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Original Article

Plantaricin LD1 Inhibits the Growth and Biofilm Formation of *Staphylococcus aureus* in Milk

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Abstract

Background and objectives: The contamination of food by spoilage microorganisms is a major concern for public health. Food-borne pathogens are commonly encapsulated by biofilms. Bacteriocins of probiotic lactic acid bacteria, such as nisin and pediocin have been used as bio-preservatives to improve food safety and extend the storage period of some food types. Therefore, there is a need to explore the potential of bacteriocins to impede the growth and biofilm formation by food spoilage bacteria in milk.

Methods: Plantaricin LD1 was purified from a potential probiotic, *Lactobacillus plantarum* LD1. Antimicrobial and anti-biofilm activity was then determined against a food-borne pathogen, *Staphylococcus aureus* subsp. *aureus* ATCC 25923. The efficacy of plantaricin LD1 against *S. aureus* subsp. *aureus* ATCC 25923 was also tested in the milk.

Results: Plantaricin LD1 had a minimum inhibitory concentration of 79.16 µg/mL and minimum bactericidal concentration of 158.33 µg/mL against *S. aureus* subsp. *aureus* ATCC 25923. Biofilm formation of *S. aureus* subsp. *aureus* ATCC 25923 was completely inhibited in the presence of 79.16 µg/mL of plantaricin LD1. Complete loss of cell viability in milk was observed after treatment with double minimum inhibitory concentration (158.33 µg/mL) of plantaricin LD1 at 48 hrs.

Conclusions: Our findings illustrate the antimicrobial and anti-biofilm activity of plantaricin LD1 against *S. aureus* subsp. *aureus* ATCC 25923 in milk. These results suggest that plantaricin LD1 can be used as a natural preservative to expand the shelf-life of milk.

Introduction

Food-borne diseases, caused by contamination of food products by spoilage bacteria, are a major concern for public health. Such contaminations not only alter the smell, taste and shelf-life of food products, but also cause significant threats to public health.[1,2] Food contaminating pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes* and *Salmonella typhi* are hard to eliminate altogether from food and the human environment.[3] *S. aureus* is a zoonotic pathogen that causes severe illnesses in humans and animals. It is also one of the major biofilm-forming pathogens responsible for mastitis in dairy animals around the world.[4] It produces enterotoxins responsible for illnesses, such as bacteremia, toxic shock syndrome, staphylococcal food-borne poisoning (nausea, vomiting, abdominal cramps and diarrhea), abscess, infective endocarditis, sepsis and pneumonia.[5] *S. aureus* is common in the milk and other dairy products because milk-producing animals are the primary sources of *S. aureus* contamination. Other sources of contamination include human handling, milking equipment and the environment.[4–6] The microbial niche generally constitutes biofilms composed of extracellular polymeric substances (EPS) like polysaccharides, proteins, lipids and extracellular DNA, which aid in microbial adhesion to various surfaces.[7] An additional benefit is that bacteria living in biofilms are several times more resistant to antimicrobials than their planktonic counterparts.[8] Inhibiting biofilm formation by pathogens is a major challenge during the treatment of various infectious diseases.[9]

Keywords: Food preservatives; *Lactobacillus plantarum* LD1; Plantaricin LD1; Biofilm; Milk.

Abbreviations: AWDA, agar well diffusion assay; BOD, biological oxygen demand; CEC, cation-exchange chromatography; CFS, cell-free supernatant; CV, crystal violet; EPS, extracellular polymeric substances; GFC, gel-filtration chromatography; LAB, lactic acid bacteria; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; MRS, de Man, Rogosa and Sharpe medium; NMWCO, nominal molecular weight cut-off; OD, optical density; PBS, phosphate-buffered saline.

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Table 1. Antimicrobial activity of plantaricin LD1 and nisin against different bacteria

Target bacteria	Zone of growth inhibition (mm)	
	Plantaricin LD1	Nisin
Micrococcus luteus MTCC 106	20	25
Escherichia coli ATCC 25922	9	Nil
Staphylococcus aureus subsp. *aureus* ATCC 25923	20	16

Food industries have adopted several types of preservation strategies to prevent the growth of spoilage microorganisms in food products.[10] Preservatives in foods primarily serve as flavor enhancers, nutritional quality boosters, spoilage delay agents, and inhibitors of microbial growth and biofilm formation.[11] In recent years, chemical preservatives have become one of the most popular and cost-efficient methods for preserving several foods.[10] Sodium benzoates, sodium nitrite, potassium sorbate, *etc.* are among the most widely used chemical food preservatives.[12] These preservatives have been effective at inhibiting food-borne pathogens, but they also cause resistance development in pathogens as well as changes to the organoleptic property of the food.[13] The increasing demand of consumers for 'safe-to-eat' food has ignited curiosity for the development of valuable natural biopreservatives.[14] Natural antimicrobials, such as bacteriocins of lactic acid bacteria (LAB), are useful alternatives to chemical-based food preservatives.[15] LAB bacteriocins in particular, are of interest because they are deemed safe for use in the food industry.

Bacteriocins of probiotic LAB are safe for human consumption, non-carcinogenic and non-allergic.[16] These bacteriocins have low molecular weights (~3 to 10 kDa), are hydrophobic and cationic peptides, and are stable in many organic solvents, polymers and detergents. They also exhibit stability at low pH and high temperature but are unstable in the presence of proteolytic enzymes.[17] Due to their proteinaceous nature and being produced by food-grade LAB, bacteriocins do not induce toxicity. The biochemical and genetic properties of bacteriocins provide a valid reason for their consideration as food preservatives.[18] Bacteriocins differ from antibiotics due to their proteinaceous nature and their ease of digestion by protease enzymes in the human digestive system.[19,20] Bacteriocins such as nisin and pediocin PA-1 have been reported as having applications in food preservation.[21] Nisin and pediocin PA-1/AcH are the only commercially available bacteriocins under the name Nisaplin™ and Alta 2341™ or Microgard™, respectively.[22] Nisin, which has been approved by the United States Food and Drug Administration (USFDA), is used for the safety of processed cheese, dairy products and canned foods.[17] Therefore, it is necessary to explore other bacteriocins which may have potential applications in food safety.

Previously, plantaricin LD1 was purified from the cell-free supernatant (CFS) of *Lactobacillus plantarum* LD1 isolated from batter of *Dosa* in our laboratory.[23] The bacteriocin was cationic, with a molecular weight of 6.5 kDa, heat stable and showed antimicrobial activity against broad range of bacteria (*Micrococcus luteus* MTCC 106, *L. delbrueckii* NRRL B-4525, *Lactococcus lactis* NRRL B-1821, *L. acidophilus* NRRL B-4495, *L. curvatus* NRRL B-4562, *E. faecium* NRRL B-2354, *L. lactis cremoris* NRRL B-634, *Pseudomonas fluorescens*, *E. coli* (urogenic), *P. aeruginosa*, *S. aureus*, *Vibrio* sp., *Shigella flexneri* and *S. typhi*).[23,24] In this study, the inhibitory effect of plantaricin LD1 was investigated against a food-borne pathogen; *S. aureus* subsp. *aureus* ATCC 25923 in milk.

Methods

Bacterial strains and growth conditions

L. plantarum LD1 was grown in MRS (de Man, Rogosa and Sharpe) medium (1% beef extract, 0.005% manganese sulphate, 2% glucose, 0.2% ammonium citrate, 0.5% yeast extract, 0.5% sodium acetate, 0.01% magnesium sulphate, 0.1% tween 80, 1% proteose peptone, 0.2% dipotassium hydrogen phosphate, pH 6.5) at 37°C for 18 hrs under the static condition in a BOD incubator (Laby Instrument, Haryana, India).[23,25] *M. luteus* MTCC 106 and *S. aureus* subsp. *aureus* ATCC 25923 were grown in Nutrient Broth (NB) medium (0.5% pepsin, 0.5% sodium chloride, 0.15% yeast extract, 0.15% beef extract, pH 7.4). *E. coli* ATCC 25922 was grown in Luria-Bertani (LB) broth medium (1.0% casein enzyme hydrolysate, 1.0% sodium chloride, 0.5% yeast extract, pH 7.5) at 37°C for 18 hrs with continuous shaking at 200 rpm in an incubator shaker (Scigenics, Tamil Nadu, India).[26] The chemicals and media components were purchased from Sisco Research Laboratory (Mumbai, India) and HiMedia (Mumbai, India). Nisin was purchased from Sigma (St. Louis, USA). Milk (*Vita*, Haryana, India) was purchased from the local market of Rohtak, Haryana, India. The ingredients of milk are carbohydrates 5.10 g, fat 1.50 g, protein 3.30 g, calcium 150 mg, vitamin A (Retinol Palmitate) 36 µg and vitamin D (Ergocalciferol) 0.62 µg.

Preparation of purified plantaricin LD1

The cell-free supernatant (CFS) of *L. plantarum* LD1 was used to purify the plantaricin LD1 as described in our previous study.[25] Briefly, sterilized CFS was passed successively through a 3 kDa nominal molecular weight cut-off (NMWCO) hollow fiber cartridge equipped with AKTA Flux-S (GE Healthcare, Uppsala, Sweden). The retentate was collected in a sterile container and loaded onto a cation-exchange HiPrep SP FF 16/10 (1.6 × 10 cm, 20 mL) column equipped with AKTAprime plus system (GE Healthcare, Uppsala, Sweden). Agar well diffusion assay (AWDA) was used to determine the antimicrobial activity after the fractions (1 mL) were collected. Gel-filtration chromatography (GFC) using a sephadex G-50 column (1.6 × 50 cm, 100 mL) equipped with AKTA prime plus system was used to desalt the pooled cation-eluted active fractions. The protein concentration of plantaricin LD1 was determined using the Bradford assay. The antimicrobial activity of plantaricin LD1 was determined against *M. luteus* MTCC 106, *E. coli* ATCC 25922 and *S. aureus* subsp. *aureus* ATCC 25923 using AWDA. Nisin was used as a positive control (Table 1).

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The sodium acetate buffer (10 mM, pH 4.5) was used to prepare a two-fold serial dilution of plantaricin LD1 and 100 µL of each dilution was transferred to the wells of microtiter plates

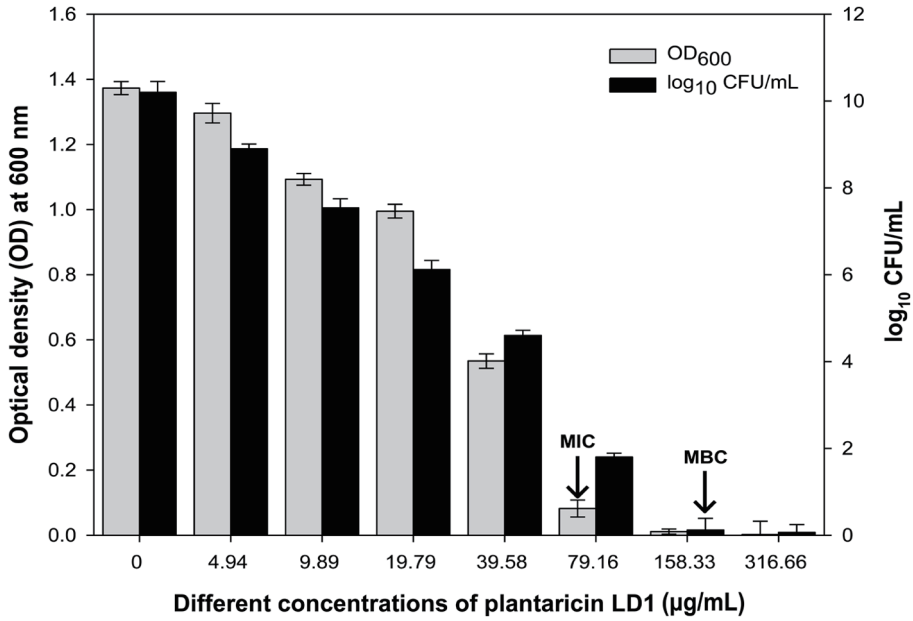

Fig. 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of plantaricin LD1 against *Staphylococcus aureus* subsp. *aureus* ATCC 25923. The results are expressed a mean ±SD (n=3). CFU, colony forming units; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; OD, optical density.

already containing 200 µL of *S. aureus* subsp. *aureus* ATCC 25923 with an initial OD_{600} of 0.02 in NB medium. The control set was supplemented with 100 µL of sodium acetate buffer in place of plantaricin LD1. The microtiter plate was incubated in a BOD incubator shaker for 18 hrs at 37°C with constant shaking at 200 rpm. Net growth was calculated by subtracting the final and initial OD_{600} using a microplate reader (Molecular Devices, San Jose, USA). The lowest concentration of plantaricin LD1 which showed no observable growth ($OD_{600} < 0.1$) of the target strains was considered the MIC and the MBC as the lowest concentration of plantaricin LD1 when complete loss of cell viability was recorded.[26]

Inhibition of biofilm formation

The crystal violet (CV) method was used to measure the inhibition of biofilm formation as described previously by Yadav *et al*.[27] Briefly, a microtiter plate was filled with 200 µL of an overnight grown culture (~10^6 CFU/mL) of *S. aureus* subsp. *aureus* ATCC 25923 and various concentrations of plantaricin LD1 (0–316.66 µg/mL) were added to each well and incubated at 37°C for 18 hrs with constant shaking at 200 rpm. For control samples, sodium acetate buffer (10 mM, pH 4.5) was added to the well instead of plantaricin LD1. After incubation, the liquid content was decanted and rinsed twice with filter-sterilized phosphate-buffered saline (PBS, pH 7.4) to remove the cells of the indicator strain and the growth medium. The biofilm was then fixed in the microtiter plate wells by adding absolute methanol (150 µL) and incubated at room temperature for 20 mins. The wells were then twice rinsed using filter sterilized double-distilled water (ddH_2O) and air dried for 30 mins in the inverted position. The biofilm was stained with 200 µL crystal violet (CV) solution (0.1%), and incubated for 15 mins at room temperature before the unbounded dye was removed by washing with ddH_2O. The bound dye was then released by adding an aliquot (150 µL) of acetic acid (33%) and incubating at room temperature for 30 mins. To determine the amount of biofilm formation, the optical density (OD) of the dye released was measured at 585 nm using a microplate reader.

Effect of plantaricin LD1 in milk

The efficacy of plantaricin LD1 against *S. aureus* subsp. *aureus* ATCC 25923 was evaluated in sterilized milk (*Vita*, Haryana, India). The overnight grown culture of *S. aureus* subsp. *aureus* ATCC 25923 (OD_{600} 0.02) was added to 5 mL of sterilized milk and treated with MIC (79.16 µg/mL) and double MIC (158.33 µg/mL) of plantaricin LD1. A set without plantaricin LD1 was used as a control. The untreated and treated sets were incubated for 48 hrs at 37°C in an incubator with constant shaking at 200 rpm. The colony-forming units per milliliter (CFU/mL) were calculated to estimate the cell viability of the target strain.[28]

Statistical analysis

Experiments were carried out in triplicate and mean values and standard deviations (mean ± SD) were plotted using SigmaPlot 11.0. Statistically significant results ($p < 0.05$) were ascertained using the student t-test.

Results

Growth inhibition of S. aureus subsp. aureus ATCC 25923

The untreated set of *S. aureus* subsp. *aureus* ATCC 25923 showed growth up to OD_{600} 1.37 ± 0.043. The plantaricin LD1-treated cells showed a reduction trend in the growth up to OD_{600} 0.00 ± 0.04 at different concentrations (4.94–316.66 µg/mL). Therefore, 79.16 µg/mL was considered MIC of plantaricin LD1. Cell viability of *S. aureus* subsp. *aureus* ATCC 25923 decreased up to $\log_{10}$ nil CFU/mL in the presence of different concentrations (4.94–316.66 µg/mL). Therefore, 158.33 µg/mL was considered as MBC of plantaricin LD1 (Fig. 1). The percentage growth inhibition and per-

Table 2. Percentage inhibition of growth, cell viability and biofilm formation of *Staphylococcus aureus* subsp. *aureus* ATCC 25923 after treatment with plantaricin LD1

S. No.	Different concentrations of plantaricin LD1 (µg/mL)	Percentage growth inhibition	Percentage inhibition of cell viability	Percentage inhibition of biofilm formation
1.	0	0	0	0
2.	4.94	15.33	12.75	21.74
3.	9.89	20.40	26.08	43.48
4.	19.79	27.74	40.00	65.22
5.	39.58	61.31	54.90	78.26
6.	79.16	94.16	85.35	97.83
7.	158.33	99.27	100.00	98.26
8.	316.66	100.00	100.00	100.00

centage inhibition of cell viability were increased to 100 percent after treatment with different concentrations of plantaricin LD1 (4.94–316.66 µg/mL) (Table 2).

Inhibition of biofilm formation

Optical density (OD_{585}) readings for the untreated set of *S. aureus* subsp. *aureus* ATCC 25923 was 0.23 ± 0.003, suggesting the formation of biofilm. Inhibition of biofilm formation by plantaricin LD1 was illustrated by a concentration dependent reduction in optical density absorbance at OD_{585} from 0.18 ± 0.008 to 0.00 ± 0.001 in the presence of different concentrations (4.94–316.66 µg/mL) (Fig. 2). At 79.16 µg/mL, plantaricin LD1 completely inhibited biofilm formation of *S. aureus* subsp. *aureus* ATCC 25923. The percentage of inhibition of biofilm formation was found 0.00 ± 0.002 to 100 ± 0.005 after treatment with various concentrations of plantaricin LD1 (4.94–316.66 µg/mL) as depicted in Table 2.

Kill kinetics of S. aureus subsp. aureus ATCC 25923 in Milk

Milk without (control) plantaricin LD1 showed increased growth of *S. aureus* subsp. *aureus* ATCC 25923 with time, and recorded $\log_{10}$ 9.5 ± 0.18 CFU/mL at 48 hrs. Milk treated with MIC (79.16 µg/mL) of plantaricin LD1 showed a decrease in cell viability to $\log_{10}$ 1.12 ± 0.20 CFU/mL at 48 hrs. The complete loss of cell viability of *S. aureus* subsp. *aureus* ATCC 25923 was found after treatment with double MIC (158.33 µg/mL) of plantaricin LD1 at

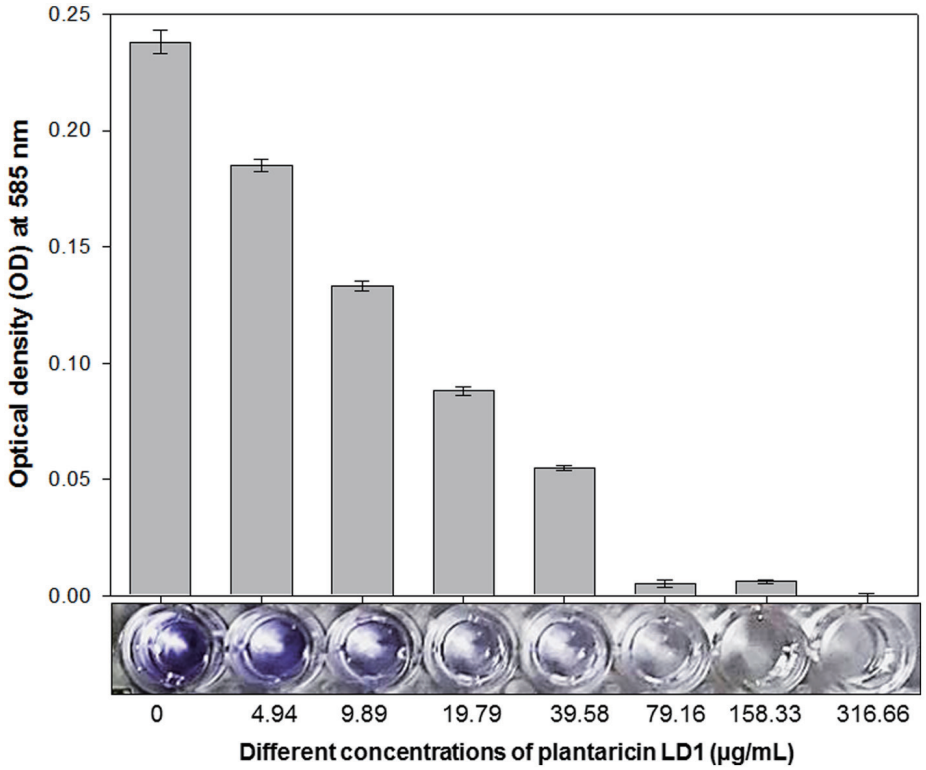

Fig. 2. Inhibition of biofilm formation of *Staphylococcus aureus* subsp. *aureus* ATCC 25923 in the presence of different concentrations of plantaricin LD1.

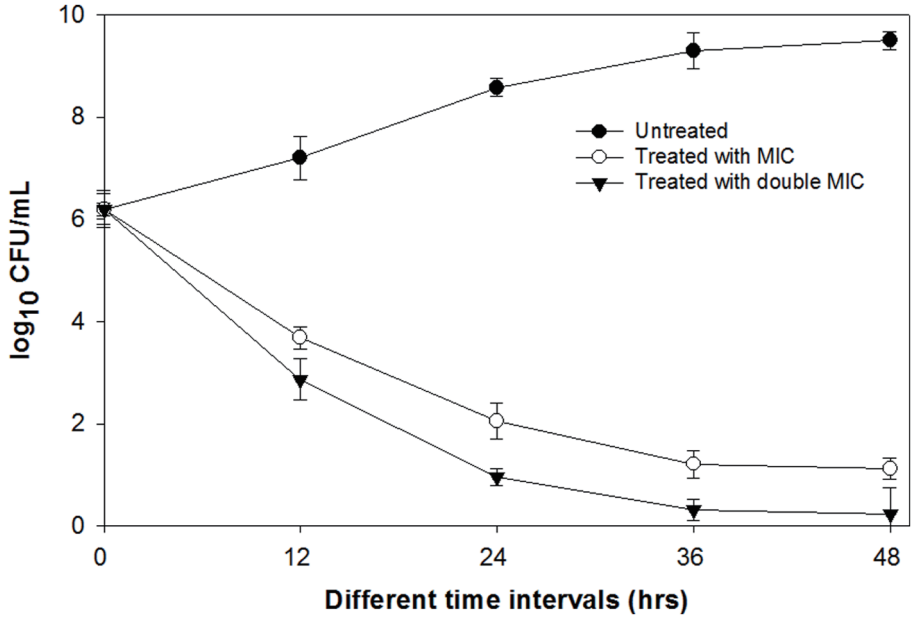

Fig. 3. Kill kinetics of *Staphylococcus aureus* subsp. *aureus* ATCC 25923 in the presence of MIC (79.16 µg/mL) and double MIC (158.33 µg/mL) of plantaricin LD1 as compared to untreated cells in the milk. CFU, colony forming units; MIC, minimum inhibitory concentration.

48 hrs (Fig. 3). This suggested that plantaricin LD1 caused killing of *S. aureus* subsp. *aureus* ATCC 25923 cells in milk.

Discussion

Food-borne pathogenic bacteria grow in a wide range of food materials such as dairy, meat and vegetable products.[29] Preservation techniques such as canning, pasteurization and addition of preservatives are standard,[30] and cause adverse effects such as allergic reactions, formation of carcinogenic products and alteration of the sensory properties of the foods.[31] The presence of *S. aureus* biofilm on food comes with a severe risk of diseases associated with the consumption of contaminated fresh or processed foods. The use of probiotic bacteria and their metabolic products has promising applications in ensuring the safety and enhancement of the shelf-life of foods by inhibiting food-borne pathogens.[32] Bacteriocins of probiotic LAB are broadly utilized in food safety because of their non-toxic nature, categorized as 'Generally Regarded as Safe' (GRAS) and inhibit the growth of food-borne pathogens.[33,34] Different species of *Staphylococcus* are responsible for causing skin and soft tissue infections that can be life-threatening. Although many bacteriocins have been reported to be active against food-borne pathogens, few of them have been diligently characterized for their application in food safety.[15]

Our previous research demonstrated that crude plantaricin LD1 from *L. plantarum* LD1 is thermostable, pH-stable, sensitive to proteolytic enzymes, stable in the presence of various organic solvents and has antimicrobial activity against related bacteria and some food-borne pathogens.[24] It also has bactericidal activity against *S. aureus* subsp. *aureus* ATCC 25923, as previously demonstrated by Gupta and Tiwari.[24] In this study, we demonstrated inhibition of growth and biofilm formation by the food-borne pathogen, *S. aureus* subsp. *aureus* ATCC 25923 in milk using purified plantaricin LD1. The MIC of purified plantaricin LD1 was 76.16 µg/mL against *S. aureus* subsp. *aureus* ATCC 25923. Similarly, bacteriocin MN047A from *L. crustorum* MN047 showed MIC 165 µg/mL against *S. aureus* ATCC 29213 which is almost double.[35] Plantaricin Pln1 required an even higher concentration (475 µg/mL) to inhibit the growth of *S. aureus* ATCC 29213.[36] In another study, the bacteriocin XN2 from *Lacticaseibacillus rhamnosus* XN2 required a higher concentration (100 µg/mL) for growth inhibition of *S. aureus* CICC 10384 as compared to plantaricin LD1.[37] Gong *et al.*[38] also suggested that the plantaricin MG produced by *L. plantarum* KLDS1.0391 required higher concentration (1,000 µg/mL) for the inhibition of growth of *S. aureus* ATCC 25923. In addition, bacteriocin SLG10 from *L. plantarum* SLG10 and plantaricin 827 from *L. plantarum* 163 inhibited *S. aureus* CICC 10384 and *S. aureus* ATCC 25923, respectively.[39,40] In contrast, Barbosa *et al.*[41] suggested that the bacteriocin MBSa4 isolated from *L. plantarum* MBSa4 did not inhibit *S. aureus* ATCC 29213.

Microbial biofilms harbor complex microbial communities which allow microbes to adhere tightly to surfaces. This is an essential characteristic for the survival of various microorganisms.[8,39] Antimicrobial peptides (AMPs) inhibit biofilm formation by interacting with extracellular DNA and facilitating surface detachment.[42] Consequently, naturally produced AMPs have become a focus point for research aimed at mitigating the spread of pathogenic organisms.[43] AMPs derived from natural sources, such as bacteriocins, also represent an attractive and interesting alternative for the treatment of bacterial infections resulting from multidrug-resistant microorganisms.[44] Their potential as supplements or replacements for antibiotics could be an environmentally friendly approach to encouraging reduction in antibiotic overuse and resistance.[45] In this study, plantaricin LD1 completely inhibited biofilm development by *S. aureus* subsp. *aureus* ATCC 25923. In another study, the biofilm of *S. aureus* ATCC 25923 was inhibited by plantaricin YKX from *L. plantarum* YKX, as suggested by Pei *et al.*[46] Plantaricin GZ1-27 purified from *L. plantarum* GZ1-27 also inhibited biofilm formation of *S. aureus* ATCC 43300 by preventing the creation of surface matrix-associated proteins features.[47] Plantaricin 827 from *L. plantarum* 163 and bacteriocin C4B from

L. lactis F01 inhibited the biofilm formation of *S. aureus* ATCC 25923, ATCC 43300 and ATCC 35395, respectively.[40,48] Milk is rich in macro and micronutrients and therefore it is a suitable medium for the growth of pathogenic bacteria.[49] *S. aureus* contamination in food has led to a significant need in the food industry for natural, secure and efficient antimicrobial agents that are capable of inactivating pathogenic bacteria from food.[40] Plantaricin LD1 treatment of *S. aureus* subsp. *aureus* ATCC 25923 cells resulted in a time-bound bactericidal effect in milk. Similarly, Zhao *et al*.[40] showed plantaricin 827 from *L. plantarum* 163 inactivating *S. aureus* ATCC 25923 in skim milk, and bacteriocin MN047A from *L. crustorum* MN047 showed loss in cell viability of *S. aureus* ATCC 29213 in a dose and time-dependent manner as suggested by Yi *et al*.[35] Other bacteriocins, such as enterocin AS-48 and bacteriocin NX371 inhibited the growth of *S. aureus* CECT 976 and *S. aureus* (MRSA) ATCC 43300 in milk.[2,50] This study is in alignment with the other studies suggesting the application of plantaricin LD1 for the safety of milk and related dairy products.

Future directions

Plantaricin LD1 is a non-toxic antimicrobial peptide purified from food-grade *L. plantarum* LD1, which is safe as a food preservative and medicine. The efficacy of plantaricin LD1 against the food-borne pathogen, *S. aureus* subsp. *aureus* ATCC 25923 in a milk model has highlighted its potential application in dairy industry food safety. The findings from this study have also emphasized the role of plantaricinLD1 as a potential antibiotic against clinical pathogens that cause infectious diseases.

Conclusions

Plantaricin LD1 purified from food isolate of *L. plantarum* LD1 has antimicrobial and anti-biofilm activity against the food-borne pathogen, *S. aureus* subsp. *aureus* ATCC 25923. Plantaricin LD1 treated milk remained fresh by inhibiting the cell viability of *S. aureus* subsp. *aureus* ATCC 25923. Therefore, plantaricin LD1 may be used as a natural food bio-preservative for the safety of milk.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

MKY and AB performed the experiments, analyzed the data and designed the manuscript. SKT, as corresponding authors, provided all laboratory facilities, chemicals and reviewed the manuscript. All authors read and approved the final version of the manuscript.

Data sharing statement

No additional data are available.

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