In-vitro and In-silico approach for characterization of antimicrobial-peptide from probiotics against *Staphylococcus aureus* and *Escherichia coli*

Amrutha Bindu

University of Mysore

Lakshmidhevi N (✉ lakshmiavina@rediffmail.com)

University of Mysore <https://orcid.org/0000-0003-0280-3029>

Research Article

Keywords: bacteriocin, lactic acid bacteria (LAB), probiotic, plantaricin, lactobacillus, MIC

Posted Date: April 21st, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-366314/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

The present aim was to determine the characteristic feature and stability of antimicrobial compound (AMC) produced by probiotic strains of *Enterococcus durans* MCC4243, *Lactiplantibacillus plantarum* (Basonym: *Lactobacillus plantarum* MCC4246) and *Limosilactobacillus fermentum* (Basonym: *Lactobacillus fermentum* MCC4233) against *Staphylococcus aureus* MTCC96 and *Escherichia coli* MTCC118. Growth kinetic assay revealed 24h of incubation to be optimum for bacteriocin production. Ammonium sulphate precipitation-dialysis was found to be favorable method for extraction of AMC compared to other methods employed. The partially purified compound after ion-exchange chromatography was found to be thermo-resistant upto 90°C and stable under wide range of pH. The compound was sensitive to proteinase-K, but resistant to trypsin, α -amylase and lipase. The apparent molecular weight of AMC from MCC4243 and MCC4246 was found to be 3.5KDa. PCR confirmed the presence of plantaricinA gene in MCC4246. Translated partial amino acid sequence of *pInA* gene of MCC4246 displayed 48 amino acid sequence which had 100% similarity with plantaricinA of *Lactobacillus plantarum* (WP 0036419). The sequence revealed 7 β sheets, 6 α sheets, 6 predicted coils and 9 predicted turns and functions on cytoplasm with 10.82 isoelectric point and 48.6% hydrophobicity. From the study, the amino acid sequence “KSSAYSLQMGATAIKQVKKLFKKWGW” of peptide was predicted to be responsible for antimicrobial activity.

Introduction

In recent years, people are being attracted towards consumption of food, positively associated with health and nutrition. The attitude towards un-acceptance of food products containing some of the artificial preservatives has increased due to growing awareness of its ill-effect on human and animal health (Anand and Sati 2013). Hence it is a major challenge in food sector to satisfy consumers demand for products free of chemical preservatives and also to ensure natural means of food safety. Further, the chemically synthesized antibiotics and their non judicious usage has resulted in emerging multidrug resistant pathogens and their devastating damage thereafter. Consequently, the need for new strategies and methodologies to control infectious pathogens is increasing. The health benefits executed by some of the traditional foods have attracted researchers to dig the mechanism and apply the knowledge for betterment of life. In this regard, antimicrobial compounds (AMC) or the bacteriocins from food grade lactic acid bacteria (LAB) have engrossed because of its safety and broad range of antibacterial spectrum.

Bacteriocins are ribosomally synthesized proteinaceous compound produced by bacteria to protect themselves as well as to competitively colonize in the gut through elimination of undesirable organisms. Bacterial ability to inhibit spoilage organisms has been exploited for its application as natural preservatives in various food formulations. LAB being the natural microflora of human intestine has added advantage for its application in food due to their GRAS (Generally recognized as Safe) status. Bacteriocins from LAB are of low molecular peptides which vary in their size, post-translation modifications, stability and mode of action (Amortegui et al. 2014). According to Drider et al (2006) and

Nes and Holo (2000) bacteriocins, have been classified into 3 groups based on presence or absence of post-translational modification: **Class I**-Lantibiotics containing lanthionine or β -methyl-lanthionine; **Class II**- heat stable unmodified bacteriocins; **Class III**-large heat labile bacteriocin. Class II bacteriocins are further subdivided into Class IIa (one peptide), Class IIb (two peptide), Class IIc (circular), Class IId (unmodified, linear, non-pediocin like) and Class IIE (Microcin like). Class IIa bacteriocins are <10 kDa in size with 37-48 amino acids that inhibit growth of food spoilage organisms such as *Listeria monocytogenes*, *Bacillus cereus*, *Clostridium perfringens*, *Staphylococcus aureus* and *E. coli* (Rodrigues et al. 2005).

Bacteriocin produced by *Lactococcus lactis* subsp. *lactis* ie., Nisin is the only bacteriocin approved by US-FDA for commercial application in food products. However the application of nisin is limited in neutral and alkaline pH. Long-time preservation of foods by biopreservative process and inhibition of pathogenic microbes through natural means is therefore universal need in the present scenario. Considering this, the present work was carried out to optimize the method for efficient extraction of antimicrobial compound (AMC) from LAB isolated from fermented foods. These LAB cultures including *Enterococcus durans* DB-1aa (MCC4231), *Enterococcus faecium* DB-b2-15b (MCC4263), *Lactiplantibacillus plantarum* (Basonym: *Lactobacillus plantarum* Cu2-PM7 (MCC4246), *Limosilactobacillus fermentum* (Basonym: *Lactobacillus fermentum* Cu3-PM8 (MCC4233) and *Lactobacillus fermentum* IB-PM15 have been previously characterized for their potential probiotic properties (Bindu and Lakshmidhevi 2020). In the present study, we report the effect of physical and chemical parameter on the activity of partially purified AMC and PCR approach has been applied for characterization of antimicrobial peptide.

Materials And Methods

Bacterial cultures and growth condition

The selected probiotic cultures *Enterococcus durans* DB-1aa (MCC4231), *Enterococcus faecium* DB-b2-15b (MCC4263), *Lactobacillus plantarum* Cu2-PM7 (MCC4246), *Lactobacillus fermentum* Cu3-PM8 (MCC4233) and *Lactobacillus fermentum* IB-PM15 were isolated from fermented food products, identified and deposited at Microbial Culture Collection Center, Pune (Bindu and Lakshmidhevi 2020). Recently, *Lactobacillus plantarum* has been classified as *Lactiplantibacillus plantarum* and *Lactobacillus fermentum* as *Limosilactobacillus fermentum* (Zheng et al. 2020). They were grown in deMann Rogosa Sharpe (MRS) medium at 37°C for 16-18 h. To study the properties of AMC two indicator organisms ie., *Staphylococcus aureus* MTCC 96 and *Escherichia coli* MTCC 118 were used. The indicator bacteria were grown in Brain Heart Infusion (BHI) media at 37°C under constant shaking condition (120 rpm). The selected cultures were stored under 40% glycerol at -20°C until use.

Antimicrobial activity of selected probiotic cultures

Antimicrobial activity was tested by the agar well diffusion assay as described by Xie et al. (2009). Each experiment was carried out in triplicates and the mean diameter of the inhibition zone was measured in mm and recorded. Antibiotic chloramphenicol (1 mg/ml) was used as positive control. The inhibitory

activity was further quantified by the two-fold dilution method. Briefly, cell suspension of pathogenic culture grown in BHI broth was adjusted to turbidity of 0.5 McFarland which is equivalent to $1-2 \times 10^5$ CFU/mL. Antibacterial activity was quantified by testing the two fold serially diluted sample of AMC by agar well diffusion assay and the results were expressed as AU/ml. Arbitrary unit (AU) is defined as the reciprocal value of the highest dilution at which the zone of inhibition is observed.

Minimum Inhibitory Concentration (MIC)

MIC was estimated by using different concentration of the sample (10-100 µg) in a 96 well ELISA plate. Freshly, grown pathogenic cultures at a final concentration of 10^5 CFU/mL was inoculated into each well and the plate was incubated at 37°C for 24 h. Chloramphenicol (5-50 µg) was used as a positive control and sample with un-inoculated broth as negative control. After incubation absorbance was measured at 600nm. MIC was defined as the lowest protein concentration (in mg/mL) which inhibited the growth of pathogenic culture completely. All assays were performed in triplicate.

Determination of proteinaceous nature of AMC

The proteinaceous nature of AMC was checked agar well diffusion assay by treating the crude AMC with proteinase K at a final concentration of 2 mg/ml for 30 min. The untreated CFS and the enzyme alone in buffer (pH 7.0) served as controls.

Probiotic growth and AMC production

Growth kinetics and AMC production was studied for a period of 5 days. The selected cultures were inoculated (1% v/v) in 250 ml MRS broth and incubated at 37°C without agitation. An aliquot of sample was drawn after every 12 h during growth and checked for bacterial growth and antimicrobial activity. Bacterial growth was determined by measuring cell density at 600nm. The antimicrobial activity was evaluated after filter sterilizing the neutralized CFS by well diffusion assay.

Extraction of the AMC

Optimization of the extraction procedure

The CFS of the selected probiotic cultures was subjected to various extraction procedures to determine a suitable protocol for maximum recovery of AMC. **Protocol 1:** CFS was mixed with ice-cold ethanol (1:1 v/v) and allowed for precipitation overnight at 4°C. The precipitate was air-dried, resuspended in phosphate buffer (pH 7.0) and checked for inhibitory activity. **Protocol 2:** CFS was blended with equal volumes of chloroform on a magnetic stirrer for 1 h. The mixture was left at room temperature undisturbed for phase separation. AMC in the interphase was collected carefully and the residual chloroform was evaporated under nitrogen and monitored for the inhibitory activity. **Protocol 3:** Fine powder of ammonium sulfate was added slowly to the CFS until a saturation level of 80% under constant stirring. The procedure was followed at 4°C under aseptic condition. The precipitate obtained was dialyzed against phosphate buffer (pH 7.0) with three changes of buffer and then analyzed for inhibitory

activity. **Protocol 4:** Butanol (1: 0.5 v/v) was added to CFS and vigorously mixed in a separating funnel. The mixture was left at room temperature for phase separation. The butanol layer was carefully collected and evaporated to dryness using rotary evaporator and checked for the activity.

Ion exchange chromatography

The dialyzed sample (60 mg protein) of each culture was loaded separately onto a DEAE cellulose column (18 X 3 cm) pre-equilibrated with sodium phosphate buffer (0.2 mM; pH 7.0). A linear gradient of 0.0-1.0M NaCl in the same buffer was used to elute the protein fractions at a flow rate of 1 ml/min. Fractions (2 ml) was collected and checked for absorbance at 280 nm. The fractions showing the highest protein content were pooled, lyophilized and examined for antimicrobial activity.

Stability of partially purified AMC

Effect of Temperature and pH

The effect of temperature was evaluated by incubating the partially purified AMC for 30 min at different temperatures (30, 40, 50, 60, 70, 80, 90°C). The residual activity was checked by agar well diffusion assay after cooling to room temperature. The effect of pH was studied by adjusting the AMC to different pH (3-9) using 1M NaOH or 1M HCl. The sample was incubated at 37°C for 30 min. The inhibitory activity was later checked after neutralization of AMC.

Effect of enzymes

The effect of enzymes such as trypsin, amylase and lipase on inhibitory activity was evaluated by treating the partially purified AMC with each enzyme at a final concentration of 1mg/ml. Samples were incubated at 37°C for 30 min. After incubation, samples were heat treated at 90°C for 5 min to inactivate the enzymes and then the residual activity was determined as described earlier.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and overlay assay to detect AMC

Crude sample, dialyzed sample as well as column purified fractions of bacteriocin were subjected to SDS-PAGE (Laemmli 1970) along with low range protein marker ranging from 3.5-43 KDa. The antimicrobial activity in the partially purified sample was determined by overlay assay (Barboza-Corona et al. 2007). The sample containing 80 µg of protein was mixed with equal volume of non-reducing loading buffer and directly loaded in the SDS-PAGE gel without boiling. Following electrophoresis, the gel was cut vertically and immersed in fixing solution (25% isopropanol, 10% acetic acid) for 1 h and washed with double-distilled water with three changes after every 30 min. Later, the gel was sterilized by exposing to UV for 30 min and then aseptically placed in a sterile petriplate and overlaid with BHI agar pre-inoculated with 1% (equivalent to 10^8 CFU/ml) overnight grown test organism (*Staphylococcus aureus* or *E. coli*). The petriplate was later incubated at 37°C for 24 h and observed for the presence of inhibition zone.

Detecting bacteriocin genes by polymerase chain reaction (PCR)

The presence of bacteriocin gene was identified in *L. plantarum* Cu2-PM7 using plantaricin A (F: GTA CAG TAC TAA TGG GAG; R: CTT ACG CCA ATC TAT ACG) and P1/P2 primers (F: AAAATATCTAACTAATACTTG; R: TAAAAAGATATTTGACCAAAA). The gene was amplified in 25 µl reaction mixture containing 2 µl of 1X PCR buffer, 1.5 µl of MgCl₂, 5 µl of dNTPs, 0.5 µl of each (Forward and reverse) primer, 2.5 U of *Taq* polymerase and 100 ng of genomic DNA. PCR conditions include denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for pln primer and 52°C for P1/P2 primer, for 45 sec and extension at 72°C for 1 min and final extension of 72°C for 10 min. The amplified PCR product was sequenced at GENESPY Research Services, Mysore. Sequence was matched with similar sequence from NCBI data base.

Bacteriocin peptide analysis

Bacteriocin peptide structure for the putative amino acid was predicted by using **Geneious Prime® 2019.2.1 Software** (Kearse et al. 2012). Using the software, secondary structure, antigenic regions, signal cleavage sites, transmembrane regions and protein domains prediction plugins was determined. Further the amino acid sequence data was analyzed. The peptide showing activity was later analyzed by using protein predict server (<https://open.predictprotein.org/>) followed by PDB database (Armstrong et al. 2020).

Statistical analysis

The experiments were conducted in triplicates and the data analysis was done using STATISTICA version 6 software (Statsoft Inc., Tulsa, USA). The significant different between the samples were analyzed by using ANOVA (analysis of variance), and the mean separation was accomplished by Duncan's multiple range test.

Results

Minimum inhibitory concentration of AMC

According to the results obtained, MIC was in the range of 2200 to 2400 µg against *S. aureus* and *E. coli*. Higher activity against *S. aureus* (228.6 AU/mg) and *E. coli* (114.3 AU/mg) was noticed in AMC of *L. plantarum* Cu2-PM7, followed by *L. fermentum* Cu3-PM8 (139.1 and 69.6 AU/mg against *S. aureus* and *E. coli* respectively). *Enterococcus faecium* DB-b2-15b displayed lowest activity (38.1 and 38.1 AU/mg against *S. aureus* and *E. coli* respectively). Based on the data, three cultures viz., *Enterococcus durans* DB-1aa, *L. plantarum* Cu2-PM7 and *L. fermentum* CU3-PM8, showing higher activities were selected for further study.

Proteinaceous nature of AMC from probiotic culture

Proteinaceous nature of the neutralized CFS was confirmed by treating with proteinase K. According to the results obtained, the activity was not lost after neutralization but was significantly reduced on treating

with proteinase K. This confirms that the inhibitory activity was not due to acidity but was due to proteinaceous compound.

Growth kinetics and bacteriocin production

The selected cultures entered the late exponential phase in 24 h and remained constant in stationary phase upto 5 days. The OD (optical density) at 600nm is presented in Table 1. Inhibition zone in the range of 12-16 mm dia was observed upto 3 days against *S. aureus*. On day 4th and 5th, no activity was observed. MIC against *S. aureus* was 2400 µg for all the tested AMC after 24 h of growth (Table 1). However on the 5th day MIC was 5200, 5000 and 3800 µg for AMC from DB-1aa, Cu2-PM7 and Cu3-PM8 respectively. Activity was highest at 24th h, later the activity gradually decreased. According to the data obtained, maximum activity (266.02 AU/mg) was noticed in *L. plantarum* Cu2-PM7. The AMC compound of Cu2-PM7 and Cu3-PM8 showed inhibitory activity against *E. coli* upto 5 days, but DB-1aa displayed zone of inhibition upto 3 days (Table 1). With increase in incubation days, a gradual reduction in the size of inhibition zone was observed. The MIC was 2400 µg on the 1st day but displayed 5600, 5200 and 3800 µg on the 5th day. The activity was maximum (133.01 AU/mg) with *L. plantarum* Cu2-PM7 after 24 h of incubation.

Extraction of AMC from selected cultures

AMC from selected cultures was extraction by four different techniques to determine the best method for extraction of AMC. Ethanol precipitation did not show any inhibitory activity against *S. aureus*, however against *E. coli* lesser zone of inhibition (10 mm dia) was observed (Fig. S1). Comparatively, sample after ammonium sulphate precipitation-dialysis displayed maximum zone of inhibition than chloroform extract and butanol extract (Table 2). MIC against *S. aureus* was in the range of 1900 – 2200 µg and 2000 – 2300 µg against *E. coli*. According to the data obtained, ammonium sulphate precipitation-dialysis was found to be the best method for extraction which showed an activity of 155.27, 212.72 and 179.80 AU/mg with *E. durans* DB-1aa, *L. plantarum* Cu2-PM7 and *L. fermentum* Cu3-PM8 respectively. Hence, further step of purification was carried out after ammonium sulphate precipitation and dialysis.

Purification of bacteriocin by ion exchange chromatography

Fig. S2 display the inhibitory activity of crude CFS and ammonium sulphate precipitated- dialyzed sample of each isolate. An increase in the inhibition zone size could be observed in the dialyzed sample. Further, ion-exchange chromatography was carried out using DEAE cellulose column as a next step of bacteriocin purification. As shown in Fig. 1, the sample from each culture was separated into three fractions. Protein fraction of DB-1aa was eluted between 0.06 – 0.2 M (fraction 1), 0.24 – 0.4 M (fraction 2) and 0.4 – 1.0 M (fraction 3). In case of Cu2-PM7 elute from 0.06-0.36 M, 0.45 – 0.6 M and 0.63 – 1.0 M was considered as fraction 1, 2 and 3 respectively. Similarly, in Cu3-PM8 fractions from 0.06 – 0.33 M, 0.48 – 0.6 M and 0.63 – 1.0 M was selected. The eluent of each peak was collected and concentrated by lyophilization. The antimicrobial activity assay confirmed the zone of inhibition in fraction 1 and 2 of all

the three culture against *S. aureus* and *E. coli* (Fig. 1). The data obtained revealed highest inhibition zone of 20 and 18 mm against *S. aureus* and *E. coli* by fraction-1 of *L. plantarum* Cu2-PM7 with MIC of 1200 and 1300 µg respectively. The details of purification data are summarized in Table 3. The final yield of bacteriocin obtained after ion-exchange chromatography was 0.4, 0.5 and 0.4% for DB-1aa, Cu2-PM7 and Cu3-PM8 respectively against *S. aureus*. Correspondingly, a final yield of 0.35, 0.76 and 0.68% was found against *E. coli*.

Effect of temperature and pH on bacteriocin activity

According to the results obtained, the bacteriocins from all the tested cultures were active in all the temperature tested (Table 4). However, bacteriocin from Cu3-PM8 showed slight reduction in the activity at higher temperature. Further, the pH stability of bacteriocin purified from selected probiotic culture was analyzed at various pH ranging from pH 3-10 (Table 5). The data revealed that the inhibitory activity was more active at acidic pH (inhibition zone ranging from 14-20 mm dia). As the pH approached neutral, the activity reduced and was completely inhibited at alkaline pH.

Effect of enzymes on bacteriocin activity

According to the data obtained, the inhibitory activity was retained in all the three bacteriocin after treating with α-amylase and lipase. However, in presence of trypsin 55, 34 and 43% activity was retained.

SDS-PAGE analysis of antimicrobial compound

Molecular weight of the antimicrobial compound from selected probiotic culture (Cu2-PM7 and Cu3-PM8) was determined by SDS-PAGE (Fig. 2). Accordingly, apparent molecular weight of both AMC was found to be 3.5 kDa. Inhibition zone corresponding to their respective protein bands was developed on performing activity assay by overlaying indicator strain (Fig. 2).

Detecting plantaricin gene by PCR

PCR analysis of plantaricin gene was carried out in Cu2-PM7 using plnA and P1/P4 primer. A good amplification of 450 bp was observed with plnA F/R primer. On the other hand, no amplification was detected with P1/P4 primer. The control culture, *E. durans* DB-1aa did not show any amplification in presence of any of the tested primer. Further, the PCR product was sequenced and the obtained sequence was subjected to BLASTn search in NCBI database and aligned with hit sequences. The results indicated 100% homology with plantaricin *plnA* sequence of *Lactobacillus plantarum* strain EG.LP 18.7 (MN172266.1) (Fig. 3).

The nucleotide sequence was translated to amino acid sequence and checked for homology with BLAST search. Translated partial amino acid sequence of *pln A* gene of Cu2-PM7 displayed 48 amino acid

sequence which had 100% similarity with plantaricin A of *L. plantarum* (WP 0036419). Fig. 4 represents the similarity of plnA-PM7 amino acid sequence with related sequence from NCBI data base.

Bacteriocin peptide analysis

The pln A gene sequence was translated and the 138 amino acid sequence thus obtained was studied for its structural and functional properties. It was observed that the partial sequence of Cu2-PM7 possessed 10.82 isoelectric point and 48.6% hydrophobicity. The sequence possessed 7 β sheets, 6 α sheets, 6 predicted coils and 9 predicted turns and functions on cytoplasm. One topological domain, which encodes the bacteriocin-like signal sequence (**MKQLSNKEMQKIVGG**) was noticed with a GG cleavage motif at their N-terminal region. Further, four antigenic regions were detected in the acquired sequence which were located at sites 6-26, 28-46, 65-77 and 122-135 with sequence read **LSILRCAVNVILSSKYVQSNQ, LFKIVTLTFIKYLIALEFH, THYPKSEVIIMKI** and **WLIYITVFQVIFRIDGV**, respectively. A 3D structure for the bacteriocin-like peptide was inferred from Phyre2 and indicated alpha and beta sheet structure fragments (Fig. 5). The protein-predict database showed maximum homology towards bacteriocin plantaricin A of *Lactobacillus plantarum* WCFS1, with the ID P80214. Hence, it can be perceived that the amino acid sequence stretch of our native isolate Cu2-PM7 possessed “**KSSAYSLQMGATAIKQVKKLFKKWGW**” peptide showing a molecular weight of 3 kDa.

Discussion

The increasing awareness among consumers about the side effect caused by some of the chemically synthesized artificial preservatives motivated the study to characterize bacteriocins from probiotic culture that can positively enhance the characteristic taste, wellness and safety of the product. The present probiotic cultures were isolated from fermented food and were shown to exhibit potential probiotic properties (Bindu and Lakshmidevi 2020). The selected cultures showed acid and bile tolerance, gastrointestinal survival, good adhesion property and also possible functional properties including antioxidant activity and industrially important enzyme production. The selected probiotic cultures displayed large spectrum of inhibitory activity against pathogenic and spoilage bacteria including *Micrococcus luteus*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus subtilis*, *Klebsiella* sp., *E. coli*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, *Salmonella* sp, and *Enterobacter aeruginosa*. In the view of their probiotic candidacy, the AMC from these cultures were characterized in the present investigation.

Each indigenous strain exhibited a variable titre and pattern of inhibition against selected pathogens. On the context of inhibitory activity of all the selected probiotic culture, studies were continued with *Staphylococcus aureus* and *E. coli* as indicator organisms because of their know pathogenecity (Croxen et al. 2013; Kadariya et al. 2014). According to the results obtained, inhibitory activity against *S. aureus* and *E. coli* was in the following increasing order: DB-b2-15b < DB-1aa < IB-PM15 < Cu3-PM8 < Cu2-PM7. Further studies were therefore continued with DB-1aa, Cu2-PM7 and Cu3-PM8.

Generally, bacteriocins are proteinaceous compound produced by certain bacteria to hinder the growth of similar or closely related strains. Hence, to confirm the proteinaceous nature of the AMC, the cell free supernatant (CFS) of the probiotic culture was treated with proteinase K. The enzyme “proteinase K” is a serine protease responsible for degradation of protein. Accordingly, the CFS treated with the enzyme was unable to inhibit the pathogen indicating the denaturation of proteinaceous compound present in CFS which is responsible for antimicrobial activity. As these probiotic cultures are lactic acid bacteria, the action of lactic acid was initially nullified by adjusting the pH of CFS to 7.0 using 1N NaOH.

Growth kinetic studies reveal that the selected probiotic cultures enter late exponential phase within 24 h and in stationary phase for the next 5 consecutive days tested. With respect to *S. aureus*, inhibitory activity was noticed upto 3 days, later reduction or no activity was seen. In case of *E. coli*, inhibitory activity was observed upto 5 day. Maximum activity was determined in Cu2-PM7 after 24 h against *S. aureus* (266.02 AU/mg) and *E. coli* (133.01 AU/mg). Bello et al. (2018) have shown bacteriocins from *L. plantarum* Z1116 with 500 AU/ml at the 9th hour. The inhibitory activity gradually increased and reached 12,000 AU/ml after 18th hour of growth in MRS broth at 30°C where high cell density (6.8 OD₆₀₀ nm) was recorded. In the same study, *Enterococcus faecium* AU02 and *Leuconostoc lactis* PKT0003 showed highest level of bacteriocins activity (3200 AU/ml and 1600 AU/ml) at 21st and 24th h of growth where highest (2.8 and 2.75 OD₆₀₀ nm) cell density was recorded. Milioni et al. (2015) compared bacteriocin production of *Lactobacillus plantarum* LpU4 grown in MRS broth and MRS broth buffered with citrate at 25°C. In normal MRS broth, the antimicrobial activity was initiated at the beginning of the exponential phase reaching a maximum (1,600 AU/ml) at late exponential phase (24 h) and remained constant during the stationary phase upto 48 h. In buffered MRS broth, the activity increased from 100 to 3,200 AU/ml during the exponential growth phase and was stable until 48 h. According to Barbosa et al. (2013), the optimum condition for bacteriocins production (1600 AU/ml) was 25°C and 20 h of incubation time.

As a first step of purification, the AMC was subjected to various extraction techniques for optimum yield. Accordingly, ammonium sulphate precipitation-dialysis technique was found to be more favorable compared to chloroform extraction, ethanol precipitation or butanol extraction. Likewise, Song et al. (2014) used ammonium sulphate precipitation as first step of bacteriocin purification from *Lactobacillus plantarum* ZJ5 and reported an activity of 317.14 AU/mg. Earlier studies have reported most of the bacteriocin to contain positively charged amino acid residues with hydrophobic features (Barbosa et al. 2013). Hence, ion exchange chromatography is the commonly applied strategy which has been successfully used for purification of bacteriocin. In the present investigation, therefore ion exchange chromatography has been applied for purification of AMC. According to the data obtained, increase in the purification fold of 2.75, 1.87 and 1.92 was noticed with respect to DB-1aa, Cu2-PM7 and Cu3-PM8 against *S. aureus*. With regard to *E. coli*, purification fold of 2.4, 2.84 and 3.26 was perceived. Song et al. (2014) obtained 1.7% yield after RP-HPLC purification of plantaricin from *Lactobacillus plantarum* ZJ5. In another study, RP-HPLC purified bacteriocin from *Lactobacillus sakei* showed 74949.6 AU/mg activity with 32% yield and 40.05 fold increase in the activity as tested against *Enterococcus faecalis* J2-2 (Barbosa et al. 2013).

Stability study of partially purified AMC at different temperature revealed that the compound is resistant upto 90°C. Earlier literature also support the data that the AMC from *Lactobacillus plantarum* are heat stable (Todorov 2009). Earlier studies have shown bacteriocins stable upto 60°C (Nowroozi et al. 2004; Sowani and Thorat 2012; Martinez et al. 2013), however at higher temperature above 80°C, a significant reduction in the antimicrobial activity has been reported. In the present study, the tested bacteriocin was stable even at 90°C. Similar results of temperature stability have also been reported for bacteriocins LPBM10, bacST202Ch, bacST216Ch, enterocin AS-48, and plantaricin OL15 (Mourad et al. 2005; Zapata et al. 2009; Todorov et al. 2010). Barbosa et al. (2013) have reported a anti-listerial bacteriocin from *Lactobacillus sakei* MBSa1 which is stable even at 121°C for 15 min. Lactocin NK24 from *Lactococcus lactis* display 87% reduction in the inhibitory activity at 100°C and gets completely inactivated after steam sterilization (Lee and Paik 2001). Ferchichi et al. (2001) reports almost 25 and 8.3% reduction in the antimicrobial activity of lactocin MMFII, from *Lactococcus lactis* at 80 and 110°C respectively. Bacteriocin tolerance to higher temperature would be an essential property for their application in thermally processed food. In this regards, the three bacteriocins purified from the selected probiotic culture indicate their possible application as biopreservative agents.

The data on pH stability showed that the bacteriocins from the selected probiotic cultures are active at acidic pH. The activity, however significantly reduced at neutral pH and alkaline pH. Similarly, previous studies have reported that bacteriocins are highly stable at acidic pH but get inactivated at alkaline pH (Todorov et al. 2010; Hernandez et al. 2005). The bacteriocin with anti-listerial activity from *Lactobacillus sakei* MBSa1 was found stable at pH 2 to 6, but lost part of the activity at pH 8 and 10 (Barbosa et al. 2013). Todorov and Dicks (2005) reports almost 50% reduction in antimicrobial activity of bacteriocins ST28MS and ST26MS from *L. plantarum* at pH values lower than 4.0. Zapata et al. (2009) observed a marked reduction in the inhibitory activity of bacteriocin from *L. plantarum* LPBM10 with increase in pH value higher than 5. However, several studies have shown bacteriocins (ST23LD, ST341LD, bacST202Ch, bacST216Ch, and ST71KS) from *Lactobacillus plantarum*, which are stable between pH 2.0 and 12.0 (Todorov and Dicks 2005; Todorov et al. 2010; Martinez et al. 2013).

The effect of digestive enzyme such as trypsin, lipase and α -amylase on the bacteriocin activity was analyzed and the residual activity was determined. According to the data obtained, bacteriocin of the all the three selected cultures were stable in presence of lipase and α -amylase indicating the lack of carbohydrate or lipid moiety. However in presence of trypsin partial inactivation was observed.

SDS-PAGE analysis and over-lay assay revealed a molecular weight of bacteriocin to be 3.5 kDa in both Cu2-PM7 and Cu3-PM8. Earlier reports confirms that the bacteriocin produced by *Lactobacillus* spp have molecular weight lower than 10 kDa (Cintas et al. 2001; Cotter et al. 2013) as well as morethan 14 kDa (Todorov et al. 2004; Todorov and Dicks 2006). In the similar line, bacteriocin-producing lactic acid bacteria (LAB) were isolated from fermented *Parkia biglobosa* seeds and were identified as *Lactobacillus plantarum* Z1116, *Enterococcus faecium* AU02 and *Leuconostoc lactis* PKT0003. They produced bacteriocins of size 3.2 kDa, 10 kDa and 10 kDa, respectively (Bello et al. 2018). Tome et al. (2009) partially purified bacteriocins from nine LAB isolated from vacuum-packaged cold-smoked salmon (CSS)

which was active against *Listeria monocytogenes*, *E. faecalis*, *E. faecium*, and *Staphylococcus aureus*. The molecular size of bacteriocins ranged from 2.8 to 4.5 kDa. Messi et al. (2001) isolated LAB from Italian sausages that produced plantaricin with a molecular weight of 4.5 kDa which showed broad spectrum activity against food pathogens including *S. aureus*, *L. monocytogenes* and *A. hydrophila*. *Lactobacillus plantarum* SA6 isolated from fermented sausage produced a plantaricin peptide with molecular mass of 3.4 kDa. Amortegui et al. (2014) isolated *Lactobacillus plantarum* from ensiled corn and purified the bacteriocin by ammonium sulphate precipitation (70%) and dialysis. They reported 5 and 10 kDa protein with antagonistic activity against *Listeria innocua*, *Listeria monocytogenes*, and *Enterococcus faecalis*. Milioni et al. [16] characterized 4.8 kDa plantaricin from *Lactobacillus plantarum* which was isolated from sheep-milk cheese. Plantaricin IIA-1A5 active against *Staphylococcus aureus* was purified which showed single band on SDS-PAGE with apparent molecular weight of 6.4 kDa (Arief et al. 2015).

In an attempt to determine the plantaricin gene in the selected probiotic strain *L. plantarum* Cu2-PM7, PCR was carried out using specific primers. As expected, an amplified product of 450 bp was found with plnA F/R primer in *L. plantarum* Cu2-PM7. However no amplification was observed with P1/P4 primer. *E. durans* DB-1aa genomic DNA was used as control where no amplification was observed. The results indicate that the present strain *L. plantarum* Cu2-PM7 harbors the plantaricin A gene. BLASTn analysis of the sequence showed highest similarity with *Lactobacillus plantarum* strain EG.LP 18.7 (MN172266.1). Similarly, Remiger et al. (1996) reported the binding position of primer plnA5p and S7, outside the structural gene of plantaricin A, which allowed the amplification of 450 bp DNA fragment in *L. plantarum* strains. Earlier studies on comparative genome analysis have reported significant variation in the plantaricin operon in the gene cluster of *Lactobacillus plantarum* strains (Maldonado et al. 2004; Rojo-Bezares et al. 2008). Ben Omar et al. (2008) isolated *Lactobacillus* strains from poto-poto, a Congolese fermented maize product and observed significant variation in the number of genes between the strains. The operon of *Pln* locus of plantaricin encoding genes is either simple or complex (Diep et al. 2009). Devi and Halami (2019) reported different plantaricin types based on the presence or absence of *pln* genes.

The translated protein sequence of plnA gene of *L. plantarum* Cu2-PM7 showed 100% homology with plantaricin A of *L. plantarum* (WP 0036419). As described by Diep et al. (1996), plantaricin system in *L. plantarum* is organized into five operon. (1) plnABCD: The regulatory operon encoding bacteriocin –like peptide (plnA), a histidine protein kinase (plnB) and two cytoplasmic response regulators (pln C and pln D). (2) pln GHSTUV: operon associated with transport (3) plnJKLR (4) plnMNOP (5) plnEF1 : related to plantaricin production and immunity. Plantaricin A is a single peptide bacteriocin without post translational modification (Diep et al. 2009). They are included in subclass IIc. *L. plantarum* CTC305 originally isolated from fermented sausage and *L. plantarum* CII isolated from cucumber fermentation were shown to share the plantaricin A encoding gene plnA (Diep et al. 1994).

In the current analysis, bioinformatic tools were used to characterize the peptide nature. Geneious Prime software predicted the protein domain **MKQLSNKEMQKIVGG** which codes for bacteriocin like protein. On further analysis of this motif, it was observed that this motif is responsible for the entry of the bacteriocin

peptide, with a GG cleavage motif at their N-terminal region. The sequence also confirmed the presence of four antigenic regions at sites 6-26, 28-46, 65-77 and 122-135.

It is well known that bacteriocin encoding genes are found along with immunity proteins and other accessory proteins which are arranged in an operon cluster (Noda et al. 2018). In continuation, the bacteriocin-like signal sequence was targeted to predict the peptide which shows activity. The bacteriocin sequence showed maximum homology towards plantaricin A of *Lactobacillus plantarum* WCFS1, with the ID P80214. Further, the peptide with bacteriocin plantaricin-A had a PBD ID of **IYTR**, this ID possessed a sequence of 26 amino acids with 2.99 molecular weight (Fig. 5) (Kristiansen et al. 2005). Hence, it can be perceived that the amino acid sequence stretch of our native isolate Cu2-PM7 possessed “**KSSAYSLQMGATAIKQVKKLFKKWGW**” peptide which is responsible for antimicrobial activity.

Conclusions

The data support the potential inhibitory activity of bacteriocin against *Staphylococcus aureus* and *E. coli*. On the basis of two important observations, i.e. proteinaceous nature of antagonistic substances and their heat stability, they can be considered as bacteriocins. The peptide analysis support the plantaricin A type bacteriocin present in the native probiotic culture *L. plantarum* Cu2-PM7. Overall the study highlights the characteristic features of bacteriocins produced from probiotic bacteria isolated from fermented foods which could be employed in food as preservative agent. The data obtained enhances our knowledge on the biocontrol of undesirable bacteria that could be used as an alternative therapy for treating infectious disease.

Declarations

Acknowledgements: This work was support by University Grants Commission for the award of NFSC research fellowship.

Declarations

Ethical Approval: This article does not contain any studies with human or animals

Consent for publication: This manuscript does not contain any individual person 's data.

Availability of data and material: Data sharing not applicable to this article as no datasets were generated or analyzed during the current study

Conflict of Interest: Dr. Lakshmidevi and Mrs. Amrutha Bindu declare that they have no conflict of interest

Funding: This work was support by University Grants Commission for the award of NFSC research fellowship.

Authors contribution: Lakshmidevi and Amrutha Bindu conceived and designed the experiment. Amrutha Bindu conducted experiment and wrote the manuscript. Both the authors read and approved the manuscript for submission.

Authors' information: Dr. Lakshmidevi N is professor from Department of Microbiology, University of Mysore. She is expertise in bacterial metabolites, immunology and peptide analysis. Mrs. Amrutha Bindu is a research scholar doing her PhD in the same department.

Informed consent: Informed consent was obtained from both the participants included in the study.

References

1. Anand SP, Sati N (2013) Artificial preservatives and their harmful effects: Looking towards nature for safer alternatives. *IJPSR* 4(7):2496-2501.
2. Drider D, Fimland G, Héchard Y, McMullen LM, Prevost H (2006) The continuing story of class IIa bacteriocins. *Microbiol Mol Biol Rev* 70:564–582.
3. Nes IF, Holo H (2000) Class II antimicrobial peptides from lactic acid bacteria. *Biopolymers (Peptide Science)* 55:50-61.
4. Amortegui J, Rodriguez-Lopez A, Rodriguez D, Carrascal AK, Almeciga-Diaz CJ, Melendez ADP, Sanchez OF (2014) Characterization of a new bacteriocin from *Lactobacillus plantarum* LE5 and LE27 isolated from Ensiled corn. *Appl Biochem Biotechnol* 172(7): 3374-3389.
5. Rodrigues E, Calzada J, Arques JK, Rodriguez JM, Nunez M, Medina M (2005) Antimicrobial activity of pediocin-producing *Lactococcus lactis* on *Listeria monocytogenes*, *Staphylococcus aureus* and *Escherichia coli* O157:H7 in cheese. *Int Dairy J* 5:51-57.
6. Bindu A, Lakshmidevi N (2020) Identification and *in-vitro* evaluation of probiotic attributes of lactic acid bacteria isolated from fermented food sources. *Arch Microbiol* DOI:10.1007/s00203-020-02037-0.
7. Zheng J, Wittouck S, Salvetti E, Franz CMAP, Harris HMB, Mattarelli P, O'Toole PW, Pot B, Vandamme P, Walter J, Watanabe K, Wuyts S, Felis GE, Gänzle MG, Lebeer S (2020) A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. *Int J Syst Evol Microbiol* 70:2782–2858.
8. Xie J, Zhang R, Shang C, Guo Y (2009) Isolation and characterization of a bacteriocin produced by an isolated *Bacillus subtilis* LFB112 that exhibits antimicrobial activity against domestic animal pathogens. *Afr J Biotechnol* 8(20):5611-5619.
9. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.
10. Barboza-Corona JE, Vazquez-Acosta H, Bideshi DK, Salcedo-Hernandez R (2007) Bacteriocin-like inhibitor substances produced by Mexican strains of *Bacillus thuringiensis*. *Arch Microbiol* 187:117-

11. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinform* 28:1647–1649.
12. Armstrong DR, Berrisford JM, Conroy MJ, Gutmanas A, Anyango S, Choudhary P, Clark AR, Dana JM, Deshpande M, Dunlop R, Gane P, Gáborová R, Gupta D, Haslam P, Koča J, Mak L, Mir S, Mukhopadhyay A, Nadzirin N, Nair S, Paysan-Lafosse T, Pravda L, Sehnal D, Salih O, Smart O, Tolchard J, Varadi M, Svobodova-Vařeková R, Zaki H, Kleywegt GJ, Velankar S (2020) PDBe: improved findability of macromolecular structure data in the PDB. *Nucleic Acids Res* 48(D1):D335-D343.
13. Croxen MA, Law RJ, Scholz R, Keeney KM, Wlodarska M, Finlay BB (2013) Recent Advances in understanding enteric pathogenic *Escherichia coli*. *Clin Microbiol Rev* 26(4):822-880.
14. Kadariya J, Smith TC, Thapaliya D (2014) *Staphylococcus aureus* and Staphylococcal food-borne disease: An ongoing challenge in public health. *Emerging microbial concerns in Food Safety and New Control Measures* Artilce ID 827965.
15. Bello OO, Babalola OO, Adegboye M, Fashola MO, Bello TK (2018) Partial purification, characterization and application of bacteriocin from bacteria isolated *Parkia biglobosa* *NE Sci* 3(2):72-94.
16. Milioni C, Martínez B, Degl'Innocenti S, Turchi B, Fratini F, Cerri D, Fischetti R (2015) A novel bacteriocin produced by *Lactobacillus plantarum* LpU4 as a valuable candidate for biopreservation in artisanal raw milk cheese. *Dairy Sci Technol* 95:479-494.
17. Barbosa MS, Todorov SD, Belguesmia Y, Choiset Y, Rabesona H, Ivanova IV, Chobert JM, Haertle T, Franco BDGM (2013) Purification and characterization of the bacteriocin produced by *Lactobacillus sakei* MBSa1 isolated from Brazilian salami. *J Appl Microbiol* 116:1195-1208.
18. Song DF, Zhu MY, Gu Q (2014) Purification and characterization of plantaricin ZJ5, a new bacteriocin produced by *Lactobacillus plantarum* *PLoS ONE* 9(8):e105549.
19. Todorov SD (2009) Bacteriocins from *Lactobacillus plantarum*-production, genetic organization and mode of action. *Braz J Microbiol* 40:209–221.
20. Nowroozi J, Mirzaii M, Norouzi M (2004) Study of *Lactobacillus* as probiotic bacteria. *Iranian Journal of Public Health* 33:1–7.
21. Sowani HM, Thorat P (2012) Antimicrobial activity studies of bacteriocin produced by *Lactobacilli* isolates from carrot kanji. *OnLine J Biol Sci* 12:6–10.
22. Martinez RC, Wachsman M, Torres NI, LeBlanc JG, Todorov SD, Franco BD (2013) Biochemical, antimicrobial and molecular characterization of a noncytotoxic bacteriocin produced by *Lactobacillus plantarum* *Food Microbiol* 34:376–381.
23. Mourad K, Halima ZK, Nour-Eddine K (2005) Detection and activity of plantaricin OL15 a bacteriocin produced by *Lactobacillus plantarum* OL15 isolated from Algerian fermented olives. *Grasas y*

Aceites 56:192–197.

24. Zapata S, Muñoz J, Ruiz O, Montoya O, Gutierrez P (2009) Isolation of *Lactobacillus plantarum* LPBM10 and partial characterization of its bacteriocin. *Vitae* 16:75–82.
25. Todorov SD, Ho P, Vaz-Velho M, Dicks LM (2010) Characterization of bacteriocins produced by two strains of *Lactobacillus plantarum* isolated from Beloura and Chouriço, traditional pork products from Portugal. *Meat Sci* 84:334–343.
26. Lee NK, Paik HD (2001) Partial characterization of lactocin NK24, a newly identified bacteriocin of *Lactococcus lactis* NK24 isolated from Jeot-gal. *Food Microbiol* 18:17–24.
27. Ferchichi M, Frère J, Mabrouk K, Manai M (2001) Lactococcin MMFII, a novel class IIa bacteriocin produced by *Lactococcus lactis* MMFII, isolated from a Tunisian dairy product. *FEMS Microbiol Lett* 205:49–55.
28. Hernández D, Cardell E, Zárate V (2005) Antimicrobial activity of lactic acid bacteria isolated from Tenerife cheese: initial characterization of plantaricin TF711, a bacteriocin-like substance produced by *Lactobacillus plantarum* J Appl Microbiol 99:77–84.
29. Todorov SD, Dicks LMT (2005) *Lactobacillus plantarum* isolated from molasses produces bacteriocins active against Gram-negative bacteria. *Enz Microbial Technol* 36:318–326.
30. Cintas L, Herranz C, Hernández P, Casaus M, Nes L (2001) Review: bacteriocins of lactic acid bacteria. *Food Sci Technol Int* 7:281–305.
31. Cotter PD, Ross RP, Hill C (2013) Bacteriocins-a viable alternative to antibiotics? *Nature Rev Microbiol* 11:95–105.
32. Todorov SD, van Reenen CA, Dicks LM (2004) Optimization of bacteriocin production by *Lactobacillus plantarum* ST13BR, a strain isolated from barley beer. *J Gen Appl Microbiol* 50:149–157.
33. Todorov SD, Dicks LMT (2006) Screening for bacteriocin-producing lactic acid bacteria from boza, a traditional cereal beverage from Bulgaria: comparison of the bacteriocins. *Process Biochem* 46:11–19.
34. Tomé E, Todorov SD, Gibbs PA, Teixeira PC (2009) Partial characterization of nine bacteriocins produced by lactic acid bacteria isolated from cold-smoked salmon with activity against *Listeria monocytogenes*. *Food Biotechnol* 23:50–73.
35. Messi P, Bondi M, Sabia C, Battini R, Manicardi G (2001) Detection and preliminary characterization of a bacteriocin (plantaricin 35d) produced by a *Lactobacillus plantarum* Int J Food Microbiol 64(1-2):193-198.
36. Arief II, Budiman C, Jenie BSL, Andreas E, Yuneni A (2015) Plantaricin IIA-1A5 from *Lactobacillus plantarum* IIA-1A5 displays bactericidal activity against *Staphylococcus aureus*. *Beneficial Microbes* 6(4):603-613.
37. Remiger A, Ehrmann MA, Vogel RF (1996) Identification of Bacteriocin-Encoding Genes in *Lactobacilli* by Polymerase Chain Reaction (PCR). *System Appl Microbiol* 19:28-34.

38. Maldonado A, Jimenez-Diaz R, Ruiz-Barba JL (2004) Induction of plantaricin production in *Lactobacillus plantarum* NC8 after co-culture with specific gram positive bacteria is mediated by an autoinduction mechanism. J Bacteriol 86:1556–1564.
39. Rojo-Bezares B, Sáenz Y, Navarro L, Jiménez-Díaz R, Zarazaga M, Ruiz-Larrea F, Torres C (2008) Characterization of a new organization of the plantaricin locus in the inducible bacteriocin-producing *Lactobacillus plantarum* J23 of grape must origin. Arch Microbiol 189:491–499.
40. Ben Omar N, Abriouel H, Keleke S, Sanchez Valenzuela A, Martinez-Canamero M, Lucas Lopez R, Ortega E, Galvez A (2008) Bacteriocin producing *Lactobacillus* strains isolated from poto poto, a Congolese fermented maize product, and genetic fingerprinting of their plantaricin operons. Int J Food Microbiol 30:18–25.
41. Diep DB, Straume D, Kjos M, Torres C, Nes IF (2009) An overview of the mosaic bacteriocin pln loci from *Lactobacillus plantarum*. Peptides 30:1562–1574.
42. Devi SM, Halami PM (2019) Genetic variation of *pln* loci among probiotic *Lactobacillus plantarum* group strains with antioxidant and cholesterol lowering ability. Probiotics Antimicrob Proteins 11:11–22.
43. Diep DB, Havarstein LS, Nes IF (1996) Characterization of the locus responsible for the bacteriocin production in *Lactobacillus plantarum* J Bacteriol 178:4472-4483.
44. Diep DB, Havarstein LS, Nissen-Meyer, Nes IF (1994) The gene encoding plantaricin A, a bacteriocin from *Lactobacillus plantarum* C11, is located on the same transcription unit as an agr-like regulatory system. Appl Environ Microbiol 60:160-166.
45. Noda M, Miyauchi R, Danshiitsoodol N, Matoba Y, Kumagai T, Sugiyama M (2018) Expression of genes involved in bacteriocin production and self-resistance in *Lactobacillus brevis* 174A is mediated by two regulatory proteins. Appl Environ Microbiol 84:e02707–02717.
46. Kristiansen PE, Fimland G, Mantzilas D, Nissen-Meyer (2005) Structure and mode of action of the membrane-permeabilizing antimicrobial peptide pheromone Plantaricin A. J Biol Chem 280(4):22945-22950.

Tables

Table 1 Antimicrobial activity of selected cultures during growth

Culture	Incubation period (hours)	Optical density at 600 nm	Inhibition zone (mm in dia)				MIC (µg)		Activity (AU/mg protein)	
			<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>
DB-1aa	24	3.45 ± 0.01	16 ± 0.00	16 ± 0.01	2400 ± 0.02	2400 ± 0.11	96.00 ± 0.06	96.00 ± 0.17		
	48	3.48 ± 0.21	14 ± 0.01	14 ± 0.01	2500 ± 0.04	2500 ± 0.05	75.90 ± 0.11	75.90 ± 0.28		
	72	3.79 ± 0.22	12 ± 0.00	12 ± 0.00	2800 ± 0.12	2800 ± 0.06	26.04 ± 0.02	26.04 ± 0.05		
	96	4.06 ± 0.12	-	-	4900 ± 0.05	4900 ± 0.11	-	-		
	120	3.76 ± 0.11	-	-	5200 ± 0.06	5600 ± 0.12	-	-		
Cu2-PM7	24	3.38 ± 0.21	16 ± 0.01	16 ± 0.00	2400 ± 0.05	2400 ± 0.11	266.02 ± 0.11	133.01 ± 0.04		
	48	3.48 ± 0.12	16 ± 0.01	16 ± 0.01	2400 ± 0.12	2400 ± 0.23	198.03 ± 0.14	99.01 ± 0.02		
	72	3.61 ± 0.03	16 ± 0.01	14 ± 0.01	2400 ± 0.22	2400 ± 0.14	80.58 ± 0.12	40.29 ± 0.18		
	96	4.18 ± 0.05	-	12 ± 0.01	4500 ± 0.11	4500 ± 0.12	-	37.30 ± 0.11		
	120	4.27 ± 0.11	-	12 ± 0.00	5000 ± 0.22	5200 ± 0.11	-	15.04 ± 0.02		
Cu3-PM8	24	3.48 ± 0.12	15 ± 0.00	16 ± 0.00	2400 ± 0.15	2400 ± 0.15	178.8 ± 0.14	89.39 ± 0.01		
	48	3.61 ± 0.22	15 ± 0.00	16 ± 0.00	2400 ± 0.12	2400 ± 0.21	141.9 ± 0.12	71.00 ± 0.01		
	72	3.70 ± 0.14	15 ± 0.01	14 ± 0.01	2500 ± 0.14	2500 ± 0.05	67.32 ± 0.17	67.32 ± 0.05		

96	4.10 ± 0.06	-	12 ± 0.01	3800 ± 0.22	3800 ± 0.06	-	52.57 ± 0.06
120	4.25 ± 0.05	-	12 ± 0.01	3800 ± 0.05	3800 ± 0.01	-	25.00 ± 0.11

Values are average of two experiments performed individually (Mean ± SD). ‘-’ indicate no activity.

Table 2 MIC and activity of AMC extracted with various methods

Culture	Extraction method	Inhibition zone (mm in dia)				MIC (μ g)				Activity (AU/ mg protein)			
		<i>S. aureus</i>		<i>E. coli</i>		<i>S. aureus</i>		<i>E. coli</i>		<i>S. aureus</i>		<i>E. coli</i>	
<i>E. durans</i> DB-1aa	1	-		10	$\pm$	6200	$\pm$	2800	$\pm$	-		60.39	$\pm$
				0.01		0.12		0.11				0.11	
	2	12	$\pm$	15	$\pm$	2400	$\pm$	2300	$\pm$	34.50	$\pm$	69.00	$\pm$
		0.01		0.02		$\pm$ 0.11		0.23		0.00		0.02	
	3	-		12	$\pm$	6800	$\pm$	2200	$\pm$	-		15.79	$\pm$
				0.01		0.21		0.12				0.00	
	4	15	$\pm$	14	$\pm$	2200	$\pm$	2300	$\pm$	155.27		155.27	$\pm$
		0.01		0.00		0.11		0.11		$\pm$ 0.02		0.15	
<i>L. plantarum</i> Cu2-PM7	1	-		10	$\pm$	6000	$\pm$	3200	$\pm$	-		80.47	$\pm$
				0.01		0.22		0.22				0.11	
	2	14	$\pm$	15	$\pm$	2300	$\pm$	2300	$\pm$	92.31	$\pm$	92.31	$\pm$
		0.01		0.01		0.03		0.11		0.01		0.09	
	3	12	$\pm$	12	$\pm$	2800	$\pm$	2800	$\pm$	20.29	$\pm$	20.29	$\pm$
		0.02		0.00		0.05		0.05		0.03		0.00	
	4	18	$\pm$	14	$\pm$	1900	$\pm$	2200	$\pm$	319.08		212.72	$\pm$
		0.01		0.01		0.06		0.21		$\pm$ 0.11		0.15	
<i>L. fermentum</i> Cu3-PM8	1	-		10	$\pm$	6000	$\pm$	3000	$\pm$	-		31.76	$\pm$
				0.01		0.09		0.03				0.11	
	2	14	$\pm$	14	$\pm$	3200	$\pm$	3200	$\pm$	83.01	$\pm$	83.01	$\pm$
		0.01		0.00		0.11		0.05		0.02		0.11	
	3	12	$\pm$	10	$\pm$	3800	$\pm$	3800	$\pm$	22.33	$\pm$	22.33	$\pm$
		0.02		0.01		0.12		0.11		0.00		0.02	
	4	18	$\pm$	18	$\pm$	2000	$\pm$	2000	$\pm$	179.80		179.80	$\pm$
		0.02		0.02		0.11		0.09		$\pm$ 0.11		0.21	

Values are average of two experiments performed individually (Mean $\pm$ SD). ‘-’ indicate no activity. Extraction methods (1) Ethanol precipitation; (2) Chloroform extract (3) Butanol extraction (4) Ammonium Sulphate precipitation-dialysis

Table 3 Summary of bacteriocin purification from selected cultures

Isolate	Purification step	Volume (ml)	Activity (AU/ml)	Total activity (AU)	Yield (%)	Protein (mg/ml)	Total protein (mg)	Specific activity (AU/mg)	Purification factor
DB-1aa	CFS	5000	160	800000	100.00	1.8	9000	88.90	1
	AS	50	160	8000	1.00	1.03	51.50	155.34	1.75
	IE	5	640	3200	0.4	2.62	13.10	244.27	2.75
Cu2-PM7	CFS	5000	320	1600000	100.00	1.4	7000	228.57	1
	AS	50	240	12000	0.75	0.75	37.50	320.00	1.40
	IE	5	1600	8000	0.5	3.74	18.70	427.81	1.87
Cu3-PM8	CFS	5000	320	1600000	100.00	2.3	11500	139.13	1
	AS	50	160	8000	0.5	0.89	44.50	179.77	1.29
	IE	5	1280	6400	0.4	4.8	24.00	266.67	1.92

Isolate	Purification step	Volume (ml)	Activity (AU/ml)	Total activity (AU)	Yield (%)	Protein (mg/ml)	Total protein (mg)	Specific activity (AU/mg)	Purification factor
DB-1aa	CFS	5000	160	800000	100.00	1.8	9000	88.9	1
	AS	50	160	8000	1.00	1.03	51.50	155.34	1.75
	IE	5	560	2800	0.35	2.62	13.10	213.74	2.4
Cu2-PM7	CFS	5000	160	800000	100.00	1.4	7000	114.29	1
	AS	50	160	8000	1.00	0.75	37.50	213.33	1.87
	IE	5	1216	6080	0.76	3.74	18.70	325.13	2.84
Cu3-PM8	CFS	5000	160	800000	100.00	2.3	11500	69.56	1
	AS	50	160	8000	1.00	0.89	44.50	179.77	2.58
	IE	5	1088	5440	0.68	4.8	24.00	226.67	3.26

Table 4 Stability of bacteriocin at different temperature

Temperature (°C)	Inhibitory activity against <i>Staphylococcus aureus</i>											
	Inhibition zone (mm dia)						Activity (AU/mg protein)					
	DB-1aa		Cu2-PM7		Cu3-PM8		DB-1aa		Cu2-PM7		Cu3-PM8	
30	15	±	15	±	15	±	240.12	±	426.20	±	263.12	±
	0.01		0.00		0.01		0.09		0.11		0.01	
40	15	±	15	±	15	±	244.12	±	429.30	±	265.73	±
	0.00		0.00		0.01		0.12		0.12		0.11	
50	15	±	15	±	15	±	242.03	±	425.20	±	261.42	±
	0.02		0.01		0.01		0.11		0.11		0.15	
60	15	±	15	±	15	±	252.02	±	419.70	±	260.12	±
	0.01		0.02		0.00		0.14		0.15		0.12	
70	15	±	15	±	15	±	241.15	±	415.60	±	261.12	±
	0.01		0.01		0.02		0.05		0.21		0.21	
80	15	±	15	±	15	±	252.05	±	411.07	±	260.09	±
	0.01		0.00		0.02		0.12		0.15		0.14	
90	15	±	15	±	15	±	226.70	±	409.10	±	255.02	±
	0.01		0.01		0.00		0.11		0.06		0.11	
Inhibitory activity against <i>E. coli</i>												
	DB-1aa		Cu2-PM7		Cu3-PM8		DB-1aa		Cu2-PM7		Cu3-PM8	
30	12	±	15	±	12	±	239.72	±	322.15	±	220.09	±
	0.00		0.00		0.01		0.12		0.11		0.11	
40	12	±	15	±	12	±	242.43	±	325.60	±	227.12	±
	0.01		0.00		0.01		0.15		0.21		0.21	
50	12	±	15	±	15	±	241.03	±	322.30	±	225.03	±
	0.02		0.01		0.01		0.16		0.11		0.11	
60	10	±	15	±	12	±	223.12	±	320.46	±	222.12	±
	0.02		0.02		0.01		0.11		0.22		0.11	
70	10	±	15	±	10	±	212.06	±	319.60	±	209.73	±
	0.11		0.02		0.00		0.16		0.06		0.11	
80	10	±	15	±	10	±	207.05	±	319.00	±	152.42	±
	0.00		0.01		0.00		0.21		0.09		0.06	
90	10	±	15	±	10	±	200.18	±	312.90	±	169.12	±
	0.00		0.01		0.01		0.11		0.11		0.09	

Values are average of two experiments performed individually (Mean ± SD).

Table 5 Stability of bacteriocin at different pH

pH	Inhibitory activity against <i>Staphylococcus aureus</i>					
	Inhibition zone (mm dia)			Activity (AU/mg protein)		
	DB-1aa	Cu2-PM7	Cu3-PM8	DB-1aa	Cu2-PM7	Cu3-PM8
3	15 ± 0.00	15 ± 0.01	18 ± 0.01	242.12 ± 0.11	422.12 ± 0.11	269.72 ± 0.02
4	15 ± 0.01	15 ± 0.00	15 ± 0.00	245.72 ± 0.05	427.32 ± 0.21	266.32 ± 0.11
5	15 ± 0.01	15 ± 0.00	15 ± 0.01	248.62 ± 0.11	426.32 ± 0.14	265.42 ± 0.22
6	14 ± 0.01	14 ± 0.01	14 ± 0.01	240.12 ± 0.14	412.43 ± 0.06	252.42 ± 0.09
7	-	10 ± 0.01	-	110.12 ± 0.21	400.23 ± 0.06	112.6 ± 0.11
8	-	-	-	12.13 ± 0.11	32.0 ± 0.05	-
9	-	-	-	-	-	-
10	-	-	-	-	-	-
	Inhibitory activity against <i>E. coli</i>					
	DB-1aa	Cu2-PM7	Cu3-PM8	DB-1aa	Cu2-PM7	Cu3-PM8
	DB-1aa	Cu2-PM7	Cu3-PM8	DB-1aa	Cu2-PM7	Cu3-PM8
3	14 ± 0.00	20 ± 0.02	12 ± 0.00	240.12 ± 0.12	326.70 ± 0.12	220.60 ± 0.14
4	14 ± 0.01	14 ± 0.01	12 ± 0.01	244.72 ± 0.06	322.42 ± 0.05	222.60 ± 0.15
5	14 ± 0.01	14 ± 0.00	12 ± 0.01	242.62 ± 0.14	319.06 ± 0.11	226.00 ± 0.12
6	14 ± 0.01	14 ± 0.00	12 ± 0.00	240.32 ± 0.06	315.20 ± 0.22	215.90 ± 0.15
7	14 ± 0.00	-	-	239.63 ± 0.15	109.20 ± 0.21	109.30 ± 0.06
8	-	-	-	62.02 ± 0.12	32.60 ± 0.11	22.60 ± 0.02
9	-	-	-	-	-	-
10	-	-	-	-	-	-

Values are average of two experiments performed individually (Mean ± SD). ‘-’ indicate no activity

Figures

Figure 1

Elution profile of protein by ion-exchange chromatography. (A) *E. durans* DB-1aa; (B) *L. plantarum* Cu2-PM7; (C) *L. fermentum* Cu3-PM8. Inhibitory activity of protein fractions (1, 2 and 3) eluted by ion-exchange chromatography against *S. aureus* and *E. coli*. A = Antibiotic chloramphenicol (1 mg/ ml) was used as positive control

Figure 2

SDS-PAGE analysis of AMC (A) Cu₂-PM7; (B) Cu₃-PM8. (1) Crude; (2) Ammonium sulphate and dialyzed sample; (3) Column fraction; (M) Protein marker. Circle indicate inhibition zone in overlay plates

Figure 3

Plantaricin gene characterization (A) phylogenetic similarity between plnA-PM7 and related sequence from NCBI database (B) Sequences aligned (dark shade indicate maximum homology).

Figure 4

Plantaricin peptide analysis (A) Amino acid sequence similarity between plnA-PM7 and related sequence from NCBI data base (B) Amino acid sequence aligned (dark shade represents maximum homology)

Figure 5

Structure and functional analysis of Cu₂-PM7 bacteriocin like peptide (A) Sequence analysis (B) 3D structure (C) Properties of active fraction

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [supplementary1.docx](#)