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ORIGINAL ARTICLE

Antibacterial property of bacteriocin produced by *Lactobacillus plantarum* LD4 isolated from a fermented food

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Abstract Bacteriocins are ribosomally synthesized antimicrobial peptides secreted by certain lactic acid bacteria, with potential application in food safety. Bacteriocin-producing *Lactobacillus plantarum* LD4 was identified using biochemical and molecular techniques including 16S rDNA amplification, followed by sequencing. Bacteriocin production started after 3 h, reaching a maximum during the early stationary phase. Bacteriocin present in cell-free supernatant showed stability in a pH range of 2.0 to 6.0, as well as at different temperatures (60–100 and 121 °C for 15 min under 15 psi pressure). Antimicrobial activity was not affected by catalase, lipase or α-amylase, but was reduced in the presence of trypsin and protease, suggesting the proteinaceous nature of the compound. Activity remained stable after treatment with different organic solvents, surfactants, and detergents. The molecular weight of bacteriocin LD4 was found to be ~6 kDa using tricine SDS-PAGE. Antimicrobial activity was demonstrated against Gram-positive and Gram-negative bacteria including *Micrococcus luteus*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Vibrio* sp., *Enterobacter cloacae*, *Enterococcus faecium*, and a few strains of lactic acid bacteria and haloarchaea. Bacteriocin LD4 caused K^+ ion efflux in target cells, suggesting the pore-forming nature of the compound. Given the stability under various conditions and the broad antimicrobial spectrum against pathogens, the bacteriocin LD4 has the potential for application in food safety as well as therapeutics.

Keywords *Lactobacillus plantarum* LD4 · Bacteriocin · Antimicrobial activity · Fermented food · Pathogens

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Introduction

Bacteriocins are ribosomally synthesized antibacterial peptides produced by bacteria that inhibit the growth of similar or closely related bacterial strains (Nishie et al. 2012). The bacteriocins of lactic acid bacteria (LAB) have certain advantages that make them good candidates for use in biopreservation. Most bacteriocins produced by LAB are generally recognized as safe (GRAS) by the US Food and Drug Administration. They have no antagonistic effects on eukaryotic cells, with the exception of cytolysin produced by *Enterococcus faecalis* (Heng et al. 2007). Because of their proteinaceous nature, they are completely inactivated by gastrointestinal enzymes and have only a slight effect on intestinal microbiota. They are resistant to acidic pH and heat, so they can be utilized in the food industry (Zaeim et al. 2014). Bacteriocins of LAB play a very important role in the food fermentation industry as natural preservatives, since they are capable of inhibiting the growth of many food-spoilage and pathogenic bacteria, such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, and *Clostridium botulinum*. Some studies have also demonstrated the use of bacteriocins to control biofilm formation in steel tanks, suggesting that they are natural and environmentally safe sanitizers (Ndlovu et al. 2015). These peptides have attracted significant attention because of their potential application as non-toxic additives in food safety and prevention of food spoilage by food-borne pathogenic bacteria (Henderson et al.

1992; Cleveland et al. 2001; Morisset et al. 2004). Klaenhammer classified LAB bacteriocins into three main groups on the basis of their structural characteristics: class I are small (<5 kDa) peptides called lantibiotics (post-translationally modified); class II are small heat-stable, non-modified membrane-active peptides; and class III are large (>10 kDa) heat-labile peptides/proteins (Song et al. 2014).

Most bacteriocins are small, heat-stable, hydrophobic cationic peptides (Aunpad and Na-Bangchang 2007; Lemos et al. 2008). Numerous bacteriocins from *Lactobacillus plantarum* have been identified, and are generally referred to as plantaricins (Chen et al. 2014). Both class I and class IIa plantaricins have been identified from various *L. plantarum* strains, e.g. plantaricins LR14 and W (Holo et al. 2001; Tiwari and Srivastava 2008a). A number of plantaricins have been purified; these include plantaricins JK, 1.25, NC8, and PASM1 (Song et al. 2014). Some show a narrow antibacterial spectrum, exhibiting antibacterial activity against bacteria closely related to the producer microorganisms. For example, plantaricins C, JK, and NC8, like nisin, appear to inhibit a few Gram-positive bacteria, including *Lactobacillus sakei*, *Enterococcus faecalis*, and *Bacillus subtilis*, but they do not inhibit Gram-negative members. Therefore, studies exploring broad-spectrum bacteriocins are needed (Song et al. 2014). Because different types of bacteria produce different bacteriocins, the investigation of LAB from new sources is important for identifying bacteria capable of producing novel bacteriocins for food safety and clinical applications. In the present study, *Lactobacillus plantarum* strain LD4, previously isolated in our laboratory from a fermented food, Dosa, was identified and its bacteriocin characterized for stability, mode of action, host range, and molecular weight.

Materials and methods

Bacterial strains and culture media

L. plantarum LD4 previously isolated from Dosa batter was available in our laboratory culture stock. For production of the bacteriocin, strain LD4 was grown in de Man–Rogosa–Sharpe (MRS) medium (1 % peptone, 1 % beef extract, 0.5 % yeast extract, 2 % dextrose, 0.1 % Tween 80, 0.2 % tri-ammonium citrate, 0.5 % sodium acetate, 0.05 % magnesium sulphate, 0.02 % manganese sulphate, and 0.2 % dipotassium phosphate) at 37 °C and pH 6.8 under static conditions, and maintained on TGYE medium (0.5 % tryptone, 0.1 % glucose, and 0.3 % yeast extract) at 37 °C and pH 6.8 (Tiwari and Srivastava 2008b). *Micrococcus luteus* MTCC106 was used as an indicator strain for determination of antimicrobial activity. A few Gram-positive and Gram-negative bacteria and haloarchaea strains were also tested against the LD4 bacteriocin. These included *Staphylococcus aureus*, *Vibrio* sp., *Salmonella typhi*, *Enterobacter cloacae* NRRL B14298, and *Micrococcus luteus* MTCC106, which were grown in nutrient broth (0.5 % peptone, 0.3 % beef extract, and 0.5 % sodium chloride) at 37 °C and pH 7.0. *Pseudomonas aeruginosa*, *P. fluorescens*, and *Escherichia coli* NCDC135 were grown in Luria-Bertani broth (0.5 % yeast extract, 1 % tryptone, and 1 % sodium chloride) at 37 °C and pH 7.0. Targeted LAB strains *Lactobacillus curvatus* NRRl B4562, *L. delbrueckii* NRRl B4525, *L. acidophilus* NRRl B4495, *L. plantarum* NRRl B4496, and *Lactococcus lactis* subsp. *lactis* NRRL B1821 were grown in MRS medium at 37 °C and pH 6.8. Haloarchaea strains were grown in *Halobacterium salinarum* (HS) medium (0.5 % casamino acids, 0.5 % yeast extract, 2 % $MgSO_4.7H_2O$, 0.3 % tri-Na-citrate, 0.002 % $FeSO_4.7H_20$, 15 % NaCl, 0.2 % KCl, and 0.0026 % $MnCl_2.4H_20$) at 42 °C and pH 7.2 (Hassanshahian and Mohamadian 2011). All media components were purchased from HiMedia Laboratories (Mumbai, India), Sisco Research Laboratories (Mumbai, India), and Sigma-Aldrich (St. Louis, MO, USA).

Identification of strain LD4

Gram staining was performed using a standard protocol. Strain LD4 was tested for catalase production by adding few drops of 3.0 % H_2O_2 onto a clean glass slide containing bacterial culture. The amount of effervescence released served as indication of the presence of catalase. *E. coli* and *L. plantarum* NRRL B4496 were used as positive and negative control, respectively. Strain LD4 was investigated for drop in pH in culture medium during growth and also for antibiotic resistance. Different antibiotic solutions including streptomycin (50 μg/mL), erythromycin (10 μg/mL), tetracycline (10 μg/mL), gentamicin (10 μg/mL), kanamycin (50 μg/mL), and chloramphenicol (25 μg/mL) were filter-sterilized using a 0.2-μm membrane (mdi, Ambala, India) and added to TGYE agar medium. Strain LD4 was streaked and incubated overnight at 37 °C. Antibacterial activity was determined by the appearance of bacterial growth on agar plates. To monitor carbohydrate fermentation, cell pellets of the overnight culture were re-suspended in 0.8 % NaCl saline solution. Aliquots of 50 μl of cell suspension were added to each well of a carbohydrate fermentation kit (HiCarbo kit; HiMedia, Mumbai, India), incubated overnight at 37 °C, and monitored for colour change. The results were interpreted following the manufacturer's instructions. The carbohydrate fermentation pattern was compared with that of standard strains described in Bergey's of Systematic Bacteriology. Halotolerance was monitored by the growth of strain LD4 in TGYE broth containing different concentrations of NaCl (0.5, 6.5, 10, 15 and 20 %), with an initial optical density (OD_{600}) of 0.02, and incubated at 37 °C for 24 h. After incubation, growth was monitored and compared to a control without NaCl.

Molecular identification was performed using 16S rDNA amplification and sequencing. The genomic DNA of strain LD4 was isolated from overnight culture using a bacterial genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA). Amplification of 16S rDNA was carried out using the GeneAmp PCR System (Applied Biosystems, Foster City, CA, USA). Universal primers of LAB (forward primer 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse primer 5′-CCGTCAATTCCTTTGAGTTT-3′) were used for amplification of the 16S rDNA sequence. PCR was carried out in a final volume of 25 μl containing 0.1 U *Taq* polymerase, 2 pmol of each primer, 200 ng of template DNA, 200 μM of deoxynucleotide (dNTP) solution, and sterile distilled water. Gene amplification was performed for 30 cycles consisting of an initial denaturation step for 5 min, cycle denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, and extension for 1 min at 72 °C, with a final extension step for 5 min at 72 °C. The PCR product thus obtained was sequenced by a custom facility available at the University of Delhi South Campus, New Delhi. The 16S rDNA sequence was compared using nucleotide BLAST (BLASTN, NCBI). Ten sequences closest to the sequence obtained were further aligned by ClustalW. A phylogenetic tree was constructed using the neighbor-joining (NJ) method in MEGA 6.06 software.

Preparation and assay of crude bacteriocin

To determine bacteriocin-producing ability, strain LD4 was grown in MRS medium with an initial OD_{600} 0.02. After overnight growth, cell-free supernatant (CFS) was collected by centrifugation (10,000 *g*, 15 min and 4 °C), filter-sterilized using a 0.2-μm membrane (Advanced Microdevices [mdi], Ambala, India) and used as crude bacteriocin for further characterization. The antimicrobial activity of the crude bacteriocin was determined by agar well diffusion assay (AWDA), as suggested by Tiwari and Srivastava (2008a). AWDA was performed by overlaying soft nutrient agar (0.8 %) seeded with indicator strain *M. luteus* (~10^6 CFU/ml) on the nutrient base agar plate. The wells cut (6.0-mm diameter) from these plates were filled with 100 μl of crude bacteriocin (pH 4.6). MRS medium (pH 4.6) was used as control. After overnight incubation, the diameter of the zone of growth inhibition was measured. The antimicrobial activity was also determined in terms of activity unit per milliliter (AU/ml). Briefly, it was the reciprocal of the highest dilution at which no visible growth occurred, as described by Pasic et al. (2008).

Growth kinetics and production of bacteriocin

The mid-log phase culture of strain LD4 was sub-cultured to an initial OD_{600} 0.02 in MRS broth and incubated at 37 °C under static conditions overnight. Samples were withdrawn at regular 1-h intervals, and growth (in terms of OD_{600} and CFU/ml), change in pH, and production of bacteriocin in terms of growth inhibition zone were monitored.

Effect of different temperatures and pH on bacteriocin activity

To assess thermal stability, the crude bacteriocin was heated at 80 and 100 °C for 15 min in a water bath and at 121 °C for 15 min under 15 psi pressure prior to activity assay. As the pH of food may vary considerably, determining the affect of different pH values on bacteriocin activity is important. For pH stability, the CFS was resuspended in a 1:1 ratio of different buffer solutions ranging from pH 2 to 10 (HCl-KCl, 10 mM, pH 2 and 4; phosphate buffer, 10 mM, pH 6 and 7; Tris-Cl, 10 mM, pH 8 and 10) and incubated for 2 h at 37 °C. The residual activity of the treated set was monitored by AWDA and compared with the control (untreated CFS, pH 4.6). For negative control, buffers of each pH were used in place of the bacteriocin.

Effect of organic solvents, surfactants, and detergents on bacteriocin activity

The effect of different chemicals on the stability of the crude bacteriocin was investigated using organic solvents including ethanol, methanol, isopropanol, acetone, ethyl acetate, and acetonitrile; surfactants including Tween 80 and Triton X-100; and detergents urea and sodium dodecyl sulphate (SDS). These chemicals were mixed at a final concentration of 1 % (v/v or w/v as appropriate) with the crude bacteriocin. Untreated bacteriocin samples and different chemicals were used as positive and negative controls, respectively. All sets were incubated at 37 °C for 2 h and tested for antimicrobial activity using AWDA.

Effect of hydrolytic enzymes on bacteriocin activity

To understand the chemical nature of the CFS, it was treated with the different hydrolytic enzymes. Catalase, α-amylase, lipase, trypsin, α-chymotrypsin, protease, and pepsin were prepared in sodium acetate buffer (10 mM, pH 4.6) and proteinase K in phosphate buffer (10 mM, pH 5.0). Enzyme solutions were mixed with CFS at a concentration of 1 mg/ml. Following incubation at 37 °C for 2 h, enzyme activity was terminated by heating the sample at 100 °C for 5 min. Untreated CFS and the various buffers were used as positive and negative controls, respectively. The residual activity was measured by AWDA. All enzymes were procured from Sigma-Aldrich (St. Louis, MO, USA) and used according to the manufacturer's instructions.

Determination of molecular mass

Bacteriocin LD4 was partially purified using CFS obtained from overnight culture of *L. plantarum* LD4. A 100-ml aliquot of CFS was precipitated by the addition of ammonium sulphate (0–90 %) and centrifuged at 8000 *g* for 15 min at 4 °C. The pellet was resuspended in the minimum amount of sodium acetate buffer (10 mM, pH 4.6). The sample was dialyzed using dialysis membrane (2 kDa cut-off; Sigma-Aldrich, St. Louis, MO, USA) against the same buffer. It was passed through a cation-exchange chromatography column packed with SP Sepharose matrix (Sigma-Aldrich). The fractions were eluted with step gradients of NaCl (0.1–0.3, 0.3–0.5, and 0.5–1.0 M). The eluted fractions demonstrating activity were loaded on a tricine sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) system, along with molecular weight marker (Merck Genei, Bangalore, India), as suggested by Schägger and von Jagow (1987). The separating gel consisted of 16.5 % T (total concentration of both acrylamide and bisacrylamide) and 3 % C (relative percentage of bisacrylamide to both monomers); the spacer consisted of 10 % T and 3 % C; and the stack 4 % T and 3 % C. The samples were mixed with 3X loading buffer and incubated at 40 °C for 30 min before loading on the gel. The bacteriocin sample was run in duplicate at 30 V for approximately 1 h until it entered the separating gel, and was further run for another 8–9 h at 120 V. After electrophoresis, the gel was cut into two parts. one part containing molecular weight marker proteins and bacteriocin was stained with coomassie brilliant blue R-250. The other half of the gel containing bacteriocin only was extensively washed with regular replacement of sterile double-distilled water and was transferred onto nutrient agar plates. The gel was overlaid with soft nutrient agar (0.8 %) seeded with indicator strain *M. luteus* (~10^6 CFU/ml), followed by overnight incubation at 37 °C. After incubation, the bioassay plate was observed for the zone of growth inhibition around the protein band and compared with the molecular weight marker.

Mode of action and host range

M. luteus and *E. coli* were grown in NB and LB medium, respectively, at 37 °C and 200 rpm, and the cells were harvested in the log phase. About 10^6 CFU/ml were resuspended in 10 ml fresh medium containing partially purified bacteriocin (64 AU/ml). The positive control was grown without treatment. Samples were withdrawn at regular 2-h intervals to determine growth and viable count in terms of OD_{600} and CFU/ml, respectively, by plating appropriate dilutions.

For measurement of potassium ion release from the target cells, the target strains *M. luteus* and *E. coli* were grown up to mid-log phase. The cells were recovered and washed three times with Tris-acetate buffer (10 mM, pH 7.4) containing 100 mM NaCl. The washed cells were resuspended in the same buffer and kept on ice until use. The bacteriocin (5 AU/ml) was added in each set of target strains *M. luteus* and *E. coli*. Nisin (5 AU/ml) was used as positive control. The diluents of bacteriocin LD4 and nisin were used as negative control. The release of K^+ ions was monitored at 1-min intervals using a digital flame photometer (Environmental & Scientific Instruments Co. [ESICO], Haryana, India) calibrated with KCl solutions (20 and 40 ppm).

To determine the host range of the bacteriocin, CFS was tested against different strains of LAB, other Gram-positive and Gram-negative bacteria, and a few strains of haloarchaea. Antimicrobial activity was determined using AWDA.

Statistical analysis

Experiments were performed in triplicate, and mean values were plotted along with standard deviation (mean ± SD). The level of statistical significance was estimated as *p* value ($p = < 0.05$) using Student's *t* test. Where necessary, three independent experiments were performed to monitor the reproducibility of the results.

Results

Identification of strain LD4

Strain LD4 was identified using morphological, biochemical, and molecular methods. The colonies of strain LD4 appeared as milky white, dome-shaped, and small, with entire margins. The strain was found to be a Gram-positive, catalase-negative, and rod-shaped bacillus. During growth of strain LD4, the pH of the medium dropped from pH 7.0 to 4.6, indicating acid production, which is a characteristic feature of LAB. The growth of strain LD4 was found to be reduced at 0.5 % NaCl, whereas complete inhibition occurred at 6.5 % and above. It was resistant to streptomycin, gentamicin, and kanamycin but sensitive to erythromycin, tetracycline, and chloramphenicol, and was able to ferment various carbohydrates including mannose, fructose, dextrose, trehalose, sucrose, maltose, cellobiose, esculin, and inulin (Table 1). The carbohydrate fermentation pattern thus obtained was compared with reference strains present in Bergey's manual. Based on these biochemical tests, strain LD4 was found to belong to the genus *Lactobacillus*.

Species-level identification was carried out using 16S rDNA amplification and sequencing. The amplified PCR product of 16S rDNA was found to be approximately 900 bp (Fig. 1a). The sequencing of 610 bp of the amplified product was performed at a custom facility available at the University of Delhi South Campus, New Delhi. Sequence homology of

Table 1 Morphological, biochemical, and physiological characteristics of *Lactobacillus plantarum* LD4

Serial Number	Tests	Characteristics
1.	Colony colour	Milky-white
2.	Catalase test	Negative
3.	Gram staining	Positive
4.	Cell shape	Rod-shaped
5.	Colony morphology	Small, dome-shaped
6.	Acid tolerance	Positive
7.	Acidifying activity	Positive
8.	Response to antibiotics:	
	a) Streptomycin, gentamicin, kanamycin	Resistance
	b) Erythromycin, tetracycline, chloramphenicol	Sensitive
9.	Ferment carbohydrates mannose, fructose, dextrose, trehalose, sucrose, maltose, cellobiose, esculin and inulin	Positive
10.	NaCl concentrations (%):	Growth (OD_{600})
	a) Control (without NaCl)	1.68 ± 0.032
	b) 0.5	0.68 ± 0.029
	c) 6.5	Nil
	d) 10	Nil
	e) 15	Nil
	f) 20	Nil

16S rDNA was performed using BLASTN (NCBI), which demonstrated 99 % homology with *Lactobacillus plantarum* WCFS1. The sequence thus obtained was submitted to GenBank (NCBI) with accession number KT004675. A phylogenetic tree constructed using the neighbor-joining (NJ) method in MEGA 6.06 software is shown in Fig. 1b.

Growth and bacteriocin production

Strain LD4 showed a typical sigmoidal growth pattern consisting of a short lag phase of 2 h, reaching a late log phase at 8 h, and stationary phase thereafter. A significant drop in the pH of the medium during growth was also observed. This

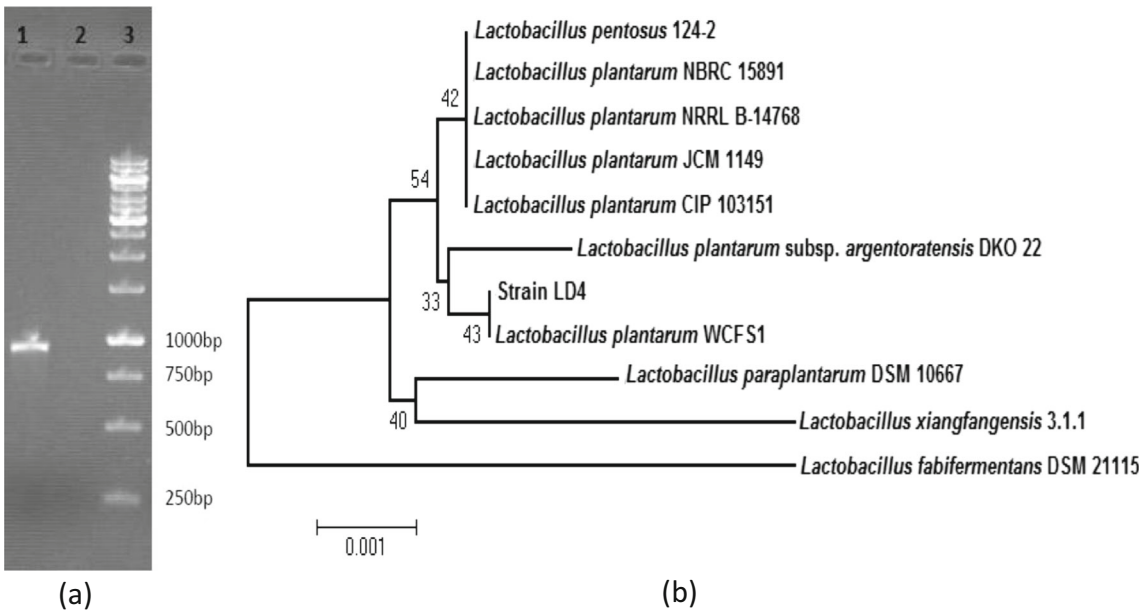

Fig. 1 Gel electrophoresis of PCR-amplified product from genomic DNA of strain LD4. Lane 1 is 16S rDNA amplicon of strain LD4, lane 2 is empty, and lane 3 shows 1 kb DNA ladder (Promega, Madison, WI, USA) (**a**) Phylogenetic tree constructed by neighbor-joining method using MEGA 6.06 software. Numerals indicate bootstrap values (**b**)

event started in the beginning of the log phase, with maximum bacteriocinogenic activity detected at pH 4.6. Bacteriocin production followed a growth-related pattern, starting during the log phase (3 h), and reaching a maximum at the beginning of the stationary phase (9 h). Thereafter, no change in antimicrobial activity was observed (Fig. 2).

Characterization of the bacteriocin

The antimicrobial activity of the CFS of strain LD4 was found to be completely stable at temperatures of 60, 80, 100, and 121 °C. While approximately 100 % activity was recorded between pH 2 and 4, 30 and 60 % loss in activity was observed at pH 6 and 7, respectively. No activity was found beyond pH 7, showing its stability in acidic pH. Buffers of the respective pH did not show zone of growth inhibition. Thus, the antimicrobial compound was found to be highly stable at higher temperatures and acidic pH. Nearly 100 % activity was retained after treatment with different organic solvents, surfactants, and detergents. When the crude bacteriocin was treated with proteolytic enzymes including proteases and trypsin, activity was reduced to a 16-mm zone of growth inhibition compared to control (20 mm). Sodium acetate buffer (10 mM, pH 4.6), used as negative control, showed no inhibition. However, α-amylase and lipase treatment did not influence activity, which suggests that the inhibitory compound is proteinaceous in nature and requires no carbohydrate or lipid moieties for its activity. The biochemical characterization of bacteriocin LD4 is shown in Table 2.

Determination of molecular mass

Tricine SDS-PAGE of bacteriocin LD4 showed a single diffused protein band, with a molecular mass of approximately 6 kDa. The protein band showed a corresponding zone of growth inhibition on the bioassay plate, confirming the presence of antimicrobial activity in the protein band, as shown in Fig. 3.

Mode of action and antimicrobial spectrum

The growth of *M. luteus* and *E. coli* was inhibited in the presence of bacteriocin LD4. Growth inhibition was found to be higher in *M. luteus* than in *E. coli*. The *M. luteus* cells treated with bacteriocin LD4 did not maintain growth throughout the incubation period, whereas treated cells of *E. coli* demonstrated slow growth. Similarly, the decrease in CFU/ml was greater in *M. luteus* than in *E. coli* cells (Fig. 4a, b). Bacteriocin LD4 caused the release of K^+ ions just after its addition to the *M. luteus* and *E. coli* cultures. This was higher in the cells of *M. luteus* than in *E. coli* cells. Similarly, nisin caused a release of K^+ ions at a lower level in *M. luteus* but not in *E. coli*. After the addition of diluents of bacteriocin LD4 and nisin, a slight increase in K^+ ion concentration was observed, suggesting the presence of K^+ ions in the formulation (Fig. 5a, b).

Bacteriocin LD4 exhibited a broad range of inhibition against Gram-positive and Gram-negative bacteria, including the pathogens *Pseudomonas aeruginosa*, *P. fluorescens*, *Staphylococcus aureus*, *Vibrio* sp., *Salmonella typhi*, *Enterobacter cloacae*, and *E. coli* (urogenic). It also inhibited the targeted LAB and haloarchaea strains (Table 3).

Discussion

Fermented food products are a major source of food materials harboring lactic acid bacteria. The production of bacteriocins from GRAS-status microorganisms is of the utmost importance (Battcock and Azam-Ali 1998). Therefore, in this study, *L. plantarum* LD4 was isolated from a traditional fermented

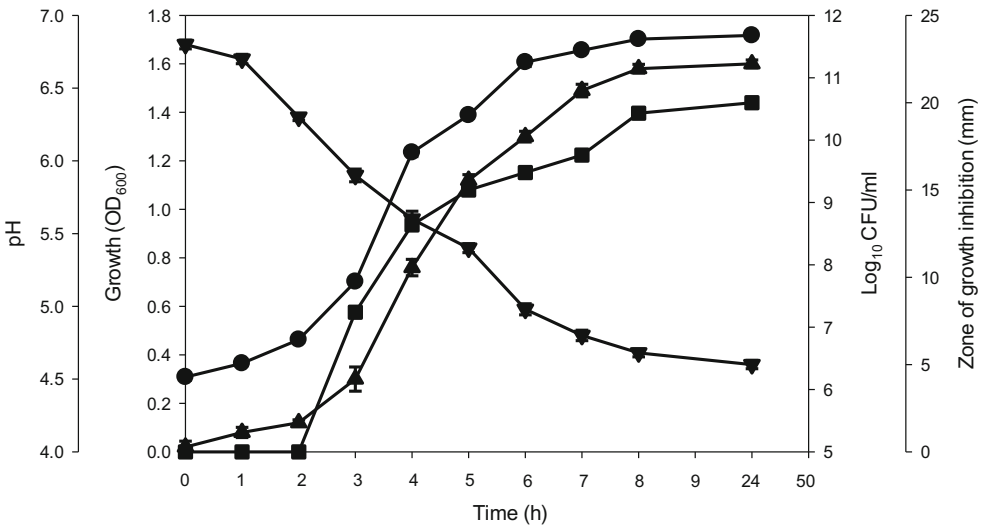

Fig. 2 Growth response of *Lactobacillus plantarum* LD4 in MRS medium demonstrated in terms of OD_{600} (filled upright triangle) and CFU/ml (closed circle). The change in pH (filled inverted triangle) and antimicrobial activity (closed square) at different stages of growth are also shown

Table 2 Biochemical properties of bacteriocin LD4

Serial Number	Treatments	Antimicrobial activity (mm)
1	Control (untreated)	20.12 ± 0.32
2	Temperatures	
	a) 60 °C, 15 min	20.14 ± 0.48
	b) 80 °C, 15 min	20.12 ± 0.64
	c) 100 °C, 15 min	20.19 ± 0.28
	d) 121 °C at 15 psi, 15 min	19.18 ± 0.33
3	pH	
	a) pH 2.0	20.08 ± 0.30
	b) pH 4.6 (control)	20.12 ± 0.40
	c) pH 6.0	14.00 ± 0.43
	d) pH 7.0	8.08 ± 0.65
	e) pH 8.0	Nil
4	Organic solvents, surfactants, and detergents	
	a) Ethanol	20.08 ± 0.32
	b) Methanol	20.08 ± 0.21
	c) Isopropanol	20.08 ± 0.28
	d) Acetone	20.08 ± 0.29
	e) Ethyl acetate	20.08 ± 0.31
	f) Acetonitrile	20.08 ± 0.40
	g) Urea	20.08 ± 0.37
	h) Tween 80	20.08 ± 0.26
	i) Triton X-100	20.08 ± 0.29
	j) SDS	20.08 ± 0.32
5	Hydrolyzing enzymes	
	a) Catalase	20.07 ± 0.21
	b) α-Amylase	20.18 ± 0.20
	c) Lipase	20.08 ± 0.18
	d) Trypsin	16.21 ± 0.29
	b) α-Chymotrypsin	19.12 ± 0.32
	c) Proteinase K	19.68 ± 0.28
	d) Protease	16.38 ± 0.27
	e) Pepsin	20.00 ± 0.28

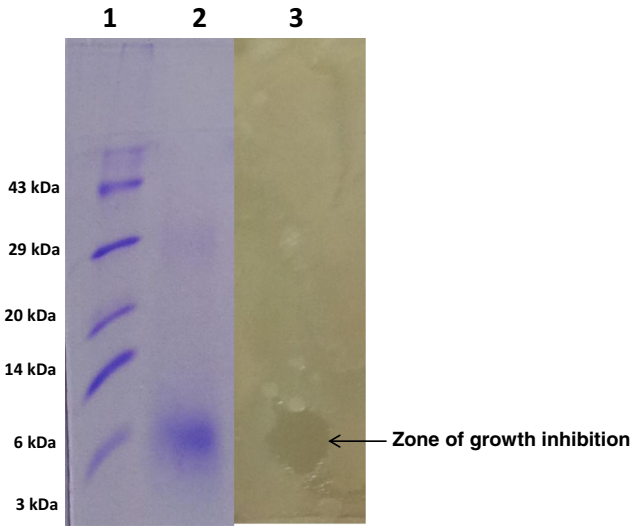

Fig. 3 Tricine SDS-PAGE analysis of partially purified bacteriocin LD4. Lane 1 is the molecular weight marker, lane 2 is partially purified bacteriocin LD4, and lane 3 shows the zone of growth inhibition against indicator strain *M. luteus* corresponding to the protein band in lane 2

food, Dosa, which has long been consumed in southern India. Strain LD4 was identified as *Lactobacillus* sp. using carbohydrate fermentation patterns, and species-level identification was carried out using 16S rDNA sequencing. Phylogenetic analysis suggested that strain LD4 belonged to *Lactobacillus plantarum*. The strain followed normal sigmoidal growth patterns, and bacteriocin production was found to be a growth-associated phenomenon. Maximum production occurred in the early stationary phase, and activity continued throughout the stationary phase, similar to plantaricin BM-1 (Zhang et al. 2013). Some reports have shown that the production of bacteriocins remained constant throughout the growth pattern, including stationary phase (Rushdy and Gomaa 2013).

CFS recovered from the early stationary phase was considered a crude bacteriocin and was used for further characterization. The stability of the bacteriocin at low pH is an important factor, as bacteriocins are used in fermented foods (Sahingil et al. 2011). The LD4 bacteriocin was active in acidic pH, but activity decreased as pH increased. Therefore, the activity of bacteriocin LD4 was tested at pH 4.6 for further characterization. During assays, the respective buffers of different pH values did not show an inhibition zone, suggesting that the activity in the CFS is not due to neutralization. Although the pH of CFS was acidic (pH 4.6), the negative controls (MRS medium, pH 4.6, and HCl-KCl buffer, pH 4.6) did not show a zone of growth inhibition, indicating that the activity in CFS is not due to acids. Various studies have reported on the pH stability of bacteriocins. Generally, under acidic conditions, bacteriocins produced by LAB are highly stable but at neutral and alkaline conditions, many of them are easily inactivated (Gupta and Tiwari 2014). For example, bacteriocin ST31 and paracaseicin A are active only in an acidic pH range, activity was highly reduced at pH 6 and completely disappeared at pH values between 7.0 and 9.0 (Todorov et al. 1999; Bendjeddou et al. 2012).

Heating of food is also an important step during food processing. Therefore, it is essential that a bacteriocin is heat-stable in order to ensure effective food biopreservation (Tuncer and Ozden 2010). Similar to bacteriocins produced by *Lactobacillus brevis* OG1 and *L. plantarum* F1, bacteriocin LD4 was found to be heat-stable up to autoclaving and boiling temperatures (Ogunbanwo et al. 2003). Paracaseicin A, on the other hand, was able to retain only 50 % of its activity after heating at 120 °C for 5 min, and activity completely

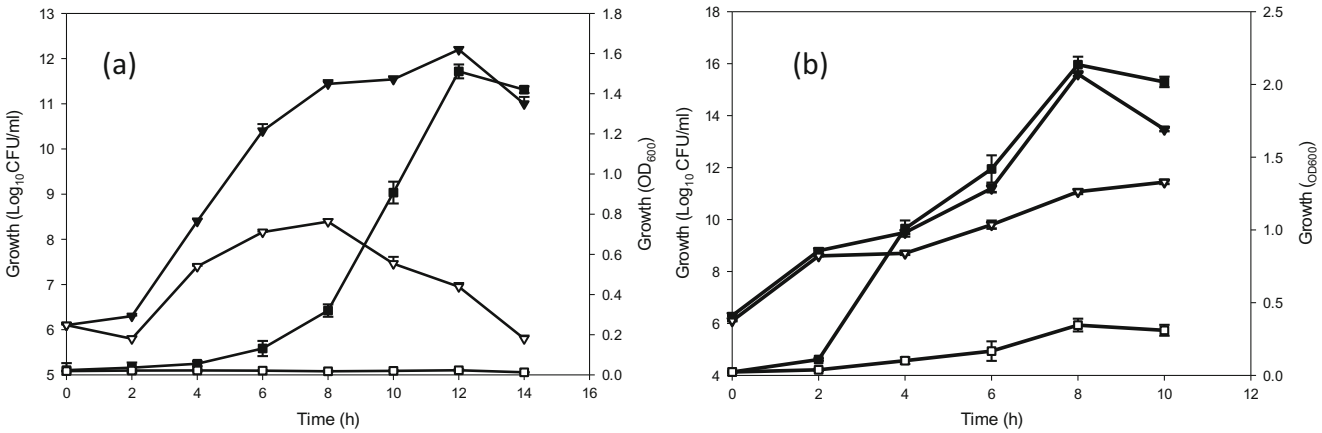

Fig. 4 Effect of bacteriocin LD4 on the growth of *M. luteus* (**a**) and *E. coli* (**b**) in terms of OD_{600} (untreated: filled square; treated: open square) and CFU/ml (untreated: filled inverted triangle, treated: open inverted triangle)

disappeared with heating at 120 °C for 10 min (Bendjeddou et al. 2012). The stability against organic solvents, surfactants, and detergents favors a broad application of the bacteriocin because of its potential to retain its structure and function during different purification steps. Reports from other studies have also suggested the stability of bacteriocins under such conditions (Rushdy and Gomaa 2013). The acid resistance and heat stability of bacteriocins are also very useful characteristics for additives used in the processing and preservation of various foods.

Treatment of the LD4 bacteriocin with trypsin and protease resulted in reduced activity, which suggests the proteinaceous nature of the compound. Similar characteristics have been reported for plantaricins C, D, MG, and BM-1 (Gong et al. 2010; Zhang et al. 2013). Different bacteriocins have shown different responses to proteolytic enzymes, such as brevicin 37 produced by *L. brevis*, which was inactivated by pronase E and trypsin (Saidi et al 2011). Moreover, treatment with catalase, α-amylase, and lipase did not affect the activity of bacteriocin LD4, indicating no role of hydrogen peroxide, carbohydrates, or lipids, respectively, in the antimicrobial activity. The low molecular mass (~6 kDa) substantiated the thermal stability of bacteriocin LD4. Small bacteriocins are generally known to diffuse on tricine SDS-PAGE, which could be due to the size and/or highly hydrophobic nature of the peptides (Tiwari and Srivastava 2008b; Kumar et al. 2010).

The growth inhibition shown by bacteriocin LD4 may be due to membrane perturbation, leading to K^+ ion efflux from treated cells, indicating the pore-forming nature of the compound. K^+ ion efflux suggests that bacteriocin LD4 may exert its antimicrobial activity by targeting the cytoplasmic membrane of sensitive cells and forming transient pores, followed by leakage of small ions. as also reported by Riazi et al. (2012). The LD4 bacteriocin showed a broad host range and

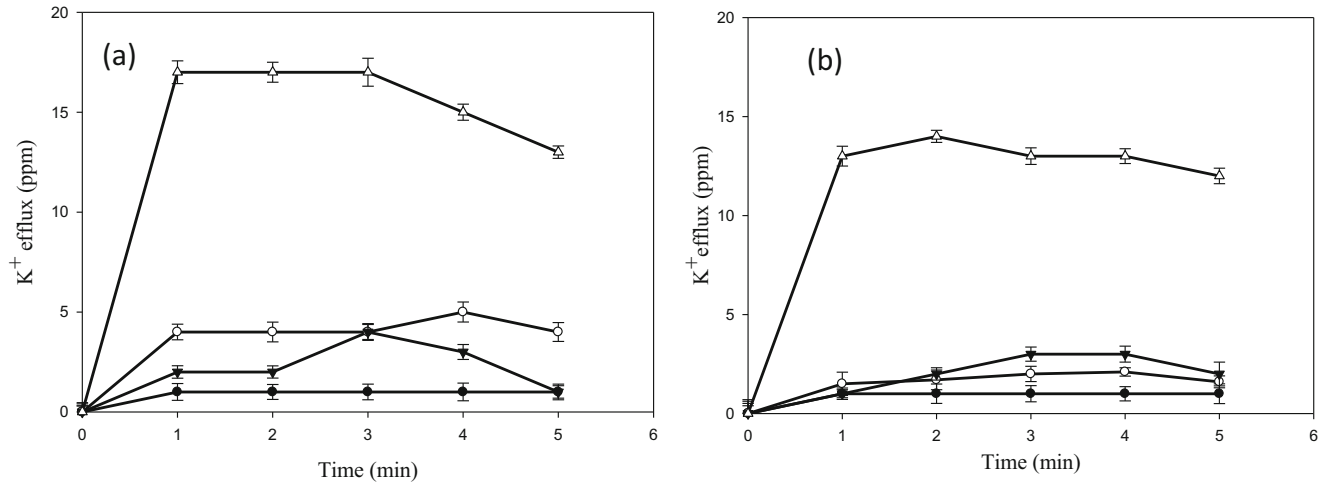

Fig. 5 Effect of bacteriocin LD4 on the efflux of K^+ ions from cytoplasmic membrane of *M. luteus* (**a**) and *E. coli* (**b**). Bacteriocin LD4 (upright triangle) caused a higher efflux of K^+ ions compared to nisin (open circle), used as positive control. Nisin (closed circle) and bacteriocin LD4 diluent (filled inverted triangle) showed only a basal level of K^+ ion concentration, and was used as negative control

Table 3 Antimicrobial spectrum of bacteriocin LD4

Serial Number	Target strains	Growth media	Zone of growth inhibition (mm)
1	*Micrococcus luteus* MTCC106	NB	20.12 ± 0.18
2	*Vibrio* sp.	do	16.21 ± 0.25
3	*Salmonella typhi*	do	16.12 ± 0.23
4	*Enterobacter cloacae* NRRL B14298	do	12.00 ± 0.19
5	*Staphylococcus aureus*	do	15.08 ± 0.19
6	*Enterococcus faecium* NRRL B2354	do	19.04 ± 0.13
7	*Pseudomonas aeruginosa*	LB	15.04 ± 0.28
8	*P. fluorescens*	do	14.21 ± 0.20
9	*E. coli* (urogenic)	do	18.03 ± 0.22
10	*Lactobacillus curvatus* NRRL B4562	MRS	Nil
11	*L. delbrueckii* NRRL B4525	do	11.42 ± 0.28
12	*L. acidophilus* NRRL B4495	do	17.02 ± 0.23
13	*L. plantarum* NRRL B4496	do	Nil
14	*Lactococcus lactis* subsp. *lactis* NRRL B1821	do	Nil
15	Haloarchaea strains HA57, 77, 80, and 83	HS	16.04 ± 0.19
16	Haloarchaea strain HA13	do	21.04 ± 0.18
17	Haloarchaea strains HA50, and 52	do	13.08 ± 0.14
18	Haloarchaea strains HA57 and 64	do	16.09 ± 0.17
19	Haloarchaea strain HA75	do	25.01 ± 0.16
20	Haloarchaea strains HA22, 32, 35-1, 35-2, 40, 49, 60, 63, 66, 71, 82, 87, 94, 96, 100, 105, 107, 109, 114-1, 114-2, 131, 115, 117, 121, 124, 127, 129, 132-1, 132-2, 133, 134, 136, 138, 139, 1342, and 67-2	do	11.04 ± 0.16
21	*Haloferax larsenii* strains HA1, 3, 4, 8, 9, and 10	do	14.05 ± 0.22

NB nutrient broth, *LB* Luria-Bertani, *MRS* de Man–Rogosa–Sharpe, *HS Halobacterium salinarum* medium

effectiveness against different LAB strains and other Gram-positive and Gram-negative bacteria. Similarly, bacteriocins produced by *L. plantarum* F1 and *L. brevis* OG1 isolated from Nigerian fermented food products showed a broad spectrum of inhibition against various LAB and pathogenic food spoilage organisms (Ogunbanwo et al. 2003; Rushdy and Gomaa 2013). Similar findings have been reported for plantaricin BM-1, S, C, C19, and -BN. as summarized by Olasupo (1996). The bacteriocins of LAB do not efficiently inhibit Gram-negative bacteria because the outer membrane of these bacteria obstructs the site used by the bacteriocin (Stevens et al. 1991). However, bacteriocin LD4 had activity against Gram-negative bacteria similar to bacteriocin BH-1 (Messi et al. 2001; Todorov and Dicks 2005). To date, only a few bacteriocins have been reported to inhibit Gram-negative bacteria including *E. coli*, *Pseudomonas fluorescens*, *P. putida*, and *Salmonella typhimurium* (Gong et al. 2010; Perez et al. 2014). The inhibition of haloarchaea strains suggests a host range of bacteriocin LD4 across the domain, and the possibility of its use in food safety applications for salted foods as well. Various strains of haloarchaea have been found to contaminate high-salt fermented foods consumed by humans (Birbir et al. 2004; Lee 2013). Therefore, bacteriocins active against haloarchaea may also be applied for the preservation of salted food products. There is growing demand for methods of manipulating bacteriocins at the gene and protein levels for enhanced effects on target cells (Perez et al. 2014; Tiwari et al. 2015). The above properties demonstrated by bacteriocin LD4 may be applied for food safety and therapeutics.

Conclusions

Lactobacillus plantarum LD4, a food isolate, was identified using morphological, biochemical, and molecular methods. Optimal bacteriocin production was found during the early stationary phase. Bacteriocin LD4 was characterized as thermo-pH stable, and active in various organic solvents, surfactants, and detergents. It was sensitive to trypsin and protease, suggesting the proteinaceous nature of the compound. The molecular mass of the bacteriocin was found to be ~6 kDa. It inhibited target cells by pore formation, as efflux of K^+ ions was found in treated cells of indicator strains. Bacteriocin LD4 was able to inhibit the growth of related LAB, certain food-borne pathogens, and a few strains of haloarchaea, suggesting its wide application in food safety and clinical usage.

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