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Purification and Characterization of Enterocin LR/6, a Bacteriocin from *Enterococcus faecium* LR/6

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Abstract Enterocin LR/6, a bacteriocin obtained from the culture filtrate of *Enterococcus faecium* strain LR/6, has been purified to homogeneity using ammonium sulfate precipitation, cation-exchange chromatography, gel-filtration, and checked on reverse-phase high-performance liquid chromatography. It is active at high temperatures (boiling as well as autoclaving) and over a wide range of pH (2.0–8.0). Also, it is sensitive to a number of proteolytic enzymes but is stable in the presence of surfactants and organic solvents. The protein could be stored at least up to 1 year at low temperatures (4 °C and −20 °C) without any loss of activity. The N-terminal sequence of enterocin LR/6 showed no homology with known enterocins or other bacteriocins present in the database, suggesting it to be a novel enterocin. Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry and tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis revealed its mass to be ~6.1 kDa. It showed a bactericidal mode of action against indicator strain, *Micrococcus luteus*.

Keywords *Enterococcus faecium* · Enterocin LR/6 · Purification · Characterization · Mode of action

Introduction

Lactic acid bacteria (LAB) and their metabolites have been consumed in cultured foods, with no adverse effects, in high quantities by countless generations of people. This group of microorganisms, hence, is a preferred source for food-use bacteriocins [1]. Bacteriocins are ribosomally synthesized, extracellularly released peptides with an antimicrobial activity usually exerted against the closely related strains [2]. Since the last decade, the number of

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reported bacteriocins, and more so, the enterocins, has increased. Interestingly, enterocins are particularly active against pathogenic bacteria, such as *Listeria*, *Clostridium*, and *Staphylococcus* [3, 4]. The established role of bacteriocin-producing strains in food fermentation and preservation has attracted increasing interest in recent years, either as the components of the natural microflora or raised under controlled conditions [5]. Bacteriocins, being biologically derived, low-molecular-weight proteinaceous compounds, are considered to be easily degraded during digestion in human beings [6] and, thus, could serve as the best alternative to the predominant method of chemical preservation. Based on their primary structure, molecular mass, and heat stability, bacteriocins produced by LAB can be subdivided into four classes [1, 7, 8]. Enterococci, as part of the natural intestinal flora of humans and animals, are known to play an important role in maintaining microbial balance [9, 10]. Many different enterocins have been described from *Enterococcus faecalis* and *Enterococcus faecium*. Some of these peptides revealed activity against *Escherichia coli* [11] and *Salmonella pullorum* [12].

Different bacteria produce different types of bacteriocins. Therefore, there is a pressing need to explore and isolate more and more bacteria from new sources capable of producing novel bacteriocins and to characterize them for further applications. In the present study, we report the purification and characterization of an enterocin produced by *E. faecium* LR/6, showing bactericidal activity against the indicator organism, *Micrococcus luteus*.

Material and Methods

Bacterial Strains, Growth Conditions, and Media

Enterococcus faecium LR/6, isolated from rhizosphere (unpublished data), was routinely propagated in tryptone–glucose–yeast extract (TGYE) medium (tryptone, 5.0 g/l; glucose, 1.0 g/l; yeast extract, 3.0 g/l, pH 7.0) as described [13]. For bacteriocin production, the strain LR/6 was grown in optimized TGYE medium (glucose, 20 g/l; yeast extract, 20.0 g/l; tryptone, 15.0 g/l; tween 80, 1.0 ml/l; triammonium citrate, 1.0 g/l; sodium acetate, 11.3 g/l; K_2HPO_4, 3.0 g/l; $MgSO_4$, 0.5 g/l; $MnSO_4$, 0.2 g/l, and pH 7.0). The indicator organism used in bacteriocin assay, *M. luteus*, was propagated in nutrient broth (peptone, 5.0 g/l; beef extract, 3.0 g/l; NaCl, 5.0 g/l, pH 7.0). All cultures were raised at 37 °C and 200 rpm in an incubator shaker. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) and all media components were purchased from Hi-media, Mumbai, India.

Bacteriocin Assay

The culture supernatant of *E. faecium* LR/6 grown for 18 h in optimized TGYE medium was filtered through a 0.2-μm-pore-size membrane (mdi, Ambala Cantt, India), and the antimicrobial activity therein was quantified by microtiter plate assay [14]. Each well of the microtiter plate contained 200 μl of nutrient broth with the indicator organism (A_{630}=0.02), to which was added bacteriocin fractions at twofold dilution in 50 μl of nutrient broth. The plates were incubated for 6 h at 37 °C, and the growth inhibition of the indicator organism was measured spectrophotometrically as A_{630} with Microplate Reader (Bio-Rad, Hercules, CA, USA). One bacteriocin unit, represented as AU/ml, was arbitrarily defined as the amount of bacteriocin that inhibited the growth of the indicator organism by 50% in comparison to an untreated control.

Bacteriocin Purification

Bacteriocin was purified from a 1,000-ml culture of *E. faecium* LR/6, grown in optimized TGYE broth as described above. Cells were removed by centrifugation at 12,000×*g* for 10 min at room temperature. Purification of bacteriocin was achieved by using a multistep protocol.

Ammonium Sulfate Precipitation

Culture supernatant was brought to 90% saturation with solid ammonium sulfate, and after stirring overnight at 4 °C, the precipitate was collected by centrifugation (GSA rotor, 6,000×*g*, 10 min, 4 °C). The precipitate was dissolved in 60 ml sodium phosphate buffer (20 mM, pH 6.0), and the bacteriocin suspension was desalted by dialyzing through a 2-kDa cut-off dialysis membrane (Sigma) against the same buffer for 24 h. The dialyzed suspension was centrifuged at 12,000×*g* for 15 min at 4 °C. The supernatant was filtered through a 0.2-μm membrane and tested for antimicrobial activity. This desalted active sample was lyophilized, resuspended in 60 ml of the same buffer, and labeled as Fraction I.

Cation Exchange Chromatography

Fraction I was injected into a HiPrep 16/10 SP XL Fast Flow cation exchange column on ÄKTA purifier system (Amersham Biosciences, Piscataway, NJ, USA), equilibrated, and washed with 20 mM sodium phosphate buffer (pH 6.0). The proteins were then eluted by a linear gradient of NaCl (0% to 100% of 1 M) in the same buffer. The flow rate was maintained at 1 ml/min and the protein concentration was monitored as absorbance at 215 nm. Collected fractions (5 ml) were assayed for antimicrobial activity. Active fractions were pooled, lyophilized, and resuspended in 6 ml sterile MQ water (Fraction II).

Gel-Filtration Chromatography

Fraction II was then loaded on a Sephadex G-25 column (HiPrep 26/10, Desalting column, Amersham Biosciences) linked to an ÄKTA purifier system equilibrated with MQ water and eluted with the same at a flow rate of 1 ml/min. In these fractions, protein concentration was monitored as described above and conductivity was observed in terms of millisiemens per centimeter. Eluted fractions (5 ml) were checked for antimicrobial activity and the active fractions were pooled.

Reverse-Phase High-Performance Liquid Chromatography

To check the homogeneity of purified active fractions eluted from the gel filtration chromatography, they were lyophilized, and resuspended in high-performance liquid chromatography (HPLC)-grade water containing 0.1% trifluoroacetic acid (TFA). These were further resolved on analytical C-18 reverse phase column (250×4.6 mm, Synergy, 4 μ Hydro RP, Phenomenex) using an HPLC system (Shimadzu, Kyoto, Japan). The column was equilibrated with solvent A (HPLC-grade water containing 0.1% TFA) and fractions were eluted with a step gradient of solvent B (acetonitrile containing 0.1% TFA). The flow rate was maintained at 1 ml/min and fractions were monitored by an ultraviolet detector at 226 nm.

Protein Concentration Determination

Protein concentration was determined by using the bicinchoninic acid protein assay kit (Sigma-Aldrich), as recommended by the supplier.

Sensitivity to Heat, pH, and Hydrolyzing Enzymes

Aliquots of purified bacteriocin sample were exposed to temperature treatments of 60, 80, and 100 °C for 30 min and one aliquot was autoclaved (121 °C at 15 psi, for 15 min). The treated samples were tested for antimicrobial activity, as described before. In a separate experiment, samples of bacteriocin were adjusted to pH values ranging from 2.0 to 8.0 in a 1:1 ratio of different buffer solutions (HCl–KCl 50 mM, pH 2.0 and 4.0, and phosphate buffer 50 mM, pH 6.0, 7.0, and 8.0), incubated at 37 °C for 4 h, and tested for antimicrobial activity. Sensitivity of the bacteriocin to proteolytic enzymes (proteinase K, pepsin, papain, α-chymotrypsin, and protease), as also to lipase and α-amylase (Sigma-Aldrich), was tested at a final concentration of 1 mg/ml, at 37 °C for 2 h. After incubation, the enzymes were heat-inactivated (5 min at 100 °C) and tested for antimicrobial activity. Untreated samples were taken as controls in all cases.

Effect of Surfactants, Organic Solvents, and Storage

Surfactants used were sodium dodecyl sulfate (SDS), Tween 80, Tween 20, Triton X-100, and urea, which were added to the purified bacteriocin at a final concentration of 1% (v/v) and incubated at 37 °C for 5 h. Surfactants at 1% in TGYE broth were used as controls.

To test the sensitivity of the purified bacteriocin to organic solvents, the same was mixed with various organic solvents (ethanol, methanol, isopropanol, acetone, ethyl acetate, and toluene) at a final concentration of 50% (v/v). After incubation for 2 h at 37 °C, the organic solvent was evaporated and the residual antimicrobial activity was determined. Untreated bacteriocin sample was taken as control.

In order to determine the stability, the purified bacteriocin sample was stored for 1 year at low temperatures (4 °C and −20 °C), and antimicrobial activity was compared with the fresh purified preparation.

Killing Kinetics

The effect of purified bacteriocin was determined on nongrowing cells of indicator organism, *Micrococcus luteus*. For this, the indicator organism was grown up to log-phase in Nutrient Broth and the cells were harvested by centrifugation (8,000×*g* for 10 min) and resuspended in sterile normal saline (0.85%). One hundred and 200 AU/ml of bacteriocin were added to the cell suspension of the indicator organism [~10^6 colony-forming units (CFU)/ml] and incubated at 37 °C. Samples were withdrawn at appropriate intervals and viable cell counts were determined by plating on nutrient agar medium. Cell suspension in normal saline was used as control.

Tricine SDS-Polyacrylamide Gel Electrophoresis and Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight Mass Spectrometry

To estimate the molecular mass of the antimicrobial peptides, tricine-SDS-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) was carried out as described [15]. This consisted

of a step gradient of 4%, 10%, and 16.5% polyacrylamide for the stacking, spacer, and separating gel, respectively. Electrophoresis was conducted at a constant voltage of 100 V for 4 h. Myoglobin fragments (Sigma) were used as molecular weight markers. After electrophoresis, the gel was cut into two parts, and one part, which contained the marker proteins and purified enterocin LR/6, was stained with Coomassie Brilliant Blue R-250. The other part, which contained the purified enterocin LR/6, was extensively washed with regular replacement of sterile Milli Q water and was transferred to a TGYE agar plate. The gel was overlaid with soft agar (0.8%) seeded with ~10^6 CFU/ml of the indicator organism, *M. luteus*, followed by overnight incubation at 37 °C [16, 17].

The molecular mass of the protein was further confirmed by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF/MS). The mass analysis was carried out by the custom Protein Sequencing Facility of IOWA State University, USA.

Amino Acid Sequencing

N-terminal amino acid sequencing was done by Edman degradation and analyzed on a protein/peptide sequencer, model 494 Procise (Perkin Elmer Applied Biosystems, Waltham, MA, USA). The sequencing was also carried out by the same facility.

Results and Discussion

Genus *Enterococcus* belongs to a group of important LAB as they participate and contribute towards different fermentation processes. Their functionality in dairy and meat products has been reported in detail [18, 19]. Recently, a review on the role and application of enterococci in food and health has also been published [20]. Several bacteriocins produced by *E. faecium* [21], or other enterococci of different origins [9], have been reported and characterized at the biochemical and genetic level. Ideas have also been forwarded to the possible use of *E. faecium* as a starter culture to enhance the safety of food products [20].

Purification

The bacteriocin enterocin LR/6 was secreted into the growth medium, and maximum activity was obtained from the cultures in the stationary phase. This bacteriocin was purified to apparent homogeneity using a multistep purification protocol, as also described for enterocin ON-157 [22] and enterocin P [23]. These methods are consistent with the conserved biochemical characteristics of many bacteriocins, i.e., they are generally small, cationic, and hydrophobic in nature. As has been noted for other bacteriocins, like enterocin I [24] and enterocin ON-157 [22], a marked increase in specific activity was observed after each purification step.

As is clear from the Table 1, at the first step, ammonium sulfate precipitation resulted in an approximate two-and-a-half-fold increase in specific activity. When this sample was applied on SP-Sepharose Fast Flow column chromatography, a single absorbance and active peak was observed between 20% and 80% of 1 M NaCl (Fig. 1a). Specific activity was increased almost six-and-a-half-fold, amounting to a 3.5% yield. Finally, the desalting of active fractions also yielded a single active peak (Fig. 1b), with the specific activity being maintained at almost sixfold. Further resolution on reverse-phase HPLC yielded a single absorbance peak, confirming the homogeneity of the purified bacteriocin, enterocin LR/6 (Fig. 2).

Table 1 Summary of the purification of enterocin LR/6 from *E. faecium* LR/6.

Fraction	Volume (ml)	Total protein (μg)	Total activity (AU/ml)	Specific activity[a]	Fold increase in sp. act.	Yield (%)
Culture supernatant	1,000	1.2×10^4	5.9×10^5	49.16	1	100
Ammonium sulfate precipitation	60	4.2×10^2	5.5×10^4	130.95	2.66	9.32
Cation exchange chromatography	6	64.32	2.1×10^4	326.49	6.64	3.55
Gel filtration chromatography	3	18.52	5.8×10^3	313.17	6.37	0.98

[a] Specific activity is the ratio of total activity to that of total protein.

Effects of Heat, pH, and Hydrolytic Enzymes

The activity of bacteriocin was fully stable upon treatment at different temperatures, including the boiling and autoclaving. While similar results have been reported for durancin L28-1A from *E. durans* [25], bacteriocin ST15 from *E. faecium* was inactivated when subjected to 121 °C for 20 min [26].

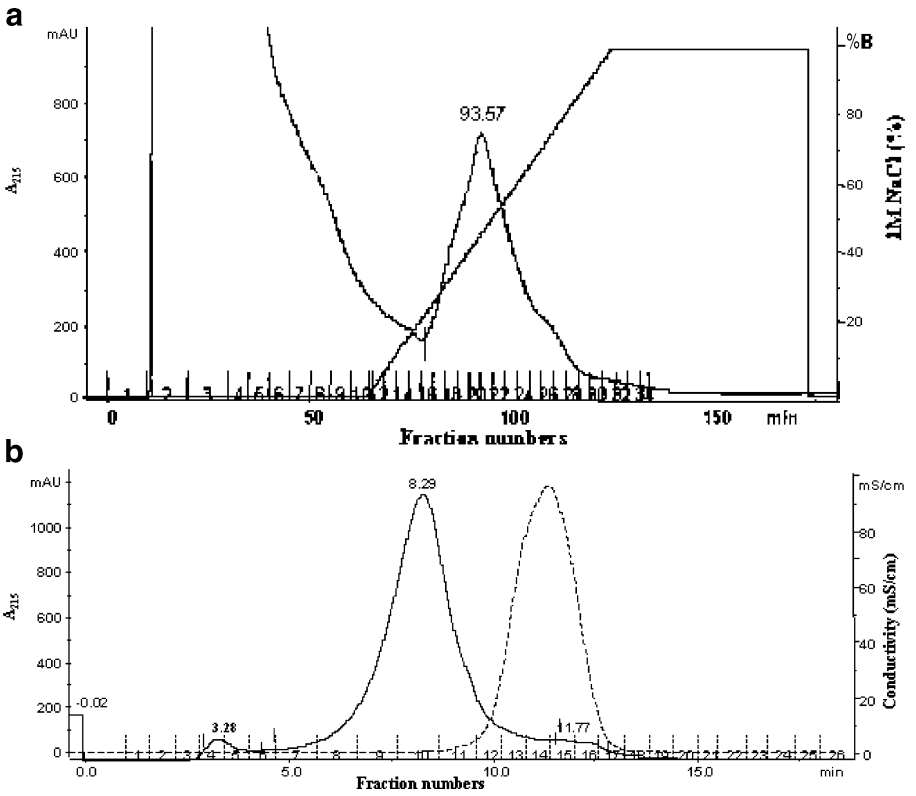

Fig. 1 **a** Elution profile of enterocin LR/6 using cation-exchange chromatography on ÄKTA purifier system. The fractions were monitored for protein concentration by absorbance at 215 nm and eluted with 0–100% linear gradient of NaCl (1 M). **b** Elution profile of enterocin LR/6 (retention time 8.29) using gel-filtration chromatography on ÄKTA purifier system. Salt concentration was measured in terms of conductivity (mS/cm) represented by the last peak

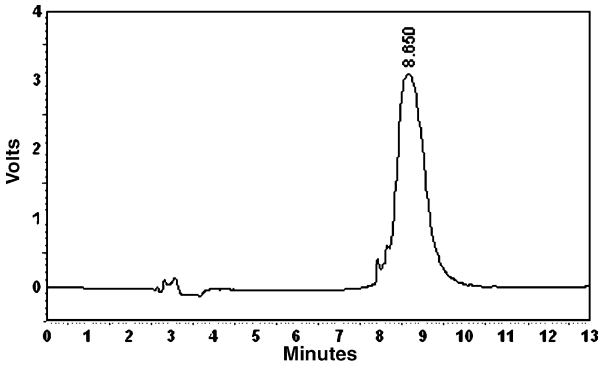

Fig. 2 Reverse-phase HPLC analysis of enterocin LR/6 (retention time 8.65). The elution was done by step gradient of 0–60% of acetonitrile and water containing 0.1% TFA

Antimicrobial property of enterocin LR/6 also remained unaffected in the pH range of 2.0–6.0. At pH 7.0 and 8.0, however, the activity was reduced by ~20%. These results are similar to that reported for the bacteriocin produced by *E. mundtii* 5 [27]. Several bacteriocins produced by enterococci are known to exhibit a wide range of pH stability [26, 28]. These properties taken together have been considered highly useful for their application as a food preservative.

The enterocin LR/6 was found to be sensitive to different proteolytic enzymes (proteinase K, pepsin, papain, α-chymotrypsin, and protease), confirming its proteinaceous nature. On the other hand, ineffectivity of α-amylase and lipase on antimicrobial activity suggested that enterocin LR/6 is not glycosylated and does not contain lipid moiety. These results are in agreement with the bacteriocin from *E. faecium* JCM 5804^T [29] and mundticin KS [30].

Effect of Surfactants, Organic Solvents, and Storage

Enterocin LR/6 remained fully active when treated with different surfactants and organic solvents as given in "Material and Methods." Such stability has been a common feature of many bacteriocins produced by enterococci and other LAB [26, 31–33].

Long-term storage (1 year) at temperatures of 4 or −20 °C did not affect the antimicrobial activity, a result that is in agreement with the bacteriocins produced from *E. faecium* EK13 [34] and *Lactobacillus gasseri* KT7 [35].

Killing Kinetics

A complete loss of viability of the target organism was recorded after 8 and 6 h when treated with 100 and 200 AU/ml of enteriocin LR/6, respectively (Fig. 3). The loss of viability in comparison to the control indicated that the bactericidal mode of action and the effectiveness appear to depend upon the concentration. A similar mode of action is employed by enterocins EJ97 [32] and CCM 4231, RZS C5, and RZS C13 [36]. Plantaricin TF711 produced by *L. plantarum* TF711, on the other hand, exerted bacteriostatic action [33].

Molecular Size Determination and MALDI-TOF/MS

In Tricine-SDS-PAGE analysis, a single band with an approximate molecular size ~6.0 kDa could be observed. That this conformed to the enterocin LR/6 was demonstrated by activity

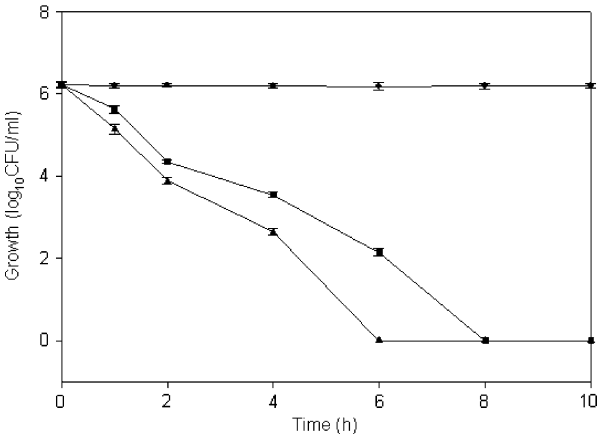

Fig. 3 Nature of antimicrobial action of enterocin LR/6 on indicator strain, *M. luteus*. Control untreated cells of *M. luteus* (*circles*) and those treated with 100 AU/ml (*squares*) and with 200 AU/ml (*triangles*) enterocin LR/6

analysis wherein a large inhibition zone was detected (Fig. 4). In addition, MALDI-TOF/MS analysis confirmed the molecular mass of ~6.1 kDa. This molecular size was similar to the bacteriocin produced by *E. avium* [37]. On the other hand, the enterocin ON-157 produced by *E. faecium* NIAI 157 [22] is known to be one of the lowest molecular weight types.

Partial N-terminal Amino Acid Sequencing

The first 10 amino acid residues of the N terminus of enterocin LR/6 were determined by Edman degradation. The following sequence was obtained: GSLXEKQXDI. In this partial sequence, X perhaps denotes the blank cycle indicating the presence of cysteine. On

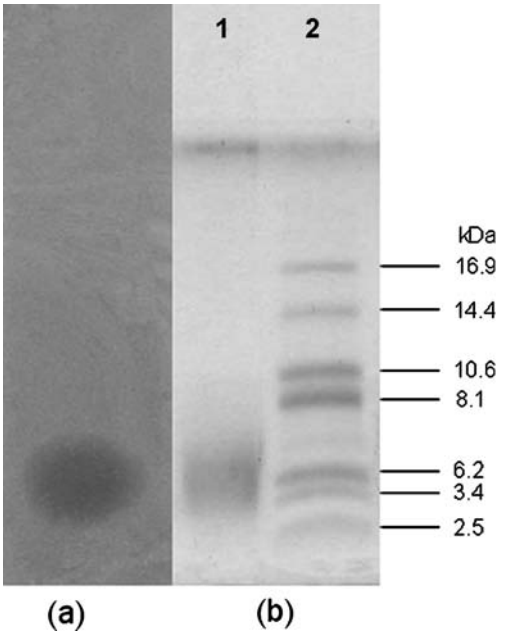

Fig. 4 Tricine-SDS-PAGE of purified enterocin LR/6 and the associated antimicrobial bioassay (**a**) corresponding zone of inhibition (**b**). *Lane 1* enterocin LR/6, *lane 2* protein molecular weight marker

comparison of this amino acid sequence with other bacteriocin sequences, available in the NCBI databank, enterocin LR/6 did not show any homology with other known bacteriocins. The presence of two or more cysteines suggests that they may form disulfide bridges, a feature often related to the antimicrobial efficiency [38]. Within the enterocins, enterocin 4 [39], bacteriocin AS-48, and ENT1 [40] are the ones that do not contain cysteine but show a broad inhibitory spectrum. Together with these properties, the lack of significant amino acid sequence similarity to other bacteriocins indicates that enterocin LR/6 may be a novel enterocin.

Conclusions

In this work, we purified a low-molecular-weight enterocin LR/6 produced from *E. faecium* LR/6. The bacteriocin was purified to homogeneity by a multistep protocol and tested by reverse-phase HPLC. The antimicrobial peptide was partially sequenced by Edman degradation, but the N-terminal amino acid sequence showed no homology with the known bacteriocins. MALDI-TOF/MS and Tricine-SDS-PAGE analysis revealed a mass of ~6.1 kDa. It showed high thermal and pH stability and sensitivity to proteolytic enzymes. Moreover, enterocin LR/6 remained stable in the presence of some surfactants and organic solvents and could be stored up to 1 year at low temperatures without losing its antimicrobial property. It showed a bactericidal mode of action on the indicator organism. The unique combination of all the above-mentioned properties revealed enterocin LR/6 to be a novel bacteriocin whose applications can be further tested.

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