Inhibitory effect of enterocin KP in combination with sublethal factors on *Escherichia coli* O157:H7 or *Salmonella* Typhimurium in BHI broth and UHT milk

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Abstract: The effects of physical and chemical sublethal treatments on the antibacterial activity of enterocin KP produced by *Enterococcus faecalis* KP against *Escherichia coli* O157:H7 and *Salmonella* Typhimurium were investigated. Enterocin KP was not active against intact cells of *E. coli* O157:H7 or *S.* Typhimurium. However, the use of enterocin KP together with ethylenediaminetetraacetic acid (50 mM), sodium tripolyphosphate (50 mM), sublethal heating (60 °C for 10 min), cold shock (–20 °C for 2 h), or acid stress (mixture of 40% lactic acid, 16% propionic acid, 16% acetic acid) in BHI medium decreased the cell number of *E. coli* O157:H7 by 7.27, 6.28, 3.39, 3.06, 4.20 log and *S.* Typhimurium by 7.21, 6.20, 3.64, 3.38, 3.98 log cfu/mL, respectively. The combination of enterocin KP with ethylenediaminetetraacetic acid decreased the cell number of *E. coli* O157:H7 in UHT milk to undetectable level, enterocin KP plus sodium tripolyphosphate or enterocin KP plus sublethal heating caused a reduction by 6.07 and 5.68 log cycles. The results of this study showed that enterocin KP could be applied as a biopreservative to inhibit *E. coli* O157:H7 and *S.* Typhimurium in combination with physical and food grade chemical hurdles.

Key words: Bacteriocin, enterocin KP, sublethal injury, gram-negative bacteria, UHT milk

1. Introduction

Over the past decades, food safety has become an important issue in many countries. Inhibition of food spoilage and foodborne pathogenic bacteria by natural, biological, or food-grade compounds is of great interest to the food industry due to public health and economic concerns. In food safety, gram-negative foodborne pathogenic and spoilage bacteria are especially problematic because of their inherent resistance to some natural antimicrobials (Helander and Mattila-Sandholm, 2000; Belfiore et al., 2007).

Biopreservation in the food industry has attracted great attention in recent years. Biopreservation can be defined as the extension and improvement of shelf life and safety of foods by natural or controlled microbiota and/or their antimicrobial compounds (Stiles, 1996). Bacteriocin producing lactic acid bacteria (LAB) and bacteriocin extracts fall within this concept. Bacteriocins are proteinaceous antimicrobial substances produced by many bacterial species. Bacteriocins produced by LAB have been widely studied due to their potential use in food preservation as natural biopreservatives. The use of bacteriocins in the food industry can help to reduce the addition of chemical preservatives as well as the intensity of heat treatments, resulting in foods that are more naturally preserved and richer in organoleptic and nutritional properties (Cleveland et al., 2001; Drider et al., 2006).

Although LAB bacteriocins have created considerable interest for use as food biopreservatives, they have several limitations. Their inhibitory activity is generally narrowed to gram-positive bacteria. Most LAB bacteriocins are not active against gram-negative bacteria. To be an effective food biopreservative, the bacteriocins preferably should have antibacterial activity against important gram-positive and gram-negative spoilage and pathogenic bacteria. Therefore, food application of bacteriocins relies on the use of multiple hurdles. The concept of hurdle technology is defined as the intelligent use of combinations of 2 or more antimicrobial factors or techniques acting synergistically and more effectively at suboptimal levels than each of them alone at the optimal level. This approach is particularly useful not only because it improves the stability and safety of foods but also because the acceptability of foods is enhanced (Leistner, 2000). The application of LAB

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bacteriocins as part of hurdle technology has received great attention in recent years. It has been verified that the antibacterial activities of LAB bacteriocins such as nisin and pediocin against gram-positive bacteria are greatly increased and their inhibitory spectrum also extends to gram-negative bacteria when used in combination with physical and chemical treatments damaging the bacterial outer membrane, which acts as a barrier against diffusion of bacteriocin molecules to the cytoplasmic membrane (Stevens et al., 1991; Kalchayanand et al., 1992, 1994; Schved et al., 1994; Cutter and Siragusa, 1995a, 1995b; Boziaris et al., 1998, Ananuo et al., 2005; Osmanağaoglu, 2005). Therefore, bacteriocins in combination with other antimicrobial factors may be useful tools for the implementation of methods intended to significantly reduce the load of food spoilage and foodborne pathogenic bacteria.

Enterocin KP produced by *Enterococcus faecalis* KP exhibits bactericidal activity against some of the gram-positive pathogenic and spoilage bacteria such as *Listeria monocytogenes*, *L. ivanovii*, *E. faecium*, and *E. faecalis* (Isleroglu et al., 2012). Researchers reported that enterocin KP did not cause a clear inhibition zone against *E. coli* O157:H7 or *Salmonella* Typhimurium on BHI agar. However, a decrease in cell density was observed at where enterocin KP was applied as a spot assay in solid medium.

The objective of the present study was to determine the efficacy of enterocin KP on *Salmonella* Typhimurium or *Escherichia coli* O157:H7 in combination with physical and chemical treatments that cause damage to the outer membrane in buffer and UHT milk.

2. Materials and methods

2.1. Organisms and culture conditions

Enterococcus faecalis KP was used as enterocin KP producer strain (Isleroglu et al., 2012). *Salmonella* Typhimurium RSSK 95091 and *E. coli* O157:H7 RSKK 232 were supplied by the Refik Saydam Hıfzıssıhha Culture Collection (Turkey). *Enterococcus faecalis* KP was propagated in De Mann Rogosa and Sharp (MRS) (Fluka, Germany) broth at 30–32 °C and the other bacteria were grown in Brain–Heart Infusion (BHI) (Fluka, Germany) broth at 35–37 °C. *Enterococcus faecalis* KP was maintained frozen at –80 °C in MRS containing 20% (v/v) glycerol, *S.* Typhimurium, and *E. coli* O157:H7 in BHI broth containing 20% (v/v) glycerol.

2.2. Preparation of enterocin KP

Enterocin KP was prepared by using the method given by Moreno et al. (2002). After the culture of *E. faecalis* KP grown in MRS broth at 32 °C for 18 h was centrifuged (8000 × *g* at 4 °C, 20 min), the pellet was discarded. The pH of cell-free culture supernatant was adjusted to 6.5 by the addition of 10 N NaOH and then it was filter-sterilized (0.45 µm). Filter-sterilized supernatant was subjected to ammonium sulfate precipitation (50% of saturation) and organic solvent precipitation (a methanol/chloroform mixture, 1:2, v/v). Finally, the pellet obtained by centrifugation was stored at –80 °C until used. The activity of enterocin KP against *Lactobacillus plantarum* in cell-free sterile supernatant and the pellet was 800 and 102,400 AU/mL, respectively.

2.3. Bacteriocin bioassay

Antimicrobial activity of enterocin KP was determined by using the agar spot test. For the bacteriocin assay test, *Lactobacillus plantarum* DSM 2601 was used as indicator bacterium, which is one of the most sensitive bacteria to enterocin KP. The antimicrobial activity was defined as the reciprocal of the highest serial 2-fold dilution showing a clear zone (at least 2 mm) of growth inhibition of the indicator strain and expressed as one arbitrary unit (AU) per milliliter of the bacteriocin preparation (AU/mL) (Bhunia et al., 1988).

2.4. Antimicrobial activity assays of enterocin KP to *E. coli* O157:H7 or *S.* Typhimurium

Enterocin KP (1600 AU/mL) was added to exponential phase cultures of *E. coli* O157:H7 or *S.* Typhimurium strains (about 10^7 cfu/mL) incubating in BHI broth at 25 °C. At desired intervals, samples were removed and serially diluted into sterile saline solution (NaCl 0.85%). The appropriate dilutions were plated on duplicate BHI plates, and the average numbers of colonies (cfu/mL) obtained after different periods of incubation at 25 °C were used to establish the growth and survival curves. In addition, the antimicrobial activity of enterocin KP against *E. coli* O157:H7 or *S.* Typhimurium was determined by agar spot test. Soft BHI agar (0.8% agar) inoculated with *E. coli* O157:H7 or *S.* Typhimurium at the level of about 10^7 cfu/mL was poured on BHI agar (1.5% agar) pre-poured plates. After solidification, 20 µL of enterocin KP (1600 AU/mL) was spotted on soft BHI agar and incubated at 35–37 °C for 24 h. At the end of the incubation, the plates were checked for inhibitory zones.

2.5. Effect of sublethal heating and cold shock on activity of enterocin KP

The overnight grown culture (10 mL) of *E. coli* O157:H7 or *S.* Typhimurium was harvested by centrifugation and resuspended in 100 mL of sterile peptone water (0.1%). Cell suspension of *E. coli* O157:H7 or *S.* Typhimurium was divided into 4 portions of 9 mL. The cell suspensions supplemented with 1 mL of enterocin KP preparation or supplemented with 1 mL of sterile water were heated at 60 °C for 10 min and then cooled immediately in water at 4 °C (Ananou et al., 2005). The final concentration of enterocin KP in the cell suspension was 1600 AU/mL.

To determine the effect of cold shock on inhibitory activity of enterocin KP, cell suspensions of *E. coli* O157:H7

or *S.* Typhimurium with/without enterocin KP were frozen at –20 °C for 2 h and then thawed immediately. The viable bacterial counts of the treated cells were determined by sampling on BHI plates in duplicate at 37 °C for 24–48 h (Boziaris and Adam, 2001). For both analyses, the initial concentration of *E. coli* O157:H7 or *S.* Typhimurium was about 4×10^8 cfu/mL.

2.6. Effect of chelating agents on activity of enterocin KP

An overnight culture of *E. coli* O157:H7 or *S.* Typhimurium cells was individually inoculated into 12 tubes containing sterile fresh BHI broth and then incubated at 35–37 °C until their OD values reached 0.1 at 600 nm. After that, each bacterial culture was centrifuged (15 min at $6000 \times g$) and supernatants were removed. The cell pellets were resuspended in 1 mL of the following solutions: (a) sterile peptone water, (b) disodium ethylenediaminetetraacetic acid (Na_2-EDTA) (50 mM), (c) sodium tripolyphosphate (STPP) (50 mM), (d) enterocin KP (1600 AU/mL), (e) EDTA plus enterocin KP, and (f) STPP plus enterocin KP. Cell suspensions were incubated at 35–37 °C for 30 and 60 min, centrifuged (at $6000 \times g$ for 15 min), washed with sterile peptone water, and resuspended again in 1 mL of sterile peptone water. Serial dilutions were made in sterile peptone water and plated in duplicate in BHI agar. After incubation at 35–37 °C for 24–48 h, the survivor cells were enumerated and bacterial counts were given as cfu/mL (Ananua et al., 2005). For both analyses, the initial concentration of *E. coli* O157:H7 or *S.* Typhimurium was about 4×10^8 cfu/mL.

2.7. Effect of acid stress on activity of enterocin KP

A cell suspension of *E. coli* O157:H7 or *S.* Typhimurium (about 4×10^8 cfu/mL) in sterile peptone water (0.1%) was exposed to a combination of enterocin KP (1600 AU/mL) and an acid solution containing 40% lactic acid, 16% propionic acid, and 16% acetic acid (pH 5.5). The acid solution was used at the 1% level, which gives a final acid concentration of 0.7%. The treated samples with enterocin KP plus acid solution and the control samples (just treated with acid solution or enterocin KP) were stored at 4–5 °C for 7 days. During the storage, samples were taken and the viable bacterial cells were determined by sampling on BHI agar plates in duplicate at 37 °C for 24–48 h (Kalchayanand et al., 1992).

2.8. Effect of enterocin KP alone or in combination with heat, EDTA, or STTP on *E. coli* O157:H7 in UHT milk

Cell suspensions of *E. coli* O157:H7 (about 2×10^8 cfu/mL) inoculated into UHT milk were exposed to the following treatments: (i) enterocin KP (1600 AU/mL), (ii) EDTA (50 mM), (iii) EDTA plus enterocin KP, (iv) STPP (50 mM), (v) STPP plus enterocin KP, (vi) sublethal heating (60 °C/10 min), and (vii) sublethal heating plus enterocin KP. During incubation at room temperature for 24 h, samples were periodically taken and viable cell counts were

determined by sampling on BHI agar plates in duplicate at 37 °C for 24–48 h. The UHT milk used in this study was obtained a retail market in Tokat (Turkey).

2.9. Statistical analyses

Data were analyzed using analysis of variance (ANOVA) performed with the Statistical Package for Social Sciences for Windows (version 11.0; SPSS, Chicago, IL, USA). The application of treatments (enterocin KP, sublethal heating, cold shock, acid stress, EDTA, STPP) was used as the factor. Differences were considered significant at $P < 0.05$. All experiments were performed 3 times.

3. Results

3.1. Inhibitory activity of enterocin KP against *E. coli* O157:H7 or S. Typhimurium

The growth of *E. coli* O157:H7 or *S.* Typhimurium in BHI broth at 25 °C was inhibited by enterocin KP (Figure 1). The decrease in the number of *E. coli* O157:H7 or *S.* Typhimurium cells was 0.66 and 0.45 log in the first hour of incubation and over the 24-h incubation period the reduction in viable cell numbers reached 1.27 and 0.98 log ($P < 0.05$), respectively. Although enterocin KP reduced the viable cell numbers, it did not give a clear inhibition zone against *E. coli* O157:H7 or *S.* Typhimurium when
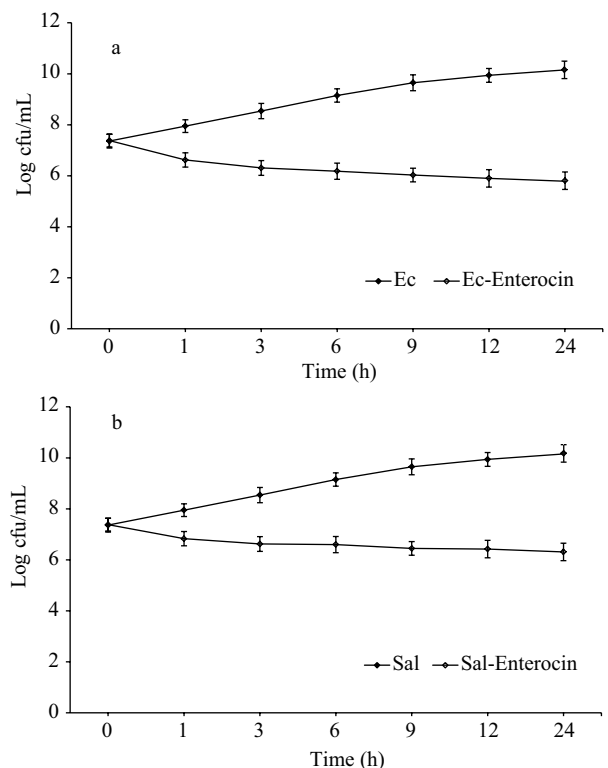

Figure 1. Effect of enterocin KP against *E. coli* O157:H7 (a) and *S.* Typhimurium (b) in BHI broth at 25 °C. Ec, *E. coli* O157:H7; Sal, *S.* Typhimurium.

tested by the agar spot test in solid medium (results not shown).

3.2. Effect of sublethal heating and cold shock on activity of enterocin KP against *E. coli* O157:H7 or S. Typhimurium

The effect of sublethal heating on survival of *E. coli* O157:H7 and *S.* Typhimurium in the presence or absence of enterocin KP is shown in Figure 2. A heat treatment of 60 °C for 10 min reduced the initial numbers of *E. coli* O157:H7 or *S.* Typhimurium by 1.09 and 1.21 log cfu/mL (P < 0.05), respectively. It was observed that surviving cells after heat treatment became sensitive to enterocin KP (P < 0.01). The combined effect of sublethal heating and enterocin KP reduced the cell counts of *E. coli* O157:H7 or *S.* Typhimurium by 3.39 and 3.64 log cfu/mL (P < 0.01), respectively.

Figure 3 shows the effect of cold shock with or without enterocin KP on the survival of *E. coli* O157:H7 and *S.* Typhimurium. The combination of enterocin KP and cold shock decreased the cell number of *E. coli* O157:H7 by 3.06 log cycles and *S.* Typhimurium by 3.38 log cycles (P < 0.01). It was observed that statistically there was not a significant difference between the sensitivity of these bacteria to treatment of enterocin KP together with cold shock (P > 0.05).

3.3 Effect of chelating agents on the activity of enterocin KP against *E. coli* O157:H7 or S. Typhimurium

The combination of enterocin KP with EDTA or STPP had a synergistic effect on the inhibitory activity of enterocin KP against *E. coli* O157:H7 or *S.* Typhimurium. Treatment of *E. coli* O157:H7 with EDTA or STTP in the presence of enterocin KP resulted in 7.27 and 6.28 log reduction in the viable counts after 60 min of incubation, respectively (P < 0.01) (Figure 4). The use of enterocin KP together with EDTA or STTP decreased the cell number of *S.* Typhimurium by 7.21 and 6.20 log at the end of incubation (P < 0.01), respectively (Figure 5). Addition of enterocin
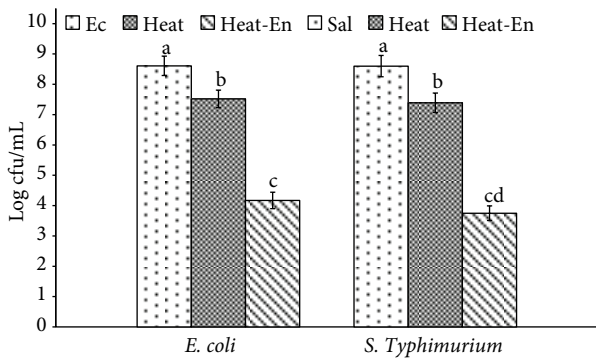
Figure 3. Effect of enterocin KP alone or in combination with freezing on *E. coli* O157:H7 or *S.* Typhimurium. Ec, *E. coli* O157:H7; En, enterocin KP; Sal, *S.* Typhimurium.

KP alone at 1600 AU/mL resulted in a decrease in the cell population of *E. coli* O157:H7 or *S.* Typhimurium by 0.71 and 0.50 log (P > 0.05), respectively (Figures 4 and 5). The application of EDTA or STTP alone did not cause any reduction in the number of cells (P > 0.05). Cell numbers of positive control samples containing only *E. coli* O157:H7 or *S.* Typhimurium increased during the incubation period (Figures 4 and 5).

3.4. Effect of acid stress on activity of enterocin KP against *E. coli* O157:H7 or S. Typhimurium

The combination of enterocin KP and acid stress decreased the cell numbers of *E. coli* O157:H7 or *S.* Typhimurium by 2.1 and 1.8 log cycles on day 1 of storage, and 4.20 and 3.98 log cycles on day 7 of storage, respectively (P < 0.01) (Figure 6). The cell numbers of *E. coli* O157:H7 and *S.* Typhimurium subjected to acid stress did not decrease significantly on day 1 of storage (0.42 and 0.37 log cycles), and the cell counts showed a further decrease with the extension of storage time (1.31 and 0.95 log cycles, P > 0.05) (Figure 6).

3.5. Effect of combination of enterocin KP with heat, EDTA, or STTP on *E. coli* O157:H7 in UHT milk

Application of enterocin KP or heat treatment alone caused only 0.50 and 0.92 log cycle decreases in the cell counts of *E. coli* O157:H7 in UHT milk after 1 h of incubation at 37 °C, respectively (Figure 7). Over the 24-h incubation period, the decrease in enterocin KP containing sample reached 1.11 log cfu/mL. EDTA or STTP did not reduce the cell counts of *E. coli* O157:H7 in UHT milk. However, the combination of enterocin KP with EDTA, STTP, or heat treatment resulted in a significant cell reduction in UHT milk. Treatment of *E. coli* O157:H7 cells with enterocin KP plus EDTA caused 6.95 log cycles reduction after 12 h of incubation, and the viable cell number was

Figure 2. Effect of enterocin KP alone or in combination with sublethal heating on *E. coli* O157:H7 or *S.* Typhimurium. Ec, *E. coli* O157:H7; En, enterocin KP; Sal, *S.* Typhimurium.
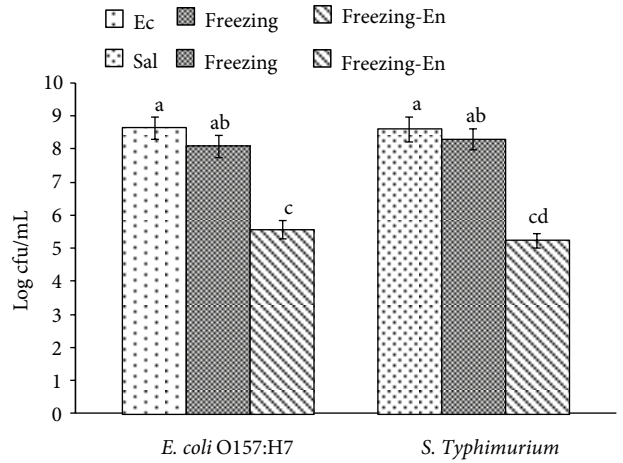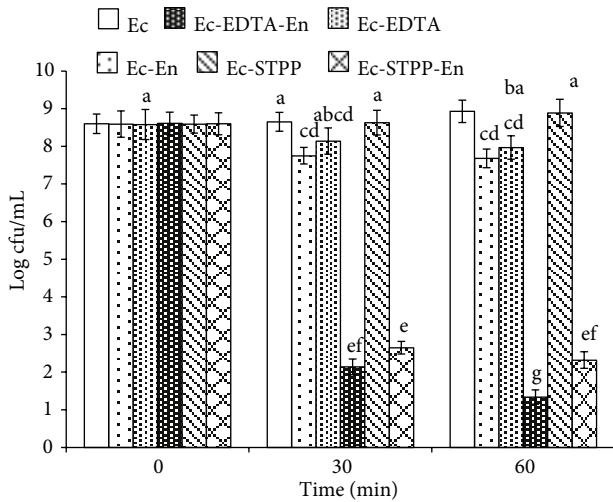
Figure 4. Effect of enterocin KP alone or in combination with EDTA or STTP on *E. coli* O157:H7. Ec, *E. coli* O157:H7; En, enterocin KP.

Figure 5. Effect of enterocin KP alone or in combination with EDTA or STTP on *S.* Typhimurium. En, enterocin KP; Sal, *S.* Typhimurium.

undetectable at the end of the incubation period (Figure 7). When *E. coli* O157:H7 was treated with enterocin KP plus STTP or enterocin KP plus heat treatment, the cell count decreased by 6.07 and 5.68 log cycles (P < 0.01) at the end of the incubation period, respectively (Figure 7).

4. Discussion

Enterocin KP decreased the viable cell number of *E. coli* O157:H7 and *S.* Typhimurium by only 1.27 and 0.98 log in BHI broth at the end of the incubation period. Although enterocin KP reduced the cell densities of both bacteria to a certain level, this reduction was not enough to cause a clear inhibition zone on BHI agar applied by the agar spot test. A similar result was reported by Isleroglu et al. (2012). Hence, it was assumed that both bacteria were resistant to enterocin KP because of their outer membrane. Therefore, in order to impair the integrity of the outer membrane and increase sensitivity of *E. coli* O157:H7 and *S.* Typhimurium to enterocin KP, both bacteria were exposed to some chemical and physical treatments.

The outer membrane of gram-negative bacteria acts as a permeability barrier to the action of antimicrobial compounds such as LAB bacteriocins, preventing their penetration to their site of action, the cytoplasmic membrane (Gao et al., 1999; Yethon and Whitfield, 2001). If the permeability of the outer membrane is altered by chemical or physical treatments, gram-negative bacteria can show sensitivity to bacteriocins (Kalchayanand et al., 1992, 1994; Boziaris et al., 1998).

EDTA and STPP, food-grade chelators, are used in a wide variety of food products to prevent oxidation and other deteriorative reactions catalyzed by metal ions. They
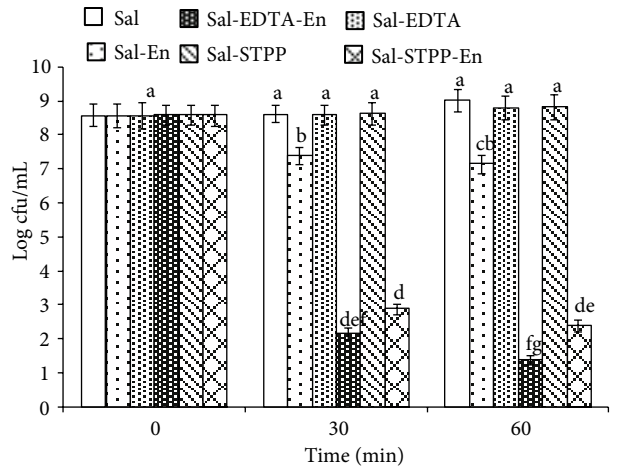
also have antimicrobial activity by enhancing the activity of antimicrobials and antibiotics, especially against gram-negative bacteria (Alakomi et al., 2003; Schrödter et al., 2008). There was a comparable loss of viability of *E. coli* O157:H7 and *S.* Typhimurium when they were treated with both enterocin KP and EDTA or enterocin KP and STTP. The use of enterocin KP together with EDTA or STTP decreased the cell number of these 2 gram-negative bacteria about 7 and 6 log cfu/mL, respectively. These results indicated that permeabilized cells became sensitive to enterocin KP. The combined effect of enterocin KP and EDTA offered a better result than enterocin KP and STTP did. Treatment of gram-negative bacteria with chelating agents generally results in removal of Ca^{2+} and Mg^{2+} cations from the lipopolysaccharide layer by chelation, destabilizing the outer membrane structure, altering its permeability, and allowing bacteriocins to reach the cytoplasmic membrane (Vaara, 1992; Helander et al., 1997; Yethon and Whitfield, 2001; Hancock and Rozek, 2002; Alakomi et al., 2003). As a result, EDTA and STPP overcome the penetration barrier in gram-negative bacteria, rendering these species sensitive to hydrophobic antibiotics and bacteriocins (Alakomi et al., 2003; Sampathkumar et al., 2003; Belfiore et al., 2007). The enhanced effect of chelators such as EDTA, disodium pyrophosphate, trisodium phosphate, hexametaphosphate, or citrate against gram-negative bacteria has been demonstrated for nisin both under laboratory conditions and in foods (Stevens et al., 1991; Cutter and Siragusa, 1995a, 1995b; Carneiro De Melo et al., 1998; Boziaris and Adams, 1999; Fang and Tsai, 2003). Brochrocin C, enterocin AS-48, pediocin P, cerein 8A, carnocyclin A, and carnobacteriocin BM1 also showed an increased antimicrobial activity on EDTA or STTP-treated gram-negative bacteria (Abriouel et al., 1998; Gao et al.,
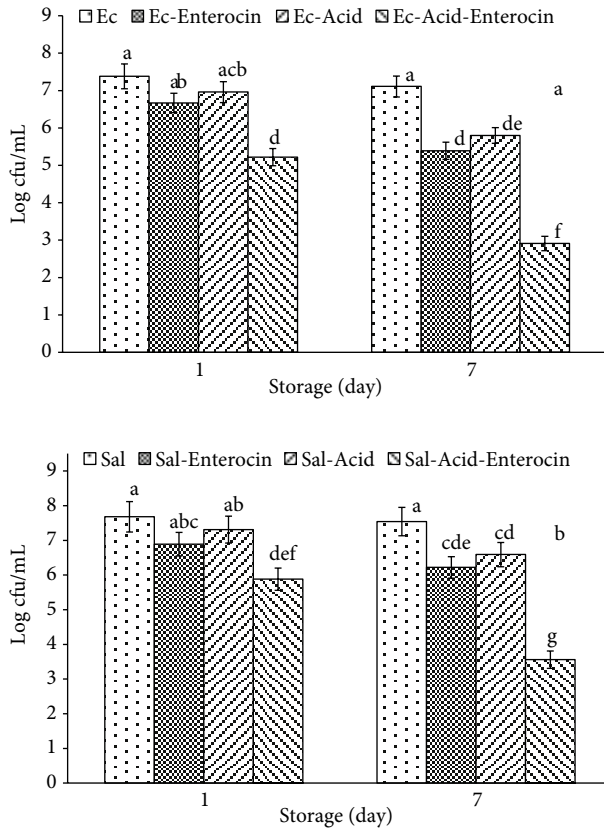
Figure 7. Effect of enterocin KP alone or in combination with EDTA, STTP, or sublethal heat treatment on *E. coli* O157:H7 in UHT milk. Ec, *E. coli* O157:H7; En, enterocin KP.

Figure 6. Effect of enterocin KP alone or in combination with acid stress on *E. coli* O157:H7 (a) and *S.* Typhimurium (b). Ec, *E. coli* O157:H7; En, enterocin KP; Sal, *S.* Typhimurium.

Cold shock treatment alone did not result in a significant decrease in the counts of *E. coli* O157:H7 (0.55 log cfu/mL) or *S.* Typhimurium (0.41 log cfu/mL) (P > 0.05). However, *E. coli* O157:H7 and *S.* Typhimurium became sensitive to enterocin KP when these cells were frozen along with enterocin KP application, and their viable cell numbers were decreased by 3.06 and 3.38 log cycles, respectively. Similar to sublethal heating, *S.* Typhimurium was more sensitive to the combination of enterocin KP and cold shock than *E. coli* O157:H7, but it was observed that statistically there was not a significant difference between the 2 bacteria (P > 0.05). Freezing causes some morphological and structural changes in the outer membrane of gram-negative bacteria, crystallizing the liquid-like lipids within membranes and creating channels (Haest et al., 1972). When the outer membrane has lost its integrity, bacteriocins may bind the phospholipid head groups and form transient transmembrane pores. These changes can cause sensitivity to hydrophobic compounds, efflux of periplasmic enzymes, and small molecules (Ter steeg et al., 1999; Boziaris and Adams, 2000; Cao-Hoang et al., 2008). The enhanced effect of cold shock and bacteriocins against gram-negative bacteria such as *Aeromonas hydrophila*, *S.* Typhimurium, *Yersinia enterocolitica*, *E. coli* 0157:H7, and *Pseudomonas fluorescens* has been demonstrated for nisin and pediocin AcH (Kalchayanand et al., 1992; Boziaris and Adams, 2000; Boziaris and Adams, 2001) and pediocin P (Osmanağaoğlu, 2005). Cao-Hoang et al. (2008) reported that the combining effect of rapid chilling and nisin application caused a dose-dependent reduction in the population of *E. coli* cells in both exponential and stationary growth phases. A reduction of 6 log of exponentially growing cells was achieved with rapid chilling in the presence of 100 IU/mL (1 IU/mL = 100 AU/

1999; Ananou et al., 2005; Osmanağaoğlu, 2005; Lappe et al., 2009; Martin-Visscher et al., 2011).

Application of enterocin KP together with sublethal heating caused the cell reduction of *E. coli* O157:H7 or *S.* Typhimurium by 3.39 and 3.64 log cycles, respectively. Based on the data obtained in this study, sublethally heated *S.* Typhimurium was more sensitive to enterocin KP than sublethally heated *E. coli* O157:H7, but this difference was not statistically significant (P > 0.05). Sublethal heat treatment causes some changes in the outer membrane, including morphological and structural changes, involving damage or release of lipopolysaccharides. These changes can alter the permeability barrier of the outer membrane, allowing the bacteriocin molecule to reach to its target side, the cytoplasmic membrane (Boziaris and Adams, 2001). Some researchers reported that gram-negative bacteria injured by heat treatment became highly sensitive to nisin (Kalchayanand et al., 1992; Boziaris et al., 1998; Boziaris and Adam, 2001), pediocin AcH (Kalchayanand et al., 1992), bacteriocin AS-48 (Abriouel et al., 1998), pediocin P (Osmanagaoglu, 2005), and enterocin AS-48 (Ananou et al., 2005).
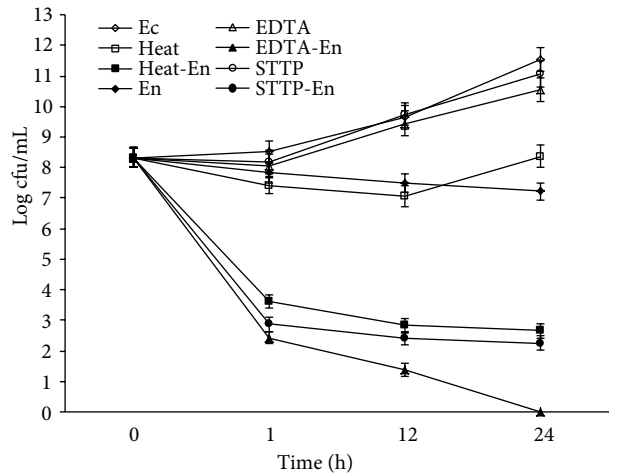
mL). Cells were more sensitive if nisin was present during stress.

Weak organic acids including lactic acid and acetic acid have been used for centuries to preserve foods. Lactic acid and acetic acid are able to cause sublethal injury to gram-negative bacteria such as *E. coli*, *P. aeruginosa*, and *S.* Typhimurium. Such injury involved disruption of the lipopolysaccharide layer (Roth and Keenan, 1971; Przybylski and Witter, 1979; Alakomi et al., 2000). In the present study, it was observed that *E. coli* O157:H7 and *S.* Typhimurium exposed to acid stress with the acid mixture containing lactic acid, propionic acid, and acetic acid gained sensitivity to enterocin KP and the level of surviving populations of these 2 gram-negative bacteria decreased with the extension of storage time. Our data are in accord with earlier findings that organic acids can sensitize gram-negative bacteria to bacteriocins by causing sublethal injury. Therefore, bacteriocins became active against gram-negative bacteria when used together with organic acids (Kalchayanand et al., 1992; Alakomi et al., 2000; Helander and Mattila-Sandholm, 2000; Osmanağaoğlu, 2005; Ananou et al., 2007).

The combination of enterocin KP with EDTA, STTP, or sublethal heat treatment caused a significant reduction in the cell number of *E. coli* O157:H7 in UHT milk. Treatment of enterocin KP together with EDTA decreased the cell number of *E. coli* O157:H7 in UHT milk to an undetectable level, while enterocin KP plus STTP or enterocin KP plus sublethal heat treatment caused a reduction by 6.07

and 5.68 log cycles at the end of the incubation period, respectively. These results indicate that the combination of enterocin KP with EDTA is more effective against *E. coli* O157:H7 in UHT milk than that of enterocin KP with STTP or with heat treatment. Ananou et al. (2005) reported that the combined application of enterocin AS-48 with 0.5% STPP or a sublethal heat shock to *E. coli* O157:H7 in apple juice caused the cells to become very sensitive to enterocin AS-48. The researchers reported that in both cases the survival cells were completely eliminated after 24-h incubation (initial *E. coli* population was 10^3 cfu/L).

From this work, we can conclude that the antimicrobial activity of enterocin KP against *E. coli* O157:H7 or *S.* Typhimurium, which are normally resistant to enterocin KP, can be enhanced by combination with one of the physical or chemical stresses. Consequently, the use of food-grade chelators, mild heat treatment, cold shock, or acid stress in combination with enterocin KP would be an ideal approach for the application of hurdle technology to inhibit the proliferation of gram-negative foodborne pathogens in foods, and enhance the shelf life and safety of foods.

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