Korean J. Food Sci. An.
Vol. 34, No. 5, pp. 614~621(2014)
DOI http://dx.doi.org/10.5851/kosfa.2014.34.5.614

ARTICLE

Screening of Bacteriocin-producing *Enterococcus faecalis* Strains for Antagonistic Activities against *Clostridium perfringens*

Sun-Kyung Han, Myeong-Su Shin, Ho-Eun Park, So-Young Kim[1], and Wan-Kyu Lee*

College of Veterinary Medicine, Chungbuk National University, Cheongju 362-763, Korea

[1]*Functional Food & Nutrition Division, Department of Agrofood Resource, National Academy of Agricultural Science, Rural Development Administration, Jeonju 560-500, Korea*

Abstract

This study was conducted to isolate and characterize bacteriocin-producing bacteria against *Clostridium perfringens* (*C. perfringens*) from domestic animals to determine their usefulness as probiotics. Bacteriocin-producing bacteria were isolated from pig feces by the spot-on-lawn method. A total of 1,370 bacterial stains were isolated, and six were tentatively selected after identifying the inhibitory activity against the pathogenic indicator *C. perfringens* KCTC 3269 and KCTC 5100. The selected strains were identified as *Enterococcus faecalis* (*E. faecalis*) by 16s rRNA sequencing. Most of the isolated bacterial strains were resistant to 0.5% bile salts for 48 h and remained viable after 2 h at pH 3.0. Some *E. faecalis* also showed strong inhibitory activity against *Listeria monocytogenes* KCTC 3569, KCTC 3586 and KCTC 3710. In the present study, we finally selected *E. faecalis* AP 216 and AP 45 strain based on probiotic selection criteria such as antimicrobial activity against *C. perfringens* and tolerance to acid and bile salts. The bacteriocins of *E. faecalis* AP 216 and AP 45 strains were highly thermostable, showing anticlostridial activities even after incubation at 121°C for 15 min. These bacteriocin-producing bacteria and/or bacteriocins could be used in feed manufacturing as probiotics as an alternative to antibiotics in the livestock industry.

Keywords: antimicrobial activity, bacteriocin, probiotics, *Clostridium perfringens*

Introduction

Lactic acid bacteria (LAB) show antagonistic actions against spoilage and pathogenic organisms because they produce organic acids, fatty acids, hydrogen peroxide, diacetyl, and substances endowed with antibiotic activity (Ouwehand and Vesterlund, 1998). LAB also produce antimicrobial substances such as bacteriocins, which are generally defined as ribosomally synthesized peptides or proteins with bactericidal actions that often target bacterial species closely related to the producer strain (Klaenhammer, 1993). These compounds have attracted great interest because of their potential use as food preservatives, therapeutic agents against Gram positive bacteria and several viruses, and importance in modifying gut microflora (Daeschel *et al.,* 1990; Gould, 1996; Klaenhammer, 1993; Shearer *et al.,* 2014). Nevertheless, little is known about the bacteriocin-producing intestinal LAB induced animal sources (Diez-Gonzalez, 2007; Strompfová, 2006).

Clostridium perfringens (*C. perfringens*) is widely distributed in the environment, food and intestine as the normal gut flora in humans and animals (Steele and Wright, 2001). This microorganism forms additional toxins that have been proposed to be important for the pathogenesis of intestinal disorders. This microorganism causes problems such as gas gangrene, phlegmon and food poisoning in humans, as well as fatal enterotoxaemia in various animals (Timoney *et al.,* 1998). Although this pathogen can be controlled through hygienic methods and antimicrobial agents, the rise of multiple antibiotic drugs has created concerns regarding the possibility of antibiotic residues, development of antibiotic-resistant bacteria, imbalance of beneficial normal gut flora, and a reduction in the ability to cure bacterial infections in humans and animals (Jensen, 1998).

Therefore, in this study, we attempted to isolate and characterize bacteriocin-producing bacteria with antagonistic activities against *C. perfringens* from domestic ani-

*Corresponding author: Wan-Kyu Lee, College of Veterinary Medicine, Chungbuk National University, Cheongju 362-763, Korea. Tel: +82-43-261-2960, Fax: +82-43-267-3150, E-mail: wklee@cbu.ac.kr

mals and to develop a potential candidate for probiotic use in domestic animals as an alternative to antibiotics.

Material and Methods

Bacterial strains and culture condition

Enterococcus faecalis (*E. faecalis*) AP 110, AP 216, AP 44, AP 45, AP 47 and AP 51 strains were isolated from the feces of pigs and maintained at -70°C in MRS broth (Difco Laboratories, Detroit, MI, USA) containing 50% glycerol. Indicator microorganisms used in this study were obtained from the Korean Collection for Type Culture (KCTC), Korean Culture Center of Microorganisms (KC CM) and our collection from domestic animals for further studies (Table 1). The organism was propagated in appropriate media such as BHI or MRS broth.

Isolation of LAB from the feces of pigs

Feces obtained from slaughterhouses and farms were put into transport anaerobic medium (BHI broth; brain heart infusion 37.0 g, yeast extract 5.0 g, 0.1% resazurin 1.0 mL, 0.1% hemin 1.0 mL, and agar 0.7 g per L) that was replaced with O_2-free CO_2 gas and transported immediately to a laboratory. The samples were then serially diluted ten-fold with sterile diluent A (KH_2PO_4 0.5 g, Na_2HPO_4 6.0 g, L-cysteine HCl 0.5 g, Tween 80 0.5 g, and agar 1.0 g per L) plated on BHI or MRS agar and incubated at 37°C for 48 h under anaerobic conditions in an anaerobic steel wool jar filled with O_2-free CO_2 gas (Mitsuoka, 1980; Parker, 1955). After incubation, approximately twenty colonies per sample were randomly selected with sterilized toothpicks and inoculated into 1 mL BHI or MRS broth in an Eppendorf tube. The isolates were subsequently grown overnight at 37°C, after which 3 μL of culture broth were spotted on BHI agar, which were closely streaked of an overnight culture of *C. perfringens* KCTC 3269 (at a level of about 1.0×10^7 CFU/mL) using a sterile cotton swap (Teo and Tan, 2005). After incubation for 24 h, colonies with a clear inhibition zone were further examined for the production of bacteriocin.

Table 1. Antimicrobial spectrum of the selected *Enterococcus faecalis* strains isolated from the intestine of pigs against various indicator organisms

Indicator organisms	*Enterococcus faecalis* AP					
	110	216	44	45	47	51
Bacillus cereus KCTC 1012	−	−	−	−	−	−
Clostridium perfringens KCTC 3269	+	+	+	+	+	+
Clostridium perfringens KCTC 5100	+	−	+	+	+	−
Enterococcus faecalis KCTC 2011	−	−	−	+	−	−
Enterococcus faecium KCTC 3122	−	−	−	−	−	−
Enterococcus durans KCTC 3121	−	−	−	−	−	−
Escherichia coli KCTC 1682	−	−	−	−	−	−
Klebsiella pneumoniae KCTC 2208	−	−	−	−	−	−
Lactobacillus brevis KCTC 3498	−	−	−	+	−	−
Lactobacillus casei KCTC 3110	−	−	−	−	−	−
Lactobacillus delbruekii KCTC 1047	−	−	−	+	−	−
Lactobacillus fermentum KCTC 3112	−	−	−	−	−	−
Lactobacillus plantarum KCTC 3108	−	−	−	+	−	−
Leuconostoc mesenteroides KCTC 3505	−	−	−	−	−	−
Listeria monocytogenes KCTC 3569	+	+	+	+	+	−
Listeria monocytogenes KCTC 3586	+	+	+	+	+	−
Listeria monocytogenes KCTC 3710	+	+	+	+	+	−
Pediococcus acidilactici KCTC 1626	−	−	−	−	−	−
Proteus mirabillis KCTC 2565	−	−	−	−	−	−
Pseudomonas aeruginosa KCTC 1750	−	−	−	−	−	−
Salmonella Enteritidis KCCM 12021	−	−	−	−	−	−
Salmonella Typhimurium KCTC 2515	−	−	−	−	−	−
Staphylococcus aureus KCTC 1621	−	−	−	−	−	−
Staphylococcus intermedius KCTC 3344	−	−	−	−	−	−
Streptococcus mutans KCTC 3300	−	−	−	−	−	−

Detection of bacteriocin-producing bacteria and spectrum of antimicrobial activity

Cells were pelleted by centrifugation (7000 *g* for 10 min), after which the supernatants were adjusted to pH 6.5 with 1 N NaOH, 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 supernatants were serially diluted, and 10 μL aliquots of samples were spotted onto the surface of soft BHI or MRS agar (0.7%) seeded with an overnight culture of an indicator strain. In the case of *Clostridium* spp., an overnight culture was closely streaked onto the surface of BHI agar using a sterile cotton swab (Teo and Tan, 2005). Following incubation for 24 h at an appropriate temperature, the 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 definite inhibition of the indicator lawn.

Identification of bacterial strains

To identify bacteriocin-producing stains, the morphological and biochemical properties of each isolate were characterized according to Bergey's manual (Holt *et al.,* 1994). Gram staining, cell morphology, catalase activity, salt tolerance, gas production, growth temperature range, and biochemical carbohydrate fermentation patterns were assessed using an API 20E kit (Biomérieux, France). The 16S rRNA was sequenced using a Big Dye terminator

cycle sequencing kit (Applied BioSystems, USA), and sequences were resolved on an automated RNA sequencing system (Applied BioSystems model 3730XL, USA). The 16S rRNA sequence of each strain was aligned to the 16S rRNA gene sequence of LAB and other related taxa to compare the levels of similarity.

Growth curve and bacteriocin production in BHI medium

The growth curve and bacteriocin production were investigated in BHI medium. Finally, selected strains (*E. faecalis* AP 216 and AP 45) were incubated in 200 mL BHI broth. Temperature was maintained at 37°C and the pH was not controlled. Samples were taken at 2 h intervals to measure cell counts and bacteriocin activity. Viable cell counts were determined by the spread plate method on BHI agar, and bacteriocin activities against *C. perfringens* KCTC 3269 were tested by the spot-on-lawn assay (Teo and Tan, 2005).

Preparation of cell-free supernatants

Cell-culture broth was centrifuged at 10,000 *g* for 10 min at 4°C, after which the supernatant was adjusted to pH 6.5 with 5 N NaOH or 6 N HCl and filter-sterilized through 0.2 μm pore size membrane filters.

Effects of heat, pH and enzymes on bacteriocin activity

The effects of heat, pH and enzymes on the activities of partially purified bacteriocin were examined as described by Lyon and Glatz (1993). Briefly, supernatants were treated with various enzymes at 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 supplier (Sigma Chemical Co., USA). Mixtures were incubated at 30°C for 1 h and then heated at 80°C for 10 min to inactivate the enzymes. Cell-free supernatants were heated for 30 min at 60°C or 90°C, or at 121°C for 15 min, after which residual bacteriocin activity against *C. perfringens* KCTC 3269 was determined by the spot-on-lawn assay (Teo and Tan, 2005). To investigate the effects of pH on antimicrobial stability, the pH of the supernatants was adjusted to between 2 and 10 with either 1 N HCl or 1 N NaOH and then incubated at 30°C for 1 h.

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

Acid and bile salt tolerance were tested as described by Shin *et al.* (1999). To test acid and heat tolerance, overnight cultures in BHI medium of four selected strains were harvested at 3,000 *g* for 10 min at 4°C and then washed twice with 50 mM phosphate buffer, after which they were resuspended in 20 mL of the same buffer and the final pH was adjusted to 2.0, 2.3, 2.5, 3.0, 4.0, 5.0, 6.0 and 7.0. The suspensions were then incubated at 37°C for 2 h, after which the viable cell counts were determined by the spread plate method on BHI agar. For the heat tolerance test, selected strains were exposed to 50°C, 60°C, 70°C, 80°C or 90°C for 30 min, after which the suspensions were properly diluted and the viable cell counts were determined by the spread plate method on BHI agar. Bile tolerance was determined by spreading the cells on BHI 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, after which the viable cell counts were determined.

Results

Isolation and identification of bacteriocin-producing bacteria

A total of 1,370 strains were isolated from pig feces, 354 of which showed inhibitory activity in the first screening step (data not shown). Cell-free supernatants of these isolates were neutralized with 1 N NaOH to eliminate the effects of organic acids, and the inhibition test against indicator organisms was conducted according to the spot-on-lawn method. Six strains were tentatively selected as anti-*Clostridium perfringens* substance-producing candidates, each of which exhibited slightly different antimicrobial activities against the indicator, *C. perfringens* KC TC 3269 and KCTC 5100. The strains were characterized as Gram-positive, catalase-negative, facultative anaerobic cocci-shaped bacteria (Fig. 1). Based on comparisons of their characteristics with Bergey's manual and the results of an API test (data not shown), the isolates were classified as *E. faecalis* AP 110, AP 216, AP 44, AP 45, AP 47 and AP 51. The six selected strains were identified as *E. faecalis* by 16s rRNA sequencing.

Spectrum of antimicrobial activity

The cell-free supernatants were tested for their antimicrobial activities against various Gram-positive and Gram-negative bacteria using the spot-on-lawn method (Table 1). All selected strains showed relatively strong inhibitory activity against the growth of *C. perfringen*s and *Listeria monocytogenes* (*L. monocytogenes*) when compared to

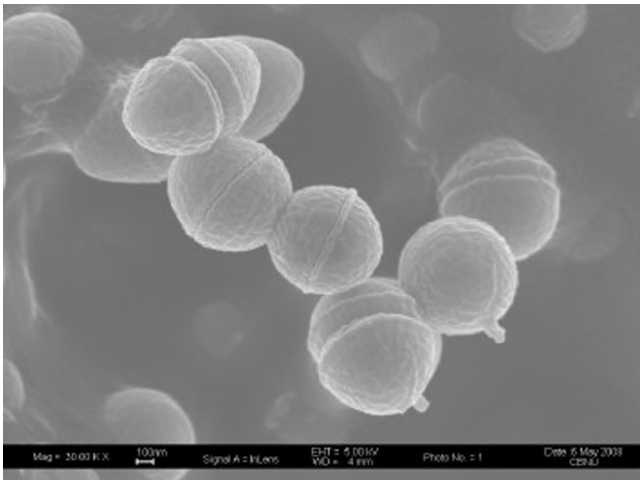

Fig. 1. Scanning electron microscopic observation of the *Enterococcus faecalis* AP 216 (×15,000).

other indicators. Additionally, *E. faecalis* AP 45 exhibited antagonistic activities against *C. perfringens*, the field isolate from domestic animals (data not shown). Particularly, *E. faecalis* AP 45 demonstrated a relatively broad spectrum of activity against *C. perfringens* KCTC 3269 and KCTC 5100, *E. faecalis* KCTC 2011, *L. brevis* KCTC 3498, *L. delbruekii* KCTC 1047, *L. plantarum* KCTC 3108 and *L. monocytogenes* KCTC 3569, KCTC 3586 and KC TC 3710 based on the spot-on-lawn method. However, they did not inhibit the growth of the Gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella* Typhimurium (Table 1).

Cell growth and bacteriocin production

The bacteriocin production of *E. faecalis* AP 216 and AP 45 in standard cultures was detected during the exponential phase of growth, and reached maximum levels (800 and 3200 AU/mL, respectively) in the stationary phase.

Table 2. Effect of enzymes, heat and pH on the activity of the cell-free supernatants produced by *Enterococcus faecalis* AP 216 and AP 45

Treatment		Relative antimicrobial activity (%)	
		AP 216	AP 45
Enzyme	Proteinase K	0	50
	Protease XIV	0	25
	Pepsin	25	0
	Trypsin	0	0
	α-Amylase	50	25
	α-Amylase	50	25
	Catalase	100	100
Heating	60, 30 min	100	50
	90, 30 min	100	25
	121, 15 min	100	25
pH	pH 2.0	50	100
	pH 3.0	100	100
	pH 4.0	100	100
	pH 5.0	100	100
	pH 6.0	100	100
	pH 7.0	100	100
	pH 8.0	100	100
	pH 9.0	100	100
	pH 10.0	100	100

In addition, the bacteriocin titer decreased markedly with further incubation, with no bacteriocin detected in culture supernatants at the end of the incubation period (Fig. 2).

Effect of enzyme, heat treatment, and pH on bacteriocin activity

Anti-*Clostridium perfringens* activities of cell-free supernatants of *E. faecalis* AP 216 and AP 45 were completely inactivated by at least one of proteinase K, protease XIV, pepsin, or trypsin, but they were not completely inactivated by treatment with α-amylase, β-amylase, or catalase (Table 2). The bacteriocins of *E. faecalis* AP 216 strains were highly thermostable, maintaining anticlostridial acti-

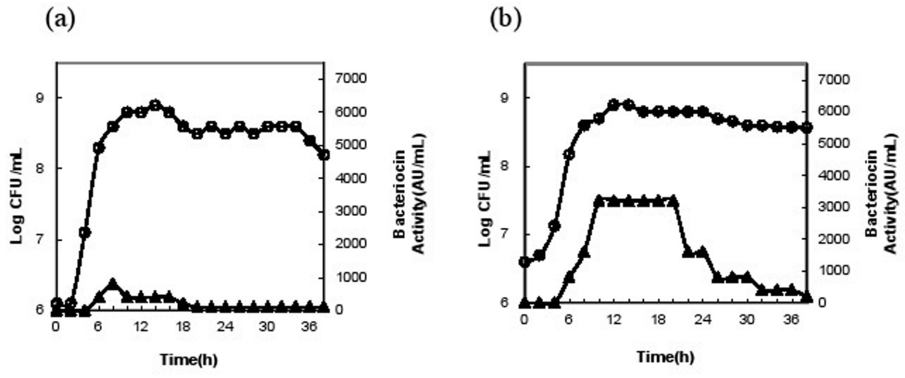

Fig. 2. Cell growth and bacteriocin production of *Enterococcus faecalis* AP 216 (a) and *Enterococcus faecalis* AP 45 (b) in BHI broth. ○, viable cell count; ▲, bacteriocin activity.

vities even after incubation at 121°C for 15 min, but the inhibitory activities of *E. faecalis* AP 45 were diminished when incubated at 60°C or 90°C for 30 min and at 121°C for 15 min. Very small or no significant decreases in the anticlostridial activities of the filtrates from selected strains were observed when they were adjusted from pH 2.0 to 10.0 for 1 h compared to the untreated filtrates.

Acid, bile, and heat tolerance

The acid tolerance study showed that *E. faecalis* AP 216 and AP 45 strains were stable at pH 3.0, although their viable cell number decreased after incubation at pH 2.3 and pH 2.5 for 2 h, respectively (Fig. 3). In contrast to acid tolerance, *E. faecalis* AP 216 and AP 45 were stable following exposure to bile salt at up to 0.5% for 48 h. To understand the influences of the thermal processing of feed containing bacteriocin-producing bacteria, preliminary examinations for heat resistance were carried out using the isolates. Two strains survived at 60°C for 30 min.

Discussion

The presence of *C. perfringens* in animals has been linked to increased incidence of bovine enterotoxaemia, diarrhea in piglets and sheep, and intestinal disorders such as necrotic enteritis in chickens (Bueschel *et al*., 2003; Garmory *et al*., 2000; Herholz *et al*., 1999; Klaasen *et al*., 1999; Manteca *et al*., 2002). Bacteriocin from Gram-positive microorganisms such as LAB has been subjected to intensive investigation in recent years because of the utility of biopreservatives or bioregulators and antibiotic resistance in pathogenic microorganisms such as *C. perfringens* (Hammerman *et al*., 2006; Saarela *et al*., 2000). In the present study, 1370 strains were isolated from the feces of domestic animals, and six *E. faecalis* strains were selected after determining that their anti-*Clostridium perfringens* activities were mediated through bacteriocin production.

Generally, most *Enterococcal* bacteriocin displays bacteriocidal effects (Fouquié Moreno *et al*., 2003; Sparo *et al*., 2013). Previous studies have shown that *Lactobacillus rhamnosus* (Alander *et al*., 1999), *Lactobacillus plantarum* (Schoster *et al*., 2013; West and Warner, 1988), *Lactococcus lactis* subsp. *lactis* (Harlender and Spelhaug 1989), and *Pediococcus pentosaceus* (Graham and McKay, 1985) are bactericidal for *Clostridium* spp. Additionally, we previously reported that bacteriocin-producing *Bacillus* strains isolated from domestic animals exhibited inhi-

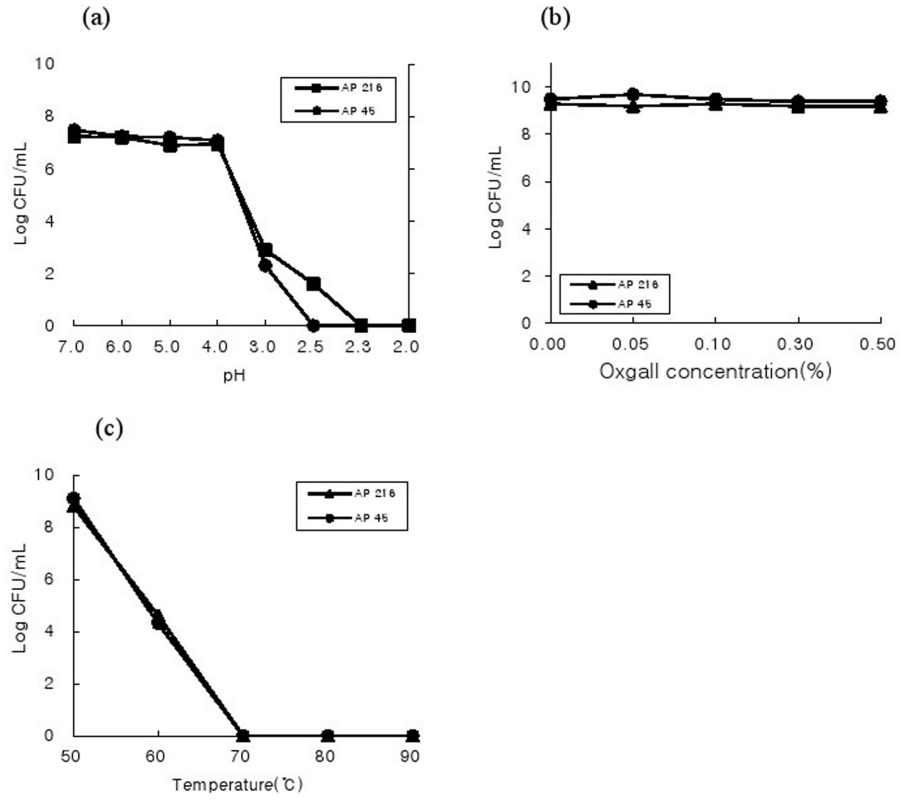

Fig. 3. Acid tolerance (a), bile salt resistance (b), and heat resistance (c) of the *Enterococcus faecalis* AP 216 and AP 45.

bitory activity against *C. perfringens* (Han *et al*. 2011). In this study, six selected *E. faecalis* strains were shown to exhibit various degrees of antimicrobial activity against indicator organisms. The results of the present study demonstrate anti-*Clostridium perfringens* bacteriocin production by *E. faecalis* for the first time. In addition, the bacteriocin of *E. faecalis* AP 45 was also inhibitory toward *E. faecalis*, *L. brevis*, *L. delbrueki*, *L. plantarum* and *L. monocytogenes.* However, it did not inhibit the growth of Gram-negative bacteria such as *E. coli*, *P. aeruginosa*, and *S.* Typhimurium.

The antimicrobial activity of bacteriocins produced by the two selected strains, *E. faecalis* AP 45 and AP 216, dramatically decreased at 36-48 h during prolonged fermentation. This pattern has been observed for other LAB bacteriocins (Aasen *et al*., 2000; Daba *et al*., 1991). Bacteriocins are often produced during the growth phase and then lost due to proteolytic degradation, protein aggregation, and adsorption by cells (Aasen *et al*., 2000; De Vuyst *et al*., 1996; Parente *et al*., 1994). Additionally, most authors have noted that good cell growth frequently goes hand in hand with bacteriocin production (Cabo *et al*., 2001; De Vuyst *et al*., 1996).

The effects of various enzymes on the supernatants of *E. faecalis* AP 45 and AP 216 were carefully investigated. As shown in Table 2, the antimicrobial activity of *E. faecalis* AP 216 and AP 45 were completely inactivated in response to at least one of proteinase K, protease XIV, pepsin, or trypsin, indicating that the antimicrobial substance has a proteinaceous property that can be classified as a bacteriocin. Similar to the reported stability of bacteriocins from *E. faecalis* and *Pediococcus acidilactici* (Bhunia *et al*., 1987; Galvez *et al*., 1986), bacteriocins of the selected strains were stable in the presence of various pHs. Furthermore, their stabilities did not decrease significantly as the pH increased to 10. Heat stability is very important for industrial applications such as manufacturing of animal feed. Because the two strains showed antimicrobial activities with heat stable bacteriocin, they can be used in feed manufacturing.

For probiotic application, it is important to select strains with high colony forming capacity, acid and bile resistance, inhibitory activity against pathogenic microorganisms and the ability to effectively regulate normal flora in the gastrointestinal tract (Chateau *et al*., 1993; Nurmi *et al*., 1983). Selected strains exhibited resistance to 0.5% bile salts and remained viable after 30 min at pH 3.0.

In the present study, we screened bacteriocin-producing *E. faecalis* strains for antagonistic activities against *C. perfringens* and finally selected *E. faecalis* AP 216 and AP 45 strain based on probiotic selection criteria such as antimicrobial activity against *C. perfringens* and tolerance to acid and bile salts. These bacteriocin-producing bacteria and/or bacteriocins can be used as probiotics as an alternative to antibiotics in the livestock industry.

Acknowledgements

This study was carried out with the support of "Cooperative Research Program for Agricultural Science & Technology Development (Project No. PJ009126)", Rural Development Administration, Republic of Korea.

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 426 87. *Appl. Microbiol. Biotechnol*. **53**, 159-166.
2. Alander, M., Satokari, R., Korpela, R., Saxelin, M., Vilpponen-Salmela, T., Mattila Sandholm, T., and von Wright, A. (1999) Persistence of colonization of human colonic mucosa by a probiotic strain, *Lactobacillus rhamnosus* GG, after oral consumption. *Appl. Environ. Microbiol*. **65**, 351-354.
3. Bhunia, A. K., Johnson, M. C., and Ray, B. (1987) Direct detection of an antimicrobial peptide of *Pediococcus acidilactici* in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Indust. Microbiol*. **2**, 319-322.
4. Bueschel, D. M., Jost, B. H., Billington, S. J., Trinh, H. T., and Songer, J. G. (2003) Prevalence of *cpb*2, encoding beta2 toxin, in *Clostridium perfringens* field isolates: correlation of genotype with phenotype. *Vet. Microbiol*. **94**, 121-129.
5. Cabo, M. L., Murado, M. A., González, M., and Pastoriza, L. (2001) Effects of aeration and pH gradient on nisin production. *A mathematical model. Enzyme Microb. Technol*. **29**, 264-273.
6. Chateau, N., Castellanos, I., and Deschamps, A. M. (1993) Distribution of pathogen inhibition in the *Lactobacillus* isolates of commercial probiotic consortium. *J. Appl. Bacteriol*. **74**, 36-40.
7. Daba, H., Pandian, S., Gosselin, J. F., Simard, R. E., Huang, J., and Lacroix, C. (1991) Detection and activity of a bacteriocin produced by *Leuconostoc mesenteroides*. *Appl. Environ. Microbiol*. **57**, 3450-3455.
8. Daeschel, M. A., McKenny, M. C., and McDonald, L. C. (1990) Bactericidal activity of *Lactobacillus plantarum* C-11. *Food Microbiol*. **7**, 91-98.
9. De Vuyst, L., Callewaert, R., and Crabbé, K. (1996) Primary metabolite kinetics of bacteriocin biosynthesis by *Lactobacillus amylovorus* and evidence for stimulation of bacteriocin under unfavourable growth conditions. *Microbiol*. **142**, 817-827.
10. Diez-Gonzalez, F. (2007) Applications of bacteriocins in live-

stock. *Curr. Issues Intestinal. Microbiol.* **8**, 15-24.
11. Fouquié Moreno, M. R., Callewaert, R., Devreese, B., Van Beeumen, J., and De Vuyst, L. (2003) Isolation and biochemical characterisation of enterocins produced by *enterococci* from different sources. *J. Appl. Microbiol*. **94**, 214-229.
12. Galvez, A., Maqueda, M., Valdivia, E., Quesada, A., and Montoya, E. (1986) Characterization and partial purification of a broad spectrum antibiotic AS-48 produced by *Streptococcus faecalis*. *Can. J. Microbiol*. **32**, 765-771.
13. Garmory, H. S., Chanter, N., French, N. P., Bueschel, D., Songer, J. G., and Titball, R. W. (2000) Occurrence of *Clostridium perfringens* beta2-toxin amongst animals, determined using genotyping and subtyping PCR assays. *Epidemiol. Infect*. **124**, 61-67.
14. Gould, G. W. (1996) Industry perspectives on the use of natural antimicrobials and inhibitors for food applications. *J. Food Prot. Suppl*. **59**, 82-86.
15. Graham, D. C. and McKay, L. L. (1985) Plasmid DNA in strains of *Pediococcus cerevisiae* and *Pediococcus pentosaceus. Appl. Environ. Microbiol*. **50**, 532-534.
16. Hammerman, C., Bin-Nun, A., and Kaplan, M. (2006) Safety of probiotics: comparison of two popular strains. *BMJ*. **333**, 1006-1008.
17. Han, S. K., Choi, H. J., Lee, S. M., Shin, M. S., and Lee, W. K. (2011) Screening of Bacteriocin-producing *Bacillu*s strains isolated from domestic animal feces for antagonistic activities against *Clostridium perfringens*. *Korean J. Food Sci. An.* **31**, 405-412.
18. Harlender, S. K. and Spelhaug, S. R. (1989) Inhibition of foodborne bacterial pathogens by bacteriocins from *Lactococcus lactis* and *Pediococcus pentosaceus*. *J. Food Prot*. **52**, 856-862.
19. Héchard, Y. and Sahl, H. G. (2002) Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. *Biochimie*. **84**, 545-557.
20. Herholz, C., Miserez, R., Nicolet, J., Frey, J., Popoff, M., Gibert, M., Gerber, H., and Straub, R. (1999) Prevalence of beta2-toxigenic *Clostridium perfringens* in horses with intestinal disorders. *J. Clin. Microbiol*. **37**, 358-361.
21. 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.
22. Jensen, B. B. (1998) The impact of feed additives on the microbial ecology of the gut in young pigs. *J Anim Feed Sci*. **7**, 45-64.
23. Klaasen, H. L., Molkenboer, M. J., Barkker, J., Miserez, R., Hani, H., Frey, J., Popoff, M. R., and van den Bosch, J. E. (1999) Detection of beta2 toxin gene of *Clostridium perfringens* in diarrhoeic piglets in the Netherlands and Switzerland. *FEMS Immunol. Med. Microbiol*. **24**, 325-332.
24. Klaenhammer, T. R. (1993) Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol. Rev.* **12**, 39-85.
25. Lyon, W. J. and Glatz, B. A. (1993) Isolation and purification of propionicin PLG-1, a bacteriocin produced by a strain of *Propionibacterium thoenii*. *Appl. Environ. Microbiol*. **59**, 83-88.
26. Manteca, C., Daude, G., Jauniaux, T., Linden, A., Pirson, V., Detilleux, J., Ginter, A., Coppe, P., Kaeckenbeeck, A., and Mainil, J. G. (2002) A role for *Clostridium perfringens* beta2-toxin in bovine enterotoxaemia. *Vet. Microbiol*. **86**, 191-202.
27. Mayr-Harting, A., Hedges, A. J., and Berkeley, R. C. W. (1972) Methods for studying bacteriocins. *Methods in Microbiology* ed. Bergen T and Norris JR. pp. 315-422.
28. Mitsuoka, T. (1980) The world of anaerobic bacteria: A color atlas of anaerobic bacteria. *Sobun press*. Tokyo, pp. 13-65.
29. Nurmi, E. V., Schneitz, C. E., and Makela, P. H. (1983) Process for the production of a bacterial preparation. Canadian Patent no. 1151066.
30. Ouwehand, A. C. and Vesterlund, S. (1998) Antimicrobial components from lactic acid bacteria. *In Lactic acid bacteria: Microbial Ecology and Functional Aspects*, 2nd ed. pp. 139-159. [Salminen S and von Wright A, editors]. New York: Marcel Dekker Inc.
31. 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.
32. Parker, C. A. (1955) Anaerobiosis with iron wool. *Aust. J Exp. Biol. Med. Sci*. 33, 33-37.
33. Saarela, M., Mogensen, G., Fondén, R., Mättö, J., and Mattila-Sandholm, T. (2000) Probiotic bacteria: safety, functional and technological properties. *J. Biotechnol.* **84**, 197-215.
34. Schoster, A., Kokotovic, B., Permin, A., Pedersen, P. D., Dal Bello, F., and Guardabassi, L. (2013) *In vitro* inhibition of *Clostridium difficile* and *Clostridium perfringens* by commercial probiotic strains. *Anaerobe*. **20**, 36-41.
35. Shearer, A. E., Hoover, D. G., and Kniel, K. E. (2014) Effect of bacterial cell-free supernatants on infectivity of norovirus surrogates. *J. Food Prot*. **77**, 145-149.
36. Shin, M. S., Kim, H. M., Kim, K. T., Huh, C. S., Bae, H. S., and Baek, Y. J. (1999) Selection and characteristics of *Lactobacillus acidophilus* isolated from Korean feces. *Kor. J. Food Sci. Technol.* **31**, 495-501.
37. Sparo, M. D., Confalonieri, A., Urbizu, L., Ceci, M., and Bruni, S. F. (2013) Bio-preservation of ground beef meat by *Enterococcus faecalis* CECT7121. *Braz. J. Microbiol*. **44**, 43-49.
38. Steele, F. M. and Wright, K. H. (2001) Cooling rate effect on outgrowth of *Clostridium perfringens* in cooked, ready-to-eat turkey breast roasts. *Poultry Sci*. **80**, 813-816.
39. Strompfová, V., Marcináková, M., Simonová, M., Gancarcíková, S., Jonecová, Z., Sciranková, L., Koscová, J., Buleca, V., Cobanová, 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.
40. Teo, A. Y. and Tan, H. M. (2005) Inhibition of *Clostridium perfringens* by a novel strain of *Bacillus subtilis* isolated from the gastrointestinal tracts of healthy chickens. *Appl. Environ. Microbiol*. **71**, 4185-4190.
41. Timoney, J. F., Gillespie, J. H., Scott, F. W., and Barlough, J. E. (1998) Hagan and Bruner's microbiology and infectious diseases of domestic animals. 8th ed, Comstock Publishing

Associates, Ithaca and London, pp. 214-240.
42. West, C. A. and Warner, P. J. (1988) Plantacin B, a bacteriocin produced by *Lactobacillus plantarum* NCDO 1193. *FEMS Microbiol. Lett*. **49**, 163-165.

(Received 2014.6.17/Revised 2014.8.7/Accepted 2014.8.18)