Anti-staphylococcal activity of bacteriocins of food isolates *Enterococcus hirae* LD3 and *Lactobacillus plantarum* LD4 in pasteurized milk

Poonam Sheoran¹ · Santosh Kumar Tiwari¹

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Abstract

Bacteriocins of *Enterococcus hirae* LD3 and *Lactobacillus plantarum* LD4 have been applied in milk for growth inhibition of *Staphylococcus aureus*. The enumeration of *S. aureus* cells in nutrient broth and milk was found $\log_{10}$ 9.7 and 10.2 CFU/mL, respectively, whereas it was reduced with increasing concentration of bacteriocins suggesting loss of cell viability. The lethal concentration (LC₅₀) of enterocin LD3 and plantaricin LD4 against *S. aureus* was 160 and 220 µg/mL, respectively. Bacteriocin-treated cells were stained red with propidium iodide (PI) indicating dead cells further confirms bactericidal nature. The enterocin LD3-treated cells showed higher infrared absorbance at 1451.82 cm^{-1} corresponding to phospholipids suggesting membrane-acting nature of the bacteriocin. However, plantaricin LD4-treated cells did not show such alterations suggesting different mode of action. Both bacteriocins caused disruption and shrinkage of target cells, and leakage of intracellular contents as observed in transmission electron microscope (TEM). The present study suggests killing of *S. aureus* in milk, therefore, enterocin LD3 and plantaricin LD4 may be applied in biopreservation of milk and related food products.

Keywords Enterocin LD3 · Plantaricin LD4 · Bactericidal · *Staphylococcus aureus* · Milk

Introduction

The contamination of dairy products by spoilage bacteria such as *Listeria monocytogenes* and *Staphylococcus aureus* is a serious concern (Pimentel-Filho et al. 2014). Such contaminations not only cause deterioration in sensory quality and shelf-life of foods, but also create a serious public health risk (Gopal et al. 2015). *S. aureus* is one of the most serious pathogen living in mucous membranes and skin of warm-blooded animals. It produces enterotoxins responsible for staphylococcal food poisoning causing bovine mastitis, abdominal cramps, nausea, vomiting and diarrhea. Modern society is more conscious of the importance of food safety, as many of the chemical additives used in food may elicit toxic concern (Yang et al. 2014; Nura et al. 2016). Thus, there is an urgent need to find safe and effective biopreservatives to

inhibit pathogenic strains for either food preservation and/or control of infectious diseases (Wang et al. 2016).

Recently, different methods have been developed to control food-borne pathogens and reduce their potential risks to human health (Fu et al. 2018). Such methods include addition of live bacteria as probiotics and/or their bacteriocins. Bacteriocins are ribosomally-synthesized antimicrobial proteins or peptides produced by bacteria, which kill or inhibit bacterial strains closely related to producer (Silva et al. 2018). Particularly, the bacteriocins produced by lactic acid bacteria (LAB) have been the center of attention as they are generally regarded as safe (GRAS) and have potential application as natural preservatives in the food industry. Further, bacteriocins have several attributes which make them suitable as food-biopreservative such as ability to inhibit pathogenic and spoilage bacteria, e.g., *L. monocytogenes*, *S. aureus*, *Bacillus cereus*, *Clostridium botulinum*, etc., susceptibility to digestive proteases, constancy in a wide range of temperature and pH, no alteration of the organoleptic properties of food, no toxicity to eukaryotic cells and simplicity to scale-up production (Perez et al. 2014).

Several bacteriocins have been characterized but only few are commonly available for industrial applications (Chi

✉ Santosh Kumar Tiwari
santoshgenetics@gmail.com

¹ Department of Genetics, Maharshi Dayanand University, Rohtak, Haryana 124001, India

and Holo 2018). In our previous study, *Enterococcus hirae* LD3 and *Lactobacillus plantarum* LD4 were isolated from a fermented food, *Dosa* and characterized for the production of bacteriocins with probiotic potential (Gupta and Tiwari 2015; Gupta et al. 2016; Kumar et al. 2016). In this study, purified bacteriocins from these strains have been applied in pasteurized milk to observe their lethal effect on *S. aureus* and assess their food safety efficacy.

Materials and methods

Bacterial strains and growth conditions

The bacteriocin-producing *E. hirae* LD3 and *L. plantarum* LD4 were grown in MRS medium for 18 h at 37 °C (Gupta and Tiwari 2015; Kumar et al. 2016). *S. aureus* ATCC259323 was obtained from Pandit Bhagwat Dayal Sharma University of Health Sciences, Rohtak and grown in Nutrient Broth (NB) and/or 10% (v/v) pasteurized milk (Anand Milk Union Limited, Gujarat, India) at 37 °C for 24 h in a BOD incubator (Scigenics Biotech, Chennai, India). All the media components were purchased from Hi-Media (Mumbai, India), Sisco Research Laboratory (SRL, Mumbai, India) and Sigma–Aldrich (St-Louis, USA).

Preparation of purified bacteriocins

Enterocin LD3 and plantaricin LD4 were purified using ammonium sulphate precipitation, cation exchange chromatography and reverse-phase HPLC as reported previously (Gupta et al. 2016) and purified bacteriocin samples were used in further experiments.

Lethality assay of bacteriocins in medium and milk

To observe the lethal concentration (LC₅₀) of enterocin LD3 and plantaricin LD4 against *S. aureus*, microdilution method was used as suggested by Omobhude et al. (2017). Briefly, twofold serial dilutions of bacteriocins were individually made in sodium acetate buffer (10 mM, pH 6.0). An aliquot of 100 µL of each dilution was added in a set containing 100 µL *S. aureus* (log₁₀ 5.2 CFU/mL) cells, in nutrient broth and 10% pasteurized milk, individually as suggested by Pinto et al. (2011). In the control set, sodium acetate buffer was added without bacteriocin. Bacteriocin-treated and control sets were incubated at 37 °C for 24 h. After incubation, 100 µL aliquot from each set was tenfold serially diluted in normal saline solution (0.8% NaCl) for determination of viable count (CFU/mL). The LC₅₀ was determined as the concentration where 50% viability loss of *S. aureus* cells was recorded.

Time-kill assay and staining of live/dead cells

Time and concentration-based killing of *S. aureus* cells was monitored in the presence of LC₅₀ and 2 × LC₅₀ of bacteriocins individually upto 24 h. The target strain, *S. aureus* (log₁₀ 5.2 CFU/mL) was grown in 10% milk in the presence of bacteriocins individually at 37 °C, for 24 h. The control set was grown without bacteriocin under same conditions. The survival of cells was enumerated in terms of CFU/mL at time intervals of 2 h upto 24 h and compared with untreated control.

Bacteriocin-treated cells were stained with 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) to assess the live and dead cells, respectively. The treated (LC₅₀) cells (~ 10⁶ CFU/mL) were recovered at 10 h of incubation. The untreated cells, washed twice with normal saline were used as positive control (live cells). For negative control (dead cells), cells (~ 10⁶ CFU/mL) were treated with 70% ethanol, washed twice with normal saline and incubated at 60 °C for 10 min. An aliquot of 10 µL of stock solution (1 mg/mL) of each DAPI and PI were added to 1 mL each bacteriocins-treated, live and dead cells. Staining was carried out at room temperature before the live/dead cells were analysed at excitation 330–380 nm and barrier filter 400 nm using fluorescent microscope (DS-Fi2, Nikon eclipse, Japan) with 40× magnification as suggested by Duan et al. (2017).

Fourier transform infrared spectroscopy (FTIR)

S. aureus cells (~ 10⁶ CFU/mL), recovered at 10 h, were treated (LC₅₀) individually with enterocin LD3 and plantaricin LD4 and washed twice with sterile normal saline. Infrared absorbance spectra of untreated and bacteriocin-treated cells were acquired using FTIR spectrophotometer (Bruker, Germany) on diamond-attenuated total reflectance accessory as suggested by Zoumpopoulou et al. (2013). Opus software was used for spectra acquisition. The cells were placed in direct contact with the internal reflecting diamond crystal. Once in contact with the crystal, multiple scans were obtained to reduce error. Each spectrum was baseline corrected and spectral range was set from 500 cm⁻¹ to 4000 cm⁻¹ at a resolution of 8 cm⁻¹.

Transmission electron microscopy (TEM)

To observe morphological changes in bacteriocin-treated *S. aureus* cells recovered at 10 h, TEM analysis was performed. Briefly, cell suspension (~ 10⁶ CFU/mL) was centrifuged at 10,000 rpm, 4 °C for 10 min and washed twice with normal saline as suggested by Okuda et al. (2013). Subsequently, cell pellets were further rinsed thrice with

normal saline and fixed in a mixture of 2% glutaraldehyde and 2% paraformaldehyde in phosphate buffer for 2 h at 4 °C. To remove the fixative, samples were centrifuged at 10,000 rpm, 4 °C for 5 min. The pellets were post-fixed for 1 h in 1% osmium tetroxide at 4 °C. Samples were dehydrated in acetone, infiltrated and embedded in resin araldite CY 212 (TAAB, UK). The sections (1 µm) were cut with an ultra-microtome, mounted on to glass slides, stained with aqueous toluidine blue and observed under a light microscope for gross observation of the area. For electron microscopic examination, the thin sections of grey-silver colour interference (70–80 nm) were further cut and mounted onto 300 mesh-copper grids. Sections were stained with alcoholic uranyl acetate and alkaline lead citrate, washed gently with distilled water and observed under a transmission electron microscope (Morgagni 268D Fei, Netherlands) at an operating voltage 200 kV with magnification 15,000×. The images were digitally acquired using a charge-coupled devices (CCD) camera attached to the microscope. The TEM was performed at Sophisticated Analytical Instrumentation Facility, All India Institutes of Medical Sciences, New Delhi, India.

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. For FTIR spectroscopy and TEM analysis, three independent experiments were performed to monitor the reproducibility of results.

Results

Lethal concentration of bacteriocins in medium and milk

The growth of *S. aureus* was monitored in medium and milk with different concentrations of bacteriocins. It was observed that the untreated cells were grown upto similar extent, log₁₀ 9.7 and 10.2 CFU/mL in medium and milk, respectively at 24 h, whereas decrease in growth was observed with increasing concentrations of bacteriocins. There was 50% loss in cell viability (LC₅₀) by enterocin LD3 recorded at 140 and 160 µg/mL in milk and medium, respectively (Fig. 1a). Similarly, LC₅₀ 200 and 220 µg/mL were recorded for plantaricin LD4 in milk and medium, respectively (Fig. 1b).

Time- and concentration-based killing of *S. aureus* cells

During time-kill assay, untreated *S. aureus* cells grew normally from log₁₀ 5.2 to 10.2 CFU/mL, whereas, viability loss was recorded in bacteriocin-treated cells over incubation period. The LC₅₀ and 2 × LC₅₀ of enterocin LD3 were able to reduce cell viability upto log₁₀ 2.7 and 2.0 CFU/mL, respectively (Fig. 2a). On the other hand, in presence of LC₅₀ and 2 × LC₅₀ of plantaricin LD4, the cell viability was reduced to log 3.8 and 2.5 CFU/mL, respectively at 24 h (Fig. 2b). Therefore, enterocin LD3 showed higher bactericidal effect as compared to plantaricin LD4. The live cells were stained blue with DAPI (Fig. 2c) and dead cells were stained red with PI (Fig. 2d). Whereas, bacteriocins-treated cells showed pinkish-red colour suggesting dead cells and few blue colour suggesting live cells (Fig. 2e, f). Therefore, it was observed that cells were killed after treatment with bacteriocins

Fig. 1 Effect of different concentrations of enterocin LD3 (a) and plantaricin LD4 (b) on growth (log₁₀ CFU/mL) of *Staphylococcus aureus* in nutrient broth (black bar) and milk (gray bar)

Fig. 2 Growth response of *Staphylococcus aureus* in milk in the presence of two concentrations (LC_{50} and $2 \times LC_{50}$) of enterocin LD3 (a) and plantaricin LD4 (b) the live cells of *S. aureus* were stained blue

with propidium iodide (c) and dead cells were stained red (d) with 4',6-diamidino-2-phenylindole. Whereas, cells treated with enterocin LD3 (e) plantaricin LD4 (f) showed mixture of live and dead cells

confirming bactericidal activity of enterocin LD3 and plantaricin LD4.

FTIR analysis suggests membrane-acting nature of bacteriocins

The FTIR analysis was performed to monitor the interaction of bacteriocins with *S. aureus* cells in milk. The variation in absorbance of bacteriocin-treated and untreated cells was compared to elucidate the possible mechanism of action. It was observed that IR absorbance was higher in enterocin LD3-treated cells at approximately 1451.82 cm^{-1} and 1094.30 cm^{-1} corresponding to phospholipids and nucleic acids, whereas untreated cells did not show such alterations in the absorbance. Contrary to enterocin LD3, plantaricin LD4-treated cells did not show changes in the absorbance (Fig. 3). These findings further suggest that enterocin LD3 may interact with lipid membrane and nucleic acids, and plantaricin LD4 kills cells using different mechanism as it did not show changes in the absorbance.

Bacteriocins disrupt *S. aureus* cells in milk

The effect of bacteriocins on cell morphology of *S. aureus* was visualized under electron microscope. Untreated cells were found to be normal cocci with intact cell boundary (Fig. 4a). Whereas, bacteriocin-treated cells were found to be ruptured with damaged cell membrane and also provided evidence of leakage of intracellular contents from *S. aureus* cells (Fig. 4b, c). This result further suggested the bactericidal nature of enterocin LD3 and plantaricin LD4 in pasteurized milk.

Discussion

Staphylococcal food poisoning is a major concern in public health. *S. aureus* present in milk collected from the animal suffering from diseases is responsible for several food-borne diseases. For control of such pathogens, use of bacteriocins is of great interest as they are generally recognized as safe natural biopreservatives (Silva et al. 2018). In our previous studies, the inhibitory effect of purified enterocin LD3

Fig. 3 The FTIR spectra of *Staphylococcus aureus* cells treated (continuous grey line) with enterocin LD3 and plantaricin LD4 in comparison with untreated cells (dotted grey line)

Fig. 4 Transmission electron microscopy (15,000 $\times$ magnification) of *Staphylococcus aureus* cells (a) showing coccus shape and uniform cytoplasmic materials. Whereas, *S. aureus* cells treated (LC₅₀) with

enterocin LD3 (b) and plantaricin LD4 (c) showed disruption of cell membrane and release of intracellular contents (as indicated by the arrows)

and plantaricin LD4 have been tested against pathogenic bacteria such as *S. aureus*, *Salmonella typhi*, *Vibrio* sp., *Pseudomonas aeruginosa* and *Escherichia coli* (Gupta et al. 2016; Kumar et al. 2016). Here, we have demonstrated the killing of *S. aureus* cells in milk using bacteriocin LD3 and LD4. Both bacteriocins were found to be effective in milk with similar extent as in medium suggesting the efficacy of the bacteriocins in food model. Enterocin LD3 showed lower LC₅₀ against *S. aureus* as compared to plantaricin LD4 may be due to different killing mechanism of bacteriocins. As reported earlier, *Lactobacillus sake* Lb706 did not inhibit the growth of *S. aureus* and plantaricin LP84 in *Idli* batter was not effective completely for the inhibition of *S. aureus* (Jama and Varadaraj 1999; Lubas et al. 2012). Therefore, there is urgent need for the investigation of new bacteriocins with potent applications in the safety of various foods.

The addition of bacteriocins to exponentially growing cells of *S. aureus* resulted in rapid decrease in cell population

suggesting the bactericidal efficacy of the bacteriocins in pasteurized milk. The time- and dose-dependent bactericidal nature of bacteriocins against *S. aureus* cells further offer opportunity to optimize the condition of growth inhibition. Fluorescent stain, DAPI and PI have been generally used for the staining of live and dead cells, respectively. Therefore, death of bacteriocin-treated *S. aureus* cells was also confirmed staining with PI. The cells treated with bacteriocin were found to be red after staining with PI indicating dead cells and bactericidal nature of bacteriocins as suggested by Johnson and Criss (2013). This observation indicated that the membrane integrity was destroyed in bacteriocin-treated cells which reflect that the cytoplasmic membrane may be the probable target of action as suggested by Chopra et al. (2015).

The membrane-acting nature of enterocin LD3 was demonstrated using FTIR spectra of treated *S. aureus* cells. The spectrum was found to be altered with higher IR absorbance

in the region $\sim 1451.82\text{ cm}^{-1}$ corresponding to phospholipids (Thangarasu et al. 2018), indicating the membrane-acting nature of enterocin LD3. Further, enterocin LD3-treated cells also showed significant variation in absorbance at $\sim 1094.30\text{ cm}^{-1}$ corresponding to nucleic acids (Depciuch et al. 2017) suggesting that enterocin LD3 may bind with nucleic acids before or after the death of cells. Therefore, it was found that enterocin LD3 possibly interact with membrane and also binds with nucleic acids of target cells. Possibly, there may be ionic interaction between enterocin LD3 and targets (phospholipids and nucleic acids) due to cationic nature of the enterocin LD3 (Gupta et al. 2016). Whereas, plantaricin LD4-treated cells did not show such alteration in the absorbance corresponds to phospholipids and nucleic acids suggesting different mode of action. However, both bacteriocins demonstrated the killing of *S. aureus* cells. These findings have provided a suitable platform to disclose the exact mechanism of action of enterocin LD3 and plantaricin LD4.

The membrane damage is typical characteristic of bacteriocins of lactic acid bacteria (Jiang et al. 2017) and therefore, bactericidal nature of enterocin LD3 and plantaricin LD4 was further supported by morphological visualization of *S. aureus* cells. The untreated *S. aureus* cells showed uniform density in cytoplasm and presented continuous-smooth cell membrane, whereas bacteriocin-treated cells revealed morphological alterations and clear visualization of disruption of cell boundary/cell membrane resulting leakage of intracellular contents as observed under TEM. Similar events have been reported for nisin and plantaricin EF-treated cells also (Pattanayaiying et al. 2014; Zhang et al. 2015). These results are in agreement with decrease in *S. aureus* counts as observed in viability loss and time-kill assays. Thus, enterocin LD3 and plantaricin LD4 caused bactericidal effect on target cells by disrupting cell membrane and cell lysis leading to cell death.

Conclusions

S. aureus showed normal growth in medium and milk. Enterocin LD3 and plantaricin LD4 caused significant loss in cell viability of *S. aureus* in pasteurized milk both time- and concentration-dependent manner. The death of cells was also conformed using differential staining where dead cells stained red with PI. The FTIR analysis suggested that enterocin LD3 may interacts with cell membrane and nucleic acids, whereas plantaricin LD4 did not show such evidence suggesting different mode of action. However, both bacteriocins caused cell disruption and leakage of intracellular contents as observed under transmission electron microscope. The present study suggests enterocin LD3 and plantaricin LD4 causing loss of *S. aureus* cell viability in pasteurized

milk and therefore, may be applied as food-biopreservative against staphylococcal milk contamination.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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