Identification and Characterisation of Antimicrobial Activity of Nisin A Produced by *Lactococcus lactis* subsp. *lactis* LL27

Pınar ŞANLIBABA[1], Nefise AKKOÇ[2] and Mustafa AKÇELIK[2]

[1]Kalecik High School, Ankara University, Kalecik, Ankara, Turkey; [2]Department of Biology, Faculty of Science, Ankara University, Tandoğan, Ankara, Turkey

Abstract

Şanlibaba P., Akkoç N., Akçelik M. (2009): **Identification and characterisation of antimicrobial activity of nisin A produced by *Lactococcus lactis* subsp. *lactis* LL27**. Czech J. Food Sci., **27**: 55–64.

In this study, bacteriocin producing lactococcal strains were isolated from Turkish raw milk samples. Among these isolates, LL27 had the highest inhibition activity against Gram-positive indicator strains, and was selected for further analyses. DNA sequencing of 16S ribosomal RNA gene demonstrated that the isolate was *Lactococcus lactis* subsp. *lactis*. The gene encoding the bacteriocin in this strain was found to be identical to that of nisin A using direct PCR sequence methods. The bacteriocin was completely inactivated by α-chymotrypsin and proteinase K and partially inactivated by lipase and α-amylase. pH and heat stability characteristics were found to be identical with those of the control nisin. The inhibitory activity of the bacteriocin produced by LL27 was also evaluated against Gram-negative bacteria in combination with heat and freezing treatments. The results obviously showed that the production level of nisin of the *Lc. lactis* LL27 had a significant inhibitory effect on the pathogenic Gram-negative strains with the heating and freezing processes which are commonly used in the food processing.

Keywords: nisin A; *Lactococcus lactis*; sublethal injury; heating; freezing

Lactic acid bacteria (LAB) have been used for centuries in the fermentation of foods, not only for flavour and texture, but also due to their ability to prevent the growth of pathogenic microorganisms (McAulife *et al.* 2001). The interest in the application of microorganisms and their metabolites for the prevention of food spoilage and the extension of shelf life of foods has increased during the last decade. Along with lactic acid, the roles of other metabolites such as bacterial growth inhibitors (e.g. hydrogen peroxide, acetic acid, acetoin, and diacetyl) have been demonstrated. However, the leading role in the explanation of the antagonism of lactic acid bacteria is assigned to specific antimicrobial substances of a protein nature, bacteriocins. Many bacteriocins produced by LAB inhibit not only the closely related species but also the growth of food borne pathogens, such as *Listeria monocytogenes* and food spoilage bacteria (Cintas *et al.* 1995; Cleveland *et al.* 2001). Out of the bacteriocins produced by LAB, nisin has been the most extensively studied. It is active against Gram-positive bacteria and is identified as four types (nisin A, Z, Q, U) that differ in both amino acid compositions and biological activities (Cleveland *et al.* 2001; Zendo *et al.* 2003; Wirawan *et al.* 2006). Nisin sensitivity to α-chymotrypsin, its heat stability at low pH and

Supported by the grant from Ankara University, Turkey, BİYEP program with the project entitled "Enhancing of Nisin Production Ability in *Lactococcus lactis* subsp. *lactis*".

non-toxic nature has promoted its industrial use. Thus far, nisin is the only bacteriocin to have found a widespread application in the food industry. It is permitted as a food additive in at least 46 countries, particularly for the inhibition of *Clostridium* species in processed cheese, dairy products, and canned products (Delves-Broughton 1990).

Nisin exhibits a broad spectrum of inhibitory activity against Gram-positive bacteria including their spore forms (Delves-Broughton 1990). However, it is not generally active against Gram-negative bacteria, yeasts and fungi due to their outer membrane permeability barrier. The alteration of this barrier by a chelator or some physical treatments leads to an induced sensitivity against nisin (Kalchayanand *et al.* 1992; Boziaris *et al.* 1998; Boziaris & Adams 2000). Induced sensitivity to nisin has been accomplished by the use of chelating agents, e.g. EDTA, as well as a heat shock or freezing and acid treatments (Kalchayanand *et al.* 1992). Therefore, using nisin producers in the fermented food production processes including heat and freezing applications has a great importance for inactivating the Gram-negative pathogenic strains (Elliason & Tatini 1999).

Although nisin has been approved as a food preservative by Food and Drug Administration (FDA), the low rate production by the producer strains has restricted its application in the fermentation systems. Therefore, the isolation and characterisation of new strains having a high production ability has been the main subject in this respect. The first nisin producers were isolated from and identified in fermented milk products, but since then they have been isolated from various dairy products (Mitra *et al.* 2005), traditional fermented vegetables (Olasupo *et al.* 1999), fermented meat products (Choi *et al.* 2000), river water (Zendo *et al.* 2003), and human milk (Beasley & Saris 2004).

In this paper, we report a new bacteriocin producing lactococcal strain isolated from cow milk samples that were collected from different provinces of Turkey. The antimicrobial spectrum and the biochemical characteristics of the bacteriocin produced by this strain were determited. After amplification of and sequencing the respective structural gene, the type of this bacteriocin was assigned to Nisin A. Also, the antimicrobial activity of nisin against Gram-negative strains, particularly *Escherichus coli* and *Salmonella typhimurium,* were evaluated under sublethal stress conditions such as heating and freezing.

MATERIAL AND METHODS

Bacterial strains and culture media. The lactococcal strains *Lc. lactis* subsp. *lactis* LL27 and *Lc. lactis* SIK83 (reference strain) were grown in M17 medium (GM17) at 30°C. The indicator strains used for the inhibitory activity tests of *Lc. lactis* subsp. *lactis* LL27 bacteriocin were obtained from Laboratory of Microbial Gene Technology NHL (As, Norway) and Culture Collection of Science Faculty of Ankara University, *Lc. innocua*, *Pediococcus pentosaceus*, *Enterecoccus* sp. and the other *Lactococcus* sp. strains were grown in GM17 broth at 30°C. *Lactobacillus* sp. strains were grown in de Man-Rogosa-Sharpe medium (Oxoid, UK) at 37°C. *Bacillus cereus, Staphylococcus carnosus, St. aureus, Pseudomonas fluorescens, S. typhimurium* and *E. coli* were grown in Luria Bertani broth (LB) at 37°C. Bacterial stocks were stored at –80°C in their respective broths supplemented with 20% glycerol.

Isolation and identification of bacteriocin producing strains. Totally 102 cow milk samples were collected from 7 individual regions (Marmara, Aegean, Mediterranean, Central Anatolia, Black Sea, Eastern Anatolia, Southeast Anatolia) of Turkey. *Lc. lactis* strains were isolated by inoculating the samples on to NRCLA medium (Harrigan & McCance 1966). The isolates, with bacteriocin production phenotypes were selected on the basis of their inhibitory spectrum and identified by their colony morphology, Gram-staining, cell morphology, and by 16S rDNA homology using a pair of bacteria-specific universal primers; forward 5'-AGA GTT TGA TCC TGG CTC AG-3' and reverse 5'-CCG TCA ATT CCT TTG AGT TT-3' (Beasley & Saris 2004).

Detection of antimicrobial activity. The antimicrobial activity was evaluated as described by Van Belkum *et al.* (1989). The bacteriocin-producing *Lactococcus* isolates were grown overnight at 30°C in M17 broth supplemented with glucose (GM17), by using sterile toothpicks the strains were transferred to GM17 plates. After incubating overnight, a layer of 5 ml of soft GM17 agar (0.5% agar) containing 100 µl of the indicator strain was poured on to the surface. The colonies were examined for inhibition zones after overnight incubation at 30°C.

Nisin activity was assayed by the critical-dilution method (Daba *et al.* 1993). The sensitive indicator *Lbc. plantarum* LMG2003 strain was chosen to assay

the activity. The supernatant of the producer strain was serially diluted two fold in 125 µl volumes of dH_2O (pH 2.5) after treating with catalase. Then 5 µl of each dilution was dropped on to the plates containing 5 ml of soft GM17 agar, the plates having been inoculated with the sensitive indicator strain. The activity was calculated using the formula $(1000 \times 5^{-1}) \times D^{-1}$, where D is the highest dilution that allowed no growth of the indicator organism after 18 h of incubation.

Effects of heat, enzymes, and pH on bacteriocin activity. To determine the effect of pH on the bacteriocin activity, cell free culture supernatants (CFCS) of the isolates were adjusted to pH values of 2 to 11 by using 6 mol/l NaOH or HCl. The samples were assayed for the activity with the bacteriocin sensitive strain of *Lbc. plantarum* LMG2003. *Lc. lactis* subsp. *lactis* SIK83 was used as the positive control, being a nisin producer. The samples treated with proteinase K before pH adjusting were also tested against the same indicator to avoid the possible pH inhibition.

To evaluate the effect of heat on the bacteriocin activity, CFCS were heated at 100°C for 5, 10, 15, and 20 min, and at 121°C for 15 minutes. CFCS were also treated with the following enzymes at the final concentration of 1 mg/ml of trypsin (pH 7, Merck, Germany), α-chymotrypsin (pH 7, type II), proteinase K (pH 7), lipase (pH 7), α-amylase (pH 7,) and lyzozyme (pH 7) (all Sigma, USA). Following the incubation at 37°C for 2 h, the enzyme activities were determined by heating at 100°C for 5 minutes. The untreated samples were used as the controls (Franz *et al.* 1997). After the heat or enzyme treatments the remaining bacteriocin activity was determined by well-diffusion assay.

DNA extraction, polymerase chain reaction (PCR), and DNA sequencing. Genomic DNA was extracted by the method of Engelke *et al.* (1992). The PCR analysis was carried out with a volume of 50 µl mixture in a DNA thermocycler (Techne A512, Great Britain). The procedure consisted of 30 cycles of 94°C for 45 s, 52.5°C for 45 s, and 72°C for 1 minute. The primers for the nisin genes comprised the following nucleotide sequences; 5'-ATG AGT ACA AAA GAT TTT AAC TTG-3' and 5'-ATT TGC TTA CGT GAA TAA TAC AA-3'.

The amplified PCR products were harvested from agarose gel and purified with gel extraction and PCR purification kits (Promega, USA). The purified product was sequenced with an autoread sequencing kit in the ABI PRISM377 DNA sequencer (Perkin Elmer, USA).

Plasmid isolation and conjugation. Plasmid DNA was isolated by the method of Anderson and McKay (1983). The plasmid DNA samples were subjected to electrophoresis in 0.7% agarose gels.

The conjugation procedure was adopted from Gasson and Davies (1980). The recipient and donor strains were grown in GM17 broth at 30°C for 18 hours. For the recipient strain *Lc. lactis* MG1363 strain, erythromycin (5 µl/ml) was added to this medium. Two ml of the donor and 3 ml of the recipient culture (both 10^{-4} diluted) were mixed and the cells were collected on sterile membrane filters (0.45 µm Sartorius, Germany). The filters containing the recipient and donor cells were placed on GM17 agar plates, supplemented with 0.5% (w/w) glucose, and kept at 30°C for 18 hours. The filters were then taken of the GM17 agar plates and washed in 1 ml of sterile Ringer solution to suspend the cells. Serial dilutions were made (up to 10^{-8}) and from each dilution the aliquots were spread on to fast slow differential agar plates containing antibiotics which were subsequently incubated at 30°C for 48 hours. The conjugation frequency was determined according to the ratio of the number of transconjugants per ml to the number of donor per ml. The stabilities of the nisin production phenotype in the LL27 and its transconjugants were determined after 70 generations according to the method proposed by Picon *et al.* (2005).

Heating and freezing stress and nisin treatment. To determine the susceptibility of Gram-negative cells (*E. coli* and *S. typhimurium*) to the produced nisin concentrations of 500 and 1000 IU/ml with heating and freezing, cell suspensions in peptone water were treated using the method proposed by Kalchayanand *et al.* (1992). The cells from 10 ml broth of each strain were harvested by centrifugation and resuspended in 0.1% sterile peptone water to a cell concentration of 10^5–10^6 CFU/ml. The experiment was carried out with three replicas and two parallels for each replica. Three sets of samples of each strain (*E. coli* and *S. typhimurium*) were prepared as follows; one with 0.9 ml cell suspension and 0.1 ml sterile water, two with 0.9 ml cell suspension and 0.1 ml nisin preparation. The final concentration of nisin was 500 and 1000 IU/ml, respectively. Three tubes were heated at 55°C for 10 min and then cooled immediately in

water (4°C). Three ones with similar treatments were frozen at –20°C for 2 h and 24 h and then thawed rapidly. All 9 samples were enumerated for the colony forming units (CFU) by preparing serial dilutions in peptone water and by plating in duplicate for each dilution on LB agar and incubated at 37°C for 48 hours.

Nisin produced by *Lc. lactis* subsp. *lactis* LL27 was prepared from the cultures by the method developed by Yang *et al.* (1992). Briefly, pH of the broth culture was adjusted to 6.0 and the cells were harvested by centrifugation at 6000 RPM for 15 minutes. The cells with the adsorbed nisin were resuspended in 100 mmol/l saline at pH 2 to release nisin. The cells were then removed by centrifugation and the nisin containing supernatant was dialysed, freeze dried, and resuspended to the desired potency.

Statistical analysis. A one-way analysis of variance (ANOVA) along with the Tukey and Hsu MCB (Multiple comparisons with the best) comparisons was carried out using the MINITAB 14.0 (Minitab Inc. State College, PA) to determine the significance effect of nisin under different heating and freezing stresses. Three repetitions of each inactivation assay were performed. For each condition, the standard deviations were calculated by converting the CFU/ml to $\log_{10}$, and the significance levels were set as $P < 0.05$ and $P < 0.001$.

RESULTS AND DISCUSSION

Isolation and identification of bacteriocin-producing lactic acid bacteria

Sixteen lactococci isolated from 102 cow milk samples were found to produce inhibition zones against the indicator strains of *Lbc. plantarum* LMG2003 and *Lbc. sake* NCDO2714. The culture supernatants of these isolates were tested for antimicrobial activity against Gram-positive and Gram-negative bacteria. Only three (LL27, LL57, LL90) of them were found to secrete bacteriocins into the culture broth. Among these isolates, LL27 had the broadest spectrum of inhibitory activity (Table 1). This isolate was a Gram-positive, catalase negative coccus. Based on these characteristics and the identity of this strain by 16S rDNA analysis, it was confirmed that LL27 has 99% homology to *Lc. lactis* subsp. *lactis* (data not shown).

Microorganisms are the major cause of food related diseases and spoilage in the production and storage of food and beverages. Antibiotics and food preservatives are generally used to combat these microorganisms. However, due to the potential danger of the antibiotic resistant bacteria and the demand by the consumers for purer and safer foods, there is a growing interest to replace these substances by natural products that are easily degraded and are harmless to the individuals and the environment. In this respect, a novel approach, using bacteriocins or bacteriocin producing strains is a convenient strategy to control the undesirable microflora in foods. Bacteriocin production is widespread among lactic acid bacteria, especially in *Lc. lactis*, which are often assumed to be mainly associated with milk and dairy products (Rodriguez *et al.* 2000). In this study, a new bacteriocin producer strain has been isolated from cow milk obtained from different provinces of Turkey. Based on the results of 16S rDNA sequencing and phenotypic tests, LL27 was confirmed to be *Lc. lactis* subsp. *lactis.* Our findings that approximately 1% of cow milk samples contain nisin-producing bacteria are consistent with previous reports (Mitra *et al.* 2005).

Characterisation of bacteriocin

The inhibitory spectrum of the bacteriocin produced by *Lc. lactis* subsp. *lactis* LL27 is presented in Table 1. Strain LL27 was found to show the inhibitory activity at different levels to 17 out of 23 indicator bacteria tested in this study. No inhibitory activity could be detected against *Lc.*

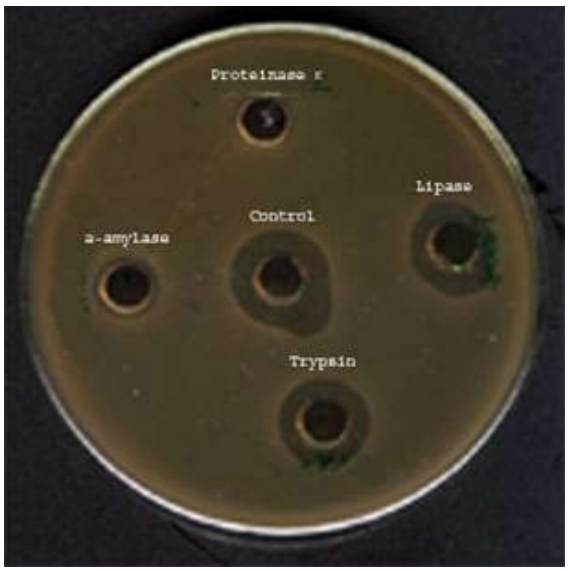

Figure 1. Inhibition zones of nisin produced by LL27 strain after treatment with different enzymes

Table 1. Inhibitory spectrum of bacteriocins produced by the strains *Lactococcus lactis* subsp. *lactis* LL27 and SIK83

Strain	Sensitivity*	
	LL27	SIK83
Lc. lactis subsp. *lactis* SIK83 (Nisin producer)	NZ	NZ
Lc. lactis subsp. *lactis* IL1403 (Nisin sensitive)	+++	+++
Lc. lactis subsp. *lactis* 105 (Lacticin producer)	++	++
Lc. lactis subsp. *lactis* LMG2908	++	++
Lc. lactis subsp. *lactis* T1	+	+
Lc. lactis subsp. *lactis* 731 (Lacticin 3147 producer)	+	++
Lc. lactis subsp. *lactis* 2 (Lacticin 3147 producer)	++	++
Lc. lactis subsp. *lactis* LMG2912 (Lacticin 3147 producer)	+	++
Lc. lactis subsp. *lactis* JC 17 (Lacticin 481 producer)	+++	+++
Lc. lactis subsp. *lactis* LMG2132 (Lactococcin producer)	++	++
Lc. lactis subsp. *lactis* LMG2088 (Lactococcin G producer)	NZ	NZ
Lbc. sakei NCDO2714 (Nisin sensitive)	+++	+++
Lbc. plantarum LMG2003	+++	+++
Ent. faecalis LMG 2708 (Nisin sensitive)	+++	++
Ent. faecalis NCDO581	++	+++
P. pentosaceus LMG2001 (Pediocin producer)	+++	+++
L. innocua LMG 2813 (Nisin sensitive)	+++	+++
St. carnosus LMG2709	++	+++
St. aureus LMG3027	NZ	++
B. cereus LMG2732	++	++
S. typhimurium SL1344	NZ	NZ
E. coli LMG 3083 (ETEC)	NZ	NZ
P. fluorescens P1	NZ	NZ

*Inhibition zone: + = 1–5 mm; ++ = 6–10 mm; +++ = 11 mm and over diameter of inhibition zone, NZ = no zone

lactis subsp. *lactis* SIK83, *Lc. lactis* subsp. *lactis* LMG2088, *S. aureus* LMG3027, *E. coli* LMG3083 (ETEC), *S. typhimurium* SL1344, and *P. fluorescens* PI. Nisin producing *Lc. lactis* SIK83 was used as an experimental control and showed an inhibitory spectrum identical to LL27.

The effects of enzymes, pH, and heat treatments on the activity of the bacteriocin produced by LL27 are presented in Table 2. Protease sensitivity assay demonstrated that the antimicrobial substance produced by LL27 is a bacteriocin-like substance since its inhibitory activity was completely eliminated by the treatment with proteinase K and α-chymotrypsin. The activity was, however, not affected by other proteases including trypsin, pepsin, and non-protease enzymes including catalase and lysozyme. When lipase and α-amylase were applied, 50% and 87.5% of its activity was lost, respectively (Table 2 and Figure 1), which was not observed in the case of nisin produced by the reference strain of SIK83. The bacteriocin was found to be active over a wide pH range between 2 and 11. At the pH between 2 and 4, the activity was much higher than at neutral and basic pH values. 50–87.5% activity decreases were obtained from pH 9 to 11. The bacteriocin was completely stable under the heat treatment at 100°C for 5, 10, 15, and 20 minutes. However, the activity decreased by 97% on

Table 2. Effect of enzymes, heat treatments, and pH on bacteriocin activity (IU/ml)

Strain	LL27	SIK83
Control	3 200	6 400
Enzymes		
Trypsin	3 200	6 400
α-chymotrypsin	0	0
Proteinase-K	0	0
Pepsin	3 200	3 200
α-amylase	400	6 400
Lipase	1 600	6 400
Catalase	3 200	6 400
Lysozyme	3 200	6 400
Heat		
100°C for 5 min	3 200	6 400
100°C for 10 min	3 200	6 400
100°C for 15 min	3 200	6 400
100°C for 20 min	3 200	6 400
121°C for 15 min	100	100
pH		
2	6 400	12 800
3	6 400	12 800
4	6 400	12 800
5	3 200	6 400
6	3 200	6 400
7	3 200	6 400
8	3 200	6 400
9	1 600	3 200
10	800	1 600
11	400	800

applying the sterilisation temperature (121°C for 15 min) in accordance with the nisin produced by the control strain *Lc. lactis* SIK83. Based on these results, it appeared that *Lc. lactis* subsp. *lactis* LL27 produced a nisin-like bacteriocin.

To prove that the bacteriocin produced by *Lc. lactis* subsp. *lactis* LL27 was nisin, PCR analysis was performed using the published sequences of the nisin structural gene (Dodd *et al.* 1990). Two primers complementary to the sequences occurring proximal to 5' and 3' ends of the nisin A structural gene were used to amplify the nisin gene from the genomic DNA of LL27 and *Lc. lactis* SIK83. A

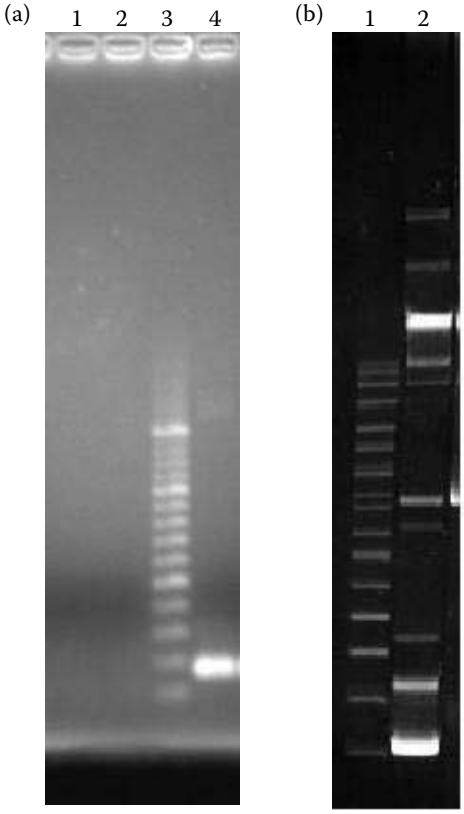

Figure 2. (a) Nisin gene amplification using specific primers: lane 1 – negative control, lane 2 – amplification from plasmid extracts of the LL27 strain, lane 3 – o'rangerular marker (fermentas) 1500, 1400, 1300, 1200, 1100, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp, lane 4 – amplification from genomic DNA of the LL27 strain; (b) the plasmid profile of the LL27 strain: lane 1 – supercoiled marker (Sigma, USA) 16.2, 14.1, 12.2, 10.2, 8.0, 7.2, 6.0, 5.0, 4.0, 2.9, 2.1 kb, lane 2 – plasmids of the LL27 strain

179 bp fragment was amplified from the genomic DNA of LL27 (Figure 2a), which was identical to that amplified from the nisin-producing strain of *Lc. lactis* SIK83. The amplified PCR product of *Lc. lactis* LL27 was subsequently sequenced using both strands as shown in Figure 3. These results indicated that the sequences were 100% identical to those of nisin A, in which instead of asparagine (AAT), histidine (CAT) residue is located at position 27 of the nisin peptide

The antimicrobial substance produced by *Lc. lactis* subsp. *lactis* LL27 strain had similar pH sensitivity and heat insensitivity patterns to nisin. However, the most distinctive property of the LL27 bacteriocin was its susceptibility to α-amylase and lipase, which was not seen in the case of nisin from the reference strain. Although, by definition, all bacteriocins are protein based, some have been

reported to consist of combinations of different proteins or proteins together with lipid or carbohydrate residues (Nes & Johnsborg 2004). As with the nisin-producing reference strain, the bacteriocin from LL27 strain exhibited a broad spectrum of antimicrobial activity (Table 1). However, a slight difference was observed in the inhibition of *St. aureus* LMG3027, which was inhibited by the nisin-producing reference strain contrary to LL27 strain. In addition, *Lc. lactis* subsp. *lactis* LL27 showed a higher inhibition activity against *E. faecalis* LMG2708, whereas the nisin-producing strain (SIK83) had a higher activity against *Lc. lactis* subsp. *lactis* 731, *Lc. lactis* subsp. *lactis* LMG2912, *S. carnosus* LMG2709, *E. faecalis* NCDO581. In the literature such difference in inhibitory spectra has generally been attributed to other products of LAB such as organic acids, hydrogen peroxide, diacetyl, and inhibitory enzymes (Nes & Johnsborg 2004). Due to the differences in biochemical activities between the bacteriocins of LL27 and SIK83 strains, the sequence of the structural gene was determined. The existence of histidine at position 27 in the nisin structure showed that the bacteriocin produced by LL27 strain is identical to nisin A.

The genetic nature of nisin production by strain LL27

In order to determine whether the nisin production ability in LL27 strain is chromosomally or plasmid DNA encoded, a PCR assay was applied by using the primers specific in the nisin A structural gene. By analysing the extracts of genomic and plasmid DNAs of LL27 separately, a 179 bp product was obtained from genomic DNA indicating that the nisin production genes were located on the chromosomal DNA (Figure 2a). The examination of the plasmids content of the LL27 strain revealed that it has 10 distinct plasmids with molecular weights varying from 2.1 to 33.1 kb (Figure 2b). As a consequence of the conjugation trials, the nisin production genes were transferred to the erythromycin resistant strain of *Lc. lactis* MG1363 with a frequency of 2×10^{-3} per donor cell. All nisin-producing transconjugants were found to be plasmid free, indicating that the nisin determinants were transferred by a chromosomally located conjugative transposon. Additionally, the nisin production level of the donor strain LL27 cannot be exceeded by the three different transconjugants, which were able to produce 800–1600 IU nisin/ml. The stability of the nisin production by LL27 strain was determined as 90% with the transconjugants as 50% in average.

Studies have shown that the nisin production genes are either located on the conjugative plasmids (Horn *et al.* 1991; Akçelik *et al.* 2006) or linked with conjugative transposons on the chromosome (Rauch & de Vos 1992). In this study, the production of nisin A by LL27 strain was found to be located on a conjugative transposon residing in the chromosome. The conjugative nature of the production facilitates the relations with genetic manipulations, providing developments in industrial starter cultures and bringing an economical gain in the fermentation industry. However, Picon *et al.* (2005) claimed that at least 50% stability is required for any traits of the starter cultures for them to be efficient at the industrial level after 70 generations. Under this circumstance, the stability of the nisin production in the transconjugants has indicated that LL27 strain can be used as a potential donor to improve the starter culture properties even in spite of the low production of transcojugants compared to LL27 producer.

Effect of the produced nisin on Gram-negative strains under heating and freezing treatments

A number of physical treatments (heating and freezing) were applied to overcome the penetration of nisin produced by LL27 into two Gram-negative bacteria, *E. coli* and *S. typhimurium*, which were assigned to be insensitive through the antimicrobial activity assays. Figure 4 show the effect of nisin on the bacterial population with three types of thermal shock; heating at 55°C for 10 min, freezing at –20°C for 2 h and 24 h, respectively, followed by thawing. All three physical treatments reduced the viable cell number, however, when nisin was present in the medium during these treatments, a dose dependent increase in lethality was observed in most cases (Figure 4). After the heat treatment at 55°C for 10 min, the amount of *E. coli* was reduced, by 1.39 log CFU/ml and 2.92 log CFU/ml in the presence of 500 IU nisin/ml and 1000 IU nisin/ml, respectively. Nisin was found to affect more this strain in the freezing treatments. Accordingly, –20°C for 2 h and 24 h reduced *E. coli* population by 2.94 and 5.08 log CFU/ml, respectively, with 500 IU nisin/ml. However, when the exposure

```
1   ATG AGT ACA AAA GAT TTT AAC TTG GAT TTG
    Met Ser Thr Lys Asp Phe Asn Leu Asp Leu

30  GTA TCT GTT TCG AAG AAA GAT TCA GGT GCA
    Val Ser Val Ser Lys Lys Asp Ser Gly Ala

60  TCA CCA CGC ATT ACA AGT ATT TCG CTA TGT
    Ser Pro Arg Ile Thr Ser Ile Ser Leu Cys

90  ACA CCC GGT TGT AAA ACA GGA GCT CTG ATG
    Thr Pro Gly Cys Lys Thr Gly Ala Leu Met

120 GGT TGT AAC ATG AAA ACA GCA ACT TGT CAT
    Gly Cys Asn Met Lys Thr Ala Thr Cys His

150 TGT AGT ATT CAC GTA AGC AAA TAA
    Cys Ser Ile His Val Ser Lys *
```

Figure 3. Nucleotide sequence and deduced amino acid sequence of the *nisA* gene isolated from *Lc. lactis* subsp. *lactis* LL27: the amino acid sequence is shown below the coding sequence: the nucleotide in the *nisA* sequence that differs with that in the *nisZ* gene sequence is in bold and codon is shown by asterisk

amount of nisin was increased to 1000 IU/ml, higher reductions (3.94 and 5.52 log CFU/ml) were yielded. While the 500 IU/ml concentration was found to be significant at the level of $P < 0.005$, 1000 IU/ml showed a more significant level ($P < 0.001$) on the viable cell reduction effect with each of the stress treatments.

S. typhimurium strain was found less sensitive to nisin than *E. coli* strain, under heating and freezing treatments. While heating at 55°C for 10 min with 500 IU nisin/ml reduced (1.47 log CFU/ml) the viability of *S. typhimurium* cells significantly ($P < 0.05$) in accordance with the control group without nisin, increasing the amount of nisin to 1000 IU/ml at the same heating treatment resulted in 3.34 log CFU/ml viable cell reduction ($P < 0.001$). Furthermore, the treatment by –20°C freezing and thawing in the presence of 500 IU nisin/ml reduced the population of *S. typhimurium* cells by about 0.78 log CFU/ml ($P > 0.05$) after 2 h and 2.91 log CFU/ml ($P < 0.05$) after 24 h of incubation. Nevertheless, 1000 IU nisin/ml reduced the population of *S. typhimurium* cells more significantly ($P < 0.001$) at the level of 3.46 and 3.58 log CFU/ml, respectively.

Apart from these results, *E. coli* cells were found to be significantly ($P < 0.05$) more sensitive than *S. typhimurium* cells in the stress treatments with both of 500 and 1000 IU/ml except heating at 55°C for 10 minutes. Additionally, the highest lethality ($P < 0.05$) was observed for both *E. coli* and *S. typhimurium* strains, when the treatment at –20°C for 24 h was applied in the presence of 500 and 1000 IU nisin/ml.

The stress treatments used with 500 and 1000 IU nisin/ml affected the viability of *E. coli* and *S. ty-*

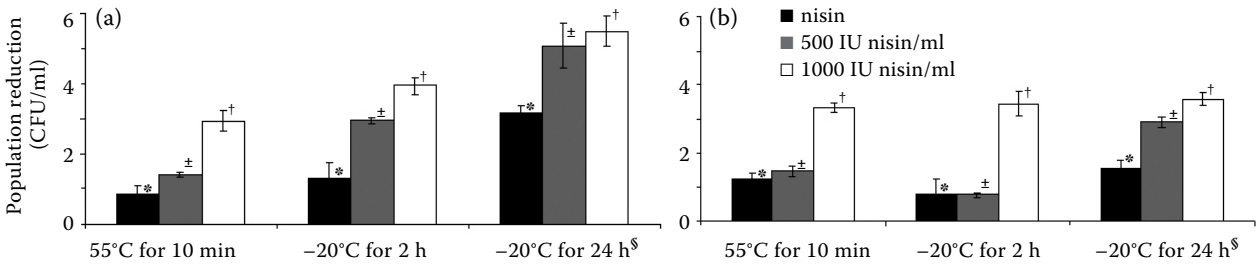

Figure 4. The effect of stress treatments and the presence of nisin on inactivation of Gram-negative (a) *E. coli* and (b) *S. typhimurium* without nisin

Significantly ($P < 0.05$): *more sensitive than *S. typhimurium* except 55°C for 10 min heating; §different at lethality with both 500 and 1000 IU nisin/ml; ±different from control group without nisin; †different from control group without nisin

phimurium cells to different extents. These reductions were found significant at the levels of $P < 0.05$ and $P < 0.001$ (Figure 4) when compared to the controls without nisin. In earlier studies, similar reductions were reported which ranged between log 2.1 to 5 for *Salmonella* and log 1.5 to 3.5 for *E. coli* using higher nisin concentrations (> 2000 IU/ml) (Kalchayanand *et al.* 1992; Boziaris & Adams 2000). This implies that the sensitivity of the injured cells is not increased by higher amounts of nisin. Likewise, Boziaris *et al.* (1998) stated that concentrations of nisin higher than 1500 IU/ml did not have any significant additional effects on *S. enteritidis* PT4. Consequently, the presence of low amounts of nisin during food processing treatments that impose stress on Gram-negative bacteria could increase the lethality of the process, enhancing both microbiological safety and stability of food. Especially, the combination of nisin and freezing/heating applications involved in food producing processes may be contributed to the hurdle technology against Gram-negative pathogen and spoilage bacteria.

CONCLUSION

The incorporation of the bacteriocin-producing lactococci in foods provides an attractive alternative to the addition of purified bacteriocin. The new LL27 strain has important characteristics to be a suitable starter culture in respect to its moderate nisin production ability together with the conjugative property while showing inhibitory activity of its bacteriocin against Gram-negative strains under different heating and freezing treatments which have often been used in food processes. Furthermore, the origin from unprocessed cow milk indicates that LL27 strain may be potentially a natural isolate. Likewise the demand on using commercial starter cultures within the developing dairy industy has resulted to loose entire natural microflora which may comprise versatile starter cultures. In this view, the other starter features of LL27 should be investigated for gaining new starter cultures.

Refrences

Akçelik O., Tukel Ç., Özcengiz G., Akçelik M. (2006): Characterization of bacteriocins from two *Lactococcus lactis* subsp. *lactis* isolates. Molecular Nutrition and Food Research, **50**: 306–313.

Anderson D.G., McKay L.L. (1983): A simple and rapid method for isolating large plasmid DNA from lactic streptococci. Applied and Environmental Microbiology, **46**: 549–552.

Beasley S.S., Saris P.E.J. (2004): Nisin producing *Lactococcus lactis* isolated from human milk. Applied and Environmental Microbiology, **70**: 5051–5053.

Boziaris I.S., Humpheson L., Adams M.R. (1998): Effect of nisin on heat injury and inactivation of *Salmonella enteritidis* PT4. International Journal of Food Microbiology, **43**: 7–13 .

Boziaris I.S., Adams M.R. (2000): Transient sensitivity to nisin in cold shocked Gram negatives. Letters in Applied Microbiology, **31**: 233–237.

Choi H.-J., Cheigh C.-I., Kim S.-B., Pyun Y.-R. (2000): Production of nisin like bacteriocin by *Lactococcus lactis* subsp. *lactis* A164 isolated from kimchi. Journal of Applied Microbiology, **88**: 563–571.

Cintas L.M., Rodriquez J.M., Fernandez M.F., Sletten K., Nes I.F., Hernandez P.E., Holo H. (1995): Isolation and characterization of pediocin L50, a new bacteriocin from *Pediococcus acidilactici* with a broad inhibitory spectrum. Applied and Environmental Microbiology, **61**: 2643–2648.

Cleveland J., Montville T.J., Nes I.F., Chikindas M.L. (2001): Bacteriocins safe natural antimicrobials for food preservation. International Journal of Food Microbiology, **71**: 1–20.

Daba H., Lacroix C., Huang J., Simard R.E. (1993): Influence of growth conditions on production and activity of mesenterocin 5 by a strain of *Lueconostoc mesenteroides*. Applied Microbiology and Biotechnology, **39**:166–173.

Delves-Broughton J. (1990): Nisin and its uses as a food preservative. Food Technology, **44**: 100–117.

Dodd H.M., Horn N., Gasson M.J. (1990): Analysis of the genetic determinant for the production of peptide antibiotic nisin. Journal of General Microbiology, **136**: 555–566.

Elliason D.J., Tatini S.R. (1999): Enhanced inactivation of *Salmonella* Typhimurium and verotoxigenic *Escherichia coli* by nisin at 6–5°C. Food Microbiology, **16**: 257–267.

Engelke G., Gutowski-Eckel Z., Hammelmann M., Entian K.D. (1992): Biosynthesis of the lanthibiotic nisin; genomic organization and membrane localization of the nisB protein. Applied and Environmental Microbiology, **58**: 3730–3743.

Franz C.M.A.P., Du Toit M., von Holy A, Schillinger U., Holzapfel W.H. (1997): Production of nisin-like bacteriocins by *Lactococcus lactis* strains isolated from vegetables. Journal of Basic Microbiology, **3**: 187–196.

Gasson M.J., Davies F.L. (1980): High frequency conjugation associated with streptococcus lactis donor cell aggregation. Journal of Bacteriology, **143**: 1260–1264.

Harrigan F.W., McCance E.M. (1966): Laboratory Methods in Microbiology. Academic Press, London, New York: 285.

Horn N., Swindell S., Dodd H., Gasson M. (1991): Nisin biosynthesis genes are encoded by a novel conjugative transposon. Molecular and General Genetics, **228**: 129–135.

Kalchayanand N., Hanlin M.B., Ray B. (1992): Sublethal injury makes Gram negative and resistant Gram positive bacteria sensitive to the bacteriocins, pediocin AcH and nisin. Letters in Applied Microbiology, **15**: 239–243.

McAulife O., Ross R.P., Hill C. (2001): Lantibiotics: structure, biosynthesis and mode of action. FEMS Miccrobiology Reviews, **25**: 285–308.

Mitra S., Chskrabartty P.K., Biswas S.R. (2005): Production and characterization of nisin-like peptide produced by a strain of *Lactococcus lactis* isolated from fermented milk. Current Microbiology, **51**: 183–187.

Nes I.N., Johnsborg O. (2004): Exploration of antimicrobial potential in LAB by genomics. Current Opinion in Biotechnology, **15**: 100–104.

Olasupo N.A., Schillinger U., Narbad A., Dodd H., Holzapfel W.H. (1999): Occurrence of nisin Z production in *Lactococcus lactis* BFE 1500 isolated from Wara, a traditional Nigerian cheese product. International Journal of Food Microbiology, **53**: 141–153.

Picon A., de Torres B., Gaya P., Nunez M. (2005): Cheese making with *Lactococcus lactis* strain expressing a mutant oligopeptide binding protein as a starter results in a different peptide profile. International Journal of Food Microbiology, **104**: 299–307.

Rauch P.J.G., de Vos M.W. (1992): Characterization of the novel nisin-sucrose conjugative transposon Tn5276 and its insertion in *Lactococcus lactis*. Journal of Bacteriology, **174**: 1280–1287.

Rodriguez E., Gonzalez B., Gaya P., Nunez M., Medina M. (2000): Diversity of bacteriocins produced by lactic acid bacteria isolated from raw milk. International Dairy Journal, **10**: 7–15.

van Belkum M.J., Hayema B.J., Geis A., Kok J., Venema G. (1989): Cloning of two bacteriocin genes from a lactococcal bacteriocin plasmid. Applied and Environmental Microbiology, **55**: 1187–1191.

Wirawan R.E., Klesse N.A., Jack R.W., Tagg J.R. (2006): Molecular and genetic characterization of a novel nisin variant produced by *Streptococcus uberis*. Applied and Environmental Microbiology, **72**: 1148–1156.

Yang R., Johnson M.C., Ray B. (1992): Novel method to extract large amounts of bacteriocins from lactic acid bacteria. Applied and Environmental Microbiology, **58**: 3355–3359.

Zendo T., Fukao M., Ueda K., Higuchi T., Nakayama J., Sonomoto K. (2003): Identification of the lantibiotic nisin Q, a new natural nisin variant produced by *Lactococcus lactis* 61-14 isolated from a river in Japan. Bioscience, Biotechnology and Biochemistry, **67**: 1616–1619.

Received for publication September 8, 2008
Accepted after corrections February 7, 2008

Corresponding author.

Prof. Dr. Mustafa Akcelik, University of Ankara, Faculty of Science, Department of Biology, Tandogan, 06100 Ankara/Turkey
tel.: + 90 312 212 67 20 ext. 1119; fax: + 90 312 223 23 95; e-mail: akcelik@science.ankara.edu.tr