

Synergistic antibacterial effects of bacteriocin produced by *Bacillus velezensis* BUU004 and medicinal plant extracts against *Escherichia coli* and *Salmonella* Typhimurium in dried, crushed, and seasoned squid

¹Soodsawaeng, P., ²Butkhot, N., ¹Boonthai, T., ³Vuthiphandchai, V. and ⁴*Nimrat S.

¹Biological Science Program, Faculty of Science, Burapha University, Chon Buri 20131, Thailand

²Environmental Science Program, Faculty of Science, Burapha University, Chon Buri 20131, Thailand

³Department of Aquatic Science, Faculty of Science, Burapha University, Chon Buri 20131, Thailand

⁴Department of Microbiology and Environmental Science Program, Faculty of Science, Burapha University, Chon Buri 20131, Thailand

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Abstract

Controlling the growth of food-borne pathogens in foodstuffs is important to enhance food safety and promote higher food safety standards. A number of naturally occurring compounds (e.g., bacteriocins and plant-derived compounds) have been widely reported to be safe and effective antimicrobial agents against spoilage and food-borne pathogenic bacteria. The aim of the present work was to evaluate the antibacterial potential of a semi-purified preparation (SPP) containing bacteriocin from *Bacillus velezensis* BUU004, a mixture of lemongrass (*Cymbopogon citratus* (DC) Stapf.), chili spur pepper (*Capsicum frutescens* L.) extracts, and their combination to control the growth of *Escherichia coli* and *Salmonella* Typhimurium in dried, crushed, and seasoned squid during 28 d of storage. The mixed herb extracts (160 mg/mL) showed stronger inhibitory activity against *E. coli* and *S. Typhimurium* than the SPP (800 AU/mL) from *B. velezensis* BUU004. Interestingly, the combination of SPP from *B. velezensis* BUU004 and the mixed herb extracts substantially decreased the numbers of both pathogens in dried seasoned squid during storage as compared to that observed with the individual additives. The bactericidal activity of the SPP from *B. velezensis* BUU004 in combination with the mixed herb extracts against the food-borne pathogens involved cell lysis as ruptured cell walls were observed by a scanning electron microscopy. Therefore, the SPP from *B. velezensis* BUU004 combined with the mixed herb extracts offers tremendous advantages as a novel, safe, natural, and effective way to improve the biosafety of dried seafood products.

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Keywords

Bacillus velezensis,
bacteriocin,
dried seafood,
E. coli,
herb extract,
Salmonella Typhimurium

Introduction

Traditional dried seafood products, which are popular in Thai cuisine, are typically stable at high temperatures due to their low water activity, high salt content, and altered physical and biochemical characteristics resulting from production processes including sun-drying, salting, fermenting, and brining (Fu *et al.*, 2007). However, these products are highly prone to becoming contaminated with spoilage and pathogenic bacteria, leading to serious food-borne illnesses which is a public health concern. In retail dried seafood products in Thailand, the incidence of viable and pathogenic bacterial counts over the allowable limit has been increasing over the past decade, which reflects the lack of hygiene in the production process (Thungkao and Muangharm, 2008; Nimrat *et al.*, 2019). The most common food-borne

pathogenic bacteria in dried seafood products include *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella* (Thungkao and Muangharm, 2008; Butkhot *et al.*, 2019a; Nimrat *et al.*, 2019).

In addition to being a food hygiene indicator, *E. coli* strains can be aetiologic agents of food-borne diarrhoea and extra-intestinal diseases in humans, and are widely distributed in animals and the environment (Balière *et al.*, 2015). The presence of *E. coli* in seafood products reflects a sanitation problem, and poses a serious risk to human health if associated with diarrhoeagenic *E. coli* (Costa, 2013). *Salmonella* is a bacterium that causes two distinct syndromes, systemic disease and gastroenteritis, the latter is frequently associated with food-borne transmission through the consumption of contaminated foods of animal origin such as meat, poultry meat, eggs, and fresh and processed seafood products (Amagliani

*Corresponding author.

Email: subunti@buu.ac.th ; subunttbt@gmail.com

et al., 2012). The number of salmonellosis cases resulting in hospitalisation due to seafood consumption has been increasing worldwide, especially in Asian countries (Amagliani et al., 2012).

A growing demand from health-conscious consumers and legal authorities for food products that are minimally processed and contain low levels or are free of potentially carcinogenic and toxic chemical preservatives has opened up new opportunities to generate robust biopreservation-based strategies to control pathogen growth and achieve high food safety standards. In recent years, bacteriocins, natural antimicrobial peptides, or proteins synthesised through the ribosomal synthesis pathway have attracted substantial attention for their ability to lengthen the shelf lives of a variety of food products (e.g., dairy products, soymilk, fish and fish products, raw meat products, sausages, fruits and vegetables, commercial sauces, and salad dressings) without causing detrimental effects to their organoleptic qualities (Johnson et al., 2018). However, some limitations including a lack of effective protection against Gram-negative bacteria, a narrow pH range activity, spontaneous loss of bactericidal activity, possible inactivation in food matrix by proteolytic enzymes, and the development of bacteriocin-resistant strains have been suggested as factors obstructing the use of bacteriocins in the food industry (Ghraiiri and Hani, 2015). To improve their antibacterial activity against several food-borne pathogens, bacteriocins have been combined with other antibacterial agents and a variety of hurdle technologies including organic acids, salts, EDTA, heat, high hydrostatic pressure, modified atmosphere packaging, and pulsed electric fields (Galvez et al., 2007). Plant essential oils and extracts have also been used in combination with bacteriocins to control pathogen growth in food products owing to their harmless side effects, broad-spectrum antibacterial activity, economic benefits, and a long history of safe use in foods as flavouring agents (Nazarizadeh et al., 2013). Notably, several studies have shown the spectrum of inhibitory activity of nisin against Gram-negative bacteria when used in combination with thyme (Solomakos et al., 2008; Turgis et al., 2012) and oregano (Govaris et al., 2010) essential oils. Similarly, the antibacterial activity of enterocin against the Gram-positive bacteria *Staphylococcus aureus* and *Listeria monocytogenes* is significantly potentiated by the presence of thyme essential oil and phenolic compounds including carvacrol, geraniol, eugenol, terpineol, caffeic acid, *p*-coumaric acid, citral, and hydrocinnamic acid (Grande et al., 2007; Ghraiiri and Hani, 2015).

Lemongrass (*Cymbopogon citratus* (DC) Stapf.), a medicinal herb in the Poaceae family, has long been used as a culinary agent in Thai cuisines as well as in folk medicine to treat coughs, malaria, ophthalmia, pneumonia, and vascular disorders. Chemical analysis of lemongrass extracts revealed the presence of geraniol, neral, and limonene as major components that are responsible for their antioxidant and antibacterial activities (Oussalah et al., 2007). Chili spur pepper (*Capsicum frutescens* L.) is a plant in the Solanaceae family with fruits that are generally dried and ground to produce powdered spice used to flavour dishes. The most important chemical compounds present in chili spur pepper are phenylpropanoid chemotypes including capsaicin, dihydrocapsaicin, cinnamic acid, *m*-coumaric acid, and *o*-coumaric acid, with cinnamic and *m*-coumaric acids contributing to the inhibition of food-borne pathogens (Dorantes et al., 2000).

In our recent study, a semi-purified preparation (SPP) containing bacteriocin from *Bacillus velezensis* BUU004 exhibited broad-spectrum antibacterial activity against food-borne Gram-positive and Gram-negative bacteria (Butkhot et al., 2019a). However, unsatisfactory activity against *E. coli* and *Salmonella* Typhimurium was observed when the SPP alone was added to dried seasoned squid. Therefore, the objective of the present work was to evaluate the antibacterial potential of SPP from *B. velezensis* BUU004, a mixture of lemongrass, chili spur pepper extracts, and their combination to control the growth of *E. coli* and *S. Typhimurium* in dried seasoned squid as a food model.

Materials and methods

Cultivation conditions and preparation of bacteriocin from B. velezensis BUU004

The *B. velezensis* BUU004 strain used in the present work was previously shown to be an excellent aquaculture probiotic (Nimrat et al., 2012), and biosynthesise bacteriocin with activity against food-borne pathogens (Butkhot et al., 2019b). The strain was maintained at -80°C in Trypticase Soy Broth (TSB; Becton BD, Sparks, MD, USA) plus 20% glycerol. A loopful of *B. velezensis* BUU004 strain was inoculated into TSB, and cultured at 30°C with shaking at 200 rpm for 18 h. Then, the supernatant containing bacteriocin was collected by centrifugation (Centrifuge 5804 R, Eppendorf, Hamburg, Germany) at 8,000 g and 4°C for 10 min. The bacteriocin-containing SPP was prepared by adding ammonium sulphate at 80% saturation to the cell-free supernatant to precipitate proteins with gentle stirring

overnight at 4°C (Butkhot *et al.*, 2019a). Subsequently, the precipitated protein was centrifuged at 10,000 g and 4°C for 30 min, and then resuspended in 50 mM sodium phosphate buffer (pH 7.0) prior to dialysis using a dialysis membrane tubing (1 kDa cut-off; Spectrum Laboratory, Los Angeles, CA, USA) at 4°C overnight. The SPP was filtered using a 0.45-µm membrane filter (Sartorius, Gottingen, Germany) and stored at -20°C in a sterile amber bottle. The filter-sterilised SPP was assessed for bacteriocin activity in arbitrary units (AUs) as previously described by Butkhot *et al.* (2019a).

Extraction of medicinal plants

Plant extraction was performed following a method described by Quave *et al.* (2008) with slight modification. Lemongrass stems and chili spur pepper fruits were harvested from a local botanical garden in Chon Buri Province. Lemongrass stems and chili spur pepper fruits exhibiting damage from insects and/or microorganisms were discarded. After being rinsed and shade-dried for 10 h, the herbs were cut into small pieces, and dried in a plate drier at 35°C for 72 h. The dried plant material was then ground using an electric blender. The plant material powders were immersed with denatured 95% ethanol as an extractant at a ratio of 1:10 of material to extractant in a shaking incubator (New Brunswick Innova 4340, Edison, NJ, USA) at 30°C, 120 rpm for 72 h. After being vacuum-filtered using a Whatman filter membrane (No. 1), the supernatant was evaporated at 40°C using a rotary evaporator (Buchi R-215, Flawil, Switzerland). The resulting ethanolic extract was then dissolved in 35% ethanol to produce a stock solution (160 mg/mL) which was then stored in a tightly sealed bottle at -20°C.

Preparation of pathogenic bacteria

Pathogenic bacteria including *S. Typhimurium* TISTR 292 obtained from the culture collection of Thailand Institute of Scientific and Technological Research, PathumThani, Thailand; and *E. coli* ATCC 25922 maintained in the Department of Microbiology, Faculty of Science, Burapha University, Chon Buri, Thailand were used to assess the antibacterial activity of the SPP from *B. velezensis* BUU004, the herb extracts, and their combination. All stock cultures were maintained at -80°C in TSB containing 20% glycerol, and grown on Trypticase Soy Agar (TSA; Becton BD, Sparks, MD, USA) at 35°C for 24 h with two consecutive transfers to produce active sub-cultures. Working cultures prepared from sub-cultures were propagated in TSB at 35°C for 24 h, and then adjusted to a density of 10⁴ CFU/mL

using the McFarland turbidity standard before being used.

Effect of SPP, the herb extracts, and their combination on food-borne pathogens in dried and seasoned squid

The efficacy of the SPP, the mixed herb extracts, and their combination on *S. Typhimurium* TISTR 292 and *E. coli* ATCC 25922 growths in dried seasoned squid was evaluated following the method described by Butkhot *et al.* (2019a). Dried, crushed, and seasoned squid were cut to 2 × 2 cm pieces using sterile scissors. Then, a prepared cell suspension (0.5 mL) of either *S. Typhimurium* TISTR 292 or *E. coli* ATCC 25922 was slowly added over the entire surface of a square piece of squid using an autopipette to a final concentration of 10⁴ CFU/g prior to air-drying in a biosafety cabinet for 15 min to allow maximum adhesion to the food matrix. The samples with each pathogen suspension were treated with (1) sterile distilled water (negative control), (2) the SPP from *B. velezensis* BUU004 (800 AU/mL), (3) a mixture of lemongrass and chili spur pepper extracts (160 mg/mL), and (4) a combination of the SPP (800 AU/mL) and the mixed herb extracts (160 mg/mL) and then air-dried for 15 min. For each treatment, small volume (0.1 mL) of the tested additive was slowly dispensed onto the entire surface of a piece of the squid samples using an autopipette to allow maximum absorption into the food matrix. Each additive was separately added onto a piece of squid sample to form the appropriate mixture of additives and to ensure that the samples received the tested additives at the desired concentrations. Subsequently, the squid samples for each batch were separately maintained in a sterile plastic zip-lock bag at 4°C. Pathogen enumeration from the dried squid samples was performed at 15 min and 1, 7, 14, 21, and 28 d post-inoculation using the most probable number (MPN) method. In addition, during storage at 4°C for 14 and 28 d, half of the samples from each batch were re-treated with the appropriate additives prior to counting viable pathogenic cells as described below.

Pathogen enumeration

A three-tube dilution MPN method was used to enumerate *S. Typhimurium* TISTR 292 and *E. coli* ATCC 25922 modified from FDA (2007). For each group, a squid sample (2 g) was aseptically taken from the zip-lock bag and diluted 10-fold with lactose broth. Subsequently, 1 mL of each dilution was transferred to three sets of three tubes containing Tetrathionate (TT; Becton BD, Sparks, MD, USA) broth, and incubated at 35 ± 2°C for 24 h. Turbid TT

broth was then streaked onto Hektoen enteric (HE) agar plates (Becton BD, Sparks, MD, USA), and then incubated at $35 \pm 2^\circ\text{C}$ for 24 h. Suspected *Salmonella* colonies (rounded, blue-green colonies with or without a black-spotted centre) were confirmed using selected biochemical tests (FDA, 2007).

To enumerate *E. coli* ATCC 25922, a portion of dried squid (2 g) was serially diluted with Butterfield's phosphate-buffered dilution water and thoroughly homogenised using a stomacher. Then, aliquots of the homogenate were transferred to three tubes containing Lauryl Tryptose Broth (LST; Becton BD, Sparks, MD, USA), and incubated at $35 \pm 1^\circ\text{C}$ for 24 - 48 h. Then, all culture broths with turbidity and gas formation were inoculated into *E. coli* broth (Becton BD, Sparks, MD, USA), and incubated at $44.5 \pm 0.2^\circ\text{C}$ for 24 - 48 h. A loopful of culture from the turbid tubes containing gas was then streaked onto Levine Eosin Methylene Blue Agar (L-EMB; Becton BD, Sparks, MD, USA). Following incubation at $35 \pm 1^\circ\text{C}$ for 18 - 24 h, typical colonies (dark centred and flat colonies with or without a metallic sheen) were analysed by IMViC reactions and other selected biochemical tests (FDA, 2002). The MPN table was used to calculate the approximate number of bacteria per gram.

Analysis of bactericidal activity against food-borne pathogens using scanning electron microscopy

Cultures of *S. Typhimurium* TISTR 292 and *E. coli* ATCC 25922 grown in TSB at 30°C for 18 h to the exponential growth phase were adjusted to a density of 10^8 CFU/mL using the 0.5 McFarland turbidity standard. Subsequently, the cell suspension was centrifuged of 8,000 g at 4°C for 10 min. Then, the cell pellets were individually mixed with either the SPP from *B. velezensis* BUU004 (800 AU/mL) or a combination of the mixed herb extracts (160 mg/mL) and the SPP (800 AU/mL). A cell pellet treated with sterile TSB was used as a negative control. After incubating at $35 \pm 1^\circ\text{C}$ for 24 h, the cell pellets were harvested by centrifugation (8,000 g at 4°C for 10 min), resuspended in phosphate-buffered saline (PBS; pH 7.2), and divided into two portions. The cell suspensions of treated *S. Typhimurium* TISTR 292 and *E. coli* ATCC 25922 were diluted 10-fold, and spread-plated onto HE agar and L-EMB plates, respectively, to assess the bactericidal activity of the tested additives. The remaining portions were prepared for scanning electron microscopy observations (Butkhot *et al.*, 2019a). In brief, the collected cell pellets were prefixed with 2.5% glutaraldehyde in PBS (pH 7.2) at 4°C for 4 h, and then post-fixed with 1% osmium tetroxide at 4°C for

2 h. After being washed thrice with PBS (pH 7.2), the fixed samples were dehydrated with a graded ethanol series (20, 50, 70, 80, 90, and 95%) at 25°C . The samples were then coated with gold in a sputter coater (Polaron Range SC 7620, Quorum Technologies, East Sussex, UK), and morphological observations of cell damages were performed using a scanning electron microscope (SEM; LEO 1450 VP, ZEISS, Oberkochen, Germany) equipped with SEM User Interface LEO-32 software (LEO Electron Microscopy Ltd., Cambridge, England).

Results

Anti-pathogenic activity of the SPP, the mixed herb extracts, and their combination in dried and seasoned squid

The *E. coli* ATCC 25922 numbers in dried squid treated with the SPP or the mixed herb extracts were 460 and 1,100 MPN/g at 15 min post-inoculation, and markedly decreased to 43 and 38 MPN/g, respectively, at 28 d of storage (Table 1). The combination of the SPP and the mixed herb extracts appeared to have greater inhibitory activity against *E. coli* ATCC 25922, as the pathogen number substantially decreased to 23 MPN/g at the end of experiment. When all three types of supplements were added to the dried seasoned squid product every 14 d, the inhibitory activity against *E. coli* ATCC 25922 was stronger than that observed for samples with a single treatment during 28 d of chilled storage. At the beginning of the experiment, the numbers of *E. coli* ATCC 25922 in dried squid treated with either the SPP or the mixed herb extracts were 460 and 1,100 MPN/g, and then progressively decreased over the assay to 23 and 3.6 MPN/g, respectively. Interestingly, the combination of the SPP and the mixed herb extracts exhibited relatively high anti-*E. coli* activity in dried and seasoned squid, because the number of *E. coli* ATCC 25922 decreased from 460 to < 3.0 MPN/g during 28 d of storage (Table 1).

Unlike the observed sensitivity of *E. coli* ATCC 25922, *S. Typhimurium* TISTR 292 appeared to be more resistant to all tested supplements. Extremely high numbers ($> 1,100$ MPN/g) of *S. Typhimurium* TISTR 292 were observed throughout the course of the 28-d incubation in the un-inoculated group. The numbers of *S. Typhimurium* TISTR 292 in dried squid treated with the SPP, the mixed herb extracts, and their combination were $> 1,100$ MPN/g after 15 min of storage, remained unchanged until 21 d of storage, and then decreased to 460, 240, and 240 MPN/g, respectively, after 28 d of storage (Table 2). When the dried seasoned squid samples were exposed

Table 1. Antibacterial of semi-purified preparation containing bacteriocin produced by *Bacillus velezensis* BUU004, the herb extracts, and their combination towards *E. coli* ATCC 25922 in dried, crushed, and seasoned squid during 28 d of storage.

Treatment	Sampling time (d post-inoculation; MPN/g)					
	15 min	1	7	14	21	28
Distilled water	> 1,100	1,100	460	240	240	75
SPP from <i>B. velezensis</i> BUU004	460	460	460	150	93	43
A mixture of lemongrass and chili spur pepper extracts	1,100	460	240	93	43	38
A combination of the SPP and the mixed herb extracts	460	93	93	93	43	23
SPP (added every 14-d)	460	460	460	93	43	23
A mixture of lemongrass and chili spur pepper extracts (added every 14-d)	1,100	460	240	43	14	3.6
A combination of the SPP and the mixed herb extracts (added every 14-d)	460	93	43	9.2	3.6	< 3.0

Table 2. Antibacterial effect of semi-purified preparation containing bacteriocin produced by *Bacillus velezensis* BUU004, the herb extracts, and their combination towards *Salmonella* Typhimurium TISTR 292 in dried, crushed, and seasoned squid during 28 d of storage.

Treatment	Sampling time (d post-inoculation; MPN/g)					
	15 min	1	7	14	21	28
Distilled water	> 1,100	> 1,100	> 1,100	> 1,100	> 1,100	> 1,100
SPP from <i>B. velezensis</i> BUU004	> 1,100	> 1,100	> 1,100	> 1,100	> 1,100	460
A mixture of lemongrass and chili spur pepper extracts	> 1,100	> 1,100	> 1,100	> 1,100	> 1,100	240
A combination of the SPP and the mixed herb extracts	> 1,100	> 1,100	> 1,100	> 1,100	> 1,100	240
SPP (added every 14-d)	> 1,100	> 1,100	> 1,100	> 1,100	240	93
A mixture of lemongrass and chili spur pepper extracts (added every 14-d)	> 1,100	> 1,100	> 1,100	> 1,100	23	23
A combination of the SPP and the mixed herb extracts (added every 14-d)	> 1,100	> 1,100	> 1,100	> 1,100	23	< 3.0

to the SPP or the mixed herb extracts every 14 d, the number of *S. Typhimurium* TISTR 292 appeared to remain constant at > 1,100 MPN/g until 14 d of storage, and then decreased to 93 and 23 MPN/g, respectively, at day 28 of storage. The addition of the mixed herb extracts in combination with the SPP resulted in a reduction in the number of *S. Typhimurium* TISTR 292 from > 1,100 MPN/g at 15 min post-storage to 23 and < 3.0 MPN/g at 21 and 28 d post-storage, respectively (Table 2).

Scanning electron microscopy

Rod shaped, regular, and intact cell walls were observed for the untreated cells of *E. coli* ATCC 25922 (Figure 1a) and *S. Typhimurium* TISTR 292

(Figure 2a). In contrast, deformed, pitted, and withered cells were observed for the SPP-treated *E. coli* ATCC 25922 (Figure 1b) and *S. Typhimurium* TISTR 292 (Figure 2b), which might have been due to the leakage of intracellular contents. *E. coli* ATCC 25922 cells treated with the combined herb extracts and SPP had a rough membrane appearance along with shrinkage and pore formation on their surfaces (Figure 1c). *S. Typhimurium* TISTR 292 cells exposed to the mixed herb extracts combined with the SPP exhibited ruptured cell walls and cell shrivelling (Figure 2c). Based on the spread-plate results, untreated *E. coli* ATCC 25922 and *S. Typhimurium* TISTR 292 grew well on the culture media. No growth of *S. Typhimurium* TISTR 292 and *E. coli* ATCC

25922 treated with either the SPP or the combined SPP and the mixed herb extracts were observed on the media, confirming the bactericidal activity of the tested additives.

Discussion

Despite the growth of most microorganisms being limited by high salt and low moisture contents, the biosafety of dried seafood products is a concern due to the presence of spoilage and pathogenic bacteria, and extensive handling during their preparation and distribution by personnel.

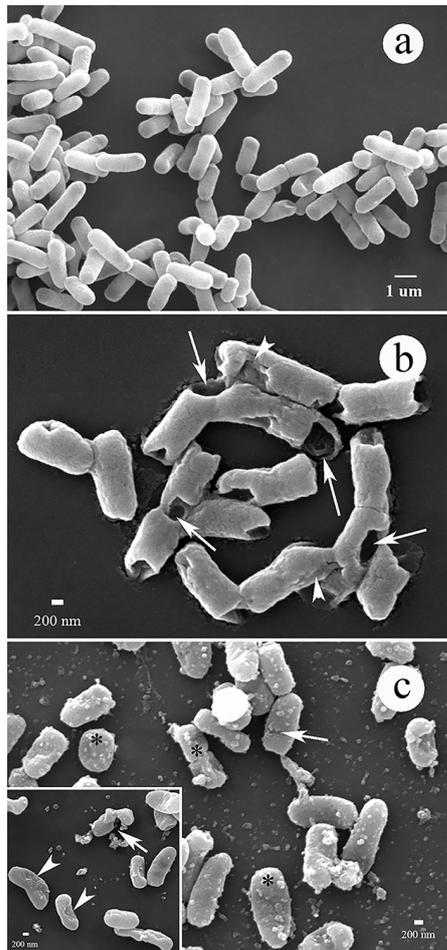


Figure 1. Scanning electron microscopy (SEM) images depicting morphological changes of *E. coli* ATCC 25922 treated with different additives. (a) smooth cell walls without any disruptions for untreated *E. coli* cells, (b) pore formation (arrow) and wrinkle (arrowhead) on the surfaces of *E. coli* cells exposed to semi-purified preparation containing bacteriocin produced by *B. velezensis* BUU004 (SPP), and (c) rough surfaces (asterisk) of *E. coli* treated with the SPP combined with lemongrass and chili spur pepper extracts, with the inset showing pore formation (arrow) and pitting deformation (arrowhead).

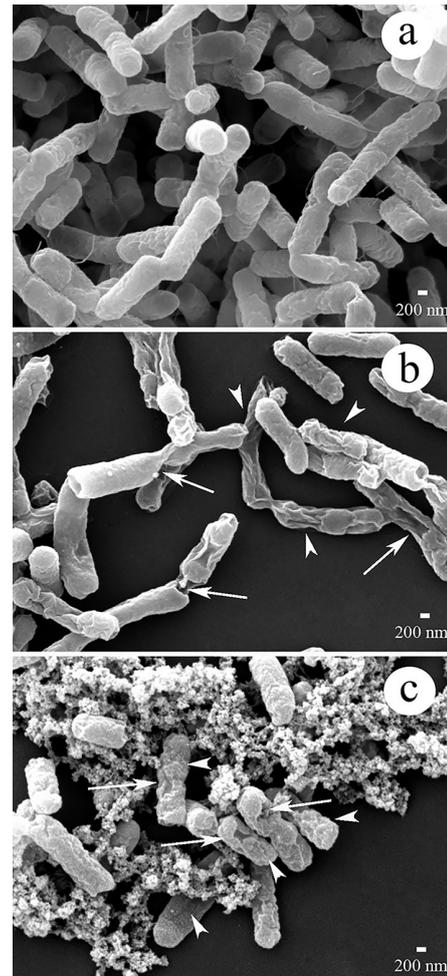


Figure 2. Scanning electron microscopy (SEM) images depicting morphological changes of *Salmonella* Typhimurium TISTR 292 treated with different additives. (a) typical shape of untreated *S. Typhimurium* cells, (b) *S. Typhimurium* exposed to the semi-purified preparation containing bacteriocin produced by *B. velezensis* BUU004 (SPP) showing pore formation (arrow) on the surfaces and cell shrinkage (arrowhead), and (c) pore formation (arrow) and corrugated surfaces (arrowhead) of *S. Typhimurium* treated with the SPP combined with lemongrass and chili spur pepper extracts.

Simultaneously, the safety of synthetic preservatives has been questioned in recent years owing to their potential carcinogenic and toxic effects. Thus, the development of economical, natural, and effective food preservative systems is needed in response to the public demand for safe, convenient, and healthy dried seafood products. In the present work, the preservative efficacy of an SPP from *B. velezensis* BUU004 alone was shown to be unlikely to ensure the complete protection of dried seasoned squid against *E. coli* ATCC 25922 and *S. Typhimurium* TISTR 292. Bacteriocins produced by members of the *Bacillus* genus have been reported to have antagonistic

activities against pathogenic Gram-negative bacteria, including *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Shigella flexneri*, *Salmonella* Enteritidis, *S. Gallinarum*, *S. Pullorum*, and *S. Typhimurium* (Lim *et al.*, 2016). Similarly, our SPP from *B. velezensis* BUU004 was previously shown to be active against *Staphylococcus aureus*, *B. cereus*, *B. coagulans*, *Listeria monocytogenes*, *Micrococcus luteus*, *E. coli*, *E. coli* O157:H7, and *S. Typhimurium* through *in vitro* experiments (Butkhot *et al.*, 2019b). This inhibition appears to be related to the disintegration of the structural architecture of the cytoplasmic membrane, as pore formation of the SPP-exposed cells was observed in the present work. However, the antibacterial activity of bacteriocins is well known to be diminished in meat products owing to their binding to proteins and fat as well as their inactivation by indigenous and/or microbial proteolytic enzymes (Aasen *et al.*, 2003; Stergiou *et al.*, 2006). These factors may explain why weak antagonistic activity against *E. coli* ATCC 25922 and *S. Typhimurium* TISTR 292 was observed in the dried squid model used in the present work.

The mixed extracts of lemongrass and chili spur pepper at 160 mg/mL produced moderate antibacterial activity against *E. coli* ATCC 25922 and comparatively weak activity against *S. Typhimurium* TISTR 292 in dried seasoned squid during storage. In an *in vitro* experiment, the inhibitory activity of lemongrass essential oil was shown to be effective in inhibiting the growth of pathogenic bacteria, including *E. coli* O157:H7, *S. Typhimurium*, *Staphylococcus aureus*, and *L. monocytogenes* (Oussalah *et al.*, 2007). In a food model study, the addition of lemongrass oil had a great inhibitory effect on pathogenic *E. coli* O157:H7 and *S. enterica* in apple juice (Friedman *et al.*, 2004). Similarly, de Oliveira *et al.* (2013) reported that supplementation with lemongrass essential oil (3.90, 7.80, and 15.60 $\mu\text{L/g}$) could prolong the refrigerated shelf life of ground beef by markedly reducing the initial inoculated population of *Salmonella enterica* serotype Enteritidis at 6 log CFU/g to approximately 1 - 3 log CFU/g after 6 d of storage depending on the oil concentration. The antagonistic activity of chili pepper extract against food-borne pathogens was also reported in a previous *in vitro* study (Dorantes *et al.*, 2000). In a study by Careaga *et al.* (2003), a *C. annuum* bell pepper extract was added to raw beef meat at concentrations of 0.02 - 2.5 mL per 100 g of raw beef to control the growth of *S. Typhimurium*. They observed that the pathogen was inactivated when at least 1.5 mL of extract was added per 100 g of meat. Herb extract/essential oil contains a variety

of compounds with different chemical structures that are responsible for their antibacterial activity. The predominant components of lemongrass extract/essential oil that have broad biological activities such as antibacterial, antifungal, antiviral, and antioxidant activities are citral chemotypes that include geranial (45%), neral (32%), and limonene (9%; Oussalah *et al.*, 2007). The active constituents of chili pepper shown to contribute to inhibitory action against food-borne pathogens are cinnamic acid and *m*-coumaric acid (Dorantes *et al.*, 2000). This is the first study to evaluate the activity of mixed lemongrass and chili spur pepper extracts against food-borne pathogens in a food product.

The combination of the SPP from *B. velezensis* BUU004 (800 AU/mL) and the mixed herb extracts (160 mg/mL) exhibited great antibacterial activity towards *E. coli* ATCC 25922 and *S. Typhimurium* TISTR 292 in dried seasoned squid by reducing their viable counts to below detectable levels (< 3.0 MPN/g) after 28 d of refrigerated storage. Our results clearly indicated their synergistic activity against the assayed food-borne pathogens. Similarly, Solomakos *et al.* (2008) reported that the use of nisin at 500 or 1,000 IU/g in combination with 0.6% thyme essential oil showed an effective potential to inhibit the growth of *E. coli* O157:H7 in minced beef during refrigerated storage at 10°C. Oregano essential oil (0.6 - 0.9%) was shown to be more effective by dramatically reducing the number of *S. Enteritidis* cells when combined with nisin at concentrations of 500 - 1,000 IU/g in minced sheep meat without organoleptic deterioration (Govaris *et al.*, 2010). Similarly, the antilisterial activity of enterocin AS-48, a cyclic bacteriocin produced by *Enterococcus faecalis*, was shown to significantly increase by the concomitant individual addition of essential oils of thyme verbena, thyme red, Spanish oregano, ajowan, tea tree, clove, and sage to a Russian salad (Molinos *et al.*, 2009). Recently, the use of essential oil (0.1 - 0.2%) from *Ziziphora clinopodioides* together with nisin resulted in a pronounced decrease in *E. coli* O157:H7 numbers in raw beef patties during 9 d of refrigerated storage (Shahbazi *et al.*, 2016). Although the exact mechanism of synergy between the tested additives used in the present work remains unknown, the combination of the SPP from *B. velezensis* BUU004 and the mixed herb extracts played a key role in the cell membrane destruction, as evidenced by observations of an irregular cell shape along with ruptured SPP-herb-exposed cells. These results indicated that cell lysis was one of bactericidal modes of action of the SPP combined with the mixed herb extracts. In a study by Sivarooban *et al.* (2008), a

combination of 6,400 IU nisin with either a green tea or a grape seed extract caused an apparent reduction in *L. monocytogenes* cell numbers from approximately 6 to 3.7 log CFU/mL, and an undetectable level, respectively, after 24 h of incubation, and alterations in cell membranes and condensed cytoplasm were detected through TEM. In another study conducted by Ettayebi *et al.* (2000), the complete inhibition of *L. monocytogenes* and *B. subtilis* growth was observed using a combination of thymol, a major active constituent of thyme and nisin Z. This group postulated that thymol induced destabilisation of the bacterial membrane structure by causing a change in the permeability of the cytoplasmic membrane, resulting in an increased concentration of intracellular nisin, alterations in the proton motive force, inhibition of enzymatic systems, and leakage of a variety of molecules and ions eventually leading to the death of thymol-nisin-exposed cells. Therefore, further studies should investigate other potential modes of action of the SPP in combination with the mixed herb extracts assayed in the present work, such as permeability of cell membrane, ion efflux, the extracellular APT concentration, and release of protein and DNA to understand the antibacterial activity and mode of action. Chemical transformation of inactive compounds to active forms when naturally occurring supplements are combined may be involved in promoting greater antibacterial activity as compared to that observed with the addition of single supplement (Wolska *et al.*, 2012).

In the present work, the addition of the SPP from *B. velezensis* BUU004, the mixed herb extracts, and the SPP in combination with the mixed herb extracts to dried seasoned squid every 14 d was more effective against the two pathogens than a single treatment of these supplements. The lower antibacterial activity observed in dried seasoned squid when a single treatment was used might be associated with the volatile properties of herb extracts and the concentrations of active compounds being reduced during 28 d of storage (Abdollahzadeh *et al.*, 2014). A decreased concentration of bacteriocin during storage to a level at which the antimicrobials are unable to inhibit pathogens may also account for such a phenomenon (Ghalfi *et al.*, 2007). In addition, intrinsic factors of the seafood and environmental parameters may influence bacterial sensitivity to the additives used in the present work (Gutierrez *et al.*, 2008). Therefore, the stability of the SPP from *B. velezensis* BUU004 and the mixed herb extracts should be further investigated during the storage of dried seafood products.

Our results revealed that all assayed additives

(the SPP from *B. velezensis* BUU004, the mixed herb extracts, and the SPP in combination with the mixed herb extracts) had a stronger bactericidal effect towards *E. coli* ATCC 25922 than *S. Typhimurium* TISTR 292. These results may be a consequence of different structural features of the lipid bilayer (types and arrangement of hydrophobic molecule chains) and metabolism machinery between the two pathogens (Abee *et al.*, 1994).

To the best of our knowledge, this is the first study to report the effects of a combination of a bacteriocin produced by a *Bacillus* species with plant-derived extracts in inhibiting food-borne pathogenic bacteria in a food model. Application of the novel combination would provide a more acceptable way for consumers to buy a minimally processed or relatively natural product, and could be an alternative to chemically synthetic counterparts in the dried seafood industry.

Conclusion

The combination of the SPP from *B. velezensis* BUU004 with the mixed herb extracts (lemongrass and chili spur pepper) decreased the growth of *E. coli* ATCC 25922 and *S. Typhimurium* TISTR 292 in dried, crushed, and seasoned squid during refrigerated storage for 28 d, particularly when added every 14 d. Scanning electron microscopy analysis showed that cell rupture was the mechanism of action of the SPP from *B. velezensis* BUU004 in combination with the mixed herb extracts. The inhibitory activity of our combined supplement would have significant implications to reduce the outbreak of food-borne illnesses, particularly those caused by *E. coli* and *Salmonella*, and enhance the bacteriological quality of dried seafood products. However, this combination should be investigated further for its effect on the organoleptic quality of foods to confirm the sensorial acceptability and its biosafety prior to use in the food industry.

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