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Screening and Characterization of Bacteriocinogenic Lactic Acid Bacteria from Jeot-Gal, a Korean Fermented Fish Food

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Abstract Bacteriocins are classified as proteins which are produced by heterogeneous groups of bacteria, having an antimicrobial effect of the closely related organisms. Recently, bacteriocins derived from lactic acid bacteria and other food-related organisms have been the subject of much research on potential food biopreservatives. The goal of this study was to screen and characterize the bacteriocinogenic lactic acid bacteria from Jeot-gal (commercial fermented fish foods). All bacteriocinogenic isolates were identified as lactic acid bacteria. Isolates NK24, NK34, and SA72 were tentatively identified as *Lactococcus lactis*, whereas isolate SA131 was recognized as *Lactobacillus brevis*, according to the API 50 CHL kit database. All antimicrobial substances produced from four lactic acid bacteria isolates completely lost their antibacterial activity after being treated with some proteases, indicating to their proteinaceous nature. The bacteriocin produced from isolates NK24, NK34, and SA72 showed a broad spectrum of activity when compared to those produced from isolate SA131. All bacteriocins isolated during the course of this study showed a bactericidal mode of inhibition.

Key words: Bacteriocin, screening, lactic acid bacteria, identification, Jeot-gal, fermented fish food

Lactic acid bacteria may be characterized as gram-positive, catalase-negative, and non-sporing bacteria. Not only do they produce both lactic and acetic acids as homo- or hetero-products, they also provide flavor and odor in dairy fermented foods, along with meat. Substances produced by lactic acid bacteria, which are used in these food fermentations, show inhibitory activities against putrefactive and pathogenic microorganisms. The inhibitory activities of lactic acid bacteria in these foods are associated with major products such as organic acids, diacetyls, hydrogen peroxide, ammonia, and bacteriocins [3, 10, 22, 23].

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Bacteriocins are proteinaceous macromolecules that can either kill susceptible bacteria by dissipating proton motif force by which essential cell processes such as protein, DNA, and ATP syntheses are interfered [22]. Even though their antibacterial efficiencies and spectra are lower and narrower than those of therapeutic antibiotics, their antagonistic specificity and easy digestibility in the human digestive tract make them good candidates as natural food preservatives or natural bioregulators of food fermentation [21]. Because bacteriocins are known as natural products derived from many microorganisms associated with foods, there is an overwhelming interest in their use as natural food preservatives today [4]. Among the bacteriocins, nisin was the first to be characterized in food bacteria, and it continues to be the most widely studied subject in terms of its chemistry, molecular biology, and application [12, 13, 18].

When compared to Europe and America, Korea is known to have more resources in relations to its use of lactic acid bacteria [11, 23]. Chang (fermented soybean sauce and pastes), Kimchi (fermented vegetables), and Jeot-gal (fermented fish foods) are the three major salt-fermented and preserved food categories in Korea. Unfortunately, domestic study of bacteriocins has been limited. Jeot-gal is the generic name of high-salt fermented fish foods, which partially contain hydrolyzed solid fish organ material immersed in liquid fish exudate. The microbiology of Jeot-gal is probably based upon the rapid fermentation of these products. Ten genera of bacteria (*Achromobacter*, *Bacillus*, *Brevibacterium*, *Flavobacterium*, *Halobacterium*, *Leuconostoc*, *Micrococcus*, *Pediococcus*, *Pseudomonas*, and *Sarcina*) and two genera of yeast (*Saccharomyces* and *Torulopsis*) have been isolated from and identified in various Jeot-gal [11]. However, not much is known about the characteristics of the lactic acid bacteria in these products or their antibacterial properties [14].

In this context, an attempt was made to isolate lactic acid bacteria which could produce bacteriocin in the Jeot-gal food environment. In this paper, the screening of novel bacteriocinogenic lactic acid bacteria from Jeot-gal, and

their tentative identification and characterization have been described in detail.

The bacterial strains used as indicator microorganisms for bacteriocin screening and antimicrobial activity evaluation were obtained from different culture collections and were cultivated in appropriate media as indicated in Table 3. Bacteriocinogenic lactic acid bacteria isolated from Jeot-gal were maintained at -70°C in lactobacilli MRS broth (Difco Laboratories, Detroit, U.S.A.) in which 20% (v/v) of glycerol was added. Working cultures were propagated in MRS broth at 30°C for 12 h before they could be used in the experiments. Various kinds of Jeot-gal were used as potential sources of novel bacteriocinogenic lactic acid bacteria. Jeot-gals were purchased from several department stores in Masan city, Korea. To isolate the bacteriocin producing bacteria, the 'general spreading method' and 'triple agar layer method' were adopted. For the general spreading method, serial dilutions were made in sterile water, spread onto MRS agar plates, and incubated at 37°C for 24 h. Colonies were then streaked onto fresh MRS agar plates. For using the triple agar layer method [8], 0.1 ml serial dilutions were poured onto 5-ml MRS agar (50°C) plates and solidified, and then 5 ml MRS (0.75% agar) was then poured over the plate. Five milliliter of MRS soft agar, seeded with fresh indicators (*Lactococcus lactis* CA170-12, *L. lactis* KCCM 40104, *Lactobacillus delbrueckii* ATCC 4797, *Leuconostoc mesenteroides* KCCM 11324, *Escherichia coli* KCCM 32396, and *E. coli* JM 109) were finally poured over the plate. After incubation for 24 h at 37°C, the plates were turned over, and the colonies showing a distinct halo were carefully dug out and purified by streaking on the MRS agar [5].

For the bacteriocin activity assay, the spot-on-lawn method was performed as described previously [16]. For the purpose of identifying bacteriocinogenic strains, the following characteristics were examined: cell morphology, Gram stain, catalase test, and biochemical carbohydrate fermentation patterns, using an API 50 CHL kit (bioMereux, France) [4, 9]. Bacteriocin-like substance (BLIS) production was performed in a 500 ml flask and MRS broth was used as a fermentation medium. Strains were cultured at 30°C for 24 h. Culture broths were adjusted to pH 7.0, and centrifuged at 8,000 ×g for 20 min at 4°C. Supernatants were filter-sterilized through 0.22 μm pore size cellulose acetate [6]. To determine the stability towards various enzymes, cell-free supernatants of bacteriocinogenic strains were treated at 37°C for 1 h with various enzymes at a final 1 mg/ml concentration. All enzymes were suspended in buffers, as recommended by its supplier (Sigma, St. Louis, U.S.A.). Untreated bacteriocin plus buffers, and buffers, and enzyme suspensions alone served as controls [6, 17]. The spot-on-lawn method was primarily used to assess the antimicrobial activity of the cell-free supernatant of the isolated bacteriocinogenic lactic acid bacteria against several gram-positive bacteria, gram-negative bacteria, yeast, and mold. All strains, which were previously subcultured in an appropriate growth agar medium, were propagated in a liquid medium, and then inoculated into a soft agar media (0.75% agar) [17]. Cells from the log-phase of *Micrococcus flavus* ATCC 10240 and *Pediococcus acidilactici* KCTC 1626 were suspended in a sterile 100 mM phosphate buffer (pH 7.0). Tests were conducted at 30°C by adding 400 AU/ml and 3,200 AU/ml of cell-free supernatant of bacteriocin producers, respectively. Samples were taken out at every 30 min, and the viable cells (CFU/ml) were determined on MRS agar plates by using the standard plate counting method [15].

It has long been suggested that lactic acid bacteria play an important role in antibiosis as well as the fermentation of Jeot-gal. Souane *et al.* [20] reported that the microflora of Jeot-gal, which is a Korean low-salt fillet-fish product, was dominated by lactic acid bacteria. To screen bacteriocinogenic lactic acid bacteria, about 400 lactic acid bacteria were successfully isolated from various kinds of Jeot-gal by using MRS agar media. They were tested for their antimicrobial activity towards six indicator strains, using the spot-on-lawn method. In this test, only two isolates (isolates NK24 and NK34) showed antimicrobial activity towards some of the indicator strains. The antagonistic activities of these two isolates were sensitive to proteolytic enzymes, which indicated that their activities were due to bacteriocins. On the other hand, the triple agar layer method was required to directly screen antimicrobials-producing lactic acid bacteria from the different types of Jeot-gals (pollack tripe, sea arrow, *et al.*). The isolates obtained using the triple agar layer method were retested for their antimicrobial activity using the spot-on-lawn method. Among them, only two isolates (isolates SA72 and SA131) proved to be sensitive to proteolytic enzymes. Upon dilution of the bacteriocins, the zones of inhibition on lawns of the indicator strain diminished in size without appearance of plaques, suggesting that the inhibition was not caused by the replication of bacteriophage.

Bacteriocin-producing strains of NK24, NK34, SA72, and SA131 were identified by gram-staining, a catalase test, morphology, and biochemical carbohydrate fermentation patterns using a API 50 CHL kit. On the basis of these results, strains NK24, NK34, and SA72 were tentatively identified as *L. lactis*, whereas SA131 was tentatively identified as *L. brevis* (Table 1).

In order to elucidate whether the antagonistic activity of isolates from Jeot-gal was due to bacteriocins or other inhibitory substances such as antibiotics, they were treated with enzymes. As is shown in Table 2, treatment of NK24 and NK34 bacteriocins with protease IX or protease XIV caused complete loss of bacteriocin activity. On the other hand, SA72 and SA131 bacteriocins were inactivated by treatment with protease XIV, α-chymotrypsin, and proteinase

Table 1. Microbiological tentative identification of bacteriocin producers by carbon source utilization pattern.

	Bacteriocin producers					Bacteriocin producers			
	NK24	NK34	SA72	SA131		NK24	NK34	SA72	SA131
Morphology	Coccus	Coccus	Coccus	Rod	Esculine	+	+	-	-
Gram staining	+	+	+	+	Salicine	+	+	+	+
Glycerol	-[a]	-	-	-	Cellobiose	+	+	+	+
Erythritol	-	-	-	-	Maltose	+	+	+	+
D-Arabinose	-	-	-	-	Lactose	+	+	+	-
L-Arabinose	+	+	+	-	Melibiose	-	-	-	-
Ribose	+	+	+	+	Saccharose	+	+	+	+
D-Xylose	+	+	+	+	Trehalose	+	+	+	+
L-Xylose	-	-	-	+	Inuline	-	-	-	+
Adonitol	-	-	-	-	Melezitose	-	-	-	-
β-Methylxyloside	-	-	-	-	D-Raffinose	-	-	-	-
Galactose	+	+	+	-	Amidon	+	+	+	-
D-Glucose	+	+	+	+	Glycogene	-	-	-	-
D-Fructose	+	+	+	+	Xylitol	-	-	-	-
D-Mannose	+	+	+	+	β-Gentibiose	+	+	+	+
L-Sorbose	-	-	-	+	D-Turanose	-	-	-	-
Rhamnose	-	-	-	-	D-Lyxose	+	+	-	-
Dulcitol	-	-	-	-	D-Tagatose	-	-	-	+
Inositol	-	-	-	-	D-Fucose	-	-	-	-
Mannitol	+	+	+	-	L-Fucose	-	-	-	-
Sorbitol	-	-	-	-	D-Arabitol	-	-	-	-
α-Methyl-D-mannoside	-	-	-	-	L-Arabitol	-	-	-	-
α-Methyl-D-glucoside	-	-	-	-	Gluconate	+	+	+	-
N-Acetylglucosamine	+	+	+	+	2-Cetogluconate	-	-	-	-
Amygdaline	+	+	+	+	5-Cetogluconate	-	-	-	-
Arbutine	+	+	+	+					

[a]Data obtained by API 50 CHL kit. +: positive; -: negative.

Table 2. Effect of various enzymes on bacteriocin activity.[a]

Enzyme treatment	Bacteriocins			
	NK24	NK34	SA72	SA131
Control	+	+	+	+
Trypsin	+	+	+	+
Protease IX	-	-	ND[b]	ND
Protease XIII	+	+	+	+
Protease XIV	-	-	-	-
Pepsin	+	+	ND	ND
α-Chymotrypsin	+	+	-	-
Proteinase K	+	+	-	-
Papain	+	+	+	+
Lipase	+	+	+	+

[a]Cell-free supernatants were digested by various enzymes.
[b]ND: Not determined.

K. A substance, which lost its inhibitory activity upon treatment by proteolytic enzyme, was considered to be a bacteriocin-like inhibitory substance (BLIS). Otherwise, the inhibitory substance was considered to be an antibiotic-like inhibitory substance (ALIS). When the cell-free supernatants were treated with lipase, the bacteriocin activity was unchanged. These results confirmed the proteinaceous nature of the antimicrobial substance and suggested that lipid moieties were not essential for bacteriocin activity.

To determine the spectrum of antimicrobial activity, the cell-free supernatant was tested against various bacteria, yeast, and mold by following the spot-on-lawn method, and the results are summarized in Table 3. The bacteriocins from strains NK24, NK34, and SA72 showed a relatively broad spectrum of activity against *Bacillus pumilis* HTD-1, *B. subtilis* IFO 12113, *L. lactis* CA170-12, *L. lactis* KCCM 40104, *Leuconostoc mesenteroides* KCCM 11324, *Micrococcus flavus* ATCC 10240, *Pediococcus acidilactici* KCTC 1626, *Propionibacterium acnes* P1, *P. acnes* P3, *Staphylococcus aureus* KCCM 32359, *E. coli* KCCM 32396, *Pseudomonas cepacia* SBA 9613, and *Sphingomonas paucimobilis* BNJ 9664, when compared with other bacteriocins of lactic acid bacteria. Accordingly, from their inhibitory spectra, these bacteriocins appeared to have more similarity with lantibiotic nisin, which inhibits the majority of gram-positive bacteria [2], than several bacteriocins from *Lactobacillus* sp., whose activity spectra include only strains of the same genus family [7, 19]. However, the bacteriocin from strain SA131 showed activity against *B. pumilis* HTD-1, *B. subtilis*

Table 3. Antimicrobial spectrum of activity of Jeot-gal bacteriocins.

	Culture Medium	Incubation Temp.	Bacteriocins			
			NK24	NK34	SA72	SA131
Gram-positive bacteria						
Bacillus cereus	NB	30	-	-	-	-
Bacillus pumilis HTD-1	NB	30	+	+	+	+
Bacillus subtilis IFO 12113	LB	37	+	-	+	+
Lactococcus lactis CA 170-12	MRS	30	ND[a]	+	-	-
Lactococcus lactis KCCM 40104	MRS	30	+	+	+	-
Lactobacillus delbrueckii ATCC 4797	MRS	37	-	-	-	+
Leuconostoc mesenteroides KCCM 11324	MRS	25	+	+	+	-
Micrococcus flavus ATCC 10240	NB	30	+	+	+	-
Pediococcus acidilactici KCTC 1626	MRS	37	+	+	-	+
Propionibacterium acnes P1	NLB	32	+	+	+	-
Propionibacterium acnes P2	NLB	32	-	-	-	+
Propionibacterium acnes P3	NLB	32	+	-	-	+
Propionibacterium acnes P4	NLB	32	-	-	-	-
Propionibacterium acnes P5	NLB	32	-	-	-	-
Staphylococcus aureus KCCM 32359	NB	37	+	+	+	-
Gram-negative bacteria						
Aeromonas hydrophila SBA 9612	NB	30	-	-	-	-
Chryseomonas luteola SBA 9634	NB	30	-	-	-	-
Escherichia coli JM109	LB	37	-	-	-	-
Escherichia coli KCCM 32396	LB	37	+	+	+	-
Pseudomonas cepacia SBA 9613	NB	30	+	+	+	-
Pseudomonas fluorescens SBB 9631	NB	30	-	-	-	-
Pseudomonas putida	NB	30	-	-	-	-
Sphingomonas paucimobilis BNJ 9664	NB	30	+	+	+	-
Xanthomonas maltophila SBC 9611	NB	30	-	-	-	-
Yeast and mold						
Aspergillus niger KCCM 11239	PDA	26	-	-	-	-
Saccharomyces cerevisiae KCCM 11201	YPD	26	-	-	-	-

[a]ND: Not determined.

IFO 12113, *L. delbrueckii* ATCC 4797, *P. acidilactici* KCTC 1626, *P. acnes* P2, and *P. acnes* P3.

Since these bacteriocins have different antimicrobial spectra, they may possess different modes of action. Because of it, each of them may show some activity of the bacteria which are resistant to other bacteriocin(s). Therefore, making use of either a mixture of these bacteriocins or of the genetically engineered bacteria producing multiple bacteriocins could provide a useful approach to reduce the frequency of resistant populations to develop, and to improve hygienic quality as well as to extend the shelf-life of raw food and food products. No indication of cell lysis due to bacteriocin was observed by microscopic investigation. Among the four bacteriocins, those produced by strains NK24 and SA72 were selected for further study. These two bacteriocins were partially purified through ammonium sulfate precipitation and their antimicrobial spectra was determined against several spoilage and pathogenic bacteria. They have attracted much attention of the food industry as potential natural food preservatives (unpublished data).

In order to test the mode of inhibition on the indicator strains in the log phase, the viable cell number of the indicator strain with and without bacteriocin were determined. A decrease in CFU per milliliter was observed after bacteriocins were exposed to indicator cells (Fig. 1). All bacteriocins from the four lactic acid bacteria showed a bactericidal action. Although Kimchi bacteriocin caseicin K319 also exhibited a bactericidal action against the indicator strain in phosphate buffer, its killing was not immediate [1]. However, all Jeot-gal bacteriocins produced an immediate effect, as is shown in Fig. 1. Unfortunately, the intrinsic nature of the bactericidal mode of inhibition exhibited by the Jeot-gal bacteriocins has not yet been identified and required further investigation for its elucidation.

Since the four bacteriocin-producing lactic acid bacteria studied in this work were isolated from Jeot-gal, it is tempting to speculate that they may be highly competitive in food systems and capable of producing bacteriocins *in situ*. Consequently, they may be more suitable as food-preserving microorganisms than microorganisms isolated

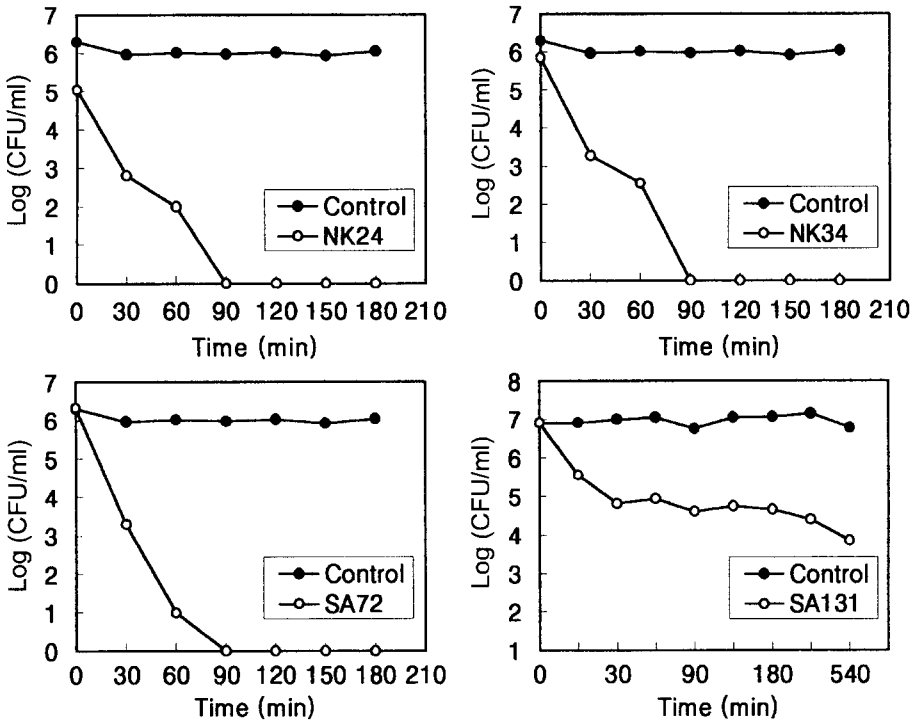

Fig. 1. Mode of inhibition of the bacteriocins produced from strains NK24, NK34, SA72, and SA131.

from different environments which produce the same bacteriocins.

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