Microbiological Communication

Determination of Molecular Weight and Antimicrobial Activities of a Purified Bacteriocin from *Lactiplantibacillus plantarum* MDP 5

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ABSTRACT

Nowadays, unlimited use of antibiotics and preservatives have become a big concern regarding the human health which turn the interest of biotech industries into their research on biologically active molecules from probiotic microbes, since they are nontoxic and suitable for many safer applications. On this background, the present investigation focused on the characterization of a bacteriocin from a Lactobacillus strain. The characterization of a bacteriocin was done using a *Lactiplantibacillus plantarum* MDP 5 isolated from the local markets of Puducherry and production was performed under standardized cultural conditions. The study on the maximum recovery of bacteriocin using ammonium sulphate precipitation method revealed that the 60% saturation rate evidenced highest activity of 6500 AU/ml with 4.113g/L dry weight followed by the purification was done with RP-HPLC method using C18 column. The purified bacteriocin revealed a novel molecular weight of 22 kDa with the help of SDS-PAGE which has not been reported from Lactobacillus species. Further, the purified bacteriocin evidenced appreciable antimicrobial activities against all the tested human bacterial pathogens of this study. The highest antimicrobial activity was recorded against Escherichia coli MTCC 443 followed by *Staphylococcus aureus* MTCC 96, *Vibrio parahemolyticus* MTCC 451, *Enterococcus faecalis* MTCC 9845, *Pseudomonas aeruginosa* MTCC 741 and *Klebsiella pneumoniae* MTCC 109 in the concentrations of 8AU/ml, 16AU/ml, 32AU/ml, 64AU/ml, 256AU/ml and 512AU/ml, respectively. From the overall observation, this study explored a novel bacteriocin purified from a probiotic bacterium represented potential antimicrobial activities against many human pathogens which suggesting its possible use for the safe therapeutic applications.

KEY WORDS: ANTIMICROBIAL, BACTERIOCIN, LACTIPLANTIBACILLUS PLANTARUM, MOLECULAR WEIGHT, PURIFICATION.

INTRODUCTION

Rapidly increasing microbial resistance to antibiotics is becoming a global threat which needs an immediate attention to resolve this massive issue on behalf of the whole mankind (Carlet et al. 2012; Balan 2012). Antibiotic resistance is not a new phenomenon and are naturally common which has been known since the discovery of Penicillin however the overwhelming resistance of microbes can be directly linked to its improper use through self-medication, excessive medical prescription, prolonged use of practice in veterinary applications, etc., over the past several years globally (Mathur et al. 2018). Further, there are more complications relating to the overuse of antibiotics such as killing of normal, beneficial and indigenous microbiota as well as environmental contamination etc. which are the root cause for many prolonged ecological issues. Hence, it is our significant urgency to develop an alternative method or substance to overcome such global crisis (Balan et al. 2019; Ng et al. 2020; Dai et al. 2021).

In the last two decades, antimicrobial peptides especially bacteriocin are in the frontline of research regarding the alternatives to the existing antibiotics but have never been

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substantially improved to the commercially available formats (Cavera et al. 2015). Bacteriocin are known as ribosomally synthesized proteinaceous compounds which can inhibit the growth of closely related microbes through various mechanisms. The bioactive properties of bacteriocin are first classified during 1925 however their role on biomedical arena have been recently reported (Inglis et al. 2013). Many extensive studies have been carried out till date by US Food and Drug Administration (FDA) on bacteriocin regarding their applications as a potential food preservative. Till 2012, 62 genera and 162 species are considered as safe microbial cultures for many fermentation purposes which warrant the research on antibiotics from the probiotic and prebiotic strains for their safe use on human health (Kareem and Razavi, 2020; Trejo-González et al. 2021).

Bacteriocin produced by lactic acid bacteria have been recognized as potential food preservative, in contrast their use in the field of pharmaceuticals is still in the underdeveloped stage (Hassan et al. 2020; Negash and Tsehai 2020). Despite the fact, bacteriocin are well known for the antimicrobial properties, they were less concentrated for their use as a therapeutic agent (Arthur et al. 2014). The recent trends on the search for potential alternatives in place of antibiotics enhance the studies on safe bioactive compounds from lactic acid bacteria, hence the present investigation characterize a bacteriocin compound from a *Lactobacillus* species. To accomplish the stated aim of this study, the bacteriocin produced from the *Lactiplantibacillus plantarum* MDP 5 was precipitated, purified and characterized their molecular weight and antimicrobial activities against a panel of various pathogenic bacteria.

MATERIAL AND METHODS

To analyse the microorganism and culture conditions: the bacteriocin producing bacterium, *Lactiplantibacillus plantarum* MDP 5 was used in this study for the molecular weight determination and antimicrobial activities against a panel of pathogenic bacterial strains. *L. plantarum* MDP 5 was originally isolated from the milk and dairy products collected from the local markets of Karaikal, Puducherry, India. This strain was molecular identified using 16S rRNA partial sequence method and the sequence was submitted in the NCBI GenBank with the accession number MW301154.1. The following cultural conditions were used in this study for the production of bacteriocin were 2% fructose, 1% peptone, 2% inoculum at 108 CFU/ml, pH 7, 35°C temperature, 200 rpm agitation, 0.1% $MgSO_4$, 0.1% $FeSO_4$ and 0.1% $KHPO_4$.

The bacteriocin precipitation was carried out under different concentrations of ammonium sulphate ranging from 10 to 100% saturation rate at 0°C for an overnight period (Green and Hughens 1955). After 60hrs incubation, the cultured broth was centrifuged at 3000rpm for 20min. and the cell free supernatant was collected and added with different saturation rates of ammonium sulphate for the precipitation of bacteriocin. The precipitated compounds were collected under centrifugation at 10,000rpm for 20 min. The collected precipitates were dialyzed against dialysis membrane with the pH 7 phosphate buffer solution (PBS) for 24h with intermediate changes of PBS to remove the salt and the final solution was subjected to lyophilization. The final lyophilized samples were assayed for bacteriocin bioassay and the dry weight of the total crude sample was also taken into account for selection of best percentage ammonium sulphate saturation rate for the maximum recovery of bacteriocin. The sample showed the highest recovery was used for the purification using the column chromatography.

To study the bacteriocin bioassay, the production of bacteriocin was evaluated by microtiter plate assay method (Kang and Lee 2005). The assay was performed in 96-well flat bottom polystyrene microtitre plates with lids (Tarsons, India). In this assay, well plates were filled with 100 µL of serially diluted bacteriocin samples followed by the addition of 20 µL indicator strain, *V. cholerae* MTCC 3906 at a concentration of 105 CFU/ml assessed using optical density at 620nm and 80 µL of 2.5x concentrated tryptone soy broth (TSB). The growth control wells have 100 µL of phosphate buffer (pH 7), 20 µL of indicator strain and 80 µL of 2.5x concentrated tryptone soya broth (TSB). The susceptibility control well plate has 100 µL of 4mg/ml streptomycin containing phosphate buffer (pH 7), 20 µL of indicator strain and 80 µL of 2.5x concentrated tryptone soya broth (TSB). After 6 hrs incubation, the well plates were covered with lids and incubated at 37 °C for 48 hrs. After incubation, the absorbance of the well broth was recorded at 620 nm for each well using a microplate reader (Biotek Elx808, WI, USA), where the assays were carried out in triplicate. The growth inhibition percentages of the tested pathogens were calculated as follows:

$$\% \text{ Growth inhibition} = [(1 - (As/Ac)] \times 100$$

where As represents the absorbance of the well with test samples and Ac represents the absorbance of the control well (without any added bioactive sample). Further, one arbitrary unit (AU) of bacteriocin activity was defined as the reciprocal of the highest dilution of supernatant causing 50% growth inhibition of indicator strain.

For the bacteriocin purification, the lyophilized sample was dissolved in 5ml distilled water, filtered using a 0.2µm syringe filter and the purification was done using a Reverse Phase (RP) - High Pressure Liquid Chromatography (HPLC). RP-HPLC was performed with a Waters 600 HPLC system (Waters, USA) equipped with an Xterra Prep RP18 OBD column (Waters, USA; 5 µl, 18 × 100 mm) held at 40°C. The solvent system consisted of distilled water (solvent A) and acetonitrile (solvent B). The compounds were eluted at a flow rate of 4ml/min with a linear gradient from the mixture A:B (100:0, vol/vol) to A:B (0:100, vol/vol) in 12min. The absorbances of the eluted fractions were measured at 210 nm (Mani et al. 2016). All the collected fractions were dried under rotary evaporation and stored at - 20°C. All the collected individual fractions were studied using bacteriocin bioassay and the fraction showed the appreciable activity was studied for the molecular weight of the purified bacteriocin using SDS PAGE.

The molecular weight of bacteriocin was identified with the help of SDS-PAGE (Hames 1998). A volume of 30 µl SDS gel loading buffer and samples of purified and crude bacteriocin were individually taken in one ml tube which were heated for 3 min at 60°C. Assembles were fixed in an electrophoresis apparatus and 15 µl of purified and crude bacteriocin and protein markers in the range between 10-44 kDa were loaded in various wells. The gel was run at 50 V and stained for the identification protein bands using Coomassie brilliant blue.

For the antimicrobial activities of bacteriocin, a battery of six different pathogenic bacterium were procured from MTCC (Microbial Type Culture Collection), CSIR-Institute of Microbial Technology, Chandigarh, India, for the antimicrobial study of the purified bacteriocin. The pathogenic bacterial strains were *Vibrio parahemolyticus* MTCC 451, *Escherichia coli* MTCC 443, *Staphylococcus aureus* MTCC 96, *Enterococcus faecalis* MTCC 9845, *Klebsiella pneumoniae* MTCC 109 and *Pseudomonas aeruginosa* MTCC 741. These strains were cultured on tryptic soy broth (TSB) at 37ºC overnight and the inoculum of each strain was adjusted to a concentration of 108 CFU/ml. The assay was carried out in a 96-well flat bottom polystyrene microtitre plates with lids (Tarsons, India). An aliquot of 250µl TSB were added in the well plates prepared with different concentrations of purified bacteriocin ranging from 8 AU mL^{-1} to 512 AU mL^{-1} and wells without any added bacteriocin concentration were used as negative and growth control. All the wells except the negative control were inoculated with 2.5 µL of the prepared test bacterial strains. The inoculated well plates were covered with lids and incubated at 37ºC for 48hrs. After incubation, the absorbance was measured at 600 nm for each well using a microplate reader (Biotek Elx808, WI, USA). The growth inhibition percentage of the tested microorganism was calculated as follows (Balan et al. 2016):

$$\% \text{ Growth inhibition} = [(1 - (Ac/Ao)] \times 100$$

where Ac represents the absorbance of the well with known bacteriocin concentration c and Ao represents the absorbance of the control well (without bacteriocin). Confocal laser scanning microscopy (CLSM) was used to visualize the bacterial cell biomass in the microtiter well plates after antimicrobial assay performed using bacteriocin at different concentrations. A loopful of broth culture after the antimicrobial treatment was smeared on glass microscopic slides and fixed with 2% (v/v) glutaraldehyde in PBS for 15min. Excess fixative was removed by washing the smears with PBS for 15min. The smears were stained for bacterial biomass with 0.01% (w/v) acridine orange in PBS for 15min, which was followed by washing with PBS for 30min to remove excess dyes. The stained films were visualized in situ by CLSM with an Olympus LSMGB200 CLSM (Olympus Optical Co. Ltd., Tokyo, Japan). The CLSM used an argon ion laser at 488 nm for excitation and a 605–632 nm band-pass filter for emission. Images were captured and processed using Olympus LSMGB200 CLSM bundled programs (Rice et al. 2005).

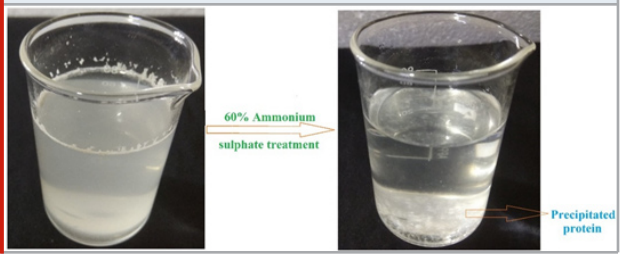
RESULTS AND DISCUSSION

Ammonium sulphate precipitation of bacteriocin: ioindustrial interest on bioactive molecules like bacteriocin from Lactic acid bacterium is becoming more because of its safer applications in the many arenas (Kranjec et al. 2021). The production of bacteriocin in this study was performed using a *L. plantarum* MDP 5 under standardized cultural conditions. After incubation, the cultured broth was centrifuge separated for the cell free supernatant. The supernatant was divided into ten equal proportions of 100ml and various percentage saturation rates of ammonium sulphate were used for the bacteriocin precipitation. After an overnight incubation, protein precipitates were separated using centrifugation methods and evaluated for the bacteriocin activity ae well as crude protein content. Among the studied ten different saturation rates, 60% ammonium sulphate revealed highest 6500AU/ml bacteriocin activity followed by 70%, 80% and 90% ammonium sulphate with 6400AU/ml, 6100AU/ml and 5600AU/ml bacteriocin activities, respectively (Table 1) (Kranjec et al. 2021).

Table 1. Crude bacteriocin precipitation using various saturation rates of ammonium sulphate

Saturation rate of Ammonium Sulphate (%)	Dry weight of crude protein (g/L)	Bacteriocin activity (AU/mg)
10	2.782	2200
20	3.127	3100
30	3.473	3900
40	3.786	4500
50	3.902	5200
60	4.113	6500
70	4.390	6400
80	4.782	6100
90	5.351	5600
100	5.672	5000

Figure 1: Appreciable precipitation of bacteriocin using 60% ammonium sulphate from cell free supernatant of *L. plantarum* MDP 5 cultured broth

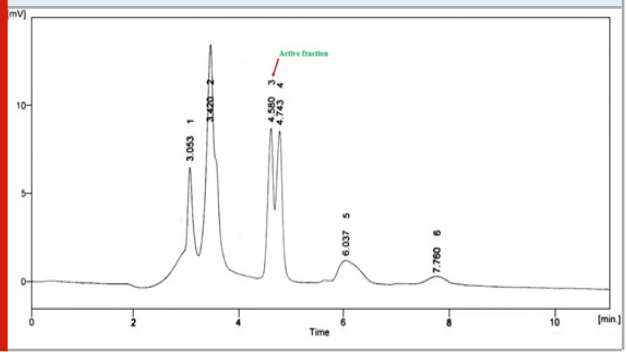


The increasing percentage saturation rates of ammonium sulphate enhance the quantity of protein precipitation however reduced the bacteriocin activity. The 60% ammonium sulphate concentration which have showed highest bacteriocin activity recorded 4.113 g of protein

per litre of cultured broth. The loss of activity may be due to the denaturation of desired compound at the higher concentration of ammonium sulphate (Balan et al. 2012; Balan et al. 2013). Further, the loss of bacteriocin activity was observed from 70% ammonium sulphate hence the 60% saturation rate was taken into account for the best recovery of bacteriocin in this study (Fig. 1). Similar to this study, different percentage saturation rates of ammonium sulphate were performed for the precipitation of bacteriocin produced by *Lactobacillus pentosus* ZFM94 however revealed highest recovery at 40% ammonium sulphate (Dai et al. 2021).

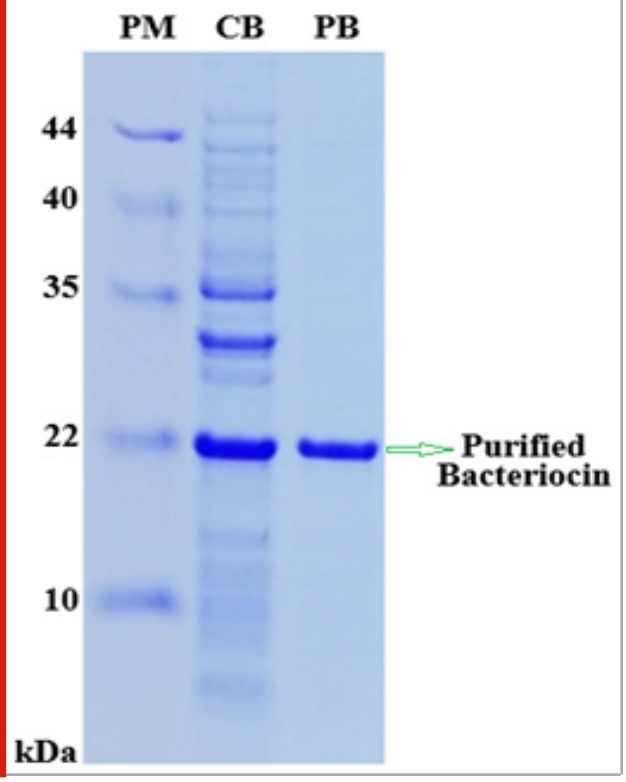
Purification and molecular weight of bacteriocin: Followed by precipitation of bacteriocin, purification was performed using RP-HPLC method. Among the collected fractions during purification procedure, six fractions revealed the presence of organic compounds, further, bacteriocin activity was evidenced only at the third fraction as shown in the figure 2 and this fraction was dried using rotary vacuum evaporation for the further analysis. The purified fraction revealed a total dry weight of 0.576 g from one litre of cultured broth and revealed the recovery rate of 90.3 % from crude precipitated form. The same RP-HPLC method using C_{18} column was used in the recent studies for the purified of two bioactive molecules namely Aneurinifactin and Staphylosan from a marine bacterium and marine yeast (Balan et al. 2017; Balan et al. 2019; Dai et al. 2021).

Figure 2: HPLC chromatogram of crude bacteriocin precipitated from 60% ammonium sulphate



The purity and the molecular weight of the purified bacteriocin was analysed with the help of SDS-PAGE. Results revealed that the purified fraction has only one molecule that is bacteriocin with a molecular mass of 22 kDa (Fig. 3). The purified bacteriocin recorded 5,030 AU activity per milligram whereas the crude precipitated form reported only 780 AU activity per milligram. Further, the purified bacteriocin evidenced 5.03 AU of bacteriocin activity per µg and a single AU of bacteriocin activity was recorded from 0.199 µg of purified bacteriocin. According to Kareem and Razavi (2020) and Moradi et al. (2021), molecular weight of most bacteriocin purified from lactic acid bacteria were reported earlier within 10 kDa. Till date, the highest molecular weight of *Lactobacillus bacteriocin* was purified from a *L. helveticus* which was about 15 kDa. Based on the above observations, the purified bacteriocin from this study is a novel bioactive compound (Kareem and Razavi 2020; Hassan et al. 2020; Moradi et al. 2021).

Figure 3: Molecular weight determination of the purified bacteriocin using SDS PAGE in which the lane "PM" represents the protein molecular weight marker, lane "CB" represents the crude bacteriocin obtained after precipitation procedure and the lane "PB" represents the purified bacteriocin after RP-HPLC



Antimicrobial activities of the purified bacteriocin: The antimicrobial activities of the purified bacteriocin were evaluated with the help of microdilution method using a series of double dilution concentrations from 8 AU/ml to 512 AU/ml. Interestingly, all the six human bacterial pathogens tested in this study revealed strong susceptibility against this purified bacteriocin and all the pathogens revealed their complete growth inhibition within these tested concentrations. The strongest antimicrobial activity was recorded against *Escherichia coli* MTCC 443 at 8 AU/ml followed by *Staphylococcus aureus* MTCC 96, Vibrio parahemolyticus MTCC 451, *Enterococcus faecalis* MTCC 9845, *Pseudomonas aeruginosa* MTCC 741 and *Klebsiella pneumoniae* MTCC 109 in the concentrations of 16 AU/ml, 32 AU/ml, 64 AU/ml, 256 AU/ml and 512 AU/ml, respectively (Table 2) (Hassan et al. 2020; Moradi et al. 2021).

Furthermore, the results revealed that this purified bacteriocin was activity against both gram positive and negative bacteria since *Staphylococcus aureus* MTCC 96 and *Enterococcus faecalis* MTCC 9845 were gram positive bacteria and rest of the strains were gram negative

bacteria. CLSM was used to study the bacterial biomass of the treated well plates at different concentrations of the purified bacteriocin. The microscopic photographs at all the significant concentrations were illustrated in the figure 4. Further, all the growth control well plates showed dense biomass of bacterial cells, the concentrations at which bacteriocin revealed completion growth inhibition showed no cell and bacteriocin activity dependent cell biomass was observed on the rest of the photographs shown which illustrated the microscopy representation of the antimicrobial activity (Hassan et al. 2020; Moradi et al. 2021).

Table 2. Antimicrobial activities of the purified bacteriocin at various concentration against different pathogenic bacteria, all the experimental values are expressed as mean ± standard deviation, (n=3) and "*" represents the repeated values of the last column

Test bacterial pathogen	Arbitrary unit per millilitre (AU/ml) of bacteriocin required to inhibit the percentage growth of human pathogens						
	8	16	32	64	128	256	512
Vibrio parahemolyticus MTCC 451	73±2.3	91±3.7	100±0	*	*	*	*
Escherichia coli MTCC 443	100±0	*	*	*	*	*	*
Staphylococcus aureus MTCC 96	88±3.3	100±0	*	*	*	*	*
Enterococcus faecalis MTCC 9845	55±2.3	76±3.3	93±2.7	100±0	*	*	*
Klebsiella pneumoniae MTCC 109	37±1.7	49±2.3	61±2.7	72±3.3	83±3.7	93±4.3	100±0
Pseudomonas aeruginosa MTCC 741	45±2	56±2.3	69±3.3	82±3.7	96±4	100±0	*

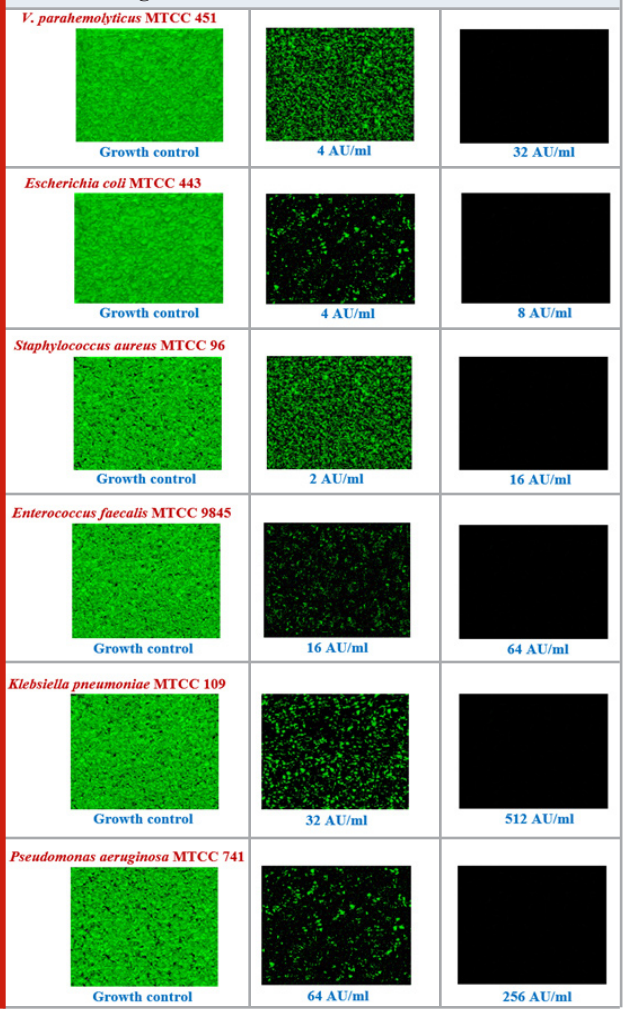


Figure 4: Antimicrobial activities of the purified bacteriocin studied using CLSM

Similarly, bacteriocin obtained from *Lactobacillus plantarum* zrx03 procured from infant's feces revealed broad antimicrobial activity against *Bacillus subtilis* CICC 10002, *Bacillus anthracis* CICC 20443, *Escherichia coli* JM109 ATCC 67387, Salmonella CMCC 541and *Staphylococcus aureus* ATCC 25923 (Lei et al. 2020). Likewise, bacteriocin of a Lactobacillus species procured from yoghurt showed appreciable antimicrobial activity against Acinetobacter baumannii and *Staphylococcus aureus*. The above studies signify the antimicrobial activity of the purified bacteriocin from this investigation (Hassan et al. 2020).

CONCLUSION

The findings of the present study produced bacteriocin using a probiotic *L. plantarum* MDP 5 which was ammonium sulphate precipitated with 60% saturation rate and further purified using RP-HPLC procedure. SDS-PAGE revealed the novel molecular weight of this purified bacteriocin having 22 kDa. Furthermore, the purified bacteriocin evidenced appreciable antimicrobial activity against all the tested six bacterial pathogens. All the above studies proved that this bacteriocin can possibly be used as a safe and potential antimicrobial agent in the field of pharmaceutical applications.

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Conflict of Interests: Authors declare no conflict of interest to disclose.

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