International Journal of Interferon, Cytokine and Mediator Research Dovepress
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ORIGINAL RESEARCH

Human gastric biopsy-derived lactobacilli suppress *Helicobacter pylori*-induced interleukin-8 production from gastric epithelial cells in vitro

This article was published in the following Dove Press journal:
International Journal of Interferon, Cytokine and Mediator Research
7 May 2011
Number of times this article has been viewed

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Abstract: In this report we evaluate the anti-inflammatory effect of a variety of *Lactobacillus* spp. isolated from human gastric biopsies of dyspeptic patients. These lactobacilli were previously shown to secrete factors that inhibit tumor necrosis factor production from human monocytoid cells in vitro. Conditioned media from these lactobacilli were tested for the ability to modulate *Helicobacter pylori*-induced interleukin-8 (IL-8) production in AGS gastric epithelial cells. Out of 26 *Lactobacillus* spp. isolated from urease-negative patients thought not to harbor *H. pylori*, five significantly inhibited IL-8 production. The IL-8-inhibitory lactobacilli were identified as *L. casei* or *L. paracasei*, *L. plantarum*, *L. rhamnosus*, and *L. salivarius* and had no antagonistic effects on *H. pylori* growth. Our findings suggest potential candidates of *Lactobacillus* for further investigation of their beneficial effects on *H. pylori*-infected patients.

Keywords: *Lactobacillus, Helicobacter pylori,* interleukin-8, stomach, gastric biopsy, probiotics

Introduction

Helicobacter pylori is a Gram-negative bacterial pathogen that infects the stomach of humans worldwide. Persistent infection with *H. pylori* results in a gastric inflammatory response, leading to the development of gastroduodenal diseases like chronic atrophic gastritis, gastric and duodenal ulcers, and gastric cancer.[1,2] *H. pylori* infection induces the production of several inflammatory mediators, including interleukin-8 (IL-8),[3–5] which is a potent neutrophil chemotactic and activating agent[6,7] and is associated with disease severity.[8,9]

Less than 80% of *H. pylori* infections are eradicated with standard triple therapy,[10–12] and the eradication rate is less than 60% if clarithromycin-resistant *H. pylori* is present.[13,14] Even with more aggressive therapy, cases of clarithromycin-resistant *H. pylori* continue to result in unsatisfactory eradication rates.[15] A potential solution to declining eradication rates of *H. pylori* infection is the administration of probiotic organisms. Others have demonstrated probiotics to be useful adjuncts in the treatment of *H. pylori* infection, resulting in higher eradication rates, reduction of severity and activity of gastritis, and reduction of antibiotic-associated side effects.[16,17]

We previously isolated lactobacilli from gastric biopsies of dyspeptic patients and demonstrated their immunomodulatory activity against tumor necrosis factor (TNF) production from lipopolysaccharide (LPS)-activated THP-1 monocytoid cells.[18] In this study, we investigated whether human stomach-derived *Lactobacillus* spp. can modulate IL-8 production from *H. pylori*-stimulated AGS gastric epithelial cells.

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Materials and methods

Bacterial strains, culture conditions, and preparation of *Lactobacillus*-conditioned media

Lactobacillus spp. were routinely grown on de Man, Rogosa, Sharpe (MRS) agar (Becton Dickinson, Sparks, MD) anaerobically (10% CO_2, 10% H_2, and 80% N_2) in an anaerobic chamber (Concept Plus, Ruskinn Technology Ltd, Bridgend, UK) at 37°C. *Lactobacillus*-conditioned media (LCM) were prepared as previously described.[19] Briefly, lactobacilli were cultured as stated previously for 24 hours. The optical density of each culture was determined at 600 nm (OD_{600}) by spectrophotometery (Bio-Rad Smart Spec™ Plus, Bio-Rad Laboratories Inc, Hercules, CA), adjusted to an OD_{600} 0.1 in MRS broth, and cultured as stated previously for 48 hours. Culture supernatant was collected by centrifugation at 4000 × g for 10 minutes at 4°C, and the collected supernatant was filtered through a 0.22 µm filter (Minisart, Sartorius Stedim Biotech GmbH, Goettingen, Germany). Filtered supernatant was concentrated in a Savant SpeedVac (Savant instruments, Farmingdale, NY) and then resuspended in an equal volume of RPMI 1640 medium (Gibco-Invitrogen, Carlsbad, CA) for further testing with AGS cells. The conditioned media were stored at –20°C until further analysis.

H. pylori ATCC 43504 was grown on Columbia blood agar (Oxoid, Hampshire, UK) supplemented with 7% (v/v) horse serum (Gibco New Zealand Ltd, Auckland, New Zealand) and 7% (v/v) sheep blood at 37°C for 48 hours under microaeorphilic conditions (6–12% O_2, 5–8% CO_2) using MGC Anaero Pack-Micro Aero boxes (Mitsubishi Gas Chemical Company, Inc, New York, NY). Bacterial cells were then transferred to, and resuspended in, antibiotic-free RPMI medium. The bacterial suspension was adjusted to the density of No. 6 McFarland standard and used in the coculture assay.

THP-1 monocytoid cell culture and bioassay for TNF activity

Monocytoid cell culture and in vitro bioassays were performed as stated previously.[18] Briefly, THP-1 human monocytoid cells (ATCC TIB- 202, Manassas, VA) were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco-Invitrogen) in 96-well flat-bottomed tissue culture plates (Nunclon D, Roskilde, Denmark) and incubated at 37°C in a humidified 5% CO_2 incubator. THP-1 cells (2.5×10^5 cells/mL) were incubated with conditioned media (5% v/v) alone or in combination with purified LPS (100 ng/mL) from *Escherichia coli* serotype O127:B8 (Sigma-Aldrich, St Louis, MO) for 3.5 hours at 37°C. Supernatants were collected from individual wells by centrifugation at 4°C and assayed for TNF with human TNF-specific sandwich quantitative enzyme-linked immunosorbent assay (DuoSet, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

AGS gastric epithelial cell culture

AGS human gastric adenocarcinoma epithelial cells (ATCC CRL-1739) were obtained from the American Type Culture Collection (Manassas, VA). AGS cells were cultured as a monolayer (>80% confluence) in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco-Invitrogen) at 37°C and 5% CO_2 atmosphere for 48 hours. Adherent cells were detached from the flask with 0.25% (v/v) trypsin in 1 mM EDTA (Gibco-Invitrogen) at 37°C for 7–10 minutes. Detached cells were suspended in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco-Invitrogen), and the cell suspension was used for coculture assay.

Bioassay for IL-8 activity in AGS gastric epithelial cells

AGS cell suspensions were seeded at a density of 1.0×10^5 cells/mL (20,000 cells per well) in a volume of 200 µL per well of a 96-well flat-bottomed tissue culture plate (Nunclon D) and preincubated as described previously. After 24 hours of incubation, the culture supernatant was replaced with 200 µL of fresh RPMI. Cells were exposed to 5% (v/v) conditioned media alone or in combination with 3×10^7 colony-forming unit (CFU)/mL viable *H. pylori* ATCC 43503 (6.0×10^6 CFU per well with a multiplicity of infection of 300). The assay plate was incubated under 5% CO_2 at 37°C for 24 hours, and cell culture supernatants were collected by centrifugation at 125 × g for 7 minutes at 4°C. The IL-8 level in the culture supernatant was determined using a Quantikine Human IL-8 Immunoassay Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Cell viability assay

Cell viability was tested by Trypan blue dye exclusion (Gibco-Invitrogen). Briefly, cell suspensions were mixed with 0.4 w/v trypan blue solution (1:1) and visually examined for the inclusion or exclusion of dye. Trypan blue stains nonviable cells and is excluded by viable cells. The number of total cells and stained cells were counted on a hemocytometer under an inverted microscope within a 1 mm^2 area. The percentage of viable cells was calculated from the ratio of viable cells over total cells.

H. pylori growth inhibition by lactobacilli

Disc diffusion method

IL-8-inhibitory strains of lactobacilli were tested for antagonistic activity against *H. pylori* ATCC 43504 as follows. Sterile 6 mm membrane discs (Whatman, Maidstone, UK) were soaked in cell-free *Lactobacillus*-conditioned media at room temperature for at least 1 hour. *H. pylori* was applied to Columbia blood agar, and discs containing *Lactobacillus*-conditioned media were subsequently placed atop the agar. After incubation under microaerophilic conditions at 37°C for 72 hours, a clear zone around a disc was scored positive for antagonistic activity against the growth of *H. pylori*. A disc soaked with MRS broth was used as a negative control. Conditioned medium from *L. salivarius* T59, our laboratory strain that can antagonize the growth of *H. pylori*, was used as a positive control. The experiments were performed in triplicate.

Spot-overlay method

Antagonism of *H. pylori* growth was also assessed using a spot-overlay method as previously described.[20] Briefly, *Lactobacillus* spp. were cultured anaerobically at 37°C for 24 hours in brain heart infusion (BHI) broth (Oxoid), spotted onto BHI agar, and incubated anaerobically at 37°C for 48 hours. The spotted plate of *Lactobacillus* was then overlaid with soft agar (0.75% agar) containing 72-hour culture of *H. pylori* in BHI broth supplemented with 10% horse serum at a final concentration of 1×10^8 CFU/mL. The overlain plate was incubated microaerophilically at 37°C for 72 hours. A clear zone around each spot of *Lactobacillus* was scored as positive. *L. salivarius* T59 was included as a positive control for inhibition of *H. pylori*. The experiments were performed in triplicate.

Statistical analysis

All experiments were performed three times in triplicate, and the results were reported as mean value with standard deviation (SD). The data were analyzed using the Student's *t*-test with one-tailed distribution and were considered statistically significant at a P value < 0.05.

Results

IL-8 production by *H. pylori*-stimulated AGS gastric epithelial cells and immunomodulatory effects of gastric biopsy-derived *Lactobacillus* spp.

Twenty-six *Lactobacillus* spp. isolated from gastric biopsies of dyspeptic patients were selected on the basis that they are able to inhibit TNF production in LPS-activated THP-1 monocytoid cells but do not stimulate TNF production in high magnitude by themselves[18] (Table 1, Figure 1). These isolates were tested for the ability to inhibit IL-8 production in *H. pylori*-stimulated AGS gastric epithelial cells. AGS gastric epithelial cells secreted increased amounts (>15-fold) of IL-8 after 24 hours of coincubation with *H. pylori* compared with cells exposed to bacterial media (MRS) alone. Cell-free conditioned media of five *Lactobacillus* spp. significantly inhibited IL-8 production in various magnitudes, whereas those of 14 lactobacilli did not (Figure 2). Conditioned media of these 19 lactobacilli did not have cytotoxic effects on AGS cells, as the percentage of viable cells in each sample was >80%. Conditioned media from seven lactobacilli (*L. casei* B13, *L.gasseri* XB68, *L. salivarius* B8 and B73, and *L. plantarum* B64, B67, and B87) killed more than 35% of AGS cells, resulting in >80% of IL-8 suppression (data not shown).

The ability of the five IL-8-suppressing *Lactobacillus* spp. (*L. plantarum* B90 and XB7, *L. salivarius* B60, *L. rhamnosus* B103, and *L. casei* or *L. paracasei* B106) to modulate IL-8 production in the presence or absence of *H. pylori* is shown in Figure 3. In the absence of *H. pylori*, the conditioned media of these five *Lactobacillus* spp. did not significantly stimulate IL-8 production.

IL-8-inhibitory *Lactobacillus* spp. did not inhibit the growth of *H. pylori*

To investigate whether IL-8-suppressive activity of these five *Lactobacillus* spp. resulted from the inhibition of *H. pylori* growth, we tested the antagonism of *H. pylori* growth by using the LCM disc diffusion and spot-overlay methods. It was found that these lactobacilli did not inhibit the growth of *H. pylori*, as shown in Table 2.

Discussion

The human gastrointestinal microbiota are an important source of potential probiotics for human use. The human stomach harbors diverse microbial flora, including lactobacilli, as determined by 16S rRNA gene sequencing,[21] and different *Lactobacillus* spp. have been cultivated from gastric biopsies of healthy humans.[22,23] These gastric lactobacilli could be promising candidate probiotics for *H. pylori*-associated diseases. Although our gastric *Lactobacillus* spp. were isolated from dyspeptic patients, they have the ability to inhibit TNF production from LPS-activated THP-1 monocytoid cells.[18] In the present study,

Table 1 Tumor necrosis factor (TNF)-inhibitory *Lactobacillus* spp. isolated from gastric biopsies of dyspeptic patients

Code	Species[a]	Source[b]	TNF value (pg/mL)[c]		% TNF suppression[d]
			No LPS	With LPS	
B13	*L. casei*	Mild gastritis	27.59 ± 1.70	502.41 ± 48.13	16.45*
XB68	*L. gasseri*	Normal	–	331.05 ± 82.13	63.64***
B90	*L. plantarum*	Normal	–	667.85 ± 133.81	47.32*
XB94	*L. gasseri*	Normal	–	662.83 ± 83.72	28.05*
B106	*L. casei* or *L. paracasei*	Normal	–	598.00 ± 179.26	35.09*
B7	*L. plantarum*	Gastritis	–	322.40 ± 248.35	74.52**
B8	*L. salivarius*	Gastritis	31.05 ± 53.78	102.67 ± 60.48	76.31***
B21	*L. salivarius*	Gastritis	218.38 ± 3.31	565.46 ± 2.65	47.47***
XB41	*L. gasseri*	Gastritis	–	959.62 ± 124.16	30.16*
XB45	*L. gasseri*	Gastritis	283.82 ± 31.37	495.33 ± 20.82	25.31*
B47	*L. salivarius*	Gastritis	183.20 ± 180.17	565.07 ± 345.54	55.34*
XB48	*L. gasseri*	Gastritis	136.55 ± 26.22	543.52 ± 92.45	18.05*
XB58	*L. gasseri*	Gastritis	–	436.48 ± 66.48	36.85*
B64	*L. plantarum*	Gastritis	–	173.49 ± 118.69	74.90**
B60	*L. salivarius*	Gastritis	224.53 ± 98.58	359.24 ± 61.67	48.03*
B67	*L. plantarum*	Gastritis	–	382.86 ± 157.58	53.39*
B73	*L. salivarius*	Gastritis	151.43 ± 19.18	383.57 ± 109.76	53.30**
XB77	*L. gasseri*	Gastritis	70.61 ± 47.29	609.78 ± 40.00	27.12*
B87	*L. plantarum*	Gastritis	–	588.54 ± 96.42	41.91**
XB95	*L. gasseri*	Gastritis	–	1272.38 ± 166.13	24.76**
XB96	*L. gasseri*	Gastritis	–	1112.16 ± 72.73	34.23***
B103	*L. rhamnosus*	Gastritis	–	1033.51 ± 27.97	21.48***
XB7	*L. plantarum*	Gastritis	–	241.22 ± 66.09	57.23***
B23	*L. salivarius*	Gastric ulcer	–	340.53 ± 87.06	39.62**
B36	*L. agilis*	Gastric ulcer	–	293.65 ± 119.97	62.76*
XB40	*L. gasseri*	Gastric ulcer	–	110.99 ± 35.10	82.41**

Notes: [a]Species was identified based on 16S rRNA gene sequence identity; [b]Denote the patient's endoscopic diagnosis. All patients were urease negative, suggesting the absence of *Helicobacter pylori* in the stomach; [c]Data are reported as the mean value ± standard deviation; – indicates TNF value below detection limit; [d]TNF suppression was calculated from the difference of TNF value of THP-1 cells cocultured with MRS media control + LPS and LCM + LPS. Significantly different from control: ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

Abbreviations: LPS, lipopolysaccharide; MRS, de Man, Rogosa, Sharpe.

we demonstrated that five gastric isolates of *Lactobacillus* spp. have the ability to inhibit IL-8 production from AGS gastric epithelial cells stimulated by *H. pylori*. This suggests that these lactobacilli secrete immunoregulatory factors known as immunomodulins,[24] which are potentially capable of inhibiting IL-8 production from their host. Previous reports have shown that viable *L. bulgaricus* or its culture supernatant inhibited IL-8 production in SGC-7901 gastric adenocarcinoma cells treated with *H. pylori*.[25] In addition, live *L. gasseri* OLL2716 (LG21), as well as its spent culture supernatant, was reported to suppress *H. pylori*-induced IL-8 production from both MKN45 gastric epithelial cells and the gastric mucosa of *H. pylori*-infected patients.[26] The fact that different strains of the same species of our gastric lactobacilli varied in their ability to suppress IL-8 production highlights the strain specificity of this inhibitory property. IL-8 secreted by gastric epithelial cells of *H. pylori*-infected patients stimulates the migration of neutrophils and lymphocytes to the inflamed mucosa, resulting in more inflammatory pathology.[2] Neutrophils and mononuclear cells recruited to patients' mucosa induce nitric oxide synthase to produce mutagenic nitric oxides that may result in DNA damage and subsequent apoptosis in the gastric mucosa.[27] Therefore, our IL-8-inhibitory gastric *Lactobacillus* may be beneficial for the reduction of mucosal inflammation.

Probiotic *Lactobacillus* are known to suppress *H. pylori*-induced IL-8 production in vitro by different mechanisms, such as the inhibition of the TLR4 pathway,[25] acid production,[26,28] activation of suppressor of cytokine signaling,[29] and inhibition of expression and function of the Cag type IV secretion system.[28] Our isolated *Lactobacillus* did not inhibit the growth of *H. pylori* as shown by either the disc diffusion or spot-overlay assays. In addition, IL-8 production was not affected by acidity, because the pH

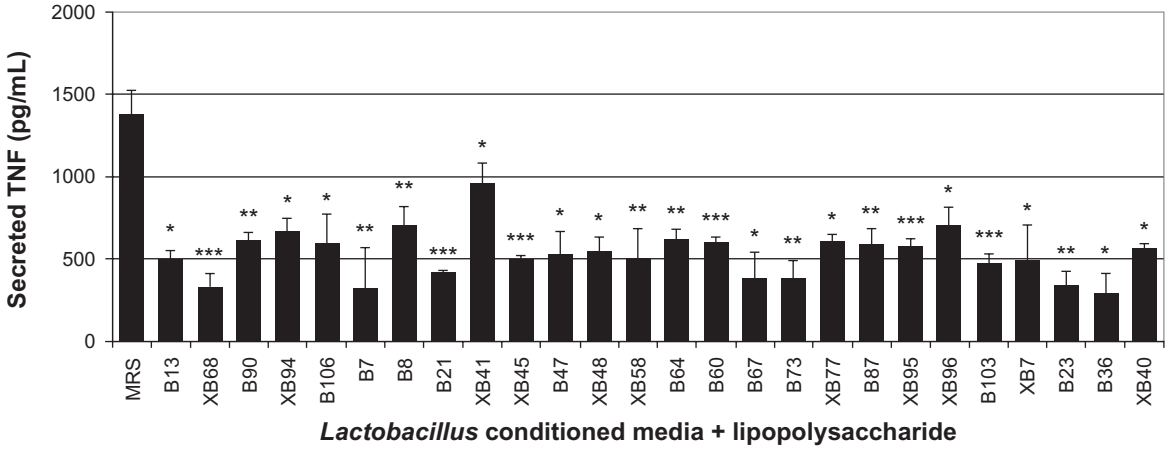

Figure 1 Effects of *Lactobacillus* conditioned media on TNF production in LPS-activated THP-1 monocytoid cells.
Notes: TNF secretions were determined by human TNF enzyme-linked immunosorbent assay. The results are expressed as the mean of triplicate determinations. Significantly different from control: ***$P<0.001$; **$P<0.01$; *$P<0.05$. Error bars indicate standard deviations.
Abbreviations: LPS, lipopolysaccharide; MRS, de Man, Rogosa, Sharpe; TNF, tumor necrosis factor.

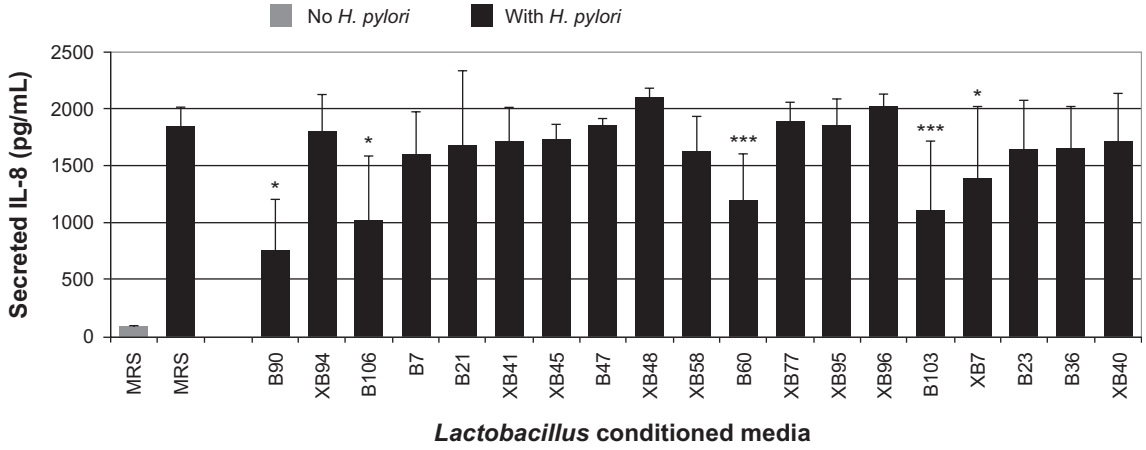

Figure 2 Effects of *Lactobacillus*-conditioned media from 19 gastric isolates on IL-8 production from *Helicobacter pylori*-stimulated AGS gastric epithelial cells.
Notes: AGS cells were coincubated with *Lactobacillus*-conditioned media and *H. pylori*. IL-8 secretions were determined by human IL-8 enzyme-linked immunosorbent assay. The results are expressed as the mean of triplicate determinations representative of three independent experiments. Significantly different from control: ***$P < 0.001$; *$P < 0.05$. Error bars indicate standard deviations.
Abbreviations: IL-8, interleukin-8; MRS, de Man, Rogosa, Sharpe.

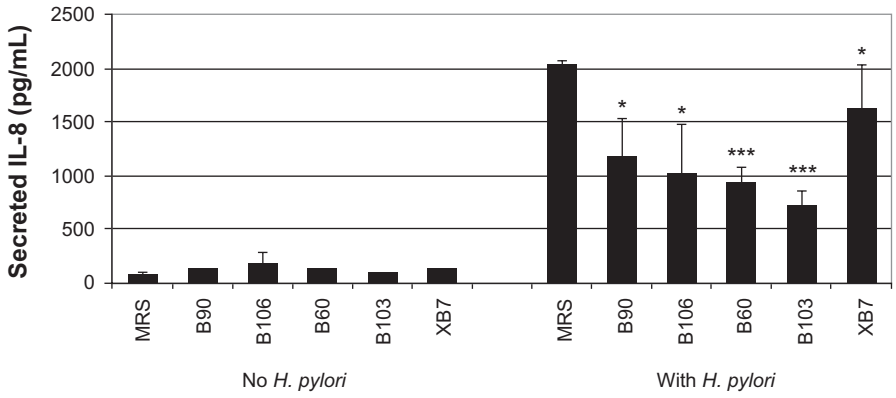

Figure 3 Immunomodulatory effects of *Lactobacillus* B90-, B106-, B60-, B103-, and XB7-conditioned media on AGS gastric epithelial cells.
Notes: Bioassays for IL-8 production were performed with or without *Helicobacter pylori* stimulation. The results are expressed as the mean of triplicate determinations representative of three independent experiments. Significantly different from control: ***$P < 0.001$; *$P < 0.05$. Error bars indicate standard deviations.
Abbreviations: IL-8, interleukin-8; MRS, de Man, Rogosa, Sharpe.

Table 2 Interleukin-8-inhibitory *Lactobacillus* spp. are not antagonistic to *Helicobacter pylori* growth

	Disc diffusion assay	Spot-overlay assay
L. salivarius T59[a]	+	+
L. casei or *L. paracasei* B106	–	–
L. plantarum B90	–	–
L. plantarum XB7	–	–
L. rhamnosus B103	–	–
L. salivarius B60	–	–

Notes: [a]Positive control strain known to inhibit *H. pylori* growth; – indicates no growth inhibition; + indicates growth inhibition.

levels of conditioned media of IL-8-inhibitory and IL-8-noninhibitory *Lactobacillus* spp. were approximately equal (data not shown).

Of the five IL-8-suppressive lactobacilli identified in the present study, *L. salivarius* is considered indigenous (autochthonous) to humans, whereas the remaining four species are considered transient (allochthonous).[30] We have identified indigenous and transient *Lactobacillus* species with anti-inflammatory activity. These isolates are potential candidates for the treatment of *H. pylori*-induced gastric illness and deserve further characterization of their probiotic mechanisms.

Acknowledgments

This work was supported by the Thailand Research Fund through the Royal Golden Jubilee PhD Program (Grant No. PHD/0100/2549) to TT and the Thai Government Research Budget to ST. We thank Jennifer K Spinler for reviewing the manuscript.

Disclosure

The authors declare no conflicts of interests in this work.

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