Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Isolation and characterization of bacteriocin-producing bacteria from the gastrointestinal tract of broiler chickens for probiotic use

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Keywords
alternatives, antimicrobial activity, bacteriocin, broiler, probiotics.

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2008/0280: received 19 February 2008, revised 11 July 2008 and accepted 6 August 2008

doi:10.1111/j.1365-2672.2008.03935.x

Abstract

Aims: To isolate and characterize the bacteriocin-producing bacteria (BPB) from the gastrointestinal tract of broiler chickens for probiotic use.

Methods and Results: In total, 291 bacterial strains were isolated from broilers and screened for bacteriocin-producing ability. The bacteriocins produced by *Enterococcus faecium* SH 528, *Ent. faecium* SH 632 and *Pediococcus pentosaceus* SH 740 displayed inhibitory activity against pathogens including *Clostridium perfringens* and *Listeria monocytogenes*. Activity of the bacteriocins remained unchanged after 30 min of heat treatment at 60°C or exposure to organic solvents, but diminished after treatment with proteolytic enzymes. PCR was used to detect the structural genes enterocin A and B in SH 528, enterocin L50 and P in SH 632, and pediocin PA-1 in SH 740. Most of them were resistant to 0·5% bile salts and remained viable after 2 h at pH 3·0. *Ent. faecium* SH 528 exhibited the highest amylase activity among the strains tested.

Conclusions: We selected *Ent. faecium* SH 528 and SH 632 and *Ped. pentosaceus* SH 740 by probiotic selection criteria including inhibition activity against pathogens.

Significance and Impact of the Study: The isolated BPB could potentially be used in the poultry industry as probiotics to control pathogens.

Introduction

Antibiotics have been widely used at subtherapeutic levels in animals used for food as animal growth promoters (AGPs) for more than 50 years (Dibner and Richards 2005). Subtherapeutic antibiotics have improved growth and feed conversion in poultry and swine production. However, in recent years, the appearance of resistant bacterial populations, residual antibiotics in meats and an increasing demand for organic production have increased interest in searching for alternatives to antibiotics. In 2006, the European Union banned antibiotics as AGP in livestock production. Among alternatives to antibiotics are competitive exclusion products, probiotics, prebiotics, organic acids, plant extracts and essential oils, feed enzymes, bacteriophages and hen egg antibodies (Dahiya *et al.* 2006).

Probiotics have been defined as 'a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance' (Fuller 1989). Lactic acid bacteria (LAB) are normal intestinal flora both in humans and animals (Gilliland *et al.* 1975; Vaughan *et al.* 2005), and some strains are used as probiotics. Probiotics used in animal feed to improve productivity are different from those used to enhance the health of humans. Micro-organisms (primarily LAB) used as feed additives should preferentially originate from the microbiota of the target animal (Kosin and Rakshit 2006). To inhibit pathogenic micro-organisms in the gastrointestinal tract, they should also produce antimicrobial substances such as organic acids, diacetyl, hydrogen peroxide and bacteriocins (Daeschel 1989; Cleveland *et al.* 2001). Bacteriocins, which are ribosomally synthesized peptides or proteins with antimicrobial properties that often target bacterial species that are closely related to the producer strain, could potentially be used in the food and feed industries as natural preservatives and as probiotics for humans and livestock (Diez-Gonzalez 2007). Enterococci and pediococci are LAB that normally live in the intestines of animals and are commonly found in fermented foods, meat and dairy products. Bacteriocins produced by the enterococci include the well-characterized enterocins A, B, P, CRL35, 1071A and B, mundticin, bacteriocin 31, T8, AS-48 and enterolysin A (Franz *et al.* 2007). Some strains of pediococci produce pediocins, members of the Class IIa bacteriocins, which are small, heat-stable and non-modified anti-listerial peptides. Despite the potential of BPB as alternatives to antibiotics, only a few studies have investigated the application of bacteriocin-producing LAB of intestinal origin in the animal industry. Gillor *et al.* (2004) reported that bacteriocins produced by *Escherichia coli* (known as colicins and microcins) can play a promising role in the prevention of *Salmonella* contamination in the poultry industry. Recent studies have shown that *Lactobacillus salivarius* strains isolated from chicken intestine produce bacteriocins with antagonistic activity against Gram-positive bacteria and *Campylobacter jejuni* (Pilasombut *et al.* 2006; Stern *et al.* 2006).

This study describes the isolation of BPB from the gastrointestinal tract of broiler chickens. We partially characterized the bacteriocin produced by three selected strains, which were evaluated according to the selection criteria of probiotics such as acid tolerance, bile salts tolerance and activitiers of digestive enzymes.

Materials and methods

Bacterial strains and culture conditions

Enterococcus faecium SH 528, *Ent. faecium* SH 632 and *Ped. pentosaceus* SH 740 were isolated from the intestines of broiler chickens and maintained at −70°C in lactobacilli MRS broth (Difco Laboratories, Detroit, MI, USA) containing 50% volume per volume (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 3.

Isolation of LAB from the gastrointestinal tract of broiler chickens

Thirty 31-day-old Cobb broilers (average body weight, 1730 g) were obtained from a slaughterhouse. The birds were allowed access to water and fed on a commercial soybean and corn-based diet without added antibiotics and probiotics. The gastrointestinal tracks were excised and the entire contents of ileum and caecum were collected. The samples of homogenized ileum or caecum were serially diluted 10-fold with saline solution, and 100 μl of them was plated on MRS agar, respectively, and incubated at 37°C for 2–3 days under anaerobic conditions. Approximately five colonies per sample were randomly selected with sterilized toothpicks and inoculated into 1 ml MRS broth in an Eppendorf tube. The isolates were grown in MRS broth for 2 days at 37°C, and then 10 μl of culture broth were spotted on MRS agar. After drying for 1 h, the plate was overlaid with soft MRS or BHI agar (0·7%) seeded with an overnight culture of the indicator strains, *E. coli* KCTC 1467, *Salm. enterica* serovar Typhimurium KCTC 2515, *Staphylococcus aureus* KCTC 1621, *L. monocytogenes* KCTC 3569 and *Lact. sake* KCCM 40264, at a level of about $5{\cdot}0 \times 10^6$ CFU ml^{-1}. After incubation for 24 h, colonies with a clear inhibition zone were further examined for production of bacteriocin.

Detection of bacteriocin-producing LAB and spectrum of antimicrobial activity

The cells were pelleted by centrifugation (10 000 ***g*** for 15 min). The supernatants were adjusted to pH 6·5 with 10 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 samples were spotted onto the surface of soft MRS or BHI agar (0·7%) seeded with an overnight culture of an indicator strain. After 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^{-1}), which was defined as the reciprocal of the highest two-fold dilution showing definite inhibition of the indicator lawn.

Identification of bacterial strains

To identify bacteriocin-producing strains, we characterized the morphological and biochemical properties of each isolate according to Bergey's manual (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, Lyon, France). We sequenced 16S rDNA using the Big Dye terminator cycle sequencing kit (Applied BioSystems, Forter City, CA, USA), and sequences were resolved on an automated DNA sequencing system (Applied BioSystems model 3730XL). The 16S rDNA sequence of each strain was aligned to the 16S rDNA gene sequence of LAB and other related taxa in order to compare the levels of similarity.

Growth curve and bacteriocin production in MRS medium

The growth curve and bacteriocin production were investigated. Selected strains were incubated in MRS broth in a 5-l jar fermenter (Fermentec Co., Cheongju, Korea). Temperature was maintained at 37°C, the agitation speed was 50 rpm 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 pour plate method on MRS agar, and bacteriocin activities were tested by the spot-on-lawn assay.

Preparation of cell-free supernatants

Cell-culture broth from the jar fermenter was centrifuged at 3000 *g* for 30 min at 4°C, and the supernatant was adjusted to pH 6·5 with 10 N NaOH and filter-sterilized through 0·2-μm pore size membrane filters.

Effects of heat, pH, enzymes and organic solvents

Cell-free supernatants were heated for 30 min at 60°C or 90°C, or at 121°C for 15 min, and then residual bacteriocin activity was determined by the spot-on-lawn assay. To investigate the effects of pH on antimicrobial stability, the pH values of the supernatants were adjusted between 2 and 10 with either 1 N HCl or 1 N NaOH and incubated at 30°C for 1 h. The supernatants were treated with various enzymes at a final concentration of 1 mg ml^{-1}. All enzymes (Proteinase K, protease type XIV, trypsin, α-amylase, β-amylase and catalase) were dissolved in buffers recommended by the supplier (Sigma Chemical Co., St Louis, MO, USA). Mixtures were incubated at 30°C for 1 h and heated at 80°C for 10 min to inactivate the enzymes. Supernatants were also treated with 50% organic solvents including ethanol, methanol, chloroform, acetone, acetonitrile, hexane and cyclohexane. The solvent-treated samples were incubated at 37°C for 1 h.

PCR amplification of known enterocin and pediocin genes

Total DNA of the selected strains was isolated by the method of Anderson and McKay (1983) and used as template in the PCR reactions. The specific primers used for the enterocin and pediocin genes are listed in Table 1. The PCR reactions were carried out with a PCR kit (SolGent, Daejeon, Korea) in a thermal cycler (Amplitron® II, Barnstead/Thermolyne, Montréal, QC, Canada). The PCR conditions included an initial denaturing step for 10 min at 95°C and 35 cycles of 95°C for 1 min, 56°C for 1 min and 72°C for 1 min, followed by 5 min at 72°C. PCR amplified products were resolved by electrophoresis on 2% weight per volume (w/v) agarose gels (0·5 × Tris-borate-ethylenediaminetetraacetic acid buffer, pH 8·0).

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

Acid and bile salt tolerance were performed as described by Shin *et al.* (1999). To test acid tolerance, overnight cultures in MRS medium of three selected strains were harvested at 4000 *g* for 10 min at 4°C and washed twice with 50 mmol phosphate buffer and resuspended in 20 ml of the same buffer. The final pH was adjusted to 2·0, 2·3, 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.

Table 1 Primers used in the PCR reactions

Primer	Sequence (5′–3′)	Target	Reference
Ent A-F	AAATATTATGGAAATGGAGTGTAT	Enterocin A	Du Toit *et al.* (2000)
Ent A-R	GCACTTCCCTGGAATTGCTC		
Ent B-F	GAAAATGATCACAGAATGCCTA	Enterocin B	Du Toit *et al.* (2000)
Ent B-R	GTTGCATTTAGAGTATACATTTG		
Ent P-F	TATGGTAATGGTGTTTATTGTAAT	Enterocin P	Du Toit *et al.* (2000)
Ent P-R	ATGTCCCATACCTGCCAAAC		
Ent L50-F	STGGGAGCAATCGCAAAATTAG	Enterocin L50	Du Toit *et al.* (2000)
Ent L50-R	ATTGCCCATCCTTCTCCAAT		
Ent Q-F	GGAATAAGAGTAGTAGTGGAATACTGATATGAGTC	Enterocin Q	Cintas *et al.* (2000)
Ent Q-R	AAAGACTGCTCTTCCGAGCAGCC		
Ped-F	TTGTGATGAAAAAAATTGAAAAATTA	Pediocin PA-1	This study
Ped-R	GCATTTATGATTACCTTGATGTCC		

Assay of enzyme activities

The supernatant was separated from the culture broth by centrifugation (10 000 *g*) and filtered through 0·2-μm pore size membrane filters. The filtrate was used in enzyme activity assays. Amylase and cellulase activity were assayed according to a modified method of Miller (1959) and Khasin *et al.* (1993). Briefly, filtered supernatants (50 μl) were mixed with 950 μl of 0·5% substrate (starch or carboxymethylcellulose) in 0·1 mol l^{-1} Tris–HCl buffer (pH 7·0) and incubated at 37°C for 10 min. The reactions were terminated by adding 1 ml of dinitrosalicylic acid (DNS) and boiling for 5–7 min. After adding 8 ml of distilled water to each reaction mixture, the absorbance was determined at 540 nm by UV spectrophotometry (Unico, Dayton, NJ, USA). One unit of amylase or cellulose was defined as the amount of enzyme that produces 1 μmol of glucose equivalent per minute. Lipase activity was assayed according to a modified method of Lesuisse *et al.* (1993). The activity was measured using 0·5 ml of culture supernatant and 0·5 ml of 1% 4-nitrophenyl butyrate in 50 mmol Tris–HCl (pH 7·0). The reaction was carried out at 37°C for 10 min, and the absorbance was determined at 405 nm. One unit of lipase was defined as the amount of enzyme that librated 1 μmol of 4-nitrophenol per minute. Protease activity was assayed according to a modified method of Yanagida *et al.* (1986). Briefly, 2·5 ml of 0·7% casein (from bovine milk, Sigma) solution were mixed with 0·5 ml of culture broth and incubated at 37°C for 30 min. The reaction was stopped by adding 2·5 ml of 10% trichloroacetic acid (TCA). The reaction mixture was centrifuged (3000 *g*) at 4°C for 30 min, and the absorbance of the supernatant was measured spectrophotometrically at 275 nm. One unit of protease activity was defined as the amount causing an increase of 1 μmol tyrosine in 1 min.

Statistical analysis

The data were statistically analysed using the software package SPSS 13·0 Window Program (SPSS Inc., Chicago, IL, 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

Screening and identification of bacteriocin-producing LAB

Among 291 strains isolated from the gastrointestinal tract of broiler chickens, 17 isolates showed antagonistic activities against more than two indicators tested in the first screening step. Cell-free supernatants of these isolates were neutralized with NaOH in order to eliminate the effect of organic acids, and the inhibition test against indicator organisms was performed according to the spot-on-lawn method. Three strains (SH 528, SH 632 and SH 740) were ultimately selected as antimicrobial substance-producing candidates, and each exhibited slightly different antimicrobial activities against the indicators. They were all Gram-positive, catalase-negative and facultatively anaerobic cocci with pair or tetrad cell organization and did not produce gas. Based on comparisons of their characteristics with Bergey's manual and the results of the API test (carbohydrate fermentation test), the isolates were classified as *Ent. faecium* SH 528, *Ent. faecium* SH 632 and *Ped. pentosaceus* SH 740 (Table 2). The 16S rDNA sequences of the SH 528 (GenBank accession number EU878169) and SH 632 (GenBank accession number EU878170) revealed 99% similarity with *Ent. faecium* ATCC 19434, and the 16S rDNA sequence of SH 740 (GenBank accession number EU878171)was 99% similar to *Ped. pentosaceus* ATCC 25745.

Table 2 General characteristics of the BPB from the gastrointestinal tract of broiler chickens

Characteristics	*Ent. faecium* SH 528	*Ent. faecium* SH 632	*Ped. pentosaceus* SH 740
Sources	Ileum	Cecum	Cecum
Morphology			
Shape	Cocci	Cocci	Cocci
Gram stain	+	+	+
Motility	–	–	–
Acid-fast staining	–	–	–
Culture characteristics			
Aerobic growth	+	+	+
Anaerobic growth	+	+	+
Growth at 25°C	+	+	+
Growth at 45°C	+	+	+
Physiological characteristics			
Catalase	–	–	–
Gas from glucose	–	–	–
Acid from			
Glucose	+	+	+
D-xylose	+	–	+
Mannitol	+	–	–
Cellobiose	+	+	+
Esculine	+	+	+
Saccharose	+	+	+
Raffinose	–	+	–
Lactose	+	+	+

+, positive; –, negative.

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 3). All selected strains demonstrated a broad spectrum of activity against all *Ent.* strains, *Leuconostoc*, *L.* and *Ped.* strains tested and relatively strong inhibition activity against the growth of *L. monocytogenes* compared to other indicators. However, they did not inhibit the growth of Gram-negative bacteria such as *E. coli* and *Salm. enterica* serovar Typhimurium. Interestingly, the *Ent. faecium* SH 528 strain exhibited antagonistic activity against *Cl. perfringens.*

Cell growth and bacteriocin production

When the pH of culture broth was not controlled, the bacteriocin production of the three strains (SH 528, SH 632 and SH 740) began in the middle of the exponential growth phase, reached maximum levels (3200, 400 and 3200 AU ml^{-1}, respectively) in the stationary phase, and then declined (Fig. 1).

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

Antimicrobial activities of cell-free supernatants of all selected strains were completely inactivated by treatment with proteolytic enzymes, Proteinase K, protease XIV and trypsin, but they were not affected by treatment with α-amylase, β-amylase and catalase (Table 4). The bacteriocins of SH 632 and SH 740 strains were highly thermostable, maintaining antibacterial activities even after incubation at 95°C for 30 min, but the inhibitory activity of SH 632 was lost when incubated at 121°C for 15 min. Bacteriocin activities of the three selected strains were stable from pH 2–9 for 1 h and were not affected by exposure to organic solvents at concentrations of 50%.

PCR amplification of enterocin and pediocin genes

PCR products of ~130 bp and 160 bp were amplified from *Ent. faecium* SH 528 with primers for enterocin A and B, while PCR products of ~130 bp and 100 bp

Table 3 Antimicrobial spectra of three strains isolated from the gastrointestinal tract of broiler chickens

Indicator strains	Medium	SH 528 (AU ml^{-1})	SH 632 (AU ml^{-1})	SH 740 (AU ml^{-1})
B. cereus KCTC 1012	BHI	–	–	–
Cl. perfringens KCTC 3269	BHI	200	–	–
E. coli KCTC 1467	BHI	–	–	–
E. coli KCTC 11682	BHI	–	–	–
Enterobacter aerogenes KCTC 2190	BHI	–	–	–
Enterococcus durans KCTC 3121	MRS	800	100	400
Enterococcus faecalis KCTC 2011	MRS	200	100	800
Enterococcus faecalis KCTC 3206	MRS	400	100	400
Enterococcus faecium KCTC 3122	MRS	400	100	100
Enterococcus faecium KCTC 3513	MRS	200	100	400
Klebsiella pneumoniae KCTC 2208	BHI	–	–	–
Lact. acidophilus KCTC 3111	MRS	–	–	–
Lact. casei KCTC 3109	MRS	–	–	–
Lact. delbrueckii KCTC 1047	MRS	–	–	–
Lact. fermentum KCTC 3112	MRS	–	–	–
Lact. plantarum KCTC 3108	MRS	–	–	–
Lact. sake KCCM 40264	MRS	100	–	–
Leuconostoc mesenteroides KCTC 3505	MRS	–	–	400
L. monocytogenes KCTC 3569	BHI	3200	800	1600
L. monocytogenes KCTC 3710	BHI	400	400	400
L. innocua KCTC 3586	BHI	400	400	400
Ped. acidilactici KCTC 1626	MRS	100	–	100
Ped. dextrinicus KCTC 3506	MRS	800	–	200
Proteus mirabillis KCTC 2566	BHI	–	–	–
Pseudomonas aeruginosa KCTC 1750	BHI	–	–	–
Salm. enterica serovar Typhimurium KCTC 2515	BHI	–	–	–
Staph. aureus KCTC 1621	BHI	–	–	–
Staph. aureus KCTC 1916	BHI	–	–	–
Staph. epidermidis KCTC 1917	BHI	–	–	–

–, no inhibition zone.

were amplified from *Ent. faecium* SH 632 with primers for enterocin P and L50 A and B. Also, a PCR product of 191 bp was obtained from *Ped. pentosaceus* SH 740 using the primer for pediocin PA-1 (Fig. 2).

Acid and bile tolerance

Results from the acid tolerance tests demonstrated that *Ent. faecium* SH 528 withstands acid better than the other two strains (Fig. 3a). SH 528 and SH 632 exhibited good survival rates (6·4–6·5 log CFU ml^{-1}) at pH 3·0. However, after 2 h of incubation at pH 2·3, SH 528 retained a moderate rate of survival (4·9 log CFU ml^{-1}), whereas viable counts of SH 632 and SH 740 were decreased to 1·3–2·0 log CFU ml^{-1}. Each strain was resistant to 0·5% bile salts (Fig. 3b).

Assay of digestive enzyme activity

The digestive enzyme activity of three selected strains isolated from intestinal contents of broiler chickens and two other enteric bacteria were tested *in vitro*. The amylase and cellulase activities of strains SH 528, SH 632 and SH 740 were significantly higher than other enteric bacteria including *E. coli* and *Bacillus subtilis* ($P < 0.05$) (Table 5). However, there were no large differences in protease or lipase activity between the strains tested.

Discussion

Probiotics have received increasing attention as an alternative to subtherapeutic antibiotics and for the purpose of improving productivity in the poultry industry. Benefi-

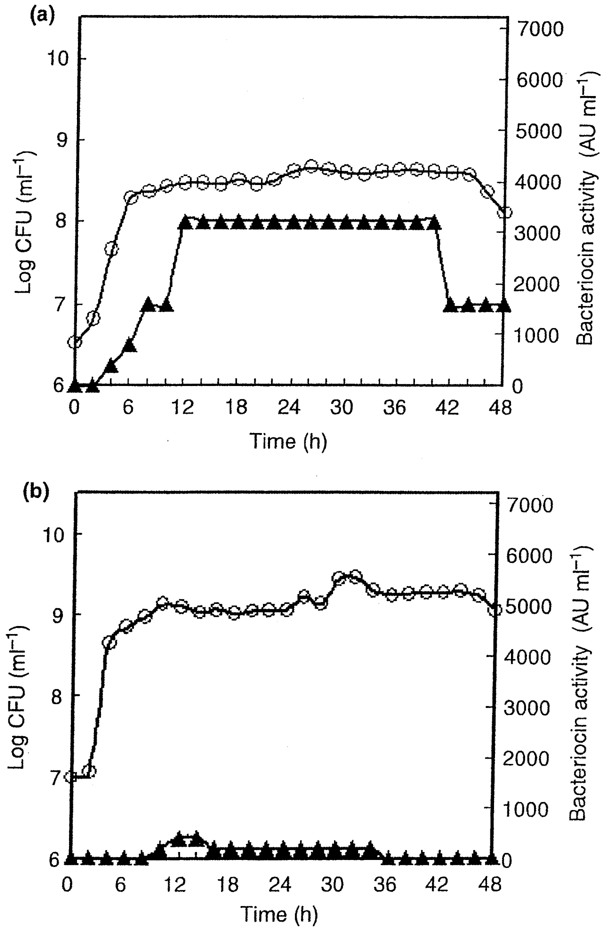

Figure 1 Cell growth and bacteriocin production of (a) *Ent. faecium* SH 528; (b) *Ent. faecium* SH 632; (c) *Ped. pentosaceus* SH 740 in MRS broth. O, viable cell count; ▲, bacteriocin activity.

Table 4 Effects of enzymes, heat, pH and organic solvents on the activity of the bacteriocins partially purified from the selected strains

	Bacteriocin activity (AU ml^{-1})		
Treatment	SH 528	SH 632	SH 740
Control	3200	400	1600
Enzymes			
Proteinase K	0	0	0
Protease XIV	0	0	0
Trypsin	0	0	0
α-Amylase	3200	400	1600
β-Amylase	3200	400	1600
Catalase	3200	400	1600
Heating			
60°C, 30 min	3200	400	1600
95°C, 30 min	3200	100	1600
121°C, 15 min	400	0	400
pH			
pH 2·0	3200	400	1600
pH 3·0	3200	400	1600
pH 4·0	3200	400	1600
pH 5·0	3200	400	1600
pH 6·0	3200	400	1600
pH 7·0	3200	400	1600
pH 8·0	3200	400	1600
pH 9·0	3200	400	1600
pH 10·0	1600	400	400
Organic solvents			
Ethanol	3200	400	1600
Methanol	3200	400	1600
Chloroform	0	200	1600
Acetone	3200	200	1600
Acetonitrile	3200	200	1600
Hexane	3200	400	1600
Ethyl acetate	3200	400	1600
Acetate	3200	400	1600

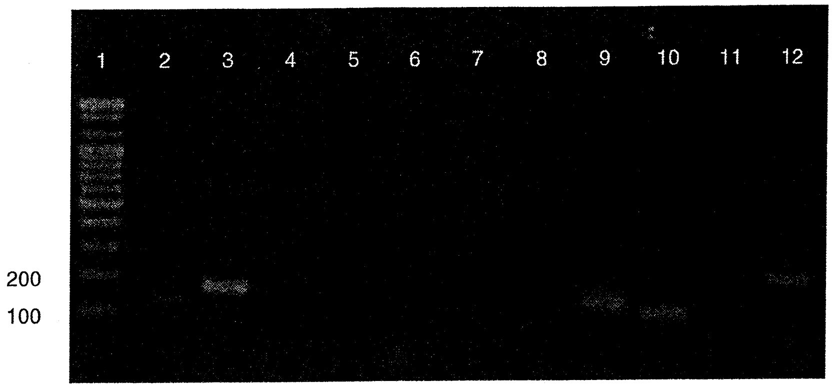

Figure 2 PCR products for the detection of enterocin and pediocin structural genes in SH 528 strain (lane 2–6), SH 632 strain (lane 7–11) and SH 740 strain (lane 12). Lanes: 1, 100-bp ladder; 2 and 7, PCR products with enterocin A primers; 3 and 8, PCR products with enterocin B primers; 4 and 9, PCR products with enterocin P primers; 5 and 10, PCR products with enterocin L50 primers; 6 and 11, PCR products with enterocin Q primers; 12, PCR products with pediocin PA-1 primers.

cial traits of probiotic bacteria in animal production are the synthesis of antimicrobial substances such as organic acids, hydrogen peroxide and bacteriocins that inhibit the growth of pathogenic micro-organisms (Kosin and Rakshit 2006), as well as producing enzymes with activities that result in increased nutrient utilization and availability within the intestinal tract (Nahashon *et al.* 1994). In total, 291 strains were isolated from the gastrointestinal tract of broiler chickens, and three strains were selected based on their antimicrobial activities mediated through bacteriocins. This study describes the partial characterization of bacteriocins produced by *Ent. faecium* SH 528, *Ent. faecium* SH 632 and *Ped. pentosaceus* SH 740 and suggests they may be useful probiotics for use in the poultry industry.

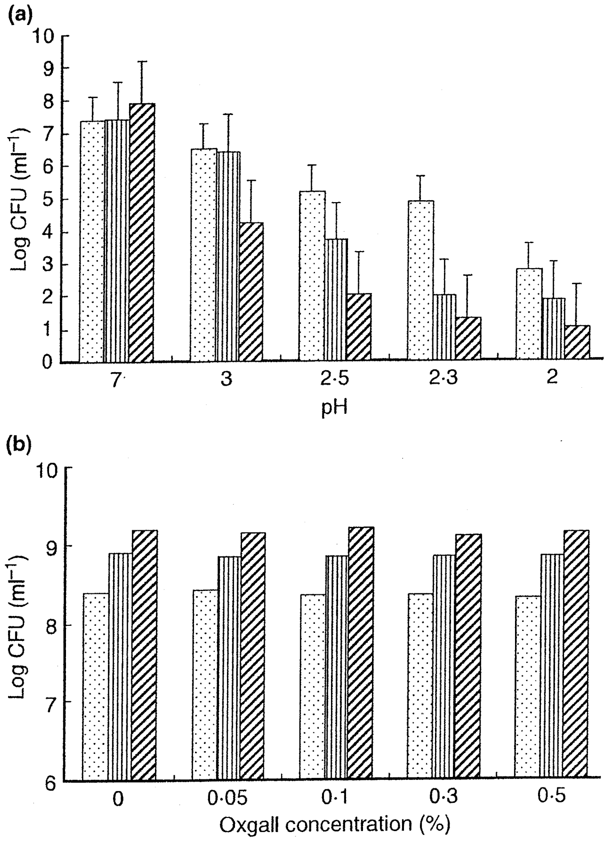

Figure 3 Acid tolerance (a) and bile-salt resistance (b) of the isolated strains in sodium phosphate buffer at various pHs for 2 h and in MRS agar containing oxgall for 48 h, respectively. (░), SH 528; (▥), SH 632; (▨), SH 740.

A number of probiotic bacteria have been studied for their abilities to inhibit poultry enteropathogenic bacteria (Barbosa *et al.* 2005; Kizerwetter-Świda and Binek 2005; Timmerman *et al.* 2006; Van Coillie *et al.* 2007). However, the LAB that were isolated from poultry and identified as bacteriocin producers with inhibitory activities against pathogens include *Lact. acidophilus* (Juven *et al.* 1992), *Lact. reuteri* (De Lima and Filho 2005), *Lact. salivarius* (De Lima and Filho 2005; Pilasombut *et al.* 2006; Stern *et al.* 2006), and *Ent. faecium* (Audisio *et al.* 2000; Strompfová *et al.* 2003). Among them, the bacteriocins produced by *Lact. salivarius* and *Ent. faecium* are inhibitory to *Camp. jejuni* and *Salm. pullorum*, respectively. In our study, bacteriocins produced by three selected strains, SH 528, SH 632 and SH 740, exhibited antagonistic activities against *L. monocytogenes* and/or *Cl. perfringens* and also had inhibitory activities against Gram-negative bacteria such as *E. coli* and *Salm. enterica* serovar Typhimurium; this latter antimicrobial activity was primarily due to the accumulation of organic acids (data not shown).

Subtherapeutic antibiotics have been used for controlling *Cl. perfringens*-associated necrotic enteritis in poultry (Dahiya *et al.* 2006). Enteric pathogens of poultry such as *Cl. perfringens*, *E. coli*, *L. monocytogenes* and *Salm.* sp., which are also considered to be responsible for human gastroenteritis, are an important concern to the poultry industry because of lost productivity, increased mortality and bacterial contamination of poultry carcasses during poultry processing. Supplementing poultry feed with BPB can have a direct effect on reducing the existing populations of foodborne pathogens, and long-term colonization

Table 5 Comparison of enzyme activities produced from the selected strains

	Total activity (unit ml^{-1})			
Strains	Amylase	Cellulase	Protease	Lipase
Enterococcus faecium SH 528	1·190 ± 0·252^{a}	0·253 ± 0·013^{a}	0·030 ± 0·006^{a}	0·150 ± 0·087^{b}
Enterococcus faecium SH 632	0·905 ± 0·156^{b}	0·280 ± 0·008^{a}	0·028 ± 0·006^{a}	0·098 ± 0·007^{b}
Pediococcus pentosaceus SH 740	0·487 ± 0·048^{c}	0·133 ± 0·072^{b}	0·027 ± 0·005^{a}	0·001 ± 0·001^{c}
Escherichia coli KCTC 1682	0·001 ± 0·001^{d}	0·001 ± 0·001^{c}	0·030 ± 0·004^{a}	0·028 ± 0·007^{c}
Bacillus subtilis KCTC 3135	0·046 ± 0·011^{d}	0·011 ± 0·007^{c}	0·034 ± 0·008^{a}	0·240 ± 0·082^{a}

Values are given as mean ± SD. Values with different superscripts in the same column differ ($P < 0{\cdot}05$).

with BPB would prevent re-introduction of pathogenic bacteria (Diez-Gonzalez 2007). Mahadeo and Tatini (1994) reported that nisin reduced the growth of the pathogen *Listeria* when added to scald water from a poultry processing plant. Multispecies or multistrain probiotics have been shown to be more effective than monospecies probiotics in growth performance and mortality of broilers (Timmerman *et al.* 2004). Therefore, a combination of bacteriocins and/or the three selected strains (SH 528, SH623 and SH 740) that show a different array of inhibition spectra against pathogens could be used in controlling enteric pathogens (especially *L. monocytogenes* and *Cl. perfringens*) in the gastrointestinal tract of broiler chickens and further reduce the contamination by pathogens in poultry processing.

The antimicrobial activity of the bacteriocins produced by the three selected strains dramatically decreased at 36–48 h in prolonged fermentation (Fig. 1). This pattern has been observed for other LAB bacteriocins (Daba *et al.* 1991; Aasen *et al.* 2000). Bacteriocins are often produced during the growth phase and then lost due to proteolytic degradation, protein aggregation and adsorption by the cells (Parente *et al.* 1994; De Vuyst *et al.* 1996; Aasen *et al.* 2000). Proteolytic enzymes abolished the antimicrobial activities against the indicator strains, indicating that the inhibitory substances produced by SH 528, SH 632 and SH 740 were proteinaceous. There was no loss of antagonistic activities following treatment with amylase, organic solvents and catalase, probably due to the absence of carbohydrate moieties in the molecule and of hydrogen peroxide. These results suggest that the inhibitory compounds are bacteriocins.

PCR amplification has been used to demonstrate the presence of structural enterocin genes among enterococci isolated from pig feces (Du Toit *et al.* 2000). Several studies have demonstrated that multiple bacteriocin production by enterococci occurs frequently (De Vuyst *et al.* 2003; Poeta *et al.* 2007). The production of multiple bacteriocins by single strains and repeated isolation of the same enterocins by different groups may reflect efficient gene transfer and their diversity in enterococci in nature (Poeta *et al.* 2007). The enterocin A and B genes were found in *Ent. faecium* SH 528. The structural gene of enterocin A is widely distributed among *Ent. faecium* strains and is generally found with enterocin B. The structural enterocin L50A/B and P gene were seen in *Ent. faecium* SH 632. Cintas *et al.* (2000) reported that enterocin L50 is maximally synthesized at 16–25°C and is not detected at 37°C or above, whereas enterocin P and Q production was optimal at higher temperatures (37–47°C). According to the above results, the bacteriocin produced by *Ent. faecium* SH 632 cultured at 37°C will not be an enterocin L50-like bacteriocin. Therefore, further studies are necessary to investigate the effects of temperature, pH and medium for the optimization of bacteriocin production. The pediocin gene was also found in *Ped. pentosaceus* SH 740. To our knowledge, *Ped. pentosaceus* SH 740 is the first pediocin producer of chicken origin.

Resistance to low pH and bile salts are prerequisites for probiotics to survive and grow in the intestinal tract (Gilliland *et al.* 1984; Ehrmann *et al.* 2002). In comparison to humans and domestic animals, the alimentary tract of chickens is shorter and the time required for feed to pass through the entire alimentary canal is as short as 2·5 h (Duke 1977). Therefore, acid tolerance of bacterial strains in chicken is not as crucial as for those in other animals where the food passage rate is much longer. Among the selected strains, two strains showed a good survival rate at pH 3·0, and all of them were tolerant to 0·5% bile salts. Recent studies have indicated that supplementation of amylolytic cultures improved digestibility of nutrients (Lee *et al.* 2001; Onderci *et al.* 2006) and that high amylase activity was an important condition for selection of probiotics in order to increase starch hydrolysis. *Ent. faecium* SH 528 and SH 632 strains had a higher amylase activity than other strains tested. Together these results suggest that the three selected strains, which exhibited high amylase activities, low pH and bile-salt tolerance, as well as host specificity, are potential candidates for use as probiotics in poultry production.

In conclusion, the bacteriocins produced by *Ent. faecium* SH 528, *Ent. faecium* SH 632 and *Ped. pentosaceus* SH 740 isolated from broiler chickens showed a wide spectrum of inhibitory activity against enteric pathogens, *L. monocytogenes* and *Cl. perfringens*. They also had desirable probiotic characteristics such as acid resistance, bile tolerance and digestive enzyme activities. Further *in vivo* field tests are necessary to investigate the effects of mixed three BPB on the production of poultry and the antimicrobial activities with synergic effects against pathogenic bacteria.

Acknowledgements

This study was supported by Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry, Republic of Korea.

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