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ARTICLE

Selection and Characterization of Bacteriocin-Producing *Lactobacillus* sp. AP 116 from the Intestine of Pig for Potential Probiotics

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Abstract

The purpose of this study was to isolate bacteriocin-producing bacteria with antagonistic activities against pathogens from the intestines of pigs for probiotic use. *Lactobacillus* sp. AP 116 possessing antimicrobial property was selected from a total of 500 isolates. The AP 116 strain showed a relatively broad spectrum of inhibitory activity against *Listeria monocytogenes*, *Clostridium perfringens*, *Pediococcus dextrinicus*, and *Enterococcus* strains using the spot-on-lawn method. Bacteriocin activity remained unchanged after 15 min of heat treatment at 121°C and exposure to organic solvents; however, it diminished after treatment with proteolytic enzymes. Maximum production of bacteriocin occurred at 34°C when a pH of 6.0 was maintained throughout the culture during fermentation. According to a tricine SDS-PAGE analysis, the molecular weight of the bacteriocin was approximately 5 kDa. The isolate tolerated bile salts and low pH, and also induced nitric oxide (NO) in mouse peritoneal macrophages. Bacteriocin and bacteriocin-producing bacteria, such as *Lactobacillus* sp. AP 116, could be potential candidates for use as probiotics as an alternative to antibiotics in the pig industry.

Key words: antimicrobial activity, bacteriocin, probiotics, alternatives, *Lactobacillus*

Introduction

Antibiotics have been widely used at subtherapeutic levels as an animal growth promoter and against pathogenic bacteria in gastrointestinal systems for more than 50 years (Dibner and Richards, 2005). Subtherapeutic antibiotics have succeeded in improving growth and feed conversion in poultry and swine production. However, resistant bacterial populations, residual antibiotics in animal meat, and the increasing consumer demand for organic production have increased interest in searching for alternatives to antibiotics in recent years.

Among these alternatives, probiotics have received much attention as the most promising substitute to in-feed antibiotics and for improving animal productivity (Byun *et al.*, 2000; Joerger, 2003; Roselli *et al.*, 2005). Probiotic bacteria (primarily lactic acid bacteria, LAB) used as feed additives should originate preferably from the target animal microflora (Kosin and Rakshit, 2006). Probiotics are mostly anticipated to function as a growth inhibitor of pathogenic bacteria in animal intestines due to their abilities to modulate the host's immune system and/or directly affect infectious microorganisms by producing antimicrobial agents including organic acids, hydrogen peroxide, and bacteriocins (Oelschlaeger, 2010). Macrophages play the central role in initiating the first defense line of host immunity. Activated macrophages may regulate immunity by enhancing secretion of proinflammatory cytokines (interleukin-6, tumor necrosis factor-α) and nitric oxide (NO). NO is a short-lived mediator that has antimicrobial actions against various phathogens via its cytotoxic or cytostatic effects (Snyder and Bredt, 1992).

Many Gram-positive and Gram-negative bacteria produce bacteriocins, which are ribosomally-synthesized peptides or proteins with antimicrobial properties that often target bacterial species that are closely related to the producer strain. Bacteriocins produced by LAB have received particular attention in recent years due to their potential application in the food industry as natural preservatives against food-borne pathogens (Cleveland *et al.*, 2001). Food-borne pathogens, such as *Listeria monocytogenes*, *Clostridium perfringens*, and *Salmonella* sp., are com-

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monly found in animal intestines (Jung *et al.*, 2003; Kim *et al.*, 2006). They are frequently associated with swine diseases and may contaminate meat during pork processing (Thévenot *et al.*, 2006; Warriner *et al.*, 2002). Therefore, to prevent gastrointestinal colonization of livestock by foodborne pathogens, the use of bacteriocin-producing bacteria in animal feed is recommended (Callaway *et al.*, 2004; Gillor *et al.*, 2004). Bacteriocin production by intestinal LAB may play an important role in their survival, thereby enabling them to compete in an environment with an abundance and diversity of microorganisms (Damelin *et al.*, 1995; Du Toit *et al.*, 2000). Despite their potential as an alternative to antibiotics, few studies have investigated the use of bacteriocin-producing intestinal LAB in the animal industry (Diez-Gonzalez, 2007; Strompfová *et al.*, 2006).

The purpose of this study was to isolate bacteriocin-producing bacteria with antagonistic activities against pathogens from the intestines of pig and to develop a potential candidate for probiotic use in the pig industry as an alternative to antibiotics. We describe the selection process and partial characteristics of *Lactobacillus* sp. AP 116 and the bacteriocin that shows inhibitory activity against several Gram-positive bacteria.

Materials and Methods

Bacterial strains and culture conditions

Lactobacillus sp. AP 116 was isolated from the intestines of pig and maintained at -70°C in lactobacilli MRS broth (Difco Laboratories, Detroit, USA) containing 50% (v/v) glycerol. Indicator organisms were obtained from the Korean Collection for Type Culture (KCTC) or Korean Culture Center of Microorganisms (KCCM), and propagated in appropriate media as indicated in Table 1.

Isolation of LAB

Intestine samples (obtained from a slaughterhouse) were homogenized and serially diluted ten-fold with saline solution, plated on MRS, and incubated at 37°C for 2 to 3 d. The antimicrobial substances producing bacteria were screened by a modification of the deferred method. For the modified deferred method, approximately five colonies per sample were randomly selected with sterilized toothpicks and inoculated into 1 mL of MRS broth in a microcentrifuge tube. After isolates were grown for 2 d at 37°C, 10 μL of culture broth were spotted on MRS agar and dried for 1 h. The plate was overlaid with 0.7% MRS or BHI agar (Difco Laboratories, USA) seeded with an overnight culture of the following indicator strains (approximately 5.0×10^6 CFU/mL each): *Escherichia coli* KCTC 1467, *Salmonella* Typhimurium KCTC 2515, *Staphylococcus aureus* KCTC 1621, *Listeria monocytogenes* KCTC 3569, and *Lactobacillus sake* KCCM 40264. After incubation for 24 h at the appropriate temperature, colonies with clear inhibition zones were further examined for bacteriocin production.

Detection of bacteriocin-producing LAB by the spot-on-lawn method

Cells were pelleted by centrifugation (10,000 g for 15 min). The supernatant was adjusted to pH 6.5 with 10 N NaOH and filtered through 0.2-μm pore size membrane filters and used to detect antagonistic activity against indicator organisms according to the spot-on-lawn method (Mayr-Harting *et al.*, 1972). The supernatant was serially diluted, and 10-μL samples were spotted onto 0.7% MRS or BHI agar seeded with an overnight culture of an indicator strain. After incubation for 24 h at the appropriate temperature, plates were checked for inhibition zones. Bacteriocin activity was expressed in terms of arbitrary units per mL (AU/mL), which was defined as the highest dilution showing a definite inhibition of the indicator lawn.

Identification of bacterial strain

Morphological and biochemical properties of the samples were examined to identify bacteriocin-producing microorganisms (Holt *et al.*, 1994). We assessed Gram staining, morphology, catalase activity, salt tolerance, gas production, growth temperature range, and biochemical carbohydrate fermentation patterns using an API 50 CHL kit (Biomérieux, France). The 16S rDNA gene was sequenced using the Big Dye terminator cycle sequencing kit (Applied BioSystems, USA) and an automated DNA sequencing system (Applied Biosystems model 3730XL, USA). This sequence was aligned with the 16S rRNA gene sequence of LAB and other related taxa to compare levels of similarity.

Bacteriocin extraction by pH-mediated adsorption and desorption

Bacteriocin extraction from *Lactobacillus* sp. AP 116 was performed using a modified method of Yang *et al.* (1992). After AP 116 was grown at 37°C in 1 L of MRS broth for 16 h, the culture was heated to 80°C for 30 min to kill the cells and inactivate proteolytic enzymes. Subsequently, the pH of the culture was adjusted to 6.5 with

5 N NaOH and was stirred for 30 min at room temperature to allow cellular adsorption of the bacteriocin. Bacterial cells were collected by centrifugation at 4,000 g for 30 min and washed twice with 5 mM sodium phosphate buffer (pH 6.5). Cells were resuspended in 50 ml of 100 mM NaCl, adjusted to pH 2.0 with 5% phosphoric acid, and then gently stirred at 4°C for 2 h to desorb the bacteriocin from the cells. Cell suspensions were then centrifuged at 4,000 g for 30 min. Supernatants were dialyzed using a membrane with a 1 kDa cutoff (Spectrum Medical Inc., Los Angeles, CA, USA) for 24 h at 4°C and then freeze-dried. Freeze-dried material was resuspended in distilled water and used as crude bacteriocin in the following experiments.

Effects of heat, pH, enzymes, and organic solvents on bacteriocin activity

Crude bacteriocin was heated for 30 min at 60°C or 90°C, or at 121°C for 15 min. Residual bacteriocin activity was subsequently determined by the spot-on-lawn assay. To study the effects of pH on antimicrobial stability, the crude bacteriocin was adjusted to a pH of 2-10 with either 1 N HCl or 1 N NaOH and incubated at 30°C for 1 h. The bacteriocin was also treated with various enzymes in a final concentration of 1 mg/mL. All enzymes (Proteinase K, protease type XIV, pepsin, trypsin, α-amylase, β-amylase, and catalase) were dissolved in buffers recommended by the manufacturer (Sigma Chemical Co., USA). The mixture was then incubated at 30°C for 1 h and heated at 80°C for 10 min to inactivate the enzymes. The bacteriocin was also treated with 50% organic solvents such as ethanol, methanol, chloroform, acetone, acetonitrile, hexane, and cyclohexane. Solvent-treated samples were incubated at 37°C for 1 h.

Determination of the molecular weight of the antimicrobial compound

Electrophoresis of the crude bacteriocin was performed on a 16.5% tricine-SDS polyacrylamide gel. One-half of the gel was stained with 0.1% Coomassie brilliant blue G250 (Bio-Rad, USA) and destained using a methanol–acetic acid–water (3:1:6) solution. The other half of the gel was washed three times in sterile water for 30 min and overlaid with BHI soft agar (0.7%) inoculated with fresh cultures of *L. monocytogenes*. The molecular weight of the bacteriocin was estimated by comparing molecular mass markers.

Activity against *L. monocytogenes*

Cells of *L. monocytogenes* at log-phase growth were centrifuged, washed, and resuspended in 10 ml of 50 mM phosphate buffer (pH 6.8) to a final concentration of 2.0 × 10^8 CFU/mL. Crude bacteriocin was added at concentrations of 200, 400, and 800 AU/mL. Viable cells were counted from aliquots taken at predetermined time intervals.

Culture conditions for bacteriocin production

The effects of temperature and pH on bacteriocin production were studied. AP 116 was incubated in MRS broth in a 5-L jar fermenter (FERMENTEC Co., Korea). Temperature and pH were maintained at 30°C, 34°C, and 37°C, and at 5.0, 6.0, and 7.0 with 10 N NaOH, respectively. The culture was agitated at 50 rpm. Cell counts and bacteriocin activity were measured at 2 h intervals. Viable cell counts were determined by the pour plate method on MRS agar, and bacteriocin activity was tested by the spot-on-lawn assay.

Nitric oxide assay

Mouse peritoneal macrophages were isolated from C57BL/6 mouse according to the method of Zhang *et al.* (2008). Peritoneal macrophages (5×10^5 cells/well) were triplicately cultured in a RPMI 1640 medium (Sigma, USA) supplemented with 10% (v/v) FBS (Invitrogen, USA) along with, 100 mg/mL streptomycin and 100 U/mL penicillin (Invitrogen, USA) and heat-killed probiotics (100 μL containing 5×10^7 CFU/mL of AP 116 or *Lactobacillus rhamnosus* GG) were added to the wells. PBS was only added to wells as a negative control, and LPS (100 ng/mL; Sigma, USA) was added in PBS-containing wells as a positive control. After 24 h, the culture supernatants were collected and the concentrations of NO in the supernatants were determined. NO levels of macrophages were measured using Griess reagent (Promega, USA). Briefly, 50 mL of macrophage culture supernatant were mixed (in triplicate) with an equal volume of 1% sulfanilamide in 5% phosphoric acid and 0.1% N-1-Naphylethylenediamine dihydrochloride at room temperature for 10 min in the dark. Absorbance was then measured at 540 nm by using a microplate reader (Molecular Devices, USA). The production levels of NO were calculated from a standard curve for a nitrite standard (0-100 μM sodium nitrite).

Survival and growth at low pH and in the presence of bile salts

Acid and bile salt tolerance were performed as describ-

ed by Shin *et al.* (2008). To test acid tolerance, overnight cultures in MRS medium of selected strain were harvested at 4000 g for 10 min at 4°C and washed twice with 50 mM phosphate buffer and resuspended in 20 ml of the same buffer. The final pH was adjusted to 2.0, 2.5, 3.0, and 7.0. The suspensions were incubated at 37°C for 2 h, and the viable cell counts were determined by the pour plate method on MRS agar. Bile tolerance was determined by spreading the cells on MRS agar plates containing oxgall bile (0%, 0.05%, 0.1%, 0.3%, and 0.5%, respectively). Plates were incubated at 37°C for 48 h, and the viable cell counts were determined.

Statistical analysis

The data were statistically analyzed using the software package SPSS 13.0 Window Program (SPSS Inc., USA). A one-way analysis of variance (ANOVA) with Duncan's multiple range test was used to distinguish treatment mean differences. Values of $p<0.05$ were considered significant.

Results and Discussion

Screening and identification of bacteriocin-producing LAB

More than 60% of the approximately 500 strains isolated from the intestines of pig showed inhibitory activity to at least one of the five indicators tested in the first screening step. The isolates that showed a larger inhibition zone (greater than 15 mm) were further tested for their antimicrobial activities. Cell-free supernatants were neutralized with NaOH to eliminate the effect of organic acids; subsequently, the inhibition test against indicator organisms was performed by the spot-on-lawn method. Five strains (AP 116, HN 52, HN 92, HN 98, and HN 235) were finally selected as antimicrobial agent-producing bacteria. AP 116 showed the largest zone of inhibition against *L. monocytogenes* and was therefore selected for further characterization. This strain was characterized as a Gram-positive, catalase-negative, and facultative anaerobic rod-shaped bacterium (Fig. 1). Based on these characteristics and on the API test results (carbohydrate fermentation test), the isolate was classified as *Lactobacillus acidophilus* AP 116 (data not shown). The 16S rDNA sequence of AP 116 showed 99% similarity with *Lactobacillus* sp. 52A and low similarity (96%) with *L. agilis*; thus, further work is required to determine species identification.

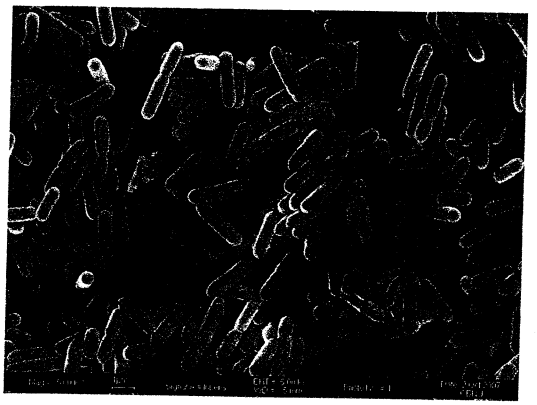

Fig. 1. Scanning electron micrograph of *Lactobacillus* sp. AP 116.

Spectrum of antimicrobial activity

Both culture and crude bacteriocin extracted by pH-mediated adsorption method were tested for their antimicrobial activities against various Gram-positive and Gram-negative bacteria using the modified deferred and spot-on-lawn methods (Table 1). AP 116 showed a broad spectrum of inhibitory activity against most of the pathogenic and non-pathogenic bacteria tested by the modified deferred method. The inhibitory activity of AP 116 against Gram-negative strains in the modified deferred analysis could be attributed primarily by the accumulation of organic acids. It also demonstrated a relatively broad spectrum of activity against all *Enterococcus* strains, *Listeria*, *Pediococcus dextrinicus*, and *Cl. perfringens* tested by the spot-on-lawn method. The bacteriocin was inactive against Gram-negative bacteria, *E. coli*, and *Salmonella* Typhimurium. However, the bacteriocin had a high anti-listeria activity compared to other sensitive strains and a low inhibitory activity against *Cl. perfringens*. The spectrum of anti-listerial activity of AP 116 bacteriocin was similar to that reported for other Class IIa bacteriocins, especially pediocin PA-1 (Gonzalez and Kunka, 1987). Growth of bacteriocin-producing bacteria may have a direct affect on reducing existing populations of food-borne pathogens. Long-term colonization with bacteriocin-producing bacteria may also prevent further re-introduction of pathogenic bacteria (Diez-Gonzalez, 2007). Therefore, AP 116, which showed antagonistic activity against pathogens, could be used in controlling enteric pathogenic bacteria in pig gastrointestinal systems and further reduce contamination in swine processing.

Effects of enzymes, heat, pH, and organic solvents on bacteriocin activity

Antimicrobial activity was completely inactivated by

Table 1. Antimicrobial spectrum of bacteriocin produced by *Lactobacillus* sp. AP 116

Indicator strains	Medium	Modified deferred method	Spot-on-lawn method
Bacillus cereus KCTC 1012	BHI	+	–
Clostridium perfringens KCTC 3269	BHI	+	200 AU/mL
Escherichia coli KCTC 1467	BHI	+	–
Escherichia coli KCTC 11682	BHI	+	–
Enterobacter aerogenes KCTC 2190	BHI	+	–
Enterococcus durans KCTC 3121	MRS	+	51,200 AU/mL
Enterococcus faecalis KCTC 2011	MRS	+	25,600 AU/mL
Enterococcus faecalis KCTC 3206	MRS	+	25,600 AU/mL
Enterococcus faecium KCTC 3513	MRS	+	51,200 AU/mL
Klebsiella pneumoniae KCTC 2208	BHI	+	–
Lactobacillus acidophilus KCTC 3111	MRS	+	–
Lactobacillus casei KCTC 3109	MRS	–	–
Lactobacillus delbrueckii KCTC 1047	MRS	–	–
Lactobacillus fermentum KCTC 3112	MRS	–	–
Lactobacillus plantarum KCTC 3108	MRS	–	–
Leuconostoc mesenteroides KCTC 3505	MRS	–	–
Listeria monocytogenes KCTC 3569	BHI	+	204,800 AU/mL
Listeria monocytogenes KCTC 3710	BHI	+	204,800 AU/mL
Listeria innocua KCTC 3586	BHI	+	204,800 AU/mL
Pediococcus acidilactici KCTC 1626	MRS	+	–
Pediococcus dextrinicus KCTC 3506	MRS	+	51,200 AU/mL
Proteus mirabillis KCTC 2566	BHI	+	–
Pseudomonas aeruginosa KCTC 1750	BHI	+	–
Salmonella Typhimurium KCTC 2515	BHI	+	–
Staphylococcus aureus KCTC 1621	BHI	+	–
Staphylococcus aureus KCTC 1916	BHI	+	–
Staphylococcus epidermidis KCTC 1917	BHI	+	–

–, no inhibition zone; +, clear inhibition zone

treatment with Proteinase K, protease type XIV, and trypsin. However, this did not occur with pepsin, α-amylase, β-amylase, and catalase (Table 2). The AP 116 bacteriocin was highly thermostable, maintaining antimicrobial activity even after treatment at 121°C for 15 min. Bacteriocin activity was also stable from pH 2 to 10 for 1 h. It was mostly unaffected by exposure to the organic solvents at 50% concentrations. Antimicrobial activity was sensitive to three proteolytic enzymes tested, indicating that the inhibitory agent produced by *Lactobacillus* sp. AP 116 was proteinaceous in structure. There was no loss of antimicrobial activity following treatment with amylase, organic solvents, and catalase due to the possible absence of carbohydrate moieties and hydrogen peroxide in its composition. Similar results have been found in previous studies (Bhunia *et al.*, 1991; Gonzalez and Kunka, 1987; Shin *et al.*, 2008). These results suggest that the inhibitory compound is a bacteriocin.

Bacteriocin extraction and determination of molecular weight

A brown, sticky contaminant was detected in pellets after treatment of ammonium sulfate saturation. Due to the presence of this substance, the next step in the purification of bacteriocin was difficult (data not shown). However, a clean and white freeze-dried powder (crude bacteriocin) was obtained by the pH-mediated adsorption and desorption method. To estimate the molecular weight, the crude bacteriocin was subjected to tricine SDS-PAGE. A single band appeared at approximately 5 kDa. When the duplicate gel was tested for antimicrobial activity against *L. monocytogenes* as an indicator, an inhibition zone was detected (Fig. 2). Further studies are necessary to purify and characterize the bacteriocin.

Activity against *L. monocytogenes*

To determine whether the bacteriocin produced by *Lactobacillus* sp. AP 116 was bactericidal or bacteristatic against sensitive strains, crude bacteriocin was added at different concentrations to *L. monocytogenes* suspended in 50 mM phosphate buffer. The number of viable bacterial cells decreased rapidly after the first 30 min and then

Table 2. Effects of enzymes, heat, pH, and organic solvents on the activity of bacteriocin partially purified from *Lactobacillus* sp. AP 116

Treatment		Bacteriocin activity (AU/mL)
Control		3,200
Enzymes	Proteinase K	0
	Protease XIV	0
	Pepsin	3,200
	Trypsin	0
	α-Amylase	3,200
	β-Amylase	3,200
	Catalase	3,200
Heating	60°C, 30 min	3,200
	95°C, 30 min	3,200
	121°C, 15 min	3,200
pH	pH 2.0	3,200
	pH 3.0	3,200
	pH 4.0	3,200
	pH 5.0	3,200
	pH 6.0	3,200
	pH 7.0	3,200
	pH 8.0	3,200
	pH 9.0	3,200
	PH 10.0	3,200
Organic solvents	Ethanol	3,200
	Methanol	3,200
	Chloroform	3,200
	Acetone	1,600
	Acetonitrile	1,600
	Hexane	3,200
	Cyclohexane	3,200

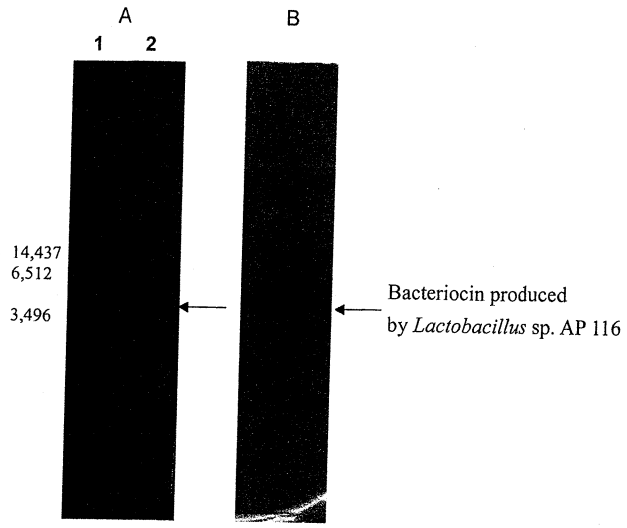

Fig. 2. SDS-PAGE and detection of antimicrobial activity of the crude bacteriocin from AP 116. (A) Gel stained with Coomassie brilliant blue G250: (lane 1) molecular weight standards, (lane 2) crude bacteriocin, and (B) gel overlaid with BHI soft agar inoculated with *L. monocytogenes*.

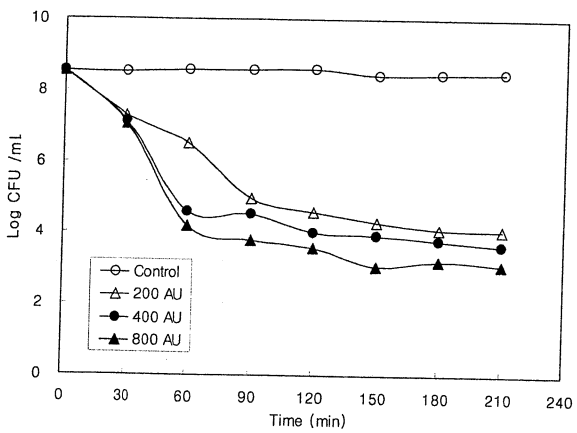

Fig. 3. Inhibitory action of AP 116 bacteriocin against *L. monocytogenes* incubated at 37°C in 10 mM phosphate buffer.

slowly for 3 h (Fig. 3). During this period, the reduction in viable cell number was approximately 4.6 Log scale (4.5×10^3 CFU/mL) at 800 AU/mL, 4.3 Log scale (8.2×10^3 CFU/mL) at 400 AU/mL, and 2.8 Log scale (2.5×10^5 CFU/mL) at 200 AU/mL. The rapid decrease in the number of viable cells treated with bacteriocin suggests a mechanism based on bactericidal activity. The effectiveness of inhibition was in proportion to bacteriocin concentration. A similar mode of action has been observed in many other bacteriocins from LAB (Bhunia *et al.*, 1991; Heo *et al.*, 2007).

Influence of pH and temperatures on cell growth and bacteriocin production

When the pH of the culture was not controlled, bacteriocin production began toward the middle of the exponential growth phase, reached maximum levels (2,400 AU/mL) during the stationary phase, and then rapidly declined (Fig. 4). The pH of the medium dropped from 6.5 to 4.1 during the 38-h incubation period. This pattern has been observed for other LAB bacteriocins (Aasen *et al.*, 2000; Daba *et al.*, 1991; Parente *et al.*, 1994). Bacteriocins are often produced during growth phase and then decrease due to proteolytic degradation, protein aggregation, and adsorption (Parente and Ricciardi, 1994; Parente *et al.*, 1994). Bacteriocin activities and cell counts were also determined at a constant pH of 5.0, 6.0, and 7.0 at 37°C. Maximum bacteriocin production was at pH 6.0 or less during fermentation, whereas bacteriocin activity was detected very low at pH 7.0 at 37°C. Optimal temperature for bacteriocin production and cell growth was at 34°C (6,400 AU/mL and 3.8×10^9 CFU/mL, respectively).

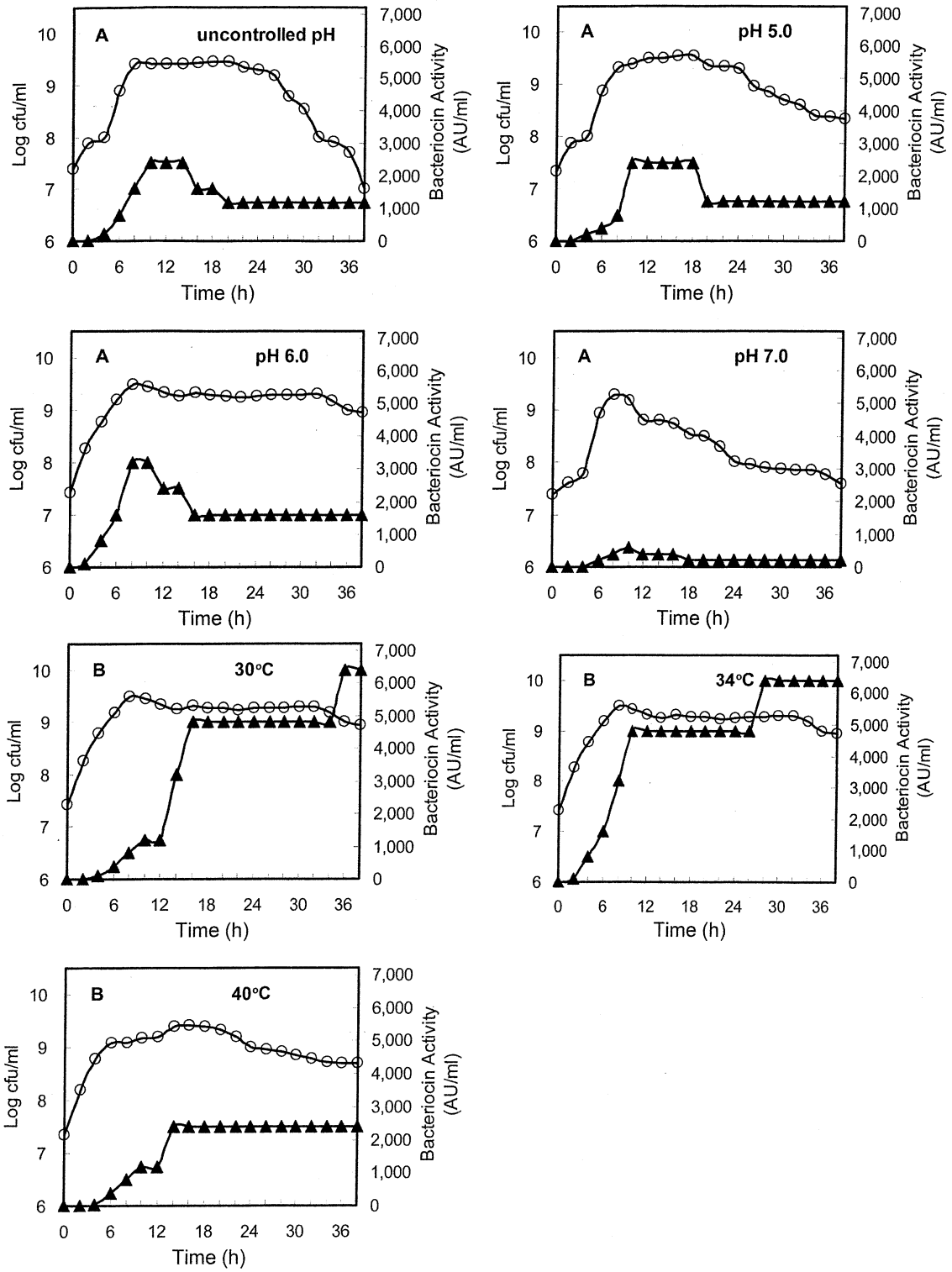

Fig. 4. Cell growth and bacteriocin production of *Lactobacillus* sp. AP 116 in MRS broth for (A) different pH at 37°C and (B) different temperatures at pH 6.0. (○) viable cell count; (▲) bacteriocin activity.

Evaluation of NO production and viability

The immunostimulatory effect of the AP 116 strain was tested via NO production by *in vitro* culture experiments using murine peritoneal macrophages. The No production induced by both AP 116 and LGG were 1.78±0.06 μM/mL and 1.67±0.18 μM/mL, respectively, which were significantly different from that in the PBS control (Fig. 5). LGG has been known to have immunomodulatory effects by inducing immune cells to produce NO and inflammatory cytokines such as IL-12, IL-17, and TNF-α (Mileti *et*

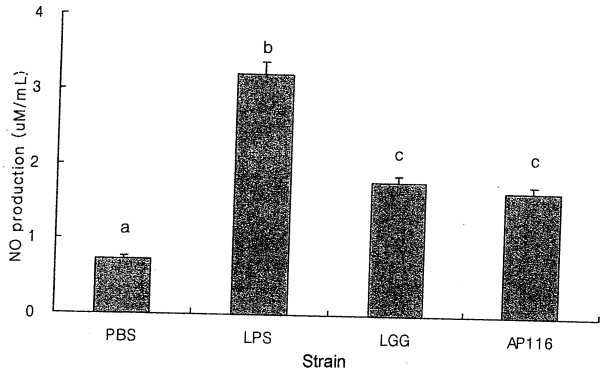

Fig. 5. Nitric oxide (NO) production by murine peritoneal macrophages induced by heat-killed *L. rhamnosus* GG (LGG) and *Lactobacillus* sp. AP 116. Murine peritoneal macrophages (5×10^5 cells/mL) were stimulated with probiotics or PBS for 24 h. Data are mean±SD values of triplicates. Different superscript letters ($^{a, b, c}$) indicate statistical differences as determined by ANOVA ($p < 0.05$).

al. 2009). The heat-killed AP 116 stimulated mouse peritoneal macrophages to increase the production of NO in a similar pattern to that of LGG. Resistance to low pH and bile salts are prerequisites for probiotics to survive and grow in the intestinal tract, as the beneficial effects of them can be expected when viable cells of these organisms are able to survive through the stomach and digestive system (Shin *et al.*, 2008). The AP 116 strain was tolerant to pH 3.0 and the residual counts were greater than 10^6 CFU/mL after 2 h incubation, while showed reduced viability after being exposed to pH 2.0 (Fig. 6A). This result is similar to previous studies, where *Lactobacillus* strains were viable even after being exposed to pH values of 2.5-4.0, but showed reduced viability at lower pH values (Mishra and Prasad, 2005). The selected strain was resistant to 0.5% bile salts (Fig. 6B). Bie salts and pH 3.0 had no effect on the AP 116.

The ability of probiotic bacteria to directly inhibit growth and proliferation of pathogenic microorganisms potentially confers the producer with a competitive advantage over other intestinal microbiota (Dahiya *et al.*, 2006; Doron and Gorbach, 2006). This antimicrobial activity includes the production of antimicrobial factors such as bacteriocins, short chain fatty acid, nitric oxide and hydrogen peroxide. The bacteriocin produced by *Lactobacillus* sp. AP 116 isolated from pig intestine showed a wide spectrum of inhibitory activity against Gram-positive food spoilage bacteria and pathogens. Bacteriocin and bacteriocin-producing *Lactobacillus* sp. AP 116 could potentially be used in both food and feed industries as natural biopreservatives and for probiotic use in livestock.

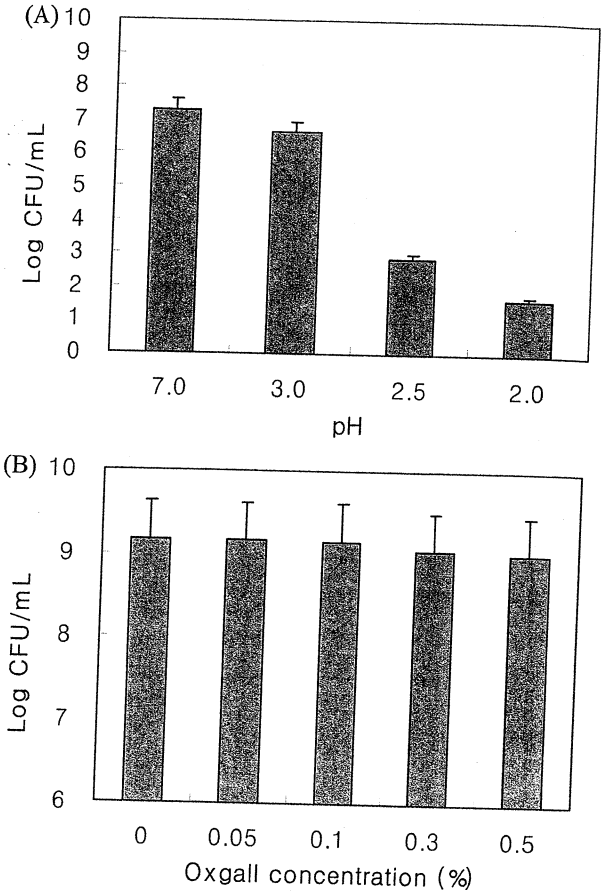

Fig. 6. Acid tolerance (A) and bile-salt resistance (B) of the AP 116 strain in sodium phosphate buffer at various pHs for 2 h and in MRS agar containing oxgall for 48 h, respectively.

Acknowledgments

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References

1. Aasen, I. M., Moretro, T., Katla, T., Axelsson, L., and Storro, I. (2000) Influence of complex nutrients, temperature and pH on bacteriocin production by *Lactobacillus sakei* CCUG 42678. *Appl. Microbiol. Biotechnol.* **53**, 159-166.
2. Bhunia, A. K., Johnson, M. C., Ray, B., and Kalchayamand, N. (1991) Mode of action of pediocin AcH from *Pediococcus acidilactici* H on sensitive bacterial strains. *J. Appl. Bacteriol.* **70**, 25-33.
3. Byun, J. W., Kim, G. T., Bae, H. S., Baek, Y. J., and Lee, W. K. (2000) *In vitro* selection of lactic acid bacteria for probiotic use in pigs. *Korea J. Vet. Res.* **40**, 701-706.
4. Callaway, T. R., Anderson, R. C., Edrington, T. S., Genovese, K. J., Harvey, R. B., Poole, T. L., and Nisbet, D. J.

(2004) Recent pre-harvest supplementation strategies to reduce carriage and shedding of zoonotic enteric bacterial pathogens in food animals. *Anim. Health Res. Rev.* **5**, 35-47.
5. Cleveland, J., Montville, T. J., Nes, I. F., and Chikindas, M. L. (2001) Bacteriocins: safe, natural antimicrobials for food preservation. *Int. J. Food Microbiol.* **71**, 1-20.
6. Daba, H., Panadian, S., Gosselin, J. F., Simard, R., Huang, J., and Lacroix, C. (1991) Detection and activity of a bacteriocin produced by *Leuconostoc mesenteroides*. *Appl. Environ. Microbiol.* **57**, 3450-3455.
7. Dahiya, J. P., Wilkie, D. C., Van Kessel, A. G., and Drew, M. D. (2006) Potential strategies for controlling necrotic enteritis in broiler chickens in post-antibiotic era. *Anim. Feed Sci. Technol.* **129**, 60-88.
8. Damelin, L. H., Dykes, G. A., and Von Holy, A. (1995) Biodiversity of lactic acid bacteria from food-related ecosystems. *Microbiol.* **83**, 13-22.
9. Diez-Gonzalez, F. (2007) Applications of bacteriocins in livestock. *Curr. Issues Intestinal Microbiol.* **8**, 15-24.
10. Dibner, J. J. and Richards, J. D. (2005) Antibiotic growth promoters in agriculture: history and mode of action. *Poultry Sci.* **84**, 634-643.
11. Doron, S. and Gorbach, S. L. (2006) Probiotics: their role in the treatment and prevention of disease. *Expert. Rev. Anti-Infect. Ther.* **4**, 261-275.
12. Du Toit, M., Franz, C. M. A. P., Dicks, L. M. T., and Holzapfel, W. H. (2000) Preliminary characterization of bacteriocins produced by *Enterococcus faecium* and *Enterococcus faecalis* isolated from pig faeces. *J. Appl. Microbiol.* **88**, 482-494.
13. Gillor, O., Kirkup, B. C., and Riley, M. A. (2004) Colicins and microcins: the next generation antimicrobials. *Adv. Appl. Microbiol.* **54**, 129-146.
14. Gonzalez, C. F. and Kunka, B. S. (1987) Plasmid-associated bacteriocin production and sucrose fermentation in *Pediococcus acidilactici*. *Appl. Environ. Microb.* **53**, 2534-2538.
15. Heo, S., Lee, S. K., Lee, C. H., Min, S. G., Park, J. S., and Kim, H. Y. (2007) Morphological changes induced in *Listeria monocytogenes* V7 by a bacteriocin produced by *Pediococcus acidilactici*. *J. Microbiol. Biotechnol.* **17**, 663-667.
16. Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., and Williams, S. T. (1994) *Bergey's Manual of Determinative Bacteriology*. 9th ed. Williams and Wilkins. Baltimore, USA
17. Joerger, R. D. (2003) Alternatives to antibiotics: bacteriocins, antimicrobial peptides and bacteriophages. *Poultry Sci.* **82**, 640-647.
18. Jung, B. Y., Lim, H. S., and Kim, B. H. (2003) Prevalence of *Listeria* spp. in cecal contents of livestock. *Kor. J. Vet. Publ. Hlth.* **27**, 41-46.
19. Kim, S. B., Lim, H. J., Lee, W. K., Hwang, I. G., Woo, G. J., and Ryu, S. R. (2006) PCR-based detection and molecular genotyping of enterotoxigenic *Clostridium perfringens* isolates from swine diarrhea in Korea. *J. Microbiol. Biotechnol.* **16**, 291-294.
20. Kosin, B. and Rakshit, S. K. (2006) Microbial and processing criteria for production of probiotics: a review. *Food Technol. Biotechnol.* **44**, 371-379.
21. Mayr-Harting, A., Hedges, A. J., and Berkeley, R. C. W. (1972) Methods for studying bacteriocins. In: Methods in Microbiology. Bergen, T. and Norris, J. R. (ed) Academic Press, London, pp. 315-422
22. Mileti, E., Matteoli, G., Iliev, I. D., and Rescigno, M. (2009) Comparison of the immunomodulatory properties of three probiotic strains of Lactobacilli using complex culture systems: prediction for *in vivo* efficacy. *PLoS One* **4**, e7056.
23. Mishra, V. and Prasad, D. N. (2005) Application of in vitro methods for selection of Lactobacillus casei strains as potential probiotics. *Int. J. Food Microbiol.* 103, 109-115.
24. Oelschlaeger, T. A. (2010) Mechanisms of probiotic actions. *Int. J. Med. Microbiol.* **300**, 57-62.
25. Parente, E. and Ricciardi, A. (1994) Influence of pH on the production of enterocin 1146 during batch fermentation. *Lett. Appl. Microbiol.* **19**, 12-15.
26. Parente, E., Ricciardi, A., and Addario, G. (1994) Influence of pH on growth and bacteriocin production by *Lactococcus lactis* subsp. *lactis* 140 NWC during batch fermentation. *Appl. Microbiol. Biotechnol.* **41**, 388-394.
27. Roselli, M., Finamore, A., Britti, M. S., Bosi, P., Oswald, I., and Mengheri, E. (2005) Alternatives to in-feed antibiotics in pigs: evaluation of probiotics, zinc or organic acids as protective agents for the intestinal mucosa. A comparison of in vitro and in vivo results. *Anim. Res.* **54**, 203-218.
28. Shin M. S., Han, S. K., Ji, A. R., Kim, K. S., and Lee, W. K. (2008) Isolation and characterization of bacteriocin-producing bacteria from the gastrointestinal tract of broiler chickens for probiotic use. *J. Appl. Microbiol.* **105**, 2203-2212.
29. Snyder, S. H. and Bredt, D. S. (1992) Biological roles of nitric oxide. *Sci. Am.* **266**, 68-77.
30. Strompfová, V., Marcièáková, M., Simonová, M., Gancarèíková, S., Jonecová, Z., Sciranková, L., Košèová, J., Buleca, V., Èobanová, K., and Lauková, A. (2006) *Enterococcus faecium* EK13–an enterocin A-producing strain with probiotic character and its effect in piglets. *Anaerobe* **12**, 242-248.
31. Thévenot, D., Dernburg, A., and Vernozy-Rozand, C. (2006) An updated review of *Listeria monocytogenes* in the pork meat industry and its products. *J. Appl. Microbiol.* **101**, 7-17.
32. Warriner, K., Aldsworth, T. G., Kaur, S., and Dodd, C. E. R. (2002) Cross-contamination of carcasses and equipment during pork processing. *J. Appl. Microbiol.* **93**, 169-177.
33. Yang, R., Johnson, M. C., and Ray, B. (1992) Novel method to extract large amounts of bacteriocins from lactic acid bacteria. *Appl. Environ. Microb.* **58**, 3355-3359.
34. Zhang, X., Goncalves, R., and Mosser, D. M. (2008) The isolation and characterization of murine macrophages. *Curr. Protoc. Immunol.* **Chapter 14**, Unit 14.1.

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