Research Article

Antibacterial Activity of Bacteriocin Produced by *Bacillus velezensis* BUU004, Herb Extracts and Their Combination for Controlling Spoilage and Pathogenic Bacteria in Dried, Seasoned and Crushed Squid

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ABSTRACT

Biopreservation is an attractive measure to maintain the quality and safety of food products simultaneously with fit in the healthy lifestyle of consumers. This study aimed to evaluate biopreservative potential of bacteriocin produced by *Bacillus velezensis* BUU004, a mixture of lemongrass and hot pepper extracts, and their combination for controlling growths of spoilage bacteria and foodborne pathogens in dried, seasoned and crushed squid. The squid samples were treated with 1) sterile distilled water (control), 2) nisin solution, 3) semi-purified preparation containing bacteriocin from *B. velezensis* BUU004 (SPP-BV), 4) a mixture of lemongrass and hot pepper extracts, and 5) a combination of the SPP-BV and the mixed herb extracts. During 28-day storage, the three types of additives were as effective as commercial nisin for securing biosafety of the dried squid evident by a significant ($p<0.05$) reduction in total

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viable count, compared to the control. Administration of the tested additives every 14 days was more effective against spoilage bacteria in dried squid than a single treatment. Then, the tested additives were investigated for their antibacterial activities against pathogenic *E. coli, Salmonella* Typhimurium and *Bacillus cereus.* The strongest inhibitory effect on all tested pathogens was observed with the combined addition of the SPP-BV and the mixed herb extracts in dried squid. SEM analysis revealed that the three additives had bactericidal activity through destruction of the bacterial cell walls. This study suggested that all tested additives had potential as an alternative for controlling food spoilage and pathogenic bacteria and enhancing the safety of dried seafood products.

Keywords: Bacteriocin, Herb extract, *Bacillus cereus, E. coli, Salmonella,* Dried seafood, Synergy

Introduction

Seafood and seafood-based products have a highly significant social, economic and nutritional role for people in Thailand. Over 80% fishermen in fishing villages along the Gulf of Thailand and shores of the Andaman Sea engage in traditional or small-scale processed seafood products. A lack of efficient control over the quality of raw materials is a main problem faced by the commercial processing factories. As for the small traditional plants, development of the products to meet evolving food qualities is limited due to a lack of appropriate technology. As a consequence, biosafety has recently become a significant public health issue in the seafood industry of Thailand. For example, retailed processed seafood products in Chon Buri province containing viable bacteria count (TVC) over the allowable limit imposed by Thailand and international food administration agencies have been incessantly reported for over the past decade [1-2]. Several foodborne pathogens have been also isolated from traditional Thai seafood products, e.g. *Bacillus cereus, Escherichia coli, Staphylococcus aureus* and *Salmonella* [1-3]. Dried, crushed and seasoned squids can be easily contaminated by spoilage and pathogenic bacteria because the production conditions in small factories or household facilities are primitive with rudimentary hygienic practices. In a retail store, the post-prepared dried and crushed squids are commonly stored at room temperature and portioned from bulk containers into small plastic bags with a poor handling manner [3]. We also observed an apparent increase in TVC and human-skin bacterial flora in the post-prepared squids during storage, indicating implementation of an effective technology for minimizing the microbial contamination needed.

Although *E. coli* is mostly harmless and part of a healthy intestinal tract, some of them can cause diseases from mild food-poisoning syndromes to severe life-threatening infections in humans. The contamination is generally spread through a fecal-oral route or a direct contact with animals or food handlers. The presence of *E. coli* in seafood products reflects a lack of proper hygienic approaches and poses a serious risk to human health if associated with diarrheagenic *E. coli* [4]. *Salmonella* strains also pose a significant threat to human health owing to causing a range of illnesses, including typhoid fever and gastroenteritis. In Thailand, *Salmonella* is one of the main causes of foodborne infection resulting in approximately 269 hospitalizations (26%), behind *Vibrio parahaemolyticus* in 2014 [5]. *Salmonella* is usually contracted through food handlers, the surfaces and tools used during food preparation, and the consumption of contaminated foods of animal origin, such as meat, poultry meat, eggs, and fresh and processed seafood products [6]. *B. cereus,* a toxin-producing bacterium, is becoming an important etiologic agent of food poisoning in the industrialized world due to heat resistance of its endospore. It causes two distinct forms of foodborne illnesses: diarrhea and emesis by means of production of the enterotoxin haemolysin BL (HBL) and

non-haemolytic enterotoxin (NHE), cytotoxin K (CytK), and an emesis-inducing toxin cereulide [7]. The bacterium is a common soil saprophyte and also frequently isolated from meat, eggs and dairy products as well as animal and plant materials [8]. Recently, it exists dominantly in traditional dried seafood products in Thailand, e.g. salted fish, dried mussels, seasoned fish strips, seasoned squids, and seasoned crabs [2].

In recent years, there is an obvious increase in a healthy lifestyle of consumers to purchase a minimally processed product simultaneously with suspicion of chemical preservatives being causes of intoxications, allergies, cancers and other degenerative diseases [9]. This has prompted a search for an effective, safe, food-compatible, and natural-based preservative to protect food products from pathogen contamination. Currently, naturally occurring compounds, e.g. bacteriocins from beneficial bacteria and plant-derived extracts, have drawn substantial attention as a biopreservation-based strategy to enhance food safety and promote higher food safety standards.

A plenty of plant species have medicinal characteristics and beneficial impact on health, such as antioxidant, anti-inflammatory, antimicrobial, anti-mutagenic and anti-carcinogenic potentials [10]. In Thailand, lemongrass (*Cymbopogon citratus* (DC) Stapf.), a tropical perennial plant in the Poaceae family, has been used for centuries as both folk medicine and culinary ingredient. It is traditionally used as a sedative, and a medicine to remedy coughs, malaria, ophthalmia, pneumonia and vascular disorders. Hot pepper (*Capsicum frutescens* L.) is a plant belonging to the Solanaceae family, with fruits that are generally dried and ground to produce powdered spice used to flavour dishes. It is rich in capsaicinoids currently employed for development of a novel medicine because of its beneficial properties, e.g. antioxidant, antimicrobial, anti-inflammatory and antitumor activities [11]. Recently, we observed that a mixture of lemongrass and hot pepper extract had inhibitory activity against foodborne pathogens, indicating its biopreservative potential in the food system [12].

Bacteriocins are natural ribosomally synthetic peptides produced by beneficial bacteria in their ecological niches for self-preservation and competitive advantage. They are easily digested in the gastrointestinal tract, thereby considered as basically safe food additives [13]. Bacteriocins biosynthesized by *Bacillus* species have attracted immense attention to form an alternative preservation technique in the food industry because of varied structural diversity, stability under a wide range of environments and broad inhibitory spectrum [14]. In our recent study, bacteriocin produced by *B. velezensis* BUU004 exhibited promising potential as a safe biopreservative together with a broad-spectrum of *in vitro* antibacterial activity against foodborne Gram-positive and Gram-negative bacteria [15]. Therefore, the purpose of this study was to investigate the antibacterial potential of bacteriocin from *B. velezensis* BUU004, a mixture of

lemongrass and hot pepper extracts and their combination to control spoilage bacteria and foodborne pathogens (*E. coli, S.* Typhimurium and *B. cereus*) in dried, seasoned and crushed squid.

Materials and Methods

Pathogen strains and growth conditions

Foodborne pathogenic bacteria, including *E. coli* ATCC 25922, *S.* Typhimurium TISTR 292, and *B. cereus* TISTR 687, were grown in Trypticase Soy Broth (TSB; Becton BD, Sparks, MD, USA) with vigorous shaking at 35°C for 24 h, and then adjusted to a concentration of 10^4 CFU/mL using the McFarland turbidity standard before use.

Preparation of ethanolic herb extracts

Lemongrass (stems) and hot pepper (fruits), harvested from a local botanical garden in Chon Buri Province, were extracted following a method described by Soodsawaeng et al. [12]. In brief, the herbs were chopped, dried, and ground using a blender. The plant material powders were extracted in 95% ethanol at a ratio of 1:10 of material to extractant with constant agitation at 30°C, 120 rpm, for 72 h. The supernatant was evaporated at 40°C using a rotary evaporator (Buchi R-215, Flawil, Switzerland). A stock solution (160 mg/mL) was produced by dissolving in 35% ethanol.

Nisin preparation

Nisin contained 2.5% active nisin (Sigma-Aldrich Chemical Co, Darmstadt, Germany). Nisin powder (100 mg) was dissolved in 0.02 N HCl solution to produce a final concentration of 10^3 IU/mL. The solution was filtered using a 0.45 μm syringe filter.

Culture and preparation of bacteriocin from *B. velezensis* BUU004

A bacteriocin producing *B. velezensis* BUU004 strain was cultured in TSB at 30°C with shaking at 200 rpm for 18 h. The cell-free supernatant was harvested by centrifugation at 8,000 *g* and 4°C for 10 min prior to addition of ammonium sulfate at 80% saturation to precipitate proteins with gentle stirring overnight at 4°C [3]. Then, after centrifuging at 10,000 *g*, 4°C for 30 min, the precipitated protein was re-suspended in 50 mM sodium phosphate buffer (pH 7.0) and the bacteriocin-containing suspension was dialysed using a dialysis membrane tubing (1 kDa cutoff, Spectrum Laboratory, Los Angeles, CA, USA) at 4°C overnight. Bacteriocin activity in arbitrary units (AUs) of the resulting semi-purified preparation containing bacteriocin from *B. velezensis* BUU004 (SPP-BV) was assessed by means of a well diffusion technique against *B. cereus* TISTR 687 used as indicator [3].

Effect of the SPP-BV, the mixture of herb extracts and their combination on food-spoilage bacteria in dried, seasoned and crushed squid

Dried, crushed and seasoned squid purchased from Nong Mon Market, Chon Buri province, was used as a food model. The dried, crushed and seasoned squid was prepared according to Butkhot *et al.* [3]. A 2x2 cm piece of the squid was added with 1) sterile distilled water (control), 2) nisin solution (10^3 IU/mL), 3) the SPP-BV (800 AU/mL), 4) a mixture of lemongrass and hot pepper extracts (160 mg/mL) and 5) a combination of the SPP-BV (800 AU/mL) and the mixed herb extracts (160 mg/mL) prior to air-drying for 15 min. For each treatment, the entire surface of a piece of the squid samples was slowly introduced with the tested additives (0.1 mL) using an autopipette. After air-drying for 15 min, the squid samples in each treatment were stored in a sterile plastic bag at room temperature and half of the samples from each treatment were retreated with the appropriate additives at 14 and 28 day of storage. TVC in the squid samples was enumerated at 15 min and 1, 7, 14, 21 and 28 day post-inoculation.

Enumeration of TVC was conducted using a spread plate method following US FDA [16] with some modifications. The squid sample (5 g) collected at defined intervals was homogenized with Butterfield's phosphate-buffered dilution water (45 mL) using a stomacher. A 10-fold dilution was made, and then an aliquot (0.1 mL) from each dilution was seeded onto Plate Count Agar (Becton BD, Sparks, Maryland, USA). After incubating at $35\pm2^{\circ}$C C for 24 h, all colonies were counted and expressed as colony forming unit (CFU) per g of sample. All measurements were performed in triplicate.

Effect of the SPP-BV, the mixture of herb extracts and their combination on foodborne pathogens in dried, seasoned and crushed squid

A prepared 2x2 cm piece of the squid was inoculated with the cell suspension (0.5 mL) of the tested pathogens, e.g., *E. coli* ATCC 25922, *S.* Typhimurium TISTR 292, or *B. cereus* TISTR 687 at 10^4 CFU/mL and air-dried in a biosafety cabinet for 15 min to allow maximum adhesion in the food matrix. Experimental design was divided into 5 treatments as described above. For each treatment, the squid sample with each pathogen was added with a minute volume (0.1 mL) of the tested additives. After drying for 15 min, the samples were maintained in a plastic bag at room temperature and aseptically taken from the bag for pathogen enumeration at 15 min, 1, 7, 14, 21 and 28 day post-inoculation. Pathogen enumeration from the dried squid samples was conducted using the most probable number (MPN) method [16]. The MPN tables were used to calculate the approximate number of bacteria per gram.

A series of three-tube dilution technique was employed to count *E. coli.* Three successive 10-fold dilution was made by adding a portion of the squid sample (2 g) in Butterfield's phosphate-buffered dilution water prior to homogenization using a stomacher for 2 min.

An aliquot of the homogenate was dispensed in a set of three tubes containing Lauryl Tryptose Broth (LST; Becton BD) and incubated at $35 \pm 1^{\circ}C$ for 24-48 h. Afterwards, cloudy broths with gas formation were transferred into *E. coli* broth (Becton BD) and incubated at $44.5 \pm 0.2^{\circ}C$ for 24-48 h. The turbid tubes with gas were then streaked onto Levine Eosin Methylene Blue Agar (Becton BD) using an inoculating loop, and incubated at $35 \pm 1^{\circ}C$ for 18-24 h. Suspicious colonies (dark centered and flat colonies with or without a metallic sheen) were subjected to IMViC reactions and other selected biochemical tests.

To count *S.* Typhimurium, the squid sample (2 g) was diluted 10-fold with lactose broth, homogenized using a stomacher and incubated at $35 \pm 2^{\circ}C$ for 24h. Small volume (1 mL) of each dilution was pipetted into three sets of three tubes of Tetrathionate (Becton BD) broth and incubated at $35 \pm 2^{\circ}C$ for 24 h. The tubes with turbidity were transferred onto Hektoen enteric agar plates (Becton BD) prior to incubating at $35 \pm 2^{\circ}C$ for 24 h. Suspected *Salmonella* colonies (rounded, blue-green colonies with or without a black-spotted center) were confirmed using selected biochemical tests.

B. cereus was enumerated by diluting the dried squid sample (2 g) with Butterfield's phosphate buffered dilution water. After three successive 10-fold dilution was prepared, a small volume (1 mL) of each dilution was inoculated into three sets of three tubes containing TSB supplemented with 0.15% Polymyxin B prior to being incubated at $30 \pm 1^{\circ}C$ for 48 ± 2 h. Cloudy culture was streaked onto Mannitol Egg Yolk Polymyxin agar and incubated at $30 \pm 1^{\circ}C$ for 18-24 h. Typical colonies (pink colony with lecithinase enzyme activity observed by the presence of an opaque zone around the colony) were subcultured onto Nutrient Agar and assessed by biochemical and morphological testes.

Scanning electron microscopy

Logarithmic-phased cells of pathogenic *S.* Typhimurium TISTR 292, *E. coli* ATCC 25922 and *B. cereus* TISTR 687 were adjusted to 10^8 CFU/mL using the 0.5 McFarland turbidity standard. The pathogen cells were exposed to the tested additives at desired concentrations as mentioned previously at $35 \pm 1^{\circ}C$ for 20 h. The bacterial cells treated with sterile TSB were considered as a negative control. Then, the untreated and treated pathogen cells were prepared for morphological observations using a scanning electron microscope (SEM; LEO 1450 VP, ZEISS, Oberkochen, Germany) [3]. The bactericidal activity of the tested additives was also evaluated by spread-plating the leftover portions of the untreated and treated cells onto Trypticase Soy Agar plates (Becton BD).

Data analysis

All data are expressed as mean ± standard deviation. Data were normalized and transformed when needed. A one-way analysis of variance (ANOVA) was used to compare

between treated groups and the control. Tukey's test was used to identify any difference of parameters found in the ANOVA at a significant level of $p < 0.05$. All statistical analyses were conducted using Minitab Version 18.1.0.

Results

Total viable bacteria

During 28-day storage at room temperature, TVC in the squid samples treated with either the SPP-BV, the mixture of herb extracts, or their combination were significantly ($p < 0.05$) lower than that of the control. At the end of the experiment, TVC in the control squid was $1.09 \pm 0.09 \times 10^4$ CFU/g while all treated groups contained TVC in the range of 6.95 ± 1.28 to $8.68 \pm 0.88 \times 10^3$ CFU/g. When all tested additives were supplemented in the dried squid every 14 days, TVC in all treated groups decreased significantly ($p < 0.05$) in comparison with those of a single addition. During 28-day storage, similar TVC were observed in the squid treated with the SPP-BV, the mixture of herb extracts, or their combination ranging from 3.30 ± 0.22 to $4.93 \pm 0.45 \times 10^3$ CFU/g. At 28 days of storage, there was no significant ($p > 0.05$) difference of TVC between the SPP-BV-treated squid and nisin-treated squid (Fig. 1).

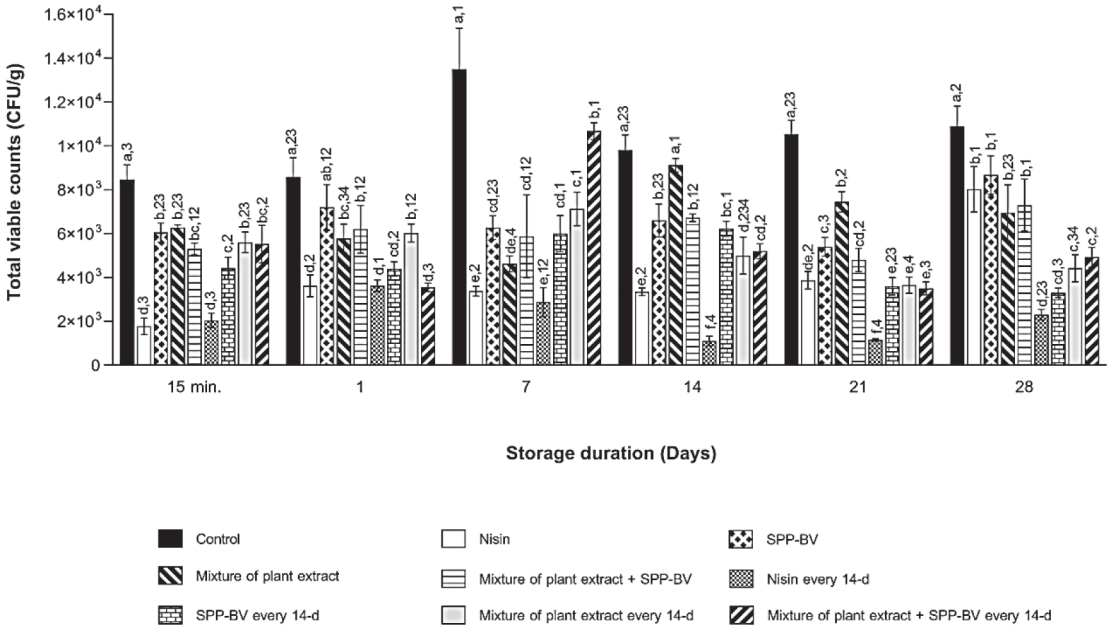

Figure 1 Change in TVC of dried, seasoned and crushed squid treated with the tested additives during 28 days of storage at room temperature.

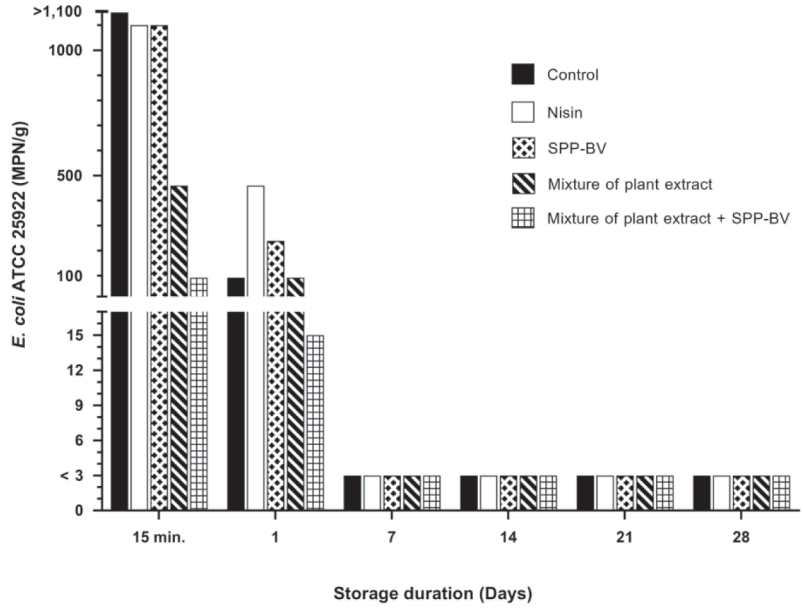

Figure 2 *E. coli* numbers in dried, seasoned and crushed squid treated with the tested additives in comparison with commercial nisin solution during 28 days of storage at room temperature.

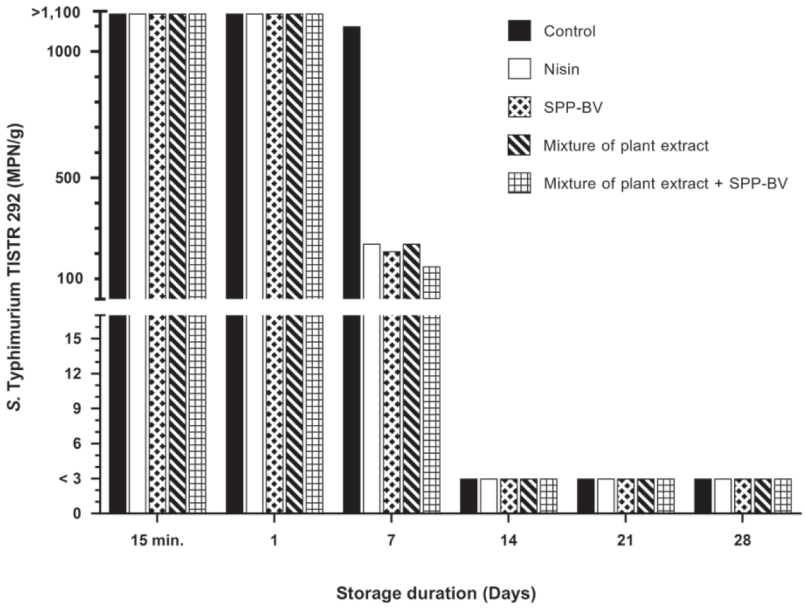

Figure 3 *S.* Typhimurium numbers in dried, seasoned and crushed squid treated with the tested additives, compared to commercial nisin solution during 28 days of storage at room temperature.

Pathogenic bacteria

E. coli in the control squid were > 1,100 and 93 MPN/g at 15 min and 1 day post-inoculation, respectively. The strongest anti-*E.coli* activity was observed with the presence of a combination of the SPP-BV and the mixed herb extracts in dried squid due to a reduction in the number of *E. coli* from 93 MPN/g at 15 min post-storage to 15 MPN/g at 1 day of storage. *E. coli* numbers in the squid treated with either the mixture of herb extracts or the SPP-BV were 93 and 240 MPN/g at 1 day post-storage. *E. coli* was absent by day 7 of storage in all treatments and the control (Fig. 2).

S. Typhimurium numbers in the untreated squid were ≥ 1,100 MPN/g during the first 7 days of storage. Similar to the observed sensitivity of *E. coli,* the combination of the SPP-BV and mixed herb extracts appeared to have greater inhibitory activity against *S.* Typhimurium since the pathogen number pronouncedly decreased from > 1,100 MPN/g at 15 min post-inoculation to 150 MPN/g after 7 days of storage. *S.* Typhimurium was vanished by 14 days of storage in all treatments and the control (Fig. 3).

The addition of the tested additives, e.g. nisin, the mixture of herb extracts, the SPP-BV, and the combination of the SPP-BV and mixed herb extracts in the dried squid resulted in a substantial reduction in *B. cereus* number in the range of 93-150 MPN/g by 15 min post-inoculation. Thereafter, comparatively similar antibacterial activities against *B. cereus* were observed in the squid samples supplemented with those additives because of a progressive decrease in *B. cereus* numbers ranging from 43-93 MPN/g at 1 day post-storage to 11-16 MPN/g at the end of storage. In contrast, *B. cereus* numbers in the control were relatively high (> 1,100 MPN/g) at 15 min of storage and slowly reduced to 23 MPN/g after 28 day of storage (Fig. 4).

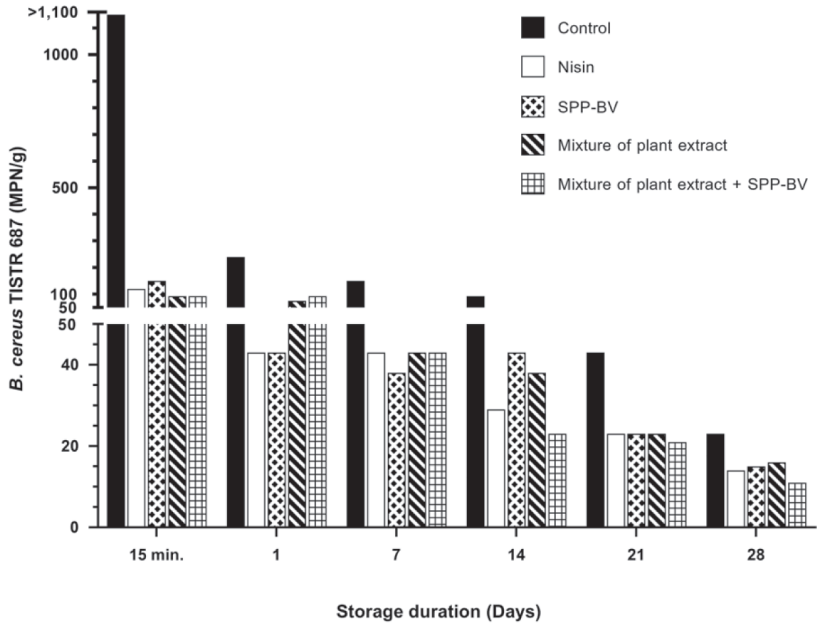

Figure 4 *B. cereus* numbers in dried, seasoned and crushed squid treated with the tested additives in comparison with commercial nisin solution during 28 days of storage at room temperature.

Scanning electron microscopy

The control cells of *E. coli, S.* Typhimurium and *B. cereus* demonstrated normal ultrastructures, intact cell walls, and smooth surfaces (Fig. 5a, 6a, 7a). On the contrary, in the presence of the tested additives, the membrane surfaces of *E. coli, S.* Typhimurium and *B. cereus* cells treated with the SPP-BV appeared to be collapsed, and their morphological shapes were severely changed, e.g. cell shrinkage, pore formation, extensive and evidently wrinkled boundary of the cells, and ghost cells with cytoplasmic material completely lost (Fig. 5b, 6b, 7b). The mixed-herb-treated cells of *E. coli, S.* Typhimurium and *B. cereus* exhibited agglutination, adhesion among adjacent cells, rough surfaces, cell shrinkage, noticeable pits on the head parts of the cells, and collapsed, flat and empty cells that may be indicative of intracellular fluid leakages (Fig. 5c, 6c, 7c). Similarly, cells of *E. coli,* S. Typhimurium and *B. cereus* exposed to the combination of the SPP-BV and the mixed herb extracts showed pore formation along with adhesion among adjacent cells, wrinkled surfaces, and pits on the head portion of the cells (Fig. 5d, 6d, 7d). The SEM images suggested that antibacterial mechanisms of the SPP-BV, the mixture of herb extracts, and their combination was associated with the disruption of cell walls, which caused cell mortality as no viable cells of the three pathogenic bacteria treated with the additives were observed on the culture medium plates (data not shown). On the contrary, the untreated cells of all three pathogens grew well on the medium.

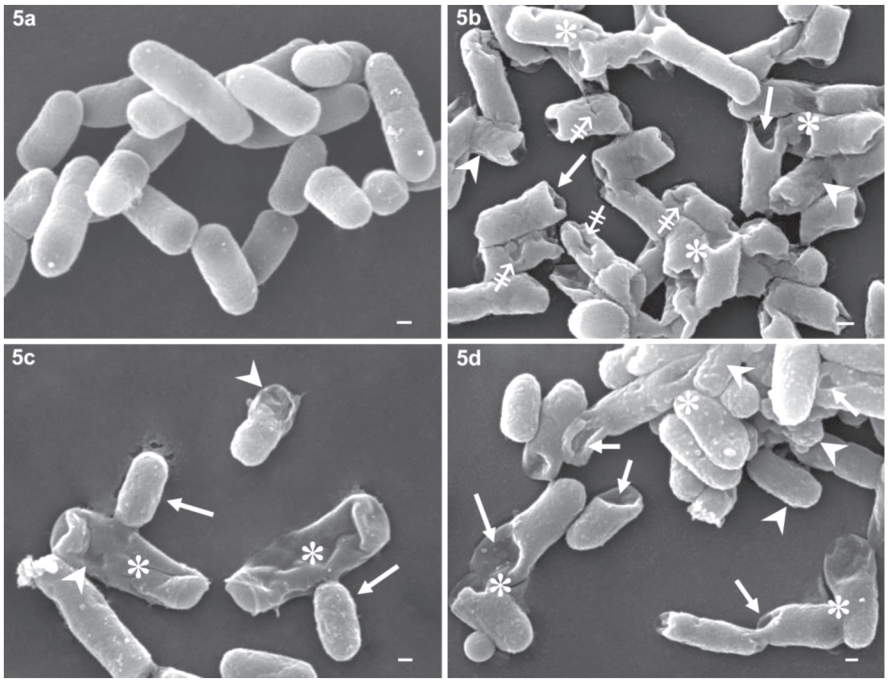

Figure 5 Microscopic images of *E. coli* treated with or without the additives for 20 h. a) normal structural cells of untreated *E. coli,* b) hollow SPP-BV-treated cells (↑) along with wrinkled surfaces (➤), noticeable pits (⇞), and adhesion among adjacent cells (⁎), c) shrunk mixed-herb-treated cells (↑) concomitant with pit formation (➤), and corrugated surfaces (⁎), and d) pore formation (↑), and severely rough surfaces (➤), and agglutination (⁎) of the cells treated with the combination of the SPP-BV and mixed herb extracts. Bar = 200 nm.

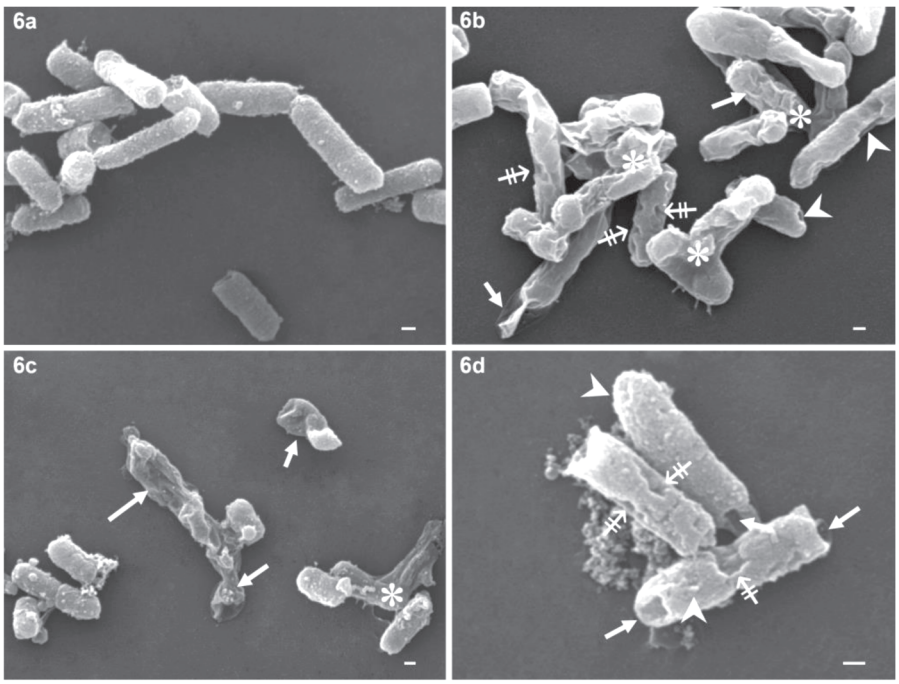

Figure 6 Scanning electron micrograph of *S.* Typhimurium treated with or without the additives for 20 h. a) undamaged morphological structure of untreated *S.* Typhimurium cells, b) severely damaged structure of the SPP-BV-treated cells with extensive and evidently wrinkled boundary (↑), hollowness (➢), pore formation (⇞), and severely corrugated surfaces (⁎), c) ghost distorted cells with intracellular components outflow (↑) and adhesion among adjacent cells (⁎) of mixed-herb-treated *S.* Typhimurium, and d) hollow cells (↑) together with severely rough surface (➢) and pore formation (⇞) of *S.* Typhimurium treated with the combination of the SPP-BV and mixed herb extracts. Bar = 200 nm.

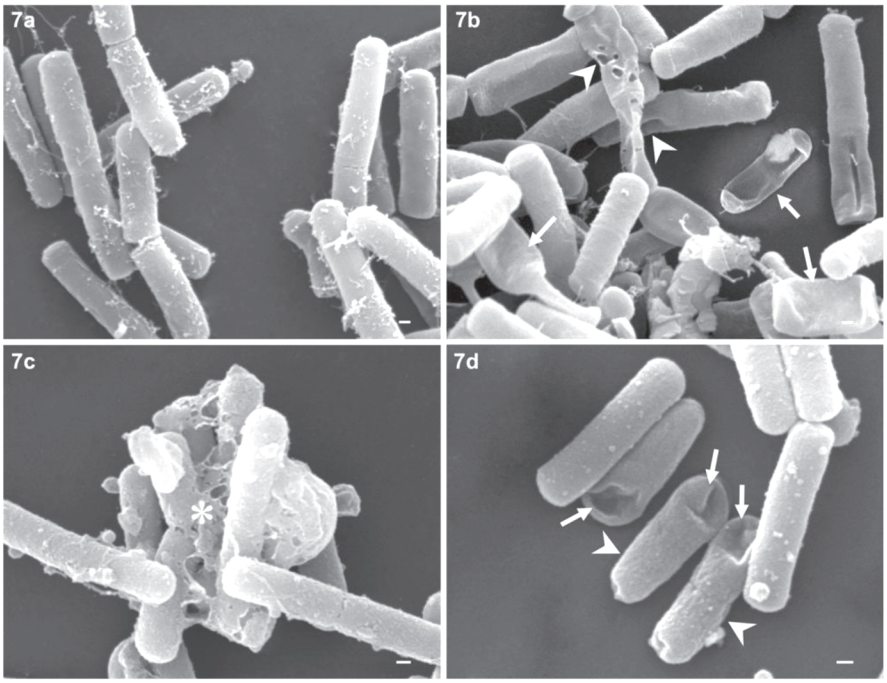

Figure 7 SEM analysis of *B. cereus* exposed to the additives for 20 h. a) normal appearance of unexposed *B. cereus* cells, b) flat and empty cells (↑) along with pore formation (➢) of the SPP-BV-treated *B. cereus*, c) agglutination and adhesion among adjacent cells (⁎) of the mixed-herb-treated *B. cereus,* and d) severely abnormal shape of *B. cereus* exposed to the combination of the SPP-BV and mixed herb extracts with pit formation on the head parts of the cells (↑) and rough surfaces (➢). Bar = 200 nm.

Conclusion and Discussion

Nisin is an antibacterial peptide with FDA-approved and GRAS (generally regarded as safe) status for use in products and currently licensed as a food preservative in over 50 countries. However, such limitation of nisin usage in food products is its expensiveness, lack of inhibitory actions against Gram-negative bacteria, and development of nisin-resistant pathogenic strains [17]. This has aroused the advent of alternative technologies for preservation of food products. This finding demonstrated that administration of the tested additives (the SPP-BV, the mixture of lemongrass and hot pepper extracts, and their combination) was as effective as commercial nisin for controlling spoilage bacteria in dried, seasoned and crushed squids during storage at room temperature. The results corroborated with a study conducted by Fangio and Fritz [18]. Addition of a bacteriocin-like substance produced by a harmless strain of *B. cereus* P9 resulted in significant reductions in mesophilic and psychrotrophic aerobic bacteria counts in fresh beef and *B. cereus* count in lettuce (*Lactuca sativa* L.) under refrigeration condition.

Likewise, Kaewklom et al. [19] reported that partially purified bacteriocin containing amycin prepared from *B. amyloliquefaciens* showed potent antilisterial effect on the growth of *Listeria monocytogenes* in sliced bologna sausage during a chilled storage. According to Kong *et al.* [20], the incorporation of a mixture of honeysuckle (*Scutellaria* sp.) and *Forsythia suspense* Thunb extracts or a mixed spice extract (cinnamon, rosemary and clove oil) caused a 1.8–2.3-log decrease in TVC of vacuum-packaged fresh pork during a refrigerated storage for 28 days. Azizkhani and Tooryan [21] also reported a significant reduction in TVC of beef sausages supplemented with a mixture of extracts from rosemary (*Rosmarinus officinalis*) and mint (*Mentha longifolia* L. Hudson) during storage. It is well known that the mode of action of bacteriocins produced by *Bacillus* species involves binding to the peptidoglycan layer that causes destabilization of the cellular structure by destroying membrane integrity and formation of transient pores resulting in leakage of intracellular fluids and eventually cell death [22] as severely damaged structure of the SPP-BV-treated cells illustrated by SEM and no viable cells on the culture medium in this study.

The major constituents of lemongrass extract/essential oil are citral, an isomeric monoterpene mixture of geranial and neral, which are responsible for its broad spectrum of antibacterial activity [23]. The monoterpene mechanism of action is mainly associated with destruction of the hydrophobic structure and function of the cell membrane due to the lipophilic characteristic [24]. Cinnamic acid and *m*-coumaric acid, the two main phenolic components of hot pepper, have been found to have inhibitory action against food-borne pathogens [25]. In this study, the mixed-herb-treated cells of the pathogens showed severe destruction of cell architecture as examined by SEM. The mixed herb extracts may affect the bacterial cells by various bactericidal mechanisms such as attacking the bilayer of cell membrane, destroying membrane integrity, increasing membrane permeability, disturbing cellular activities, and ultimately leading to cell mortality [26]. The bactericidal activity of the mixed herb extracts observed in this study may also be related to synergistic interactions between the components in the extracts.

Our results confirmed that the supplementation of the three types of additives every 14 days was more effective against spoilage bacteria in dried seasoned squid than a single addition of these additives. As a result of volatile characteristics of herb extracts, a decrease in the concentration of active compounds during storage may account for such a phenomenon when a single treatment is employed [27]. The lower inhibitory efficacy observed in the SPP-BV- treated dried seasoned squid with a single addition is possibly due, in part to the fact that bacteriocin concentration reduces during storage to a level at which the active compositions lack an inhibitory activity against pathogen cells [28]. Even though a significant reduction in viable bacterial count was observed in the dried squid treated with our additives (the SPP-BV,

the mixture of lemongrass and hot pepper extracts, and their combination), eradication of indigenous spoilage bacteria in dried squid was comparatively more difficult than experimentally inoculated pathogens (*E. coli, S.* Typhimurium and *B. cereus*). Typically, biofilms play an important role in physiological alteration of bacterial cells living in complex microbial ecosystems resulting in an increased tolerance to environmental stresses. The extracellular polymeric matrix of biofilms can also facilitate the bacteria's attachment to a surface and impede penetration of active compounds into the deeper layer and bacterial cells inside the biofilms [29]. This may explain high inherent resistance to our tested additives of indigenous flora in dried, seasoned and crushed squid. Our results suggested that preservatives should be promptly administered in food products prior to establishment and biofilm formation of pathogenic bacteria for securing entire protection and enhancing biosafety of foods.

Such combination of food-compatible biopreservative represents a feasible alternative for ensuring food safety. In this study, a combination of the SPP-BV and the mixed herb extracts exhibited a strong potential for controlling spoilage bacteria and eliminating foodborne pathogenic bacteria, especially *E. coli* and *S.* Typhimurium in dried, seasoned and crushed squid during storage. Similarly, Field et al. [30] reported that supplementation of a combination of bioengineered derivative nisin V and low concentrations of either carvacrol or trans-cinnamaldehyde resulted in a substantial delay of lag phase and a significant reduction in viable cells of *L. monocytogenes* in laboratory media, chocolate milk drink and chicken noodle soup. In accordance with Grande et al. [31], antibacterial potential of bacteriocin, enterocin AS-48, against *Staphylococcus aureus* was markedly extended with the presence of essential oils, namely carvacrol, geraniol, eugenol, terpineol, caffeic acid, *p*-coumaric acid, citral or hydrocinnamic acid in carbonara sauce during 30 days of storage. Abdollahzadeh *et al.* [27] revealed synergistic activity against *L. monocytogenes* in minced fish meat during refrigerated storage as the *L. monocytogenes* viable count reduced to below 2 log CFU/g after 6 days with presence of thyme essential oil while the pathogen count was below 2 log CFU/g after the second day of storage with simultaneous use of thyme essential oil and nisin. Our results clearly showed that the combination of the SPP-BV and the mixed herb extracts had a bactericidal effect on the pathogen cells through deconstruction of the cell wall and cell lysis as the presence of pore formation along with other obviously severe damages observed by SEM. These phenomena may be attributed to a synergy. Synergistic action against bacterial cells can occur through pore formation by activity of antimicrobial peptides whereas active constituents in plant essential oils/extracts have a fluidifying effect on the cell membrane that allows depolarization, increased membrane permeability, changing in the proton motive force, and inhibiting cellular activities [32]. Likewise, Pol and Smid [33] postulated a reduction of viable counts of *L. monocytogenes*

and *B. cereus* exposed to carvacrol simultaneously with nisin possibly due to carvacrol function by prolonging the lifetime of pores created by nisin or increasing the number and size of the pores on the cell membranes. The actual mode of mechanisms of the combination of the SPP-BV and the herb extracts remained unknown and merit to be investigated further.

In conclusion, this study demonstrated the potential use of the natural-derived compounds including the SPP-BV, the mixture of lemongrass and hot pepper extracts, and their combination, with powerful antibacterial activity for controlling spoilage and foodborne bacterial growths in dried seafood products. Controlling the growth of foodborne pathogens in foodstuffs using our tested additives has a significant implication to prevent gastrointestinal illnesses, particularly caused by *E. coli, S.* Typhimurium and *B. cereus* and promote higher food safety standards. Application of the three types of additives can meet consumers' demand for natural preservatives and prevent the potential prejudicial hazards of synthetic counterparts. This may also have financial benefits by reducing the costs of treatment derived from an expensive commercial nisin. However, an additional investigation related to the effect of the three tested supplements on organoleptic quality is required to ensure the consumer acceptability of dried, seasoned and crushed squid in terms of sensorial properties, e.g. flavor, taste and color. In recent study, the SPP-BV has been confirmed as its basic biosafety characteristic due to low cytotoxic effect and absence of the enterotoxin genes in its genome, indicating a great potential as a natural food biopreservative [15].

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