Research Article

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Anti-*Helicobacter pylori* activity of fermented milk with lactic acid bacteria

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Abstract

BACKGROUND: Ten strains of lactic acid bacteria (LAB) were investigated for their anti-*Helicobacter pylori* effects. The bactericidal activity and organic acid content in spent culture supernatants (SCS) from fermented milk were measured. In addition, the exclusion effect of SCS against *H. pylori* infection of human gastric epithelial AGS cells was assayed.

RESULTS: Three LAB strains, LY1, LY5 and IF22, showed better anti-*Helicobacter* effects than the other strains. There were no significant differences in the bactericidal activity of LAB strains between original SCS, artificial SCS and SCS treated by heating or protease digestion. However, neutralised SCS lost this activity. These results suggest that the anti-*H. pylori* activity of SCS may be related to the concentration of organic acids and the pH value but not to protein components. In the AGS cell culture test, both fermented LY5-SCS and artificial LY5-SCS significantly reduced *H. pylori* infection and urease activity ($P < 0.05$).

CONCLUSION: In this study, *in vitro* methods were used to screen potential probiotics with anti-*H. pylori* activity. This may provide an excellent and rapid system for studying probiotics in the functional food and dairy industries.

Keywords: lactic acid bacteria; *Helicobacter pylori*; bactericidal activity; SCS; AGS

INTRODUCTION

Helicobacter pylori, a Gram-negative, spiral-shaped microaerophilic pathogen, has the ability to colonise mucous layers of the human gastric epithelium. Humans have been infected by *H. pylori* for at least 50 000 years and probably throughout their evolution.[1] Several reports have suggested the possibility of waterborne transmission, as the organism can survive for several days in fresh cold water, salt water, distilled water or tap water.[2] Long-term *H. pylori* infection is thought to be a major causative factor in peptic ulcer disease, gastric adenocarcinoma and chronic gastritis in humans.[3–5] *Helicobacter pylori* infection is acquired in childhood and persists throughout life, often without inducing symptoms. The *H. pylori* infection rate has remained high in developing countries and is still about 40% in developed countries.[6] However, the treatment of *H. pylori* infection faces many obstacles, not only the growing number of antibiotic-resistant species but also the accompanying unwanted side effects of clinical therapy.[7–9]

Most probiotics contain lactic acid bacteria (LAB) such as *Lactobacillus, Bifidobacterium* and *Enterococcus* species, and it has been shown that probiotics are beneficial in humans and animals.[10,11] Potential probiotics must have certain properties, including adhesion, competitive exclusion capacity and immunomodulation, to prevent infection of the gastrointestinal epithelium by pathogens.[12–14] Recent studies have found that certain LAB strains, i.e. *Lactobacillus rhamnosus* GG (LGG), *Lactobacillus salivarius* and *Lactobacillus gasseri*, inhibit the growth of *H. pylori* both *in vitro* and *in vivo*.[15–19] It has been reported that lactic acid and other potentially inhibitory metabolites in fermented milk and culture supernatant fractions have a direct effect on pathogens and parasites.[18,20–22] In addition, several clinical studies showed that probiotic supplementation reduced therapy-related side effects or increased *H. pylori* eradication.[23–27] *Lactobacillus acidophilus* strain LA1 secretes an antibacterial compound that can be used as an adjuvant to antibiotic treatment to prevent the re-emergence of *H. pylori* infection in the human gastrointestinal tract.[28] It is believed that the mechanism for the observed prevention by LAB of infection in mice and humans involves competition for binding sites.[29,30] Furthermore, Aiba *et al*.[29] found

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that the amount of lactic acid increased and the number of *H. pylori* decreased in parallel with an increase in the number of colonising *L. salivarius*. This is likely due to the latter's high affinity for binding to gastric epithelial cells, thereby enabling the production of a sufficient amount of lactic acid to interfere with *H. pylori* infection. Therefore the antagonistic effect of spent culture supernatants (SCS) is considered to be due to the production of different factors such as organic acids or bacteriocins or due to a protein-mediated mechanism.[29,31–34] In another study, Rokka *et al*.[35] found that strains of *Lactobacillus plantarum* showed anti-*Helicobacter* activity *in vitro*, which seemed to be associated with the cell wall rather than with the SCS or intracellular fraction.

Reports on the bactericidal activity of SCS from fermented milk with LAB (LAB-SCS) against *H. pylori* are rare. This study provides evidence that anti-*Helicobacter* substances are partially present in LAB-SCS. Ten strains of LAB isolated from different sources, including yogurt, commercial probiotic powder, pickled vegetables and faecal specimens of healthy infants or adults, were evaluated for their anti-*Helicobacter* activity. The organic acids in fermented milk produced by these LAB strains were analysed and compared. In addition, the exclusion effect of LAB-SCS against *H. pylori* infection of human gastric epithelial AGS cells was investigated.

EXPERIMENTAL

Bacterial strains and culture conditions

We used the API 50 CHL *Lactobacillus* identification system (Biomerieux, Marcy L'Etoile, France) to identify LAB strains, confirmed by 16S rRNA gene sequences in the GenBank Database. The identification and source of each strain are listed in Table 1. For the studies, bacteria were cultured in de Man–Rogosa–Sharpe (MRS) medium (Difco, Detroit, MI, USA) at 37 °C. All strains were serially transferred at least three times prior to use. For the preparation of milk medium, 10 g of powdered non-fat milk was mixed with 90 mL of distilled water and heated to 100 °C for 30 min. A 1 mL starter of each LAB strain was transferred into 100 mL of reconstituted milk medium and incubated at 37 °C for 48 h. *Helicobacter pylori* strain BCRC 17 021 was obtained from the Bioresources Collection and Research Center (BCRC; Hsin-Chu, Taiwan), while strain CMU83 was an isolate from a patient suffering from gastric ulcer disease at the Taichung Veterans General Hospital in Taiwan. Both *H. pylori* strains were grown in *Brucella* broth (Difco) supplemented with 50 mL L^{-1} heat-inactivated foetal bovine serum (FBS; Hyclone, BRL, New York, NY, USA) in CO_2 incubators (Thermo 3130, Forma Scientific, Marietta, OH, USA) at 37 °C for 48 h under microaerophilic conditions (5% (v/v) O_2, 10% (v/v) CO_2, 85% (v/v) N_2).

Determination of cell number, pH and titratable acidity

The test LAB strains were cultured in milk medium for 12, 24 and 48 h. Viable cell counts were determined by the standard plate-counting method. The cell numbers were counted after incubation at 37 °C for 48 h. The pH values of the 12, 24 and 48 h supernatants of fermented milk were measured with a pH meter before plating for enumeration. For the titratable acidity assay, 10 g samples were blended with 90 mL of distilled water, then the mixed suspensions were titrated with 0.1 mol L^{-1} NaOH to a final pH of 8.3 and the titratable volume was recorded. The titratable acidity (TA) of each LAB-SCS was expressed as[36]

$$\text{TA (lactic acid \%)} = \frac{\text{titratable volume (mL) of 0.1 mol L}^{-1}\ \text{NaOH} \times 0.009 \times \text{titre of 0.1 mol L}^{-1}\ \text{NaOH}}{\text{weight of sample (g)}}$$

Table 1. LAB strains used in study

Strain	Species identified	Sample
LGG	*Lactobacillus rhamnosus*	Yogurt
LY1	*Lactobacillus bulgaricus*	Yogurt
LY5	*Lactobacillus acidophilus*	Yogurt
IF3	*Lactobacillus acidophilus*	Infant faeces
IF9	*Lactobacillus plantarum*	Infant faeces
IF22	*Lactobacillus paracasei*	Infant faeces
AF3	*Enterococcus faecalis*	Infant faeces
GK31	*Enterococcus faecium*	Granule type of probiotic food
V2	*Lactobacillus plantarum*	Traditional pickled vegetables
V15	*Pediococcus pentosaceus*	Traditional pickled vegetables

Agar well diffusion assay to determine susceptibility of *H. pylori*

Samples of SCS in fermented milk were obtained by centrifugation (4000 × *g*, 10 min, 4 °C) of the 12, 24 and 48 h LAB cultures, followed by filtration of the supernatants through sterile filters of 0.45 μm pore size (Millipore, Bedford, MA, USA). The filtered supernatants were stored at −80 °C until assayed. The modified agar diffusion method described by Sgouras *et al*.[37] was used. *Helicobacter pylori* strains BCRC 17 021 and CMU83 were cultured in *Brucella* broth containing 50 mL L^{-1} FBS for 48 h and then diluted to 10^8 colony-forming units (CFU) mL^{-1} with sterile phosphate-buffered saline (PBS; pH 7.2). Aliquots of 100 μL of the bacterial dilution were spread on the *Brucella* agar plates, and wells (7 mm in diameter) were made in the agar with a sterile glass rod. Aliquots of 70 μL of the LAB-SCS obtained from the 12, 24 and 48 h cultures were dropped into the wells. The plates were incubated at 37 °C for 72 h under microaerophilic conditions. The diameters of the inhibition zones around the wells were then measured with callipers. Results are expressed as the mean diameter of triplicate independent experiments for each sample.

Organic acid analysis

The organic acid analysis method modified by Zeppa *et al*.[38] was used. Organic acids in LAB-SCS were determined by high-performance liquid chromatography. Following protein precipitation with 1 mol L^{-1} $HClO_4$, LAB-SCS samples were filtered (0.22 μm pore size). The filtered supernatants were diluted tenfold, and aliquots of 10 μL were injected into a 250 mm × 4.6 mm Synergi 4 μm C18/ODS Hydro-RP column (Phenomenex, Torrance, CA, USA). Elution was performed at 35 °C with 5 mmol L^{-1} H_2SO_4 at a flow rate of 0.5 mL min^{-1}. The optical density (OD) of organic acids at 220 nm was measured with an L-7405 UV detector (Hitachi, Tokyo, Japan). Solutions containing different organic acids (10 mmol L^{-1}), including lactic acid, acetic acid, formic acid, propionic acid and butyric acid, were used as standards. Quantification of organic acids in LAB-SCS was based on the external standard method.

Time-kill assay of *H. pylori* by LAB-SCS

The time-kill assay used to determine the viability of *H. pylori* after exposure to LAB-SCS was performed according to the method reported by Koga *et al.*[39] with modification. Briefly, *H. pylori* strains BCRC 17 021 and CMU83 were grown in *Brucella* broth supplemented with 50 mL L^{-1} FBS at 37 °C for 48 h under microaerophilic conditions. The cells were centrifuged at 4000 × *g* for 10 min and washed twice with sterile PBS. The final concentration of *H. pylori* was adjusted to 1×10^7 CFU mL^{-1} in 5 mL of various conditioning solutions, including different LAB-SCS from the 48 h culture: neutralised LY5-SCS (adjusted to pH 7 with 1.0 mol L^{-1} NaOH), LY5-SCS heat treated at 100 °C for 30 min, artificial LY5-SCS (111.5 mmol L^{-1} lactic acid and 11.2 mmol L^{-1} acetic acid in PBS), 100 mmol L^{-1} lactic acid in PBS, 100 mmol L^{-1} acetic acid in PBS, 100 mmol L^{-1} formic acid in PBS, and PBS (pH 7.2) as the control. In addition, LY5-SCS was digested with 0.5 mg mL^{-1} proteinase K (Sigma, St. Louis, MO, USA) at 25 °C for 30 min. Viable cell counts of *H. pylori* were determined after co-incubation at 37 °C with different conditioning solutions in a shaker rotating at 110 rpm for 4 h. A 1 mL aliquot of each test solution was withdrawn at different incubation times (0, 1, 2 and 4 h) and serially diluted in PBS (tenfold dilutions). Finally, 100 µL of each dilution was spread on *Brucella* agar containing 50 mL L^{-1} FBS and incubated at 37 °C for 48 h under microaerophilic conditions. Each assay was carried out in triplicate independent experiments.

Exclusion effect of LAB-SCS against *H. pylori* adhering to AGS cells

A human gastric epithelial cell line, AGS (gastric adenocarcinoma, ATCC CRL 1739), was purchased from BCRC and routinely cultured in 900 mL L^{-1} F-12 medium (Gibco, Gaithersburg, MD, USA) with 100 mL L^{-1} heat-inactivated FBS. A 1 mL aliquot of a suspension of 1×10^5 AGS cells mL^{-1} in fresh tissue culture medium was added to a 24-well multi-dish tissue culture plate and cultured at 37 °C in a humidified atmosphere of 5% (v/v) CO_2 in air. After the cells had grown into a confluent monolayer, 100 µL of viable *H. pylori* CMU83 at 1×10^5 CFU mL^{-1} was added to each well and incubated for 1 h to allow *H. pylori* to adhere to the AGS cells. After incubation the cells in each well were washed three times with sterile PBS. A 1 mL aliquot of fresh F-12 medium containing 100 µL of PBS (pH 7.2), LY5-SCS, neutralised LY5-SCS (pH 7) or artificial LY5-SCS was added to each well in triplicate and allowed to incubate for 1 h to exclude adherent *H. pylori* from the AGS cells. Subsequently, each well was washed five times to remove non-adherent *H. pylori*. The AGS cells were then lysed with 1 mL aliquots of 1 g L^{-1} Triton X-100. Appropriate dilutions of lysed cell solution were pipetted onto *Brucella* agar containing 50 mL L^{-1} FBS to determine the number of adherent *H. pylori* CMU83. Finally, the exclusion rate (%) of each treatment was calculated as

$$\text{exclusion rate (\%)} = (1 - \text{number of adherent } H.\ pylori \text{ after each SCS treatment/number of adherent } H.\ pylori \text{ after PBS treatment}) \times 100$$

Urease activity of *H. pylori* adhering to AGS cells

The urease activity of *H. pylori* adhering to AGS cells was determined by a modified phenol red method described by Sgouras *et al.*[37] The conditions for incubation of cultured AGS cells with different treatments (PBS, neutralised LY5-SCS, LY5-SCS and artificial LY5-SCS) were the same as those described previously for the exclusion assay. After washing the cells five times with PBS, 300 µL of urease reaction buffer (200 g L^{-1} urea and 0.12 g L^{-1} phenol red in phosphate buffer, pH adjusted to 6.5) was added to each well. The 24-well multi-dish plates were incubated at 37 °C for 3 h according to the Berthelot reaction, with modifications to allow the *H. pylori* adhering to AGS cells to produce ammonia.[40] Finally, the OD value at 580 nm ($OD_{580\ nm}$) was measured with a spectrophotometer.

Statistical analysis

Data are presented as mean ± standard deviation (SD) from triplicate trials. Data analysis was carried out using SPSS Version 12.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance was used to determine significant differences between means at a significance level of $P < 0.05$.

RESULTS

Cell count, pH, titratable acidity and well diffusion assay

The LAB strains used in this study were isolated from various samples such as yogurt and infant faeces (Table 1). The cell counts, pH, TA during LAB culture and the inhibition zone of the SCS of these LAB cultures are shown in Table 2. Two *H. pylori* strains, a type strain BCRC 17 021 and a clinically isolated strain CMU83, were used for the inhibition study. After 48 h of incubation the viable counts of strains LY1, LY5 and IF22 showed their higher growth ability compared with the other strains (Table 2). Owing to the utilisation of carbohydrates in fermentation, the pH of all culture media decreased after 48 h of incubation. TA increased continuously with incubation time. Therefore the decline in pH is associated with the growth of LAB and the production of organic acids. The inhibition activity of these LAB strains against *H. pylori* was determined by a well diffusion assay (Table 2). The results showed that the SCS of LY1, LY5 and IF22 strains after 48 h of incubation possessed the antagonistic activity to inhibit the growth of both *H. pylori* BCRC 17 021 and CMU83. However, the other LAB-SCS were ineffective. In addition, the results showed that the *H. pylori* strain CMU83 isolated from a clinical patient was more tolerant than the type strain BCRC 17 021. Furthermore, LAB strain LY5 had a lower pH and higher TA after 48 h of culture in milk medium than the other LAB strains tested. These results showed that the anti-*H. pylori* activity was closely correlated with acid production and TA. Finally, it should be mentioned that the production of organic acids depended on the different strains, growth rate and incubation time of LAB cells.

Organic acids in LAB-SCS

The concentrations of lactic acid and acetic acid were determined after 12, 24 and 48 h culture of the LAB cells (Table 3). As expected, the production of lactic acid by strains LY1, LY5 and IF22 was higher than that by the other LAB strains during incubation. The concentration of lactic acid in LY5-SCS was highest after 48 h of incubation. In addition, the production of acetic acid was low in the SCS of strains LY1, LY5 and IF22 after 48 h of incubation.

Time-kill assay of LAB-SCS

To understand the bactericidal activity against both *H. pylori* strains, a time-kill assay was performed. Each strain of *H. pylori*, BCRC 17 021 and CMU83, was co-cultured with SCS of LGG, IF22, LY1 and LY5 for 4 h. The results are shown in Fig. 1. The SCS of LY1 and LY5 showed dramatic bactericidal ability after co-incubation with the type strain or clinical isolate of *H. pylori*.

Table 2. Viable counts of LAB, pH, titratable acidity (TA) and inhibition of *Helicobacter pylori* by LAB-SCS[a]

	Viable counts (log CFU mL^{-1})		pH		TA (lactic acid %)[b]		Diameter of inhibition zone (mm)[c]			
							BCRC 17 021		CMU83	
LAB strain	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
LGG	8.1 ± 0.2	8.2 ± 0.3	6.3 ± 0.1	6.2 ± 0.1	0.16 ± 0.02	0.16 ± 0.01	–	–	–	–
LY1	9.4 ± 0.3	9.5 ± 0.4	4.0 ± 0.2	3.6 ± 0.2	0.79 ± 0.04	1.42 ± 0.05	–	9.7 ± 0.5	–	8.3 ± 0.5
LY5	9.2 ± 0.2	8.3 ± 0.5	3.7 ± 0.1	3.6 ± 0.2	1.18 ± 0.06	1.42 ± 0.08	8.7 ± 0.5	10.0 ± 0.8	8.0 ± 0.0	8.3 ± 0.5
IF3	7.8 ± 0.4	7.0 ± 0.5	5.7 ± 0.2	5.1 ± 0.2	0.33 ± 0.03	0.50 ± 0.02	–	–	–	–
IF9	8.0 ± 0.1	8.3 ± 0.3	6.4 ± 0.1	5.9 ± 0.1	0.17 ± 0.01	0.48 ± 0.02	–	–	–	–
IF22	9.3 ± 0.2	9.0 ± 0.6	4.5 ± 0.1	4.3 ± 0.3	0.78 ± 0.01	1.37 ± 0.12	–	10.0 ± 0.0	–	8.7 ± 0.5
AF3	7.6 ± 0.5	7.5 ± 0.3	6.5 ± 0.2	6.5 ± 0.1	0.15 ± 0.05	0.15 ± 0.01	–	–	–	–
GK31	7.9 ± 0.3	7.9 ± 0.5	6.4 ± 0.2	6.4 ± 0.2	0.16 ± 0.06	0.19 ± 0.05	–	–	–	–
V2	7.4 ± 0.3	7.2 ± 0.4	6.5 ± 0.1	6.5 ± 0.1	0.15 ± 0.01	0.15 ± 0.02	–	–	–	–
V15	8.9 ± 0.1	8.3 ± 0.3	6.4 ± 0.1	5.5 ± 0.2	0.18 ± 0.02	0.19 ± 0.04	–	–	–	–
Milk[d]			6.6 ± 0.1	6.6 ± 0.1	0.13 ± 0.01	0.13 ± 0.01	–	–	–	–

[a] Values are presented as mean ± SD from triplicate experiments.
[b] Calculated as TA (lactic acid %) = titratable volume (mL) of 0.1 mol L^{-1} NaOH × 0.009 × titre of 0.1 mol L^{-1} NaOH/weight of sample (g).
[c] The inhibition activity of each LAB strain on *H. pylori* was determined by an agar well diffusion assay as described in the text. *Helicobacter pylori* strains BCRC 17 021 and CMU83 were used as indicators. Inhibition activity is presented as diameter of inhibition zone; –, no inhibition zone. The wells on the *Brucella* agar plates were made with a sterile glass rod (7 mm in diameter).
[d] Negative control.

Table 3. Organic acids in LAB-SCS after culturing LAB in milk medium[a]

	Lactic acid (mmol L^{-1})		Acetic acid (mmol L^{-1})	
LAB strain	24 h	48 h	24 h	48 h
LGG	7.9 ± 2.2	8.1 ± 2.8	ND	24.6 ± 4.3
LY1	77.9 ± 3.7	103.3 ± 5.3	ND	8.6 ± 2.1
LY5	91.7 ± 6.2	111.5 ± 3.9	3.8 ± 1.2	11.2 ± 3.5
IF3	19.6 ± 2.5	31.7 ± 2.4	ND	ND
IF9	12.8 ± 1.2	16.2 ± 2.6	ND	ND
IF22	82.1 ± 5.5	83.6 ± 1.8	9.5 ± 1.9	12.8 ± 2.8
AF3	14.4 ± 3.0	27.8 ± 4.2	ND	ND
GK31	5.7 ± 2.7	5.8 ± 2.5	ND	ND
V2	4.4 ± 3.1	5.0 ± 2.1	ND	ND
V15	11.5 ± 3.2	28.3 ± 2.6	ND	ND

[a] Values are presented as mean ± SD from triplicate experiments. ND, not detected.

Moreover, the *H. pylori* clinical strain CMU83 was more tolerant to LAB-SCS than the type strain BCRC 17 021. *Helicobacter pylori* CMU83 still had viable cells (~5–6 log CFU mL^{-1}) after 4 h of co-incubation with LY1-SCS or LY5-SCS, while the viable count of *H. pylori* BCRC 17 021 was almost undetectable (Fig. 1). However, the bactericidal ability of LGG-SCS and IF22-SCS was not obvious. Comparing the relationship between organic acid production and bactericidal activity, the results showed that LY5-SCS produced a higher concentration of lactic acid after 48 h of incubation than the other LAB strains (Table 3). It also showed stronger bactericidal activity against both *H. pylori* strains.

In the well diffusion and time-kill assays, LAB strain LY5 showed the best inhibitory effect against the growth of both *H. pylori* strains. In order to ascertain the anti-*H. pylori* substance in the fermented milk, LY5-SCS was subjected to neutralisation, heating and protease digestion treatments. In addition, artificial LY5-SCS was prepared to confirm the bactericidal effect of organic acids. The results are shown in Fig. 2. The inhibition activity of LY5-SCS against *H. pylori* was lost when the SCS was neutralised to pH 7. However, there were no differences in bactericidal activity between the LY5-SCS that had been subjected to heating and protease treatments and the original SCS. These results suggest that the anti-*H. pylori* activity of LY5-SCS is not related to protein components but is associated with the concentration of organic acids and the pH value. To further substantiate this, time-kill assays were performed with artificial solutions containing organic acids similar to those in LY5-SCS. The results also showed that artificial LY5-SCS had obvious bactericidal activity (Fig. 2), even stronger than that of cultured LY5-SCS from fermented milk. Under the same conditions the *H. pylori* clinical isolate CMU83 showed better acid tolerance than *H. pylori* BCRC 17 021. The acid tolerance of this clinical strain may be an important factor in infection. In addition, we found that the bactericidal activity of formic acid was more effective than that of lactic acid or acetic acid against both *H. pylori* strains (Fig. 2). These results prove that the bactericidal activity of organic acids against *H. pylori* is related to pH, concentration and category of organic acids.

Effect of LAB-SCS treatment on *H. pylori* adhesion to AGS cells

Treatment of *H. pylori* CMU83 with cultured LY5-SCS and artificial LY5-SCS significantly reduced the number of *H. pylori* adhering to monolayers of cultured human gastric epithelial AGS cells ($P < 0.05$) (Table 4). In addition, the exclusion rate of artificial LY5-SCS was slightly higher than that of cultured LY5-SCS. However, neutralised SCS (pH 7) did not show an exclusion effect for *H. pylori* infection of AGS cells. This finding further indicated that organic acids in SCS play a key role in inhibiting the adhesion of *H. pylori* to AGS cells. Furthermore, the urease activity of *H. pylori* CMU83 adhering to AGS cells was examined (Table 4). After treatment

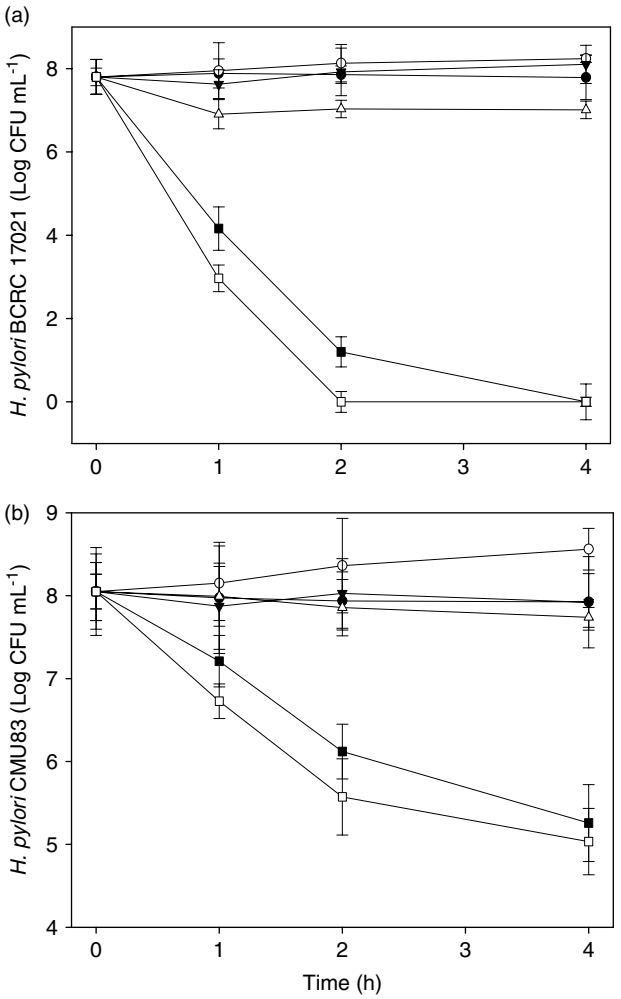

Figure 1. Effect of different treatments on growth of *Helicobacter pylori* (a) BCRC 17 021 and (b) CMU83. *Helicobacter pylori* cells were cultured in different solutions: ●, PBS (pH 7.2); ○, milk; ▼, LAB-SCS strain LGG from 48 h culture; △, LAB-SCS strain IF22 from 48 h culture; ■, LAB-SCS strain LY1 from 48 h culture; □, LAB-SCS strain LY5 from 48 h culture. Viable cell counts were determined after incubation at 37 °C in a shaker rotating at 110 rpm for 4 h. Data are presented as mean and SD from triplicate independent experiments ($n = 3$).

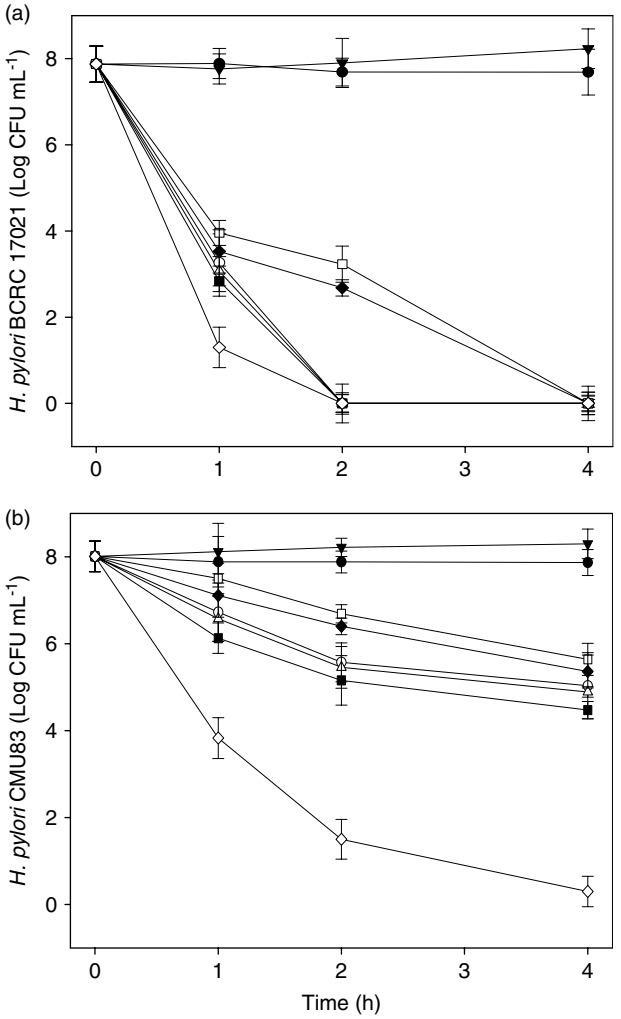

Figure 2. Effect of different treatments on growth of *Helicobacter pylori* (a) BCRC 17 021 and (b) CMU83. *Helicobacter pylori* cells were cultured in different solutions: ●, PBS (pH 7.2); ○, LY5-SCS (pH 3.62) from 48 h culture; ▼, neutralised LY5-SCS (pH 7); △, LY5-SCS heat treated at 100 °C for 30 min; ■, artificial LY5-SCS (111.5 mmol L^{-1} lactic acid and 11.2 mmol L^{-1} acetic acid in PBS, pH 2.91); □, 100 mmol L^{-1} lactic acid in PBS (pH 3.06); ♦, 100 mmol L^{-1} acetic acid in PBS (pH 3.71), ◊, 100 mmol L^{-1} formic acid in PBS (pH 3). Viable cell counts were determined after incubation at 37 °C in a shaker rotating at 110 rpm for 4 h. Data are presented as mean and SD from triplicate independent experiments ($n = 3$).

with cultured LY5-SCS and artificial LY5-SCS the urease activity of *H. pylori* was significantly reduced ($P < 0.05$).

DISCUSSION

In this study the inhibition zone of LAB-SCS from fermented milk against *H. pylori* was measured (Table 2). The pH, viable count, TA and organic acid content of SCS were also assayed (Table 3). The results showed that three LAB strains, LY1, LY5 and IF22, had better anti-*Helicobacter* effects than the other strains, with lower pH and more efficient bactericidal activity against both *H. pylori* strains according to the time-kill assay (Fig. 1). In addition, there were no obvious differences in bactericidal activity between cultured LY5-SCS, heat-treated LY5-SCS, protease-digested LY5-SCS and artificial LY5-SCS. However, this activity against *H. pylori* was lost with neutralised LY5-SCS (Fig. 2). These results suggest that the anti-*H. pylori* activity of SCS is not related to protein components but is associated with the concentration of organic acids and the pH value. Based on these results, the exclusion effect of LY5-SCS against *H. pylori* infection on AGS cells was investigated (Table 4). Both cultured LY5-SCS and artificial LY5-SCS significantly reduced *H. pylori* CMU83 infection and urease activity ($P < 0.05$). Thus we conclude that the concentration of organic acids in fermented milk produced by probiotic LAB strains is an important factor in inhibiting *H. pylori* infection.

Recently, several studies have found that SCS from LAB exhibit antibacterial activity against *H. pylori* both *in vitro* and *in vivo*.[15–18,41] It has been reported that lactic acid and other potentially inhibitory metabolites such as acetic acid, formic acid and proteins in fermented milk or culture supernatant fractions have a direct effect on pathogens.[18,20,22,28,42–44] In our study, only lactic acid and acetic acid were detected in the LAB-SCS of the LAB strains tested. This does not mean these LAB strains produced only lactic acid and acetic acid. In order to evaluate whether organic acids in cultured SCS are the only factor responsible for

Table 4. Exclusion effect of LAB-SCS and artificial SCS against infection of *Helicobacter pylori* CMU83 to AGS cells[a]

Treatment	Exclusion rate (%)[b]	Number of adherent *H. pylori* (CFU mL^{-1})	Urease activity ($OD_{580\ nm}$)
PBS (pH 7.2)	–	$(1.54 \pm 0.20) \times 10^6$z	0.38 ± 0.02y
LY5-SCS (pH 7)	−9.09	$(1.68 \pm 0.23) \times 10^6$z	0.40 ± 0.03y
LY5-SCS	58.6	$(8.37 \pm 0.31) \times 10^5$y	0.24 ± 0.03x
Artificial LY5-SCS	61.4	$(5.94 \pm 0.17) \times 10^5$x	0.22 ± 0.01x

[a] Values are presented as mean ± SD from three trials. Means in the same column followed by different letters are significantly different ($P < 0.05$) between treatments.
[b] Exclusion rate (%) = (1 − number of adherent *H. pylori* after each SCS treatment/number of adherent *H. pylori* after PBS treatment) × 100.

the inhibitory activity against *H. pylori*, we assayed the bactericidal activity of artificial SCS and compared it with that of cultured SCS. The results showed that both cultured SCS and artificial SCS significantly decreased the viability of *H. pylori* (Fig. 1). In the time-kill assay, lactic acid, acetic acid and formic acid at 100 mmol L^{-1} were also confirmed as having bactericidal activity. The results showed that formic acid had the strongest activity against *H. pylori* strains BCRC 17 021 and CMU83 (Fig. 2). These results support those of Oh *et al*.[42] However, this bactericidal activity was lost after adjusting the pH of cultured LGG-SCS to 7. Midolo *et al*.[45] and Boyanova *et al*.[18] also found that the bactericidal effect against *H. pylori* was lost or decreased when the pH of SCS was adjusted from the original 2.3 to 5.3. Aiba *et al*.[29] found that a 1% solution of lactic acid (∼100 mmol L^{-1}) was sufficient to inhibit the growth and urease activity of *H. pylori*. The bactericidal activity of LAB-SCS after heating or protease treatment was similar to that of cultured LAB-SCS. These results further verify that pH and organic acids are important factors inhibiting the growth of *H. pylori in vitro*.[45] Aiba *et al*.[29] also reported that, as the amount of lactic acid increased, the number of *H. pylori* decreased in parallel with the increase in the number of colonising *L. salivarius*. This is due to the latter's high affinity for binding to gastric epithelial cells and producing organic acids to inhibit *H. pylori* infection. In the same study the authors also validated that a strain of *L. acidophilus* was unable to suppress the growth of *H. pylori in vivo* because of the low level of lactic acid production in the stomach.[29]

It has been demonstrated that the coccoid form of *H. pylori* causes a loss of infectivity.[46,47] Nam *et al*.[48] found that, when *H. pylori* was treated with SCS, the cells changed from helical to coccoid form and became necrotic. Coconnier *et al*.[49] observed the same result for *L. acidophilus* after treatment with SCS. It is known that the undissociated forms of organic acids play a critical role in their inhibitory power.[50] Garrote *et al*.[51] reported that the inhibitory effect of kefir on *Escherichia coli* could be attributed to lactic acid and acetic acid produced during the fermentation process. Meanwhile, Jin *et al*.[52] also found that the major organic acids of *Lactobacillus* culture supernatants inhibiting the growth of enterotoxigenic *E. coli* were acetic acid and lactic acid. In addition, several studies have reported that the antagonistic activity of LAB-SCS against foodborne pathogens involves both organic acid and non-lactic acid molecules,[53–56] and they are able to inhibit *H. pylori* in a concentration-dependent manner.[57]

The AGS cell line was isolated from a Chinese patient with gastric cancer and has been widely used to study the relationship between *H. pylori* infection and human gastric diseases such as gastric ulcer and gastric cancer.[14,58,59] In this study, AGS cells were used to evaluate the efficacy of LAB-SCS to inhibit the adhesion of *H. pylori* to gastric cells. The results showed that the high content of organic acids in SCS is efficacious in inhibiting *H. pylori* adhesion to AGS cells. The urease activity of *H. pylori* adhering to AGS cells after treatment with SCS of milk culture was also measured. Urease produced by *H. pylori* degrades urea to produce ammonia, which neutralises the acidic environment, thereby allowing *H. pylori* to survive in the stomach.[60] Comparing the results for exclusion effect and urease activity in this study, it was found that there was an inverse relationship between the two. For example, the exclusion rate of artificial LY5-SCS against *H. pylori* infection was highest (61.4%) but the urease activity of *H. pylori* was lowest (Table 4). Therefore LAB-SCS are able to inhibit *H. pylori* infection in AGS cells.

In conclusion, the results of this study show that the concentration and category of organic acids from probiotics affect the viability of *H. pylori in vitro*. The production of organic acids is dependent on the LAB strain, growth rate and fermentation pathway. The *in vitro* methods used here might provide for the rapid screening of potential probiotics with anti-*H. pylori* activity in the functional food and dairy industries. Finally, the anti-*H. pylori* activity of LY5-SCS that was fermented in milk by *L. acidophilus* LY5 should be investigated in future *in vivo* studies.

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