International Food Research Journal 25(6): 2362-2371 (December 2018)
Journal homepage: *http://www.ifrj.upm.edu.my*

Characterization of *Listeria*-active bacteriocin produced by a new strain *Lactobacillus plantarum* subsp. *plantarum* SKI19 isolated from "sai krok e-san mu"

Botthoulath, V., *Upaichit, A. and Thumarat, U.

Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Songkhla 90112, Thailand

Article history

Received: 6 January 2018
Received in revised form: 13 April 2018
Accepted: 19 April 2018

Keywords

Listeria-active bacteriocin
Lactobacillus plantarum subsp. plantarum
Inhibition
Sausage.

Abstract

Bacteriocin produced by *Lactobacillus plantarum* subsp. *plantarum* SKI19 was harvested for purification during early stationary phase (optimum bacteriocin production). Purification of bacteriocin SKI19 was achieved by ammonium sulfate precipitation at 60% saturation followed by SP-Sepharose Fast Flow column. The molecular mass of bacteriocin SKI19 determined by Tricine-SDS-PAGE analysis was estimated to 2.5 kDa. Physical and biochemical studies revealed that purified bacteriocin SKI19 was heat-stable even after autoclaving at 121°C for 15 mins, and was active over a wide pH range (2.0-10.0). A reduction in antibacterial activity was observed after treatment of the bacteriocin SKI19 with proteolytic enzymes (α-chymotrypsin, trypsin, proteinase K, and pronase E), confirming that bacteriocin SKI19 is proteinaceous in nature. When viewed under SEM, cell membranes of the indicator strain appeared to collapse after exposure to bacteriocin SKI19. It was found that bacteriocin SKI19 inhibited not only closely related species, but was also effective against *Listeria monocytogenes* DMST 17303. Moreover, complete inhibition (*L. monocytogenes* DMST 17303) or significant inhibition (*Escherichia coli* DMST 4212, and *Staphylococcus aureus* DMST 8840) by SKI19 was observed in co-cultivation under anaerobic conditions. The results suggest that bacteriocin SKI19 as well as the direct use of SKI19 as a starter culture might be applied as biocontrol agents to improve the shelf-life of certain food products.

Introduction

Lactic acid bacteria (LAB) are a group of Gram-positive bacteria that are non-respiring, non-spore forming, catalase negative, acid-resistant, low G+C content, and anaerobic-aerotolerant. They have various shapes, like rods (bacilli) and spheres (coccus). They can be found mainly in natural environments such as the gastrointestinal tract of various animals, dairy products, seafood products, soils and on some plant surfaces (Aureli *et al.*, 2011). Substrate utilization, metabolic capabilities, and probiotic properties are associated principally with their physiological characteristics. LAB do not only produce lactic acid during their fermentation, but they also liberate antimicrobial substances, such as antimicrobial peptides (AMPs) or bacteriocins. These, degraded by proteases in the gastrointestinal tract (GI), have gained much attention as safe biopreservatives, since bacteriocins produced by LAB are generally considered as safe (GRAS) (Pingitore *et al.*, 2007). These antimicrobial compounds are ribosomally synthesized, extracellularly, released low molecular mass peptides or peptide complexes (usually 30-60 amino acids), having bactericidal or biostatic activity. Some bacteriocins appear to inhibit different indicator bacteria, including food poisoning bacteria, foodborne pathogenic bacteria, and closely related species (Swetwiwathana and Visessanguan, 2015).

Sai krok e-san mu is one of the most popular and accepted sausages among consumers, in particularly Northeastern region of Thailand. Sai krok e-san mu is made up of basic ingredients like pork, cooked rice, fresh garlic, salt, and sugar. The product is allowed to ferment at ambient temperature for approximately 2 or 3 days, during which time it attains a low pH (Phithakpol *et al.*, 1995). The growth of various microorganisms is associated with the fermentation process of sai krok e-san mu. Among them, LAB dominates as a natural microflora in fermentation comprising of species such as *Lactobacillus plantarum, Lactobacillus pentosus, Lactobacillus sakei, Pediococcus pentosaceus,* and *Pediococcus acidilactici* (Visessangua *et al.*, 2015). However, Paukatong and Kunawasen (2001) reported that fermented meat products produced by naturally occurring LAB are often contaminated by pathogenic

*Corresponding author.
Email: *apichat.u@psu.ac.th*

bacteria such as *Salmonella* spp., *S. aureus, Bacillus cereus, E. coli,* and *L. monocytogenes.* Though chemical preservatives have been applied in inhibiting these harmful bacteria, long-term consumption of the artificial chemical preservatives may risk the health of consumers (Lü *et al.*, 2014). Thus, the demand to improve the products' stability and shelf-life by inhibiting undesirable spoilage and pathogenic microorganisms is imperative and being given increased attention by Thai fermented sausage producers. This suggests that bacteriocin-producing strains could be used as natural preservative agents. There are several reports on the isolation and characterization of bacteriocin-producing LAB from various sources (Ogunbanwo *et al*., 2003), and there are several types of bacteriocins from food-associated LAB that are involved in numerous food fermentations, and have many potential applications of protective cultures in various food systems (Cleveland *et al*., 2001; Cotter *et al.*, 2005; Zachar of and Lovitt, 2012). From this point of view, the industrial processes require specific characteristics of bacteriocins for particular products. Therefore, it is vital to characterize the antimicrobial substances produced by the bacterial strains, to obtain the desired characteristics.

The objectives of the present study were to purify and characterize bacteriocin produced by *L. plantarum* subsp. *plantarum* SKI19 isolated from sai krok e-san mu. The strain was also tested in co-cultivation with pathogenic bacteria, with the aim of using this selected LAB strain as a starter bio-protective culture for further application in certain food products.

Materials and Methods

Bacterial strains and culture conditions

L. plantarum subsp. *plantarum* SKI19 (GenBank accession number is KY762264) used in this study was previously isolated from 10 freshly prepared sai krok e-san mu samples, which were bought from local markets in Songkhla province. The indicator strains of food spoilage and foodborne pathogenic bacteria used in this research were based on Thai Community Product Standards. All of these indicator strains were purchased from the Department of Medical Science, Ministry of Public Health, Thailand (DMST). The genetically closely related species used as indicator strains were purchased from the Thailand Institute of Scientific and Technological Research (TISTR), the Japan Collection of Microorganisms (JCM), and were also obtained from our own cultured stocks. The *Enterococcus faecalis* CM6CR07 and *Enterococcus faecalis* CF1GI14 were kindly provided by Dr. Noraphat Hwanhlem (Hwanhlem *et al.*, 2017). Growth media and incubation conditions of these strains are summarized in Table 1. All strains used in this study were maintained as stock cultures at -80°C in 30% glycerol. The strains were subcultured at least twice prior to the experiments.

Table 1. Antibacterial spectrum of bacteriocin produced by *L. plantarum* subsp. *plantarum* SKI19

Indicator strain	Growth* medium	Growth condition (°C/h)	Activity**
Closely related species			
E. faecalis CF1GI14	MRS agar, MRS broth	37°C/24-48 h, Aerobic	++
E. faecalis CM6CR07	MRS agar, MRS broth	37°C/24-48 h, Aerobic	++
E. faecium CKF12	MRS agar, MRS broth	37°C/24-48 h, Aerobic	++
E. lactis CKF16	MRS agar, MRS broth	37°C/24-48 h, Aerobic	++
L. plantarum TISTR 875	MRS agar, MRS broth	37°C/24-48 h, Aerobic	–
L. plantarum JR21	MRS agar, MRS broth	37°C/24-48 h, Aerobic	–
L. sakei subsp. *sakei* JCM 1157	MRS agar, MRS broth	37°C/24-48 h, Aerobic	++
L. lactic FMK14	MRS agar, MRS broth	37°C/24-48 h, Aerobic	–
L. mesenteroides TISTR 053	MRS agar, MRS broth	37°C/24-48 h, Aerobic	–
P. pentosaceus TISTR 413	MRS agar, MRS broth	37°C/24-48 h, Aerobic	–
Food spoilage and foodborne pathogens			
B. cereus DMST 5040	NA, NB	37°C/24-48 h, Aerobic	–
B. coagulans AGM-BB2	NA, NB	37°C/24-48 h, Aerobic	–
B. subtilis AGM-BB3	NA, NB	37°C/24-48 h, Aerobic	–
C. perfringens DMST 16637	TA, TB	37°C/24-48 h, Anaerobic	–
E. coli DMST 4212	LA, LB	37°C/24-48 h, Aerobic	–
L. monocytogenes DMST 17303	TSA, TSB	37°C/24-48 h, Aerobic	++
S. Enteritidis DMST 15676	TSA, TSB	37°C/24-48 h, Aerobic	–
S. Typhimurium DMST 15674	TSA, TSB	37°C/24-48 h, Aerobic	–
S. aureus DMST 8840	NA, NB	37°C/24-48 h, Aerobic	–

*Growth medium: LA, Luria Bertani agar; LB, Luria Bertani broth; MRS, de Man Rogosa and Sharpe medium; NA, Nutrient agar; NB, Nutrient broth; TA, Thioglycolate agar; TB, Thioglycolate broth; TSA, Trypticase soy agar; TSB, Trypticase soy broth.

**Diameter of inhibition zone: -, no inhibition zone; +, 5 – 10 mm; ++, 11 – 20 mm; +++, > 20 mm.

Bacteriocin SKI19 production during growth

The seed culture was prepared by streaking the glycerol stock (-80°C) of strain SKI19 on MRS agar and incubated for 24-48 hr at 37°C to obtain the bacterial colony. One loop full of colony was inoculated into 5 ml MRS broth and incubated for 24 hr at 37°C. After the cell growth, 10% of cell culture (10^8-10^9 CFU/ml) was inoculated into 4.5 ml MRS broth and incubated at 37°C for 24 hr. Then, 1% of active culture of SKI19 was inoculated into 1,000 ml of MRS broth and incubated at 37°C. Aliquots of 10 ml were examined for growth (OD 600 nm) and pH changes were monitored every 3 hr, up to 48 hr. The amount of bacteriocin in the neutralized cell free

culture supernatant (NCFS) was determined using two-fold dilution by agar well diffusion method. The antagonistic activity was expressed in arbitrary unit per milliliter (AU/ml). An arbitrary unit (AU) was calculated as 50 µl of the highest dilution of the two-fold serial dilution that gives result of a minimal visible inhibition zone against *L. monocytogenes* DMST 17303 (10^6 CFU/ml). The arbitrary unit per milliliter was calculated according to the formula below:

$$AU/ml=(1000/V)\times D$$ (H-Kittikun *et al.*, 2015)

Where, D is the dilution factor.

V is the volume of neutralized cell free culture supernatant (NCFS) (50 µl).

Purification of bacteriocin produced by SKI19

Ten percent of seed culture was inoculated into MRS broth (1,000 ml) and incubated for 24 hr at 37°C. After incubation, the whole culture broth was collected by centrifugation at 10,000 xg for 20 mins at 4°C. Cell free culture supernatant was submitted to saturate with ammonium sulfate in ranking from 0-60% and kept stirring for 30 mins at 4°C to precipitate out the proteins. After centrifugation, the pellet was collected to suspend in 20 mM phosphate buffer, pH 6.8 (buffer A) and dialyzed using a 1 kDa cut-off membrane (Spectra/Por® 6 Dialysis Membrane, USA) against the same buffer at 4°C overnight. The dialyzed protein of 3 ml was loaded onto an SP-Sepharose Fast Flow cation-exchange column (GE Healthcare, Sweden), equilibrated with the same buffer. Then the column was washed with buffer A and eluted stepwise using different concentrations of NaCl (0.2-1 M in 20 mM sodium phosphate buffer, pH 6.8) at a flow rate of 1 ml/min. The protein fractions were measured at 280 nm and checked for antagonistic activity against *L. monocytogenes* DMST 17303 (10^6 CFU/ml) by agar well diffusion method. The active fractions were pooled and subjected to HiTrap Desalting column (GE Healthcare, Sweden) to desalt. The column was equilibrated with the buffer A, the active fractions were loaded manually using a 5 ml disposable syringe, and washed with the buffer A. The protein was eluted by using 20 mM sodium phosphate buffer (pH 6.8), and tested for antibacterial activity against *L. monocytogenes* DMST 17303 (10^6 CFU/ml) by agar well diffusion method. After that, purified bacteriocin SKI19 was submitted to be lyophilized and kept at -20°C for further experiments. The protein concentration of the bacteriocin SKI19 was performed by Lowry's method (Lowry and Rosenbrough, 1951), using bovine serum albumin as the standard.

Molecular weight determination of bacteriocin SKI19

The molecular weight of purified bacteriocin SKI19 was determined by Tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis (Tricine-SDS-PAGE), described by Schägger and Von Jagow (1987). Polyacrylamide concentration in stacking and separating gel were 5.0% and 16.5%, respectively. A 20 µl aliquot of the sample was mixed with 20 µl of sample buffer and heated for 10 mins at 70°C. Electrophoresis was performed at a constant 100 volts on a Mini-PROTEIN Tetra Electrophoresis System (Bio-rad laboratories, USA) at a constant current of 20 mA in stacking gel and 30 mA in running gel. The molecular weight markers Thermo Scientific Spectra Multicolor Low Range Protein Ladder (2-40 kDa) was used as a standard marker (Thermo Fisher Scientific, USA).

After electrophoresis, the gel which contained the molecular marker was cut and stained with Coomassie Brilliant Blue G-250 (Thermo Fisher Scientific, USA), and destained by washing overnight with a mixture of acetic acid-methyl alcohol-water (5:5:1) until the background stain disappeared. The other half of the gel was immersed in a fixation solution (12.5% trichloroacetic acid) for 30 mins. Subsequently, the gel was washed twice in sterilized distilled water for 30 mins. The gel was then placed into a Petri dish and overlaid with 25 ml of TSA soft agar (0.8%) seeded with *L. monocytogenes* DMST 17303 (10^6 CFU/ml). The dish was incubated at 37°C for 24 hr. The result was analyzed for an inhibition zone and compared with the Coomassie Brilliant Blue G-250 stained gel.

Inhibitory spectrum of bacteriocin SKI19

Antibacterial activity spectrum was determined using an agar well diffusion assay (Parente and Hill, 1992). Soft agar (0.8% agar, 48°C) was first seeded with the indicator strains (10^6 CFU/ml) and then mixed. The mixture was poured into a sterile Petri dish, and after solidification, wells of uniform diameter (5 mm) were punched using a cork borer. Aliquots (50 µl) of purified bacteriocin SKI19 was dispensed into each well and incubated at 37°C for 24 hr. Fifty microliters of MRS broth was applied as a control, and the results are expressed as the diameter of inhibition zone, measured using Vernier calipers. The indicator strains are shown in Table 1.

Effect of pH, temperature, and enzymes on bacteriocin SKI19 activity

The pH effect on purified bacteriocin SKI19 stability

was investigated according to Lü *et al*. (2014) by adjusting the pH of bacteriocin solution (2,560 AU/ml) in a range of pH values from 2.0 to 12.0 with the following buffers in the concentration of 20 mM: glycine-HCl (pH 2.0), citric acid phosphate (pH 3.0, 4.0, 5.0, 6.0), sodium phosphate (pH 7.0), Tris-HCl (pH 8.0, 9.0) and glycine-NaOH (pH 10.0, 11.0, 12.0). The untreated bacteriocin SKI19 was used as a control. The samples were then incubated at 37°C for 3 hr and readjusted to pH 6.5, thereafter the residual bacteriocin activity was expressed in arbitrary unit per milliliter (AU/ml) that showed in a clear zone of growth inhibition against *L. monocytogenes* DMST 17303 (10^6 CFU/ml) by agar well diffusion assay.

The effect of temperature on purified bacteriocin SKI19 stability was tested by keeping 100 µl (2,560 AU/ml) of purified bacteriocin in 20 mM phosphate buffer (pH 7.0). The bacteriocin stability was performed in various temperatures (4°C, 30°C, 40°C, 60°C, 70°C, 80°C, and 100°C) for 15, 30, 60, and 120 mins in temperature-controlled water bath. Also, the autoclaving at 121°C for 15 and 30 mins were performed. The untreated bacteriocin SKI19 was used as a control. The residual bacteriocin activity was then presented in arbitrary unit per milliliter (AU/ml) that indicated the inhibition zone against *L. monocytogenes* DMST 17303 (10^6 CFU/ml) by agar well diffusion assay.

The following enzymes were tested, including trypsin, α-chymotrypsin, proteinase K, and pronase E (Sigma, USA). These enzymes were dissolved in 50 mM potassium phosphate buffer (pH 7.8). Lipase and α-amylase (Sigma, USA) were dissolved in 50 mM potassium phosphate buffer (pH 7.0). Pepsin (Sigma, USA) was dissolved in 50 mM glycin-HCl buffer (pH 2.2). All enzymes were prepared as stock solutions in a concentration of 10 mg/ml and used at a final concentration of 1 mg/ml. A 45 µl aliquot (2,560 AU/ml) of purified bacteriocin SKI19 was mixed with 5 µl of each enzyme solution. The mixtures were incubated at 37°C for 3 hr. The bacteriocin SKI19 in buffer (without enzyme), enzyme-buffer, and buffer alone were used as controls. All samples were readjusted to pH 6.5 and heated for 5 mins at 100°C to inhibit activity of used enzymes. After cooling, the residual bacteriocin activity was defined in arbitrary unit per milliliter (AU/ml) that displayed a clear zone of growth inhibition against *L. monocytogenes* DMST 17303 (10^6 CFU/ml) by agar well diffusion assay.

Scanning electron microscopy (SEM)

Bacterial cells for SEM were prepared as described by Wen *et al*. (2016) with minor modifications. *L. monocytogenes* DMST 17303 was used as the test indicator and cultured in trypticase soy broth at 37°C for 7 hr to approximately 10^6 CFU/ml. Thereafter, the bacterial cells were treated with or without purified bacteriocin SKI19 (2,560 AU/ml) and incubated at 37°C for 3 hr. Then, cells were collected by centrifugation at 5000 xg for 15 mins at 4°C and washed three times using 0.1 M phosphate buffer saline (PBS) (90 g/L NaCl, 10.9 g/L NaH_2PO_4, 3.2 g/L NaH_2-PO_4) (pH 7.2). A thin smear was prepared on a glass slide and fixed in 2.5% glutaraldehyde for 1 hr. Then, the fixed samples were washed twice with 0.1 M PBS and followed by washed twice with sterile distilled water for 15 mins. In the next step, the dehydration was performed using series ethanol from 50% to 100%, kept at 4°C and dried with CO_2. The dried cells were coated with gold in a sputter coater. Samples were observed under SEM (Quanta 400, Czech Republic).

Inhibitory effect of SKI19 against pathogenic bacteria in co-cultivation

Co-cultivation of SKI19 with pathogenic bacteria was determined according to the method described by Hongpattarakere *et al*. (2012). The co-cultivation was evaluated in minimal medium containing 22.0 g/L peptone water, 2.0 g/L yeast extract, 0.1 g/L NaCl, 0.04 g/L K_2HPO_4, 0.01 g/L $CaCl_2.6H_2O$, 2 g/L $NaHCO_3$, 0.01 g/L $MgSO_4.7H_2O$, 0.5 g/L bile salt, 2 mL Tween-80, 0.5 g/L L-cysteine and supplemented with 0.1% glucose (pH 6.5). For each experiment, strains were inoculated in equal proportions with 10^5 CFU/ml in the co-culture and the monoculture, which was used as a control. All samples were incubated at 37°C for 48 hr under anaerobic conditions. After 0, 6, 12, 18, 24, 36, and 48 hr of incubation, samples were collected to analyze for pH, SKI19, and remaining pathogenic bacteria. An appropriate ten-fold serial dilution was spread-plated onto selective agar for enumeration of the individual strain. Growth of SKI19 was enumerated on MRS agar, while the enumeration of pathogenic bacteria including *E. coli* DMST 4212, *L. monocytogenes* DMST 17303, and *S. aureus* DMST 8840 were determined on Violet red bile agar, Palcam agar, and Baird-parker agar (Himedia, India), respectively. The results were expressed as $\log_{10}$ CFU/ml.

Results and Discussion

Growth and bacteriocin SKI19 production

L. plantarum subsp. *plantarum* SKI19 was monitored for cell growth, pH reduction, and bacteriocin production (AU/ml) as the results

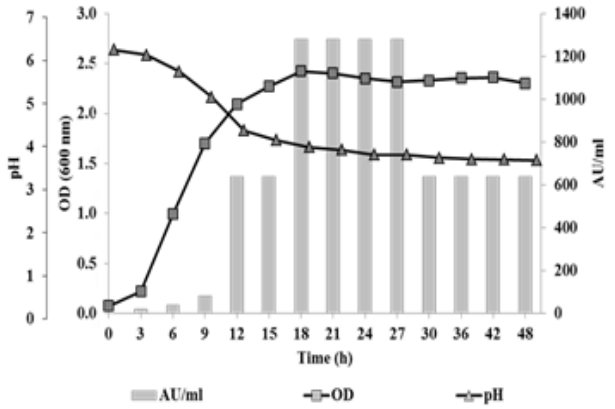

Figure 1. Growth (OD 600 nm), bactericocin production (AU/ml) and pH reduction of *L. plantarum* subsp. *plantarum* SKI19 in MRS broth at 37°C.

presented in Figure 1. The cell density of SKI19 increased at log phase after 6 hr of incubation, subsequently reached maximum at 18 hr, and then entered the stationary phase, which lasted till 48 hr of growth at 37°C. Production of bacteriocin produced by SKI19 was first detected after 3 hr of incubation at the value of 20 AU/ml, increased to the highest level of 1,280 AU/ml during in the following 18 hr, remained stable for the next 8 hr, and moderately declined to 640 AU/ml for the rest of the fermentation period. SKI19 produced the optimum bacteriocin during early stationary growth, which may revealed that the peptide is a primary metabolite (da Silva Sabo *et al.*, 2014). Similar trends have been reported for bacteriocin ST8KF (Powell *et al.*, 2007), plantaricin MG (Gong *et al.,* 2010), and bacteriocin ST22Ch, ST153Ch, and ST154Ch (Todorov *et al*., 2013). The level of pH in the fermentation was decreased from 6.4 to 4.3 during the first 15 hr of incubation, continuously reduced to around 3.96 during the next 9 hr. This presents the maximum period of the bacteriocin production. The sharp increase in bacteriocin activity for SKI19 from 640 AU/ml to 1,280 AU/ml occurred at pH between 4.3 and 3.96. In the late stationary-phase culture, bacteriocin production was moderately dropped with pH levels, appeared steady until the end of the incubation period. Yang *et al.* (1992), Van Reenen *et al*. (1998) and Powell *et al*. (2007) reported that such a small change in culture pH may not facilitate the release of bacteriocin SKI19 from the outer surface of the producer cells. The metabolism of residual nutrients or medium compositions could be influential for the enhanced activity of bacteriocin, not for cell growth (Todorov *et al.*, 2013). The partial digestion of the antibacterial compound by proteolytic enzymes secreted from the cells in late stationary phase might have affected the diminishing antibacterial activity (Gong *et al.*, 2010). Our results revealed that from 18-27 hr was an appropriate period to collect the sample for bacteriocin purification.

Purification of bacteriocin SKI19

The cell free supernatant (CFS) obtained from a 18 hr culture of SKI19 was collected for purification of bacteriocin. The first step of bacteriocin purification was performed by 60% ammonium sulphate precipitation. Recovery of partial purified bacteriocin SKI19 was 24% of the initial total activity with an approximately 6.14-fold increase in the specific activity. The dialyzed sample obtained from Spectra/Por® 6 Dialysis Membrane was purified by cation exchange chromatography using a SP-Sepharose Fast Flow column. The active fractions were eluted with 20 mM sodium phosphate buffer containing 0.4 M NaCl. Therefore, based on two purification steps, the purified bacteriocin SKI19 had a yield of 10% and a purification of 15.52-fold. It showed the highest antagonistic activity (6,918.91 AU/ml) against *L. monocytogenes* DMST 17303 by agar well diffusion assay. Most bacteriocin purification strategies are based on ion-exchange and hydrophobic-interaction chromatography's (Barbosa *et al.*, 2013), due to their being able to separate the peptides based on their electric charge by manipulating buffer pH. Also, most LAB-bacteriocins contain positively charged amino acid residues and present hydrophobic characteristics at pH lower than their isoelectric point. This suggests that the use of cation-exchange resin is appropriate for their purification (Pingitore *et al.*, 2007; Nishie *et al*., 2012). These methods were successfully used in the purification of bacteriocins produced by *Lactobacillus* spp. including plantaricin MG (Gong *et al.*, 2010), bacteriocin MBSa4 (Barbosa *et al.*, 2016), and plantaricin K25 (Wen *et al*., 2016).

Molecular weight of bacteriocin SKI19

Purified bacteriocin SKI19 from SP-Sepharose Fast Flow column was submitted to determine the molecular weight by Tricine-SDS-PAGE and achieved an assay for checking activity against *L. monocytogenes* DMST 17303. The molecular weight of purified bacteriocin SKI19 was estimated at 2.5 kDa with the inhibition zone corresponding to the position of the bacteriocin SKI19 band (Figure 2a). Uymaz *et al.* (2011) reported that the bacteriocin produced by *L. plantarum* BG33 isolated from traditional Turkish Tulum cheese, which has a molecular weight of 2.7 kDa. *L. plantarum* MBSa4 isolated from Brazilian salami produced a two-peptide plantaricin with a molecular mass of 2.3 kDa (Barbosa *et al*., 2016).

Inhibitory spectrum of bacteriocin SKI19

The inhibitory spectrum of antibacterial activity of purified bacteriocin SKI19 was assessed by agar well diffusion method, as displayed in Table 1. The bacteriocin SKI19 exhibited inhibitory activity against the closely related species namely, *E. faecalis* CF1GI14, *E. faecalis* CM6CR07, *E. faecium* CKF12, *E. lactis* CKF16, and *L. sakei* subsp. *sakei* JCM 1157. Moreover, the inhibition by bacteriocin SKI19 also suppressed the growth of foodborne bacterium like *L. monocytogenes* DMST 17303. Cleveland *et al.* (2001) theorized that the class IIa bacteriocins include bacteriocin-like Listeria-active peptides, dominated a conserved N-terminal sequence Tyr–Gly–Asn–Gly–Val, and two cysteines forming a disulfide bridge in the N-terminal half of the peptide. Thus, our results reveal that the anti-Listeria organism in the inhibition region is predominantly this bacteriocin SKI19. Furthermore, bacteriocin SKI19 did not inhibit the tested probiotic strains such *L. plantarum* TISTR 875, *L. plantarum* JR21, *L. lactic* FMK14, *L. mesenteroides* TISTR 053, and *P. pentosaceus* TISTR 413, suggesting the high possibility of using this bacteriocin SKI19 as a biopreservative in fermented foods for control of *L. monocytogenes,* without affecting probiotic cultures (Barbosa *et al.*, 2013). However, no activity was observed towards Gram-positive bacteria (*B. cereus* DMST 5040, *B. coagulan* AGM-BB2, *B. subtilis* AGM-BB3, *C. perfringens* DMST 1663, and *S. aureus* DMST 8840) and Gram-negative bacteria (*E. coli* DMST 4212, *S.* Enteritidis DMST 15676, and *S.* Typhimurium DMST 15674), due to their supplementary layers (phospholipids, proteins, and lipopolysaccharides) that make the outer membrane of this class of bacteria to act as a permeability barrier and hinder the site for bacteriocin action (Parada *et al.*, 2007; Gong *et al.*, 2010). A similar observation has been reported regarding some of these bacteriocins, notably plantaricin NC8 (Maldonado *et al.*, 2003), bacteriocin PMU33 (Noonpakdee *et al.*, 2009), and bacteriocin MBSa4 (Barbosa *et al.*, 2016), produced by *L. plantarum* strains, which appeared to inhibit only Gram-positive bacteria, but showed no activity against Gram-negative bacteria. In contrast, plantaricin MG (Gong *et al.*, 2010), plantaricins 163 (Hu *et al.*, 2013), and plantaricin K25 (Wen *et al.*, 2016) were active against both Gram-positive and Gram-negative bacteria. This may depend on the different bacteriocin-producing strain (Powell *et al.*, 2007; Xie *et al.,* 2011).

Effects of pH, temperature, and enzymes on bacteriocin SKI19 activity

The activity of bacteriocin SKI19 was unaffected after 2 hr incubation in the pH range from 2.0 to 10.0 (2,560 AU/ml). But, part of its activity was complete inactivation when treated at pH 11.0-12.0. Furthermore, the activity of bacteriocin SKI19 remained steady at 4, 30, 40, 60, 70, 80, 100°C after 2 hr incubation, and after autoclaving at 121°C for 15 mins. A moderate decrease in activity was observed after heating at 121°C for 30 mins (Table 2). Oscariz and Pisabarro (2001) theorized that a strong positive net charge and a high isoelectric point make bacteriocins stable and working in a wide range of pH. This facilitates them to interact at physiological pH values with an anionic surface of Gram-positive bacteria. In addition, their complex structure of mono-sulfide and disulfide intra-molecular bonds enhances the secondary structures to have more stability and higher bacteriocin activity. This is in agreement with the studies by Gong *et al.* (2010) that plantaricin MG produced by *L. plantarum* KLDS1.0391 was shown to be thermo-stable and maintained activity within a range of pH 2.0 to 10.0. Also, Todorov *et al.* (2013) tested antimicrobial activity produced by *L. sakei* and found its bacteriocin activity remained stable after incubation at pH 2.0-10.0, and was heat-stable. Our results indicate that bacteriocin produced by *L. plantarum* subsp. *plantarum* SKI19 provided thermo-

Table 2. Effect of pH, temperature, and enzymes on activity of purified bacteriocin produced by *L. plantarum* subsp. *plantarum* SKI19.

Treatment	Bacteriocin activity (AU/ml)[a]
Enzymes	
α-chymotrypsin	0
Trypsin	0
Pronase E	0
Proteinase K	0
Pepsin	2,560
Lipase	2,560
α-amylase	2,560
Catalase	2,560
Control (without enzyme)	2,560
Temperature	
4°C/15-120 mins	2,560
30°C/15-120 mins	2,560
40°C/15-120 mins	2,560
60°C/15-120 mins	2,560
70°C/15-120 mins	2,560
80°C/15-120 mins	2,560
100°C/15-120 mins	2,560
121°C/15 mins	2,560
121°C/30 mins	640
pH	
2.0-10.0	2,560
11.0-12.0	0

[a]Triplicate performance.

tolerant and pH stable characteristics. This strain thus shows some promise of possible application as a bacteriocin for food preservation in the food industry. Moreover, low pH bacteriocin SKI19 stability shows the capacity for application in acidified products with long shelf-life (Barbosa *et al*., 2016).

Purified bacterioicn SKI19 was completely inactivated when treated with proteolytic enzymes, notably trypsin, α-chymotrypsin, proteinase K, and pronase E, confirming it is basically proteinaceous in nature. Treatment of purified bacteriocin SKI19 with pepsin did not affect activity (Table 2). A similar observation was reported by González *et al*. (1994) that plantaricin C did not result in loss of activity by some of proteases, including pepsin and proteinase K, which depends on strain-specific characteristics. When bacteriocin SKI19 was treated with catalase, it did not result in a loss of antibacterial activity indicating that the inhibitory activity was not attributed to hydrogen peroxide. In addition, fully antibacterial activity was retained when treated with α-amylase and lipase. This finding revealed that bacteriocin SKI19 was not composed of carbohydrate and lipid. This result was similar with that of other authors by Gong *et al*. (2010); Barbosa *et al.* (2016); and Wen *et al.* (2016). Our results suggest that the inhibitory activity is contributed by the effect of proteinaceous compounds.

SEM

The SEM study revealed the morphological cell shape of *L. monocytogenes* DMST 17303 treated with purified bacteriocin SKI19, and with untreated *L. monocytogenes* DMST 17303 serving as a control (Figures 2b and 2c). The cell surfaces of the control were smooth and intact with regular structure. After incubation with bacteriocin SKI19 at the MIC (2,560 AU/ml) for 2 hr, the morphological shape of the membrane of the indicator strain appeared to change, in which cell wall is seemed shrunken, corrugated, damaged, and pores were formed in the end part of the target cells (white arrows). This leads to cytoplasm outflow, resulting in cell death. Our result insists that the bactericidal activity released by bacteriocin SKI19 was related to damage to the cell membranes, as it destroyed the integrity of the membrane and caused subsequent cell lysis. This result matches observation on other bacteriocins (Wen *et al*., 2016; Khan and Kang, 2016).

Competitiveness of SKI19 in co-cultivation with pathogenic bacteria

Recent approaches in the preservation of meat products have concentrated more on biocontrol, as bacteriocinogenic *Lactobacillus* strains are being utilized as protective microbiota to inhibit growth

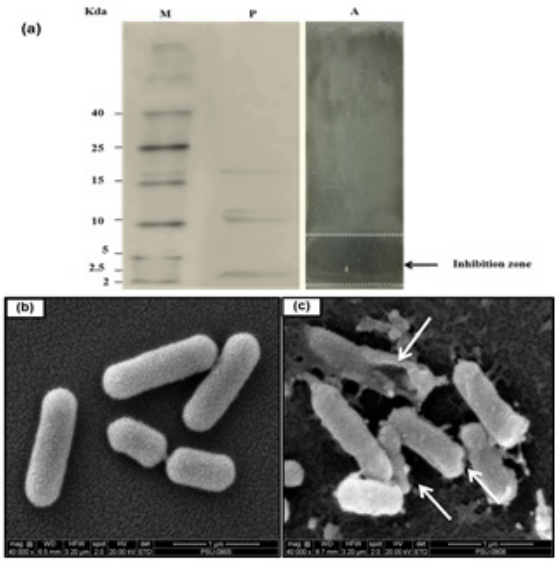

Figure 2. Tricine-SDS-PAGE of purified bacteriocin SKI19 produced by *L. plantarum* subsp. *plantarum* SKI19 (a). Lane P: purified bacteriocin from SP-Sepharose Fast Flow; Lane A: antibacterial zone of bacteriocin SKI19, the gel was overlaid with *L. monocytogenes* DMST 17303 (10^6 CFU/ml) in TSA and incubated at 37°C for 24 hr; Lane M: Molecular weight marker. Morphological changes of untreated (b) and treated *L. monocytogenes* DMST 17303 (c) with purified bacteriocin SKI19 for 2 hr viewed by SEM.

of undesired microorganisms (Todorov *et al*., 2013). Although, only 0.1% of glucose presented in minimal medium for co-cultivation, SKI19 showed the ability to decrease the growth of *E. coli* DMST 4212 and *S. aureus* DMST 8840 (Figures 3a and 3b), as the pathogen cells reduced from values of 5 log units at 36 hr to 4 log units at 48 hr in co-culture system. Moreover, SKI19 exhibited a highly significant inhibitory effect against *L. monocytogenes* DMST 17303. The cell concentration of *L. monocytogenes* DMST 17303 sharply declined from about 3 log units at 24 hr to complete inhibition after 36 hr of incubation in co-culture studies (Figure 3c), indicating the synergistic effect of lower pH and bacteriocin produced by SKI19 led to the eradication of the stressed cells of *L. monocytogenes* DMST 17303. This observation correlates with the spectrum of activity determination of purified bacteriocin SKI19, as mentioned in Table 1. Swetwiwathana *et al.* (2007) reported the synergistic effect of high concentrations of weak acid (pH 4.5) and pediocin PA-1 produced by *P. pentosaceus* TISTR 536 caused the reduction of the cells of *S. anatum*. However, our results revealed the pH level dropped to approximately 5.0 for all of the co-culture studies (Figures 3a, 3b, and 3c). Furthermore, the preliminary characterization of antibacterial compounds produced by SKI19 exhibited that the direct use of cell free supernatant

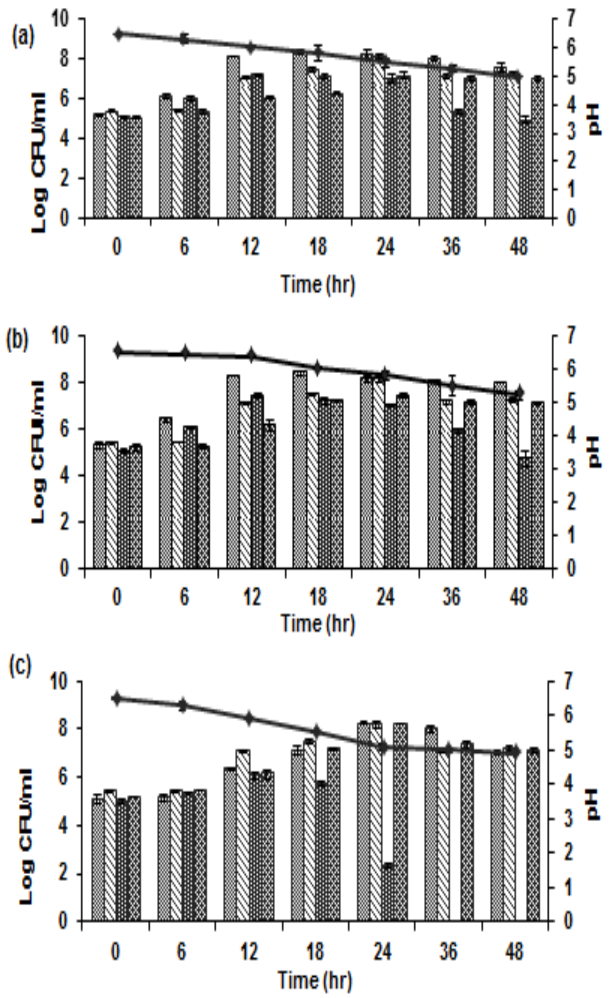

Figure 3. Growth of *E. coli* DMST 4212 (a), *S. aureus* DMST 8840 (b), and *L. monocytogenes* DMST 17303 (c) in co-culture system. Pathogen alone; SKI19 alone; Pathogen in co-culture; SKI19 in co-culture; pH in co-cultivation. The results (Mean ± SD) of three experiments.

(CFS) revealed inhibitory activity against the closely related bacteria *Enterococcus* spp. and *L. sakei* subsp. *sakei* JCM 1157. Also, it inhibited food spoilage and foodborne pathogenic bacteria including Gram-positive and Gram-negative bacteria (data not presented). The inhibition is probably due to the production of antibacterial compounds, like lactic and acetic acids, diacetyl, hydrogen peroxide (H_2O_2), bacteriocin, or their combination (Barbosa *et al.*, 2013). However, after excluding the effects of organic acids and hydrogen peroxide in the CFS, SKI19 did not inhibit Gram-negative pathogenic bacteria. It could be hypothesized that organic acids produced by SKI19 are the main agents to inhibit the growth of *E. coli* DMST 4212 and *S. aureus* DMST 8840. Kongnum and Hongpattarakere (2012) also observed a similar phenomenon, with *L. plantarum* MR03.12 completely inactivating *Vibrio harveyi* in broth co-culture experiments. Also, *L. plantarum* or *L. brevis* totally inactivated *Salmonella* spp. in mixed cultures after 24 hr or 48 hr, respectively (Szala *et al.*, 2012), indicating that organic acids produced by LAB are the core factors inhibiting the growth of Gram-negative bacteria, as observed also in our study. In addition, SKI19 growths in co-culture and in monoculture still remain at stable levels 10^6–10^7 CFU/ml until the end of fermentation. Our results thus clearly highlighted that SKI19 has a very high competitive ability against foodborne pathogenic bacteria and is likely for application as protective culture in fermented meat products.

Conclusion

Bacteriocin SKI19 has action against the closely related bacteria as well as the foodborne pathogenic bacterium, *L. monocytogenes* DMST 17303. Moreover, bacteriocin SKI19 showed considerable heat stability (121°C), pH stability (2.0-10.0), and was sensitive to proteolytic enzymes. Evidenced inhibitory effect of SKI19 in co-cultivation with pathogenic bacteria creates the possibility for its application as protective culture in fermented meat products. However, further studies are necessary to apply bacteriocin SKI19 with a view to the biopreservation of meat products and related foods.

Acknowledgements

This work was supported by the Higher Education Research Promotion and the Thailand's Education Hub for Southern Region of ASEAN Countries Project Office of the Higher Education Commission. Also, the research was funded by the Graduate School, Prince of Songkla University, and the Research and Development Office (RDO), Prince of Songkla University.

References

Aureli, P., Capurso, L., Castellazzi, A. M., Clerici, M., Giovannini, M., Morelli, L., Poli, A., Pregliasco, F. and Zuccotti, F. 2011. Probiotics and health. Review of Pharmaceutical Research 63: 366-376.

Barbosa, M. S., Todorov, S. D., Belguesmia, Y., Choiset, Y., Rabesona, H., Ivanova, I. V., Chobert, J. M., Haertl, T. and Franco, B. D. G. M. 2013. Purification and characterization of the bacteriocin produced by *Lactobacillus sakei* MBSa1 isolated from Brazilian salami. Journal of Applied Microbiology 116: 1364-5072.

Barbosa, M. S., Todorov, S. D., Ivanova, I. V., Belguesmia, Y., Choiset, Y., Rabesona, H., Chobert, J. M., Haertl, T. and Franco, B. D. G. M. 2016. Characterization of a two-peptide plantaricin produced by *Lactobacillus plantarum* MBSa4 isolated from Brazilian salami. Food Control 60: 103-11.

Cleveland, J., Montville, T. J., Nes, I. F. and Chikindas, M. L. 2001. Bacteriocins: safe, natural antimicrobials for food preservation. International of Food Microbiology 71: 1-20.

Cotter, P. D., Hil, C. and Ross, R. P. 2005. Bacterial lantibiotics: strategies to improve therapeutic potential. Current Protein and Peptide Science 6: 61-75.

da Silva Sabo, S., Vitolo, M., González , J. M. D. and Oliveira, R. P. D. S. 2014. Overview of *Lactobacillus plantarum* as a promising bacteriocin producer among lactic acid bacteria. Food Research International 64: 527-536.

Gong, H. S., Meng, X. C. and Wang, H. 2010. Plantaricin MG active against Gram-negative bacteria produced by *Lactobacillus plantarum* KLDS1.0391 isolated from "Jiaoke", a traditional fermented cream from China. Food Control 21: 89-96.

González, B., Arca, P., Mayo, B. and Suárez, J. 1994. Detection, purification, and partial characterization of plantaricin C, a bacteriocin produced by a *Lactobacillus plantarum* strain of dairy origin. Applied and Environmental Microbiology 60: 2158-2163.

H-Kittikun, A., Biscola, V., El-Ghaish, S., Jaffrès, E., Dousset, X., Pillot, G., Haertlé, T., Chobert, J. M. and Hwanhlem, N. 2015. Bacteriocin-producing *Enterococcus faecalis* KT2W2G isolated from mangrove forests in southern Thailand: Purification, characterization and safety evaluation. Food Control 54: 126-134.

Hongpattarakere, T., Cherntong, N., Wichienchot, S., Kolida, S. and Rastall, R. A. 2012. *In vitro* prebiotic evaluation of exopolysaccharides produced by marine isolated lactic acid bacteria. Carbohydrate Polymer 87: 846-852.

Hu, M., Zhao, H., Zhang, C., Yu, J. and Lu, Z. 2013. Purification and characterization of plantaricin 163, a novel bacteriocin produced by *Lactobacillus plantarum* 163 isolated from traditional chinese fermented vegetables. Journal of Agriculture and Food Chemistry 61: 11676-11682.

Hwanhlem, N., Ivanova, T., Biscola, V., Choiset, Y. and Haertlé, T. 2017. Bacteriocin producing *Enterococcus faecalis* isolated from chicken gastrointestinal tract originating from Phitsanulok, Thailand: Isolation, screening, safety evaluation and probiotic properties. Food Control 78: 187-195.

Khan, I. and Kang, S. C. 2016. Probiotic potential of nutritionally improved *Lactobacillus plantarum* DGK-17 isolated from Kimchi – A traditional Korean fermented food. Food Control 60: 88-94.

Kongnum, K. and Hongpattarakere, T. 2012. Effect of Lactobacillus plantarum isolated from digestive tract of wild shrimp on growth and survival of white shrimp *(Litopenaeus vannamei)* challenged with Vibrio harveyi. Fish and Shellfish Immunology 32: 170-177.

Lowry, O. H. and Rosenbrough, N. J. 1951. Protein measurement with the folin-phenol reagent. Journal of Biological Chemistry193: 265-271.

Lü, X., Hu, P., Dang, Y. and Liu, B. 2014. Purification and partial characterization of a novel bacteriocin produced by *Lactobacillus casei* TN-2 isolated from fermented camel milk (Shubat) of Xinjiang Uygur Autonomous region, China. Food Control 43: 276-283.

Maldonado, A., Ruiz-Barba, J. L. and Jiménez-Díaz, R. 2003. Purification and genetic characterization of plantaricin NC8, a novel coculture-inducible two-peptide bacteriocin from *Lactobacillus plantarum* NC8. Applied and Environmental Microbiology 69:383-389.

Nishie, M., Nagao, J. I. and Sonomoto, K. 2012. Antibacterial peptides "Bacteriocins": an overview of their diverse characteristics and applications. Biocontrol Science 17: 1-16.

Noonpakdee, W., Jumriangrit, P., Wittayakom, K., Zendo, J., Nakayama, J and Sonomoto, K. 2009. Two-*peptide bacteriocin from* Lactobacillus plantarum PMU 33 strain isolated from som-fak, a Thai low salt fermented fish product. Asia Pacific journal of Molecular Biology and Biotechnology 17: 19-25.

Ogunbanwo, S. T., Sanni A. I. and Onilude, A. A. 2003. Characterization of bacteriocin produced by Lactobacillus plantarum F1 and *Lactobacillus brevis* OG1. African Journal of Biotechnology 2: 219-227.

Oscariz, J. C. and Pisabarro, A. G. 2001. Classification and mode of action of membrane-active bacteriocins produces by Gram-positive bacteria. International Microbiology 4: 13-19.

Parada, J. L., Caron, C. R., Medeiros, A. B. P. and Soccol, C. R. 2007. Bacteriocins from lactic acid bacteria: Purification, properties and use as biopreservatives. Brazilian archives of Biology and Technology 50: 521-542.

Parente, E. and Hill, C. 1992. A comparison of factors affecting the production of two bacteriocins from lactic acid bacteria. Journal of Applied Bacteriology 73: 290-298.

Paukatong, K. V. and Kunawasen S. 2001. The Hazard Analysis and Critical Control Points (HACCP) generic model for the production of Thai pork sausage (Nham). Berliner Und Munch Tierarztliche Wochenschriift 114: 1-4.

Phithakpol, B., Varanyanond, W., Reungmaneepaitoon, S. and Wood, H. 1995. The traditional fermented foods of Thailand. Bangkok: Institute of Food Research and Product Development, Kasetsart University, Thailand.

Pingitore, E. V., Salvucci, E., Sesma, F. and Nader-Macías, M. E. 2007. Different strategies for purification of antimicrobial peptides from lactic acid bacteria (LAB). In Communicating Current Research and Educational Topics and Trends in Applied Microbiology, Méndez-Vilas, A. eds, p. 557-568. Spain: Formatex.

Powell, J. E., Witthuhn, R. C., Todorov, S. D. and Dicks, L. M. T. 2007 Characterization of bacteriocin ST8KF produced by a kefir isolate *Lactobacillus plantarum* ST8KF. International Dairy Journal 17: 190-198.

Schägger, H. and von Jagow, G. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Analytical Biochemistry 166: 368-379.

Swetwiwathana, A., Lotong, N., Nakayama, J. and Sonomoto, K. 2007. Maturation of nham – a Thai fermented meat product; effect of pediocin PA-1 producer (*Pediococcus pentosaceus* TISTR 536) as starter culture, nitrite and garlic on *Salmonella anatum* during nham fermentation. Fleischwirtschaft international : journal for meat production and meat processing 3: 46-49.

Swetwiwathana, A. and Visessanguan, W. 2015. Potential of bacteriocin-producing lactic acid bacteria for safety improvements of traditional Thai fermented meat and human health. Meat Science 109: 101-105.

Szala, B., Paluszak, Z. and Motyl, I. 2012. Antagonistic effect of lactic acid bacteria on *Salmonella senftenberg* in mixed cultures. Polish journal Environment Studies 21: 1399-1403.

Todorov, S. D., Vaz-Velho, M., Franco, B. D. G. M. and Holzapfel, W. H. 2013. Partial characterization of bacteriocins produced by three strains of *Lactobacillus sakei,* isolated from salpicao, a fermented meat product from North-West of Portugal. Food Control 30: 111-121.

Uymaz, B., Akkoç, N. and Akçelik, M. 2011. Partial characterization of bacteriocins produced by two *Lactobacilus* strains with probiotic properties. Acta Biologica Hungarica 62: 95-105.

van Reenen, C. A., Dicks, L. M. T. and Chikindas, M. L. 1998. Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum.* Journal of Applied Microbiology 84: 31131-31137.

Visessanguan, W., Plengvidhya, V., Chokesajjawatee, N. and Bakar, J. A. 2015. Lactic meat fermentation. In J.D. Owens (Ed.), Indigenous fermented foods of Southeast Asia, p. 313-358. New York, USA: CRC Press.

Wen, L. S., Philip, K. and Ajam, N. 2016. Purification, characterization and mode of action of plantaricin K25 produced by *Lactobacillus plantarum*. Food Control 60: 430-439.

Xie, Y., An, H., Hao, Y., Qin, Q., Huang, Y. and Luo, Y. 2011. Characterization of an anti-Listeria bacteriocin produced by *Lactobacillus plantarum* LB-B1 isolated from koumiss, a traditionally fermented dairy product from China. Food Control 22: 1027-1031.

Yang, R., Johnson, M. and Ray, B. 1992. Novel method to extract large amounts of bacteriocins from lactic acid bacteria. Applied and Environmental Microbiology 58: 3355-3359.

Zacharof, M. P. and Lovitt, R. W. 2012. Bacteriocins produced by lactic acid bacteria a review article. APCBEE Procedia 2: 50-56.