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보 문

Evaluation of the anti-*Helicobacter pylori* and cytotoxic properties of the antimicrobial substances from *Lactobacillus acidophilus* BK13 and *Lactobacillus paracasei* BK57

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Lactobacillus acidophilus BK13 and *Lactobacillus paracasei* BK57 균주가 생산한 항균물질의 anti-*Helicobacter pylori* 활성 및 위장상피세포에 대한 세포독성 평가

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ABSTRACT: The objective of this study is to investigate the anti-*Helicobacter pylori* and anti-cancer activities of the live cells (LC), cell-free culture supernatants (CFCS), and bacteriocin solution (BS) obtained from *Lactobacillus acidophilus* BK13 and *Lactobacillus paracasei* BK57 strains. After incubation for 30 h in MRS broth, the concentration of lactic acid produced by *L. paracasei* BK57 (155.9±10.2 mM) was higher than in MRS broth using *L. acidophilus* BK13 (126.8±7.9 mM). Maximum bacteriocin activity (128 AU/ml) of BK13 strain was observed after 30 h of cultivation at 37°C, however its magnitude was significantly lower than that of BK57 strain (256 AU/ml). The LC of *L. acidophilus* BK13 and *L. paracasei* BK57 were able to inhibit the growth of *H. pylori* ATCC 43504 at different incubation times, depending on the initial inoculum of the LAB. These CFCS and BS obtained from BK13 and BK57 strains dramatically inhibited the growth, adhesive ability, and enzymatic activity of *H. pylori*. Meanwhile, the anti-cancer effect of the lactic acid from *L. acidophilus* BK13 and *L. paracasei* BK57 strains on AGS cells had significant differences with the control group. Therefore, these antagonistic substances-producing strains are potentially useful as new potential antimicrobial agents for the management and prevention of *H. pylori* infections.

Key words: *Helicobacter pylori*, adhesion, anti-cancer activity, antimicrobial activity, urease

Helicobacter pylori is a helix-shaped Gram-negative found in the upper gastrointestinal tract and is present in more than 50% of the world's population. As a general rule, infection rate appears to be higher in developing than in developed countries and it seems to be decreasing with improvements in hygiene practices (Brown, 2000). Possible routes of infection include either oral-oral or fecal-oral contact, use of unsterile endoscopes, and crowed or high-density living conditions (Cave, 1997). Chronic infection with *H. pylori* causes gastric mucous membrane damage and atrophic and even metaplastic changes in the stomach, thereby inducing inflammation of the gastric mucosa. Furthermore, gastric colonization with *H. pylori* can lead to variety of upper gastrointestinal disorders, such as nonulcer dyspepsia, peptic ulcer disease, distal gastric adenocarcinomas, gastric mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric cancer. Bacterial virulence factors such as the cytotoxin-associated gene pathogenicity island-encoded protein CagA and the vacuolating cytotoxin VacA can cause inflammation, damage of gastric epithelial cells, and/or apoptosis and are potentially a carcinogen (Kusters

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et al., 2006).

Medications used to eradicate *H. pylori* include antibiotics (amoxicillan, clarithromycin, metronidazole or tetracycline), proton pump inhibitor (PPI, lansoprarzole and omeprazole), and anti-diarrheal medicine (bismuth subsalicylate). Although these standard therapies are commonly used, increasing antimicrobial resistance and side effects and falling eradication rates are the result of the widespread use of antibiotics (Egan *et al*., 2007). For this reason, antibiotic therapy is not recommended in patients infected with *H. pylori*, therefore the use of probiotics as alternative strategies has been suggested. According to previous studies, probiotics which is defined as a living microbial species have been proven to be useful in the treatment of *H. pylori*-associated disorders and diseases (Sgouras *et al*., 2004; Lesbros-Pantoflickova *et al*., 2007). Ingesting lactic acid bacteria (LAB) exerted a suppressive effect on *H. pylori* infection in both animals and humans, and probiotic preparations of yogurt containing *Lactobacillus acidophilus* La5 and *Bifidobacterium lactis* Bb12 were effective in reducing the rates of eradication of *H. pylori* in humans (Wang *et al*., 2004).

Some probiotic strains are able to inhibit *H. pylori* growth through the release of the antimicrobial agent called bacteriocins or organic acids (lactic, acetic, and butyric acid) and the competition for the limited nutrients required for bacterial growth or the receptors on epithelial cells (Boirivant and Strober, 2007). In addition, they prevent the adherence of the pathogenic bacteria to the host cells by enhancing the barrier function of the intestinal mucosa, stimulating the immune response, and releasing gut-protective metabolites such as arginine, glutamine, short-chain fatty acids, and conjugated linoleic acids (Hemaiswarya *et al*., 2013). Probiotic treatment reduced the inflammatory effects of infection by this pathogen and the side effects caused by traditional anti-*H. pylori* therapy, so adjuvant therapy with probiotics is currently becoming more popular than the traditional eradication methods.

L. acidophilus BK13 and *L. paracasei* BK57 strains used in this study were originally isolated from Baikkimchi and these strains showed relatively good survival property in artificial gastric juice and the antimicrobial activity against *H. pylori* in the previous experiment (Lim, 2014). The objective of this study is to investigate the anti-*H. pylori* and anti-cancer activities of the live cells (LC), cell-free culture supernatants (CFCS), and bacteriocin solution (BS) obtained from *L. acidophilus* BK13 and *L. paracasei* BK57 strains.

Materials and Methods

Bacterial strains and culture conditions

LAB strains were grown in Lactobacilli MRS broth (Difco) for 24 h at 37°C. The tested strains were stored in Lactobacilli MRS broth (Difco) with 20% glycerol at -80°C. These strains were recovered from frozen stock and cultivated twice on MRS agar aerobically at 37°C for 24 h.

Culture conditions of *H. pylori*

H. pylori ATCC 43504 strain used in this study was obtained from American Type Culture Collection (ATCC). *H. pylori* was cultured under microaerophilic conditions (10% CO_2, Anoxomat system, MART Co.) on Brucella agar (Difco) plates supplemented with 5% (v/v) fetal bovine serum (FBS, Gibco BRL), 0.2% (w/v) 2,6-di-o-methyl-β-cyclodextrin (CD), and antibiotics (cefsulodine, vancomycin, trimethoprim, and amphotericin B, Sigma-Aldrich) at 37°C for 48 h. The strain was stored in Brucella broth (Difco) containing 20% (v/v) glycerol at -80°C. And then, it was recovered from frozen stock and sub-cultured twice in Brucella broth supplemented with 5% FBS at 37°C for 48 h with agitation on a rotary shaker at 100 rpm in a microaerobic condition prior to experimental use.

Microbiological and chemical analysis during the incubation

The tested LAB strains were propagated in MRS for 30 h at 37°C aerobically. At convenient time intervals, culture samples were aseptically withdrawn from the fermentation vessel to determine viable cell number, pH, and titratable acidity. Viable cell counts of LAB culture were determined by the standard plate count (SPC) method with MRS medium at 37°C after 48 h of incubation. The pH value of samples was measured separately by a digital pH meter (Metrohm 744). Finally, titratable acidity of the culture was determined by titration with 0.1 N NaOH and expressed in terms of milliliters of NaOH.

Preparation of antimicrobial substances

Lactic acid: Cultures of LAB strains were grown in MRS broth at 37°C under the optimum conditions. After incubation, the CFCS was derived from fresh overnight cultures, centrifuged at 7,000 × *g* for 10 min at 4°C, and freezed until used. The CFCS was filtered using a 0.22 μm membrane filter (Millipore) and precipitated protein by the addition of $HClO_4$ (1 M). And then, lactic acid was determined by high-pressure liquid chromatography (HPLC, Shimadzu), with Aminex HPX-87H column (300 mm by 7.8 mm: Bio-Rad) and a refractive index detector (GBC Scientific Equipment Pty Ltd.). The operating conditions were the following: H_2SO_4 solution (5 mM) was used as eluent at a flow rate of 0.5 ml/min and column temperature of 35°C. The sample injection volume was 50 μl. Lactic acid was monitored by measuring the optical density at 220 nm and its concentration was calculated from a standard curve of known L-lactate concentration.

Crude bacteriocin: The obtained CFCS was filter-sterilized using 0.45 μm pore size filter to eliminate the possible presence of viable cells and their pH was adjusted to 6.5 by means of 1 N NaOH to exclude the antimicrobial effect of organic acid. Inhibitory activity from hydrogen peroxide was eliminated by the addition of 1 mg/ml catalase (Sigma-Aldrich). The CFCS were treated with ammonium sulfate to 50% saturation and agitated at 4°C for overnight. The crude BS was then precipitated from the CFCS by centrifugation (12,000 × *g* for 30 min at 4°C) and the precipitates were re-suspended in 20 mM sodium phosphate buffer (pH 6.5). Dialysis was followed in a dialysis bag (Spectrum Medical Industries, Inc.) with a molecular weight cut-off of 1,000 Da against the same buffer for 24 h at 4°C.

Bacteriocin activity

Bacteriocin activity was measured using a microtiter plate assay (Hole *et al*., 1991) described previously against *H. pylori* as the target organism. Cells of *H. pylori* ATCC 43504 were obtained by centrifugation (7,000 × *g*, 4°C, 10 min) from 48 h-old cultures, washed twice with sterile phosphate buffer saline (PBS, pH 7.0), and resuspended in the same buffer. The bacterial suspension (1.0×10^5 CFU/ml), the diluted BS of different concentrations, and Brucella broth were individually placed in microtiter plate wells (Falcon). Plates were incubated at 37°C for 24 h and the growth inhibition of the indicator organism was measured spectrophotometrically at 600 nm with a microplate reader (BioTek, Inc.). The bacteriocin activity (arbitrary units, AU) was defined as the reciprocal of the highest dilution inhibiting the growth of the indicator strain by 50%.

Growth of *H. pylori* co-cultured with LC, CFCS, and BS

LAB strains were grown in MRS broth at 37°C overnight. The LC of LAB strain were prepared by centrifuging (7,000 × *g*, 10 min, 4°C) the active cultures and washing two times by PBS (pH 7.0) and diluted to a concentration of 10^6, 10^7, and 10^8 CFU/ml. Meanwhile, 48 h-old *H. pylori* cultures were resuspended in sterile PBS (pH 7.0) and centrifuged at 7,000 × *g* for 10 min at 4°C. The cell pellet was collected, and the fresh *H. pylori* cells (1.0×10^6 CFU/ml) suspended in antibiotic-free Brucella broth containing 5% FBS were incubated under microaerophilic conditions for 48 h at 37°C in the presence of the LC, CFCS or BS at different concentration. Initially and then at predetermined intervals, aliquots of the cultures were removed, serially diluted, and cultured at 37°C under microaerophilic conditions for 48 h on Brucella agar containing 5% FBS to determine the viable cell counts of *H. pylori*. Finally, the inhibition (%) of viability of *H. pylori* by the LC, CFCS, and BS treatment was calculated as follows; Inhibition (%) = (1-cell counts of *H. pylori* after each treatment/cell counts of *H. pylori* after PBS treatment) × 100.

Cell culture

Human gastric cancer cell lines (AGS and SNU-1) were from American Type Culture Collection (USA). Cells were routinely grown in RPMI 1640 medium (Gibco, BRL), supplemented with 10% (v/v) inactivated (30 min, 56°C) FBS, l-glutamine, $NaHCO_3$, kanamycin (60 μg/ml), and streptomycin (20 μg/ml) (Nikken bio medical). Monolayers of cells were grown in six-well tissue culture plates (Falcon) at the density of 5×10^4 cells/well. Experiments and maintenance of the cells were carried out at 37°C in humidified atmosphere of 5% CO_2 and 95% air. Culture medium was replaced every other day. Cultures were used at late post-confluence, i.e., after 15 days in culture.

Adhesion of *H. pylori* against AGS cells in the presence of LC, CFCS, and BS

The inhibition of adhesion of *H. pylori* by the tested LAB strains were examined as previously described by Wang *et al.* (2014) with some modifications. AGS cells (4×10^5 cells/ml) were seeded into six-well tissue culture plates (Gibco) and cultured at 37°C in an incubator with 5% CO_2-95% air until monolayers of the cells was nearly confluent. Monolayers were washed twice with PBS (pH 7.0) before inhibition assays. The LAB and *H. pylori* cultures propagated in MRS broth and Brucella broth containing 5% FBS, respectively, were harvested by centrifugation at 7,000 × *g* for 10 min at 4°C and washed two times with PBS (pH 7.0).

In the exclusion assay, 1 ml of the LC (1.0×10^8 CFU/ml) suspended in antibiotic-free RPMI 1640 medium were added to each well seeded with AGS cells and incubated for 2 h at 37°C in 5% CO_2-95% air atmosphere. And then 1 ml of *H. pylori* (1.0×10^8 CFU/ml) suspended in cell culture medium was added to wells and left to incubate for an additional 2 h. In the competition assays, 1 ml of the LC suspension (1.0×10^8 CFU/ml) of LAB strains and 1 ml *H. pylori* suspension (1.0×10^8 CFU/ml) were added to each well seeded with AGS cells simultaneously and incubated for 4 h at 37°C in 5% CO_2-95% air. In the displacement assay, 1 ml of *H. pylori* (1.0×10^8 CFU/ml) suspended in antibiotic-free cell culture medium were added to wells seeded with AGS cells. After incubation for 2 h at 37°C in 5% CO_2-95% air, 1 ml of LAB-LC suspension (1.0×10^8 CFU/ml) prepared in antibiotic-free cell culture medium were added to wells and left to incubate for an additional 2 h. Wells containing *H. pylori* alone served as controls.

Meanwhile, 1 ml aliquot of fresh cell culture medium containing the CFCS or BS adjusted to a suitable concentration was added to each wells seeded with AGS cells and allowed to incubate for 2 h to remove adherent *H. pylori* from the epithelial cells. In all assays, monolayers were washed three times with PBS (pH 7.0) to release unbound bacteria after incubation. To determine the number of adherent *H. pylori*, AGS cells were lysed with 0.1% Triton X-100 for 5 min. And then, aliquots of serial 10-fold dilutions were plated onto antibiotic-selective agar for 48 h at 37°C.

Urease assay of *H. pylori* co-cultured with CFCS or BS

Urease activity in *H. pylori* adhering to AGS cells was determined by measuring the release of ammonia by a modification of phenol red method (Sgouras *et al*., 2004). The incubation conditions for the adherence of *H. pylori* to AGS cells in the presence of PBS, CFCS, and BS were the same as those described previously for the adhesion assay. The incubated cell cultures were washed five times with PBS (pH 7.0), and then urease reaction buffer (200 g/L urea and 0.12 g/L phenol red in PBS, pH adjusted to 6.5) was added to each well of the microtiter plate. The well plates were incubated for 3 h at 37°C in order to induce the production of ammonia from *H. pylori* adhering to AGS cells. After incubation, the absorbance at 550 nm was measured with a microtiter plate reader.

Cytotoxicity assay for gastric cancer cell lines

Cytotoxic effect of the CFCS and BS from *L. acidophilus* BK13 and *L. paracasei* BK57 against AGS and SNU-1 cells was determined using an MTT assay described by Zheng *et al*. (1996). A cell suspension (with cell density of 1.0×10^4 cell/well) was seeded into each well of the 96-well culture plates (Falcon) and incubated for 24 h at 37°C in a 5% CO_2 humidified incubator. After obtaining a semi confluent cell layer, the exhausted media were discarded. And then, the cultivated cells were exposed to various concentrations of the CFCS (50, 100, and 150 μl/ml) and BS (100, 200, and 300 AU/ml) prepared in RPMI 1640 medium and incubated at 37°C in humidified 5% CO_2 for 24 h. The wells containing the same amount of the cell culture medium and having no the antimicrobial substance were used as control. The medium of each well after incubation was replaced with equally fresh media containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (2 mg/ml) and samples were incubated under dark conditions for 4 h at 37°C. After discarding all media from the plates, 100 μl of dimethyl sulfoxide (DMSO) was added to all wells. The plates were held for 5 min at room temperature with shaking to achieve complete dissolution of formazan. The absorbance of the MTT formazan was determined at 540 nm using a spectrophotometric plate reader. The percentage cell viability was determined based on the formula: Viability (%)= (optical density of sample/optical density of control) × 100.

Statistical analysis

All experiments were performed in triplicate and results were expressed as mean±standard deviations (SD). Differences between the means of the test and control groups were examined for significance by Student's *t*-test and $P<0.05$ was considered to be statistically significant. In particular, the results on cytotoxicity for gastric cancer cell lines were subjected to one-way analysis of variance (ANOVA) and means were separated by Duncan's multiple range test ($P<0.05$) using the software package SPSS 12.0 Window Program (SPSS Inc.).

Results and Discussion

Changes of cell number, pH, titratable acidity, lactic acid content, and bacteriocin activity during incubation period

Changes of the viable cell counts, pH, titratable acidity, lactic acid content, and bacteriocin activity during incubation by the selected *L. acidophilus* BK 13 and *L. paracasei* BK57 in MRS broth at 37°C are shown in Table 1. The cell growth rate of the two LAB varied slightly depending on the species. *L. acidophilus* BK13 reached $1.1\pm0.2 \times 10^9$ CFU/ml after 30 h at 37°C. The cell population of *L. paracasei* BK57 rapidly increased and reached 10^8 CFU/ml in MRS broth after 24 h of fermentation and constant to 30 h fermentation. After 30 h of incubation, the pH values of *L. acidophilus* BK13 and *L. paracasei* BK57 cultures were 3.50±0.18 and 3.38±0.14, respectively. The two strains produced lactic acid followed by the decreasing of pH. After incubation for 30 h, the concentration of lactic acid produced by *L. paracasei* BK57 (155.9±10.2 mM) was higher than in MRS broth using *L. acidophilus* BK13 (126.8±7.9 mM). *L. acidophilus* BK13 and *L. paracasei* BK57 cultures showed a time-dependent decrease and increase in the pH and titratable acidity, respectively, when grown on MRS medium. The production of titratable acid was different among the two strains at different times of incubation. After 30 h incubation, the titratable acidity of BK13 and BK57 strains reached 1.36±0.23 and 1.61±0.13%, respectively. The bacteriocin activity of *L. acidophilus* BK13 and *L. paracasei* BK57 started in the log phase of growth and increased with increase in incubation time. Maximum bacteriocin activity (128 AU/ml) of BK13 strain was observed after 30 h of cultivation at 37°C. Although BK13 strain demonstrated similar antimicrobial activity on *H. pylori*, its magnitude was significantly lower than those of BK57 strain ($P<0.05$). The production of the bacteriocin from BK57 strain was first detected after 12 h of incubation (4 AU/ml), whereas the peak of bacteriocin production was observed at 24-30 h (256 AU/ml), during the late exponential growth phase.

In recent decades, it has become clear that the overall inhibitory action of LAB is due to a variety of antagonistic factors that include organic (lactic, acetic, and formic) acids, ethanol, hydrogen peroxide, diacetyl, acetoin, 2,3-butanediol, acetaldehyde, benzoate, and bacteriolytic enzymes (Klaenhammer,

Table 1. Changes of viable cell counts, pH, titratable acidity, lactic acid content, and bacteriocin activity during incubation by the selected *L. acidophilus* BK 13 and *L. paracasei* BK57 in MRS broth at 37°C

Strain	Incubation time (h)	Viable cell counts (CFU/ml)	pH	Lactic acid (mM)	Titratable acidity (%)	Bacteriocin activity (AU/ml)
Lactobacillus acidophilus BK13	0	$5.2\pm2.1\times10^3$	6.84±0.04	ND	ND	ND
	12	$4.9\pm3.7\times10^6$	5.46±0.26	23.6±5.2	0.29±0.17	ND
	18	$5.6\pm31\times10^7$	4.40±0.30	55.1±4.8	0.75±0.26	16
	24	$3.0\pm1.8\times10^8$	3.87±0.07	83.9±12.0	0.87±0.06	64
	30	$1.1\pm0.2\times10^9$	3.50±0.18	126.8±7.9	1.36±0.23	128
Lactobacillus paracasei BK57	0	$6.0\pm1.9\times10^3$	6.79±0.03	ND	ND	ND
	12	$8.2\pm5.5\times10^5$	5.12±0.27	40.6±6.1	0.36±0.16	4
	18	$6.9\pm4.1\times10^7$	4.09±0.12	81.3±3.9	0.84±0.31	32
	24	$7.1\pm6.0\times10^8$	3.62±0.10	120.1±9.8	1.29±0.07	256
	30	$1.6\pm4.8\times10^8$	3.38±0.14	155.9±10.2	1.61±0.13	256

ND, not detected.

1988). In addition, certain strains of LAB are further known to produce bactericidal proteins, termed bacteriocins, which are antagonistic to a wide spectrum of microorganisms, and thus can make significant contributions to food preservation and intestinal ecology (De Vuyst and Leroy, 2007). Several studies have shown that the bacteriocin production in LAB is dependent on the biomass (Powell *et al*., 2007; Settanni *et al*., 2008). The bacteriocin biosynthesis is affected by type and level of the carbon, nitrogen and phosphate sources, cations surfactants, and inhibitors. Primary metabolite kinetics of the bacteriocin production with a peak activity usually occur at the end of exponential growth phase, followed by a decrease during the stationary phase (Savadogo *et al*., 2006). The decrease in bacteriocin activity at the end of exponential growth phase might be explained by the degradation of the bacteriocin by extracellular proteolytic enzymes (Todorov and Dicks, 2005). New proteinaceous active substance produced by *L. paracasei* subsp. *paracasei* strain M3 displayed the bactericidal activity against *H. pylori* NCIPD 230. The synthesis of this antimicrobial substance was detected in the late logarithmic growth phase during batch fermentation (Atanassova *et al*., 2003).

LAB have their ability to produce lactic acid as the major product of sugar (e.g., glucose) fermentation by either the homo- or heterofermentative pathway (Wee *et al*., 2006). Chen *et al*. (2011) showed that the main organic acid present in the CFCS of *Lactobacillus gasseri* Chen and *Lactobacillus plantarum* 18 having significant anti-*H. pylori* activity was lactic acid with the range of content from 114 to 150 mM. Among the strains showing antagonistic activity against *H. pylori*, lactic acid levels were 13.4 to 18.6 mg/ml for *Lactobacillus delbrueckii* subsp. *bulgaricus* and 0.6 to 11.5 mg/ml for *Streptococcus thermophilus* (Aslim *et al*., 2011).

Antagonistic activity of LC, CFCS, and BS obtained from the LAB against *H. pylori* ATCC 43504

The antagonistic activity of the LC, CFCS, and BS obtained from *L. acidophilus* BK13 and *L. paracasei* BK57 against *H. pylori* ATCC 43504 are presented in Table 2. The LC of *L. acidophilus* BK13 and *L. paracasei* BK57 were able to inhibit the growth of *H. pylori* ATCC 43504 at different incubation times, depending on the initial inoculum of the LAB. Specially, the LC of BK57 strain exhibited higher inhibition capacity on *H. pylori* than that of BK13 strain. When the inoculum of *L. paracasei* BK57 was 10^8 CFU/ml, 93.5±5.1% inhibition of the pathogen growth was observed after 48 h. Furthermore, these CFCS and BS obtained from BK13 and BK57 strains exhibited varying degrees of inhibitory activity against *H. pylori* strain and inhibited the growth of *H. pylori* dramatically after 48 h of incubation. The CFCS and BS of the two LAB tested significantly reduced the viable cell counts of *H. pylori* in dose-dependent manner. At the same concentration, the CFCS of BK13 strain showed a weaker inhibitory effect compared to BK57-CFCS. These results noticed that the high degree of the antimicrobial activity of *L. paracasei* BK57 may be partly attributed to their higher acid production. Using time-kill studies, the inhibition of *H. pylori* observed in the presence of the BS (300 AU/ml) of *L. acidophilus* BK13 ranged between 89.6±3.0 and 93.9±1.1%, while 60.5±2.8 – 64.6±3.7% reduction was observed in the BS of BK57 strain during incubation. This implied that low pH values and high titratable acidity are important for anti-*H. pylori* activity. In a study by Boyanova *et*

Table 2. Time-kill assay of *H. pylori* ATCC 43504 with LC, CFCS, and BS obtained from the selected LAB

Strains	Incubation time (h)	Inhibition (%)								
		Initial cell counts of LAB (CFU/ml)			Concentration of CFCS (μl/ml)			Concentration of BS (AU/ml)		
		10^6	10^7	10^8	50	100	150	100	200	300
Lactobacillus acidophilus BK13	12	73.1±2.6	76.0±3.8	82.1±7.0	23.1±5.3	34.5±0.9	40.9±1.5	32.9±2.5	64.7±2.2	89.6±3.0
	24	82.4±4.8	84.1±5.2	88.2±6.5	28.7±4.7	40.4±2.5	47.8±4.6	36.1±4.1	70.5±4.0	91.4±4.7
	48	75.6±9.1	84.6±3.7	86.1±5.7	24.7±3.6	38.3±3.6	52.7±5.7	38.0±3.7	73.1±5.8	93.9±1.1
Lactobacillus paracasei BK57	12	84.9±2.9	86.1±5.3	90.6±4.0	35.1±4.2	45.2±1.7	49.5±3.2	12.5±5.5	34.8±6.0	64.6±3.7
	24	85.3±6.0	89.5±4.7	92.2±1.2	39.8±3.6	48.7±0.8	53.6±4.0	13.4±4.7	33.9±3.7	66.7±6.2
	48	83.5±4.5	88.1±8.0	93.5±5.1	40.4±1.5	49.5±0.9	59.5±6.8	15.9±0.7	36.2±2.8	60.5±2.8

Data were presented as mean±SD from three independent experiments.
*Statistical analysis preformed with s Student *t*-test between control and treated *H. pylori* with the bacteriocin showed a highly significant difference ($P<0.05$).

al. (2009), the anti-*Helicobacter* activity of *L. delbrueckii* subsp. *bulgaricus* cultures was strain-dependent and these tested strains secreted metabolic products such as lactic acid and bacteriocin which exert activity against *H. pylori*.

Several probiotic strains affect the host beneficially, have exhibited antagonistic properties against *H. pylori in vitro* without antibiotic-associated gastrointestinal side-effects (Pacifico *et al*., 2014). In previous study, BK13 and BK57 strains showed relatively high tolerance to artificial gastric juice (Lim, 2014) and good adhesive properties to the human epithelial cells (data not shown), so these isolates met partially the selection criteria for potential probiotics. These are several putative mechanisms for probiotic efficacy against *H. pylori*; enhancement of barrier function, competition for the limited nutrients, inhibition of the epithelial and mucosal adherence of pathogens, production of inhibitory compounds (hydrogen peroxide, diacetyl, organic acids, and bacteriocin-like substances), and stimulation of the immune response (Hamilton-Miller, 2003).

Two main types of substances such as short chain fatty acids (SCFAs) and bacteriocin proteins have been implicated in the inhibition of *H. pylori* by LAB. SCFAs such as formic, acetic, propionic, butyric and lactic acids are produced as a result of the metabolism of carbohydrates by probiotics and play an important role in decreasing the pH *in vitro*. Their antimicrobial activity could be due to the inhibition of urease activity by high lactic acid producers, such as *Lactobacillus salivarius* and *Lactobacillus casei* Shirota (Gotteland *et al*., 2006). Lactic acid is produced in largest amount during the metabolism of carbohydrate *Lactobacillus* and have an important role in decreasing pH, which is related with respect to H^+ ions, important in the inhibition of *H. pylori in vitro* (Midolo *et al*., 1995). The antimicrobial activity occurs through the diffusion of lactic molecules into microbial cells until equilibrium is reached. Acids are generally thought to exert their antimicrobial effect by interfering with the maintenance of cell membrane potential, active transports, and a variety of metabolic reactions, causing membrane disruption, reducing intracellular pH homeostasis, and accumulating of toxic anions (Brul and Coote, 1999). Previous studies have indicated that the inhibitory role of *L. acidophilus* against *H. pylori* was related to the acid production and the low pH attained (Lorca *et al*., 2001). The supernatant of *L. acidophilus* culture has been shown to greatly suppress the viability of *H. pylori* both *in vitro* and *in vivo*, dependent on pH and lactic acid level (Coconnier *et al*., 1998).

However, the antimicrobial activity of certain probiotic lactobacilli may even be based on the production of bacteriocins. The proteinaceous bacteriocins with anti-*H. pylori* activity were synthesized by probiotic strains of *Lactobacillus*, *Enterococcus faecium*, *Bacillus subtilis*, and *Bifidobacterium* (Gotteland *et al*., 2006). In the mode of action, all types of bacteriocins show their antimicrobial effects on the target cell surface via various mechanisms including the deficiencies in the cell wall synthesis, changes in the membrane permeability, and/or formation of pores causing the death of the target cells (Moll *et al*., 1999).

Chen *et al*. (2011) reported that the count of *H. pylori* decreased in the co-culturing live lactobacilli and LAB-CFCS. At 48 h, the count of the pathogen was reduced only in co-cultures with the *L. gasseri* Chen and *L. plantarum* 18 and their CFCS; the cells of live *Lactobacillus rhamnosus* GG could not inhibit the growth of *H. pylori*. The CFCS of *L. plantarum* 18 had a greater ability to inhibit the growth of *H. pylori* than the other two strains. Michetti *et al*. (1999) and Aiba *et al*. (1998) showed that the supernatants from *L. johnsonii* La1 and *Lactobacillus salivarius* cultures inhibit the growth of *H. pylori in vitro* whether or not *H. pylori* is bound to epithelial cells. This effect was due to the production of a large amount of lactic acid since the inhibitory effect could be reproduced after the incubation of *H. pylori* in the presence of lactic acid. Furthermore, the urease activity became almost undetectable at a lactic acid concentration of 10 mM. Midolo *et al*. (1995) reported that lactic, acetic, and hydrochloric acids demonstrated an inhibition of *H. pylori* growth in a concentration-dependent manner, with lactic acid resulting in the highest inhibition. Lorca *et al*. (2001) demonstrated that the antibacterial activity of the spent broth obtained from 17 strains of lactobacilli against 10 strains of *H. pylori* was associated with organic acids or intracellular proteinaceous component. De Vuyst *et al*. (2004) demonstrated that the specific activity of the CFCS (pH 6.5) of *Lactobacillus johnsonii* La1 was also observed in time-kill assays. Also, the concentrated CFCS, containing no organic acids, of *L. johnsonii* La1, *Lactobacillus casei* Shirota, and *Lactobacillus amylovorus* DCE471 inhibited *H. pylori* ATCC 43504 in a killing assay. These data indicate that the antibacterial substance(s) other than lactic acid or acetic acid is (are)

responsible for the inhibitory activity. Kim *et al*. (2003) suggested that lacticins A164 and BH5 produced by *Lactococcus lactis* subsp. *lactis* A164 and *L. lactis* BH5, respectively, showed the anti-*H. pylori* activity of lacticin A164 was dependent on initial inoculum size as well as concentration of the bacteriocin added.

Inhibition of the adhesion of *H. pylori* by LC, CFCS, and BS obtained from the LAB

The inhibition capacities of two *Lactobacillus* strains on adhesion of *H. pylori* to AGS cells were examined. The adhesion of the pathogen to AGS cells without interruption by lactobacilli was assigned as 100%. As shown in Fig. 1, *H. pylori* adhesion was reduced significantly by the LC of the two lactobacilli strains in all assays. The level of inhibition of adhesion performed in exclusion assay was stronger than the competition and displacement assays. In the exclusion assay, *L. acidophilus* BK13 was found to significantly reduce the adherence of *H. pylori* to 53.8%, and this strain also showed better inhibition activity in adherence of the pathogen than *L. paracasei* BK57 in competition and displacement assays. Meanwhile, the incubation of *H. pylori* with each of the active compounds (lactic acid and bacteriocin) produced by the tested strains inhibited its adhesion to AGS cells, but there were some differences between the compounds in their *H. pylori* inhibition kinetics. The CFCSs of these strains showed the concentration-dependent anti-*H. pylori* activity, however, the number of *H. pylori* adhering to the AGS cells of the CFCS (> 100 μl/ml) of BK13 strain was significantly lower than that of the CFCS prepared from the culture of BK57 strain. In addition, the adhesion of *H. pylori* decreased by about 50% from the control after exposure to 300 AU/ml of the BS produced by BK13 strain, whereas, there was no significant difference (P>0.05) between the control and the treatment with the BS (300 AU/ml) of BK57 strain. These results suggested that their antimicrobial substances in the cultures as well as viable cells of the selected *L. acidophilus* BK13 and *L. paracasei* BK57 strains play a key role in inhibiting the adhesion of *H. pylori* to AGS cells and could help to prevent infection in an early stage of colonization of the gastric mucosa by this pathogen.

Numerous studies have investigated the binding properties of *H. pylori*. *In vitro* experiments have provided evidence that the

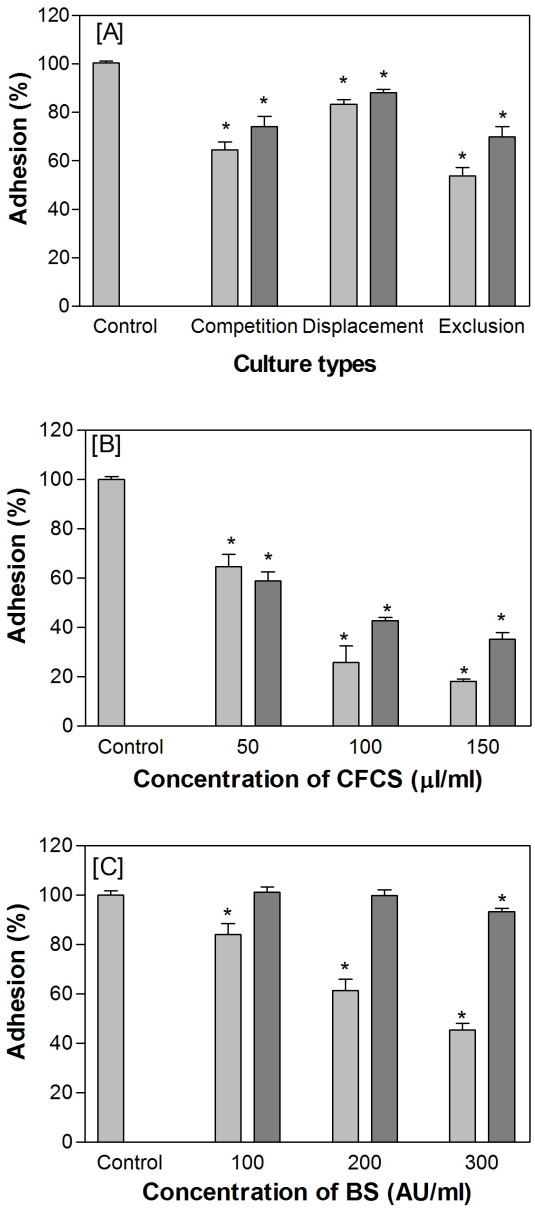

Fig. 1. Effects of the LC [A], CFCS [B], and BS [C] obtained from *L. acidophilus* (□) BK13 and *L. paracasei* (■) BK57 strains on the adhesion of *H. pylori* ATCC 43504 to AGS cells. Experimental conditions were described in the text. Each value shown was the mean±standard error of the three experiments. *Statistical analysis preformed with Student's *t*-test between control and treated *H. pylori* with the antimicrobial substance showed a highly significant difference (P<0.05).

adhesion of *H. pylori* to epithelial cell lines and to mucins may involve sialic acid and sulfated oligosaccharides (Sutton, 2001). This has been supported *in vivo* by Genta *et al.* (1996), who have found that the adhesion of *H. pylori* to areas of intestinal metaplasia is associated with the expression of sulfomucins on the gastric tissue. In the gastric mucosa, the adhesion of *H.*

pylori which interacts with epithelial cells via multiple bacterial surface components is important in determining the outcome in *H. pylori*-associated gastrointestinal diseases (Lesbros-Pantoflickova *et al*., 2007).

However, some probiotic bacteria with beneficial health effects are able to reduce the bacterial load and inflammation of *H. pylori* in animal and human studies. Two mechanisms of probiotic action have been identified to mediate the maintenance of the gastrointestinal microbial balance: production of antibacterial substances and competitive inhibition of pathogen and toxin adherence to the intestinal epithelium, and the suppression effect of probiotic strains is strain dependent (Servin and Coconnier, 2003). *In vitro* studies showed that certain lactobacilli including *L. johnsonii* La1 or *L. acidophilus* LB inhibit the attachment of *H. pylori* to intestinal HT-29 cells or to MKN-45 gastric cell lines by secreting the antimicrobial substances (Lesbros-Pantoflickova *et al*., 2007). In addition, the anti-adhesive effect might be the result of competition between probiotic strains such as *Lactobacillus reuteri* or *Weissella confuse* and pathogen for the same receptor or the induction of mucin production by probiotics. The ability to inhibit the adhesion of pathogens to immobilized human mucus appears to depend on both the specific probiotic strains and the pathogens (Oelschlaeger, 2010). Furthermore, a non-specific rather than a specific blockage of receptor sites is the most likely mechanism because lactobacilli can inhibit adhesion of the large varieties of pathogenic bacteria, although each adheres to its particular receptor on the cells (Pacifico *et al*., 2014).

Similar results were noted in other studies; certain lactobacilli such as *L. johnsonii* La1 or *L. acidophilus* LB can exert their anti-adhesion activity by secreting the antimicrobial substances (Lesbros-Pantoflickova *et al*., 2007). Lin *et al*. (2009) reported that the spent culture supernatants from LAB inhibit *H. pylori* infection and adhesion to AGS cells. In addition, treatment by the bacteriocin or the cells of *E. faecium* TM39 significantly reduced the binding of *H. pylori* to monolayers of AGS cell line (Tsai *et al*., 2004).

Inhibition of *H. pylori* urease activity of antimicrobial substances obtained from the LAB

The effect of the CFCS and BS of *L. acidophilus* BK13 and *L. paracasei* BK57 strains on the urease activity of *H. pylori* ATCC 43504 adhered against AGS cells was examined. As shown in Fig. 2, the urease activity of *H. pylori* was inhibited prominently by the CFCS from BK13 and BK57 strains. After treatment with the CFCS (150 μl/ml) of BK13 strain, the urease activity of the *H. pylori* cells decreased to 50% of the control level and no significant difference in the inhibitory activity of these CFCSs was observed between these strains. Furthermore, the urease activity in *H. pylori* culture was significantly inhibited by the presence of the BSs of the tested strains. The BS (300 AU/ml) of *L. acidophilus* BK13 exhibited high inhibitory effect on the urease activity of *H. pylori* (0.25±0.02), while the BS (300 AU/ml) of *L. paracasei* BK57 was ineffective to inhibit the urease activity of *H. pylori*. Our *in vitro* study found that the antimicrobial substances of the LAB inhibited the urease

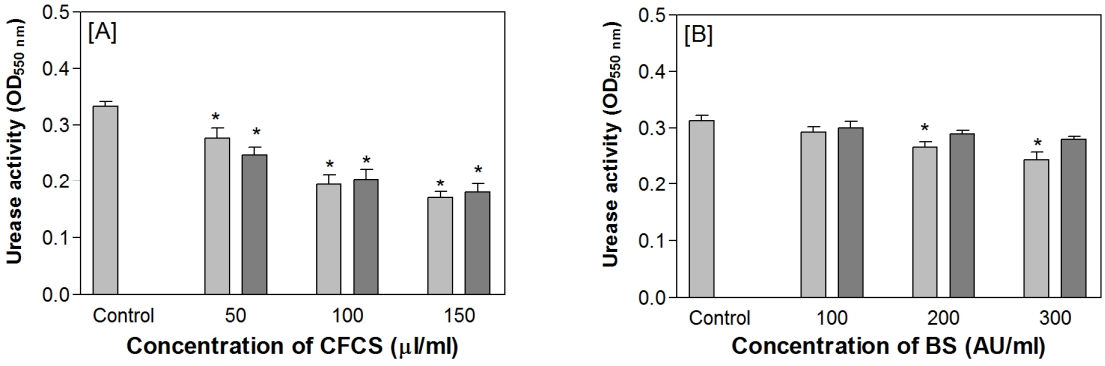

Fig. 2. Urease activity of *H. pylori* ATCC 43504 in the co-culture with the CFCS (A) and BS (B) obtained from *L. acidophilus* BK13 (□) and *L. paracasei* BK57 (■) strains. Experimental conditions were described in the text. Each value shown was the mean±standard error of the three experiments. *Statistical analysis preformed with Student's *t*-test between control and treated *H. pylori* with the antimicrobial substance showed a highly significant difference (P<0.05).

activity of *H. pylori* in a dose-dependent manner, therefore, the amount of antimicrobial substance released by the lactobacilli strains correlated with the intensity of the inhibitory effect against *H. pylori*. These findings suggested that the lactic acid in the CFCS prepared from the two selected lactobacilli strains possess effective activity to inhibit the urease of *H. pylori* adhered to AGS cells.

The spiral morphology and flagellar motility of *H. pylori* allow this bacterium to penetrate in the viscous mucus layer of the stomach. Meanwhile, *H. pylori* urease catalyzes the hydrolysis of urea into bicarbonate and ammonia to neutralize the acidic environment of the gastrointestinal tract (Luo *et al*., 1999). This enzyme plays a central role in *H. pylori* pathogenesis and is critical for bacterial colonization of the human gastric mucosa. The ammonia produced by urease can damage the gastric mucosa through the disruption of tight junctions and the alteration of permeability of gastric epithelium. Moreover, urease stimulates activation of mononuclear phagocytes and production of inflammatory cytokines (Cellini and Donelli, 2000).

Lin *et al*. (2009) found that there was an inverse relationship between the exclusion rate and urease activity; that is the point when the exclusion rate of LAB-SCS against *H. pylori* infection was highest (41.1%), the urease activity of *H. pylori* was lowest. Therefore, LAB-SCS is able to inhibit *H. pylori* infection in AGS cells. Examining whether lactic acid participates in inhibition of the *H. pylori* urease activity, it was found that a concentration of 200 mM D, L-lactic acid totally inhibited the *H. pylori* urease activity, whereas a range of concentrations from 60 to 100 mM, similar to that determined in the *L. acidophilus* LB-SCS (84 mM), failed to inhibit urease activity of *H. pylori*. These results demonstrate that lactic acid does not participate in the action of LB-SCS against the *H. pylori* urease (Coconnier *et al*., 1998). A similar inhibition pattern was found in the other studies. Yoon and Won (2002) suggested that both *Lactobacillus helveticus* CU631 and CFCS obtained from this strain had strong inhibitory activities in urease of *H. pylori* NCTC 11637 and CJH12. Tsai *et al*. (2004) demonstrated that after 2 h contact of the *H. pylori* cells with the bacteriocin from *E. faecium* TM39, the urease activity of *H. pylori* adhered to AGS cells decreased to one tenth of its original level. Thus, the inhibitory activity of the antibacterial compound(s) secreted from strain TM39 could result in the inhibition on *H. pylori* urease activity.

Cytotoxicity of antimicrobial substances obtained from the LAB

The cytotoxic activity of the antimicrobial substances from the LAB strains against human gastric cancer cells was measured using the MTT method. Inhibition of AGS and SNU-1 cells proliferation by the CFCSs from *L. acidophilus* BK13 *and L. paracasei* BK57 is shown in Fig. 3. The CFCSs of the tested *Lactobacillus* strains potently inhibited the viability of each cell line, in a dose-dependent manner. According to these results, the strongest effect of BK13 strain was found at a concentration of 150 μl/ml of the CFCS at 24 h of incubation (70.0±4.8% inhibition). Whereas, the cytotoxicity inhibition rate of BK57-CFCS was 56.6±5.3% for a concentration of 150 μl/ml at 24 h of incubation. In addition, the viability of SNU-1

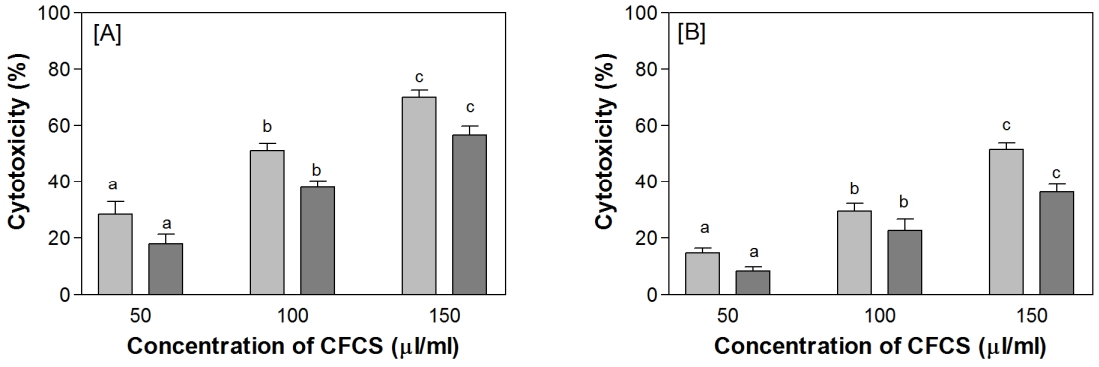

Fig. 3. Proliferation of AGS [A] and SNU-1 [B] cells in the presence of the CFCS obtained from *L. acidophilus* BK13 (■) and *L. paracasei* BK57 (■) strains. Experimental conditions were described in the text. Each value shown was the mean±standard error of the three experiments. Means with different superscript letters indicate statistically significant differences as determined by ANOVA ($P < 0.05$).

carcinoma cell was inhibited by the CFCSs from *L. acidophilus* BK13 and *L. paracasei* BK57 strains after 24 h incubation. However, the anti-proliferative effect of these strains on SNU-1 carcinoma cell was lower than that on AGS cell. In contrast, the BSs from the two lactobacilli strains did not have any effect on the cell viability of the tested cancer cells (data not shown). Our findings demonstrated that the anti-cancer effect of the lactic acid from *L. acidophilus* BK13 and *L. paracasei* BK57 strains on AGS cells had significant differences in the concentration of the lactic acid (P<0.05).

Lactobacilli and bifidobacteria which are the most prominent probiotic bacteria have been reported to possess certain anti-cancer properties. The precise mechanisms by which LAB may inhibit cancer are presently unknown. However, the anti-carcinogenic effect of probiotics may be attributable to alteration of the metabolic activities of intestinal microflora, binding and degrading potential carcinogens, quantitative and/or qualitative alterations in the intestinal microflora incriminated in producing putative carcinogen(s) and promoters, production of anti-carcinogenic compounds, enhancing the host's immune response, and effects on physiology of the host (Rafter, 2002).

Sevda *et al*. (2015) demonstrated that the CFCS and the cell-free lyophilized filtrate of *Pediococcus pentosaceus*, *L. plantarum*, and *W. confuse* were found to inhibit the growth of colon cancer cells in a dose-dependent manner as detected by the MTT assay. Anti-cancer activities were found in peptidoglycans isolated from *L. casei* (Fichera and Giese, 1994). Furthermore, it has been reported that the polysaccharide fractions originating from *Lactobacillus* cultures and glycoproteins found in the supernatants of *Lactobacillus* cultures have the same effect (Manjunath and Ranganathan, 1989). Sadeghi-Aliabadi *et al*. (2014) reported that *L. plantarum* A7 (2.5, 5, and 10 mg/ml) and *L. rhamnosus* GG (5 and 10 mg/ml) CFCSs that displayed significant (P<0.05) inhibitory effects on Caco-2 cells compared with the control groups could be considered as colon cancer biological product, most likely due to its advantages in significant organic acid production.

Our results disagree with a previous study by Joo *et al*. (2012); they reported that nisin, a bacteriocin and commonly used food preservative, may serve as a novel potential therapeutic for treating head and neck squamous cell carcinoma (HNSCC), as it induces preferential apoptosis, cell cycle arrest, and reduces cell proliferation in HNSCC cells, compared with primary keratinocytes. Fermenticin HV6b, a class Ⅱ antimicrobial peptide produced by *Lactobacillus fermentum* HV6b MTCC 10770 isolated from human vaginal ecosystem induced apoptosis in cancerous cells (Kaur *et al*., 2013). Shaikh *et al*. (2012) revealed that *L. salivarius* bacteriocins possessed functional properties very similar to that of Azurin, a bacteriocin with proven cytostatic and apoptotic effect against human cancer cell, therefore, these bacteriocins may be an effective therapeutic candidate to control the cancer cells.

In conclusion, the antimicrobial substances obtained from *L. acidophilus* BK13 and *L. paracasei* BK57 could inhibit the viability, adhesive ability against human gastric epithelial cells, and urease activity of *H. pylori* ATCC43504. Therefore, these antagonistic substances-producing strains are potentially useful as new potential antimicrobial agents for the management and prevention of *H. pylori* infections. In future, we will undertake further microbiological and clinical trials to evaluate whether these two strains can be used as an alternative or complementary therapy for the treatment of *H. pylori* infection without causing side effects.

적 요

Lactobacillus acidophilus BK13과 *Lactobacillus paracasei* BK57 균주로부터 얻은 세포, 배양상등액 및 박테리오신 용액의 anti-*Helicobacter pylori* 활성과 위장상피세포에 대한 세포독성을 평가하였다. 실험균주를 MRS 배지 상에서 30시간 배양한 결과, *L. acidophilus* BK57 (126.8±7.9 mM) 보다 *L. paracasei* BK57 (155.9±7.9 mM)가 더 많은 양의 유산을 생산하였다. 또한, BK13 균주의 최대 박테리오신 활성(128 AU/ml)은 37°C에서 30시간 배양 후 관찰되었으나, 이는 BK57의 활성(256 AU/ml) 보다는 낮았다. BK13 및 BK57 균주의 살아있는 세포를 *H. pylori*와 혼합 배양한 결과, 유산균의 초기균수에 의존하여 *H. pylori*의 저해효과가 나타났다. 게다가 BK13과 BK57로부터 얻은 배양상등액과 박테리오신은 *H. pylori*의 성장을 억제할 뿐만 아니라 위장상피세포에 대한 부착력과 urease 활성도 저해하였다. 한편, 이러한 균주들이 생산한 유산은 위암세포에 대한 세포독성 효과가 대조구보다 유의한 수준으로 높게 나타났다. 따라서 BK13과 BK57 균주의 항균물

질은 위장질환의 원인균인 *H. pylori*를 저해시키는데 효과적이므로 이들 유산균은 *H. pylori* 감염으로부터 위장을 보호하는데 유용할 것으로 사료된다.

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