Original paper

In Vitro and *In Vivo* Anti-*Helicobacter pylori* Activity of Probiotics Isolated from Mongolian Dairy Products

Shiro TAKEDA^{1*}, Masahiko TAKESHITA¹, Tastuya MATSUSAKI¹, Yukiharu KIKUCHI¹, Chuluunbat TSEND-AYUSH², Tsendesuren OYUNSUREN³, Masahiko MIYATA⁴, Ken MAEDA⁴, Shin YASUDA⁴, Yuji AIBA⁵, Yasuhiro KOGA⁵ and Keiji IGOSHI^{4*}

¹Research and Development Division, Minami Nihon Rakuno Kyodo Co. Ltd., 5282 Takagi, Miyakonojo, Miyazaki, 885-0003 Japan

²Food and Biotechnology School, Mongolian University of Science and Technology, Ulaanbaatar, Mongolia

³Mongolian Biotechnology Association, Ulaanbaatar, Mongolia

⁴Department of Bioscience, School of Agriculture, Tokai University, Kawayo, Minamiaso, Aso, Kumamoto, Japan

⁵Laboratory for Infectious Diseases, School of Medicine, Tokai University, 143 Shimokasuya, Isehara, Kanagawa, 259-1193 Japan

Received October 3, 2014 ; Accepted January 26, 2015

Helicobacter (H.) pylori is known to be a bacterial risk factor for gastric cancer. In this study, 16 strains of lactic acid bacteria (LAB) isolated from Mongolian dairy products were screened for potential probiotic activity against *H. pylori* strain no. 130 *in vitro*. *Lactobacillus (L.) paracasei* 06TCa19 and *L. plantarum* 07MR044 were identified as LAB strains with anti-*H. pylori* activities. Compared to strain 07MR044, strain 06TCa19 was more potent in reducing *H. pylori* counts in co-culture. The ability of strain 06TCa19 to inhibit the growth of *H. pylori* was attributable to its rapid and excessive generation of lactic acid. Moreover, oral administration of strain 06TCa19 was found to significantly reduce the number of colonizing *H. pylori* in the stomach of *H. pylori* strain no. 130-infected mice. Thus, strain 06TCa19 is potentially effective against *H. pylori* infection.

Keywords: anti-*Helicobacter pylori*, probiotics, *Lactobacillus paracasei*, lactic acid, mongolian dairy products

Introduction

Helicobacter (H.) pylori, which inhabits more than 50% of the world's population, is a Gram-negative, spiral-shaped, microaerophilic bacterium that colonizes the mucosa of the human gastric epithelium (de Vries *et al.*, 2008). *H. pylori* infection is the major factor inducing chronic gastritis, peptic ulcer, and even gastric cancer in humans (Uemura *et al.*, 2001). Although eradication therapies such as the administration of antibiotics are presently available, issues such as side effects of chemotherapy and increased antibiotic resistance of *H. pylori* have been reported (Cheng *et al.*, 2012; Gerrits *et al.*, 2006).

Therefore, prevention of *H. pylori* infection or eliminating its colonization in the stomach is critical. In addition, development of alternative medicinal products toward preventing the infection and proliferation of *H. pylori*, such as functional foods and natural compounds, is required. The anti-*H. pylori* activities of some natural products and foods have already been reported (Kudo *et al.*, 2011; Lee *et al.*, 2009; Pastene *et al.*, 2010).

Lactic acid bacteria (LAB) have been used worldwide in the preparation of several kinds of food, especially dairy products. Some LAB, termed probiotics, are living microorganisms that

*To whom correspondence should be addressed.

E-mail: Shiro Takeda ; s-takeda@dairy-milk.co.jp
Kenji Igoshi ; igoshi@agri.u-tokai.ac.jp

confer health benefits on the host when administered in adequate amounts (i). Several probiotic LAB have been shown to inhibit *H. pylori* growth *in vitro* (Hamilton-Miller, 2003). Some probiotics are reportedly effective in both suppressing *H. pylori* colonization and reducing gastric mucosal inflammation in humans (Boonyaritichai *et al.*, 2009; Fujimura *et al.*, 2006; Patel *et al.*, 2014; Tamura *et al.*, 2006). Thus, the identification of LAB strains possessing anti-*H. pylori* activity appears promising in developing effective foods, such as yogurt, capable of preventing *H. pylori* infection and proliferation.

We previously isolated LAB strains from traditional Mongolian dairy products (Takeda *et al.*, 2011). Several of these strains exhibited tolerance to artificial gastric acids and adhered to human intestinal epithelial cells *in vitro*, hence these LAB strains were considered probiotic LAB candidates (Takeda *et al.*, 2011). In the present study, we aimed to investigate the anti-*H. pylori* activities of these probiotic LAB candidates. To the best of our knowledge, this is the first work to investigate the anti-*H. pylori* activity of LAB from Mongolian dairy products, and we identified an LAB strain that exhibited high anti-*H. pylori* activity *in vitro* and *in vivo*.

Materials and Methods

Bacterial strains and culture conditions The LAB strains used in this study were isolated from Mongolian dairy products, and their tolerance to gastric acid *in vitro* was confirmed previously (Takeda *et al.*, 2011). The species and origins of the LAB strains are shown in Table 1. The LAB were cultured in de Man, Rogosa, and Sharpe (MRS) broth (Merck, Darmstadt, Germany) at 37°C for 18 h. *H. pylori* strain no. 130 (cag A⁺, vacuolating toxin⁺) used in this study was previously isolated from a gastric biopsy specimen (Kabir *et al.*, 1997). *H. pylori* was cultured in brain heart infusion (BHI) medium (Difco Laboratories, MI, USA) containing 5% inactivated horse serum (HS) under microaerobic conditions, i.e., in an anaerobic jar with Anaero Pack MicroAero (Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan), at 37°C for 48 h.

Estimation of growth inhibition of *H. pylori* co-cultured with LAB *H. pylori* and LAB strains were grown in BHI broth with 5% HS and in MRS broth, respectively. *H. pylori* and LAB cultures were centrifuged at 10,000 × *g* for 5 min and washed twice with sterilized phosphate-buffered saline (PBS). Then, the cells were suspended in the same volume of PBS as that in BHI broth with 5% HS or MRS broth. *H. pylori* and LAB suspensions were inoculated into fresh BHI broth with 5% HS (1% v/v). In screening for *H. pylori* growth inhibition by LAB, the initial cell density of each tested LAB strain in co-culture was 5.9 – 6.4 log₁₀ colony-forming units (CFU)/mL, as determined by colony counts using MRS agar. The initial concentration of *H. pylori* used in co-culture was adjusted to 6.0 log₁₀ CFU/mL in reference to a predetermined plot of CFU versus optical density at 660 nm of the *H. pylori* suspension prepared in PBS. Similarly, the cell densities of LAB were also adjusted in a similar manner, when necessary. The co-

culture of LAB and *H. pylori* was performed at 37°C for 48 h under microaerobic conditions. As a control, PBS was used instead of LAB. After incubation, the co-culture was serially diluted with PBS, and the diluent was surface spread onto modified Skirrow's agar for selective culturing of *H. pylori*. Modified Skirrow's agar was prepared from BHI agar with 5% HS by supplementing the medium with 25 µg/mL tetrazolium violet (Sigma-Aldrich, MO, USA), 5 µg/mL trimethoprim (Wako Pure Chemical, Osaka, Japan), 2.5 U/mL polymyxin B sulfate (Sigma-Aldrich), 10 µg/mL vancomycin hydrochloride (Wako Pure Chemical), and 5 µg/mL bacitracin (Nacalai Tesque, Inc., Kyoto, Japan). Skirrow's agar plates were incubated at 37°C for 4 d under microaerobic conditions. MRS agar plates were incubated at 37°C for 3 d, and subsequently LAB counts were determined.

Preparation of LAB culture fractions and estimation of anti-*H. pylori* activity The anti-*H. pylori* activity of LAB culture fractions was investigated by a modified well diffusion assay, as reported by Simova *et al.* (2009). Briefly, LAB were cultured in BHI broth with 5% HS, the broth was centrifuged at 10,000 × *g* for 5 min, and the LAB cells were collected separately from the supernatant. The harvested LAB cells were washed with PBS. Then, a portion of the cell suspension was heated at 100°C for 15 min. The pH of the separated supernatant was adjusted to 6.5 with 1 mol/L sodium hydroxide. A portion of this neutralized supernatant was treated with 10 mmol/L of phosphate buffer (pH 6.5) containing 1 mg/mL catalase (Sigma-Aldrich). The LAB cells, heat-treated LAB cells, and untreated and treated supernatants were separately lyophilized. Then, each lyophilizate was suspended in 10% of their original volume using sterilized distilled water and stored at –30°C until use. The supernatant was filtered through a 0.22-µm pore-size filter (Millipore, MA, USA) before use. *H. pylori* was inoculated into BHI broth with 5% HS containing 25 µg/mL tetrazolium violet and 0.7% (w/v) agar at a final concentration of 7.0 log₁₀ CFU/mL and then poured into Petri dishes. Wells (10 mm diameter) were made on the solidified agar and then filled with 100 µL of each sample. After incubation at 37°C for 48 h under microaerobic conditions, the diameter of *H. pylori* inhibition zones was measured.

Measurement of pH and organic acids in co-cultures The pH of co-cultures was measured using a pH meter (F-12; Horiba Ltd., Kyoto, Japan). Co-cultures were centrifuged at 10,000 × *g* for 5 min. Then, 0.5 mL of the supernatant was ultrafiltered at 15,000 × *g* for 20 min to obtain the fraction with a MW <10 kDa (Nanosep 10K OMEGA; Pall Corp., NY, USA). The obtained fraction was diluted two-fold with distilled water and then subjected to an analysis of organic acids using the HPLC Agilent SERIES 1100 System (Agilent Technologies Inc., CA, USA). For the analysis of organic acids, the diluted solution was injected into a reversed-phase column (Inertsil ODS-3; GL Sciences Inc., Tokyo, Japan). Elution was performed at 30°C with 50 mmol/L phosphate buffer (pH 2.8) at a flow rate of 0.7 mL/min. Organic acids were detected using absorbance at 210 nm. A solution containing 5.13 mmol/L

lactic acid and 24.84 mmol/L acetic acid was used as the standard solution. Quantification of organic acids was based on the external standard method.

Growth inhibition of *H. pylori* by lactic acid L-lactic acid (Wako Pure Chemical), DL-lactic acid (Wako Pure Chemical), and D-lactic acid (Bachem AG, Bubendorf, Switzerland) were filtered through a filter (0.22 µm pore size) and used for the test. *H. pylori* was cultured in BHI broth with 5% HS at an initial cell density of $6.0 \log_{10}$ CFU/mL at 37°C under microaerobic conditions. To investigate *H. pylori* growth inhibition by lactic acid during the early culture stage, each lactic acid solution was added to the cultures at 12 h incubation and cultures were further incubated at 37°C under microaerobic conditions. The *H. pylori* counts in cultures were estimated by using modified Skirrow's agar, as described above. In addition, to investigate the anti-*H. pylori* activity of L-, DL-, and D-lactic acid, strain no. 130, which was pre-cultured in BHI broth with 5% HS, was inoculated into fresh culture medium containing different levels of lactic acid and incubated at 37°C microaerobically. After incubation, *H. pylori* counts were estimated using modified Skirrow's agar, as described above. The inhibitory lactic acid concentration for a 50% reduction in *H. pylori* (IC₅₀) was also determined from a curve depicting the concentrations of lactic acid and the CFU obtained after incubation with these lactic acid concentrations.

***H. pylori* infection in mice** *H. pylori* infection was induced in mice as described by Aiba *et al.* (1998). Briefly, 4-week-old male, germ-free BALB/c mice were obtained from CLEA Japan, Inc. (Tokyo, Japan). The mice were orally inoculated with *H. pylori* strain no. 130 at a concentration of $9.0 \log_{10}$ CFU for 3 consecutive days. For LAB administration, *H. pylori*-infected mice were orally administered LAB at $9.0 \log_{10}$ CFU once a day for 3 consecutive days at the age of 8 weeks and thereafter administered once at 9, 10, and 11 weeks. These doses were in reference to a previous report (Aiba *et al.*, 1998). The mice were sacrificed at 12 weeks of examination. Their gastric organs were removed and homogenized in PBS on ice. The homogenate was diluted serially with PBS (10^{-1} – 10^{-4}) and cultured in modified Skirrow's agar for *H. pylori* and in MRS agar for LAB. The experimental protocol was approved by the Animal Experiment Committee of Tokai University, Japan (Permission no. 133005). The animal experimentation guidelines of the university were followed in the animal studies.

Statistical analysis The ratio of *H. pylori* growth in screening, the inhibition zones of *H. pylori* by LAB culture fractions, and the bacterial counts *in vivo* were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's test. The bacterial counts and pH of cultures inoculated with LAB or lactic acid were analyzed by two-way ANOVA, followed by Bonferroni's test. The organic acid levels in the co-cultures of *H. pylori* and LAB were also analyzed by two-way ANOVA, followed by Bonferroni's test. $p < 0.05$ was considered as statistically significant.

Results and Discussion

Effects of LAB on *H. pylori* growth in vitro In screening the tested LAB for anti-*H. pylori* activity, LAB were co-cultured with *H. pylori* for 2 days, and *H. pylori* counts were subsequently determined. The growth ratio of *H. pylori* in the co-cultures is shown in Table 1. All of the tested LAB strains demonstrated a decrease in the growth ratio of *H. pylori*. In particular, significant differences were observed in the growth ratio of *H. pylori* co-cultured with *Lactobacillus* (*L.*) *plantarum* strains 05AM23 (37.3%) and 07MR044 (35.4%) compared to PBS (control) ($p < 0.05$, one-way ANOVA, followed by Tukey's test). For *L. paracasei*, strain 06TCa19 showed the lowest *H. pylori* growth ratio (46.2%), but a significant difference was not observed.

It was demonstrated that the inhibitory effects of LAB on *H. pylori* growth showed LAB species inter- and intra-variability (Hütt *et al.*, 2006; Rokka *et al.*, 2006). In this study, *L. paracasei* strain

Table 1. Tested LAB and their effects on the growth of *H. pylori*.

Species	Strain	Growth ratio of <i>H. pylori</i> (%) ^a
PBS		100.0 ± 8.1
<i>Lactobacillus paracasei paracasei</i>	06TCa19 ^b	46.2 ± 11.2
	06TCa22 ^b	56.8 ± 19.0
	06TCa43 ^b	49.3 ± 11.8
<i>Lactobacillus paracasei tolerans</i>	06TCa39 ^b	53.5 ± 15.0
	07LC080 ^c	57.1 ± 22.7
	08LY034 ^d	59.5 ± 18.0
	08LY065 ^c	86.1 ± 6.4
<i>Lactobacillus plantarum</i>	08LY075 ^c	66.2 ± 5.8
	05AM23 ^c	37.3 ± 2.9*
	06CC2 ^d	48.6 ± 4.4
	06CC9 ^d	41.4 ± 3.6
	06TCa8 ^b	41.6 ± 4.8
	06TCa40 ^b	42.6 ± 10.0
	07MR044 ^b	35.4 ± 7.3*
<i>Lactobacillus delbrueckii lactis</i>	08MR029 ^c	61.7 ± 2.7
	06TC3 ^b	61.3 ± 11.9

Data represent mean ± standard deviation (SD).

The experiment was independently carried out three times. The asterisks indicate significant differences from the control (PBS) by one-way ANOVA, followed by Tukey's test ($p < 0.05$). ^aGrowth inhibition ratios were expressed as a percentage of the control. *H. pylori* strain no. 130 counts in co-culture with each LAB were determined on modified Skirrow's agar. ^bLAB strains were isolated from *tarag*, a traditional Mongolian yogurt. ^cLAB strains were isolated from *airag*, an alcoholic, fermented horse milk. ^dLAB strains were isolated from *aaruul*, a traditional Mongolian cheese. ^eLAB strain was isolated from *urum*, a clotted cream made from milk. The tested LAB strains were isolated from ninety-five tarags, thirty-one airags, nine aaruuls and two urums.

Fig. 1. Effects of LAB in co-culture with *H. pylori*.

(A) The count of *H. pylori* in co-cultures with the strain 06TCa19 (●), 07MR044 (▲), and PBS (□). (B) The count of LAB in the co-cultures of *H. pylori* strain no. 130 with the strain 06TCa19 (●) and 07MR044 (▲). (C) pH in the co-cultures of *H. pylori* strain no. 130 with the 06TCa19 strain (●), 07MR044 strain (▲), and PBS (□). The experiments were independently carried out three times. Vertical bars represent SD. Values with different small letters indicate a significant difference at each incubation time by two-way ANOVA, followed by the Bonferroni's test ($p < 0.05$).

06TCa19 and *L. plantarum* strain 07MR044 exhibited potent abilities to inhibit the growth of *H. pylori* (Table 1). However, other strains, including *L. paracasei* strain 08LY065 and *L. plantarum* strain 08MR029, did not inhibit the growth of *H. pylori* compared to strains 06TCa19 or 07MR044. *L. delbrueckii lactis* strain 06TC3 also did not show *H. pylori* growth inhibition. Thus, the inhibitory effects of LAB on *H. pylori* growth were also thought to vary between different species as well as within the

same species in this study.

Potent growth inhibition of *H. pylori* by strains 06TCa19 and 07MR044 was noted among *L. paracasei* and *L. plantarum* species, respectively, in the screening process; therefore, these strains were used in subsequent experiments. To compare the *H. pylori* growth-inhibition abilities of strains 06TCa19 and 07MR044, the initial counts of LAB and *H. pylori* in the co-culture were adjusted to 6.0 log₁₀ CFU/mL, and bacterial counts and medium pH were estimated during the time course of co-culturing. As shown in Fig. 1A, the counts of *H. pylori* co-cultured with strain 06TCa19 were significantly lower than the control and with strain 07MR044 at 24 h and 48 h incubation ($p < 0.05$, two-way ANOVA, followed by Bonferroni's test). The LAB counts at 6 h, 12 h and 24 h incubation were significantly higher, and pH at 12 h was significantly lower, in co-culture with strain 06TCa19 than with strain 07MR044 ($p < 0.05$, two-way ANOVA, followed by Bonferroni's test) (Figs. 1B and 1C). There was a correlation between the reduction in *H. pylori* counts and the reduction in pH, which is related to the increase in LAB counts in the co-culture with strain 06TCa19. Therefore, the inhibition of *H. pylori* growth may be attributed to the release of products by strain 06TCa19 growth or the increase in cell counts of strain 06TCa19.

Identification of the active anti-*H. pylori* component(s) in LAB culture To identify the substance(s) that contributes to the anti-*H. pylori* activity of strains 06TCa19 and 07MR044, six fractions from the respective BHI broth with 5% HS cultures were prepared and subjected to the well diffusion assay. The culture solutions of strains 06TCa19 and 07MR044 and their supernatants exhibited large inhibitory zones against *H. pylori* (Table 2). However, the fractions of pH-neutralized supernatant and pH-neutralized supernatant treated with catalase showed significantly smaller inhibitory zones than those of the culture solutions and the untreated supernatants ($p < 0.05$, one-way ANOVA, followed by Tukey's test). The inhibitory zones from the fractions of 06TCa19 and 07MR044 cells were also significantly smaller than those of the fractions of the culture solutions or the untreated supernatants ($p < 0.05$, one-way ANOVA, followed by Tukey's test). In addition, heat-killed 06TCa19 and 07MR044 cells did not produce inhibitory zones, respectively.

Some *H. pylori* growth-inhibiting activities by LAB are reportedly attributable to the production of organic acids (Aiba *et al.*, 1998; Lin *et al.*, 2009; Midolo *et al.*, 1995). On the other hand, *H. pylori* growth inhibition by LAB has been reported to depend on the cell wall fraction and bacteriocin (Rokka *et al.*, 2006; Simova *et al.*, 2009). In this study, because the supernatant fractions of strains 06TCa19 and 07MR044 exhibited high inhibitory activity against *H. pylori*, the main inhibitory substances were thought to be contained in the supernatants (Table 2). In addition, neutralization of pH and catalase treatment were conducted to exclude the effects of organic acids and to eliminate the effects of hydrogen peroxide on anti-*H. pylori* activity, respectively; and

Table 2. Effect of LAB culture fractions on *H. pylori* growth.

Fraction of LAB culture	Diameter of the inhibition zone (mm)	
	06TCa19	07MR044
PBS	ND	ND
Culture solution (containing cells)	17.1 ± 2.3 ^a	18.0 ± 0.2 ^a
Supernatant	17.7 ± 1.8 ^a	18.7 ± 2.8 ^a
Supernatant pH neutralized to 6.5	3.9 ± 0.9 ^b	2.7 ± 0.7 ^b
Supernatant pH neutralized to 6.5 and treated with catalase	2.9 ± 1.1 ^b	1.6 ± 0.6 ^b
LAB cells	2.8 ± 0.8 ^b	2.2 ± 0.1 ^b
Heat-killed LAB cells	ND	ND

Samples were prepared from three independent experiments and subjected to a well diffusion assay. Inhibition zones were expressed by subtracting the diameter of the well from that of each sample. Data represent the mean ± SD. ND, not detected. Values with different superscripts indicate a significant difference in each LAB group by one-way ANOVA, followed by Tukey's test ($p < 0.05$).

Table 3. Levels of organic acids in the co-cultures of LAB and *H. pylori*.

Strain	Lactic acid (mmol/L)				Acetic acid (mmol/L)			
	6 h	12 h	24 h	48 h	6 h	12 h	24 h	48 h
PBS	ND	ND	ND	ND	72.9 ± 2.3	68.2 ± 3.3	69.4 ± 2.9	69.8 ± 2.8
06TCa19	9.0 ± 1.0	25.0 ± 0.5*	24.5 ± 0.6	23.3 ± 0.3	71.2 ± 2.6	73.4 ± 3.4	74.8 ± 4.9	79.3 ± 2.6
07MR044	8.7 ± 1.1	15.1 ± 2.2	22.6 ± 2.5	22.2 ± 1.5	69.6 ± 2.2	66.8 ± 2.5	71.0 ± 2.1	72.8 ± 5.3

Samples were collected from three independent experiments for analysis by HPLC. Data represent the mean ± SD. ND, not detected.

Levels of lactic and acetic acids in the initial incubation were ND and 63.2 ± 2.0 mmol/L, respectively. Lower limits of detection were 2.5 mmol/L for lactic acid and 12.4 mmol/L for acetic acid. The asterisk indicates significantly different from the lactic acid level of strain 07MR044 at 12 h by two-way ANOVA, followed by Bonferroni's test ($p < 0.05$).

these fractions of strains 06TCa19 and 07MR044 showed remarkably small inhibitory zones against *H. pylori*. Therefore, the inhibitory activities of strains 06TCa19 and 07MR044 against *H. pylori* were thought to be caused by the production of organic acids. Also, the level of organic acids, including lactic acid, produced by the LAB strains that did not inhibit *H. pylori* growth (Table 1) may be lower than that produced by strains 06TCa19 and 07MR044.

Analysis of organic acids in co-cultured *H. pylori* and LAB
The organic acid levels in the supernatants of co-cultured *H. pylori* and LAB were analyzed. The lactic acid concentrations in the co-cultures of *H. pylori* and LAB increased in a time-dependent manner, but no lactic acid was detected in the supernatants of PBS (control) (Table 3). The concentration of lactic acid in the co-culture with strain 06TCa19 reached 25.0 mmol/L at 12 h incubation, and the lactic acid concentration was significantly higher than that in the co-culture with strain 07MR044 at 12 h ($p < 0.05$, two-way ANOVA, followed by Bonferroni's test). On the other hand, no significant differences were observed in the concentrations of acetic acid of the control and co-cultures inoculated with the LAB strains.

As shown in Table 3, the increase in lactic acid concentrations in the co-culture of *H. pylori* and LAB was due to the growth of

LAB, as no lactic acid was detected in the control. In addition, the lactic acid concentration of the co-culture inoculated with strain 06TCa19 was significantly higher than that with strain 07MR044 at 12 h after LAB inoculation ($p < 0.05$). Thus, strain 06TCa19 produced a larger amount of lactic acid compared to strain 07MR044 during early-stage co-culture with *H. pylori*. This ability of 06TCa19 may contribute to the anti-*H. pylori* activity. On the other hand, Lin *et al.* (2009) stated that the anti-*H. pylori* activity was due to the acetic acid as well as the lactic acid produced in the culture medium. Acetic acid was detected in the *H. pylori* culture inoculated with PBS, and no significant differences were observed in the concentrations of acetic acid in this study (Table 3). Therefore, it is suggested that acetic acid was not produced by strains 06TCa19 and 07MR044 and did not contribute to the inhibition of *H. pylori* in this study.

Effects of lactic acid produced by LAB during early-stage co-culture on *H. pylori* growth
To investigate the effect of lactic acid produced by strains 06TCa19 and 07MR044 during early-stage co-culture on *H. pylori* growth, 25 mmol/L L-lactic acid and 15 mmol/L DL-lactic acid were added to the *H. pylori* culture at 12 h incubation, and *H. pylori* was enumerated. The concentrations and schedules for L- and DL-lactic acid addition were based on the results given in Table 3, which showed the inoculation of strains

Fig. 2. Effect of lactic acid on *H. pylori* counts.

H. pylori strain no. 130 counts in cultures inoculated with 25 mmol/L L-lactic acid (●), 15 mmol/L DL-lactic acid (▲), and PBS (□) at 12 h incubation are shown. The experiment was independently carried out three times. Vertical bars represent SD. Values with different small letters indicate a significant difference at each incubation time by two-way ANOVA, followed by Bonferroni's test ($p < 0.05$).

06TCa19 and 07MR044 to *H. pylori* cultures, respectively. It was also confirmed that strains 06TCa19 and 07MR044 produced L-lactic acid and DL-lactic acid, respectively (data not shown). As shown in Fig. 2, the addition of 25 mmol/L L-lactic acid at 12 h incubation significantly reduced *H. pylori* counts in the medium to lower than that observed with PBS or 15 mmol/L DL-lactic acid addition at 24 h and 48 h incubation ($p < 0.05$, two-way ANOVA, followed by Bonferroni's test). On the other hand, the addition of 15 mmol/L DL-lactic acid did not produce a time-dependent reduction in *H. pylori* counts.

The anti-*H. pylori* activity mediated by lactic acid has been described in previous reports (Midolo *et al.*, 1995; Zheng *et al.*, 2014). The effect of lactic acid on *H. pylori* strain was also determined in this study. As shown in Fig. 2, the addition of 25 mmol/L L-lactic acid at 12 h incubation significantly reduced *H. pylori* counts as compared to the addition of 15 mmol/L DL-lactic acid at the same time-point or the control. This result was consistent with the notable reduction in *H. pylori* counts upon inoculation with strain 06TCa19 *in vitro* (Fig. 1A). Also, *H. pylori* growth inhibition mediated by the activities of D- and L-lactic acids was found to be similar (IC₅₀ of approximately 23.0 mmol/L for both). Therefore, the large amount of L-lactic acid produced by strain 06TCa19 during early-stage co-culture was inferred to contribute to the growth inhibition of *H. pylori*. In addition, the large amount of organic acids, including lactic acid, produced by LAB in culture can reportedly inhibit *H. pylori* adhesion and invasion of gastric cell lines *in vitro* (Lin *et al.*, 2009). The large amount of L-lactic acid produced by strain 06TCa19 is expected to inhibit the adhesion of *H. pylori* to human gastric cells.

Effects of oral administration of LAB on bacterial counts in the stomach of H. pylori-infected mice To investigate the effects of

Fig. 3. Effects of oral administration of LAB on bacterial counts in the stomach of *H. pylori*-infected mice.

(A) *H. pylori* counts in the stomach of *H. pylori* strain no. 130-infected mice ($n = 7$). Striped, hollow, and solid columns show the numbers of *H. pylori* in the stomach of *H. pylori* strain no. 130-infected mice administered PBS, strain 06TCa19, and strain 07MR044, respectively. (B) LAB counts in the stomach of infected mice ($n = 7$). Hollow and solid columns show the numbers of LAB in the stomach of infected mice administered strain 06TCa19 and strain 07MR044, respectively. Vertical bars represent standard error (SE). Values with different small letters indicate a significant difference by one-way ANOVA, followed by Tukey's test ($p < 0.05$). ND indicates the lower limit of detection ($< 2.0 \log_{10}$ CFU/g).

strains 06TCa19 and 07MR044 on *H. pylori* colonization in the stomach of mice, infected mice were orally administered the respective strains. *H. pylori* counts in the stomach of infected mice administered strain 06TCa19 were significantly lower than in those administered PBS (control) or strain 07MR044 ($p < 0.05$, one-way ANOVA, followed by Tukey's test) (Fig. 3A). Further, no reduction was noted in *H. pylori* counts in the stomach of infected mice administered strain 07MR044. On the other hand, LAB counts in the stomach of infected mice administered strain 06TCa19 were higher than in those administered strain 07MR044, although not significantly so (Fig. 3B).

Administration of several LAB strains has been demonstrated to reduce the number of *H. pylori* in the stomach of infected mice (Aiba *et al.*, 1998; Kabir *et al.*, 1997; Kimura *et al.*, 2003; Ushiyama *et al.*, 2003). In this study, oral administration of strain

06TCa19 to infected mice produced a significant reduction in *H. pylori* levels, and the oral administration of strain 06TCa19 showed a beneficial effect on reducing or eliminating *H. pylori* in the stomach *in vivo* (Fig. 3A). The beneficial effect of *L. salivarius* WB1004 administration on the eradication of *H. pylori* in the stomach of infected mice was due to its ability to adhere to gastric epithelial cells and produce high levels of lactic acid, in parallel with its rapid growth in the stomach (Aiba *et al.*, 1998). In addition, *L. gasseri* OLL2716 showed strong anti-acid properties and tolerance to gastric juices *in vitro*, leading to the suppression of *H. pylori* infection (Fujimura *et al.*, 2012; Kimura 2004). In our previous study, the tolerance of strain 06TCa19 to gastric acid was confirmed *in vitro* (Takeda *et al.*, 2011), and the strain potentially adhered to the stomach of *H. pylori*-infected mice in this study (Fig. 3B). Furthermore, strain 06TCa19 was observed to produce L-lactic acid at a high rate and in large amounts *in vitro* (Table 3). Therefore, the administration of strain 06TCa19 is suggested to reduce the number of colonized *H. pylori* in the stomach of infected mice. Moreover, it is suggested that the L-lactic acid produced in the stomach by this strain can contribute to reductions in *H. pylori* levels, as shown by *L. salivarius* WB1004 and *L. gasseri* OLL2716. Further *in vivo* studies are required to clarify the mechanism of anti-*H. pylori* activity by strain 06TCa19. Notably, strain 06TCa19 can grow favorably in milk, and milk fermented using this strain has a good flavor and has been reported to improve human bowel habits (Takeda *et al.*, 2013). Thus, strain 06TCa19 may be an effective LAB starter for the production of fermented milk with anti-*H. pylori* activity, and potentially aiding in intestinal regulation. Future investigations on whether oral administration of dairy products such as yogurt prepared with strain 06TCa19 can exert anti-*H. pylori* activity in humans are warranted.

In conclusion, *L. paracasei* strain 06TCa19 isolated from Mongolian dairy products was suggested to possess anti-*H. pylori* activity, in addition to potentially aiding human intestinal regulation. Strain 06TCa19 inhibited the growth of *H. pylori* strain no. 130 and produced significant amounts of L-lactic acid, likely contributing to the anti-*H. pylori* activity *in vitro*. Furthermore, strain 06TCa19 tended to adhere to gastric epithelial cells in *H. pylori*-infected mice in this study, leading to a reduction in *H. pylori* colonization.

Acknowledgements We thank Mr. Yuuta Watanabe (School of Agriculture, Tokai University) for his technical assistance in this study.

References

- Aiba, Y., Suzuki, N., Kabir, A.M., Takagi, A., and Koga, Y. (1998). Lactic acid-mediated suppression of *Helicobacter pylori* by the oral administration of *Lactobacillus salivarius* as a probiotic in a gnotobiotic murine model. *Am. J. Gastroenterol.*, **93**, 2097-2101.
- Boonyaritichai, S., Kuwabara, K., Nagano, J., Kobayashi, K., and Koga, Y. (2009). Long-term administration of probiotics to asymptomatic pre-schoolchildren for either the eradication or the prevention of *Helicobacter pylori* infection. *Helicobacter*, **14**, 202-207.
- Cheng, A., Sheng, W.H., Liou, J.M., Wang, H.P., Wu, M.S., Lin, J.T., and Chang, S.C. (2012). Comparative *in vitro* antimicrobial susceptibility and synergistic activity of antimicrobial combinations against *Helicobacter pylori* isolates in Taiwan. *J. Microbiol. Immunol. Infect.*, In press.
- de Vries, A.C., van Driel, H.F., Richardus, J.H., Ouwendijk, M., van Vuuren, A.J., de Man, R.A., and Kuipers, E.J. (2008). Migrant communities constitute a possible target population for primary prevention of *Helicobacter pylori*-related complications in low incidence countries. *Scand. J. Gastroenterol.*, **43**, 403-409.
- Fujimura, S., Kato, S., Oda, M., Miyahara, M., Ito, Y., Kimura, K., Kawamura, T., Ohnuma, M., Tateno, H., and Watanabe, A. (2006). Detection of *Lactobacillus gasseri* OLL2716 strain administered with yogurt drink in gastric mucus layer in humans. *Lett. Appl. Microbiol.*, **43**, 578-581.
- Fujimura, S., Watanabe, A., Kimura, K., and Kaji, M. (2012). Probiotic mechanism of *Lactobacillus gasseri* OLL2716 strain against *Helicobacter pylori*. *J. Clin. Microbiol.*, **50**, 1134-1136.
- Gerrits, M.M., van Vliet, A.H., Kuipers, E.J., and Kusters, J.G. (2006). *Helicobacter pylori* and antimicrobial resistance: molecular mechanisms and clinical implications. *Lancet Infect. Dis.*, **6**, 699-709.
- Hamilton-Miller, J.M. (2003). The role of probiotics in the treatment and prevention of *Helicobacter pylori* infection. *Int. J. Antimicrob. Agents*, **22**, 360-366.
- Hütt, P., Shchepetova, J., Löivukene, K., Kullisaar, T., and Mikelsaar, M. (2006). Antagonistic activity of probiotic lactobacilli and bifidobacteria against entero- and uropathogens. *J. Appl. Microbiol.*, **100**, 1324-1332.
- Kabir, A.M., Aiba, Y., Takagi, A., Kamiya, S., Miwa, T., and Koga, Y. (1997). Prevention of *Helicobacter pylori* infection by lactobacilli in a gnotobiotic murine model. *Gut*, **41**, 49-55.
- Kimura, K. (2004). Health benefits of probiotics: probiotics for *Helicobacter pylori* infection. *Food Sci. Technol. Res.*, **10**, 1-5.
- Kimura, K., Sakamoto, I., Igarashi, M., Takagi, A., Miwa, T., Aiba, Y., and Koga, Y. (2003). Development of probiotics for *Helicobacter pylori* infection. *Biosci. Microflora*, **22**, 1-4.
- Kudo, H., Takeuchi, H., Shimamura, T., Kadota, Y., Sugiura, T., and Ukeda, H. (2011). *In vitro* anti-*Helicobacter pylori* activity of Chinese chive (*Allium tuberosum*). *Food Sci. Technol. Res.*, **17**, 505-513.
- Lee, J.H., Shim, J.S., Chung, M.S., Lim, S.T., and Kim, K.H. (2009). *In vitro* anti-adhesive activity of green tea extract against pathogen adhesion. *Phytother. Res.*, **23**, 460-466.
- Lin, W.H., Lin, C.K., Sheu, S.J., Hwang, C.F., Ye, W.T., Hwang, W.Z., and Tsen, H.Y. (2009). Antagonistic activity of spent culture supernatants of lactic acid bacteria against *Helicobacter pylori* growth and infection in human gastric epithelial AGS cells. *J. Food Sci.*, **74**, M225-M230.
- Midolo, P.D., Lambert, J.R., Hull, R., Luo, F., and Grayson, M.L. (1995). *In vitro* inhibition of *Helicobacter pylori* NCTC 11637 by organic acids

- and lactic acid bacteria. *J. Appl. Bacteriol.*, **79**, 475-479.
- Pastene, E., Speisky, H., Garcia, A., Moreno, J., Troncoso, M., and Figueroa, G. (2010). *In vitro* and *in vivo* effects of apple peel polyphenols against *Helicobacter pylori*. *J. Agric. Food Chem.*, **58**, 7172-7179.
- Patel, A., Shah, N., and Prajapati, J.B. (2014). Clinical appliance of probiotics in the treatment of *Helicobacter pylori* infection-A brief review. *J. Microbiol. Immunol. Infect.*, **47**, 429-437.
- Rokka, S., Pihlanto, A., Korhonen, H., and Joutsjoki, V. (2006). *In vitro* growth inhibition of *Helicobacter pylori* by lactobacilli belonging to the *Lactobacillus plantarum* group. *Lett. Appl. Microbiol.*, **43**, 508-513.
- Simova, E.D., Beshkova, D.B., and Dimitrov, Zh.P. (2009). Characterization and antimicrobial spectrum of bacteriocins produced by lactic acid bacteria isolated from traditional Bulgarian dairy products. *J. Appl. Microbiol.*, **106**, 692-701.
- Takeda, S., Fujimoto, R., Takenoyama, S., Takeshita, M., Kikuchi, Y., Tsend-Ayush, C., Dashnyam, B., Muguruma, M., and Kawahara, S. (2013). Application of probiotics from Mongolian dairy products to fermented dairy products and its effects on human defecation. *Food Sci. Technol. Res.*, **19**, 245-253.
- Takeda, S., Yamasaki, K., Takeshita, M., Kikuchi, Y., Tsend-Ayush, C., Dashnyam, B., Ahmmed, A.M., Kawahara, S., and Muguruma, M. (2011). The investigation of probiotic potential of lactic acid bacteria isolated from traditional Mongolian dairy products. *Anim. Sci. J.*, **82**, 571-579.
- Tamura, A., Kumai, H., Nakamichi, N., Sugiyama, T., Deguchi, R., Takagi, A., and Koga, Y. (2006). Suppression of *Helicobacter pylori*-induced interleukin-8 production *in vitro* and within the gastric mucosa by a live *Lactobacillus* strain. *J. Gastroenterol. Hepatol.*, **21**, 1399-406.
- Uemura, N., Okamoto, S., Yamamoto, S., Matsumura, N., Yamaguchi, S., Yamakido, M., Taniyama, K., Sasaki, N., and Schlemper, R.J. (2001). *Helicobacter pylori* infection and the development of gastric cancer. *N. Engl. J. Med.*, **345**, 784-789.
- Ushiyama, A., Tanaka, K., Aiba, Y., Shiba, T., Takagi, A., Mine, T., and Koga, Y. (2003). *Lactobacillus gasseri* OLL2716 as a probiotic in clarithromycin-resistant *Helicobacter pylori* infection. *J. Gastroenterol. Hepatol.*, **18**, 986-991.
- Zheng P.X., Fang H.Y., Yang H.B., Tien N.Y., Wang M.C., and Wu J.J. (2014). *Lactobacillus pentosus* strain LPS16 produces lactic acid, inhibiting multidrug-resistant *Helicobacter pylori*. *J. Microbiol. Immunol. Infect.*, In press.

URL Cited

- i) http://www.who.int/foodsafety/publications/fs_management/probiotics/en/index.html (July 13, 2009).