Synergistic Effect of Enterocin NKR-5-3 and Sodium Citrate against *Listeria innocua* ATCC 33090 in Culture Media and Chilled Shrimp

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ABSTRACT

Enterocin NKR-5-3, an antimicrobial peptide and class II bacteriocin, is produced by *Enterococcus faecium* NKR-5-3 isolated from Thai fermented fish. However, it displays low antimicrobial activity against *Listeria* spp., which are recognized as significant food-borne bacteria in seafood industries. In this study, commercial food additives with potential ability to enhance bacteriocin activity, including ethylenediaminetetraacetic acid (EDTA), sodium lactate (SL), and sodium citrate (SC), were applied to enhance antimicrobial activity of enterocin NKR-5-3 against *L. innocua* ATCC 33090. The results showed that only 1.5% (w/v) SC could produce a significantly more effective ($p \leq 0.05$) synergistic interaction with enterocin NKR-5-3 against *L. innocua* ATCC 33090 when comparing with EDTA (250 ppm) and SL (2% v/v). The combination of enterocin NKR-5-3 at final concentration of 3,200 $AU \cdot mL^{-1}$ and 1.5% (w/v) SC could reduce the count of *L. innocua* ATCC 33090 by 1.33, 1.14, 0.97 and 1.13 log $CFU \cdot mL^{-1}$ in culture broth at 4 °C after 16 h of incubation period, when compared to the control, treatment with only SC, treatment with only enterocin NKR-5-3, and initial loading number, respectively. The synergistic interaction of enterocin NKR-5-3 and SC enhanced the bactericidal mode of action against *L. innocua* ATCC 33090, confirmed by total viable count in culture media together with morphological damage of target cells observed by SEM. In artificially contaminated shrimp meat samples kept at 4 °C for 48 h, the combination of enterocin NKR-5-3 and SC could reduce the count of *L. innocua* ATCC 33090 by 1.89, 1.78, 1.30 and 0.81 log $CFU \cdot g^{-1}$ compared to the control, treatment with only SC, only enterocin NKR-5-3, and initial loading number, respectively.

Keywords: Bacteriocin, Chilled shrimp, Enterocin NKR-5-3, *Listeria innocua*, Sodium citrate, Synergistic interaction

INTRODUCTION

Listeriosis is a disease caused by the infection with *Listeria monocytogenes*. It has become a significant public health concern worldwide due to high hospitalization and mortality rates after infection, particularly in pregnant women, adults aged 65 or older, and people with weakened immune systems (Muriana, 1996; Miettinen *et al*., 1999; Freitag *et al*., 2009; European Food Safety Authority and European Centre for Disease Prevention and Control, 2021; Centers for Disease Control and Prevention, 2022). *Listeria monocytogenes* prevalently contaminates chilled fresh fish and fishery products (Jørgensen and Huss, 1998; Buchanan *et al*., 2017). Listeriosis cases have been reported in people consuming cold-smoked salmon, trout, and gravad salmon (Schjørring *et al*., 2017; European Centre for Disease Prevention and Control and European Food Safety Authority, 2019),

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crabmeat (Elson *et al*., 2019), and marinated herring cutlet (Aichinger, 2010).

Bacteriocins are ribosomally synthesized proteinaceous antimicrobial compounds with bactericidal or bacteriostatic activity against closely related bacteria (De Vuyst and Vandamme, 1994; Nes *et al*., 2013). In particular, bacteriocins that are produced by lactic acid bacteria (LAB) are accepted to be safe and have potential as a natural food bio-preservative (Schillinger *et al*., 1996; Cleveland *et al*., 2001; O' Sullivan *et al*., 2002; Ng *et al*., 2020; Kirtonia *et al*., 2021). The main inhibition mechanism of bacteriocin against target bacterial cells is pore formation, induced by binding of the cationic region in bacteriocin molecules to negatively charged phospholipids, which increases the permeability of the cytoplasmic membrane, leading to an efflux of intracellular components (Kumariya *et al*., 2019; Ennahar *et al*., 2000). Furthermore, some bacteriocins inhibit cell wall and nucleic acid synthesis as well as essential enzyme reactions of sensitive target bacteria (Simons *et al*., 2020).

Recently, the activity of bacteriocin against some Gram-negative and Gram-positive bacteria was found to be improved through synergistic interaction with other bioactive molecules such as phages, antibiotics, nanoparticles and essential oils (Zgheib *et al*., 2020). Moreover, some commercial food additives have also been applied for enhancing the antimicrobial activity of various target bacteriocins. Buncic *et al*. (1995) reported on enhancing antimicrobial activity of nisin against *L. monocytogenes* at refrigeration temperatures by combining it with sodium lactate. Lacticin 3147 in combination with either sodium lactate or sodium citrate significantly reduced the aerobic plate count in pork sausage (Scannell *et al*., 2000). The antimicrobial activity of enterocin AS-48 against *B. cereus* in rice gruel was increased by combining it with sodium lactate (Grande *et al*., 2006). The combination of sodium citrate or sodium lactate with nisin produced an increase in antimicrobial activity of nisin against *Arcobacter butzleri* in simulated chicken meats (Long and Phillips, 2003). The antimicrobial activity of enterocin AS-48 and Brochrocin C was also increased against EDTA-treated Gram-negative bacteria (Abriouel *et al*., 1998; Gao *et al*., 1999; Ananou *et al*., 2005). Ascorbic acid and EDTA showed a synergistic effect with nisin in inhibiting growth of *Salmonella* Enteritidis ATCC 13076 (Sangcharoen *et al*., 2017).

Enterococcus faecium NKR-5-3, a lactic acid bacterium isolated from Thai fermented fish (Pla-Ra) sample and characterized as a non-pathogenic strain in our previous study, produced enterocin NKR-5-3, which is categorized in the group of bacteriocins (Wilaipun *et al*., 2004). Enterocin NKR-5-3 consists of five different antimicrobial peptide units (Ent 53A, B, C, D and Z), and this is the most interesting characteristic of this bacteriocin (Ishibashi *et al*., 2021). From our previous work, enterocin NKR-5-3 exhibited strong inhibitory effect against some food-borne pathogenic bacteria such as *Bacillus cereus*, *Clostridium perfringens*, and some LAB strains. However, it had low antimicrobial activity against *Listeria* spp. and had no antimicrobial activity against any Gram-negative bacteria (Wilaipun *et al*., 2004).

In this research, we aimed to enhance the antimicrobial activity of enterocin NKR-5-3 by combining it with target commercial food additives including EDTA, sodium citrate and sodium lactate to control *L. innocua* ATCC 33090. The non-pathogenic *L. innocua* was used to represent the pathogenic *L. monocytogenes* to ensure laboratory safety. Several other studies on *L. monocytogenes* used *L. innocua* as the surrogate of this severely pathogenic bacteria due to its close genetic relationship, high similarity in ecological cohabitation and physiological properties, and because of safety concerns in the laboratory (Giraffa *et al*., 1995; Kathariou *et al*., 1995; Kamat and Nair, 1996; Costa *et al*., 2018; He *et al*., 2021). In addition, we also studied the selected food additive that showed the highest synergistic antimicrobial activity when combined with enterocin NKR-5-3 in controlling *L. innocua* ATCC 33090 at refrigeration temperature, and the physical morphology of *L. innocua* ATCC 33090 cells after being treated with the mixtures. Finally, we also studied the application of enterocin NKR-5-3 and/or selected food additive in controlling *L. innocua* ATCC 33090 in the artificial contamination of chilled shrimp meat samples.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Enterocin NKR-5-3-producing lactic acid bacteria, *Enterococcus faecium* NKR-5-3, was maintained as frozen stock at -20 °C in M17 broth (Merck, Darmstadt, Germany) containing 20% (v/v) glycerol. The culture was propagated twice in M17 broth at 35 °C for 18 h before use. *E. faecium* NKR-5-3 was cultured in M17 broth at 35 °C for 24 h for bacteriocin production and *Enterococcus faecalis* ATCC 19433 was used as the indicator strain (positive control) for bacteriocin activity determination.

Listeria innocua ATCC 33090 and *E. faecalis* ATCC 19433 were maintained as frozen stocks at -20 °C in TSBYE broth (Tryptic soy broth (Bacto™, USA) supplemented with 6 $g \cdot L^{-1}$ Yeast extract (Bacto™, USA)) containing 20% (v/v) glycerol. Both of them were subcultured every two weeks on TSAYE (Tryptic soy agar supplemented with 6 $g \cdot L^{-1}$ Yeast extract) slants and kept at 4 °C. The cultures were activated twice in TSBYE at 35 °C for 12 h before use.

Determination of bacteriocin activity

The cell-free neutralized supernatant (CFNS) of *Enterococcus faecium* NKR-5-3 was prepared as described by Wilaipun *et al*. (2004). The CFNS was concentrated by evaporator to obtain antimicrobial activity against *E. faecalis* ATCC 19433 at 128,000 $AU \cdot mL^{-1}$, then kept in storage by freezing at -20 °C and defrosted at 4 °C for 24 h before use. The critical dilution assay was used to determine bacteriocin activity of enterocin NKR-5-3 in CFNS form (Mayr-Harting *et al*., 1972). Briefly, CFNS was serially diluted two-fold with sterile distilled water, and the aliquots (10 µL) of each dilution were spotted onto TSAYE plates overlaid with 5 mL of TSAYE soft agar media containing 10^7 log $CFU \cdot mL^{-1}$ of overnight (12 h) cultured *E. faecalis* ATCC 19433 (indicator strain). Plates were incubated at 35 ºC for 24 h. The arbitrary activity unit (AU) was defined as the reciprocal of the highest dilution producing an inhibition zone on the indicator lawn and was multiplied by a factor of 100 to obtain the $AU \cdot mL^{-1}$ of the original sample.

Chemicals

Three target commercial food additives including ethylenediaminetetraacetic acid (EDTA; Merck, Darmstadt, Germany), sodium lactate (SL; Wako, Tokyo, Japan), and sodium citrate tribasic dihydrate (SC; Sigma-Aldrich, St. Louis, USA) have been reported to be capable of enhancing bacteriocin activity, and thus were used to enhance enterocin NKR-5-3 activity against target bacterial strain. Stock solutions (20X of specified final concentration) of each target food additive was prepared by dissolving in deionized water, then sterilized by filtering through 0.2 µm membrane syringe filter (Sartorius, Germany) and kept at 4 °C.

Effect of enterocin NKR-5-3 and/or target food additives on Listeria innocua ATCC 33090 in culture media

Listeria innocua ATCC 33090 was cultured in TSBYE at 35 °C for 12 h to obtain approximately 10^7 log $CFU \cdot mL^{-1}$. *L. innocua* ATCC 33090 in TSBYE (2 µL) was inoculated to 200 µL of TSBYE containing enterocin NKR-5-3 and/or target food additives under specified final concentration in the following combinations: (1) enterocin NKR-5-3 (3,200 $AU \cdot mL^{-1}$), (2) EDTA (250 ppm), (3) SC (1.5% w/v), (4) SL (2% v/v), (5) enterocin NKR-5-3 (3,200 $AU \cdot mL^{-1}$) and EDTA (250 ppm), (6) enterocin NKR-5-3 (3,200 $AU \cdot mL^{-1}$) and SC (1.5% w/v), (7) enterocin NKR-5-3 (3,200 $AU \cdot mL^{-1}$) and SL (2% v/v), and (8) TSBYE without enterocin NKR-5-3 or target food additives. All treatments were performed in 96-well microplates (Corning star, USA) and incubated at 35 °C for 10 h. During incubation, growth of *L. innocua* ATCC 33090 in each treatment was evaluated every 1 h by turbidity measurement at 600 nm (OD_{600}) with X52 Power Wave microplate spectrophotometer (BioTex, USA).

Effect of enterocin NKR-5-3 and/or SC on Listeria innocua ATCC 33090 at refrigeration temperature

One mL of log phase *Listeria innocua* ATCC 33090 (approximately 10^7 log $CFU \cdot mL^{-1}$) in TSBYE was added into 100 mL of TSBYE containing different target antimicrobial substances at specified final concentrations: (1) 3,200 $AU \cdot mL^{-1}$

enterocin NKR-5-3, (2) 1.5% (w/v) SC, (3) the combination of 3,200 AU·mL^{-1} enterocin NKR-5-3 and 1.5% (w/v) SC, and (4) TSBYE without adding any antimicrobial substance. All treatments were incubated at 4±2 °C for 96 h. During incubation, culture broth samples were taken every 8 h, then total viable number of *L. innocua* ATCC 33090 were determined by using PALCAM agar (Merck, Darmstadt, Germany) and incubating at 35 °C for 24 h.

Effect of enterocin NKR-5-3 and/or SC on physical morphology of Listeria innocua ATCC 33090 cells

Listeria innocua ATCC 33090 was cultured in TSBYE and incubated at 35 °C for 12 h. After incubation, 0.5 mL of bacterial cell suspension (approximately 10^7 log CFU·mL^{-1}) was added into 50 mL of each target antimicrobial solution, consisting of (1) 3,200 AU·mL^{-1} enterocin NKR-5-3, (2) 1.5% (w/v) SC, (3) the combination of 3,200 AU·mL^{-1} enterocin NKR-5-3 and 1.5% (w/v) SC, and (4) 0.85% (w/v) NaCl. All treatments were then incubated at 35 °C for 1 h. A sample from each treatment was centrifuged at 6,000 g at 4 °C for 10 min, and precipitated cells of *L. innocua* ATCC 33090 were collected. The precipitated cells of *L. innocua* ATCC 33090 were fixed in 0.1 M phosphate buffer with 2.5% (v/v) glutaraldehyde (pH 7.2) at 4 °C for 2 h. The specimen was rinsed twice by 0.1 M phosphate buffer for 10 min in each rinsing, and once in distilled water for 10 min. Target cells were dehydrated by using serial ethanol concentrations (50, 75, 90 and 100% v/v), placed on stubs, dried with CPD 020 critical point dryer (Balzers, USA), coated with gold in SCD 040 sputter coater (Balzers, USA), and observed under JSM-5410LV scanning microscope (JEOL, Japan).

Effect of enterocin NKR-5-3 and/or SC against Listeria innocua ATCC 33090 in the artificial contamination of chilled shrimp meat samples

Whole fresh white shrimp (*Litopenaeus vannamei*) purchased from a local supermarket was chilled in an ice box during transport to the laboratory. The shrimp was rinsed with tap water, de-headed and peeled. Shrimp meat was subsequently sterilized by heating in an autoclave at 121 °C for 10 min and homogenized in a sterilized blender (Waring 32BL80, USA). The homogenized shrimp meat sample (300 g) was transferred into a sterile stomacher bag (Seward, UK) and filled with 300 mL of different target antimicrobial solutions at specified final concentrations: (1) 3,200 AU·mL^{-1} enterocin NKR-5-3, (2) 1.5% (w/v) SC, (3) the combination of 3,200 AU·mL^{-1} enterocin NKR-5-3 and 1.5% (w/v) SC, and (4) 0.85% (w/v) NaCl. Then, prepared shrimp samples were inoculated with log phase *Listeria innocua* ATCC 33090 at final concentration of 10^5 log CFU·g^{-1}. The inoculated samples were mixed thoroughly by stomacher (Seward BA 7021, UK) and incubated at 4±2 °C for 48 h. During incubation, inoculated shrimp samples were taken at every 8 h, then total viable number of *L. innocua* ATCC 33090 were determined by using PALCAM agar (Merck, Darmstadt, Germany) and incubating at 35 °C for 24 h.

Statistical analysis

All experiments were done in triplicate and the data were presented as mean±SD. Analysis of variance was performed to detect significant differences among treatments, and means were compared using Duncan's multiple range tests. The tests were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Effect of enterocin NKR-5-3 and/or target food additives on Listeria innocua ATCC 33090 in culture media

In our previous study, enterocin NKR-5-3 produced by *Enterococcus faecium* NKR-5-3 showed broad antimicrobial spectrum with strong antimicrobial activity against various target Gram-positive bacteria. However, it had low antimicrobial activity against *Listeria* spp., significant pathogens that often contaminate seafood and other food products kept at low temperature (Wilaipun *et al*., 2004). Because of this limitation, three food additives (EDTA, SL, and SC) with potential to enhance bacteriocin efficacy were used in our trials to assess their synergistic interaction with enterocin NKR-5-3 against *L. innocua* ATCC 33090 in culture media. The results of the trials are shown in Figure 1.

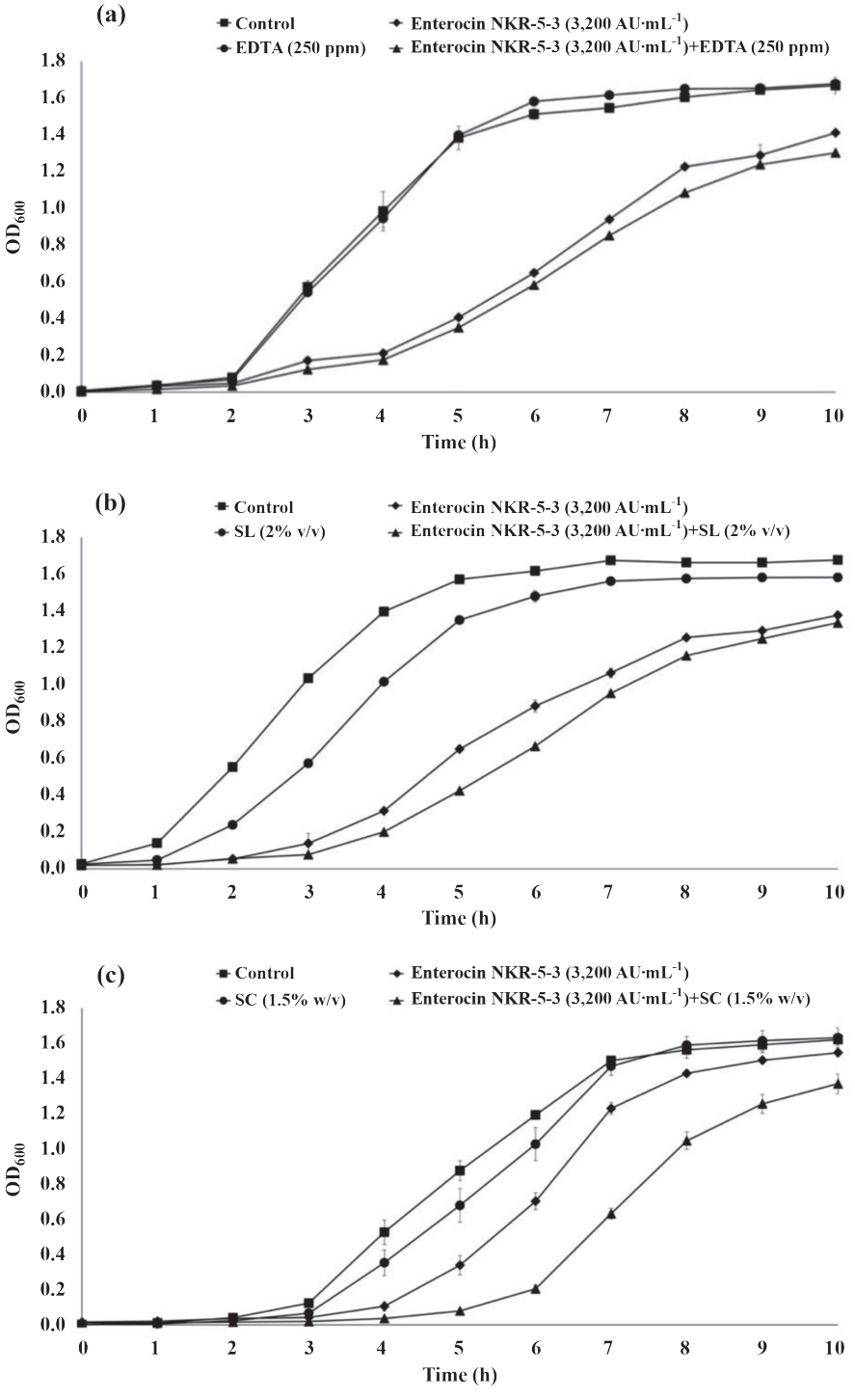

Figure 1. Optical density (OD_{600}) at hourly time intervals of *Listeria innocua* ATCC 33090 cultured in TSBYE medium containing each target substance and incubated at 35 °C for 10 h (a) enterocin NKR-5-3 and/or EDTA; (b) enterocin NKR-5-3 and/or SL; (c) enterocin NKR-5-3 and/or SC.

For EDTA at the final concentration of 250 ppm (Figure 1a), there was no significant effect ($p>0.05$) on retarding the growth (OD_{600}) of *L. innocua* ATCC 33090 cultured in TSBYE+ EDTA when comparing with growth in TSBYE without EDTA (control). Meanwhile, growth of *L. innocua* ATCC 33090 was retarded when cultured in TSBYE+enterocin NKR-5-3 and TSBYE+enterocin NKR-5-3+EDTA, although no significant difference ($p>0.05$) was observed in either of these treatments after 10 h of incubation. It indicated that EDTA could not enhance antimicrobial activity of enterocin NKR-5-3 against *L. innocua* ATCC 33090. EDTA is a chelating agent with high affinity for metal ions and is not recognized as an important antimicrobial agent by itself (Lambert *et al*., 2004). Moreover, minimum bactericidal concentrations (MBCs) of EDTA against *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *E. coli* were all reported to be more than 16,384 ppm (Belfiore *et al*., 2007). EDTA has been reported to exhibit a synergistic interaction with some cationic antimicrobial peptides and bacteriocins (Walkenhorst *et al*., 2014; Prudêncio *et al*., 2015). EDTA has the potential to counteract the antagonistic effect of divalent cations on bacteriocin molecules by complex formation, which causes a reduction of bacteriocin efficacy. However, EDTA is known to disturb the structure of the outer membrane and allow hydrophobic molecules such as bacteriocin to insert into the cytoplasmic membrane, which causes some bacterial target cells to be more sensitive to bacteriocin (Ganzle *et al*., 1999).

EDTA at final concentration of 40 mM was reported to show a synergistic antimicrobial effect with nisin and gallidermin against *Salmonella* Typhimurium ATCC 23564 in culture media (Martin-Visscher *et al*., 2011). In addition, the combination of EDTA and SL at final concentrations of 500-1,000 mM and 200 mM, respectively, showed a synergistic antimicrobial effect with lactocin 705 in inhibiting *E. coli* O157:H7 (Prudêncio *et al*., 2015). However, the concentration of EDTA used in this study did not exhibit any antimicrobial activity by itself, nor did it enhance antimicrobial activity of enterocin NKR-5-3 against *L. innocua* ATCC 33090. We chose to use EDTA at a final concentration of only 250 ppm (0.86 mM), which is the maximum level permitted for use in food applications by Thai Food and Drug Administration regulation (Thai Food and Drug Administration, 2011). This low concentration of EDTA was apparently inadequate for disturbing the membrane structure of target bacteria cells, as suggested by Lappe *et al*. (2009).

Similar to EDTA, SL at final concentration of 2% (v/v) could neither retard the growth of *L. innocua* ATCC 33090 nor enhance antimicrobial activity of enterocin NKR-5-3 (Figure 1b). Sodium lactate has generally recognized as safe (GRAS) status and is used as a preservative in various food products (Doores, 1993). The antibacterial mechanism of SL is attributed to its ability in the undissociated form to penetrate through cytoplasmic membranes, causing reduced intracellular pH and disrupting the transmembrane proton motive force of target cells. Moreover, it may act as a potentiator of the effects of other antimicrobial substances (Alakomi *et al*., 2000). Previously, SL at final concentration of 2% (v/v) was used for enhancing antimicrobial activity of various known bacteriocins against target pathogenic bacteria (Scannell *et al*., 2000; Long and Phillips, 2003; Bari *et al*., 2005). Belfiore *et al*. (2007) and Prudêncio *et al*. (2015) described the synergistic interactions of some target bacteriocins such as nisin, lactocin 705 and cerein 8A when incorporated with the combination of SL and EDTA, but did not mention any synergistic interaction between each target bacteriocin and SL alone. Meanwhile, SL at final concentration of 2% had synergistic interaction with nisin in reducing the *L. monocytogenes* population in contaminated cabbage, broccoli and mung bean sprout samples (Bari *et al*., 2005). The dissimilarity in synergistic interaction of SL with enterocin NKR-5-3 in inhibiting *Listeria* sp. compared to nisin, as reported by Bari *et al*. (2005), was possibly due to the difference in molecular structure and antimicrobial properties of each target bacteriocin. Nisin is a lantibiotic bacteriocin (class I bacteriocin), whereas enterocin NKR-5-3 is a multi-peptide bacteriocin in class II (Wilaipun *et al*., 2004; Prudêncio *et al*., 2015; Ishibashi *et al*., 2021). Furthermore, temperature was also reported to influence the sensitivity of *Listeria* sp. to lactate. Lactate at final concentrations of 2 and 2.5% showed more potential to inhibit *L. monocytogenes* at 5 ºC than at 10 ºC (Qvist *et al*., 1994; Mbandi and Shelef, 2002).

As illustrated in Figure 1c, SC at final concentration of 1.5% (w/v) showed no significant effect ($p>0.05$) on retarding the growth (OD_{600}) of *L. innocua* ATCC 33090 cultured in TSBYE+SC when comparing with growth in TSBYE without SC (control). However, growth of *L. innocua* ATCC 33090 was retarded when cultured in TSBYE+enterocin NKR-5-3 and TSBYE+enterocin NKR-5-3+SC. Interestingly, the combination of TSBYE+enterocin NKR-5-3+SC clearly retarded the growth of *L. innocua* ATCC 33090 more than TSBYE+enterocin NKR-5-3 ($p\leq0.05$). This evidence suggests that SC at final concentration of 1.5% (w/v) had a synergistic interaction with enterocin NKR-5-3 in inhibiting *L. innocua* ATCC 33090. As such, the combination of enterocin NKR-5-3 and SC at final concentrations of 3,200 $AU{\cdot}mL^{-1}$ and 1.5% (w/v), respectively, was selected to control *L. innocua* ATCC 33090 under various specified conditions in further experimental steps. Similar to our finding, SC also displayed a synergistic interaction with various known bacteriocins in inhibiting their specific target indicator strains, such as nisin against *A. butzleri* (Long and Phillips, 2003), *L. innocua* (Scannell *et al*., 2000), *Staphylococcus aureus* (Khudhir, 2019), and *L. monocytogenes* (Oladunjoye *et al*., 2016), Lacticin 3147 against *C. perfringens* (Scannell *et al*., 2000), and bovicin HC5 against *Salmonella* Typhimurium (Prudêncio *et al*., 2014). SC, as citric acid salt, is recognized as a GRAS food additive with chelating property and possesses a bacteriostatic effect against foodborne pathogens such as *Listeria*, *Salmonella* and *Escherichia coli* O157:H7 (Morey *et al*., 2014). The general mechanisms of organic salts in their inhibition of target bacteria are due to a pH-lowering effect on the intracellular environment, alteration in cell membrane permeability with a disruption of substrate transportation, and depletion of the reducing agent level that is necessary for electron transport systems (Miller *et al*., 1993). In addition, the outstanding characteristic of citrate is its chelating property, which allows it to react with divalent metal ions (Ca^{2+}, Mg^{2+}, and Mn^{2+} ions), causing the alteration in the cell membrane, and finally, lysis of the cell (Ayres *et al*., 1999). The presumed mechanism for the synergistic interaction of SC and enterocin NKR-5-3 found in this experiment is the cooperation between cation chelation and bacteriocin functions. The chelating by SC on the cell membrane of *L. innocua* ATCC 33090 caused cell membrane permeability alterations and weakened cells (Miller *et al*., 1993; Ayres *et al*., 1999). The chelating mechanism of SC supported the efficiency of enterocin NKR-5-3 in pore-forming within the cell membrane of *L. innocua* ATCC 33090. Thereby, *L. innocua* ATCC 33090 cells leaked together with deprived of essential mineral and energy sources, resulting in cell death (De Vuyst and Vandamme, 1994; Ennahar *et al*., 2000).

Effect of enterocin NKR-5-3 and/or SC on Listeria innocua ATCC 33090 at refrigeration temperature

In general, *Listeria* spp. is capable of growing at low temperatures, such as in ice storage or refrigeration (Seeliger and Jones, 1986; Kontominas *et al*., 2021). Therefore, antimicrobial efficacy of enterocin NKR-5-3 and/or SC against *L. innocua* ATCC 33090 at low temperature is necessary for further application of these target compounds in food- and seafood-related industries. The results in Figure 2 show that total viable number of *L. innocua* ATCC 33090 increased from 5.77 log $CFU{\cdot}mL^{-1}$ to 7.10 log $CFU{\cdot}mL^{-1}$ when cultured in TSBYE, and from 5.71 log $CFU{\cdot}mL^{-1}$ to 7.09 log $CFU{\cdot}mL^{-1}$ when cultured in TSBYE+SC at 4 °C for 96 h. These results indicate that there was no significant difference ($p>0.05$) in total viable number of *L. innocua* ATCC 33090 between the treatments during 96 h of incubation, and suggests that SC could not inhibit *L. innocua* ATCC 33090 growth even in a low-temperature environment. Similarly, SC at 2% (w/v) did not show any antimicrobial effect against *L. innocua* and *C. perfringens* at 4 ºC (Scannell *et al*., 2000). However, Miller *et al*. (1993) reported that SC at final concentration of 6% (w/v) was required to inhibit the growth of *C. botulinum*.

Meanwhile, TSBYE+enterocin NKR-5-3 and TSBYE+enterocin NKR-5-3+SC could retard *L. innocua* ATCC 33090 growth, indicated by total viable number, compared with growth in TSBYE (control). Moreover, TSBYE+enterocin NKR-5-3 and TSBYE+enterocin NKR-5-3+SC could clearly reduce total viable number of *L. innocua* ATCC 33090 during the first 8-16 h of incubation compared

to the initial total viable number at 0 h. This evidence indicates that enterocin NKR-5-3 and enterocin NKR-5-3+SC had bactericidal activity against *L. innocua* ATCC 33090 cells.

The comparison between growth of *L. innocua* ATCC 33090 in TSBYE+enterocin NKR-5-3+SC and TSBYE+enterocin NKR-5-3 was addressed. The results showed that total viable number of *L. innocua* ATCC 33090 cultured in TSBYE+enterocin NKR-5-3+SC was dramatically reduced (by 1.13 log CFU·mL^{-1}) in the first 16 h of incubation period compared to the initial loading number (0 h). At the same time, total number of *L. innocua* ATCC 33090 cultured in TSBYE+ enterocin NKR-5-3+SC was 1.33, 1.14 and 0.97 log CFU·mL^{-1} lower than the bacterium cultured in TSBYE (control), TSBYE+SC and TSBYE+ enterocin NKR-5-3, respectively. Furthermore, the same inhibition effect of TSBYE+enterocin NKR-5-3+SC against *L. innocua* ATCC 33090 compared to each of the other treatments was detected throughout the incubation period. This finding confirmed that TSBYE+enterocin NKR-5-3+SC had stronger bactericidal mode of action against *L. innocua* ATCC 33090 cells than TSBYE+ enterocin NKR-5-3. Moreover, the synergistic effect between SC and enterocin NKR-5-3 in inhibiting *L. innocua* ATCC 33090 still continued even at low temperature. Likewise, SC at final concentration of 2% (w/v) was shown to have synergistic interactions with nisin and lacticin in inhibiting *Salmonella* Kentucky and *L. innocua* at 4 ºC (Scannell *et al*., 2000). This evidence suggested that SC is capable of enhancing the antimicrobial activity of some target bacteriocins for controlling foodborne pathogenic bacteria at low temperature. Therefore, scanning electron microscopy (SEM) was applied to observe *L. innocua* ATCC 33090 cell morphology after being treated with enterocin NKR-5-3 in combination with SC and to confirm the mode of action of this mixture.

Effect of enterocin NKR-5-3 and/or SC on physical morphology of Listeria innocua ATCC 33090 cells

The physical morphology of *Listeria innocua* ATCC 33090 cells after treatment with enterocin NKR-5-3 and/or SC was investigated by using SEM. After being treated with 1.5% (w/v) SC for 1 h, the *L. innocua* ATCC 33090 cells were not altered and still maintained their typical rod shape (Figure 3c). Meanwhile, treatment with enterocin NKR-5-3 caused an alteration of *L. innocua* ATCC

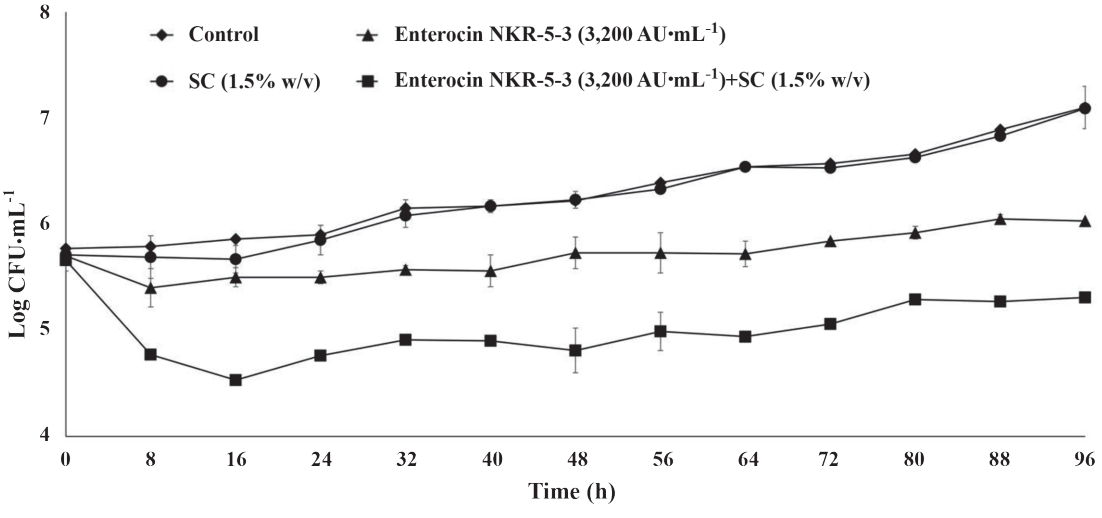

Figure 2. Total viable cell counts (Log CFU·mL^{-1}) at selected time intervals of *Listeria innocua* ATCC 33090 cultured in TSBYE media containing enterocin NKR-5-3 and/or SC at 4±2 °C.

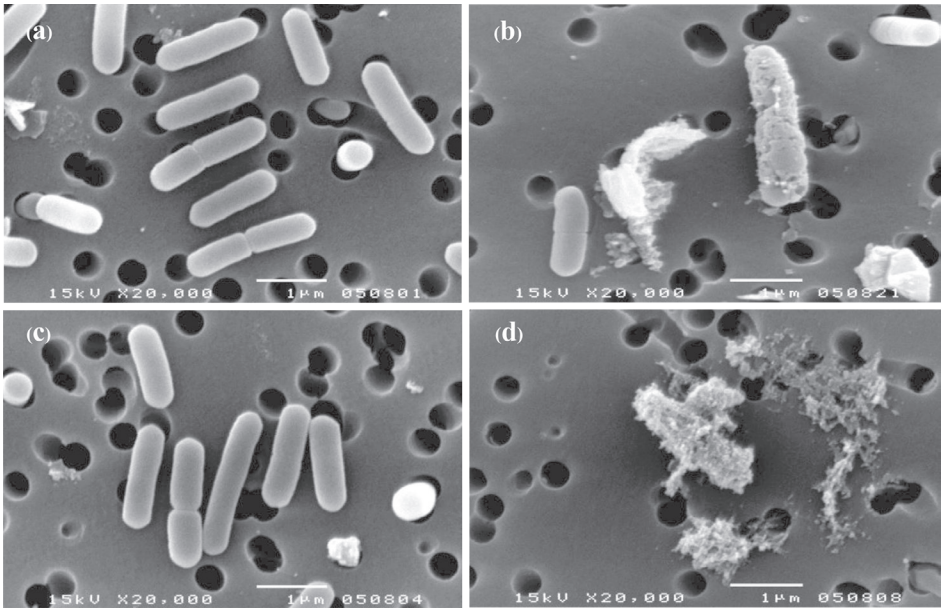

Figure 3. Physical morphology of *Listeria innocua* ATCC 33090 cells after treating with (a) control, (b) enterocin NKR-5-3 (3,200 AU·mL^{-1}), (c) SC (1.5% w/v), and (d) the combination of enterocin NKR-5-3 (3,200 AU·mL^{-1}) and SC (1.5% w/v), as visualized under scanning electron microscopy (×20,000).

33090 cell morphology, indicated by damaged cells (Figure 3b); however, some undamaged cells were observed in this sample. Furthermore, treatment with enterocin NKR-5-3+SC caused extensive damage to *L. innocua* ATCC 33090 cells; membranes were ruptured, the surface pitted, and cells were completely destroyed (Figure 3d). These observations confirm the results from our previous studies, which indicated that a combination of enterocin NKR-5-3 and SC at the final concentrations of 3,200 AU·mL^{-1} and 1.5% (w/v), respectively, had synergistic antimicrobial activity against *L. innocua* ATCC 33090. Moreover, the cell damage apparent under SEM confirms the bactericidal activity of enterocin NKR-5-3 and SC.

Enterococcus faecium NKR-5-3 produces enterocin NKR-5-3 in the culture supernatant, which consists of five different antimicrobial peptides, namely enterocin NKR-5-3A, B, C, D, and Z (Ent 53A, Ent 53B, Ent 53C, Ent 53D and Ent 53Z), and all of them belong to class II bacteriocin (Ishibashi *et al*., 2012). Most class II bacteriocin molecules have an amphiphilic helical structure which can insert into the target bacterial cell, and form pores within cell membrane, causing cell leakage, dissipation of cell proton motive force, and cell damage (Wu *et al*., 2022). Several other studies used SEM to observe the effects of pore formation and cell damage in target bacterial cells after treatment with different class II bacteriocins; examples include *L. monocytogenes*, *S. aureus* and *B. cereus* treated with plantaricin LPL-1, lactocin MXJ and plantaricin GZ1-27, respectively (Lü *et al*., 2014; Du *et al*., 2018; Wang *et al*., 2018).

Effect of enterocin NKR-5-3 and/or SC against Listeria innocua ATCC 33090 in the artificial contamination of chilled shrimp meat samples

Efficacy of enterocin NKR-5-3 in combination with SC against *Listeria innocua* ATCC 33090 in the artificial contamination of chilled shrimp meat samples is necessary baseline information for further application of these target compounds in fish and fishery products. In the first 8 h of storing artificial contaminated shrimp meat samples at 4±2 °C, treatment with the combination

of enterocin NKR-5-3 and SC showed the highest antimicrobial activity against *L. innocua* ATCC 33090 by reducing total viable number by 0.67 and 0.81 log CFU·g^{-1} when compared to the initial loading number and the control, respectively (Table 1). Meanwhile, treatment with only SC had no antimicrobial effect against *L. innocua* ATCC 33090 and no significant difference ($p>0.05$) from the control. On the other hand, treatment with only enterocin NKR-5-3 reduced total viable number by 0.17 and 0.24 log CFU·g^{-1} when compared to the initial loading number and the control at the same storage time, but the amount of reduction was significantly lower than by using the combination of enterocin NKR-5-3 and SC.

After storing artificially contaminated shrimp meat samples at 4±2 °C for 48 h, the treatment with the combination of enterocin NKR-5-3 (3,200 AU·mL^{-1}) and SC (1.5% w/v) still showed the highest antimicrobial activity against *L. innocua* ATCC 33090. It reduced total viable number of *L. innocua* ATCC 33090 by 0.81 and 1.89 log CFU·g^{-1} when compared to the initial contamination value and the control sample, respectively. Meanwhile, treatment with only SC had no antimicrobial effect against *L. innocua* ATCC 33090 and no significant difference from the control ($p>0.05$). In addition, treatment with only enterocin NKR-5-3 was only able to reduce the population of *L. innocua* ATCC 33090 by 0.59 log CFU·g^{-1} when compared to the control. This result suggests that 1.5% (w/v) SC has potential as an alternative commercial food additive that can be used for enhancing the antimicrobial activity of enterocin NKR-5-3 against *L. innocua* ATCC 33090, particularly in refrigerated products. In addition, the combination of enterocin NKR-5-3 (3,200 AU·mL^{-1}) and SC (1.5% w/v) had a synergistic bactericidal mode of action against *L. innocua* ATCC 33090 not only in culture broth but also in shrimp meat samples during storage at a temperature of 4±2 °C for 48 h.

The enhancement of antimicrobial activity of enterocins by food additives against *Listeria* sp. have been studied in various foods. Molinos *et al*. (2005) found that a washing solution containing the combination of enterocin AS-48 with acetic acid, citric acid, sodium propionate, and potassium sorbate showed significant increase in antimicrobial activity against *L. monocytogenes* on fresh alfalfa sprouts, soybean sprouts, and green asparagus. Citric acid, lactic acid, and p-hydroxybenzoic methylester acid (PHBME) had the capacity to improve the antimicrobial activity of enterocin AS-48 against *L. monocytogenes* in ready-to-eat salad stored at 10 °C (Molinos *et al*., 2009).

Table 1. Total viable count of *Listeria innocua* ATCC 33090 (mean±SD) at selected time intervals in artificially contaminated chilled shrimp meat samples after treatment with enterocin NKR-5-3 and/or SC at 4±2 °C.

Time (h)	***Listeria innocua* ATCC 33090 counts (log CFU·g^{-1})**			
	Control	**enterocin NKR-5-3 (3,200 AU·mL^{-1})**	**SC (1.5% w/v)**	**enterocin NKR-5-3 (3,200 AU·mL^{-1})+SC (1.5% w/v)**
0	5.60±0.06^{a}	5.65±0.00^{a}	5.69±0.02^{a}	5.59±0.09^{a}
8	5.72±0.09^{a}	5.48±0.07^{b}	5.70±0.03^{a}	4.92±0.06^{c}
16	6.15±0.06^{a}	5.48±0.07^{c}	5.70±0.03^{b}	4.92±0.06^{d}
24	6.15±0.01^{a}	5.67±0.10^{b}	6.05±0.01^{a}	4.49±0.01^{c}
32	6.51±0.05^{a}	5.84±0.09^{b}	6.30±0.18^{a}	4.95±0.05^{c}
40	6.61±0.02^{a}	5.97±0.01^{b}	6.52±0.08^{a}	4.83±0.09^{c}
48	6.67±0.04^{a}	6.08±0.03^{b}	6.56±0.10^{a}	4.78±0.02^{c}

Note: Means in the same row with different lowercase superscript letters are significantly ($p\leq0.05$) different.

The findings from this study can be of great benefit in controlling *Listeria* sp. in shrimp and another food products that are stored at low temperature. However, further application of the mixture of enterocin NKR-5-3 and SC in target food products should also address (1) strain, physiological stage and number of target bacteria; and (2) surrounding environment, such as pH, temperature, salt, some positively charged metal ions, and proteolytic enzymes (Maina *et al*., 2017; Sidhu and Nehra, 2019).

CONCLUSION

Enterocin NKR-5-3 is a multi-peptide bacteriocin produced by *Enterococcus faecium* NKR-5-3 and exhibits weak antimicrobial activity against *Listeria* spp. According to our study, only SC at final concentration of 1.5% (w/v) showed a synergistic interaction with enterocin NKR-5-3 (3,200 $AU \cdot mL^{-1}$) in inhibiting *L. innocua* ATCC 33090 in culture broth, when compared with EDTA (250 ppm) and SL (2% v/v). The bactericidal activity of the combination of SC and enterocin NKR-5-3 was confirmed by total viable count in culture media as well as morphological damage to target cells observed by SEM. This synergistic bactericidal action against *L. innocua* ATCC 33090 was also demonstrated in artificially contaminated shrimp meat stored at 4 °C. This finding can benefit further study or application of enterocin NKR-5-3 and other known bacteriocins for control of *Listeria* spp. in ready-to-eat or chilled fresh fish and fishery products.

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