Synergistically-acting Enterocin LD3 and Plantaricin LD4 Against Gram-Positive and Gram-Negative Pathogenic Bacteria

Poonam Sheoran[1] · Santosh Kumar Tiwari[1]

Abstract

The efficacy of antimicrobials is an important aspect during their applications in food and therapeutics. In this study, combination of two bacteriocins, enterocin LD3 and plantaricin LD4, was studied against two pathogenic bacteria, *Staphylococcus aureus* subsp. *aureus* ATCC25923 and *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC13311 for increasing their potency and bactericidal activity. The minimal inhibitory concentrations (MICs) of enterocin LD3 and plantaricin LD4 against *Staph. aureus* subsp. *aureus* ATCC25923 were 180 and 220 µg/mL, whereas in combination, reduced to 115 µg/mL, respectively. The MICs of enterocin LD3 and plantaricin LD4 against *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 were 240 and 320 µg/mL, respectively, whereas in combination, these were found to be 130 µg/mL, respectively. The fractional inhibitory concentration (FIC) indices calculated as 0.50 against *Staph. aureus* subsp. *aureus* ATCC25923 and 0.43 against *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 were found to be ≤ 0.5 indicating the synergy. The isobologram showed MIC of combined bacteriocins falls below the plotted straight line further signifies synergy. The growth response of *Staph. aureus* subsp. *aureus* ATCC25923 and *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 was significantly reduced in the presence of combined bacteriocins in comparison with their individual effects. The number of dead cells was higher as a result of combined effect as compared with their independent effect evidenced by fluorescent microscopy. Transmission electron microscopy (TEM) revealed the higher disruption of cell membrane in the combined bacteriocin-treated cells as compared with alone effects. The FTIR spectra of enterocin LD3-treated cells showed alteration at ~ 1,451.82 and ~ 1,094.30/cm corresponding to nucleic acids and phospholipids suggesting its interaction with cell membrane and nucleic acids. In contrast, plantaricin LD4-treated cells did not show such alterations suggesting plantaricin LD4 may kill target cells using other mechanism. Our data suggest that different mode of action of both bacteriocins results in division of labour and may be responsible for their synergistic activity against target cells. Similarly, the synergistic effect of bacteriocins was also observed against other pathogenic bacteria such as *Proteus mirabilis* ATCC43071, *Pseudomonas aeruginosa* ATCC27853 and *Escherichia coli* ATCC25922. These bacteriocins, therefore, act synergistically against target pathogens and may be applied in appropriate combinations for food safety and medical applications.

Keywords Enterocin LD3 · Plantaricin LD4 · Synergistic effect · *Staphylococcus aureus* · *Salmonella* Typhimurium · Fluorescence microscopy · Transmission electron microscope

Introduction

Microbial infections are major concern for the public health. In present situation of COVID-19 pandemic, the chances of secondary infections remain higher and become complicated during treatment procedures [1]. The most common pathogens, *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* Typhimurium, etc., are hard to be eliminated from food and human environments. *Staph. aureus* is the major human pathogen causing a wide range of infections such as bacteremia and infective endocarditis. The treatment remains challenging due to the emergence of multidrug resistant strains such as MRSA (methicillin-resistant *Staph. aureus*) [2]. *Salm.* Typhimurium is the most dominant serovar around the world associated with food-borne outbreaks in both developing and high-income countries [3]. Human infection with *Salm.* Typhimurium normally occurs through consumption of undercooked meat, dairy

✉ Santosh Kumar Tiwari
santoshgenetics@gmail.com

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

products and especially raw eggs. Outbreaks by *Salm.* Enteritidis and *Salm.* Heidelberg have been mainly associated with consumption of raw eggs, whereas outbreak caused by *Salm.* Newport has been linked to uncooked ground beef, runny scrambled eggs or omelettes [4]. Salmonellosis is one of the most common food-borne diseases worldwide, accounting around 93.8 million food-borne illnesses and 155,000 deaths per year worldwide [5]. Therefore, it is essential to use effective and safe antimicrobials for the control of such pathogens.

The use of chemical preservatives has been recognized as effective in limiting food-borne pathogens but causes severe toxicity to the consumers. Similarly, overdose of antibiotics during the treatment of infectious diseases has resulted development of multidrug resistance in pathogens [2, 6]. Since the past, several compounds have been searched in food safety and clinical applications, but nature-derived products have always been the preference. Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria exhibiting narrow spectra of activity against related species, whereas others display broader activity spectra against unrelated species [7]. In particular, most bacteriocins from lactic acid bacteria (LAB) have been attractive as potential natural antimicrobials due to the non-toxic nature of the compounds [8]. Although, bacteriocins are mostly non-toxic and considered safe, exceptions do exist; e.g. cytolysin produced by several enterococci has known cytotoxic activity [9]. In most cases, cytotoxicity has only been noted at levels much higher than the MIC required to inhibit food spoiling microorganisms [10, 11]. Generally, bacteriocins demonstrate strong activity against sensitive strains in the nanomolar range rendering them more effective antimicrobials. However, perhaps an even better option is to combine bacteriocins along with other existing antimicrobials for effective killing of the target strains [11]. It is plausible that the use of synergistically acting bacteriocins with other antimicrobials may accelerate each other's effects, thereby dropping the likelihood of resistance development by target strains [12]. As reported earlier, the combined effect of nisin and pediocin displayed a synergistic activity against *Lactobacillus sakei* and also resulted in additive effect against *Bacillus cereus* and *L. monocytogenes.* These combined bacteriocins may represent potential candidates applied in food systems for controlling pathogenic or food spoilage bacteria [13]. Furthermore, the combined use of these compounds may also reduce the concentration and thus decrease financial burden related with the synthesis and management of expensive antibiotics [14].

Keeping these views in consideration, enterocin LD3 and plantaricin LD4 have been studied to evaluate their individual and combined effects against pathogenic bacteria. These bacteriocins are produced from *Lactobacillus plantarum* LD4 and *Enterococcus hirae* LD3 isolated from indigenous fermented food, *Dosa*, which is most commonly consumed in the

southern part of India. Strain LD3 and LD4 have been previously characterized for their probiotic efficacy in vitro and found to lower cholesterol, tolerance to low pH, higher hydrophobicity and antimicrobial activity against food-borne and clinical pathogens [6, 15]. Later, enterocin LD3 purified from cell-free supernatant (CFS) of *Ent. hirae* LD3 showed novel characteristics such as unique mass (4114.62 Da), N-terminal sequence (H2NQGGQANQ–COOH), pH and heat stability with broad host-range activity. In laboratory conditions, enterocin LD3 demonstrated antimicrobial activity against different pathogens and related LAB [16]. Further, mode of action of enterocin LD3 was found to be bactericidal; involving dissipation of membrane potential and efflux of ATP, ions, proteins and nucleic acids was recorded [16, 17]. Plantaricin LD4 was found to be approx. 6 kDa in size, stable up to 121 °C and below pH 7.0. It was also able to inhibit several pathogens, haloarchaea and related strains as described previously [6]. Recently, we have also demonstrated antistaphylococal activity of enterocin LD3 and plantaricin LD4 in pasteurized milk indicating their efficacy in food safety [18]. The aim of the present study is to explore the synergistic effect of these bacteriocins against selected food-borne pathogens, *Staph. aureus* subsp. *aureus* ATCC25923 and *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 for their applications in food safety and/or as clinical antimicrobials.

Materials and Methods

Bacterial Strains and Growth Conditions

Ent. hirae LD3 and *L. plantarum* LD4 were grown in MRS medium at 37 °C for 18 h. *Staph. aureus* subsp. *aureus* ATCC25923 and *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 were obtained from Pandit Bhagwat Dayal Sharma University of Health Sciences, Rohtak, Haryana, India, and grown in nutrient broth (NB) at 37 °C overnight in a BOD incubator (Scigenics Biotech, Chennai, India). All the media components were purchased from Hi-Media, Mumbai, India.

Determination of Minimum Inhibitory Concentrations

Enterocin LD3 and plantaricin LD4 were purified using activity-guided multistep chromatographic techniques such as cation exchange chromatography, gel-filtration chromatography and reverse-phase ultra-performance liquid chromatography as reported previously [16]. The minimum inhibitory concentrations (MICs) of purified bacteriocins against *Staph. aureus* subsp. *aureus* ATCC25923 and *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 were determined using the broth microdilution method as suggested by Weeks

et al. [19]. Briefly, different concentrations (0–340 μg/mL) of enterocin LD3 and plantaricin LD4 were added individually into the wells of 96-well polypropylene plates containing 100 μL cell suspension of *Staph. aureus* subsp. *aureus* ATCC25923 and *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 in NB medium (OD600 0.02) and incubated at 37 °C for 24 h as mentioned in CLSI manual [20]. The MIC was determined as the lowest concentration of bacteriocin showing no visible bacterial growth (OD600 < 0.05) as suggested previously [17].

Estimation of the Synergistic Effect of Bacteriocins

The interaction between enterocin LD3 and plantaricin LD4 against *Staph. aureus* subsp. *aureus* ATCC25923 and *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 was performed using a 'checkerboard' assay as reported by Acosta et al. [21]. Different concentrations of enterocin LD3 (5, 10, 15, 20, 25, 30, 35 and 40 μg/mL) and plantaricin LD4 (50, 60, 70, 80, 90, 100, 110 and 120 μg/mL) were added into the wells of microplate in vertical and horizontal orientation, respectively, so that the wells would contain mixed concentrations of the two bacteriocins in different proportions. Wells with cells alone without bacteriocin were used as controls. Wells containing bacteriocin alone were used as an additional control (as a blank). Each well was inoculated with 100 μL (OD600 0.02) NB medium containing *Staph. aureus* subsp. *aureus* ATCC25923 and *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 individually and incubated at 37 °C for 24 h in a microplate reader (Molecular devices, Sunnyvale, USA). An automated 30-min shake was performed to assure even distribution of bacteriocins and cells in each well. The net absorbance was determined by subtracting the initial OD600 from the final OD600. The fractional inhibitory concentration (FIC) was calculated by dividing the MIC of the bacteriocin in the mixture by the MIC of respective bacteriocin independently. The FIC index (FICI) was calculated as FICindex = FICenterocin LD3 + FICplantaricin LD4. According to European Committee for Antimicrobial Susceptibility Testing (EUCAST) [22], a synergistic effect is observed when FICI value ≤ 0.5, an additive effect when 0.5 < FICI value ≤ 1, an indifferent effect (IndE) when 1 < FICI value < 2 and an antagonistic effect (AntE) when FICI value ≥ 2.

The results of the checkerboard assay were represented graphically by plotting the meeting points formed by pairs of concentrations of enterocin LD3 and plantaricin LD4 on a graph known as an isobologram. MIC values of the bacteriocins used alone were plotted on the x- and y-axes and joined by a line. Then, effective combined concentrations (MIC) of bacteriocins were plotted and compared with the previous line. The isobologram was interpreted examining the position of the ratio points and extrapolating synergy (below the line),

antagonism (above the line) and additive effect (on the line) as reported by Amrouche et al. [23].

Growth Inhibition Assays

The interactive effect of bacteriocins was also studied in terms of time- and concentration-bound growth response of target cells. *Staph. aureus* subsp. *aureus* ATCC25923 was treated with enterocin LD3 (25 μg/mL) and plantaricin LD4 (90 μg/mL) individually and in combination (115 μg/mL). Similarly, *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 was treated with enterocin LD3 (30 μg/mL) and plantaricin LD4 (100 μg/mL) individually and in combination (130 μg/mL). These sets were grown in NB medium (100 μL) with initial OD600 0.02 and incubated for 24 h at 37 °C with intermittent mixing. The absorbance was monitored at regular intervals of 2 h up to 24 h using a microplate reader (Molecular devices, Sunnyvale, USA).

Estimation of Live/Dead Cells

The concentration-based killing of *Staph. aureus* subsp. *aureus* ATCC25923 and *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 cells was further confirmed by staining with a mixture of 4′, 6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) (Merck, Darmstadt, Germany, USA) as reported by Lee et al. [24]. For viable staining, 10 μL of a 1 mg/mL stock solution of each PI and DAPI was added to 1 mL of target cells treated with bacteriocin independently and in combination for 24 h. Here, to observe the independent effect of bacteriocins on *Staph. aureus* subsp. *aureus* ATCC25923, 25 μg/mL of enterocin LD3 and 90 μg/mL of plantaricin LD4 were used, and for combined effect, a total of 115 μg/mL were added. On the other hand, enterocin LD3 as 30 μg/mL and plantaricin LD4 as 100 μg/mL were used to observe the individual effect on *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311, whereas for obtaining the synergistic effect, a mixture of bacteriocins (130 μg/mL) was used. As positive control, cells grown for 24 h (~ 10^6 CFU/mL) in normal saline (0.8% NaCl) were used. The treated and untreated cell suspensions were incubated with the mixture of PI and DAPI for 10 min. The staining was carried out at room temperature before the live/dead cells were analysed at excitation 330–380 nm using a fluorescent microscope (DS-Fi2, Nikon Eclipse, Japan) with × 40 magnification.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy is capable of monitoring conformational, compositional and quantitative differences of biochemical compounds in microbial cells. Therefore, this technique was used to evaluate the cellular response of bacteriocin-treated

and untreated cells as reported by Zoumpopoulou et al. [25]. Briefly ~10^6 CFU/mL cells of *Staph. aureus* subsp. *aureus* ATCC25923 were treated with enterocin LD3 (25 μg/mL) and plantaricin LD4 (90 μg/mL) individually and in combination (115 μg/mL). Similarly, *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 was treated with enterocin LD3 (30 μg/mL) and plantaricin LD4 (100 μg/mL) individually and in combination (130 μg/mL) and incubated at 37 °C, 200 rpm for 10 h. The untreated cells were used as controls. After incubation for 10 h, cell suspensions were washed twice with sterile saline (0.85% NaCl). Infrared absorbance spectra of bacteriocin-treated and untreated target cells were monitored using an FTIR spectrophotometer (Bruker, Bremen, Germany) on diamond-attenuated total reflectance accessory. For spectra acquisition, Opus software was used. The cells were placed in direct contact with the internal reflecting diamond crystal. Multiple scans were obtained to reduce error. Each spectrum was baseline corrected, and the spectral range was set from 500/cm to 4000/cm at a resolution of 8/cm.

Transmission Electron Microscopy (TEM)

TEM analysis was performed to explore alterations in the morphology of target cells treated individually and with a mixture of bacteriocins. The cells of *Staph. aureus* subsp. *aureus* ATCC25923 and *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 were harvested from mid-log phase (~10^6 CFU/mL) by centrifugation at 7000 *g*, for 10 min at 4 °C, and used for TEM analysis as reported by Zhang et al. [26]. The cells were washed individually twice with normal saline and individually treated with bacteriocins alone and in combination for 24 h. For analysis of bacteriocins acting independently, *Staph. aureus* subsp. *aureus* ATCC25923 cells were treated with enterocin LD3 (25 μg/mL) and plantaricin LD4 (90 μg/mL), whereas *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 cells were treated with enterocin LD3 (30 μg/mL) and plantaricin LD4 (100 μg/mL) for independent effect of bacteriocins. On the other hand, to evaluate the synergistic effect, 115 and 130 μg/mL mixed concentrations of bacteriocins were used for *Staph. aureus* subsp. *aureus* ATCC25923 and *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311, respectively. Untreated cells were resuspended in normal saline and used as controls. After treatment, cells were processed to visualize under transmission electron microscope as reported previously [17].

Antibacterial Spectrum

To observe the host-range of bacteriocins alone and in combination against other pathogenic bacteria such as *Proteus mirabilis* ATCC43071, *Pseudomonas aeruginosa* ATCC27853, *E. coli* ATCC25922, *Staph. aureus* subsp. *aureus* ATCC25923 and *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311, *Serratia marcescens* ATCC27137 and indicator strain, *Micrococcus luteus* MTCC106, agar well diffusion assay (AWDA) was performed overlaying soft nutrient agar (0.8%) seeded with respective pathogenic strain (~10^6 CFU/mL) on the nutrient base agar as reported previously [27]. On such plates, the wells were cut out (6.0 mm diameter) and filled with aliquots of 100 μL of enterocin LD3 (50 μg/mL) and plantaricin LD4 (50 μg/mL) independent and in combination (25 μg/mL each, total 50 μg/mL) in the wells. The plate was incubated for 18 h at 37 °C. The zone of growth inhibition (mm) was measured.

Statistical Analysis

The data presented are means ± standard deviation (SD) of three independent experiments. The level of statistical significance was estimated as $p < 0.05$ using Student's *t* test. Florescence microscopy and TEM analysis were performed three times for reproducibility of the results.

Results

Synergistic Effect of Enterocin LD3 and Plantaricin LD4

The growth of target strains was drastically reduced as the concentration of bacteriocin was increased. The growth of *Staph. aureus* subsp. *aureus* ATCC25923 was completely inhibited in the presence of 180 and 220 μg/mL enterocin LD3 and plantaricin LD4, respectively. Therefore, MICs of these bacteriocins were considered 180 and 220 μg/mL against *Staph. aureus* subsp. *aureus* ATCC25923 (Fig. 1a). Similarly, the MIC of enterocin LD3 and plantaricin LD4 were found to be 240 and 320 μg/mL, respectively, against *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 (Fig. 1b). The corresponding values of MICs of enterocin LD3 and plantaricin LD4 in combination was observed as 25 and 90 μg/mL (115 μg/mL), respectively, against *Staph. aureus* subsp. *aureus* ATCC25923 (Fig. 2a) whereas 30 and 100 μg/mL (130 μg/mL) against *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 (Fig. 2b). Thus, MIC of enterocin LD3 in combination was reduced to approx. 1/8th against *Staph. aureus* subsp. *aureus* ATCC25923 and *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311. Similarly, the MIC of plantaricin LD4 in combination was reduced to approx. 1/3rd against both target strains. Therefore, enterocin LD3 and plantaricin LD4 showed lower MICs in combination as compared with their individual effect against *Staph. aureus* subsp. *aureus* ATCC25923 and *Salm. enterica* subsp. *enterica* serovar
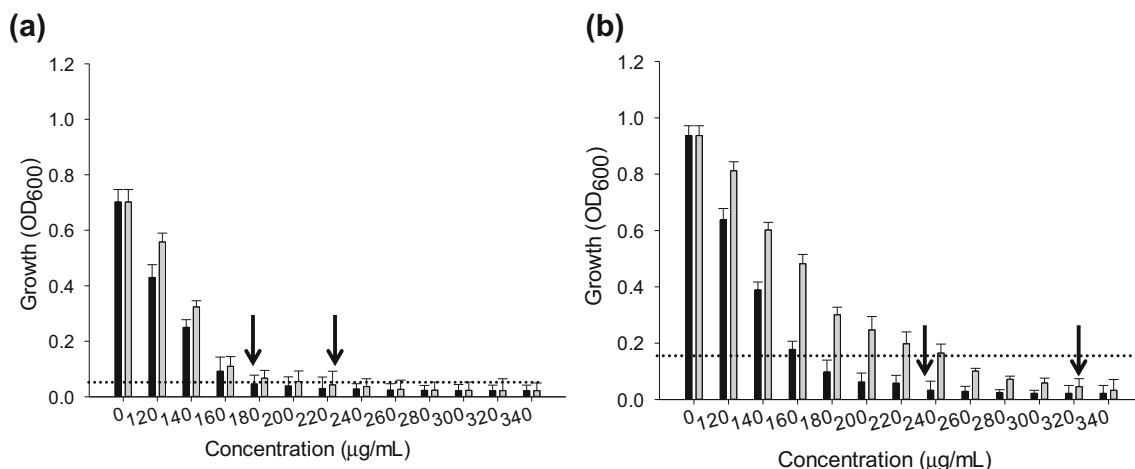
(a)

(b)

Fig. 1 Effect of different concentrations of enterocin LD3 (black bar) and plantaricin LD4 (grey bar) on *Staphylococcus aureus* subsp. *aureus* ATCC25923 (**a**) and *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC13311 (**b**). Enterocin LD3 showed MIC 180 and 220 µg/mL against *Staph. aureus* subsp. *aureus* ATCC25923 and *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 as compared with plantaricin LD4 240 and 320 µg/mL, respectively

Typhimurium ATCC13311. The FICs of enterocin LD3 and plantaricin LD4 against *Staph. aureus* subsp. *aureus* ATCC25923 were 0.10 and 0.40, whereas corresponding FICs were 0.12 and 0.31 against *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311, respectively. Therefore, the FIC index was calculated as 0.50 against *Staph. aureus* subsp. *aureus* ATCC25923 and 0.43 against *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311. Since the FIC index of both bacteriocins is ≤ 0.5, the synergistic interaction of enterocin LD3 and plantaricin LD4 was apparent. The isobologram provided a graphical representation of the nature of bacteriocin interaction. It was constructed showing that the combination of two bacteriocins inhibiting target strains falls below the plotted straight line, of which the end point reflects the independent MICs of the bacteriocins (Fig. 3a, b). This further signifies synergy between enterocin LD3 and plantaricin LD4 against *Staph. aureus* subsp. *aureus* ATCC25923 and *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311. Therefore, enterocin LD3 and plantaricin LD4 showed synergistic effect against the target bacteria tested.

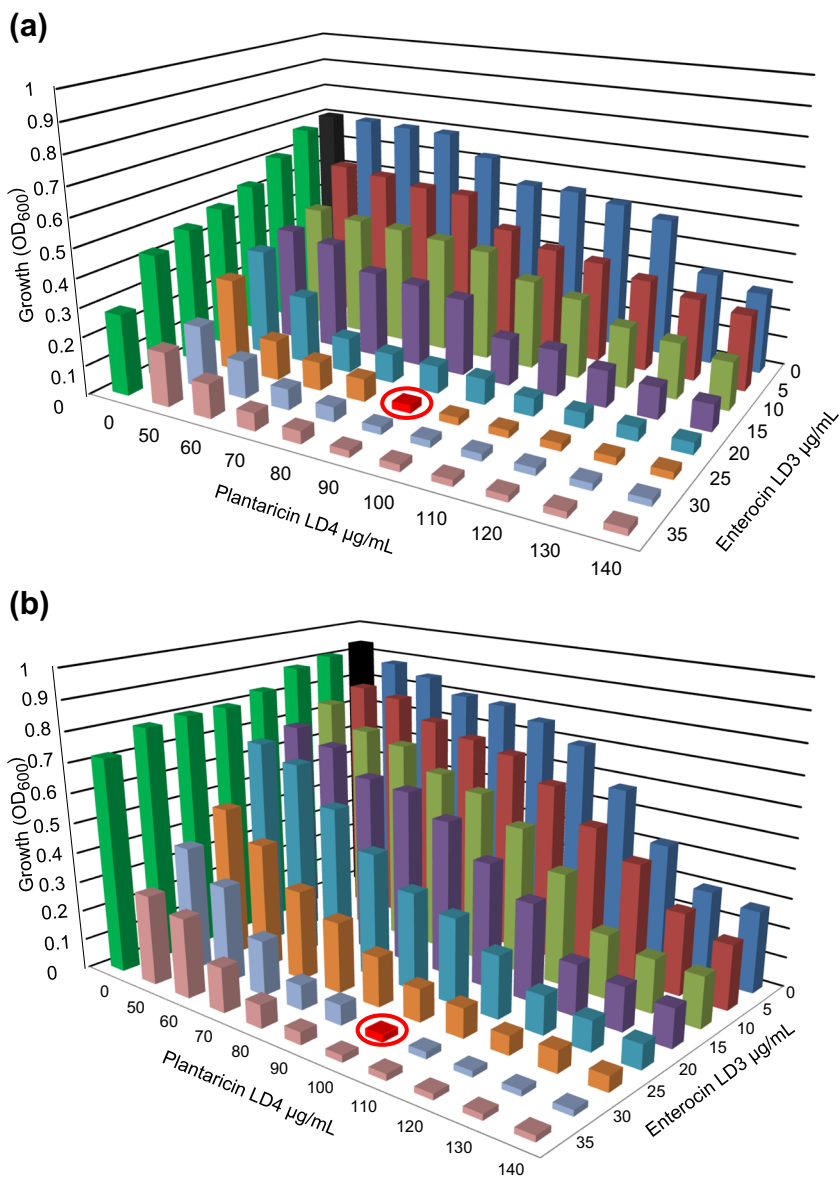## Combined Bacteriocins Caused Higher Growth Inhibition

It was found that untreated *Staph. aureus* subsp. *aureus* ATCC25923 and *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 cells followed normal growth pattern and grew up to OD600 0.702 and 0.937, respectively. When *Staph. aureus* subsp. *aureus* ATCC25923 cells were treated with enterocin LD3 (25 µg/mL) and plantaricin LD4 (90 µg/mL) individually, growth was partially inhibited and found to be OD600 0.47 and 0.54, respectively, at 24 h,

whereas combined effect of bacteriocins (115 µg/mL) on *Staph. aureus* subsp. *aureus* ATCC25923 showed complete inhibition (OD600 0.028) as compared with untreated cells. Similarly, *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 showed growth up to OD600 0.79 and 0.70 in the presence of individual enterocin LD3 (30 µg/mL) and plantaricin LD4 (100 µg/mL), but the growth (OD600 0.023) was completely inhibited in the presence of combination (130 µg/mL) of bacteriocins (data not shown). Therefore, significant growth inhibition of target strains was observed when bacteriocins were used in combination than alone.

Combined Bacteriocins Caused Higher Lethality

The cell membrane permeability was also determined by fluorescent microscopy using DAPI and PI which could distinguish intact cells with ruptured membranes. The untreated cells (live) were stained blue (Fig. 4a, e) with DAPI and dead cells stained red (Fig. 4d, h) with PI. After treatment of cells with enterocin LD3 (Fig. 4b, f) or plantaricin LD4 (Fig. 4c, g) individually, light pink fluorescence was observed in *Staph. aureus* subsp. *aureus* ATCC25923 and *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 cells, indicating that the cell membrane of the treated cells was damaged. When cells were treated with combined bacteriocins, dead cells were found to be red indicating higher effect of bacteriocins (Fig. 4d, h). However, there is possible explanation for the reduction in DAPI fluorescence, and increase in PI fluorescence of target cells indicating the combined bacteriocin effect was more potent than the effects independently.

Fig. 2 Checkerboard assay layout allows for simultaneous testing of several mixed concentrations of enterocin LD3 and plantaricin LD4 against *Staphylococcus aureus* subsp. *aureus* ATCC25923 (**a**) and *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC13311 (**b**). The upper row and the left column are used to make bacteriocin solution at increasing concentrations, then the top row (plantaricin LD4) is spread downward, and the first left column (enterocin LD3) is spread across the right side of the 96-well plate. The result shows an increasing order of inhibition from no inhibition to delayed growth through partial inhibition and finally complete inhibition. The first well of upper row served as control without bacteriocin. The red circle represents MIC in combination

Enterocin LD3 Interacts with Cell Membrane and Nucleic Acids, Whereas Plantaricin LD4 Does Not

To observe the effect of bacteriocins independently and in combination on the cell membranes and nucleic acids of *Staph. aureus* subsp. *aureus* ATCC25923 and *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 cells, FTIR analysis was performed. The *Staph. aureus* subsp. *aureus* ATCC25923 cells after treatment with enterocin LD3 alone showed increased absorbance at ∼ 1094.30 and ∼ 1451.82/cm corresponding to nucleic acids and phospholipids, respectively (Fig. 5a), whereas plantaricin LD4-treated *Staph. aureus* subsp. *aureus* ATCC25923 cells did not show such increase in the wavelength corresponding to nucleic acids and phospholipids (Fig. 5b). When *Staph. aureus* subsp. *aureus* ATCC25923 cells were treated with a mixture of enterocin LD3 and plantaricin LD4, higher absorbance at ∼ 1094.30 and ∼ 1451.82/cm was observed as compared with their effects alone (Fig. 5c). Similarly, *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 cells after treatment with enterocin LD3 independently showed increase in the absorbance at ∼ 1094.30 and ∼ 1451.82/cm corresponding to nucleic acids and phospholipids, respectively (Fig. 6a). The plantaricin LD4-treated *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 cells did not show such alteration in the wavelength corresponding to nucleic acids and phospholipids (Fig. 6b). The *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311cells treated with a mixture of enterocin LD3 and plantaricin LD4 showed higher absorbance at ∼ 1094.30 and ∼ 1451.82/cm as compared with the effects of the bacteriocins independently (Fig. 6c) indicating synergy between two
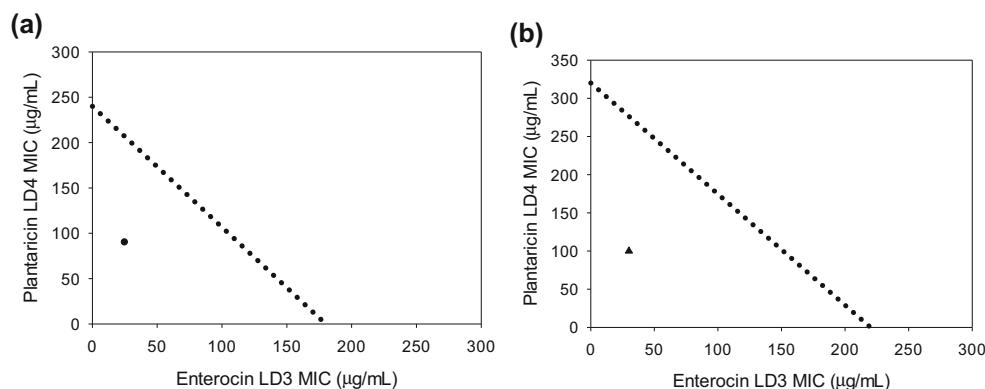
(a)

(b)

Fig. 3 Isobologram showing synergistic interaction (indicated by closed filled circle) between enterocin LD3 (25 μg/mL) and plantaricin LD4 (90 μg/mL) against *Staphylococcus aureus* subsp. *aureus* ATCC25923 (**a**). For the *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC13311, synergistic concentrations (indicated by closed filled upright triangle) of enterocin LD3 and plantaricin LD4 were found to be 30 and 100 μg/mL, respectively (**b**). MIC values of the bacteriocins used alone were plotted on the x- and y-axes and joined by a dotted straight line. It was constructed showing that the combination of two bacteriocins inhibiting target strains falls below the plotted straight line indicating synergy

bacteriocins. Enterocin LD3 and plantaricin LD4 also showed higher absorbance in the range of 2800 and 3000/cm against both cells exhibiting typical C–H stretching and vibrations corresponding to the CH3- and CH2- functional groups.

The Morphological Damage of Cell Was Higher in Combined Treatment

The TEM analysis was used to visualize the morphology and intracellular images of target cells. Untreated *Staph. aureus* subsp. *aureus* ATCC25923 cells displayed a highly homogenous intracellular density and round shape (Fig. 7a), but enterocin LD3-treated cells showed disruption of the cell membrane and release of intracellular contents (Fig. 7b). *Staph. aureus* subsp. *aureus* ATCC25923 cells treated with plantaricin LD4 also exhibited cellular damages (Fig. 7c), while enterocin LD3 and plantaricin LD4 combined treatment caused higher damage to most cells, showing sunken cell surface and shrinking cytoplasm (Fig. 7d). Irregularity of the cell surface and the increase of the cell mass were undoubtedly
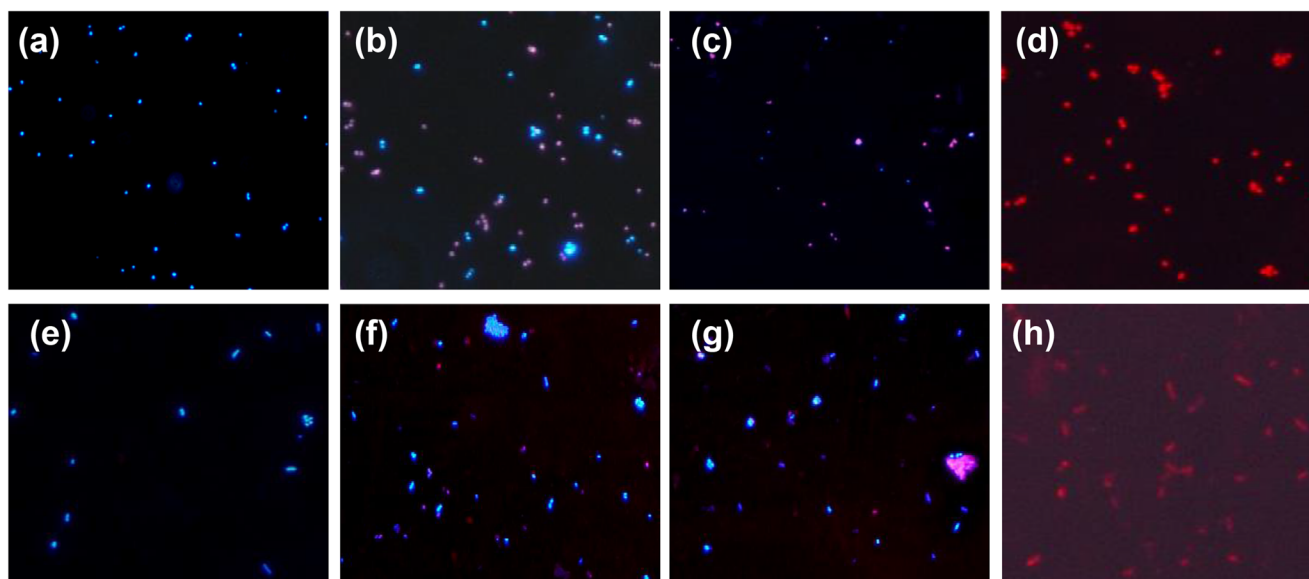

Fig. 4 Untreated *Staphylococcus aureus* subsp. *aureus* ATCC25923 (**a**) and *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC13311 (**e**) cells showed blue colour indicating live cells. *Staph. aureus* subsp. aureus ATCC25923 cells treated with enterocin LD3 (25 μg/mL) independent (**b**) and plantaricin LD4 (90 μg/mL) independent (**f**); and *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 cells treated with enterocin LD3 (30 μg/ mL) independent (**c**) and plantaricin LD4 (100 μg/mL) independent (**g**) showed the mixture of blue and pink cells indicating partially killed cells, whereas combined effects of bacteriocins against *Staph. aureus* subsp. *aureus* ATCC25923 (115 μg/mL) (**d**) and *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 (130 μg/mL) (**h**) showed red colour indicating completely dead cells
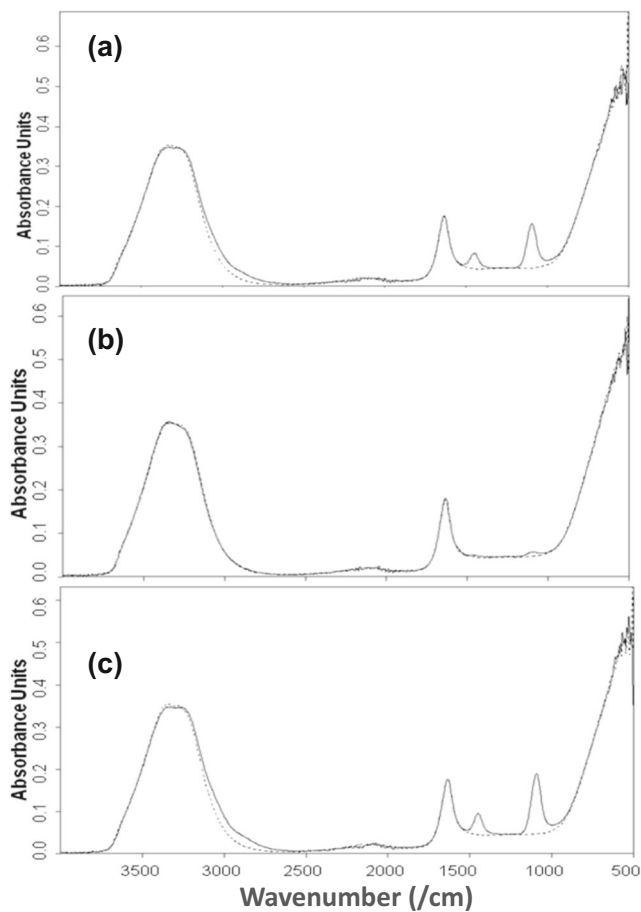
Fig. 5 The Fourier transforms infrared spectra of bacteriocin-treated (continuous grey line) and untreated cells (dotted line) of *Staphylococcus aureus* subsp. *aureus* ATCC25923. The cells treated with enterocin LD3 independent (**a**) showed higher absorbance corresponding to phospholipids (~ 1451.82/cm) and nucleic acids (1094.30/cm) shown as peaks, whereas cells treated with plantaricin LD4 independent (**b**) did not show such increased absorbance. When both bacteriocins have been used together (**c**), the absorbance was higher as compared with their independent effects. This indicated that enterocin LD3 directly interact with cell membrane and nucleic acids of the target cells, whereas plantaricin LD4 interacts with neither cell membrane nor nucleic acids and may use other mechanisms for cell killing. Therefore, their combined use causes synergy in cell killing due to different mode of action against target strain

Fig. 6 The Fourier transforms infrared spectra of bacteriocin-treated (continuous grey line) and untreated cells (dotted line) of *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC13311. The cells treated with enterocin LD3 independent (**a**) showed higher absorbance corresponding to phospholipids (~ 1451.82/cm) and nucleic acids (1094.30/cm) shown as peaks, whereas cells treated with plantaricin LD4 independent (**b**) did not show such increased absorbance. When both bacteriocins have been used together (**c**), the absorbance was higher as compared with their independent effects. This indicated that enterocin LD3 directly interacts with cell membrane and nucleic acids of the target cells, whereas plantaricin LD4 interacts with neither cell membrane nor nucleic acids and may use other mechanism for cell killing. Therefore, their combined use causes synergy in cell killing due to different mode of action against target strain

evident which indicated the perforation of cell membrane with consequent cell deformation after combined bacteriocin treatment. The untreated *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311cells maintained typical rods and intact status (Fig. 7e). When held in contact with enterocin LD3, cells showed an alternation in morphology and ruptured cells were observed (Fig. 7f). Similarly, plantaricin LD4 induced a dramatic change in the *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311cells showing various alterations (Fig. 7g). Therefore, enterocin LD3 and plantaricin LD4 showed cellular damages of both Gram-positive (*Staph. aureus* subsp. *aureus* ATCC25923) and Gram-negative (*Salm. enterica* subsp. *enterica* serovar Typhimurium
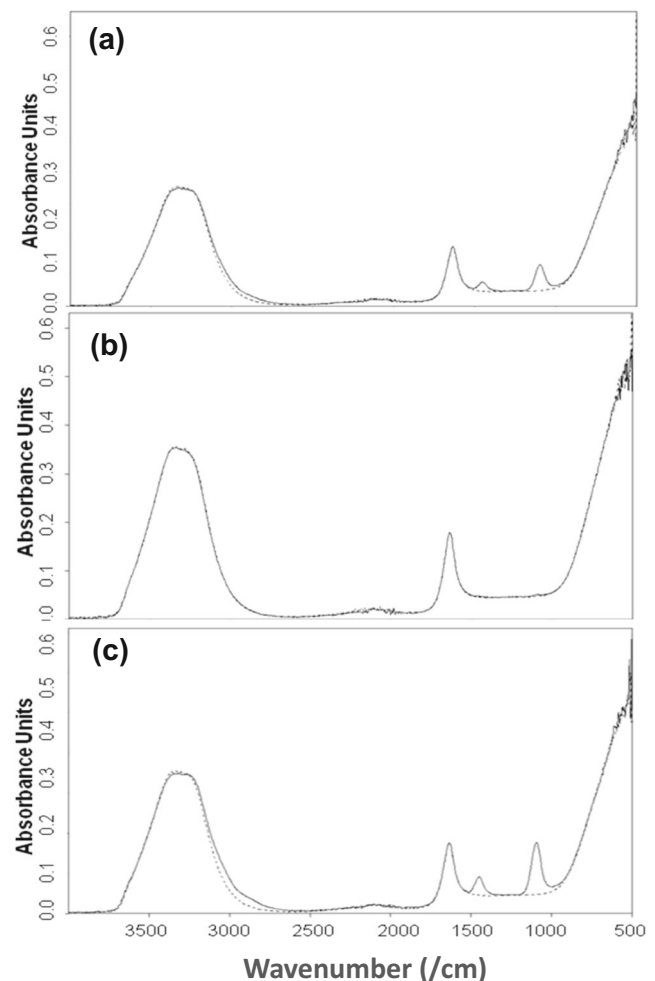
ATCC13311) bacteria. In the presence of combined concentrations of these bacteriocins, the outer membrane of *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 cells was highly damaged, cell membrane was found to be protruding into the cytoplasm, and several cells displayed ruptures and loss of cytoplasm, indicating cytoplasmic membrane was severely affected when both bacteriocins were present together (Fig. 7h). Thus, combined effects of enterocin LD3 and plantaricin LD4 caused higher cell damage and bactericidal effect as compared with their activity alone.
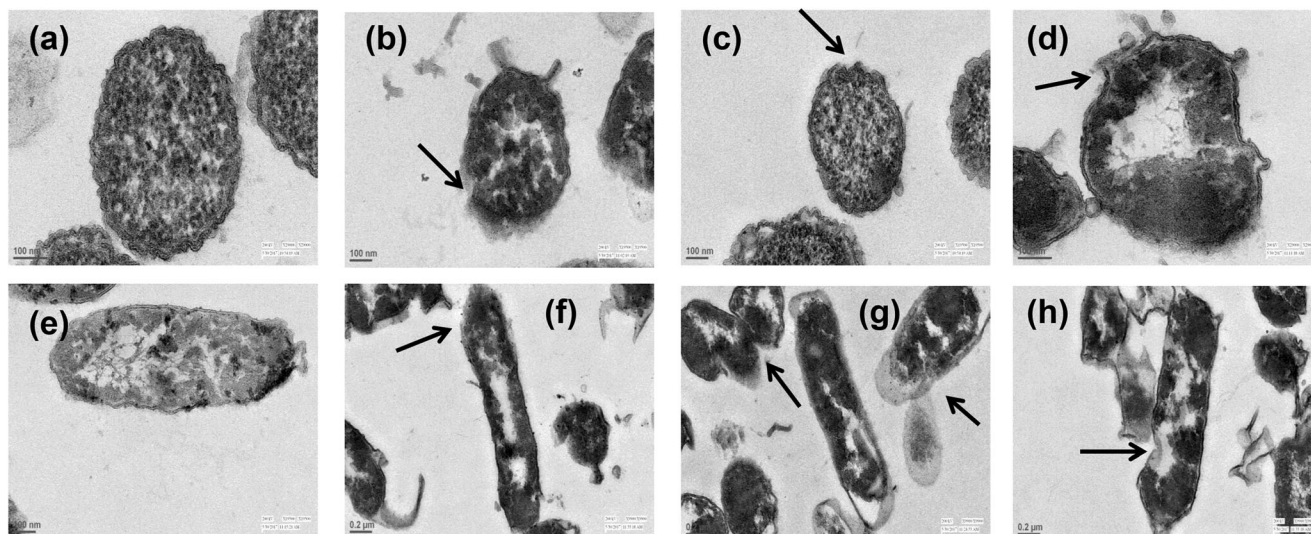
different producers, enterocin LD3 and plantaricin LD4, were studied to evaluate their individual and combined effects.

In this study, we have shown the efficacy of these bacteriocins in combination against two important food-borne pathogens, *Staph. aureus* subsp. *aureus* ATCC25923 and *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311. The MICs of enterocin LD3 and plantaricin LD4 (180 and 220 μg/mL against *Staph. aureus* subsp. *aureus* ATCC25923 and 240 and 320 μg/mL against *Salm. aureus* subsp. *aureus* ATCC25923 and *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311), when compared with other bacteriocins, were found to be comparatively lower than enterocin DD93 (200 μg/mL) against *Staph. aureus* and plantaricin MG (500 μg/mL) against *Salm.* Typhimurium [20, 29]. Furthermore, the MIC of enterocin LD3 and plantaricin LD4 in combination was significantly reduced. The reduced combined MICs of enterocin LD3 and plantaricin LD4 (130 μg/mL, respectively) against *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311are in the range of CLSI susceptible breakpoints (≤256 μg/mL for sulphamethoxazole against *Salmonella* and *E. coli*). The breakpoint for ceftaroline against *Staph. aureus* is ≤1 μg mL [19, 20] which is much lower than the combined MICs of enterocin LD3 and plantaricin LD4 (115 μg/mL) against *Staph. aureus* subsp. *aureus* ATCC25923. These observations have indicated that there is further scope to reduce the MICs of these bacteriocins using genetic modifications, increasing purity level and testing synergy with existing antimicrobials. Though the breakpoints are different for different microorganisms and different antibiotics, these are universally adopted susceptibility concentrations useful in clinical settings. Other studies have reported that nisin and pediocin in combination showed synergistic activity against *L. sakei* and caused an additive effect against *B. cereus* and *L. monocytogenes*. Similarly, the mixture of nisin and enterocin MT104B displayed a synergistic activity against *Staph. aureus* [13]. Ferreira et al. [29] showed that a combination of pediocin 34, nisin and enterocin F99 was highly effective with lowered concentrations against target strains as compared with individual effects. However, there are no such reports on use of combined enterocin and plantaricin against food-borne or clinical pathogens.

According to EUCAST [19], a synergistic effect is observed when FICI value ≤0.5. The FIC indices calculated as 0.50 against *Staph. aureus* subsp. *aureus* ATCC25923 and 0.43 against *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311were found to be ≤0.5 indicating the synergy between two enterocin LD3 and plantaricin LD4. The FIC index defines the nature of interaction of two antimicrobials and indicates full synergy if ≤0.5 [20, 21]. The isobologram further signifies synergy between two bacteriocins against *Staph. aureus* subsp. *aureus* ATCC25923 and *Salm. enterica* subsp. *enterica* serovar Typhimurium

ATCC13311 where combined MICs fall below the strait line. The isobologram interpretation examining the position of the ratio points suggests synergy if it is below the line [23]. These findings here indicated that synergistic interaction between the two bacteriocins could be due to their different mode of action against target bacteria. One of them could be involved in pore formation/disruption of the outer cell membrane allowing access of the other bacteriocin in target cells as also suggested by Chi and Holo [8]. The mechanism of action of plantaricin LD4 is not exactly known, but our recent findings suggest that enterocin LD3 causes membrane disruption and enters inside the cells interacting with nucleic acids [17].

The effect of bacteriocins was also observed on the growth response of *Staph. aureus* subsp. *aureus* ATCC25923 and *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311. The untreated cells of *Staph. aureus* subsp. *aureus* ATCC25923 and *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 followed a normal growth pattern. When the cells were treated with individual bacteriocin, there was partial inhibition of growth recorded, whereas complete growth inhibition was found when both bacteriocins were applied in combination. Therefore, significant growth inhibition of target strains was observed when bacteriocins were used in combination than alone. Therefore, synergistic property of antimicrobials may improve kill kinetics. Further the synergistic interaction of antimicrobials may also reduce the development of resistance in pathogenic bacterial strains as suggested by Al Atya et al. [30]. In earlier reports, the combination of lactocin 705, enterocin CRL35 and nisin was found to be synergistically active against *L. monocytogenes* FBUNT where viability loss occurred after incubation for 3 h [31]. However, to the best of our knowledge, there are no studies where enterocin and plantaricin reported to show synergistic interactions against Gram-negative bacteria. In the present study, we are reporting not only inhibition of Gram-positive and Gram-negative bacteria by enterocin LD3 and plantaricin LD4 but also their synergy against Gram-negative pathogens. The cell killing was further confirmed by the use of membrane permeable stain, PI, which is a nucleic acid staining dye, enters in to cells with compromised membranes and binds to DNA, giving a red fluorescence. Thus, bacterial cells with intact membranes exclude PI while being stained by DAPI emit blue fluorescence, whereas bacterial cells with damaged membranes are stained with PI and emit a red fluorescence [32, 33]. After treatment with enterocin LD3 or plantaricin LD4, light pink fluorescence was observed in *Staph. aureus* subsp. *aureus* ATCC25923 and *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 cells, indicating that the cell membrane of the treated cells was damaged. In combination treatment, dead cells were found to be red suggesting higher effect of bacteriocins. The synergy among the bacteriocin is possible explanation for the increase in PI fluorescence of target cells.

The FTIR analysis of target cells was performed to monitor the specific interaction of the bacteriocins with cellular targets. The valuable information about the biochemical composition of the target bacteria may be used to evaluate the mechanism of action of bacteriocins in treated cells [34]. Therefore, bacteriocin-treated cells were scanned using FTIR to monitor the interaction of bacteriocins with cellular targets. The FTIR spectra of enterocin LD3-treated cells showed alteration at ~ 1094.30/cm corresponding to nucleic acids [35] suggesting enterocin LD3 may enter inside the cells and interacts with nucleic acids. The spectrum was also found to be altered (increased absorbance) at the region ~ 1451.82/cm corresponding to phospholipids [36] indicating the interaction of enterocin LD3 with membrane lipids. These observations indicated that enterocin LD3 not only interacts with cell membrane but also enters inside the target cells interacting with nucleic acids. In contrast, plantaricin LD4-treated cells did not show such alteration in the wavelength corresponding to phospholipids and nucleic acids suggesting plantaricin LD4 kills target cells using other mechanism which is different to enterocin LD3. Our data suggest that different mode of action of both bacteriocins results in division of labour and may be responsible for their synergistic activity against target cells. Since enterocin LD3 interacts directly with phospholipids, it may be involved in pore formation or cell membrane damage allowing both/other bacteriocins access inside the cells causing cell death.

The membrane-acting nature of these bacteriocins was also confirmed using TEM analysis. It was used to visualize the morphology and capture intracellular images of the target cells. Untreated *Staph. aureus* subsp. *aureus* ATCC25923 cells displayed a highly homogenous intracellular density and round shape, but enterocin LD3-treated cells showed disruption of cell membrane and release of intracellular contents. *Staph. aureus* subsp. *aureus* ATCC25923 cells treated with plantaricin LD4 also exhibited cellular damages, while enterocin LD3 and plantaricin LD4 combination treatment caused wide surface damage to most cells, showing sunken cell surface and shrinking cytoplasm. Irregularity of the cell surface and the increase of cell mass were undoubtedly evident indicating perforation of the cell wall with consequent cell deformation after combined bacteriocin treatment. The untreated *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311cells maintained typical rods and intact status. When exposed to enterocin LD3, the cells showed an alteration in morphology, and ruptured cells were observed. Similarly, plantaricin LD4 induced a dramatic change in the *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311cells showing various alterations [37] reported that plantaricin MG damaged outer membrane of *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 with release of cellular contents in the surrounding, but nisin did not show any effect on cell morphology of

Salm. enterica subsp. *enterica* serovar Typhimurium ATCC13311. Other reports showed that nisin did not inhibit *Salmonella* spp. Due to their external membrane consisting of extensive amounts of protein, phospholipids and polysaccharide may act as a barrier to the action of nisin on the cytoplasmic membrane [38, 39]. Yildirim et al. [40] explained that enterocin KP alone did not act against intact cells of *Salm.* Typhimurium and *E. coli* but in the presence of physiochemical sublethal treatments decreases their cell number. However, in the present study, enterocin LD3 and plantaricin LD4 showed bactericidal effects on both Gram-positive and Gram-negative bacteria. In the presence of combined concentration of these bacteriocins, the outer membrane of *Salm. enterica* subsp. *enterica* serovar Typhimurium ATCC13311 cells was damaged, cell membrane protruded into the cytoplasm, and several cells displayed ruptures and loss of cytoplasm, indicating the cytoplasmic membrane was severely affected by the mixture of bacteriocins. The activity of these bacteriocins alone and in combination was also observed against other pathogens such as *P. mirabilis* ATCC43071, *P. aeruginosa* ATCC27853 and *E. coli* ATCC25922 suggesting their wider applications. However, their effect against *S. marcescens* ATCC27137 was not recorded either alone or in combination suggesting the possibility to explore other strains of this species for activity testing. In contrast, bacteriocins produced by *E. mundtii* 115 and *E. faecium* DSH20 did not inhibit *E. coli*, *Staph. aureus*, *Salm.* Typhimurium, *M. luteus* and *P. aeruginosa* [29, 41]. Furthermore, synergistic interaction may also reduce the costs of application of these bacteriocins. In addition, combinatorial therapies with bacteriocins and/or other antimicrobials may broaden antimicrobial spectra and reduce the probability of resistance development probably due to contribution of two different mechanisms of bacteriocin action as also suggested by Gulluce et al. [42].

Conclusions

In the present study, two synergistically acting bacteriocins, enterocin LD3 and plantaricin LD4 have been used against known food-borne pathogens, *Staphylococcus aureus* subsp. *aureus* ATCC25923 and *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC13311. It was found that enterocin LD3 was more effective on target cells than plantaricin LD4. When both bacteriocins were used in combination, their effect was manyfold higher than their individual effects suggesting synergy between the two bacteriocins. The synergetic effect was observed in terms of reduced MIC, FICI, isobologram interpretation, higher growth inhibition, loss of cell viability and damage to the cell membrane of the target cells. Further, inhibition of Gram-positive and Gram-negative pathogenic bacteria is other unique feature suggesting their wider applications in food safety and clinical settings. Use of

synergistically acting compounds may reduce the likelihood of resistance development against target bacteria and may also be economical for industrial applications.

Compliance with Ethical Standards

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

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent For this type of study, formal consent is not required.

References

1. Cox MJ, Loman N, Bogaert D, O'Grady J (2020) Co-infections: potentially lethal and unexplored in COVID-19. Lancet Microbe 1: E11. https://doi.org/10.1016/S2666-5247(20)30009-4

2. Tong SY, Davis JS, Eichenberger E, Holland TL, Fowler VG Jr (2015) Staphylococcus aureus infections: epidemiology, pathophysiology, clinical manifestations, and management. Clin Microbiol Rev 28(3):603–661. https://doi.org/10.1128/CMR.00134-14

3. Mohammed M (2017) Phage typing or CRISPR typing for epidemiological surveillance of Salmonella Typhimurium? BMC Res Notes 10:578. https://doi.org/10.1186/s13104-017-2878-0

4. DuPont HL (2007) The growing threat of foodborne bacterial enteropathogens of animal origin. Clin Infect Dis 45:1353–1361. https://doi.org/10.1086/522662

5. Eng SK, Pusparajah P, Mutalib N-SA, Ser H, Chan K, Lee L-H (2015) Salmonella: a review on pathogenesis, epidemiology and antibiotic resistance. Front Life Sc 8:284–293. https://doi.org/10.1080/21553769.2015.1051243

6. Kumar V, Sheoran P, Gupta A, Yadav JP, Tiwari SK (2016) Antibacterial property of bacteriocin produced by Lactobacillus plantarum LD4 isolated from a fermented food. Ann Microbiol 66:1431–1440. https://doi.org/10.1007/s13213-016-1230-6

7. Alvarez-Sieiro P, Montalbán-López M, Mu D, Kuipers OP (2016) Bacteriocins of lactic acid bacteria: extending the family. Appl Microbiol Biotechnol 100:2939–2951. https://doi.org/10.1007/s00253-016-7343-9

8. Chi H, Holo H (2018) Synergistic antimicrobial activity between the broad spectrum bacteriocin garvicin KS and nisin, farnesol and polymyxin B against gram -positive and gram-negative bacteria. Curr Microbiol 75:272–277. https://doi.org/10.1007/s00284-017-1375-y

9. Lohans CT, Vederas JC (2012) Development of class IIa bacteriocins as therapeutic agents. Int J Microbiol 2012:386410. https://doi.org/10.1155/2012/386410

10. Dicks LMT, Dreyer L, Smith C, van Staden AD (2018) A review: the fate of bacteriocins in the human gastro-intestinal tract: do they cross the gut–blood barrier? Front Microbiol 9:2297. https://doi.org/10.3389/fmicb.2018.02297

11. Chikindas M, Weeks R, Drider D, Chistyakov V, Dicks L (2018) Functions and emerging applications of bacteriocins. Curr Opin Biotechnol 49:23–28. https://doi.org/10.1016/j.copbio.2017.07.011

12. Doern CD (2014) When does 2 plus 2 equal 5? A review of antimicrobial synergy testing. J Clin Microbiol 52:4124–4128. https://doi.org/10.1128/JCM.01121-14

13. Turgis M, Vu KD, Jamshidian M, Maherani B, Lacroix M (2016) Synergistic antimicrobial effect of combined bacteriocins against food pathogens and spoilage bacteria. Microbio Res Inter 4:1–5 https://pdfs.semanticscholar.org/d239/70f5eb2e8c0ffa916b6b1d12b1d9087fe8ac.pdf

14. Pillet F, Formosa DC, Baaziz H, Dague E, Rols MP (2016) Cell wall as a target for bacteria inactivation by pulsed electric fields. Sci Rep 6:1–8. https://doi.org/10.1038/srep19778

15. Gupta A, Tiwari SK (2015) Probiotic potential of bacteriocin producing Enterococcus hirae strain LD3 isolated from Dosa batter. Ann Microbiol 65:2333–2342. https://doi.org/10.1007/s13213-015-1075-4

16. Gupta A, Tiwari SK, Netrebov V, Chikindas ML (2016) Biochemical properties and mechanism of action of enterocin LD3 purified from Enterococcus hirae LD3. Probiotics Antimicrob Proteins 83:161–169. https://doi.org/10.1007/s12602-016-9217-y

17. Sheoran P, Tiwari SK (2019a) Enterocin LD3 from Enterococcus hirae LD3 causing efflux of intracellular ions and UV absorbing materials in gram-negative bacteria. J Appl Microbiol 126:1059–1069. https://doi.org/10.1111/jam.14203

18. Sheoran P, Tiwari SK (2019b) Anti-staphylococcal activity of bacteriocins of food isolates Enterococcus hirae LD3 and Lactobacillus plantarum LD4 in pasteurized milk. 3Biotech 9(1):1–7. https://doi.org/10.1007/s13205-018-1546-y

19. Weeks RM, Moretti A, Song S, Uhrich KE, Karlyshev AV, Chikindas ML (2019) Cationic amphiphiles against Gardnerella vaginalis resistant strains and bacterial vaginosis-associated pathogens. Pathog Dis 77(8):ftz059. https://doi.org/10.1093/femspd/ftz059

20. Clinical and Laboratory Standards Institute (CLSI) (2017) Performance standards for antimicrobial susceptibility test. 27th Edition. CLSI Supplement M100. Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite (2500) Wayne, PA 19087. In: USA

21. Acosta MP, Ruzal SM, Allievi MC, Palomino MM, Rivas CS (2010) Synergistic effects of the Lactobacillus acidophilus surface layer and nisin on bacterial growth. Appl Environ Microbiol 76:974–977. https://doi.org/10.1128/AEM.01427-09

22. European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) (2000) Terminology relating to methods for the determination of susceptibility of bacteria to antimicrobial agents. Clin Microbiol Infect 6:503–508. https://doi.org/10.1046/j.1469-0691.2000.00149.x

23. Amrouche T, Noll KS, Wang Y, Huang Q, Chikindas ML (2010) Antibacterial activity of subtilosin alone and combined with curcumin, poly-lysine and zinc lactate against Listeria monocytogenes strains. Probiotics Antimicrob Proteins 2:250–257. https://doi.org/10.1007/s12602-010-9042-7

24. Lee CR, Cho IH, Jeong BCJ, Lee SH (2013) Strategies to minimize antibiotic resistance. Int J Environ Res Public Health 10:4274–4305. https://doi.org/10.3390/ijerph10094274

25. Zoumpopoulou G, Pepelassi E, Papaioannou W, Georgalaki M, Maragkoudakis PA, Tarantilis PA, Polissiou M, Tsakaldou E, Papadimitriou K (2013) Incidence of bacteriocins produced by food-related lactic acid bacteria active towards oral pathogens. Int J Mol Sci 14:4640–4646. https://doi.org/10.3390/ijms14034640

26. Zhang X, Wang Y, Liu L, Wei Y, Shang N, Zhang X, Li P (2016) Two-peptide bacteriocins PlnEF causes cell membrane damage to *Lactobacillus plantarum*. Biochim Biophys Acta 1858:274–280. https://doi.org/10.1016/j.bbamem.2015.11.018

27. Kaur G, Singh T, Malik R, Bhardwaj A, De S (2014) Antibacterial efficacy of nisin, pediocin 34, and enterocin FH99 against *L. monocytogenes*, *E. faecium*, and *E. faecalis* and bacteriocin cross resistance and antibiotic susceptibility of their bacteriocin resistant variants. J Food Sci Technol 51:233–244. https://doi.org/10.1007/s13197-011-0500-3

28. Favaro L, Penna ALB, Todorov SD (2015) Bacteriocinogenic LAB from cheeses– application in biopreservation? Trends Food Sci Technol 41:37–48. https://doi.org/10.1016/j.tifs.2014.09.001

29. Ferreira AE, Canal N, Morales D, Fuentefria DB, Corcao G (2007) Characterization of enterocins produced by *Enterococcus mundtii* isolated from humans feces. Braz Arch Biol Technol 50:249–258. https://doi.org/10.1590/S1516-89132007000200010

30. Al Atya AK, Belguesmia Y, Chataigne G, Ravallec R, Vachee A, Szunerits S, Boukhrroub R, Drider D (2016) Anti-MRSA activities of enterocins DD28 and DD39 and evidences on their role in the inhibition of biofilm formation. Front Microbiol 7:1–12. https://doi.org/10.3389/fmicb.2016.00817

31. Vignolo G, Palacios JC, Farias ME, Sesma F, Schillinger U, Holzapfel W (2000) Combined effect of bacteriocins on the survival of various *Listeria* species in broth and meat systems. Curr Microbiol 41:410–416. https://doi.org/10.1007/s002840010159

32. Johnson M, Brittany CAK (2013) Fluorescence microscopy methods for determining the viability of bacteria in association with mammalian cells. J Vis Exp 79:50729–50738 10/3791/50729

33. Sun Z, Li P, Liu F, Bian H, Wang D, Wang X, Zou Y, Sun C, Xu W (2017) Synergistic antibacterial mechanism of the *Lactobacillus crispatus* surface layer protein and nisin on *Staphylococcus saprophyticus*. Sci Rep 7:265–277. https://doi.org/10.1038/s41598-017-00303-8

34. Senbagam D, Gurusamy R, Senthil KB (2013) Physical chemical and biological characterization of a new bacteriocin produced by *Bacillus cereus* NS02. Asian Pac J Trop Med 6:934–941. https://doi.org/10.1016/S1995-7645(13)60167-4

35. Depciuch J, Kasprzyk I, Sadik O, Parlinska-Wojtan M (2017) FTIR analysis of molecular composition changes in hazel pollen from unpolluted and urbanized areas. Aerobiologia (Bologna) 33:1–12. https://doi.org/10.1007/s10453-016-9445-3

36. Eberhardt K, Beleites C, Marthandan S, Matthaus C, Diekmann S, Popp J (2017) Raman and infrared spectroscopy distinguishing replicative senescent from proliferating primary human fibrobalt cells by detecting spectral differences mainly due to biomolecular alterations. Anal Chem 89:2937–2947. https://doi.org/10.1021/acs.analchem.6b04264

37. Gong HS, Meng XC, Wang H (2010) Mode of action of plantaricin MG, a bacteriocin active against *Salmonella* typhimurium. J Basic Microbiol 50:S37–S45. https://doi.org/10.1002/jobm.201000130

38. Rattanachaikunsopon P, Phumkhachorn P (2010a) Antimicrobial activity of basil (*Ocimum basilicum*) oil against *Salmonella enteritidis in vitro* and in food. Biosci Biotechnol Biochem 74:1–6. https://doi.org/10.1271/bbb.90939

39. Prudencio CV, Vanetti MCD, Prieto M (2015) Tolerance of *Salmonella enterica* serovar typhimurium to nisin combined with EDTA is accompanied by changes in cellular composition. Food Res Int 69:281–288. https://doi.org/10.1080/19476337.2018.1523810

40. Yildirim Z, Ilk Y, Yildirim M, Tokatli K, Oncul N (2014) Inhibitory effect of enterocin KP in combination with sublethal factors on *Escherichia coli* O157:H7 or *Salmonella* Typhimurium in BHI broth and UHT milk. Turk J Biol 38:412–419. https://doi.org/10.3906/biy-1310-69

41. Shokri D, Zaghian S, Khodabakhsh F, Fazeli H, Mobasherizadeh S, Ataei B (2014) Antimicrobial activity of a UV-stable bacteriocin-like inhibitory substance (BLIS) produced by *Enterococcus faecium* strain DSH20 against vancomycin-resistant enterococcus (VRE) strains. J Microbiol Immunol Infect 47:371–376. https://doi.org/10.1016/j.jmii.2013.05.004

42. Gulluce M, Karadayi M, Baris O (2013) Bacteriocins: promising antimicrobials. Microbial pathogens and strategies for combating them. Sci Technol Edu 10:1016–1027. https://doi.org/10.9790/264X-03022833