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Inhibition of *Listeria monocytogenes* by Bacteriocin(s) from Lactic Acid Bacteria Isolated from Kimchi

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Abstract : Four strains of lactic acid bacteria which produced bacteriocins inhibitory to *Listeria* species were isolated from Kimchi, and were identified as *Leuconostoc mesenteroides* subsp. *mesenteroides* (2 strains), *Leuconostoc paramesenteroides* and *Pediococcus pentosaceus*. The bacteriocins produced by the isolates inhibited all of the *Listeria monocytogenes* strains tested, but *L. denigrificans* 28 and *L. welchimeri* 89 were not inhibited by the bacteriocin produced by the *Leu. paramesenteroides* isolate. The bacteriocin produced by the *P. pentosaceus* isolate was more inhibitory against sensitive strains and showed broader spectrum of antimicrobial activity than those produced by other isolates. The bacteriocins produced by *Leuconostoc* isolates were sensitive to pronase E treatment, but that produced by the *P. pentosaceus* isolate was not completely inactivated. The bacteriocins produced by all of the isolates were not sensitive to catalase, α-amylase and lysozyme and heat (30 min at 100℃) treatments(Received April 3, 1995; accepted April 27, 1995).

Introduction

Listeria monocytogenes has become a great concern to food industry because of its psychrotrophic nature and relatively high resistance to heat, to acid and alkali, and to high concentrations of salt as well as high mortality of listeriosis.[6,13,23] Food preservatives such as nitrite and nisin are not very effective against *L. monocytogenes*.[17,24,25] Recently, food industry has been greatly concerned with the development of natural substances which can replace synthetic preservatives and harsh heat treatments due to the consumers' preference to less heat-treated and synthetic preservative-free foods and to the occurrence of heat-resistant psychrotrophic food-borne pathogens like *L. monocytogenes*.

Lactic acid bacteria are able to inhibit other microorganisms by producing a variety of antimicrobial agents such as organic acids, diacetyl, and hydrogen peroxide.[9] Moreover, some lactic acid bacteria produce bacteriocins which inhibit a variety of food-borne pathogens, including *Bacillus cereus, Clostridium perfringens, Listeria* species, and *Staphylococcus aureus*, suggesting the usefulness of lactic acid bacteria or their bacteriocin as natural food preservatives.[4,11,15,18,26,30] Thus, there have been great efforts to isolate bacteriocin-producing lactic acid bacteria from food, but most of the efforts have been concentrated on the isolation of such organisms from meat or dairy products.[18]

Lactic acid bacteria such as *Leuconostoc, Lactobacillus, Streptococcus* and *Pediococcus* are known to be associated with the fermentation of Kimchi.[14,27] It is reasonable to assume that at least some of the lactic acid bacteria associated with Kimchi fermentation might be able to produce bacteriocin-like inhibitors of food-borne pathogens. However, only a few reports on the inhibition of food-borne pathogens by lactic acid bacteria from Kimchi have been published.[10,20,21,22,29] This study was conducted to isolate lactic acid bacteria which produces bacteriocin-like inhibitors of *L. moncytogenes* from Kimchi.

Materials and Methods

Bacterial strains

The bacterial strains and their sources are listed in Table 3 and 4.

Isolation of lactic acid bacteria inhibiting *Listeria*

Lactic acid bacteria were isolated from Kimchi samples by streaking serial dilutions of the juice of the samples in 1% peptone water on MRS (Difco) agar plates.

Primary screening for the selection of bacteriocin-like inhibitor producing lactic acid bacteria isolated from Kimchi was done by spot-on-the lawn deferred antagonism method by Fleming *et al.*[7] using *L. monocytogenes* Scott A3 and *L. ivanovii* 28 as indicator organisms. In the screening, MRS agar medium containing 0.2% dextrose

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(MRS-0.2) was used to prevent the production of excessive amount of acids, and the cultures were incubated at 30℃ in an anaerobic chamber (GasPak, BBL) to rule out any inhibition due to hydrogen peroxide production. Colonies showing clear zone of growth inhibition after 24 to 48 h incubation were selected.

Secondary screening was done by agar well diffusion method of Schillinger and Lücke[26] with concentrated neutralized cell-free supernatants of the culture broth and the same indicator organisms as in the primary screening. Concentrated neutralized cell-free supernatants were prepared by neutralizing the overnight culture broths with 3 N NaOH, filter sterilizing with sterile 0.2 μm-pore size cellulose acetate filters (Corning) and then concentrating with Centricon concentrators (3-kDa cut off, Amicon) to 1/10 of the original volume, and used to exclude inhibitory effect due to lactic acid production. Portions (100 μl) of neutralized cell-free supernatants were placed in the wells and allowed to diffuse into the agar at 4℃ for 1 h, the plates were incubated for 24 to 48 h at 30℃ in an anaerobic chamber (GasPak, BBL) and checked for inhibition zones.

Identification of the isolates

General characteristics of the isolates were determined according to the Manual of Methods for General and Molecular Bacteriology[8] and Bergey's Manual of Systematic Bacteriology.[28] Utilization (oxidation) of different carbon sources was tested by Biolog MicroStation™ 2 System (Biolog, Inc., Hayward, CA) using BLA™ (Biolog Lactic Acid) agar, BLA™ suspension broth and Biolog GP MicroPlate™ as in the manufacturer's instruction manual.[13]

Partial purification of bacteriocin

A partially purified bacteriocin was prepared by ammonium sulfate precipitation. Overnight culture broth grown in MRS broth at 30℃ was centrifuged at 10,000 ×*g* for 20 min at 4℃, and the culture supernatant was made up to 70% saturation by stepwise addition of ammonium sulfate and kept overnight at 4℃ with gentle stirring. After centrifugation (10,000×*g*, 20 min, 4℃), the sedimented pellet was recovered and suspended in 1/10 of the starting volume of 10 mM potassium phosphate buffer, pH 7.0. The pellet suspension was dialyzed at 4℃ with a dialysis membrane with a 3.5-kDa cut off against the same buffer for at least 18 h with two changes of buffer. After dialysis, the solution in the dialysis bag was sterilized by filtration through a 0.2 μm-pore size cellulose acetate filter (Corning) and used as crude bacteriocin preparation.

Characterization of the bacteriocin

Crude bacteriocin preparations were tested for sensitivity to heat, chloroform and enzyme treatments. Heat treatments were carried out for 10, 30 and 60 min at 100℃. Chloroform treatment was carried out by adding 50 μl chloroform to 200 μl crude bacteriocin solution and leaving at room temperature for 1 h. Catalase (bovine liver, Sigma), protease (pronase E, Sigma), α-amylase (*Bacillus licheniformis*, Sigma) and lysozyme (chicken egg white, Sigma) treatments were carried out by adding 100 μl enzyme solution (2 mg/ml in 10 mM phosphate buffer, pH 7.0) to 100 μl crude bacteriocin solution and incubating at 37℃ for 1 h. The remaining activity was measured by the agar well diffusion method using *L. ivanovii* 28 and *L. monocytogenes* Scott A3 as indicator organisms. An untreated preparation of bacteriocin served as the control.

To test for lysozyme-like activity, a lawn of *Micrococcus lysodeikticus* cells was prepared on TSA by pouring 3 ml of TSA soft agar containing 0.5 g of lyophilized *M. lysodeikticus* cells (Sigma) onto a TSA plate. Six-microliter samples of lysozyme (50 mg/ml), pronase E (1 mg/ml), and crude bacteriocin solutions were spotted onto this lawn. After overnight incubation at 37℃, the inhibition zones were measured.

Results and Discussion

Isolation and identification of bacteriocin-producing strains

Twenty seven isolates which showed inhibitory activity against *L. ivanovii* 28 and *L. monocytogenes* Scott A3 were selected by spot-on-the lawn deferred antagonism assay among about 600 isolates from 25 Kimchi samples (Fig. 1). From the 27 isolates, 14 isolates showed inhibitory activity in agar well diffusion assay. Four isolates (isolate no. 48, 167, 194, 311) with greater antimicrobial activity were used for further studies. Characteristics of the four isolates are shown in Table 1 and 2. On the basis of their morphological and physiological properties, the isolates were identified as *Leuconostoc mesenteroides* subsp. *mesenteroides* (no. 48 and no. 194), *Leuconostoc paramesenteroides* (no. 167) and *Pediococcus pentosaseus* (no. 311). *P. pentosaceus* and *Leu. mesenteroides* had been isolated from Kimchi and found to have antimicrobial activity.[20,22,29]

Among the bacteriocin producing lactic acid bacteria, *Enterococcus faecium*,[1,12,16,19] *Leuconostoc mesenteroides*,[5] *Pediococcus acidilactici*,[2] *Pediococcus pentosaceus*,[30] *Lactococcus lactis*,[30] *Streptococcus lactis*[4] and *Lactobacillus sake*[26] were reported to produce bacteriocins inhibitory to *L. monocytogenes*. However, most of the strains were isolated from meat or dairy products and not much effort has been concerted to isolate such organisms from vegetable products. Ha *et al.*[10] isolated 17 strains of bacteriocin-producing lactic acid bacteria from Kimchi. Most of

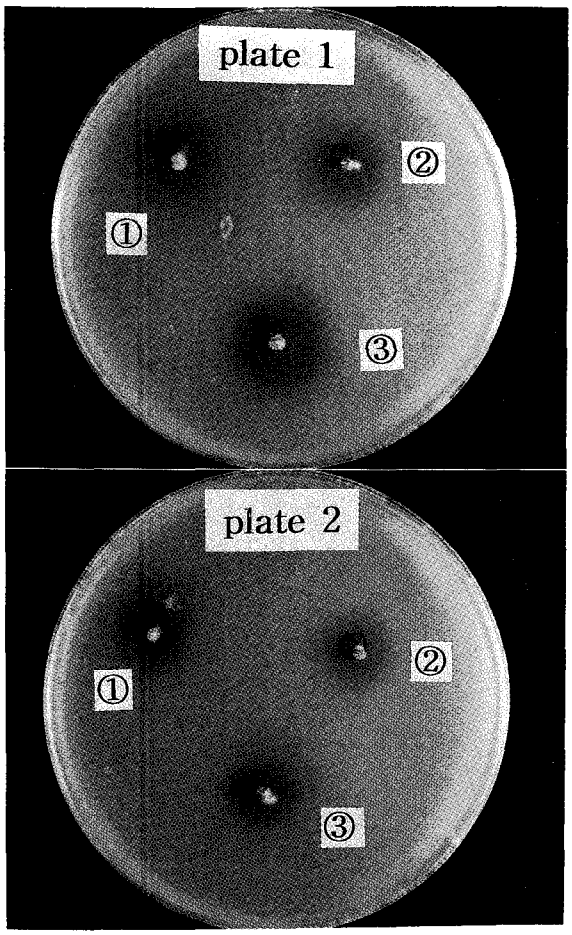

Fig 1. Inhibition of *Listeria* spp. by lactic acid bacteria isolated from Kimchi in spot-on-the lawn deferred antagonism assay. The isolates are ①, *Leuconostoc mesenteroides* subsp. *mesenteroides* (isolate no. 48); ②, *Leuconostoc paramesenteroides* (isolate no. 167); ③, *Pediococcus pentosaseus* (isolate no. 311). Indicator organisms are *L. ivanovii* 28 (plate 1) and *L. monocytogenes* Scott A3 (plate 2).

the strains were *Lactobacillus* (12 strains) or *Enterococcus* (6 strains), and only one strain was *Leu. mesenteroides* subsp. *mesenteroides*. Among the strains, only *Enterococcus faecium* species (6 strains) produced bacteriocin inhibitory to *L. monocytogenes* ATCC 19111, and *L. monocytogens* was less sensitive to the bacteriocin than other indicator organisms. In this study, no lactobacilli or enterococci were isolated. The difference is thought to be due to the difference in the screening procedure. They used *Enterococcus faecium* KCTC 3095 as an indicator organism in the screening. On the other hand, *L. ivanovii* 28 and *L. monocytogenes* Scott A3 wereused as indicator organisms in this study.

Inhibitory spectrum of the isolates

The inhibitory spectrum of the bacteriocins of the isolates against various *Listeria* strains are shown in Table 3. All of the *Listeria* strains tested were inhibited by all of the 4 isolates in the spot-on-the lawn deferred antagonism assay. However, *L. monocytogenes* ATCC

Table 1. General characteristics of bacteriocin-producing lactic acid bacteria isolated from Kimchi

Characteristics	Isolate No.			
	48	167	194	311
Cell form	Cocci	Cocci	Cocci	Cocci
Cell arrangement	Pairs, chains	Pairs, chains	Pairs, chains	Tetrads, pairs
Gram reaction	+[a]	+	+	+
Motility	−	−	−	−
Spore formation	−	−	−	−
Catalase	−	−	−	−
Dextran from sucrose	+	−	+	−
Hydrolysis of esculin	+	−	+	−
Gas from glucose	+	−	+	−
Ammonia from agrinine	−	+	+	+
Growth at 10℃	+	+	+	+
at 40℃	+	+	+	+
at 45℃	−	−	−	+
at 50℃	−	−	−	−
Growth at pH 4.5	−	+	−	+
at pH 6.5	+	+	+	+
at pH 7.5	+	+	+	+
at pH 8.5	+	+	+	+
Growth at 3.5% NaCl	+	+	+	+
at 6.5% NaCl	+[W]	+	+[W]	+
at 10% NaCl	−	−	−	+

[a]Symbols: +, positive; +[W], weakly positive; −, negative

Table 2. Utilization of carbohydrates by bacteriocin-producing lactic acid bacteria isolated from Kimchi[a]

Characteristics	Isolate No.			
	48	167	194	311
Amygdalin	+[b]	(+)	+	−
L-Arabinose	+	−	+	+
Arbutin	+	−	+	+
Cellobiose	+	−	+	+
D-Fructose	+	+	+	+
D-Galactose	+	+	+	+
D-Glucose	+	+	+	+
D-Gluconic acid	+	+	+	−
α-D-Lactose	(+)	(+)	(+)	−
Maltose	+	+	+	+
D-Mannitol	+	(+)	(+)	−
D-Mannose	+	+	+	+
D-Melezitose	−	−	−	−
D-Melibiose	+	+	+	−
D-Raffinose	+	−	−	−
L-Rhamnose	−	−	−	+
D-Ribose	+	−	(+)	+
Salicin	+	+	+	−
D-Sorbitol	+[W]	−	+[W]	−
Sucrose	+	+	+	+
Trehalose	+	+	+	+
Xylose	+	−	−	+

[a]Carbohydrate utilization test was performed by Biolog MicroStation™ 2 System (Biolog, Inc., Hayward, CA) as described in Materials and Methods. [b]Symbols : +, positive in 4 h; (+), delayed positive in 24 h; +[W], weakly positive in 24 h; −, negative.

15313 and *L. seeligeri* 62 were not inhibited by all of the 4 isolates in agar well diffusion assay, and *L. denigrificans* 28 and *L. welchimeri* 89 were not inhibited by the *Leu. paramesenteroides* isolate. *P. pentosaceus* isolate was more inhibitory against sensitive strains than *Leu. mesenteroides* subsp. *mesenteroides* or *Leu. paramesenteroides*, and *L. ivanovii* 28, *L. monocytogenes* Scott A3 and *L. monocytogenes* ATCC 19116 were more sensitive than other *Listeria* strains (data not shown).

Table 3. Inhibitory spectrum of bacteriocins of lactic acid bacteria isolated from Kimchi against *Listeria* spp.

Tagret strain	Reaction to LAB[a,b]				Sourcec
	48[d]	167[e]	194[f]	311[g]	
L. denigrificans 28	S(S)	S(R)	S(S)	S(S)	HW
L. grayi 29	S(S)	S(S)	S(S)	S(S)	HW
L. innocua 13	S(S)	S(S)	S(S)	S(S)	HW
L. ivanovii 28	S(S)	S(S)	S(S)	S(S)	HW
L. monocytogenes Lm8	S(S)	S(S)	S(S)	S(S)	FRDC
L. monocytogenes Lm13	S(S)	S(S)	S(S)	S(S)	FRDC
L. monocytogenes Lm21	S(S)	S(S)	S(S)	S(S)	FRDC
L. monocytogenes 1089	S(S)	S(S)	S(S)	S(S)	5)
L. monocytogenes Scott A3	S(S)	S(S)	S(S)	S(S)	5)
L. monocytogenes ATCC 15313	S(S)	S(S)	S(S)	S(S)	NIHK
L. monocytogenes ATCC 11916	S(S)	S(S)	S(S)	S(S)	NIHK
L. monocytogenes ATCC 11917	S(S)	S(S)	S(S)	S(S)	NIHK
L. monocytogenes ATCC 11918	S(S)	S(S)	S(S)	S(S)	NIHK
L. murrayi 30	S(S)	S(S)	S(S)	S(S)	HW
L. seeligeri 62	S(S)	S(S)	S(S)	S(S)	HW
L. welchimeri 89	S(S)	S(R)	S(S)	(S)	HW

[a]S, sensitive; R, resistant [b]Spot-on-the lawn deferred antagonism assay (Agar well diffusion assay) [c]Abbreviations: ATCC, American Type Culture Collection (Rockville, Md, USA); HW, Health and Welfare Canada (Ottawa, Ontario); FRDC, Food Research and Development Center (St. Hyacinthe, Quebbec, Canada); IFO, Institute for Fermentation (Osaka, Japan); KCCM, Korean Culture Center of Microorganisms (Seoul, Korea); NIHK, National Institute of Health Korea (Seoul, Korea). [d]*Leuconostoc mesenteroides* subsp. *mesenteroides* [e]*Leuconostoc paramesenteroides* [f]*Leuconostoc mesenteroides* subsp. *mesenteroides* [g]*Pediococcus pentosaceus*

The inhibitory spectrum of the bacteriocins of the isolates against lactic acid bacteria are shown in Table 4. All of the 4 isolates inhibited *Lactobacillus acidophilus* KCCM 32820, *Streptococcus lactis* KCCM 32406 and *Enterococcus faecalis* Lb 475, but none of the isolates inhibited *Pediococcus acidilactici* KCCM 11902 and *Pediococcus pentosaseus* ATCC 43200. *P. pentosaceus* isolate inhibited *Leu. lactis* ATCC 19256 and *Leu. paramesenteroides* ATCC 33313, but *Leu. mesenteroides* subsp. *mesenteroides* and *Leu. paramesenteroides* isolates did not.

P. pentosaceus isolate inhibited Gram-negative pathogenic bacteria, *Pseudomonas aeruginosa* KCCM 11328, *Serratia marcecens* KCCM 11809 and *Vibrio parahaemolyticus* KCCM 11965 and *Leu. paramesenteroides* isolate inhibited *P. aeruginosa* KCCM 11328 and *S. marcecens* KCCM 11809 in the spot-on-the lawn deferred antagonism assay (Table 4). However, their antimicrobial activity was low and none of the pathogenic strains were inhibited in the agar well diffusion assay. The nature of the inhibition needs to be further studied.

Daba *et al.*[5] isolated bacteriocin-producing *Leu. mesenteroides* UL5 from Cheddar cheese using *L. ivanovii* 28 as an indicator organism. The bacteriocin inhibited all of the *Listeria* strains tested, but did not inhibit most of the lactic acid bacteria tested except *P. pentosaceus* and *Streptococcus faecalis*. Ha *et al.*[10] isolated bacteriocin-producing *Lactobacillus, Enterococcus* and *Leuconostoc* strains from Kimchi using *Enterococcus faecium* as an indicator organism. Among the strains, only *E. faecium* species produced bacteriocin inhibitory to *L. monocytogenes*. In this study, no lactobacilli or enterococci were isolated.

The results of this and other studies suggest that there might be a lot of bacteriocin-producing lactic acid

Table 4. Inhibitory spectrum of bacteriocins of lactic acid bacteria isolated from Kimchi against lactic acid bacteria and Gram-negative pathogens

Target strain	Reaction to LAB[a,b]				Sourece or other designation[c]
	48[d]	167[e]	194[f]	311[g]	
Lactobacillus acidophilus KCCM 32820	S(R)	S(S)	S(R)	S(S)	ATCC 4356
Leuconostoc lactis ATCC 19256	R(R)	R(R)	R(R)	S(R)	
Leuconostoc paramesenteroides ATCC 33313	R(R)	R(R)	R(R)	S(S)	
Pediococcus acidilactici KCCM 11902	R(R)	R(R)	R(R)	R(R)	ATCC 8081
Pediococccus pentosaceus ATCC 43200	R(R)	R(R)	R(R)	R(R)	
Streptococcus faecalis KCCM 11814	S(S)	R(R)	S(R)	S(S)	ATCC 29212
Streptococcus lactis KCCM 32406	S(S)	S(S)	S(S)	S(S)	IFO 12007
Enterococcus faecalis Lb 475	S(S)	S(S)	S(S)	S(S)	26)
Pseudomonas aeruginosa KCCM 11328	R(R)	S(R)	R(R)	S(R)	ATCC 27853
Serratia marcecens KCCM 11809	R(R)	S(R)	R(R)	S(R)	ATCC 13380
Vibrio parahaemolyticus KCCM 11965	R(R)	R(R)	R(R)	S(R)	ATCC 17802

[a,b,c,d,e,f,g]same as in Table 1

Table 5. Some characteristics of crude bacteriocin preparations of the isolates

Treatment	Isolate			
	48[a]	167[b]	194[c]	311[d]
Heat treatment at 100℃				
10 min	+++[e]	+++	+++	+++
30 min	+++	++	++	+++
60 min	++	++	+	++
Chloroform treatment	++	+	+	++
Enzyme treatment				
Catalase	+++	+++	+++	+++
Protease (pronase E)	−	−	−	+
α-amylase	+++	+++	+++	+++
Lysozyme	+++	+++	+++	+++
Lysozyme-like activity	−[f]	−	−	−

[a] *Leuconostoc mesenteroides* subsp. *mesenteroides*. [b] *Leuconostoc paramesenteroides*. [c] *Leuconostoc mesenteroides* subsp. *mesenteroides*. [d] *Pediococcus pentosaceus*. [e] +++, zone of inhibition was greater than 2/3 of the control after treatment; ++, zone of inhibition was 1/3 to 2/3 of the control after treatment; +, zone of inhibition was smaller than 1/3 of the control; −, no activity retained. [f] +, lysozyme-like activity on *M. lysodeikticus*; −, no lysozyme-like activity.

bacteria in Kimchi and that some of the bacteriocins be effective against various foodborne pathogens. The use of Kimchi lactic acid bacteria and/or their bacteriocins as novel food preservatives should be thoroughly investigated.

Characteristics of the bacteriocins produced by the isolate strains

Some characteristics of crude bacteriocins produced by the isolate strains are shown in Table 5. The inhibitory activities of the crude bacteriocins produced by *Leuconostoc* isolates against *L. ivanovii* 28 and *L. monocytogenes* Scott A3 were completely neutralized by protease (pronase E) treatment, but the activity of crude baceriocin produced by *P. pentosaceus* isolate was not completely inactivated. Lewus *et al.*[15] reported that bacteriocins produced by *P. pentosaceus* ATCC 43200 and *P. pentosaceus* ATCC 43201 were inhibitory against *L. monocytogenes* strains and their activities were not inactivated by pronase E treatment. They suggested that these bacteriocins might contain only a minor component of proteinaceous character or that the active domains of these substances might not be affected by the enzyme. Whether the *P. pentosaceus* isolated in this study is identical to either of *P. pentosaceus* ATCC 43200 or *P. pentosaceus* ATCC 43201 is to be determined and the exact nature of the bacteriocin produced by the isolate needs to be further studied.

The activities of crude bacteriocins produced by all of the isolates were not reduced by catalase, α-amylase and lysozyme treatments. The inhibitory activities of the crude bacteriocins were not significantly affected after heating for 30 min at 100℃, clearly indicating that the active substances are heat-stable proteins. The inhibitory activities were partially reduced by chloroform treatment. None of the crude bacteriocin preparations showed lysozyme-like action on *M. lysodeikticus*, while lysozyme produced a prominent and clear zone of lysis.

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김치에서 분리한 젖산균 bacteriocin에 의한 *Listeria monocytogenes*의 억제

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초록 : 김치로부터 *Listeria* spp.를 억제하는 박테리오신을 생산하는 젖산균 4균주를 선발하여 *Leuconostoc mesenteroides* subsp. *mesenteroides* (2균주), *Leuconostoc paramesenteroides* 및 *Pediococcus pentosaceus*로 동정하였다. 실험한 모든 *Listeria monocytogenes* 균주들은 모든 분리 균주의 박테리오신에 의해 억제되었으나, *L. denigrificans* 28과 *L. welchimeri* 89는 *Leu. paramesenteroides* 분리균주에 의해 억제되지 않았다. 분리균주들 중에서 *P. pentosaceus*의 박테리오신이 가장 항균활성이 높았고, 항균범위도 넓었다. *Leuconostoc* 분리균주들의 박테리오신의 항균활성은 pronase E에 의해 완전히 불활성화 되었으나 *P. pentosaceus*의 것은 완전히 불활성화되지 않았다. 분리균주들의 박테리오신은 catalase, α-amylase, lysozyme 및 100℃ 30분의 열처리에 대해서 안정하였다.

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