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ARTICLE

Screening of Bacteriocin-producing *Bacillus* Strains Isolated from Domestic Animal Feces for Antagonistic Activities against *Clostridium perfringens*

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Abstract

The purpose of this study was to isolate and characterize bacteriocin-producing bacteria against *Clostridium perfringens* from domestic animals to determine their usefulness as probiotics. The feces of cattle and chicken were used as sources to isolate bacteriocin-producing bacteria using the spot-on-lawn method. In total, 900 bacterial stains were isolated from domestic animal feces, and 19 strains were finally selected after determining the inhibitory activity against the pathogenic indicator *C. perfringens* KCTC 3269. Eighteen strains of *Bacillus subtilis* and one strain of *Brevibacillus parabrevis* were identified by 16s rRNA sequencing. Most of the bacterial strains isolated were resistant to 0.5% bile salts and remained viable after 2 h at pH 3.0. Additionally, some *B. subtilis* strains showed strong inhibitory activity against *Listeria monocytogenes*. We isolated and screened *B. subtilis* strains CB 153 and CB 189 from cattle and *B. subtilis* MSC 156 and *B. parabrevis* MSC 164 from chickens using probiotic selection criteria such as inhibition activity against *C. perfringens* and tolerance to acid and bile salts. The isolated bacteriocin-producing bacteria and/or bacteriocin have the potential to be used as probiotics in the livestock industry.

Key words: bacteriocin, *Bacillus*, *Clostridium perfringens,* probiotics

Introduction

Clostridium perfringens, known as the 'flesh eating' bacteria, is widely distributed in the environment, food and intestine as the normal gut flora in human and animals (Steele and Wright, 2001). The *C. perfringens* strain is a gram-positive, rod-shaped, spore-forming, anaerobic bacterium that is capable of causing a broad spectrum of diseases in both human and animals (Hatheway, 1990; Rood and Cole, 1991). The pathogenic importance of this organism as the causative agent of gas gangrene was discovered at the end of the 20th century and since then the organism has been the object of intensive study (Ispolatovskaya, 1971). Besides its involvement in gas gangrene and food poisoning in human, various forms of acute enteritis and fatal enterotoxaemia in animals have been attributed to *C. perfringens*. The disease is routinely controlled by the prophylactic supplementation of feed or water with a variety of antibacterial drugs. However, the continued feeding of antibiotics at sub-therapeutic levels has created concerns about the extent to which usage increases the possibilities of antibiotic residue in meat, the development of antibiotic-resistant bacteria, imbalance of beneficial normal gut flora, and a reduction in the ability to cure bacterial infections in human and animals (Jensen, 1998). Increased awareness of the potential problems associated with the use of antibiotics has stimulated research efforts to identify alternatives to their use as feed additives. Among these alternatives, probiotics have received much attention as the most promising substitute to in-feed antibiotics and for improving animal productivity (Park and Ryu, 1995).

Probiotics are live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance (Fuller, 1989). These effects were due to their ability to produce a variety of inhibitory substances including organic acids, fatty acids, hydrogen peroxide, diacetyl, and bacteriocins (Ouwehand and Vesterlund, 1998). Bacteriocins are ribosomally synthesized peptides

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or proteins with antimicrobial properties that often target bacterial species that are closely related to the producer strain (Klaenhammer, 1993). Therefore, bacteriocins can be distinguished from peptide antibiotics in that they are ribosomally synthesized rather than secondary metabolites (Hurst, 1981). On account of the increased antibiotic resistance among pathogens, bacteriocins have attracted attention as an alternative means to prevent infection by pathogens. A large number of bacteriocins from lactic acid bacteria (LAB) in fermented foods and human sources to inhibit foodborne pathogens have been isolated and characterized (Cleveland *et al*., 2001; Vlaemynck *et al*., 1994). For instance, colicins and microcins produced by *E. coli* can play a promising role in preventing *Salmonella* contamination in the poultry industry (Gillor *et al*., 2004). In addition, nisin and lacticin 3147, have been found useful in preventing mastitis in animals. Recent studies have shown that *Lactobacillus salivarius* strains isolated from chicken intestine produced bacteriocins with antagonistic effect against *Campylobacter jejuni* and Gram-positive bacteria (Pilasombut *et al*., 2006; Stern *et al*., 2006). Spore probiotics are being used extensively in humans as dietary supplements and in animal as growth promoters and competitive exclusion agents due to the resistance to heat and low pH of gastric barrier (Cutting, 2011). Bacteriocin-producing *Bacillus* could be used as probiotics in livestock to improve animal health and inhibit pathogenic bacteria (Abriouel *et al*., 2011). Despite of their potentials as an alternative to antibiotics, few studies have investigated the use of bacteriocin-producing *Bacillus* in the animal industry (Diez-Gonzalez, 2007).

The purpose of this study was to isolate and characterize the bacteriocin-producing bacteria with antagonistic activities against *C. perfringens* from domestic animals, and to develop a potential candidate for probiotic use in the domestic animals as an alternative to antibiotics.

Materials and Methods

Bacterial strains and culture condition

Bacillus subtilis (*B. subtilis*) CB 153, CB 174, CB 175, CB 181, CB 186, CB 189, CB 191, CB 192, CB 193 (isolates from cattle), *B. subtilis* MSC 151, MSC 156, MSC 176, MSC 177, MSC 181, MSC 182, MSC 183, MSC 187, MSC 199 and *Brevibacillus parabrevis* MSC 164 (isolates from chicken) strains were isolated from the feces of domestic animals and maintained at -70°C in Tryptic Soy broth (Difco Laboratories, USA) containing 50% glycerol. Twenty three strains for indicator organism were obtained from the Korean Collection for Type Culture (KCTC), Korean Culture Center of Microorganisms (KCCM) and our collection from domestic animals for further studies. The strains were propagated in appropriate media such as Brain Heart Infusion or deMan Rogosa Sharpe broth (Difco Laboratories).

Isolation of antimicrobial substance-producing *Bacillus* spp. from the feces of domestic animals

The fresh feces samples, which were obtained from farms, were heated at 80°C for 20 min in sterile saline, serially diluted ten-fold with saline solution, and then plated onto TS agar plus 0.6% yeast extract and incubated at 37°C. After incubation, approximately twenty colonies per sample were randomly selected with sterilized toothpicks and inoculated into 1 mL TS broth in microcentrifuge tubes. The isolates were grown for 1 day at 37°C and 3 μL of culture broth were spotted on BHI agar prepared by inoculating 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 production of bacteriocin.

Inhibitory spectrum of activity

Cells were pelleted by centrifugation (7,000 g for 10 min). The supernatants were adjusted to pH 6.5 with 1N NaOH or HCl in order to eliminate the effect of organic acid, and filtered through 0.2 μm pore size membrane filters (Sartorius stedim biotech, Germany), and used to detect antagonistic activity against indicator organisms according to the spot-on-lawn method (Mayr-Harting *et al*., 1972; Varadaraj *et al*., 1993). The supernatants were serially diluted, and 10 μL samples were spotted onto the surface of soft BHI or MRS agar (0.7 %) seeded with an overnight culture of an indicator strain. In case of *Clostridium* spp. an overnight culture was closely streaked onto the surface of BHI agar using a sterile cotton swap (Teo and Tan, 2005). After incubation for 24 h, 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 using an API 20E kit (Biomérieux, France) were assessed. The 16S rRNA was sequenced using the 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 *Bacillus* and other related taxa in order to compare the levels of similarity.

Growth curve and bacteriocin production in TS medium

The growth curve and bacteriocin production were investigated in TS medium. Selected strains (*B. subtilis* CB 153, *B. subtilis* CB 189, *B. subtilis* MSC 156 and *B. parabrevis* MSC 164) were incubated in 200 mL of TS 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 TS 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 were centrifuged at 10,000 g for 10 min at 4°C, and the supernatant was adjusted to pH 6.5 with 5N NaOH or 6N HCl and filter-sterilized through 0.2 μm pore size membrane filters.

Effects of heat, pH and enzyme on bacteriocin activity

The effect of heat, pH and enzymes on the activities of the partially purified bacteriocin was examined as described by Lyon and Glatz (1993). 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 against *C. perfringens* KCTC 3269 was determined by the spot-on-lawn assay. To investigate the effects of pH on antimicrobial stability, the pHs of the supernatants were adjusted between 2 and 10 with either 1N HCl or 1N NaOH and incubated at 30°C for 1 h. The 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 heated at 80°C for 10 min to inactivate the enzymes.

Survival and growth at low pHs, bile salts and various temperatures

Acid and bile salt tolerance were performed as described by Shin *et al*. (1999). To test acid and heat tolerance, overnight cultures in TS broth of four selected strains were harvested at 3,000 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, 2.3, 2.5, 3.0, 4.0, 5.0, 6.0 and 7.0 with 1 N HCl. The suspensions were incubated at 37°C for 2 h and the viable cell counts were determined by the spread plate method on TS agar. For the heat tolerance test, selected strains were exposed at each temperature range (50, 60, 70, 80, and 90°C) for 30 min. Then, the suspensions were properly diluted and the viable cell counts were determined on TS agar by spreading method. Bile tolerance was determined by spreading the cells on TS agar plates containing ox gall 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.

Results

Isolation and identification of antimicrobial substance-producing bacteria

A total of 900 strains were isolated from the feces of cattle and chicken, and 44 strains out of them showed inhibitory activities in the first screening step (data not shown). Nineteen strains were ultimately selected as antimicrobial substance-producing candidates, and each exhibited slightly different antimicrobial activities against the indicator, *C. perfringens* KCTC 3269. The strains were characterized as a Gram-positive, catalase-positive, and strict aerobic rod-shaped bacterium. Gram staining confirmed that these strains posses endospore and are spore formers. Based on comparisons of their characteristics with Bergey's manual and the results of the API test (data not shown), the isolates were classified as *B. subtilis* CB 153, CB 174, CB 175, CB 181, CB 186, CB 189, CB 191, CB 192, CB 193, *B. subtilis* MSC 151, MSC 156, MSC 176, MSC 177, MSC 181, MSC 182, MSC 183, MSC 187, MSC 199 and *B. parabrevis* MSC 164 (Fig. 1). The selected strains were identified as 18 strains of *B. subtilis* (from cattle and layer chicken) and 1 strain of *B. parabrevis* (from chicken) by 16s rRNA sequencing.

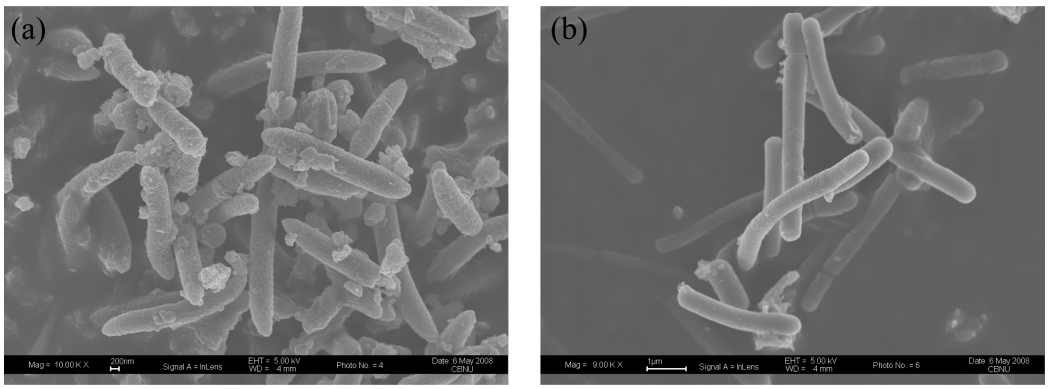

Fig. 1. Scanning electron microscopic observation of the selected isolates. (a) *B. subtilis* CB 153 (× 10,000); (b) *B. parabrevis* MSC 164 (× 9,000)

Spectrum of antimicrobial activity

The cell-free supernatants were tested for their antimicrobial activities against various Gram-positive and Gram-negative indicator organisms using the spot-on-lawn method (Table 1). All selected strains have relatively strong inhibition activity against the growth of *C. perfringens* and *Listeria monocytogenes* compared to other indicators. Also, *B. parabrevis* MSC 164 strain exhibited antagonistic activity against *C. perfringens*, a field isolate from domestic animals. However, they did not inhibit the growth of Gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella* "Typhimurium".

Table 1. Antimicrobial spectrum of selected strains isolated from cattle and chicken against various indicator organisms

Indicator organisms	Cattle		Chicken	
	CB 153	CB 189	MSC 156	MSC 164
Bacillus cereus KCTC 1012	–[a]	–	–	–
Clostridium perfringens KCTC 3269	+	+	+	+
Clostridium perfringens C2 [b]	–	–	–	+
Enterococcus faecalis KCTC 2011	–	–	–	–
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	–	–	–	–

[a]–, no inhibition zone; +, clear inhibition zone
[b]Field isolates from domestic chicken

Cell growth and bacteriocin production

When the pH of culture broth was not controlled, the antimicrobial substances were secreted at the beginning (CB 189 and MSC 164) or middle (CB 153 and MSC 156) of exponential phase (Fig. 2). The maximum antimicrobial activities of CB 189, MSC 156, and MSC 164 against *C. perfringens* were 3,200 AU/mL during the stationary phase, and then rapidly declined.

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

The anticlostridial activities of cell-free supernatants of all selected strains were completely inactivated by at least one of the protein-degrading enzymes tested, but they were not completely affected by treatment with α-amylase, β-amylase, and catalase (Table 2). No inhibition zones were observed with enzyme controls without cell-free supernatant (data not shown). The bacteriocins of *B. parabrevis* MSC 164 strains were highly thermostable, maintaining anticlostridial activities even after incubation at 121°C for 15 min, but the inhibitory activities of other strains were diminished when incubated at 60°C or 90°C for 30 min. Very small or no significant decreases in the anticlostridial activities of the filtrates from four selected strains were observed when they were adjusted from pH 2.0 to 10.0 for 1 h.

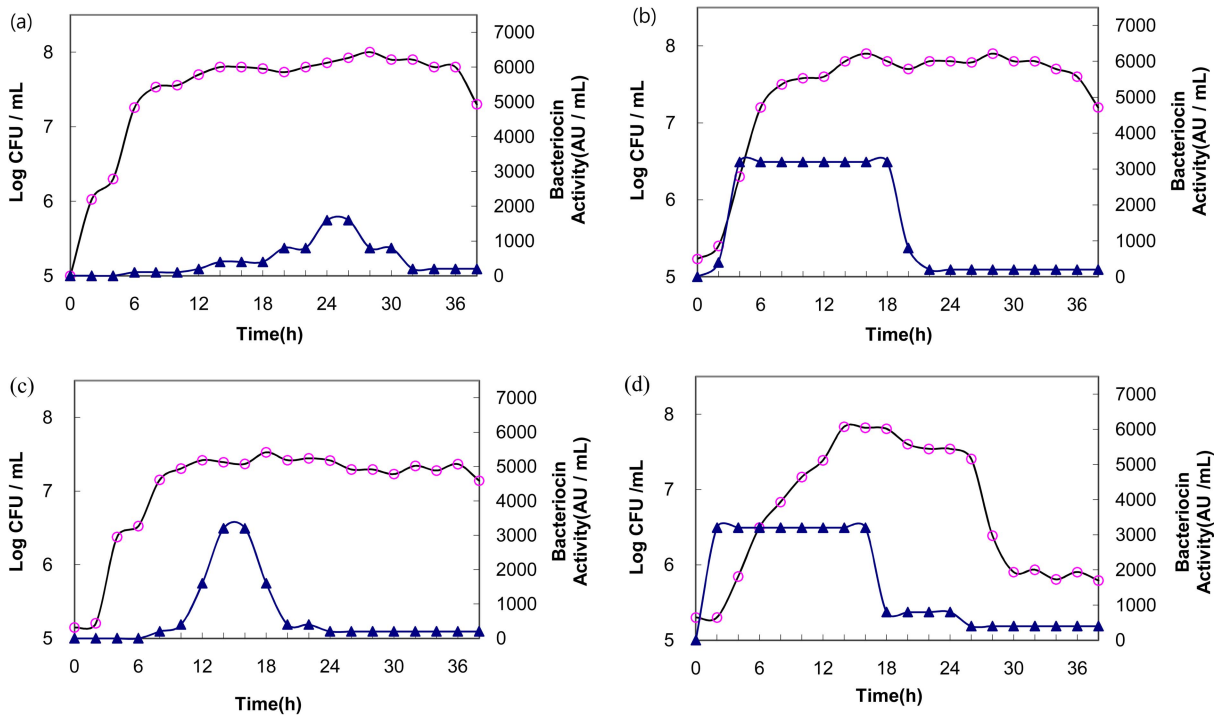

Fig. 2. Cell growth and bacteriocin production of the selected isolates in TS broth. (a) *B. subtilis* CB 153; (b) *B. subtilis* CB 189; (c) *B. subtilis* MSC 156; (d) *B. parabrevis* MSC 164. ○, viable cell count; ▲, bacteriocin activity

Acid, bile, and heat tolerance

Acid and bile tolerance were tested to evaluate the survival of probiotic bacteria after oral administration, and heat tolerance was important to retain high viability for feed preparation and storage. The acid tolerance study showed that *B. subtilis* CB 153, CB 189, *B. subtilis* MSC 156, and *B. parabrevis* MSC 164 have moderate acid tolerance at pH 2.0 as compared to initial bacterial counts (Fig. 3). Each strain was resistant to 0.5% bile salts except *B. parabrevis* MSC 164. In order to understand the influences of the thermal processing of feed containing bacteriocin-producing bacteria, preliminary examinations for heat resistance were carried out using the four isolates. All strains survived at 60°C for 30 min, but, three of the four isolates (CB 153, CB 189, and MSC 156) survived at 90°C for 30 min.

Table 2. Effect of enzyme, heat and pH on the activity of the cell-free supernatants produced by selected strains from cattle and chicken

Treatment		Relative bacteriocin activity (%)			
		Cattle		Chicken	
		CB 153	CB 189	MSC 156	MSC 164
Enzyme	Proteinase K	50	50	100	0
	Protease XIV	0	0	50	100
	Pepsin	50	50	0	100
	Trypsin	12.5	50	100	25
	α-Amylase	6.25	50	100	25
	β-Amylase	3.1	50	50	50
	Catalase	100	100	100	100
Heating	60°C, 30 min	50	50	100	100
	95°C, 30 min	50	50	50	100
	121°C, 15 min	25	50	50	100
pH	pH 2.0	100	50	100	100
	pH 3.0	100	100	100	100
	pH 4.0	100	100	100	100
	pH 5.0	100	100	100	100
	pH 6.0	100	100	100	100
	pH 7.0	100	100	100	100
	pH 8.0	100	100	100	100
	pH 9.0	100	100	100	100
	pH 10.0	100	100	100	100

Discussion

Recently health promoting microorganisms, e.g., probiotics, have been increasingly included in various food products and proposed for use as a food supplement or therapeutic tools for several infectious diseases (Hammerman *et al*., 2006; Saarela *et al*., 2000). Probiotic therapy is very attractive because it is an effective against pathogenic bacterial infection and non-invasive low cost approach on synthesis of antimicrobial substances, which attempts to recreate natural flora rather than its disruption.

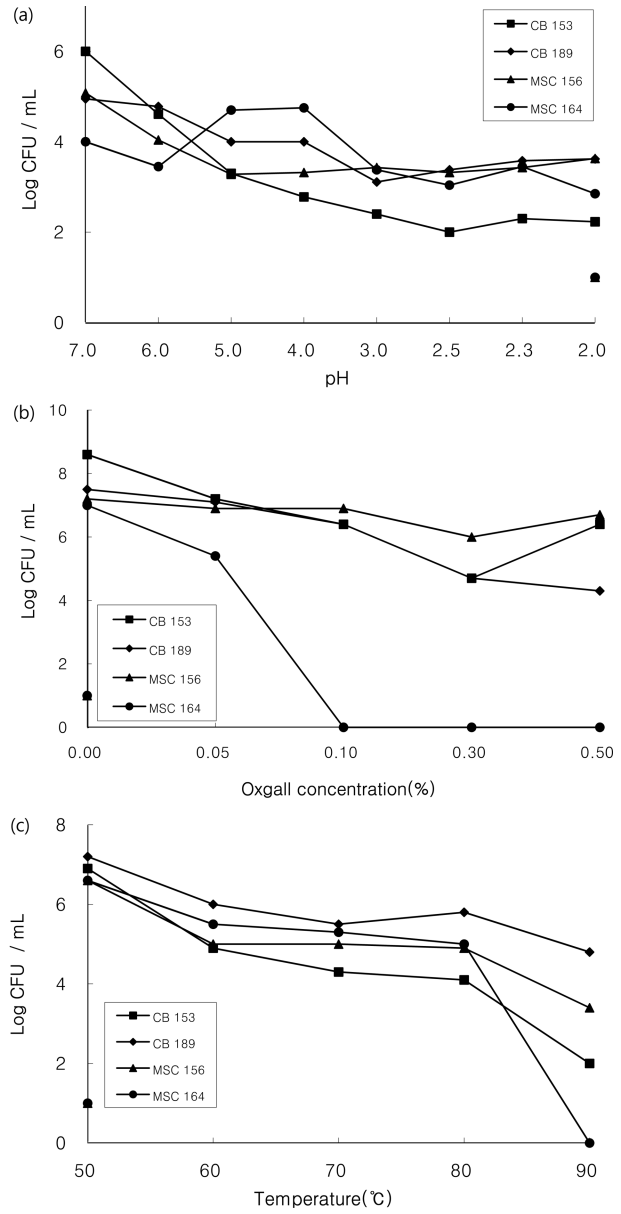

Fig. 3. Acid tolerance, bile salt resistance, and heat resistance of the selected strains in sodium phosphate buffer at various pHs for 2 h (a) and in TS agar containing oxgall for 48 h (b) and at various temperatures for 2 h (c), respectively.

In the present study, 900 strains were isolated from the feces of domestic animals, and nineteen strains were selected based on their anticlostridial activities mediated through bacteriocin. The isolates were identified as 18 strains of *B. subtilis* and 1 strain of *B. parabrevis* by 16s rRNA sequencing. Vlaemynck *et al* (1994) reported that only eight of the almost 4,000 strains, were isolated from milk, cheese and from different samples taken on the farm (silage and faeces), exhibited inhibitory activity (*L. monocytogenes*, *C. perfringens* and some *Bacillus* spp.) in the cell-free supernatant fluid. However, present studies showed higher screening ratio than Vlaemynck's experiment regardless of a kind of samples and indicator strains (Vlaemynck *et al*., 1994) because spore-forming bacteria were selected by adding a heating-step in the pretreatment of samples to kill vegetable cells before plating.

The majority of well-characterized bacteriocins are heat-stable, nontoxic, and susceptible to degradation by proteolytic enzymes of the gastrointestinal tract (Kosin and Rakshit, 2006). In our study, antimicrobial activities of cell-free supernatants of all selected strains were completely inactivated by at least one of proteolytic enzymes tested, and also partially decreased by the treatment of amylase. These results indicate that the inhibitory compound is a bacteriocin which a carbohydrate moiety is essential for the activity. The bacteriocins of the selected strains contained a proteinaceous antimicrobial factor that was stable in the presence of high heat and various pHs. It can also be inferred that the antimicrobial activity of cell-free supernatants could not be due to the production of hydrogen peroxide of selected strains.

A number of lactic acid bacteria have been shown to exhibit various degrees of antimicrobial activity against *Clostridium* spp. Previous studies have shown that *Lactobacillus rhamnosus* (Alander *et al*., 1999), *Lactobacillus plantarum* (West and Warner, 1988), and *Pediococcus pentosaceus* (Graham and McKay, 1985) are bactericidal for *Clostridium* spp. Despite extensive screening of these bacteriocins against a wide spectrum of pathogenic microorganisms, there have been few reports that demonstrated the direct effect of metabolites of *Bacillus* strains on *C. perfringens*. In this way, our results carry an important meaning. In addition, the bacteriocins of some selected strains were also inhibitory toward *L. monocytogenes* or *C. perfringens* field strain isolated from diseased chicken.

The probiotic bacteria must be able to colonize the gastrointestinal tract, survive the low pH of the stomach and bile acids in the intestines, and compete against other microorganisms in the gastrointestinal tract (Chateau *et al*., 1993; Nurmi *et al*., 1983). Most of the bacterial strains isolated exhibited resistant to 0.5% bile salts and remained viable after 2 h at pH 3.0. Interestingly *B. subtilis* CB 153, *B. subtilis* CB 189 and *B. subtilis* MSC 156, and *B. parabrevis* MSC 164 showed moderate acid tolerance at pH 2.0 as compared to initial counts. Among them, *B. subtilis* CB 153, *B. subtilis* 189, and *B. subtilis* MSC 156 are spore-forming bacteria, survived at 90°C for 30 min that can offer some advantages over the more common *Lactobacillus* products in that they can be stored

indefinitely in a desiccated form (Mazza, 1994). Moreover, the heat-stability and anti-listerial activity seem to fit the criteria of a class II bacteriocin (Héchard and Sahl, 2002).

Nineteen bacteriocin-producing bacteria against *C. perfringens* were isolated and identified from domestic animal feces. These isolates were characterized by antimicrobial spectrum, cell growth and bacteriocin production, acid, bile acid and heat tolerance. These results suggest that a combination of bacteriocins or multispecies probiotics of the selected strains has a strong potential of alternative to antibiotics in domestic animals.

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