm *H. pylori* colonization in mice, we measured serum levels of anti-*H. pylori* IgG-1, since the serological absorbance index of IgG against *H. pylori* is related to the degree of *H. pylori* colonization [25]. Serum antibody titers were elevated 4 weeks after *H. pylori* inoculation, with values of 1.33±0.04 and 0.76±0.02 pg/mL in the *H. pylori* infection (Group II) and *H. pylori* infection/*L. paracasei* HP7 (Group IV) pre-treatment groups, respectively, as compared to 0.25±0.005 in control animals (Group I) (Figure 3). These results indicate that *H. pylori* infection is reduced by pre-treatment with *L. paracasei* HP7.

L. paracasei HP7 reduces *H. pylori* colonization

Repeated intragastric inoculation of C57BL/6 mice with *H. pylori* (1.0×10^9 CFU/mouse, three times) yielded a positive reaction in the campylobacter-like organism (CLO) test of gastric mucosa (Table 1). The stomachs of *H. pylori*-infected mice orally treated with *L. paracasei* HP7 at a dose of 2.0×10^7 CFU/kg/day during a 4-week period showed a positive reaction rate of 50%. CLO scores were decreased by *L. paracasei* HP7 pre-treatment (Group IV) relative to *H. pylori*-infected animals without pre-treatment (Group II) (P<0.05; Figure 4). Thus, *L. paracasei* HP7 can decrease the rate of *H. pylori* colonization.

Table 1. Reactivity in the CLO test of gastric mucosa from mice infected with *H. pylori* followed by treatment with *Lactobacilllus paracasei* HP7 or vehicle

Group	Treatment	n	Positive %[a]	Therapeutic %
I	No treatment	10	0% (CI[b] 0-27.6)	-
II	*H. pylori*	10	100% (CI 72.2-100)	0%, CI (0-27.6)
III	HP7	10	0% (CI 0-27.6)	-
IV	*H. pylori*+HP7	10	50% (CI 23.7-76.3)	50% (CI 23.7-76.3)

[a]A positive percentage reflects *H. pylori* colonization, which was observed as medium color change from yellow to red.
[b]Incidence (95% confidential interval [CI]) was calculated using MiniTab statistical software.

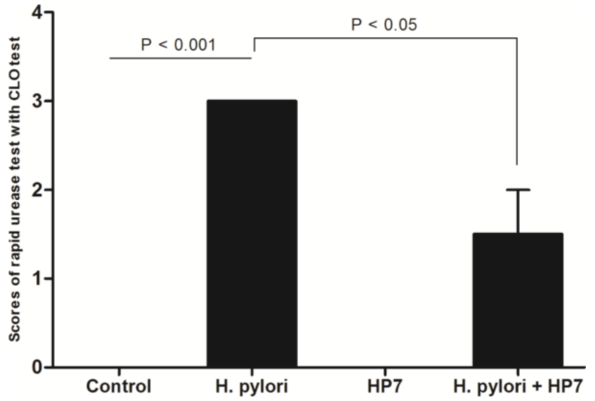

Figure 4. CLO scores for gastric mucosa of mice infected with *H. pylori* followed by treatment with *L. paracasei* HP7.

Table 2. Histopathological lesion scores of mice infected with *H. pylori* followed by treatment with *Lactobacilllus paracasei* HP7 or with no treatment

Group	Treatment	n	Histopathological lesion score
I	No treatment	10	0±0
II	*H. pylori*	10	5.6±0.34
III	HP7	10	0±0
IV	*H. pylori*+HP7	10	3.2±0.25*

*Significantly different from the control group II (P<0.05).

L. paracasei HP7 alleviates gastric mucosa lesions caused by *H. pylori*

Pathological changes in the gastric mucosa were negligible in animals without *H. pylori* infection (groups I and III). In contrast, mice in group II (*H. pylori* inoculation) showed gastric atrophy and ulceration and widespread mucosal destruction. However, mice in group IV (*H. pylori*+*L. paracasei* HP7) showed a significant improvement in villi lesions. These results were confirmed by the lower histopathological lesion score in group IV as compared to group II (Table 2).

Discussion

Lactic acid bacteria suppress the growth of human bacterial pathogens by secreting compounds such as antibiotic agents, organic acids, and bacteriocins and by decreasing environmental pH, thereby inhibiting gastrointestinal infections [26,27]. The inhibitory activity of *H. pylori* has been reported in several *Lactobacillus* spp., including *L. acidophilus* [27], *Lactobacillus casei* [29], *Lactobacillus johnsonii* [30], *Lactobacillus reuteri* [31], and *Lactobacillus salivarius* [32].

A new *Lactobacillus* spp. isolated from kimchi by Korea Yakult Co. Ltd. was identified as *L. paracasei* and was named strain HP7. Kimchi is considered a healthy food since it is enriched in vitamins A, B, and C and is high in fiber, but also contains a number of lactic acid bacteria [33].

In this study, we identified that the adhesion of *H. pylori* to human gastric epithelial cells was inhibited by *L. paracasei* HP7, which also suppressed *H. pylori*-induced inflammation by reducing IL-8 expression in *H. pylori*-infected AGS cells. The inhibitory activity of *L. paracasei* HP7 against *H. pylori* was confirmed in a mouse model; a rapid urease test of mouse stomachs showed decreased *H. pylori* colonization, mucosal inflammation, and epithelial damage. Thus, eradicating *H. pylori* reduced inflammation in the stomach, although it is also possible that *L. paracasei* HP7 has direct anti-inflammatory effects on gastric mucosa.

Although triple therapy consisting of two antibiotics and a proton pump inhibitor is effective over a short term and helps to maintain patient compliance, many patients experience undesirable side effects such as diarrhea, epigastric pain, nausea, and bloating [34]. By comparison, *L. paracasei* HP7 is safe and therefore appropriate for the prevention and treatment of *H. pylori* infection. In this study, the therapeutic effect of *L. paracasei* HP7 was partial showing 50%. However, it revealed *H. pylori* adhesion and reduce the inflammatory response. Other researchers reported also that probiotics alone cannot completely eliminate *H. pylori* but can reduce the amount of *H. pylori* load in the stomach, and alleviate gastric mucosal inflammation [35,36]. Chronic inflammation and increased cell proliferation are features of many human cancers, and their suppression by *L. paracasei* HP7 can potentially prevent *H. pylori*-induced carcinogenesis in the stomach.

In summary, our results show that *L. paracasei* HP7 inhibits *H. pylori* growth and adhesion to gastric epithelial cells *in vitro* and *in vivo*. Thus, *L. paracasei* HP7 can be used to treat patients with gastric symptoms including ulcers caused by *H. pylori*.

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Conflict of interests The authors declare that there is no financial conflict of interests to publish these results.

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