Journal of Microbiology, Immunology and Infection (2016) 49, 168–174

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ORIGINAL ARTICLE

Lactobacillus pentosus strain LPS16 produces lactic acid, inhibiting multidrug-resistant *Helicobacter pylori*

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Received 9 January 2014; received in revised form 14 April 2014; accepted 16 April 2014
Available online 27 May 2014

KEYWORDS
Acid;
Bactericidal;
Helicobacter;
Lactobacillus

Background/Purpose: Helicobacter pylori is a human gastric pathogen. Antibiotic resistance of *H. pylori* has become a problem increasing the failure of *H. pylori* eradication. Therefore alternative approaches are required. The aim of this study was to evaluate the anti-*H. pylori* activity of *Lactobacillus pentosus* strain LPS16 and the mechanism of its killing effect.
Methods: The anti-*H. pylori* activity of LPS16 was determined by the disc diffusion test and time killing assay. High-performance liquid chromatography analysis was used to analyze the secreted compounds of LPS16. Sixty *H. pylori* strains isolated from different gastric diseases, having different antibiotic susceptibility were collected to analyze the spectrum of anti-*H. pylori* activity of LPS16. Adhesion ability of LPS16 to gastric epithelial cell lines was assayed by flow cytometry.
Results: The anti-*H. pylori* activity of LPS16 depended on the secreted component, and lactic acid mediated bactericidal activity against *H. pylori*. The bactericidal activity did not vary

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http://dx.doi.org/10.1016/j.jmii.2014.04.014

significantly among the strains isolated from different diseases having different antibiotic susceptibility. Moreover, LPS16 can adhere on gastric epithelial cell lines AKG and MKN45.
Conclusion: L. pentosus strain LPS16 had the broad-spectrum anti-*H. pylori* activity, suggesting that it can be used to prevent *H. pylori* infection.

Introduction

Helicobacter pylori is one of the major human pathogens. It can colonize the surface of gastric mucosa and increase the risk of gastroduodenal ulcers. Colonization is also correlated with gastric adenocarcinoma.[1] Therefore, eradication of *H. pylori* is important to prevent peptic ulceration and gastric carcinogenesis. Triple therapy, which is a proton-pump inhibitor combined with two antibiotics, including clarithromycin and amoxicillin, or clarithromycin and metronidazole, is highly recommended as the first line treatment.[2] However, resistance to amoxicillin, clarithromycin, and metronidazole in *H. pylori* has already increased.[3–5] Therefore, new therapies are urgently required.

Many studies have suggested that lactic acid bacteria (LAB), including *Lactobacillus* and *Bifidobacterium*, increase the eradication rate of *H. pylori* infection. LAB inhibit *H. pylori* growth through secretion of protein components or organic acid, decrease the adherence capacity of *H. pylori* to gastric epithelial cells, reduce mucosal inflammation, and stabilize the gastric barrier.[6–9] Clinical trials have demonstrated that LAB increase the eradication rate and decrease the adverse effects when combined with antibiotic treatment.[10]

Lactobacillus pentosus has been shown to inhibit the growth of *Escherichia coli*, *Salmonella*, *Aspergillus niger*, and *Aspergillus oryzae*.[11–13] It also reduced the mortality of influenza virus-infected mice and mice with pneumonia caused by *Streptococcus pneumoniae*.[14–16] The inhibitory activity against *H. pylori* was found in *L. pentosus*, but the mechanism and spectrum of inhibitory activity are still unclear.[17,18]

In this study, we demonstrate that *L. pentosus* strain LPS16 has anti-*H. pylori* activity, and determine its mechanism and spectrum against *H. pylori* isolated from different diseases, and against strains with different antimicrobial susceptibility. In addition, the adhesion ability of LPS16 to gastric epithelial cells was also evaluated.

Methods

Bacterial strains and cell lines

L. pentosus strain LPS16 was isolated from traditional salted bamboo sprouts and provided by Synbiotech Inc. (Kaohsiung, Taiwan). *Lactobacillus reuteri* ATCC55730 was used as a control strain. These strains were cultured in MRS broth (Becton, Dickinson and Company, Sparks, MD, USA) for 24 hours at 37°C prior to usage. This was stored at −80°C in tryptic soy broth supplemented with 0.5% yeast extract and 15% glycerol.

Sixty *H. pylori* strains were collected from the National Cheng-Kung University Hospital, Tainan, Taiwan. *H. pylori* J99, 26695, and ATCC43054 were purchased from ATCC. *H. pylori* was grown on CDC anaerobic blood agar or *Brucella* agar plates (Becton, Dickinson and Company) containing 10% horse serum (abbreviated as BAH) at 37°C under microaerophilic conditions. All bacteria were stored at −80°C in brain–heart infusion (BHI) with 30% glycerol.

The human gastric adenocarcinoma cell line AGS was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan) and was maintained in Ham's F-12 medium (GIBCO BRL, Grand Island, NY, USA) with 10% fetal calf serum (FCS). The other human gastric cancer cell line, MKN45, was obtained from the Health Science Research Resources Bank in Japan and was maintained in RPMI 1640 medium (GIBCO BRL) containing 10% FCS. The cells were subcultured every 2 days.

Time killing assay

Overnight culture of LPS16 and *L. reuteri* ATCC55730 in MRS broth were centrifuged at 2330 g for 10 minutes at 4°C, and cell-free cultured supernatants were obtained from filtration through 0.22 μm-pore-size filters (Millipore, Molsheim, France). They were further checked for absence of bacteria by plating on MRS agar plates. The boiled cell-free supernatant was collected from boiling the cell-free supernatant for 20 minutes and filtration through 0.22 μm-pore-size filters. The neutralized cell-free supernatant was obtained by adjusting to pH 7.0 with NaOH and filtration through 0.22 μm-pore-size filters. Protein concentration of these supernatants was adjusted to 300 ng/μL. The acidified fresh MRS broth was adjusted by lactic acid (LA) or hydrochloric acid (HCl) to pH 3.8 and pH 4.5, and filtered through 0.22 μm-pore-size filters. *H. pylori* from CDC anaerobic blood agar was washed and suspended in BHI broth (OD_{600} = 1). Aliquots of *H. pylori* and cell-free supernatant (250 μL each) were incubated for different periods and viable *H. pylori* cells were counted by plating on BAH.

Measurement of organic acid concentration

The cell-free supernatant of LPS16 overnight culture medium was collected by centrifugation and filtration through 0.22 μm-pore-size filters. The organic acid concentrations in LPS16 and fresh MRS broth were detected by high-performance liquid chromatography, which was performed by the Food Industry Research and Development Institute

(Hsinchu, Taiwan). The organic acids included acetic acid, citric acid, fumaric acid, lactic acid, malic acid, oxalic acid, succinic acid, and tartaric acid.

Disc diffusion assay

Fresh MRS broth and cell-free supernatant of LPS16 were prepared as described above and concentrated to 3 μg/μL by lyophilizer (Labconco, MO, USA). For LPS16 bacterial cell, overnight culture LPS16 was washed with phosphate-buffered saline and adjusted to $OD_{600} = 1.5$. Six-mm paper discs (Becton, Dickinson and Company) were soaked with 20 μL samples and dried for 5 minutes. *H. pylori* (McFarland No. 2.0) was spread on BAH and discs were placed. Clear zone sizes were measured after 2 days culture at 37°C under microaerophilic conditions.

Determination of minimal inhibitory concentration against *H. pylori*

Minimal inhibitory concentrations (MICs) of amoxicillin (GlaxoSmithKline, Middlesex, UK), clarithromycin (Abbott Laboratories, Abbott Park, IL, USA), metronidazole, tetracycline, and ciprofloxacin (Sigma–Aldrich, St Louis, MO, USA) were determined by the agar dilution assay according to the guidelines of the Clinical and Laboratory Standards Institute.[19] Briefly, *H. pylori* (McFarland No. 2.0) was inoculated on antibiotic-containing Mueller–Hinton agar with 5% sheep blood. After incubation for 72 hours, the MIC of each antibiotic was determined. *H. pylori* ATCC43504 was used as a control strain.

Clarithromycin resistance was defined as ≥1.0 μg/mL.[19] The resistances of amoxicillin, ciprofloxacin, metronidazole, and tetracycline were defined as ≥0.5 μg/mL, >1.0 μg/mL, ≥8 μg/mL, and ≥4 μg/mL, respectively.[20]

Bacterium labeling with PKH2 green fluorescence

To estimate the adhesion ability of bacteria to gastric epithelial cells by flow cytometry, the bacteria were labeled with the PKH2 Green Fluorescent Cell Linker Kit (Sigma–Aldrich) following the manufacturer's instructions. Briefly, bacteria were suspended in F12 medium and adjusted to $OD_{600} = 1.0$. A 500-μL sample of the bacterial suspension was centrifuged and suspended in 1 mL diluent A containing 1.5 μL PKH2 dye. After 4 minutes' incubation, the labeling reaction was stopped by adding 1 mL FCS. The labeled bacteria were washed with F12 medium with 2% FCS and 0.1% bovine serum albumin, and finally suspended in 0.1% bovine serum albumin.

Adhesion assay measured by flow cytometry

The adhesion abilities of LPS16 and *H. pylori* were estimated by flow cytometry as previous described.[21] Briefly, AGS and MKN45 cells were treated with 0.25% EDTA and suspended in serum-free F12 and RPMI medium, respectively. AGS and MKN45 cells (5×10^5 cells) were incubated with adequate PKH2-labeled bacteria in 250 μL F12 or RPMI medium. After a 1 hour incubation at 37°C, 500 μL FACS (Fluorescence-activated cell sorting) scan (Becton, Dickinson and Company) was added and the numbers of adhered bacteria were analyzed by flow cytometry.

Statistical analysis

To compare the anti-*H. pylori* activities, unpaired *t* test, one-way analysis of variance (one-way ANOVA) with Bonferroni post test, Mann–Whitney *U* test, Pearson, or Spearman's correlation were used to estimate the statistical significance. A p value <0.05 was considered as statistical significance. All of the statistical analyses were performed in SPSS software, version 17.0 (SPSS Inc., Chicago, IL, USA) and Prism, version 5 (GraphPad software, San Diego, CA, USA).

Results

Lactic acid mediated bactericidal activity of LPS16

A disc diffusion assay was used to estimate the anti-*H. pylori* activities of LPS16 cell and supernatant. Results showed that bacterial cells alone had no inhibition zone on *H. pylori* ATCC43505 and J99 (Fig. 1A), whereas concentrated cell-free supernatant of LPS16 and concentrated MRS showed inhibition zones (Fig. 1B). Concentrated cell-free supernatant of LPS16 produced significantly larger zones than concentrated MRS did (one-way ANOVA with Bonferroni post test, $p < 0.01$).

To confirm the anti-*H. pylori* activity of LPS16 further, a cell-free culture supernatant of LPS16 was incubated with *H. pylori* ATCC43504. After 15 minutes' incubation, viable *H. pylori* were reduced to 10^1 colony-forming units (CFU; Fig. 1C), whereas viable *H. pylori* in MRS broth remained at 10^8 CFU even after 60 minutes (Fig. 1C). The viable *H. pylori* in the cell-free culture supernatant from *L. reuteri* ATCC55730 was also reduced, but more than in LPS16 supernatant after 15 minutes' incubation (unpaired *t* test, $p < 0.01$, Fig. 1C).

To determine if the protein component was required for bactericidal activity in LPS16, cell-free culture supernatant of LPS16 was boiled and incubated with *H. pylori* ATCC43504. No significant difference was found between boiled and un-boiled LPS16 cultured supernatant (unpaired *t* test, $p > 0.05$, Fig. 1C).

The average pH value of LPS16 culture supernatant was 3.8, which was lower than fresh MRS broth (pH = 6.0). To analyze the contribution of acid on bactericidal activity, *H. pylori* ATCC43504 was incubated with neutralized LPS16 cell-free cultured supernatant. Viable *H. pylori* in neutralized LPS16 supernatant and MRS broth were not significantly different (Fig. 1D, unpaired *t* test, $p > 0.05$).

The acid component in LPS culture supernatant was further characterized. Compared to fresh MRS broth, the LA concentration showed dramatic increase after overnight culture of LPS16, whereas other organic acids remained nondetectable or not greatly changed (Table 1). To further determine the role of LA in anti-*H. pylori* activity, fresh MRS broth was adjusted to pH 4.5 or pH 3.8 by LA (MRS-LA). After 15 minutes' incubation with MRS-LA (pH = 3.8), no viable *H. pylori* remained (Fig. 1D), which was similar to the bactericidal effect of LPS16 (Fig. 1C). However, when incubated

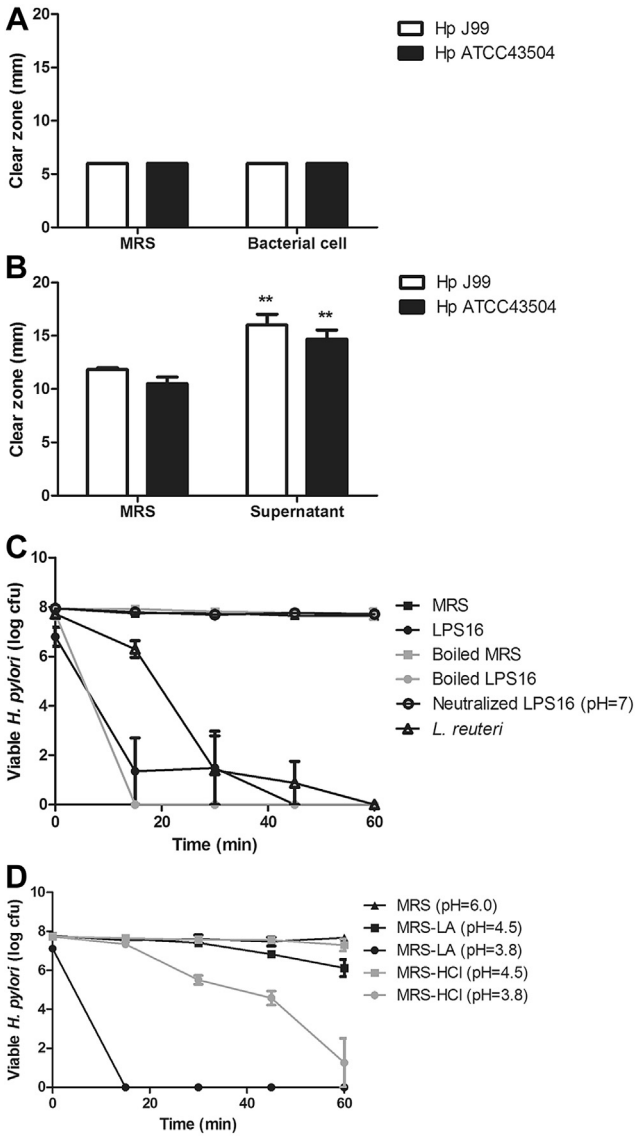

Figure 1. The acid-mediated bactericidal activity of *Lactobacillus pentosus* strain LPS16 on *Helicobacter pylori*. The (A) bacterial cells and (B) concentrated cell-free supernatant were prepared from LPS16 and soaked into 6 mm discs. The anti-*H. pylori* activities on reference strains J99 (white bar) and ATCC43504 (black bar) were estimated by disc diffusion assay. (C) A 250 μL sample of *H. pylori* ATCC43504 (OD_{600} = 1) was incubated with 250 μL fresh MRS broth, LPS16 cell-free culture supernatant, boiled fresh MRS broth, boiled LPS16 culture supernatant, neutralized culture supernatant of LPS16, or cell-free culture supernatant of *Lactobacillus reuteri* ATCC 55730. After 15 minutes, 30 minutes, 45 minutes, or 60 minutes, *H. pylori* was plated on *Brucella* plates with 10% horse serum, and viable *H. pylori* was calculated from at least three independent experiments. (D) A 250 μL sample of *H. pylori* ATCC43504 (OD_{600} = 1) was incubated with 250 μL fresh MRS broth (pH = 6.0), MRS-LA (pH = 4.5 and pH = 3.8), or MRS-HCl (pH = 4.5 and pH = 3.8). After 15 minutes, 30 minutes, 45 minutes, or 60 minutes, *H. pylori* was plated on *Brucella* plates with 10% horse serum, and viable *H. pylori* was calculated from at least three independent experiments. $^{**}p < 0.01$.

Table 1 The concentration of organic acids in fresh MRS broth and *Lactobacillus pentosus* LPS16 culture supernatant

Organic acid	Concentration (g/100 g) MRS	LPS16
Acetic acid	0.40	0.39
Citric acid	0.19	0.15
Fumaric acid	ND	ND
Lactic acid	0.06	2.74
Malic acid	ND	ND
Oxalic acid	ND	ND
Succinic acid	ND	ND
Tartaric acid	ND	ND

ND = not detectable.

with MRS-LA (pH = 4.5), after 60 minutes' incubation there were still 1.3×10^6 CFU viable *H. pylori* (Fig. 1D).

To exclude the nonspecific effect from low pH, HCl was used to adjust the pH value of fresh MRS broth. Compared to lactic acid, the HCl produced lower bactericidal activity in both pH 3.8 and pH 4.5 conditions (Fig. 1D).

The anti-*H. pylori* activity of LPS16 was independent of the diseases and antibiotic susceptibility of *H. pylori*

To verify the anti-*H. pylori* activity further, 60 *H. pylori* strains isolated from duodenal ulcer (29 strains), gastric cancer (24 strains), gastric ulcer (3 strains), and gastritis (4 strains) were used to assay the anti-*H. pylori* activity of LPS16. The results showed that LPS16 supernatant had a larger inhibition zone than MRS broth did (Fig. 2A, unpaired *t* test, $p < 0.05$). Susceptibilities of *H. pylori* from different diseases to LPS16 supernatant were not different (Fig. 2A, one-way ANOVA, $p > 0.05$). However, MRS broth produced smaller clear zones on the strains isolated from gastric ulcer and gastritis (Fig. 2A, one-way ANOVA, $p < 0.05$).

To analyze if antibiotic-resistant *H. pylori* was more resistant to LPS16 treatment, 35 *H. pylori* strains with different antibiotic susceptibility were chosen for further analysis. The antibiotic susceptibilities of *H. pylori* to amoxicillin, clarithromycin, ciprofloxacin, metronidazole, and tetracycline were determined by agar dilution. Among 35 *H. pylori* strains, 17 strains were sensitive to all antibiotics, and 18 strains were resistant to at least one of the antibiotics. Antibiotic-sensitive and antibiotic-resistant strains had similar susceptibility to MRS and LPS16 (Fig. 2B, unpaired *t* test, $p > 0.05$), and LPS16 had significantly larger inhibition zones than MRS did (Fig. 2B, unpaired t test, $p < 0.05$).

To elucidate the susceptibility of antibiotic-resistant *H. pylori* to LPS16 treatment further, 18 antibiotic-resistant *H. pylori* strains were further divided according to their resistant patterns. Eight, one, seven, and two strains were resistant to one, two, three, and four antibiotics, respectively (Table 2). The number of resistances was not correlated to the susceptibility to LPS16 (Pearson and Spearman correlation, $p > 0.05$), and the MIC of each antibiotic was not correlated to the susceptibility to LPS16, either ($p > 0.05$).

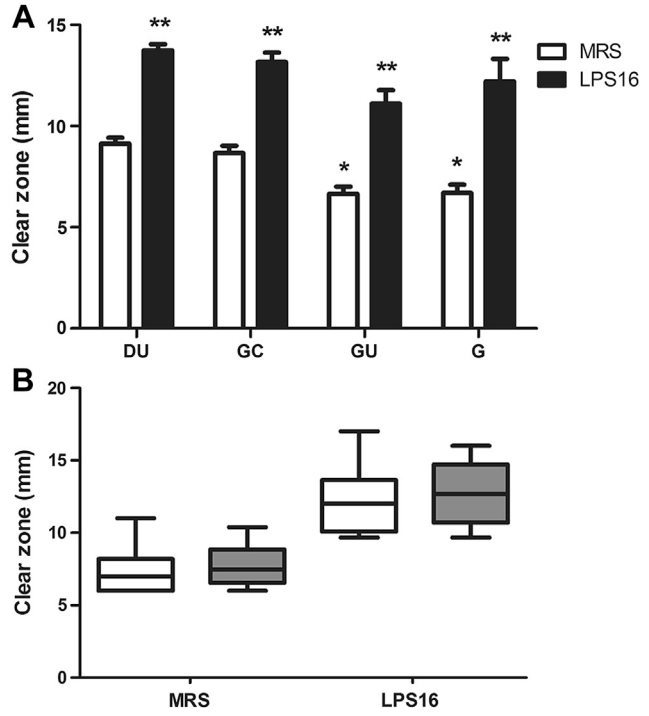

Figure 2. The antibacterial activity of *Lactobacillus pentosus* strain LPS16 on clinical *Helicobacter pylori*. (A) Sixty *H. pylori* strains isolated from duodenal ulcer (DU, 29 strains), gastric cancer (GC, 24 strains), gastric ulcer (GU, 3 strains), and gastritis (G, 4 strains) were used to estimate the anti-*H. pylori* activity of LPS16 by disc diffusion assay. (B) Thirty-five *H. pylori* strains, including 17 antibiotic-sensitive strains (white boxes) and 18 strains (gray boxes) resistant to at least one antibiotic were chosen to analyze the anti-*H. pylori* activity of LPS16 by disc diffusion assay. The clear zone size was calculated from at least three independent experiments. Box plot was used to present the distribution of clear zone size. The error bar describes the minimum and maximum zone sizes. $^{*}p < 0.05$. $^{**}p < 0.01$.

LPS16 adhered to epithelial cell lines

To test whether LPS16 can adhere to gastric epithelial cells, PKH2-labeled LPS16 was incubated with gastric epithelial cells AGS and MKN45, and adherence ability was determined by flow cytometry. LPS16 [multiplicity of infection (MOI) = 100] showed significant adherence to two cell lines when compared to epithelial cells only (Fig. 3, Mann–Whitney *U* test, $p < 0.05$). However, LPS16 (MOI = 100) showed less adhesion ability than *H. pylori* J99 and 26695 (MOI = 10 and MOI = 20) on the two cell lines (Fig. 3, Mann–Whitney *U* test, $p < 0.05$).

Discussion

In this study, cell-free supernatant of *L. pentosus* strain LPS16 showed a bactericidal effect against *H. pylori*, and LA played a key role. The bactericidal activity of LPS16 was similar among antibiotic-sensitive and -resistant *H. pylori* and strains isolated from different diseases. LPS16 can adhere to gastric epithelial cell lines AGS and MKN45, which suggests that LPS16

Table 2 The susceptibility of 35 *Helicobacter pylori* strains to antimicrobial agents and *Lactobacillus pentosus* strain LPS16 culture supernatant

No. of resistant antibiotics	Resistant antibiotics	No. of strains	Average clear zone size, mm (SD)		MIC range (μg/mL)*				
			MRS	LPS16	AMX	CIP	CLR	MTZ	TET
0		17	7.45 (1.58)	12.13 (2.04)	0.03–0.12	0.03–0.50	0.03–0.50	0.03–4.00	0.03–0.50
1	CLR	1	10.4	15.6	0.03	0.50	64.00	4.00	0.12
	CIP	3	9.21 (1.25)	13.60 (1.04)	0.03–0.03	1.00–32.00	0.06–0.06	1.00–4.00	0.12–1.00
	MTZ	4	7.99 (1.06)	13.09 (1.21)	0.03–0.03	0.12–0.50	0.03–0.06	8.00–32.00	0.03–0.50
2	MTZ + CIP	1	7.67	15.00	0.12	16.00	0.03	64.00	0.12
3	AMX + CLR + MTZ	2	6.00 (0.00)	10.25 (0.12)	1.00–1.00	0.50–0.50	64.00–64.00	64.00–64.00	0.24–0.50
	CLR + MTZ + CIP	5	6.91 (1.11)	11.98 (2.56)	0.12–0.24	1.00–16.00	32.00–64.00	16.00–256.00	0.12–1.00
4	AMX + CLR + MTZ + CIP	1	7.20	12.40	0.50	16.00	64.00	32.00	0.50
	CLR + MTZ + TET + CIP	1	7.25	12.25	0.24	32.00	64.00	64.00	4.00

AMX = amoxicillin; CIP = ciprofloxacin; CLR = clarithromycin; MIC = minimal inhibitory concentration; MTZ = metronidazole; SD = standard deviation; TET = tetracycline.

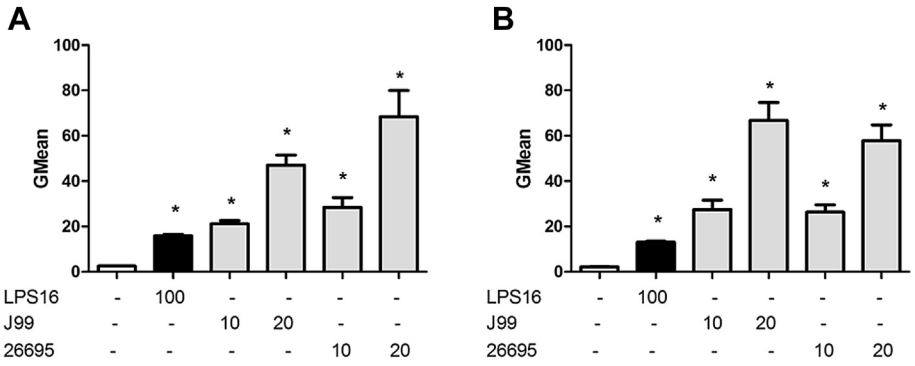

Figure 3. The adhesion abilities of *Lactobacillus pentosus* strain LPS16, *Helicobacter pylori* J99 and 26695 on gastric epithelial cells. Bacteria were labeled with green fluorescence PKH2 and the adhesion ability of each strain on gastric epithelial cell lines (A) AGS and (B) MKN45 were analyzed by FACS scan. The multiplicity of infection 10, 20, and 100 of each experiment are shown and the results are expressed by geometric mean of fluorescence (GMean) ± standard error from three independent experiments. *$p < 0.05$.

can retain in the gastric environment. These data suggest that LPS16 can be useful in preventing *H. pylori* infection.

It is known that bacterial cell-associated compounds had anti-*H. pylori* activity, and bactericidal activity may be the result of autolysis of *Lactobacillus acidophilus* and release of cytosolic autolysins.[22,23] However, in *L. pentosus* strain LPS16, bacterial cells did not have anti-*H. pylori* activity, and the bactericidal activity was mainly dependent on secreted LA. *L. pentosus* was classified as a heterofermentative LAB, and genes involved in producing acetate and LA are present in its genome.[24] However, when LPS16 is cultured in MRS broth, only LA showed a dramatic increase, suggesting that production of acetic acid is not favored in MRS broth. It has been shown that LA had higher bactericidal activity than acetic acid and HCl, and increased bactericidal activity against *H. pylori* was correlated with increased concentration of acid.[9,25] Because LPS16 mainly produced LA, it suggests LPS16 may have higher bactericidal activity against *H. pylori*. As shown in Fig. 1C, LPS16 had higher anti-*H. pylori* activity than *L. reuteri* ATCC55730, which has been demonstrated to show an increased eradication rate of *H. pylori* infection and decreased side effects of antibiotic treatment,[26,27] suggesting LPS16 can be useful to prevent *H. pylori* infection.

The metronidazole- and clarithromycin-resistant *H. pylori* strains showed sensitivity to treatment with *Lactobacillus salivarius*, *Lactobacillus gasseri*, *Lactobacillus delbruckii* subsp. *bulgaricus*.[7,28,29] In this study, although the susceptibilities of *H. pylori* varied, LPS16 showed anti-*H. pylori* activity on antibiotic-sensitive and -resistant strains; even the strains with multidrug resistance and higher MICs were still susceptible to LPS16 treatment. This indicates that LPS16 can be used to treat antibiotic-resistant *H. pylori* infection.

In summary, this study showed that LPS16 secretes a large amount of LA, which has a broad spectrum of anti-*H. pylori* activity. LPS16 can be used to prevent *H. pylori* infection.

Conflicts of interest

This work was supported in part by the Southern Taiwan Science Park, Taiwan, and Synbiotech Inc (grant 100CB03, production improvement and clinical study on gastrointestinal function of Kefir lactic acid bacteria).

Acknowledgments

We thank Robert Jonas for helpful comments on the manuscript. This work was supported in part by the Southern Taiwan Science Park, Taiwan and Synbiotech Inc., (grant 100CB03), and the National Science Council, Taiwan (grant NSC-99-3112-B-006-015).

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