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Antagonistics of *Lactobacillus plantarum* ZDY2013 against *Helicobacter pylori* SS1 and its infection *in vitro* in human gastric epithelial AGS cells

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In this study, the anti-*Helicobacter pylori* activity of *Lactobacillus plantarum* ZDY2013 was investigated and *Lactobacillus rhamnosus* GG was used as a positive control. The anti-*H. pylori* mechanism *in vitro* was also examined. Results revealed that either the viable cells or supernatant of *L. plantarum* ZDY2013 could suppress the growth or urease activity of *H. pylori*. The inhibitory effects of *L. plantarum* ZDY2013 were relatively higher than those of *L. rhamnosus* GG ($P < 0.05$), and such effects might be a result of their lactic acid production (e.g., 51.105 ± 0.097 mmol/L for *L. plantarum* ZDY2013 and 33.113 ± 0.063 mmol/L for *L. rhamnosus* GG). The anti-adhesion capacity of *L. plantarum* ZDY2013 against *H. pylori* was also stronger than that of *L. rhamnosus* GG in terms of inhibition, competition, and displacement. Among these inhibitory strategies, competition exhibited the best performance, with an inhibition ratio of 92.65%. Upon inhibition and anti-adhesion, the cells and supernatant of *L. plantarum* ZDY2013 significantly strengthened the expression of the anti-inflammatory cytokine IL-10, but attenuated the expression of the pro-inflammatory cytokine TNF-α in AGS cells induced by *H. pylori* SS1. Remarkably, the supernatant of ZDY2013 achieved a relatively higher anti-inflammatory effect than that exerted by its cells. With excellent lactic acid yield and antagonistic and anti-inflammatory effects against *H. pylori* SS1 infection, *L. plantarum* ZDY2013 shows potential to be used as a probiotics candidate.

[**Key words:** *Lactobacillus plantarum* ZDY2013; *Helicobacter pylori*; Organic acid; Urease activity; Anti-adhesion; Inflammatory property]

Helicobacter pylori, a Gram-negative microorganism, is recognized as a major cause of most gastroduodenal diseases, including chronic gastritis, peptic ulcer, duodenal ulcer, gastric adenocarcinoma, and gastric cancer (1–3). The prevalence rates of *H. pylori* infection in the adult population are approximately 20%–50% in industrialized countries and as high as 80% in developing countries (4). A prevalent therapy (5) involving a proton pump inhibitor and antibiotics (amoxicillin and clarithromycin) has emerged as the primary treatment of *H. pylori* infection, but this treatment may cause serious side effects. Hence, natural food substances, such as apple peel polyphenols (6), green tea extract (7), and probiotics (8), have been investigated as modes of adjuvant therapy.

Probiotics, defined as "live microorganisms which, when administered in adequate amounts, may exert a health benefit to the host" (9), have been used to fight several gastrointestinal diseases, such as irritable bowel syndrome (10) and infectious diarrhea (11), and *H. pylori*-related gastrointestinal diseases (12). A recent meta-analysis found that the adjunct use of few probiotic strains has improved *H. pylori* eradication rates and prevented the development of adverse events and antibiotic-associated diarrhea in individuals treated with standard eradication therapies (13). For probiotic usage, *Lactobacillus* should possess certain properties, including adhesive ability, competitive and exclusion capacity, and immune modulation, to prevent pathogenic infection in the gastrointestinal epithelium (14,15). Certain *Lactobacillus* species, such as *Lactobacillus rhamnosus* GG (16), *L. acidophilus* (17), *L. casei* (18), *L. johnsonii* (19), *L. reuteri* (20), and *L. salivarius* (21) eradicate *H. pylori* through specific mechanisms, including inhibiting *H. pylori* attachment to mucosal cells (22), reducing *H. pylori*-associated gastric inflammation (23), or eliciting other physiological effects through the production of organic acids or antioxidant substances (24). As a broadly applied species, *Lactobacillus plantarum* exert antagonistic effects against *H. pylori* because the former has organic acids, bacteriocins, or uncertain antimicrobial components. For instance, *L. plantarum* MG208 inhibits the adhesion of *H. pylori* by secreting antimicrobial substances or competing with adhesion receptors (21). To our knowledge, the anti-*H. pylori* activity of *L. plantarum* may be strain specific and associated with different manners of co-existence on epithelial cells and expression of cytokines in the mucosal immune system of the gastrointestinal tract. To date, information regarding the mechanisms of the competition and inhibition of *H. pylori* by *L. plantarum* is limited.

In the present study, *L. plantarum* ZDY2013 was isolated from traditional Chinese fermented soybeans and its probiotic attributes (e.g., acid tolerance, bile tolerance, antimicrobial activity, antibiotic sensitivity, adherence ability) have been evaluated (25,26). Due to its excellent probiotics attributes, it is necessary to further explore whether it has antagonistic and anti-inflammatory effects against *H. pylori* SS1 infection. In this study, therefore, *L. plantarum*

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ZDY2013 was evaluated in terms of its modulation of cytokine expression and anti-*H. pylori* SS1 activity as measured by inhibition, competition, and displacement for adhesion in an AGS cell line. *L. rhamnosus* GG was used as a positive control.

MATERIALS AND METHODS

Bacterial strains and cultural conditions The *L. plantarum* ZDY2013 strain used in this study was isolated from fermented soybeans as described previously (25) and *L. rhamnosus* GG was used as a positive control. For *in vitro* experiments, *L. plantarum* ZDY2013 and *L. rhamnosus* GG were cultured at 37°C overnight in de Man Rogosa Sharpe (MRS) broth (Beijing Solarbio Science & Technology Co. Ltd., Beijing, China) under anaerobic conditions (5% H_2, 10% CO_2, and 85% N_2).

The Sydney strain of *H. pylori* SS1 was cultured on a *Campylobacter* agar base (Oxoid, Basingstoke, England) supplemented with 5% defibrinated sheep blood (Solarbio, Beijing, China), 10 μg/mL vancomycin, 2.5 units/mL polymyxin B, 5 μg/mL amphotericin and 5 μg/mL trimethoprim under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) at 37°C for 48 h.

Human gastric cancer cell lines (AGS cells, BCRC 60102) were cultured using a RPMI-1640 nutrition mixture medium containing 10% fetal bovine serum and 1% penicillin–streptomycin at 37°C in a humidified incubator containing 5% CO_2 (Thermo Scientific).

Determination of organic acid production by *L. plantarum* ZDY2013 through high-performance liquid chromatography The organic acid analysis method was modified as described by (27). The organic acids in the supernatant of *Lactobacillus* were determined by high-performance liquid chromatography (HPLC) (Waters Corporation, Milford, MA, USA). Briefly, the supernatant of overnight culture of *Lactobacillus* was collected by centrifugation and filtration through 0.22 μm filters. Standard solutions containing 10 mM of different organic acids, including acetic acid, fumaric acid, lactic acid, malic acid and oxalic acid, were used. The organic acid quantification of the supernatants of *Lactobacillus* was based on the external standard method (28).

***H. pylori* inhibition assay** Anti-*H. pylori* activity was investigated by agar diffusion assay as previously described (29,30), with minor modification. Briefly, 100 μL aliquots of *H. pylori* SS1 as indicator microorganism (adjusted to 10^8 cfu/mL) were spread onto *Campylobacter* agar. Then, 200 μL of cells (washed twice with 0.01 mM PBS and re-suspended in fresh MRS broth) and supernatants of *Lactobacillus* spp. (pH adjusted to 6.5 or 3.5), dead cells of *Lactobacillus* spp. (by boiling in a water bath for 20 min), and fresh MRS broth were added into Oxford cups (stainless steel cylinder with outer diameter 7.8 ± 0.1 mm, inner diameter 6.0 ± 0.1 mm, and height 10.0 ± 0.1 mm) individually. The plates were incubated at 37°C for 48 h under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2), the diameter of the inhibition zone was measured. The experiment was performed in triplicate.

***H. pylori* urease activity assay** Urease activity was determined by a modified phenol red method (31). Briefly, fresh cells (10^8 CFU/mL) of *H. pylori* SS1 suspended in antibiotic-free brain heart infusion broth (BHIB) containing 5% serum were incubated at 37°C for 24 h under microaerophilic conditions in the presence of a 10% volume of the living cells (10^8 CFU/mL) or supernatants of *L. plantarum* ZDY2013. The cells of *H. pylori* SS1 alone served as the control group. Then, 50 μL of *H. pylori* SS1 cell suspension was added to 200 μL of urease reaction buffer (i.e., 20% (w/v) urea and 0.012% phenol red in phosphate buffer, with the final pH adjusted to 6.5) on a microtiter plate. The plate was incubated at 37°C for 1 h, and its absorbance was measured at 562 nm with a microplate reader (Thermo, USA). Urease activity was adopted as a viability measure of *H. pylori* SS1 (32). The bacteriostatic ratio was calculated as follows:

$$(OD_{562} \text{ of } H.\ pylori\text{- } OD_{562} \text{ of sample group})/OD_{562} \text{ of } H.\ pylori \times 100\% \quad (1)$$

Exclusion effect of *L. plantarum* ZDY2013 against *H. pylori* SS1 adherent onto AGS cells The adhesion assay of gastric epithelial cells (AGS) was determined in accordance with the method reported by Whorwell et al. (33). Briefly, AGS cells were seeded at a concentration of 10^5 cells/mL in 24-well microtiter plates and incubated at 37°C under 5% CO_2 until 95% confluence. For the adhesion assay of *Lactobacillus* spp., 0.5 mL of cells of *Lactobacillus* spp. (10^8 CFU/mL) suspended in fresh FBMI-1640 medium without antibiotics was incubated with AGS cells for 1.5 h. Non-adhering *Lactobacillus* spp. was washed off in triplicate with 0.01 mM PBS, and the AGS cells were lysed using pancreatic enzymes. Appropriate dilutions of the lysate were plated on MRS agar to determine the number of viable cells.

The ability of *Lactobacillus* spp. to inhibit the adhesion of *H. pylori* SS1 to AGS cells was investigated in accordance with the method reported by Pryde et al. (34). Briefly, three different experimental types were performed: (i) Competitive assay simultaneously was incubated for AGS cells (10^5 cells) with *Lactobacillus* spp. (10^8 CFU/mL) and *H. pylori* SS1 (10^8 CFU/mL) for 2 h. (ii) Inhibition assay was pre-incubated for AGS cells (10^5 cells) with *Lactobacillus* (10^8 CFU/mL) for 1.5 h and then added *H. pylori* SS1 (10^8 CFU/mL) and further incubated for 2 h. (iii) Displacement assay was pre-incubated for AGS cells with *H. pylori* SS1 (10^8 CFU/mL) for 2 h and then added *Lactobacillus* (10^8 CFU/mL) and further incubated for 1.5 h. Competitiveness was calculated as the percentage of adhesion of the *H. pylori* added with *Lactobacillus* relative to the number of *H. pylori* -bound bacteria in the absence of *Lactobacillus* (control). The adhesion inhibition of *H. pylori* was expressed as a percentage through the following formula: inhibition of adhesion = 100 × (1 − T1/T2), where T1 and T2 are the percentages of adhesion by *H. pylori* cells in the presence and absence of *Lactobacillus*, respectively. *H. pylori* displacement was expressed as the percentage of adhesion by *H. pylori* cells in the presence and absence of the *Lactobacillus* strain, as described above.

Quantification of cytokine in AGS cultures The levels of tumor necrosis factor alpha (TNF-α) as pro-inflammatory cytokine and interleukin 10 (IL-10) as anti-inflammatory cytokine in AGS cells were determined by quantitative PCR (qPCR) as described by Kirjavainen et al. (35). Briefly, AGS cells (10^5 cells) were pre-treated with the cells or supernatants of *Lactobacillus* (10^8 CFU/mL) for 1.5 h; then *H. pylori* SS1 (10^8 CFU/mL) was incubated for 2 h. Untreated AGS was used as control.

The transcription levels of the cytokines in the AGS cells were evaluated by qPCR. Total RNA was isolated from cells by using the TRIzol reagent (TransGen Biotech, Beijing, China). Single-stranded cDNA was synthesized from the total RNA by using the PrimeScript RT reagent kit with a gDNA Eraser for qPCR (Takara, Shiga, Japan) in accordance with the manufacturer's instructions. The qPCR was conducted with a SYBR Premix Ex Taq II (TliRNaseH Plus) (Takara) and ABI 7500 fast real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). The cycling profile used for qPCR was as follows: a pre-heating step for enzyme activation at 95°C for 1 min, followed by 40 cycles of 95°C for 15 s, 58°C for 60 s, and 72°C for 15 s. The relative transcription level was calculated using the $2^{-\Delta\Delta Ct}$ method (36). The β-2-microglobulin gene was used as reference gene. Oligonucleotides specific to IL-10 and TNF-α (Table S1) were used for qPCR.

Statistical analysis Statistical analysis was performed using the GraphPad Prism v5.0 software. Data were expressed as mean ± SD and subjected to one-way ANOVA. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Comparison of organic acids produced by *L. plantarum* ZDY2013 and *L. rahmnosus* GG In a previous work, *L. plantarum* ZDY2013 was observed to inhibit the activity of *H. pylori* (36). To probe how ZDY2013 inhibits *H. pylori*, we considered whether the supernatant possess anti-*H. pylori* activity. Therefore, we compared the organic acids produced by *L. plantarum* ZDY2013 and *Lactobacillus rahmnosus* GG. The amount of organic acids produced were quantified by HPLC, and the results are shown in Table 1. *L. plantarum* ZDY2013 produced the higher amount for lactic acid (51.105 ± 0.097 mmol/L), followed by malic acid, acetic acid, and oxalic acid. Likewise, *L. rhamnosus* GG produced the higher amount for lactic acid (33.113 ± 0.063 mmol/L). By contrast, *L. plantarum* ZDY2013 produced a significantly higher lactic acid amount than that yield by *L. rhamnosus* GG ($P < 0.001$).

Inhibition of the growth and urease activity of *H. pylori* by *L. plantarum* ZDY2013 Next, we tested the antibacterial activity of *L. plantarum* ZDY 2013 (ZDY) and its supernatant (Sn) against

TABLE 1. Organic acids in the cell-free supernatants of overnight culture of *Lactobacillus* in MRS broth.

Bacterial strain	Oxalic acid (mmol/L)	Malic acid (mmol/L)	Lactic acid (mmol/L)	Acetic acid (mmol/L)	Fumaric acid (mmol/L)
LGG	2.051 ± 0.003	11.857 ± 0.084	33.113 ± 0.063	8.34 ± 0.028	0.008
ZDY	1.661 ± 0.003	11.605 ± 0.083	51.105 ± 0.097*	9.633 ± 0.032	0.016

L. rhamnosus GG, represented with LGG. *L. plantarum* ZDY2013, represented with ZDY. *$P < 0.05$.

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